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CA 2946003 A1 2015/11/05

(21) 2 946 003

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) **Date de dépôt PCT/PCT Filing Date:** 2015/05/01
(87) **Date publication PCT/PCT Publication Date:** 2015/11/05
(85) **Entrée phase nationale/National Entry:** 2016/10/14
(86) **N° demande PCT/PCT Application No.:** US 2015/028837
(87) **N° publication PCT/PCT Publication No.:** 2015/168589
(30) **Priorités/Priorities:** 2014/05/01 (US61/987,467); 2014/09/11 (US62/049,230)

(51) **Cl.Int./Int.Cl.** ***C12N 15/11*** (2006.01), ***C07H 21/04*** (2006.01)

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(54) **Titre : COMPOSITIONS ET METHODES DE MODULATION DE L'EXPRESSION DE L'ANGIOPOIETINE DE TYPE 3**
(54) **Title: COMPOSITIONS AND METHODS FOR MODULATING ANGIOPOIETIN-LIKE 3 EXPRESSION**

(57) **Abrégé/Abstract:**
Provided herein are methods, compounds, and compositions for reducing expression of an ANGPTL3 mRNA and protein in an animal. Also provided herein are methods, compounds, and compositions for reducing lipids and/or glucose in an animal. Such methods, compounds, and compositions are useful to treat, prevent, delay, or ameliorate any one or more of cardiovascular disease and/or metabolic disease, or a symptom thereof, in an individual in need thereof.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 November 2015 (05.11.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/168589 A3

(51) **International Patent Classification**:
C12N 15/11 (2006.01) *C07H 21/04* (2006.01)

(21) **International Application Number**:
PCT/US2015/028837

(22) **International Filing Date**:
1 May 2015 (01.05.2015)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
61/987,467 1 May 2014 (01.05.2014) US
62/049,230 11 September 2014 (11.09.2014) US

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(88) **Date of publication of the international search report**:
30 December 2015

WO 2015/168589 A3

(54) **Title**: COMPOSITIONS AND METHODS FOR MODULATING ANGIOPOIETIN-LIKE 3 EXPRESSION

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DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 379

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NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

COMPOSITIONS AND METHODS FOR MODULATING ANGIOPOIETIN-LIKE 3 EXPRESSION

Sequence Listing

The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled BIOL0254WOSEQ_ST25.txt, created on April 28, 2015 which is 0.98 MB in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

Field of the Invention

Provided herein are methods, compounds, and compositions for reducing expression of angiopoietin-like 3 (ANGPTL3) mRNA and protein in an animal. Also, provided herein are methods, compounds, and compositions having an ANGPTL3 inhibitor for reducing ANGPTL3 related diseases or conditions in an animal. Such methods, compounds, and compositions are useful, for example, to treat, prevent, delay or ameliorate any one or more of cardiovascular disease or metabolic syndrome, or a symptom thereof, in an animal.

Background

Diabetes and obesity (sometimes collectively referred to as "diabesity") are interrelated in that obesity is known to exacerbate the pathology of diabetes and greater than 60% of diabetics are obese. Most human obesity is associated with insulin resistance and leptin resistance. In fact, it has been suggested that obesity may have an even greater impact on insulin action than diabetes itself (Sindelka et al., *Physiol Res.*, 2002, *51*, 85-91). Additionally, several compounds on the market for the treatment of diabetes are known to induce weight gain, a very undesirable side effect to the treatment of this disease.

Cardiovascular disease is also interrelated to obesity and diabetes. Cardiovascular disease encompasses a wide variety of etiologies and has an equally wide variety of causative agents and interrelated players. Many causative agents contribute to symptoms such as elevated plasma levels of cholesterol, including non-high density lipoprotein cholesterol (non-HDL-C), as well as other lipid-related disorders. Such lipid-related disorders, generally referred to as dyslipidemia, include hyperlipidemia, hypercholesterolemia and hypertriglyceridemia among other indications. Elevated non-HDL cholesterol is associated with atherogenesis and its sequelae, including cardiovascular diseases such as arteriosclerosis, coronary artery disease, myocardial infarction, ischemic stroke, and other forms of heart disease. These rank as the most prevalent types of illnesses in industrialized countries. Indeed, an estimated 12 million people in the United States suffer with coronary artery disease and about 36 million require treatment for elevated cholesterol levels.

Epidemiological and experimental evidence has shown that high levels of circulating triglyceride (TG) can contribute to cardiovascular disease and a myriad of metabolic disorders (Valdivielso et al., 2009,

Atherosclerosis Zhang et al., 2008, *Circ Res.* 1;102(2):250-6). TG derived from either exogenous or endogenous sources is incorporated and secreted in chylomicrons from the intestine or in very low density lipoproteins (VLDL) from the liver. Once in circulation, TG is hydrolyzed by lipoprotein lipase (LpL) and the resulting free fatty acids can then be taken up by local tissues and used as an energy source. Due to the profound effect LpL has on plasma TG and metabolism in general, discovering and developing compounds that affect LpL activity are of great interest.

Metabolic syndrome is a combination of medical disorders that increase one's risk for cardiovascular disease and diabetes. The symptoms, including high blood pressure, high triglycerides, decreased HDL and obesity, tend to appear together in some individuals. It affects a large number of people in a clustered fashion. In some studies, the prevalence in the USA is calculated as being up to 25% of the population. Metabolic syndrome is known under various other names, such as (metabolic) syndrome X, insulin resistance syndrome, Reaven's syndrome or CHAOS. With the high prevalence of cardiovascular disorders and metabolic disorders there remains a need for improved approaches to treat these conditions

The angiopoietins are a family of secreted growth factors. Together with their respective endothelium-specific receptors, the angiopoietins play important roles in angiogenesis. One family member, angiopoietin-like 3 (also known as angiopoietin-like protein 3, ANGPT5, ANGPTL3, or angiopoietin 5), is predominantly expressed in the liver, and is thought to play a role in regulating lipid metabolism (Kaplan et al., *J. Lipid Res.*, **2003**, *44*, 136-143). Genome-wide association scans (GWAS) surveying the genome for common variants associated with plasma concentrations of HDL, LDL and triglyceride found an association between triglycerides and single-nucleotide polymorphisms (SNPs) near ANGPTL3 (Willer et al., Nature Genetics, 2008, 40(2):161-169). Individuals with homozygous ANGPTL3 loss-of-function mutations present with low levels of all atherogenic plasma lipids and lipoproteins, such as total cholesterol (TC) and TG, low density lipoprotein cholesterol (LDL-C), apoliprotein B (apoB), non-HDL-C, as well as HDL-C (Romeo *et al.* 2009, *J Clin Invest*, 119(1):70-79; Musunuru *et al.* 2010 *N Engl J Med*, 363:2220-2227; Martin-Campos et al. 2012, *Clin Chim Acta*, 413:552-555; Minicocci *et al.* 2012, *J Clin Endocrinol Metab*, 97:e1266-1275; Noto *et al.* 2012, *Arterioscler Thromb Vasc Biol*, 32:805-809; Pisciotta *et al.* 2012, *Circulation Cardiovasc Genet*, 5:42-50). This clinical phenotype has been termed familial combined hypolipidemia (FHBL2). Despite reduced secretion of VLDL, subjects with FHBL2 do not have increased hepatic fat content. They also appear to have lower plasma glucose and insulin levels, and importantly, both diabetes and cardiovascular disease appear to be absent from these subjects. No adverse clinical phenotypes have been reported to date (Minicocci *et al.* 2013, *J of Lipid Research*, 54:3481-3490). Reduction of ANGPTL3 has been shown to lead to a decrease in TG, cholesterol and LDL levels in animal models (U.S. Serial Number 13/520,997; PCT Publication WO 2011/085271). Mice deficient in ANGPTL3 have very low plasma triglyceride (TG) and cholesterol levels, while overpexpression produces the opposite effects (Koishi et al. 2002; Koster 2005; Fujimoto 2006). Accordingly, the potential role of ANGPTL3 in lipid metabolism makes it an attractive target for therapeutic intervention.

To date, therapeutic strategies to treat cardiometabolic disease by directly targeting ANGPTL3 levels have been limited. ANGPTL3 polypeptide fragments (U.S. Serial Number 12/128,545), anti-ANGPTL3 antibodies (U.S. Serial Number 12/001,012) and ANGPTL3 nucleic acid inhibitors including antisense oligonucleotides (U.S. Serial Number 13/520,997; PCT Publication WO 2011/085271; incorporated by reference herein, in their entirety) have previously been suggested or developed, but none of the compounds directly targeting ANGPTL3 have been approved for treating cardiometabolic disease. Accordingly, there is an unmet need for highly potent and tolerable compounds to inhibit ANGPTL3. The invention disclosed herein relates to the discovery of novel, highly potent inhibitors of ANGPTL3 expression and their use in treatment.

Summary of the Invention

Provided herein are compositions and methods for modulating expression of ANGPTL3 mRNA and protein. In certain embodiments, the composition is an ANGPTL3 specific inhibitor. In certain embodiments, the ANGPTL3 specific inhibitor decreases expression of ANGPTL3 mRNA and protein.

In certain embodiments, the composition is an ANGPTL3 specific inhibitor. In certain embodiments, the ANGPTL3 specific inhibitor is a nucleic acid. In certain embodiments, the nucleic acid is an antisense compound. In certain embodiments, the antisense compound is a modified oligonucleotide. In certain embodiments, the antisense compound is a modified oligonucleotide with a conjugate group attached.

In certain embodiments, the ANGPTL3 specific inhibitor is a modified oligonucleotide with a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and having a nucleobase sequence comprising at least 8, least 9, least 10, least 11, at least 12, least 13, at least 14, at least 15, at least 16, least 17, least 18, least 19, or 20 contiguous nucleobases of the nucleobase sequence of SEQ ID NO: 77.

In certain embodiments, the ANGPTL3 specific inhibitor is a modified oligonucleotide with a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprising a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to an equal length portion of nucleobases 1140-1159 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

In certain embodiments, the ANGPTL3 specific inhibitor is a modified oligonucleotide with a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprising a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to an equal length portion of nucleobases 9715-9734 of SEQ ID NO: 2, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 2.

In certain embodiments, the ANGPTL3 specific inhibitor is a modified oligonucleotide with a conjugate group, wherein the modified oligonucleotide consists of 20 linked nucleosides and having a nucleobase sequence comprising at least 8 contiguous nucleobases of SEQ ID NO: 77, wherein the modified oligonucleotide comprises: (a) a gap segment consisting of ten linked deoxynucleosides; (b) a 5' wing

segment consisting of five linked nucleosides; (c) a 3' wing segment consisting of five linked nucleosides; and wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar, wherein each internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine.

In certain embodiments, the ANGPTL3 specific inhibitor is a modified oligonucleotide with a conjugate group, wherein the modified oligonucleotide consists of 20 linked nucleosides and having a nucleobase sequence consisting of at least 8 contiguous nucleobases of SEQ ID NO: 77, wherein the modified oligonucleotide consists of: (a) a gap segment consisting of ten linked deoxynucleosides; (b) a 5' wing segment consisting of five linked nucleosides; (c) a 3' wing segment consisting of five linked nucleosides; and wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar, wherein each internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine.

In certain embodiments, the present disclosure provides conjugated antisense compounds. In certain embodiments, the present disclosure provides conjugated antisense compounds comprising an antisense oligonucleotide complementary to a nucleic acid transcript. In certain embodiments, the present disclosure provides methods comprising contacting a cell with a conjugated antisense compound comprising an antisense oligonucleotide complementary to a nucleic acid transcript. In certain embodiments, the present disclosure provides methods comprising contacting a cell with a conjugated antisense compound comprising an antisense oligonucleotide and reducing the amount or activity of a nucleic acid transcript in a cell.

The asialoglycoprotein receptor (ASGP-R) has been described previously. See e.g., Park et al., PNAS vol. 102, No. 47, pp 17125-17129 (2005). Such receptors are expressed on liver cells, particularly hepatocytes. Further, it has been shown that compounds comprising clusters of three N-acetylgalactosamine (GalNAc) ligands are capable of binding to the ASGP-R, resulting in uptake of the compound into the cell. See e.g., Khorev et al., Bioorganic and Medicinal Chemistry, 16, 9, pp 5216-5231 (May 2008). Accordingly, conjugates comprising such GalNAc clusters have been used to facilitate uptake of certain compounds into liver cells, specifically hepatocytes. For example it has been shown that certain GalNAc-containing conjugates increase activity of duplex siRNA compounds in liver cells in vivo. In such instances, the GalNAc-containing conjugate is typically attached to the sense strand of the siRNA duplex. Since the sense strand is discarded before the antisense strand ultimately hybridizes with the target nucleic acid, there is little concern that the conjugate will interfere with activity. Typically, the conjugate is attached to the 3' end of the sense strand of the siRNA. See e.g., U.S. Patent 8,106,022. Certain conjugate groups described herein are more active and/or easier to synthesize than conjugate groups previously described.

In certain embodiments of the present invention, conjugates are attached to single-stranded antisense compounds, including, but not limited to RNase H based antisense compounds and antisense compounds that alter splicing of a pre-mRNA target nucleic acid. In such embodiments, the conjugate should remain attached

to the antisense compound long enough to provide benefit (improved uptake into cells) but then should either be cleaved, or otherwise not interfere with the subsequent steps necessary for activity, such as hybridization to a target nucleic acid and interaction with RNase H or enzymes associated with splicing or splice modulation. This balance of properties is more important in the setting of single-stranded antisense compounds than in siRNA compounds, where the conjugate may simply be attached to the sense strand. Disclosed herein are conjugated single-stranded antisense compounds having improved potency in liver cells in vivo compared with the same antisense compound lacking the conjugate. Given the required balance of properties for these compounds such improved potency is surprising.

In certain embodiments, conjugate groups herein comprise a cleavable moiety. As noted, without wishing to be bound by mechanism, it is logical that the conjugate should remain on the compound long enough to provide enhancement in uptake, but after that, it is desirable for some portion or, ideally, all of the conjugate to be cleaved, releasing the parent compound (e.g., antisense compound) in its most active form. In certain embodiments, the cleavable moiety is a cleavable nucleoside. Such embodiments take advantage of endogenous nucleases in the cell by attaching the rest of the conjugate (the cluster) to the antisense oligonucleotide through a nucleoside via one or more cleavable bonds, such as those of a phosphodiester linkage. In certain embodiments, the cluster is bound to the cleavable nucleoside through a phosphodiester linkage. In certain embodiments, the cleavable nucleoside is attached to the antisense oligonucleotide (antisense compound) by a phosphodiester linkage. In certain embodiments, the conjugate group may comprise two or three cleavable nucleosides. In such embodiments, such cleavable nucleosides are linked to one another, to the antisense compound and/or to the cluster via cleavable bonds (such as those of a phosphodiester linkage). Certain conjugates herein do not comprise a cleavable nucleoside and instead comprise a cleavable bond. It is shown that that sufficient cleavage of the conjugate from the oligonucleotide is provided by at least one bond that is vulnerable to cleavage in the cell (a cleavable bond).

In certain embodiments, conjugated antisense compounds are prodrugs. Such prodrugs are administered to an animal and are ultimately metabolized to a more active form. For example, conjugated antisense compounds are cleaved to remove all or part of the conjugate resulting in the active (or more active) form of the antisense compound lacking all or some of the conjugate.

In certain embodiments, conjugates are attached at the 5' end of an oligonucleotide. Certain such 5'-conjugates are cleaved more efficiently than counterparts having a similar conjugate group attached at the 3' end. In certain embodiments, improved activity may correlate with improved cleavage. In certain embodiments, oligonucleotides comprising a conjugate at the 5' end have greater efficacy than oligonucleotides comprising a conjugate at the 3' end (see, for example, Examples 56, 81, 83, and 84). Further, 5'-attachment allows simpler oligonucleotide synthesis. Typically, oligonucleotides are synthesized on a solid support in the 3' to 5' direction. To make a 3'-conjugated oligonucleotide, typically one attaches a pre-conjugated 3' nucleoside to the solid support and then builds the oligonucleotide as usual. However, attaching that conjugated nucleoside to the solid support adds complication to the synthesis. Further, using

that approach, the conjugate is then present throughout the synthesis of the oligonucleotide and can become degraded during subsequent steps or may limit the sorts of reactions and reagents that can be used. Using the structures and techniques described herein for 5'-conjugated oligonucleotides, one can synthesize the oligonucleotide using standard automated techniques and introduce the conjugate with the final (5'-most) nucleoside or after the oligonucleotide has been cleaved from the solid support.

In view of the art and the present disclosure, one of ordinary skill can easily make any of the conjugates and conjugated oligonucleotides herein. Moreover, synthesis of certain such conjugates and conjugated oligonucleotides disclosed herein is easier and/or requires few steps, and is therefore less expensive than that of conjugates previously disclosed, providing advantages in manufacturing. For example, the synthesis of certain conjugate groups consists of fewer synthetic steps, resulting in increased yield, relative to conjugate groups previously described. Conjugate groups such as GalNAc3-10 in Example 46 and GalNAc3-7 in Example 48 are much simpler than previously described conjugates such as those described in U.S. 8,106,022 or U.S. 7,262,177 that require assembly of more chemical intermediates . Accordingly, these and other conjugates described herein have advantages over previously described compounds for use with any oligonucleotide, including single-stranded oligonucleotides and either strand of double-stranded oligonucleotides (e.g., siRNA).

Similarly, disclosed herein are conjugate groups having only one or two GalNAc ligands. As shown, such conjugates groups improve activity of antisense compounds. Such compounds are much easier to prepare than conjugates comprising three GalNAc ligands. Conjugate groups comprising one or two GalNAc ligands may be attached to any antisense compounds, including single-stranded oligonucleotides and either strand of double-stranded oligonucleotides (e.g., siRNA).

In certain embodiments, the conjugates herein do not substantially alter certain measures of tolerability. For example, it is shown herein that conjugated antisense compounds are not more immunogenic than unconjugated parent compounds. Since potency is improved, embodiments in which tolerability remains the same (or indeed even if tolerability worsens only slightly compared to the gains in potency) have improved properties for therapy.

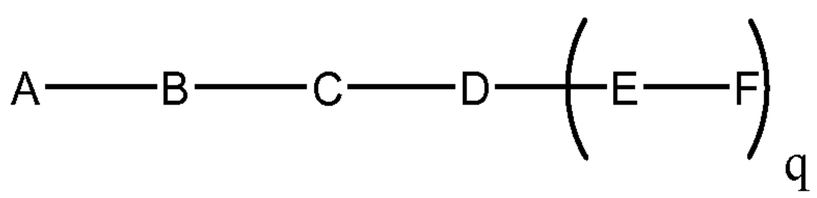
In certain embodiments, conjugation allows one to alter antisense compounds in ways that have less attractive consequences in the absence of conjugation. For example, in certain embodiments, replacing one or more phosphorothioate linkages of a fully phosphorothioate antisense compound with phosphodiester linkages results in improvement in some measures of tolerability. For example, in certain instances, such antisense compounds having one or more phosphodiester are less immunogenic than the same compound in which each linkage is a phosphorothioate. However, in certain instances, as shown in Example 26, that same replacement of one or more phosphorothioate linkages with phosphodiester linkages also results in reduced cellular uptake and/or loss in potency. In certain embodiments, conjugated antisense compounds described herein tolerate such change in linkages with little or no loss in uptake and potency when compared to the conjugated full-phosphorothioate counterpart. In fact, in certain embodiments, for example, in Examples 44,

57, 59, and 86, oligonucleotides comprising a conjugate and at least one phosphodiester internucleoside linkage actually exhibit increased potency in vivo even relative to a full phosphorothioate counterpart also comprising the same conjugate. Moreover, since conjugation results in substantial increases in uptake/potency a small loss in that substantial gain may be acceptable to achieve improved tolerability. Accordingly, in certain embodiments, conjugated antisense compounds comprise at least one phosphodiester linkage.

In certain embodiments, conjugation of antisense compounds herein results in increased delivery, uptake and activity in hepatocytes. Thus, more compound is delivered to liver tissue. However, in certain embodiments, that increased delivery alone does not explain the entire increase in activity. In certain such embodiments, more compound enters hepatocytes. In certain embodiments, even that increased hepatocyte uptake does not explain the entire increase in activity. In such embodiments, productive uptake of the conjugated compound is increased. For example, as shown in Example 102, certain embodiments of GalNAc-containing conjugates increase enrichment of antisense oligonucleotides in hepatocytes versus non-parenchymal cells. This enrichment is beneficial for oligonucleotides that target genes that are expressed in hepatocytes.

In certain embodiments, conjugated antisense compounds herein result in reduced kidney exposure. For example, as shown in Example 20, the concentrations of antisense oligonucleotides comprising certain embodiments of GalNAc-containing conjugates are lower in the kidney than that of antisense oligonucleotides lacking a GalNAc-containing conjugate. This has several beneficial therapeutic implications. For therapeutic indications where activity in the kidney is not sought, exposure to kidney risks kidney toxicity without corresponding benefit. Moreover, high concentration in kidney typically results in loss of compound to the urine resulting in faster clearance. Accordingly for non-kidney targets, kidney accumulation is undesired.

In certain embodiments, the present disclosure provides conjugated antisense compounds represented by the formula:



wherein

A is the antisense oligonucleotide;

B is the cleavable moiety

C is the conjugate linker

D is the branching group

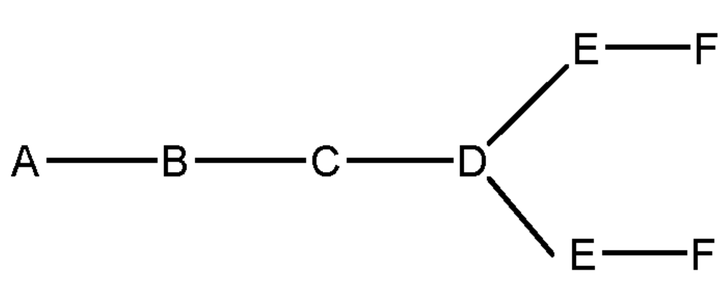
each E is a tether;

each F is a ligand; and

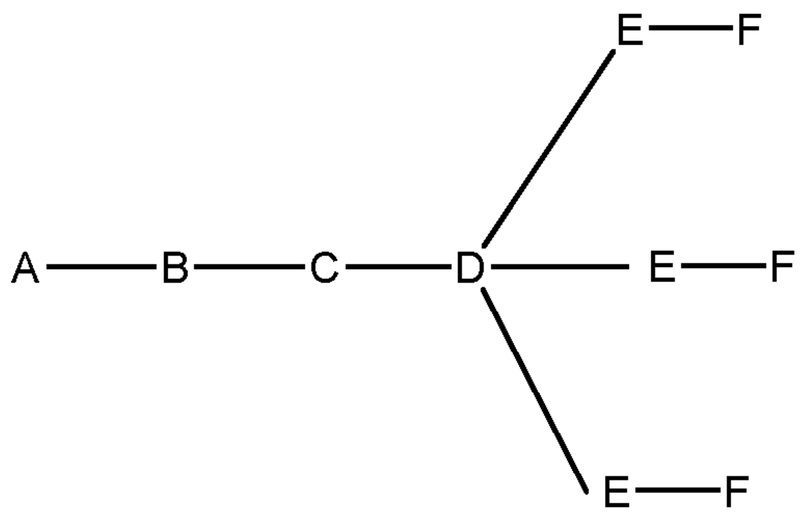
q is an integer between 1 and 5.

In the above diagram and in similar diagrams herein, the branching group "D" branches as many times as is necessary to accommodate the number of (E-F) groups as indicated by "q". Thus, where q = 1, the formula is:

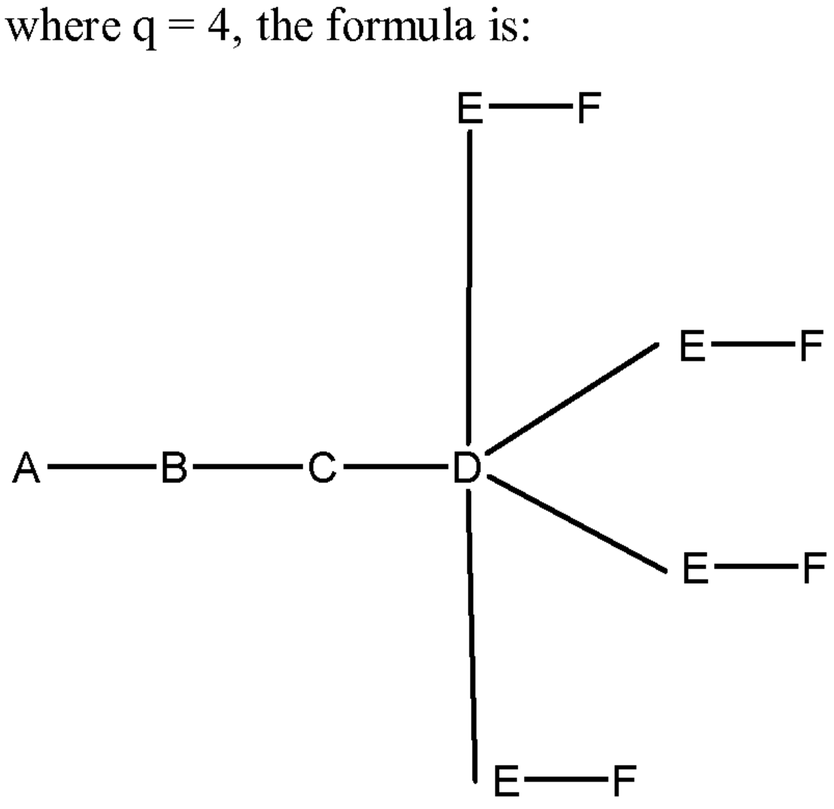
where q = 2, the formula is:



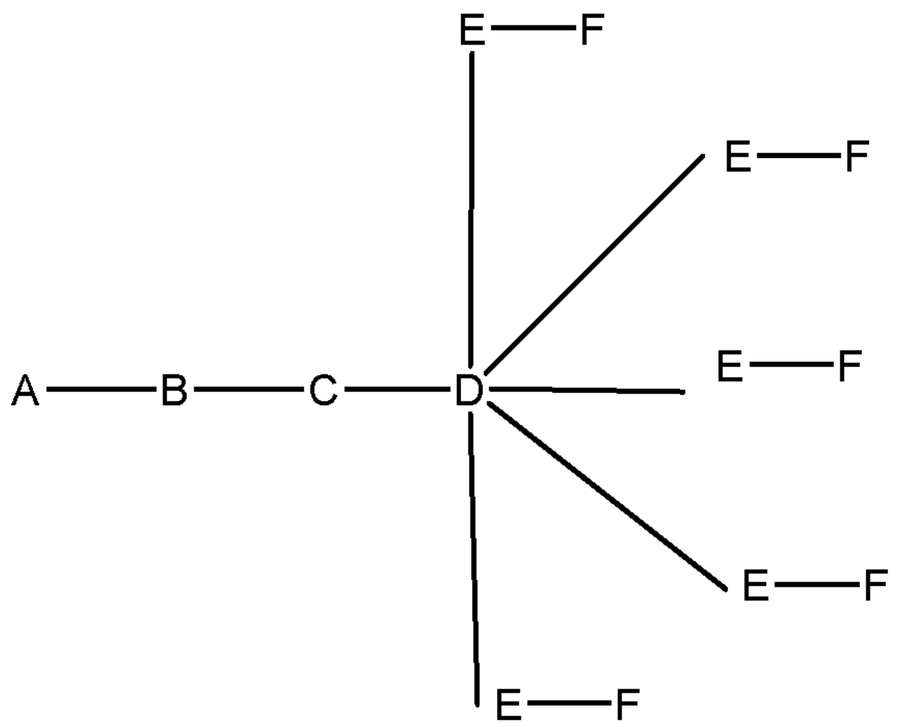
where q = 3, the formula is:



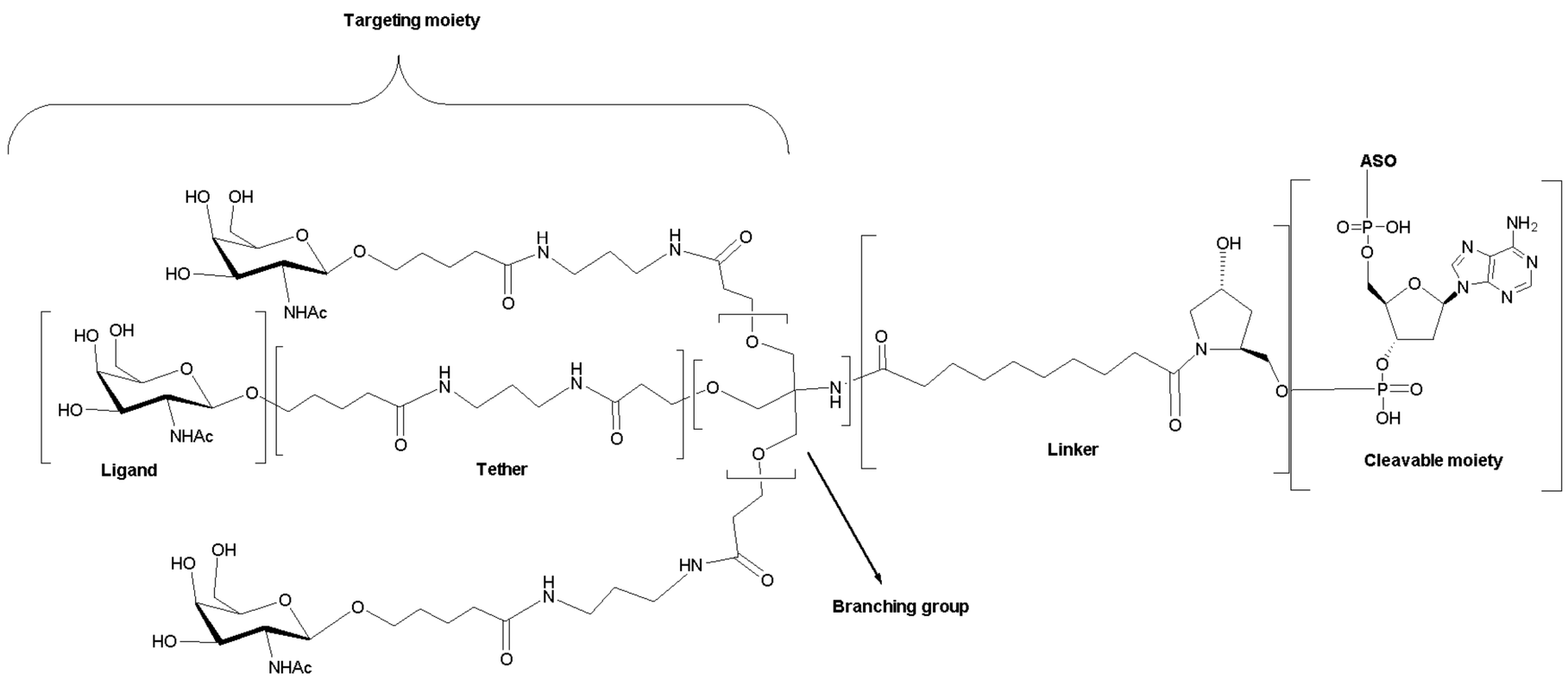
where q = 4, the formula is:



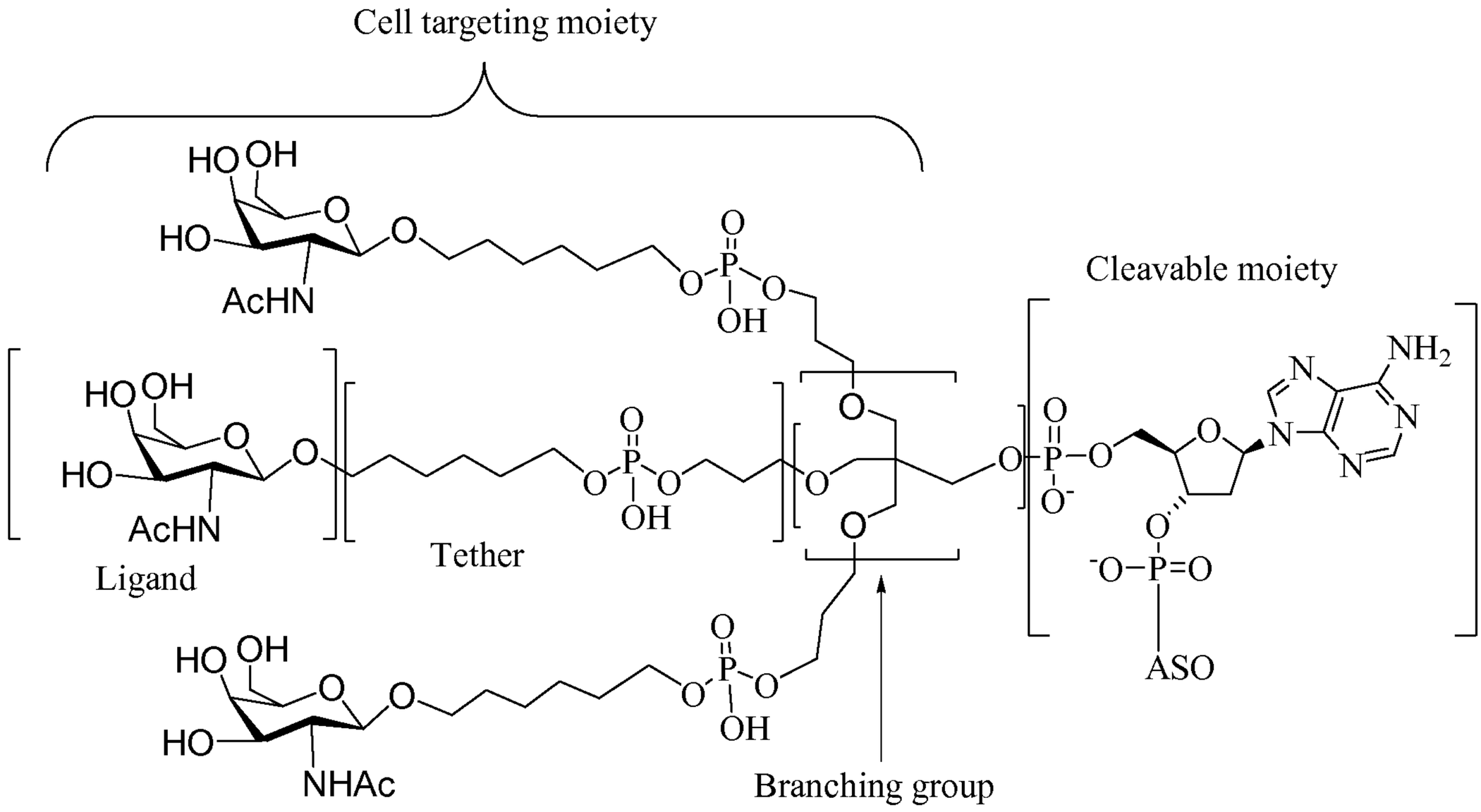
where q = 5, the formula is:



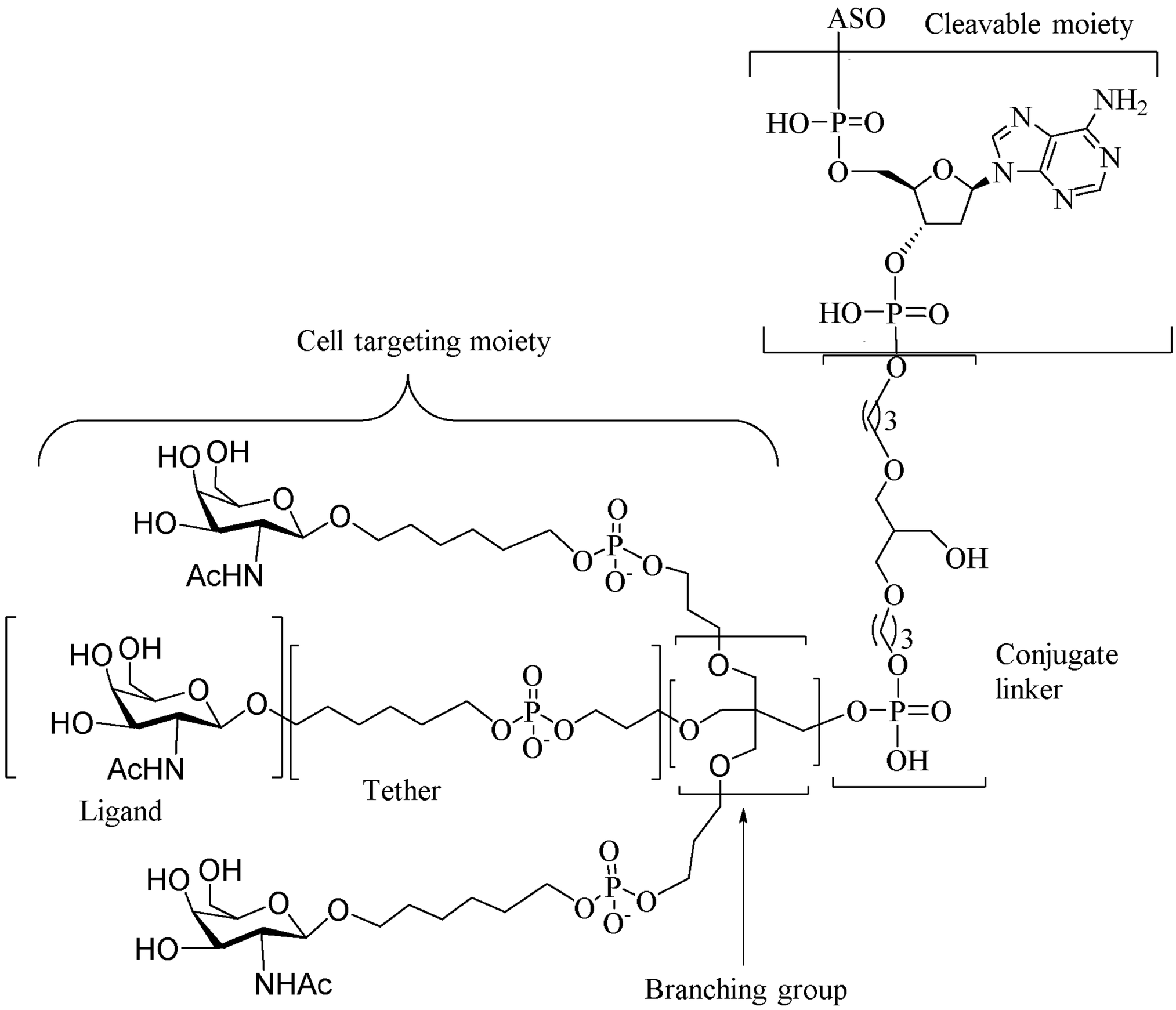
In certain embodiments, conjugated antisense compounds are provided having the structure:



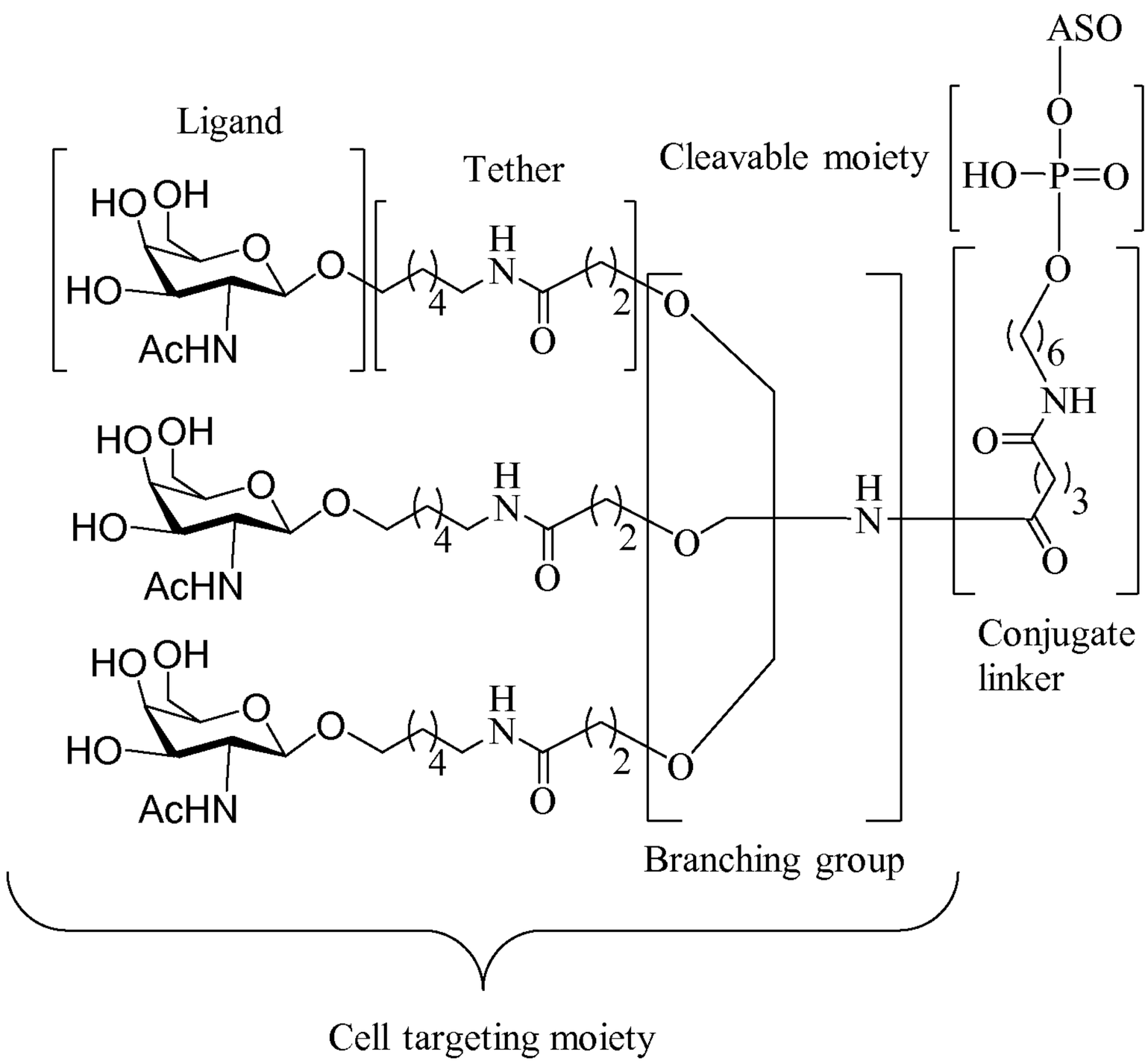
In certain embodiments, conjugated antisense compounds are provided having the structure:



In certain embodiments, conjugated antisense compounds are provided having the structure:

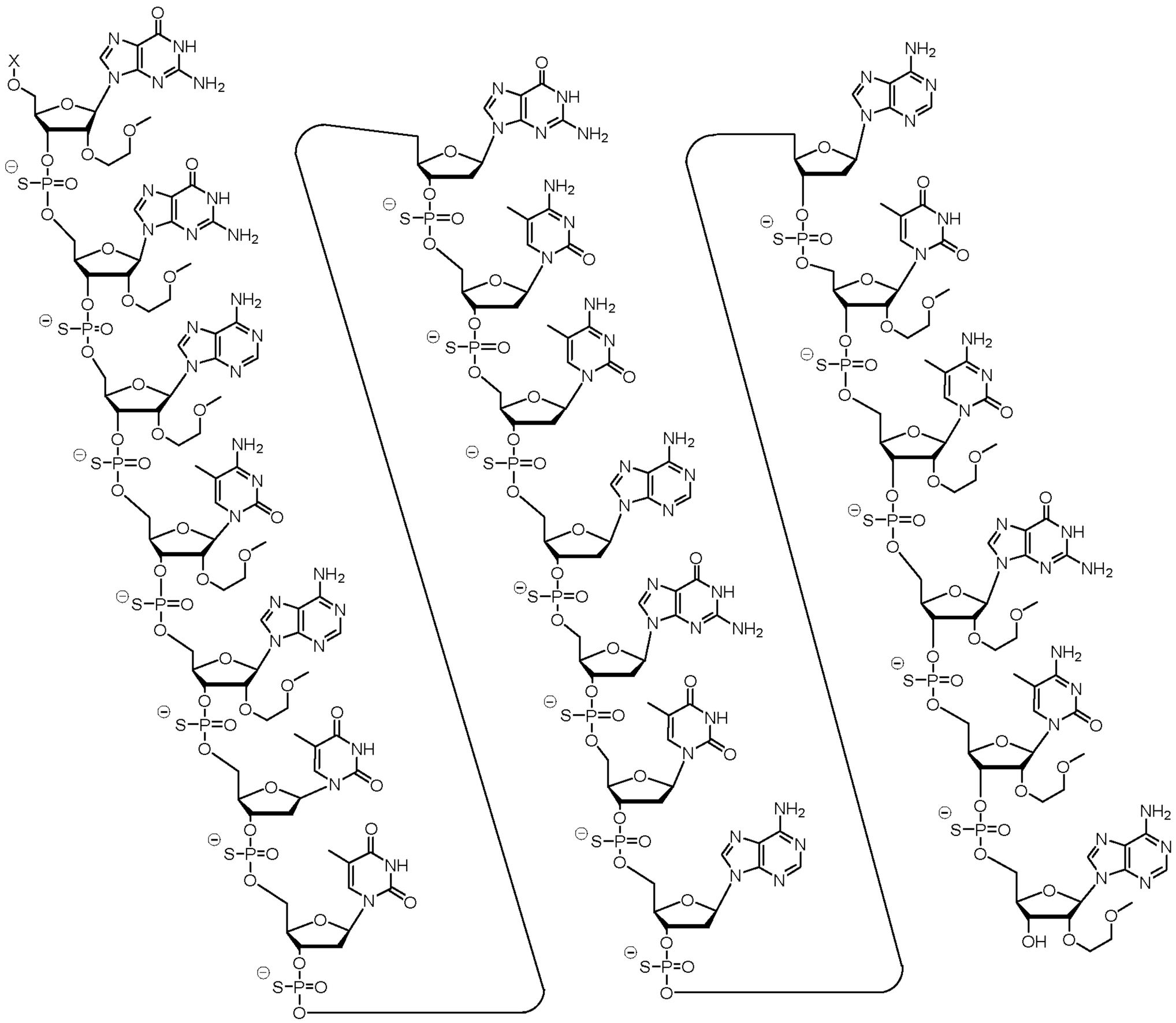


In certain embodiments, conjugated antisense compounds are provided having the structure:

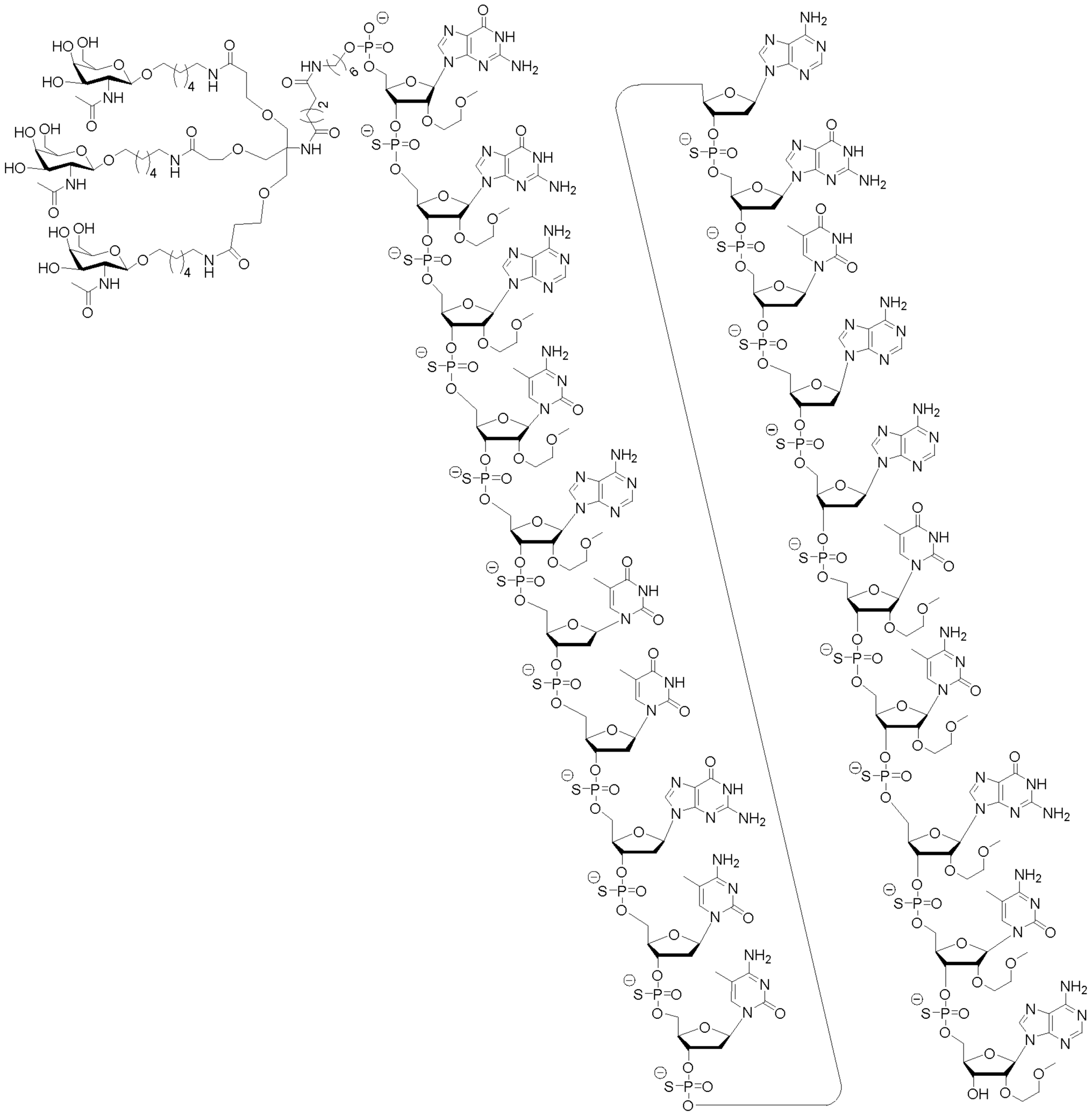


In embodiments having more than one of a particular variable (e.g., more than one "m" or "n"), unless otherwise indicated, each such particular variable is selected independently. Thus, for a structure having more than one n, each n is selected independently, so they may or may not be the same as one another.

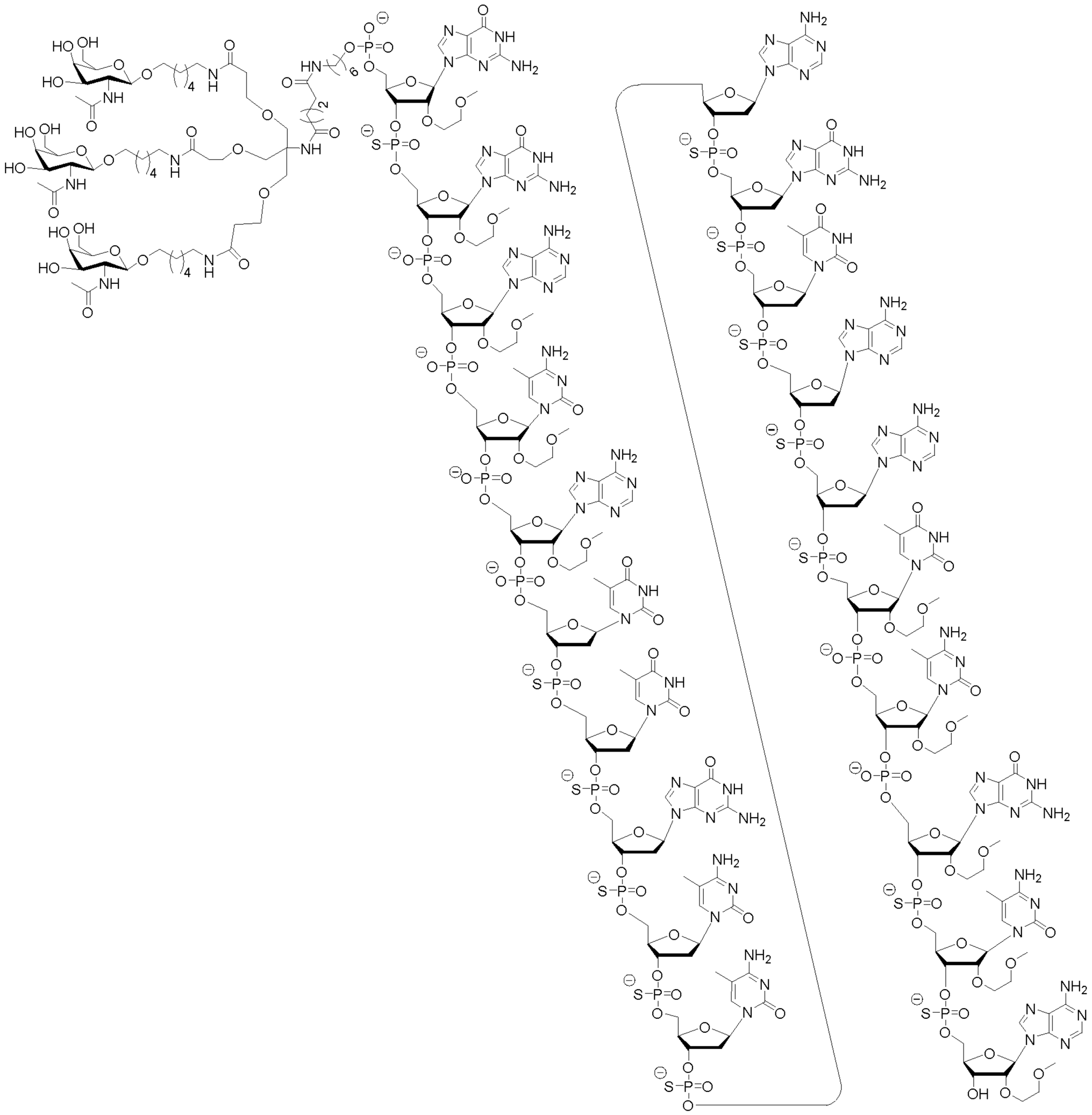
In certain embodiments, the present disclosure provides conjugated antisense compounds represented by the following structure. In certain embodiments, the antisense compound comprises the modified oligonucleotide ISIS 563580 with a 5'-X, wherein X is a conjugate group comprising GalNAc. In certain embodiments, the antisense compound consists of the modified oligonucleotide ISIS 563580 with a 5'-X, wherein X is a conjugate group comprising GalNAc.



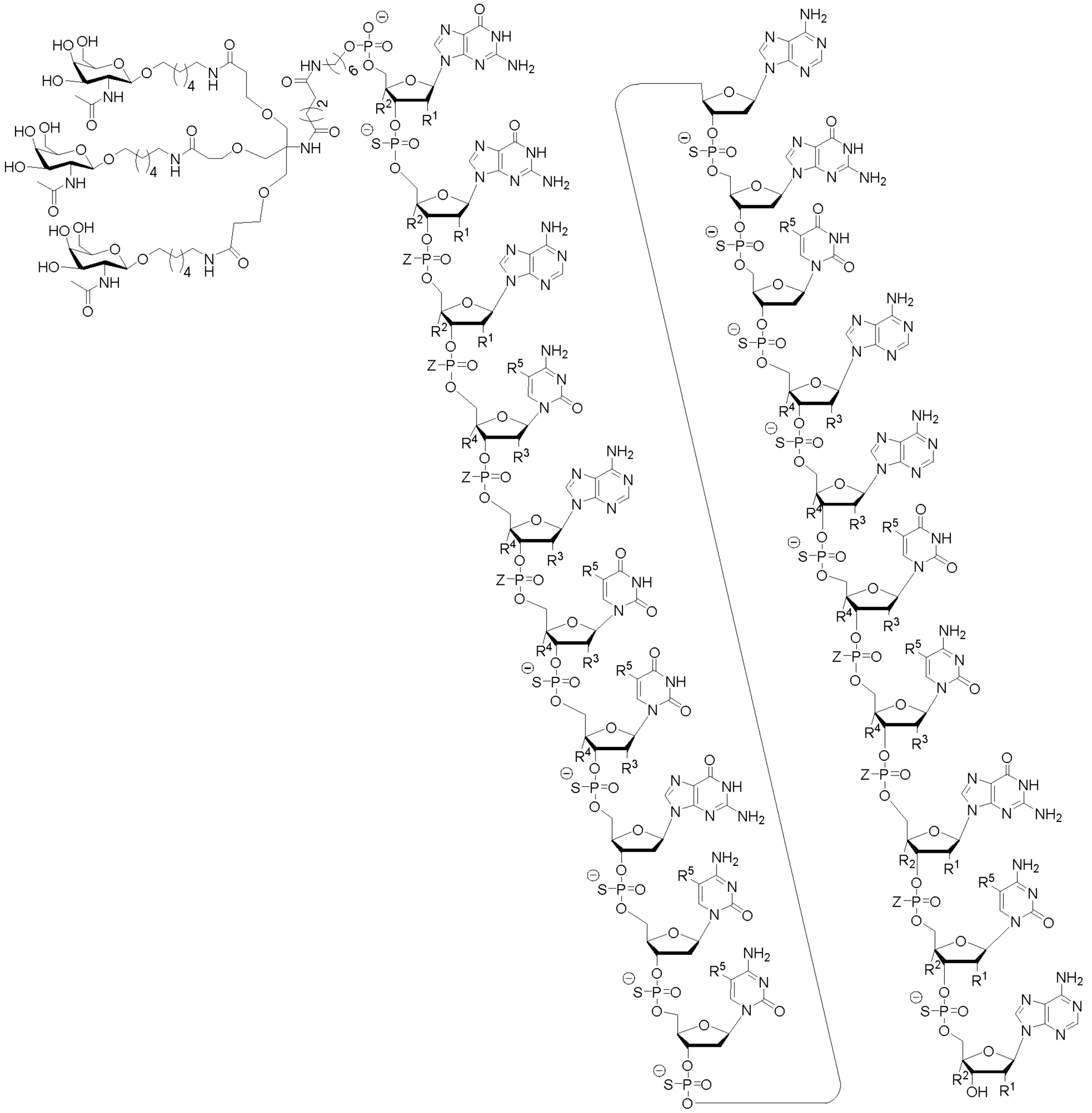
In certain embodiments, the present disclosure provides conjugated antisense compounds represented by the following structure. In certain embodiments, the antisense compound comprises the conjugated modified oligonucleotide ISIS 703801. In certain embodiments, the antisense compound consists of the conjugated modified oligonucleotide ISIS 703801.



In certain embodiments, the present disclosure provides conjugated antisense compounds represented by the following structure. In certain embodiments, the antisense compound comprises the conjugated modified oligonucleotide ISIS 703802. In certain embodiments, the antisense compound consists of the conjugated modified oligonucleotide ISIS 703802.



In certain embodiments, the present disclosure provides conjugated antisense compounds represented by the following structure. In certain embodiments, the antisense compound comprises a modified oligonucleotide with the nucleobase sequence of SEQ ID NO: 77 with a 5'-GalNAc with variability in the sugar mods of the wings. In certain embodiments, the antisense compound consists of a modified oligonucleotide with the nucleobase sequence of SEQ ID NO: 77 with a 5'-GalNAc with variability in the sugar mods of the wings.



wherein either R^1 is $-OCH_2CH_2OCH_3$ (MOE)and R^2 is H; or R^1 and R^2 together form a bridge, wherein R^1 is –O- and R^2 is $-CH_2$-, -$CH(CH_3)$-, or -CH_2CH_2-, and R^1 and R^2 are directly connected such that the resulting bridge is selected from: -O-CH_2-, -O-$CH(CH_3)$-, and –O-CH_2CH_2-;

and for each pair of R^3 and R^4 on the same ring, independently for each ring: either R^3 is selected from H and -$OCH_2CH_2OCH_3$ and R^4 is H; or R^3 and R^4 together form a bridge, wherein R^3 is –O-, and R^4 is –CH_2-, -$CH(CH_3)$-, or -CH_2CH_2-and R^3 and R^4 are directly connected such that the resulting bridge is selected from: -O-CH_2-, -O-$CH(CH_3)$-, and –O-CH_2CH_2-;

and R^5 is selected from H and –CH_3;

and Z is selected from S^- and O^-.

Certain embodiments provide a composition comprising a conjugated antisense compound described herein, or a salt thereof, and a pharmaceutically acceptable carrier or diluent.

In certain embodiments, the modulation of ANGPTL3 expression occurs in a cell or tissue. In certain embodiments, the modulations occur in a cell or tissue in an animal. In certain embodiments, the animal is a human. In certain embodiments, the modulation is a reduction in ANGPTL3 mRNA level. In certain embodiments, the modulation is a reduction in ANGPTL3 protein level. In certain embodiments, both ANGPTL3 mRNA and protein levels are reduced. Such reduction may occur in a time-dependent or in a dose-dependent manner.

Certain embodiments provide compositions and methods for use in therapy. Certain embodiments provide compositions and methods for preventing, treating, delaying, slowing the progression and/or ameliorating ANGPTL3 related diseases, disorders, and conditions. In certain embodiments, such diseases, disorders, and conditions are cardiovascular and/or metabolic diseases, disorders, and conditions. In certain embodiments, the compositions and methods for therapy include administering an ANGPTL3 specific inhibitor to an individual in need thereof. In certain embodiments, the ANGPTL3 specific inhibitor is a nucleic acid. In certain embodiments, the nucleic acid is an antisense compound. In certain embodiments, the antisense compound is a modified oligonucleotide. In certain embodiments, the antisense compound is a modified oligonucleotide with a conjugate group attached.

Detailed Description of the Invention

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. Herein, the use of the singular includes the plural unless specifically stated otherwise. As used herein, the use of "or" means "and/or" unless stated otherwise. Furthermore, the use of the term "including" as well as other forms, such as "includes" and "included", is not limiting. Also, terms such as "element" or "component" encompass both elements and components comprising one unit and elements and components that comprise more than one subunit, unless specifically stated otherwise.

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including, but not limited to, patents, patent applications, articles, books, and treatises, are hereby expressly incorporated-by-reference for the portions of the document discussed herein, as well as in their entirety.

Definitions

Unless specific definitions are provided, the nomenclature utilized in connection with, and the procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques can be used for chemical synthesis, and chemical analysis. Certain such techniques and procedures may be found for example in "Carbohydrate Modifications in Antisense Research" Edited by Sangvi and Cook, American Chemical Society , Washington D.C., 1994; "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., 21st edition, 2005; and "Antisense Drug Technology, Principles, Strategies, and Applications" Edited by Stanley T. Crooke, CRC Press, Boca Raton, Florida; and Sambrook et al., "Molecular Cloning, A laboratory Manual," 2nd Edition, Cold Spring Harbor Laboratory Press, 1989, which are hereby incorporated by reference for any purpose. Where permitted, all patents, applications, published applications and other publications, GENBANK Accession Numbers and associated sequence information obtainable through databases such as National Center for Biotechnology Information (NCBI) and other data referred to throughout in the disclosure herein are incorporated by reference for the portions of the document discussed herein, as well as in their entirety.

Unless otherwise indicated, the following terms have the following meanings:

As used herein, "nucleoside" means a compound comprising a nucleobase moiety and a sugar moiety. Nucleosides include, but are not limited to, naturally occurring nucleosides (as found in DNA and RNA) and modified nucleosides. Nucleosides may be linked to a phosphate moiety.

As used herein, "chemical modification" means a chemical difference in a compound when compared to a naturally occurring counterpart. Chemical modifications of oligonucleotides include nucleoside modifications (including sugar moiety modifications and nucleobase modifications) and internucleoside linkage modifications. In reference to an oligonucleotide, chemical modification does not include differences only in nucleobase sequence.

As used herein, "furanosyl" means a structure comprising a 5-membered ring comprising four carbon atoms and one oxygen atom.

As used herein, "naturally occurring sugar moiety" means a ribofuranosyl as found in naturally occurring RNA or a deoxyribofuranosyl as found in naturally occurring DNA.

As used herein, "sugar moiety" means a naturally occurring sugar moiety or a modified sugar moiety of a nucleoside.

As used herein, "modified sugar moiety" means a substituted sugar moiety or a sugar surrogate.

As used herein, "substituted sugar moiety" means a furanosyl that is not a naturally occurring sugar moiety. Substituted sugar moieties include, but are not limited to furanosyls comprising substituents at the 2'-position, the 3'-position, the 5'-position and/or the 4'-position. Certain substituted sugar moieties are bicyclic sugar moieties.

As used herein, "2'-substituted sugar moiety" means a furanosyl comprising a substituent at the 2'-

position other than H or OH. Unless otherwise indicated, a 2'-substituted sugar moiety is not a bicyclic sugar moiety (i.e., the 2'-substituent of a 2'-substituted sugar moiety does not form a bridge to another atom of the furanosyl ring.

As used herein, "MOE" means $-OCH_2CH_2OCH_3$.

As used herein, "2'-F nucleoside" refers to a nucleoside comprising a sugar comprising fluorine at the 2' position. Unless otherwise indicated, the fluorine in a 2'-F nucleoside is in the ribo position (replacing the OH of a natural ribose).

As used herein the term "sugar surrogate" means a structure that does not comprise a furanosyl and that is capable of replacing the naturally occurring sugar moiety of a nucleoside, such that the resulting nucleoside sub-units are capable of linking together and/or linking to other nucleosides to form an oligomeric compound which is capable of hybridizing to a complementary oligomeric compound. Such structures include rings comprising a different number of atoms than furanosyl (e.g., 4, 6, or 7-membered rings); replacement of the oxygen of a furanosyl with a non-oxygen atom (e.g., carbon, sulfur, or nitrogen); or both a change in the number of atoms and a replacement of the oxygen. Such structures may also comprise substitutions corresponding to those described for substituted sugar moieties (e.g., 6-membered carbocyclic bicyclic sugar surrogates optionally comprising additional substituents). Sugar surrogates also include more complex sugar replacements (e.g., the non-ring systems of peptide nucleic acid). Sugar surrogates include without limitation morpholinos, cyclohexenyls and cyclohexitols.

As used herein, "bicyclic sugar moiety" means a modified sugar moiety comprising a 4 to 7 membered ring (including but not limited to a furanosyl) comprising a bridge connecting two atoms of the 4 to 7 membered ring to form a second ring, resulting in a bicyclic structure. In certain embodiments, the 4 to 7 membered ring is a sugar ring. In certain embodiments the 4 to 7 membered ring is a furanosyl. In certain such embodiments, the bridge connects the 2'-carbon and the 4'-carbon of the furanosyl.

As used herein, "nucleotide" means a nucleoside further comprising a phosphate linking group. As used herein, "linked nucleosides" may or may not be linked by phosphate linkages and thus includes, but is not limited to "linked nucleotides." As used herein, "linked nucleosides" are nucleosides that are connected in a continuous sequence (i.e. no additional nucleosides are present between those that are linked).

As used herein, "nucleobase" means a group of atoms that can be linked to a sugar moiety to create a nucleoside that is capable of incorporation into an oligonucleotide, and wherein the group of atoms is capable of bonding with a complementary naturally occurring nucleobase of another oligonucleotide or nucleic acid. Nucleobases may be naturally occurring or may be modified. "Nucleobase sequence" means the order of contiguous nucleobases independent of any sugar, linkage, or nucleobase modification.

As used herein the terms, "unmodified nucleobase" or "naturally occurring nucleobase" means the naturally occurring heterocyclic nucleobases of RNA or DNA: the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) (including 5-methyl C), and uracil (U).

As used herein, "modified nucleobase" means any nucleobase that is not a naturally occurring

nucleobase.

As used herein, “modified nucleoside” means a nucleoside comprising at least one chemical modification compared to naturally occurring RNA or DNA nucleosides. Modified nucleosides comprise a modified sugar moiety and/or a modified nucleobase.

As used herein, “bicyclic nucleoside” or “BNA” means a nucleoside comprising a bicyclic sugar moiety.

As used herein, “constrained ethyl nucleoside” or “cEt” means a nucleoside comprising a bicyclic sugar moiety comprising a 4’-$CH(CH_3)$-O-2’bridge.

As used herein, “locked nucleic acid nucleoside” or “LNA” means a nucleoside comprising a bicyclic sugar moiety comprising a 4’-CH_2-O-2’bridge.

As used herein, “2’-substituted nucleoside” means a nucleoside comprising a substituent at the 2’-position other than H or OH. Unless otherwise indicated, a 2’-substituted nucleoside is not a bicyclic nucleoside.

As used herein, “deoxynucleoside” means a nucleoside comprising 2’-H furanosyl sugar moiety, as found in naturally occurring deoxyribonucleosides (DNA). In certain embodiments, a 2’-deoxynucleoside may comprise a modified nucleobase or may comprise an RNA nucleobase (e.g., uracil).

As used herein, "oligonucleotide" means a compound comprising a plurality of linked nucleosides. In certain embodiments, an oligonucleotide comprises one or more unmodified ribonucleosides (RNA) and/or unmodified deoxyribonucleosides (DNA) and/or one or more modified nucleosides.

As used herein “oligonucleoside” means an oligonucleotide in which none of the internucleoside linkages contains a phosphorus atom. As used herein, oligonucleotides include oligonucleosides.

As used herein, “modified oligonucleotide” means an oligonucleotide comprising at least one modified nucleoside and/or at least one modified internucleoside linkage.

As used herein, “linkage” or “linking group” means a group of atoms that link together two or more other groups of atoms.

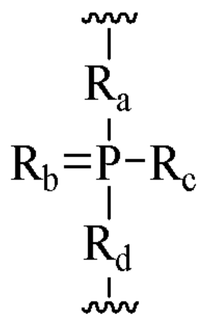
As used herein “internucleoside linkage” means a covalent linkage between adjacent nucleosides in an oligonucleotide.

As used herein “naturally occurring internucleoside linkage” means a 3' to 5' phosphodiester linkage.

As used herein, “modified internucleoside linkage” means any internucleoside linkage other than a naturally occurring internucleoside linkage.

As used herein, “terminal internucleoside linkage” means the linkage between the last two nucleosides of an oligonucleotide or defined region thereof.

As used herein, “phosphorus linking group” means a linking group comprising a phosphorus atom. Phosphorus linking groups include without limitation groups having the formula:



wherein:

R_a and R_d are each, independently, O, S, CH_2, NH, or NJ_1 wherein J_1 is C_1-C_6 alkyl or substituted C_1-C_6 alkyl;

R_b is O or S;

R_c is OH, SH, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, amino or substituted amino; and

J_1 is R_b is O or S.

Phosphorus linking groups include without limitation, phosphodiester, phosphorothioate, phosphorodithioate, phosphonate, phosphoramidate, phosphorothioamidate, thionoalkylphosphonate, phosphotriesters, thionoalkylphosphotriester and boranophosphate.

As used herein, “internucleoside phosphorus linking group” means a phosphorus linking group that directly links two nucleosides.

As used herein, “non-internucleoside phosphorus linking group” means a phosphorus linking group that does not directly link two nucleosides. In certain embodiments, a non-internucleoside phosphorus linking group links a nucleoside to a group other than a nucleoside. In certain embodiments, a non-internucleoside phosphorus linking group links two groups, neither of which is a nucleoside.

As used herein, "neutral linking group" means a linking group that is not charged. Neutral linking groups include without limitation phosphotriesters, methylphosphonates, MMI (-CH_2-N(CH_3)-O-), amide-3 (-CH_2-C(=O)-N(H)-), amide-4 (-CH_2-N(H)-C(=O)-), formacetal (-O-CH_2-O-), and thioformacetal (-S-CH_2-O-). Further neutral linking groups include nonionic linkages comprising siloxane (dialkylsiloxane), carboxylate ester, carboxamide, sulfide, sulfonate ester and amides (See for example: Carbohydrate Modifications in Antisense Research; Y.S. Sanghvi and P.D. Cook Eds. ACS Symposium Series 580; Chapters 3 and 4, (pp. 40-65)). Further neutral linking groups include nonionic linkages comprising mixed N, O, S and CH_2 component parts.

As used herein, “internucleoside neutral linking group” means a neutral linking group that directly links two nucleosides.

As used herein, “non-internucleoside neutral linking group” means a neutral linking group that does not directly link two nucleosides. In certain embodiments, a non-internucleoside neutral linking group links a nucleoside to a group other than a nucleoside. In certain embodiments, a non-internucleoside neutral linking group links two groups, neither of which is a nucleoside.

As used herein, "oligomeric compound" means a polymeric structure comprising two or more sub-

structures. In certain embodiments, an oligomeric compound comprises an oligonucleotide. In certain embodiments, an oligomeric compound comprises one or more conjugate groups and/or terminal groups. In certain embodiments, an oligomeric compound consists of an oligonucleotide. Oligomeric compounds also include naturally occurring nucleic acids. In certain embodiments, an oligomeric compound comprises a backbone of one or more linked monomeric subunits where each linked monomeric subunit is directly or indirectly attached to a heterocyclic base moiety. In certain embodiments, oligomeric compounds may also include monomeric subunits that are not linked to a heterocyclic base moiety, thereby providing abasic sites. In certain embodiments, the linkages joining the monomeric subunits, the sugar moieties or surrogates and the heterocyclic base moieties can be independently modified. In certain embodiments, the linkage-sugar unit, which may or may not include a heterocyclic base, may be substituted with a mimetic such as the monomers in peptide nucleic acids.

As used herein, "terminal group" means one or more atom attached to either, or both, the 3' end or the 5' end of an oligonucleotide. In certain embodiments a terminal group is a conjugate group. In certain embodiments, a terminal group comprises one or more terminal group nucleosides.

As used herein, "conjugate" or "conjugate group" means an atom or group of atoms bound to an oligonucleotide or oligomeric compound. In general, conjugate groups modify one or more properties of the compound to which they are attached, including, but not limited to pharmacodynamic, pharmacokinetic, binding, absorption, cellular distribution, cellular uptake, charge and/or clearance properties.

As used herein, "conjugate linker" or "linker" in the context of a conjugate group means a portion of a conjugate group comprising any atom or group of atoms and which covalently link (1) an oligonucleotide to another portion of the conjugate group or (2) two or more portions of the conjugate group.

Conjugate groups are shown herein as radicals, providing a bond for forming covalent attachment to an oligomeric compound such as an antisense oligonucleotide. In certain embodiments, the point of attachment on the oligomeric compound is the 3'-oxygen atom of the 3'-hydroxyl group of the 3' terminal nucleoside of the oligomeric compound. In certain embodiments the point of attachment on the oligomeric compound is the 5'-oxygen atom of the 5'-hydroxyl group of the 5' terminal nucleoside of the oligomeric compound. In certain embodiments, the bond for forming attachment to the oligomeric compound is a cleavable bond. In certain such embodiments, such cleavable bond constitutes all or part of a cleavable moiety.

In certain embodiments, conjugate groups comprise a cleavable moiety (e.g., a cleavable bond or cleavable nucleoside) and a carbohydrate cluster portion, such as a GalNAc cluster portion. Such carbohydrate cluster portion comprises: a targeting moiety and, optionally, a conjugate linker. In certain embodiments, the carbohydrate cluster portion is identified by the number and identity of the ligand. For example, in certain embodiments, the carbohydrate cluster portion comprises 3 GalNAc groups and is designated "$GalNAc_3$". In certain embodiments, the carbohydrate cluster portion comprises 4 GalNAc groups and is designated "$GalNAc_4$". Specific carbohydrate cluster portions (having specific tether, branching

and conjugate linker groups) are described herein and designated by Roman numeral followed by subscript "a". Accordingly "GalNac3-1_a" refers to a specific carbohydrate cluster portion of a conjugate group having 3 GalNac groups and specifically identified tether, branching and linking groups. Such carbohydrate cluster fragment is attached to an oligomeric compound via a cleavable moiety, such as a cleavable bond or cleavable nucleoside.

As used herein, "cleavable moiety" means a bond or group that is capable of being split under physiological conditions. In certain embodiments, a cleavable moiety is cleaved inside a cell or sub-cellular compartments, such as a lysosome. In certain embodiments, a cleavable moiety is cleaved by endogenous enzymes, such as nucleases. In certain embodiments, a cleavable moiety comprises a group of atoms having one, two, three, four, or more than four cleavable bonds.

As used herein, "cleavable bond" means any chemical bond capable of being split. In certain embodiments, a cleavable bond is selected from among: an amide, a polyamide, an ester, an ether, one or both esters of a phosphodiester, a phosphate ester, a carbamate, a di-sulfide, or a peptide.

As used herein, "carbohydrate cluster" means a compound having one or more carbohydrate residues attached to a scaffold or linker group. (*see*, e.g., Maier et al., "Synthesis of Antisense Oligonucleotides Conjugated to a Multivalent Carbohydrate Cluster for Cellular Targeting," *Bioconjugate Chemistry*, 2003, (14): 18-29, which is incorporated herein by reference in its entirety, or Rensen et al., "Design and Synthesis of Novel *N*-Acetylgalactosamine-Terminated Glycolipids for Targeting of Lipoproteins to the Hepatic Asiaglycoprotein Receptor," *J. Med. Chem.* 2004, (47): 5798-5808, for examples of carbohydrate conjugate clusters).

As used herein, "modified carbohydrate" means any carbohydrate having one or more chemical modifications relative to naturally occurring carbohydrates.

As used herein, "carbohydrate derivative" means any compound which may be synthesized using a carbohydrate as a starting material or intermediate.

As used herein, "carbohydrate" means a naturally occurring carbohydrate, a modified carbohydrate, or a carbohydrate derivative.

As used herein "protecting group" means any compound or protecting group known to those having skill in the art. Non-limiting examples of protecting groups may be found in "Protective Groups in Organic Chemistry", T. W. Greene, P. G. M. Wuts, ISBN 0-471-62301-6, John Wiley & Sons, Inc, New York, which is incorporated herein by reference in its entirety.

As used herein, "single-stranded" means an oligomeric compound that is not hybridized to its complement and which lacks sufficient self-complementarity to form a stable self-duplex.

As used herein, "double stranded" means a pair of oligomeric compounds that are hybridized to one another or a single self-complementary oligomeric compound that forms a hairpin structure. In certain embodiments, a double-stranded oligomeric compound comprises a first and a second oligomeric compound.

As used herein, "antisense compound" means a compound comprising or consisting of an

oligonucleotide at least a portion of which is complementary to a target nucleic acid to which it is capable of hybridizing, resulting in at least one antisense activity.

As used herein, "antisense activity" means any detectable and/or measurable change attributable to the hybridization of an antisense compound to its target nucleic acid. In certain embodiments, antisense activity includes modulation of the amount or activity of a target nucleic acid transcript (e.g. mRNA). In certain embodiments, antisense activity includes modulation of the splicing of pre-mRNA.

As used herein, "RNase H based antisense compound" means an antisense compound wherein at least some of the antisense activity of the antisense compound is attributable to hybridization of the antisense compound to a target nucleic acid and subsequent cleavage of the target nucleic acid by RNase H.

As used herein, "RISC based antisense compound" means an antisense compound wherein at least some of the antisense activity of the antisense compound is attributable to the RNA Induced Silencing Complex (RISC).

As used herein, "detecting" or "measuring" means that a test or assay for detecting or measuring is performed. Such detection and/or measuring may result in a value of zero. Thus, if a test for detection or measuring results in a finding of no activity (activity of zero), the step of detecting or measuring the activity has nevertheless been performed.

As used herein, "detectable and/or measureable activity" means a statistically significant activity that is not zero.

As used herein, "essentially unchanged" means little or no change in a particular parameter, particularly relative to another parameter which changes much more. In certain embodiments, a parameter is essentially unchanged when it changes less than 5%. In certain embodiments, a parameter is essentially unchanged if it changes less than two-fold while another parameter changes at least ten-fold. For example, in certain embodiments, an antisense activity is a change in the amount of a target nucleic acid. In certain such embodiments, the amount of a non-target nucleic acid is essentially unchanged if it changes much less than the target nucleic acid does, but the change need not be zero.

As used herein, "expression" means the process by which a gene ultimately results in a protein. Expression includes, but is not limited to, transcription, post-transcriptional modification (e.g., splicing, polyadenlyation, addition of 5'-cap), and translation.

As used herein, "target nucleic acid" means a nucleic acid molecule to which an antisense compound is intended to hybridize to result in a desired antisense activity. Antisense oligonucleotides have sufficient complementarity to their target nucleic acids to allow hybridization under physiological conditions.

As used herein, "nucleobase complementarity" or "complementarity" when in reference to nucleobases means a nucleobase that is capable of base pairing with another nucleobase. For example, in DNA, adenine (A) is complementary to thymine (T). For example, in RNA, adenine (A) is complementary to uracil (U). In certain embodiments, complementary nucleobase means a nucleobase of an antisense compound that is capable of base pairing with a nucleobase of its target nucleic acid. For example, if a

nucleobase at a certain position of an antisense compound is capable of hydrogen bonding with a nucleobase at a certain position of a target nucleic acid, then the position of hydrogen bonding between the oligonucleotide and the target nucleic acid is considered to be complementary at that nucleobase pair. Nucleobases comprising certain modifications may maintain the ability to pair with a counterpart nucleobase and thus, are still capable of nucleobase complementarity.

As used herein, "non-complementary" in reference to nucleobases means a pair of nucleobases that do not form hydrogen bonds with one another.

As used herein, "complementary" in reference to oligomeric compounds (e.g., linked nucleosides, oligonucleotides, or nucleic acids) means the capacity of such oligomeric compounds or regions thereof to hybridize to another oligomeric compound or region thereof through nucleobase complementarity. Complementary oligomeric compounds need not have nucleobase complementarity at each nucleoside. Rather, some mismatches are tolerated. In certain embodiments, complementary oligomeric compounds or regions are complementary at 70% of the nucleobases (70% complementary). In certain embodiments, complementary oligomeric compounds or regions are 80% complementary. In certain embodiments, complementary oligomeric compounds or regions are 90% complementary. In certain embodiments, complementary oligomeric compounds or regions are 95% complementary. In certain embodiments, complementary oligomeric compounds or regions are 100% complementary.

As used herein, "mismatch" means a nucleobase of a first oligomeric compound that is not capable of pairing with a nucleobase at a corresponding position of a second oligomeric compound, when the first and second oligomeric compound are aligned. Either or both of the first and second oligomeric compounds may be oligonucleotides.

As used herein, "hybridization" means the pairing of complementary oligomeric compounds (e.g., an antisense compound and its target nucleic acid). While not limited to a particular mechanism, the most common mechanism of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleobases.

As used herein, "specifically hybridizes" means the ability of an oligomeric compound to hybridize to one nucleic acid site with greater affinity than it hybridizes to another nucleic acid site.

As used herein, "fully complementary" in reference to an oligonucleotide or portion thereof means that each nucleobase of the oligonucleotide or portion thereof is capable of pairing with a nucleobase of a complementary nucleic acid or contiguous portion thereof. Thus, a fully complementary region comprises no mismatches or unhybridized nucleobases in either strand.

As used herein, "percent complementarity" means the percentage of nucleobases of an oligomeric compound that are complementary to an equal-length portion of a target nucleic acid. Percent complementarity is calculated by dividing the number of nucleobases of the oligomeric compound that are complementary to nucleobases at corresponding positions in the target nucleic acid by the total length of the oligomeric compound.

As used herein, “percent identity” means the number of nucleobases in a first nucleic acid that are the same type (independent of chemical modification) as nucleobases at corresponding positions in a second nucleic acid, divided by the total number of nucleobases in the first nucleic acid.

As used herein, "modulation" means a change of amount or quality of a molecule, function, or activity when compared to the amount or quality of a molecule, function, or activity prior to modulation. For example, modulation includes the change, either an increase (stimulation or induction) or a decrease (inhibition or reduction) in gene expression. As a further example, modulation of expression can include a change in splice site selection of pre-mRNA processing, resulting in a change in the absolute or relative amount of a particular splice-variant compared to the amount in the absence of modulation.

As used herein, “chemical motif” means a pattern of chemical modifications in an oligonucleotide or a region thereof. Motifs may be defined by modifications at certain nucleosides and/or at certain linking groups of an oligonucleotide.

As used herein, “nucleoside motif” means a pattern of nucleoside modifications in an oligonucleotide or a region thereof. The linkages of such an oligonucleotide may be modified or unmodified. Unless otherwise indicated, motifs herein describing only nucleosides are intended to be nucleoside motifs. Thus, in such instances, the linkages are not limited.

As used herein, “sugar motif” means a pattern of sugar modifications in an oligonucleotide or a region thereof.

As used herein, “linkage motif” means a pattern of linkage modifications in an oligonucleotide or region thereof. The nucleosides of such an oligonucleotide may be modified or unmodified. Unless otherwise indicated, motifs herein describing only linkages are intended to be linkage motifs. Thus, in such instances, the nucleosides are not limited.

As used herein, “nucleobase modification motif” means a pattern of modifications to nucleobases along an oligonucleotide. Unless otherwise indicated, a nucleobase modification motif is independent of the nucleobase sequence.

As used herein, “sequence motif” means a pattern of nucleobases arranged along an oligonucleotide or portion thereof. Unless otherwise indicated, a sequence motif is independent of chemical modifications and thus may have any combination of chemical modifications, including no chemical modifications.

As used herein, “type of modification” in reference to a nucleoside or a nucleoside of a “type” means the chemical modification of a nucleoside and includes modified and unmodified nucleosides. Accordingly, unless otherwise indicated, a “nucleoside having a modification of a first type” may be an unmodified nucleoside.

As used herein, “differently modified” mean chemical modifications or chemical substituents that are different from one another, including absence of modifications. Thus, for example, a MOE nucleoside and an unmodified DNA nucleoside are “differently modified,” even though the DNA nucleoside is unmodified. Likewise, DNA and RNA are “differently modified,” even though both are naturally-occurring unmodified

nucleosides. Nucleosides that are the same but for comprising different nucleobases are not differently modified. For example, a nucleoside comprising a 2'-OMe modified sugar and an unmodified adenine nucleobase and a nucleoside comprising a 2'-OMe modified sugar and an unmodified thymine nucleobase are not differently modified.

As used herein, "the same type of modifications" refers to modifications that are the same as one another, including absence of modifications. Thus, for example, two unmodified DNA nucleosides have "the same type of modification," even though the DNA nucleoside is unmodified. Such nucleosides having the same type modification may comprise different nucleobases.

As used herein, "separate regions" means portions of an oligonucleotide wherein the chemical modifications or the motif of chemical modifications of any neighboring portions include at least one difference to allow the separate regions to be distinguished from one another.

As used herein, "pharmaceutically acceptable carrier or diluent" means any substance suitable for use in administering to an animal. In certain embodiments, a pharmaceutically acceptable carrier or diluent is sterile saline. In certain embodiments, such sterile saline is pharmaceutical grade saline.

As used herein the term "metabolic disorder" means a disease or condition principally characterized by dysregulation of metabolism – the complex set of chemical reactions associated with breakdown of food to produce energy.

As used herein, the term "Cardiovascular disease" or "cardiovascular disorder" means a disease or condition principally characterized by impaired function of the heart or blood vessels. Examples of cardiovascular diseases or disorders include, but are not limited to, aneurysm, angina, arrhythmia, atherosclerosis, cerebrovascular disease (stroke), coronary heart disease, hypertension, dyslipidemia, hyperlipidemia, and hypercholesterolemia.

As used herein the term "mono or polycyclic ring system" is meant to include all ring systems selected from single or polycyclic radical ring systems wherein the rings are fused or linked and is meant to be inclusive of single and mixed ring systems individually selected from aliphatic, alicyclic, aryl, heteroaryl, aralkyl, arylalkyl, heterocyclic, heteroaryl, heteroaromatic and heteroarylalkyl. Such mono and poly cyclic structures can contain rings that each have the same level of saturation or each, independently, have varying degrees of saturation including fully saturated, partially saturated or fully unsaturated. Each ring can comprise ring atoms selected from C, N, O and S to give rise to heterocyclic rings as well as rings comprising only C ring atoms which can be present in a mixed motif such as for example benzimidazole wherein one ring has only carbon ring atoms and the fused ring has two nitrogen atoms. The mono or polycyclic ring system can be further substituted with substituent groups such as for example phthalimide which has two =O groups attached to one of the rings. Mono or polycyclic ring systems can be attached to parent molecules using various strategies such as directly through a ring atom, fused through multiple ring atoms, through a substituent group or through a bifunctional linking moiety.

As used herein, “prodrug” means an inactive or less active form of a compound which, when administered to a subject, is metabolized to form the active, or more active, compound (e.g., drug).

As used herein, "substituent" and "substituent group," means an atom or group that replaces the atom or group of a named parent compound. For example a substituent of a modified nucleoside is any atom or group that differs from the atom or group found in a naturally occurring nucleoside (e.g., a modified 2’-substuent is any atom or group at the 2’-position of a nucleoside other than H or OH). Substituent groups can be protected or unprotected. In certain embodiments, compounds of the present disclosure have substituents at one or at more than one position of the parent compound. Substituents may also be further substituted with other substituent groups and may be attached directly or via a linking group such as an alkyl or hydrocarbyl group to a parent compound.

Likewise, as used herein, “substituent” in reference to a chemical functional group means an atom or group of atoms that differs from the atom or a group of atoms normally present in the named functional group. In certain embodiments, a substituent replaces a hydrogen atom of the functional group (e.g., in certain embodiments, the substituent of a substituted methyl group is an atom or group other than hydrogen which replaces one of the hydrogen atoms of an unsubstituted methyl group). Unless otherwise indicated, groups amenable for use as substituents include without limitation, halogen, hydroxyl, alkyl, alkenyl, alkynyl, acyl (-C(O)R_{aa}), carboxyl (-C(O)O-R_{aa}), aliphatic groups, alicyclic groups, alkoxy, substituted oxy (-O-R_{aa}), aryl, aralkyl, heterocyclic radical, heteroaryl, heteroarylalkyl, amino (-N(R_{bb})(R_{cc})), imino(=NR_{bb}), amido (-C(O)N(R_{bb})(R_{cc}) or -N(R_{bb})C(O)R_{aa}), azido (-N_3), nitro (-NO_2), cyano (-CN), carbamido (-OC(O)N(R_{bb})(R_{cc}) or -N(R_{bb})C(O)OR_{aa}), ureido (-N(R_{bb})C(O)N(R_{bb})(R_{cc})), thioureido (-N(R_{bb})C(S)N(R_{bb})-(R_{cc})), guanidinyl (-N(R_{bb})C(=NR_{bb})N(R_{bb})(R_{cc})), amidinyl (-C(=NR_{bb})N(R_{bb})(R_{cc}) or -N(R_{bb})C(=NR_{bb})(R_{aa})), thiol (-SR_{bb}), sulfinyl (-S(O)R_{bb}), sulfonyl (-S(O)$_2$$R_{bb}$) and sulfonamidyl (-S(O)$_2$N(R_{bb})(R_{cc}) or -N(R_{bb})S-(O)$_2$$R_{bb}$). Wherein each R_{aa}, R_{bb} and R_{cc} is, independently, H, an optionally linked chemical functional group or a further substituent group with a preferred list including without limitation, alkyl, alkenyl, alkynyl, aliphatic, alkoxy, acyl, aryl, aralkyl, heteroaryl, alicyclic, heterocyclic and heteroarylalkyl. Selected substituents within the compounds described herein are present to a recursive degree.

As used herein, "alkyl," as used herein, means a saturated straight or branched hydrocarbon radical containing up to twenty four carbon atoms. Examples of alkyl groups include without limitation, methyl, ethyl, propyl, butyl, isopropyl, n-hexyl, octyl, decyl, dodecyl and the like. Alkyl groups typically include from 1 to about 24 carbon atoms, more typically from 1 to about 12 carbon atoms (C_1-C_{12} alkyl) with from 1 to about 6 carbon atoms being more preferred.

As used herein, "alkenyl," means a straight or branched hydrocarbon chain radical containing up to twenty four carbon atoms and having at least one carbon-carbon double bond. Examples of alkenyl groups include without limitation, ethenyl, propenyl, butenyl, 1-methyl-2-buten-1-yl, dienes such as 1,3-butadiene and the like. Alkenyl groups typically include from 2 to about 24 carbon atoms, more typically from 2 to

about 12 carbon atoms with from 2 to about 6 carbon atoms being more preferred. Alkenyl groups as used herein may optionally include one or more further substituent groups.

As used herein, "alkynyl," means a straight or branched hydrocarbon radical containing up to twenty four carbon atoms and having at least one carbon-carbon triple bond. Examples of alkynyl groups include, without limitation, ethynyl, 1-propynyl, 1-butynyl, and the like. Alkynyl groups typically include from 2 to about 24 carbon atoms, more typically from 2 to about 12 carbon atoms with from 2 to about 6 carbon atoms being more preferred. Alkynyl groups as used herein may optionally include one or more further substituent groups.

As used herein, "acyl," means a radical formed by removal of a hydroxyl group from an organic acid and has the general Formula -C(O)-X where X is typically aliphatic, alicyclic or aromatic. Examples include aliphatic carbonyls, aromatic carbonyls, aliphatic sulfonyls, aromatic sulfinyls, aliphatic sulfinyls, aromatic phosphates, aliphatic phosphates and the like. Acyl groups as used herein may optionally include further substituent groups.

As used herein, "alicyclic" means a cyclic ring system wherein the ring is aliphatic. The ring system can comprise one or more rings wherein at least one ring is aliphatic. Preferred alicyclics include rings having from about 5 to about 9 carbon atoms in the ring. Alicyclic as used herein may optionally include further substituent groups.

As used herein, "aliphatic" means a straight or branched hydrocarbon radical containing up to twenty four carbon atoms wherein the saturation between any two carbon atoms is a single, double or triple bond. An aliphatic group preferably contains from 1 to about 24 carbon atoms, more typically from 1 to about 12 carbon atoms with from 1 to about 6 carbon atoms being more preferred. The straight or branched chain of an aliphatic group may be interrupted with one or more heteroatoms that include nitrogen, oxygen, sulfur and phosphorus. Such aliphatic groups interrupted by heteroatoms include without limitation, polyalkoxys, such as polyalkylene glycols, polyamines, and polyimines. Aliphatic groups as used herein may optionally include further substituent groups.

As used herein, "alkoxy" means a radical formed between an alkyl group and an oxygen atom wherein the oxygen atom is used to attach the alkoxy group to a parent molecule. Examples of alkoxy groups include without limitation, methoxy, ethoxy, propoxy, isopropoxy, *n*-butoxy, sec-butoxy, *tert*-butoxy, n-pentoxy, neopentoxy, n-hexoxy and the like. Alkoxy groups as used herein may optionally include further substituent groups.

As used herein, "aminoalkyl" means an amino substituted C_1-C_{12} alkyl radical. The alkyl portion of the radical forms a covalent bond with a parent molecule. The amino group can be located at any position and the aminoalkyl group can be substituted with a further substituent group at the alkyl and/or amino portions.

As used herein, "aralkyl" and "arylalkyl" mean an aromatic group that is covalently linked to a C_1-C_{12} alkyl radical. The alkyl radical portion of the resulting aralkyl (or arylalkyl) group forms a covalent bond

with a parent molecule. Examples include without limitation, benzyl, phenethyl and the like. Aralkyl groups as used herein may optionally include further substituent groups attached to the alkyl, the aryl or both groups that form the radical group.

As used herein, "aryl" and "aromatic" mean a mono- or polycyclic carbocyclic ring system radicals having one or more aromatic rings. Examples of aryl groups include without limitation, phenyl, naphthyl, tetrahydronaphthyl, indanyl, idenyl and the like. Preferred aryl ring systems have from about 5 to about 20 carbon atoms in one or more rings. Aryl groups as used herein may optionally include further substituent groups.

As used herein, "halo" and "halogen," mean an atom selected from fluorine, chlorine, bromine and iodine.

As used herein, "heteroaryl," and "heteroaromatic," mean a radical comprising a mono- or polycyclic aromatic ring, ring system or fused ring system wherein at least one of the rings is aromatic and includes one or more heteroatoms. Heteroaryl is also meant to include fused ring systems including systems where one or more of the fused rings contain no heteroatoms. Heteroaryl groups typically include one ring atom selected from sulfur, nitrogen or oxygen. Examples of heteroaryl groups include without limitation, pyridinyl, pyrazinyl, pyrimidinyl, pyrrolyl, pyrazolyl, imidazolyl, thiazolyl, oxazolyl, isooxazolyl, thiadiazolyl, oxadiazolyl, thiophenyl, furanyl, quinolinyl, isoquinolinyl, benzimidazolyl, benzooxazolyl, quinoxalinyl and the like. Heteroaryl radicals can be attached to a parent molecule directly or through a linking moiety such as an aliphatic group or hetero atom. Heteroaryl groups as used herein may optionally include further substituent groups.

As used herein, “conjugate compound” means any atoms, group of atoms, or group of linked atoms suitable for use as a conjugate group. In certain embodiments, conjugate compounds may possess or impart one or more properties, including, but not limited to pharmacodynamic, pharmacokinetic, binding, absorption, cellular distribution, cellular uptake, charge and/or clearance properties.

As used herein, unless otherwise indicated or modified, the term “double-stranded” refers to two separate oligomeric compounds that are hybridized to one another. Such double stranded compounds may have one or more or non-hybridizing nucleosides at one or both ends of one or both strands (overhangs) and/or one or more internal non-hybridizing nucleosides (mismatches) provided there is sufficient complementarity to maintain hybridization under physiologically relevant conditions.

As used herein, “2’-O-methoxyethyl” (also 2’-MOE and 2’-$O(CH_2)_2$-OCH_3) refers to an O-methoxy-ethyl modification of the 2’ position of a furosyl ring. A 2’-O-methoxyethyl modified sugar is a modified sugar.

As used herein, “2’-O-methoxyethyl nucleotide” means a nucleotide comprising a 2’-O-methoxyethyl modified sugar moiety.

“3’ target site” or “3’ stop site” refers to the nucleotide of a target nucleic acid which is complementary to the 3’-most nucleotide of a particular antisense compound.

As used herein, “5’ target site” or “5 start site” refers to the nucleotide of a target nucleic acid which is complementary to the 5’-most nucleotide of a particular antisense compound.

As used herein, “5-methylcytosine” means a cytosine modified with a methyl group attached to the 5’ position. A 5-methylcytosine is a modified nucleobase.

As used herein, “about” means within ±10% of a value. For example, if it is stated, “a marker may be increased by about 50%”, it is implied that the marker may be increased between 45%-55%

As used herein, “active pharmaceutical agent” means the substance or substances in a pharmaceutical composition that provide a therapeutic benefit when administered to an individual. For example, in certain embodiments an antisense oligonucleotide targeted to ANGPTL3 is an active pharmaceutical agent.

As used herein, “active target region” or “target region” means a region to which one or more active antisense compounds is targeted.

As used herein, “active antisense compounds” means antisense compounds that reduce target nucleic acid levels or protein levels.

As used herein, “adipogenesis” means the development of fat cells from preadipocytes. “Lipogenesis” means the production or formation of fat, either fatty degeneration or fatty infiltration.

As used herein, “adiposity” or “obesity” refers to the state of being obese or an excessively high amount of body fat or adipose tissue in relation to lean body mass. The amount of body fat includes concern for both the distribution of fat throughout the body and the size and mass of the adipose tissue deposits. Body fat distribution can be estimated by skin-fold measures, waist-to-hip circumference ratios, or techniques such as ultrasound, computed tomography, or magnetic resonance imaging. According to the Center for Disease Control and Prevention, individuals with a body mass index (BMI) of 30 or more are considered obese. The term "Obesity" as used herein includes conditions where there is an increase in body fat beyond the physical requirement as a result of excess accumulation of adipose tissue in the body. The term “obesity” includes, but is not limited to, the following conditions: adult-onset obesity; alimentary obesity; endogenous or metabolic obesity; endocrine obesity; familial obesity; hyperinsulinar obesity; hyperplastic-hypertrophic obesity; hypogonadal obesity; hypothyroid obesity; lifelong obesity; morbid obesity and exogenous obesity.

As used herein, “administered concomitantly” refers to the co-administration of two agents in any manner in which the pharmacological effects of both are manifest in the patient at the same time. Concomitant administration does not require that both agents be administered in a single pharmaceutical composition, in the same dosage form, or by the same route of administration. The effects of both agents need not manifest themselves at the same time. The effects need only be overlapping for a period of time and need not be coextensive.

As used herein, “administering” means providing an agent to an animal, and includes, but is not limited to, administering by a medical professional and self-administering.

As used herein, “agent” means an active substance that can provide a therapeutic benefit when administered to an animal. “First Agent” means a therapeutic compound of the invention. For example, a first

agent can be an antisense oligonucleotide targeting ANGPTL3. “Second agent” means a second therapeutic compound of the invention (e.g. a second antisense oligonucleotide targeting ANGPTL3) and/or a non-ANGPTL3 therapeutic compound.

As used herein, “amelioration” refers to a lessening of at least one indicator, sign, or symptom of an associated disease, disorder, or condition. The severity of indicators can be determined by subjective or objective measures, which are known to those skilled in the art.

As used herein, “ANGPTL3” means any nucleic acid or protein of ANGPTL3.

As used herein, “ANGPTL3 expression” means the level of mRNA transcribed from the gene encoding ANGPTL3 or the level of protein translated from the mRNA. ANGPTL3 expression can be determined by art known methods such as a Northern or Western blot.

As used herein, “ANGPTL3 nucleic acid” means any nucleic acid encoding ANGPTL3. For example, in certain embodiments, an ANGPTL3 nucleic acid includes a DNA sequence encoding ANGPTL3, a RNA sequence transcribed from DNA encoding ANGPTL3 (including genomic DNA comprising introns and exons), and a mRNA sequence encoding ANGPTL3. “ANGPTL3 mRNA” means a mRNA encoding an ANGPTL3 protein.

As used herein, “animal” refers to a human or non-human animal, including, but not limited to, mice, rats, rabbits, dogs, cats, pigs, and non-human primates, including, but not limited to, monkeys and chimpanzees.

As used herein, “apoB-containing lipoprotein” means any lipoprotein that has apolipoprotein B as its protein component, and is understood to include LDL, VLDL, IDL, and lipoprotein(a) and can be generally targeted by lipid lowering agent and therapies. “ApoB-100-containing LDL” means ApoB-100 isoform containing LDL.

As used herein, “atherosclerosis” means a hardening of the arteries affecting large and medium-sized arteries and is characterized by the presence of fatty deposits. The fatty deposits are called "atheromas" or “plaques,” which consist mainly of cholesterol and other fats, calcium and scar tissue, and damage the lining of arteries.

As used herein, “cardiometabolic disease” or “cardiometabolic disorder” are diseases or disorders concerning both the cardiovascular system and the metabolic system. Examples of cardiometabolic diseases or disorders include, but are not limited to, diabetes and dyslipidemias.

As used herein, “co-administration” means administration of two or more agents to an individual. The two or more agents can be in a single pharmaceutical composition, or can be in separate pharmaceutical compositions. Each of the two or more agents can be administered through the same or different routes of administration. Co-administration encompasses parallel or sequential administration.

As used herein, “cholesterol” is a sterol molecule found in the cell membranes of all animal tissues. Cholesterol must be transported in an animal’s blood plasma by lipoproteins including very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density

lipoprotein (HDL). "Plasma cholesterol" refers to the sum of all lipoproteins (VDL, IDL, LDL, HDL) esterified and/or non-estrified cholesterol present in the plasma or serum.

As used herein, "cholesterol absorption inhibitor" means an agent that inhibits the absorption of exogenous cholesterol obtained from diet.

As used herein, "coronary heart disease (CHD)" means a narrowing of the small blood vessels that supply blood and oxygen to the heart, which is often a result of atherosclerosis.

As used herein, "diabetes mellitus" or "diabetes" is a syndrome characterized by disordered metabolism and abnormally high blood sugar (hyperglycemia) resulting from insufficient levels of insulin or reduced insulin sensitivity. The characteristic symptoms are excessive urine production (polyuria) due to high blood glucose levels, excessive thirst and increased fluid intake (polydipsia) attempting to compensate for increased urination, blurred vision due to high blood glucose effects on the eye's optics, unexplained weight loss, and lethargy.

As used herein, "diabetic dyslipidemia" or "type 2 diabetes with dyslipidemia" means a condition characterized by Type 2 diabetes, reduced HDL-C, elevated triglycerides, and elevated small, dense LDL particles.

As used herein, "diluent" means an ingredient in a composition that lacks pharmacological activity, but is pharmaceutically necessary or desirable. For example, the diluent in an injected composition can be a liquid, e.g. saline solution.

As used herein, "dyslipidemia" refers to a disorder of lipid and/or lipoprotein metabolism, including lipid and/or lipoprotein overproduction or deficiency. Dyslipidemias may be manifested by elevation of lipids such as cholesterol and triglycerides as well as lipoproteins such as low-density lipoprotein (LDL) cholesterol.

As used herein, "dosage unit" means a form in which a pharmaceutical agent is provided, e.g. pill, tablet, or other dosage unit known in the art. In certain embodiments, a dosage unit is a vial containing lyophilized antisense oligonucleotide. In certain embodiments, a dosage unit is a vial containing reconstituted antisense oligonucleotide.

As used herein, "dose" means a specified quantity of a pharmaceutical agent provided in a single administration, or in a specified time period. In certain embodiments, a dose can be administered in one, two, or more boluses, tablets, or injections. For example, in certain embodiments where subcutaneous administration is desired, the desired dose requires a volume not easily accommodated by a single injection, therefore, two or more injections can be used to achieve the desired dose. In certain embodiments, the pharmaceutical agent is administered by infusion over an extended period of time or continuously. Doses can be stated as the amount of pharmaceutical agent per hour, day, week, or month. Doses can be expressed as mg/kg or g/kg.

As used herein, "effective amount" or "therapeutically effective amount" means the amount of active pharmaceutical agent sufficient to effectuate a desired physiological outcome in an individual in need of the agent. The effective amount can vary among individuals depending on the health and physical condition of

the individual to be treated, the taxonomic group of the individuals to be treated, the formulation of the composition, assessment of the individual's medical condition, and other relevant factors.

As used herein, "glucose" is a monosaccharide used by cells as a source of energy and metabolic intermediate. "Plasma glucose" refers to glucose present in the plasma.

As used herein, "high density lipoprotein-C (HDL-C)" means cholesterol associated with high density lipoprotein particles. Concentration of HDL-C in serum (or plasma) is typically quantified in mg/dL or nmol/L. "serum HDL-C" and "plasma HDL-C" mean HDL-C in serum and plasma, respectively.

As used herein, "HMG-CoA reductase inhibitor" means an agent that acts through the inhibition of the enzyme HMG-CoA reductase, such as atorvastatin, rosuvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin.

As used herein, "hypercholesterolemia" means a condition characterized by elevated cholesterol or circulating (plasma) cholesterol, LDL-cholesterol and VLDL-cholesterol, as per the guidelines of the Expert Panel Report of the National Cholesterol Educational Program (NCEP) of Detection, Evaluation of Treatment of high cholesterol in adults (see, Arch. Int. Med. (1988) 148, 36-39).

As used herein, "hyperlipidemia" or "hyperlipemia" is a condition characterized by elevated serum lipids or circulating (plasma) lipids. This condition manifests an abnormally high concentration of fats. The lipid fractions in the circulating blood are cholesterol, low density lipoproteins, very low density lipoproteins and triglycerides.

As used herein, "hypertriglyceridemia" means a condition characterized by elevated triglyceride levels.

As used herein, "identifying" or "selecting a subject having a metabolic or cardiovascular disease" means identifying or selecting a subject having been diagnosed with a metabolic disease, a cardiovascular disease, or a metabolic syndrome; or, identifying or selecting a subject having any symptom of a metabolic disease, cardiovascular disease, or metabolic syndrome including, but not limited to, hypercholesterolemia, hyperglycemia, hyperlipidemia, hypertriglyceridemia, hypertension, increased insulin resistance, decreased insulin sensitivity, above normal body weight, and/or above normal body fat content or any combination thereof. Such identification may be accomplished by any method, including but not limited to, standard clinical tests or assessments, such as measuring serum or circulating (plasma) cholesterol, measuring serum or circulating (plasma) blood-glucose, measuring serum or circulating (plasma) triglycerides, measuring blood-pressure, measuring body fat content, measuring body weight, and the like.

As used herein, "identifying" or "selecting a diabetic subject" means identifying or selecting a subject having been identified as diabetic or identifying or selecting a subject having any symptom of diabetes (type 1 or type 2) such as, but not limited to, having a fasting glucose of at least 110 mg/dL, glycosuria, polyuria, polydipsia, increased insulin resistance, and/or decreased insulin sensitivity.

As used herein, “identifying” or “selecting an obese subject” means identifying or selecting a subject having been diagnosed as obese or identifying or selecting a subject with a BMI over 30 and/or a waist circumference of greater than 102 cm in men or greater than 88 cm in women.

As used herein, “identifying” or “selecting a subject having dyslipidemia” means identifying or selecting a subject diagnosed with a disorder of lipid and/or lipoprotein metabolism, including lipid and/or lipoprotein overproduction or deficiency. Dyslipidemias may be manifested by elevation of lipids such as cholesterol and triglycerides as well as lipoproteins such as low-density lipoprotein (LDL) cholesterol.

As used herein, “identifying” or “selecting” a subject having increased adiposity” means identifying or selecting a subject having an increased amount of body fat (or adiposity) that includes concern for one or both the distribution of fat throughout the body and the size and mass of the adipose tissue deposits. Body fat distribution can be estimated by skin-fold measures, waist-to-hip circumference ratios, or techniques such as ultrasound, computer tomography, or magnetic resonance imaging. According to the Center for Disease Control and Prevention, individuals with a body mass index (BMI) of 30 or more are considered obese.

As used herein, “improved cardiovascular outcome” means a reduction in the occurrence of adverse cardiovascular events, or the risk thereof. Examples of adverse cardiovascular events include, without limitation, death, reinfarction, stroke, cardiogenic shock, pulmonary edema, cardiac arrest, and atrial dysrhythmia.

As used herein, “immediately adjacent” means there are no intervening elements between the immediately adjacent elements.

As used herein, “individual” or “subject” or “animal” means a human or non-human animal selected for treatment or therapy.

As used herein, “insulin resistance” is defined as the condition in which normal amounts of insulin are inadequate to produce a normal insulin response from cells, e.g., fat, muscle and/or liver cells. Insulin resistance in fat cells results in hydrolysis of stored triglycerides, which elevates free fatty acids in the blood plasma. Insulin resistance in muscle reduces glucose uptake whereas insulin resistance in liver reduces glucose storage, with both effects serving to elevate blood glucose. High plasma levels of insulin and glucose due to insulin resistance often leads to metabolic syndrome and type 2 diabetes.

As used herein, “insulin sensitivity” is a measure of how effectively an individual processes glucose. An individual having high insulin sensitivity effectively processes glucose whereas an individual with low insulin sensitivity does not effectively process glucose.

As used herein, “intravenous administration” means administration into a vein.

As used herein, “lipid-lowering” means a reduction in one or more lipids in a subject. Lipid-lowering can occur with one or more doses over time.

As used herein, “lipid-lowering agent” means an agent, for example, an ANGPTL3-specific modulator, provided to a subject to achieve a lowering of lipids in the subject. For example, in certain embodiments, a lipid-lowering agent is provided to a subject to reduce one or more of apoB, apoC-III, total

cholesterol, LDL-C, VLDL-C, IDL-C, non-HDL-C, triglycerides, small dense LDL particles, and Lp(a) in a subject.

As used herein, "lipid-lowering therapy" means a therapeutic regimen provided to a subject to reduce one or more lipids in a subject. In certain embodiments, a lipid-lowering therapy is provided to reduce one or more of apoB, apoC-III, total cholesterol, LDL-C, VLDL-C, IDL-C, non-HDL-C, triglycerides, small dense LDL particles, and Lp(a) in a subject.

As used herein, "lipoprotein", such as VLDL, LDL and HDL, refers to a group of proteins found in the serum, plasma and lymph and are important for lipid transport. The chemical composition of each lipoprotein differs in that the HDL has a higher proportion of protein versus lipid, whereas the VLDL has a lower proportion of protein versus lipid.

As used herein, "low density lipoprotein-cholesterol (LDL-C)" means cholesterol carried in low density lipoprotein particles. Concentration of LDL-C in serum (or plasma) is typically quantified in mg/dL or nmol/L. "Serum LDL-C" and "plasma LDL-C" mean LDL-C in the serum and plasma, respectively.

As used herein, "major risk factors" refers to factors that contribute to a high risk for a particular disease or condition. In certain embodiments, major risk factors for coronary heart disease include, without limitation, cigarette smoking, hypertension, low HDL-C, family history of coronary heart disease, age, and other factors disclosed herein.

As used herein, "metabolic disorder" or "metabolic disease" refers to a condition characterized by an alteration or disturbance in metabolic function. "Metabolic" and "metabolism" are terms well known in the art and generally include the whole range of biochemical processes that occur within a living organism. Metabolic disorders include, but are not limited to, hyperglycemia, prediabetes, diabetes (type I and type 2), obesity, insulin resistance, metabolic syndrome and dyslipidemia due to type 2 diabetes.

As used herein, "metabolic syndrome" means a condition characterized by a clustering of lipid and non-lipid cardiovascular risk factors of metabolic origin. In certain embodiments, metabolic syndrome is identified by the presence of any 3 of the following factors: waist circumference of greater than 102 cm in men or greater than 88 cm in women; serum triglyceride of at least 150 mg/dL; HDL-C less than 40 mg/dL in men or less than 50 mg/dL in women; blood pressure of at least 130/85 mmHg; and fasting glucose of at least 110 mg/dL. These determinants can be readily measured in clinical practice (JAMA, 2001, 285: 2486-2497).

As used herein, "mixed dyslipidemia" means a condition characterized by elevated cholesterol and elevated triglycerides.

As used herein, "MTP inhibitor" means an agent inhibits the enzyme microsomal triglyceride transfer protein.

As used herein, "non-alcoholic fatty liver disease" or "NAFLD" means a condition characterized by fatty inflammation of the liver that is not due to excessive alcohol use (for example, alcohol consumption of over 20 g/day). In certain embodiments, NAFLD is related to insulin resistance and metabolic syndrome.

NAFLD encompasses a disease spectrum ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation (steatohepatitis), fibrosis, and cirrhosis.

As used herein, "nonalcoholic steatohepatitis" (NASH) occurs from progression of NAFLD beyond deposition of triglycerides. A "second hit" capable of inducing necrosis, inflammation, and fibrosis is required for development of NASH. Candidates for the second-hit can be grouped into broad categories: factors causing an increase in oxidative stress and factors promoting expression of proinflammatory cytokines. It has been suggested that increased liver triglycerides lead to increased oxidative stress in hepatocytes of animals and humans, indicating a potential cause-and-effect relationship between hepatic triglyceride accumulation, oxidative stress, and the progression of hepatic steatosis to NASH (Browning and Horton, *J Clin Invest,* **2004**, *114*, 147-152). Hypertriglyceridemia and hyperfattyacidemia can cause triglyceride accumulation in peripheral tissues (Shimamura et al., *Biochem Biophys Res Commun,* **2004**, *322*, 1080-1085).

As used herein, "nucleic acid" refers to molecules composed of monomeric nucleotides. A nucleic acid includes ribonucleic acids (RNA), deoxyribonucleic acids (DNA), single-stranded nucleic acids, double-stranded nucleic acids, small interfering ribonucleic acids (siRNA), and microRNAs (miRNA). A nucleic acid can also comprise a combination of these elements in a single molecule.

As used herein, "parenteral administration" means administration by a manner other than through the digestive tract. Parenteral administration includes topical administration, subcutaneous administration, intravenous administration, intramuscular administration, intraarterial administration, intraperitoneal administration, or intracranial administration, e.g. intrathecal or intracerebroventricular administration. Administration can be continuous, or chronic, or short or intermittent.

As used herein, "pharmaceutical agent" means a substance that provides a therapeutic benefit when administered to an individual. For example, in certain embodiments, an antisense oligonucleotide targeted to ANGPTL3 is pharmaceutical agent.

As used herein, "pharmaceutical composition" means a mixture of substances suitable for administering to an individual. For example, a pharmaceutical composition can comprise one or more active agents and a sterile aqueous solution.

As used herein, "pharmaceutically acceptable carrier" means a medium or diluent that does not interfere with the structure or function of the oligonucleotide. Certain, of such carries enable pharmaceutical compositions to be formulated as, for example, tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspension and lozenges for the oral ingestion by a subject. Certain of such carriers enable pharmaceutical compositions to be formulated for injection or infusion. For example, a pharmaceutically acceptable carrier can be a sterile aqueous solution.

As used herein, "pharmaceutically acceptable salts" means physiologically and pharmaceutically acceptable salts of antisense compounds, i.e., salts that retain the desired biological activity of the parent oligonucleotide and do not impart undesired toxicological effects thereto.

As used herein, "portion" means a defined number of contiguous (i.e. linked) nucleobases of a nucleic acid. In certain embodiments, a portion is a defined number of contiguous nucleobases of a target nucleic acid. In certain embodiments, a portion is a defined number of contiguous nucleobases of an antisense compound.

As used herein, "prevent" refers to delaying or forestalling the onset or development of a disease, disorder, or condition for a period of time from minutes to indefinitely. Prevent also means reducing risk of developing a disease, disorder, or condition.

As used herein, "side effects" means physiological responses attributable to a treatment other than the desired effects. In certain embodiments, side effects include injection site reactions, liver function test abnormalities, renal function abnormalities, liver toxicity, renal toxicity, central nervous system abnormalities, myopathies, and malaise. For example, increased aminotransferase levels in serum can indicate liver toxicity or liver function abnormality. For example, increased bilirubin can indicate liver toxicity or liver function abnormality.

As used herein, "statin" means an agent that inhibits the activity of HMG-CoA reductase.

As used herein, "subcutaneous administration" means administration just below the skin.

As used herein, "targeting" or "targeted" means the process of design and selection of an antisense compound that will specifically hybridize to a target nucleic acid and induce a desired effect.

As used herein, "target nucleic acid," "target RNA," and "target RNA transcript" all refer to a nucleic acid capable of being targeted by antisense compounds.

As used herein, "target region" is defined as a portion of the target nucleic acid having at least one identifiable structure, function, or characteristic.

As used herein, "target segment" means the sequence of nucleotides of a target nucleic acid to which one or more antisense compound is targeted. "5' target site" or "5' start site" refers to the 5'-most nucleotide of a target segment. "3' target site" or "3' stop site" refers to the 3'-most nucleotide of a target segment.

As used herein, "therapeutically effective amount" means an amount of an agent that provides a therapeutic benefit to an individual.

As used herein, "therapeutic lifestyle change" means dietary and lifestyle changes intended to lower fat /adipose tissue mass and/or cholesterol. Such change can reduce the risk of developing heart disease, and may include recommendations for dietary intake of total daily calories, total fat, saturated fat, polyunsaturated fat, monounsaturated fat, carbohydrate, protein, cholesterol, insoluble fiber, as well as recommendations for physical activity.

As used herein, "triglyceride" means a lipid or neutral fat consisting of glycerol combined with three fatty acid molecules.

As used herein, "type 2 diabetes" (also known as "type 2 diabetes mellitus" or "diabetes mellitus, type 2", and formerly called "diabetes mellitus type 2", "non-insulin-dependent diabetes (NIDDM)", "obesity

related diabetes", or "adult-onset diabetes") is a metabolic disorder that is primarily characterized by insulin resistance, relative insulin deficiency, and hyperglycemia.

As used herein, "treat" refers to administering a pharmaceutical composition to effect an alteration or improvement of a disease, disorder, or condition.

Certain Embodiments

In certain embodiments disclosed herein, ANGPTL3 has the sequence as set forth in GenBank Accession No. NM_014495.2 (incorporated herein as SEQ ID NO: 1). In certain embodiments, ANGPTL3 has the sequence as set forth in GenBank Accession No. NT_032977.9 nucleotides 33032001 to 33046000 (incorporated herein as SEQ ID NO: 2).

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 nucleosides having a nucleobase sequence comprising at least 8 contiguous nucleobases complementary to an equal length portion of SEQ ID NOs: 1-2.

In certain embodiments, a compound comprises a siRNA or antisense oligonucleotide targeted to ANGPTL3 known in the art and a conjugate group described herein. Examples of antisense oligonucleotides targeted to ANGPTL3 suitable for conjugation include but are not limited to those disclosed in US 8,653,047 (WO 2011/085271), which is incorporated by reference in its entirety herein. In certain embodiments, a compound comprises an antisense oligonucleotide having a nucleobase sequence of any of SEQ ID NOs: 34-111 disclosed in US 8,653,047 and a conjugate group described herein. In certain embodiments, a compound comprises a siRNA sense or antisense strand having a nucleobase sequence of any of SEQ ID NOs: 34-111 disclosed in US 8,653,047 and a conjugate group described herein. The nucleobase sequences of all of the aforementioned referenced SEQ ID NOs are incorporated by reference herein.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides in length targeted to ANGPTL3. The ANGPTL3 target can have a sequence selected from any one of SEQ ID NOs: 1-2.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprising a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to an equal length portion of nucleobases 1140 to 1159 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1. In certain embodiments, the modified oligonucleotide is at least 8, least 9, least 10, least 11, at least 12, least 13, at least 14, at least 15, at least 16, least 17, least 18, least 19, or 20 contiguous nucleobases complementary to an equal length portion of nucleobases 1140 to 1159 of SEQ ID NO: 1.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked

nucleosides and comprising a nucleobase sequence complementary to nucleobases 1140 to 1159 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprising a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to an equal length portion of nucleobases 1907 to 1926 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1. In certain embodiments, the modified oligonucleotide is at least 8, least 9, least 10, least 11, at least 12, least 13, at least 14, at least 15, at least 16, least 17, least 18, least 19, or 20 contiguous nucleobases complementary to an equal length portion of nucleobases 1907 to 1926 of SEQ ID NO: 1.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprising a nucleobase sequence complementary to nucleobases 1907 to 1926 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprising a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to an equal length portion of nucleobases 147 to 162 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1. In certain embodiments, the modified oligonucleotide is at least 8, least 9, least 10, least 11, at least 12, least 13, at least 14, at least 15, or 16 contiguous nucleobases complementary to an equal length portion of nucleobases 147 to 162 of SEQ ID NO: 1.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprising a nucleobase sequence complementary to nucleobases 147 to 162 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

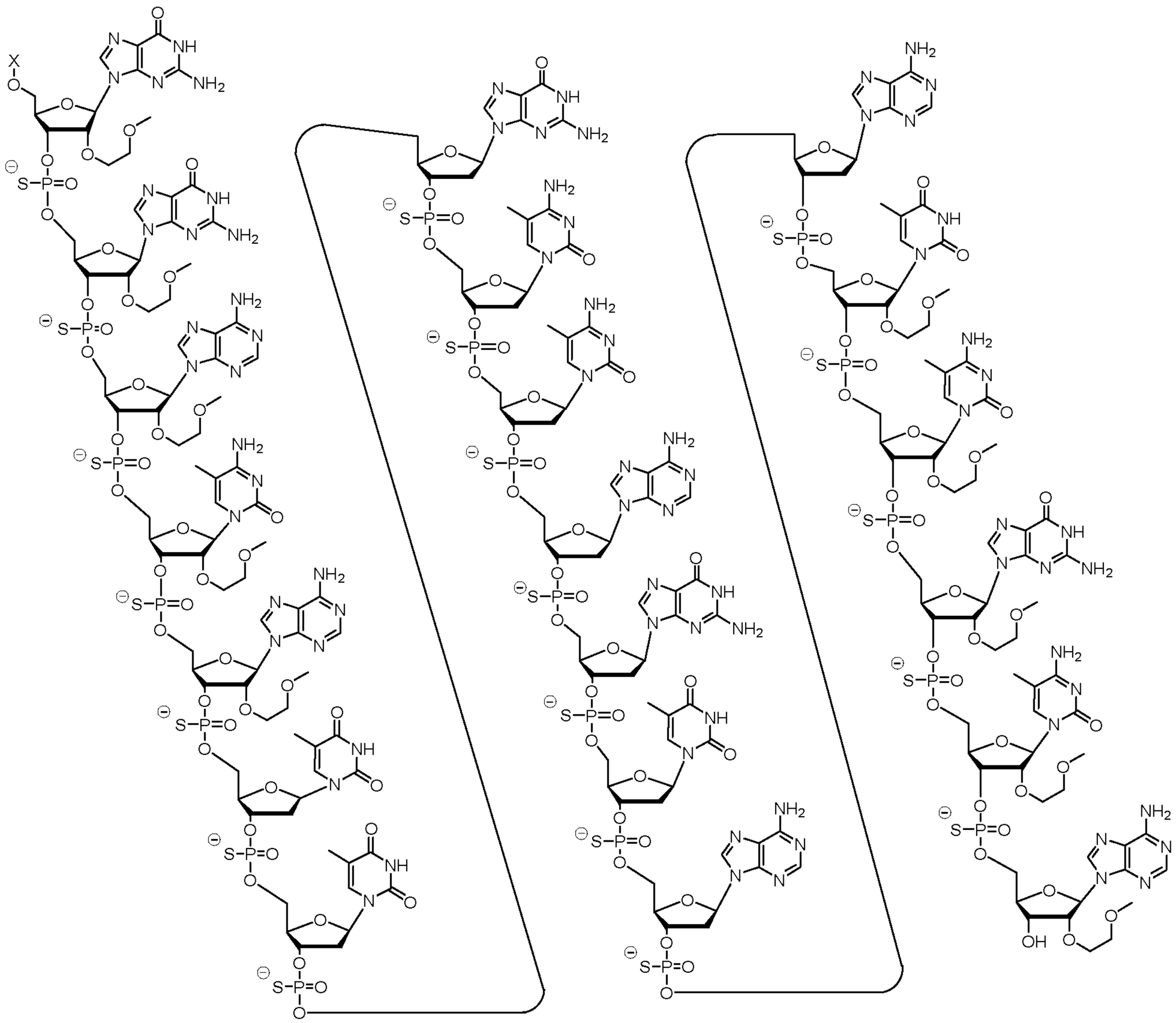
In certain embodiments, the modified oligonucleotide consists of 12 to 30, 15 to 30, 18 to 24, 19 to 22, 13 to 25, 14 to 25, 15 to 25 or 16 to 24 linked nucleosides. In certain embodiments, the modified oligonucleotide consists of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 linked nucleosides or a range defined by any two of these values. In certain embodiments, the modified oligonucleotide is 16 linked nucleosides in length. In certain embodiments, the modified oligonucleotide is 20 linked nucleosides in length.

In certain embodiments, the modified oligonucleotide comprises a nucleobase sequence comprising a portion of at least 8, least 9, least 10, least 11, at least 12, least 13, at least 14, at least 15, at least 16, least 17, least 18, least 19, or 20 contiguous nucleobases complementary to an equal length portion of SEQ ID NO: 1 or 2.

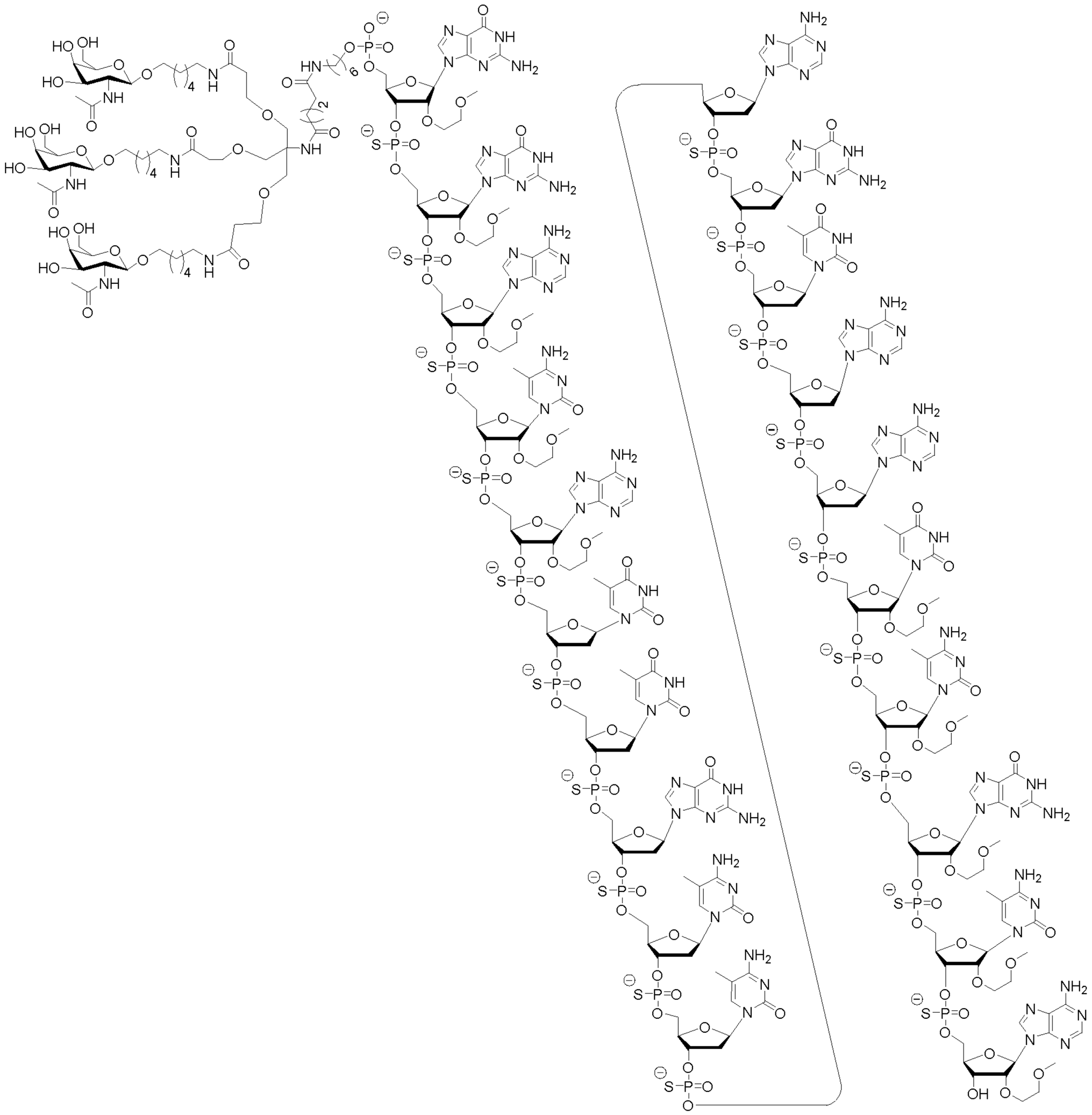
Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and having a nucleobase sequence comprising at least 8, least 9, least 10, least 11, at least 12, least 13, at least 14, at least 15, at least 16, least 17, least 18, least 19, or 20 contiguous nucleobases of a nucleobase sequence selected from any one of SEQ ID NOs: 15-27, 30-73, 75-85, 87-232, 238, 240-243, 245-247, 249-262, 264-397, 399-469, 471-541, 543-600, 604-760, 762-819, 821-966, 968-971, 973-975, 977-990, 992-1110, 1112-1186, 1188-1216, 1218-1226, 1228-1279, 1281-1293, 1295-1304, 1306-1943, 1945-1951, 1953-1977, 1979-1981, 1983-2044, 2046-2097, 2099-2181, 2183-2232, 2234-2238, 2240-2258, 2260-2265, 2267-2971, 2973-2976, 2978-4162, 4164-4329, 4331-4389, 4391-4394, 4396-4877.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of the nucleobase sequences of SEQ ID NO: 77. In certain embodiments, the compound comprises ISIS 563580 and a conjugate group. In certain embodiments, the compound consists of ISIS 563580 and a conjugate group.

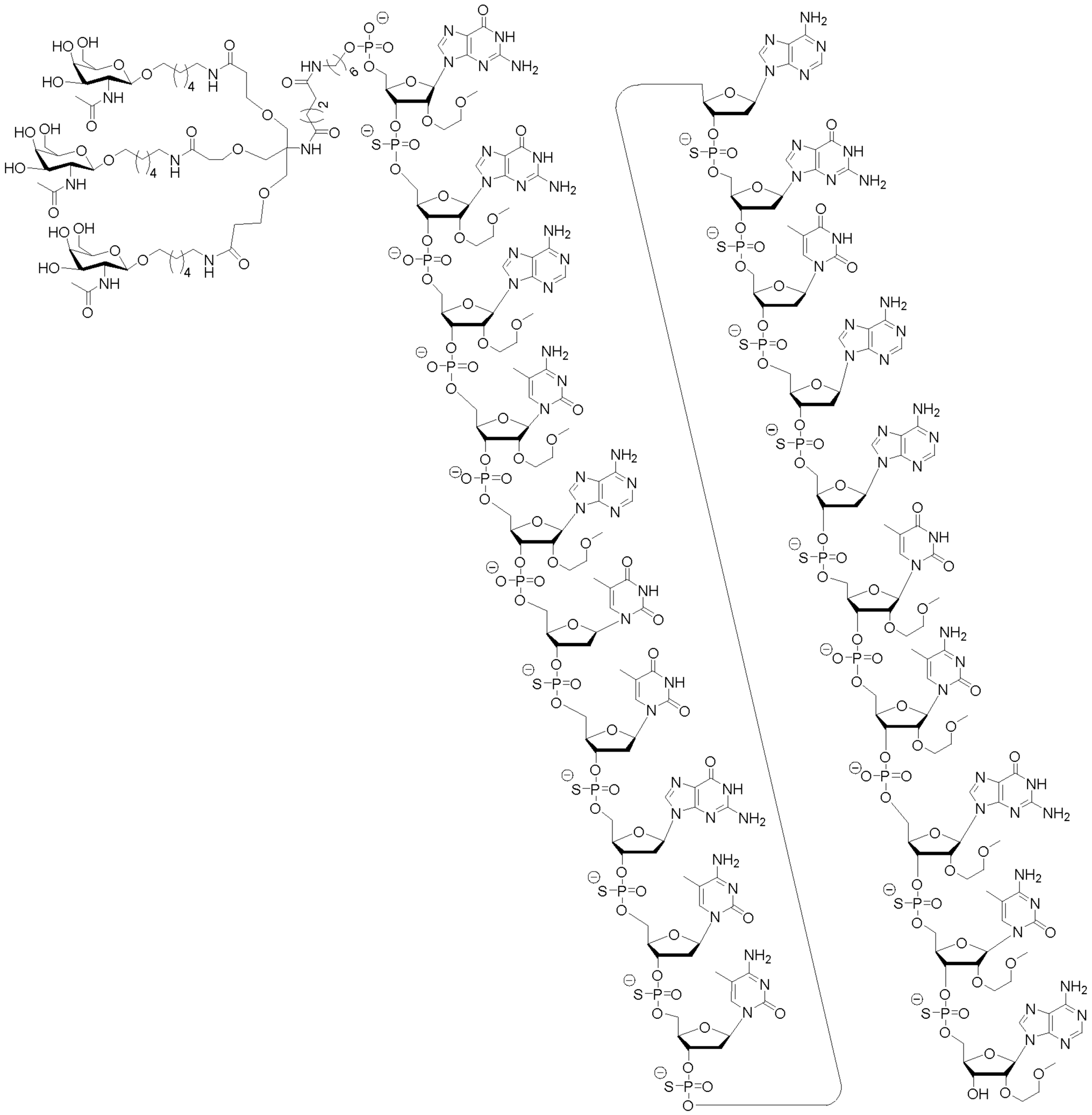
In certain embodiments, the present disclosure provides conjugated antisense compounds represented by the following structure. In certain embodiments, the antisense compound comprises the modified oligonucleotide ISIS 563580 with a 5'-X, wherein X is a conjugate group comprising GalNAc. In certain embodiments, the antisense compound consists of the modified oligonucleotide ISIS 563580 with a 5'-X, wherein X is a conjugate group comprising GalNAc.



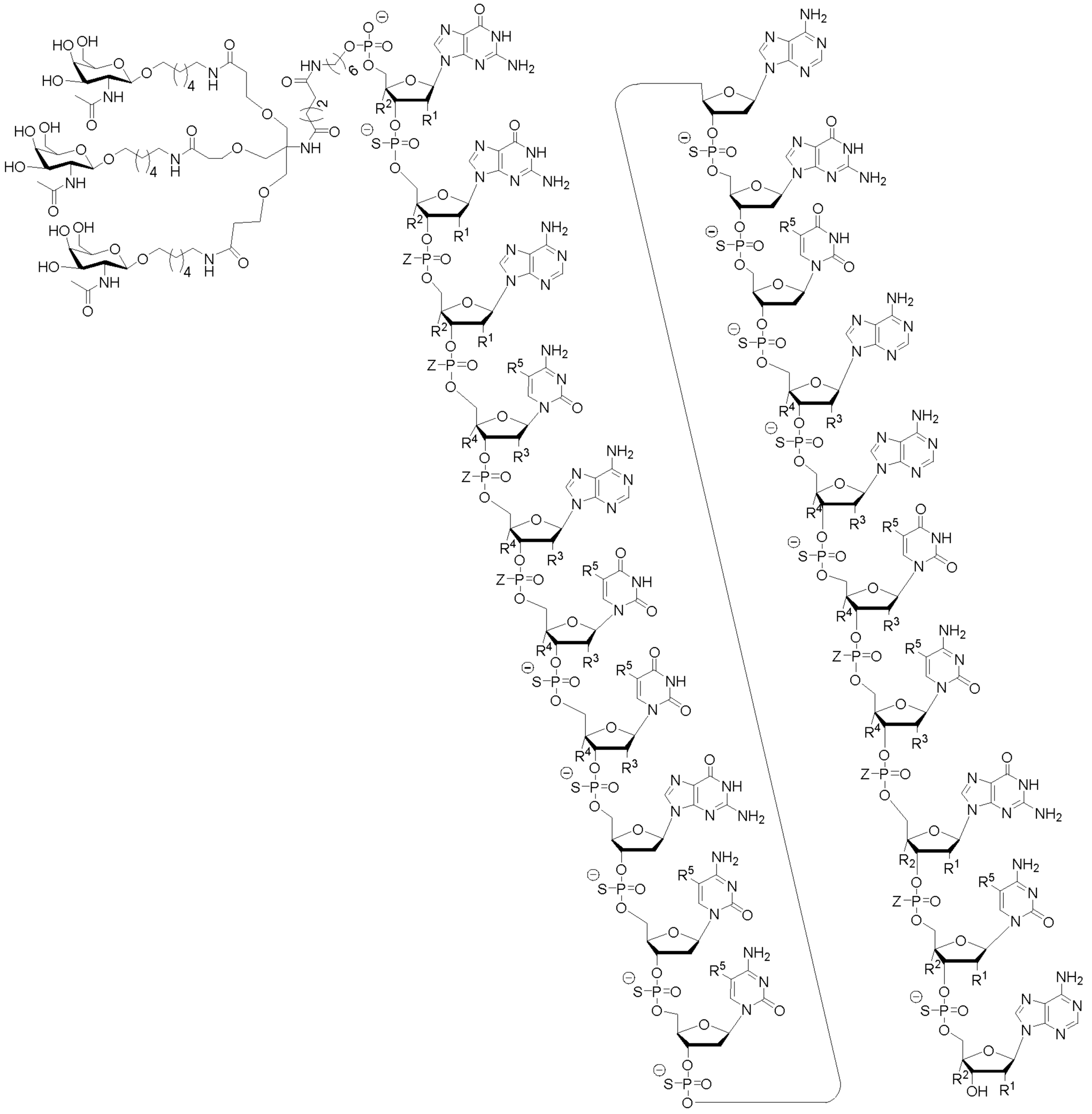
In certain embodiments, the present disclosure provides conjugated antisense compounds represented by the following structure. In certain embodiments, the antisense compound comprises the conjugated modified oligonucleotide ISIS 703801. In certain embodiments, the antisense compound consists of the conjugated modified oligonucleotide ISIS 703801.



In certain embodiments, the present disclosure provides conjugated antisense compounds represented by the following structure. In certain embodiments, the antisense compound comprises the conjugated modified oligonucleotide ISIS 703802. In certain embodiments, the antisense compound consists of the conjugated modified oligonucleotide ISIS 703802.



In certain embodiments, the present disclosure provides conjugated antisense compounds represented by the following structure. In certain embodiments, the antisense compound comprises a modified oligonucleotide with the nucleobase sequence of SEQ ID NO: 77 with a 5'-GalNAc with variability in the sugar mods of the wings. In certain embodiments, the antisense compound consists of a modified oligonucleotide with the nucleobase sequence of SEQ ID NO: 77 with a 5'-GalNAc with variability in the sugar mods of the wings.



wherein either R^1 is $-OCH_2CH_2OCH_3$ (MOE)and R^2 is H; or R^1 and R^2 together form a bridge, wherein R^1 is –O- and R^2 is $-CH_2$-, -$CH(CH_3)$-, or -CH_2CH_2-, and R^1 and R^2 are directly connected such that the resulting bridge is selected from: -O-CH_2-, -O-$CH(CH_3)$-, and –O-CH_2CH_2-;

and for each pair of R^3 and R^4 on the same ring, independently for each ring: either R^3 is selected from H and -$OCH_2CH_2OCH_3$ and R^4 is H; or R^3 and R^4 together form a bridge, wherein R^3 is –O-, and R^4 is –CH_2-, -$CH(CH_3)$-, or -CH_2CH_2-and R^3 and R^4 are directly connected such that the resulting bridge is selected from: -O-CH_2-, -O-$CH(CH_3)$-, and –O-CH_2CH_2-;

and R^5 is selected from H and –CH_3;

and Z is selected from S^- and O^-.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of the nucleobase sequence of SEQ ID NO: 20. In certain embodiments, the compound comprises ISIS 544199 and a conjugate group. In certain embodiments, the compound consists of ISIS 544199 and a conjugate group.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of the nucleobase sequence of SEQ ID NO: 35. In certain embodiments, the compound comprises ISIS 560400 and a conjugate group. In certain embodiments, the compound consists of ISIS 560400 and a conjugate group.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of the nucleobase sequence of SEQ ID NO: 90. In certain embodiments, the compound comprises ISIS 567233 and a conjugate group. In certain embodiments, the compound consists of ISIS 567233 and a conjugate group.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of the nucleobase sequence of SEQ ID NO: 93. In certain embodiments, the compound comprises ISIS 567320 and a conjugate group. In certain embodiments, the compound consists of ISIS 567320 and a conjugate group.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked

nucleosides and having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of the nucleobase sequence of SEQ ID NO: 94. In certain embodiments, the compound comprises ISIS 567321 and a conjugate group. In certain embodiments, the compound consists of ISIS 567321 and a conjugate group.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or 16 contiguous nucleobases of the nucleobase sequence of SEQ ID NO: 110. In certain embodiments, the compound comprises ISIS 559277 and a conjugate group. In certain embodiments, the compound consists of ISIS 559277 and a conjugate group.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or 16 contiguous nucleobases of the nucleobase sequence of SEQ ID NO: 114. In certain embodiments, the compound comprises ISIS 561011 and a conjugate group. In certain embodiments, the compound consists of ISIS 561011 and a conjugate group.

In certain embodiments, the nucleobase sequence of the modified oligonucleotide is at least 70%, 75%, 80%, 85%, 90%, 95% or 100% complementary to any one of SEQ ID NO: 1-2 as measured over the entirety of the modified oligonucleotide.

In certain embodiments, the compound disclosed herein is a single-stranded oligonucleotide. In certain embodiments, the compound disclosed herein is a single-stranded modified oligonucleotide.

In certain embodiments, at least one internucleoside linkage of said modified oligonucleotide is a modified internucleoside linkage. In certain embodiments, the modified internucleoside linkage is a phosphorothioate internucleoside linkage. In certain embodiments, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10 internucleoside linkages of said modified oligonucleotide are phosphorothioate internucleoside linkages. In certain embodiments, each internucleoside linkage is a phosphorothioate internucleoside linkage. In certain embodiments, the modified oligonucleotide comprises at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10 phosphodiester internucleoside linkages. In certain embodiments, each internucleoside linkage of the modified oligonucleotide is selected from a phosphodiester internucleoside linkage and a phosphorothioate internucleoside linkage.

In certain embodiments, at least one nucleoside of the modified oligonucleotide comprises a modified sugar. In certain embodiments, at least one modified sugar is a bicyclic sugar. In certain embodiments, at

least one modified sugar comprises a 2'-O-methoxyethyl, a constrained ethyl, a 3'-fluoro-HNA or a 4'- $(CH_2)_n$-O-2' bridge, wherein n is 1 or 2.

In certain embodiments, at least one nucleoside of said modified oligonucleotide comprises a modified nucleobase. In certain embodiments, the modified nucleobase is a 5-methylcytosine.

Certain embodiments disclosed herein provide compounds or compositions comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide has: a) a gap segment consisting of linked deoxynucleosides; b) a 5' wing segment consisting of linked nucleosides; and c) a 3' wing segment consisting of linked nucleosides. The gap segment is positioned between the 5' wing segment and the 3' wing segment and each nucleoside of each wing segment comprises a modified sugar.

In certain embodiments, the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar.

In certain embodiments, the compounds or compositions disclosed herein comprise a modified oligonucleotide consisting of 20 linked nucleosides having a nucleobase sequence comprising at least 8 contiguous nucleobases complementary to an equal length portion of SEQ ID NO: 1-2, wherein the modified oligonucleotide comprises: a gap segment consisting of ten linked deoxynucleosides; a 5' wing segment consisting of five linked nucleosides; and a 3' wing segment consisting of five linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment; wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar; wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, each internucleoside linkage is a phosphorothioate linkage.

In certain embodiments, the modified oligonucleotide consists of 20 linked nucleosides and comprises: a gap segment consisting of ten linked deoxynucleosides; a 5' wing segment consisting of five linked nucleosides; a 3' wing segment consisting of five linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment; wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar; wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, each internucleoside linkage is a phosphorothioate linkage.

In certain embodiments, the compounds or compositions disclosed herein comprise a modified oligonucleotide consisting of 20 linked nucleosides having a nucleobase sequence comprising at least 8 contiguous nucleobases of a nucleobase sequence selected of SEQ ID NO: 77, wherein the modified oligonucleotide comprises: a gap segment consisting of ten linked deoxynucleosides; a 5' wing segment consisting of five linked nucleosides; and a 3' wing segment consisting of five linked nucleosides; wherein

the gap segment is positioned between the 5’ wing segment and the 3’ wing segment; wherein each nucleoside of each wing segment comprises a 2’-O-methoxyethyl sugar; wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, each internucleoside linkage is a phosphorothioate linkage.

In certain embodiments, the modified oligonucleotide consists of 20 linked nucleosides with the nucleobase sequence of SEQ ID NO: 77 and comprises: a gap segment consisting of ten linked deoxynucleosides; a 5’ wing segment consisting of five linked nucleosides; a 3’ wing segment consisting of five linked nucleosides; wherein the gap segment is positioned between the 5’ wing segment and the 3’ wing segment; wherein each nucleoside of each wing segment comprises a 2’-O-methoxyethyl sugar; wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, each internucleoside linkage is a phosphorothioate linkage.

In certain embodiments, the compounds or compositions disclosed herein comprise a modified oligonucleotide consisting of 20 linked nucleosides having a nucleobase sequence comprising at least 8 contiguous nucleobases of a nucleobase sequence selected of SEQ ID NO: 20, wherein the modified oligonucleotide comprises: a gap segment consisting of ten linked deoxynucleosides; a 5’ wing segment consisting of five linked nucleosides; and a 3’ wing segment consisting of five linked nucleosides; wherein the gap segment is positioned between the 5’ wing segment and the 3’ wing segment; wherein each nucleoside of each wing segment comprises a 2’-O-methoxyethyl sugar; wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, each internucleoside linkage is a phosphorothioate linkage.

In certain embodiments, the modified oligonucleotide consists of 20 linked nucleosides with the nucleobase sequence of SEQ ID NO: 20 and comprises: a gap segment consisting of ten linked deoxynucleosides; a 5’ wing segment consisting of five linked nucleosides; a 3’ wing segment consisting of five linked nucleosides; wherein the gap segment is positioned between the 5’ wing segment and the 3’ wing segment; wherein each nucleoside of each wing segment comprises a 2’-O-methoxyethyl sugar; wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, each internucleoside linkage is a phosphorothioate linkage.

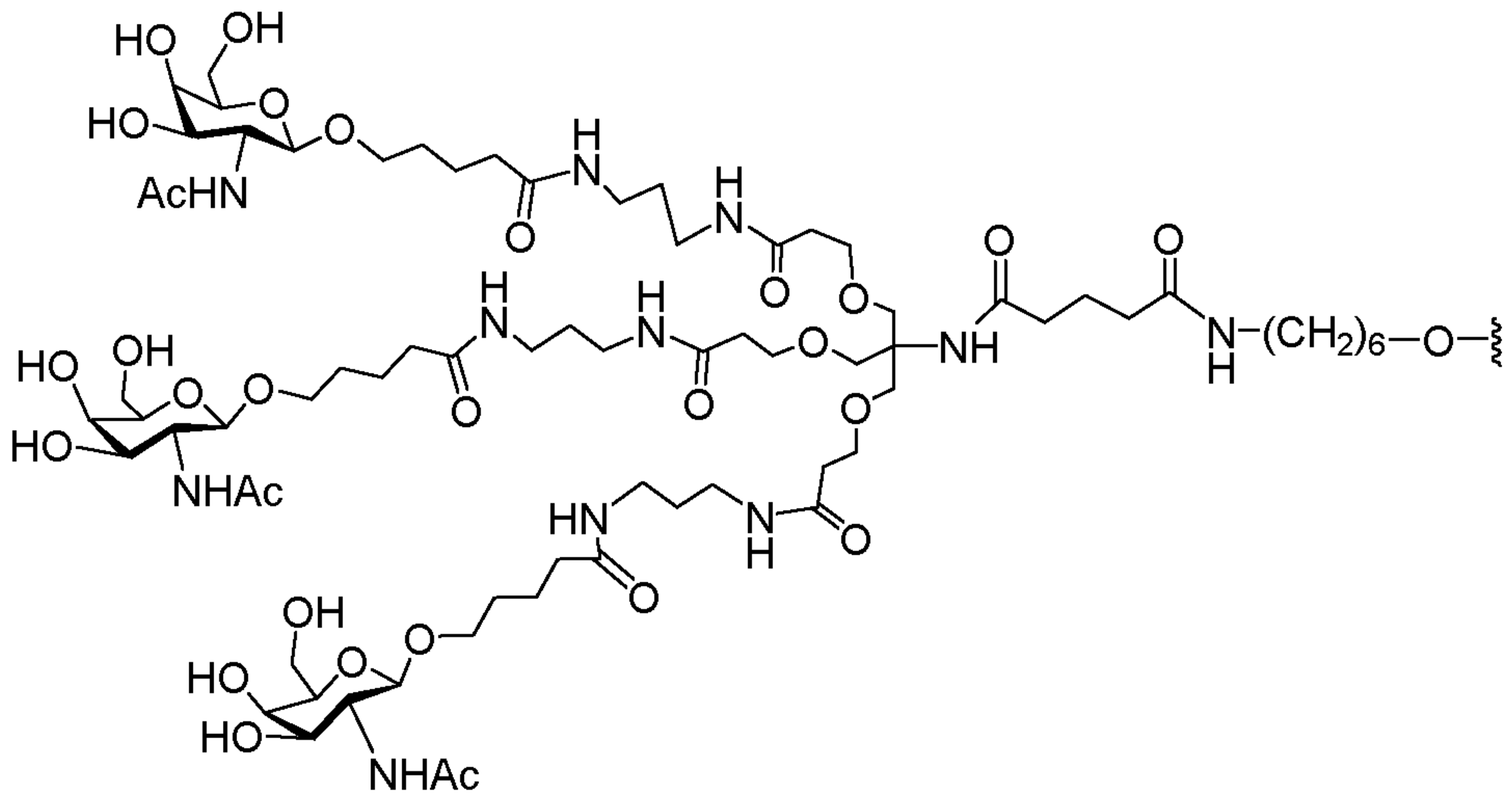
In certain embodiments, the compounds or compositions disclosed herein comprise a modified oligonucleotide consisting of 16 linked nucleosides having a nucleobase sequence comprising at least 8 contiguous nucleobases of a nucleobase sequence of SEQ ID NO: 110, wherein the modified oligonucleotide comprises: a gap segment consisting of ten linked deoxynucleosides; a 5’ wing segment consisting of three linked nucleosides; and a 3’ wing segment consisting of three linked nucleosides; wherein the gap segment is positioned between the 5’ wing segment and the 3’ wing segment; wherein each wing segment comprises at least one 2’-O-methoxyethyl sugar and at least one cEt sugar; wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, each internucleoside linkage is a phosphorothioate linkage.

In certain embodiments, the modified oligonucleotide consists of 16 linked nucleosides with the nucleobase sequence of SEQ ID NO: 110 and comprises: a gap segment consisting of ten linked deoxynucleosides; a 5' wing segment consisting of three linked nucleosides; a 3' wing segment consisting of three linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment; wherein each wing segment comprises at least one 2'-O-methoxyethyl sugar and at least one cEt sugar; wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, each internucleoside linkage is a phosphorothioate linkage.

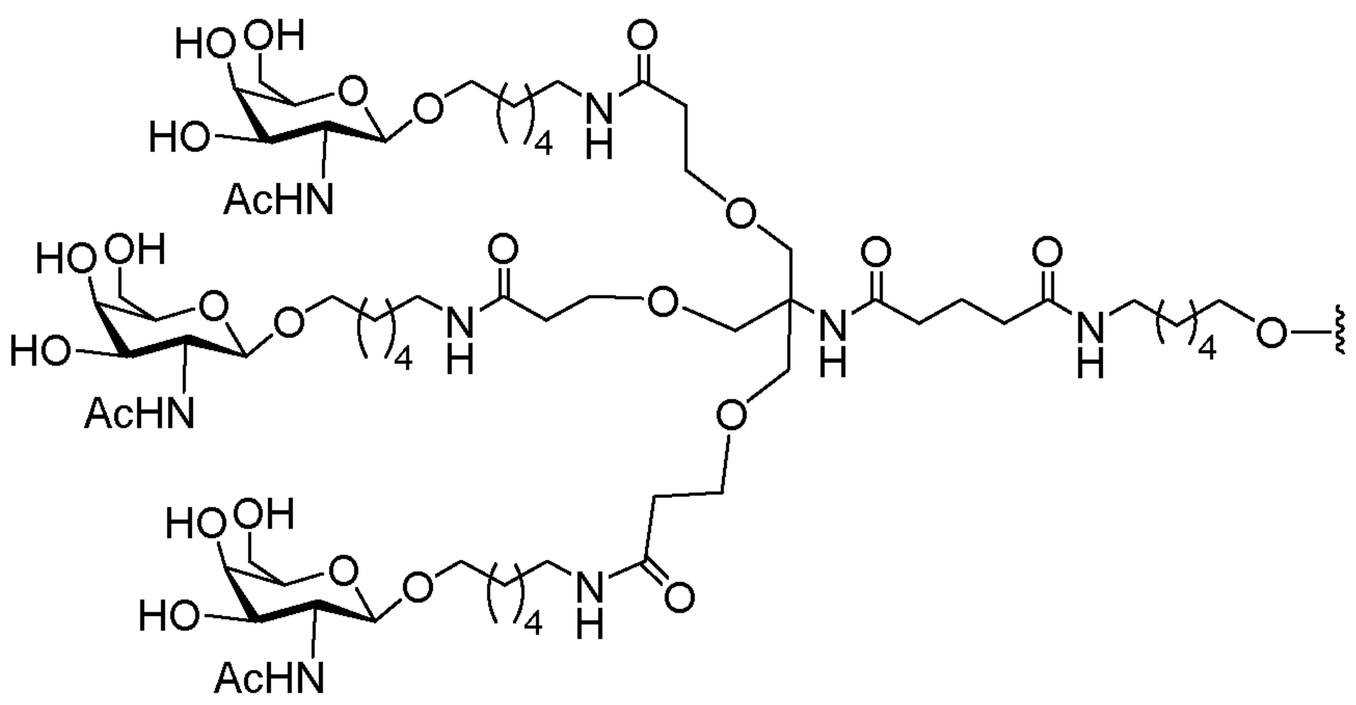
In certain embodiments, the conjugate group is linked to the modified oligonucleotide at the 5' end of the modified oligonucleotide. In certain embodiments, the conjugate group is linked to the modified oligonucleotide at the 3' end of the modified oligonucleotide.

In certain embodiments, the conjugate group comprises exactly one ligand. In certain embodiments, the conjugate group comprises one or more ligands. In certain embodiments, the conjugate group comprises exactly two ligands. In certain embodiments, the conjugate group comprises two or more ligands. In certain embodiments, the conjugate group comprises three or more ligands. In certain embodiments, the conjugate group comprises exactly three ligands. In certain embodiments, each ligand is selected from among: a polysaccharide, modified polysaccharide, mannose, galactose, a mannose derivative, a galactose derivative, D-mannopyranose, L-Mannopyranose, D-Arabinose, L-Galactose, D-xylofuranose, L-xylofuranose, D-glucose, L-glucose, D-Galactose, L-Galactose, α-D-Mannofuranose, β-D-Mannofuranose, α-D-Mannopyranose, β-D-Mannopyranose, α-D-Glucopyranose, β-D-Glucopyranose, α-D-Glucofuranose, β-D-Glucofuranose, α-D-fructofuranose, α-D-fructopyranose, α-D-Galactopyranose, β -D-Galactopyranose, α-D-Galactofuranose, β -D-Galactofuranose, glucosamine, sialic acid, α-D-galactosamine, N-Acetylgalactosamine, 2-Amino-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-β-D-glucopyranose, 2-Deoxy-2-methylamino-L-glucopyranose, 4,6-Dideoxy-4-formamido-2,3-di-*O*-methyl-D-mannopyranose, 2-Deoxy-2-sulfoamino-D-glucopyranose, *N*-Glycoloyl-α-neuraminic acid, 5-thio-β-D-glucopyranose, methyl 2,3,4-tri-*O*-acetyl-1-thio-6-*O*-trityl-α-D-glucopyranoside, 4-Thio-β-D-galactopyranose, ethyl 3,4,6,7-tetra-*O*-acetyl-2-deoxy-1,5-dithio-α-D-*gluco*-heptopyranoside, 2,5-Anhydro-D-allononitrile, ribose, D-ribose, D-4-thioribose, L-ribose, L-4-thioribose. In certain embodiments, each ligand is N-acetyl galactosamine.

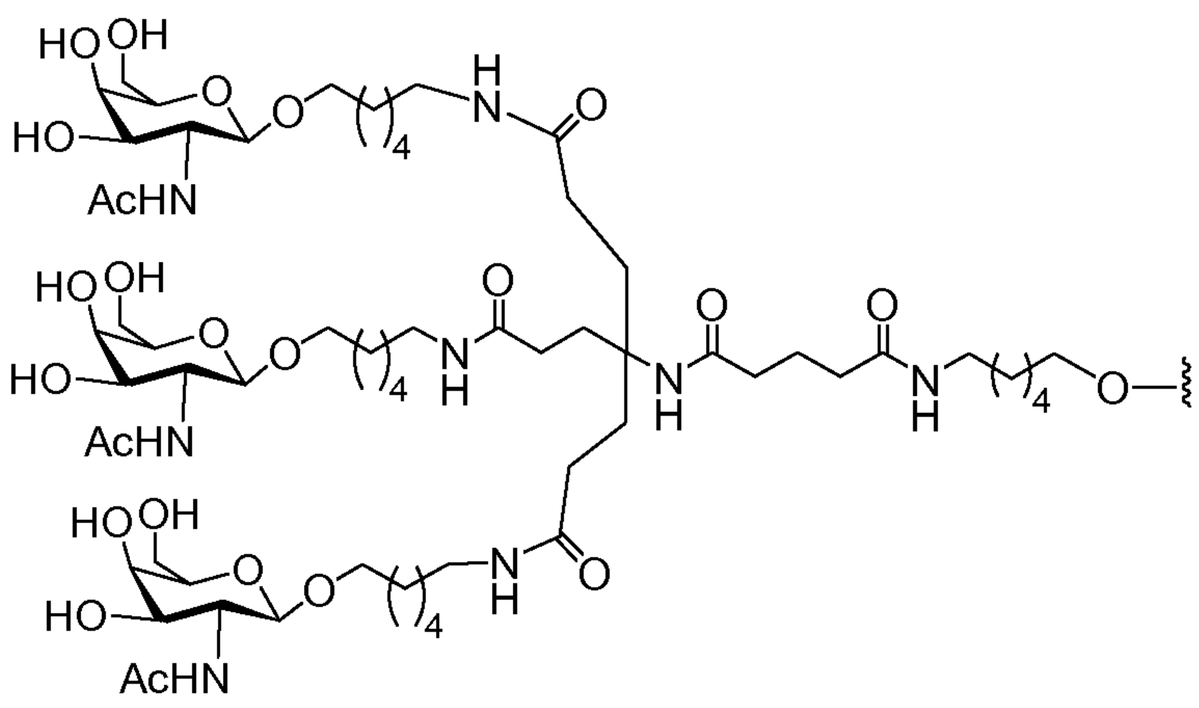
In certain embodiments, the conjugate group comprises:



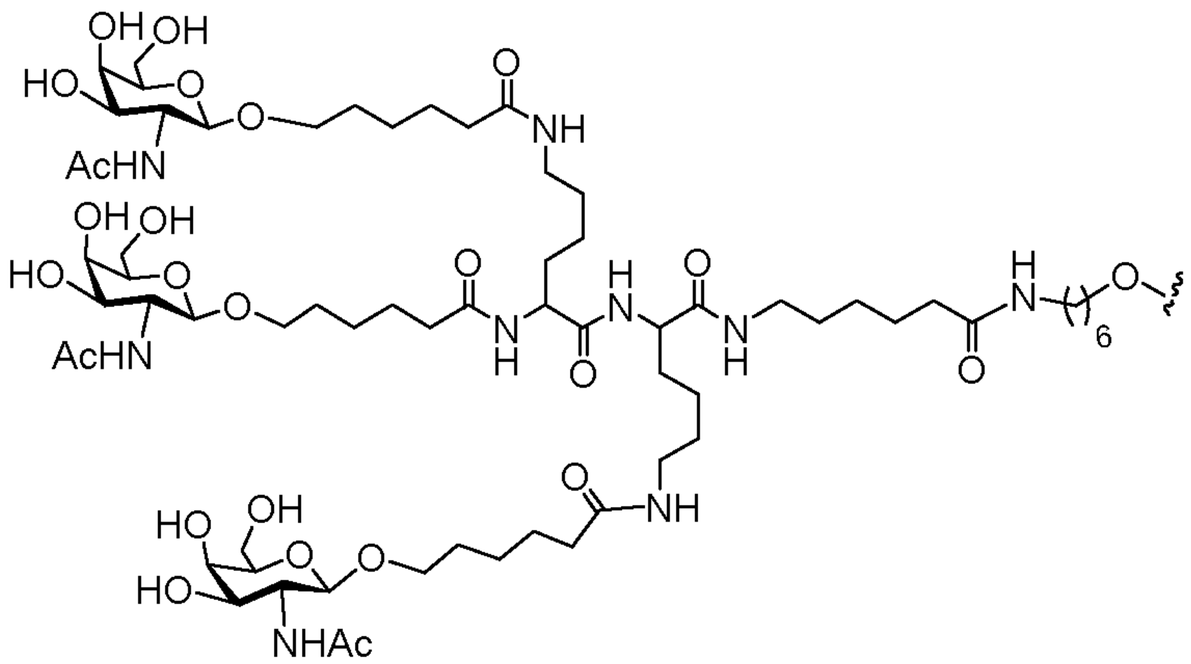
In certain embodiments, the conjugate group comprises:



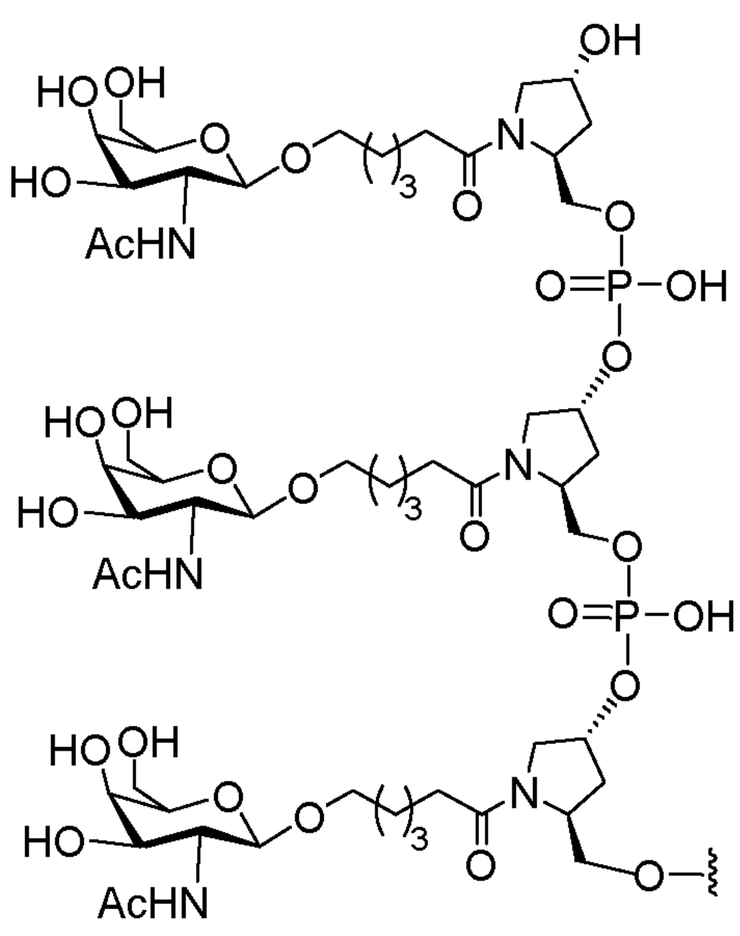
In certain embodiments, the conjugate group comprises:



In certain embodiments, the conjugate group comprises:

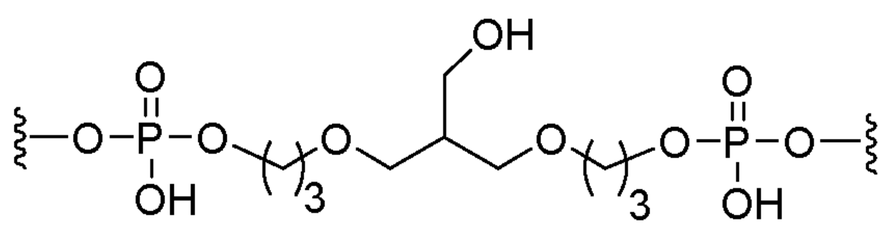


In certain embodiments, the conjugate group comprises:



In certain embodiments, the conjugate group comprises at least one phosphorus linking group or neutral linking group.

In certain embodiments, the conjugate group comprises a structure selected from among: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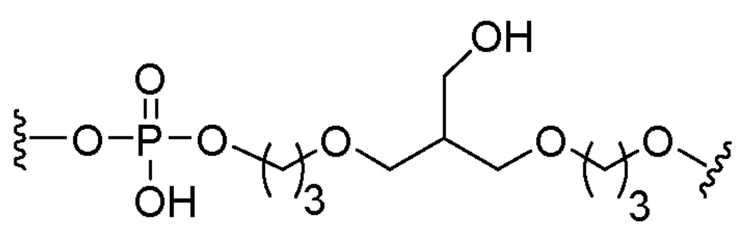
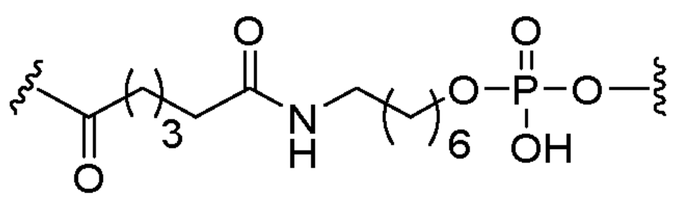
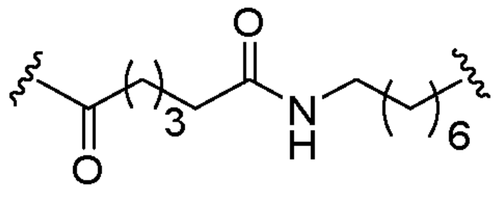
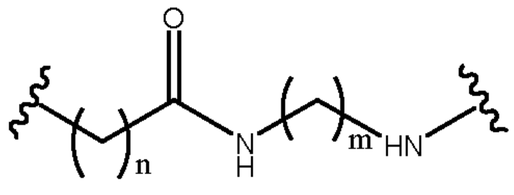
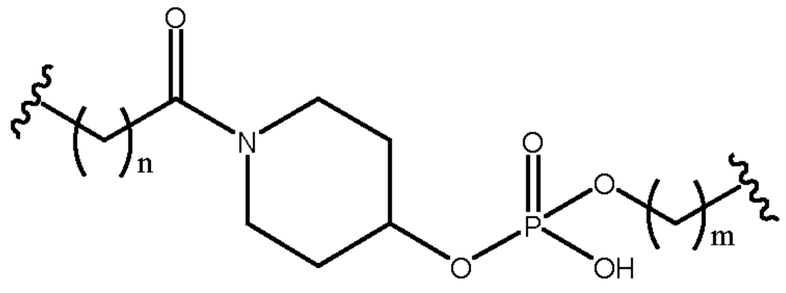
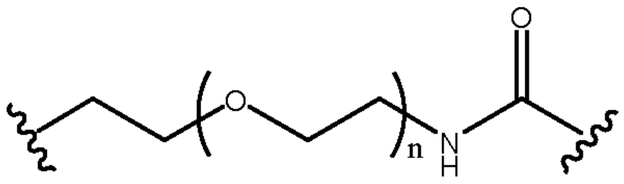
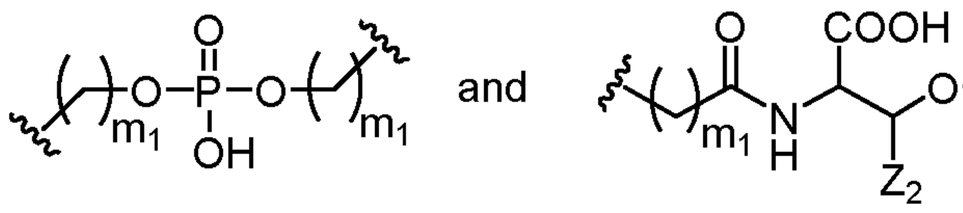


wherein n is from 1 to 12; and

wherein m is from 1 to 12.

In certain embodiments, the conjugate group has a tether having a structure selected from among:



wherein L is either a phosphorus linking group or a neutral linking group;

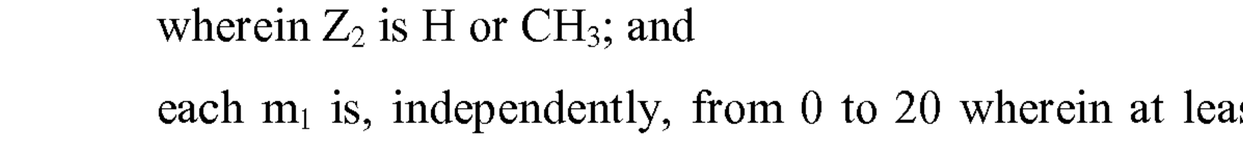
Z_1 is $C(=O)O\text{-}R_2$;

Z_2 is H, C_1-C_6 alkyl or substituted C_1-C_6 alky;

R_2 is H, C_1-C_6 alkyl or substituted C_1-C_6 alky; and

each m_1 is, independently, from 0 to 20 wherein at least one m_1 is greater than 0 for each tether.

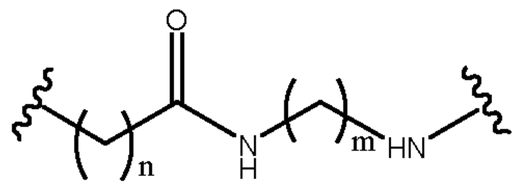
In certain embodiments, the conjugate group has a tether having a structure selected from among:



wherein Z_2 is H or CH_3; and

each m_1 is, independently, from 0 to 20 wherein at least one m_1 is greater than 0 for each tether.

In certain embodiments, the conjugate group has tether having a structure selected from among: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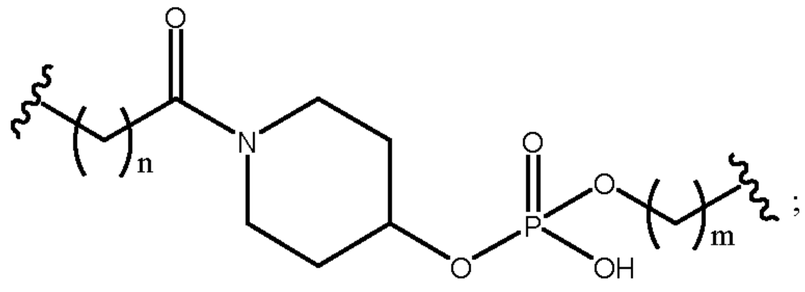
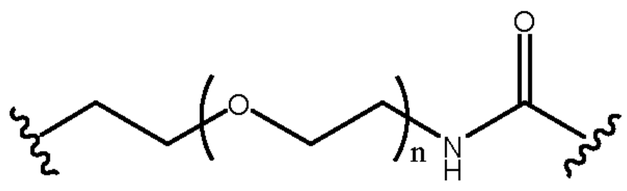
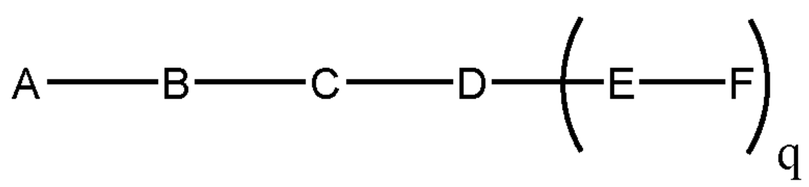

;

;

wherein n is from 1 to 12; and

wherein m is from 1 to 12.

In certain embodiments, the conjugate group is covalently attached to the modified oligonucleotide.

In certain embodiments, the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

B is the cleavable moiety

C is the conjugate linker

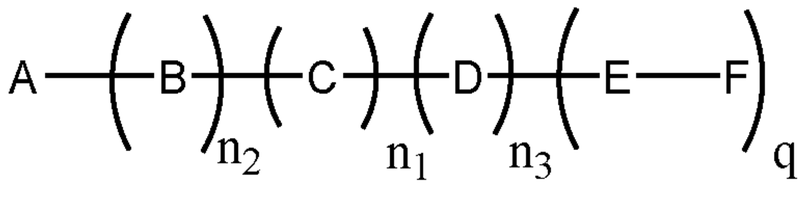
D is the branching group

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

In certain embodiments, the compound has a structure represented by the formula:



wherein:

A is the modified oligonucleotide;

B is the cleavable moiety

C is the conjugate linker

D is the branching group

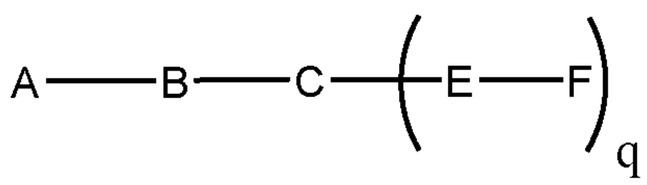
each E is a tether;

each F is a ligand;

each n is independently 0 or 1; and

q is an integer between 1 and 5.

In certain embodiments, the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

B is the cleavable moiety;

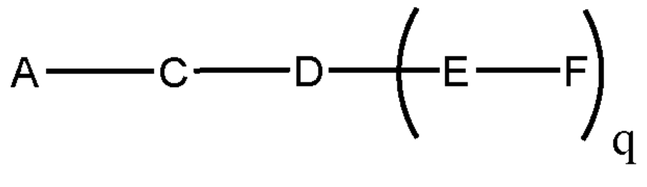
C is the conjugate linker;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

In certain embodiments, the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

C is the conjugate linker;

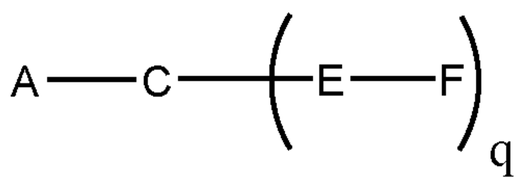
D is the branching group;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

In certain embodiments, the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

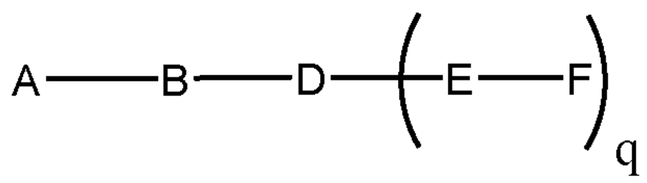
C is the conjugate linker;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

In certain embodiments, the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

B is the cleavable moiety;

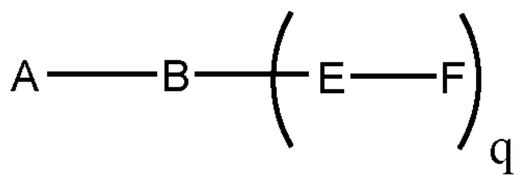
D is the branching group;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

In certain embodiments, the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

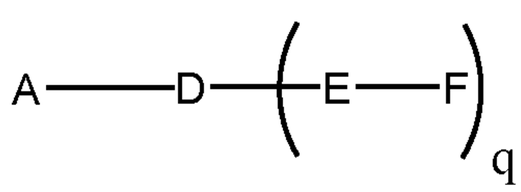
B is the cleavable moiety;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

In certain embodiments, the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

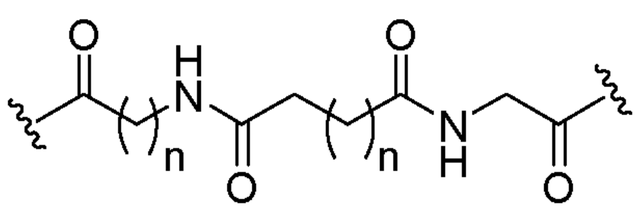
D is the branching group;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

In certain embodiments, the conjugate linker has a structure selected from among: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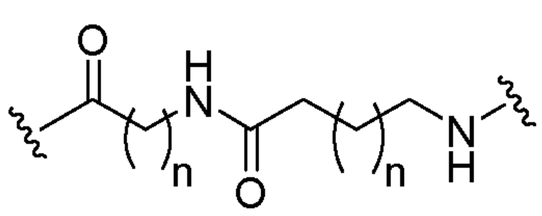
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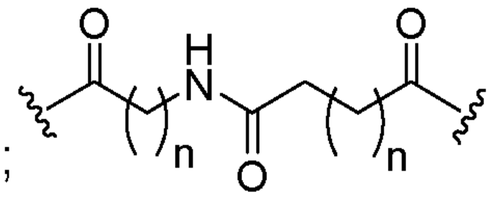
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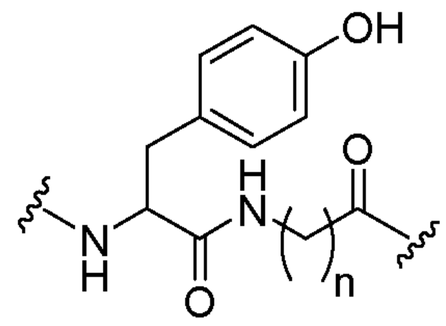
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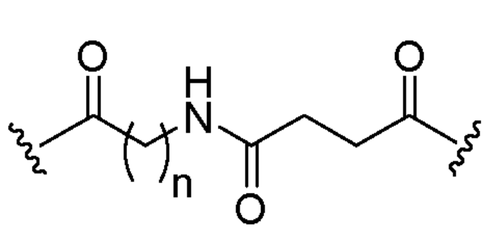
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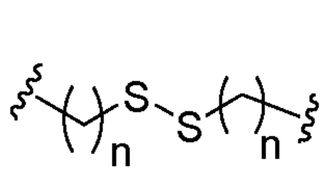
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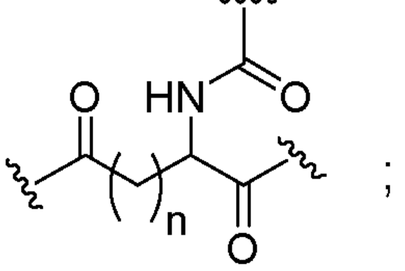
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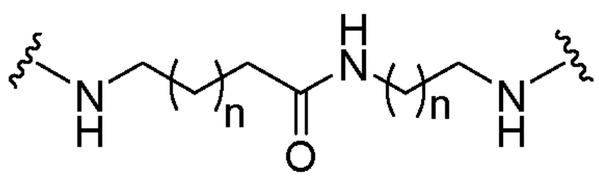
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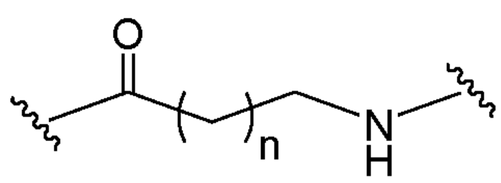
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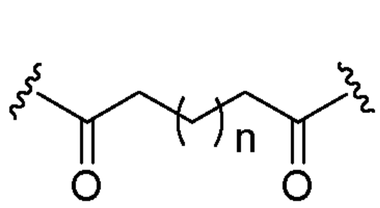
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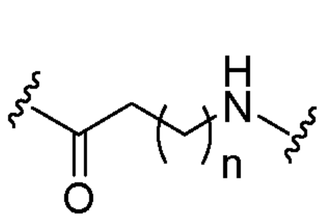
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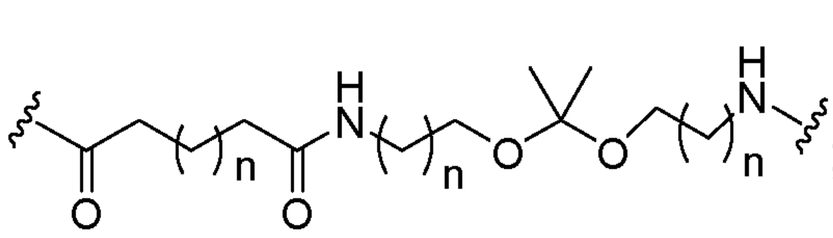
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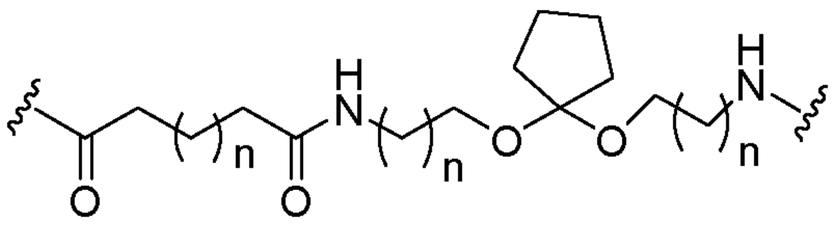
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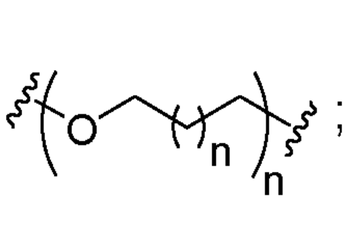
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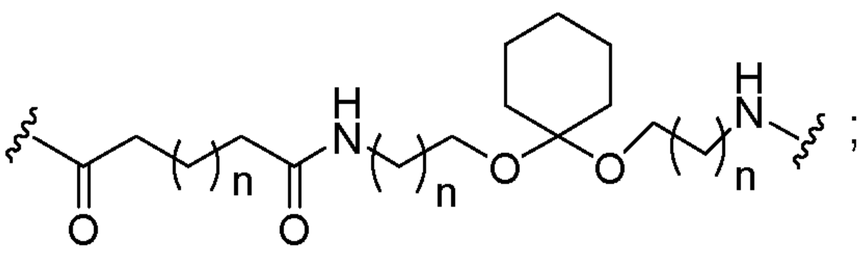
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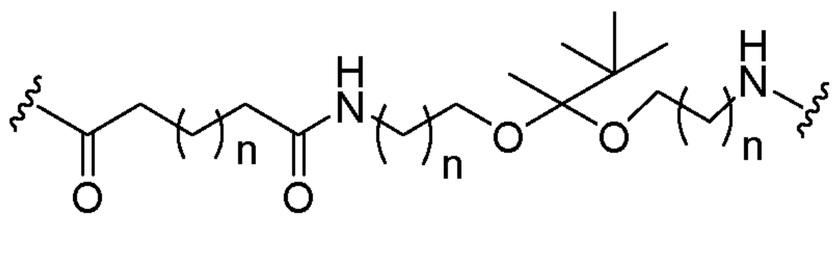
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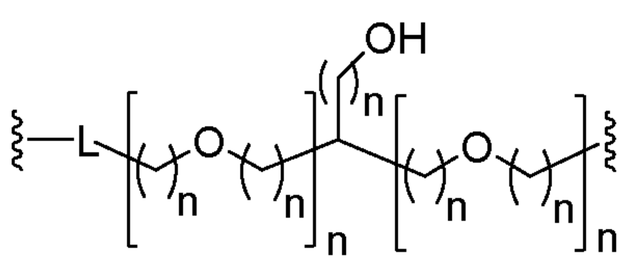
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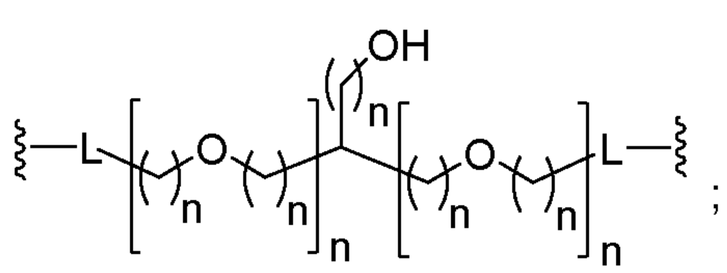
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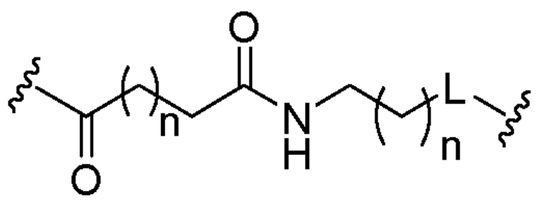
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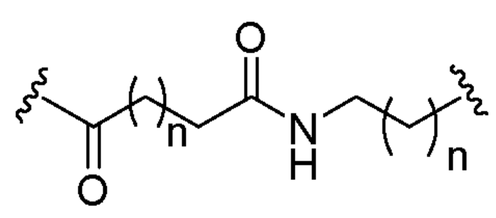
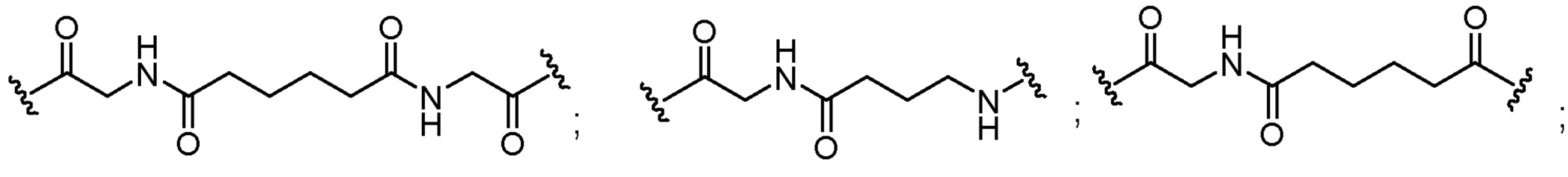
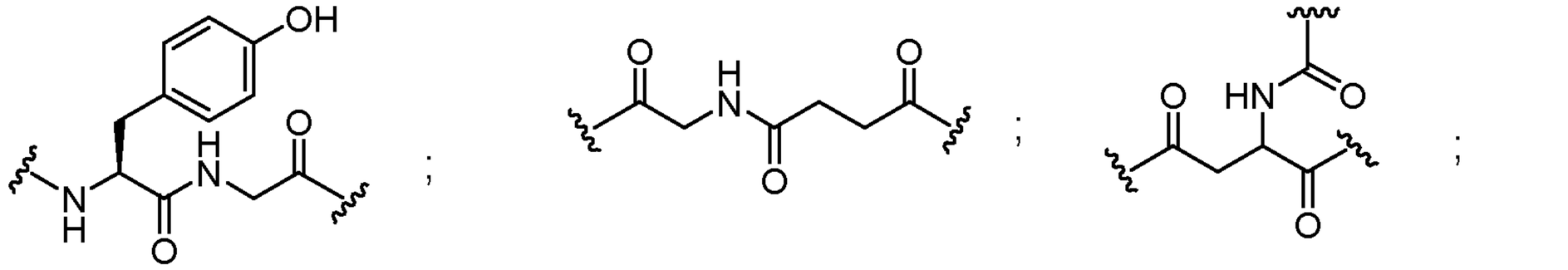

.

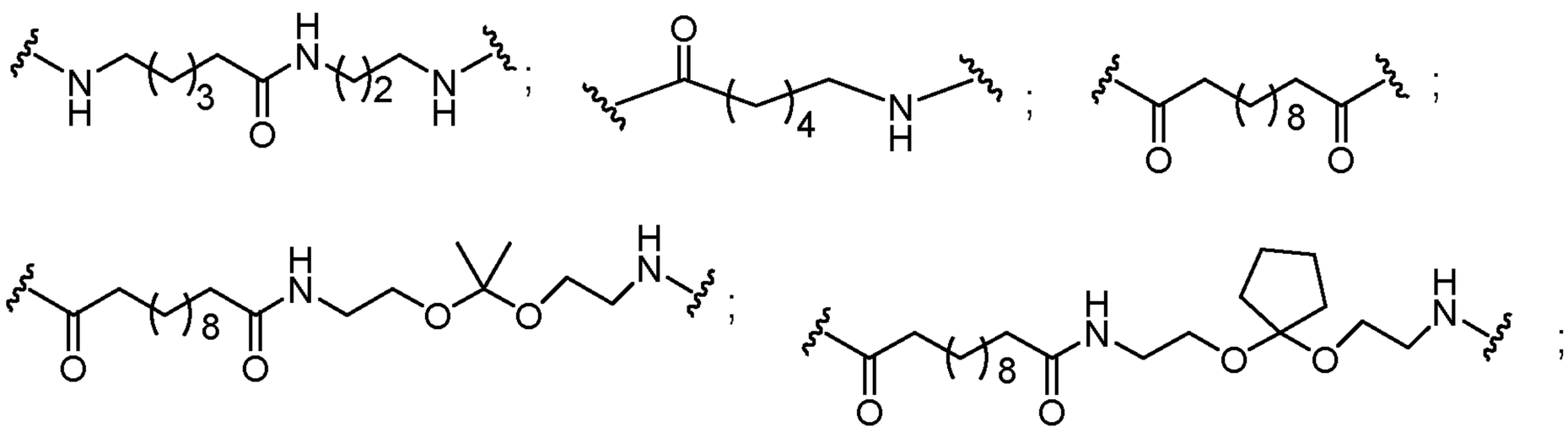
wherein each L is, independently, a phosphorus linking group or a neutral linking group; and

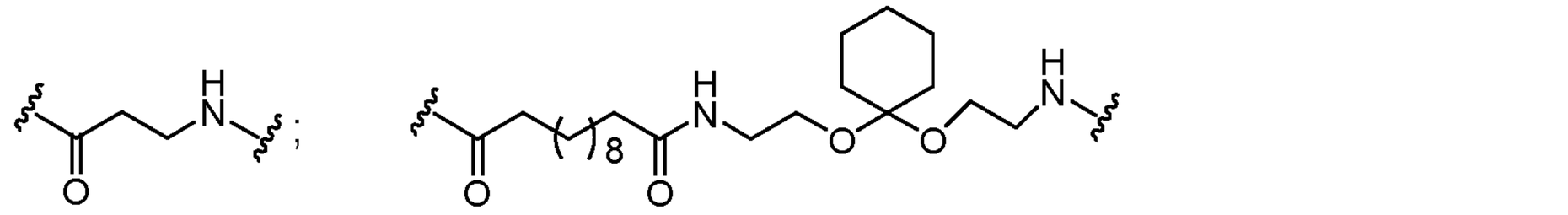
each n is, independently, from 1 to 20.

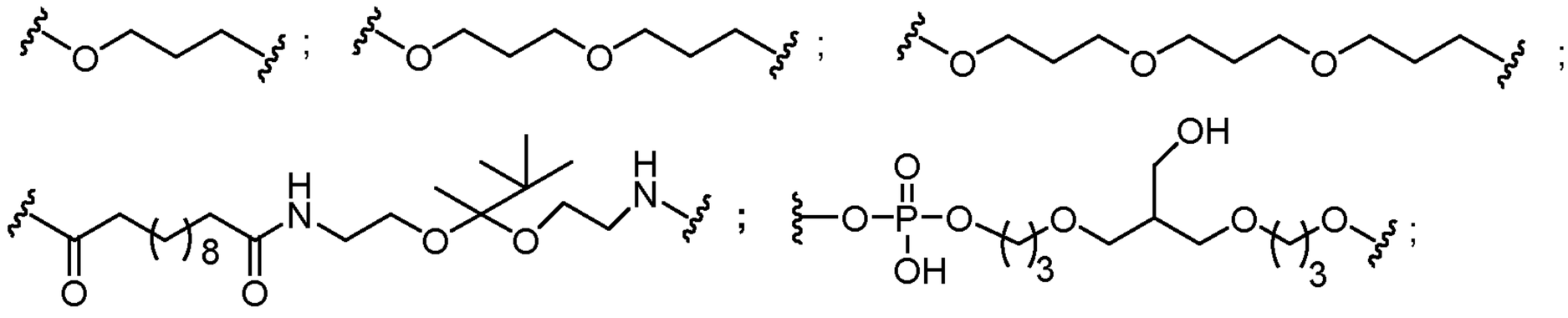
In certain embodiments, the conjugate linker has a structure selected from among:



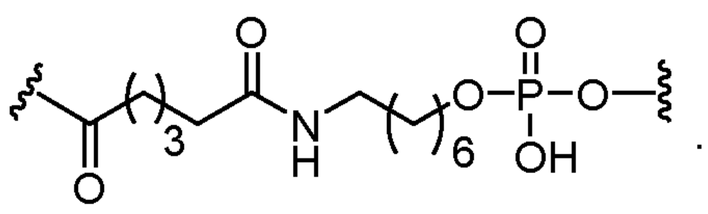






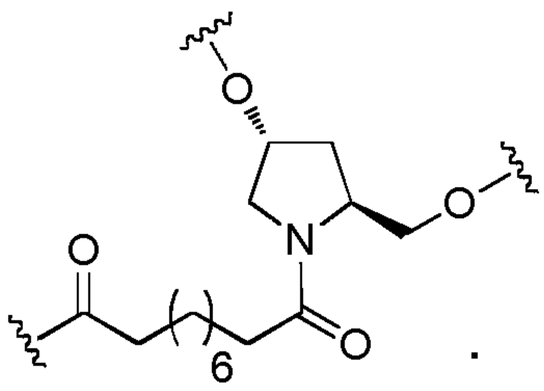


and



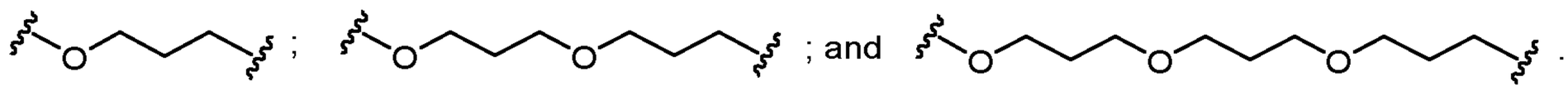
.

In certain embodiments, the conjugate linker has the followingstructure:

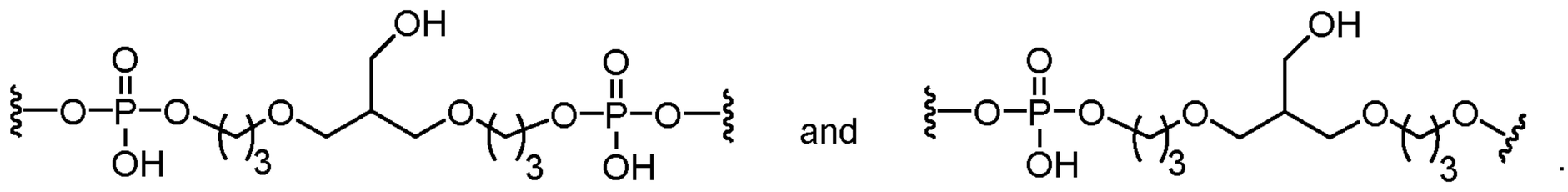


.

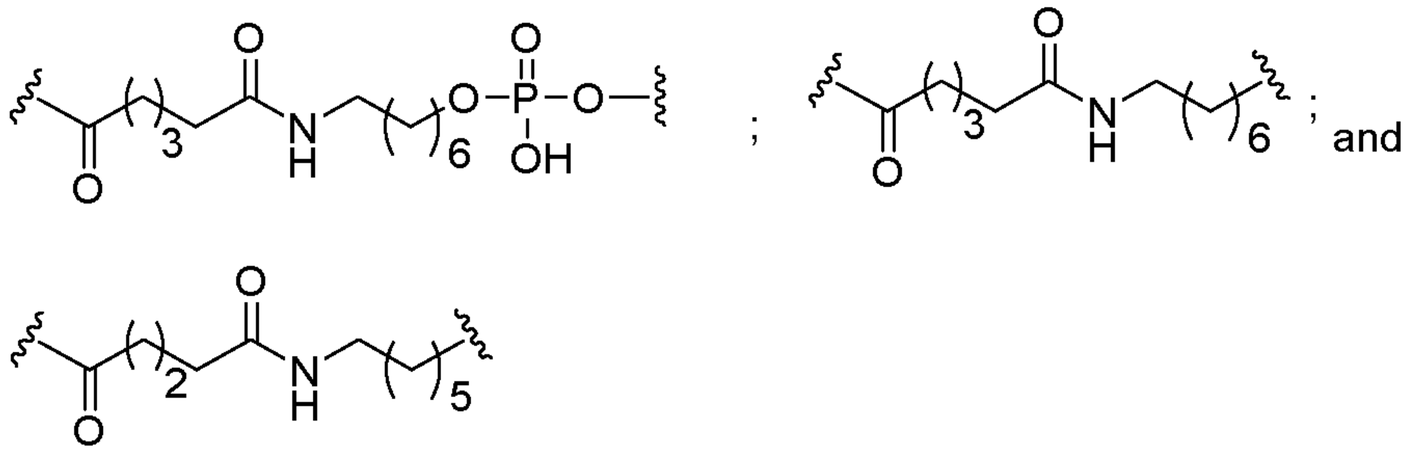
In certain embodiments, the conjugate linker has a structure selected from among:



In certain embodiments, the conjugate linker has a structure selected from among:



In certain embodiments, the conjugate linker has a structure selected from among:



In certain embodiments, the conjugate linker comprises a pyrrolidine. In certain embodiments, the conjugate linker does not comprise a pyrrolidine.

In certain embodiments, the conjugate linker comprises PEG.

In certain embodiments, the conjugate linker comprises an amide. In certain embodiments, the conjugate linker comprises at least two amides. In certain embodiments, the conjugate linker does not comprise an amide. In certain embodiments, the conjugate linker comprises a polyamide.

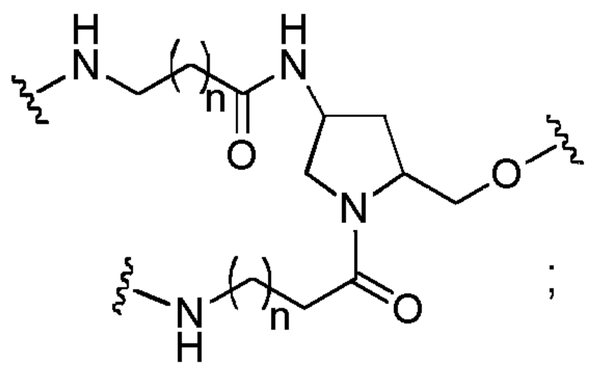
In certain embodiments, the conjugate linker comprises an amine.

In certain embodiments, the conjugate linker comprises one or more disulfide bonds.

In certain embodiments, the conjugate linker comprises a protein binding moiety. In certain embodiments, the protein binding moiety comprises a lipid. In certain embodiments, the protein binding moiety is selected from among: cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid,

dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine), a vitamin (e.g., folate, vitamin A, vitamin E, biotin, pyridoxal), a peptide, a carbohydrate (e.g., monosaccharide, disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, polysaccharide), an endosomolytic component, a steroid (e.g., uvaol, hecigenin, diosgenin), a terpene (e.g., triterpene, e.g., sarsasapogenin, friedelin, epifriedelanol derivatized lithocholic acid), or a cationic lipid. In certain embodiments, the protein binding moiety is selected from among: a C16 to C22 long chain saturated or unsaturated fatty acid, cholesterol, cholic acid, vitamin E, adamantane or 1-pentafluoropropyl.

In certain embodiments, the conjugate linker has a structure selected from among: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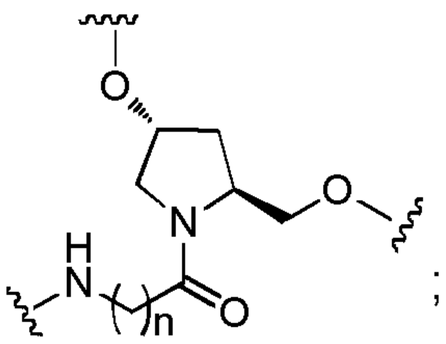
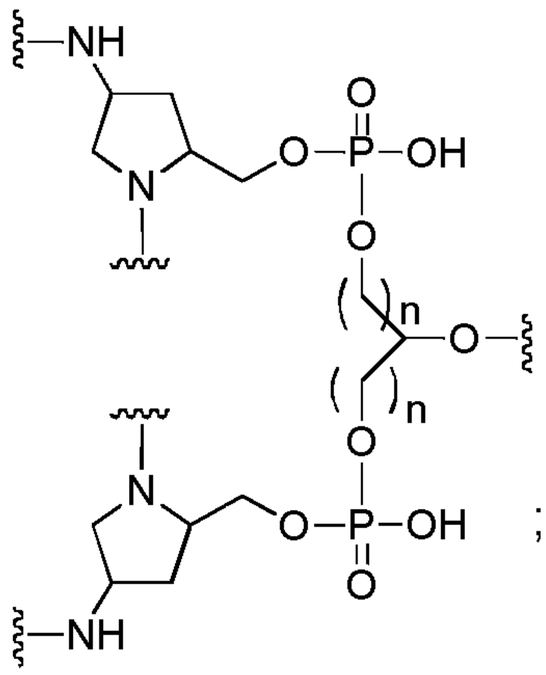

;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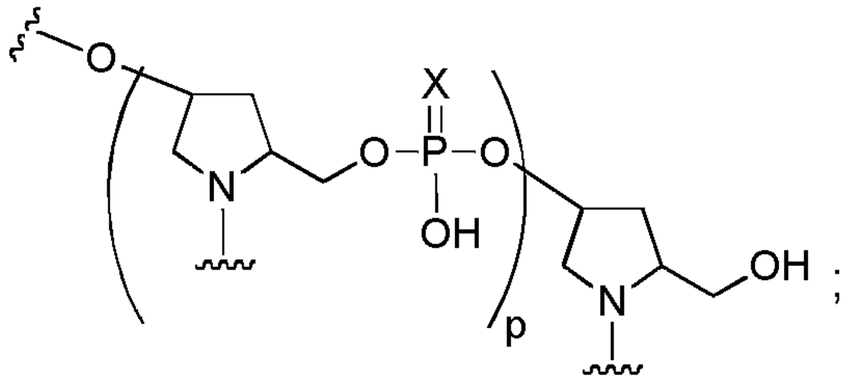
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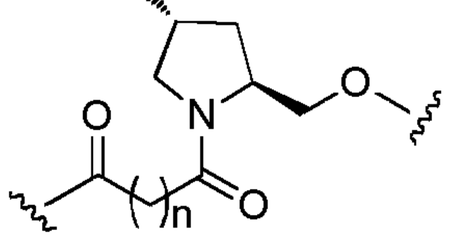
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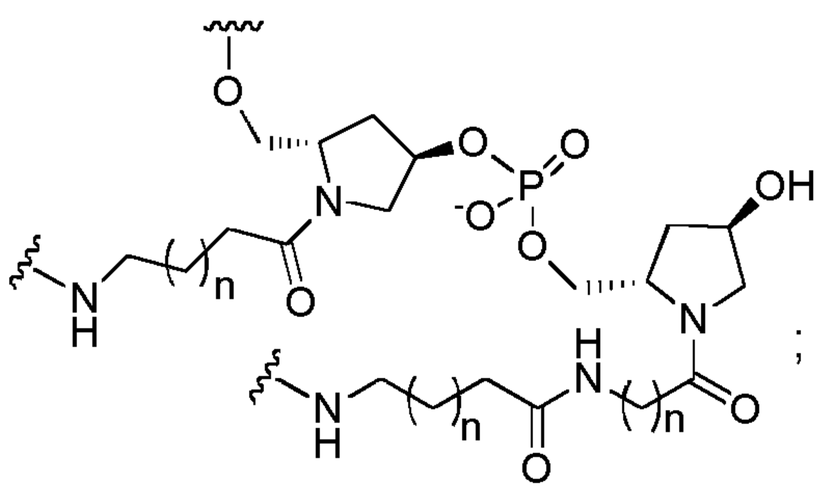
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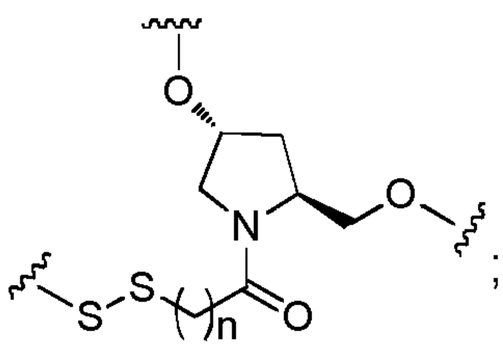
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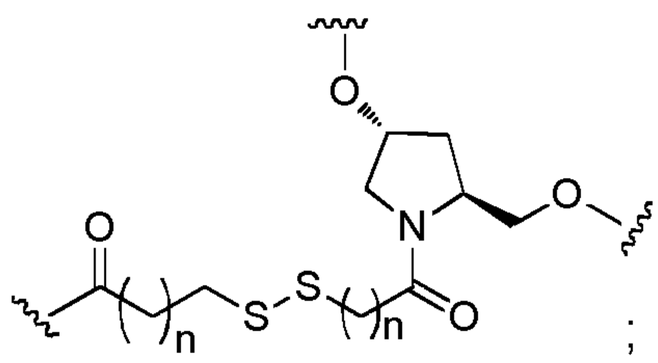
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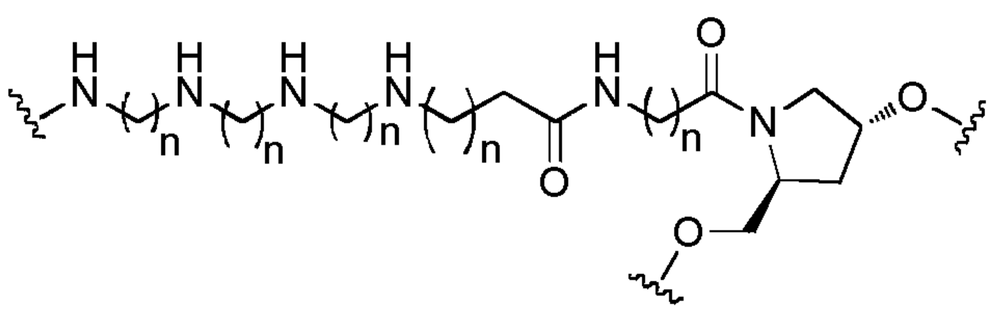
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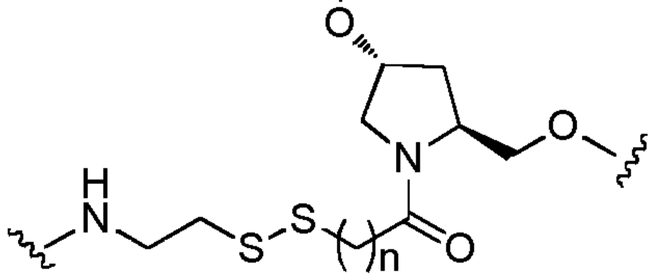
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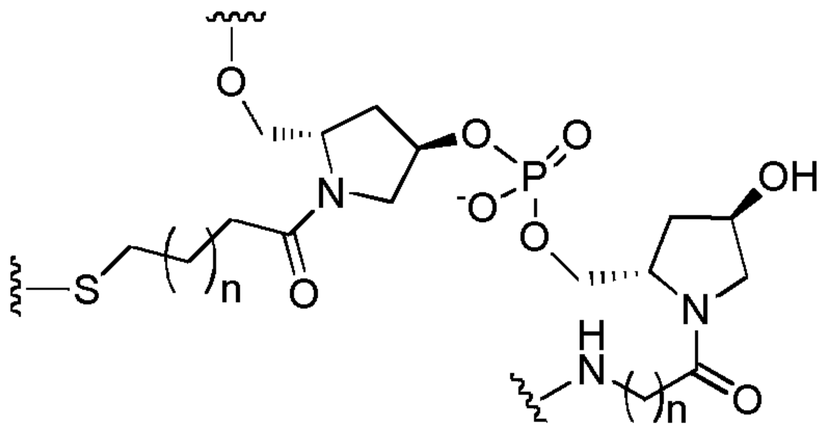
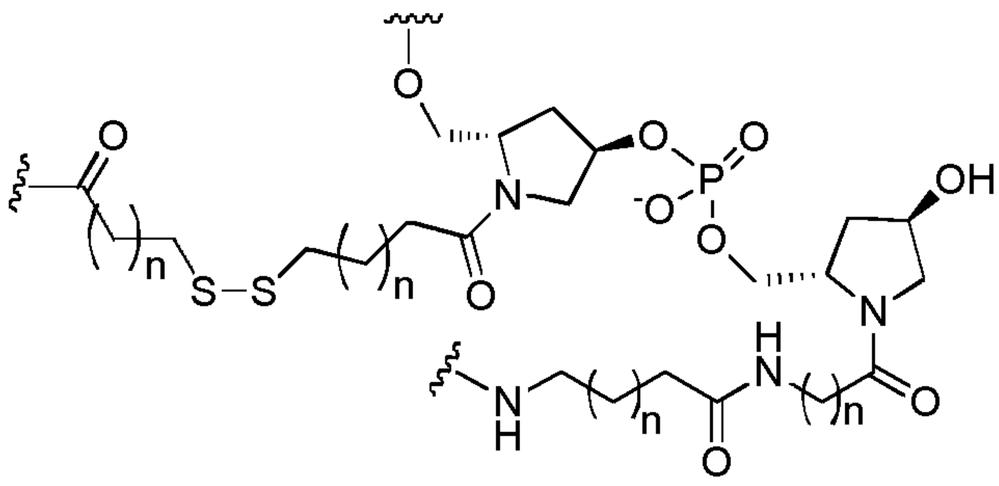
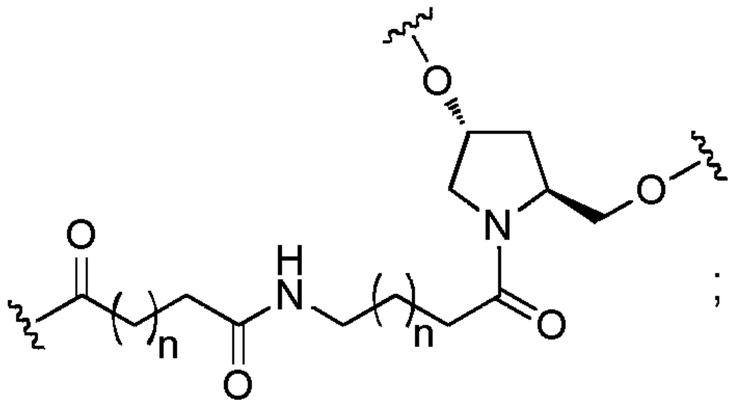

; and



wherein each n is, independently, is from 1 to 20; and p is from 1 to 6.

In certain embodiments, the conjugate linker has a structure selected from among:



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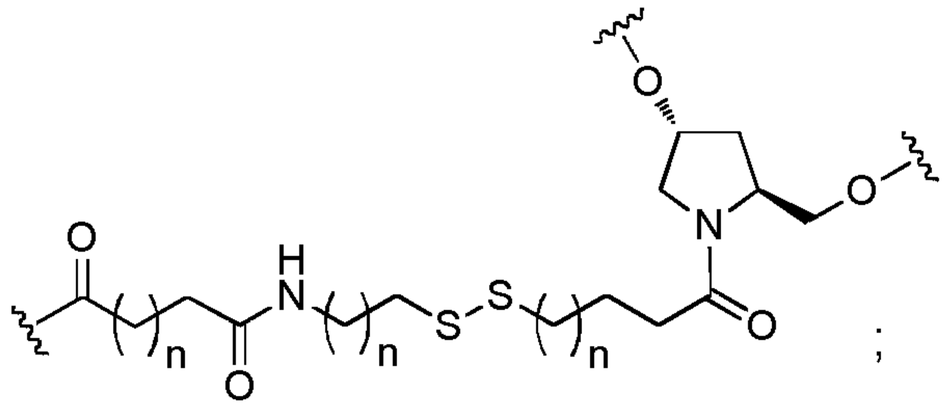

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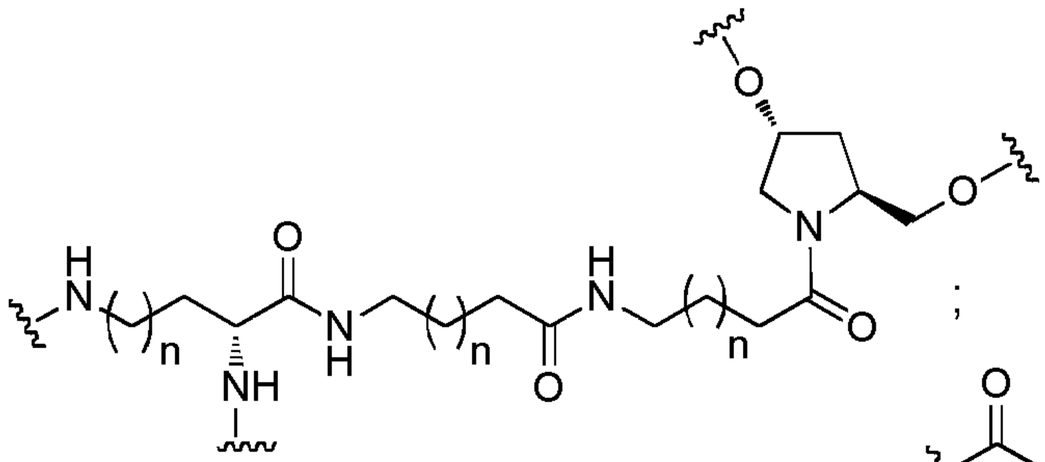
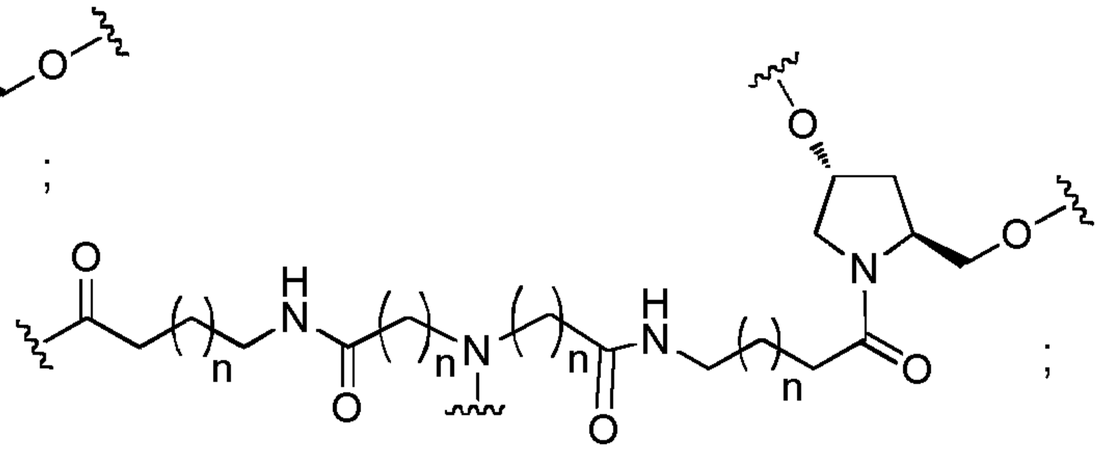


;



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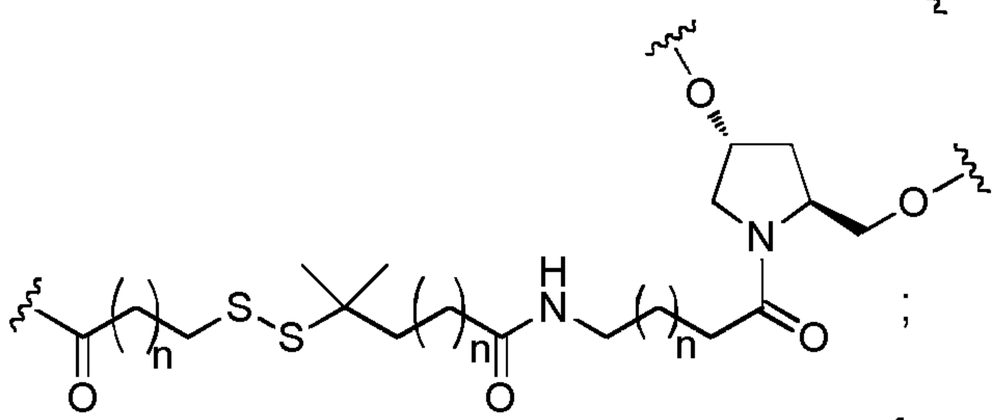

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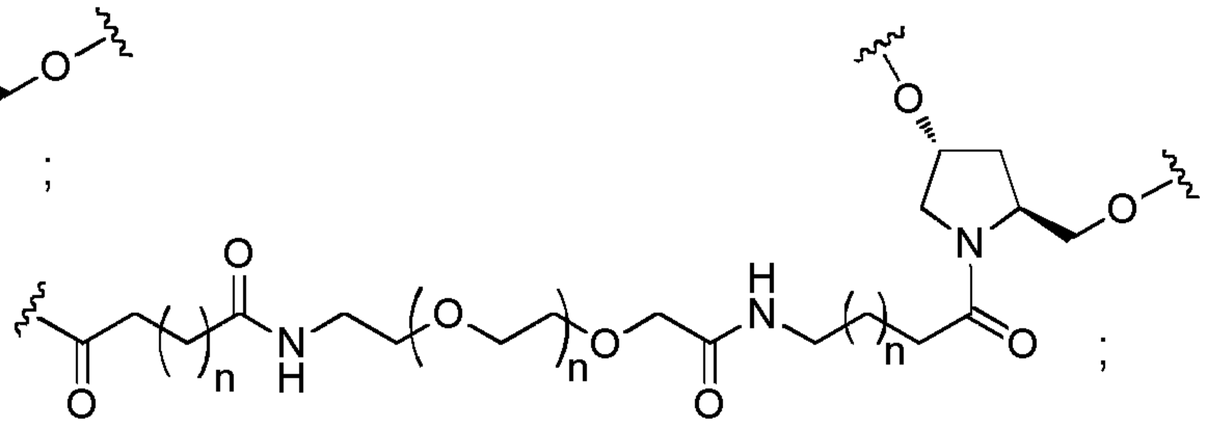

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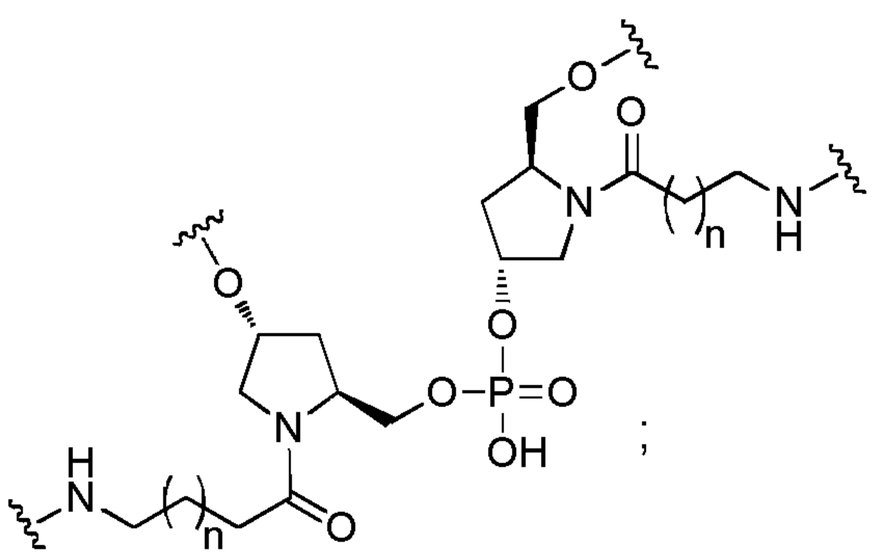
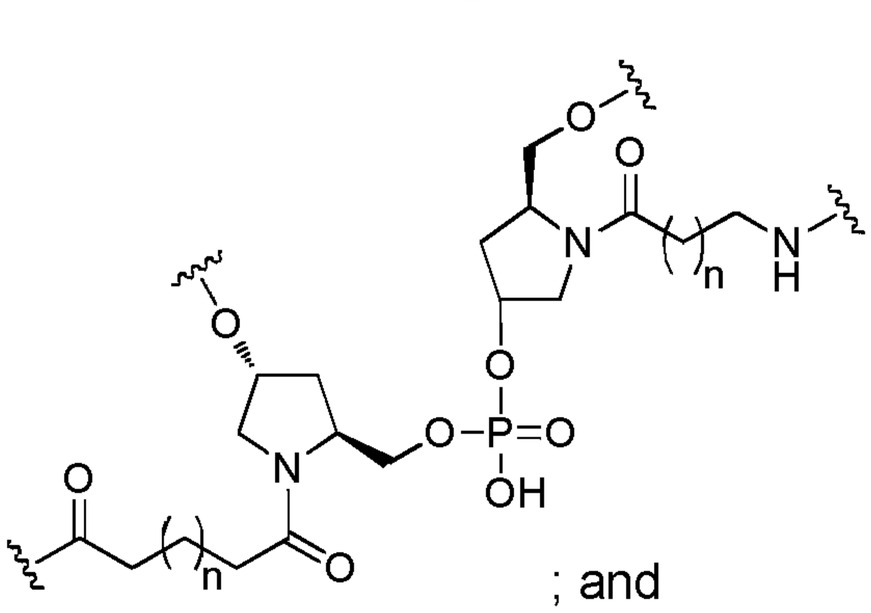
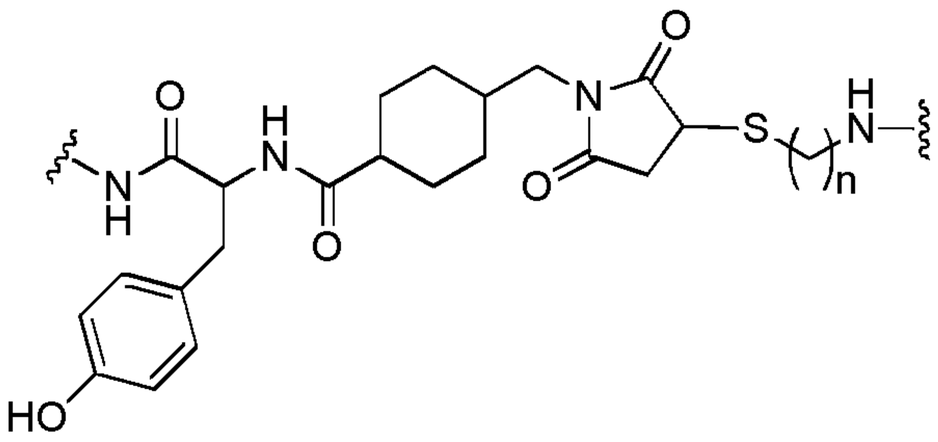


;

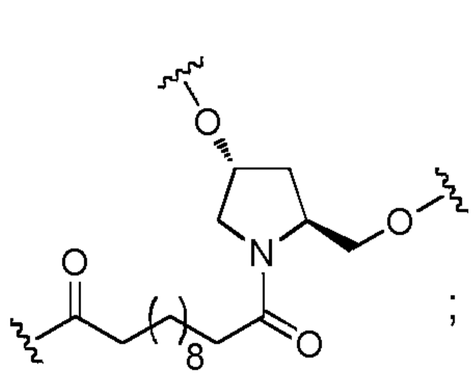


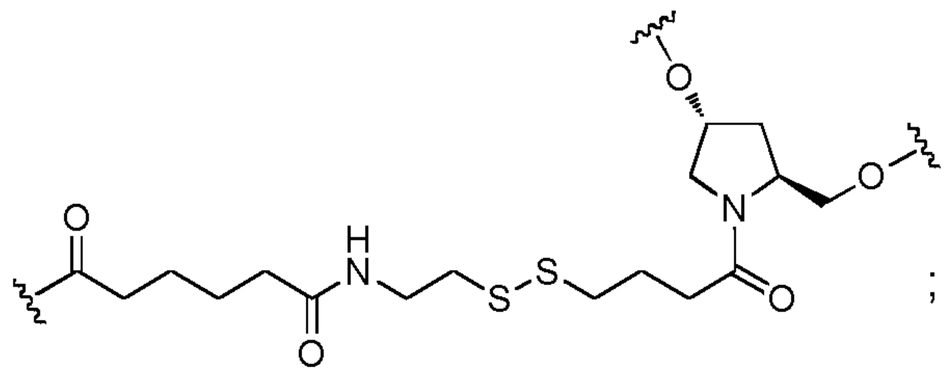
; and

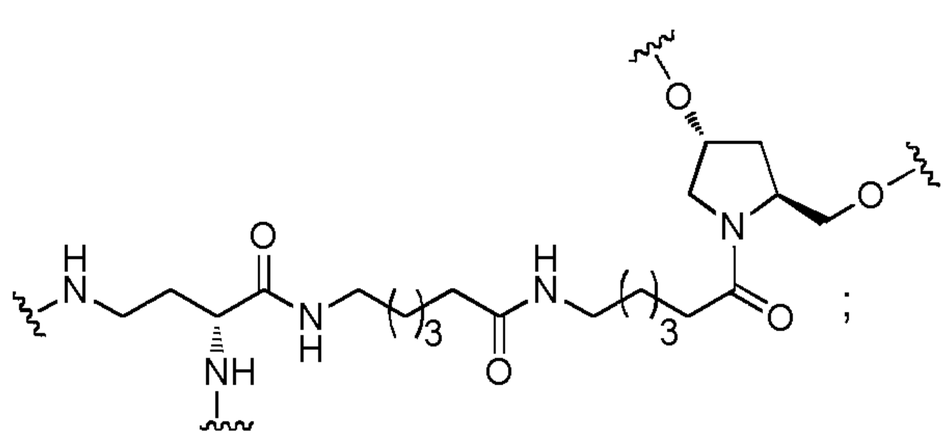


wherein each n is, independently, from 1 to 20.

In certain embodiments, the conjugate linker has a structure selected from among:


;


;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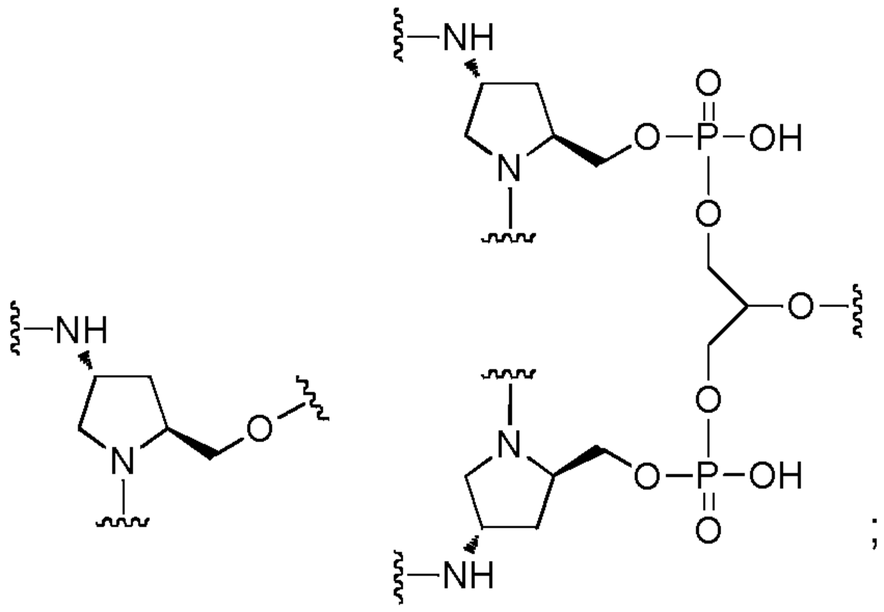
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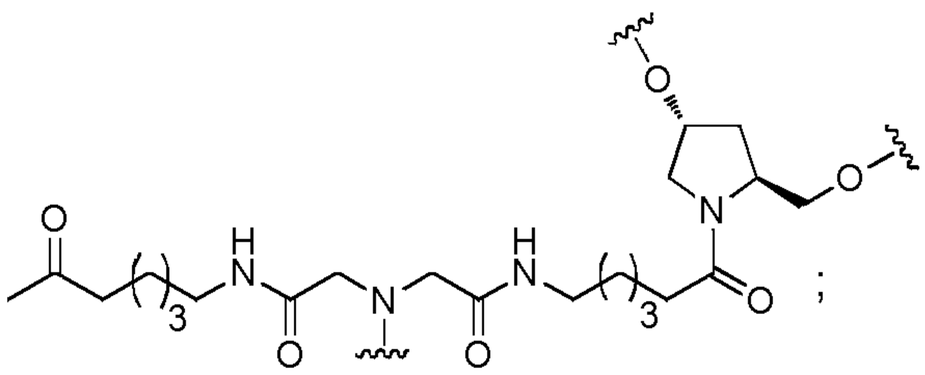
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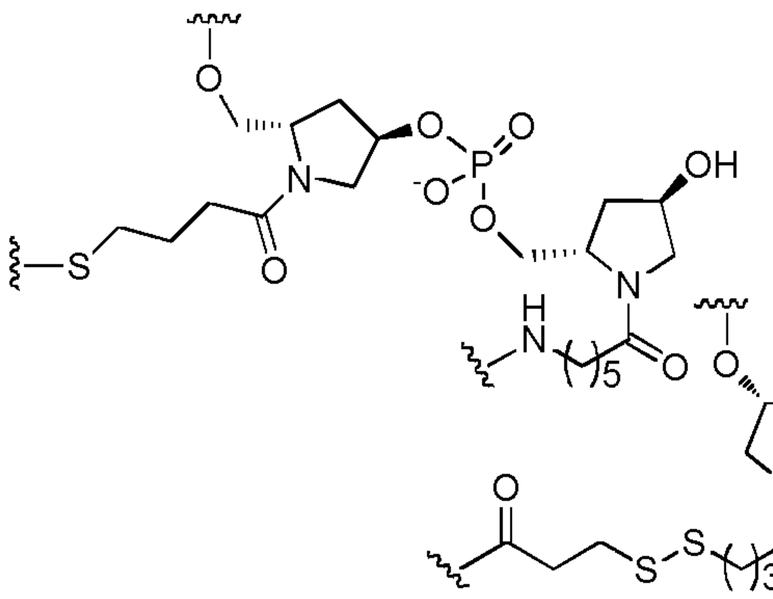
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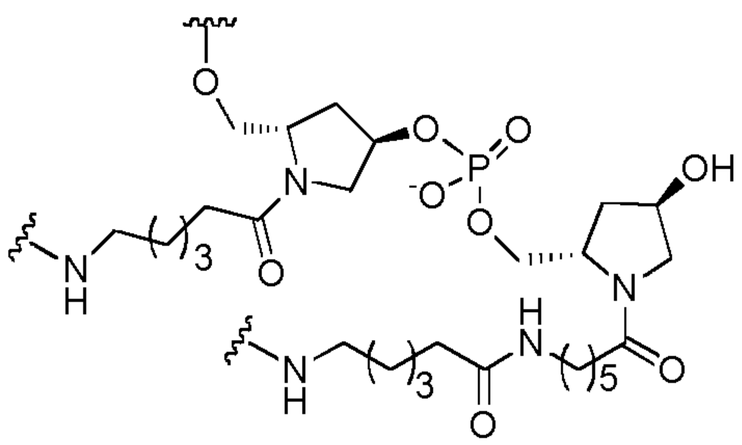
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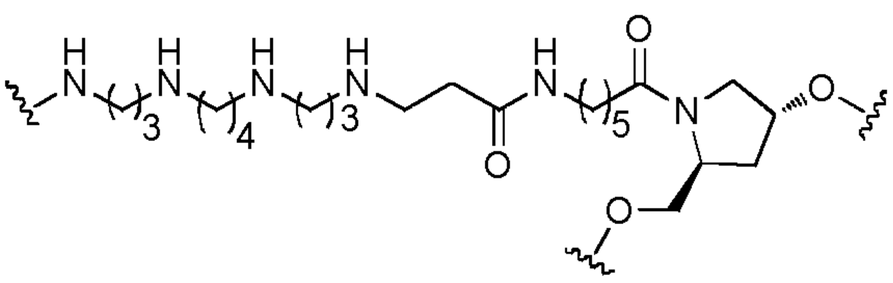
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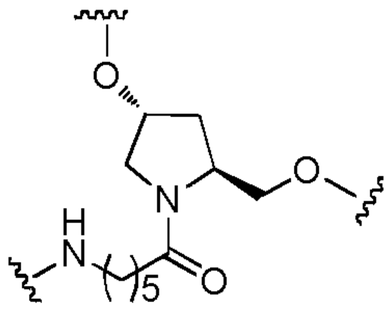
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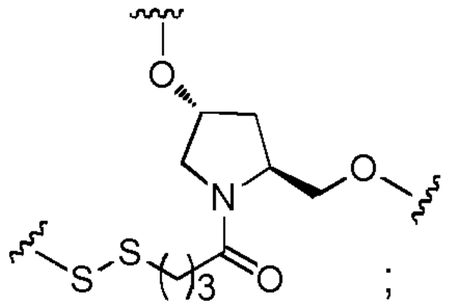
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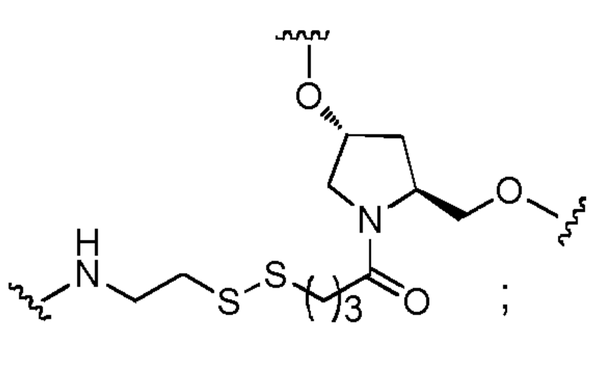
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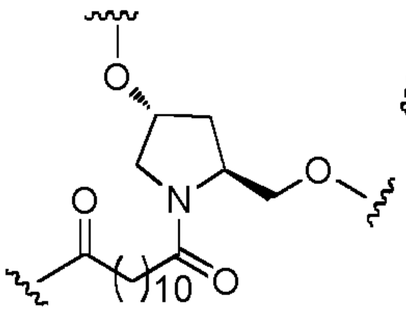
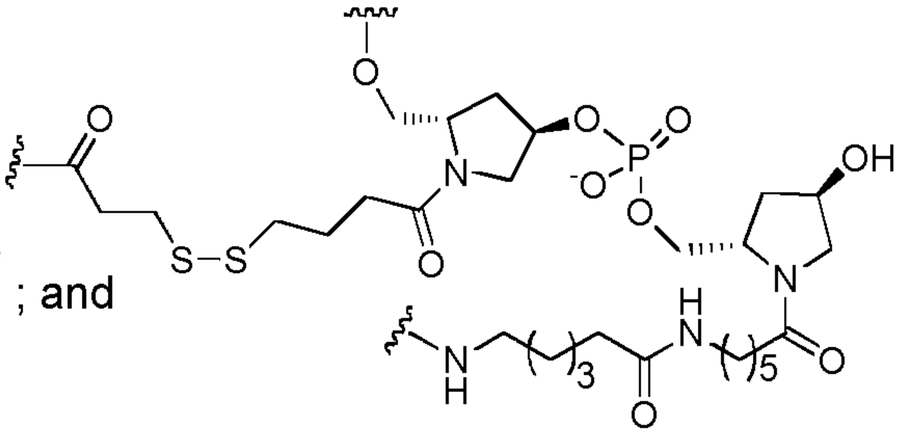
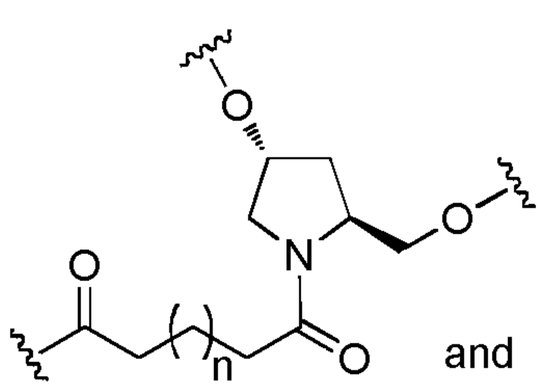

; and


.

