



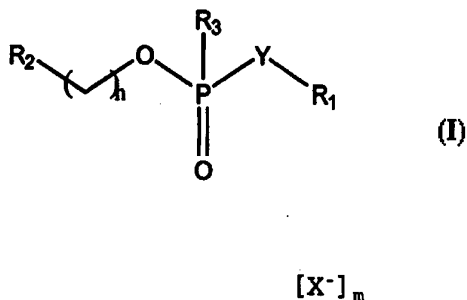
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<p>(21) International Application Number: PCT/US96/09478 (22) International Filing Date: 5 June 1996 (05.06.96) (30) Priority Data: 08/484,716 7 June 1995 (07.06.95) US (71) Applicant: GENTA INCORPORATED [US/US]; 3550 General Atomics Court, San Diego, CA 92121 (US). (72) Inventors: DWYER, Brian, Patrick; 13639 Comuna Drive, Poway, CA 92064 (US). LEBEDEV, Alexander, V.; 4015 Camino Lindo, San Diego, CA 92122 (US). BROWN, Bob, Dale; 445 North Willow Spring Drive, Encinitas, CA 92024 (US). SCHWARTZ, David, Aaron; 1544 Valeda, Encinitas, CA 92024 (US). (74) Agents: BIGGS, Suzanne, L. et al.; Lyon & Lyon, First Interstate World Center, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).</p>	<p>(81) Designated States: AU, CA, JP, KR, NZ, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	

(54) Title: PHOSPHONIC ACID-BASED CATIONIC LIPIDS

(57) Abstract

The present invention provides novel phosphonic acid-based cationic lipids of general structure (I), or a salt, or solvate, or enantiomers thereof wherein: (a) R₁ is a lipophilic moiety; (b) R₂ is a positively charged moiety; (c) R₃ is a lipophilic moiety of 1 to about 24 carbon atoms, a positively charged moiety, or a negatively charged moiety; (d) n is an integer from 0 to 8; (e) X⁻ is an anion or polyanion; (f) Y is N or O, and (g) m is an integer from 0 to a number equivalent to the positive charge(s) present on the lipid. The present invention further provides compositions of these lipids with polyanionic macromolecules, methods for interfering with protein expression in a cell utilizing these compositions and a kit for preparing the same.



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DESCRIPTION

PHOSPHONIC ACID-BASED CATIONIC LIPIDS

Technical Field of the Invention

The present invention relates to the field of cationic lipid compounds and their uses, including the delivery of macromolecules into cells.

5 Background of the Invention

None of the following discussion of the background of the invention is admitted to be prior art to the invention.

Lipid aggregates, such as liposomes, have been reported to be useful as agents for the delivery of macromolecules (such as DNA, RNA, oligonucleotides, proteins, and pharmaceutical compounds) into cells. In particular, lipid aggregates, which include charged as well as uncharged lipids, have been described as being especially effective for delivering polyanionic molecules to cells. The reported effectiveness of cationic lipids may result from charge interactions with cells which are said to bear a net negative charge. It has also been postulated that the net positive charge on the cationic lipid aggregates may enable them to bind polyanions, such as nucleic acids. For example, lipid aggregates containing DNA have been reported to be effective agents for efficient transfection of cells.

The structure of a lipid aggregate depends on

factors which include composition of the lipid and the method of forming the aggregate. Lipid aggregates include, for example, liposomes, unilamellar vesicles, multilamellar vesicles, micelles and the like, and may
5 have particle sizes in the nanometer to micrometer range. Various methods of making lipid aggregates have been reported in the art. One type of lipid aggregate includes phospholipid containing liposomes. An important drawback to the use of this type of aggregate as a cell
10 delivery vehicle is that the liposome has a negative charge that reduces the efficiency of binding to a negatively charged cell surface. It has been reported that positively charged liposomes that are able to bind DNA may be formed by combining cationic lipid compounds
15 with phospholipids. These liposomes then be utilized to transfer DNA into target cells. (See, e.g. Felgner et al., *Proc. Nat. Acad. Sci.* 84:7413-7417, 1987; Eppstein et al. U.S. Patent No. 4,897,355; Felgner et al. U.S. Patent No. 5,264,618; and Gebeyehu et al. U.S. Patent
20 No. 5,334,761).

Known cationic lipids include N[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium chloride ("DOTMA") and combinations of DOTMA with dioleoylphosphatidylethanolamine ("DOPE") have been
25 commercially available. Formulation of DOTMA, either by itself or in 1:1 combination with DOPE, into liposomes by conventional techniques has been reported. However, compositions comprising DOTMA have been reported to show some toxicity to cells.

30 Another commercially available cationic lipid, 1,2-

bis(oleoyloxy)-3,3-(trimethylammonia)propane ("DOTAP") differs from DOTMA in structure in that the oleoyl moieties are linked by ester, rather than ether, linkages to the propylamine. However, DOTAP is reported
5 to be more readily degraded by target cells. Other cationic lipids which represent structural modifications of DOTMA and DOTAP have also been reported.

Other reported cationic lipid compounds include those in which carboxyspermine has been conjugated to
10 one of two types of lipids and includes compounds such as 5-carboxyspermylglycine dioctaoleoylamide ("DOGS") and dipalmitoyl-phosphatidylethanolamine 5-carboxyspermyl-amide ("DPPES") (See, e.g. Behr et al., U.S. Patent No. 5,171,678).

15 Another reported cationic lipid composition is a cationic cholesterol derivative ("DC-Chol") which has been formulated into liposomes in combination with DOPE. (See, Gao, X. and Huang, L., *Biochim. Biophys. Res. Commun.* 179:280, 1991). For certain cell lines, these
20 liposomes were said to exhibit lower toxicity and provide more efficient transfection than the DOTMA-containing compositions.

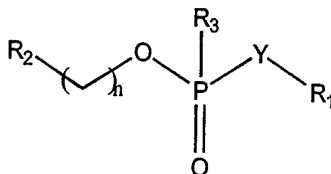
Lipopolylysine, made by conjugating polylysine to DOPE has been reported to be effective for transfection
25 in the presence of serum. (Zhou, X. et al., *Biochim. Biophys. Acta* 1065:8, 1991).

However, of the cationic lipids which have been proposed for use in delivering macromolecules to cells, no particular cationic lipid has been reported to work
30 well with a wide variety of cell types. Since cell types

differ from one another in membrane composition, different cationic lipid compositions and different types of lipid aggregates may be effective for different cell types, either due to their ability to contact and fuse with target cell membranes directly or due to different interactions with intracellular membranes or the intracellular environment. For these and other reasons, design of effective cationic lipids has largely been empirical. In addition to content and transfer, other factors believed important include, for example, ability to form lipid aggregates suited to the intended purpose, toxicity of the composition to the target cell, stability as a carrier for the macromolecule to be delivered, and function in an *in vivo* environment. Thus, there remains a need for improved cationic lipids which are capable of delivering macromolecules to a wide variety cell types with greater efficiency.

Summary of the Invention

In one aspect of the present invention novel phosphonic acid-based cationic lipids having the structure:



$[X^-]_m$

or a salt, or solvate, or enantiomers thereof are provided wherein; (a) R_1 is a lipophilic moiety; (b) R_2 is a positively charged moiety; (c) R_3 is a lipophilic moiety of about 1 to about 24 carbon atoms, a positively charged moiety, or a negatively charged moiety; (d) n is an integer from 0 to 8; (e) X^- is an anion or polyanion; (f) Y is N or O, and (g) m is an integer from 0 to a number equivalent to the positive charge(s) present on the lipid.

10 In one embodiment R_1 may be selected from a variety of lipophilic moieties including a straight chain alkyl of 1 to about 24 carbon atoms, a straight chain alkenyl of 2 to about 24 carbon atoms, a symmetrical branched alkyl or alkenyl of about 10 to about 50 carbon atoms
15 (preferably 25-40), an unsymmetrical branched alkyl or alkenyl of about 10 to about 50 carbon atoms, a steroidyl moiety, an amine derivative, a glyceryl derivative, or $OCH(R_4R_5)$ or $N(R_4R_5)$, wherein R_4 and R_5 are straight chain or branched alkyl moieties of about 10 to
20 about 30 carbon atoms, or a glyceryl derivative.

Preferably when R_1 is a steroidyl moiety it is a cholesteryl moiety.

Preferably when R_1 is an amine derivative it is a straight chain, branched or cyclic acylamine or
25 alkylamine. In particular, R_1 is a 3-N-1,2-diacyl-1,2-propanediol-3-amino moiety, a 3-N-1,2-dialkyl-1,2-propanediol-3-amino moiety, or a 3-N-1,2 N,N-diacyl-1,2,3-triaminopropanyl moiety. It is preferable that when the amine derivative is an 3-N-1,2-diacyl-1,2-
30 propanediol-3-amino moiety that the diacyl moiety be an

alkanoic acid of about 10 to about 30 carbon atoms or an alkenoic acid of about 10 to about 30 carbon atoms. It is also preferable that when the amine moiety is a 3-N-1,2-dialkyl-1, 2-propanediol-3-amino moiety that the
5 alkyl moieties be alkyl groups of about 10 to about 30 carbon atoms or alkenyl groups of about 10 to about 30 carbon atoms.

When R_1 is a glyceryl derivative it is preferable that it be a 3-O-1,2-diacylglyceryl moiety or a 3-O-1,2-
10 dialkylglyceryl moiety. In particular when R_1 is 3-O-1,2-diacylglyceryl moiety it is preferable that the diacyl group be an alkanolic acid of about 10 to about 30 carbon atoms or an alkenoic acid of about 10 to about 30 carbon atoms. It is also preferred that when the glyceryl moiety
15 is 3-O-1,2-dialkylglyceryl moiety that the alkyl moieties be alkyl groups of about 10 to about 30 carbon atoms or alkenyl groups of about 10 to about 30 carbon atoms.

It is particularly preferred that when a moiety
20 contains an alkanolic acid that the acid be stearic acid and when a moiety contains an alkenoic acid that the acid be palmitic acid or oleic acid.

In another embodiment R_2 may be selected from a variety of positively charged moieties including an
25 amino acid residue having a positively charged group on the side chain, an alkylamine moiety, a fluoroalkylamine moiety, or a perfluoroalkylamine moiety of 1 to about 6 carbon atoms, an arylamine moiety or an aralkylamine moiety of 5 to about 10 carbon atoms, a guanidinium
30 moiety, an enamine moiety, a aromatic or non-aromatic

cyclic amine moiety of about 5 to about 10 carbon atoms, an amidine moiety, an isothiourea moiety, a heterocyclic amine moiety, or a substituted heterocyclic moiety and a substituted alkyl moiety of 1 to about 6 carbon atoms
5 substituted with a substituent selected from the group consisting of NH_2 , $\text{C}(=\text{O})\text{NH}_2$, NHR_6 , $\text{C}(=\text{O})\text{NHR}_6$, NHR_6R_7 , or $\text{C}(=\text{O})\text{NHR}_6\text{R}_7$, wherein R_6 and R_7 are independently selected from an alkyl moiety of 1 to about 24 carbon atoms, an alkenyl moiety of 2 to about 24 carbon atoms, an aryl
10 moiety of about 5 to about 20 carbon atoms, and an aralkyl moiety of about 6 to about 25 carbon atoms.

Preferably when R_2 is an amino acid residue it is lysine, arginine, histidine, ornithine, or an amino acid analog. In particular, when R_2 is an amino acid analog it
15 is preferably 3-carboxyspermidine, 5-carboxyspermidine, 6-carboxyspermine or a monoalkyl, dialkyl, or peralkyl substituted derivative which is substituted on one or more amine nitrogens with an alkyl group of 1 to about 6 carbon atoms.

20 In still another embodiment R_3 may be selected from a variety of moieties including a lipophilic moiety of about 3 to about 24 carbon atoms, a positively charged moiety, or a negatively charged moiety. When R_3 is a lipophilic moiety it is preferable that it be a straight
25 chain alkyl moiety of about 1 to about 24 carbon atoms (most preferably a one carbon methyl group), a straight chain alkenyl moiety of 2 to about 24 carbon atoms, a symmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, a unsymmetrical branched alkyl
30 or alkenyl moiety of about 10 to about 50 carbon

atoms, an aryl of about 5 to about 20 carbon atoms, an aralkyl moiety of about 6 to about 25 carbon atoms, or a steroidyl moiety.

Preferably when the R_3 is a positively charged moiety
5 it is an amino acid residue having a positively charged group on the side chain, an alkylaminoalkyl moiety, a fluoroalkylaminoalkyl moiety, a perfluoroalkylaminoalkyl moiety, a guanidiniumalkyl moiety, an enaminoalkyl moiety, a cyclic aminoalkyl moiety, an amidinoalkyl
10 moiety, an isothioureä alkyl moiety, or a heterocyclic amine moiety

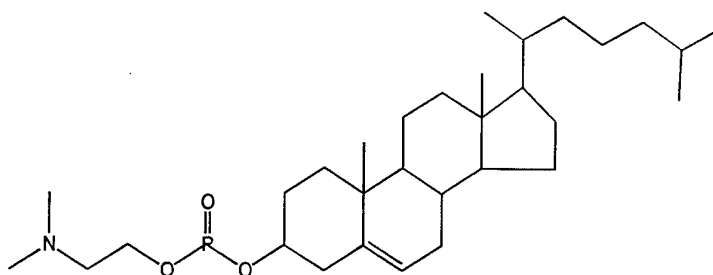
When R_3 is a negatively charged moiety it is preferable that it be a carboxyalkyl moiety, a phosphonoalkyl moiety, a sulfonoalkyl moiety, or a
15 phosphatidylalkyl moiety of 1 to about 24 carbon atoms more preferably between 6 and 12 carbon atoms, most preferably between 8 and 10.

It is further preferred that n be an integer from 1 to 8, more preferably 2 to 6 and most preferably 2, 3,
20 or 4.

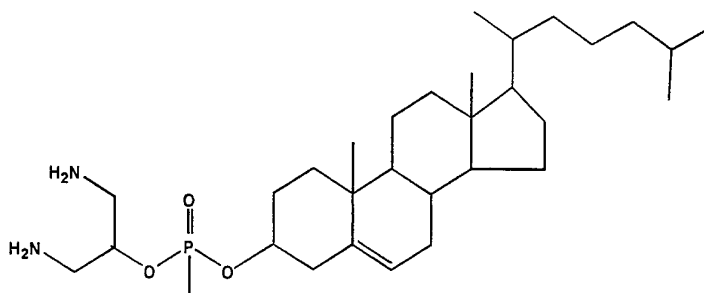
It is also preferred that X^- be a pharmaceutically acceptable anion or polyanion.

