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(72) Inventeurs/Inventors:
RILATT, IAN, FR;
PEREZ, MICHEL, FR;
GOETSCH, LILIANE, FR;
BROUSSAS, MATTHIEU, FR;
BEAU-LARVOR, CHARLOTTE, FR;
HAEUW, JEAN-FRANCOIS, FR

(73) Propriétaire/Owner:
PIERRE FABRE MEDICAMENT, FR

(74) Agent: NORTON ROSE FULBRIGHT CANADA

(54) Titre : CONJUGUE ANTICORPS-MEDICAMENT ET SON UTILISATION POUR LE TRAITEMENT DU CANCER
(54) Title: ANTIBODY-DRUG-CONJUGATE AND ITS USE FOR THE TREATMENT OF CANCER

(57) **Abrégé/Abstract:**

The present invention relates to an antibody-drug-conjugate. From one aspect, the invention relates to an antibody-drug-conjugate comprising an antibody capable of binding to a Target, said antibody being conjugated to at least one drug selected from derivatives of dolastatin 10 and auristatins. The invention also comprises method of treatment and the use of said antibody-drug-conjugate for the treatment of cancer.

(74) Agent(suite/continued): LLP/S.E.N.C.R.L., S.R.L.

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- (71) **Applicant:** PIERRE FABRE MEDICAMENT [FR/FR];
45, place Abel Gance, F-92100 Boulogne-Billancourt (FR).
- (72) **Inventors:** RILATT, Ian; 67 chemin de Montplaisir, F-81100 Castres (FR). PEREZ, Michel; 45 chemin de la Pique, F-81100 Castres (FR). GOETSCH, Liliane; 15 route de Cluze, F-74130 Ayze (FR). BROUSSAS, Matthieu; 13b rue du Lieutenant Bovagne, Immeuble Les Terrasses, F-01420 Seyssel (FR). BEAU-LARVOR, Charlotte; 156 rue du Champs à la Moine, Hameau de Vigny, F-74520 Jonzier Epagny (FR). HAEUW, Jean-François; 30 Domaine du Salève, Route du Salève, F-74160 Beaumont (FR).
- (74) **Agent:** REGIMBEAU; Regimbeau, 20, rue de Chazelles, F-75847 Paris Cedex 17 (FR).
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(54) **Title:** ANTIBODY-DRUG-CONJUGATE AND ITS USE FOR THE TREATMENT OF CANCER(57) **Abstract:** The present invention relates to an antibody-drug-conjugate. From one aspect, the invention relates to an antibody-drug-conjugate comprising an antibody capable of binding to a Target, said antibody being conjugated to at least one drug selected from derivatives of dolastatin 10 and auristatins. The invention also comprises method of treatment and the use of said antibody-drug-conjugate for the treatment of cancer.

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ANTIBODY-DRUG-CONJUGATE
AND ITS USE FOR THE TREATMENT OF CANCER

The present invention relates to an antibody-drug-conjugate. From one aspect, the invention relates to an antibody-drug-conjugate comprising an antibody capable of binding to a target, said antibody being conjugated to at least one drug selected from derivatives of dolastatin 10 and auristatins. The invention also comprises method of
5 treatment and the use of said antibody-drug-conjugate for the treatment of cancer.

Background of the invention

The invention relates to an antibody-drug-conjugate (ADC) or conjugate and its use for the treatment of cancer.

10 ADCs combine the binding specificity of an antibody with the potency of drugs such as, for example, cytotoxic agents. The technology associated with the development of monoclonal antibodies, the use of more effective drugs and the design of chemical linkers to covalently bind these components, has progressed rapidly in recent years .

The use of ADCs allows the local delivery of drugs which, if administered as
15 unconjugated drugs, may result in unacceptable levels of toxicity to normal cells.

In other words, maximal efficacy with minimal toxicity is sought thereby. Efforts to design and refine ADC have focused on the selectivity of antibody as well as drug mechanism of action, drug-linking, drug/antibody ratio (loading or DAR), and drug-releasing properties. Drug moieties may impart their cytotoxic and cytostatic
20 effects by mechanisms including tubulin binding, DNA binding, proteasome, impairment of ribosome function, protein synthesis and/or topoisomerase inhibition. Some cytotoxic drugs tend to be inactive or less active when conjugated to large antibody.

Each antibody must be characterized separately, an appropriate linker designed,
25 and a suitable cytotoxic agent identified that retains its potency upon delivery to tumor cells. One must consider the antigen density on the cancer target and whether normal tissues express the target antigen. Other considerations include whether the entire ADC is internalized upon binding the target; whether a cytostatic or cytotoxic drug is

preferable when considering possible normal tissue exposure and/or the type and stage of the cancer being treated; and, whether the linker connecting the antibody to the drug payload is a cleavable or a non-cleavable linkage. Furthermore, the antibody to drug moiety conjugation ratio must be sufficient without compromising the binding activity of the antibody and/or the potency of the drug and without modifying physicochemical properties of the ADC resulting on aggregation or deleterious properties regarding to the future development process of the compound.

An ADC is a complex biological molecule and the challenges to develop an effective ADC remain a significant issue.

10

Summary of the invention

In a first aspect, the present invention is directed to an antibody-drug-conjugate of the following formula (I):

15



(I)

or a pharmaceutically acceptable salt thereof,

20

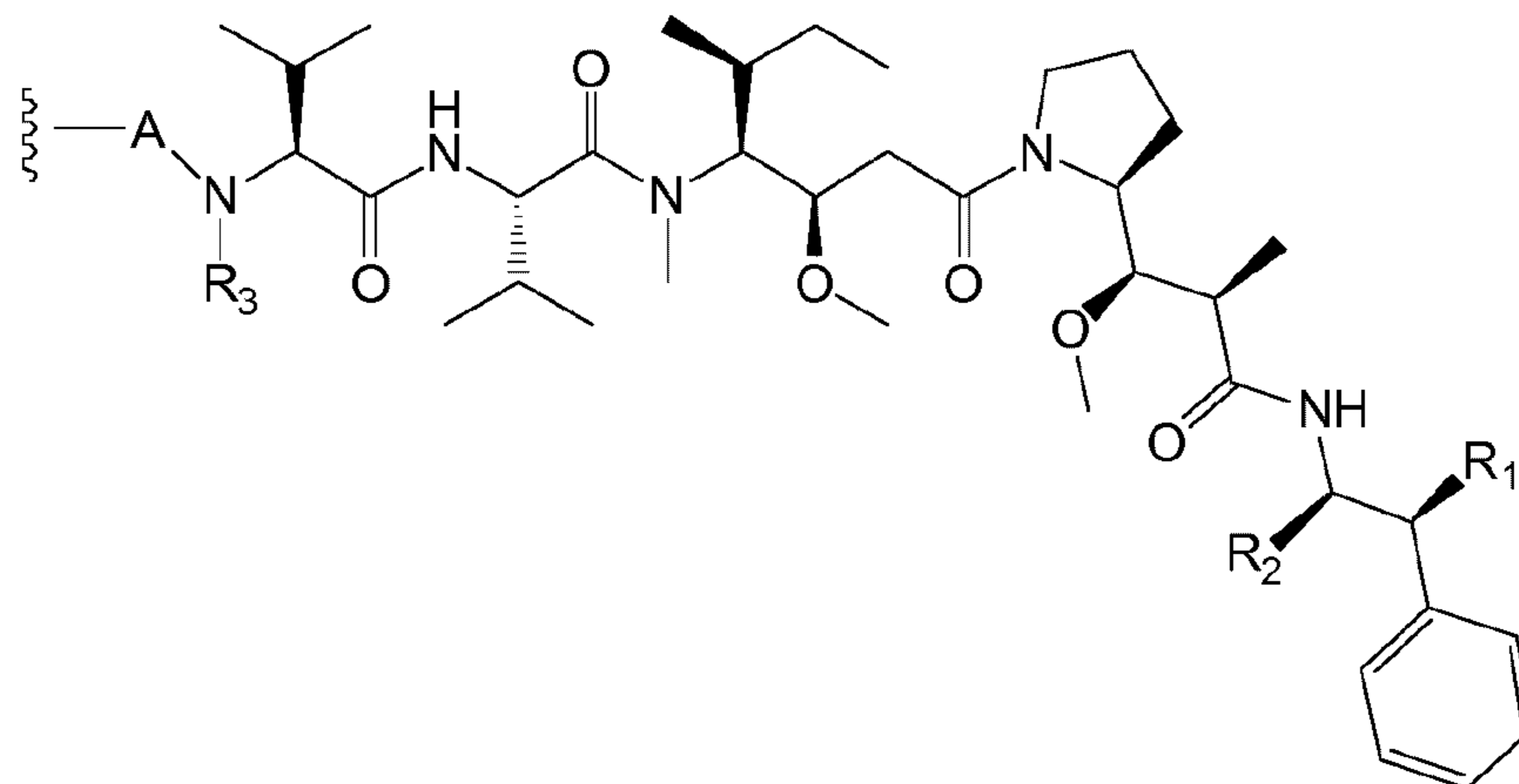
wherein

Ab is a [Target] antibody or an antigen antibody, or a [Target] binding fragment thereof or an antigen binding fragment thereof;

L is a linker;

D is a drug moiety of the following formula (II):

3



(II)

wherein:

R_1 is H or OH;

5 R_2 is a group: (C₁-C₆)alkyl, COOH, COO-((C₁-C₆)alkyl) or thiazolyl;

R_3 is H or a (C₁-C₆)alkyl group;

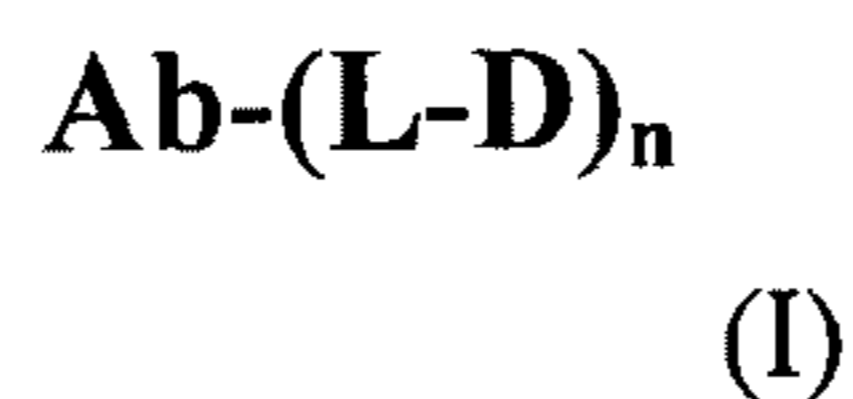
A is:

- a group of formula -Het-Alk- wherein Alk is a (C₁-C₈)alkanediyl group and is linked to NR₃, and Het is a heterocycle optionally substituted by a (C₁-C₆)alkyl group and containing at least one nitrogen atom, said nitrogen atom being linked to L, or
- a group of formula -A_a-A_b- wherein A_a is linked to L and is O or NR₉ with R₉ being H or (C₁-C₆)alkyl and A_b is linked to NR₃ and is:
 - a (C₁-C₈)alkanediyl group,
 - 15 ▪ a -(CH₂CH₂X₁)_{a1}(CH₂CH₂X₂)_{a2}(CH₂CH₂X₃)_{a3}(CH₂CH₂X₄)_{a4}CH₂CH₂- group with X₁, X₂, X₃ and X₄ each independently of one another representing O or NR₈; a₁, a₂, a₃ and a₄ each independently of one another representing 0 or 1; and R₈ representing H or a (C₁-C₆)alkyl group,
 - 20 ▪ an aryl-(C₁-C₈)alkanediyl or heterocycle-(C₁-C₈)alkanediyl group, said group being optionally substituted by a (C₁-C₆)alkyl group, the aryl or heterocycle moiety being linked to A_a and the (C₁-C₈)alkanediyl moiety being linked to NR₃;

the wavy line indicates the point of attachment to L; and

25 n is 1 to 12.

In accordance with one aspect of the present invention there is provided an antibody-drug-conjugate of the following formula (I):

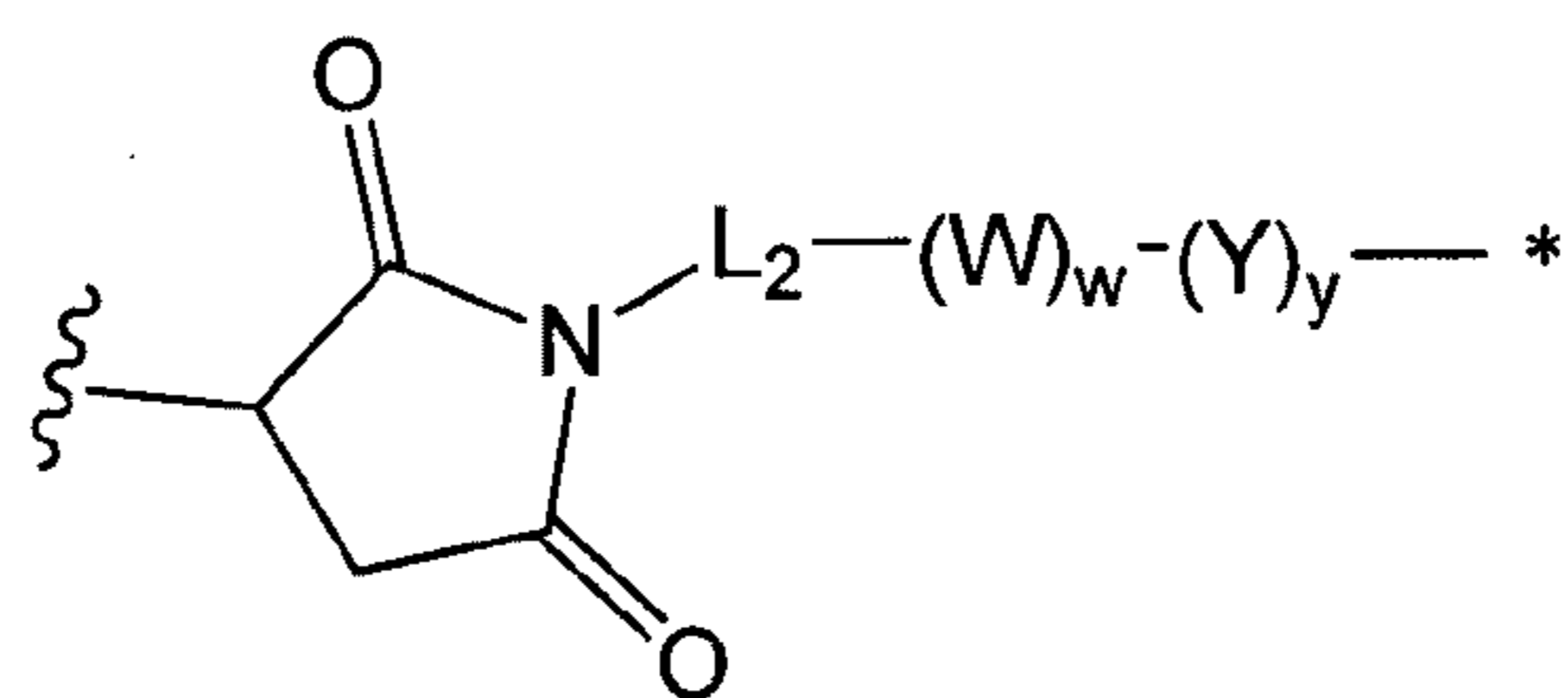


or a pharmaceutically acceptable salt thereof,

wherein

Ab is a [Target] antibody, or a [Target] binding fragment thereof;

L is a linker of the following formula (III):

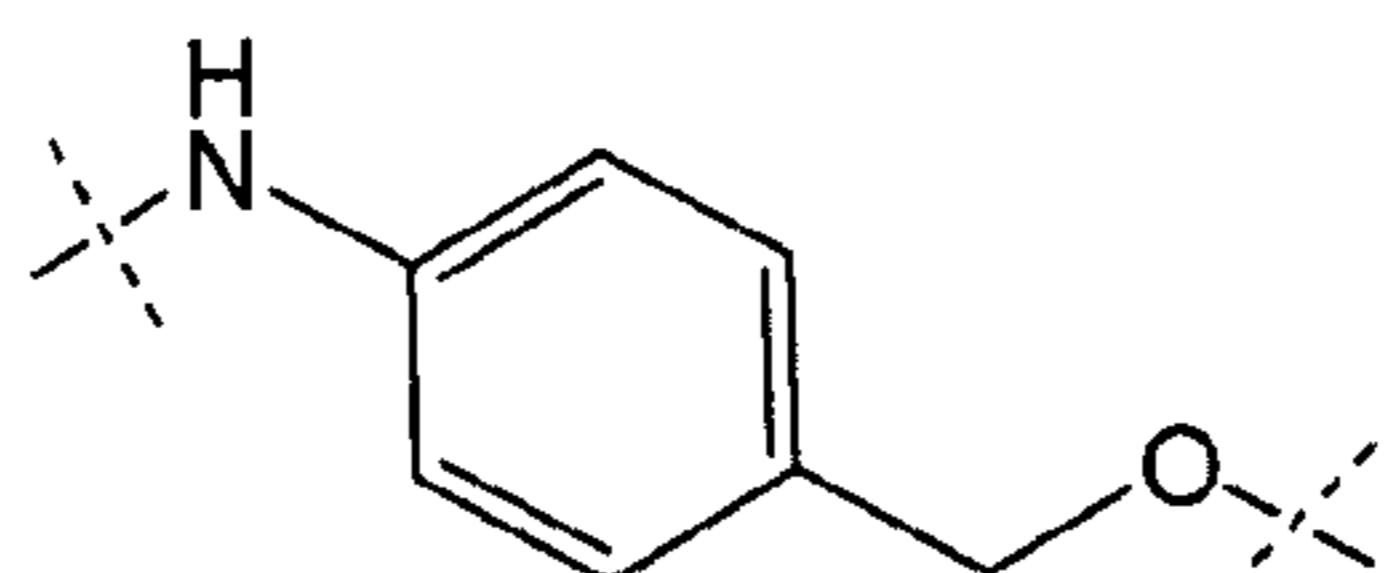


(III)

wherein

L_2 is (C₄-C₁₀)cycloalkyl-carbonyl, (C₂-C₆)alkyl, or (C₂-C₆)alkyl-carbonyl,

W is an amino acid unit; **w** is an integer comprised between 0 and 5;

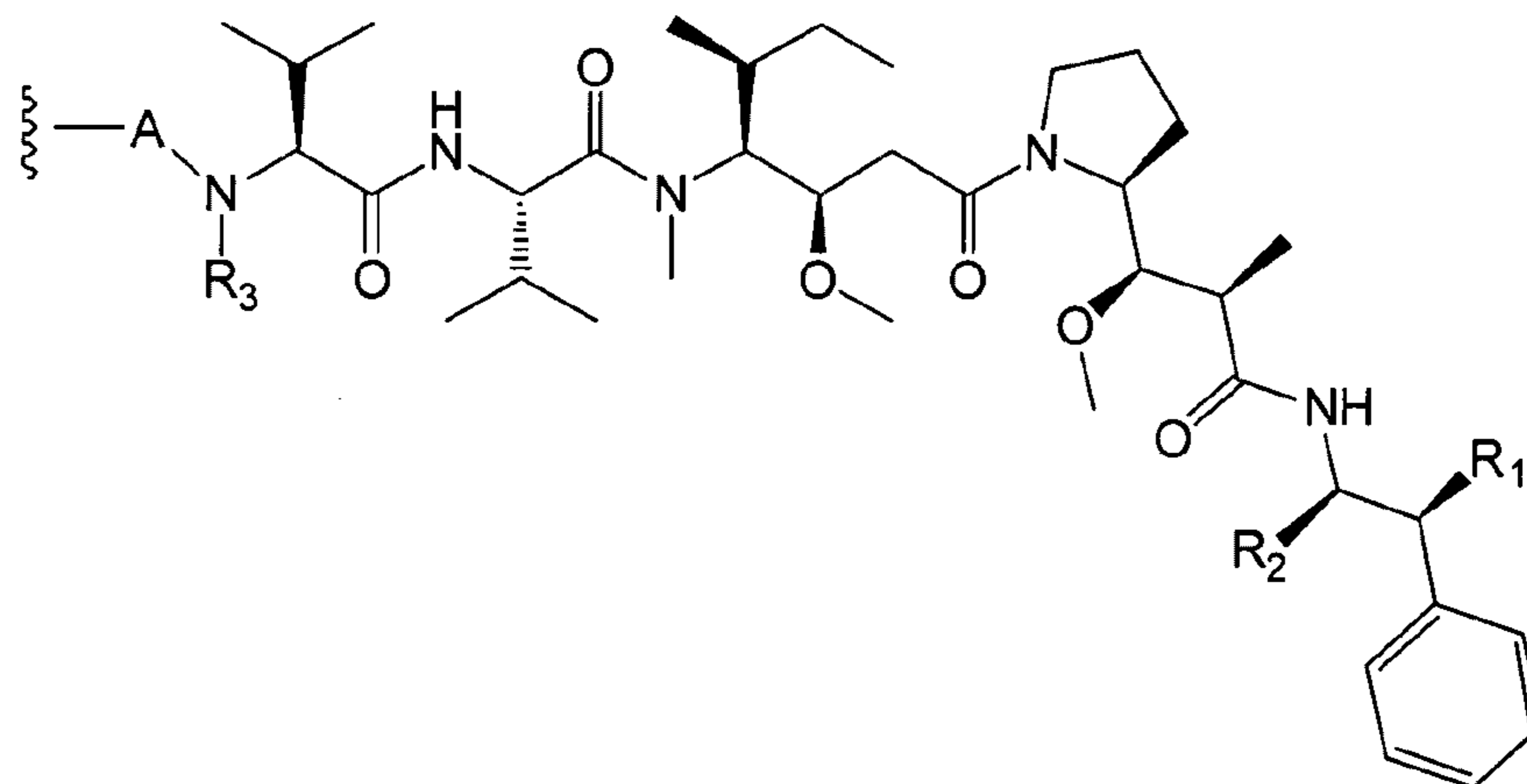


Y is PAB-carbonyl with PAB being

the asterisk indicates the point of attachment to **D**;

the wavy line indicates the point of attachment to **Ab**;

D is a drug moiety of the following formula (II):



(II)

wherein:

R_1 is H or OH;

R_2 is a group: (C₁–C₆)alkyl, COOH, COO–((C₁–C₆)alkyl) or thiazolyl;

R_3 is H or a (C₁–C₆)alkyl group;

A is:

- a group of formula -Het-Alk- wherein Alk is a (C₁–C₈)alkanediyl group and is linked to NR₃, and Het is a heterocycle optionally substituted by a (C₁–C₆)alkyl group and containing at least one nitrogen atom, said nitrogen atom being linked to L, or
- a group of formula –A_a-A_b- wherein A_a is linked to L and is O or NR₉ with R₉ being H or (C₁–C₆)alkyl and A_b is linked to NR₃ and is:
 - a (C₁–C₈)alkanediyl group,
 - a $-(CH_2CH_2X_1)_{a1}(CH_2CH_2X_2)_{a2}(CH_2CH_2X_3)_{a3}(CH_2CH_2X_4)_{a4}CH_2CH_2-$ group with X₁, X₂, X₃ and X₄ each independently of one another representing O or NR₈; a₁, a₂, a₃ and a₄ each independently of one another representing 0 or 1; and R₈ representing H or a (C₁–C₆)alkyl group, or
 - an aryl-(C₁–C₈)alkanediyl or heterocycle-(C₁–C₈)alkanediyl group, said group being optionally substituted by a (C₁–C₆)alkyl group, the aryl or heterocycle moiety being linked to A_a and the (C₁–C₈)alkanediyl moiety being linked to NR₃;

the wavy line indicates the point of attachment to L; and

n is 1 to 12.

In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein the [Target] or antigen of said [Target] antibody or antigen antibody according to the invention, or a [Target] or an antigen binding fragment thereof, is selected from CD19, CD20, CD22, CD25, CD30, CD33, CD40, 5 CD56, CD64, CD70, CD74, CD79, CD105, CD138, CD174, CD205, CD227, CD326, CD340, MUC16, GPNMB, PSMA, Cripto, ED-B, TMEFF2, EphB2, EphA2, FAP, av integrin, Mesothelin, EGFR, TAG-72, GD2, CAIX, 5T4, HER1, HER3, HER2, IGF-1R, Axl and their extra cellular membrane (ECD) fragment.

10 In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein the [Target] or antigen of said [Target] antibody or antigen antibody according to the invention, or a [Target] or an antigen binding fragment thereof, is selected from the HER2, the IGF-1R and the protein Axl, preferably the human HER2, human IGF-1R and the human protein Axl, and their extra cellular 15 membrane (ECD) fragment.

In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein said **Ab** is an antibody, or an antigen binding fragment thereof, capable of binding to the human IGF-1R selected from: 20 i) the antibodies 208F2, 212A11, 214F8, 219D6 and 213B10; ii) the antibodies which compete for binding to IGF-1R with the antibodies of i); and iii) the antibodies which bind to the same epitope of IGF-1R as the antibodies of i).

In an embodiment, the present invention relates to an antibody-drug-conjugate 25 according to the invention, wherein said **Ab** is an antibody, or an antigen binding fragment thereof, capable of binding to the human IGF-1R selected from: i) an antibody which comprises the three heavy chain CDRs of sequence SEQ ID No. 1, 2 and 3 and the three light chain CDRs of sequence SEQ ID No. 4, 5 and 6; ii) an antibody which competes for binding to IGF-1R with the antibody of i); and 30 iii) an antibody which binds to the same epitope of IGF-1R as the antibody of i).

In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein said **Ab** is an antibody which comprises:

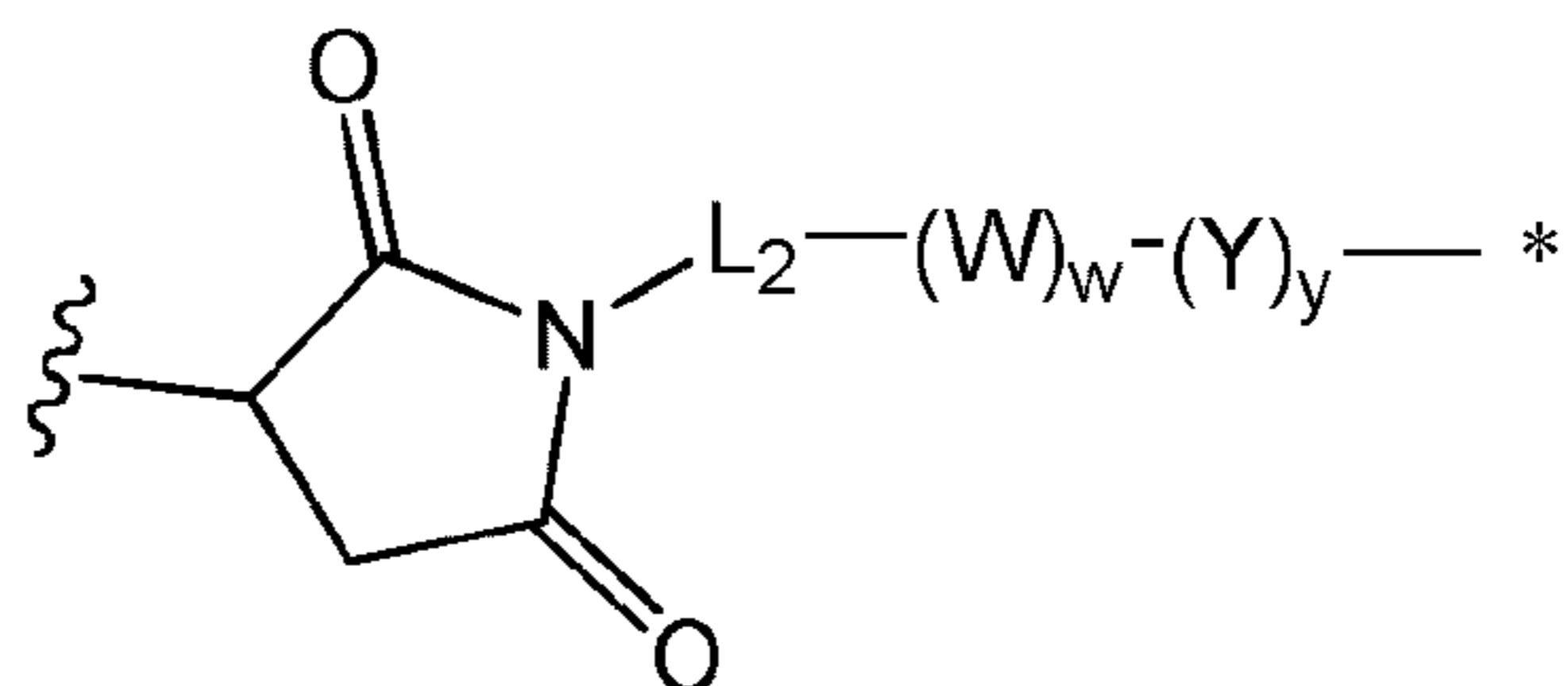
- a) a heavy chain variable domain (VH) of sequence SEQ ID No. 33 wherein said sequence SEQ ID No. 33 comprises at least 1 back-mutation selected from the residues
 5 20, 34, 35, 38, 48, 50, 59, 61, 62, 70, 72, 74, 76, 77, 79, 82 or 95; and
 b) a light chain variable domain (VL) of sequence SEQ ID No. 35, wherein said sequence SEQ ID No. 35 comprises at least 1 back-mutation selected from the residues
 22, 53, 55, 65, 71, 72, 77 or 87.

10 In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein said **Ab** is an antibody, or an antigen binding fragment thereof, capable of binding to the human protein Axl selected from:

- i) an antibody which comprises the three heavy chain CDRs of sequence SEQ ID No. 59, 60 and 61 and the three light chain CDRs of sequence SEQ ID No. 56, 57 and 58;
 15 ii) an antibody which competes for binding to Axl with the antibody of i); and
 iii) an antibody which binds to the same epitope of Axl as the antibody of i).

In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein said **Ab** is an antibody, or an antigen binding
 20 fragment thereof, capable of binding to the human HER2, preferably consisting of Trastuzumab.

In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein **L** is a linker of the following formula (III):



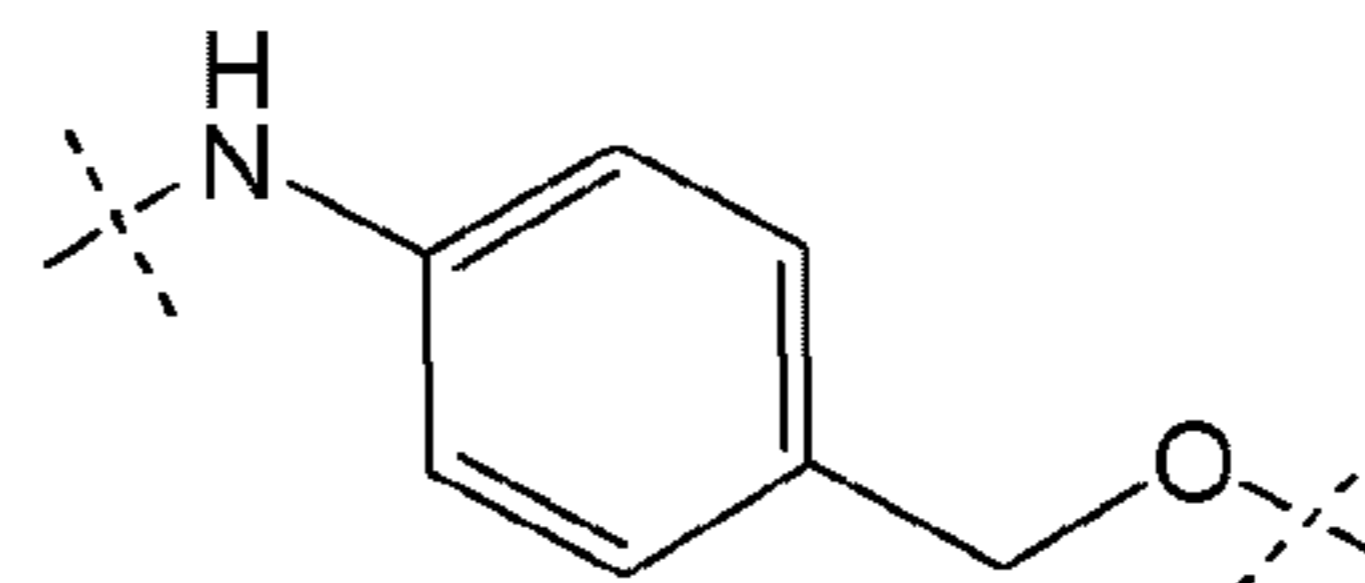
(III)

25 wherein

L_2 is (C₄-C₁₀)cycloalkyl-carbonyl, (C₂-C₆)alkyl, (C₂-C₆)alkyl-carbonyl,

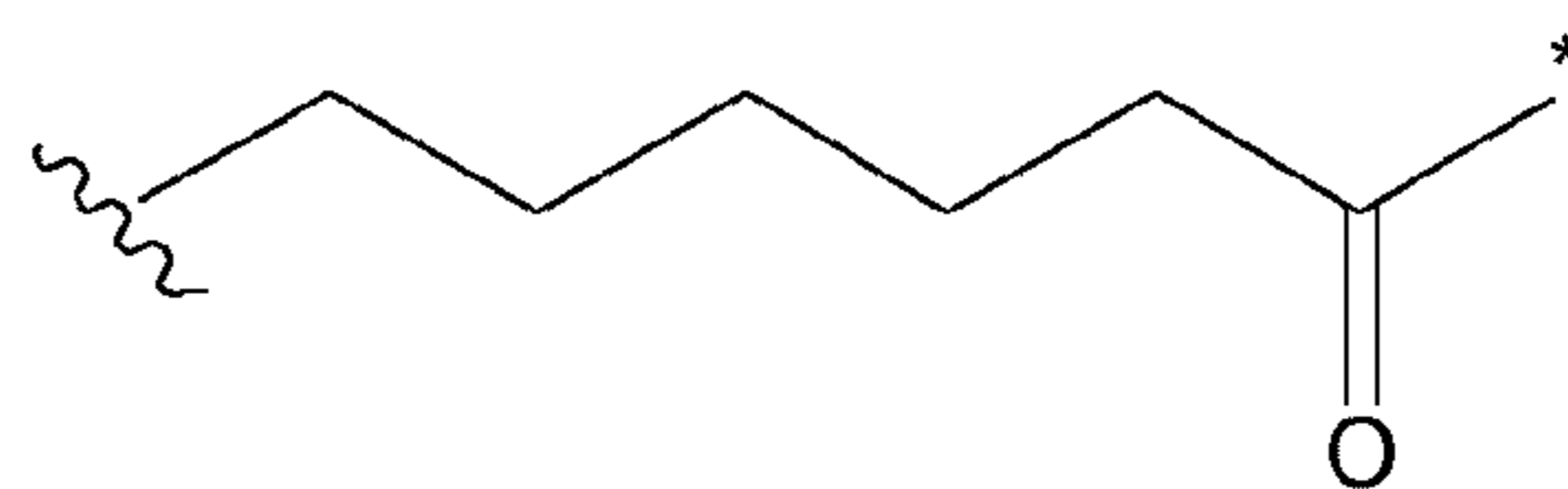
W is an amino acid unit; w is an integer comprised between 0 and 5;

6



Y is PAB-carbonyl with PAB being
the asterisk indicates the point of attachment to **D**; and
the wavy line indicates the point of attachment to **Ab**.

5 In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein L_2 is of the following formula:

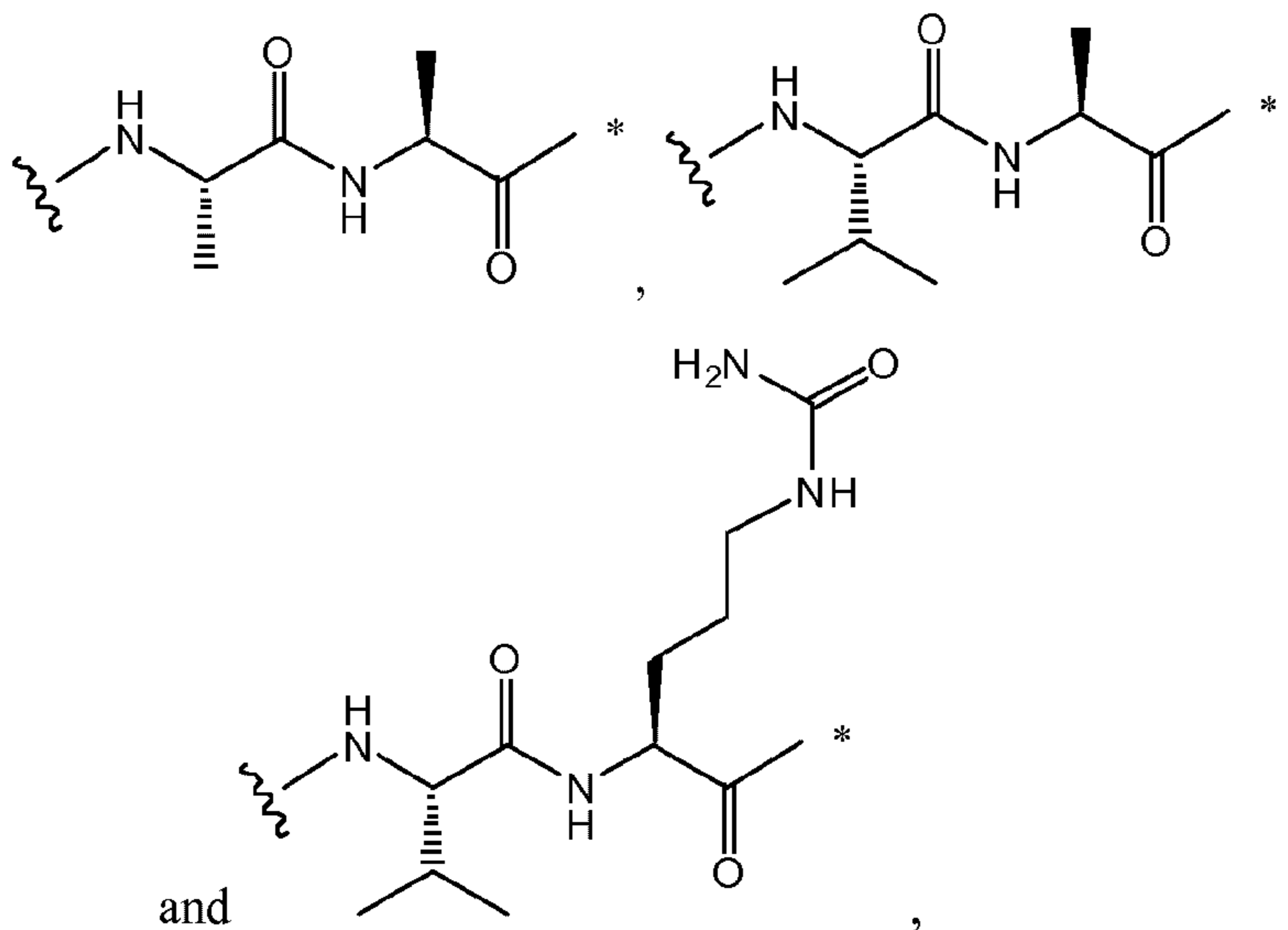


wherein

the asterisk indicates the point of attachment to $(W)_w$; and

10 the wavy line indicates the point of attachment to the nitrogen atom of the maleimide moiety.

In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein $w = 0$, or $w = 2$ and then $(W)_w$ is selected from:



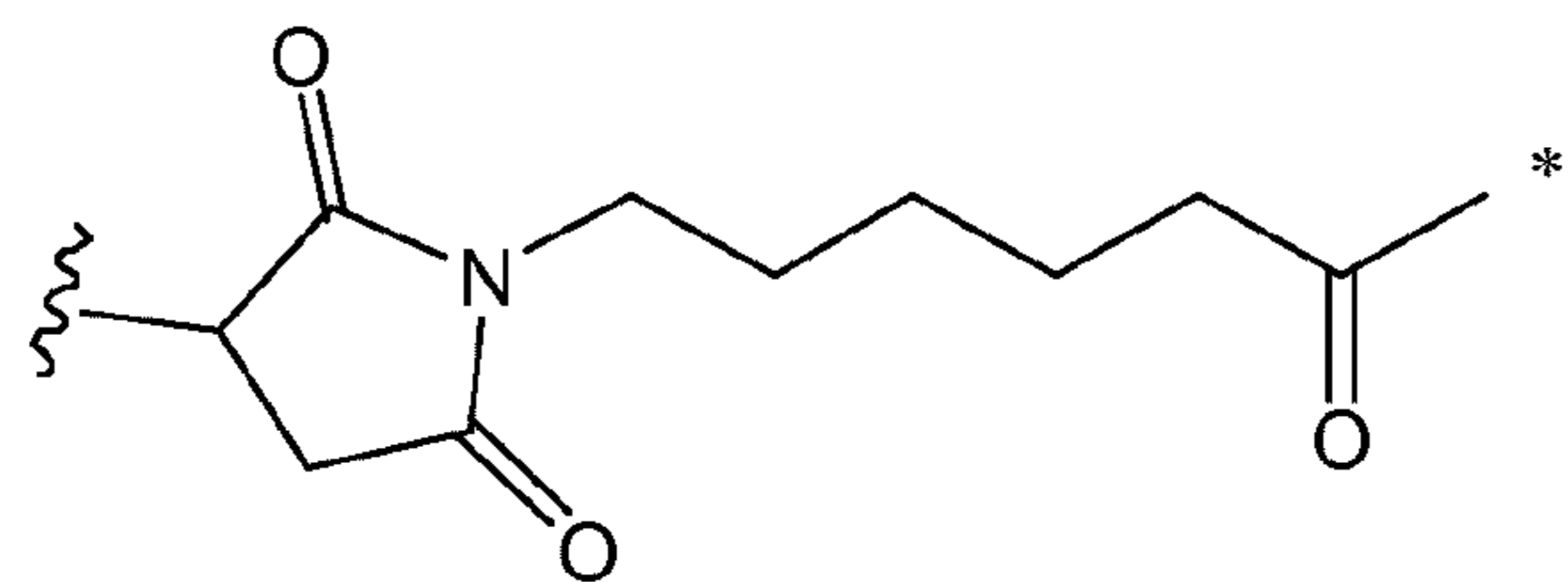
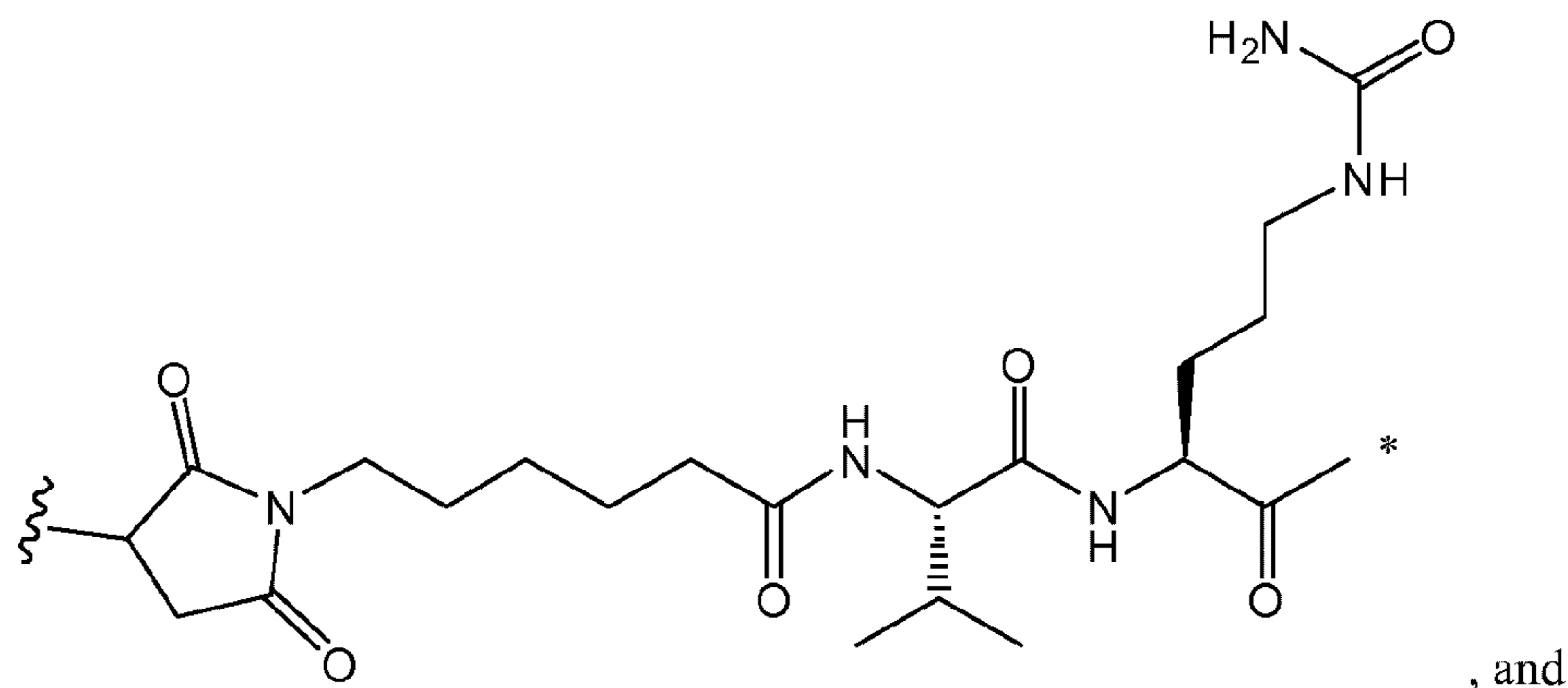
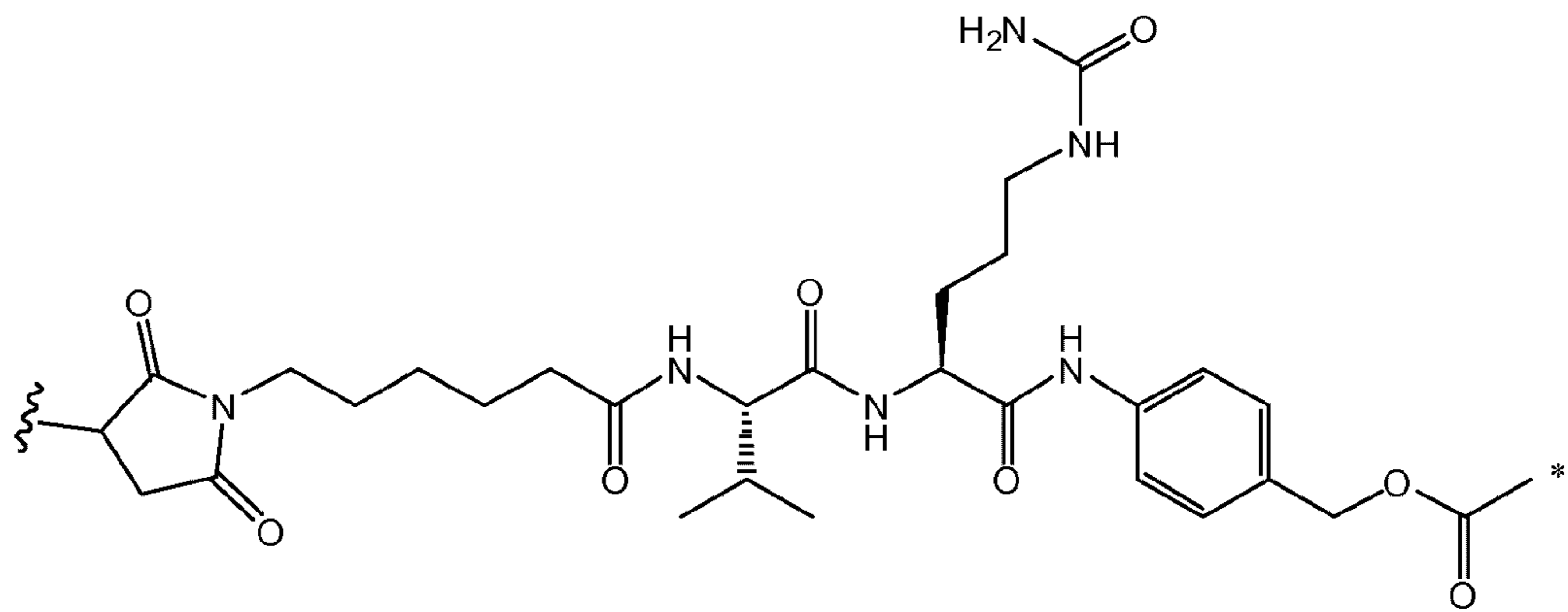
and

15

wherein

the asterisk indicates the point of attachment to $(Y)_y$; and
the wavy line indicates the point of attachment to L_2 .

In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein **L** is selected from:



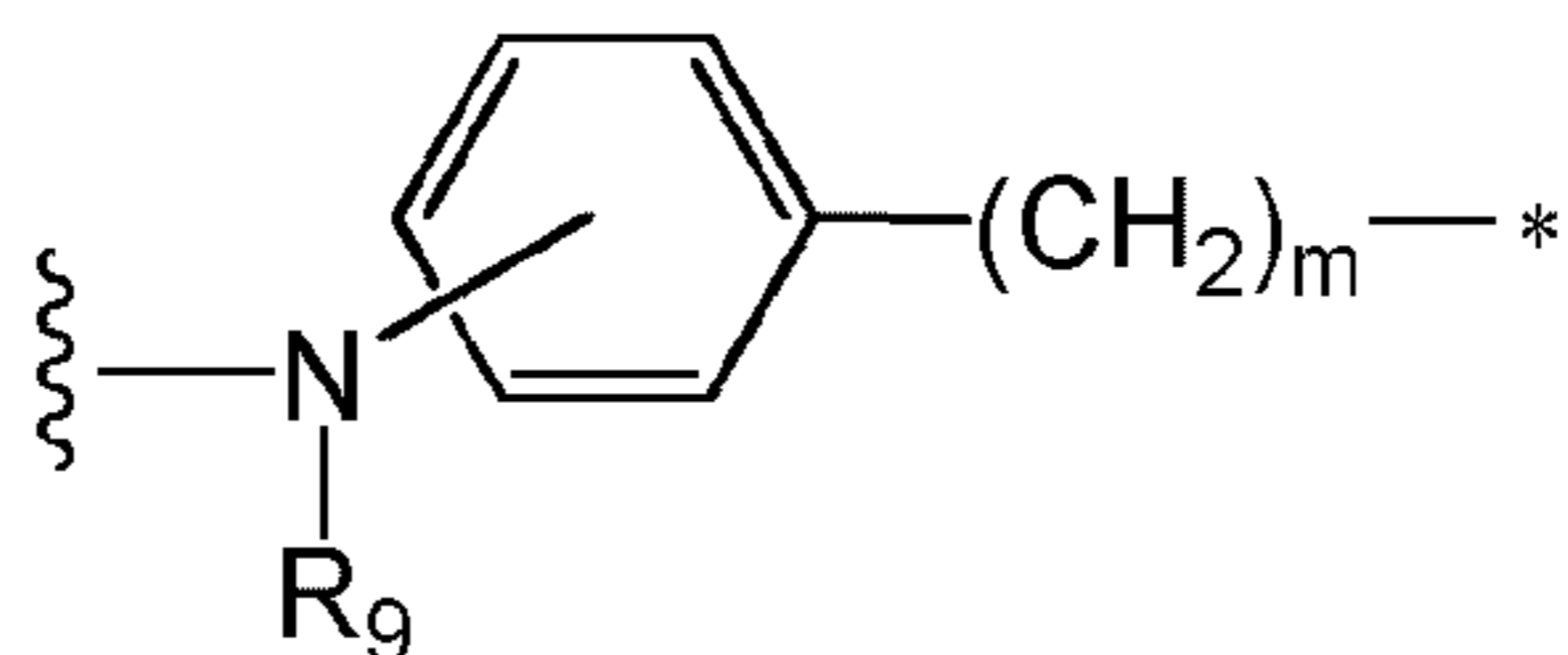
5 wherein the asterisk indicates the point of attachment to **D**, and the wavy line indicates the point of attachment to **Ab**.

In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein **A** is a group of formula $-A_a-A_b-$ in which A_a is as defined in claim 1 and A_b is a group:

- 10
- phenyl-(C₁-C₂)alkanediyl, or
 - heterocycle-(C₁-C₂)alkanediyl optionally substituted by a (C₁-C₆)alkyl group (notably unsubstituted), the heterocycle being a saturated, unsaturated or aromatic

ring with 5 or 6 members comprising 1 or 2 nitrogen atoms, chosen in particular from among pyridine, piperidine and imidazole, and being preferably a pyridine.

In an embodiment, the present invention relates to an antibody-drug-conjugate
5 according to the invention, wherein A is a group of the following formula:

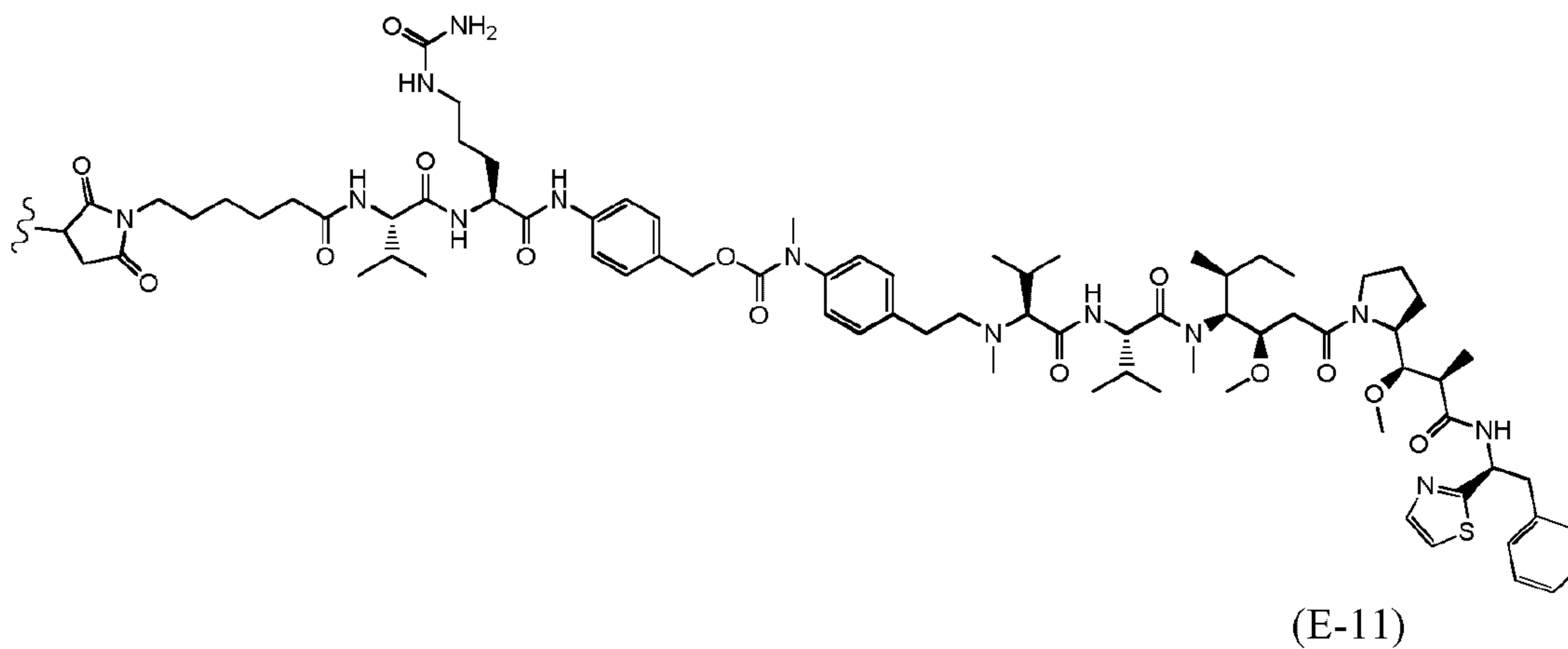


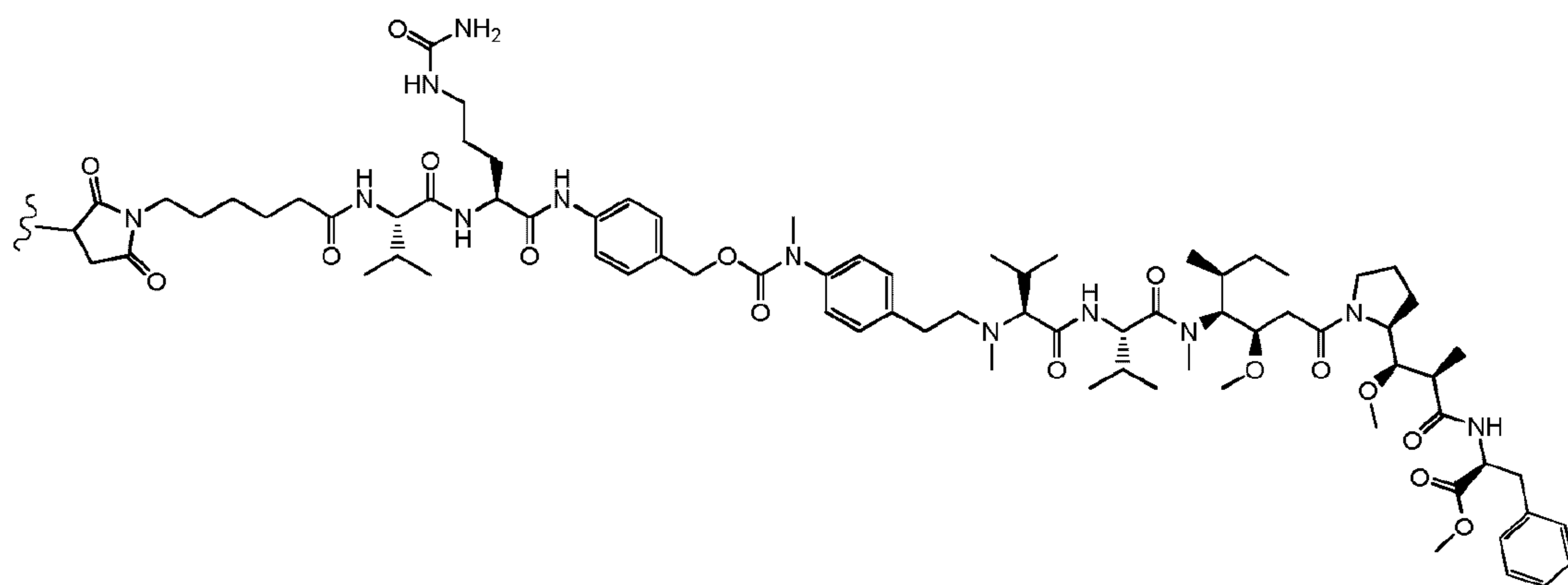
wherein:

R₉ is as defined in claim 1 and m is an integer comprised between 1 and 8, and preferably wherein R₉ = H or Me and m = 1 or 2,

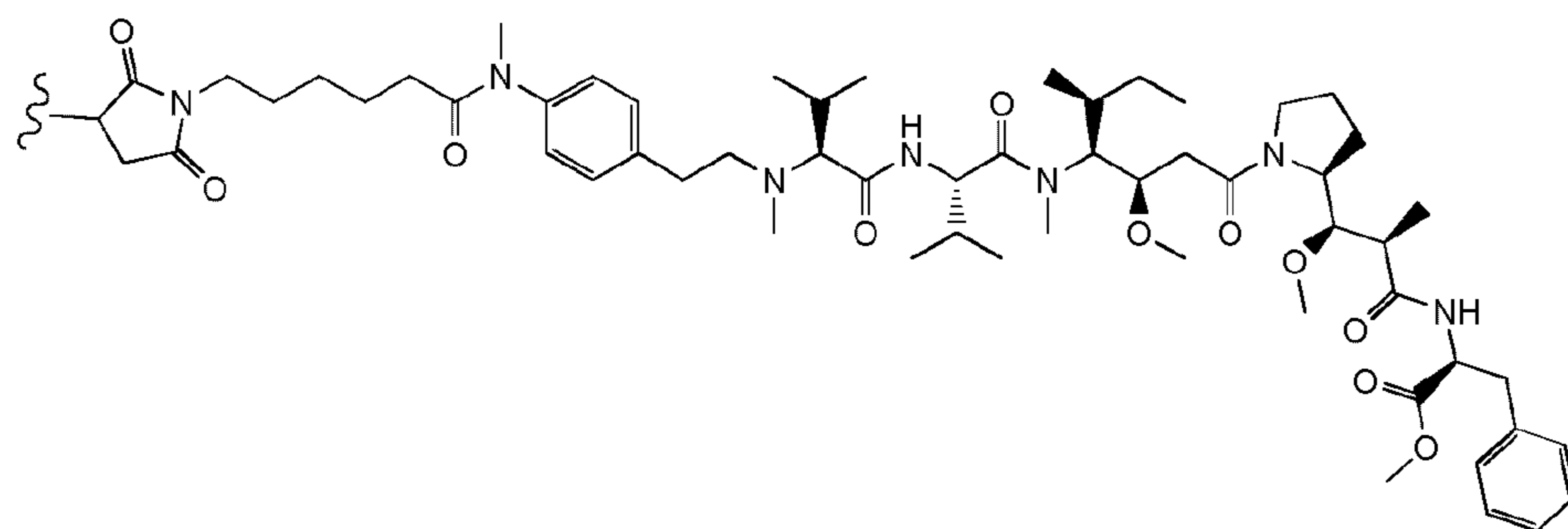
10 the wavy line indicates the point of attachment to L, and the asterisk indicates the point of attachment to NR₃.