5

In certain embodiments, the conjugate linker has a structure selected from among: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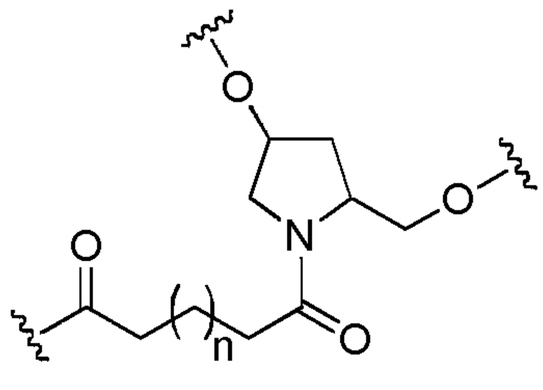
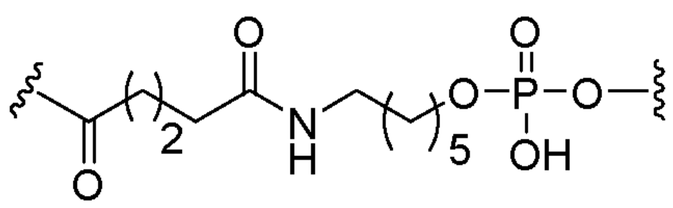
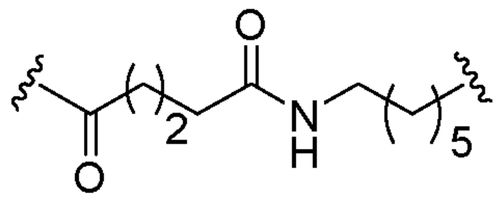
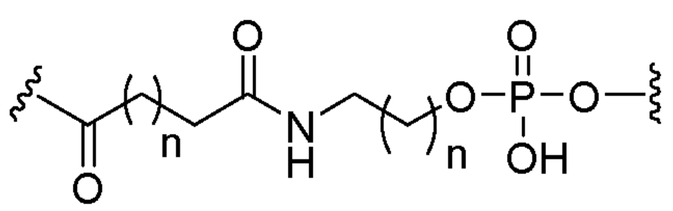
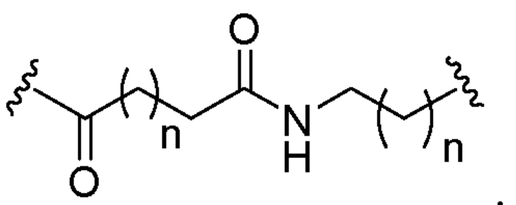


wherein n is from 1 to 20.

In certain embodiments, the conjugate linker has a structure selected from among:

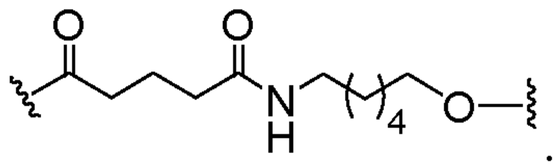

and

.

In certain embodiments, the conjugate linker has a structure selected from among:

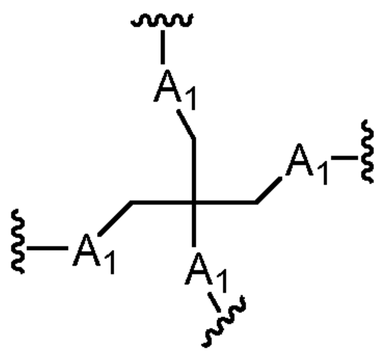
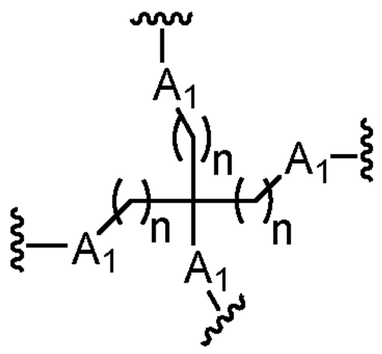
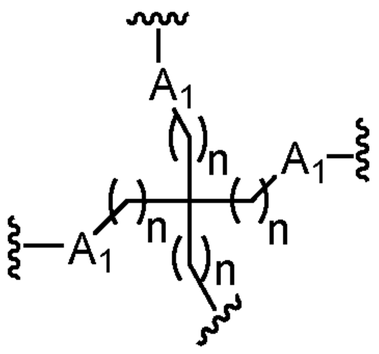

and

;

wherein each n is independently, 0, 1, 2, 3, 4, 5, 6, or 7.

In certain embodiments, the conjugate linker has the following structure:


.

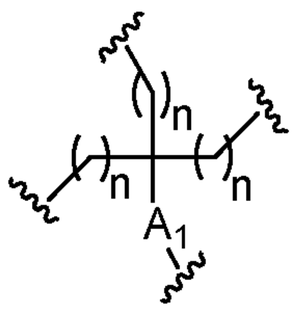
In certain embodiments, the branching group has one of the following structures:

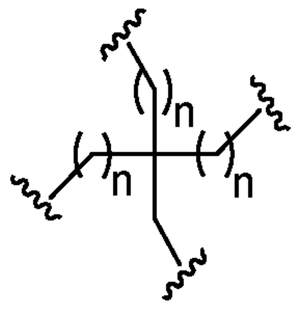

;

and


wherein each A_1 is independently, O, S, C=O or NH; and

each n is, independently, from 1 to 20.

In certain embodiments, the branching group has one of the following structures:

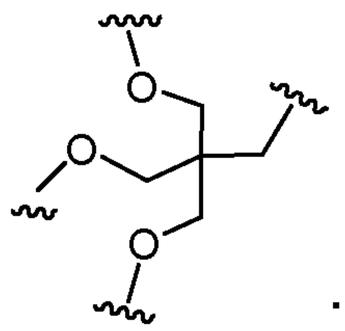


and


wherein each A_1 is independently, O, S, C=O or NH; and

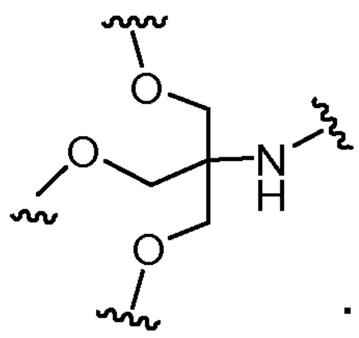
each n is, independently, from 1 to 20.

In certain embodiments, the branching group has the following structure:



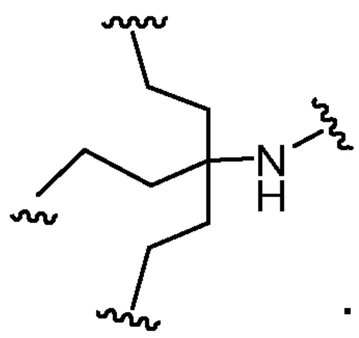
.

In certain embodiments, the branching group has the following structure:



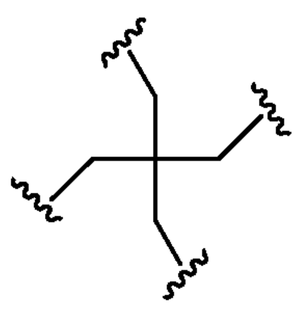
.

In certain embodiments, the branching group has the following structure:



.

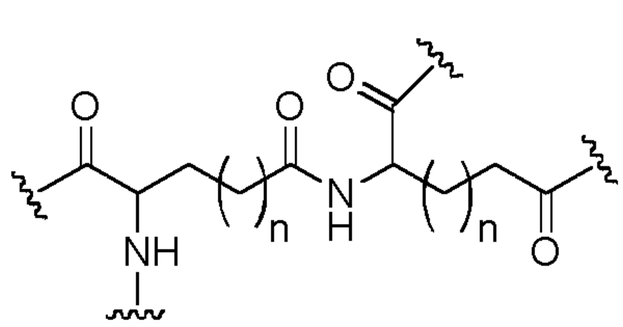
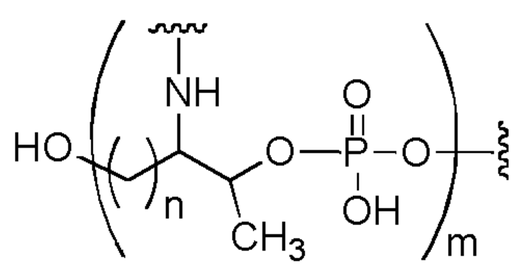
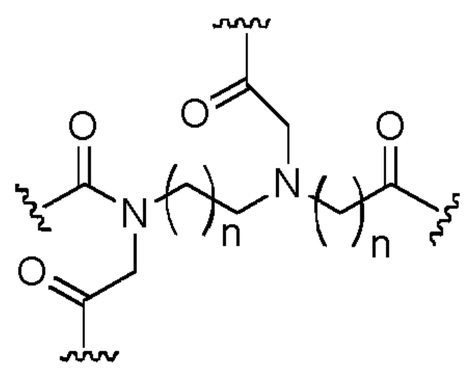
In certain embodiments, the branching group has the following structure:

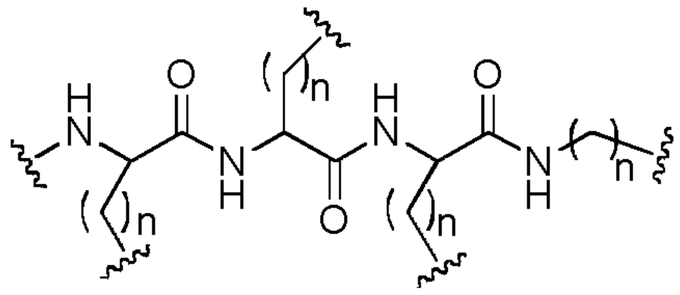
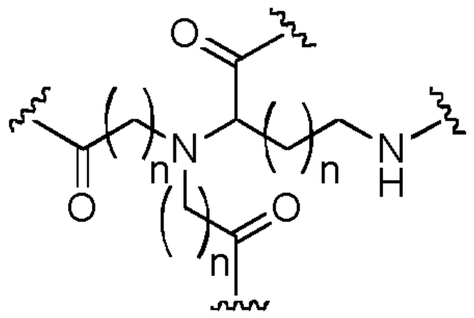
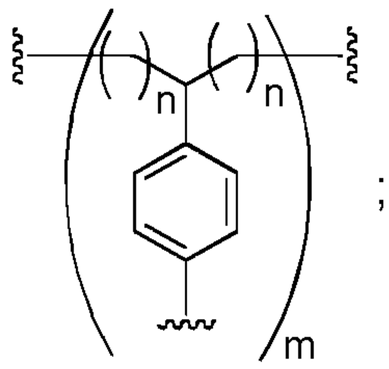


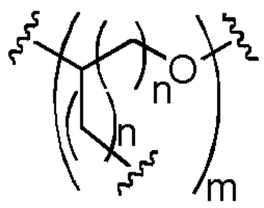
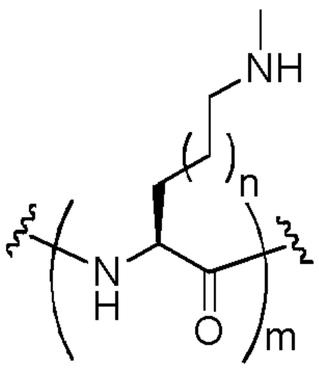
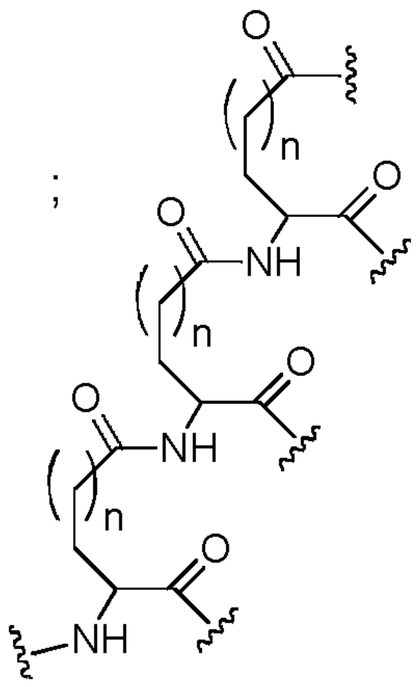
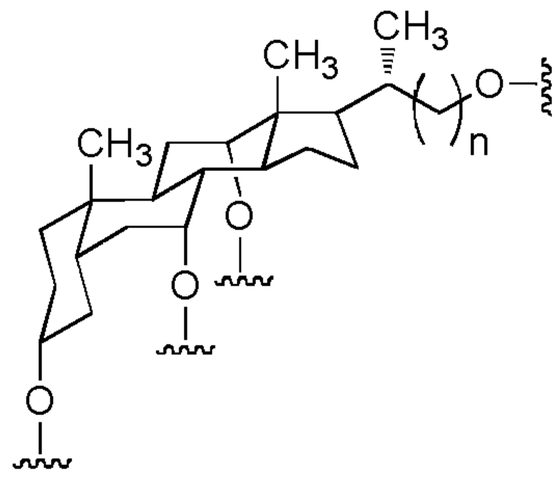
.

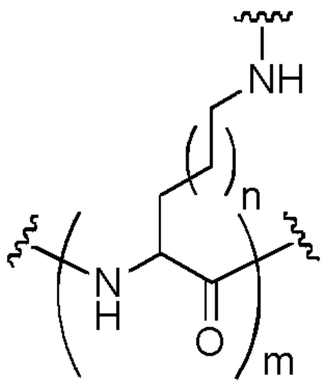
In certain embodiments, the branching group comprises an ether.

In certain embodiments, the branching group has the following structure:


; 
; 
;


; 
; 
;

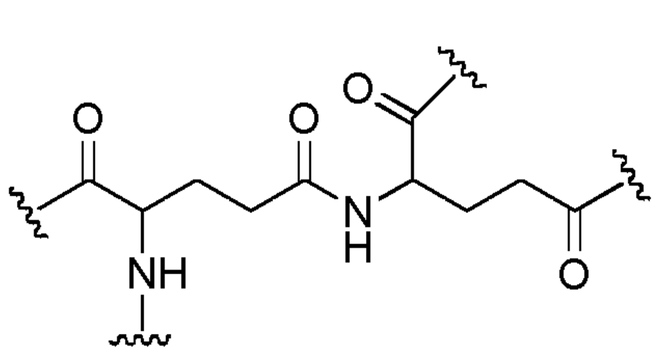
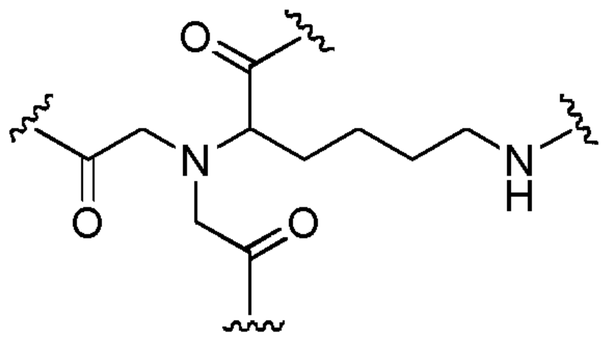
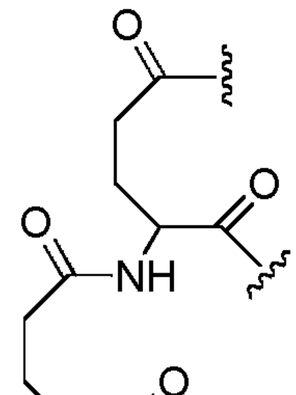

; 
; 
; 
; and

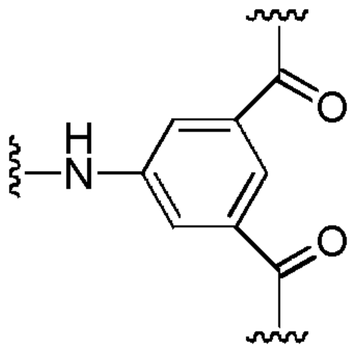
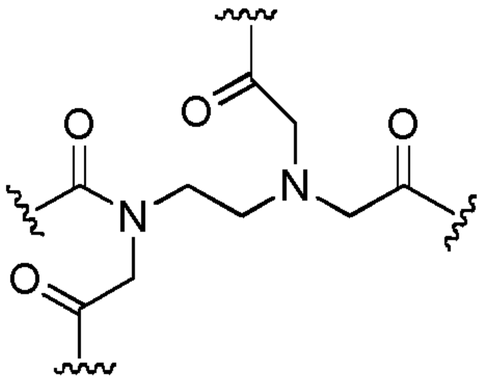
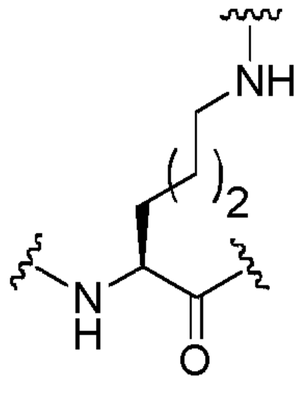
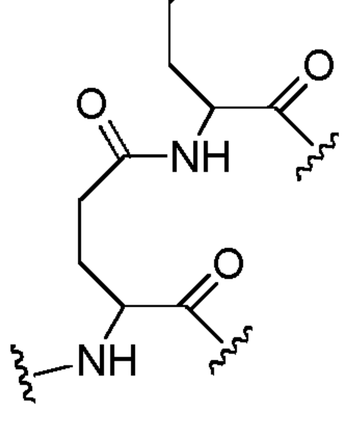


each n is, independently, from 1 to 20; and

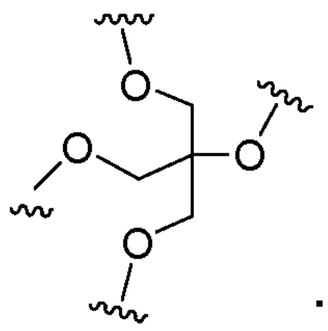
m is from 2 to 6.

In certain embodiments, the branching group has the following structure:


; 
; 
;

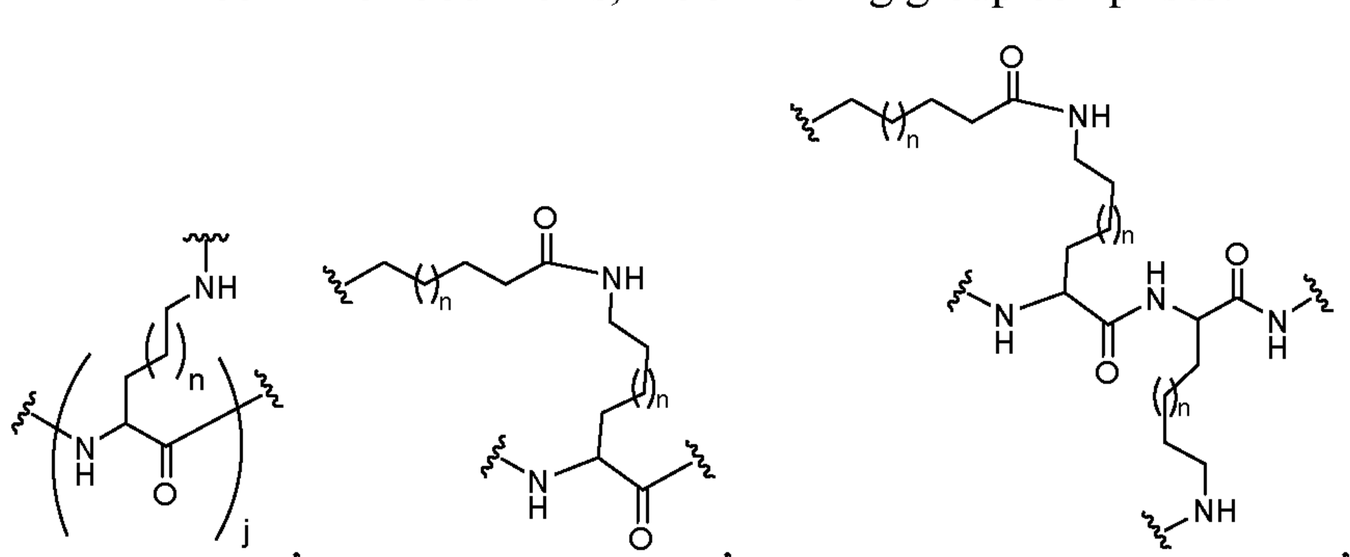

; 
; 
; and 
.

In certain embodiments, the branching group has the following structure:



.

In certain embodiments, the branching group comprises:



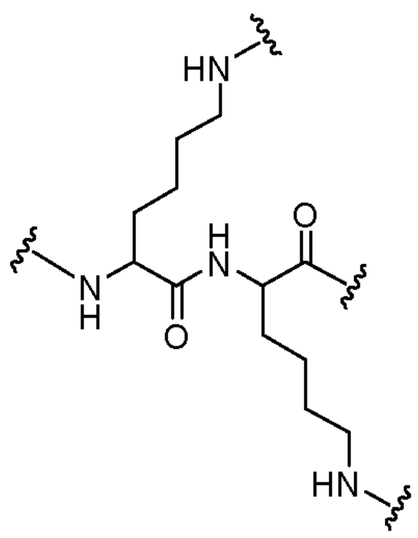
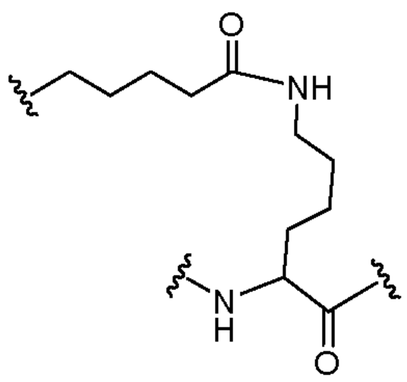
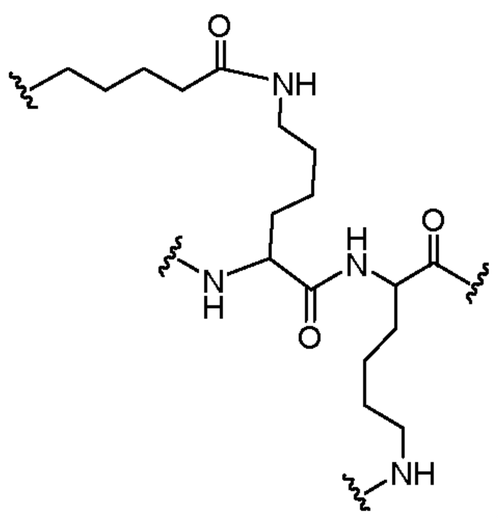
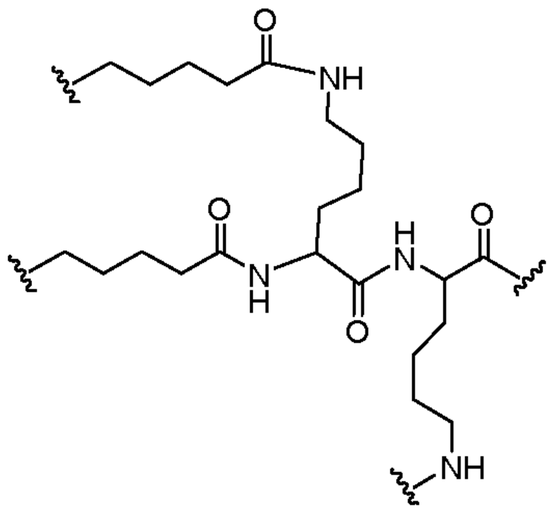
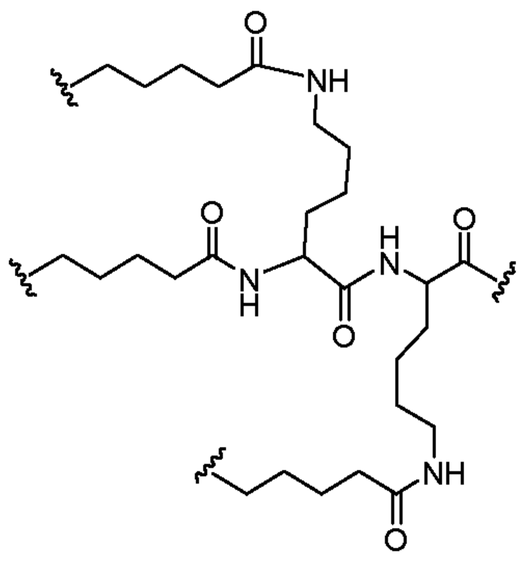
, or

;

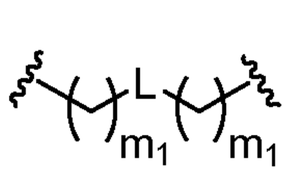
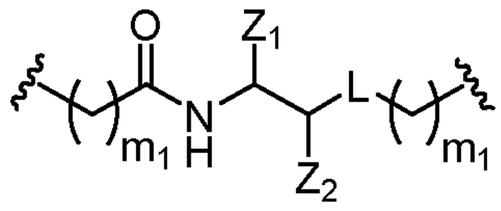
wherein each j is an integer from 1 to 3; and

wherein each n is an integer from 1 to 20.

In certain embodiments, the branching group comprises:





, or

.

In certain embodiments, each tether is selected from among:


and


wherein L is selected from a phosphorus linking group and a neutral linking group;

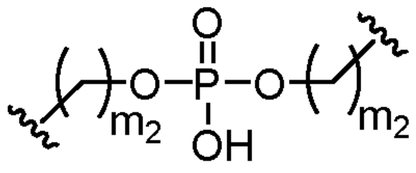
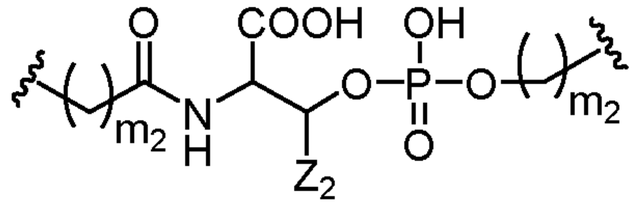
Z_1 is C(=O)O-R_2;

Z_2 is H, C_1-C_6 alkyl or substituted C_1-C_6 alky;

R_2 is H, C_1-C_6 alkyl or substituted C_1-C_6 alky; and

each m_1 is, independently, from 0 to 20 wherein at least one m_1 is greater than 0 for each tether.

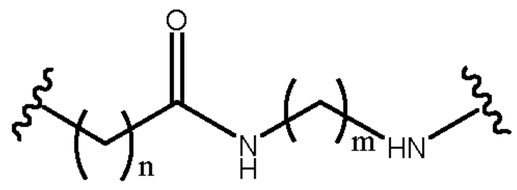
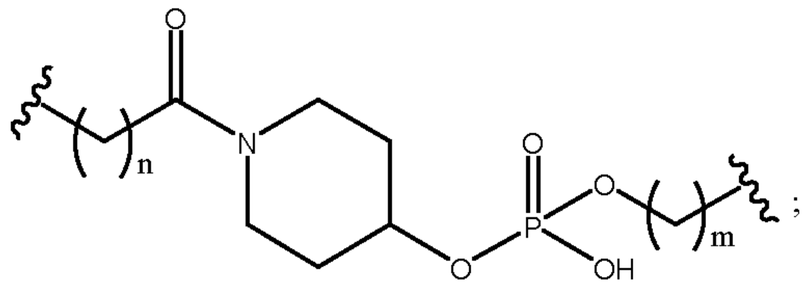
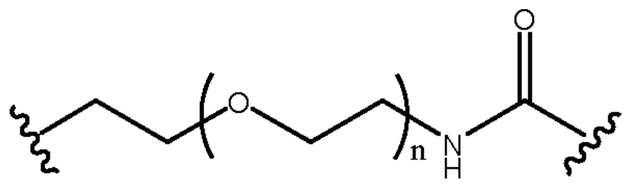
In certain embodiments, each tether is selected from among:


and


wherein Z_2 is H or CH_3; and

each m_2 is, independently, from 0 to 20 wherein at least one m_2 is greater than 0 for each tether.

In certain embodiments, each tether is selected from among:

.

wherein n is from 1 to 12; and

wherein m is from 1 to 12.

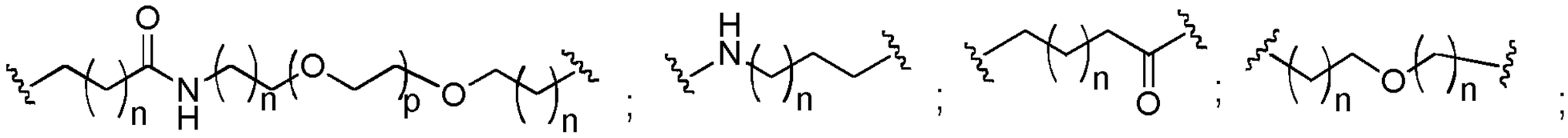
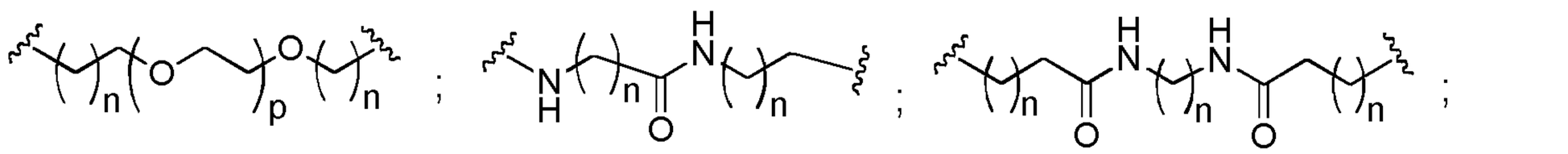
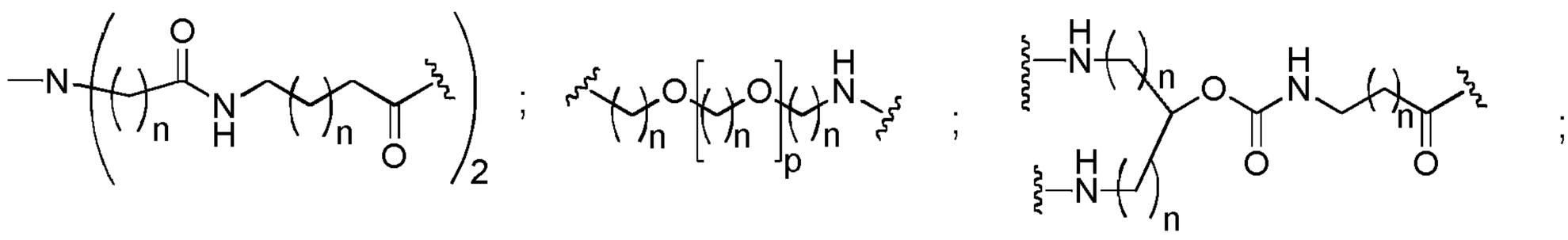
In certain embodiments, at least one tether comprises ethylene glycol.

In certain embodiments, at least one tether comprises an amide. In certain embodiments, at least one tether comprises a polyamide.

In certain embodiments, at least one tether comprises an amine.

In certain embodiments, at least two tethers are different from one another. In certain embodiments, all of the tethers are the same as one another.

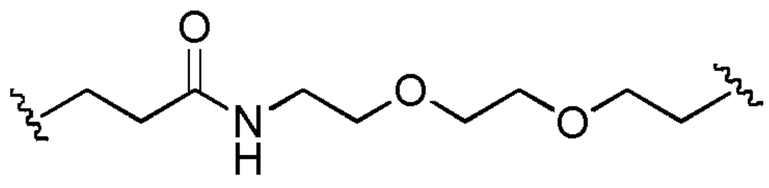
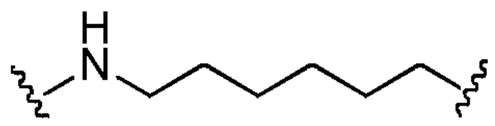
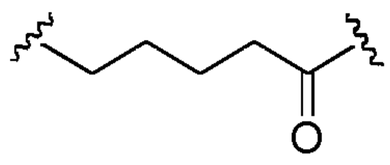
In certain embodiments, each tether is selected from among:

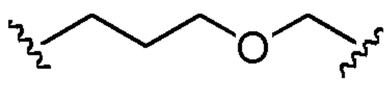
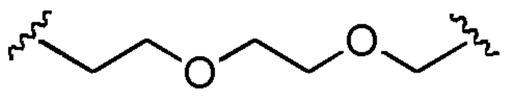
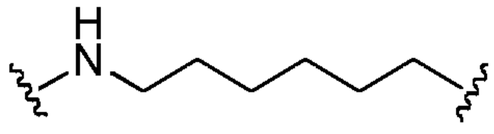
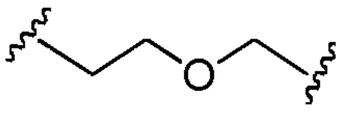




wherein each n is, independently, from 1 to 20; and

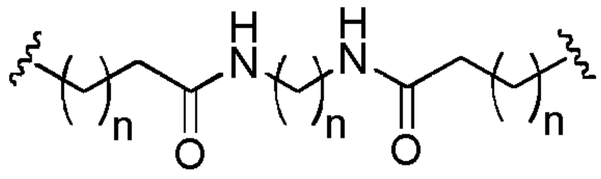
each p is from 1 to about 6.

In certain embodiments, each tether is selected from among:


; 
; 
;

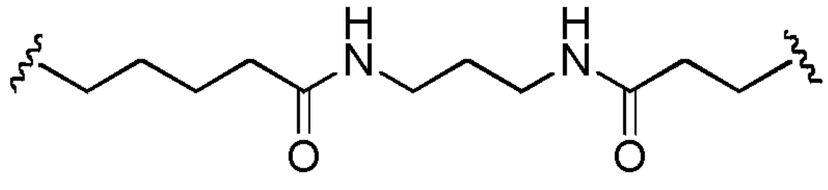

; 
; 
; and 
.

In certain embodiments, each tether has the following structure:

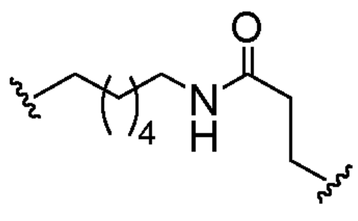
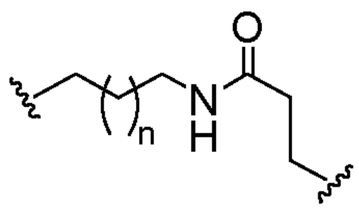


wherein each n is, independently, from 1 to 20.

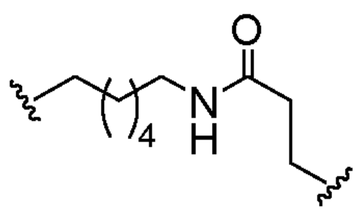
In certain embodiments, each tether has the following structure:


.

In certain embodiments, the tether has a structure selected from among:


, or 
; wherein each n is independently, 0, 1, 2, 3, 4, 5, 6, or 7.

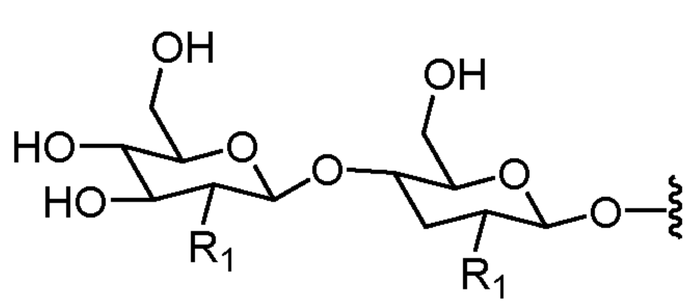
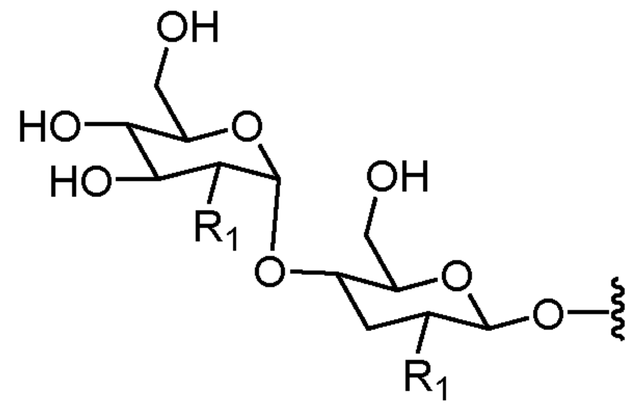
In certain embodiments, the tether has a structure selected from among:


.

In certain embodiments, the ligand is galactose.

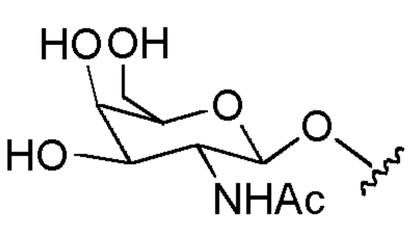
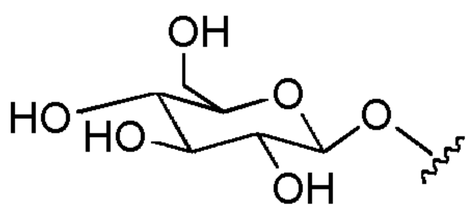
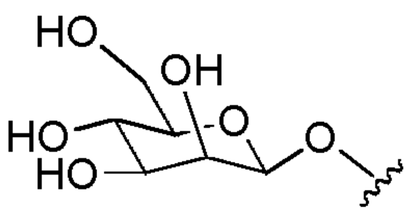
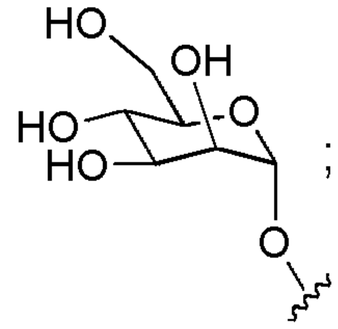
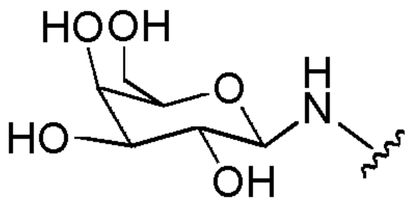
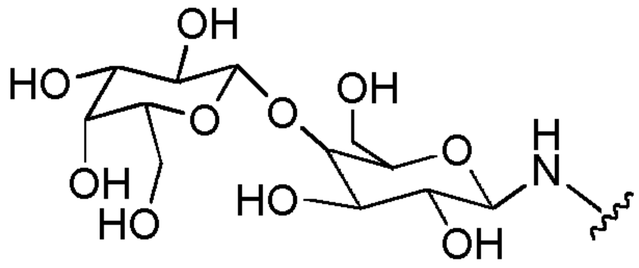
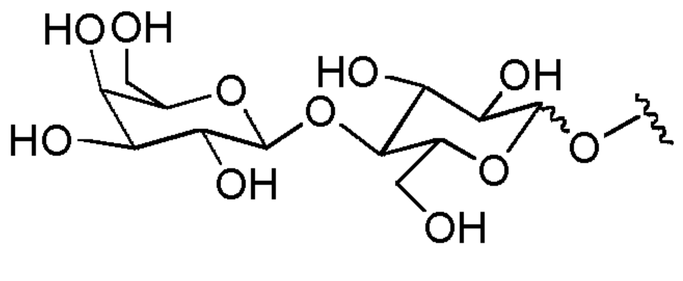
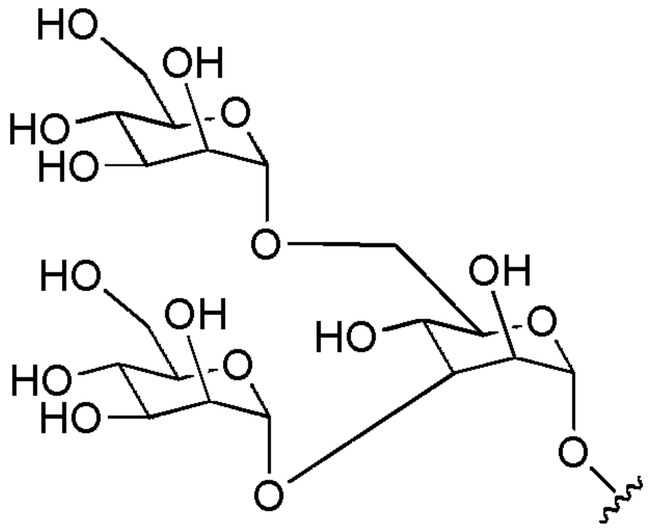
In certain embodiments, the ligand is mannose-6-phosphate.

In certain embodiments, each ligand is selected from among:


and


wherein each R_1 is selected from OH and NHCOOH.

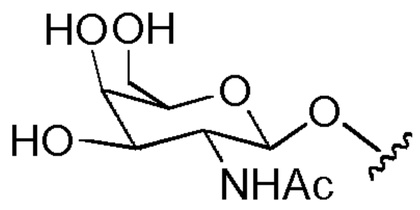
In certain embodiments, each ligand is selected from among:


;

;

;

;

;

;

; and

.

In certain embodiments, each ligand has the following structure:

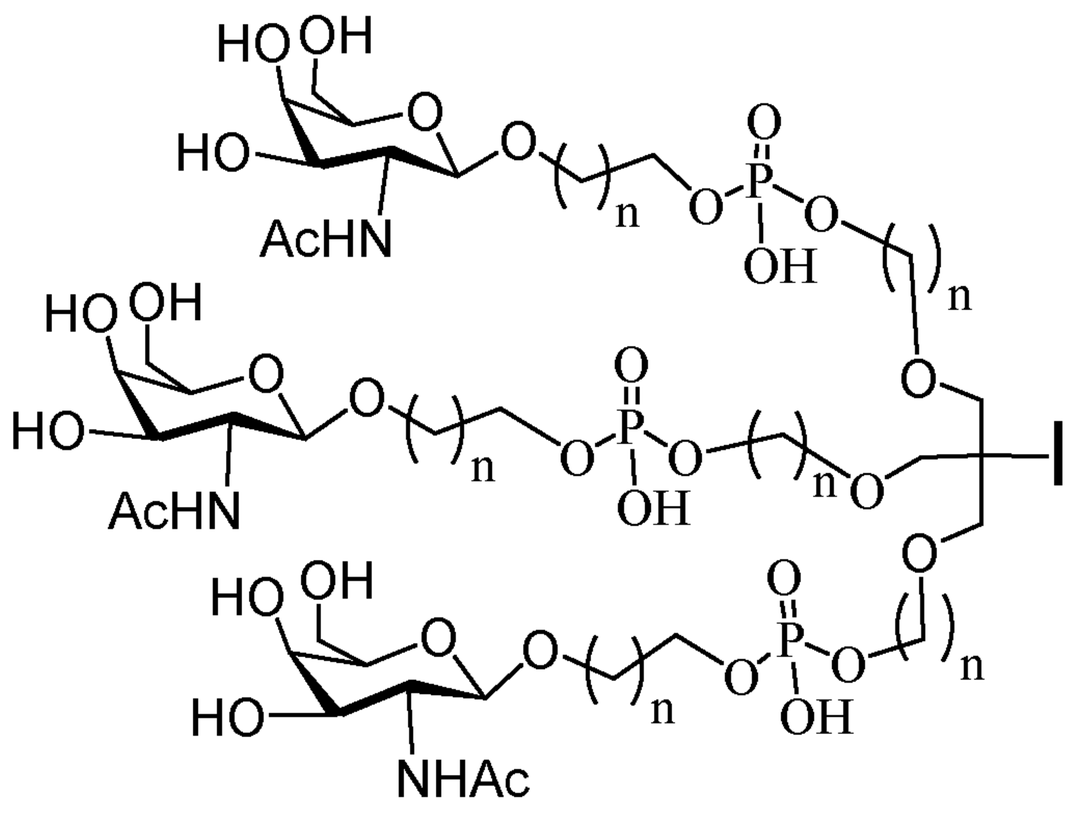
.

In certain embodiments, each ligand has the following structure:



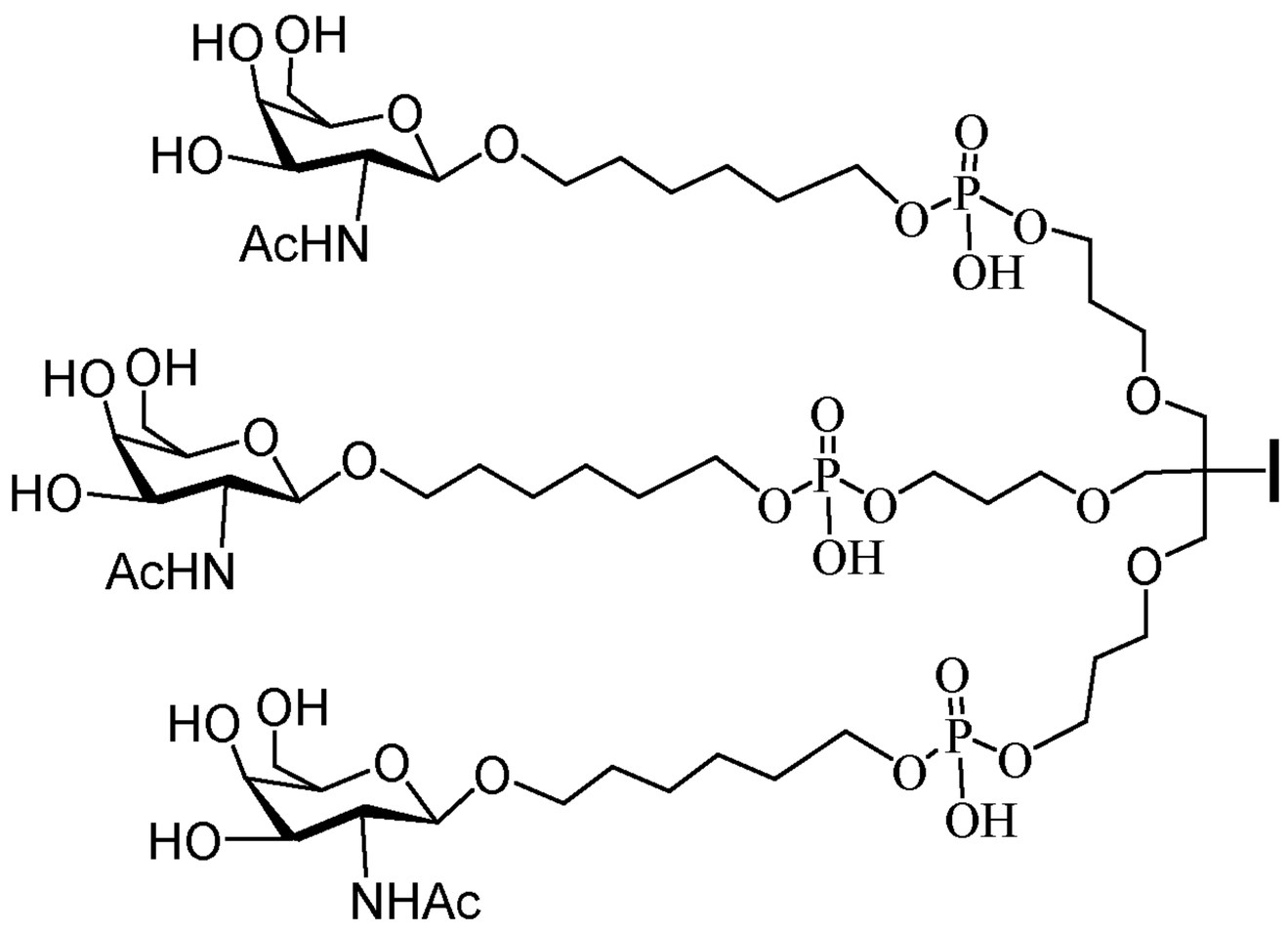
In certain embodiments, the conjugate group comprises a cell-targeting moiety.

In certain embodiments, the conjugate group comprises a cell-targeting moiety having the following structure:

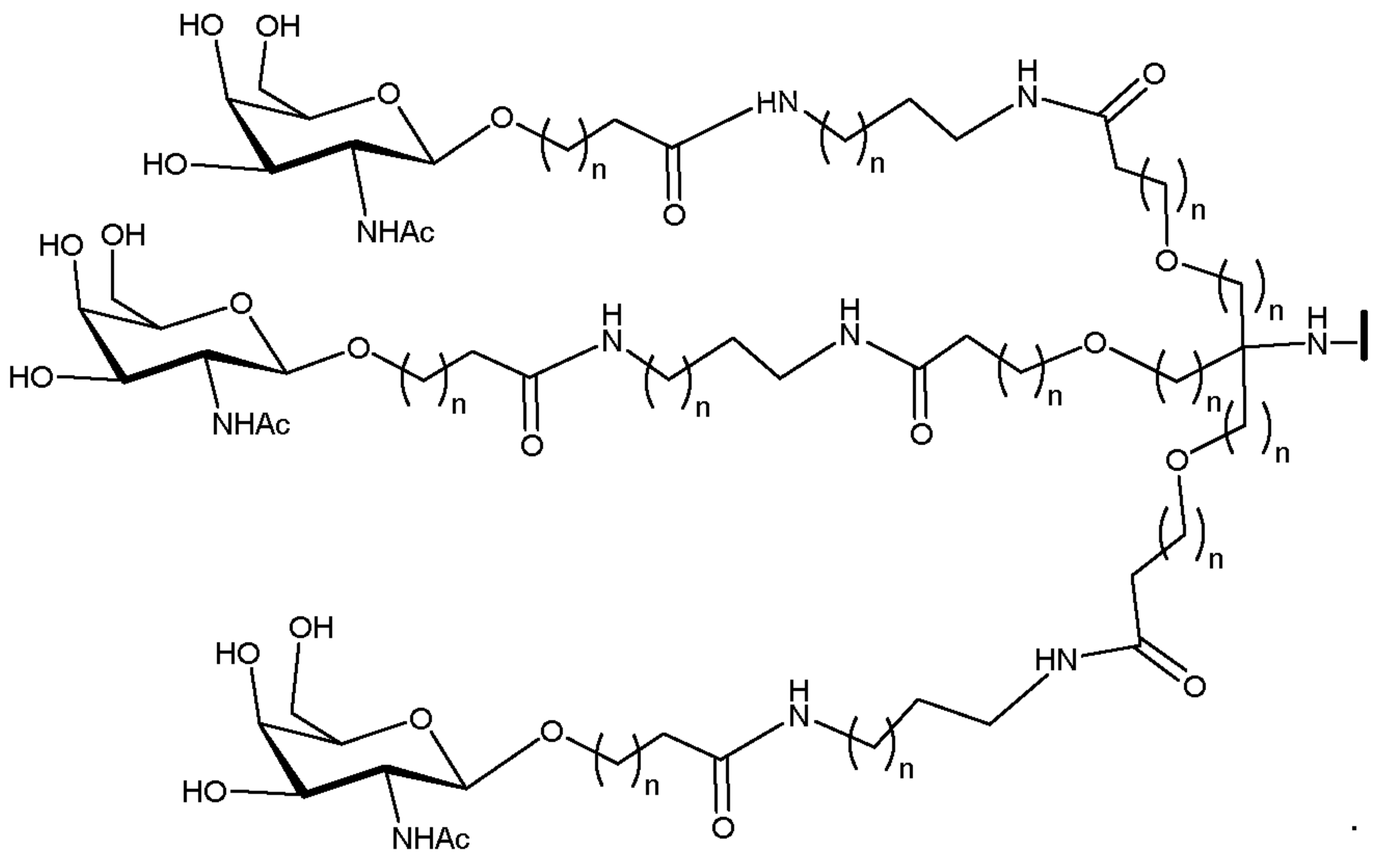


wherein each n is, independently, from 1 to 20.

In certain embodiments, the cell-targeting moiety has the following structure:

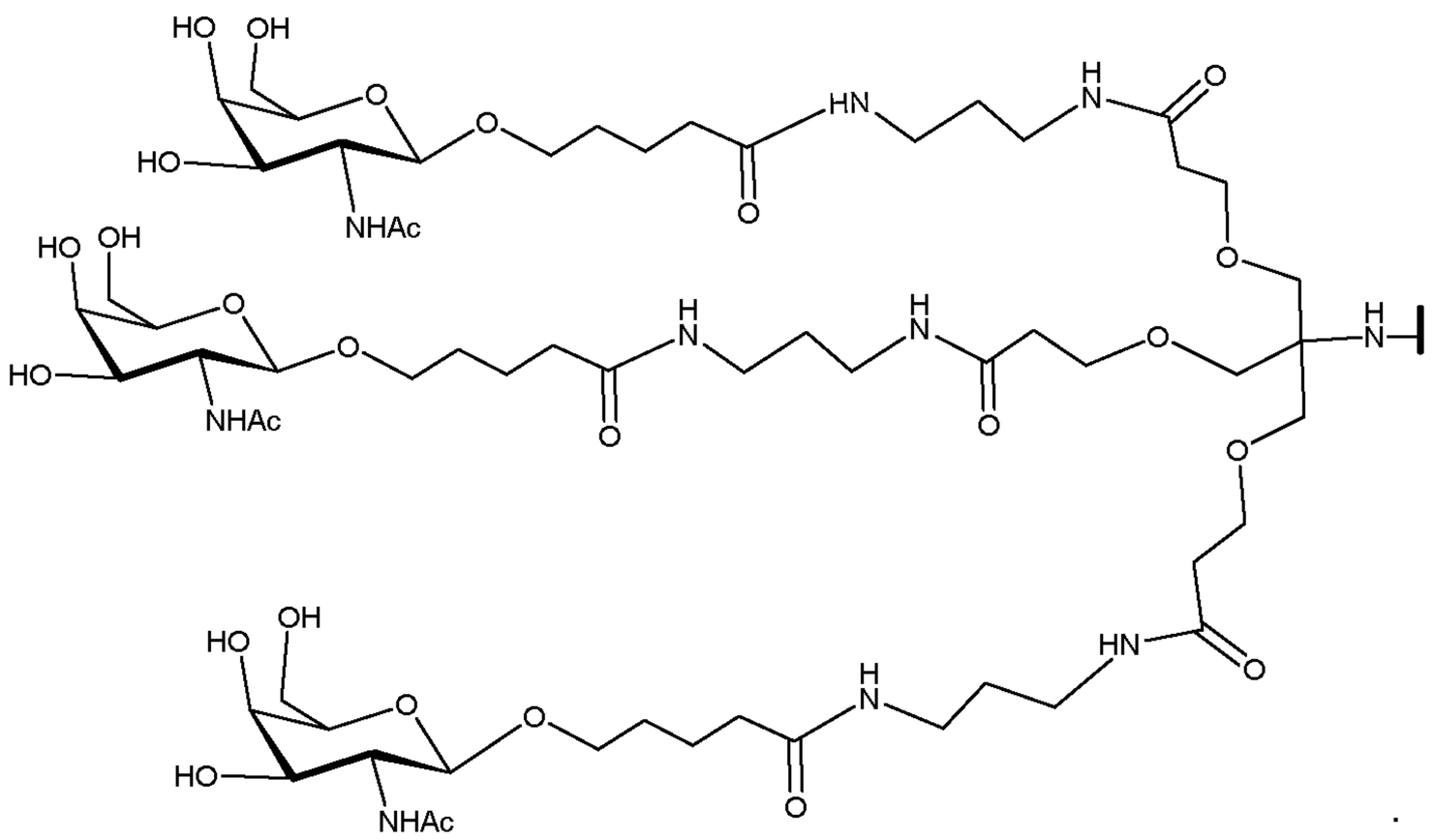


In certain embodiments, the cell-targeting moiety has the following structure:

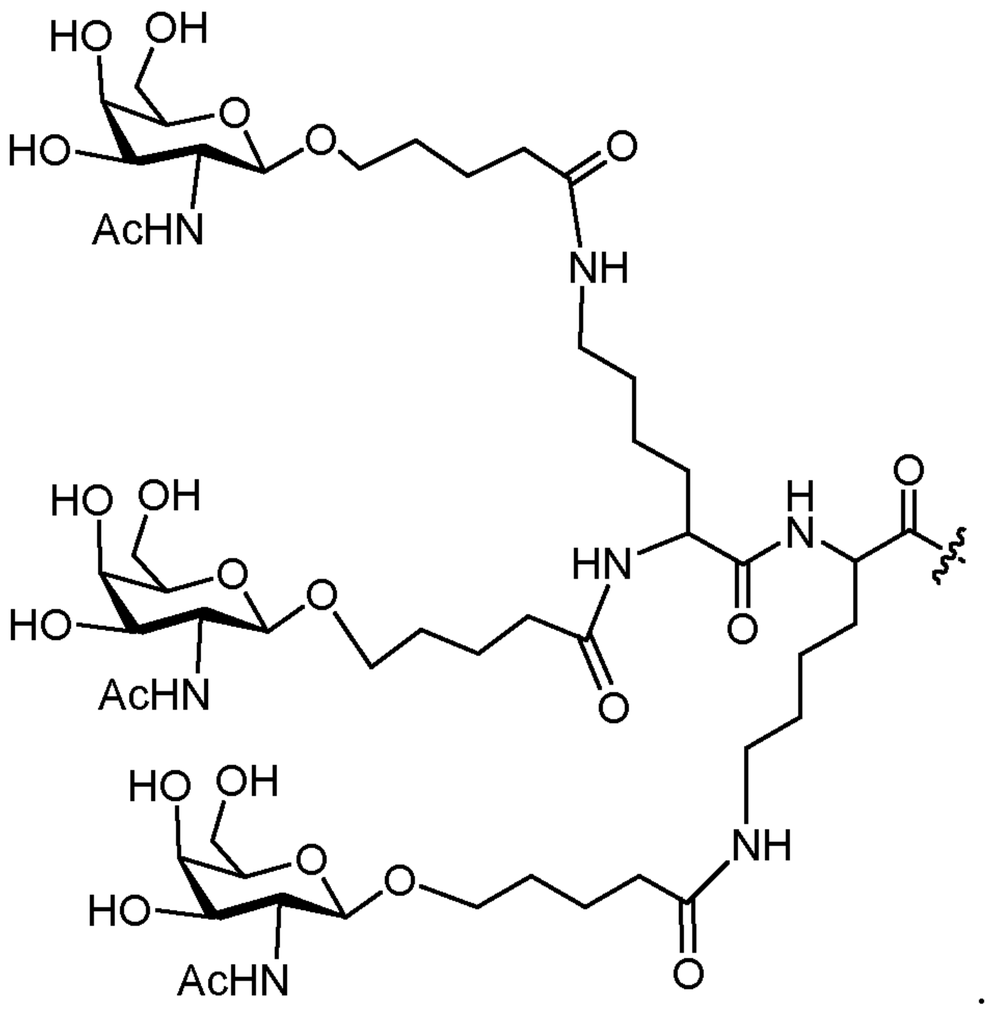


wherein each n is, independently, from 1 to 20.

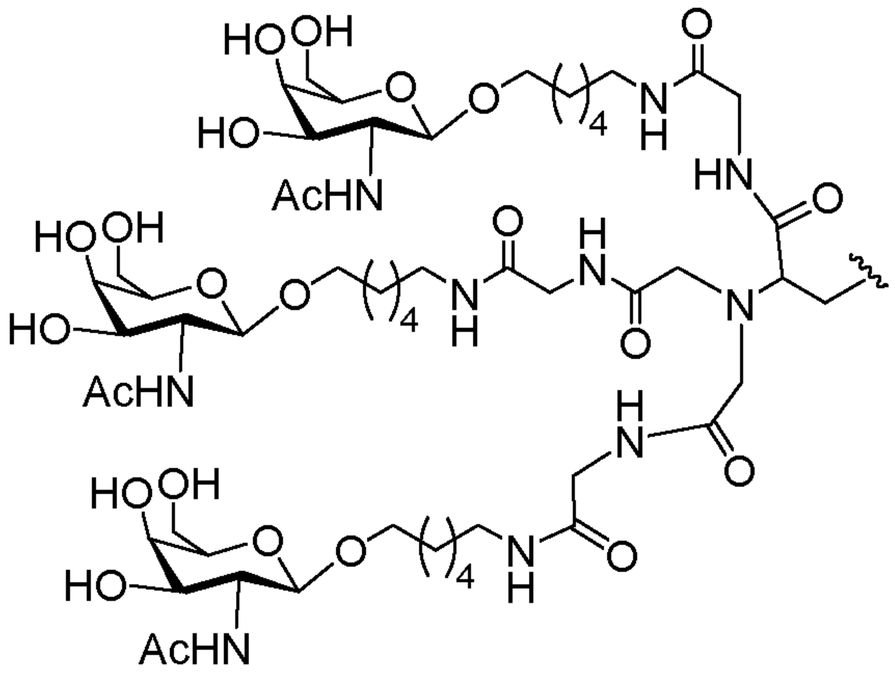
In certain embodiments, the cell-targeting moiety has the following structure:



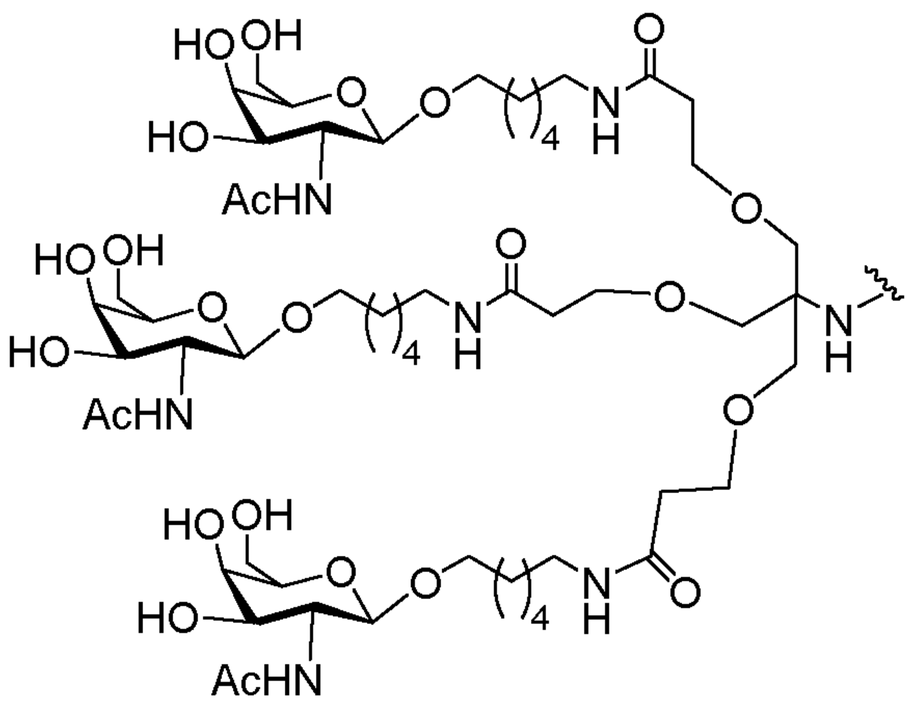
In certain embodiments, the cell-targeting moiety comprises:



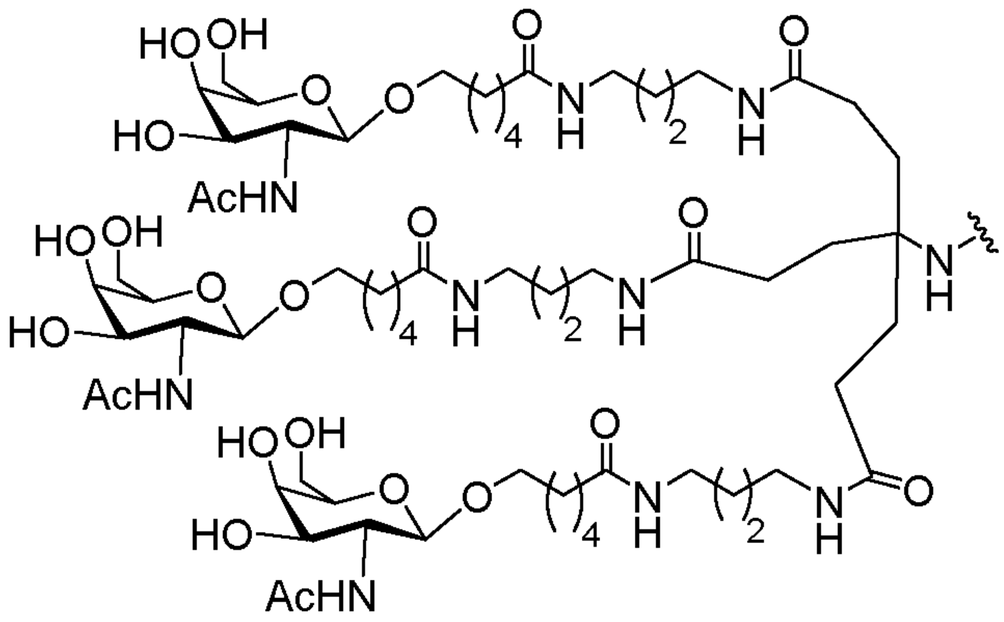
In certain embodiments, the cell-targeting moiety comprises:



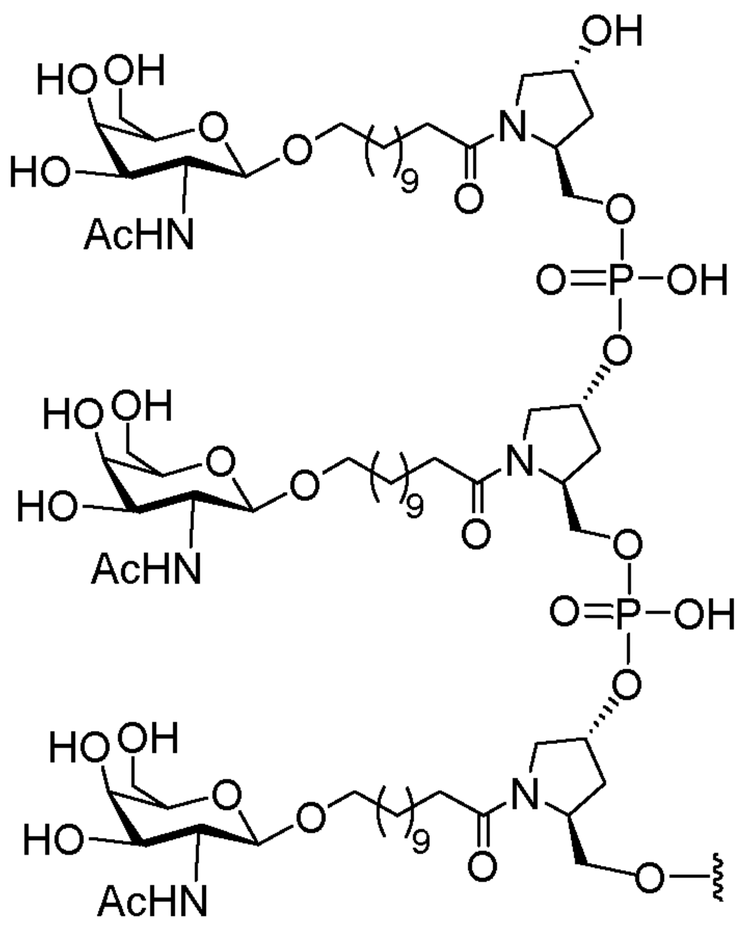
In certain embodiments, the cell-targeting moiety has the following structure:



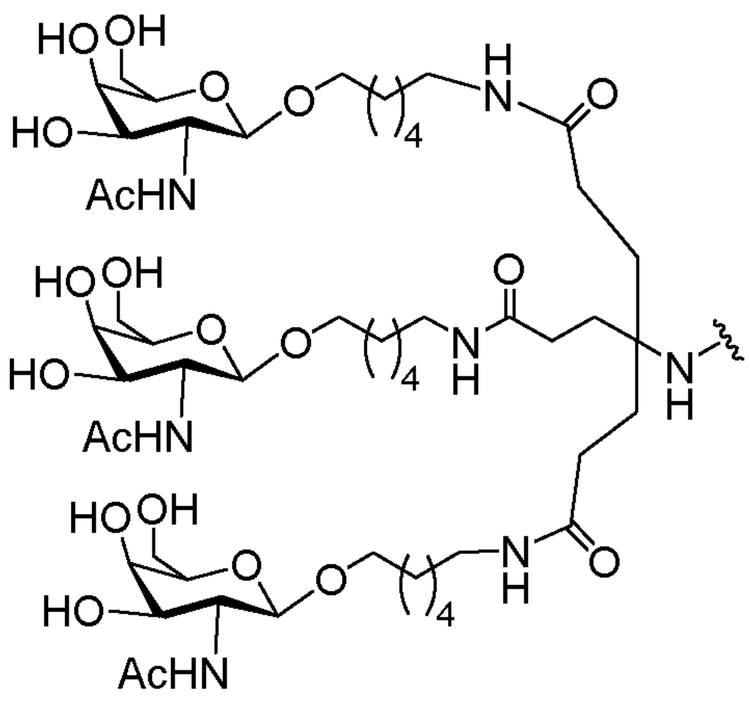
In certain embodiments, the cell-targeting moiety has the following structure:



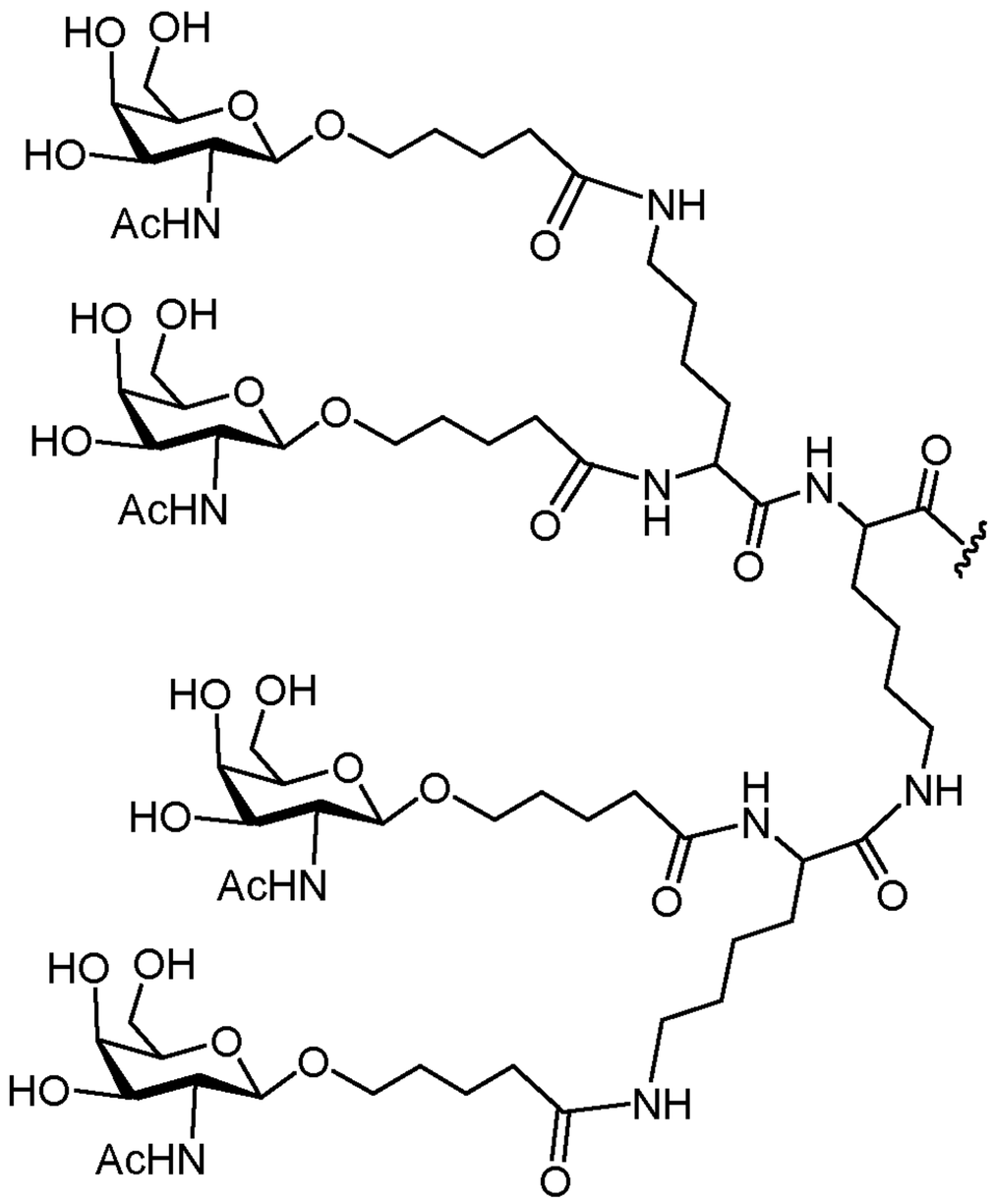
In certain embodiments, the cell-targeting moiety comprises:



In certain embodiments, the cell-targeting moiety has the following structure:

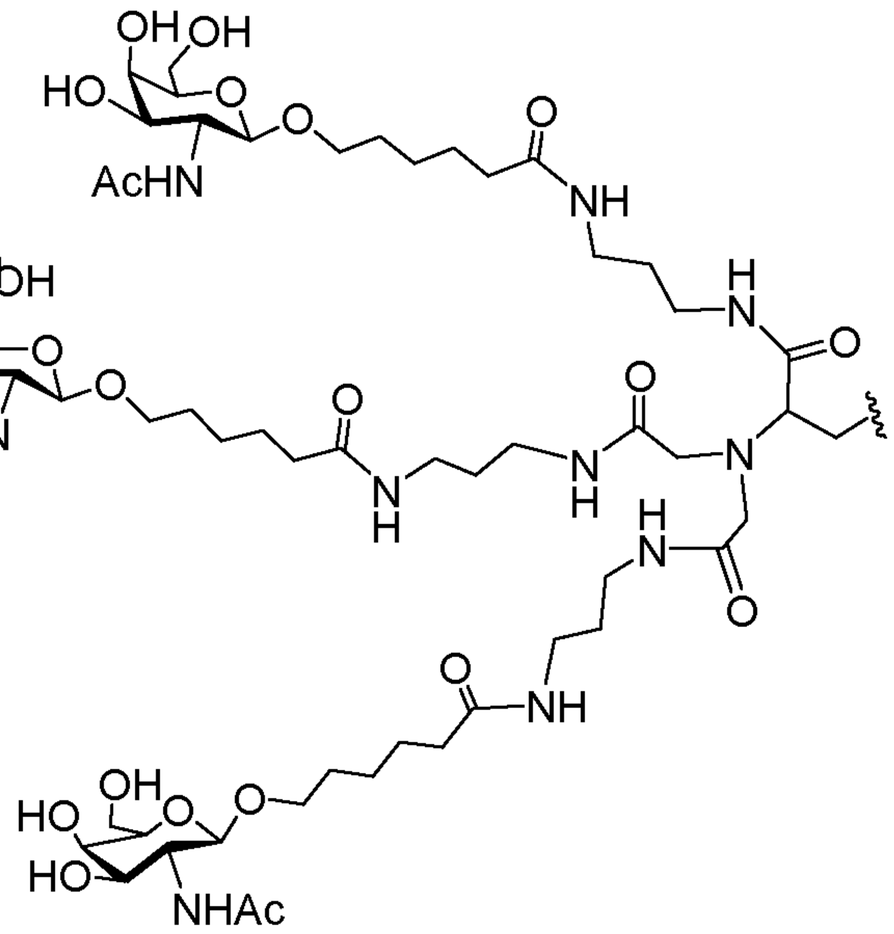


In certain embodiments, the cell-targeting moiety comprises:

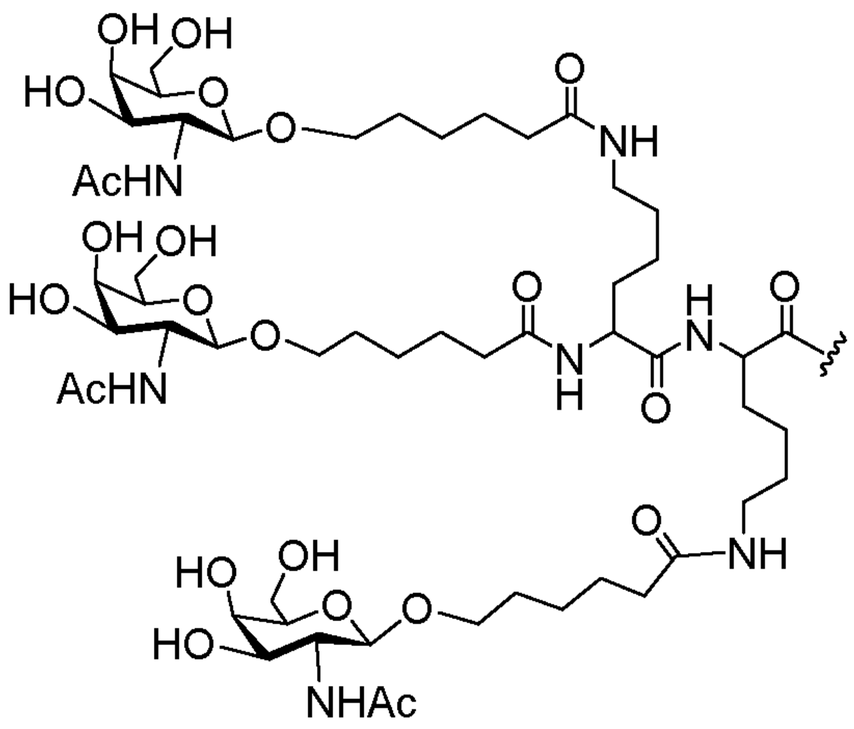


.

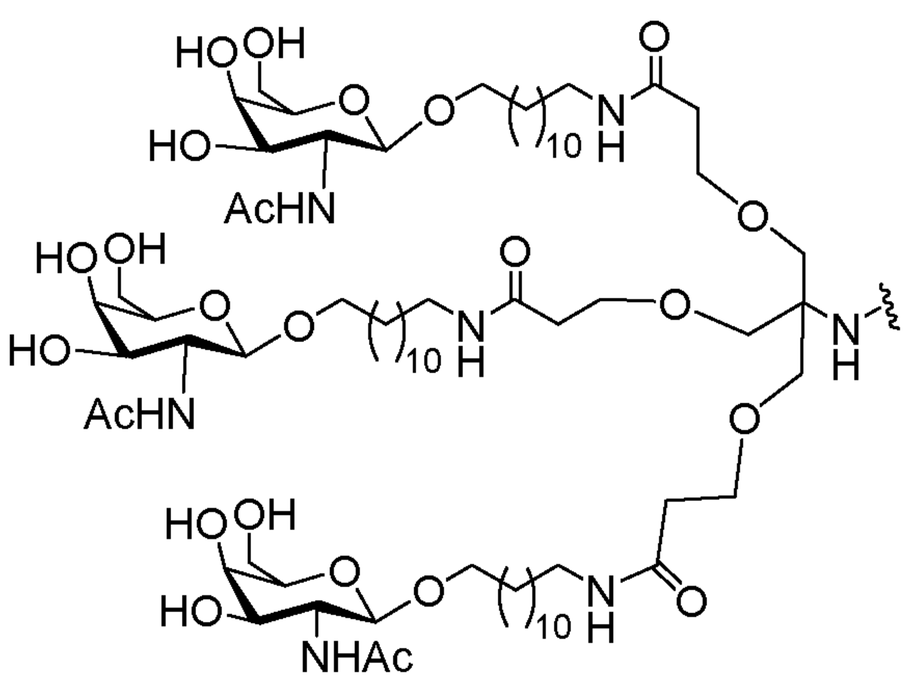
In certain embodiments, the cell-targeting moiety comprises:



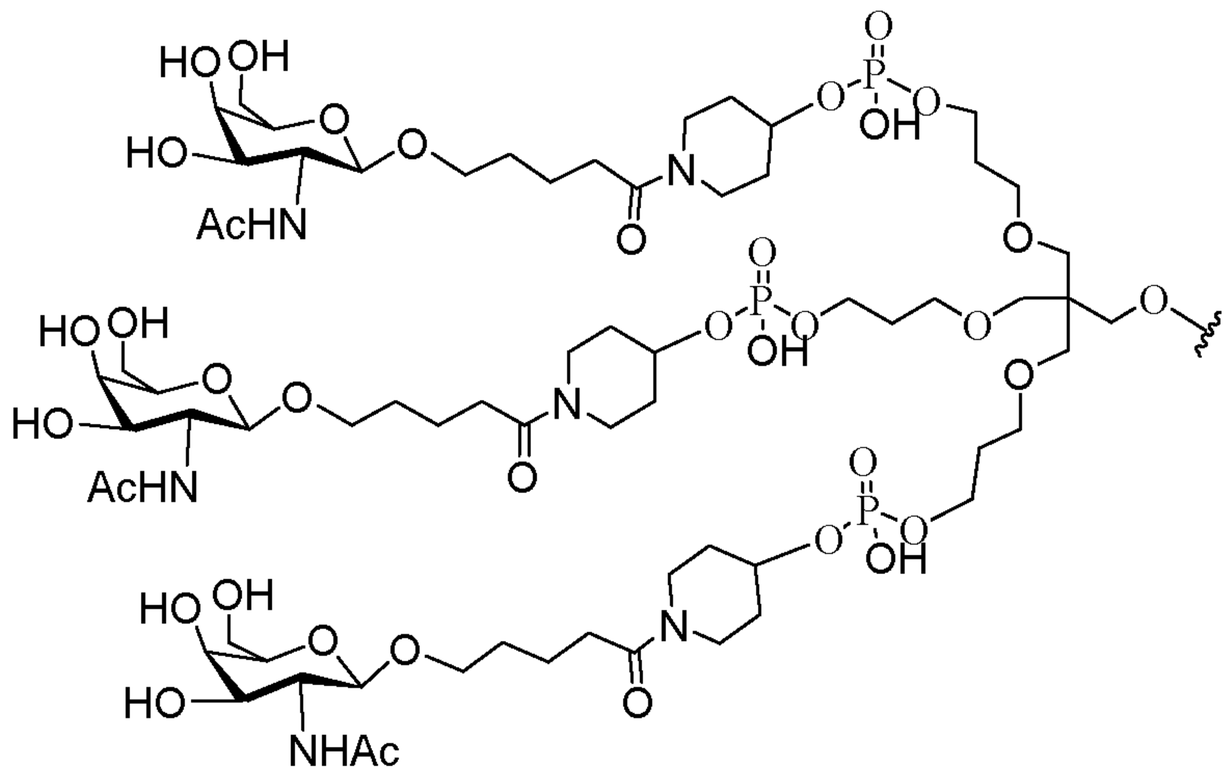
In certain embodiments, the cell-targeting moiety comprises:



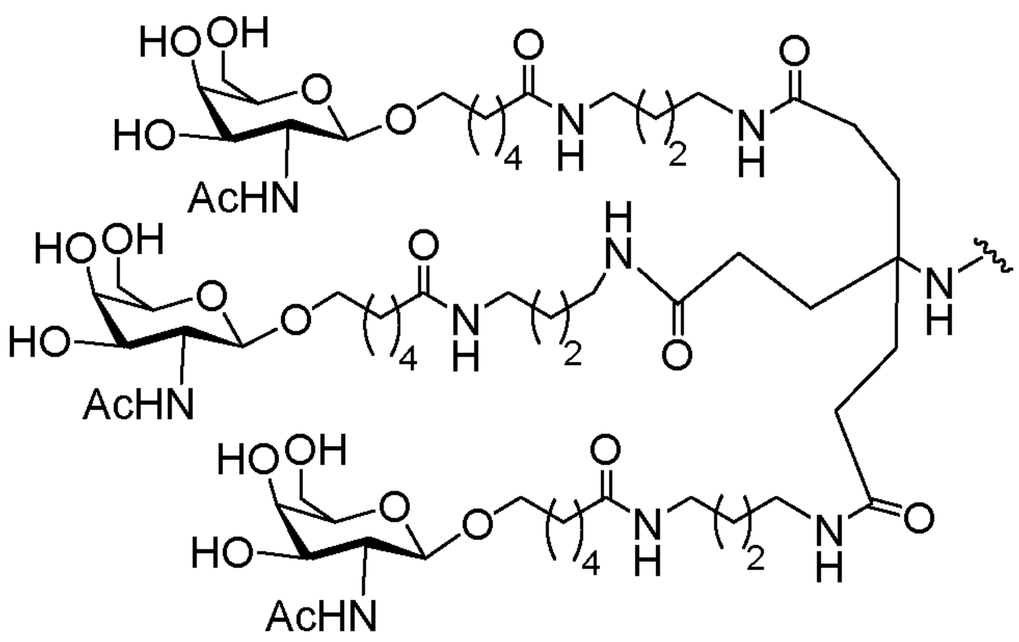
In certain embodiments, the cell-targeting moiety has the following structure:



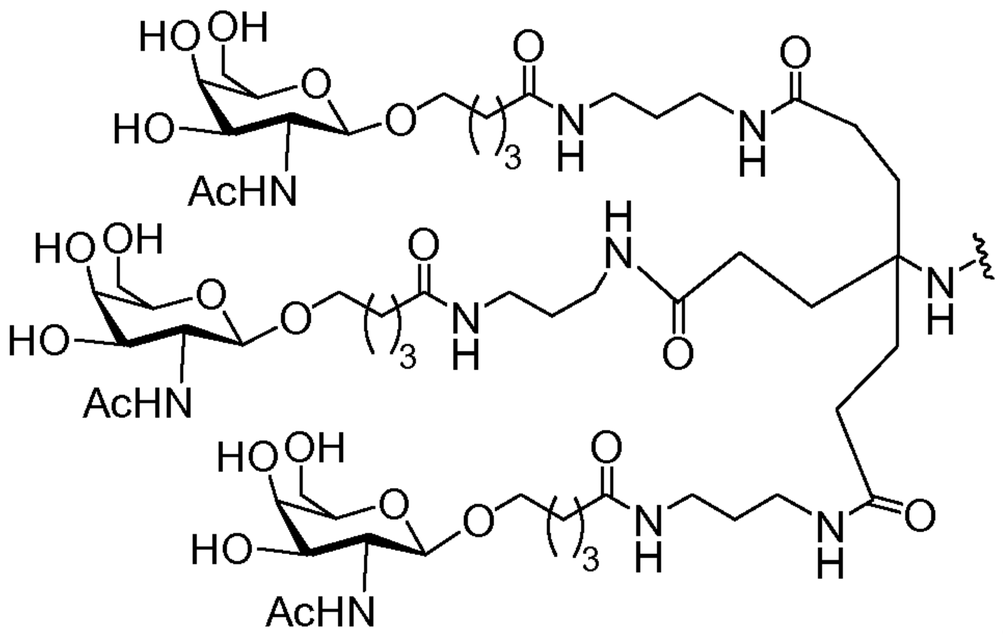
In certain embodiments, the cell-targeting moiety has the following structure:



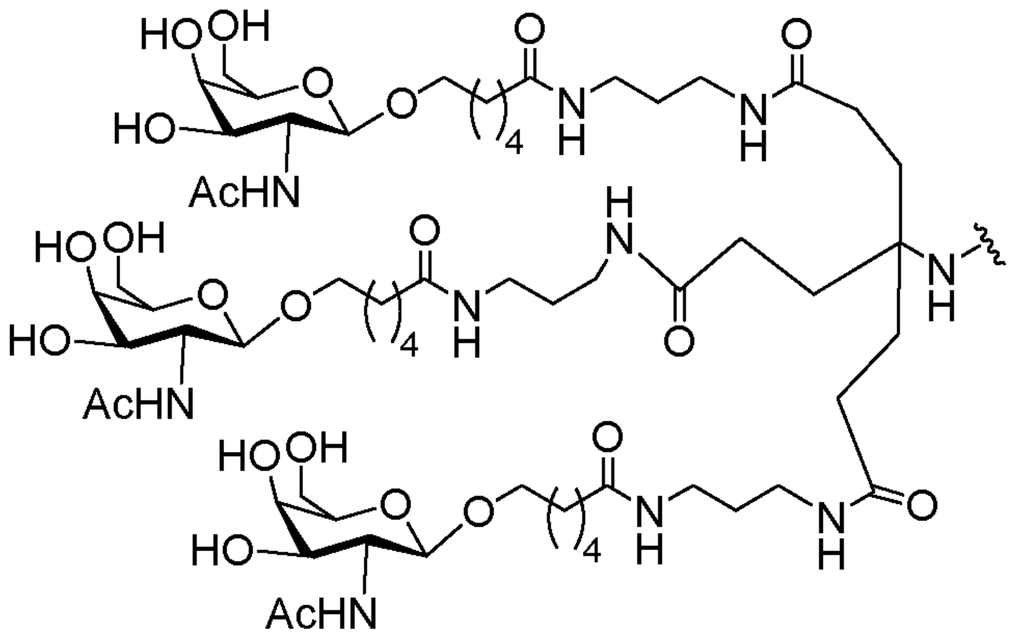
In certain embodiments, the cell-targeting moiety has the following structure:



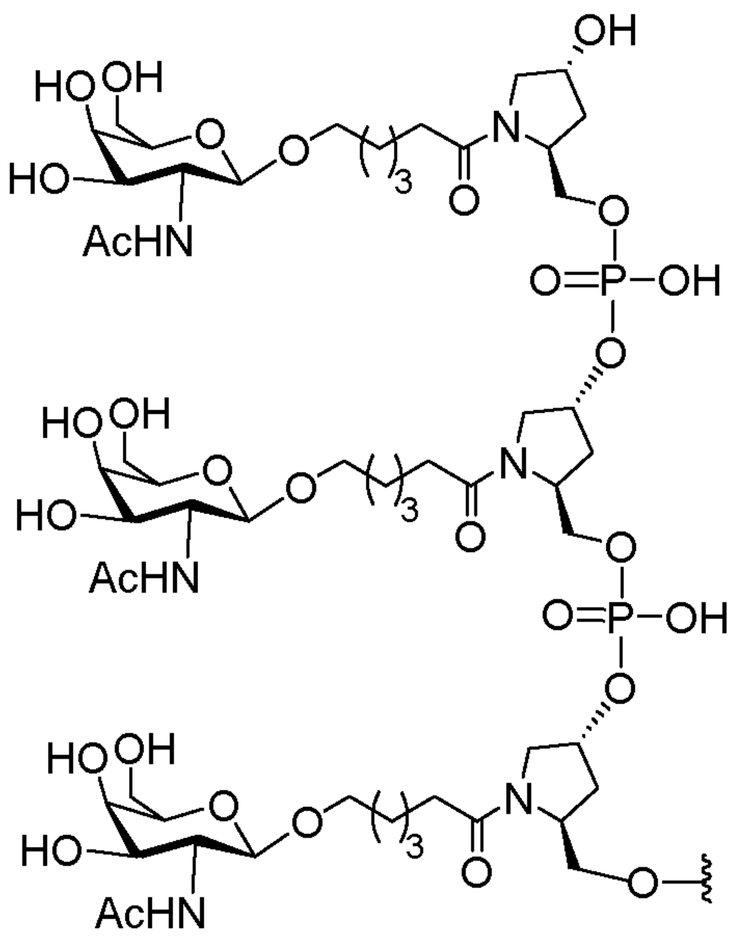
In certain embodiments, the cell-targeting moiety has the following structure:



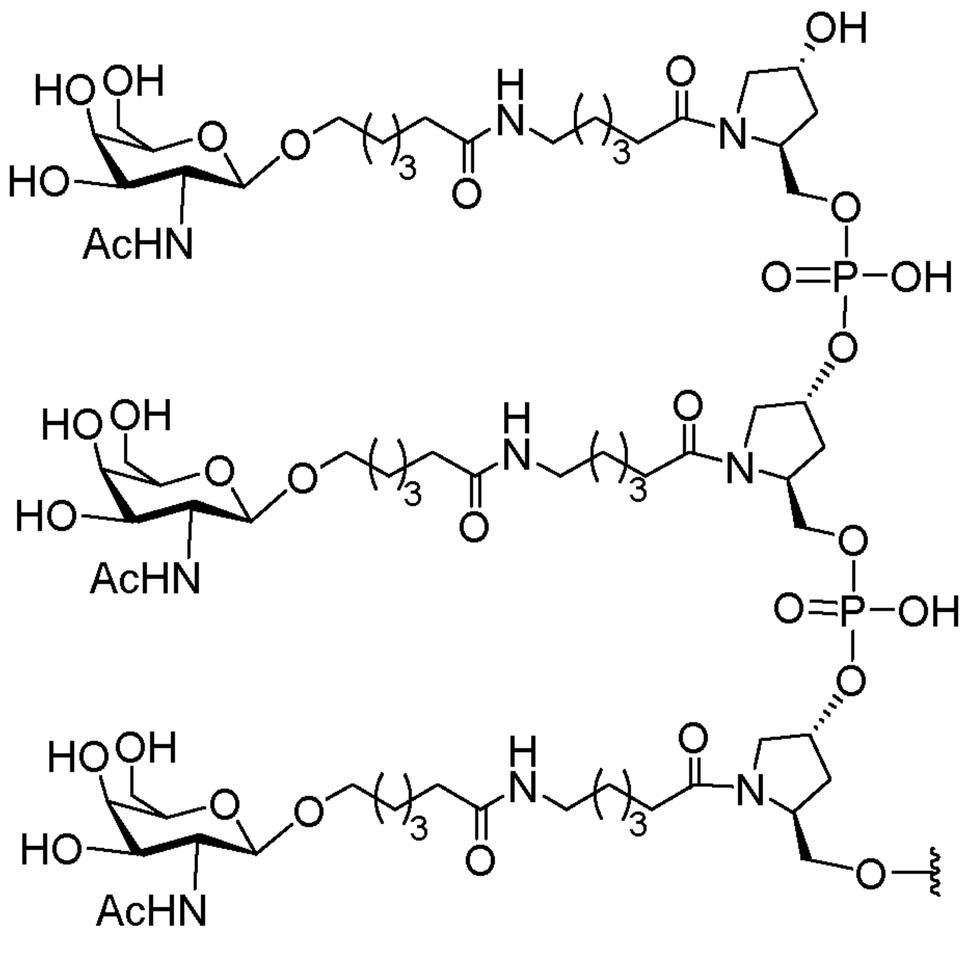
In certain embodiments, the cell-targeting moiety has the following structure:



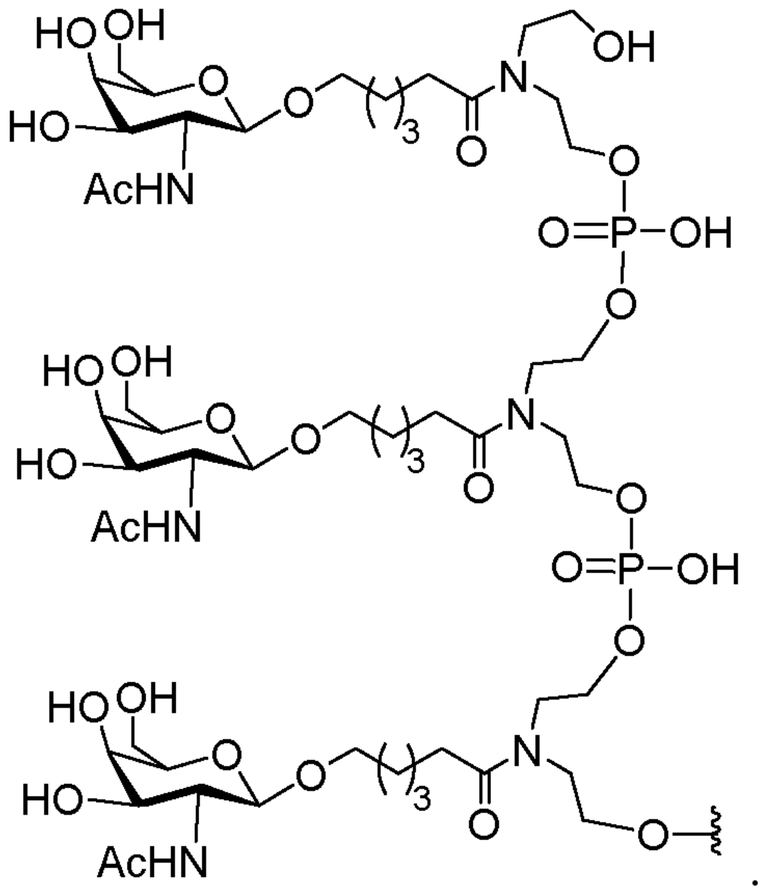
In certain embodiments, the cell-targeting moiety comprises:



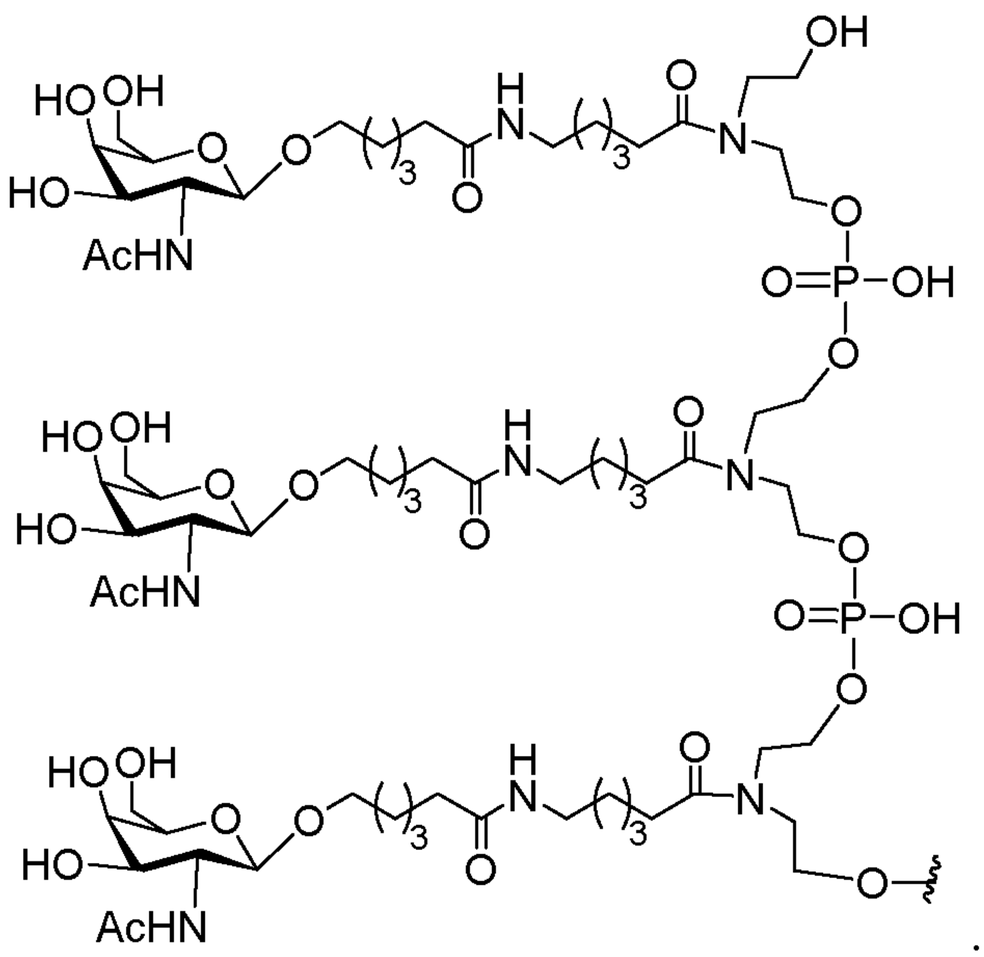
In certain embodiments, the cell-targeting moiety comprises:



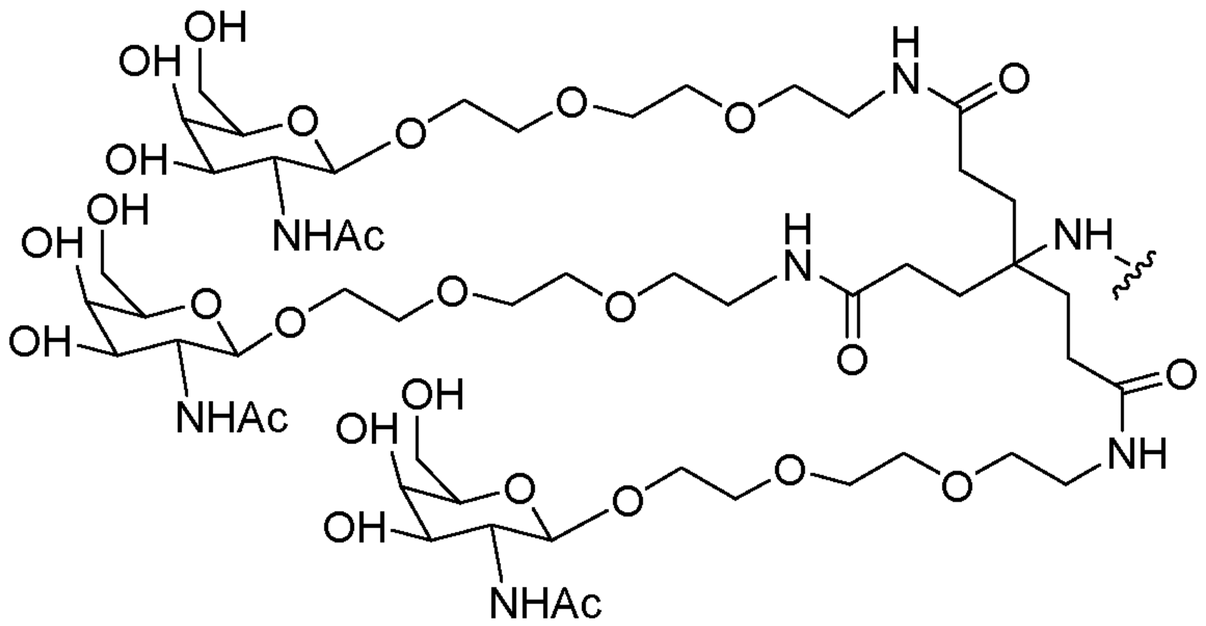
In certain embodiments, the cell-targeting moiety comprises:



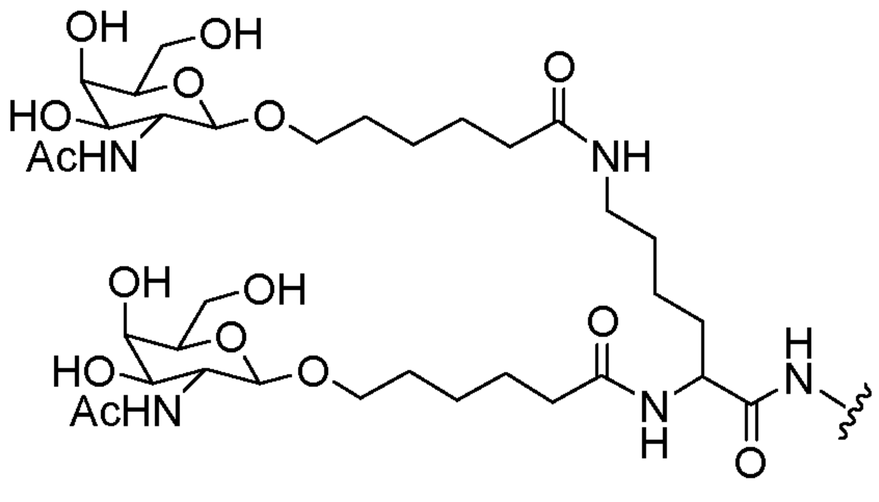
In certain embodiments, the cell-targeting moiety comprises:



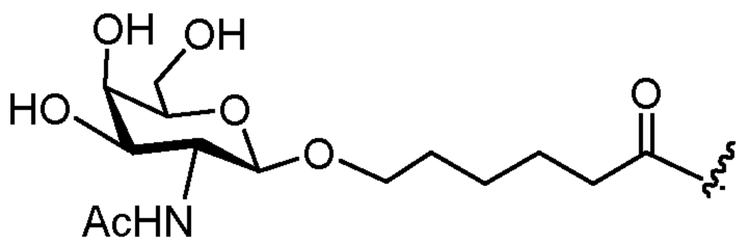
In certain embodiments, the cell-targeting moiety has the following structure:



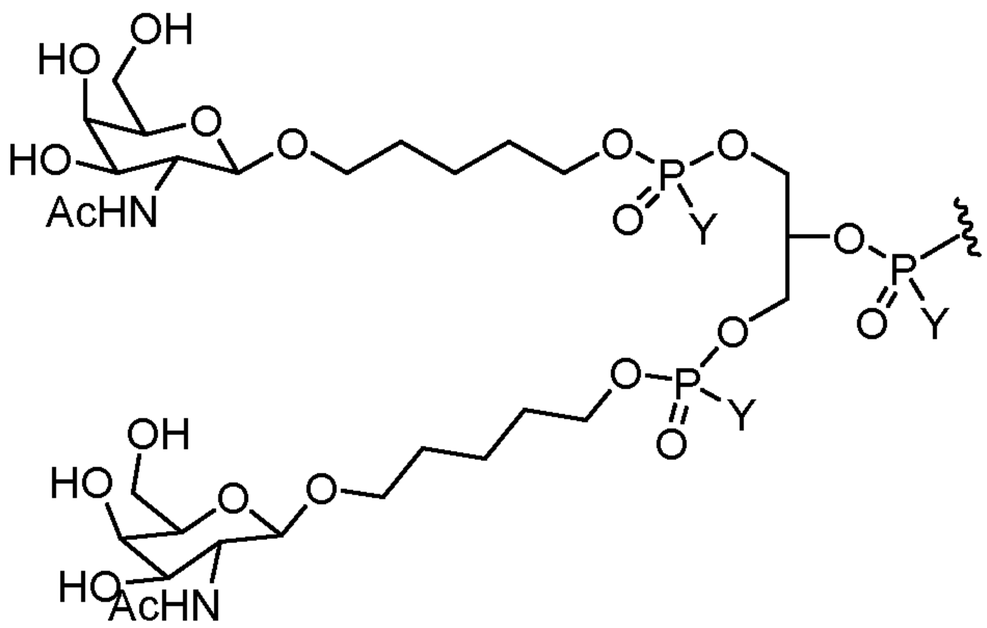
In certain embodiments, the cell-targeting moiety comprises:



In certain embodiments, the cell-targeting moiety has the following structure:

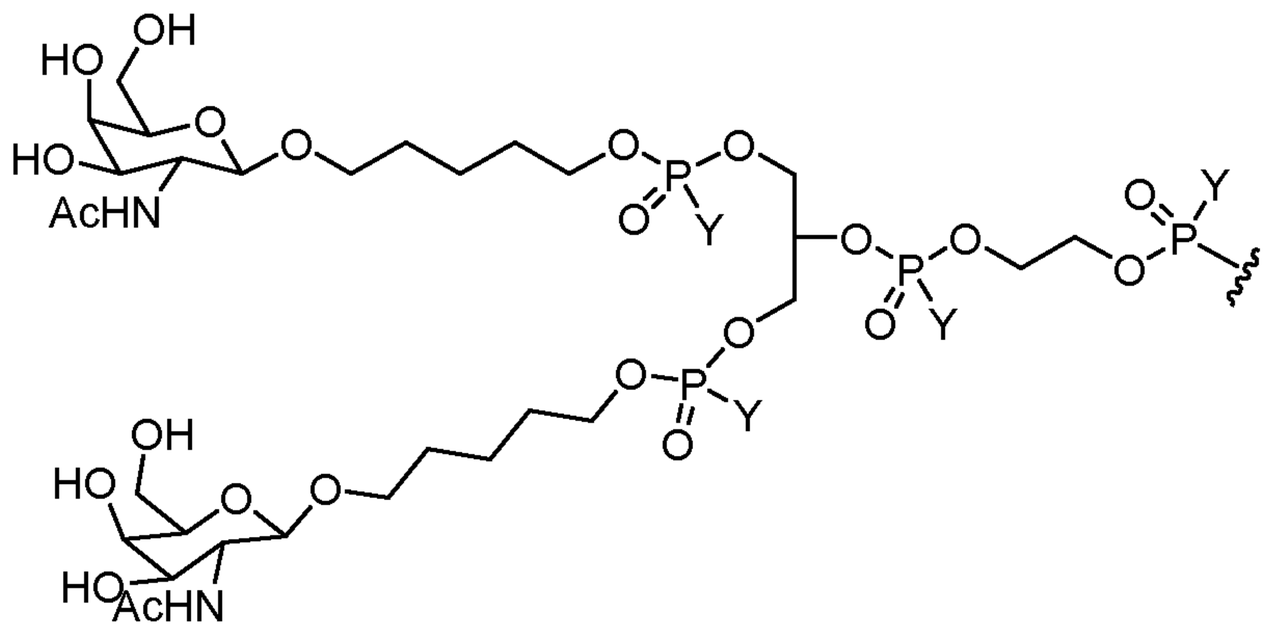


In certain embodiments, the cell-targeting moiety comprises:



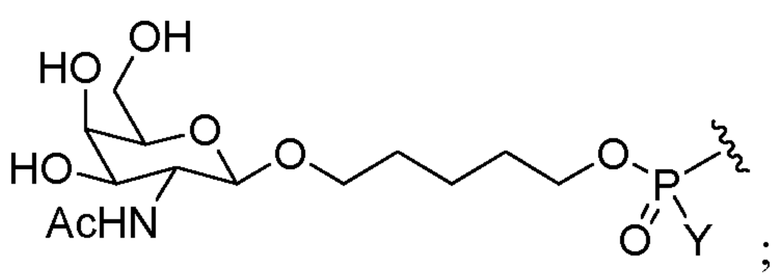
wherein each Y is selected from O, S, a substituted or unsubstituted C_1-C_{10} alkyl, amino, substituted amino, azido, alkenyl or alkynyl.

In certain embodiments, the conjugate group comprises:



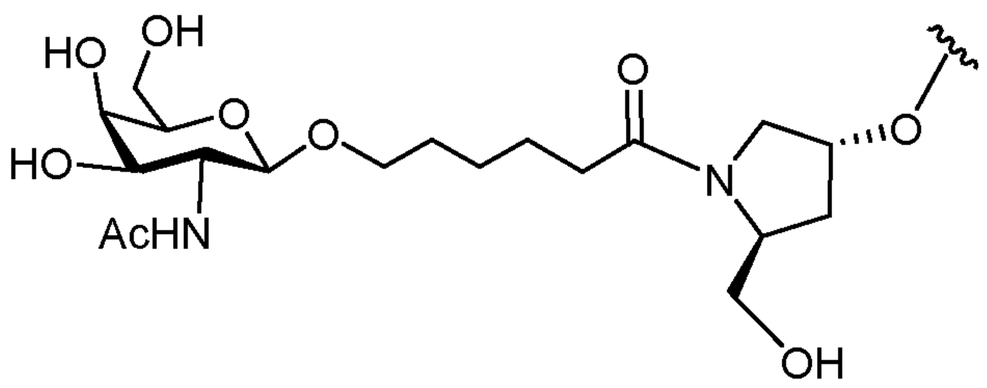
wherein each Y is selected from O, S, a substituted or unsubstituted C_1-C_{10} alkyl, amino, substituted amino, azido, alkenyl or alkynyl.

In certain embodiments, the cell-targeting moiety has the following structure:

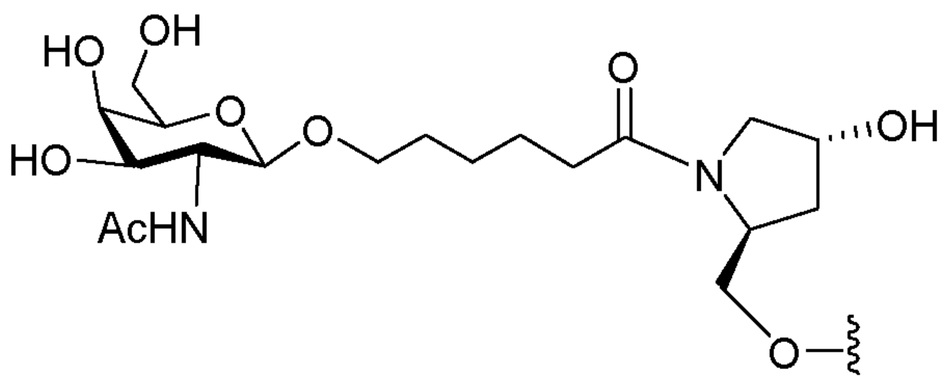


wherein each Y is selected from O, S, a substituted or unsubstituted C_1-C_{10} alkyl, amino, substituted amino, azido, alkenyl or alkynyl.

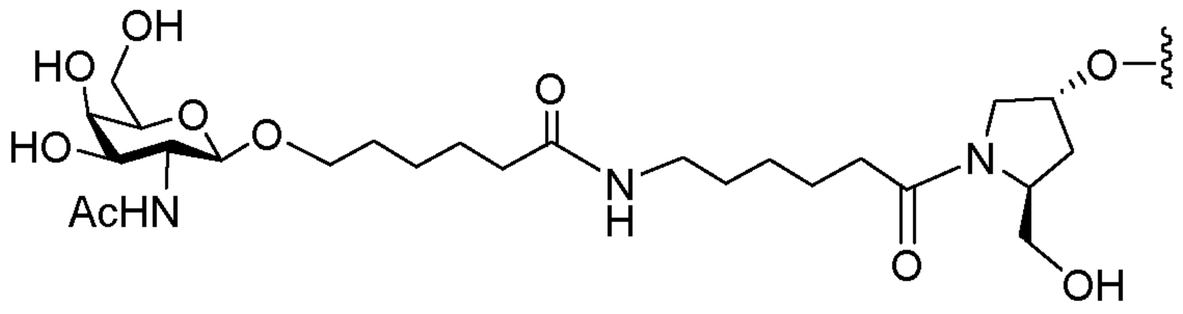
In certain embodiments, the conjugate group comprises:



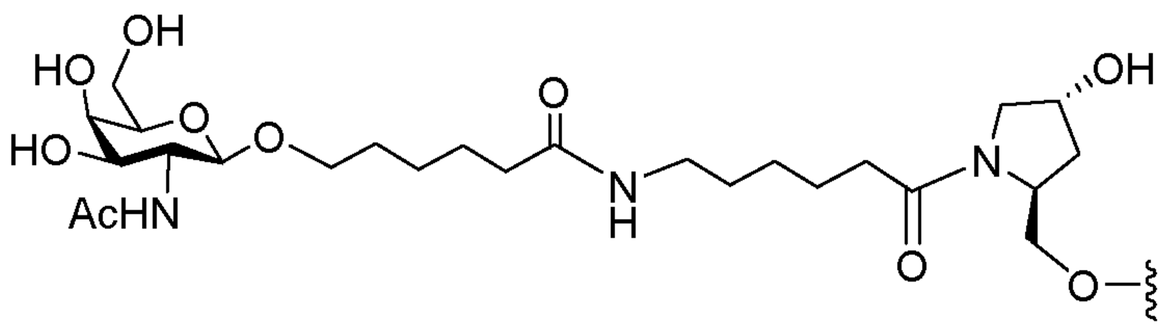
In certain embodiments, the conjugate group comprises:



T In certain embodiments, the conjugate group comprises:



In certain embodiments, the conjugate group comprises:



In certain embodiments, the conjugate group comprises a cleavable moiety selected from among: a phosphodiester, an amide, or an ester.

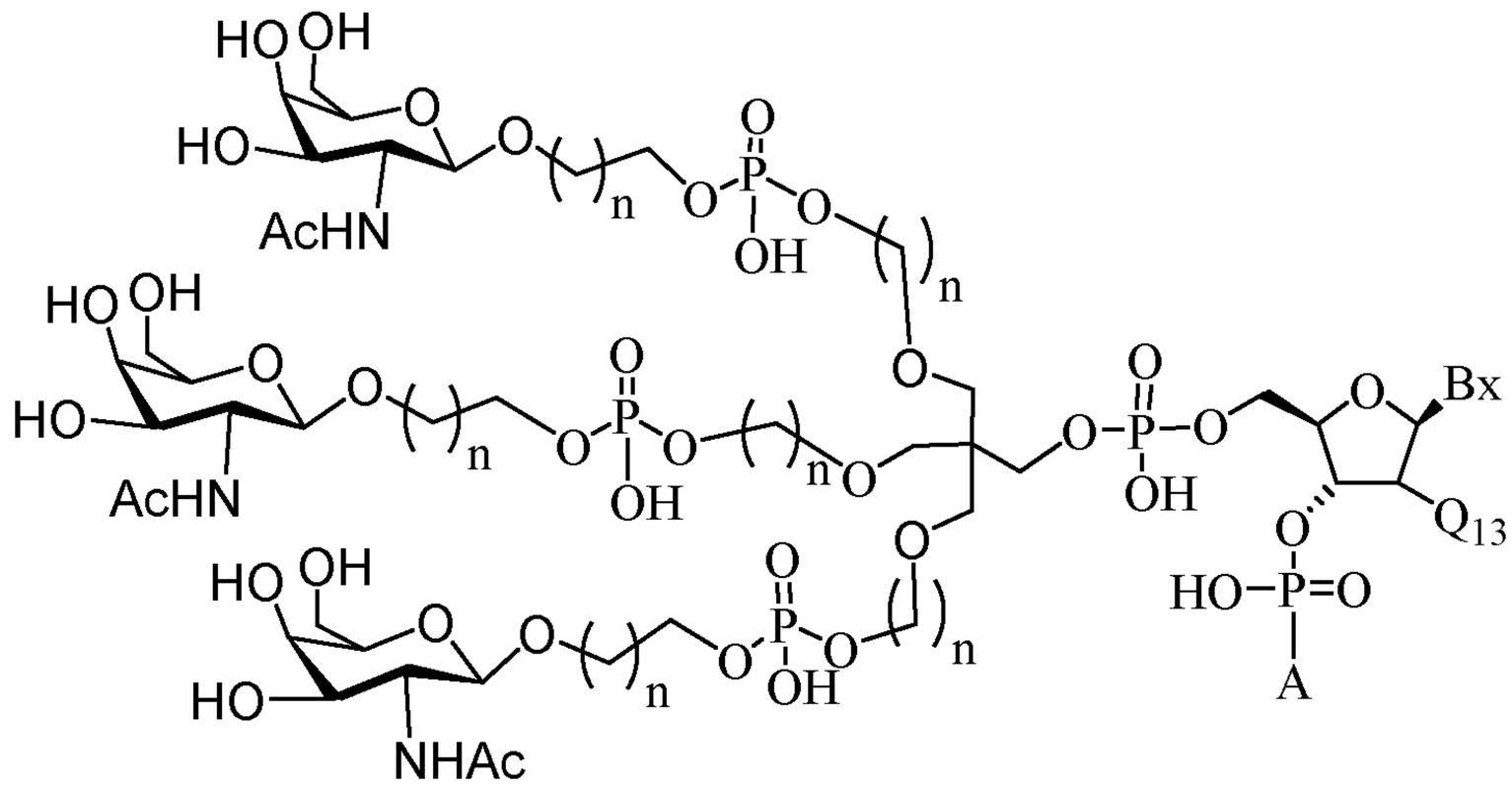
In certain embodiments, the conjugate group comprises a phosphodiester cleavable moiety.

In certain embodiments, the conjugate group does not comprise a cleavable moiety, and wherein the conjugate group comprises a phosphorothioate linkage between the conjugate group and the oligonucleotide.

In certain embodiments, the conjugate group comprises an amide cleavable moiety.

In certain embodiments, the conjugate group comprises an ester cleavable moiety.

In certain embodiments, the compound has the following structure:



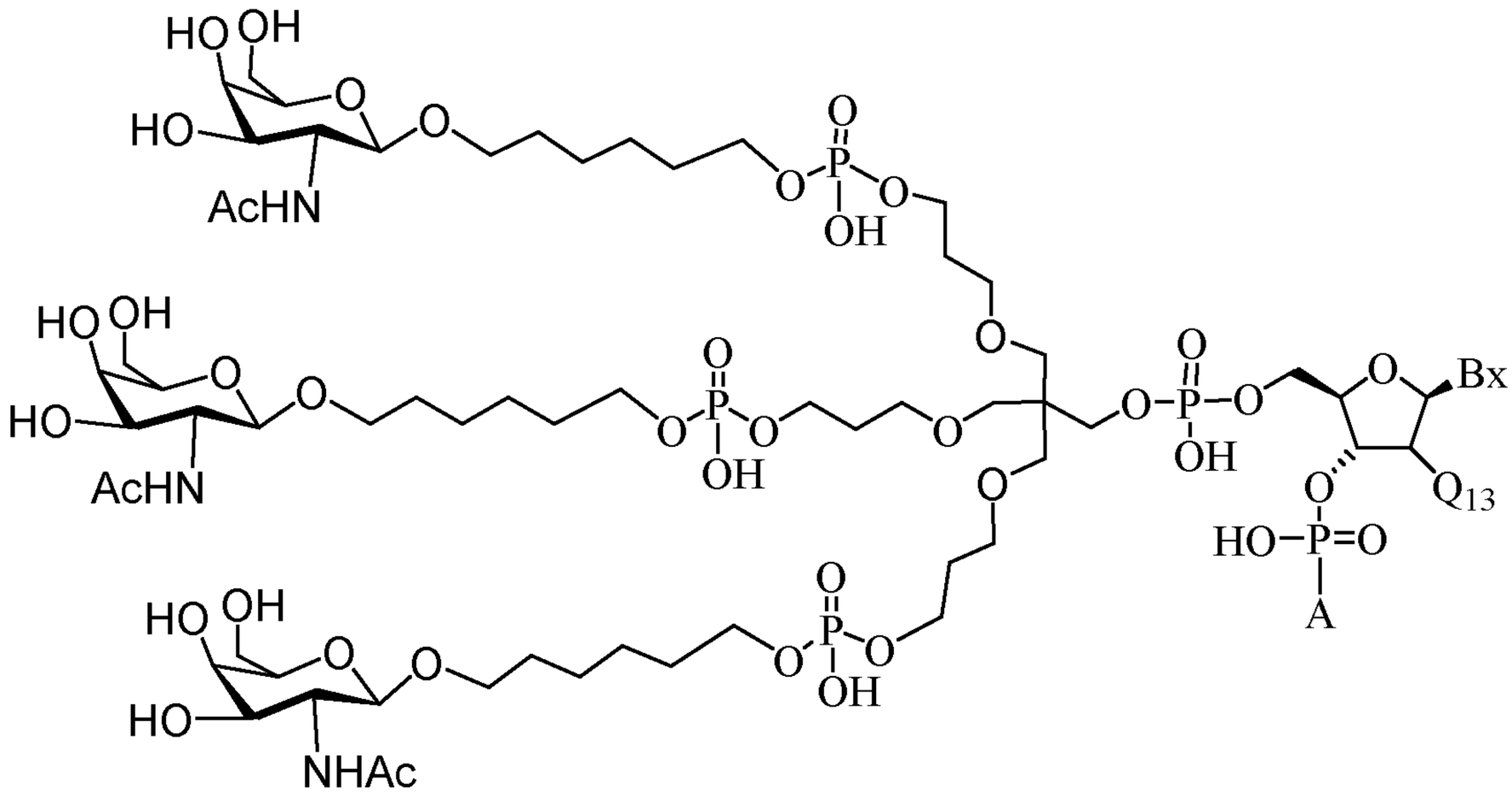
wherein each n is, independently, from 1 to 20;

Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:



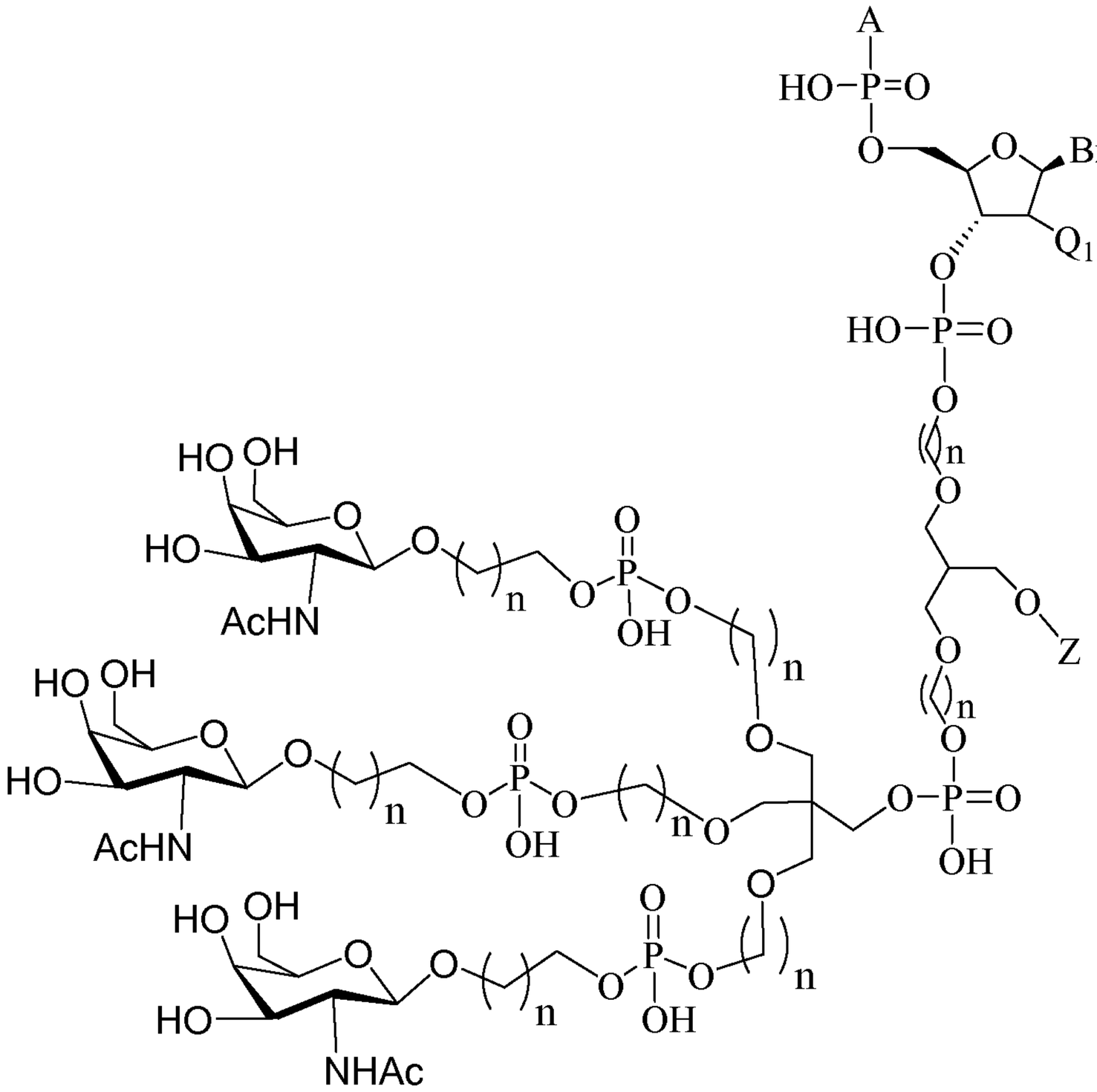
wherein each n is, independently, from 1 to 20;

Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:



wherein each n is, independently, from 1 to 20;

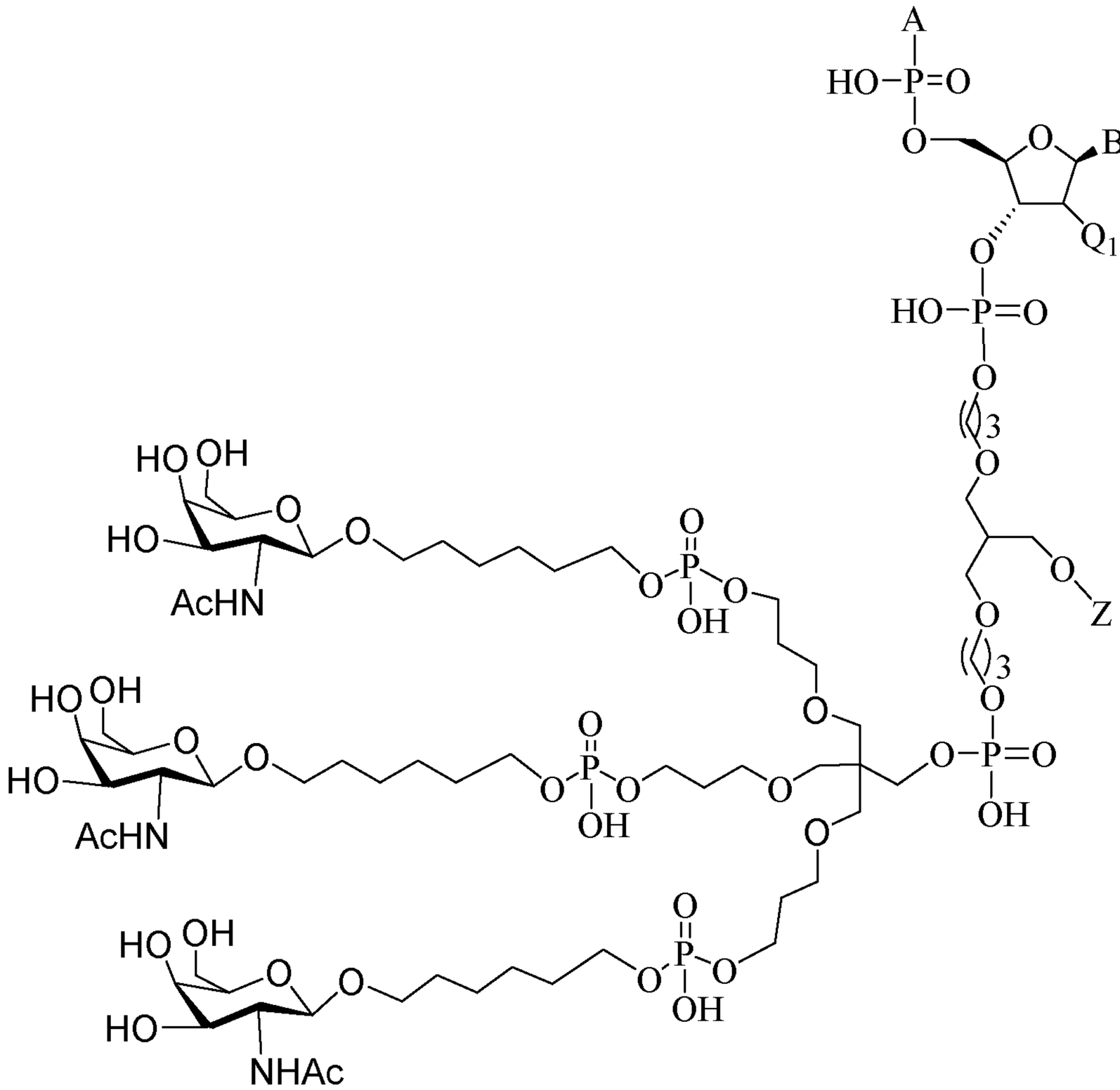
Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide;

Z is H or a linked solid support; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:



wherein each n is, independently, from 1 to 20;

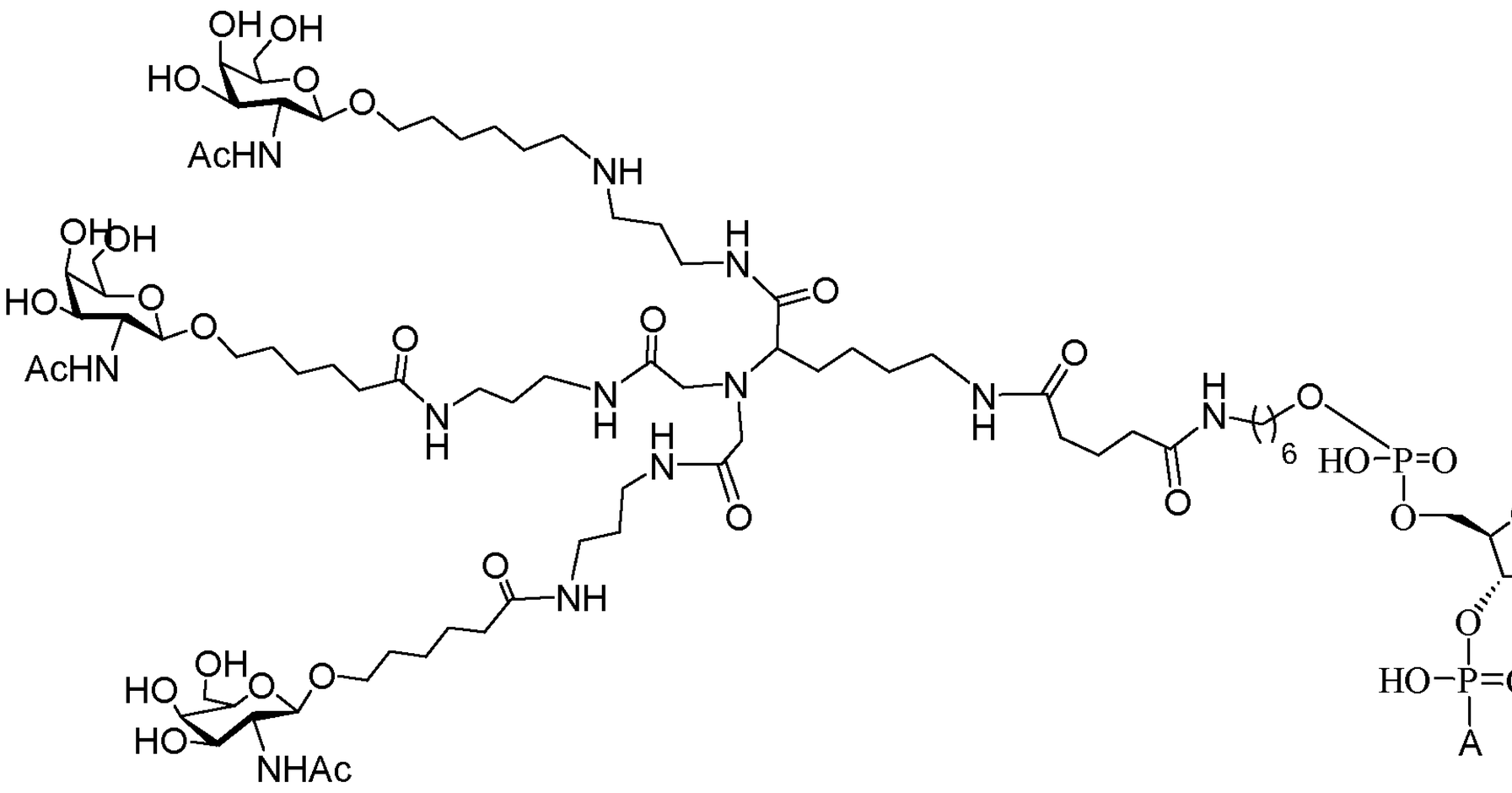
Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide;

Z is H or a linked solid support; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:

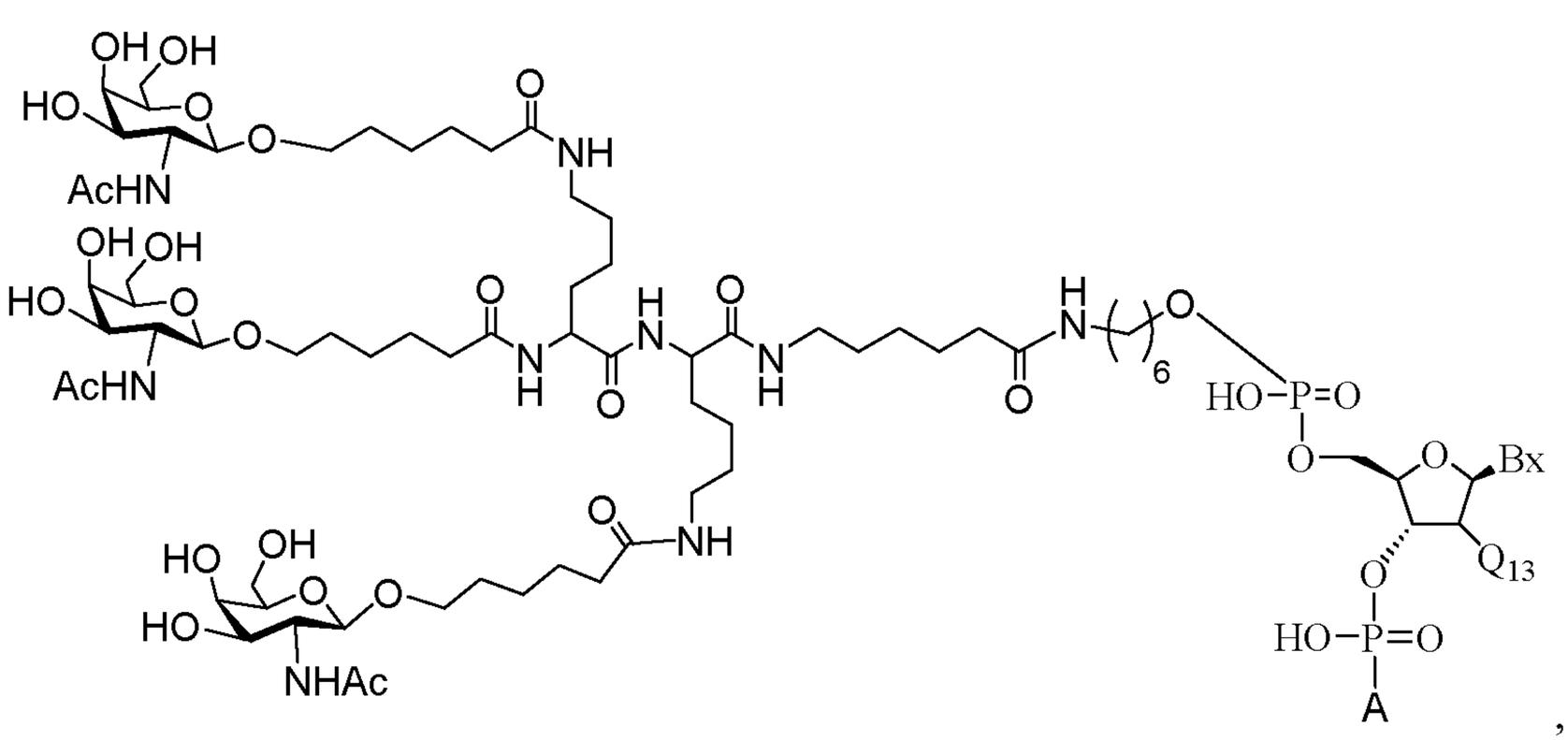


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide;and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:

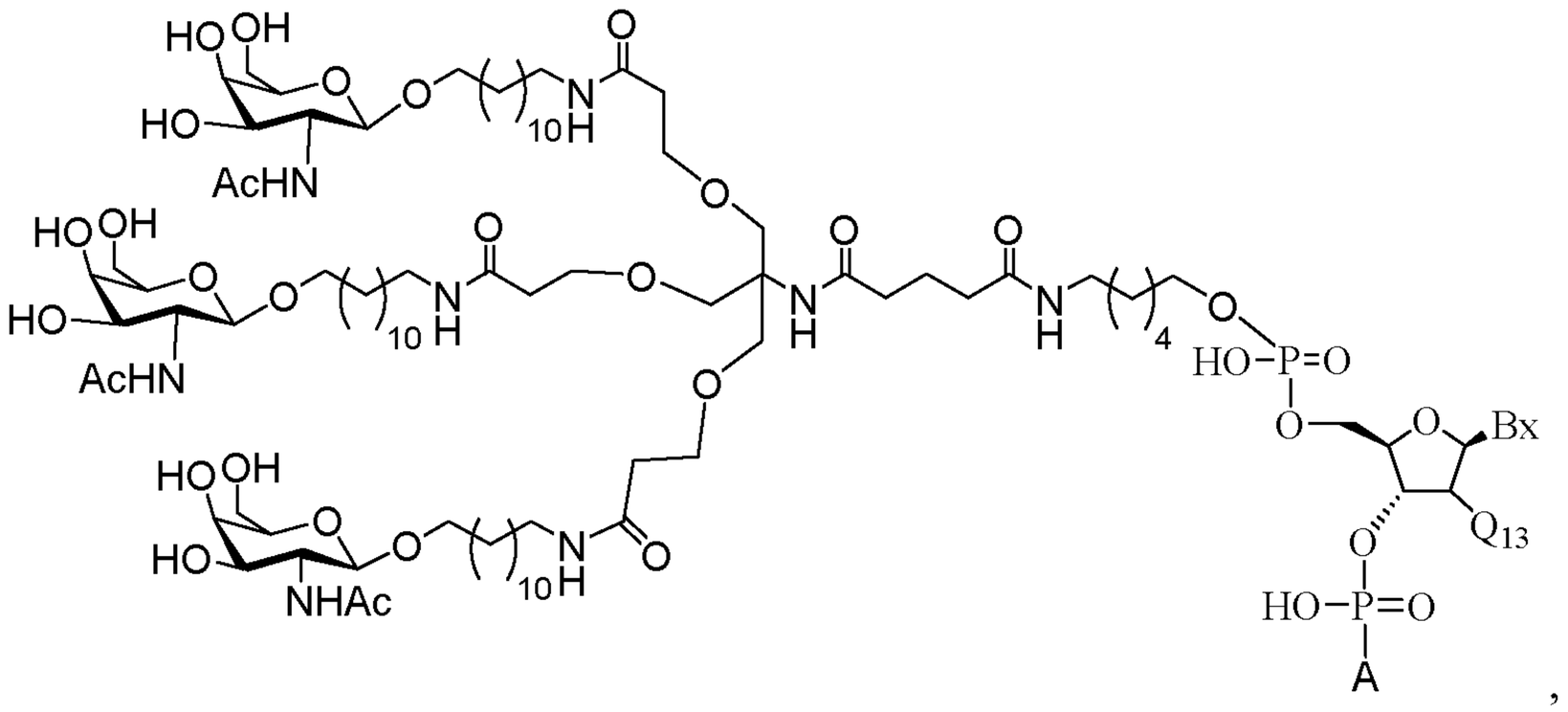


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide;and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:

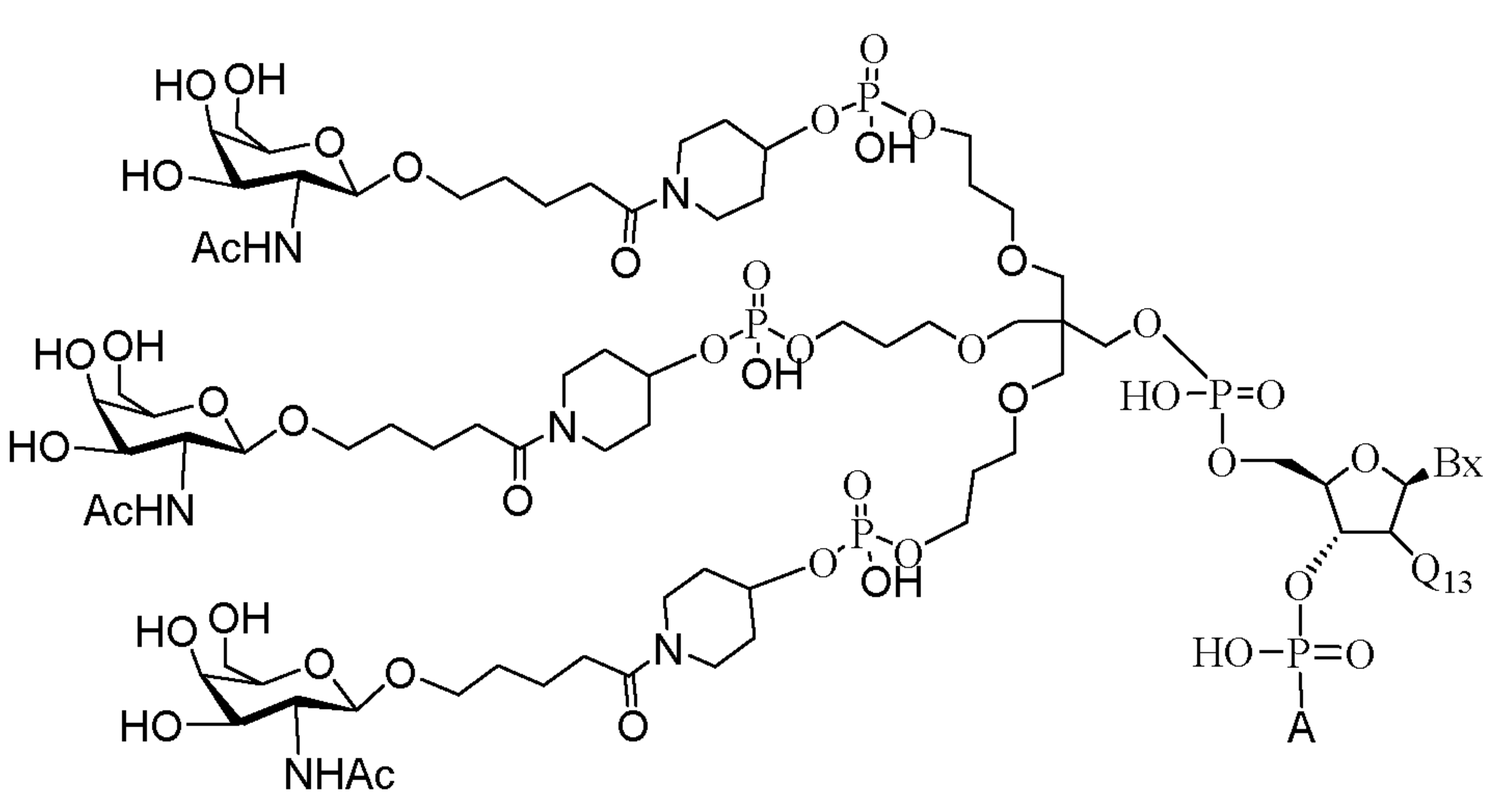


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:

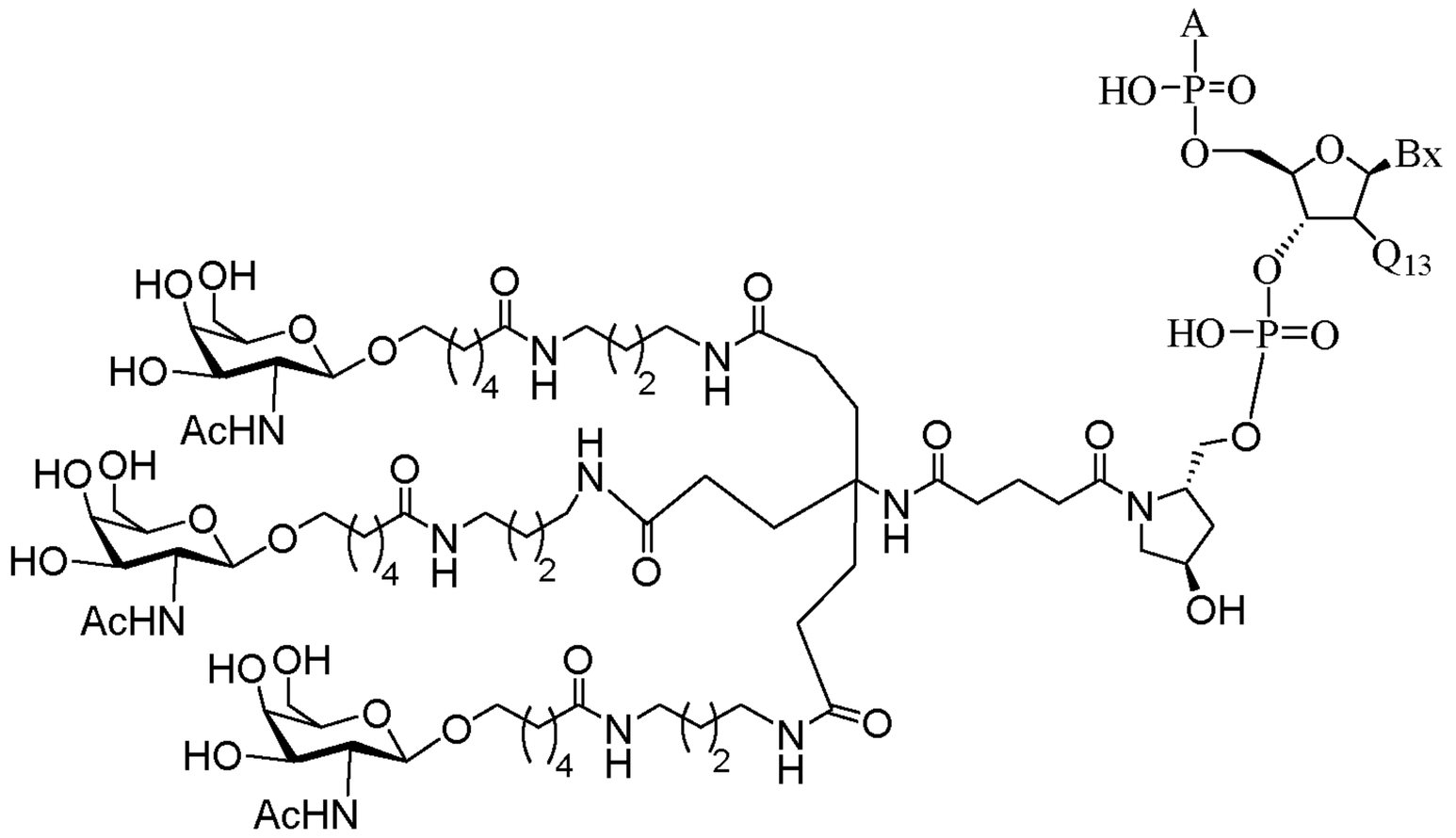


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:

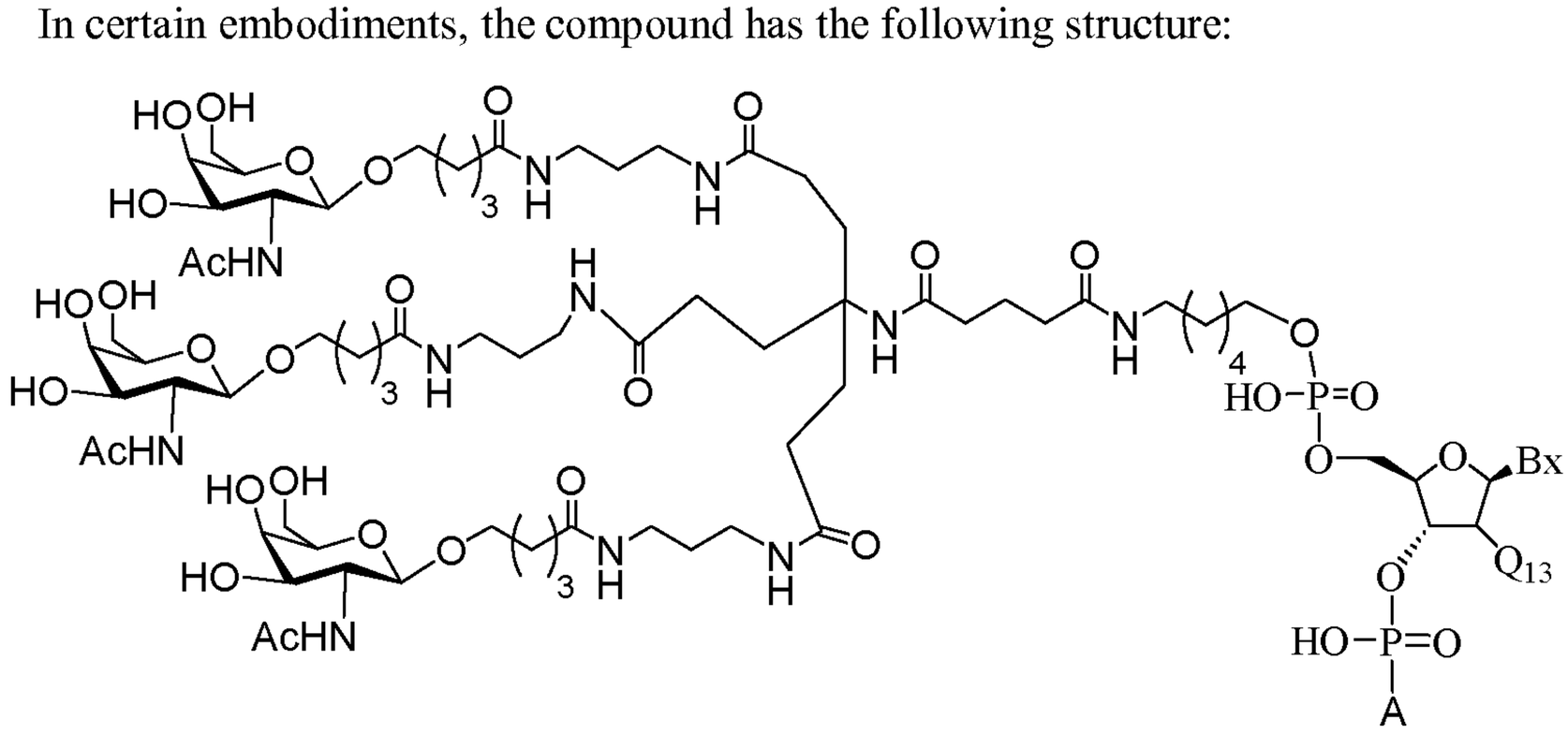


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:

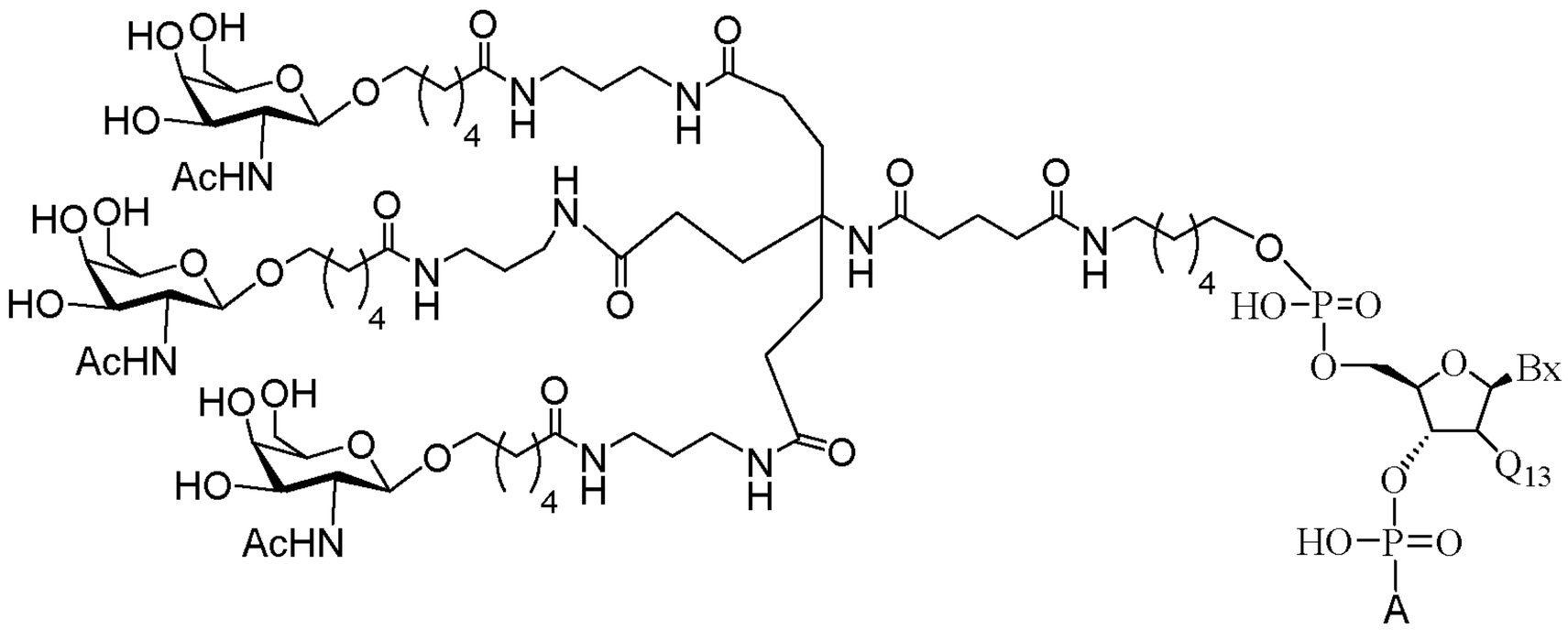


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:

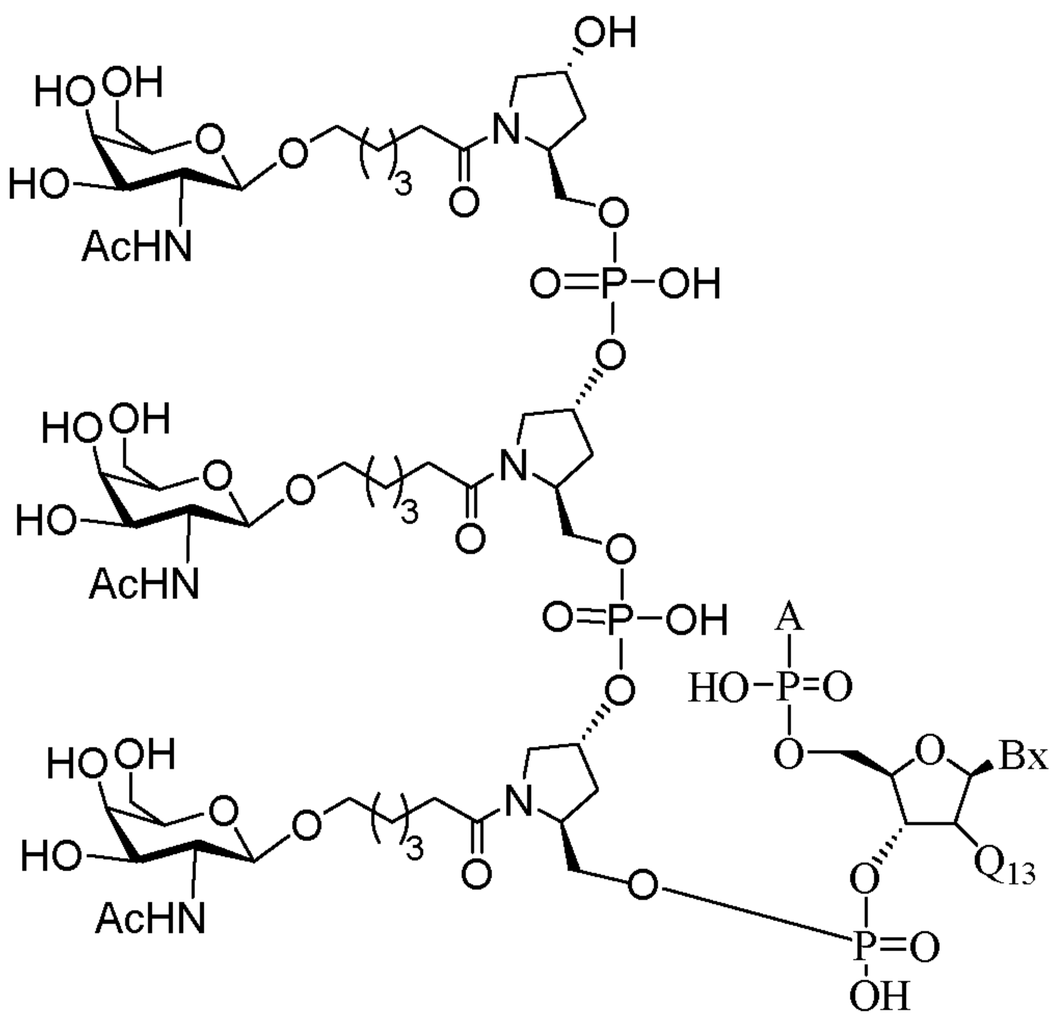


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:

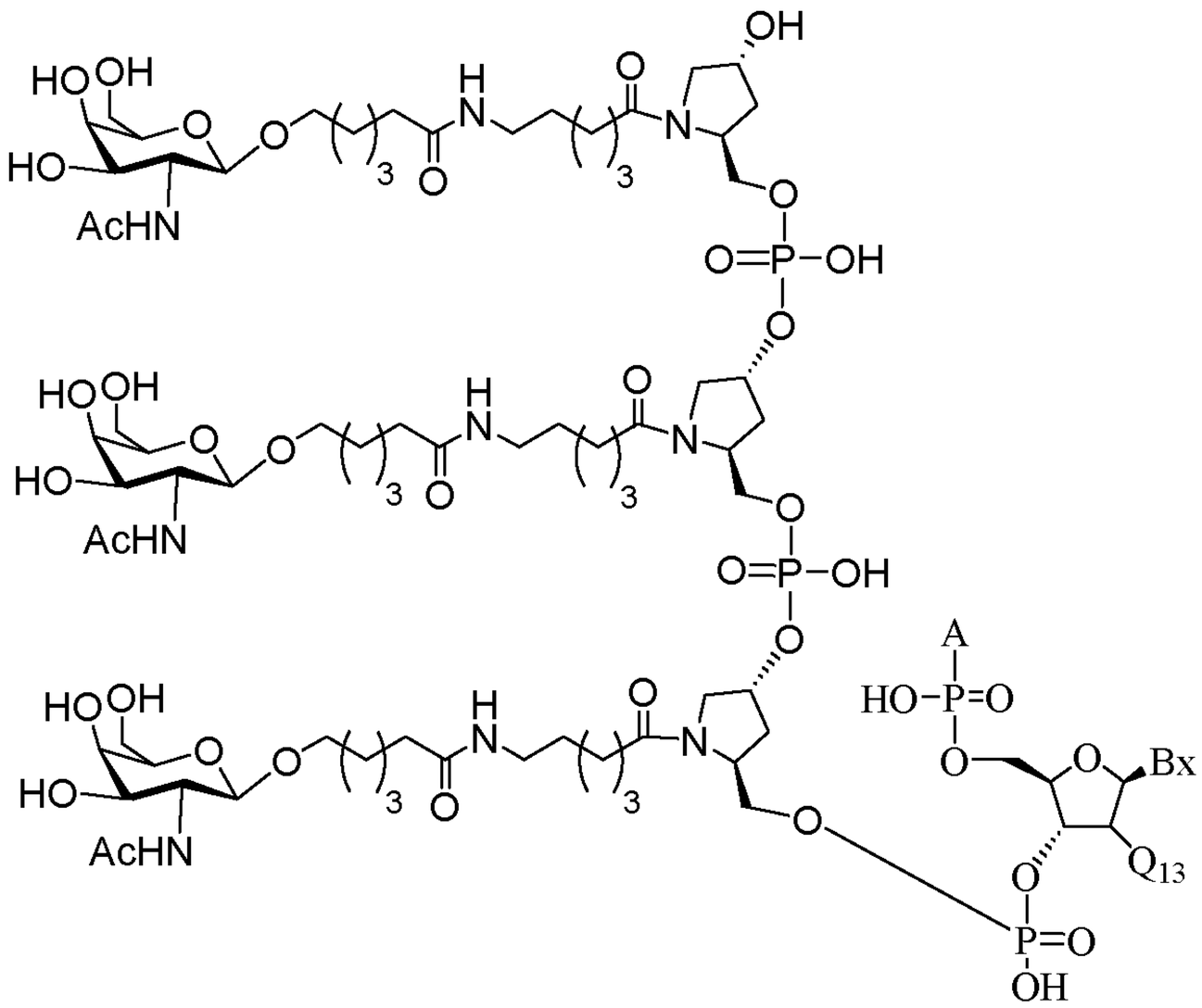


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:

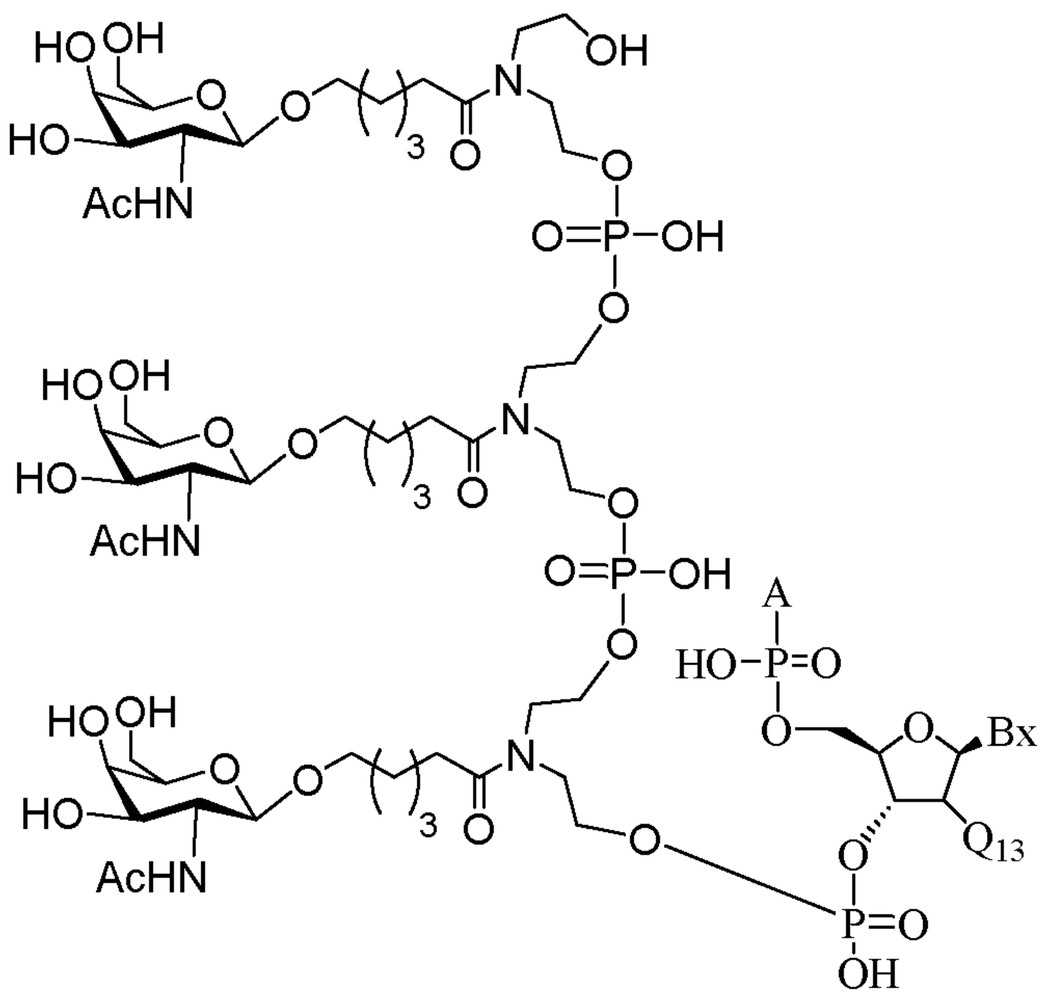


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:

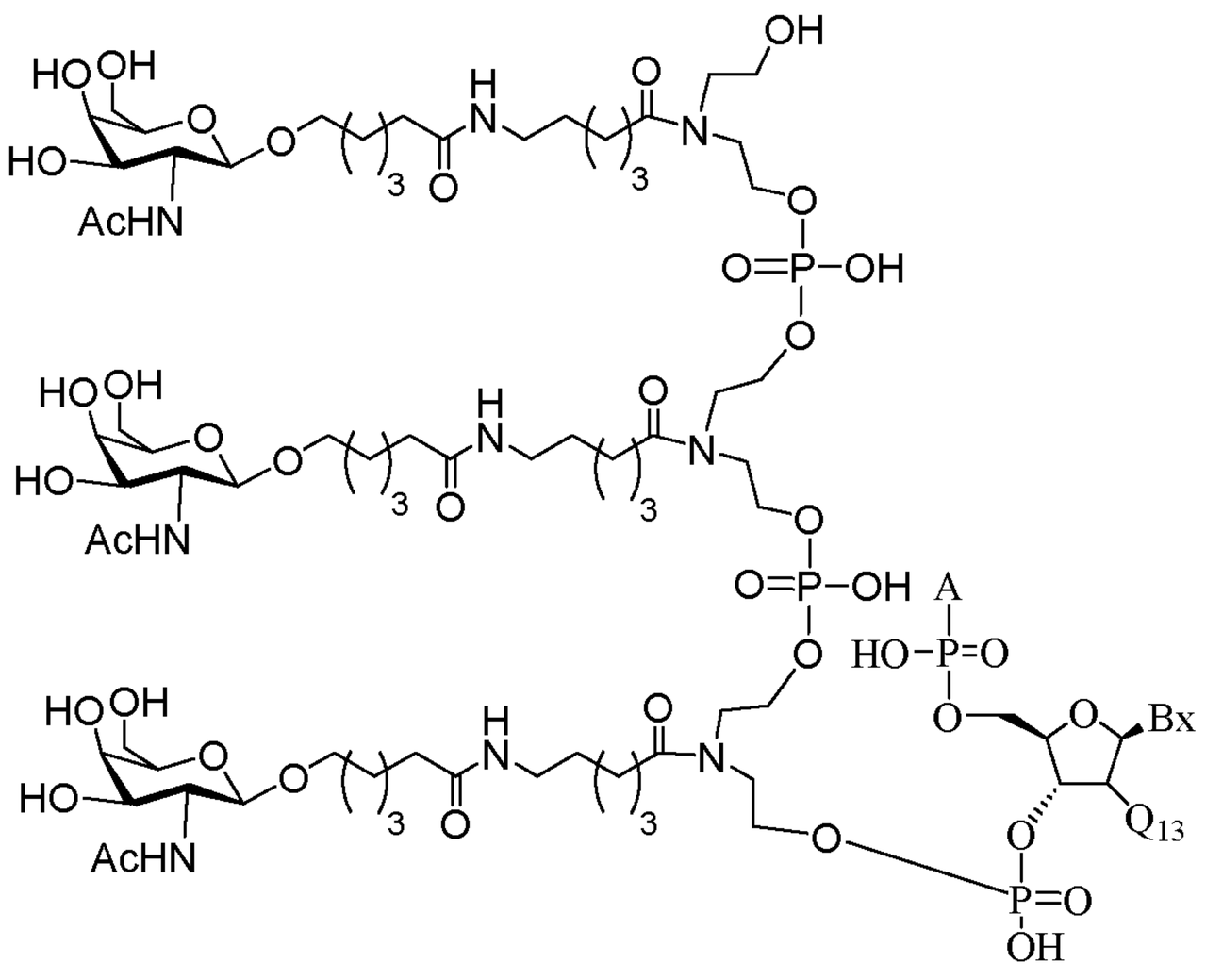


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the compound has the following structure:

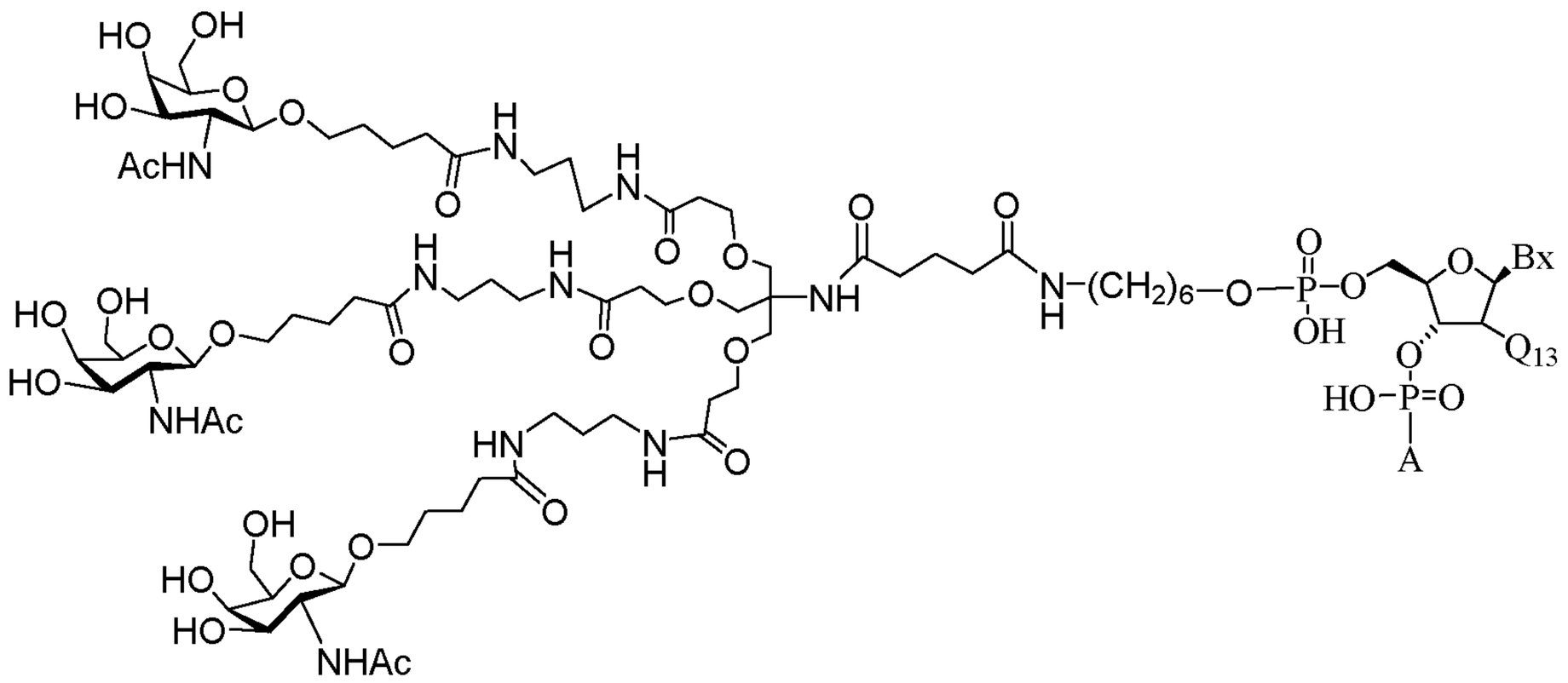


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the conjugate group comprises:

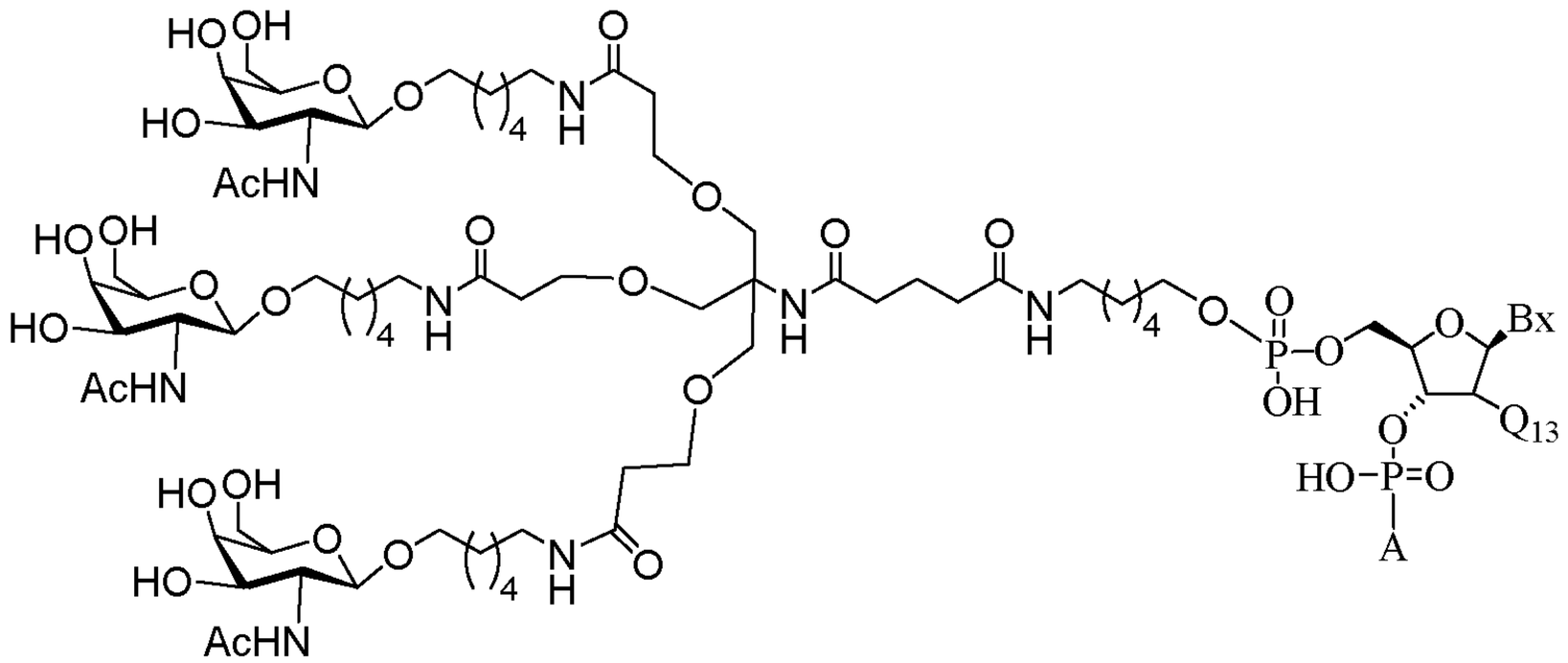


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the conjugate group comprises:

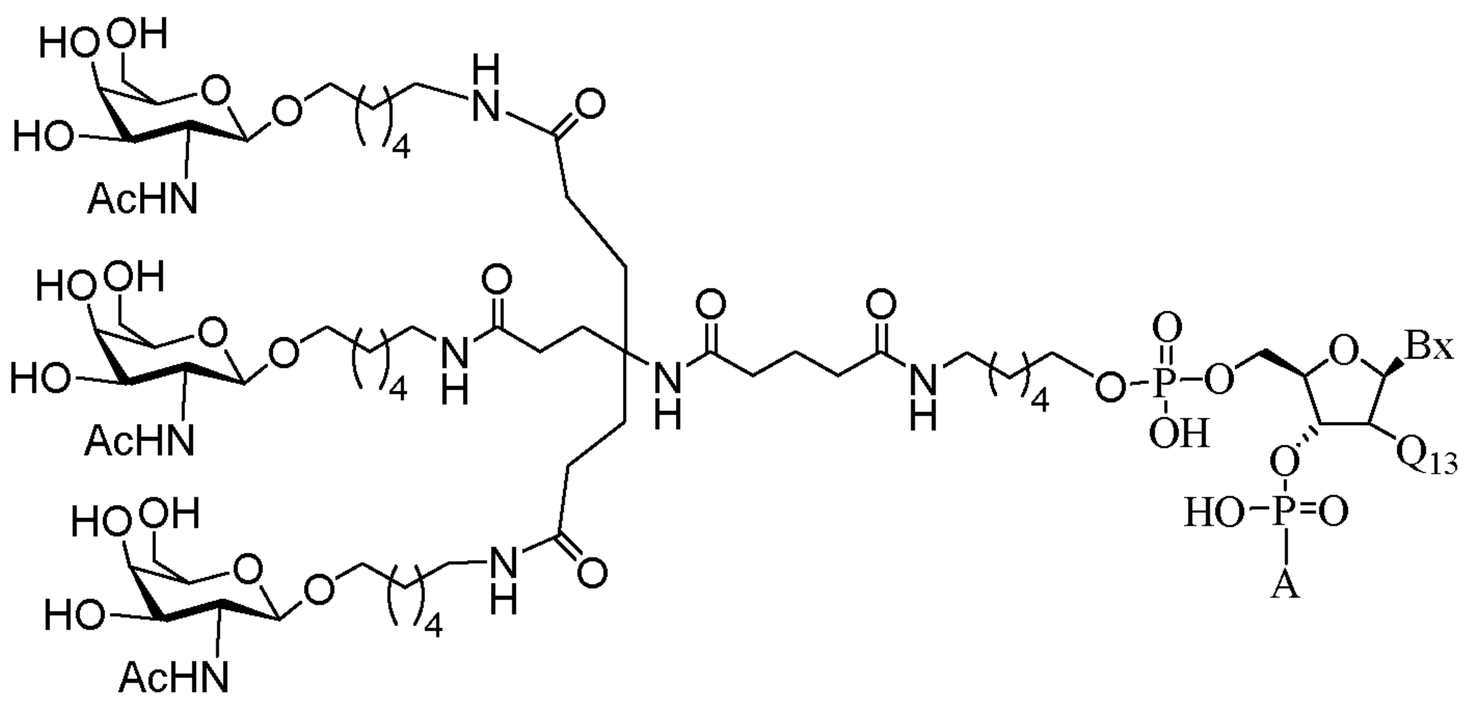


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, the conjugate group comprises:



wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

In certain embodiments, B_x is selected from among from adenine, guanine, thymine, uracil, or cytosine, or 5-methyl cytosine. In certain embodiments, B_x is adenine. In certain embodiments, B_x is thymine.

In certain embodiments, Q_{13} is $O(CH_2)_2$-OCH_3. In certain embodiments, Q_{13} is H.

Certain embodiments of the invention provide a prodrug comprising the compositions or compounds disclosed herein. Certain embodiments provide methods of using the conjugated antisense compounds and compositions described herein for inhibiting ANGPTL3 expression. In certain embodiments, the conjugated antisense compounds or compositions inhibit ANGPTL3 by at least 5%, at least 10%, at least 20%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95%. In a preferred embodiment, the

antisense compound comprising a modified oligonucleotide and a conjugate group decreases ANGPTL3 by at least 50%. In a preferred embodiment, the antisense compound comprising a modified oligonucleotide and a conjugate group decreases ANGPTL3 by at least 55%. In a preferred embodiment the antisense compound comprising a modified oligonucleotide and a conjugate group decreases ANGPTL3 by at least 60%. In a preferred embodiment, the antisense compound comprising a modified oligonucleotide and a conjugate group decreases ANGPTL3 by at least 65%. In a preferred embodiment, the antisense compound comprising a modified oligonucleotide and a conjugate group decreases ANGPTL3 by at least 70%. In a preferred embodiment, the antisense compound comprising a modified oligonucleotide and a conjugate group decreases ANGPTL3 by at least 75%. In a preferred embodiment, the antisense compound comprising a modified oligonucleotide and a conjugate group decreases ANGPTL3 by at least 80%. In a preferred embodiment, the antisense compound comprising a modified oligonucleotide and a conjugate group decreases ANGPTL3 by at least 85%. In a preferred embodiment, the antisense compound comprising a modified oligonucleotide and a conjugate group decreases ANGPTL3 by at least 90%. In a preferred embodiment, the antisense compound comprising a modified oligonucleotide and a conjugate group decreases ANGPTL3 by at least 95%.

In certain embodiments, the conjugated antisense compounds or compositions disclosed herein have an IC_{50} of less than 20 μM, less than 10 μM, less than 8 μM, less than 5μM, less than 2 μM, less than 1 μM, or less than 0.8 μM, when tested human cells, for example, in the Hep3B cell line as described in Examples 2-3 and 7-10.

In certain embodiments, the conjugated antisense compounds or compositions disclosed herein are efficacious by virtue of having a viscosity of less than 40 cP, less than 35 cP, less than 30 cP, less than 25 cP, less than 20 cP or less than 15 cP when measured by the parameters as described in Example 13.

In certain embodiments, the conjugated antisense compounds or compositions disclosed herein are highly tolerable, as demonstrated by the *in vivo* tolerability measurements described in the examples. In certain embodiments, the conjugated antisense compounds as described herein are highly tolerable, as demonstrated by having an increase in ALT and/or AST value of no more than 4 fold, 3 fold, 2 fold or 1.5 fold over saline treated animals.

Certain embodiments disclosed herein provide a salt of the conjugated antisense compounds disclosed herein. In certain embodiments, the compounds or compositions disclosed herein comprise a salt of the modified oligonucleotide with the conjugate group.

In certain embodiments, the conjugated antisense compounds or compositions disclosed herein further comprise a pharmaceutically acceptable carrier or diluent.

In certain embodiments, the animal is a human.

Certain embodiments disclosed herein provide methods comprising administering to an animal the conjugated antisense compounds or compositions disclosed herein. In certain embodiments, administering the conjugated antisense compound or composition is therapeutic. In certain embodiments, administering the

conjugated antisense compound or composition treats, prevents, or slows progression of a disease related to ANGPTL3. In certain embodiments, the disease is related to elevated ANGPTL3. In certain embodiments, administering the conjugated antisense compound or composition prevents, treats, ameliorates, or slows progression of a cardiovascular and/or metabolic disease.

Certain embodiments disclosed herein provide methods for treating a human with a cardiovascular and/or metabolic disease comprising identifying a human with cardiovascular and/or metabolic disease and administering to the human a therapeutically effective amount of any of the conjugated antisense compounds or compositions disclosed herein, so as to treat the human for cardiovascular and/or metabolic disease.

Certain embodiments provide conjugated antisense compounds and compositions described herein for use in therapy. In certain embodiments, the therapy is used in treating, preventing, or slowing progression of a disease related to ANGPTL3. In certain embodiments, the therapy is used in treating, preventing, or slowing progression of a disease related to elevated ANGPTL3.

In certain embodiments, the disease is a cardiovascular and/or metabolic disease, disorder or condition. In certain embodiments, the metabolic and/or cardiovascular disease includes, but is not limited to, obesity, diabetes, insulin resistance, atherosclerosis, dyslipidemia, lipodystrophy, coronary heart disease, non-alcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH) hyperfattyacidemia or metabolic syndrome, or a combination thereof. The dyslipidemia can be hyperlipidemia. The hyperlipidemia can be combined hyperlipidemia (CHL), hypercholesterolemia, hypertriglyceridemia, or both hypercholesterolemia and hypertriglyceridemia. The combined hyperlipidemia can be familial or non-familial. The hypercholesterolemia can be familial homozygous hypercholesterolemia (HoFH), familial heterozygous hypercholesterolemia (HeFH). The hypertriglyceridemia can be familial chylomicronemia syndrome (FCS) or hyperlipoproteinemia Type IV. The NAFLD can be hepatic steatosis or steatohepatitis. The diabetes can be type 2 diabetes or type 2 diabetes with dyslipidemia. The insulin resistance can be insulin resistance with dyslipidemia.

In certain embodiments, the conjugated antisense compounds or compositions disclosed herein are designated as a first agent and the methods or uses disclosed herein further comprise administering a second agent. In certain embodiments, the first agent and the second agent are co-administered. In certain embodiments the first agent and the second agent are co-administered sequentially or concomitantly.

In certain embodiments, the second agent is a glucose-lowering agent. The glucose lowering agent can include, but is not limited to, a therapeutic lifestyle change, PPAR agonist, a dipeptidyl peptidase (IV) inhibitor, a GLP-1 analog, insulin or an insulin analog, an insulin secretagogue, a SGLT2 inhibitor, a human amylin analog, a biguanide, an alpha-glucosidase inhibitor, or a combination thereof. The glucose-lowering agent can include, but is not limited to metformin, sulfonylurea, rosiglitazone, meglitinide, thiazolidinedione, alpha-glucosidase inhibitor or a combination thereof. The sulfonylurea can be acetohexamide, chlorpropamide, tolbutamide, tolazamide, glimepiride, a glipizide, a glyburide, or a gliclazide. The

meglitinide can be nateglinide or repaglinide. The thiazolidinedione can be pioglitazone or rosiglitazone. The alpha-glucosidase can be acarbose or miglitol.

In certain embodiments, the second agent is a lipid-lowering therapy. In certain embodiments the lipid lowering therapy can include, but is not limited to, a therapeutic lifestyle change, HMG-CoA reductase inhibitor, cholesterol absorption inhibitor, MTP inhibitor (e.g., a small molecule, polypeptide, antibody or antisense compound targeted to MTP), ApoB inhibitor (e.g., a small molecule, polypeptide, antibody or antisense compound targeted to ApoB), ApoC3 inhibitor (e.g., a small molecule, polypeptide, antibody or antisense compound targeted to ApoC3), PCSK9 inhibitor (e.g., a small molecule, polypeptide, antibody or antisense compound targeted to PCSK9),CETP inhibitor (e.g., a small molecule, polypeptide, antibody or antisense compound targeted to CETP), fibrate, beneficial oil (e.g., krill or fish oils (e.g., Vascepa^{R}), flaxseed oil, or other oils rich in omega-3 fatty acids such as α-linolenic acid (ALA), docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA)), or any combination thereof. The HMG-CoA reductase inhibitor can be atorvastatin, rosuvastatin, fluvastatin, lovastatin, pravastatin, or simvastatin. The cholesterol absorption inhibitor can be ezetimibe. The fibrate can be fenofibrate, bezafibrate, ciprofibrate, clofibrate, gemfibrozil and the like.

In certain embodiments, administration comprises parenteral administration. In certain embodiments, administration comprises subcutaneous administration.

In certain embodiments, administering a conjugated antisense compound disclosed herein results in a reduction of lipid levels, including triglyceride levels, cholesterol levels, insulin resistance, glucose levels or a combination thereof. One or more of the levels can be independently reduced by at least 5%, at least 10%, at least 20%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95%. Administering the conjugated antisense compound can result in improved insulin sensitivity or hepatic insulin sensitivity. Administering the conjugated antisense compound disclosed herein can result in a reduction in atherosclerotic plaques, obesity, glucose, lipids, glucose resistance, cholesterol, or improvement in insulin sensitivity or any combination thereof.

Certain embodiments provide the use of a conjugated antisense compound as described herein in the manufacture of a medicament for treating, ameliorating, delaying or preventing one or more of a disease related to ANGPTL3. Certain embodiments provide the use of a conjugated antisense compound as described herein in the manufacture of a medicament for treating, ameliorating, delaying or preventing one or more of a metabolic disease or a cardiovascular disease.

Certain embodiments provide a kit for treating, preventing, or ameliorating one or more of a metabolic disease or a cardiovascular disease as described herein wherein the kit comprises: a) a conjugated antisense compound as described herein; and optionally b) an additional agent or therapy as described herein. The kit can further include instructions or a label for using the kit to treat, prevent, or ameliorate one or more of a metabolic disease or a cardiovascular disease.

Antisense Compounds

Oligomeric compounds include, but are not limited to, oligonucleotides, oligonucleosides, oligonucleotide analogs, oligonucleotide mimetics, antisense compounds, antisense oligonucleotides, and siRNAs. An oligomeric compound can be "antisense" to a target nucleic acid, meaning that is capable of undergoing hybridization to a target nucleic acid through hydrogen bonding.

In certain embodiments, an antisense compound has a nucleobase sequence that, when written in the 5' to 3' direction, comprises the reverse complement of the target segment of a target nucleic acid to which it is targeted. In certain such embodiments, an antisense oligonucleotide has a nucleobase sequence that, when written in the 5' to 3' direction, comprises the reverse complement of the target segment of a target nucleic acid to which it is targeted.

In certain embodiments, an antisense compound targeted to ANGPTL3 nucleic acid is 10 to 30 nucleotides in length. In other words, antisense compounds are from 10 to 30 linked nucleobases. In other embodiments, the antisense compound comprises a modified oligonucleotide consisting of 8 to 80, 10 to 80, 12 to 50, 12 to 30, 15 to 30, 18 to 24, 19 to 22, or 20 linked nucleobases. In certain such embodiments, the antisense compound comprises a modified oligonucleotide consisting of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 linked nucleobases in length, or a range defined by any two of the above values.

In certain embodiments, the antisense compound comprises a shortened or truncated modified oligonucleotide. The shortened or truncated modified oligonucleotide can have a single nucleoside deleted from the 5' end (5' truncation), or alternatively from the 3' end (3' truncation). A shortened or truncated oligonucleotide can have two or more nucleosides deleted from the 5' end, or alternatively can have two or more nucleosides deleted from the 3' end. Alternatively, the deleted nucleosides can be dispersed throughout the modified oligonucleotide, for example, in an antisense compound having one or more nucleoside deleted from the 5' end and one or more nucleoside deleted from the 3' end.

When a single additional nucleoside is present in a lengthened oligonucleotide, the additional nucleoside can be located at the 5', 3' end or central portion of the oligonucleotide. When two or more additional nucleosides are present, the added nucleosides can be adjacent to each other, for example, in an oligonucleotide having two nucleosides added to the 5' end (5' addition), or alternatively to the 3' end (3' addition) or the central portion, of the oligonucleotide. Alternatively, the added nucleoside can be dispersed throughout the antisense compound, for example, in an oligonucleotide having one or more nucleoside added to the 5' end, one or more nucleoside added to the 3' end, and/or one or more nucleoside added to the central portion.

It is possible to increase or decrease the length of an antisense compound, such as an antisense oligonucleotide, and/or introduce mismatch bases without eliminating activity. For example, in Woolf et al. (Proc. Natl. Acad. Sci. USA 89:7305-7309, 1992), a series of antisense oligonucleotides 13-25 nucleobases in

length were tested for their ability to induce cleavage of a target RNA in an oocyte injection model. Antisense oligonucleotides 25 nucleobases in length with 8 or 11 mismatch bases near the ends of the antisense oligonucleotides were able to direct specific cleavage of the target mRNA, albeit to a lesser extent than the antisense oligonucleotides that contained no mismatches. Similarly, target specific cleavage was achieved using 13 nucleobase antisense oligonucleotides, including those with 1 or 3 mismatches.

Gautschi et al (J. Natl. Cancer Inst. 93:463-471, March 2001) demonstrated the ability of an oligonucleotide having 100% complementarity to the bcl-2 mRNA and having 3 mismatches to the bcl-xL mRNA to reduce the expression of both bcl-2 and bcl-xL in vitro and in vivo. Furthermore, this oligonucleotide demonstrated potent anti-tumor activity in vivo.

Maher and Dolnick (Nuc. Acid. Res. 16:3341-3358, 1988) tested a series of tandem 14 nucleobase antisense oligonucleotides, and a 28 and 42 nucleobase antisense oligonucleotides comprised of the sequence of two or three of the tandem antisense oligonucleotides, respectively, for their ability to arrest translation of human DHFR in a rabbit reticulocyte assay. Each of the three 14 nucleobase antisense oligonucleotides alone was able to inhibit translation, albeit at a more modest level than the 28 or 42 nucleobase antisense oligonucleotides.

Certain Antisense Compound Motifs and Mechanisms

In certain embodiments, antisense compounds have chemically modified subunits arranged in patterns, or motifs, to confer to the antisense compounds properties such as enhanced inhibitory activity, increased binding affinity for a target nucleic acid, or resistance to degradation by *in vivo* nucleases.

Chimeric antisense compounds typically contain at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, increased binding affinity for the target nucleic acid, and/or increased inhibitory activity. A second region of a chimeric antisense compound may confer another desired property e.g., serve as a substrate for the cellular endonuclease RNase H, which cleaves the RNA strand of an RNA:DNA duplex.

Antisense activity may result from any mechanism involving the hybridization of the antisense compound (e.g., oligonucleotide) with a target nucleic acid, wherein the hybridization ultimately results in a biological effect. In certain embodiments, the amount and/or activity of the target nucleic acid is modulated. In certain embodiments, the amount and/or activity of the target nucleic acid is reduced. In certain embodiments, hybridization of the antisense compound to the target nucleic acid ultimately results in target nucleic acid degradation. In certain embodiments, hybridization of the antisense compound to the target nucleic acid does not result in target nucleic acid degradation. In certain such embodiments, the presence of the antisense compound hybridized with the target nucleic acid (occupancy) results in a modulation of antisense activity. In certain embodiments, antisense compounds having a particular chemical motif or pattern of chemical modifications are particularly suited to exploit one or more mechanisms. In certain embodiments, antisense compounds function through more than one mechanism and/or through mechanisms

that have not been elucidated. Accordingly, the antisense compounds described herein are not limited by particular mechanism.

Antisense mechanisms include, without limitation, RNase H mediated antisense; RNAi mechanisms, which utilize the RISC pathway and include, without limitation, siRNA, ssRNA and microRNA mechanisms; and occupancy based mechanisms. Certain antisense compounds may act through more than one such mechanism and/or through additional mechanisms.

RNase H-Mediated Antisense

In certain embodiments, antisense activity results at least in part from degradation of target RNA by RNase H. RNase H is a cellular endonuclease that cleaves the RNA strand of an RNA:DNA duplex. It is known in the art that single-stranded antisense compounds which are "DNA-like" elicit RNase H activity in mammalian cells. Accordingly, antisense compounds comprising at least a portion of DNA or DNA-like nucleosides may activate RNase H, resulting in cleavage of the target nucleic acid. In certain embodiments, antisense compounds that utilize RNase H comprise one or more modified nucleosides. In certain embodiments, such antisense compounds comprise at least one block of 1-8 modified nucleosides. In certain such embodiments, the modified nucleosides do not support RNase H activity. In certain embodiments, such antisense compounds are gapmers, as described herein. In certain such embodiments, the gap of the gapmer comprises DNA nucleosides. In certain such embodiments, the gap of the gapmer comprises DNA-like nucleosides. In certain such embodiments, the gap of the gapmer comprises DNA nucleosides and DNA-like nucleosides.

Certain antisense compounds having a gapmer motif are considered chimeric antisense compounds. In a gapmer an internal region having a plurality of nucleotides that supports RNaseH cleavage is positioned between external regions having a plurality of nucleotides that are chemically distinct from the nucleosides of the internal region. In the case of an antisense oligonucleotide having a gapmer motif, the gap segment generally serves as the substrate for endonuclease cleavage, while the wing segments comprise modified nucleosides. In certain embodiments, the regions of a gapmer are differentiated by the types of sugar moieties comprising each distinct region. The types of sugar moieties that are used to differentiate the regions of a gapmer may in some embodiments include β-D-ribonucleosides, β-D-deoxyribonucleosides, 2'-modified nucleosides (such 2'-modified nucleosides may include 2'-MOE and 2'-O-CH_3, among others), and bicyclic sugar modified nucleosides (such bicyclic sugar modified nucleosides may include those having a constrained ethyl). In certain embodiments, nucleosides in the wings may include several modified sugar moieties, including, for example 2'-MOE and bicyclic sugar moieties such as constrained ethyl or LNA. In certain embodiments, wings may include several modified and unmodified sugar moieties. In certain embodiments, wings may include various combinations of 2'-MOE nucleosides, bicyclic sugar moieties such as constrained ethyl nucleosides or LNA nucleosides, and 2'-deoxynucleosides.

Each distinct region may comprise uniform sugar moieties, variant, or alternating sugar moieties. The wing-gap-wing motif is frequently described as "X-Y-Z", where "X" represents the length of the 5'-wing, "Y" represents the length of the gap, and "Z" represents the length of the 3'-wing. "X" and "Z" may comprise uniform, variant, or alternating sugar moieties. In certain embodiments, "X" and "Y" may include one or more 2'-deoxynucleosides. "Y" may comprise 2'-deoxynucleosides. As used herein, a gapmer described as "X-Y-Z" has a configuration such that the gap is positioned immediately adjacent to each of the 5'-wing and the 3' wing. Thus, no intervening nucleotides exist between the 5'-wing and gap, or the gap and the 3'-wing. Any of the antisense compounds described herein can have a gapmer motif. In certain embodiments, "X" and "Z" are the same; in other embodiments they are different. In certain embodiments, "Y" is between 8 and 15 nucleosides. X, Y, or Z can be any of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30 or more nucleosides.

In certain embodiments, the antisense compound targeted to an ANGPTL3 nucleic acid has a gapmer motif in which the gap consists of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 linked nucleosides.

In certain embodiments, the antisense oligonucleotide has a sugar motif described by Formula A as follows: $(J)_m$-$(B)_n$-$(J)_p$-$(B)_r$-$(A)_t$-$(D)_g$-$(A)_v$-$(B)_w$-$(J)_x$-$(B)_y$-$(J)_z$

wherein:

each A is independently a 2'-substituted nucleoside;

each B is independently a bicyclic nucleoside;

each J is independently either a 2'-substituted nucleoside or a 2'-deoxynucleoside;

each D is a 2'-deoxynucleoside;

m is 0-4; n is 0-2; p is 0-2; r is 0-2; t is 0-2; v is 0-2; w is 0-4; x is 0-2; y is 0-2; z is 0-4; g is 6-14;

provided that:

at least one of m, n, and r is other than 0;

at least one of w and y is other than 0;

the sum of m, n, p, r, and t is from 2 to 5; and

the sum of v, w, x, y, and z is from 2 to 5.

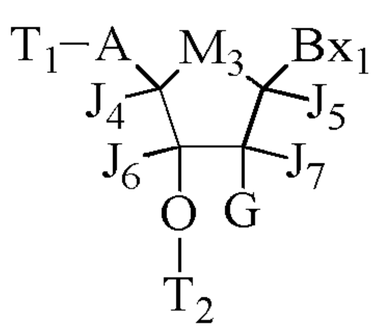
RNAi Compounds

In certain embodiments, antisense compounds are interfering RNA compounds (RNAi), which include double-stranded RNA compounds (also referred to as short-interfering RNA or siRNA) and single-stranded RNAi compounds (or ssRNA). Such compounds work at least in part through the RISC pathway to degrade and/or sequester a target nucleic acid (thus, include microRNA/microRNA-mimic compounds). In certain embodiments, antisense compounds comprise modifications that make them particularly suited for such mechanisms.

1. ssRNA compounds

In certain embodiments, antisense compounds including those particularly suited for use as single-stranded RNAi compounds (ssRNA) comprise a modified 5'-terminal end. In certain such embodiments, the 5'-terminal end comprises a modified phosphate moiety. In certain embodiments, such modified phosphate is stabilized (e.g., resistant to degradation/cleavage compared to unmodified 5'-phosphate). In certain embodiments, such 5'-terminal nucleosides stabilize the 5'-phosphorous moiety. Certain modified 5'-terminal nucleosides may be found in the art, for example in WO/2011/139702.

In certain embodiments, the 5'-nucleoside of an ssRNA compound has Formula IIc:



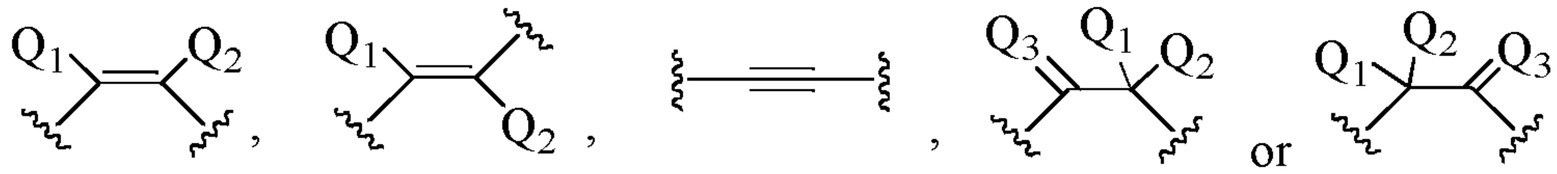
IIc

wherein:

T_1 is an optionally protected phosphorus moiety;

T_2 is an internucleoside linking group linking the compound of Formula IIc to the oligomeric compound;

A has one of the formulas:



Q_1 and Q_2 are each, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl, substituted C_2-C_6 alkynyl or $N(R_3)(R_4)$;

Q_3 is O, S, $N(R_5)$ or $C(R_6)(R_7)$;

each R_3, R_4 R_5, R_6 and R_7 is, independently, H, C_1-C_6 alkyl, substituted C_1-C_6 alkyl or C_1-C_6 alkoxy;

M_3 is O, S, NR_{14}, $C(R_{15})(R_{16})$, $C(R_{15})(R_{16})C(R_{17})(R_{18})$, $C(R_{15})=C(R_{17})$, $OC(R_{15})(R_{16})$ or $OC(R_{15})(Bx_2)$;

R_{14} is H, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

R_{15}, R_{16}, R_{17} and R_{18} are each, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

Bx_1 is a heterocyclic base moiety;

or if Bx_2 is present then Bx_2 is a heterocyclic base moiety and Bx_1 is H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

J_4, J_5, J_6 and J_7 are each, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

or J_4 forms a bridge with one of J_5 or J_7 wherein said bridge comprises from 1 to 3 linked biradical groups selected from O, S, NR_{19}, $C(R_{20})(R_{21})$, $C(R_{20})=C(R_{21})$, $C[=C(R_{20})(R_{21})]$ and C(=O) and the other two of J_5, J_6 and J_7 are each, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

each R_{19}, R_{20} and R_{21} is, independently, H, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

G is H, OH, halogen or $O\text{-}[C(R_8)(R_9)]_n\text{-}[(C=O)_m\text{-}X_1]_j\text{-}Z$;

each R_8 and R_9 is, independently, H, halogen, C_1-C_6 alkyl or substituted C_1-C_6 alkyl;

X_1 is O, S or $N(E_1)$;

Z is H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl, substituted C_2-C_6 alkynyl or $N(E_2)(E_3)$;

E_1, E_2 and E_3 are each, independently, H, C_1-C_6 alkyl or substituted C_1-C_6 alkyl;

n is from 1 to about 6;

m is 0 or 1;

j is 0 or 1;

each substituted group comprises one or more optionally protected substituent groups independently selected from halogen, OJ_1, $N(J_1)(J_2)$, $=NJ_1$, SJ_1, N_3, CN, $OC(=X_2)J_1$, $OC(=X_2)N(J_1)(J_2)$ and $C(=X_2)N(J_1)(J_2)$;

X_2 is O, S or NJ_3;

each J_1, J_2 and J_3 is, independently, H or C_1-C_6 alkyl;

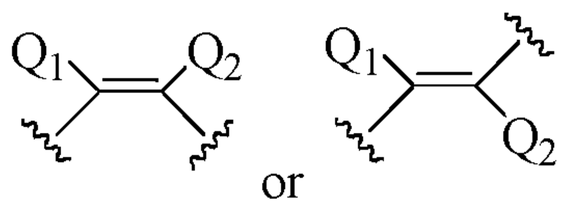
when j is 1 then Z is other than halogen or $N(E_2)(E_3)$; and

wherein said oligomeric compound comprises from 8 to 40 monomeric subunits and is hybridizable to at least a portion of a target nucleic acid.

In certain embodiments, M_3 is O, CH=CH, OCH_2 or $OC(H)(Bx_2)$. In certain embodiments, M_3 is O.

In certain embodiments, J_4, J_5, J_6 and J_7 are each H. In certain embodiments, J_4 forms a bridge with one of J_5 or J_7.

In certain embodiments, A has one of the formulas:

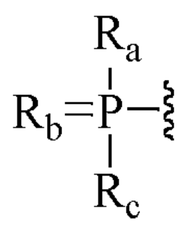


or

wherein:

Q_1 and Q_2 are each, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy or substituted C_1-C_6 alkoxy. In certain embodiments, Q_1 and Q_2 are each H. In certain embodiments, Q_1 and Q_2 are each, independently, H or halogen. In certain embodiments, Q_1 and Q_2 is H and the other of Q_1 and Q_2 is F, CH_3 or OCH_3.

In certain embodiments, T_1 has the formula:



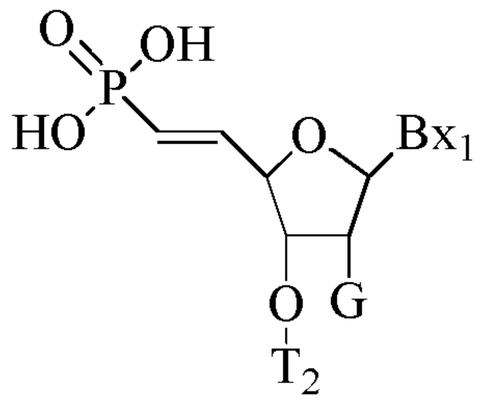
wherein:

R_a and R_c are each, independently, protected hydroxyl, protected thiol, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, protected amino or substituted amino; and

R_b is O or S. In certain embodiments, R_b is O and R_a and R_c are each, independently, OCH_3, OCH_2CH_3 or $CH(CH_3)_2$.

In certain embodiments, G is halogen, OCH_3, OCH_2F, $OCHF_2$, OCF_3, OCH_2CH_3, $O(CH_2)_2F$, OCH_2CHF_2, OCH_2CF_3, OCH_2-CH=CH_2, $O(CH_2)_2$-OCH_3, $O(CH_2)_2$-SCH_3, $O(CH_2)_2$-OCF_3, $O(CH_2)_3$-$N(R_{10})(R_{11})$, $O(CH_2)_2$-$ON(R_{10})(R_{11})$, $O(CH_2)_2$-$O(CH_2)_2$-$N(R_{10})(R_{11})$, $OCH_2C(=O)$-$N(R_{10})(R_{11})$, $OCH_2C(=O)$-$N(R_{12})$-$(CH_2)_2$-$N(R_{10})(R_{11})$ or $O(CH_2)_2$-$N(R_{12})$-$C(=NR_{13})[N(R_{10})(R_{11})]$ wherein R_{10}, R_{11}, R_{12} and R_{13} are each, independently, H or C_1-C_6 alkyl. In certain embodiments, G is halogen, OCH_3, OCF_3, OCH_2CH_3, OCH_2CF_3, OCH_2-CH=CH_2, $O(CH_2)_2$-OCH_3, $O(CH_2)_2$-$O(CH_2)_2$-$N(CH_3)_2$, $OCH_2C(=O)$-$N(H)CH_3$, $OCH_2C(=O)$-$N(H)$-$(CH_2)_2$-$N(CH_3)_2$ or OCH_2-$N(H)$-$C(=NH)NH_2$. In certain embodiments, G is F, OCH_3 or $O(CH_2)_2$-OCH_3. In certain embodiments, G is $O(CH_2)_2$-OCH_3.

In certain embodiments, the 5'-terminal nucleoside has Formula IIe:



IIe

In certain embodiments, antisense compounds, including those particularly suitable for ssRNA comprise one or more type of modified sugar moieties and/or naturally occurring sugar moieties arranged along an oligonucleotide or region thereof in a defined pattern or sugar modification motif. Such motifs may include any of the sugar modifications discussed herein and/or other known sugar modifications.

In certain embodiments, the oligonucleotides comprise or consist of a region having uniform sugar modifications. In certain such embodiments, each nucleoside of the region comprises the same RNA-like sugar modification. In certain embodiments, each nucleoside of the region is a 2'-F nucleoside. In certain

embodiments, each nucleoside of the region is a 2'-OMe nucleoside. In certain embodiments, each nucleoside of the region is a 2'-MOE nucleoside. In certain embodiments, each nucleoside of the region is a cEt nucleoside. In certain embodiments, each nucleoside of the region is an LNA nucleoside. In certain embodiments, the uniform region constitutes all or essentially all of the oligonucleotide. In certain embodiments, the region constitutes the entire oligonucleotide except for 1-4 terminal nucleosides.

In certain embodiments, oligonucleotides comprise one or more regions of alternating sugar modifications, wherein the nucleosides alternate between nucleotides having a sugar modification of a first type and nucleotides having a sugar modification of a second type. In certain embodiments, nucleosides of both types are RNA-like nucleosides. In certain embodiments the alternating nucleosides are selected from: 2'-OMe, 2'-F, 2'-MOE, LNA, and cEt. In certain embodiments, the alternating modificatios are 2'-F and 2'-OMe. Such regions may be contiguous or may be interupted by differently modified nucleosides or conjugated nucleosides.

In certain embodiments, the alternating region of alternating modifications each consist of a single nucleoside (i.e., the patern is $(AB)_xA_y$ wheren A is a nucleoside having a sugar modification of a first type and B is a nucleoside having a sugar modification of a second type; x is 1-20 and y is 0 or 1). In certan embodiments, one or more alternating regions in an alternating motif includes more than a single nucleoside of a type. For example, oligonucleotides may include one or more regions of any of the following nucleoside motifs:

AABBAA;

ABBABB;

AABAAB;

ABBABAABB;

ABABAA;

AABABAB;

ABABAA;

ABBAABBABABAA;

BABBAABBABABAA; or

ABABBAABBABABAA;

wherein A is a nucleoside of a first type and B is a nucleoside of a second type. In certain embodiments, A and B are each selected from 2'-F, 2'-OMe, BNA, and MOE.

In certain embodiments, oligonucleotides having such an alternating motif also comprise a modified 5' terminal nucleoside, such as those of formula IIc or IIe.

In certain embodiments, oligonucleotides comprise a region having a 2-2-3 motif. Such regions comprises the following motif:

$-(A)_2-(B)_x-(A)_2-(C)_y-(A)_3-$

wherein: A is a first type of modifed nucleosde;

B and C, are nucleosides that are differently modified than A, however, B and C may have the same or different modifications as one another;

x and y are from 1 to 15.

In certain embodiments, A is a 2'-OMe modified nucleoside. In certain embodiments, B and C are both 2'-F modified nucleosides. In certain embodiments, A is a 2'-OMe modified nucleoside and B and C are both 2'-F modified nucleosides.

In certain embodiments, oligonucleosides have the following sugar motif:

5'- (Q)- $(AB)_xA_y$-$(D)_z$

wherein:

Q is a nucleoside comprising a stabilized phosphate moiety. In certain embodiments, Q is a nucleoside having Formula IIc or IIe;

A is a first type of modifed nucleoside;

B is a second type of modified nucleoside;

D is a modified nucleoside comprising a modification different from the nucleoside adjacent to it. Thus, if y is 0, then D must be differently modified than B and if y is 1, then D must be differently modified than A. In certain embodiments, D differs from both A and B.

X is 5-15;

Y is 0 or 1;

Z is 0-4.

In certain embodiments, oligonucleosides have the following sugar motif:

5'- (Q)- $(A)_x$-$(D)_z$

wherein:

Q is a nucleoside comprising a stabilized phosphate moiety. In certain embodiments, Q is a nucleoside having Formula IIc or IIe;

A is a first type of modifed nucleoside;

D is a modified nucleoside comprising a modification different from A.

X is 11-30;

Z is 0-4.

In certain embodiments A, B, C, and D in the above motifs are selected from: 2'-OMe, 2'-F, 2'-MOE, LNA, and cEt. In certain embodiments, D represents terminal nucleosides. In certain embodiments, such terminal nucleosides are not designed to hybridize to the target nucleic acid (though one or more might hybridize by chance). In certiain embodiments, the nucleobase of each D nucleoside is adenine, regardless of the identity of the nucleobase at the corresponding position of the target nucleic acid. In certain embodiments the nucleobase of each D nucleoside is thymine.

In certain embodiments, antisense compounds, including those particularly suited for use as ssRNA comprise modified internucleoside linkages arranged along the oligonucleotide or region thereof in a defined

pattern or modified internucleoside linkage motif. In certain embodiments, oligonucleotides comprise a region having an alternating internucleoside linkage motif. In certain embodiments, oligonucleotides comprise a region of uniformly modified internucleoside linkages. In certain such embodiments, the oligonucleotide comprises a region that is uniformly linked by phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide is uniformly linked by phosphorothioate internucleoside linkages. In certain embodiments, each internucleoside linkage of the oligonucleotide is selected from phosphodiester and phosphorothioate. In certain embodiments, each internucleoside linkage of the oligonucleotide is selected from phosphodiester and phosphorothioate and at least one internucleoside linkage is phosphorothioate.

In certain embodiments, the oligonucleotide comprises at least 6 phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least 8 phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least 10 phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least one block of at least 6 consecutive phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least one block of at least 8 consecutive phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least one block of at least 10 consecutive phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least one block of at least one 12 consecutive phosphorothioate internucleoside linkages. In certain such embodiments, at least one such block is located at the 3' end of the oligonucleotide. In certain such embodiments, at least one such block is located within 3 nucleosides of the 3' end of the oligonucleotide.

Oligonucleotides having any of the various sugar motifs described herein, may have any linkage motif. For example, the oligonucleotides, including but not limited to those described above, may have a linkage motif selected from non-limiting the table below:

5' most linkage	Central region	3'-region
PS	Alternating PO/PS	6 PS
PS	Alternating PO/PS	7 PS
PS	Alternating PO/PS	8 PS

2. siRNA compounds

In certain embodiments, antisense compounds are double-stranded RNAi compounds (siRNA). In such embodiments, one or both strands may comprise any modification motif described above for ssRNA. In certain embodiments, ssRNA compounds may be unmodified RNA. In certain embodiments, siRNA compounds may comprise unmodified RNA nucleosides, but modified internucleoside linkages.

Several embodiments relate to double-stranded compositions wherein each strand comprises a motif defined by the location of one or more modified or unmodified nucleosides. In certain embodiments,

compositions are provided comprising a first and a second oligomeric compound that are fully or at least partially hybridized to form a duplex region and further comprising a region that is complementary to and hybridizes to a nucleic acid target. It is suitable that such a composition comprise a first oligomeric compound that is an antisense strand having full or partial complementarity to a nucleic acid target and a second oligomeric compound that is a sense strand having one or more regions of complementarity to and forming at least one duplex region with the first oligomeric compound.

The compositions of several embodiments modulate gene expression by hybridizing to a nucleic acid target resulting in loss of its normal function. In some embodiments, the target nucleic acid is ANGPTL3. In certain embodiment, the degradation of the targeted ANGPTL3 is facilitated by an activated RISC complex that is formed with compositions disclosed herein.

Several embodiments are directed to double-stranded compositions wherein one of the strands is useful in, for example, influencing the preferential loading of the opposite strand into the RISC (or cleavage) complex. The compositions are useful for targeting selected nucleic acid molecules and modulating the expression of one or more genes. In some embodiments, the compositions of the present invention hybridize to a portion of a target RNA resulting in loss of normal function of the target RNA.

Certain embodiments are drawn to double-stranded compositions wherein both the strands comprises a hemimer motif, a fully modified motif, a positionally modified motif or an alternating motif. Each strand of the compositions of the present invention can be modified to fulfil a particular role in for example the siRNA pathway. Using a different motif in each strand or the same motif with different chemical modifications in each strand permits targeting the antisense strand for the RISC complex while inhibiting the incorporation of the sense strand. Within this model, each strand can be independently modified such that it is enhanced for its particular role. The antisense strand can be modified at the 5'-end to enhance its role in one region of the RISC while the 3'-end can be modified differentially to enhance its role in a different region of the RISC.

The double-stranded oligonucleotide molecules can be a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The double-stranded oligonucleotide molecules can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e. each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double-stranded structure, for example wherein the double-stranded region is about 15 to about 30, e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 base pairs; the antisense strand comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof (e.g., about 15 to about 25 or more nucleotides of the

double-stranded oligonucleotide molecule are complementary to the target nucleic acid or a portion thereof). Alternatively, the double-stranded oligonucleotide is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the siRNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s).

The double-stranded oligonucleotide can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The double-stranded oligonucleotide can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either in vivo or in vitro to generate an active siRNA molecule capable of mediating RNAi.

In certain embodiments, the double-stranded oligonucleotide comprises separate sense and antisense sequences or regions, wherein the sense and antisense regions are covalently linked by nucleotide or non-nucleotide linkers molecules as is known in the art, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, van der waals interactions, hydrophobic interactions, and/or stacking interactions. In certain embodiments, the double-stranded oligonucleotide comprises nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the double-stranded oligonucleotide interacts with nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene.

As used herein, double-stranded oligonucleotides need not be limited to those molecules containing only RNA, but further encompasses chemically modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules lack 2'-hydroxy (2'-OH) containing nucleotides. In certain embodiments short interfering nucleic acids optionally do not include any ribonucleotides (e.g., nucleotides having a 2'-OH group). Such double-stranded oligonucleotides that do not require the presence of ribonucleotides within the molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, double-stranded oligonucleotides can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. As used herein, the term siRNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and

others. In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, translational inhibition, or epigenetics. For example, double-stranded oligonucleotides can be used to epigenetically silence genes at both the post-transcriptional level and the pre-transcriptional level. In a non-limiting example, epigenetic regulation of gene expression by siRNA molecules of the invention can result from siRNA mediated modification of chromatin structure or methylation pattern to alter gene expression (see, for example, Verdel et al., 2004, Science, 303, 672-676; Pal-Bhadra et al., 2004, Science, 303, 669-672; Allshire, 2002, Science, 297, 1818-1819; Volpe et al., 2002, Science, 297, 1833-1837; Jenuwein, 2002, Science, 297, 2215-2218; and Hall et al., 2002, Science, 297, 2232-2237).

It is contemplated that compounds and compositions of several embodiments provided herein can target ANGPTL3 by a dsRNA-mediated gene silencing or RNAi mechanism, including, e.g., "hairpin" or stem-loop double-stranded RNA effector molecules in which a single RNA strand with self-complementary sequences is capable of assuming a double-stranded conformation, or duplex dsRNA effector molecules comprising two separate strands of RNA. In various embodiments, the dsRNA consists entirely of ribonucleotides or consists of a mixture of ribonucleotides and deoxynucleotides, such as the RNA/DNA hybrids disclosed, for example, by WO 00/63364, filed Apr. 19, 2000, or U.S. Ser. No. 60/130,377, filed Apr. 21, 1999. The dsRNA or dsRNA effector molecule may be a single molecule with a region of self-complementarity such that nucleotides in one segment of the molecule base pair with nucleotides in another segment of the molecule. In various embodiments, a dsRNA that consists of a single molecule consists entirely of ribonucleotides or includes a region of ribonucleotides that is complementary to a region of deoxyribonucleotides. Alternatively, the dsRNA may include two different strands that have a region of complementarity to each other.

In various embodiments, both strands consist entirely of ribonucleotides, one strand consists entirely of ribonucleotides and one strand consists entirely of deoxyribonucleotides, or one or both strands contain a mixture of ribonucleotides and deoxyribonucleotides. In certain embodiments, the regions of complementarity are at least 70, 80, 90, 95, 98, or 100% complementary to each other and to a target nucleic acid sequence. In certain embodiments, the region of the dsRNA that is present in a double-stranded conformation includes at least 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 50, 75,100, 200, 500, 1000, 2000 or 5000 nucleotides or includes all of the nucleotides in a cDNA or other target nucleic acid sequence being represented in the dsRNA. In some embodiments, the dsRNA does not contain any single stranded regions, such as single stranded ends, or the dsRNA is a hairpin. In other embodiments, the dsRNA has one or more single stranded regions or overhangs. In certain embodiments, RNA/DNA hybrids include a DNA strand or region that is an antisense strand or region (e.g, has at least 70, 80, 90, 95, 98, or 100% complementarity to a target nucleic acid) and an RNA strand or region that is a sense strand or region (e.g, has at least 70, 80, 90, 95, 98, or 100% identity to a target nucleic acid), and vice versa.

In various embodiments, the RNA/DNA hybrid is made in vitro using enzymatic or chemical synthetic methods such as those described herein or those described in WO 00/63364, filed Apr. 19, 2000, or U.S. Ser. No. 60/130,377, filed Apr. 21, 1999. In other embodiments, a DNA strand synthesized in vitro is complexed with an RNA strand made in vivo or in vitro before, after, or concurrent with the transformation of the DNA strand into the cell. In yet other embodiments, the dsRNA is a single circular nucleic acid containing a sense and an antisense region, or the dsRNA includes a circular nucleic acid and either a second circular nucleic acid or a linear nucleic acid (see, for example, WO 00/63364, filed Apr. 19, 2000, or U.S. Ser. No. 60/130,377, filed Apr. 21, 1999.) Exemplary circular nucleic acids include lariat structures in which the free 5' phosphoryl group of a nucleotide becomes linked to the 2' hydroxyl group of another nucleotide in a loop back fashion.

In other embodiments, the dsRNA includes one or more modified nucleotides in which the 2' position in the sugar contains a halogen (such as fluorine group) or contains an alkoxy group (such as a methoxy group) which increases the half-life of the dsRNA in vitro or in vivo compared to the corresponding dsRNA in which the corresponding 2' position contains a hydrogen or an hydroxyl group. In yet other embodiments, the dsRNA includes one or more linkages between adjacent nucleotides other than a naturally-occurring phosphodiester linkage. Examples of such linkages include phosphoramide, phosphorothioate, and phosphorodithioate linkages. The dsRNAs may also be chemically modified nucleic acid molecules as taught in U.S. Pat. No. 6,673,661. In other embodiments, the dsRNA contains one or two capped strands, as disclosed, for example, by WO 00/63364, filed Apr. 19, 2000, or U.S. Ser. No. 60/130,377, filed Apr. 21, 1999.

In other embodiments, the dsRNA can be any of the at least partially dsRNA molecules disclosed in WO 00/63364, as well as any of the dsRNA molecules described in U.S. Provisional Application 60/399,998; and U.S. Provisional Application 60/419,532, and PCT/US2003/033466, the teaching of which is hereby incorporated by reference. Any of the dsRNAs may be expressed in vitro or in vivo using the methods described herein or standard methods, such as those described in WO 00/63364.

Occupancy

In certain embodiments, antisense compounds are not expected to result in cleavage or the target nucleic acid via RNase H or to result in cleavage or sequestration through the RISC pathway. In certain such embodiments, antisense activity may result from occupancy, wherein the presence of the hybridized antisense compound disrupts the activity of the target nucleic acid. In certain such embodiments, the antisense compound may be uniformly modified or may comprise a mix of modifications and/or modified and unmodified nucleosides.

Target Nucleic Acids, Target Regions and Nucleotide Sequences

Nucleotide sequences that encode ANGPTL3 include, without limitation, the following: the human sequence as set forth in GenBank Accession No. NM_014495.2 (incorporated herein as SEQ ID NO: 1) or GenBank Accession No. NT_032977.9 nucleotides 33032001 to 33046000 (incorporated herein as SEQ ID NO: 2). It is understood that the sequence set forth in each SEQ ID NO in the Examples contained herein is independent of any modification to a sugar moiety, an internucleoside linkage, or a nucleobase. As such, antisense compounds defined by a SEQ ID NO can comprise, independently, one or more modifications to a sugar moiety, an internucleoside linkage, or a nucleobase. Antisense compounds described by Isis Number (Isis No) indicate a combination of nucleobase sequence and motif.

In certain embodiments, a target region is a structurally defined region of the target nucleic acid. For example, a target region can encompass a 3' UTR, a 5' UTR, an exon, an intron, an exon/intron junction, a coding region, a translation initiation region, translation termination region, or other defined nucleic acid region. The structurally defined regions for ANGPTL3 can be obtained by accession number from sequence databases such as NCBI and such information is incorporated herein by reference. In certain embodiments, a target region can encompass the sequence from a 5' target site of one target segment within the target region to a 3' target site of another target segment within the target region.

In certain embodiments, a "target segment" is a smaller, sub-portion of a target region within a nucleic acid. For example, a target segment can be the sequence of nucleotides of a target nucleic acid to which one or more antisense compound is targeted. "5' target site" or "5' start stie" refers to the 5'-most nucleotide of a target segment. "3' target site" or "3' stop site" refers to the 3'-most nucleotide of a target segment.

Targeting includes determination of at least one target segment to which an antisense compound hybridizes, such that a desired effect occurs. In certain embodiments, the desired effect is a reduction in mRNA target nucleic acid levels. In certain embodiments, the desired effect is reduction of levels of protein encoded by the target nucleic acid or a phenotypic change associated with the target nucleic acid.

A target region can contain one or more target segments. Multiple target segments within a target region can be overlapping. Alternatively, they can be non-overlapping. In certain embodiments, target segments within a target region are separated by no more than about 300 nucleotides. In certain embodiments, target segments within a target region are separated by a number of nucleotides that is, is about, is no more than, is no more than about, 250, 200, 150, 100, 90, 80, 70, 60, 50, 40, 30, 20, or 10 nucleotides on the target nucleic acid, or is a range defined by any two of the preceding values. In certain embodiments, target segments within a target region are separated by no more than, or no more than about, 5 nucleotides on the target nucleic acid. In certain embodiments, target segments are contiguous. Contemplated are target regions defined by a range having a starting nucleic acid that is any of the 5' target sites or 3' target sites listed herein.

Suitable target segments can be found within a 5' UTR, a coding region, a 3' UTR, an intron, an exon, or an exon/intron junction. Target segments containing a start codon or a stop codon are also suitable

target segments. A suitable target segment can specifically exclude a certain structurally defined region such as the start codon or stop codon.

The determination of suitable target segments can include a comparison of the sequence of a target nucleic acid to other sequences throughout the genome. For example, the BLAST algorithm can be used to identify regions of similarity amongst different nucleic acids. This comparison can prevent the selection of antisense compound sequences that can hybridize in a non-specific manner to sequences other than a selected target nucleic acid (i.e., non-target or off-target sequences).

There can be variation in activity (e.g., as defined by percent reduction of target nucleic acid levels) of the antisense compounds within an active target region. In certain embodiments, reductions in ANGPTL3 mRNA levels are indicative of inhibition of ANGPTL3 protein expression. Reductions in levels of an ANGPTL3 protein are also indicative of inhibition of target mRNA expression. Further, phenotypic changes, such as a reduction of the level of cholesterol, LDL, triglyceride, or glucose, can be indicative of inhibition of ANGPTL3 mRNA and/or protein expression.

Hybridization

In some embodiments, hybridization occurs between an antisense compound disclosed herein and an ANGPTL3 nucleic acid. The most common mechanism of hybridization involves hydrogen bonding (e.g., Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding) between complementary nucleobases of the nucleic acid molecules.

Hybridization can occur under varying conditions. Stringent conditions are sequence-dependent and are determined by the nature and composition of the nucleic acid molecules to be hybridized.

Methods of determining whether a sequence is specifically hybridizable to a target nucleic acid are well known in the art (Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed., 2001). In certain embodiments, the antisense compounds provided herein are specifically hybridizable with an ANGPTL3 nucleic acid.

Complementarity

An antisense compound and a target nucleic acid are complementary to each other when a sufficient number of nucleobases of the antisense compound can hydrogen bond with the corresponding nucleobases of the target nucleic acid, such that a desired effect will occur (e.g., antisense inhibition of a target nucleic acid, such as an ANGPTL3 nucleic acid).

An antisense compound can hybridize over one or more segments of an ANGPTL3 nucleic acid such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure, mismatch or hairpin structure).

In certain embodiments, the antisense compounds provided herein, or a specified portion thereof, are, or are at least, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% complementary to an ANGPTL3 nucleic acid, a target region, target segment, or specified portion thereof. In certain embodiments, the antisense compounds provided herein, or a specified

portion thereof, are, or are at least, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% complementary to the sequence of one or more of SEQ ID NOs: 1-2. Percent complementarity of an antisense compound with a target nucleic acid can be determined using routine methods.