In a particularly preferred embodiment the phosphonic acid-based cationic lipid has the structure:

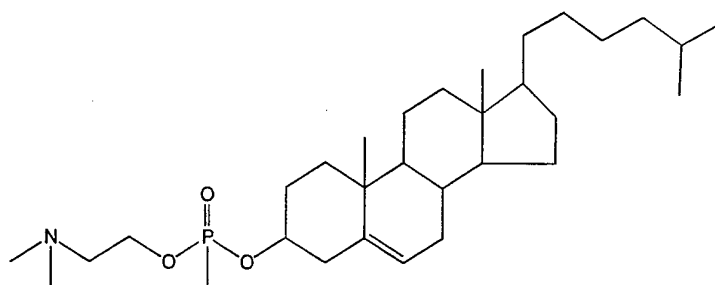
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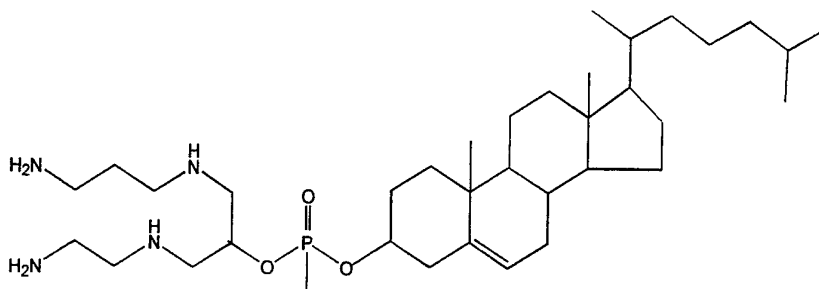
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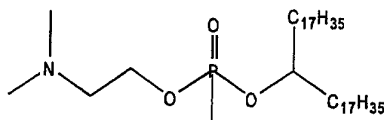
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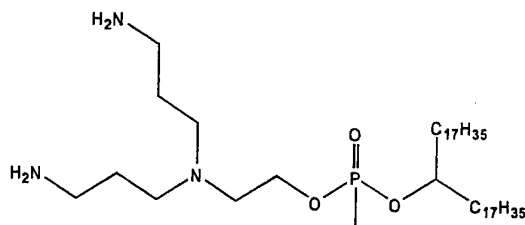


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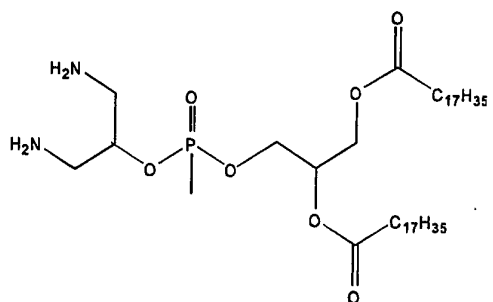
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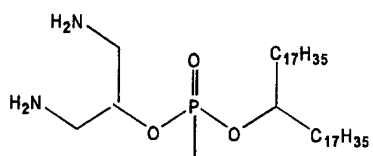


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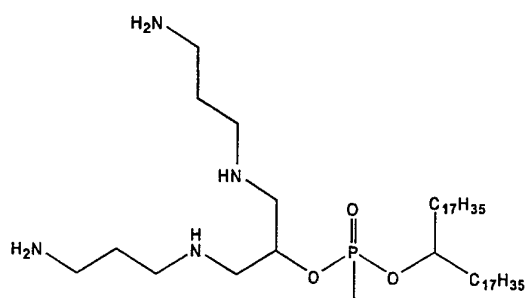
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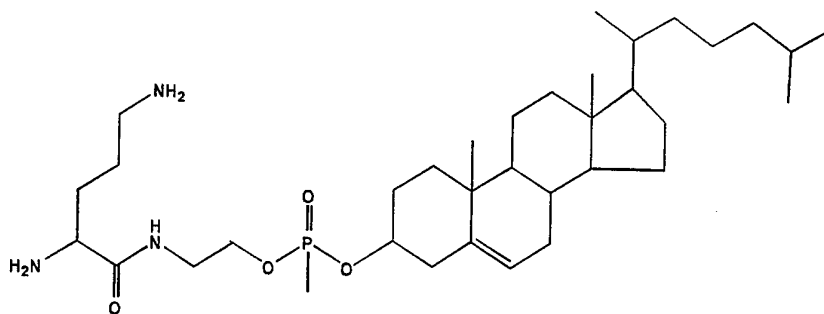
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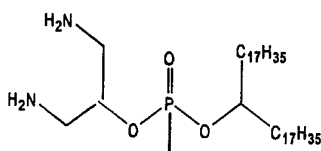
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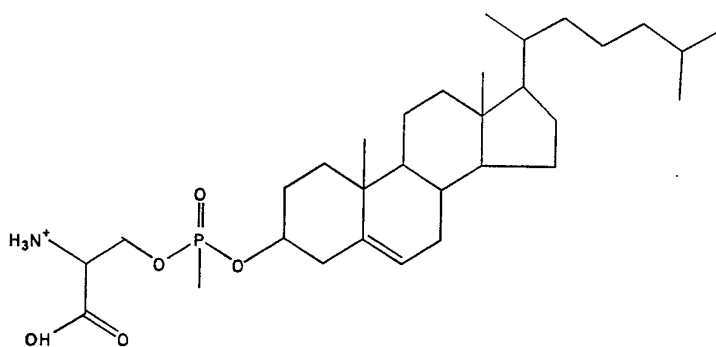
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X



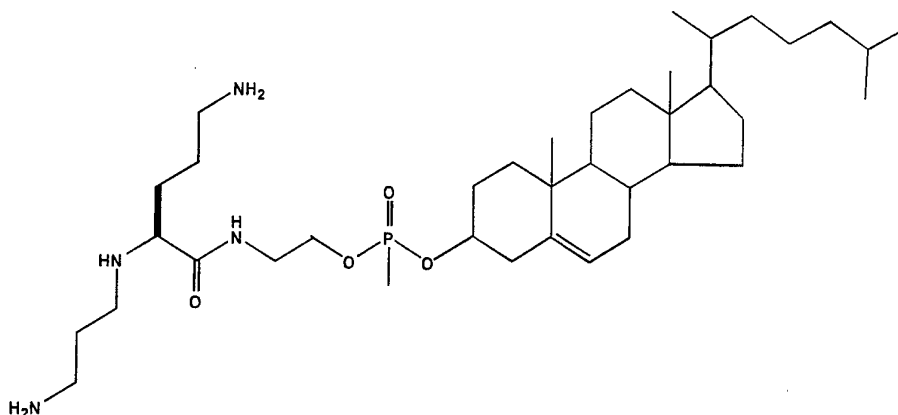
XI



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XII

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**XIII**

In another aspect of the present invention, compositions comprising a polyanionic macromolecule and any of the lipid structures described above are provided. In particular, the polyanionic macromolecule may be a variety of macromolecules including an expression vector capable of expressing a polypeptide in a cell. In a preferred embodiment the polyanionic macromolecule is DNA.

In still another aspect of the invention methods for the delivery of a polyanionic macromolecule into a cell by contacting any of the compositions above with the cell are provided. In particular, a method is provided to interfere with the expression of a protein in a cell by contacting any of the the compositions described above with a cell wherein the composition comprises an oligomer having a base sequence that is

substantially complimentary to an RNA sequence in the cell that encodes the protein.

The present invention further provides a kit for delivering a polyanionic macromolecule into a cell
5 comprising any of the compositions described above.

Detailed Description of the Invention

Prior to setting forth the remaining description invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that
10 will be used herein after. These terms will have the following meanings unless explicitly stated otherwise:

"Lipophilic Moiety" refers to a moiety which demonstrates one or more of the following characteristics:

- 15 A. tend to be water insoluble
 B. tend to be soluble in non-polar solvent
 C. tend to favor octanol in octanol/water partition measurements, and
 D. tend to be compatible with lipid bilayers
20 and may be bilayer forming.

In particular, lipophilic moieties having an octanol/water partition coefficient of 0.5 or lower are preferable, where octanol/water partition coefficient is measured by the concentration in water divided by
25 concentration in octanol.

"Positively Charged Moiety" refers to "positively charged moiety and negatively charged moiety" means a moiety, independent of the cationic lipid for which it is a substituent, having a net positive or negative

charge within the pH range of 2 to 12. The net charge of the cationic lipid is the summation of all charged moieties occurring on the lipid, such that the net charge may be positive, neutral or negative.

5 The term "alkyl" refers to saturated aliphatic groups including straight-chain, branched-chain and cyclic groups. Suitable alkyl groups include, but are not limited to, cycloalkyl groups such as cyclohexyl and cyclohexylmethyl. These groups may be optionally
10 substituted with one or more functional groups which are attached commonly to such chains, such as, e.g., hydroxyl, bromo, fluoro, chloro, iodo, mercapto or thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, alkoxy-carbonyl, alkyl, alkenyl, nitro, amino,
15 alkoxy, amido, and optionally substituted isothioureido, amidino, guanidino, and the like to form alkyl groups such as trifluoromethyl, 3-hydroxyhexyl, 2-carboxypropyl, 2-fluoroethyl, carboxymethyl, 4-cyanobutyl, 2-guanidinoethyl,
20 3-N,N'-dimethylisothiuroniumpropyl, and the like. "Lower alkyl" refers to alkyl groups of 1 to 6 carbon atoms. Fluoroalkyl or perfluoroalkyl refers to singly, partially, or fully fluorinated alkyl groups.

 The term "alkenyl" refers to an unsaturated
25 aliphatic group having at least one double bond.

 The term "arylamine" refers to an amine linked to aromatic groups that have at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which
30 may be optionally substituted with one or more

functional groups which are attached commonly to such chains, such as, e.g., hydroxyl, bromo, fluoro, chloro, iodo, mercapto or thio, cyano, cyanoamido, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, alkoxycarbonyl, 5 alkyl, alkenyl, nitro, amino, alkoxyl, amido, and the like to form aryl groups such as biphenyl, iodobiphenyl, methoxybiphenyl, anthryl, bromophenyl, iodophenyl, chlorophenyl, hydroxyphenyl, methoxyphenyl, formylphenyl, acetylphenyl, trifluoromethylthiophenyl, 10 trifluoromethoxyphenyl, alkylthiophenyl, trialkylammoniumphenyl, amidophenyl, thiazolylphenyl, oxazolylphenyl, imidazolylphenyl, imidazolylmethylphenyl, cyanophenyl, pyridylphenyl, pyrrolylphenyl, pyrazolylphenyl, triazolylphenyl, 15 tetrazolylphenyl and the like.

The term "aralkylamine" refers to an alkyl group substituted with an aryl group. Suitable aralkyl groups include benzyl, picolyl, and the like, all of which may be optionally substituted.

20 The term "oligonucleoside" or "oligomer" refers to a chain of nucleosides that are linked by internucleoside linkages that is generally from about 4 to about 100 nucleosides in length, but which may be greater than about 100 nucleosides in length. They are 25 usually synthesized from nucleoside monomers, but may also be obtained by enzymatic means. Thus, the term "oligomer" refers to a chain of oligonucleosides that have internucleosidyl linkages linking the nucleoside monomers and, thus, includes oligonucleotides, nonionic 30 oligonucleoside alkyl- and aryl-phosphonate analogs,

alkyl- and aryl-phosphonothioates, phosphorothioate or phosphorodithioate analogs of oligonucleotides, phosphoramidate analogs of oligonucleotides, neutral phosphate ester oligonucleoside analogs, such as

5 phosphotriesters and other oligonucleoside analogs and modified oligonucleosides, and also includes nucleoside/non-nucleoside polymers. The term also includes nucleoside/non-nucleoside polymers wherein one or more of the phosphorus group linkages between

10 monomeric units has been replaced by a non-phosphorous linkage such as a formacetal linkage, a thioformacetal linkage, a morpholino linkage, a sulfamate linkage, a silyl linkage, a carbamate linkage, an amide linkage, a guanidine linkage, a nitroxide linkage or a substituted

15 hydrazine linkage. It also includes nucleoside/non-nucleoside polymers wherein both the sugar and the phosphorous moiety have been replaced or modified such as morpholino base analogs, or polyamide base analogs. It also includes nucleoside/non-nucleoside polymers

20 wherein the base, the sugar, and the phosphate backbone of the non-nucleoside are either replaced by a non-nucleoside moiety or wherein a non-nucleoside moiety is inserted into the nucleoside/non-nucleoside polymer. Optionally, said non-nucleoside moiety may serve to link

25 other small molecules which may interact with target sequences or alter uptake into target cells.

Lipid Aggregate is a term that includes liposomes of all types both unilamellar and multilamellar as well as micelles and more amorphous aggregates of cationic

30 lipid or lipid mixed with amphipathic lipids such as

phospholipids.

Target Cell refers to any cell to which a desired compound is delivered, using a lipid aggregate as carrier for the desired compound.

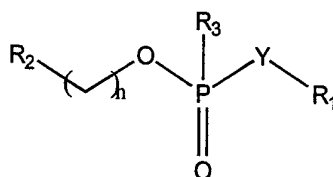
5 Transfection is used herein to mean the delivery of expressible nucleic acid to a target cell, such that the target cell is rendered capable of expressing said nucleic acid. It will be understood that the term "nucleic acid" includes both DNA and RNA without regard
10 to molecular weight, and the term "expression" means any manifestation of the functional presence of the nucleic acid within the cell, including without limitation, both transient expression and stable expression.

Delivery is used to denote a process by which a
15 desired compound is transferred to a target cell such that the desired compound is ultimately located inside the target cell or in, or on the target cell membrane. In many uses of the compounds of the invention, the desired compound is not readily taken up by the target
20 cell and delivery via lipid aggregates is a means for getting the desired compound into the cell. In certain uses, especially under *in vivo* conditions, delivery to a specific target cell type is preferable and can be facilitated by compounds of the invention.

25 The generic structure of functionally active cationic lipids requires three contiguous moieties, e.g. cationic-head-group/ linker/lipid-tail group. While a wide range of structures can be envisioned for each of the three moieties, it has been demonstrated that there

is no *a priori* means to predict which cationic lipid will successfully transfect anionic macromolecules into a particular cell line. The property of a cationic lipid to be formulated with an anionic macromolecule which will then successfully transfect a cell line is empirical. We demonstrate the abilities of novel cationic lipids which are chemically linked into multimeric constructions to enhance the uptake of macromolecules.