In an embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, wherein (L-D) is selected from:

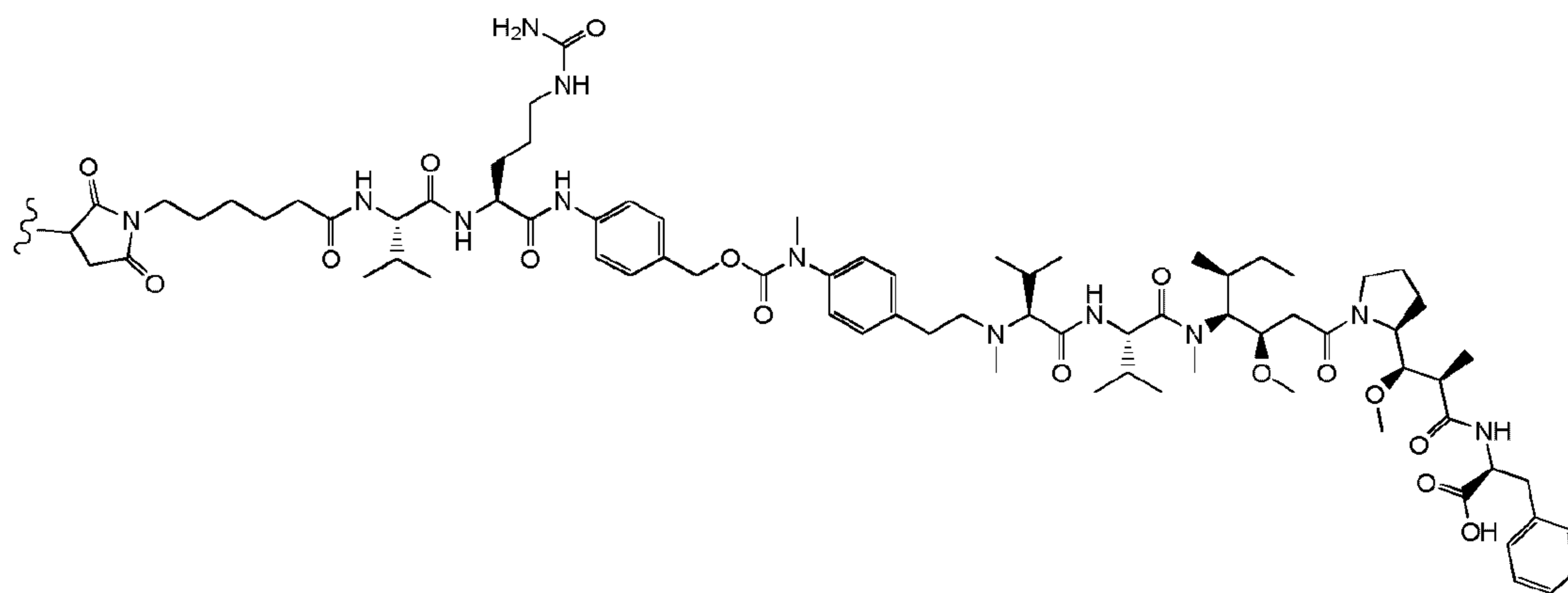




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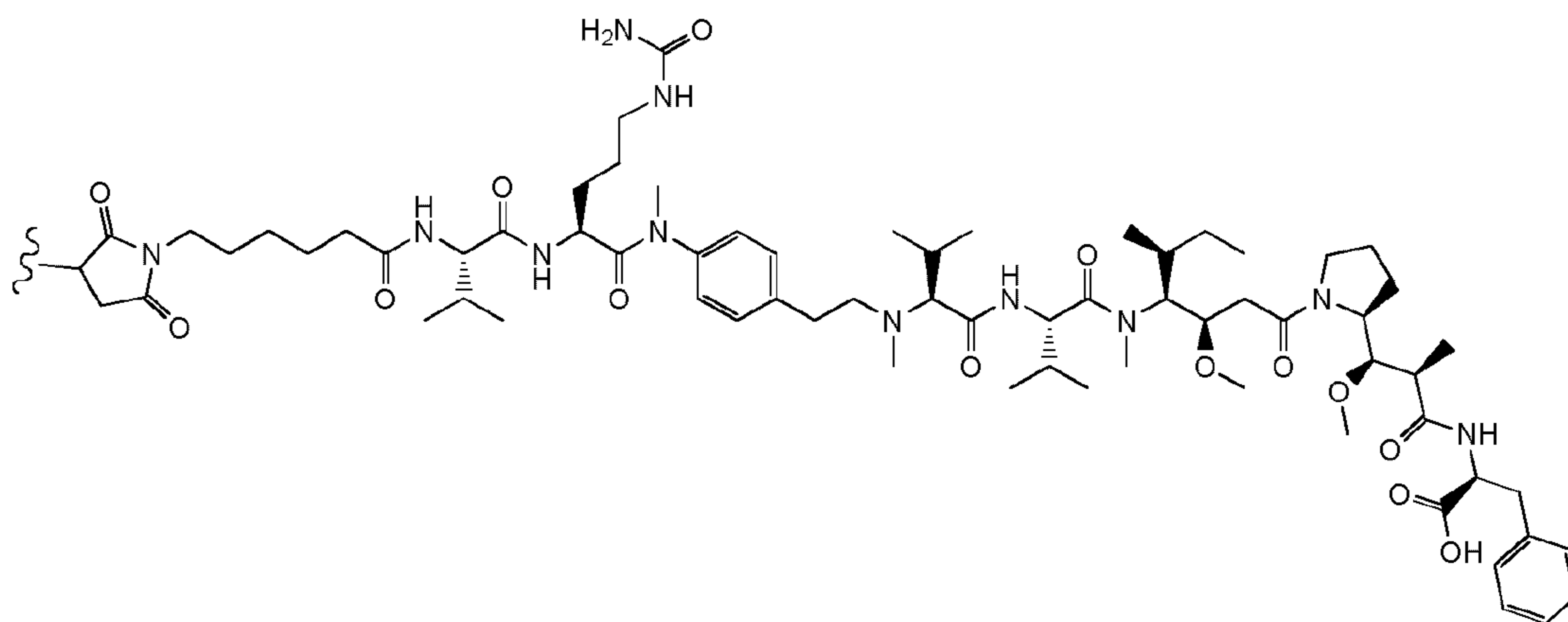


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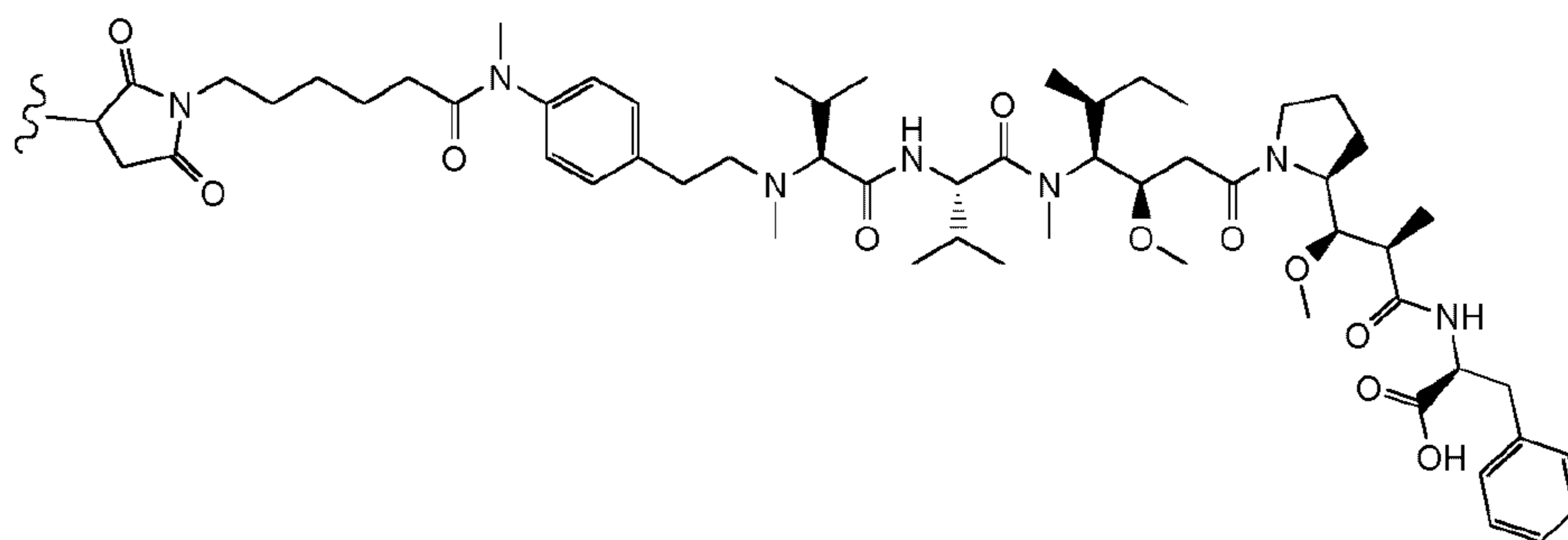


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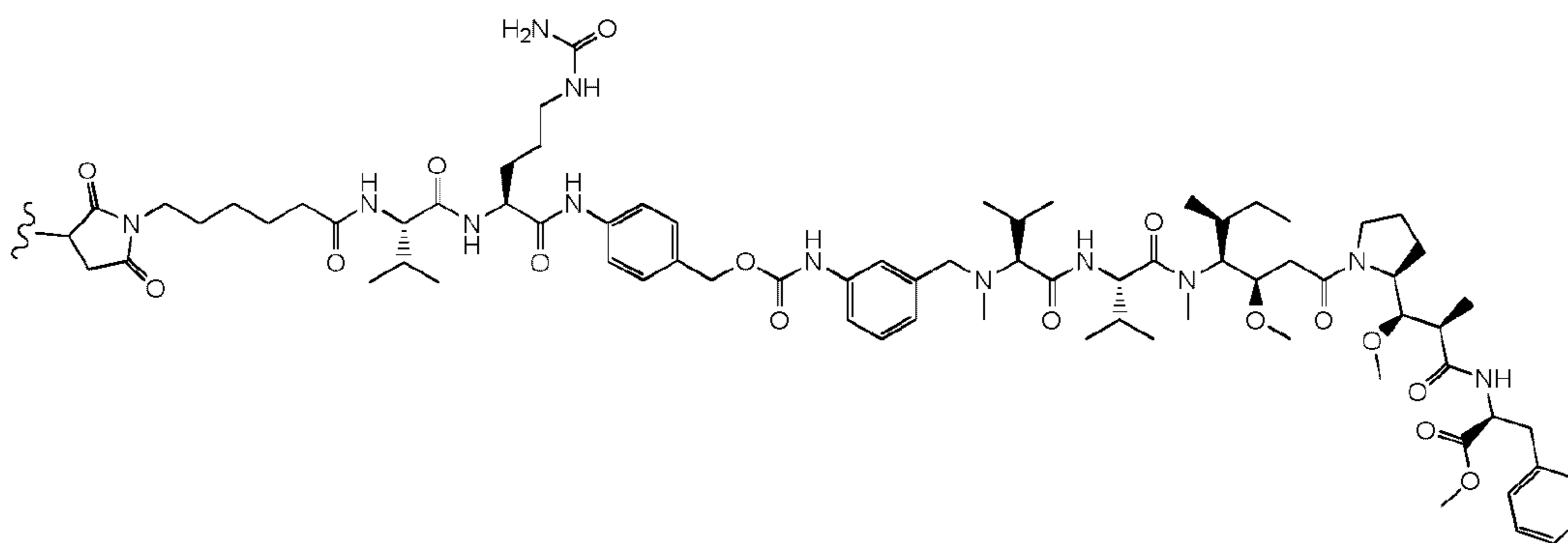
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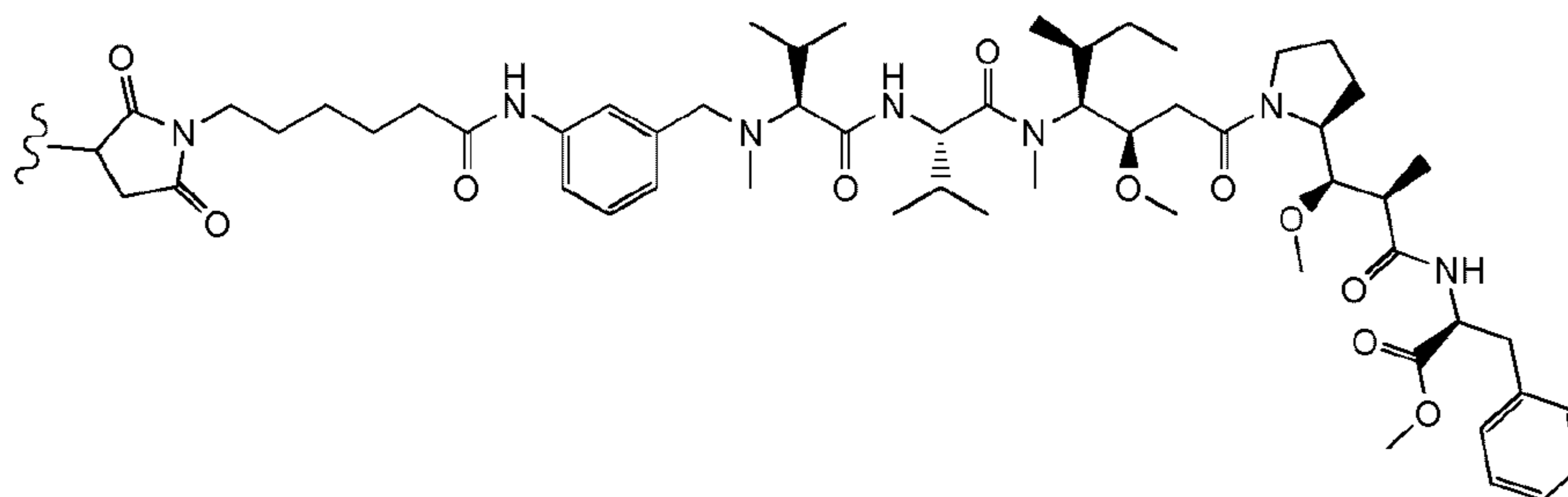
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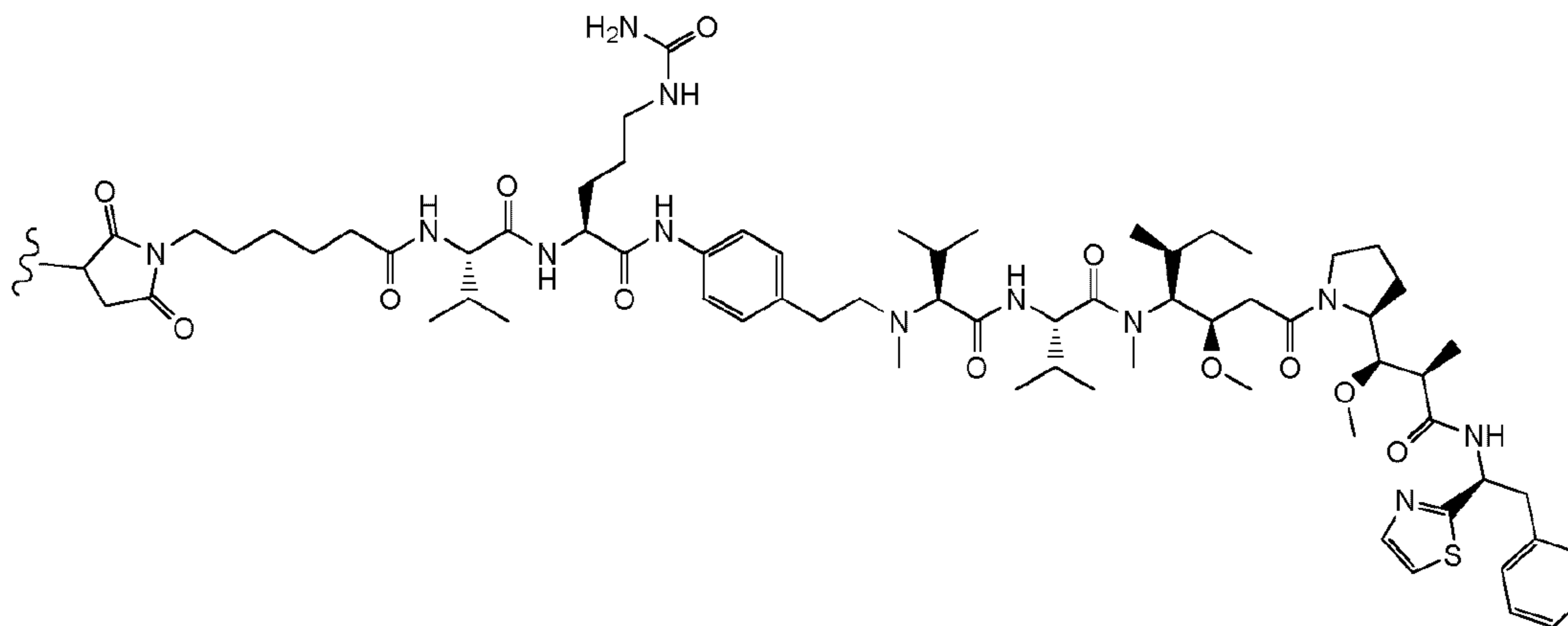


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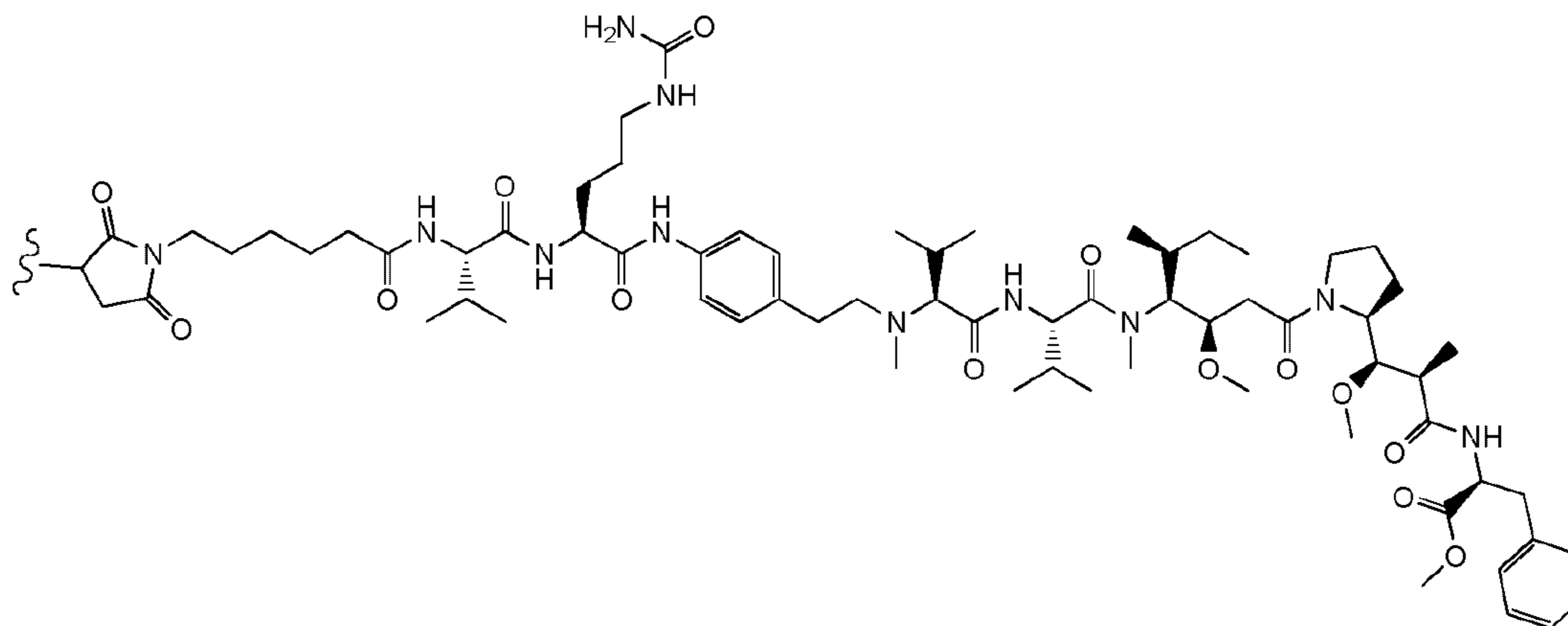


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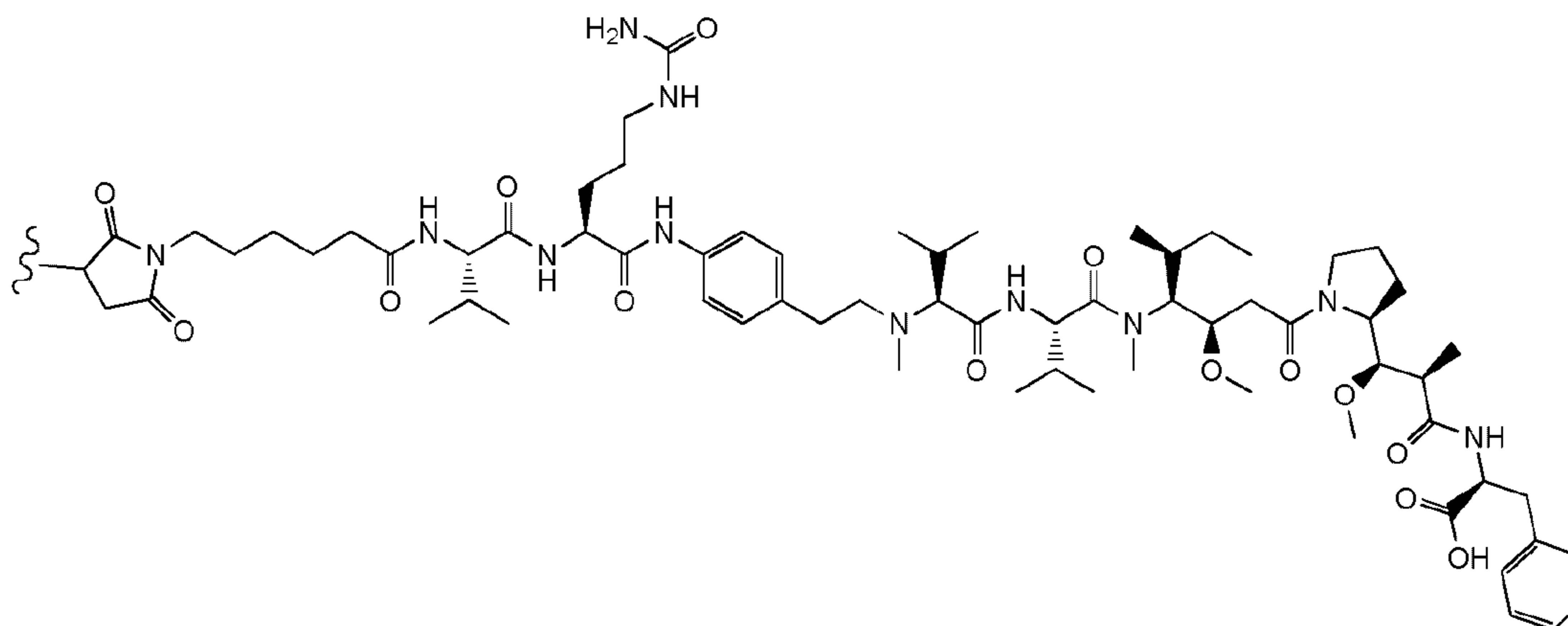


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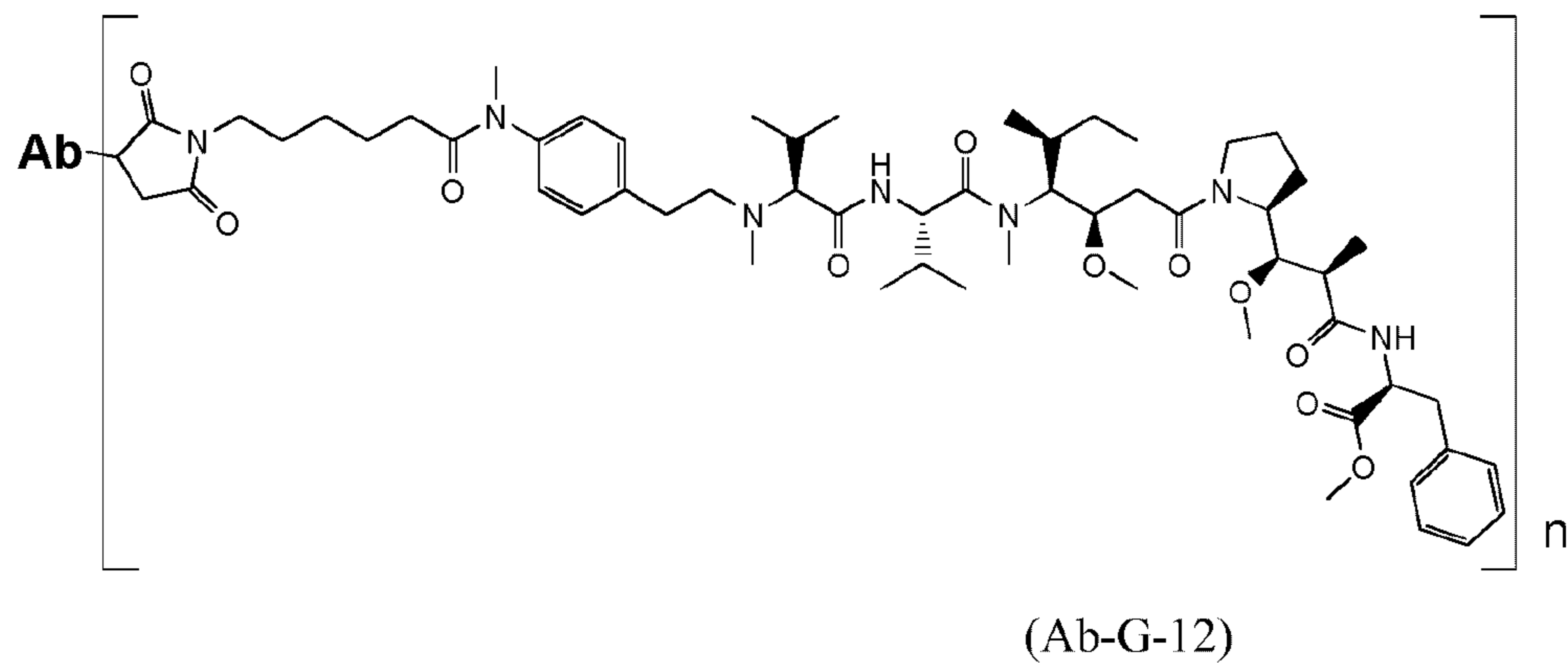
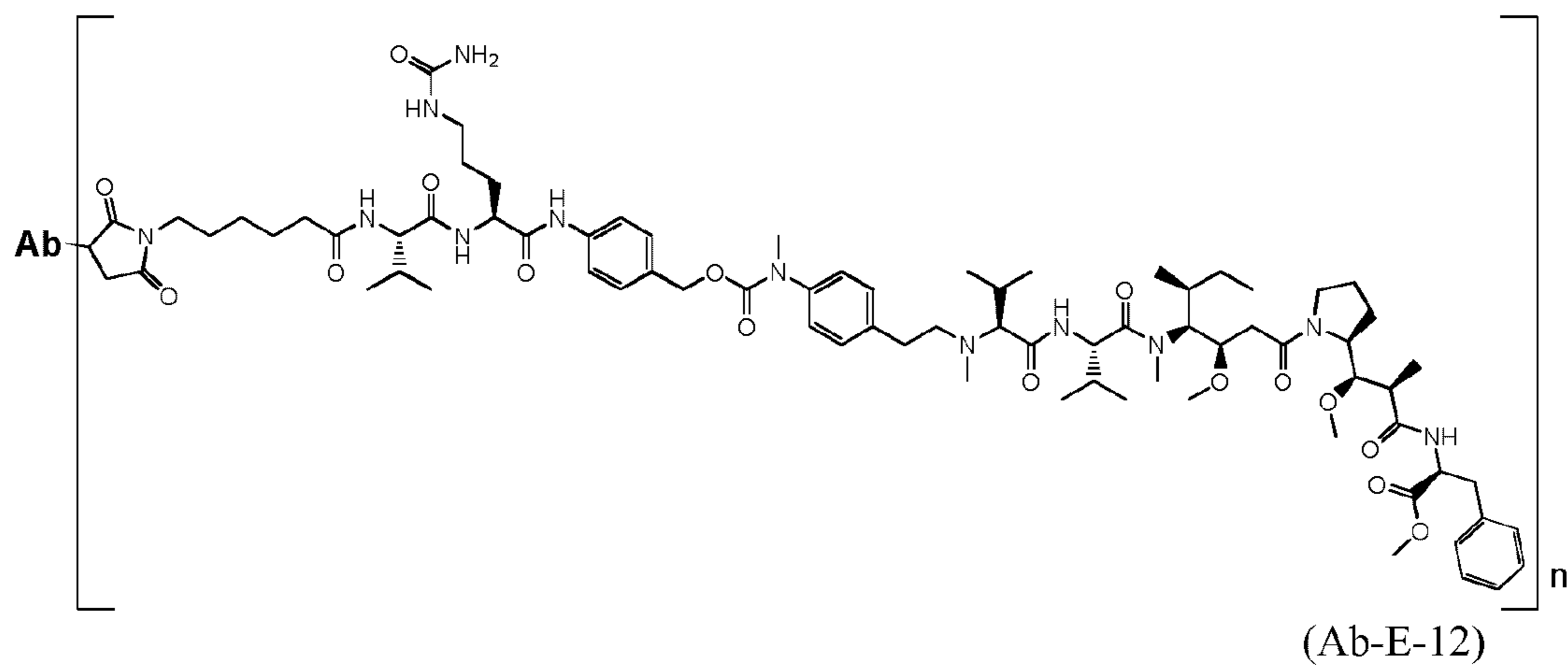
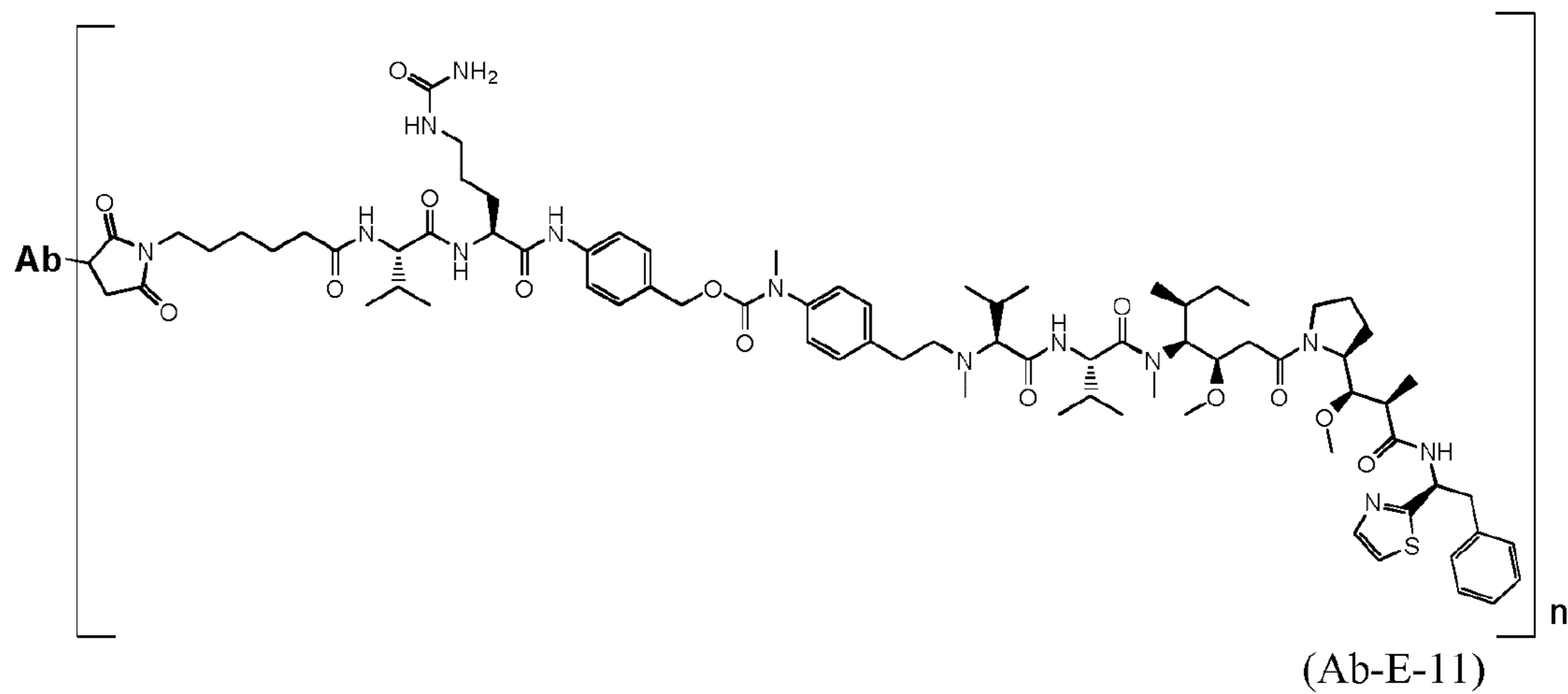
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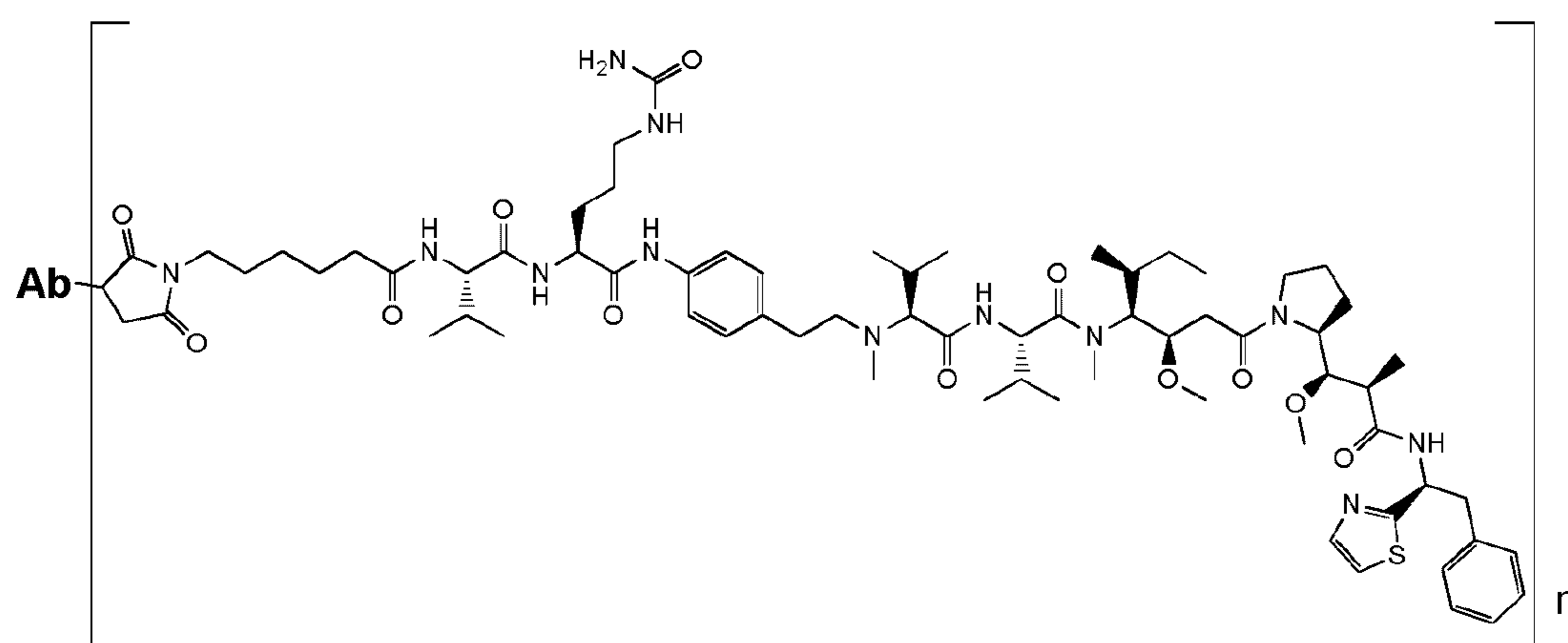
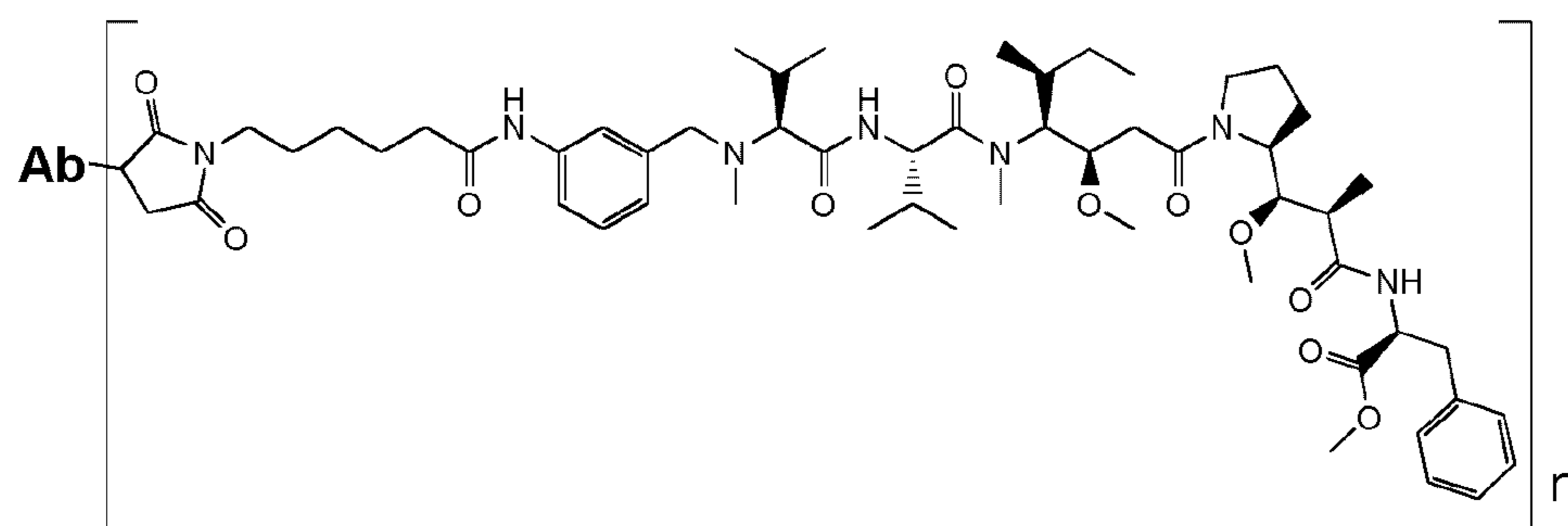
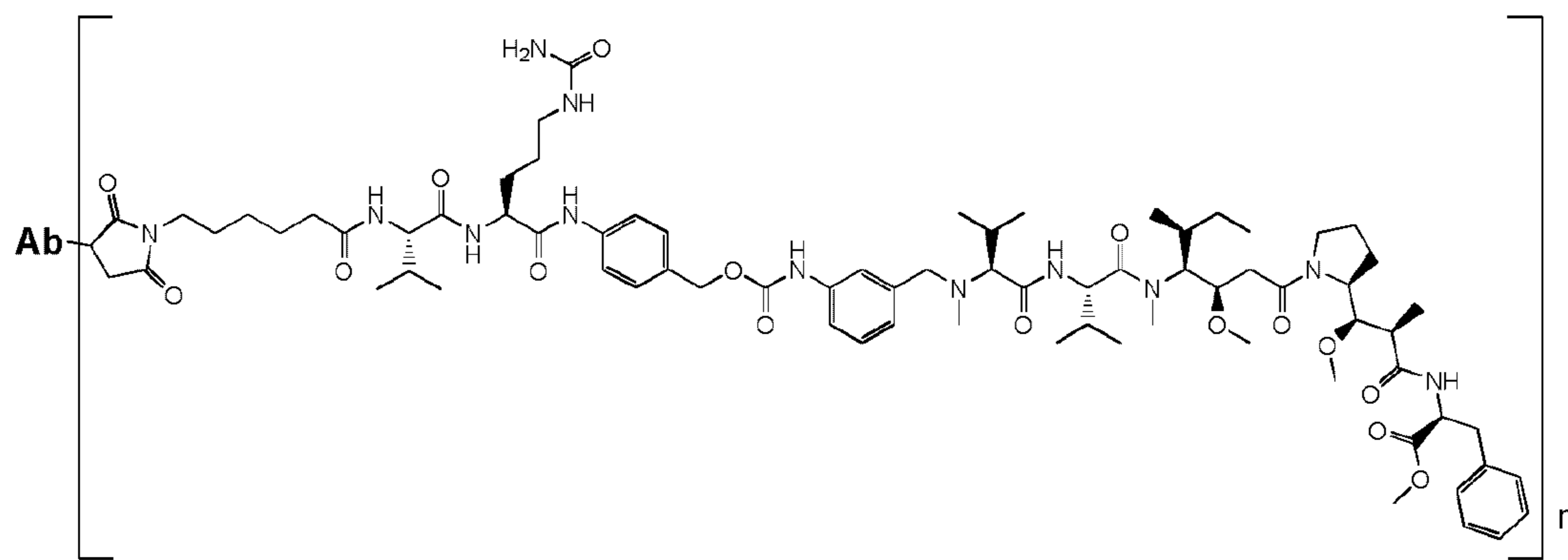
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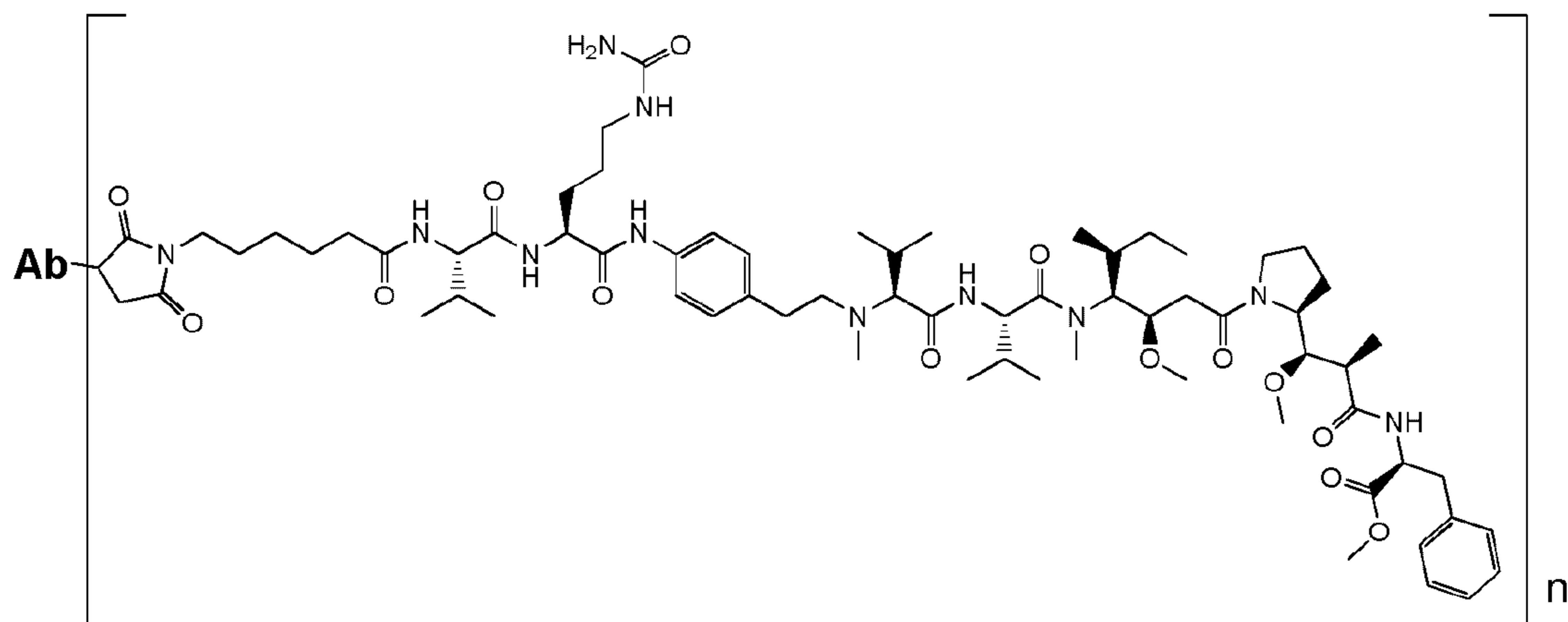
wherein the wavy line indicates the point of attachment to **Ab**.

In another embodiment, the present invention relates to an antibody-drug-conjugate according to the invention, having the formula selected from:

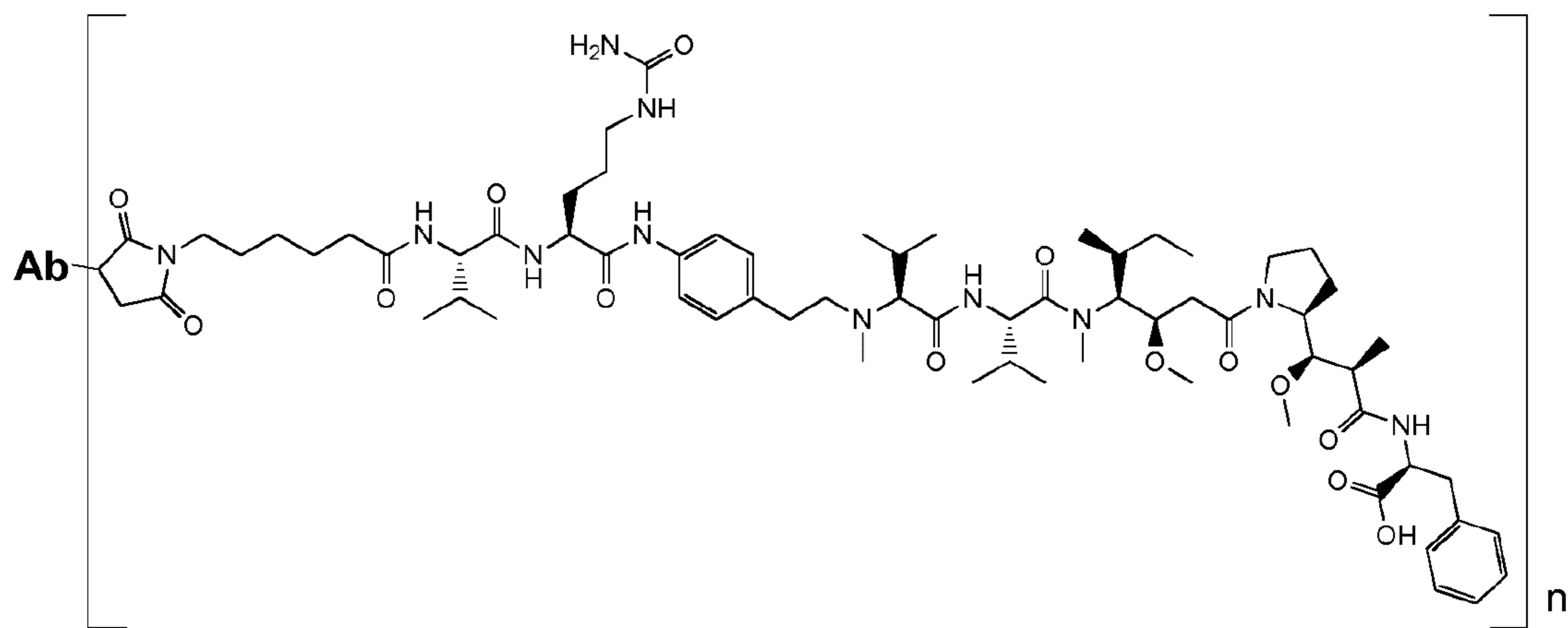


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(Ab-F-62)



(Ab-F-63)

and the pharmaceutically acceptable salts thereof,

wherein **Ab** is a [Target] antibody or an antigen antibody, or a [Target] binding fragment thereof or an antigen binding fragment thereof.

- 5 In an embodiment, and according to the invention, said **Ab** is a [Target] antibody or an antigen antibody, or a [Target] binding fragment thereof or an antigen binding fragment thereof wherein said [Target] or antigen is selected from CD19, CD20, CD22, CD25, CD30, CD33, CD40, CD56, CD64, CD70, CD74, CD79, CD105, CD138, CD174, CD205, CD227, CD326, CD340, MUC16, GPNMB, PSMA, Cripto, ED-B,
- 10 TMEFF2, EphB2, EphA2, FAP, av integrin, Mesothelin, EGFR, TAG-72, GD2, CAIX, 5T4, HER1, HER3, HER2, IGF-1R, Axl and their extra cellular membrane (ECD) fragment.

In an embodiment, and according to the invention, said **Ab** is a [Target] antibody or antigen antibody, or a [Target] or an antigen binding fragment thereof, wherein said [Target] or antigen is selected from the HER2, the IGF-1R and the protein Axl, preferably the human HER2, the human IGF-1R and the human protein Axl, and their extra cellular membrane (ECD) fragment.

In an embodiment, and according to the invention, said **Ab** is an antibody, or an antigen binding fragment thereof, and is selected from:

- 10 a) an **Ab**, or an antigen binding fragment thereof, capable of binding to the human IGF-1R selected from:
 - i) the antibodies 208F2, 212A11, 214F8, 219D6 and 213B10;
 - ii) the antibodies which compete for binding to IGF-1R with the antibodies of i); and
 - iii) the antibodies which bind to the same epitope of IGF-1R as the antibodies of i),
- 15 b) an **Ab**, or an antigen binding fragment thereof, capable of binding to the human IGF-1R selected from:
 - i) an antibody which comprises the three heavy chain CDRs of sequence SEQ ID No. 1, 2 and 3 and the three light chain CDRs of sequence SEQ ID No. 4, 5 and 6;
 - ii) an antibody which competes for binding to IGF-1R with the antibody of i); and
 - 20 iii) an antibody which binds to the same epitope of IGF-1R as the antibody of i),
- c) an **Ab** which comprises:
 - i) a heavy chain variable domain (VH) of sequence SEQ ID No. 33 wherein said sequence SEQ ID No. 33 comprises at least 1 back-mutation selected from the residues 20, 34, 35, 38, 48, 50, 59, 61, 62, 70, 72, 74, 76, 77, 79, 82 or 95; and
 - 25 ii) a light chain variable domain (VL) of sequence SEQ ID No. 35, wherein said sequence SEQ ID No. 35 comprises at least 1 back-mutation selected from the residues 22, 53, 55, 65, 71, 72, 77 or 87,
- d) an **Ab**, or an antigen binding fragment thereof, capable of binding to the human protein Axl selected from:
 - 30 i) an antibody which comprises the three heavy chain CDRs of sequence SEQ ID No. 59, 60 and 61 and the three light chain CDRs of sequence SEQ ID No. 56, 57 and 58;
 - ii) an antibody which competes for binding to Axl with the antibody of i); and

iii) an antibody which binds to the same epitope of Axl as the antibody of i), and

e) an **Ab**, or an antigen binding fragment thereof, capable of binding to the human HER2, preferably consisting of Trastuzumab or Pertuzumab..

5 In an embodiment, and according to the invention, **n** is 2 or **n** is 4.

In another embodiment, the present invention is directed to an antibody-drug-conjugate according to the present invention for use as a medicament.

10 In another embodiment, the present is directed to a composition comprising at least one antibody-drug-conjugate according to the present invention.

In an embodiment, the composition according to the present invention further comprises a pharmaceutically acceptable vehicle.

15

In another embodiment, the present invention is directed to a composition according to the present invention for use in the treatment of a [Target]- or an antigen-expressing cancer, said Target]- or an antigen being preferably selected from CD19, CD20, CD22, CD25, CD30, CD33, CD40, CD56, CD64, CD70, CD74, CD79, CD105, 20 CD138, CD174, CD205, CD227, CD326, CD340, MUC16, GPNMB, PSMA, Cripto, ED-B, TMEFF2, EphB2, EphA2, FAP, av integrin, Mesothelin, EGFR, TAG-72, GD2, CAIX, 5T4, HER1, HER3, HER2, IGF-1R, Axl and their extra cellular membrane (ECD) fragment, more preferably from the HER2, the IGF-1R and the protein Axl, also more preferably the human HER2, the human IGF-1R and the human protein Axl, and 25 their extra cellular membrane (ECD) fragment.

In an embodiment, said [Target]- or antigen-expressing cancer is a cancer chosen from breast, colon, esophageal carcinoma, hepatocellular, gastric, glioma, lung, melanoma, osteosarcoma, ovarian, prostate, rhabdomyosarcoma, renal, thyroid, uterine 30 endometrial cancer, mesothelioma, oral squamous carcinoma, Kaposi sarcoma, acute leukemia, colorectal carcinoma, melanoma, pancreatic ductal adenocarcinoma and any drug resistant cancer.