For example, an antisense compound in which 18 of 20 nucleobases of the antisense compound are complementary to a target region, and would therefore specifically hybridize, would represent 90 percent complementarity. In this example, the remaining noncomplementary nucleobases can be clustered or interspersed with complementary nucleobases and need not be contiguous to each other or to complementary nucleobases. As such, an antisense compound which is 18 nucleobases in length having 4 (four) noncomplementary nucleobases which are flanked by two regions of complete complementarity with the target nucleic acid would have 77.8% overall complementarity with the target nucleic acid and would thus fall within the scope of the present invention. Percent complementarity of an antisense compound with a region of a target nucleic acid can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., J. Mol. Biol., 1990, 215, 403 410; Zhang and Madden, Genome Res., 1997, 7, 649 656). Percent homology, sequence identity or complementarity, can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wis.), using default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482 489).

In certain embodiments, the antisense compounds provided herein, or specified portions thereof, are fully complementary (i.e. 100% complementary) to a target nucleic acid, or specified portion thereof. For example, an antisense compound can be fully complementary to an ANGPTL3 nucleic acid, or a target region, or a target segment or target sequence thereof. As used herein, "fully complementary" means each nucleobase of an antisense compound is capable of precise base pairing with the corresponding nucleobases of a target nucleic acid. For example, a 20 nucleobase antisense compound is fully complementary to a target sequence that is 400 nucleobases long, so long as there is a corresponding 20 nucleobase portion of the target nucleic acid that is fully complementary to the antisense compound. Fully complementary can also be used in reference to a specified portion of the first and /or the second nucleic acid. For example, a 20 nucleobase portion of a 30 nucleobase antisense compound can be "fully complementary" to a target sequence that is 400 nucleobases long. The 20 nucleobase portion of the 30 nucleobase oligonucleotide is fully complementary to the target sequence if the target sequence has a corresponding 20 nucleobase portion wherein each nucleobase is complementary to the 20 nucleobase portion of the antisense compound. At the same time, the entire 30 nucleobase antisense compound can be fully complementary to the target sequence, depending on whether the remaining 10 nucleobases of the antisense compound are also complementary to the target sequence.

The location of a non-complementary nucleobase can be at the 5' end or 3' end of the antisense compound. Alternatively, the non-complementary nucleobase or nucleobases can be at an internal position of

the antisense compound. When two or more non-complementary nucleobases are present, they can be either contiguous (i.e. linked) or non-contiguous. In one embodiment, a non-complementary nucleobase is located in the wing segment of a gapmer antisense oligonucleotide.

In certain embodiments, antisense compounds that are, or are up to 10, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleobases in length comprise no more than 4, no more than 3, no more than 2, or no more than 1 non-complementary nucleobase(s) relative to a target nucleic acid, such as an ANGPTL3 nucleic acid, or specified portion thereof.

In certain embodiments, antisense compounds that are, or are up to 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleobases in length comprise no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 non-complementary nucleobase(s) relative to a target nucleic acid, such as an ANGPTL3 nucleic acid, or specified portion thereof.

The antisense compounds provided herein also include those which are complementary to a portion of a target nucleic acid. As used herein, "portion" refers to a defined number of contiguous (i.e. linked) nucleobases within a region or segment of a target nucleic acid. A "portion" can also refer to a defined number of contiguous nucleobases of an antisense compound. In certain embodiments, the antisense compounds, are complementary to at least an 8 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 10 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 15 nucleobase portion of a target segment. Also contemplated are antisense compounds that are complementary to at least an 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more nucleobase portion of a target segment, or a range defined by any two of these values.

Identity

The antisense compounds provided herein can also have a defined percent identity to a particular nucleotide sequence, SEQ ID NO, or the sequence of a compound represented by a specific Isis number, or portion thereof. As used herein, an antisense compound is identical to the sequence disclosed herein if it has the same nucleobase pairing ability. For example, a RNA which contains uracil in place of thymidine in a disclosed DNA sequence would be considered identical to the DNA sequence since both uracil and thymidine pair with adenine. Shortened and lengthened versions of the antisense compounds described herein as well as compounds having non-identical bases relative to the antisense compounds provided herein also are contemplated. The non-identical bases can be adjacent to each other or dispersed throughout the antisense compound. Percent identity of an antisense compound is calculated according to the number of bases that have identical base pairing relative to the sequence to which it is being compared.

In certain embodiments, the antisense compounds, or portions thereof, are at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to one or more of the antisense compounds or SEQ ID NOs, or a portion thereof, disclosed herein.

Modifications

A nucleoside is a base-sugar combination. The nucleobase (also known as base) portion of the nucleoside is normally a heterocyclic base moiety. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to the 2', 3' or 5' hydroxyl moiety of the sugar. Oligonucleotides are formed through the covalent linkage of adjacent nucleosides to one another, to form a linear polymeric oligonucleotide. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside linkages of the oligonucleotide.

Modifications to antisense compounds encompass substitutions or changes to internucleoside linkages, sugar moieties, or nucleobases. Modified antisense compounds are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target, increased stability in the presence of nucleases, or increased inhibitory activity.

Chemically modified nucleosides can also be employed to increase the binding affinity of a shortened or truncated antisense oligonucleotide for its target nucleic acid. Consequently, comparable results can often be obtained with shorter antisense compounds that have such chemically modified nucleosides.

Modified Internucleoside Linkages

The naturally occurring internucleoside linkage of RNA and DNA is a 3' to 5' phosphodiester linkage. Antisense compounds having one or more modified, i.e. non-naturally occurring, internucleoside linkages are often selected over antisense compounds having naturally occurring internucleoside linkages because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for target nucleic acids, and increased stability in the presence of nucleases.

Oligonucleotides having modified internucleoside linkages include internucleoside linkages that retain a phosphorus atom as well as internucleoside linkages that do not have a phosphorus atom. Representative phosphorus containing internucleoside linkages include, but are not limited to, phosphodiesters, phosphotriesters, methylphosphonates, phosphoramidate, and phosphorothioates. Methods of preparation of phosphorous-containing and non-phosphorous-containing linkages are well known.

In certain embodiments, antisense compounds targeted to an ANGPTL3 nucleic acid comprise one or more modified internucleoside linkages. In certain embodiments, the modified internucleoside linkages are phosphorothioate linkages. In certain embodiments, each internucleoside linkage of an antisense compound is a phosphorothioate internucleoside linkage.

In certain embodiments, oligonucleotides comprise modified internucleoside linkages arranged along the oligonucleotide or region thereof in a defined pattern or modified internucleoside linkage motif. In certain embodiments, internucleoside linkages are arranged in a gapped motif. In such embodiments, the internucleoside linkages in each of two wing regions are different from the internucleoside linkages in the gap region. In certain embodiments the internucleoside linkages in the wings are phosphodiester and the internucleoside linkages in the gap are phosphorothioate. The nucleoside motif is independently selected, so such oligonucleotides having a gapped internucleoside linkage motif may or may not have a gapped

nucleoside motif and if it does have a gapped nucleoside motif, the wing and gap lengths may or may not be the same.

In certain embodiments, oligonucleotides comprise a region having an alternating internucleoside linkage motif. In certain embodiments, oligonucleotides of the present invention comprise a region of uniformly modified internucleoside linkages. In certain such embodiments, the oligonucleotide comprises a region that is uniformly linked by phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide is uniformly linked by phosphorothioate. In certain embodiments, each internucleoside linkage of the oligonucleotide is selected from phosphodiester and phosphorothioate. In certain embodiments, each internucleoside linkage of the oligonucleotide is selected from phosphodiester and phosphorothioate and at least one internucleoside linkage is phosphorothioate.

In certain embodiments, the oligonucleotide comprises at least 6 phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least 8 phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least 10 phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least one block of at least 6 consecutive phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least one block of at least 8 consecutive phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least one block of at least 10 consecutive phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least block of at least one 12 consecutive phosphorothioate internucleoside linkages. In certain such embodiments, at least one such block is located at the 3' end of the oligonucleotide. In certain such embodiments, at least one such block is located within 3 nucleosides of the 3' end of the oligonucleotide.

In certain embodiments, oligonucleotides comprise one or more methylphosponate linkages. In certain embodiments, oligonucleotides having a gapmer nucleoside motif comprise a linkage motif comprising all phosphorothioate linkages except for one or two methylphosponate linkages. In certain embodiments, one methylphosponate linkage is in the central gap of an oligonucleotide having a gapmer nucleoside motif.

In certain embodiments, it is desirable to arrange the number of phosphorothioate internucleoside linkages and phosphodiester internucleoside linkages to maintain nuclease resistance. In certain embodiments, it is desirable to arrange the number and position of phosphorothioate internucleoside linkages and the number and position of phosphodiester internucleoside linkages to maintain nuclease resistance. In certain embodiments, the number of phosphorothioate internucleoside linkages may be decreased and the number of phosphodiester internucleoside linkages may be increased. In certain embodiments, the number of phosphorothioate internucleoside linkages may be decreased and the number of phosphodiester internucleoside linkages may be increased while still maintaining nuclease resistance. In certain embodiments it is desirable to decrease the number of phosphorothioate internucleoside linkages while

retaining nuclease resistance. In certain embodiments it is desirable to increase the number of phosphodiester internucleoside linkages while retaining nuclease resistance.

Modified Sugar Moieties

Antisense compounds of the invention can optionally contain one or more nucleosides wherein the sugar group has been modified. Such sugar modified nucleosides may impart enhanced nuclease stability, increased binding affinity, or some other beneficial biological property to the antisense compounds. In certain embodiments, nucleosides comprise chemically modified ribofuranose ring moieties. Examples of chemically modified ribofuranose rings include without limitation, addition of substitutent groups (including 5' and 2' substituent groups, bridging of non-geminal ring atoms to form bicyclic nucleic acids (BNA), replacement of the ribosyl ring oxygen atom with S, N(R), or $C(R_1)(R_2)$ (R, R_1 and R_2 are each independently H, C_1-C_{12} alkyl or a protecting group) and combinations thereof. Examples of chemically modified sugars include 2'-F-5'-methyl substituted nucleoside (see PCT International Application WO 2008/101157 Published on 8/21/08 for other disclosed 5',2'-bis substituted nucleosides) or replacement of the ribosyl ring oxygen atom with S with further substitution at the 2'-position (see published U.S. Patent Application US2005-0130923, published on June 16, 2005) or alternatively 5'-substitution of a BNA (see PCT International Application WO 2007/134181 Published on 11/22/07 wherein LNA is substituted with for example a 5'-methyl or a 5'-vinyl group).

Examples of nucleosides having modified sugar moieties include without limitation nucleosides comprising 5'-vinyl, 5'-methyl (*R* or *S*), 4'-S, 2'-F, 2'-OCH_3, 2'-OCH_2CH_3, 2'-OCH_2CH_2F and 2'-$O(CH_2)_2OCH_3$ substituent groups. The substituent at the 2' position can also be selected from allyl, amino, azido, thio, O-allyl, O-C_1-C_{10} alkyl, OCF_3, OCH_2F, $O(CH_2)_2SCH_3$, $O(CH_2)_2$-O-$N(R_m)(R_n)$, O-CH_2-C(=O)-$N(R_m)(R_n)$, and O-CH_2-C(=O)-$N(R_l)$-$(CH_2)_2$-$N(R_m)(R_n)$, where each R_l, R_m and R_n is, independently, H or substituted or unsubstituted C_1-C_{10} alkyl.

As used herein, "bicyclic nucleosides" refer to modified nucleosides comprising a bicyclic sugar moiety. Examples of bicyclic nucleic acids (BNAs) include without limitation nucleosides comprising a bridge between the 4' and the 2' ribosyl ring atoms. In certain embodiments, antisense compounds provided herein include one or more BNA nucleosides wherein the bridge comprises one of the formulas: 4'-(CH_2)-O-2' (LNA); 4'-(CH_2)-S-2'; 4'-$(CH_2)_2$-O-2' (ENA); 4'-$CH(CH_3)$-O-2' and 4'-$CH(CH_2OCH_3)$-O-2' (and analogs thereof see U.S. Patent 7,399,845, issued on July 15, 2008); 4'-$C(CH_3)(CH_3)$-O-2' (and analogs thereof see PCT/US2008/068922 published as WO 2009/006478, published January 8, 2009); 4'-CH_2-$N(OCH_3)$-2' (and analogs thereof see PCT/US2008/064591 published as WO/2008/150729, published December 11, 2008); 4'-CH_2-O-$N(CH_3)$-2' (see published U.S. Patent Application US2004-0171570, published September 2, 2004); 4'-CH_2-N(R)-O-2', wherein R is H, C_1-C_{12} alkyl, or a protecting group (see U.S. Patent 7,427,672, issued on September 23, 2008); 4'-CH_2-$C(H)(CH_3)$-2' (see Zhou *et al., J. Org. Chem.,* 2009, *74*, 118-134); and 4'-CH_2-$C(=CH_2)$-2' (and analogs thereof see PCT/US2008/066154 published as WO 2008/154401, published on December 8, 2008).

Further bicyclic nucleosides have been reported in published literature (see for example: Srivastava et al., *J. Am. Chem. Soc.*, 2007, *129(26)* 8362-8379; Frieden *et al., Nucleic Acids Research,* 2003, *21*, 6365-6372; Elayadi *et al., Curr. Opinion Invens. Drugs,* 2001, *2*, 558-561; Braasch *et al., Chem. Biol.,* 2001, *8*, 1-7; Orum *et al., Curr. Opinion Mol. Ther.,* 2001, *3*, 239-243; Wahlestedt et al., *Proc. Natl. Acad. Sci. U. S. A.*, 2000, *97*, 5633-5638; Singh et al., *Chem. Commun.*, 1998, *4*, 455-456; Koshkin et al., *Tetrahedron*, 1998, *54*, 3607-3630; Kumar et al., *Bioorg. Med. Chem. Lett.*, 1998, *8*, 2219-2222; Singh et al., *J. Org. Chem.*, 1998, *63*, 10035-10039; U.S. Patents Nos.: 7,741,457; 7,399,845; 7,053,207; 7,034,133; 6,794,499; 6,770,748; 6,670,461; 6,525,191; 6,268,490; U.S. Patent Publication Nos.: US2008-0039618; US2007-0287831; US2004-0171570; U.S. Patent Applications, Serial Nos.: 61/097,787; 61/026,995; and International applications: WO 2009/006478; WO 2008/154401; WO 2008/150729; WO 2009/100320; WO 2011/017521; WO 2009/067647; WO 2010/036698; WO 2007/134181; WO 2005/021570; WO 2004/106356; WO 99/14226. Each of the foregoing bicyclic nucleosides can be prepared having one or more stereochemical sugar configurations including for example α-L-ribofuranose and β-D-ribofuranose (see PCT international application PCT/DK98/00393, published on March 25, 1999 as WO 99/14226).

As used herein, "monocylic nucleosides" refer to nucleosides comprising modified sugar moieties that are not bicyclic sugar moieties. In certain embodiments, the sugar moiety, or sugar moiety analogue, of a nucleoside may be modified or substituted at any position.

As used herein, "4'-2' bicyclic nucleoside" or "4' to 2' bicyclic nucleoside" refers to a bicyclic nucleoside comprising a furanose ring comprising a bridge connecting two carbon atoms of the furanose ring connects the 2' carbon atom and the 4' carbon atom of the sugar ring.

In certain embodiments, bicyclic sugar moieties of BNA nucleosides include, but are not limited to, compounds having at least one bridge between the 4' and the 2' carbon atoms of the pentofuranosyl sugar moiety including without limitation, bridges comprising 1 or from 1 to 4 linked groups independently selected from $-[C(R_a)(R_b)]_n-$, $-C(R_a)=C(R_b)-$, $-C(R_a)=N-$, $-C(=NR_a)-$, $-C(=O)-$, $-C(=S)-$, $-O-$, $-Si(R_a)_2-$, $-S(=O)_x-$, and $-N(R_a)-$; wherein: x is 0, 1, or 2; n is 1, 2, 3, or 4; each R_a and R_b is, independently, H, a protecting group, hydroxyl, C_1-C_{12} alkyl, substituted C_1-C_{12} alkyl, C_2-C_{12} alkenyl, substituted C_2-C_{12} alkenyl, C_2-C_{12} alkynyl, substituted C_2-C_{12} alkynyl, C_5-C_{20} aryl, substituted C_5-C_{20} aryl, heterocycle radical, substituted heterocycle radical, heteroaryl, substituted heteroaryl, C_5-C_7 alicyclic radical, substituted C_5-C_7 alicyclic radical, halogen, OJ_1, NJ_1J_2, SJ_1, N_3, $COOJ_1$, acyl (C(=O)-H), substituted acyl, CN, sulfonyl ($S(=O)_2$-J_1), or sulfoxyl ($S(=O)$-J_1); and

each J_1 and J_2 is, independently, H, C_1-C_{12} alkyl, substituted C_1-C_{12} alkyl, C_2-C_{12} alkenyl, substituted C_2-C_{12} alkenyl, C_2-C_{12} alkynyl, substituted C_2-C_{12} alkynyl, C_5-C_{20} aryl, substituted C_5-C_{20} aryl, acyl (C(=O)-H), substituted acyl, a heterocycle radical, a substituted heterocycle radical, C_1-C_{12} aminoalkyl, substituted C_1-C_{12} aminoalkyl or a protecting group.

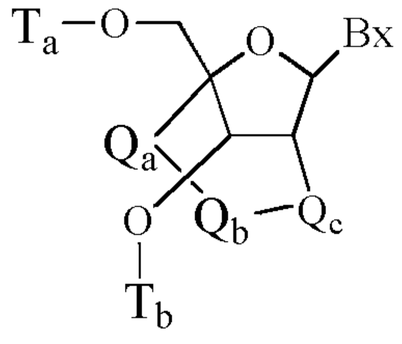
In certain embodiments, the bridge of a bicyclic sugar moiety is , $-[C(R_a)(R_b)]_n-$, $-[C(R_a)(R_b)]_n-O-$, $-C(R_aR_b)-N(R)-O-$ or $-C(R_aR_b)-O-N(R)-$. In certain embodiments, the bridge is 4'-CH_2-2', 4'-$(CH_2)_2$-2', 4'-

$(CH_2)_3$-2', 4'-CH_2-O-2', 4'-$(CH_2)_2$-O-2', 4'-CH_2-O-N(R)-2' and 4'-CH_2-N(R)-O-2'- wherein each R is, independently, H, a protecting group or C_1-C_{12} alkyl.

In certain embodiments, bicyclic nucleosides are further defined by isomeric configuration. For example, a nucleoside comprising a 4'-(CH_2)-O-2' bridge, may be in the α-L configuration or in the β-D configuration. Previously, α-L-methyleneoxy (4'-CH_2-O-2') BNA's have been incorporated into antisense oligonucleotides that showed antisense activity (Frieden *et al., Nucleic Acids Research,* 2003, *21*, 6365-6372).

In certain embodiments, bicyclic nucleosides include those having a 4' to 2' bridge wherein such bridges include without limitation, α-L-4'-(CH_2)-O-2', β-D-4'-CH_2-O-2', 4'-$(CH_2)_2$-O-2', 4'-CH_2-O-N(R)-2', 4'-CH_2-N(R)-O-2', 4'-$CH(CH_3)$-O-2', 4'-CH_2-S-2', 4'-CH_2-N(R)-2', 4'-CH_2-$CH(CH_3)$-2', and 4'-$(CH_2)_3$-2', wherein R is H, a protecting group or C_1-C_{12} alkyl.

In certain embodiment, bicyclic nucleosides have the formula:



wherein:

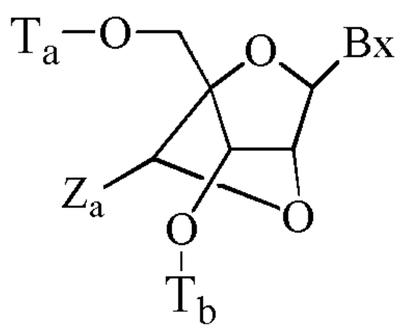
Bx is a heterocyclic base moiety;

-Q_a-Q_b-Q_c- is -CH_2-N(R_c)-CH_2-, -C(=O)-N(R_c)-CH_2-, -CH_2-O-N(R_c)-, -CH_2-N(R_c)-O- or -N(R_c)-O-CH_2;

R_c is C_1-C_{12} alkyl or an amino protecting group; and

T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium.

In certain embodiments, bicyclic nucleosides have the formula:



wherein:

Bx is a heterocyclic base moiety;

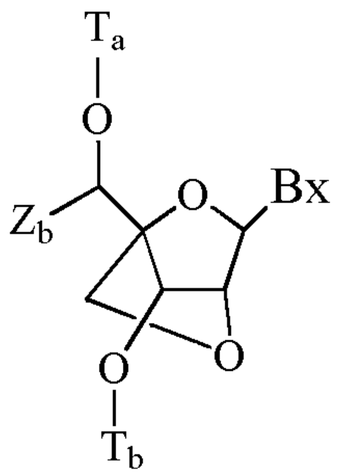
T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium;

Z_a is C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, substituted C_1-C_6 alkyl, substituted C_2-C_6 alkenyl, substituted C_2-C_6 alkynyl, acyl, substituted acyl, substituted amide, thiol or substituted thiol.

In one embodiment, each of the substituted groups, is, independently, mono or poly substituted with substituent groups independently selected from halogen, oxo, hydroxyl, OJ_c, NJ_cJ_d, SJ_c, N_3, $OC(=X)J_c$, and

$NJ_eC(=X)NJ_cJ_d$, wherein each J_c, J_d and J_e is, independently, H, C_1-C_6 alkyl, or substituted C_1-C_6 alkyl and X is O or NJ_c.

In certain embodiments, bicyclic nucleosides have the formula:



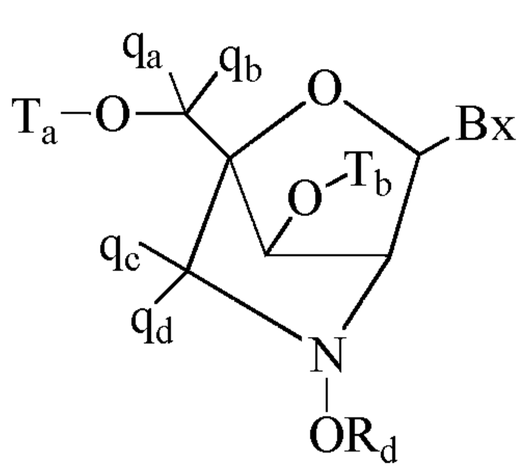
wherein:

Bx is a heterocyclic base moiety;

T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium;

Z_b is C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, substituted C_1-C_6 alkyl, substituted C_2-C_6 alkenyl, substituted C_2-C_6 alkynyl or substituted acyl (C(=O)-).

In certain embodiments, bicyclic nucleosides have the formula:



wherein:

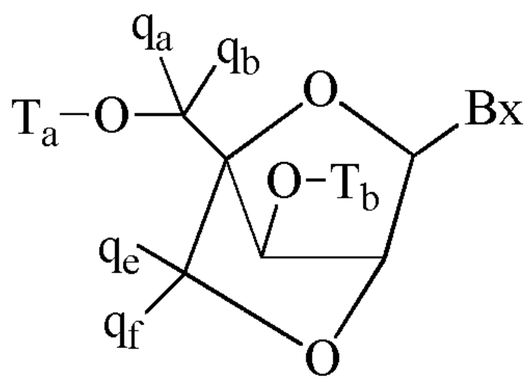
Bx is a heterocyclic base moiety;

T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium;

R_d is C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

each q_a, q_b, q_c and q_d is, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl, C_1-C_6 alkoxyl, substituted C_1-C_6 alkoxyl, acyl, substituted acyl, C_1-C_6 aminoalkyl or substituted C_1-C_6 aminoalkyl;

In certain embodiments, bicyclic nucleosides have the formula:



wherein:

Bx is a heterocyclic base moiety;

T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium;

q_a, q_b, q_e and q_f are each, independently, hydrogen, halogen, C_1-C_{12} alkyl, substituted C_1-C_{12} alkyl, C_2-C_{12} alkenyl, substituted C_2-C_{12} alkenyl, C_2-C_{12} alkynyl, substituted C_2-C_{12} alkynyl, C_1-C_{12} alkoxy, substituted C_1-C_{12} alkoxy, OJ_j, SJ_j, SOJ_j, SO_2J_j, NJ_jJ_k, N_3, CN, $C(=O)OJ_j$, $C(=O)NJ_jJ_k$, $C(=O)J_j$, $O\text{-}C(=O)NJ_jJ_k$, $N(H)C(=NH)NJ_jJ_k$, $N(H)C(=O)NJ_jJ_k$ or $N(H)C(=S)NJ_jJ_k$;

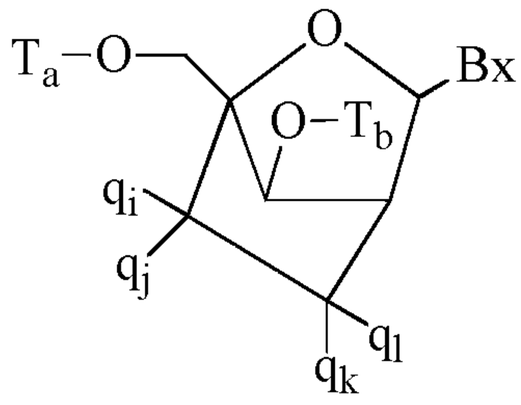
or q_e and q_f together are $=C(q_g)(q_h)$;

q_g and q_h are each, independently, H, halogen, C_1-C_{12} alkyl or substituted C_1-C_{12} alkyl.

The synthesis and preparation of adenine, cytosine, guanine, 5-methyl-cytosine, thymine and uracil bicyclic nucleosides having a 4'-CH_2-O-2' bridge, along with their oligomerization, and nucleic acid recognition properties have been described (Koshkin et al., *Tetrahedron*, 1998, *54*, 3607-3630). The synthesis of bicyclic nucleosides has also been described in WO 98/39352 and WO 99/14226.

Analogs of various bicyclic nucleosides that have 4' to 2' bridging groups such as 4'-CH_2-O-2' and 4'-CH_2-S-2', have also been prepared (Kumar *et al., Bioorg. Med. Chem. Lett.*, 1998, *8*, 2219-2222). Preparation of oligodeoxyribonucleotide duplexes comprising bicyclic nucleosides for use as substrates for nucleic acid polymerases has also been described (Wengel et al., WO 99/14226). Furthermore, synthesis of 2'-amino-BNA, a novel conformationally restricted high-affinity oligonucleotide analog has been described in the art (Singh et al., *J. Org. Chem.*, 1998, *63*, 10035-10039). In addition, 2'-amino- and 2'-methylamino-BNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

In certain embodiments, bicyclic nucleosides have the formula:



wherein:

Bx is a heterocyclic base moiety;

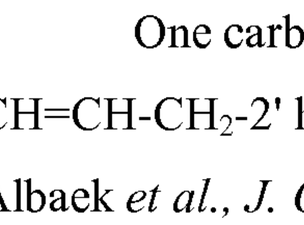
T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium;

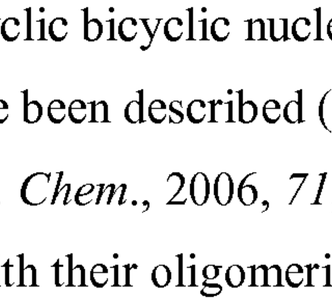
each q_i, q_j, q_k and q_l is, independently, H, halogen, C_1-C_{12} alkyl, substituted C_1-C_{12} alkyl, C_2-C_{12} alkenyl, substituted C_2-C_{12} alkenyl, C_2-C_{12} alkynyl, substituted C_2-C_{12} alkynyl, C_1-C_{12} alkoxyl, substituted C_1-C_{12} alkoxyl, OJ_j, SJ_j, SOJ_j, SO_2J_j, NJ_jJ_k, N_3, CN, $C(=O)OJ_j$, $C(=O)NJ_jJ_k$, $C(=O)J_j$, $O\text{-}C(=O)NJ_jJ_k$, $N(H)C(=NH)NJ_jJ_k$, $N(H)C(=O)NJ_jJ_k$ or $N(H)C(=S)NJ_jJ_k$; and

q_i and q_j or q_l and q_k together are $=C(q_g)(q_h)$, wherein q_g and q_h are each, independently, H, halogen, C_1-C_{12} alkyl or substituted C_1-C_{12} alkyl.

One carbocyclic bicyclic nucleoside having a 4'-$(CH_2)_3$-2' bridge and the alkenyl analog bridge 4'-CH=CH-CH_2-2' have been described (Frier *et al., Nucleic Acids Research*, 1997, *25*(*22*), 4429-4443 and Albaek *et al., J. Org. Chem.*, 2006, *71*, 7731-7740). The synthesis and preparation of carbocyclic bicyclic nucleosides along with their oligomerization and biochemical studies have also been described (Srivastava *et al., J. Am. Chem. Soc.* 2007, *129(26)*, 8362-8379).

In certain embodiments, bicyclic nucleosides include, but are not limited to, (A) α-L-methyleneoxy (4'-CH_2-O-2') BNA , (B) β-D-methyleneoxy (4'-CH_2-O-2') BNA , (C) ethyleneoxy (4'-$(CH_2)_2$-O-2') BNA, (D) aminooxy (4'-CH_2-O-N(R)-2') BNA, (E) oxyamino (4'-CH_2-N(R)-O-2') BNA, (F) methyl(methyleneoxy) (4'-$CH(CH_3)$-O-2') BNA (also referred to as constrained ethyl or cEt), (G) methylene-thio (4'-CH_2-S-2') BNA, (H) methylene-amino (4'-CH_2-N(R)-2') BNA, (I) methyl carbocyclic (4'-CH_2-$CH(CH_3)$-2') BNA, (J) propylene carbocyclic (4'-$(CH_2)_3$-2') BNA, and (K) vinyl BNA as depicted below.


(A)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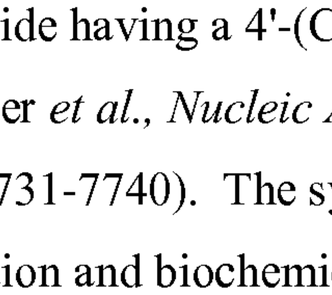
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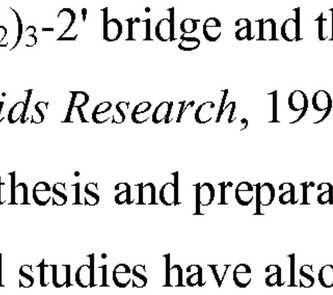
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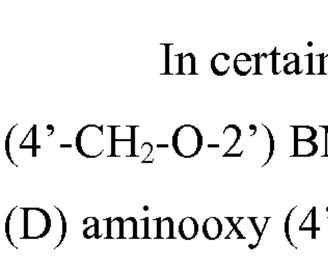
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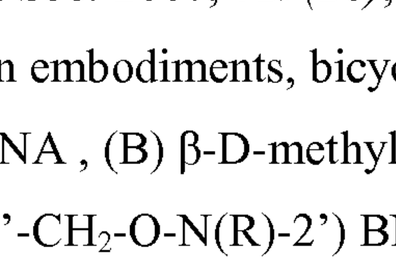
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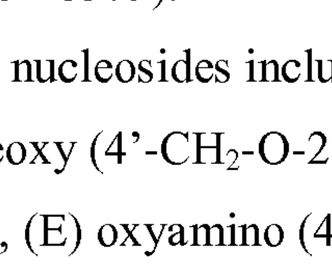
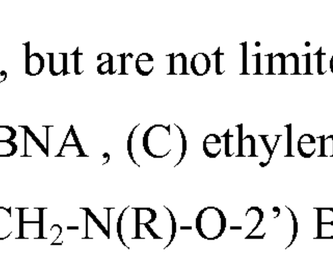

(G)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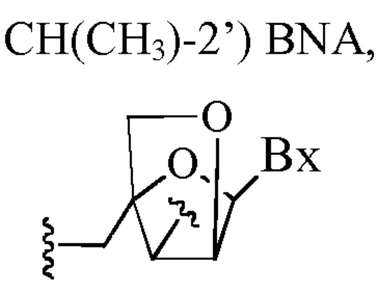
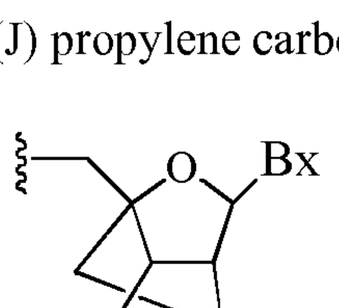

(I)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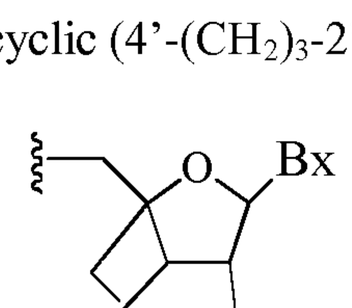
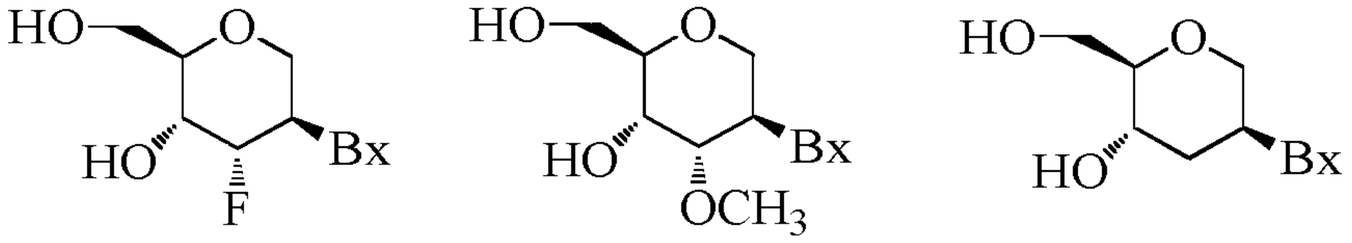
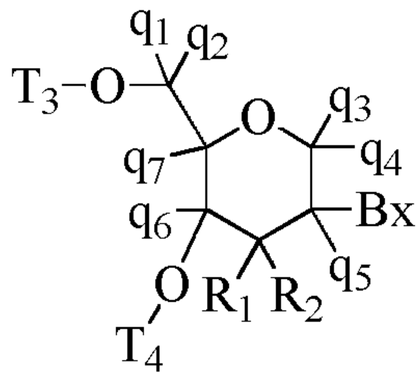

(K)

wherein Bx is the base moiety and R is, independently, H, a protecting group, C_1-C_6 alkyl or C_1-C_6 alkoxy.

As used herein, the term "modified tetrahydropyran nucleoside" or "modified THP nucleoside" means a nucleoside having a six-membered tetrahydropyran "sugar" substituted for the pentofuranosyl residue in normal nucleosides and can be referred to as a sugar surrogate. Modified THP nucleosides include, but are not limited to, what is referred to in the art as hexitol nucleic acid (HNA), anitol nucleic acid (ANA), manitol nucleic acid (MNA) (see Leumann, *Bioorg. Med. Chem.*, 2002, *10*, 841-854) or fluoro HNA (F-HNA) having a tetrahydropyranyl ring system as illustrated below.



In certain embodiment, sugar surrogates are selected having the formula:



wherein:

Bx is a heterocyclic base moiety;

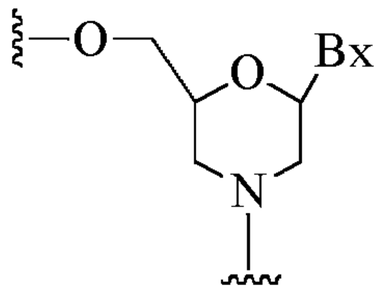
T_3 and T_4 are each, independently, an internucleoside linking group linking the tetrahydropyran nucleoside analog to the oligomeric compound or one of T_3 and T_4 is an internucleoside linking group linking the tetrahydropyran nucleoside analog to an oligomeric compound or oligonucleotide and the other of T_3 and T_4 is H, a hydroxyl protecting group, a linked conjugate group or a 5' or 3'-terminal group;

q_1, q_2, q_3, q_4, q_5, q_6 and q_7 are each independently, H, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl; and

one of R_1 and R_2 is hydrogen and the other is selected from halogen, substituted or unsubstituted alkoxy, NJ_1J_2, SJ_1, N_3, $OC(=X)J_1$, $OC(=X)NJ_1J_2$, $NJ_3C(=X)NJ_1J_2$ and CN, wherein X is O, S or NJ_1 and each J_1, J_2 and J_3 is, independently, H or C_1-C_6 alkyl.

In certain embodiments, q_1, q_2, q_3, q_4, q_5, q_6 and q_7 are each H. In certain embodiments, at least one of q_1, q_2, q_3, q_4, q_5, q_6 and q_7 is other than H. In certain embodiments, at least one of q_1, q_2, q_3, q_4, q_5, q_6 and q_7 is methyl. In certain embodiments, THP nucleosides are provided wherein one of R_1 and R_2 is F. In certain embodiments, R_1 is fluoro and R_2 is H; R_1 is methoxy and R_2 is H, and R_1 is methoxyethoxy and R_2 is H.

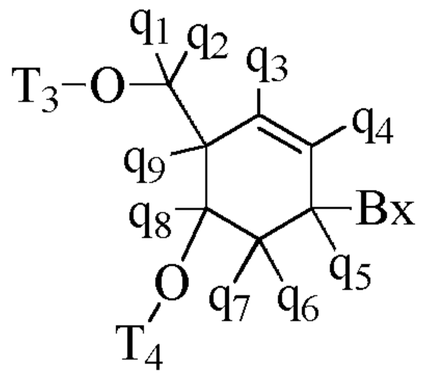
In certain embodiments, sugar surrogates comprise rings having more than 5 atoms and more than one heteroatom. For example nucleosides comprising morpholino sugar moieties and their use in oligomeric compounds has been reported (see for example: Braasch *et al.*, *Biochemistry*, 2002, *41*, 4503-4510; and U.S. Patents 5,698,685; 5,166,315; 5,185,444; and 5,034,506). As used here, the term "morpholino" means a sugar surrogate having the following formula:



In certain embodiments, morpholinos may be modified, for example by adding or altering various substituent groups from the above morpholino structure. Such sugar surrogates are referred to herein as "modifed morpholinos."

Combinations of modifications are also provided without limitation, such as 2'-F-5'-methyl substituted nucleosides (see PCT International Application WO 2008/101157 published on 8/21/08 for other disclosed 5', 2'-bis substituted nucleosides) and replacement of the ribosyl ring oxygen atom with S and further substitution at the 2'-position (see published U.S. Patent Application US2005-0130923, published on June 16, 2005) or alternatively 5'-substitution of a bicyclic nucleic acid (see PCT International Application WO 2007/134181, published on 11/22/07 wherein a 4'-CH_2-O-2' bicyclic nucleoside is further substituted at the 5' position with a 5'-methyl or a 5'-vinyl group). The synthesis and preparation of carbocyclic bicyclic nucleosides along with their oligomerization and biochemical studies have also been described (*see, e.g.,* Srivastava *et al., J. Am. Chem. Soc.* 2007, *129(26)*, 8362-8379).

In certain embodiments, antisense compounds comprise one or more modified cyclohexenyl nucleosides, which is a nucleoside having a six-membered cyclohexenyl in place of the pentofuranosyl residue in naturally occurring nucleosides. Modified cyclohexenyl nucleosides include, but are not limited to those described in the art (see for example commonly owned, published PCT Application WO 2010/036696, published on April 10, 2010, Robeyns *et al., J. Am. Chem. Soc.,* 2008, *130(6)*, 1979-1984; Horváth *et al., Tetrahedron Letters,* 2007, *48*, 3621-3623; Nauwelaerts *et al., J. Am. Chem. Soc.*, 2007, *129(30)*, 9340-9348; Gu *et al.,*, *Nucleosides, Nucleotides & Nucleic Acids*, 2005, *24(5-7)*, 993-998; Nauwelaerts *et al., Nucleic Acids Research,* 2005, *33(8)*, 2452-2463; Robeyns et al., *Acta Crystallographica, Section F: Structural Biology and Crystallization Communications,* 2005, *F61(6)*, 585-586; Gu *et al., Tetrahedron*, 2004, *60(9)*, 2111-2123; Gu *et al., Oligonucleotides*, 2003, *13(6)*, 479-489; Wang *et al., J. Org. Chem.,* 2003, *68*, 4499-4505; Verbeure *et al., Nucleic Acids Research*, 2001, *29(24)*, 4941-4947; Wang *et al., J. Org. Chem.,* 2001, *66*, 8478-82; Wang *et al., Nucleosides, Nucleotides & Nucleic Acids*, 2001, *20(4-7)*, 785-788; Wang *et al., J. Am. Chem.,* 2000, *122*, 8595-8602; Published PCT application, WO 06/047842; and Published PCT Application WO 01/049687; the text of each is incorporated by reference herein, in their entirety). Certain modified cyclohexenyl nucleosides have Formula X.



X

wherein independently for each of said at least one cyclohexenyl nucleoside analog of Formula X:

Bx is a heterocyclic base moiety;

T_3 and T_4 are each, independently, an internucleoside linking group linking the cyclohexenyl nucleoside analog to an antisense compound or one of T_3 and T_4 is an internucleoside linking group linking the tetrahydropyran nucleoside analog to an antisense compound and the other of T_3 and T_4 is H, a hydroxyl protecting group, a linked conjugate group, or a 5'-or 3'-terminal group; and

q_1, q_2, q_3, q_4, q_5, q_6, q_7, q_8 and q_9 are each, independently, H, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl, substituted C_2-C_6 alkynyl or other sugar substituent group.

Many other monocyclic, bicyclic and tricyclic ring systems are known in the art and are suitable as sugar surrogates that can be used to modify nucleosides for incorporation into oligomeric compounds as provided herein (see for example review article: Leumann, Christian J. *Bioorg. & Med. Chem.*, 2002, *10*, 841-854). Such ring systems can undergo various additional substitutions to further enhance their activity.

As used herein, "2'-modified sugar" means a furanosyl sugar modified at the 2' position. In certain embodiments, such modifications include substituents selected from: a halide, including, but not limited to substituted and unsubstituted alkoxy, substituted and unsubstituted thioalkyl, substituted and unsubstituted amino alkyl, substituted and unsubstituted alkyl, substituted and unsubstituted allyl, and substituted and unsubstituted alkynyl. In certain embodiments, 2' modifications are selected from substituents including, but not limited to: $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nF$, $O(CH_2)_nONH_2$, $OCH_2C(=O)N(H)CH_3$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10. Other 2'-substituent groups can also be selected from: C_1-C_{12} alkyl, substituted alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, F, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving pharmacokinetic properties, or a group for improving the pharmacodynamic properties of an antisense compound, and other substituents having similar properties. In certain embodiments, modifed nucleosides comprise a 2'-MOE side chain (Baker *et al.*, *J. Biol. Chem.*, 1997, *272*, 11944-12000). Such 2'-MOE substitution have been described as having improved binding affinity compared to unmodified nucleosides and to other modified nucleosides, such as 2'- *O*-methyl, *O*-propyl, and *O*-aminopropyl. Oligonucleotides having the 2'-MOE substituent also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, *Helv.*

Chim. Acta, 1995, *78*, 486-504; Altmann *et al.*, *Chimia*, 1996, *50*, 168-176; Altmann *et al.*, *Biochem. Soc. Trans.*, 1996, *24*, 630-637; and Altmann *et al.*, *Nucleosides Nucleotides*, 1997, *16*, 917-926).

As used herein, "2'-modified" or "2'-substituted" refers to a nucleoside comprising a sugar comprising a substituent at the 2' position other than H or OH. 2'-modified nucleosides, include, but are not limited to, bicyclic nucleosides wherein the bridge connecting two carbon atoms of the sugar ring connects the 2' carbon and another carbon of the sugar ring; and nucleosides with non-bridging 2'substituents, such as allyl, amino, azido, thio, O-allyl, O-C_1-C_{10} alkyl, -OCF_3, O-$(CH_2)_2$-O-CH_3, 2'-O$(CH_2)_2SCH_3$, O-$(CH_2)_2$-O-$N(R_m)(R_n)$, or O-CH_2-C(=O)-$N(R_m)(R_n)$, where each R_m and R_n is, independently, H or substituted or unsubstituted C_1-C_{10} alkyl. 2'-modifed nucleosides may further comprise other modifications, for example at other positions of the sugar and/or at the nucleobase.

As used herein, "2'-F" refers to a nucleoside comprising a sugar comprising a fluoro group at the 2' position of the sugar ring.

As used herein, "2'-OMe" or "2'-OCH_3", "2'-O-methyl" or "2'-methoxy" each refers to a nucleoside comprising a sugar comprising an -OCH_3 group at the 2' position of the sugar ring.

As used herein, "MOE" or "2'-MOE" or "2'-$OCH_2CH_2OCH_3$" or "2'-O-methoxyethyl" each refers to a nucleoside comprising a sugar comprising a -$OCH_2CH_2OCH_3$ group at the 2' position of the sugar ring.

Methods for the preparations of modified sugars are well known to those skilled in the art. Some representative U.S. patents that teach the preparation of such modified sugars include without limitation, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,670,633; 5,700,920; 5,792,847 and 6,600,032 and International Application PCT/US2005/019219, filed June 2, 2005 and published as WO 2005/121371 on December 22, 2005, and each of which is herein incorporated by reference in its entirety.

As used herein, "oligonucleotide" refers to a compound comprising a plurality of linked nucleosides. In certain embodiments, one or more of the plurality of nucleosides is modified. In certain embodiments, an oligonucleotide comprises one or more ribonucleosides (RNA) and/or deoxyribonucleosides (DNA).

In nucleotides having modified sugar moieties, the nucleobase moieties (natural, modified or a combination thereof) are maintained for hybridization with an appropriate nucleic acid target.

In certain embodiments, antisense compounds comprise one or more nucleosides having modified sugar moieties. In certain embodiments, the modified sugar moiety is 2'-MOE. In certain embodiments, the 2'-MOE modified nucleosides are arranged in a gapmer motif. In certain embodiments, the modified sugar moiety is a bicyclic nucleoside having a (4'-$CH(CH_3)$-O-2') bridging group. In certain embodiments, the (4'-$CH(CH_3)$-O-2') modified nucleosides are arranged throughout the wings of a gapmer motif.

Modified Nucleobases

Nucleobase (or base) modifications or substitutions are structurally distinguishable from, yet functionally interchangeable with, naturally occurring or synthetic unmodified nucleobases. Both natural and modified nucleobases are capable of participating in hydrogen bonding. Such nucleobase modifications can impart nuclease stability, binding affinity or some other beneficial biological property to antisense compounds. Modified nucleobases include synthetic and natural nucleobases such as, for example, 5-methylcytosine (5-me-C). Certain nucleobase substitutions, including 5-methylcytosine substitutions, are particularly useful for increasing the binding affinity of an antisense compound for a target nucleic acid. For example, 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278).

Additional modified nucleobases include 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH3) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Heterocyclic base moieties can also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Nucleobases that are particularly useful for increasing the binding affinity of antisense compounds include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2 aminopropyladenine, 5-propynyluracil and 5-propynylcytosine.

In certain embodiments, antisense compounds targeted to an ANGPTL3 nucleic acid comprise one or more modified nucleobases. In certain embodiments, shortened or gap-widened antisense oligonucleotides targeted to an ANGPTL3 nucleic acid comprise one or more modified nucleobases. In certain embodiments, the modified nucleobase is 5-methylcytosine. In certain embodiments, each cytosine is a 5-methylcytosine.

Compositions and Methods for Formulating Pharmaceutical Compositions

Antisense oligonucleotides can be admixed with pharmaceutically acceptable active or inert substance for the preparation of pharmaceutical compositions or formulations. Compositions and methods for the formulation of pharmaceutical compositions are dependent upon a number of criteria, including, but not limited to, route of administration, extent of disease, or dose to be administered.

Antisense compound targeted to an ANGPTL3 nucleic acid can be utilized in pharmaceutical compositions by combining the antisense compound with a suitable pharmaceutically acceptable diluent or carrier. A pharmaceutically acceptable diluent includes phosphate-buffered saline (PBS). PBS is a diluent suitable for use in compositions to be delivered parenterally. Accordingly, in one embodiment, employed in the methods described herein is a pharmaceutical composition comprising an antisense compound targeted to an ANGPTL3 nucleic acid and a pharmaceutically acceptable diluent. In certain embodiments, the pharmaceutically acceptable diluent is PBS. In certain embodiments, the antisense compound is an antisense oligonucleotide.

Pharmaceutical compositions comprising antisense compounds encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other oligonucleotide which, upon administration to an animal, including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to pharmaceutically acceptable salts of antisense compounds, prodrugs, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents. Suitable pharmaceutically acceptable salts include, but are not limited to, sodium and potassium salts.

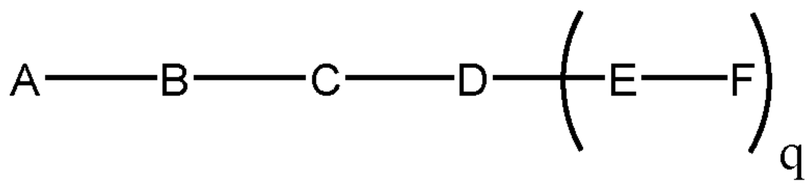
A prodrug can include the incorporation of additional nucleosides at one or both ends of an antisense compound which are cleaved by endogenous nucleases within the body, to form the active antisense compound.

Conjugated Antisense Compounds

Antisense compounds can be covalently linked to one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the resulting antisense oligonucleotides. Typical conjugate groups include cholesterol moieties and lipid moieties. Additional conjugate groups include carbohydrates, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes.

Antisense compounds can also be modified to have one or more stabilizing groups that are generally attached to one or both termini of antisense compounds to enhance properties such as, for example, nuclease stability. Included in stabilizing groups are cap structures. These terminal modifications protect the antisense compound having terminal nucleic acids from exonuclease degradation, and can help in delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap), or at the 3'-terminus (3'-cap), or can be present on both termini. Cap structures are well known in the art and include, for example, inverted deoxy abasic caps. Further 3' and 5'-stabilizing groups that can be used to cap one or both ends of an antisense compound to impart nuclease stability include those disclosed in WO 03/004602 published on January 16, 2003.

In certain embodiments, the present disclosure provides conjugated antisense compounds represented by the formula:



wherein

A is the antisense oligonucleotide;

B is the cleavable moiety

C is the conjugate linker

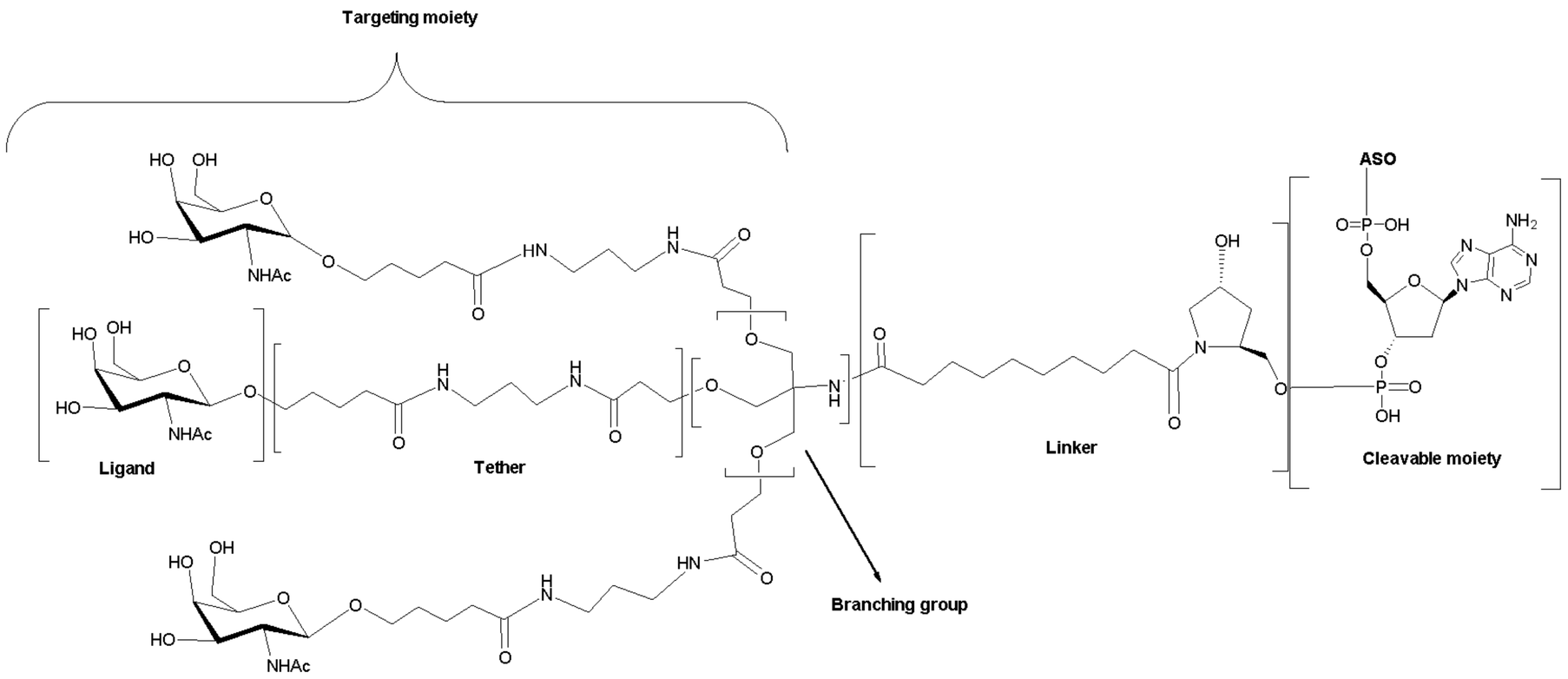
D is the branching group

each E is a tether;

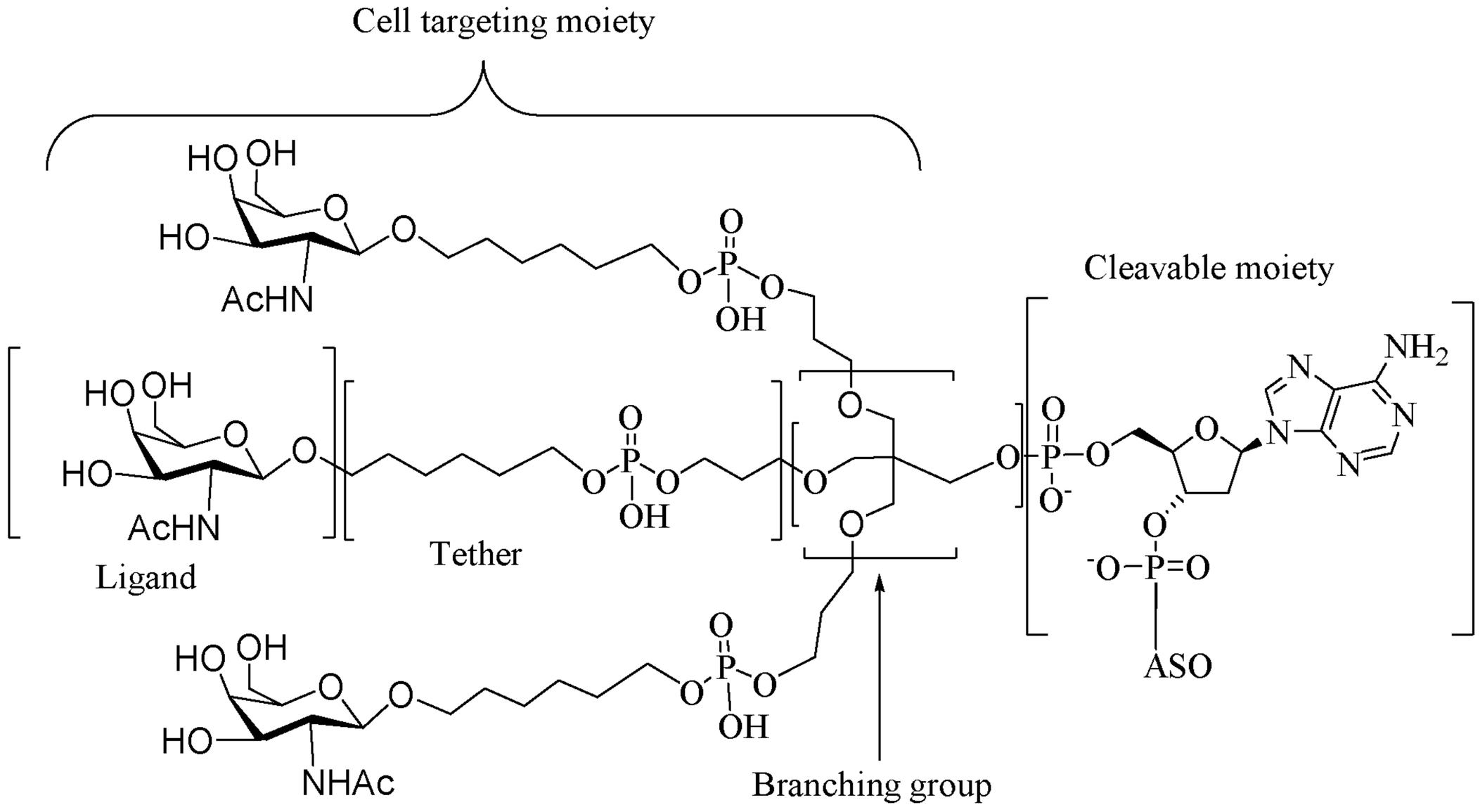
each F is a ligand; and

q is an integer between 1 and 5.

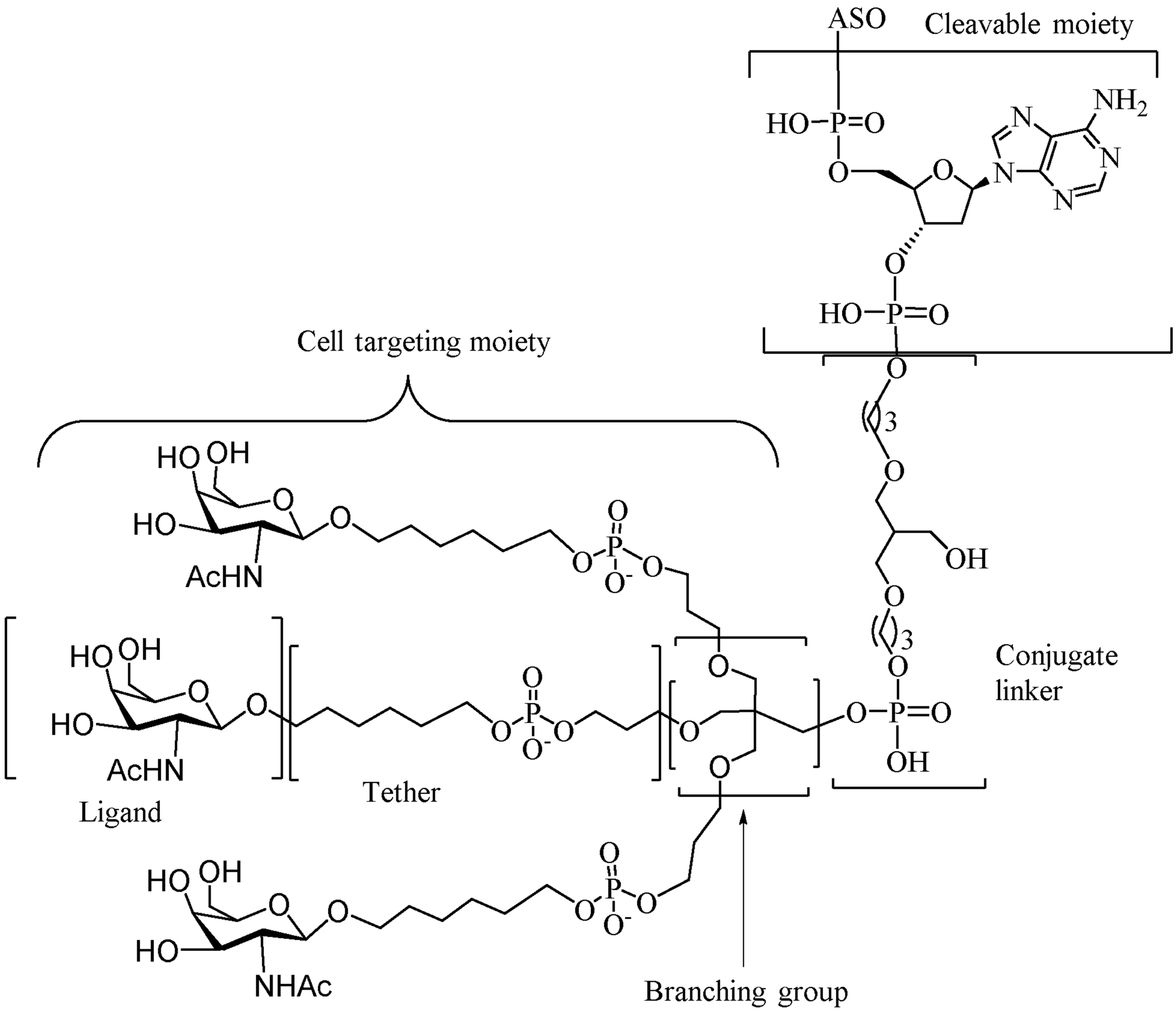
In certain embodiments, conjugated antisense compounds are provided having the structure:



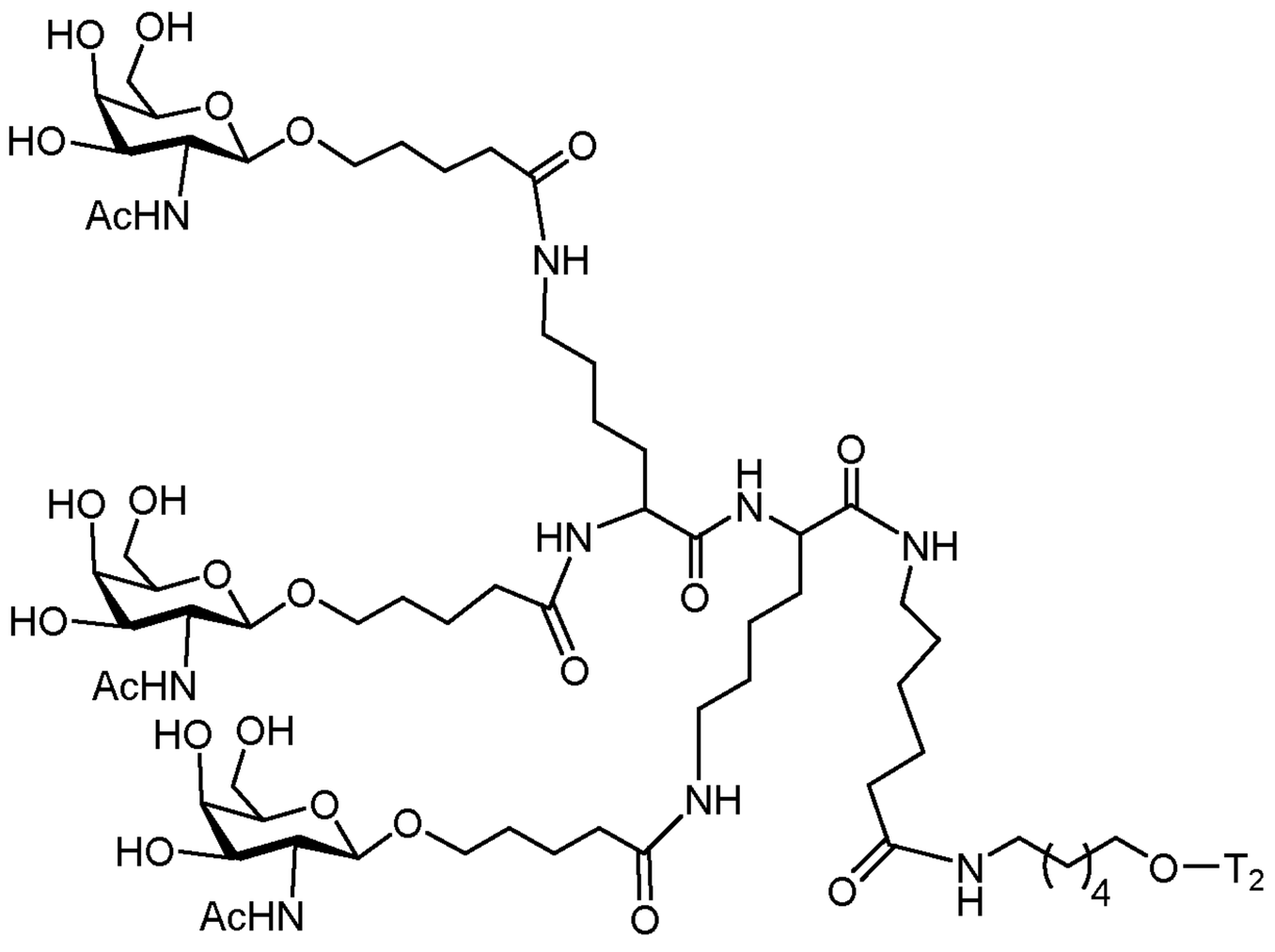
In certain embodiments, conjugated antisense compounds are provided having the structure:



In certain embodiments, conjugated antisense compounds are provided having the structure:



The present disclosure provides the following non-limiting numbered embodiments:



wherein:

T_2 is a nucleoside, a nucleotide, a monomeric subunit, or an oligomeric compound.

In embodiments having more than one of a particular variable (e.g., more than one "m" or "n"), unless otherwise indicated, each such particular variable is selected independently. Thus, for a structure having more than one n, each n is selected independently, so they may or may not be the same as one another.

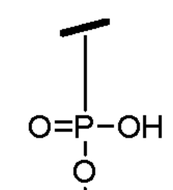
i. <u>Certain Cleavable Moieties</u>

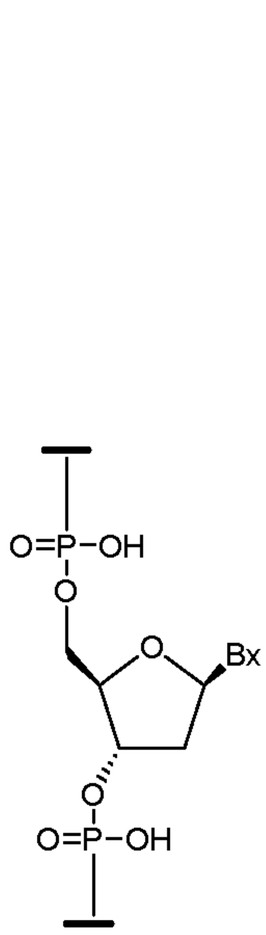
In certain embodiments, a cleavable moiety is a cleavable bond. In certain embodiments, a cleavable moiety comprises a cleavable bond. In certain embodiments, the conjugate group comprises a cleavable moiety. In certain such embodiments, the cleavable moiety attaches to the antisense oligonucleotide. In certain such embodiments, the cleavable moiety attaches directly to the cell-targeting moiety. In certain such embodiments, the cleavable moiety attaches to the conjugate linker. In certain embodiments, the cleavable moiety comprises a phosphate or phosphodiester. In certain embodiments, the cleavable moiety is a cleavable nucleoside or nucleoside analog. In certain embodiments, the nucleoside or nucleoside analog comprises an optionally protected heterocyclic base selected from a purine, substituted purine, pyrimidine or substituted pyrimidine. In certain embodiments, the cleavable moiety is a nucleoside comprising an optionally protected heterocyclic base selected from uracil, thymine, cytosine, 4-N-benzoylcytosine, 5-methylcytosine, 4-N-benzoyl-5-methylcytosine, adenine, 6-N-benzoyladenine, guanine and 2-N-isobutyrylguanine. In certain embodiments, the cleavable moiety is 2'-deoxy nucleoside that is attached to the 3' position of the antisense oligonucleotide by a phosphodiester linkage and is attached to the linker by a phosphodiester or phosphorothioate linkage. In certain embodiments, the cleavable moiety is 2'-deoxy adenosine that is attached to the 3' position of the antisense oligonucleotide by a phosphodiester linkage and is attached to the linker by a phosphodiester or phosphorothioate linkage. In certain

embodiments, the cleavable moiety is 2'-deoxy adenosine that is attached to the 3' position of the antisense oligonucleotide by a phosphodiester linkage and is attached to the linker by a phosphodiester linkage.

In certain embodiments, the cleavable moiety is attached to the 3' position of the antisense oligonucleotide. In certain embodiments, the cleavable moiety is attached to the 5' position of the antisense oligonucleotide. In certain embodiments, the cleavable moiety is attached to a 2' position of the antisense oligonucleotide. In certain embodiments, the cleavable moiety is attached to the antisense oligonucleotide by a phosphodiester linkage. In certain embodiments, the cleavable moiety is attached to the linker by either a phosphodiester or a phosphorothioate linkage. In certain embodiments, the cleavable moiety is attached to the linker by a phosphodiester linkage. In certain embodiments, the conjugate group does not include a cleavable moiety.

In certain embodiments, the cleavable moiety is cleaved after the complex has been administered to an animal only after being internalized by a targeted cell. Inside the cell the cleavable moiety is cleaved thereby releasing the active antisense oligonucleotide. While not wanting to be bound by theory it is believed that the cleavable moiety is cleaved by one or more nucleases within the cell. In certain embodiments, the one or more nucleases cleave the phosphodiester linkage between the cleavable moiety and the linker. In certain embodiments, the cleavable moiety has a structure selected from among the following:





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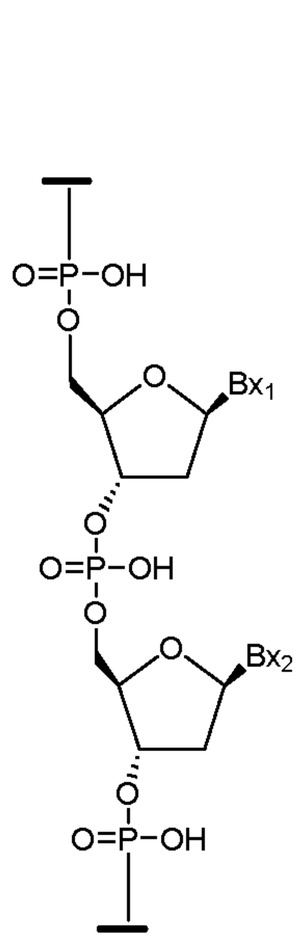
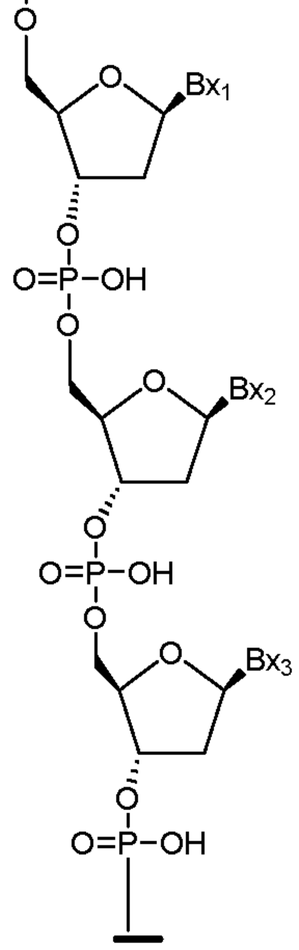
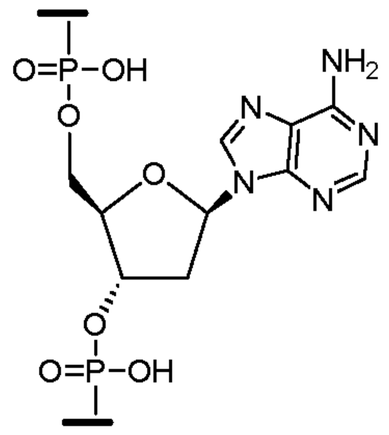


; and



wherein each of Bx, Bx_1, Bx_2, and Bx_3 is independently a heterocyclic base moiety. In certain embodiments, the cleavable moiety has a structure selected from among the following:



i. **Certain Linkers**

In certain embodiments, the conjugate groups comprise a linker. In certain such embodiments, the linker is covalently bound to the cleavable moiety. In certain such embodiments, the linker is covalently bound to the antisense oligonucleotide. In certain embodiments, the linker is covalently bound to a cell-targeting moiety. In certain embodiments, the linker further comprises a covalent attachment to a solid support. In certain embodiments, the linker further comprises a covalent attachment to a protein binding moiety. In certain embodiments, the linker further comprises a covalent attachment to a solid support and further comprises a covalent attachment to a protein binding moiety. In certain embodiments, the linker includes multiple positions for attachment of tethered ligands. In certain embodiments, the linker includes multiple positions for attachment of tethered ligands and is not attached to a branching group. In certain embodiments, the linker further comprises one or more cleavable bond. In certain embodiments, the conjugate group does not include a linker.

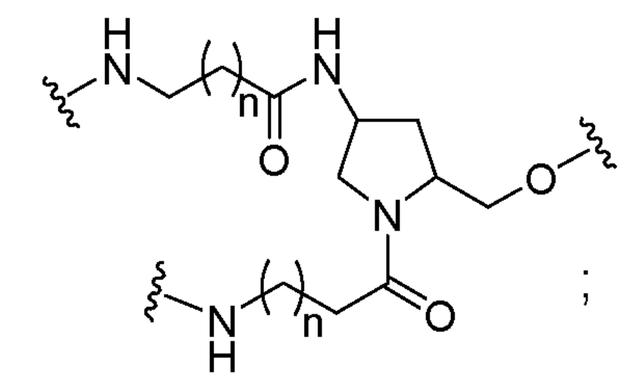
In certain embodiments, the linker includes at least a linear group comprising groups selected from alkyl, amide, disulfide, polyethylene glycol, ether, thioether (-S-) and hydroxylamino (-O-N(H)-) groups. In certain embodiments, the linear group comprises groups selected from alkyl, amide and ether groups. In certain embodiments, the linear group comprises groups selected from alkyl and ether groups. In certain embodiments, the linear group comprises at least one phosphorus linking group. In certain embodiments, the linear group comprises at least one phosphodiester group. In certain embodiments, the linear group includes at least one neutral linking group. In certain embodiments, the linear group is covalently attached to the cell-targeting moiety and the cleavable moiety. In certain embodiments, the linear group is covalently attached to the cell-targeting moiety and the antisense oligonucleotide. In certain embodiments, the linear group is covalently attached to the cell-targeting moiety, the cleavable moiety and a solid support. In certain embodiments, the linear group is covalently attached to the cell-targeting moiety, the cleavable moiety, a solid support and a protein binding moiety. In certain embodiments, the linear group includes one or more cleavable bond.

In certain embodiments, the linker includes the linear group covalently attached to a scaffold group. In certain embodiments, the scaffold includes a branched aliphatic group comprising groups selected from alkyl, amide, disulfide, polyethylene glycol, ether, thioether and hydroxylamino groups. In certain

embodiments, the scaffold includes a branched aliphatic group comprising groups selected from alkyl, amide and ether groups. In certain embodiments, the scaffold includes at least one mono or polycyclic ring system. In certain embodiments, the scaffold includes at least two mono or polycyclic ring systems. In certain embodiments, the linear group is covalently attached to the scaffold group and the scaffold group is covalently attached to the cleavable moiety and the linker. In certain embodiments, the linear group is covalently attached to the scaffold group and the scaffold group is covalently attached to the cleavable moiety, the linker and a solid support. In certain embodiments, the linear group is covalently attached to the scaffold group and the scaffold group is covalently attached to the cleavable moiety, the linker and a protein binding moiety. In certain embodiments, the linear group is covalently attached to the scaffold group and the scaffold group is covalently attached to the cleavable moiety, the linker, a protein binding moiety and a solid support. In certain embodiments, the scaffold group includes one or more cleavable bond.

In certain embodiments, the linker includes a protein binding moiety. In certain embodiments, the protein binding moiety is a lipid such as for example including but not limited to cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine), a vitamin (e.g., folate, vitamin A, vitamin E, biotin, pyridoxal), a peptide, a carbohydrate (e.g., monosaccharide, disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, polysaccharide), an endosomolytic component, a steroid (e.g., uvaol, hecigenin, diosgenin), a terpene (e.g., triterpene, e.g., sarsasapogenin, friedelin, epifriedelanol derivatized lithocholic acid), or a cationic lipid. In certain embodiments, the protein binding moiety is a C16 to C22 long chain saturated or unsaturated fatty acid, cholesterol, cholic acid, vitamin E, adamantane or 1-pentafluoropropyl.

In certain embodiments, a linker has a structure selected from among: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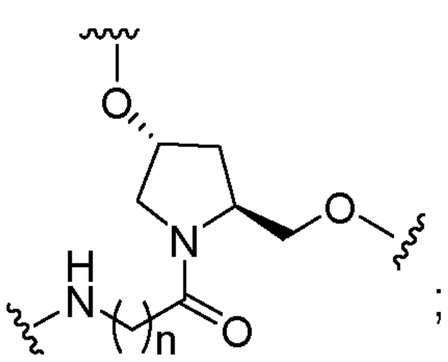
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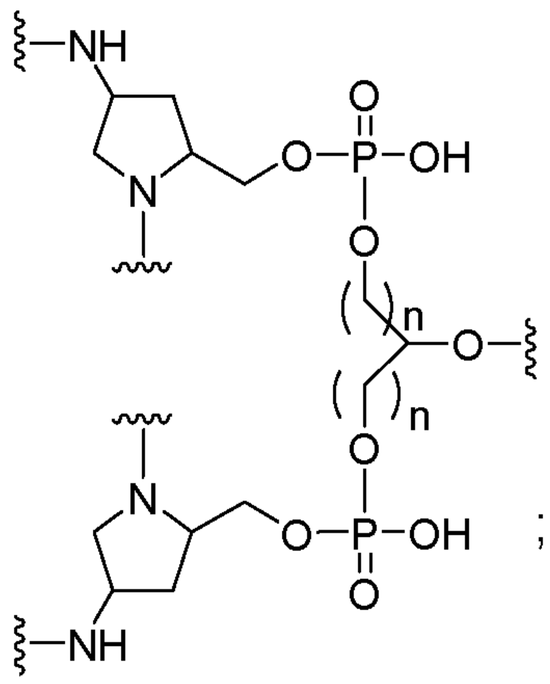
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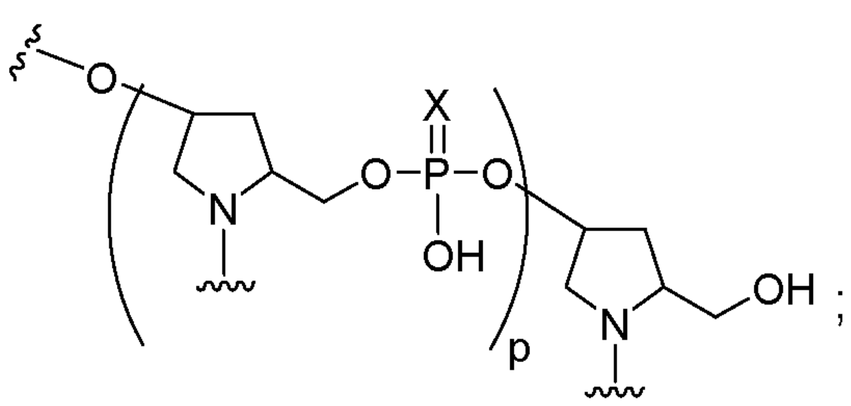
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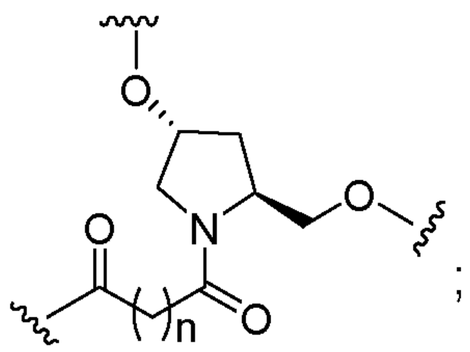
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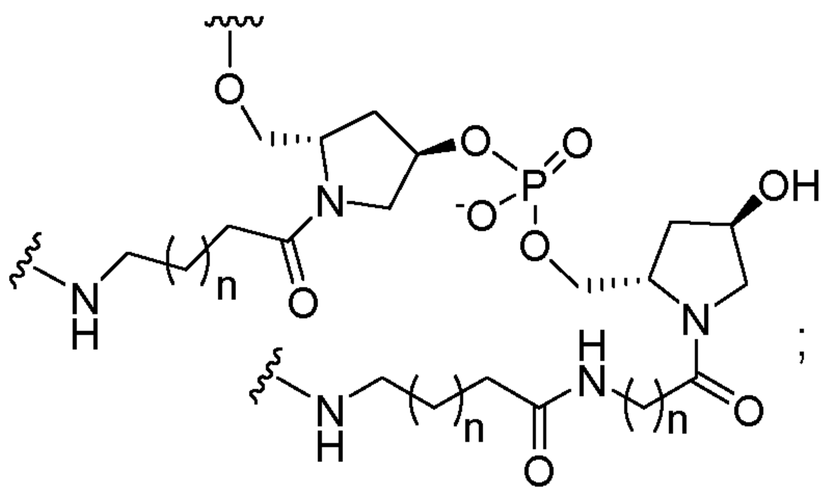
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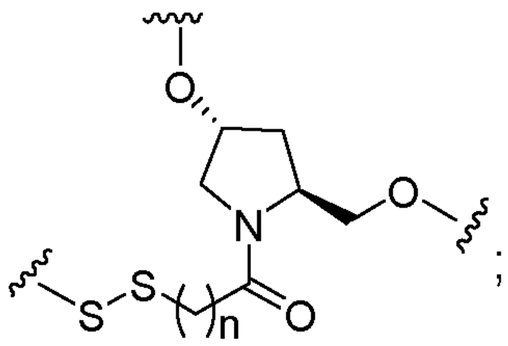
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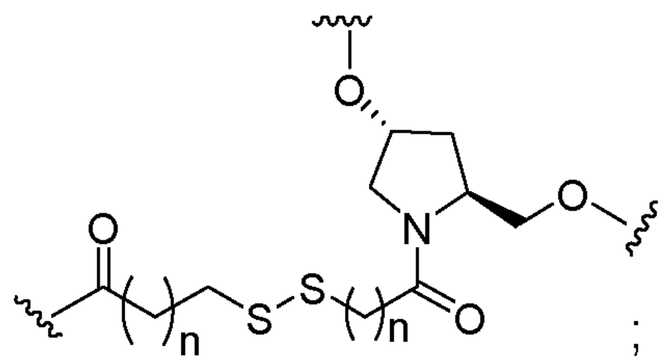
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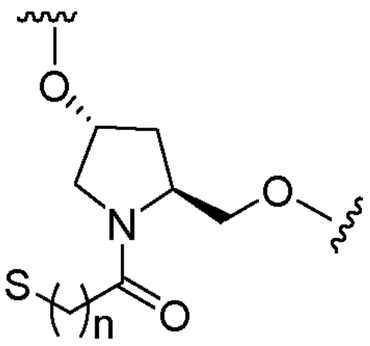
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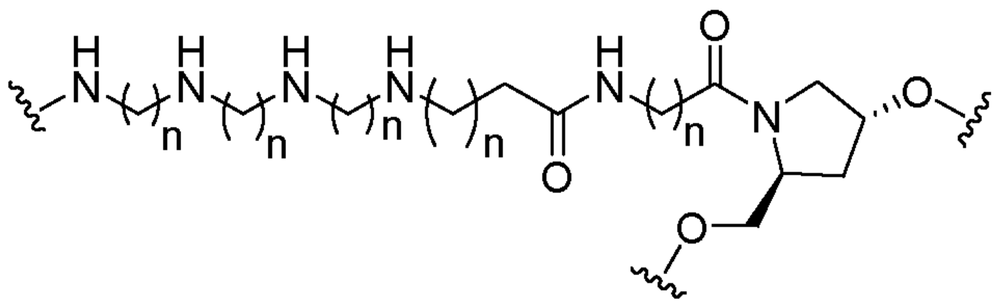
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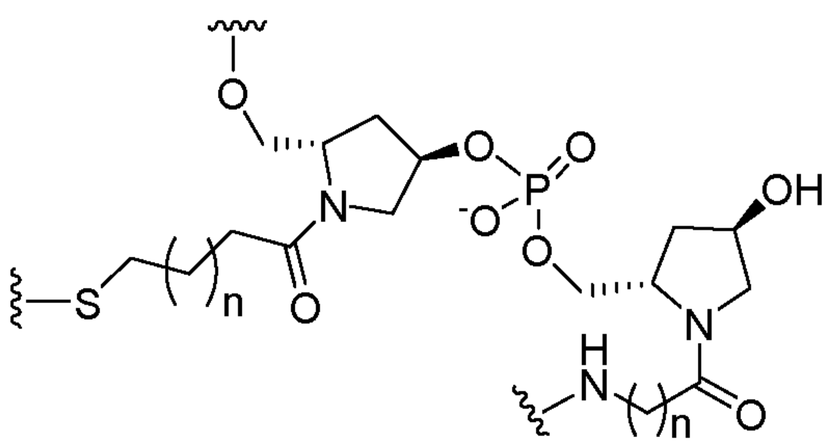
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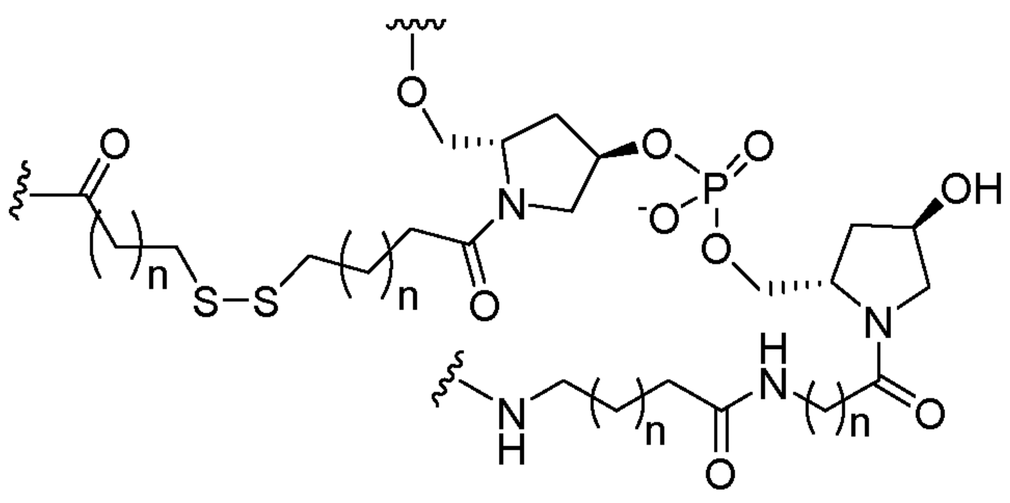
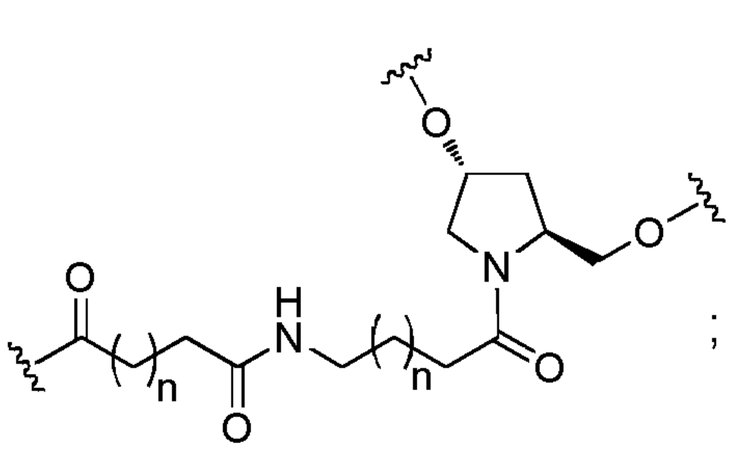


wherein each n is, independently, from 1 to 20; and p is from 1 to 6.

In certain embodiments, a linker has a structure selected from among: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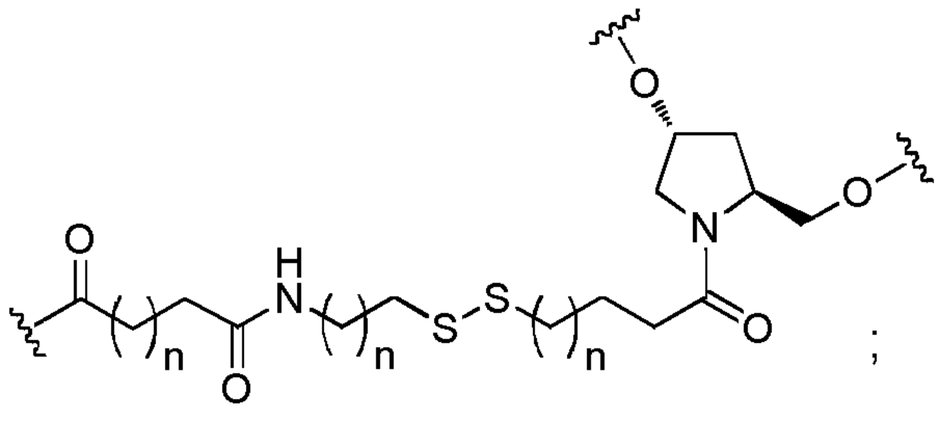
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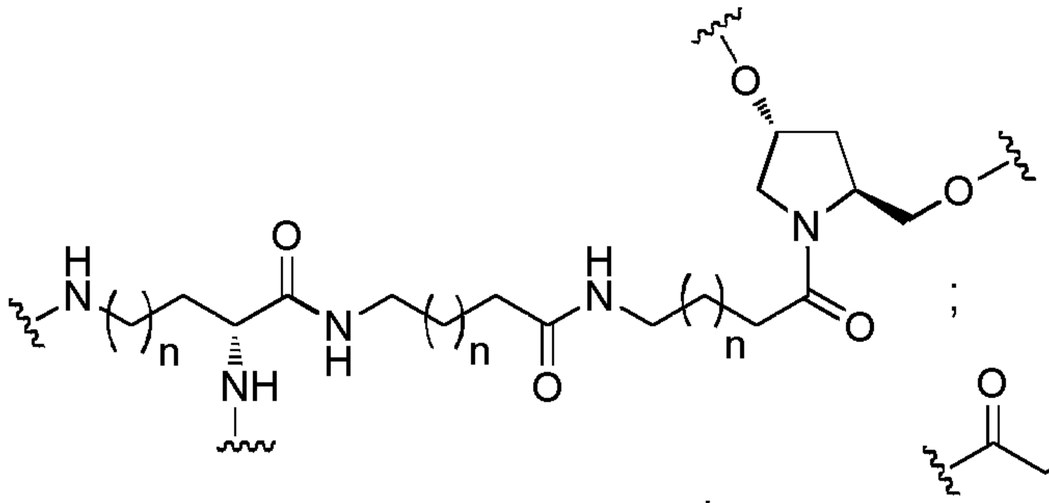
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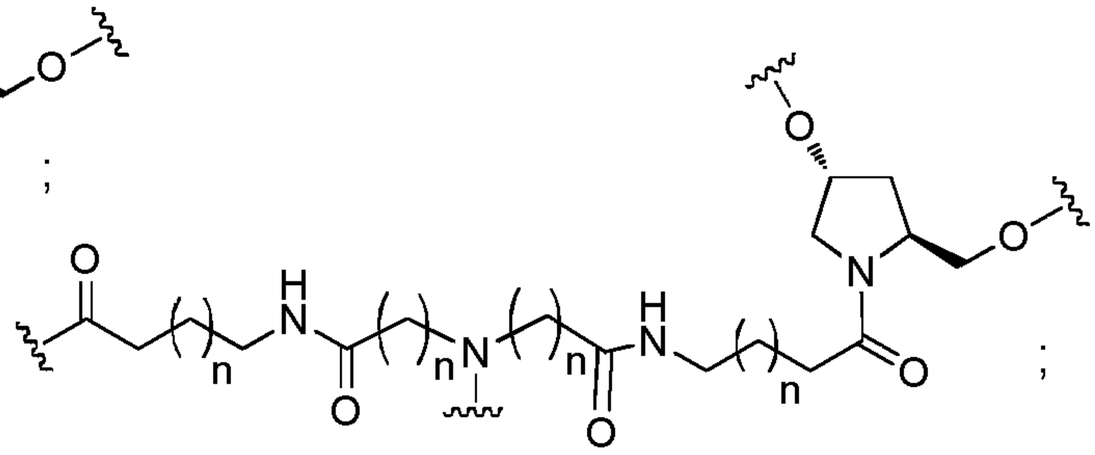
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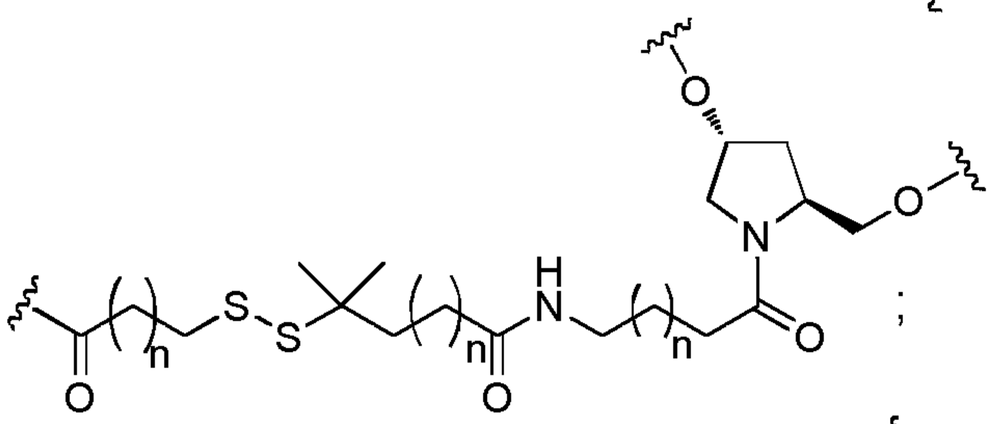
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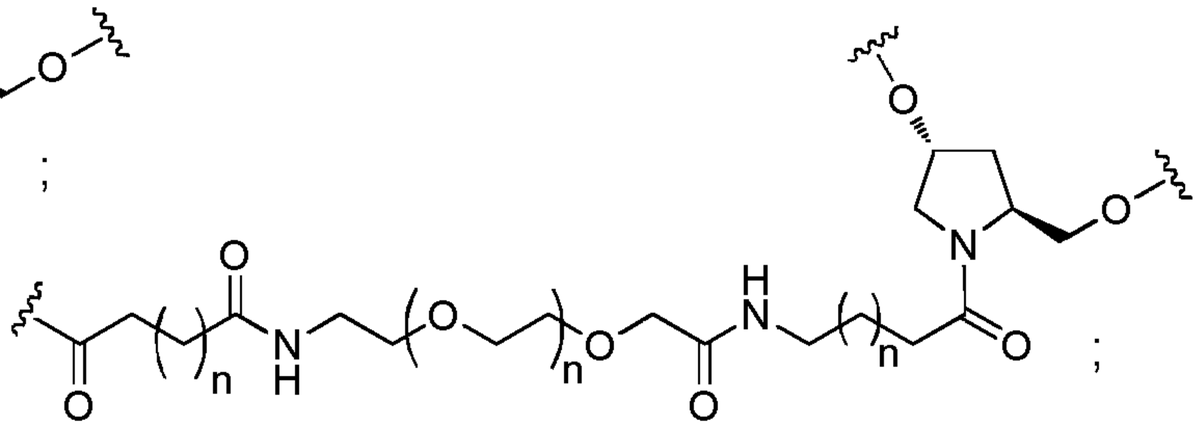
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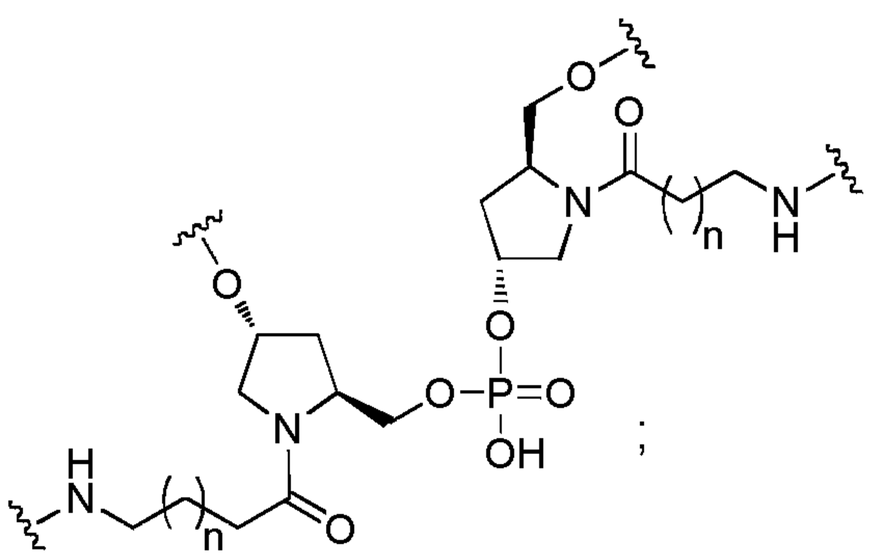
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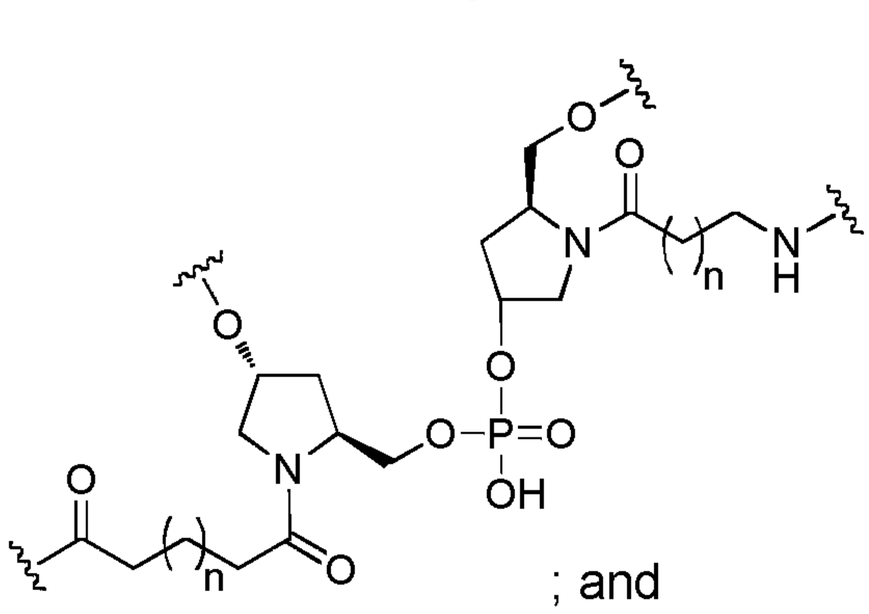
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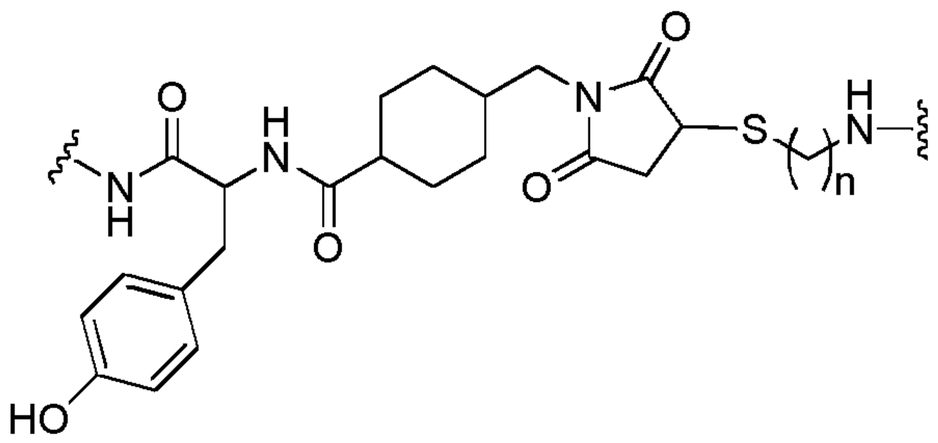
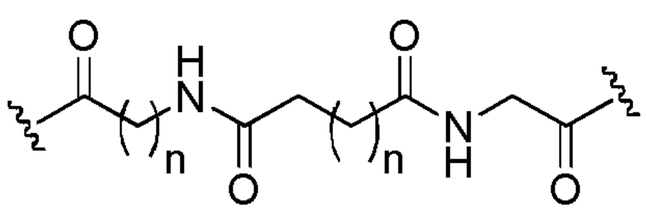


wherein each n is, independently, from 1 to 20.

In certain embodiments, a linker has a structure selected from among: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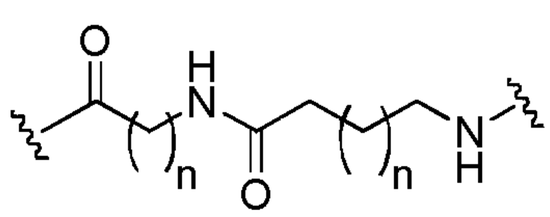
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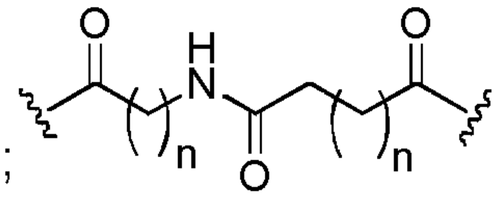
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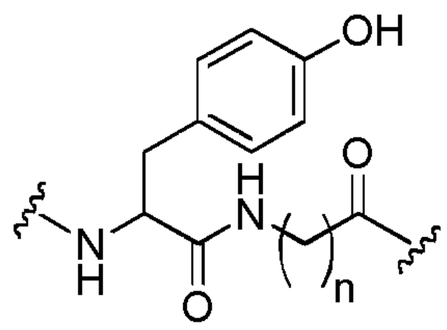
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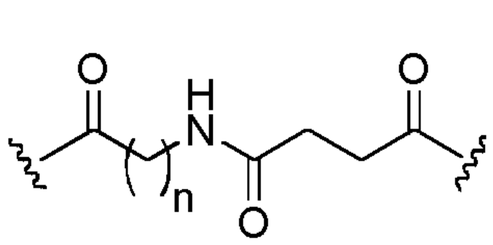
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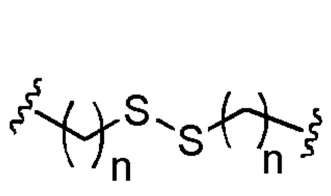
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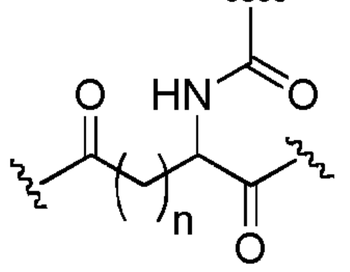
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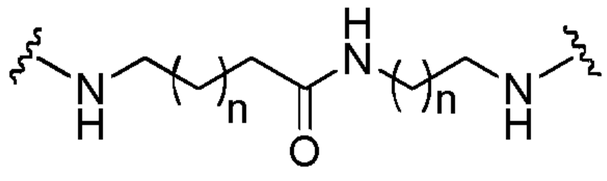
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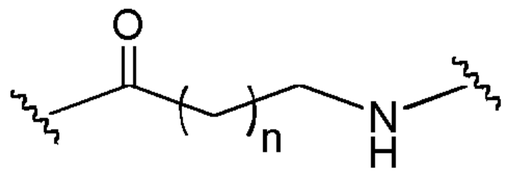
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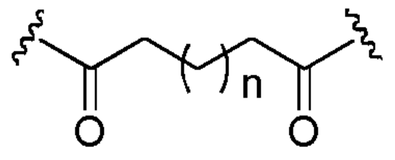
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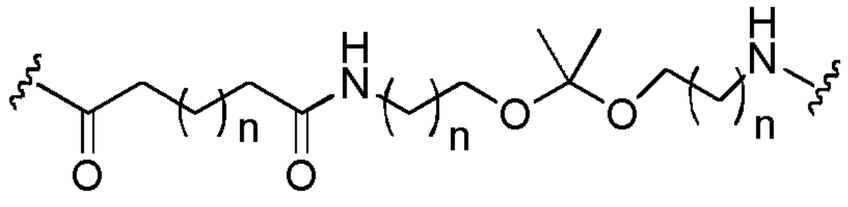
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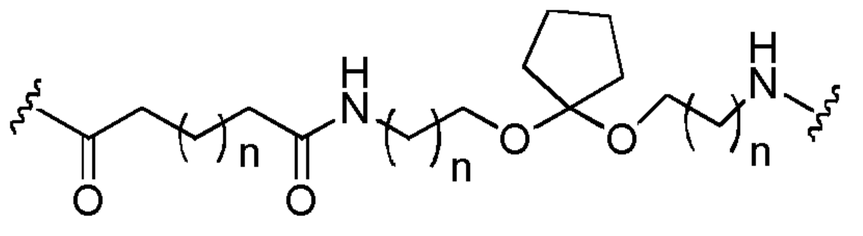
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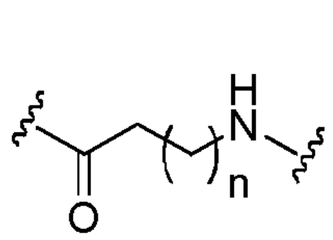
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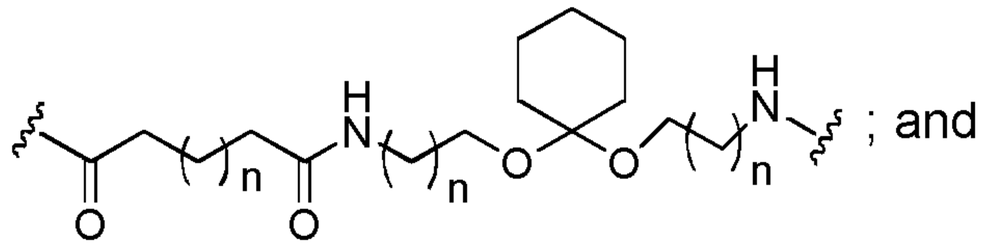
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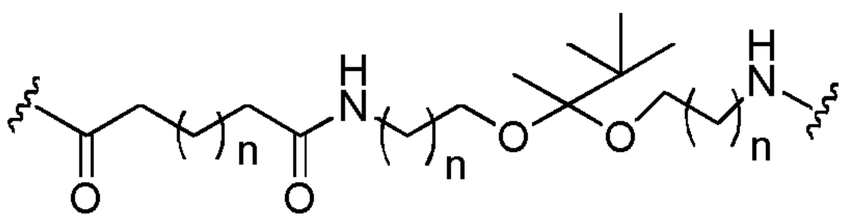
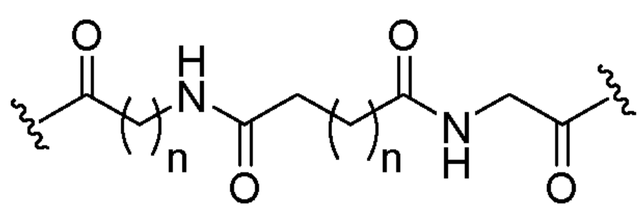


wherein n is from 1 to 20.

In certain embodiments, a linker has a structure selected from among: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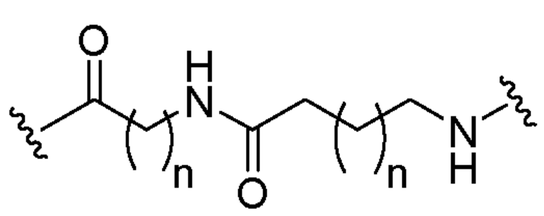
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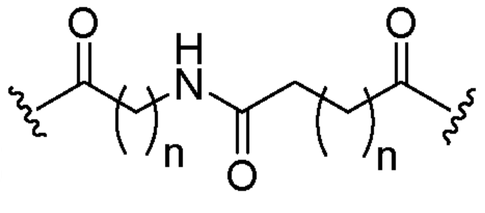
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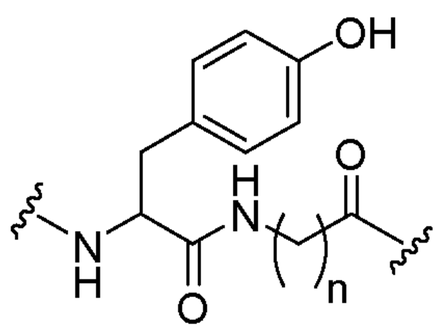
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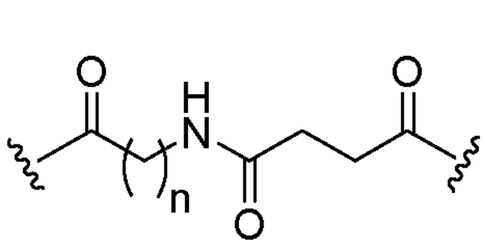
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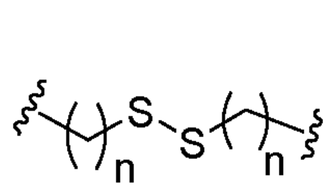
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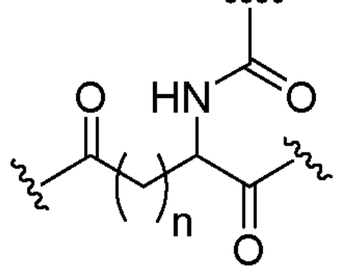
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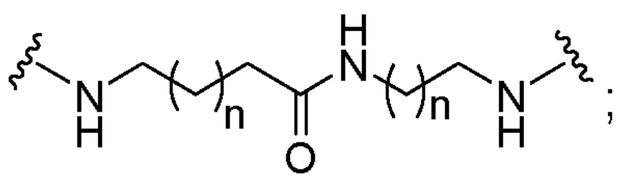
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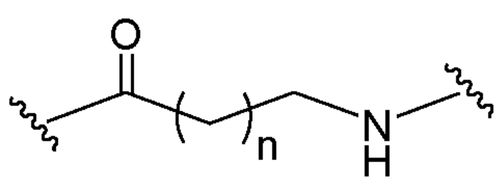
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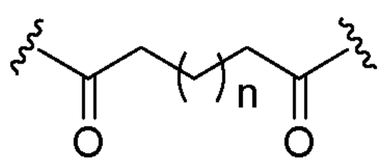
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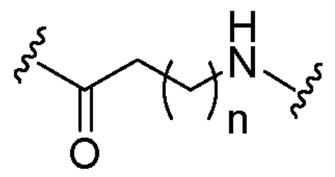
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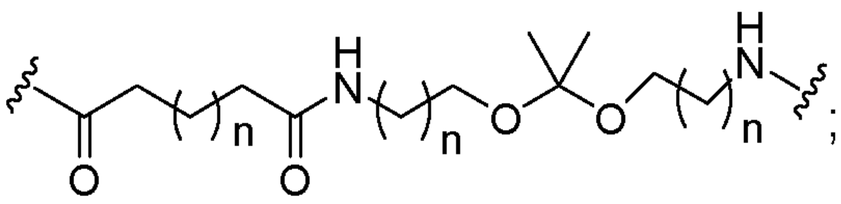
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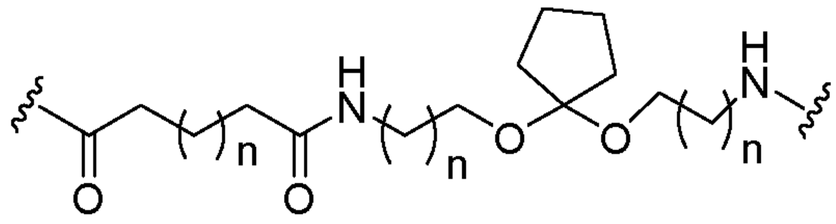
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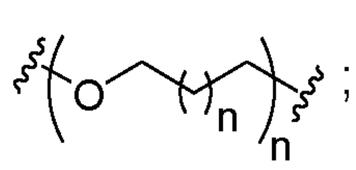
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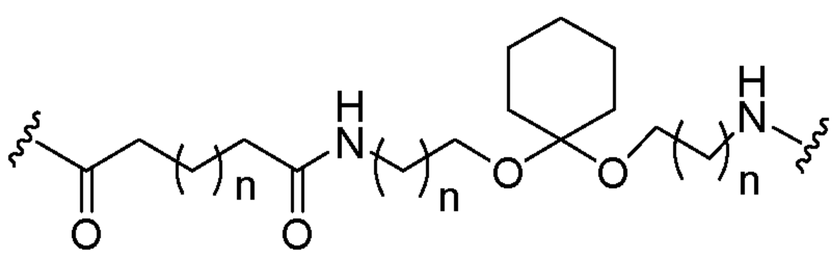
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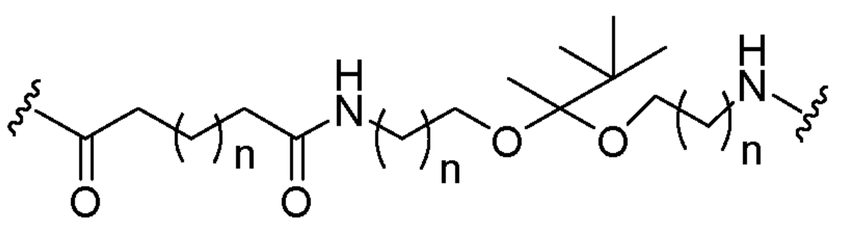
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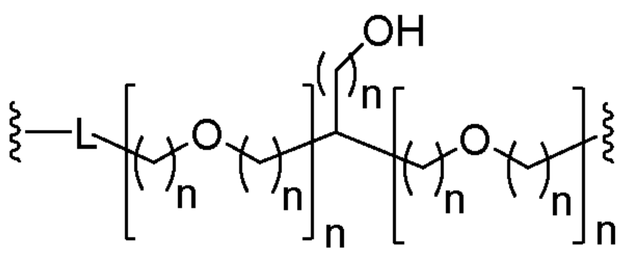
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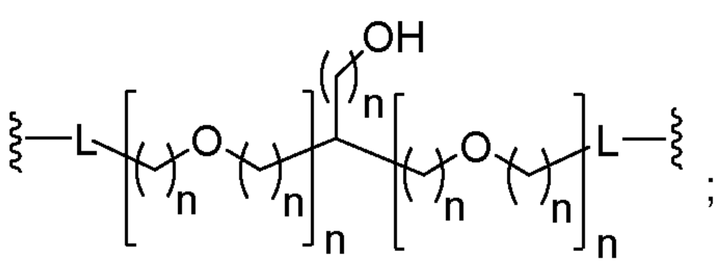
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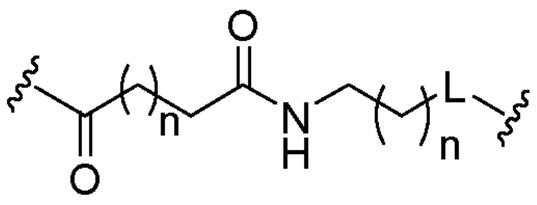
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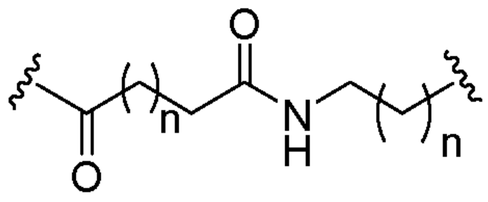
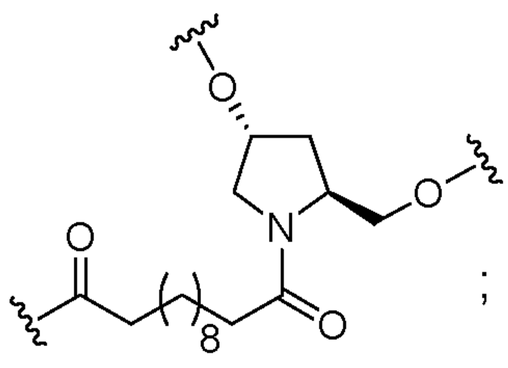

.

wherein each L is, independently, a phosphorus linking group or a neutral linking group; and

each n is, independently, from 1 to 20.

In certain embodiments, a linker has a structure selected from among: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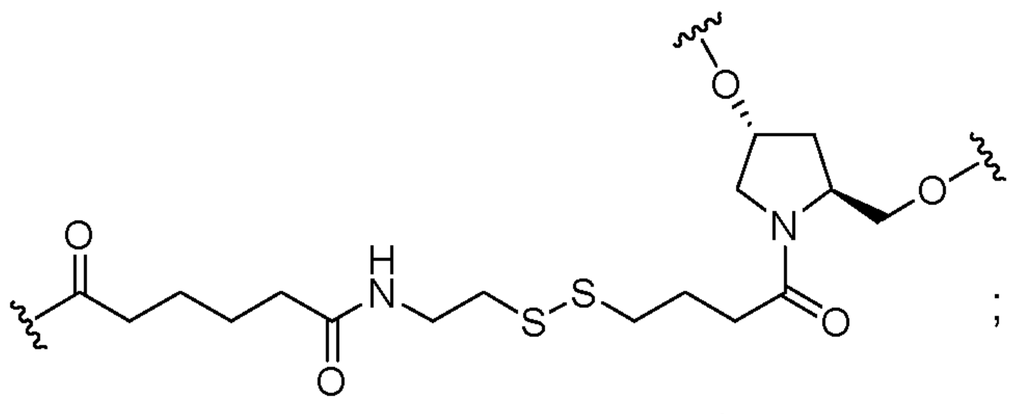
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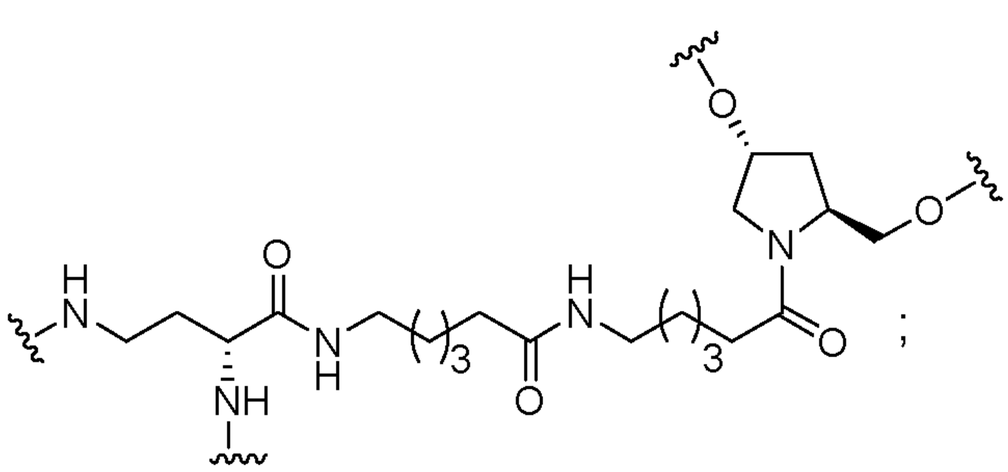
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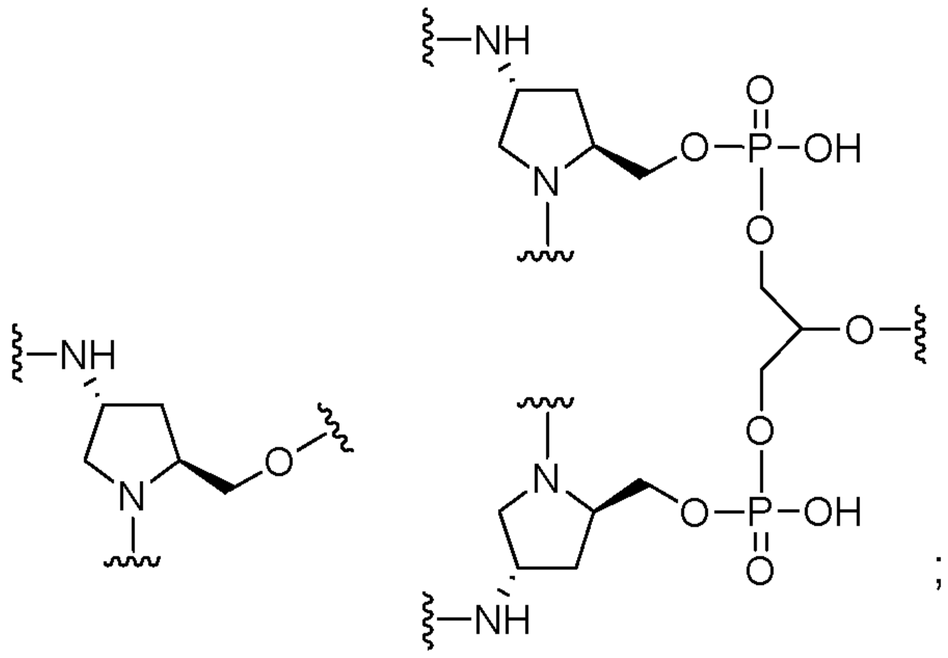
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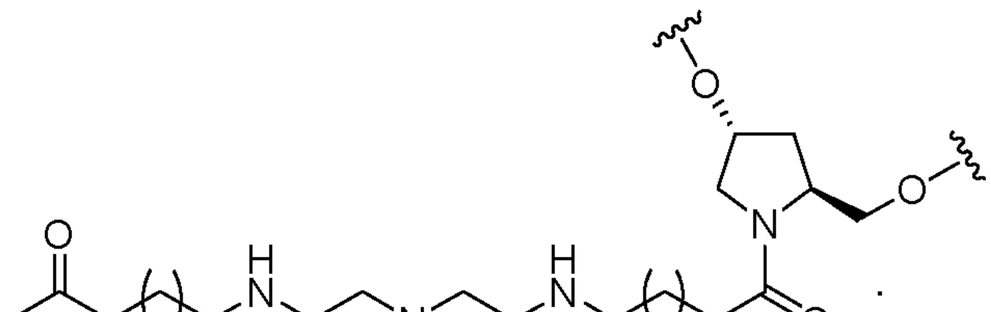
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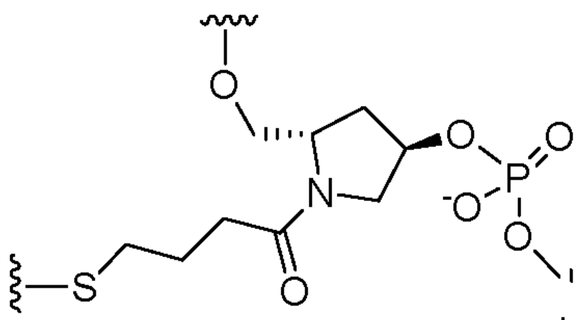
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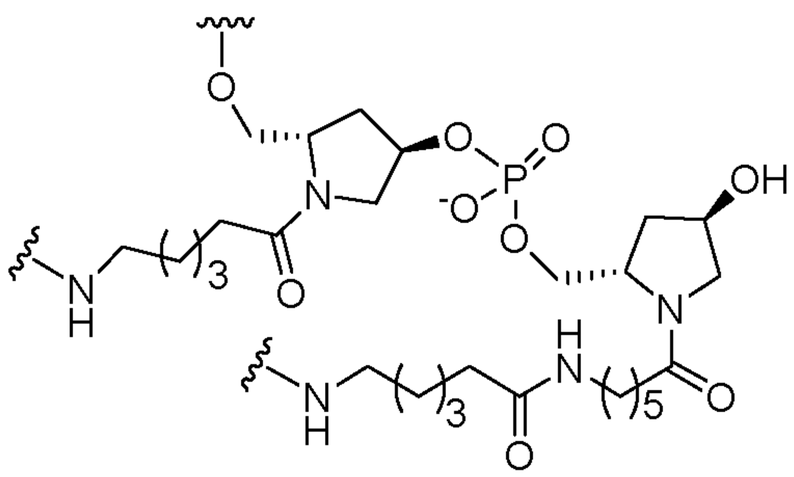
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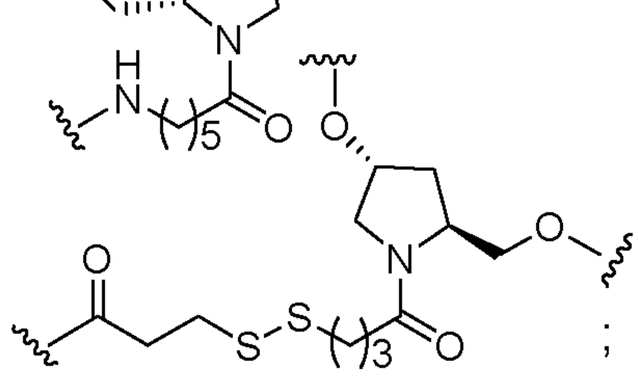
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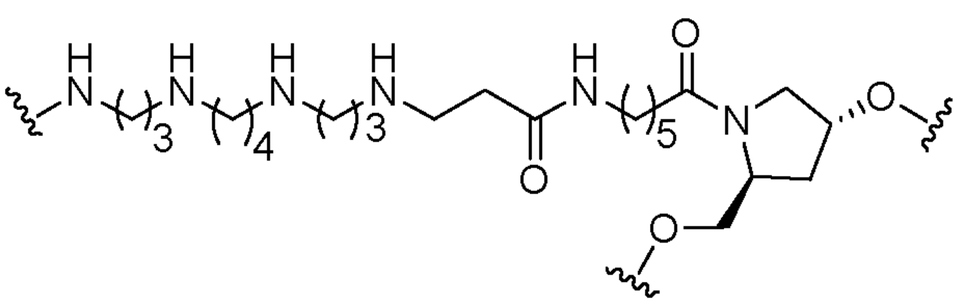
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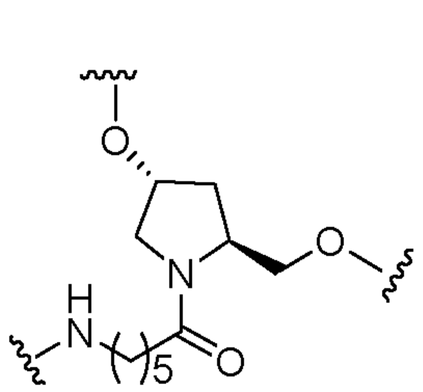
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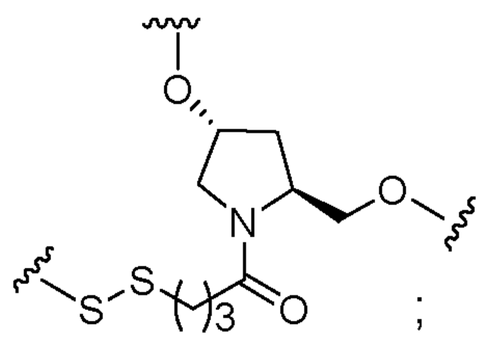
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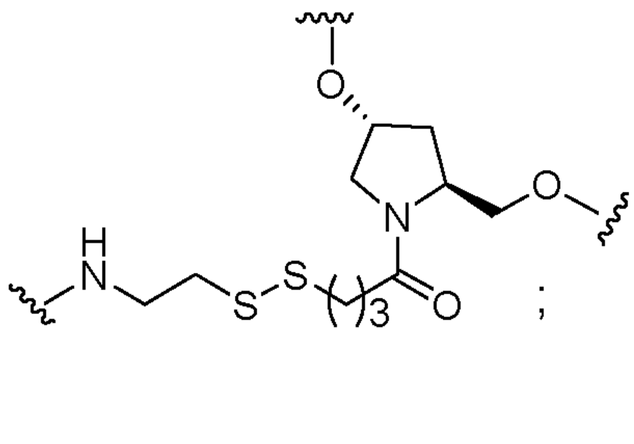
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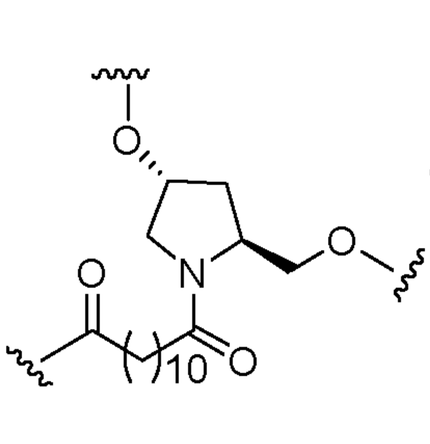
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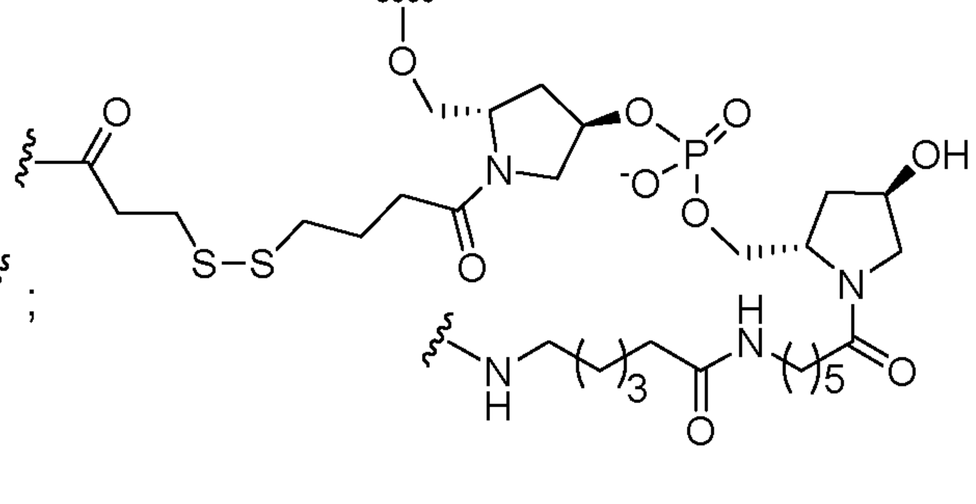
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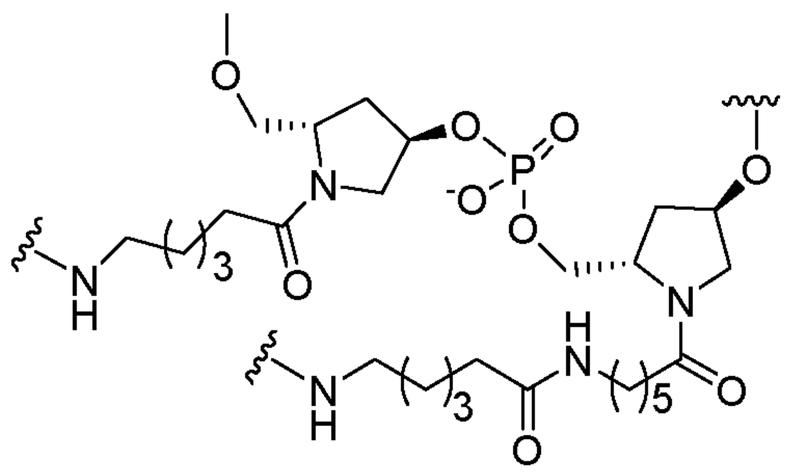

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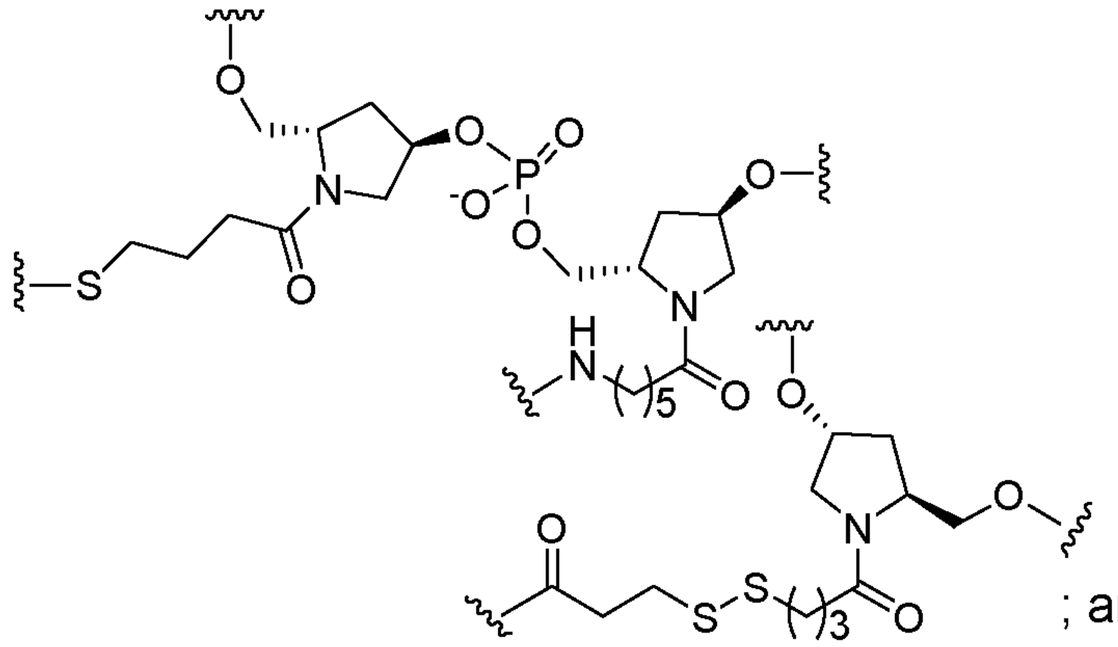

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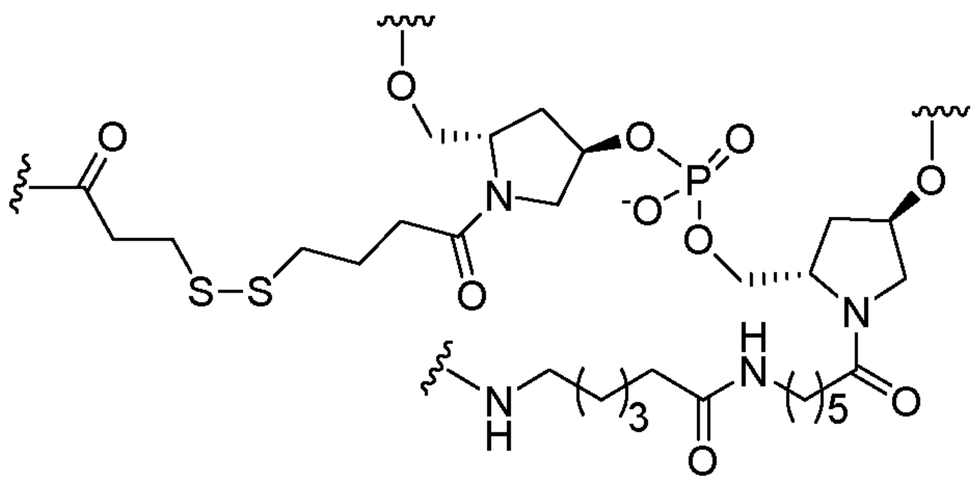

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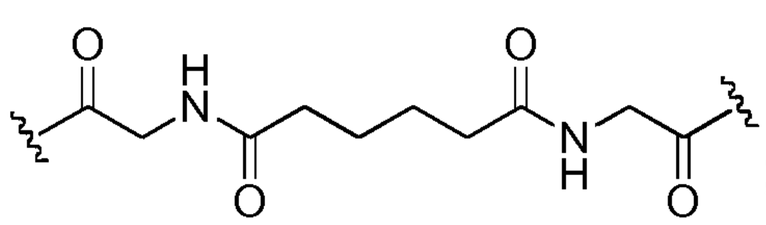
In certain embodiments, a linker has a structure selected from among: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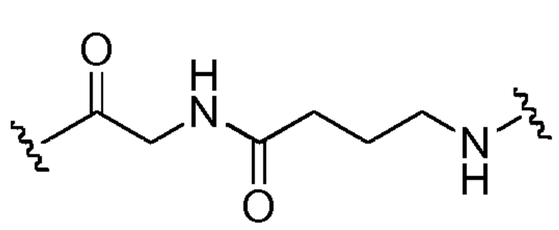
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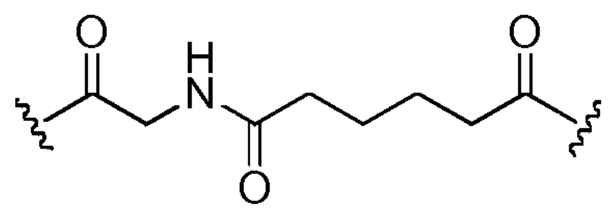
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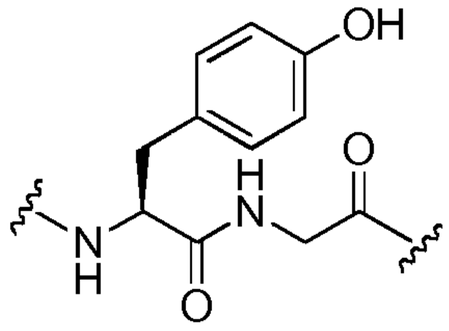
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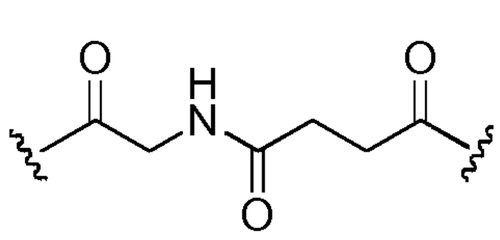
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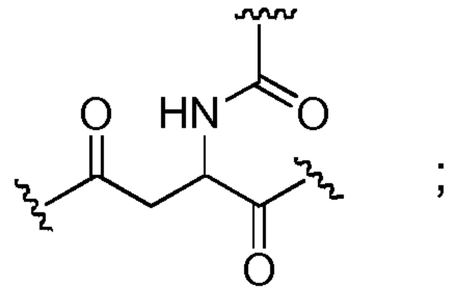
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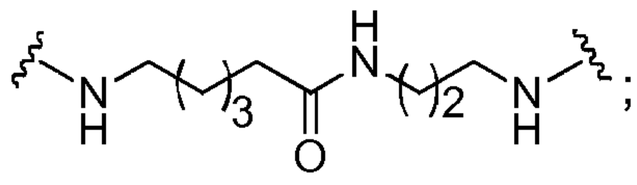
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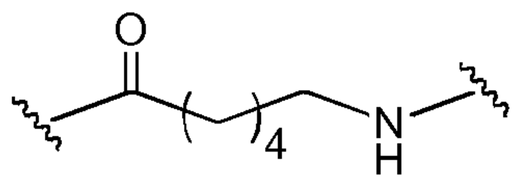
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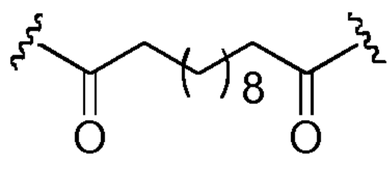
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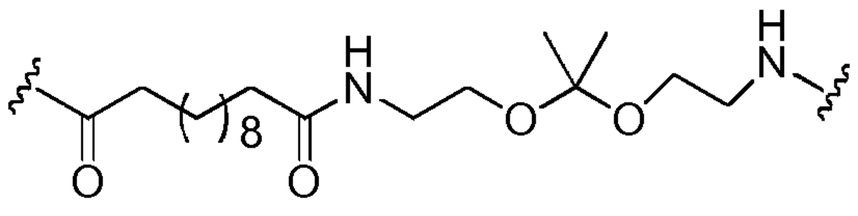
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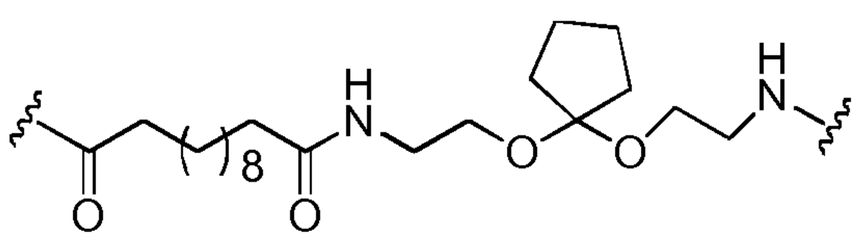
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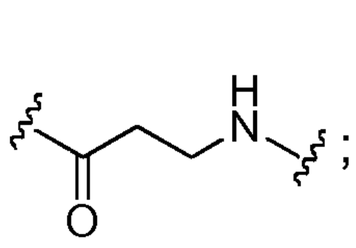
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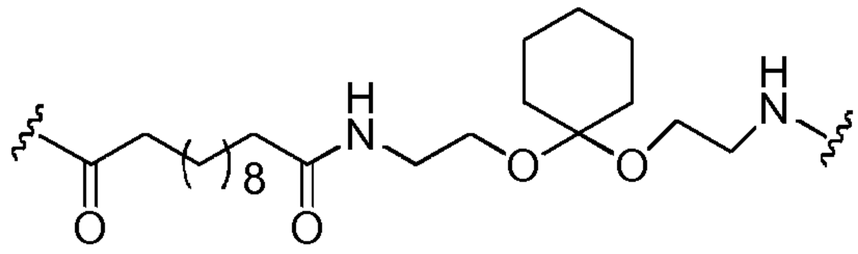
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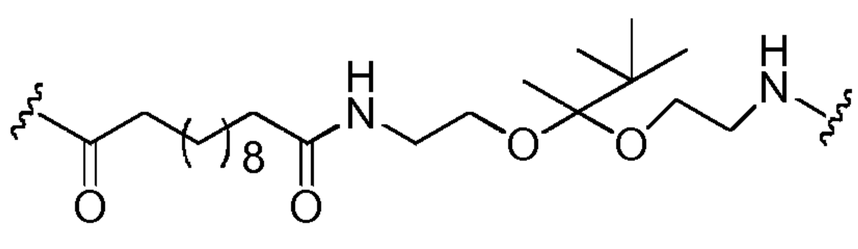
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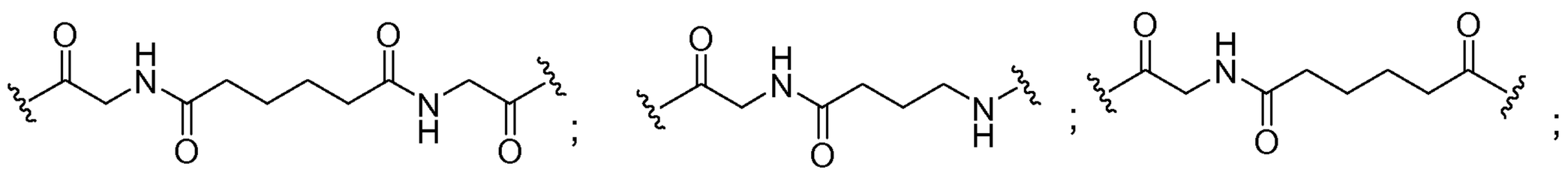
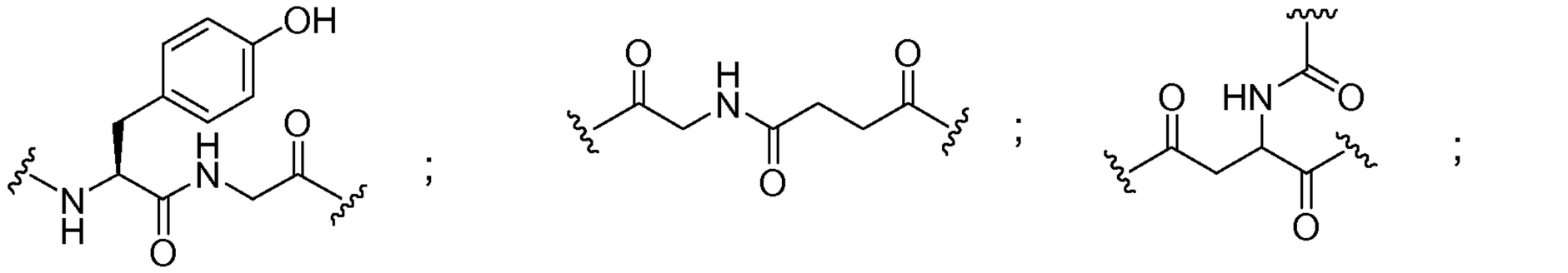
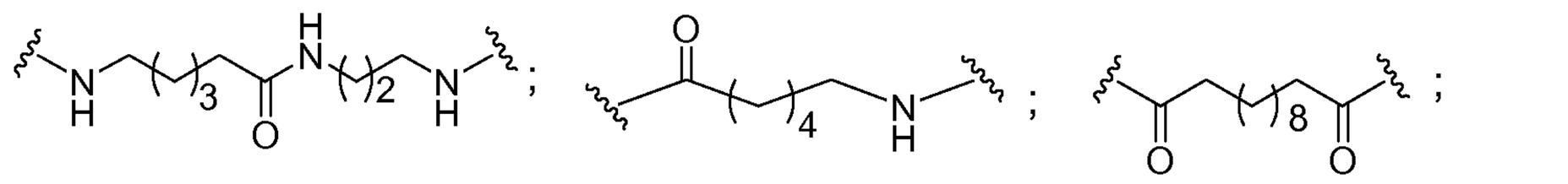
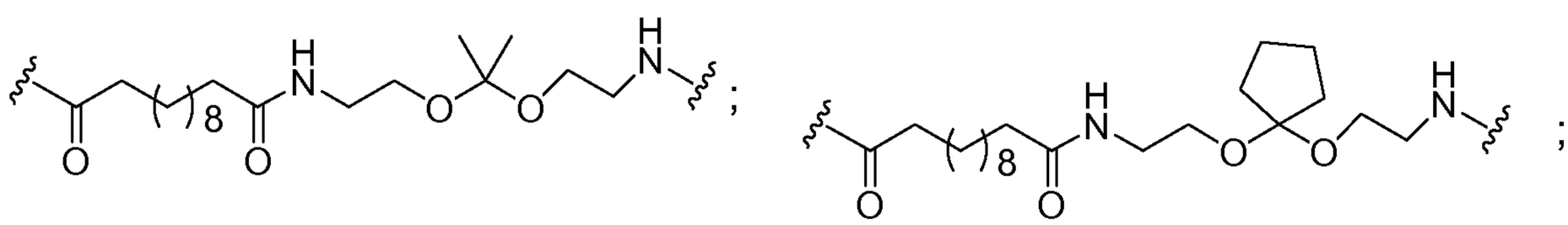
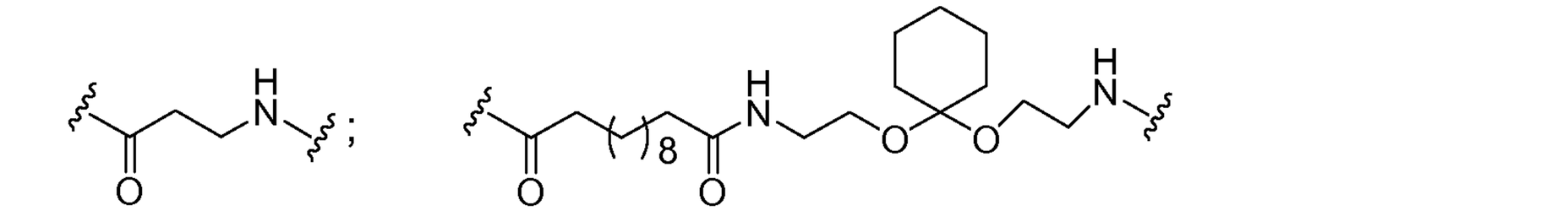
In certain embodiments, a linker has a structure selected from among:

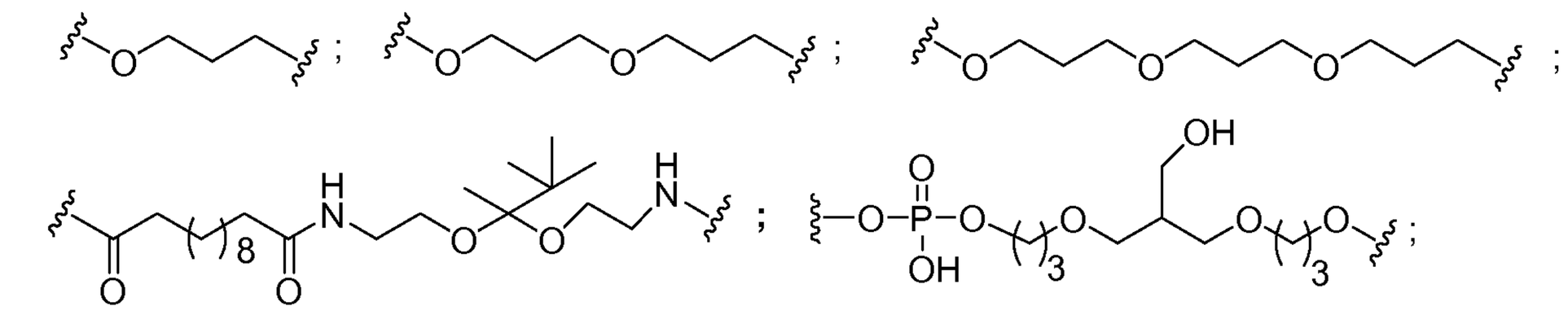


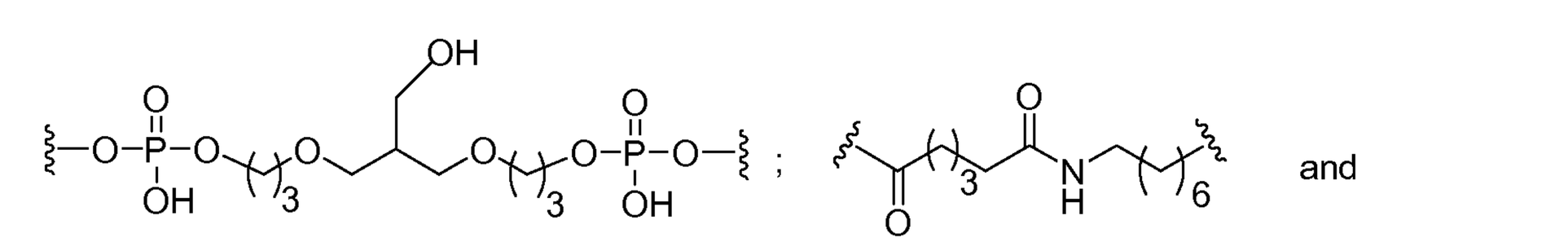


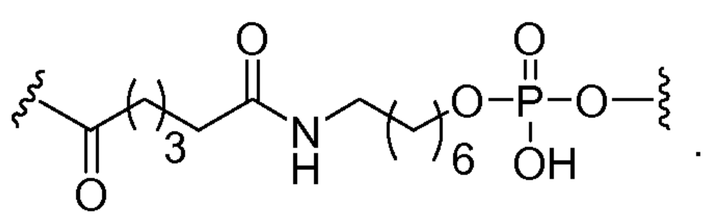








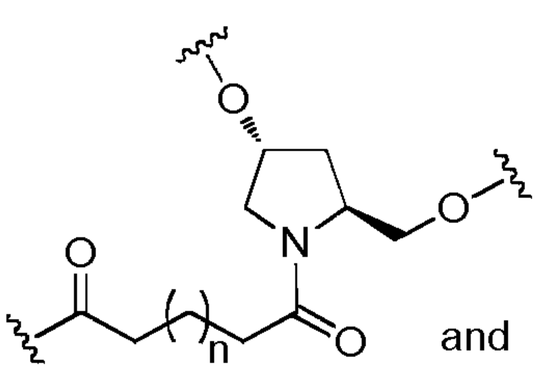
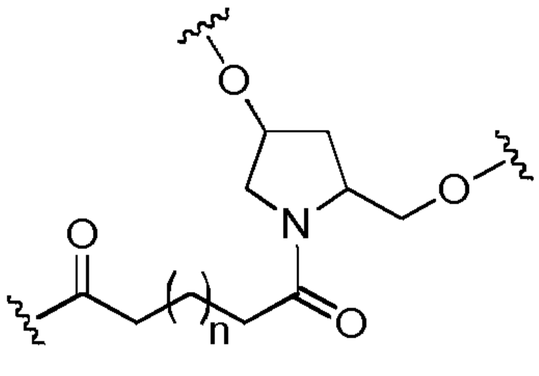




and

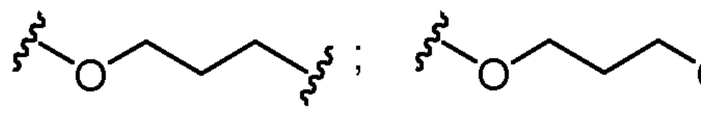
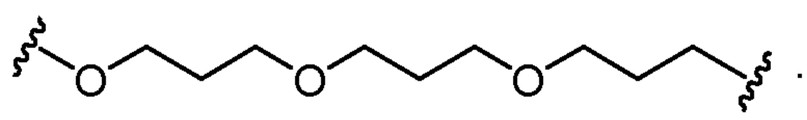
.

In certain embodiments, a linker has a structure selected from among:

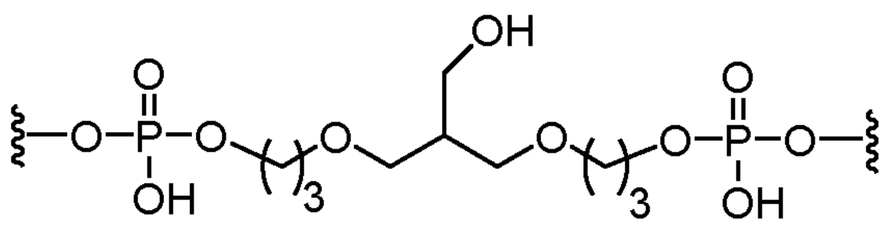
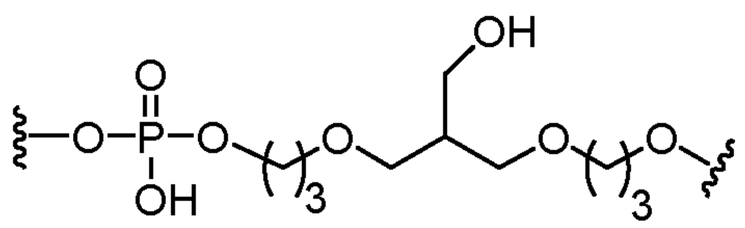

and


wherein n is from 1 to 20.

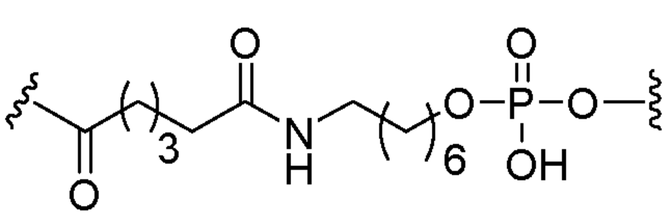
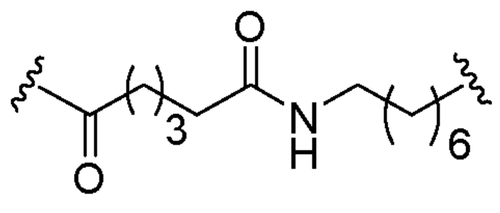
In certain embodiments, a linker has a structure selected from among:


; and

.

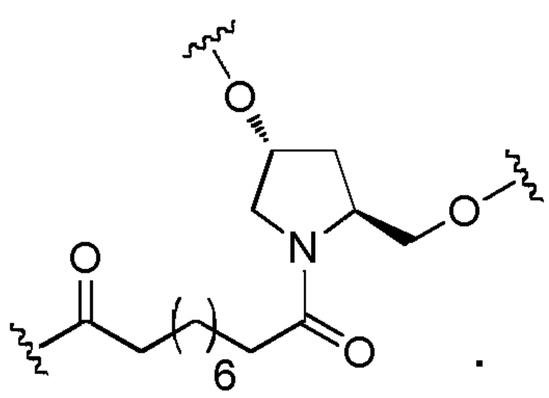
In certain embodiments, a linker has a structure selected from among:


and

.

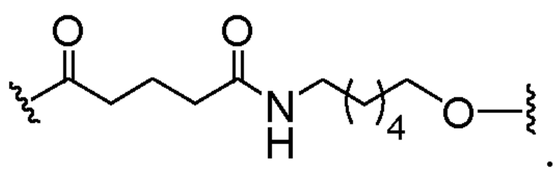
In certain embodiments, a linker has a structure selected from among:


and

.

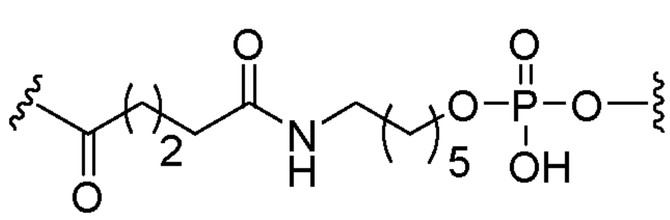
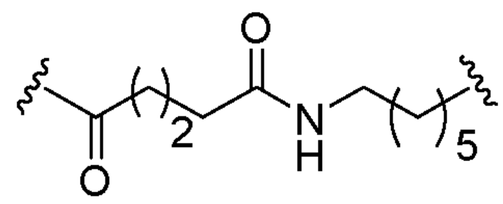
In certain embodiments, the conjugate linker has the structure:


.

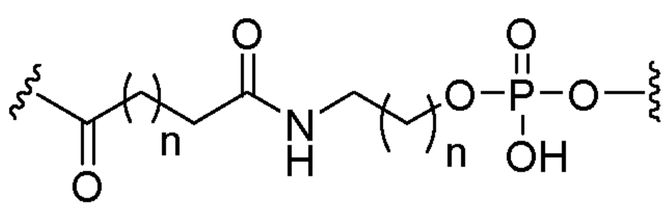
In certain embodiments, the conjugate linker has the structure:


.

In certain embodiments, a linker has a structure selected from among:


and

.

In certain embodiments, a linker has a structure selected from among: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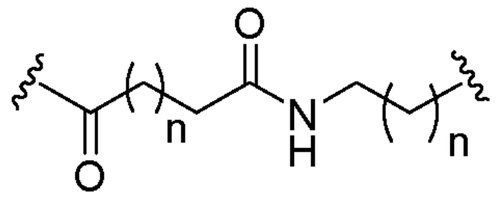

;

wherein each n is independently, 0, 1, 2, 3, 4, 5, 6, or 7.

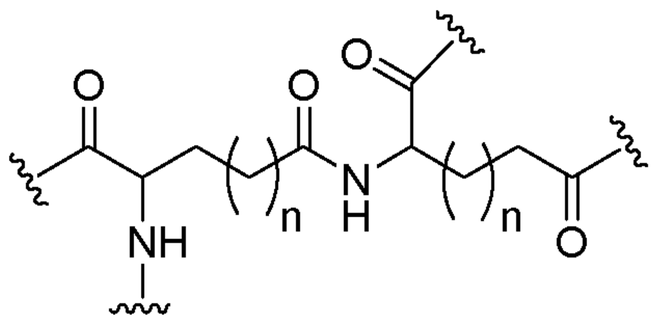
ii. Certain Cell-Targeting Moieties

In certain embodiments, conjugate groups comprise cell-targeting moieties. Certain such cell-targeting moieties increase cellular uptake of antisense compounds. In certain embodiments, cell-targeting moieties comprise a branching group, one or more tether, and one or more ligand. In certain embodiments, cell-targeting moieties comprise a branching group, one or more tether, one or more ligand and one or more cleavable bond.

1. Certain Branching Groups

In certain embodiments, the conjugate groups comprise a targeting moiety comprising a branching group and at least two tethered ligands. In certain embodiments, the branching group attaches the conjugate linker. In certain embodiments, the branching group attaches the cleavable moiety. In certain embodiments, the branching group attaches the antisense oligonucleotide. In certain embodiments, the branching group is covalently attached to the linker and each of the tethered ligands. In certain embodiments, the branching group comprises a branched aliphatic group comprising groups selected from alkyl, amide, disulfide, polyethylene glycol, ether, thioether and hydroxylamino groups. In certain embodiments, the branching group comprises groups selected from alkyl, amide and ether groups. In certain embodiments, the branching group comprises groups selected from alkyl and ether groups. In certain embodiments, the branching group comprises a mono or polycyclic ring system. In certain embodiments, the branching group comprises one or more cleavable bond. In certain embodiments, the conjugate group does not include a branching group.

In certain embodiments, a branching group has a structure selected from among: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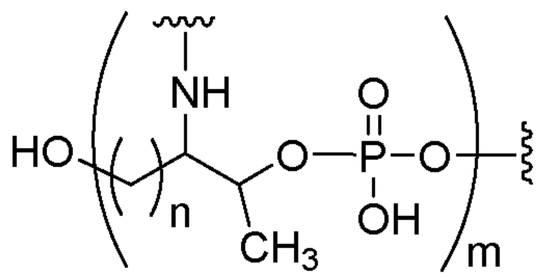
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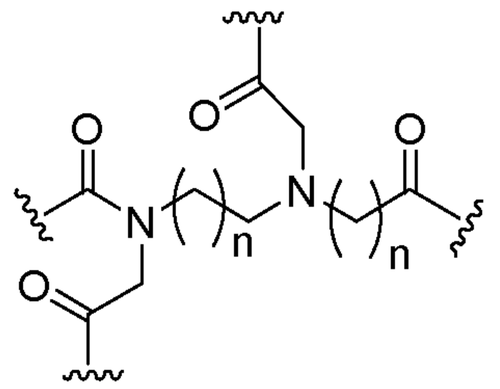
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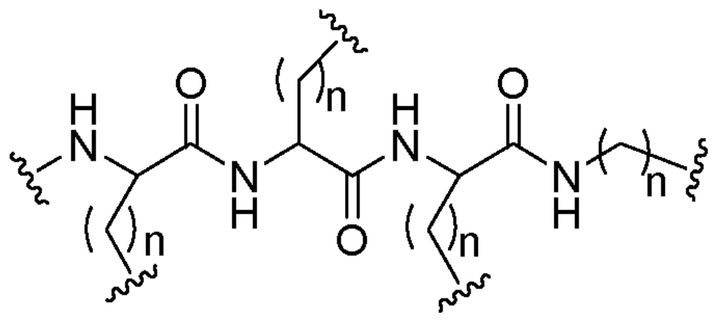
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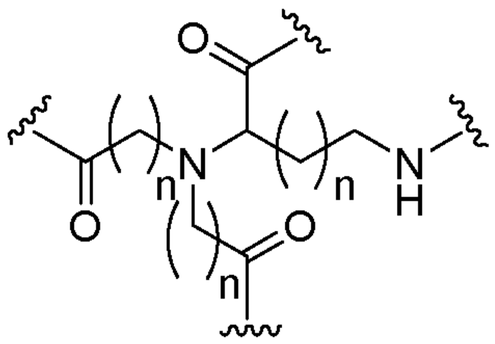
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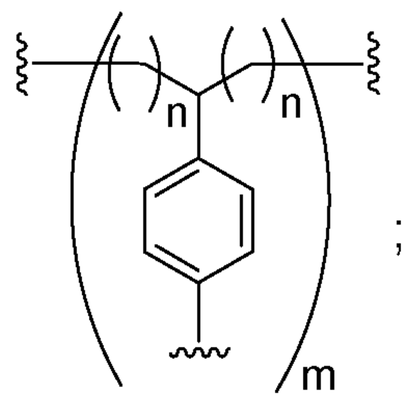
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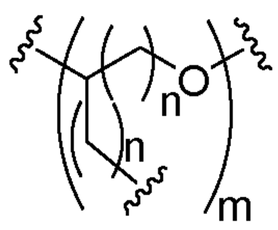
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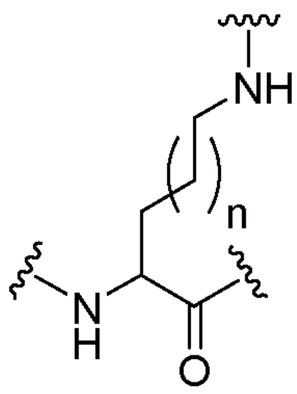
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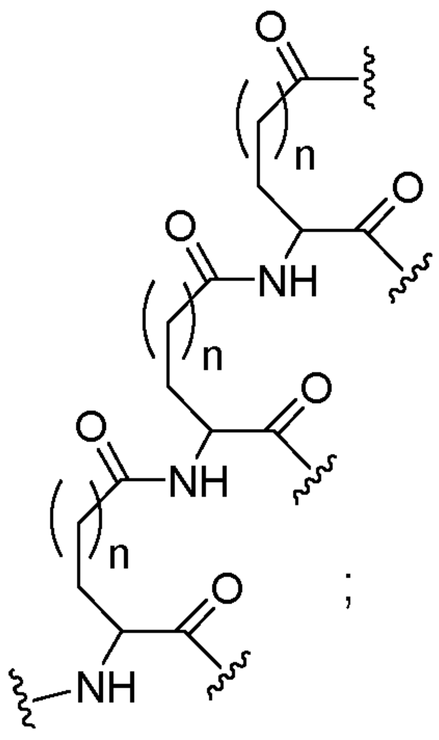
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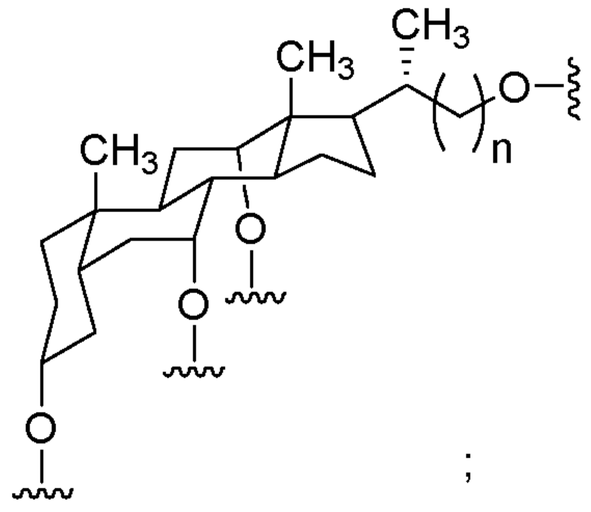
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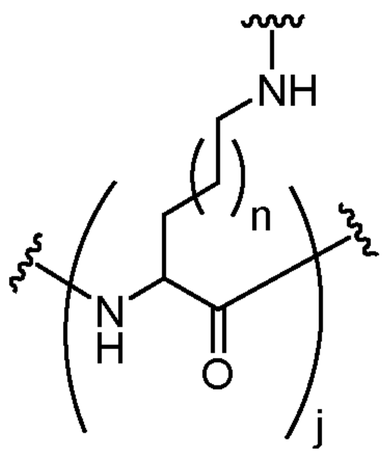
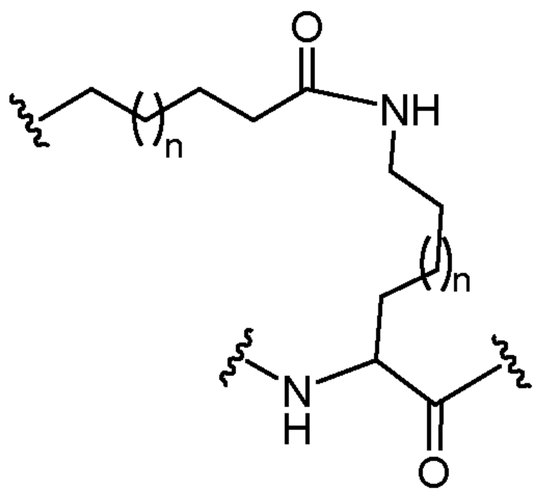
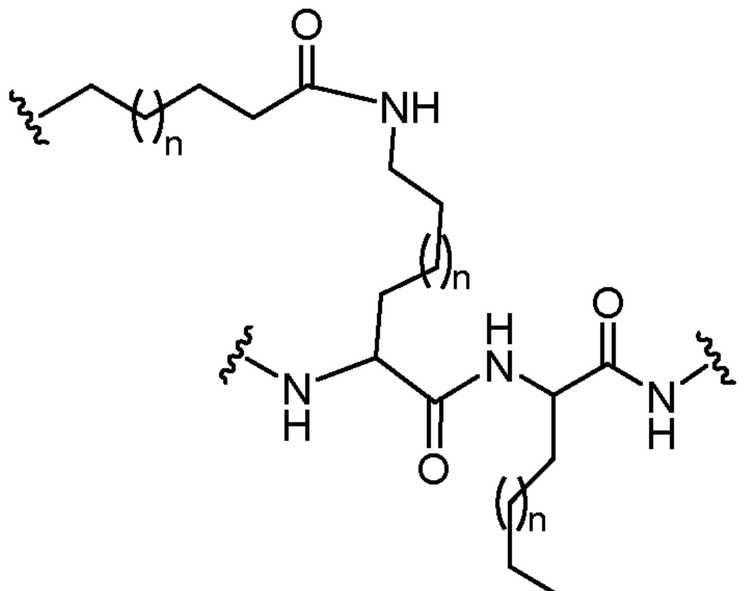
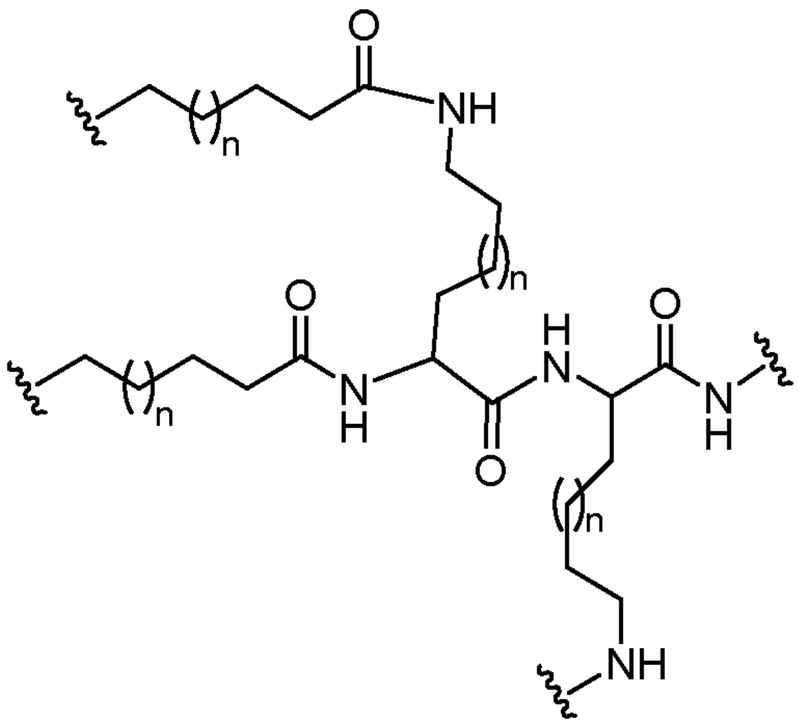
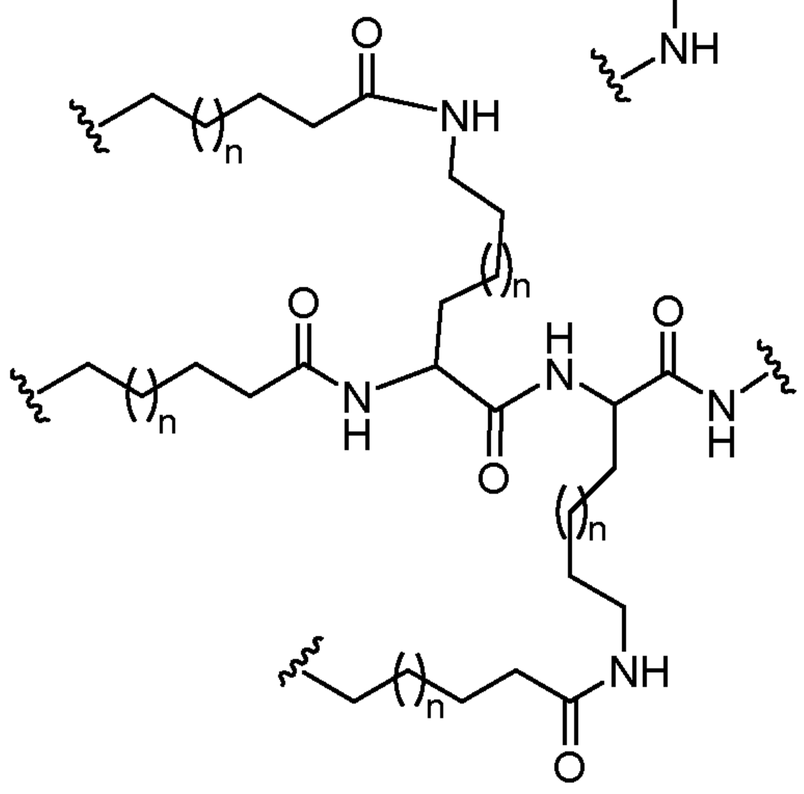
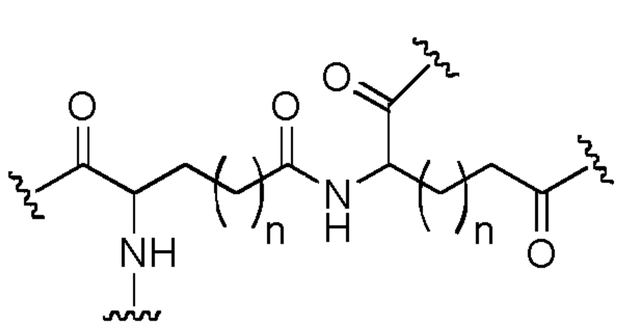






wherein each n is, independently, from 1 to 20;

j is from 1 to 3; and

m is from 2 to 6.

In certain embodiments, a branching group has a structure selected from among: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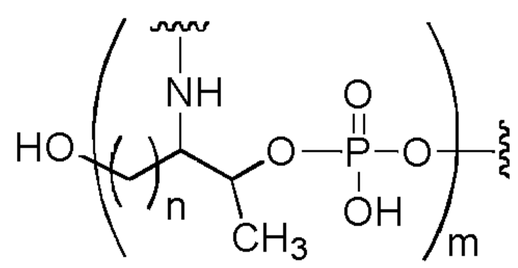
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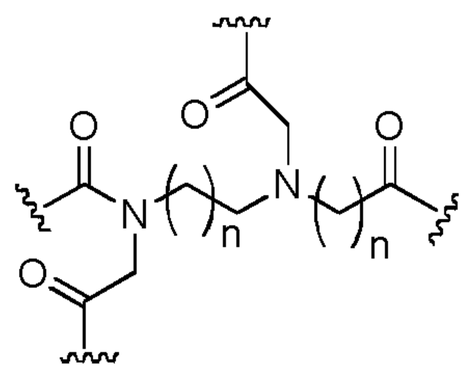
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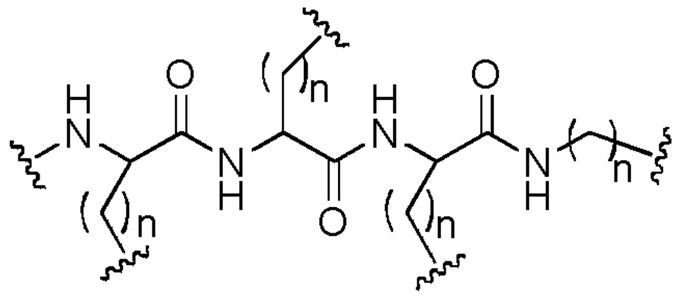
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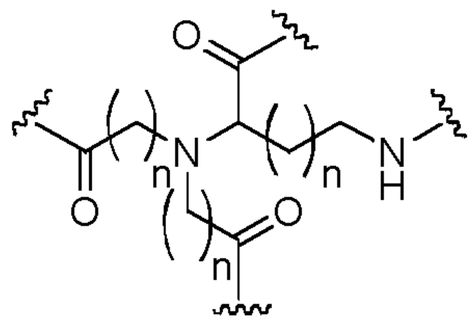
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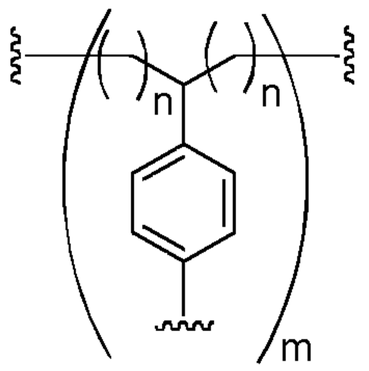
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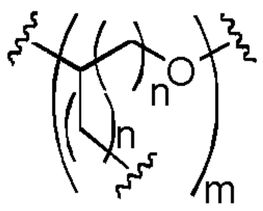
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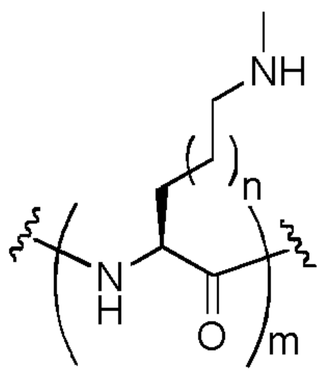
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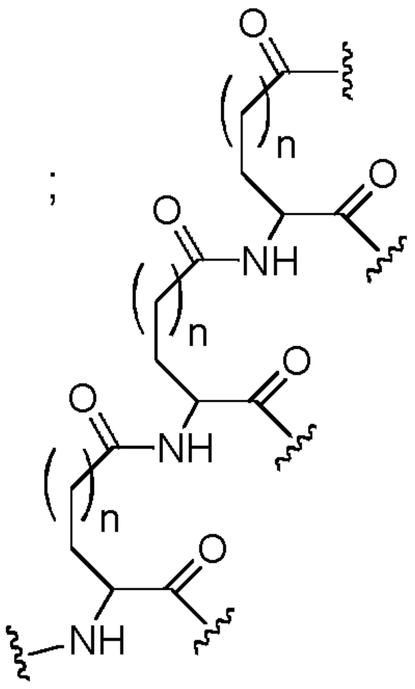
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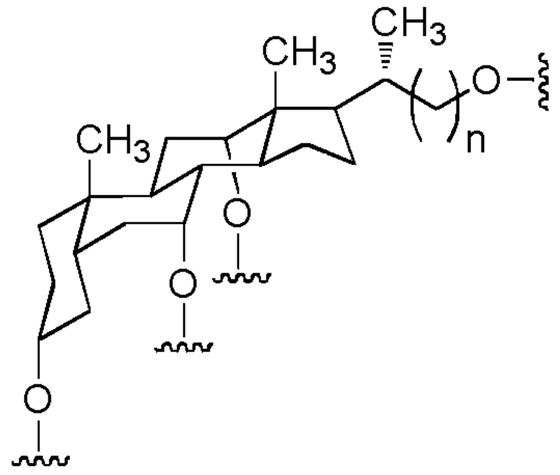
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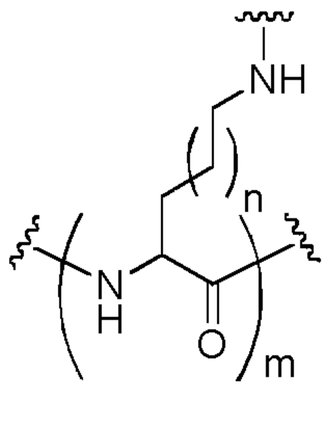
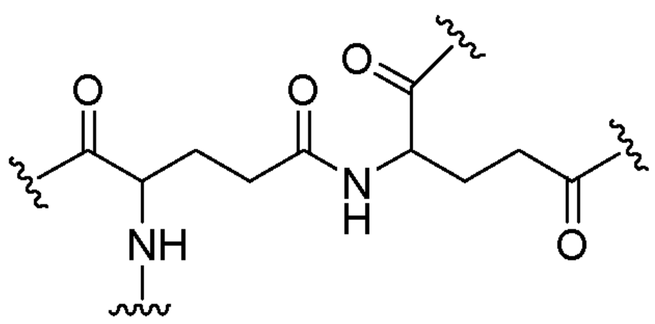


wherein each n is, independently, from 1 to 20; and

m is from 2 to 6.

In certain embodiments, a branching group has a structure selected from among: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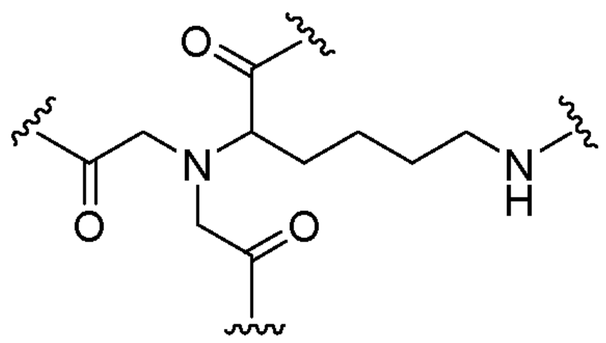
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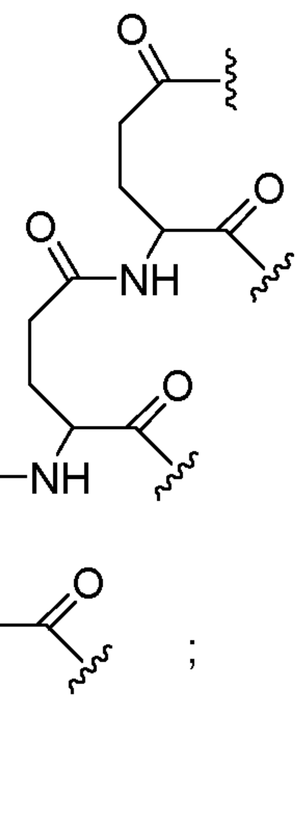
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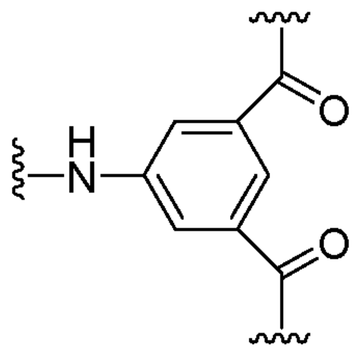
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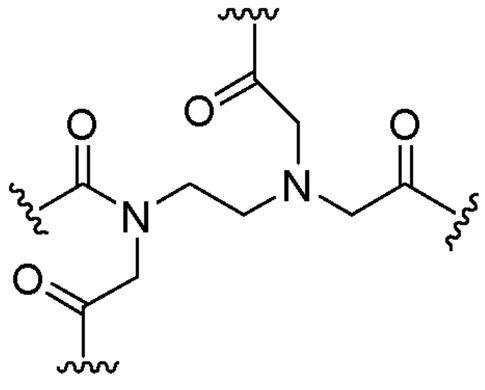
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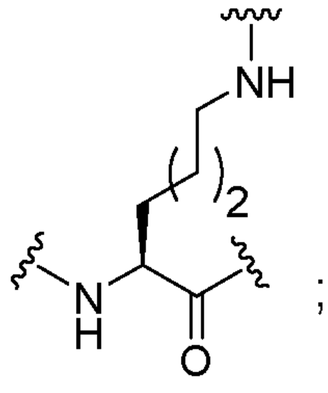
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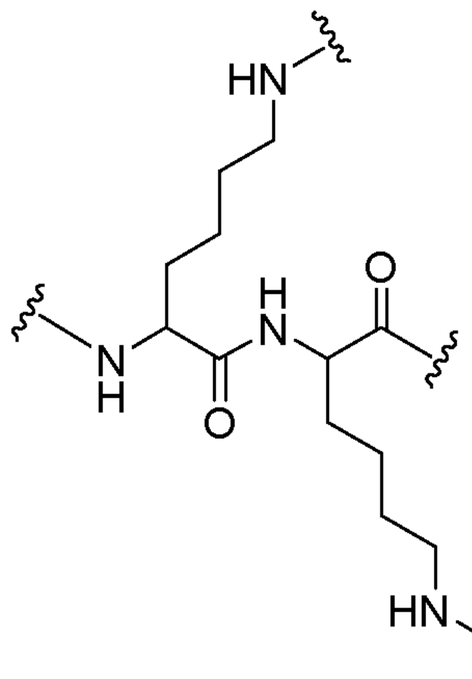
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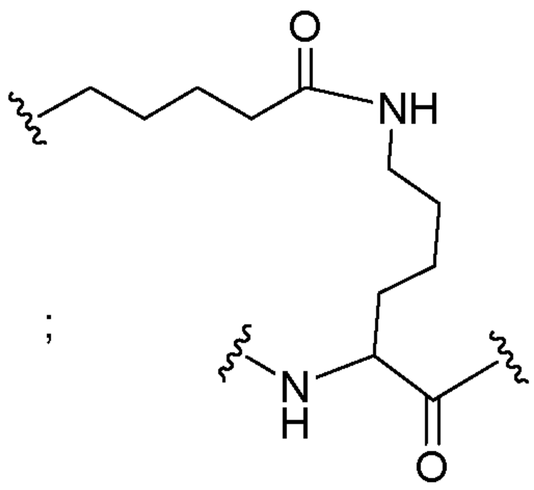
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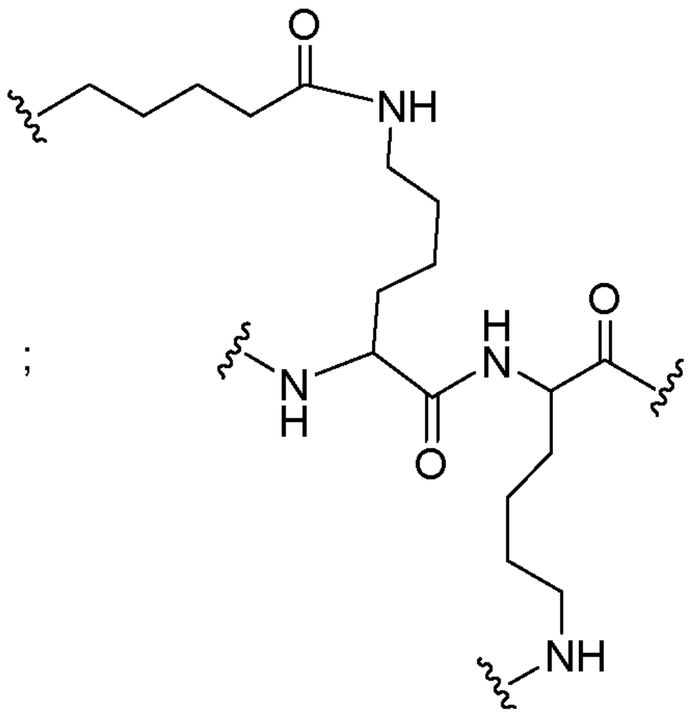
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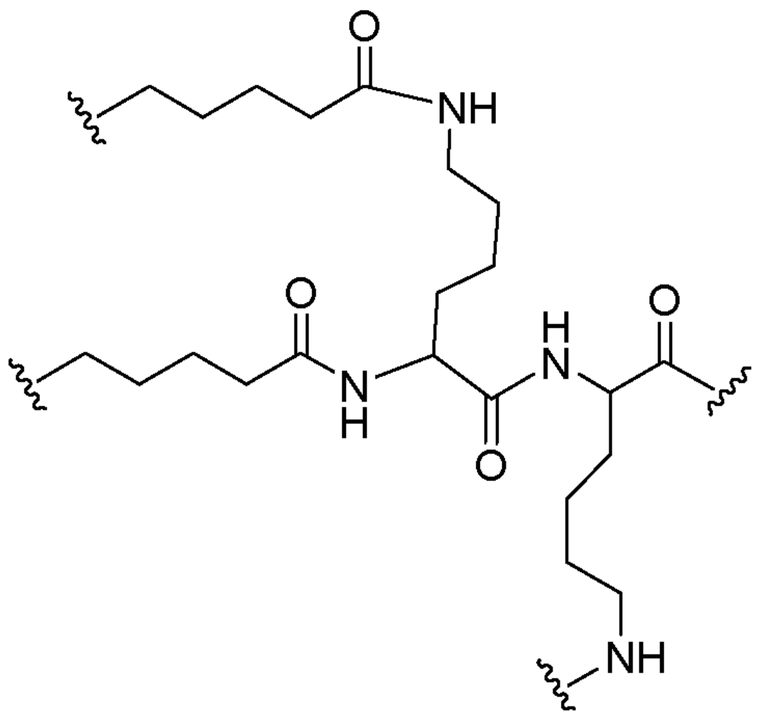
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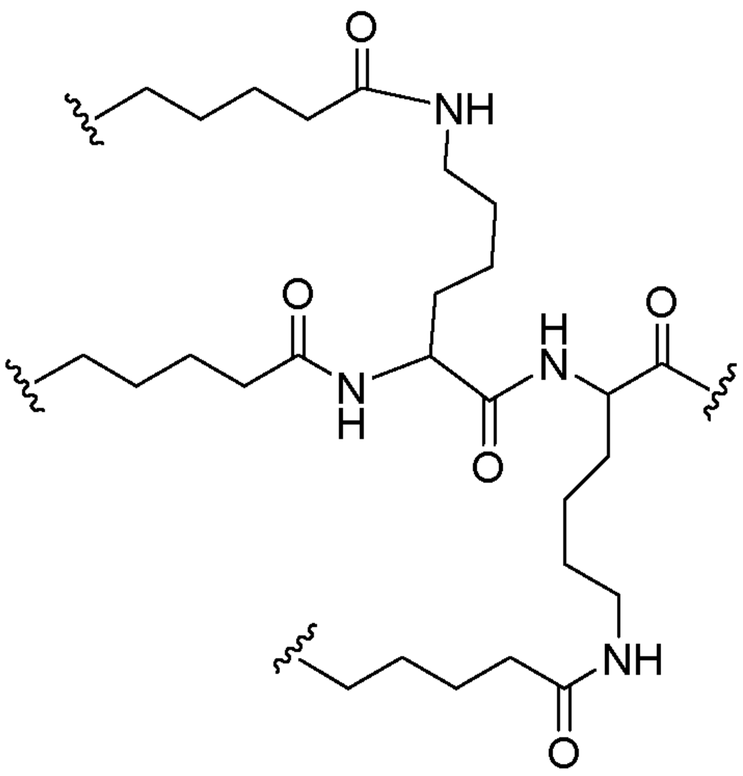
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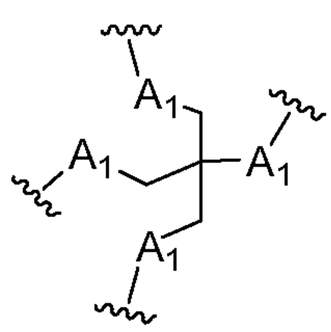
In certain embodiments, a branching group has a structure selected from among: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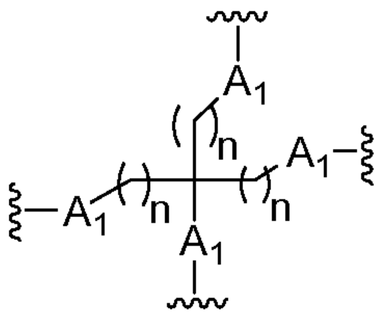
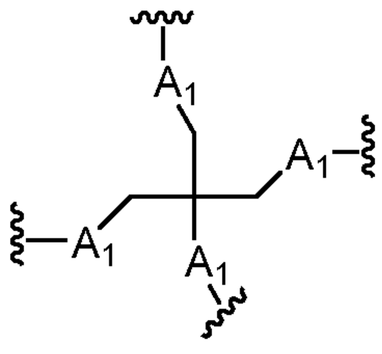


wherein each A_1 is independently, O, S, C=O or NH; and

each n is, independently, from 1 to 20.

In certain embodiments, a branching group has a structure selected from among: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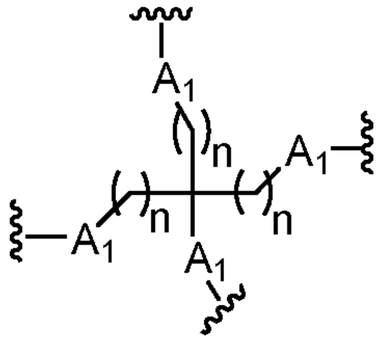
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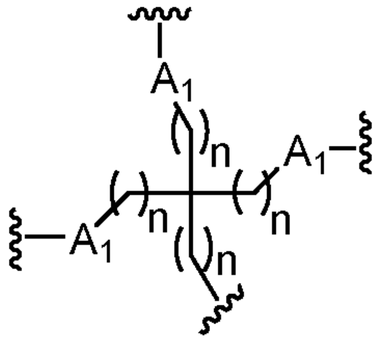
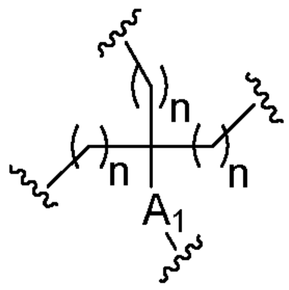


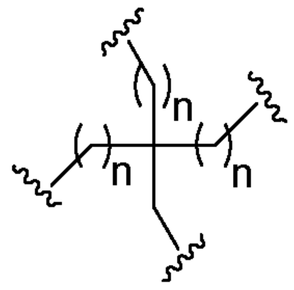
wherein each A_1 is independently, O, S, C=O or NH; and

each n is, independently, from 1 to 20.

In certain embodiments, a branching group has a structure selected from among:



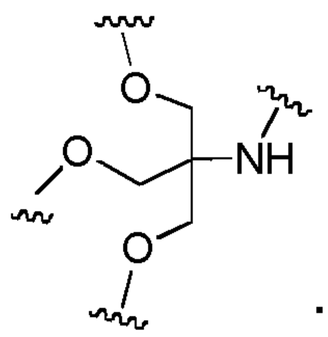
and



wherein A_1 is O, S, C=O or NH; and

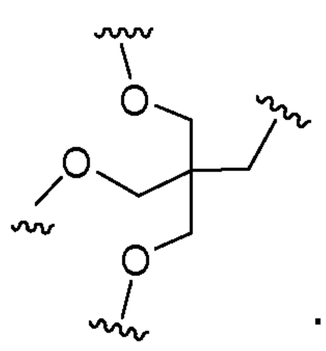
each n is, independently, from 1 to 20.

In certain embodiments, a branching group has a structure selected from among:



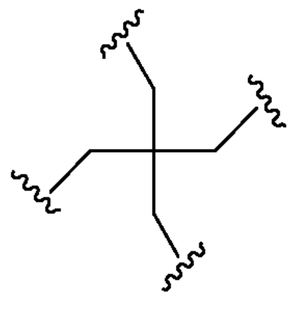
.

In certain embodiments, a branching group has a structure selected from among:



.

In certain embodiments, a branching group has a structure selected from among:



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2. Certain Tethers

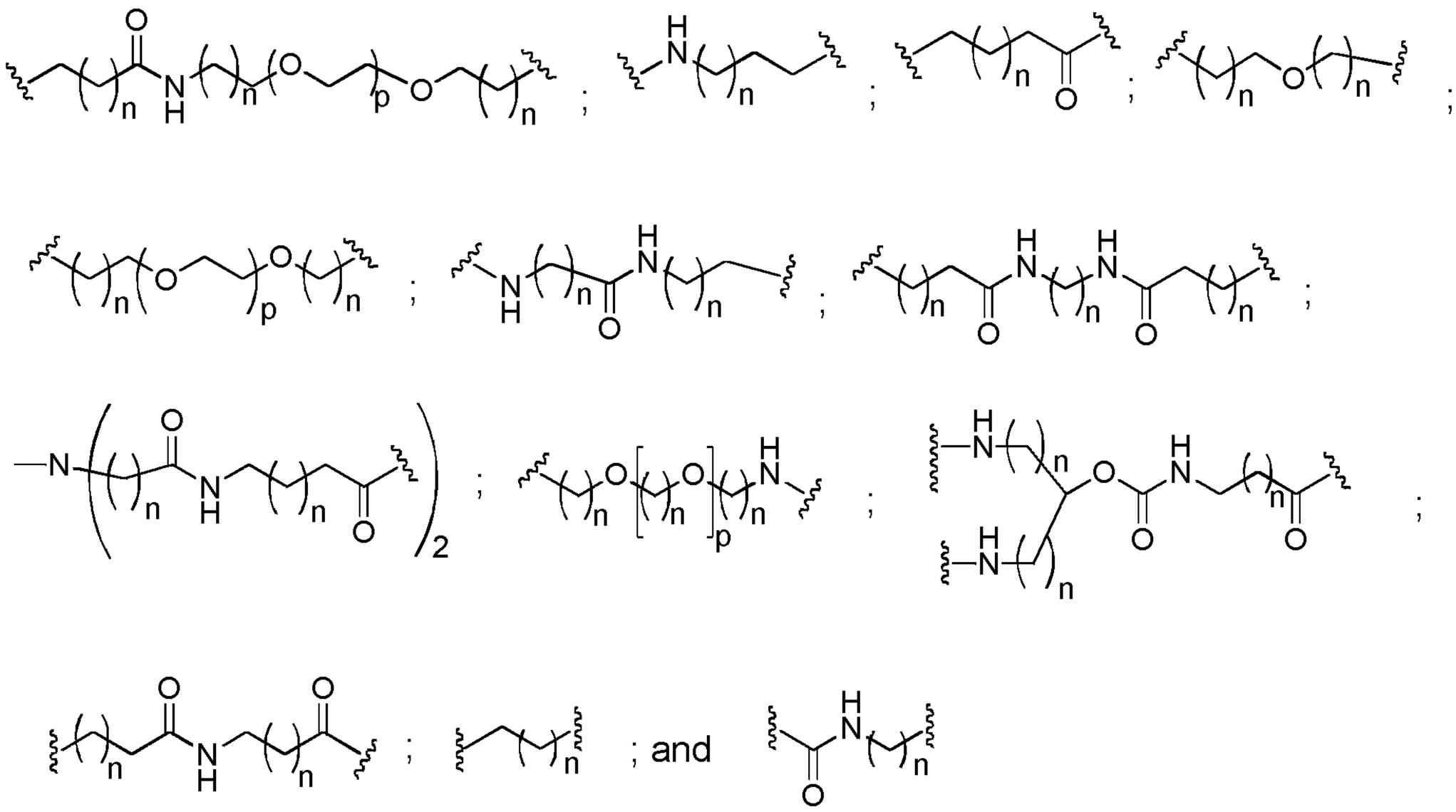
In certain embodiments, conjugate groups comprise one or more tethers covalently attached to the branching group. In certain embodiments, conjugate groups comprise one or more tethers covalently attached to the linking group. In certain embodiments, each tether is a linear aliphatic group comprising one or more groups selected from alkyl, ether, thioether, disulfide, amide and polyethylene glycol groups in any combination. In certain embodiments, each tether is a linear aliphatic group comprising one or more groups selected from alkyl, substituted alkyl, ether, thioether, disulfide, amide, phosphodiester and polyethylene glycol groups in any combination. In certain embodiments, each tether is a linear aliphatic group comprising one or more groups selected from alkyl, ether and amide groups in any combination. In certain embodiments,

each tether is a linear aliphatic group comprising one or more groups selected from alkyl, substituted alkyl, phosphodiester, ether and amide groups in any combination. In certain embodiments, each tether is a linear aliphatic group comprising one or more groups selected from alkyl and phosphodiester in any combination. In certain embodiments, each tether comprises at least one phosphorus linking group or neutral linking group.

In certain embodiments, the tether includes one or more cleavable bond. In certain embodiments, the tether is attached to the branching group through either an amide or an ether group. In certain embodiments, the tether is attached to the branching group through a phosphodiester group. In certain embodiments, the tether is attached to the branching group through a phosphorus linking group or neutral linking group. In certain embodiments, the tether is attached to the branching group through an ether group. In certain embodiments, the tether is attached to the ligand through either an amide or an ether group. In certain embodiments, the tether is attached to the ligand through an ether group. In certain embodiments, the tether is attached to the ligand through either an amide or an ether group. In certain embodiments, the tether is attached to the ligand through an ether group.

In certain embodiments, each tether comprises from about 8 to about 20 atoms in chain length between the ligand and the branching group. In certain embodiments, each tether group comprises from about 10 to about 18 atoms in chain length between the ligand and the branching group. In certain embodiments, each tether group comprises about 13 atoms in chain length.

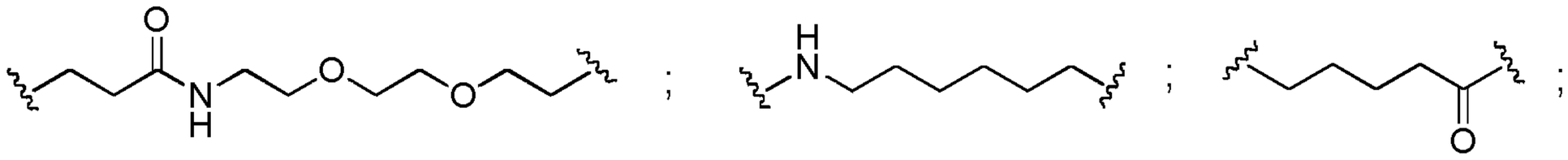
In certain embodiments, a tether has a structure selected from among:



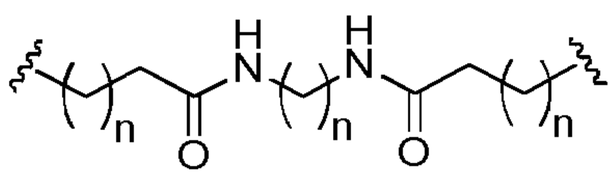
wherein each n is, independently, from 1 to 20; and

each p is from 1 to about 6.

In certain embodiments, a tether has a structure selected from among:

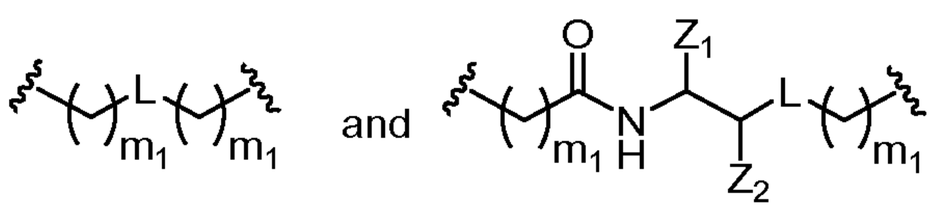


In certain embodiments, a tether has a structure selected from among:



wherein each n is, independently, from 1 to 20.

In certain embodiments, a tether has a structure selected from among:



wherein L is either a phosphorus linking group or a neutral linking group;

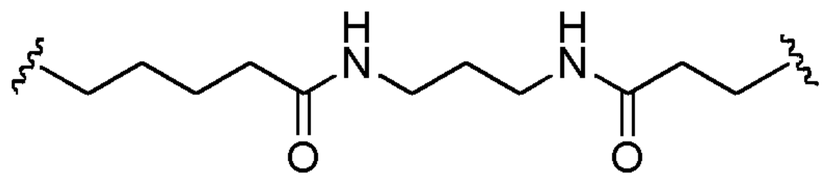
Z_1 is C(=O)O-R_2;

Z_2 is H, C_1-C_6 alkyl or substituted C_1-C_6 alky;

R_2 is H, C_1-C_6 alkyl or substituted C_1-C_6 alky; and

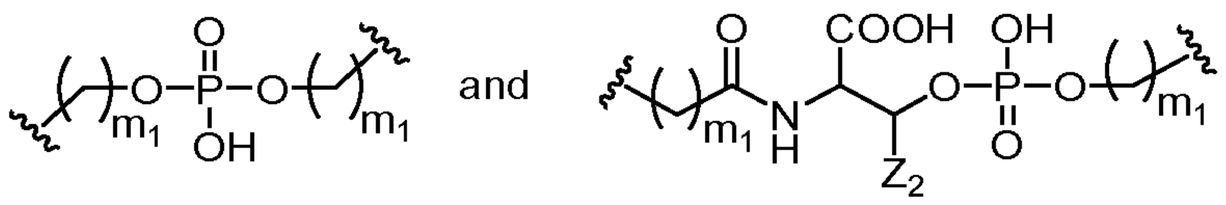
each m_1 is, independently, from 0 to 20 wherein at least one m_1 is greater than 0 for each tether.

In certain embodiments, a tether has a structure selected from among:



.

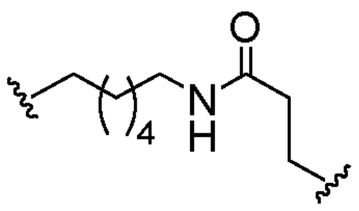
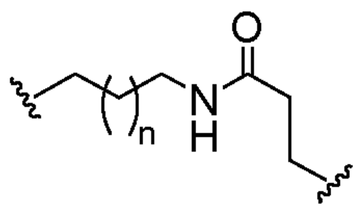
In certain embodiments, a tether has a structure selected from among:



wherein Z_2 is H or CH_3; and

each m_1 is, independently, from 0 to 20 wherein at least one m_1 is greater than 0 for each tether.

In certain embodiments, a tether has a structure selected from among:


, or

; wherein each n is independently, 0, 1, 2, 3, 4, 5, 6, or 7.

In certain embodiments, a tether comprises a phosphorus linking group. In certain embodiments, a tether does not comprise any amide bonds. In certain embodiments, a tether comprises a phosphorus linking group and does not comprise any amide bonds.

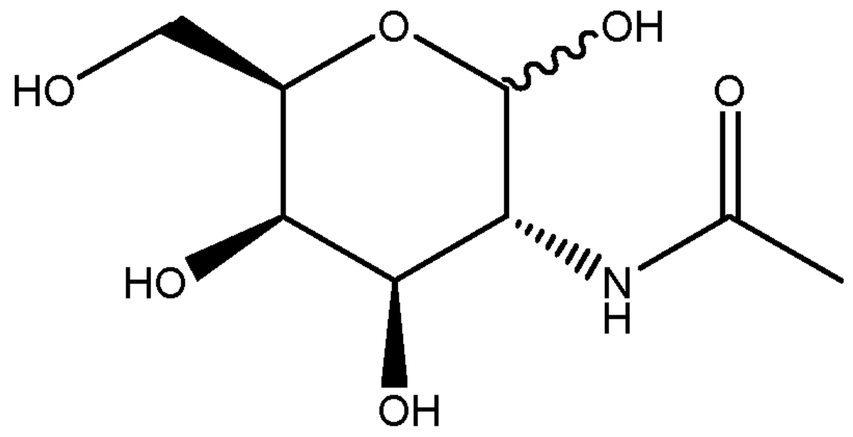
3. Certain Ligands

In certain embodiments, the present disclosure provides ligands wherein each ligand is covalently attached to a tether. In certain embodiments, each ligand is selected to have an affinity for at least one type of receptor on a target cell. In certain embodiments, ligands are selected that have an affinity for at least one type of receptor on the surface of a mammalian liver cell. In certain embodiments, ligands are selected that have an affinity for the hepatic asialoglycoprotein receptor (ASGP-R). In certain embodiments, each ligand is a carbohydrate. In certain embodiments, each ligand is, independently selected from galactose, N-acetyl galactoseamine, mannose, glucose, glucosamone and fucose. In certain embodiments, each ligand is N-acetyl galactoseamine (GalNAc). In certain embodiments, the targeting moiety comprises 2 to 6 ligands. In certain embodiments, the targeting moiety comprises 3 ligands. In certain embodiments, the targeting moiety comprises 3 N-acetyl galactoseamine ligands.

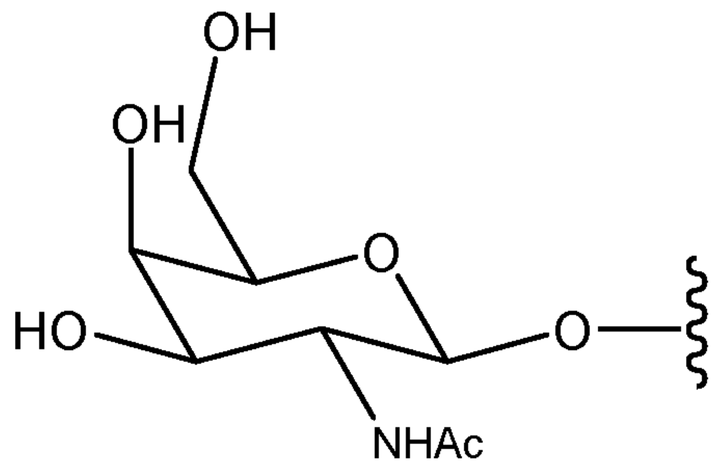
In certain embodiments, the ligand is a carbohydrate, carbohydrate derivative, modified carbohydrate, multivalent carbohydrate cluster, polysaccharide, modified polysaccharide, or polysaccharide derivative. In certain embodiments, the ligand is an amino sugar or a thio sugar. For example, amino sugars may be selected from any number of compounds known in the art, for example glucosamine, sialic acid, α-D-galactosamine, N-Acetylgalactosamine, 2-acetamido-2-deoxy-D-galactopyranose (GalNAc), 2-Amino-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-β-D-glucopyranose (β-muramic acid), 2-Deoxy-2-methylamino-L-glucopyranose, 4,6-Dideoxy-4-formamido-2,3-di-*O*-methyl-D-mannopyranose, 2-Deoxy-2-sulfoamino-D-glucopyranose and *N*-sulfo-D-glucosamine, and *N*-Glycoloyl-α-neuraminic acid. For example, thio sugars may be selected from the group consisting of 5-Thio-β-D-glucopyranose, Methyl 2,3,4-tri-*O*-acetyl-1-thio-6-*O*-trityl-α-D-glucopyranoside, 4-Thio-β-D-galactopyranose, and ethyl 3,4,6,7-tetra-*O*-acetyl-2-deoxy-1,5-dithio-α-D-*gluco*-heptopyranoside.

In certain embodiments, "GalNac" or "Gal-NAc" refers to 2-(Acetylamino)-2-deoxy-D-galactopyranose, commonly referred to in the literature as N-acetyl galactosamine. In certain embodiments, "N-acetyl galactosamine" refers to 2-(Acetylamino)-2-deoxy-D-galactopyranose. In certain embodiments, "GalNac" or "Gal-NAc" refers to 2-(Acetylamino)-2-deoxy-D-galactopyranose. In certain embodiments,

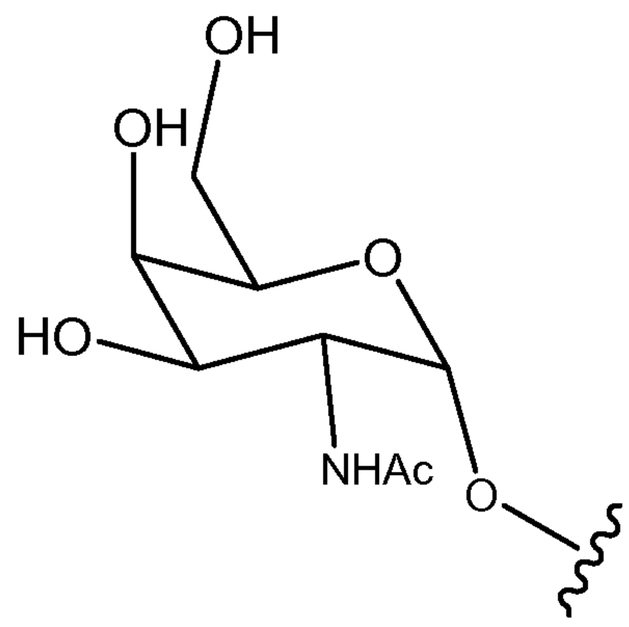
"GalNac" or "Gal-NAc" refers to 2-(Acetylamino)-2-deoxy-D-galactopyranose, which includes both the β-form: 2-(Acetylamino)-2-deoxy-β-D-galactopyranose and α-form: 2-(Acetylamino)-2-deoxy-D-galactopyranose. In certain embodiments, both the β-form: 2-(Acetylamino)-2-deoxy-β-D-galactopyranose and α-form: 2-(Acetylamino)-2-deoxy-D-galactopyranose may be used interchangeably. Accordingly, in structures in which one form is depicted, these structures are intended to include the other form as well. For example, where the structure for an α-form: 2-(Acetylamino)-2-deoxy-D-galactopyranose is shown, this structure is intended to include the other form as well. In certain embodiments, In certain preferred embodiments, the β-form 2-(Acetylamino)-2-deoxy-D-galactopyranose is the preferred embodiment.



2-(Acetylamino)-2-deoxy-D-galactopyranose

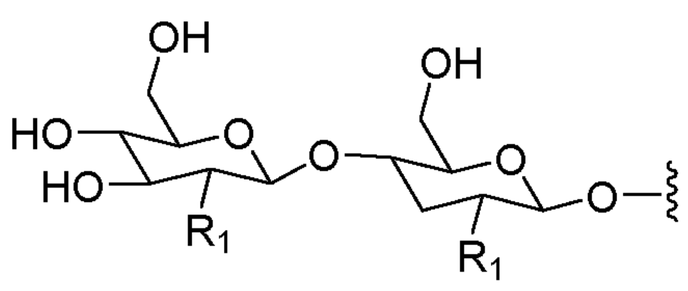
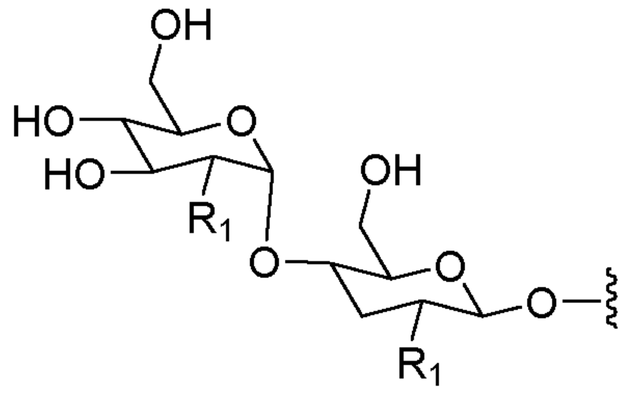


2-(Acetylamino)-2-deoxy-β-D-galactopyranose



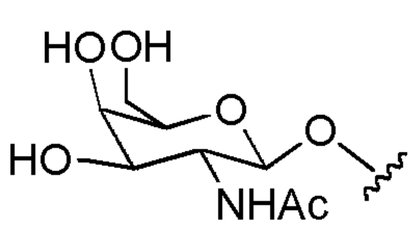
2-(Acetylamino)-2-deoxy-α-D-galactopyranose

In certain embodiments one or more ligand has a structure selected from among:

wherein each R_1 is selected from OH and NHCOOH.

In certain embodiments one or more ligand has a structure selected from among: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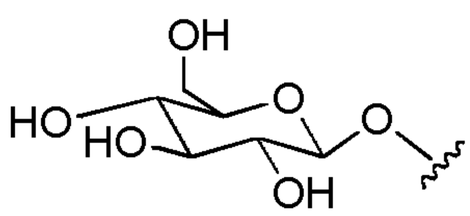
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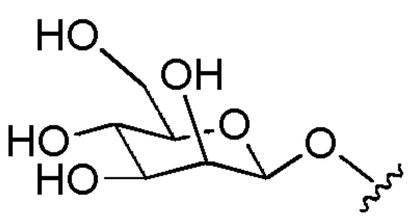
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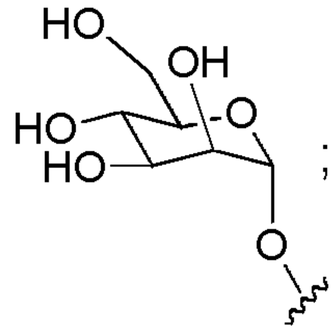
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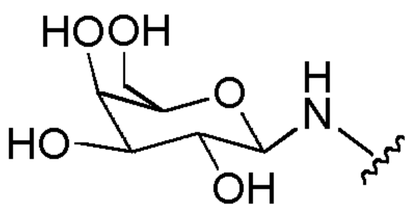
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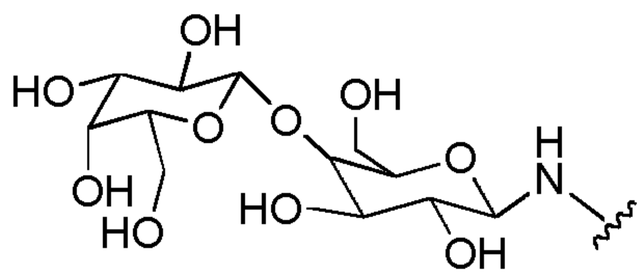
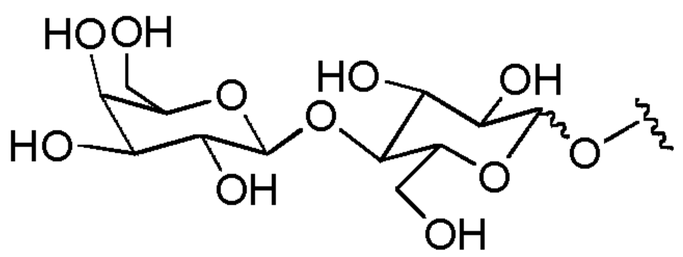
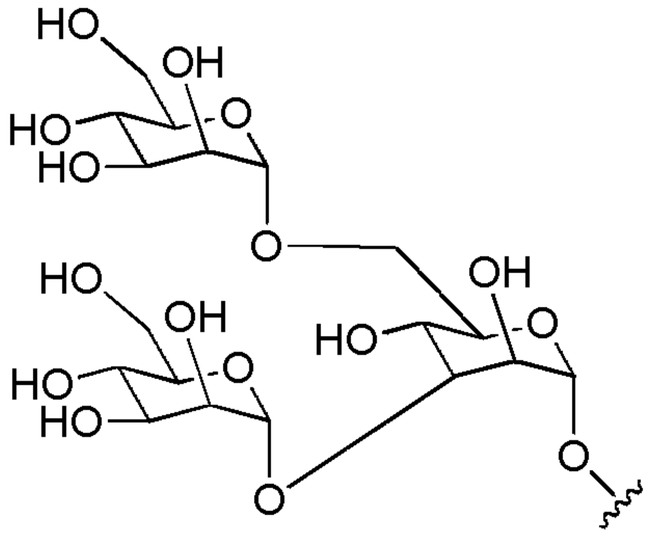
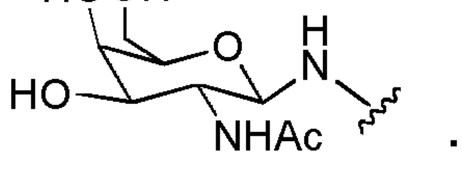
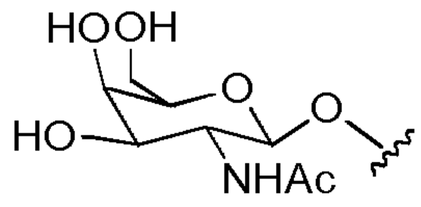

;

; and

.

In certain embodiments one or more ligand has a structure selected from among:


.

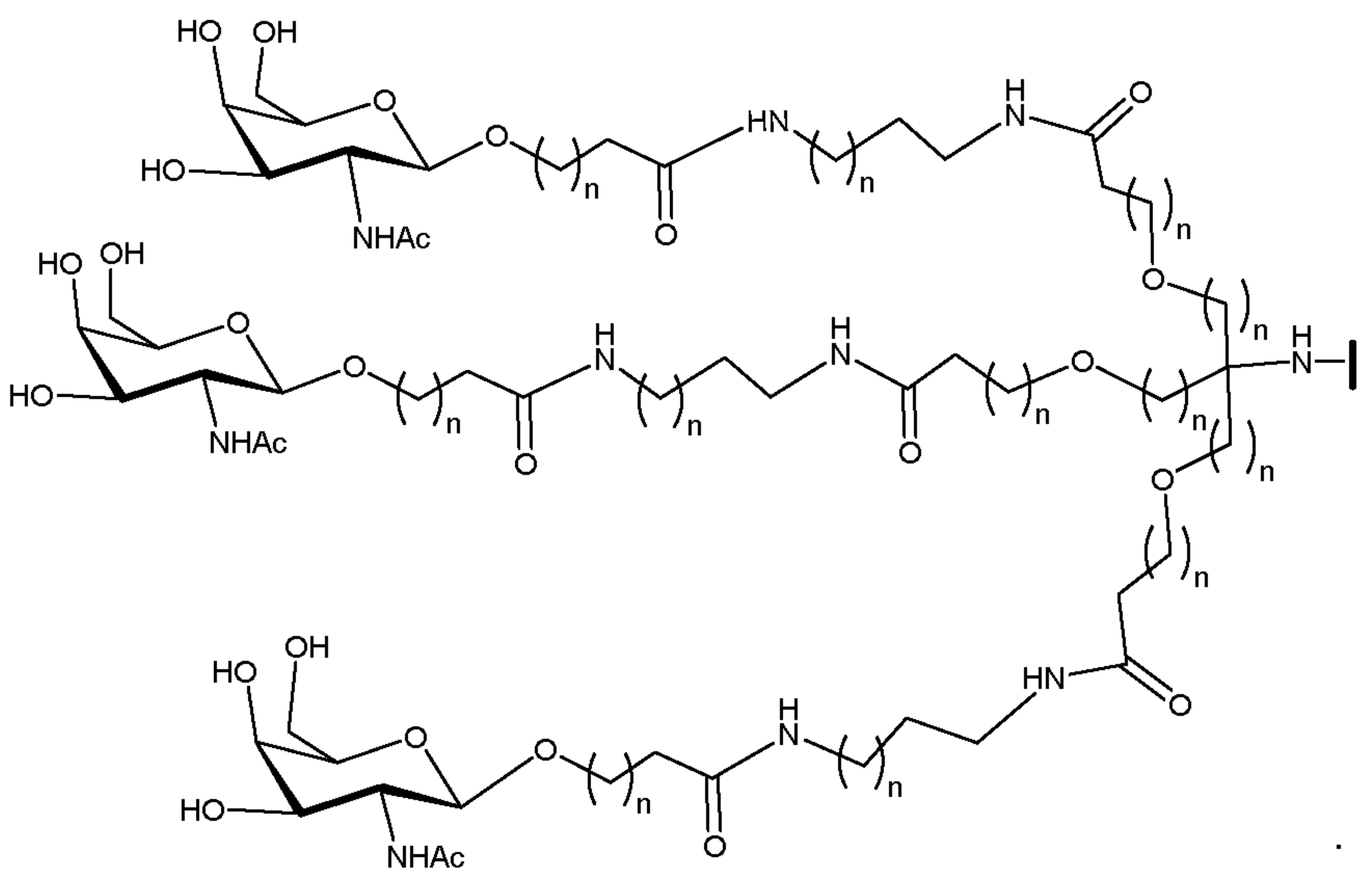
In certain embodiments one or more ligand has a structure selected from among:



.

iii. Certain Conjugates

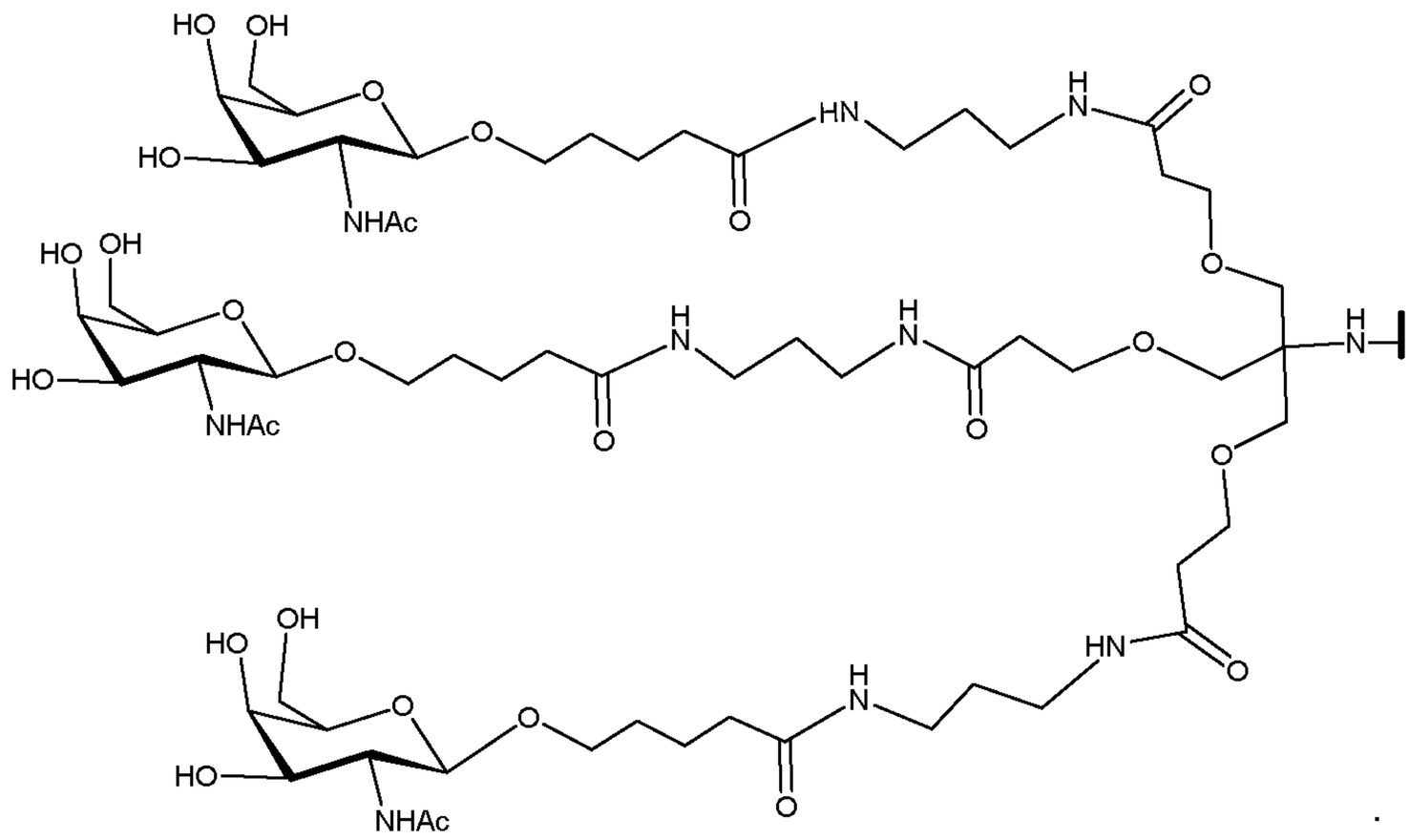
In certain embodiments, conjugate groups comprise the structural features above. In certain such embodiments, conjugate groups comprise the following structure:



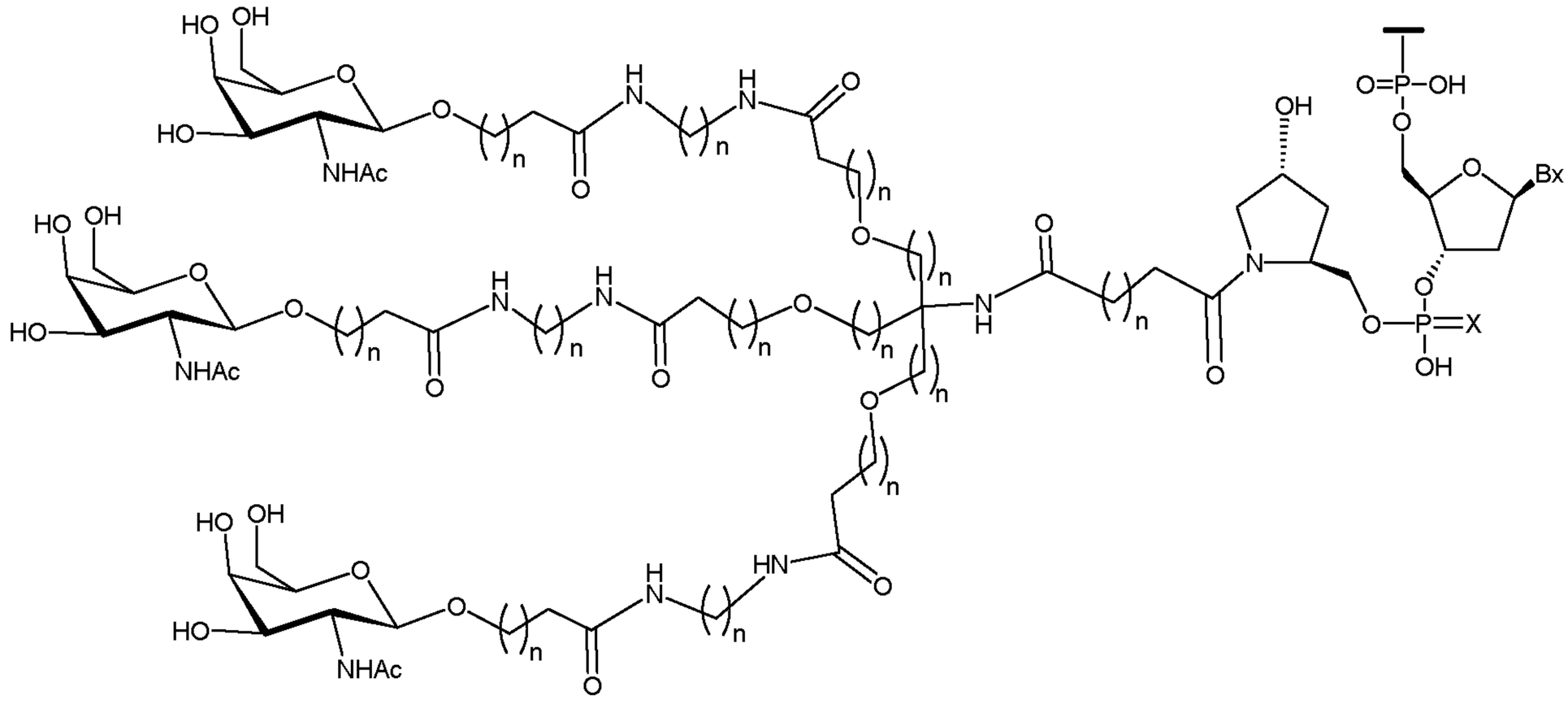
.

wherein each n is, independently, from 1 to 20.

In certain such embodiments, conjugate groups comprise the following structure:



In certain such embodiments, conjugate groups have the following structure:



wherein each n is, independently, from 1 to 20;

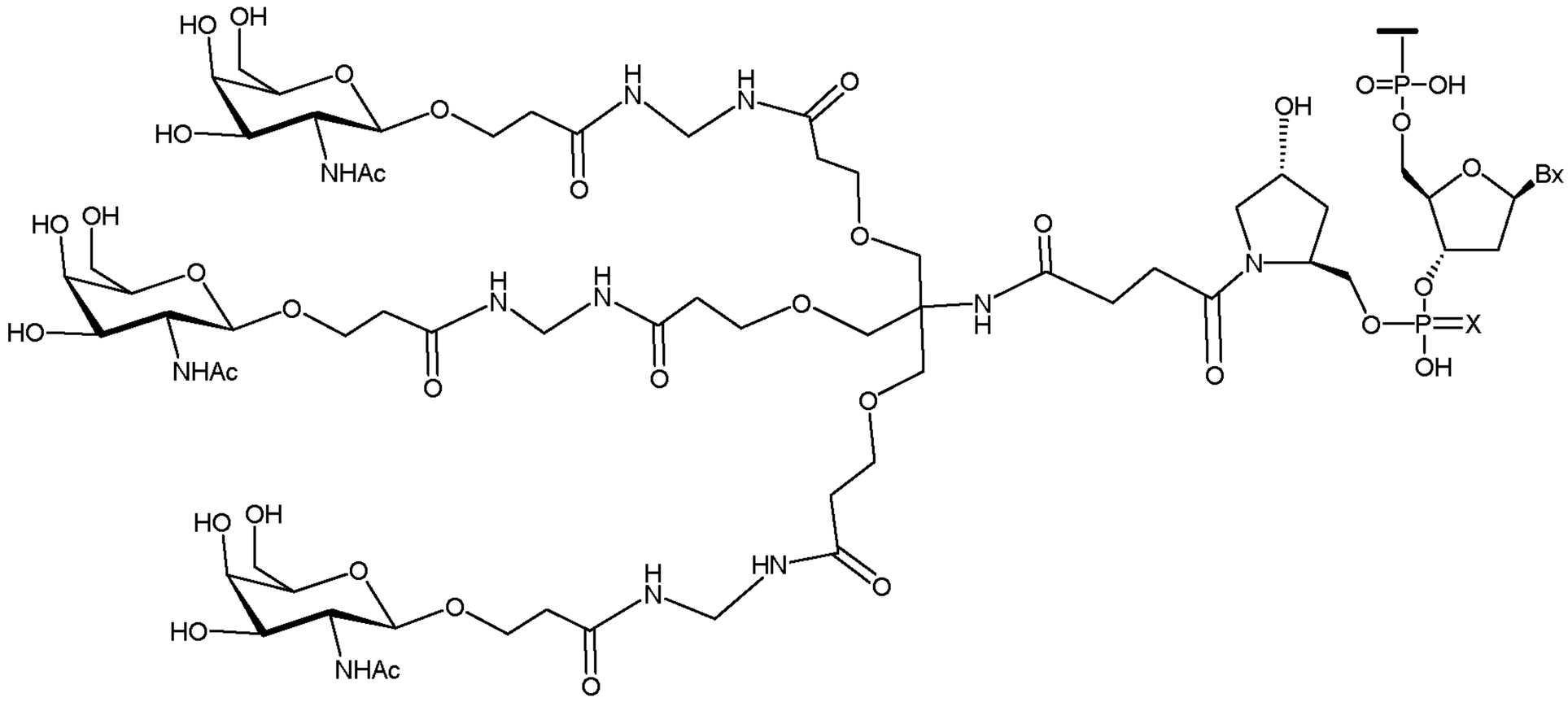
Z is H or a linked solid support;

Q is an antisense compound;

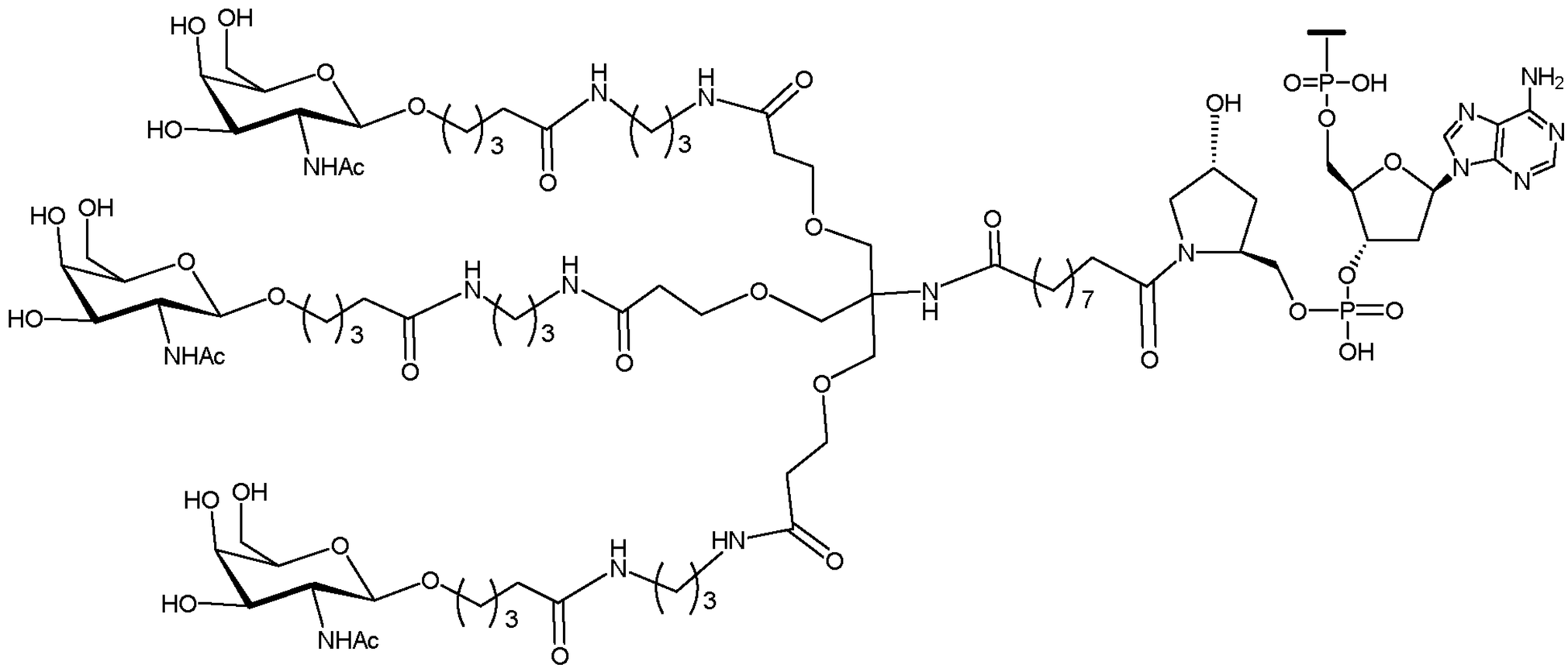
X is O or S; and

Bx is a heterocyclic base moiety.