The novel phosphonic acid-based cationic lipids of the present invention have the general structure:



$[X^-]_m$

comprising any salt, solvate, or enantiomers thereof.

The symbols R_1 , R_2 , R_3 , Y , X , n and m are described as follows.

R_1 represents the lipid-tail group of the phosphonic acid-based cationic lipid and may be a variety of lipophilic moieties. In particular, these include for example, a straight chain alkyl of 1 to about 24 carbon atoms, a straight chain alkenyl of 2 to about 24 carbon atoms, a symmetrical branched alkyl or alkenyl of about 10 to about 50 carbon atoms, a unsymmetrical branched

alkyl or alkenyl of about 10 to about 50 carbon atoms, a steroidyl moiety, a glyceryl derivative, a amine derivative, and $OCH(R_4R_5)$ or $N(R_4R_5)$, wherein R_4 and R_5 are straight chain or branched alkyl moieties of about 10 to
5 about 30 carbon atoms.

In the case where R_1 is a steriodal moiety a variety of such moieties may be utilized including for example pregnenolone, progesterone, cortisol, corticosterone, aldosterone, androstenedione, testosterone, or
10 cholesterol or analogs thereof. A cholesteryl moiety is particularly preferred.

When R_1 is an amine derivative a variety of such derivatives may be utilized, for example, a straight chain, branched or cyclic acylamine or alkylamine.
15 Particular amine derivatives include a 3-N-1,2-diacyl-1,2-propanediol-3-amino moiety, a 3-N-1,2-dialkyl-1,2-propanediol-3-amino moiety, and a 3-N-1,2 N,N-diacyl-1,2,3-triaminopropanyl moiety. In the case when R_1 is an 3-N-1,2-diacyl-1,2-propanediol-3-amino moiety the diacyl
20 moiety may be an alkanolic acid of about 10 to about 30 carbon atoms or an alkenolic acid of about 10 to about 30 carbon atoms. Similarly when R_1 is an 3-N-1,2-dialkyl-1, 2-propanediol-3-amino moiety the alkyl moieties may be alkyl groups of about 10 to about 30 carbon atoms or
25 alkenyl groups of about 10 to about 30 carbon atoms. These groups may be straight chain, symmetrically, or unsymmetrically branched alkyl and alkenyl groups.

When R_1 is a glyceryl derivative a variety of such derivatives may be utilized including more specifically
30 a 3-O-1,2-diacylglyceryl moiety or a 3-O-1,2-

dialkylglyceryl moiety. In particular when R_1 is a 3-O-1,2-diacylglyceryl moiety the diacyl group may be an alkanolic acid of about 10 to about 30 carbon atoms or an alkenolic acid of about 10 to about 30 carbon atoms.

5 Similarly when R_1 is a 3-O-1,2-dialkylglyceryl moiety the alkyl moieties may be alkyl groups of about 10 to about 30 carbon atoms or alkenyl groups of about 10 to about 30 carbon atoms. These groups may be straight chain, symmetrically, or unsymmetrically branched alkyl and
10 alkenyl groups.

In either case when R_1 is an amine derivative or a glyceryl derivative comprising an alkanolic acid the acid is preferably a stearic acid. Similarly, when these derivative comprise an alkenolic acid the acid is
15 preferably a palmitic acid or an oleic acid.

R_2 represents the cationic head group of the phosphonic acid-based cationic lipid and may be a variety of positively charged moieties including, for example, an amino acid residue having a positively charged group
20 on the side chain, an alkylamine moiety, a fluoroalkylamine moiety, or a perfluoroalkylamine moiety of 1 to about 6 carbon atoms, an arylamine moiety or an aralkylamine moiety of 5 to about 10 carbon atoms, a guanidinium moiety, an enamine moiety, a aromatic or
25 non-aromatic cyclic amine moiety of about 5 to about 10 carbon atoms, an amidine moiety, an isothiourea moiety, a heterocyclic amine moiety, and a substituted heterocyclic moiety and a substituted alkyl moiety of 1 to about 6 carbon atoms substituted with a substituent
30 selected from the group consisting of NH_2 , $C(=O)NH_2$, NHR_6 ,

$C(=O)NHR_6$, NHR_6R_7 , or $C(=O)NHR_6R_7$, wherein R_6 and R_7 are independently selected from an alkyl moiety of 1 to about 24 carbon atoms, an alkenyl moiety of 2 to about 24 carbon atoms, an aryl moiety of about 5 to about 20
5 carbon atoms, or an aralkyl moiety of about 6 to about 25 carbon atoms.

In particular when R_2 is an amino acid residue it may be, for example, lysine, arginine, histidine, ornithine, or an amino acid analog. Although R_2 may be a
10 variety of positively charged amino acid analogs, specific examples include 3-carboxyspermidine, 5-carboxyspermidine, 6-carboxyspermine and a monoalkyl, dialkyl, or peralkyl substituted derivative which is substituted on one or more amine nitrogens with an alkyl
15 group of 1 to about 6 carbon atoms.

R_3 may be a wide variety of moieties including for example a lipophilic moiety, a positively charged moiety and a negatively charged moiety. In particular when R_3 is a lipophilic moiety it may be a straight chain alkyl
20 moiety of 1 to about 24 carbon atoms, a straight chain alkenyl moiety of 2 to about 24 carbon atoms, a symmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, a unsymmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms,
25 an aryl moiety of about 5 to about 20 carbon atoms, an aralkyl moiety of about 6 to about 25 carbon atoms, or a steroidyl moiety.

When R_3 is a positively charged it may be an amino acid residue having a positively charged group on the
30 side chain, an alkylaminoalkyl moiety, a

fluoroalkylaminoalkyl moiety, a perfluoroalkylaminoalkyl moiety, a guanidiniumalkyl moiety, an enaminoalkyl moiety, a cyclicaminoalkyl moiety, an amidinoalkyl moiety, an isothioureia alkyl moiety, or a heterocyclic
5 amine. Other amino acids such as tyrosine and cysteine can serve to attach pendant amino containing functional groups. The functional side groups can also be reactive sites for incorporation of dendritic branching. (see, e.g. Szoka, *Bioconjugate Chem.* 4:372, 1993).

10 In the case when R_3 is a negatively charged moiety it may be a carboxyalkyl moiety, a phosphonoalkyl moiety, a sulfonoalkyl moiety, or a phosphatidylalkyl moiety of 1 to about 24 carbon atoms.

The linker comprises the structure joining the head
15 group, R_1 to the lipid-tail group, R_2 . This structure includes Y which may be an oxygen or a nitrogen and a series of $-CH_2-$ groups, the number of which, is indicated by the letter n. n is an integer ranging from 0 to 8, in particular cases it ranges from 2 to 6 and in specific
20 instances the integer is 2, 3 or 4.

The counterion represented by X^- is an anion or a polyanion that binds to the positively charged groups present on the phosphonic acid-based cationic lipid via charge-charge interactions. When these cationic lipids
25 are to be used *in vivo* the anion or polyanion should be pharmaceutically acceptable.

m is an integer indicating the number of anions or polyanions associated with the cationic lipid. In particular this integer ranges in magnitude from 0 to a
30 number equivalent to the positive charge(s) present on

the lipid.

The cationic lipids of the present invention include salts, solvates, or enantiomeric isomers resulting from any or all asymmetric atoms present in
5 the lipid. Included in the scope of the invention are racemic mixtures, diastereomeric mixtures, optical isomers or synthetic optical isomers which are isolated or substantially free of their enantiomeric or diastereomeric partners. The racemic mixtures may be
10 separated into their individual, substantially optically pure isomers by techniques known in the art, such as, for example, the separation of diastereomeric salts formed with optically active acid or base adjuncts followed by conversion back to the optically active
15 substances. In most instances, the desired optical isomer is synthesized by means of stereospecific reactions, beginning with the appropriate stereoisomer of the desired starting material. Methods and theories used to obtain enriched and resolved isomers have been
20 described (Jacques *et al.*, "Enantiomers, Racemates and Resolutions." Kreiger, Malabar, FL, 1991).

The salts include pharmaceutically or physiologically acceptable non-toxic salts of these compounds. Such salts may include those derived by
25 combination of appropriate cations such as alkali and alkaline earth metal ions or ammonium and quaternary amino ions with the acid anion moiety of the phosphate or phosphorothioate acid group present in polynucleotides. Suitable salts include for example,
30 acid addition salts such as HCl, HBr, HF, HI, H₂SO₄, and

trifluoroacetate. The salts may be formed from acid addition of certain organic and inorganic acids, e.g., HCl, Hbr, H₂SO₄, amino acids or organic sulfonic acids, with basic centers, (e.g. amines), or with acidic
5 groups. The composition herein also comprise compounds of the invention in their un-ionized, as well as zwitterionic forms.

Exemplary invention cationic lipids have the structures that are provided in the Summary of the
10 Invention above.

The cationic lipids form aggregates with polyanionic macromolecules such as oligonucleotides, oligomers, peptides, or polypeptides through attraction between the positively charged lipid and the negatively
15 charged polyanionic macromolecule. The aggregates may comprise multiamellar or unilamellar liposomes or other particles. Hydrophobic interactions between the cationic lipids and the hydrophobic substituents in the polyanionic macromolecule such as aromatic and alkyl
20 moieties may also facilitate aggregate formation.

Cationic lipids have been shown to efficiently deliver nucleic acids and peptides into cells in the presence of serum and thus are suitable for use *in vivo* or *ex vivo*.

Cationic lipid-polyanionic macromolecule aggregates
25 may be formed by a variety of method known in the art. Representative methods are disclosed by Felgner *et al.*, *supra*; Eppstein *et al. supra*; Behr *et al. supra*; Bangham, A. *et al. M. Mol. Biol.* 23:238, 1965; Olson, F. *et al. Biochim. Biophys. Acta* 557:9, 1979; Szoka, F. *et al. Proc. Natl. Acad. Sci.* 75: 4194, 1978; Mayhew, E. *et*
30

al. Biochim. Biophys. Acta 775:169, 1984; Kim, S. *et al. Biochim. Biophys. Acta* 728:339, 1983; and Fukunaga, M. *et al. Endocrinol.* 115:757, 1984. Commonly used techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion. See, e.g., Mayer, L. *et al. Biochim. Biophys. Acta* 858:161, 1986. Microfluidization is used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew, E., *supra*). In general aggregates may be formed by preparing lipid particles consisting of either (1) a cationic lipid of the invention or (2) a cationic lipid mixed with a colipid, followed by adding a polyanionic macromolecule to the lipid particles at about room temperature (about 18 to 26° C). In general, conditions are chosen that are not conducive to deprotection of protected groups. The mixture is then allowed to form an aggregate over a period of about 10 minutes to about 20 hours, with about 15 to 60 minutes most conveniently used. The complexes may be formed over a longer period, but additional enhancement of transfection efficiency will not usually be gained by a longer period of complexing. Colipids that are suitable for preparing lipid aggregates with the cationic lipids of the present invention are dimyristoylphosphatidylethanolamine, dipalmitoyl-phosphatidylethanolamine, palmitoyloleolphosphatidyl-ethanolamine, cholesterol, distearoyalphosphatidyl-ethanolamine, phosphatidylethanolamine covalently linked to polyethylene glycol and mixtures of these colipids.

The optimal cationic lipid:colipid ratios for a given cationic lipid is determined by mixing experiments to prepare lipid mixtures for aggregation with a polyanionic macromolecule using cationic
5 lipid:colipid ratios between about 1:0 and 1:10. Methods to determine optimal cationic lipid:colipid ratios have been described (see, Felgner, *infra*). Each lipid mixture is optionally tested using more than one oligonucleotide-lipid mixture having different nucleic
10 acid:lipid molar ratios to optimize the oligonucleotide:lipid ratio.

Suitable molar ratios of cationic lipid:colipid are about 0.1:1 to 1:0.1, 0.2:1 to 1:0.2, 0.4:1 to 1:0.4, or 0.6:1 to 1:0.6. Lipid particle preparation containing
15 increasing molar proportions of colipid have been found to enhance oligonucleotide transfection into cells with increasing colipid concentrations.

In addition, the cationic lipids can be used together in admixture, or different concentrations of
20 two or more cationic lipids in admixture, with or without colipid.

Liposomes or aggregates are conveniently prepared by first drying the lipids in solvent (such as chloroform) under reduced pressure. The lipids are then
25 hydrated and converted to liposomes or aggregates by adding water or low ionic strength buffer (usually less than about 200 mM total ion concentration) followed by agitating (such as vortexing and/or sonication) and/or freeze/thaw treatments. The size of the aggregates or
30 liposomes formed range from about 40 nm to 600 nm in

diameter.

The amount of an oligonucleotide delivered to a representative cell by at least some of the cationic lipids was found to be significantly greater than the amount delivered by commercially available transfection lipids. The amount of oligonucleotide delivered into cells was estimated to be about 2- to 100-fold greater for these cationic lipids of the invention based on the observed fluorescence intensity of transfected cells after transfection using a fluorescently labeled oligonucleotide. The cationic lipids described herein also transfect some cell types that are not detectably transfected by commercial lipids. Functionality of cationic lipid-DNA aggregates was demonstrated by assaying for the gene product of the exogenous DNA. Similarly the functionality of cationic lipid-oligonucleotide aggregates were demonstrated by antisense inhibition of a gene product.

The cationic lipids described herein also differed from commercially available lipids by efficiently delivering an oligonucleotide into cells in tissue culture over a range of cell confluency from about 50 to 100%. Most commercially available lipids require cells that are at a relatively narrow confluency range for optimal transfection efficiency. For example, Lipofectin™ requires cells that are 70-80% confluent for transfecting the highest proportion of cells in a population. The cationic lipids described herein may be used to transfect cells that are about 10-50% confluent, but toxicity of the lipids was more pronounced, relative

to that seen using cells that are about 50-100% confluent. In general, the cationic lipids transfected cells that were about 60-100% confluent with minimal toxicity and optimal efficiency. Confluency ranges of 5 60-95% or 60-90% are thus convenient for transfection protocols with most cell lines in tissue culture.

The cationic lipid aggregates were used to transfect cells in tissue culture and the RNA and the DNA encoded gene products were expressed in the 10 transfected cells.