In an embodiment, the present invention relates to a method for the treatment of a [Target]- or antigen-expressing cancer in a subject in need thereof, comprising administering to the subject an effective amount of at least one antibody-drug-conjugate according to the present invention or of a composition according to the present invention.

Detailed description of the invention

10 I – The Antibody (Ab)

The terms "antibody", "antibodies" "ab", "MAb" or "immunoglobulin" are used interchangeably in the broadest sense and include monoclonal antibodies, preferably isolated, engineered or recombinant antibodies (e.g., full length or intact monoclonal antibodies), polyclonal antibodies, multivalent antibodies or multispecific antibodies (e.g., bispecific antibodies) and also antibody fragment thereof, so long as they exhibit the desired biological activity.

In an embodiment, the antibody of the invention consists of a recombinant antibody. The term "recombinant antibody" refers to an antibody that results from the expression of recombinant DNA within living cells. A recombinant antibody of the invention is obtained by using laboratory methods of genetic recombination, well known by a person skilled in the art, creating DNA sequences that would not be found in biological organisms.

In another embodiment, the antibody of the invention consists of a chemically synthesized antibody.

25 More particularly, such a molecule consists of a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain comprises a heavy chain variable region (or domain) (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region comprises three domains, CH1, CH2 and CH3. Each light chain comprises a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. 30 The light chain constant region comprises one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity

determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g. effector cells) and the first component (Clq) of the classical complement system.

By “antigen binding fragment” or “target binding fragment” of an antibody according to the invention, it is intended to indicate any peptide, polypeptide, or protein retaining the ability to bind to the target (also generally referred as antigen) of the antibody, and comprising an amino acid sequence of at least 10 contiguous amino acid residues, at least 15 contiguous amino acid residues, at least 20 contiguous amino acid residues, at least 25 contiguous amino acid residues, at least 40 contiguous amino acid residues, at least 50 contiguous amino acid residues, at least 60 contiguous amino acid residues, at least 70 contiguous amino acid residues, at least contiguous 80 amino acid residues, at least contiguous 90 amino acid residues, at least contiguous 100 amino acid residues, at least contiguous 125 amino acid residues, at least 150 contiguous amino acid residues, at least contiguous 175 amino acid residues, at least contiguous 200 amino acid residues, or at least contiguous 250 amino acid residues of the amino acid sequence of the antibody.

In an embodiment, such “antigen binding fragments” are selected in the group consisting of Fv, scFv (sc for single chain), Fab, F(ab')₂, Fab', scFv-Fc fragments or diabodies, or any fragment of which the half-life time would have been increased by chemical modification, such as the addition of poly(alkylene) glycol such as poly(ethylene) glycol (“PEGylation”) (pegylated fragments called Fv-PEG, scFv-PEG, Fab-PEG, F(ab')₂-PEG or Fab'-PEG) (“PEG” for Poly(Ethylene) Glycol), or by incorporation in a liposome, said fragments having at least one of the characteristic CDRs of the antibody according to the invention. Preferably, said “antigen binding fragments” will be constituted or will comprise a partial sequence of the heavy or light variable chain of the antibody from which they are derived, said partial sequence being sufficient to retain the same specificity of binding as the antibody from which it is

descended and a sufficient affinity, preferably at least equal to 1/100, in a more preferred manner to at least 1/10, of the affinity of the antibody from which it is descended, with respect to the target.

By “binding”, “binds”, or the like, it is intended that antibody, or any antigen
5 binding fragment thereof, forms a complex with an antigen that is relatively stable under physiologic conditions. Specific binding can be characterized by an equilibrium dissociation constant of at least about $1 \cdot 10^{-6}$ M. Methods for determining whether two molecules bind are well known in the art and include, for example, equilibrium dialysis, surface plasmon resonance, radiolabelled assays and the like. For the avoidance of
10 doubt, it does not mean that the said antibody could not bind or interfere, at a low level, to another antigen. Nevertheless, as a preferred embodiment, the said antibody binds only to the said antigen.

As used in the present specification, the expression “[Target] antibody” should be interpreted as similar to “anti-[Target] antibody” and means an antibody capable of
15 binding to the [Target].

The expression “Target” or [Target] shall be interpreted as any molecule present at the surface of cells, preferably tumoral cells, more preferably mammals and human cells, and which can be used for drug delivery. Preferably, the Target is specifically express or overexpress on the surface of tumoral cells in comparison with normal cells.

20 More particularly, the Target can be selected from CD19, CD20, CD22, CD25, CD30, CD33, CD40, CD56, CD64, CD70, CD74, CD79, CD105, CD138, CD174, CD205, CD227, CD326, CD340, MUC16, GPNMB, PSMA, Cripto, ED-B, TMEFF2, EphB2, EphA2, FAP, av integrin, Mesothelin, EGFR, TAG-72, GD2, CAIX, 5T4, HER1, HER3, HER2, IGF-1R or Axl (B.A. Teicher, Current Cancer Drug Targets,
25 2009, 9, 982-1004).

As preferred Target, it can be mentioned HER2, IGF-1R and Axl, preferably human HER2, IGF-1R and Axl.

As non limitative example, the expression “HER2 antibody”, “IGF-1R antibody” or “Axl antibody” should be interpreted as similar to “anti-HER2 antibody”, “anti-IGF-
30 1R antibody” or “anti-Axl antibody” and means an antibody capable of binding to HER2, IGF-1R or Axl, respectively.

In the present application, the epitope of the antibody is preferentially localized into the extracellular domain of the Target.

In a particular embodiment, the antibody, or any antigen binding fragment thereof, is capable of binding to the Target with an EC_{50} comprised between $10 \cdot 10^{-10}$ to 1×10^{-10} , and more preferentially between 8×10^{-10} to 2×10^{-10} .

The term half maximal effective concentration (EC_{50}) corresponds to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after some specified exposure time. It is commonly used as a measure of drug's potency. The EC_{50} of a graded dose response curve therefore represents the concentration of a compound where 50% of its maximal effect is observed. The EC_{50} of a quantal dose response curve represents the concentration of a compound where 50% of the population exhibits a response, after specified exposure duration. Concentration measures typically follow a sigmoidal curve, increasing rapidly over a relatively small change in concentration. This can be determined mathematically by derivation of the best-fit line.

As a preferred embodiment, the EC_{50} , determined in the present invention, characterizes the potency of antibody to bind on the Target ECD exposed on human tumor cells. The EC_{50} parameter is determined using FACS analysis. The EC_{50} parameter reflects the antibody concentration for which 50% of the maximal binding on the human Target expressed on tumor cells is obtained. Each EC_{50} value was calculated as the midpoint of the dose response curve using a four-parameter regression curve fitting program (Prism Software). This parameter has been selected as to be representative of physiological/pathological conditions.

The term "epitope" is a region of an antigen that is bound by an antibody. Epitopes may be defined as structural or functional. Functional epitopes are generally a subset of the structural epitopes and have those residues that directly contribute to the affinity of the interaction. Epitopes may also be conformational, that is, composed of non-linear amino acids. In certain embodiments, epitopes may include determinants that are chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl groups, or sulfonyl groups, and, in certain embodiments, may have specific three-dimensional structural characteristics, and/or specific charge characteristics.

The competition for binding to the Target can be determined by any methods or techniques known by the person skilled in the art such as, without limitation, radioactivity, Biacore, ELISA, Flow cytometry, etc. As “which competes for binding to the Target” it is meant a competition of at least 20%, preferentially at least 50% and
5 more preferentially at least 70%.

The determination of the binding to the same epitope can be determined by any methods or techniques known by the person skilled in the art such as, without limitation in the examples, radioactivity, Biacore, ELISA, Flow cytometry. As “which bind to the same epitope of the Target”, it is meant a competition of at least 20%, preferentially at
10 least 50% and more preferentially at least 70%.

As above mentioned, and contrary to the general knowledge, the present invention focuses on specific antibodies presenting a high ability to be internalized following their binding to the Target. As used herein, an antibody that “is internalized” or that “internalized” (the two expressions being similar) is one that is taken up by
15 (meaning it “enters”) the cell upon binding to the Target on a mammalian cell. Such an antibody is interesting as part of the ADC, so it addresses the linked cytotoxic into the targeted cancer cells. Once internalized the cytotoxic triggers cancer cell death.

Important keys to success with ADC therapy are thought to be the target antigen specificity and the internalization of the antigen-antibody complexes into the cancer
20 cells. Obviously non-internalizing antigens are less effective than internalizing antigens to delivers cytotoxic agents. Internalization processes are variable across antigens and depend on multiple parameters that can be influenced by antibodies.

In the ADC, the cytotoxic confers the cytotoxic activity and the used antibody is responsible for the specificity against cancer cells, as well as a vector for entering
25 within the cells to correctly address the cytotoxic. Thus to improve the ADC, the antibody must exhibit high ability to internalize into the targeted cancer cells. The efficiency of the antibody mediated internalisation differs significantly depending on the epitope targeted. Selection of potent internalizing antibodies requires various experimental data studying not only the Target downregulation but also following the
30 antibody penetration into the cells.

In a preferred embodiment, the internalization of the antibody according to the invention can be evaluated by immunofluorescence or FACS (Flow Cytometry) (as

exemplified hereinafter in the present application) or any method or process known by the person skilled in the art specific for the internalization mechanism. In a preferred embodiment, the antibody according to the invention can induce internalization after binding to the Target of at least 30%, preferentially 50% and more preferentially 80%.

5 The complex Target/antibody is internalized after binding of the antibody to the ECD of said Target, and a reduction in the quantity of Target at the surface of the cells is induced. This reduction can be quantified by any method known by the person skilled in the art (western-blot, FACS, immunofluorescence, etc).

In one embodiment, this reduction, thus reflecting the internalization, can be preferably measured by FACS and expressed as the difference or delta between the Mean Fluorescence Intensity (MFI) measured at 4°C with the MFI measured at 37°C after 4 hours incubation with the antibody.

As non limitative example, this delta is determined based on MFIs obtained with untreated cells and cells treated with the antibody using i) cancer cells, such as for example MCF,7 after a 4 hour incubation period with the antibody herein described and ii) a secondary antibody labelled with Alexa488. This parameter is defined as calculated with the following formula: $\Delta(\text{MFI}_{4^{\circ}\text{C}} - \text{MFI}_{37^{\circ}\text{C}})$. Other embodiments will be detailed in the following examples.

This difference between MFIs reflects the Target downregulation as MFIs are proportional to Target expressed on the cell-surface.

In an advantageous aspect, the antibodies consist of antibodies triggering, as an example, a $\Delta(\text{MFI}_{4^{\circ}\text{C}} - \text{MFI}_{37^{\circ}\text{C}})$ on cancer cell, such as MCF-7, of at least 280, preferably of at least 400.

In more details, the above mentioned delta can be measured according to the following process, which must be considered as an illustrative and non limitative example:

- a) Treating and incubating tumor cells of interest with the antibody of the invention in either cold (4°C) or warm (37°C) complete culture medium;
- 30 b) Treating the treated cells of step a) and, in parallel, untreated cells with a secondary antibody;

- c) Measuring the MFI (representative of the quantity of IGF-1R present at the surface) for the treated and the non treated cells with a secondary labeled antibody capable of binding to the antibody of the invention; and
- 5 d) Calculating the delta as the subtraction of the MFI obtained with the treated cells from the MFI obtained with the non treated cells.

From this delta MFI, an internalization percentage can be determined as:

$$100 \times (\text{MFI}_{4^{\circ}\text{C}} - \text{MFI}_{37^{\circ}\text{C}}) / \text{MFI}_{4^{\circ}\text{C}}$$

The antibodies, according to the invention, present, preferably, an internalization
10 percentage comprised between 50% and 99%, 70% and 90%, preferentially between 75% and 87%.

A particular advantage of the antibodies herein described relies on their rate of internalization.

It is generally known that, for an ADC, it is desirable that the used antibodies
15 exhibit a rapid rate of internalization, preferably within 24 hours from administration of the antibody and, more preferably within 12 hours and, even more preferably within 6 hours.

In the present invention, the internalization rate, also referred as cell surface bound antibody decrease or cell surface antibody decay, is expressed as t1/2 (half life)
20 and corresponds as the time necessary to obtain a decrease of 50% of the ΔMFI (this aspect will be clearly understood regarding the following examples).

A particular advantage is that the antibodies of the invention have a t1/2 comprised between 5 and 25 minutes, and preferentially between 10 and 20 minutes.

In the following specification, two preferred Target will be exemplified.

25 For these two preferred Target, by “antibody according to the invention” or “antibody of the invention” it must be understood: “antibody of the ADC according to the invention” or “antibody of the ADC of the invention” respectively.

I.1: IGF-1R antibodies

30 A particular embodiment of the invention relates to an ADC wherein the antibody Ab comprises three heavy chain CDRs with CDR-H2 of sequence SEQ ID No.

2 and CDR-H3 of sequence SEQ ID No. 3, and three light chain CDRs with CDR-L2 of sequence SEQ ID No. 5.

A particular embodiment of the invention relates to an ADC wherein the antibody Ab comprises the three heavy chain CDRs of sequences SEQ ID Nos. 1, 2 and 3 and the three light chain CDRs of sequences SEQ ID Nos. 4, 5 and 6.

An embodiment of the ADC comprises an antibody comprising the three heavy chain CDRs comprising the sequences SEQ ID Nos. 1, 2 and 3, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID Nos. 1, 2 and 3; and the three light chain CDRs comprising the sequences SEQ ID Nos. 4, 5 and 6, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID Nos. 4, 5 and 6.

In another embodiment, the antibody, or any antigen binding fragment thereof, comprises the three light chain CDRs comprising the sequences SEQ ID Nos. 1, 2 and 3; and the three heavy chain CDRs comprising the sequences SEQ ID Nos. 4, 5 and 6.

The IMGT unique numbering has been defined to compare the variable domains whatever the antigen receptor, the chain type, or the species [Lefranc M.-P., Immunology Today 18, 509 (1997) / Lefranc M.-P., The Immunologist, 7, 132-136 (1999) / Lefranc, M.-P., Pommié, C., Ruiz, M., Giudicelli, V., Foulquier, E., Truong, L., Thouvenin-Contet, V. and Lefranc, Dev. Comp. Immunol., 27, 55-77 (2003)]. In the IMGT unique numbering, the conserved amino acids always have the same position, for instance cystein 23 (1st-CYS), tryptophan 41 (CONSERVED-TRP), hydrophobic amino acid 89, cystein 104 (2nd-CYS), phenylalanine or tryptophan 118 (J-PHE or J-TRP). The IMGT unique numbering provides a standardized delimitation of the framework regions (FR1-IMGT: positions 1 to 26, FR2-IMGT: 39 to 55, FR3-IMGT: 66 to 104 and FR4-IMGT: 118 to 128) and of the complementarity determining regions: CDR1-IMGT: 27 to 38, CDR2-IMGT: 56 to 65 and CDR3-IMGT: 105 to 117. As gaps represent unoccupied positions, the CDR-IMGT lengths (shown between brackets and separated by dots, e.g. [8.8.13]) become crucial information. The IMGT unique numbering is used in 2D graphical representations, designated as IMGT Colliers de Perles [Ruiz, M. and Lefranc, M.-P., Immunogenetics, 53, 857-883 (2002) / Kaas, Q. and Lefranc, M.-P., Current Bioinformatics, 2, 21-30 (2007)], and in 3D structures in

IMGT/3Dstructure-DB [Kaas, Q., Ruiz, M. and Lefranc, M.-P., T cell receptor and MHC structural data. Nucl. Acids. Res., 32, D208-D210 (2004)].

It must be understood that, without contradictory specification in the present specification, complementarity-determining regions or CDRs, mean the hypervariable
5 regions of the heavy and light chains of immunoglobulins as defined according to the IMGT numbering system.

Nevertheless, CDRs can also be defined according to the Kabat numbering system (Kabat *et al.*, Sequences of proteins of immunological interest, 5th Ed., U.S. Department of Health and Human Services, NIH, 1991, and later editions). There are
10 three heavy chain CDRs and three light chain CDRs. Here, the terms “CDR” and “CDRs” are used to indicate, depending on the case, one or more, or even all, of the regions containing the majority of the amino acid residues responsible for the antibody’s binding affinity for the antigen or epitope it recognizes. In order to simplify the reading of the present application, the CDRs according to Kabat are not defined.
15 Nevertheless, it would be obvious for the person skilled in the art, using the definition of the CDRs according to IMGT, to define the CDRs according to Kabat.

In the sense of the present invention, the “identity” or “percentage identity” between two sequences of nucleic acids or amino acids means the percentage of identical nucleotides or amino acid residues between the two sequences to be compared,
20 obtained after optimal alignment, this percentage being purely statistical and the differences between the two sequences being distributed randomly along their length. The comparison of two nucleic acid or amino acid sequences is traditionally carried out by comparing the sequences after having optimally aligned them, said comparison being able to be conducted by segment or by using an “alignment window”. Optimal
25 alignment of the sequences for comparison can be carried out, in addition to comparison by hand, by means of the local homology algorithm of Smith and Waterman (1981) [Ad. App. Math. 2:482], by means of the local homology algorithm of Needleman and Wunsch (1970) [J. Mol. Biol. 48:443], by means of the similarity search method of Pearson and Lipman (1988) [Proc. Natl. Acad. Sci. USA 85:2444] or by means of
30 computer software using these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI, or by the comparison software BLAST NR or BLAST P).

Percentage identity is calculated by determining the number of positions at which the amino acid nucleotide or residue is identical between the two sequences, preferably between the two complete sequences, dividing the number of identical positions by the total number of positions in the alignment window and multiplying the result by 100 to obtain the percentage identity between the two sequences.

For example, the BLAST program, "BLAST 2 sequences" (Tatusova et al., "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol., 1999, Lett. 174:247-250) available on the site <http://www.ncbi.nlm.nih.gov/gorf/bl2.html>, can be used with the default parameters (notably for the parameters "open gap penalty": 5, and "extension gap penalty": 2; the selected matrix being for example the "BLOSUM 62" matrix proposed by the program); the percentage identity between the two sequences to compare is calculated directly by the program.

For the amino acid sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with a reference amino acid sequence, preferred examples include those containing the reference sequence, certain modifications, notably a deletion, addition or substitution of at least one amino acid, truncation or extension. In the case of substitution of one or more consecutive or non-consecutive amino acids, substitutions are preferred in which the substituted amino acids are replaced by "equivalent" amino acids. Here, the expression "equivalent amino acids" is meant to indicate any amino acids likely to be substituted for one of the structural amino acids without however modifying the biological activities of the corresponding antibodies and of those specific examples defined below.

Equivalent amino acids can be determined either on their structural homology with the amino acids for which they are substituted or on the results of comparative tests of biological activity between the various antibodies likely to be generated.

As a non-limiting example, table 1 below summarizes the possible substitutions likely to be carried out without resulting in a significant modification of the biological activity of the corresponding modified antibody; inverse substitutions are naturally possible under the same conditions.

Table 1

| Original residue | Substitution(s) |
|------------------|-----------------|
| Ala (A) | Val, Gly, Pro |
| Arg (R) | Lys, His |
| Asn (N) | Gln |
| Asp (D) | Glu |
| Cys (C) | Ser |
| Gln (Q) | Asn |
| Glu (E) | Asp |
| Gly (G) | Ala |
| His (H) | Arg |
| Ile (I) | Leu |
| Leu (L) | Ile, Val, Met |
| Lys (K) | Arg |
| Met (M) | Leu |
| Phe (F) | Tyr |
| Pro (P) | Ala |
| Ser (S) | Thr, Cys |
| Thr (T) | Ser |
| Trp (W) | Tyr |
| Tyr (Y) | Phe, Trp |
| Val (V) | Leu, Ala |

A particular aspect of the invention is that the antibody, does not bind to the Insulin receptor (IR). This aspect is of interest as the antibody herein described will not have any negative impact on the IR, meaning the Insulin metabolism.

5 Still another advantageous aspect of the antibody of the ADC of the invention is that it is capable of binding not only to the human IGF-1R but also to the monkey IGF-1R, and more particularly to the cynomolgus IGF-1R. This aspect is also of interest as it will facilitate the toxicity assessment required for clinical trials.

In still another embodiment, the antibody of the ADC of the invention consists of a monoclonal antibody.

10 The term "monoclonal antibody" or "Mab" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e. the individual antibodies of the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single epitope. Such monoclonal antibody may be
15 produced by a single clone of B cells or hybridoma. Monoclonal antibodies may also

be recombinant, i.e. produced by protein engineering or chemical synthesis. Monoclonal antibodies may also be isolated from phage antibody libraries. In addition, in contrast with preparations of polyclonal antibodies which typically include various antibodies directed against various determinants, or epitopes, each monoclonal antibody is directed against a single epitope of the antigen.

The monoclonal antibody herein includes murine, chimeric and humanized antibody, such as described after.

The antibody is preferably derived from an hybridoma of murine origin filed within the French collection for microorganism cultures (CNCM, Pasteur Institute, 25 Rue du Docteur Roux, 75724 Paris Cedex 15, France), said hybridoma being obtained by the fusion of Balb/C immunized mice splenocytes/lymphocytes and cells of the myeloma Sp 2/O-Ag 14 cell line.

In an embodiment, the IGF-1R antibody of the ADC of the invention consists of a murine antibody, then referred as m[*name of the antibody*].

In an embodiment, the IGF-1R antibody consists of a chimeric antibody, then referred as c[*name of the antibody*].

In an embodiment, the IGF-1R antibody consists of a humanized antibody, then referred as hz[*name of the antibody*].

For the avoidance of doubt, in the following specification, the expressions “IGF-1R antibody” and “[*name of the antibody*]” are similar and include (without contrary specification) the murine, the chimeric and the humanized versions of the said IGF-1R antibody or of the said “[*name of the antibody*]”. When necessary, the prefix m- (murine), c- (chimeric) or hz- (humanized) is used.

For more clarity, the following table 2 illustrates the CDR sequences, defined according to IMGT, for the preferred antibodies.

Table 2

| | Heavy chain | Light chain | SEQ ID No. |
|----------------------|-------------|-------------|------------|
| Consensus | CDR-H1 | | 1 |
| | CDR-H2 | | 2 |
| | CDR-H3 | | 3 |
| | | CDR-L1 | 4 |
| | | CDR-L2 | 5 |
| | | CDR-L3 | 6 |
| 208F2 | CDR-H1 | | 7 |
| | CDR-H2 | | 2 |
| | CDR-H3 | | 3 |
| | | CDR-L1 | 9 |
| | | CDR-L2 | 5 |
| | | CDR-L3 | 11 |
| 212A11 | CDR-H1 | | 7 |
| | CDR-H2 | | 2 |
| | CDR-H3 | | 3 |
| | | CDR-L1 | 10 |
| | | CDR-L2 | 5 |
| | | CDR-L3 | 11 |
| 214F8 & 213B10 | CDR-H1 | | 7 |
| | CDR-H2 | | 2 |
| | CDR-H3 | | 3 |
| | | CDR-L1 | 9 |
| | | CDR-L2 | 5 |
| | | CDR-L3 | 12 |
| 219D6 | CDR-H1 | | 8 |
| | CDR-H2 | | 2 |
| | CDR-H3 | | 3 |
| | | CDR-L1 | 9 |
| | | CDR-L2 | 5 |
| | | CDR-L3 | 11 |

It will be obvious for the man skilled in the art that any combination of 6 CDRs as above described should be considered as part of the present invention.

As can be observed from this table 2, all the antibodies herein described have the same sequences for the CDR-H2, CDR-H3 and CDR-L2, this property being of particular interest as above described.

A specific aspect relates to a murine antibody characterized in that said antibody also comprises light chain and heavy chain constant regions derived from an antibody of a species heterologous with the mouse, notably man.

Another specific aspect relates to a chimeric (c) antibody characterized in that said antibody also comprises light chain and heavy chain constant regions derived from an antibody of a species heterologous with the mouse, notably human.

A chimeric antibody is one containing a natural variable region (light chain and heavy chain) derived from an antibody of a given species in combination with constant regions of the light chain and the heavy chain of an antibody of a species heterologous to said given species.

5 The chimeric antibodies can be prepared by using the techniques of recombinant genetics. For example, the chimeric antibody could be produced by cloning recombinant DNA containing a promoter and a sequence coding for the variable region of a nonhuman monoclonal antibody of the ADC of the invention, notably murine, and a sequence coding for heterologous species antibody constant region, preferably human.

10 A chimeric antibody the ADC according to the invention coded by one such recombinant gene could be, for example, a mouse-human chimera, the specificity of this antibody being determined by the variable region derived from the murine DNA and its isotype determined by the constant region derived from human DNA.

In a preferred, but not limitative, embodiment, the antibody of the ADC of the invention is selected from:

- a) an antibody comprising a heavy chain variable domain of sequence SEQ ID No. 13 or any sequence exhibiting at least 80% identity with SEQ ID No. 13 and the three light chain CDRs of sequences SEQ ID Nos. 9, 5 and 11;
- b) an antibody comprising a heavy chain variable domain of sequence
20 SEQ ID No. 14 or any sequence exhibiting at least 80% identity with SEQ ID No. 14 and the three light chain CDRs of sequences SEQ ID Nos. 10, 5 and 11;
- c) an antibody comprising a heavy chain variable domain of sequence SEQ ID No. 15 or any sequence exhibiting at least 80% identity with SEQ ID No. 15 and the three light chain CDRs of sequences SEQ ID Nos. 9, 5 and 12;
- 25 d) an antibody comprising a heavy chain variable domain of sequence SEQ ID No. 16 or any sequence exhibiting at least 80% identity with SEQ ID No. 16 and the three light chain CDRs of sequences SEQ ID Nos. 9, 5 and 11; and
- e) an antibody comprising a heavy chain variable domain of sequence SEQ ID No. 17 or any sequence exhibiting at least 80% identity with SEQ ID No. 17
30 and the three light chain CDRs of sequences SEQ ID Nos. 9, 5 and 12.

By “any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 13 to 17”, it is intended to designate the sequences exhibiting

the three heavy chain CDRs SEQ ID Nos. 1, 2 and 3 and, in addition, exhibiting at least 80%, preferably 85%, 90%, 95% and 98%, identity with the full sequence SEQ ID No. 13 to 17 outside the sequences corresponding to the CDRs (i.e. SEQ ID No. 1, 2 and 3).

In another preferred, but not limitative, embodiment, the antibody of the ADC of the invention is selected from:

- a) an antibody comprising a light chain variable domain of sequence SEQ ID No. 18 or any sequence exhibiting at least 80% identity with SEQ ID No. 18 and the three heavy chain CDRs of sequences SEQ ID Nos. 7, 2 and 3;
- b) an antibody comprising a light chain variable domain of sequence SEQ ID No. 19 or any sequence exhibiting at least 80% identity with SEQ ID No. 19 and the three heavy chain CDRs of sequences SEQ ID Nos. 7, 2 and 3;
- c) an antibody comprising a light chain variable domain of sequence SEQ ID No. 20 or any sequence exhibiting at least 80% identity with SEQ ID No. 20 and the three heavy chain CDRs of sequences SEQ ID Nos. 7, 2 and 3;
- d) an antibody comprising a light chain variable domain of sequence SEQ ID No. 21 or any sequence exhibiting at least 80% identity with SEQ ID No. 21 and the three heavy chain CDRs of sequences SEQ ID Nos. 8, 2 and 3; and
- e) an antibody comprising a light chain variable domain of sequence SEQ ID No. 22 or any sequence exhibiting at least 80% identity with SEQ ID No. 22 and the three heavy chain CDRs of sequences SEQ ID Nos. 7, 2 and 3.

By “any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 18 to 22”, it is intended to designate the sequences exhibiting the three light chain CDRs SEQ ID Nos. 4, 5 and 6 and, in addition, exhibiting at least 80%, preferably 85%, 90%, 95% and 98% , identity with the full sequence SEQ ID No. 18 to 22 outside the sequences corresponding to the CDRs (i.e. SEQ ID No. 4, 5 and 6).

An embodiment of the invention relates to an ADC wherein Ab is an antibody selected from:

- a) an antibody comprising a heavy chain variable domain of sequence SEQ ID No. 13 or any sequence exhibiting at least 80% identity with SEQ ID No. 13 and a light chain variable domain of sequence SEQ ID No. 18 or any sequence exhibiting at least 80% identity with SEQ ID No. 18;

b) an antibody comprising a heavy chain variable domain of sequence SEQ ID No. 14 or any sequence exhibiting at least 80% identity with SEQ ID No. 14 and a light chain variable domain of sequence SEQ ID No. 19 or any sequence exhibiting at least 80% identity with SEQ ID NO. 19;

5 c) an antibody comprising a heavy chain variable domain of sequence SEQ ID No. 15 or any sequence exhibiting at least 80% identity with SEQ ID No. 15 and a light chain variable domain of sequence SEQ ID No. 20 or any sequence exhibiting at least 80% identity with SEQ ID No. 20;

d) an antibody comprising a heavy chain variable domain of sequence
10 SEQ ID No. 16 or any sequence exhibiting at least 80% identity with SEQ ID No. 16 and a light chain variable domain of sequence SEQ ID No. 21 or any sequence exhibiting at least 80% identity with SEQ ID No. 21; and

e) an antibody comprising a heavy chain variable domain of sequence
15 SEQ ID No. 17 or any sequence exhibiting at least 80% identity with SEQ ID No. 17 and a light chain variable domain of sequence SEQ ID No. 22 or any sequence exhibiting at least 80% identity with SEQ ID No. 22.

Chimeric antibodies herein described can be also characterized by the constant domain and , more particularly, said chimeric antibodies can be selected or designed such as, without limitation, IgG1, IgG2, IgG3, IgM, IgA, IgD or IgE. More preferably,
20 in the context of the present invention, said chimeric antibodies are IgG1 or IgG4.

An embodiment of the invention relates to an ADC wherein Ab is a chimeric antibody comprising variable domains VH and VL as above described in the format IgG1. More preferably, said chimeric antibody comprises a constant domain for the VH of sequence SEQ ID No. 43 and a Kappa domain for the VL of sequence SEQ ID No.
25 45.

An embodiment of the invention relates to an ADC wherein Ab is a chimeric antibody comprising variable domains VH and VL as above described in the format IgG4. More preferably, said chimeric antibody comprises a constant domain for the VH of sequence SEQ ID No. 44 and a Kappa domain for the VL of sequence SEQ ID No.
30 45.

In another preferred, but not limitative, embodiment, the antibody of the ADC of the invention is selected from:

- a) an antibody comprising or consisting of a heavy chain of sequence SEQ ID No. 23 or any sequence exhibiting at least 80% identity with SEQ ID No. 23 and a light chain of sequence SEQ ID No. 28 or any sequence exhibiting at least 80% identity with SEQ ID No. 28;
- 5 b) an antibody comprising or consisting of a heavy chain of sequence SEQ ID No. 24 or any sequence exhibiting at least 80% identity with SEQ ID No. 24 and a light chain of sequence SEQ ID No. 29 or any sequence exhibiting at least 80% identity with SEQ ID No. 29;
- c) an antibody comprising or consisting of a heavy chain of sequence
10 SEQ ID No. 25 or any sequence exhibiting at least 80% identity with SEQ ID No. 25 and a light chain of sequence SEQ ID No. 30 or any sequence exhibiting at least 80% identity with SEQ ID No. 30;
- d) an antibody comprising or consisting of a heavy chain of sequence
15 SEQ ID No. 26 or any sequence exhibiting at least 80% identity with SEQ ID No. 26 and a light chain of sequence SEQ ID No. 31 or any sequence exhibiting at least 80% identity with SEQ ID No. 31; and
- e) an antibody comprising or consisting of a heavy chain of sequence
20 SEQ ID No. 27 or any sequence exhibiting at least 80% identity with SEQ ID No. 27 and a light chain of sequence SEQ ID No. 32 or any sequence exhibiting at least 80% identity with SEQ ID No. 32.

For more clarity, the following table 3 illustrates the sequences of the VH and VL, respectively, for the preferred chimeric antibodies.

Table 3

| | Heavy Chain | Light chain | SEQ ID No. |
|---------|----------------------|----------------------|-------------------|
| c208F2 | Variable domain (VH) | | 13 |
| | | Variable domain (VL) | 18 |
| | Full length | | 23 |
| | | Full length | 28 |
| c212A11 | Variable domain (VH) | | 14 |
| | | Variable domain (VL) | 19 |
| | Full length | | 24 |
| | | Full length | 29 |
| c214F8 | Variable domain (VH) | | 15 |
| | | Variable domain (VL) | 20 |
| | Full length | | 25 |
| | | Full length | 30 |
| c219D6 | Variable domain (VH) | | 16 |
| | | Variable domain (VL) | 21 |
| | Full length | | 26 |
| | | Full length | 31 |
| c213B10 | Variable domain (VH) | | 17 |
| | | Variable domain (VL) | 22 |
| | Full length | | 27 |
| | | Full length | 32 |

Yet another specific aspect of the present invention relates to an ADC wherein Ab is a humanized antibody characterized in that the constant regions of the light chain and the heavy chain derived from human antibody are, respectively, the lambda or kappa region and the gamma-1, gamma-2 or gamma-4 region.

5 “Humanized antibodies” means an antibody that contains CDR regions derived from an antibody of nonhuman origin, the other parts of the antibody molecule being derived from one (or several) human antibodies. In addition, some of the skeleton segment residues (called FR) can be modified to preserve binding affinity.

10 The humanized antibodies or fragments of same can be prepared by techniques known to a person skilled in the art. Such humanized antibodies are preferred for their use in methods involving *in vitro* diagnoses or preventive and/or therapeutic treatment

in vivo. Other humanization techniques, also known to a person skilled in the art, such as, for example, the “CDR grafting” technique described by PDL in patents EP 0 451 216, EP 0 682 040, EP 0 939 127, EP 0 566 647 or US 5,530,101, US 6,180,370, US 5,585,089 and US 5,693,761. US patents 5,639,641 or 6,054,297, 5,886,152 and
5 5,877,293 can also be cited.

As a particular embodiment of the invention, and as it will be explicated in more details in the examples after, it is herein described an antibody consisting of the hz208F2. Such humanization can also be applied to the other antibodies part of the present invention.

10 In a preferred embodiment, the antibody of the ADC according to the present invention comprises a heavy chain variable domain (VH) having:

i) the CDR-H1, CDR-H2 and CDR-H3 of sequences SEQ ID Nos. 7, 2 and 3, respectively, and

15 ii) the FR1, FR2 and FR3 derived from the human germline IGHV1-46*01 (SEQ ID No. 46), and

iii) the FR4 derived from the human germline IGHJ4*01 (SEQ ID No. 48).

In a preferred embodiment, the antibody of the ADC according to the present invention comprises a light chain variable domain (VL) having:

20 i) the CDR-L1, CDR-L2 and CDR-L3 of sequences SEQ ID Nos. 9, 5 and 11, respectively, and

ii) the FR1, FR2 and FR3 derived from the human germline IGKV1-39*01 (SEQ ID No. 47), and

iii) the FR4 derived from the human germline IGKJ4*01 (SEQ ID No. 49).

25 In a preferred, but not limitative, embodiment of the invention, the antibody of the ADC comprises:

a) a heavy chain having CDR-H1, CDR-H2 and CDR-H3 of sequences SEQ ID Nos. 7, 2 and 3, respectively, and FR1, FR2 and FR3 derived from the human germline IGHV1-46*01 (SEQ ID No. 46), and the FR4 derived from the human germline IGHJ4*01 (SEQ ID No. 48); and

30 b) a light chain having CDR-L1, CDR-L2 and CDR-L3 of sequences SEQ ID Nos. 9, 5 and 11, respectively, and FR1, FR2 and FR3 derived from the human germline

IGKV1-39*01 (SEQ ID No. 47), and the FR4 derived from the human germline IGKJ4*01 (SEQ ID No. 49).

In an embodiment, the antibody of the ADC according to the invention comprises a heavy chain variable domain (VH) of sequence SEQ ID No. 33 and a light chain variable domain (VL) of sequence SEQ ID No. 35. Said humanized antibody will be called thereafter hz208F2 (“Variant 1” or “Var. 1”).

In another embodiment, the antibody of the ADC according to the present invention comprises a heavy chain variable domain (VH) of sequence SEQ ID No. 33 wherein said sequence SEQ ID No. 33 comprises at least 1 back-mutation selected from the residues 20, 34, 35, 38, 48, 50, 59, 61, 62, 70, 72, 74, 76, 77, 79, 82 or 95.

By the expressions “back-mutation” or “back mutation” it is meant a mutation or replacement of the human residue present in the germline by the corresponding residue initially present in the murine sequence.

In another embodiment, the antibody of the ADC according to the present invention comprises a heavy chain variable domain (VH) of sequence SEQ ID No. 33 wherein said sequence SEQ ID No. 33 comprises 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or 17 back-mutations selected from the residues 20, 34, 35, 38, 48, 50, 59, 61, 62, 70, 72, 74, 76, 77, 79, 82 or 95.

For more clarity, the following table 4 illustrates the preferred back-mutations.

20

Table 4

| | | | | | | | | | |
|-----------|----|----|----|----|----|----|----|----|----|
| N° résidu | 20 | 34 | 35 | 38 | 48 | 50 | 59 | 61 | |
| Murin | M | I | Y | K | L | W | K | N | |
| humain | V | M | H | R | M | I | S | A | |
| N° résidu | 62 | 70 | 72 | 74 | 76 | 77 | 79 | 82 | 95 |
| Murin | E | L | A | K | S | N | A | F | F |
| humain | Q | M | R | T | T | S | V | E | Y |

In an embodiment, the antibody of the ADC according to the present invention comprises a light chain variable domain (VL) of sequence SEQ ID No. 35, wherein said sequence SEQ ID No. 35 comprises at least 1 back-mutation selected from the residues 22, 53, 55, 65, 71, 72, 77 or 87.

In an embodiment, the antibody of the ADC according to the present invention comprises a light chain variable domain (VL) of sequence SEQ ID No. 35, wherein said

sequence SEQ ID No. 35 comprises 2, 3, 4, 5, 6, 7 or 8 back-mutations selected from the residues 22, 53, 55, 65, 71, 72, 77 or 87.

In another embodiment, the antibody of the ADC according to the present invention comprises:

- 5 a) a heavy chain variable domain (VH) of sequence SEQ ID No. 33 wherein said sequence SEQ ID No. 33 comprises at least 1 back-mutation selected from the residues 20, 34, 35, 38, 48, 50, 59, 61, 62, 70, 72, 74, 76, 77, 79, 82 or 95; and
- b) a light chain variable domain (VL) of sequence SEQ ID No. 35, wherein said sequence SEQ ID No. 35 comprises at least 1 back-mutation selected from the residues
- 10 22, 53, 55, 65, 71, 72, 77 or 87.

For more clarity, the following table 5 illustrates the preferred back-mutations.

Table 5

| N° résidu | 22 | 53 | 55 | 65 | 71 | 72 | 77 | 87 |
|-----------|----|----|----|----|----|----|----|----|
| Murin | S | R | H | R | Y | S | N | F |
| humain | T | S | Q | S | F | T | S | Y |

15

In such an embodiment, the antibody of the ADC according to the invention comprises all the back-mutations above mentioned and corresponds to an antibody comprising a heavy chain variable domain (VH) of sequence SEQ ID No. 34 and a light chain variable domain (VL) of sequence SEQ ID No. 36. Said humanized antibody will

20 be called thereafter hz208F2 (“Variant 3” or “Var. 3”).

In another embodiment, all the humanized forms comprised between the Variant 1 and the Variant 3 are also encompassed by the present invention. In other words, the antibody of the ADC according to the invention corresponds to an antibody comprising a heavy chain variable domain (VH) of “consensus” sequence SEQ ID No. 41 and a

25 light chain variable domain (VL) of “consensus” sequence SEQ ID No. 42. Said humanized antibody, as a whole, will be called thereafter hz208F2 (“Variant2” or “Var.2”).

In a preferred, but not limitative, embodiment, the antibody of the ADC of the invention is selected from:

a) an antibody comprising a heavy chain variable domain of sequence SEQ ID No. 33 or any sequence exhibiting at least 80% identity with SEQ ID No. 33 and the three light chain CDRs of sequences SEQ ID Nos. 9, 5 and 11; or

b) an antibody comprising a heavy chain variable domain of sequence
5 SEQ ID No. 34 or any sequence exhibiting at least 80% identity with SEQ ID No. 34 and the three light chain CDRs of sequences SEQ ID Nos. 9, 5 and 11.

By “any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 33 or 34”, it is intended to designate the sequences exhibiting the three heavy chain CDRs SEQ ID Nos. 1, 2 and 3 and, in addition,
10 exhibiting at least 80%, preferably 85%, 90%, 95% and 98%, identity with the full sequence SEQ ID No. 33 or 34 outside the sequences corresponding to the CDRs (i.e. SEQ ID Nos. 1, 2 and 3).

In a preferred, but not limitative, embodiment, the antibody of the ADC of the invention is selected from:

15 a) an antibody comprising a light chain variable domain of sequence SEQ ID No. 35 or any sequence exhibiting at least 80% identity with SEQ ID No. 35 and the three heavy chain CDRs of sequences SEQ ID Nos. 7, 2 and 3; and

b) an antibody comprising a heavy chain variable domain of sequence
20 SEQ ID No. 36 or any sequence exhibiting at least 80% identity with SEQ ID No. 36 and the three heavy chain CDRs of sequences SEQ ID Nos. 7, 2 and 3.

By “any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 35 or 36”, it is intended to designate the sequences exhibiting the three light chain CDRs SEQ ID Nos. 4, 5 and 6 and, in addition,
25 exhibiting at least 80%, preferably 85%, 90%, 95% and 98%, identity with the full sequence SEQ ID No. 35 or 36 outside the sequences corresponding to the CDRs (i.e. SEQ ID Nos. 4, 5 and 6).

Humanized antibodies herein described can be also characterized by the constant domain and, more particularly, said humanized antibodies can be selected or designed such as, without limitation, IgG1, IgG2, IgG3, IgM, IgA, IgD or IgE. More preferably,
30 in the context of the present invention, said humanized antibodies are IgG1 or IgG4.

An embodiment of the invention relates to an ADC wherein Ab is a humanized antibody comprising variable domains VH and VL as above described in the format

IgG1. More preferably, said humanized antibody comprises a constant domain for the VH of sequence SEQ ID No. 43 and a Kappa domain for the VL of sequence SEQ ID No. 45.

5 An embodiment of the invention relates to an ADC wherein Ab is a humanized antibody comprising variable domains VH and VL as above described in the format IgG4. More preferably, said humanized antibody comprises a constant domain for the VH of sequence SEQ ID No. 44 and a Kappa domain for the VL of sequence SEQ ID No. 45.

10 Still another embodiment of the invention relates to an ADC wherein Ab is an antibody selected from:

- a) an antibody comprising a heavy chain of sequence SEQ ID No. 37 or any sequence exhibiting at least 80% identity with SEQ ID No. 37 and a light chain of sequence SEQ ID No. 39 or any sequence exhibiting at least 80% identity with SEQ ID No. 39; and
- 15 b) an antibody comprising a heavy chain of sequence SEQ ID No. 38 or any sequence exhibiting at least 80% identity with SEQ ID No. 38 and a light chain of sequence SEQ ID No. 40 or any sequence exhibiting at least 80% identity with SEQ ID No. 40.

20 For more clarity, the following table 6 illustrates non limitative examples of sequences of the VH and VL for the variant 1 (Var. 1) and the variant 3 (Var. 3) of the humanized antibody hz208F2. It also comprises the consensus sequence for the variant 2 (Var. 2).

Table 6

| | Heavy Chain | Light chain | SEQ ID No. |
|---------------------|----------------------|----------------------|-------------------|
| hz208F2 (var. 1) | Variable domain (VH) | | 33 |
| | | Variable domain (VL) | 35 |
| | Full length | | 37 |
| | | Full length | 39 |
| hz208F2 (Var. 3) | Variable domain (VH) | | 34 |
| | | Variable domain (VL) | 36 |
| | Full length | | 38 |
| | | Full length | 40 |
| hz208F2 (Var. 2) | Variable domain (VH) | | 41 |
| | | Variable domain (VL) | 42 |

Another aspect of the present invention is an ADC wherein Ab is an antibody selected from i) an antibody produced by the hybridoma I-4757, I-4773, I-4775, I-4736 or I-4774 deposited at the CNCM, Institut Pasteur France on the 30 May 2013, 26 June 2013, 26 June 2013, 24 April 2013 and 26 June 2013, respectively, ii) an antibody which competes for binding to IGF-1R with the antibody of i); or iii) an antibody which binds to the same epitope of IGF-1R as does the antibody of i).

It is described herein a murine hybridoma selected from the hybridoma I-4757, I-4773, I-4775, I-4736 and I-4774 deposited at the CNCM, Institut Pasteur France on the 30 May 2013, 26 June 2013, 26 June 2013, 24 April 2013 and 26 June 2013, respectively.

It is also described an isolated nucleic acid coding for an antibody, or for an antigen binding fragment thereof, according to the invention.

The terms “nucleic acid”, “nucleic sequence”, “nucleic acid sequence”, “polynucleotide”, “oligonucleotide”, “polynucleotide sequence” and “nucleotide sequence”, used interchangeably in the present description, mean a precise sequence of nucleotides, modified or not, defining a fragment or a region of a nucleic acid, containing unnatural nucleotides or not, and being either a double-strand DNA, a single-strand DNA or transcription products of said DNAs.

The nucleic sequences of the antibodies of the ADC of present invention have been isolated and/or purified, i.e., they were sampled directly or indirectly, for example

by a copy, their environment having been at least partially modified. Isolated nucleic acids obtained by recombinant genetics, by means, for example, of host cells, or obtained by chemical synthesis should also be mentioned here.

It is also described a vector comprising a nucleic acid coding for an antibody of the ADC of the invention, or for an antigen binding fragment thereof, according to the invention.

It is disclosed notably targets cloning and/or expression vectors that contain such a nucleotide sequence.

The vectors preferably contain elements which allow the expression and/or the secretion of nucleotide sequences in a given host cell. The vector thus may contain a promoter, translation initiation and termination signals, as well as suitable transcription regulation regions. It must be able to be maintained in a stable manner in the host cell and may optionally have specific signals which specify secretion of the translated protein. These various elements are selected and optimized by a person skilled in the art according to the host cell used. For this purpose, the nucleotide sequences can be inserted in self-replicating vectors within the chosen host or be integrative vectors of the chosen host.

The vectors are, for example, vectors of plasmid or viral origin. They are used to transform host cells in order to clone or express the nucleotide sequences of the invention.

Such vectors are prepared by methods typically used by a person skilled in the art and the resulting clones can be introduced into a suitable host by standard methods such as lipofection, electroporation, conjugation, heat shock or chemical methods.

It is disclosed also isolated host cells transformed by or comprising a vector as above described.

The host cell can be selected among prokaryotic or eukaryotic systems such as bacterial cells, for example, but also yeast cells or animal cells, notably mammal cells (with the exception of human). Insect or plant cells can also be used.

It is also disclosed a method for the production of an antibody according to the invention, or an antigen binding fragment thereof, characterized in that said method comprises the following steps:

a) the culture in a medium with the suitable culture conditions for a host cell as disclosed above; and

b) the recovery of the antibody thus produced from the culture medium or from said cultured cells.

5 The transformed cells are of use in methods for the preparation of recombinant antibodies. Methods for the preparation of antibodies in recombinant form using a vector and/or a cell transformed by a vector, are also comprised in the present specification. Preferably, a cell transformed by a vector as above described is cultured under conditions that allow the expression of the aforesaid antibody and recovery of
10 said antibody.

As already mentioned, the host cell can be selected among prokaryotic or eukaryotic systems. In particular, it is possible to identify the nucleotide sequences that facilitate secretion in such a prokaryotic or eukaryotic system. A vector according to the invention carrying such a sequence can thus be used advantageously for the production
15 of recombinant proteins to be secreted. Indeed, the purification of these recombinant proteins of interest will be facilitated by the fact that they are present in the supernatant of the cellular culture rather than inside host cells.

The antibody of the ADC of the invention can also be prepared by chemical synthesis. One such method of preparation is also an object of the invention. A person
20 skilled in the art knows methods for chemical synthesis, such as solid-phase techniques or partial solid-phase techniques, by condensation of fragments or by conventional synthesis in solution. Polypeptides obtained by chemical synthesis and capable of containing corresponding unnatural amino acids are also comprised in the invention.

The ADC comprising antibody likely to be obtained by the method above
25 described are also comprised in the present invention.

According to a particular aspect, the invention concerns an ADC wherein Ab is an antibody, or an antigen binding fragment thereof, as above described for use as an addressing vehicle for delivering a cytotoxic agent at a host target site, said host target site consisting of an epitope localized into IGF-1R, preferably the IGF-1R extracellular
30 domain, more preferably the human IGF-1R (SEQ ID No. 50) and still more preferably the human IGF-1R extracellular domain (SEQ ID No. 51), and still more preferably to

the N-terminal of the human IGF-1R extracellular domain (SEQ ID No. 52), or any natural variant sequence thereof.

In a preferred embodiment, said host target site is a target site of a mammalian cell, more preferably of a human cell, more preferably cells which naturally or by way of genetic recombination, express IGF-1R.

I.2: Axl antibodies

Without any contradictory indication, the definition or expression used and defined in the paragraph "I.1, IGF-1R antibodies" are the same in the present paragraph and can be applied to all the ADC of the present invention.

The Axl antibody is capable of binding to the human protein Axl. More particularly, the said target is an epitope located into the extracellular domain of Axl (referred as the Axl ECD for "Axl Extra Cellular Domain").

The Axl ECD is a 451 amino acids fragment, corresponding to amino acids 1-451 of the sequence SEQ ID No. 103, which sequence is represented in the sequence listing as SEQ ID No. 105. Amino acids 1-25 corresponding to the signal peptide, the ECD of the human protein Axl without the signal peptide corresponds to the amino acids 26-451 of the sequence SEQ ID No.104, represented by the sequence SEQ ID No. 106.

The Axl antibody of the ADC of the invention comprises the three light chain CDRs comprising the sequences SEQ ID Nos. 56, 57 and 58, or any sequence exhibiting at least 90%, preferably 95% and 98% identity with SEQ ID Nos. 56, 57 and 58; and the three heavy chain CDRs comprising the sequences SEQ ID Nos. 59, 60 and 61, or any sequence exhibiting at least 90%, preferably 95% and 98% identity with SEQ ID Nos. 59, 60 and 61.

In an embodiment, the Axl antibody comprises the three light chain CDRs comprising respectively the sequences SEQ ID Nos. 56, 57 and 58; and the three heavy chain CDRs comprising respectively the sequences SEQ ID Nos. 59, 60 and 61.

In an embodiment, the Axl antibody consists of the m1613F12 comprising i) a light chain variable domain of sequence SEQ ID No. 62, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 62; and/or ii)

a heavy chain variable domain of sequence SEQ ID No. 63, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 63.

For more clarity, table 7a below summarizes the various amino acid sequences corresponding to the Axl antibody.

5

Table 7a

| | CDR numbering | Heavy chain | Light chain | SEQ ID NO. |
|---------|---------------|-----------------|-----------------|------------|
| 1613F12 | IMGT | | CDR-L1 | 56 |
| | | | CDR-L2 | 57 |
| | | | CDR-L3 | 58 |
| | | CDR-H1 | | 59 |
| | | CDR-H2 | | 60 |
| | | CDR-H3 | | 61 |
| | | | variable domain | 62 |
| | | variable domain | | 63 |

In an embodiment, the Axl antibody consists of the c1613F12 comprising the three light chain CDRs comprising the sequences SEQ ID No. 56, 57 and 58, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 56, 57 and 58; and the three heavy chain CDRs comprising the sequences
 10 SEQ ID No. 59, 60 and 61, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 59, 60 and 61.

In an embodiment, c1613F12 comprises the three light chain CDRs comprising respectively the sequences SEQ ID Nos. 56, 57 and 58; and the three heavy chain CDRs comprising respectively the sequences SEQ ID Nos. 59, 60 and 61.

15 In an embodiment, the Axl antibody consists of the c1613F12 comprising i) a light chain variable domain of sequence SEQ ID No. 62, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 62; and/or ii) a heavy chain variable domain of sequence SEQ ID No. 63, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 63.

In an embodiment, the Axl antibody consists of the hz1613F12 comprising the three light chain CDRs comprising the sequences SEQ ID No. 56, 57 and 58, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 56, 57 and 58; and the three heavy chain CDRs comprising the sequences
5 SEQ ID No. 59, 60 and 61, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 59, 60 and 61.

In an embodiment, hz1613F12 comprises the three light chain CDRs comprising respectively the sequences SEQ ID Nos. 56, 57 and 58; and the three heavy chain CDRs comprising respectively the sequences SEQ ID Nos. 59, 60 and 61.

10 In an embodiment, hz1613F12 comprises a light chain variable domain consisting of the sequence SEQ ID No. 70, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 70; and the three heavy chain CDRs consisting of sequences SEQ ID No. 59, 60 and 61.

15 In another embodiment of the invention, hz1613F12 comprises a light chain variable domain of sequence selected in the group consisting of SEQ ID No. 71 to 81 or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 71 to 81; and the three heavy chain CDRs consisting of SEQ ID No. 59, 60 and 61.

20 In order to illustrate the identity percentage as defined before, by “any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 70 to 81”, its is intended to designate the sequences exhibiting the three light chain CDRs SEQ ID No. 56, 57 and 58 and, in addition, exhibiting at least 80%, preferably 85%, 90%, 95% and 98% , identity with the full sequence SEQ ID No. 70 to 81 outside the sequences corresponding to the CDRs (i.e. SEQ ID No. 56, 57 and 58).

25 For more clarity, table 7b below summarizes the various amino acid sequences corresponding to the humanized Axl antibody light chain (VL) of the ADC of the invention (with hz. = humanized)

Table 7b

| | Version | SEQ ID NO. |
|--------------|----------------|-------------------|
| hz1613F12 VL | consensus | 70 |
| | VL1 | 71 |
| | VL1 I2V | 72 |
| | VL1 M4I | 73 |
| | VL2.1 | 74 |
| | VL2.1 V49T | 75 |
| | VL2.1 P50N | 76 |
| | VL2.2 | 77 |
| | VL2.2 V49T | 78 |
| | VL2.2 P50N | 79 |
| | VL2.3 | 80 |
| | VL3 | 81 |

In an embodiment, hz1613F12 comprises a heavy chain variable domain consisting of the sequence SEQ ID No. 82, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 82; and the three light chain CDRs consisting of sequences SEQ ID No. 56, 57 and 58.

5 In another embodiment, hz1613F12 comprises a heavy chain variable domain of sequence selected in the group consisting of SEQ ID No. 83 to 102, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 83 to 102; and the three light chain CDRs consisting of SEQ ID No. 56, 57 and 58.

10 In order to illustrate the identity percentage as defined before, by “any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 82 to 102”, it is intended to designate the sequences exhibiting the three heavy chain CDRs SEQ ID No. 59, 60 and 61 and, in addition, exhibiting at least 80%, preferably 85%, 90%, 95% and 98% , identity with the full sequence SEQ ID No. 82 to 102 outside the sequences corresponding to the CDRs (i.e. SEQ ID No. 59, 60 and 61).

15 For more clarity, table 7c below summarizes the various amino acid sequences corresponding to the humanized antigen binding protein heavy chain (VH) of the invention (with hz. = humanized)

Table 7c

| | Version | SEQ ID NO. |
|--------------|-----------------|-------------------|
| hz1613F12 VH | consensus | 82 |
| | VH1 | 83 |
| | VH1 M39I | 84 |
| | VH1 W55R N66K | 85 |
| | VH1 I84S | 86 |
| | VH1 S85N | 87 |
| | VH1 I84N S85N | 88 |
| | VH2.1 | 89 |
| | VH2.1 Q3H | 90 |
| | VH2.1 W55R | 91 |
| | VH2.1 N66K | 92 |
| | VH2.1 W55R N66K | 93 |
| | VH2.1 R80S | 94 |
| | VH2.1 N66K R80S | 95 |
| | VH2.2 | 96 |
| | VH2.2 M89L | 97 |
| | VH2.3 | 98 |
| | VH2.3 W55R | 99 |
| | VH2.3 Q3H W55R | 100 |
| | VH2.4 | 101 |
| VH3 | 102 | |

In an embodiment, hz1613F12 comprises a light chain variable domain of sequence selected in the group consisting of SEQ ID No. 70 and any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 70; and a heavy chain variable domain of sequence selected in the group consisting of

5 SEQ ID No. 82 and any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 82.

In an embodiment, hz1613F12 comprises a light chain variable domain of sequence selected in the group consisting of SEQ ID No. 71 to 81, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 71 to 81; and a heavy chain variable domain of sequence selected in the group
 5 consisting of SEQ ID No. 83 to 102, or any sequence exhibiting at least 80%, preferably 85%, 90%, 95% and 98% identity with SEQ ID No. 83 to 102.

Another aspect of the present invention is an ADC wherein Ab is an antibody selected from i) an antibody produced by the hybridoma I-4505, deposited at the CNCM, Institut Pasteur France on the 28 July 2011, ii) an antibody which competes for
 10 binding to Axl with the antibody of i); or iii) an antibody which binds to the same epitope of Axl as does the antibody of i).

According to another aspect, It is disclosed a murine hybridoma selected from the hybridoma I-4505.

Table 8 below summarizes the nucleotide sequences concerning CDRs of the
 15 1613F12.