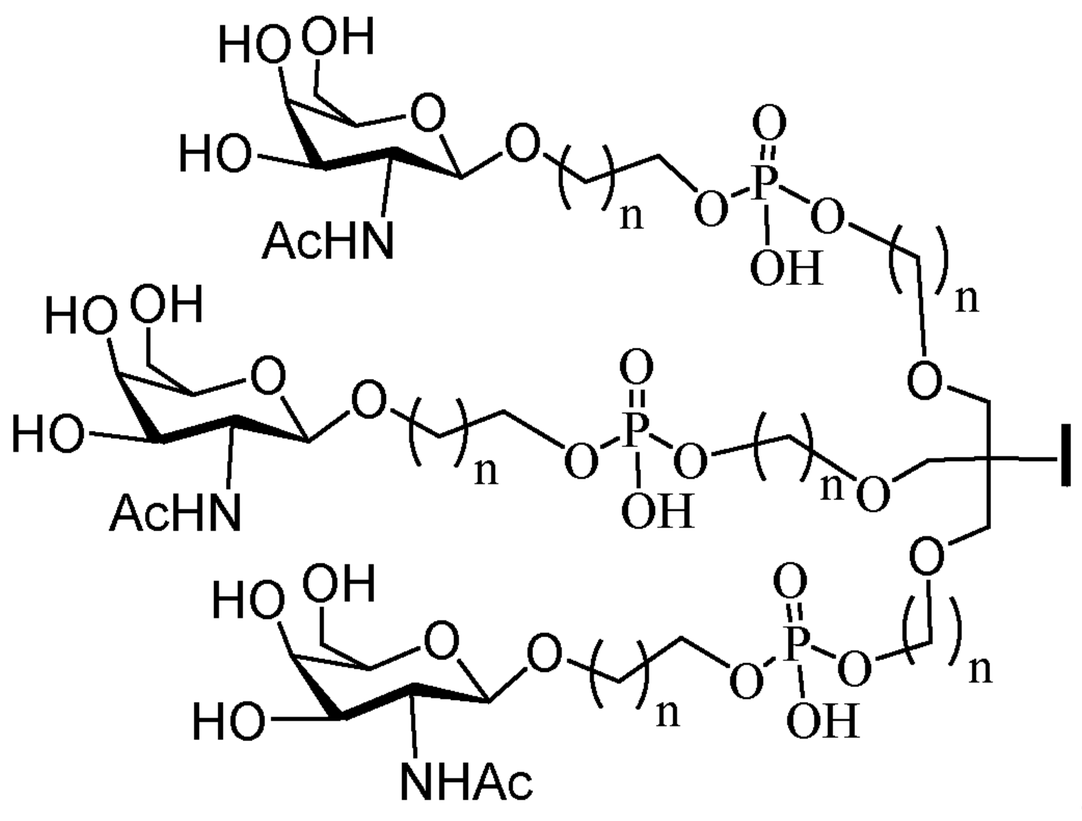
In certain such embodiments, conjugate groups have the following structure:



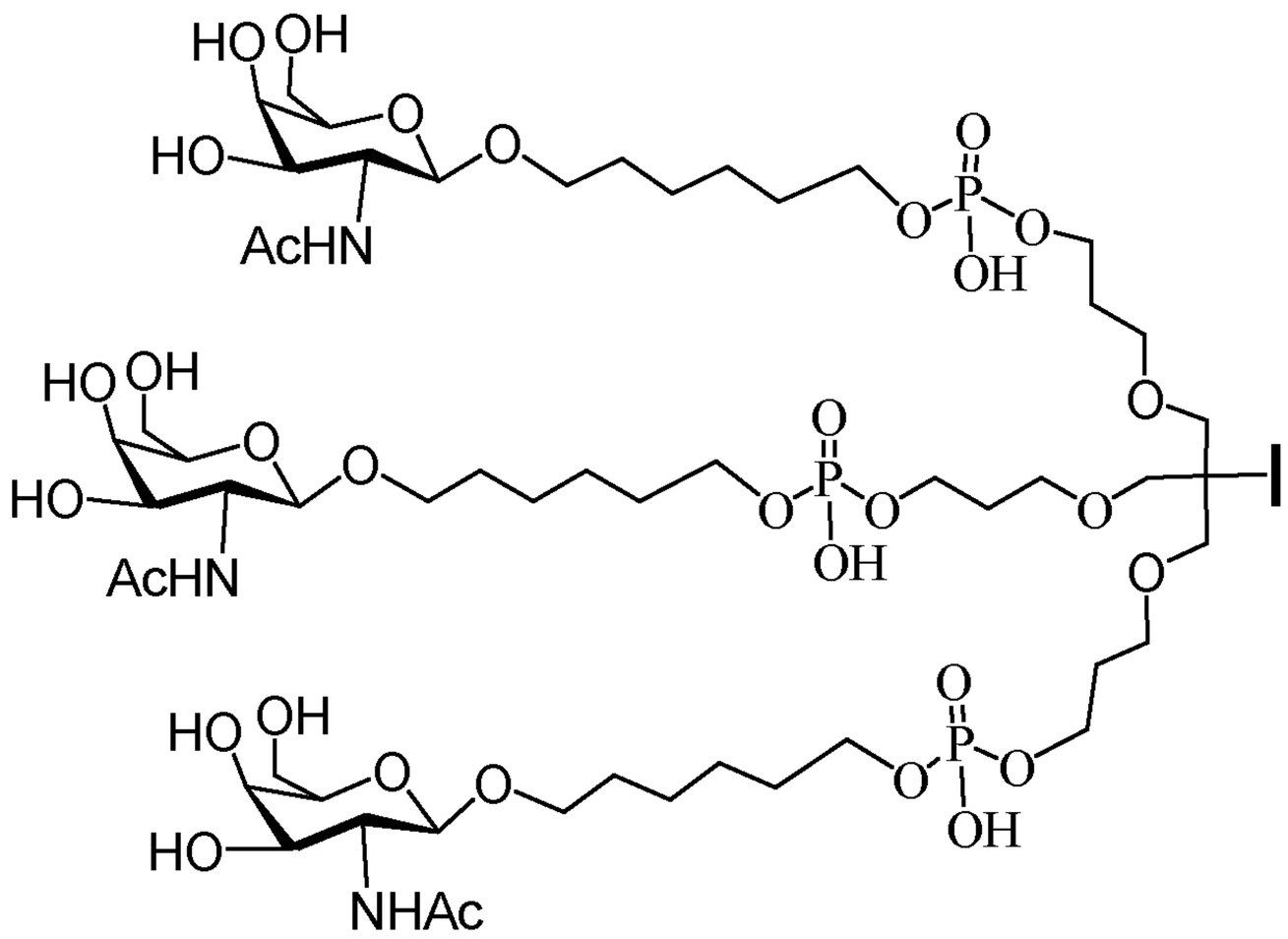
In certain such embodiments, conjugate groups have the following structure:



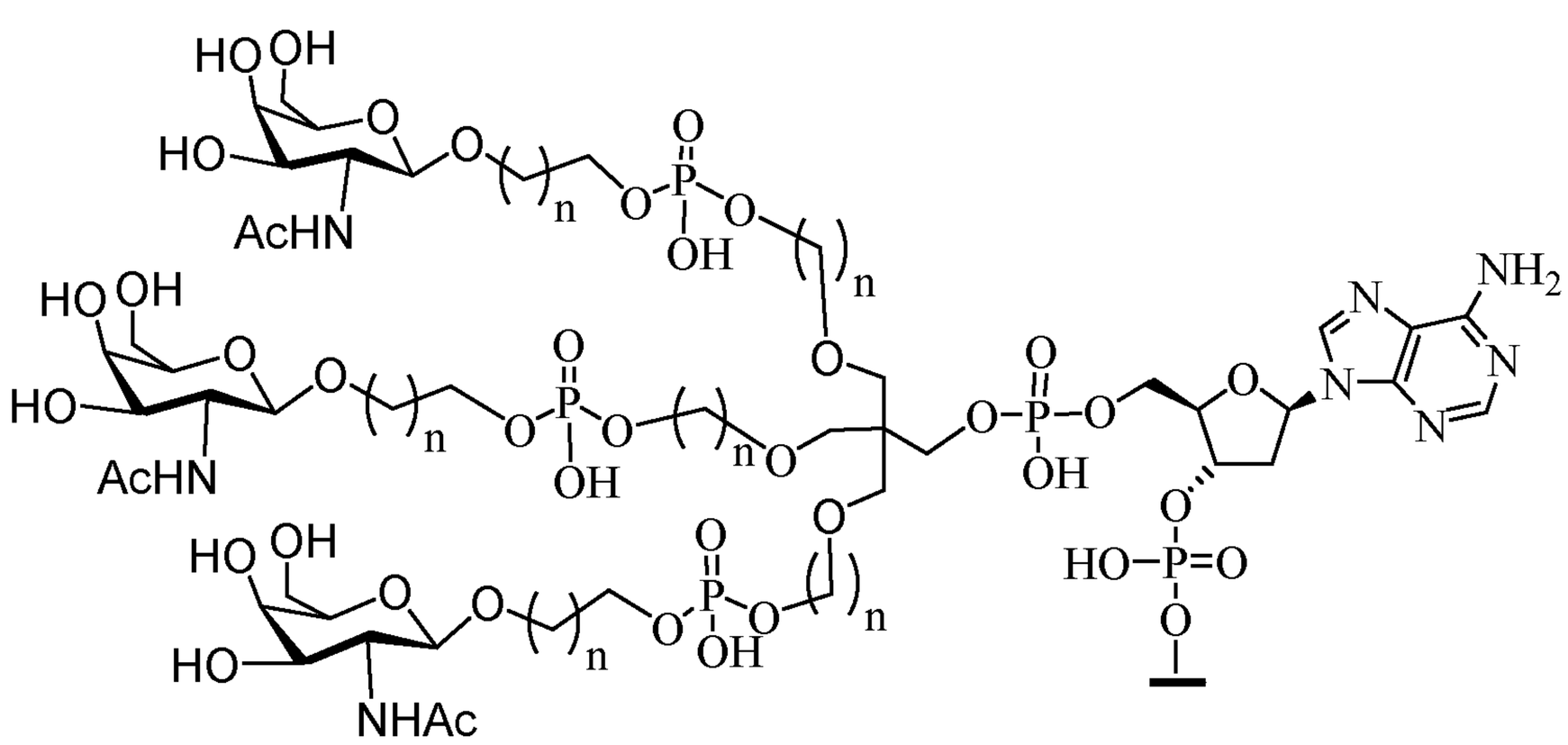
In certain such embodiments, conjugate groups comprise the following structure:



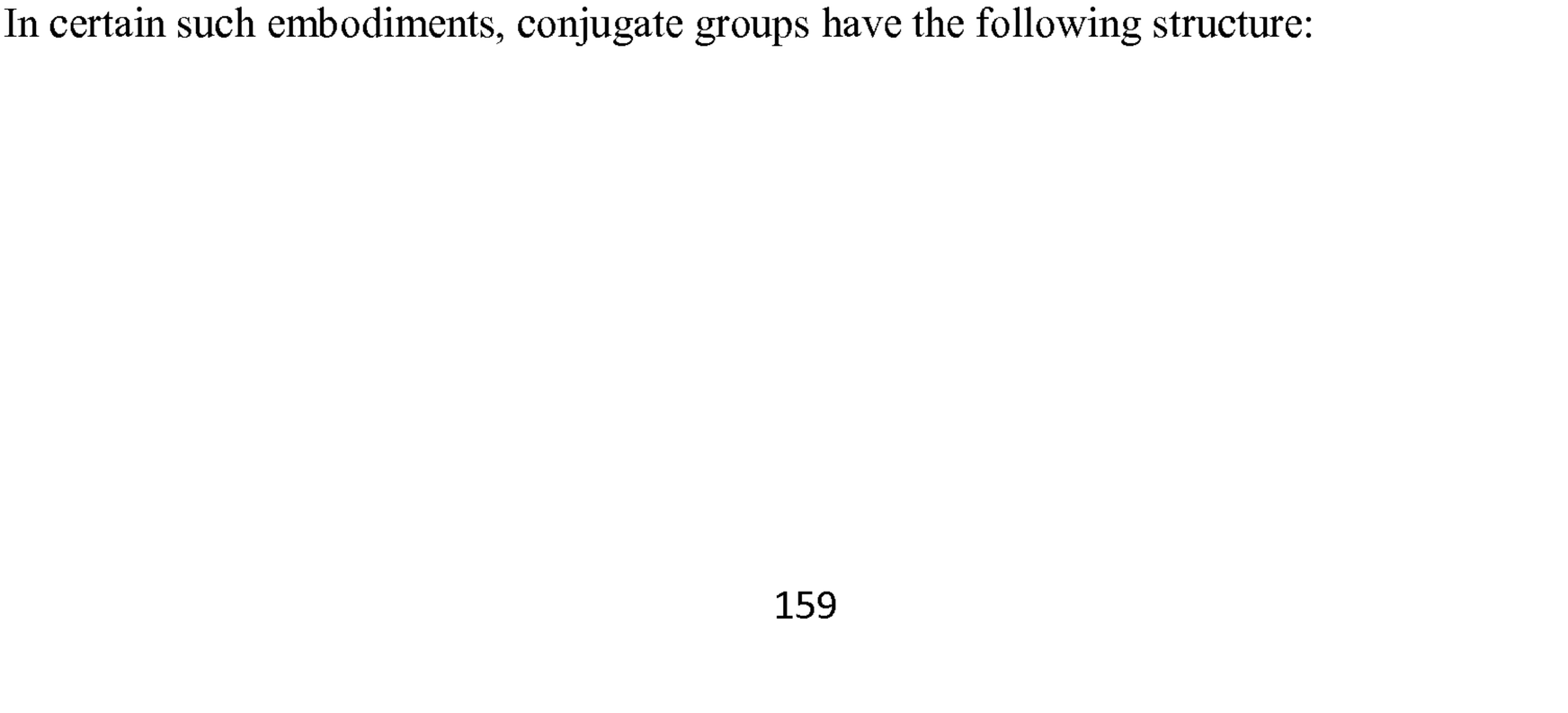
In certain such embodiments, conjugate groups comprise the following structure:

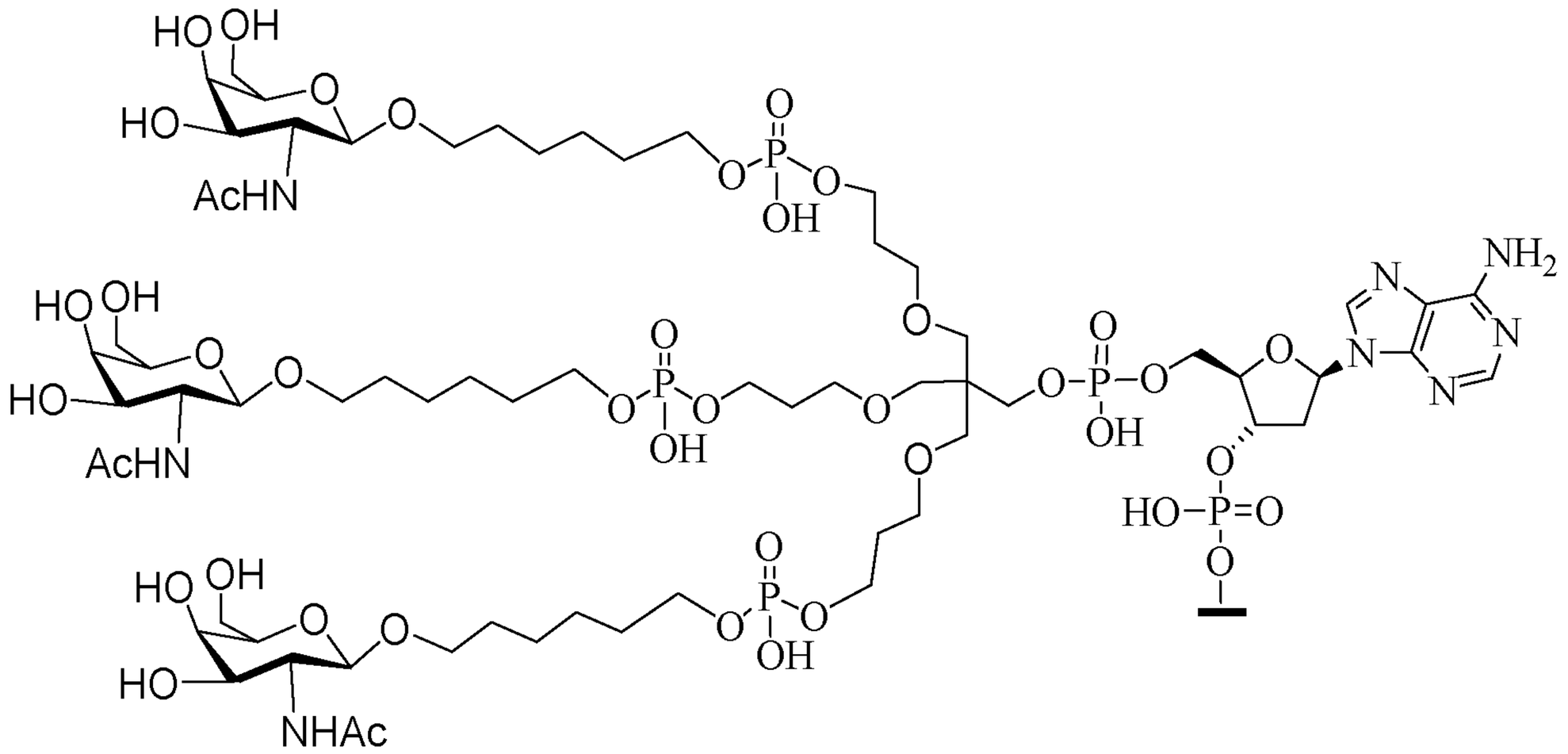


In certain such embodiments, conjugate groups have the following structure:

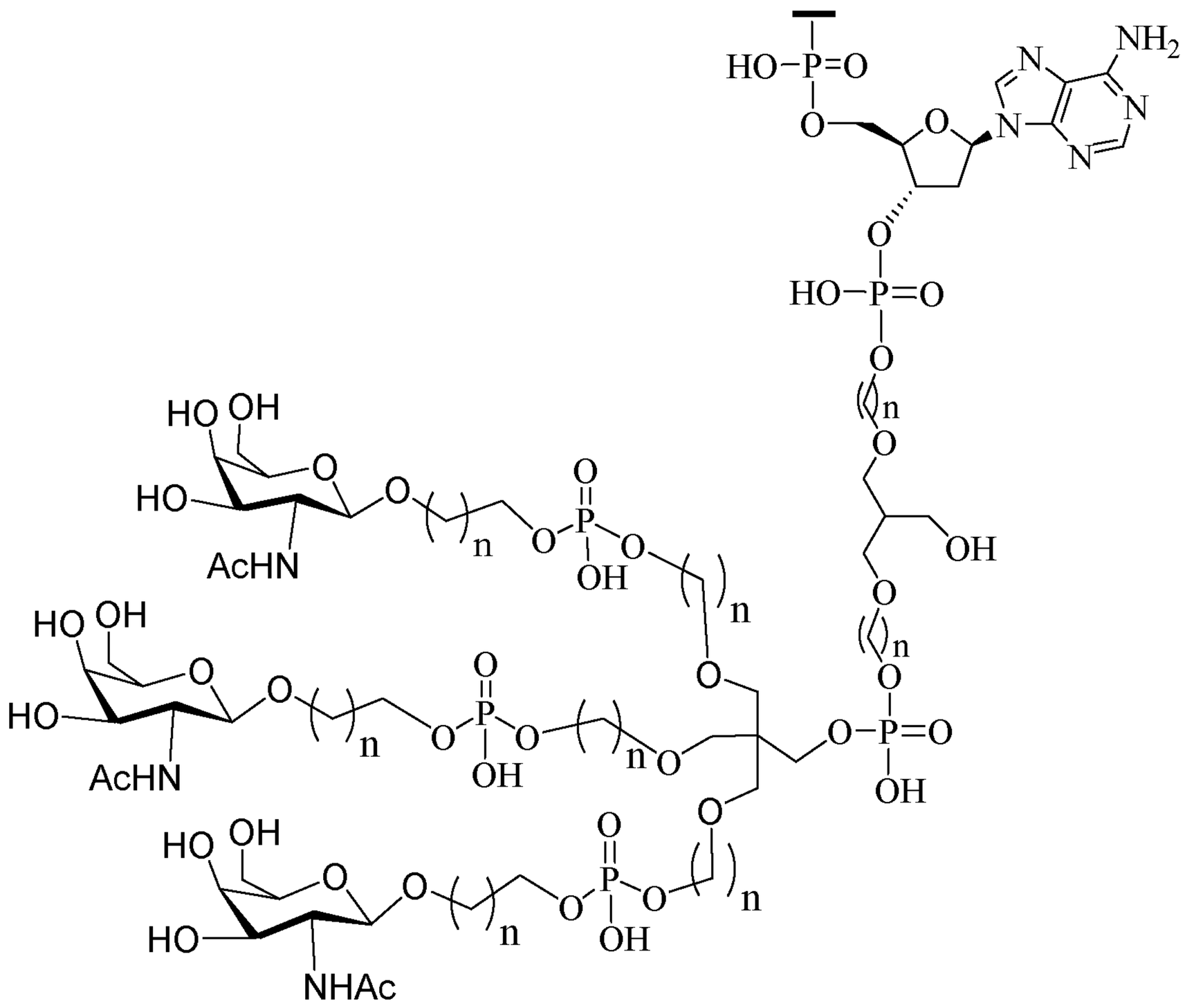


In certain such embodiments, conjugate groups have the following structure:

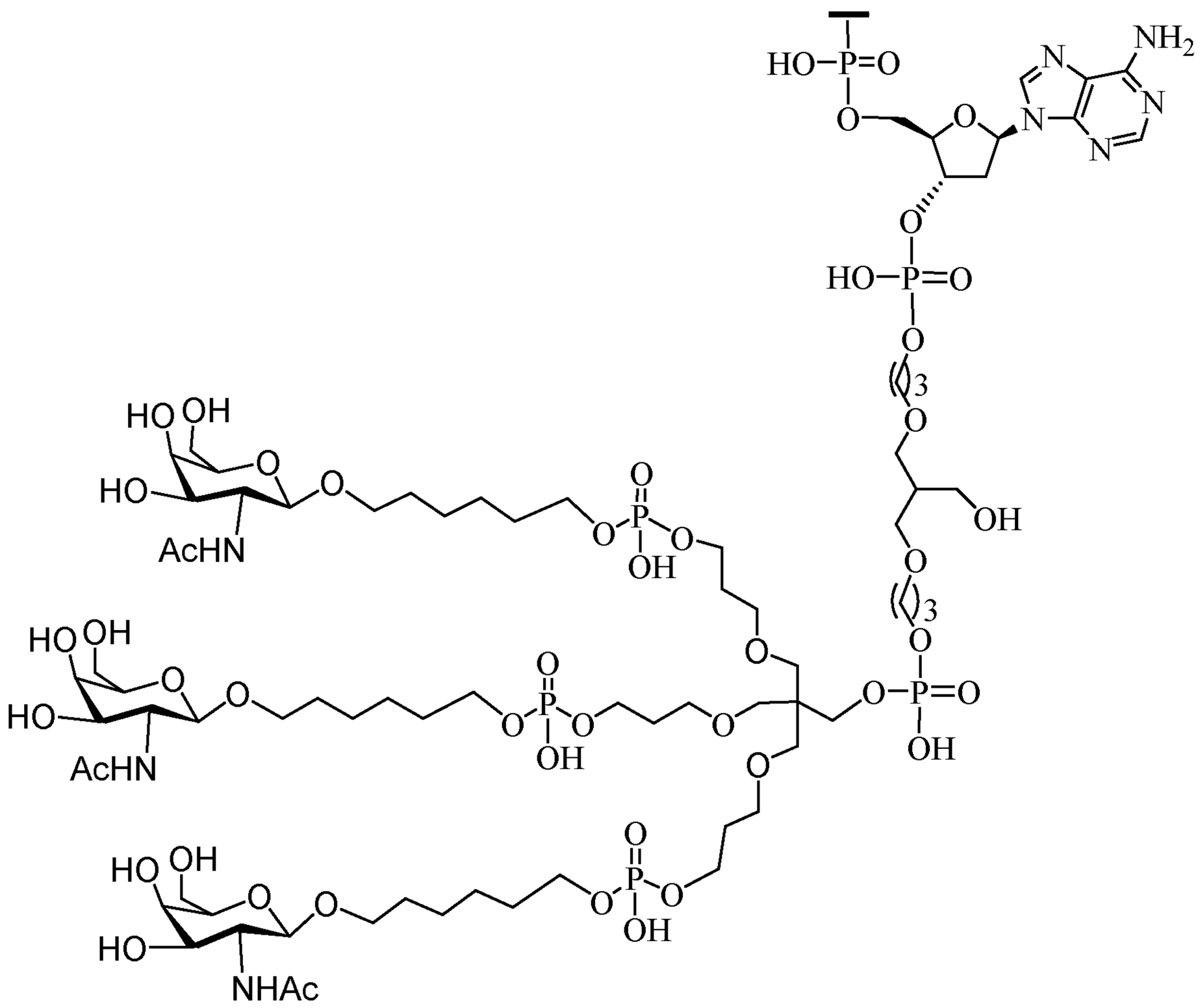




In certain such embodiments, conjugate groups have the following structure:



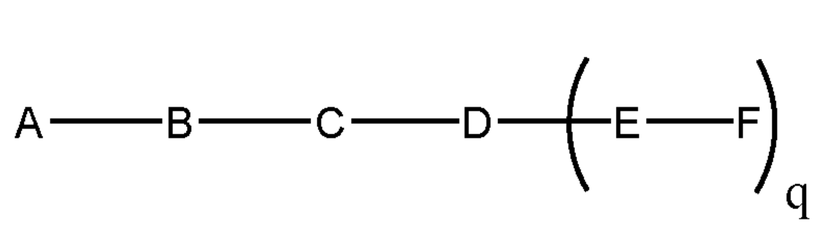
In certain such embodiments, conjugate groups have the following structure:



In certain embodiments, conjugates do not comprise a pyrrolidine.

b. Certain conjugated antisense compounds

In certain embodiments, the conjugates are bound to a nucleoside of the antisense oligonucleotide at the 2', 3', of 5' position of the nucleoside. In certain embodiments, a conjugated antisense compound has the following structure:



wherein

A is the antisense oligonucleotide;

B is the cleavable moiety

C is the conjugate linker

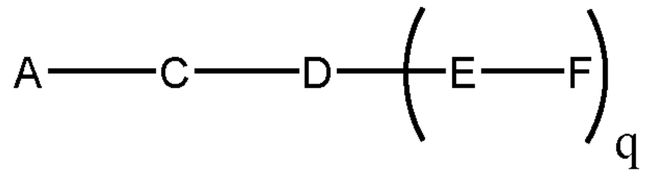
D is the branching group

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

In certain embodiments, a conjugated antisense compound has the following structure:



wherein

A is the antisense oligonucleotide;

C is the conjugate linker

D is the branching group

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

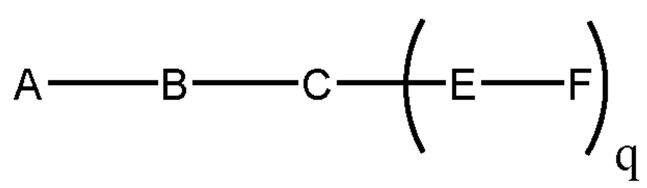
In certain such embodiments, the conjugate linker comprises at least one cleavable bond.

In certain such embodiments, the branching group comprises at least one cleavable bond.

In certain embodiments each tether comprises at least one cleavable bond.

In certain embodiments, the conjugates are bound to a nucleoside of the antisense oligonucleotide at the 2', 3', of 5' position of the nucleoside.

In certain embodiments, a conjugated antisense compound has the following structure:



wherein

A is the antisense oligonucleotide;

B is the cleavable moiety

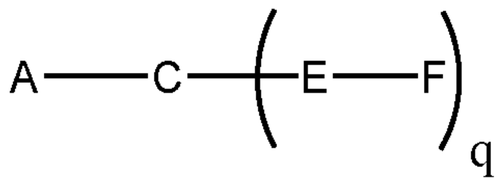
C is the conjugate linker

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

In certain embodiments, the conjugates are bound to a nucleoside of the antisense oligonucleotide at the 2', 3', of 5' position of the nucleoside. In certain embodiments, a conjugated antisense compound has the following structure:



wherein

A is the antisense oligonucleotide;

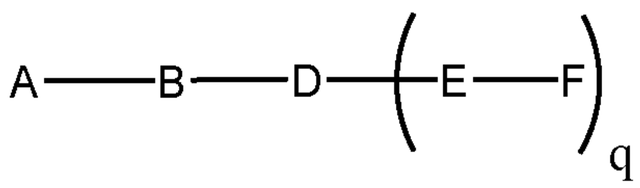
C is the conjugate linker

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

In certain embodiments, a conjugated antisense compound has the following structure:



wherein

A is the antisense oligonucleotide;

B is the cleavable moiety

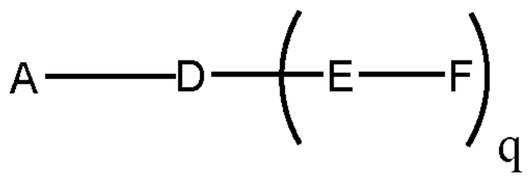
D is the branching group

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

In certain embodiments, a conjugated antisense compound has the following structure:



wherein

A is the antisense oligonucleotide;

D is the branching group

each E is a tether;

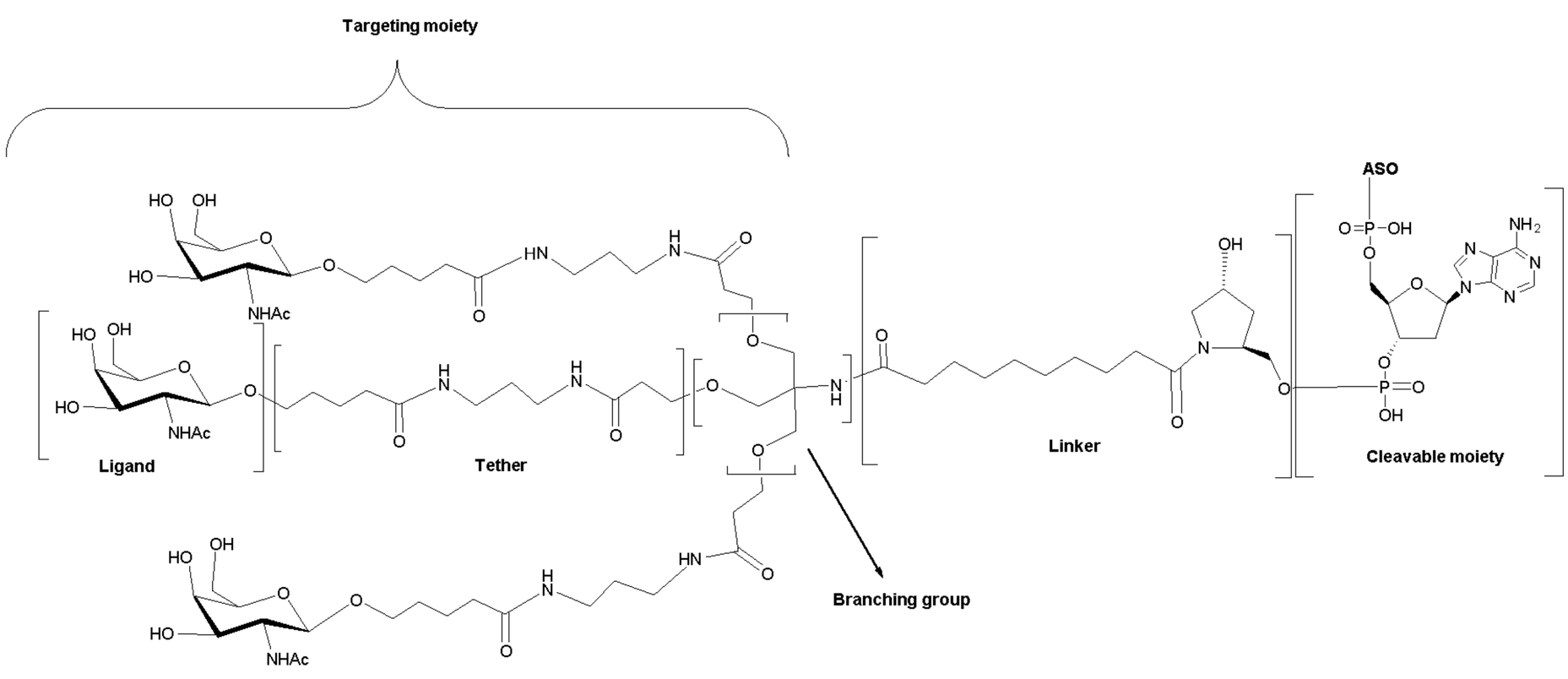
each F is a ligand; and

q is an integer between 1 and 5.

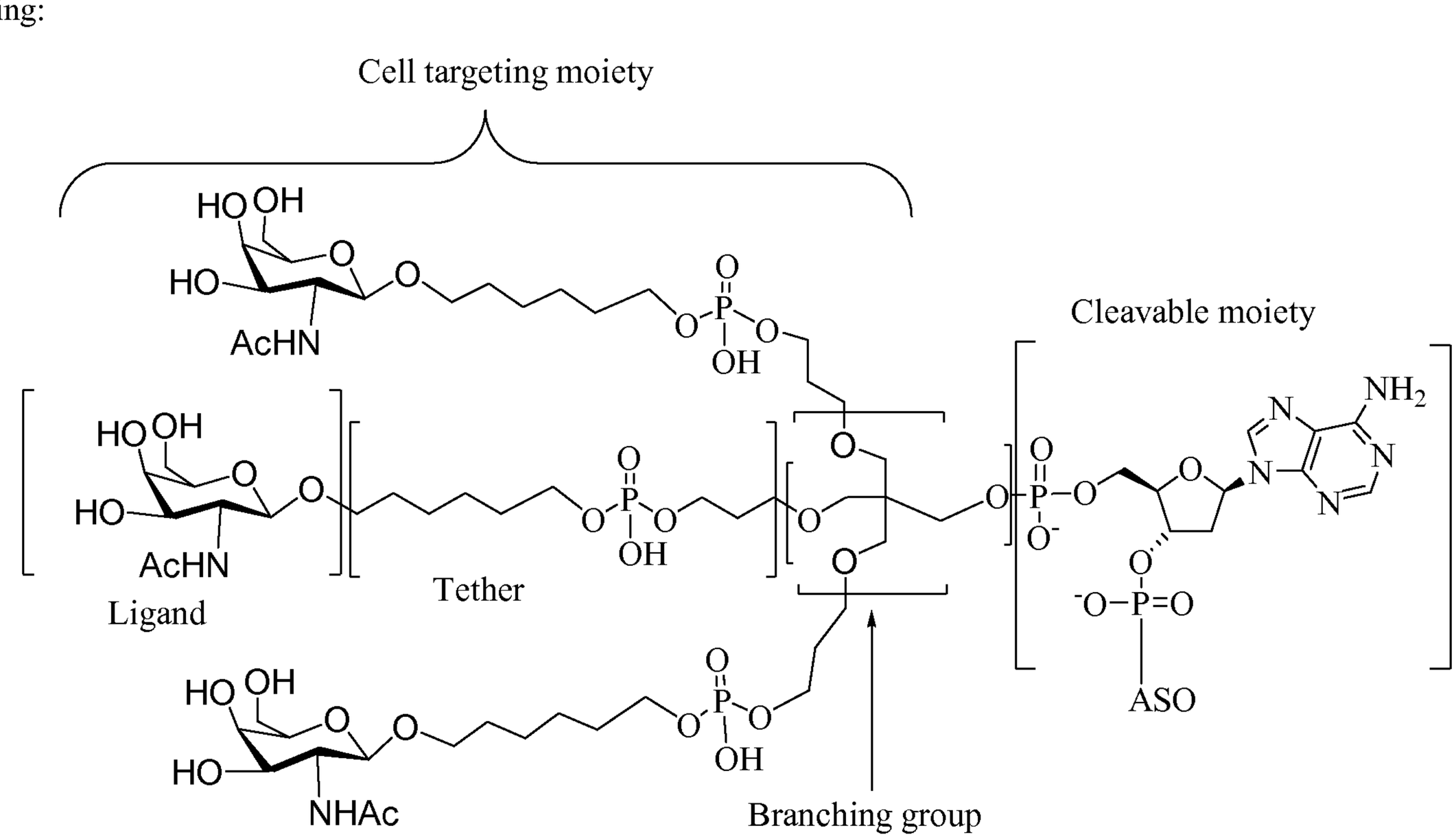
In certain such embodiments, the conjugate linker comprises at least one cleavable bond.

In certain embodiments each tether comprises at least one cleavable bond.

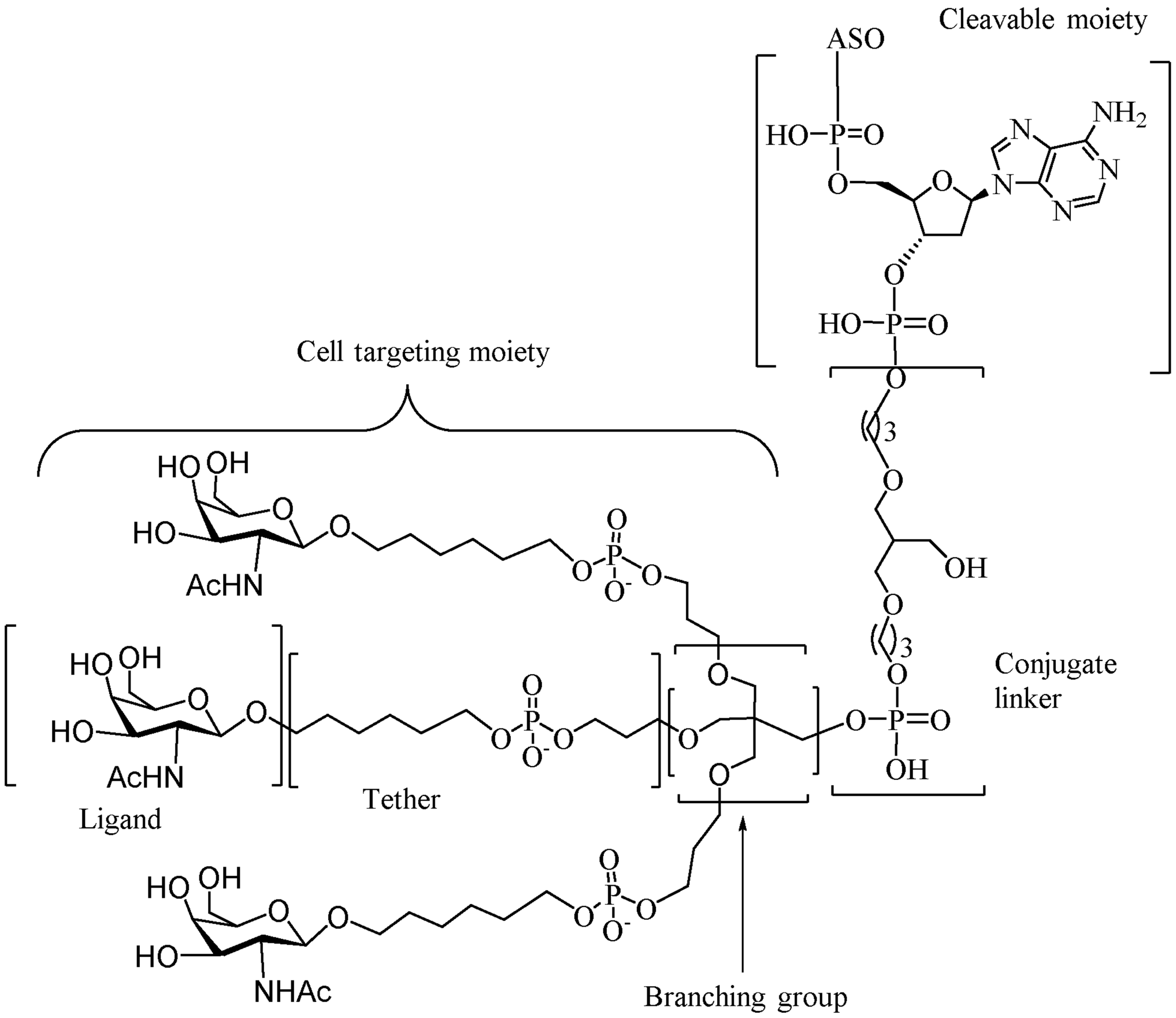
In certain embodiments, a conjugated antisense compound has a structure selected from among the following:



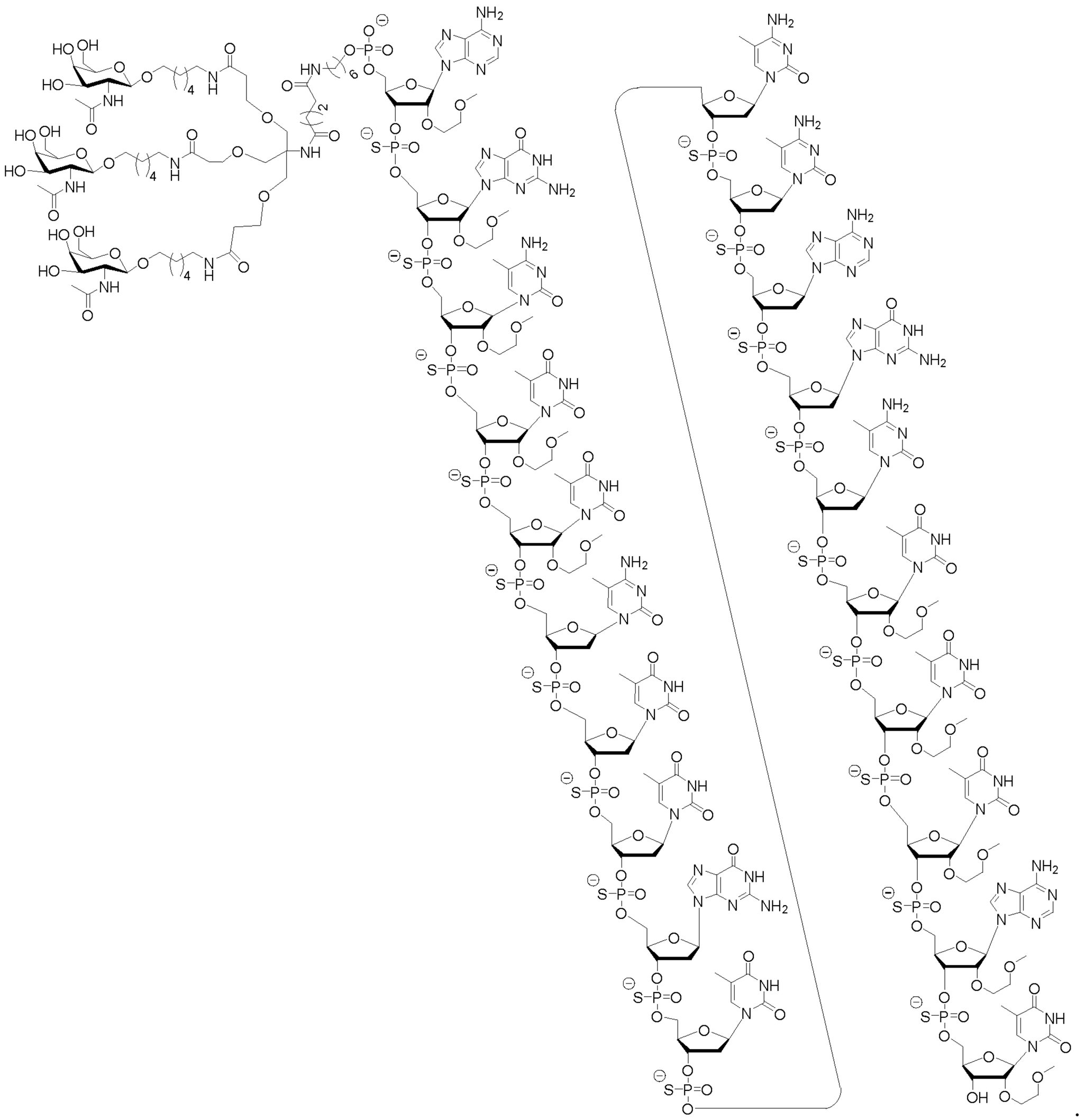
In certain embodiments, a conjugated antisense compound has a structure selected from among the following:



In certain embodiments, a conjugated antisense compound has a structure selected from among the following:



In certain embodiments, the conjugated antisense compound has the following structure:



Representative United States patents, United States patent application publications, and international patent application publications that teach the preparation of certain of the above noted conjugates, conjugated antisense compounds, tethers, linkers, branching groups, ligands, cleavable moieties as well as other modifications include without limitation, US 5,994,517, US 6,300,319, US 6,660,720, US 6,906,182, US 7,262,177, US 7,491,805, US 8,106,022, US 7,723,509, US 2006/0148740, US 2011/0123520, WO 2013/033230 and WO 2012/037254, each of which is incorporated by reference herein in its entirety.

Representative publications that teach the preparation of certain of the above noted conjugates, conjugated antisense compounds, tethers, linkers, branching groups, ligands, cleavable moieties as well as other modifications include without limitation, BIESSEN et al., "The Cholesterol Derivative of a

Triantennary Galactoside with High Affinity for the Hepatic Asialoglycoprotein Receptor: a Potent Cholesterol Lowering Agent" J. Med. Chem. (1995) 38:1846-1852, BIESSEN et al., "Synthesis of Cluster Galactosides with High Affinity for the Hepatic Asialoglycoprotein Receptor" J. Med. Chem. (1995) 38:1538-1546, LEE et al., "New and more efficient multivalent glyco-ligands for asialoglycoprotein receptor of mammalian hepatocytes" Bioorganic & Medicinal Chemistry (2011) 19:2494-2500, RENSEN et al., "Determination of the Upper Size Limit for Uptake and Processing of Ligands by the Asialoglycoprotein Receptor on Hepatocytes in Vitro and in Vivo" J. Biol. Chem. (2001) 276(40):37577-37584, RENSEN et al., "Design and Synthesis of Novel N-Acetylgalactosamine-Terminated Glycolipids for Targeting of Lipoproteins to the Hepatic Asialoglycoprotein Receptor" J. Med. Chem. (2004) 47:5798-5808, SLIEDREGT et al., "Design and Synthesis of Novel Amphiphilic Dendritic Galactosides for Selective Targeting of Liposomes to the Hepatic Asialoglycoprotein Receptor" J. Med. Chem. (1999) 42:609-618, and Valentijn *et al.,* "Solid-phase synthesis of lysine-based cluster galactosides with high affinity for the Asialoglycoprotein Receptor" *Tetrahedron,* 1997, *53(2),* 759-770, each of which is incorporated by reference herein in its entirety.

In certain embodiments, conjugated antisense compounds comprise an RNase H based oligonucleotide (such as a gapmer) or a splice modulating oligonucleotide (such as a fully modified oligonucleotide) and any conjugate group comprising at least one, two, or three GalNAc groups. In certain embodiments a conjugated antisense compound comprises any conjugate group found in any of the following references: Lee, *Carbohydr Res*, 1978, 67, 509-514; Connolly et al., *J Biol Chem*, 1982, 257, 939-945; Pavia et al., *Int J Pep Protein Res*, 1983, 22, 539-548; Lee et al., *Biochem*, 1984, 23, 4255-4261; Lee et al., *Glycoconjugate J*, 1987, 4, 317-328; Toyokuni et al., *Tetrahedron Lett*, 1990, 31, 2673-2676; Biessen et al., *J Med Chem*, 1995, 38, 1538-1546; Valentijn et al., *Tetrahedron*, 1997, 53, 759-770; Kim et al., *Tetrahedron Lett*, 1997, 38, 3487-3490; Lee et al., *Bioconjug Chem*, 1997, 8, 762-765; Kato et al., *Glycobiol*, 2001, 11, 821-829; Rensen et al., *J Biol Chem*, 2001, 276, 37577-37584; Lee et al., *Methods Enzymol*, 2003, 362, 38-43; Westerlind et al., *Glycoconj J*, 2004, 21, 227-241; Lee et al., *Bioorg Med Chem Lett*, 2006, 16(19), 5132-5135; Maierhofer et al., *Bioorg Med Chem*, 2007, 15, 7661-7676; Khorev et al., *Bioorg Med Chem*, 2008, 16, 5216-5231; Lee et al., *Bioorg Med Chem*, 2011, 19, 2494-2500; Kornilova et al., Analyt Biochem, 2012, 425, 43-46; Pujol et al., *Angew Chemie Int Ed Engl*, 2012, 51, 7445-7448; Biessen et al., *J Med Chem*, 1995, 38, 1846-1852; Sliedregt et al., *J Med Chem*, 1999, 42, 609-618; Rensen et al., *J Med Chem*, 2004, 47, 5798-5808; Rensen et al., *Arterioscler Thromb Vasc Biol*, 2006, 26, 169-175; van Rossenberg et al., *Gene Ther*, 2004, 11, 457-464; Sato et al., *J Am Chem Soc*, 2004, 126, 14013-14022; Lee et al., *J Org Chem*, 2012, 77, 7564-7571; Biessen et al., *FASEB J*, 2000, 14, 1784-1792; Rajur et al., *Bioconjug Chem*, 1997, 8, 935-940; Duff et al., *Methods Enzymol*, 2000, 313, 297-321; Maier et al., *Bioconjug Chem*, 2003, 14, 18-29; Jayaprakash et al., *Org Lett*, 2010, 12, 5410-5413; Manoharan, *Antisense Nucleic Acid Drug Dev*, 2002, 12, 103-128; Merwin et al., *Bioconjug Chem*, 1994, 5, 612-620; Tomiya et al., Bioorg Med Chem, 2013, 21, 5275-5281; International applications WO1998/013381; WO2011/038356; WO1997/046098;

WO2008/098788; WO2004/101619; WO2012/037254; WO2011/120053; WO2011/100131; WO2011/163121; WO2012/177947; WO2013/033230; WO2013/075035; WO2012/083185; WO2012/083046; WO2009/082607; WO2009/134487; WO2010/144740; WO2010/148013; WO1997/020563; WO2010/088537; WO2002/043771; WO2010/129709; WO2012/068187; WO2009/126933; WO2004/024757; WO2010/054406; WO2012/089352; WO2012/089602; WO2013/166121; WO2013/165816; U.S. Patents 4,751,219; 8,552,163; 6,908,903; 7,262,177; 5,994,517; 6,300,319; 8,106,022; 7,491,805; 7,491,805; 7,582,744; 8,137,695; 6,383,812; 6,525,031; 6,660,720; 7,723,509; 8,541,548; 8,344,125; 8,313,772; 8,349,308; 8,450,467; 8,501,930; 8,158,601; 7,262,177; 6,906,182; 6,620,916; 8,435,491; 8,404,862; 7,851,615; Published U.S. Patent Application Publications US2011/0097264; US2011/0097265; US2013/0004427; US2005/0164235; US2006/0148740; US2008/0281044; US2010/0240730; US2003/0119724; US2006/0183886; US2008/0206869; US2011/0269814; US2009/0286973; US2011/0207799; US2012/0136042; US2012/0165393; US2008/0281041; US2009/0203135; US2012/0035115; US2012/0095075; US2012/0101148; US2012/0128760; US2012/0157509; US2012/0230938; US2013/0109817; US2013/0121954; US2013/0178512; US2013/0236968; US2011/0123520; US2003/0077829; US2008/0108801; and US2009/0203132; each of which is incorporated by reference in its entirety.

Cell culture and antisense compounds treatment

The effects of antisense compounds on the level, activity or expression of ANGPTL3 nucleic acids can be tested in vitro in a variety of cell types. Cell types used for such analyses are available from commercial vendors (e.g. American Type Culture Collection, Manassus, VA; Zen-Bio, Inc., Research Triangle Park, NC; Clonetics Corporation, Walkersville, MD) and cells are cultured according to the vendor's instructions using commercially available reagents (e.g. Invitrogen Life Technologies, Carlsbad, CA). Illustrative cell types include, but are not limited to, HepG2 cells, Hep3B cells, Huh7 (hepatocellular carcinoma) cells, primary hepatocytes, A549 cells, GM04281 fibroblasts and LLC-MK2 cells.

In vitro testing of antisense oligonucleotides

Described herein are methods for treatment of cells with antisense oligonucleotides, which can be modified appropriately for treatment with other antisense compounds.

In general, cells are treated with antisense oligonucleotides when the cells reach approximately 60-80% confluence in culture.

One reagent commonly used to introduce antisense oligonucleotides into cultured cells includes the cationic lipid transfection reagent LIPOFECTIN® (Invitrogen, Carlsbad, CA). Antisense oligonucleotides are mixed with LIPOFECTIN® in OPTI-MEM® 1 (Invitrogen, Carlsbad, CA) to achieve the desired final concentration of antisense oligonucleotide and a LIPOFECTIN® concentration that typically ranges 2 to 12 ug/mL per 100 nM antisense oligonucleotide.

Another reagent used to introduce antisense oligonucleotides into cultured cells includes LIPOFECTAMINE 2000® (Invitrogen, Carlsbad, CA). Antisense oligonucleotide is mixed with LIPOFECTAMINE 2000® in OPTI-MEM® 1 reduced serum medium (Invitrogen, Carlsbad, CA) to achieve the desired concentration of antisense oligonucleotide and a LIPOFECTAMINE® concentration that typically ranges 2 to 12 ug/mL per 100 nM antisense oligonucleotide.

Another reagent used to introduce antisense oligonucleotides into cultured cells includes Cytofectin® (Invitrogen, Carlsbad, CA). Antisense oligonucleotide is mixed with Cytofectin® in OPTI-MEM® 1 reduced serum medium (Invitrogen, Carlsbad, CA) to achieve the desired concentration of antisense oligonucleotide and a Cytofectin® concentration that typically ranges 2 to 12 ug/mL per 100 nM antisense oligonucleotide.

Another reagent used to introduce antisense oligonucleotides into cultured cells includes Oligofectamine™ (Invitrogen Life Technologies, Carlsbad, CA). Antisense oligonucleotide is mixed with Oligofectamine™ in Opti-MEM™-1 reduced serum medium (Invitrogen Life Technologies, Carlsbad, CA) to achieve the desired concentration of oligonucleotide with an Oligofectamine™ to oligonucleotide ratio of approximately 0.2 to 0.8 µL per 100 nM.

Another reagent used to introduce antisense oligonucleotides into cultured cells includes FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN). Antisense oligomeric compound was mixed with FuGENE 6 in 1 mL of serum-free RPMI to achieve the desired concentration of oligonucleotide with a FuGENE 6 to oligomeric compound ratio of 1 to 4 µL of FuGENE 6 per 100 nM.

Another technique used to introduce antisense oligonucleotides into cultured cells includes electroporation (Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed., 2001).

Cells are treated with antisense oligonucleotides by routine methods. Cells are typically harvested 16-24 hours after antisense oligonucleotide treatment, at which time RNA or protein levels of target nucleic acids are measured by methods known in the art and described herein. In general, when treatments are performed in multiple replicates, the data are presented as the average of the replicate treatments.

The concentration of antisense oligonucleotide used varies from cell line to cell line. Methods to determine the optimal antisense oligonucleotide concentration for a particular cell line are well known in the art. Antisense oligonucleotides are typically used at concentrations ranging from 1 nM to 300 nM when transfected with LIPOFECTAMINE2000®, Lipofectin or Cytofectin. Antisense oligonucleotides are used at higher concentrations ranging from 625 to 20,000 nM when transfected using electroporation.

RNA Isolation

RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are well known in the art (Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed., 2001). RNA is prepared using methods well known in the art, for example, using the TRIZOL® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocols.

Analysis of inhibition of target levels or expression

Inhibition of levels or expression of an ANGPTL3 nucleic acid can be assayed in a variety of ways known in the art (Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed., 2001). For example, target nucleic acid levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or quantitative real-time PCR. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are well known in the art. Northern blot analysis is also routine in the art. Quantitative real-time PCR can be conveniently accomplished using the commercially available ABI PRISM® 7600, 7700, or 7900 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Quantitative Real-Time PCR Analysis of Target RNA Levels

Quantitation of target RNA levels can be accomplished by quantitative real-time PCR using the ABI PRISM® 7600, 7700, or 7900 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Methods of quantitative real-time PCR are well known in the art.

Prior to real-time PCR, the isolated RNA is subjected to a reverse transcriptase (RT) reaction, which produces complementary DNA (cDNA) that is then used as the substrate for the real-time PCR amplification. The RT and real-time PCR reactions are performed sequentially in the same sample well. RT and real-time PCR reagents are obtained from Invitrogen (Carlsbad, CA). RT and real-time-PCR reactions are carried out by methods well known to those skilled in the art.

Gene (or RNA) target quantities obtained by real time PCR can be normalized using either the expression level of a gene whose expression is constant, such as cyclophilin A or GADPH or by quantifying total RNA using RIBOGREEN® (Life Technologies™, Inc. Carlsbad, CA). Cyclophilin A or GADPH expression can be quantified by real time PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA can be quantified using RIBOGREEN® RNA quantification reagent. Methods of RNA quantification by RIBOGREEN® are taught in Jones, L.J., et al, (Analytical Biochemistry, 1998, 265, 368-374). A CYTOFLUOR® 4000 instrument (PE Applied Biosystems) can be used to measure RIBOGREEN® fluorescence.

Methods for designing real-time PCR probes and primers are well known in the art, and can include the use of software such as PRIMER EXPRESS® Software (Applied Biosystems, Foster City, CA). Probes and primers used in real-time PCR were designed to hybridize to ANGPTL3 specific sequences and are disclosed in the Examples below. The target specific PCR probes can have FAM covalently linked to the 5' end and TAMRA or MGB covalently linked to the 3' end, where FAM is the fluorescent dye and TAMRA or MGB is the quencher dye.

Analysis of Protein Levels

Antisense inhibition of ANGPTL3 nucleic acids can be assessed by measuring ANGPTL3 protein levels. Protein levels of ANGPTL3 can be evaluated or quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), enzyme-linked immunosorbent assay (ELISA), quantitative protein assays, protein activity assays (for example, caspase activity assays), immunohistochemistry, immunocytochemistry or fluorescence-activated cell sorting (FACS) (Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed., 2001). Antibodies directed to a target can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional monoclonal or polyclonal antibody generation methods well known in the art.

In vivo testing of antisense compounds

Antisense compounds, for example, antisense oligonucleotides, are tested in animals to assess their ability to inhibit expression of ANGPTL3 and produce phenotypic changes. Testing can be performed in normal animals, or in experimental disease models. For administration to animals, antisense oligonucleotides are formulated in a pharmaceutically acceptable diluent, such as phosphate-buffered saline. Administration includes parenteral routes of administration. Following a period of treatment with antisense oligonucleotides, RNA is isolated from tissue and changes in ANGPTL3 nucleic acid expression are measured. Changes in ANGPTL3 protein levels are also measured.

Certain Indications

In certain embodiments, provided herein are methods of treating an individual comprising administering one or more pharmaceutical compositions as described herein. In certain embodiments, the individual has a metabolic disease and/or cardiovascular disease. In certain embodiments, the individual has combined hyperlipidemia (e.g., familial or non-familial), hypercholesterolemia (e.g., familial homozygous hypercholesterolemia (HoFH), familial heterozygous hypercholesterolemia (HeFH)), dyslipidemia, lipodystrophy, hypertriglyceridemia (e.g., heterozygous LPL deficiency, homozygous LPL deficiency), coronary artery disease (CAD), familial chylomicronemia syndrome (FCS), hyperlipoproteinemia Type IV), metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), diabetes (e.g., Type 2 diabetes, Type 2 diabetes with dyslipidemia), insulin resistance (e.g., insulin resistance with dyslipidemia), vascular wall thickening, high blood pressure (e.g., pulmonary arterial hypertension), sclerosis (e.g., atherosclerosis, systemic sclerosis, progressive skin sclerosis and proliferative obliterative vasculopathy such as digital ulcers and pulmonary vascular involvement), or a combination thereof.

In certain embodiments, the compounds targeted to ANGPTL3 described herein modulate lipid and/or energy metabolism in an animal. In certain embodiments, the compounds targeted to ANGPTL3 described herein modulate physiological markers or phenotypes of hypercholesterolemia, dyslipidemia, lipodystrophy, hypertriglyceridemia, metabolic syndrome, NAFLD, NASH and/or diabetes. For example,

administration of the compounds to animals can modulate one or more of VLDL, non-esterified fatty acids (NEFA), LDL, cholesterol, triglyceride, glucose, insulin or ANGPTL3 levels. In certain embodiments, the modulation of the physiological markers or phenotypes can be associated with inhibition of ANGPTL3 by the compounds.

In certain embodiments, the compounds targeted to ANGPTL3 described herein reduce and/or prevent one or more of hepatic TG accumulation (i.e. hepatic steatosis), atherosclerosis, vascular wall thinkening (e.g., arterial intima-media thickening), combined hyperlipidemia (e.g., familial or non-familial), hypercholesterolemia (e.g., familial homozygous hypercholesterolemia (HoFH), familial heterozygous hypercholesterolemia (HeFH)), dyslipidemia, lipodystrophy, hypertriglyceridemia (e.g., heterozygous LPL deficiency, homozygous LPL deficiency, familial chylomicronemia syndrome (FCS), hyperlipoproteinemia Type IV), metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), diabetes (e.g., Type 2 diabetes, Type 2 diabetes with dyslipidemia), insulin resistance (e.g., insulin resistance with dyslipidemia), high blood pressure and sclerosis, or any combination thereof. In certain embodiments, the compounds targeted to ANGPTL3 described herein improve insulin sensitivity.

In certain embodiments, administration of an antisense compound targeted to an ANGPTL3 nucleic acid results in reduction of ANGPTL3 expression by about at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99%, or a range defined by any two of these values.

In certain embodiments, pharmaceutical compositions comprising an antisense compound targeted to ANGPTL3 are used for the preparation of a medicament for treating a patient suffering from, or susceptible to, a metabolic disease or cardiovascular disease. In certain embodiments, pharmaceutical compositions comprising an antisense compound targeted to ANGPTL3 are used in the preparation of a medicament for treating a patient suffering from, or susceptible to, one or more of combined hyperlipidemia (e.g., familial or non-familial), hypercholesterolemia (e.g., familial homozygous hypercholesterolemia (HoFH), familial heterozygous hypercholesterolemia (HeFH)), dyslipidemia, lipodystrophy, hypertriglyceridemia (e.g., familial chylomicronemia syndrome (FCS), hyperlipoproteinemia Type IV), metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), diabetes (e.g., Type 2 diabetes, Type 2 diabetes with dyslipidemia), insulin resistance (e.g., insulin resistance with dyslipidemia), vascular wall thickening, high blood pressure and sclerosis, or a combination thereof.

Administration

In certain embodiments, the compounds and compositions as described herein are administered parenterally.

In certain embodiments, parenteral administration is by infusion. Infusion can be chronic or continuous or short or intermittent. In certain embodiments, infused pharmaceutical agents are delivered with a pump.

In certain embodiments, parenteral administration is by injection. The injection can be delivered with a syringe or a pump. In certain embodiments, the injection is a bolus injection. In certain embodiments, the injection is administered directly to a tissue or organ. In certain embodiments, the injection is subcutaneous.

Certain Combination Therapies

In certain embodiments, a first agent comprising the modified oligonucleotide disclosed herein is co-administered with one or more secondary agents. In certain embodiments, such second agents are designed to treat the same disease, disorder or condition as the first agent described herein. In certain embodiments, such second agents are designed to treat a different disease, disorder, or condition as the first agent described herein. In certain embodiments, such second agents are designed to treat an undesired side effect of one or more pharmaceutical compositions as described herein. In certain embodiments, second agents are co-administered with the first agent to treat an undesired effect of the first agent. In certain embodiments, second agents are co-administered with the first agent to produce a combinational effect. In certain embodiments, second agents are co-administered with the first agent to produce a synergistic effect.

In certain embodiments, a first agent and one or more second agents are administered at the same time. In certain embodiments, the first agent and one or more second agents are administered at different times. In certain embodiments, the first agent and one or more second agents are prepared together in a single pharmaceutical formulation. In certain embodiments, the first agent and one or more second agents are prepared separately.

In certain embodiments, second agents include, but are not limited to a glucose-lowering agent or a lipid-lowering agent. The glucose lowering agent can include, but is not limited to, a therapeutic lifestyle change, PPAR agonist, a dipeptidyl peptidase (IV) inhibitor, a GLP-1 analog, insulin or an insulin analog, an insulin secretagogue, a SGLT2 inhibitor, a human amylin analog, a biguanide, an alpha-glucosidase inhibitor, or a combination thereof. The glucose-lowering agent can include, but is not limited to metformin, sulfonylurea, rosiglitazone, meglitinide, thiazolidinedione, alpha-glucosidase inhibitor or a combination thereof. The sulfonylurea can be acetohexamide, chlorpropamide, tolbutamide, tolazamide, glimepiride, a glipizide, a glyburide, or a gliclazide. The meglitinide can be nateglinide or repaglinide. The thiazolidinedione can be pioglitazone or rosiglitazone. The alpha-glucosidase can be acarbose or miglitol. In certain embodiments the lipid lowering therapy can include, but is not limited to, a therapeutic lifestyle change, niacin, HMG-CoA reductase inhibitor, cholesterol absorption inhibitor, MTP inhibitor (e.g., a small molecule, polypeptide, antibody or antisense compound targeted to MTP), fibrate, PCSK9 inhibitor (e.g., PCSK9 antibodies, polypeptides, small molecules nucleic acid compounds targeting PCSK9), CETP inhibitor

(e.g., small molecules such as torcetrapib and anacetrapib, polypeptides, antibodies or nucleic acid compounds targeted to CETP), apoC-III inhibitor (e.g., a small molecule, polypeptide, antibody or nucleic acid compounds targeted to apoC-III), apoB inhibitor (e.g., a small molecule, polypeptide, antibody or nucleic acid compounds targeted to apoB), beneficial oils rich in omega-3 fatty acids, omega-3 fatty acids or any combination thereof. The HMG-CoA reductase inhibitor can be atorvastatin, rosuvastatin, fluvastatin, lovastatin, pravastatin, simvastatin and the like. The cholesterol absorption inhibitor can be ezetimibe. The fibrate can be fenofibrate, bezafibrate, ciprofibrate, clofibrate, gemfibrozil and the like. The beneficial oil rich in omega-3 fatty acids can be krill, fish (e.g., Vascepa^{R}), flaxseed oil and the like. The omega-3 fatty acid can be ALA, DHA, EPA and the like.

Certain Compounds

Antisense oligonucleotides targeting human ANGPTL3 were described in an earlier publication (see PCT Patent Publication No. WO 2011/085271 published July 14, 2011, incorporated by reference herein, in its entirety). Several oligonucleotides (233676, 233690, 233710, 233717, 233721, 233722, 337459, 337460, 337474, 337477, 337478, 337479, 337481, 337484, 337487, 337488, 337490, 337491, 337492, 337497, 337498, 337503, 337505, 337506, 337508, 337513, 337514, 337516, 337520, 337521, 337525, 337526 and 337528) described therein, including the top ten most potent antisense compounds *in vitro*, were used as benchmarks throughout select *in vitro* screens for antisense compounds described hereinbelow and in US Serial Number 61/920,652. Of the most potent compounds described in WO 2011/085271, ISIS 233722 was found to be highly variable in its ability to inhibit ANGPTL3. According, although initially included in some *in vitro* studies, 233722 was not selected as a benchmark for further studies. Of the previously identified potent *in vitro* benchmark compounds, five (233710, 233717, 337477, 337478, 337479 and 337487) were selected for testing *in vivo*, as described hereinbelow, in huANGPTL3 transgenic mice to assess the most potent in reducing human mRNA transcript and protein expression (Example 126). The antisense oligonucleotide with the highest initial *in vivo* potency in reducing ANGPTL3 levels (233710) was used as a benchmark for *in vivo* assessment of the new antisense compounds described hereinbelow.

In certain embodiments, the antisense compounds described herein benefit from one or more improved properties relative to the antisense compounds described in WO 2011/085271 and in US Serial Number 61/920,652. These improved properties are demonstrated in the examples herein, and a non-exhaustive summary of the examples is provided below for ease of reference.

In a first screen described herein, about 3000 newly designed 5-10-5 MOE gapmer antisense compounds targeting human ANGPTL3 were tested in Hep3B cells for their effect on human ANGPTL3 mRNA *in vitro* (Example 116). The mRNA inhibition levels of the new antisense compounds were assessed with some previously designed antisense compounds (233717, 337484, 337487, 337492 and 337516) used as benchmarks in select studies. Of the about 3000 newly designed antisense compounds from this first screen, about 85 antisense compounds were selected for *in vitro* dose-dependent inhibition studies to determine their

half maximal inhibitory concentration (IC_{50}) (Examples 117-118). Of the about 85 new antisense compounds tested for their half maximal inhibitory concentration (IC_{50}), about 38 antisense compounds that demonstrated potent dose-dependent reduction of ANGPTL3 were selected for *in vivo* potency and tolerability (ALT and AST) testing in mice (Examples 126-127) with antisense compound 233710 used as a benchmark.

In a second screen described herein, about 2000 newly designed antisense compounds targeting human ANGPTL3 with a MOE gapmer motif or a mixed motif (deoxy, 5-10-5 MOE and cET gapmers) were also tested in Hep3B cells for their effect on human ANGPTL3 mRNA *in vitro* (Examples 119-121). The inhibition levels of the new antisense compounds were assessed with some previously designed antisense compounds (233717, 337487, 337513, 337514 and 337516) used as benchmarks in select studies. Of the about 2000 newly designed antisense compounds from this second screen, about 147 antisense compounds were selected for *in vitro* dose-dependent inhibition studies to determine their half maximal inhibitory concentration (IC_{50}) (Examples 122-125). Of the about 147 new antisense compounds from tested for their half maximal inhibitory concentration (IC_{50}), about 73 antisense compounds that demonstrated potent dose-dependent reduction of ANGPTL3 were selected for *in vivo* potency and tolerability (e.g., ALT and AST) testing in mice (Examples 126-127) with antisense compound 233710 used as a benchmark.

Of the about 111 antisense compounds from screens one and two that were tested for potency and tolerability in mice, 24 were selected for more extensive tolerability testing in mice by assessing liver metabolic markers, such as alanine transaminase (ALT), aspartate transaminase (AST), albumin and bilirubin, as well as kidney metabolic markers BUN and creatinine and organ weight (Example 127).

In parallel with the *in vivo* murine studies seventeen antisense compounds were selected for viscosity testing (Example 128). Generally, antisense compounds that were not optimal for viscosity were not taken forward in further studies.

Based on the results of the mice tolerability study, twenty antisense compounds were selected for *in vivo* tolerability testing in rats (Example 129). In the rats, liver metabolic markers, such as ALT, AST, albumin and bilirubin, body and organ weights, as well as kidney metabolic markers, such as BUN, creatinine and total protein/creatinine ratio, were measured to determine the tolerability of a compound *in vivo*.

The twenty antisense compounds tested in the rats were also assessed for cross-reactivity to a rhesus monkey ANGPTL3 gene sequence (Example 130). Although the antisense compounds in this study were tested in cynomolgus monkeys, the cynomolgus monkey ANGPTL3 sequence was not available for comparison to the sequences of the full-length compounds, therefore the sequences of the antisense compounds were compared to that of the closely related rhesus monkey. The sequences of eight antisense compounds were found to have 0-2 mismatches with the rhesus ANGPTL3 gene sequence and were further studied in cynomolgus monkeys (Example 130). The eight antisense compounds (ISIS 563580, ISIS 560400, ISIS 567320, ISIS 567321, ISIS 544199, ISIS 567233, ISIS 561011 and ISIS 559277) were tested for inhibition of ANGPTL3 mRNA and protein expression as well as tolerability in the monkeys. In the tolerability studies, body weights, liver metabolic markers (ALT, AST and bilirubin), kidney metabolic

markers (BUN and creatinine), hematology parameters (blood cell counts, hemoglobin and hematocrit), and pro-inflammatory markers (CRP and C3) were measured. Additionally, the full-length oligonucleotide concentration present in liver and kidney was measured and the ratio of full-length oligonucleotide in the kidney/liver was calculated.

The sequence of a potent and tolerable antisense compound, ISIS 563580, assessed in cynomolgus monkeys was further modified with a GalNAc conjugate and/or changes in the backbone chemistry as shown in Examples 113-115 and 131 and evaluated for increase potency.

Accordingly, provided herein are antisense compounds with any one or more improved characteristics e.g., improved relative to the antisense compounds described in WO 2011/085271 and in US Serial Number 61/920,652. In certain embodiments, provided herein are antisense compounds comprising a modified oligonucleotide as described herein targeted to, or specifically hybridizable with, a region of nucleotides of any one of SEQ ID NOs: 1-2.

In certain embodiments, certain antisense compounds as described herein are efficacious by virtue of their potency in inhibiting ANGPTL3 expression. In certain embodiments, the compounds or compositions inhibit ANGPTL3 by at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95%.

In certain embodiments, certain antisense compounds as described herein are efficacious by virtue of an *in vitro* IC_{50} of less than 20 μM, less than 10 μM, less than 8 μM, less than 5μM, less than 2 μM, less than 1 μM, less than 0.9 μM, less than 0.8 μM, less than 0.7 μM, less than 0.6 μM, or less than 0.5 μM when tested in human cells, for example, in the Hep3B cell line (as described in Examples 117-118 and 122-125). In certain embodiments, preferred antisense compounds having an IC_{50} <1.0 μM include SEQ ID NOs: 15, 20, 24, 34, 35, 36, 37, 42, 43, 44, 47, 50, 51, 57, 58, 60, 77, 79, 82, 87, 88, 90, 91, 93, 94, 100, 101, 104, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 169, 170, 177, 188, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, and 232. In certain embodiments, preferred antisense compounds having an IC_{50} <0.9 μM include SEQ ID NOs: 15, 20, 35, 36, 42, 43, 44, 50, 57, 60, 77, 79, 87, 88, 90, 91, 93, 94, 101, 104, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 177, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, and 232. In certain embodiments, preferred antisense compounds having an IC_{50} <0.8 μM include SEQ ID NOs: 15, 20, 35, 36, 42, 43, 44, 50, 57, 60, 77, 79, 87, 88, 90, 91, 93, 94, 101, 104, 110, 111, 112, 113, 114, 115, 116, 117, 118, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 177, 209, 210, 211, 212, 213, 214, 215, 217, 218, 219, 220, 221, 222, 223, 224, 225, 228, 229, 230, 231, and 232. In certain embodiments, preferred antisense compounds having

an IC_{50} <0.7 μM include SEQ ID NOs: 15, 20, 36, 42, 43, 57, 60, 114, 117, 127, 131, 177, 209, 210, 211, 212, 213, 214, 215, 217, 218, 219, 220, 221, 222, 223, 224, 225, 228, 229, 230, 231, and 232. In certain embodiments, preferred antisense compounds having an IC_{50} <0.6 μM include SEQ ID NOs: 15, 20, 36, 42, 43, 57, 60, 114, 117, 127, 131, 177, 209, 210, 211, 212, 213, 215, 217, 218, 219, 220, 221, 222, 224, 225, 228, 229, 230, 231, and 232. In certain embodiments, preferred antisense compounds having an IC_{50} <0.5 μM include SEQ ID NOs: 43, 114, 117, 127, 131, 177, 209, 210, 211, 212, 215, 217, 218, 219, 220, 221, 222, 229, 230, and 232.

In certain embodiments, certain antisense compounds as described herein are efficacious by virtue of having a viscosity of less than 40 cP, less than 35 cP, less than 30 cP, less than 25 cP, less than 20 cP, less than 15 cP, or less than 10 cP when measured by an assay (as described in Example 128). Oligonucleotides having a viscosity greater than 40 cP would have less than optimal viscosity. In certain embodiments, preferred antisense compounds having a viscosity <20 cP include SEQ ID NOs: 16, 18, 20, 34, 35, 36, 38, 49, 77, 90, 93, and 94. In certain embodiments, preferred antisense compounds having a viscosity <15 cP include SEQ ID NOs: 16, 18, 20, 34, 35, 38, 49, 90, 93, and 94. In certain embodiments, preferred antisense compounds having a viscosity <10 cP include SEQ ID NOs: 18, 34, 35, 49, 90, 93, and 94.

In certain embodiments, certain antisense compounds as described herein are highly tolerable, as demonstrated by the *in vivo* tolerability measurements described in the examples. In certain embodiments, the certain antisense compounds as described herein are highly tolerable, as demonstrated by having an increase in ALT and/or AST value of no more than 3 fold, 2 fold or 1.5 fold over saline treated animals.

In certain embodiments, certain antisense compounds as described herein are efficacious by virtue of having one or more of an inhibition potency of greater than 50%, an *in vitro* IC_{50} of less than 1 μM, a viscosity of less than 20 cP, and no more than a 3 fold increase in ALT and/or AST.

In certain embodiments, ISIS 563580 (SEQ ID NO: 77) is preferred. This compound was found to be a potent inhibitor in ANGPTL3 transgenic mice and the most tolerable antisense compound. It had an acceptable viscosity of about 16.83 cP and an IC_{50} value of <0.8 μM *in vitro*. In mice it had no more than a 3 fold increase in ALT and/or AST levels over saline treated animals. Also, in monkeys, it was among the most tolerable and potent compounds in inhibiting ANGPTL3 and had the best ratio of full-length oligonucleotide concentration.

In certain embodiments, ISIS 544199 (SEQ ID NO: 20) is preferred. This compound was found to be a potent and tolerable antisense compound. It had an acceptable viscosity of 1.7 cP and an IC_{50} value of <0.5 μM *in vitro*. In mice it had no more than a 3 fold increase in ALT and/or AST levels over saline treated animals. Also, in monkeys, it was among the most potent compounds in inhibiting ANGPTL3 and had a good ratio of full-length oligonucleotide concentration.

In certain embodiments, ISIS 559277 (SEQ ID NO: 110) is preferred. This compound was found to be a potent and tolerable antisense compound. It had an IC_{50} value of <0.8 μM *in vitro*. In mice it had no more than a 3 fold increase in ALT and/or AST levels over saline treated animals. Also, in monkeys, it was

among the most potent compounds in inhibiting ANGPTL3 and had a good ratio of full-length oligonucleotide concentration.

In certain embodiments, a GalNAc conjugated antisense compound, ISIS 658501 (SEQ ID NO: 4912), is preferred. This antisense compound was found to be more potent than its parent compound ISIS 563580 (SEQ ID NO: 77) as shown by the inhibition levels.

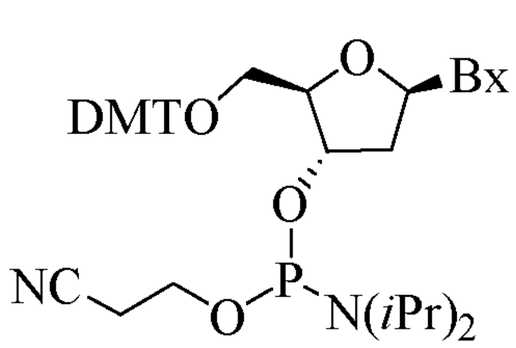
In certain embodiments, a GalNAc conjugated antisense compound, ISIS 703801 (SEQ ID NO: 77), is preferred. This antisense compound was found to be several fold more potent than its parent compound ISIS 563580 (SEQ ID NO: 77). ISIS 703801 had an ID50 value of 1 while ISIS 563580 had an ID50 value of 6.

In certain embodiments, a GalNAc conjugated antisense compound, ISIS 703802 (SEQ ID NO: 77), is preferred. This antisense compound was found to be several fold more potent than its parent compound ISIS 563580 (SEQ ID NO: 77). ISIS 703802 had an ID50 value of 0.3 while ISIS 563580 had an ID50 value of 6.

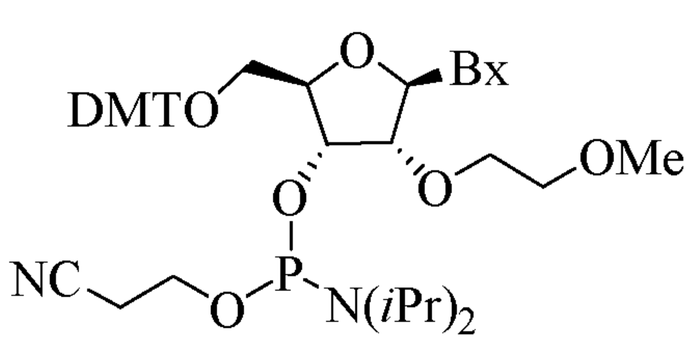
EXAMPLES

The following examples illustrate certain embodiments of the present disclosure and are not limiting. Moreover, where specific embodiments are provided, the inventors have contemplated generic application of those specific embodiments. For example, disclosure of an oligonucleotide having a particular motif provides reasonable support for additional oligonucleotides having the same or similar motif. And, for example, where a particular high-affinity modification appears at a particular position, other high-affinity modifications at the same position are considered suitable, unless otherwise indicated.

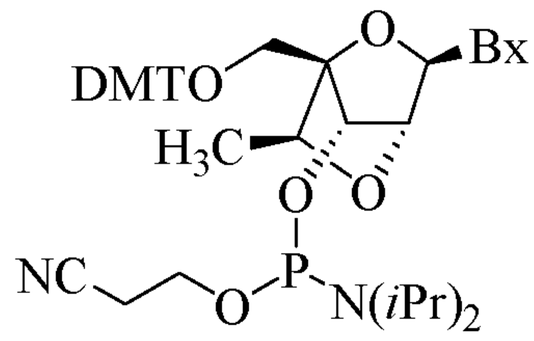
Example 1: General Method for the Preparation of Phosphoramidites, Compounds 1, 1a and 2



1



1a

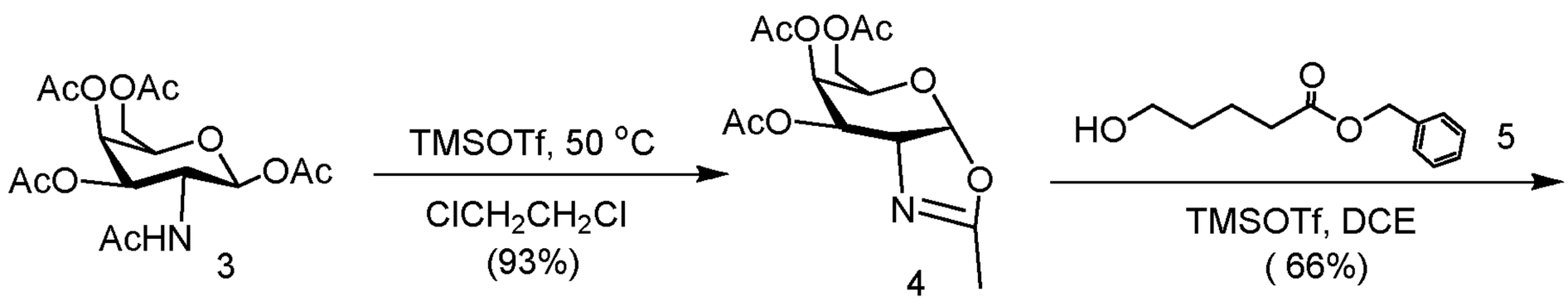


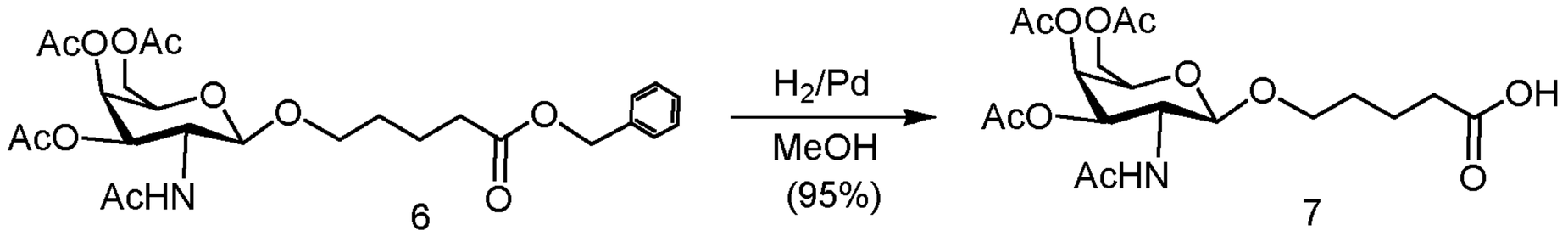
2

Bx is a heterocyclic base;

Compounds 1, 1a and 2 were prepared as per the procedures well known in the art as described in the specification herein (see Seth et al., Bioorg. Med. Chem., 2011, 21(4), 1122-1125, J. Org. Chem., 2010, 75(5), 1569-1581, Nucleic Acids Symposium Series, 2008, 52(1), 553-554); and also see published PCT International Applications (WO 2011/115818, WO 2010/077578, WO2010/036698, WO2009/143369, WO 2009/006478, and WO 2007/090071), and US patent 7,569,686).

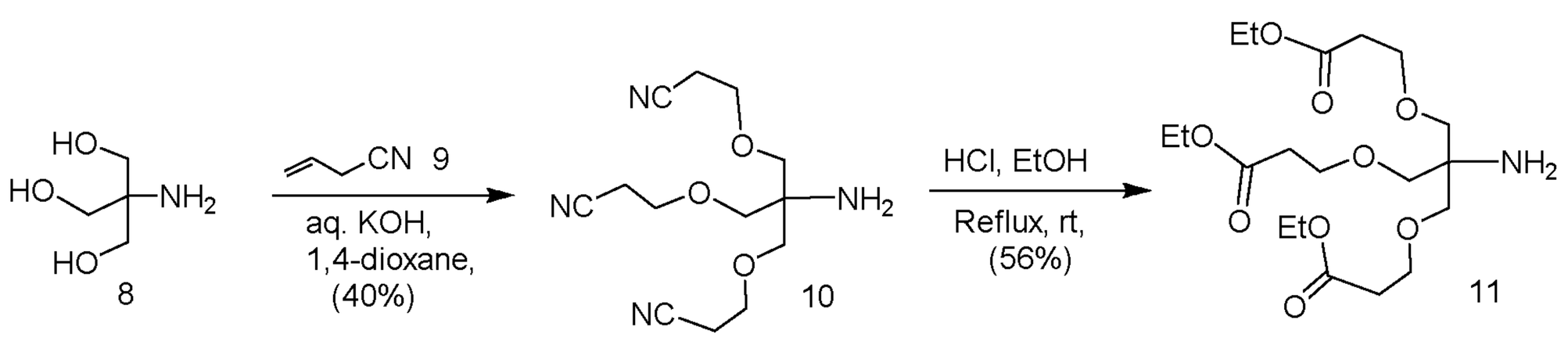
Example 2: Preparation of Compound 7





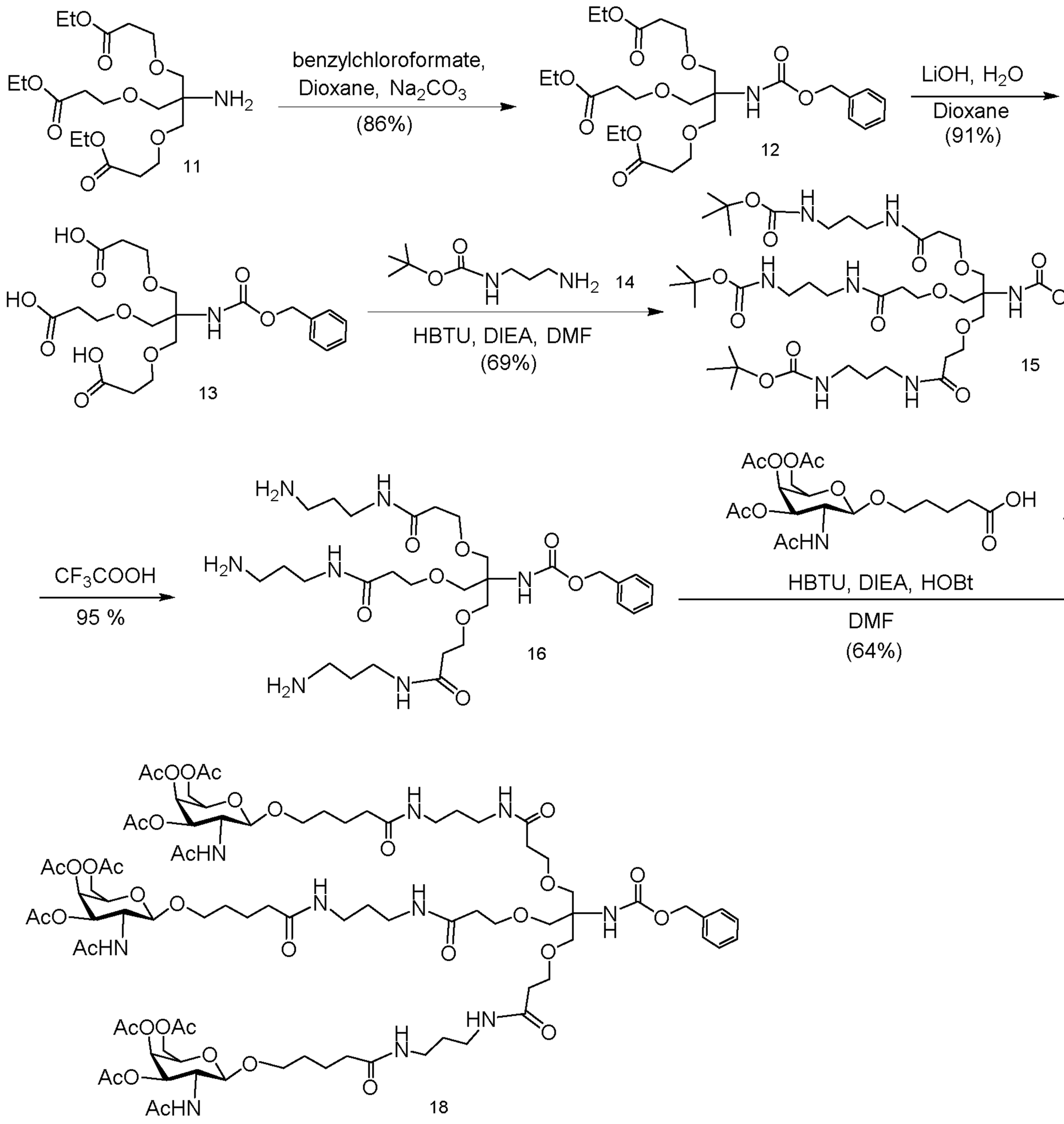
Compounds 3 (2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-Dgalactopyranose or galactosamine pentaacetate) is commercially available. Compound 5 was prepared according to published procedures (Weber *et al., J. Med. Chem.*, 1991, *34*, 2692).

Example 3: Preparation of Compound 11



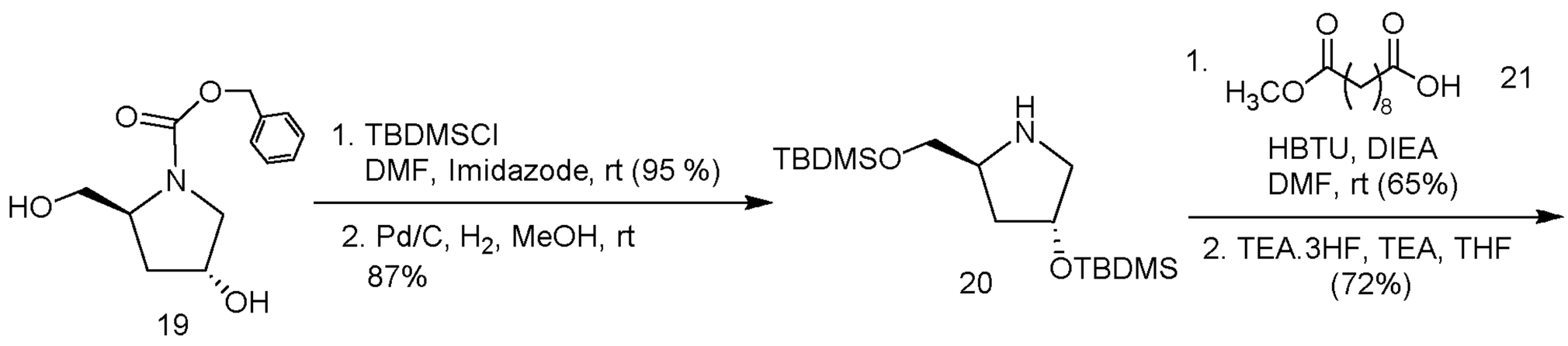
Compounds 8 and 9 are commercially available.

Example 4: Preparation of Compound 18



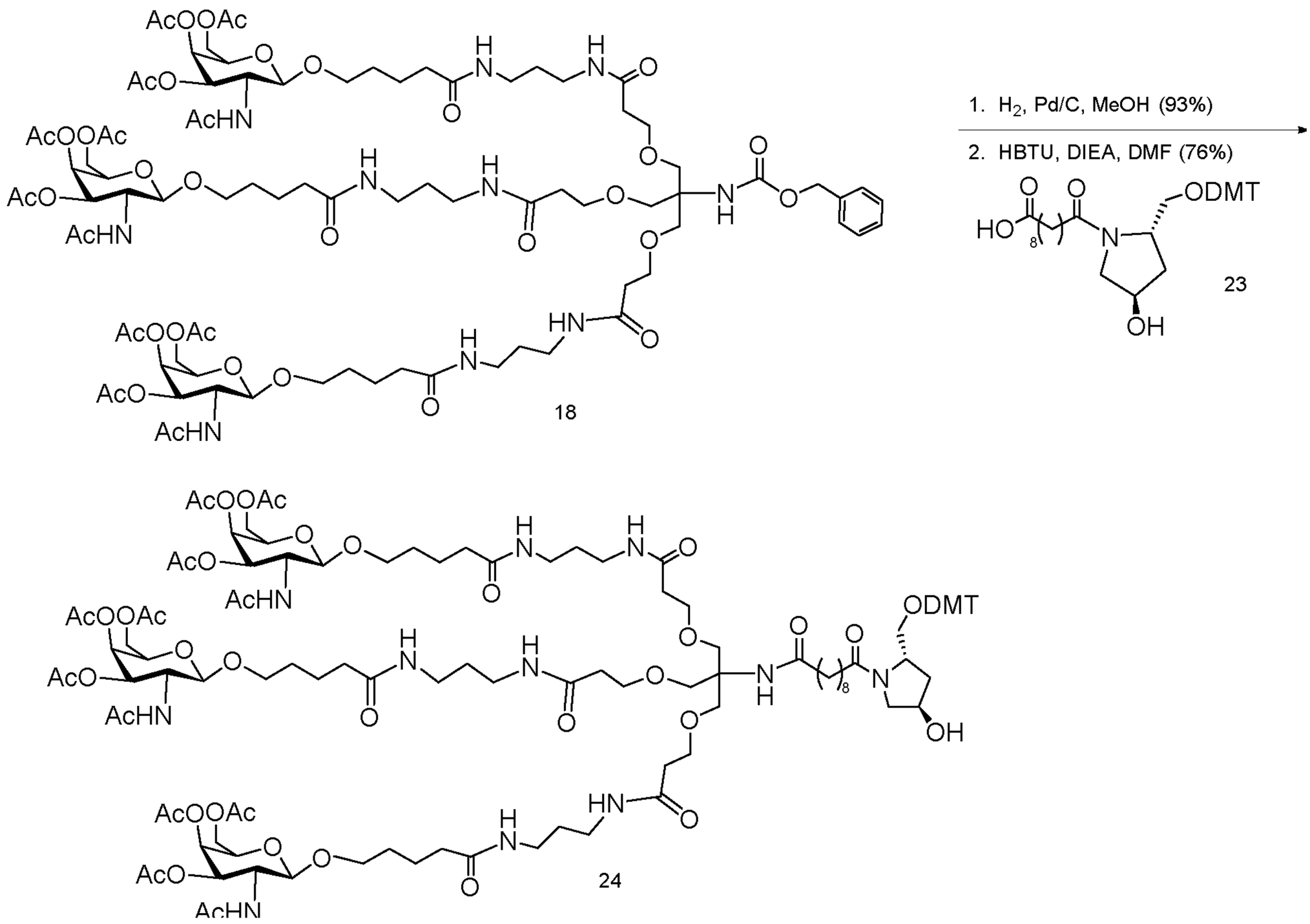
Compound 11 was prepared as per the procedures illustrated in Example 3. Compound 14 is commercially available. Compound 17 was prepared using similar procedures reported by Rensen *et al., J. Med. Chem.*, 2004, *47*, 5798-5808.

Example 5: Preparation of Compound 23



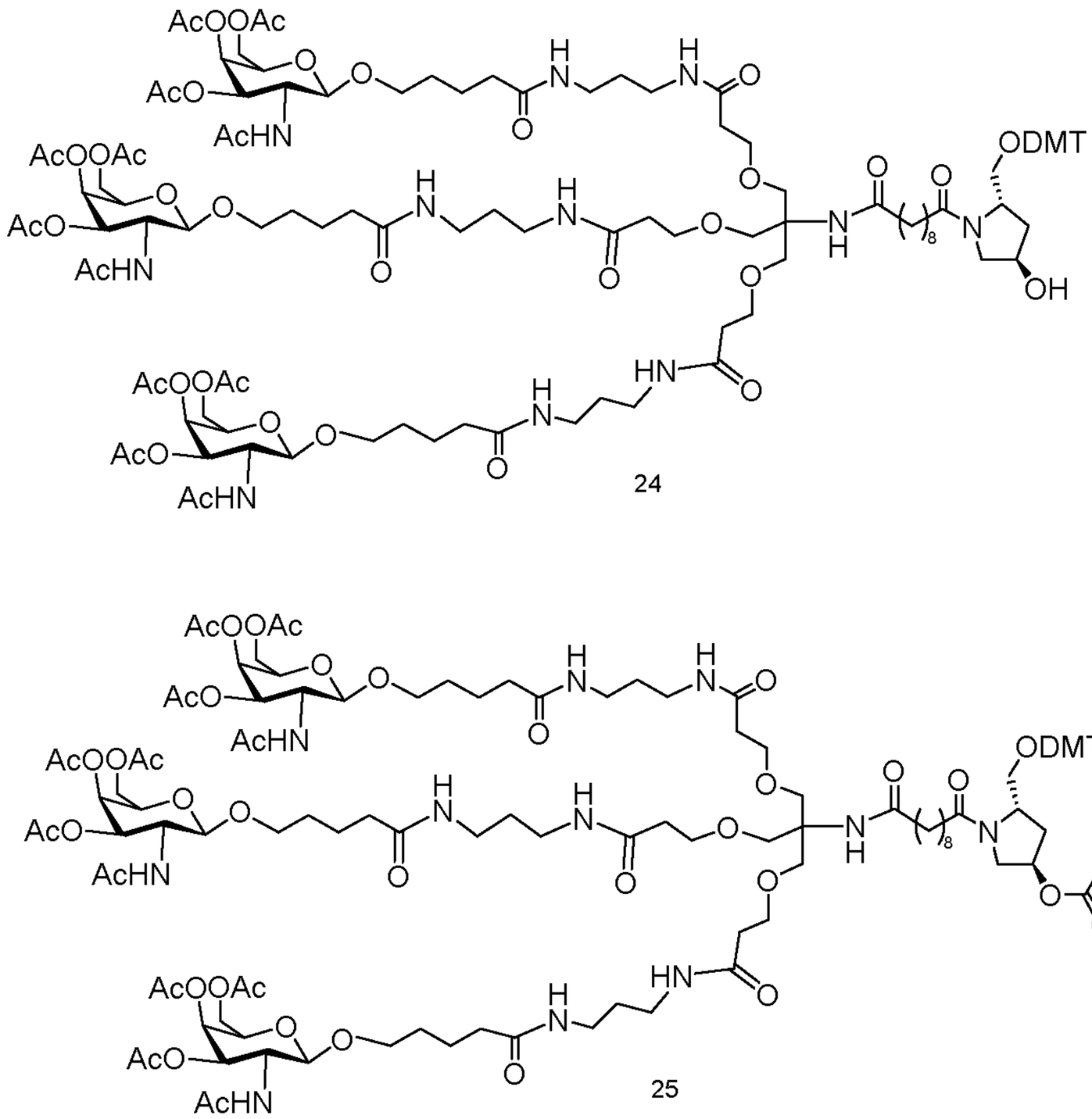
Compounds 19 and 21 are commercially available.

Example 6: Preparation of Compound 24



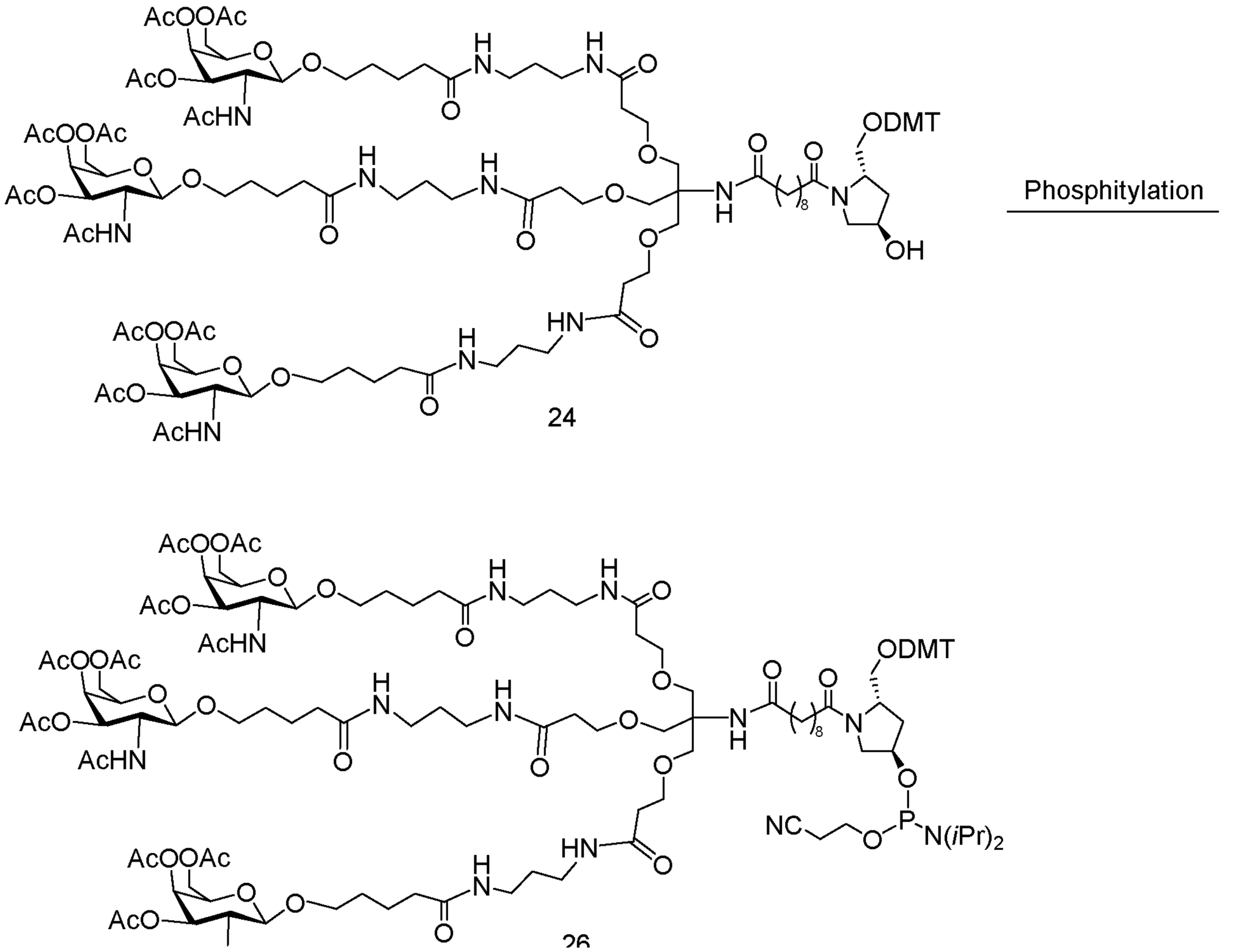
Compounds 18 and 23 were prepared as per the procedures illustrated in Examples 4 and 5.

Example 7: Preparation of Compound 25



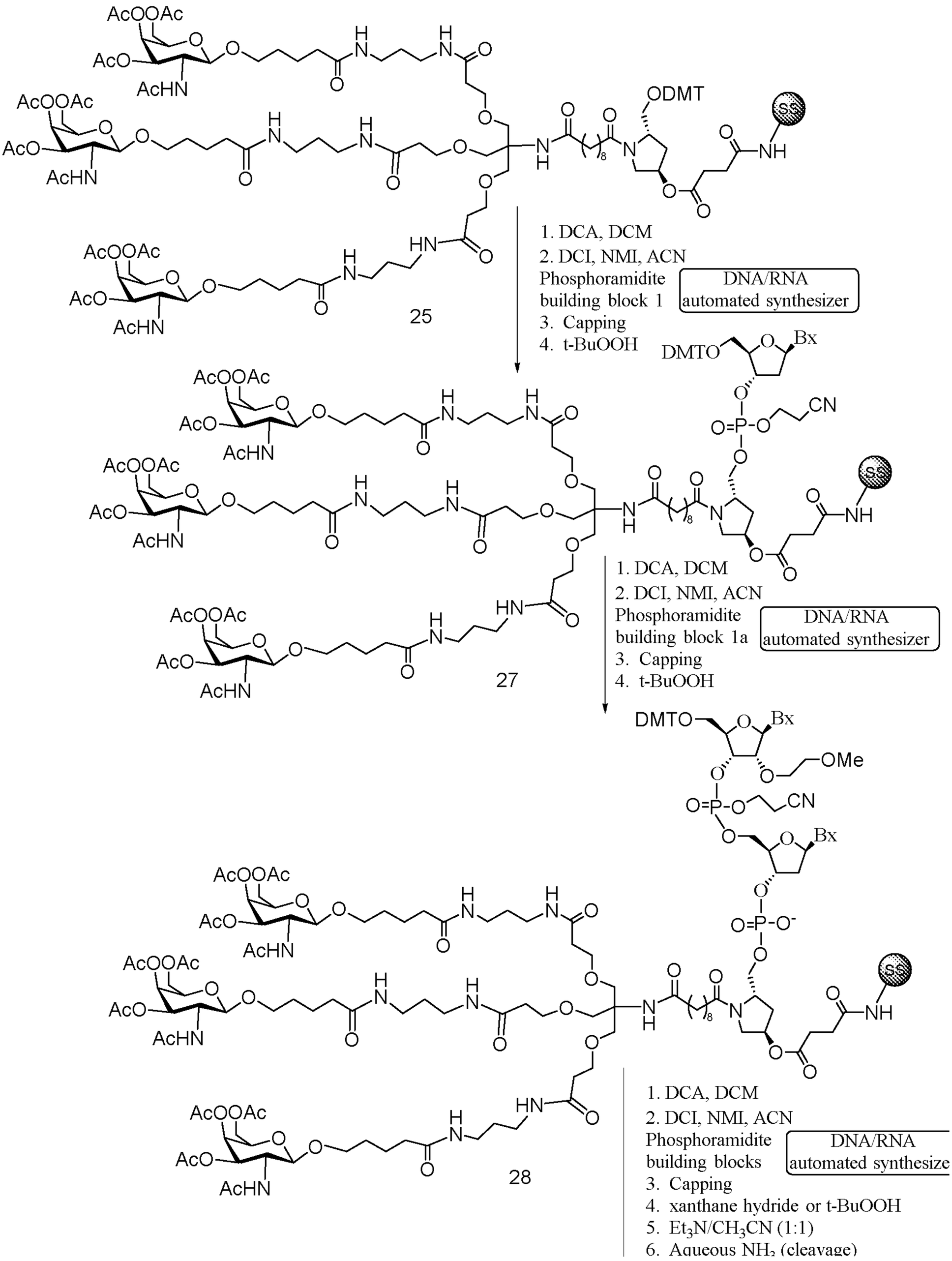
Compound 24 was prepared as per the procedures illustrated in Example 6.

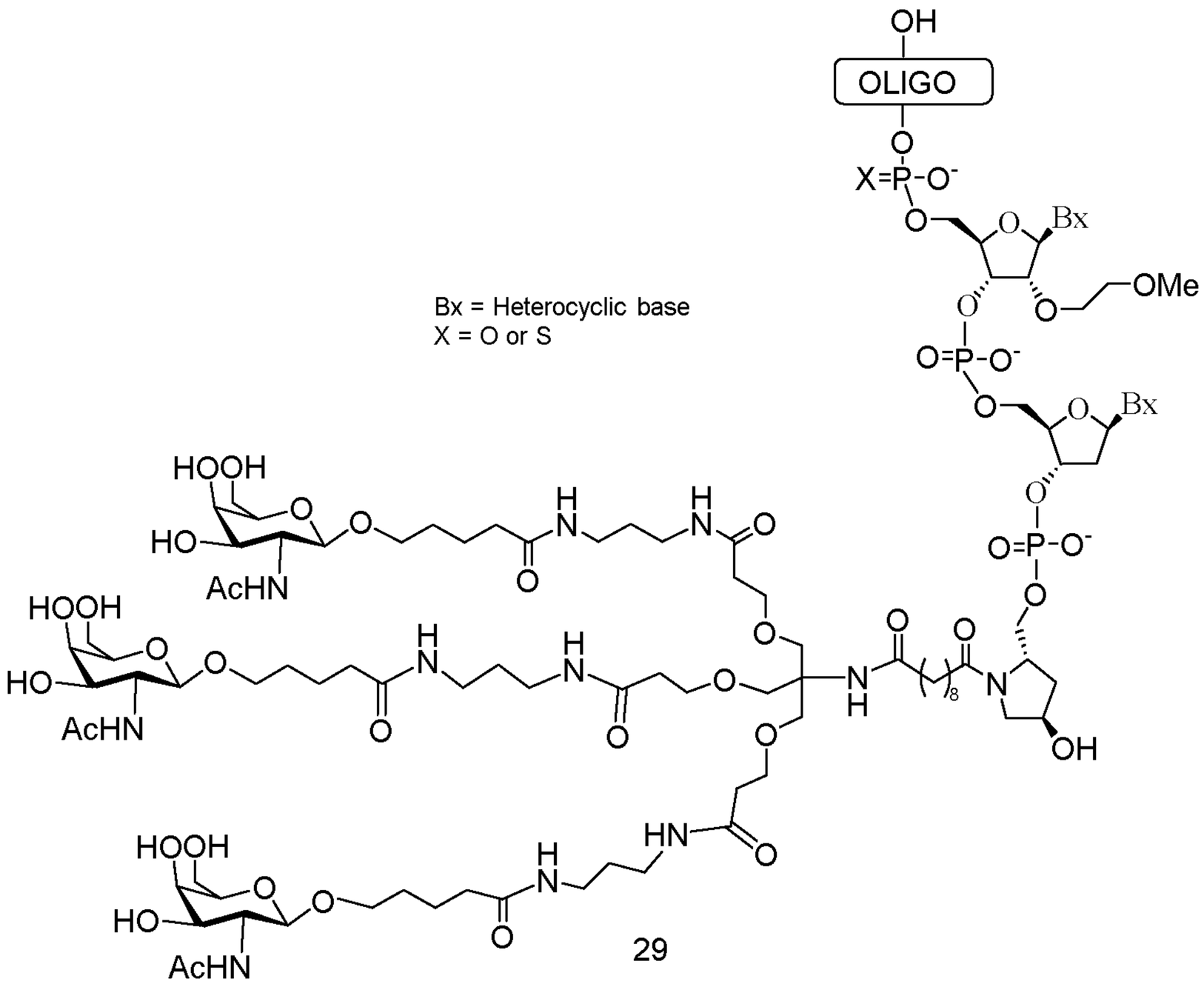
Example 8: Preparation of Compound 26



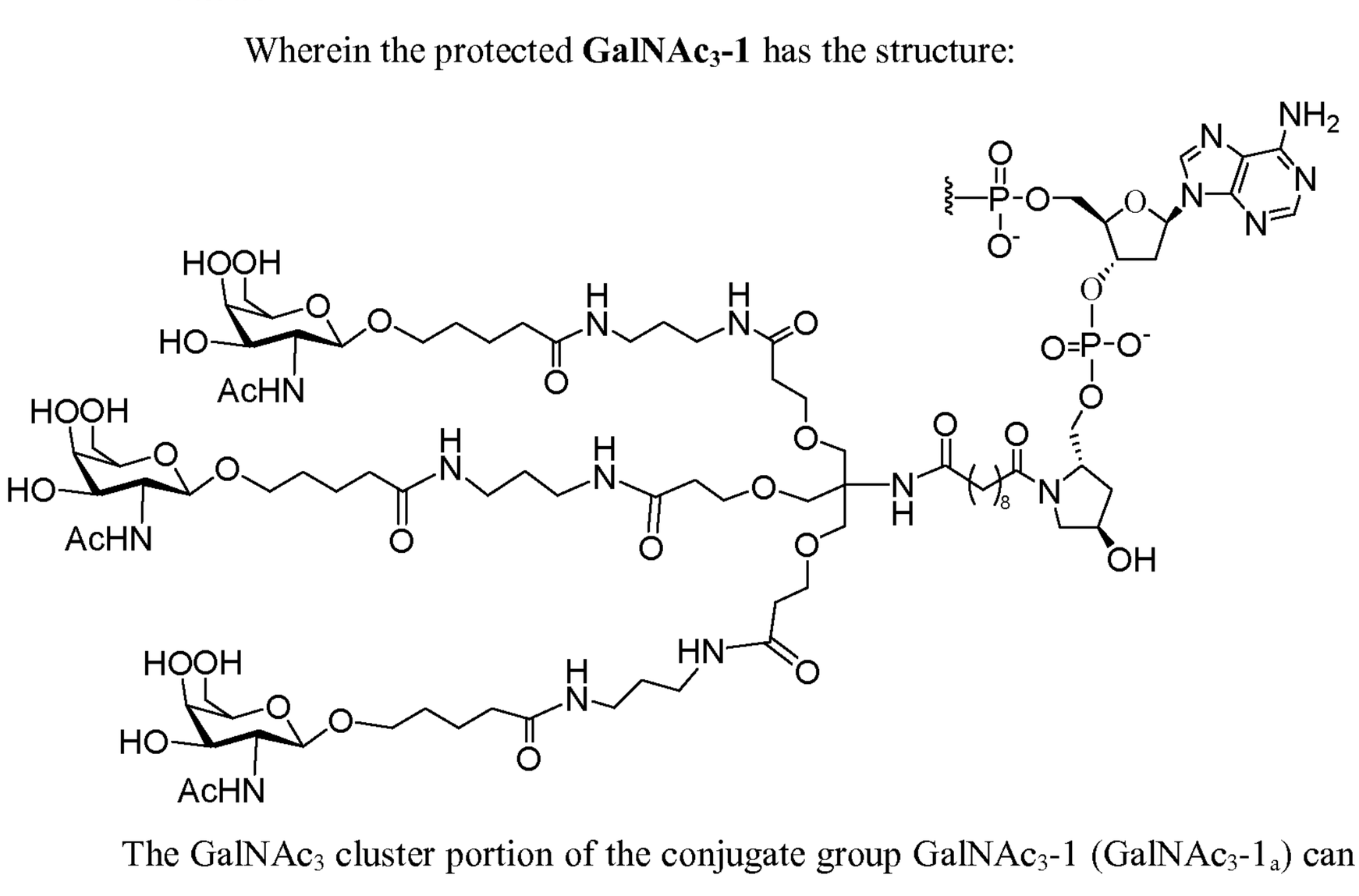
Compound 24 is prepared as per the procedures illustrated in Example 6.

Example 9: General preparation of conjugated ASOs comprising $GalNAc_3$-1 at the 3' terminus, Compound 29

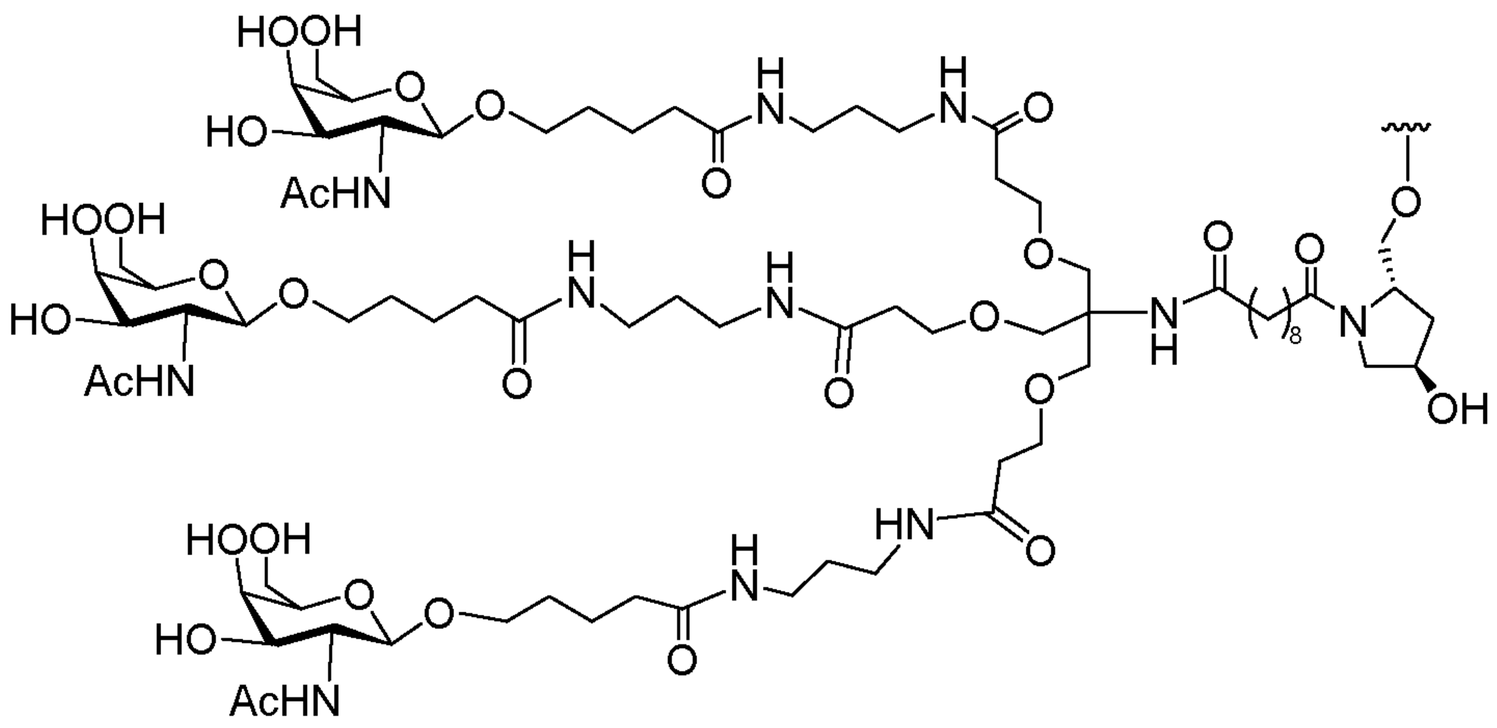




Wherein the protected **GalNAc$_3$-1** has the structure:

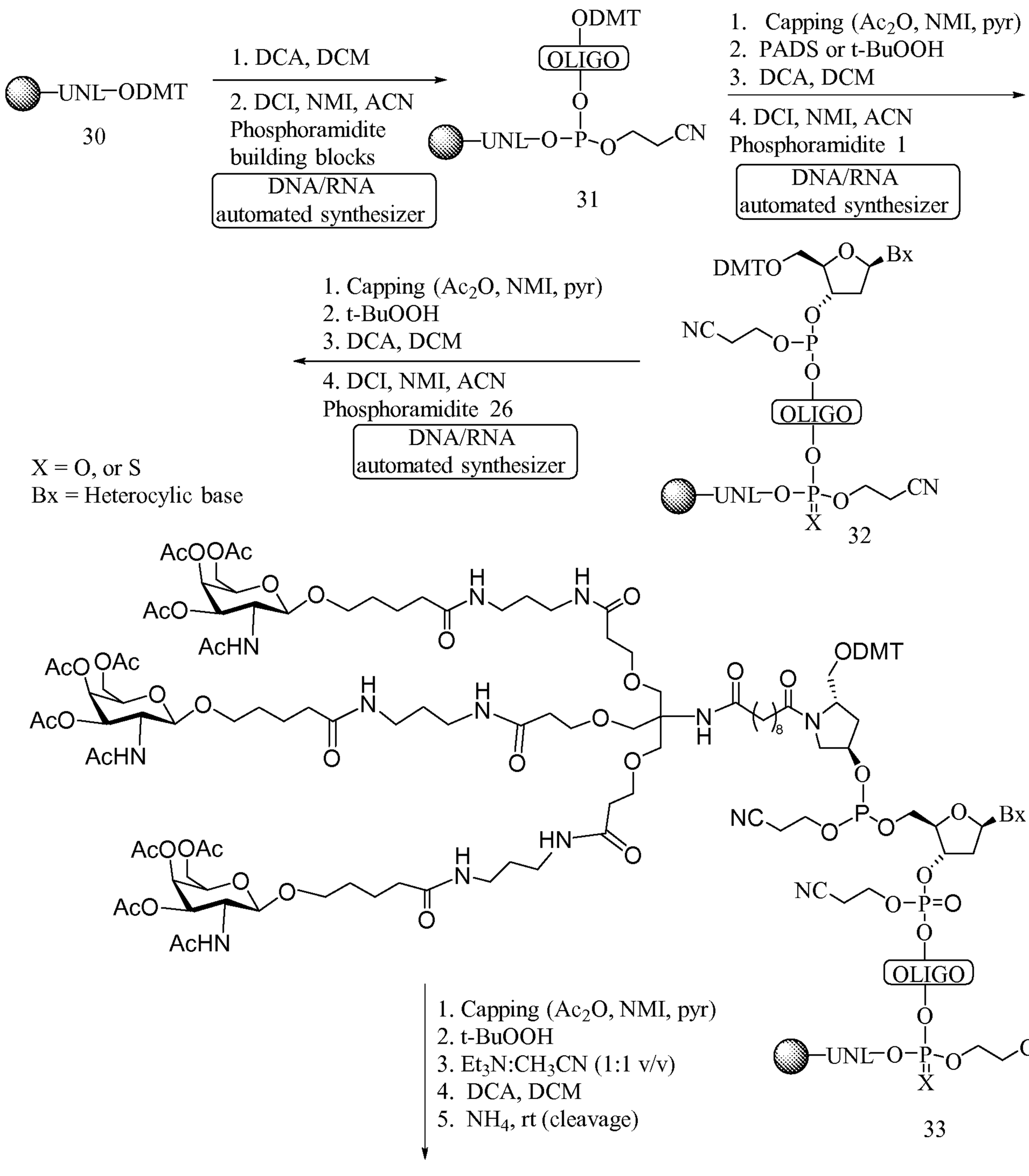


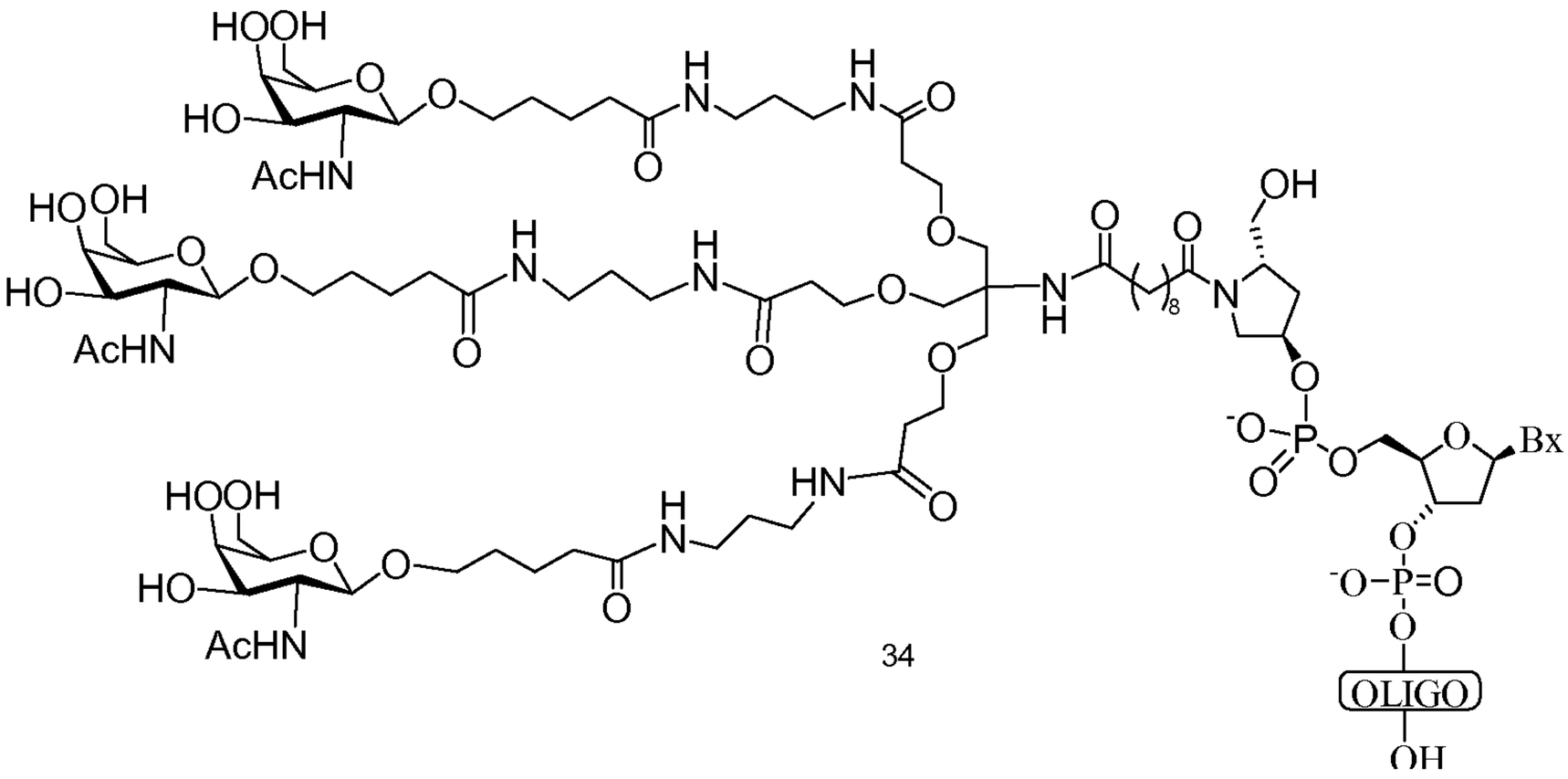
The GalNAc$_3$ cluster portion of the conjugate group GalNAc$_3$-1 (GalNAc$_3$-1$_a$) can be combined with any cleavable moiety to provide a variety of conjugate groups. Wherein GalNAc$_3$-1$_a$ has the formula:



The solid support bound protected **GalNAc$_3$-1**, Compound 25, was prepared as per the procedures illustrated in Example 7. Oligomeric Compound 29 comprising **GalNAc$_3$-1** at the 3' terminus was prepared using standard procedures in automated DNA/RNA synthesis (see Dupouy *et al., Angew. Chem. Int. Ed.,* 2006, *45,* 3623-3627). Phosphoramidite building blocks, Compounds 1 and 1a were prepared as per the procedures illustrated in Example 1. The phosphoramidites illustrated are meant to be representative and not intended to be limiting as other phosphoramidite building blocks can be used to prepare oligomeric compounds having a predetermined sequence and composition. The order and quantity of phosphoramidites added to the solid support can be adjusted to prepare gapped oligomeric compounds as described herein. Such gapped oligomeric compounds can have predetermined composition and base sequence as dictated by any given target.

Example 10: General preparation conjugated ASOs comprising GalNAc$_3$-1 at the 5' terminus, Compound 34

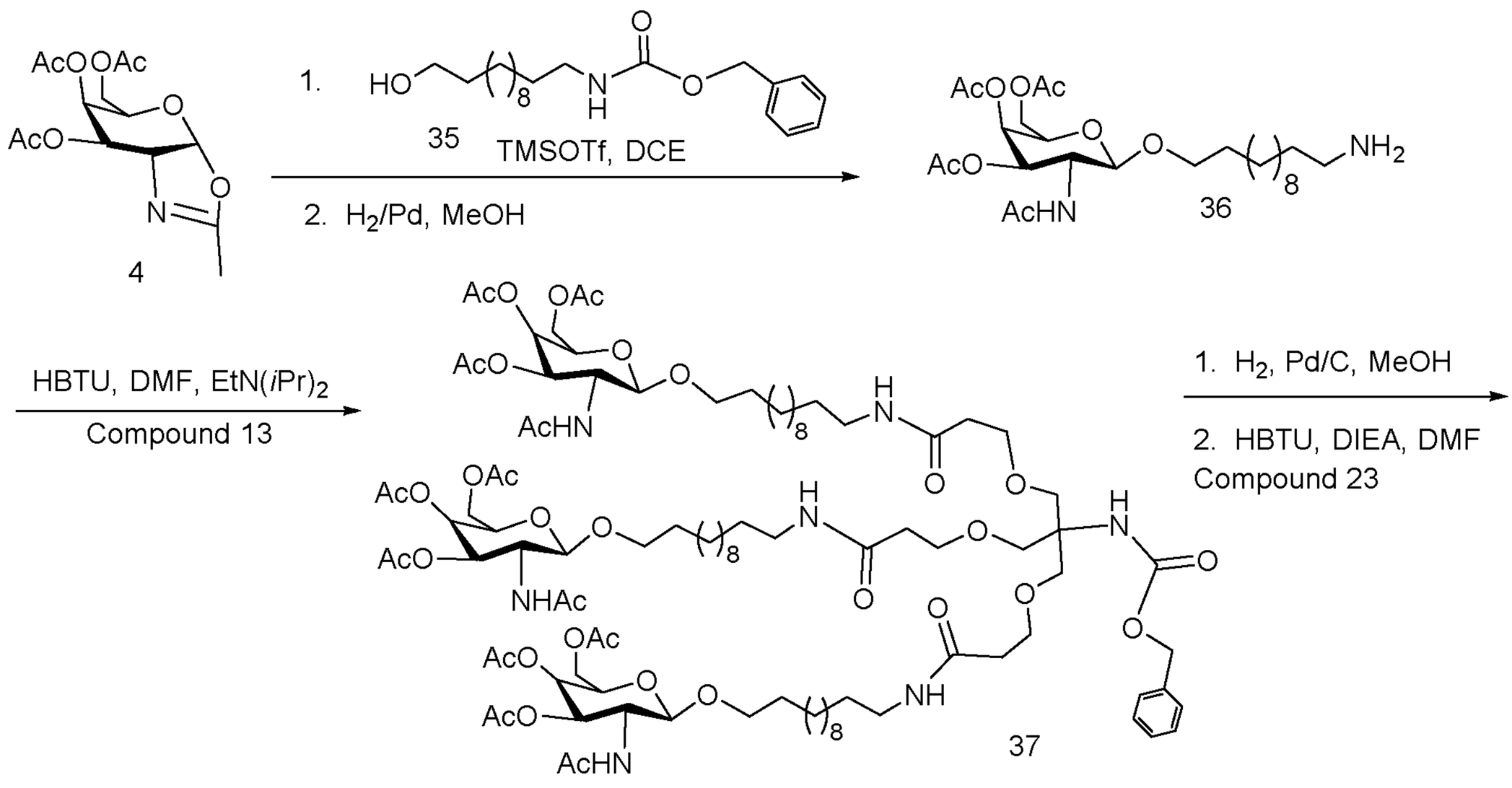


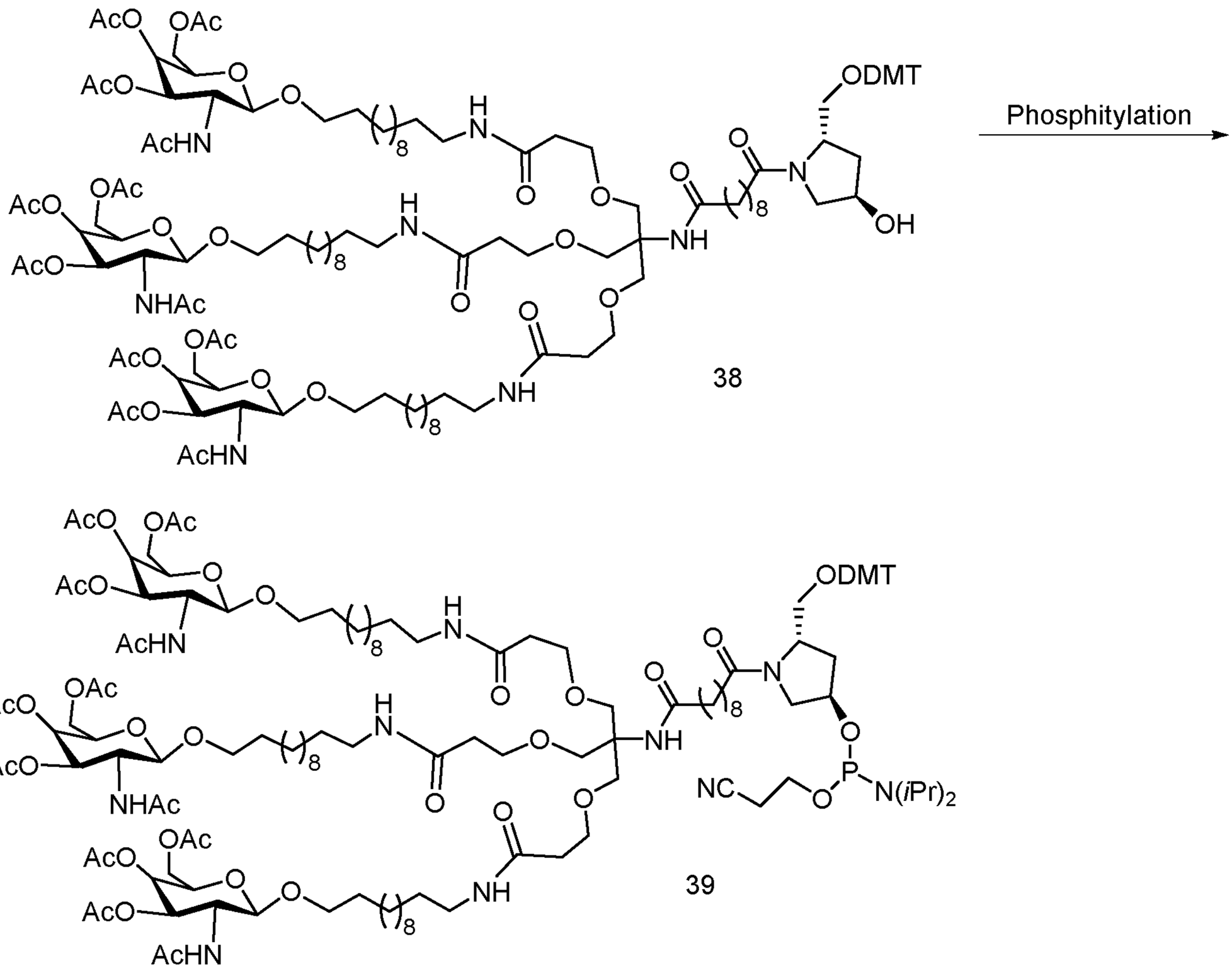


34

The Unylinker™ 30 is commercially available. Oligomeric Compound 34 comprising a **GalNAc$_3$-1** cluster at the 5' terminus is prepared using standard procedures in automated DNA/RNA synthesis (see Dupouy *et al., Angew. Chem. Int. Ed.,* 2006, *45,* 3623-3627). Phosphoramidite building blocks, Compounds 1 and 1a were prepared as per the procedures illustrated in Example 1. The phosphoramidites illustrated are meant to be representative and not intended to be limiting as other phosphoramidite building blocks can be used to prepare an oligomeric compound having a predetermined sequence and composition. The order and quantity of phosphoramidites added to the solid support can be adjusted to prepare gapped oligomeric compounds as described herein. Such gapped oligomeric compounds can have predetermined composition and base sequence as dictated by any given target.

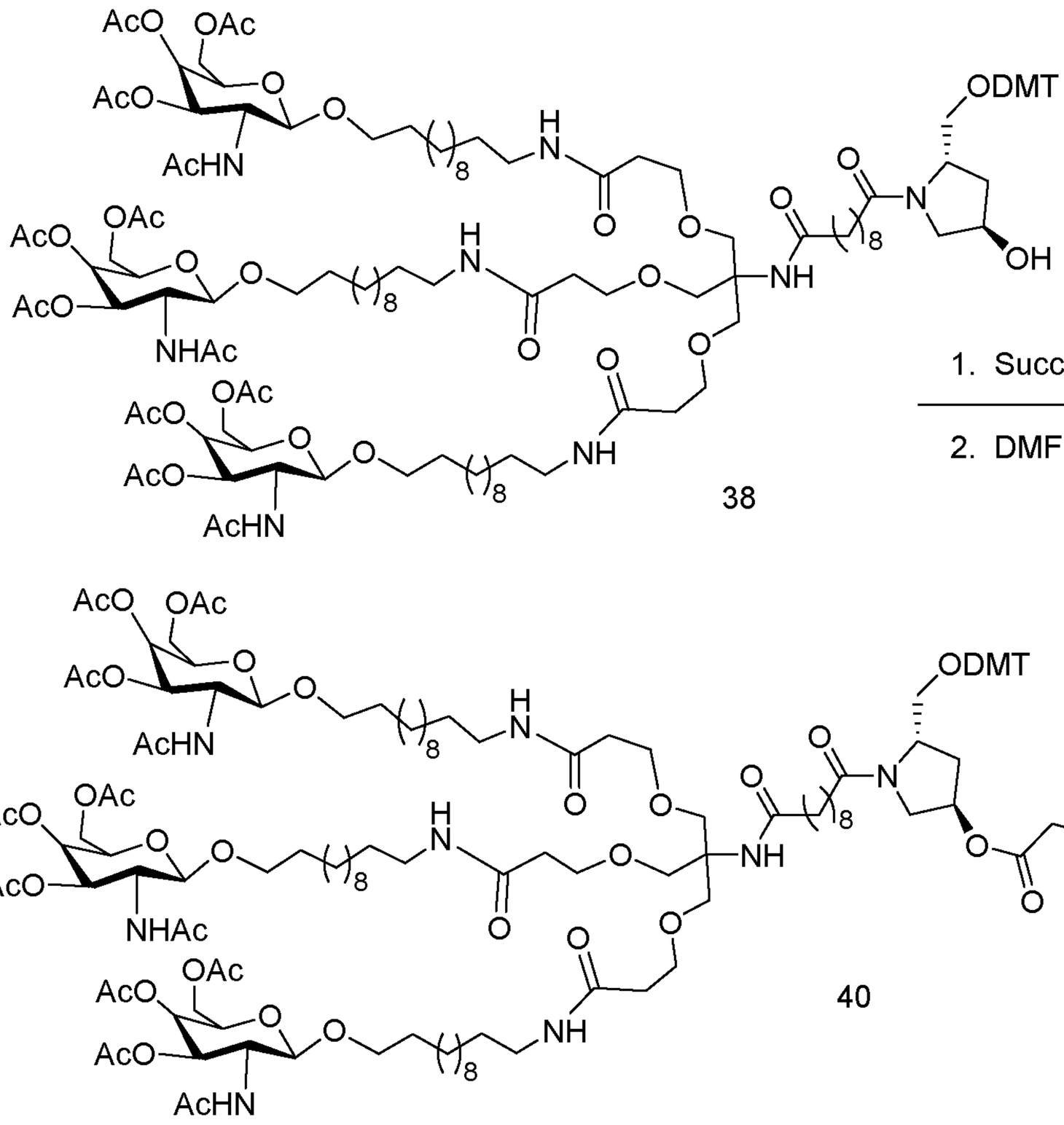
Example 11: Preparation of Compound 39





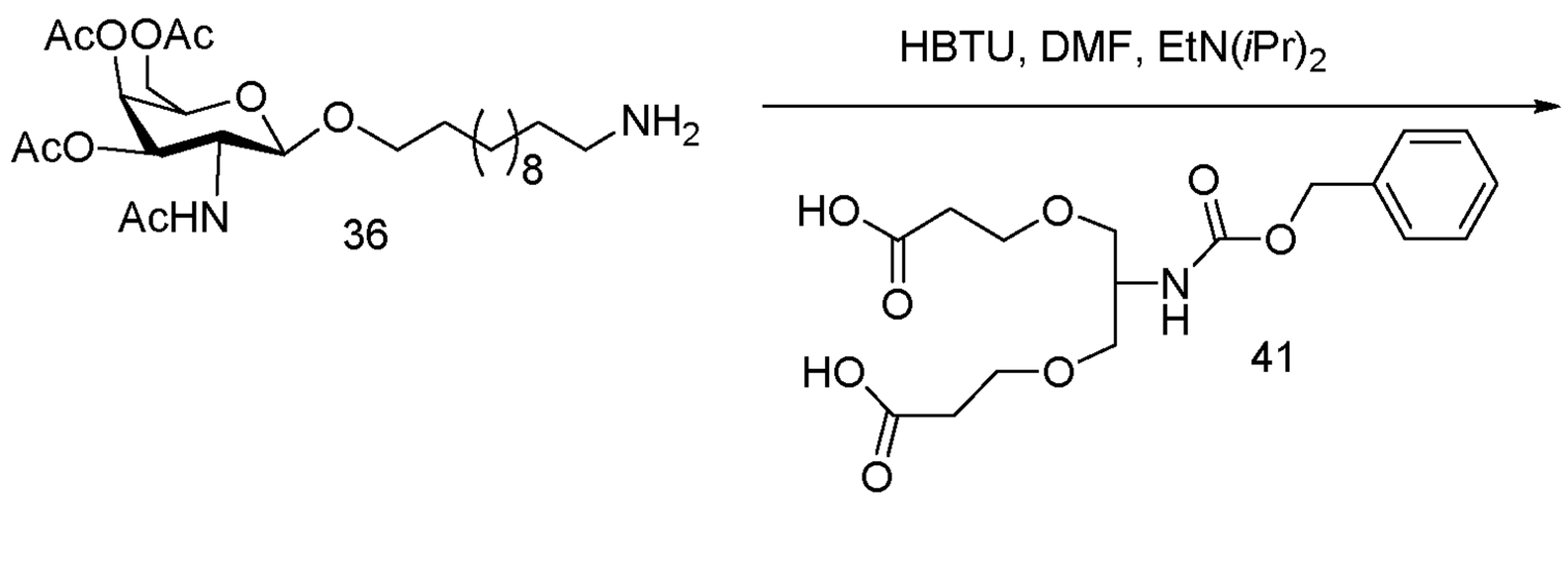
Compounds 4, 13 and 23 were prepared as per the procedures illustrated in Examples 2, 4, and 5. Compound 35 is prepared using similar procedures published in Rouchaud *et al., Eur. J. Org. Chem.,* 2011, *12*, 2346-2353.

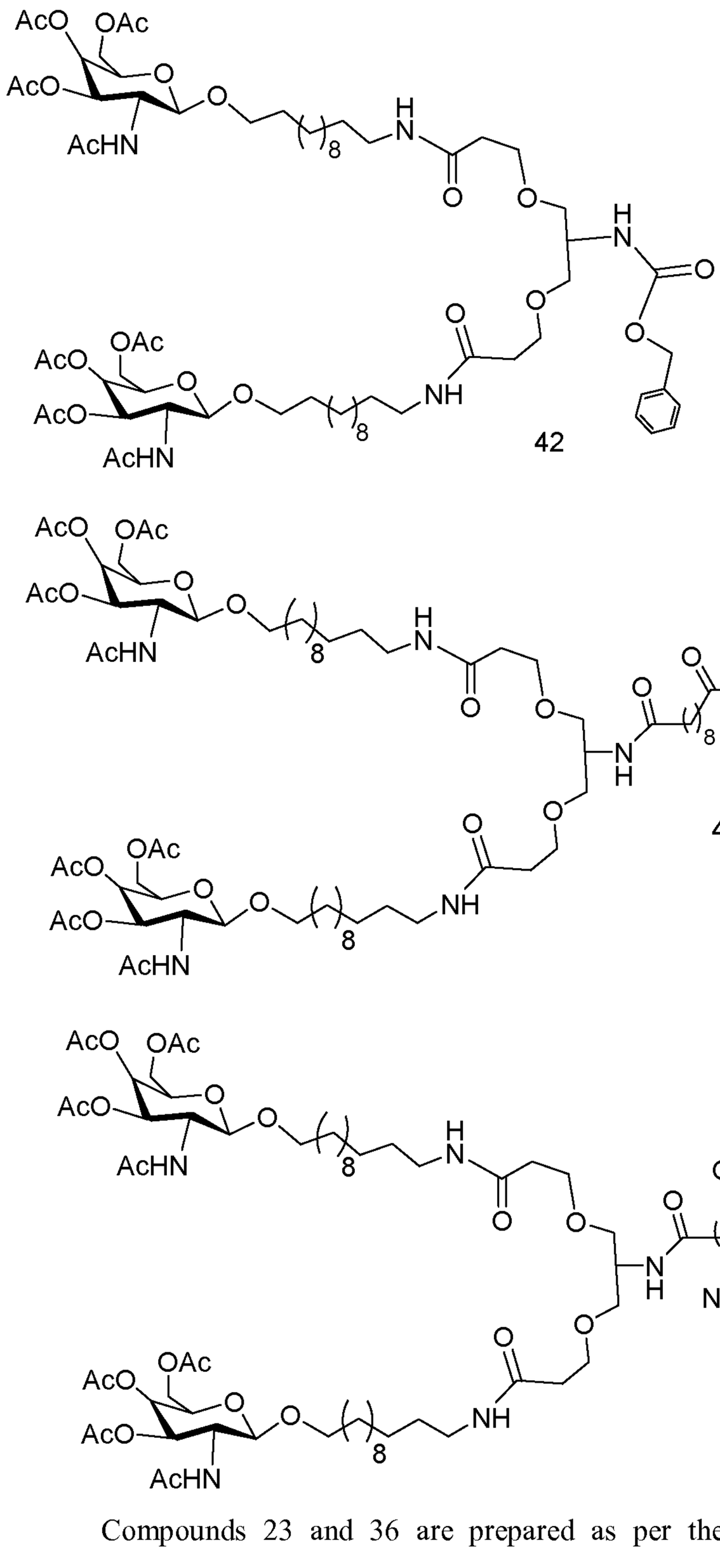
Example 12: Preparation of Compound 40



Compound 38 is prepared as per the procedures illustrated in Example 11.

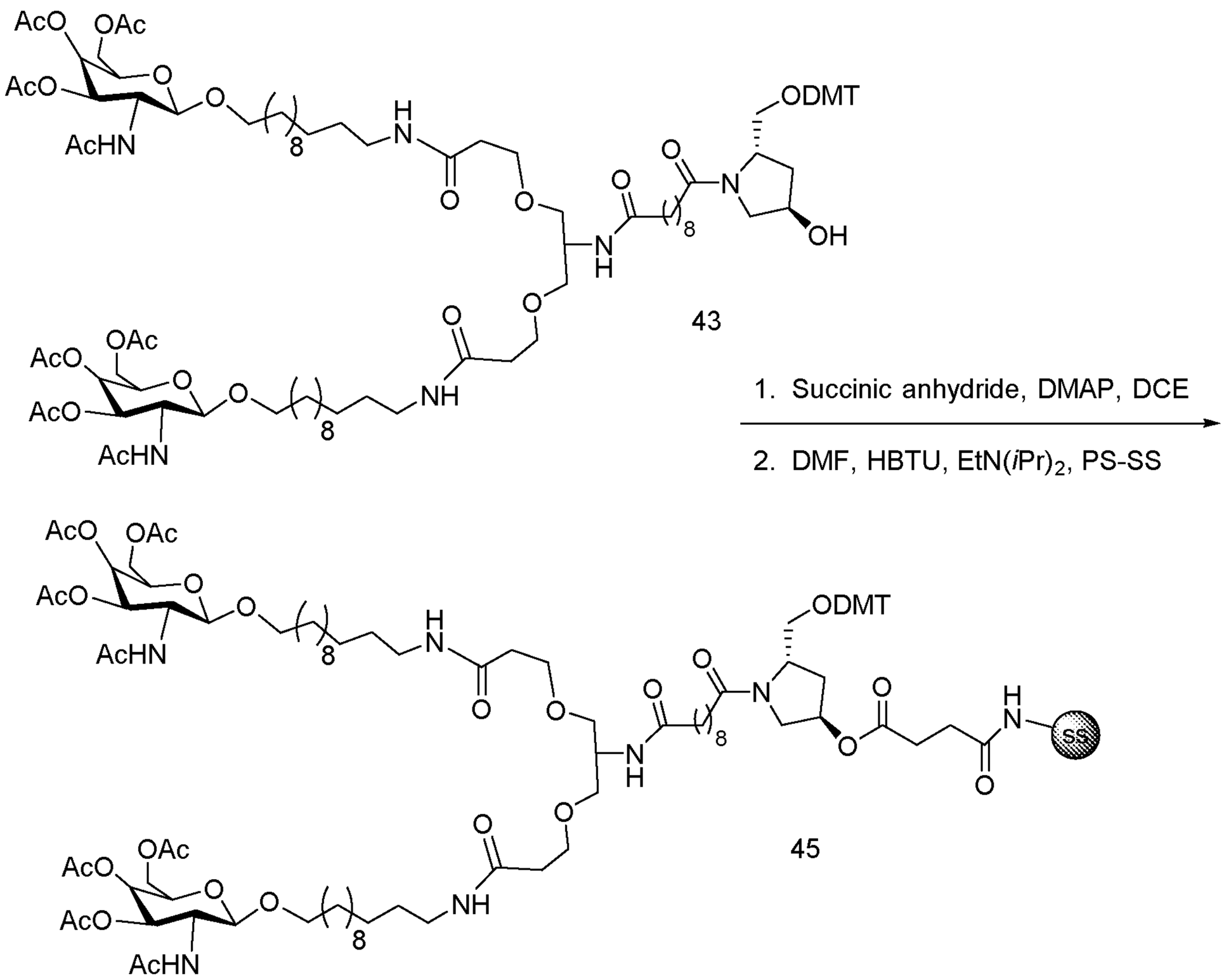
Example 13: Preparation of Compound 44





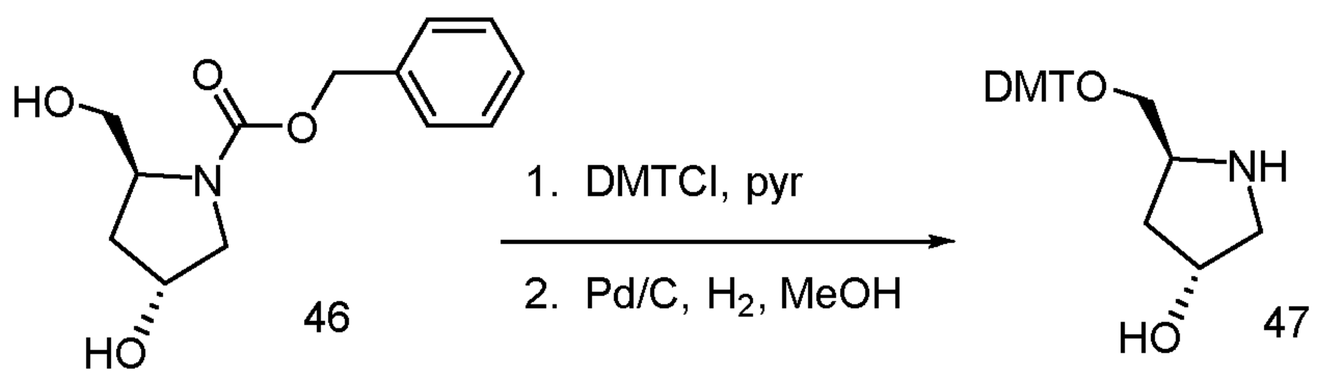
Compounds 23 and 36 are prepared as per the procedures illustrated in Examples 5 and 11. Compound 41 is prepared using similar procedures published in WO 2009082607.

Example 14: Preparation of Compound 45



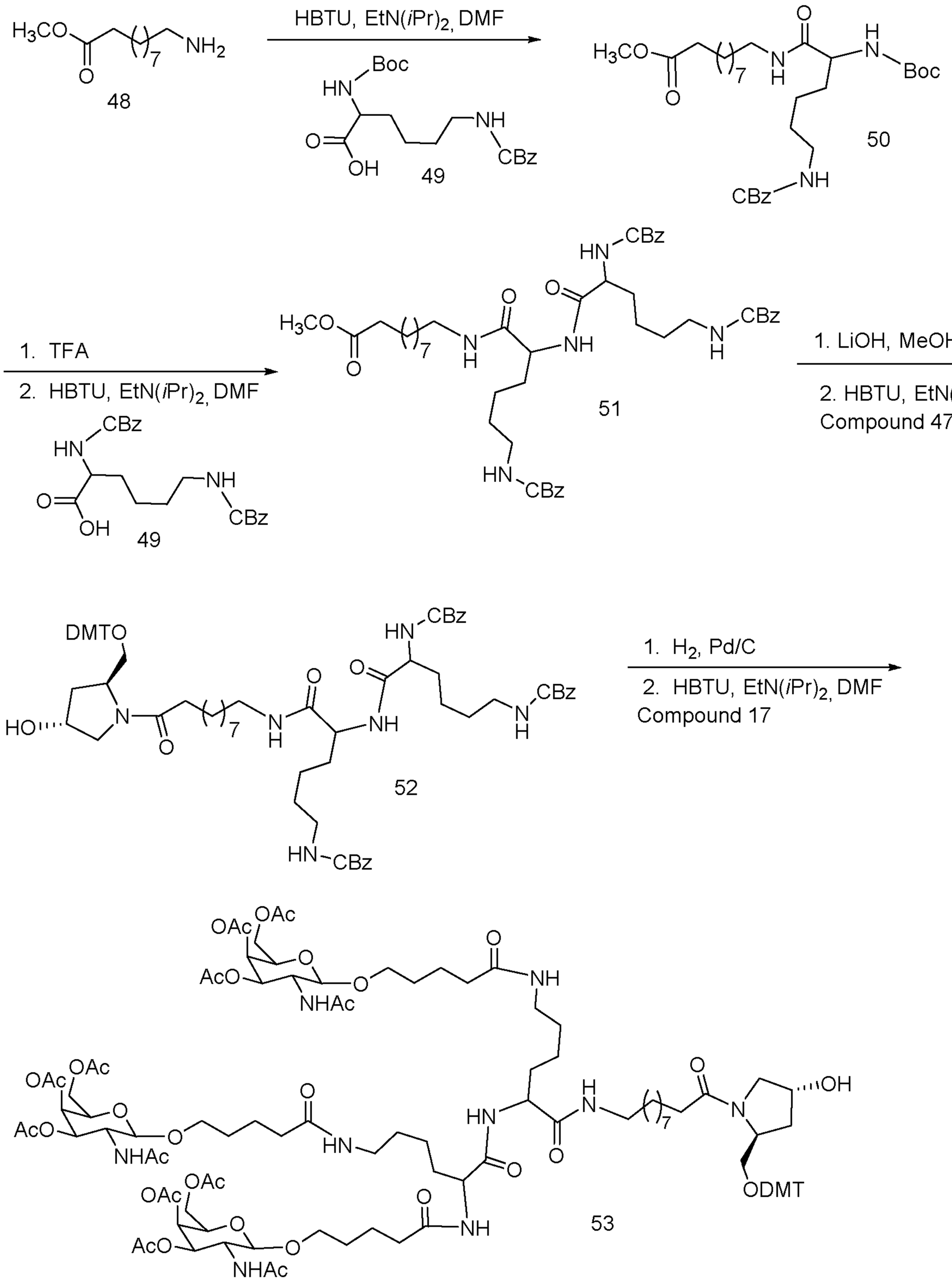
Compound 43 is prepared as per the procedures illustrated in Example 13.

Example 15: Preparation of Compound 47



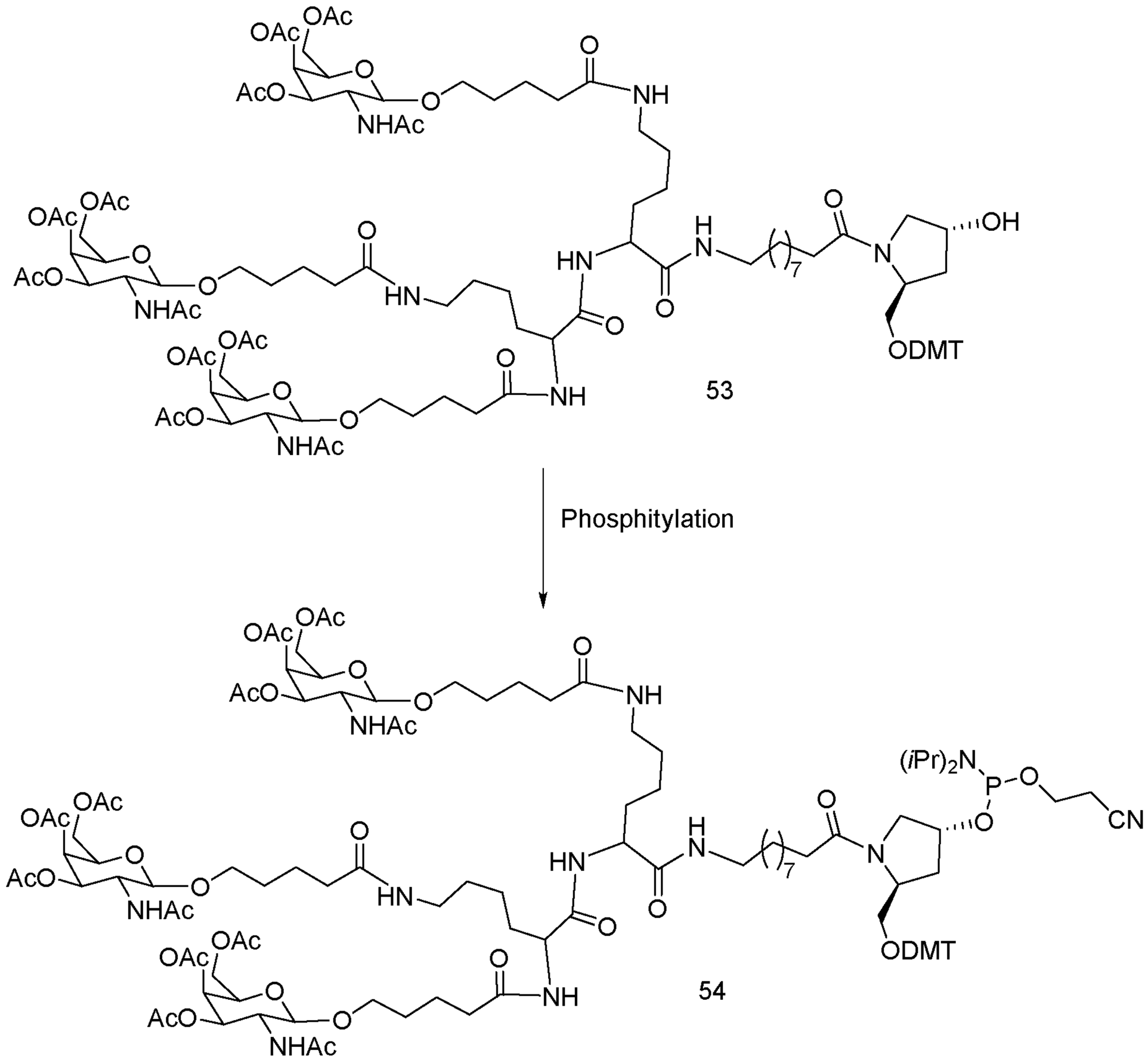
Compound 46 is commercially available.

Example 16: Preparation of Compound 53



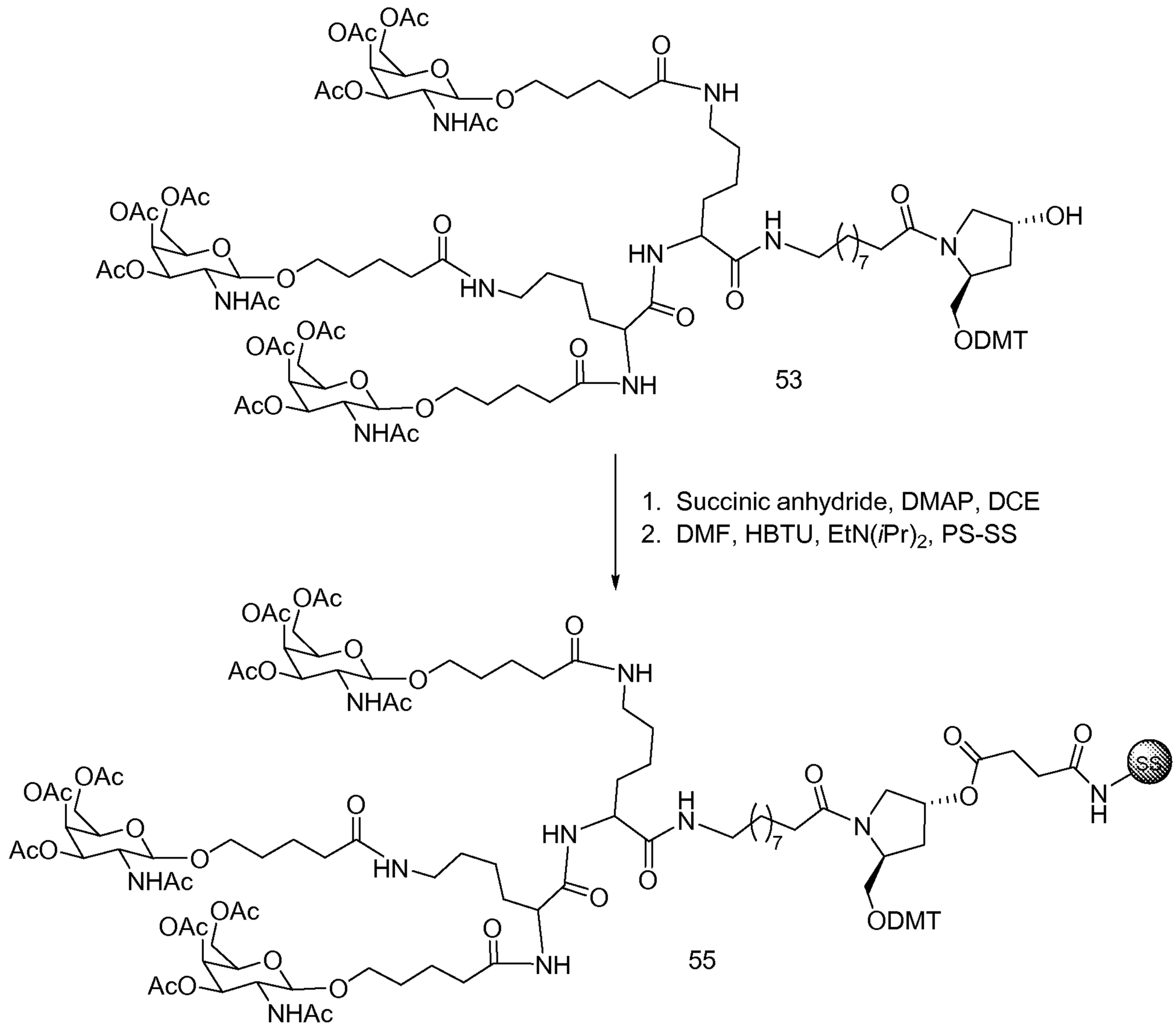
Compounds 48 and 49 are commercially available. Compounds 17 and 47 are prepared as per the procedures illustrated in Examples 4 and 15.

Example 17: Preparation of Compound 54



Compound 53 is prepared as per the procedures illustrated in Example 16.

Example 18: Preparation of Compound 55



Compound 53 is prepared as per the procedures illustrated in Example 16.

Example 19: General method for the preparation of conjugated ASOs comprising GalNAc$_3$-1 at the 3' position via solid phase techniques (preparation of ISIS 647535, 647536 and 651900)

Unless otherwise stated, all reagents and solutions used for the synthesis of oligomeric compounds are purchased from commercial sources. Standard phosphoramidite building blocks and solid support are used for incorporation nucleoside residues which include for example T, A, G, and ^{m}C residues. A 0.1 M solution of phosphoramidite in anhydrous acetonitrile was used for β-D-2'-deoxyribonucleoside and 2'-MOE.

The ASO syntheses were performed on ABI 394 synthesizer (1-2 μmol scale) or on GE Healthcare Bioscience ÄKTA oligopilot synthesizer (40-200 μmol scale) by the phosphoramidite coupling method on an **GalNAc$_3$-1** loaded VIMAD solid support (110 μmol/g, Guzaev *et al.*, 2003) packed in the column. For the coupling step, the phosphoramidites were delivered 4 fold excess over the loading on the solid support and phosphoramidite condensation was carried out for 10 min. All other steps followed standard protocols

supplied by the manufacturer. A solution of 6% dichloroacetic acid in toluene was used for removing dimethoxytrityl (DMT) group from 5'-hydroxyl group of the nucleotide. 4,5-Dicyanoimidazole (0.7 M) in anhydrous CH_3CN was used as activator during coupling step. Phosphorothioate linkages were introduced by sulfurization with 0.1 M solution of xanthane hydride in 1:1 pyridine/CH_3CN for a contact time of 3 minutes. A solution of 20% *tert*-butylhydroperoxide in CH_3CN containing 6% water was used as an oxidizing agent to provide phosphodiester internucleoside linkages with a contact time of 12 minutes.

After the desired sequence was assembled, the cyanoethyl phosphate protecting groups were deprotected using a 1:1 (v/v) mixture of triethylamine and acetonitrile with a contact time of 45 minutes. The solid-support bound ASOs were suspended in aqueous ammonia (28-30 wt %) and heated at 55 °C for 6 h.

The unbound ASOs were then filtered and the ammonia was boiled off. The residue was purified by high pressure liquid chromatography on a strong anion exchange column (GE Healthcare Bioscience, Source 30Q, 30 µm, 2.54 x 8 cm, A = 100 mM ammonium acetate in 30% aqueous CH_3CN, B = 1.5 M NaBr in A, 0-40% of B in 60 min, flow 14 mL min-1, λ = 260 nm). The residue was desalted by HPLC on a reverse phase column to yield the desired ASOs in an isolated yield of 15-30% based on the initial loading on the solid support. The ASOs were characterized by ion-pair-HPLC coupled MS analysis with Agilent 1100 MSD system.

Antisense oligonucleotides not comprising a conjugate were synthesized using standard oligonucleotide synthesis procedures well known in the art.

Using these methods, three separate antisense compounds targeting ApoC III were prepared. As summarized in Table 17, below, each of the three antisense compounds targeting ApoC III had the same nucleobase sequence; ISIS 304801 is a 5-10-5 MOE gapmer having all phosphorothioate linkages; ISIS 647535 is the same as ISIS 304801, except that it had a **$GalNAc_3$-1** conjugated at its 3'end; and ISIS 647536 is the same as ISIS 647535 except that certain internucleoside linkages of that compound are phosphodiester linkages. As further summarized in Table 17, two separate antisense compounds targeting SRB-1 were synthesized. ISIS 440762 was a 2-10-2 cEt gapmer with all phosphorothioate internucleoside linkages; ISIS 651900 is the same as ISIS 440762, except that it included a **$GalNAc_3$-1** at its 3'-end.

Table 17
Modified ASO targeting ApoC III and SRB-1

ASO	Sequence (5' to 3')	Target	CalCd Mass	Observed Mass	SEQ ID No.
ISIS 304801	$A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{e}$	ApoC III	7165.4	7164.4	4878
ISIS 647535	$A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{eo}$**$A_{do'}$-$GalNAc_3$-$1_a$**	ApoC III	9239.5	9237.8	4879
ISIS 647536	$A_{es}G_{eo}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{eo}T_{eo}T_{es}A_{es}T_{eo}$**$A_{do'}$-$GalNAc_3$-$1_a$**	ApoC III	9142.9	9140.8	4879
ISIS 440762	$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{k}$	SRB-1	4647.0	4646.4	4880
ISIS 651900	$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{ko}$**$A_{do'}$-$GalNAc_3$-$1_a$**	SRB-1	6721.1	6719.4	4881

Subscripts: "e" indicates 2'-MOE modified nucleoside; "d" indicates β-D-2'-deoxyribonucleoside; "k" indicates 6'-*(S)*-CH_3 bicyclic nucleoside (e.g. cEt); "s" indicates phosphorothioate internucleoside linkages (PS); "o" indicates phosphodiester internucleoside linkages (PO); and "o'" indicates -O-P(=O)(OH)-. Superscript "m" indicates 5-methylcytosines. "**GalNAc$_3$-1**" indicates a conjugate group having the structure shown previously in Example 9. Note that **GalNAc$_3$-1** comprises a cleavable adenosine which links the ASO to remainder of the conjugate, which is designated "**GalNAc$_3$-1$_a$.**" This nomenclature is used in the above table to show the full nucleobase sequence, including the adenosine, which is part of the conjugate. Thus, in the above table, the sequences could also be listed as ending with "**GalNAc$_3$-1**" with the "**A$_{do}$**" omitted. This convention of using the subscript "a" to indicate the portion of a conjugate group lacking a cleavable nucleoside or cleavable moiety is used throughout these Examples. This portion of a conjugate group lacking the cleavable moiety is referred to herein as a "cluster" or "conjugate cluster" or "GalNAc$_3$ cluster." In certain instances it is convenient to describe a conjugate group by separately providing its cluster and its cleavable moiety.

Example 20: Dose-dependent antisense inhibition of human ApoC III in huApoC III transgenic mice

ISIS 304801 and ISIS 647535, each targeting human ApoC III and described above, were separately tested and evaluated in a dose-dependent study for their ability to inhibit human ApoC III in human ApoC III transgenic mice.

Treatment

Human ApoCIII transgenic mice were maintained on a 12-hour light/dark cycle and fed *ad libitum* Teklad lab chow. Animals were acclimated for at least 7 days in the research facility before initiation of the experiment. ASOs were prepared in PBS and sterilized by filtering through a 0.2 micron filter. ASOs were dissolved in 0.9% PBS for injection.

Human ApoC III transgenic mice were injected intraperitoneally once a week for two weeks with ISIS 304801 or 647535 at 0.08, 0.25. 0.75, 2.25 or 6.75 μmol/kg or with PBS as a control. Each treatment group consisted of 4 animals. Forty-eight hours after the administration of the last dose, blood was drawn from each mouse and the mice were sacrificed and tissues were collected.

ApoC III mRNA Analysis

ApoC III mRNA levels in the mice's livers were determined using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. ApoC III mRNA levels were determined relative to total RNA (using Ribogreen), prior to normalization to PBS-treated control. The results below are presented as the average percent of ApoC III mRNA levels for each treatment group, normalized to PBS-treated control and are denoted as "% PBS". The half maximal effective dosage (ED_{50}) of each ASO is also presented in Table 18, below.

As illustrated, both antisense compounds reduced ApoC III RNA relative to the PBS control. Further, the antisense compound conjugated to **GalNAc$_3$-1** (ISIS 647535) was substantially more potent than the antisense compound lacking the **GalNAc$_3$-1** conjugate (ISIS 304801).

Table 18

Effect of ASO treatment on ApoC III mRNA levels in human ApoC III transgenic mice

ASO	Dose (µmol/kg)	% PBS	ED_{50} (µmol/kg)	3' Conjugate	Internucleoside linkage/Length	SEQ ID No.
PBS	0	100	--	-	--	
ISIS 304801	0.08	95	0.77	None	PS/20	4878
	0.75	42				
	2.25	32				
	6.75	19				
ISIS 647535	0.08	50	0.074	**$GalNAc_3$-1**	PS/20	4879
	0.75	15				
	2.25	17				
	6.75	8				

ApoC III Protein Analysis (Turbidometric Assay)

Plasma ApoC III protein analysis was determined using procedures reported by Graham *et al, Circulation Research,* published online before print March 29, 2013.

Approximately 100 µl of plasma isolated from mice was analyzed without dilution using an Olympus Clinical Analyzer and a commercially available turbidometric ApoC III assay (Kamiya, Cat# KAI-006, Kamiya Biomedical, Seattle, WA). The assay protocol was performed as described by the vendor.

As shown in the Table 19 below, both antisense compounds reduced ApoC III protein relative to the PBS control. Further, the antisense compound conjugated to **$GalNAc_3$-1** (ISIS 647535) was substantially more potent than the antisense compound lacking the **$GalNAc_3$-1** conjugate (ISIS 304801).

Table 19

Effect of ASO treatment on ApoC III plasma protein levels in human ApoC III transgenic mice

ASO	Dose (µmol/kg)	% PBS	ED_{50} (µmol/kg)	3' Conjugate	Internucleoside Linkage/Length	SEQ ID No.
PBS	0	100	--	--	--	
ISIS 304801	0.08	86	0.73	None	PS/20	4878
	0.75	51				
	2.25	23				
	6.75	13				
ISIS 647535	0.08	72	0.19	**$GalNAc_3$-1**	PS/20	4879
	0.75	14				
	2.25	12				
	6.75	11				

Plasma triglycerides and cholesterol were extracted by the method of Bligh and Dyer (Bligh, E.G. and Dyer, W.J. Can. J. Biochem. Physiol. 37: 911-917, 1959)(Bligh, E and Dyer, W, *Can J Biochem Physiol*, 37, 911-917, 1959)(Bligh, E and Dyer, W, *Can J Biochem Physiol*, 37, 911-917, 1959) and measured by using a Beckmann Coulter clinical analyzer and commercially available reagents.

The triglyceride levels were measured relative to PBS injected mice and are denoted as "% PBS". Results are presented in Table 20. As illustrated, both antisense compounds lowered triglyceride

levels. Further, the antisense compound conjugated to **GalNAc$_3$-1** (ISIS 647535) was substantially more potent than the antisense compound lacking the **GalNAc$_3$-1** conjugate (ISIS 304801).

Table 20

Effect of ASO treatment on triglyceride levels in transgenic mice

ASO	Dose (µmol/kg)	% PBS	ED_{50} (µmol/kg)	3' Conjugate	Internucleoside Linkage/Length	SEQ ID No.
PBS	0	100	--	--	--	
ISIS 304801	0.08	87	0.63	None	PS/20	4878
	0.75	46				
	2.25	21				
	6.75	12				
ISIS 647535	0.08	65	0.13	**GalNAc$_3$-1**	PS/20	4879
	0.75	9				
	2.25	8				
	6.75	9				

Plasma samples were analyzed by HPLC to determine the amount of total cholesterol and of different fractions of cholesterol (HDL and LDL). Results are presented in Tables 21 and 22. As illustrated, both antisense compounds lowered total cholesterol levels; both lowered LDL; and both raised HDL. Further, the antisense compound conjugated to **GalNAc$_3$-1** (ISIS 647535) was substantially more potent than the antisense compound lacking the **GalNAc$_3$-1** conjugate (ISIS 304801). An increase in HDL and a decrease in LDL levels is a cardiovascular beneficial effect of antisense inhibition of ApoC III.

Table 21

Effect of ASO treatment on total cholesterol levels in transgenic mice

ASO	Dose (µmol/kg)	Total Cholesterol (mg/dL)	3' Conjugate	Internucleoside Linkage/Length	SEQ ID No.
PBS	0	257	--	--	
ISIS 304801	0.08	226	None	PS/20	4878
	0.75	164			
	2.25	110			
	6.75	82			
ISIS 647535	0.08	230	**GalNAc$_3$-1**	PS/20	4879
	0.75	82			
	2.25	86			
	6.75	99			

Table 22

Effect of ASO treatment on HDL and LDL cholesterol levels in transgenic mice

ASO	Dose (µmol/kg)	HDL (mg/dL)	LDL (mg/dL)	3' Conjugate	Internucleoside Linkage/Length	SEQ ID No.
PBS	0	17	28	--	--	
ISIS 304801	0.08	17	23	None	PS/20	4878
	0.75	27	12			

	2.25	50	4			
	6.75	45	2			
ISIS 647535	0.08	21	21	**GalNAc$_3$-1**	PS/20	4879
	0.75	44	2			
	2.25	50	2			
	6.75	58	2			

Pharmacokinetics Analysis (PK)

The PK of the ASOs was also evaluated. Liver and kidney samples were minced and extracted using standard protocols. Samples were analyzed on MSD1 utilizing IP-HPLC-MS. The tissue level (μg/g) of full-length ISIS 304801 and 647535 was measured and the results are provided in Table 23. As illustrated, liver concentrations of total full-length antisense compounds were similar for the two antisense compounds. Thus, even though the **GalNAc$_3$-1** -conjugated antisense compound is more active in the liver (as demonstrated by the RNA and protein data above), it is not present at substantially higher concentration in the liver. Indeed, the calculated EC_{50} (provided in Table 23) confirms that the observed increase in potency of the conjugated compound cannot be entirely attributed to increased accumulation. This result suggests that the conjugate improved potency by a mechanism other than liver accumulation alone, possibly by improving the productive uptake of the antisense compound into cells.

The results also show that the concentration of **GalNAc$_3$-1** conjugated antisense compound in the kidney is lower than that of antisense compound lacking the GalNAc conjugate. This has several beneficial therapeutic implications. For therapeutic indications where activity in the kidney is not sought, exposure to kidney risks kidney toxicity without corresponding benefit. Moreover, high concentration in kidney typically results in loss of compound to the urine resulting in faster clearance. Accordingly, for non-kidney targets, kidney accumulation is undesired. These data suggest that **GalNAc$_3$-1** conjugation reduces kidney accumulation.

Table 23

PK analysis of ASO treatment in transgenic mice

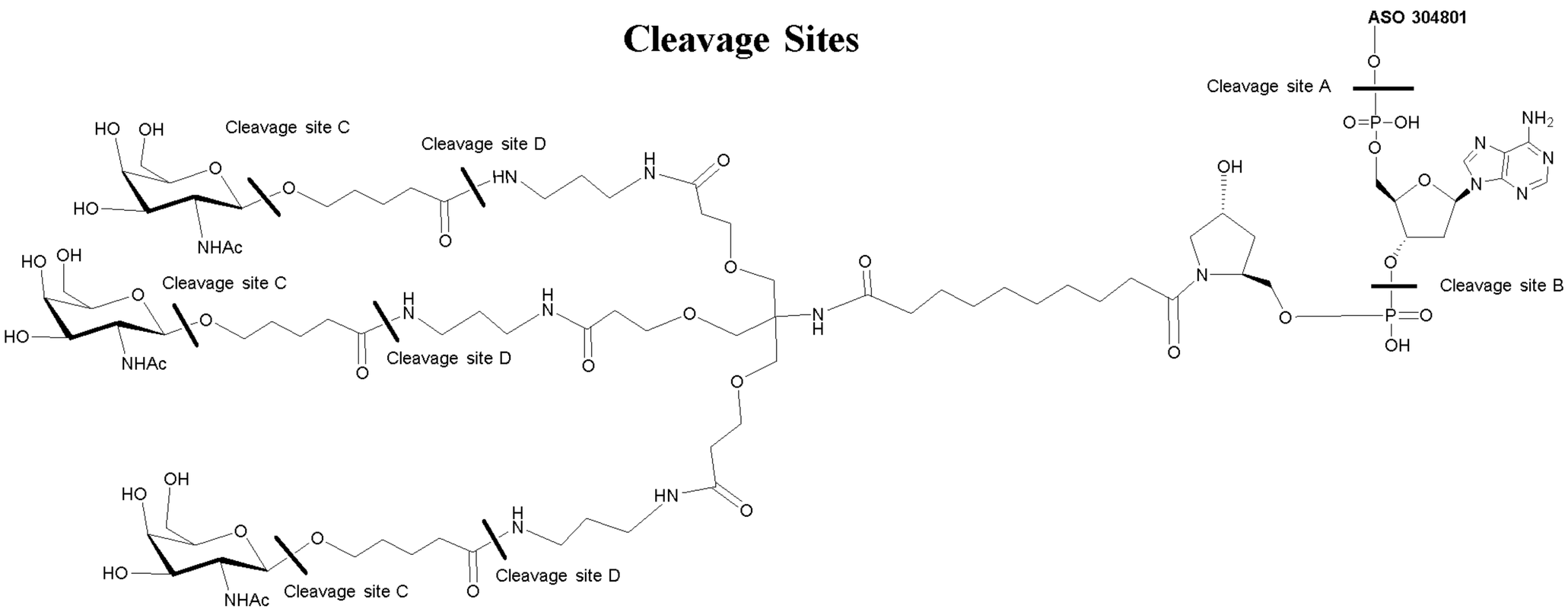
ASO	Dose (μmol/kg)	Liver (μg/g)	Kidney (μg/g)	Liver EC_{50} (μg/g)	3' Conjugate	Internucleoside Linkage/Length	SEQ ID No.
ISIS 304801	0.1	5.2	2.1	53	None	PS/20	4878
	0.8	62.8	119.6				
	2.3	142.3	191.5				
	6.8	202.3	337.7				
ISIS 647535	0.1	3.8	0.7	3.8	**GalNAc$_3$-1**	PS/20	4879
	0.8	72.7	34.3				
	2.3	106.8	111.4				
	6.8	237.2	179.3				

Metabolites of ISIS 647535 were also identified and their masses were confirmed by high resolution mass spectrometry analysis. The cleavage sites and structures of the observed metabolites are shown below. The relative % of full length ASO was calculated using standard procedures and the results are presented in Table 23a. The major metabolite of ISIS 647535 was full-length ASO lacking the entire conjugate (i.e. ISIS 304801), which results from cleavage at cleavage site A, shown below. Further, additional metabolites resulting from other cleavage sites were also observed. These results suggest that introducing other cleabable bonds such as esters, peptides, disulfides, phosphoramidates or acyl-hydrazones between the **GalNAc$_3$-1** sugar and the ASO, which can be cleaved by enzymes inside the cell, or which may cleave in the reductive environment of the cytosol, or which are labile to the acidic pH inside endosomes and lyzosomes, can also be useful.

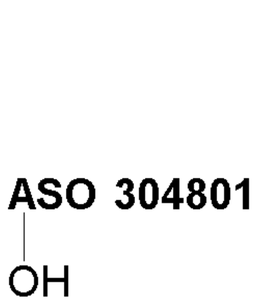
Table 23a
Observed full length metabolites of ISIS 647535

Metabolite	ASO	Cleavage site	Relative %
1	ISIS 304801	A	36.1
2	ISIS 304801 + dA	B	10.5
3	ISIS 647535 minus [3 GalNAc]	C	16.1
4	ISIS 647535 minus [3 GalNAc + 1 5-hydroxy-pentanoic acid tether]	D	17.6
5	ISIS 647535 minus [2 GalNAc + 2 5-hydroxy-pentanoic acid tether]	D	9.9
6	ISIS 647535 minus [3 GalNAc + 3 5-hydroxy-pentanoic acid tether]	D	9.8

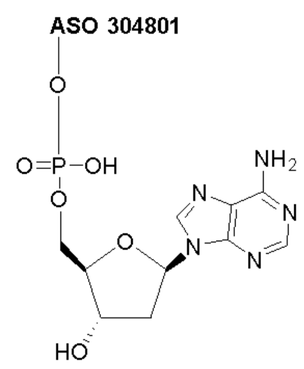
Cleavage Sites



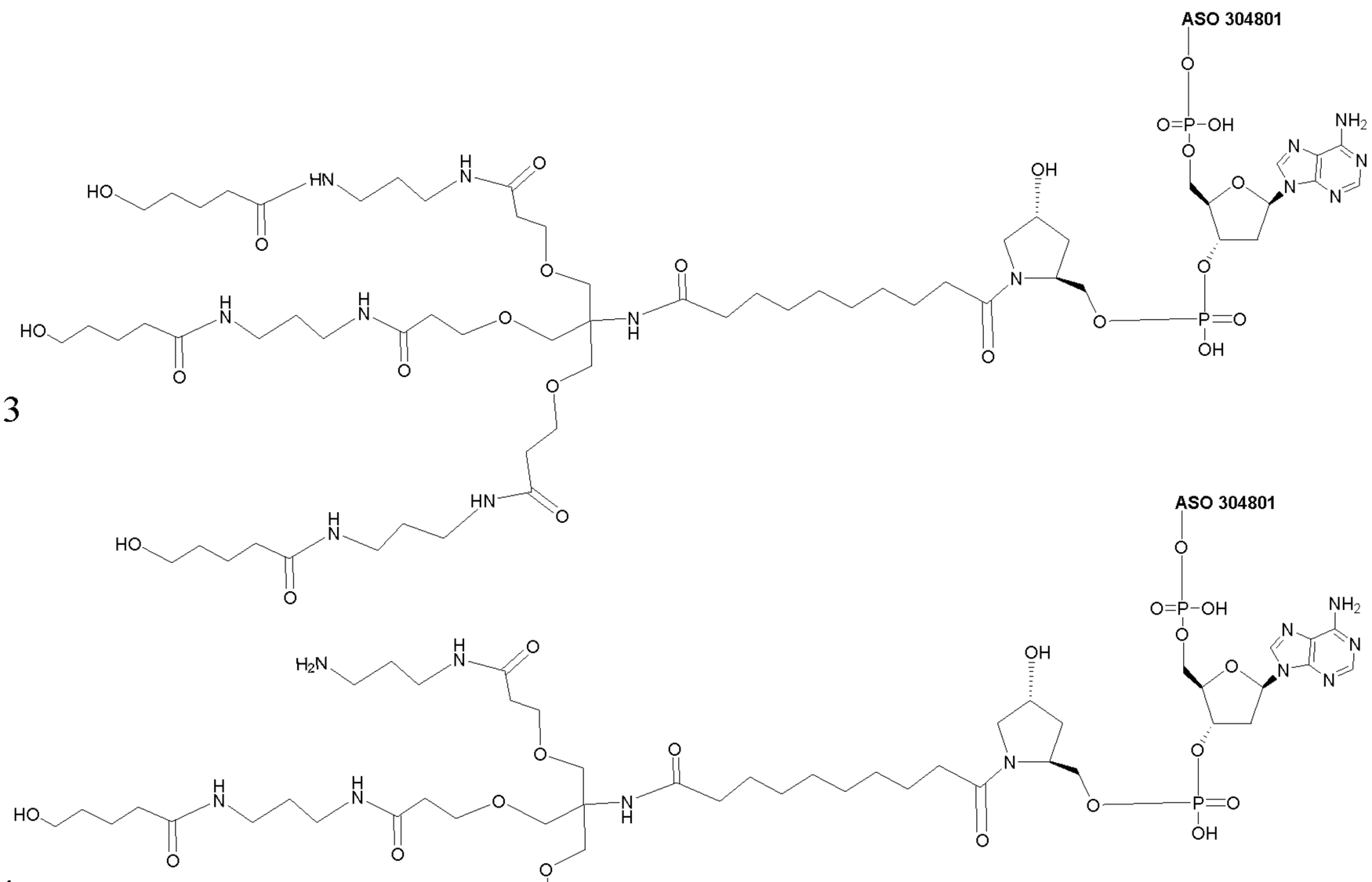
Metabolite 1



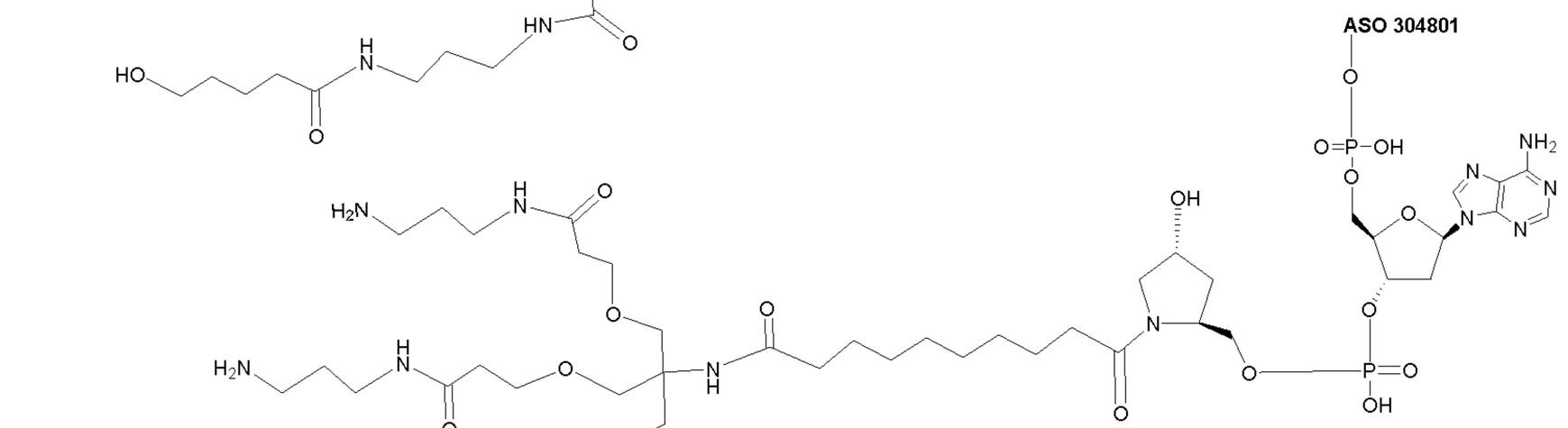
Metabolite 2



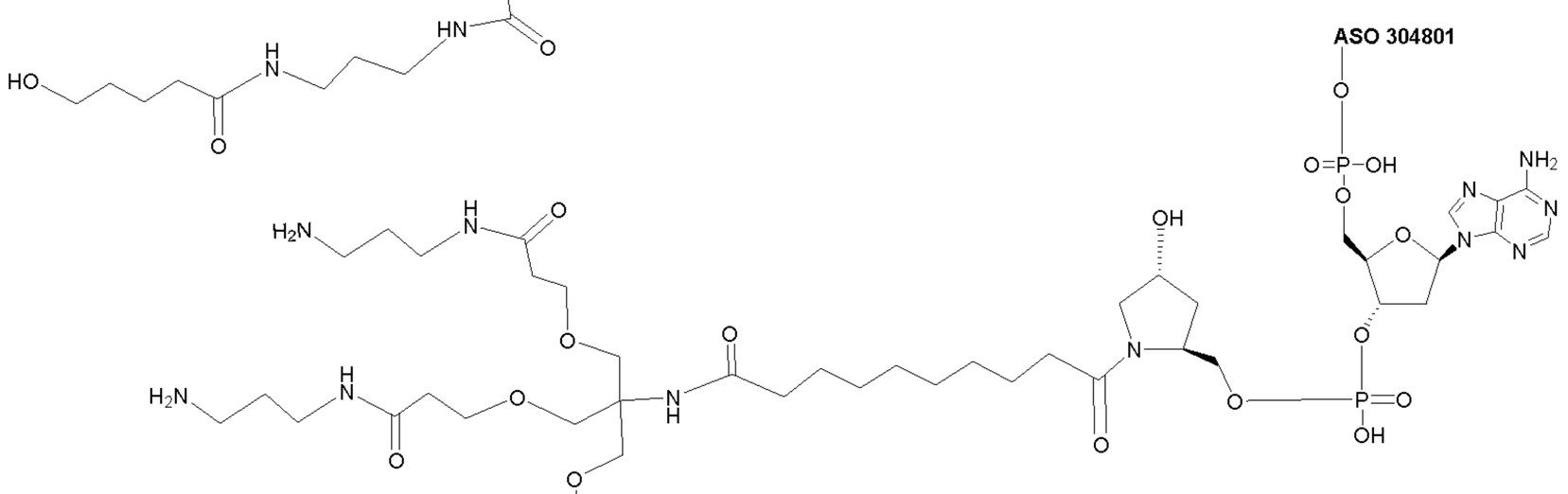
Metabolite 3



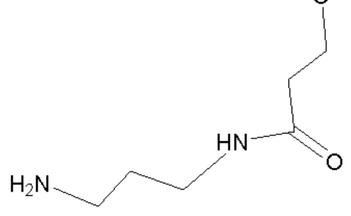
Metabolite 4



Metabolite 5



Metabolite 6



Example 21: Antisense inhibition of human ApoC III in human ApoC III transgenic mice in single administration study

ISIS 304801, 647535 and 647536 each targeting human ApoC III and described in Table 17, were further evaluated in a single administration study for their ability to inhibit human ApoC III in human ApoC III transgenic mice.

Treatment

Human ApoCIII transgenic mice were maintained on a 12-hour light/dark cycle and fed *ad libitum* Teklad lab chow. Animals were acclimated for at least 7 days in the research facility before initiation of the experiment. ASOs were prepared in PBS and sterilized by filtering through a 0.2 micron filter. ASOs were dissolved in 0.9% PBS for injection.

Human ApoC III transgenic mice were injected intraperitoneally once at the dosage shown below with ISIS 304801, 647535 or 647536 (described above) or with PBS treated control. The treatment group consisted of 3 animals and the control group consisted of 4 animals. Prior to the treatment as well as after the last dose, blood was drawn from each mouse and plasma samples were analyzed. The mice were sacrificed 72 hours following the last administration .

Samples were collected and analyzed to determine the ApoC III mRNA and protein levels in the liver; plasma triglycerides; and cholesterol, including HDL and LDL fractions were assessed as described above (Example 20). Data from those analyses are presented in Tables 24-28, below. Liver transaminase levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in serum were measured relative to saline injected mice using standard protocols. The ALT and AST levels showed that the antisense compounds were well tolerated at all administered doses.

These results show improvement in potency for antisense compounds comprising a **GalNAc$_3$-1** conjugate at the 3' terminus (ISIS 647535 and 647536) compared to the antisense compound lacking a **GalNAc$_3$-1** conjugate (ISIS 304801). Further, ISIS 647536, which comprises a **GalNAc$_3$-1** conjugate and some phosphodiester linkages was as potent as ISIS 647535, which comprises the same conjugate and all internucleoside linkages within the ASO are phosphorothioate.

Table 24

Effect of ASO treatment on ApoC III mRNA levels in human ApoC III transgenic mice

ASO	Dose (mg/kg)	% PBS	ED_{50} (mg/kg)	3' Conjugate	Internucleoside linkage/Length	SEQ ID No.
PBS	0	99	--	-	--	
ISIS 304801	1	104	13.2	None	PS/20	4878
	3	92				
	10	71				
	30	40				
ISIS 647535	0.3	98	1.9	**GalNAc$_3$-1**	PS/20	4879
	1	70				
	3	33				
	10	20				
ISIS	0.3	103	1.7	**GalNAc$_3$-1**	PS/PO/20	4879

647536	1	60				
	3	31				
	10	21				

Table 25

Effect of ASO treatment on ApoC III plasma protein levels in human ApoC III transgenic mice

ASO	Dose (mg/kg)	% PBS	ED_{50} (mg/kg)	3' Conjugate	Internucleoside Linkage/Length	SEQ ID No.
PBS	0	99	--	--	--	
ISIS 304801	1	104	23.2	None	PS/20	4878
	3	92				
	10	71				
	30	40				
ISIS 647535	0.3	98	2.1	**$GalNAc_3$-1**	PS/20	4879
	1	70				
	3	33				
	10	20				
ISIS 647536	0.3	103	1.8	**$GalNAc_3$-1**	PS/PO/20	4879
	1	60				
	3	31				
	10	21				

Table 26

Effect of ASO treatment on triglyceride levels in transgenic mice

ASO	Dose (mg/kg)	% PBS	ED_{50} (mg/kg)	3' Conjugate	Internucleoside Linkage/Length	SEQ ID No.
PBS	0	98	--	--	--	
ISIS 304801	1	80	29.1	None	PS/20	4878
	3	92				
	10	70				
	30	47				
ISIS 647535	0.3	100	2.2	**$GalNAc_3$-1**	PS/20	4879
	1	70				
	3	34				
	10	23				
ISIS 647536	0.3	95	1.9	**$GalNAc_3$-1**	PS/PO/20	4879
	1	66				
	3	31				
	10	23				

Table 27

Effect of ASO treatment on total cholesterol levels in transgenic mice

ASO	Dose (mg/kg)	% PBS	3' Conjugate	Internucleoside Linkage/Length	SEQ ID No.
PBS	0	96	--	--	
ISIS 304801	1	104	None	PS/20	4878
	3	96			
	10	86			
	30	72			

ISIS 647535	0.3	93	**GalNAc$_3$-1**	PS/20	4879
	1	85			
	3	61			
	10	53			
ISIS 647536	0.3	115	**GalNAc$_3$-1**	PS/PO/20	4879
	1	79			
	3	51			
	10	54			

Table 28

Effect of ASO treatment on HDL and LDL cholesterol levels in transgenic mice

ASO	Dose (mg/kg)	HDL % PBS	LDL % PBS	3' Conjugate	Internucleoside Linkage/Length	SEQ ID No.
PBS	0	131	90	--	--	
ISIS 304801	1	130	72	None	PS/20	4878
	3	186	79			
	10	226	63			
	30	240	46			
ISIS 647535	0.3	98	86	**GalNAc$_3$-1**	PS/20	4879
	1	214	67			
	3	212	39			
	10	218	35			
ISIS 647536	0.3	143	89	**GalNAc$_3$-1**	PS/PO/20	4879
	1	187	56			
	3	213	33			
	10	221	34			

These results confirm that the **GalNAc$_3$-1** conjugate improves potency of an antisense compound. The results also show equal potency of a **GalNAc$_3$-1** conjugated antisense compounds where the antisense oligonucleotides have mixed linkages (ISIS 647536 which has six phosphodiester linkages) and a full phosphorothioate version of the same antisense compound (ISIS 647535).

Phosphorothioate linkages provide several properties to antisense compounds. For example, they resist nuclease digestion and they bind proteins resulting in accumulation of compound in the liver, rather than in the kidney/urine. These are desirable properties, particularly when treating an indication in the liver. However, phosphorothioate linkages have also been associated with an inflammatory response. Accordingly, reducing the number of phosphorothioate linkages in a compound is expected to reduce the risk of inflammation, but also lower concentration of the compound in liver, increase concentration in the kidney and urine, decrease stability in the presence of nucleases, and lower overall potency. The present results show that a **GalNAc$_3$-1** conjugated antisense compound where certain phosphorothioate linkages have been replaced with phosphodiester linkages is as potent against a target in the liver as a counterpart having full

phosphorothioate linkages. Such compounds are expected to be less proinflammatory (See Example 24 describing an experiment showing reduction of PS results in reduced inflammatory effect).

Example 22: Effect of $GalNAc_3$-1 conjugated modified ASO targeting SRB-1 *in vivo*

ISIS 440762 and 651900, each targeting SRB-1 and described in Table 17, were evaluated in a dose-dependent study for their ability to inhibit SRB-1 in Balb/c mice.

Treatment

Six week old male Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously once at the dosage shown below with ISIS 440762, 651900 or with PBS treated control. Each treatment group consisted of 4 animals. The mice were sacrificed 48 hours following the final administration to determine the SRB-1 mRNA levels in liver using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. SRB-1 mRNA levels were determined relative to total RNA (using Ribogreen), prior to normalization to PBS-treated control. The results below are presented as the average percent of SRB-1 mRNA levels for each treatment group, normalized to PBS-treated control and is denoted as "% PBS".

As illustrated in Table 29, both antisense compounds lowered SRB-1 mRNA levels. Further, the antisense compound comprising the **$GalNAc_3$-1** conjugate (ISIS 651900) was substantially more potent than the antisense compound lacking the **$GalNAc_3$-1** conjugate (ISIS 440762). These results demonstrate that the potency benefit of **$GalNAc_3$-1** conjugates are observed using antisense oligonucleotides complementary to a different target and having different chemically modified nucleosides, in this instance modified nucleosides comprise constrained ethyl sugar moieties (a bicyclic sugar moiety).

Table 29

Effect of ASO treatment on SRB-1 mRNA levels in Balb/c mice

ASO	Dose (mg/kg)	Liver % PBS	ED_{50} (mg/kg)	3' Conjugate		Internucleoside linkage/Length	SEQ ID No.
PBS	0	100		-		--	
ISIS 440762	0.7	85	2.2	None		PS/14	4880
	2	55					
	7	12					
	20	3					
ISIS 651900	0.07	98	0.3	**$GalNAc_3$-1**		PS/14	4881
	0.2	63					
	0.7	20					
	2	6					
	7	5					

Example 23: Human Peripheral Blood Mononuclear Cells (hPBMC) Assay Protocol

The hPBMC assay was performed using BD Vautainer CPT tube method. A sample of whole blood from volunteered donors with informed consent at US HealthWorks clinic (Faraday & El Camino Real,

Carlsbad) was obtained and collected in 4-15 BD Vacutainer CPT 8 ml tubes (VWR Cat.# BD362753). The approximate starting total whole blood volume in the CPT tubes for each donor was recorded using the PBMC assay data sheet.

The blood sample was remixed immediately prior to centrifugation by gently inverting tubes 8-10 times. CPT tubes were centrifuged at rt (18-25 °C) in a horizontal (swing-out) rotor for 30 min. at 1500-1800 RCF with brake off (2700 RPM Beckman Allegra 6R). The cells were retrieved from the buffy coat interface (between Ficoll and polymer gel layers); transferred to a sterile 50 ml conical tube and pooled up to 5 CPT tubes/50 ml conical tube/donor. The cells were then washed twice with PBS (Ca^{++}, Mg^{++} free; GIBCO). The tubes were topped up to 50 ml and mixed by inverting several times. The sample was then centrifuged at 330 x g for 15 minutes at rt (1215 RPM in Beckman Allegra 6R) and aspirated as much supernatant as possible without disturbing pellet. The cell pellet was dislodged by gently swirling tube and resuspended cells in RPMI+10% FBS+pen/strep (~1 ml / 10 ml starting whole blood volume). A 60 µl sample was pipette into a sample vial (Beckman Coulter) with 600 µl VersaLyse reagent (Beckman Coulter Cat# A09777) and was gently vortexed for 10-15 sec. The sample was allowed to incubate for 10 min. at rt and being mixed again before counting. The cell suspension was counted on Vicell XR cell viability analyzer (Beckman Coulter) using PBMC cell type (dilution factor of 1:11 was stored with other parameters). The live cell/ml and viability were recorded. The cell suspension was diluted to 1 x 10^7 live PBMC/ml in RPMI+ 10% FBS+pen/strep.

The cells were plated at 5 x 10^5 in 50 µl/well of 96-well tissue culture plate (Falcon Microtest). 50 µl/well of 2x concentration oligos/controls diluted in RPMI+10% FBS+pen/strep. was added according to experiment template (100 µl/well total). Plates were placed on the shaker and allowed to mix for approx. 1 min. After being incubated for 24 hrs at 37 °C; 5% CO_2, the plates were centrifuged at 400 x g for 10 minutes before removing the supernatant for MSD cytokine assay (i.e. human IL-6, IL-10, IL-8 and MCP-1).

Example 24: Evaluation of Proinflammatory Effects in hPBMC Assay for $GalNAc_3$-1 conjugated ASOs

The antisense oligonucleotides (ASOs) listed in Table 30 were evaluated for proinflammatory effect in hPBMC assay using the protocol described in Example 23. ISIS 353512 is an internal standard known to be a high responder for IL-6 release in the assay. The hPBMCs were isolated from fresh, volunteered donors and were treated with ASOs at 0, 0.0128, 0.064, 0.32, 1.6, 8, 40 and 200 µM concentrations. After a 24 hr treatment, the cytokine levels were measured.

The levels of IL-6 were used as the primary readout. The EC_{50} and E_{max} was calculated using standard procedures. Results are expressed as the average ratio of E_{max}/EC_{50} from two donors and is denoted as "E_{max}/EC_{50}." The lower ratio indicates a relative decrease in the proinflammatory response and the higher ratio indicates a relative increase in the proinflammatory response.

With regard to the test compounds, the least proinflammatory compound was the PS/PO linked ASO (ISIS 616468). The **$GalNAc_3$-1** conjugated ASO, ISIS 647535 was slightly less proinflammatory than its

non-conjugated counterpart ISIS 304801. These results indicate that incorporation of some PO linkages reduces proinflammatory reaction and addition of a **GalNAc$_3$-1** conjugate does not make a compound more proinflammatory and may reduce proinflammatory response. Accordingly, one would expect that an antisense compound comprising both mixed PS/PO linkages and a **GalNAc$_3$-1** conjugate would produce lower proinflammatory responses relative to full PS linked antisense compound with or without a **GalNAc$_3$-1** conjugate. These results show that **GalNAc$_3$-1** conjugated antisense compounds, particularly those having reduced PS content are less proinflammatory.

Together, these results suggest that a **GalNAc$_3$-1** conjugated compound, particularly one with reduced PS content, can be administered at a higher dose than a counterpart full PS antisense compound lacking a **GalNAc$_3$-1** conjugate. Since half-life is not expected to be substantially different for these compounds, such higher administration would result in less frequent dosing. Indeed such administration could be even less frequent, because the **GalNAc$_3$-1** conjugated compounds are more potent (See Examples 20-22) and re-dosing is necessary once the concentration of a compound has dropped below a desired level, where such desired level is based on potency.

Table 30
Modified ASOs

ASO	Sequence (5' to 3')	Target	SEQ ID No.
ISIS 104838	$G_{es}{}^{m}C_{es}T_{es}G_{es}A_{es}T_{ds}T_{ds}A_{ds}G_{ds}A_{ds}G_{ds}$ $A_{ds}G_{ds}A_{ds}G_{ds}G_{es}T_{es}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	TNFα	4882
ISIS 353512	$T_{es}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{ds}A_{ds}T_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{ds}G_{ds}$ $G_{ds}A_{ds}G_{ds}A_{ds}{}^{m}C_{ds}{}^{m}C_{ds}T_{es}G_{es}G_{e}$	CRP	4883
ISIS 304801	$A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}$ $^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{e}$	ApoC III	4878
ISIS 647535	$A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}$ $^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{eo}$**$A_{do'}$-GalNAc$_3-1_a$**	ApoC III	4879
ISIS 616468	$A_{es}G_{eo}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}$ $^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{eo}T_{eo}T_{es}A_{es}T_{e}$	ApoC III	4878

Subscripts: "e" indicates 2'-MOE modified nucleoside; "d" indicates β-D-2'-deoxyribonucleoside; "k" indicates 6'-*(S)*-CH_3 bicyclic nucleoside (e.g. cEt); "s" indicates phosphorothioate internucleoside linkages (PS); "o" indicates phosphodiester internucleoside linkages (PO); and "o'" indicates -O-P(=O)(OH)-. Superscript "m" indicates 5-methylcytosines. "**$A_{do'}$-GalNAc$_3$-1$_a$**" indicates a conjugate having the structure **GalNAc$_3$-**1 shown in Example 9 attached to the 3'-end of the antisense oligonucleotide, as indicated.

Table 31
Proinflammatory Effect of ASOs targeting ApoC III in hPBMC assay

ASO	EC_{50} (μM)	E_{max} (μM)	E_{max}/EC_{50}	3' Conjugate	Internucleoside Linkage/Length	SEQ ID No.
ISIS 353512 (high responder)	0.01	265.9	26,590	None	PS/20	4883
ISIS 304801	0.07	106.55	1,522	None	PS/20	4878
ISIS 647535	0.12	138	1,150	**$GalNAc_3$-1**	PS/20	4879
ISIS 616468	0.32	71.52	224	None	PS/PO/20	4878

Example 25: Effect of $GalNAc_3$-1 conjugated modified ASO targeting human ApoC III *in vitro*

ISIS 304801 and 647535 described above were tested *in vitro*. Primary hepatocyte cells from transgenic mice at a density of 25,000 cells per well were treated with 0.03,0.08, 0.24, 0.74, 2.22, 6.67 and 20 μM concentrations of modified oligonucleotides. After a treatment period of approximately 16 hours, RNA was isolated from the cells and mRNA levels were measured by quantitative real-time PCR and the hApoC III mRNA levels were adjusted according to total RNA content, as measured by RIBOGREEN.

The IC_{50} was calculated using the standard methods and the results are presented in Table 32. As illustrated, comparable potency was observed in cells treated with ISIS 647535 as compared to the control, ISIS 304801.

Table 32
Modified ASO targeting human ApoC III in primary hepatocytes

ASO	IC_{50} (μM)	3' Conjugate	Internucleoside linkage/Length	SEQ ID No.
ISIS 304801	0.44	None	PS/20	4878
ISIS 647535	0.31	**$GalNAc_3$-1**	PS/20	4879

In this experiment, the large potency benefits of **$GalNAc_3$-1** conjugation that are observed *in vivo* were not observed *in vitro*. Subsequent free uptake experiments in primary hepatocytes *in vitro* did show increased potency of oligonucleotides comprising various GalNAc conjugates relative to oligonucleotides that lacking the GalNAc conjugate.(see Examples 60, 82, and 92)

Example 26: Effect of PO/PS linkages on ApoC III ASO Activity

Human ApoC III transgenic mice were injected intraperitoneally once at 25 mg/kg of ISIS 304801, or ISIS 616468 (both described above) or with PBS treated control once per week for two weeks. The treatment group consisted of 3 animals and the control group consisted of 4 animals. Prior to the treatment as

well as after the last dose, blood was drawn from each mouse and plasma samples were analyzed. The mice were sacrificed 72 hours following the last administration.

Samples were collected and analyzed to determine the ApoC III protein levels in the liver as described above (Example 20). Data from those analyses are presented in Table 33, below.

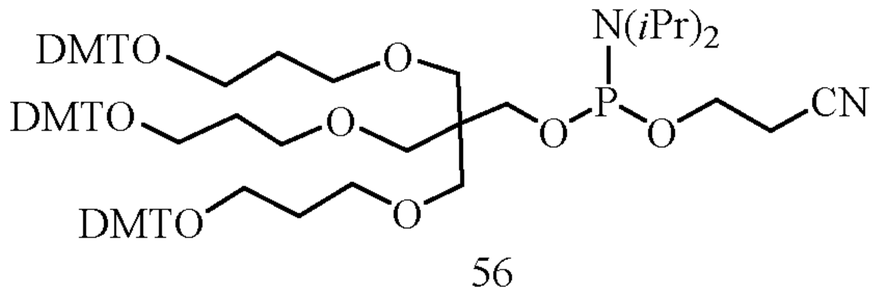
These results show reduction in potency for antisense compounds with PO/PS (ISIS 616468) in the wings relative to full PS (ISIS 304801).

Table 33

Effect of ASO treatment on ApoC III protein levels in human ApoC III transgenic mice

ASO	Dose (mg/kg)	% PBS	3' Conjugate	Internucleoside linkage/Length	SEQ ID No.
PBS	0	99	-	--	
ISIS 304801	25 mg/kg/wk for 2 wks	24	None	Full PS	4878
ISIS 616468	25 mg/kg/wk for 2 wks	40	None	14 PS/6 PO	4878

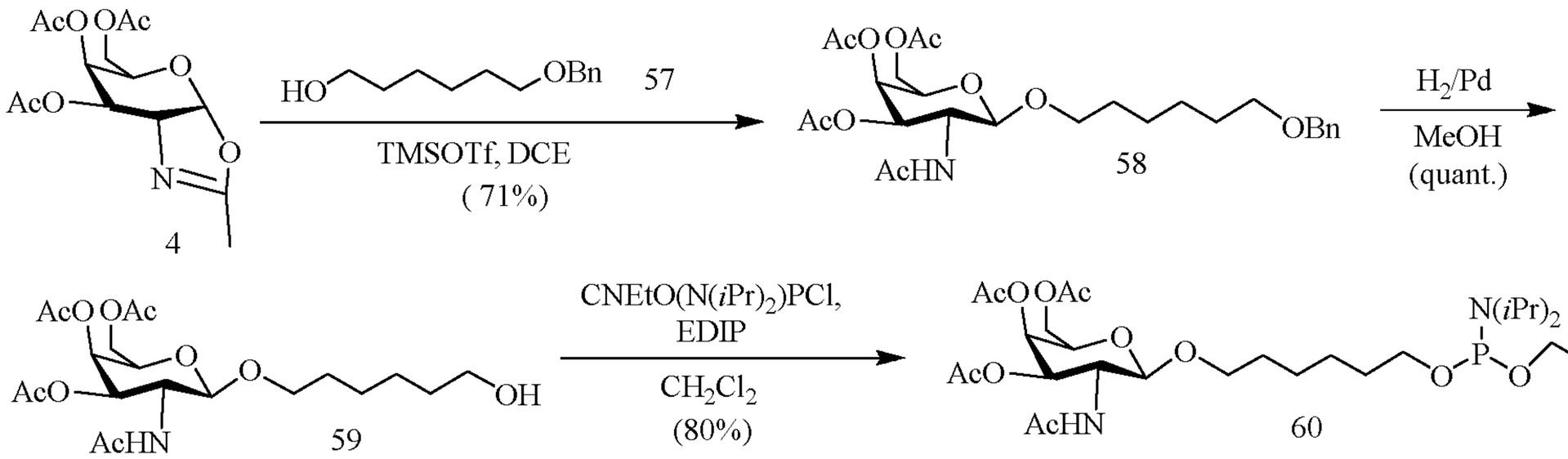
Example 27: Compound 56



56

Compound 56 is commercially available from Glen Research or may be prepared according to published procedures reported by Shchepinov *et al., Nucleic Acids Research,* 1997, *25(22)*, 4447-4454.

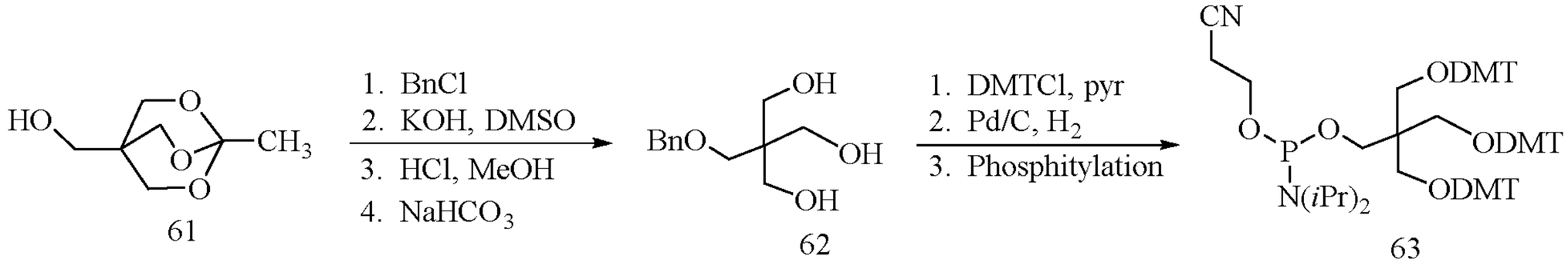
Example 28: Preparation of Compound 60



Compound 4 was prepared as per the procedures illustrated in Example 2. Compound 57 is commercially available. Compound 60 was confirmed by structural analysis.

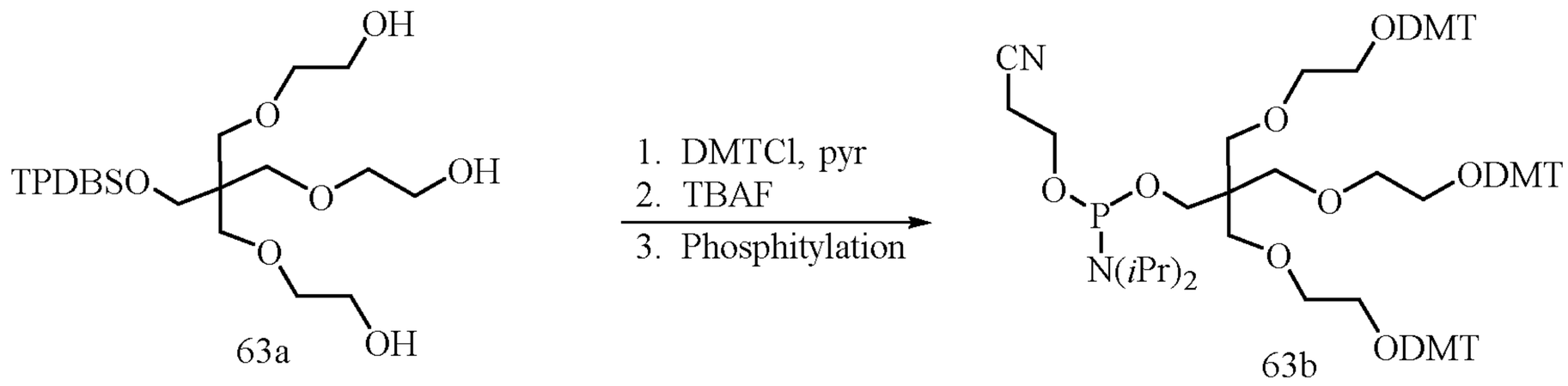
Compound 57 is meant to be representative and not intended to be limiting as other monoprotected substituted or unsubstituted alkyl diols including but not limited to those presented in the specification herein can be used to prepare phosphoramidites having a predetermined composition.

Example 29: Preparation of Compound 63



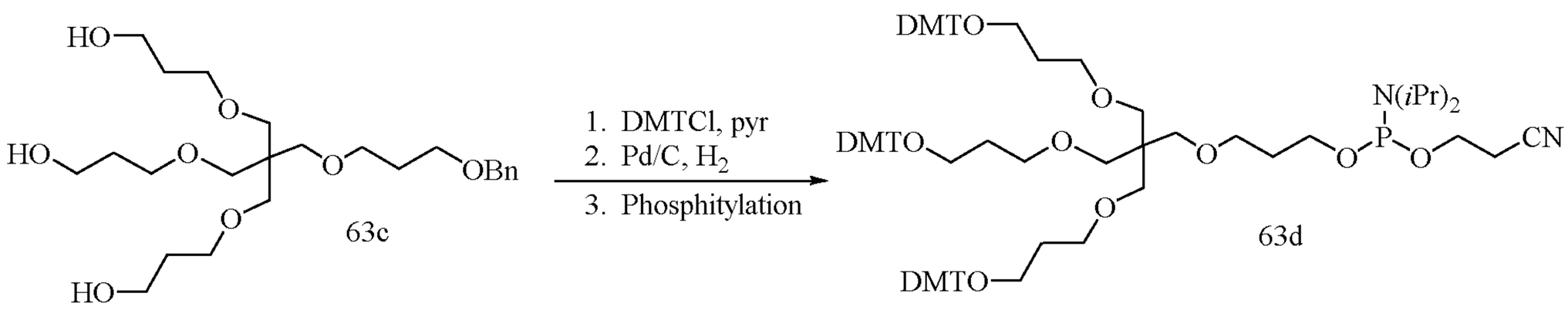
Compounds 61 and 62 are prepared using procedures similar to those reported by Tober *et al., Eur. J. Org. Chem.*, 2013, *3*, 566-577; and Jiang *et al., Tetrahedron,* 2007, *63(19),* 3982-3988.

Alternatively, Compound 63 is prepared using procedures similar to those reported in scientific and patent literature by Kim *et al., Synlett,* 2003, *12,* 1838-1840; and Kim *et al.,* published PCT International Application, WO 2004063208.**Example 30: Preparation of Compound 63b**



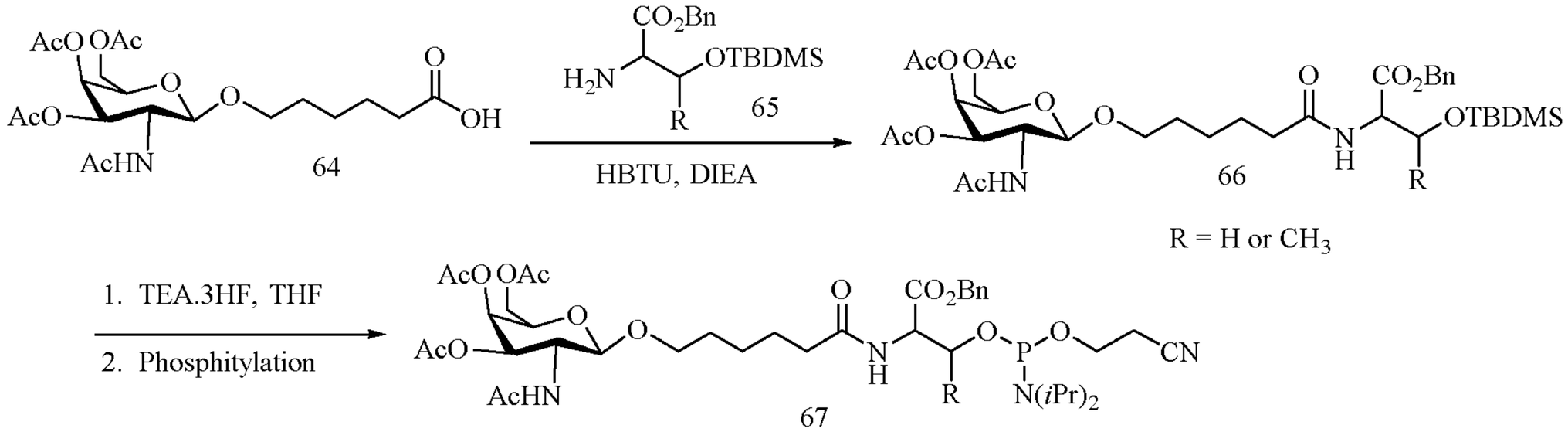
Compound 63a is prepared using procedures similar to those reported by Hanessian *et al., Canadian Journal of Chemistry,* 1996, *74(9),* 1731-1737.

Example 31: Preparation of Compound 63d



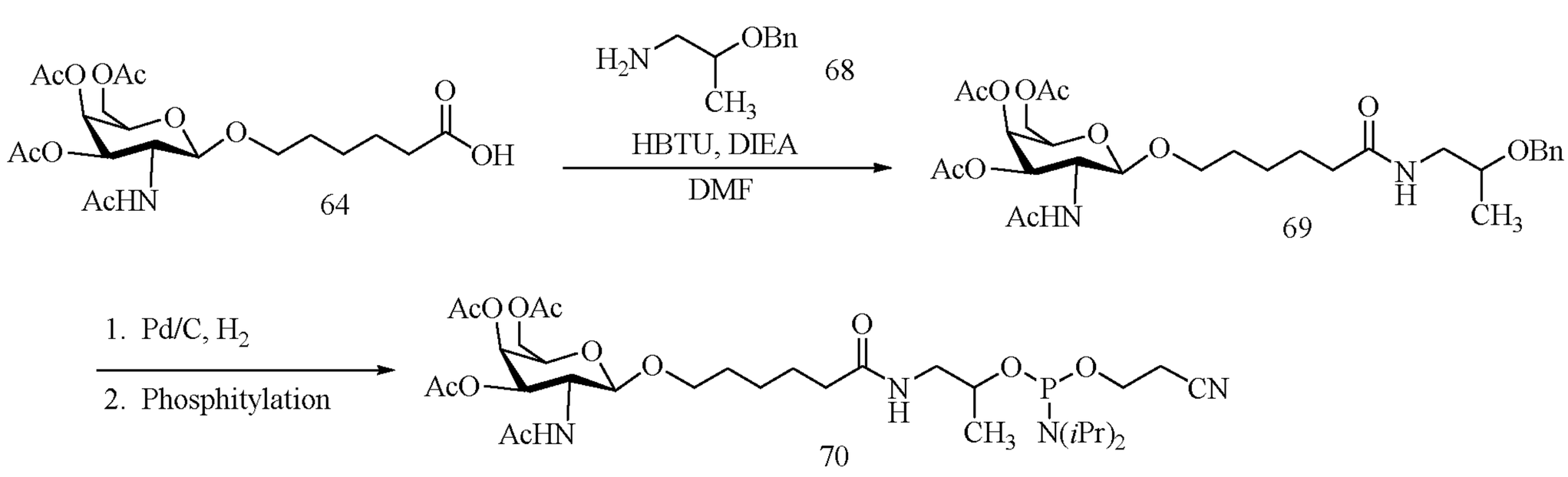
Compound 63c is prepared using procedures similar to those reported by Chen *et al., Chinese Chemical Letters,* 1998, *9(5),* 451-453.

Example 32: Preparation of Compound 67



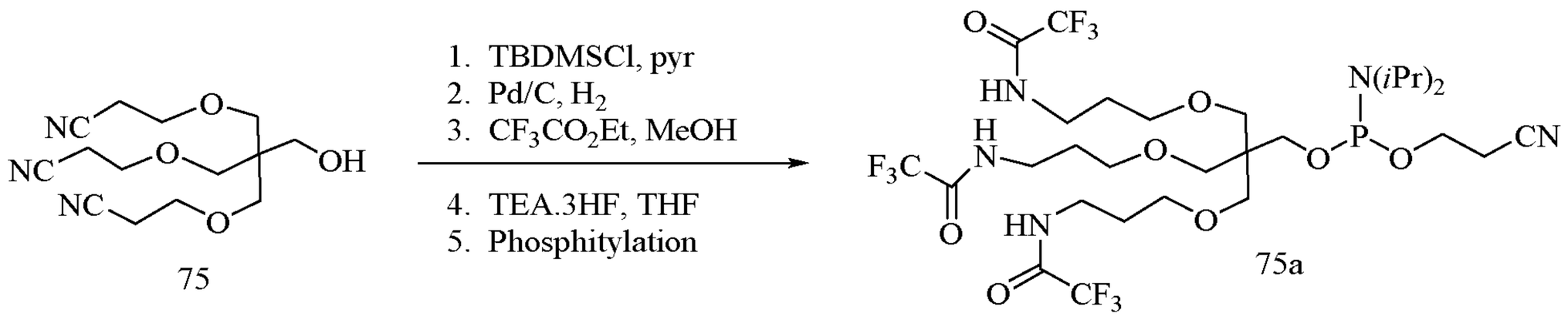
Compound 64 was prepared as per the procedures illustrated in Example 2. Compound 65 is prepared using procedures similar to those reported by Or *et al.*, published PCT International Application, WO 2009/003009. The protecting groups used for Compound 65 are meant to be representative and not intended to be limiting as other protecting groups including but not limited to those presented in the specification herein can be used.

Example 33: Preparation of Compound 70



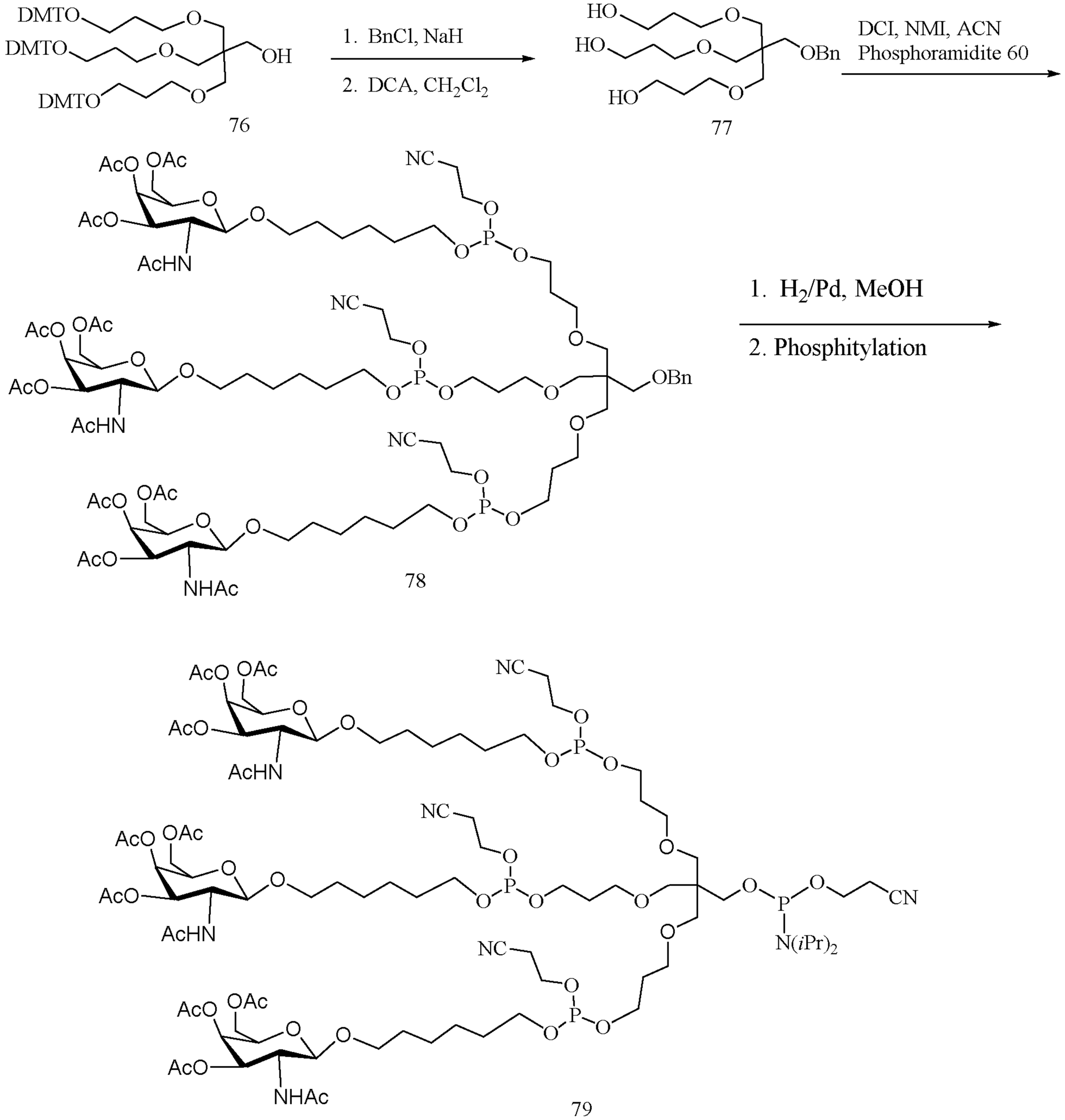
Compound 64 was prepared as per the procedures illustrated in Example 2. Compound 68 is commercially available. The protecting group used for Compound 68 is meant to be representative and not intended to be limiting as other protecting groups including but not limited to those presented in the specification herein can be used.

Example 34: Preparation of Compound 75a



Compound 75 is prepared according to published procedures reported by Shchepinov *et al., Nucleic Acids Research,* 1997, *25(22)*, 4447-4454.