The cationic lipid aggregates may be formed with a variety of macromolecules such as oligonucleotides and oligomers. Oligonucleotides used in aggregate formation may be single stranded or double stranded DNA or RNA, 15 oligonucleotide analogs, and plasmids.

In general, relatively large oligonucleotides such as plasmids or mRNAs will carry one or more genes that are to be expressed in a transfected cell, while comparatively small oligonucleotides will comprise (1) a 20 base sequence that is complementary (via Watson Crick or Hoogsteen binding) to a DNA or RNA sequence present in the cell or (2) a base sequence that permits oligonucleotide binding to a molecule inside a cell such as a peptide, protein or glycoprotein. Exemplary RNAs 25 include ribozymes and antisense RNA sequences that are complementary to a target RNA sequence in a cell.

An Oligonucleotide may be a single stranded unmodified DNA or RNA comprising (a) the purine or pyrimidine bases guanine, adenine, cytosine, thymine 30 and/or uracil: (b) ribose or deoxyribose; and (c) a

phosphodiester group that linkage adjacent nucleoside moieties. Oligonucleotides typically comprise 2 to about 100 linked nucleosides. Typical oligonucleotides range in size from 2-10, 2-15, 2-20, 2-25, 2-30, 2-50, 8-20, 5 8-30 or 2-100 linked nucleotides. Oligonucleotides are usually linear with uniform polarity and, when regions of inverted polarity are present, such regions comprise no more than one polarity inversion per 10 nucleotides. One inversion per 20 nucleotides is typical.

10 Oligonucleotides can also be circular, branched or double-stranded. Antisense oligonucleotides generally will comprise a sequence of about from 8-30 bases or about 8-50 bases that is substantially complementary to a DNA or RNA base sequence present in the cell. The 15 size of oligonucleotide that is delivered into a cell is limited only by the size of polyanionic macromolecules that can reasonably be prepared and thus DNA or RNA that is 0.1 to 1 Kilobase (Kb), 1 to 20 Kb, 20 Kb to 40 Kb or 40 Kb to 1,000 Kb in length may be delivered into cells.

20 Oligonucleotides also include DNA or RNA comprising one or more covalent modifications. Covalent modifications include (a) substitution of an oxygen atom in the phosphodiester linkage of an polynucleotide with a sulfur atom, a methyl group or the like, (b) 25 replacement of the phosphodiester group with a nonphosphorus moiety such as $-O-CH_2O-$, $-S-CH_2O-$ or $-O-CH_2O-S$, and (c) replacement of the phosphodiester group with a phosphate analog such as $-O-P(S)(O)-O-$, $-O-P(S)(S)-O-$, $-O-P(CH_3)(O)-O-$ or $-O-P(NHR^{10})(O)-O-$ where R^{10} 30 is alkyl (C_{1-6}), or an alkyl ether (C^{1-6}). Such

substitutions may constitute from about 10% to 100% or about 20 to about 80% of the phosphodiester groups in unmodified DNA or RNA. Other modifications include substitutions of or on sugar moiety such as morpholino, arabinose 2'-fluororibose, 2'-fluoroarabinose, 2'-O-methylribose or 2'-O-allylribose. Oligonucleotides and methods to synthesize them have been described (for example see: U.S. Patent Application Serial Nos. 08/154,013, filed November 16, 1993, 08/154,014, filed November 16, 1993, 08/001,179, filed June 6, 1993, and 08/233,778 filed May 4, 1994, PCT/US90/03138, PCT/US90/06128, PCT/US90/06090, PCT/US90/06110, PCT/US92/03385, PCT/US91/08811, PCT/US91/03680, PCT/US91/06855, PCT/US91/01141, PCT/US92/10115, PCT/US92/10793, PCT/US93/05110, PCT/US93/05202, PCT/US92/04294, WO 86/05518, WO 89/12060, WO 91/08213, WO 90/15065, WO 91/15500, WO 92/02258, WO 92/20702, WO 92/20822, WO 92/20823, U.S. Patent No.: 5,214,136 and Uhlmann, *Chem Rev.* 90:543, 1990). Oligonucleotides are usually linear with uniform polarity and, when regions of inverted polarity are present, such regions comprise no more than one polarity inversion per 10 nucleotides. One inversion per 20 nucleotides is typical. Oligonucleotides may also be circular, branched or double-stranded.

The linkage between the nucleotides of the oligonucleotide may be a variety of moieties including both phosphorus-containing moieties and non phosphorus-containing moieties such as formacetal, thioformacetal, riboacetal and the like. A linkage usually comprises 2

or 3 atoms between the 5' position of a nucleotide and the 2' or 3' position of an adjacent nucleotide. However, other synthetic linkers may contain greater than 3 atoms.

5 The bases contained in the oligonucleotide may be unmodified or modified or natural or unnatural purine or pyrimidine bases and may be in the α or β anomer. Such bases may be selected to enhance the affinity of oligonucleotide binding to its complementary sequence
10 relative to bases found in native DNA or RNA. However, it is preferable that modified bases are not incorporated into an oligonucleotide to an extent that it is unable to bind to complementary sequences to produce a detectably stable duplex or triplex.

15 Exemplary bases include adenine, cytosine, guanine, hypoxanthine, inosine, thymine, uracil, xanthine, 2-aminopurine, 2,6-diaminopurine, 5-(4-methylthiazol-2-yl)uracil, 5-(5-methylthiazol-2-yl)uracil, 5-(4-methylthiazol-2-yl)cytosine, 5-(5-methylthiazol-2-yl)cytosine and the like. Other exemplary bases include
20 alkylated or alkynylated bases having substitutions at, for example, the 5 position of pyrimidines that results in a pyrimidine base other than uracil, thymine or cytosine, (*i.e.*, 5-methylcytosine, 5-(1-propynyl)cytosine, 5-(1-butynyl)cytosine, 5-(1-butynyl)uracil, 5-(1-propynyl)uracil and the like). The use of modified bases or base analogs in oligonucleotides have been previously described (see
25 PCT/US92/10115; PCT/US91/08811; PCT/US92/09195; WO 92/09705; WO 92/02258; Nikiforov, *et al.*, *Tet. Lett.*

33:2379, 1992; Clivio, et al., *Tet. Lett.* 33:65, 1992; Nikiforov, et al., *Tet. Lett.* 32:2505, 1991; Xu, et al., *Tet. Lett.* 32:2817, 1991; Clivio, et al., *Tet. Lett.* 33:69, 1992; Connolly, et al., *Nucl. Acids Res.* 17:4957, 5 1989).

Aggregates may comprise oligonucleotides or oligomers encoding a therapeutic or diagnostic polypeptide. Examples of such polypeptides include histocompatibility antigens, cell adhesion molecules, 10 cytokines, antibodies, antibody fragments, cell receptor subunits, cell receptors, intracellular enzymes and extracellular enzymes or a fragment of any of these. The oligonucleotides also may optionally comprise expression control sequences and generally will comprise 15 a transcriptional unit comprising a transcriptional promoter, an enhancer, a transcriptional terminator, an operator or other expression control sequences.

Oligonucleotides used to form aggregates for transfecting a cell may be present as more than one 20 expression vector. Thus, 1, 2, or 3 or more different expression vectors may be delivered into a cell as desired. Expression vectors will typically express 1, 2, or 3 genes when transfected into a cell, although many genes may be present such as when a herpes virus vector 25 or a yeast artificial chromosome is delivered into a cell. Expression vectors that are introduced into a cell can encode selectable markers (e.g. neomycin phosphotransferase, thymidine kinase, xanthine-guanine phosphoribosyl-transferase, and the like) or 30 biologically active proteins such as metabolic enzymes

or functional proteins (e.g. immunoglobulin genes, cell receptor genes, cytokines (e.g. IL-2, IL-4, GM-CSF, γ -INF and the like), or genes that encode enzymes that mediate purine or pyrimidine metabolism and the like).

5 The nucleic acid sequence of the oligonucleotide coding for specific genes of interest may be retrieved, without undue experimentation, from the GenBank of EMBL DNA libraries. Such sequences may include coding sequences, for example, the coding sequences for
10 structural proteins, hormones, receptors and the like, and the DNA sequences for other DNAs of interest, for example, transcriptional and translational regulatory elements (promoters, enhancers, terminators, signal sequences and the like), vectors (integrating or
15 autonomous) and the like. Non-limiting examples of DNA sequences which may be introduced into cells with the reagent of the invention include those sequences coding for fibroblast growth factor (see WO 87/01728); ciliary neurotrophic factor (Lin et al., *Science*, 246:1023,
20 1989; human interferon- α receptor (Uze, et al., *Cell*, 60:225, 1990; the interleukins and their receptors (reviewed in Mizal, *FASEB J.*, 3:2379, 1989; hybrid interferons (see EPO 051,873); the RNA genome of human rhinovirus (Callahan, *Proc. Natl. Acad. Sci.*, 82:732,
25 1985; antibodies including chimeric antibodies (see U.S. Pat. No.: 4,816,567); reverse transcriptase (see Moelling, et al., *J. Virol.*, 32:370, 1979; human CD4 and soluble forms thereof (Maddon et al., *Cell*, 47:333, 1986, see WO 88/01304 and WO 89/01940); and EPO 330,191,
30 which discloses a rapid immunoselection cloning method

useful for the cloning of a large number of desired proteins.

Aggregates can be used in antisense inhibition of gene expression in a cell by delivering an antisense
5 oligonucleotide into the cell (see Wagner, *Science* 260:1510, 1993 and WO 93/10820). Such oligonucleotides will generally comprise a base sequence that is complementary to a target RNA sequence that is expressed by the cell. However, the oligonucleotide may regulate
10 intracellular gene expression by binding to an intracellular nucleic acid binding protein (see Clusel, *Nucl. Acids Res.* 21:3405, 1993) or by binding to an intracellular protein or organelle that is not known to bind to nucleic acids (see WO 92/14843). A cell that is
15 blocked for expression of a specific gene(s) is useful for manufacturing and therapeutic applications. Exemplary manufacturing uses include inhibiting protease synthesis in a cell to increase production of a protein for a therapeutic or diagnostic application (e.g.,
20 reduce target protein degradation caused by the protease). Exemplary therapeutic applications include inhibiting synthesis of cell surface antigens to reduce rejection and/or to induce immunologic tolerance of the cell either after it is implanted into a subject or when
25 the cell is transfected *in vivo* (e.g. histocompatibility antigens, such as MHC class II genes, and the like).

Methods to introduce aggregates into cells *in vitro* and *in vivo* have been previously described (see U.S. Patent Nos.: 5,283,185 and 5,171,678; WO 94/00569; WO
30 93/24640; WO 91/16024; Felgner, *J. Biol Chem* 269:2550,

1994; Nabel, *Proc. Natl. Acad. Sci.* 90:11307, 1993;
Nabel, *Human Gene Ther.* 3:649, 1992; Gershon, *Biochem.*
32:7143, 1993; and Strauss *EMBO J.* 11:417, 1992.

Entry of liposomes or aggregates into cells may be
5 by endocytosis or by fusion of the liposome or aggregate
with the cell membrane. When fusion takes place, the
liposomal membrane is integrated into the cell membrane
and the aqueous contents of the liposome merge with the
fluid in the cell.

10 Endocytosis of liposomes occurs in a limited class
of cells; those that are phagocytic, or able to ingest
foreign particles. When phagocytic cells take up
liposomes or aggregates, the cells move the spheres into
subcellular organelles known as lysosomes, where the
15 liposomal membranes are thought to be degraded. From
the lysosome, the liposomal lipid components probably
migrate outward to become part of cell's membranes and
other liposomal components that resist lysosomal
degradation (such as modified oligonucleotides or
20 oligomers) may enter the cytoplasm.

Lipid fusion involves the transfer of individual
lipid molecules from the liposome or aggregate into the
plasma membrane (and vice versa); the aqueous contents
of the liposome may then enter the cell. For lipid
25 exchange to take place, the liposomal lipid must have a
particular chemistry in relation to the target cell.
Once a liposomal lipid joins the cell membrane it can
either remain in the membrane for a period of time or be
redistributed to a variety of intracellular membranes.
30 The invention lipids can be used to deliver an

expression vector into a cell for manufacturing or therapeutic use. The expression vectors can be used in gene therapy protocols to deliver a therapeutically useful protein to a cell or for delivering nucleic acids encoding molecules that encode therapeutically useful proteins or proteins that can generate an immune response in a host for vaccine or other immunomodulatory purposes according to known methods (see U.S. Patent Nos.: 5,399,346 and 5,336,615, WO 94/21807 and WO 94/12629). The vector-transformed cell can be used to produce commercially useful cell lines, such as a cell line for producing therapeutic proteins or enzymes (e.g. erythropoietin, and the like), growth factors (e.g. human growth hormone, and the like) or other proteins. The aggregates may be utilized to develop cell lines for gene therapy applications in humans or other species including murine, feline, bovine, equine, ovine or non-human primate species. The aggregates may be used in the presence of serum and will thus deliver polyanionic macromolecules into cells in tissue culture medium containing serum *in vitro* or in an animal *in vivo*.

Description of the Figures

Figure 1 depicts a synthetic scheme for the preparation of compounds 1, 2 and 3.

Figure 2 depicts the structure of 4 (Cholest-5-en-3-ol (3 β)-, [2-2,3-diamino-1-oxopentyl)amino]ethyl]carbamate, dihydrochloride) and 5 (N- α --(6-carboxyspermino)-N,N-dioctadecylglutamic acid- γ -amide).

Figure 3 depicts the combination of cationic lipids 1 and 4 delivers plasmids and oligodeoxynucleotides to cells.

Figure 4 depicts the demonstration of nuclear delivery of oligonucleotides of varying charge densities by novel cationic lipids in COS-7 and SNB-19 cells.

The following examples are offered by way of illustration and not by way of limitation.

Examples

10 General Methods

All reactions were run under a positive pressure of dry argon. Reactions requiring anhydrous conditions were performed in flame-dried glassware which was cooled under argon. Tetrahydrofuran (THF, Aldrich Milwaukee, WI) was distilled from potassium/benzophenone ketyl immediately prior to use. Methylene chloride, pyridine, toluene, heptane, methanol, and ethanol were obtained as anhydrous reagent (<0.005% water) or reagent grade and were used without further purification. TLC was performed on 0.2 mm E. Merck precoated silica gel 60 F₂₅₄ TLC plates (20 x 20 cm aluminum sheets, Fisher, PA). Flash chromatography was performed using E. Merck 230-400 mesh silica gel. All ¹H, ¹³C and ³¹P NMR spectra were recorded on a 300 MHz Bruker ARX Spectrometer (Bruker, Boston, MA) and were obtained in CDCl₃, unless otherwise indicated. Mass spectra were provided by The Scripps Research Institute Mass Spectrometry Facility of La Jolla, CA. FAB mass spectra were obtained on a FISON S VG

ZAB-VSE double focussing mass spectrometer (Fisons, Altrincham UK) equipped with a cesium ion gun. ESI mass spectra were obtained on an API III PE Sciex triple-quadrupole mass spectrometer (Sciex, Toronto CA).