Table 8

| Antibody | CDR numbering | Heavy chain | Light chain | SEQ ID NO. |
|-----------------|----------------------|--------------------|--------------------|-------------------|
| 1613F12 | IMGT | | CDR-L1 | 64 |
| | | | CDR-L2 | 65 |
| | | | CDR-L3 | 66 |
| | | CDR-H1 | | 67 |
| | | CDR-H2 | | 68 |
| | | CDR-H3 | | 69 |

In an embodiment, nucleic acid sequences of the CDRs can be selected from the sequences SEQ ID Nos. 64 to 69.

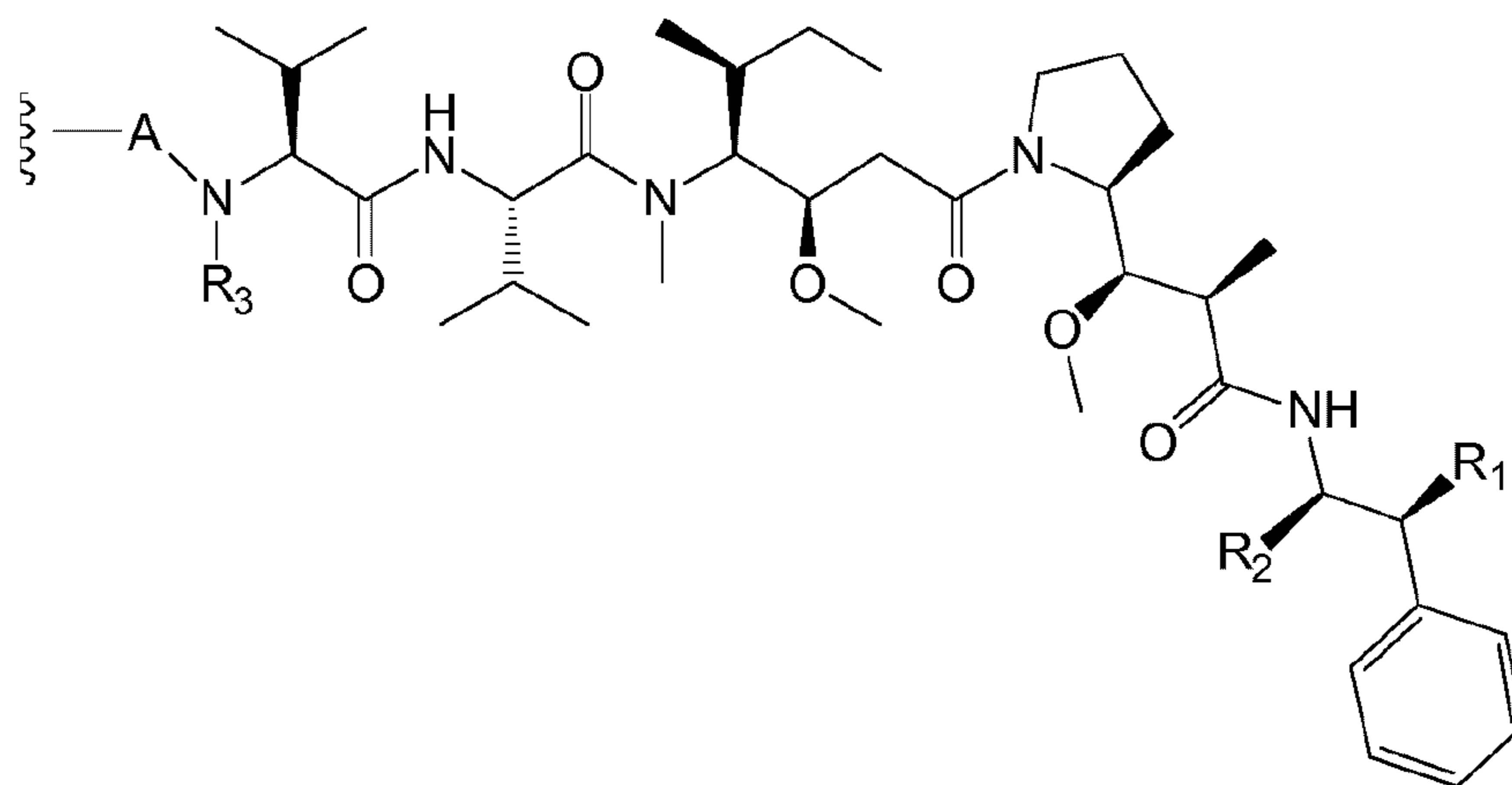
A non limitative embodiment it is disclosed herein an isolated nucleic acid or a combination thereof coding for an antibody characterized in that said nucleic acid or its
 20 combination comprises the 6 CDRs sequences SEQ ID Nos. 64 to 69.

The HER2 antibodies can consist of any antibody capable of binding to HER2 such as, without limitation, TrasGEX (Glycotope), Trastuzumab, Pertuzumab or any biosimilar thereof.

In a preferred embodiment, the HER2 antibody is the Trastuzumab (Herceptin®; 5 4D5; Genentech, San Francisco, CA).

II – The drug (D)

The drug moiety according to the invention has the following formula (II)



(II)

10

wherein:

R₁ is H or OH;

R₂ is a group: (C₁-C₆)alkyl (e.g. methyl), COOH, COO-((C₁-C₆)alkyl) (such as COOMe) or thiazolyl (such as thiazol-2-yl);

15

R₃ is H or a (C₁-C₆)alkyl group (such as methyl), in particular a (C₁-C₆)alkyl group,

A is:

20

- a group of formula -Het-Alk- wherein Alk is a (C₁-C₈)alkanediyl group and is linked to NR₃, and Het is a heterocycle optionally substituted by a (C₁-C₆)alkyl group and containing at least one nitrogen atom, said nitrogen atom being linked to L, or
- a group of formula -A_a-A_b- wherein A_a is linked to L and is O or NR₉ with R₉ being H or (C₁-C₆)alkyl (such as methyl) and A_b is linked to NR₃ and is:

- a (C₁-C₈)alkanediyl group (such as -(CH₂)_m- with m comprised between 1 and 8),
- a -(CH₂CH₂X₁)_{a1}(CH₂CH₂X₂)_{a2}(CH₂CH₂X₃)_{a3}(CH₂CH₂X₄)_{a4}CH₂CH₂-group with X₁, X₂, X₃ and X₄ each independently of one another representing O or NR₈; a₁, a₂, a₃ and a₄ each independently of one another representing 0 or 1 (in particular with a₁ + a₂ + a₃ + a₄ = 1 or 2, in particular 1); and R₈ representing H or a (C₁-C₆)alkyl group (such as methyl),
- an aryl-(C₁-C₈)alkanediyl or heterocycle-(C₁-C₈)alkanediyl group, said group being optionally substituted by a (C₁-C₆)alkyl group, the aryl or heterocycle moiety being linked to A_a and the (C₁-C₈)alkanediyl moiety being linked to NR₃;

the wavy line indicates the point of attachment to L.

The radicals R₂, R₃ and A may be chiral groups and may be in the form of their different stereoisomers and optionally in the form of a mixture of stereoisomers.

By « stereoisomer », in the meaning of the present invention is meant a geometric isomer or an optical isomer.

Geometrical isomers result from the different position of the substituents on a double bond which may therefore have a Z or E configuration.

Optical isomers result in particular from the different position in space of the substituents on a carbon atom comprising 4 different substituents. This carbon atom then forms a chiral or asymmetric centre. Optical isomers comprise diastereoisomers and enantiomers. Optical isomers which are images of one another in a mirror but which cannot be superimposed are called « enantiomers ». Optical isomers which are not superimposable images of one another in a mirror are called « diastereoisomers ».

A mixture containing equal quantities of two individual enantiomer forms of opposite chirality is called a « racemic mixture ».

By « alkyl » in the present invention is meant a monovalent linear or branched, saturated hydrocarbon chain. For example, mention can be made of methyl, ethyl, propyl, isopropyl, butyl, isobutyl, *sec*-butyl, *tert*-butyl, pentyl or hexyl groups.

By « (C_x-C_y)alkyl » in the meaning of the present invention is meant an alkyl chain such as defined above comprising x to y carbon atoms. Therefore, a (C₁-C₆)alkyl

group is an alkyl chain having 1 to 6 carbon atoms. The (C₁-C₆)alkyl is advantageously a (C₁-C₄)alkyl, preferably a (C₁-C₂)alkyl.

By « alkanediyl » in the present invention is meant a divalent linear or branched, saturated hydrocarbon chain. For example, mention can be made of methanediyl, ethanediyl, propanediyl, butanediyl, pentanediyl, hexanediyl, and the like.

By « (C_x-C_y)alkanediyl » in the meaning of the present invention is meant an alkanediyl chain such as defined above comprising x to y carbon atoms. Therefore, a (C₁-C₈)alkanediyl group is an alkanediyl chain having 1 to 8 carbon atoms.

By « aryl » in the meaning of present invention is meant an aromatic hydrocarbon group preferably having 6 to 10 carbon atoms and able to comprise one or two fused rings. For example a phenyl or a naphthyl can be cited. Advantageously it is a phenyl.

By « heterocycle » in the meaning of present invention is meant a saturated, unsaturated or aromatic hydrocarbon group having 1 or 2 fused rings and in which one or more, advantageously 1 to 4, more advantageously 1 or 2 of the carbon atoms are each replaced by a heteroatom chosen from among oxygen, nitrogen and sulfur. Advantageously the heterocycle comprises 5 to 10 carbon atoms and heteroatoms. For example, mention can be made of furan, pyrrole, thiophene, thiazole, isothiazole, oxadiazole, imidazole, oxazole, isoxazole, pyridine, pyrimidine, piperazine, piperidine, quinazoline, quinoline, quinoxaline, benzofuran, benzothiophene, indoline, indolizine, benzothiazole, benzothiophene, benzopyran, benzoxazole, benzo[1,3]dioxole, benzoisoxazole, benzimidazole, chromane, chromene, dihydrobenzofuran, dihydrobenzothiophene, dihydroisoxazole, isoquinoline, dihydrobenzo[1,4]dioxin, imidazo[1,2-a]pyridine, furo[2,3-c]pyridine, 2,3-dihydro-1*H*-indene, [1,3]dioxolo[4,5-c]pyridine, pyrrolo[1,2-c]pyrimidine, pyrrolo[1,2-a]pyrimidine, tetrahydronaphthalene and benzo[b][1,4]oxazin.

In the present invention, the heterocycle is more particularly a saturated, unsaturated or aromatic ring with 5 to 6 members comprising 1 or 2 nitrogen atoms. For example, mention can be made of pyrrole, imidazole, pyridine, pyrimidine, piperazine and piperidine rings. Preferably it is a pyridine, a piperidine, or an imidazole.

Among the drug moieties of the invention, one particularly appreciated class of drug moieties corresponds to the formula (II) drug moieties in which R_1 is OH and R_2 represents a (C_1-C_6) alkyl group, such as methyl.

Another particularly appreciated class of drug moieties corresponds to the formula (II) drug moieties in which R_1 is a hydrogen and R_2 is a thiazolyl (in particular a thiazol-2-yl group).

Another class of particularly appreciated drug moieties corresponds to the formula (II) drug moieties in which R_1 is a hydrogen and R_2 is a $COO-((C_1-C_6)alkyl)$ group such as COOMe.

Another class of particularly appreciated compounds corresponds to the formula (II) drug moieties in which R_1 is a hydrogen and R_2 is a COOH group.

According to one particular embodiment of the present invention, R_2 is a methyl, COOH, COOMe or thiazol-2-yl group.

Therefore the drug moieties of the invention are advantageously formula (II) drug moieties in which:

- $R_1=OH$ and $R_2=Me$ (methyl), or
- $R_1=H$ and $R_2=COOH$, COOMe or thiazol-2-yl.

According to a preferred embodiment, $R_1=H$ and $R_2=COOH$, COOMe or thiazol-2-yl, preferably $R_2=COOH$ or COOMe, and more preferably $R_2=COOH$.

R_3 particularly represents H or a methyl group, advantageously a methyl group.

In a preferred embodiment, R_1 is H, R_2 is COOH and R_3 is a methyl group.

In the definition of A:

- the $(C_1-C_8)alkanediyl$ group is advantageously a $(C_1-C_6)alkanediyl$ group, preferably a $(C_1-C_4)alkanediyl$ group and more preferably a $(C_1-C_2)alkanediyl$ group, and in particular it is a straight chain having the formula $-(CH_2)_m-$ wherein m is an integer comprised between 1 and 8, advantageously between 1 and 6, preferably between 1 and 4, more preferably m is 1 or 2,
- the aryl group is advantageously a phenyl group, and
- the heterocycle is advantageously a saturated, unsaturated or aromatic ring with 5 or 6 members having 1 or 2 nitrogen atoms; for example, a pyrrole, imidazole, pyridine, pyrimidine, piperazine, or piperidine ring; preferably a pyridine, piperidine or imidazole; more preferably a pyridine.

When A is a group of formula -Het-Alk-, Het is more particularly a heterocycle chosen among a piperidine and an imidazole.

When A is a group of formula $-A_a-A_b-$ wherein A_b is a heterocycle-(C₁-C₈)alkanediyl group optionally substituted by a (C₁-C₆)alkyl group, the heterocycle is more particularly a pyridine.

According to a preferred embodiment, A is a group of formula $-A_a-A_b-$ as defined above.

A_a is O or NR₉, and preferably NR₉, with R₉ as defined above, and preferably with R₉ = H or Me.

According to a particular embodiment of the invention, A_b represents a group:

- (C₁-C₆)alkanediyl, in particular (C₂-C₆)alkanediyl (such as $-(CH_2)_m-$ with m comprised between 1 and 6, preferably between 2 and 6),
- $-(CH_2CH_2X_1)_{a_1}(CH_2CH_2X_2)_{a_2}CH_2CH_2-$ with $a_1 + a_2$ advantageously representing 1 or 2, in particular 1,
- aryl-(C₁-C₆)alkanediyl, or
- heterocycle-(C₁-C₆)alkanediyl optionally substituted by a (C₁-C₆)alkyl group (notably unsubstituted).

According to another particular embodiment of the invention, A_b represents a group:

- (C₁-C₄)alkanediyl, in particular (C₂-C₄)alkanediyl (such as $-(CH_2)_m-$ with m comprised between 1 and 4 and preferably being 2, 3 or 4),
- $-(CH_2CH_2X_1)CH_2CH_2-$ or $-(CH_2CH_2X_1)(CH_2CH_2X_2)CH_2CH_2-$,
- aryl-(C₁-C₄)alkanediyl, or
- heterocycle-(C₁-C₄)alkanediyl optionally substituted by a (C₁-C₆)alkyl group (notably unsubstituted).

According to another particular embodiment of the invention, A_b represents a group:

- (C₁-C₄)alkanediyl, in particular (C₂-C₄)alkanediyl (such as $-(CH_2)_m-$ with m comprised between 1 and 4 and preferably being 2, 3 or 4),
- $-(CH_2CH_2X_1)CH_2CH_2-$,
- aryl-(C₁-C₂)alkanediyl, or

- heterocycle-(C₁-C₂)alkanediyl optionally substituted by a (C₁-C₆)alkyl group (notably unsubstituted).

According to another particular embodiment, A_b represents:

- an aryl-(C₁-C₈)alkanediyl group; or a heterocycle-(C₁-C₈)alkanediyl group optionally substituted by a (C₁-C₆)alkyl group (notably unsubstituted);
- notably an aryl-(C₁-C₆)alkanediyl group; or a heterocycle-(C₁-C₆)alkanediyl group optionally substituted by a (C₁-C₆)alkyl group (notably unsubstituted);
- advantageously an aryl-(C₁-C₄)alkanediyl group; or a heterocycle-(C₁-C₄)alkanediyl group optionally substituted by a (C₁-C₆)alkyl group (notably unsubstituted);
- and preferably an aryl-(C₁-C₂)alkanediyl group; or a heterocycle-(C₁-C₂)alkanediyl group optionally substituted by a (C₁-C₆)alkyl group (notably unsubstituted).

According to yet another particular embodiment, A_b is an aryl-(C₁-C₈)alkanediyl group; notably an aryl-(C₁-C₆)alkanediyl group; advantageously an aryl-(C₁-C₄)alkanediyl group; and preferably an aryl-(C₁-C₂)alkanediyl group.

- In the above particular embodiments for A_b, the aryl group is advantageously a phenyl group.

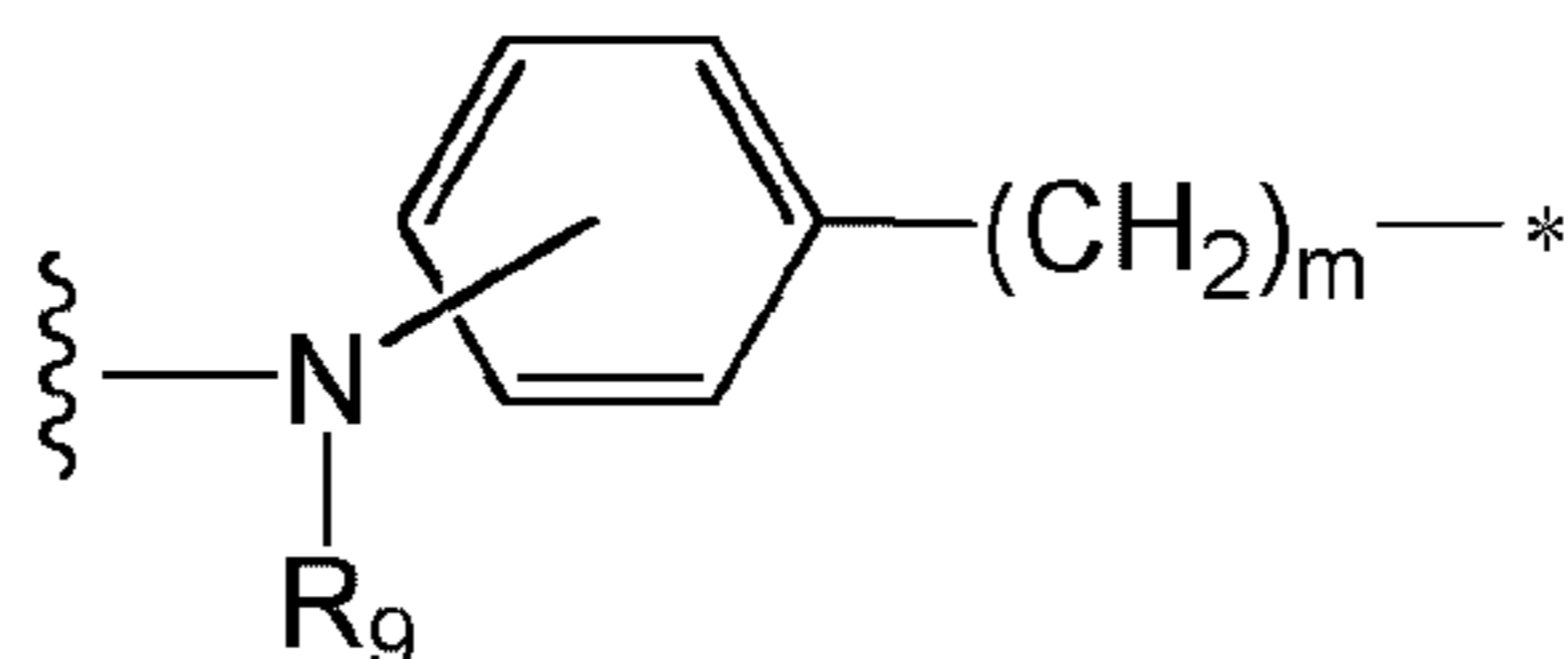
- In the above particular embodiments for A_b, the heterocycle is advantageously a saturated, unsaturated or aromatic ring with 5 or 6 members having 1 or 2 nitrogen atoms. For example, mention can be made of pyrrole, imidazole, pyridine, pyrimidine, piperazine, or piperidine rings. Preferably it is a pyridine, piperidine or imidazole, and more preferably, it is a pyridine.

Advantageously, A_b represents a group:

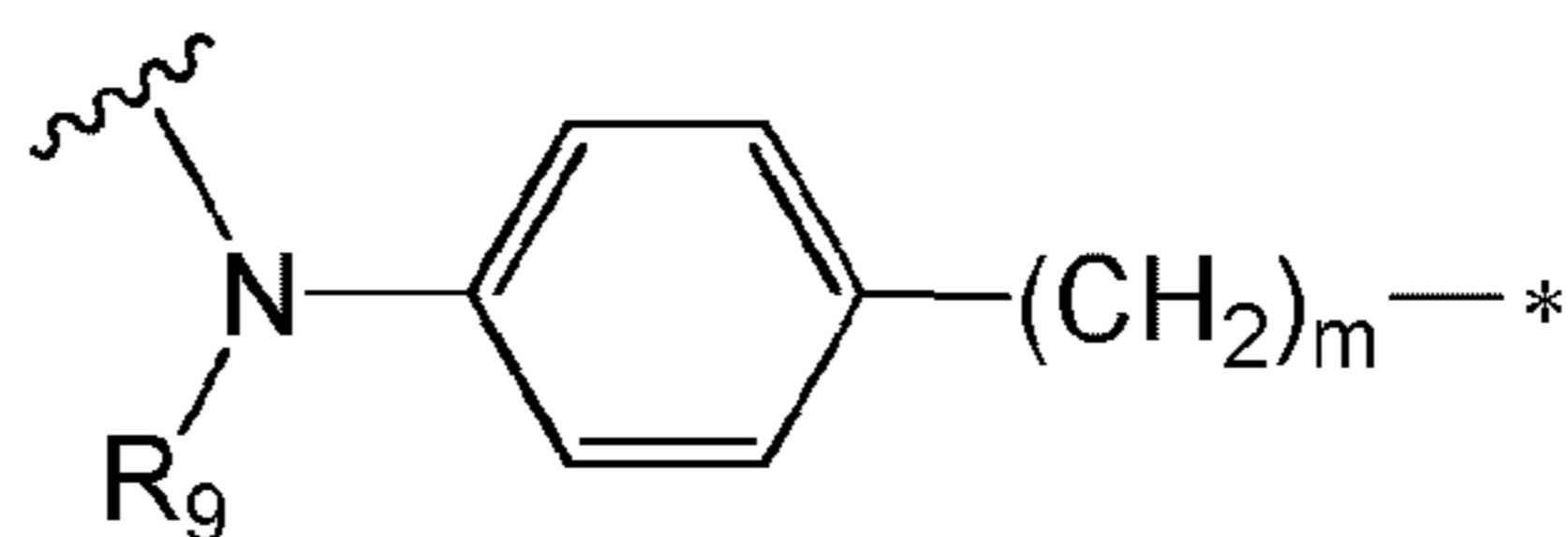
- phenyl-(C₁-C₂)alkanediyl, or
- heterocycle-(C₁-C₂)alkanediyl optionally substituted by a (C₁-C₆)alkyl group (notably unsubstituted), the heterocycle being a saturated, unsaturated or aromatic ring with 5 or 6 members comprising 1 or 2 nitrogen atoms, chosen in particular from among pyridine, piperidine and imidazole, and being preferably a pyridine.

In a preferred embodiment, A_b is a phenyl-(C₁-C₂)alkanediyl group.

According to a preferred embodiment, A has the following formula:



and preferably the following formula:



wherein:

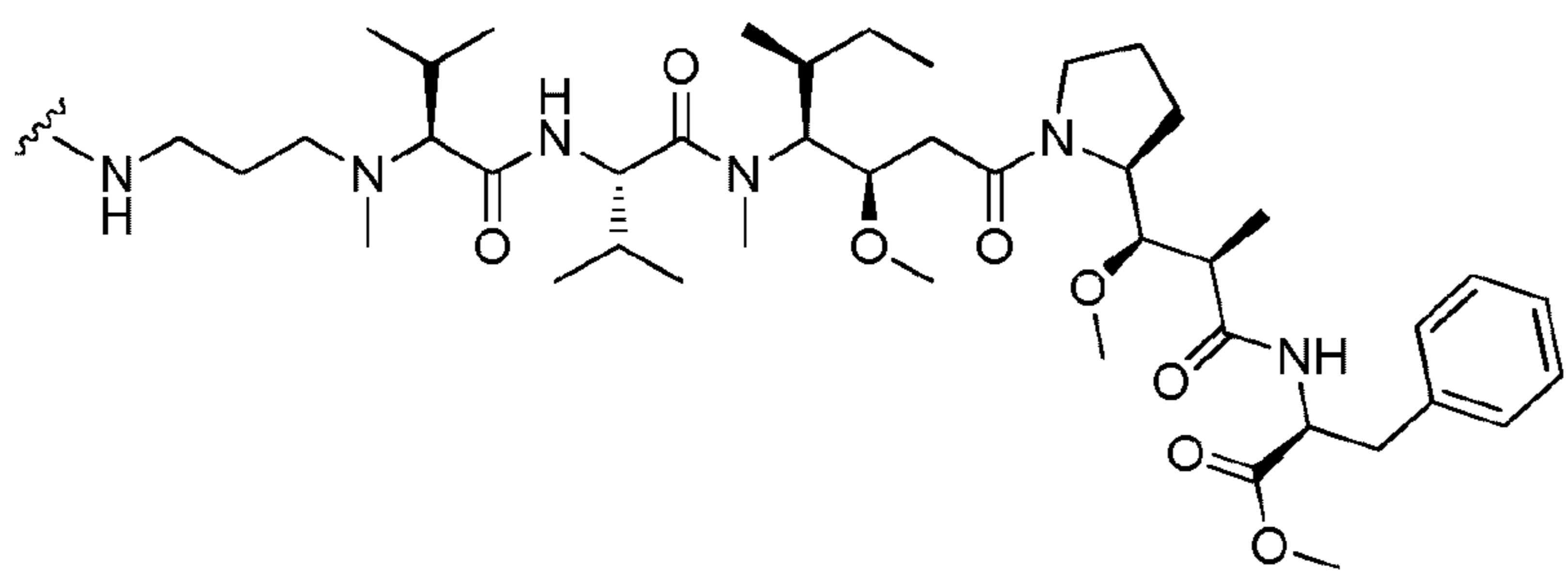
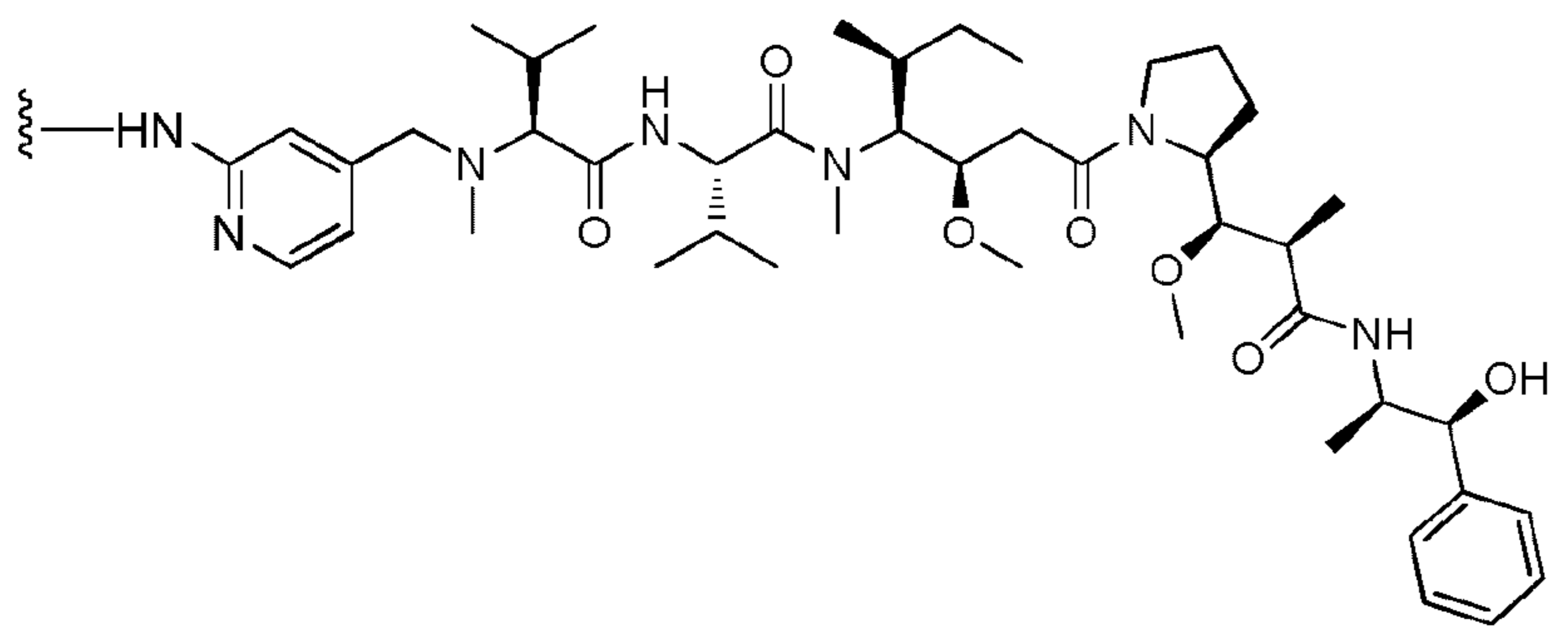
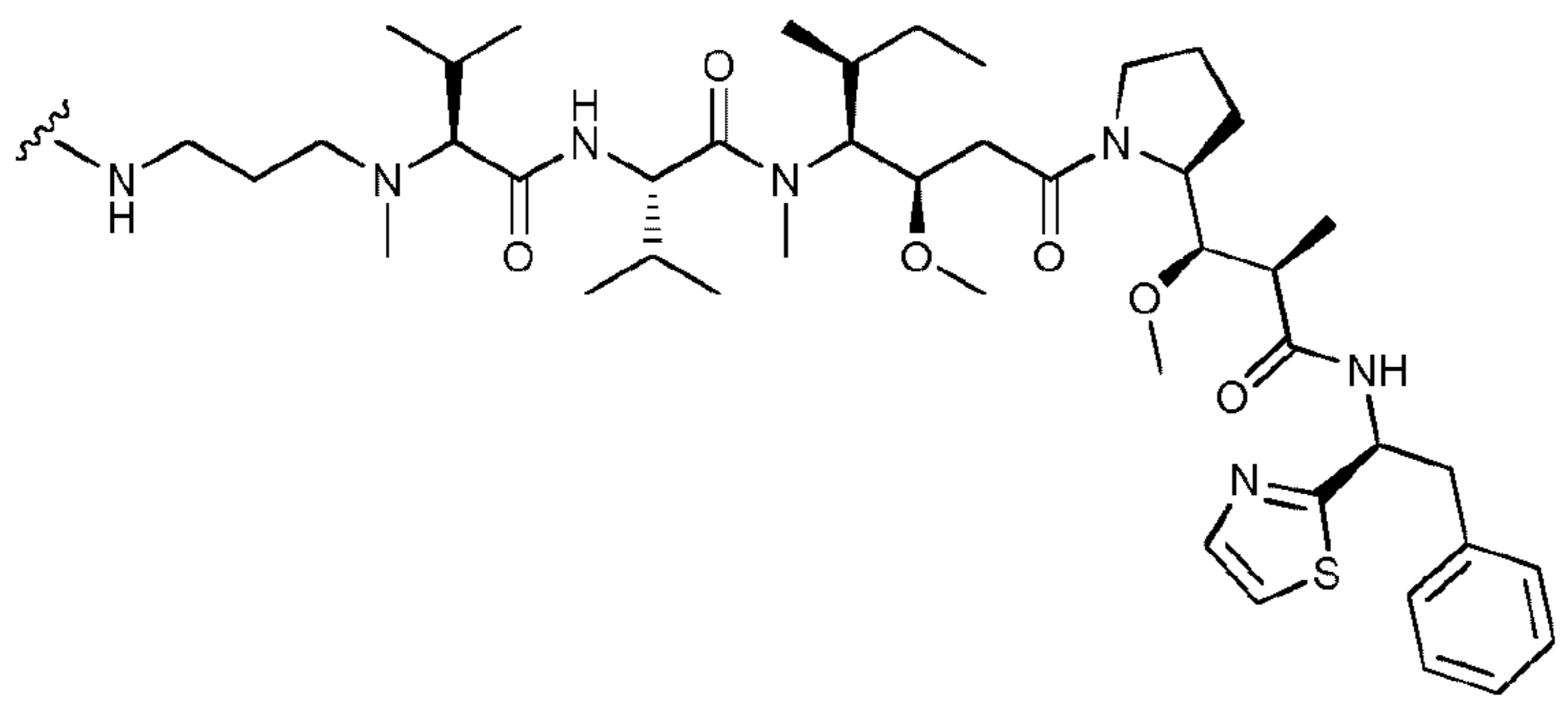
R₉ and m are as defined previously, and preferably with R₉ = H or Me and m = 1

5 or 2,

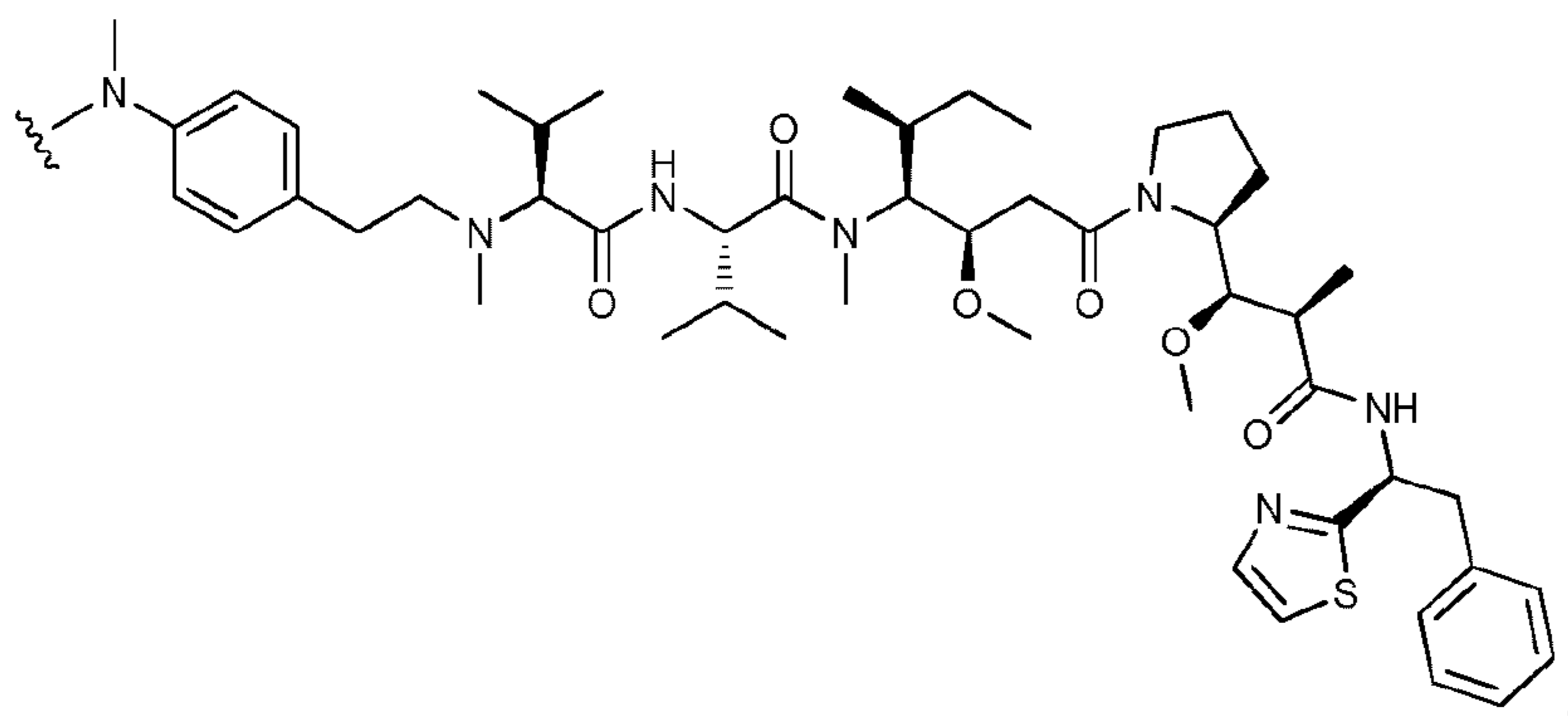
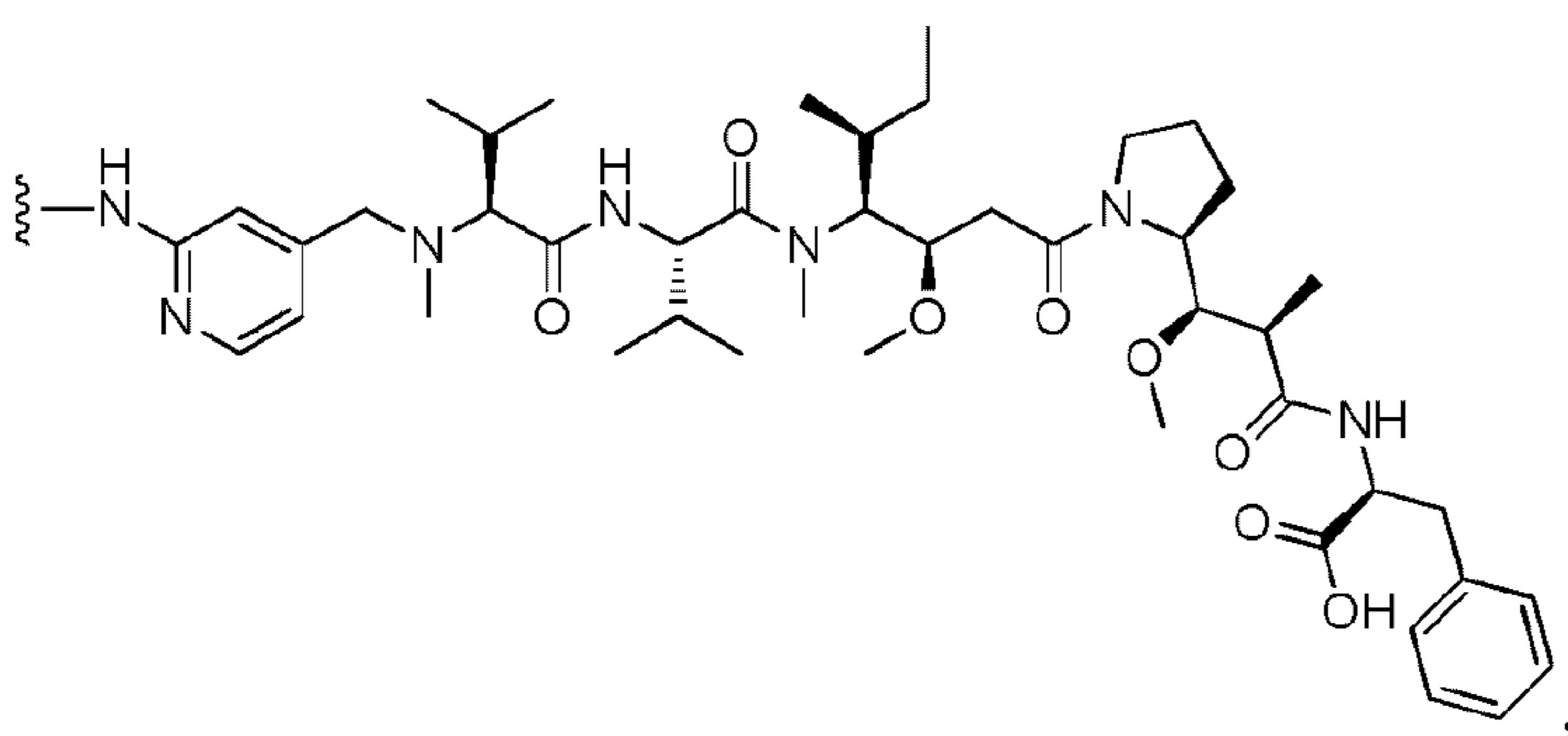
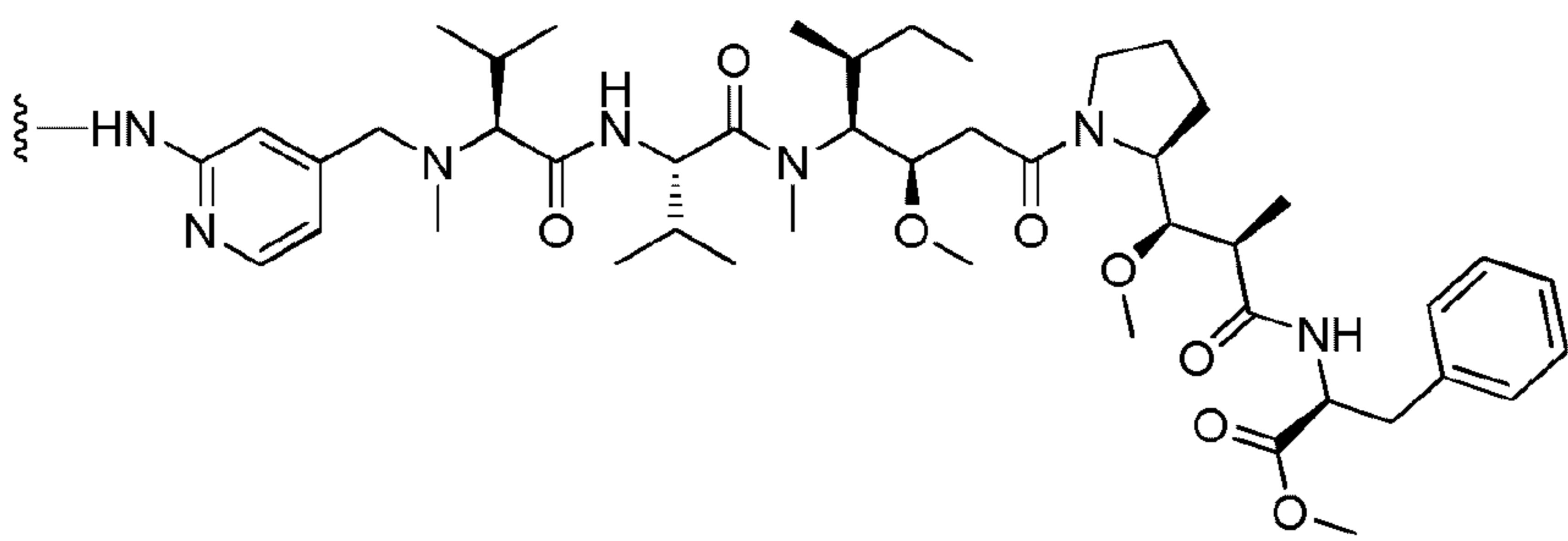
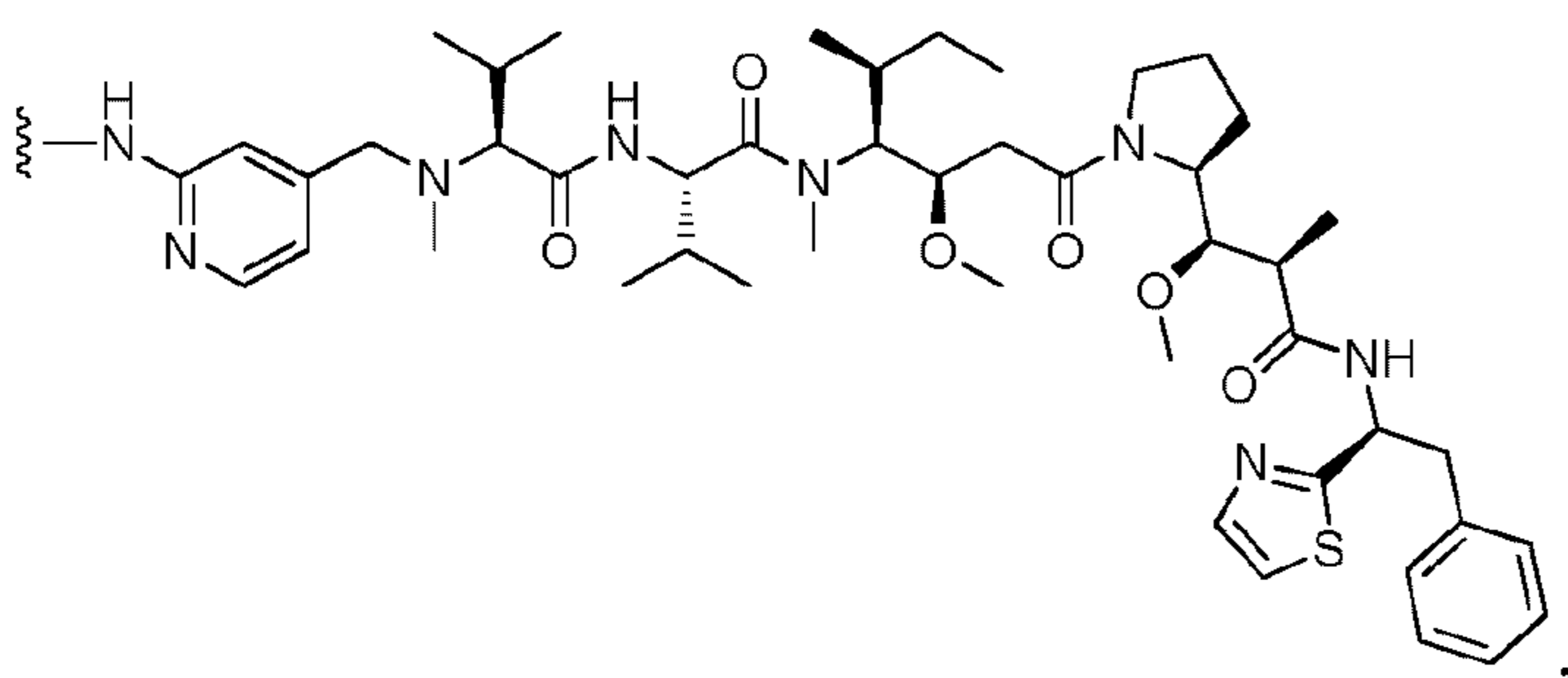
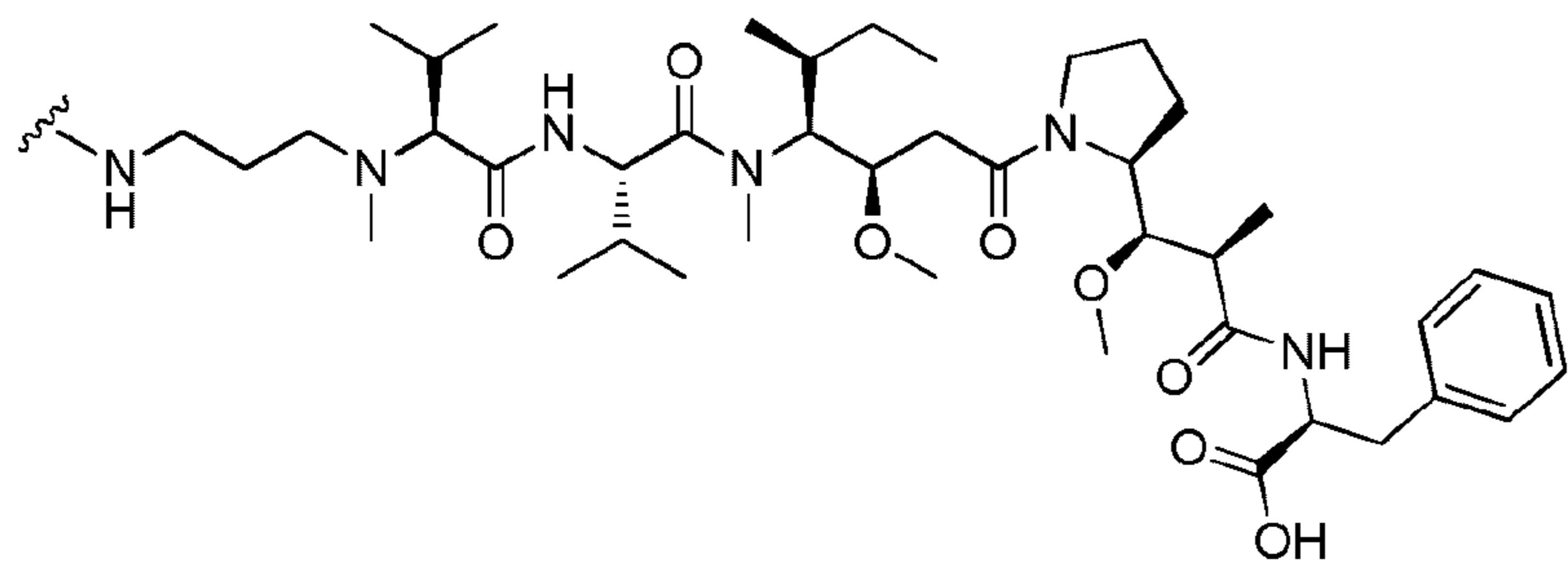
the wavy line indicates the point of attachment to L, and

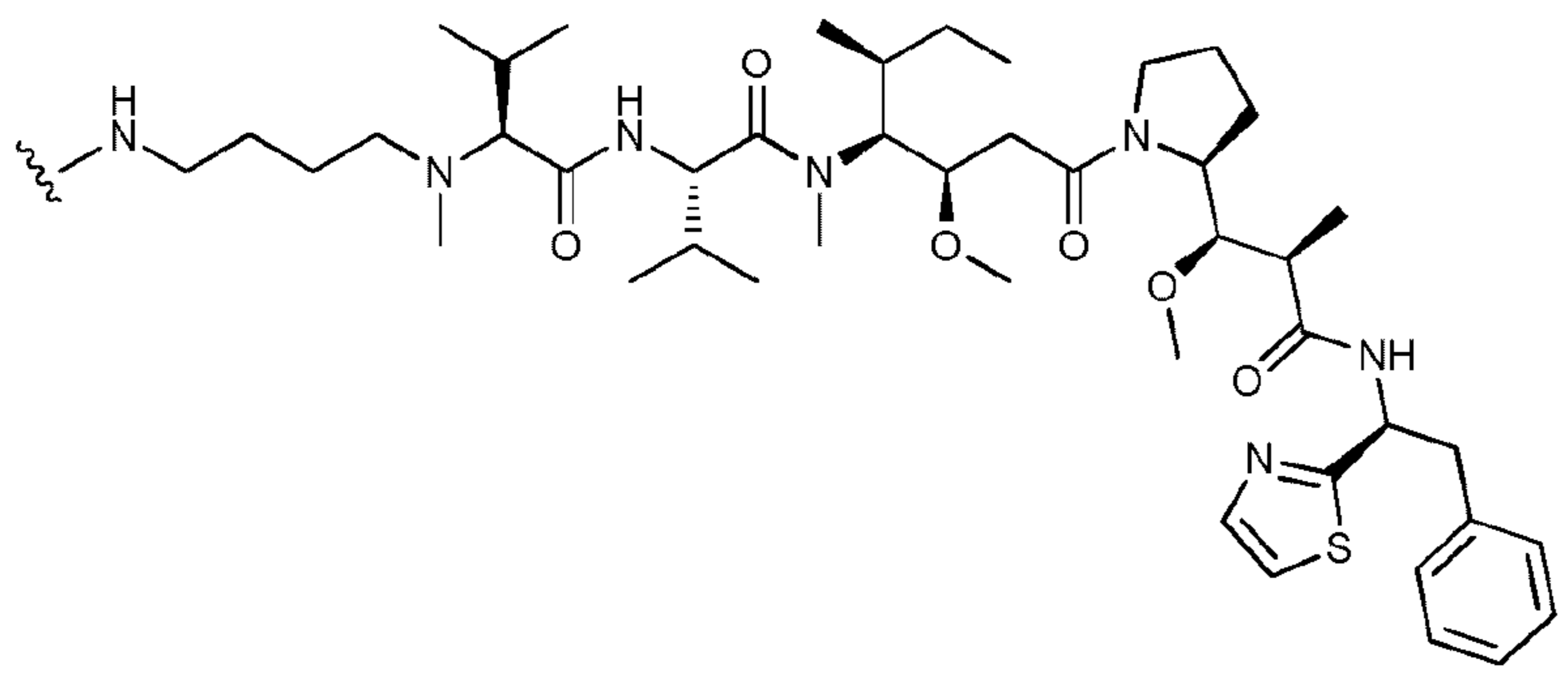
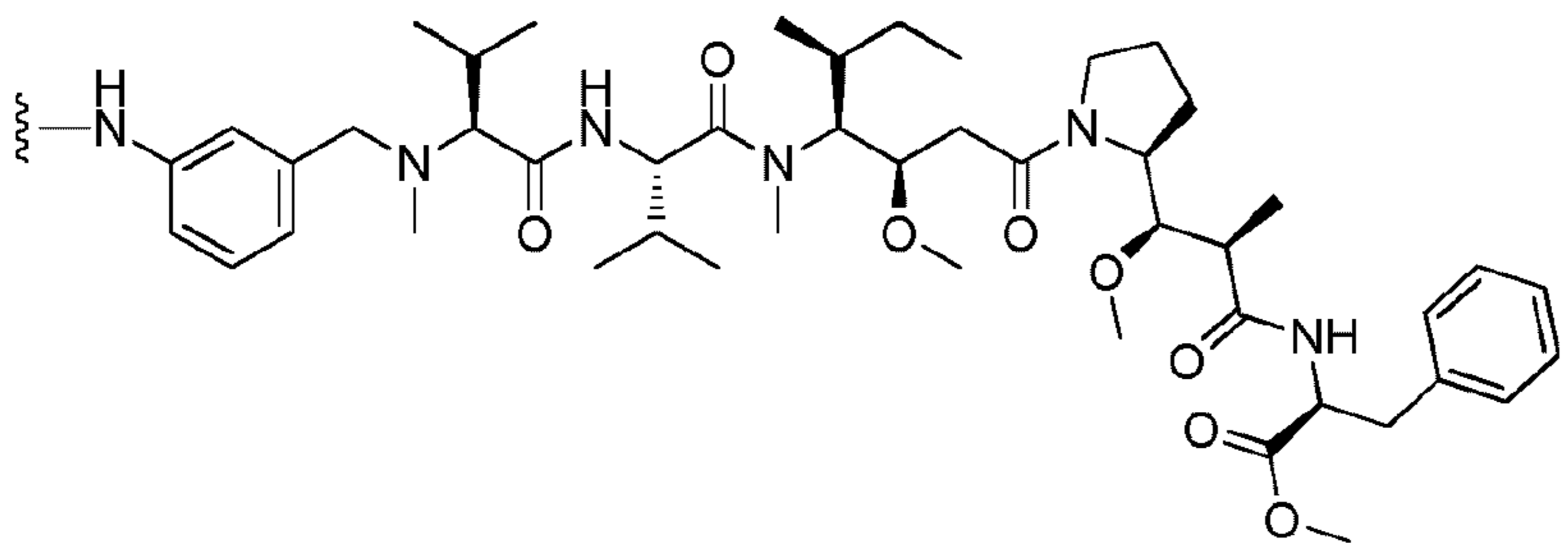
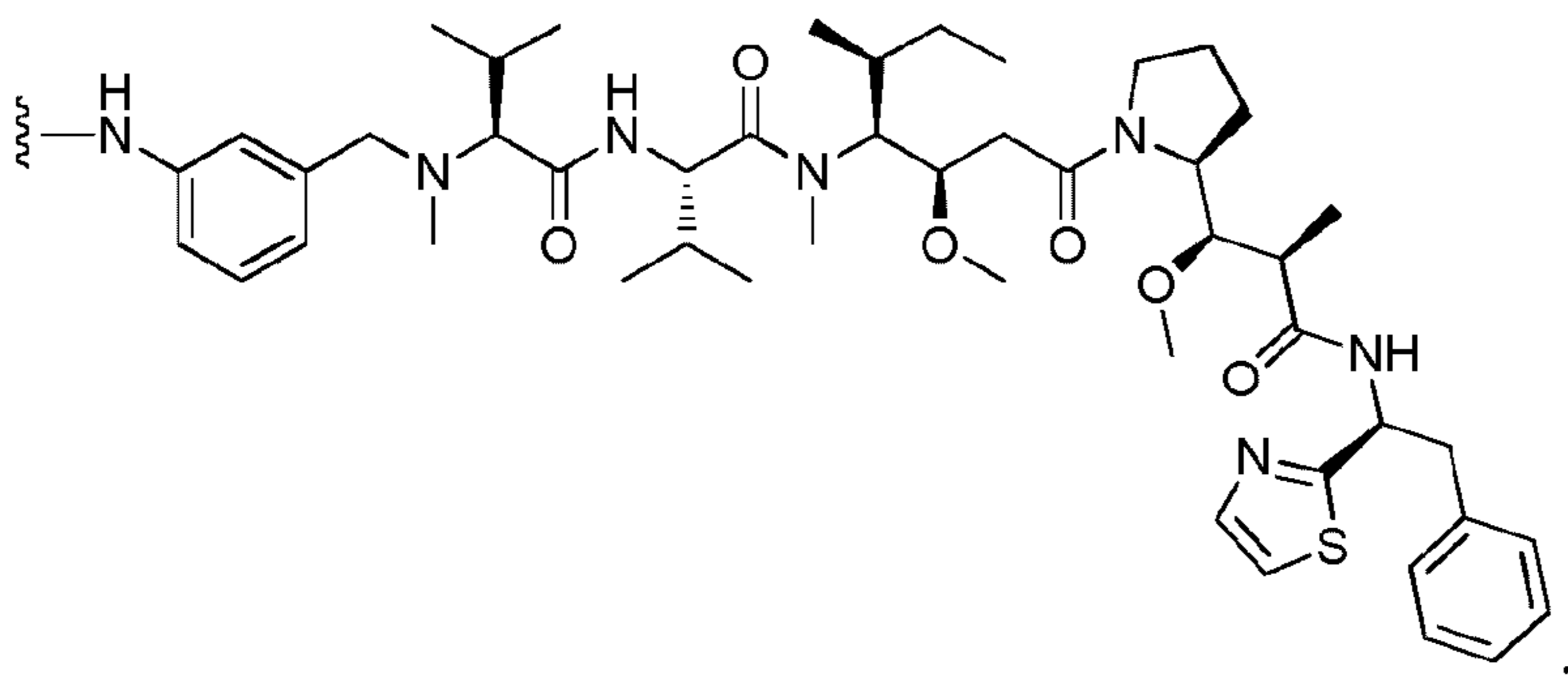
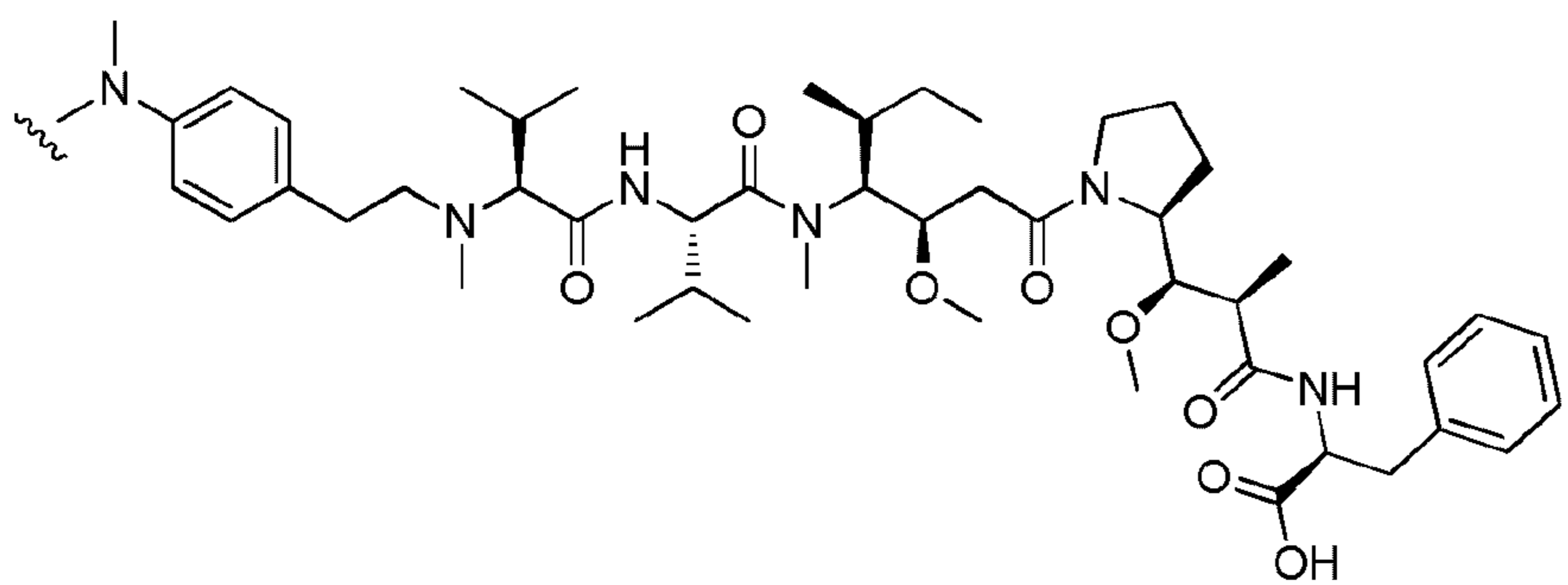
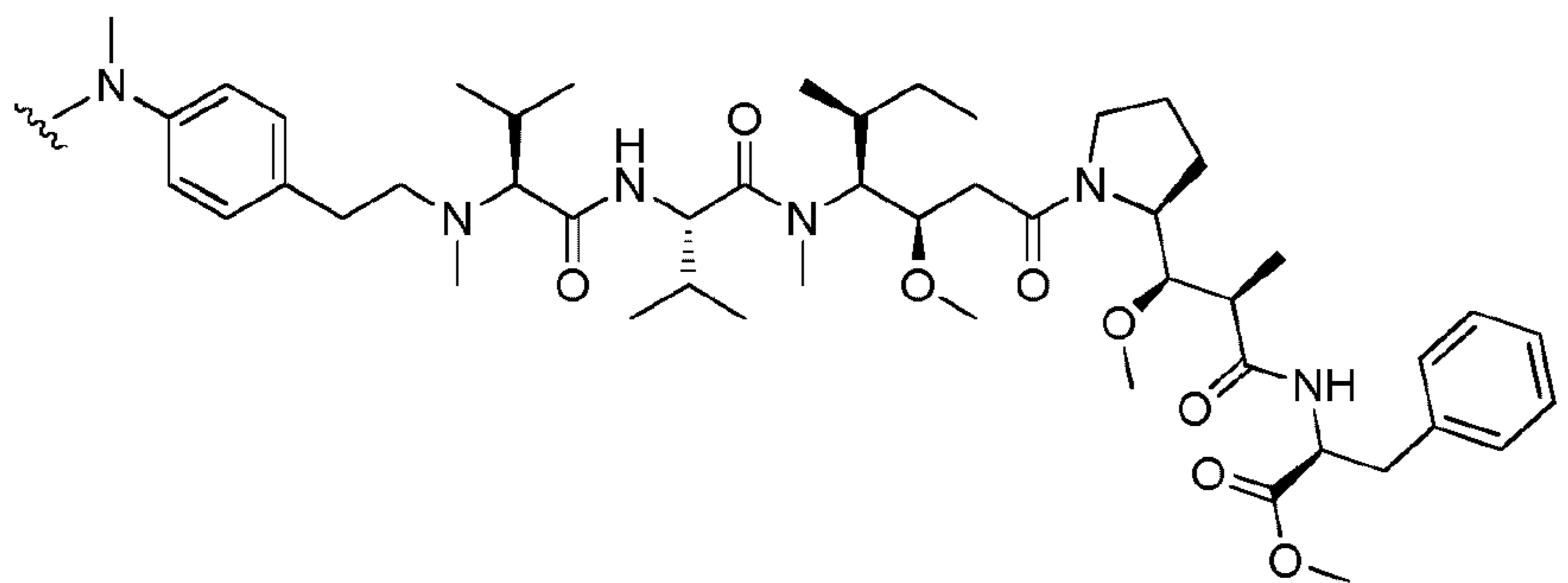
the asterisk indicates the point of attachment to NR₃.