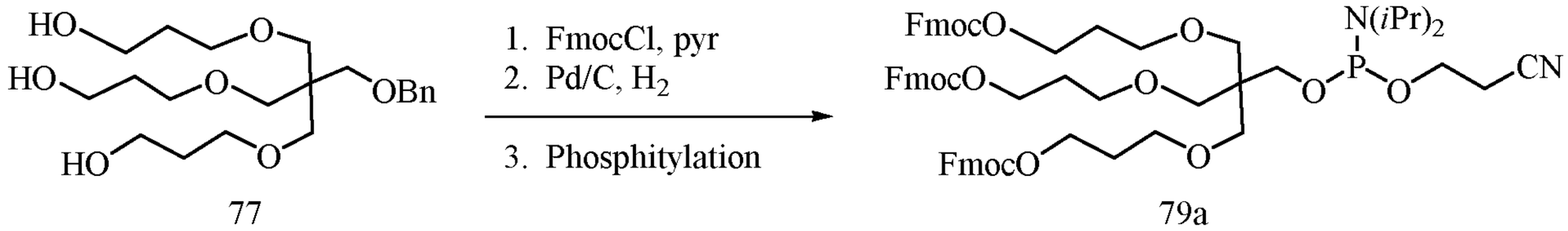
Example 35: Preparation of Compound 79



76 77 78 79

Compound 76 was prepared according to published procedures reported by Shchepinov *et al., Nucleic Acids Research,* 1997, *25(22)*, 4447-4454.

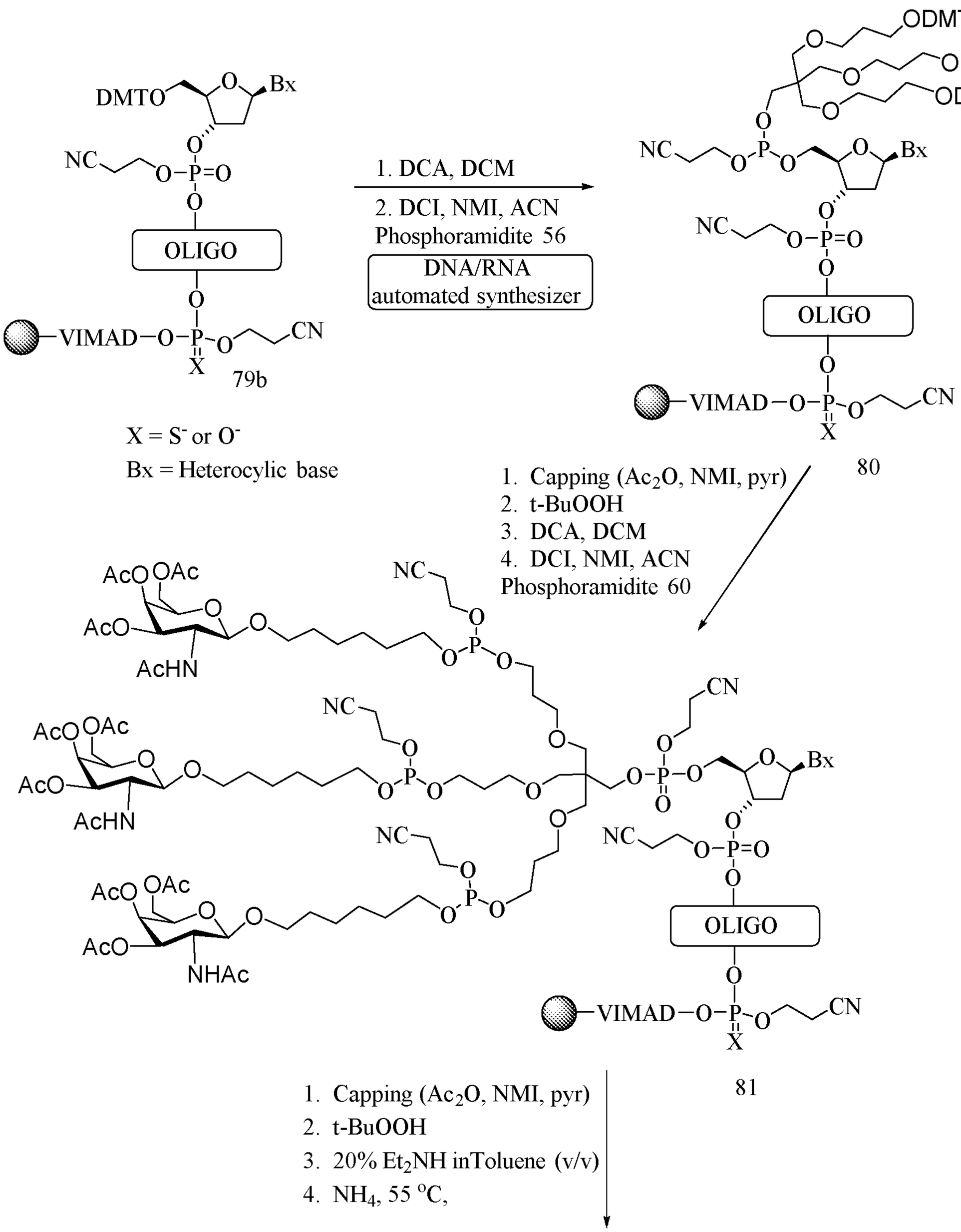
Example 36: Preparation of Compound 79a

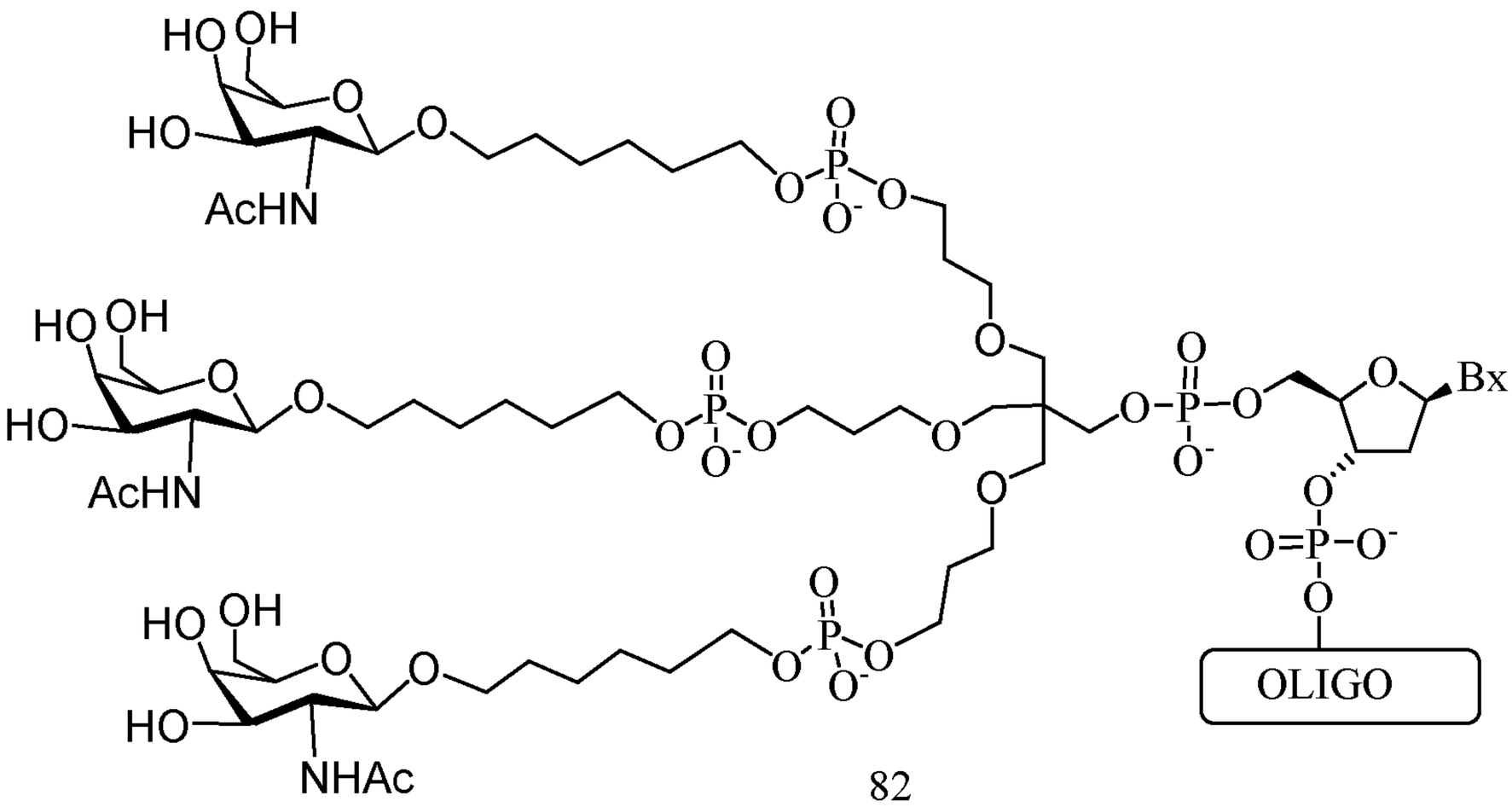


77 79a

Compound 77 is prepared as per the procedures illustrated in Example 35.

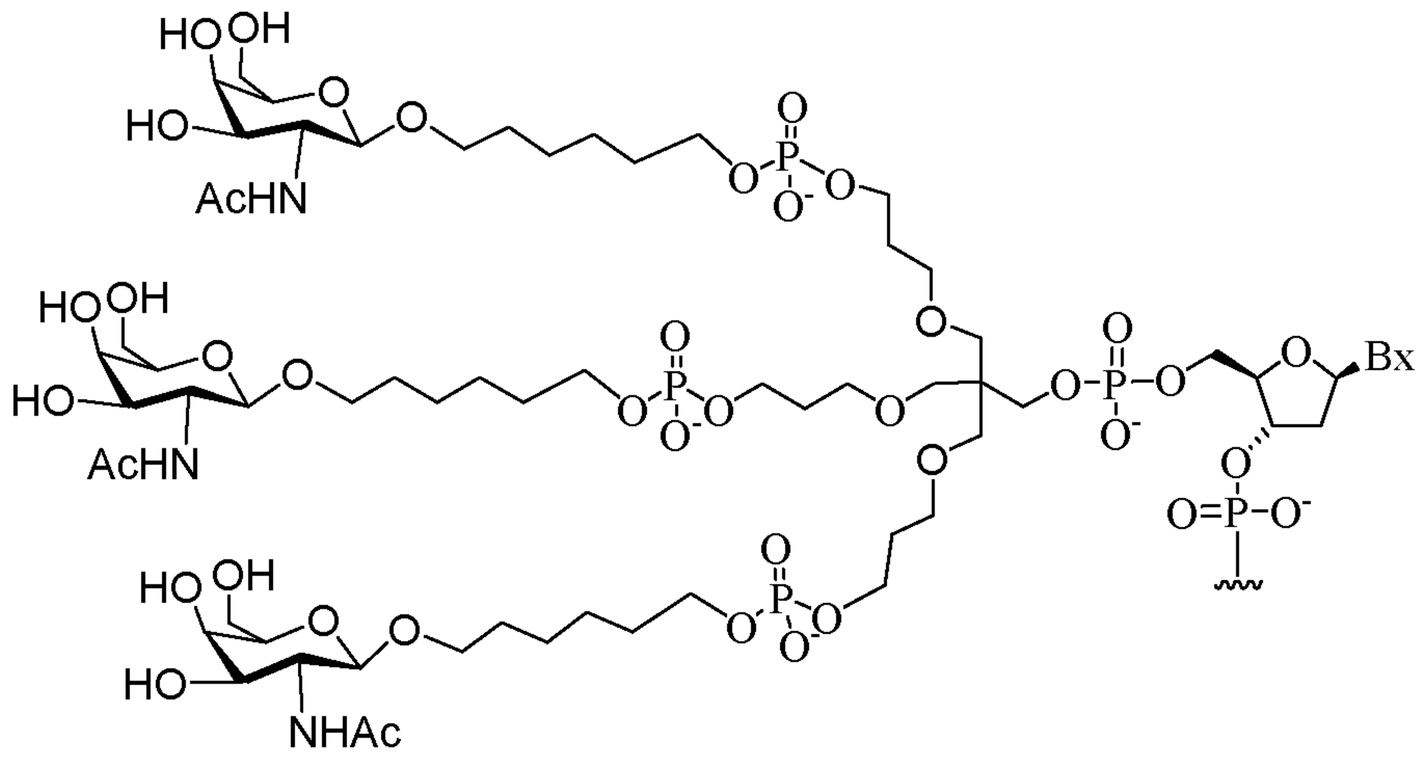
Example 37: General method for the preparation of conjugated oligomeric compound 82 comprising a phosphodiester linked $GalNAc_3$-2 conjugate at 5' terminus *via* solid support (Method I)



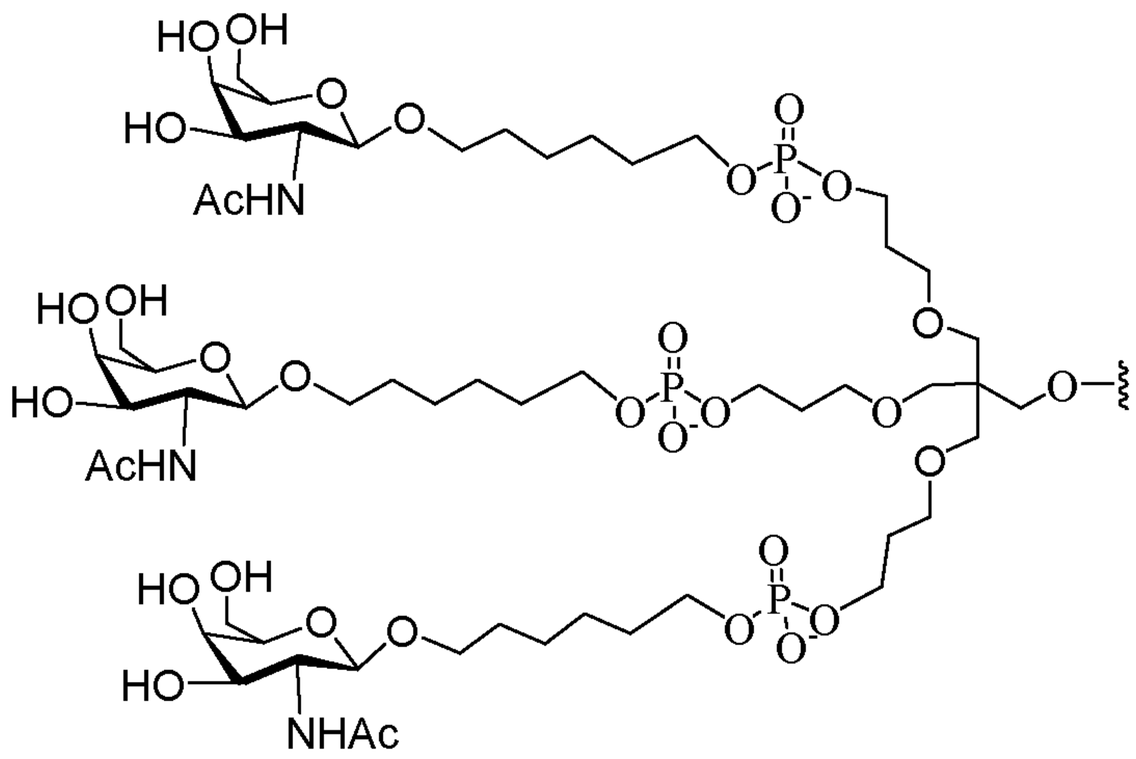


82

wherein $GalNAc_3$-2 has the structure:



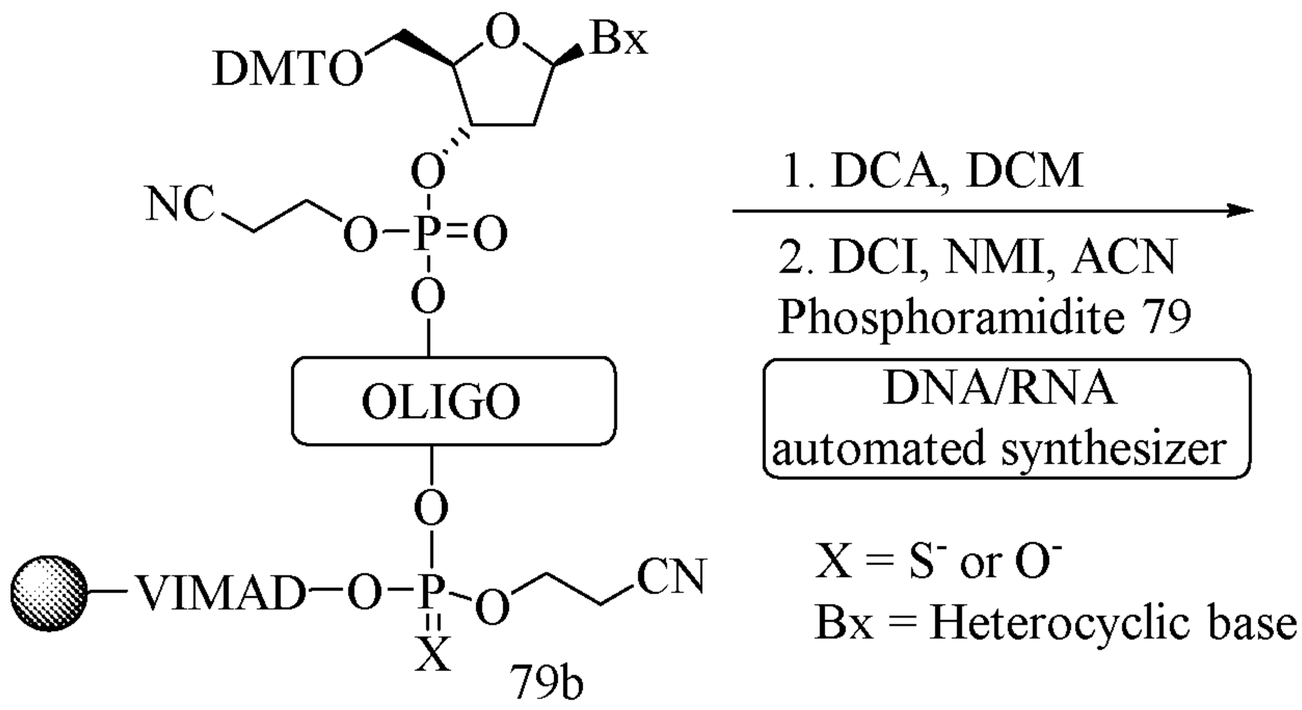
The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-2 ($GalNAc_3$-2_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. Wherein $GalNAc_3$-2_a has the formula:

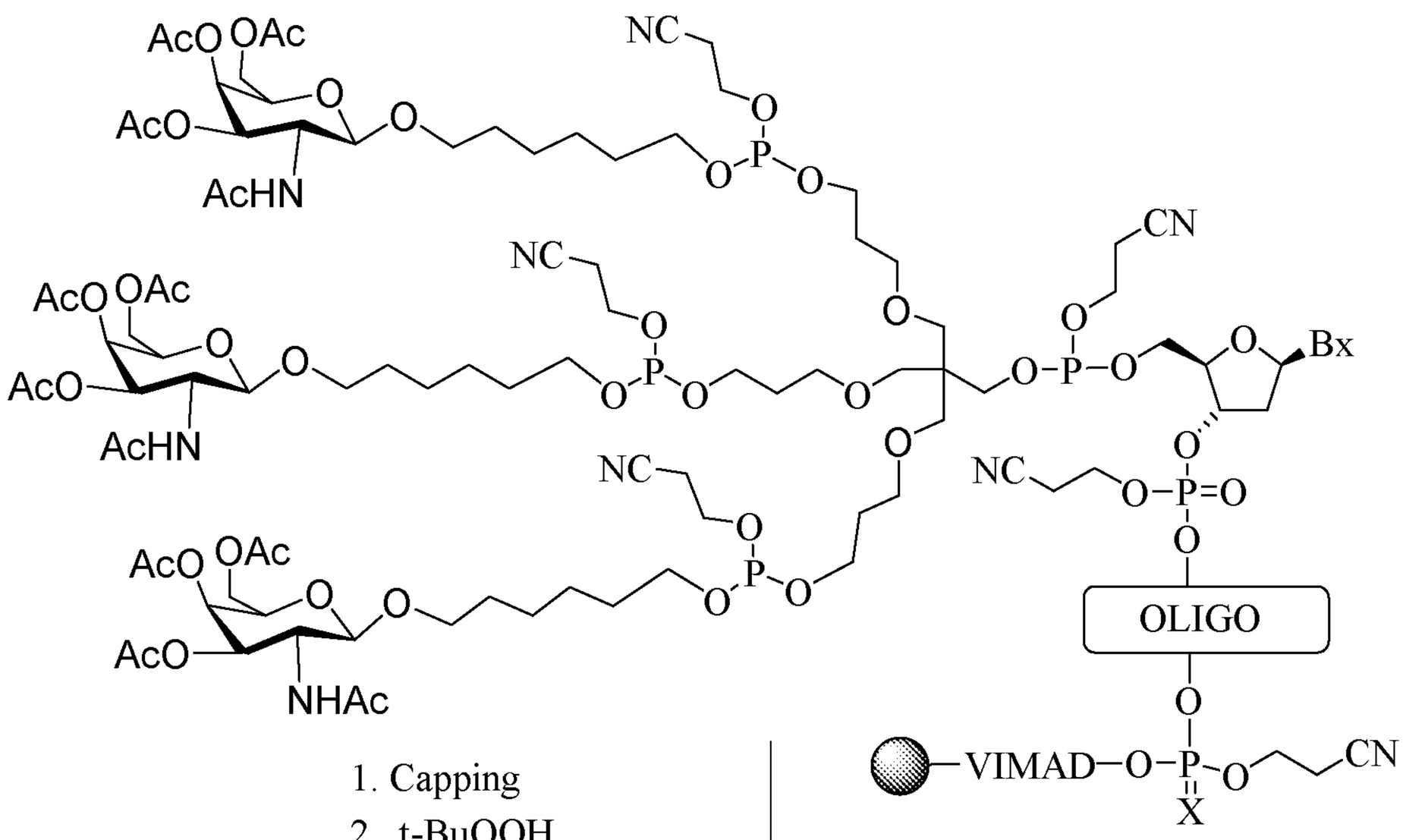


216

The VIMAD-bound oligomeric compound 79b was prepared using standard procedures for automated DNA/RNA synthesis (see Dupouy *et al., Angew. Chem. Int. Ed.,* 2006, *45,* 3623-3627). The phosphoramidite Compounds 56 and 60 were prepared as per the procedures illustrated in Examples 27 and 28, respectively. The phosphoramidites illustrated are meant to be representative and not intended to be limiting as other phosphoramidite building blocks including but not limited those presented in the specification herein can be used to prepare an oligomeric compound having a phosphodiester linked conjugate group at the 5' terminus. The order and quantity of phosphoramidites added to the solid support can be adjusted to prepare the oligomeric compounds as described herein having any predetermined sequence and composition.

Example 38: Alternative method for the preparation of oligomeric compound 82 comprising a phosphodiester linked GalNAc$_3$-2 conjugate at 5' terminus (Method II)



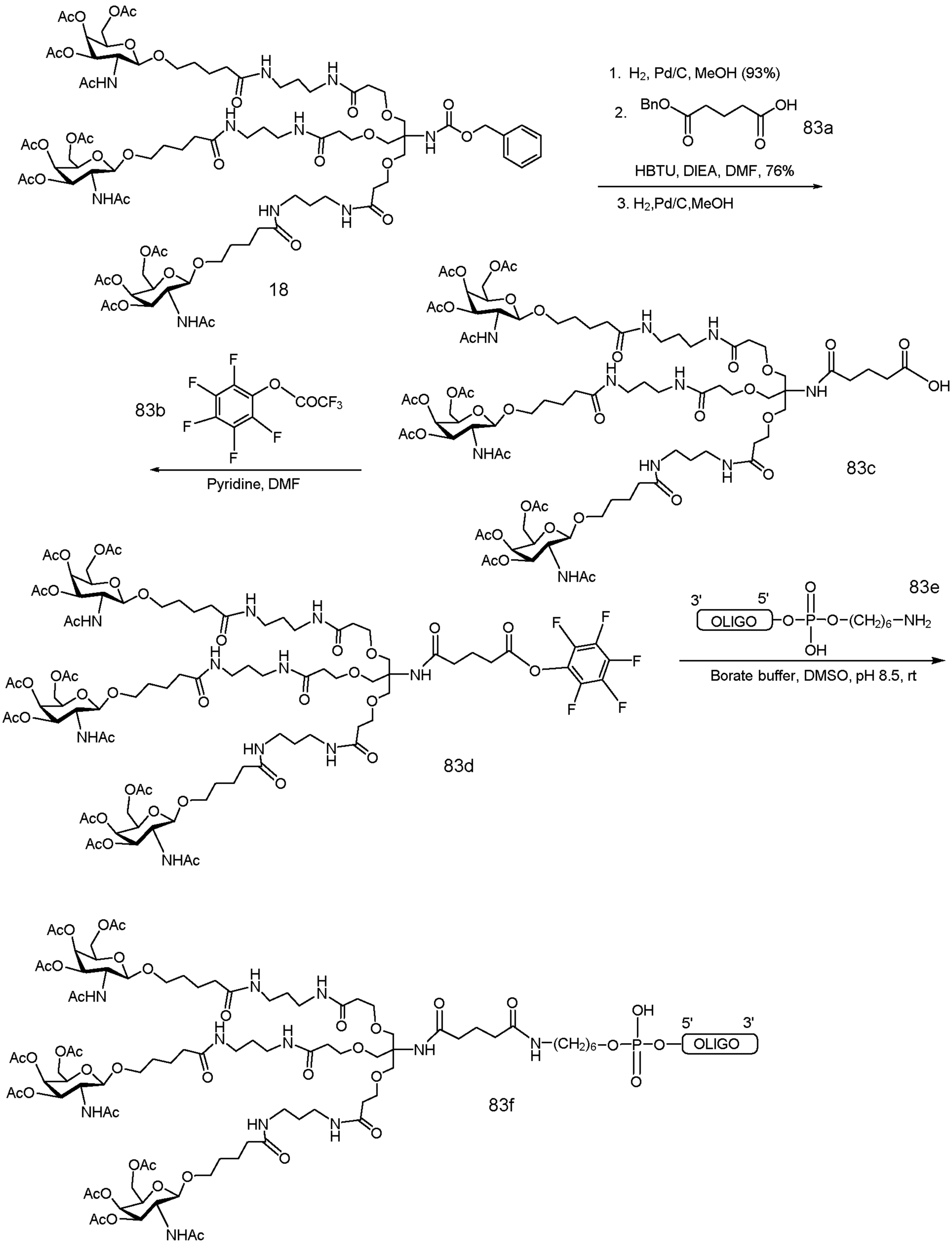


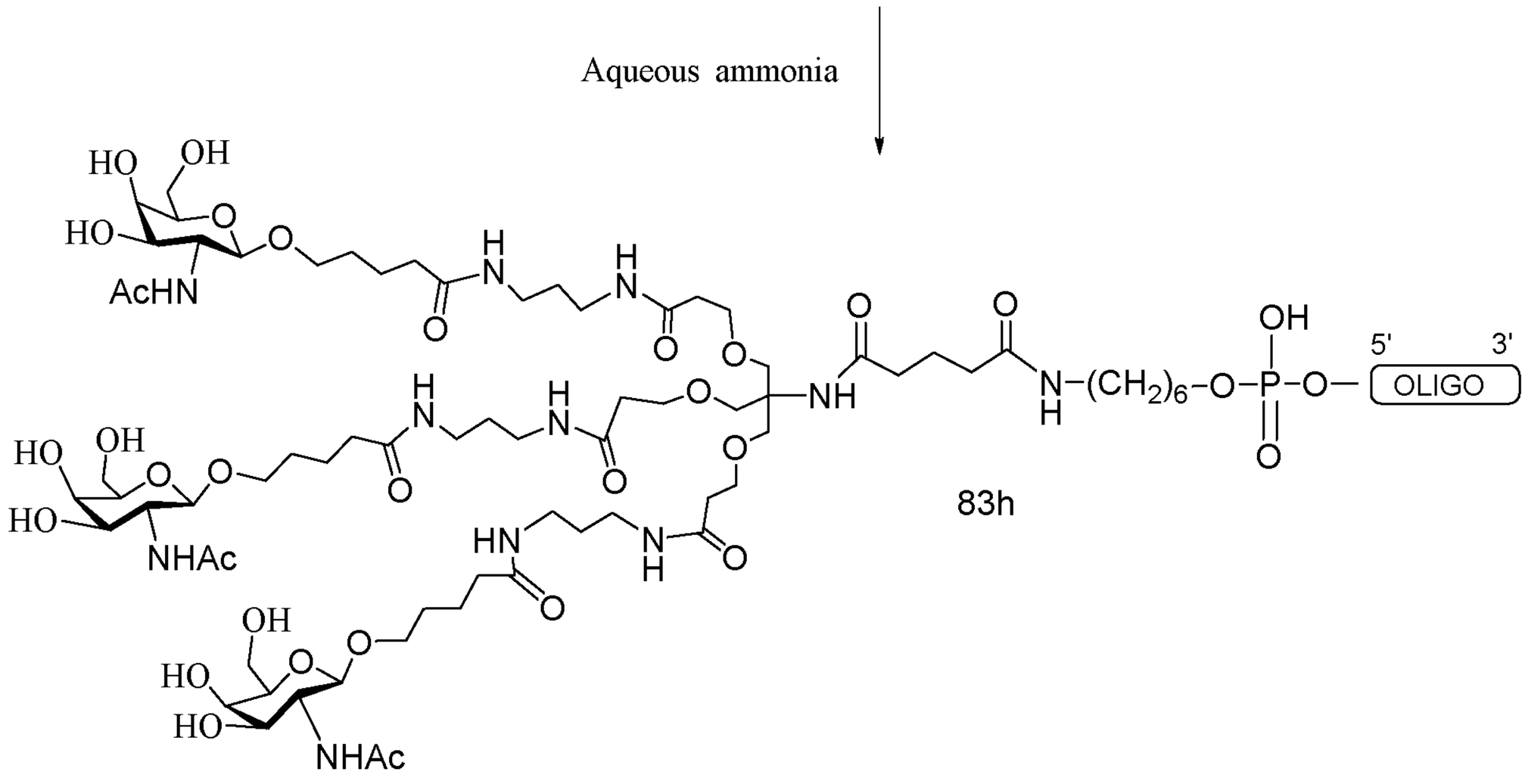
Oligomeric Compound 82

The VIMAD-bound oligomeric compound 79b was prepared using standard procedures for automated DNA/RNA synthesis (see Dupouy *et al., Angew. Chem. Int. Ed.,* 2006, *45,* 3623-3627). The GalNAc$_3$-2 cluster phosphoramidite, Compound 79 was prepared as per the procedures illustrated in Example 35. This alternative method allows a one-step installation of the phosphodiester linked GalNAc$_3$-2 conjugate to the oligomeric compound at the final step of the synthesis. The phosphoramidites illustrated are meant to

be representative and not intended to be limiting, as other phosphoramidite building blocks including but not limited to those presented in the specification herein can be used to prepare oligomeric compounds having a phosphodiester conjugate at the 5' terminus. The order and quantity of phosphoramidites added to the solid support can be adjusted to prepare the oligomeric compounds as described herein having any predetermined sequence and composition.

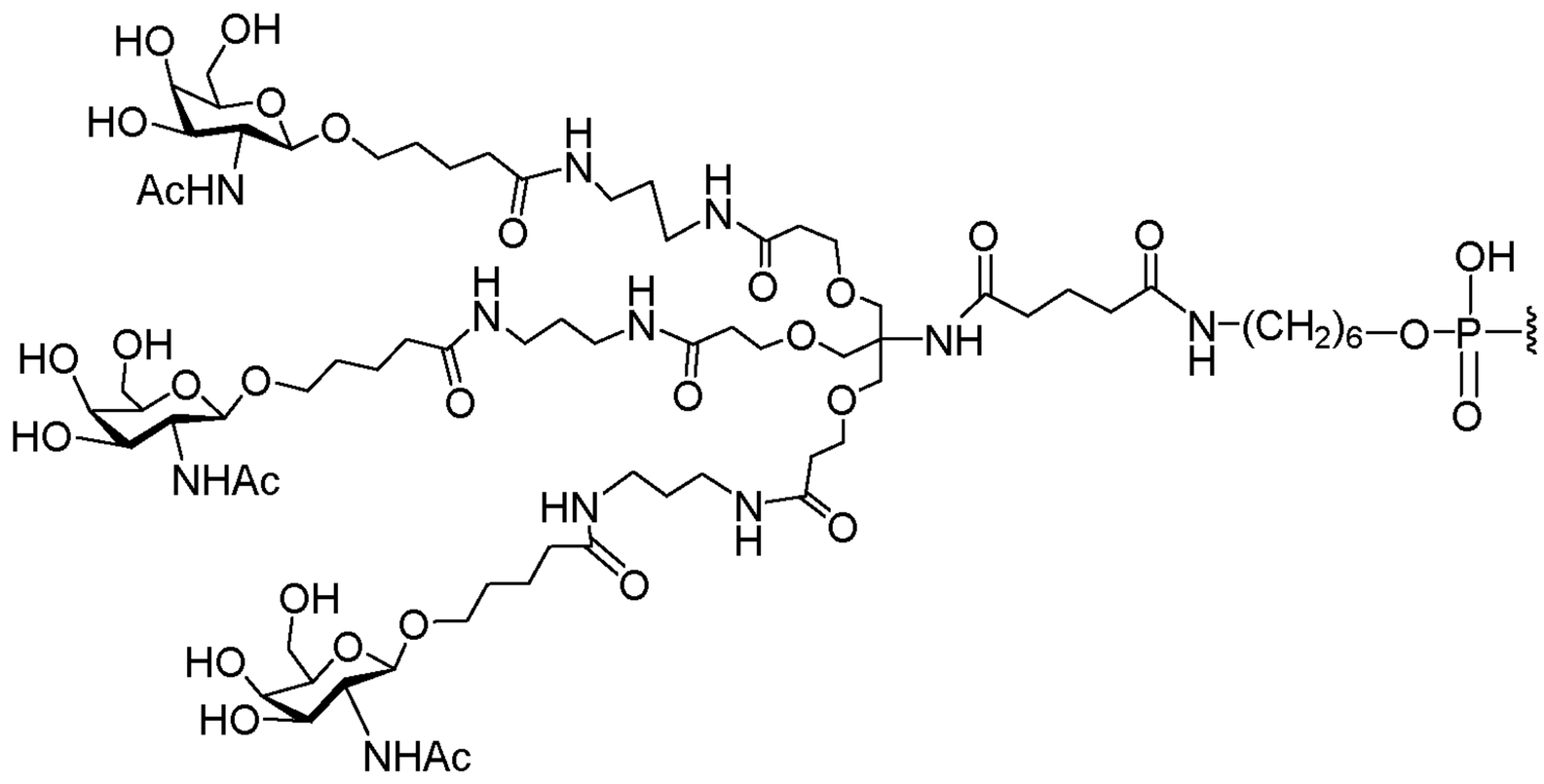
Example 39: General method for the preparation of oligomeric compound 83h comprising a $GalNAc_3$-3 Conjugate at the 5' Terminus ($GalNAc_3$-1 modified for 5' end attachment) *via* Solid Support





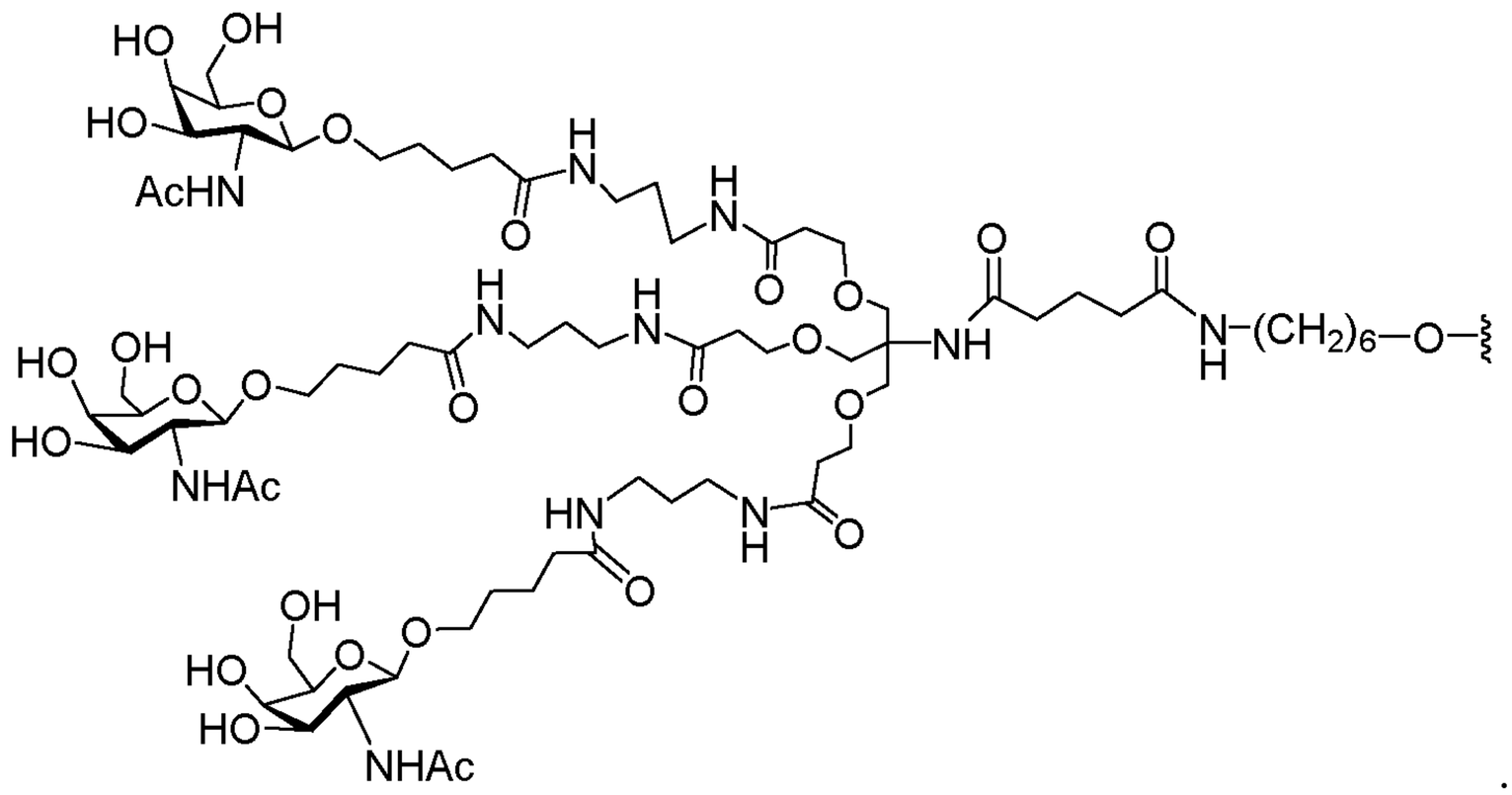
Compound 18 was prepared as per the procedures illustrated in Example 4. Compounds 83a and 83b are commercially available. Oligomeric Compound 83e comprising a phosphodiester linked hexylamine was prepared using standard oligonucleotide synthesis procedures. Treatment of the protected oligomeric compound with aqueous ammonia provided the 5'-$GalNAc_3$-3 conjugated oligomeric compound (83h).

Wherein $GalNAc_3$-3 has the structure:

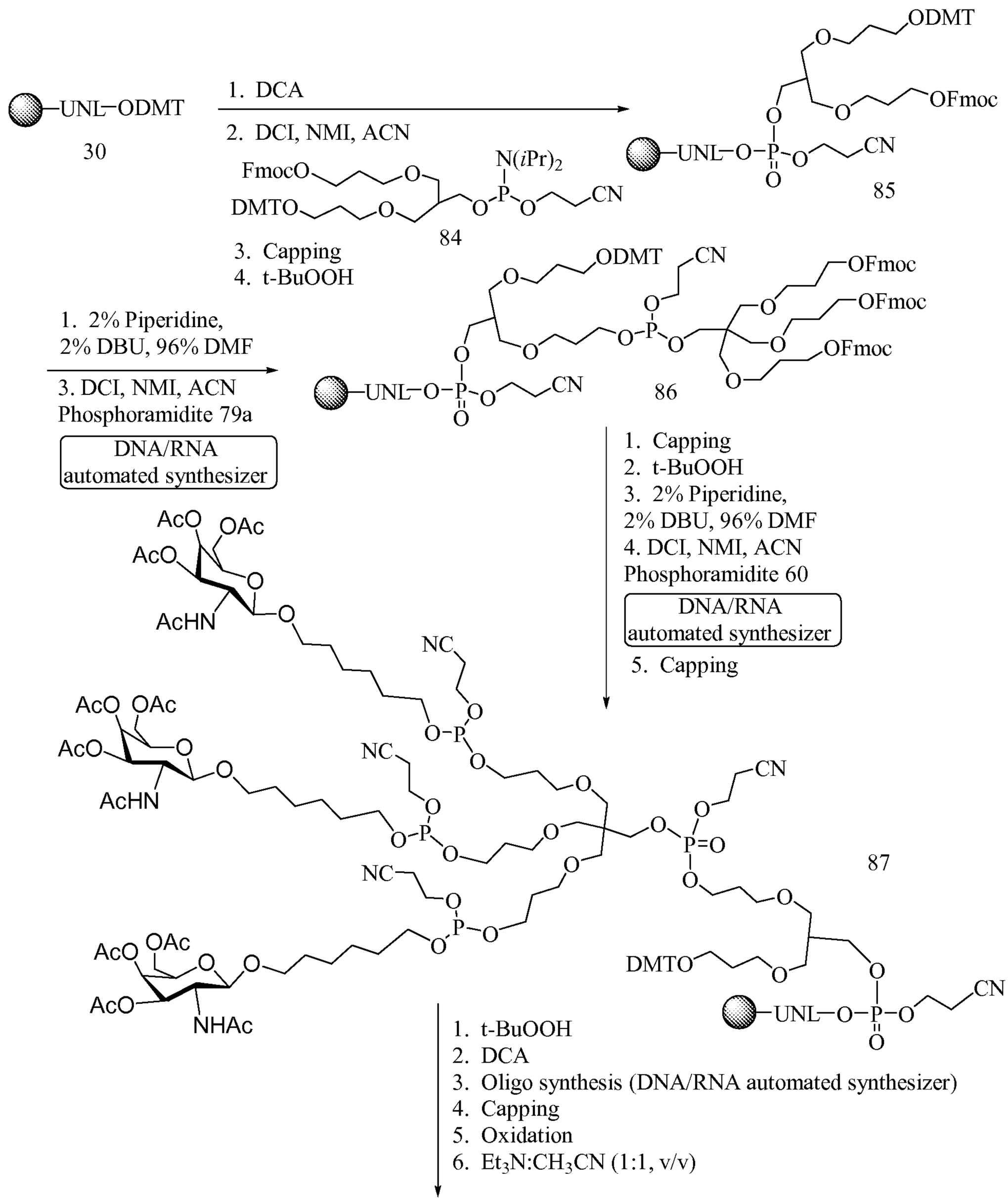


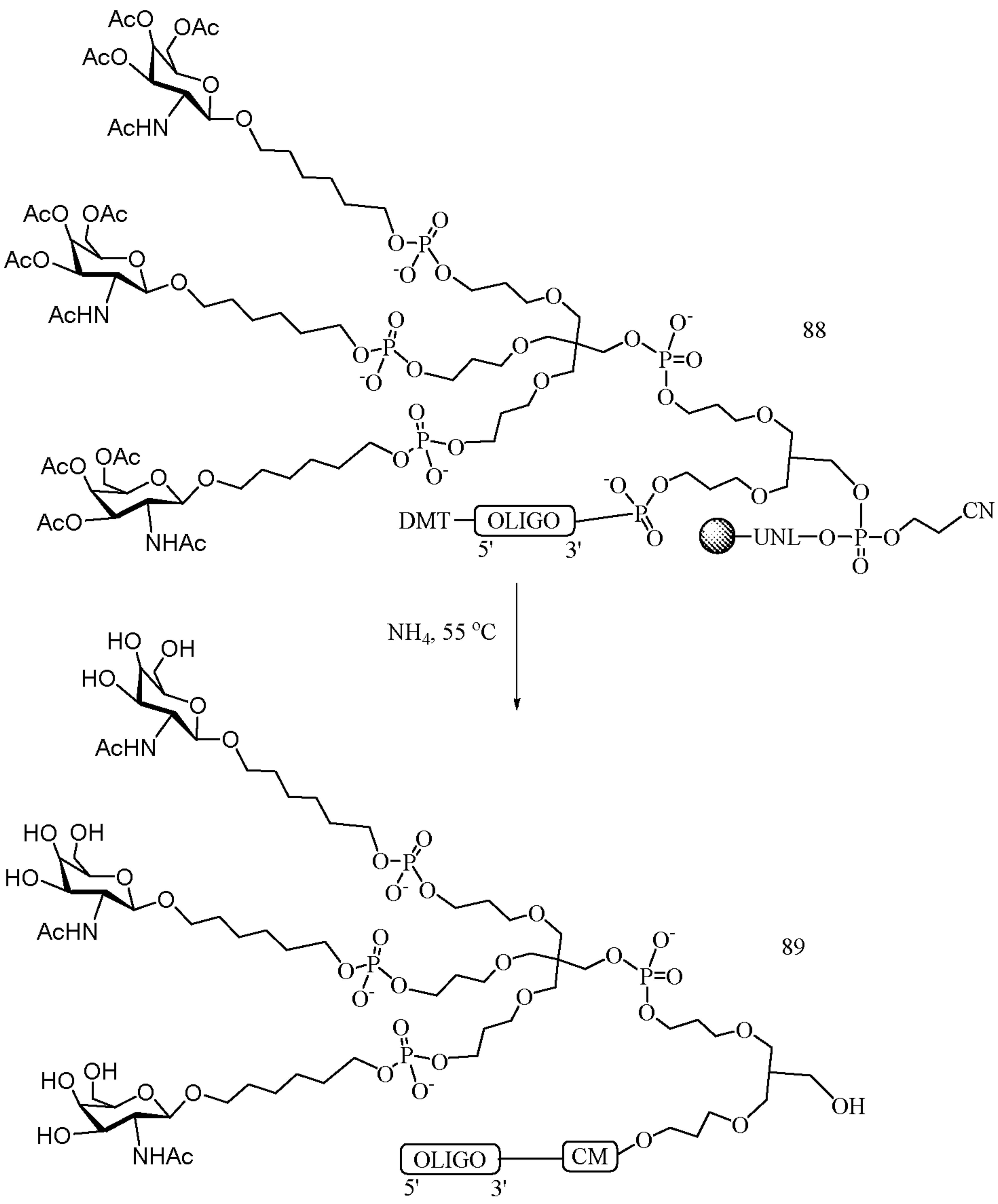
.

The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-3 ($GalNAc_3$-3_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. Wherein $GalNAc_3$-3_a has the formula:



Example 40: General method for the preparation of oligomeric compound 89 comprising a phosphodiester linked $GalNAc_3$-4 conjugate at the 3' terminus *via* solid support

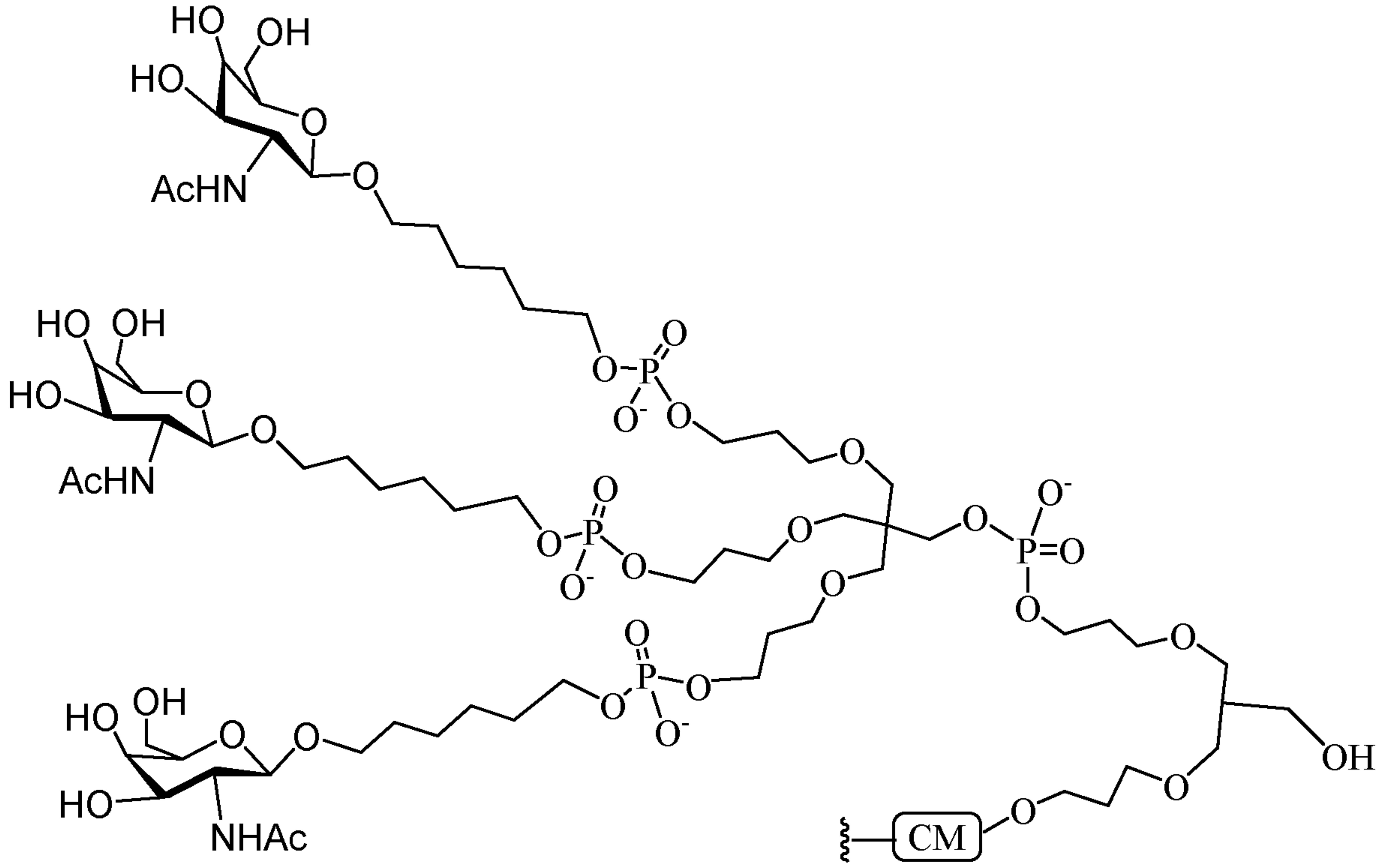



AcO
OAc
AcO
O
AcHN
O
O
P
O
O-
O
DMT
OLIGO
5'
3'
UNL
CN
NH4, 55 °C
HO
OH
NHAc
CM
OH

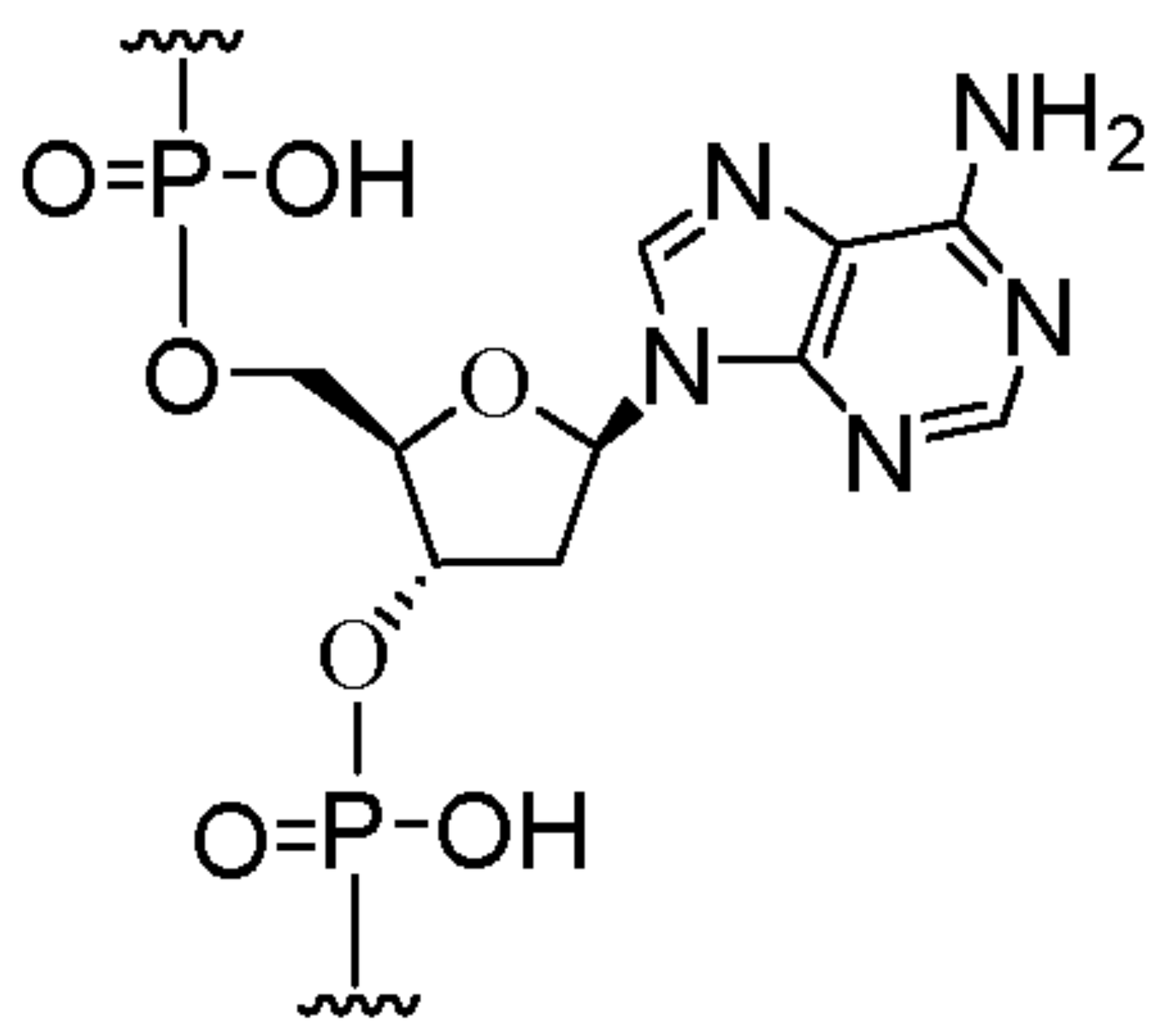
88

89

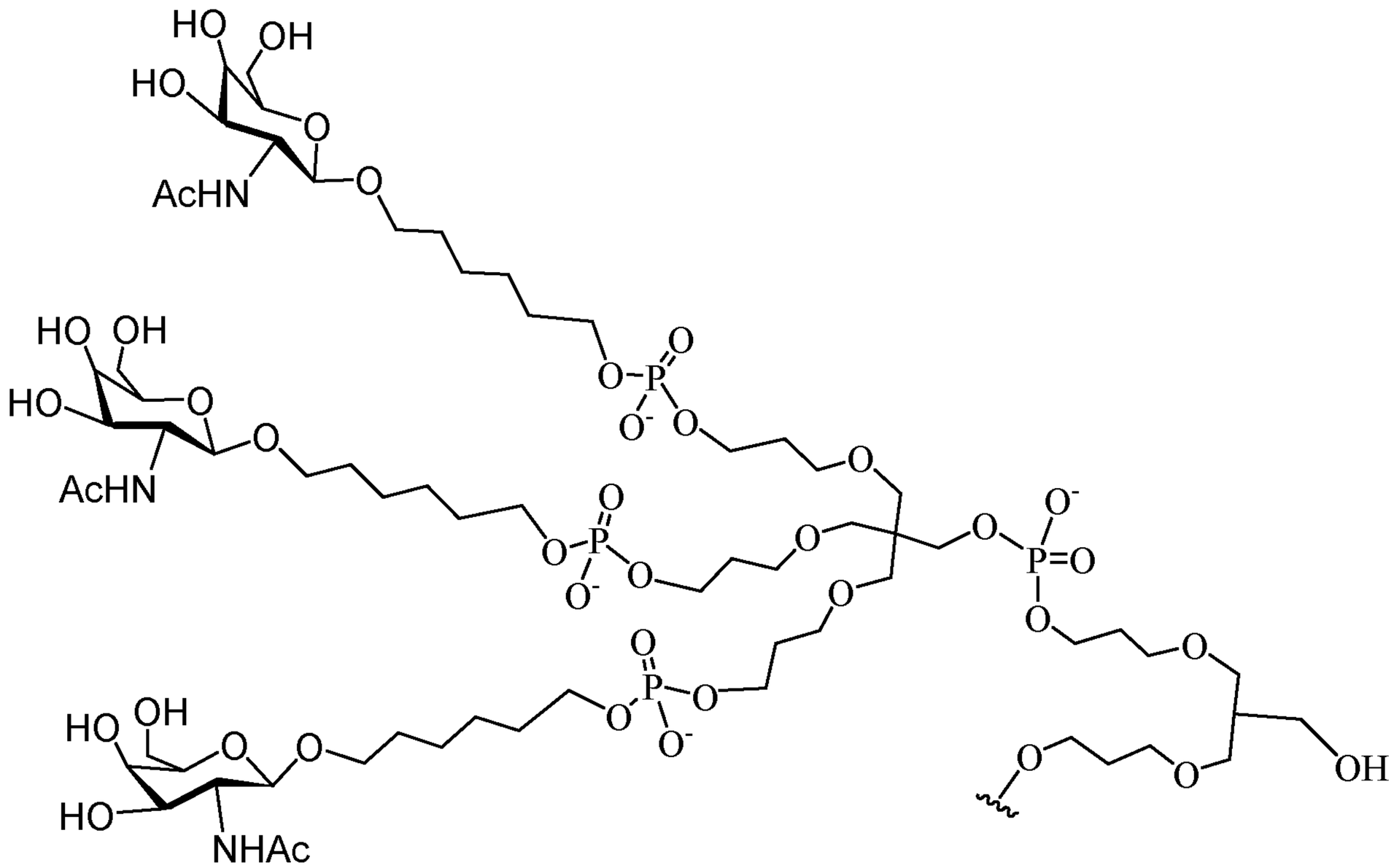
Wherein GalNAc$_3$-4 has the structure:



Wherein CM is a cleavable moiety. In certain embodiments, cleavable moiety is:



The GalNAc$_3$ cluster portion of the conjugate group GalNAc$_3$-4 (GalNAc$_3$-4$_a$) can be combined with any cleavable moiety to provide a variety of conjugate groups. Wherein GalNAc$_3$-4$_a$ has the formula:



The protected Unylinker functionalized solid support Compound 30 is commercially available. Compound 84 is prepared using procedures similar to those reported in the literature (*see* Shchepinov *et al., Nucleic Acids Research,* 1997, *25(22)*, 4447-4454; Shchepinov *et al., Nucleic Acids Research,* 1999, *27*, 3035-3041; and Hornet *et al., Nucleic Acids Research,* 1997, *25*, 4842-4849).

The phosphoramidite building blocks, Compounds 60 and 79a are prepared as per the procedures illustrated in Examples 28 and 36. The phosphoramidites illustrated are meant to be representative and not intended to be limiting as other phosphoramidite building blocks can be used to prepare an oligomeric compound having a phosphodiester linked conjugate at the 3' terminus with a predetermined sequence and composition. The order and quantity of phosphoramidites added to the solid support can be adjusted to prepare the oligomeric compounds as described herein having any predetermined sequence and composition.

Example 41: General method for the preparation of ASOs comprising a phosphodiester linked $GalNAc_3$-2 (see Example 37, Bx is adenine) conjugate at the 5' position *via* solid phase techniques (preparation of ISIS 661134)

Unless otherwise stated, all reagents and solutions used for the synthesis of oligomeric compounds are purchased from commercial sources. Standard phosphoramidite building blocks and solid support are used for incorporation nucleoside residues which include for example T, A, G, and ^{m}C residues. Phosphoramidite compounds 56 and 60 were used to synthesize the phosphodiester linked $GalNAc_3$-2

conjugate at the 5' terminus. A 0.1 M solution of phosphoramidite in anhydrous acetonitrile was used for β-D-2'-deoxyribonucleoside and 2'-MOE.

The ASO syntheses were performed on ABI 394 synthesizer (1-2 μmol scale) or on GE Healthcare Bioscience ÄKTA oligopilot synthesizer (40-200 μmol scale) by the phosphoramidite coupling method on VIMAD solid support (110 μmol/g, Guzaev *et al.*, 2003) packed in the column. For the coupling step, the phosphoramidites were delivered at a 4 fold excess over the initial loading of the solid support and phosphoramidite coupling was carried out for 10 min. All other steps followed standard protocols supplied by the manufacturer. A solution of 6% dichloroacetic acid in toluene was used for removing the dimethoxytrityl (DMT) groups from 5'-hydroxyl groups of the nucleotide. 4,5-Dicyanoimidazole (0.7 M) in anhydrous CH_3CN was used as activator during the coupling step. Phosphorothioate linkages were introduced by sulfurization with 0.1 M solution of xanthane hydride in 1:1 pyridine/CH_3CN for a contact time of 3 minutes. A solution of 20% *tert*-butylhydroperoxide in CH_3CN containing 6% water was used as an oxidizing agent to provide phosphodiester internucleoside linkages with a contact time of 12 minutes.

After the desired sequence was assembled, the cyanoethyl phosphate protecting groups were deprotected using a 20% diethylamine in toluene (v/v) with a contact time of 45 minutes. The solid-support bound ASOs were suspended in aqueous ammonia (28-30 wt %) and heated at 55 °C for 6 h.

The unbound ASOs were then filtered and the ammonia was boiled off. The residue was purified by high pressure liquid chromatography on a strong anion exchange column (GE Healthcare Bioscience, Source 30Q, 30 μm, 2.54 x 8 cm, A = 100 mM ammonium acetate in 30% aqueous CH_3CN, B = 1.5 M NaBr in A, 0-40% of B in 60 min, flow 14 mL min-1, λ = 260 nm). The residue was desalted by HPLC on a reverse phase column to yield the desired ASOs in an isolated yield of 15-30% based on the initial loading on the solid support. The ASOs were characterized by ion-pair-HPLC coupled MS analysis with Agilent 1100 MSD system.

Table 34

ASO comprising a phosphodiester linked $GalNAc_3$-2 conjugate at the 5' position targeting SRB-1

ISIS No.	Sequence (5' to 3')	CalCd Mass	Observed Mass	SEQ ID No.
661134	**$GalNAc_3$-$2_{a-o'}$$A_{do}$**$T_{ks}$$^{m}C_{ks}A_{ds}G_{ds}T_{ds}$$^{m}C_{ds}A_{ds}T_{ds}$ $G_{ds}A_{ds}$$^{m}C_{ds}T_{ds}T_{ks}$$^{m}C_{k}$	6482.2	6481.6	4884

Subscripts: "e" indicates 2'-MOE modified nucleoside; "d" indicates β-D-2'-deoxyribonucleoside; "k" indicates 6'-*(S)*-CH_3 bicyclic nucleoside (e.g. cEt); "s" indicates phosphorothioate internucleoside linkages (PS); "o" indicates phosphodiester internucleoside linkages (PO); and "o'" indicates -O-P(=O)(OH)-. Superscript "m" indicates 5-methylcytosines. The structure of $GalNAc_3$-2_a is shown in Example 37.

Example 42: General method for the preparation of ASOs comprising a $GalNAc_3$-3 conjugate at the 5' position *via* solid phase techniques (preparation of ISIS 661166)

The synthesis for ISIS 661166 was performed using similar procedures as illustrated in Examples 39 and 41.

ISIS 661166 is a 5-10-5 MOE gapmer, wherein the 5' position comprises a $GalNAc_3$-3 conjugate. The ASO was characterized by ion-pair-HPLC coupled MS analysis with Agilent 1100 MSD system.

Table 34a
ASO comprising a $GalNAc_3$-3 conjugate at the 5' position via a hexylamino phosphodiester linkage targeting Malat-1

ISIS No.	Sequence (5' to 3')	Conjugate	Calcd Mass	Observed Mass	SEQ ID No.
661166	5'-**$GalNAc_3$-$3_{a\text{-}o}$**-$^{m}C_{es}G_{es}G_{es}T_{es}G_{es}$ $^{m}C_{ds}A_{ds}A_{ds}G_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{ds}A_{ds}G_{ds}$ $G_{es}A_{es}A_{es}T_{es}T_{e}$	**5'-$GalNAc_3$-3**	8992.16	8990.51	4885

Subscripts: "e" indicates 2'-MOE modified nucleoside; "d" indicates β-D-2'-deoxyribonucleoside; "s" indicates phosphorothioate internucleoside linkages (PS); "o" indicates phosphodiester internucleoside linkages (PO); and "o'" indicates -O-P(=O)(OH)-. Superscript "m" indicates 5-methylcytosines. The structure of "5'-$GalNAc_3$-3a" is shown in Example 39.

Example 43: Dose-dependent study of phosphodiester linked $GalNAc_3$-2 (see examples 37 and 41, Bx is adenine) at the 5' terminus targeting SRB-1 *in vivo*

ISIS 661134 (see Example 41) comprising a phosphodiester linked $GalNAc_3$-2 conjugate at the 5' terminus was tested in a dose-dependent study for antisense inhibition of SRB-1 in mice. Unconjugated ISIS 440762 and 651900 ($GalNAc_3$-1 conjugate at 3' terminus, see Example 9) were included in the study for comparison and are described previously in Table 17.

Treatment

Six week old male Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously once at the dosage shown below with ISIS 440762, 651900, 661134 or with PBS treated control. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration to determine the liver SRB-1 mRNA levels using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. SRB-1 mRNA levels were determined relative to total RNA (using Ribogreen), prior to normalization to PBS-treated control. The results below are presented as the average percent of SRB-1 mRNA levels for each treatment group, normalized to PBS-treated control and is denoted as "% PBS". The ED_{50}s were measured using similar methods as described previously and are presented below.

As illustrated in Table 35, treatment with antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner. Indeed, the antisense oligonucleotides comprising the phosphodiester linked $GalNAc_3$-2 conjugate at the 5' terminus (ISIS 661134) or the $GalNAc_3$-1 conjugate linked at the 3' terminus (ISIS 651900) showed substantial improvement in potency compared to the unconjugated antisense oligonucleotide (ISIS 440762). Further, ISIS 661134, which comprises the phosphodiester linked $GalNAc_3$-2 conjugate at the 5' terminus was equipotent compared to ISIS 651900, which comprises the $GalNAc_3$-1 conjugate at the 3' terminus.

Table 35

ASOs containing $GalNAc_3$-1 or $GalNAc_3$-2 targeting SRB-1

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA levels (% PBS)	ED_{50} (mg/kg)	Conjugate	SEQ ID No.
PBS	0	100	--	--	
440762	0.2	116	2.58	No conjugate	4880
	0.7	91			
	2	69			
	7	22			
	20	5			
651900	0.07	95	0.26	**3' $GalNAc_3$-1**	4881
	0.2	77			
	0.7	28			
	2	11			
	7	8			
661134	0.07	107	0.25	**5' $GalNAc_3$-2**	4881
	0.2	86			
	0.7	28			
	2	10			
	7	6			

Structures for 3' $GalNAc_3$-1 and 5' $GalNAc_3$-2 were described previously in Examples 9 and 37.

Pharmacokinetics Analysis (PK)

The PK of the ASOs from the high dose group (7 mg/kg) was examined and evaluated in the same manner as illustrated in Example 20. Liver sample was minced and extracted using standard protocols. The full length metabolites of 661134 (5' $GalNAc_3$-2) and ISIS 651900 (3' $GalNAc_3$-1) were identified and their masses were confirmed by high resolution mass spectrometry analysis. The results showed that the major metabolite detected for the ASO comprising a phosphodiester linked $GalNAc_3$-2 conjugate at the 5' terminus (ISIS 661134) was ISIS 440762 (data not shown). No additional metabolites, at a detectable level, were observed. Unlike its counterpart, additional metabolites similar to those reported previously in Table 23a were observed for the ASO having the $GalNAc_3$-1 conjugate at the 3' terminus (ISIS 651900). These results suggest that having the phosphodiester linked $GalNAc_3$-1 or $GalNAc_3$-2 conjugate may improve the PK profile of ASOs without compromising their potency.

Example 44: Effect of PO/PS linkages on antisense inhibition of ASOs comprising GalNAc$_3$-1 conjugate (see Example 9) at the 3' terminus targeting SRB-1

ISIS 655861 and 655862 comprising a GalNAc$_3$-1 conjugate at the 3' terminus each targeting SRB-1 were tested in a single administration study for their ability to inhibit SRB-1 in mice. The parent unconjugated compound, ISIS 353382 was included in the study for comparison.

The ASOs are 5-10-5 MOE gapmers, wherein the gap region comprises ten 2'-deoxyribonucleosides and each wing region comprises five 2'-MOE modified nucleosides. The ASOs were prepared using similar methods as illustrated previously in Example 19 and are described Table 36, below.

Table 36
Modified ASOs comprising GalNAc$_3$-1 conjugate at the 3' terminus targeting SRB-1

ISIS No.	Sequence (5' to 3')	Chemistry	SEQ ID No.
353382 (parent)	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	Full PS no conjugate	4886
655861	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{eo}\mathbf{A_{do'}\text{-}GalNAc_3\text{-}1_a}$	Full PS with **GalNAc$_3$-1** conjugate	4887
655862	$G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T_{eo}\mathbf{A_{do'}\text{-}GalNAc_3\text{-}1_a}$	Mixed PS/PO with **GalNAc$_3$-1** conjugate	4887

Subscripts: "e" indicates 2'-MOE modified nucleoside; "d" indicates β-D-2'-deoxyribonucleoside; "s" indicates phosphorothioate internucleoside linkages (PS); "o" indicates phosphodiester internucleoside linkages (PO); and "o'" indicates -O-P(=O)(OH)-. Superscript "m" indicates 5-methylcytosines. The structure of "GalNAc$_3$-1" is shown in Example 9.

Treatment

Six week old male Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously once at the dosage shown below with ISIS 353382, 655861, 655862 or with PBS treated control. Each treatment group consisted of 4 animals. Prior to the treatment as well as after the last dose, blood was drawn from each mouse and plasma samples were analyzed. The mice were sacrificed 72 hours following the final administration to determine the liver SRB-1 mRNA levels using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. SRB-1 mRNA levels were determined relative to total RNA (using Ribogreen), prior to normalization to PBS-treated control. The results below are presented as the average percent of SRB-1 mRNA levels for each treatment group, normalized to PBS-treated control and is denoted as "% PBS". The ED$_{50}$s were measured using similar methods as described previously and are reported below.

As illustrated in Table 37, treatment with antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner compared to PBS treated control. Indeed, the antisense oligonucleotides comprising the GalNAc$_3$-1 conjugate at the 3' terminus (ISIS 655861 and 655862) showed substantial improvement in potency comparing to the unconjugated antisense oligonucleotide (ISIS 353382). Further,

ISIS 655862 with mixed PS/PO linkages showed an improvement in potency relative to full PS (ISIS 655861).

Table 37
Effect of PO/PS linkages on antisense inhibition of ASOs comprising $GalNAc_3$-1 conjugate at 3' terminus targeting SRB-1

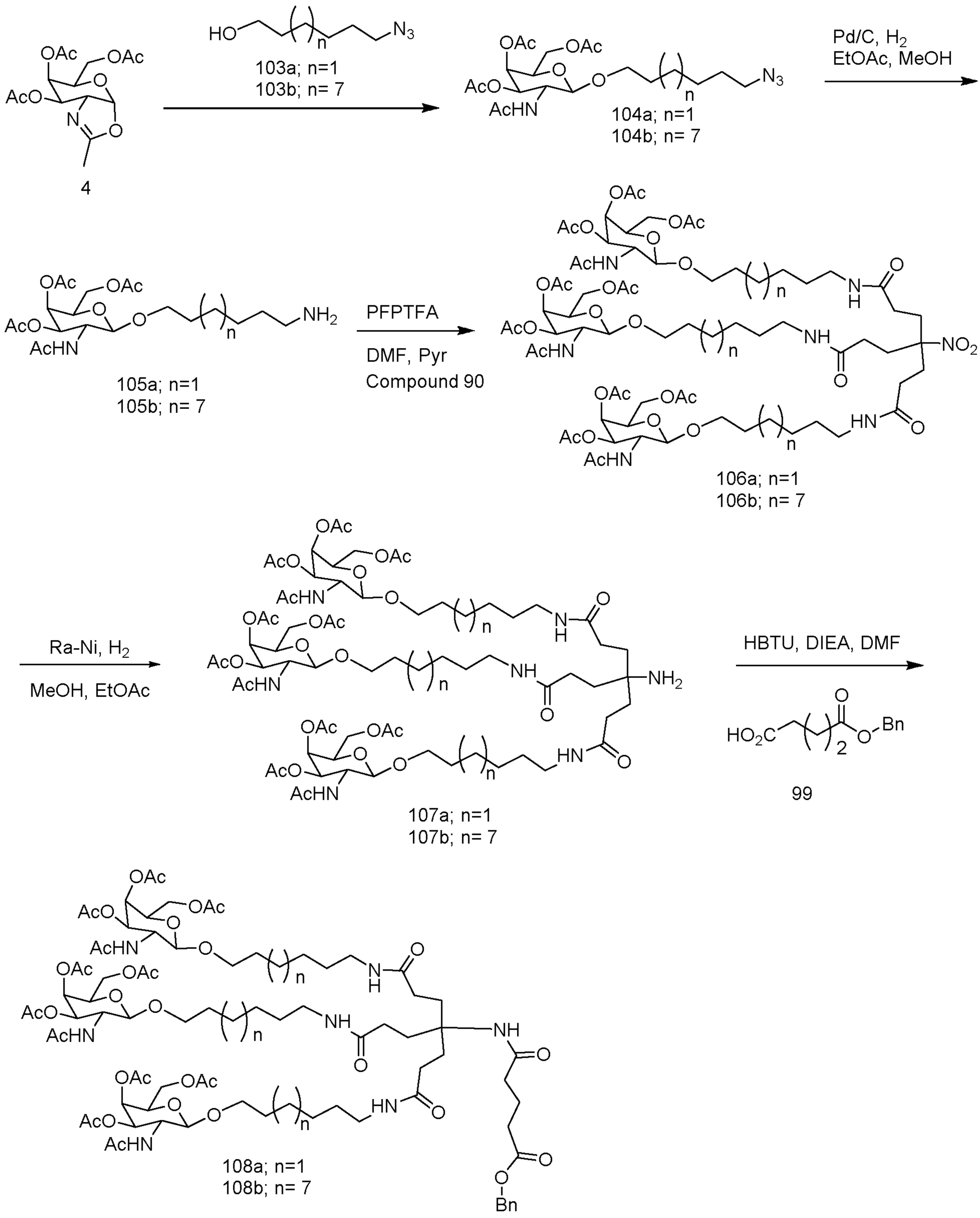
ISIS No.	Dosage (mg/kg)	SRB-1 mRNA levels (% PBS)	ED_{50} (mg/kg)	Chemistry	SEQ ID No.
PBS	0	100	--	--	
353382 (parent)	3	76.65	10.4	Full PS without conjugate	4886
	10	52.40			
	30	24.95			
655861	0.5	81.22	2.2	Full PS with $GalNAc_3$-1 conjugate	4887
	1.5	63.51			
	5	24.61			
	15	14.80			
655862	0.5	69.57	1.3	Mixed PS/PO with $GalNAc_3$-1 conjugate	4887
	1.5	45.78			
	5	19.70			
	15	12.90			

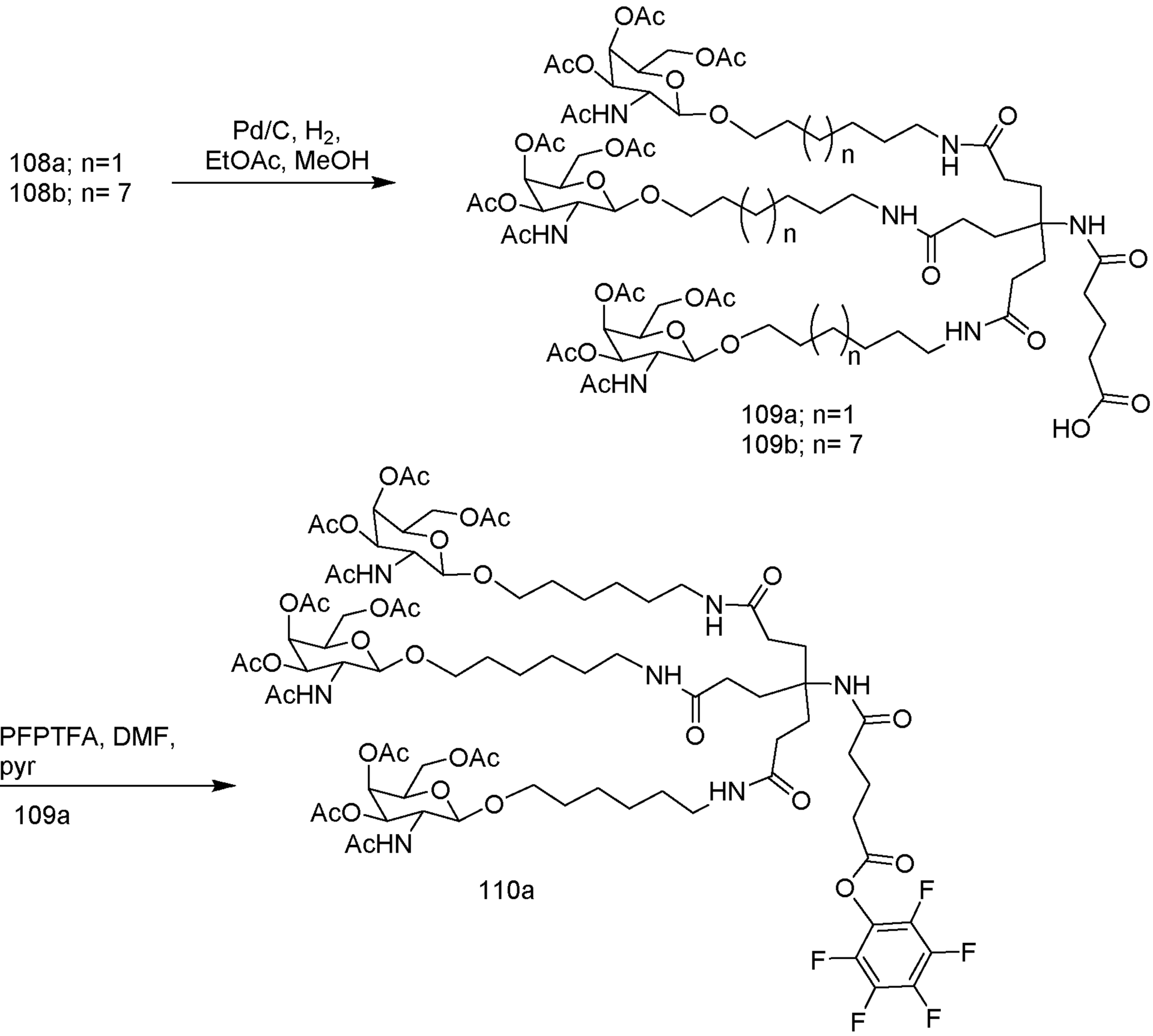
Liver transaminase levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in serum were measured relative to saline injected mice using standard protocols. Organ weights were also evaluated. The results demonstrated that no elevation in transaminase levels (Table 38) or organ weights (data not shown) were observed in mice treated with ASOs compared to PBS control. Further, the ASO with mixed PS/PO linkages (ISIS 655862) showed similar transaminase levels compared to full PS (ISIS 655861).

Table 38
Effect of PO/PS linkages on transaminase levels of ASOs comprising $GalNAc_3$-1 conjugate at 3' terminus targeting SRB-1

ISIS No.	Dosage (mg/kg)	ALT (U/L)	AST (U/L)	Chemistry	SEQ ID No.
PBS	0	28.5	65	--	
353382 (parent)	3	50.25	89	Full PS without conjugate	4886
	10	27.5	79.3		
	30	27.3	97		
655861	0.5	28	55.7	Full PS with **$GalNAc_3$-1**	4887
	1.5	30	78		
	5	29	63.5		
	15	28.8	67.8		
655862	0.5	50	75.5	Mixed PS/PO with **$GalNAc_3$-1**	4887
	1.5	21.7	58.5		
	5	29.3	69		
	15	22	61		

Example 45: Preparation of PFP Ester, Compound 110a





Compound 4 (9.5g, 28.8 mmoles) was treated with compound 103a or 103b (38 mmoles), individually, and TMSOTf (0.5 eq.) and molecular sieves in dichloromethane (200 mL), and stirred for 16 hours at room temperature. At that time, the organic layer was filtered thru celite, then washed with sodium bicarbonate, water and brine. The organic layer was then separated and dried over sodium sulfate, filtered and reduced under reduced pressure. The resultant oil was purified by silica gel chromatography (2%-->10% methanol/dichloromethane) to give compounds 104a and 104b in >80% yield. LCMS and proton NMR was consistent with the structure.

Compounds 104a and 104b were treated to the same conditions as for compounds 100a-d (Example 47), to give compounds 105a and 105b in >90% yield. LCMS and proton NMR was consistent with the structure.

Compounds 105a and 105b were treated, individually, with compound 90 under the same conditions as for compounds 901a-d, to give compounds 106a (80%) and 106b (20%). LCMS and proton NMR was consistent with the structure.

Compounds 106a and 106b were treated to the same conditions as for compounds 96a-d (Example 47), to give 107a (60%) and 107b (20%). LCMS and proton NMR was consistent with the structure.

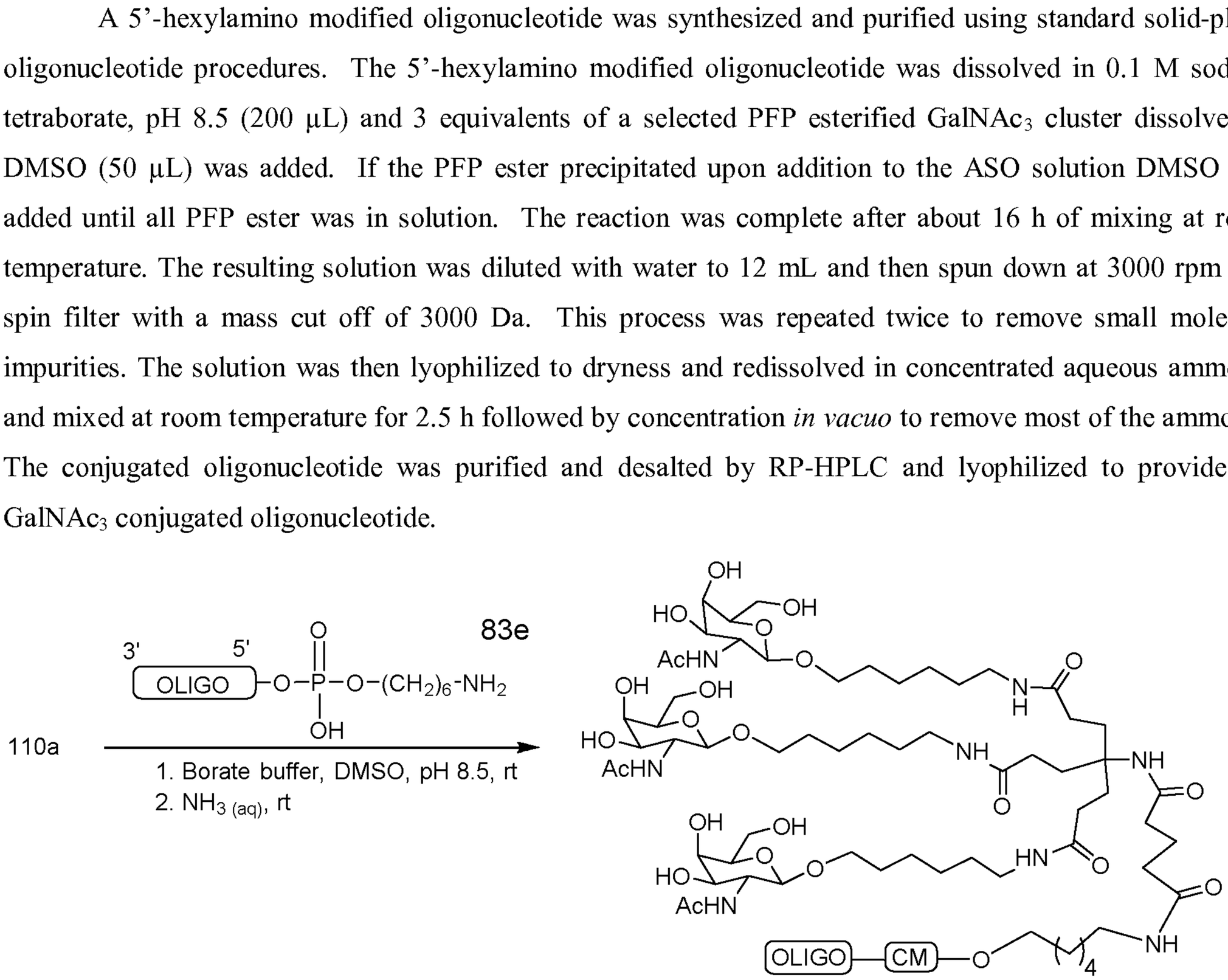
Compounds 107a and 107b were treated to the same conditions as for compounds 97a-d (Example 47), to give compounds 108a and 108b in 40-60% yield. LCMS and proton NMR was consistent with the structure.

Compounds 108a (60%) and 108b (40%) were treated to the same conditions as for compounds 100a-d (Example 47), to give compounds 109a and 109b in >80% yields. LCMS and proton NMR was consistent with the structure.

Compound 109a was treated to the same conditions as for compounds 101a-d (Example 47), to give Compound 110a in 30-60% yield. LCMS and proton NMR was consistent with the structure. Alternatively, Compound 110b can be prepared in a similar manner starting with Compound 109b.

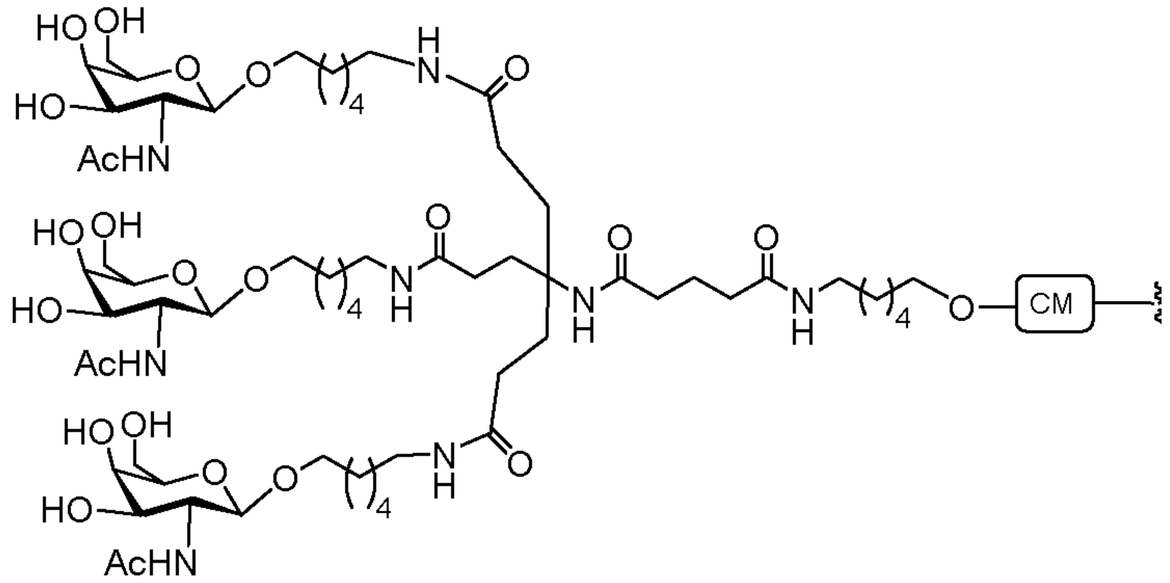
Example 46: General Procedure for Conjugation with PFP Esters (Oligonucleotide 111); Preparation of ISIS 666881 ($GalNAc_3$-10)

A 5'-hexylamino modified oligonucleotide was synthesized and purified using standard solid-phase oligonucleotide procedures. The 5'-hexylamino modified oligonucleotide was dissolved in 0.1 M sodium tetraborate, pH 8.5 (200 μL) and 3 equivalents of a selected PFP esterified $GalNAc_3$ cluster dissolved in DMSO (50 μL) was added. If the PFP ester precipitated upon addition to the ASO solution DMSO was added until all PFP ester was in solution. The reaction was complete after about 16 h of mixing at room temperature. The resulting solution was diluted with water to 12 mL and then spun down at 3000 rpm in a spin filter with a mass cut off of 3000 Da. This process was repeated twice to remove small molecule impurities. The solution was then lyophilized to dryness and redissolved in concentrated aqueous ammonia and mixed at room temperature for 2.5 h followed by concentration *in vacuo* to remove most of the ammonia. The conjugated oligonucleotide was purified and desalted by RP-HPLC and lyophilized to provide the $GalNAc_3$ conjugated oligonucleotide.



111

Oligonucleotide 111 is conjugated with $GalNAc_3$-10. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-10 ($GalNAc_3$-10_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)- as shown in the oligonucleotide (ISIS 666881) synthesized with $GalNAc_3$-10 below. The structure of $GalNAc_3$-10 ($GalNAc_3$-10_a-CM-) is shown below:



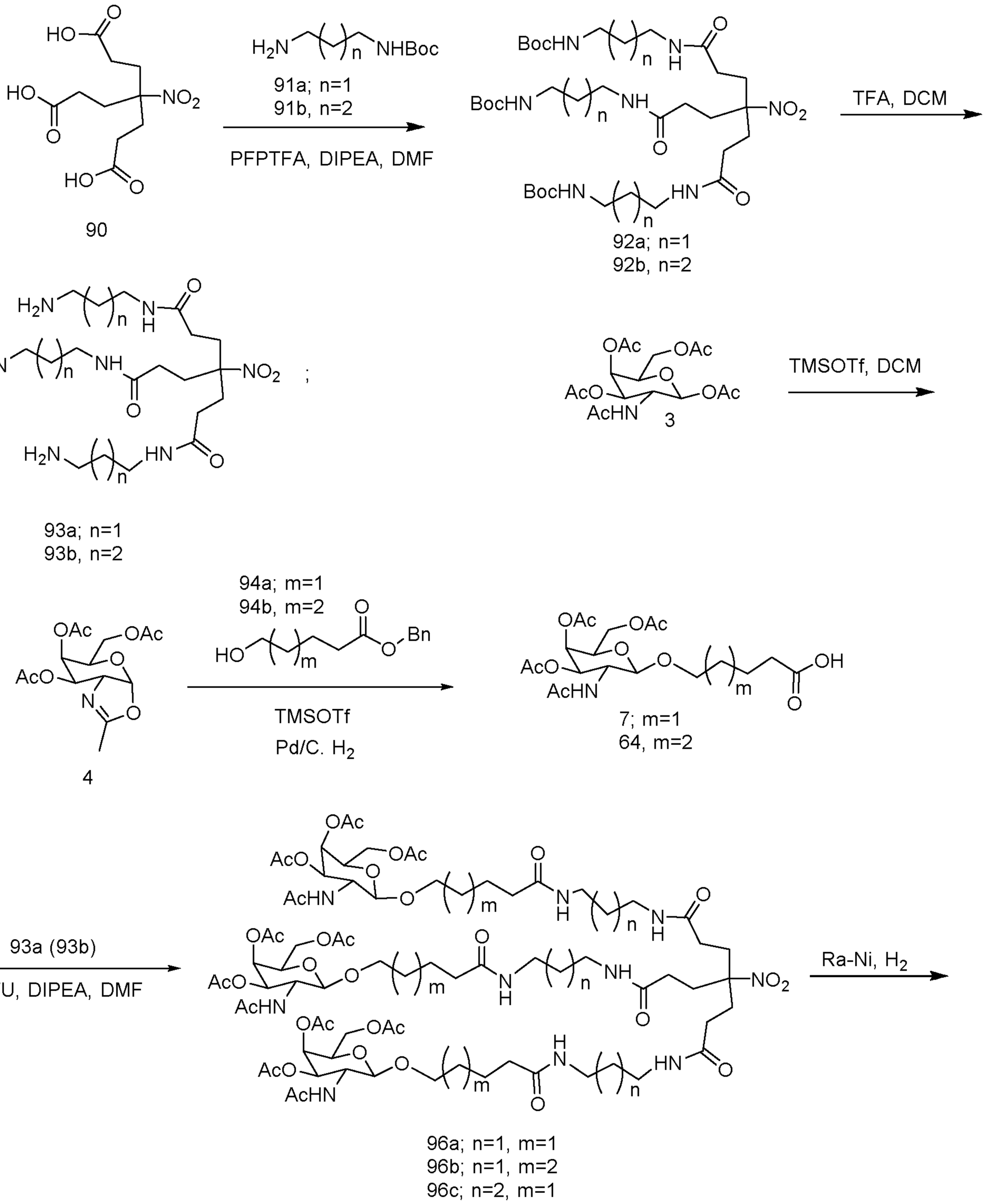
Following this general procedure ISIS 666881 was prepared. 5'-hexylamino modified oligonucleotide, ISIS 660254, was synthesized and purified using standard solid-phase oligonucleotide procedures. ISIS 660254 (40 mg, 5.2 μmol) was dissolved in 0.1 M sodium tetraborate, pH 8.5 (200 μL) and 3 equivalents PFP ester (Compound 110a) dissolved in DMSO (50 μL) was added. The PFP ester precipitated upon addition to the ASO solution requiring additional DMSO (600 μL) to fully dissolve the PFP ester. The reaction was complete after 16 h of mixing at room temperature. The solution was diluted with water to 12 mL total volume and spun down at 3000 rpm in a spin filter with a mass cut off of 3000 Da. This process was repeated twice to remove small molecule impurities. The solution was lyophilized to dryness and redissolved in concentrated aqueous ammonia with mixing at room temperature for 2.5 h followed by concentration *in vacuo* to remove most of the ammonia. The conjugated oligonucleotide was purified and desalted by RP-HPLC and lyophilized to give ISIS 666881 in 90% yield by weight (42 mg, 4.7 μmol).

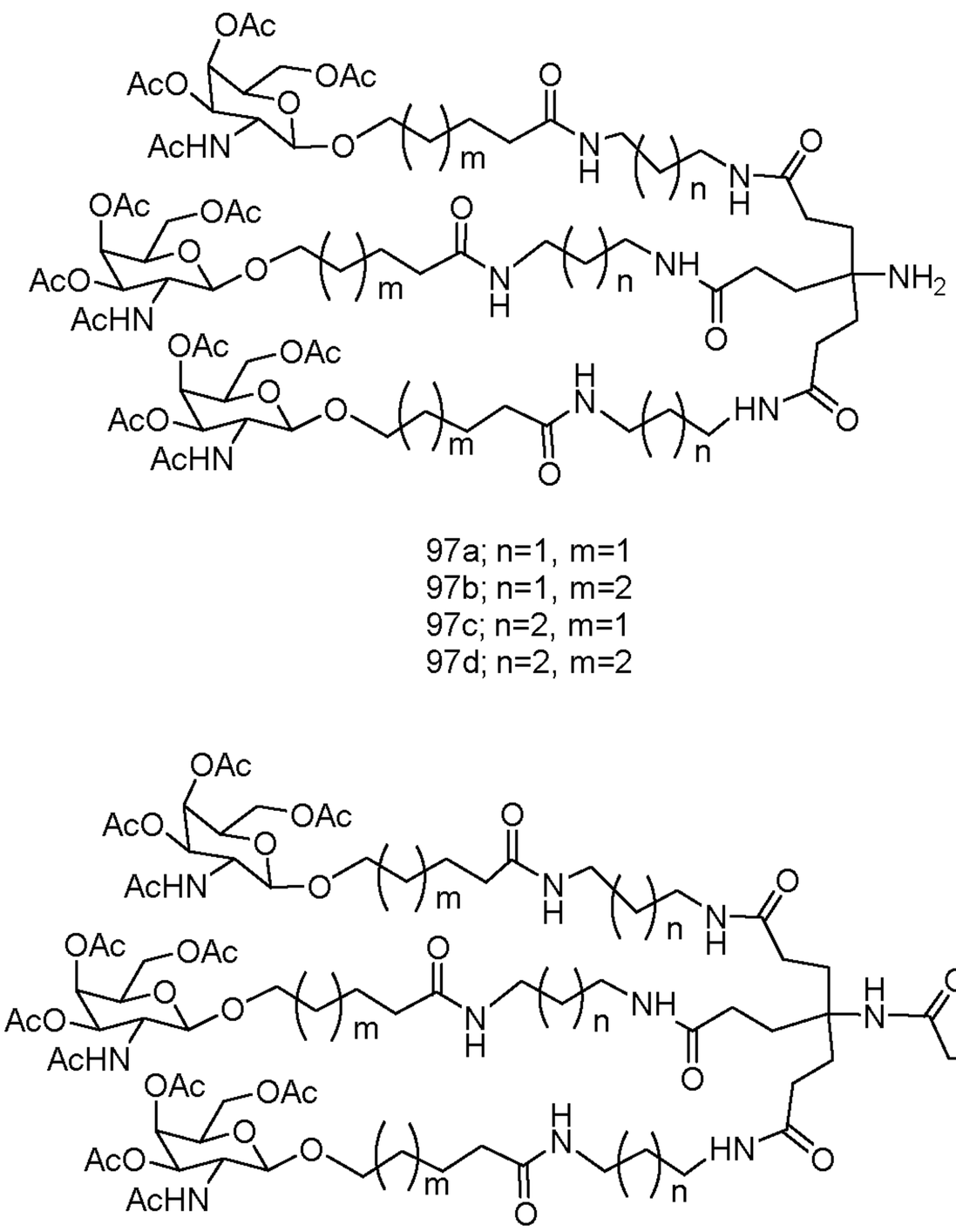
$GalNAc_3$-10 conjugated oligonucleotide

ASO	Sequence (5' to 3')	5' group	SEQ ID No.
ISIS 660254	$NH_2(CH_2)_{6\text{-}o}A_{do}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}$ $^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	Hexylamine	4888
ISIS 666881	**$GalNAc_3$-$10_{a\text{-}o'}A_{do}$**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}$ $^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	**$GalNAc_3$-10**	4888

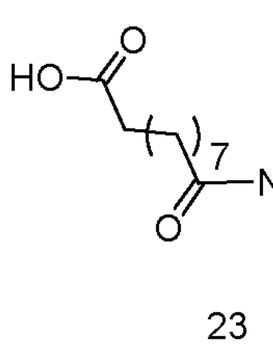
Capital letters indicate the nucleobase for each nucleoside and ^{m}C indicates a 5-methyl cytosine. Subscripts: "e" indicates a 2'-MOE modified nucleoside; "d" indicates a β-D-2'-deoxyribonucleoside; "s" indicates a phosphorothioate internucleoside linkage (PS); "o" indicates a phosphodiester internucleoside linkage (PO); and "o'" indicates -O-P(=O)(OH)-. Conjugate groups are in bold.

Example 47: Preparation of Oligonucleotide 102 Comprising GalNAc$_3$-8



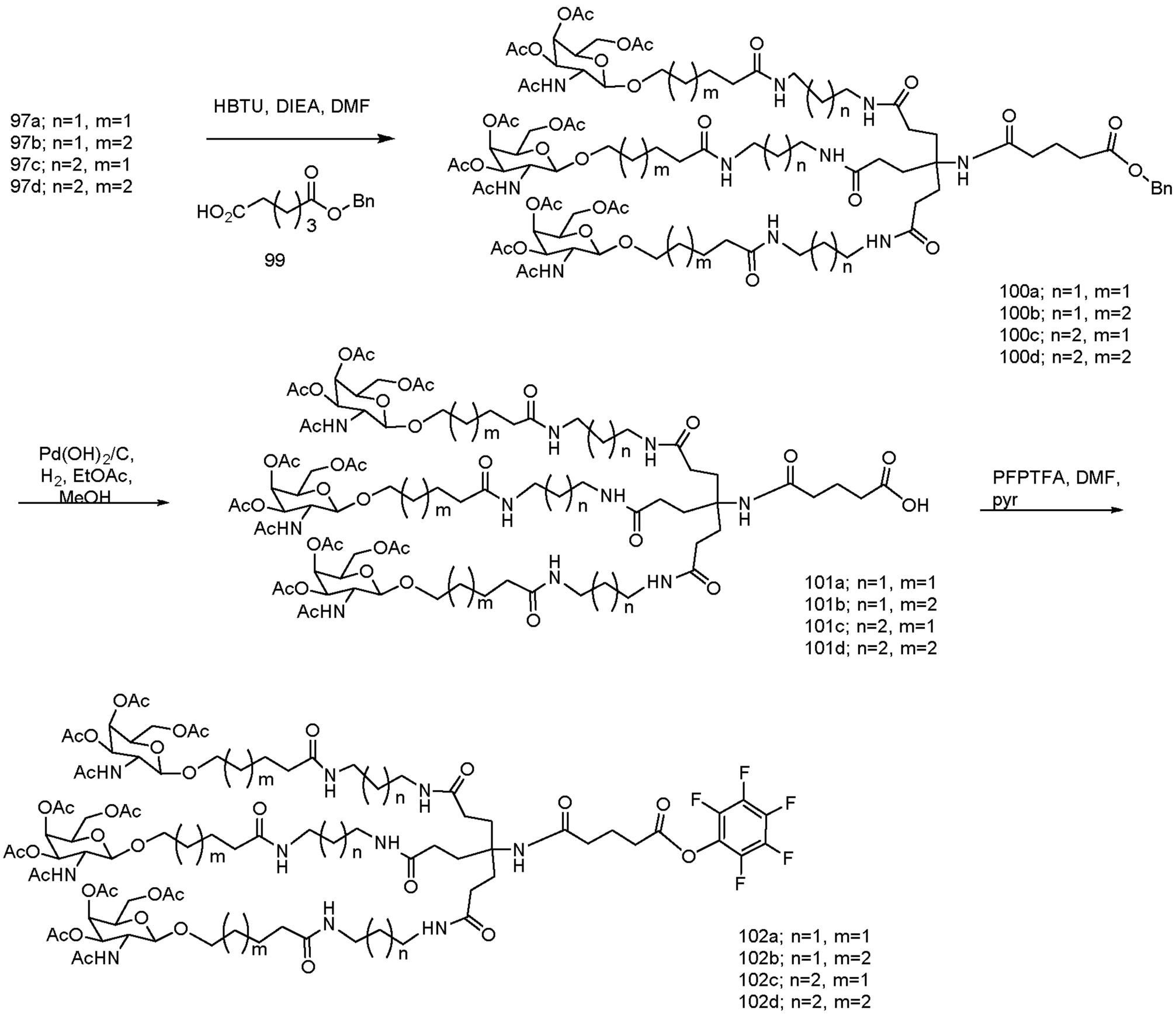


HBTU, DIEA, DMF



97a; n=1, m=1
97b; n=1, m=2
97c; n=2, m=1
97d; n=2, m=2

98a; n=1, m=1
98b; n=1, m=2
98c; n=2, m=1
98d; n=2, m=2



The triacid 90 (4 g, 14.43 mmol) was dissolved in DMF (120 mL) and *N*,*N*-Diisopropylethylamine (12.35 mL, 72 mmoles). Pentafluorophenyl trifluoroacetate (8.9 mL, 52 mmoles) was added dropwise, under argon, and the reaction was allowed to stir at room temperature for 30 minutes. Boc-diamine 91a or 91b (68.87 mmol) was added, along with *N*,*N*-Diisopropylethylamine (12.35 mL, 72 mmoles), and the reaction was allowed to stir at room temperature for 16 hours. At that time, the DMF was reduced by >75% under reduced pressure, and then the mixture was dissolved in dichloromethane. The organic layer was washed with sodium bicarbonate, water and brine. The organic layer was then separated and dried over sodium sulfate, filtered and reduced to an oil under reduced pressure. The resultant oil was purified by silica gel chromatography (2%-->10% methanol/dichloromethane) to give compounds 92a and 92b in an approximate 80% yield. LCMS and proton NMR were consistent with the structure.

Compound 92a or 92b (6.7 mmoles) was treated with 20 mL of dichloromethane and 20 mL of trifluoroacetic acid at room temperature for 16 hours. The resultant solution was evaporated and then

dissolved in methanol and treated with DOWEX-OH resin for 30 minutes. The resultant solution was filtered and reduced to an oil under reduced pressure to give 85-90% yield of compounds 93a and 93b.

Compounds 7 or 64 (9.6 mmoles) were treated with HBTU (3.7g, 9.6 mmoles) and *N*,*N*-Diisopropylethylamine (5 mL) in DMF (20 mL) for 15 minutes. To this was added either compounds 93a or 93b (3 mmoles), and allowed to stir at room temperature for 16 hours. At that time, the DMF was reduced by >75% under reduced pressure, and then the mixture was dissolved in dichloromethane. The organic layer was washed with sodium bicarbonate, water and brine. The organic layer was then separated and dried over sodium sulfate, filtered and reduced to an oil under reduced pressure. The resultant oil was purified by silica gel chromatography (5%-->20% methanol/dichloromethane) to give compounds 96a-d in 20-40% yield. LCMS and proton NMR was consistent with the structure.

Compounds 96a-d (0.75 mmoles), individually, were hydrogenated over Raney Nickel for 3 hours in Ethanol (75 mL). At that time, the catalyst was removed by filtration thru celite, and the ethanol removed under reduced pressure to give compounds 97a-d in 80-90% yield. LCMS and proton NMR were consistent with the structure.

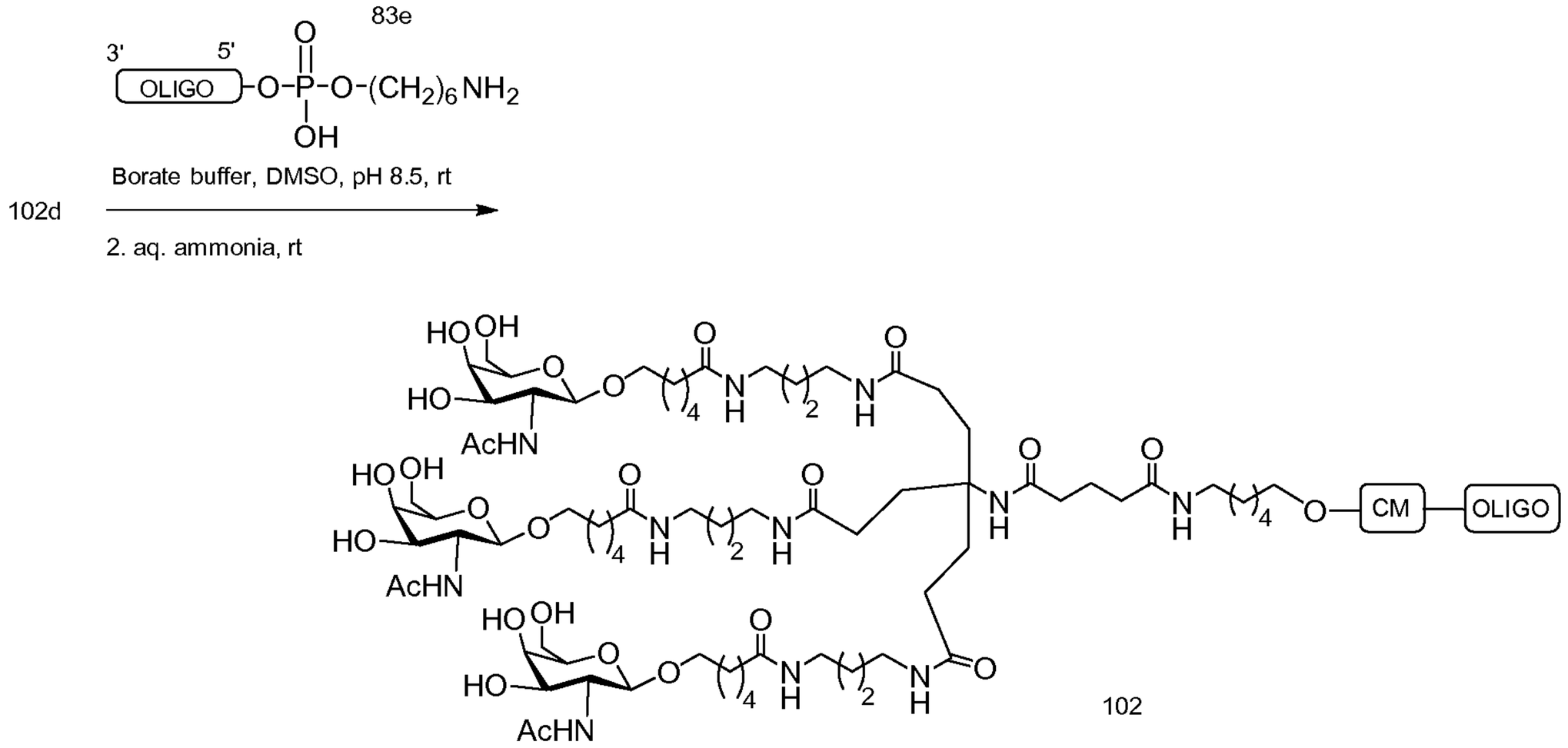
Compound 23 (0.32g, 0.53 mmoles) was treated with HBTU (0.2g, 0.53 mmoles) and *N*,*N*-Diisopropylethylamine (0.19 mL, 1.14 mmoles) in DMF (30mL) for 15 minutes. To this was added compounds 97a-d (0.38 mmoles), individually, and allowed to stir at room temperature for 16 hours. At that time, the DMF was reduced by >75% under reduced pressure, and then the mixture was dissolved in dichloromethane. The organic layer was washed with sodium bicarbonate, water and brine. The organic layer was then separated and dried over sodium sulfate, filtered and reduced to an oil under reduced pressure. The resultant oil was purified by silica gel chromatography (2%-->20% methanol/dichloromethane) to give compounds 98a-d in 30-40% yield. LCMS and proton NMR was consistent with the structure.

Compound 99 (0.17g, 0.76 mmoles) was treated with HBTU (0.29 g, 0.76 mmoles) and *N*,*N*-Diisopropylethylamine (0.35 mL, 2.0 mmoles) in DMF (50mL) for 15 minutes. To this was added compounds 97a-d (0.51 mmoles), individually, and allowed to stir at room temperature for 16 hours. At that time, the DMF was reduced by >75% under reduced pressure, and then the mixture was dissolved in dichloromethane. The organic layer was washed with sodium bicarbonate, water and brine. The organic layer was then separated and dried over sodium sulfate, filtered and reduced to an oil under reduced pressure. The resultant oil was purified by silica gel chromatography (5%-->20% methanol/ dichloromethane) to give compounds 100a-d in 40-60% yield. LCMS and proton NMR was consistent with the structure.

Compounds 100a-d (0.16 mmoles), individually, were hydrogenated over 10% $Pd(OH)_2/C$ for 3 hours in methanol/ethyl acetate (1:1, 50 mL). At that time, the catalyst was removed by filtration thru celite, and the organics removed under reduced pressure to give compounds 101a-d in 80-90% yield. LCMS and proton NMR was consistent with the structure.

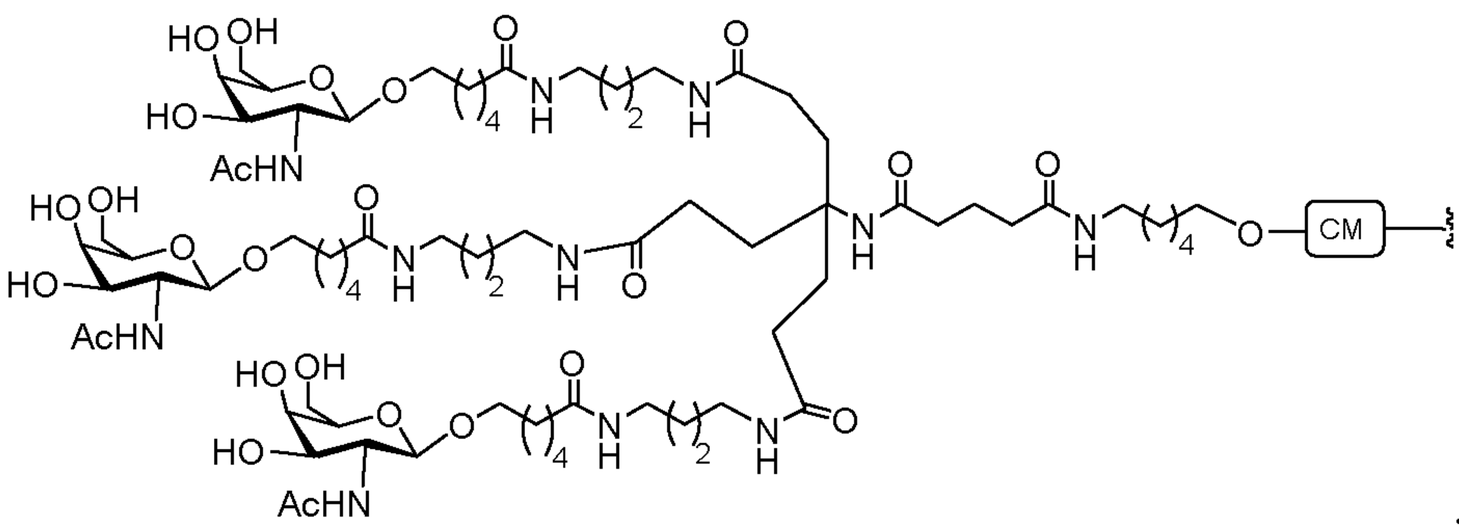
Compounds 101a-d (0.15 mmoles), individually, were dissolved in DMF (15 mL) and pyridine (0.016 mL, 0.2 mmoles). Pentafluorophenyl trifluoroacetate (0.034 mL, 0.2 mmoles) was added dropwise,

under argon, and the reaction was allowed to stir at room temperature for 30 minutes. At that time, the DMF was reduced by >75% under reduced pressure, and then the mixture was dissolved in dichloromethane. The organic layer was washed with sodium bicarbonate, water and brine. The organic layer was then separated and dried over sodium sulfate, filtered and reduced to an oil under reduced pressure. The resultant oil was purified by silica gel chromatography (2%-->5% methanol/dichloromethane) to give compounds 102a-d in an approximate 80% yield. LCMS and proton NMR were consistent with the structure.

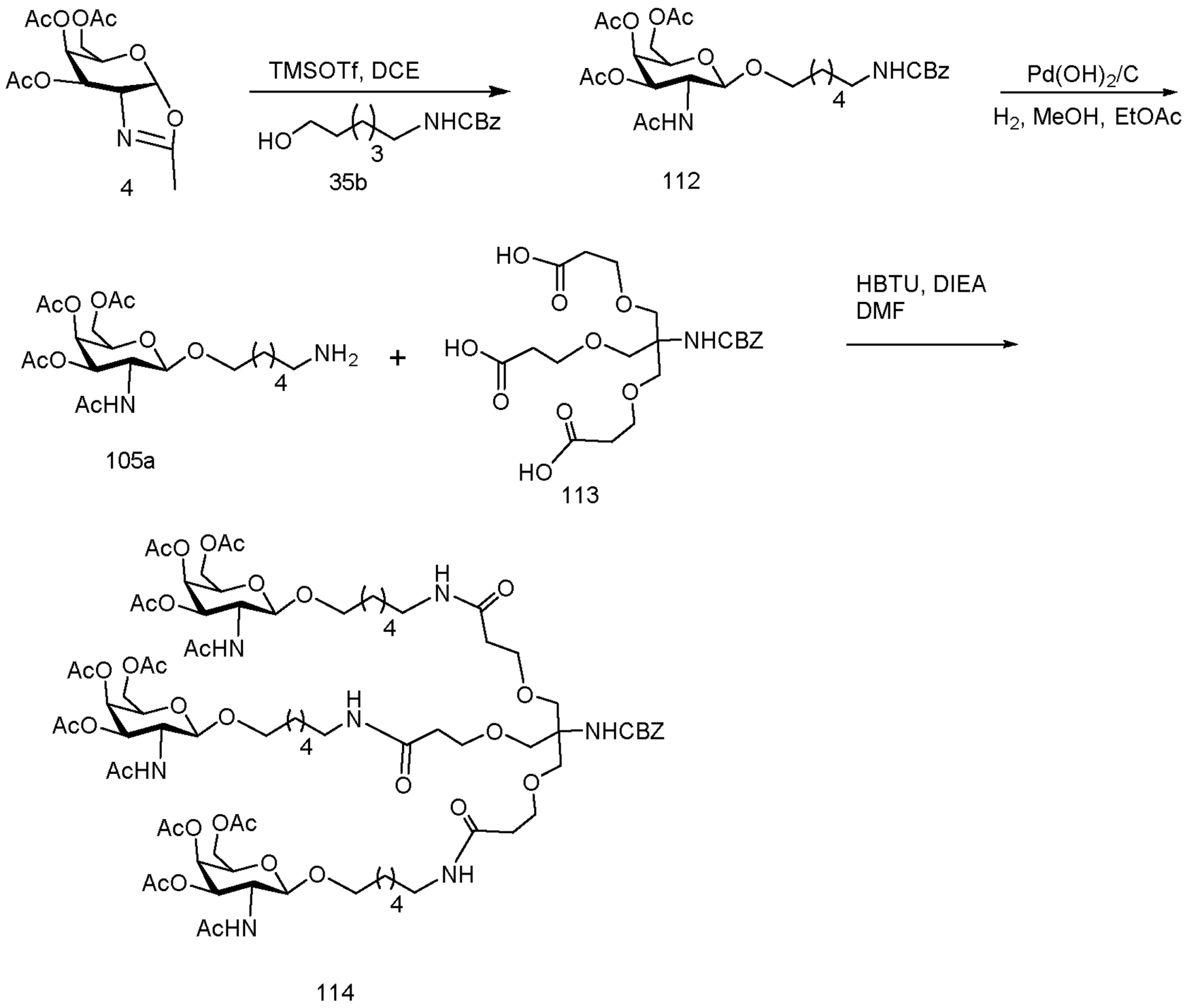


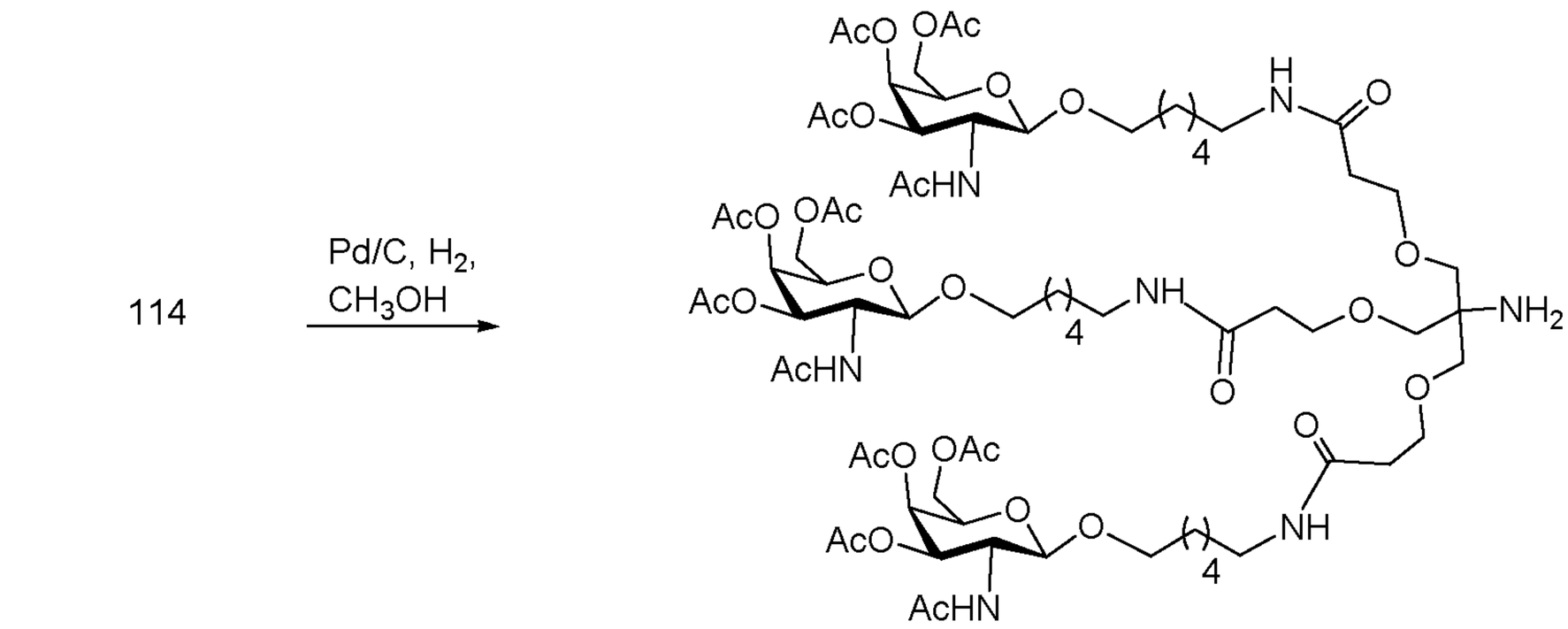
Oligomeric Compound 102, comprising a $GalNAc_3$-8 conjugate group, was prepared using the general procedures illustrated in Example 46. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-8 ($GalNAc_3$-8_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In a preferred embodiment, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-.

The structure of $GalNAc_3$-8 ($GalNAc_3$-8_a-CM-) is shown below:



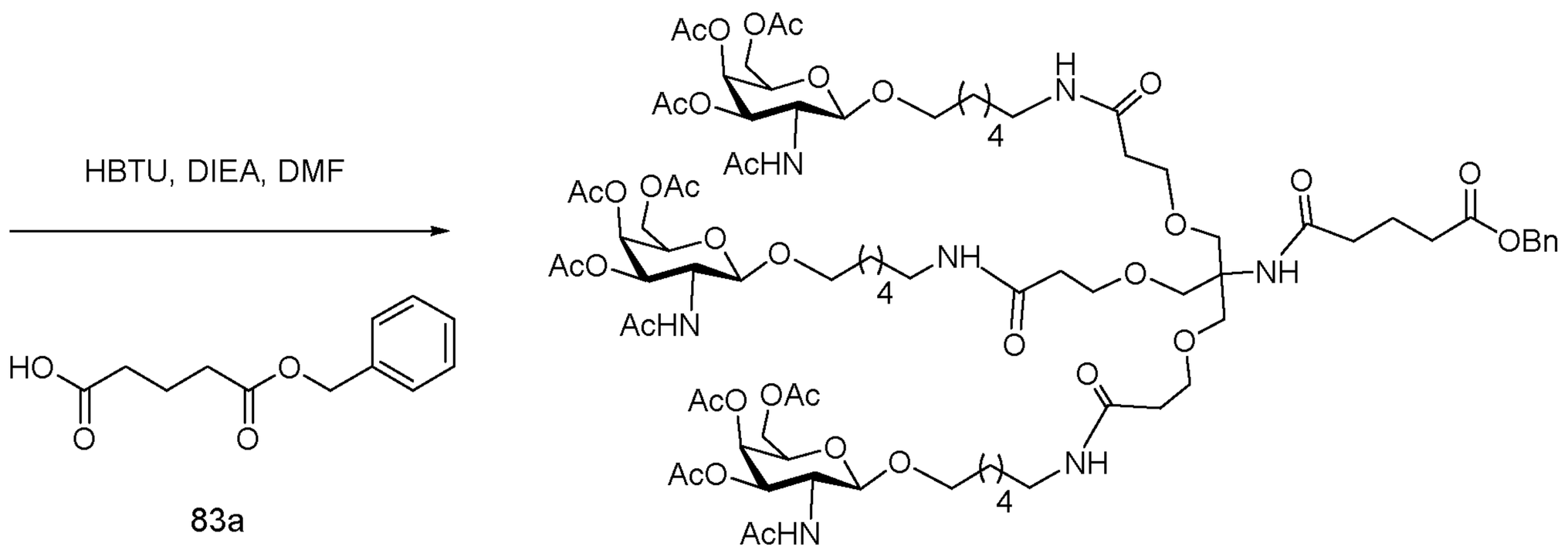
Example 48: Preparation of Oligonucleotide 119 Comprising GalNAc$_3$-7





114

115



83a

116

Compound 112 was synthesized following the procedure described in the literature (*J. Med. Chem.* 2004, *47*, 5798-5808).

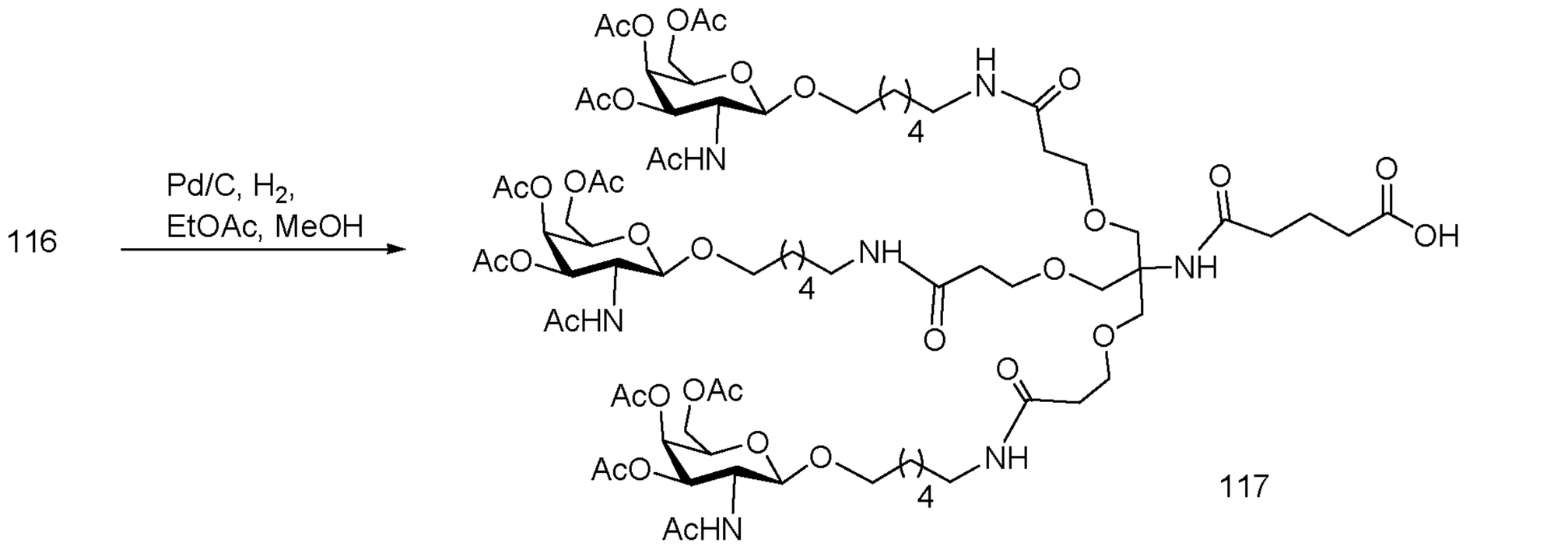
Compound 112 (5 g, 8.6 mmol) was dissolved in 1:1 methanol/ethyl acetate (22 mL/22 mL). Palladium hydroxide on carbon (0.5 g) was added. The reaction mixture was stirred at room temperature under hydrogen for 12 h. The reaction mixture was filtered through a pad of celite and washed the pad with 1:1 methanol/ethyl acetate. The filtrate and the washings were combined and concentrated to dryness to yield Compound 105a (quantitative). The structure was confirmed by LCMS.

Compound 113 (1.25 g, 2.7 mmol), HBTU (3.2 g, 8.4 mmol) and DIEA (2.8 mL, 16.2 mmol) were dissolved in anhydrous DMF (17 mL) and the reaction mixture was stirred at room temperature for 5 min. To this a solution of Compound 105a (3.77 g, 8.4 mmol) in anhydrous DMF (20 mL) was added. The reaction was stirred at room temperature for 6 h. Solvent was removed under reduced pressure to get an oil. The residue was dissolved in CH_2Cl_2 (100 mL) and washed with aqueous saturated $NaHCO_3$ solution (100 mL) and brine (100 mL). The organic phase was separated, dried (Na_2SO_4), filtered and evaporated. The residue

was purified by silica gel column chromatography and eluted with 10 to 20 % MeOH in dichloromethane to yield Compound 114 (1.45 g, 30%). The structure was confirmed by LCMS and ^{1}H NMR analysis.

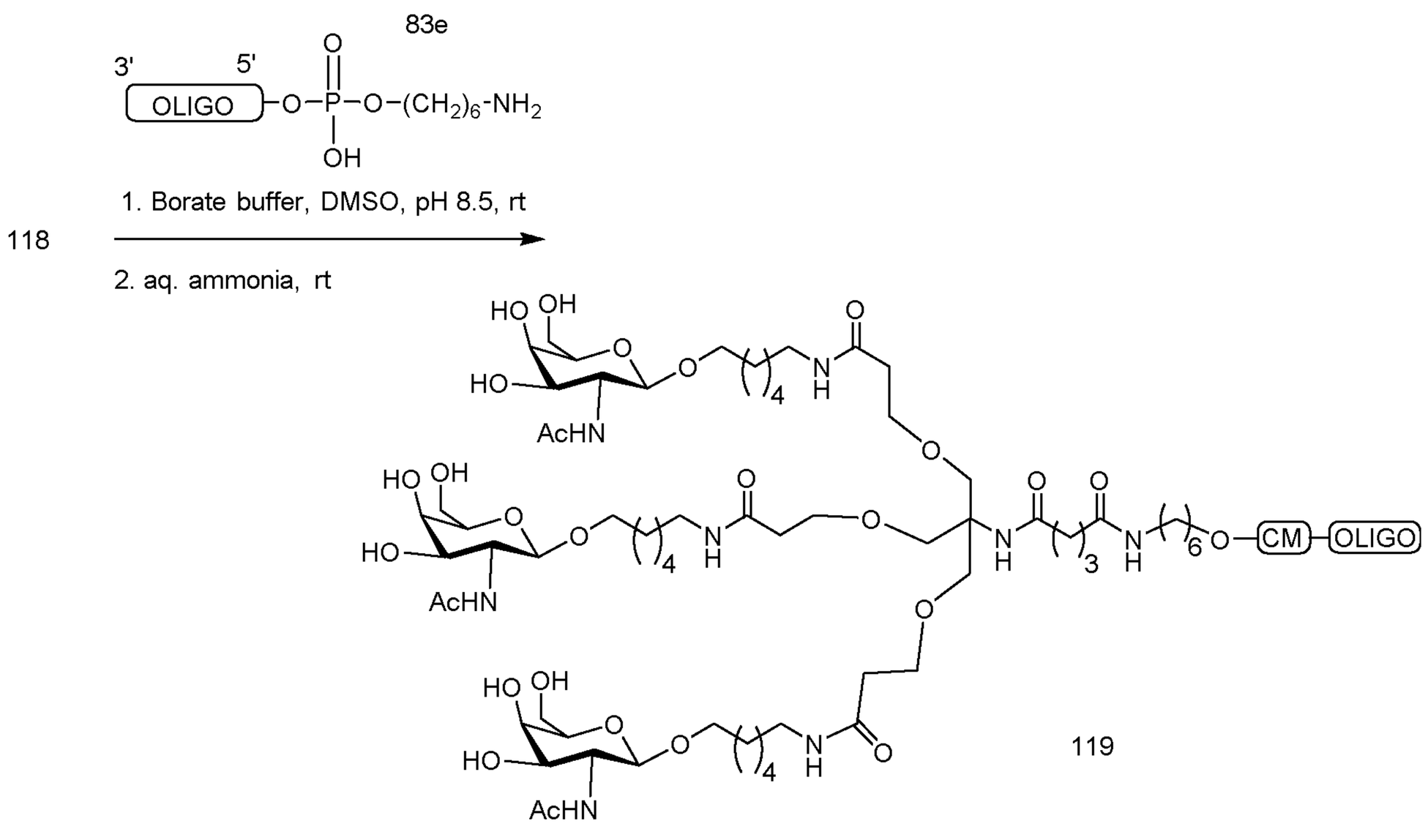
Compound 114 (1.43 g, 0.8 mmol) was dissolved in 1:1 methanol/ethyl acetate (4 mL/4 mL). Palladium on carbon (wet, 0.14 g) was added. The reaction mixture was flushed with hydrogen and stirred at room temperature under hydrogen for 12 h. The reaction mixture was filtered through a pad of celite. The celite pad was washed with methanol/ethyl acetate (1:1). The filtrate and the washings were combined together and evaporated under reduced pressure to yield Compound 115 (quantitative). The structure was confirmed by LCMS and ^{1}H NMR analysis.

Compound 83a (0.17 g, 0.75 mmol), HBTU (0.31 g, 0.83 mmol) and DIEA (0.26 mL, 1.5 mmol) were dissolved in anhydrous DMF (5 mL) and the reaction mixture was stirred at room temperature for 5 min. To this a solution of Compound 115 (1.22 g, 0.75 mmol) in anhydrous DMF was added and the reaction was stirred at room temperature for 6 h. The solvent was removed under reduced pressure and the residue was dissolved in CH_2Cl_2. The organic layer was washed aqueous saturated $NaHCO_3$ solution and brine and dried over anhydrous Na_2SO_4 and filtered. The organic layer was concentrated to dryness and the residue obtained was purified by silica gel column chromatography and eluted with 3 to 15 % MeOH in dichloromethane to yield Compound 116 (0.84 g, 61%). The structure was confirmed by LC MS and ^{1}H NMR analysis.



Compound 116 (0.74 g, 0.4 mmol) was dissolved in 1:1 methanol/ethyl acetate (5 mL/5 mL). Palladium on carbon (wet, 0.074 g) was added. The reaction mixture was flushed with hydrogen and stirred at room temperature under hydrogen for 12 h. The reaction mixture was filtered through a pad of celite. The celite pad was washed with methanol/ethyl acetate (1:1). The filtrate and the washings were combined together and evaporated under reduced pressure to yield compound 117 (0.73 g, 98%). The structure was confirmed by LCMS and ^{1}H NMR analysis.

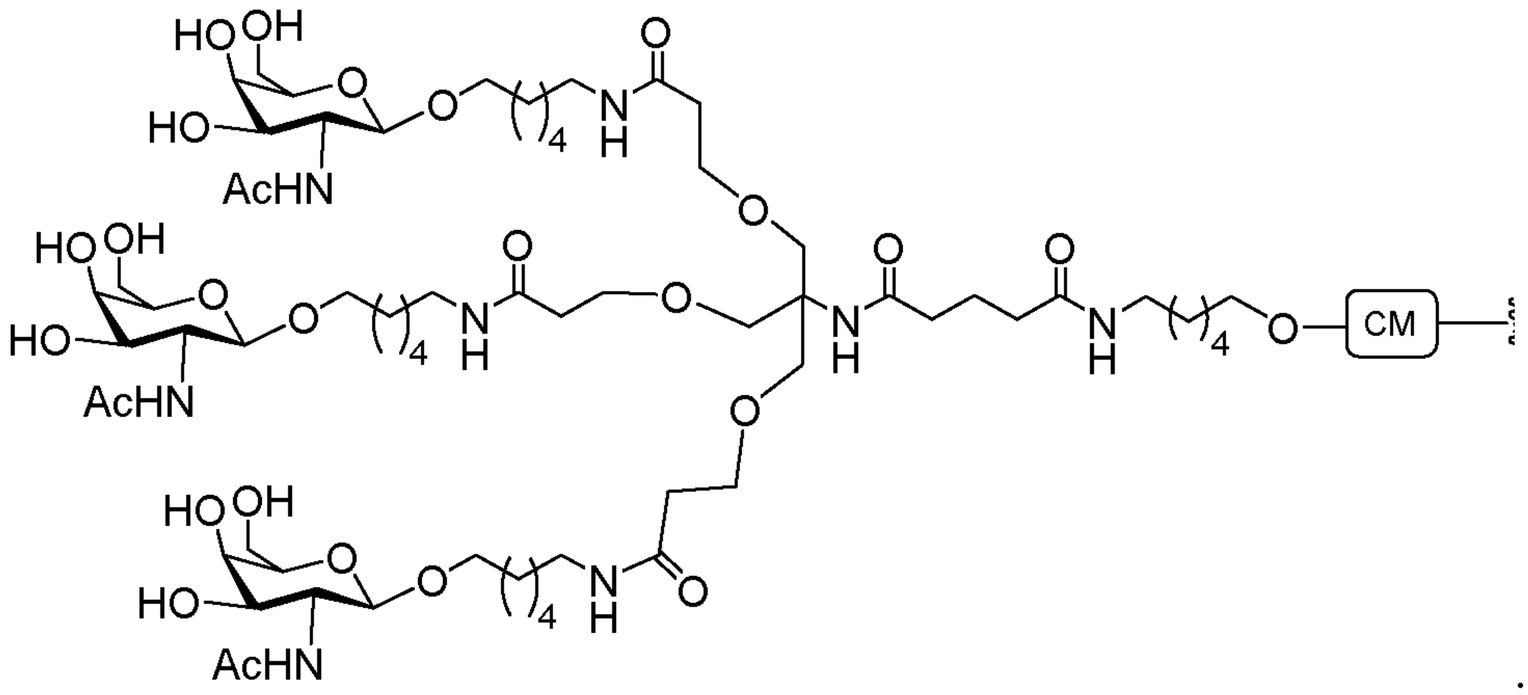
Compound 117 (0.63 g, 0.36 mmol) was dissolved in anhydrous DMF (3 mL). To this solution *N*,*N*-Diisopropylethylamine (70 μL, 0.4 mmol) and pentafluorophenyl trifluoroacetate (72 μL, 0.42 mmol) were added. The reaction mixture was stirred at room temperature for 12 h and poured into a aqueous saturated $NaHCO_3$ solution. The mixture was extracted with dichloromethane, washed with brine and dried over anhydrous Na_2SO_4. The dichloromethane solution was concentrated to dryness and purified with silica gel column chromatography and eluted with 5 to 10 % MeOH in dichloromethane to yield compound 118 (0.51 g, 79%). The structure was confirmed by LCMS and ^{1}H and ^{1}H and ^{19}F NMR.



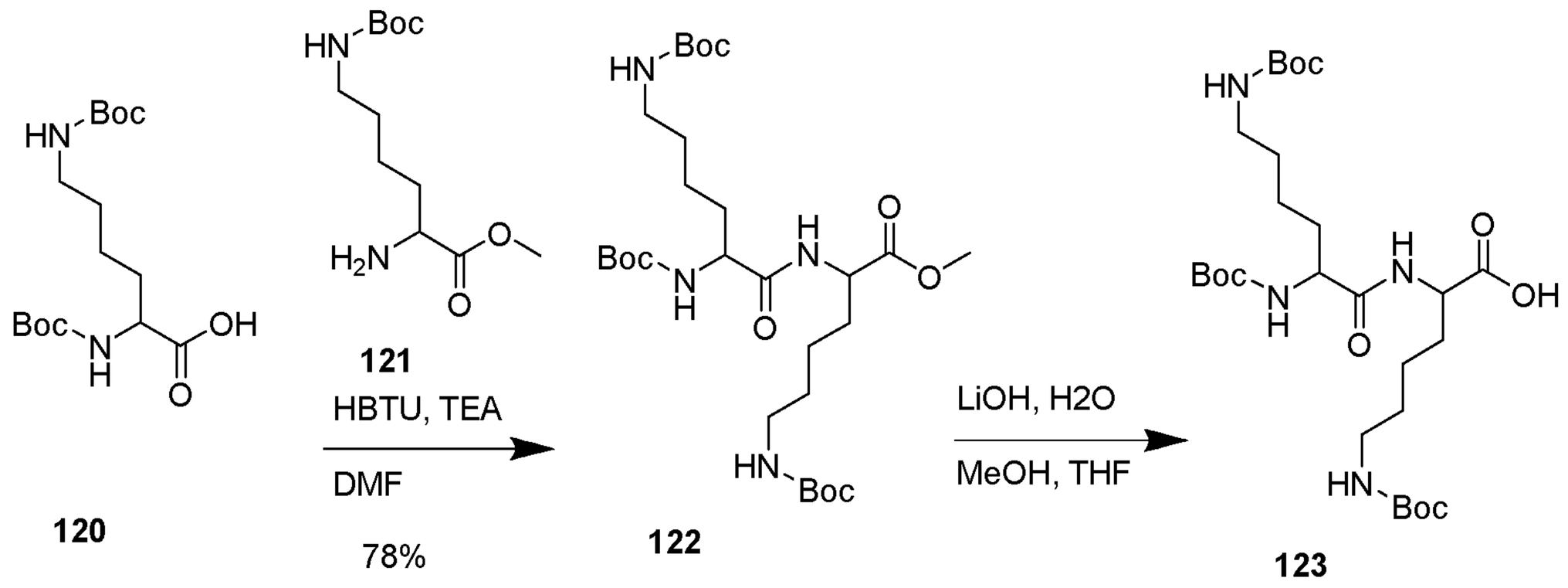
118 119

Oligomeric Compound 119, comprising a $GalNAc_3$-7 conjugate group, was prepared using the general procedures illustrated in Example 46. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-7 ($GalNAc_3$-7_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-.

The structure of $GalNAc_3$-7 ($GalNAc_3$-7_a-CM-) is shown below:



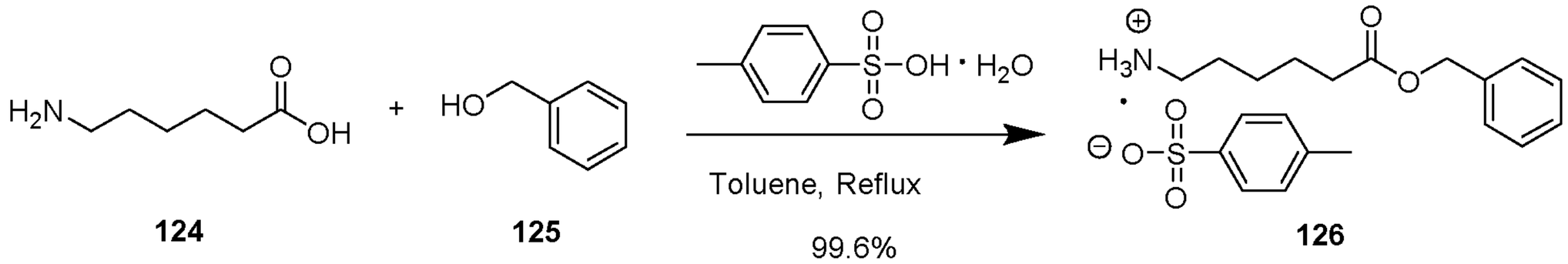
Example 49: Preparation of Oligonucleotide 132 Comprising GalNAc$_3$-5



Compound 120 (14.01 g, 40 mmol) and HBTU (14.06 g, 37 mmol) were dissolved in anhydrous DMF (80 mL). Triethylamine (11.2 mL, 80.35 mmol) was added and stirred for 5 min. The reaction mixture was cooled in an ice bath and a solution of compound 121 (10 g, mmol) in anhydrous DMF (20 mL) was added. Additional triethylamine (4.5 mL, 32.28 mmol) was added and the reaction mixture was stirred for 18 h under an argon atmosphere. The reaction was monitored by TLC (ethyl acetate:hexane; 1:1; Rf = 0.47). The solvent was removed under reduced pressure. The residue was taken up in EtOAc (300 mL) and washed with 1M $NaHSO_4$ (3 x 150 mL), aqueous saturated $NaHCO_3$ solution (3 x 150 mL) and brine (2 x 100 mL). Organic layer was dried with Na_2SO_4. Drying agent was removed by filtration and organic layer was concentrated by rotary evaporation. Crude mixture was purified by silica gel column chromatography and eluted by using 35 – 50% EtOAc in hexane to yield a compound 122 (15.50 g, 78.13%). The structure was confirmed by LCMS and ^{1}H NMR analysis. Mass *m/z* 589.3 $[M + H]^+$.

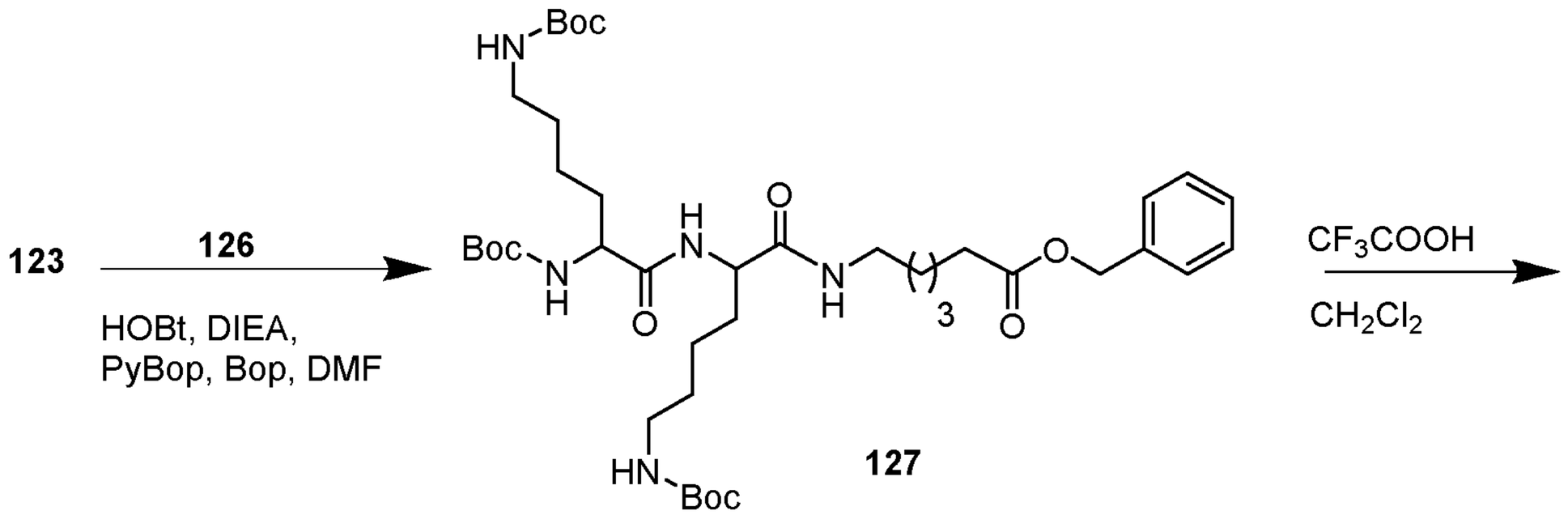
A solution of LiOH (92.15 mmol) in water (20 mL) and THF (10 mL) was added to a cooled solution of Compound 122 (7.75 g,13.16 mmol) dissolved in methanol (15 mL). The reaction mixture was stirred at room temperature for 45 min. and monitored by TLC (EtOAc:hexane; 1:1). The reaction mixture was

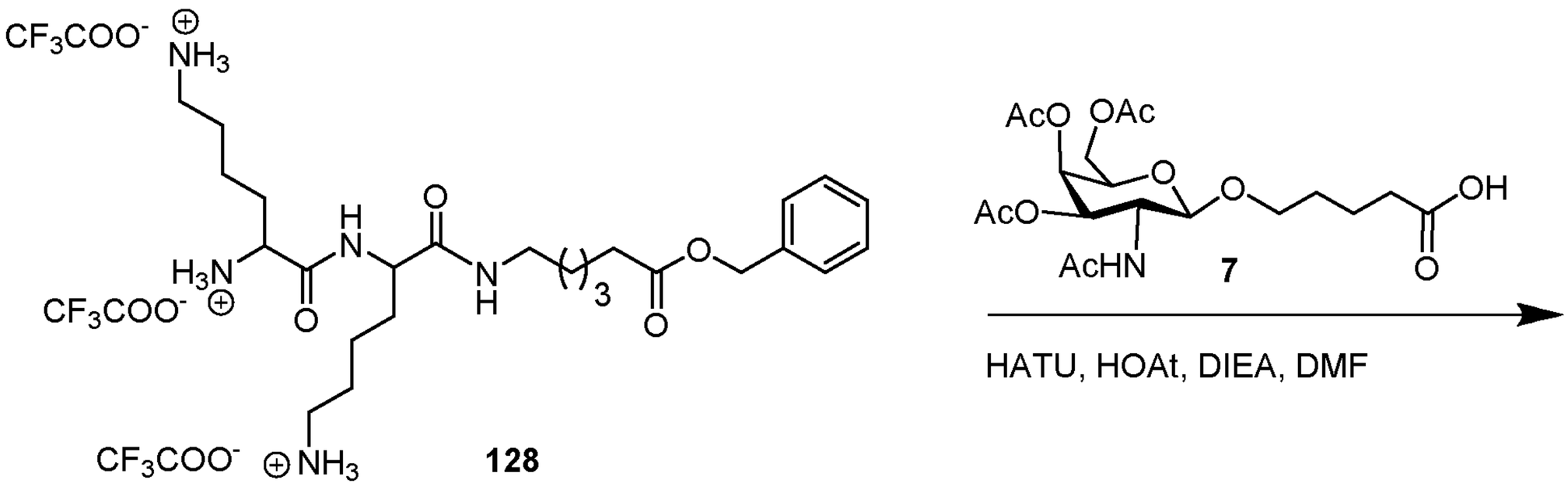
concentrated to half the volume under reduced pressure. The remaining solution was cooled an ice bath and neutralized by adding concentrated HCl. The reaction mixture was diluted, extracted with EtOAc (120 mL) and washed with brine (100 mL). An emulsion formed and cleared upon standing overnight. The organic layer was separated dried (Na_2SO_4), filtered and evaporated to yield Compound 123 (8.42 g). Residual salt is the likely cause of excess mass. LCMS is consistent with structure. Product was used without any further purification. M.W.cal:574.36; M.W.fd:575.3 $[M + H]^+$.

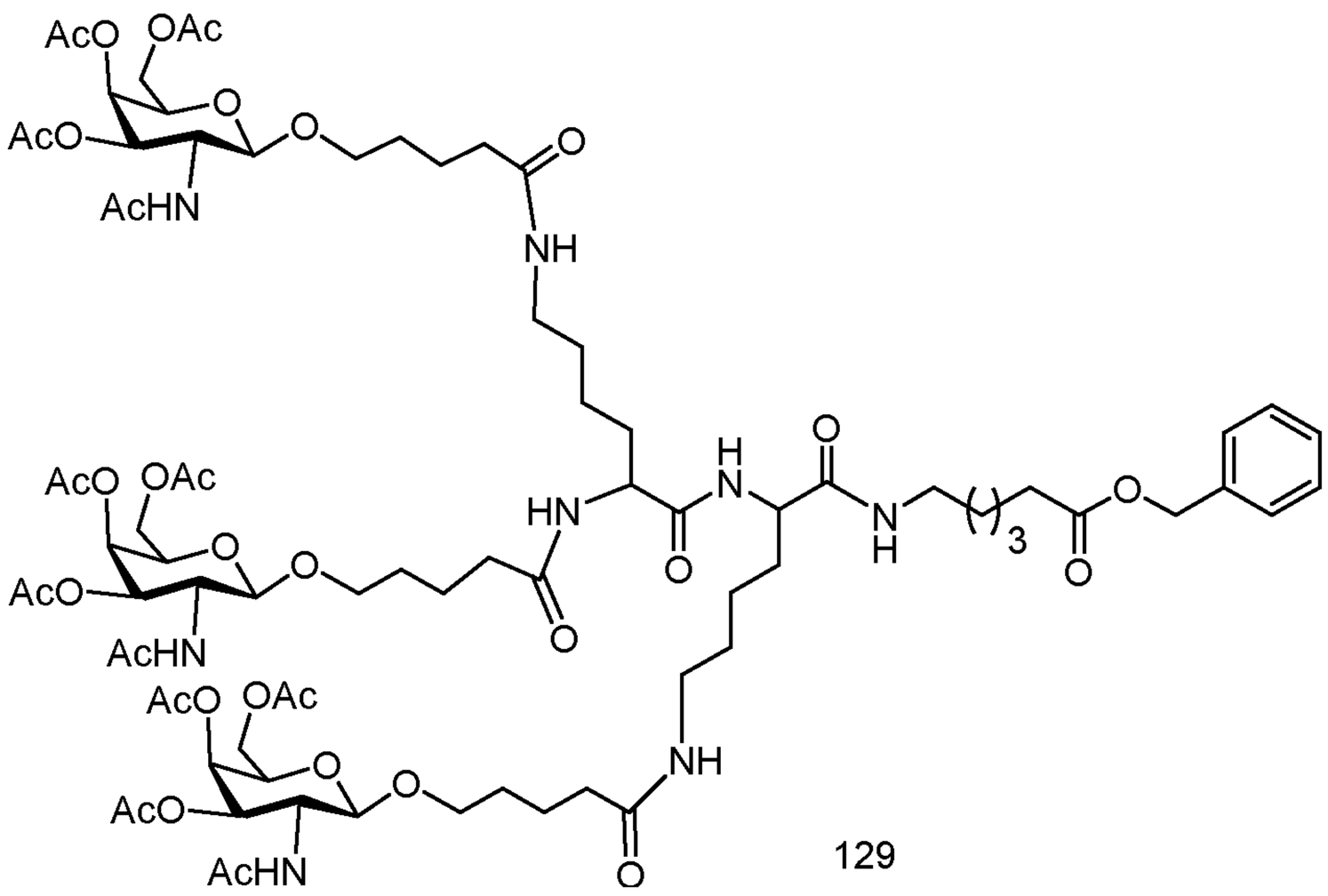


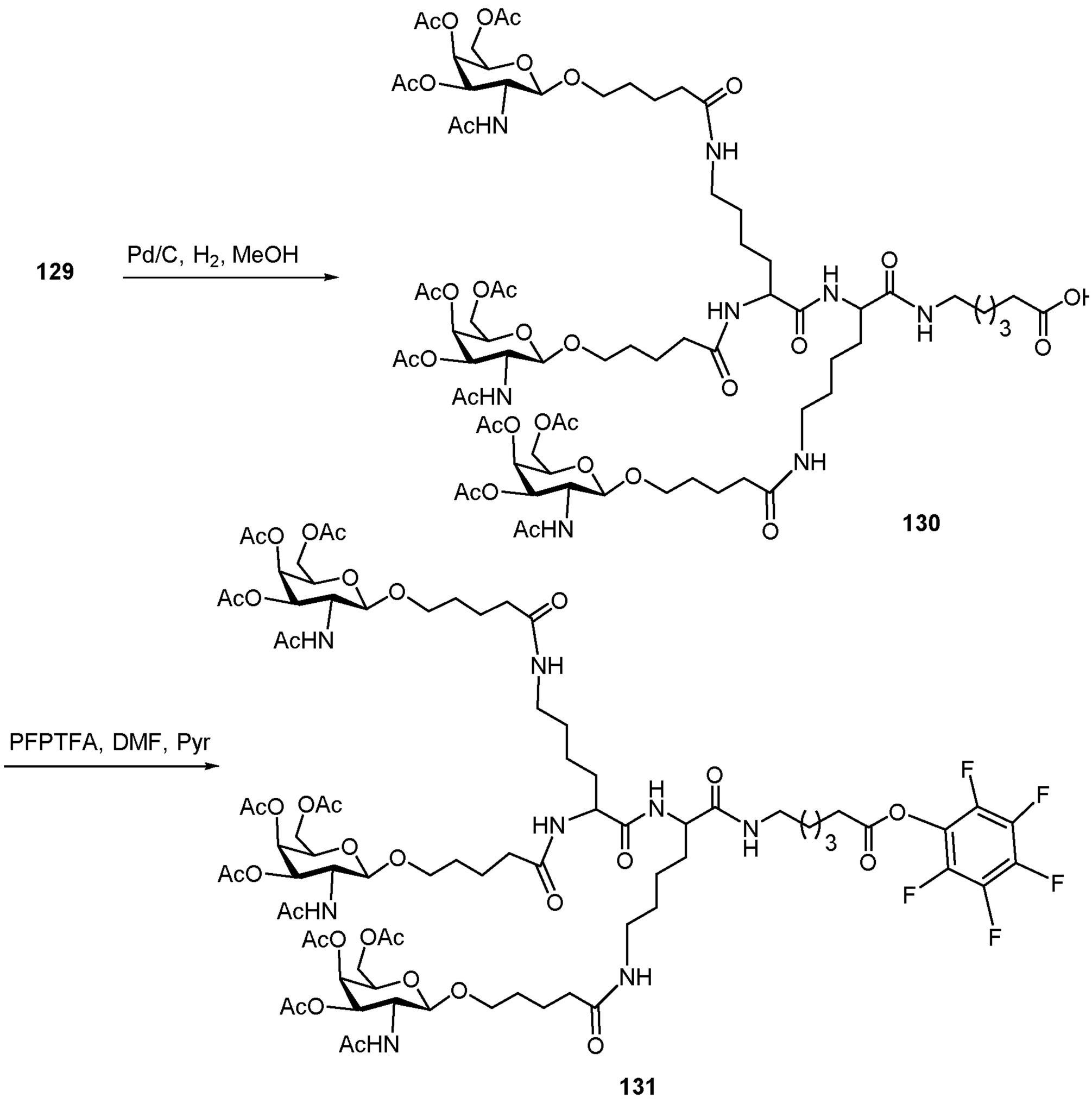
124 **125** **126**

Compound 126 was synthesized following the procedure described in the literature (*J. Am. Chem. Soc.* 2011, *133*, 958-963).


123
126
HOBt, DIEA,
PyBop, Bop, DMF
Boc
HN
Boc
N
H
H
N
O
O
N
H
3
O
O
HN
Boc
127
CF3COOH
CH2Cl2


CF3COO-
NH3
H3N
CF3COO-
CF3COO-
NH3
128
AcO
OAc
O
AcO
AcHN
7
OH
HATU, HOAt, DIEA, DMF


AcO
OAc
O
AcO
AcHN
NH
HN
O
H
N
O
N
H
3
O
O
NH
O
129



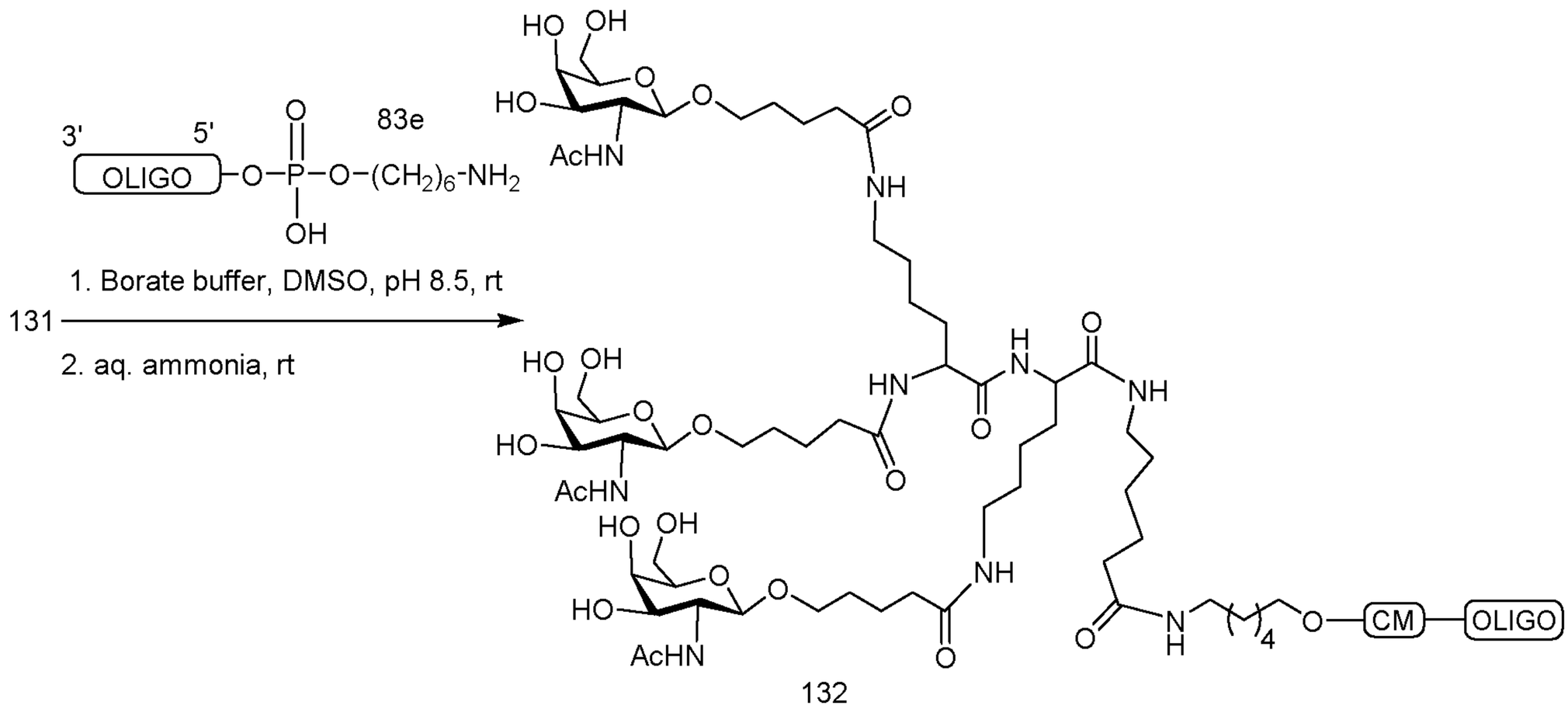
Compound 123 (7.419 g, 12.91 mmol), HOBt (3.49 g, 25.82 mmol) and compound 126 (6.33 g, 16.14 mmol) were dissolved in and DMF (40 mL) and the resulting reaction mixture was cooled in an ice bath. To this *N*,*N*-Diisopropylethylamine (4.42 mL, 25.82 mmol), PyBop (8.7 g, 16.7 mmol) followed by Bop coupling reagent (1.17 g, 2.66 mmol) were added under an argon atmosphere. The ice bath was removed and the solution was allowed to warm to room temperature. The reaction was completed after 1 h as determined by TLC (DCM:MeOH:AA; 89:10:1). The reaction mixture was concentrated under reduced pressure. The residue was dissolved in EtOAc (200 mL) and washed with 1 M $NaHSO_4$ (3x100 mL), aqueous saturated $NaHCO_3$ (3x100 mL) and brine (2x100 mL). The organic phase separated dried (Na_2SO_4), filtered and concentrated. The residue was purified by silica gel column chromatography with a gradient of 50% hexanes/EtOAC to 100% EtOAc to yield Compound 127 (9.4 g) as a white foam. LCMS and ^1H NMR were consistent with structure. Mass *m/z* 778.4 $[M + H]^+$.

Trifluoroacetic acid (12 mL) was added to a solution of compound 127 (1.57 g, 2.02 mmol) in dichloromethane (12 mL) and stirred at room temperature for 1 h. The reaction mixture was co-evaporated with toluene (30 mL) under reduced pressure to dryness. The residue obtained was co-evaporated twice with acetonitrile (30 mL) and toluene (40 mL) to yield Compound 128 (1.67 g) as trifluoro acetate salt and used for next step without further purification. LCMS and ^{1}H NMR were consistent with structure. Mass *m/z* 478.2 $[M + H]^+$.

Compound 7 (0.43 g, 0.963 mmol), HATU (0.35 g, 0.91 mmol), and HOAt (0.035 g, 0.26 mmol) were combined together and dried for 4 h over P_2O_5 under reduced pressure in a round bottom flask and then dissolved in anhydrous DMF (1 mL) and stirred for 5 min. To this a solution of compound 128 (0.20 g, 0.26 mmol) in anhydrous DMF (0.2 mL) and *N*,*N*-Diisopropylethylamine (0.2 mL) was added. The reaction mixture was stirred at room temperature under an argon atmosphere. The reaction was complete after 30 min as determined by LCMS and TLC (7% MeOH/DCM). The reaction mixture was concentrated under reduced pressure. The residue was dissolved in DCM (30 mL) and washed with 1 M $NaHSO_4$ (3x20 mL), aqueous saturated $NaHCO_3$ (3 x 20 mL) and brine (3x20 mL). The organic phase was separated, dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography using 5-15% MeOH in dichloromethane to yield Compound 129 (96.6 mg). LC MS and ^{1}H NMR are consistent with structure. Mass *m/z* 883.4 $[M + 2H]^+$.

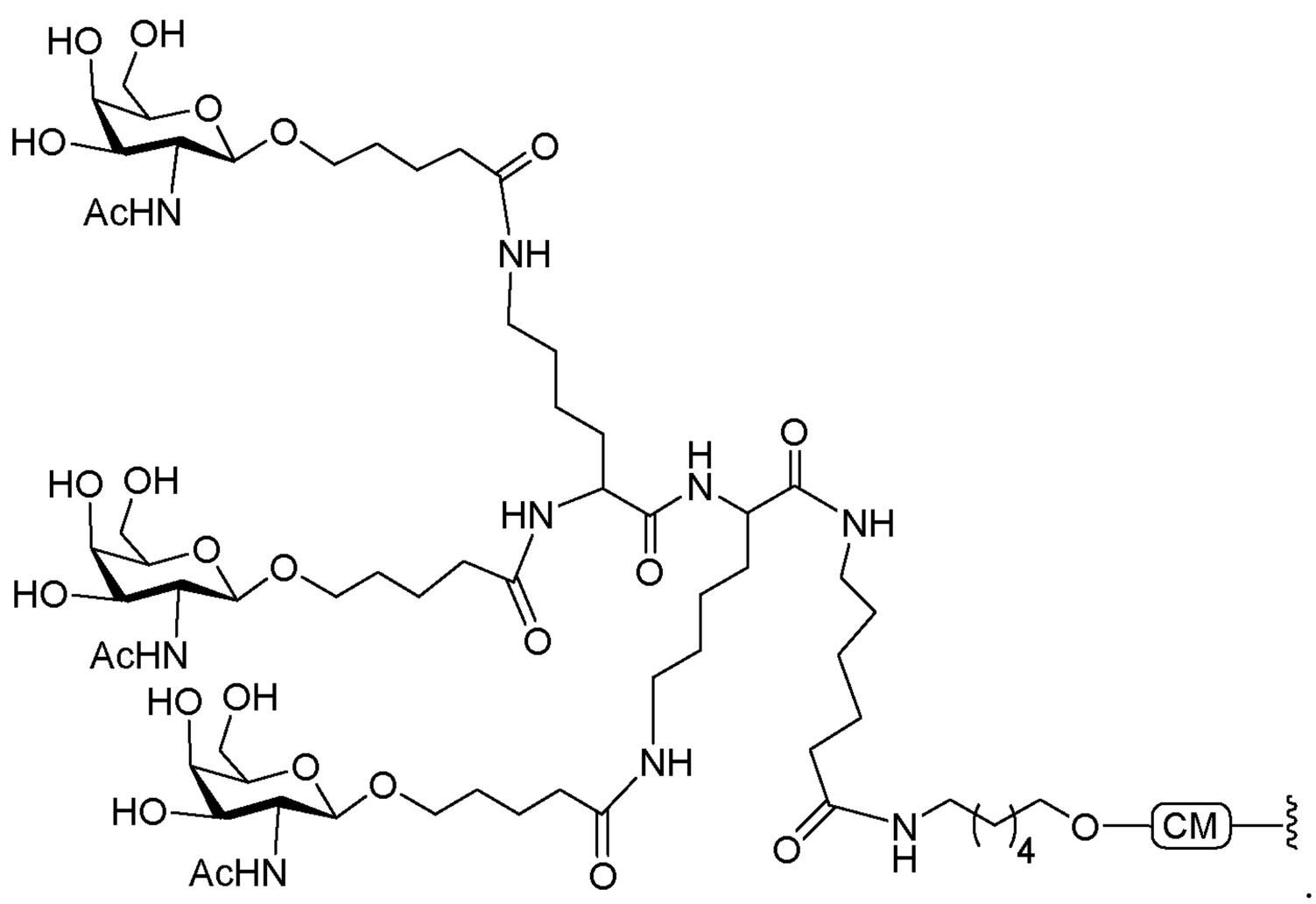
Compound 129 (0.09 g, 0.051 mmol) was dissolved in methanol (5 mL) in 20 mL scintillation vial. To this was added a small amount of 10% Pd/C (0.015 mg) and the reaction vessel was flushed with H_2 gas. The reaction mixture was stirred at room temperature under H_2 atmosphere for 18 h. The reaction mixture was filtered through a pad of Celite and the Celite pad was washed with methanol. The filtrate washings were pooled together and concentrated under reduced pressure to yield Compound 130 (0.08 g). LCMS and ^{1}H NMR were consistent with structure. The product was used without further purification. Mass *m/z* 838.3 $[M + 2H]^+$.

To a 10 mL pointed round bottom flask were added compound 130 (75.8 mg, 0.046 mmol), 0.37 M pyridine/DMF (200 µL) and a stir bar. To this solution was added 0.7 M pentafluorophenyl trifluoroacetate/DMF (100 µL) drop wise with stirring. The reaction was completed after 1 h as determined by LC MS. The solvent was removed under reduced pressure and the residue was dissolved in $CHCl_3$ (~ 10 mL). The organic layer was partitioned against $NaHSO_4$ (1 M, 10 mL) , aqueous saturated $NaHCO_3$ (10 mL) and brine (10 mL) three times each. The organic phase separated and dried over Na_2SO_4, filtered and concentrated to yield Compound 131 (77.7 mg). LCMS is consistent with structure. Used without further purification. Mass *m/z* 921.3 $[M + 2H]^+$.

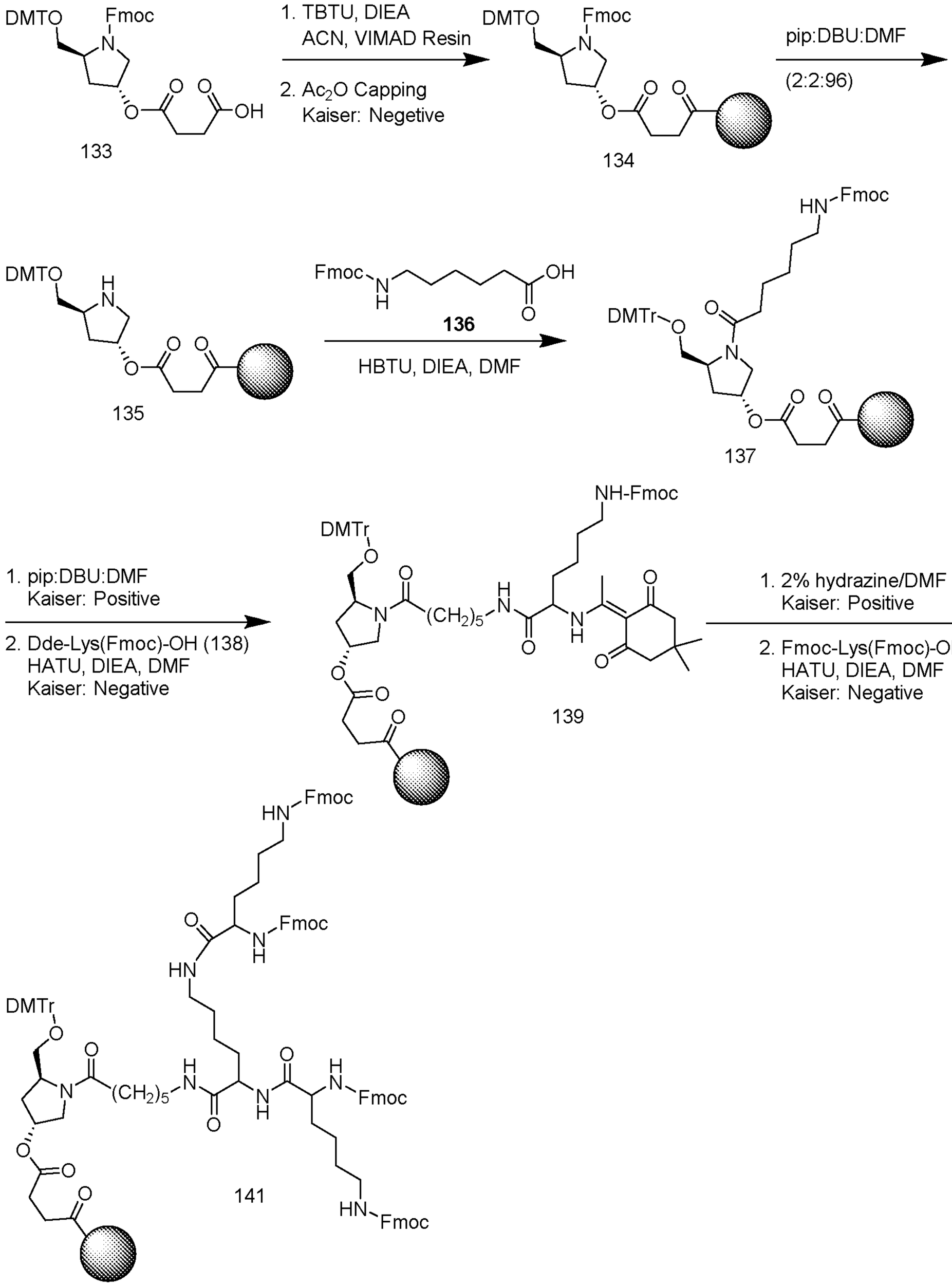


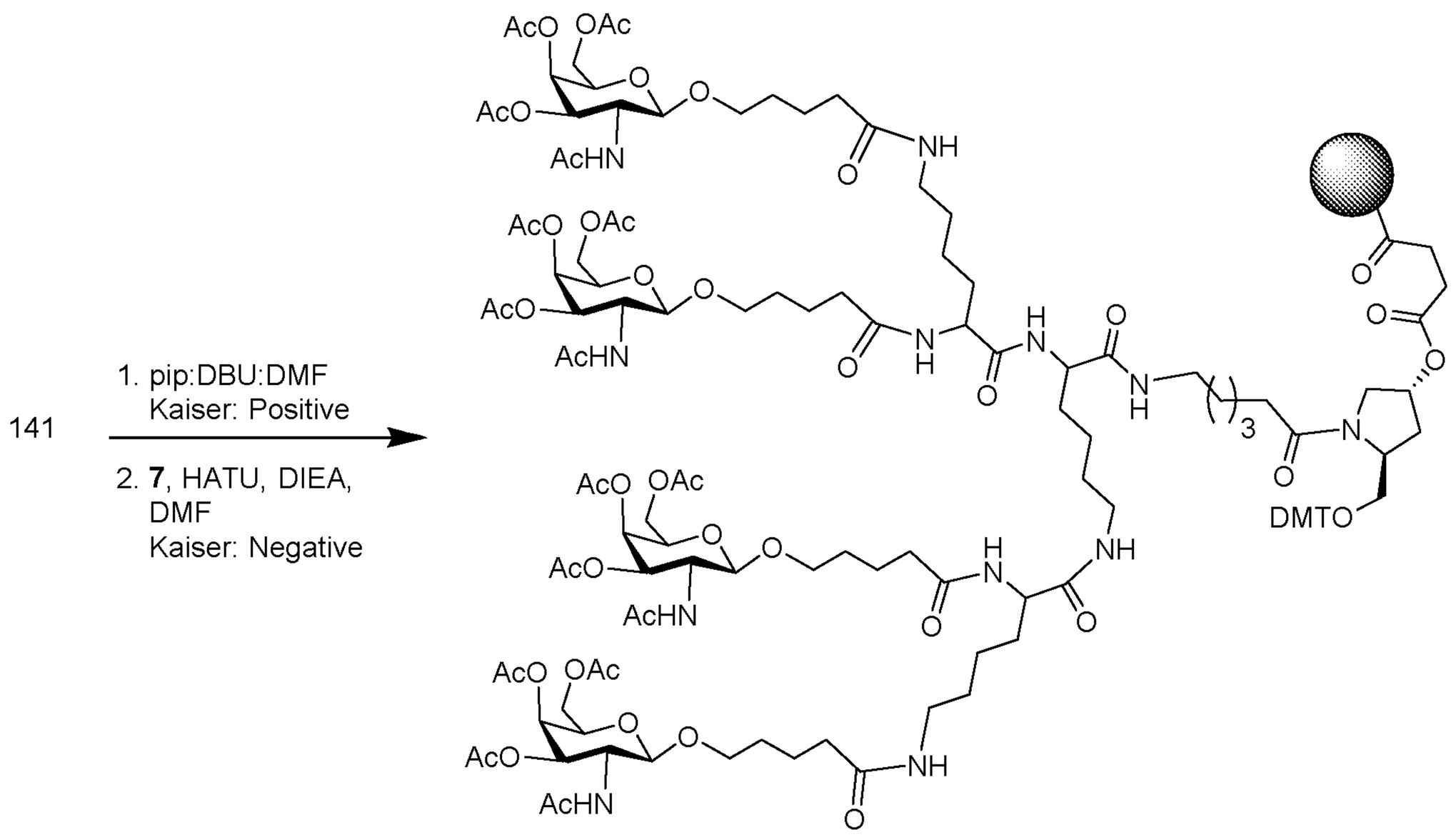
Oligomeric Compound 132, comprising a $GalNAc_3$-5 conjugate group, was prepared using the general procedures illustrated in Example 46. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-5 ($GalNAc_3$-5_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-.

The structure of $GalNAc_3$-5 ($GalNAc_3$-5_a-CM-) is shown below:



Example 50: Preparation of Oligonucleotide 144 Comprising GalNAc$_4$-11



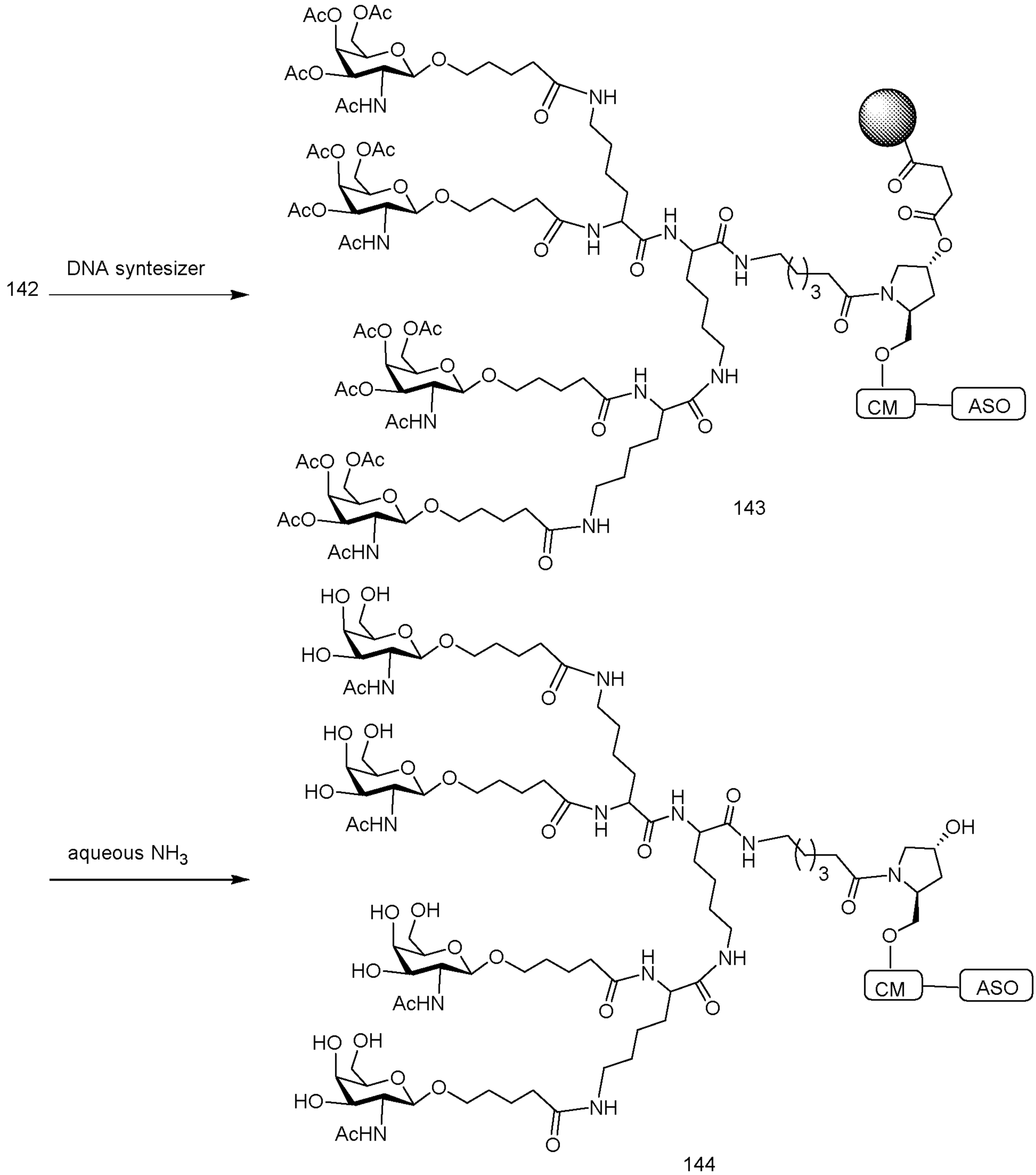


141 142

Synthesis of Compound 134. To a Merrifield flask was added aminomethyl VIMAD resin (2.5 g, 450 μmol/g) that was washed with acetonitrile, dimethylformamide, dichloromethane and acetonitrile. The resin was swelled in acetonitrile (4 mL). Compound 133 was pre-activated in a 100 mL round bottom flask by adding 20 (1.0 mmol, 0.747 g), TBTU (1.0 mmol, 0.321 g), acetonitrile (5 mL) and DIEA (3.0 mmol, 0.5 mL). This solution was allowed to stir for 5 min and was then added to the Merrifield flask with shaking. The suspension was allowed to shake for 3 h. The reaction mixture was drained and the resin was washed with acetonitrile, DMF and DCM. New resin loading was quantitated by measuring the absorbance of the DMT cation at 500 nm (extinction coefficient = 76000) in DCM and determined to be 238 μmol/g. The resin was capped by suspending in an acetic anhydride solution for ten minutes three times.

The solid support bound compound 141 was synthesized using iterative Fmoc-based solid phase peptide synthesis methods. A small amount of solid support was withdrawn and suspended in aqueous ammonia (28-30 wt%) for 6 h. The cleaved compound was analyzed by LC-MS and the observed mass was consistent with structure. Mass *m/z* 1063.8 $[M + 2H]^+$.

The solid support bound compound 142 was synthesized using solid phase peptide synthesis methods.



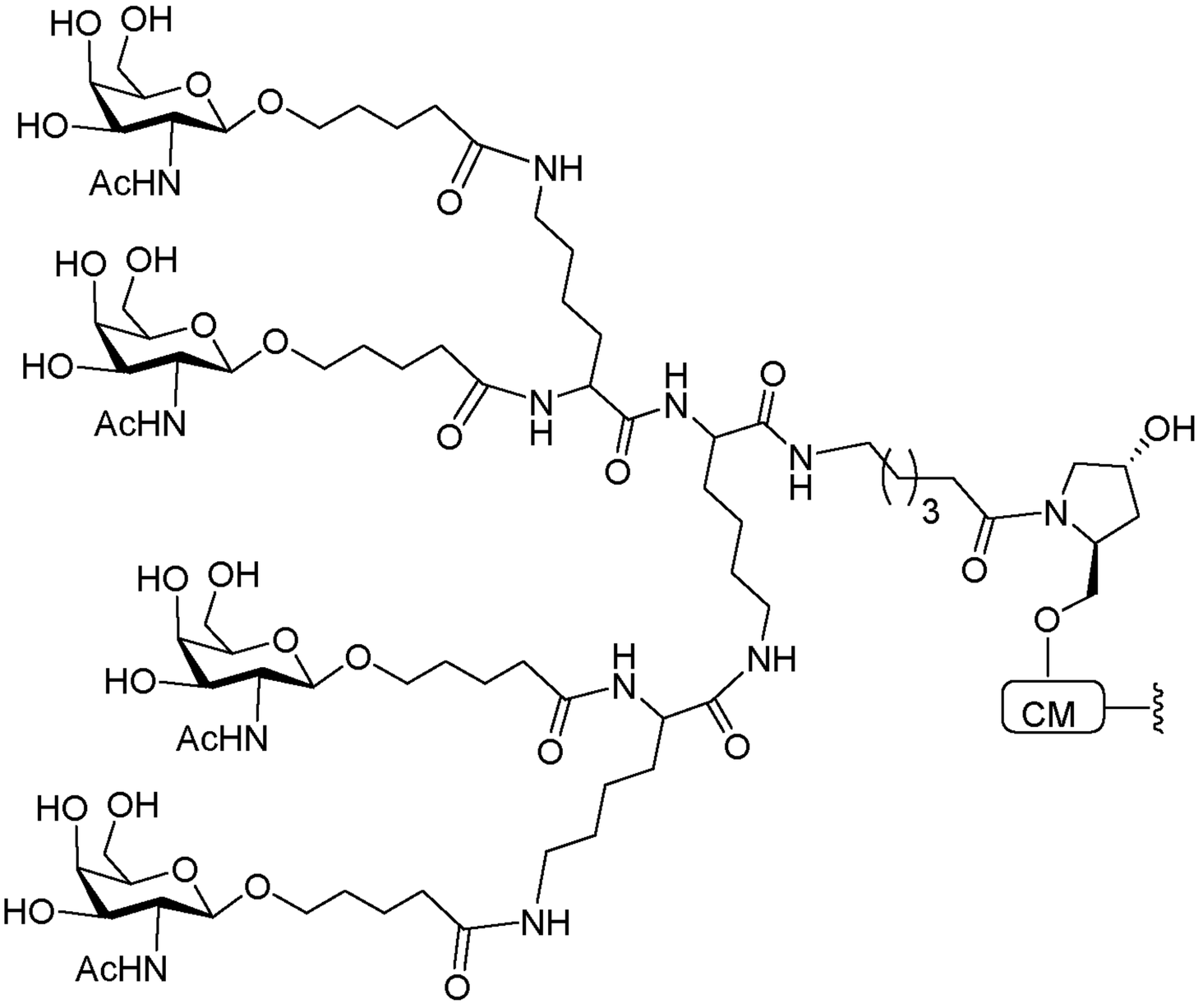
The solid support bound compound 143 was synthesized using standard solid phase synthesis on a DNA synthesizer.

The solid support bound compound 143 was suspended in aqueous ammonia (28-30 wt%) and heated at 55 °C for 16 h. The solution was cooled and the solid support was filtered. The filtrate was concentrated and the residue dissolved in water and purified by HPLC on a strong anion exchange column. The fractions containing full length compound 144 were pooled together and desalted. The resulting $GalNAc_4$-11

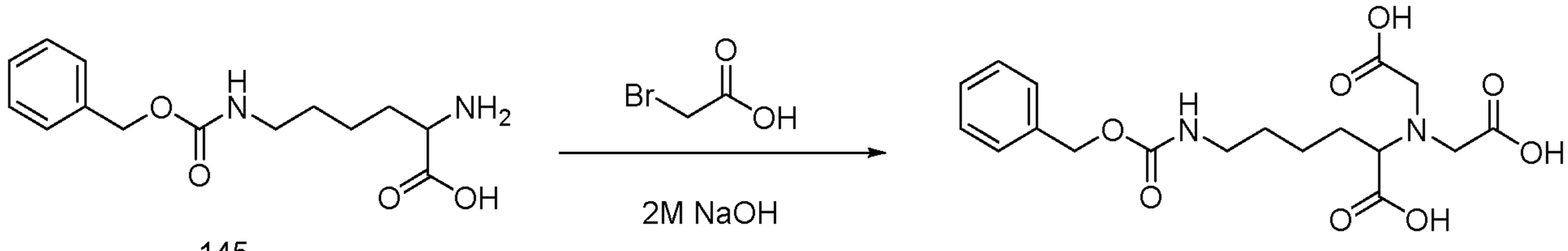
conjugated oligomeric compound was analyzed by LC-MS and the observed mass was consistent with structure.

The $GalNAc_4$ cluster portion of the conjugate group $GalNAc_4$-11 ($GalNAc_4$-11_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-.

The structure of $GalNAc_4$-11 ($GalNAc_4$-11_a-CM) is shown below:

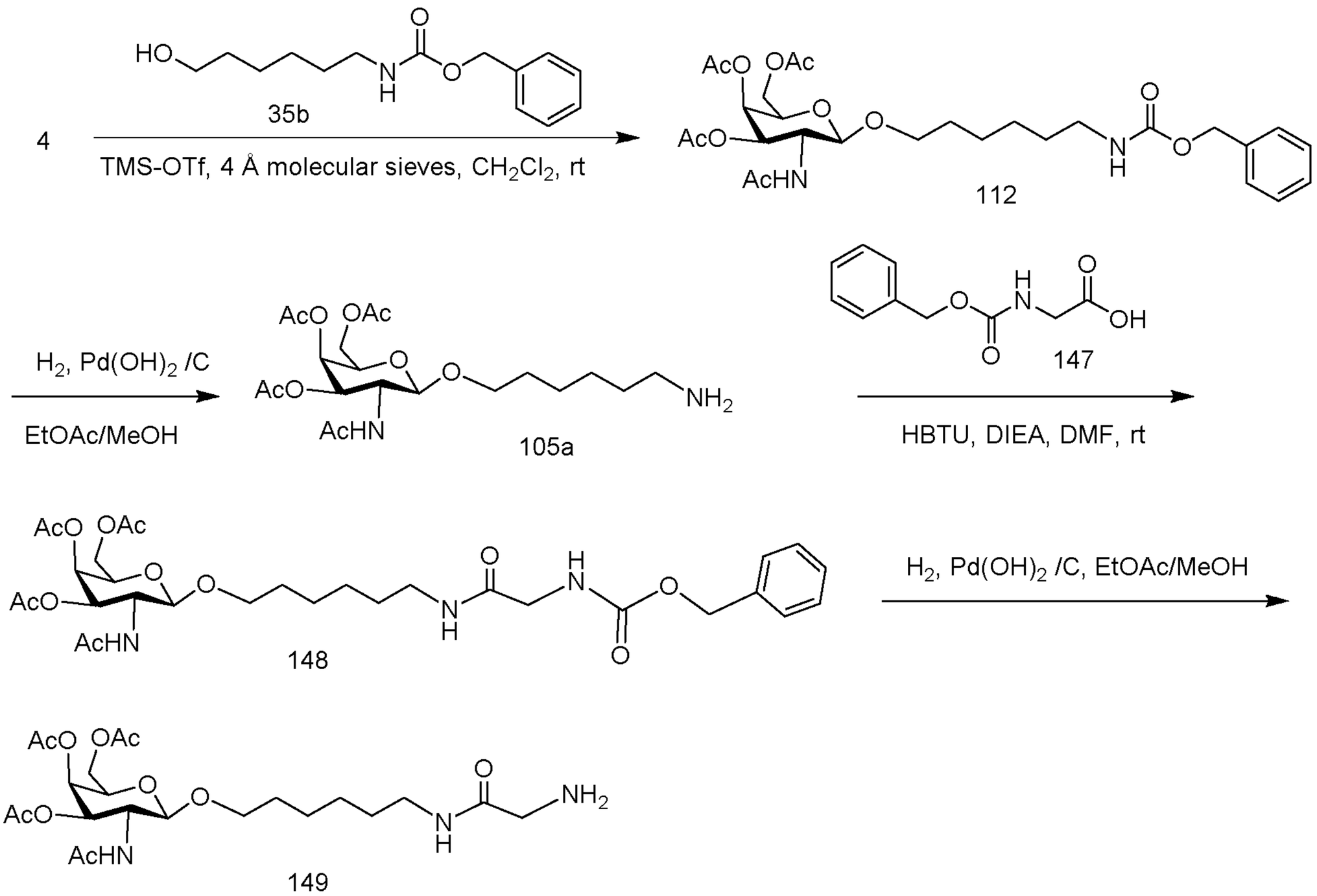


Example 51: Preparation of Oligonucleotide 155 Comprising $GalNAc_3$-6



145 146

Compound 146 was synthesized as described in the literature (*Analytical Biochemistry* 1995, *229*, 54-60).



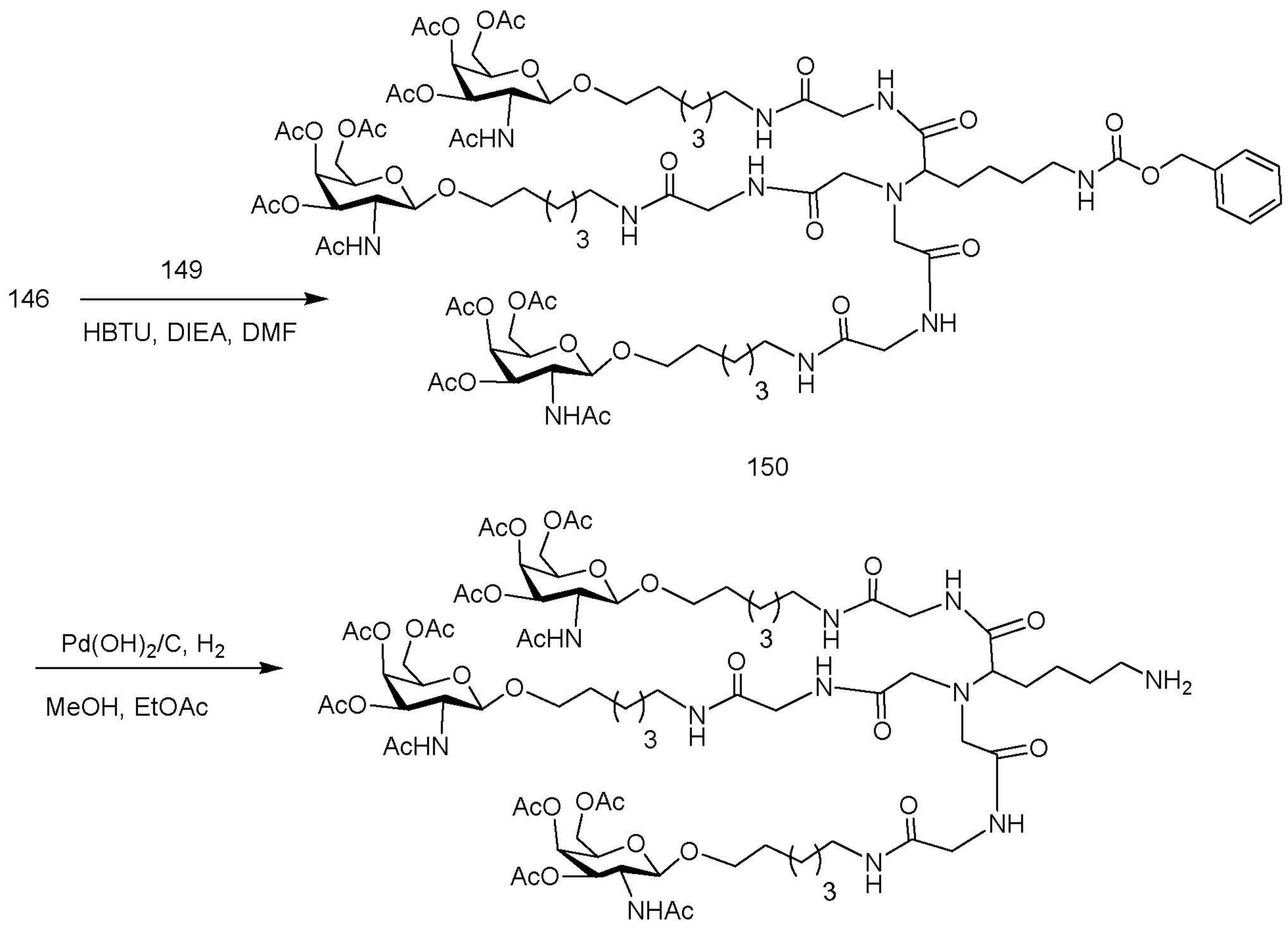
Compound 4 (15 g, 45.55 mmol) and compound 35b (14.3 grams, 57 mmol) were dissolved in CH_2Cl_2 (200 ml). Activated molecular sieves (4 Å. 2 g, powdered) were added, and the reaction was allowed to stir for 30 minutes under nitrogen atmosphere. TMS-OTf was added (4.1 ml, 22.77 mmol) and the reaction was allowed to stir at room temp overnight. Upon completion, the reaction was quenched by pouring into solution of saturated aqueous $NaHCO_3$ (500 ml) and crushed ice (~ 150 g). The organic layer was separated, washed with brine, dried over $MgSO_4$, filtered, and was concentrated to an orange oil under reduced pressure. The crude material was purified by silica gel column chromatography and eluted with 2-10 % MeOH in CH_2Cl_2 to yield Compound 112 (16.53 g, 63 %). LCMS and ^{1}H NMR were consistent with the expected compound.

Compound 112 (4.27 g, 7.35 mmol) was dissolved in 1:1 MeOH/EtOAc (40 ml). The reaction mixture was purged by bubbling a stream of argon through the solution for 15 minutes. Pearlman's catalyst (palladium hydroxide on carbon, 400 mg) was added, and hydrogen gas was bubbled through the solution for 30 minutes. Upon completion (TLC 10% MeOH in CH_2Cl_2, and LCMS), the catalyst was removed by filtration through a pad of celite. The filtrate was concentrated by rotary evaporation, and was dried briefly under high vacuum to yield Compound 105a (3.28 g). LCMS and 1H NMR were consistent with desired product.

Compound 147 (2.31 g, 11 mmol) was dissolved in anhydrous DMF (100 mL). *N,N*-Diisopropylethylamine (DIEA, 3.9 mL, 22 mmol) was added, followed by HBTU (4 g, 10.5 mmol). The

reaction mixture was allowed to stir for ~ 15 minutes under nitrogen. To this a solution of compound 105a (3.3 g, 7.4 mmol) in dry DMF was added and stirred for 2 h under nitrogen atmosphere. The reaction was diluted with EtOAc and washed with saturated aqueous $NaHCO_3$ and brine. The organics phase was separated, dried ($MgSO_4$), filtered, and concentrated to an orange syrup. The crude material was purified by column chromatography 2-5 % MeOH in CH_2Cl_2 to yield Compound 148 (3.44 g, 73 %). LCMS and ^1H NMR were consistent with the expected product.

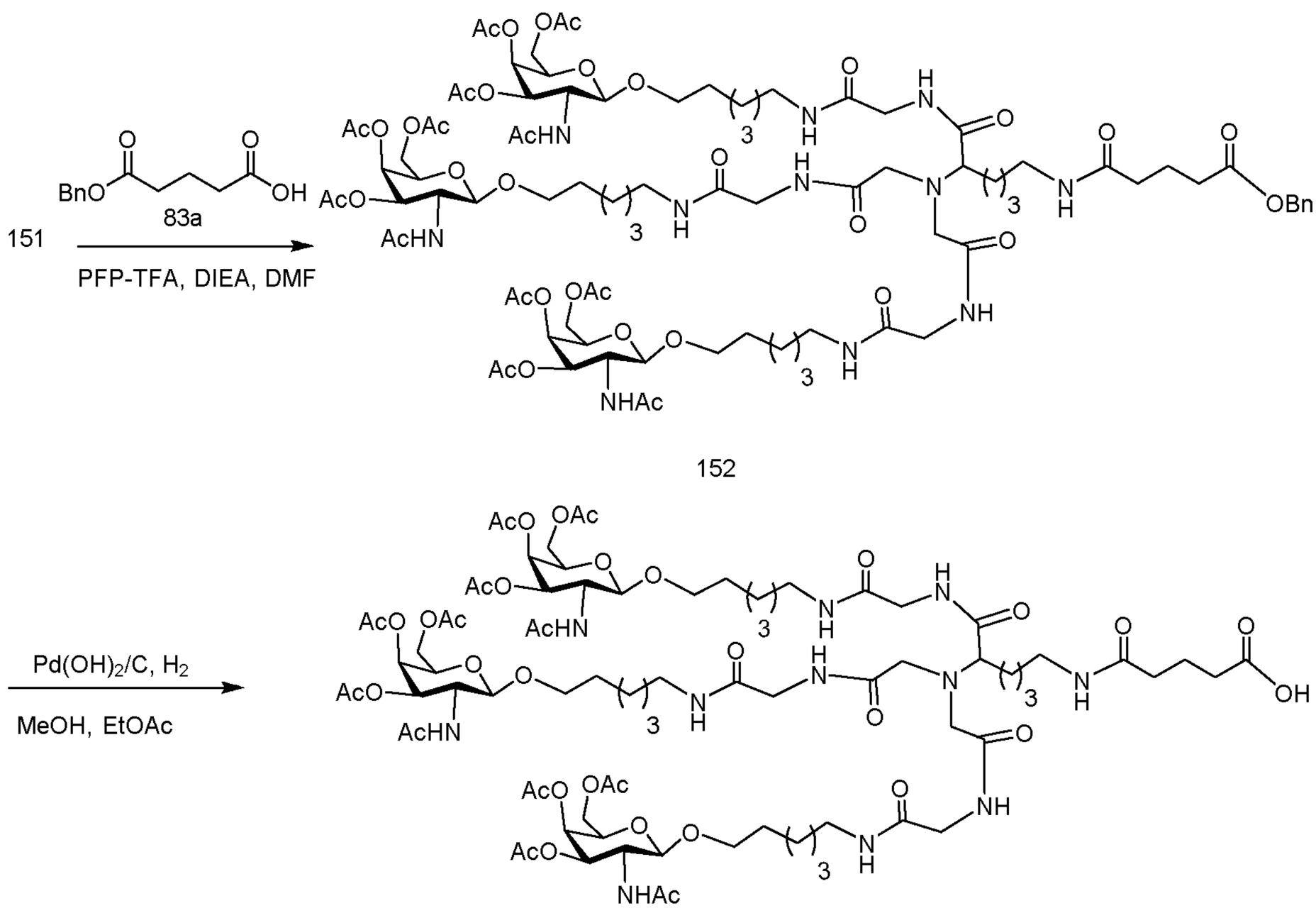
Compound 148 (3.3 g, 5.2 mmol) was dissolved in 1:1 MeOH/EtOAc (75 ml). The reaction mixture was purged by bubbling a stream of argon through the solution for 15 minutes. Pearlman's catalyst (palladium hydroxide on carbon) was added (350 mg). Hydrogen gas was bubbled through the solution for 30 minutes. Upon completion (TLC 10% MeOH in DCM, and LCMS), the catalyst was removed by filtration through a pad of celite. The filtrate was concentrated by rotary evaporation, and was dried briefly under high vacuum to yield Compound 149 (2.6 g). LCMS was consistent with desired product. The residue was dissolved in dry DMF (10 ml) was used immediately in the next step.



Compound 146 (0.68 g, 1.73 mmol) was dissolved in dry DMF (20 ml). To this DIEA (450 µL, 2.6 mmol, 1.5 eq.) and HBTU (1.96 g, 0.5.2 mmol) were added. The reaction mixture was allowed to stir for 15

minutes at room temperature under nitrogen. A solution of compound 149 (2.6 g) in anhydrous DMF (10 mL) was added. The pH of the reaction was adjusted to pH = 9-10 by addition of DIEA (if necessary). The reaction was allowed to stir at room temperature under nitrogen for 2 h. Upon completion the reaction was diluted with EtOAc (100 mL), and washed with aqueous saturated aqueous $NaHCO_3$, followed by brine. The organic phase was separated, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel column chromatography and eluted with 2-10 % MeOH in CH_2Cl_2 to yield Compound 150 (0.62 g, 20 %). LCMS and ^1H NMR were consistent with the desired product.

Compound 150 (0.62 g) was dissolved in 1:1 MeOH/ EtOAc (5 L). The reaction mixture was purged by bubbling a stream of argon through the solution for 15 minutes. Pearlman's catalyst (palladium hydroxide on carbon) was added (60 mg). Hydrogen gas was bubbled through the solution for 30 minutes. Upon completion (TLC 10% MeOH in DCM, and LCMS), the catalyst was removed by filtration (syringe-tip Teflon filter, 0.45 μm). The filtrate was concentrated by rotary evaporation, and was dried briefly under high vacuum to yield Compound 151 (0.57 g). The LCMS was consistent with the desired product. The product was dissolved in 4 mL dry DMF and was used immediately in the next step.

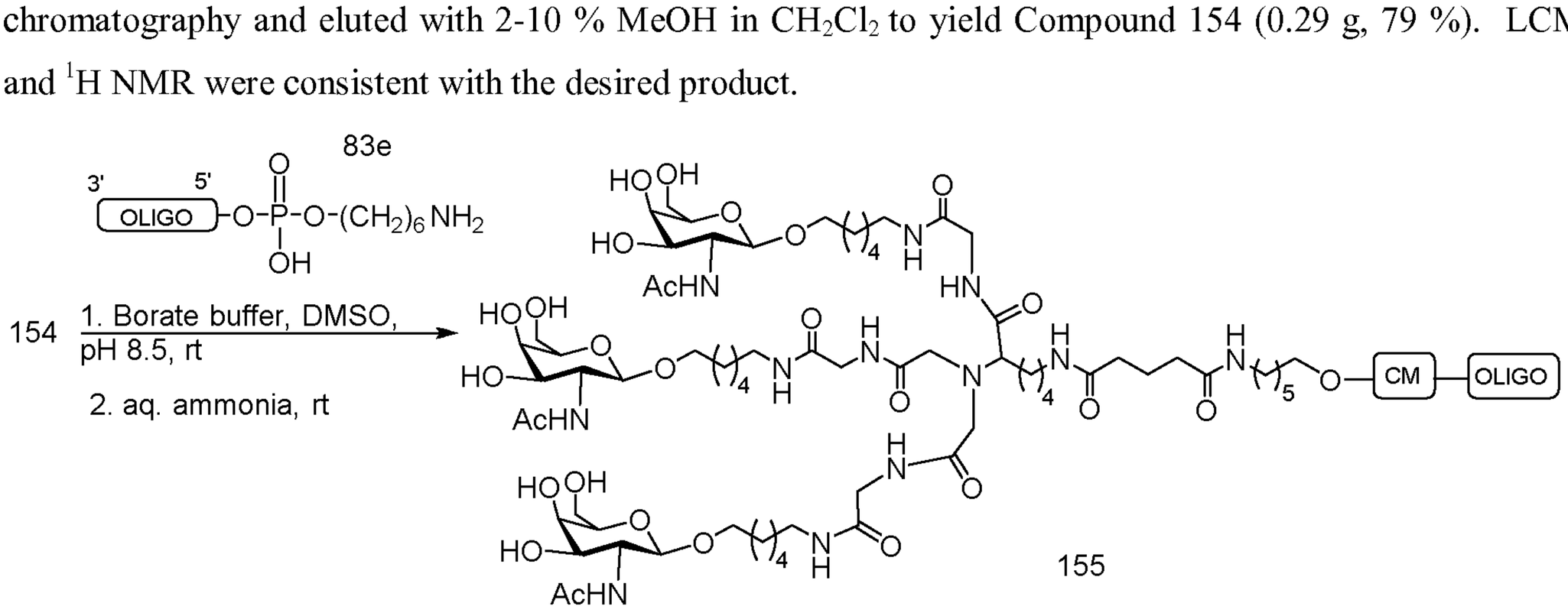


Compound 83a (0.11 g, 0.33 mmol) was dissolved in anhydrous DMF (5 mL) and *N*,*N*-Diisopropylethylamine (75 µL, 1 mmol) and PFP-TFA (90 µL, 0.76 mmol) were added. The reaction mixture turned magenta upon contact, and gradually turned orange over the next 30 minutes. Progress of reaction was monitored by TLC and LCMS. Upon completion (formation of the PFP ester), a solution of compound 151 (0.57 g, 0.33 mmol) in DMF was added. The pH of the reaction was adjusted to pH = 9-10 by addition of *N*,*N*-Diisopropylethylamine (if necessary). The reaction mixture was stirred under nitrogen for ~ 30 min. Upon completion, the majority of the solvent was removed under reduced pressure. The residue was diluted with CH_2Cl_2 and washed with aqueous saturated $NaHCO_3$, followed by brine. The organic phase separated, dried over $MgSO_4$, filtered, and concentrated to an orange syrup. The residue was purified by

silica gel column chromatography (2-10 % MeOH in CH_2Cl_2) to yield Compound 152 (0.35 g, 55 %). LCMS and ^{1}H NMR were consistent with the desired product.

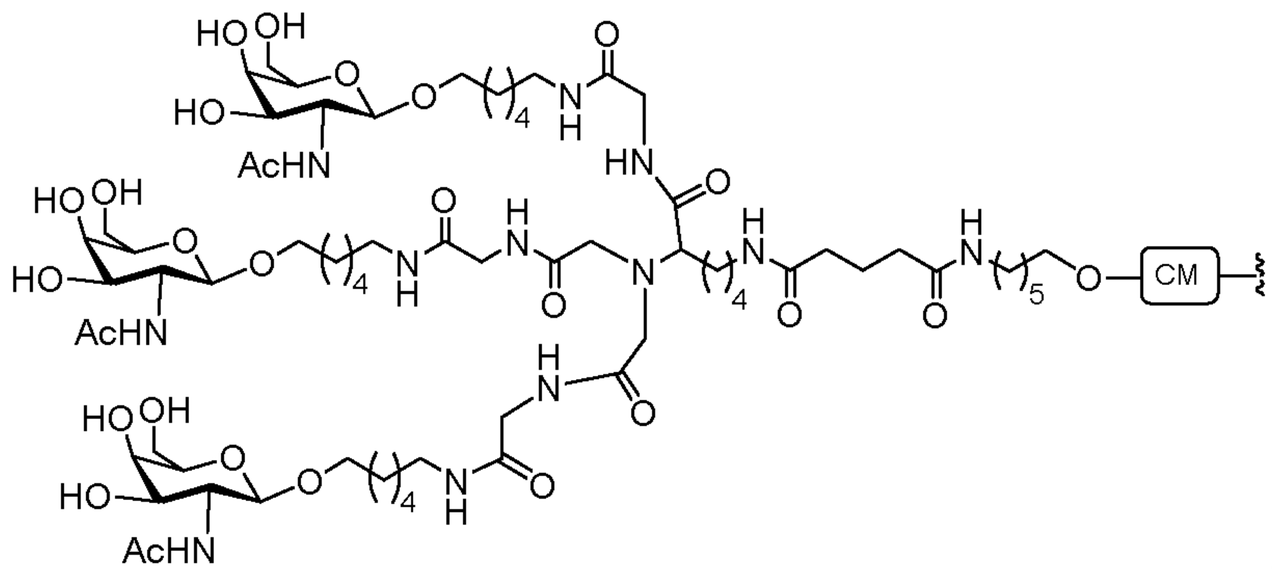
Compound 152 (0.35 g, 0.182 mmol) was dissolved in 1:1 MeOH/EtOAc (10 mL). The reaction mixture was purged by bubbling a stream of argon thru the solution for 15 minutes. Pearlman's catalyst (palladium hydroxide on carbon) was added (35 mg). Hydrogen gas was bubbled thru the solution for 30 minutes. Upon completion (TLC 10% MeOH in DCM, and LCMS), the catalyst was removed by filtration (syringe-tip Teflon filter, 0.45 μm). The filtrate was concentrated by rotary evaporation, and was dried briefly under high vacuum to yield Compound 153 (0.33 g, quantitative). The LCMS was consistent with desired product.

Compound 153 (0.33 g, 0.18 mmol) was dissolved in anhydrous DMF (5 mL) with stirring under nitrogen. To this *N*,*N*-Diisopropylethylamine (65 μL, 0.37 mmol) and PFP-TFA (35 μL, 0.28 mmol) were added. The reaction mixture was stirred under nitrogen for ~ 30 min. The reaction mixture turned magenta upon contact, and gradually turned orange. The pH of the reaction mixture was maintained at pH = 9-10 by adding more *N*,-Diisopropylethylamine. The progress of the reaction was monitored by TLC and LCMS. Upon completion, the majority of the solvent was removed under reduced pressure. The residue was diluted with CH_2Cl_2 (50 mL), and washed with saturated aqueous $NaHCO_3$, followed by brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated to an orange syrup. The residue was purified by column chromatography and eluted with 2-10 % MeOH in CH_2Cl_2 to yield Compound 154 (0.29 g, 79 %). LCMS and ^{1}H NMR were consistent with the desired product.

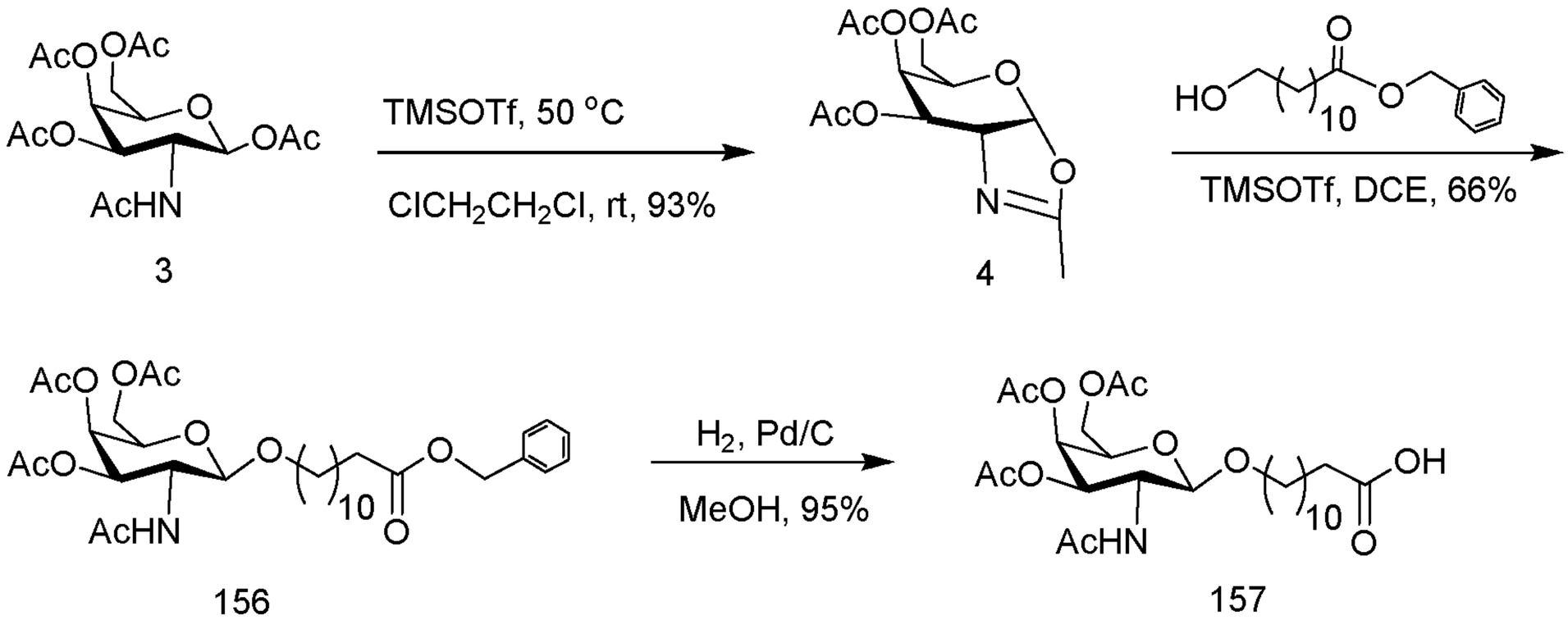


Oligomeric Compound 155, comprising a $GalNAc_3$-6 conjugate group, was prepared using the general procedures illustrated in Example 46. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-6 ($GalNAc_3$-6_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-.

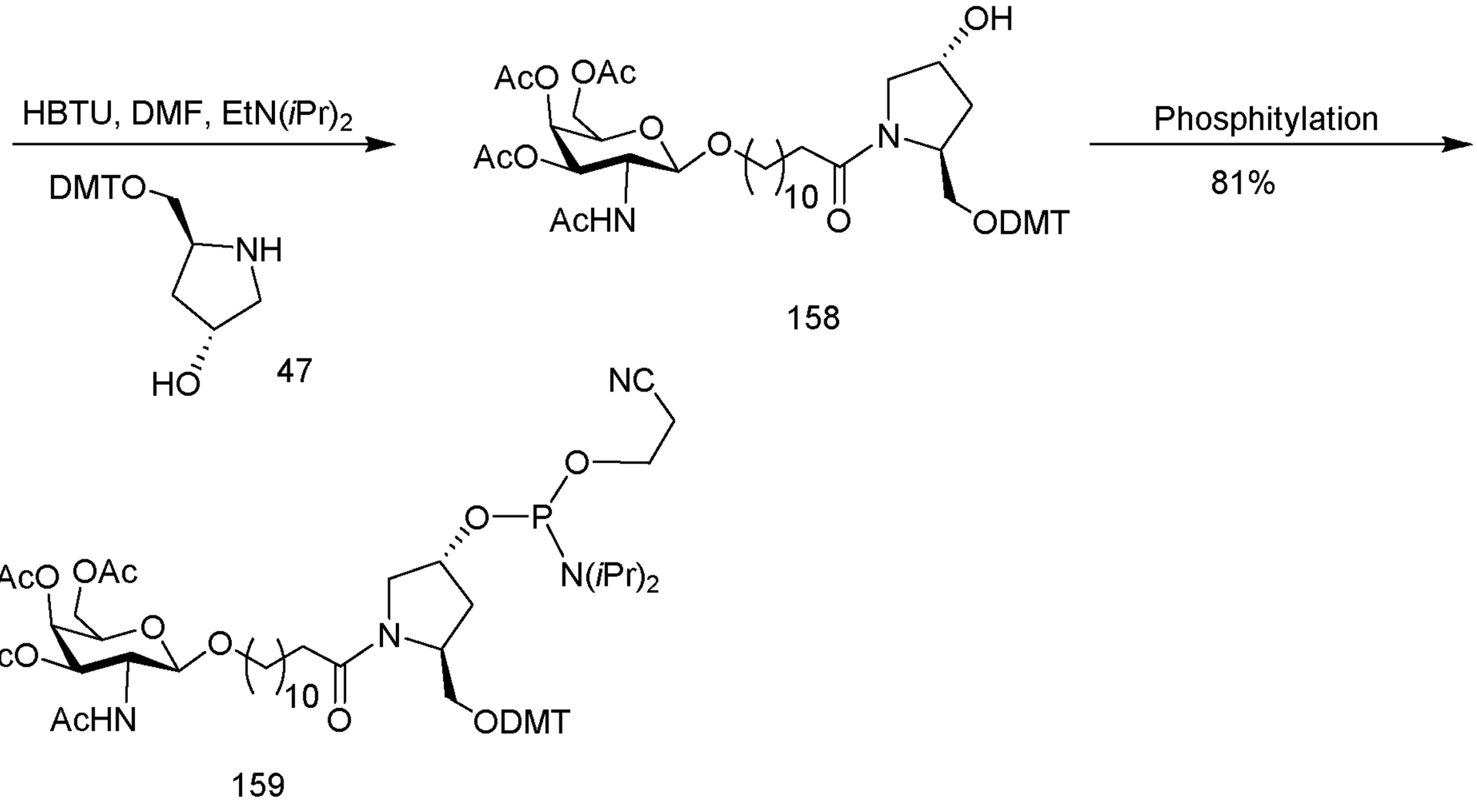
The structure of $GalNAc_3$-6 ($GalNAc_3$-6_a-CM-) is shown below:



Example 52: Preparation of Oligonucleotide 160 Comprising GalNAc$_3$-9



3 4 156 157



47 158 159

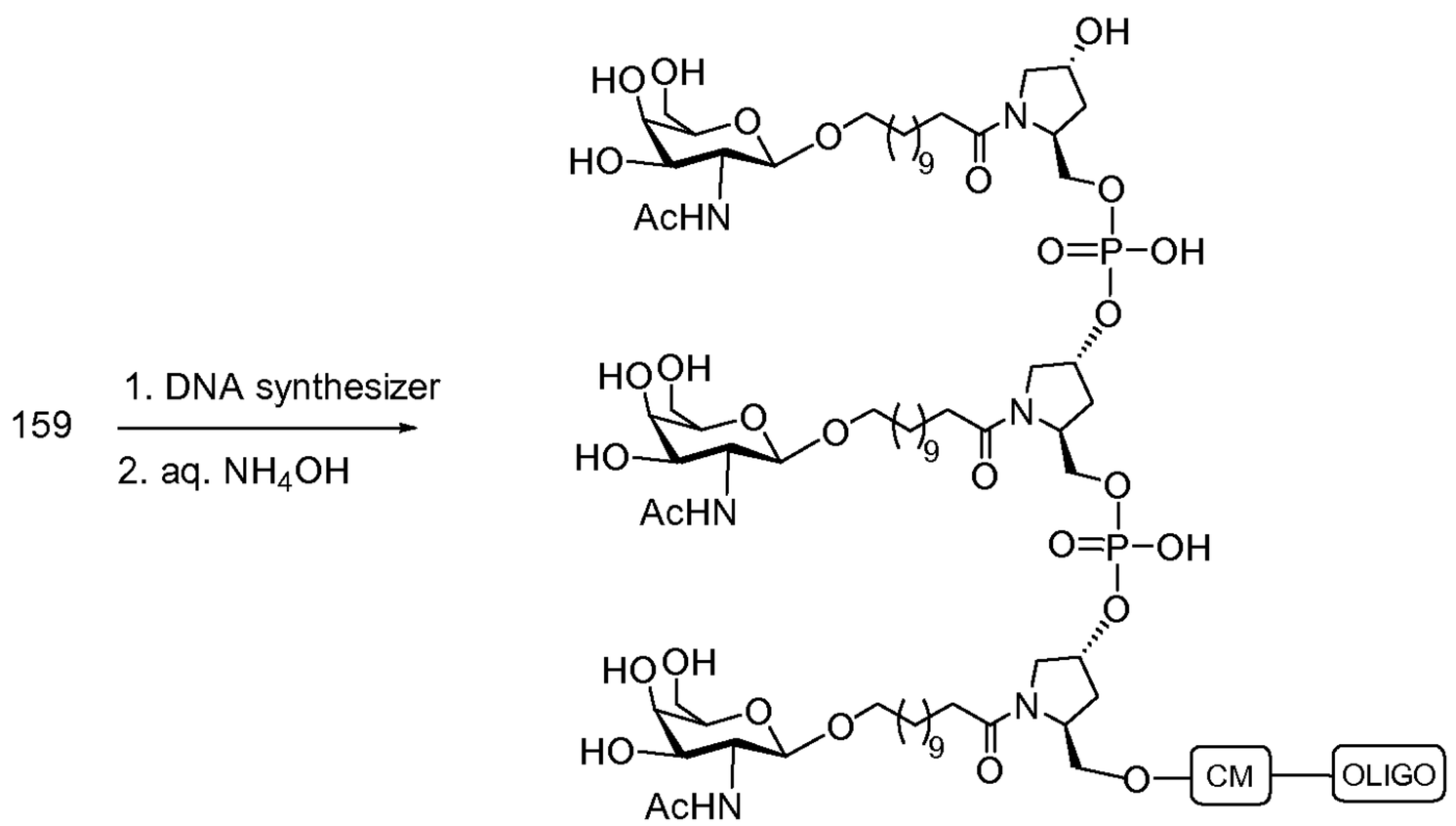
Compound 156 was synthesized following the procedure described in the literature (*J. Med. Chem.* 2004, *47*, 5798-5808).

Compound 156, (18.60 g, 29.28 mmol) was dissolved in methanol (200 mL). Palladium on carbon (6.15 g, 10 wt%, loading (dry basis), matrix carbon powder, wet) was added. The reaction mixture was stirred at room temperature under hydrogen for 18 h. The reaction mixture was filtered through a pad of

celite and the celite pad was washed thoroughly with methanol. The combined filtrate was washed and concentrated to dryness. The residue was purified by silica gel column chromatography and eluted with 5-10 % methanol in dichloromethane to yield Compound 157 (14.26 g, 89%). Mass *m/z* 544.1 $[M-H]^-$.

Compound 157 (5 g, 9.17 mmol) was dissolved in anhydrous DMF (30 mL). HBTU (3.65 g, 9.61 mmol) and *N*,*N*-Diisopropylethylamine (13.73 mL, 78.81 mmol) were added and the reaction mixture was stirred at room temperature for 5 minutes. To this a solution of compound 47 (2.96 g, 7.04 mmol) was added. The reaction was stirred at room temperature for 8 h. The reaction mixture was poured into a saturated $NaHCO_3$ aqueous solution. The mixture was extracted with ethyl acetate and the organic layer was washed with brine and dried (Na_2SO_4), filtered and evaporated. The residue obtained was purified by silica gel column chromatography and eluted with 50% ethyl acetate in hexane to yield compound 158 (8.25g, 73.3%). The structure was confirmed by MS and ^1H NMR analysis.

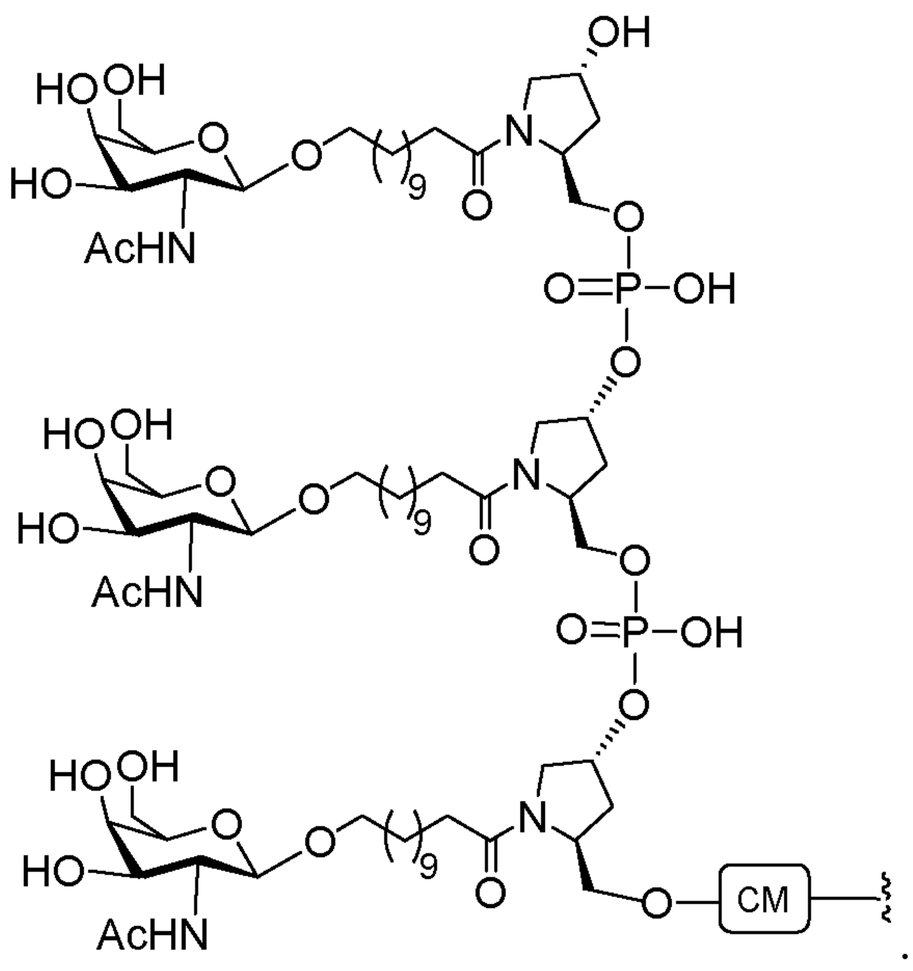
Compound 158 (7.2 g, 7.61 mmol) was dried over P_2O_5 under reduced pressure. The dried compound was dissolved in anhydrous DMF (50 mL). To this 1H-tetrazole (0.43 g, 6.09 mmol) and *N*-methylimidazole (0.3 mL, 3.81 mmol) and 2-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropyl phosphorodiamidite (3.65 mL, 11.50 mmol) were added. The reaction mixture was stirred t under an argon atmosphere for 4 h. The reaction mixture was diluted with ethyl acetate (200 mL). The reaction mixture was washed with saturated $NaHCO_3$ and brine. The organic phase was separated, dried (Na_2SO_4), filtered and evaporated. The residue was purified by silica gel column chromatography and eluted with 50-90 % ethyl acetate in hexane to yield Compound 159 (7.82 g, 80.5%). The structure was confirmed by LCMS and ^{31}P NMR analysis.



160

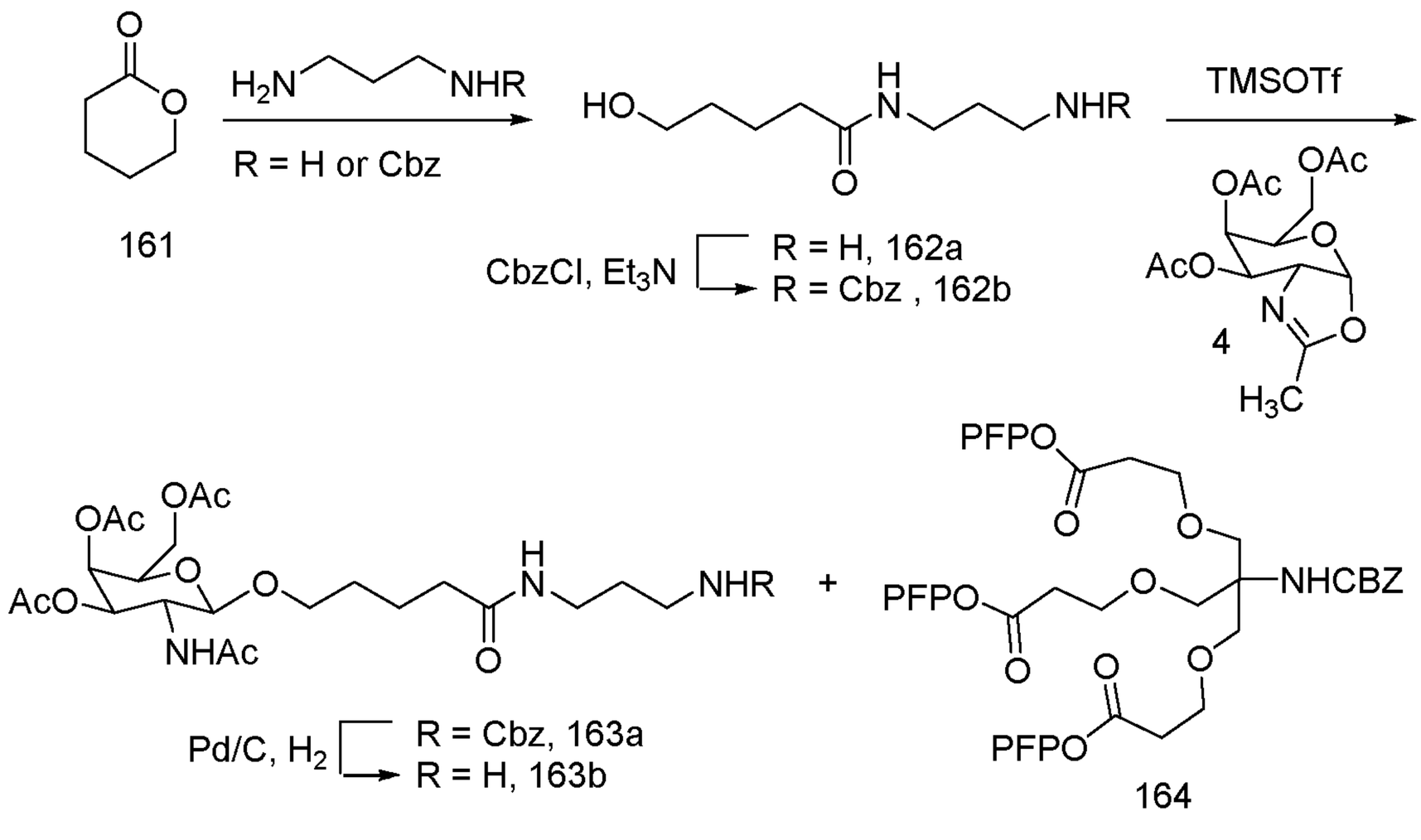
Oligomeric Compound 160, comprising a $GalNAc_3$-9 conjugate group, was prepared using standard oligonucleotide synthesis procedures. Three units of compound 159 were coupled to the solid support, followed by nucleotide phosphoramidites. Treatment of the protected oligomeric compound with aqueous ammonia yielded compound 160. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-9 ($GalNAc_3$-

9_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-. The structure of $GalNAc_3$-9 ($GalNAc_3$-9_a-CM) is shown below:



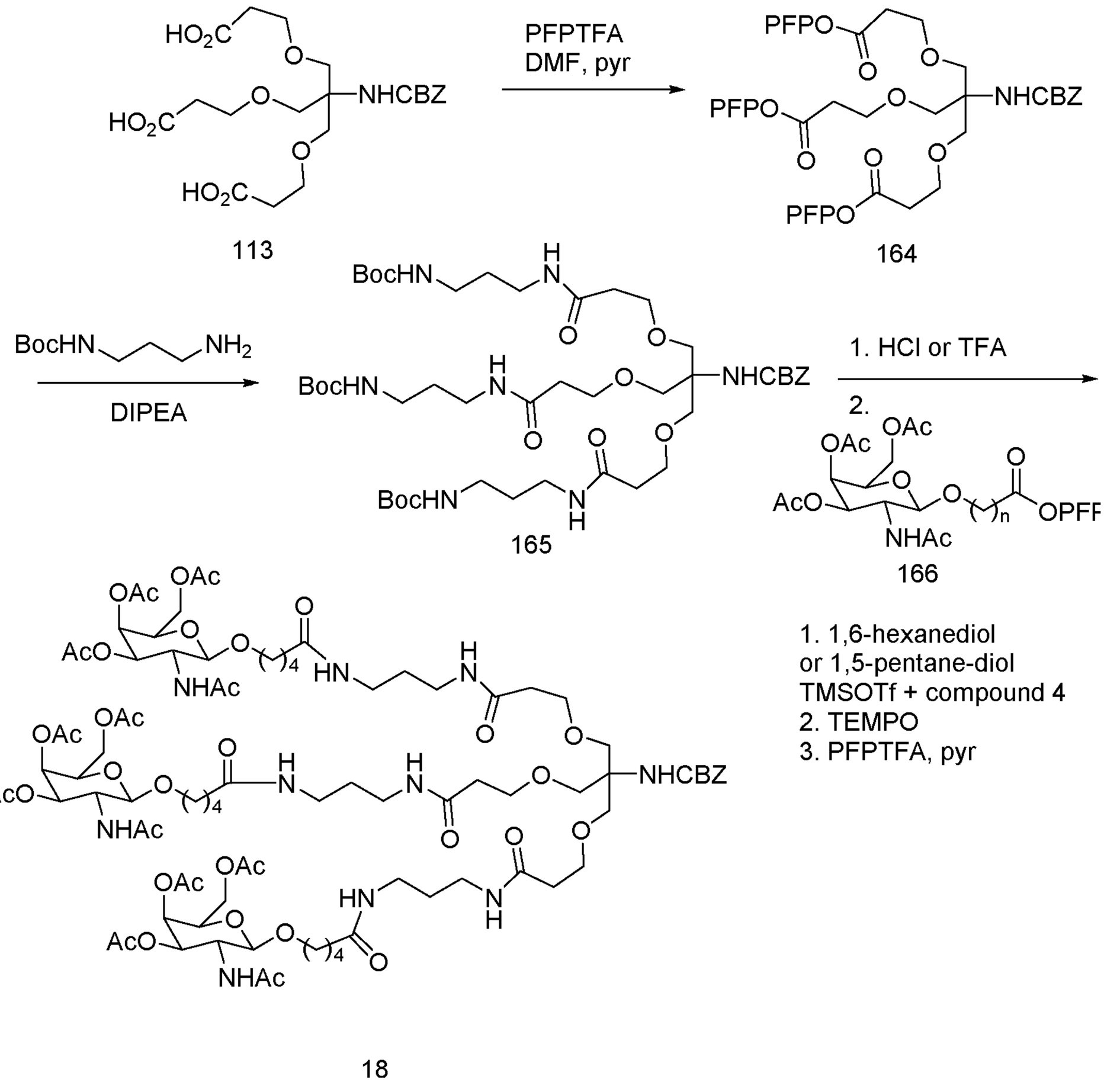
.