5 **Example 1: Synthesis of 1 ((3 β)-Cholest-5-en-3-ol, 2-(dimethylamino)ethyl methylphosphonate)**

Reagents were purchased from Aldrich (Milwaukee, WI) and used without further purification: cholesterol (36,279-4); methylphosphonic dichloride (22,805-2);
10 imidazole (I-20-2); acetonitrile anhydrous (27,100-4); dichloromethane anhydrous (27,099-7). N,N-dimethylethanolamine (D15,740-6) was distilled at reduced pressure prior use.

All steps of the process were monitored by ³¹P NMR
15 spectroscopy.

To a solution of imidazole (5.5 g; (81 mmol)) in hot (50° C) acetonitrile (16 mL) was added a solution of methylphosphonic dichloride (2.7 g; (20.3 mmol)) in warm (40° C) acetonitrile (4 mL). After 2-3 minutes a white
20 precipitate of imidazolium chloride appeared. The reaction mixture was allowed to stand for 15 minutes more followed by addition of N,N-dimethylethanolamine (R₁OH, 1.8 mL; (20.3 mmol)). After 2-3 minutes at room temperature all the precipitate dissolved and a
25 homogeneous solution was obtained. The solution was kept at room temperature for 30 minutes followed by the addition of a solution of cholesterol (R₂OH, 5.4 g; (13.9 mmol)) in dichloromethane (20 mL). After 3 hours at room

temperature the mixture was evaporated to yield an oil. The oil was dissolved in dichloromethane (100 mL) and the organic layer was washed with water (2 x 50 mL), evaporated from absolute ethanol (30 mL) separated from
5 water, and evaporated 3 times to an oil state adding 20-30 mL dichloromethane. The crude product was purified by flash chromatography on silica gel (5% methanol in dichloromethane). Fractions containing the product were combined and evaporated under high vacuum to give 3.4 g
10 (6.3 mmol); 46% yield; calculated on cholesterol used) of a white solid. Rf 0.26 (1:19 methanol:dichloromethane). ^{31}P - $\{^1\text{H}\}$ NMR (121.4 MHz; CDCl_3 ; mixture of diastereomers) ppm: 29.98 and 30.58. ^1H NMR (300 MHz; CDCl_3 ; mixture of diastereomers) ppm: 5.33
15 (d, 1H); 4.23 (m, 1H); 4.04 (m, 2H); 2.53 (d-t, 2H); 2.40 (d, 2H), 2.24 (d, 6H); 1.5-2.0 (overlap m, 6H); 1.0-1.3 (overlap m, 11H) 1.45 (d-d, 3H; P- CH_3 , J=17 Hz); 0.8-0.9 (d+d, 10H); 0.96 (s, 3H), 0.63 (s, 3H).

**Example 2: Synthesis of 2 ((3 β)-Cholest-5-en-3-ol, 2-
20 ((2,5-diamino-1-oxopentyl)amino)ethyl methylphosphonate, dihydrochloride)**

Reagents were purchased from Aldrich and used without purification: cholesterol (36,279-4); methylphosphonic dichloride (22,805-2) ; imidazole (I-
25 20-2); acetonitrile (anhydrous) (27,100-4); dichloromethane anhydrous (27,099-7).

N- α -N- δ -Bis-Boc-L-Ornithine-[N-(2-hydroxyethyl)]amide was prepared as follows and used

without further purification. To a solution of Bis-Boc-L-ornithine (15 mmol) in acetonitrile (150 mL) was added N-hydroxysuccinimide (15 mmol) followed by dicyclohexylcarbodiimide (16.5 mmol). After 1 hour the
5 dicyclohexylurea was filtered away, and ethanolamine (22.5 mmol) was added. The reaction proceeded overnight at room temperature. The solvent was removed, and the residue was taken up in ethylacetate. The organic layer was washed with 10 % citric acid, water, saturated
10 sodium bicarbonate, and brine. After drying with anhydrous magnesium sulfate the solvent was removed under reduced pressure. ¹H NMR (300 MHz, CDCl₃, TMS = 0), δ 7.3 (bm, 1H), 5.68 (bd, 1H, J= 7.0), 5.06 (bm, 1H), 4.21 (bm, 1H), 3.68 (t, 2H, J = 4.9), 3.41 (m, 2H),
15 3.30-3.00 (m, 2H), 1.90-1.48 (m, 4H), 1.43 (s, 18H).

All main steps of the process were monitored by ³¹P NMR spectroscopy.

To a solution of of imidazole (2.2 g; (32.3 mmol)) in 5 mL of hot (50° C) acetonitrile was added a solution
20 of methylphosphonic dichloride (1.0 g; (7.5 mmol)) in of warm (40° C) acetonitrile (3 mL). After 2-3 minutes a white precipitate of imidazolium chloride appeared. The reaction mixture was allowed to stand for an additional 15 minutes more followed by addition of cholesterol (3.1
25 g (8.0 mmol)) in dichloromethane (8 mL). After stirring the mixture for 1 hour at room temperature a solution of N-α-N-δ-Bis-Boc-L-ornithine-[N-(2-hydroxyethyl)]amide (1.95 g (5.2 mmol)) in acetonitrile (5 mL) was added. After stirring for 2 hours at room temperature mixture
30 was evaporated to an oil state and dissolved in 100 mL

of dichloromethane. Organic layer was washed 2 times with water (50 mL), separated from water and interface layers, and evaporated 3 times to an oil state adding 20-30 mL dichloromethane. The crude product was purified
5 by flash chromatography on silica gel (4% methanol in dichloromethane). Fractions containing the product were combined and evaporated under high vacuum to give a colorless oil. The residue was dissolved in 10 mL of dry dioxane and 10 mL of 7M HCl in dry dioxane was added.
10 Shortly thereafter a white precipitate appeared. After 1 hour at room temperature the mixture was evaporated to dryness and evaporated under high vacuum to give 1.56 g (1.75 mmol); 34% yield of a white powder. R_f 0.22 (1:24 methanol:dichloromethane) ^{31}P - $\{^1\text{H}\}$ NMR (121.4 MHz; CDCl_3 ; mixture of diastereomers) ppm: 30.53 and 30.64. ^1H NMR
15 (300 MHz; $\text{CD}_3\text{OD}:\text{CDCl}_3 = 2:1$; mixture of diastereomers) ppm: 5.05 (d, 1H); 3.83 (m, 2H); 3.62 (m, 2H); 2.63 (bt, 2H); 2.05 (m, 1H), 1.3-1.8 (overlap m, 6H); 0.8-1.3 (overlap m, 8H) 1.23 (d-d, 3H; P- CH_3 , $J=17$ Hz); 0.69 (s,
20 3H); 0.5-0.7 (d+d, 10H); 0.96 (s, 3H), 0.35 (s, 3H).

Example 3: Synthesis of 3 (Phosphonic acid, methyl-, 2-(dimethylamino)ethyl 1-hepadecyloctadecyl ester)

Reagents were purchased from Aldrich and used without further purification: methylphosphonic
25 dichloride (22,805-2) ; imidazole (I-20-2); acetonitrile anhydrous (27,100-4); dichloromethane anhydrous (27,099-7).

N,N-dimethylethanolamine (D15,740-6) was distilled

at reduced pressure prior use.

18-Hydroxypentatriacontane was prepared as follows and used without further purification. In a dry flask charged with argon was prepared a solution of lithium
5 aluminum hydride (0.75 g, (19.8 mmol)) in freshly distilled tetrahydrofuran (400 mL). To this solution was added stearone (5.0 g, (9.9 mmol)). The reaction mixture was slowly brought to reflux (and allowed to stir at reflux overnight). After cooling to room
10 temperature, the remaining lithium aluminum hydride was quenched by the dropwise addition of water (0.76 mL), followed by 15 % NaOH (0.76 mL), and water again (2.2 mL). The reaction mixture was allowed to stir for 1 hour. The solvent was evaporated, and the resulting
15 solids were triturated with hot toluene (300 mL). The toluene was evaporated to yield the desired alcohol (4.9g, 97%). ¹H NMR (300 MHz, CDCl₃, TMS = 0), δ 3.58 (m, 1H), 1.38 (m, 4H), 1.25 (bs, 60H), 0.88 (t, 6H, J = 6.5).

20 All main steps of the process were monitored by ³¹P NMR spectroscopy.

To a solution of imidazole (2.9 g (42.5 mmol)) in hot (50° C) acetonitrile (6 mL) was added a solution of methylphosphonic dichloride (1.4 g; (10.4
25 mmol)) in warm (40° C) acetonitrile (4 mL). After 2-3 minutes a white precipitate of imidazolium chloride appeared. The reaction mixture was allowed to stay 15 minutes more followed by addition of N,N-dimethylethanolamine (1.04 mL (10.4 mmol)). After 2-3
30 minutes at room temperature all precipitate dissolved

and a clean solution was obtained. The solution was kept at room temperature for 30 minutes followed by the addition of a solution of 18-hydroxypentatriacontane (5.3 g (10.4 mmol)) in of hot (50° C) dichloromethane (50 mL). The mixture was refluxed for 3 hours until all 18-hydroxypentatriacontane solid dissolved. The reaction mixture was evaporated to yield an oil which was evaporated from ethanol (30 mL) dissolved in dichloromethane (100 mL). The organic layer was washed with water (2 x 50 mL), and evaporated to yield an oil which was evaporated from dichloromethane (3 x 20-30 mL). The crude product was purified by flash chromatography on silica gel (5% methanol in dichloromethane), fractions containing the product were combined, evaporated and left to stand under high vacuum to give colorless oil. The oil was dissolved in dry dioxane (10 mL) and 7 M HCl in dry dioxane (10 mL) in dry dioxane were added. After 15 minutes the mixture was evaporated to dryness and left to stand under high vacuum to give 2.45 g (3.7 mmol; 36% yield) of white solid. Rf 0.32 (1:19 methanol:dichloromethane); ³¹P-{¹H} NMR (121.4 MHz; CDCl₃) ppm: 29.98. ¹H NMR (300 MHz; CDCl₃) ppm: 4.38 (m, 1H); 3.95-4.20 (m, 2H); 2.54 (t, 2H); 2.24 (s, 6H); 1.54 (overlap m, 4H); 1.44 (d, 3H; P-CH₃, J=17 Hz); 1.21 (bs, 62H); 0.84 (t, 6H).

Example 4: Cell Preparation and Treatment

COS 7 cells (ATCC # 1651) were plated at 1.5 x 10⁵ cells/well in a 12 well plate format on the day before transfections began. All cultures were maintained at

37°C in 5% CO₂. On the next day, the transfection mixes were prepared as follows: 4 µg of the target CAT plasmid (pG1040, the UCAT chloramphenicol acetyl transferase gene is inserted into pRc/CMV from Invitrogen, San Diego, CA, see Example 5), was combined with 10, 50, 100, or 400 nanomolar final phosphorothioate oligomer (synthesized by JBL Scientific, San Luis Obispo, CA) in 2 mL Opti-MEM® (Gibco/BRL, Gaithersburg, MD) The oligomer/plasmid mixes were combined with 24 micrograms of lipid and gently vortexed. The final plasmid concentration was 2 µg/mL and the final lipid concentration was 12 mg/mL. The final result was four sets of transfection mixes tested in duplicate:

- 15 pG1040 plasmid + 2519-1 oligomer + Lipofectin
 pG1040 plasmid + 2520-1 oligomer + Lipofectin
 pG1040 plasmid + 2519-1 oligomer + GC-001/GC-003
 lipid mix
 pG1040 plasmid + 2520-1 oligomer + GC-001/GC-003
 20 lipid mix

Phosphorothioate oligomer 2519-1 (5'-tag-ctt-cct-tag-ctc-ctg-cat) is complimentary to pG1040 from +1 to +21 on the CAT mRNA.

Phosphorothioate oligomer 2520-1 (5' tag-ctt-ccg-
 25 caa-ctc-ttg-cat) is a 4 mismatch control phosphorothioate, also from +1 to +21 on the CAT mRNA.

The culture medium was aspirated and the cells were rinsed twice in one mL Opti-MEM® per well, followed by

the addition of 1 mL of transfection to each well. The cells were cultured in the transfection mix for 16 hours. The mix was removed and replaced with 1 mL of complete culture medium (DMEM plus 10% fetal bovine serum and 1/100 dilution of penicillin/streptomycin stock, all from Gibco/BRL, Gaithersburg, MD) and the cells were incubated another 5 hours.

Cell lysates were prepared by rinsing twice in PBS and then treated with 0.5 mL of 1X Reporter Lysis Buffer (Promega, Madison, WI). The lysed cells were pipetted into 1.5 mL tubes and frozen in CO₂/EtOH once and thawed. The crude lysate was then clarified by microcentrifugation at 14,000 rpm for 10 minutes to pellet cell debris. The supernatant was recovered and assayed directly or frozen at -20°C.

The cell lysates were then assayed for CAT activity and the total protein concentration was determined as described below. The CAT activity was normalized to total protein and plotted as shown.

20

Chloramphenicol Acetyltransferase Assay

The following reaction mixture was prepared for each sample:

65 mL 0.23 M Tris, pH 8/0.5% BSA (Sigma, St. Louis, MO),

4 μ L ¹⁴C-chloramphenicol, 50 nCi/ μ L (Dupont, Boston, MA), and

5 μ L mg/mL n-butyryl coenzyme A (Pharmacia, Piscataway, NJ)

A CAT activity standard curve was prepared by

serially diluting CAT stock (Promega, Madison, WI) 1:1000, 1:10,000 and 1:90,000 in 0.25 M Tris, pH 8/0.5% BSA. The original stock CAT was at 7000 Units/mL. CAT lysate was then added in a labeled tube with Tris/BSA
5 buffer for final volume of 50 μ L.