Advantageously, the drug moiety is chosen from among the following moieties:

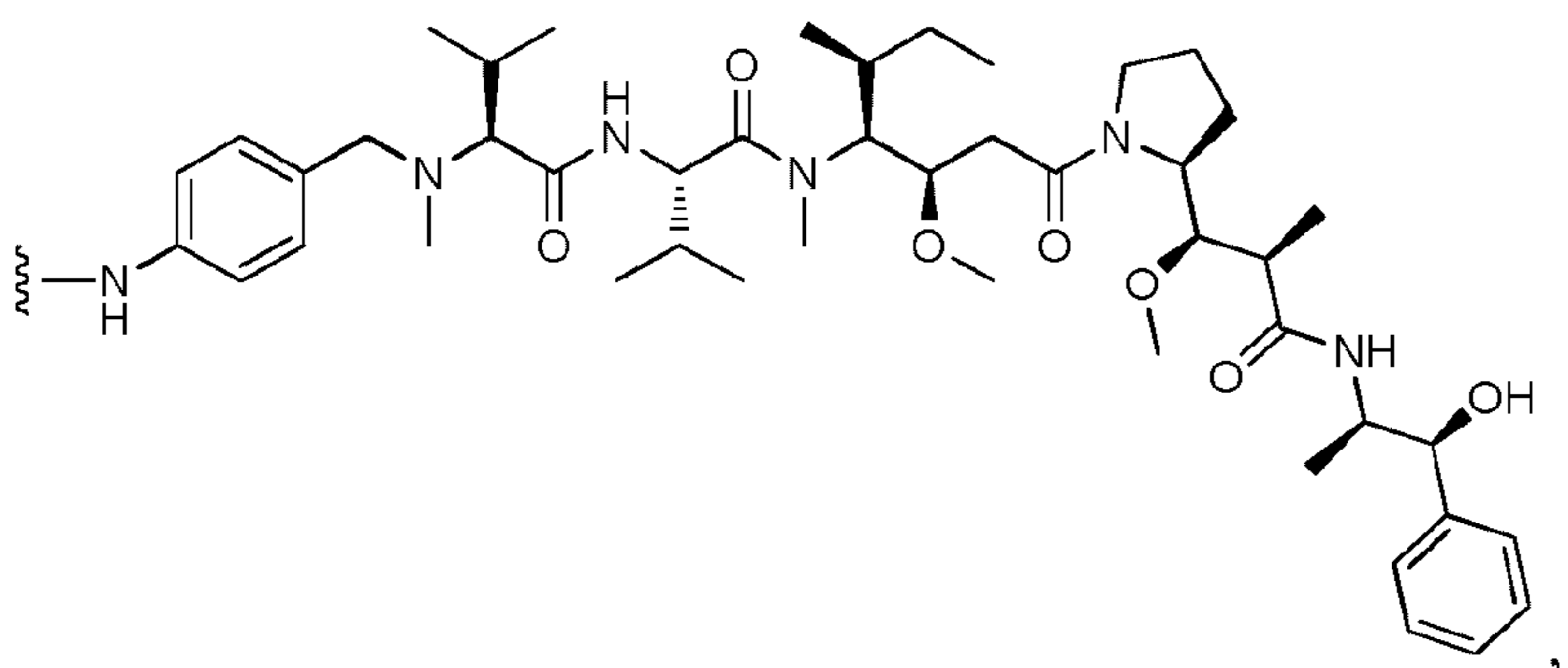
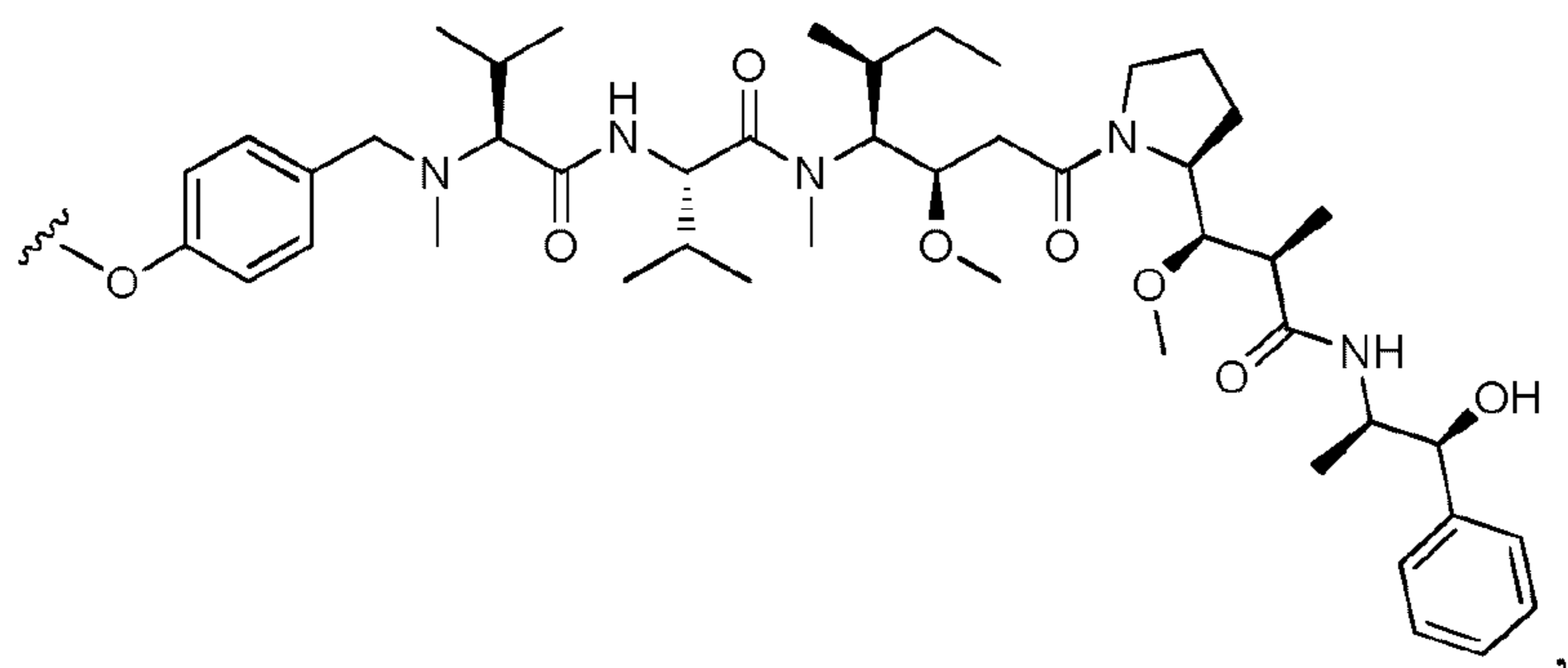
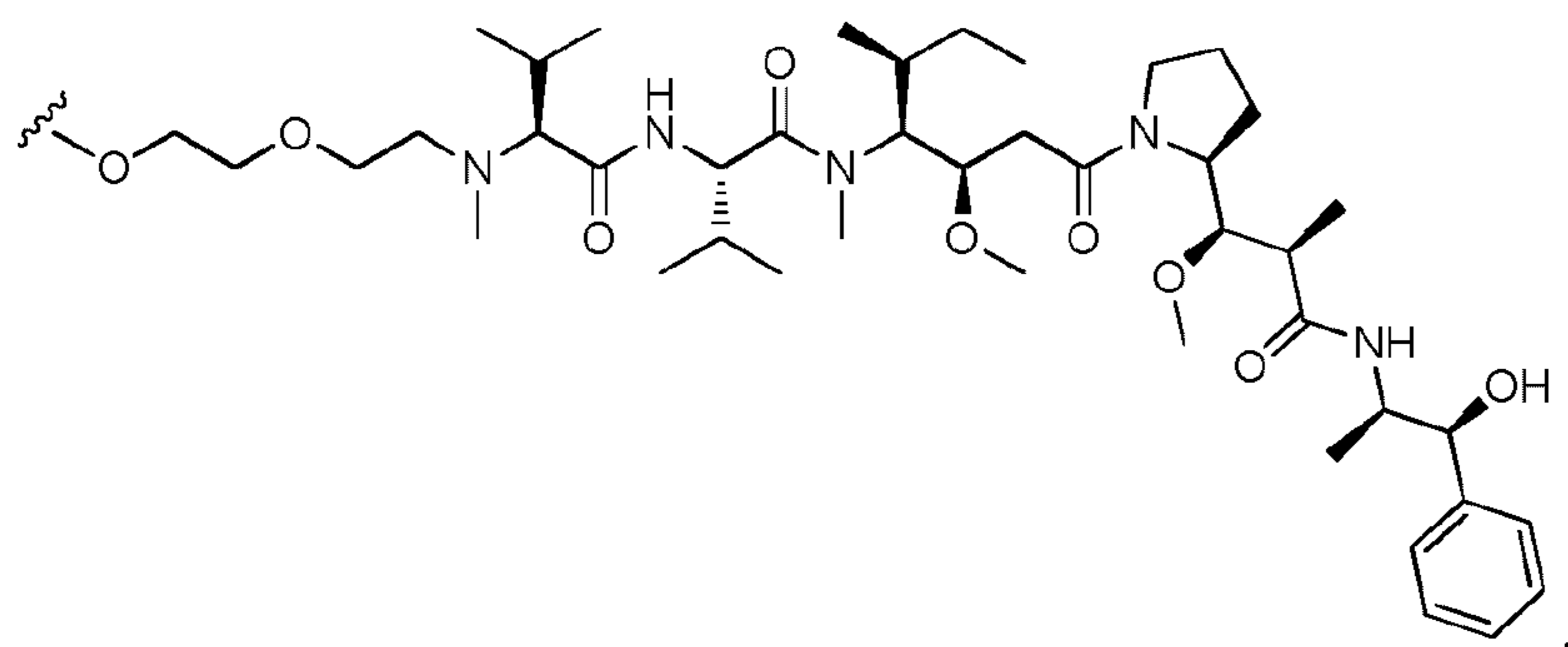
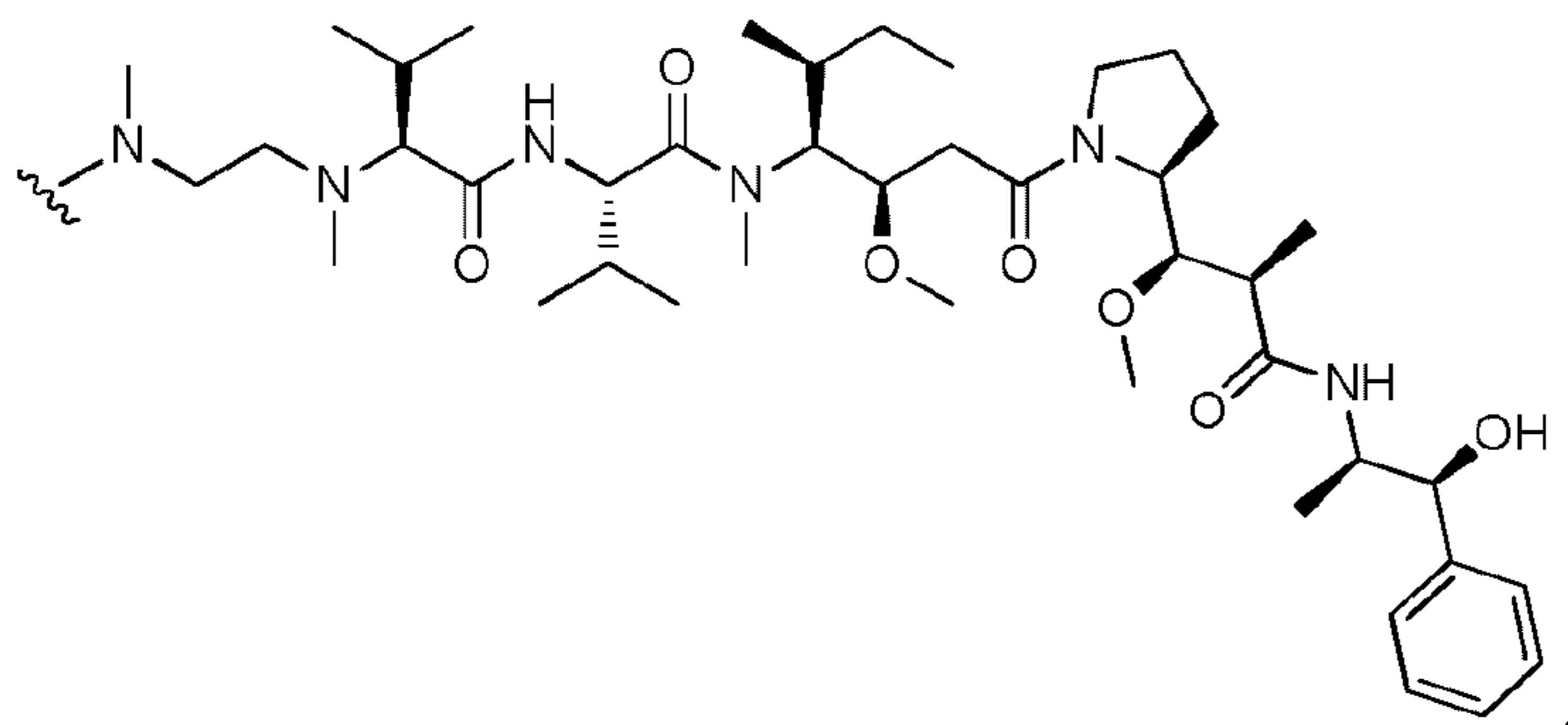


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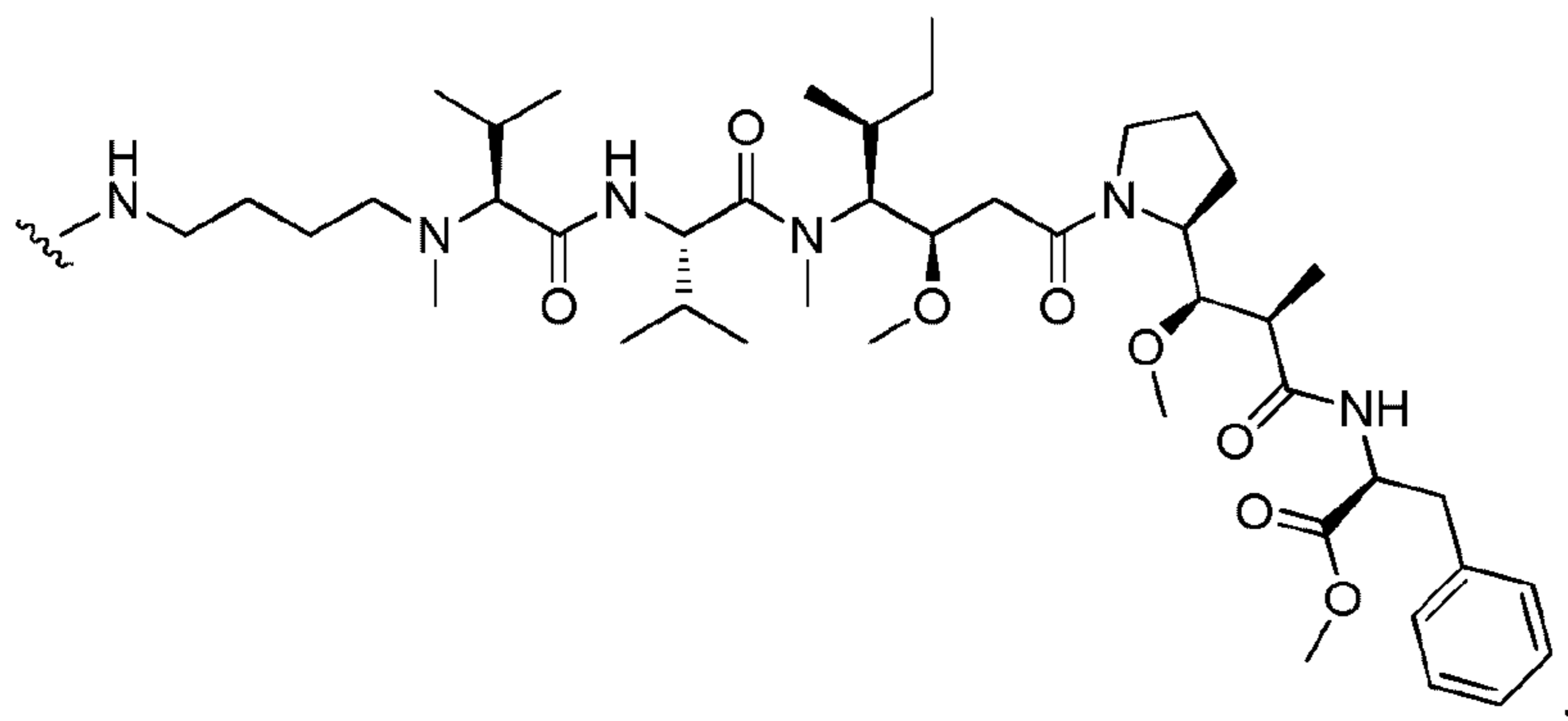
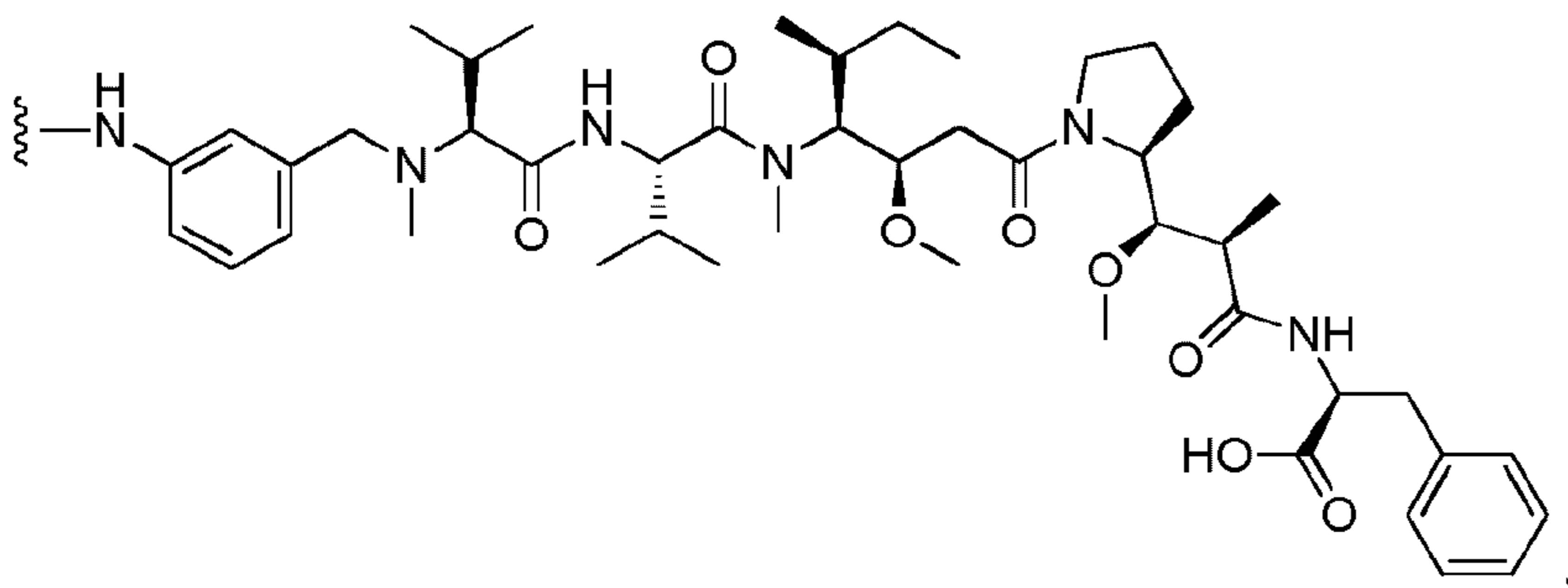
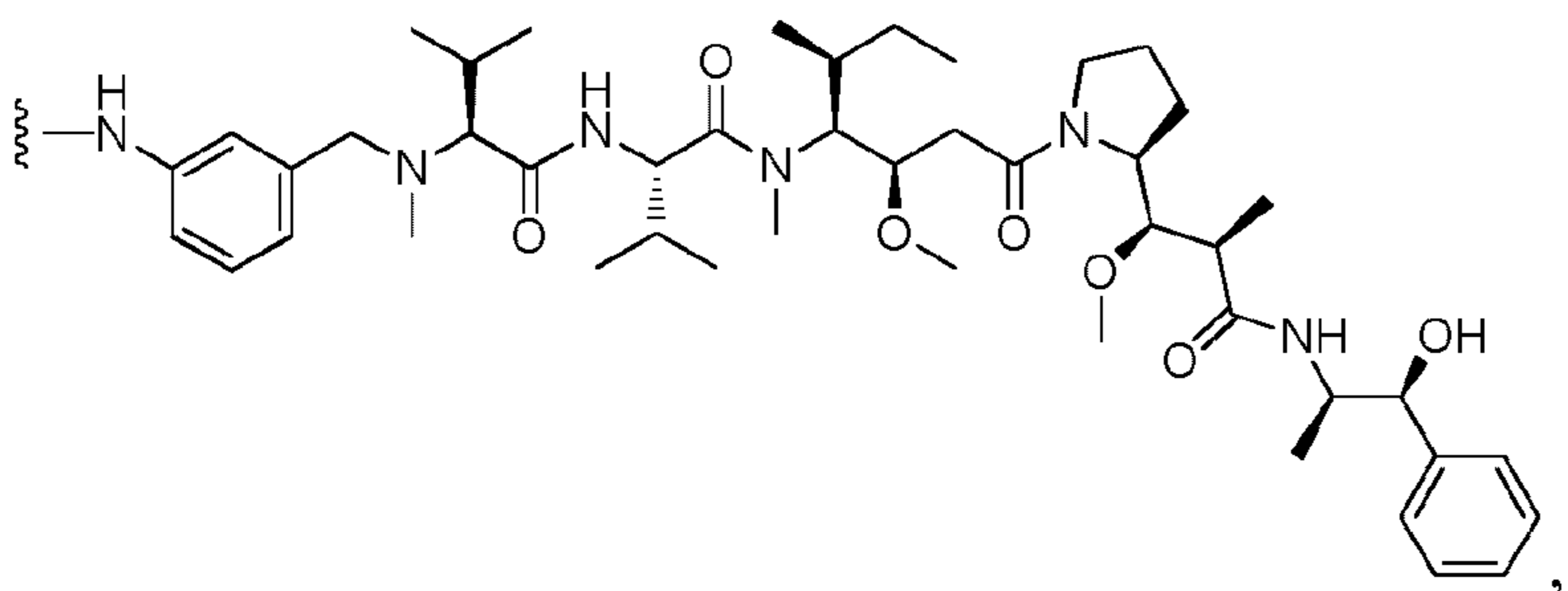
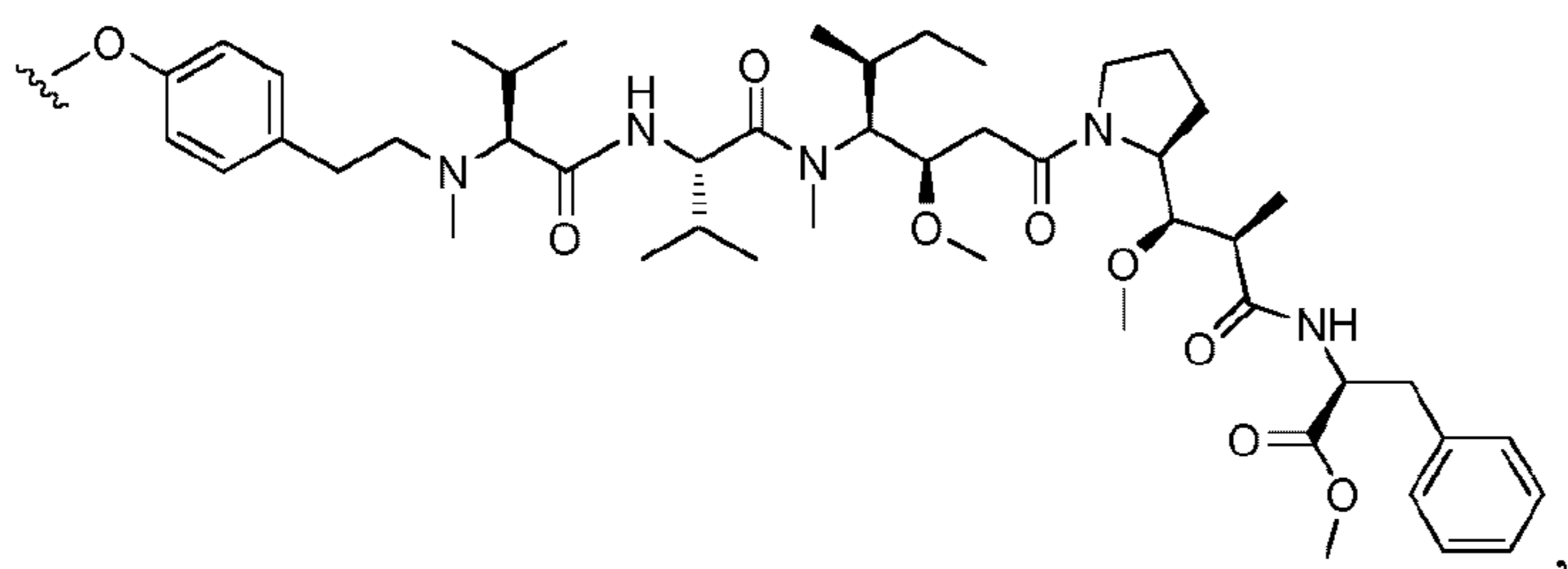
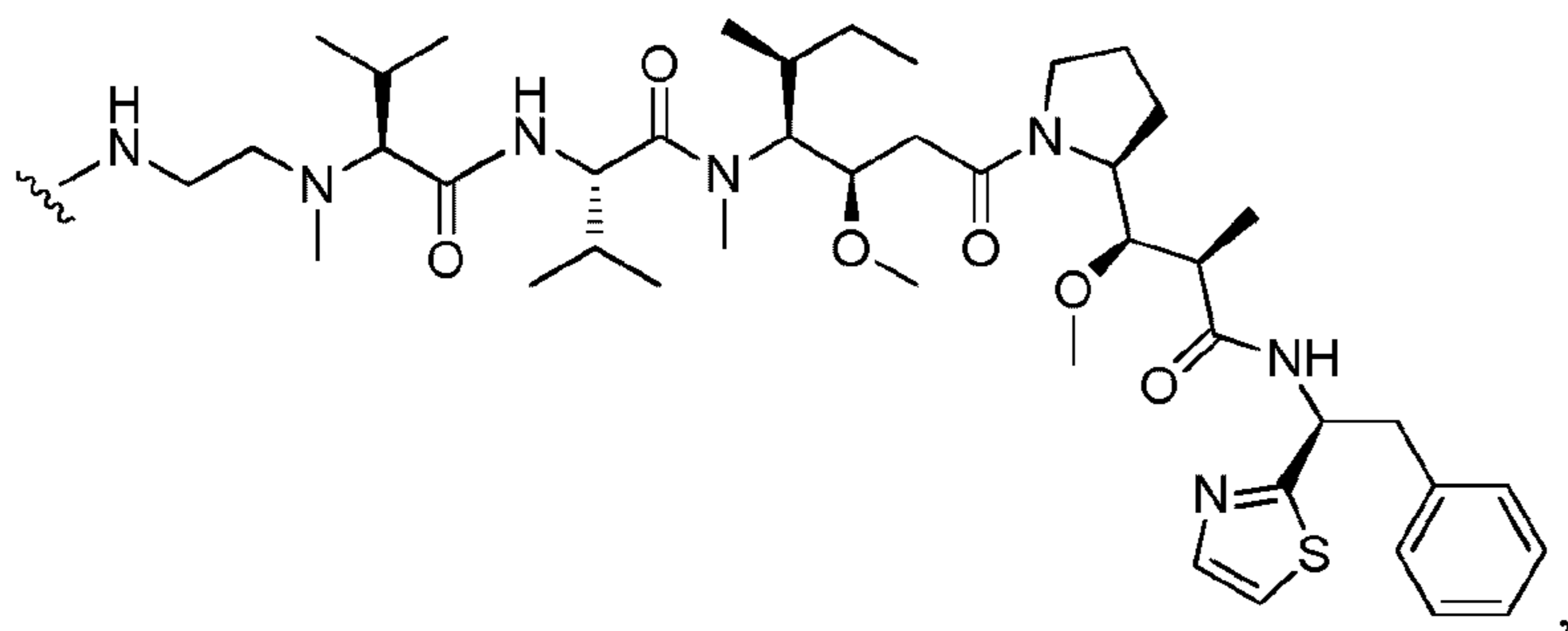


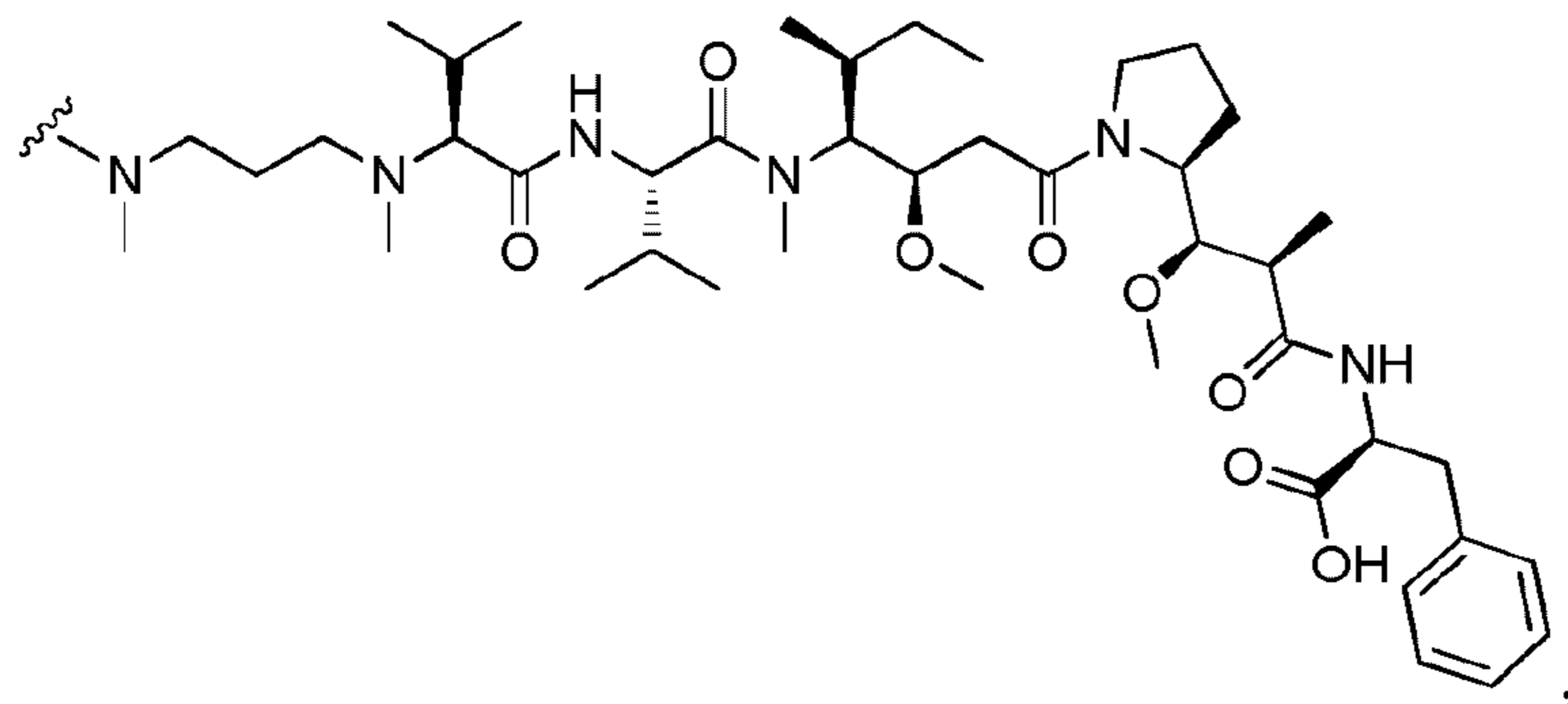
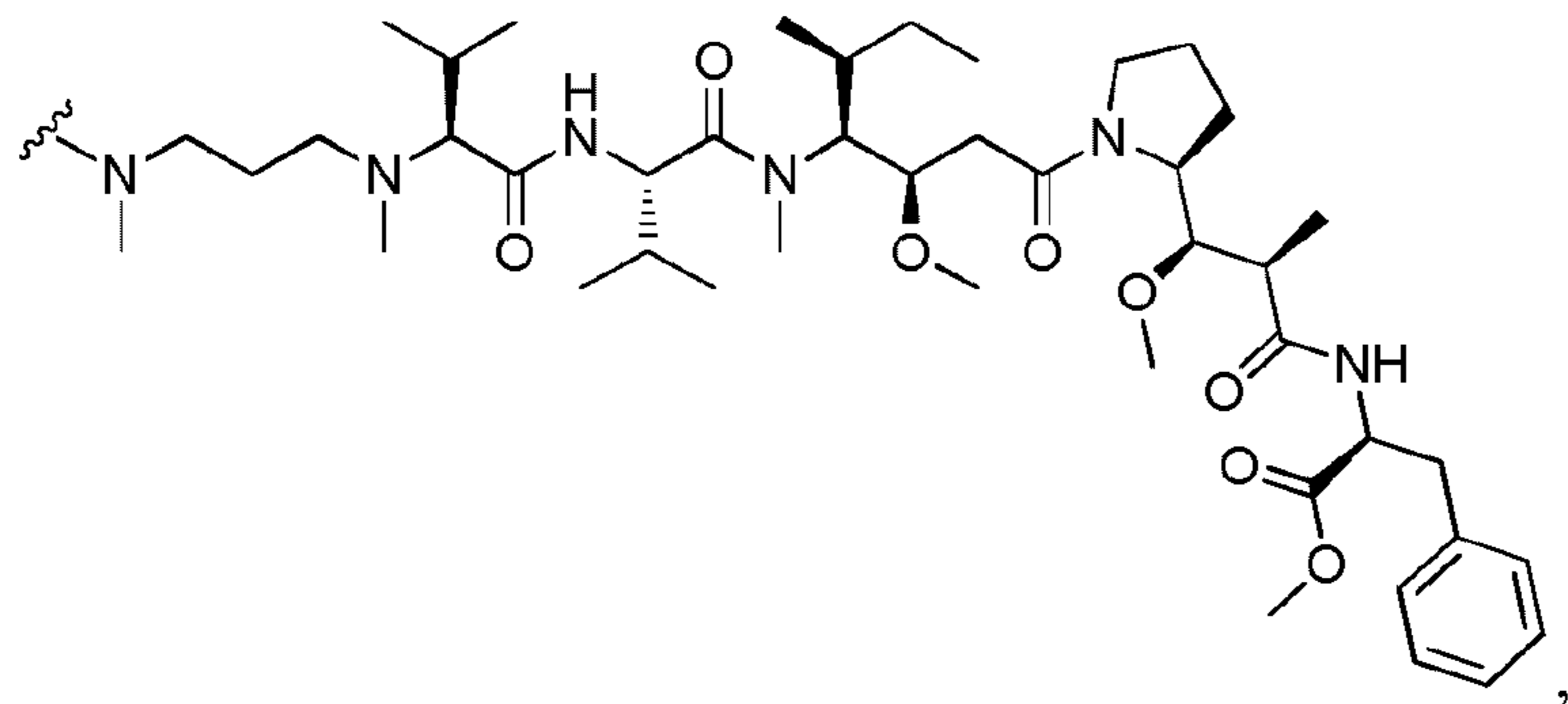
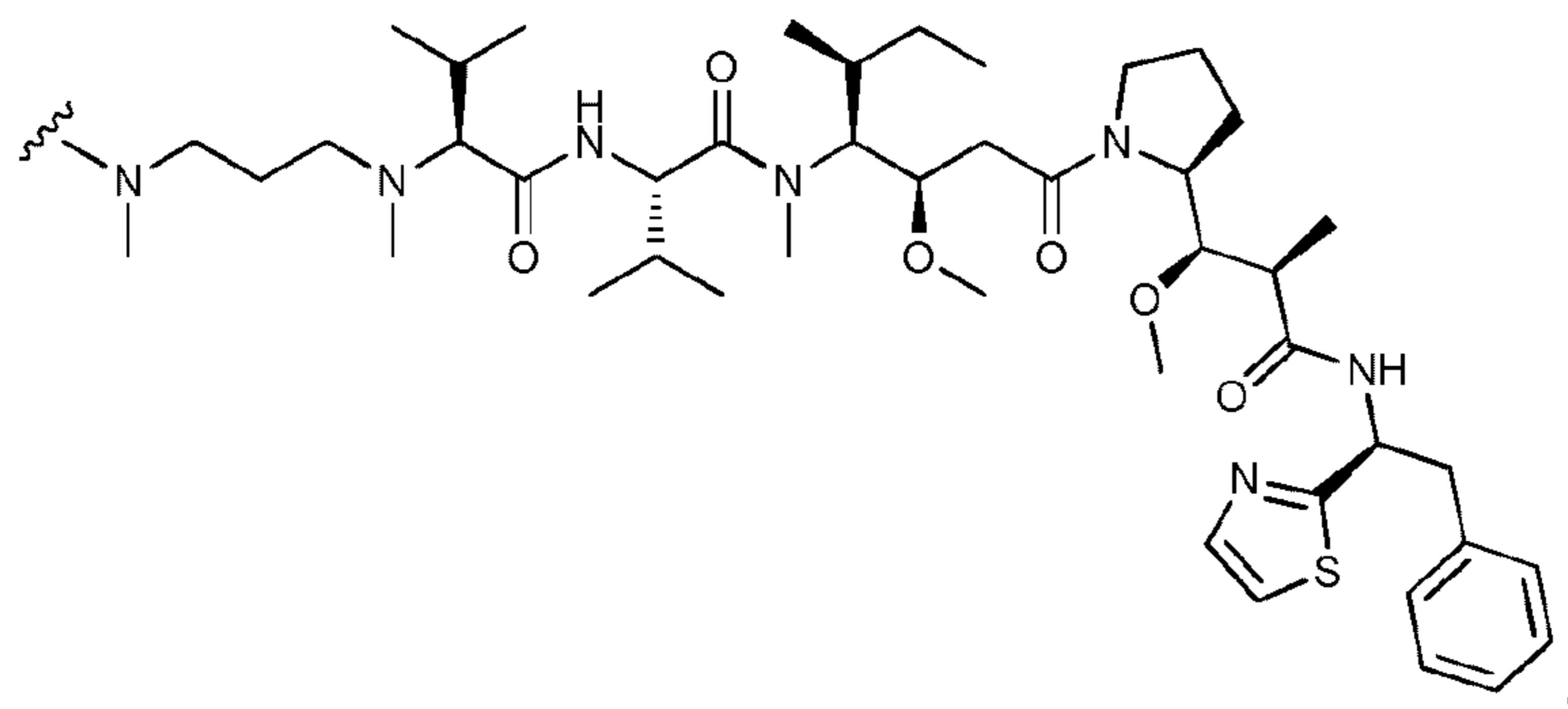
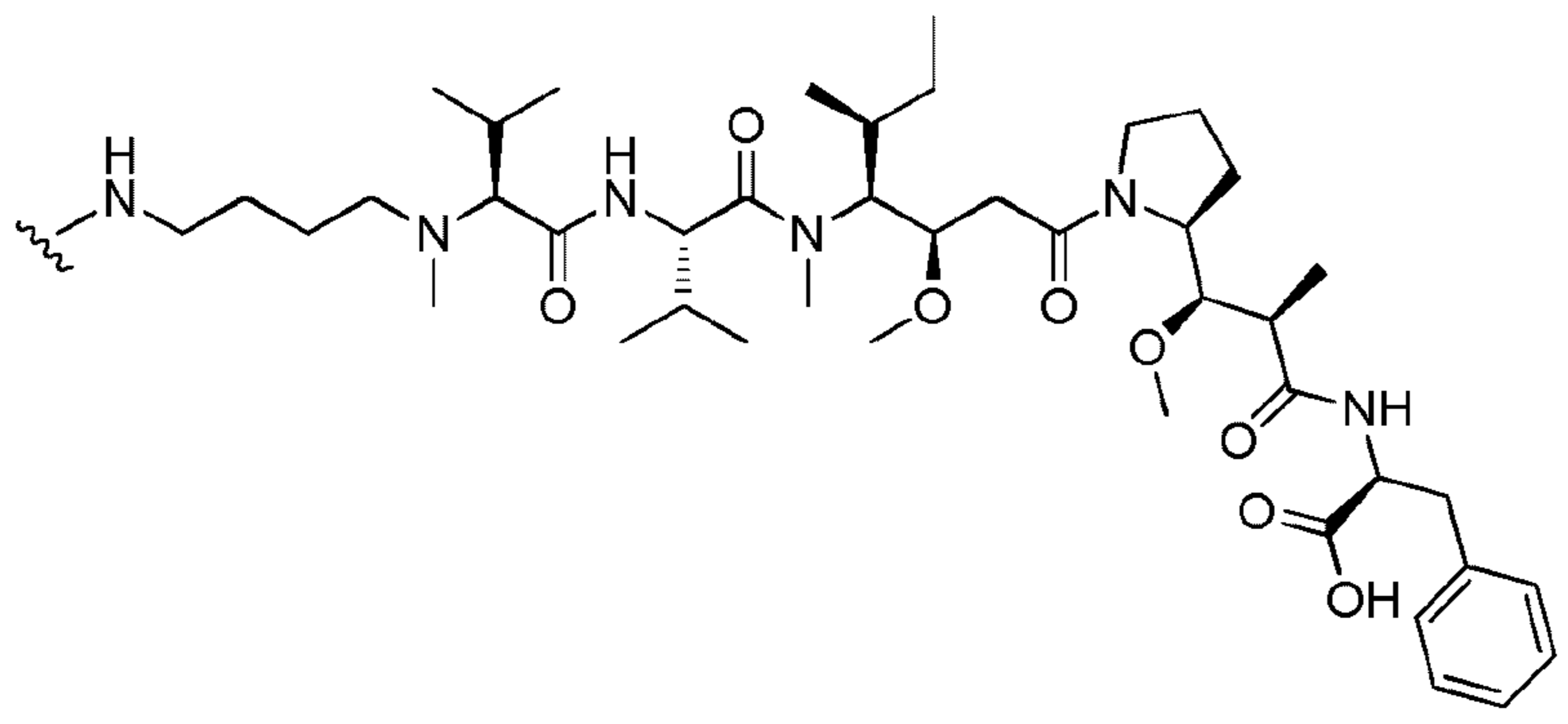


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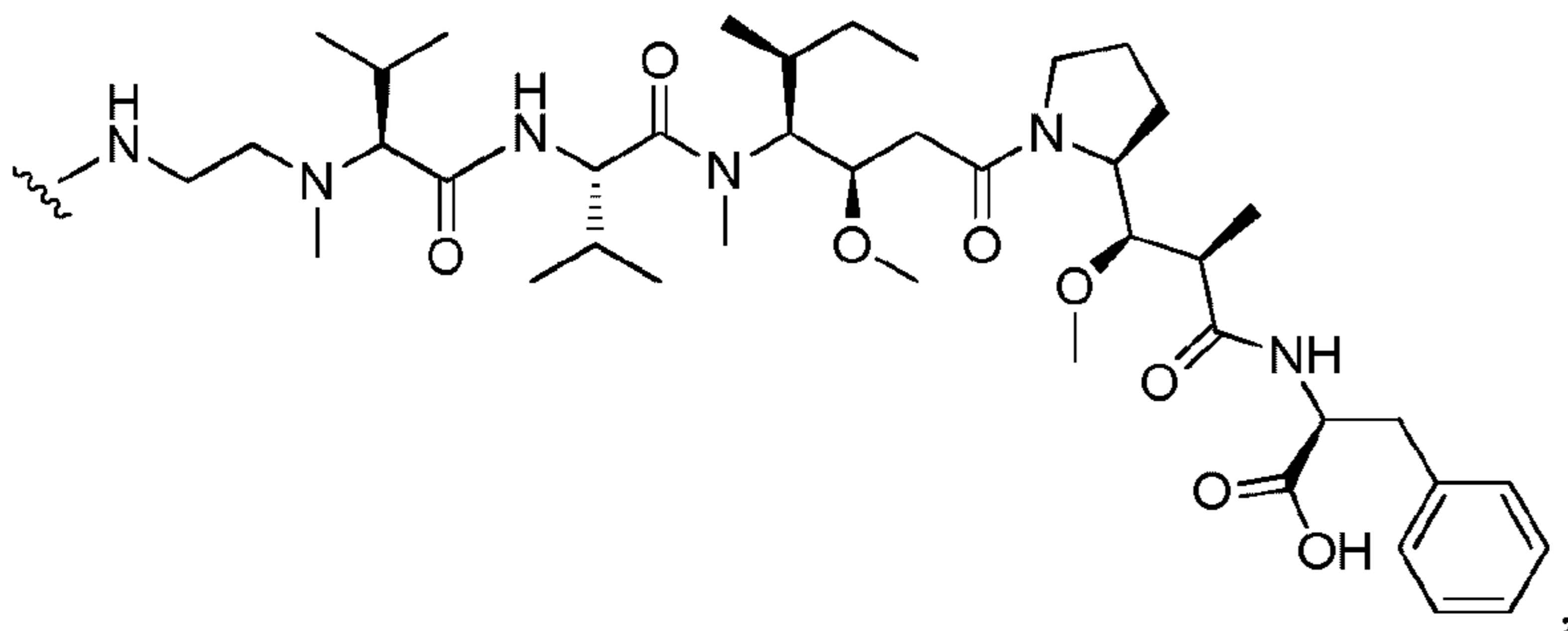
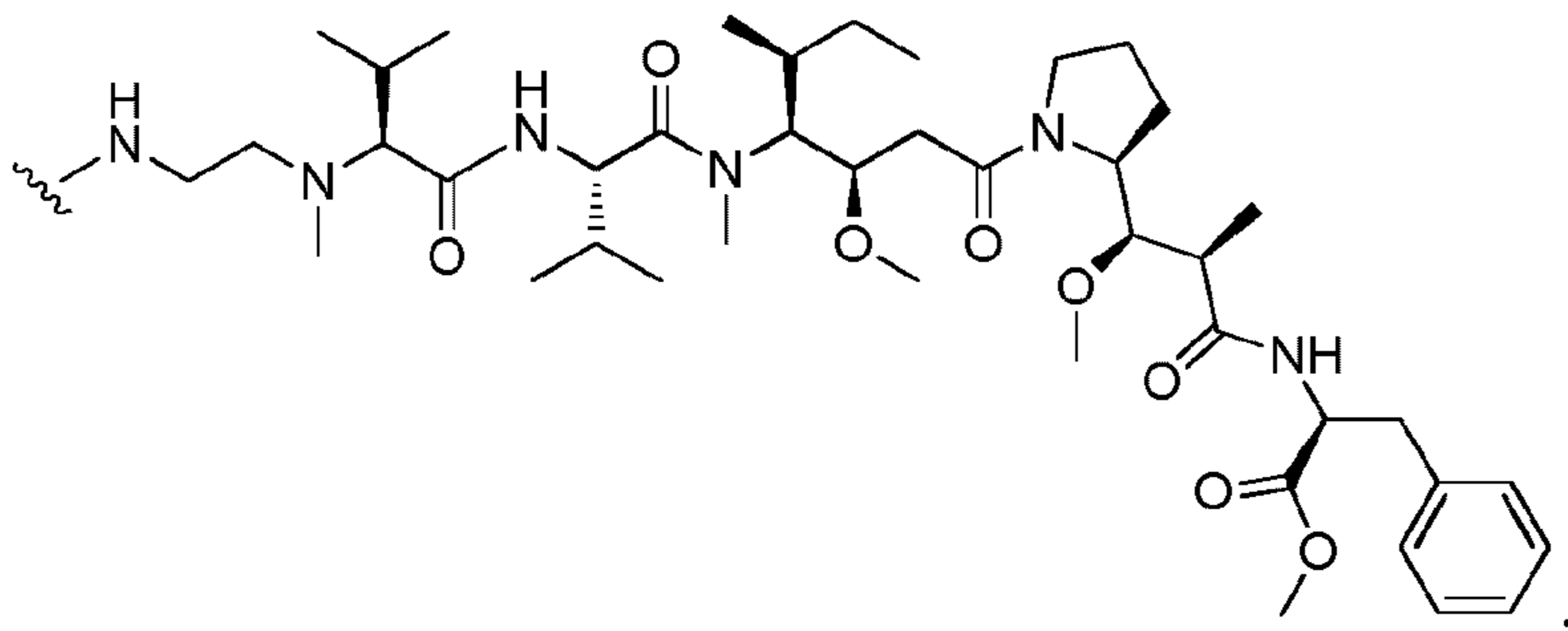
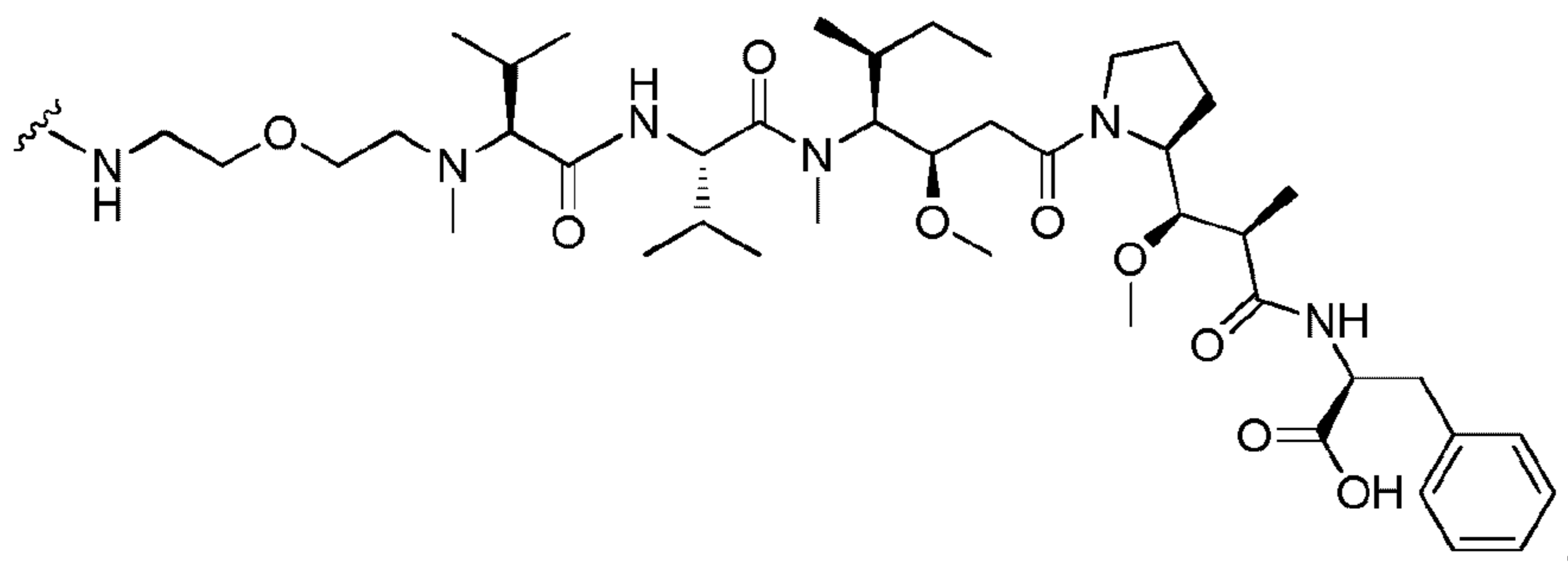
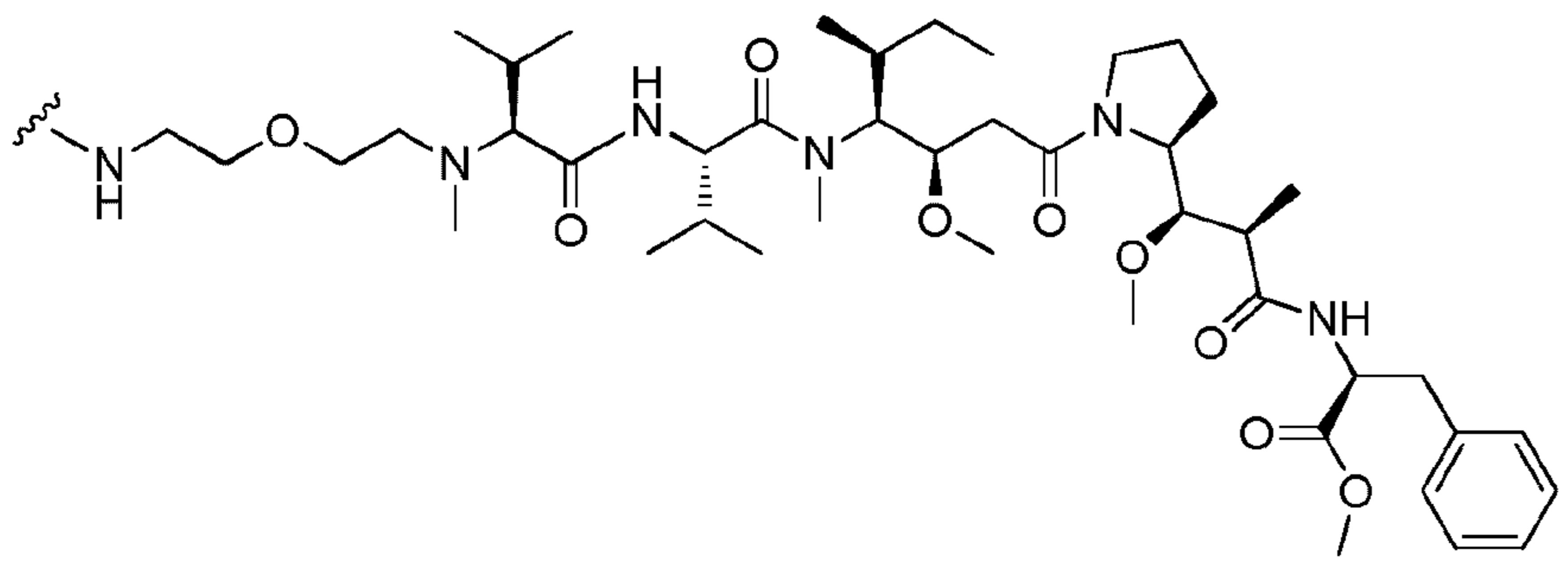
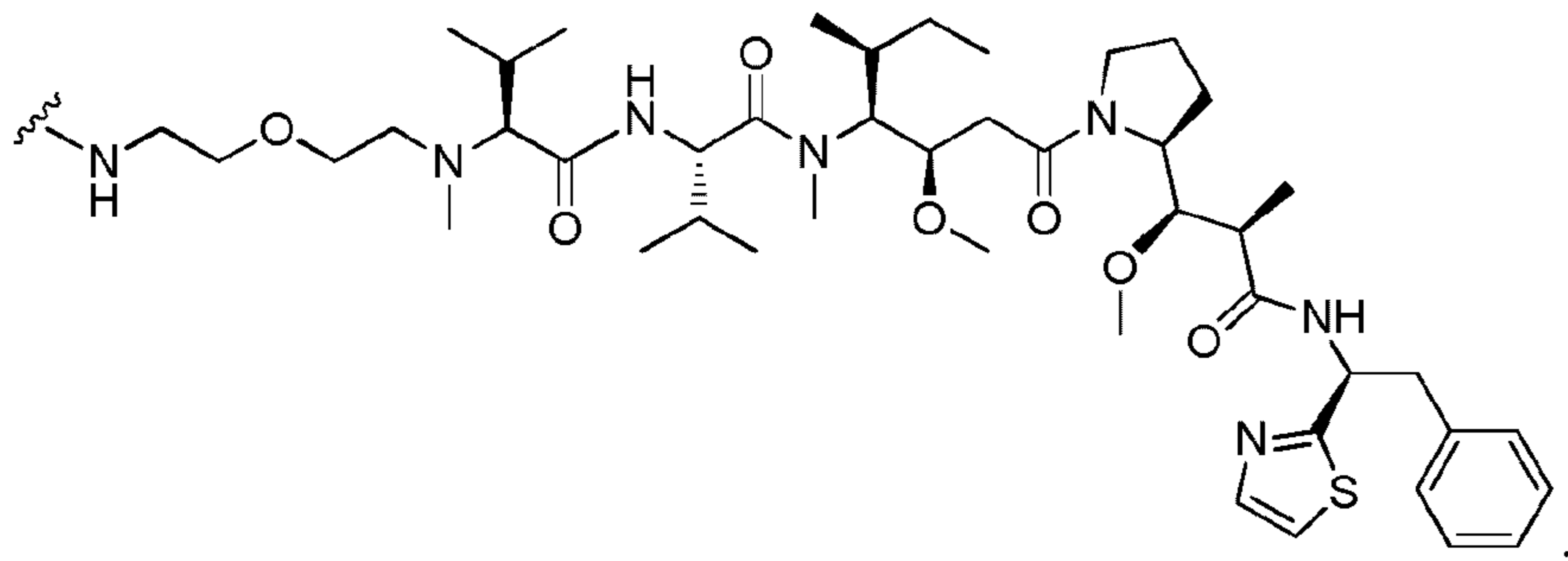