Example 53: Alternate procedure for preparation of Compound 18 (GalNAc$_3$-1a and GalNAc$_3$-3a)



Lactone 161 was reacted with diamino propane (3-5 eq) or Mono-Boc protected diamino propane (1 eq) to provide alcohol 162a or 162b. When unprotected propanediamine was used for the above reaction, the excess diamine was removed by evaporation under high vacuum and the free amino group in 162a was protected using CbzCl to provide 162b as a white solid after purification by column chromatography. Alcohol 162b was further reacted with compound 4 in the presence of TMSOTf to provide 163a which was converted to 163b by removal of the Cbz group using catalytic hydrogenation. The pentafluorophenyl (PFP) ester 164 was prepared by reacting triacid 113 (see Example 48) with PFPTFA (3.5 eq) and pyridine (3.5 eq) in DMF (0.1 to 0.5 M). The triester 164 was directly reacted with the amine 163b (3–4 eq) and DIPEA (3–4 eq) to provide Compound 18. The above method greatly facilitates purification of intermediates and minimizes the formation of byproducts which are formed using the procedure described in Example 4.

Example 54: Alternate procedure for preparation of Compound 18 (GalNAc$_3$-1a and GalNAc$_3$-3a)



113 164

165

166

18

The triPFP ester 164 was prepared from acid 113 using the procedure outlined in example 53 above and reacted with mono-Boc protected diamine to provide 165 in essentially quantitative yield. The Boc groups were removed with hydrochloric acid or trifluoroacetic acid to provide the triamine which was reacted with the PFP activated acid 166 in the presence of a suitable base such as DIPEA to provide Compound 18.

The PFP protected Gal-NAc acid 166 was prepared from the corresponding acid by treatment with PFPTFA (1-1.2 eq) and pyridine (1-1.2 eq) in DMF. The precursor acid in turn was prepared from the corresponding alcohol by oxidation using TEMPO (0.2 eq) and BAIB in acetonitrile and water. The precursor alcohol was prepared from sugar intermediate 4 by reaction with 1,6-hexanediol (or 1,5-pentanediol or other diol for other n values) (2-4 eq) and TMSOTf using conditions described previously in example 47.

Example 55: Dose-dependent study of oligonucleotides comprising either a 3' or 5'-conjugate group (comparison of $GalNAc_3$-1, 3, 8 and 9) targeting SRB-1 *in vivo*

The oligonucleotides listed below were tested in a dose-dependent study for antisense inhibition of SRB-1 in mice. Unconjugated ISIS 353382 was included as a standard. Each of the various $GalNAc_3$ conjugate groups was attached at either the 3' or 5' terminus of the respective oligonucleotide by a phosphodiester linked 2'-deoxyadenosine nucleoside (cleavable moiety).

Table 39

Modified ASO targeting SRB-1

ASO	Sequence (5' to 3')	Motif	Conjugate	SEQ ID No.
ISIS 353382 (parent)	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	none	4886
ISIS 655861	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{eo}$**$A_{do'}$-GalNAc$_3-1_a$**	5/10/5	**$GalNAc_3$-1**	4887
ISIS 664078	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{eo}$**$A_{do'}$-GalNAc$_3-9_a$**	5/10/5	**$GalNAc_3$-9**	4887
ISIS 661161	**GalNAc$_3$-3$_a$-$_o$,A_{do}** $G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**$GalNAc_3$-3**	4888
ISIS 665001	**GalNAc$_3$-8$_a$-$_o$,A_{do}** $G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**$GalNAc_3$-8**	4888

Capital letters indicate the nucleobase for each nucleoside and ^{m}C indicates a 5-methyl cytosine. Subscripts: "e" indicates a 2'-MOE modified nucleoside; "d" indicates a β-D-2'-deoxyribonucleoside; "s" indicates a phosphorothioate internucleoside linkage (PS); "o" indicates a phosphodiester internucleoside linkage (PO); and "o'" indicates -O-P(=O)(OH)-. Conjugate groups are in bold.

The structure of $GalNAc_3$-1_a was shown previously in Example 9. The structure of $GalNAc_3$-9 was shown previously in Example 52. The structure of $GalNAc_3$-3 was shown previously in Example 39. The structure of $GalNAc_3$-8 was shown previously in Example 47.

Treatment

Six week old male Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously once at the dosage shown below with ISIS 353382, 655861, 664078, 661161, 665001 or with saline. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration to determine the liver SRB-1 mRNA levels using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. The results below are presented as the average percent of SRB-1 mRNA levels for each treatment group, normalized to the saline control.

As illustrated in Table 40, treatment with antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner. Indeed, the antisense oligonucleotides comprising the phosphodiester linked

GalNAc$_3$-1 and GalNAc$_3$-9 conjugates at the 3’ terminus (ISIS 655861 and ISIS 664078) and the GalNAc$_3$-3 and GalNAc$_3$-8 conjugates linked at the 5’ terminus (ISIS 661161 and ISIS 665001) showed substantial improvement in potency compared to the unconjugated antisense oligonucleotide (ISIS 353382). Furthermore, ISIS 664078, comprising a GalNAc$_3$-9 conjugate at the 3' terminus was essentially equipotent compared to ISIS 655861, which comprises a GalNAc$_3$-1 conjugate at the 3’ terminus. The 5' conjugated antisense oligonucleotides, ISIS 661161 and ISIS 665001, comprising a GalNAc$_3$-3 or GalNAc$_3$-9, respectively, had increased potency compared to the 3' conjugated antisense oligonucleotides (ISIS 655861 and ISIS 664078).

Table 40

ASOs containing GalNAc$_3$-1, 3, 8 or 9 targeting SRB-1

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA (% Saline)	Conjugate
Saline	n/a	100	
353382	3	88	none
	10	68	
	30	36	
655861	0.5	98	**GalNac$_3$ -1** (3')
	1.5	76	
	5	31	
	15	20	
664078	0.5	88	**GalNac$_3$-9** (3')
	1.5	85	
	5	46	
	15	20	
661161	0.5	92	**GalNac$_3$-3** (5')
	1.5	59	
	5	19	
	15	11	
665001	0.5	100	**GalNac$_3$-8** (5')
	1.5	73	
	5	29	
	15	13	

Liver transaminase levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in serum were measured relative to saline injected mice using standard protocols. Total bilirubin and BUN were also evaluated. The change in body weights was evaluated with no significant change from the saline group. ALTs, ASTs, total bilirubin and BUN values are shown in the table below.

Table 41

ISIS No.	Dosage mg/kg	ALT	AST	Total Bilirubin	BUN	Conjugate
Saline		24	59	0.1	37.52	
353382	3	21	66	0.2	34.65	none
	10	22	54	0.2	34.2	
	30	22	49	0.2	33.72	

655861	0.5	25	62	0.2	30.65	**GalNac$_3$-1** (3')
	1.5	23	48	0.2	30.97	
	5	28	49	0.1	32.92	
	15	40	97	0.1	31.62	
664078	0.5	40	74	0.1	35.3	**GalNac$_3$-9** (3')
	1.5	47	104	0.1	32.75	
	5	20	43	0.1	30.62	
	15	38	92	0.1	26.2	
661161	0.5	101	162	0.1	34.17	**GalNac$_3$-3** (5')
	1.5 g	42	100	0.1	33.37	
	5 g	23	99	0.1	34.97	
	15	53	83	0.1	34.8	
665001	0.5	28	54	0.1	31.32	**GalNac$_3$-8** (5')
	1.5	42	75	0.1	32.32	
	5	24	42	0.1	31.85	
	15	32	67	0.1	31.	

Example 56: Dose-dependent study of oligonucleotides comprising either a 3' or 5'-conjugate group (comparison of GalNAc$_3$-1, 2, 3, 5, 6, 7 and 10) targeting SRB-1 *in vivo*

The oligonucleotides listed below were tested in a dose-dependent study for antisense inhibition of SRB-1 in mice. Unconjugated ISIS 353382 was included as a standard. Each of the various GalNAc$_3$ conjugate groups was attached at the 5' terminus of the respective oligonucleotide by a phosphodiester linked 2'-deoxyadenosine nucleoside (cleavable moiety) except for ISIS 655861 which had the GalNAc$_3$ conjugate group attached at the 3' terminus.

Table 42
Modified ASO targeting SRB-1

ASO	Sequence (5' to 3')	Motif	**Conjugate**	SEQ ID No.
ISIS 353382 (parent)	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	no conjugate	4886
ISIS 655861	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{eo}\mathbf{A_{do'}\text{-}GalNAc_3\text{-}1_a}$	5/10/5	**GalNAc$_3$-1**	4887
ISIS 664507	$\mathbf{GalNAc_3\text{-}2_{a\text{-}o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-2**	4888
ISIS 661161	$\mathbf{GalNAc_3\text{-}3_{a\text{-}o'}A_{do}}$ $G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-3**	4888
ISIS 666224	$\mathbf{GalNAc_3\text{-}5_{a\text{-}o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-5**	4888
ISIS 666961	$\mathbf{GalNAc_3\text{-}6_{a\text{-}o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-6**	4888
ISIS 666981	$\mathbf{GalNAc_3\text{-}7_{a\text{-}o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-7**	4888
ISIS 666881	$\mathbf{GalNAc_3\text{-}10_{a\text{-}o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-10**	4888

Capital letters indicate the nucleobase for each nucleoside and ^{m}C indicates a 5-methyl cytosine. Subscripts: "e" indicates a 2'-MOE modified nucleoside; "d" indicates a β-D-2'-deoxyribonucleoside; "s" indicates a phosphorothioate internucleoside linkage (PS); "o" indicates a phosphodiester internucleoside linkage (PO); and "o'" indicates -O-P(=O)(OH)-. Conjugate groups are in bold.

The structure of GalNAc$_3$-1$_a$ was shown previously in Example 9. The structure of GalNAc$_3$-2$_a$ was shown previously in Example 37. The structure of GalNAc$_3$-3$_a$ was shown previously in Example 39. The structure of GalNAc$_3$-5$_a$ was shown previously in Example 49. The structure of GalNAc$_3$-6$_a$ was shown previously in Example 51. The structure of GalNAc$_3$-7$_a$ was shown previously in Example 48. The structure of GalNAc$_3$-10$_a$ was shown previously in Example 46.

Treatment

Six week old male Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously once at the dosage shown below with ISIS 353382, 655861, 664507, 661161, 666224, 666961, 666981, 666881 or with saline. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration to determine the liver SRB-1 mRNA levels using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. The results below are presented as the average percent of SRB-1 mRNA levels for each treatment group, normalized to the saline control.

As illustrated in Table 43, treatment with antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner. Indeed, the conjugated antisense oligonucleotides showed substantial improvement in potency compared to the unconjugated antisense oligonucleotide (ISIS 353382). The 5' conjugated antisense oligonucleotides showed a slight increase in potency compared to the 3' conjugated antisense oligonucleotide.

Table 43

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA (% Saline)	Conjugate
Saline	n/a	100.0	
353382	3	96.0	none
	10	73.1	
	30	36.1	
655861	0.5	99.4	**GalNac$_3$-1** (3')
	1.5	81.2	
	5	33.9	
	15	15.2	
664507	0.5	102.0	**GalNac$_3$-2** (5')
	1.5	73.2	
	5	31.3	
	15	10.8	
661161	0.5	90.7	**GalNac$_3$-3** (5')
	1.5	67.6	
	5	24.3	

	15	11.5	
666224	0.5	96.1	**GalNac$_3$-5** (5')
	1.5	61.6	
	5	25.6	
	15	11.7	
666961	0.5	85.5	**GalNAc$_3$-6** (5')
	1.5	56.3	
	5	34.2	
	15	13.1	
666981	0.5	84.7	**GalNAc$_3$-7** (5')
	1.5	59.9	
	5	24.9	
	15	8.5	
666881	0.5	100.0	**GalNAc$_3$-10** (5')
	1.5	65.8	
	5	26.0	
	15	13.0	

Liver transaminase levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in serum were measured relative to saline injected mice using standard protocols. Total bilirubin and BUN were also evaluated. The change in body weights was evaluated with no significant change from the saline group. ALTs, ASTs, total bilirubin and BUN values are shown in Table 44 below.

Table 44

ISIS No.	Dosage mg/kg	ALT	AST	Total Bilirubin	BUN	Conjugate
Saline		26	57	0.2	27	
353382	3	25	92	0.2	27	none
	10	23	40	0.2	25	
	30	29	54	0.1	28	
655861	0.5	25	71	0.2	34	**GalNac$_3$-1** (3')
	1.5	28	60	0.2	26	
	5	26	63	0.2	28	
	15	25	61	0.2	28	
664507	0.5	25	62	0.2	25	**GalNac$_3$-2** (5')
	1.5	24	49	0.2	26	
	5	21	50	0.2	26	
	15	59	84	0.1	22	
661161	0.5	20	42	0.2	29	**GalNac$_3$-3** (5')
	1.5 g	37	74	0.2	25	
	5 g	28	61	0.2	29	
	15	21	41	0.2	25	
666224	0.5	34	48	0.2	21	**GalNac$_3$-5** (5')
	1.5	23	46	0.2	26	
	5	24	47	0.2	23	
	15	32	49	0.1	26	
666961	0.5	17	63	0.2	26	**GalNAc$_3$-6** (5')
	1.5	23	68	0.2	26	
	5	25	66	0.2	26	
	15	29	107	0.2	28	

666981	0.5	24	48	0.2	26	**GalNAc$_3$-7** (5')
	1.5	30	55	0.2	24	
	5	46	74	0.1	24	
	15	29	58	0.1	26	
666881	0.5	20	65	0.2	27	**GalNAc$_3$-10** (5')
	1.5	23	59	0.2	24	
	5	45	70	0.2	26	
	15	21	57	0.2	24	

Example 57: Duration of action study of oligonucleotides comprising a 3'-conjugate group targeting ApoC III *in vivo*

Mice were injected once with the doses indicated below and monitored over the course of 42 days for ApoC-III and plasma triglycerides (Plasma TG) levels. The study was performed using 3 transgenic mice that express human APOC-III in each group.

Table 45
Modified ASO targeting ApoC III

ASO	Sequence (5' to 3')	Linkages	SEQ ID No.
ISIS 304801	$A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}$ $^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{e}$	PS	4878
ISIS 647535	$A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}$ $A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{eo}$**$A_{do'}$-GalNAc$_3-1_a$**	PS	4879
ISIS 647536	$A_{es}G_{eo}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}$ $A_{ds}G_{ds}{}^{m}C_{ds}T_{eo}T_{eo}T_{es}A_{es}T_{eo}$**$A_{do'}$-GalNAc$_3-1_a$**	PO/PS	4879

Capital letters indicate the nucleobase for each nucleoside and ^{m}C indicates a 5-methyl cytosine. Subscripts: "e" indicates a 2'-MOE modified nucleoside; "d" indicates a β-D-2'-deoxyribonucleoside; "s" indicates a phosphorothioate internucleoside linkage (PS); "o" indicates a phosphodiester internucleoside linkage (PO); and "o'" indicates -O-P(=O)(OH)-. Conjugate groups are in bold.

The structure of GalNAc$_3$-1$_a$ was shown previously in Example 9.

Table 46
ApoC III mRNA (% Saline on Day 1) and Plasma TG Levels (% Saline on Day 1)

ASO	Dose	Target	Day 3	Day 7	Day 14	Day 35	Day 42
Saline	0 mg/kg	ApoC-III	98	100	100	95	116
ISIS 304801	30 mg/kg	ApoC-III	28	30	41	65	74
ISIS 647535	10 mg/kg	ApoC-III	16	19	25	74	94
ISIS 647536	10 mg/kg	ApoC-III	18	16	17	35	51
Saline	0 mg/kg	Plasma TG	121	130	123	105	109
ISIS 304801	30 mg/kg	Plasma TG	34	37	50	69	69
ISIS 647535	10 mg/kg	Plasma TG	18	14	24	18	71
ISIS 647536	10 mg/kg	Plasma TG	21	19	15	32	35

As can be seen in the table above the duration of action increased with addition of the 3'-conjugate group compared to the unconjugated oligonucleotide. There was a further increase in the duration of action for the conjugated mixed PO/PS oligonucleotide 647536 as compared to the conjugated full PS oligonucleotide 647535.

Example 58: Dose-dependent study of oligonucleotides comprising a 3'-conjugate group (comparison of $GalNAc_3$-1 and $GalNAc_4$-11) targeting SRB-1 *in vivo*

The oligonucleotides listed below were tested in a dose-dependent study for antisense inhibition of SRB-1 in mice. Unconjugated ISIS 440762 was included as an unconjugated standard. Each of the conjugate groups were attached at the 3' terminus of the respective oligonucleotide by a phosphodiester linked 2'-deoxyadenosine nucleoside cleavable moiety.

The structure of $GalNAc_3$-1_a was shown previously in Example 9. The structure of $GalNAc_3$-11_a was shown previously in Example 50.

Treatment

Six week old male Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously once at the dosage shown below with ISIS 440762, 651900, 663748 or with saline. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration to determine the liver SRB-1 mRNA levels using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. The results below are presented as the average percent of SRB-1 mRNA levels for each treatment group, normalized to the saline control.

As illustrated in Table 47, treatment with antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner. The antisense oligonucleotides comprising the phosphodiester linked $GalNAc_3$-1 and $GalNAc_4$-11 conjugates at the 3' terminus (ISIS 651900 and ISIS 663748) showed substantial improvement in potency compared to the unconjugated antisense oligonucleotide (ISIS 440762). The two conjugated oligonucleotides, $GalNAc_3$-1 and $GalNAc_4$-11, were equipotent.

Table 47
Modified ASO targeting SRB-1

ASO	Sequence (5' to 3')	Dose mg/kg	% Saline control	SEQ ID No.
Saline			100	
ISIS 440762	$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{k}$	0.6	73.45	4880
		2	59.66	
		6	23.50	
ISIS 651900	$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{ko}\mathbf{A_{do'}\text{-}GalNAc_3\text{-}1_a}$	0.2	62.75	4881
		0.6	29.14	
		2	8.61	
		6	5.62	
ISIS 663748	$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{ko}\mathbf{A_{do'}\text{-}GalNAc_4\text{-}11_a}$	0.2	63.99	4881
		0.6	33.53	

		2	7.58	
		6	5.52	

Capital letters indicate the nucleobase for each nucleoside and ^{m}C indicates a 5-methyl cytosine. Subscripts: "e" indicates a 2'-MOE modified nucleoside; "k" indicates 6'-*(S)*-CH_3 bicyclic nucleoside; "d" indicates a β-D-2'-deoxyribonucleoside; "s" indicates a phosphorothioate internucleoside linkage (PS); "o" indicates a phosphodiester internucleoside linkage (PO); and "o'" indicates -O-P(=O)(OH)-. Conjugate groups are in bold.

Liver transaminase levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in serum were measured relative to saline injected mice using standard protocols. Total bilirubin and BUN were also evaluated. The change in body weights was evaluated with no significant change from the saline group. ALTs, ASTs, total bilirubin and BUN values are shown in Table 48 below.

Table 48

ISIS No.	Dosage mg/kg	ALT	AST	Total Bilirubin	BUN	Conjugate
Saline		30	76	0.2	40	
440762	0.60	32	70	0.1	35	none
	2	26	57	0.1	35	
	6	31	48	0.1	39	
651900	0.2	32	115	0.2	39	**GalNac$_3$-1** (3')
	0.6	33	61	0.1	35	
	2	30	50	0.1	37	
	6	34	52	0.1	36	
663748	0.2	28	56	0.2	36	**GalNac$_4$-11** (3')
	0.6	34	60	0.1	35	
	2	44	62	0.1	36	
	6	38	71	0.1	33	

Example 59: Effects of GalNAc$_3$-1 conjugated ASOs targeting FXI *in vivo*

The oligonucleotides listed below were tested in a multiple dose study for antisense inhibition of FXI in mice. ISIS 404071 was included as an unconjugated standard. Each of the conjugate groups was attached at the 3' terminus of the respective oligonucleotide by a phosphodiester linked 2'-deoxyadenosine nucleoside cleavable moiety.

Table 49

Modified ASOs targeting FXI

ASO	Sequence (5' to 3')	Linkages	SEQ ID No.
ISIS 404071	$T_{es}G_{es}G_{es}T_{es}A_{es}A_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}{}^{m}C_{ds}$ $T_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{es}G_{es}A_{es}G_{es}G_{e}$	PS	4889
ISIS 656172	$T_{es}G_{es}G_{es}T_{es}A_{es}A_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}{}^{m}C_{ds}$ $T_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{es}G_{es}A_{es}G_{es}G_{eo}$**$A_{do'}$-GalNAc$_3-1_a$**	PS	4890
ISIS 656173	$T_{es}G_{eo}G_{eo}T_{eo}A_{eo}A_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}{}^{m}C_{ds}$ $T_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{eo}G_{eo}A_{es}G_{es}G_{eo}$**$A_{do'}$-GalNAc$_3-1_a$**	PO/PS	4890

Capital letters indicate the nucleobase for each nucleoside and ^{m}C indicates a 5-methyl cytosine. Subscripts: "e" indicates a 2'-MOE modified nucleoside; "d" indicates a β-D-2'-deoxyribonucleoside; "s" indicates a phosphorothioate internucleoside linkage (PS); "o" indicates a phosphodiester internucleoside linkage (PO); and "o'" indicates -O-P(=O)(OH)-. Conjugate groups are in bold.

The structure of GalNAc$_3$-1$_a$ was shown previously in Example 9.

Treatment

Six week old male Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously twice a week for 3 weeks at the dosage shown below with ISIS 404071, 656172, 656173 or with PBS treated control. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration to determine the liver FXI mRNA levels using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. Plasma FXI protein levels were also measured using ELISA. FXI mRNA levels were determined relative to total RNA (using RIBOGREEN®), prior to normalization to PBS-treated control. The results below are presented as the average percent of FXI mRNA levels for each treatment group. The data was normalized to PBS-treated control and is denoted as "% PBS". The ED$_{50}$s were measured using similar methods as described previously and are presented below.

Table 50
Factor XI mRNA (% Saline)

ASO	Dose mg/kg	% Control	Conjugate	Linkages
Saline		100	none	
ISIS 404071	3	92	none	PS
	10	40		
	30	15		
ISIS 656172	0.7	74	**GalNAc$_3$-1**	PS
	2	33		
	6	9		
ISIS 656173	0.7	49	**GalNAc$_3$-1**	PO/PS
	2	22		
	6	1		

As illustrated in Table 50, treatment with antisense oligonucleotides lowered FXI mRNA levels in a dose-dependent manner. The oligonucleotides comprising a 3'-GalNAc$_3$-1 conjugate group showed substantial improvement in potency compared to the unconjugated antisense oligonucleotide (ISIS 404071). Between the two conjugated oligonucleotides an improvement in potency was further provided by substituting some of the PS linkages with PO (ISIS 656173).

As illustrated in Table 50a, treatment with antisense oligonucleotides lowered FXI protein levels in a dose-dependent manner. The oligonucleotides comprising a 3'-GalNAc$_3$-1 conjugate group showed

substantial improvement in potency compared to the unconjugated antisense oligonucleotide (ISIS 404071). Between the two conjugated oligonucleotides an improvement in potency was further provided by substituting some of the PS linkages with PO (ISIS 656173).

Table 50a
Factor XI protein (% Saline)

ASO	Dose mg/kg	Protein (% Control)	Conjugate	Linkages
Saline		100	none	
ISIS 404071	3	127	none	PS
	10	32		
	30	3		
ISIS 656172	0.7	70	**GalNAc$_3$-1**	PS
	2	23		
	6	1		
ISIS 656173	0.7	45	**GalNAc$_3$-1**	PO/PS
	2	6		
	6	0		

Liver transaminase levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in serum were measured relative to saline injected mice using standard protocols. Total bilirubin, total albumin, CRE and BUN were also evaluated. The change in body weights was evaluated with no significant change from the saline group. ALTs, ASTs, total bilirubin and BUN values are shown in the table below.

Table 51

ISIS No.	Dosage mg/kg	ALT	AST	Total Albumin	Total Bilirubin	CRE	BUN	Conjugate
Saline		71.8	84.0	3.1	0.2	0.2	22.9	
404071	3	152.8	176.0	3.1	0.3	0.2	23.0	none
	10	73.3	121.5	3.0	0.2	0.2	21.4	
	30	82.5	92.3	3.0	0.2	0.2	23.0	
656172	0.7	62.5	111.5	3.1	0.2	0.2	23.8	**GalNac$_3$-1** (3')
	2	33.0	51.8	2.9	0.2	0.2	22.0	
	6	65.0	71.5	3.2	0.2	0.2	23.9	
656173	0.7	54.8	90.5	3.0	0.2	0.2	24.9	**GalNac$_3$-1** (3')
	2	85.8	71.5	3.2	0.2	0.2	21.0	
	6	114.0	101.8	3.3	0.2	0.2	22.7	

Example 60: Effects of conjugated ASOs targeting SRB-1 *in vitro*

The oligonucleotides listed below were tested in a multiple dose study for antisense inhibition of SRB-1 in primary mouse hepatocytes. ISIS 353382 was included as an unconjugated standard. Each of the conjugate groups were attached at the 3' or 5' terminus of the respective oligonucleotide by a phosphodiester linked 2'-deoxyadenosine nucleoside cleavable moiety.

Table 52
Modified ASO targeting SRB-1

ASO	Sequence (5' to 3')	Motif	Conjugate	SEQ ID No.
ISIS 353382	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	none	4886
ISIS 655861	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{eo}$**$A_{do'}$-GalNAc$_3-1_a$**	5/10/5	**GalNAc$_3$-1**	4887
ISIS 655862	$G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T_{eo}$**$A_{do'}$-GalNAc$_3-1_a$**	5/10/5	**GalNAc$_3$-1**	4887
ISIS 661161	**GalNAc$_3$-3$_{a-o}$,A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-3**	4888
ISIS 665001	**GalNAc$_3$-8$_{a-o}$,A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-8**	4888
ISIS 664078	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{eo}$**$A_{do'}$-GalNAc$_3-9_a$**	5/10/5	**GalNAc$_3$-9**	4887
ISIS 666961	**GalNAc$_3$-6$_{a-o}$,A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-6**	4888
ISIS 664507	**GalNAc$_3$-2$_{a-o}$,A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-2**	4888
ISIS 666881	**GalNAc$_3$-10$_{a-o}$,A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-10**	4888
ISIS 666224	**GalNAc$_3$-5$_{a-o}$,A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-5**	4888
ISIS 666981	**GalNAc$_3$-7$_{a-o}$,A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	5/10/5	**GalNAc$_3$-7**	4888

Capital letters indicate the nucleobase for each nucleoside and ^{m}C indicates a 5-methyl cytosine. Subscripts: "e" indicates a 2'-MOE modified nucleoside; "d" indicates a β-D-2'-deoxyribonucleoside; "s" indicates a phosphorothioate internucleoside linkage (PS); "o" indicates a phosphodiester internucleoside linkage (PO); and "o'" indicates -O-P(=O)(OH)-. Conjugate groups are in bold.

The structure of GalNAc$_3$-1$_a$ was shown previously in Example 9. The structure of GalNAc$_3$-3a was shown previously in Example 39. The structure of GalNAc$_3$-8a was shown previously in Example 47. The structure of GalNAc$_3$-9a was shown previously in Example 52. The structure of GalNAc$_3$-6a was shown previously in Example 51. The structure of GalNAc$_3$-2a was shown previously in Example 37. The structure of GalNAc$_3$-10a was shown previously in Example 46. The structure of GalNAc$_3$-5a was shown previously in Example 49. The structure of GalNAc$_3$-7a was shown previously in Example 48.

Treatment

The oligonucleotides listed above were tested *in vitro* in primary mouse hepatocyte cells plated at a density of 25,000 cells per well and treated with 0.03, 0.08, 0.24, 0.74, 2.22, 6.67 or 20 nM modified oligonucleotide. After a treatment period of approximately 16 hours, RNA was isolated from the cells and mRNA levels were measured by quantitative real-time PCR and the SRB-1 mRNA levels were adjusted according to total RNA content, as measured by RIBOGREEN®.

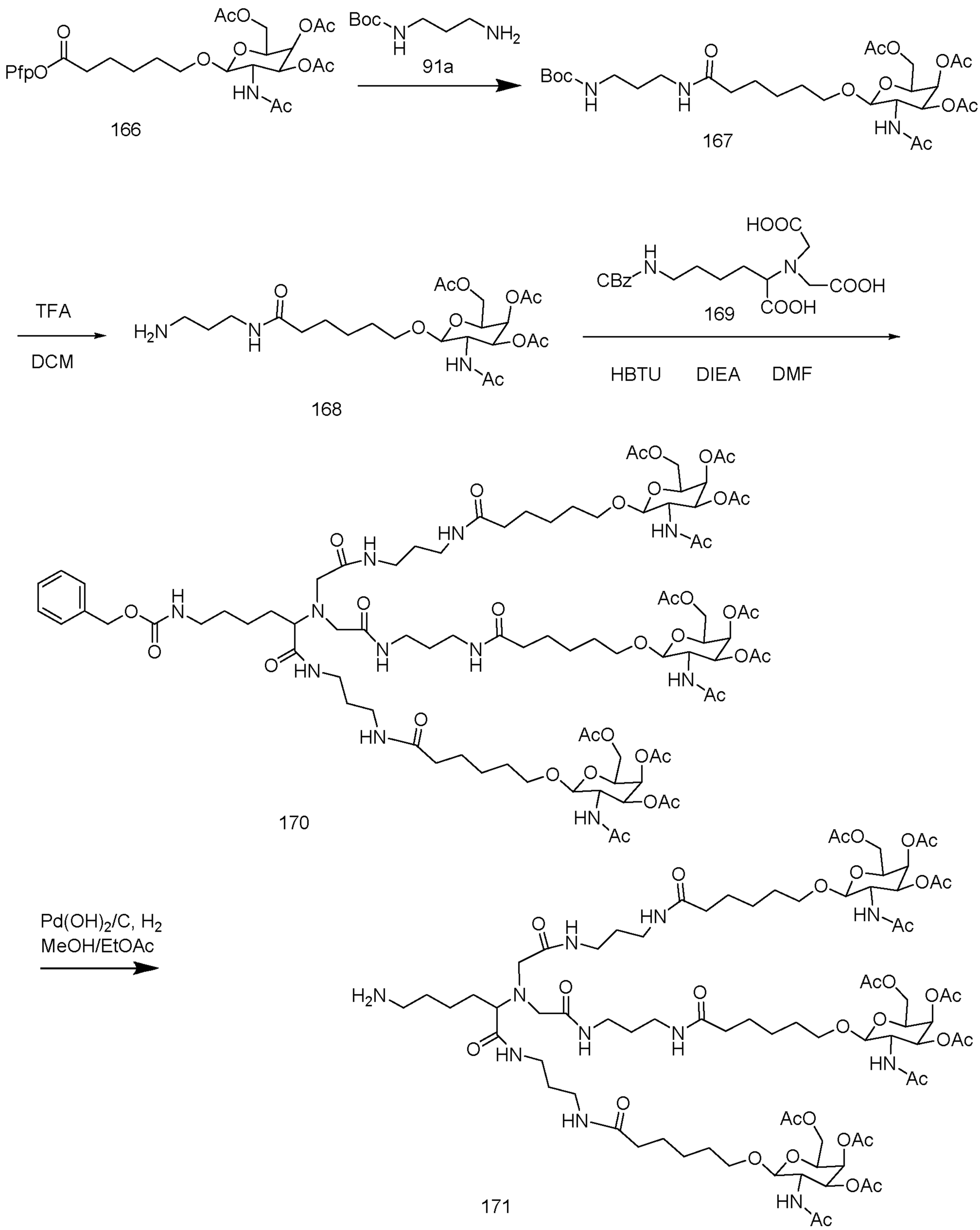
The IC_{50} was calculated using standard methods and the results are presented in Table 53. The results show that, under free uptake conditions in which no reagents or electroporation techniques are used to artificially promote entry of the oligonucleotides into cells, the oligonucleotides comprising a GalNAc conjugate were significantly more potent in hepatocytes than the parent oligonucleotide (ISIS 353382) that does not comprise a GalNAc conjugate.

Table 53

ASO	IC_{50} (nM)	Internucleoside linkages	Conjugate	SEQ ID No.
ISIS 353382	190[a]	PS	none	4886
ISIS 655861	11[a]	PS	**$GalNAc_3$-1**	4887
ISIS 655862	3	PO/PS	**$GalNAc_3$-1**	4887
ISIS 661161	15[a]	PS	**$GalNAc_3$-3**	4888
ISIS 665001	20	PS	**$GalNAc_3$-8**	4888
ISIS 664078	55	PS	**$GalNAc_3$-9**	4887
ISIS 666961	22[a]	PS	**$GalNAc_3$-6**	4888
ISIS 664507	30	PS	**$GalNAc_3$-2**	4888
ISIS 666881	30	PS	**$GalNAc_3$-10**	4888
ISIS 666224	30[a]	PS	**$GalNAc_3$-5**	4888
ISIS 666981	40	PS	**$GalNAc_3$-7**	4888

[a]Average of multiple runs.

Example 61: Preparation of oligomeric compound 175 comprising $GalNAc_3$-12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PfpO
O
OAc
HN
Ac
166
Boc
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H
NH2
91a
Boc
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N
H
O
AcO
OAc
O
OAc
HN
Ac
167
TFA
DCM
H2N
N
H
O
AcO
OAc
O
OAc
HN
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168
HOOC
CBz
N
H
N
COOH
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HBTU
DIEA
DMF
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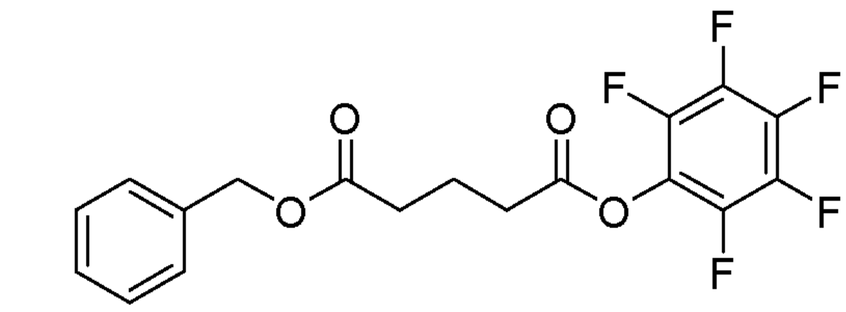
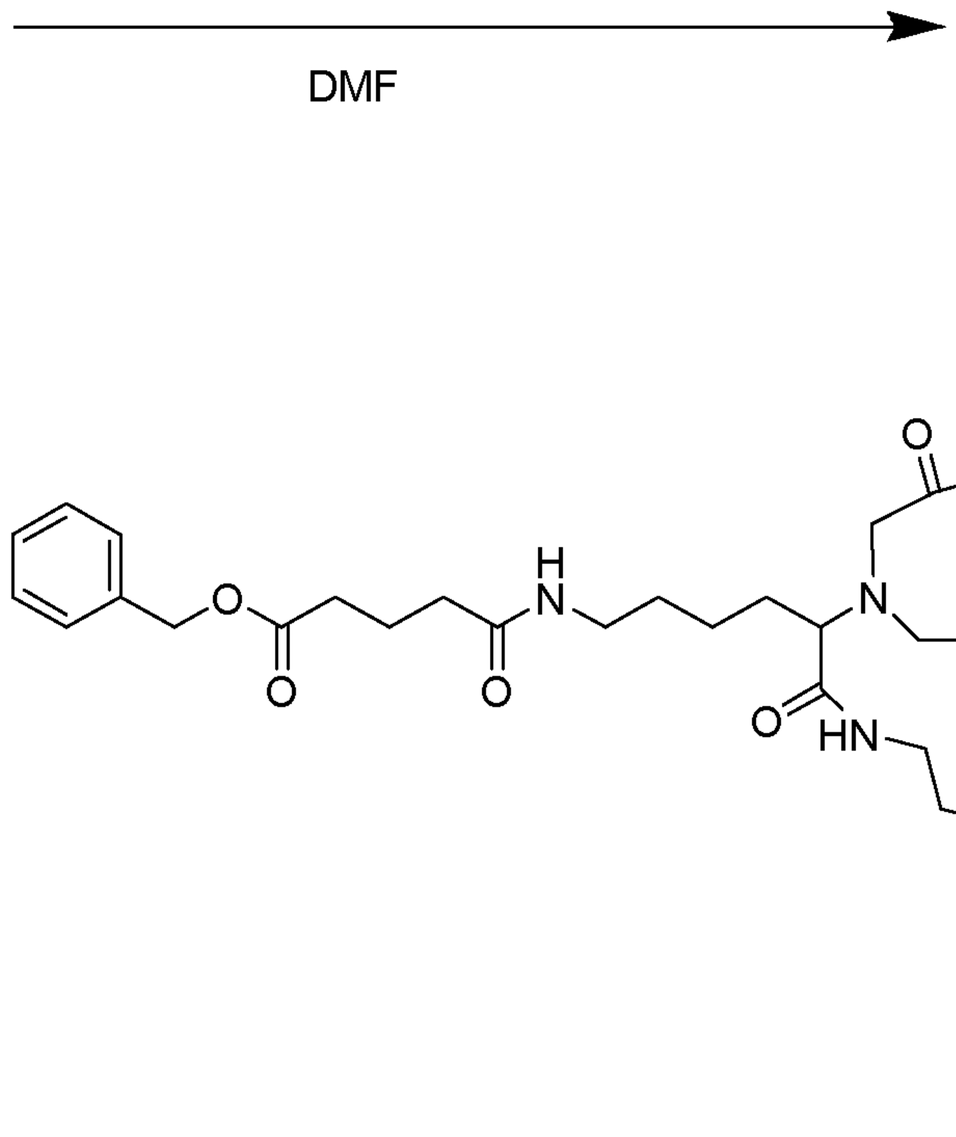
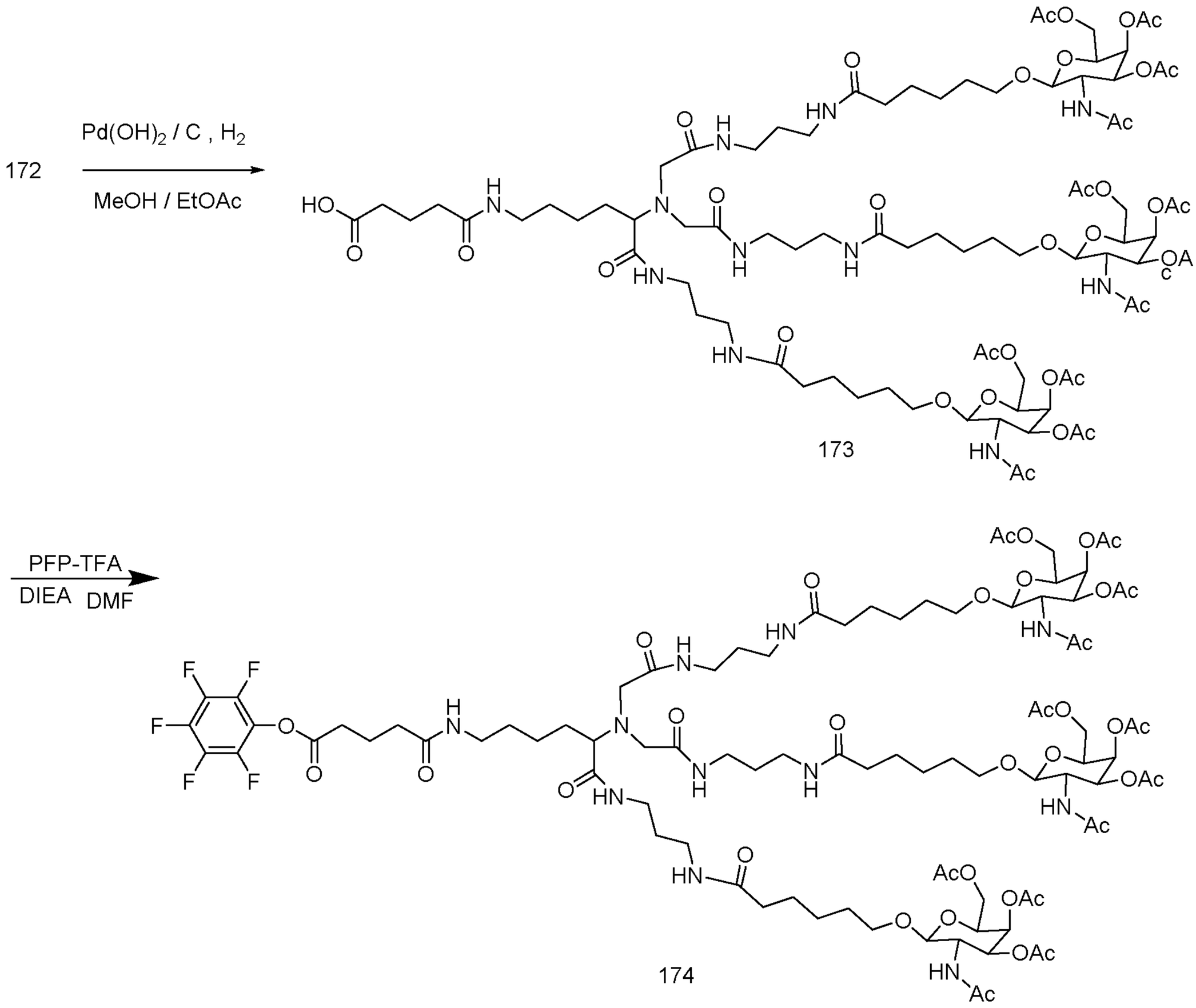
Pd(OH)2/C, H2
MeOH/EtOAc
H2N
171



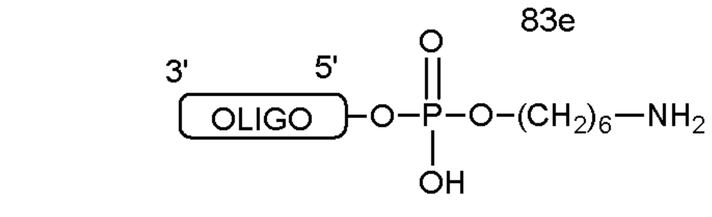
benzyl (perfluorophenyl) glutarate



DMF



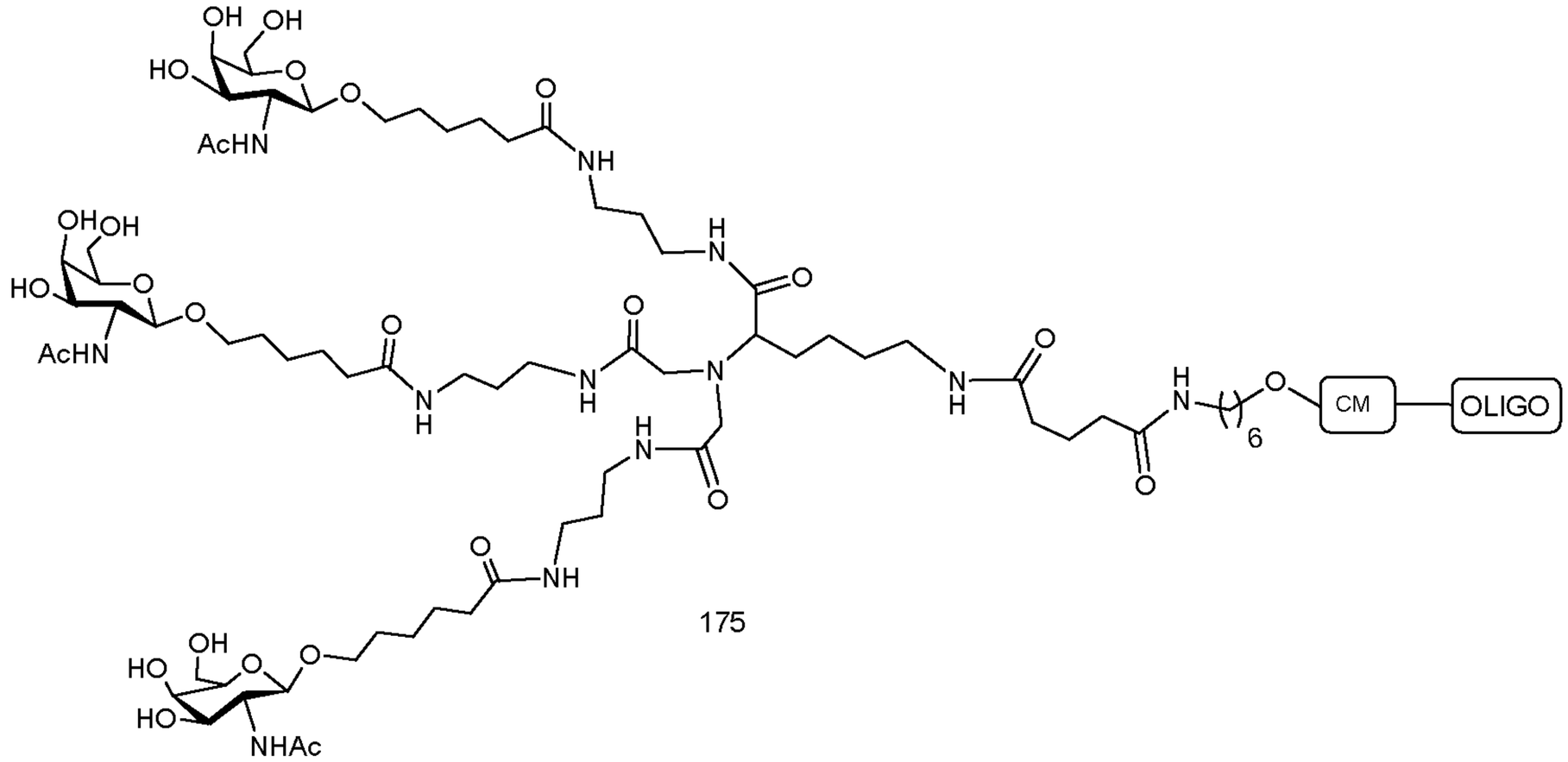
172 173 174



174

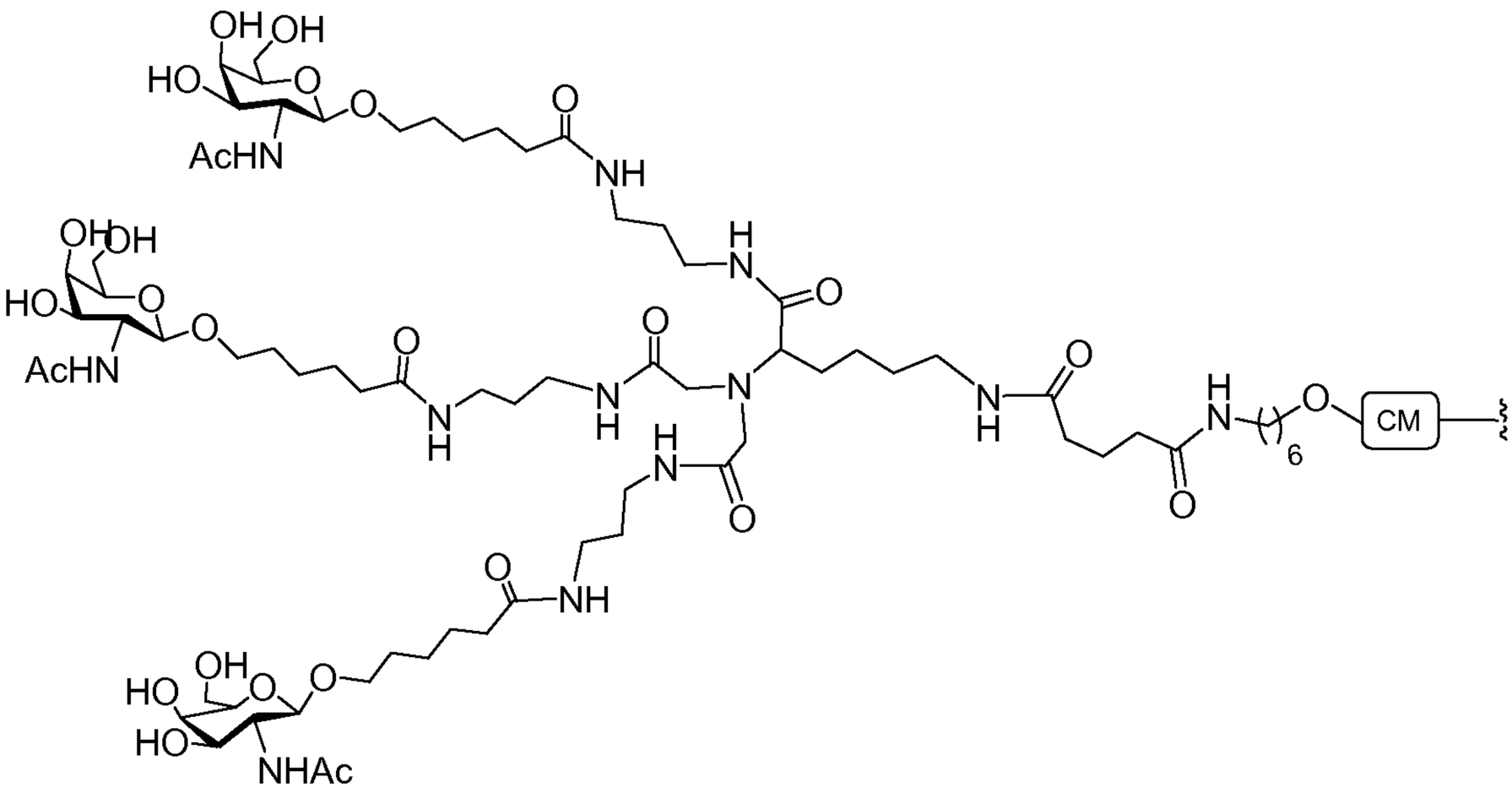
1. Borate buffer, DMSO, pH 8.5, rt

2. aq. ammonia, rt



175

Compound 169 is commercially available. Compound 172 was prepared by addition of benzyl (perfluorophenyl) glutarate to compound 171. The benzyl (perfluorophenyl) glutarate was prepared by adding PFP-TFA and DIEA to 5-(benzyloxy)-5-oxopentanoic acid in DMF. Oligomeric compound 175, comprising a $GalNAc_3$-12 conjugate group, was prepared from compound 174 using the general procedures illustrated in Example 46. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-12 ($GalNAc_3$-12_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In a certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-. The structure of $GalNAc_3$-12 ($GalNAc_3$-12_a-CM-) is shown below: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H
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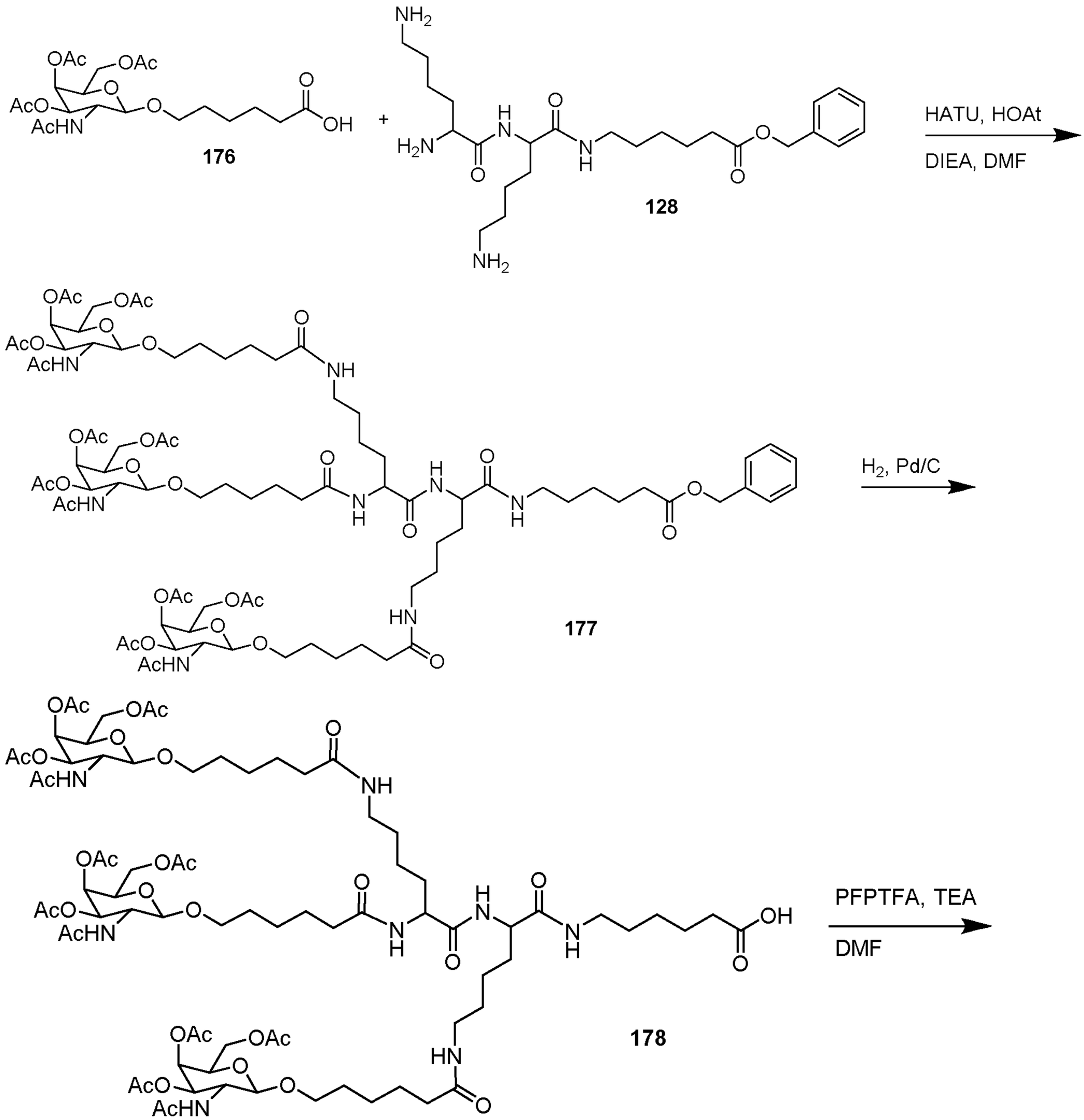
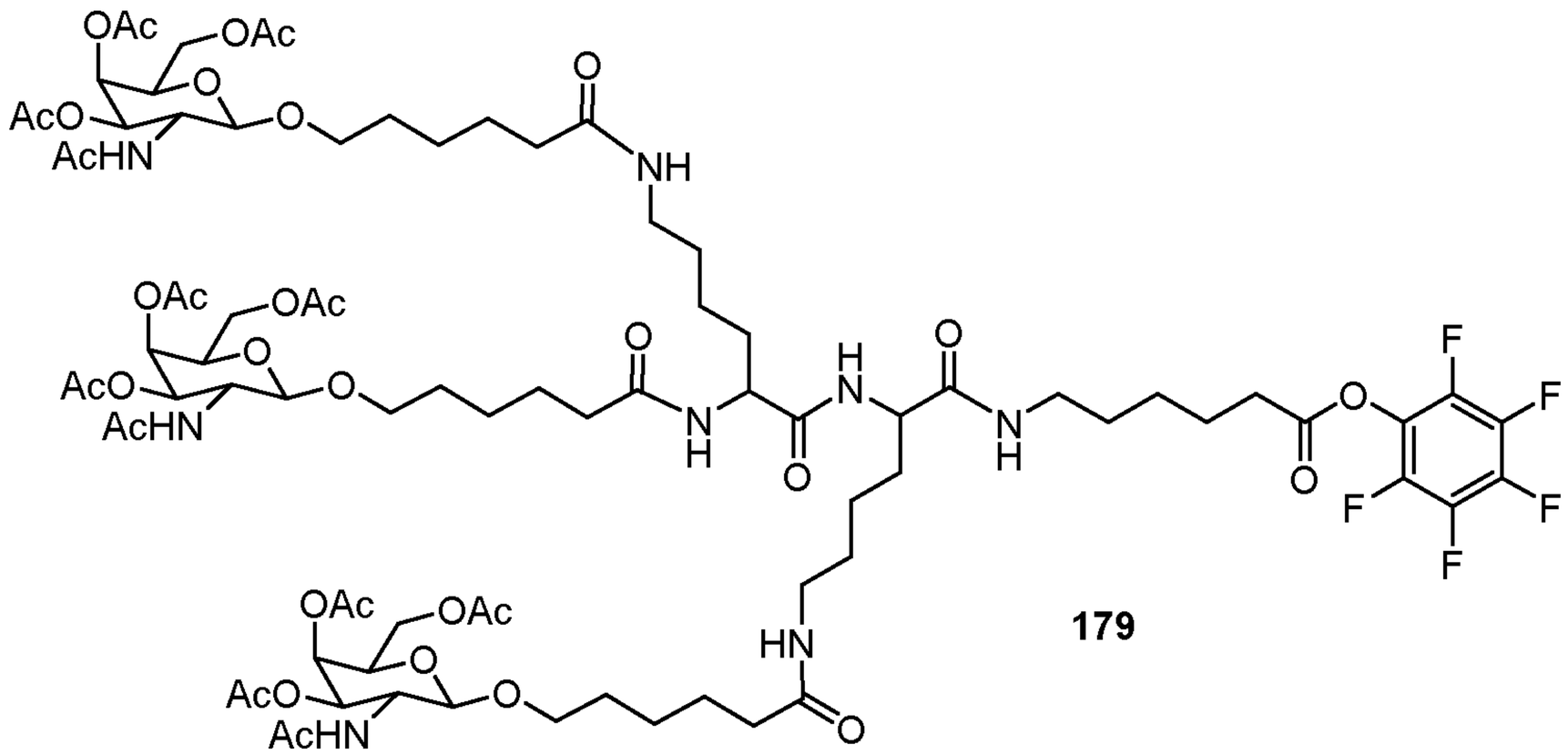
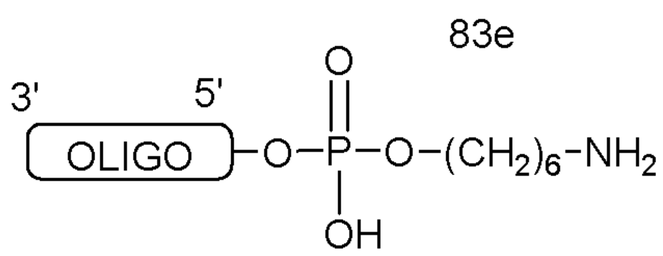
N
H
O
N
H
O
O
NH
6
O
CM
O
NH
OH
HO
O
O
HO
NHAc

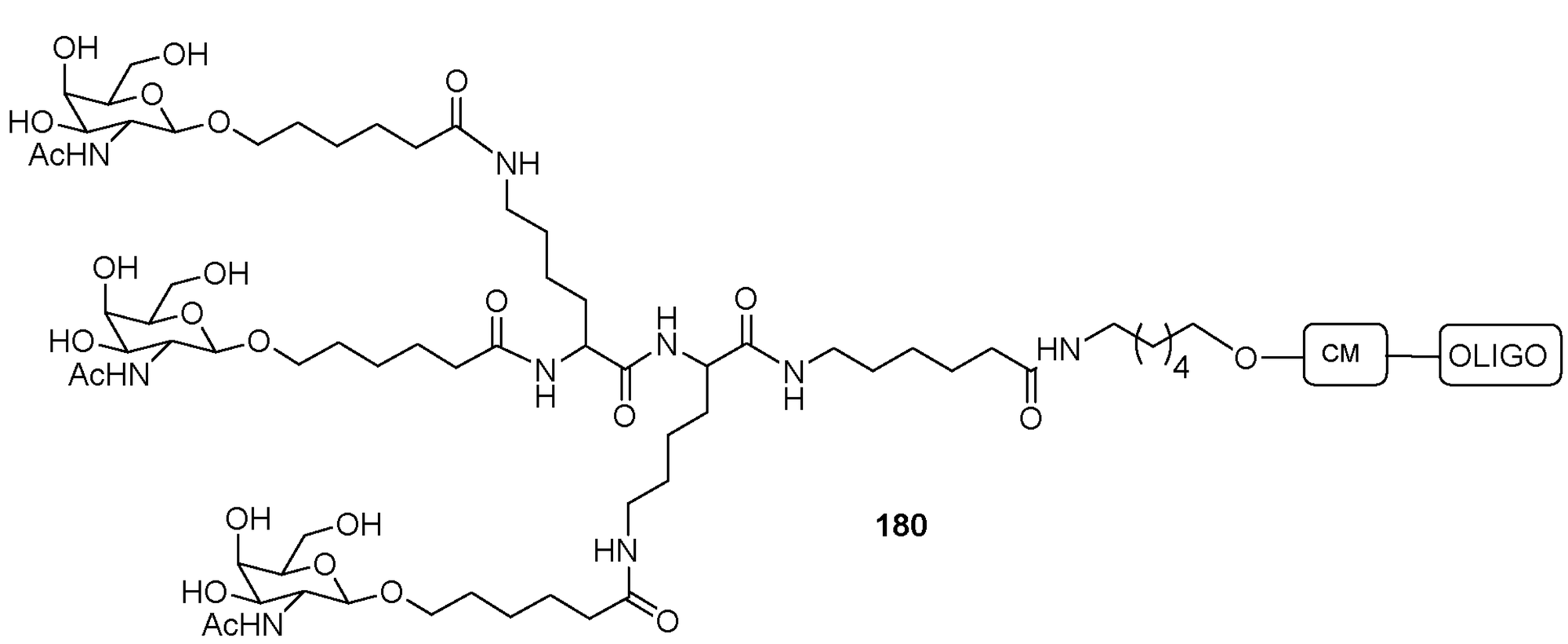
Example 62: Preparation of oligomeric compound 180 comprising GalNAc$_3$-13





179



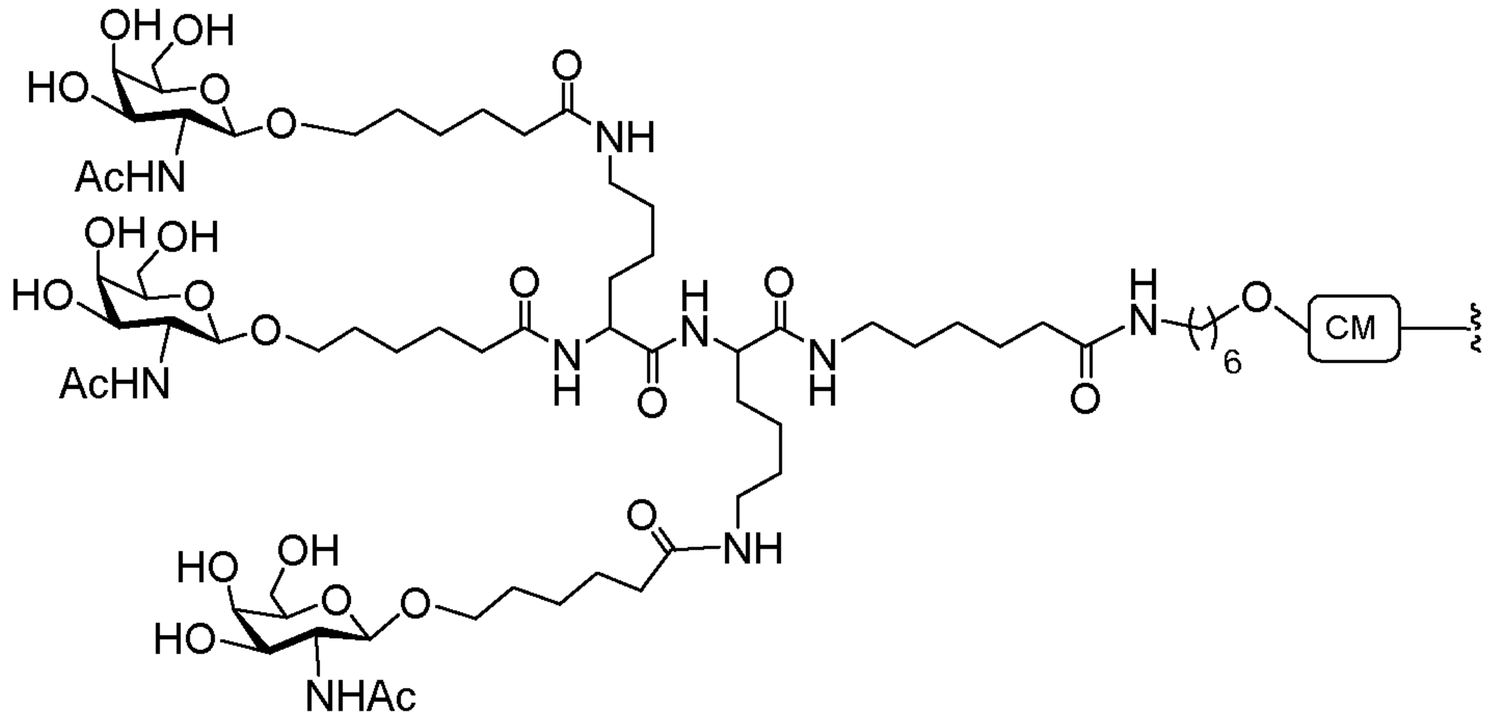


180

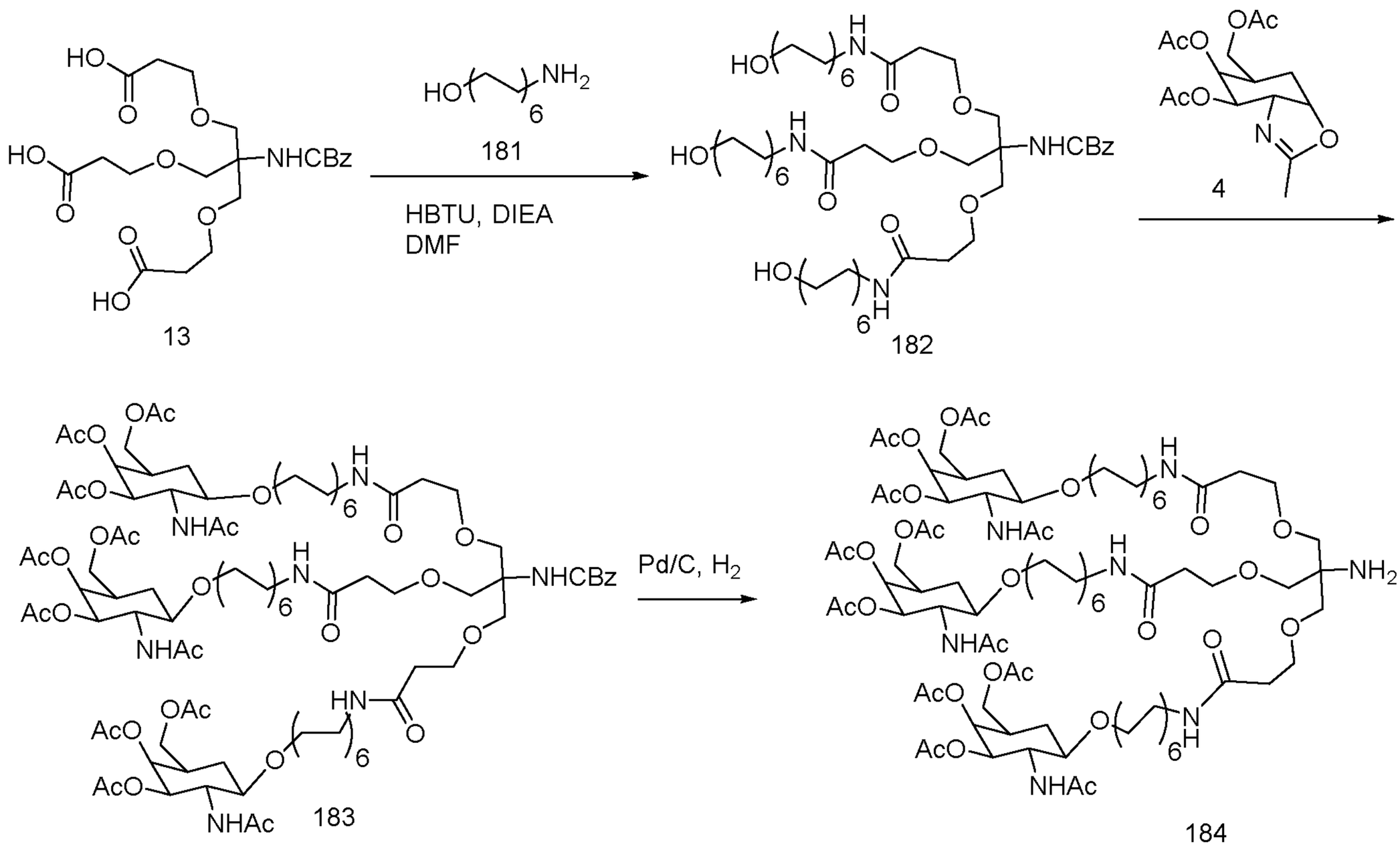
5

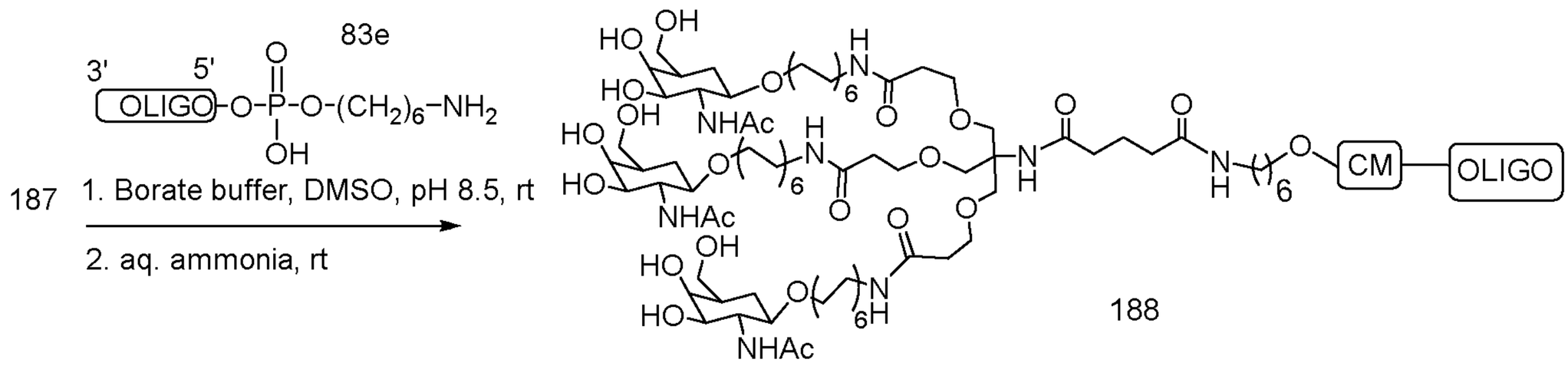
Compound 176 was prepared using the general procedure shown in Example 2. Oligomeric compound 180, comprising a $GalNAc_3$-13 conjugate group, was prepared from compound 177 using the general procedures illustrated in Example 49. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-13 ($GalNAc_3$-13_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In a

certainembodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-. The structure of $GalNAc_3$-13 ($GalNAc_3$-13_a-CM-) is shown below:

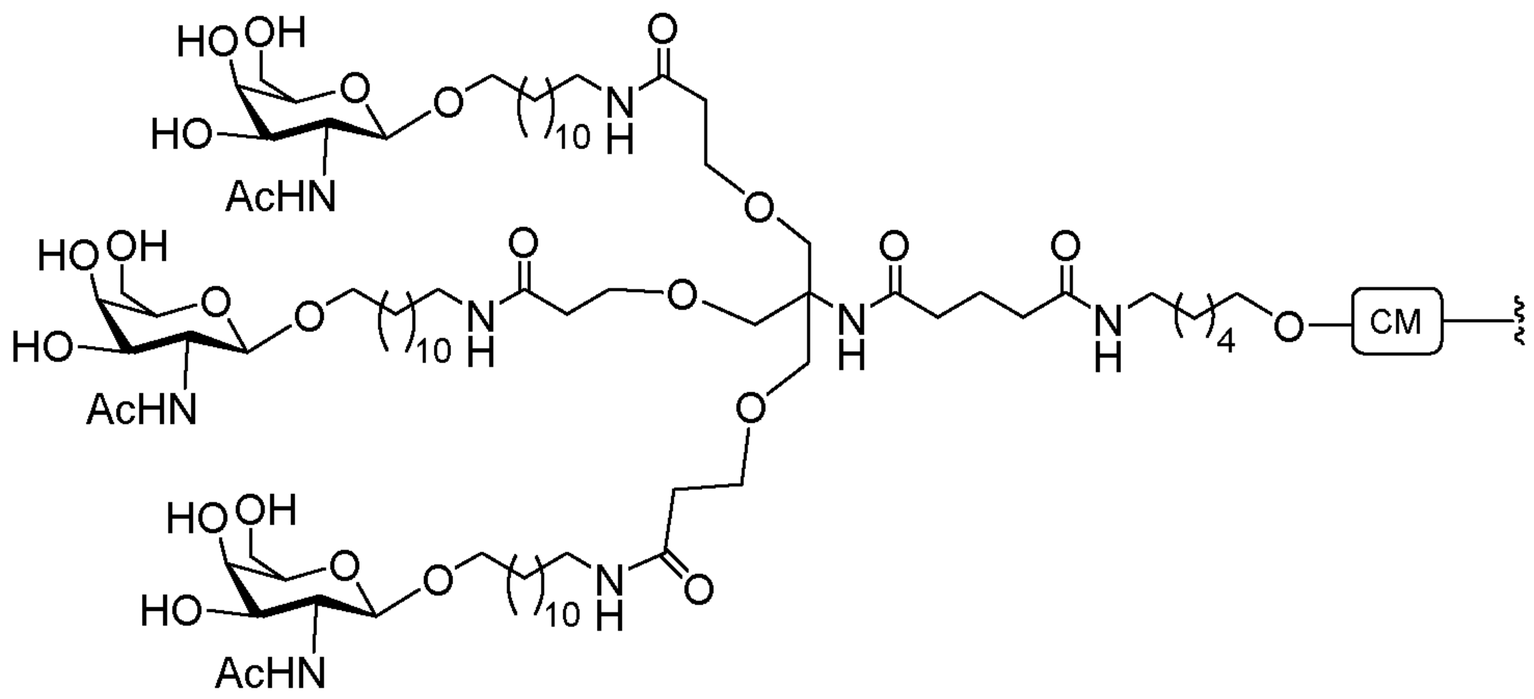


Example 63: Preparation of oligomeric compound 188 comprising GalNAc$_3$-14

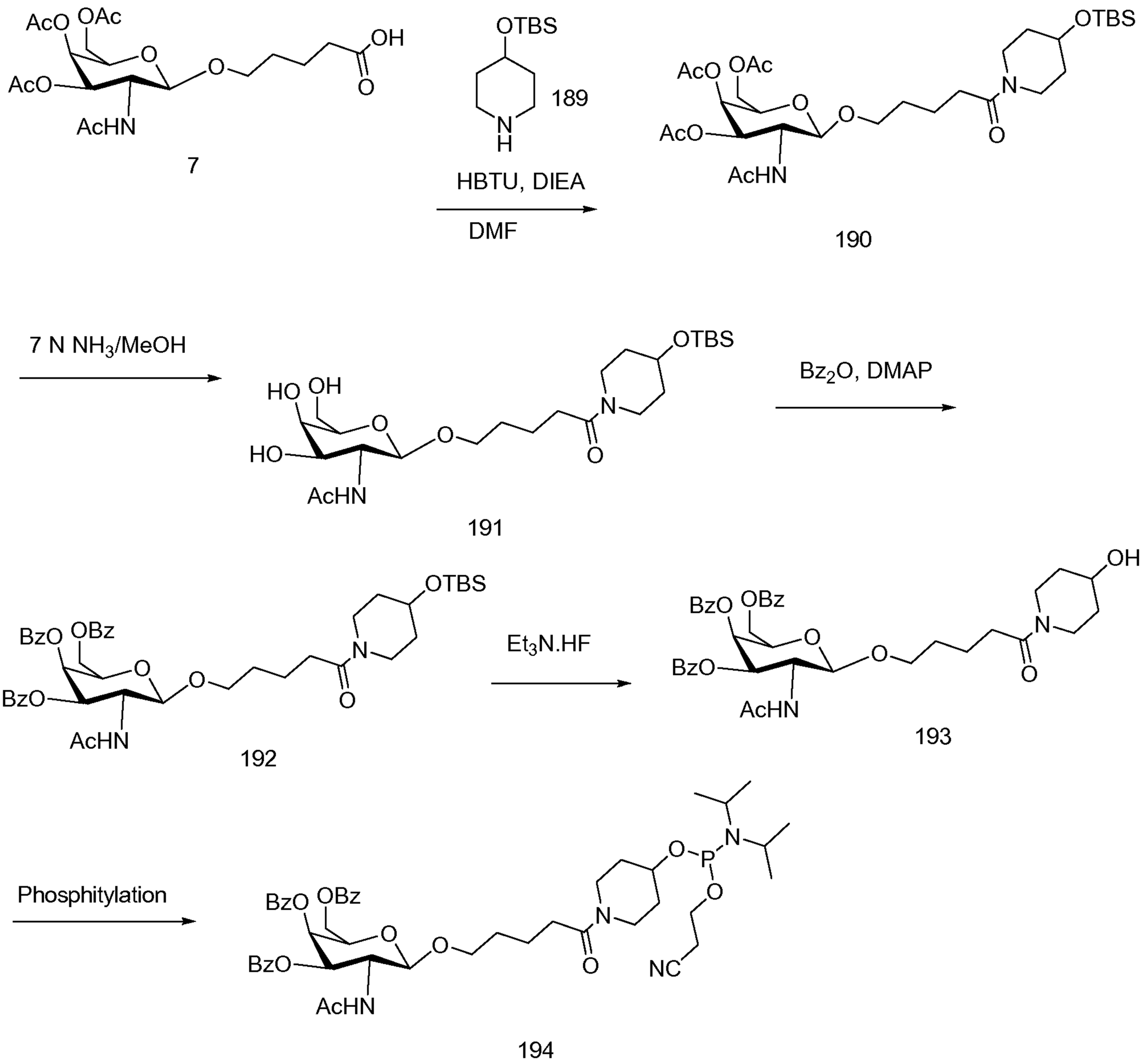


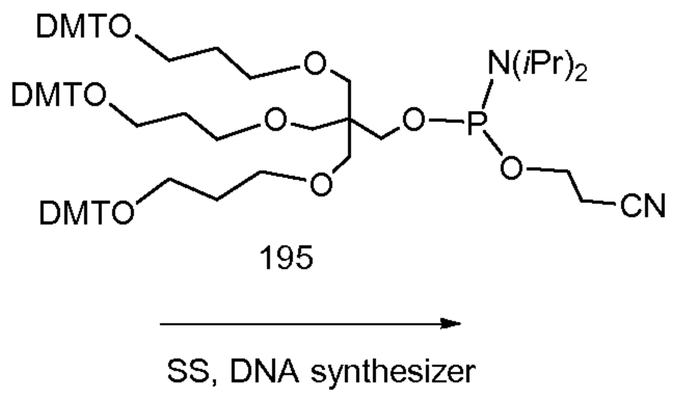


Compounds 181 and 185 are commercially available. Oligomeric compound 188, comprising a $GalNAc_3$-14 conjugate group, was prepared from compound 187 using the general procedures illustrated in Example 46. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-14 ($GalNAc_3$-14_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-. The structure of $GalNAc_3$-14 ($GalNAc_3$-14_a-CM-) is shown below:



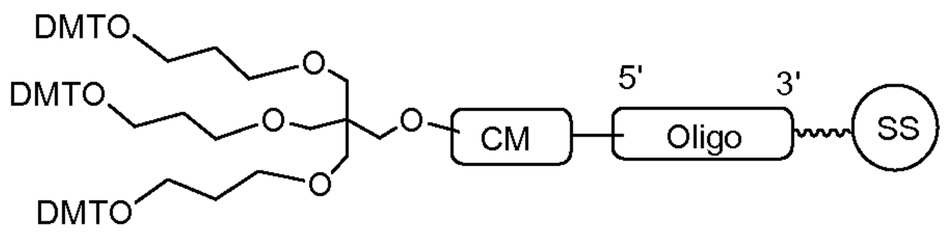
Example 64: Preparation of oligomeric compound 197 comprising $GalNAc_3$-15


AcO
OAc
AcO
AcHN
O
O
OH
O
7
OTBS
N
H
189
HBTU, DIEA
DMF
OTBS
N
O
190
7 N NH3/MeOH
HO
OH
HO
AcHN
191
Bz2O, DMAP
BzO
OBz
BzO
AcHN
192
Et3N.HF
OH
193
Phosphitylation
P
NC



195

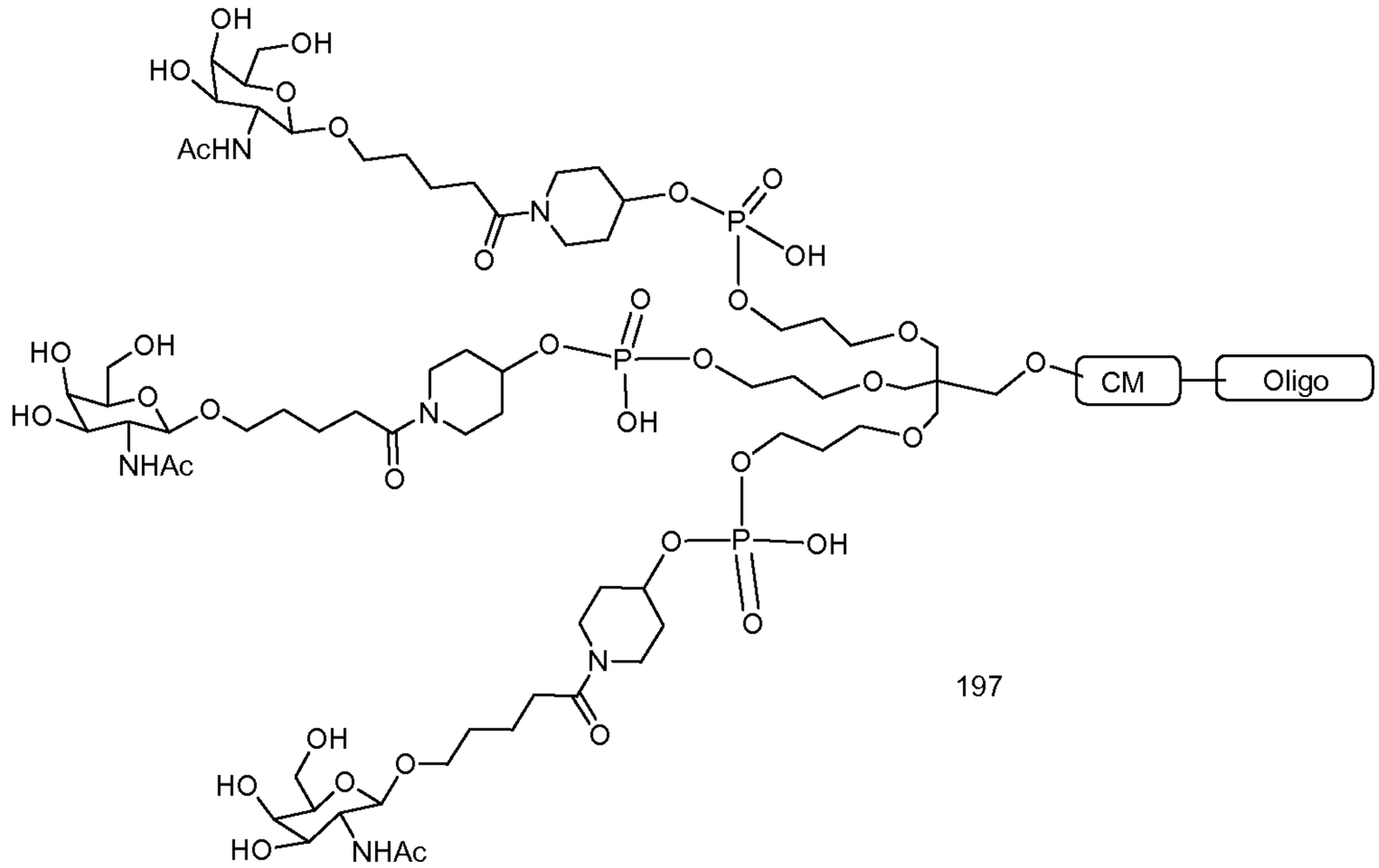
SS, DNA synthesizer



196

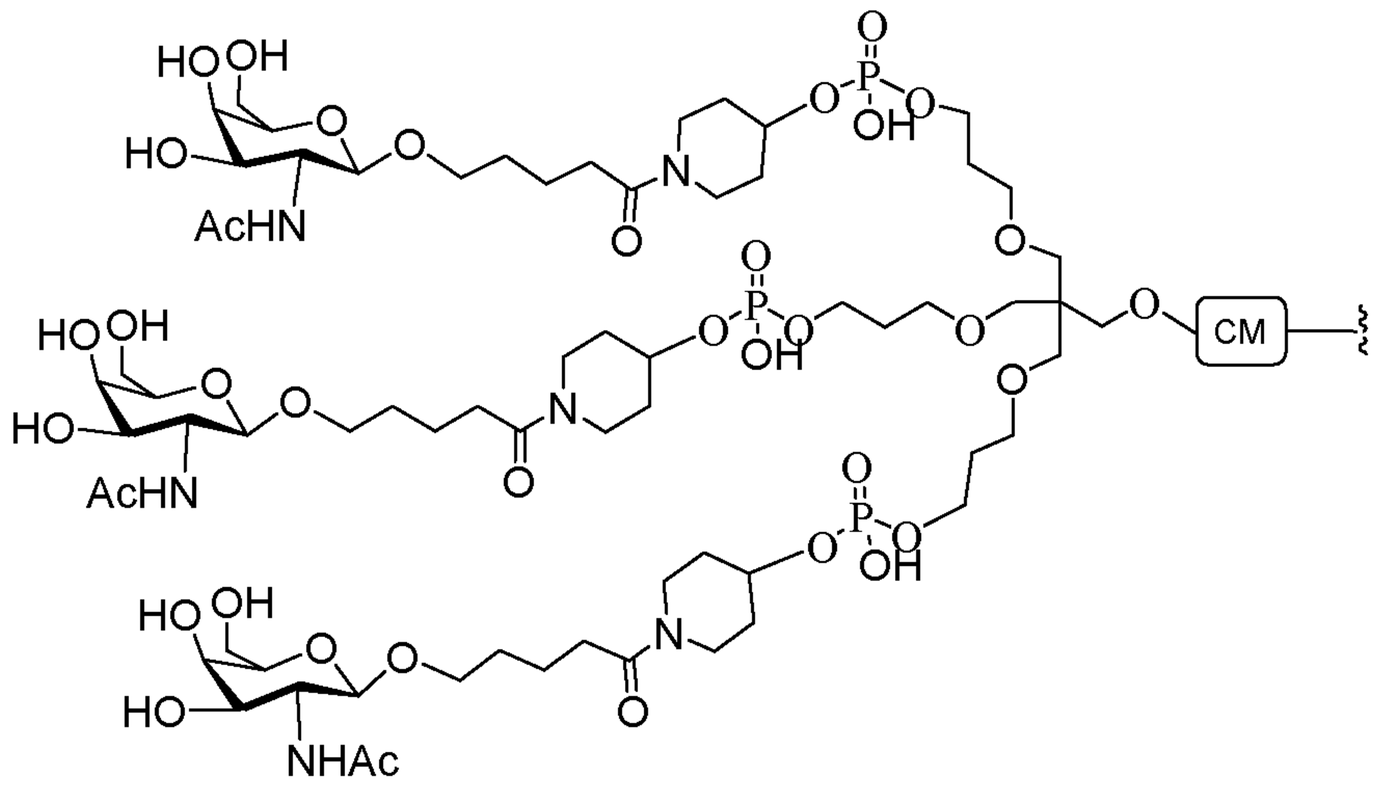
1. 194, DNA synthesizer

2. Aq NH_3 55 °C, 18 h



197

Compound 189 is commercially available. Compound 195 was prepared using the general procedure shown in Example 31. Oligomeric compound 197, comprising a $GalNAc_3$-15 conjugate group, was prepared from compounds 194 and 195 using standard oligonucleotide synthesis procedures. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-15 ($GalNAc_3$-15_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-. The structure of $GalNAc_3$-15 ($GalNAc_3$-15_a-CM-) is shown below:



Example 65: Dose-dependent study of oligonucleotides comprising a 5'-conjugate group (comparison of $GalNAc_3$-3, 12, 13, 14, and 15) targeting SRB-1 *in vivo*

The oligonucleotides listed below were tested in a dose-dependent study for antisense inhibition of SRB-1 in mice. Unconjugated ISIS 353382 was included as a standard. Each of the $GalNAc_3$ conjugate groups was attached at the 5' terminus of the respective oligonucleotide by a phosphodiester linked 2'-deoxyadenosine nucleoside (cleavable moiety).

Table 54
Modified ASOs targeting SRB-1

ISIS No.	Sequences (5' to 3')	Conjugate	SEQ ID No.
353382	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	none	4886
661161	**$GalNAc_3$-3_{a}-$_{o}$'A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-3	4888
671144	**$GalNAc_3$-12_{a}-$_{o}$'A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-12	4888
670061	**$GalNAc_3$-13_{a}-$_{o}$'A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-13	4888
671261	**$GalNAc_3$-14_{a}-$_{o}$'A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-14	4888
671262	**$GalNAc_3$-15_{a}-$_{o}$'A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-15	4888

Capital letters indicate the nucleobase for each nucleoside and ^{m}C indicates a 5-methyl cytosine. Subscripts: "e" indicates a 2'-MOE modified nucleoside; "d" indicates a β-D-2'-deoxyribonucleoside; "s" indicates a phosphorothioate internucleoside linkage (PS); "o" indicates a phosphodiester internucleoside linkage (PO); and "o'" indicates -O-P(=O)(OH)-. Conjugate groups are in bold.

The structure of $GalNAc_3$-3_a was shown previously in Example 39. The structure of $GalNAc_3$-12a was shown previously in Example 61. The structure of $GalNAc_3$-13a was shown previously in Example 62. The structure of $GalNAc_3$-14a was shown previously in Example 63. The structure of $GalNAc_3$-15a was shown previously in Example 64.

Treatment

Six to eight week old C57bl6 mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously once or twice at the dosage shown below with ISIS 353382, 661161, 671144, 670061, 671261, 671262, or with saline. Mice that were dosed twice received the second dose three days after the first dose. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration to determine the liver SRB-1 mRNA levels using real-time PCR and RIBOGREEN®

RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. The results below are presented as the average percent of SRB-1 mRNA levels for each treatment group, normalized to the saline control.

As illustrated in Table 55, treatment with antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner. No significant differences in target knockdown were observed between animals that received a single dose and animals that received two doses (see ISIS 353382 dosages 30 and 2 x 15 mg/kg; and ISIS 661161 dosages 5 and 2 x 2.5 mg/kg). The antisense oligonucleotides comprising the phosphodiester linked $GalNAc_3$-3, 12, 13, 14, and 15 conjugates showed substantial improvement in potency compared to the unconjugated antisense oligonucleotide (ISIS 335382).

Table 55
SRB-1 mRNA (% Saline)

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA (% Saline)	ED_{50} (mg/kg)	Conjugate
Saline	n/a	100.0	n/a	n/a
353382	3	85.0	22.4	none
	10	69.2		
	30	34.2		
	2 x 15	36.0		
661161	0.5	87.4	2.2	$GalNAc_3$-3
	1.5	59.0		
	5	25.6		
	2 x 2.5	27.5		
	15	17.4		
671144	0.5	101.2	3.4	$GalNAc_3$-12
	1.5	76.1		
	5	32.0		
	15	17.6		
670061	0.5	94.8	2.1	$GalNAc_3$-13
	1.5	57.8		
	5	20.7		
	15	13.3		
671261	0.5	110.7	4.1	$GalNAc_3$-14
	1.5	81.9		
	5	39.8		
	15	14.1		
671262	0.5	109.4	9.8	$GalNAc_3$-15
	1.5	99.5		
	5	69.2		
	15	36.1		

Liver transaminase levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in serum were measured relative to saline injected mice using standard protocols. Total bilirubin and BUN were also evaluated. The changes in body weights were evaluated with no significant differences from the saline group (data not shown). ALTs, ASTs, total bilirubin and BUN values are shown in Table 56 below.

Table 56

ISIS No.	Dosage (mg/kg)	ALT (U/L)	AST (U/L)	Total Bilirubin (mg/dL)	BUN (mg/dL)	Conjugate
Saline	n/a	28	60	0.1	39	n/a
353382	3	30	77	0.2	36	none
	10	25	78	0.2	36	
	30	28	62	0.2	35	
	2 x 15	22	59	0.2	33	
661161	0.5	39	72	0.2	34	$GalNAc_3$-3
	1.5	26	50	0.2	33	
	5	41	80	0.2	32	
	2 x 2.5	24	72	0.2	28	
	15	32	69	0.2	36	
671144	0.5	25	39	0.2	34	$GalNAc_3$-12
	1.5	26	55	0.2	28	
	5	48	82	0.2	34	
	15	23	46	0.2	32	
670061	0.5	27	53	0.2	33	$GalNAc_3$-13
	1.5	24	45	0.2	35	
	5	23	58	0.1	34	
	15	24	72	0.1	31	
671261	0.5	69	99	0.1	33	$GalNAc_3$-14
	1.5	34	62	0.1	33	
	5	43	73	0.1	32	
	15	32	53	0.2	30	
671262	0.5	24	51	0.2	29	$GalNAc_3$-15
	1.5	32	62	0.1	31	
	5	30	76	0.2	32	
	15	31	64	0.1	32	

Example 66: Effect of various cleavable moieties on antisense inhibition *in vivo* by oligonucleotides targeting SRB-1 comprising a 5'-$GalNAc_3$ cluster

The oligonucleotides listed below were tested in a dose-dependent study for antisense inhibition of SRB-1 in mice. Each of the $GalNAc_3$ conjugate groups was attached at the 5' terminus of the respective oligonucleotide by a phosphodiester linked nucleoside (cleavable moiety (CM)).

Table 57

Modified ASOs targeting SRB-1

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No.
661161	**$GalNAc_3$-3_{a-o},A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}$ $G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-3a	A_d	4888
670699	**$GalNAc_3$-3_{a-o},T_{do}**$G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}$ $G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-3a	T_d	4891
670700	**$GalNAc_3$-3_{a-o},A_{eo}**$G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}$	$GalNAc_3$-3a	A_e	4888

	$G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T_{e}$			
670701	**$GalNAc_3$-3_{a-o},T_{eo}**$G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}$ $G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-3a	T_e	4891
671165	**$GalNAc_3$-13_{a-o},A_{do}**$G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}$ $G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-13a	A_d	4888

Capital letters indicate the nucleobase for each nucleoside and ^{m}C indicates a 5-methyl cytosine. Subscripts: "e" indicates a 2'-MOE modified nucleoside; "d" indicates a β-D-2'-deoxyribonucleoside; "s" indicates a phosphorothioate internucleoside linkage (PS); "o" indicates a phosphodiester internucleoside linkage (PO); and "o"" indicates -O-P(=O)(OH)-. Conjugate groups are in bold.

The structure of $GalNAc_3$-3_a was shown previously in Example 39. The structure of $GalNAc_3$-13a was shown previously in Example 62.

Treatment

Six to eight week old C57bl6 mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously once at the dosage shown below with ISIS 661161, 670699, 670700, 670701, 671165, or with saline. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration to determine the liver SRB-1 mRNA levels using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. The results below are presented as the average percent of SRB-1 mRNA levels for each treatment group, normalized to the saline control.

As illustrated in Table 58, treatment with antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner. The antisense oligonucleotides comprising various cleavable moieties all showed similar potencies.

Table 58
SRB-1 mRNA (% Saline)

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA (% Saline)	$GalNAc_3$ Cluster	CM
Saline	n/a	100.0	n/a	n/a
661161	0.5	87.8	$GalNAc_3$-3a	A_d
	1.5	61.3		
	5	33.8		
	15	14.0		
670699	0.5	89.4	$GalNAc_3$-3a	T_d
	1.5	59.4		
	5	31.3		
	15	17.1		
670700	0.5	79.0	$GalNAc_3$-3a	A_e
	1.5	63.3		

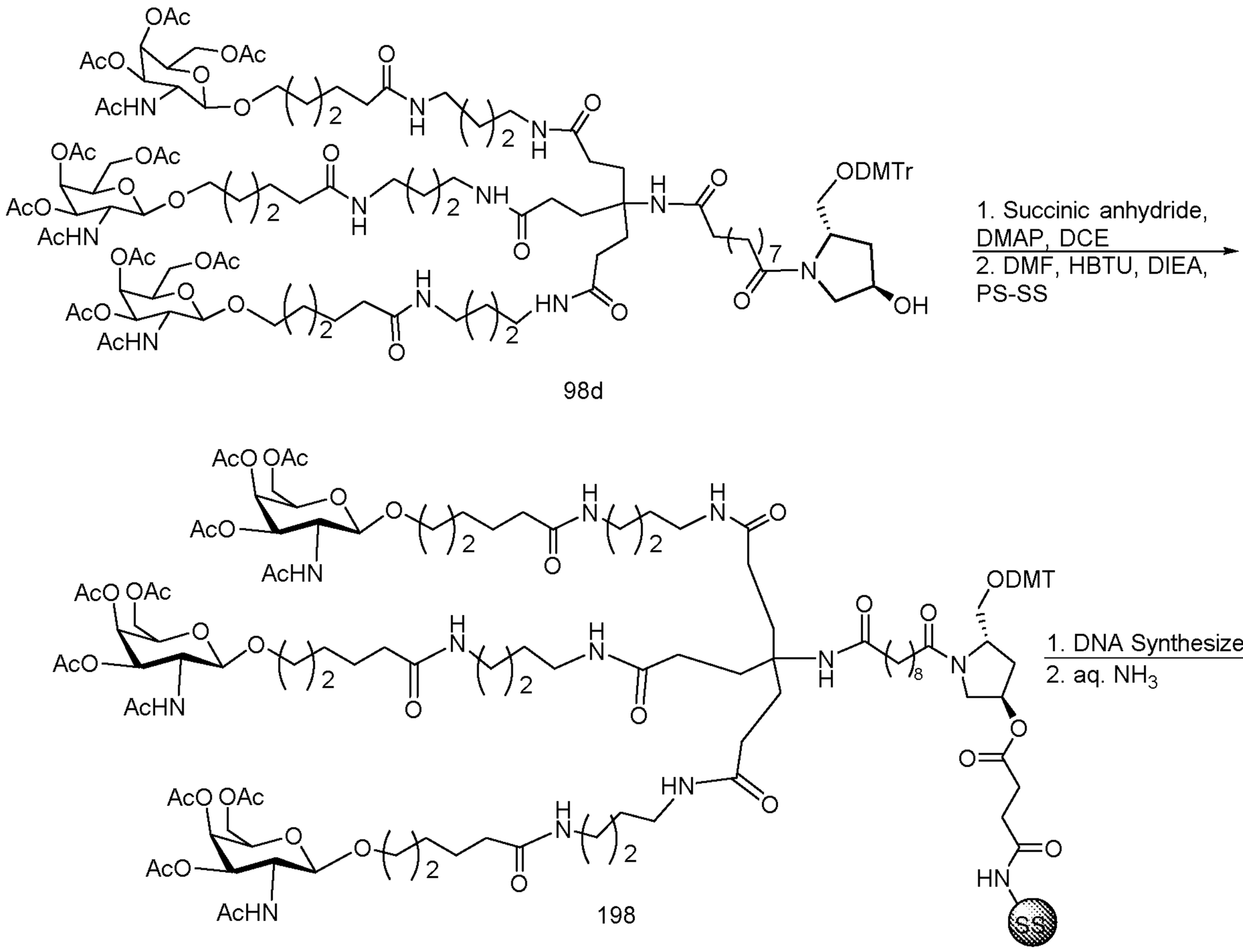
	5	32.8		
	15	17.9		
670701	0.5	79.1	$GalNAc_3$-3a	T_e
	1.5	59.2		
	5	35.8		
	15	17.7		
671165	0.5	76.4	$GalNAc_3$-13a	A_d
	1.5	43.2		
	5	22.6		
	15	10.0		

Liver transaminase levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in serum were measured relative to saline injected mice using standard protocols. Total bilirubin and BUN were also evaluated. The changes in body weights were evaluated with no significant differences from the saline group (data not shown). ALTs, ASTs, total bilirubin and BUN values are shown in Table 56 below.

Table 59

ISIS No.	Dosage (mg/kg)	ALT (U/L)	AST (U/L)	Total Bilirubin (mg/dL)	BUN (mg/dL)	$GalNAc_3$ Cluster	CM
Saline	n/a	24	64	0.2	31	n/a	n/a
661161	0.5	25	64	0.2	31	$GalNAc_3$-3a	A_d
	1.5	24	50	0.2	32		
	5	26	55	0.2	28		
	15	27	52	0.2	31		
670699	0.5	42	83	0.2	31	$GalNAc_3$-3a	T_d
	1.5	33	58	0.2	32		
	5	26	70	0.2	29		
	15	25	67	0.2	29		
670700	0.5	40	74	0.2	27	$GalNAc_3$-3a	A_e
	1.5	23	62	0.2	27		
	5	24	49	0.2	29		
	15	25	87	0.1	25		
670701	0.5	30	77	0.2	27	$GalNAc_3$-3a	T_e
	1.5	22	55	0.2	30		
	5	81	101	0.2	25		
	15	31	82	0.2	24		
671165	0.5	44	84	0.2	26	$GalNAc_3$-13a	A_d
	1.5	47	71	0.1	24		
	5	33	91	0.2	26		
	15	33	56	0.2	29		

Example 67: Preparation of oligomeric compound 199 comprising GalNAc$_3$-16

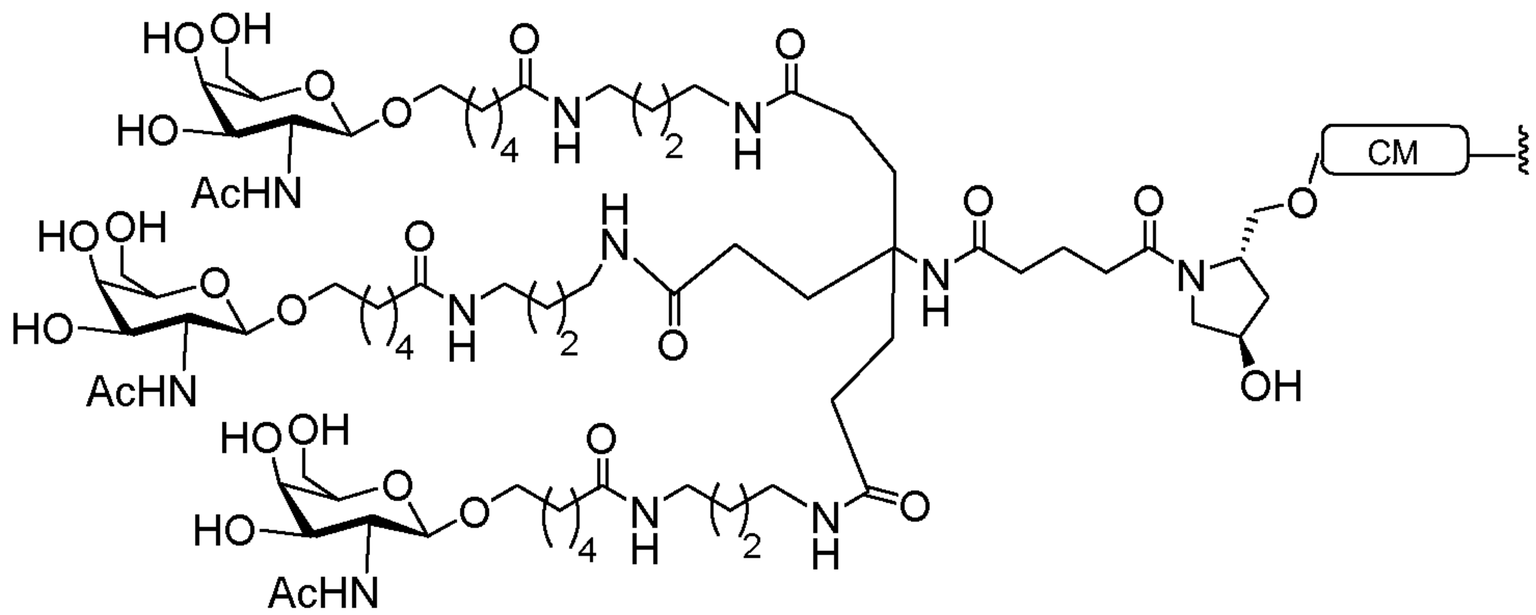


98d

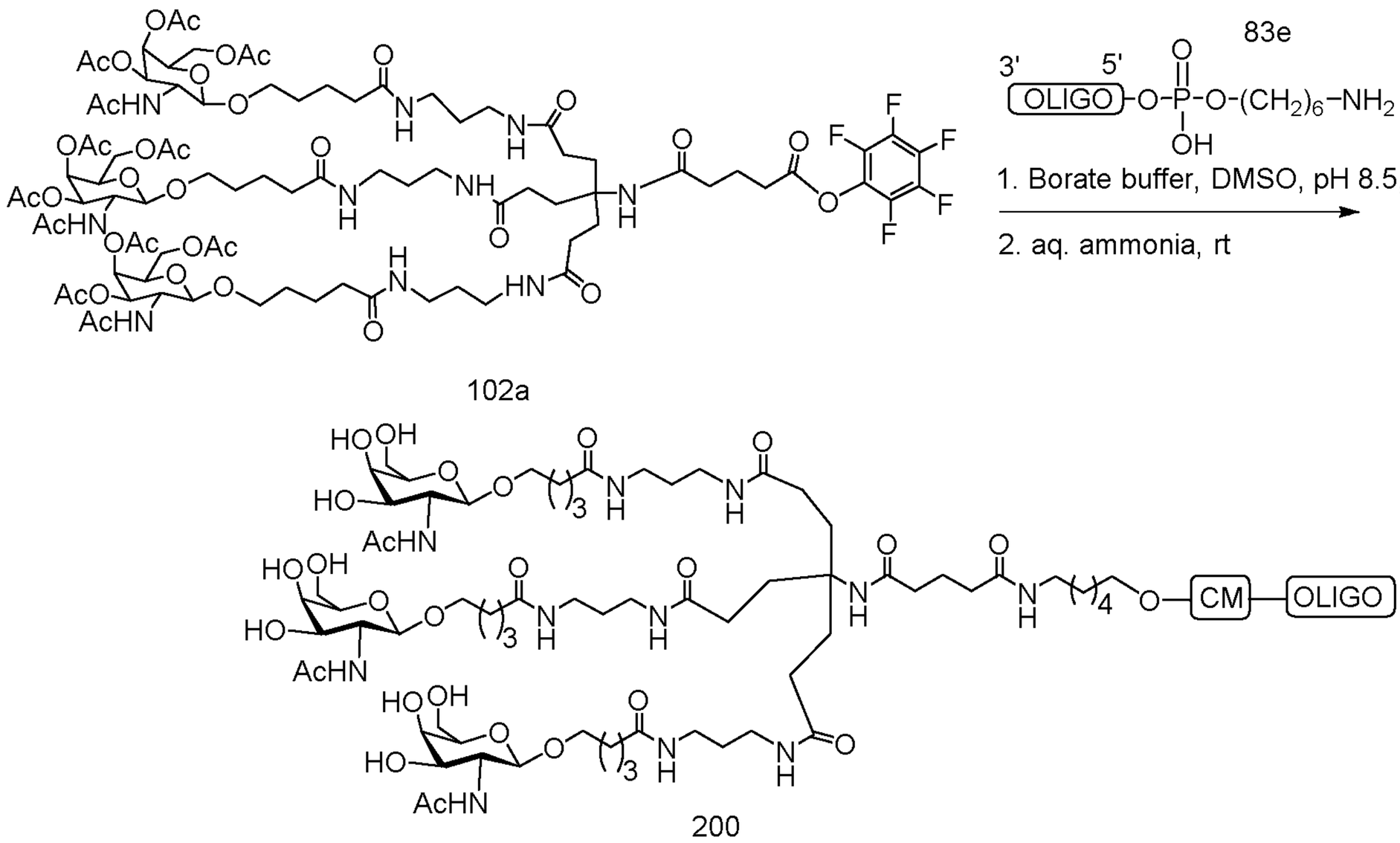
198

199

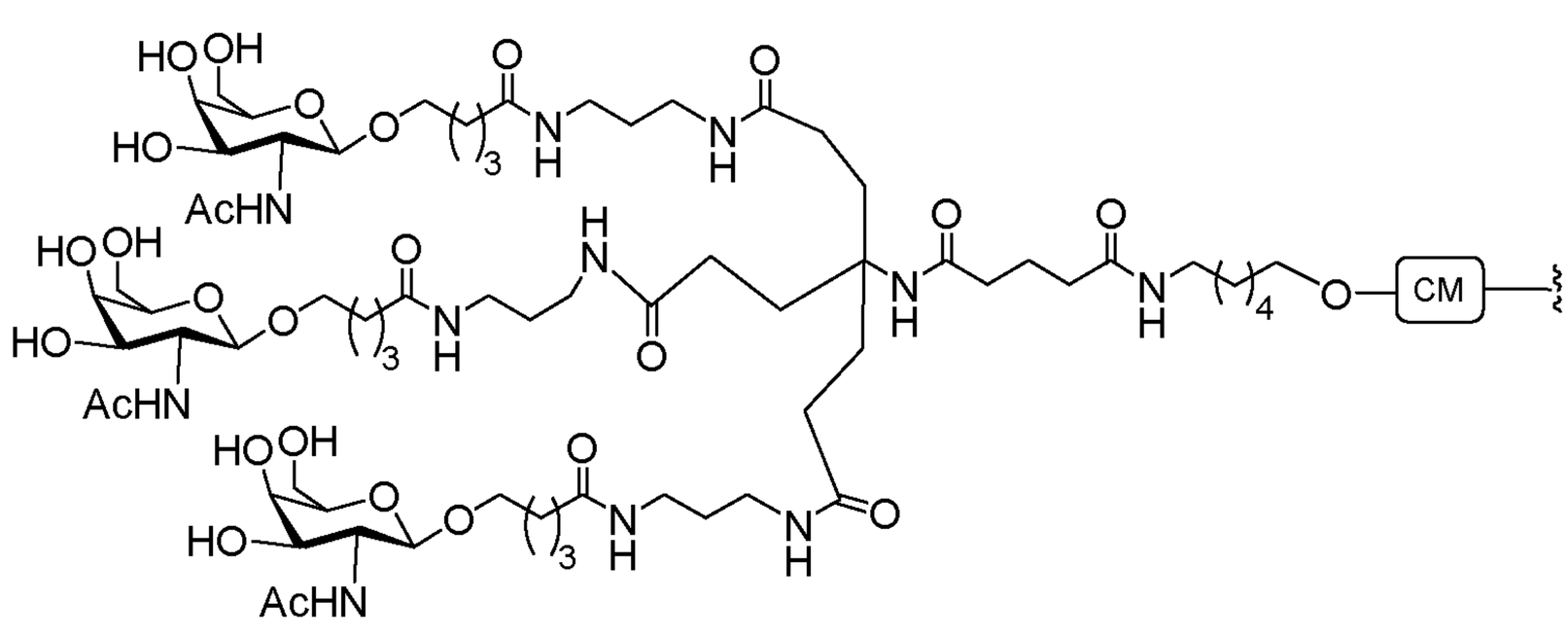
Oligomeric compound 199, comprising a GalNAc$_3$-16 conjugate group, is prepared using the general procedures illustrated in Examples 7 and 9. The GalNAc$_3$ cluster portion of the conjugate group GalNAc$_3$-16 (GalNAc$_3$-16$_a$) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A$_d$-P(=O)(OH)-.The structure of GalNAc$_3$-16 (GalNAc$_3$-16$_a$-CM-) is shown below:



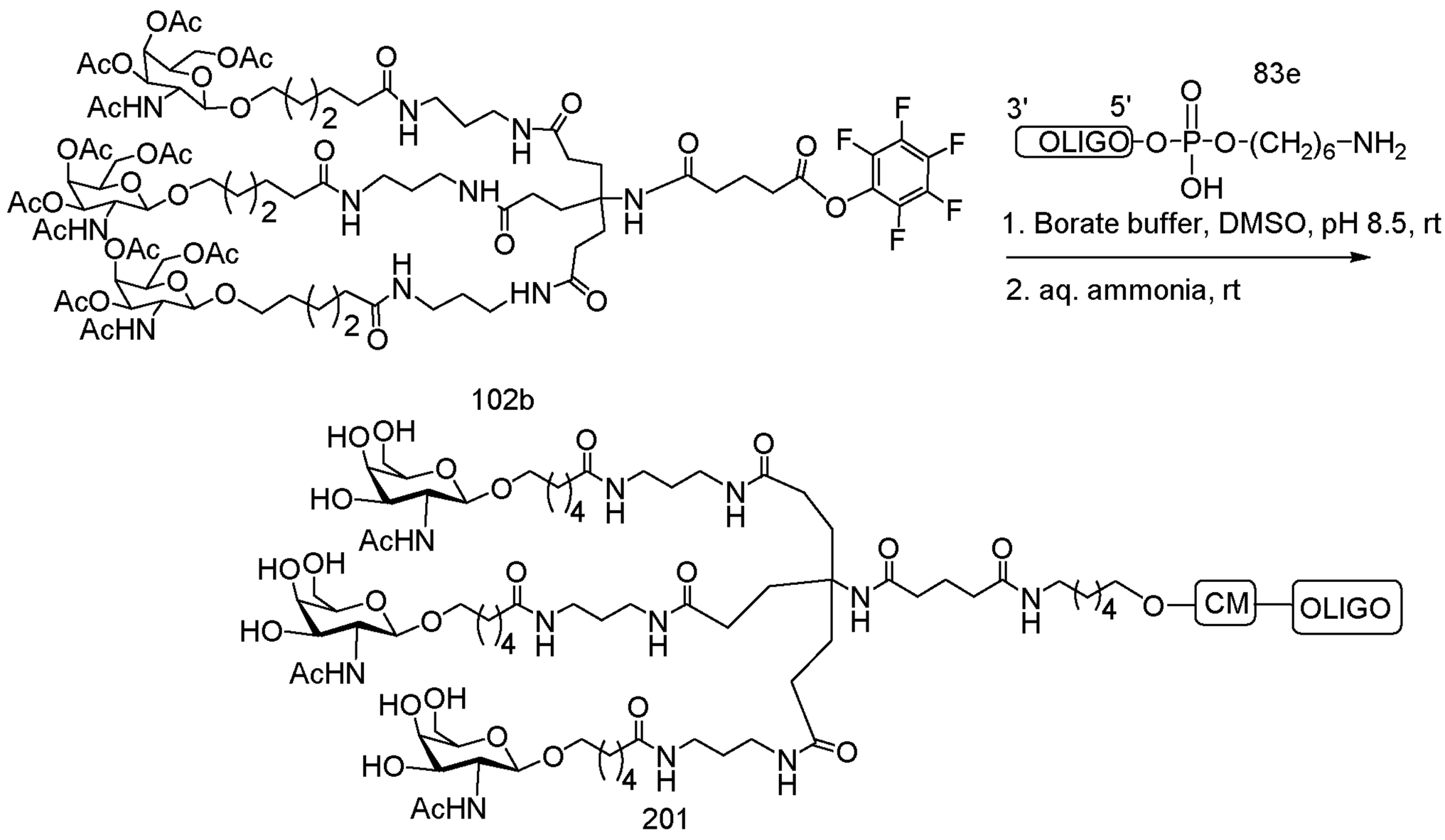
Example 68: Preparation of oligomeric compound 200 comprising GalNAc$_3$-17



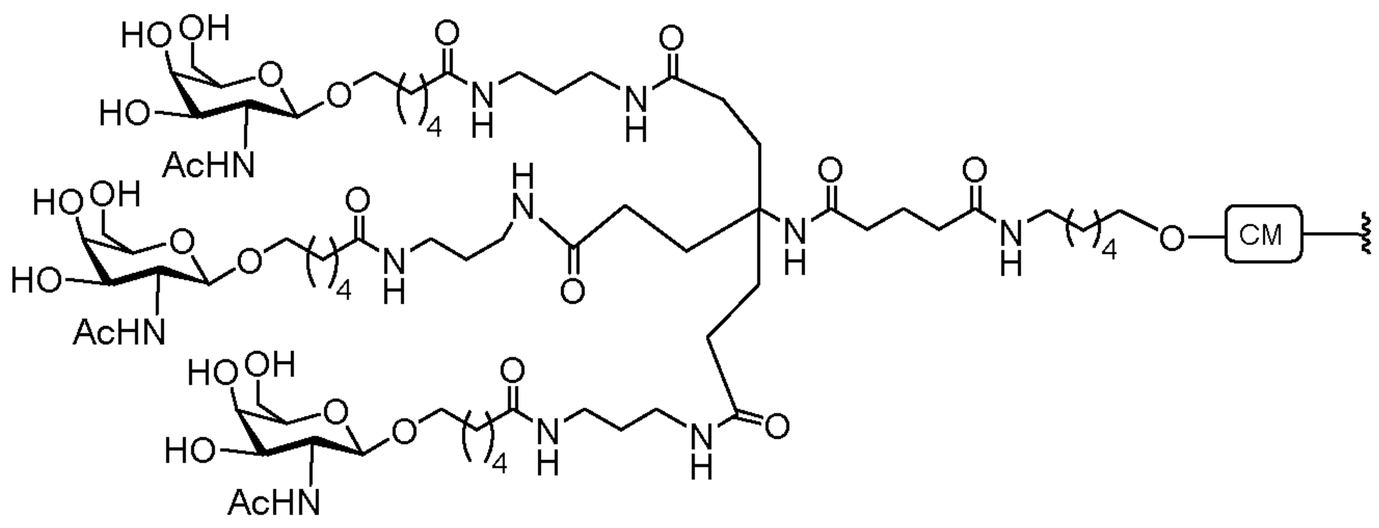
Oligomeric compound 200, comprising a GalNAc$_3$-17 conjugate group, was prepared using the general procedures illustrated in Example 46. The GalNAc$_3$ cluster portion of the conjugate group GalNAc$_3$-17 (GalNAc$_3$-17$_a$) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A$_d$-P(=O)(OH)-. The structure of GalNAc$_3$-17 (GalNAc$_3$-17$_a$-CM-) is shown below:



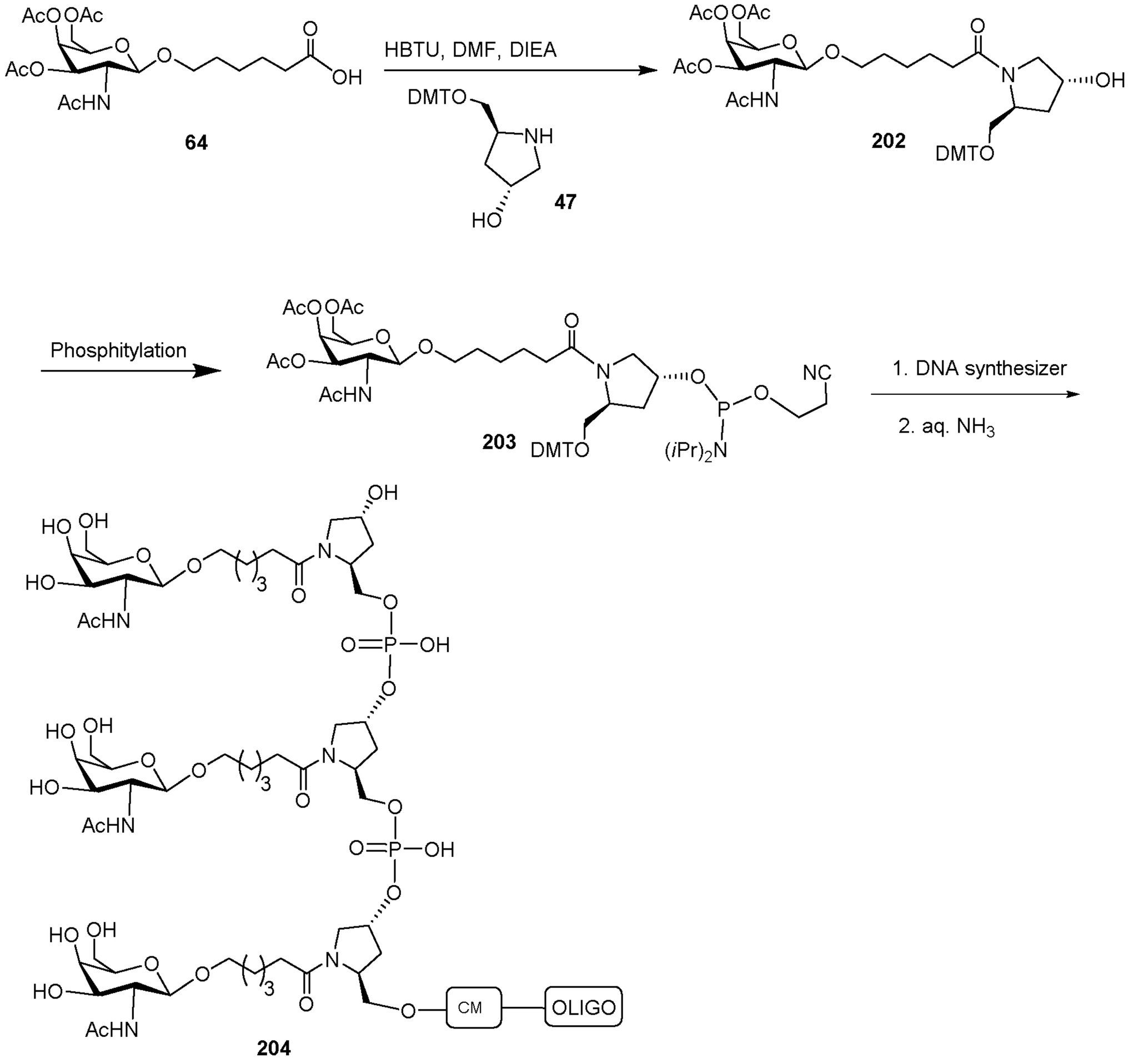
Example 69: Preparation of oligomeric compound 201 comprising GalNAc$_3$-18



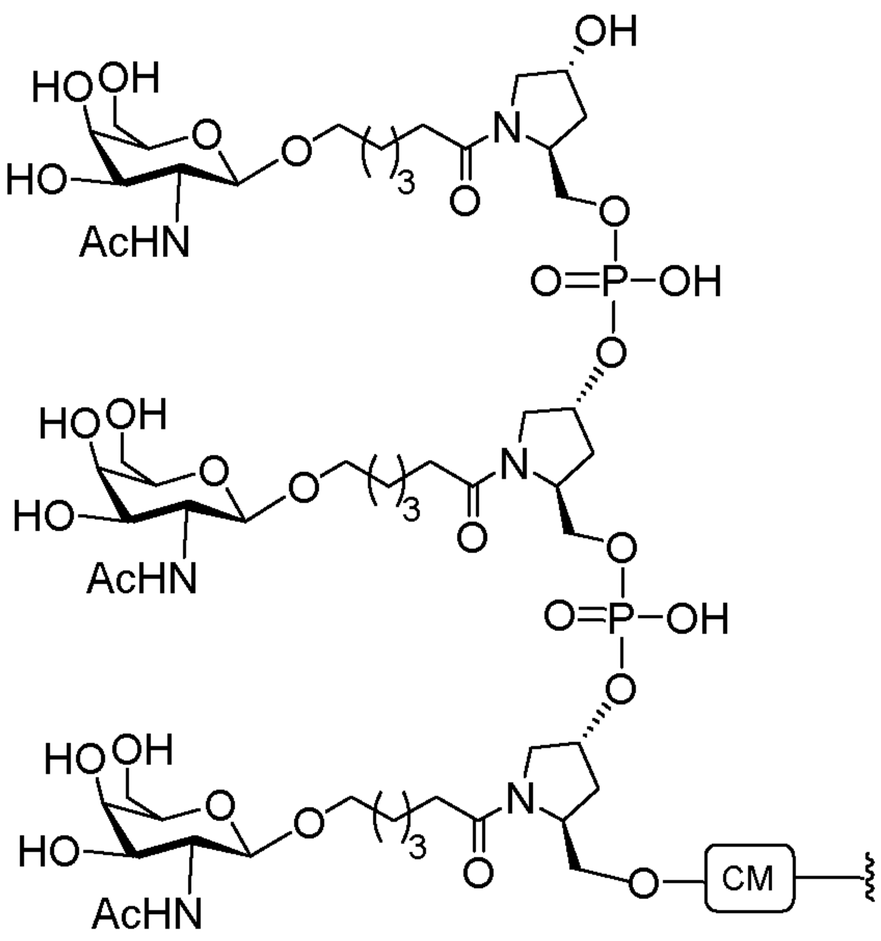
Oligomeric compound 201, comprising a GalNAc$_3$-18 conjugate group, was prepared using the general procedures illustrated in Example 46. The GalNAc$_3$ cluster portion of the conjugate group GalNAc$_3$-18 (GalNAc$_3$-18$_a$) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A$_d$-P(=O)(OH)-. The structure of GalNAc$_3$-18 (GalNAc$_3$-18$_a$-CM-) is shown below:



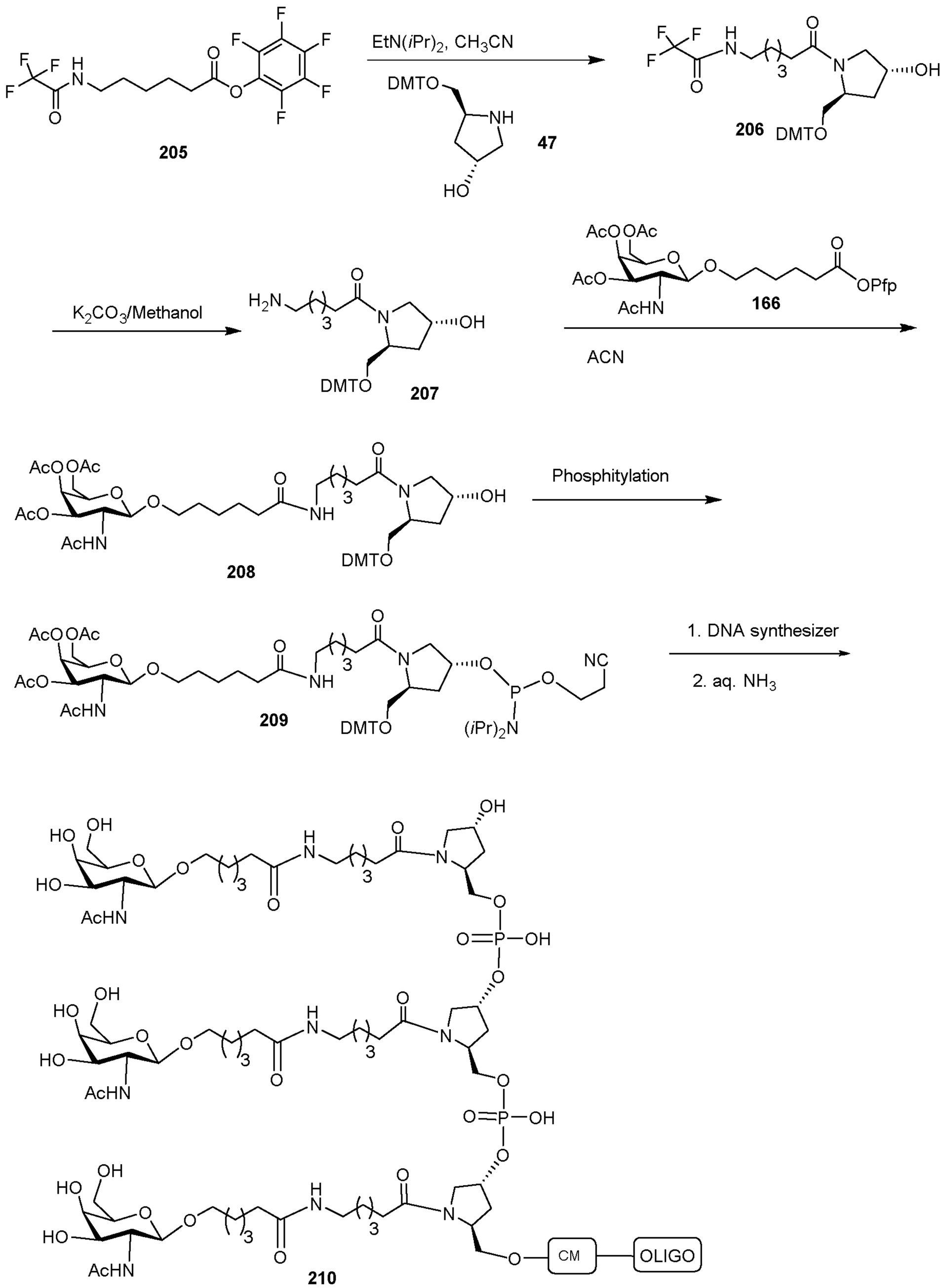
Example 70: Preparation of oligomeric compound 204 comprising GalNAc$_3$-19



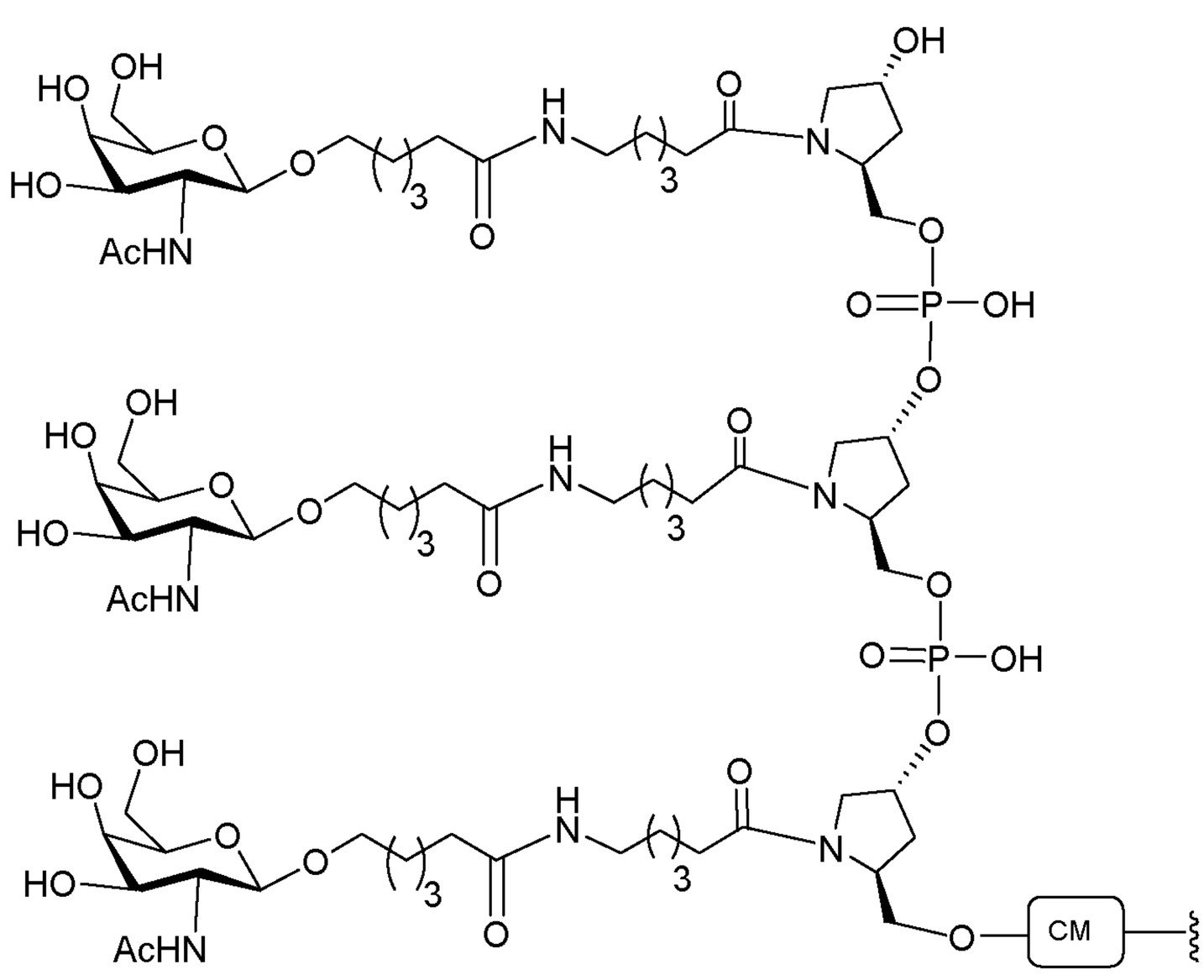
Oligomeric compound 204, comprising a $GalNAc_3$-19 conjugate group, was prepared from compound 64 using the general procedures illustrated in Example 52. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-19 ($GalNAc_3$-19_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-. The structure of $GalNAc_3$-19 ($GalNAc_3$-19_a-CM-) is shown below:


HO
OH
HO
AcHN
O
O
3
O
N
OH
O
O=P–OH
O
HO
OH
HO
AcHN
O
O
3
O
N
O
O=P–OH
HO
OH
HO
AcHN
O
O
3
O
N
O
O
CM

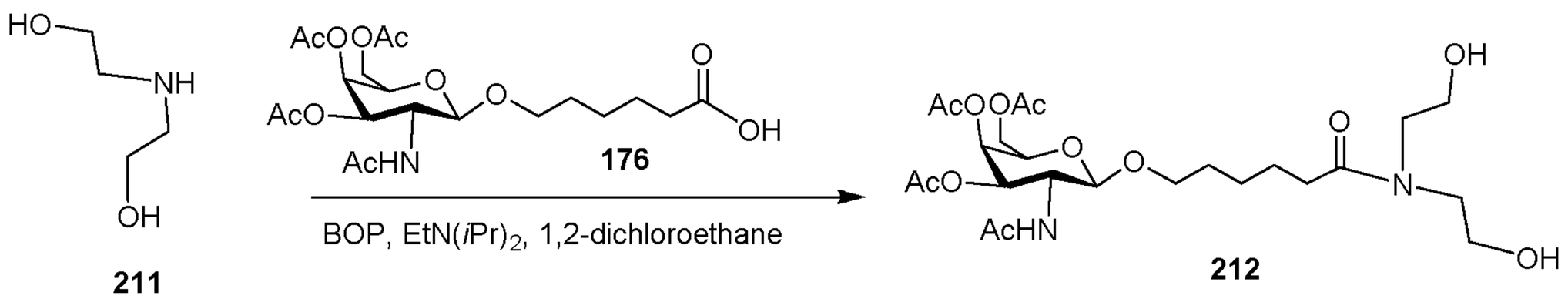
Example 71: Preparation of oligomeric compound 210 comprising GalNAc$_3$-20



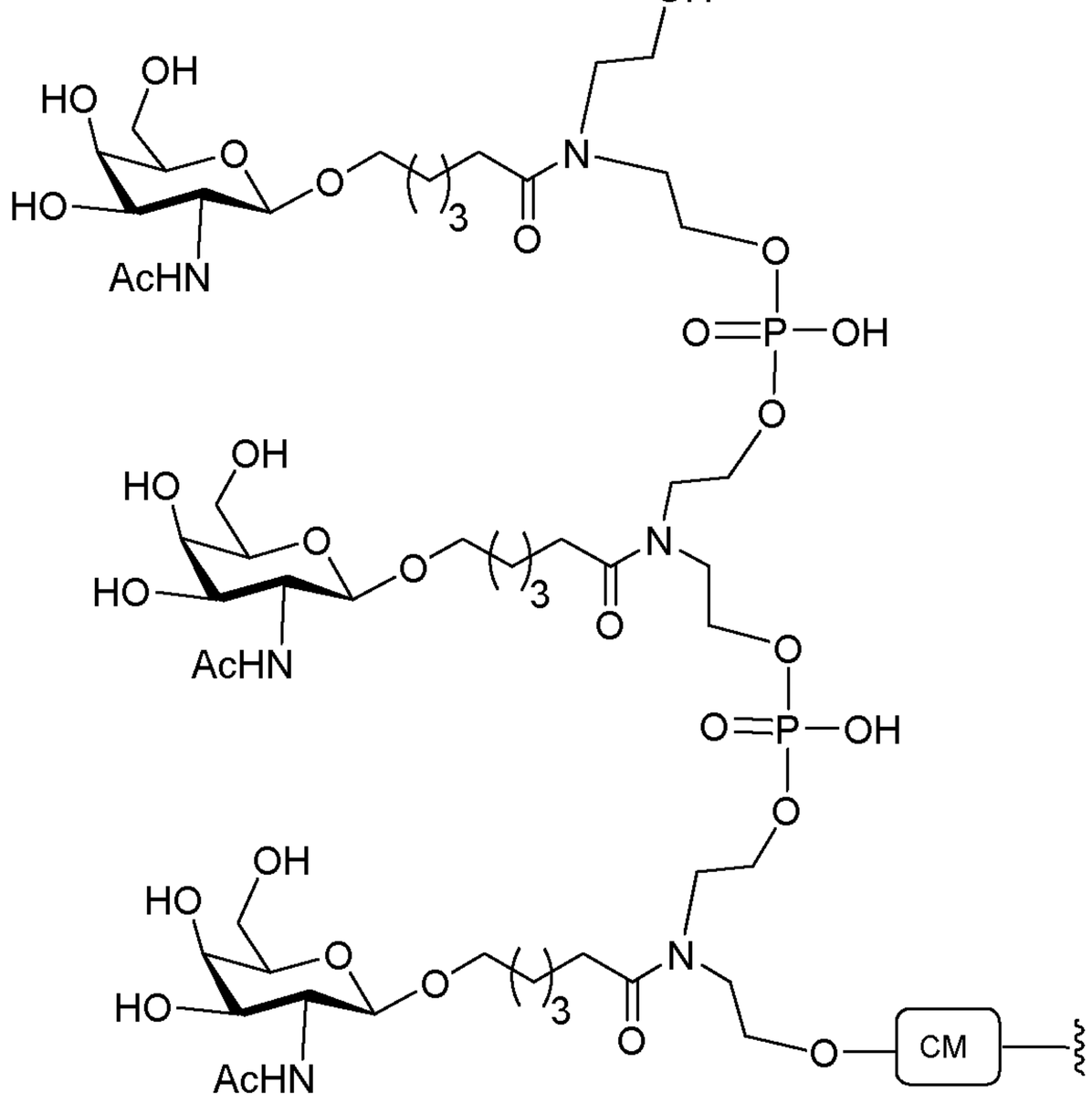
Compound 205 was prepared by adding PFP-TFA and DIEA to 6-(2,2,2-trifluoroacetamido)hexanoic acid in acetonitrile ,which was prepared by adding triflic anhydride to 6-aminohexanoic acid. The reaction mixture was heated to 80 °C, then lowered to rt. Oligomeric compound 210, comprising a $GalNAc_3$-20 conjugate group, was prepared from compound 208 using the general procedures illustrated in Example 52. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-20 ($GalNAc_3$-20_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-. The structure of $GalNAc_3$-20 ($GalNAc_3$-20_a-CM-) is shown below:



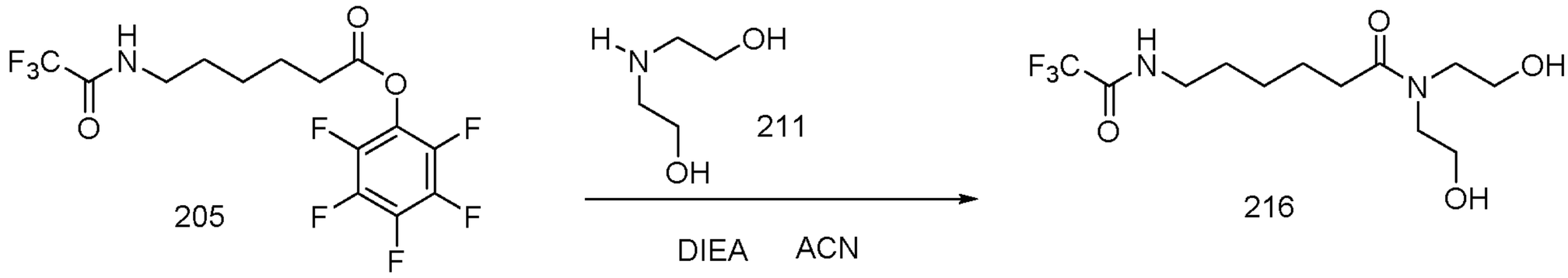
Example 72: Preparation of oligomeric compound 215 comprising GalNAc$_3$-21

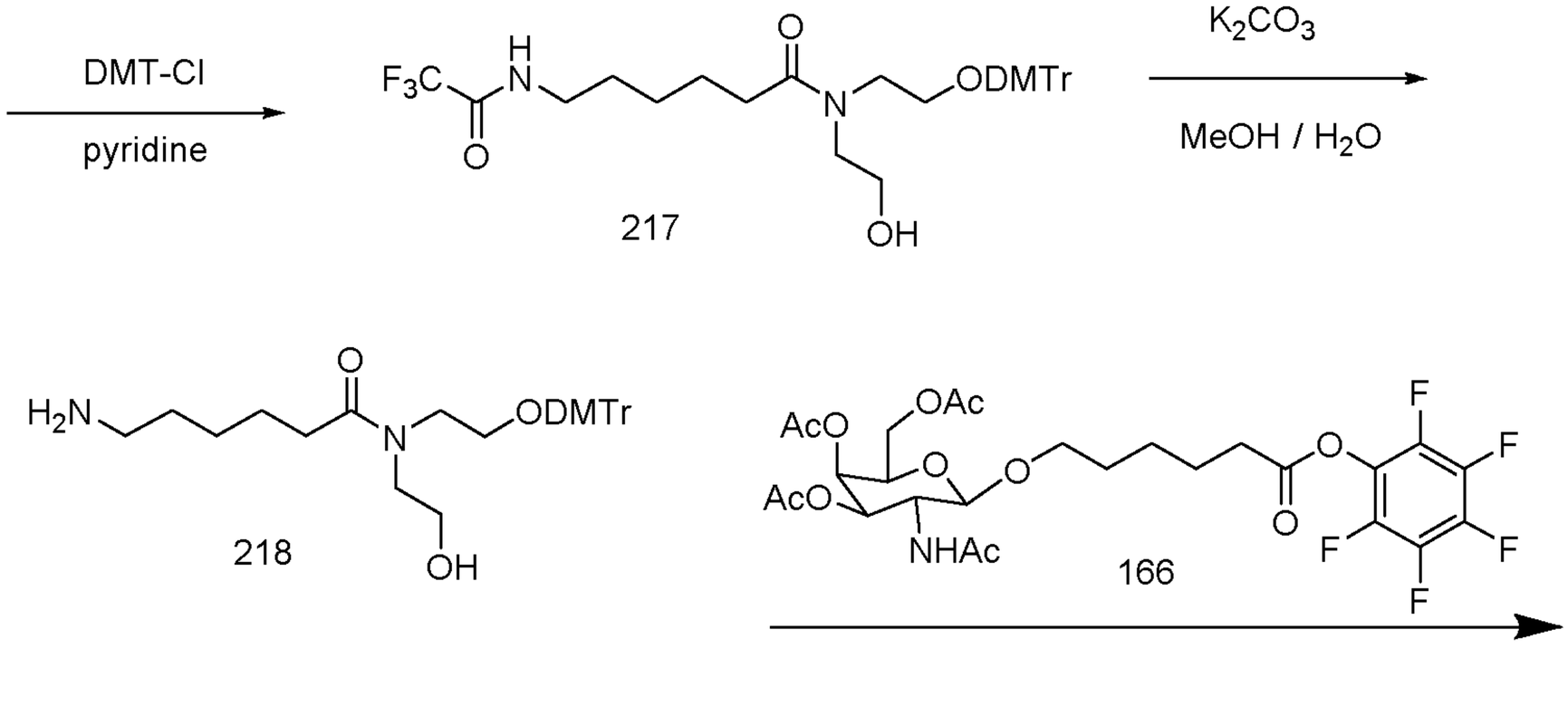


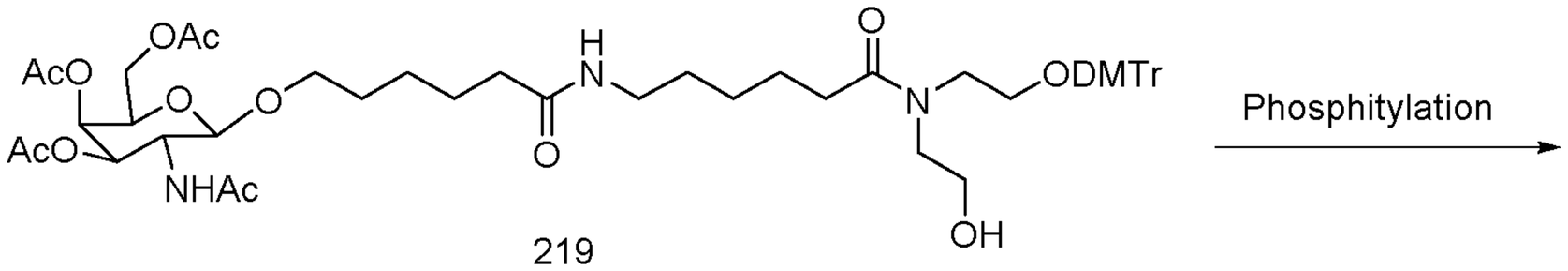
Compound 211 is commercially available. Oligomeric compound 215, comprising a $GalNAc_3$-21 conjugate group, was prepared from compound 213 using the general procedures illustrated in Example 52. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-21 ($GalNAc_3$-21_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-. The structure of $GalNAc_3$-21 ($GalNAc_3$-21_a-CM-) is shown below:

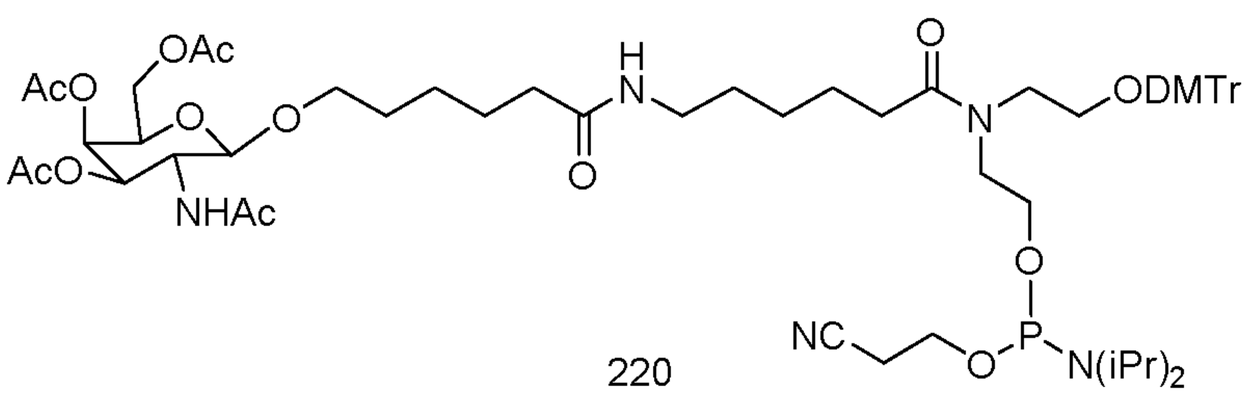


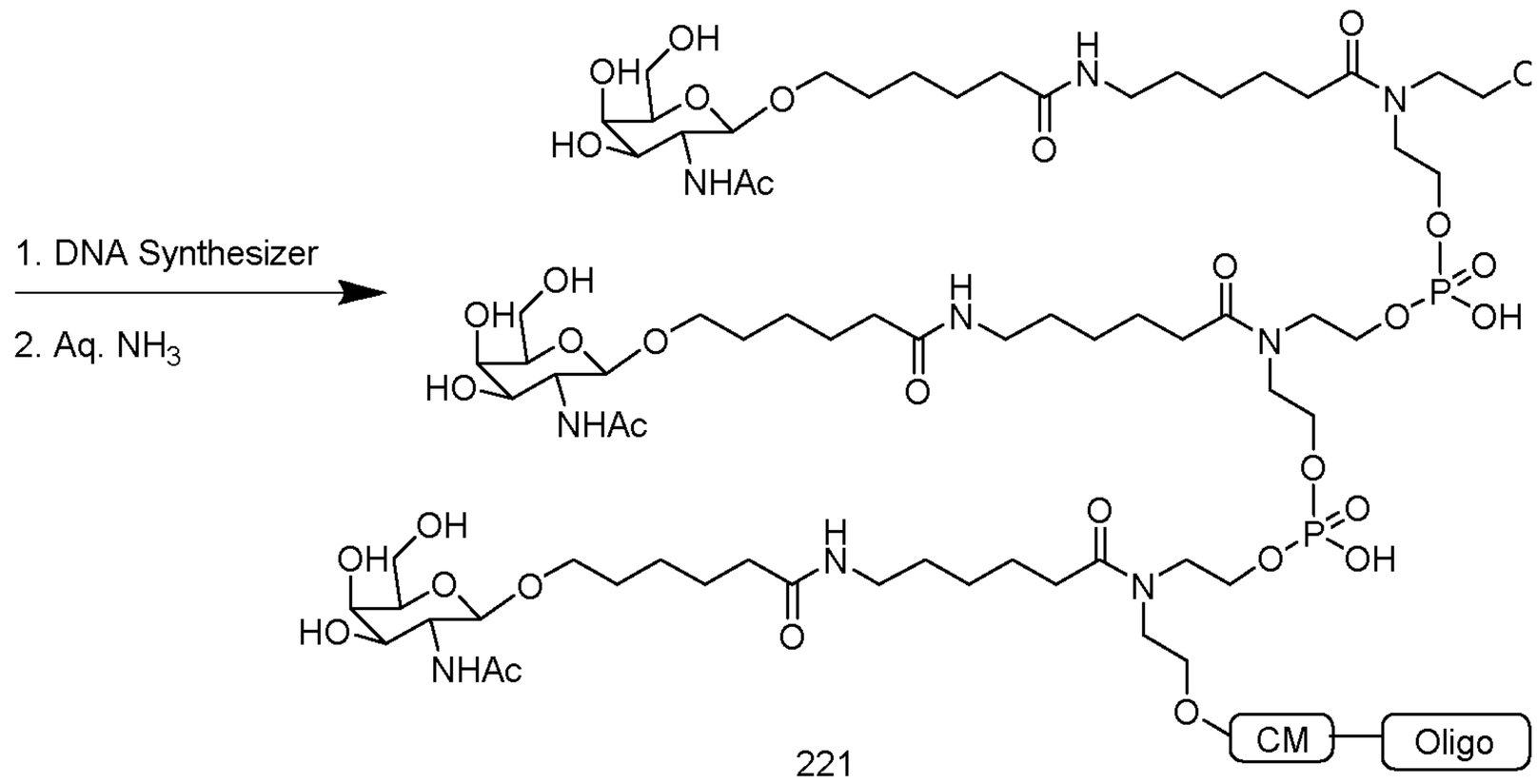
Example 73: Preparation of oligomeric compound 221 comprising GalNAc$_3$-22





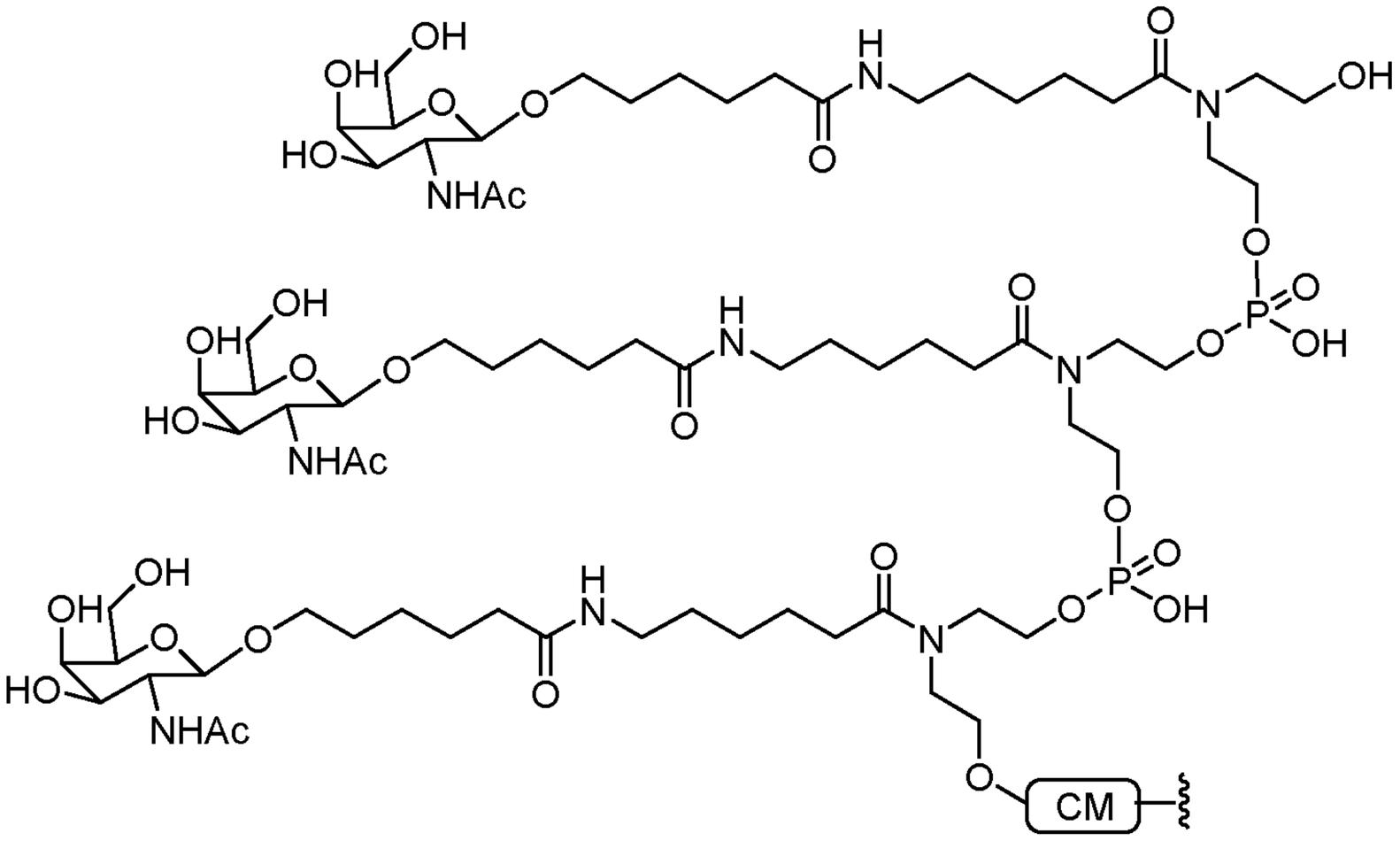






221

Compound 220 was prepared from compound 219 using diisopropylammonium tetrazolide. Oligomeric compound 221, comprising a $GalNAc_3$-21 conjugate group, is prepared from compound 220 using the general procedure illustrated in Example 52. The $GalNAc_3$ cluster portion of the conjugate group $GalNAc_3$-22 ($GalNAc_3$-22_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the cleavable moiety is -P(=O)(OH)-A_d-P(=O)(OH)-. The structure of $GalNAc_3$-22 ($GalNAc_3$-22_a-CM-) is shown below:



Example 74: Effect of various cleavable moieties on antisense inhibition *in vivo* by oligonucleotides targeting SRB-1 comprising a 5'-$GalNAc_3$ conjugate

The oligonucleotides listed below were tested in a dose-dependent study for antisense inhibition of SRB-1 in mice. Each of the $GalNAc_3$ conjugate groups was attached at the 5' terminus of the respective oligonucleotide.

Table 60
Modified ASOs targeting SRB-1

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No.
353382	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	n/a	n/a	4886
661161	**$GalNAc_3$-$3_{a\text{-}o}$,A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-3a	A_d	4888
666904	**$GalNAc_3$-$3_{a\text{-}o}$,**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-3a	PO	4886
675441	**$GalNAc_3$-$17_{a\text{-}o}$,A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-17a	A_d	4888
675442	**$GalNAc_3$-$18_{a\text{-}o}$,A_{do}**$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-18a	A_d	4888

In all tables, capital letters indicate the nucleobase for each nucleoside and ^{m}C indicates a 5-methyl cytosine. Subscripts: "e" indicates a 2'-MOE modified nucleoside; "d" indicates a β-D-2'-deoxyribonucleoside; "s" indicates a phosphorothioate internucleoside linkage (PS); "o" indicates a phosphodiester internucleoside linkage (PO); and "o'" indicates -O-P(=O)(OH)-. Conjugate groups are in bold.

The structure of $GalNAc_3$-3_a was shown previously in Example 39. The structure of $GalNAc_3$-17a was shown previously in Example 68, and the structure of $GalNAc_3$-18a was shown in Example 69.

Treatment

Six to eight week old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously once at the dosage shown below with an oligonucleotide listed in Table 60 or with saline. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration to determine the SRB-1 mRNA levels using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. The results below are presented as the average percent of SRB-1 mRNA levels for each treatment group, normalized to the saline control.

As illustrated in Table 61, treatment with antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner. The antisense oligonucleotides comprising a GalNAc conjugate showed similar potencies and were significantly more potent than the parent oligonucleotide lacking a GalNAc conjugate.

Table 61
SRB-1 mRNA (% Saline)

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA (% Saline)	$GalNAc_3$ Cluster	CM
Saline	n/a	100.0	n/a	n/a
353382	3	79.38	n/a	n/a

	10	68.67		
	30	40.70		
661161	0.5	79.18	$GalNAc_3$-3a	A_d
	1.5	75.96		
	5	30.53		
	15	12.52		
666904	0.5	91.30	$GalNAc_3$-3a	PO
	1.5	57.88		
	5	21.22		
	15	16.49		
675441	0.5	76.71	$GalNAc_3$-17a	A_d
	1.5	63.63		
	5	29.57		
	15	13.49		
675442	0.5	95.03	$GalNAc_3$-18a	A_d
	1.5	60.06		
	5	31.04		
	15	19.40		

Liver transaminase levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in serum were measured relative to saline injected mice using standard protocols. Total bilirubin and BUN were also evaluated. The change in body weights was evaluated with no significant change from the saline group (data not shown). ALTs, ASTs, total bilirubin and BUN values are shown in Table 62 below.

Table 62

ISIS No.	Dosage (mg/kg)	ALT (U/L)	AST (U/L)	Total Bilirubin (mg/dL)	BUN (mg/dL)	$GalNAc_3$ Cluster	CM
Saline	n/a	26	59	0.16	42	n/a	n/a
353382	3	23	58	0.18	39	n/a	n/a
	10	28	58	0.16	43		
	30	20	48	0.12	34		
661161	0.5	30	47	0.13	35	$GalNAc_3$-3a	A_d
	1.5	23	53	0.14	37		
	5	26	48	0.15	39		
	15	32	57	0.15	42		
666904	0.5	24	73	0.13	36	$GalNAc_3$-3a	PO
	1.5	21	48	0.12	32		
	5	19	49	0.14	33		
	15	20	52	0.15	26		
675441	0.5	42	148	0.21	36	$GalNAc_3$-17a	A_d
	1.5	60	95	0.16	34		
	5	27	75	0.14	37		
	15	24	61	0.14	36		
675442	0.5	26	65	0.15	37	$GalNAc_3$-18a	A_d
	1.5	25	64	0.15	43		
	5	27	69	0.15	37		
	15	30	84	0.14	37		

Example 75: Pharmacokinetic analysis of oligonucleotides comprising a 5'-conjugate group

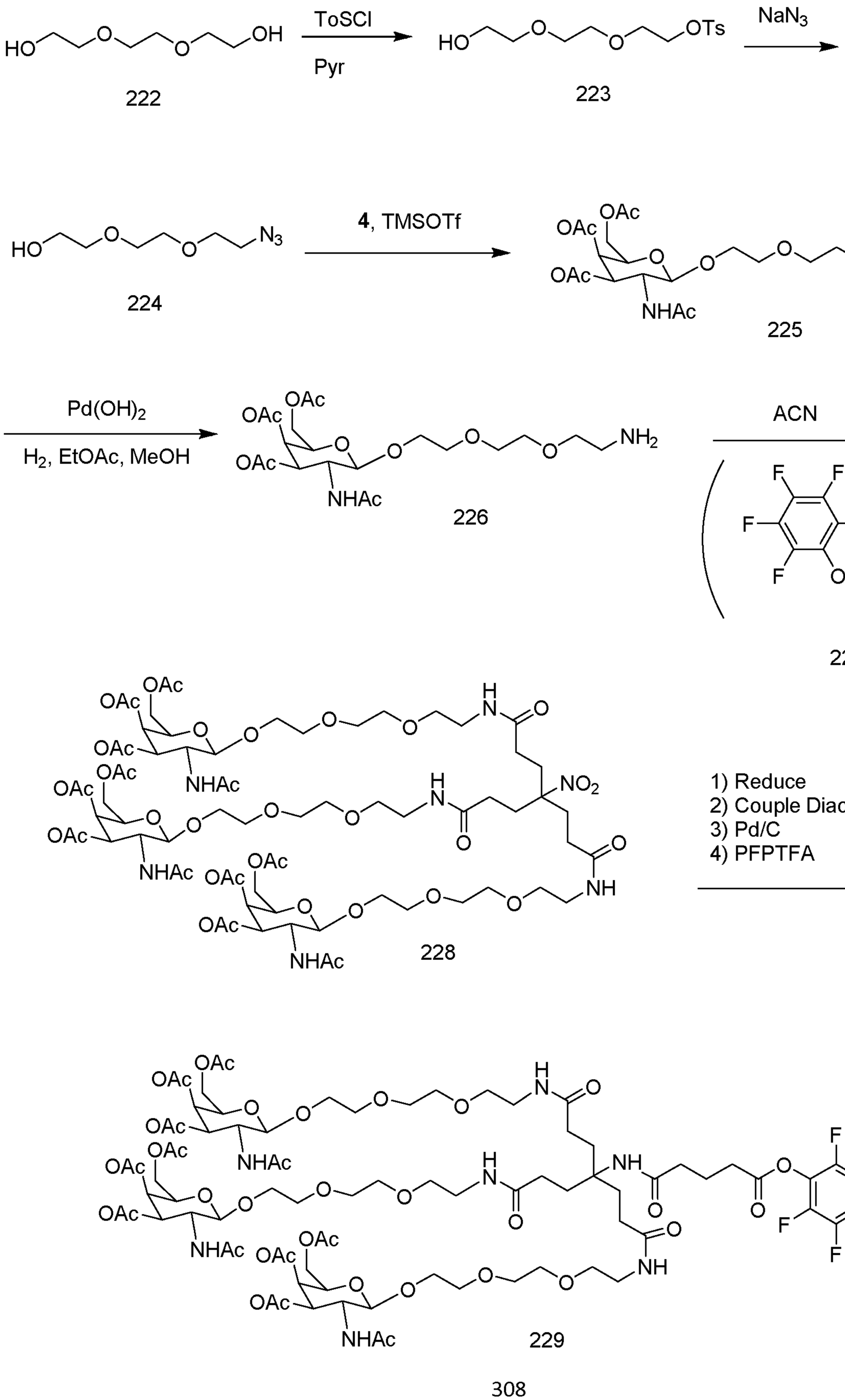
The PK of the ASOs in Tables 54, 57 and 60 above was evaluated using liver samples that were obtained following the treatment procedures described in Examples 65, 66, and 74. The liver samples were minced and extracted using standard protocols and analyzed by IP-HPLC-MS alongside an internal standard. The combined tissue level (μg/g) of all metabolites was measured by integrating the appropriate UV peaks, and the tissue level of the full-length ASO missing the conjugate ("parent," which is Isis No. 353382 in this case) was measured using the appropriate extracted ion chromatograms (EIC).

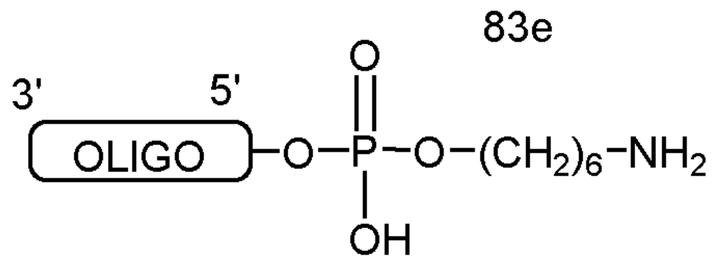
Table 63
PK Analysis in Liver

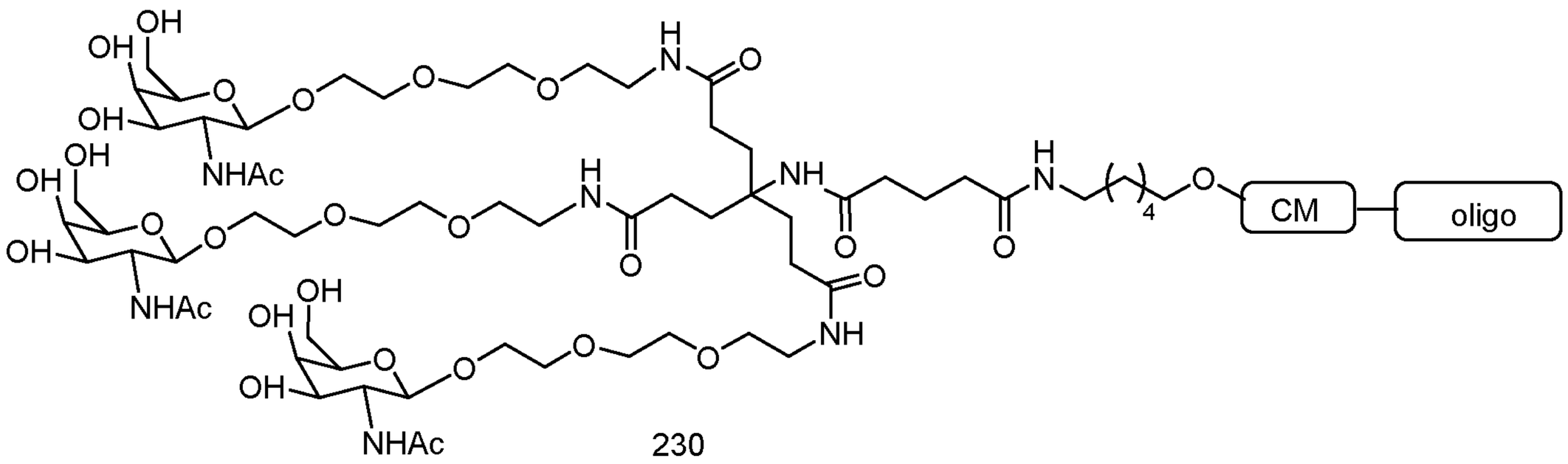
ISIS No.	Dosage (mg/kg)	Total Tissue Level by UV (μg/g)	Parent ASO Tissue Level by EIC (μg/g)	$GalNAc_3$ Cluster	CM
353382	3	8.9	8.6	n/a	n/a
	10	22.4	21.0		
	30	54.2	44.2		
661161	5	32.4	20.7	$GalNAc_3$-3a	A_d
	15	63.2	44.1		
671144	5	20.5	19.2	$GalNAc_3$-12a	A_d
	15	48.6	41.5		
670061	5	31.6	28.0	$GalNAc_3$-13a	A_d
	15	67.6	55.5		
671261	5	19.8	16.8	$GalNAc_3$-14a	A_d
	15	64.7	49.1		
671262	5	18.5	7.4	$GalNAc_3$-15a	A_d
	15	52.3	24.2		
670699	5	16.4	10.4	$GalNAc_3$-3a	T_d
	15	31.5	22.5		
670700	5	19.3	10.9	$GalNAc_3$-3a	A_e
	15	38.1	20.0		
670701	5	21.8	8.8	$GalNAc_3$-3a	T_e
	15	35.2	16.1		
671165	5	27.1	26.5	$GalNAc_3$-13a	A_d
	15	48.3	44.3		
666904	5	30.8	24.0	$GalNAc_3$-3a	PO
	15	52.6	37.6		
675441	5	25.4	19.0	$GalNAc_3$-17a	A_d
	15	54.2	42.1		
675442	5	22.2	20.7	$GalNAc_3$-18a	A_d
	15	39.6	29.0		

The results in Table 63 above show that there were greater liver tissue levels of the oligonucleotides comprising a $GalNAc_3$ conjugate group than of the parent oligonucleotide that does not comprise a $GalNAc_3$ conjugate group (ISIS 353382) 72 hours following oligonucleotide administration, particularly when taking into consideration the differences in dosing between the oligonucleotides with and without a $GalNAc_3$ conjugate group. Furthermore, by 72 hours, 40-98% of each oligonucleotide comprising a $GalNAc_3$ conjugate group was metabolized to the parent compound, indicating that the $GalNAc_3$ conjugate groups were cleaved from the oligonucleotides.

Example 76: Preparation of oligomeric compound 230 comprising GalNAc$_3$-23







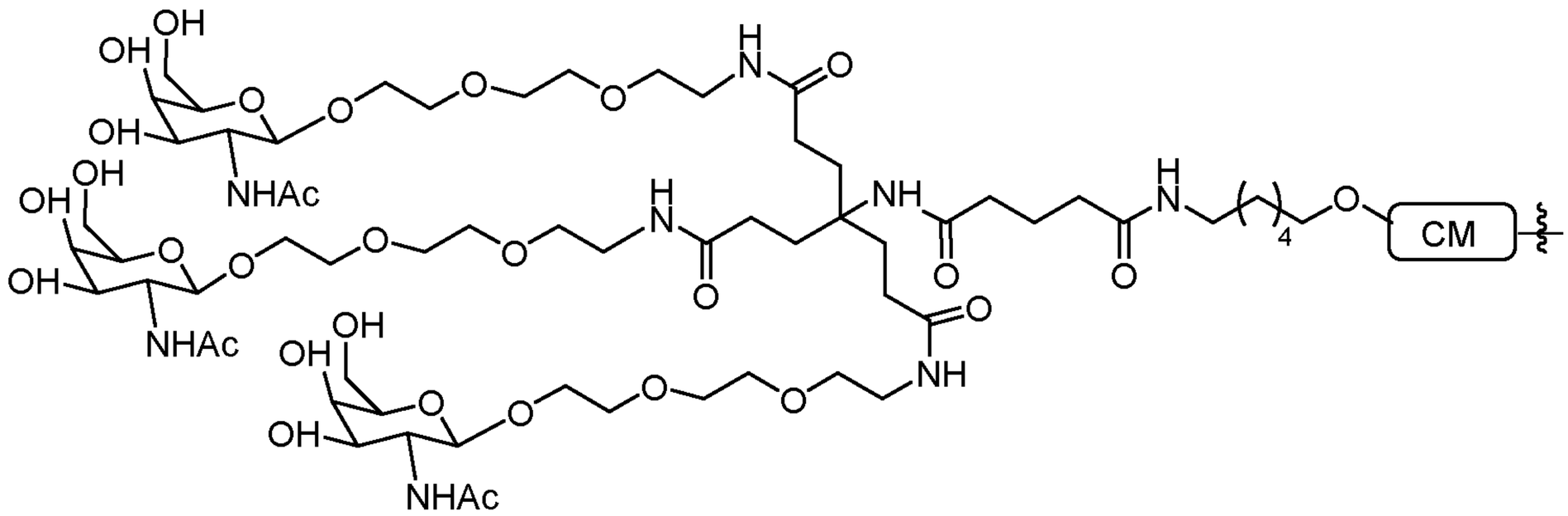
230

Compound 222 is commercially available. 44.48 ml (0.33 mol) of compound 222 was treated with tosyl chloride (25.39 g, 0.13 mol) in pyridine (500mL) for 16 hours. The reaction was then evaporated to an oil, dissolved in EtOAc and washed with water, sat. $NaHCO_3$, brine, and dried over Na_2SO_4. The ethyl acetate was concentrated to dryness and purified by column chromatography, eluted with EtOAc/hexanes (1:1) followed by 10% methanol in CH_2Cl_2 to give compound 223 as a colorless oil. LCMS and NMR were consistent with the structure. 10 g (32.86 mmol) of 1-Tosyltriethylene glycol (compound 223) was treated with sodium azide (10.68 g, 164.28 mmol) in DMSO (100mL) at room temperature for 17 hours. The reaction mixture was then poured onto water, and extracted with EtOAc. The organic layer was washed with water three times and dried over Na_2SO_4. The organic layer was concentrated to dryness to give 5.3g of compound 224 (92%). LCMS and NMR were consistent with the structure. 1-Azidotriethylene glycol (compound 224, 5.53 g, 23.69 mmol) and compound 4 (6 g, 18.22 mmol) were treated with 4A molecular sieves (5g), and TMSOTf (1.65 ml, 9.11 mmol) in dichloromethane (100mL) under an inert atmosphere. After 14 hours, the reaction was filtered to remove the sieves, and the organic layer was washed with sat. $NaHCO_3$, water, brine, and dried over Na_2SO_4. The organic layer was concentrated to dryness and purified by column chromatography, eluted with a gradient of 2 to 4% methanol in dichloromethane to give compound 225. LCMS and NMR were consistent with the structure. Compound 225 (11.9 g, 23.59 mmol) was hydrogenated in EtOAc/Methanol (4:1, 250mL) over Pearlman's catalyst. After 8 hours, the catalyst was

removed by filtration and the solvents removed to dryness to give compound 226. LCMS and NMR were consistent with the structure.

In order to generate compound 227, a solution of nitromethanetrispropionic acid (4.17 g, 15.04 mmol) and Hunig's base (10.3 ml, 60.17 mmol) in DMF (100mL) were treated dropwise with pentaflourotrifluoro acetate (9.05 ml, 52.65 mmol). After 30 minutes, the reaction was poured onto ice water and extracted with EtOAc. The organic layer was washed with water, brine, and dried over Na_2SO_4. The organic layer was concentrated to dryness and then recrystallized from heptane to give compound 227 as a white solid. LCMS and NMR were consistent with the structure. Compound 227 (1.5 g, 1.93 mmol) and compound 226 (3.7 g, 7.74 mmol) were stirred at room temperature in acetonitrile (15 mL) for 2 hours. The reaction was then evaporated to dryness and purified by column chromatography, eluting with a gradient of 2 to10% methanol in dichloromethane to give compound 228. LCMS and NMR were consistent with the structure. Compound 228 (1.7 g, 1.02 mmol) was treated with Raney Nickel (about 2g wet) in ethanol (100mL) in an atmosphere of hydrogen. After 12 hours, the catalyst was removed by filtration and the organic layer was evaporated to a solid that was used directly in the next step. LCMS and NMR were consistent with the structure. This solid (0.87 g, 0.53 mmol) was treated with benzylglutaric acid (0.18 g, 0.8 mmol), HBTU (0.3 g, 0.8 mmol) and DIEA (273.7 µl, 1.6 mmol) in DMF (5mL). After 16 hours, the DMF was removed under reduced pressure at 65°C to an oil, and the oil was dissolved in dichloromethane. The organic layer was washed with sat. $NaHCO_3$, brine, and dried over Na_2SO_4. After evaporation of the organic layer, the compound was purified by column chromatography and eluted with a gradient of 2 to 20% methanol in dichloromethane to give the coupled product. LCMS and NMR were consistent with the structure. The benzyl ester was deprotected with Pearlman's catalyst under a hydrogen atmosphere for 1 hour. The catalyst was them removed by filtration and the solvents removed to dryness to give the acid. LCMS and NMR were consistent with the structure. The acid (486 mg, 0.27 mmol) was dissolved in dry DMF (3 mL). Pyridine (53.61 µl, 0.66 mmol) was added and the reaction was purged with argon. Pentaflourotriflouro acetate (46.39 µl, 0.4 mmol) was slowly added to the reaction mixture. The color of the reaction changed from pale yellow to burgundy, and gave off a light smoke which was blown away with a stream of argon. The reaction was allowed to stir at room temperature for one hour (completion of reaction was confirmed by LCMS). The solvent was removed under reduced pressure (rotovap) at 70 °C. The residue was diluted with DCM and washed with 1N $NaHSO_4$, brine, saturated sodium bicarbonate and brine again. The organics were dried over Na_2SO_4, filtered, and were concentrated to dryness to give 225 mg of compound 229 as a brittle yellow foam. LCMS and NMR were consistent with the structure.

Oligomeric compound 230, comprising a $GalNAc_3$-23 conjugate group, was prepared from compound 229 using the general procedure illustrated in Example 46. The $GalNAc_3$ cluster portion of the $GalNAc_3$-23 conjugate group ($GalNAc_3$-23_a) can be combined with any cleavable moiety to provide a variety of conjugate groups. The structure of $GalNAc_3$-23 ($GalNAc_3$-23_a-CM) is shown below:



Example 77: Antisense inhibition *in vivo* by oligonucleotides targeting SRB-1 comprising a GalNAc$_3$ conjugate

The oligonucleotides listed below were tested in a dose-dependent study for antisense inhibition of SRB-1 in mice.

Table 64
Modified ASOs targeting SRB-1

ISIS No.	Sequences (5' to 3')	GalNAc$_3$ Cluster	CM	SEQ ID No.
661161	**GalNAc$_3$-3$_{a-o}$,A$_{do}$**G$_{es}$^{m}C$_{es}$T$_{es}$T$_{es}$^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$^{m}C$_{ds}$A$_{ds}$T$_{ds}$ G$_{ds}$A$_{ds}$^{m}C$_{ds}$T$_{ds}$T$_{es}$^{m}C$_{es}$^{m}C$_{es}$T$_{es}$T$_{e}$	GalNAc$_3$-3a	A$_d$	4888
666904	**GalNAc$_3$-3$_{a-o}$,**G$_{es}$^{m}C$_{es}$T$_{es}$T$_{es}$^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$^{m}C$_{ds}$A$_{ds}$T$_{ds}$ G$_{ds}$A$_{ds}$^{m}C$_{ds}$T$_{ds}$T$_{es}$^{m}C$_{es}$^{m}C$_{es}$T$_{es}$T$_{e}$	GalNAc$_3$-3a	PO	4886
673502	**GalNAc$_3$-10$_{a-o}$,A$_{do}$**G$_{es}$^{m}C$_{eo}$T$_{eo}$T$_{eo}$^{m}C$_{eo}$A$_{ds}$G$_{ds}$T$_{ds}$^{m}C$_{ds}$A$_{ds}$T$_{ds}$ G$_{ds}$A$_{ds}$^{m}C$_{ds}$T$_{ds}$T$_{eo}$^{m}C$_{eo}$^{m}C$_{es}$T$_{es}$T$_{e}$	GalNAc$_3$-10a	A$_d$	4888
677844	**GalNAc$_3$-9$_{a-o}$,A$_{do}$**G$_{es}$^{m}C$_{es}$T$_{es}$T$_{es}$^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$^{m}C$_{ds}$A$_{ds}$T$_{ds}$ G$_{ds}$A$_{ds}$^{m}C$_{ds}$T$_{ds}$T$_{es}$^{m}C$_{es}$^{m}C$_{es}$T$_{es}$T$_{e}$	GalNAc$_3$-9a	A$_d$	4888
677843	**GalNAc$_3$-23$_{a-o}$,A$_{do}$**G$_{es}$^{m}C$_{es}$T$_{es}$T$_{es}$^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$^{m}C$_{ds}$A$_{ds}$T$_{ds}$ G$_{ds}$A$_{ds}$^{m}C$_{ds}$T$_{ds}$T$_{es}$^{m}C$_{es}$^{m}C$_{es}$T$_{es}$T$_{e}$	GalNAc$_3$-23a	A$_d$	4888
655861	G$_{es}$^{m}C$_{es}$T$_{es}$T$_{es}$^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$^{m}C$_{ds}$A$_{ds}$T$_{ds}$G$_{ds}$A$_{ds}$^{m}C$_{ds}$T$_{ds}$T$_{es}$^{m}C$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{eo}$**A$_{do}$,-GalNAc$_3$-1$_a$**	GalNAc$_3$-1a	A$_d$	4887
677841	G$_{es}$^{m}C$_{es}$T$_{es}$T$_{es}$^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$^{m}C$_{ds}$A$_{ds}$T$_{ds}$G$_{ds}$A$_{ds}$^{m}C$_{ds}$T$_{ds}$T$_{es}$^{m}C$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{eo}$**A$_{do}$,-GalNAc$_3$-19$_a$**	GalNAc$_3$-19a	A$_d$	4887
677842	G$_{es}$^{m}C$_{es}$T$_{es}$T$_{es}$^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$^{m}C$_{ds}$A$_{ds}$T$_{ds}$G$_{ds}$A$_{ds}$^{m}C$_{ds}$T$_{ds}$T$_{es}$^{m}C$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{eo}$**A$_{do}$,-GalNAc$_3$-20$_a$**	GalNAc$_3$-20a	A$_d$	4887

The structure of GalNAc$_3$-1$_a$ was shown previously in Example 9, GalNAc$_3$-3$_a$ was shown in Example 39, GalNAc$_3$-9a was shown in Example 52, GalNAc$_3$-10a was shown in Example 46, GalNAc$_3$-19$_a$ was shown in Example 70, GalNAc$_3$-20$_a$ was shown in Example 71, and GalNAc$_3$-23$_a$ was shown in Example 76.

Treatment

Six to eight week old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were each injected subcutaneously once at a dosage shown below with an oligonucleotide listed in Table 64 or with saline. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration to determine the SRB-1 mRNA levels using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. The results below are presented as the average percent of SRB-1 mRNA levels for each treatment group, normalized to the saline control.

As illustrated in Table 65, treatment with antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner.

Table 65
SRB-1 mRNA (% Saline)

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA (% Saline)	$GalNAc_3$ Cluster	CM
Saline	n/a	100.0	n/a	n/a
661161	0.5	89.18	$GalNAc_3$-3a	A_d
	1.5	77.02		
	5	29.10		
	15	12.64		
666904	0.5	93.11	$GalNAc_3$-3a	PO
	1.5	55.85		
	5	21.29		
	15	13.43		
673502	0.5	77.75	$GalNAc_3$-10a	A_d
	1.5	41.05		
	5	19.27		
	15	14.41		
677844	0.5	87.65	$GalNAc_3$-9a	A_d
	1.5	93.04		
	5	40.77		
	15	16.95		
677843	0.5	102.28	$GalNAc_3$-23a	A_d
	1.5	70.51		
	5	30.68		
	15	13.26		
655861	0.5	79.72	$GalNAc_3$-1a	A_d
	1.5	55.48		
	5	26.99		
	15	17.58		
677841	0.5	67.43	$GalNAc_3$-19a	A_d
	1.5	45.13		
	5	27.02		
	15	12.41		
677842	0.5	64.13	$GalNAc_3$-20a	A_d
	1.5	53.56		
	5	20.47		
	15	10.23		

Liver transaminase levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in serum were also measured using standard protocols. Total bilirubin and BUN were also evaluated. Changes in body weights were evaluated, with no significant change from the saline group (data not shown). ALTs, ASTs, total bilirubin and BUN values are shown in Table 66 below.

Table 66

ISIS No.	Dosage (mg/kg)	ALT (U/L)	AST (U/L)	Total Bilirubin (mg/dL)	BUN (mg/dL)	$GalNAc_3$ Cluster	CM
Saline	n/a	21	45	0.13	34	n/a	n/a
661161	0.5	28	51	0.14	39	$GalNAc_3$-3a	A_d
	1.5	23	42	0.13	39		
	5	22	59	0.13	37		
	15	21	56	0.15	35		
666904	0.5	24	56	0.14	37	$GalNAc_3$-3a	PO
	1.5	26	68	0.15	35		
	5	23	77	0.14	34		
	15	24	60	0.13	35		
673502	0.5	24	59	0.16	34	$GalNAc_3$-10a	A_d
	1.5	20	46	0.17	32		
	5	24	45	0.12	31		
	15	24	47	0.13	34		
677844	0.5	25	61	0.14	37	$GalNAc_3$-9a	A_d
	1.5	23	64	0.17	33		
	5	25	58	0.13	35		
	15	22	65	0.14	34		
677843	0.5	53	53	0.13	35	$GalNAc_3$-23a	A_d
	1.5	25	54	0.13	34		
	5	21	60	0.15	34		
	15	22	43	0.12	38		
655861	0.5	21	48	0.15	33	$GalNAc_3$-1a	A_d
	1.5	28	54	0.12	35		
	5	22	60	0.13	36		
	15	21	55	0.17	30		
677841	0.5	32	54	0.13	34	$GalNAc_3$-19a	A_d
	1.5	24	56	0.14	34		
	5	23	92	0.18	31		
	15	24	58	0.15	31		
677842	0.5	23	61	0.15	35	$GalNAc_3$-20a	A_d
	1.5	24	57	0.14	34		
	5	41	62	0.15	35		
	15	24	37	0.14	32		

Example 78: Antisense inhibition *in vivo* by oligonucleotides targeting Angiotensinogen comprising a $GalNAc_3$ conjugate

The oligonucleotides listed below were tested in a dose-dependent study for antisense inhibition of Angiotensinogen (AGT) in normotensive Sprague Dawley rats.

Table 67
Modified ASOs targeting AGT

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No.
552668	$^{m}C_{es}A_{es}{}^{m}C_{es}T_{es}G_{es}A_{ds}T_{ds}T_{ds}T_{ds}T_{ds}T_{ds}G_{ds}{}^{m}C_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{es}G_{es}G_{es}A_{es}T_{e}$	n/a	n/a	4892
669509	$^{m}C_{es}A_{es}{}^{m}C_{es}T_{es}G_{es}A_{ds}T_{ds}T_{ds}T_{ds}T_{ds}T_{ds}G_{ds}{}^{m}C_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{es}G_{es}G_{es}A_{es}T_{eo}\mathbf{A_{do'}\text{-}GalNAc_3\text{-}1_a}$	$GalNAc_3\text{-}1_a$	A_d	4893

The structure of $GalNAc_3\text{-}1_a$ was shown previously in Example 9.

Treatment

Six week old, male Sprague Dawley rats were each injected subcutaneously once per week at a dosage shown below, for a total of three doses, with an oligonucleotide listed in Table 67 or with PBS. Each treatment group consisted of 4 animals. The rats were sacrificed 72 hours following the final dose. AGT liver mRNA levels were measured using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. AGT plasma protein levels were measured using the Total Angiotensinogen ELISA (Catalog # JP27412, IBL International, Toronto, ON) with plasma diluted 1:20,000. The results below are presented as the average percent of AGT mRNA levels in liver or AGT protein levels in plasma for each treatment group, normalized to the PBS control.

As illustrated in Table 68, treatment with antisense oligonucleotides lowered AGT liver mRNA and plasma protein levels in a dose-dependent manner, and the oligonucleotide comprising a GalNAc conjugate was significantly more potent than the parent oligonucleotide lacking a GalNAc conjugate.

Table 68
AGT liver mRNA and plasma protein levels

ISIS No.	Dosage (mg/kg)	AGT liver mRNA (% PBS)	AGT plasma protein (% PBS)	$GalNAc_3$ Cluster	CM
PBS	n/a	100	100	n/a	n/a
552668	3	95	122	n/a	n/a
	10	85	97		
	30	46	79		
	90	8	11		
669509	0.3	95	70	$GalNAc_3\text{-}1a$	A_d
	1	95	129		
	3	62	97		
	10	9	23		

Liver transaminase levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in plasma and body weights were also measured at time of sacrifice using standard protocols. The results are shown in Table 69 below.

Table 69
Liver transaminase levels and rat body weights

ISIS No.	Dosage (mg/kg)	ALT (U/L)	AST (U/L)	Body Weight (% of baseline)	$GalNAc_3$ Cluster	CM
PBS	n/a	51	81	186	n/a	n/a
552668	3	54	93	183	n/a	n/a
	10	51	93	194		
	30	59	99	182		
	90	56	78	170		
669509	0.3	53	90	190	$GalNAc_3$-1a	A_d
	1	51	93	192		
	3	48	85	189		
	10	56	95	189		

Example 79: Duration of action *in vivo* of oligonucleotides targeting APOC-III comprising a $GalNAc_3$ conjugate

The oligonucleotides listed in Table 70 below were tested in a single dose study for duration of action in mice.

Table 70
Modified ASOs targeting APOC-III

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No.
304801	$A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{e}$	n/a	n/a	4878
647535	$A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{eo}\mathbf{A_{do'}\text{-}GalNAc_3\text{-}1_a}$	$GalNAc_3$-1a	A_d	4879
663083	$\mathbf{GalNAc_3\text{-}3_a\text{-}_o{}'A_{do}}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{e}$	$GalNAc_3$-3a	A_d	4894
674449	$\mathbf{GalNAc_3\text{-}7_a\text{-}_o{}'A_{do}}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{e}$	$GalNAc_3$-7a	A_d	4894
674450	$\mathbf{GalNAc_3\text{-}10_a\text{-}_o{}'A_{do}}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{e}$	$GalNAc_3$-10a	A_d	4894
674451	$\mathbf{GalNAc_3\text{-}13_a\text{-}_o{}'A_{do}}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{e}$	$GalNAc_3$-13a	A_d	4894

The structure of $GalNAc_3$-1_a was shown previously in Example 9, $GalNAc_3$-3_a was shown in Example 39, $GalNAc_3$-7_a was shown in Example 48, $GalNAc_3$-10_a was shown in Example 46, and $GalNAc_3$-13_a was shown in Example 62.

Treatment

Six to eight week old transgenic mice that express human APOC-III were each injected subcutaneously once with an oligonucleotide listed in Table 70 or with PBS. Each treatment group consisted of 3 animals. Blood was drawn before dosing to determine baseline and at 72 hours, 1 week, 2 weeks, 3

weeks, 4 weeks, 5 weeks, and 6 weeks following the dose. Plasma triglyceride and APOC-III protein levels were measured as described in Example 20. The results below are presented as the average percent of plasma triglyceride and APOC-III levels for each treatment group, normalized to baseline levels, showing that the oligonucleotides comprising a GalNAc conjugate group exhibited a longer duration of action than the parent oligonucleotide without a conjugate group (ISIS 304801) even though the dosage of the parent was three times the dosage of the oligonucleotides comprising a GalNAc conjugate group.

Table 71

Plasma triglyceride and APOC-III protein levels in transgenic mice

ISIS No.	Dosage (mg/kg)	Time point (days post-dose)	Triglycerides (% baseline)	APOC-III protein (% baseline)	$GalNAc_3$ Cluster	CM
PBS	n/a	3	97	102	n/a	n/a
		7	101	98		
		14	108	98		
		21	107	107		
		28	94	91		
		35	88	90		
		42	91	105		
304801	30	3	40	34	n/a	n/a
		7	41	37		
		14	50	57		
		21	50	50		
		28	57	73		
		35	68	70		
		42	75	93		
647535	10	3	36	37	$GalNAc_3$-1a	A_d
		7	39	47		
		14	40	45		
		21	41	41		
		28	42	62		
		35	69	69		
		42	85	102		
663083	10	3	24	18	$GalNAc_3$-3a	A_d
		7	28	23		
		14	25	27		
		21	28	28		
		28	37	44		
		35	55	57		
		42	60	78		
674449	10	3	29	26	$GalNAc_3$-7a	A_d
		7	32	31		
		14	38	41		
		21	44	44		
		28	53	63		
		35	69	77		
		42	78	99		
674450	10	3	33	30	$GalNAc_3$-10a	A_d
		7	35	34		

		14	31	34		
		21	44	44		
		28	56	61		
		35	68	70		
		42	83	95		
674451	10	3	35	33	GalNAc$_3$-13a	A_d
		7	24	32		
		14	40	34		
		21	48	48		
		28	54	67		
		35	65	75		
		42	74	97		

Example 80: Antisense inhibition *in vivo* by oligonucleotides targeting Alpha-1 Antitrypsin (A1AT) comprising a GalNAc$_3$ Conjugate

The oligonucleotides listed in Table 72 below were tested in a study for dose-dependent inhibition of A1AT in mice.

Table 72
Modified ASOs targeting A1AT

ISIS No.	Sequences (5' to 3')	GalNAc$_3$ Cluster	CM	SEQ ID No.
476366	$A_{es}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{es}A_{es}A_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{ds}G_{ds}A_{ds}A_{ds}G_{ds}G_{ds}A_{es}A_{es}G_{es}G_{es}A_e$	n/a	n/a	4895
656326	$A_{es}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{es}A_{es}A_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{ds}G_{ds}A_{ds}A_{ds}G_{ds}G_{ds}A_{es}A_{es}G_{es}G_{es}A_{eo}$**$A_{do'}$-GalNAc$_3-1_a$**	GalNAc$_3$-1a	A_d	4896
678381	**GalNAc$_3$-3$_{a-o}$,A_{do}**$A_{es}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{es}A_{es}A_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{ds}G_{ds}A_{ds}A_{ds}G_{ds}G_{ds}A_{es}A_{es}G_{es}G_{es}A_e$	GalNAc$_3$-3a	A_d	4897
678382	**GalNAc$_3$-7$_{a-o}$,A_{do}**$A_{es}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{es}A_{es}A_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{ds}G_{ds}A_{ds}A_{ds}G_{ds}G_{ds}A_{es}A_{es}G_{es}G_{es}A_e$	GalNAc$_3$-7a	A_d	4897
678383	**GalNAc$_3$-10$_{a-o}$,A_{do}**$A_{es}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{es}A_{es}A_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{ds}G_{ds}A_{ds}A_{ds}G_{ds}G_{ds}A_{es}A_{es}G_{es}G_{es}A_e$	GalNAc$_3$-10a	A_d	4897
678384	**GalNAc$_3$-13$_{a-o}$,A_{do}**$A_{es}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{es}A_{es}A_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{ds}G_{ds}A_{ds}A_{ds}G_{ds}G_{ds}A_{es}A_{es}G_{es}G_{es}A_e$	GalNAc$_3$-13a	A_d	4897

The structure of GalNAc$_3$-1$_a$ was shown previously in Example 9, GalNAc$_3$-3$_a$ was shown in Example 39, GalNAc$_3$-7$_a$ was shown in Example 48, GalNAc$_3$-10$_a$ was shown in Example 46, and GalNAc$_3$-13$_a$ was shown in Example 62.

Treatment

Six week old, male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were each injected subcutaneously once per week at a dosage shown below, for a total of three doses, with an oligonucleotide listed in Table 72 or with PBS. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration. A1AT liver mRNA levels were determined using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard

protocols. A1AT plasma protein levels were determined using the Mouse Alpha 1-Antitrypsin ELISA (catalog # 41-A1AMS-E01, Alpco, Salem, NH). The results below are presented as the average percent of A1AT liver mRNA and plasma protein levels for each treatment group, normalized to the PBS control.

As illustrated in Table 73, treatment with antisense oligonucleotides lowered A1AT liver mRNA and A1AT plasma protein levels in a dose-dependent manner. The oligonucleotides comprising a GalNAc conjugate were significantly more potent than the parent (ISIS 476366).

Table 73
A1AT liver mRNA and plasma protein levels

ISIS No.	Dosage (mg/kg)	A1AT liver mRNA (% PBS)	A1AT plasma protein (% PBS)	$GalNAc_3$ Cluster	CM
PBS	n/a	100	100	n/a	n/a
476366	5	86	78	n/a	n/a
	15	73	61		
	45	30	38		
656326	0.6	99	90	$GalNAc_3$-1a	A_d
	2	61	70		
	6	15	30		
	18	6	10		
678381	0.6	105	90	$GalNAc_3$-3a	A_d
	2	53	60		
	6	16	20		
	18	7	13		
678382	0.6	90	79	$GalNAc_3$-7a	A_d
	2	49	57		
	6	21	27		
	18	8	11		
678383	0.6	94	84	$GalNAc_3$-10a	A_d
	2	44	53		
	6	13	24		
	18	6	10		
678384	0.6	106	91	$GalNAc_3$-13a	A_d
	2	65	59		
	6	26	31		
	18	11	15		

Liver transaminase and BUN levels in plasma were measured at time of sacrifice using standard protocols. Body weights and organ weights were also measured. The results are shown in Table 74 below. Body weight is shown as % relative to baseline. Organ weights are shown as % of body weight relative to the PBS control group.

Table 74

ISIS No.	Dosage (mg/kg)	ALT (U/L)	AST (U/L)	BUN (mg/dL)	Body weight (% baseline)	Liver weight (Rel % BW)	Kidney weight (Rel % BW)	Spleen weight (Rel % BW)
PBS	n/a	25	51	37	119	100	100	100

476366	5	34	68	35	116	91	98	106
	15	37	74	30	122	92	101	128
	45	30	47	31	118	99	108	123
656326	0.6	29	57	40	123	100	103	119
	2	36	75	39	114	98	111	106
	6	32	67	39	125	99	97	122
	18	46	77	36	116	102	109	101
678381	0.6	26	57	32	117	93	109	110
	2	26	52	33	121	96	106	125
	6	40	78	32	124	92	106	126
	18	31	54	28	118	94	103	120
678382	0.6	26	42	35	114	100	103	103
	2	25	50	31	117	91	104	117
	6	30	79	29	117	89	102	107
	18	65	112	31	120	89	104	113
678383	0.6	30	67	38	121	91	100	123
	2	33	53	33	118	98	102	121
	6	32	63	32	117	97	105	105
	18	36	68	31	118	99	103	108
678384	0.6	36	63	31	118	98	103	98
	2	32	61	32	119	93	102	114
	6	34	69	34	122	100	100	96
	18	28	54	30	117	98	101	104

Example 81: Duration of action *in vivo* of oligonucleotides targeting A1AT comprising a $GalNAc_3$ cluster

The oligonucleotides listed in Table 72 were tested in a single dose study for duration of action in mice.

Treatment

Six week old, male C57BL/6 mice were each injected subcutaneously once with an oligonucleotide listed in Table 72 or with PBS. Each treatment group consisted of 4 animals. Blood was drawn the day before dosing to determine baseline and at 5, 12, 19, and 25 days following the dose. Plasma A1AT protein levels were measured via ELISA (see Example 80). The results below are presented as the average percent of plasma A1AT protein levels for each treatment group, normalized to baseline levels. The results show that the oligonucleotides comprising a GalNAc conjugate were more potent and had longer duration of action than the parent lacking a GalNAc conjugate (ISIS 476366). Furthermore, the oligonucleotides comprising a 5'-GalNAc conjugate (ISIS 678381, 678382, 678383, and 678384) were generally even more potent with even longer duration of action than the oligonucleotide comprising a 3'-GalNAc conjugate (ISIS 656326).

Table 75
Plasma A1AT protein levels in mice

ISIS No.	Dosage (mg/kg)	Time point (days post-dose)	A1AT (% baseline)	$GalNAc_3$ Cluster	CM
PBS	n/a	5	93	n/a	n/a
		12	93		
		19	90		
		25	97		
476366	100	5	38	n/a	n/a
		12	46		
		19	62		
		25	77		
656326	18	5	33	$GalNAc_3$-1a	A_d
		12	36		
		19	51		
		25	72		
678381	18	5	21	$GalNAc_3$-3a	A_d
		12	21		
		19	35		
		25	48		
678382	18	5	21	$GalNAc_3$-7a	A_d
		12	21		
		19	39		
		25	60		
678383	18	5	24	$GalNAc_3$-10a	A_d
		12	21		
		19	45		
		25	73		
678384	18	5	29	$GalNAc_3$-13a	A_d
		12	34		
		19	57		
		25	76		

Example 82: Antisense inhibition *in vitro* by oligonucleotides targeting SRB-1 comprising a $GalNAc_3$ conjugate

Primary mouse liver hepatocytes were seeded in 96 well plates at 15,000 cells/well 2 hours prior to treatment. The oligonucleotides listed in Table 76 were added at 2, 10, 50, or 250 nM in Williams E medium and cells were incubated overnight at 37 °C in 5% CO_2. Cells were lysed 16 hours following oligonucleotide addition, and total RNA was purified using RNease 3000 BioRobot (Qiagen). SRB-1 mRNA levels were determined using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. IC_{50} values were determined using Prism 4 software (GraphPad). The results show that oligonucleotides comprising a variety of different GalNAc conjugate groups and a variety of different cleavable moieties are significantly more potent in an *in vitro* free uptake experiment than the parent oligonucleotides lacking a GalNAc conjugate group (ISIS 353382 and 666841).

Table 76
Inhibition of SRB-1 expression *in vitro*

ISIS No.	Sequence (5' to 3')	Linkages	GalNAc cluster	CM	IC_{50} (nM)	SEQ ID No.
353382	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	n/a	n/a	250	4886
655861	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{eo}\mathbf{A_{do'}\text{-}GalNAc_3\text{-}1_a}$	PS	$GalNAc_3$ $\text{-}1_a$	A_d	40	4887
661161	$\mathbf{GalNAc_3\text{-}3_a\text{-}_{o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	$GalNAc_3$ $\text{-}3_a$	A_d	40	4888
661162	$\mathbf{GalNAc_3\text{-}3_a\text{-}_{o'}A_{do}}G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T_{e}$	PO/PS	$GalNAc_3$ $\text{-}3_a$	A_d	8	4888
664078	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{eo}\mathbf{A_{do'}\text{-}GalNAc_3\text{-}9_a}$	PS	$GalNAc_3$ $\text{-}9_a$	A_d	20	4887
665001	$\mathbf{GalNAc_3\text{-}8_a\text{-}_{o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	$GalNAc_3$ $\text{-}8_a$	A_d	70	4888
666224	$\mathbf{GalNAc_3\text{-}5_a\text{-}_{o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	$GalNAc_3$ $\text{-}5_a$	A_d	80	4888
666841	$G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T_{e}$	PO/PS	n/a	n/a	>250	4886
666881	$\mathbf{GalNAc_3\text{-}10_a\text{-}_{o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	$GalNAc_3$ $\text{-}10_a$	A_d	30	4888
666904	$\mathbf{GalNAc_3\text{-}3_a\text{-}_{o'}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	$GalNAc_3$ $\text{-}3_a$	PO	9	4886
666924	$\mathbf{GalNAc_3\text{-}3_a\text{-}_{o'}T_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	$GalNAc_3$ $\text{-}3_a$	T_d	15	4891
666961	$\mathbf{GalNAc_3\text{-}6_a\text{-}_{o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	$GalNAc_3$ $\text{-}6_a$	A_d	150	4888
666981	$\mathbf{GalNAc_3\text{-}7_a\text{-}_{o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	$GalNAc_3$ $\text{-}7_a$	A_d	20	4888
670061	$\mathbf{GalNAc_3\text{-}13_a\text{-}_{o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	$GalNAc_3$ $\text{-}13_a$	A_d	30	4888
670699	$\mathbf{GalNAc_3\text{-}3_a\text{-}_{o'}T_{do}}G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T_{e}$	PO/PS	$GalNAc_3$ $\text{-}3_a$	T_d	15	4891
670700	$\mathbf{GalNAc_3\text{-}3_a\text{-}_{o'}A_{eo}}G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T$	PO/PS	$GalNAc_3$ $\text{-}3_a$	A_e	30	4888
670701	$\mathbf{GalNAc_3\text{-}3_a\text{-}_{o'}T_{eo}}G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T_{e}$	PO/PS	$GalNAc_3$ $\text{-}3_a$	T_e	25	4891
671144	$\mathbf{GalNAc_3\text{-}12_a\text{-}_{o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	$GalNAc_3$ $\text{-}12_a$	A_d	40	4888
671165	$\mathbf{GalNAc_3\text{-}13_a\text{-}_{o'}A_{do}}G_{es}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}T$	PO/PS	$GalNAc_3$ $\text{-}13_a$	A_d	8	4888
671261	$\mathbf{GalNAc_3\text{-}14_a\text{-}_{o'}A_{do}}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	PS	$GalNAc_3$ $\text{-}14_a$	A_d	>250	4888

671262	**GalNAc$_3$-15$_a$-o,A$_{do}$**G$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{es}$ ^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{ds}$T$_{ds}$G$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$ T$_{es}$ ^{m}C$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{e}$	PS	GalNAc$_3$ -15$_a$	A$_d$	>250	4888
673501	**GalNAc$_3$-7$_a$-o,A$_{do}$**G$_{es}$ ^{m}C$_{eo}$T$_{eo}$T$_{eo}$ ^{m}C$_{eo}$A$_{ds}$G$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{ds}$T$_{ds}$G$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$T$_{eo}$ ^{m}C$_{eo}$ ^{m}C$_{es}$T$_{es}$T$_{e}$	PO/PS	GalNAc$_3$ -7$_a$	A$_d$	30	4888
673502	**GalNAc$_3$-10$_a$-o,A$_{do}$**G$_{es}$ ^{m}C$_{eo}$T$_{eo}$T$_{eo}$ ^{m}C$_{eo}$A$_{ds}$G$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{ds}$T$_{ds}$G$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$T$_{eo}$ ^{m}C$_{eo}$ ^{m}C$_{es}$T$_{es}$T$_{e}$	PO/PS	GalNAc$_3$ -10$_a$	A$_d$	8	4888
675441	**GalNAc$_3$-17$_a$-o,A$_{do}$**G$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{es}$ ^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{ds}$T$_{ds}$G$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$ T$_{es}$ ^{m}C$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{e}$	PS	GalNAc$_3$ -17$_a$	A$_d$	30	4888
675442	**GalNAc$_3$-18$_a$-o,A$_{do}$**G$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{es}$ ^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{ds}$T$_{ds}$G$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$ T$_{es}$ ^{m}C$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{e}$	PS	GalNAc$_3$ -18$_a$	A$_d$	20	4888
677841	G$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{es}$ ^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{ds}$T$_{ds}$G$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$T$_{es}$ ^{m}C$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{eo}$**A$_{do}$'-GalNAc$_3$-19$_a$**	PS	GalNAc$_3$ -19$_a$	A$_d$	40	4887
677842	G$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{es}$ ^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{ds}$T$_{ds}$G$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$T$_{es}$ ^{m}C$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{eo}$**A$_{do}$'-GalNAc$_3$-20$_a$**	PS	GalNAc$_3$ -20$_a$	A$_d$	30	4887
677843	**GalNAc$_3$-23$_a$-o,A$_{do}$**G$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{es}$ ^{m}C$_{es}$A$_{ds}$G$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{ds}$T$_{ds}$G$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$ T$_{es}$ ^{m}C$_{es}$ ^{m}C$_{es}$T$_{es}$T$_{e}$	PS	GalNAc$_3$ -23$_a$	A$_d$	40	4888

The structure of GalNAc$_3$-1$_a$ was shown previously in Example 9, GalNAc$_3$-3$_a$ was shown in Example 39, GalNAc$_3$-5$_a$ was shown in Example 49, GalNAc$_3$-6$_a$ was shown in Example 51, GalNAc$_3$-7$_a$ was shown in Example 48, GalNAc$_3$-8$_a$ was shown in Example 47, GalNAc$_3$-9$_a$ was shown in Example 52, GalNAc$_3$-10$_a$ was shown in Example 46, GalNAc$_3$-12$_a$ was shown in Example 61, GalNAc$_3$-13$_a$ was shown in Example 62, GalNAc$_3$-14$_a$ was shown in Example 63, GalNAc$_3$-15$_a$ was shown in Example 64, GalNAc$_3$-17$_a$ was shown in Example 68, GalNAc$_3$-18$_a$ was shown in Example 69, GalNAc$_3$-19$_a$ was shown in Example 70, GalNAc$_3$-20$_a$ was shown in Example 71, and GalNAc$_3$-23$_a$ was shown in Example 76.

Example 83: Antisense inhibition *in vivo* by oligonucleotides targeting Factor XI comprising a GalNAc$_3$ cluster

The oligonucleotides listed in Table 77 below were tested in a study for dose-dependent inhibition of Factor XI in mice.

Table 77
Modified oligonucleotides targeting Factor XI

ISIS No.	Sequence (5' to 3')	GalNAc cluster	CM	SEQ ID No.
404071	T$_{es}$G$_{es}$G$_{es}$T$_{es}$A$_{es}$A$_{ds}$T$_{ds}$ ^{m}C$_{ds}$ ^{m}C$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$T$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{es}$G$_{es}$ A$_{es}$G$_{es}$G$_{e}$	n/a	n/a	4889
656173	T$_{es}$G$_{eo}$G$_{eo}$T$_{eo}$A$_{eo}$A$_{ds}$T$_{ds}$ ^{m}C$_{ds}$ ^{m}C$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$T$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{eo}$G$_{eo}$ A$_{es}$G$_{es}$G$_{eo}$**A$_{do}$'-GalNAc$_3$-1$_a$**	GalNAc$_3$-1$_a$	A$_d$	4890
663086	**GalNAc$_3$-3$_a$-o,A$_{do}$**T$_{es}$G$_{eo}$G$_{eo}$T$_{eo}$A$_{eo}$A$_{ds}$T$_{ds}$ ^{m}C$_{ds}$ ^{m}C$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$ T$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{eo}$G$_{eo}$A$_{es}$G$_{es}$G$_{e}$	GalNAc$_3$-3$_a$	A$_d$	4898
678347	**GalNAc$_3$-7$_a$-o,A$_{do}$**T$_{es}$G$_{eo}$G$_{eo}$T$_{eo}$A$_{eo}$A$_{ds}$T$_{ds}$ ^{m}C$_{ds}$ ^{m}C$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$ T$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{eo}$G$_{eo}$A$_{es}$G$_{es}$G$_{e}$	GalNAc$_3$-7$_a$	A$_d$	4898
678348	**GalNAc$_3$-10$_a$-o,A$_{do}$**T$_{es}$G$_{eo}$G$_{eo}$T$_{eo}$A$_{eo}$A$_{ds}$T$_{ds}$ ^{m}C$_{ds}$ ^{m}C$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$ T$_{ds}$T$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{eo}$G$_{eo}$A$_{es}$G$_{es}$G$_{e}$	GalNAc$_3$-10$_a$	A$_d$	4898
678349	**GalNAc$_3$-13$_a$-o,A$_{do}$**T$_{es}$G$_{eo}$G$_{eo}$T$_{eo}$A$_{eo}$A$_{ds}$T$_{ds}$ ^{m}C$_{ds}$ ^{m}C$_{ds}$A$_{ds}$ ^{m}C$_{ds}$T$_{ds}$ T$_{ds}$T$_{ds}$T$_{ds}$ ^{m}C$_{ds}$A$_{eo}$G$_{eo}$A$_{es}$G$_{es}$G$_{e}$	GalNAc$_3$-13$_a$	A$_d$	4898

The structure of $GalNAc_3$-1_a was shown previously in Example 9, $GalNAc_3$-3_a was shown in Example 39, $GalNAc_3$-7_a was shown in Example 48, $GalNAc_3$-10_a was shown in Example 46, and $GalNAc_3$-13_a was shown in Example 62.

Treatment

Six to eight week old mice were each injected subcutaneously once per week at a dosage shown below, for a total of three doses, with an oligonucleotide listed below or with PBS. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final dose. Factor XI liver mRNA levels were measured using real-time PCR and normalized to cyclophilin according to standard protocols. Liver transaminases, BUN, and bilirubin were also measured. The results below are presented as the average percent for each treatment group, normalized to the PBS control.

As illustrated in Table 78, treatment with antisense oligonucleotides lowered Factor XI liver mRNA in a dose-dependent manner. The results show that the oligonucleotides comprising a GalNAc conjugate were more potent than the parent lacking a GalNAc conjugate (ISIS 404071). Furthermore, the oligonucleotides comprising a 5'-GalNAc conjugate (ISIS 663086, 678347, 678348, and 678349) were even more potent than the oligonucleotide comprising a 3'-GalNAc conjugate (ISIS 656173).

Table 78

Factor XI liver mRNA, liver transaminase, BUN, and bilirubin levels

ISIS No.	Dosage (mg/kg)	Factor XI mRNA (% PBS)	ALT (U/L)	AST (U/L)	BUN (mg/dL)	Bilirubin (mg/dL)	$GalNAc_3$ Cluster	SEQ ID No.
PBS	n/a	100	63	70	21	0.18	n/a	n/a
404071	3	65	41	58	21	0.15	n/a	4889
	10	33	49	53	23	0.15		
	30	17	43	57	22	0.14		
656173	0.7	43	90	89	21	0.16	$GalNAc_3$-1a	4890
	2	9	36	58	26	0.17		
	6	3	50	63	25	0.15		
663086	0.7	33	91	169	25	0.16	$GalNAc_3$-3a	4898
	2	7	38	55	21	0.16		
	6	1	34	40	23	0.14		
678347	0.7	35	28	49	20	0.14	$GalNAc_3$-7a	4898
	2	10	180	149	21	0.18		
	6	1	44	76	19	0.15		
678348	0.7	39	43	54	21	0.16	$GalNAc_3$-10a	4898
	2	5	38	55	22	0.17		
	6	2	25	38	20	0.14		
678349	0.7	34	39	46	20	0.16	$GalNAc_3$-13a	4898
	2	8	43	63	21	0.14		
	6	2	28	41	20	0.14		

Example 84: Duration of action *in vivo* of oligonucleotides targeting Factor XI comprising a $GalNAc_3$ Conjugate

The oligonucleotides listed in Table 77 were tested in a single dose study for duration of action in mice.

Treatment

Six to eight week old mice were each injected subcutaneously once with an oligonucleotide listed in Table 77 or with PBS. Each treatment group consisted of 4 animals. Blood was drawn by tail bleeds the day before dosing to determine baseline and at 3, 10, and 17 days following the dose. Plasma Factor XI protein levels were measured by ELISA using Factor XI capture and biotinylated detection antibodies from R & D Systems, Minneapolis, MN (catalog # AF2460 and # BAF2460, respectively) and the OptEIA Reagent Set B (Catalog # 550534, BD Biosciences, San Jose, CA). The results below are presented as the average percent of plasma Factor XI protein levels for each treatment group, normalized to baseline levels. The results show that the oligonucleotides comprising a GalNAc conjugate were more potent with longer duration of action than the parent lacking a GalNAc conjugate (ISIS 404071). Furthermore, the oligonucleotides comprising a 5'-GalNAc conjugate (ISIS 663086, 678347, 678348, and 678349) were even more potent with an even longer duration of action than the oligonucleotide comprising a 3'-GalNAc conjugate (ISIS 656173).

Table 79
Plasma Factor XI protein levels in mice

ISIS No.	Dosage (mg/kg)	Time point (days post-dose)	Factor XI (% baseline)	$GalNAc_3$ Cluster	CM	SEQ ID No.
PBS	n/a	3	123	n/a	n/a	n/a
		10	56			
		17	100			
404071	30	3	11	n/a	n/a	4889
		10	47			
		17	52			
656173	6	3	1	$GalNAc_3$-1a	A_d	4890
		10	3			
		17	21			
663086	6	3	1	$GalNAc_3$-3a	A_d	4898
		10	2			
		17	9			
678347	6	3	1	$GalNAc_3$-7a	A_d	4898
		10	1			
		17	8			
678348	6	3	1	$GalNAc_3$-10a	A_d	4898
		10	1			
		17	6			
678349	6	3	1	$GalNAc_3$-13a	A_d	4898
		10	1			
		17	5			

Example 85: Antisense inhibition *in vivo* by oligonucleotides targeting SRB-1 comprising a $GalNAc_3$ Conjugate

Oligonucleotides listed in Table 76 were tested in a dose-dependent study for antisense inhibition of SRB-1 in mice.

Treatment

Six to eight week old C57BL/6 mice were each injected subcutaneously once per week at a dosage shown below, for a total of three doses, with an oligonucleotide listed in Table 76 or with saline. Each treatment group consisted of 4 animals. The mice were sacrificed 48 hours following the final administration to determine the SRB-1 mRNA levels using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. The results below are presented as the average percent of liver SRB-1 mRNA levels for each treatment group, normalized to the saline control.

As illustrated in Tables 80 and 81, treatment with antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner.

Table 80
SRB-1 mRNA in liver

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA (% Saline)	$GalNAc_3$ Cluster	CM
Saline	n/a	100	n/a	n/a
655861	0.1	94	$GalNAc_3$-1a	A_d
	0.3	119		
	1	68		
	3	32		
661161	0.1	120	$GalNAc_3$-3a	A_d
	0.3	107		
	1	68		
	3	26		
666881	0.1	107	$GalNAc_3$-10a	A_d
	0.3	107		
	1	69		
	3	27		
666981	0.1	120	$GalNAc_3$-7a	A_d
	0.3	103		
	1	54		
	3	21		
670061	0.1	118	$GalNAc_3$-13a	A_d
	0.3	89		
	1	52		
	3	18		
677842	0.1	119	$GalNAc_3$-20a	A_d
	0.3	96		
	1	65		
	3	23		

Table 81
SRB-1 mRNA in liver

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA (% Saline)	$GalNAc_3$ Cluster	CM
661161	0.1	107	$GalNAc_3$-3a	A_d
	0.3	95		
	1	53		
	3	18		
677841	0.1	110	$GalNAc_3$-19a	A_d
	0.3	88		
	1	52		
	3	25		

Liver transaminase levels, total bilirubin, BUN, and body weights were also measured using standard protocols. Average values for each treatment group are shown in Table 82 below.

Table 82

ISIS No.	Dosage (mg/kg)	ALT (U/L)	AST (U/L)	Bilirubin (mg/dL)	BUN (mg/dL)	Body Weight (% baseline)	$GalNAc_3$ Cluster	CM
Saline	n/a	19	39	0.17	26	118	n/a	n/a
655861	0.1	25	47	0.17	27	114	$GalNAc_3$-1a	A_d
	0.3	29	56	0.15	27	118		
	1	20	32	0.14	24	112		
	3	27	54	0.14	24	115		
661161	0.1	35	83	0.13	24	113	$GalNAc_3$-3a	A_d
	0.3	42	61	0.15	23	117		
	1	34	60	0.18	22	116		
	3	29	52	0.13	25	117		
666881	0.1	30	51	0.15	23	118	$GalNAc_3$-10a	A_d
	0.3	49	82	0.16	25	119		
	1	23	45	0.14	24	117		
	3	20	38	0.15	21	112		
666981	0.1	21	41	0.14	22	113	$GalNAc_3$-7a	A_d
	0.3	29	49	0.16	24	112		
	1	19	34	0.15	22	111		
	3	77	78	0.18	25	115		
670061	0.1	20	63	0.18	24	111	$GalNAc_3$-13a	A_d
	0.3	20	57	0.15	21	115		
	1	20	35	0.14	20	115		
	3	27	42	0.12	20	116		
677842	0.1	20	38	0.17	24	114	$GalNAc_3$-20a	A_d
	0.3	31	46	0.17	21	117		
	1	22	34	0.15	21	119		
	3	41	57	0.14	23	118		

Example 86: Antisense inhibition *in vivo* by oligonucleotides targeting TTR comprising a $GalNAc_3$ cluster

Oligonucleotides listed in Table 83 below were tested in a dose-dependent study for antisense inhibition of human transthyretin (TTR) in transgenic mice that express the human TTR gene.

Treatment

Eight week old TTR transgenic mice were each injected subcutaneously once per week for three weeks, for a total of three doses, with an oligonucleotide and dosage listed in the tables below or with PBS. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration. Tail bleeds were performed at various time points throughout the experiment, and plasma TTR protein, ALT, and AST levels were measured and reported in Tables 85-87. After the animals were sacrificed, plasma ALT, AST, and human TTR levels were measured, as were body weights, organ weights, and liver human TTR mRNA levels. TTR protein levels were measured using a clinical analyzer (AU480, Beckman Coulter, CA). Real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) were used according to standard protocols to determine liver human TTR mRNA levels. The results presented in Tables 84-87 are the average values for each treatment group. The mRNA levels are the average values relative to the average for the PBS group. Plasma protein levels are the average values relative to the average value for the PBS group at baseline. Body weights are the average percent weight change from baseline until sacrifice for each individual treatment group. Organ weights shown are normalized to the animal's body weight, and the average normalized organ weight for each treatment group is then presented relative to the average normalized organ weight for the PBS group.

In Tables 84-87, "BL" indicates baseline, measurements that were taken just prior to the first dose. As illustrated in Tables 84 and 85, treatment with antisense oligonucleotides lowered TTR expression levels in a dose-dependent manner. The oligonucleotides comprising a GalNAc conjugate were more potent than the parent lacking a GalNAc conjugate (ISIS 420915). Furthermore, the oligonucleotides comprising a GalNAc conjugate and mixed PS/PO internucleoside linkages were even more potent than the oligonucleotide comprising a GalNAc conjugate and full PS linkages.

Table 83
Oligonucleotides targeting human TTR

Isis No.	Sequence 5' to 3'	Linkages	GalNAc cluster	CM	SEQ ID No.
420915	$T_{es}{}^{m}C_{es}T_{es}T_{es}G_{es}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{es}T_{es}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	PS	n/a	n/a	4899
660261	$T_{es}{}^{m}C_{es}T_{es}T_{es}G_{es}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{es}T_{es}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{eo}\mathbf{A_{do'}\text{-}GalNAc_3\text{-}1_a}$	PS	$GalNAc_3$-1a	A_d	4900
682883	$\mathbf{GalNAc_3\text{-}3_{a\text{-}o}},T_{es}{}^{m}C_{eo}T_{eo}T_{eo}G_{eo}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{eo}T_{eo}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	PS/PO	$GalNAc_3$-3a	PO	4899
682884	$\mathbf{GalNAc_3\text{-}7_{a\text{-}o}},T_{es}{}^{m}C_{eo}T_{eo}T_{eo}G_{eo}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{eo}T_{eo}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	PS/PO	$GalNAc_3$-7a	PO	4899
682885	$\mathbf{GalNAc_3\text{-}10_{a\text{-}o}},T_{es}{}^{m}C_{eo}T_{eo}T_{eo}G_{eo}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{eo}T_{eo}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	PS/PO	$GalNAc_3$-10a	PO	4899
682886	$\mathbf{GalNAc_3\text{-}13_{a\text{-}o}},T_{es}{}^{m}C_{eo}T_{eo}T_{eo}G_{eo}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{eo}T_{eo}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	PS/PO	$GalNAc_3$-13a	PO	4899
684057	$T_{es}{}^{m}C_{eo}T_{eo}T_{eo}G_{eo}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{eo}T_{eo}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{eo}\mathbf{A_{do'}\text{-}GalNAc_3\text{-}19_a}$	PS/PO	$GalNAc_3$-19a	A_d	4900

The legend for Table 85 can be found in Example 74. The structure of $GalNAc_3$-1 was shown in Example 9. The structure of $GalNAc_3$-3_a was shown in Example 39. The structure of $GalNAc_3$-7_a was shown in Example 48. The structure of $GalNAc_3$-10_a was shown in Example 46. The structure of $GalNAc_3$-13_a was shown in Example 62. The structure of $GalNAc_3$-19_a was shown in Example 70.

Table 84
Antisense inhibition of human TTR *in vivo*

Isis No.	Dosage (mg/kg)	TTR mRNA (% PBS)	Plasma TTR protein (% PBS)	GalNAc cluster	CM	SEQ ID No.
PBS	n/a	100	100	n/a	n/a	
420915	6	99	95	n/a	n/a	4899
	20	48	65			
	60	18	28			
660261	0.6	113	87	$GalNAc_3$-1a	A_d	4900
	2	40	56			
	6	20	27			
	20	9	11			

Table 85
Antisense inhibition of human TTR *in vivo*

Isis No.	Dosage (mg/kg)	TTR mRNA (% PBS)	Plasma TTR protein (% PBS at BL)				GalNAc cluster	CM	SEQ ID No.
			BL	Day 3	Day 10	Day 17 (After sac)			
PBS	n/a	100	100	96	90	114	n/a	n/a	
420915	6	74	106	86	76	83	n/a	n/a	4899
	20	43	102	66	61	58			
	60	24	92	43	29	32			
682883	0.6	60	88	73	63	68	$GalNAc_3$-3a	PO	4899
	2	18	75	38	23	23			
	6	10	80	35	11	9			
682884	0.6	56	88	78	63	67	$GalNAc_3$-7a	PO	4899
	2	19	76	44	25	23			
	6	15	82	35	21	24			
682885	0.6	60	92	77	68	76	$GalNAc_3$-10a	PO	4899
	2	22	93	58	32	32			
	6	17	85	37	25	20			
682886	0.6	57	91	70	64	69	$GalNAc_3$-13a	PO	4899
	2	21	89	50	31	30			
	6	18	102	41	24	27			
684057	0.6	53	80	69	56	62	$GalNAc_3$-19a	A_d	4900
	2	21	92	55	34	30			
	6	11	82	50	18	13			

Table 86
Transaminase levels, body weight changes, and relative organ weights

Isis No.	Dosage (mg/kg)	ALT (U/L)				AST (U/L)				Body (% BL)	Liver (% PBS)	Spleen (% PBS)	Kidney (% PBS)	SEQ ID No.
		BL	Day 3	Day 10	Day 17	BL	Day 3	Day 10	Day 17					
PBS	n/a	33	34	33	24	58	62	67	52	105	100	100	100	n/a
420915	6	34	33	27	21	64	59	73	47	115	99	89	91	4899
	20	34	30	28	19	64	54	56	42	111	97	83	89	
	60	34	35	31	24	61	58	71	58	113	102	98	95	
660261	0.6	33	38	28	26	70	71	63	59	111	96	99	92	4900
	2	29	32	31	34	61	60	68	61	118	100	92	90	
	6	29	29	28	34	58	59	70	90	114	99	97	95	
	20	33	32	28	33	64	54	68	95	114	101	106	92	

Table 87
Transaminase levels, body weight changes, and relative organ weights

Isis No.	Dosage (mg/kg)	ALT (U/L)				AST (U/L)				Body (% BL)	Liver (% PBS)	Spleen (% PBS)	Kidney (% PBS)	SEQ ID No.
		BL	Day 3	Day 10	Day 17	BL	Day 3	Day 10	Day 17					
PBS	n/a	32	34	37	41	62	78	76	77	104	100	100	100	n/a
420915	6	32	30	34	34	61	71	72	66	102	103	102	105	4899
	20	41	34	37	33	80	76	63	54	106	107	135	101	
	60	36	30	32	34	58	81	57	60	106	105	104	99	
682883	0.6	32	35	38	40	53	81	74	76	104	101	112	95	4899
	2	38	39	42	43	71	84	70	77	107	98	116	99	
	6	35	35	41	38	62	79	103	65	105	103	143	97	
682884	0.6	33	32	35	34	70	74	75	67	101	100	130	99	4899
	2	31	32	38	38	63	77	66	55	104	103	122	100	
	6	38	32	36	34	65	85	80	62	99	105	129	95	
682885	0.6	39	26	37	35	63	63	77	59	100	109	109	112	4899
	2	30	26	38	40	54	56	71	72	102	98	111	102	
	6	27	27	34	35	46	52	56	64	102	98	113	96	
682886	0.6	30	40	34	36	58	87	54	61	104	99	120	101	4899
	2	27	26	34	36	51	55	55	69	103	91	105	92	
	6	40	28	34	37	107	54	61	69	109	100	102	99	
684057	0.6	35	26	33	39	56	51	51	69	104	99	110	102	4900
	2	33	32	31	40	54	57	56	87	103	100	112	97	
	6	39	33	35	40	67	52	55	92	98	104	121	108	

Example 87: Duration of action *in vivo* by single doses of oligonucleotides targeting TTR comprising a $GalNAc_3$ cluster

ISIS numbers 420915 and 660261 (see Table 83) were tested in a single dose study for duration of action in mice. ISIS numbers 420915, 682883, and 682885 (see Table 83) were also tested in a single dose study for duration of action in mice.

Treatment

Eight week old, male transgenic mice that express human TTR were each injected subcutaneously once with 100 mg/kg ISIS No. 420915 or 13.5 mg/kg ISIS No. 660261. Each treatment group consisted of 4 animals. Tail bleeds were performed before dosing to determine baseline and at days 3, 7, 10, 17, 24, and 39 following the dose. Plasma TTR protein levels were measured as described in Example 86. The results below are presented as the average percent of plasma TTR levels for each treatment group, normalized to baseline levels.

Table 88
Plasma TTR protein levels

ISIS No.	Dosage (mg/kg)	Time point (days post-dose)	TTR (% baseline)	$GalNAc_3$ Cluster	CM	SEQ ID No.
420915	100	3	30	n/a	n/a	4899
		7	23			
		10	35			
		17	53			
		24	75			
		39	100			
660261	13.5	3	27	$GalNAc_3$-1a	A_d	4900
		7	21			
		10	22			
		17	36			
		24	48			
		39	69			

Treatment

Female transgenic mice that express human TTR were each injected subcutaneously once with 100 mg/kg ISIS No. 420915, 10.0 mg/kg ISIS No. 682883, or 10.0 mg/kg 682885. Each treatment group consisted of 4 animals. Tail bleeds were performed before dosing to determine baseline and at days 3, 7, 10, 17, 24, and 39 following the dose. Plasma TTR protein levels were measured as described in Example 86. The results below are presented as the average percent of plasma TTR levels for each treatment group, normalized to baseline levels.

Table 89
Plasma TTR protein levels

ISIS No.	Dosage (mg/kg)	Time point (days post-dose)	TTR (% baseline)	$GalNAc_3$ Cluster	CM	SEQ ID No.
420915	100	3	48	n/a	n/a	4899
		7	48			
		10	48			
		17	66			
		31	80			
682883	10.0	3	45	$GalNAc_3$-3a	PO	4899
		7	37			
		10	38			
		17	42			
		31	65			

682885	10.0	3	40	$GalNAc_3$-10a	PO	4899
		7	33			
		10	34			
		17	40			
		31	64			

The results in Tables 88 and 89 show that the oligonucleotides comprising a GalNAc conjugate are more potent with a longer duration of action than the parent oligonucleotide lacking a conjugate (ISIS 420915).