Approximately, 74 μ L of reaction mixture was added to each tube, which was then incubated for approximately 1 hour in a 37°C oven. The reaction was terminated by adding 500 μ L pristane/mixed xylenes (2:1) (Sigma, St.
10 Louis, MO) to each tube. The tubes were then vortexed for 2 minutes and spun for 5 minutes. Approximately 400 μ L of the upper phase was transferred to a scintillation vial with 5 mL Scintiverse (Fisher, Pittsburgh, PA). The sample was then counted in a scintillation counter
15 (Packard).

Coomassie Protein Assay

The total protein content of the clarified cell lysates was determined by mixing 6 μ L of each cell lysate to 300 μ L of Coomassie protein assay reagent
20 (Pierce, Rockford, IL) in the wells of an untreated microtiter assay plate. Concentration curve standards were prepared using 6 μ L of 0, 75, 100, 200, 250, 400, 500, 1000, and 1500 mg/mL BSA stock solutions and 300 μ L of the Coomassie reagent. The assay samples were
25 allowed to sit for approximately 30 minutes before reading the optical absorbance at 570 nm in a microplate reader (Molecular Probes).

Results indicate that the combination of 1 and 4 was able to deliver oligo/plasmid better than

lipofectamine. Virtually no effect was seen with lipofectamine from 0.01 to 0.1 micromolar. With the combination of 1 and 4, specific inhibition was very clear at 0.1 μ M oligomer, and the trend from 0.01 to 0.1
5 indicates a reasonable dose response. From these data, it seemed that the combination of 1 and 4 was able to deliver phosphorothioate oligonucleotides better than Lipofectin or plasmid alone.

The lack of activity against pG1040 by the control
10 oligomer, 2520, indicates that this is an actual antisense effect, and not due to an artifact such as oligomer interference with plasmid delivery.

**Example 5: Preparation and transfection protocol of COS-7, SNB-19, RD and C8161 cells with the combination
15 of cationic lipids 3 and 5 and CAT plasmid.**

All cell lines were plated at 1.5×10^5 cells/well in a 12 well plate format on the day before tranfection. All cultures were maintained at 37°C in 5% CO₂. On the next day, when the cells reached approximately 80%
20 confluence, the transfection mixes were prepared as follows: 126 μ g of the target CAT plasmid (pG1035, another chloramphenicol acetyl transferase genes in the was inserted into pRc/CMV from Invitrogen, see below), was added to 36.0 mL of Opti-MEM® to make a plasmid
25 stock solution. 63 μ g of each lipid mix (from a high concetration stock in 100% ethanlol) was added to individual 1.5 mL aliquotes of Opti-MEM® and mixed thoroughly. Then 2 mL of the DNA stock (containing 7 μ g of plasmid) were added to each 1.5 mL aliquote of lipid/

Opti-MEM® and gently vortexed. This procedure yielded 3.5 mL of plasmid/lipid mixture at 2 µg/mL plasmid and 18 µg/mL lipid for a 9 to 1 lipid to DNA ratio. The quantity of ethanol in the final cell cultures was 2% or less. This small quantity of ethanol was confirmed to have no adverse effect on any of the cell lines. The cells were assayed for CAT protein as described in Example 4.

Example 6: Methods for FITC-Oligonucleotide Uptake

10 Assay

The oligonucleotides used for the determination of cationic lipid mediated oligonucleotide uptake in all cell lines tested were:

#3498-PS: 5' FITC-ggt-ata-tcc-agt-gat-ctt-ctt-ctc,
15 all-phosphorothioate backbone. This oligonucleotide has 23 negative charges on the backbone, and is considered 100% negatively charged.

#3498: 5' FITC-ggt-ata-tcc-agt-gat-ctt-ctt-ctc,
chimeric oligonucleotide. The underlined bases were
20 linked by a phosphorothioate backbone, while the other linkages in the oligomer consisted of alternating chirally pure Rp-methylphosphonates and phosphodiester. The oligomer had 11 methylphosphate, 7 diester, and 5 phosphorothioates linkages. The total charge density
25 was 57% of 3498-PS.

#3793-2: 5' FITC-ggu-aua-ucc-agu-gau-cuu-cut,
alternating chirally pure Rp methylphosphonate and diester backbone with all 2'-O-methyl groups on each

ribose in the oligonucleotide. The total charge density was 50% of 3498-PS.

Oligonucleotides 3498-PS and 3498 stocks were at 300 μ M, while the 3793-2 stock was at 440 mM.

5 The commercially available lipids used in the tests were:

	Lipofectin®	Lot#EF3101	1 mg/mL, as supplied by Gibco/BRL, (Gaithersburg, MD)
10	LipofectAMINE®	Lot#EFN101	2 mg/mL, as supplied by Gibco/BRL (Gaithersburg, MD)
15	Transfectam®	Lot#437121	1 mg dry, from Promega (Madison, WI) and resuspended in 100% ethanol.

Every phosphonic acid-based lipid used in these evaluations, as listed in the data tables, was at 1 mg/mL in 100% ethanol.

20 Tissue culture cell stocks, SNB-19 (human glioblastoma), C8161 (human tumor of unknown tissue origin), RD (human rhabdomyosarcoma, ATCC # CCL-136) and COS-7 (African green monkey kidney cells, ATCC # CRL 1651) were maintained in standard cell culture media.:
25 DMEM /F12 (1:1) mix from Mediatech, Lot#150901126, 10% fetal bovine serum from Gemini Bioproducts, Lot#A1089K, 100 units/mL penicillin and 100 μ g/mL streptomycin, from Mediatech, Lot#30001044 and 365 μ g/mL L-glutamine. The

cells were maintained under standard conditions at all times, 37 degrees C, 5% CO₂ atmosphere prior to fixation and microscopic examination.

Each FITC-labeled oligonucleotide delivery
5 determination was begun by plating the appropriate cells as listed in the data tables, into 16 well slides (Nunc #178599, glass microscope slide with 16 removable plastic wells attached to the slide surface with a silicone gasket) according to standard tissue culture methods.
10 Each cell line was plated at a starting density (approximately 20,000 cells/well) that allowed them to be healthy and 60-80% confluent one to two days after plating. The cells were allowed to adhere to the glass and recover from the plating procedure in normal
15 growth medium for 24 to 48 hours before beginning the transfection procedure.

Oligonucleotide transfection mixes were made up in Opti-MEM[®] without antibiotics as follows: 500 μ L aliquots of Opti-MEM[®] containing 0.25 μ M of 3498-PS,
20 3498, or 3793-2 (2 μ g of oligonucleotide) were pipetted into 1.5 mL Eppendorf tubes. Each cationic lipid or lipid mixture was then added to the oligonucleotide solution to give a final 9:1 or 6:1 ratio (18 or 12 micrograms of lipid total) of cationic lipid to
25 oligonucleotide by weight, as listed in the data tables. The tubes were mixed by vortexing immediately after the addition of lipids.

Transfections were begun by rinsing the cells in 200 μ L Opti-MEM[®] and then the cells were rinsed with
30 Dulbecco's phosphate buffered saline (PBS) solution.

Then 200 μ L of each oligonucleotide transfection mix was then added directly to each well to begin each transfection reaction. Transfections continued for four to six hours. The cells were then rinsed in PBS from
5 Mediatech and fixed for ten minutes in 200 μ L of 3.7% formaldehyde (Sigma, St. Louis, MO) to terminate the transfections and then rinsed again in PBS. The formaldehyde was quenched with 200 μ L of 50 mM glycine (Sigma, St. Louis, MO) for ten minutes. Finally, the
10 wells were then emptied by shaking out the glycine solution, the plastic chambers and silicone gasket were removed and the cells were covered with Fluoromount-G mounting medium (from Fisher, with photobleaching inhibitors) and a cover slip.

15 Intracellular fluorescence was evaluated under 200X magnification with a Nikon Labophot-2 microscope with an episcopic-fluorescence attachment. Using this equipment we could easily distinguish extracellular from nuclear and endosomal fluorescence. The cells were
20 scored for uptake as follows: No nuclear fluorescence, 0; up to 20% fluorescent nuclei, 1; up to 40% fluorescent nuclei, 2; up to 60% fluorescent nuclei, 3; up to 80% fluorescent nuclei, 4; and up to 100% fluorescent nuclei, 5.

25 **Example 7: Plasmids**

The following plasmids were used in certain examples.

pG1035: Splicer CAT, inserted into a pRc/CMV vector

- pG1036: Wild-type CAT, inserted into a pRc/CMV vector
 pG1040: UCAT, inserted into a pRc/CMV vector
 pGL2: Luciferase expressing plasmid (Promega)
 pSVb: b-galactosidase expressing plasmid (Clonetech)
- 5 A description of plasmids pG1035, pG1036 and pG1040 follows.

1. pG1035 (SplicerCAT) and pG1036 (wild-type CAT) and the sequences of the synthetic splice sites:

- A. Sequence of the wild type CAT gene used to
 10 create plasmid pG1036:

+409 +410

½ ½

GCC UAU UUC CCU AUU UCC CUA AAG GGU UUA UUG AGA AUA

[SEQ. ID. NO. 1]

- 15 B. Full sequence of the intron inserted within the CAT coding sequence to create SplicerCAT and plasmid pG1035:

+409 1

½ ½

20 ... ACC UGG CCU AUU UCC CUA AAG[^]gug agu gac uaa cua agu

39

½

cga cug cag acu agu cau ug(a) uug agu gua aca aga ccg

87 +410

25

½ ½

gau auc uuc gaa ccu cuc ucu cuc ucu cag[^]GGU UUA UUG AGA

...

[SEQ. ID. NO. 2]

The region of the CAT gene into which the intron was inserted is shown in sequence A above. Wild type
5 CAT DNA (Pharmacia, Piscataway, NJ) was inserted into pRc/CMV (Invitrogen, San Diego, CA) to create plasmid pG1036. The sequence is shown as the mRNA. Bases 409 and 410 are labeled for comparison to pG1035. A synthetic intron, shown as sequence B above, was
10 inserted into the CAT DNA to create plasmid pG1035. Mature mRNA sequences are shown uppercase, intronic sequences are lower case. The canonical guanosine of the splice donor is labeled +409, which corresponds to base 409 of the CAT open reading frame. The first base
15 of the intron is labeled 1. The canonical branchpoint adenosine is base 39 and the canonical intronic splice acceptor guanosine is base 87 of the intron. Base 410 marks the resumption of the CAT open reading frame. The sequences against which the oligomers are targeted are
20 underlined. The consensus splice site bases are given in bold face italics (Smith *et al.*, *Ann. Rev. Genet.* 23:527, 1989; Green, *Ann. Rev. Genet.* 20:671, 1986).

The clone pG1035 was created using synthetic DNA PCR primers to create a Hind III-Spe I 5' fragment
25 containing the first 2/3 of the open reading frame and half of the synthetic intron and an Spe I-Not I fragment containing the second half of the intron and the last 1/3 of the open reading frame. These were combined with

55

Hind III-Not I cut pRc/CMV in a 3-way ligation to yield the final plasmid. The artificial CAT gene containing the intron is named SplicerCAT. References applicable to the foregoing include Smith, et. al. *supra* and Green
5 *supra*.

2. pG1040 (UCAT) 5' untranslated regions and amino terminus:

Wild-type CAT:

5' +1
10 **Met Glu Lys Lys Ile Ser**
uuu uca gga gcu aag gaa gcu aaa aug gag aaa aaa auc acu

3'
Gly Tyr Thr Thr [SEQ. ID. NO. 3]

gga uau acc acc [SEQ. ID. NO. 4]

15 pG1040, UCAT:

5' +1
Met Glu Lys Lys Ile Ser
agu gca gga gcu aag gaa gcu acc aug gag aag aag auc acu

	5' AUG site	3' AUG site
20	3258-1	3261-1
	3260-1	3262-1

3'

Gly Tyr Thr Thr [SEQ. ID. NO. 5]

gga uau acc acc [SEQ. ID. NO. 6]

The sequences of wild type and pG1040 UCAT around
5 the AUG start codon are shown. The target sites for the
oligomers are named and underlined, and the numbers of
the chimeric oligomers against each target site are
shown beneath.

UCAT was made from wild-type CAT DNA (Pharmacia)
10 using synthetic DNA PCR primers. The resulting fragment
was cloned as a Hind III (5' end), Not I (3' end)
fragment into the vector pRc/CMV (Invitrogen). The
first adenosine of the open reading frame is designated
+1. The amino acid changes between wild-type and pG1040
15 are conservative.

The summary of the invention described above is
non-limiting and other features and advantages of the
invention will be apparent from the following descrip-
tion of the preferred embodiments, and from the claims.

20 All references which have been cited herein are
hereby incorporated by reference in their entirety.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

5 (ii) TITLE OF INVENTION:

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:

10	(A) ADDRESSEE:	Lyon & Lyon
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		Suite 4700
	(C) CITY:	Los Angeles
15	(D) STATE:	California
	(E) COUNTRY:	U.S.A.
	(F) ZIP:	90071-2066

(v) COMPUTER READABLE FORM:

20	1.44 Mb	(A) MEDIUM TYPE:	3.5" Diskette,
			storage
		(B) COMPUTER:	IBM Compatible
		(C) OPERATING SYSTEM:	IBM P.C. DOS 5.0
		(D) SOFTWARE:	Word Perfect 5.1

25 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:	To Be Assigned
(B) FILING DATE:	
(C) CLASSIFICATION:	

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

- 5
- (A) NAME: Warburg,
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 - (B) REGISTRATION NUMBER: 32,327
 - (C) REFERENCE/DOCKET NUMBER:

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- 10
- (A) TELEPHONE: (213) 489-1600
 - (B) TELEFAX: (213) 955-0440
 - (C) TELEX: 67-3510

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- 15
- (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCCUAUUUCC CUAUUUCCCU AAAGGGUUUA UUGAGAAUA
39

(2) INFORMATION FOR SEQ ID NO:2:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 120 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

59

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACCUGGCCUA UUUCCCUAAA GGUGAGUGAC UAACUAAGUC GACUGCAGAC
 5 50
 UAGUCAUUGA UUGAGUGUAA CAAGACCGGA UAUCUUCGAA CCUCUCUCUC
 100
 UCUCUCAGGG UUUAUUGAGA
 120

10 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 15 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

20 Met Glu Lys Lys Ile Ser Gly Tyr Thr Thr
 1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 54 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

UUUUCAGGAG CUAAGGAAGC UAAAAUGGAG AAAAAAUAUC CUGGAUUAUC
 CACC 54

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

5	(A) LENGTH:	10 amino acids
	(B) TYPE:	amino acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear

(ii) MOLECULE TYPE: peptide

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met	Glu	Lys	Lys	Ile	Ser	Gly	Tyr	Thr	Thr
1				5					10

15 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

20	(A) LENGTH:	54 base pairs
	(B) TYPE:	nucleic acid
	(C) STRANDEDNESS:	single
	(D) TOPOLOGY:	linear

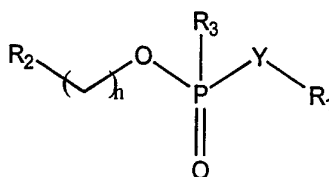
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

25	AGUGCAGGA	GCUAAGGAAG	CUACCAUGGA	GAAGAAGAU	ACUGGAUAUA
	CCACC		54		

Claims

We claim:

1. A lipid having the structure:



5

$[X^-]_m$

or a salt, or solvate, or enantiomers thereof wherein;
 (a) R_1 is a lipophilic moiety; (b) R_2 is a positively
 charged moiety; (c) R_3 is a lipophilic moiety of 1 to
 about 24 carbon atoms, a positively charged moiety, or a
 10 negatively charged moiety; (d) n is an integer from 0 to
 8; (e) X^- is an anion or polyanion; (f) Y is N or O, and
 (g) m is an integer from 0 to a number equivalent to the
 positive charge(s) present on the lipid.