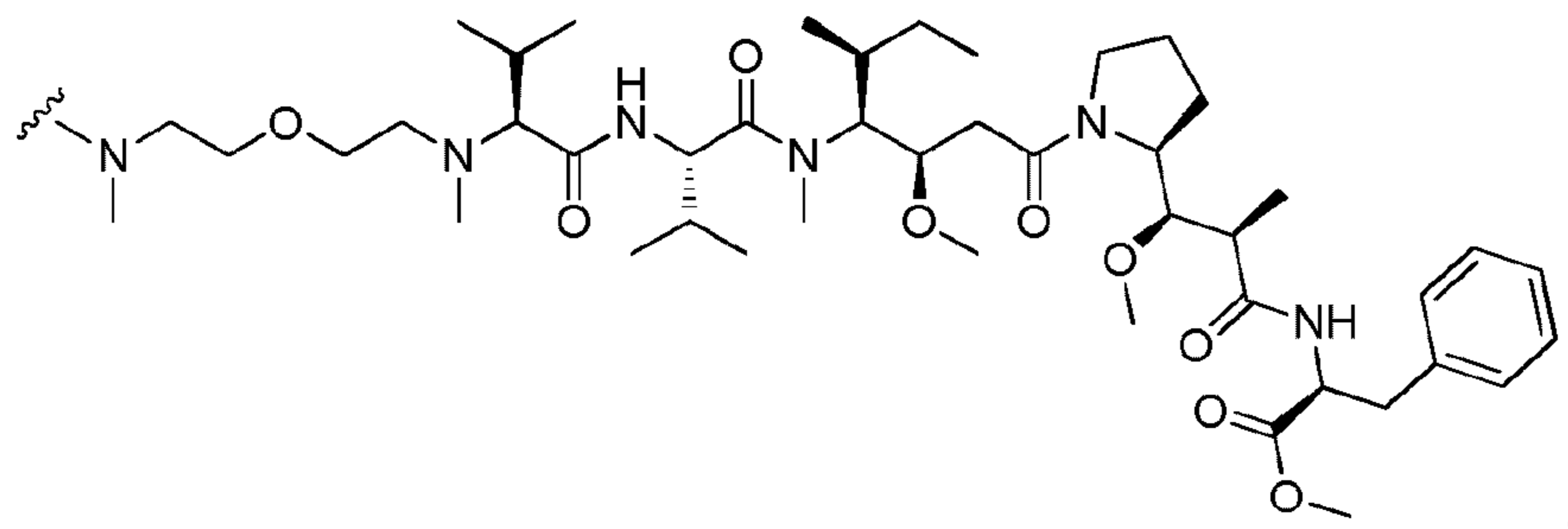
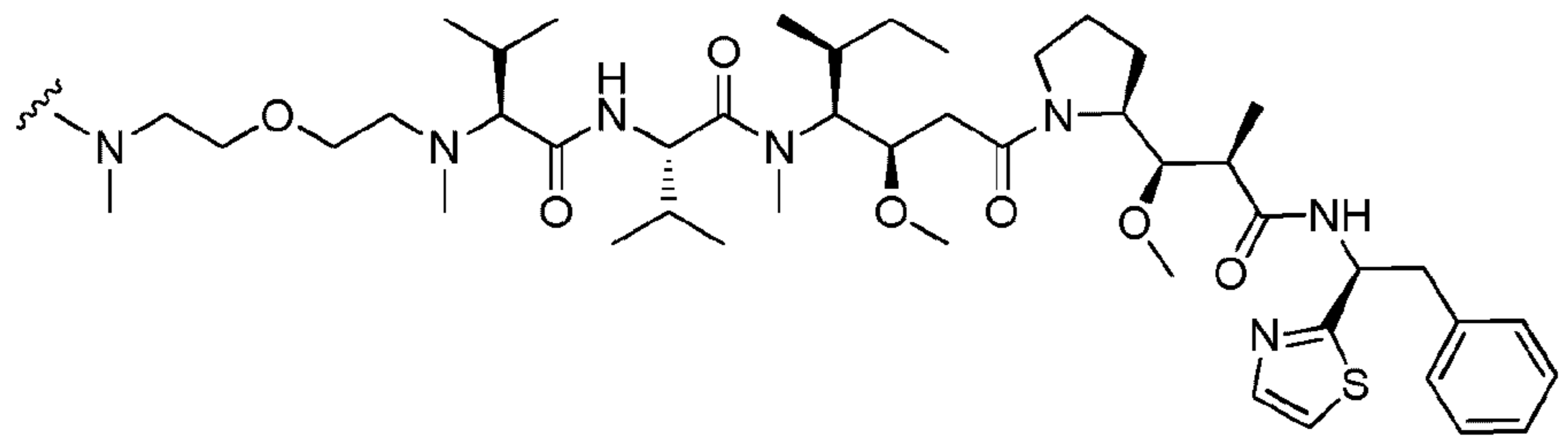
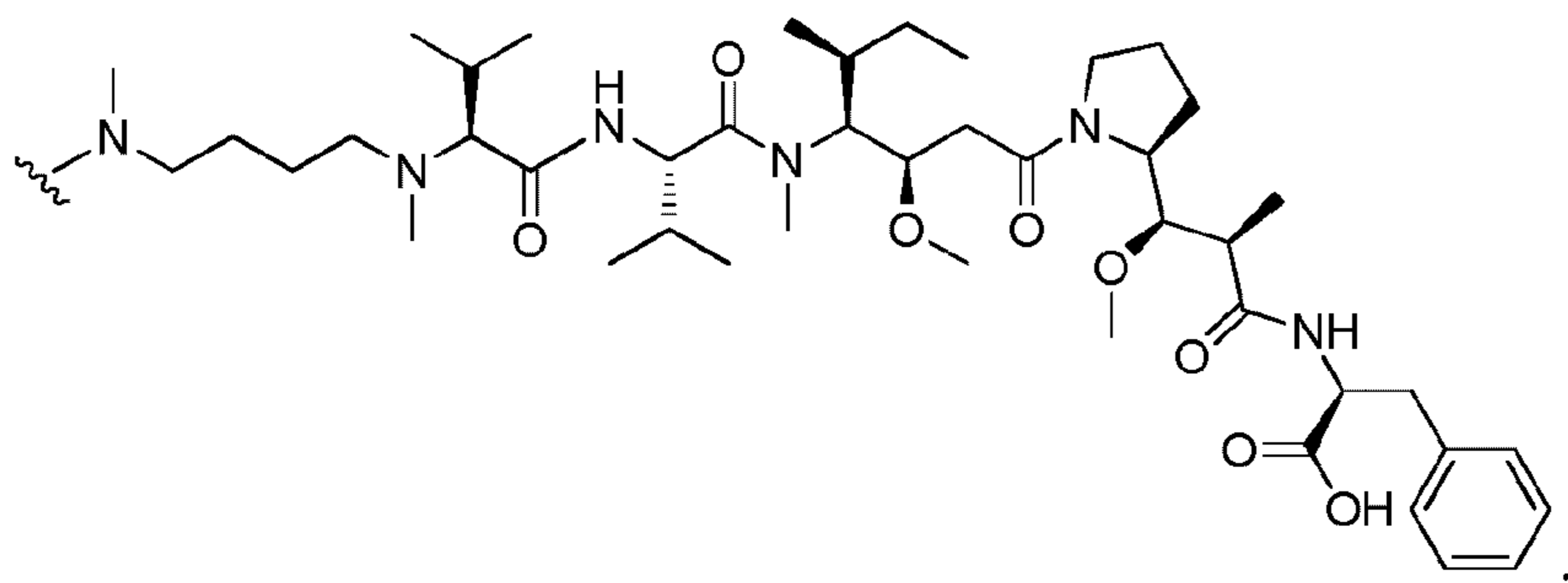
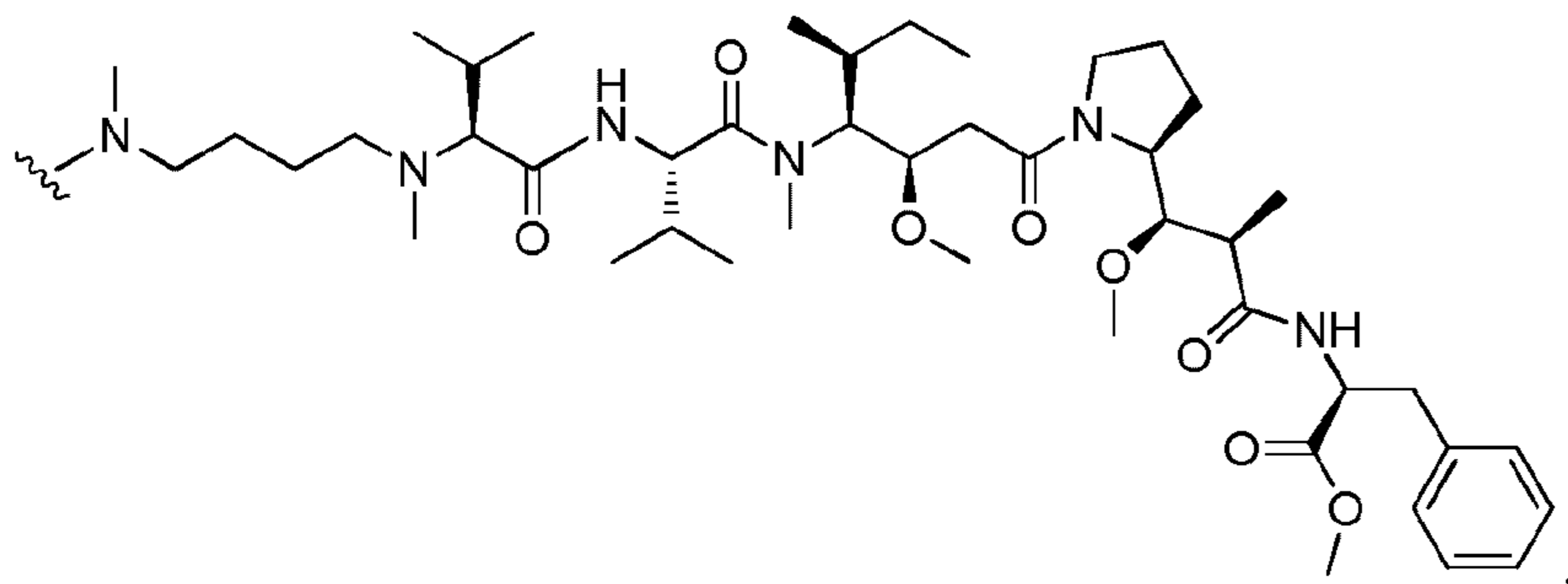
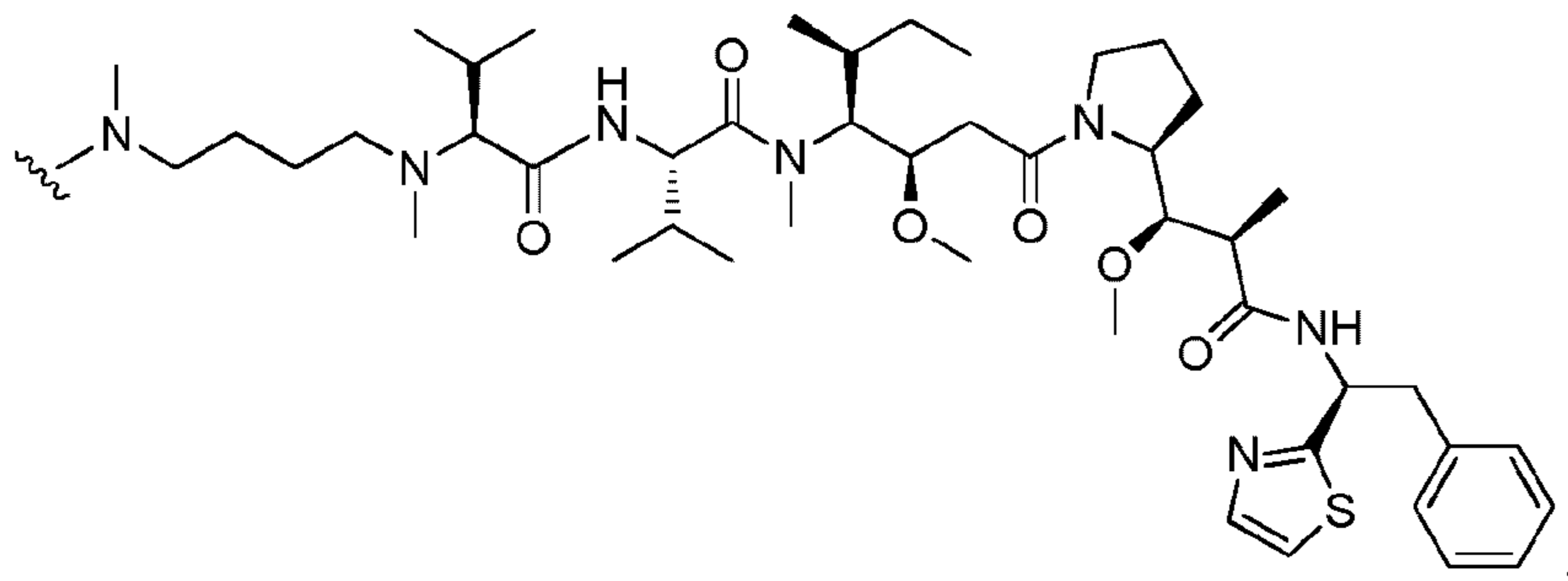
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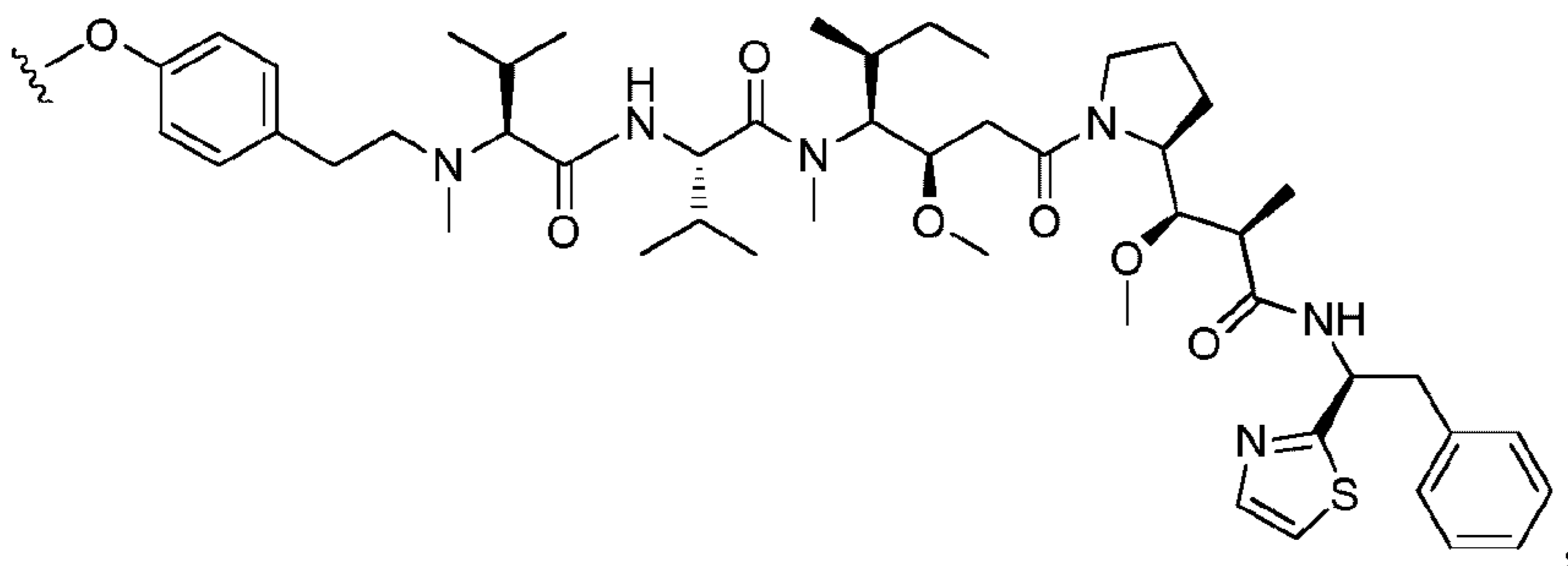
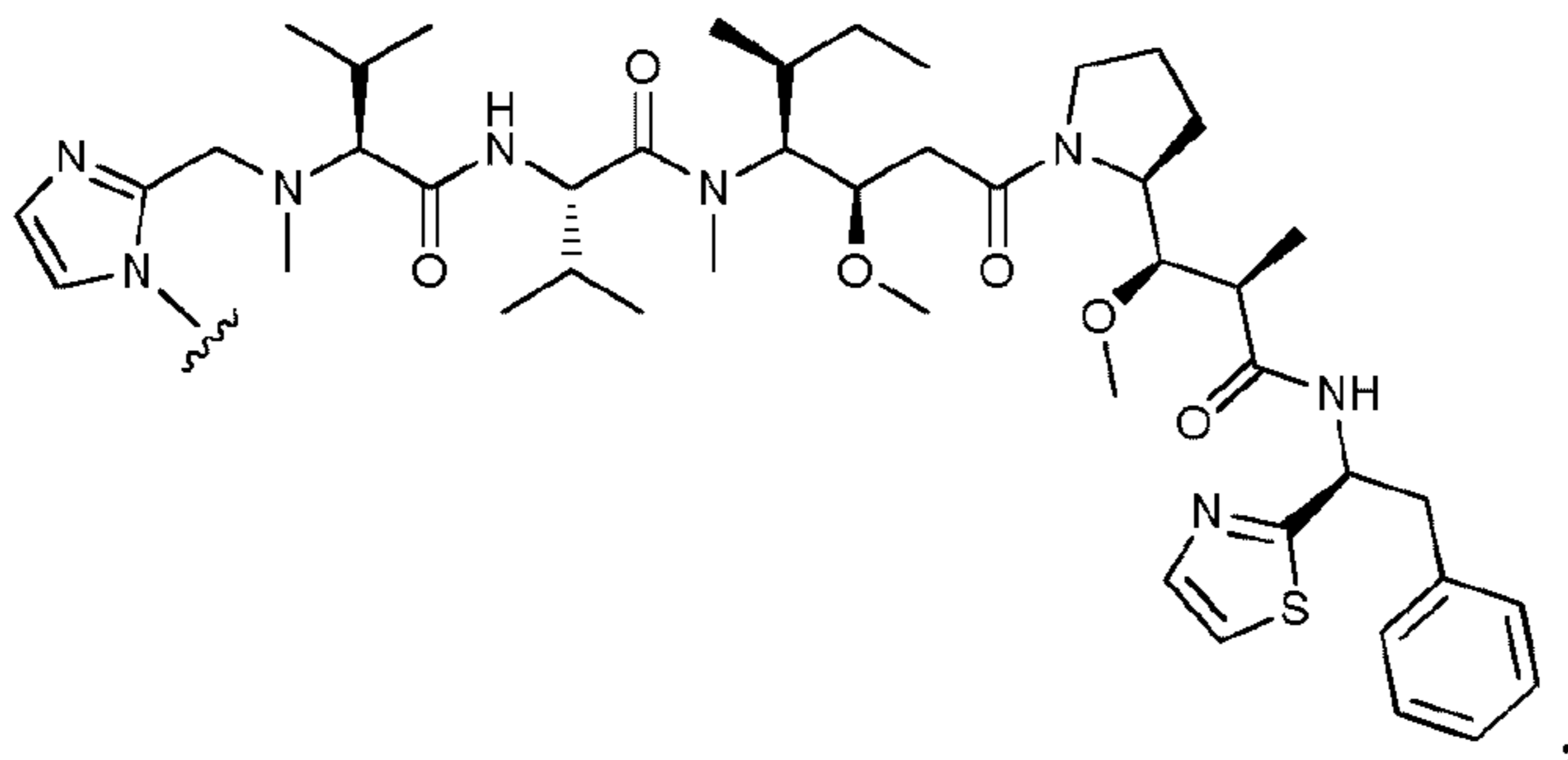
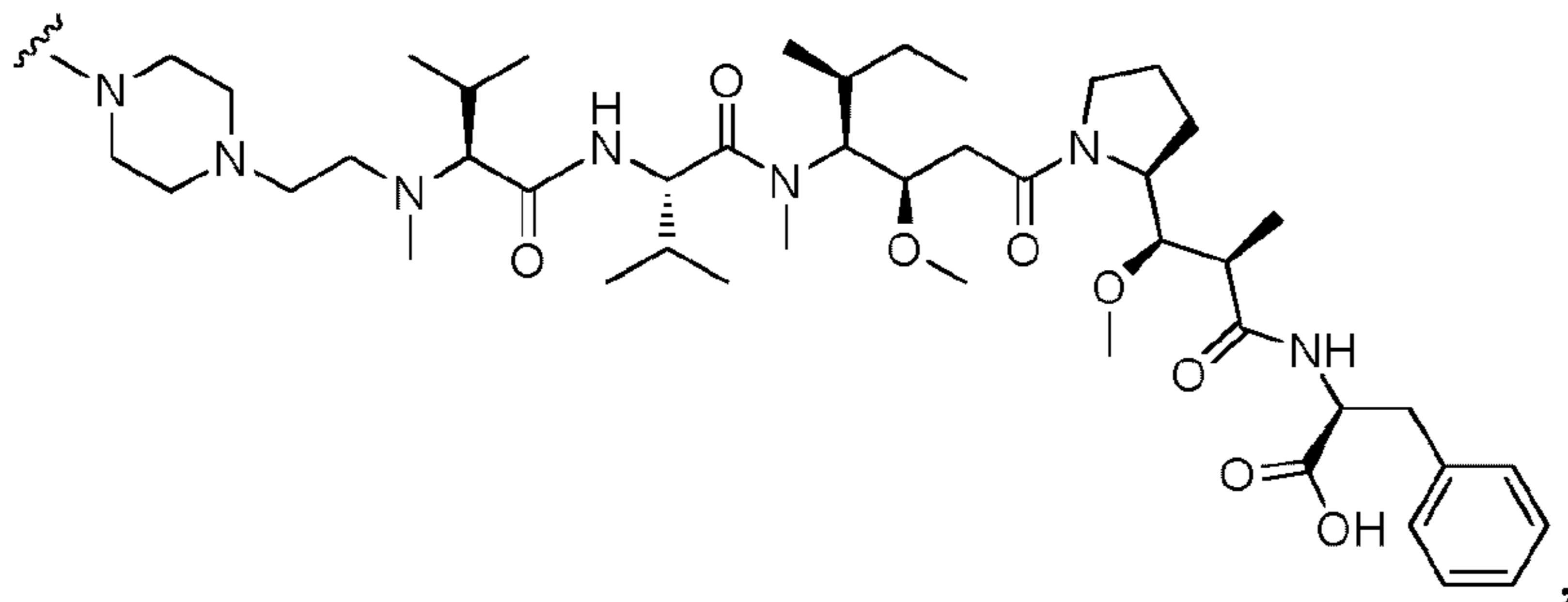
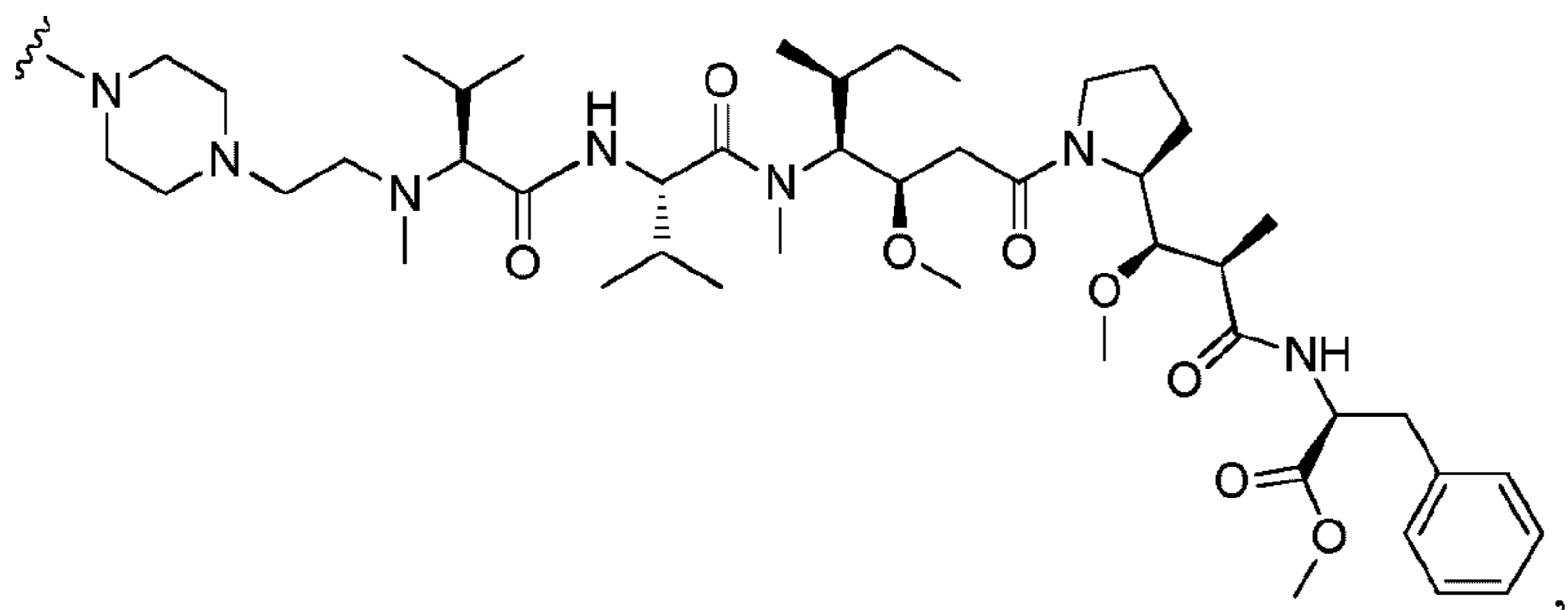
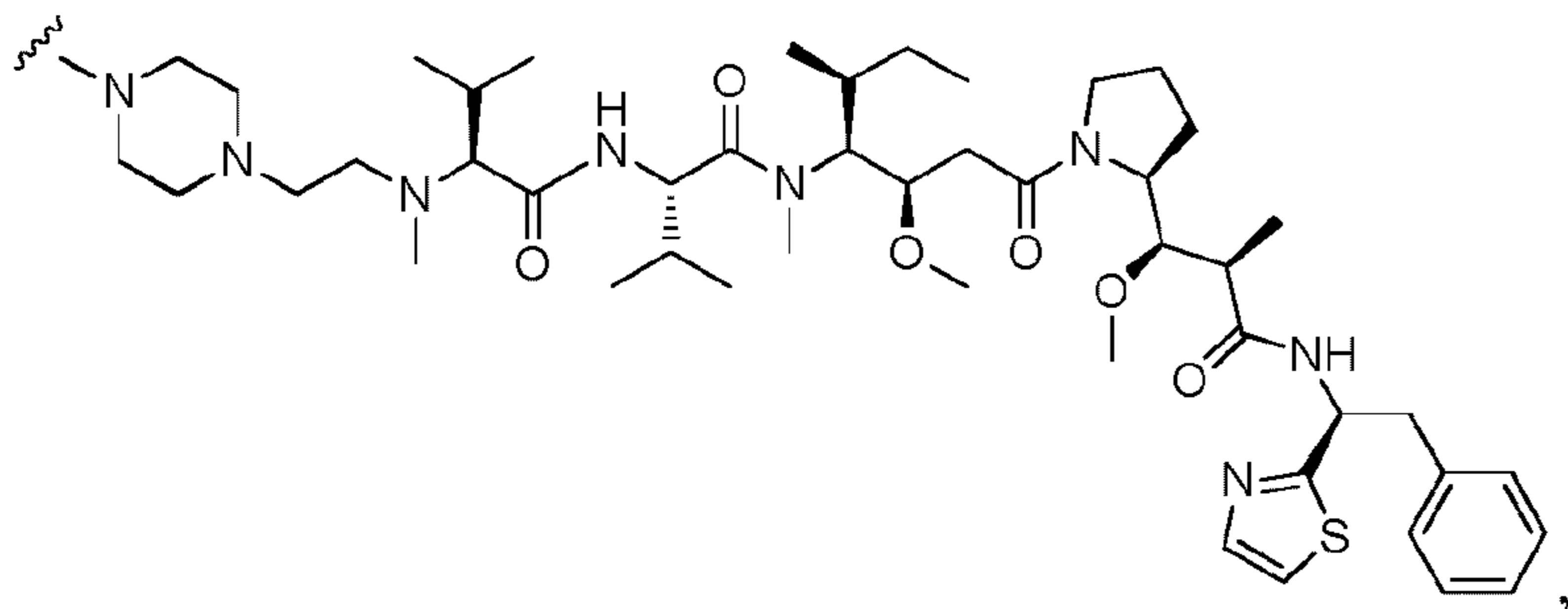


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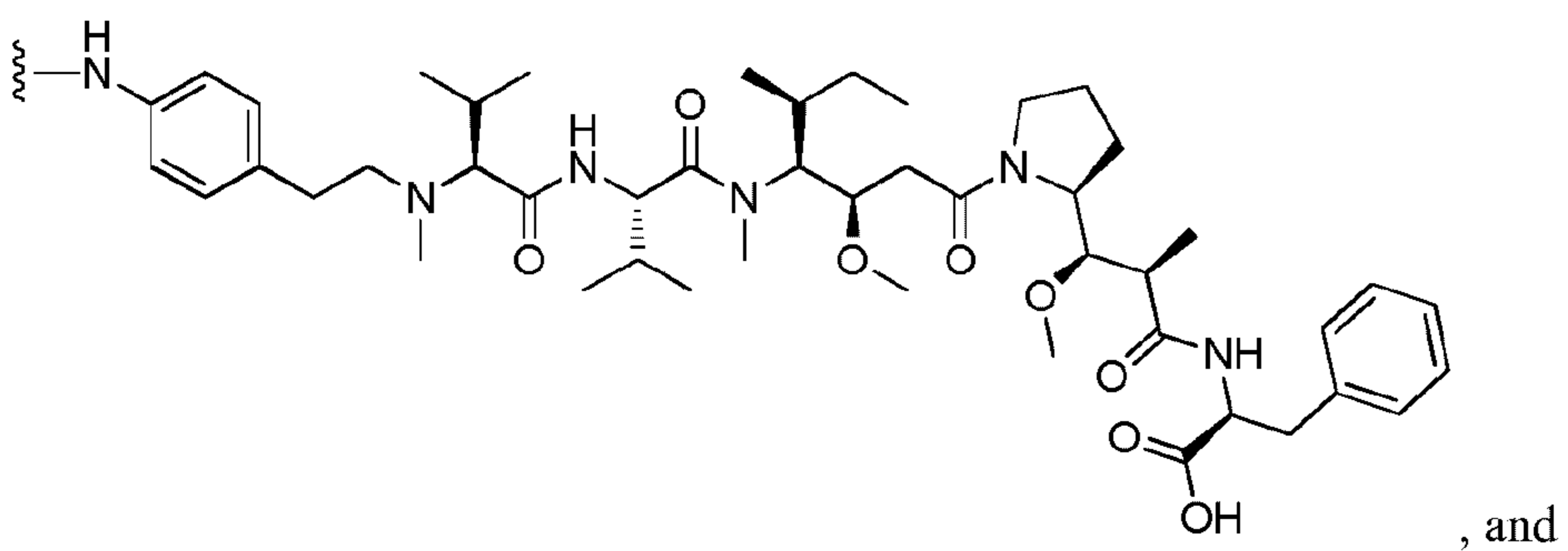
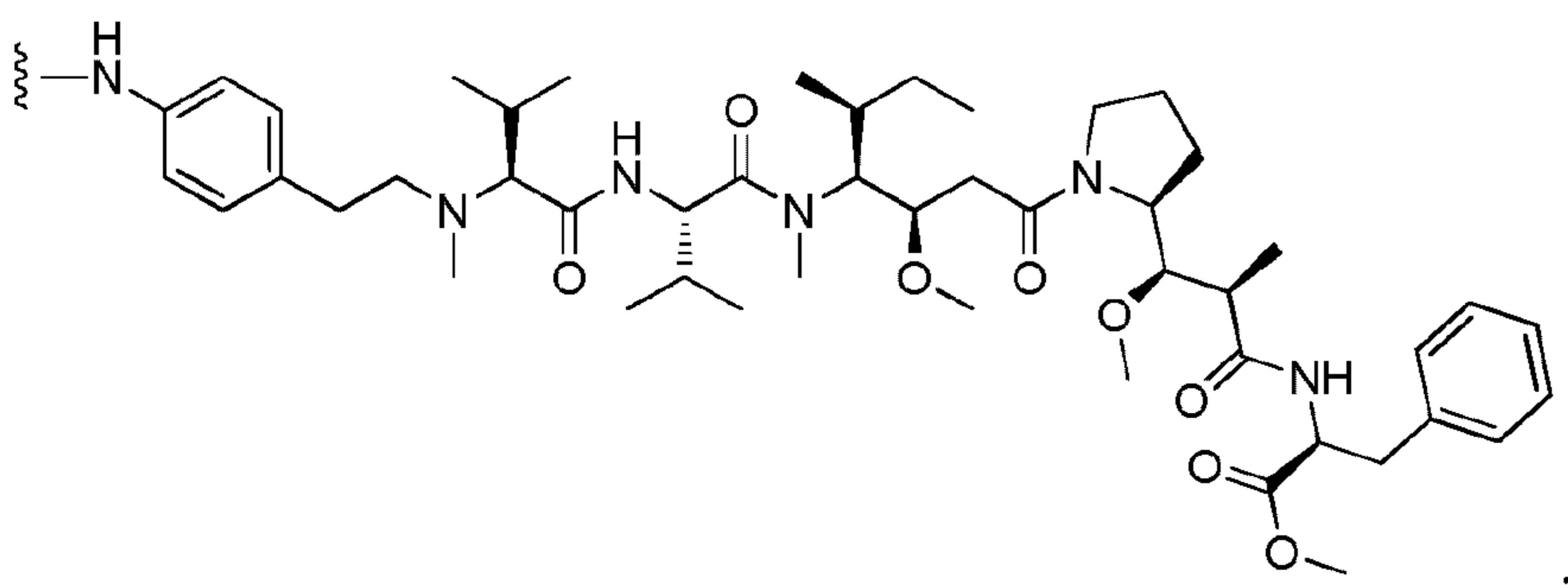
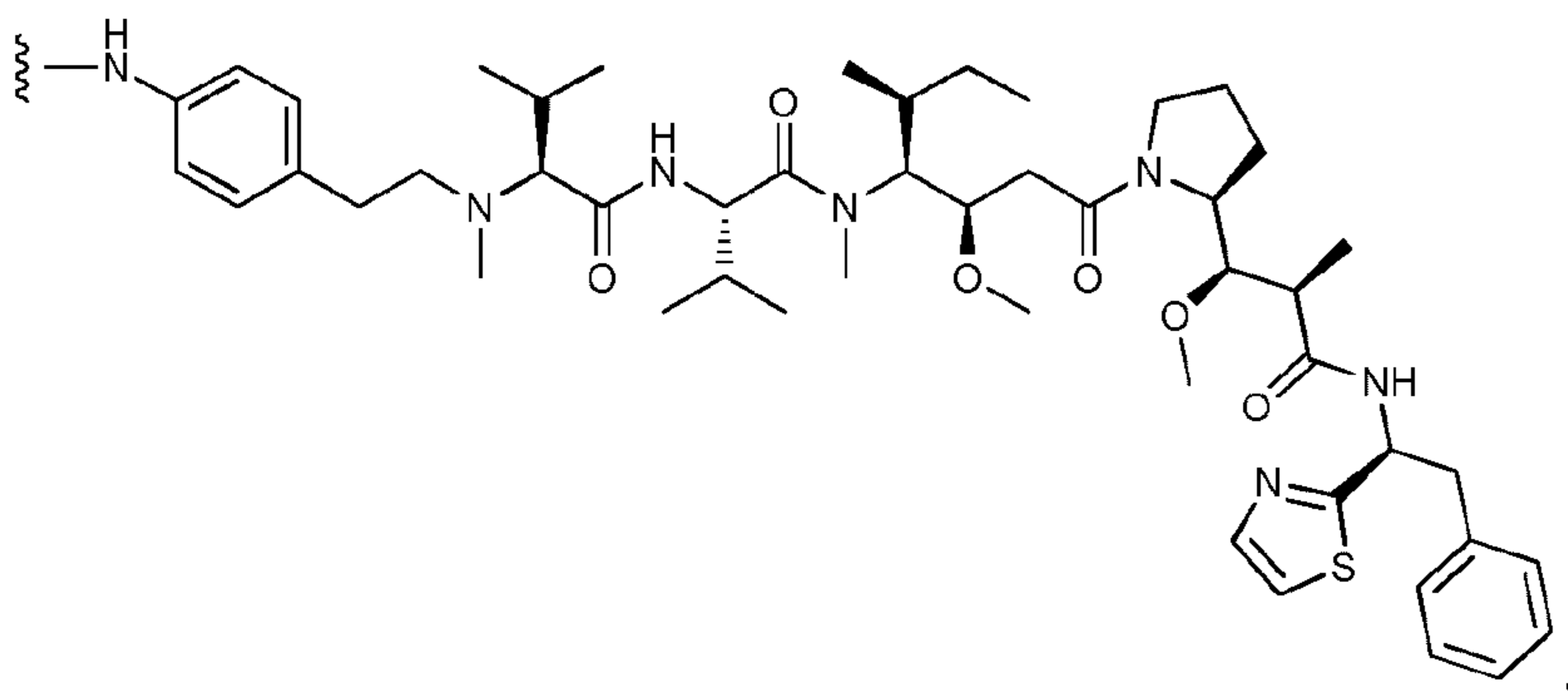
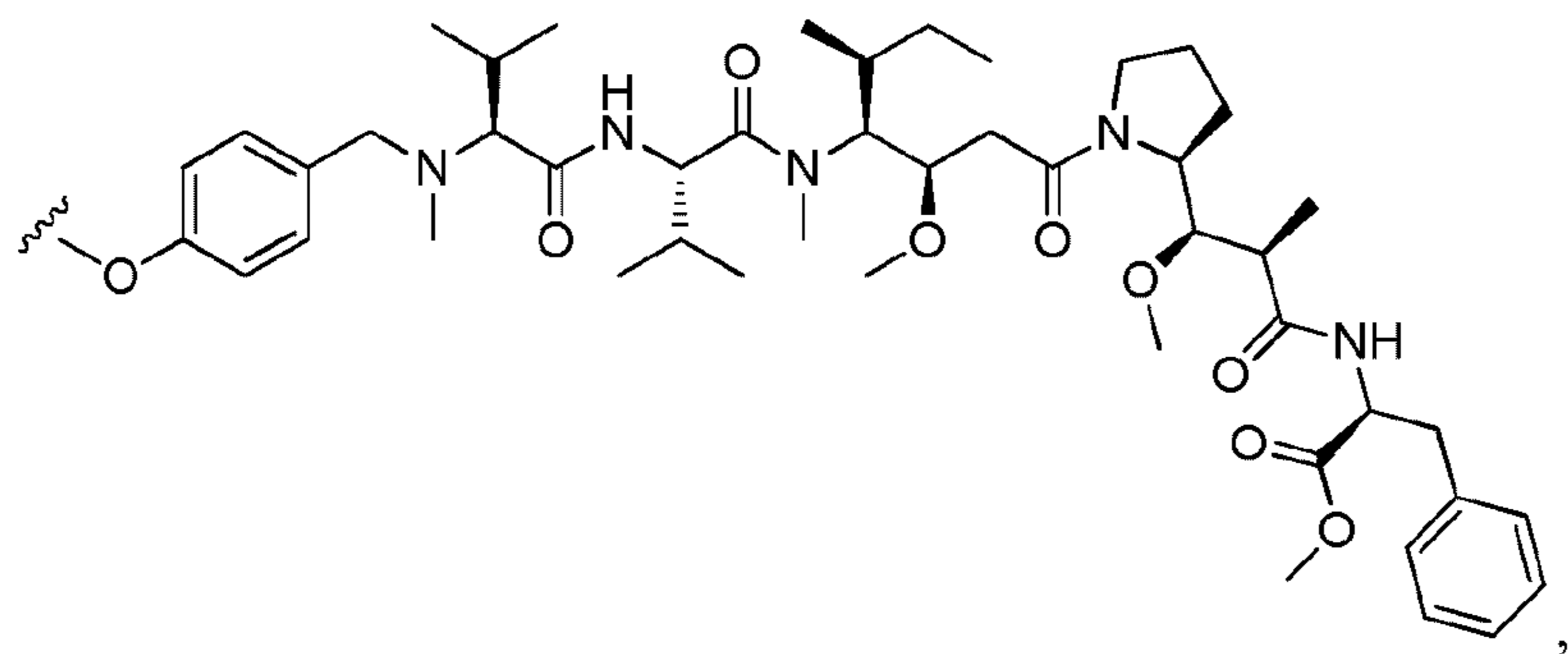
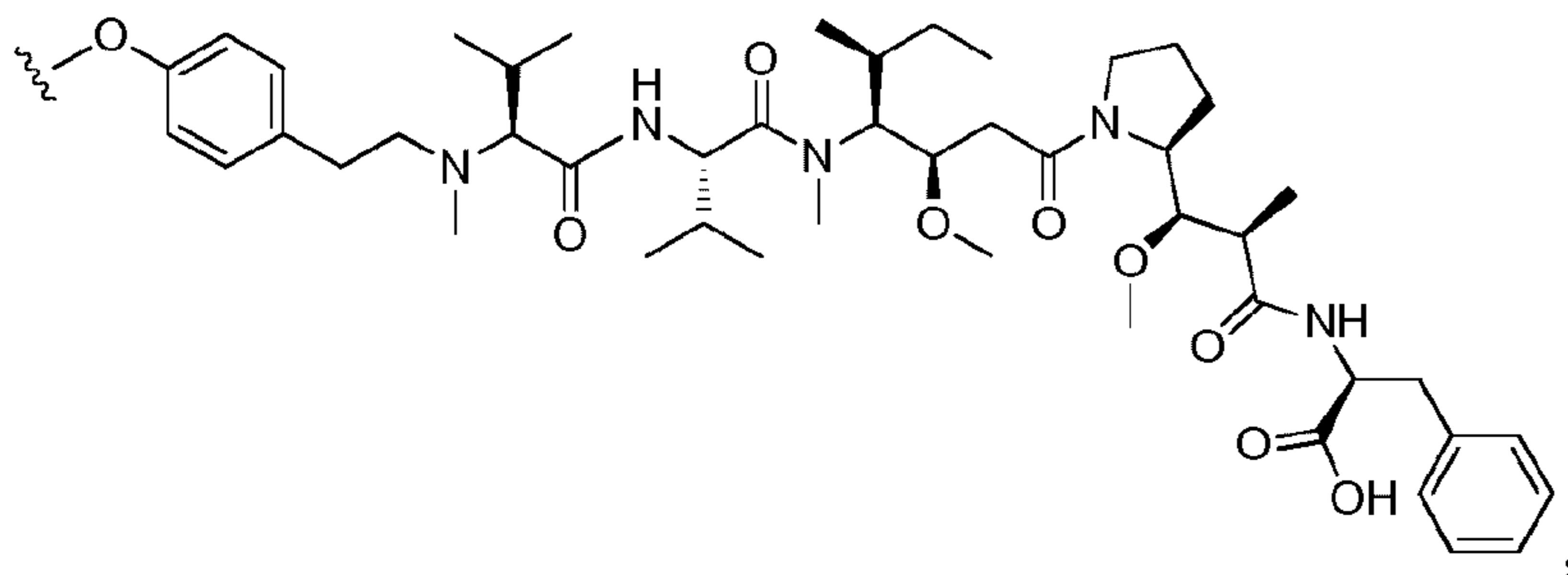


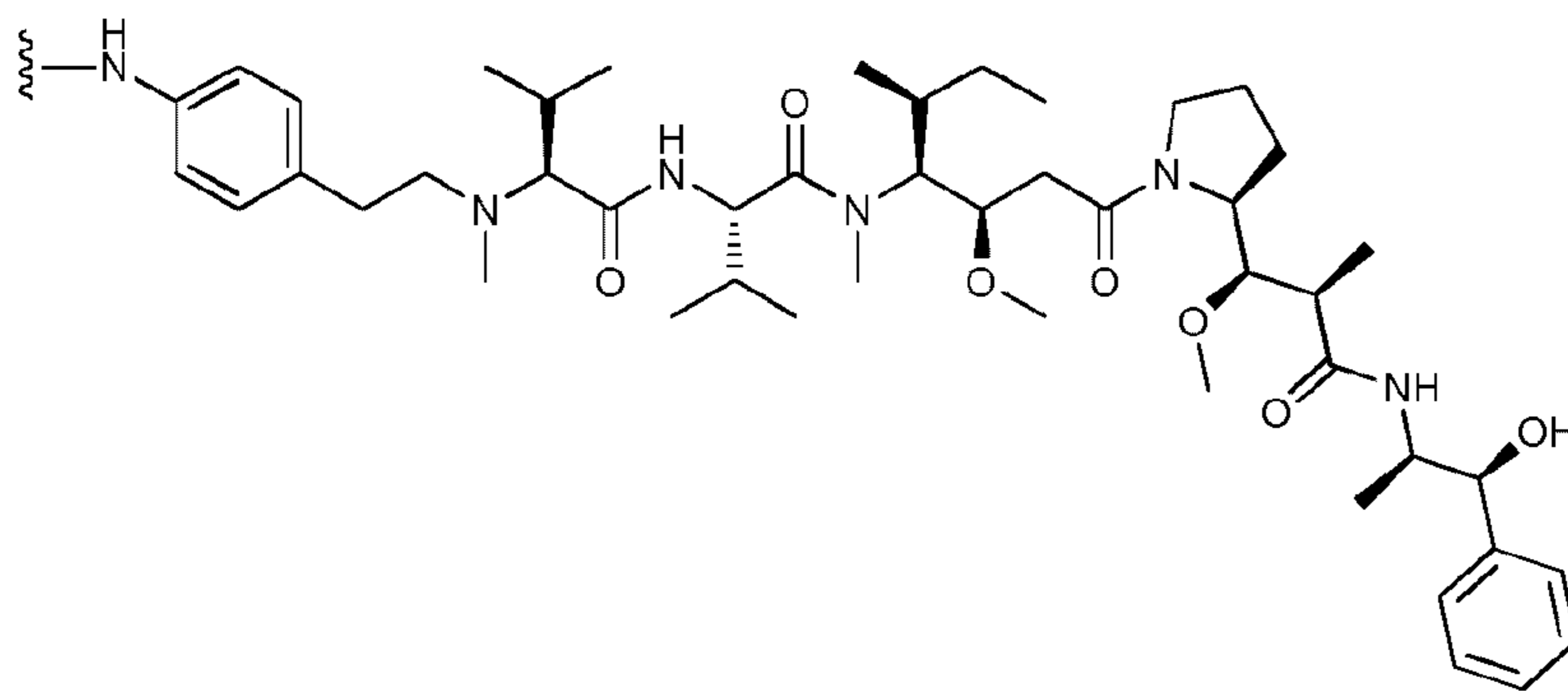


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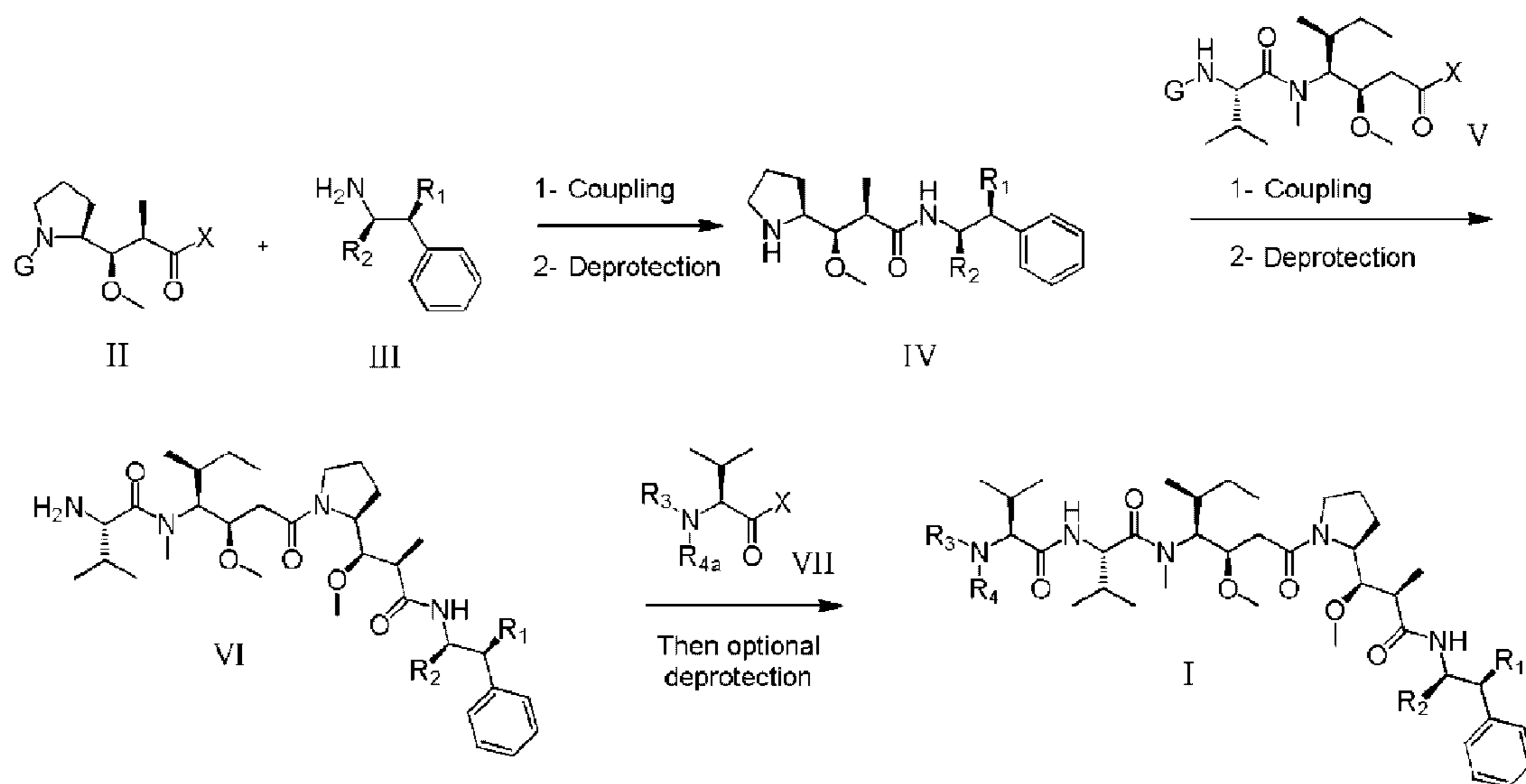
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Preparation of the drug (of formula DH):

The drug can be prepared using the general methods described in the following synthesis schemes, optionally supplemented by any standard operation when needed that is described in the literature or well known to persons skilled in the art, or described in the examples in the experimental part hereof.