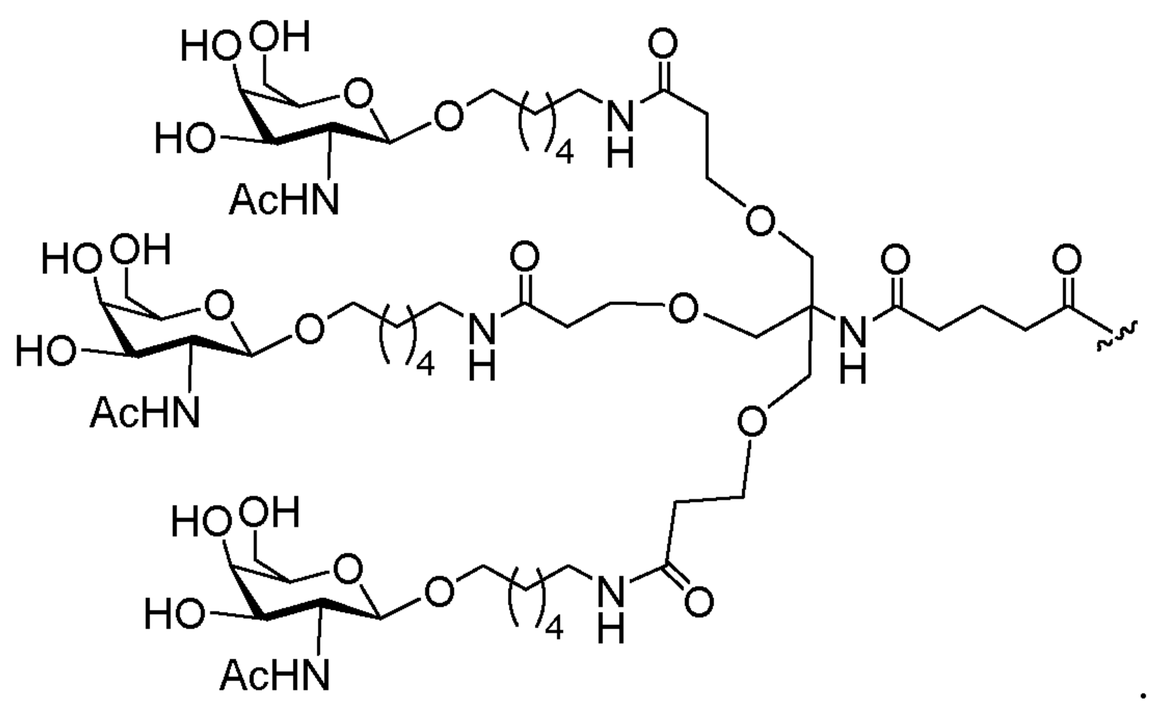
Example 88: Splicing modulation *in vivo* by oligonucleotides targeting SMN comprising a $GalNAc_3$ conjugate

The oligonucleotides listed in Table 90 were tested for splicing modulation of human survival of motor neuron (SMN) in mice.

Table 90
Modified ASOs targeting SMN

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No.
387954	$A_{es}T_{es}T_{es}{}^{m}C_{es}A_{es}{}^{m}C_{es}T_{es}T_{es}T_{es}{}^{m}C_{es}A_{es}T_{es}A_{es}A_{es}T_{es}G_{es}{}^{m}C_{es}T_{es}G_{es}G_{e}$	n/a	n/a	4901
699819	**$GalNAc_3$-7_{a}-$_{o}$**,$A_{es}T_{es}T_{es}{}^{m}C_{es}A_{es}{}^{m}C_{es}T_{es}T_{es}T_{es}{}^{m}C_{es}A_{es}T_{es}A_{es}A_{es}T_{es}G_{es}{}^{m}C_{es}T_{es}G_{es}G_{e}$	$GalNAc_3$-7a	PO	4901
699821	**$GalNAc_3$-7_{a}-$_{o}$**,$A_{es}T_{eo}T_{eo}{}^{m}C_{eo}A_{eo}{}^{m}C_{eo}T_{eo}T_{eo}T_{eo}{}^{m}C_{eo}A_{eo}T_{eo}A_{eo}A_{eo}T_{eo}G_{eo}{}^{m}C_{eo}T_{es}G_{es}G_{e}$	$GalNAc_3$-7a	PO	4901
700000	$A_{es}T_{es}T_{es}{}^{m}C_{es}A_{es}{}^{m}C_{es}T_{es}T_{es}T_{es}{}^{m}C_{es}A_{es}T_{es}A_{es}A_{es}T_{es}G_{es}{}^{m}C_{es}T_{es}G_{es}G_{eo}$**$A_{do}$'-$GalNAc_3$-$1_{a}$**	$GalNAc_3$-1a	A_d	4902
703421	X-$ATT^{m}CA^{m}CTTT^{m}CATAATG^{m}CTGG$	n/a	n/a	4901
703422	**$GalNAc_3$-7_{b}-**X-$ATT^{m}CA^{m}CTTT^{m}CATAATG^{m}CTGG$	$GalNAc_3$-7b	n/a	4901

The structure of $GalNAc_3$-7_a was shown previously in Example 48. "X" indicates a 5' primary amine generated by Gene Tools (Philomath, OR), and $GalNAc_3$-7_b indicates the structure of $GalNAc_3$-7_a lacking the –NH-C_6-O portion of the linker as shown below:



ISIS numbers 703421 and 703422 are morphlino oligonucleotides, wherein each nucleotide of the two oligonucleotides is a morpholino nucleotide.

Treatment

Six week old transgenic mice that express human SMN were injected subcutaneously once with an oligonucleotide listed in Table 91 or with saline. Each treatment group consisted of 2 males and 2 females. The mice were sacrificed 3 days following the dose to determine the liver human SMN mRNA levels both with and without exon 7 using real-time PCR according to standard protocols. Total RNA was measured using Ribogreen reagent. The SMN mRNA levels were normalized to total mRNA, and further normalized to the averages for the saline treatment group. The resulting average ratios of SMN mRNA including exon 7 to SMN mRNA missing exon 7 are shown in Table 91. The results show that fully modified oligonucleotides that modulate splicing and comprise a GalNAc conjugate are significantly more potent in altering splicing in the liver than the parent oligonucleotides lacking a GlaNAc conjugate. Furthermore, this trend is maintained for multiple modification chemistries, including 2'-MOE and morpholino modified oligonucleotides.

Table 91
Effect of oligonucleotides targeting human SMN *in vivo*

ISIS No.	Dose (mg/kg)	+Exon 7 / -Exon 7	$GalNAc_3$ Cluster	CM	SEQ ID No.
Saline	n/a	1.00	n/a	n/a	n/a
387954	32	1.65	n/a	n/a	4901
387954	288	5.00	n/a	n/a	4901
699819	32	7.84	$GalNAc_3$-7a	PO	4901
699821	32	7.22	$GalNAc_3$-7a	PO	4901
700000	32	6.91	$GalNAc_3$-1a	A_d	4902
703421	32	1.27	n/a	n/a	4901
703422	32	4.12	$GalNAc_3$-7b	n/a	4901

Example 89: Antisense inhibition *in vivo* by oligonucleotides targeting Apolipoprotein A (Apo(a)) comprising a $GalNAc_3$ conjugate

The oligonucleotides listed in Table 92 below were tested in a study for dose-dependent inhibition of Apo(a) in transgenic mice.

Table 92
Modified ASOs targeting Apo(a)

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No.
494372	$T_{es}G_{es}{}^{m}C_{es}T_{es}{}^{m}C_{es}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}T_{ds}G_{ds}{}^{m}C_{ds}$ $T_{ds}T_{es}G_{es}T_{es}T_{es}{}^{m}C_{e}$	n/a	n/a	4903
681257	**$GalNAc_3$-7_a-$_o$**,$T_{es}G_{eo}{}^{m}C_{eo}T_{eo}{}^{m}C_{eo}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}$ $T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{eo}G_{eo}T_{es}T_{es}{}^{m}C_{e}$	$GalNAc_3$-7a	PO	4903

The structure of $GalNAc_3$-7_a was shown in Example 48.

Treatment

Eight week old, female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were each injected subcutaneously once per week at a dosage shown below, for a total of six doses, with an oligonucleotide listed in Table 92 or with PBS. Each treatment group consisted of 3-4 animals. Tail bleeds were performed the day before the first dose and weekly following each dose to determine plasma Apo(a) protein levels. The mice were sacrificed two days following the final administration. Apo(a) liver mRNA levels were determined using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. Apo(a) plasma protein levels were determined using ELISA, and liver transaminase levels were determined. The mRNA and plasma protein results in Table 93 are presented as the treatment group average percent relative to the PBS treated group. Plasma protein levels were further normalized to the baseline (BL) value for the PBS group. Average absolute transaminase levels and body weights (% relative to baseline averages) are reported in Table 94.

As illustrated in Table 93, treatment with the oligonucleotides lowered Apo(a) liver mRNA and plasma protein levels in a dose-dependent manner. Furthermore, the oligonucleotide comprising the GalNAc conjugate was significantly more potent with a longer duration of action than the parent oligonucleotide lacking a GalNAc conjugate. As illustrated in Table 94, transaminase levels and body weights were unaffected by the oligonucleotides, indicating that the oligonucleotides were well tolerated.

Table 93

Apo(a) liver mRNA and plasma protein levels

ISIS No.	Dosage (mg/kg)	Apo(a) mRNA (% PBS)	Apo(a) plasma protein (% PBS)						
			BL	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
PBS	n/a	100	100	120	119	113	88	121	97
494372	3	80	84	89	91	98	87	87	79
	10	30	87	72	76	71	57	59	46
	30	5	92	54	28	10	7	9	7
681257	0.3	75	79	76	89	98	71	94	78
	1	19	79	88	66	60	54	32	24
	3	2	82	52	17	7	4	6	5
	10	2	79	17	6	3	2	4	5

Table 94

ISIS No.	Dosage (mg/kg)	ALT (U/L)	AST (U/L)	Body weight (% baseline)
PBS	n/a	37	54	103
494372	3	28	68	106
	10	22	55	102
	30	19	48	103
681257	0.3	30	80	104
	1	26	47	105
	3	29	62	102
	10	21	52	107

Example 90: Antisense inhibition *in vivo* by oligonucleotides targeting TTR comprising a $GalNAc_3$ cluster

Oligonucleotides listed in Table 95 below were tested in a dose-dependent study for antisense inhibition of human transthyretin (TTR) in transgenic mice that express the human TTR gene.

Treatment

TTR transgenic mice were each injected subcutaneously once per week for three weeks, for a total of three doses, with an oligonucleotide and dosage listed in Table 96 or with PBS. Each treatment group consisted of 4 animals. Prior to the first dose, a tail bleed was performed to determine plasma TTR protein levels at baseline (BL). The mice were sacrificed 72 hours following the final administration. TTR protein levels were measured using a clinical analyzer (AU480, Beckman Coulter, CA). Real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) were used according to standard protocols to determine liver human TTR mRNA levels. The results presented in Table 96 are the average values for each treatment group. The mRNA levels are the average values relative to the average for the PBS group. Plasma protein levels are the average values relative to the average value for the PBS group at baseline. "BL" indicates baseline, measurements that were taken just prior to the first dose. As illustrated in Table 96, treatment with antisense oligonucleotides lowered TTR expression levels in a dose-dependent manner. The oligonucleotides comprising a GalNAc conjugate were more potent than the parent lacking a GalNAc conjugate (ISIS 420915), and oligonucleotides comprising a phosphodiester or deoxyadenosine cleavable moiety showed significant improvements in potency compared to the parent lacking a conjugate (see ISIS numbers 682883 and 666943 vs 420915 and see Examples 86 and 87).

Table 95

Oligonucleotides targeting human TTR

Isis No.	Sequence 5' to 3'	Linkages	GalNAc cluster	CM	SEQ ID No.
420915	$T_{es}{}^{m}C_{es}T_{es}T_{es}G_{es}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{es}T_{es}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	PS	n/a	n/a	4899
682883	**$GalNAc_3$-$3_{a\text{-}o}$**,$T_{es}{}^{m}C_{eo}T_{eo}T_{eo}G_{eo}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{eo}T_{eo}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	PS/PO	$GalNAc_3$-3a	PO	4899
666943	**$GalNAc_3$-$3_{a\text{-}o}$,A_{do}**$T_{es}{}^{m}C_{eo}T_{eo}T_{eo}G_{eo}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{eo}T_{eo}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	PS/PO	$GalNAc_3$-3a	A_d	4904
682887	**$GalNAc_3$-$7_{a\text{-}o}$,A_{do}**$T_{es}{}^{m}C_{eo}T_{eo}T_{eo}G_{eo}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{eo}T_{eo}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	PS/PO	$GalNAc_3$-7a	A_d	4904
682888	**$GalNAc_3$-$10_{a\text{-}o}$,A_{do}**$T_{es}{}^{m}C_{eo}T_{eo}T_{eo}G_{eo}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{eo}T_{eo}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	PS/PO	$GalNAc_3$-10a	A_d	4904
682889	**$GalNAc_3$-$13_{a\text{-}o}$,A_{do}**$T_{es}{}^{m}C_{eo}T_{eo}T_{eo}G_{eo}G_{ds}T_{ds}T_{ds}A_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}A_{ds}A_{eo}T_{eo}{}^{m}C_{es}{}^{m}C_{es}{}^{m}C_{e}$	PS/PO	$GalNAc_3$-13a	A_d	4904

The legend for Table 95 can be found in Example 74. The structure of $GalNAc_3$-3_a was shown in Example 39. The structure of $GalNAc_3$-7_a was shown in Example 48. The structure of $GalNAc_3$-10_a was shown in Example 46. The structure of $GalNAc_3$-13_a was shown in Example 62.

Table 96
Antisense inhibition of human TTR *in vivo*

Isis No.	Dosage (mg/kg)	TTR mRNA (% PBS)	TTR protein (% BL)	GalNAc cluster	CM
PBS	n/a	100	124	n/a	n/a
420915	6	69	114	n/a	n/a
	20	71	86		
	60	21	36		
682883	0.6	61	73	$GalNAc_3$-3a	PO
	2	23	36		
	6	18	23		
666943	0.6	74	93	$GalNAc_3$-3a	A_d
	2	33	57		
	6	17	22		
682887	0.6	60	97	$GalNAc_3$-7a	A_d
	2	36	49		
	6	12	19		
682888	0.6	65	92	$GalNAc_3$-10a	A_d
	2	32	46		
	6	17	22		
682889	0.6	72	74	$GalNAc_3$-13a	A_d
	2	38	45		
	6	16	18		

Example 91: Antisense inhibition *in vivo* by oligonucleotides targeting Factor VII comprising a $GalNAc_3$ conjugate in non-human primates

Oligonucleotides listed in Table 97 below were tested in a non-terminal, dose escalation study for antisense inhibition of Factor VII in monkeys.

Treatment

Non-naïve monkeys were each injected subcutaneously on days 0, 15, and 29 with escalating doses of an oligonucleotide listed in Table 97 or with PBS. Each treatment group consisted of 4 males and 1 female. Prior to the first dose and at various time points thereafter, blood draws were performed to determine plasma Factor VII protein levels. Factor VII protein levels were measured by ELISA. The results presented in Table 98 are the average values for each treatment group relative to the average value for the PBS group at baseline (BL), the measurements taken just prior to the first dose. As illustrated in Table 98, treatment with antisense oligonucleotides lowered Factor VII expression levels in a dose-dependent manner, and the oligonucleotide comprising the GalNAc conjugate was significantly more potent in monkeys compared to the oligonucleotide lacking a GalNAc conjugate.

Table 97
Oligonucleotides targeting Factor VII

Isis No.	Sequence 5' to 3'	Linkages	GalNAc cluster	CM	SEQ ID No.
407935	$A_{es}T_{es}G_{es}{}^{m}C_{es}A_{es}T_{ds}G_{ds}G_{ds}T_{ds}G_{ds}A_{ds}T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}T_{es}G_{es}A_{e}$	PS	n/a	n/a	4905
686892	**$GalNAc_3$-$10_{a\text{-}o}$**,$A_{es}T_{es}G_{es}{}^{m}C_{es}A_{es}T_{ds}G_{ds}G_{ds}T_{ds}G_{ds}A_{ds}T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}T_{es}G_{es}A_{e}$	PS	$GalNAc_3$-10a	PO	4905

The legend for Table 97 can be found in Example 74. The structure of $GalNAc_3$-10_a was shown in Example 46.

Table 98
Factor VII plasma protein levels

ISIS No.	Day	Dose (mg/kg)	Factor VII (% BL)
407935	0	n/a	100
	15	10	87
	22	n/a	92
	29	30	77
	36	n/a	46
	43	n/a	43
686892	0	3	100
	15	10	56
	22	n/a	29
	29	30	19
	36	n/a	15
	43	n/a	11

Example 92: Antisense inhibition in primary hepatocytes by antisense oligonucleotides targeting Apo-CIII comprising a $GalNAc_3$ conjugate

Primary mouse hepatocytes were seeded in 96-well plates at 15,000 cells per well, and the oligonucleotides listed in Table 99, targeting mouse ApoC-III, were added at 0.46, 1.37, 4.12, or 12.35, 37.04, 111.11, or 333.33 nM or 1.00 μM. After incubation with the oligonucleotides for 24 hours, the cells were lysed and total RNA was purified using RNeasy (Qiagen). ApoC-III mRNA levels were determined using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc.) according to standard protocols. IC_{50} values were determined using Prism 4 software (GraphPad). The results show that regardless of whether the cleavable moiety was a phosphodiester or a phosphodiester-linked deoxyadensoine, the oligonucleotides comprising a GalNAc conjugate were significantly more potent than the parent oligonucleotide lacking a conjugate.

Table 99
Inhibition of mouse APOC-III expression in mouse primary hepatocytes

ISIS No.	Sequence (5' to 3')	CM	IC_{50} (nM)	SEQ ID No.
440670	$^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}A_{e}$	n/a	13.20	4906
661180	$^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}A_{eo}$ **$A_{do'}$-$GalNAc_3$-1_a**	A_d	1.40	4907

680771	**GalNAc$_3$-3$_{a\text{-}o}$**,$^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}A_{e}$	PO	0.70	4906
680772	**GalNAc$_3$-7$_{a\text{-}o}$**,$^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}A_{e}$	PO	1.70	4906
680773	**GalNAc$_3$-10$_{a\text{-}o}$**,$^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}A_{e}$	PO	2.00	4906
680774	**GalNAc$_3$-13$_{a\text{-}o}$**,$^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}A_{e}$	PO	1.50	4906
681272	**GalNAc$_3$-3$_{a\text{-}o}$**,$^{m}C_{es}A_{eo}G_{eo}{}^{m}C_{eo}T_{eo}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{eo}A_{eo}G_{es}{}^{m}C_{es}A_{e}$	PO	< 0.46	4906
681273	**GalNAc$_3$-3$_{a\text{-}o}$,A$_{do}$**$^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}A_{e}$	A$_d$	1.10	4908
683733	$^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}A_{eo}$**A$_{do}$-GalNAc$_3-19_a$**	A$_d$	2.50	4907

The structure of GalNAc$_3$-1$_a$ was shown previously in Example 9, GalNAc$_3$-3$_a$ was shown in Example 39, GalNAc$_3$-7$_a$ was shown in Example 48, GalNAc$_3$-10$_a$ was shown in Example 46, GalNAc$_3$-13$_a$ was shown in Example 62, and GalNAc$_3$-19$_a$ was shown in Example 70.

Example 93: Antisense inhibition *in vivo* by oligonucleotides targeting SRB-1 comprising mixed wings and a 5'-GalNAc$_3$ conjugate

The oligonucleotides listed in Table 100 were tested in a dose-dependent study for antisense inhibition of SRB-1 in mice.

Table 100

Modified ASOs targeting SRB-1

ISIS No.	Sequences (5' to 3')	GalNAc$_3$ Cluster	CM	SEQ ID No.
449093	$T_{ks}T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}\ A_{ds}T_{ds}\ G_{ds}\ A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{ks}{}^{m}C_{k}$	n/a	n/a	4909
699806	**GalNAc$_3$-3$_{a\text{-}o}$**,$T_{ks}T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}\ A_{ds}T_{ds}\ G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{ks}{}^{m}C_{k}$	GalNAc$_3$-3a	PO	4909
699807	**GalNAc$_3$-7$_{a\text{-}o}$**,$T_{ks}T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}\ A_{ds}T_{ds}\ G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{ks}{}^{m}C_{k}$	GalNAc$_3$-7a	PO	4909
699809	**GalNAc$_3$-7$_{a\text{-}o}$**, $T_{ks}T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}\ A_{ds}T_{ds}\ G_{ds}\ A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{e}$	GalNAc$_3$-7a	PO	4909
699811	**GalNAc$_3$-7$_{a\text{-}o}$**,$T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}\ A_{ds}T_{ds}\ G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{ks}{}^{m}C_{k}$	GalNAc$_3$-7a	PO	4909
699813	**GalNAc$_3$-7$_{a\text{-}o}$**,$T_{ks}T_{ds}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}\ A_{ds}T_{ds}\ G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{ds}{}^{m}C_{k}$	GalNAc$_3$-7a	PO	4909
699815	**GalNAc$_3$-7$_{a\text{-}o}$**,$T_{es}T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}\ A_{ds}T_{ds}\ G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{ks}{}^{m}C_{e}$	GalNAc$_3$-7a	PO	4909

The structure of GalNAc$_3$-3$_a$ was shown previously in Example 39, and the structure of GalNAc$_3$-7a was shown previously in Example 48. Subscripts: "e" indicates 2'-MOE modified nucleoside; "d" indicates β-D-2'-deoxyribonucleoside; "k" indicates 6'-*(S)*-CH_3 bicyclic nucleoside (cEt); "s" indicates phosphorothioate internucleoside linkages (PS); "o" indicates phosphodiester internucleoside linkages (PO). Supersript "m" indicates 5-methylcytosines.

Treatment

Six to eight week old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously once at the dosage shown below with an oligonucleotide listed in Table 100 or with saline. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration. Liver SRB-1 mRNA levels were measured using real-time PCR. SRB-1 mRNA levels were normalized to cyclophilin mRNA levels according to standard protocols. The results are presented as the average percent of SRB-1 mRNA levels for each treatment group relative to the saline control group. As illustrated in Table 101, treatment with antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner, and the gapmer oligonucleotides comprising a GalNAc conjugate and having wings that were either full cEt or mixed sugar modifications were significantly more potent than the parent oligonucleotide lacking a conjugate and comprising full cEt modified wings.

Body weights, liver transaminases, total bilirubin, and BUN were also measured, and the average values for each treatment group are shown in Table 101. Body weight is shown as the average percent body weight relative to the baseline body weight (% BL) measured just prior to the oligonucleotide dose.

Table 101

SRB-1 mRNA, ALT, AST, BUN, and total bilirubin levels and body weights

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA (% PBS)	ALT (U/L)	AST (U/L)	Bil	BUN	Body weight (% BL)
PBS	n/a	100	31	84	0.15	28	102
449093	1	111	18	48	0.17	31	104
	3	94	20	43	0.15	26	103
	10	36	19	50	0.12	29	104
699806	0.1	114	23	58	0.13	26	107
	0.3	59	21	45	0.12	27	108
	1	25	30	61	0.12	30	104
699807	0.1	121	19	41	0.14	25	100
	0.3	73	23	56	0.13	26	105
	1	24	22	69	0.14	25	102
699809	0.1	125	23	57	0.14	26	104
	0.3	70	20	49	0.10	25	105
	1	33	34	62	0.17	25	107
699811	0.1	123	48	77	0.14	24	106
	0.3	94	20	45	0.13	25	101
	1	66	57	104	0.14	24	107
699813	0.1	95	20	58	0.13	28	104
	0.3	98	22	61	0.17	28	105
	1	49	19	47	0.11	27	106
699815	0.1	93	30	79	0.17	25	105
	0.3	64	30	61	0.12	26	105
	1	24	18	41	0.14	25	106

Example 94: Antisense inhibition *in vivo* by oligonucleotides targeting SRB-1 comprising 2'-sugar modifications and a 5'-GalNAc$_3$ conjugate

The oligonucleotides listed in Table 102 were tested in a dose-dependent study for antisense inhibition of SRB-1 in mice.

Table 102
Modified ASOs targeting SRB-1

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No.
353382	$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	n/a	n/a	4886
700989	$G_{ms}C_{ms}U_{ms}U_{ms}C_{ms}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}U_{ms}C_{ms}C_{ms}U_{ms}U_{m}$	n/a	n/a	4910
666904	**$GalNAc_3$-3_{a}-$_{o}$**,$G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{es}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}T_{e}$	$GalNAc_3$-3a	PO	4886
700991	**$GalNAc_3$-7_{a}-$_{o}$**,$G_{ms}C_{ms}U_{ms}U_{ms}C_{ms}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}U_{ms}C_{ms}C_{ms}U_{ms}U_{m}$	$GalNAc_3$-7a	PO	4910

Subscript "m" indicates a 2'-O-methyl modified nucleoside. See Example 74 for complete table legend. The structure of $GalNAc_3$-3_a was shown previously in Example 39, and the structure of $GalNAc_3$-7a was shown previously in Example 48.

Treatment

The study was completed using the protocol described in Example 93. Results are shown in Table 103 below and show that both the 2'-MOE and 2'-OMe modified oligonucleotides comprising a GalNAc conjugate were significantly more potent than the respective parent oligonucleotides lacking a conjugate. The results of the body weights, liver transaminases, total bilirubin, and BUN measurements indicated that the compounds were all well tolerated.

Table 103
SRB-1 mRNA

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA (% PBS)
PBS	n/a	100
353382	5	116
	15	58
	45	27
700989	5	120
	15	92
	45	46
666904	1	98
	3	45
	10	17
700991	1	118
	3	63
	10	14

Example 95: Antisense inhibition *in vivo* by oligonucleotides targeting SRB-1 comprising bicyclic nucleosides and a 5'-GalNAc$_3$ conjugate

The oligonucleotides listed in Table 104 were tested in a dose-dependent study for antisense inhibition of SRB-1 in mice.

Table 104
Modified ASOs targeting SRB-1

ISIS No.	Sequences (5' to 3')	GalNAc$_3$ Cluster	CM	SEQ ID No
440762	$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{k}$	n/a	n/a	4880
666905	**GalNAc$_3$-3$_a$-$_o$**,$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{k}$	GalNAc$_3$-3$_a$	PO	4880
699782	**GalNAc$_3$-7$_a$-$_o$**,$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{k}$	GalNAc$_3$-7$_a$	PO	4880
699783	**GalNAc$_3$-3$_a$-$_o$**,$T_{ls}{}^{m}C_{ls}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ls}{}^{m}C_{l}$	GalNAc$_3$-3$_a$	PO	4880
653621	$T_{ls}{}^{m}C_{ls}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ls}{}^{m}C_{lo}$**A$_{do}$'-GalNAc$_3-1_a$**	GalNAc$_3$-1$_a$	A$_d$	4881
439879	$T_{gs}{}^{m}C_{gs}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{d}\ G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{gs}{}^{m}C_{g}$	n/a	n/a	4880
699789	**GalNAc$_3$-3$_a$-$_o$**,$T_{gs}{}^{m}C_{gs}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{d}\ G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{gs}{}^{m}C_{g}$	GalNAc$_3$-3$_a$	PO	4880

Subscript "g" indicates a fluoro-HNA nucleoside, subscript "l" indicates a locked nucleoside comprising a 2'-O-CH_2-4' bridge. See the Example 74 table legend for other abbreviations. The structure of GalNAc$_3$-1$_a$ was shown previously in Example 9, the structure of GalNAc$_3$-3$_a$ was shown previously in Example 39, and the structure of GalNAc$_3$-7a was shown previously in Example 48.

Treatment

The study was completed using the protocol described in Example 93. Results are shown in Table 105 below and show that oligonucleotides comprising a GalNAc conjugate and various bicyclic nucleoside modifications were significantly more potent than the parent oligonucleotide lacking a conjugate and comprising bicyclic nucleoside modifications. Furthermore, the oligonucleotide comprising a GalNAc conjugate and fluoro-HNA modifications was significantly more potent than the parent lacking a conjugate and comprising fluoro-HNA modifications. The results of the body weights, liver transaminases, total bilirubin, and BUN measurements indicated that the compounds were all well tolerated.

Table 105
SRB-1 mRNA, ALT, AST, BUN, and total bilirubin levels and body weights

ISIS No.	Dosage (mg/kg)	SRB-1 mRNA (% PBS)
PBS	n/a	100
440762	1	104
	3	65
	10	35
666905	0.1	105
	0.3	56
	1	18
699782	0.1	93
	0.3	63
	1	15
699783	0.1	105
	0.3	53

	1	12
653621	0.1	109
	0.3	82
	1	27
439879	1	96
	3	77
	10	37
699789	0.1	82
	0.3	69
	1	26

Example 96: Plasma protein binding of antisense oligonucleotides comprising a $GalNAc_3$ conjugate group

Oligonucleotides listed in Table 70 targeting ApoC-III and oligonucleotides in Table 106 targeting Apo(a) were tested in an ultra-filtration assay in order to assess plasma protein binding.

Table 106

Modified oligonucleotides targeting Apo(a)

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No
494372	$T_{es}G_{es}{}^{m}C_{es}T_{es}{}^{m}C_{es}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{es}G_{es}T_{es}T_{es}{}^{m}C_{e}$	n/a	n/a	4903
693401	$T_{es}G_{eo}{}^{m}C_{eo}T_{eo}{}^{m}C_{eo}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{eo}G_{eo}T_{es}T_{es}{}^{m}C_{e}$	n/a	n/a	4903
681251	**$GalNAc_3$-7_a-$_o$**,$T_{es}G_{es}{}^{m}C_{es}T_{es}{}^{m}C_{es}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{es}G_{es}T_{es}T_{es}{}^{m}C_{e}$	$GalNAc_3$-7_a	PO	4903
681257	**$GalNAc_3$-7_a-$_o$**,$T_{es}G_{eo}{}^{m}C_{eo}T_{eo}{}^{m}C_{eo}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{eo}G_{eo}T_{es}T_{es}{}^{m}C_{e}$	$GalNAc_3$-7_a	PO	4903

See the Example 74 for table legend. The structure of $GalNAc_3$-7a was shown previously in Example 48.

Ultrafree-MC ultrafiltration units (30,000 NMWL, low-binding regenerated cellulose membrane, Millipore, Bedford, MA) were pre-conditioned with 300 µL of 0.5% Tween 80 and centrifuged at 2000 g for 10 minutes, then with 300µL of a 300 µg/mL solution of a control oligonucleotide in H_2O and centrifuged at 2000 g for 16 minutes. In order to assess non-specific binding to the filters of each test oligonucleotide from Tables 70 and 106 to be used in the studies, 300 µL of a 250 ng/mL solution of oligonucleotide in H_2O at pH 7.4 was placed in the pre-conditioned filters and centrifuged at 2000 g for 16 minutes. The unfiltered and filtered samples were analyzed by an ELISA assay to determine the oligonucleotide concentrations. Three replicates were used to obtain an average concentration for each sample. The average concentration of the filtered sample relative to the unfiltered sample is used to determine the percent of oligonucleotide that is recovered through the filter in the absence of plasma (% recovery).

Frozen whole plasma samples collected in K3-EDTA from normal, drug-free human volunteers, cynomolgus monkeys, and CD-1 mice, were purchased from Bioreclamation LLC (Westbury, NY). The test oligonucleotides were added to 1.2 mL aliquots of plasma at two concentrations (5 and 150 µg/mL). An

aliquot (300 μL) of each spiked plasma sample was placed in a pre-conditioned filter unit and incubated at 37°C for 30 minutes, immediately followed by centrifugation at 2000 g for 16 minutes. Aliquots of filtered and unfiltered spiked plasma samples were analyzed by an ELISA to determine the oligonucleotide concentration in each sample. Three replicates per concentration were used to determine the average percentage of bound and unbound oligonucleotide in each sample. The average concentration of the filtered sample relative to the concentration of the unfiltered sample is used to determine the percent of oligonucleotide in the plasma that is not bound to plasma proteins (% unbound). The final unbound oligonucleotide values are corrected for non-specific binding by dividing the % unbound by the % recovery for each oligonucleotide. The final % bound oligonucleotide values are determined by subtracting the final % unbound values from 100. The results are shown in Table 107 for the two concentrations of oligonucleotide tested (5 and 150 μg/mL) in each species of plasma. The results show that GalNAc conjugate groups do not have a significant impact on plasma protein binding. Furthermore, oligonucleotides with full PS internucleoside linkages and mixed PO/PS linkages both bind plasma proteins, and those with full PS linkages bind plasma proteins to a somewhat greater extent than those with mixed PO/PS linkages.

Table 107

Percent of modified oligonucleotide bound to plasma proteins

ISIS No.	Human plasma		Monkey plasma		Mouse plasma	
	5 μg/mL	150 μg/mL	5 μg/mL	150 μg/mL	5 μg/mL	150 μg/mL
304801	99.2	98.0	99.8	99.5	98.1	97.2
663083	97.8	90.9	99.3	99.3	96.5	93.0
674450	96.2	97.0	98.6	94.4	94.6	89.3
494372	94.1	89.3	98.9	97.5	97.2	93.6
693401	93.6	89.9	96.7	92.0	94.6	90.2
681251	95.4	93.9	99.1	98.2	97.8	96.1
681257	93.4	90.5	97.6	93.7	95.6	92.7

Example 97: Modified oligonucleotides targeting TTR comprising a GalNAc$_3$ conjugate group

The oligonucleotides shown in Table 108 comprising a GalNAc conjugate were designed to target TTR.

Table 108

Modified oligonucleotides targeting TTR

ISIS No.	Sequences (5' to 3')	GalNAc$_3$ Cluster	CM	SEQ ID No
666941	**GalNAc$_3$-3$_{a\text{-}o}$,A$_{do}$** T_{es} $^{m}C_{es}$ T_{es} T_{es} G_{es} G_{ds} T_{ds} T_{ds} A_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} A_{ds} A_{es} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ $^{m}C_{e}$	GalNAc$_3$-3	A$_d$	4904
666942	T_{es} $^{m}C_{eo}$ T_{eo} T_{eo} G_{eo} G_{ds} T_{ds} T_{ds} A_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} A_{ds} A_{eo} T_{eo} $^{m}C_{es}$ $^{m}C_{es}$ $^{m}C_{eo}$ **A$_{do}$,-GalNAc$_3$-3$_a$**	GalNAc$_3$-1	A$_d$	4904
682876	**GalNAc$_3$-3$_{a\text{-}o}$,**T_{es} $^{m}C_{es}$ T_{es} T_{es} G_{es} G_{ds} T_{ds} T_{ds} A_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} A_{ds} A_{es} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ $^{m}C_{e}$	GalNAc$_3$-3	PO	4899
682877	**GalNAc$_3$-7$_{a\text{-}o}$,**T_{es} $^{m}C_{es}$ T_{es} T_{es} G_{es} G_{ds} T_{ds} T_{ds} A_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} A_{ds} A_{es} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ $^{m}C_{e}$	GalNAc$_3$-7	PO	4899
682878	**GalNAc$_3$-10$_{a\text{-}o}$,**T_{es} $^{m}C_{es}$ T_{es} T_{es} G_{es} G_{ds} T_{ds} T_{ds} A_{ds} $^{m}C_{ds}$ A_{ds}	GalNAc$_3$-10	PO	4899

	T_{ds} G_{ds} A_{ds} A_{ds} A_{es} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ $^{m}C_{e}$			
682879	**$GalNAc_3$-$13_{a\text{-}o}$,**T_{es} $^{m}C_{es}$ T_{es} T_{es} G_{es} G_{ds} T_{ds} T_{ds} A_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} A_{ds} A_{es} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ $^{m}C_{e}$	$GalNAc_3$-13	PO	4899
682880	**$GalNAc_3$-$7_{a\text{-}o}$,A_{do}** T_{es} $^{m}C_{es}$ T_{es} T_{es} G_{es} G_{ds} T_{ds} T_{ds} A_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} A_{ds} A_{es} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ $^{m}C_{e}$	$GalNAc_3$-7	A_d	4904
682881	**$GalNAc_3$-$10_{a\text{-}o}$,A_{do}** T_{es} $^{m}C_{es}$ T_{es} T_{es} G_{es} G_{ds} T_{ds} T_{ds} A_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} A_{ds} A_{es} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ $^{m}C_{e}$	$GalNAc_3$-10	A_d	4904
682882	**$GalNAc_3$-$13_{a\text{-}o}$,A_{do}** T_{es} $^{m}C_{es}$ T_{es} T_{es} G_{es} G_{ds} T_{ds} T_{ds} A_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} A_{ds} A_{es} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ $^{m}C_{e}$	$GalNAc_3$-13	A_d	4904
684056	T_{es} $^{m}C_{es}$ T_{es} T_{es} G_{es} G_{ds} T_{ds} T_{ds} A_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} A_{ds} A_{es} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ $^{m}C_{eo}$ **A_{do},-$GalNAc_3$-19_a**	$GalNAc_3$-19	A_d	4900

The legend for Table 108 can be found in Example 74. The structure of $GalNAc_3$-1 was shown in Example 9. The structure of $GalNAc_3$-3_a was shown in Example 39. The structure of $GalNAc_3$-7_a was shown in Example 48. The structure of $GalNAc_3$-10_a was shown in Example 46. The structure of $GalNAc_3$-13_a was shown in Example 62. The structure of $GalNAc_3$-19_a was shown in Example 70.

Example 98: Evaluation of pro-inflammatory effects of oligonucleotides comprising a GalNAc conjugate in hPMBC assay

The oligonucleotides listed in Table 109 and were tested for pro-inflammatory effects in an hPMBC assay as described in Examples 23 and 24. (See Tables 30, 83, 95, and 108 for descriptions of the oligonucleotides.) ISIS 353512 is a high responder used as a positive control, and the other oligonucleotides are described in Tables 83, 95, and 108. The results shown in Table 109 were obtained using blood from one volunteer donor. The results show that the oligonucleotides comprising mixed PO/PS internucleoside linkages produced significantly lower pro-inflammatory responses compared to the same oligonucleotides having full PS linkages. Furthermore, the GalNAc conjugate group did not have a significant effect in this assay.

Table 109

ISIS No.	E_{max}/EC_{50}	$GalNAc_3$ cluster	Linkages	CM
353512	3630	n/a	PS	n/a
420915	802	n/a	PS	n/a
682881	1311	$GalNAc_3$-10	PS	A_d
682888	0.26	$GalNAc_3$-10	PO/PS	A_d
684057	1.03	$GalNAc_3$-19	PO/PS	A_d

Example 99: Binding affinities of oligonucleotides comprising a GalNAc conjugate for the asialoglycoprotein receptor

The binding affinities of the oligonucleotides listed in Table 110 (see Table 76 for descriptions of the oligonucleotides) for the asialoglycoprotein receptor were tested in a competitive receptor binding assay. The competitor ligand, α1-acid glycoprotein (AGP), was incubated in 50 mM sodium acetate buffer (pH 5) with 1 U neuraminidase-agarose for 16 hours at 37°C, and > 90% desialylation was confirmed by either sialic acid

assay or size exclusion chromatography (SEC). Iodine monochloride was used to iodinate the AGP according to the procedure by Atsma et al. (*see* J Lipid Res. 1991 Jan; 32(1):173-81.) In this method, desialylated α1-acid glycoprotein (de-AGP) was added to 10 mM iodine chloride, $Na^{125}I$, and 1 M glycine in 0.25 M NaOH. After incubation for 10 minutes at room temperature, ^{125}I -labeled de-AGP was separated from free ^{125}I by concentrating the mixture twice utilizing a 3 KDMWCO spin column. The protein was tested for labeling efficiency and purity on a HPLC system equipped with an Agilent SEC-3 column (7.8x300mm) and a ß-RAM counter. Competition experiments utilizing ^{125}I -labeled de-AGP and various GalNAc-cluster containing ASOs were performed as follows. Human HepG2 cells (10^6 cells/ml) were plated on 6-well plates in 2 ml of appropriate growth media. MEM media supplemented with 10% fetal bovine serum (FBS), 2 mM L-Glutamine and 10mM HEPES was used. Cells were incubated 16-20 hours @ 37°C with 5% and 10% CO_2 respectively. Cells were washed with media without FBS prior to the experiment. Cells were incubated for 30 min @37°C with 1ml competition mix containing appropriate growth media with 2% FBS, 10^{-8} M ^{125}I -labeled de-AGP and GalNAc-cluster containing ASOs at concentrations ranging from 10^{-11} to 10^{-5} M. Non-specific binding was determined in the presence of 10^{-2} M GalNAc sugar. Cells were washed twice with media without FBS to remove unbound ^{125}I -labeled de-AGP and competitor GalNAc ASO. Cells were lysed using Qiagen's RLT buffer containing 1% ß-mercaptoethanol. Lysates were transferred to round bottom assay tubes after a brief 10 min freeze/thaw cycle and assayed on a ɣ-counter. Non-specific binding was subtracted before dividing ^{125}I protein counts by the value of the lowest GalNAc-ASO concentration counts. The inhibition curves were fitted according to a single site competition binding equation using a nonlinear regression algorithm to calculate the binding affinities (K_D's).

The results in Table 110 were obtained from experiments performed on five different days. Results for oligonucleotides marked with superscript "a" are the average of experiments run on two different days. The results show that the oligonucleotides comprising a GalNAc conjugate group on the 5'-end bound the asialoglycoprotein receptor on human HepG2 cells with 1.5 to 16-fold greater affinity than the oligonucleotides comprising a GalNAc conjugate group on the 3'-end.

Table 110
Asialoglycoprotein receptor binding assay results

ISIS No.	GalNAc conjugate	Oligonucleotide end to which GalNAc conjugate is attached	K_D (nM)
661161[a]	$GalNAc_3$-3	5'	3.7
666881[a]	$GalNAc_3$-10	5'	7.6
666981	$GalNAc_3$-7	5'	6.0
670061	$GalNAc_3$-13	5'	7.4
655861[a]	$GalNAc_3$-1	3'	11.6
677841[a]	$GalNAc_3$-19	3'	60.8

Example 100: Antisense inhibition *in vivo* by oligonucleotides comprising a GalNAc conjugate group targeting Apo(a) *in vivo*

The oligonucleotides listed in Table 111a below were tested in a single dose study for duration of action in mice.

Table 111a
Modified ASOs targeting APO(a)

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No.
681251	**$GalNAc_3$-7_{a}-$_{o}$**,$T_{es}G_{es}{}^{m}C_{es}T_{es}{}^{m}C_{es}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}$ $T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{es}G_{es}T_{es}T_{es}{}^{m}C_{e}$	$GalNAc_3$-7a	PO	4903
681257	**$GalNAc_3$-7_{a}-$_{o}$**,$T_{es}G_{eo}{}^{m}C_{eo}T_{eo}{}^{m}C_{eo}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}$ $T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{eo}G_{eo}T_{es}T_{es}{}^{m}C_{e}$	$GalNAc_3$-7a	PO	4903

The structure of $GalNAc_3$-7_a was shown in Example 48.

Treatment

Female transgenic mice that express human Apo(a) were each injected subcutaneously once per week, for a total of 6 doses, with an oligonucleotide and dosage listed in Table 111b or with PBS. Each treatment group consisted of 3 animals. Blood was drawn the day before dosing to determine baseline levels of Apo(a) protein in plasma and at 72 hours, 1 week, and 2 weeks following the first dose. Additional blood draws will occur at 3 weeks, 4 weeks, 5 weeks, and 6 weeks following the first dose. Plasma Apo(a) protein levels were measured using an ELISA. The results in Table 111b are presented as the average percent of plasma Apo(a) protein levels for each treatment group, normalized to baseline levels (% BL), The results show that the oligonucleotides comprising a GalNAc conjugate group exhibited potent reduction in Apo(a) expression. This potent effect was observed for the oligonucleotide that comprises full PS internucleoside linkages and the oligonucleotide that comprises mixed PO and PS linkages.

Table 111b
Apo(a) plasma protein levels

ISIS No.	Dosage (mg/kg)	Apo(a) at 72 hours (% BL)	Apo(a) at 1 week (% BL)	Apo(a) at 3 weeks (% BL)
PBS	n/a	116	104	107
681251	0.3	97	108	93
	1.0	85	77	57
	3.0	54	49	11
	10.0	23	15	4
681257	0.3	114	138	104
	1.0	91	98	54
	3.0	69	40	6
	10.0	30	21	4

Example 101: Antisense inhibition by oligonucleotides comprising a GalNAc cluster linked via a stable moiety

The oligonucleotides listed in Table 112 were tested for inhibition of mouse APOC-III expression *in vivo*. C57Bl/6 mice were each injected subcutaneously once with an oligonucleotide listed in Table 112 or with PBS. Each treatment group consisted of 4 animals. Each mouse treated with ISIS 440670 received a dose of 2, 6, 20, or 60 mg/kg. Each mouse treated with ISIS 680772 or 696847 received 0.6, 2, 6, or 20 mg/kg. The GalNAc conjugate group of ISIS 696847 is linked via a stable moiety, a phosphorothioate linkage instead of a readily cleavable phosphodiester containing linkage. The animals were sacrificed 72 hours after the dose. Liver APOC-III mRNA levels were measured using real-time PCR. APOC-III mRNA levels were normalized to cyclophilin mRNA levels according to standard protocols. The results are presented in Table 112 as the average percent of APOC-III mRNA levels for each treatment group relative to the saline control group. The results show that the oligonucleotides comprising a GalNAc conjugate group were significantly more potent than the oligonucleotide lacking a conjugate group. Furthermore, the oligonucleotide comprising a GalNAc conjugate group linked to the oligonucleotide via a cleavable moiety (ISIS 680772) was even more potent than the oligonucleotide comprising a GalNAc conjugate group linked to the oligonucleotide via a stable moiety (ISIS 696847).

Table 112

Modified oligonucleotides targeting mouse APOC-III

ISIS No.	Sequences (5' to 3')	CM	Dosage (mg/kg)	APOC-III mRNA (% PBS)	SEQ ID No.
440670	$^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}A_{e}$	n/a	2	92	4906
			6	86	
			20	59	
			60	37	
680772	**GalNAc$_3$-7$_{a\text{-}o}$**,$^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}A_{e}$	PO	0.6	79	4906
			2	58	
			6	31	
			20	13	
696847	**GalNAc$_3$-7$_{a\text{-}s}$**,$^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}T_{es}T_{ds}T_{ds}A_{ds}T_{ds}T_{ds}A_{ds}G_{ds}G_{ds}G_{ds}A_{ds}{}^{m}C_{es}A_{es}G_{es}{}^{m}C_{es}A_{e}$	n/a (PS)	0.6	83	4906
			2	73	
			6	40	
			20	28	

The structure of GalNAc$_3$-7$_a$ was shown in Example 48.

Example 102: Distribution in liver of antisense oligonucleotides comprising a GalNAc conjugate

The liver distribution of ISIS 353382 (see Table 36) that does not comprise a GalNAc conjugate and ISIS 655861 (see Table 36) that does comprise a GalNAc conjugate was evaluated. Male balb/c mice were subcutaneously injected once with ISIS 353382 or 655861 at a dosage listed in Table 113. Each treatment group consisted of 3 animals except for the 18 mg/kg group for ISIS 655861, which consisted of 2 animals. The animals were sacrificed 48 hours following the dose to determine the liver distribution of the

oligonucleotides. In order to measure the number of antisense oligonucleotide molecules per cell, a Ruthenium (II) tris-bipyridine tag (MSD TAG, Meso Scale Discovery) was conjugated to an oligonucleotide probe used to detect the antisense oligonucleotides. The results presented in Table 113 are the average concentrations of oligonucleotide for each treatment group in units of millions of oligonucleotide molecules per cell. The results show that at equivalent doses, the oligonucleotide comprising a GalNAc conjugate was present at higher concentrations in the total liver and in hepatocytes than the oligonucleotide that does not comprise a GalNAc conjugate. Furthermore, the oligonucleotide comprising a GalNAc conjugate was present at lower concentrations in non-parenchymal liver cells than the oligonucleotide that does not comprise a GalNAc conjugate. And while the concentrations of ISIS 655861 in hepatocytes and non-parenchymal liver cells were similar per cell, the liver is approximately 80% hepatocytes by volume. Thus, the majority of the ISIS 655861 oligonucleotide that was present in the liver was found in hepatocytes, whereas the majority of the ISIS 353382 oligonucleotide that was present in the liver was found in non-parenchymal liver cells.

Table 113

ISIS No.	Dosage (mg/kg)	Concentration in whole liver (molecules*10^6 per cell)	Concentration in hepatocytes (molecules*10^6 per cell)	Concentration in non-parenchymal liver cells (molecules*10^6 per cell)
353382	3	9.7	1.2	37.2
	10	17.3	4.5	34.0
	20	23.6	6.6	65.6
	30	29.1	11.7	80.0
	60	73.4	14.8	98.0
	90	89.6	18.5	119.9
655861	0.5	2.6	2.9	3.2
	1	6.2	7.0	8.8
	3	19.1	25.1	28.5
	6	44.1	48.7	55.0
	18	76.6	82.3	77.1

Example 103: Duration of action *in vivo* of oligonucleotides targeting APOC-III comprising a $GalNAc_3$ conjugate

The oligonucleotides listed in Table 114 below were tested in a single dose study for duration of action in mice.

Table 114

Modified ASOs targeting APOC-III

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No.
304801	$A_{es}G_{es}{}^{m}C_{es}T_{es}T_{es}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{es}T_{es}T_{es}A_{es}T_{e}$	n/a	n/a	4878
663084	**$GalNAc_3$-3_a-$_o$'A_{do}**$A_{es}G_{eo}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{eo}T_{eo}T_{es}A_{es}T_{e}$	$GalNAc_3$-3a	A_d	4894
679241	$A_{es}G_{eo}{}^{m}C_{eo}T_{eo}T_{eo}{}^{m}C_{ds}T_{ds}T_{ds}G_{ds}T_{ds}{}^{m}C_{ds}{}^{m}C_{ds}A_{ds}G_{ds}{}^{m}C_{ds}T_{eo}T_{eo}T_{es}A_{es}T_{eo}$**$A_{do}$'-$GalNAc_3$-$19_a$**	$GalNAc_3$-19a	A_d	4879

The structure of $GalNAc_3$-3_a was shown in Example 39, and $GalNAc_3$-19_a was shown in Example 70.

Treatment

Female transgenic mice that express human APOC-III were each injected subcutaneously once with an oligonucleotide listed in Table 114 or with PBS. Each treatment group consisted of 3 animals. Blood was drawn before dosing to determine baseline and at 3, 7, 14, 21, 28, 35, and 42 days following the dose. Plasma triglyceride and APOC-III protein levels were measured as described in Example 20. The results in Table 115 are presented as the average percent of plasma triglyceride and APOC-III levels for each treatment group, normalized to baseline levels. A comparison of the results in Table 71 of example 79 with the results in Table 115 below show that oligonucleotides comprising a mixture of phosphodiester and phosphorothioate internucleoside linkages exhibited increased duration of action than equivalent oligonucleotides comprising only phosphorothioate internucleoside linkages.

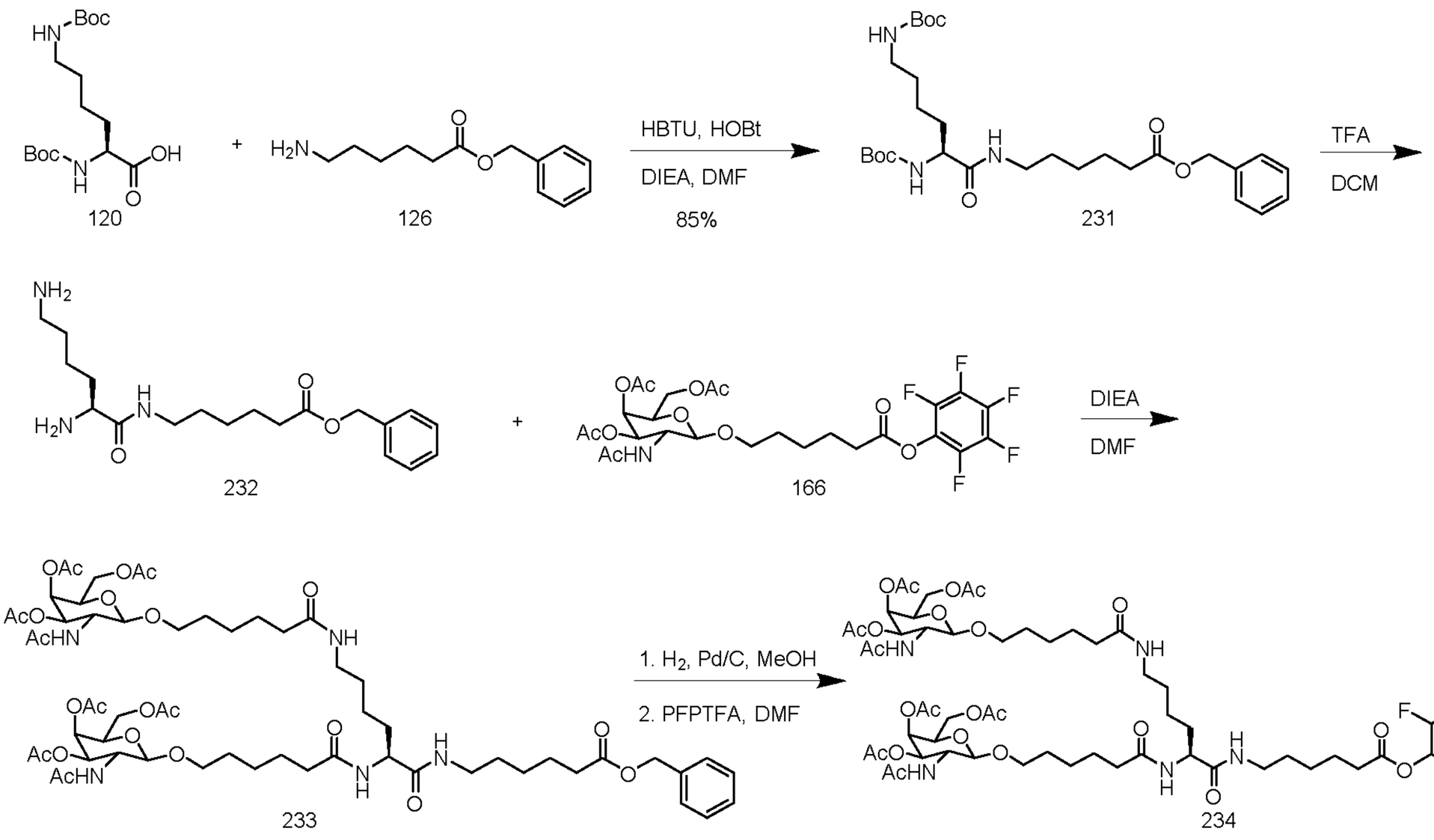
Table 115

Plasma triglyceride and APOC-III protein levels in transgenic mice

ISIS No.	Dosage (mg/kg)	Time point (days post-dose)	Triglycerides (% baseline)	APOC-III protein (% baseline)	$GalNAc_3$ Cluster	CM
PBS	n/a	3	96	101	n/a	n/a
		7	88	98		
		14	91	103		
		21	69	92		
		28	83	81		
		35	65	86		
		42	72	88		
304801	30	3	42	46	n/a	n/a
		7	42	51		
		14	59	69		
		21	67	81		
		28	79	76		
		35	72	95		
		42	82	92		
663084	10	3	35	28	$GalNAc_3$-3a	A_d
		7	23	24		
		14	23	26		
		21	23	29		
		28	30	22		
		35	32	36		
		42	37	47		
679241	10	3	38	30	$GalNAc_3$-19a	A_d
		7	31	28		
		14	30	22		
		21	36	34		
		28	48	34		
		35	50	45		

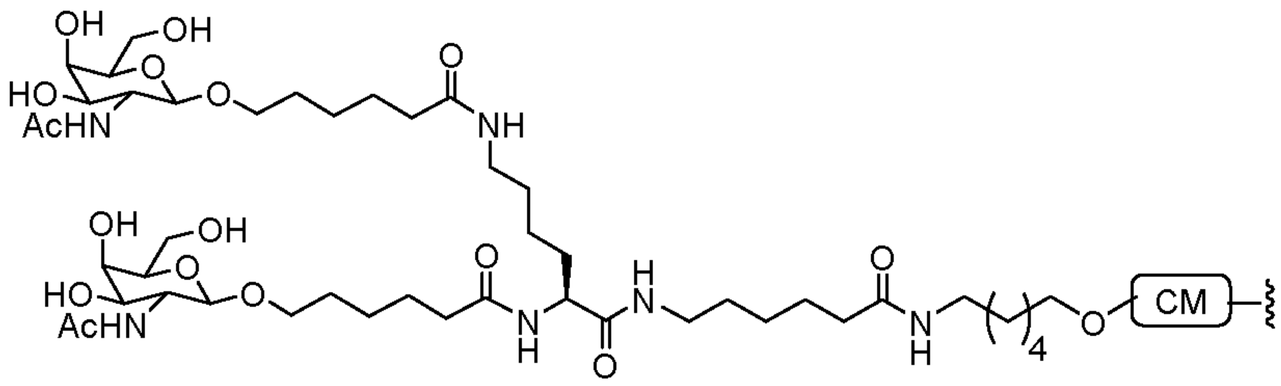
		42	72	64		

Example 104: Synthesis of oligonucleotides comprising a 5'-GalNAc$_2$ conjugate

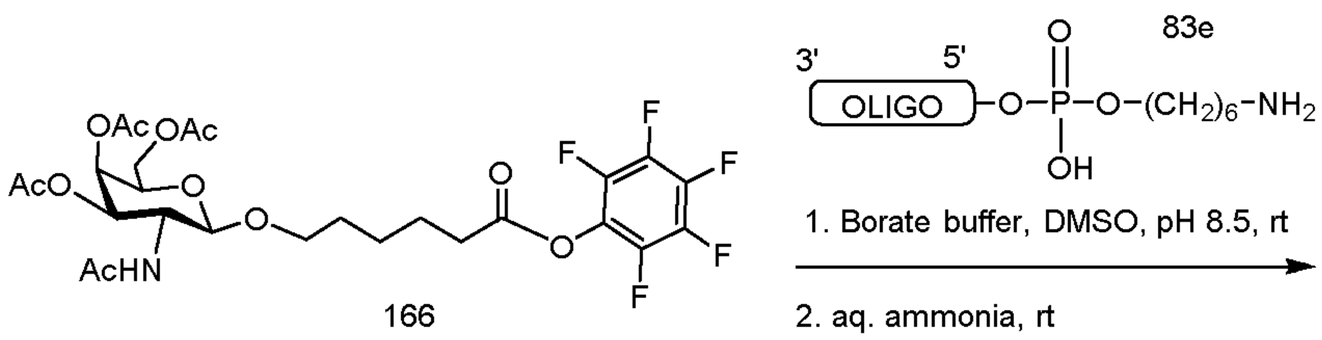


Compound 120 is commercially available, and the synthesis of compound 126 is described in Example 49. Compound 120 (1 g, 2.89 mmol), HBTU (0.39 g, 2.89 mmol), and HOBt (1.64 g, 4.33 mmol) were dissolved in DMF (10 mL. and *N*,*N*-diisopropylethylamine (1.75 mL, 10.1 mmol) were added. After about 5 min, aminohexanoic acid benzyl ester (1.36 g, 3.46 mmol) was added to the reaction. After 3h, the reaction mixture was poured into 100 mL of 1 M NaHSO4 and extracted with 2 x 50 mL ethyl acetate. Organic layers were combined and washed with 3 x 40 mL sat $NaHCO_3$ and 2 x brine, dried with Na_2SO_4, filtered and concentrated. The product was purified by silica gel column chromatography (DCM:EA:Hex , 1:1:1) to yield compound 231. LCMS and NMR were consistent with the structure. Compounds 231 (1.34 g, 2.438 mmol) was dissolved in dichloromethane (10 mL) and trifluoracetic acid (10 mL) was added. After stirring at room temperature for 2h, the reaction mixture was concentrated under reduced pressure and co-evaporated with toluene (3 x 10 mL). The residue was dried under reduced pressure to yield compound 232 as the trifuloracetate salt. The synthesis of compound 166 is described in Example 54. Compound 166 (3.39 g, 5.40 mmol) was dissolved in DMF (3 mL). A solution of compound 232 (1.3 g, 2.25 mmol) was dissolved

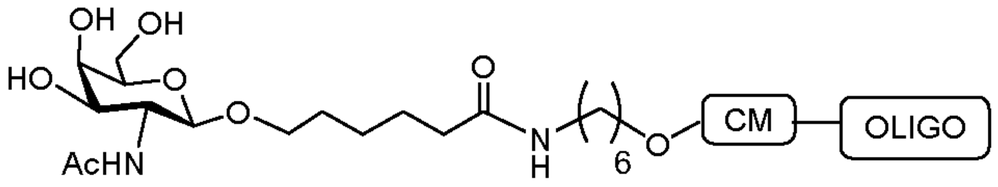
in DMF (3 mL) and *N*,*N*-diisopropylethylamine (1.55 mL) was added. The reaction was stirred at room temperature for 30 minutes, then poured into water (80 mL) and the aqueous layer was extracted with EtOAc (2x100 mL). The organic phase was separated and washed with sat. aqueous $NaHCO_3$ (3 x 80 mL), 1 M $NaHSO_4$ (3 x 80 mL) and brine (2 x 80 mL), then dried (Na_2SO_4), filtered, and concentrated. The residue was purified by silica gel column chromatography to yield compound 233. LCMS and NMR were consistent with the structure. Compound 233 (0.59 g, 0.48 mmol) was dissolved in methanol (2.2 mL) and ethyl acetate (2.2 mL). Palladium on carbon (10 wt% Pd/C, wet , 0.07 g) was added, and the reaction mixture was stirred under hydrogen atmosphere for 3 h. The reaction mixture was filtered through a pad of Celite and concentrated to yield the carboxylic acid. The carboxylic acid (1.32 g, 1.15 mmol, cluster free acid) was dissolved in DMF (3.2 mL). To this *N*,*N*-diisopropylehtylamine (0.3 mL, 1.73 mmol) and PFPTFA (0.30 mL, 1.73 mmol) were added. After 30 min stirring at room temperature the reaction mixture was poured into water (40 mL) and extracted with EtOAc (2 x 50 mL). A standard work-up was completed as described above to yield compound 234. LCMS and NMR were consistent with the structure. Oligonucleotide 235 was prepared using the general procedure described in Example 46. The $GalNAc_2$ cluster portion ($GalNAc_2$-24_a) of the conjugate group $GalNAc_2$-24 can be combined with any cleavable moiety present on the oligonucleotide to provide a variety of conjugate groups. The structure of $GalNAc_2$-24 ($GalNAc_2$-24_a-CM) is shown below:



Example 105: Synthesis of oligonucleotides comprising a $GalNAc_1$-25 conjugate



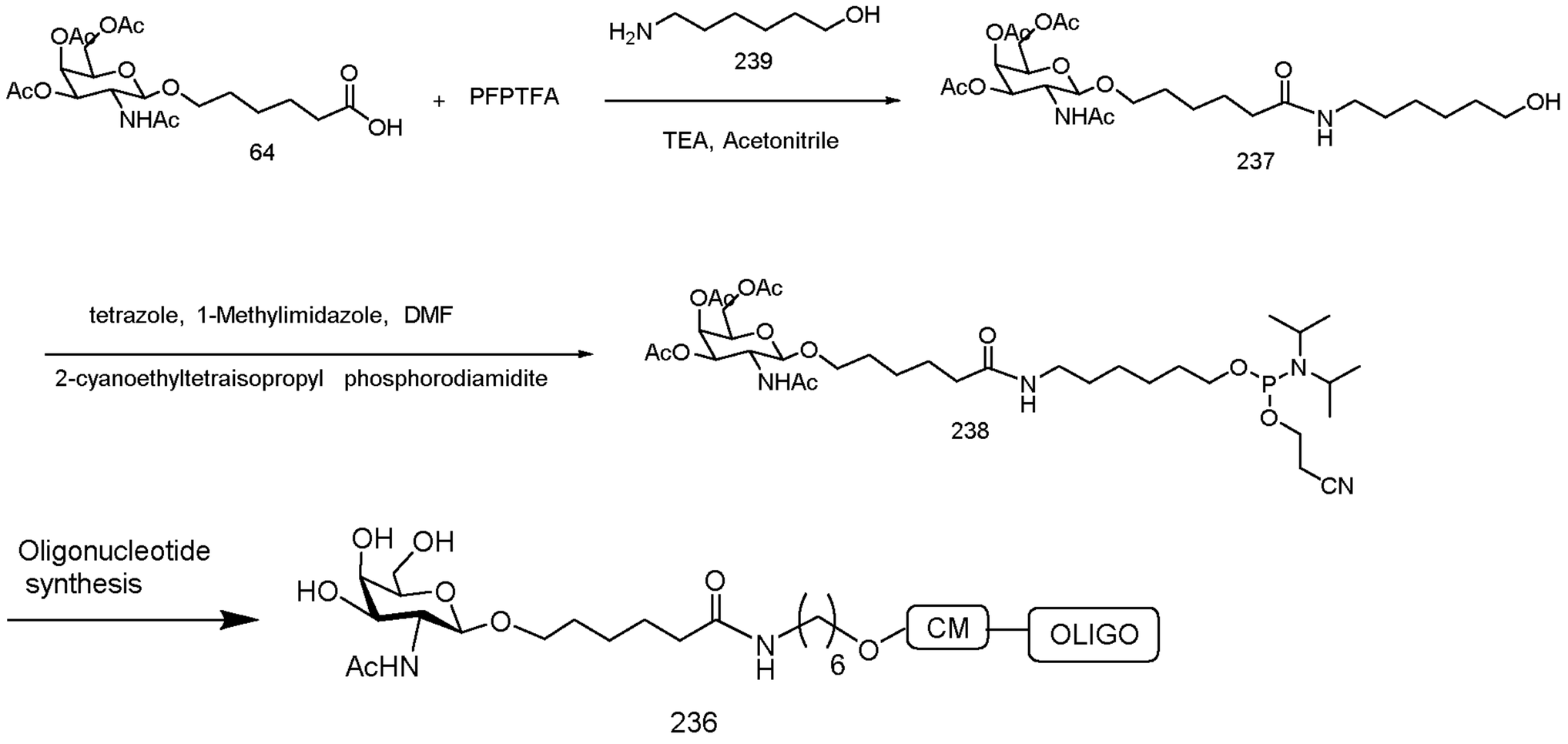
166



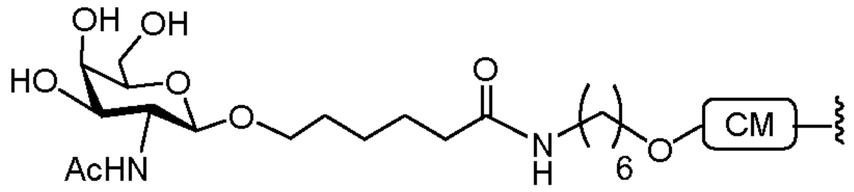
236

The synthesis of compound 166 is described in Example 54. Oligonucleotide 236 was prepared using the general procedure described in Example 46.

Alternatively, oligonucleotide 236 was synthesized using the scheme shown below, and compound 238 was used to form the oligonucleotide 236 using procedures described in Example 10.



The $GalNAc_1$ cluster portion ($GalNAc_1$-25_a) of the conjugate group $GalNAc_1$-25 can be combined with any cleavable moiety present on the oligonucleotide to provide a variety of conjugate groups. The structure of $GalNAc_1$-25 ($GalNAc_1$-25_a-CM) is shown below:



Example 106: Antisense inhibition *in vivo* by oligonucleotides targeting SRB-1 comprising a 5'-$GalNAc_2$ or a 5'-$GalNAc_3$ conjugate

Oligonucleotides listed in Tables 116 and 117 were tested in dose-dependent studies for antisense inhibition of SRB-1 in mice.

Treatment

Six to week old, male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously once with 2, 7, or 20 mg/kg of ISIS No. 440762; or with 0.2, 0.6, 2, 6, or 20 mg/kg of ISIS No. 686221, 686222, or 708561; or with saline. Each treatment group consisted of 4 animals. The mice were sacrificed 72 hours following the final administration. Liver SRB-1 mRNA levels were measured using real-time PCR. SRB-1 mRNA levels were normalized to cyclophilin mRNA levels according to standard protocols. The antisense oligonucleotides lowered SRB-1 mRNA levels in a dose-dependent manner, and the ED_{50} results are presented in Tables 116 and 117. Although previous studies showed that trivalent GalNAc-conjugated oligonucleotides were significantly more potent than divalent GalNAc-conjugated oligonucleotides, which were in turn significantly more potent than monovalent GalNAc conjugated oligonucleotides (*see*, e.g., Khorev et al., *Bioorg. & Med. Chem.*, Vol. 16, 5216-5231 (2008)), treatment with

antisense oligonucleotides comprising monovalent, divalent, and trivalent GalNAc clusters lowered SRB-1 mRNA levels with similar potencies as shown in Tables 116 and 117.

Table 116
Modified oligonucleotides targeting SRB-1

ISIS No.	Sequences (5' to 3')	GalNAc Cluster	ED_{50} (mg/kg)	SEQ ID No
440762	$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{k}$	n/a	4.7	4880
686221	**$GalNAc_2$-24_{a}-$_{o}$'A_{do}**$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{k}$	$GalNAc_2$-24_{a}	0.39	4884
686222	**$GalNAc_3$-13_{a}-$_{o}$'A_{do}**$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{k}$	$GalNAc_3$-13_{a}	0.41	4884

See Example 93 for table legend. The structure of $GalNAc_3$-13a was shown in Example 62, and the structure of $GalNAc_2$-24a was shown in Example 104.

Table 117
Modified oligonucleotides targeting SRB-1

ISIS No.	Sequences (5' to 3')	GalNAc Cluster	ED_{50} (mg/kg)	SEQ ID No
440762	$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{k}$	n/a	5	4880
708561	**$GalNAc_1$-25_{a}-$_{o}$'**$T_{ks}{}^{m}C_{ks}A_{ds}G_{ds}T_{ds}{}^{m}C_{ds}A_{ds}T_{ds}G_{ds}A_{ds}{}^{m}C_{ds}T_{ds}T_{ks}{}^{m}C_{k}$	$GalNAc_1$-25_{a}	0.4	4880

See Example 93 for table legend. The structure of $GalNAc_1$-25a was shown in Example 105.

The concentrations of the oligonucleotides in Tables 116 and 117 in liver were also assessed, using procedures described in Example 75. The results shown in Tables 117a and 117b below are the average total antisense oligonucleotide tissues levels for each treatment group, as measured by UV in units of μg oligonucleotide per gram of liver tissue. The results show that the oligonucleotides comprising a GalNAc conjugate group accumulated in the liver at significantly higher levels than the same dose of the oligonucleotide lacking a GalNAc conjugate group. Furthermore, the antisense oligonucleotides comprising one, two, or three GalNAc ligands in their respective conjugate groups all accumulated in the liver at similar levels. This result is surprising in view of the Khorev et al. literature reference cited above and is consistent with the activity data shown in Tables 116 and 117 above.

Table 117a

Liver concentrations of oligonucleotides comprising a $GalNAc_2$ or $GalNAc_3$ conjugate group

ISIS No.	Dosage (mg/kg)	[Antisense oligonucleotide] (μg/g)	GalNAc cluster	CM
440762	2	2.1	n/a	n/a
	7	13.1		
	20	31.1		
686221	0.2	0.9	$GalNAc_2$-24_{a}	A_d
	0.6	2.7		
	2	12.0		
	6	26.5		

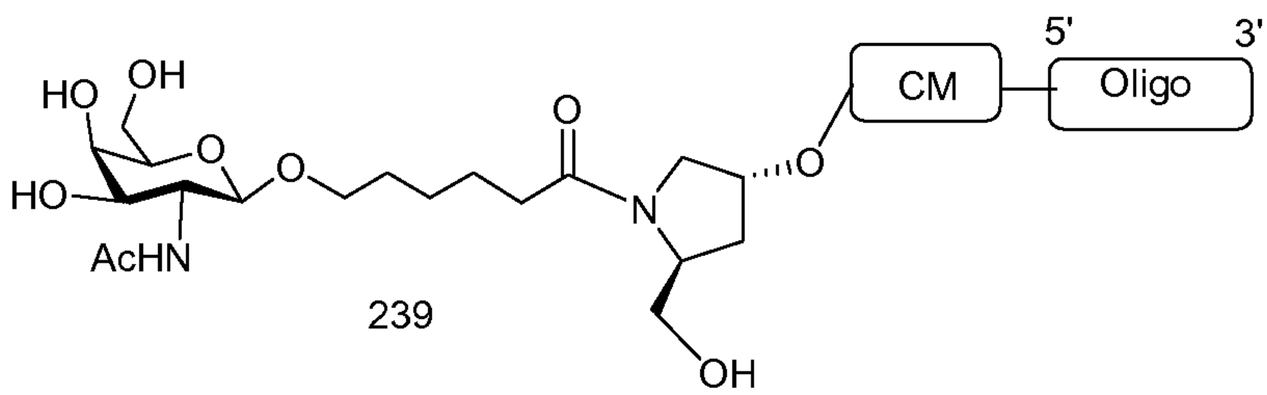
686222	0.2	0.5	$GalNAc_3$-13_a	A_d
	0.6	1.6		
	2	11.6		
	6	19.8		

Table 117b

Liver concentrations of oligonucleotides comprising a $GalNAc_1$ conjugate group

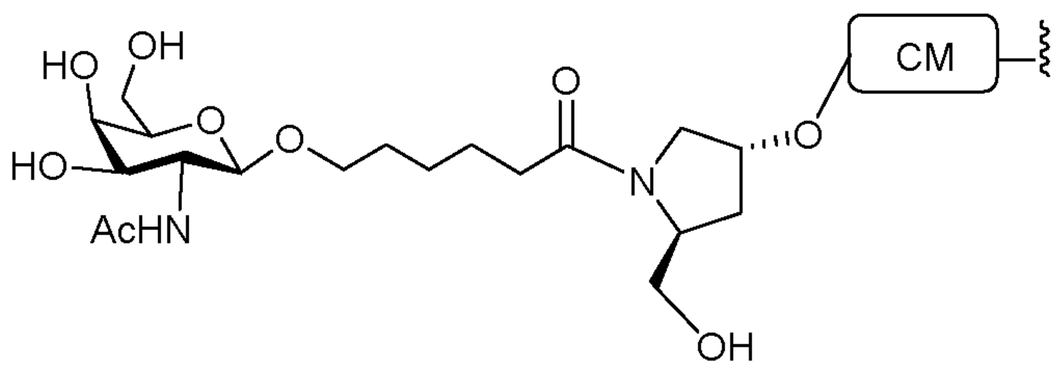
ISIS No.	Dosage (mg/kg)	[Antisense oligonucleotide] (μg/g)	GalNAc cluster	CM
440762	2	2.3	n/a	n/a
	7	8.9		
	20	23.7		
708561	0.2	0.4	$GalNAc_1$-25_a	PO
	0.6	1.1		
	2	5.9		
	6	23.7		
	20	53.9		

Example 107: Synthesis of oligonucleotides comprising a $GalNAc_1$-26 or $GalNAc_1$-27 conjugate

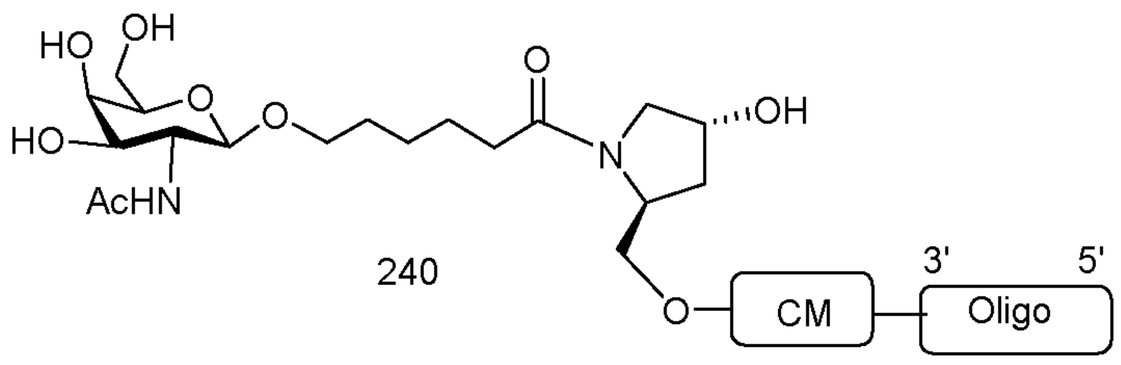


239

Oligonucleotide 239 is synthesized via coupling of compound 47 (see Example 15) to acid 64 (see Example 32) using HBTU and DIEA in DMF. The resulting amide containing compound is phosphitylated, then added to the 5'-end of an oligonucleotide using procedures described in Example 10. The $GalNAc_1$ cluster portion ($GalNAc_1$-26_a) of the conjugate group $GalNAc_1$-26 can be combined with any cleavable moiety present on the oligonucleotide to provide a variety of conjugate groups. The structure of $GalNAc_1$-26 ($GalNAc_1$-26_a-CM) is shown below:

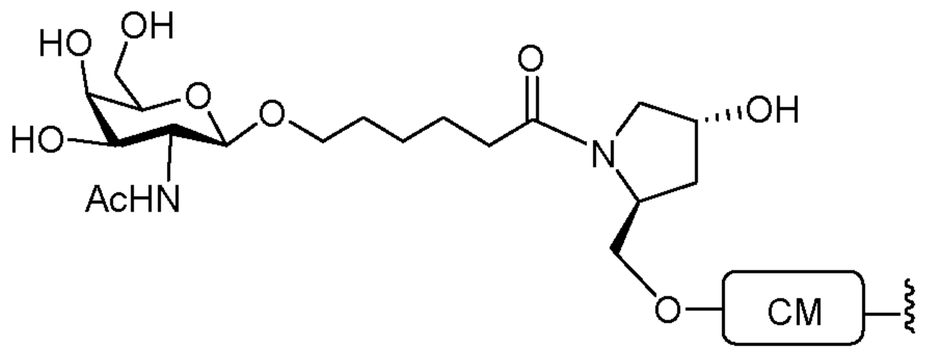


In order to add the $GalNAc_1$ conjugate group to the 3'-end of an oligonucleotide, the amide formed from the reaction of compounds 47 and 64 is added to a solid support using procedures described in Example 7. The oligonucleotide synthesis is then completed using procedures described in Example 9 in order to form oligonucleotide 240.



240

The $GalNAc_1$ cluster portion ($GalNAc_1$-27_a) of the conjugate group $GalNAc_1$-27 can be combined with any cleavable moiety present on the oligonucleotide to provide a variety of conjugate groups. The structure of $GalNAc_1$-27 ($GalNAc_1$-27_a-CM) is shown below:



Example 108: Antisense inhibition *in vivo* by oligonucleotides comprising a GalNAc conjugate group targeting Apo(a) *in vivo*

The oligonucleotides listed in Table 118 below were tested in a single dose study in mice.

Table 118

Modified ASOs targeting APO(a)

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No.
494372	$T_{es}G_{es}{}^{m}C_{es}T_{es}{}^{m}C_{es}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}T_{ds}G_{ds}{}^{m}C_{ds}$ $T_{ds}T_{es}G_{es}T_{es}T_{es}{}^{m}C_{e}$	n/a	n/a	4903
681251	**$GalNAc_3$-7_a-$_o$**,$T_{es}G_{es}{}^{m}C_{es}T_{es}{}^{m}C_{es}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}$ $T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{es}G_{es}T_{es}T_{es}{}^{m}C_{e}$	$GalNAc_3$-7a	PO	4903
681255	**$GalNAc_3$-3_a-$_o$**,$T_{es}G_{eo}{}^{m}C_{eo}T_{eo}{}^{m}C_{eo}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}$ $T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{eo}G_{eo}T_{es}T_{es}{}^{m}C_{e}$	$GalNAc_3$-3a	PO	4903
681256	**$GalNAc_3$-10_a-$_o$**,$T_{es}G_{eo}{}^{m}C_{eo}T_{eo}{}^{m}C_{eo}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}$ $T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{eo}G_{eo}T_{es}T_{es}{}^{m}C_{e}$	$GalNAc_3$-10a	PO	4903
681257	**$GalNAc_3$-7_a-$_o$**,$T_{es}G_{eo}{}^{m}C_{eo}T_{eo}{}^{m}C_{eo}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}$ $T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{eo}G_{eo}T_{es}T_{es}{}^{m}C_{e}$	$GalNAc_3$-7a	PO	4903
681258	**$GalNAc_3$-13_a-$_o$**,$T_{es}G_{eo}{}^{m}C_{eo}T_{eo}{}^{m}C_{eo}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}$ $T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{eo}G_{eo}T_{es}T_{es}{}^{m}C_{e}$	$GalNAc_3$-13a	PO	4903
681260	$T_{es}G_{eo}{}^{m}C_{eo}T_{eo}{}^{m}C_{eo}{}^{m}C_{ds}G_{ds}T_{ds}T_{ds}G_{ds}G_{ds}T_{ds}G_{ds}{}^{m}C_{ds}T_{ds}T_{eo}G_{eo}$ $T_{es}T_{es}{}^{m}C_{eo}$**A_{do}'-$GalNAc_3$-19**	$GalNAc_3$-19a	A_d	4911

The structure of $GalNAc_3$-7_a was shown in Example 48.

Treatment

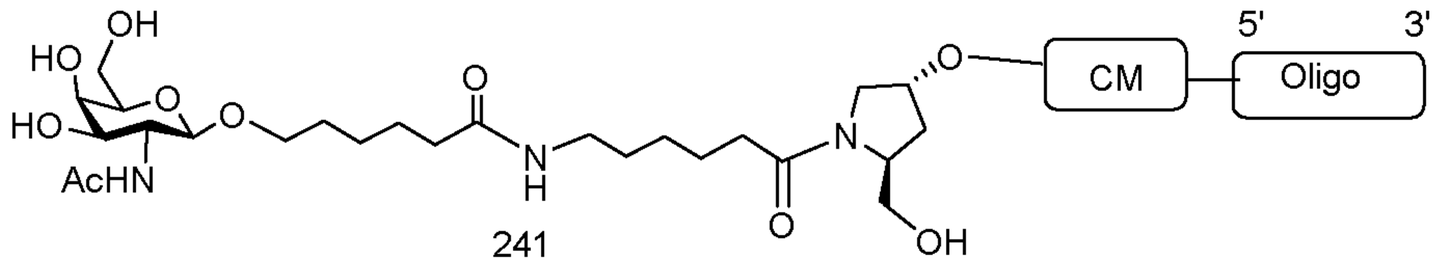
Male transgenic mice that express human Apo(a) were each injected subcutaneously once with an oligonucleotide and dosage listed in Table 119 or with PBS. Each treatment group consisted of 4 animals. Blood was drawn the day before dosing to determine baseline levels of Apo(a) protein in plasma and at 1

week following the first dose. Additional blood draws will occur weekly for approximately 8 weeks. Plasma Apo(a) protein levels were measured using an ELISA. The results in Table 119 are presented as the average percent of plasma Apo(a) protein levels for each treatment group, normalized to baseline levels (% BL), The results show that the antisense oligonucleotides reduced Apo(a) protein expression. Furthermore, the oligonucleotides comprising a GalNAc conjugate group exhibited even more potent reduction in Apo(a) expression than the oligonucleotide that does not comprise a conjugate group.

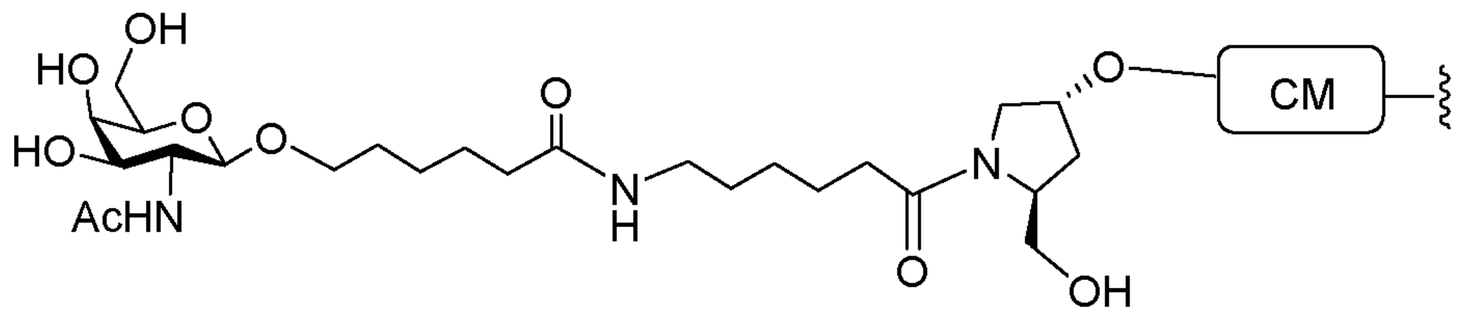
Table 119
Apo(a) plasma protein levels

ISIS No.	Dosage (mg/kg)	Apo(a) at 1 week (% BL)
PBS	n/a	143
494372	50	58
681251	10	15
681255	10	14
681256	10	17
681257	10	24
681258	10	22
681260	10	26

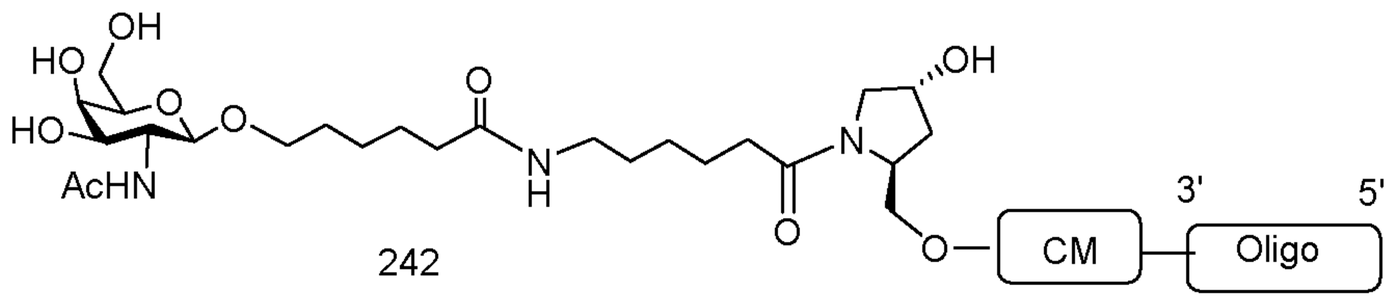
Example 109: Synthesis of oligonucleotides comprising a GalNAc$_1$-28 or GalNAc$_1$-29 conjugate



Oligonucleotide 241 is synthesized using procedures similar to those described in Example 71 to form the phosphoramidite intermediate, followed by procedures described in Example 10 to synthesize the oligonucleotide. The GalNAc$_1$ cluster portion (GalNAc$_1$-28$_a$) of the conjugate group GalNAc$_1$-28 can be combined with any cleavable moiety present on the oligonucleotide to provide a variety of conjugate groups. The structure of GalNAc$_1$-28 (GalNAc$_1$-28$_a$-CM) is shown below:

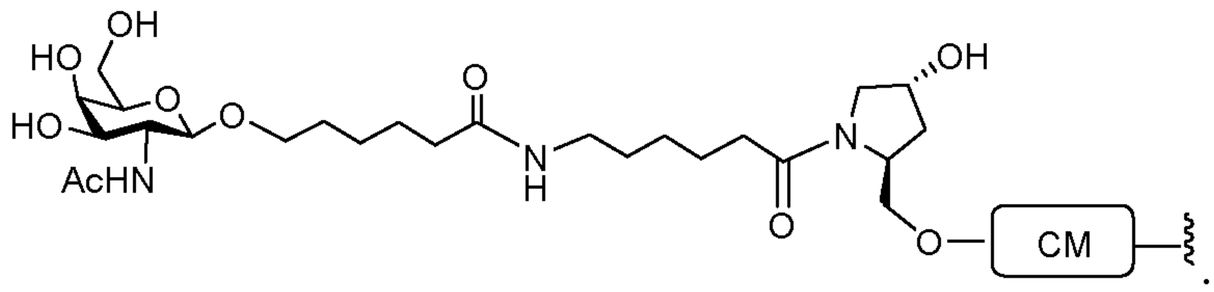


In order to add the GalNAc$_1$ conjugate group to the 3'-end of an oligonucleotide, procedures similar to those described in Example 71 are used to form the hydroxyl intermediate, which is then added to the solid support using procedures described in Example 7. The oligonucleotide synthesis is then completed using procedures described in Example 9 in order to form oligonucleotide 242.

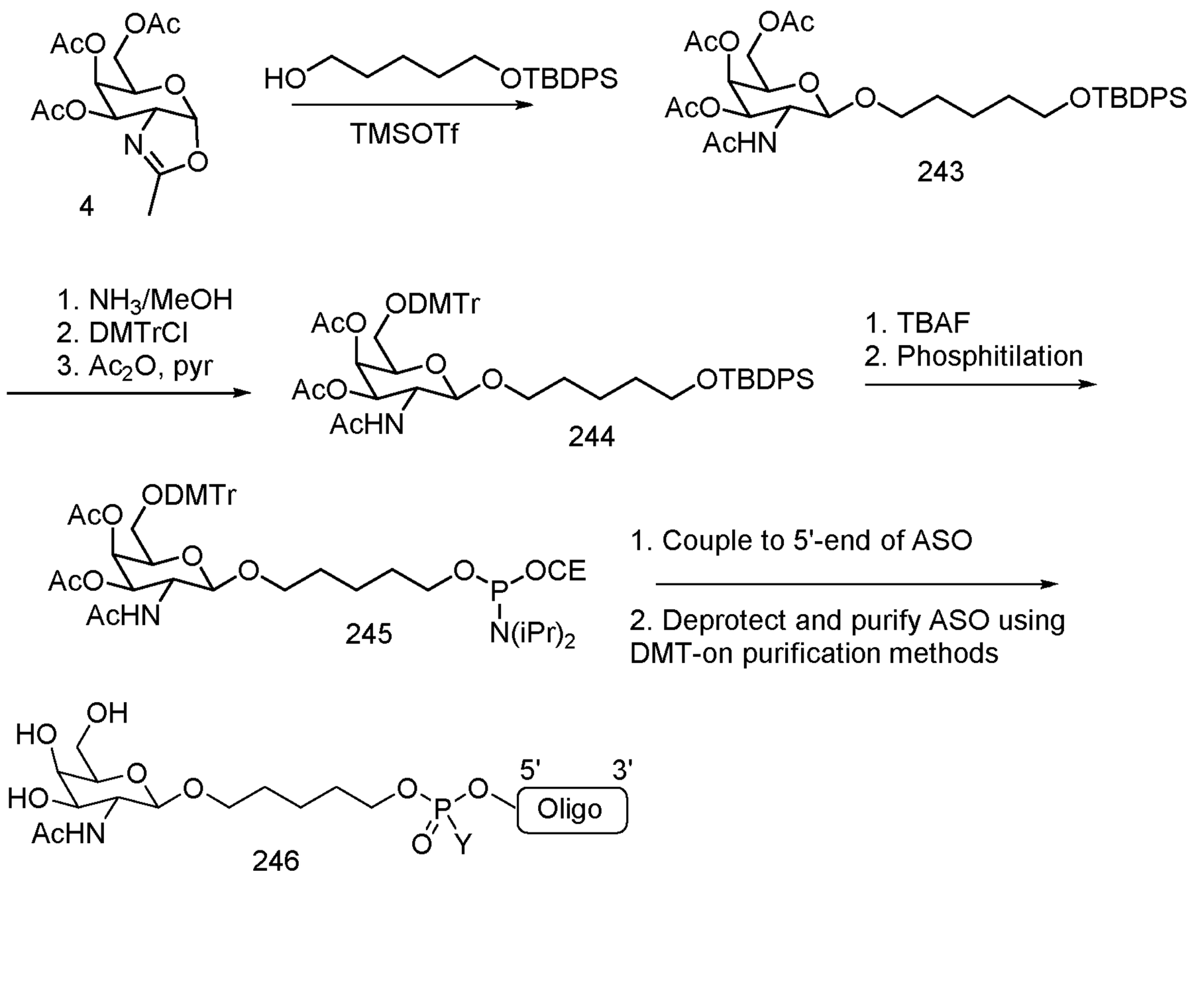


242

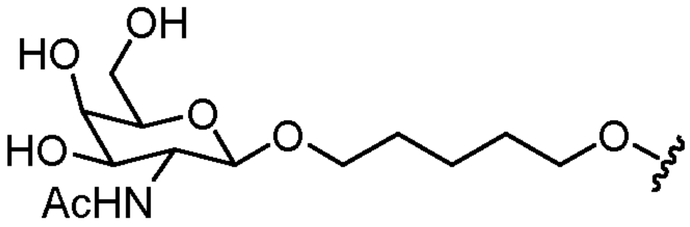
The $GalNAc_1$ cluster portion ($GalNAc_1$-29_a) of the conjugate group $GalNAc_1$-29 can be combined with any cleavable moiety present on the oligonucleotide to provide a variety of conjugate groups. The structure of $GalNAc_1$-29 ($GalNAc_1$-29_a-CM) is shown below:



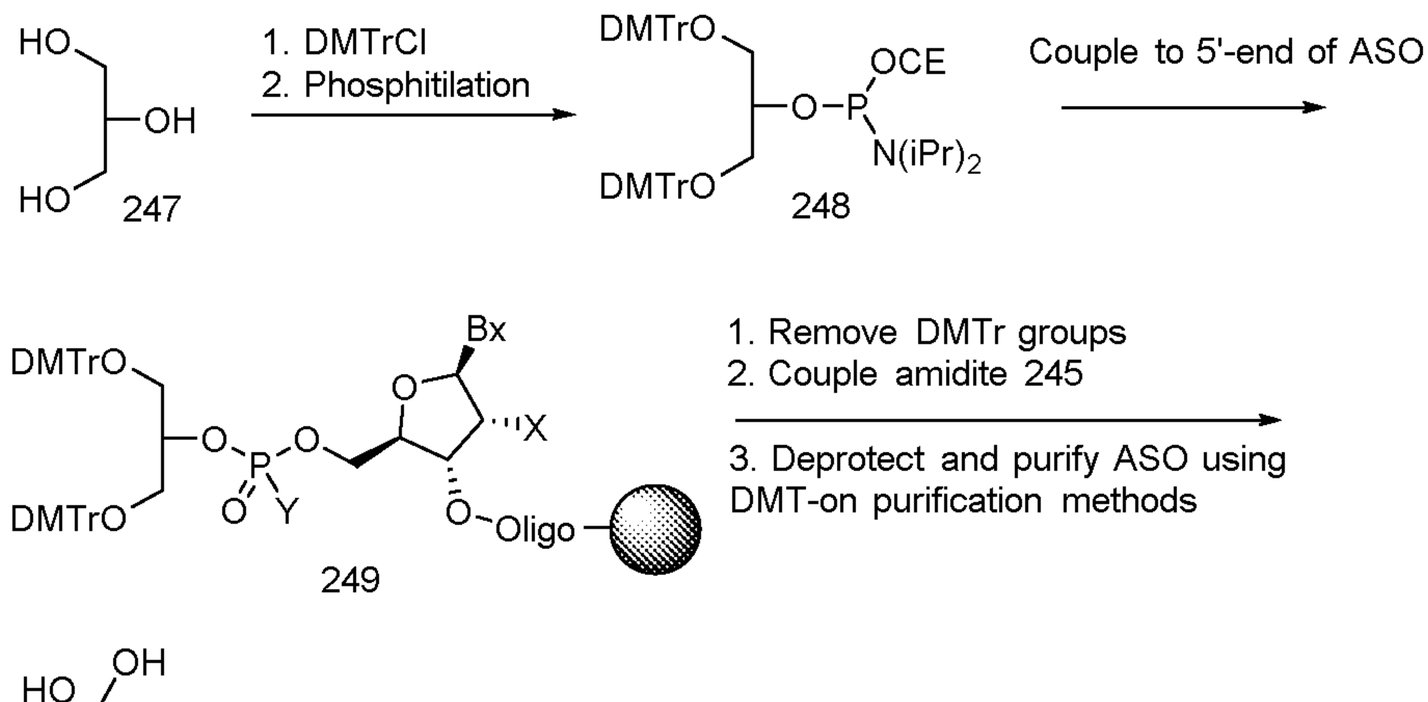
Example 110: Synthesis of oligonucleotides comprising a $GalNAc_1$-30 conjugate

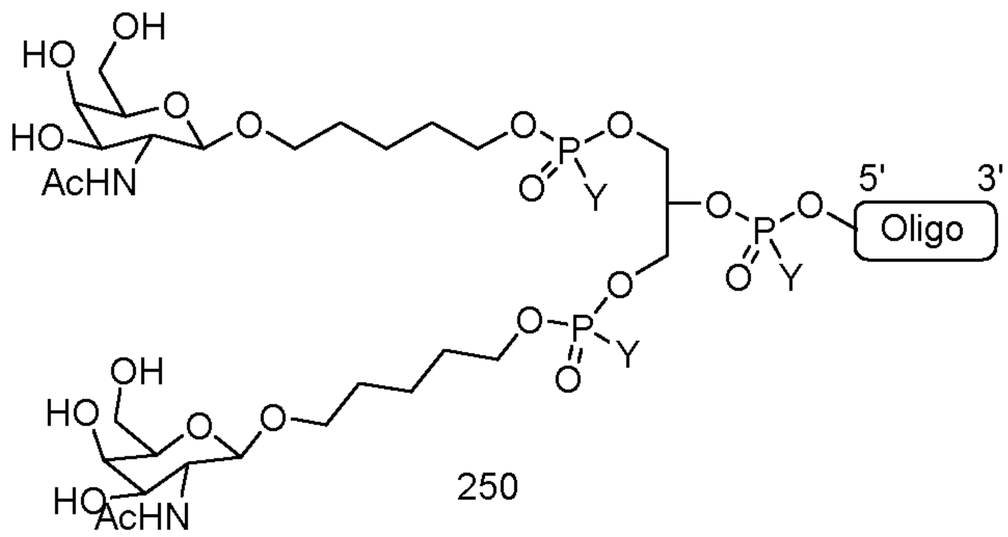


Oligonucleotide 246 comprising a GalNAc$_1$-30 conjugate group, wherein Y is selected from O, S, a substituted or unsubstituted C_1-C_{10} alkyl, amino, substituted amino, azido, alkenyl or alkynyl, is synthesized as shown above. The GalNAc$_1$ cluster portion (GalNAc$_1$-30$_a$) of the conjugate group GalNAc$_1$-30 can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, Y is part of the cleavable moiety. In certain embodiments, Y is part of a stable moiety, and the cleavable moiety is present on the oligonucleotide. The structure of GalNAc$_1$-30$_a$ is shown below:

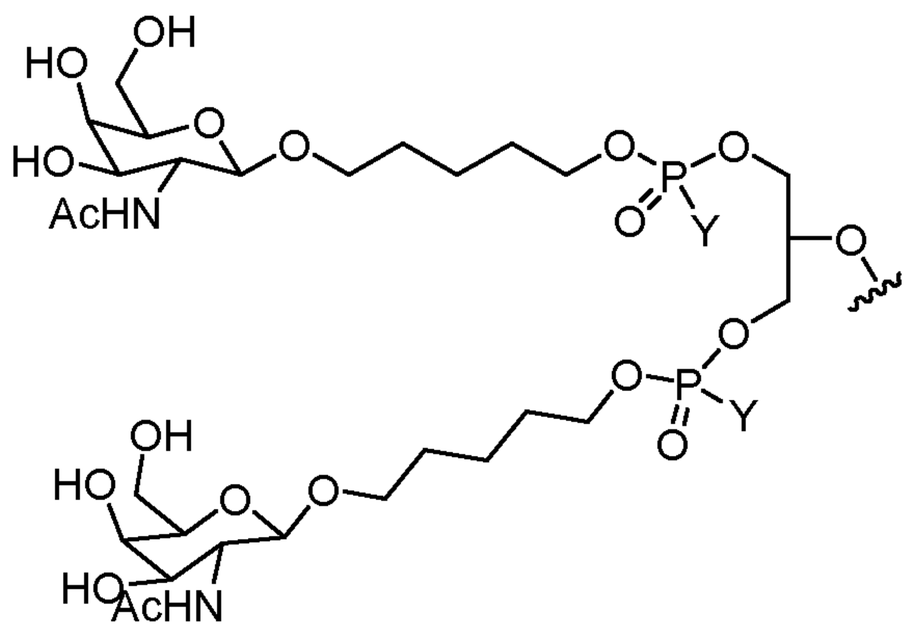


Example 111: Synthesis of oligonucleotides comprising a GalNAc$_2$-31 or GalNAc$_2$-32 conjugate

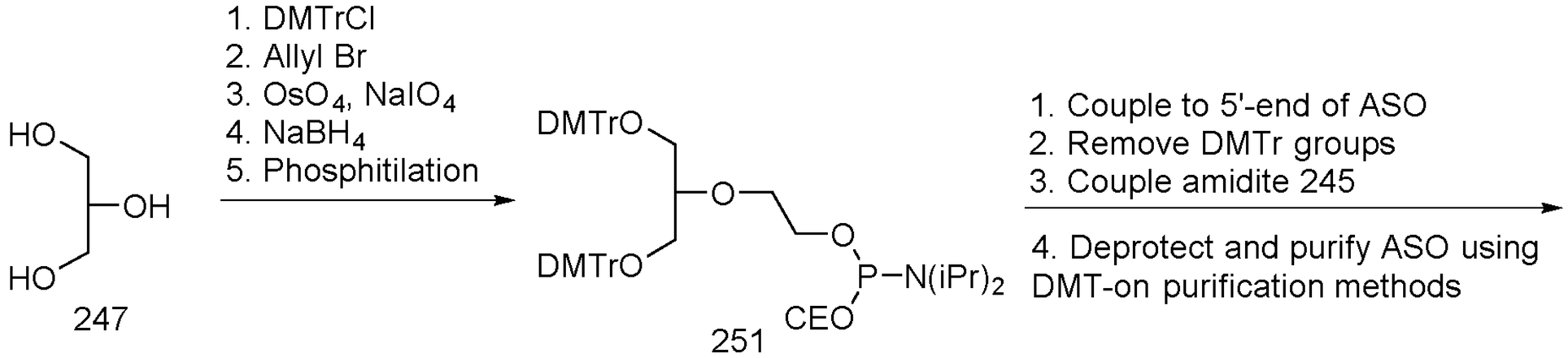


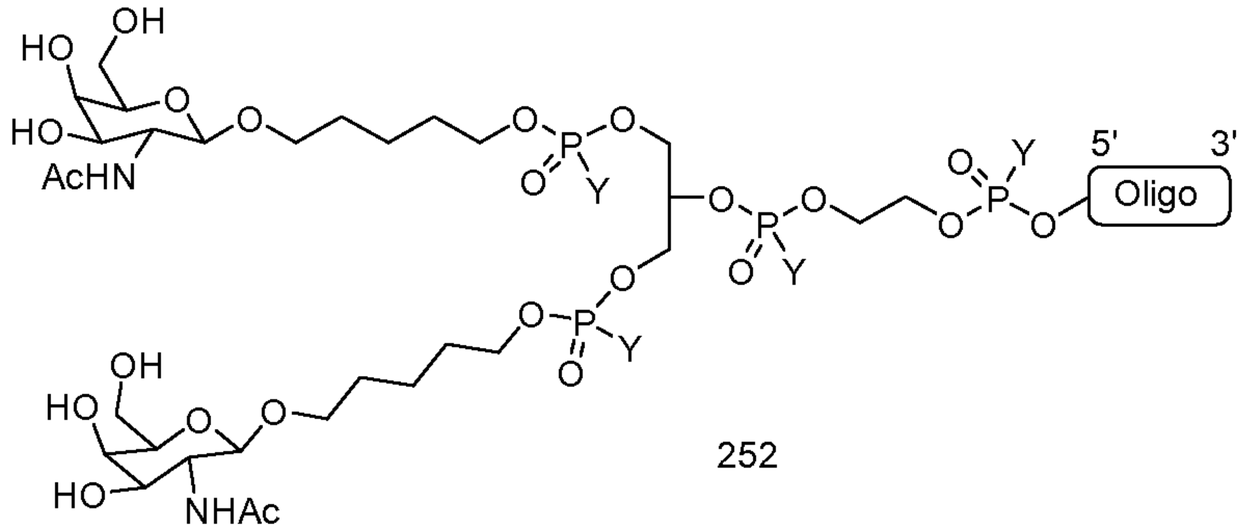


Oligonucleotide 250 comprising a GalNAc$_2$-31 conjugate group, wherein Y is selected from O, S, a substituted or unsubstituted C_1-C_{10} alkyl, amino, substituted amino, azido, alkenyl or alkynyl, is synthesized as shown above. The GalNAc$_2$ cluster portion (GalNAc$_2$-31$_a$) of the conjugate group GalNAc$_2$-31 can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the Y-containing group directly adjacent to the 5'-end of the oligonucleotide is part of the cleavable moiety. In certain embodiments, the Y-containing group directly adjacent to the 5'-end of the oligonucleotide is part of a stable moiety, and the cleavable moiety is present on the oligonucleotide. The structure of GalNAc$_2$-31$_a$ is shown below:


OH
HO
O
HO
AcHN
O
O
P
O
Y
O
O
O
P
O
Y
OH
HO
O
HO
AcHN
O

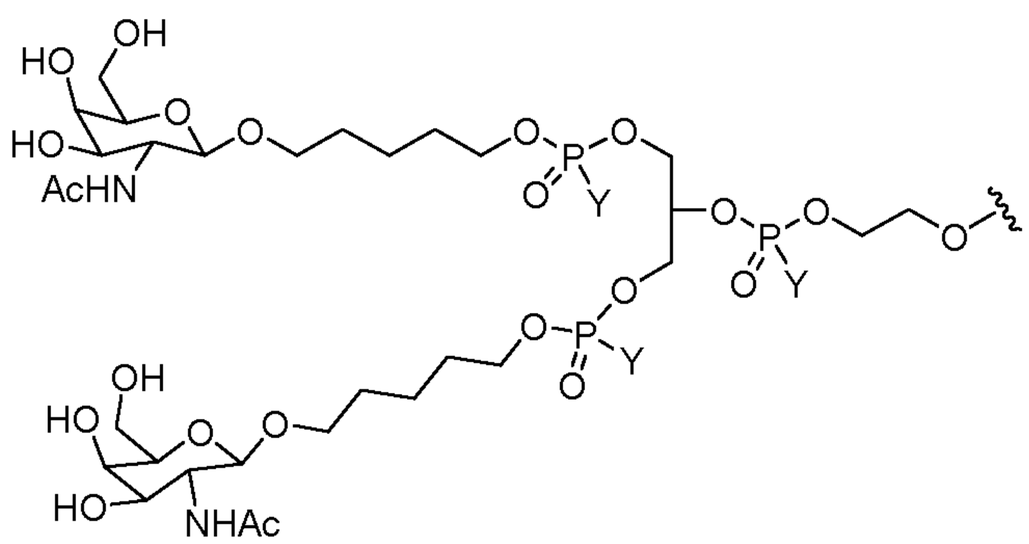
The synthesis of an oligonucleotide comprising a $GalNAc_2$-32 conjugate is shown below.





252

Oligonucleotide 252 comprising a $GalNAc_2$-32 conjugate group, wherein Y is selected from O, S, a substituted or unsubstituted C_1-C_{10} alkyl, amino, substituted amino, azido, alkenyl or alkynyl, is synthesized as shown above. The $GalNAc_2$ cluster portion ($GalNAc_2$-32_a) of the conjugate group $GalNAc_2$-32 can be combined with any cleavable moiety to provide a variety of conjugate groups. In certain embodiments, the Y-containing group directly adjacent to the 5'-end of the oligonucleotide is part of the cleavable moiety. In certain embodiments, the Y-containing group directly adjacent to the 5'-end of the oligonucleotide is part of a stable moiety, and the cleavable moiety is present on the oligonucleotide. The structure of $GalNAc_2$-32_a is shown below:



Example 112: Modified oligonucleotides comprising a $GalNAc_1$ conjugate

The oligonucleotides in Table 120 targeting SRB-1 were synthesized with a $GalNAc_1$ conjugate group in order to further test the potency of oligonucleotides comprising conjugate groups that contain one GalNAc ligand.

Table 120

ISIS No.	Sequence (5' to 3')	GalNAc cluster	CM	SEQ ID NO.
711461	**$GalNAc_1$-$25_{a\text{-}o}$,A_{do}** G_{es} $^{m}C_{es}$ T_{es} T_{es} $^{m}C_{es}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ T_{es} T_e	$GalNAc_1$-25_a	A_d	4888

711462	**GalNAc$_1$-25$_{a-o}$**,G_{es} $^{m}C_{es}$ T_{es} T_{es} $^{m}C_{es}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ T_{es} T_e	GalNAc$_1$-25$_a$	PO	4886
711463	**GalNAc$_1$-25$_{a-o}$**,G_{es} $^{m}C_{eo}$ T_{eo} T_{eo} $^{m}C_{eo}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{eo} $^{m}C_{eo}$ $^{m}C_{es}$ T_{es} T_e	GalNAc$_1$-25$_a$	PO	4886
711465	**GalNAc$_1$-26$_{a-o}$,A$_{do}$** G_{es} $^{m}C_{es}$ T_{es} T_{es} $^{m}C_{es}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ T_{es} T_e	GalNAc$_1$-26$_a$	A$_d$	4888
711466	**GalNAc$_1$-26$_{a-o}$**,G_{es} $^{m}C_{es}$ T_{es} T_{es} $^{m}C_{es}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ T_{es} T_e	GalNAc$_1$-26$_a$	PO	4886
711467	**GalNAc$_1$-26$_{a-o}$**,G_{es} $^{m}C_{eo}$ T_{eo} T_{eo} $^{m}C_{eo}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{eo} $^{m}C_{eo}$ $^{m}C_{es}$ T_{es} T_e	GalNAc$_1$-26$_a$	PO	4886
711468	**GalNAc$_1$-28$_{a-o}$,A$_{do}$** G_{es} $^{m}C_{es}$ T_{es} T_{es} $^{m}C_{es}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ T_{es} T_e	GalNAc$_1$-28$_a$	A$_d$	4888
711469	**GalNAc$_1$-28$_{a-o}$**,G_{es} $^{m}C_{es}$ T_{es} T_{es} $^{m}C_{es}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ T_{es} T_e	GalNAc$_1$-28$_a$	PO	4886
711470	**GalNAc$_1$-28$_{a-o}$**,G_{es} $^{m}C_{eo}$ T_{eo} T_{eo} $^{m}C_{eo}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{eo} $^{m}C_{eo}$ $^{m}C_{es}$ T_{es} T_e	GalNAc$_1$-28$_a$	PO	4886
713844	G_{es} $^{m}C_{es}$ T_{es} T_{es} $^{m}C_{es}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ T_{es} T_{eo}-**GalNAc$_1$-27$_a$**	GalNAc$_1$-27$_a$	PO	4886
713845	G_{es} $^{m}C_{eo}$ T_{eo} T_{eo} $^{m}C_{eo}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{eo} $^{m}C_{eo}$ $^{m}C_{es}$ T_{es} T_{eo}-**GalNAc$_1$-27$_a$**	GalNAc$_1$-27$_a$	PO	4886
713846	G_{es} $^{m}C_{eo}$ T_{eo} T_{eo} $^{m}C_{eo}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{eo} $^{m}C_{eo}$ $^{m}C_{es}$ T_{es} T_{eo} **A$_{do}$**-**GalNAc$_1$-27$_a$**	GalNAc$_1$-27$_a$	A$_d$	4887
713847	G_{es} $^{m}C_{es}$ T_{es} T_{es} $^{m}C_{es}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ T_{es} T_{eo}-**GalNAc$_1$-29$_a$**	GalNAc$_1$-29$_a$	PO	4886
713848	G_{es} $^{m}C_{eo}$ T_{eo} T_{eo} $^{m}C_{eo}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{eo} $^{m}C_{eo}$ $^{m}C_{es}$ T_{es} T_{eo}-**GalNAc$_1$-29$_a$**	GalNAc$_1$-29$_a$	PO	4886
713849	G_{es} $^{m}C_{es}$ T_{es} T_{es} $^{m}C_{es}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{es} $^{m}C_{es}$ $^{m}C_{es}$ T_{es} T_{eo} **A$_{do}$**-**GalNAc$_1$-29$_a$**	GalNAc$_1$-29$_a$	A$_d$	4887
713850	G_{es} $^{m}C_{eo}$ T_{eo} T_{eo} $^{m}C_{eo}$ A_{ds} G_{ds} T_{ds} $^{m}C_{ds}$ A_{ds} T_{ds} G_{ds} A_{ds} $^{m}C_{ds}$ T_{ds} T_{eo} $^{m}C_{eo}$ $^{m}C_{es}$ T_{es} T_{eo} **A$_{do}$**-**GalNAc$_1$-29$_a$**	GalNAc$_1$-29$_a$	A$_d$	4887

Example 113: Antisense oligonucleotides targeting angiopoietin-like 3 and comprising a GalNAc conjugate group

The oligonucleotides in Table 121 were designed to target human angiopoietin-like 3 (ANGPTL3).

Table 121

ISIS No.	Sequences (5' to 3')	SEQ ID No.
563580 (parent)	$G_{es}G_{es}A_{es}$ $^{m}C_{es}A_{es}T_{ds}T_{ds}G_{ds}$ $^{m}C_{ds}$ $^{m}C_{ds}A_{ds}G_{ds}T_{ds}A_{ds}A_{ds}T_{es}$ $^{m}C_{es}G_{es}$ $^{m}C_{es}A_e$	77
658501	$G_{es}G_{es}A_{es}$ $^{m}C_{es}A_{es}T_{ds}T_{ds}G_{ds}$ $^{m}C_{ds}$ $^{m}C_{ds}A_{ds}G_{ds}T_{ds}A_{ds}A_{ds}T_{es}$ $^{m}C_{es}G_{es}$ $^{m}C_{es}A_{eo}$**A$_{do}$-GalNAc$_3$-1$_a$**	4912
666944	**GalNAc$_3$-3$_{a-o}$,A$_{do}$**$G_{es}G_{es}A_{es}$ $^{m}C_{es}A_{es}T_{ds}T_{ds}G_{ds}$ $^{m}C_{ds}$ $^{m}C_{ds}A_{ds}G_{ds}T_{ds}A_{ds}A_{ds}T_{es}$ $^{m}C_{es}G_{es}$ $^{m}C_{es}A_e$	4913
666945	$G_{es}G_{eo}A_{eo}$ $^{m}C_{eo}A_{eo}T_{ds}T_{ds}G_{ds}$ $^{m}C_{ds}$ $^{m}C_{ds}A_{ds}G_{ds}T_{ds}A_{ds}A_{ds}T_{eo}$ $^{m}C_{eo}G_{es}$ $^{m}C_{es}A_{eo}$**A$_{do}$-GalNAc$_3$-1$_a$**	4912
666946	**GalNAc$_3$-3$_{a-o}$,A$_{do}$**$G_{es}G_{eo}A_{eo}$ $^{m}C_{eo}A_{eo}T_{ds}T_{ds}G_{ds}$ $^{m}C_{ds}$ $^{m}C_{ds}A_{ds}G_{ds}T_{ds}A_{ds}A_{ds}T_{eo}$ $^{m}C_{eo}G_{es}$ $^{m}C_{es}A_e$	4913
703801	**GalNAc$_3$-7$_{a-o}$**,$G_{es}G_{es}A_{es}$ $^{m}C_{es}A_{es}T_{ds}T_{ds}G_{ds}$ $^{m}C_{ds}$ $^{m}C_{ds}A_{ds}G_{ds}T_{ds}A_{ds}A_{ds}T_{es}$ $^{m}C_{es}G_{es}$ $^{m}C_{es}A_e$	77
703802	**GalNAc$_3$-7$_{a-o}$**,$G_{es}G_{eo}A_{eo}$ $^{m}C_{eo}A_{eo}T_{ds}T_{ds}G_{ds}$ $^{m}C_{ds}$ $^{m}C_{ds}A_{ds}G_{ds}T_{ds}A_{ds}A_{ds}T_{eo}$ $^{m}C_{eo}G_{es}$ $^{m}C_{es}A_e$	77

Example 114: Antisense inhibition *in vivo* by oligonucleotides comprising a GalNAc conjugate group targeting human ANGPTL3

Six week old male, transgenic C57Bl/6 mice that express human ANGPTL3 were each injected intraperitoneally once per week at a dosage shown below, for a total of two doses, with an oligonucleotide listed in Table 122 (and described in Table 121) or with PBS. Each treatment group consisted of 4 animals. The mice were sacrificed two days following the final dose. ANGPTL3 liver mRNA levels were measured using real-time PCR and RIBOGREEN® RNA quantification reagent (Molecular Probes, Inc. Eugene, OR) according to standard protocols. The results below are presented as the average percent of ANGPTL3 mRNA levels in liver for each treatment group, normalized to the PBS control.

As illustrated in Table 122, treatment with antisense oligonucleotides lowered ANGPTL3 liver mRNA levels in a dose-dependent manner, and the oligonucleotide comprising a GalNAc conjugate was significantly more potent than the parent oligonucleotide lacking a GalNAc conjugate.

Table 122
ANGPTL3 liver mRNA levels

ISIS No.	Dosage (mg/kg)	mRNA (% PBS)	$GalNAc_3$ Cluster	CM
563580	5	58	n/a	n/a
	10	56		
	15	36		
	25	23		
	50	20		
658501	0.3	78	$GalNAc_3$-1a	A_d
	1	60		
	3	27		
	10	19		

Liver alanine aminotransferase (ALT) levels were also measured at time of sacrifice using standard protocols. The results are showed that none of the treatment groups had elevated ALT levels, indicating that the oligonucleotides were well tolerated.

Example 115: Antisense inhibition *in vivo* by oligonucleotides comprising a GalNAc conjugate group targeting mouse ANGPTL3

The oligonucleotides listed in Table 123 below were tested in a dose-dependent study in mice.

Table 123
Modified ASOs targeting mouse ANGPTL3

ISIS No.	Sequences (5' to 3')	$GalNAc_3$ Cluster	CM	SEQ ID No.
233693	$G_{es}A_{es}{}^{m}C_{es}A_{es}T_{es}G_{ds}T_{ds}T_{ds}{}^{m}C_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{ds}{}^{m}C_{ds}{}^{m}C_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}{}^{m}C_{e}$	n/a	n/a	4914

703803	**GalNAc$_3$-7$_a$-$_o$**,$G_{es}A_{es}{}^{m}C_{es}A_{es}T_{es}G_{ds}T_{ds}T_{ds}{}^{m}C_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{ds}{}^{m}C_{ds}{}^{m}C_{ds}T_{es}{}^{m}C_{es}{}^{m}C_{es}T_{es}{}^{m}C_{e}$	GalNAc$_3$-7a	PO	4914
703804	**GalNAc$_3$-7$_a$-$_o$**,$G_{es}A_{eo}{}^{m}C_{eo}A_{eo}T_{eo}G_{ds}T_{ds}T_{ds}{}^{m}C_{ds}T_{ds}T_{ds}{}^{m}C_{ds}A_{ds}{}^{m}C_{ds}{}^{m}C_{ds}T_{eo}{}^{m}C_{eo}{}^{m}C_{es}T_{es}{}^{m}C_{e}$	GalNAc$_3$-7a	PO	4914

The structure of GalNAc$_3$-7$_a$ was shown in Example 48.

Low density lipoprotein receptor knock-out (LDLR$^{-/-}$) mice were fed a western diet for 1 week before being injected intraperitoneally once per week at a dosage shown below with an oligonucleotide listed in Table 123 or with PBS. Each treatment group consisted of 5 animals. Blood was drawn before the first dose was administered in order to determine baseline levels of triglycerides in plasma and at 2 weeks following the first dose. The results in Table 124 are presented as the average percent of plasma triglyceride levels for each treatment group, normalized to baseline levels (% BL), The results show that the antisense oligonucleotides reduced triglycerides in a dose dependent manner. Furthermore, the oligonucleotides comprising a GalNAc conjugate group exhibited even more potent reduction in triglycerides than the oligonucleotide that does not comprise a conjugate group.

Table 124
Plasma triglyceride (TG) levels

ISIS No.	Dosage (mg/kg)	TG (% BL)	ED_{50} (mg/kg)	GalNAc$_3$ Cluster	CM
PBS	n/a	110	n/a	n/a	n/a
233693	1	92	16	n/a	n/a
	3	71			
	10	57			
	30	42			
703803	0.3	96	2	GalNAc$_3$-7a	PO
	1	69			
	3	39			
	10	27			
703804	0.3	97	2	GalNAc$_3$-7a	PO
	1	54			
	3	38			
	10	26			

Example 116: Antisense inhibition of human Angiopoietin-like 3 in Hep3B cells by MOE gapmers

Antisense oligonucleotides were designed targeting an Angiopoietin-like 3 (ANGPTL3) nucleic acid and were tested for their effects on ANGPTL3 mRNA in vitro. The antisense oligonucleotides were tested in a series of experiments that had similar culture conditions. The results for each experiment are presented in separate tables shown below. Cultured Hep3B cells at a density of 20,000 cells per well were transfected using electroporation with 4,500 nM antisense oligonucleotide. After a treatment period of approximately 24 hours, RNA was isolated from the cells and ANGPTL3mRNA levels were measured by quantitative real-time PCR. Human primer probe set RTS3492_MGB (forward sequence CCGTGGAAGACCAATATAAACAATT, designated herein as SEQ ID NO: 4;

AGTCCTTCTGAGCTGATTTTCTATTTCT; reverse sequence, designated herein as SEQ ID NO: 5; probe sequence AACCAACAGCATAGTCAAATA, designated herein as SEQ ID NO: 6) was used to measure mRNA levels. ANGPTL3 mRNA levels were adjusted according to total RNA content, as measured by RIBOGREEN®. Results are presented as percent inhibition of ANGPTL3, relative to untreated control cells.

The newly designed chimeric antisense oligonucleotides in the Tables below were designed as 5-10-5 MOE gapmers. The 5-10-5 MOE gapmers are 20 nucleosides in length, wherein the central gap segment comprises of ten 2'-deoxynucleosides and is flanked by wing segments on the 5' direction and the 3' direction comprising five nucleosides each. Each nucleoside in the 5' wing segment and each nucleoside in the 3' wing segment has a 2'-MOE modification. The internucleoside linkages throughout each gapmer are phosphorothioate (P=S) linkages. All cytosine residues throughout each gapmer are 5-methylcytosines. "Start site" indicates the 5'-most nucleoside to which the gapmer is targeted in the human gene sequence. "Stop site" indicates the 3'-most nucleoside to which the gapmer is targeted human gene sequence. Each gapmer listed in the Tables below is targeted to either the human ANGPTL3 mRNA, designated herein as SEQ ID NO: 1 (GENBANK Accession No. NM_014495.2) or the human ANGPTL3 genomic sequence, designated herein as SEQ ID NO: 2 (GENBANK Accession No. NT_032977.9 truncated from nucleotides 33032001 to 33046000). 'n/a' indicates that the antisense oligonucleotide does not target that particular gene sequence with 100% complementarity.

Table 125

Inhibition of ANGPTL3 mRNA by 5-10-5 MOE gapmers targeting SEQ ID NO: 1 and 2

ISIS NO	SEQ ID NO: 1 Start Site	SEQ ID NO: 1 Stop Site	Sequence	% inhibition	SEQ ID NO: 2 Start Site	SEQ ID NO: 2 Stop Site	SEQ ID NO
544059	23	42	GATTTTCAATTTCAAGCAAC	40	3127	3146	238
337459	49	68	AGCTTAATTGTGAACATTTT	47	3153	3172	239
544060	54	73	GAAGGAGCTTAATTGTGAAC	1	3158	3177	240
544061	63	82	CAATAAAAAGAAGGAGCTTA	37	3167	3186	241
544062	66	85	GAACAATAAAAAGAAGGAGC	38	3170	3189	242
544063	85	104	CTGGAGGAAATAACTAGAGG	30	3189	3208	243
337460	88	107	ATTCTGGAGGAAATAACTAG	39	3192	3211	244
544064	112	131	TCAAATGATGAATTGTCTTG	36	3216	3235	245
544065	138	157	TTGATTTTGGCTCTGGAGAT	26	3242	3261	246
544066	145	164	GCAAATCTTGATTTTGGCTC	56	3249	3268	247
233676	148	167	ATAGCAAATCTTGATTTTGG	69	3252	3271	248
544067	156	175	CGTCTAACATAGCAAATCTT	64	3260	3279	249
544068	174	193	TGGCTAAAATTTTTACATCG	28	3278	3297	250
544069	178	197	CCATTGGCTAAAATTTTTAC	0	3282	3301	251
544070	184	203	AGGAGGCCATTGGCTAAAAT	7	3288	3307	252
544071	187	206	TGAAGGAGGCCATTGGCTAA	32	3291	3310	253

544072	195	214	GTCCCAACTGAAGGAGGCCA	9	3299	3318	254
544073	199	218	CCATGTCCCAACTGAAGGAG	6	3303	3322	255
544074	202	221	AGACCATGTCCCAACTGAAG	18	3306	3325	256
544075	206	225	TTTAAGACCATGTCCCAACT	0	3310	3329	257
544076	209	228	GTCTTTAAGACCATGTCCCA	0	3313	3332	258
544077	216	235	GGACAAAGTCTTTAAGACCA	0	3320	3339	259
544078	222	241	TCTTATGGACAAAGTCTTTA	0	3326	3345	260
544079	245	264	TATGTCATTAATTTGGCCCT	0	3349	3368	261
544080	270	289	GATCAAATATGTTGAGTTTT	27	3374	3393	262
233690	274	293	GACTGATCAAATATGTTGAG	49	3378	3397	263
544081	316	335	TCTTCTTTGATTTCACTGGT	62	3420	3439	264
544082	334	353	CTTCTCAGTTCCTTTTCTTC	35	3438	3457	265
544083	337	356	GTTCTTCTCAGTTCCTTTTC	60	3441	3460	266
544084	341	360	TGTAGTTCTTCTCAGTTCCT	51	3445	3464	267
544431	345	364	TATATGTAGTTCTTCTCAGT	9	3449	3468	268
544086	348	367	GTTTATATGTAGTTCTTCTC	39	3452	3471	269
544087	352	371	TGTAGTTTATATGTAGTTCT	30	3456	3475	270
544088	356	375	GACTTGTAGTTTATATGTAG	12	3460	3479	271
544089	364	383	TCATTTTTGACTTGTAGTTT	31	3468	3487	272
544090	369	388	CCTCTTCATTTTTGACTTGT	61	3473	3492	273
544091	375	394	TCTTTACCTCTTCATTTTTG	48	3479	3498	274
544092	380	399	CATATTCTTTACCTCTTCAT	35	3484	3503	275
544093	384	403	GTGACATATTCTTTACCTCT	63	3488	3507	276
544094	392	411	GAGTTCAAGTGACATATTCT	53	3496	3515	277
544095	398	417	TGAGTTGAGTTCAAGTGACA	31	3502	3521	278
544096	403	422	AGTTTTGAGTTGAGTTCAAG	14	3507	3526	279
544097	406	425	TCAAGTTTTGAGTTGAGTTC	38	3510	3529	280
544098	414	433	GGAGGCTTTCAAGTTTTGAG	39	3518	3537	281
544099	423	442	TTTCTTCTAGGAGGCTTTCA	57	3527	3546	282
544100	427	446	ATTTTTTCTTCTAGGAGGCT	39	3531	3550	283
544101	432	451	GTAGAATTTTTTCTTCTAGG	28	3536	3555	284
544102	462	481	GCTCTTCTAAATATTTCACT	60	3566	3585	285
544103	474	493	AGTTAGTTAGTTGCTCTTCT	40	3578	3597	286
544104	492	511	CAGGTTGATTTTGAATTAAG	38	3596	3615	287
544105	495	514	TTTCAGGTTGATTTTGAATT	28	3599	3618	288
544106	499	518	GGAGTTTCAGGTTGATTTTG	38	3603	3622	289
544107	504	523	GTTCTGGAGTTTCAGGTTGA	50	3608	3627	290
544108	526	545	TTAAGTGAAGTTACTTCTGG	20	3630	3649	291
544109	555	574	TGCTATTATCTTGTTTTTCT	23	4293	4312	292
544110	564	583	GGTCTTTGATGCTATTATCT	67	4302	4321	293
544111	567	586	GAAGGTCTTTGATGCTATTA	49	4305	4324	294
544112	572	591	CTGGAGAAGGTCTTTGATGC	52	4310	4329	295
544113	643	662	CTGAGCTGATTTTCTATTTC	12	n/a	n/a	296

337477	664	683	GGTTCTTGAATACTAGTCCT	70	6677	6696	234
544114	673	692	ATTTCTGTGGGTTCTTGAAT	32	6686	6705	297
337478	675	694	AAATTTCTGTGGGTTCTTGA	51	6688	6707	235
544115	678	697	GAGAAATTTCTGTGGGTTCT	54	6691	6710	298
544116	682	701	GATAGAGAAATTTCTGTGGG	25	6695	6714	299
544117	689	708	CTTGGAAGATAGAGAAATTT	16	6702	6721	300
337479	692	711	TGGCTTGGAAGATAGAGAAA	34	6705	6724	236
544118	699	718	GTGCTCTTGGCTTGGAAGAT	64	6712	6731	301
544119	703	722	CTTGGTGCTCTTGGCTTGGA	70	6716	6735	302
544120	707	726	AGTTCTTGGTGCTCTTGGCT	82	6720	6739	15
233710	710	729	AGTAGTTCTTGGTGCTCTTG	63	6723	6742	233
544121	713	732	GGGAGTAGTTCTTGGTGCTC	64	6726	6745	303
544122	722	741	CTGAAGAAAGGGAGTAGTTC	24	6735	6754	304
544123	752	771	ATCATGTTTTACATTTCTTA	0	6765	6784	305
544124	755	774	GCCATCATGTTTTACATTTC	35	n/a	n/a	306
544125	759	778	GAATGCCATCATGTTTTACA	8	n/a	n/a	307
544126	762	781	CAGGAATGCCATCATGTTTT	6	n/a	n/a	308
337487	804	823	CACTTGTATGTTCACCTCTG	65	7389	7408	28
233717	889	908	TGAATTAATGTCCATGGACT	33	7876	7895	14

Table 126

Inhibition of ANGPTL3 mRNA by 5-10-5 MOE gapmers targeting SEQ ID NO: 1 and 2

ISIS NO	SEQ ID NO: 1 Start Site	SEQ ID NO: 1 Stop Site	Sequence	% inhibition	SEQ ID NO: 2 Start Site	SEQ ID NO: 2 Stop Site	SEQ ID NO
544204	n/a	n/a	GACTTCTTAACTCTATATAT	0	3076	3095	309
544205	n/a	n/a	CTAGACTTCTTAACTCTATA	0	3079	3098	310
544206	n/a	n/a	GACCTAGACTTCTTAACTCT	0	3082	3101	311
544207	n/a	n/a	GGAAGCAGACCTAGACTTCT	21	3089	3108	312
544208	n/a	n/a	TCTGGAAGCAGACCTAGACT	23	3092	3111	313
544209	n/a	n/a	TCTTCTGGAAGCAGACCTAG	7	3095	3114	314
544210	n/a	n/a	CTAATCTTTAGGGATTTAGG	24	11433	11452	315
544211	n/a	n/a	TGTATCTAATCTTTAGGGAT	2	11438	11457	316
544213	n/a	n/a	TAACTTGGGCACTATATCCT	44	11553	11572	317
544214	n/a	n/a	ATTGACAAAGGTAGGTCACC	59	11576	11595	318
544215	n/a	n/a	ATATGACATGTATATTGGAT	41	11620	11639	319
544216	n/a	n/a	TTTTGTACTTTTCTGGAACA	34	11704	11723	320
544217	n/a	n/a	TAGTCTGTGGTCCTGAAAAT	32	11748	11767	321
544218	n/a	n/a	AGCTTAGTCTGTGGTCCTGA	20	11752	11771	322
544219	n/a	n/a	GACAGCTTAGTCTGTGGTCC	45	11755	11774	323
544220	n/a	n/a	GTATTCTGGCCCTAAAAAAA	2	11789	11808	324
544221	n/a	n/a	ATTTTGGTATTCTGGCCCTA	39	11795	11814	325

544223	n/a	n/a	TTTGCATTTGAAATTGTCCA	32	11837	11856	326
544224	n/a	n/a	GGAAGCAACTCATATATTAA	39	11869	11888	327
544225	n/a	n/a	TATCAGAAAAAGATACCTGA	0	9821	9840	328
544226	n/a	n/a	ATAATAGCTAATAATGTGGG	15	9875	9894	329
544227	n/a	n/a	TGCAGATAATAGCTAATAAT	31	9880	9899	330
544228	n/a	n/a	TGTCATTGCAGATAATAGCT	61	9886	9905	331
544229	n/a	n/a	TAAAAGTTGTCATTGCAGAT	38	9893	9912	332
544230	n/a	n/a	CGGATTTTTAAAAGTTGTCA	45	9901	9920	333
544231	n/a	n/a	GGGATTCGGATTTTTAAAAG	0	9907	9926	334
544232	n/a	n/a	TTTGGGATTCGGATTTTTAA	24	9910	9929	335
544233	n/a	n/a	ACGCTTATTTGGGATTCGGA	53	9917	9936	336
544251	n/a	n/a	TTTAAGAGATTTACAAGTCA	11	2811	2830	337
544252	n/a	n/a	GACTACCTGTTTTTAAAAGC	6	2851	2870	338
544253	n/a	n/a	TATGGTGACTACCTGTTTTT	12	2857	2876	339
544254	n/a	n/a	ACTTTGCTGTATTATAAACT	12	2890	2909	340
544255	n/a	n/a	ATTGTATTTAACTTTGCTGT	0	2900	2919	341
544256	n/a	n/a	GAGCAACTAACTTAATAGGT	13	2928	2947	342
544257	n/a	n/a	GAAATGAGCAACTAACTTAA	25	2933	2952	343
544258	n/a	n/a	AATCAAAGAAATGAGCAACT	0	2940	2959	344
544259	n/a	n/a	ACCTTCTTCCACATTGAGTT	8	2977	2996	345
544260	n/a	n/a	CACGAATGTAACCTTCTTCC	0	2987	3006	346
544261	n/a	n/a	TTAACTTGCACGAATGTAAC	27	2995	3014	347
544262	n/a	n/a	TATATATACCAATATTTGCC	0	3063	3082	348
544263	n/a	n/a	TCTTAACTCTATATATACCA	0	3072	3091	349
544264	n/a	n/a	CTTTAAGTGAAGTTACTTCT	17	3632	3651	350
544265	n/a	n/a	TCTACTTACTTTAAGTGAAG	9	3640	3659	351
544266	n/a	n/a	GAACCCTCTTTATTTTCTAC	1	3655	3674	352
544267	n/a	n/a	ACATAAACATGAACCCTCTT	6	3665	3684	353
544268	n/a	n/a	CCACATTGAAAACATAAACA	25	3676	3695	354
544269	n/a	n/a	GCATGCCTTAGAAATATTTT	7	3707	3726	355
544270	n/a	n/a	CAATGCAACAAAGTATTTCA	0	3731	3750	356
544271	n/a	n/a	CTGGAGATTATTTTTCTTGG	34	3768	3787	357
544272	n/a	n/a	TTCATATATAACATTAGGGA	0	3830	3849	358
544273	n/a	n/a	TCAGTGTTTTCATATATAAC	18	3838	3857	359
544274	n/a	n/a	GACATAGTGTTCTAGATTGT	14	3900	3919	360
544275	n/a	n/a	CAATAGTGTAATGACATAGT	21	3912	3931	361
544276	n/a	n/a	TTACTTACCTTCAGTAATTT	0	3933	3952	362
544277	n/a	n/a	ATCTTTTCCATTTACTGTAT	8	4005	4024	363
544278	n/a	n/a	AGAAAAAGCCCAGCATATTT	11	4037	4056	364
544279	n/a	n/a	GTATGCTTCTTTCAAATAGC	36	4130	4149	365
544280	n/a	n/a	CCTTCCCCTTGTATGCTTCT	41	4140	4159	366
544281	n/a	n/a	CCTGTAACACTATCATAATC	1	4207	4226	367
544282	n/a	n/a	TGACTTACCTGATTTTCTAT	6	4384	4403	368

544283	n/a	n/a	GATGGGACATACCATTAAAA	0	4407	4426	369
544284	n/a	n/a	GTGAAAGATGGGACATACCA	20	4413	4432	370
544285	n/a	n/a	CCTGTGTGAAAGATGGGACA	6	4418	4437	371
544286	n/a	n/a	CATTGGCTGCTATGAATTAA	41	4681	4700	372
544287	n/a	n/a	GATGACATTGGCTGCTATGA	40	4686	4705	373
544288	n/a	n/a	GAGAAACATGATCTAATTTG	12	4717	4736	374
544289	n/a	n/a	ATGGAAAGCTATTGTGTGGT	0	4747	4766	375
544290	n/a	n/a	GTCTAAAGAGCCAATATGAG	22	4771	4790	376
544291	n/a	n/a	AATCTTGGTCTAAAGAGCCA	46	4778	4797	377
544433	n/a	n/a	GAGATTTACAAGTCAAAAAT	4	2806	2825	378
544434	n/a	n/a	ATTTAACTTTGCTGTATTAT	0	2895	2914	379
544435	n/a	n/a	ATCAATGCTAAATGAAATCA	0	2955	2974	380
544436	n/a	n/a	TATTTTCTGGAGATTATTTT	0	3774	3793	381
544437	n/a	n/a	AAAATGAATATTGGCAATTC	0	4159	4178	382
233717	889	908	TGAATTAATGTCCATGGACT	36	7876	7895	14
544202	2081	2100	AAAGTCAATGTGACTTAGTA	42	11053	11072	383
544203	2104	2123	AAGGTATAGTGATACCTCAT	56	11076	11095	384

Table 127

Inhibition of ANGPTL3 mRNA by 5-10-5 MOE gapmers targeting SEQ ID NO: 1 and 2

ISIS NO	SEQ ID NO: 1 Start Site	SEQ ID NO: 1 Stop Site	Sequence	% inhibition	SEQ ID NO: 2 Start Site	SEQ ID NO: 2 Stop Site	SEQ ID NO
544127	765	784	CAGCAGGAATGCCATCATGT	4	N/A	N/A	385
544128	819	838	TGATGGCATACATGCCACTT	0	7404	7423	386
544129	828	847	TGCTGGGTCTGATGGCATAC	44	7413	7432	387
544130	832	851	GAGTTGCTGGGTCTGATGGC	16	7417	7436	388
544131	841	860	AAAACTTGAGAGTTGCTGGG	0	7426	7445	389
544132	848	867	GACATGAAAAACTTGAGAGT	0	7433	7452	390
544133	859	878	ACATCACAGTAGACATGAAA	25	7444	7463	391
233717	889	908	TGAATTAATGTCCATGGACT	36	7876	7895	14
544134	915	934	AGTTTTGTGATCCATCTATT	46	7902	7921	392
544135	918	937	TGAAGTTTTGTGATCCATCT	42	7905	7924	393
544136	926	945	CGTTTCATTGAAGTTTTGTG	45	7913	7932	394
544137	946	965	CCATATTTGTAGTTCTCCCA	44	7933	7952	395
544138	949	968	AAACCATATTTGTAGTTCTC	25	7936	7955	396
544139	970	989	AATTCTCCATCAAGCCTCCC	35	N/A	N/A	397
233722	991	1010	ATCTTCTCTAGGCCCAACCA	65	9566	9585	398
544432	997	1016	GAGTATATCTTCTCTAGGCC	0	9572	9591	399
544140	1002	1021	CTATGGAGTATATCTTCTCT	6	9577	9596	400
544141	1008	1027	GCTTCACTATGGAGTATATC	63	9583	9602	401
544142	1013	1032	AGATTGCTTCACTATGGAGT	52	9588	9607	402
544143	1046	1065	CCAGTCTTCCAACTCAATTC	35	9621	9640	403

544144	1052	1071	GTCTTTCCAGTCTTCCAACT	64	9627	9646	404
544145	1055	1074	GTTGTCTTTCCAGTCTTCCA	80	9630	9649	16
544146	1059	1078	GTTTGTTGTCTTTCCAGTCT	59	9634	9653	405
544147	1062	1081	AATGTTTGTTGTCTTTCCAG	12	9637	9656	406
544148	1095	1114	CGTGATTTCCCAAGTAAAAA	56	9670	9689	407
544149	1160	1179	GTTTTCCGGGATTGCATTGG	33	9735	9754	408
544150	1165	1184	TCTTTGTTTTCCGGGATTGC	54	9740	9759	409
544151	1170	1189	CCAAATCTTTGTTTTCCGGG	64	9745	9764	410
544152	1173	1192	ACACCAAATCTTTGTTTTCC	37	9748	9767	411
544153	1178	1197	AGAAAACACCAAATCTTTGT	32	9753	9772	412
544154	1183	1202	CAAGTAGAAAACACCAAATC	13	9758	9777	413
544155	1188	1207	GATCCCAAGTAGAAAACACC	0	9763	9782	414
544156	1195	1214	GCTTTGTGATCCCAAGTAGA	74	9770	9789	17
544157	1198	1217	TTTGCTTTGTGATCCCAAGT	73	9773	9792	415
544158	1202	1221	TCCTTTTGCTTTGTGATCCC	62	9777	9796	416
544159	1208	1227	GAAGTGTCCTTTTGCTTTGT	30	9783	9802	417
544160	1246	1265	TGCCACCACCAGCCTCCTGA	60	N/A	N/A	418
544161	1253	1272	CTCATCATGCCACCACCAGC	73	10225	10244	419
544162	1269	1288	GGTTGTTTTCTCCACACTCA	76	10241	10260	18
544163	1276	1295	CCATTTAGGTTGTTTTCTCC	25	10248	10267	420
544164	1283	1302	ATATTTACCATTTAGGTTGT	25	10255	10274	421
544165	1294	1313	CTTGGTTTGTTATATTTACC	63	10266	10285	422
544166	1353	1372	ACCTTCCATTTTGAGACTTC	75	10325	10344	19
544167	1363	1382	ATAGAGTATAACCTTCCATT	71	10335	10354	423
544168	1367	1386	TTTTATAGAGTATAACCTTC	37	10339	10358	424
544169	1374	1393	TGGTTGATTTTATAGAGTAT	37	10346	10365	425
544170	1378	1397	ATTTTGGTTGATTTTATAGA	3	10350	10369	426
544171	1383	1402	TCAACATTTTGGTTGATTTT	16	10355	10374	427
544172	1390	1409	GGATGGATCAACATTTTGGT	51	10362	10381	428
544173	1393	1412	GTTGGATGGATCAACATTTT	62	10365	10384	429
544174	1396	1415	TCTGTTGGATGGATCAACAT	5	10368	10387	430
544175	1401	1420	CTGAATCTGTTGGATGGATC	55	10373	10392	431
544176	1407	1426	AGCTTTCTGAATCTGTTGGA	65	10379	10398	432
544177	1414	1433	CATTCAAAGCTTTCTGAATC	21	10386	10405	433
544178	1417	1436	GTTCATTCAAAGCTTTCTGA	66	10389	10408	434
544179	1420	1439	TCAGTTCATTCAAAGCTTTC	6	10392	10411	435
544180	1423	1442	GCCTCAGTTCATTCAAAGCT	68	10395	10414	436
544181	1427	1446	ATTTGCCTCAGTTCATTCAA	53	10399	10418	437
544182	1431	1450	TTAAATTTGCCTCAGTTCAT	40	10403	10422	438
544183	1436	1455	GCCTTTTAAATTTGCCTCAG	70	10408	10427	439
544184	1498	1517	AGGATTTAATACCAGATTAT	38	10470	10489	440

544185	1502	1521	CTTAAGGATTTAATACCAGA	56	10474	10493	441
544186	1505	1524	TCTCTTAAGGATTTAATACC	33	10477	10496	442
544187	1546	1565	GACAGTGACTTTAAGATAAA	35	10518	10537	443
544188	1572	1591	TGTGATTGTATGTTTAATCT	48	10544	10563	444
544189	1578	1597	AGGTTATGTGATTGTATGTT	48	10550	10569	445
544190	1583	1602	CTTTAAGGTTATGTGATTGT	48	10555	10574	446
544191	1589	1608	GGTATTCTTTAAGGTTATGT	62	10561	10580	447
544192	1656	1675	ATTGATTCCCACATCACAAA	47	10628	10647	448
544193	1661	1680	CTAAAATTGATTCCCACATC	67	10633	10652	449
544194	1665	1684	CCATCTAAAATTGATTCCCA	63	10637	10656	450
544195	1771	1790	TTGTGATATTAGCTCATATG	59	10743	10762	451
544196	1794	1813	ACTAGTTTTTTAAACTGGGA	28	10766	10785	452
544197	1820	1839	GTCAAGTTTAGAGTTTTAAC	44	10792	10811	453
544198	1826	1845	TATTTAGTCAAGTTTAGAGT	14	10798	10817	454
544199	1907	1926	TACACATACTCTGTGCTGAC	82	10879	10898	20
544200	1913	1932	GATTTTTACACATACTCTGT	57	10885	10904	455
544201	2008	2027	CTGCTTCATTAGGTTTCATA	61	10980	10999	456

Table 128

Inhibition of ANGPTL3 mRNA by 5-10-5 MOE gapmers targeting SEQ ID NO: 1 and 2

ISIS NO	SEQ ID NO: 1 Start Site	SEQ ID NO: 1 Stop Site	Sequence	% inhibition	SEQ ID NO: 2 Start Site	SEQ ID NO: 2 Stop Site	SEQ ID NO
544127	765	784	CAGCAGGAATGCCATCATGT	0	N/A	N/A	457
544128	819	838	TGATGGCATACATGCCACTT	13	7404	7423	458
544129	828	847	TGCTGGGTCTGATGGCATAC	49	7413	7432	459
544130	832	851	GAGTTGCTGGGTCTGATGGC	27	7417	7436	460
544131	841	860	AAAACTTGAGAGTTGCTGGG	0	7426	7445	461
544132	848	867	GACATGAAAAACTTGAGAGT	0	7433	7452	462
544133	859	878	ACATCACAGTAGACATGAAA	18	7444	7463	463
233717	889	908	TGAATTAATGTCCATGGACT	55	7876	7895	14
544134	915	934	AGTTTTGTGATCCATCTATT	68	7902	7921	464
544135	918	937	TGAAGTTTTGTGATCCATCT	77	7905	7924	465
544136	926	945	CGTTTCATTGAAGTTTTGTG	60	7913	7932	466
544137	946	965	CCATATTTGTAGTTCTCCCA	64	7933	7952	467
544138	949	968	AAACCATATTTGTAGTTCTC	45	7936	7955	468
544139	970	989	AATTCTCCATCAAGCCTCCC	70	N/A	N/A	469
233722	991	1010	ATCTTCTCTAGGCCCAACCA	96	9566	9585	470
544432	997	1016	GAGTATATCTTCTCTAGGCC	69	9572	9591	471
544140	1002	1021	CTATGGAGTATATCTTCTCT	37	9577	9596	472
544141	1008	1027	GCTTCACTATGGAGTATATC	65	9583	9602	473
544142	1013	1032	AGATTGCTTCACTATGGAGT	55	9588	9607	474

544143	1046	1065	CCAGTCTTCCAACTCAATTC	31	9621	9640	475
544144	1052	1071	GTCTTTCCAGTCTTCCAACT	72	9627	9646	476
544145	1055	1074	GTTGTCTTTCCAGTCTTCCA	86	9630	9649	16
544146	1059	1078	GTTTGTTGTCTTTCCAGTCT	66	9634	9653	477
544147	1062	1081	AATGTTTGTTGTCTTTCCAG	21	9637	9656	478
544148	1095	1114	CGTGATTTCCCAAGTAAAAA	63	9670	9689	479
544149	1160	1179	GTTTTCCGGGATTGCATTGG	32	9735	9754	480
544150	1165	1184	TCTTTGTTTTCCGGGATTGC	48	9740	9759	481
544151	1170	1189	CCAAATCTTTGTTTTCCGGG	72	9745	9764	482
544152	1173	1192	ACACCAAATCTTTGTTTTCC	39	9748	9767	483
544153	1178	1197	AGAAAACACCAAATCTTTGT	39	9753	9772	484
544154	1183	1202	CAAGTAGAAAACACCAAATC	22	9758	9777	485
544155	1188	1207	GATCCCAAGTAGAAAACACC	5	9763	9782	486
544156	1195	1214	GCTTTGTGATCCCAAGTAGA	79	9770	9789	17
544157	1198	1217	TTTGCTTTGTGATCCCAAGT	80	9773	9792	487
544158	1202	1221	TCCTTTTGCTTTGTGATCCC	73	9777	9796	488
544159	1208	1227	GAAGTGTCCTTTTGCTTTGT	33	9783	9802	489
544160	1246	1265	TGCCACCACCAGCCTCCTGA	67	N/A	N/A	490
544161	1253	1272	CTCATCATGCCACCACCAGC	79	10225	10244	491
544162	1269	1288	GGTTGTTTTCTCCACACTCA	84	10241	10260	18
544163	1276	1295	CCATTTAGGTTGTTTTCTCC	34	10248	10267	492
544164	1283	1302	ATATTTACCATTTAGGTTGT	17	10255	10274	493
544165	1294	1313	CTTGGTTTGTTATATTTACC	76	10266	10285	494
544166	1353	1372	ACCTTCCATTTTGAGACTTC	79	10325	10344	19
544167	1363	1382	ATAGAGTATAACCTTCCATT	73	10335	10354	495
544168	1367	1386	TTTTATAGAGTATAACCTTC	41	10339	10358	496
544169	1374	1393	TGGTTGATTTTATAGAGTAT	53	10346	10365	497
544170	1378	1397	ATTTTGGTTGATTTTATAGA	28	10350	10369	498
544171	1383	1402	TCAACATTTTGGTTGATTTT	19	10355	10374	499
544172	1390	1409	GGATGGATCAACATTTTGGT	66	10362	10381	500
544173	1393	1412	GTTGGATGGATCAACATTTT	71	10365	10384	501
544174	1396	1415	TCTGTTGGATGGATCAACAT	35	10368	10387	502
544175	1401	1420	CTGAATCTGTTGGATGGATC	68	10373	10392	503
544176	1407	1426	AGCTTTCTGAATCTGTTGGA	70	10379	10398	504
544177	1414	1433	CATTCAAAGCTTTCTGAATC	35	10386	10405	505
544178	1417	1436	GTTCATTCAAAGCTTTCTGA	76	10389	10408	506
544179	1420	1439	TCAGTTCATTCAAAGCTTTC	15	10392	10411	507
544180	1423	1442	GCCTCAGTTCATTCAAAGCT	68	10395	10414	508
544181	1427	1446	ATTTGCCTCAGTTCATTCAA	67	10399	10418	509
544182	1431	1450	TTAAATTTGCCTCAGTTCAT	51	10403	10422	510
544183	1436	1455	GCCTTTTAAATTTGCCTCAG	80	10408	10427	511

544184	1498	1517	AGGATTTAATACCAGATTAT	54	10470	10489	512
544185	1502	1521	CTTAAGGATTTAATACCAGA	69	10474	10493	513
544186	1505	1524	TCTCTTAAGGATTTAATACC	58	10477	10496	514
544187	1546	1565	GACAGTGACTTTAAGATAAA	34	10518	10537	515
544188	1572	1591	TGTGATTGTATGTTTAATCT	47	10544	10563	516
544189	1578	1597	AGGTTATGTGATTGTATGTT	68	10550	10569	517
544190	1583	1602	CTTTAAGGTTATGTGATTGT	62	10555	10574	518
544191	1589	1608	GGTATTCTTTAAGGTTATGT	66	10561	10580	519
544192	1656	1675	ATTGATTCCCACATCACAAA	50	10628	10647	520
544193	1661	1680	CTAAAATTGATTCCCACATC	73	10633	10652	521
544194	1665	1684	CCATCTAAAATTGATTCCCA	73	10637	10656	522
544195	1771	1790	TTGTGATATTAGCTCATATG	57	10743	10762	523
544196	1794	1813	ACTAGTTTTTTAAACTGGGA	21	10766	10785	524
544197	1820	1839	GTCAAGTTTAGAGTTTTAAC	53	10792	10811	525
544198	1826	1845	TATTTAGTCAAGTTTAGAGT	11	10798	10817	526
544199	1907	1926	TACACATACTCTGTGCTGAC	84	10879	10898	20
544200	1913	1932	GATTTTTACACATACTCTGT	53	10885	10904	527
544201	2008	2027	CTGCTTCATTAGGTTTCATA	67	10980	10999	528

Table 129

Inhibition of ANGPTL3 mRNA by 5-10-5 MOE gapmers targeting SEQ ID NO: 1 and 2

ISIS NO	SEQ ID NO: 1 Start Site	SEQ ID NO: 1 Stop Site	Sequence	% inhibition	SEQ ID NO: 2 Start Site	SEQ ID NO: 2 Stop Site	SEQ ID NO
544127	765	784	CAGCAGGAATGCCATCATGT	18	N/A	N/A	529
544128	819	838	TGATGGCATACATGCCACTT	0	7404	7423	530
544129	828	847	TGCTGGGTCTGATGGCATAC	48	7413	7432	531
544130	832	851	GAGTTGCTGGGTCTGATGGC	14	7417	7436	532
544131	841	860	AAAACTTGAGAGTTGCTGGG	5	7426	7445	533
544132	848	867	GACATGAAAAACTTGAGAGT	0	7433	7452	534
544133	859	878	ACATCACAGTAGACATGAAA	28	7444	7463	535
233717	889	908	TGAATTAATGTCCATGGACT	51	7876	7895	14
544134	915	934	AGTTTTGTGATCCATCTATT	36	7902	7921	536
544135	918	937	TGAAGTTTTGTGATCCATCT	61	7905	7924	537
544136	926	945	CGTTTCATTGAAGTTTTGTG	54	7913	7932	538
544137	946	965	CCATATTTGTAGTTCTCCCA	67	7933	7952	539
544138	949	968	AAACCATATTTGTAGTTCTC	39	7936	7955	540
544139	970	989	AATTCTCCATCAAGCCTCCC	77	N/A	N/A	541
233722	991	1010	ATCTTCTCTAGGCCCAACCA	95	9566	9585	542
544432	997	1016	GAGTATATCTTCTCTAGGCC	86	9572	9591	543
544140	1002	1021	CTATGGAGTATATCTTCTCT	57	9577	9596	544
544141	1008	1027	GCTTCACTATGGAGTATATC	52	9583	9602	545

544142	1013	1032	AGATTGCTTCACTATGGAGT	40	9588	9607	546
544143	1046	1065	CCAGTCTTCCAACTCAATTC	32	9621	9640	547
544144	1052	1071	GTCTTTCCAGTCTTCCAACT	53	9627	9646	548
544145	1055	1074	GTTGTCTTTCCAGTCTTCCA	80	9630	9649	16
544146	1059	1078	GTTTGTTGTCTTTCCAGTCT	59	9634	9653	549
544147	1062	1081	AATGTTTGTTGTCTTTCCAG	42	9637	9656	550
544148	1095	1114	CGTGATTTCCCAAGTAAAAA	76	9670	9689	551
544149	1160	1179	GTTTTCCGGGATTGCATTGG	29	9735	9754	552
544150	1165	1184	TCTTTGTTTTCCGGGATTGC	50	9740	9759	553
544151	1170	1189	CCAAATCTTTGTTTTCCGGG	56	9745	9764	554
544152	1173	1192	ACACCAAATCTTTGTTTTCC	26	9748	9767	555
544153	1178	1197	AGAAAACACCAAATCTTTGT	22	9753	9772	556
544154	1183	1202	CAAGTAGAAAACACCAAATC	29	9758	9777	557
544155	1188	1207	GATCCCAAGTAGAAAACACC	16	9763	9782	558
544156	1195	1214	GCTTTGTGATCCCAAGTAGA	71	9770	9789	17
544157	1198	1217	TTTGCTTTGTGATCCCAAGT	55	9773	9792	559
544158	1202	1221	TCCTTTTGCTTTGTGATCCC	51	9777	9796	560
544159	1208	1227	GAAGTGTCCTTTTGCTTTGT	8	9783	9802	561
544160	1246	1265	TGCCACCACCAGCCTCCTGA	68	N/A	N/A	562
544161	1253	1272	CTCATCATGCCACCACCAGC	48	10225	10244	563
544162	1269	1288	GGTTGTTTTCTCCACACTCA	74	10241	10260	18
544163	1276	1295	CCATTTAGGTTGTTTTCTCC	33	10248	10267	564
544164	1283	1302	ATATTTACCATTTAGGTTGT	0	10255	10274	565
544165	1294	1313	CTTGGTTTGTTATATTTACC	52	10266	10285	566
544166	1353	1372	ACCTTCCATTTTGAGACTTC	69	10325	10344	19
544167	1363	1382	ATAGAGTATAACCTTCCATT	72	10335	10354	567
544168	1367	1386	TTTTATAGAGTATAACCTTC	27	10339	10358	568
544169	1374	1393	TGGTTGATTTTATAGAGTAT	39	10346	10365	569
544170	1378	1397	ATTTTGGTTGATTTTATAGA	7	10350	10369	570
544171	1383	1402	TCAACATTTTGGTTGATTTT	0	10355	10374	571
544172	1390	1409	GGATGGATCAACATTTTGGT	48	10362	10381	572
544173	1393	1412	GTTGGATGGATCAACATTTT	51	10365	10384	573
544174	1396	1415	TCTGTTGGATGGATCAACAT	46	10368	10387	574
544175	1401	1420	CTGAATCTGTTGGATGGATC	58	10373	10392	575
544176	1407	1426	AGCTTTCTGAATCTGTTGGA	57	10379	10398	576
544177	1414	1433	CATTCAAAGCTTTCTGAATC	0	10386	10405	577
544178	1417	1436	GTTCATTCAAAGCTTTCTGA	62	10389	10408	578
544179	1420	1439	TCAGTTCATTCAAAGCTTTC	21	10392	10411	579
544180	1423	1442	GCCTCAGTTCATTCAAAGCT	73	10395	10414	580
544181	1427	1446	ATTTGCCTCAGTTCATTCAA	46	10399	10418	581
544182	1431	1450	TTAAATTTGCCTCAGTTCAT	52	10403	10422	582

544183	1436	1455	GCCTTTTAAATTTGCCTCAG	66	10408	10427	583
544184	1498	1517	AGGATTTAATACCAGATTAT	31	10470	10489	584
544185	1502	1521	CTTAAGGATTTAATACCAGA	49	10474	10493	585
544186	1505	1524	TCTCTTAAGGATTTAATACC	49	10477	10496	586
544187	1546	1565	GACAGTGACTTTAAGATAAA	27	10518	10537	587
544188	1572	1591	TGTGATTGTATGTTTAATCT	30	10544	10563	588
544189	1578	1597	AGGTTATGTGATTGTATGTT	35	10550	10569	589
544190	1583	1602	CTTTAAGGTTATGTGATTGT	50	10555	10574	590
544191	1589	1608	GGTATTCTTTAAGGTTATGT	54	10561	10580	591
544192	1656	1675	ATTGATTCCCACATCACAAA	47	10628	10647	592
544193	1661	1680	CTAAAATTGATTCCCACATC	69	10633	10652	593
544194	1665	1684	CCATCTAAAATTGATTCCCA	74	10637	10656	594
544195	1771	1790	TTGTGATATTAGCTCATATG	54	10743	10762	595
544196	1794	1813	ACTAGTTTTTTAAACTGGGA	27	10766	10785	596
544197	1820	1839	GTCAAGTTTAGAGTTTTAAC	18	10792	10811	597
544198	1826	1845	TATTTAGTCAAGTTTAGAGT	12	10798	10817	598
544199	1907	1926	TACACATACTCTGTGCTGAC	83	10879	10898	20
544200	1913	1932	GATTTTTACACATACTCTGT	58	10885	10904	599
544201	2008	2027	CTGCTTCATTAGGTTTCATA	62	10980	10999	600

Table 130

Inhibition of ANGPTL3 mRNA by 5-10-5 MOE gapmers targeting SEQ ID NO: 1 and 2

ISIS NO	SEQ ID NO: 1 Start Site	SEQ ID NO: 1 Stop Site	Sequence	% inhibition	SEQ ID NO: 2 Start Site	SEQ ID NO: 2 Stop Site	SEQ ID NO
337520	N/A	N/A	CAGTGTTATTCAGATTGTAC	64	6517	6536	601
337521	N/A	N/A	AGTGTCTTACCATCATGTTT	40	6776	6795	602
337525	N/A	N/A	CACCAGCCTCCTAAAGGAGA	39	10212	10231	603
544292	N/A	N/A	GAGGAGGTGAAGTCAGTGAG	35	4815	4834	604
544293	N/A	N/A	TAGAGTAGAGGAGGTGAAGT	23	4822	4841	605
544294	N/A	N/A	TGTTTGATGTGTTTGAATAC	19	4863	4882	606
544295	N/A	N/A	GAAACAACAAGGGCAAAGGC	23	4898	4917	607
544296	N/A	N/A	TGTTTGATAACGACCCTAAG	43	4974	4993	608
544297	N/A	N/A	TTTTTGGTTAAGTGACCTTG	48	5016	5035	609
544298	N/A	N/A	GTAGAAGTTTTCAGGGATGG	23	5052	5071	610
544299	N/A	N/A	AGGAAGTAGAAGTTTTCAGG	5	5057	5076	611
544300	N/A	N/A	AGGTGAGTGTGCAGGAGAAA	11	5085	5104	612
544301	N/A	N/A	TTAAATAAAGGTGAGTGTGC	14	5093	5112	613
544302	N/A	N/A	AGTGCAGGAATAGAAGAGAT	35	5136	5155	614
544303	N/A	N/A	CATTTTAGTGCAGGAATAGA	21	5142	5161	615
544306	N/A	N/A	CTATATTCTGGAGTATATAC	39	5216	5235	616
544307	N/A	N/A	CAGTATTCTATATTCTGGAG	72	5223	5242	617

544308	N/A	N/A	GTGCCATACAGTATTCTATA	50	5231	5250	618
544309	N/A	N/A	CTGTGTGAATATGACATTAC	52	5281	5300	619
544310	N/A	N/A	TGAGGCACACTATTTCTAGT	47	5333	5352	620
544311	N/A	N/A	GACCTTTAATTATGAGGCAC	67	5345	5364	621
544312	N/A	N/A	GAATGTTGACCTTTAATTAT	23	5352	5371	622
544313	N/A	N/A	TTGTTGAATGTTGACCTTTA	69	5357	5376	623
544314	N/A	N/A	TCTACTAAGTAACTATGTGA	37	5915	5934	624
544315	N/A	N/A	CTCTTTTCTACTAAGTAACT	31	5921	5940	625
544316	N/A	N/A	AAGGATCTATTGTAAAGTTT	24	5956	5975	626
544317	N/A	N/A	CTAGGACCTTATTTAAAAGG	24	5972	5991	627
544318	N/A	N/A	ATTTCCTAGGACCTTATTTA	8	5977	5996	628
544319	N/A	N/A	TTGACAGTAAGAAAAGCAGA	28	6051	6070	629
544320	N/A	N/A	TTCTCATTGACAGTAAGAAA	56	6057	6076	630
544321	N/A	N/A	AGTTTTTCTCATTGACAGTA	50	6062	6081	631
544322	N/A	N/A	ATTGAATGATAGTTTTTCTC	42	6072	6091	632
544323	N/A	N/A	TTGGGTTTGCAATTTATTGA	36	6087	6106	633
544324	N/A	N/A	AGTGTGTTGGGTTTGCAATT	25	6093	6112	634
544325	N/A	N/A	TATTTAAGTGTGTTGGGTTT	27	6099	6118	635
544326	N/A	N/A	ATATATTCAGTAGTTTATCG	25	6145	6164	636
544327	N/A	N/A	AGATGTTGGCAGGTTGGCAA	51	6184	6203	637
544328	N/A	N/A	TCTGTAGATGTTGGCAGGTT	48	6189	6208	638
544329	N/A	N/A	TTGATAATTTTTGACCTGTA	34	6215	6234	639
544330	N/A	N/A	GGCTTTCTTGATAATTTGAT	52	6230	6249	640
544331	N/A	N/A	GTCTTACTGATCTTCAGACC	27	6282	6301	641
544332	N/A	N/A	TTTAGGTCTTACTGATCTTC	14	6287	6306	642
544333	N/A	N/A	TCAGTTTTAGGTCTTACTGA	28	6292	6311	643
544334	N/A	N/A	TGATATTCTGTTCAGATTTT	44	6326	6345	644
544335	N/A	N/A	TAGAGACTGCTTTGCTTAGA	31	6388	6407	645
544336	N/A	N/A	AGGCCAAAAGTAGAGACTGC	29	6398	6417	646
544337	N/A	N/A	GGCAAAAAAGCAGACATTGG	38	6433	6452	647
544338	N/A	N/A	AATCAGGGACATTATTTAAT	13	6473	6492	648
544339	N/A	N/A	TATTTAATCAGGGACATTAT	28	6478	6497	649
544340	N/A	N/A	CTCAAAATATTTAATCAGGG	27	6485	6504	650
544341	N/A	N/A	TACCTGTTCTCAAAATATTT	18	6493	6512	651
544342	N/A	N/A	GTACAGATTACCTGTTCTCA	68	6501	6520	652
544343	N/A	N/A	GGTGTTTGATATTTAGATAA	25	6538	6557	653
544344	N/A	N/A	TTGTCTTTCAGTTCATAATG	29	6565	6584	654
544345	N/A	N/A	ACAGTTTGTCTTTCAGTTCA	23	6570	6589	655
544346	N/A	N/A	TCTGAGCTGATAAAAGAATA	15	6657	6676	656
544347	N/A	N/A	CCCACCAAAGTGTCTTACCA	49	6784	6803	657
544348	N/A	N/A	CTTCAAGAAGGAAACCCACC	39	6798	6817	658

544349	N/A	N/A	AATAGCTTCAAGAAGGAAAC	12	6803	6822	659
544350	N/A	N/A	ACAAGTCCTAAGAATAGGGA	25	6833	6852	660
544351	N/A	N/A	GTCTAGAACAAGTCCTAAGA	53	6840	6859	661
544352	N/A	N/A	TCTAATAATCAAGTCCATAT	33	6972	6991	662
544353	N/A	N/A	ACCTTCTATATTATCTAATA	19	6985	7004	663
544354	N/A	N/A	GCATGTATCTCTTAAACAGG	50	7060	7079	664
544355	N/A	N/A	TTTCAGCATGTATCTCTTAA	79	7065	7084	21
544356	N/A	N/A	GTCCAGTGACCTTTAACTCC	69	7092	7111	665
544357	N/A	N/A	TCTTACCAAACTATTTTCTT	28	7166	7185	666
544358	N/A	N/A	GTAATGTTTATGTTAAAGCA	17	7226	7245	667
544359	N/A	N/A	TTGTGGCAAATGTAGCATTT	52	7251	7270	668
544360	N/A	N/A	GAGATTTCACTTGACATTTT	30	7277	7296	669
544361	N/A	N/A	GGAGCTTGAGATTTCACTTG	30	7284	7303	670
544362	N/A	N/A	CATCAGATTTAGTAATAGGA	0	7315	7334	671
544363	N/A	N/A	GTTATTACATCAGATTTAGT	6	7322	7341	672
544365	N/A	N/A	CAGCAGGAATGCCTAGAATC	32	7350	7369	673
544366	N/A	N/A	CTCCTTAGACAGGTTTTACC	31	7471	7490	674
544367	N/A	N/A	GTCTATTCTCCTTAGACAGG	23	7478	7497	675
544368	N/A	N/A	ACCAGGTTAATCTTCCTAAT	71	7526	7545	22
544369	N/A	N/A	ATGAATGATTGAATGTAGTC	26	7977	7996	676
544370	N/A	N/A	ATATGAAGGCTGAGACTGCT	58	8072	8091	677
544371	N/A	N/A	ATAAATTATATGAAGGCTGA	7	8079	8098	678
544372	N/A	N/A	ATATTTAAGAACAGACATGT	12	8175	8194	679
544373	N/A	N/A	AGTTATGATCATTGTAAGCC	60	8217	8236	23
544374	N/A	N/A	ATTTGTAACAGTTACTACTT	51	8276	8295	680
544375	N/A	N/A	CACAGCTTATTTGTAACAGT	70	8284	8303	681
544376	N/A	N/A	GGAGTGGTTCTTTTCACAGC	71	8298	8317	24
544377	N/A	N/A	GTGACTAATGCTAGGAGTGG	34	8311	8330	682
544378	N/A	N/A	GAATAGAGTGACTAATGCTA	45	8318	8337	683
544379	N/A	N/A	ATGAGAGAATAGAGTGACTA	58	8324	8343	684
544380	N/A	N/A	TGGTCCTTTTAACTTCCAAT	70	8365	8384	25
544381	N/A	N/A	TATACTGTATGTCTGAGTTT	66	8387	8406	685
544382	N/A	N/A	AACTAATTCATTATAAGCCA	67	8450	8469	686
544383	N/A	N/A	GCATTGAGTTAACTAATTCA	64	8460	8479	26
544385	N/A	N/A	TTTGGATTTTAAACATCTGT	61	8528	8547	687
544386	N/A	N/A	TGTATGTGCTTTTTGGATTT	37	8539	8558	688
544387	N/A	N/A	CATGGATTTTTGTATGTGCT	62	8549	8568	689
544388	N/A	N/A	TCATTCATGGATTTTTGTAT	34	8554	8573	690
544389	N/A	N/A	ACTTAGACATCATTCATGGA	55	8563	8582	691
544390	N/A	N/A	GTGAGTACTTAGACATCATT	66	8569	8588	692
544391	N/A	N/A	TTTATAAGTGAGTACTTAGA	36	8576	8595	693

544392	N/A	N/A	GTCTTCTACTTTATAAGTGA	65	8585	8604	694
544393	N/A	N/A	ATGAATGTCTTCTACTTTAT	34	8591	8610	695
544394	N/A	N/A	CAAATAGTACTGAGCATTTA	30	8627	8646	696
544395	N/A	N/A	TTAGAAGATTTGGAGCTACA	54	8718	8737	697
544396	N/A	N/A	TCACTATTAGAAGATTTGGA	37	8724	8743	698
544397	N/A	N/A	GGGTTACACTCACTATTAGA	36	8733	8752	699
544398	N/A	N/A	ACTTACCTGTCAGCCTTTTA	54	8758	8777	700
544399	N/A	N/A	CTTACCAGAATTAAGTGAGT	26	8785	8804	701
544400	N/A	N/A	AATACAAGTACAAATGGGTT	22	8810	8829	702
544401	N/A	N/A	CTGGTAAATACAAGTACAAA	55	8816	8835	703
544402	N/A	N/A	GGATTGCTGGTAAATACAAG	40	8822	8841	704
544403	N/A	N/A	TCATTTTAAGGATTGCTGGT	62	8831	8850	705
544404	N/A	N/A	AGTTAGTAGGAAGCTTCATT	56	8846	8865	706
544405	N/A	N/A	GCTATTGAGTTAGTAGGAAG	67	8853	8872	707
544407	N/A	N/A	AGCATGGTTCTTAATAACTT	67	9012	9031	708
544408	N/A	N/A	CTTTGTAGAAAAAGACAGGA	27	9062	9081	709
544409	N/A	N/A	ACCTGGCCTTTGGTATTTGC	49	9096	9115	710
544410	N/A	N/A	CATCCATATACAGTCAAGAG	80	9174	9193	27
544411	N/A	N/A	AGTCTTTATATGGATAAACT	15	9215	9234	711
544412	N/A	N/A	CGTCATTGGTAGAGGAATAT	51	9240	9259	712
544413	N/A	N/A	GATTATCCTTTCTATAATGC	48	9321	9340	713
544414	N/A	N/A	GTCTTGAATCCCTTGATCAT	40	9436	9455	714
544415	N/A	N/A	GGTGCAACTAATTGAGTTGT	27	9459	9478	715
544416	N/A	N/A	GTGTTTTTTATTGGTGCAAC	31	9471	9490	716
544417	N/A	N/A	ATTCTCCTGAAAAGAAAAGT	24	9544	9563	717
544418	N/A	N/A	ATGCCACCACCAGCCTCCTA	73	10219	10238	718
544419	N/A	N/A	ATATCCTTTAACAAATGGGT	62	11540	11559	719
544420	N/A	N/A	GCACTATATCCTTTAACAAA	50	11545	11564	720
544421	N/A	N/A	ACTTGGGCACTATATCCTTT	68	11551	11570	721
544422	N/A	N/A	GAAACATGTCCTATGAGAGT	32	11918	11937	722
544424	N/A	N/A	TTGAGCACTTTAAGCAAAGT	7	12070	12089	723
544425	N/A	N/A	GGAATTTGAGCACTTTAAGC	34	12075	12094	724
544426	N/A	N/A	TAGATTAGACAACTGTGAGT	52	12101	12120	725
544427	N/A	N/A	AAAATGAAGGTCAAGTTTGA	17	12197	12216	726
544428	N/A	N/A	GTGAAAGCAAAATGAAGGTC	33	12205	12224	727
544429	N/A	N/A	GTATTGTGAAAGCAAAATGA	39	12210	12229	728
544430	N/A	N/A	TGGAGAGTATAGTATTGTGA	35	12221	12240	729
544438	N/A	N/A	AGGAATAGAAGAGATAAATA	10	5131	5150	730
544439	N/A	N/A	TGGAGTATATACAAATAATG	30	5208	5227	731
544440	N/A	N/A	TGTTTACATTGTAGATTAAT	15	5381	5400	732
544441	N/A	N/A	CAGAATATATAATATCTTGC	57	6035	6054	733

544442	N/A	N/A	TGCAATTTATTGAATGATAG	31	6080	6099	734
544443	N/A	N/A	CATAATACATAATTTGAACC	0	6251	6270	735
544444	N/A	N/A	ATAATTTTCAGTTTTAGGTC	0	6299	6318	736
544445	N/A	N/A	TTTCAGTAATGTTTATGTTA	9	7231	7250	737
544446	N/A	N/A	AATGCCTAGAATCAATAAAA	36	7343	7362	738
544447	N/A	N/A	GTAAATATTTGTAGATTAGC	49	8003	8022	739
544448	N/A	N/A	ACAAATGTGTAATTGTTTGA	25	8101	8120	740
544449	N/A	N/A	TACTAACAAATGTGTAATTG	35	8106	8125	741
544450	N/A	N/A	TGATAAGTATATTTAAGAAC	35	8183	8202	742
544451	N/A	N/A	TTAACTTCCAATTAATTGAT	29	8357	8376	743
544452	N/A	N/A	TCTGTTATTTTATCTTGCTT	67	8513	8532	744
544453	N/A	N/A	ATCACAATCCTTTTTATTAA	18	8921	8940	745
544454	N/A	N/A	AGAGACTTGAGTAATAATAA	25	9137	9156	746
544455	N/A	N/A	AACAAAATGAAACATGTCCT	59	11926	11945	747
544127	765	784	CAGCAGGAATGCCATCATGT	33	N/A	N/A	748
544128	819	838	TGATGGCATACATGCCACTT	13	7404	7423	749
544129	828	847	TGCTGGGTCTGATGGCATAC	53	7413	7432	750
544130	832	851	GAGTTGCTGGGTCTGATGGC	22	7417	7436	751
544131	841	860	AAAACTTGAGAGTTGCTGGG	13	7426	7445	752
544132	848	867	GACATGAAAAACTTGAGAGT	0	7433	7452	753
544133	859	878	ACATCACAGTAGACATGAAA	27	7444	7463	754
233717	889	908	TGAATTAATGTCCATGGACT	58	7876	7895	14
544134	915	934	AGTTTTGTGATCCATCTATT	46	7902	7921	755
544135	918	937	TGAAGTTTTGTGATCCATCT	54	7905	7924	756
544136	926	945	CGTTTCATTGAAGTTTTGTG	40	7913	7932	757
544137	946	965	CCATATTTGTAGTTCTCCCA	45	7933	7952	758
544138	949	968	AAACCATATTTGTAGTTCTC	41	7936	7955	759
544139	970	989	AATTCTCCATCAAGCCTCCC	43	N/A	N/A	760
233722	991	1010	ATCTTCTCTAGGCCCAACCA	65	9566	9585	761
544432	997	1016	GAGTATATCTTCTCTAGGCC	40	9572	9591	762
544140	1002	1021	CTATGGAGTATATCTTCTCT	28	9577	9596	763
544141	1008	1027	GCTTCACTATGGAGTATATC	55	9583	9602	764
544142	1013	1032	AGATTGCTTCACTATGGAGT	47	9588	9607	765
544143	1046	1065	CCAGTCTTCCAACTCAATTC	33	9621	9640	766
544144	1052	1071	GTCTTTCCAGTCTTCCAACT	59	9627	9646	767
544145	1055	1074	GTTGTCTTTCCAGTCTTCCA	77	9630	9649	16
544146	1059	1078	GTTTGTTGTCTTTCCAGTCT	58	9634	9653	768
544147	1062	1081	AATGTTTGTTGTCTTTCCAG	43	9637	9656	769
544148	1095	1114	CGTGATTTCCCAAGTAAAAA	57	9670	9689	770
544149	1160	1179	GTTTTCCGGGATTGCATTGG	44	9735	9754	771
544150	1165	1184	TCTTTGTTTTCCGGGATTGC	53	9740	9759	772

544151	1170	1189	CCAAATCTTTGTTTTCCGGG	57	9745	9764	773
544152	1173	1192	ACACCAAATCTTTGTTTTCC	44	9748	9767	774
544153	1178	1197	AGAAAACACCAAATCTTTGT	36	9753	9772	775
544154	1183	1202	CAAGTAGAAAACACCAAATC	29	9758	9777	776
544155	1188	1207	GATCCCAAGTAGAAAACACC	29	9763	9782	777
544156	1195	1214	GCTTTGTGATCCCAAGTAGA	71	9770	9789	17
544157	1198	1217	TTTGCTTTGTGATCCCAAGT	66	9773	9792	778
544158	1202	1221	TCCTTTTGCTTTGTGATCCC	53	9777	9796	779
544159	1208	1227	GAAGTGTCCTTTTGCTTTGT	10	9783	9802	780
544160	1246	1265	TGCCACCACCAGCCTCCTGA	65	N/A	N/A	781
544161	1253	1272	CTCATCATGCCACCACCAGC	59	10225	10244	782
544162	1269	1288	GGTTGTTTTCTCCACACTCA	74	10241	10260	18
544163	1276	1295	CCATTTAGGTTGTTTTCTCC	38	10248	10267	783
544164	1283	1302	ATATTTACCATTTAGGTTGT	13	10255	10274	784
544165	1294	1313	CTTGGTTTGTTATATTTACC	53	10266	10285	785
544166	1353	1372	ACCTTCCATTTTGAGACTTC	70	10325	10344	19
544167	1363	1382	ATAGAGTATAACCTTCCATT	69	10335	10354	786
544168	1367	1386	TTTTATAGAGTATAACCTTC	34	10339	10358	787
544169	1374	1393	TGGTTGATTTTATAGAGTAT	38	10346	10365	788
544170	1378	1397	ATTTTGGTTGATTTTATAGA	0	10350	10369	789
544171	1383	1402	TCAACATTTTGGTTGATTTT	12	10355	10374	790
544172	1390	1409	GGATGGATCAACATTTTGGT	58	10362	10381	791
544173	1393	1412	GTTGGATGGATCAACATTTT	66	10365	10384	792
544174	1396	1415	TCTGTTGGATGGATCAACAT	49	10368	10387	793
544175	1401	1420	CTGAATCTGTTGGATGGATC	60	10373	10392	794
544176	1407	1426	AGCTTTCTGAATCTGTTGGA	64	10379	10398	795
544177	1414	1433	CATTCAAAGCTTTCTGAATC	21	10386	10405	796
544178	1417	1436	GTTCATTCAAAGCTTTCTGA	60	10389	10408	797
544179	1420	1439	TCAGTTCATTCAAAGCTTTC	18	10392	10411	798
544180	1423	1442	GCCTCAGTTCATTCAAAGCT	72	10395	10414	799
544181	1427	1446	ATTTGCCTCAGTTCATTCAA	51	10399	10418	800
544182	1431	1450	TTAAATTTGCCTCAGTTCAT	48	10403	10422	801
544183	1436	1455	GCCTTTTAAATTTGCCTCAG	70	10408	10427	802
544184	1498	1517	AGGATTTAATACCAGATTAT	44	10470	10489	803
544185	1502	1521	CTTAAGGATTTAATACCAGA	47	10474	10493	804
544186	1505	1524	TCTCTTAAGGATTTAATACC	44	10477	10496	805
544187	1546	1565	GACAGTGACTTTAAGATAAA	38	10518	10537	806
544188	1572	1591	TGTGATTGTATGTTTAATCT	47	10544	10563	807
544189	1578	1597	AGGTTATGTGATTGTATGTT	43	10550	10569	808
544190	1583	1602	CTTTAAGGTTATGTGATTGT	42	10555	10574	809
544191	1589	1608	GGTATTCTTTAAGGTTATGT	60	10561	10580	810

544192	1656	1675	ATTGATTCCCACATCACAAA	46	10628	10647	811
544193	1661	1680	CTAAAATTGATTCCCACATC	65	10633	10652	812
544194	1665	1684	CCATCTAAAATTGATTCCCA	70	10637	10656	813
544195	1771	1790	TTGTGATATTAGCTCATATG	56	10743	10762	814
544196	1794	1813	ACTAGTTTTTTAAACTGGGA	33	10766	10785	815
544197	1820	1839	GTCAAGTTTAGAGTTTTAAC	39	10792	10811	816
544198	1826	1845	TATTTAGTCAAGTTTAGAGT	21	10798	10817	817
544199	1907	1926	TACACATACTCTGTGCTGAC	80	10879	10898	20
544200	1913	1932	GATTTTTACACATACTCTGT	56	10885	10904	818
544201	2008	2027	CTGCTTCATTAGGTTTCATA	65	10980	10999	819

Table 131

Inhibition of ANGPTL3 mRNA by 5-10-5 MOE gapmers targeting SEQ ID NO: 1 and 2

ISIS NO	SEQ ID NO: 1 Start Site	SEQ ID NO: 1 Stop Site	Sequence	% inhibition	SEQ ID NO: 2 Start Site	SEQ ID NO: 2 Stop Site	SEQ ID NO
337525	N/A	N/A	CACCAGCCTCCTAAAGGAGA	58	10212	10231	820
544204	N/A	N/A	GACTTCTTAACTCTATATAT	67	3076	3095	821
544205	N/A	N/A	CTAGACTTCTTAACTCTATA	61	3079	3098	822
544206	N/A	N/A	GACCTAGACTTCTTAACTCT	54	3082	3101	823
544207	N/A	N/A	GGAAGCAGACCTAGACTTCT	58	3089	3108	824
544208	N/A	N/A	TCTGGAAGCAGACCTAGACT	48	3092	3111	825
544209	N/A	N/A	TCTTCTGGAAGCAGACCTAG	54	3095	3114	826
544210	N/A	N/A	CTAATCTTTAGGGATTTAGG	57	11433	11452	827
544211	N/A	N/A	TGTATCTAATCTTTAGGGAT	53	11438	11457	828
544213	N/A	N/A	TAACTTGGGCACTATATCCT	74	11553	11572	829
544214	N/A	N/A	ATTGACAAAGGTAGGTCACC	79	11576	11595	830
544215	N/A	N/A	ATATGACATGTATATTGGAT	66	11620	11639	831
544216	N/A	N/A	TTTTGTACTTTTCTGGAACA	61	11704	11723	832
544217	N/A	N/A	TAGTCTGTGGTCCTGAAAAT	56	11748	11767	833
544218	N/A	N/A	AGCTTAGTCTGTGGTCCTGA	72	11752	11771	834
544219	N/A	N/A	GACAGCTTAGTCTGTGGTCC	74	11755	11774	835
544220	N/A	N/A	GTATTCTGGCCCTAAAAAAA	52	11789	11808	836
544221	N/A	N/A	ATTTTGGTATTCTGGCCCTA	56	11795	11814	837
544222	N/A	N/A	GAAATTGTCCAATTTTTGGG	56	N/A	N/A	838
544223	N/A	N/A	TTTGCATTTGAAATTGTCCA	61	11837	11856	839
544224	N/A	N/A	GGAAGCAACTCATATATTAA	57	11869	11888	840
544225	N/A	N/A	TATCAGAAAAAGATACCTGA	56	9821	9840	841
544226	N/A	N/A	ATAATAGCTAATAATGTGGG	59	9875	9894	842
544227	N/A	N/A	TGCAGATAATAGCTAATAAT	60	9880	9899	843
544228	N/A	N/A	TGTCATTGCAGATAATAGCT	79	9886	9905	844
544229	N/A	N/A	TAAAAGTTGTCATTGCAGAT	59	9893	9912	845

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 379

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 379

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

CLAIMS

1. A compound comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprises a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to an equal length portion of nucleobases 1140 to 1159 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

2. The compound of claim 1, wherein the modified oligonucleotide comprises a nucleobase sequence comprising a portion of at least 10, at least 12, at least 14, at least 16, at least 18, at least 19, or at least 20 contiguous nucleobases complementary to an equal length portion of SEQ ID NO: 1

3. A compound comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprises a nucleobase sequence comprising a portion of at least 8, least 9, least 10, least 11, at least 12, least 13, at least 14, at least 15, at least 16, least 17, least 18, least 19, or 20 contiguous nucleobases complementary to an equal length portion of nucleobases 1140 to 1159 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

4. The compound of any preceding claim, wherein the nucleobase sequence of the modified oligonucleotide is at least 85%, at least 90%, at least 95%, or 100% complementary to SEQ ID NO: 1.

5. A compound comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and has a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of any of the nucleobase sequences of SEQ ID NOs: 77, 20, 35, 90, 93 or 94.

6. A compound comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and has a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, least 13, at least 14, at least 15, or 16 contiguous nucleobases of any of the nucleobase sequences of SEQ ID NOs: 110 or 114.

7. The compound of any preceding claim, wherein the modified oligonucleotide is single-stranded or double stranded.

8. The compound of any preceding claim, wherein the modified oligonucleotide comprises at least one modified internucleoside linkage.

9. The compound of claim 8, wherein the modified internucleoside linkage is a phosphorothioate internucleoside linkage.

10. The compound of claim 9, wherein the modified oligonucleotide comprises at least one phosphodiester internucleoside linkage.

11. The compound of claim 9, wherein the modified oligonucleotide comprises at least 2 phosphodiester internucleoside linkages.

12. The compound of claim 9, wherein the modified oligonucleotide comprises at least 3 phosphodiester internucleoside linkages.

13. The compound of claim 9, wherein the modified oligonucleotide comprises at least 4 phosphodiester internucleoside linkages.

14. The compound of claim 9, wherein the modified oligonucleotide comprises at least 5 phosphodiester internucleoside linkages.

15. The compound of claim 9, wherein the modified oligonucleotide comprises at least 6 phosphodiester internucleoside linkages.

16. The compound of claim 9, wherein the modified oligonucleotide comprises at least 7 phosphodiester internucleoside linkages.

17. The compound of any of claims 10 to 16, wherein each internucleoside linkage of the modified oligonucleotide is selected from a phosphodiester internucleoside linkage and a phosphorothioate internucleoside linkage.

18. The compound of claim 8, wherein each internucleoside linkage of the modified oligonucleotide comprises is a phosphorothioate internucleoside linkage.

19. The compound of any preceding claim, wherein the modified oligonucleotide comprises at least one modified sugar.

20. The compound of claim 19, wherein at least one modified sugar is a bicyclic sugar.

21. The compound of claim 19, wherein at least one modified sugar comprises a 2'-O-methoxyethyl, a constrained ethyl, a 3'-fluoro-HNA or a 4'- $(CH_2)_n$-O-2' bridge, wherein n is 1 or 2.

22. The compound of any preceding claim, wherein at least one nucleoside comprises a modified nucleobase.

23. The compound of claim 22, wherein the modified nucleobase is a 5-methylcytosine.

24. The compound of any preceding claim, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprises:

a gap segment consisting of linked deoxynucleosides;

a 5' wing segment consisting of linked nucleosides;

a 3' wing segment consisting of linked nucleosides;

wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar.

25. The compound of any preceding claim, wherein the modified oligonucleotide consists of 15 to 30, 18 to 24, 19 to 22, 13 to 25, 14 to 25, 15 to 25, 16 or 20 linked nucleosides.

27. A compound comprising a modified oligonucleotide and a conjugate group, wherein the modified oligonucleotide consists of 20 linked nucleosides and has a nucleobase sequence comprising at least 8 contiguous nucleobases complementary to an equal length portion of any of SEQ ID NO: 77, wherein the modified oligonucleotide comprises:

a gap segment consisting of ten linked deoxynucleosides;

a 5' wing segment consisting of five linked nucleosides;

a 3' wing segment consisting of five linked nucleosides;

wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar, wherein each internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine.

28. A compound consisting of ISIS 563580 and a conjugate group, ISIS 544199 and a conjugate group, ISIS 560400 and a conjugate group, ISIS 567233 and a conjugate group, ISIS 567320 and a conjugate group, ISIS 567321 and a conjugate group, ISIS 559277 and a conjugate group, ISIS 561011 and a conjugate group.

29. The compound of any of claims 1 to 28, wherein the conjugate group is linked to the modified oligonucleotide at the 5' end of the modified oligonucleotide.

30. The compound of any of claims 1 to 28, wherein the conjugate group is linked to the modified oligonucleotide at the 3' end of the modified oligonucleotide.

31. The compound of any of claims 1-30, wherein the conjugate group comprises exactly one ligand.

32. The compound of any of claims 1-30, wherein the conjugate group comprises exactly two ligands.

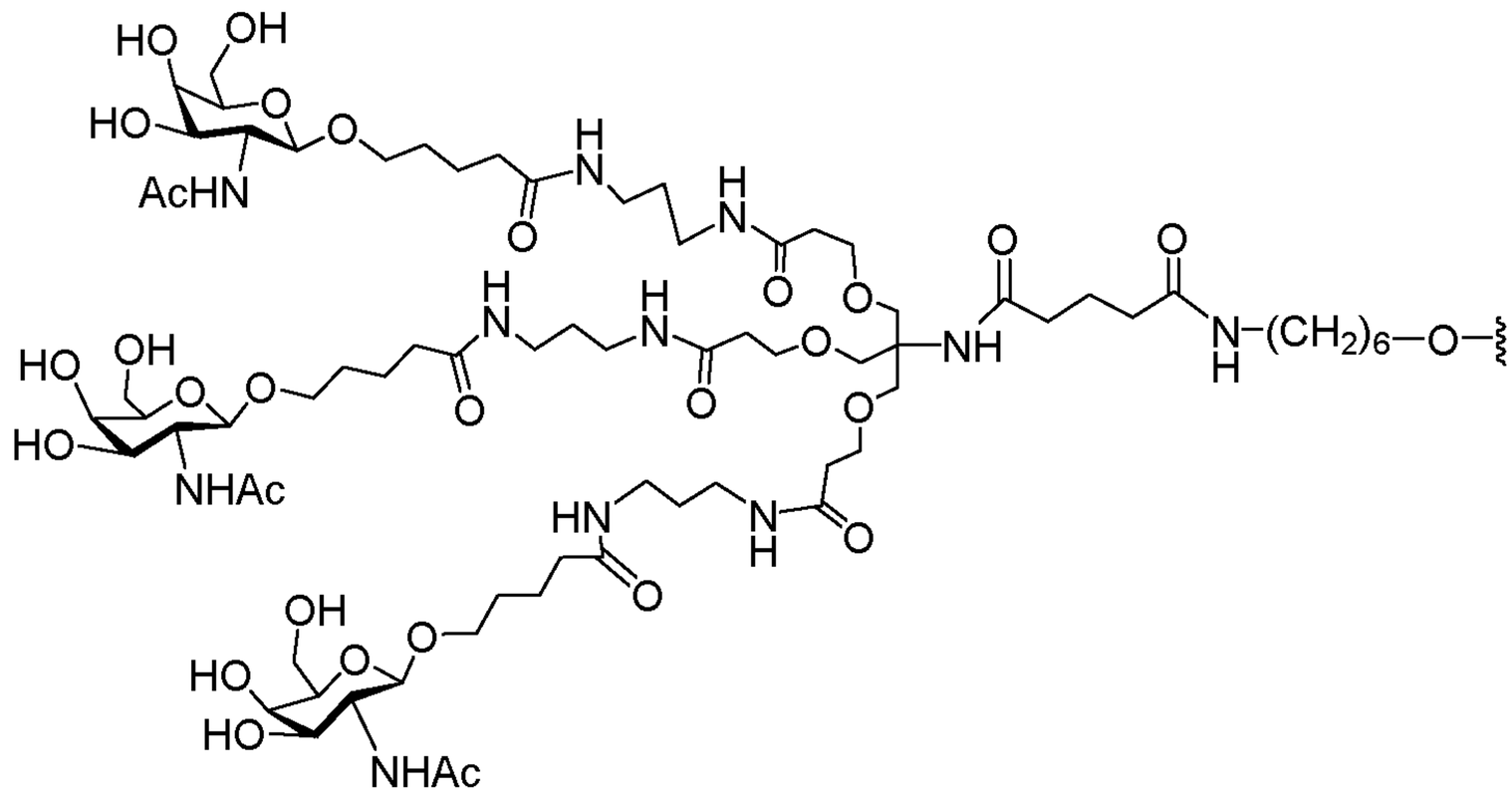
33. The compound of any of claims 1-30, wherein the conjugate group comprises three or more ligands.

34. The compound of any of claims 1-30, wherein the conjugate group comprises exactly three ligands.

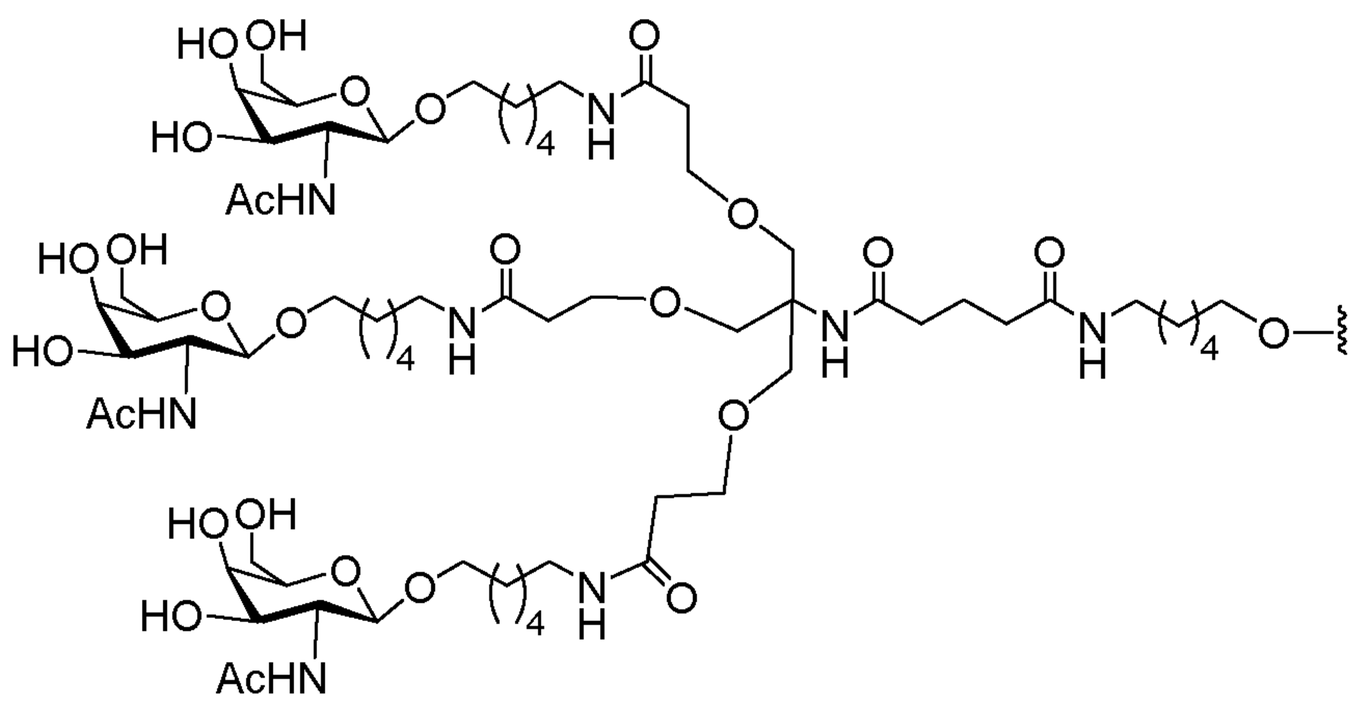
35. The compound of any of claims 31-34, wherein each ligand is selected from among: a polysaccharide, modified polysaccharide, mannose, galactose, a mannose derivative, a galactose derivative, D-mannopyranose, L-Mannopyranose, D-Arabinose, L-Galactose, D-xylofuranose, L-xylofuranose, D-glucose, L-glucose, D-Galactose, L-Galactose, α-D-Mannofuranose, β-D-Mannofuranose, α-D-Mannopyranose, β-D-Mannopyranose, α-D-Glucopyranose, β-D-Glucopyranose, α-D-Glucofuranose, β-D-Glucofuranose, α-D-fructofuranose, α-D-fructopyranose, α-D-Galactopyranose, β -D-Galactopyranose, α-D-Galactofuranose, β -D-Galactofuranose, glucosamine, sialic acid, α-D-galactosamine, N-Acetylgalactosamine, 2-Amino-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-β-D-glucopyranose, 2-Deoxy-2-methylamino-L-glucopyranose, 4,6-Dideoxy-4-formamido-2,3-di-*O*-methyl-D-mannopyranose, 2-Deoxy-2-sulfoamino-D-glucopyranose, *N*-Glycoloyl-α-neuraminic acid, 5-thio-β-D-glucopyranose, methyl 2,3,4-tri-*O*-acetyl-1-thio-6-*O*-trityl-α-D-glucopyranoside, 4-Thio-β-D-galactopyranose, ethyl 3,4,6,7-tetra-*O*-acetyl-2-deoxy-1,5-dithio-α-D-*gluco*-heptopyranoside, 2,5-Anhydro-D-allononitrile, ribose, D-ribose, D-4-thioribose, L-ribose, L-4-thioribose.

36. The compound of claim 35, wherein each ligand is N-acetyl galactosamine.

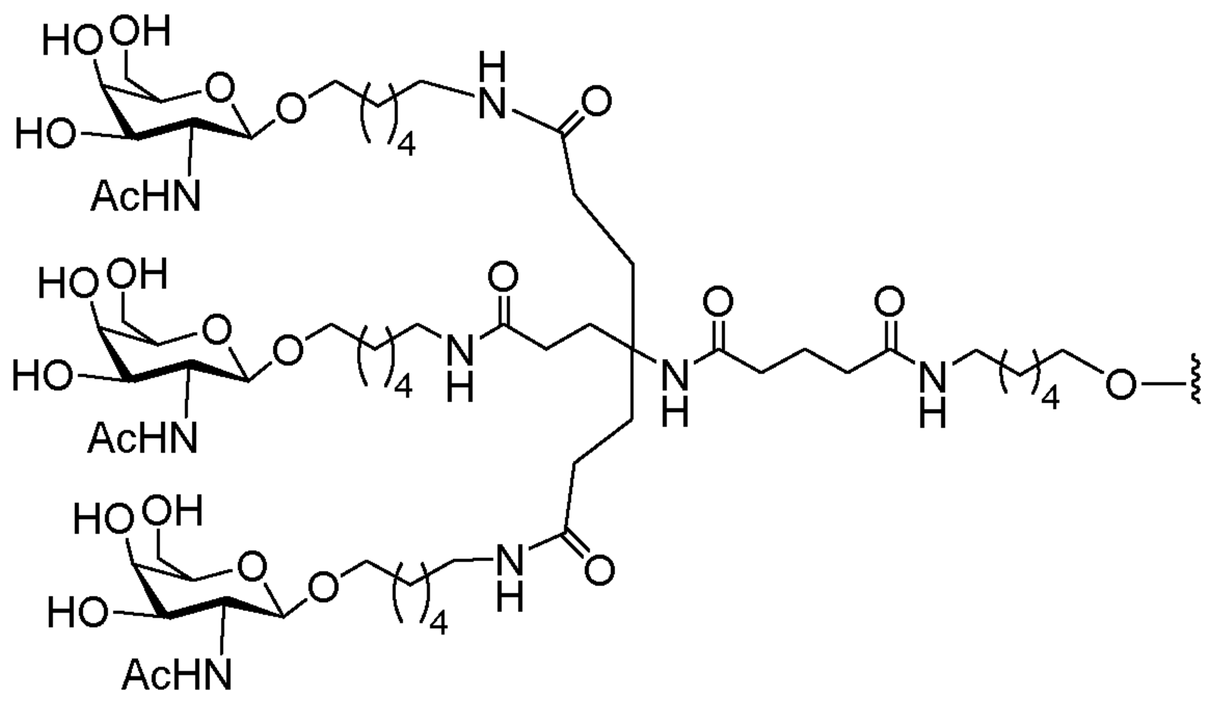
37. The compound of any of claims 1 to 30, wherein the conjugate group comprises:



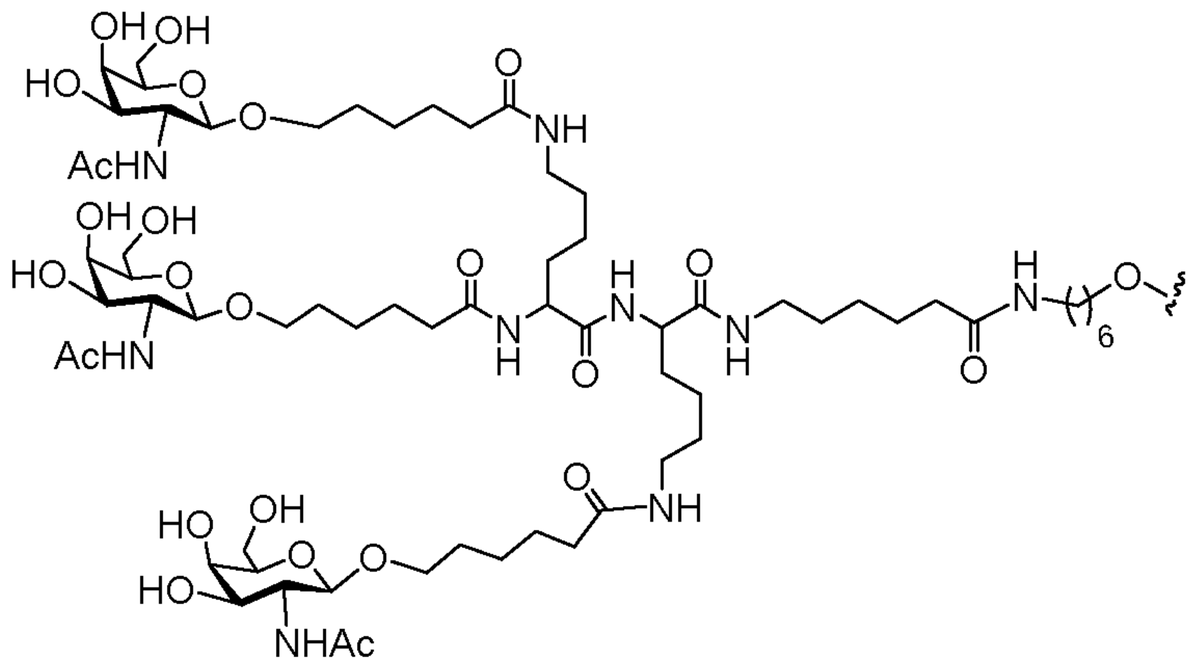
38. The compound of any of claims 1 to 30, wherein the conjugate group comprises:



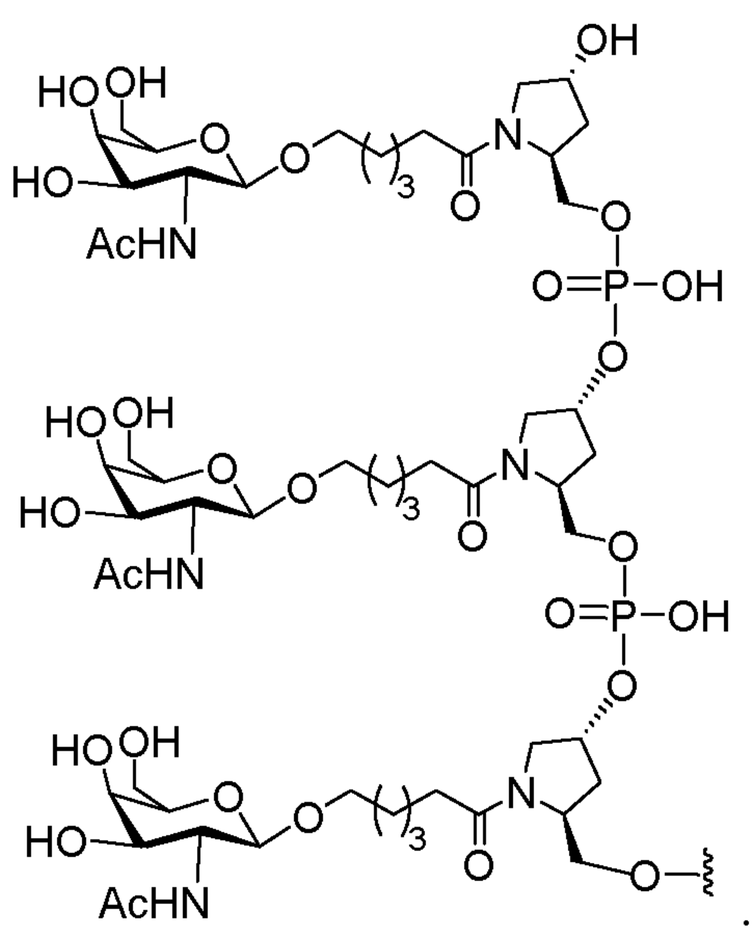
39. The compound of any of claims 1 to 30, wherein the conjugate group comprises:



40. The compound of any of claims 1 to 30, wherein the conjugate group comprises:

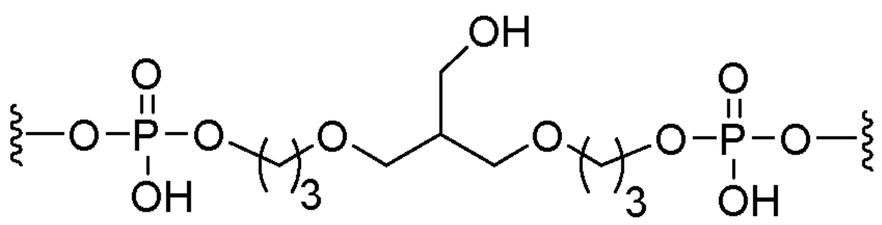
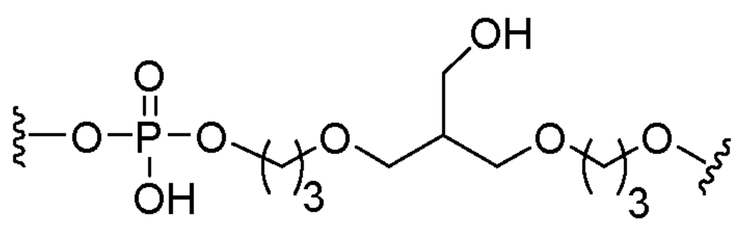
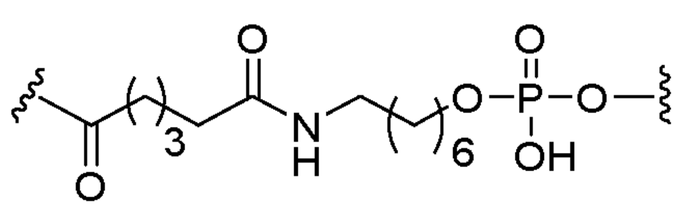
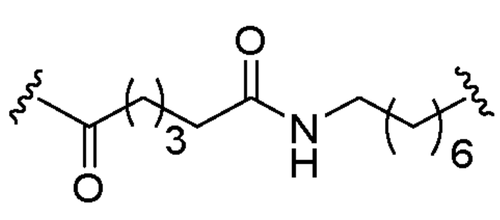
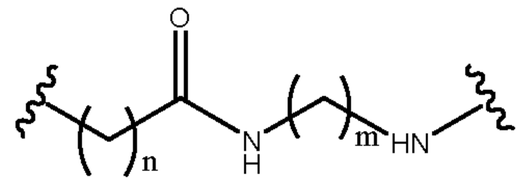
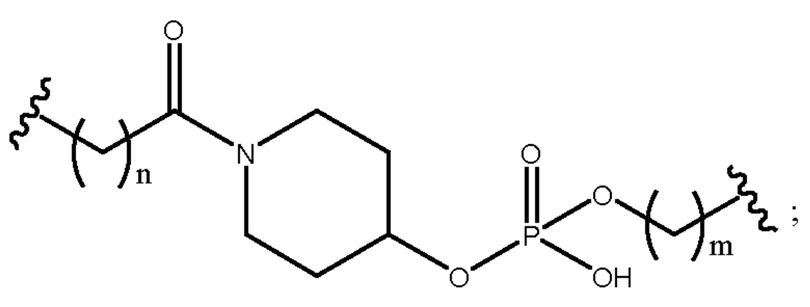
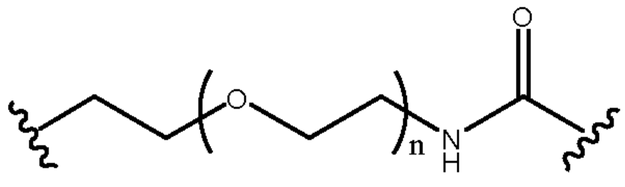


41. The compound of any of claims 1 to 30, wherein the conjugate group comprises:



42. The compound of any of claims 30 to 36, wherein the conjugate group comprises at least one phosphorus linking group or neutral linking group.

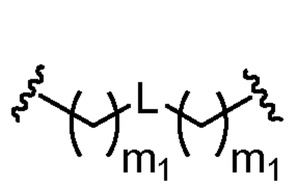
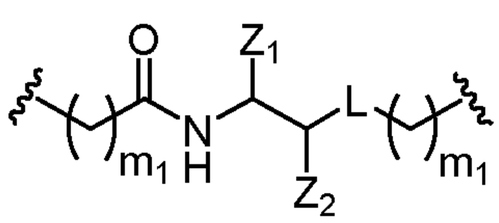
43. The compound of any of claims 1 to 42, wherein the conjugate group comprises a structure selected from among:




and





wherein n is from 1 to 12; and

wherein m is from 1 to 12.

44. The compound of any of claims 1 to 42, wherein the conjugate group has a tether having a structure selected from among:


and


wherein L is either a phosphorus linking group or a neutral linking group;

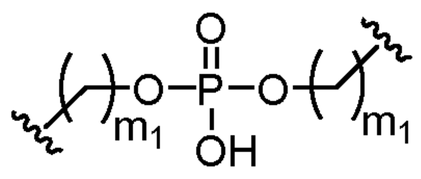
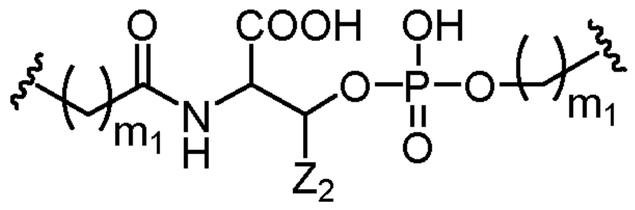
Z_1 is C(=O)O-R_2;

Z_2 is H, C_1-C_6 alkyl or substituted C_1-C_6 alky;

R_2 is H, C_1-C_6 alkyl or substituted C_1-C_6 alky; and

each m_1 is, independently, from 0 to 20 wherein at least one m_1 is greater than 0 for each tether.

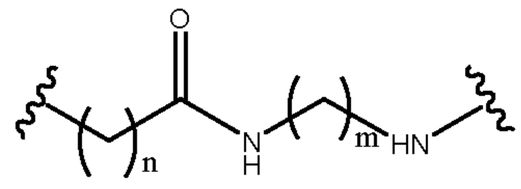
45. The compound of claim 44, wherein conjugate group has a tether having a structure selected from among:


and


wherein Z_2 is H or CH_3; and

each m_1 is, independently, from 0 to 20 wherein at least one m_1 is greater than 0 for each tether.

46. The compound of any of claims 30 to 36, wherein the conjugate group has tether having a structure selected from among:



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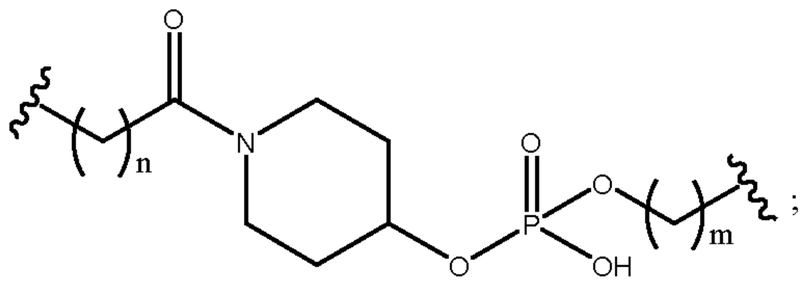

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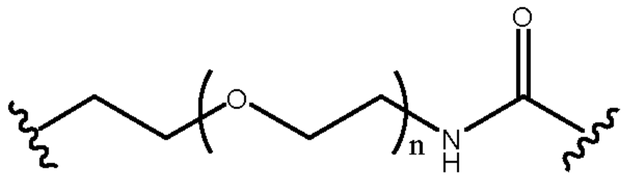


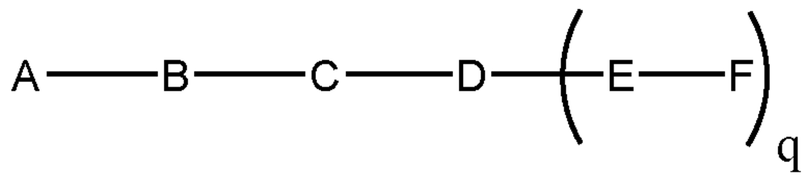
;

wherein n is from 1 to 12; and

wherein m is from 1 to 12.

47. The compound of any of claims 1 to 46, wherein the conjugate group is covalently attached to the modified oligonucleotide.

48. The compound of any of claims 1 to 47, wherein the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

B is the cleavable moiety

C is the conjugate linker

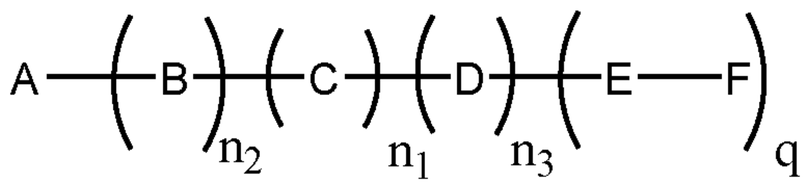
D is the branching group

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

49. The compound of any of claims 1 to 47, wherein the compound has a structure represented by the formula:



wherein:

A is the modified oligonucleotide;

B is the cleavable moiety

C is the conjugate linker

D is the branching group

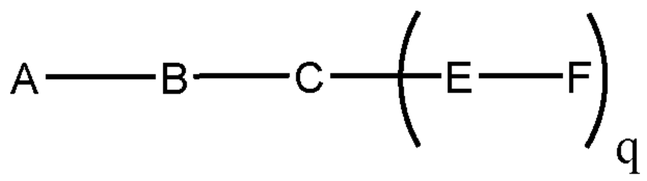
each E is a tether;

each F is a ligand;

each n is independently 0 or 1; and

q is an integer between 1 and 5.

50. The compound of any of claims 1 to 47, wherein the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

B is the cleavable moiety;

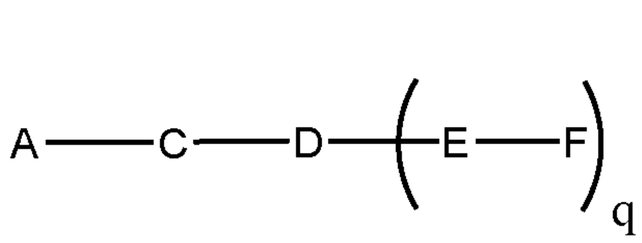
C is the conjugate linker;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

51. The compound of any of claims 1 to 47, wherein the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

C is the conjugate linker;

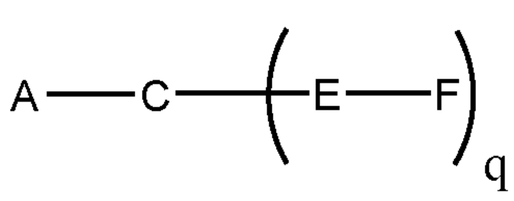
D is the branching group;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

52. The compound of any of claims 1 to 47, wherein the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

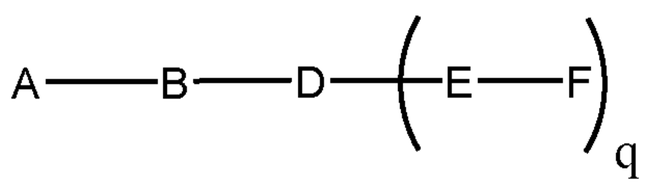
C is the conjugate linker;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

53. The compound of any of claims 1 to 47, wherein the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

B is the cleavable moiety;

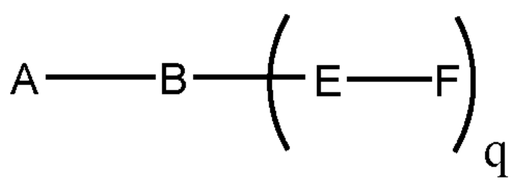
D is the branching group;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

54. The compound of any of claims 1 to 47, wherein the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

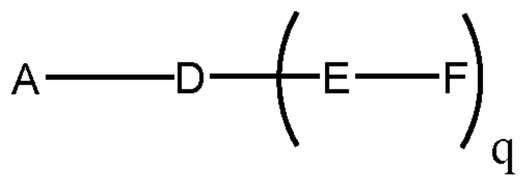
B is the cleavable moiety;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

55. The compound of any of claims 1 to 47, wherein the compound has a structure represented by the formula:



wherein

A is the modified oligonucleotide;

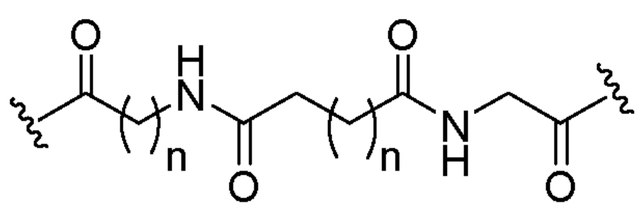
D is the branching group;

each E is a tether;

each F is a ligand; and

q is an integer between 1 and 5.

56. The compound of any of claims 48 to 55, wherein the conjugate linker has a structure selected from among: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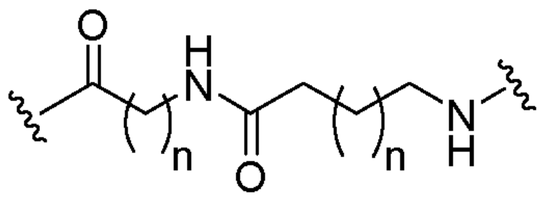
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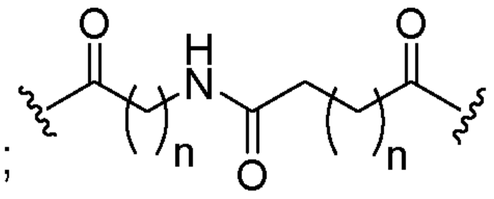
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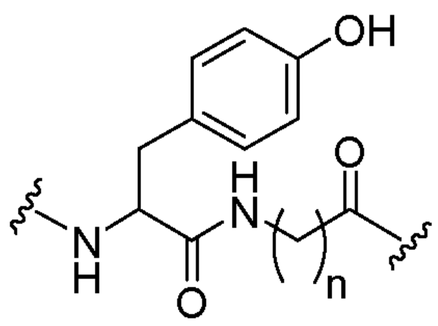
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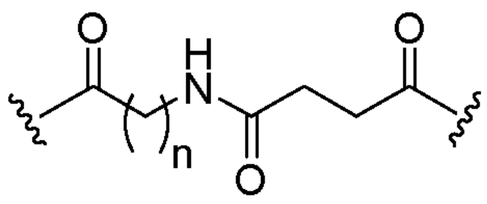
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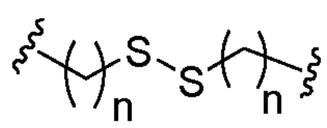
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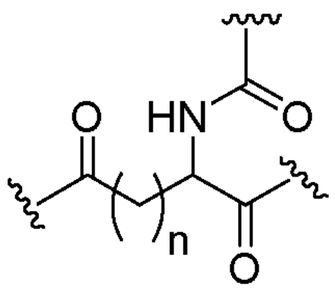
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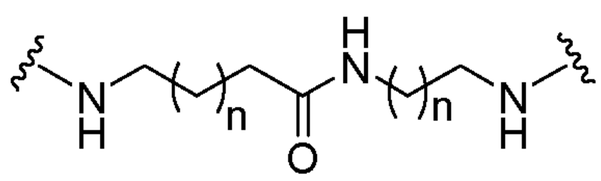
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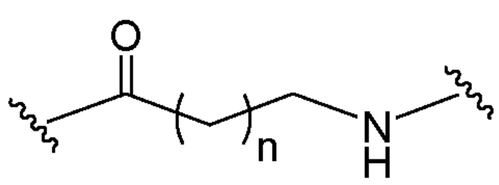
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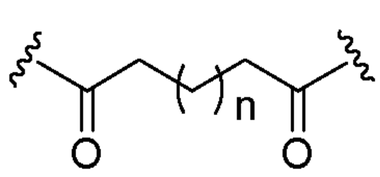
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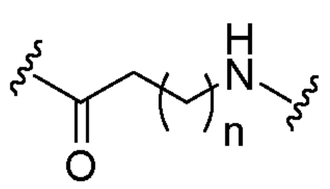
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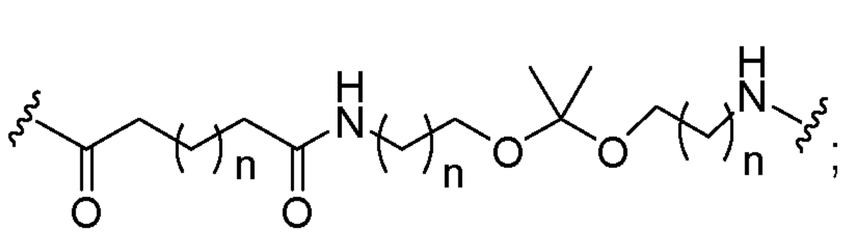
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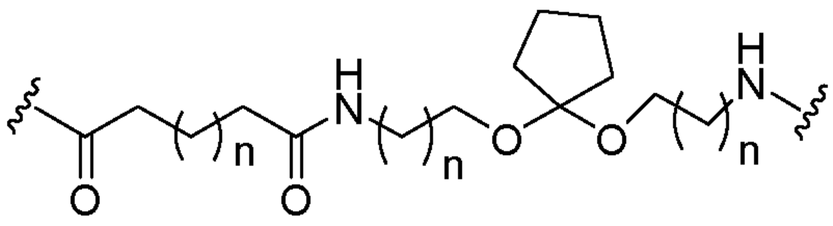
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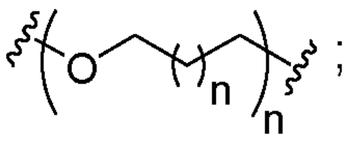
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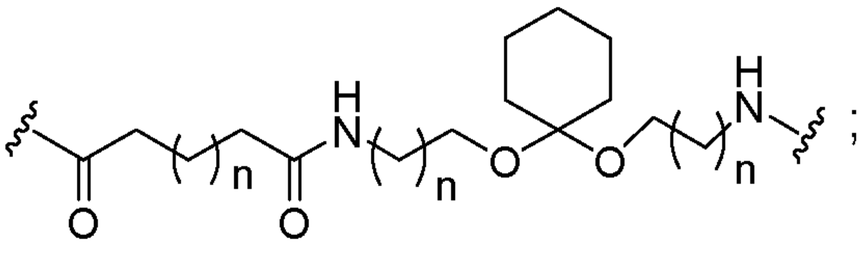
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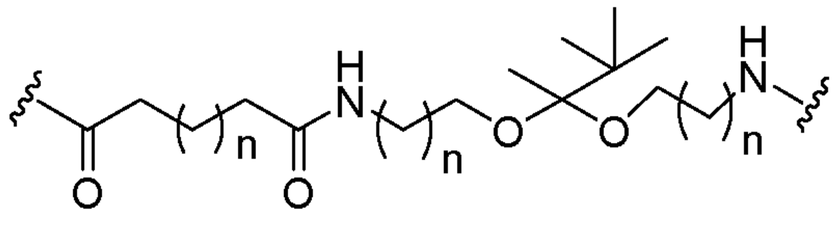
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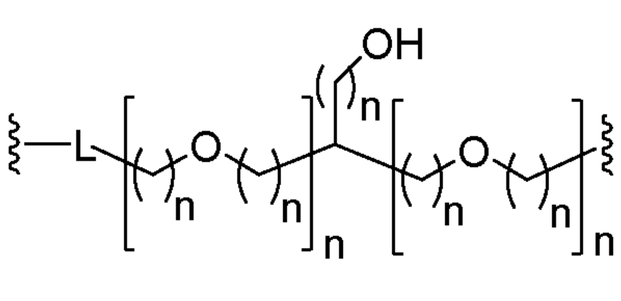
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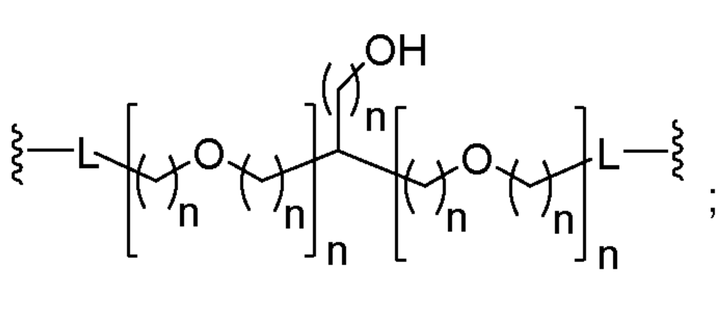
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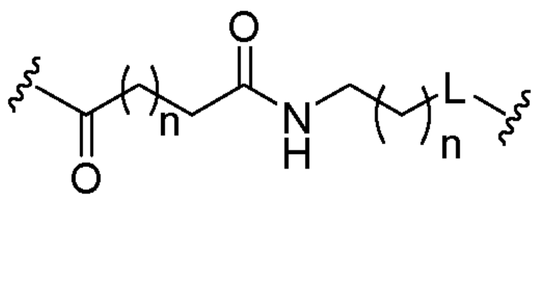
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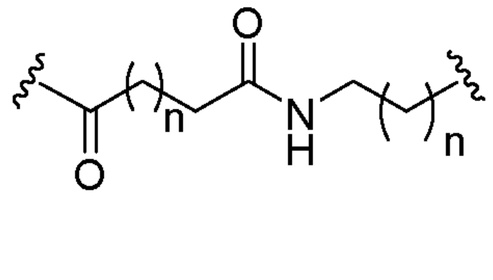
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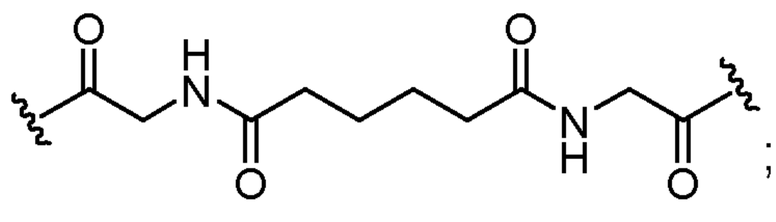
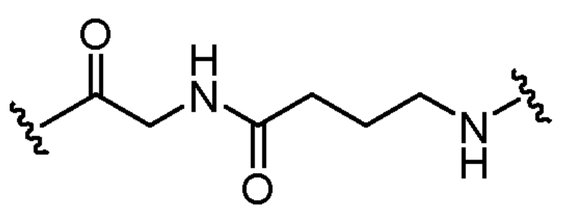
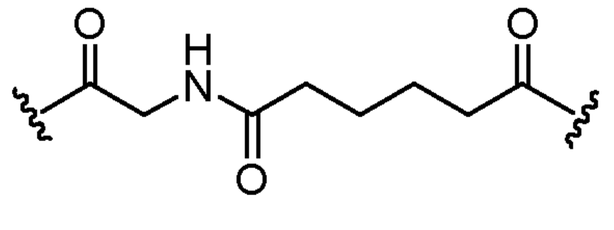
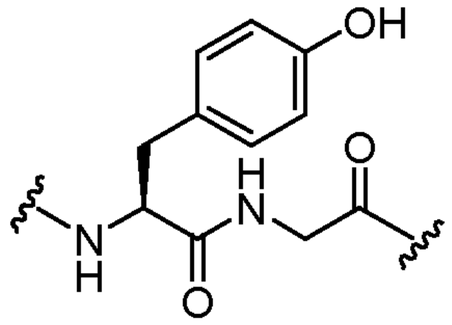
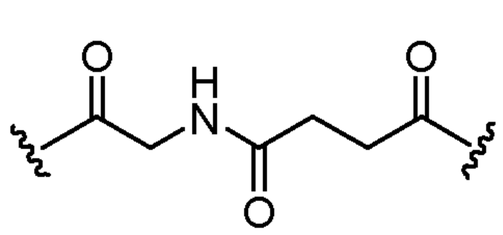
wherein each L is, independently, a phosphorus linking group or a neutral linking group; and each n is, independently, from 1 to 20.