2. A lipid according to claim 1 wherein R_1 is a
 15 lipophilic moiety selected from the group consisting of
 a straight chain alkyl of 1 to about 24 carbon atoms, a
 straight chain alkenyl of 2 to about 24 carbon atoms, a
 symmetrical branched alkyl or alkenyl of about 10 to
 about 50 carbon atoms, a unsymmetrical branched alkyl or
 20 alkenyl of about 10 to about 50 carbon atoms, a
 steroidyl moiety, a glyceryl derivative, a amine

derivative, and $\text{OCH}(\text{R}_4\text{R}_5)$ or $\text{N}(\text{R}_4\text{R}_5)$, wherein R_4 and R_5 are straight chain or branched alkyl moieties of about 10 to about 30 carbon atoms.

3. A lipid according to claim 2 wherein the
5 steroidyl moiety is cholesteryl.

4. A lipid according to claim 2 wherein R_1 is an amine derivative selected from a straight chain, branched or cyclic acylamine or alkylamine.

5. A lipid according to claim 2 wherein R_1 is an
10 amine derivative selected from the group consisting of a 3-N-1,2-diacyl-1,2-propanediol-3-amino moiety, a 3-N-1,2-dialkyl-1,2-propanediol-3-amino moiety, and a 3-N-1,2 N,N-diacyl-1,2,3-triaminopropanyl moiety.

6. A lipid according to claim 5 wherein the 3-N-
15 1,2-diacyl-1,2-propanediol-3-amino moiety has a diacyl moiety selected from an alkanolic acid of about 10 to about 30 carbon atoms or an alkenolic acid of about 10 to about 30 carbon atoms.

7. A lipid according to claim 5 wherein 3-N-1,2-
20 dialkyl-1, 2-propanediol-3-amino moiety has alkyl moieties selected from alkyl groups of about 10 to about 30 carbon atoms or alkenyl groups of about 10 to about 30 carbon atoms.

8. A lipid according to claim 2 wherein R_1 is a 3-

O-1,2-diacylglyceryl moiety or a 3-O-1,2-dialkylglyceryl moiety.

9. A lipid according to claim 8 wherein the 3-O-1,2-diacylglyceryl moiety has a diacyl group of alkanolic acid of about 10 to about 30 carbon atoms or an alkenolic acid of about 10 to about 30 carbon atoms.

10. A lipid according to any of claims 6 or 9 wherein the alkanolic acid is stearic acid.

11. A lipid according to any of claims 6 or 9 wherein the alkenolic acid is palmitic acid or oleic acid.

12. A lipid according to claim 8 wherein the 3-O-1,2-dialkylglyceryl moiety has alkyl moieties selected from alkyl groups of about 10 to about 30 carbon atoms or alkenyl groups of about 10 to about 30 carbon atoms.

13. A lipid according to claim 1 wherein R_2 is a positively charged moiety selected from the group consisting of an amino acid residue having a positively charged group on the side chain, an alkylamine moiety, a fluoroalkylamine moiety, or a perfluoroalkylamine moiety of 1 to about 6 carbon atoms, an arylamine moiety or an aralkylamine moiety of 5 to about 10 carbon atoms, a guanidinium moiety, an enamine moiety, an aromatic or non-aromatic cyclic amine moiety of about 5 to about 10 carbon atoms, an amidine moiety, an isothiourea moiety,

a heterocyclic amine moiety, and a substituted heterocyclic moiety and a substituted alkyl moiety of 1 to about 6 carbon atoms substituted with a substituent selected from the group consisting of NH_2 , $\text{C}(=\text{O})\text{NH}_2$, NHR_6 ,
5 $\text{C}(=\text{O})\text{NHR}_6$, NHR_6R_7 , or $\text{C}(=\text{O})\text{NHR}_6\text{R}_7$, wherein R_6 and R_7 are independently selected from an alkyl moiety of 1 to about 24 carbon atoms, an alkenyl moiety of 2 to about 24 carbon atoms, an aryl moiety of about 5 to about 20 carbon atoms, or an aralkyl moiety of about 6 to about
10 25 carbon atoms.

14. A lipid according to claim 13 wherein R_2 is an amino acid residue selected from the group consisting of lysine, arginine, histidine, ornithine, and an amino acid analog.

15 15. A lipid according to claim 14 wherein the amino acid analog is selected from the group consisting of 3-carboxyspermidine, 5-carboxyspermidine, 6-carboxyspermine and a monoalkyl, dialkyl, or peralkyl substituted derivative which is substituted on one or
20 more amine nitrogens with an alkyl group of 1 to about 6 carbon atoms.

16. A lipid according to claim 1 wherein R_3 is a lipophilic moiety selected from the group consisting of a straight chain alkyl moiety of about 3 to about 24
25 carbon atoms, a straight chain alkenyl moiety of 2 to about 24 carbon atoms, a symmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, a

unsymmetrical branched alkyl or alkenyl moiety of about 10 to about 50 carbon atoms, an aryl moiety of about 5 to about 20 carbon atoms, an aralkyl moiety of about 6 to about 25 carbon atoms, and a steroidyl moiety.

5 17. A lipid according to claim 1 wherein R_3 is a positively charged moiety selected from the group consisting of an amino acid residue having a positively charged group on the side chain, an alkylaminoalkyl moiety, a fluoroalkylaminoalkyl moiety, a
10 perfluoroalkylaminoalkyl moiety, a guanidiniumalkyl moiety, an enaminoalkyl moiety, a cyclicaminoalkyl moiety, an amidinoalkyl moiety, an isothioureia alkyl moiety, and a heterocyclic amine.

15 18. A lipid according to claim 1 wherein R_3 is a negatively charged moiety selected from the group consisting of a carboxyalkyl moiety, a phosphonoalkyl moiety, a sulfonoalkyl moiety, and a phosphatidylalkyl moiety of 1 to about 24 carbon atoms.

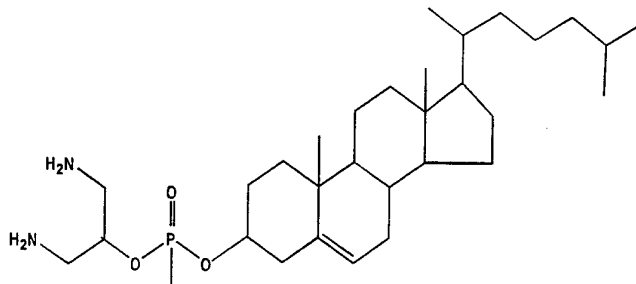
20 19. A lipid according to claim 1 wherein n is an integer from 1 to 8.

 20. A lipid according to claim 1 wherein n is an integer from 2 to 6.

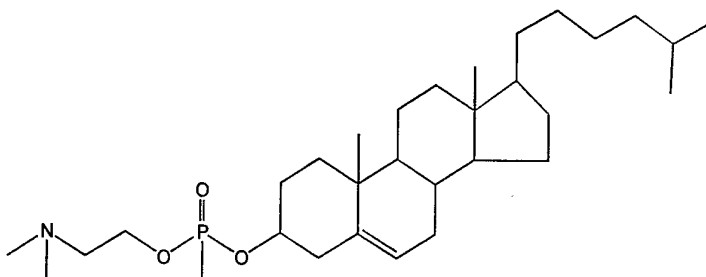
 21. A lipid according to claim 1 wherein n is 2,
25 3, or 4.

22. A lipid according to claim 1 wherein X⁻ is a pharmaceutically acceptable anion or polyanion.

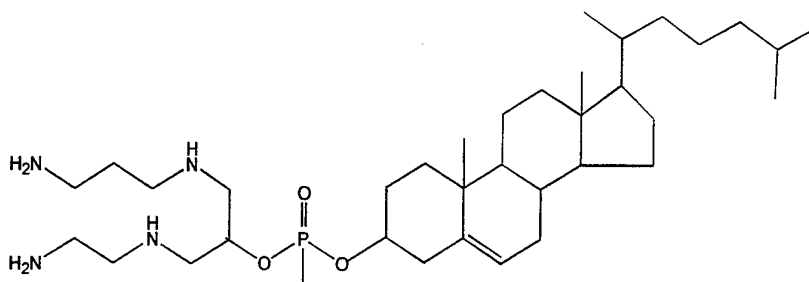
23. A methylphosphonate of the structure:



5 24. A methylphosphonate of the structure:

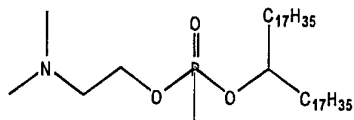


25. A methylphosphonate of the structure:

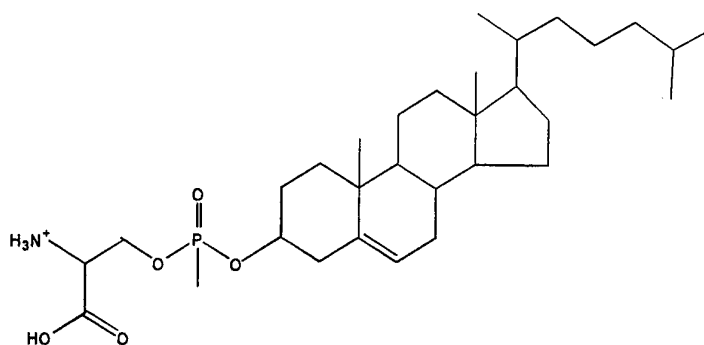


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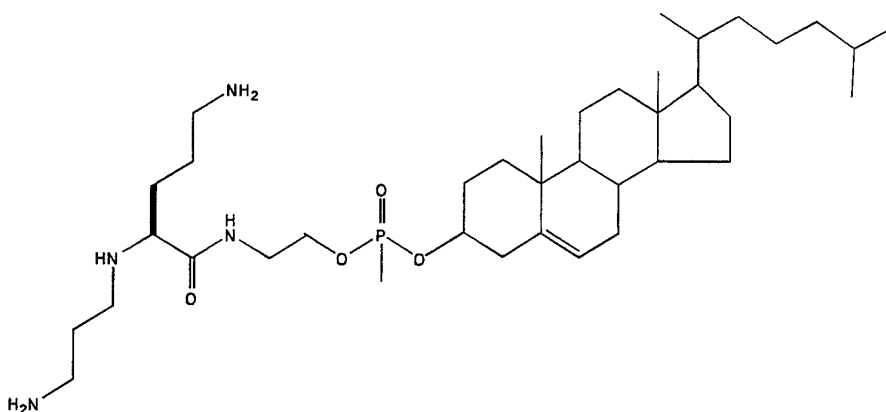
26. A phosphonic acid ester of the structure:



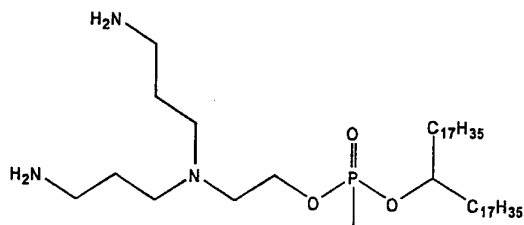
27. A phosphonic acid ester of the structure:



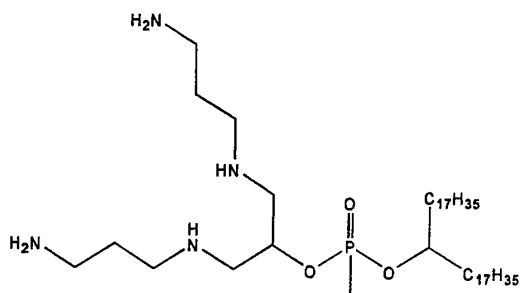
5 28. A phosphonic acid ester of the structure:



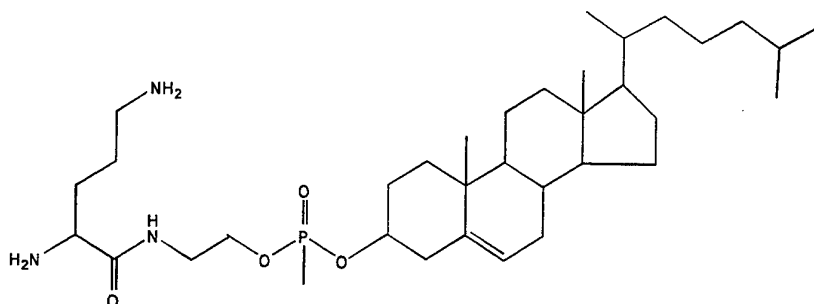
29. A phosphonic acid ester of the structure:



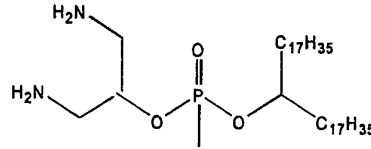
30. A phosphonic acid ester of the structure:



5 31. A phosphonic acid ester of the structure:



32. A phosphonic acid ester of the structure:



33. A composition comprising a polyanionic macromolecule and a lipid according to claim 1.

5 34. A composition according to claim 33 wherein the polyanionic macromolecule comprises an expression vector, capable of expressing a polypeptide in a cell.

35. A composition according to claim 33 wherein the polyanionic macromolecule is an oligonucleotide or
10 an oligomer.

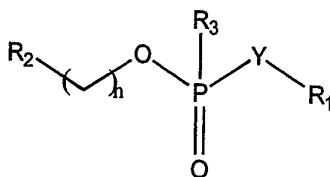
36. A composition according to claim 33 wherein the polyanionic macromolecule is DNA.