Scheme 1

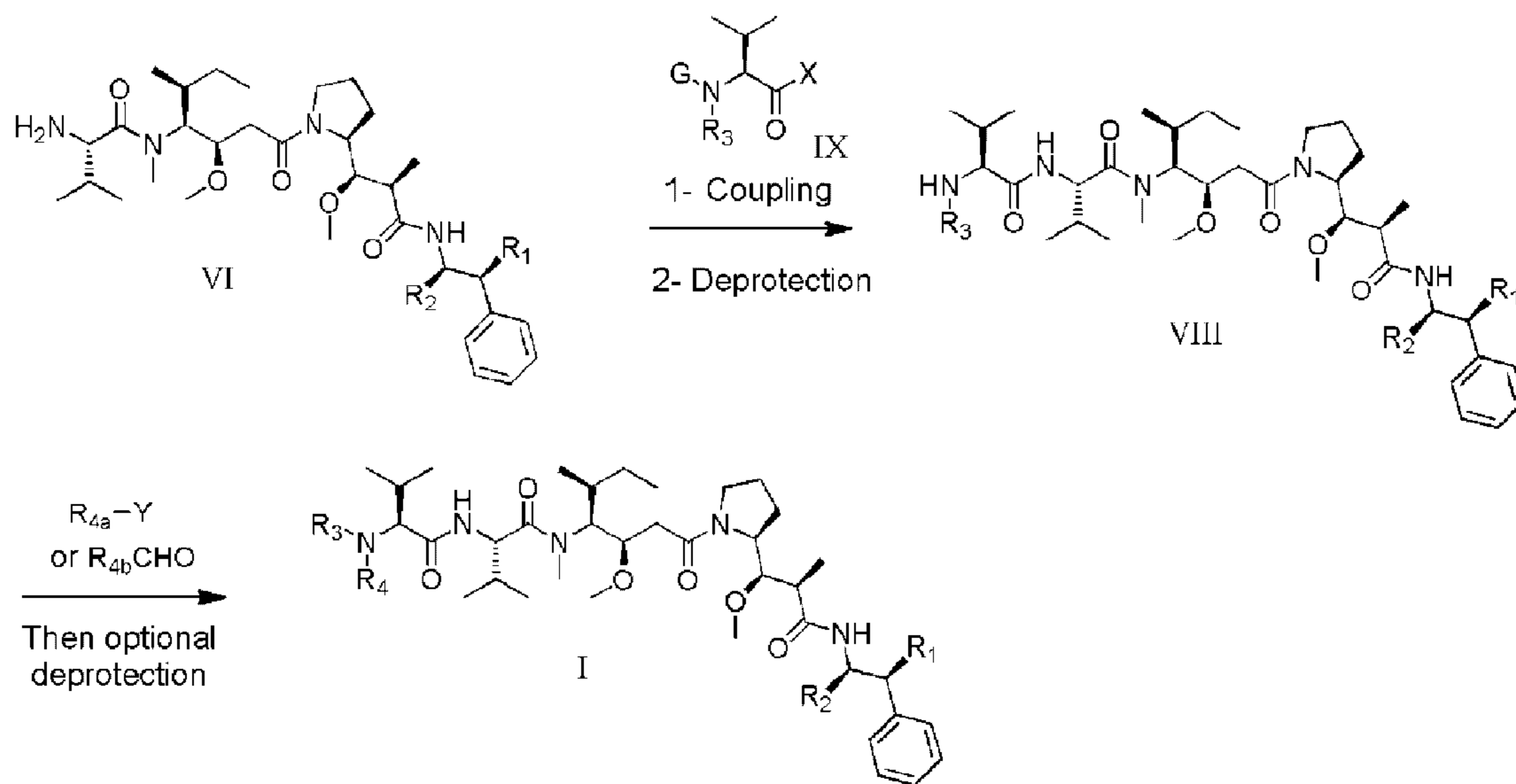
Scheme 1 illustrates the first general method which can be used to prepare the drug. In the above general formulas, R_1 , R_2 and R_3 are such as previously defined, R_4 represents $-AH$, and R_{4a} represents a R_4 group such as previously defined optionally in protected form and G is a protective group.

The first step consists of the condensing of compound (II), protected on its amine function by a protective group G , with compound (III). X may represent a leaving

group such as a chlorine. In this case the first step consists of the reaction between an acid chloride and an amine. This reaction can be conducted using methods and techniques well known to those skilled in the art. In one particularly appreciated method, the two entities are caused to react in the presence of an organic or inorganic base e.g. Et₃N, iPr₂NEt, pyridine, NaH, Cs₂CO₃, K₂CO₃ in a solvent such as THF, dichloromethane, DMF, DMSO, at a temperature notably between -20°C and 100°C. X may also be a hydroxyl (OH). In this case, the first step is a condensation reaction between the carboxylic acid (II) and the amine (III). This reaction can be performed following methods and techniques well known to skilled persons. In one particularly appreciated method, these two entities are caused to react in the presence of a coupling agent such as 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC), 3-hydroxy-1,2,3-benzotriazin-4(3H)-one, a tertiary amine such as diisopropylethylamine, in a polar aprotic solvent such as dichloromethane or DMF, at a temperature notably between -15°C and 40°C. In another particularly appreciated method, these two entities are caused to react in the presence of diethyl phosphorocyanidate (DEPC), a tertiary amine such as triethylamine, in a polar aprotic solvent such as dichloromethane or DMF, at a temperature of between -15°C and 40°C. Another particularly appreciated method consists of causing these two entities to react in the presence of O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl-uroniumhexafluorophosphate (HATU), a tertiary amine such as diisopropylethylamine, in a polar aprotic solvent such as dichloromethane or DMF, at a temperature of between -15°C and 100°C.

After deprotection of the intermediate using techniques well known to those skilled in the art (« Protective Groups in Organic Synthesis », T.W. Greene, John Wiley & Sons, 2006 and « Protecting Groups », P.J. Kocienski, Thieme Verlag, 1994), compound (IV) can be condensed with compound (V) following the methods and techniques described above to lead to compound (VI) after a deprotection step. This compound can then, after condensation with the intermediate (VII) and optional deprotection, lead to the formation of the drug. Compound (VI) can also be coupled with a compound (VII') in which R'₃ is a precursor of R₃, in particular an R₃ group protected by a protective group. Coupling followed by deprotection of group R'₃ to lead to R₃ can be carried out following the same procedures as described previously.

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Scheme 2 illustrates the second general method which can be used to prepare the
 5 drug. In the above general formulas, G is a protective group, R₁, R₂, R₃ and R_{4a} are such
 as previously defined, and R_{4b} represents -A_c-A_aH with A_a as defined previously and A_c
 representing:

- a (C₁-C₇)alkanediyl group,
- a $-(\text{CH}_2\text{CH}_2\text{X}_1)_{a1}(\text{CH}_2\text{CH}_2\text{X}_2)_{a2}(\text{CH}_2\text{CH}_2\text{X}_3)_{a3}(\text{CH}_2\text{CH}_2\text{X}_4)_{a4}\text{CH}_2^*$ group
 10 wherein the CH₂ group marked with an asterisk is linked to the CHO group of
 R_{4b}CHO,
- an aryl or heterocycle group,
- an aryl-(C₁-C₇)alkanediyl or heterocycle-(C₁-C₇)alkanediyl group, said group
 being optionally substituted by a (C₁-C₆)alkyl group, the (C₁-C₇)alkanediyl
 15 moiety being linked to the CHO group of R_{4b}CHO.

At the first step, compound (IX) protected on its amine function by a protective
 group G is condensed with compound (VI). X may represent a leaving group e.g. a
 chlorine. In this case, the first step consists of the reaction between an acid chloride and
 an amine. This reaction can be performed using methods and techniques well known to
 20 persons skilled in the art. In one particularly appreciated method the two entities are
 caused to react in the presence of an organic or inorganic base such as Et₃N, iPr₂NEt,
 pyridine, NaH, Cs₂CO₃, K₂CO₃ in a solvent such as THF, dichloromethane, DMF,

DMSO at a temperature notably between -20° and 100°C . X may also represent a hydroxyl. In this case, the first step is a condensation reaction between the carboxylic acid (IX) and the amine (VI). This reaction can be conducted following methods and techniques well known to skilled persons. In one particularly appreciated method, the two entities are caused to react in the presence of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC), 3-hydroxy-1,2,3-benzotriazin-4(3H)-one, a tertiary amine such as diisopropylethylamine, in a polar aprotic solvent such as dichloromethane or DMF, at a temperature notably between -15°C and 40°C . In another particularly appreciated method, these two entities are caused to react in the presence of diethyl phosphorocyanidate (DEPC), a tertiary amine such as triethylamine, in a polar aprotic solvent such as dichloromethane or DMF, at a temperature notably between -15°C and 40°C .

After deprotection of the intermediate, using techniques well known to skilled persons, the obtained compound (VIII) can lead to the drug after reaction with R_4Y . In this case, Y is a leaving group such as Cl, Br, I, OSO_2CH_3 , OSO_2CF_3 or O-Tosyl. The reaction is conducted in the presence of an organic or inorganic base such as Et_3N , $i\text{Pr}_2\text{NEt}$, NaH, Cs_2CO_3 , K_2CO_3 , in a polar anhydrous solvent such as dichloromethane, THF, DMF, DMSO at a temperature notably between -20° and 100°C . In another particularly appreciated method, compound (VIII) is caused to react with an aldehyde of formula $\text{R}_{4b}\text{-CHO}$ where R_{4b} corresponds to a precursor of R_4 . In this case, the reaction is a reductive amination in the presence of a reducing agent such as NaBH_4 , NaBH_3CN , $\text{NaBH}(\text{OAc})_3$, in a polar solvent such as 1,2-dichloroethane, dichloromethane, THF, DMF, MeOH, in the optional presence of titanium isopropoxide (IV), at a pH which can be controlled by the addition of an acid such as acetic acid at a temperature notably between -20°C and 100°C .

In the foregoing synthesis schemes, a drug may lead to another drug after an additional reaction step such as saponification for example using methods well known to skilled persons whereby an R_2 group representing an ester (COOMe), is changed to an R_2 group representing a carboxylic acid (COOH).

If it is desired to isolate a drug containing at least one base function in the state of an acid addition salt, this is possible by treating the free base of the drug (containing

at least one base function) with a suitable acid, preferably in equivalent quantity. The suitable acid may in particular be trifluoroacetic acid.

III – The linker (L)

5

"Linker", "Linker Unit", "L" or "link" means, in the present invention, a chemical moiety comprising a covalent bond or a chain of atoms that covalently attaches an antibody to at least one drug.

Linkers may be made using a variety of bifunctional protein coupling agents
10 such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-
15 (p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of cytotoxic agents to the addressing system. Other cross-linker reagents may be
20 BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, Ill., U.S.A).

25 The linker may be a "non cleavable" or "cleavable".

In a preferred embodiment, it consists in a "cleavable linker" facilitating release of the drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker may be used. The linker is, in a preferred embodiment, cleavable under intracellular conditions, such that
30 cleavage of the linker releases the drug from the antibody in the intracellular environment.

For example, in some embodiments, the linker is cleavable by a cleaving agent that is present in the intracellular environment (e.g., within a lysosome or endosome or caveolea). The linker can be, for example, a peptidyl linker that is cleaved by an intracellular peptidase or protease enzyme, including, but not limited to, a lysosomal or endosomal protease. Typically, the peptidyl linker comprises at least two successive amino acids or at least three successive amino acids or is at least two amino acids long or at least three amino acids long. Cleaving agents can include cathepsins B and D and plasmin, all of which are known to hydrolyze dipeptide drug derivatives resulting in the release of active drug inside target cells. For example, a peptidyl linker that is cleavable by the thiol-dependent protease cathepsin-B, which is highly expressed in cancerous tissue, can be used (e.g., a linker comprising or being Phe-Leu or Gly-Phe-Leu-Gly). In specific embodiments, the peptidyl linker cleavable by an intracellular protease comprises or is Val-Cit or Phe-Lys. One advantage of using intracellular proteolytic release of the drug is that the drug is typically attenuated when conjugated and the serum stabilities of the conjugates are typically high.

In other embodiments, the cleavable linker is pH-sensitive, i.e., sensitive to hydrolysis at certain pH values. Typically, the pH-sensitive linker is hydrolyzable under acidic conditions. For example, an acid-labile linker that is hydrolyzable in the lysosome (e.g., a hydrazone, semicarbazone, thiosemicarbazone, cis-aconitic amide, orthoester, acetal, ketal, or the like) can be used. Such linkers are relatively stable under neutral pH conditions, such as those in the blood, but are unstable at below pH 5.5 or 5.0, the approximate pH of the lysosome. In certain embodiments, the hydrolyzable linker is a thioether linker (such as, e.g., a thioether attached to the drug via an acylhydrazone bond).

In yet other embodiments, the linker is cleavable under reducing conditions (e.g., a disulfide linker). A variety of disulfide linkers are known in the art, including, for example, those that can be formed using SATA (N-succinimidyl-S-acetylthioacetate), SPDP (N-succinimidyl-3-(2-pyridyldithio)propionate), SPDB (N-succinimidyl-3-(2-pyridyldithio)butyrate) and SMPT (N-succinimidyl-oxycarbonyl-alpha-methyl-alpha-(2-pyridyl-dithio)toluene).

In certain preferred embodiments, the linker unit may have the following general formula:



wherein:

T is a stretcher unit;

a is 0 or 1;

5 W is an amino acid unit;

w is an integer ranging from 0 to 12;

Y is a spacer unit;

y is 0, 1 or 2.

10 The stretcher unit (T), when present, links the antibody to an amino acid unit (W) when present, or to the spacer unit when present, or directly to the drug. Useful functional groups that can be present on the antibody, either naturally or via chemical manipulation, include sulfhydryl, amino, hydroxyl, the anomeric hydroxyl group of a carbohydrate, and carboxyl. Suitable functional groups are sulfhydryl and amino.

15 Sulfhydryl groups can be generated by reduction of the intramolecular disulfide bonds of the antibody, if present. Alternatively, sulfhydryl groups can be generated by reaction of an amino group of a lysine moiety of the antibody with 2-iminothiolane or other sulfhydryl generating reagents. In specific embodiments, the antibody is engineered to carry one or more lysines. More preferably, the antibody can be engineered to carry one

20 or more Cysteines (cf. ThioMabs).

In certain specific embodiments, the stretcher unit forms a bond with a sulfur atom of the antibody. The sulfur atom can be derived from a sulfhydryl (-SH) group of a reduced antibody.

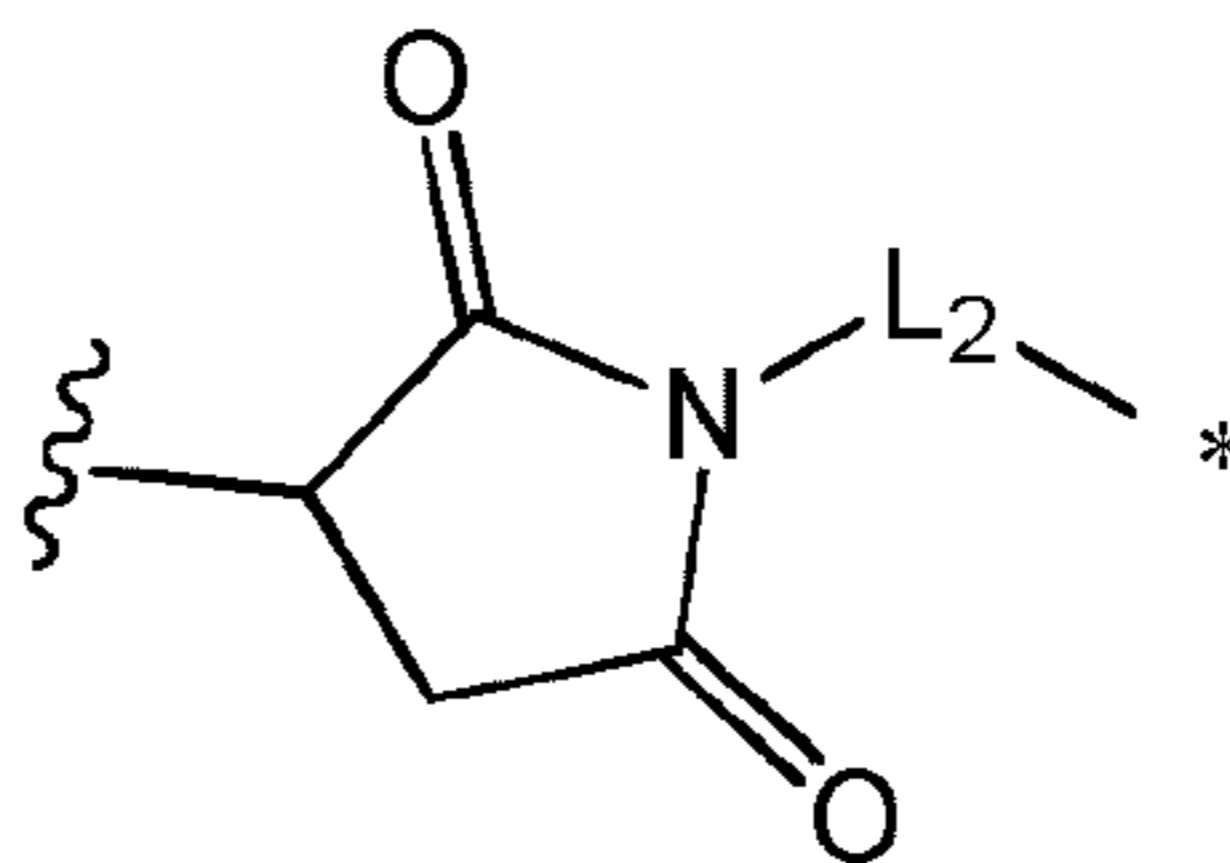
25 In certain other specific embodiments, the stretcher unit is linked to the antibody via a disulfide bond between a sulfur atom of the antibody and a sulfur atom of the stretcher unit.

In other specific embodiments, the reactive group of the stretcher contains a reactive site that can be reactive to an amino group of the antibody. The amino group can be that of an arginine or a lysine. Suitable amine reactive sites include, but are not

30 limited to, activated esters (such as succinimide esters, 4-nitrophenyl esters, pentafluorophenyl esters), anhydrides, acid chlorides, sulfonyl chlorides, isocyanates and isothiocyanates.

In yet another aspect, the reactive function of the stretcher contains a reactive site that is reactive to a modified carbohydrate group that can be present on the antibody. In a specific embodiment, the antibody is glycosylated enzymatically to provide a carbohydrate moiety or is naturally glycosylated. The carbohydrate may be mildly oxidized with a reagent such as sodium periodate and the resulting carbonyl unit of the oxidized carbohydrate can be condensed with a stretcher that contains a functionality such as a hydrazide, an oxime, a reactive amine, a hydrazine, a thiosemicarbazide, a hydrazine carboxylate, or an arylhydrazide.

According to a particular embodiment, the stretcher unit has the following formula:

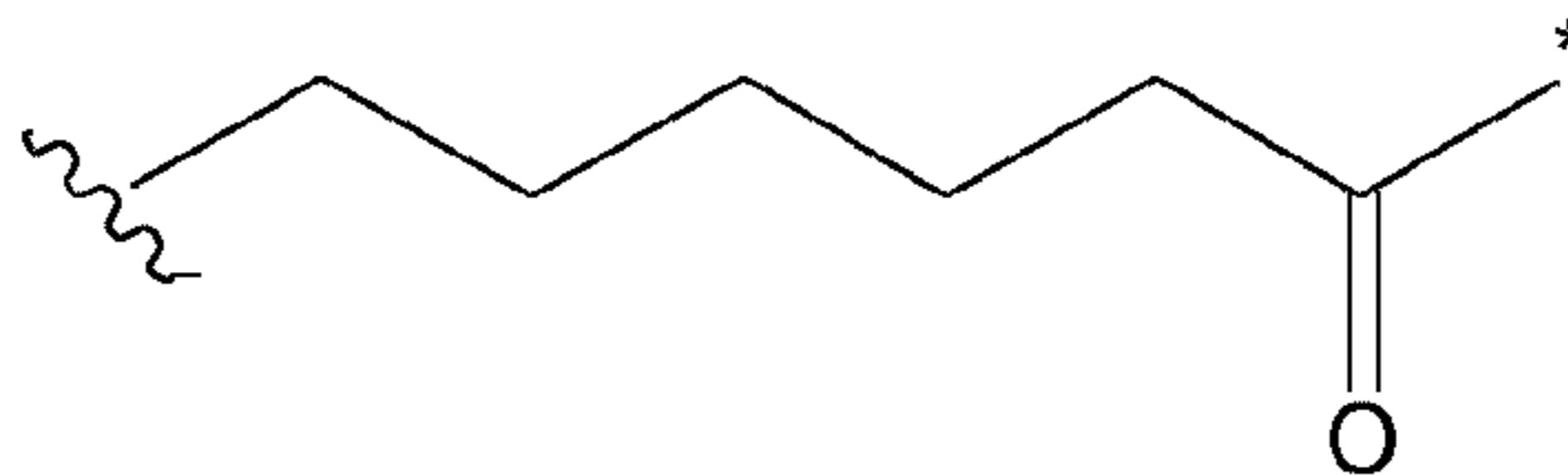


wherein

L_2 is (C_4-C_{10}) cycloalkyl-carbonyl, (C_2-C_6) alkyl or (C_2-C_6) alkyl-carbonyl (the cycloalkyl or alkyl moieties being linked to the nitrogen atom of the maleimide moiety), the asterisk indicates the point of attachment to the amino acid unit, if present, to the spacer unit, if present, or to the drug **D**, and the wavy line indicates the point of attachment to the antibody **Ab**.

By “ (C_4-C_{10}) cycloalkyl” in the present invention is meant a hydrocarbon cycle having 4 to 10 carbon atoms including, but not limited to, cyclopentyl, cyclohexyl and the like.

L_2 can be advantageously (C_2-C_6) alkyl-carbonyl such as a pentyl-carbonyl of the following formula:



wherein

the asterisk indicates the point of attachment to the amino acid unit, if present, to the spacer unit, if present, or to the drug **D**; and
the wavy line indicates the point of attachment to the nitrogen atom of the maleimide moiety.

5 The amino acid unit (W), when present, links the stretcher unit (T) if present, or otherwise the antibody to the spacer unit (Y) if the spacer unit is present, or to the drug if the spacer unit is absent.

As above mentioned, (W)_w is absent (w = 0) or may be a dipeptide, tripeptide, tetrapeptide, pentapeptide, hexapeptide, heptapeptide, octapeptide, nonapeptide,
10 decapeptide, undecapeptide or dodecapeptide unit, wherein the amino acids forming the peptides can be different from one another.

Thus (W)_w can be represented by the following formula:
(W1)_{w1}(W2)_{w2}(W3)_{w3}(W4)_{w4}(W5)_{w5}, wherein each W1 to W5 represents, independently
from one another, an amino acid unit and each w1 to w5 is 0 or 1.

15 In some embodiments, the amino acid unit (W)_w may comprise amino acid residues such as those occurring naturally, as well as minor amino acids and non-naturally occurring amino acid analogs, such as citrulline.

The amino acid residues of the amino acid unit (W)_w include, without limitation, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline,
20 lysine protected or not with acetyl or formyl, arginine, arginine protected or not with tosyl or nitro groups, histidine, ornithine, ornithine protected with acetyl or formyl, and citrulline. Exemplary amino acid linker components include preferably a dipeptide, a tripeptide, a tetrapeptide or a pentapeptide, notably a dipeptide or a tripeptide.

Exemplary dipeptides include: Val-Cit, Ala-Val, Ala-Ala, Val-Ala, Lys-Lys,
25 Cit-Cit, Val-Lys, Ala-Phe, Phe-Lys, Ala-Lys, Phe-Cit, Leu-Cit, Ile-Cit, Trp-Cit, Phe-Ala, Phe-N⁹-tosyl-Arg, Phe-N⁹-Nitro-Arg.

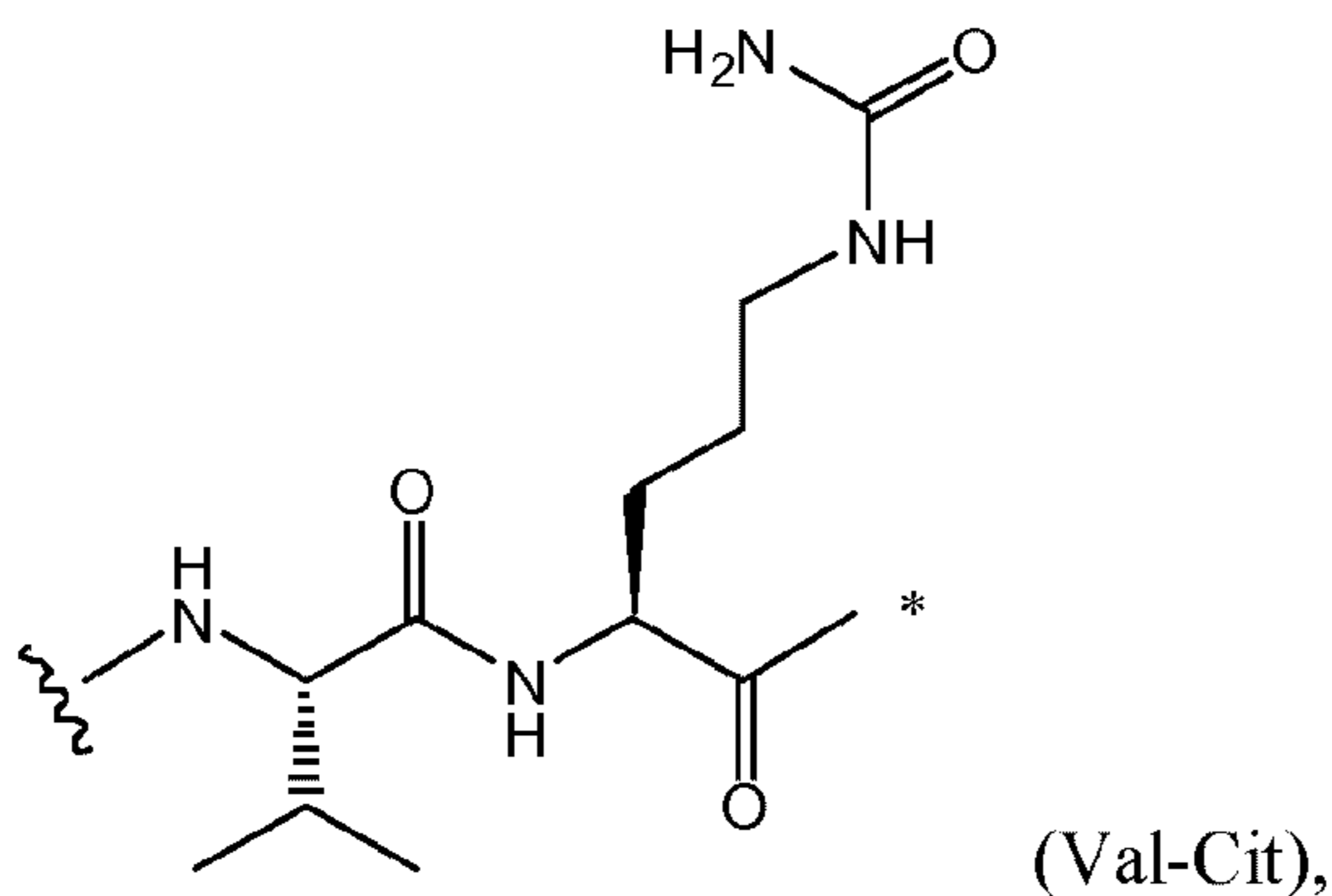
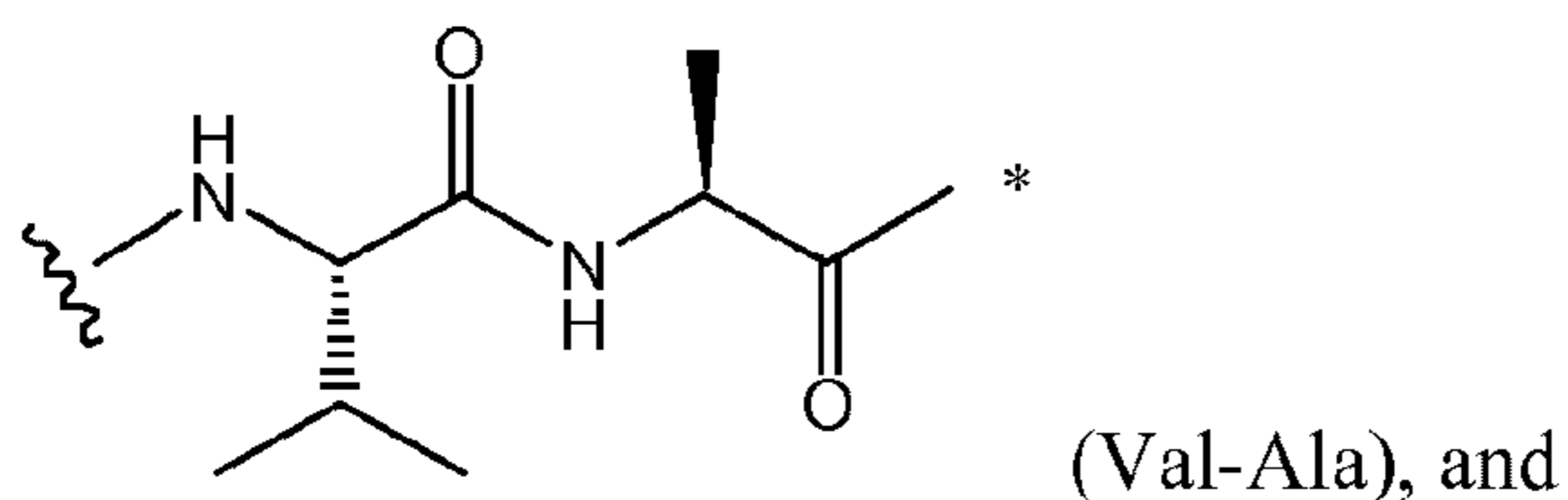
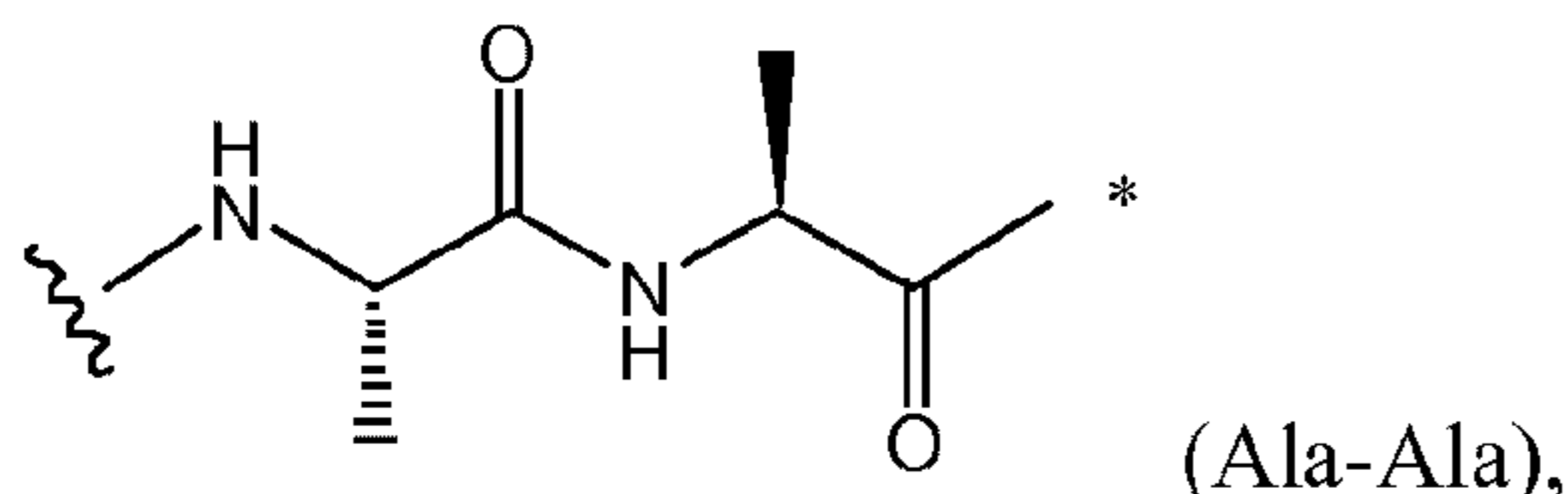
Exemplary tripeptides include: Val-Ala-Val, Ala-Asn-Val, Val-Leu-Lys, Ala-Ala-Asn, Phe-Phe-Lys, Gly-Gly-Gly, D-Phe-Phe-Lys, Gly-Phe-Lys.

Exemplary tetrapeptide include: Gly-Phe-Leu-Gly (SEQ ID NO. 53), Ala-Leu-
30 Ala-Leu (SEQ ID NO. 54).

Exemplary pentapeptide include: Pro-Val-Gly-Val-Val (SEQ ID NO. 55).

According to a particular embodiment, $(W)_w$ can be a dipeptide (i.e. $w = 2$) such as Val-Cit, or the linker lacks an amino acid unit ($w=0$). When the linker lacks an amino acid unit, preferably it lacks also a spacer unit.

According to a preferred embodiment, $w = 0$ (i.e. $(W)_w$ is a single bond) or $w =$
5 2 (i.e. $(W)_w$ is a dipeptide) and $(W)_w$ can thus be selected from:



and in particular is Val-Cit,

10 wherein

the asterisk indicates the point of attachment to the spacer unit if present, or to the drug **D**; and

the wavy line indicates the point of attachment to L_2 .

Amino acid linker components can be designed and optimized in their selectivity
15 for enzymatic cleavage by a particular enzyme, for example, a tumor-associated protease, cathepsin B, C and D, or a plasmin protease.

The amino acid unit of the linker can be enzymatically cleaved by an enzyme including, but not limited to, a tumor-associated protease to liberate the drug.

The amino acid unit can be designed and optimized in its selectivity for
20 enzymatic cleavage by a particular tumor-associated protease. The suitable units are those whose cleavage is catalyzed by the proteases, cathepsin B, C and D, and plasmin.

The spacer unit (Y), when present, links an amino acid unit if present, or the stretcher unit if present, or otherwise the antibody to the drug. Spacer units are of two general types: self-immolative and non self-immolative. A non self-immolative spacer unit is one in which part or all of the spacer unit remains bound to the drug after enzymatic cleavage of an amino acid unit from the antibody-drug conjugate. Examples of a non self-immolative spacer unit include, but are not limited to a (glycine-glycine) spacer unit and a glycine spacer unit. To liberate the drug, an independent hydrolysis reaction should take place within the target cell to cleave the glycine-drug unit bond.

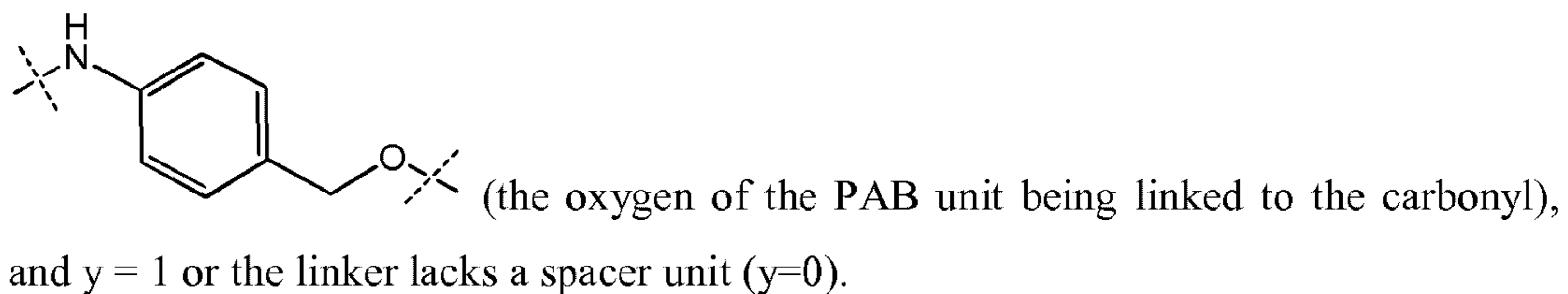
In a particular embodiment, a non self-immolative the spacer unit (Y) is Gly.

Alternatively, an antibody-drug conjugate containing a self-immolative spacer unit can release the drug without the need for a separate hydrolysis step. In these embodiments, (Y) is a residue of p-aminobenzyl alcohol (PAB) unit that is linked to (W)_w via the nitrogen atom of the PAB group, and connected directly to the drug via an ester, carbonate, carbamate or ether group.

Other examples of self-immolative spacers include, but are not limited to, aromatic compounds that are electronically equivalent to the PAB group such as residues of 2-aminoimidazol-5-methanol derivatives and ortho or para-aminobenzylacetals. Spacers can be used that undergo facile cyclization upon amide bond hydrolysis, such as substituted and unsubstituted 4-aminobutyric acid amides, appropriately substituted bicyclo[2.2.1] and bicyclo[2.2.2] ring systems and 2-aminophenylpropionic acid amides.

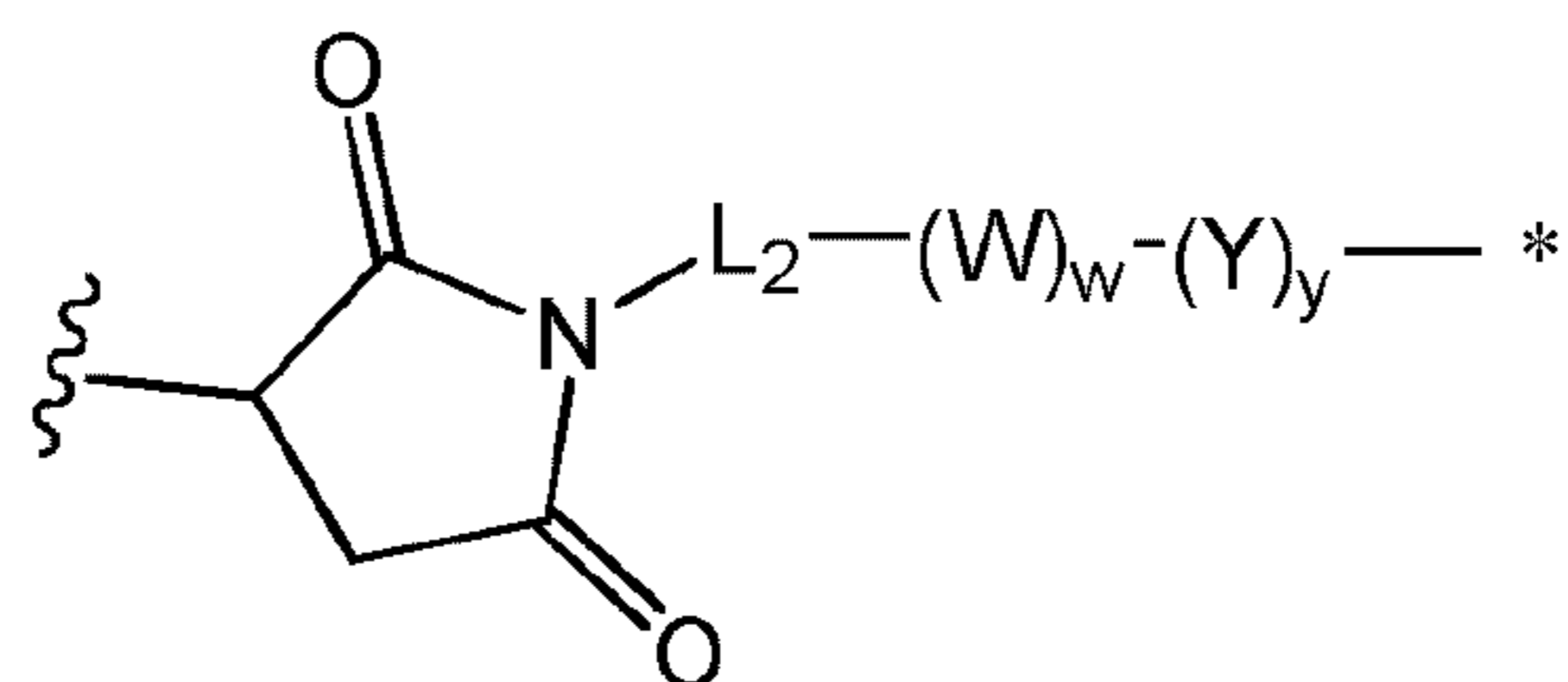
In an alternate embodiment, the spacer unit is a branched bis(hydroxymethyl)styrene (BHMS) unit, which can be used to incorporate additional drugs.

In a particular embodiment, the spacer unit (Y) is PAB-carbonyl with PAB being



In a particular embodiment, the linker has the following formula (III):

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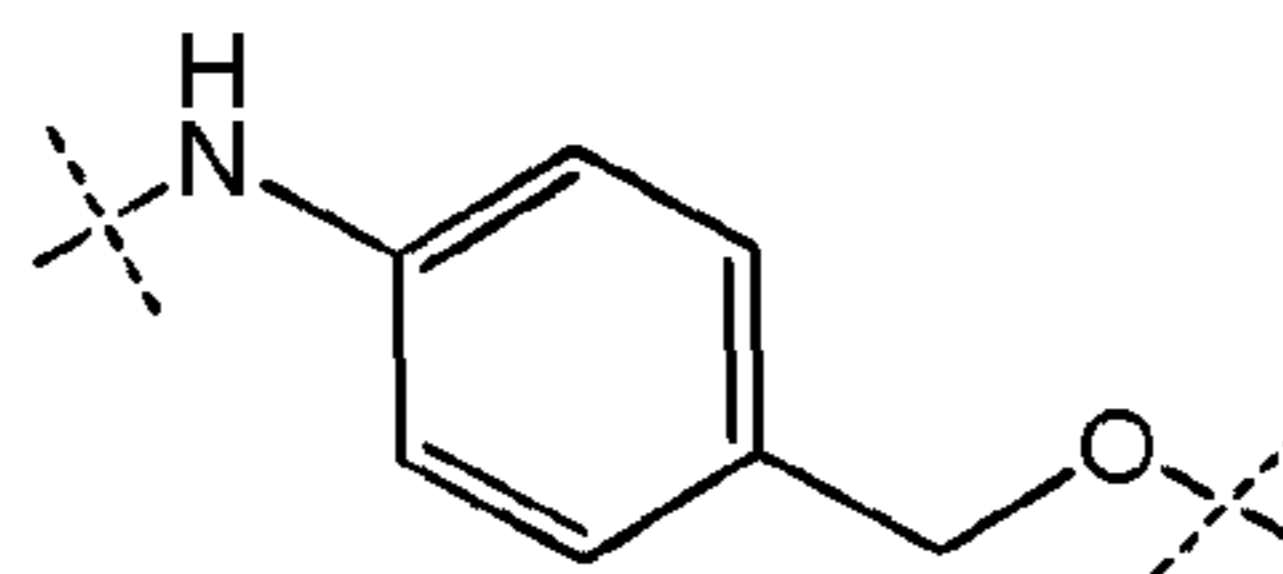


(III)

wherein

L_2 is (C₄-C₁₀)cycloalkyl-carbonyl, (C₂-C₆)alkyl or (C₂-C₆)alkyl-carbonyl (the
5 carbonyl of these moieties, when present, being linked to (W)_w),

W represents an amino acid unit, with w representing an integer comprised
between 0 and 5,

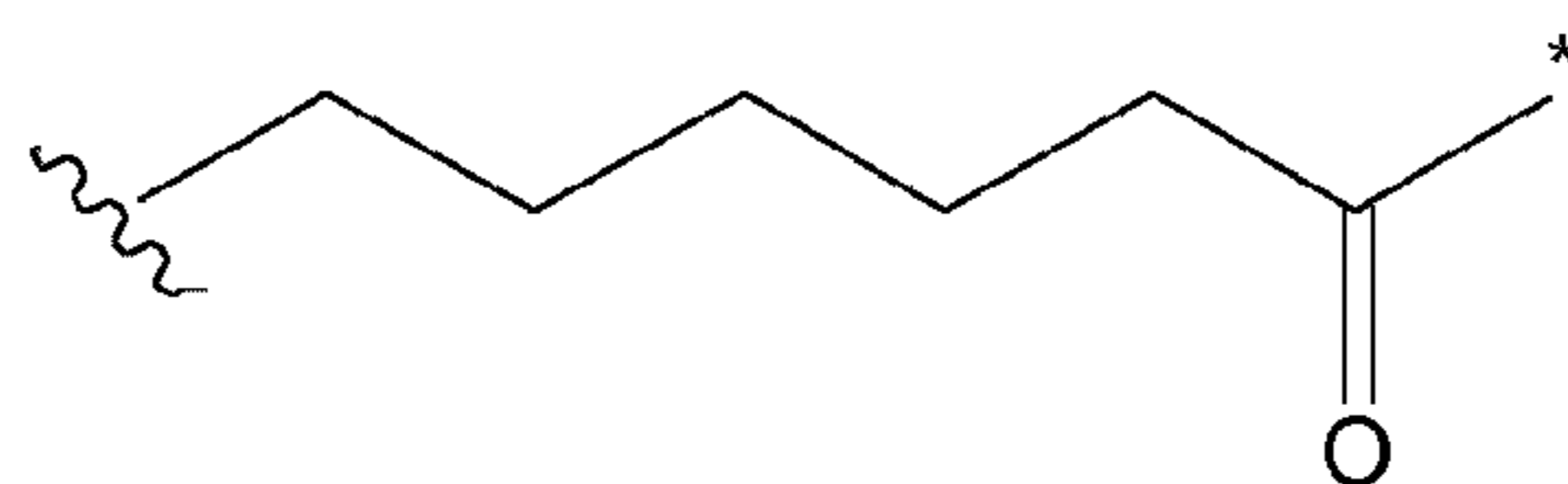


Y is PAB-carbonyl, with PAB being
the PAB unit being linked to the carbonyl), and y is 0 or 1 (preferably y is 0 when w is 0
10 and y is 0 or 1 when w is comprised between 1 and 5),

the asterisk indicates the point of attachment to the drug **D**, and

the wavy line indicates the point of attachment to the antibody **Ab**.

Advantageously, L_2 is (C₂-C₆)alkyl-carbonyl such as a pentyl-carbonyl of the
following formula:

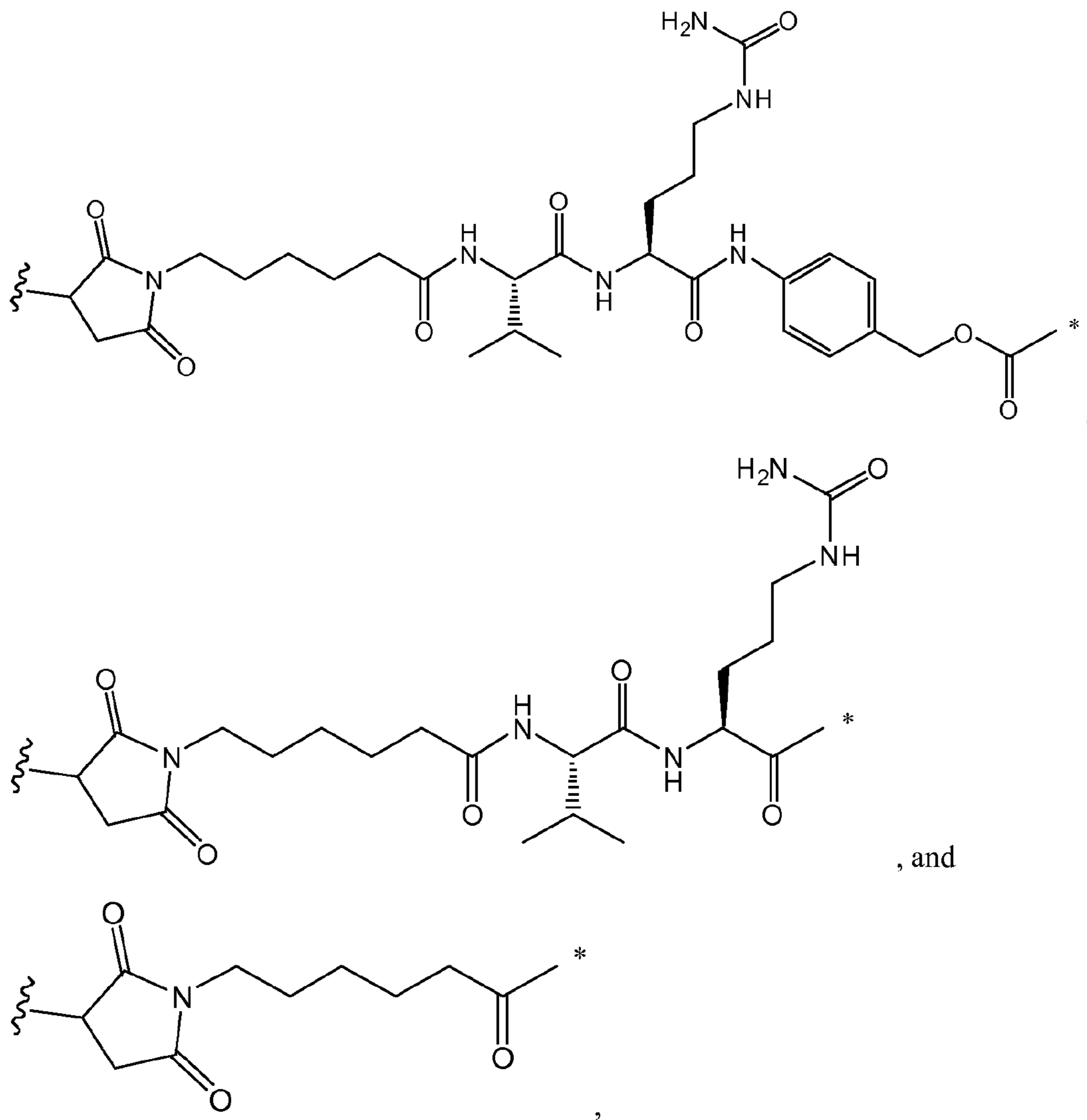


15 wherein

the asterisk indicates the point of attachment to (W)_w; and

the wavy line indicates the point of attachment to the nitrogen atom of the
maleimide moiety.

20 According to a preferred embodiment, the linker **L** is selected from:



wherein the asterisk indicates the point of attachment to the drug **D**, and the wavy line indicates the point of attachment to the antibody **Ab**.

IV – The Antibody-Drug-Conjugate (ADC)

5

In a preferred embodiment, the antibody-drug conjugate of the invention may be prepared by any method known by the person skilled in the art such as, without limitation, i) reaction of a nucleophilic group of the antibody with a bivalent linker reagent followed by reaction with a nucleophilic group of the drug or ii) reaction of a nucleophilic group of the drug with a bivalent linker reagent followed by reaction with a nucleophilic group of the antibody.

10

Nucleophilic groups on antibody include, without limitation, N-terminal amine groups, side chain amine groups (e.g. lysine), side chain thiol groups, and sugar hydroxyl or amino groups when the antibody is glycosylated.

5 Nucleophilic groups on the drug include, without limitation, amine, thiol, and hydroxyl groups, and preferably amine groups.

Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including, without limitation, active esters such as NHS esters, HOBt esters, 10 haloformates, and acid halides; alkyl and benzyl halides such as haloacetamides; aldehydes; ketones; carboxyl; and maleimide groups. The antibody may have reducible interchain disulfides, i.e. cysteine bridges. The antibody may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (dithiothreitol). Each cysteine bridge will thus form, theoretically, two reactive thiol 15 nucleophiles. Additional nucleophilic groups can be introduced into the antibody through any reaction known by the person skilled in the art. As non limitative example, reactive thiol groups may be introduced into the antibody by introducing one or more cysteine residues.

Antibody-drug conjugates may also be produced by modification of the antibody 20 to introduce electrophilic moieties, which can react with nucleophilic substituents on the linker reagent. The sugars of glycosylated antibody may be oxidized to form aldehyde or ketone groups which may react with the amine group of linker reagents or drug. The resulting imine Schiff base groups may form a stable linkage, or may be reduced to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of 25 a glycosylated antibody with either galactose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the protein that can react with appropriate groups on the drug. In another embodiment, proteins containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino acid.

30 In a preferred embodiment, the antibody-drug conjugate of the invention is prepared by preparation of the drug-linker moiety followed by coupling between a

nucleophilic group of the antibody (for ex. the SH group of a cysteine moiety) and an electrophilic group of the drug-linker moiety (for ex. a maleimide).

1. Drug-Linker

5 The Drug-Linker moiety can be prepared by coupling:

- the linker with the drug,
- a part of the linker with the drug before completing the synthesis of the linker,
- the linker with a part or a precursor of the drug before completing the synthesis of the drug, or
- 10 – a part of the linker with a part or a precursor of the drug before completing the synthesis of the linker and the drug.

The coupling reactions are well known reactions for the one skilled in the art between a nucleophilic group and an electrophilic group.

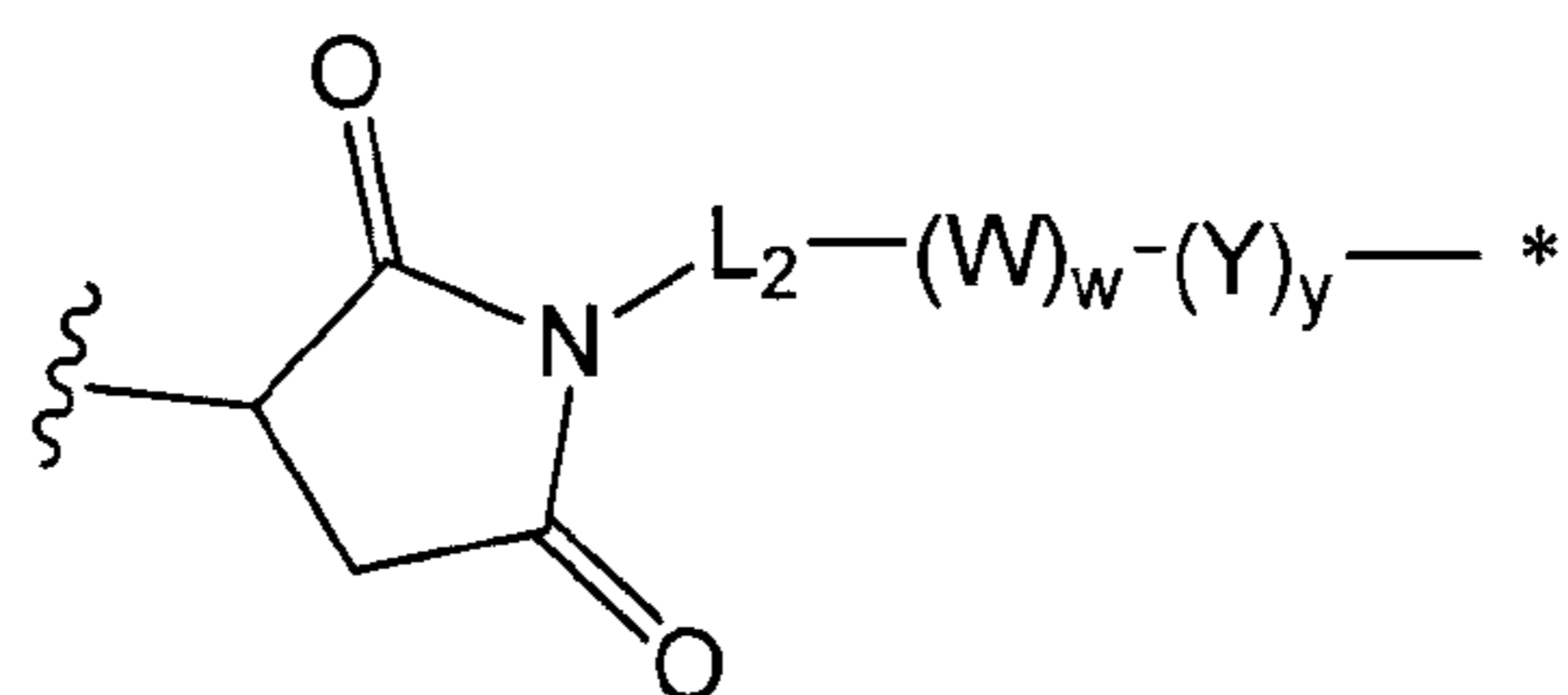
15 The nucleophilic group can be in particular an amine, thiol or hydroxyl group, and notably an amine or hydroxyl group. In a preferred embodiment it is a primary or secondary amine group.

The electrophilic group can be a carboxylic acid group (COOH) optionally in an activated form or an activated carbonate ester moiety.

20 By “activated form” of a carboxylic acid is meant a carboxylic acid in which the OH moiety of the COOH function has been replaced with an activated leaving group (LG) enabling coupling of the activated carboxylic acid group with an amino group in order to form an amide bond and release the compound LG-H. Activated forms may be activated esters, activated amides, anhydrides or acyl halides such as acyl chlorides. Activated esters include derivatives formed by reaction of the carboxylic acid group
25 with N-hydroxybenzotriazole or N-hydroxysuccinimide.