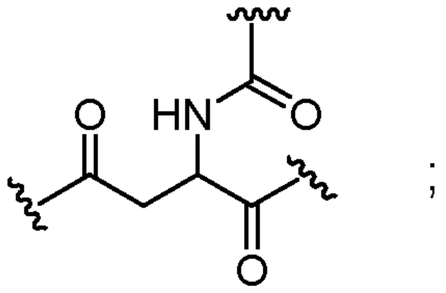


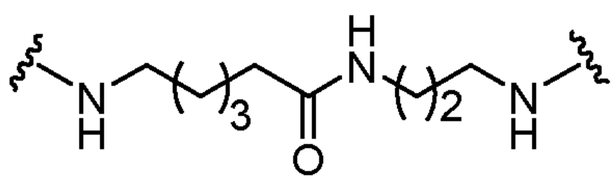


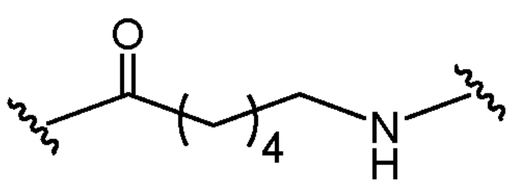


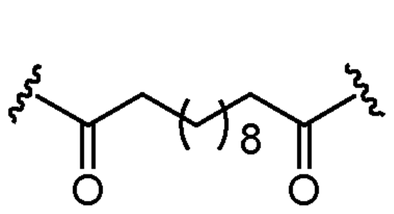


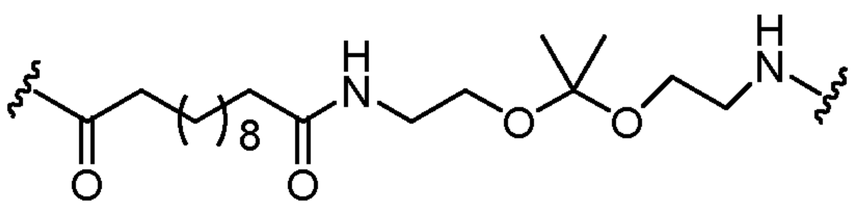


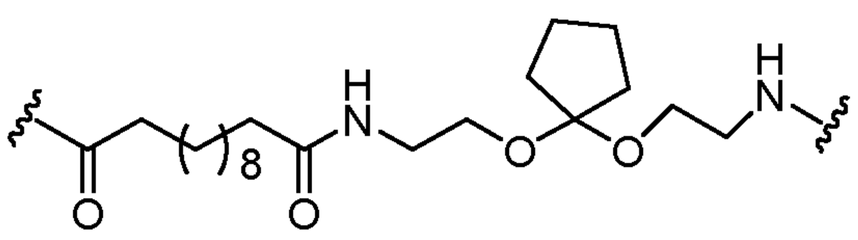


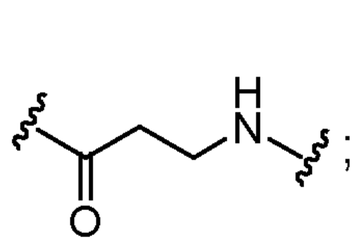


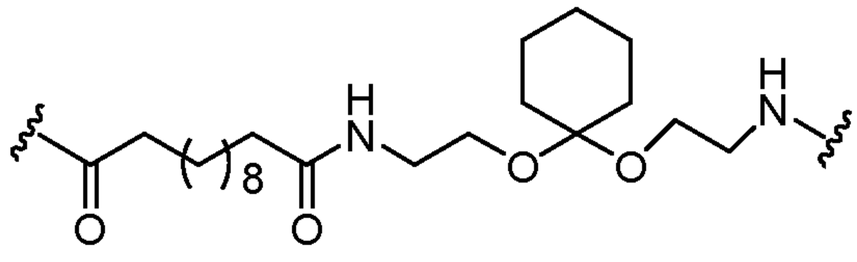


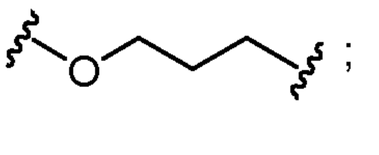


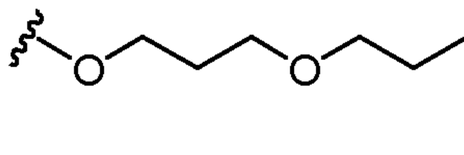


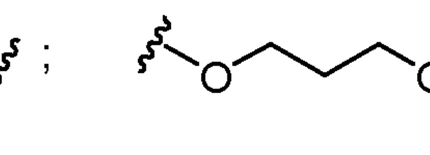


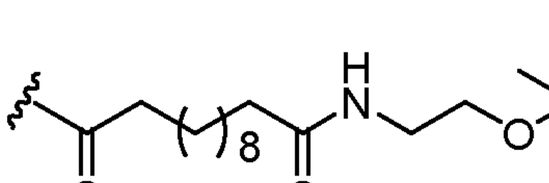


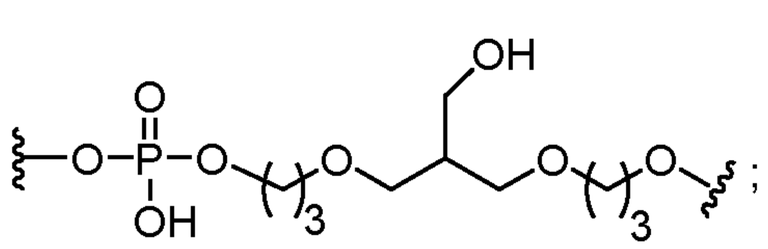


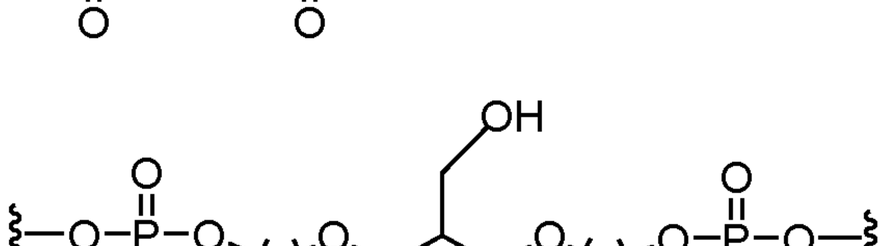


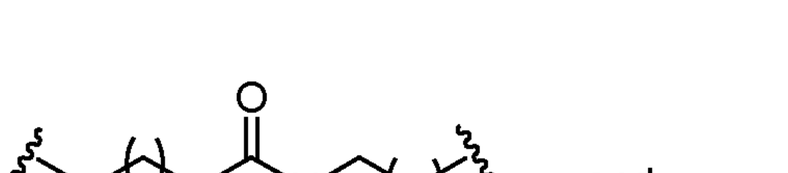












57. The compound of any of claims 48 to 55, wherein the conjugate linker has a structure selected from among:

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and

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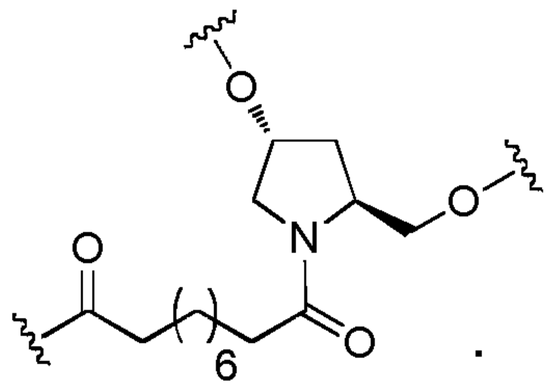
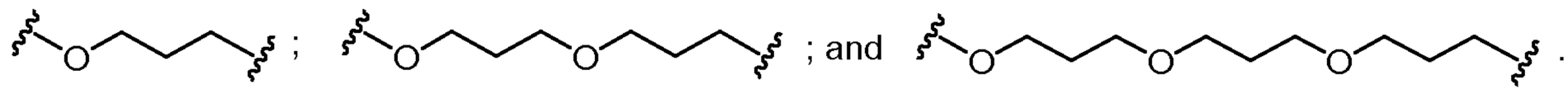
58. The compound of any of claims 48 to 55, wherein the conjugate linker has the followingstructure:



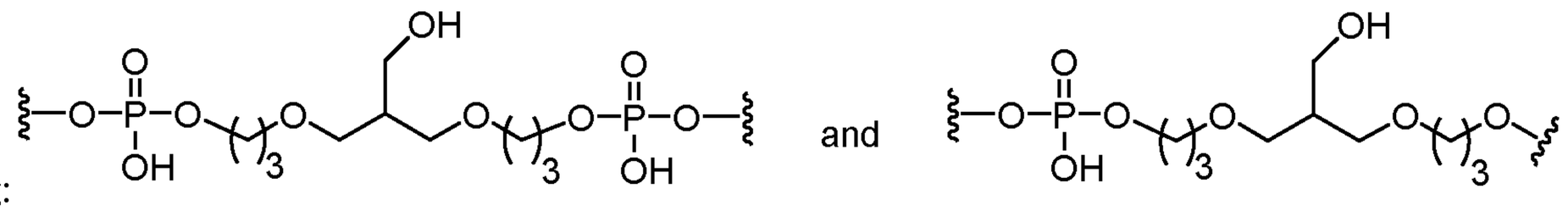
.

59. The compound of any of claims 48 to 55, wherein the conjugate linker has a structure selected from among:



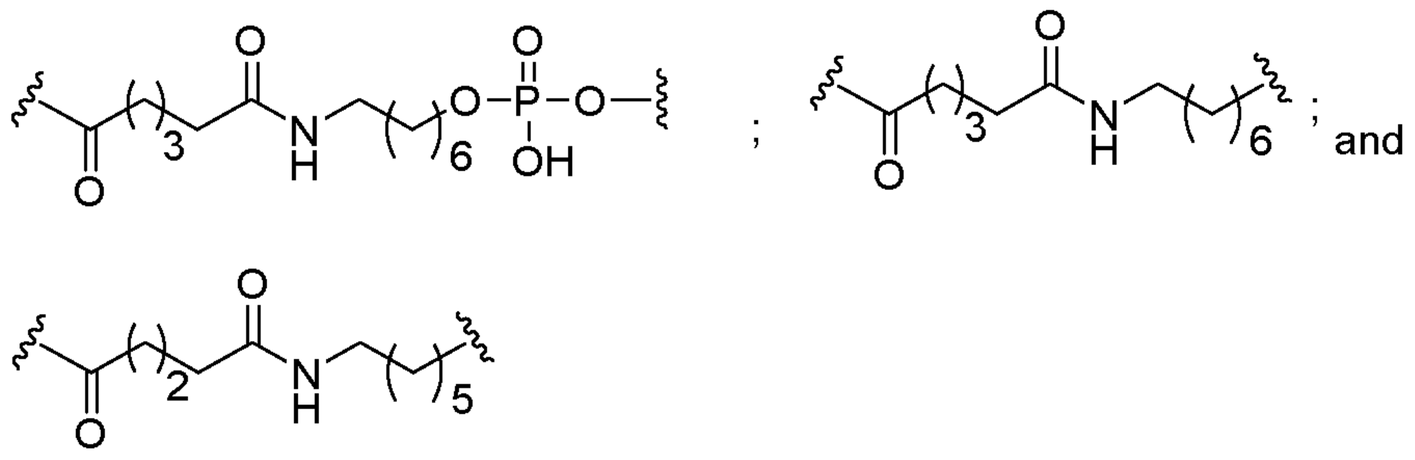
.

60. The compound of any of claims 48 to 55, wherein the conjugate linker has a structure selected from among:



.

61. The compound of any of claims 48 to 55, wherein the conjugate linker has a structure selected from among:



62. The compound of any of claims 48 to 61, wherein the conjugate linker comprises a pyrrolidine.

63. The compound of any of claims 48 to 61, wherein the conjugate linker does not comprise a pyrrolidine.

64. The compound of any of claims 48 to 63, wherein the conjugate linker comprises PEG.

65. The compound of any of claims 48 to 64, wherein the conjugate linker comprises an amide.

66. The compound of any of claims 48 to 64, wherein the conjugate linker comprises at least two amides.

67. The compound of any of claims 48 to 64, wherein the conjugate linker does not comprise an amide.

68. The compound of any of claims 48 to 67, wherein the conjugate linker comprises a polyamide.

69. The compound of any of claims 48 to 68, wherein the conjugate linker comprises an amine.

70. The compound of any of claims 48to 69, wherein the conjugate linker comprises one or more disulfide bonds.

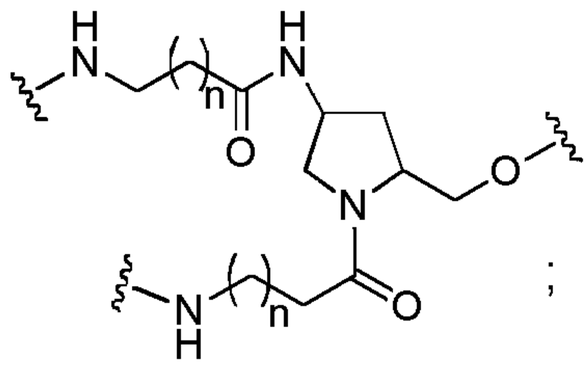
71. The compound of any of claims 48 to 70, wherein the conjugate linker comprises a protein binding moiety.

72. The compound of claim 71, wherein the protein binding moiety comprises a lipid.

73. The compound of claim 71, wherein the protein binding moiety is selected from among: cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine), a vitamin (e.g., folate, vitamin A, vitamin E, biotin, pyridoxal), a peptide, a carbohydrate (e.g., monosaccharide, disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, polysaccharide), an endosomolytic component, a steroid (e.g., uvaol, hecigenin, diosgenin), a terpene (e.g., triterpene, e.g., sarsasapogenin, friedelin, epifriedelanol derivatized lithocholic acid), or a cationic lipid.

74. The compound of claim 71, wherein the protein binding moiety is selected from among: a C16 to C22 long chain saturated or unsaturated fatty acid, cholesterol, cholic acid, vitamin E, adamantane or 1-pentafluoropropyl.

75. The compound of any of claims 48 to 74, wherein the conjugate linker has a structure selected from among: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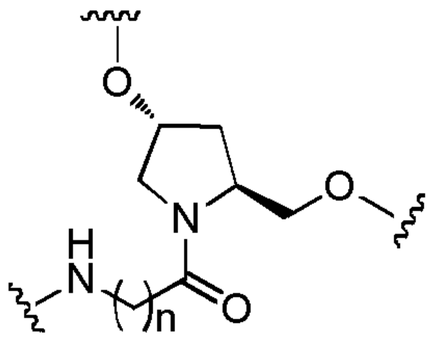
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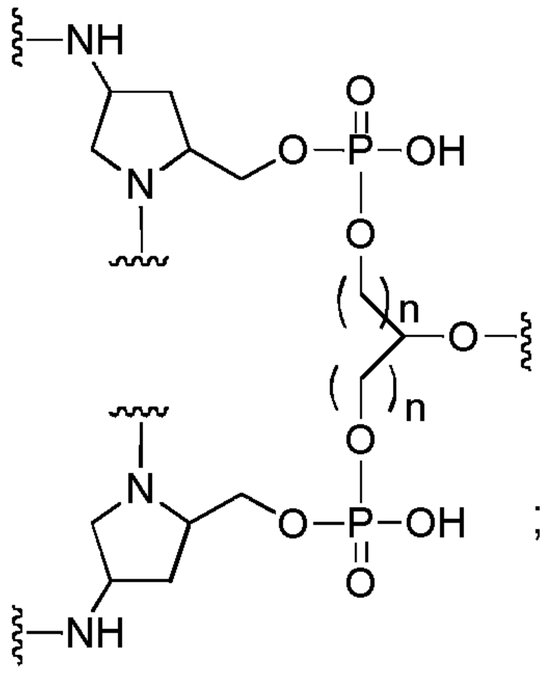
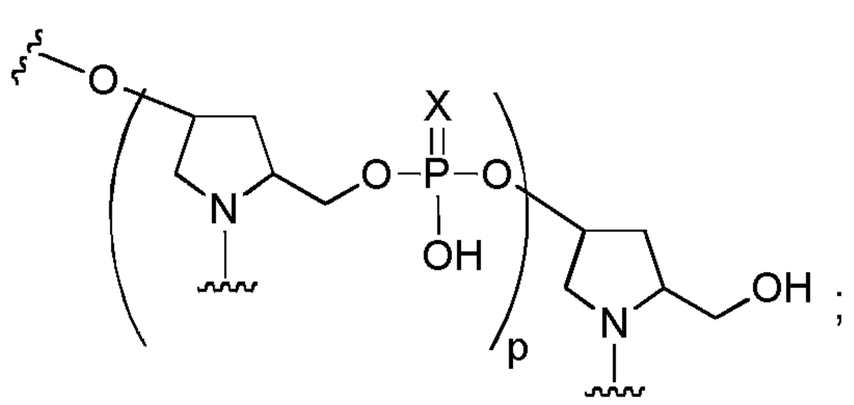

;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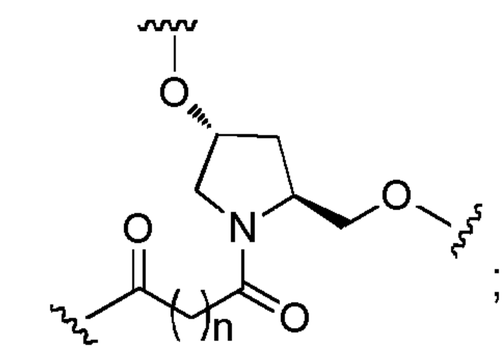
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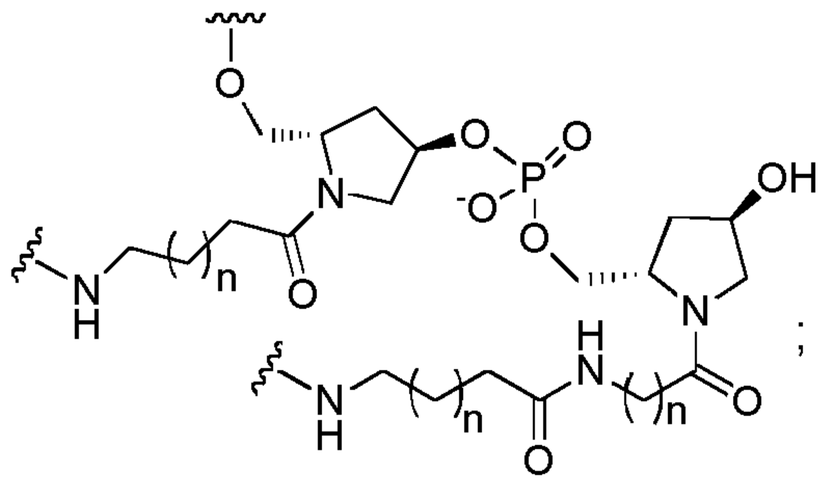
;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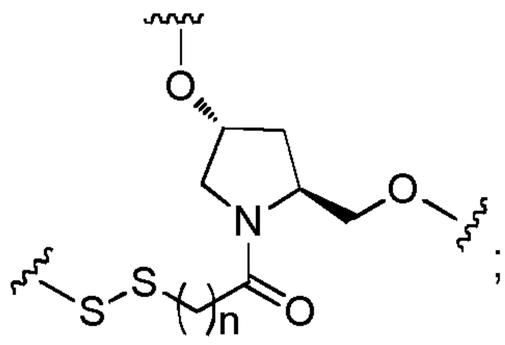
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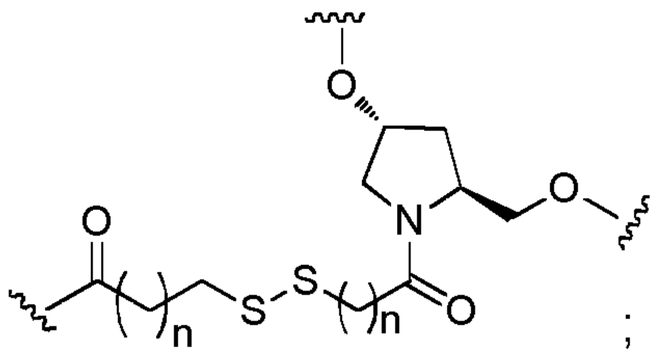
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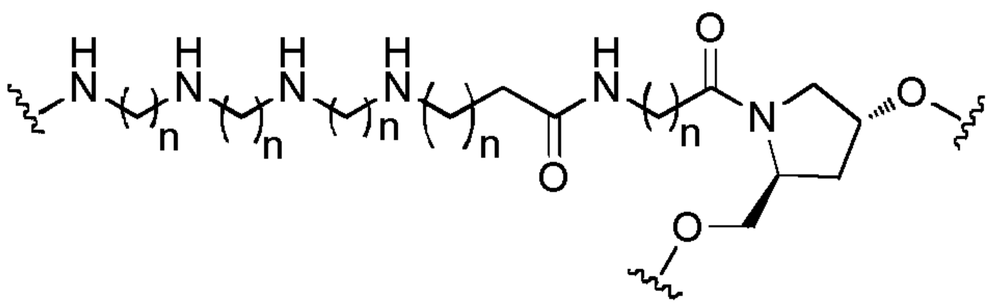
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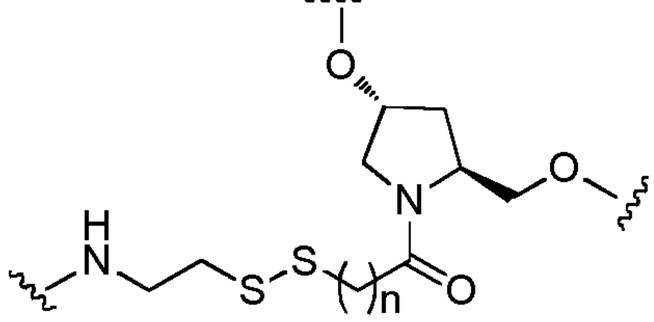
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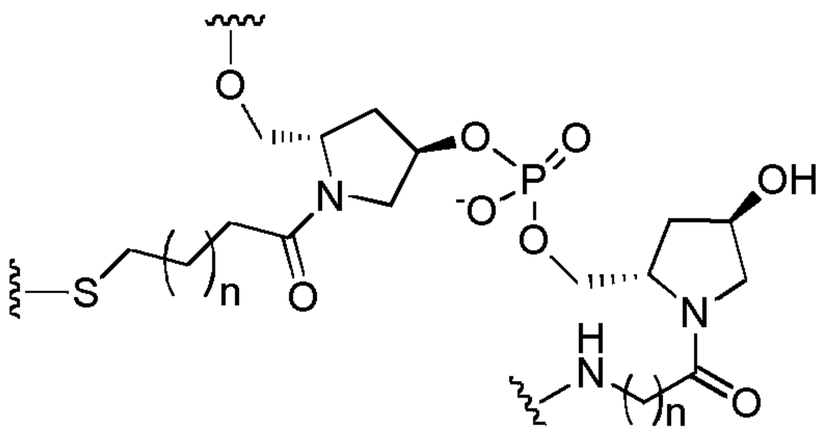
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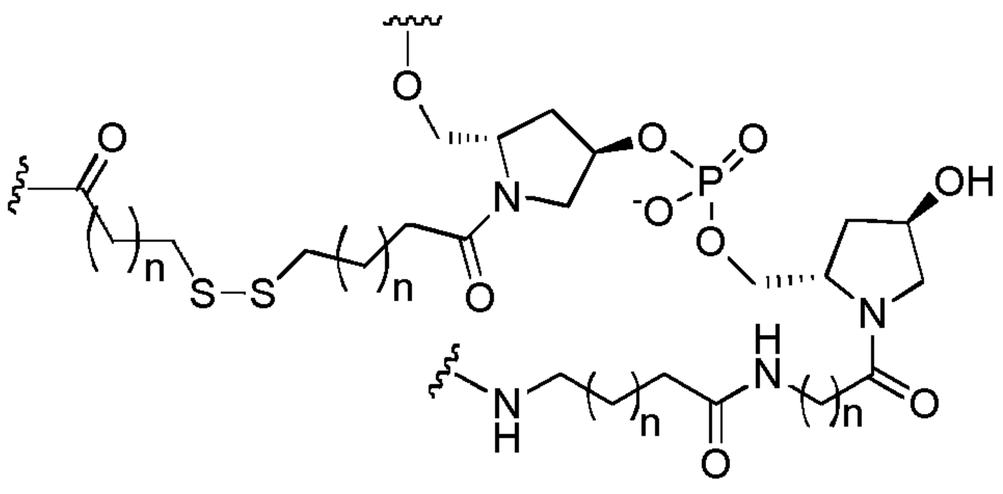
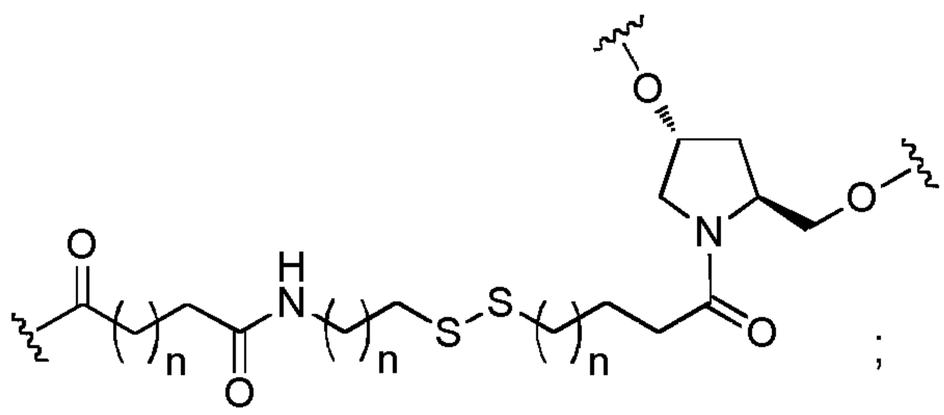


wherein each n is, independently, is from 1 to 20; and p is from 1 to 6.

76. The compound of any of claims 48 to 75, wherein the conjugate linker has a structure selected from among:

;



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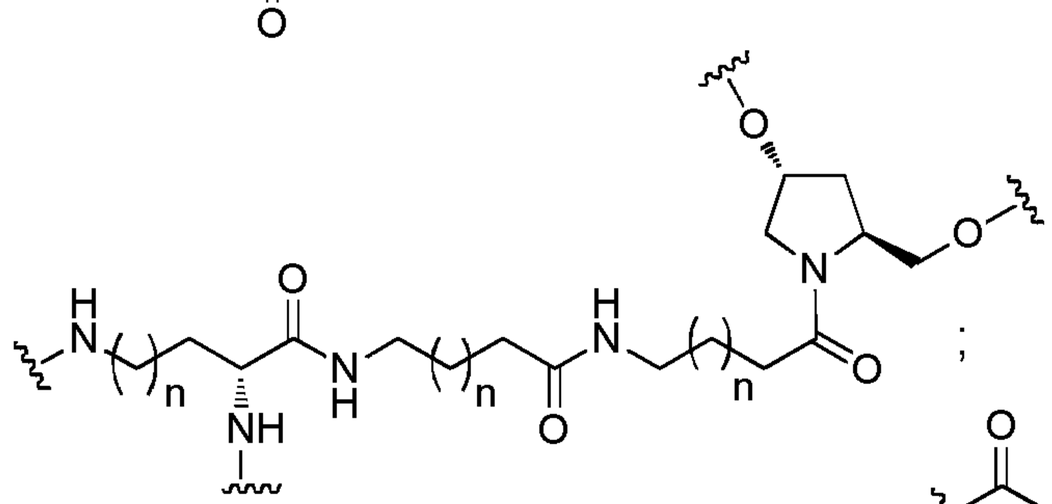

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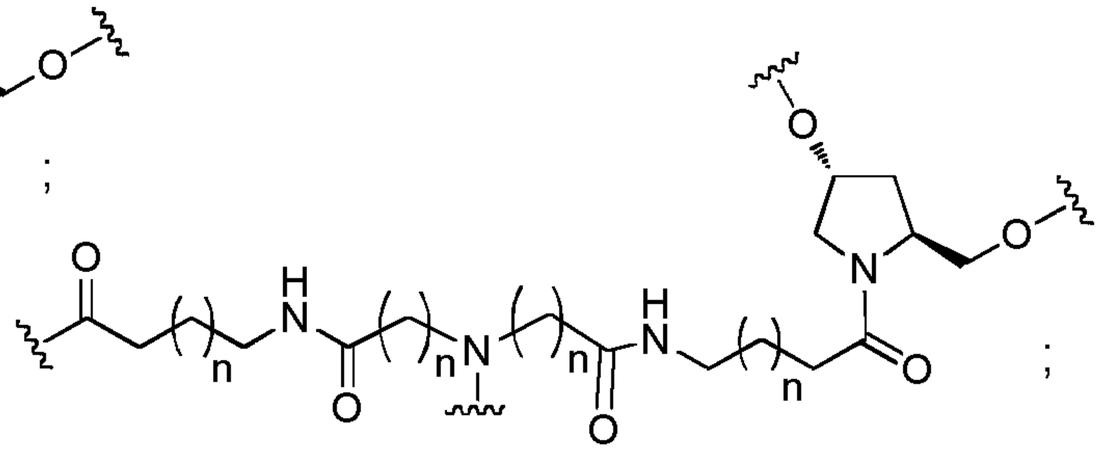

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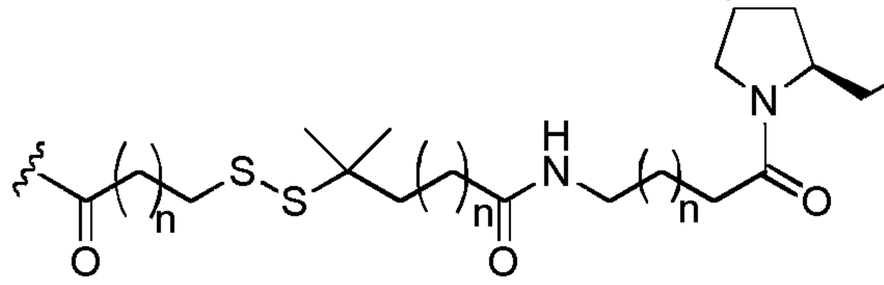

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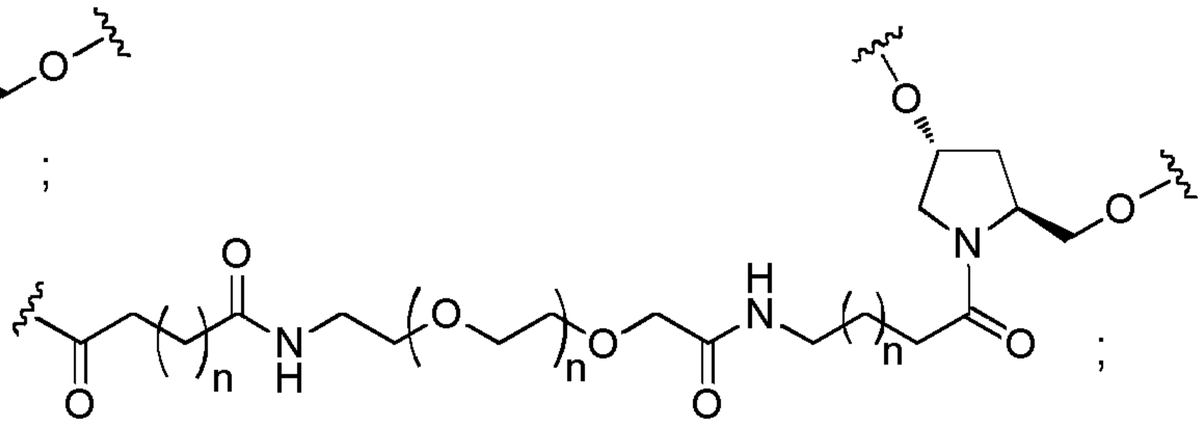

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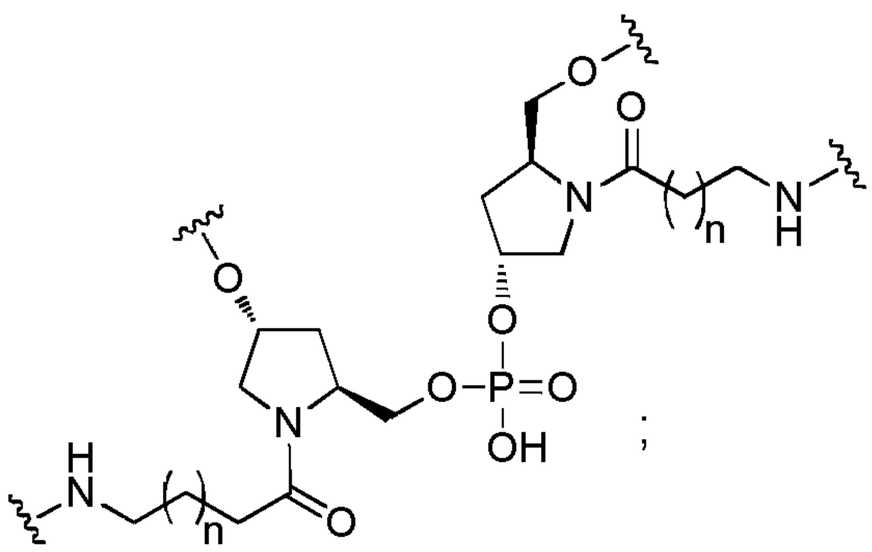

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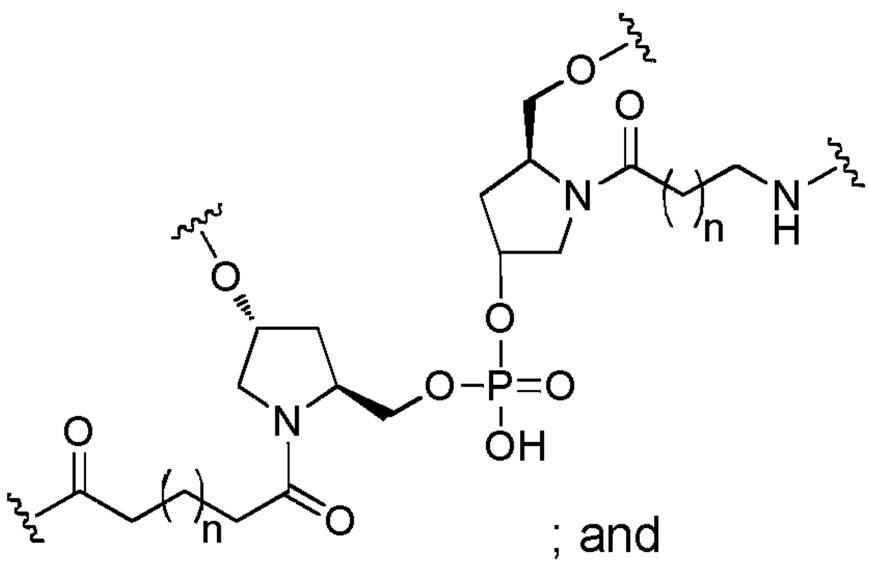
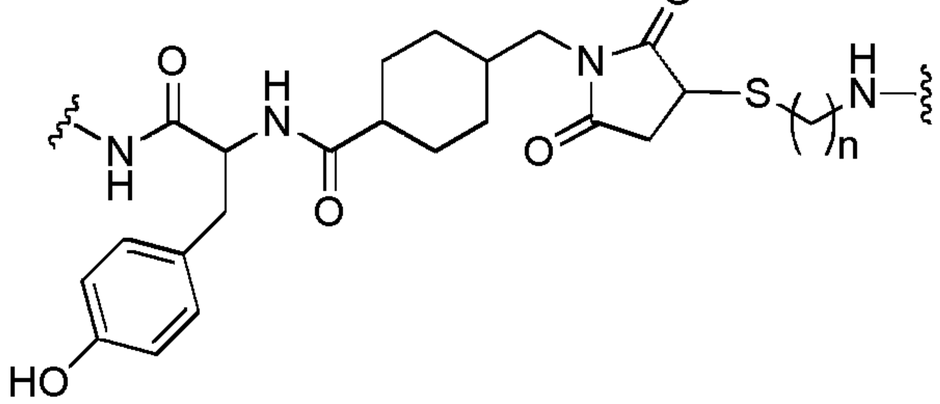
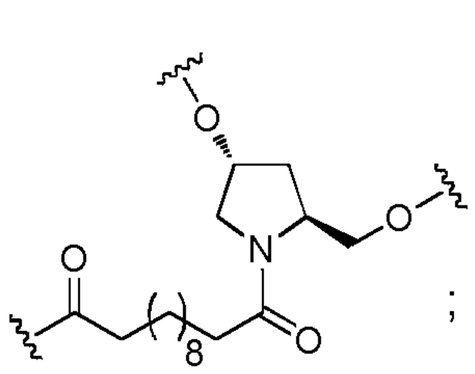


; and



wherein each n is, independently, from 1 to 20.

77. The compound of any of claims 48 to 75, wherein the conjugate linker has a structure selected from among: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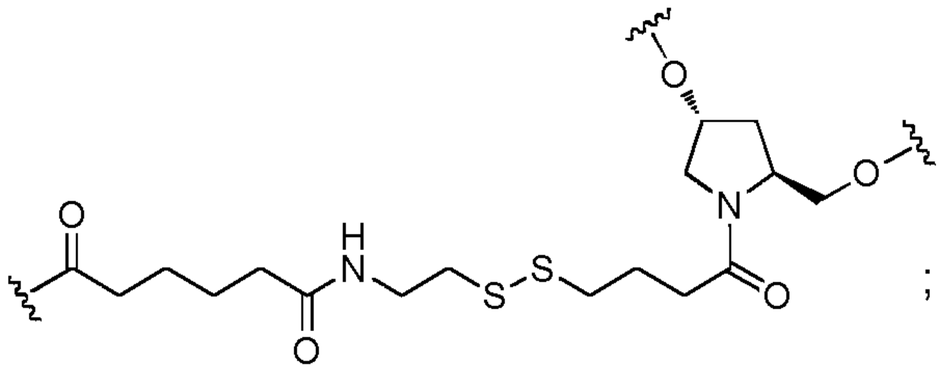
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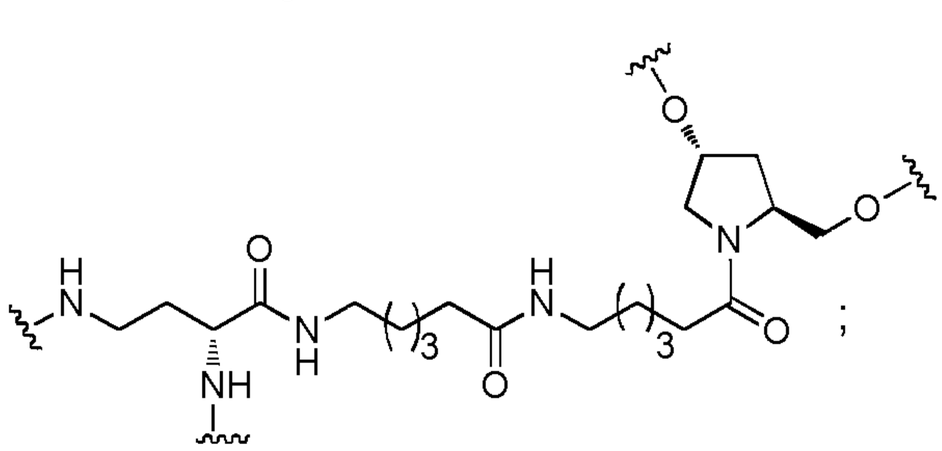
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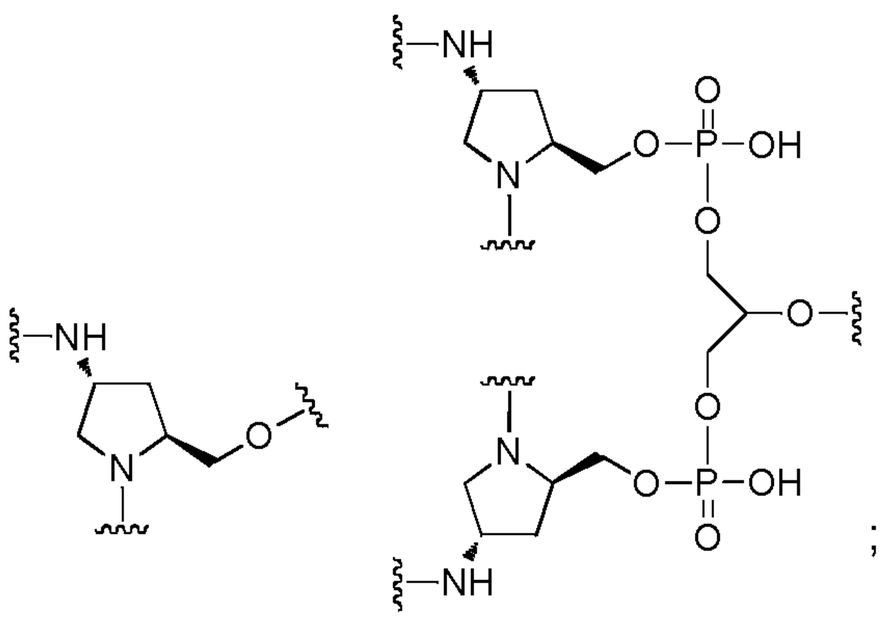
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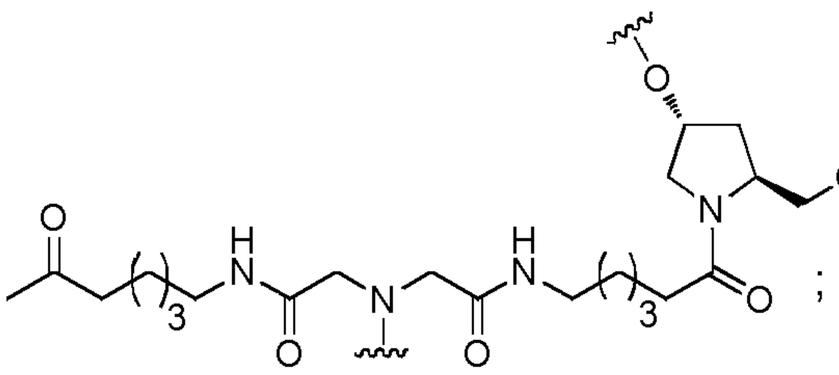
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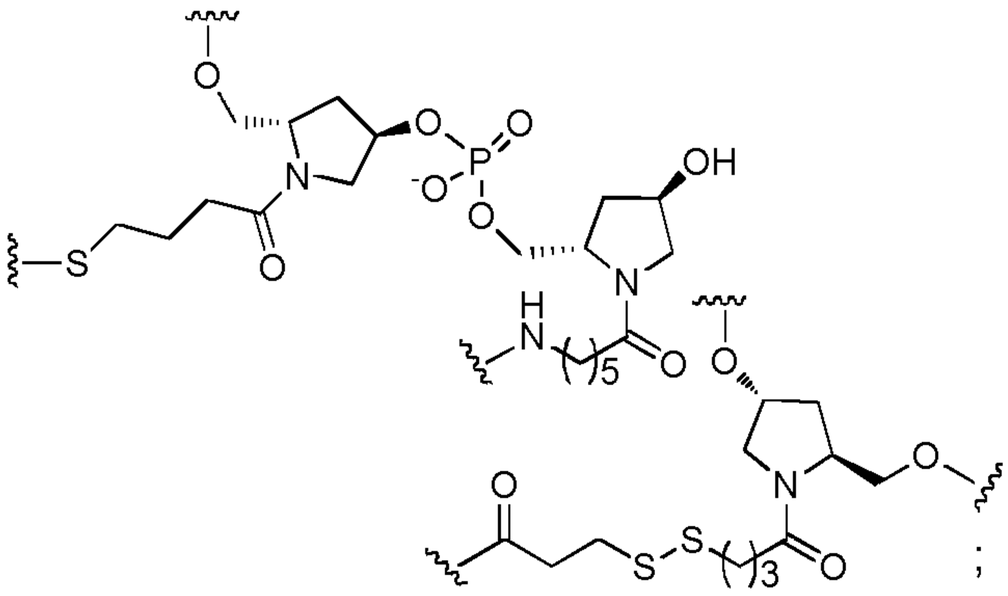
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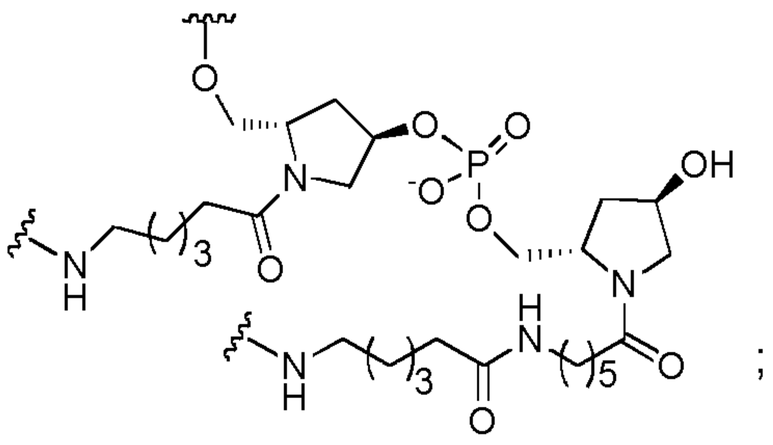
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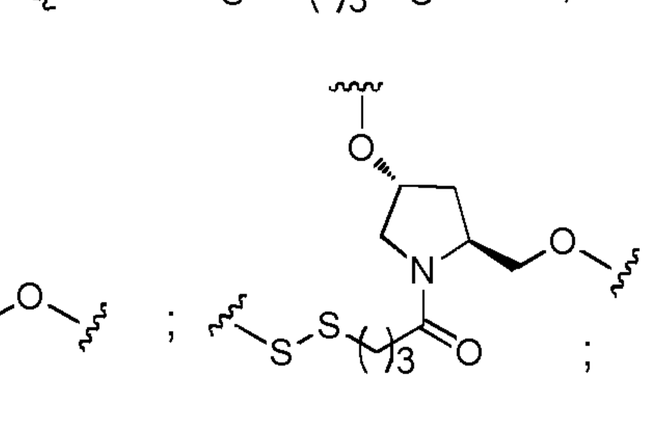
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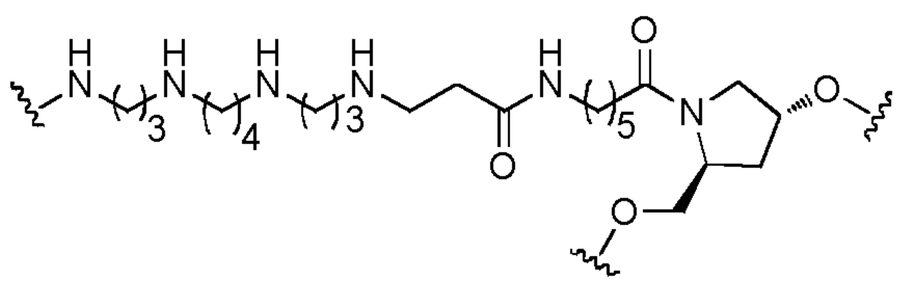
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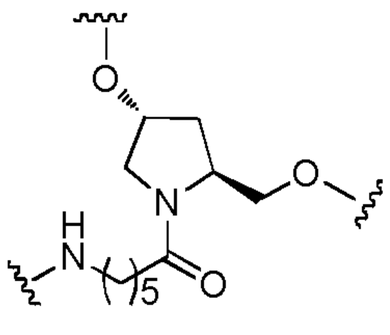
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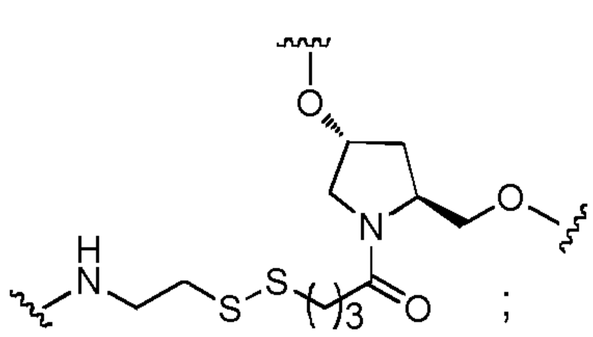
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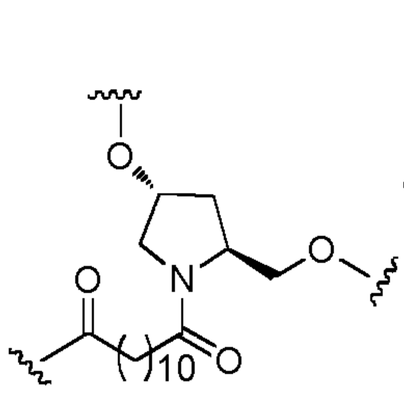
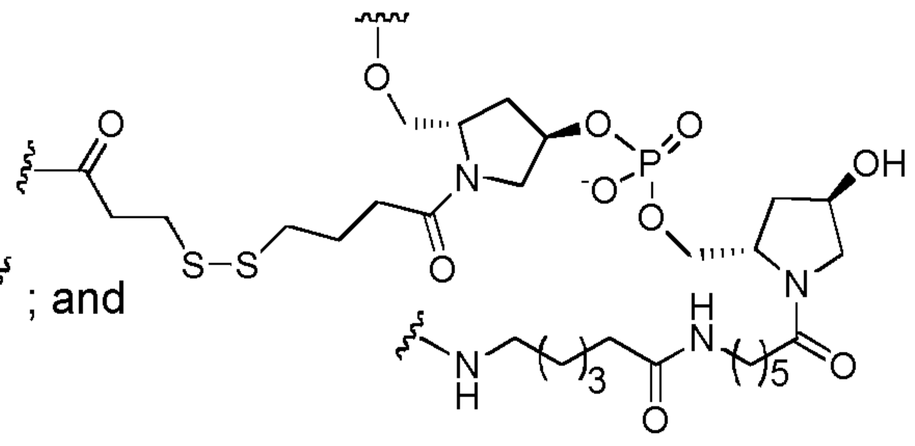
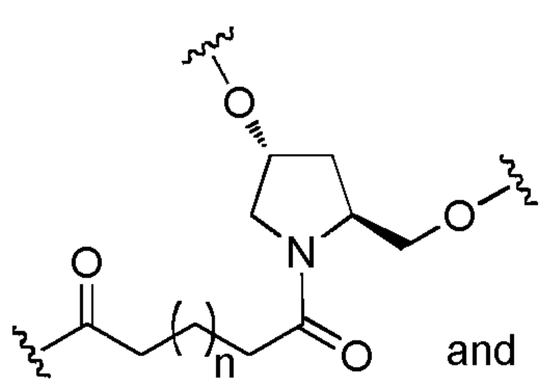
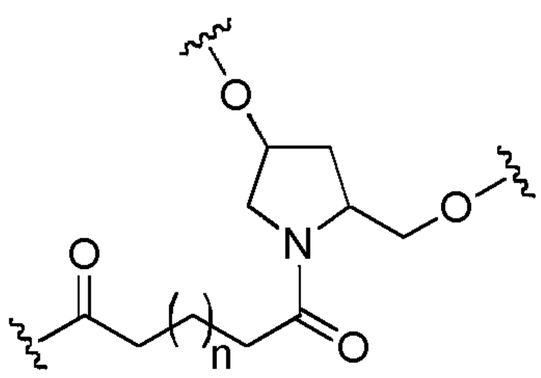

; and

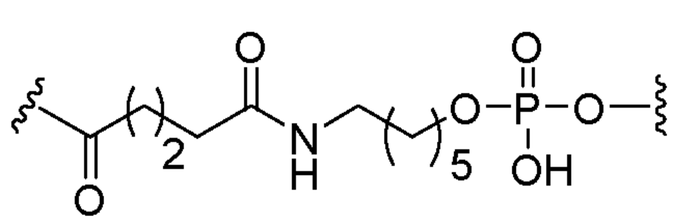
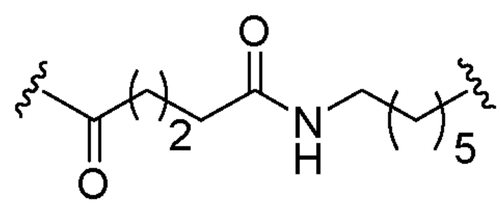

.

78. The compound of any of claims 48 to 75, wherein the conjugate linker has a structure selected from among:

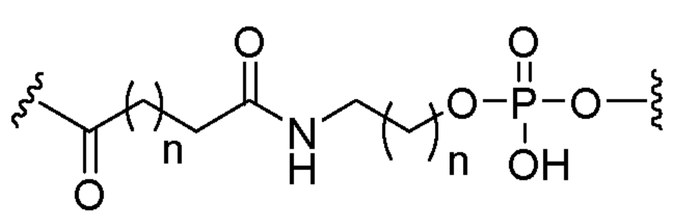
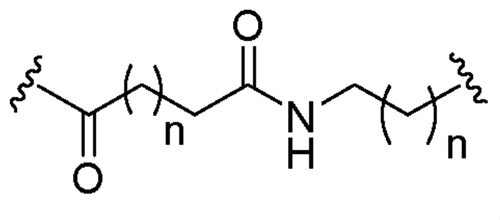

and


wherein n is from 1 to 20.

79. The compound of any of claims 48 to 75, wherein the conjugate linker has a structure selected from among:

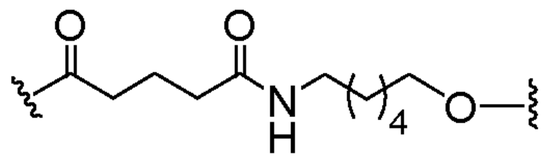

and

.

80. The compound of any of claims 48 to 75, wherein the conjugate linker has a structure selected from among:

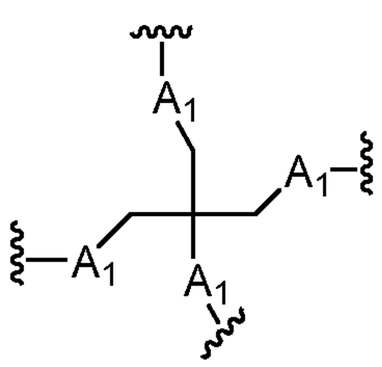
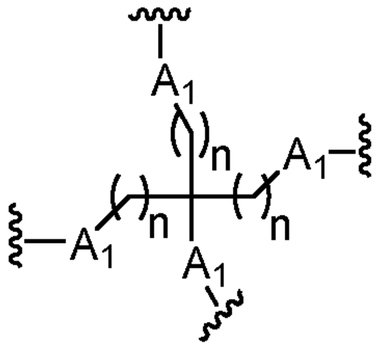
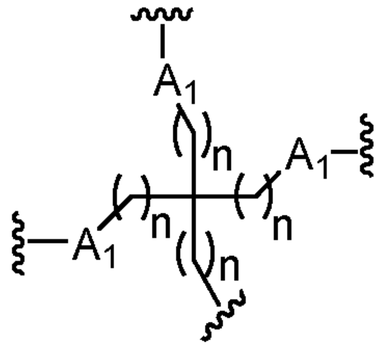

and

;

wherein each n is independently, 0, 1, 2, 3, 4, 5, 6, or 7.

81. The compound of any of claims 48 to 75, wherein the conjugate linker has the following structure:


.

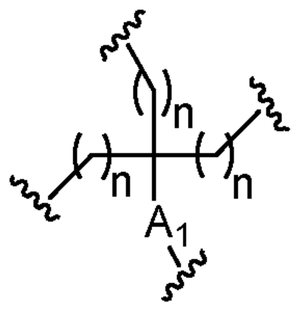
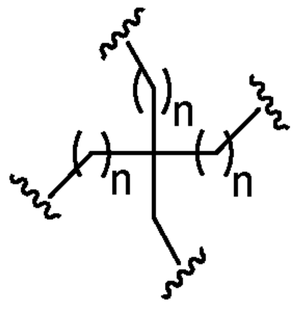
82. The compound of any of claims 48 to 81, wherein the branching group has one of the following structures:



and


wherein each A_1 is independently, O, S, C=O or NH; and

each n is, independently, from 1 to 20.

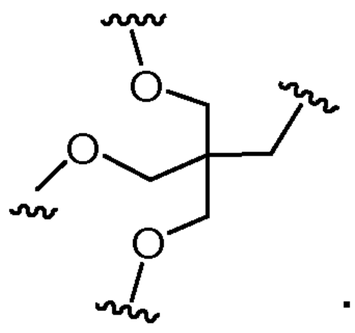
83. The compound of any of claims 48 to 81, wherein the branching group has one of the following structures:


and


wherein each A_1 is independently, O, S, C=O or NH; and

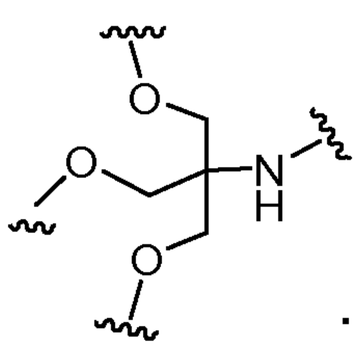
each n is, independently, from 1 to 20.

84. The compound of any of claims 48 to 81, wherein the branching group has the following structure:



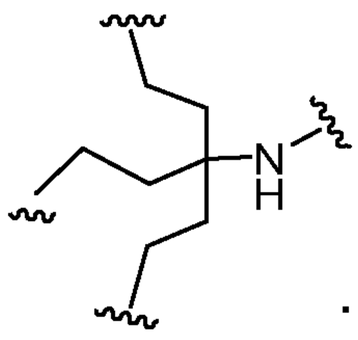
.

85. The compound of any of claims 48 to 81, wherein the branching group has the following structure:

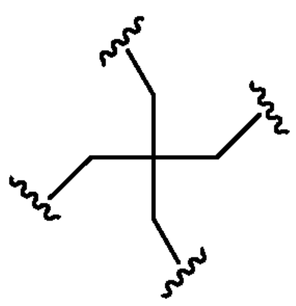


.

86. The compound of any of claims 48 to 81, wherein the branching group has the following structure:

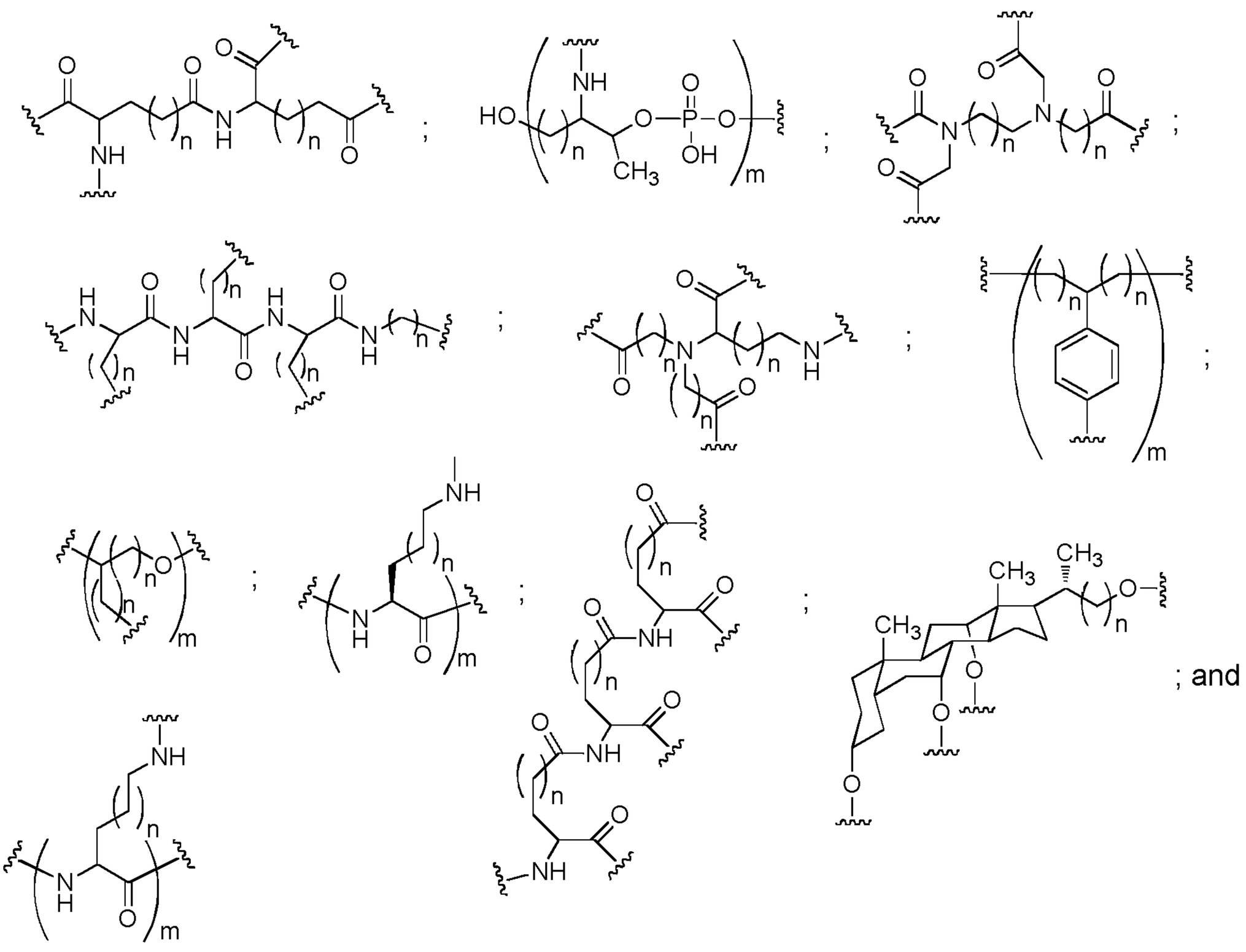


87. The compound of any of claims 48 to 81, wherein the branching group has the following structure:



88. The compound of any of claims 48 to 81, wherein the branching group comprises an ether.

89. The compound of any of claims 48 to 81, wherein the branching group has the following structure:

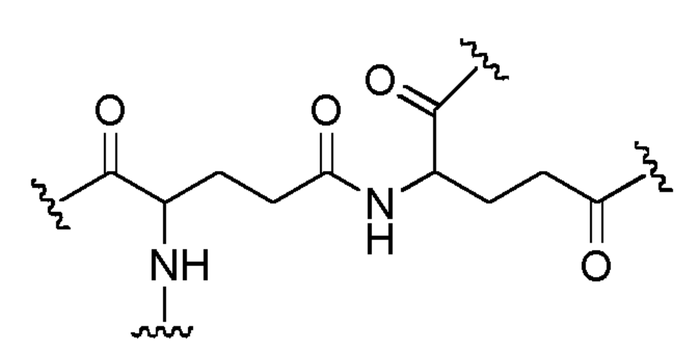
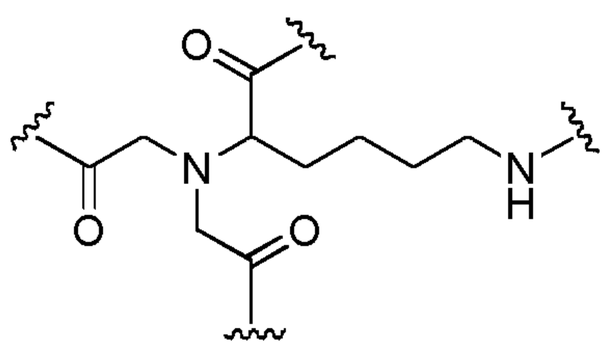
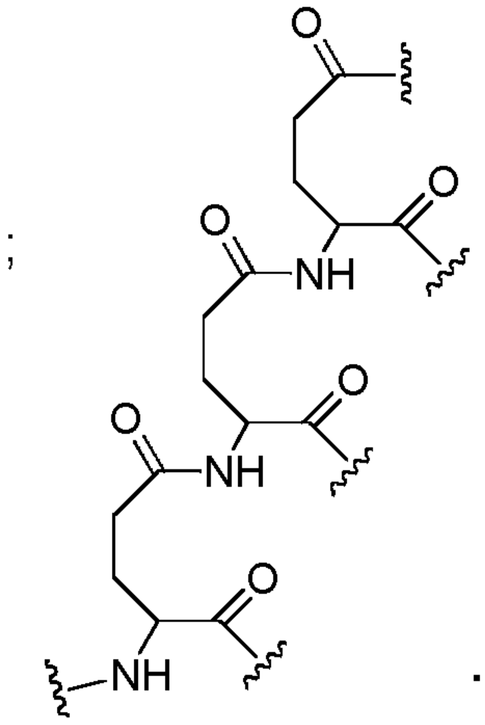
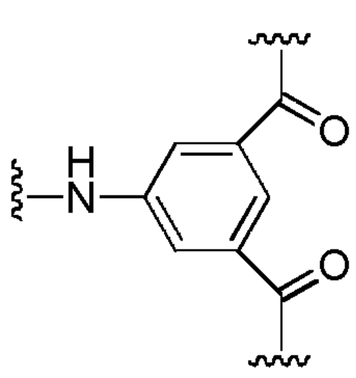
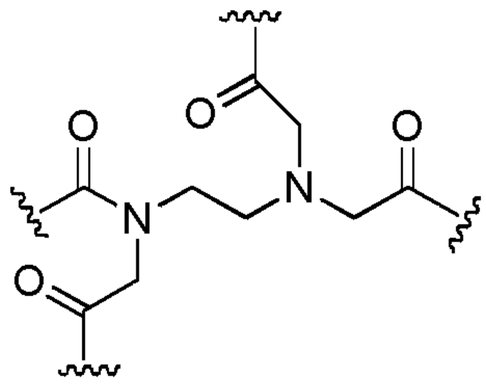
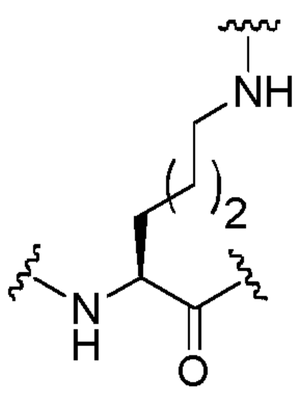


; and

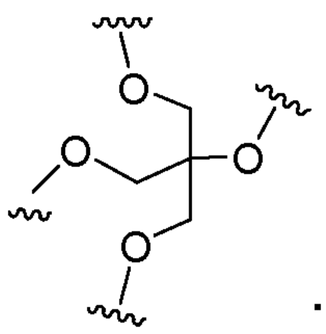
each n is, independently, from 1 to 20; and

m is from 2 to 6.

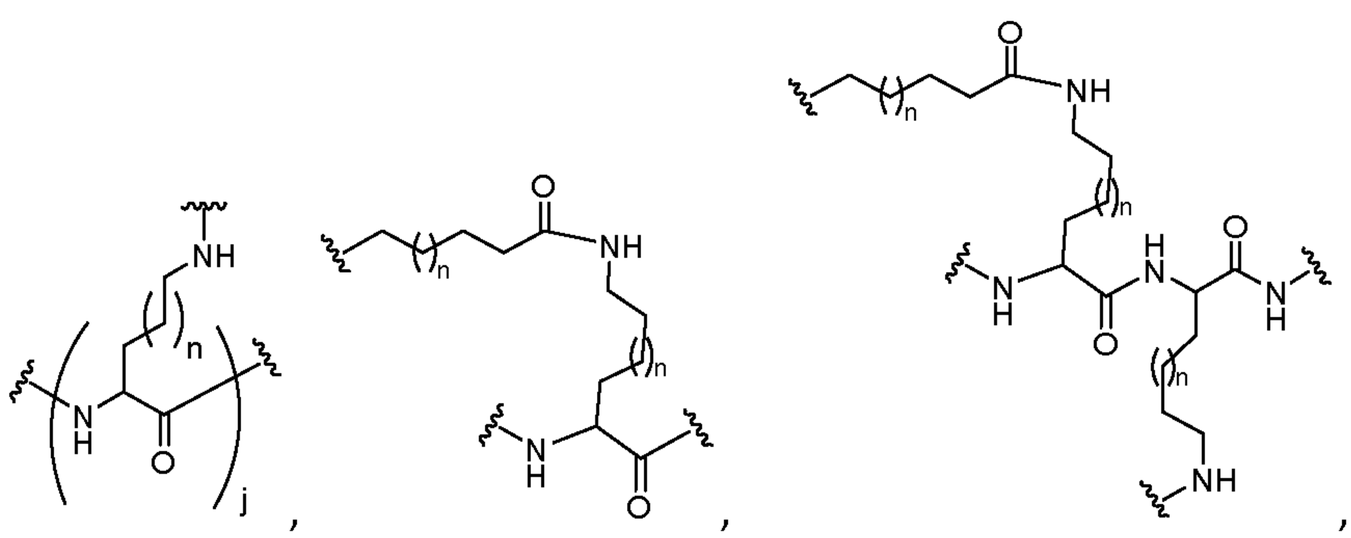
90. The compound of any of claims 48 to 81, wherein the branching group has the following structure:

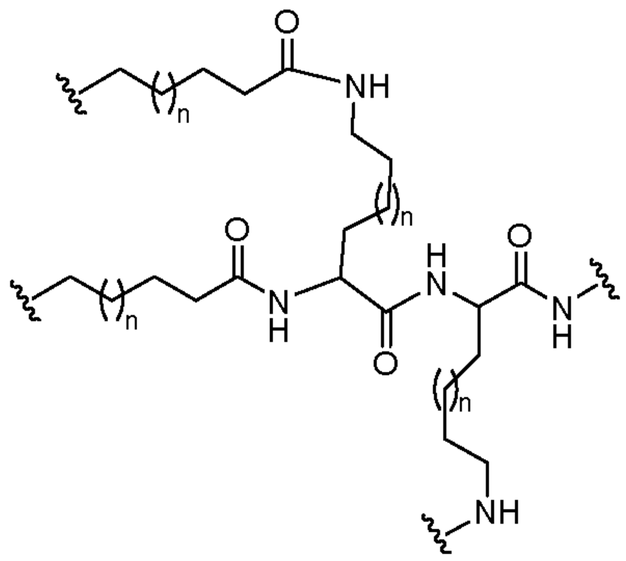
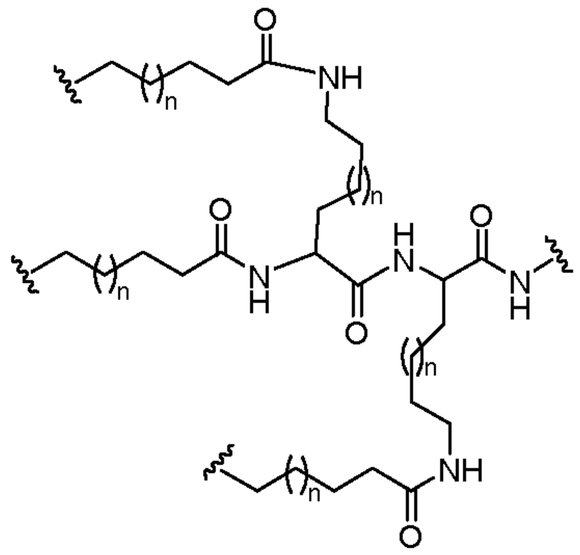

; 
; 
; 
; 
; 
; and
.

91. The compound of any of claims 48 to 81, wherein the branching group has the following structure:


.

92. The compound of any of claims 48 to 81, wherein the branching group comprises:



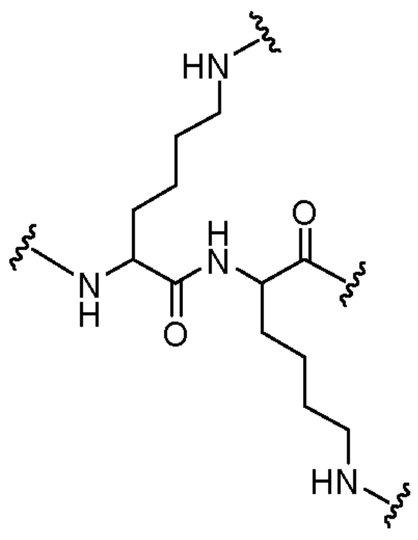
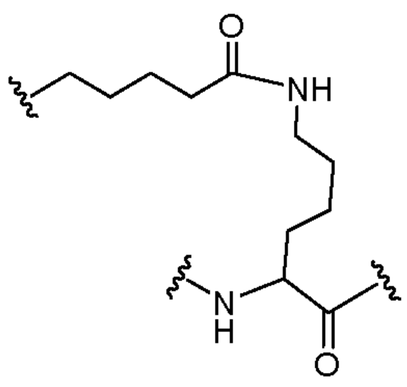
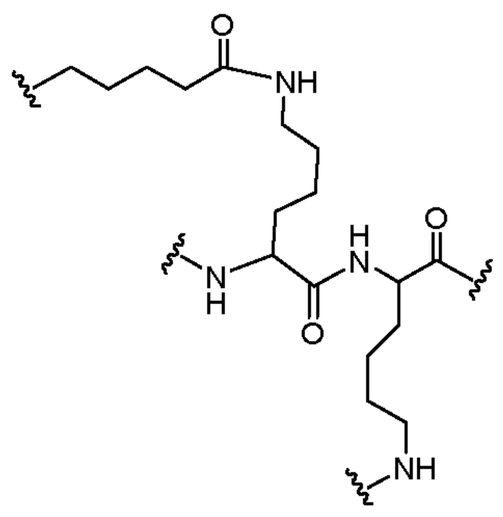



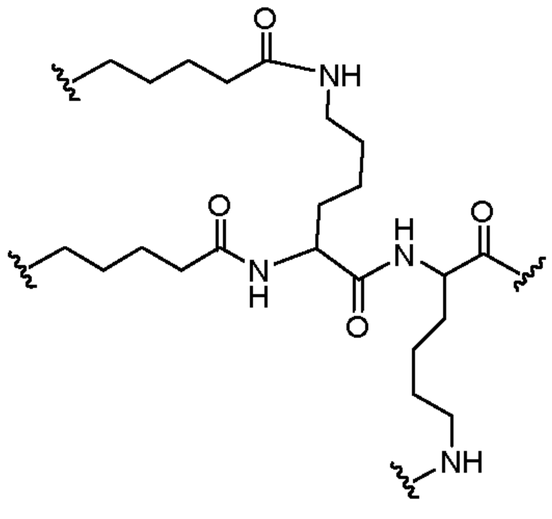
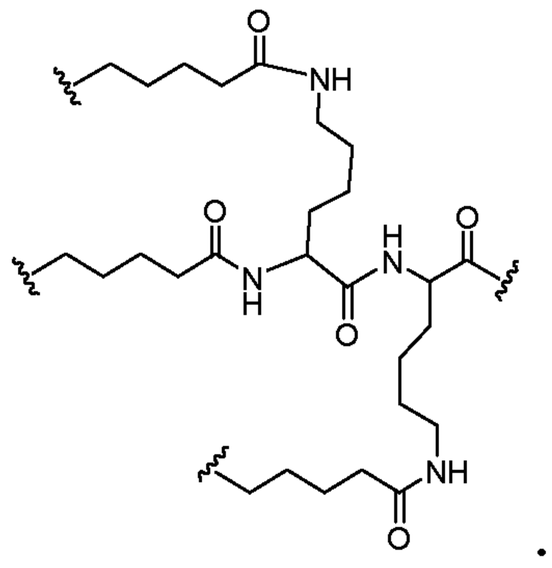
, or ;

wherein each j is an integer from 1 to 3; and

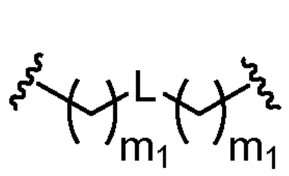
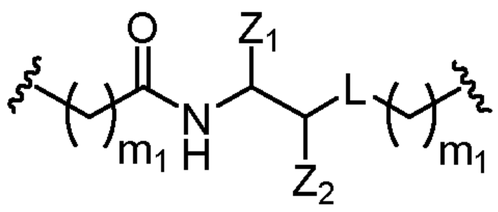
wherein each n is an integer from 1 to 20.

93. The compound of any of claims 48 to 81, wherein the branching group comprises:


, or  .

94. The compound of any of claims 48 to 93, wherein each tether is selected from among:


and 

wherein L is selected from a phosphorus linking group and a neutral linking group;

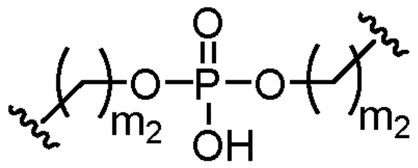
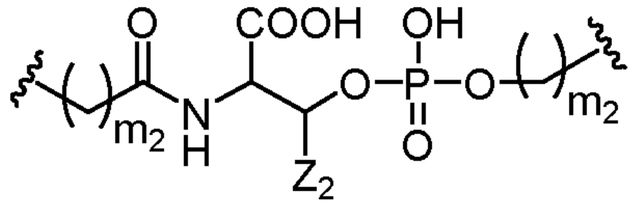
Z_1 is C(=O)O-R_2;

Z_2 is H, C_1-C_6 alkyl or substituted C_1-C_6 alky;

R_2 is H, C_1-C_6 alkyl or substituted C_1-C_6 alky; and

each m_1 is, independently, from 0 to 20 wherein at least one m_1 is greater than 0 for each tether.

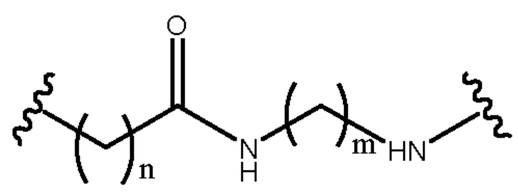
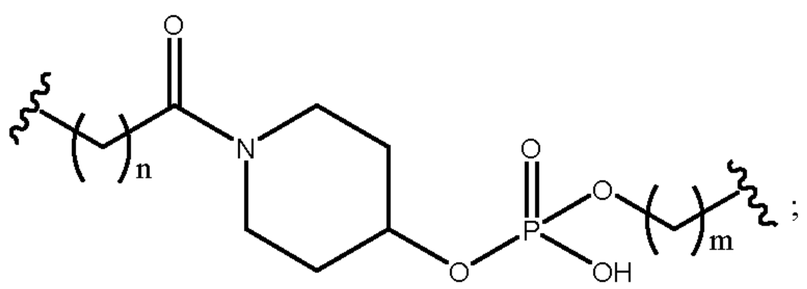
95. The compound of any of claims 48 to 93, wherein each tether is selected from among:

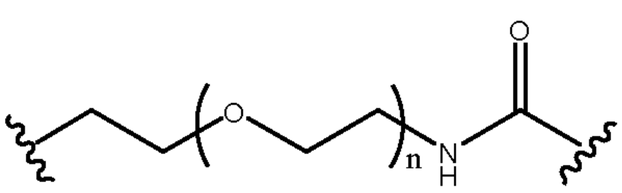

and


wherein Z_2 is H or CH_3; and

each m_2 is, independently, from 0 to 20 wherein at least one m_2 is greater than 0 for each tether.

96. The compound of any of claims 48 to 93, wherein each tether is selected from among:


;

;


;

wherein n is from 1 to 12; and

wherein m is from 1 to 12.

97. The compound of any of claims 48 to 93, wherein at least one tether comprises ethylene glycol.

98. The compound of any of claims 48 to 93 or 95, wherein at least one tether comprises an amide.

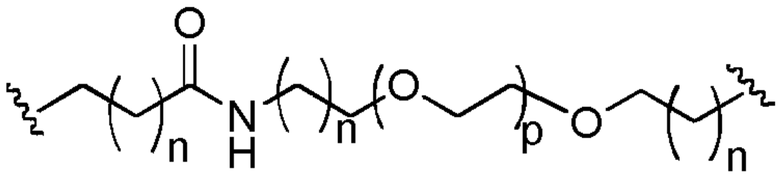
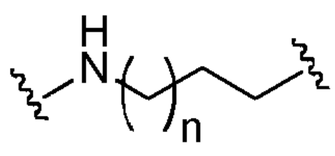
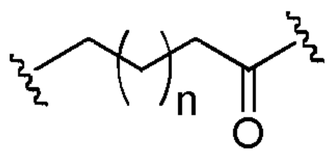
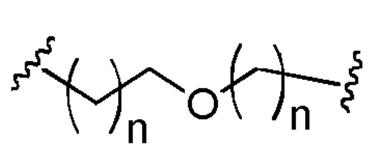
99. The compound of any of claims 48 to 93 or 95, wherein at least one tether comprises a polyamide.

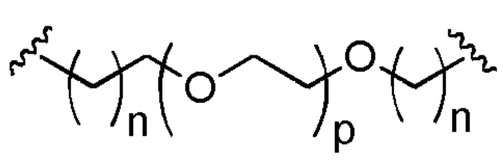
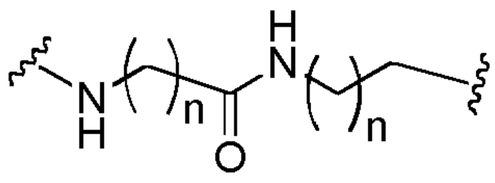
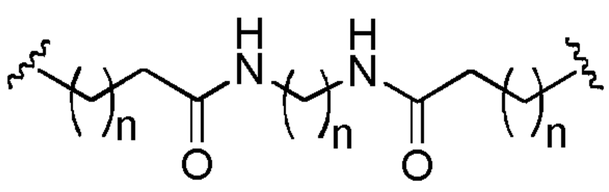
100. The compound of any of claims 48 to 93 or 95, wherein at least one tether comprises an amine.

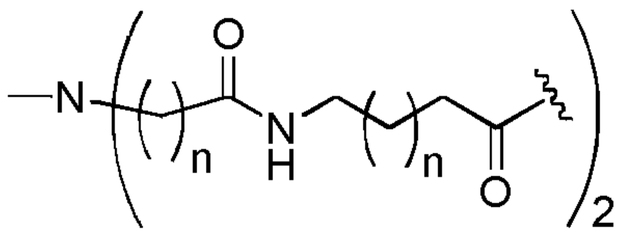
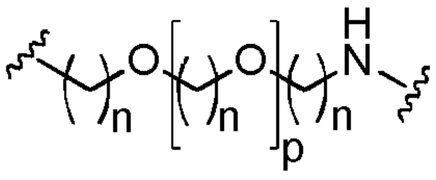
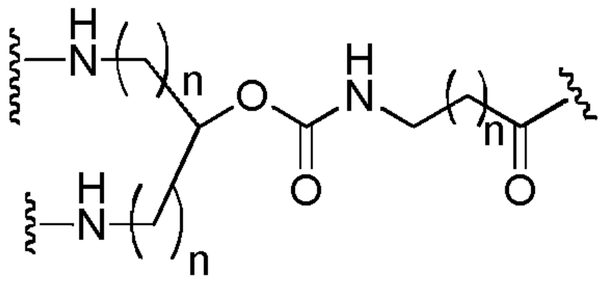
101. The compound of any of claims 48 to 93 or 95, wherein at least two tethers are different from one another.

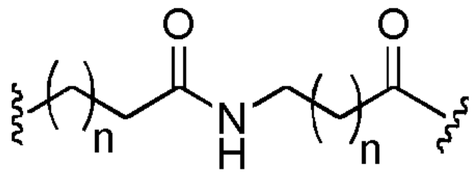
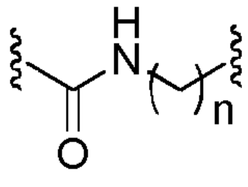
102. The compound of any of claims 48 to 93 or 95, wherein all of the tethers are the same as one another.

103. The compound of any of claims 48 to 93, wherein each tether is selected from among:


; 
; 
; 
;


; 
; 
;

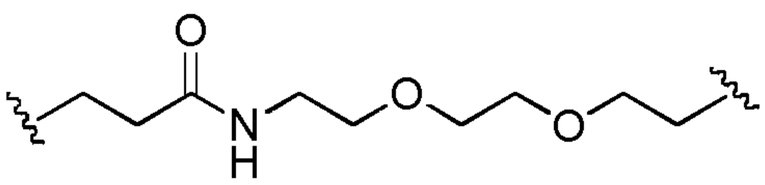
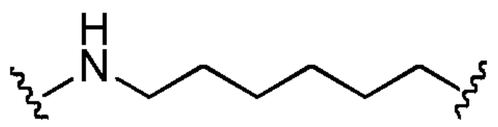
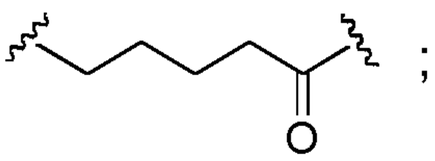

; 
; 
;

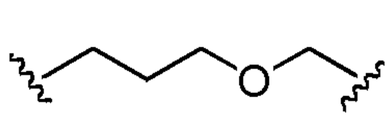
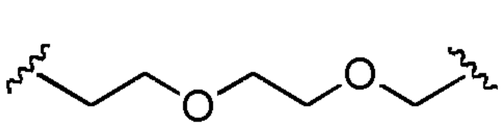
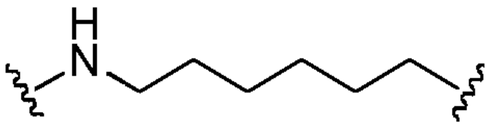
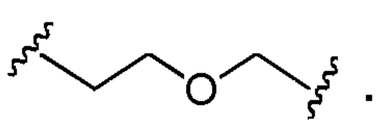

; 
; and 

wherein each n is, independently, from 1 to 20; and

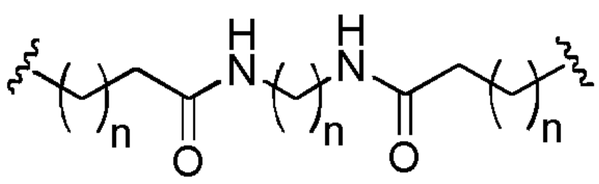
each p is from 1 to about 6.

104. The compound of any of claims 48 to 93, wherein each tether is selected from among:


; 
; 
;

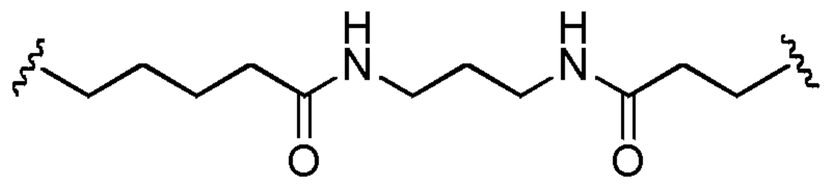

; 
; 
; and 
.

105. The compound of any of claims 48 to 93, wherein each tether has the following structure:

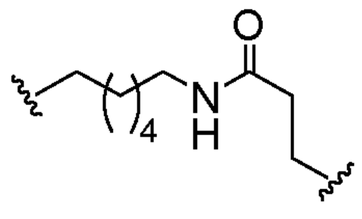
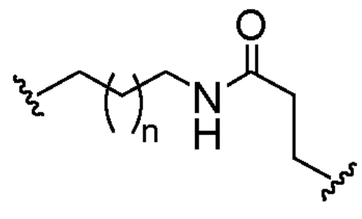


wherein each n is, independently, from 1 to 20.

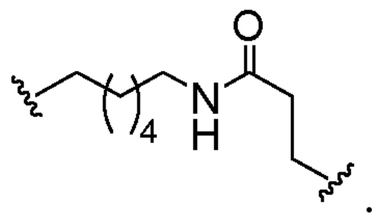
106. The compound of any of claims 48 to 93, wherein each tether has the following structure:



107. The compound of any of claims 48 to 93, wherein the tether has a structure selected from among:


, or

; wherein each n is independently, 0, 1, 2, 3, 4, 5, 6, or 7.

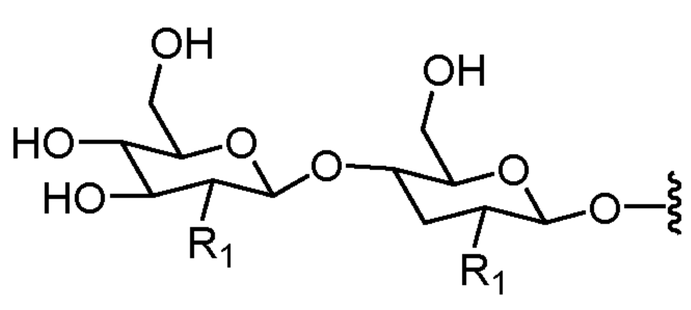
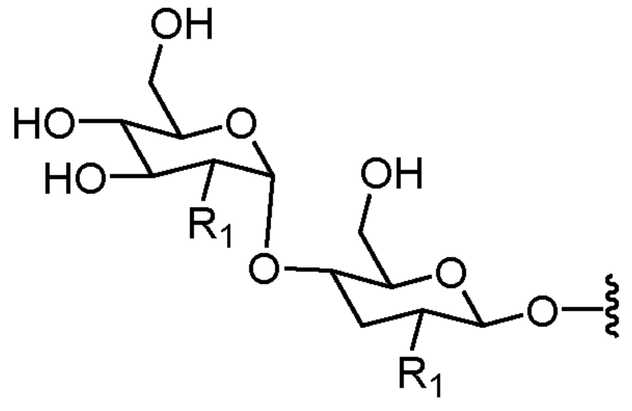
108. The compound of any of claims 48 to 93, wherein the tether has a structure selected from among:



109. The compound of any of claims 105 to 108, wherein the ligand is galactose.

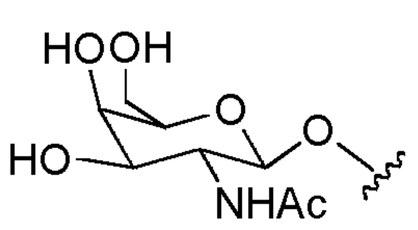
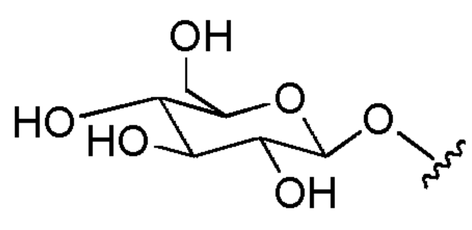
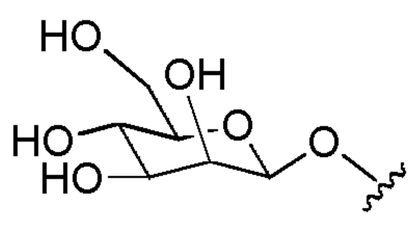
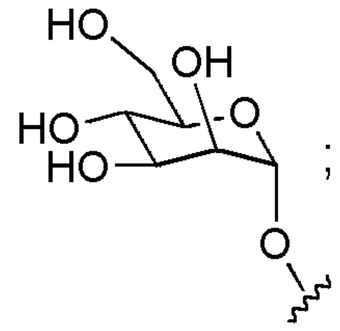
110. The compound of any of claims 105 to 108, wherein the ligand is mannose-6-phosphate.

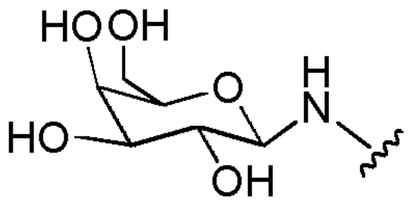
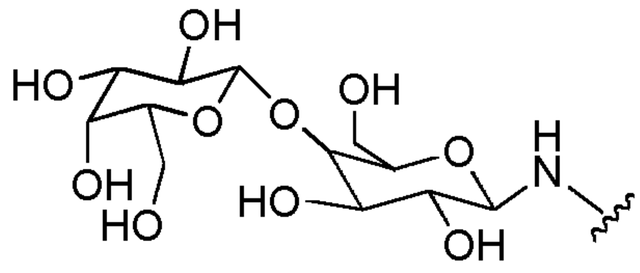
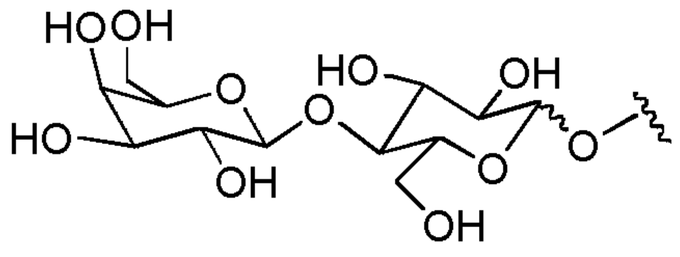
111. The compound of any of claims 105 to 108, wherein each ligand is selected from among:

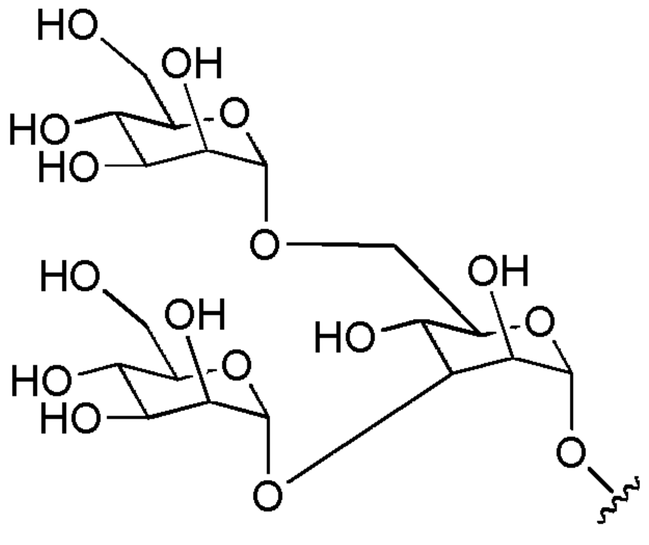

and


wherein each R_1 is selected from OH and NHCOOH.

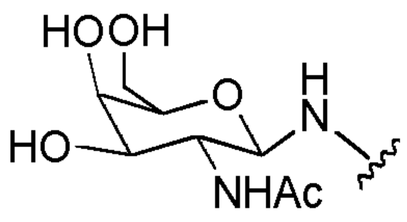
112. The compound of any of claims 105 to 108, wherein each ligand is selected from among:


; 
; 
; 
;

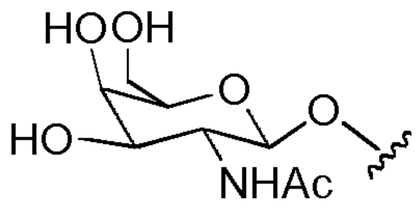

; 
; 
; and


.

113. The compound of any of claims 105 to 108, wherein each ligand has the following structure:

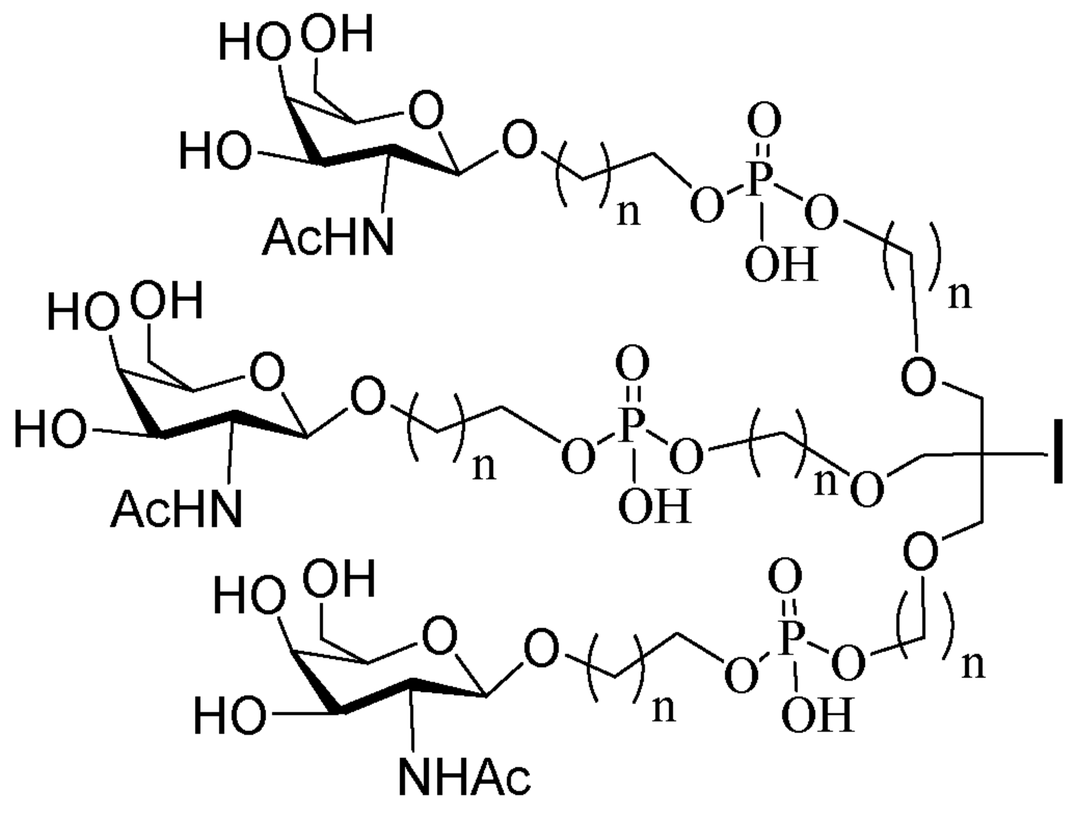

.

114. The conjugated antisense compound of any of claims 105 to 108, wherein each ligand has the following structure:


.

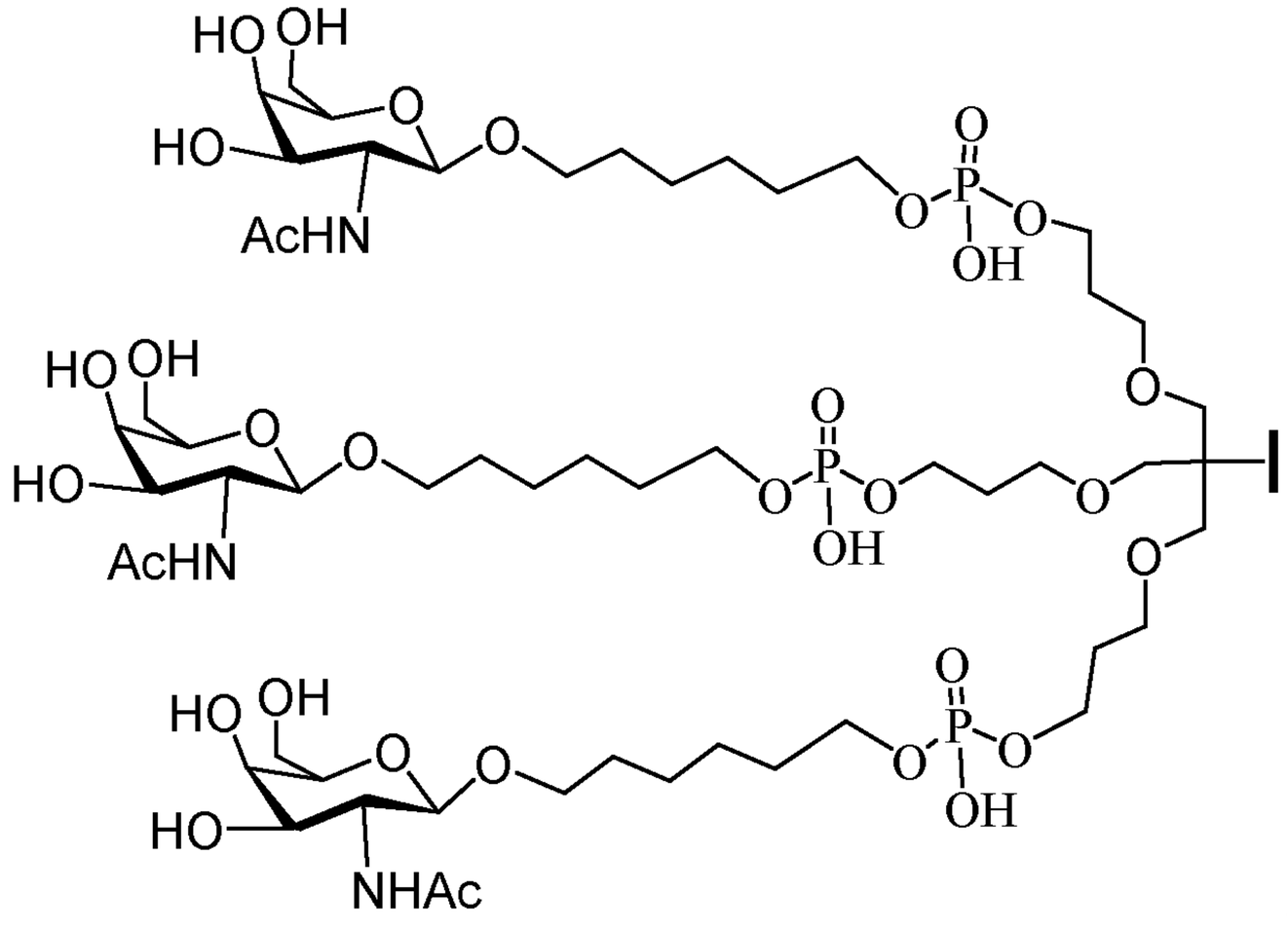
115. The compound of any of claims 1 to 30 or 56 to 81, wherein the conjugate group comprises a cell-targeting moiety.

116. The compound of claim 116, wherein the conjugate group comprises a cell-targeting moiety having the following structure:



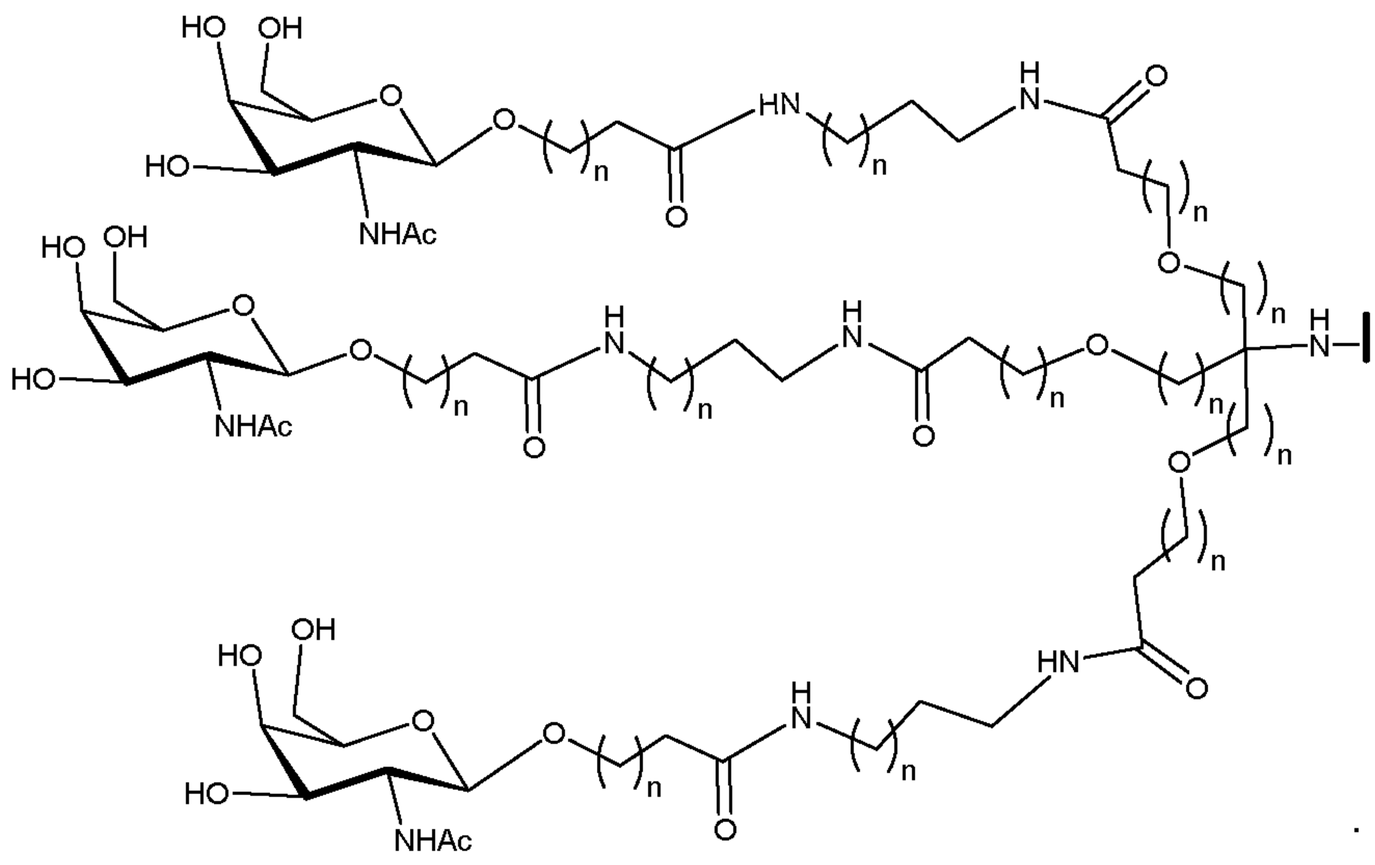
wherein each n is, independently, from 1 to 20.

117. The compound of any of claims 116, wherein the cell-targeting moiety has the following structure:



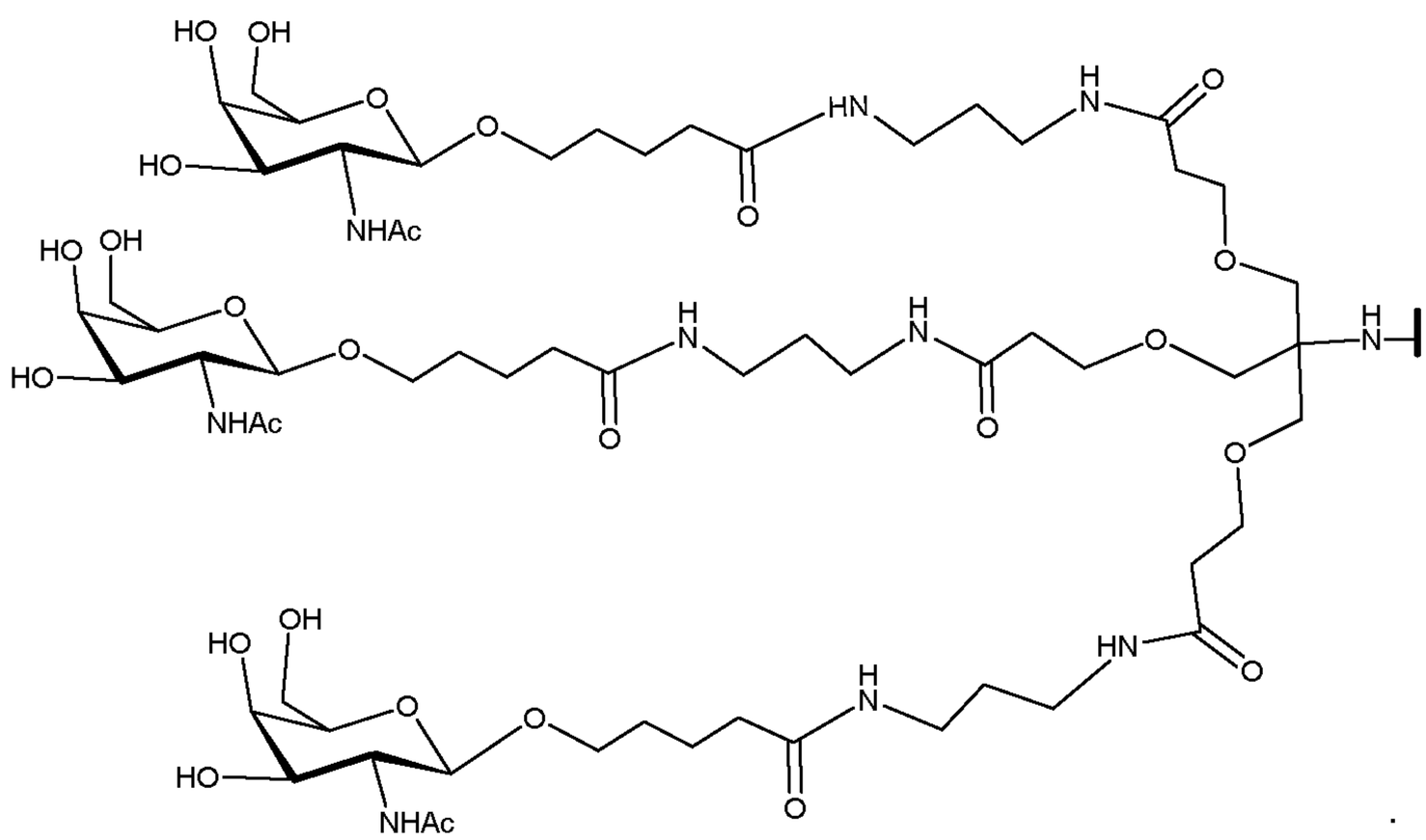
.

118. The compound of claim 116, wherein the cell-targeting moiety has the following structure:

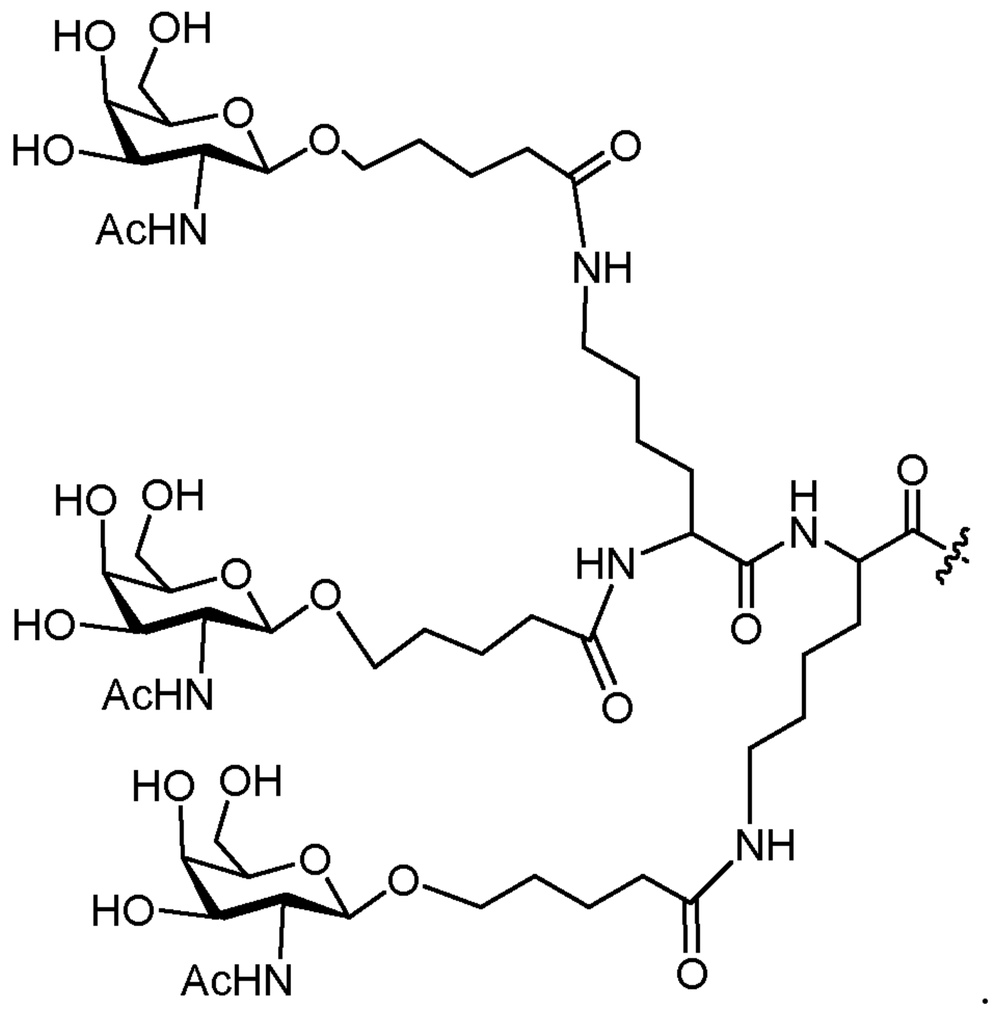


wherein each n is, independently, from 1 to 20.

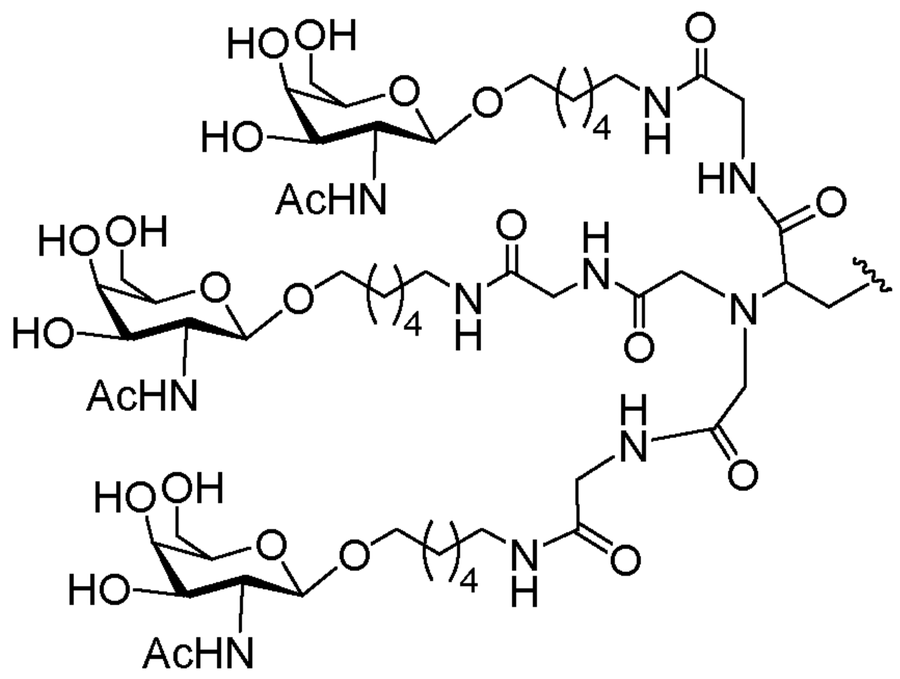
119. The compound of claim 116, wherein the cell-targeting moiety has the following structure:



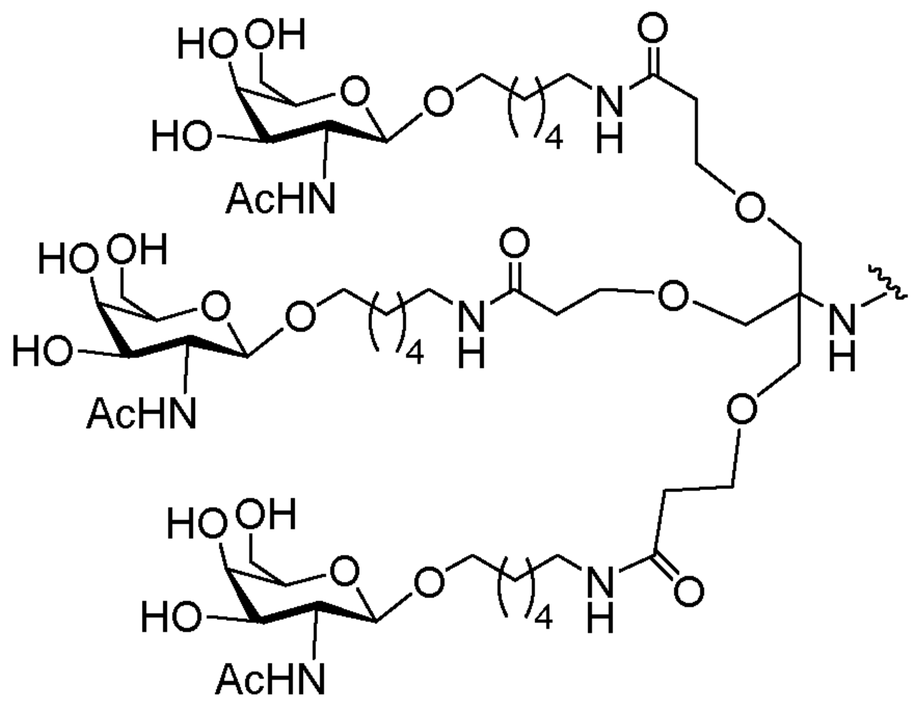
120. The compound of claim 116, wherein the cell-targeting moiety comprises:



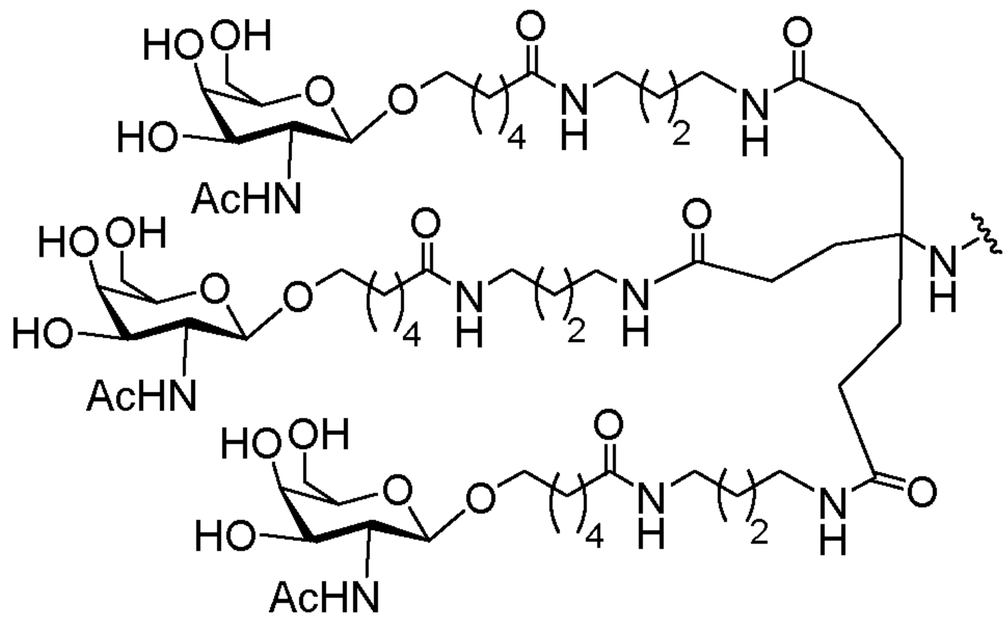
121. The compound of claim 116, wherein the cell-targeting moiety comprises:



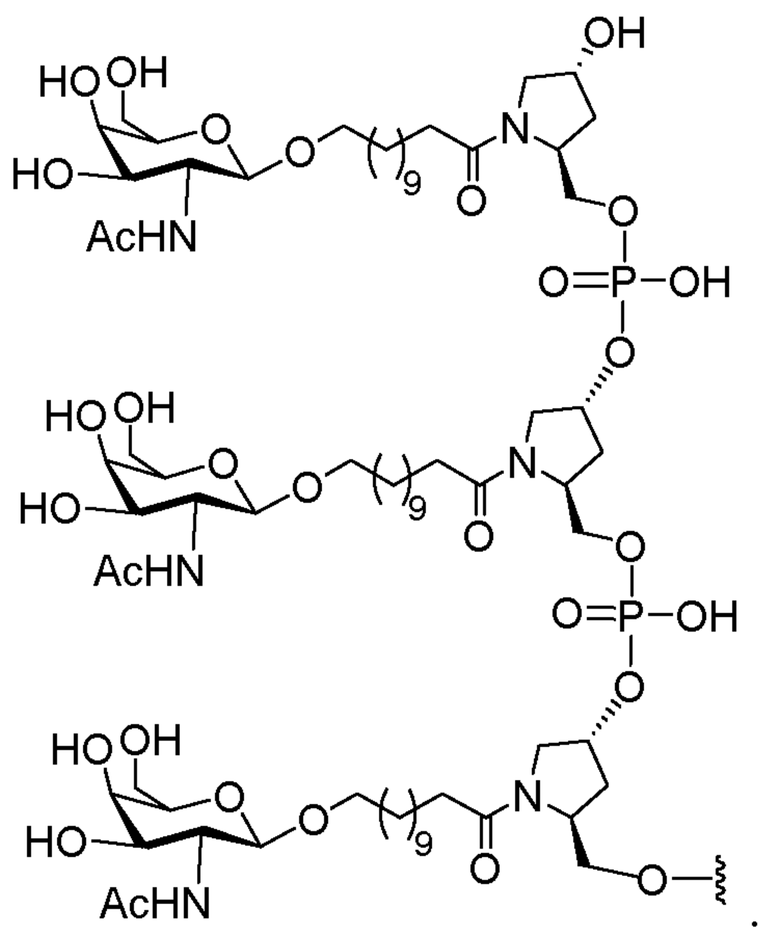
122. The compound of claim 116, wherein the cell-targeting moiety has the following structure:



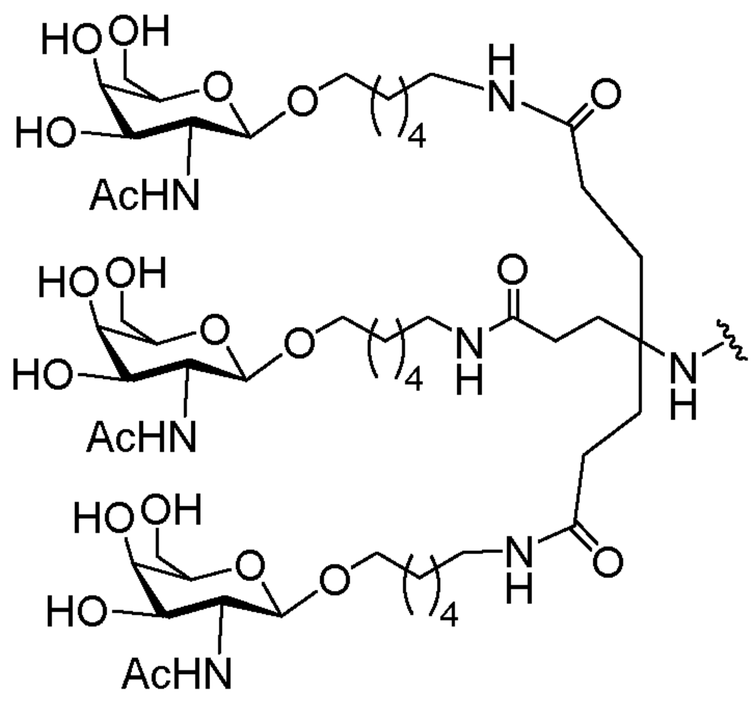
123. The compound of claim 116, wherein the cell-targeting moiety has the following structure:



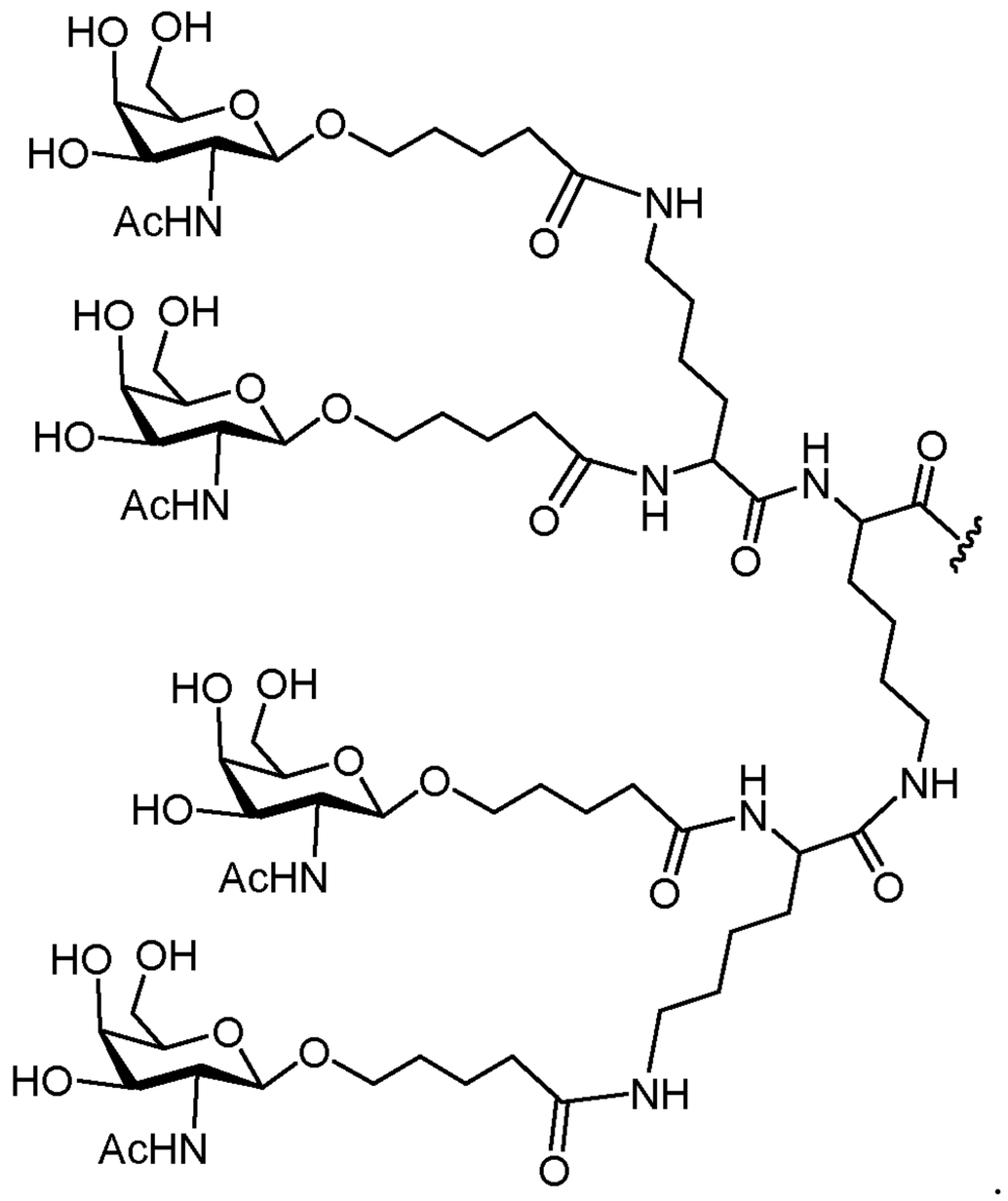
124. The compound of claim 116, wherein the cell-targeting moiety comprises:



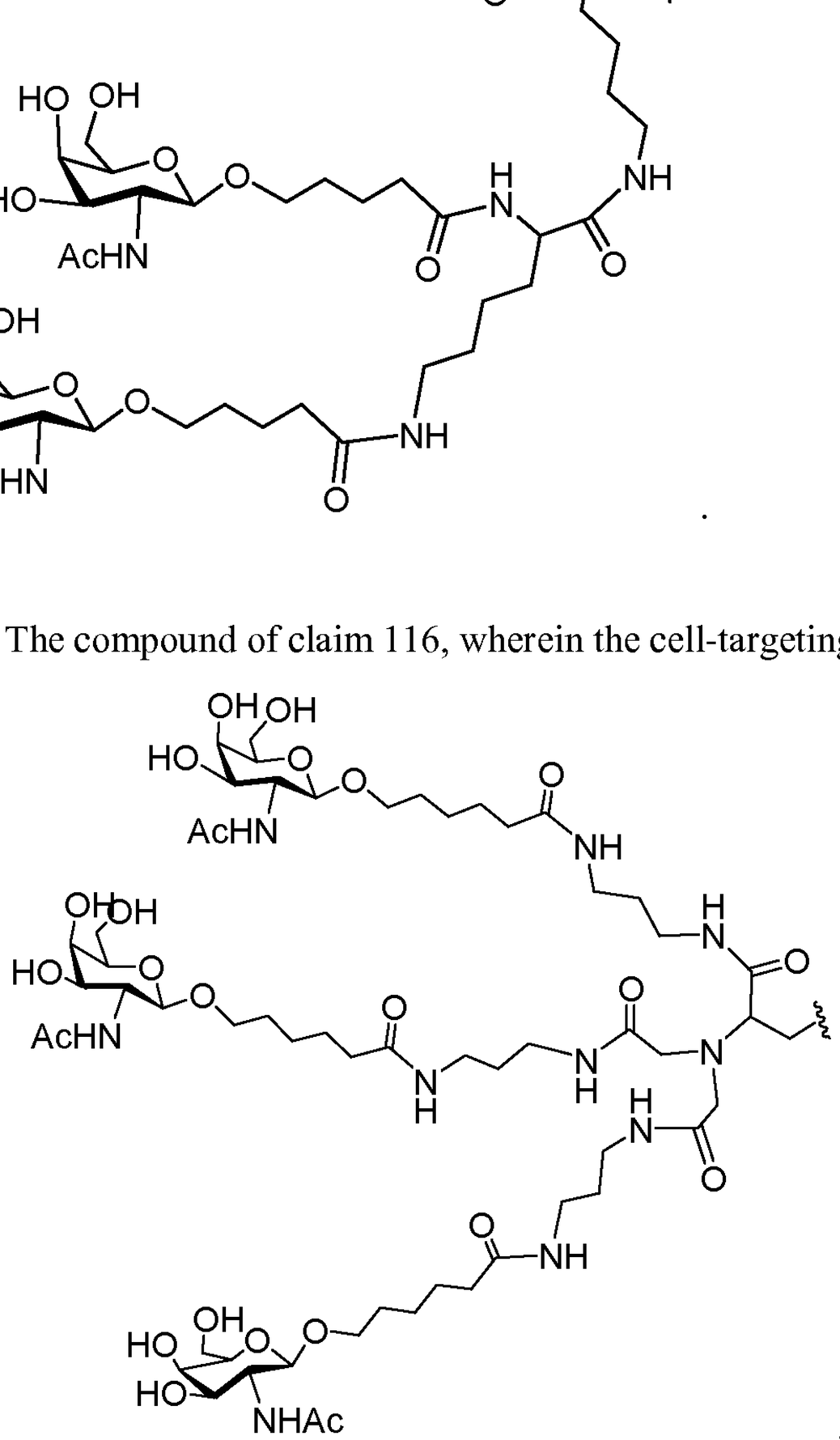
125. The compound of claim 116, wherein the cell-targeting moiety has the following structure:



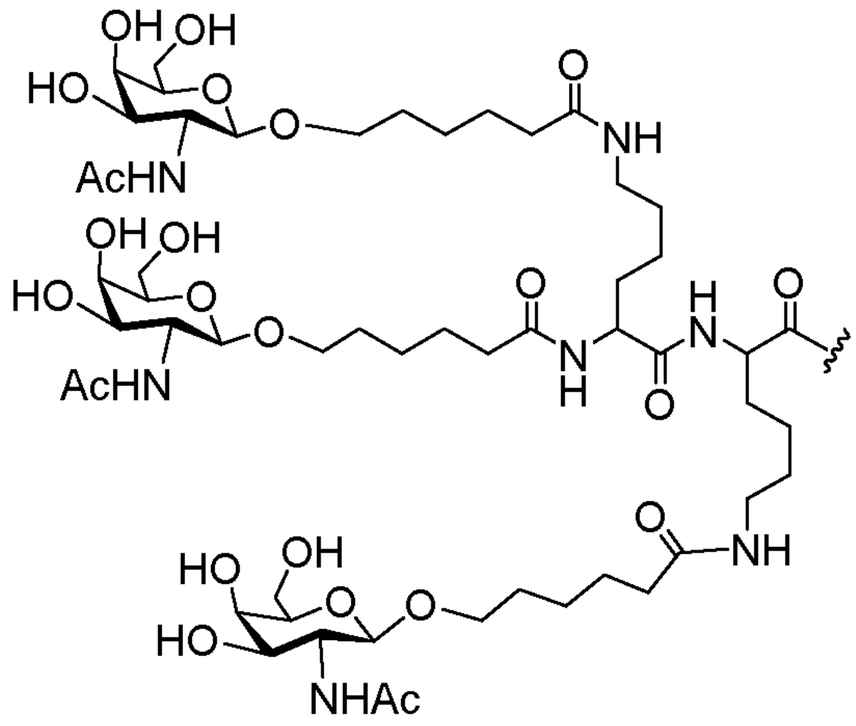
126. The compound of claim 116, wherein the cell-targeting moiety comprises:



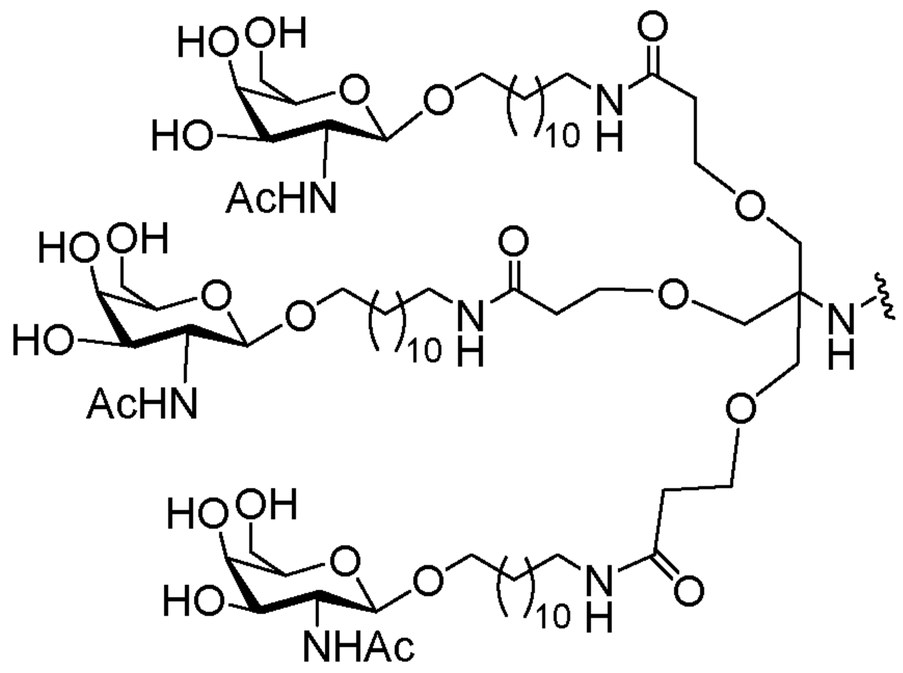
127. The compound of claim 116, wherein the cell-targeting moiety comprises:



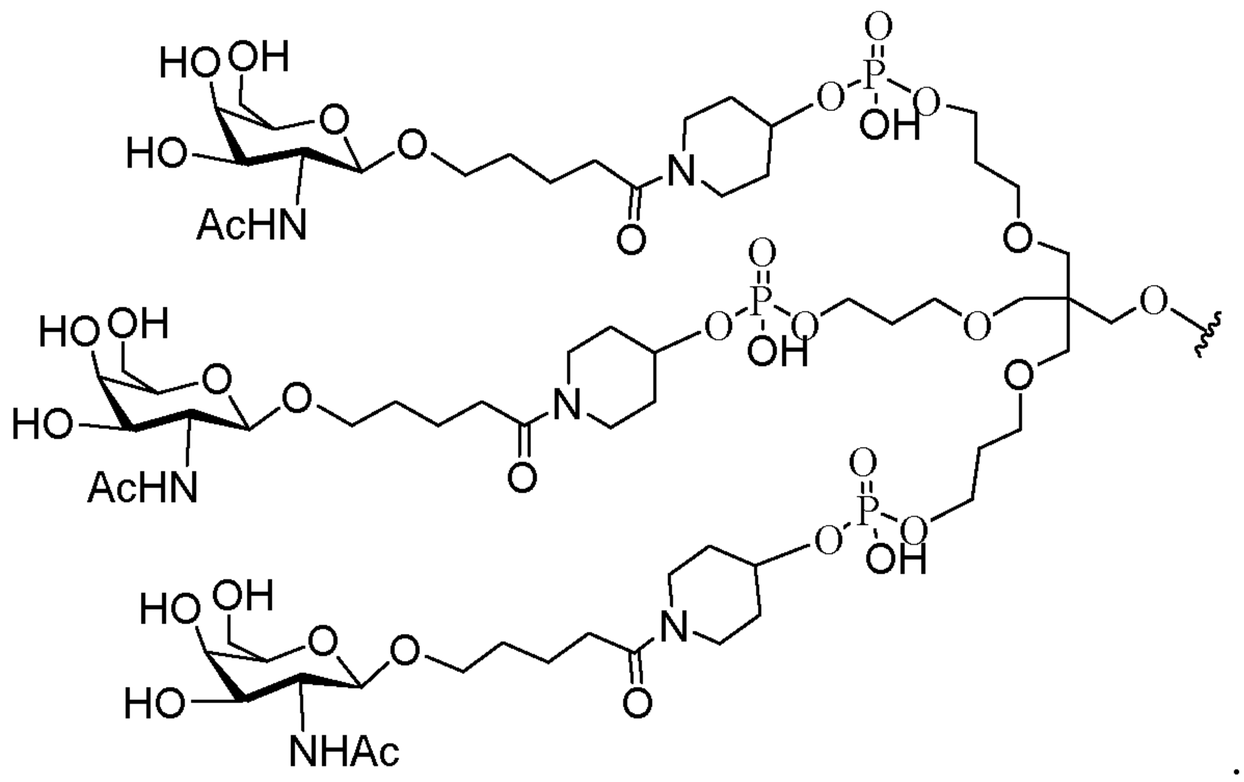
128. The compound of claim 116, wherein the cell-targeting moiety comprises:



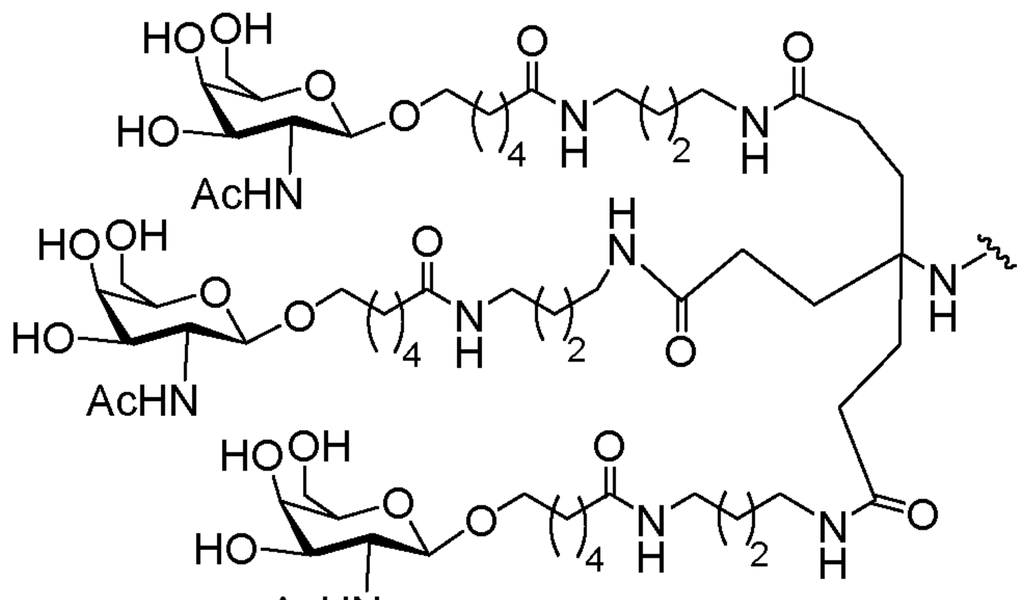
129. The compound of claim 116, wherein the cell-targeting moiety has the following structure:



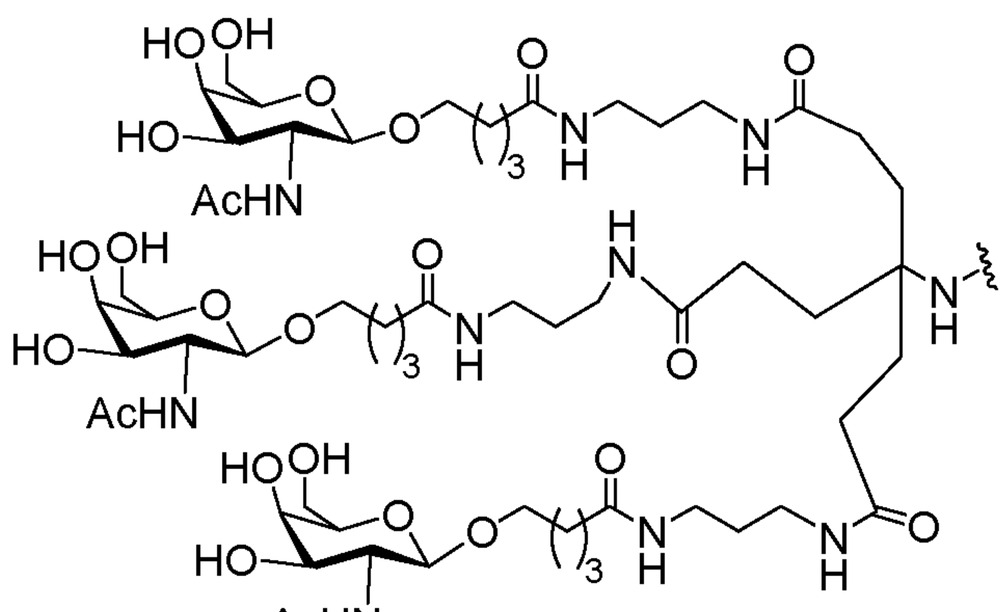
130. The compound of claim 116, wherein the cell-targeting moiety has the following structure:



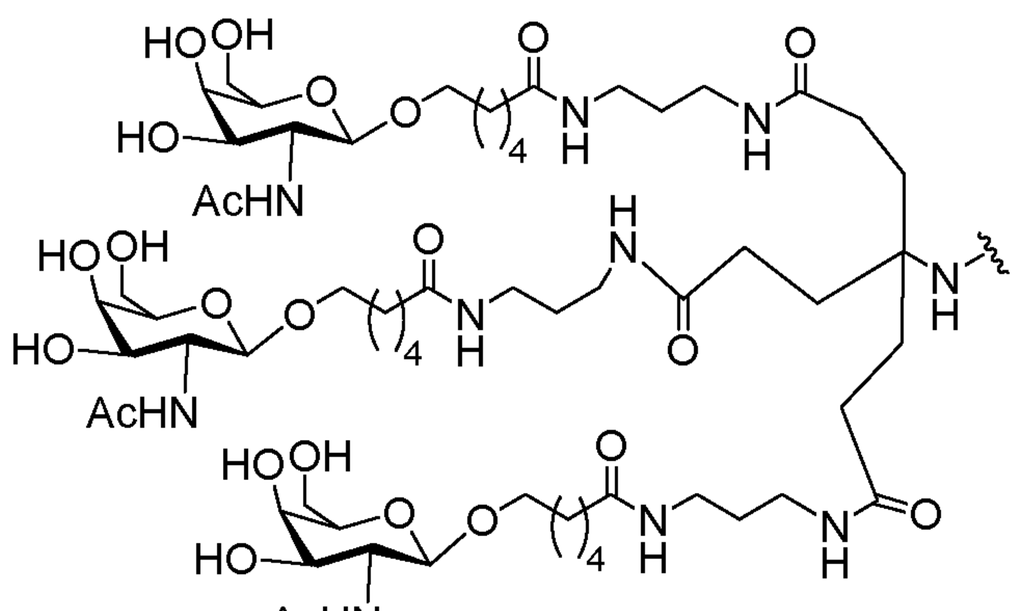
131. The compound of claim 116, wherein the cell-targeting moiety has the following structure:



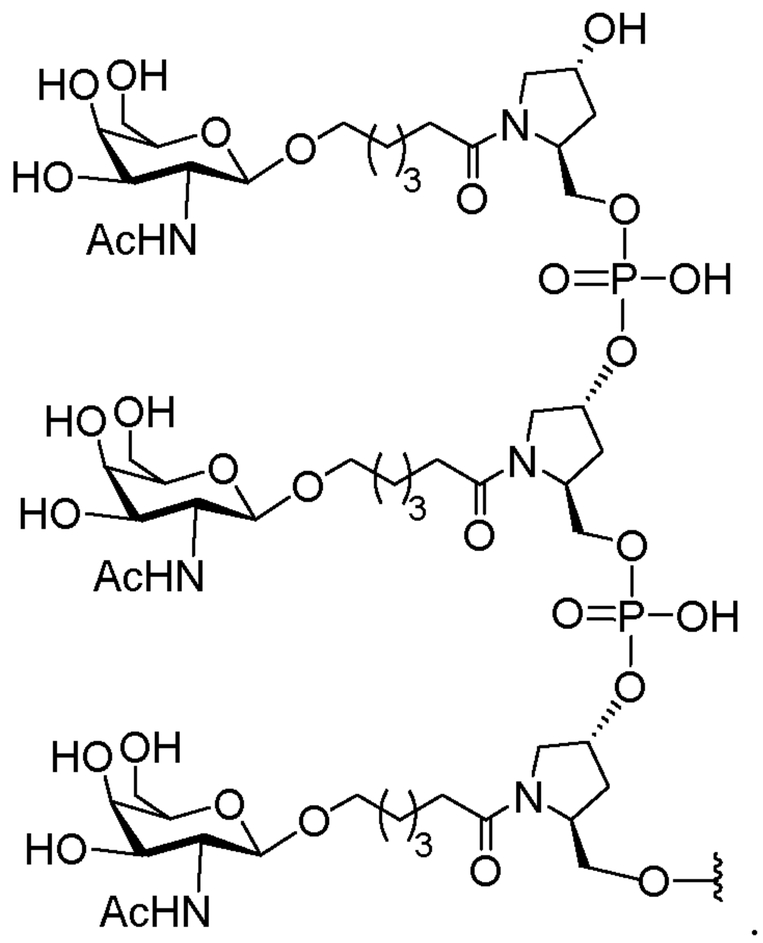
132. The compound of claim 116, wherein the cell-targeting moiety has the following structure:



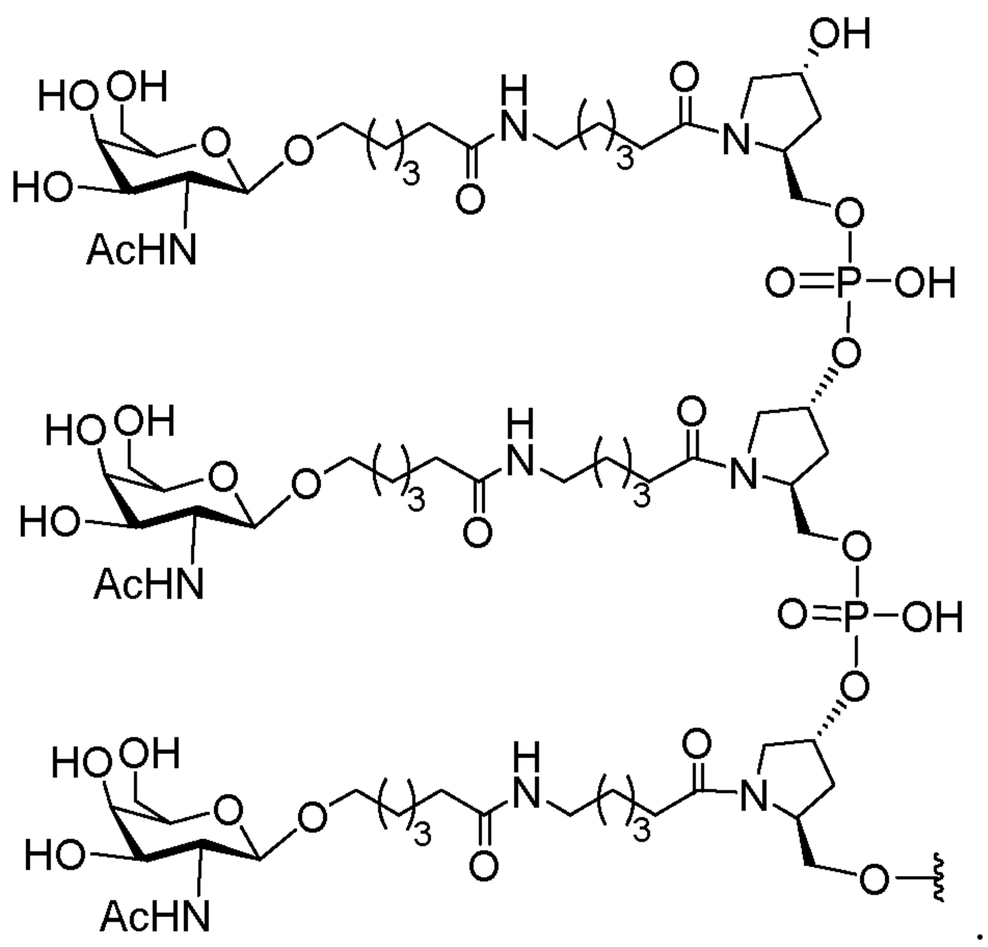
133. The compound of claim 116, wherein the cell-targeting moiety has the following structure:



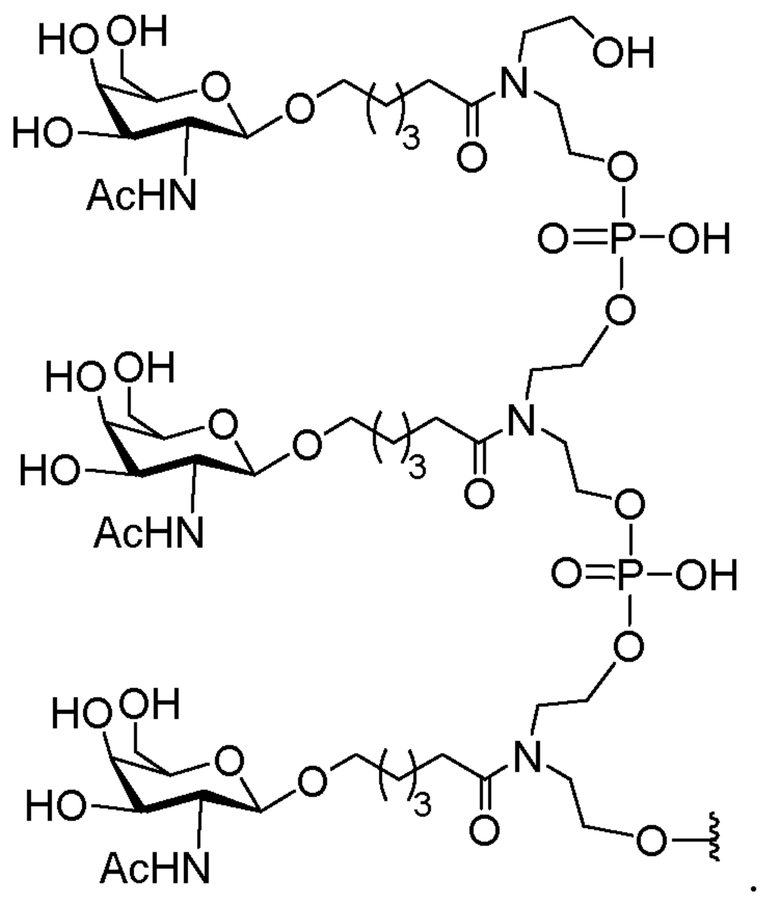
134. The compound of claim 116, wherein the cell-targeting moiety comprises:



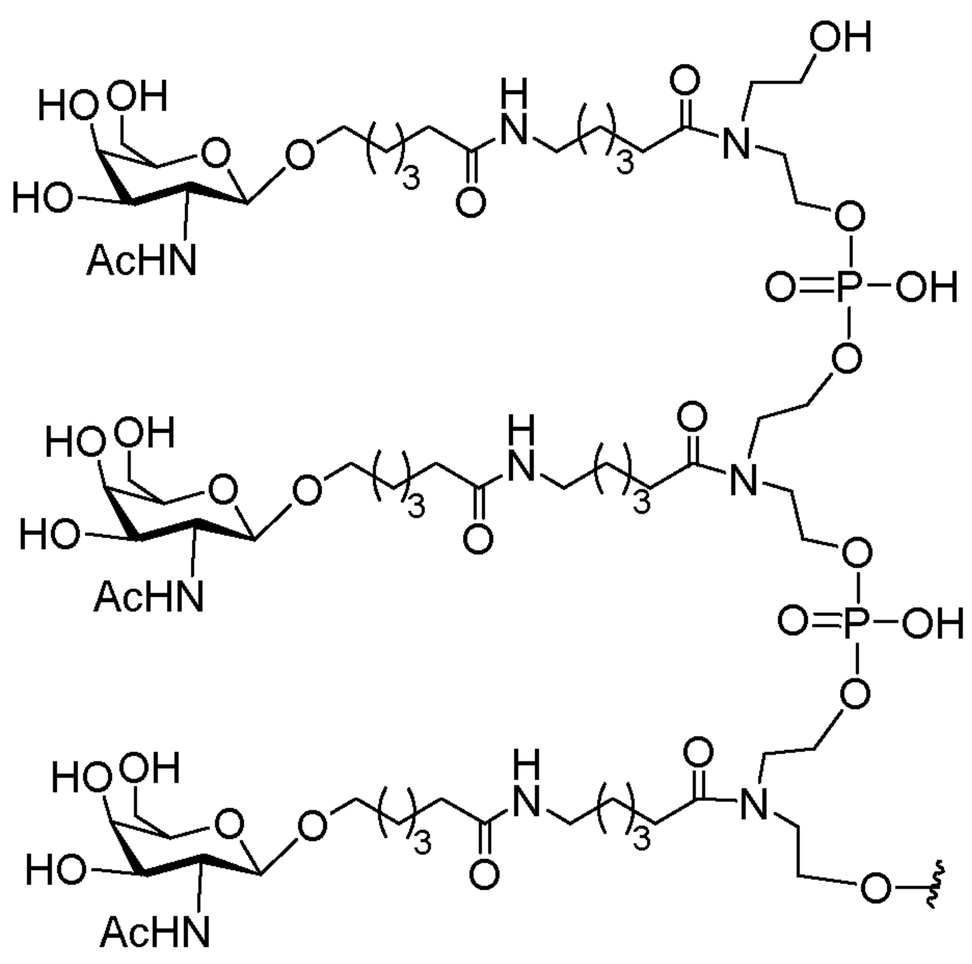
135. The compound of claim 116, wherein the cell-targeting moiety comprises:



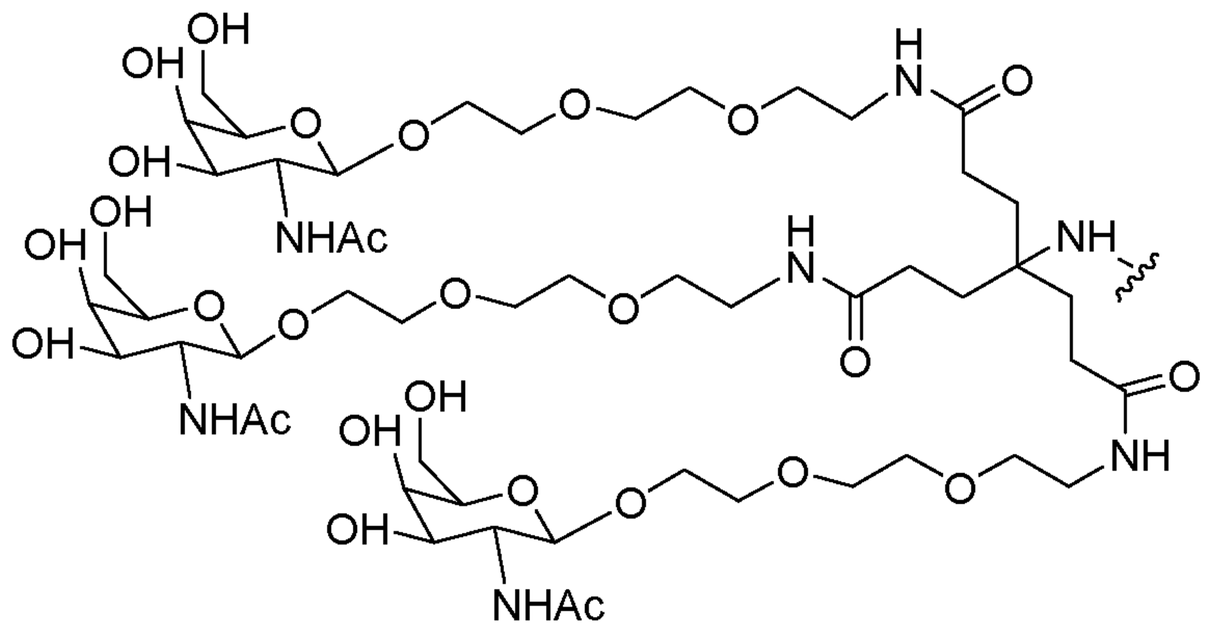
136. The compound of claim 116, wherein the cell-targeting moiety comprises:



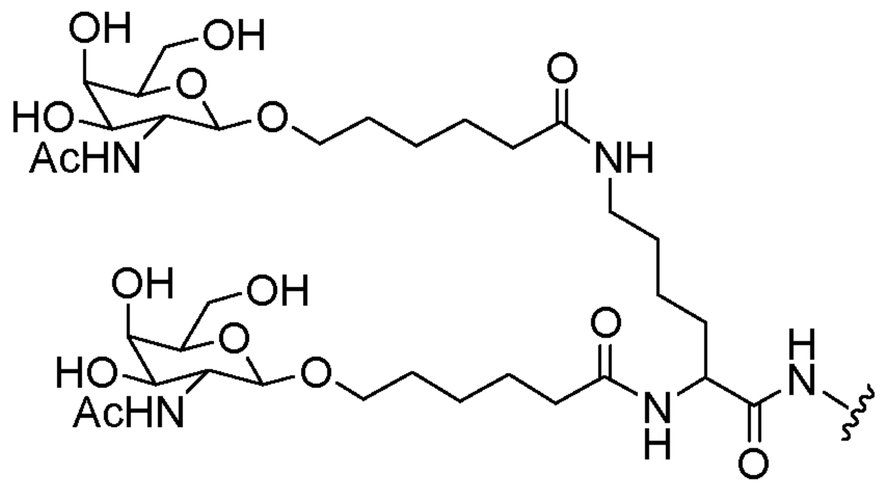
137. The compound of claim 116, wherein the cell-targeting moiety comprises:



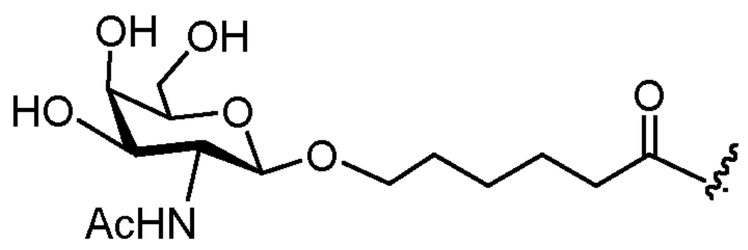
138. The compound of claim 116, wherein the cell-targeting moiety has the following structure:



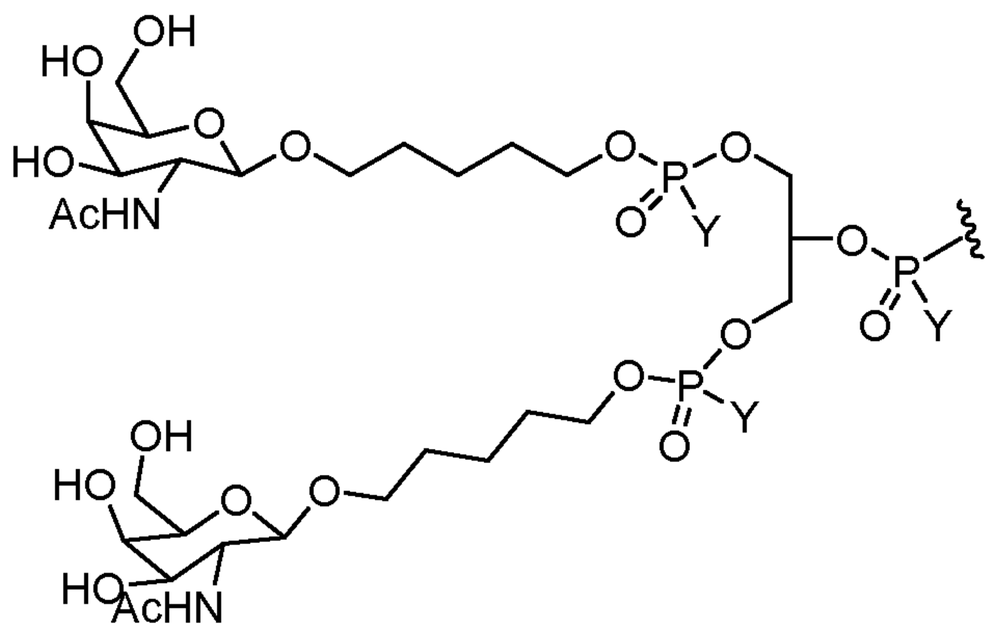
139. The compound of claim 116, wherein the cell-targeting moiety comprises:



140. The compound of claim 116, wherein the cell-targeting moiety has the following structure:



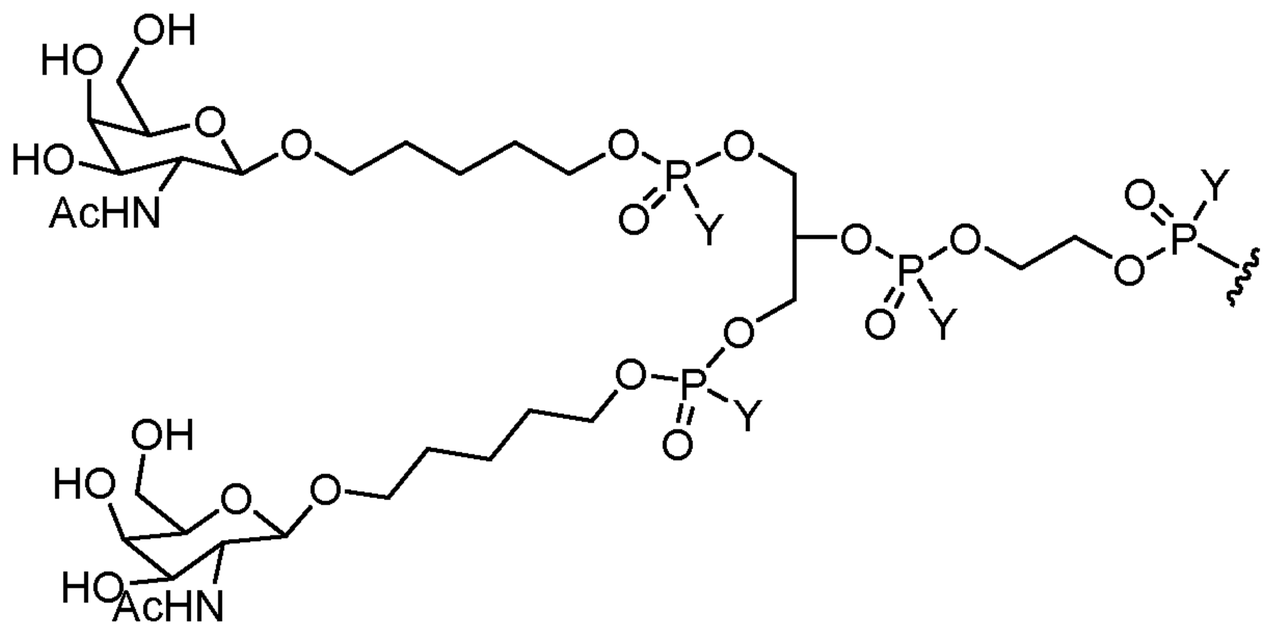
141. The compound of claim 116, wherein the cell-targeting moiety comprises:



;

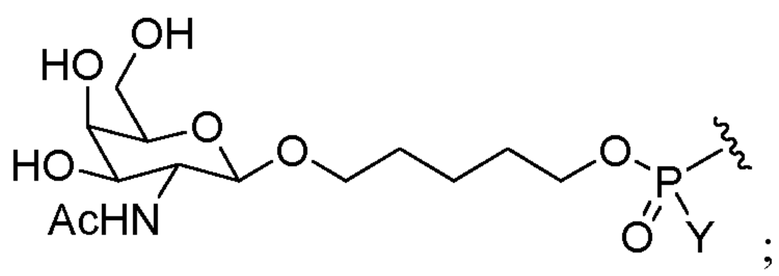
wherein each Y is selected from O, S, a substituted or unsubstituted C_1-C_{10} alkyl, amino, substituted amino, azido, alkenyl or alkynyl.

142. The compound of claim 116, wherein the conjugate group comprises:



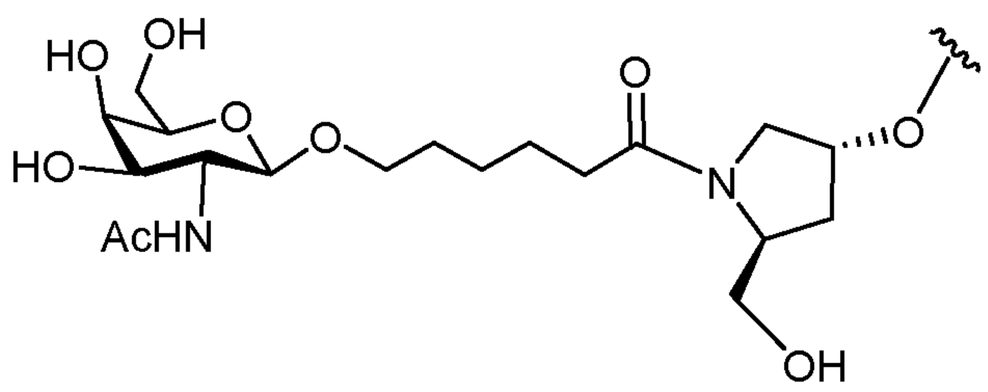
wherein each Y is selected from O, S, a substituted or unsubstituted C_1-C_{10} alkyl, amino, substituted amino, azido, alkenyl or alkynyl.

143. The compound of claim 116, wherein the cell-targeting moiety has the following structure:

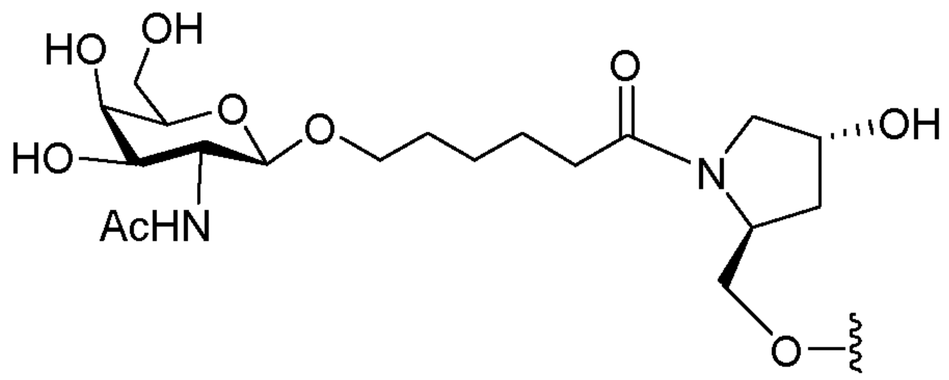


wherein each Y is selected from O, S, a substituted or unsubstituted C_1-C_{10} alkyl, amino, substituted amino, azido, alkenyl or alkynyl.

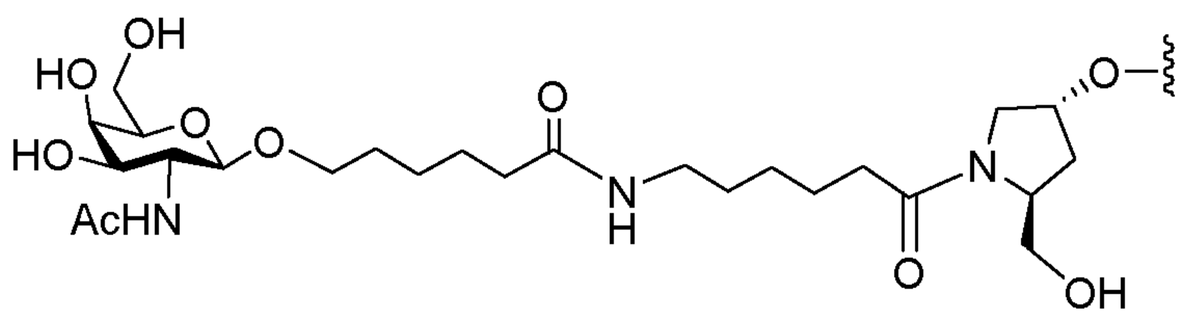
144. The compound of any of claims 1 to 30, wherein the conjugate group comprises:



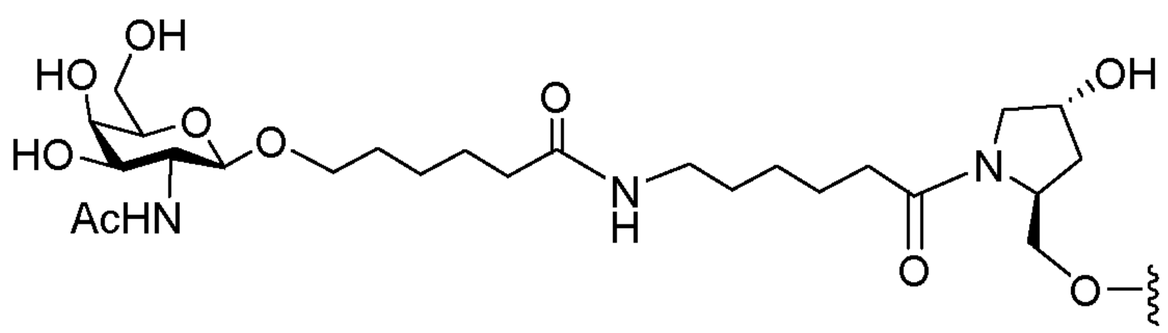
145. The compound of any of claims 1 to 30, wherein the conjugate group comprises:



146. The compound of any of claims 1 to 30, wherein the conjugate group comprises:



147. The compound of claim 117, wherein the conjugate group comprises:



148. The compound of any of claims 1 to 147, wherein the conjugate group comprises a cleavable moiety selected from among: a phosphodiester, an amide, or an ester.

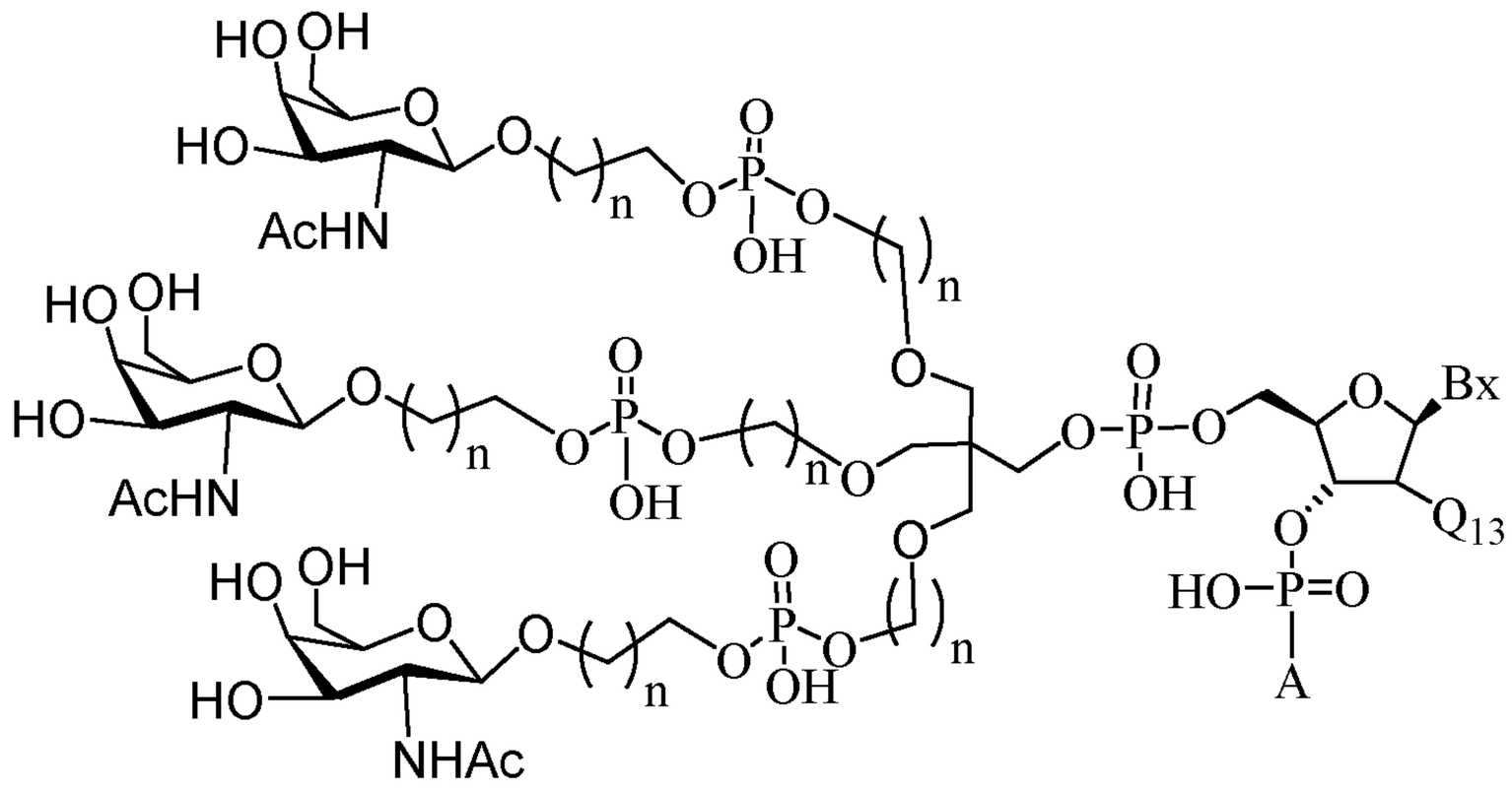
149. The compound of any of claims 1 to 147, wherein the conjugate group comprises a phosphodiester cleavable moiety.

150. The compound of any of claims 1 to 147, wherein the conjugate group does not comprise a cleavable moiety, and wherein the conjugate group comprises a phosphorothioate linkage between the conjugate group and the oligonucleotide.

151. The compound of any of claims 1 to 148, wherein the conjugate group comprises an amide cleavable moiety.

152. The compound of any of claims 1 to 148, wherein the conjugate group comprises an ester cleavable moiety.

153. The compound of any of claims 1 to 30, wherein the compound has the following structure:



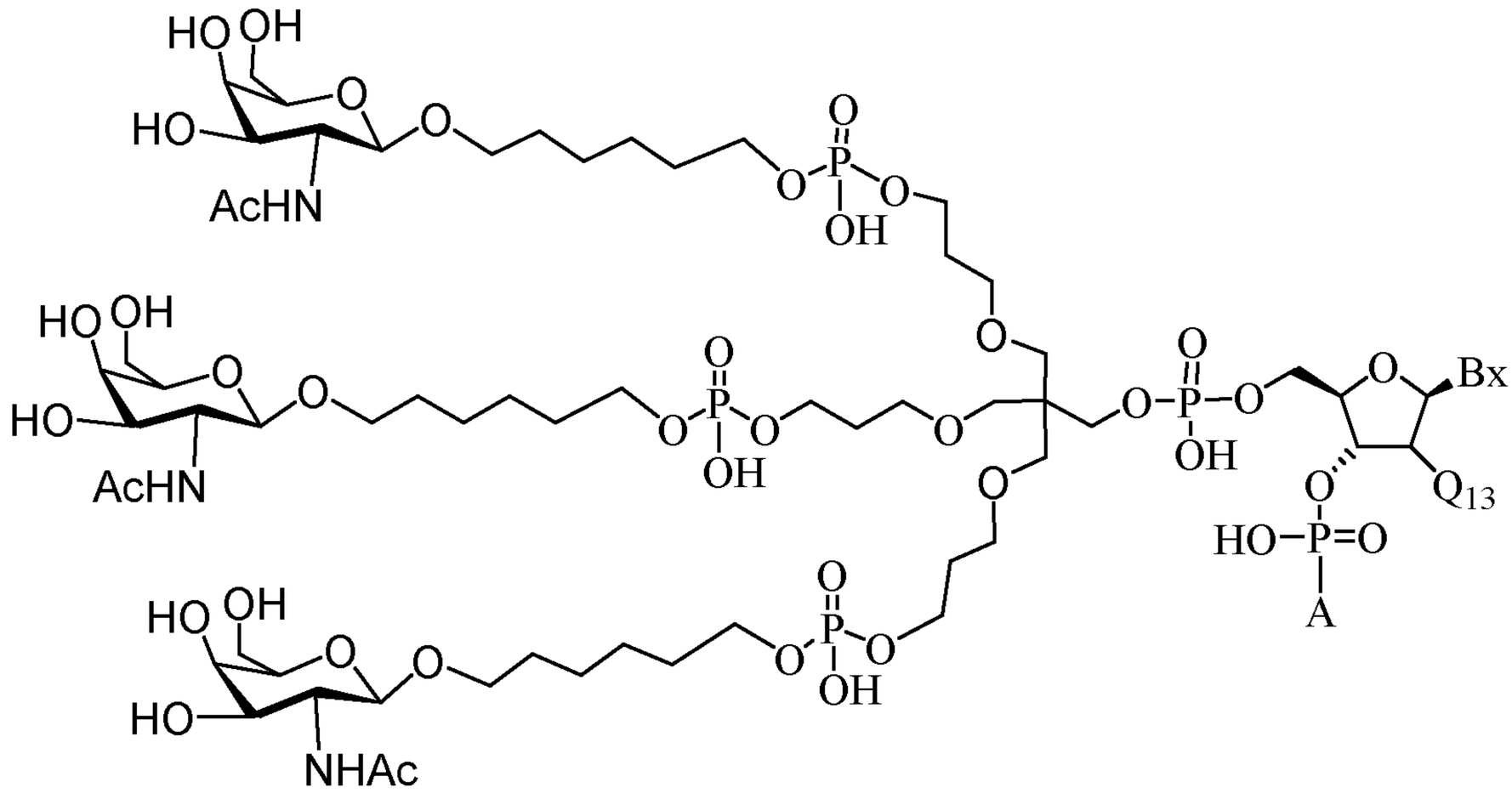
wherein each n is, independently, from 1 to 20;

Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

154. The compound of any of claims 1 to 30, wherein the compound has the following structure:



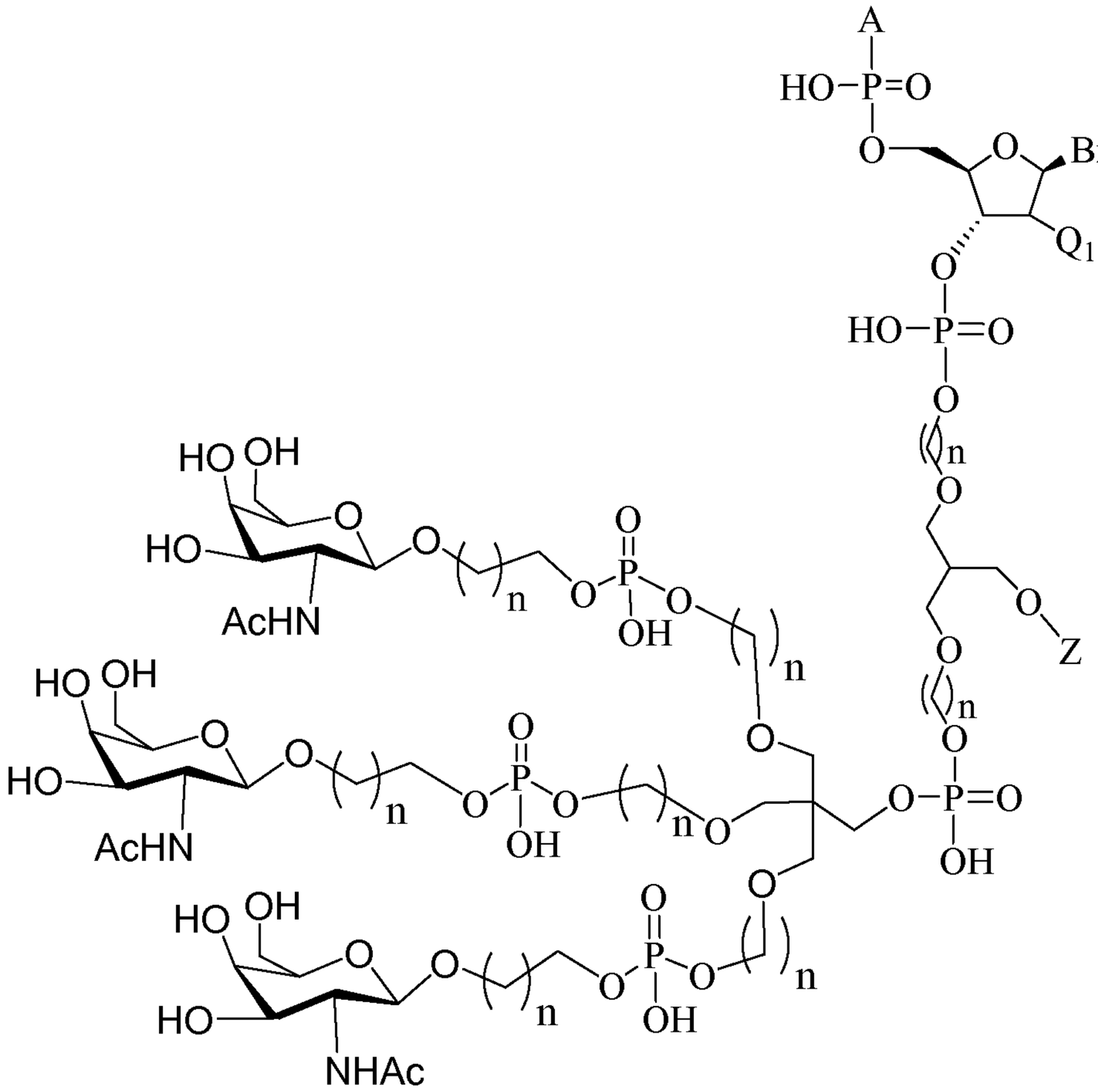
wherein each n is, independently, from 1 to 20;

Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

155. The compound of any of claims 1 to 30, wherein the compound has the following structure:



wherein each n is, independently, from 1 to 20;

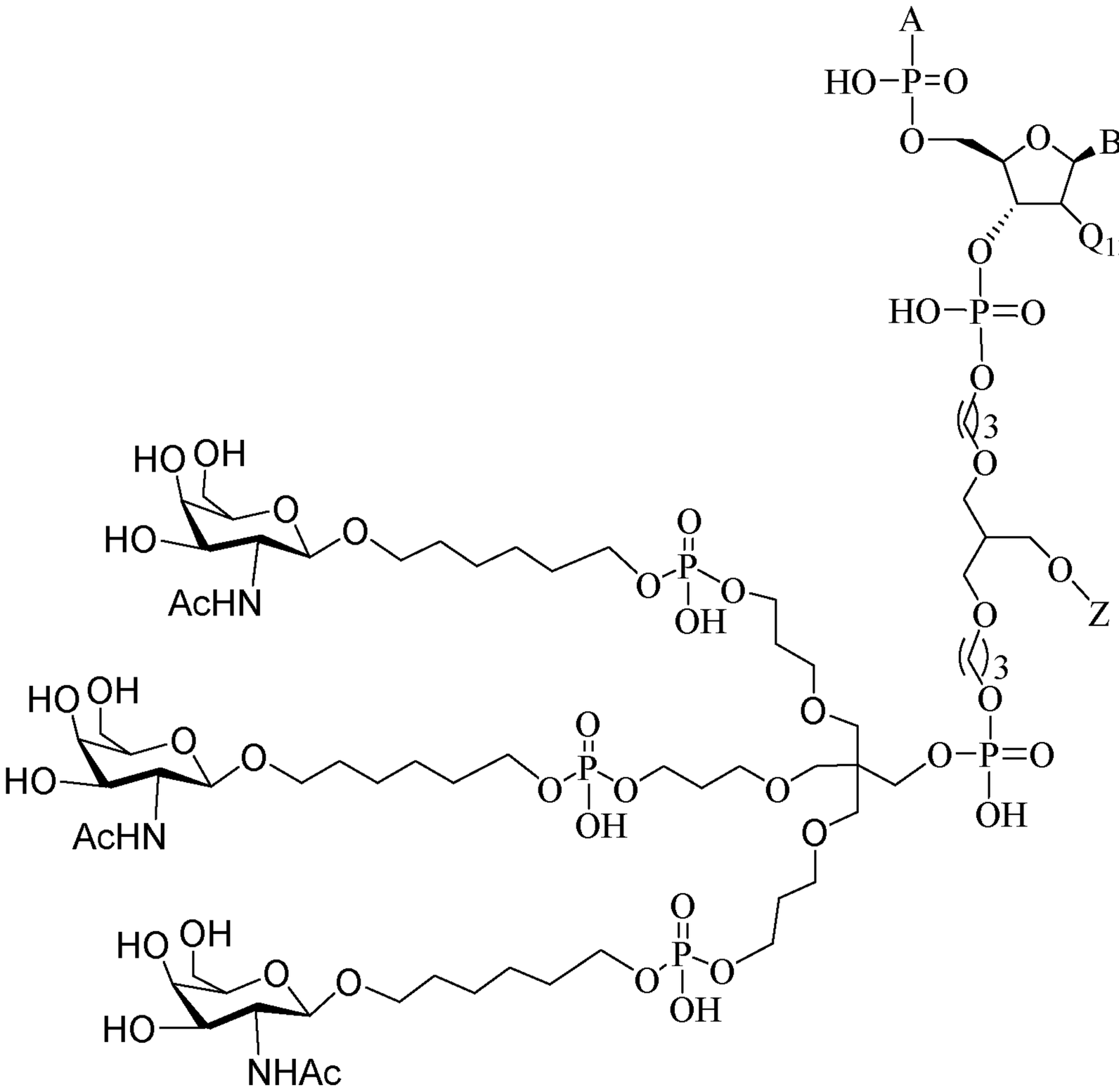
Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide;

Z is H or a linked solid support; and

Bx is a heterocyclic base moiety.

156. The compound of any of claims 1 to 30, wherein the compound has the following structure:



wherein each n is, independently, from 1 to 20;

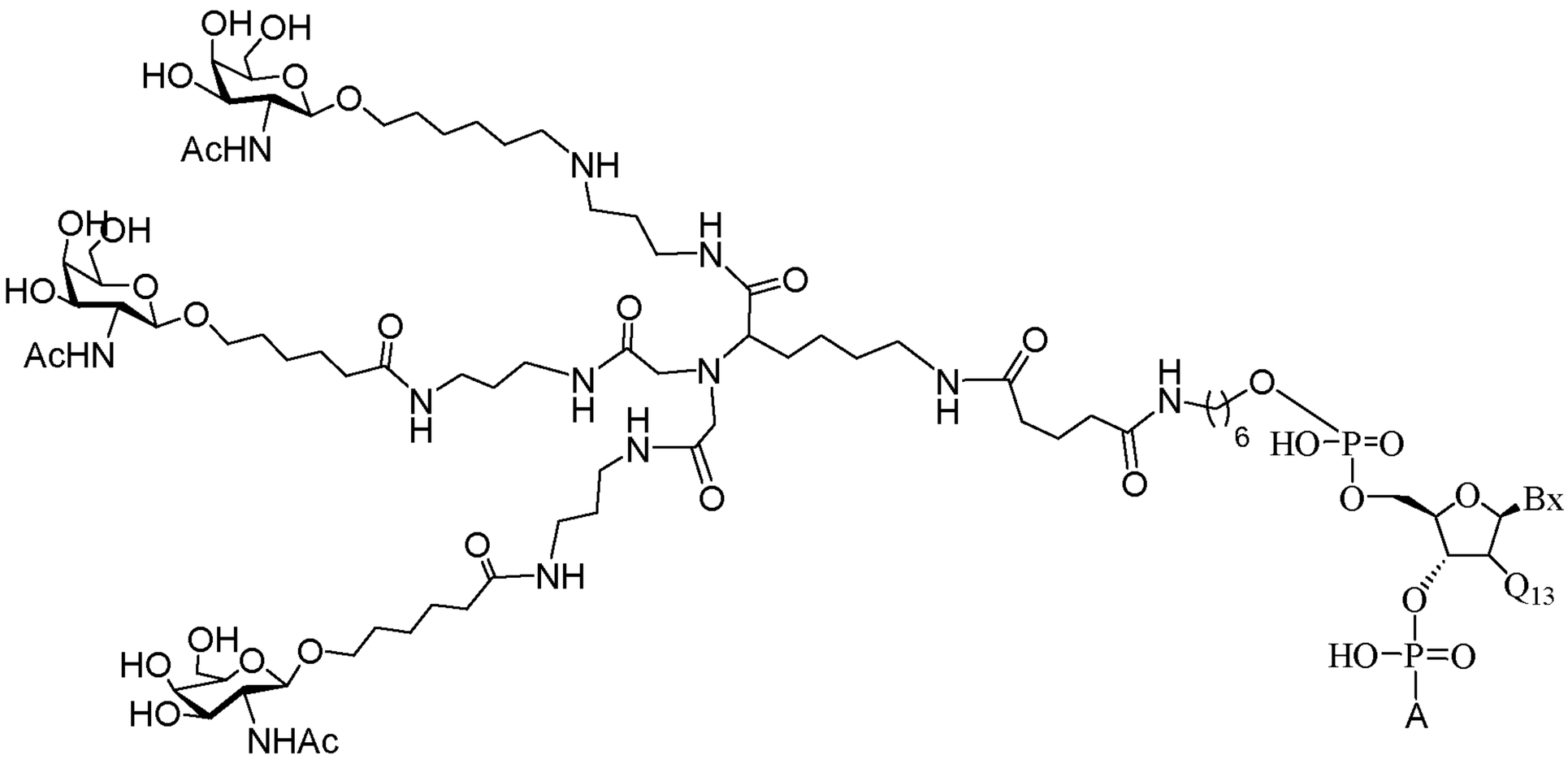
Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide;

Z is H or a linked solid support; and

Bx is a heterocyclic base moiety.

157. The compound of any of claims 1 to 30, wherein the compound has the following structure:

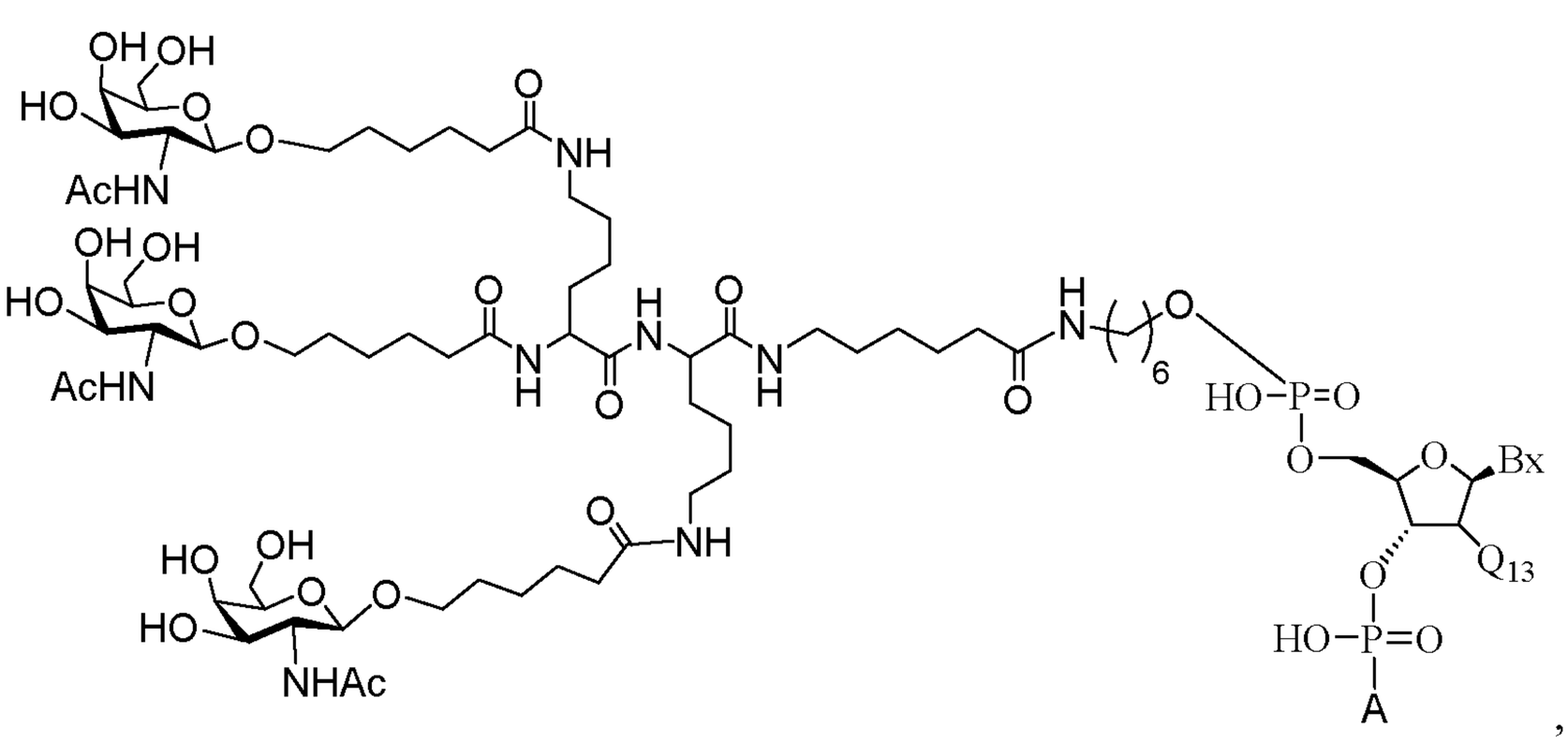


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide;and

Bx is a heterocyclic base moiety.

158. The compound of any of claims 1 to 30, wherein the compound has the following structure:

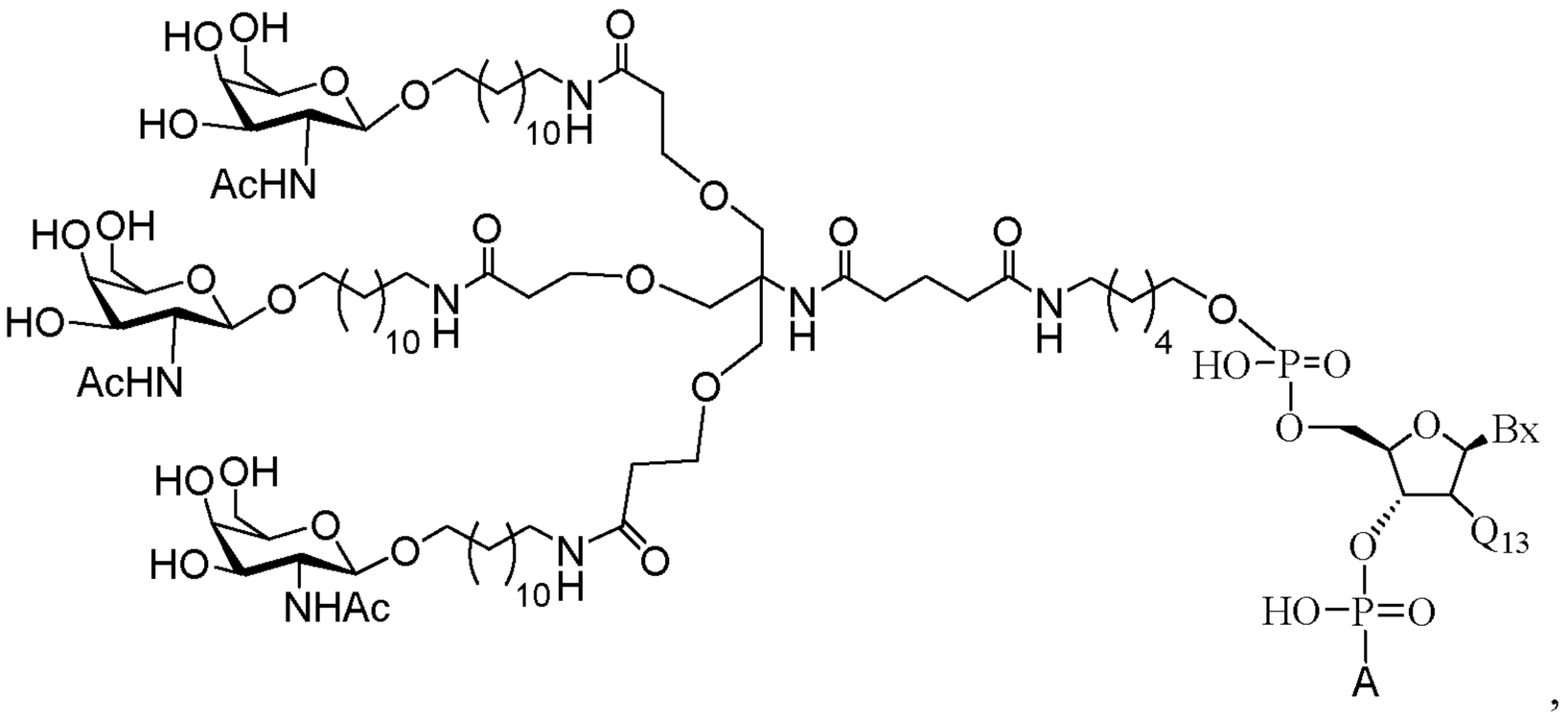


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide;and

Bx is a heterocyclic base moiety.

159. The compound of any of claims 1 to 30, wherein the compound has the following structure:

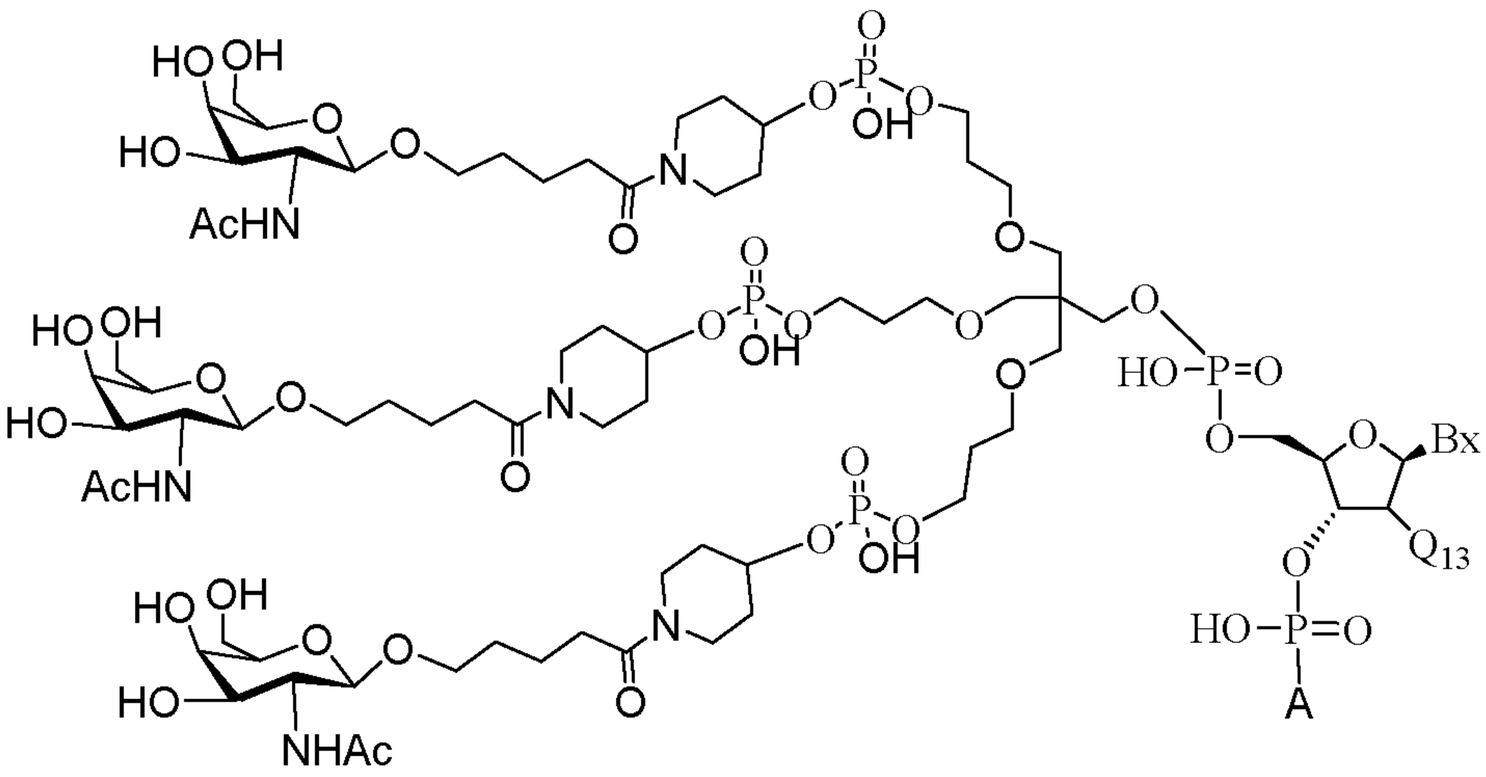


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

160. The compound of any of claims 1 to 30, wherein the compound has the following structure:

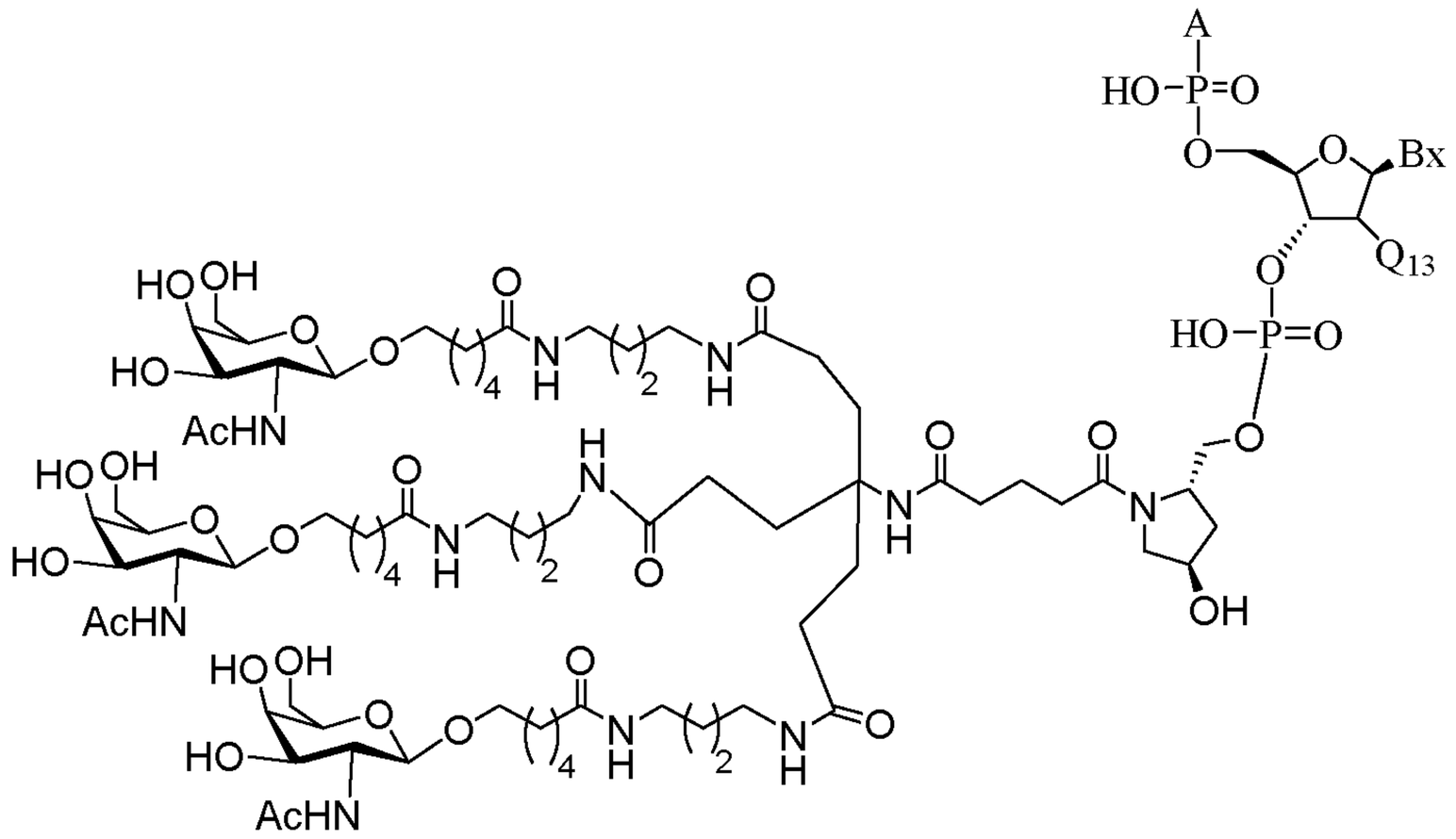


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

161. The compound of any of claims 1 to 30, wherein the compound has the following structure:

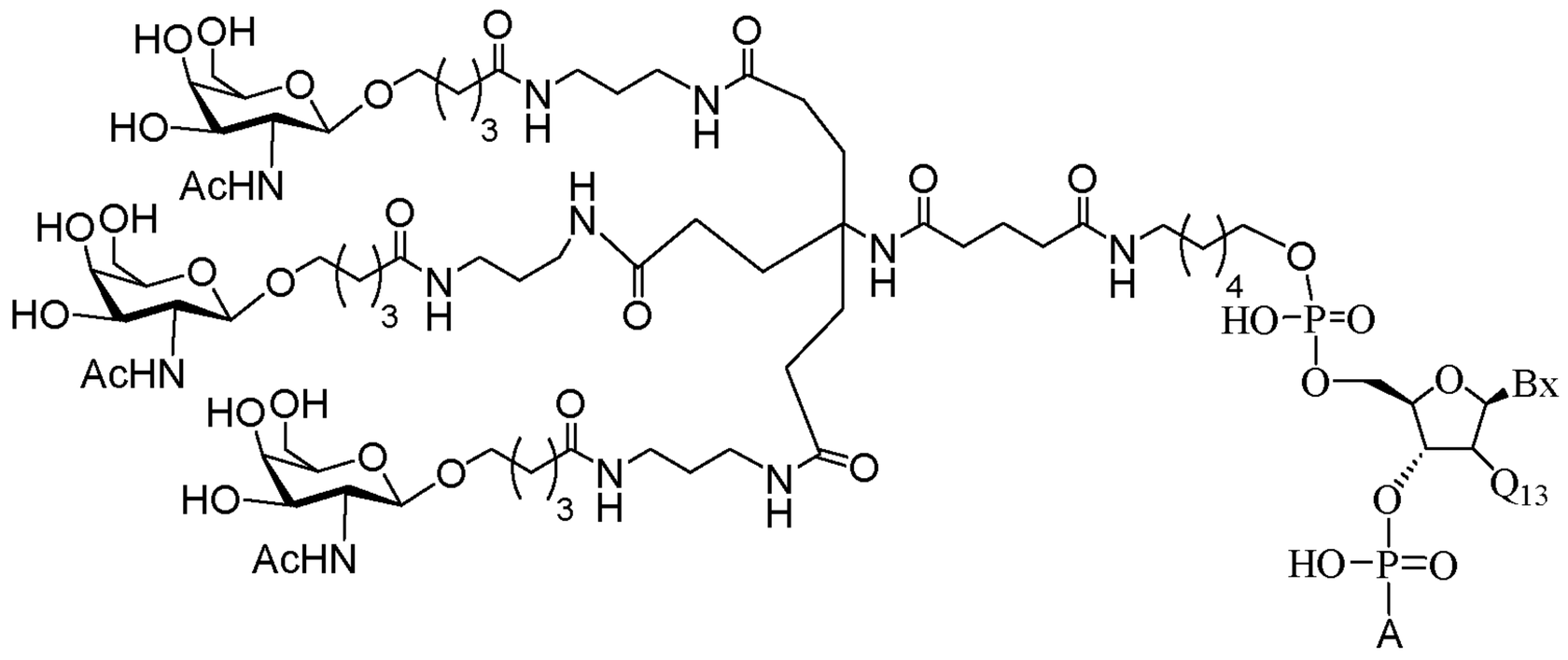


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

162. The compound of any of claims 1 to 30, wherein the compound has the following structure:

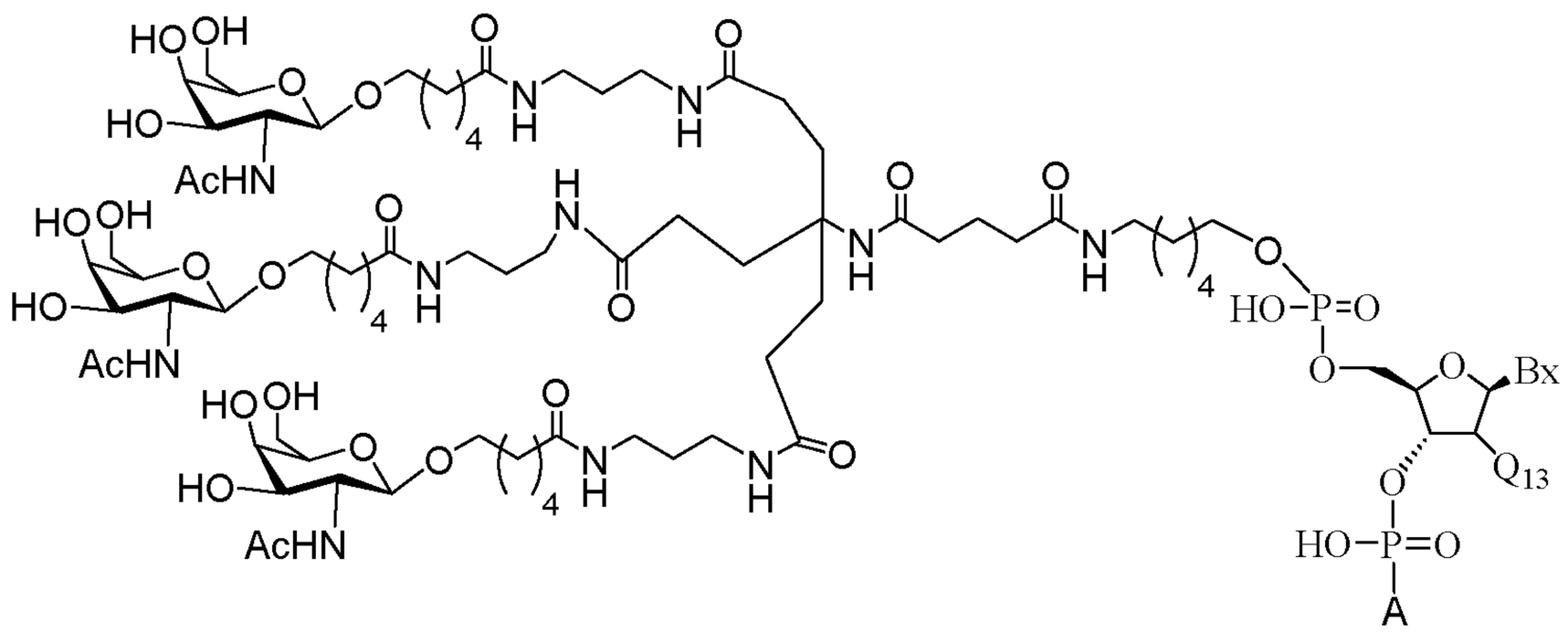


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

163. The compound of any of claims 1 to 30, wherein the compound has the following structure:

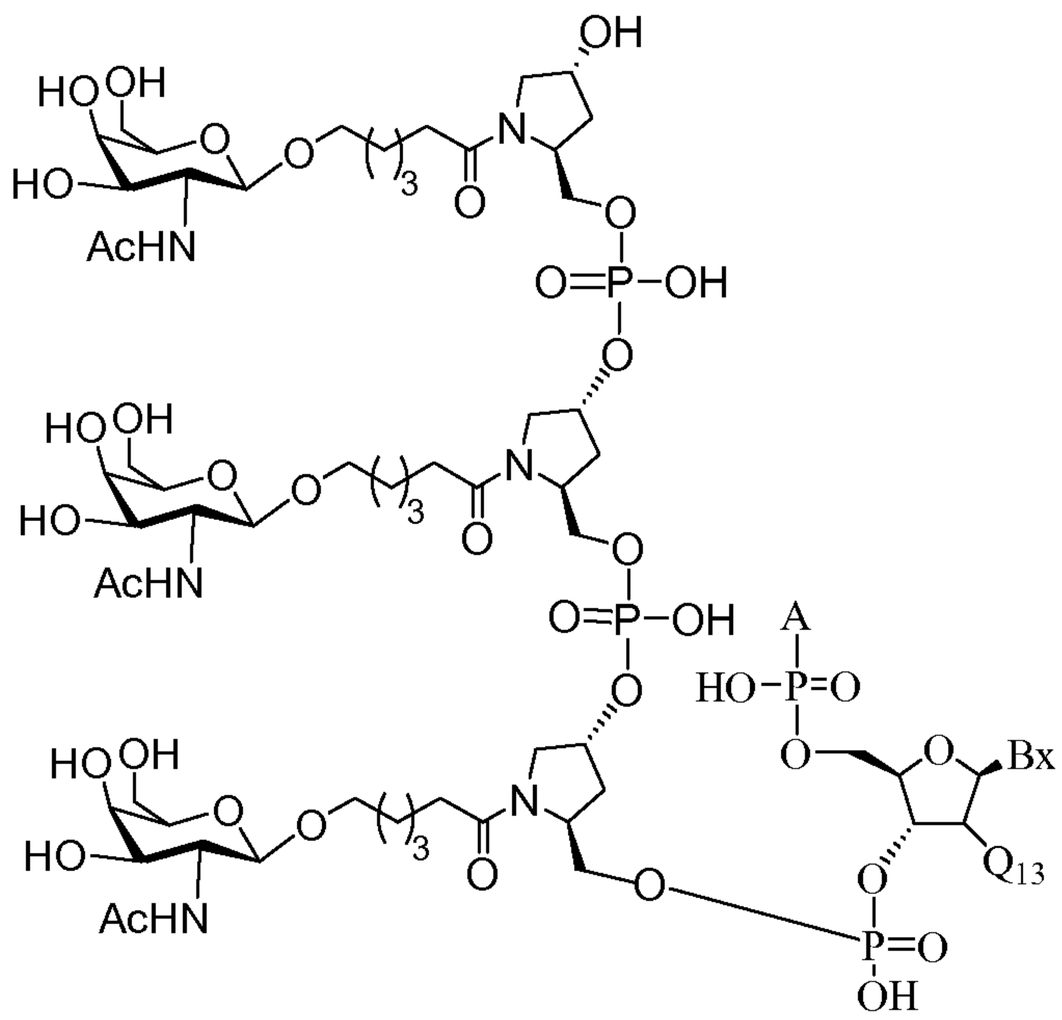


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

164. The compound of any of claims 1 to 30, wherein the compound has the following structure:

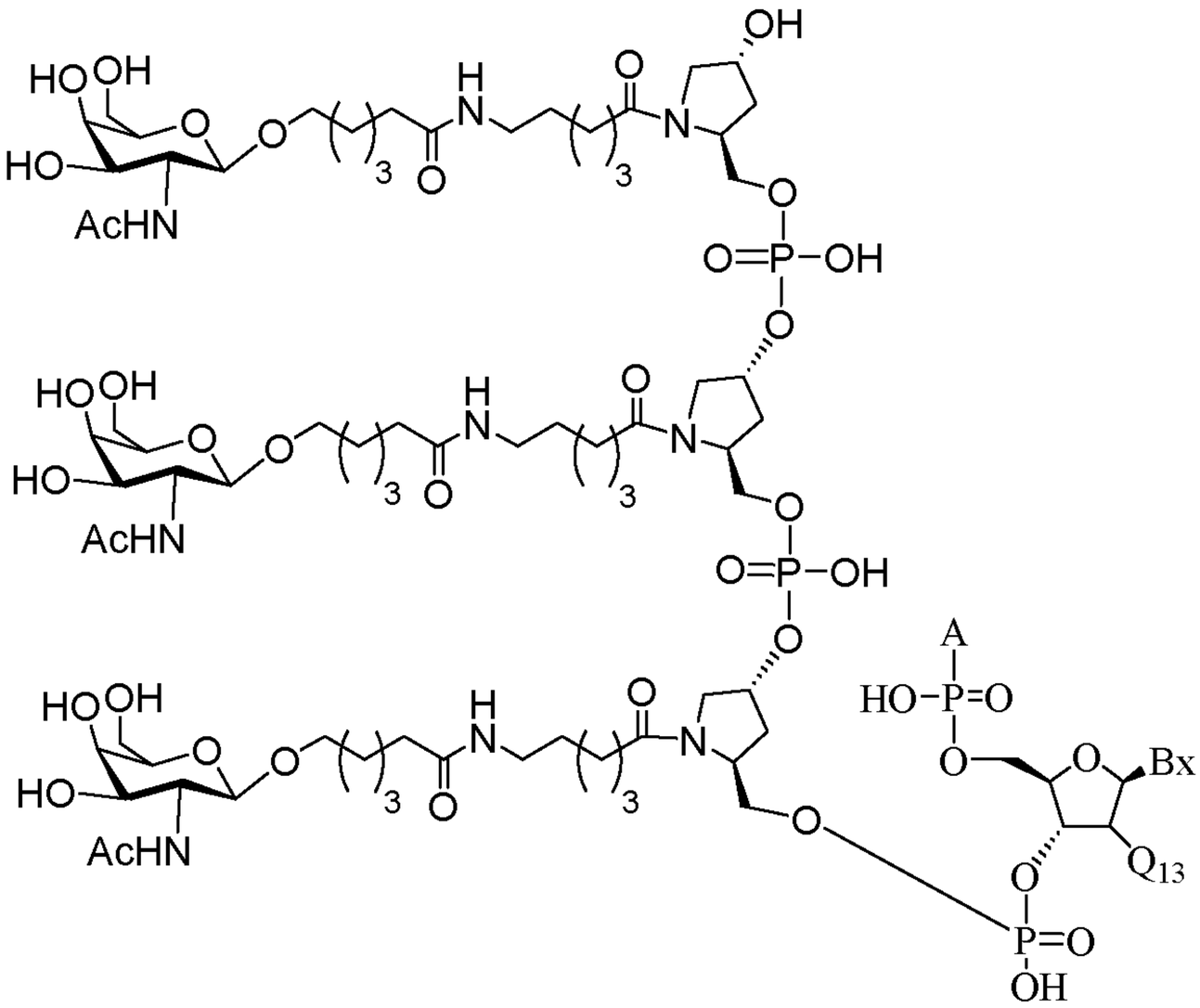


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

165. The compound of any of claims 1 to 30, wherein the compound has the following structure:

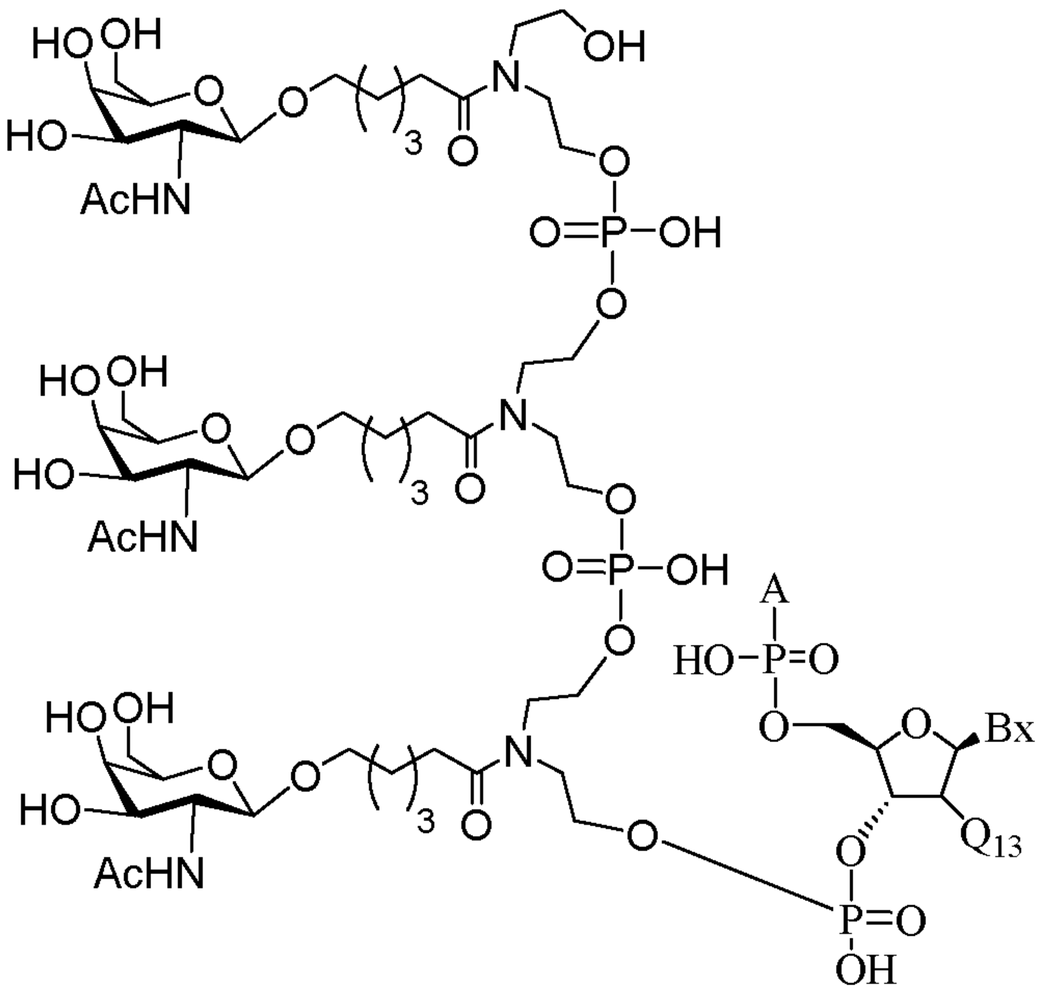


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

166. The compound of any of claims 1 to 30, wherein the compound has the following structure:

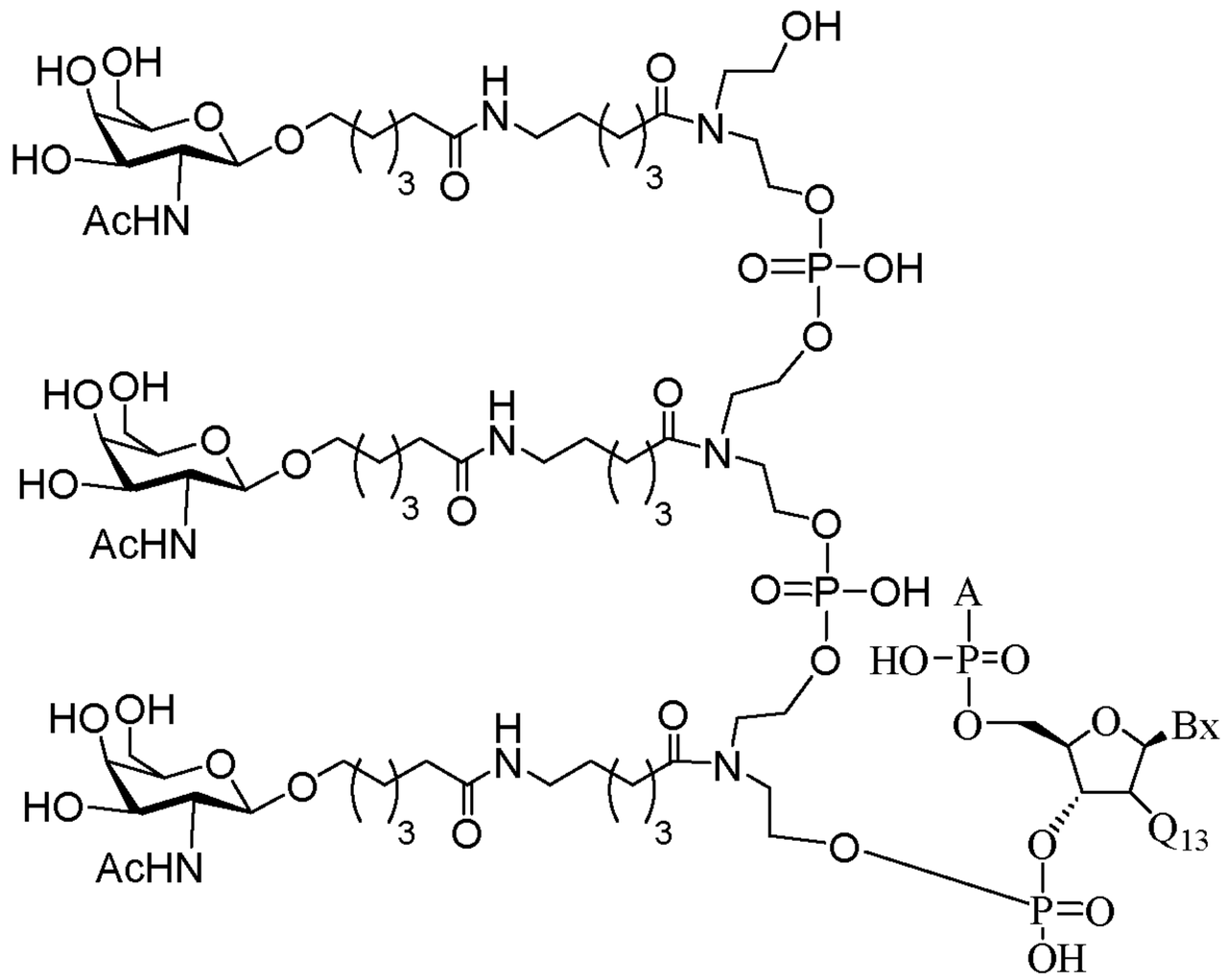


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

167. The compound of any of claims 1 to 30, wherein the compound has the following structure:

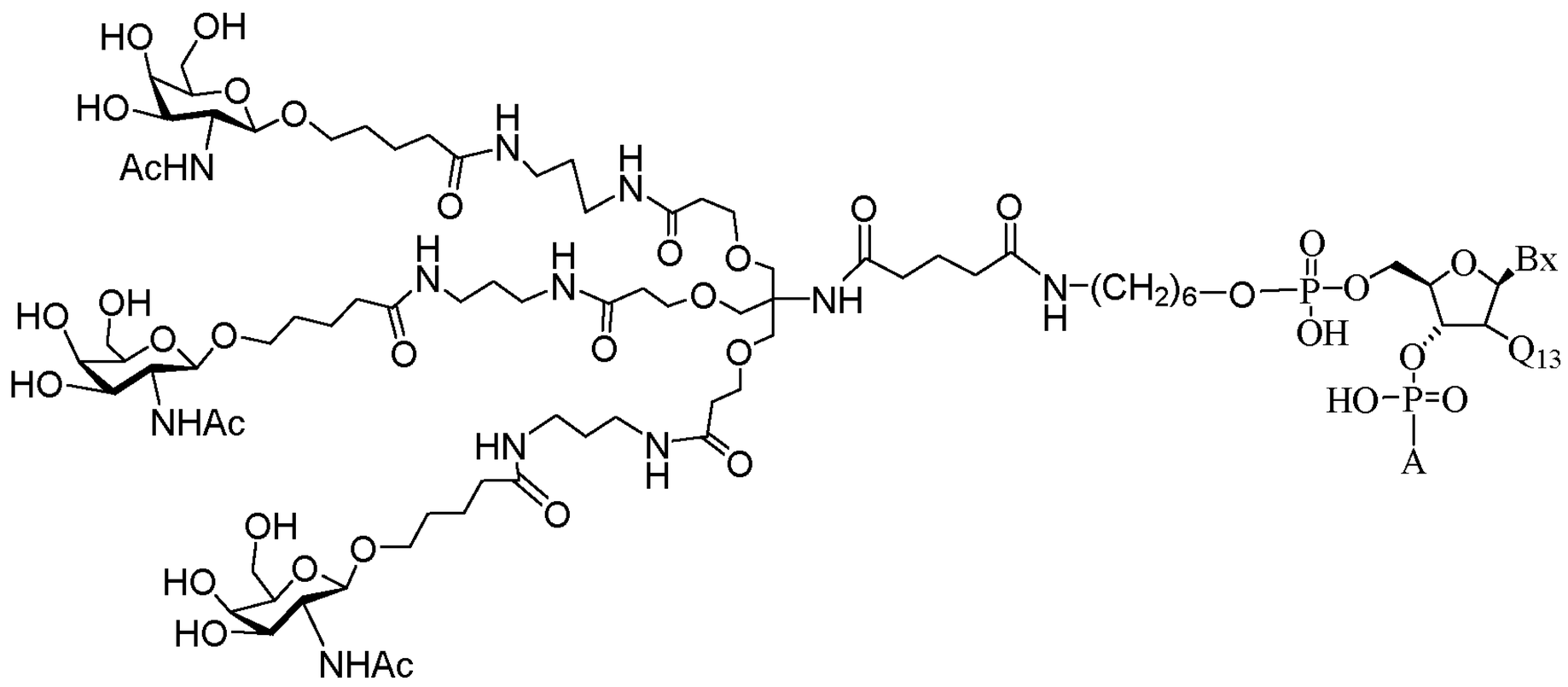


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

168. The compound of any of claims 1 to 30, wherein the conjugate group comprises:

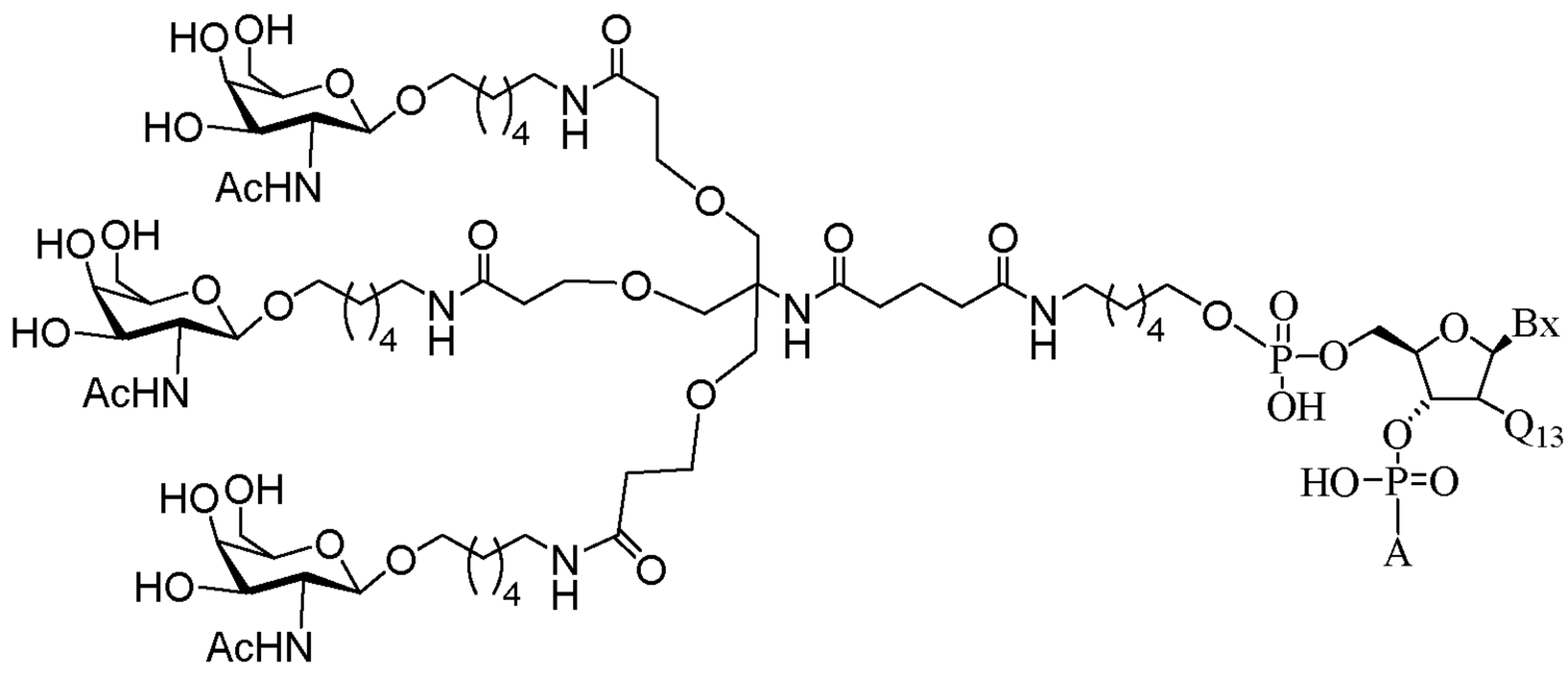


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

169. The compound of any of claims 1 to 30, wherein the conjugate group comprises:

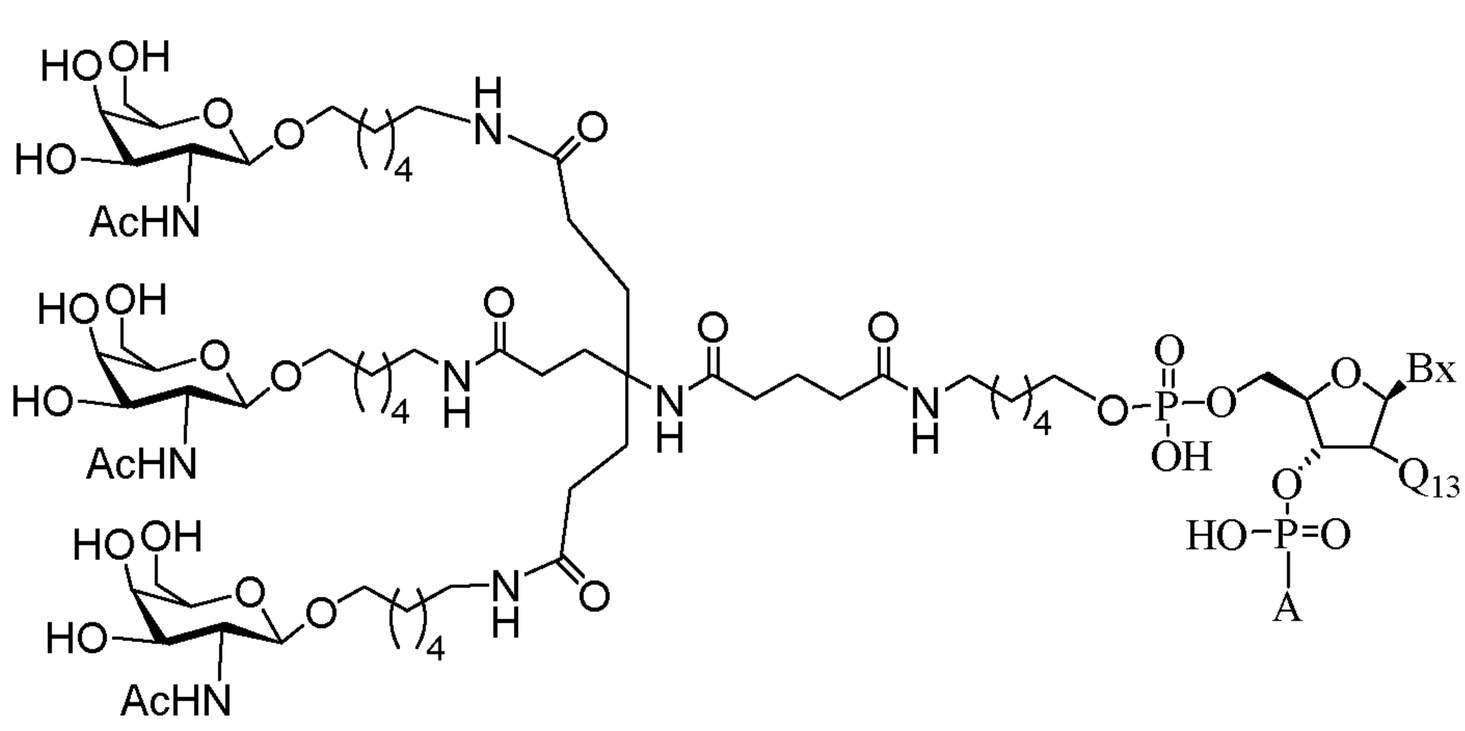


wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

170. The compound of any of claims 1 to 30, wherein the conjugate group comprises:



wherein Q_{13} is H or $O(CH_2)_2$-OCH_3;

A is the modified oligonucleotide; and

Bx is a heterocyclic base moiety.

171. The compound of any of claims 153 to 170, wherein B_x is selected from among from adenine, guanine, thymine, uracil, or cytosine, or 5-methyl cytosine.

172. The compound of any of claims 153 to 170, wherein B_x is adenine.

173. The compound of any of claims 153 to 170, wherein B_x is thymine.

174. The compound of any of claims 153 to 170, wherein Q_{13} is $O(CH_2)_2$-OCH_3.

175. The compound of any of claims 153 to 170, wherein Q_{13} is H.

176. A composition comprising the compound of any of claims 1-175 or salt thereof and at least one of a pharmaceutically acceptable carrier or diluent.

177. A prodrug comprising the compound of any of claims 1 to 176.

178. A method comprising administering to an animal the compound, composition, or prodrug of any of claims 1-177.

179. The method of claim 178, wherein the animal is a human.

180. The method of claim 178, wherein administering the compound prevents, treats, ameliorates, or slows progression of a cardiovascular and/or metabolic disease.

181. The method of claim 178, comprising co-administering the compound or composition and a second agent.

182. The method of claim 181, wherein the compound or composition and the second agent are administered concomitantly.

183. The method of claim 178, wherein the administering is parenteral.

184. The method of claim 178, wherein the administering is subcutaneous.

185. A method for treating a human with a cardiovascular and/or metabolic disease comprising identifying the human with cardiovascular and/or metabolic disease and administering to the human a therapeutically effective amount of the compound or composition of any of claims 1-177, so as to treat the human for cardiovascular and/or metabolic disease.

186. A composition comprising a compound according to any preceding claim, for use in therapy.

187. The composition of claim 185, for use in treating, preventing, or slowing progression of a disease related to elevated ANGPTL3.

188. The composition of claim 185, wherein the disease is a cardiovascular and/or metabolic disease, disorder or condition.