37. A method of delivering a polyanionic macromolecule into a cell comprising contacting a
15 composition of claim 33 with the cell.

38. A method of interfering with the expression of a protein in a cell comprising contacting a composition of claim 35 with the cell wherein the oligomer has a base sequence that is substantially complimentary to an
20 RNA sequence in the cell that encodes the protein.

38. A kit for delivering a polyanionic macromolecule into a cell comprising a composition of claim 33.

39. A lipid having the structure:



5

or a salt, or solvate, or enantiomers thereof wherein;

(a) R_1 is selected from the group consisting of

(i) a symmetrical branched alkyl or alkenyl of 25 to 40 carbon atoms; (ii) a steroidyl moiety; and (iii) a glycerol derivative;

10

(b) n is 0 to 4;

(c) R_2 is a positively charged moiety selected from the group consisting of (i) an alkylamine moiety; and (ii) a substituted alkyl moiety 1 to about 6 carbon atoms substituted with a substituent selected from the group consisting of NH_2 , $C(=O)NH_2$, NHR_6 , $C(=O)NHR_6$, NHR_6R_7 , or $C(=O)NHR_6R_7$, wherein R_6 and R_7 are independently

15

selected from an alkyl moiety of 1 to about 24 carbon atoms, an alkenyl moiety of 2 to about 24 carbon atoms, an aryl moiety of about 5 to about 20 carbon atoms, or an aralkyl moiety of about 6 to about 25 carbon atoms;

20

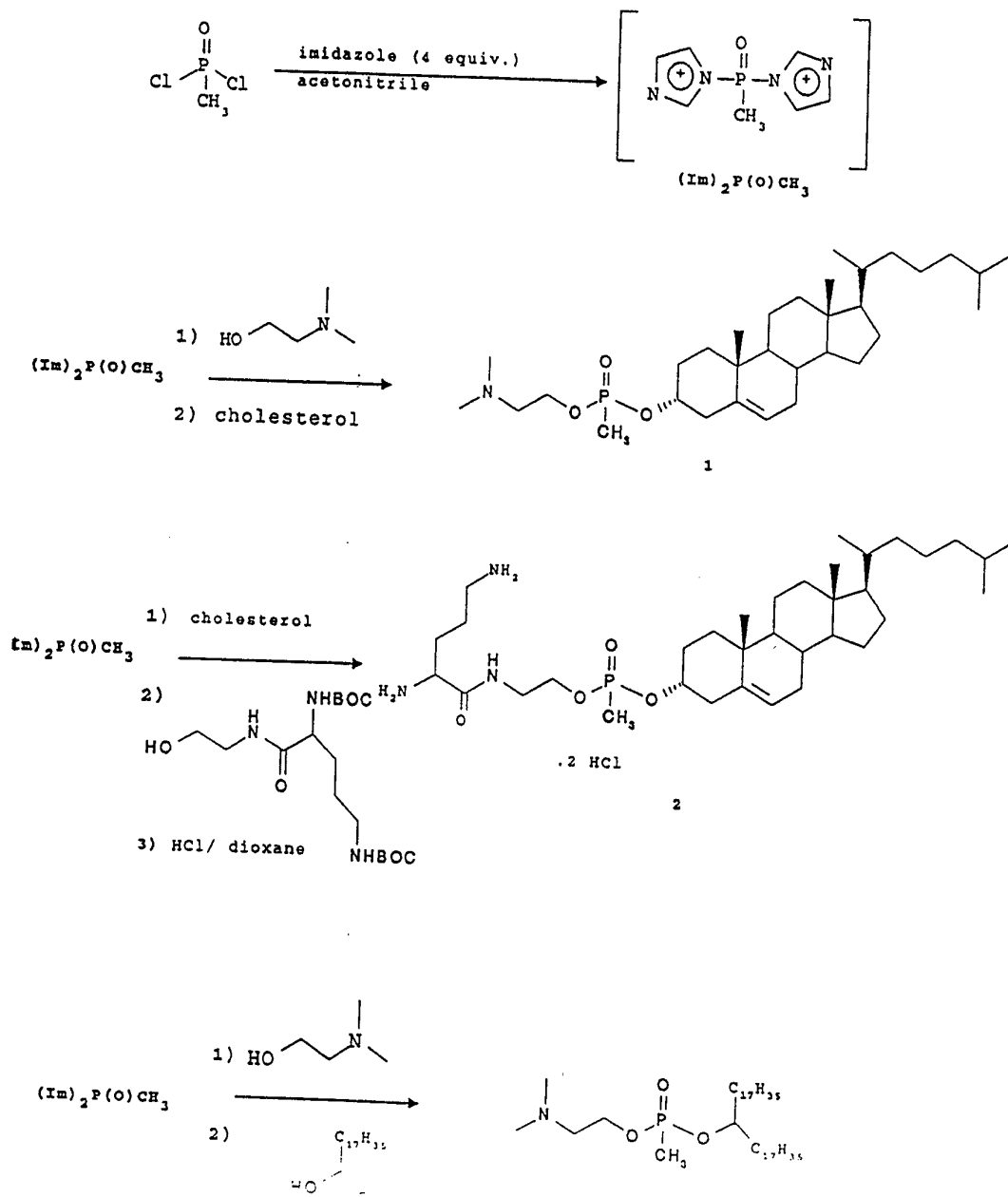
(d) X is an anion or polyanion; and

25

(e) m is an integer 0 to a number equivalent

to the positive charges present in the lipid.

Figure 1: Synthetic scheme for the synthesis of compounds 1, 2 and 3



2/4

Figure 2: Structure of 4 (Cholest-5-en-3-ol (3b)-, β -[2,3-diamino-1-oxopentyl]amino]ethyl]carbamate, dihydrochloride)

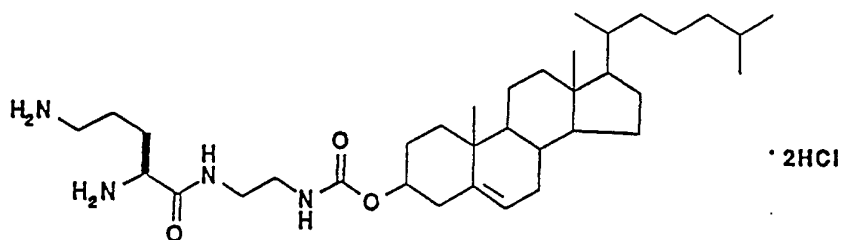


Figure 3: The combination of cationic lipids 1 and 4 delivers plasmids and oligodeoxynucleotides to cells

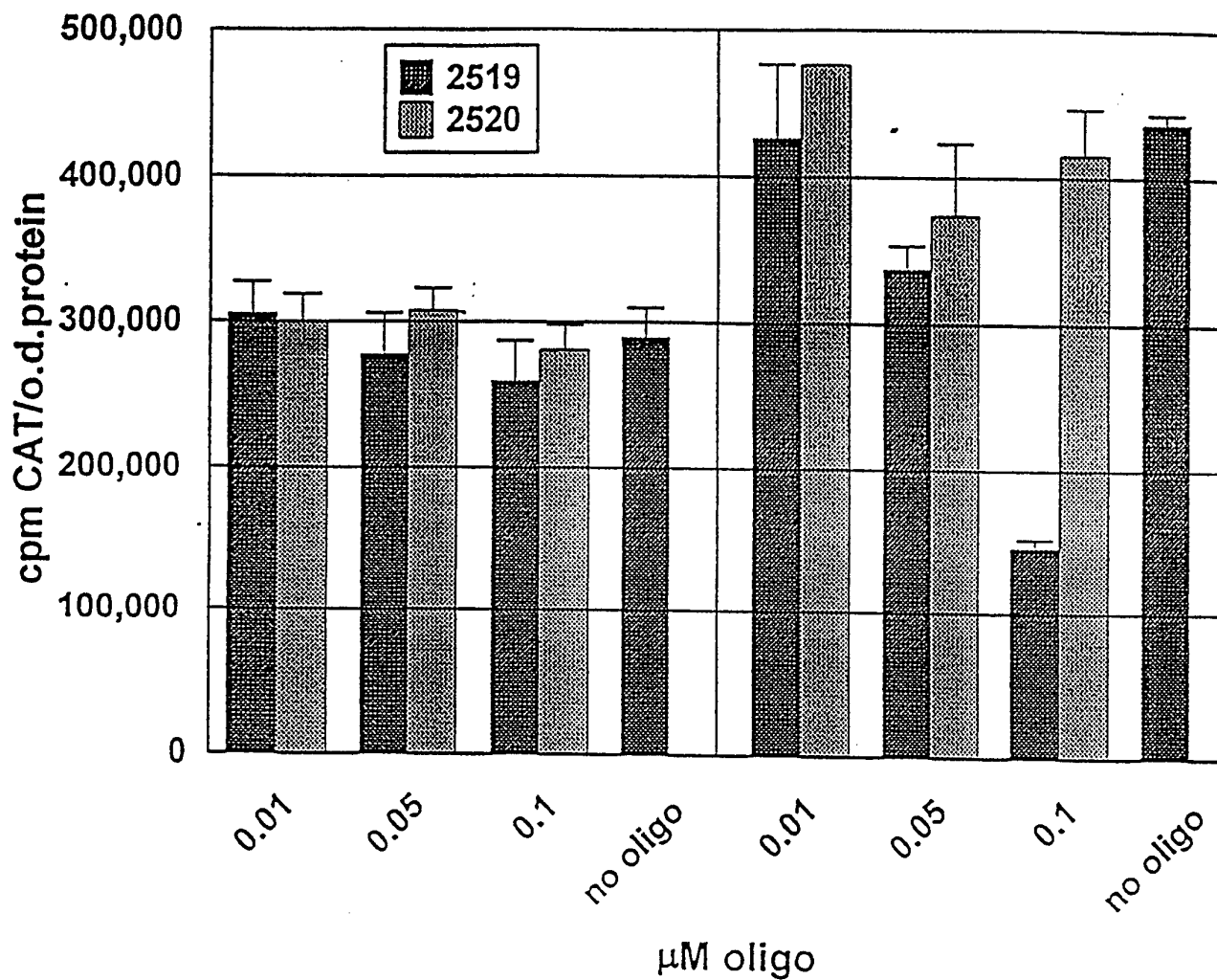


Figure 4: Demonstration of nuclear delivery of oligonucleotides of varying charge densities by novel cationic lipids to COS-7 and SNB-19 cells.

COS-7 Cells		Delivery	T'fection	Delivery	T'fectio	Delivery	T'fection	
		3498	Time	3498PS	Time	3793-2	Time	
10	Commercial Lipids							<i>delivery=</i>
	Lipofectamine	4, 5*	5 hr	3*, 4*	5 hr	5*	5hr	
	Lipofectin	1, 1*	5 hr	4*, 5*	5hr	0*	5hr	
	Transfectam	5	5 hr	5(9:1)	5hr	5*	5hr	
15	Novel Lipids							<i>nuclear</i>
	1	0	5 hr					
	1/4	2.5	5 hr	3	5hr			
20	2/DOPE	0.5	5hr	3	5hr			
	2/4	3	5hr	3.5	5 hr			
	2/1	1.5	5hr	5	5hr			
25	3	0	5 hr	0	5 hr			
	3/DOPE	0	5hr	0	5hr			
30	SNB 19		Delivery	T'fection	Delivery	T'fectio	Delivery	T'fection
			3498	Time	3498PS	Time	3793-2	Time
	Commercial Lipids							
	Transfectam	5	5hr	4.5	5hr	4.5	5hr	* = 9:1
35	Lipofectamine	4	5hr	4*	5hr	4.5	5hr	lipid:oligo
	Lipofectin	1	5hr	4*	5hr	1	5hr	
40	Novel Lipids							
	1/4	1	5hr	4*	5hr			
	2/DOPE	1	5hr	2.5	5hr			
	2/1	0	5hr	2*	5hr			
	2/4	1	5hr	1*	5hr			
45	3	0	5hr	0*	5hr	0	5hr	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/09478

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) : Please See Extra Sheet. US CL : 552/506, 507; 514/114, 119, 182; 558/169, 170 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 552/506, 507; 514/114, 119, 182; 558/169, 170 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Tetrahedron, Volume 50, Number 22, issued 1994, PANNECOUCKE, et al., "Synthesis of Phosphoric Acid Diesters of 7 β -Hydroxycholesterol and of Carbohydrates," pages 6569-6578.	1-3, 8-9, 13, 16, 19, 22, 39
X	Collection of Czechkloslovakian Chemical Communications, Volume 59, issued 1994, ALEXANDER, et al., "Preparation of 9-(2-Phosphonomethoxyethyl) adenine Esters as Potential Prodrugs," pages 1853-1869.	1, 2, 4, 13-14, 16, 19-22, 39
X	Anticancer Research, Volume 13, issued 1993, ALLEMAND, et al., "Effect of Oxysterol Derivatives on the Time Course Development of Hepatocarcinoma in Transgenic Mice, pages 1097-1101.	1-4, 13, 16-17, 19, 39
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A	document defining the general state of the art which is not considered to be part of particular relevance	*X
*E	earlier document published on or after the international filing date	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y
*O	document referring to an oral disclosure, use, exhibition or other means	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*P	document published prior to the international filing date but later than the priority date claimed	*&
document member of the same patent family		
Date of the actual completion of the international search	Date of mailing of the international search report	
06 SEPTEMBER 1996	11 OCT 1996	
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer KIMBERLY J. PRIOR aco	
Facsimile No. (703) 305-3230	Telephone No. (703) 308-1235	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09478

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,680,290 (CASSAL) 14 July 1987, see entire document.	1-4, 16, 19-22, 24, 39
X	US, A, 5,215,972 (CASSAL et al.) 01 June 1993, see entire document.	1-4, 16, 19-22, 24, 39
X	Nucleosides & Nucleotides, Volume 13, Number 9, issued 1994, BALAGOPALA, et al., "Synthesis and Anti-HIV Activity of Alkyl Steroidal 3'-Azido-3'-Deoxythymidin-5'-yl Phosphotriesters as Prodrugs of AZT, pages 1843-1853.	1-3, 8-9, 13, 16, 19, 22, 39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09478

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07J 9/00, 41/00, 43/00; C07C 229/00; A61K 31/575, 31/58, 31/66, 31/675