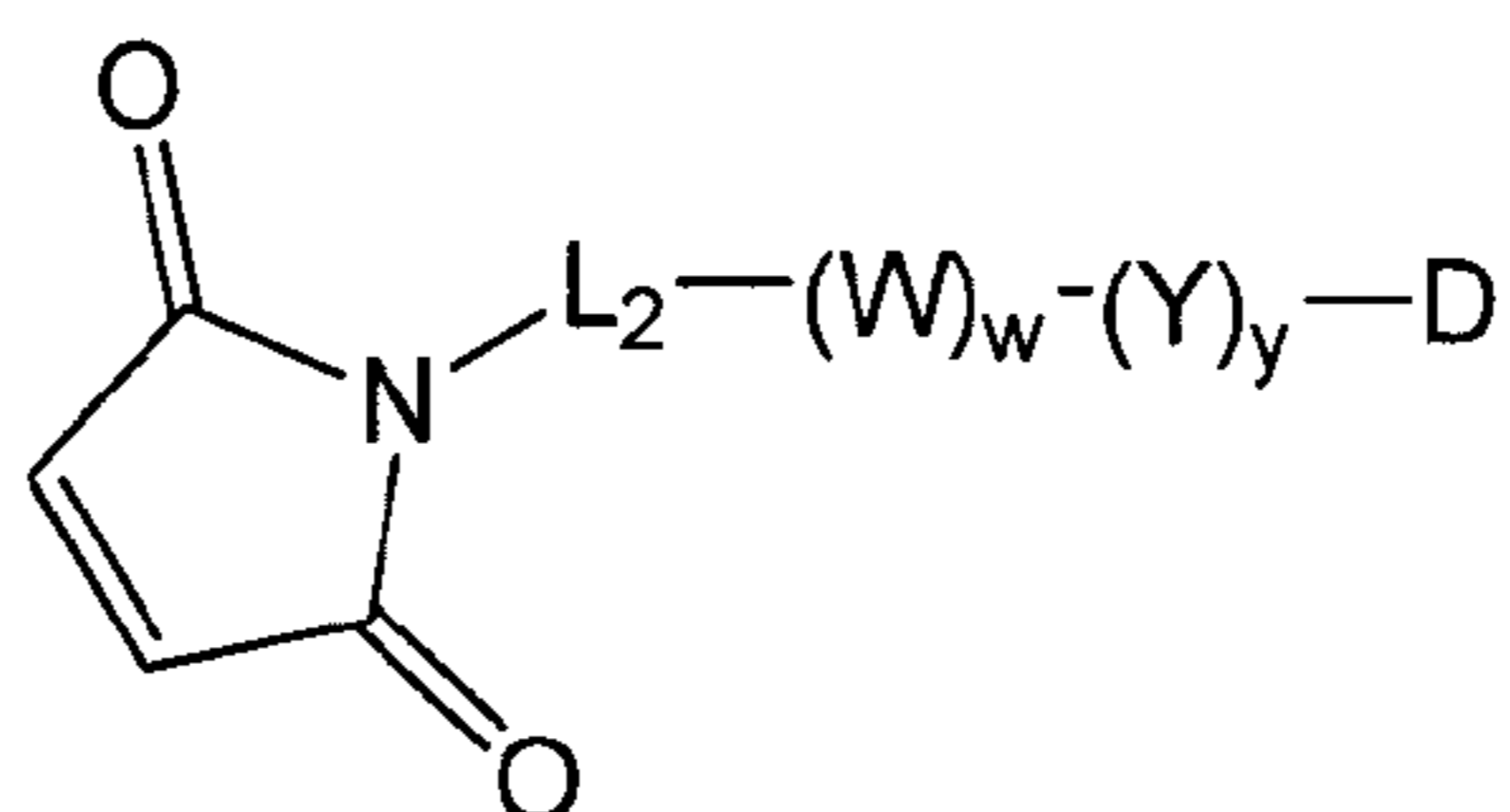
By “activated carbonate ester” is meant a carbonate ester comprising a -OC(O)OR moiety in which OR represents a good leaving group enabling coupling of the activated carbonate ester with an amino group in order to form a carbamate moiety and release the compound ROH. The R group of the activated carbonate ester includes,
30 without limitation, the p-nitro-phenyl, pentafluorophenyl, 2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl and benzyl groups, preferably the p-nitro-phenyl and pentafluorophenyl groups.

When the linker has the following formula (III):



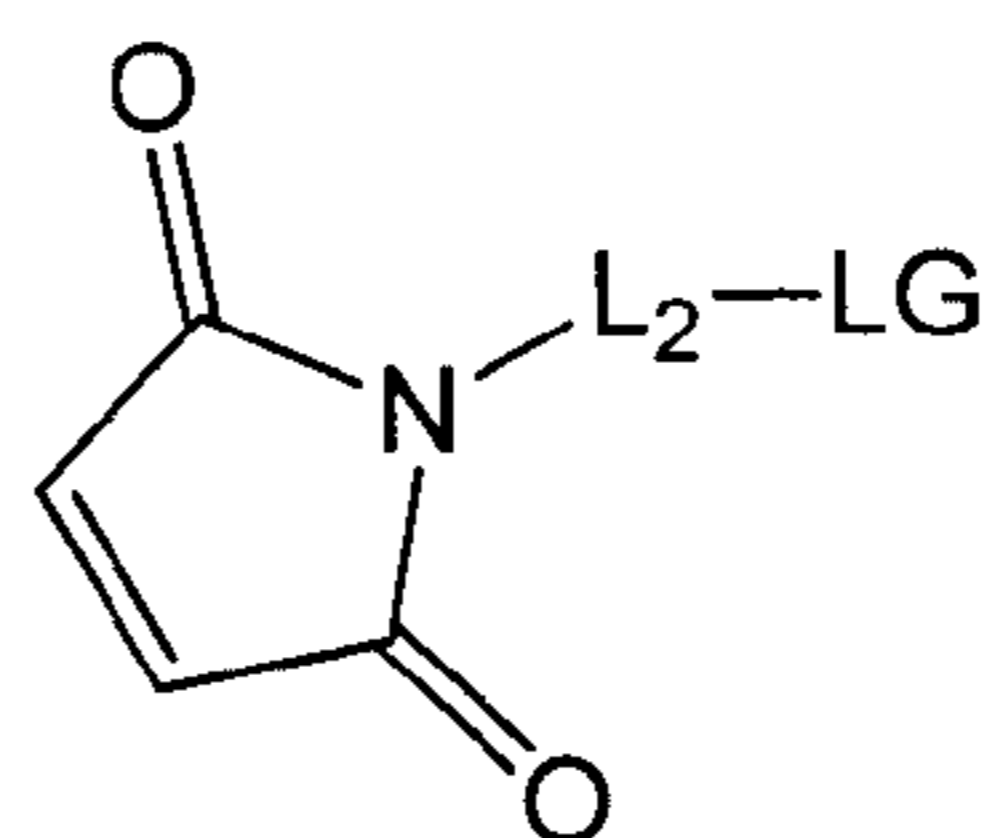
(III)

the Drug-Linker moiety has the following formula (IV):



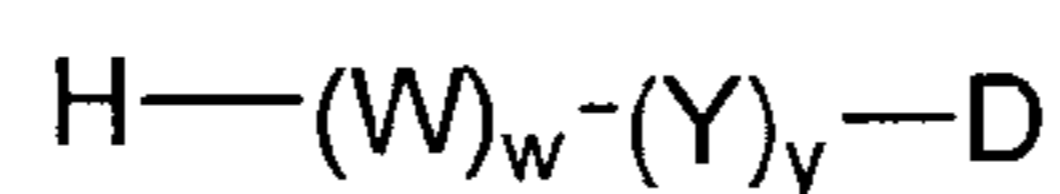
(IV)

and the last step of the synthesis of the Drug-Linker moiety is generally the coupling between a compound of the following formula (V):



(V)

- 5 where L_2 is as defined previously and LG represents a leaving group notably a halide such as a chloride or a group derived from N-hydroxysuccinimide, and a compound of the following formula (VI):



(VI).

- 10 When $y = 1$ and $Y = \text{PAB-carbonyl}$, the compound of formula (VI) can be prepared by the coupling between the drug (DH) and a compound of the following formula (VII) or a protected form thereof:



(VII)

where W and w are as defined previously and R is as defined in the definition of the “activated carbonate ester”.

When the compound of formula (VII) is in a protected form, final step of deprotection is necessary.

5 When $y = 0$, the compound (VI) has the formula $H-(W)_w-D$, wherein $(W)_w$ and D are composed of amino acid units. Consequently, the compound (VI) can be prepared in this case by a conventional peptide synthesis method well known to the one skilled in the art.

10 2. Ab-Linker-Drug

A preferred embodiment according to the invention consists of a coupling between a cysteine present on the antibody and an electrophilic group of the Drug-Linker moiety, preferably with a maleimide moiety present on the Drug-Linker moiety.

15 The maleimide-cysteine coupling can be performed by methods well known to the person skilled in the art.

Generally, antibodies do not contain many, if any, free and reactive cysteine thiol groups which can be linked to a drug moiety. Most cysteine thiol residues in antibodies exist as disulfide bridges and must be reduced with a reducing agent such as dithiothreitol (DTT) or TCEP, under partial or total reducing conditions. The loading
20 (drug/antibody ratio) of an ADC may be controlled in several different manners, including: (i) limiting the molar excess of drug-linker intermediate (D-L) or linker reagent relative to antibody, (ii) limiting the conjugation reaction time or temperature, and (iii) partial or limited reducing conditions for cysteine thiol modification.

The disulfide bond structure of human IgGs is now well established (reviewed in
25 Liu and May, mAbs 4 (2012): 17-23). There are in fact many similarities and some differences with regard to the disulfide bond structures of the 4 human IgG subclasses, namely IgG1, IgG2, IgG3 and IgG4. All IgG subclasses contain invariably 12 intra-chain disulfide bridges and the differences reside in their inter-chain disulfide bonds formed between heavy and light chains. Each intra-chain disulfide bond is associated
30 with an individual IgG domain, i.e. variable (VL and VH) and constant (CL, CH1, CH2 and CH3) domains. The 2 heavy chains are linked in their hinge region by a variable number of disulfide bridges: 2 for IgG1 and IgG4, 4 for IgG2 and 11 for IgG3. The

heavy and light chains of the IgG1 are connected by a disulfide bond between the last cysteine residue of the light chain and the fifth residue of the heavy chain, whereas for the other subclasses, IgG2, IgG3 and IgG4, the light chain is linked to the heavy chain by a disulfide bond between the last cysteine residue of the light chain and the third
5 cysteine residue of the heavy chain, which is located at the interface of VH and CH1 domains. Disulfide bond structures other than these classical structures have been described for IgG2 and IgG4 (reviewed in Liu and May, *mAbs* 4 (2012): 17-23). Inter-chain disulfide bonds are highly solvent exposed and are consequently much more reactive than the intra-chain disulfide bonds, which are buried in anti-parallel beta-sheet
10 structures within each domain and are not solvent exposed. For these reasons, whatever the antibody isotype, coupling will take place on inter-chain exposed cysteine residues after mild reduction. Each inter-chain disulfide bridge can thus form, theoretically, two sites of conjugation.

Additional nucleophilic groups can be introduced into antibodies through the
15 reaction of lysines with 2-iminothiolane (Traut's reagent) resulting in the conversion of an amine into a thiol. Reactive thiol groups may also be introduced into the antibody (or fragment thereof) by engineering one, two, three, four, or more cysteine residues (e.g., preparing mutant antibodies comprising one or more non-native cysteine amino acid residues). US 7521541 teaches engineering antibodies by introduction of reactive
20 cysteine amino acids.

Cysteine amino acids may be engineered at reactive sites in an antibody and which do not form intrachain or intermolecular disulfide linkages (Junutula, et al., 2008b *Nature Biotech.*, 26(8):925-932; Dornan et al (2009) *Blood* 114(13):2721-2729; US 7521541; US 7723485; WO2009/052249). The engineered cysteine thiols may
25 react with linker reagents or the drug-linker reagents of the present invention which have thiol-reactive, electrophilic groups such as maleimide or alpha-halo amides to form ADC with cysteine engineered antibodies and the drug moieties. The location of the drug moiety can thus be designed, controlled, and known. The drug loading can be controlled since the engineered cysteine thiol groups typically react with thiol-reactive
30 linker reagents or drug-linker reagents in high yield. Engineering an IgG antibody to introduce a cysteine amino acid by substitution at a single site on the heavy or light

chain gives two new cysteines on the symmetrical antibody. A drug loading near 2 can be achieved with near homogeneity of the conjugation product ADC.

Where more than one nucleophilic or electrophilic group of the antibody reacts with a drug-linker intermediate, or linker reagent followed by drug moiety reagent, then
5 the resulting product is a mixture of ADC compounds with a distribution of drug moieties attached to an antibody, e.g. 1, 2, 3, etc. Liquid chromatography methods such as polymeric reverse phase (PLRP) and hydrophobic interaction (HIC) may separate compounds in the mixture by drug loading value. Preparations of ADC with a single drug loading value (p) may be isolated, however, these single loading value ADCs may
10 still be heterogeneous mixtures because the drug moieties may be attached, via the linker, at different sites on the antibody.

For some antibody-drug conjugates, drug ratio may be limited by the number of attachment sites on the antibody. High drug loading, e.g. drug ratio >5 , may cause aggregation, insolubility, toxicity, or loss of cellular permeability of certain antibody-
15 drug conjugates. Typically, less drug moieties than the theoretical maximum are conjugated to an antibody during a conjugation reaction.

The drug loading also referred as the Drug-Antibody ratio (DAR) is the average number of drugs per cell binding agent.

In the case of antibody IgG1 and IgG4 isotypes, where the drugs are bound to cysteines after partial antibody reduction, drug loading may range from 1 to 8 drugs (D)
20 per antibody, i.e. where 1, 2, 3, 4, 5, 6, 7, and 8 drug moieties are covalently attached to the antibody.

In the case of an antibody IgG2 isotype, where the drugs are bound to cysteines after partial antibody reduction, drug loading may range from 1 to 12 drugs (D) per
25 antibody, i.e. where 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 drug moieties are covalently attached to the antibody.

Compositions of ADC include collections of cell binding agents, e.g. antibodies, conjugated with a range of drugs, from 1 to 8 or 1 to 12.

The average number of drugs per antibody in preparations of ADC from conjugation reactions may be characterized by conventional means such as UV, reverse
30 phase HPLC, HIC, mass spectrometry, ELISA assay, and electrophoresis.

As non limitative embodiment, it is presented herein the conjugation with the IGF-1R antibody c208F2. In this case, the drug is coupled to at least one cysteine selected from i) for the light chain of sequence SEQ ID No. 28, the residue Cys. in position 214 and ii) for the heavy chain of sequence SEQ ID No. 23, the residues Cys. in position 223, 229 and 232.

As non limitative embodiment, it is presented herein the conjugation with the IGF-1R antibody c208F2. In this case, the drug is coupled to two, three or four, cysteines selected from i) for the light chain of sequence SEQ ID No. 28, the residue Cys. in position 214 and ii) for the heavy chain of sequence SEQ ID No. 23, the residues Cys. in position 223, 229 and 232

As non limitative embodiment, it is presented herein the conjugation with the IGF-1R antibody hz208F2 (var. 1). In this case, the drug is coupled to at least one cysteine selected from i) for the light chain of sequence SEQ ID No. 39, the residue Cys. in position 214 and ii) for the heavy chain of sequence SEQ ID No. 37, the residues Cys. in position 223, 229 and 232.

As non limitative embodiment, it is presented herein the conjugation with the IGF-1R antibody hz208F2 (var. 3). In this case, the drug is coupled to two, three or four, cysteines selected from i) for the light chain of sequence SEQ ID No. 40, the residue Cys. in position 214 and ii) for the heavy chain of sequence SEQ ID No. 38, the residues Cys. in position 223, 229 and 232.

The same approach could be easily adapted to other antibodies such as, for example, the Axl antibodies.

An alternative consists of lysine coupling. An antibody may contain, for example, many lysine residues that do not react with the drug-linker intermediate (D-L) or linker reagent. Only the most reactive lysine groups may react with an amine-reactive linker reagent. Also, only the most reactive cysteine thiol groups may react with a thiol-reactive linker reagent.

Where the compounds of the invention are bound to lysines, drug loading may range from 1 to 80 drugs (D) per cell antibody, although an upper limit of 40, 20, 10 or 8 may be preferred. Compositions of ADC include collections of cell binding agents,

e.g. antibodies, conjugated with a range of drugs, from 1 to 80, 1 to 40, 1 to 20, 1 to 10 or 1 to 8.

The ADC of formula (I) according to the invention can be in the form of a
5 pharmaceutically acceptable salt.

In the present invention by “pharmaceutically acceptable” is meant that which can be used in the preparation of a pharmaceutical composition which is generally, safe non-toxic and neither biologically nor otherwise undesirable, and which is acceptable for veterinary use as well as for human pharmaceutical use.

10 By “pharmaceutically acceptable salt” of a compound is meant a salt which is pharmaceutically acceptable as defined herein and which has the desired pharmacological activity of the parent compound.

Pharmaceutically acceptable salts notably comprise:

15 (1) the addition salts of a pharmaceutically acceptable acid formed with pharmaceutically acceptable inorganic acids such as hydrochloric, hydrobromic, phosphoric, sulfuric and similar acids; or formed with pharmaceutically acceptable organic acids such as acetic, trifluoroacetic, propionic, succinic, fumaric, malic, tartaric, citric, ascorbic, maleic, glutamic, benzoic, salicylic, toluenesulfonic, methanesulfonic, stearic, lactic and similar acids; and

20 (2) the addition salts of a pharmaceutically acceptable base formed when an acid proton present in the parent compound is either replaced by a metallic ion e.g. an alkaline metal ion, an alkaline-earth metal ion or an aluminium ion; or coordinated with a pharmaceutically acceptable organic base such as lysine, arginine and similar; or with a pharmaceutically acceptable inorganic base such as sodium hydroxide, potash,
25 calcium hydroxide and similar.

These salts can be prepared from the compounds of the invention containing a base or acid function, and the corresponding acids or bases using conventional chemical methods.

30 V – Treatment

Finally, the invention relates to an ADC as above described for use as a medicament, in particular in the treatment of cancer.

A further subject of the present invention is a formal (I) compound such as defined above for use as medicinal product, in particular for the treatment of cancer.

The present invention also concerns the use of a formula (I) compound such as defined above for producing a medicinal product, particularly intended for the treatment
5 of cancer.

The present invention also concerns a method for treating cancer comprising the administration to a person in need thereof of an effective amount of a formula (I) compound such as defined above.

Cancers can be preferably selected through Target-related cancers including
10 tumoral cells expressing or over-expressing whole or part of the Target at their surface.

More particularly, said cancers are breast cancer, colon cancer, esophageal carcinoma, hepatocellular cancer, gastric cancer, glioma, lung cancer, melanoma, osteosarcoma, ovarian cancer, prostate cancer, rhabdomyosarcoma, renal cancer, thyroid cancer, uterine endometrial cancer, schwannoma, neuroblastoma, oral squamous
15 cancer, mesothelioma, leiomyosarcoma, Kaposi sarcoma, acute leukemia, colorectal carcinoma, melanoma, pancreatic ductal adenocarcinoma and any drug resistance phenomena or cancers.

For the avoidance of doubt, by drug resistance Target-expressing cancers, it must be understood not only resistant cancers which initially express the Target but also
20 cancers which initially do not express or overexpress the Target but which express the Target once they have become resistant to a previous treatment.

Another object of the invention is a pharmaceutical composition comprising the ADC as described in the specification.

More particularly, the invention relates to a pharmaceutical composition
25 comprising the ADC of the invention with at least an excipient and/or a pharmaceutical acceptable vehicle.

In the present description, the expression “pharmaceutically acceptable vehicle” or “excipient” is intended to indicate a compound or a combination of compounds entering into a pharmaceutical composition not provoking secondary reactions and
30 which allows, for example, facilitation of the administration of the active compound(s), an increase in its lifespan and/or in its efficacy in the body, an increase in its solubility in solution or else an improvement in its conservation. These pharmaceutically

acceptable vehicles and excipients are well known and will be adapted by the person skilled in the art as a function of the nature and of the mode of administration of the active compound(s) chosen.

The active ingredient can be administered in unit forms of administration, in a mixture with conventional pharmaceutical carriers, to animals or to human beings. Suitable unit forms of administration comprise forms via oral route and forms for administration via parenteral route (subcutaneous, intradermal, intramuscular or intravenous).

As solid compositions, for oral administration, use can be made of tablets, pills, powders (hard or soft gelatine capsules) or granules. In these compositions, the active ingredient of the invention is mixed with one or more inert diluents such as starch, cellulose, sucrose, lactose or silica, in a stream of argon. These compositions may also comprise substances other than diluents, for example one or more lubricants such as magnesium stearate or talc, a colouring agent, a coating (coated tablets) or a varnish.

The sterile compositions for parenteral administration may preferably be aqueous or non-aqueous solutions, suspensions or emulsions. As solvent or vehicle, use can be made of water, propylene glycol, a polyethylene glycol, vegetable oils, in particular olive oil, injectable organic esters e.g. ethyl oleate or other suitable organic solvents. These compositions may also contain adjuvants, in particular wetting, isotonic, emulsifying, dispersing and stabilising agents. Sterilisation can be performed in several manners, for example by sanitising filtration, by incorporating sterilising agents into the composition, by radiation or by heating. They can also be prepared in the form of solid sterile compositions which can be dissolved at the time of use in sterile water or any other injectable sterile medium.

Preferably, these ADCs will be administered by the systemic route, in particular by the intravenous route, by the intramuscular, intradermal, intraperitoneal or subcutaneous route, or by the oral route. In a more preferred manner, the composition comprising the ADCs according to the invention will be administered several times, in a sequential manner.

The invention concerns thus also a kit comprising at least i) an antibody-drug-conjugate according to the invention and/or a pharmaceutical composition according to

the invention and ii) a syringe or vial or ampoule in which the said antibody-drug-conjugate and/or pharmaceutical composition is disposed.

Their modes of administration, dosages and optimum pharmaceutical forms can be determined according to the criteria generally taken into account in the establishment
5 of a treatment adapted to a patient such as, for example, the age or the body weight of the patient, the seriousness of his/her general condition, the tolerance to the treatment and the secondary effects noted.

Other characteristics and advantages of the invention appear in the continuation of the description with the examples and the figures whose legends are represented
10 below.

FIGURE LEGENDS

Figures 1A-1C: Antibody binding to the human native IGF-1R by FACS
15 analyses. Figure 1A represents the titration curve, on MCF-7 cell line. MFI represents the mean of fluorescent intensity. Figure 1B represents the EC_{50} of both murine and chimeric anti-IGF-1R antibodies on the MCF-7 cell line. Figure 1C represents the B_{max} of chimeric anti-IGF-1R antibodies on MCF-7 cell line.

Figures 2A-2B: Evaluation of hIGF-1R recognition using transfected *vs* non
20 transfected cells. Figure 2A) Represents titration curves of one chimeric anti-IGF-1R Ab on IGF-1R⁺ cell line. MFI represents the mean of fluorescent intensity. Figure 2B represents the binding of chimeric anti-IGF-1R Abs on the human IGF-1R⁻ cell line.

Figures 3A-3B: Evaluation of the specificity of Abs to IGF-1R *vs* hIR using
transfected cells. Figure 3A represents the binding of murine anti-IGF-1R Ab on the
25 hIR⁺ transfected cell line. Figure 3B represents the binding of chimeric anti-IGF-1R Ab on the IR⁺ cell line. MFI represents the mean of fluorescent intensity. GRO5 anti-hIR Mab (Calbiochem) was introduced as a positive control.

Figure 4: Binding of murine anti-IGF-1R Ab on the IM-9 cell line. MFI
30 represents the mean of fluorescent intensity. GRO5 anti-hIR Mab was introduced as a positive control.

Figures 5A-5C: Evaluation of recognition of the monkey IGF-1R. Figure 5A represents the titration curves of chimeric anti-IGF-1R Ab on the COS-7 cell line. MFI

represents the mean of fluorescent intensity. Figure 5B represents the EC₅₀ of both murine and chimeric anti-IGF-1R antibodies on COS-7 cell line. Figure 5C represents the EC₅₀ of chimeric anti-IGF-1R antibodies on both NIH 3T3 transfected cells hIGF1R+ and COS-7 cell lines.

5 Figure 6: Sensorgrams obtained on a SPR technology based Biacore X100 using a CM5 sensorchip activated with more the 11000 RU of mouse anti-Tag His antibody chemically grafted to the carboxymethyl dextran matrix. The experiment was run at a flow rate of 30 μ l/min at 25°C using the HBS-EP+ as the running and samples diluting buffer. The figure showed the superposition of 4 independent sensorgrams aligned on
10 the x-axis at the beginning of the first injection of the analytes and on the y-axis by the baseline defined just before this first injection. The sensorgrams obtained with the capture of the human based sequence of the recombinant soluble IGF1R are marked by diamonds. The sensorgrams obtained with the capture of the cynomolgus based sequence of the recombinant soluble IGF1R are marked by triangles. White symbols
15 correspond to the blank cycles (5 injections of the running buffer) and black symbols correspond to the injections of the growing range of concentrations of c208F2 (5, 10, 20, 40 and 80 nM).

Figure 7: Evaluation of the intrinsic effect of anti-hIGF-1R antibodies on the receptor phosphorylation compared to IGF1.

20 Figure 8: Inhibition of IGF-1R phosphorylation in response to IGF-1 by murine anti-hIGF-1R

Figure 9: Cell surface signal intensity of anti-IGF-1R antibodies is down-regulated after cell incubation at 37°C. MCF-7 cells were incubated at 4°C or 37°C for 4 h with 10 μ g/ml of Abs. The figure represents the Δ MFI.

25 Figures 10A-10B: Antibody surface decay. Cell surface bound antibody was assessed after 10, 20, 30, 60 and 120 min at 37°C. Figure 10A represents the % of remaining IGF-1R in comparison to the signal intensity measured at 4°C. Figure 10B represents Half Life calculation using Prims Software and using exponential decay fitting.

30 Figure 11: Anti-hIGF-1R Abs are internalized. Cells were incubated with 10 μ g/ml of murine Abs for 0, 30 or 60 min at 37°C. cells were permeabilized or not and incubated with a secondary anti-mouse IgG-Alexa 488. Membrane corresponds to the

signal intensity w/o permeabilization. Total correspond to the signal intensity after cell permeabilization and cytoplasmic corresponds to internalized Ab. The name of each evaluated antibody is depicted on the top of each graph.

Figures 12A-12B: Imaging Ab internalization. Figure 12A: MCF-7 cells
 5 incubated with m208F2 for 20 min. at 4°C and washed before incubation (W) at 37°C for 15 (X), 30 (Y) and 60 (Z) min. Cells were fixed and permeabilized. The m208F2 Ab was revealed using an anti-mouse IgG Alexa488 and Lamp-1 using a rabbit anti-Lamp-1 antibody and with a secondary anti-rabbit IgG Alexa 555. Figure 12B: MCF-7 cells were incubated for 30 minutes at 37°C with anti-hIGF-1R murine antibodies and stained
 10 as described above. Colocalization was identified using the colocalization highlighter plug-in of the ImageJ software.

Figure 13: Involvement of the lysosome pathway in antibody degradation

Figure 14: Acidic pH decreases binding capacity of the five murine anti-IGF-1R antibodies.

Figures 15A-15D: Binding characteristic of the first humanized form of the
 15 c208F2 Mab. Binding properties of the hz208F2 VH3/VL3 mAb was evaluated on the human cell line MCF-7 (A), on the monkey cell line COS-7 (B) and on the transfected murine cell line expressing the human insulin receptor (C). The binding of both the murine and the chimeric 208F2 mAbs was evaluated in parallel. The anti-hIR antibody
 20 clone GRO5 was used to verify the expression of the hIR on the transefected cell line (D).

Figure 16: hz208F2 VH3/VL3 antibody surface decay

Figure 17: Superposition of to sensorgrammes obtained with a SPR based
 25 Biacore X100 device at a temperature of 25°C with a CM5 sensor chip activated on both flowcells with around 12.000 RU of a mouse anti-TagHis monoclonal antibodies chemically grafted to the carboxymethyl dextran matrix using a HBS-EP+ as the running buffer at a flow rate of 30 µl/min. Each sensorgrammes (the first one marked by triangles and the second one marked by diamonds) correspond to a complete cycle:

- 1- Injection during one minute of a solution of recombinant h-IGF1R
 30 (10 µg/ml) on the second flowcell.
- 2- For the first sensorgramme: 5 injections of running buffer during 90s each

For the second sensorgramme: five injections in the growing range of concentrations of the anti-IGF1R c208F2 antibody solutions during 90 s each.

3- A delay of 300 s for the determination of the dissociation kinetic rates.

5 4- A regeneration of the surface by an injection during 45 s of a 10 mM Glycine, HCl pH 1.5 buffer.

Figure 18: The sensorgramme corresponding to the subtraction of the blank sensorgramme (5 injections of HBS-EP+) to the sensorgramme obtained with the growing range of concentrations of the anti-IGF1R c208F2 solutions is presented in grey. The theoretical sensorgramme corresponding to the 1:1 model with the following parameters: $k_{on} = (1.206 \pm 0.036) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{off} = (7.81 \pm 0.18) \times 10^{-5} \text{ s}^{-1}$, $R_{max} = 307.6 \pm 0.3 \text{ RU}$ is presented by a thin black line. The calculated concentrations of c208F2 are reported on the graph: only the highest concentration (24 nM) is considered as a constant).

15 Figure 19: The dissociation constants correspond to the mean of the four experiments run for each antibody and correspond to the ratio: $k_{off}/k_{on} \times 10^{12}$ to be express in the pM unit. The error bars correspond to the standard error (n=4).

Figure 20: the half-lives correspond to the mean of the four experiments run for each antibody and correspond to the ratio: $\text{Ln}(2)/k_{off}/3600$ to be express in the h unit. The error bars correspond to the standard error (n=4).

Figure 21: Cell cytotoxicity of anti-IGF-1R coupled with three different compounds. Five chimeric antibodies anti-IGF-1R were coupled with either E-13, G-13 or F-63. An irrelevant antibody c9G4 was also coupled with the same compounds.

25 Figures 22A-22C: *in vivo* evaluation of c208F2-E-13 (Figure 22A), c208F2-G-13 (Figure 22B) and c208F2-F-63 (Figure 22C) in the MCF-7 xenograft model.

Figures 23A-23B: *in vivo* evaluation of both c208F2-E-13 (Figure 23A) and c208F2-G-13 (Figure 23B) compared to ADCs control (c9G4-E13 and c9G4-G-13) in the MCF-7 xenograft model.

30 Figures 24A, 24B and 24C: Binding specificity of 1613F12 on the immobilized rhAxl-Fc protein (24A), rhDtk-Fc (24B) or rhMer-Fc (24C) proteins by ELISA.

Figure 25: FACS analysis of the 1613F12 binding on human tumor cells

Figure 26: ELISA experiments studying binding on rhAxl-Fc protein of both m1613F12 and hz1613F12.

Figures 27A, 27B and 27C: Immunofluorescence microscopy of SN12C cells after incubation with 1613F12 Figure 27A- Photographs of the mIgG1 isotype control conditions both for the membrane and the intracellular staining. Figure 27B- Membrane staining. Figure 27C- Intracellular staining of both Axl receptor using 1613F12 and of the early endosome marker EEA1. Image overlays are presented below and co-localizations visualized are indicated by the arrows.

Figures 28A and 28B: Figure 28A represents the cytotoxic activity of the hz1613F12-E-13 on both SN12c (Axl⁺) and MCF-7 (Axl⁻) cell lines. Figure 28B represents the cytotoxic activity of the hz1613F12-G-13 on both SN12c (Axl⁺) and MCF-7 (Axl⁻) cell lines.

Figures 29A and B: *In vivo* activity of the trastuzumab antibody conjugated to either E-13 (29A) or G-13 (29B) compounds in the Calu-3 xenograft model.

Figures 30A to 30C: *In vivo* activity of the trastuzumab antibody conjugated to either E-13 (Fig. 30A) or G-13 (Fig. 30B) compounds or alone (Fig. 30C) in the JIMT-1 xenograft model.

EXAMPLES

Example 1: Generation of murine antibodies raised against IGF-1R ECD

To generate murine monoclonal antibodies (Mabs) against human extracellular domain (ECD) of the human IGF-1 receptor (hIGF1R), 5 BALB/c mice were immunized 3-times s.c. with 10 µg of the rhIGF-1R protein (R&D Systems, Cat N°391-GR). As an alternative, three additional immunizations with 10 µg of the murine extracellular domain (ECD) of IGF-1R (R&D Systems, Cat N° 6630-GR /Fc) were performed on some animals. The first immunization was done in presence of Complete Freund Adjuvant (Sigma, St Louis, MD, USA). Incomplete Freund adjuvant (Sigma) was added for following immunizations. Three days prior to the fusion, immunized mice were boosted with 10 µg of the rhIGF-1R protein. Then splenocytes and lymphocytes were prepared by perfusion of the spleen and by mincing of the proximal lymph nodes, respectively, harvested from 1 out of the 5 immunized mice (selected after sera titration of all mice) and fused to SP2/0-Ag14 myeloma cells (ATCC, Rockville, MD, USA). The fusion protocol is described by Kohler and Milstein (Nature, 256:495-497, 1975). Fused cells are then subjected to HAT selection. In general, for the preparation of monoclonal antibodies or their functional fragments, especially of murine origin, it is possible to refer to techniques which are described in particular in the manual "Antibodies" (Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor NY, pp. 726, 1988). Approximately 10 days after the fusion, colonies of hybrid cells were screened. For the primary screen, supernatants of hybridomas were evaluated for the secretion of Mabs raised against the IGF-1R ECD protein by FACS analysis using human breast MCF7 tumor cells (ATCC) and/or monkey COS7 cells (African green monkey kidney-SV40 transformed) which express monkey IGF-1R on their cell surface. More precisely, for the selection by flow cytometry, 10^5 cells (either MCF7 or COS7) were plated in each well of a 96 well-plate in PBS containing 1% BSA and 0.01% sodium azide (FACS buffer) at 4°C. After a 2 min centrifugation at 2000 rpm, the buffer was removed and hybridoma supernatants to be tested were added. After 20 min of incubation at 4°C, cells were washed twice and an Alexa 488-conjugated goat anti-mouse antibody 1/500° diluted in FACS buffer (#A11017, Molecular Probes Inc., Eugene, USA) was added and incubated for 20 min

at 4°C. After a final wash with FACS buffer, cells were analyzed by FACS (FacsCalibur™, Becton-Dickinson) after addition of propidium iodide to each tube at a final concentration of 40 µg/ml. Wells containing cells alone and cells incubated with the secondary Alexa 488-conjugated antibody were included as negative controls. Isotype controls were used in each experiment (Sigma, ref M90351MG). At least 5000 cells were assessed to calculate the mean value of fluorescence intensity (MFI).

Additionally an internalization assay was performed in order to select only internalizing antibodies. For this assay, MCF7 tumor cell line was cultured in RPMI 1640 without phenol red with 1% L-glutamine and 10% of FACS for 3 days before experiment. Cells were then detached using trypsin and 100 µl of a cell suspension at $4 \cdot 10^5$ cell/ml are plated in 96-multiwell plates in RPMI1640 without phenol red with 1% L-glutamine and 5% FBS. After a 2 min centrifugation at 2000 rpm, cells were resuspended in 50 µl of either hybridoma supernatants or control antibody solutions (positive and isotype controls at 1 µg/ml). After a 20 min incubation time at 4°C, cells were centrifuged 2 min at 2000 rpm and resuspended in either cold (4°C) or warm (37°C) complete culture medium. Cells were then incubated for 2 hours either at 37°C or at 4°C. Then cells were washed three times with FACS buffer. An Alexa 488-labeled goat anti-mouse IgG antibody was incubated for 20 minutes and cells were washed three times before FACS analysis on propidium iodide negative cell population.

Following the FACS analysis, two parameters were determined: (i) the difference of the fluorescent signal detected on the surface of cells incubated at 4°C with those obtained with the cells incubated at 37°C with one hybridoma supernatant and (ii) the percentage of remaining IGF1R on the cell surface.

The percentage of remaining hIGF 1R is calculated as follows: % remaining IGF-1R = $(\text{MFI}_{\text{Ab } 37^\circ\text{C}} / \text{MFI}_{\text{Ab } 4^\circ\text{C}}) \times 100$.

In addition three ELISAs were performed (either before or after cloning) to study the binding of antibodies on the recombinant human (hIGF-1R) and murine (mIGF-1R) proteins, and on the recombinant human Insulin Receptor (hIR) protein. Hybridoma secreting antibody showing binding on rh- and/or rm-IGF-1R and no binding on rhIR were retained. Briefly, 96-well ELISA plates (Costar™ 3690, Corning, NY, USA) were coated 100 µl/well of either the rhIGF-1R protein (R&D Systems, cat N° 391-GR) at 0.6 µg/ml or rmIGF-1R protein (R&D Systems, cat N°6630-GR/Fc) at 1

µg/ml or rhIR protein (R&D Systems, cat N°1544-IR/CF) at 1 µg/ml in PBS overnight at 4°C. The plates were then blocked with PBS containing 0.5% gelatin (#22151, Serva Electrophoresis GmbH, Heidelberg, Germany) for 2 h at 37°C. Once the saturation buffer discarded by flicking plates, 100 µl of each supernatant dilution were added to each well (either undiluted hybridoma supernatant either supernatant serial dilutions) and incubated for 1 h at 37°C. After three washes, 100 µl horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (#115-035-164, Jackson Immuno-Research Laboratories, Inc., West Grove, PA, USA) was added at a 1/5000 dilution in PBS containing 0.1% gelatin and 0.05% TweenTM 20 (w:w) for 1 h at 37°C. Then, ELISA plates were washed 3-times and the TMB (#UP664782, Uptima, Interchim, France) substrate is added. After a 10 min incubation time at room temperature, the reaction was stopped using 1 M sulfuric acid and the optical density at 450 nm is measured.

Hybridoma secreting antibody of interest were expanded and cloned by limit dilution. Once isotyped, one clone of each code was expanded and frozen. Each antibody of interest was produced in *in vitro* production systems named CellLine (Integra Biosciences) for further characterization.

Additional assays to address binding specificity FACS analyses were performed on IM9 cells (human IR expressing B lymphoblasts) as well as on hIGF-1R transfected cells *versus* non transfected cells.

All the data corresponding to the selected antibodies were summarized in Table 9 and demonstrated that the five selected antibodies strongly recognize the native human IGF-1R expressed either on MCF-7 breast cancer cells or on transfected cells. They also recognize monkey IGF-1R on COS-7 cells. These antibodies do not cross react with the human insulin receptor highly expressed on IM9 cells. It has to be noticed that these antibodies poorly recognize the rhIGF-1R ECD protein when directly coated to ELISA plates.

98

Table 9

| hybridoma name | Isotype | CNCM | ELISA (SNT at 5 µg/ml) | | | | MCF7 Internalisation Assay (SNT at 5 µg/ml) | | | | FACS (SNT at 5 µg/ml) | | | |
|----------------|---------|--------|------------------------|-----------|--------------|-----|---|----|----------------------|------------------------|--------------------------|------------------------------------|------------------------|--------------------------------------|
| | | | D.O 450 nm | | | | MFI | | % remaining rh IGF1R | Δ (MFI 4°C - MFI 37°C) | IM9 (h IR ⁺) | Cos-7 (monkey IGF1R ⁺) | Tf hIGF1R ⁺ | non Tf cells (h IGF1R ⁺) |
| | | | rh IGF-1R | rm IGF-1R | rh Insulin R | 4°C | 37°C | | | | | | | |
| 208F2 | IgG1 K | I-4757 | 0.163 | 0.099 | 0.140 | 355 | 94 | 27 | 261 | 4 | 106 | 2197 | 22 | |
| 212A11 | IgG1 K | I-4773 | 0.232 | 0.102 | 0.141 | 390 | 106 | 27 | 284 | 7 | 125 | 2187 | 23 | |
| 213B10 | IgG1 K | I-4774 | 0.399 | 0.127 | 0.110 | 386 | 115 | 30 | 271 | 7 | 122 | 2055 | 23 | |
| 214F8 | IgG1 K | I-4775 | 0.349 | 0.102 | 0.115 | 386 | 111 | 29 | 275 | 7 | 132 | 2137 | 20 | |
| 219D6 | IgG1 K | I-4736 | 0.329 | 0.112 | 0.106 | 349 | 106 | 30 | 243 | 7 | 114 | 2110 | 21 | |

Example 2: Antibody binding to the human native IGF-1R by FACS analyses

The five murine IGF-1R antibodies were chimerized. The binding properties of both the murine and the chimeric IGF-1R antibodies were evaluated by FACS analyses on the human MCF-7 breast adenocarcinoma cell line (ATCC#HTB-22) using increasing antibody concentrations. For that purpose, cells (1×10^6 cells/ml) were incubated with IGF-1R antibodies for 20 min. at 4°C in FACS buffer (PBS, 0.1% BSA, 0.01% NaN₃). They were then washed 3 times and incubated with the appropriate secondary antibody coupled with Alexa 488 for 20 additional minutes at 4°C in the dark before being washed 3 times in FACS buffer. The binding of anti-IGF-1R antibodies was immediately performed on viable cells which were identified using propidium iodide (that stains dead cells). The maximum of signal intensity obtained with each antibody was designed as B_{max} and expressed in mean of fluorescence intensity (MFI). The EC₅₀ of binding expressed in molarity (M) was calculated using a nonlinear regression analysis (GraphPad Prims 4.0).

The titration curve of each murine or chimeric Ab demonstrated that all generated antibodies are capable to recognize the native IGF-1R form with a typical saturation profile (Figure 1A). In order to rank antibodies and to compare the binding properties of both murine and chimeric Abs, the binding EC₅₀ of each compound was determined using a non linear regression analysis. The comparison of the EC₅₀ of each murine Ab with its corresponding chimeric form showed that the 2 forms displayed the same binding properties demonstrating that the Ab chimerization did not affect IGF-1R recognition (Figure 1B-C). EC₅₀ and B_{max} values of chimeric antibodies were summarized in Table 10.

25

Table 10

| AC | B _{max} | EC ₅₀ |
|----------------|------------------|------------------|
| c208F2 | 981 | 6.7E-10 |
| c212A11 | 991 | 6.7E-10 |
| c214F8 | 1069 | 5.0E-10 |
| c219D6 | 993 | 4.7E-10 |
| c213B10 | 1103 | 4.4E-10 |

Example 3: Confirmation of Antibody specificity by using either IGF-1R or IR transfected cells or IM9 cells that express significant levels of IR

In order to confirm the specificity of the generated antibodies for IGF-1R *versus* IR, stable transfectants expressing either hIGF-1R or hIR were evaluated by FACS analyses. Briefly, increasing concentrations of chimeric mAbs were incubated with cells for 20 min at 4°C in FACS buffer (PBS, 0.1%BSA, 0.01% NaN₃). Cells were then washed 3 times and incubated with the appropriate secondary antibody coupled with Alexa 488 before being incubated for 20 additional minutes at 4°C in the dark and then washed 3 times in FACS buffer. The binding of anti-IGF-1R antibodies was immediately performed on viable cells which were identified using propidium iodide (that stains dead cells). The binding EC₅₀ expressed in molarity (M) was calculated using a nonlinear regression analysis (GraphPad Prims 4.0).

Titration curves obtained on the hIGF-1R transfected cell line (Figure 2A) *versus* untransfected cells (Figure 2B) confirmed the binding specificity of chimeric Abs for the human IGF-1R. EC₅₀ and B_{max} values were summarized in Table 11.

Table 11

| Ac | B _{max} | EC ₅₀ (M) |
|---------|------------------|----------------------|
| c208F2 | 2008 | 3.2E-10 |
| c212A11 | 2513 | 4.4E-10 |
| c214F8 | 2094 | 2.7E-10 |
| c219D6 | 2521 | 5.5E-10 |
| c213B10 | 2029 | 3.3E-10 |

In order to verify the absence of binding of both murine and chimeric antibodies on hIR, a stable cell line expressing the human IR (hIR) was used. The recognition of human cell surface hIR by both murine and chimeric Ab was performed by FACS analyses. Increasing concentration of either the murine or the chimeric mAbs were incubated on the hIR⁺ transfected cell line for 20 minutes at 4°C in FACS buffer (PBS, 0.1%BSA, 0.01% NaN₃). Cells were then washed 3 times and incubated with the appropriate secondary antibody coupled with Alexa 488 before being incubated for 20 additional minutes at 4°C in the dark and then washed 3 times in FACS buffer. The binding of anti-IGF-1R antibodies was immediately performed on viable cells which

were identified using propidium iodide (that stains dead cells). The binding EC_{50} expressed in molarity (M) was calculated using a nonlinear regression analysis (GraphPad Prims 4.0). The anti-hIR antibody clone GRO5 was used as positive controls. The murine and chimeric 9G4 antibodies were introduced as irrelevant
5 antibodies.

The high level of expression of hIR on cell surface of the transfected cells was confirmed using the commercial anti-hIR antibody GRO5 (Figure 3A and 3B). Even using high concentrations of either the murine (Figure 3A) or the chimeric (Figure 3B) hIGF-1R Abs, no binding on cell surface of hIR⁺ transfected cells was observed. These
10 results demonstrated that neither murine nor chimeric anti-hIGF-1R Abs did recognized the hIR.

This specificity of recognition of hIGF-1R *versus* IR has also been demonstrated, by FACS analyses, using IM9 cells, a B-lymphoma cell line that expresses hIR (Figure 4). For this FACS analyses, the protocol was the same as the one
15 described above and murine antibodies were used in order to prevent the cross reactivity of the secondary anti-human Ab (IM9 cells express human Ig on their cell surface). Results presented in Figure 4 demonstrated once again that the expected signal was observed using the GRO5 anti-hIR antibody while none of the murine antibody evaluated displayed any significant binding signal on this cell line.

20

Example 4: Antibody binding to the monkey native IGF-1R by FACS and Biacore analyses

One of the first pre-requisite for regulatory toxicology studies is to find a relevant animal specie in order to evaluate the selected compound. As the series of
25 antibodies described herein is not able to recognize murine IGF-1R, the most likely specie for toxicological evaluation is the non human primate (NHP).

In order to evaluate the binding of anti-IGF-1R antibodies on monkey IGF-1R, the binding of both murine and chimeric anti-hIGF-1R antibodies was first evaluated by FACS analyses on COS-7 cell line using increasing antibody concentrations. Cells
30 (1x10⁶ cells/ml) were incubated with anti-IGF-1R antibodies for 20 minutes at 4°C in FACS buffer (PBS, 0.1%, BSA, 0.01% NaN₃). Then, cells were washed 3 times and incubated with the appropriate secondary antibody coupled with Alexa 488 before being

incubated for 20 additional minutes at 4°C in the dark and finally washed 3 times in FACS buffer. The binding of anti-IGF-1R antibodies was immediately evaluated on viable cells identified using propidium iodide (that stains dead cells). The binding EC₅₀ expressed in molarity (M) was calculated using a nonlinear regression analysis
5 (GraphPad Prims 4.0).

The titration curves obtained on the COS-7 monkey cell line showed that, all the anti-hIGF-1R Abs recognized specifically the IGF-1R expressed on the surface of the monkey cell line (Figure 5A). Determination of the, EC₅₀ for each murine and chimeric Abs showed that the 2 forms compared well regarding to their binding properties on
10 monkey IGF-1R (Figure 5B). Those results showed that all the generated anti-hIGF-1R recognized the monkey IGF-1R.

A comparison of binding EC₅₀ on COS-7 cells *versus* transfected IGF-1R cells was performed in order to verify the magnitude of chimeric antibody recognition on human *versus* monkey IGF-1R. Results shown in Figure 5C demonstrated a similar
15 recognition of human and monkey IGF-1Rs by all antibodies.

In order to confirm the recognition on another type of monkey, cells were transfected with the IGF-1R form Cynomolgus monkey to produce soluble monkey IGF-1R ECD and Biacore experiments were performed with one of the chimeric antibodies (c208F2) in order to compare its binding properties either the hIGF-1R or the
20 Cynomolgus IGF-1R.

The recognition experiments were run on a Biacore X100 device using a CM5 sensor chip activated by an anti-Tag His antibody (His capture kit GE Healthcare catalogue number 28-9950-56). More than 11000 RU of antibodies are chemically grafted on the carboxymethyl dextran matrix using the amine kit chemistry. The
25 experiments were carried out at 25°C with a flow rate of 30 µl/min using the HBS-EP buffer (GE Healthcare) as the running and sample dilution buffer. The single cycle kinetic scheme was used to defined the kinetic parameters of the binding of the chimeric form of the 208F2 antibody (c208F2) on hIGF-1R compared to Macaca IGF-1R

A solution of a soluble recombinant version of the IGF1R hetero-tetramere
30 composed of 2α chains and the extracellular domains of 2β chains expressed with an additional C-terminal 10-His tag, based either on the sequence of the human (R&D Systems catalogue number 305-GR-50) or of the one of cynomolgus (produced in

house) was injected 1 minute on the second flowcell at a dilution defined to capture around 160 RU of antigen. After the capture phase, either the running buffer was injected 5 times (90 s each injection) or a growing range of 5 concentrations of c208F2 were injected (90s each injection) on both flowcells. At the end of the fifth injection the running buffer was passed in order to define the dissociation rate.

The surface was then regenerated with an injection of a 10 mM Glycine,HCl pH 1.5 buffer during 30 s.

The computed signal corresponds to the difference between the response of the flowcell 2 (with captured IGF-1R) and the response of the flowcell 1 (without any IGF-1R molecules) (Figure 6).

For each IGF1R molecule (human or cyno), the signal due to the injections of the growing range of concentrations of c208F2 was corrected by subtraction of the signal obtained with the 5 injections of the buffer (double reference). The resulting sensorgrams were analysed using the Biaevaluation software with a 1:1 model. The kinetic rates are evaluated either independently (2 kinetics rates of the binding of c208F2 on each IGF-1R) or commonly (the same kinetic rates of the binding of c208F2 on the human and the cynomolgus IGF1R). The quality of the fitting was assessed by a Chi2/Rmax ratio lower than 0.05 RU.

The kinetics rates of the binding (see Table 12) defined separately for each IGF-1R are close and a fitting of both sensorgrams with the same kinetic rates is of good quality.

The c208F2 antibody recognizes as well the recombinant human and cynomolgus IGF-1Rs with a dissociation constant (KD) about 0.2 nM. The affinities defined in tis study correspond to the functional affinities (avidities) of the antibodies for a level of captured human and cynomolgus IGF-1R around 160 RU.

Table 12

| IGF1R | kon [1/M.s] | koff [1/s] | Kd [nM] | Chi2/Rmax |
|--------------|-------------|------------|---------|-----------|
| human | 1,52E+06 | 3,40E-04 | 0,23 | 0,045 |
| cynomogus | 1,85E+06 | 3,10E-04 | 0,17 | 0,032 |
| Hum. & Cyno. | 1,52E+06 | 3,33E-04 | 0,22 | 0,039 |

Example 5: Intrinsic effect of generated antibodies on IGF-1R phosphorylation

It is well known that antibodies could induce an agonistic effect when they bind to tyrosine kinase receptors. As we would not like to select such agonist antibodies, the
5 evaluation of hIGF-1R phosphorylation was studied using the chimeric antibodies.

For that purpose, MCF-7 cells were incubated in serum-free medium overnight. Then, either IGF-1 (100 nM) or Abs to be tested were added (10 μ g/ml) for 10 minutes at 37°C. Medium was discarded and cells were scraped in a lysis buffer (pH 7.5) containing 10 mM Tris HCl buffer (pH 7.5), 15% NaCl (1 M), 10% detergent mix (10
10 mM Tris-HCl, 10% Igepal lysis buffer) (Sigma Chemical Co.), 5% sodium deoxycholate (Sigma Chemical Co.), 1 protease inhibitor cocktail complete TM tablet (Roche), 1% phosphatase inhibitor Cocktail Set II (Calbiochem), for 90 min at 4°C. The lysates were clarified by centrifugation at 4°C, heated for 5 min at 100°C and kept at -20°C or directly loaded on 4–12% SDS-PAGE gels. Incubation of the primary antibody
15 was performed for 2 hr at room temperature and then incubation with HRP-linked secondary antibodies was done for 1 hr at room temperature. Membranes were washed in TBST prior to visualization of proteins with ECL. Blots were quantified using Image J software. Phospho- protein values were normalized with GAPDH. Phosphorylation of hIGF-1R in response to IGF-1 was considered as 100 % of stimulation. The effect of
20 anti-hIGF-1R Abs on the phosphorylation of hIGF-1R was determined as % of phosphorylation induced by IGF-1.

The results described in Figure 7 represent the mean of the % of pIGF-1R in response to the chimeric anti-IGF-1R Abs of 3 independent experiments +/- S.D. compared to IGF-1. As illustrated no significant or minor (<10%) phosphorylation of
25 hIGF-1R was detected when MCF-7 cells were incubated with 10 μ g of anti-IGF-1R Abs.

Example 6: Inhibition of IGF-1R phosphorylation in response to IGF-1 by murine IGF-1R antibodies

30 In order to characterize the selected antibodies, their ability to inhibit IGF1-induced phosphorylation was studied. For that purpose, MCF-7 cells were incubated in serum-free medium overnight. Then, cells were incubated for 5 minutes with murine

anti-hIGF-1R Abs before addition of IGF-1 for 2 minutes at 37°C. Medium was discarded and cells were scraped in a lysis buffer (pH 7.5) containing 10 mM Tris HCl buffer (pH 7.5), 15% NaCl (1 M), 10% detergent mix (10 mM Tris-HCl, 10% Igepal lysis buffer) (Sigma Chemical Co.), 5% sodium deoxycholate (Sigma Chemical Co.), 1
5 protease inhibitor cocktail complete TM tablet (Roche), 1% phosphatase inhibitor Cocktail Set II (Calbiochem), for 90 min at 4°C. The lysates were clarified by centrifugation at 4°C, heated for 5 min at 100°C and kept at -20°C or directly loaded on 4–12% SDS-PAGE gels. Incubation of the primary antibody was performed for 2 h at room temperature and then incubation with HRP-linked secondary antibodies was
10 performed for 1 hr at room temperature. Membranes were washed in TBST prior to visualization of proteins with ECL. Blots were quantified using Image J software. Phospho- protein values were normalized with GAPDH. Phosphorylation of hIGF-1R in response to IGF-1 was considered as 100 % of stimulation. The effect of anti-hIGF-1R Abs on the phosphorylation of hIGF-1R was determined as % of phosphorylation
15 induced by IGF-1.

All anti-IGF-1R Abs inhibited strongly hIGF-1R phosphorylation in response to IGF-1 (decrease > 80%) (Figure 8). The best inhibitors of IGF1-induced phosphorylation of hIGF-1R are the m208F2, m212A11 and m214F8 Mabs.

20 **Example 7: Study of IGF-1R internalization after binding of the generated IGF-1R antibodies by FACS analyses**

MCF-7 cells were incubated with 10μg/ml of chimeric antibodies at 4°C for 20 min. Then, cells were washed and incubated at 4°C or 37°C for 4 h. The quantity of cell-surface bound antibody was determined using a secondary antibody. The ΔMFI
25 defined as the difference between MFI measured at 4°C and MFI measured at 37°C after a 4 hour incubation time corresponded to the quantity of internalized Ab. The ΔMFI was presented in Figure 9 and Table 11. The percentage of internalization at 10 μg/ml of Ab were calculated as followed $100 \cdot (MFI \text{ at } 4^\circ\text{C} - MFI \text{ at } 37^\circ\text{C}) / MFI \text{ at } 4^\circ\text{C}$ and presented in Table 13.

Table 13

| Abs | % Internalization | Δ MFI | Δ MFI_ EC ₅₀ |
|---------|-------------------|--------------|--------------------------------|
| c208F2 | 83 | 288 | 1.8E-10 |
| c212A11 | 80 | 322 | 2.7E-10 |
| c214F8 | 87 | 403 | 2.2E-10 |
| c219D6 | 80 | 353 | 4.4E-10 |
| c231B10 | 85 | 369 | 2.3E-10 |

In order to determine whether antibodies that also recognized the monkey IGF-1R were able to internalize this receptor, the same internalization experiment was performed. Results summarized in Table 14 demonstrated that all tested antibodies were able to mediate monkey IGF-1R internalization.

Table 14

| Abs | Murine Abs | | Chimeric Abs | |
|--------|--------------|-------------------|--------------|-------------------|
| | Δ MFI | % internalisation | Δ MFI | % internalisation |
| 208F2 | 53 | 74 | 52 | 67 |
| 212A11 | 83 | 73 | 98 | 75 |
| 214F8 | 76 | 71 | 98 | 72 |
| 219D6 | 80 | 71 | 102 | 74 |
| 213B10 | 84 | 74 | 101 | 73 |

5 The kinetic of cell surface bound antibody decrease was further evaluated. For that purpose, MCF-7 cells were seeded in 96-well plates and incubated with 10 μ g/ml of murine for 20min at 4°C. Cells were then washed to remove unbound antibody and in media at 37°C for 10, 20, 30, 60 or 120 min. At each time point, cells were centrifuged and then surface labeled on ice with a secondary anti-mouse IgG-Alexa488 to determine
10 the amount of antibody remaining on the cell surface. The fluorescence intensity for each murine Ab and for each time point was normalized by the signal at 4°C (% remaining IGF-1R) and fitted to an exponential decay to determine the half life (t_{1/2}). t_{1/2} was considered as the time needed to obtain a decrease of 50% of the signal. As

illustrated in Figure 10, the surface level of all murine Abs dropped rapidly over the first 30 min and the decrease was almost maximum after 60 min of incubation (Figure 10A). The calculated half life was comprised between 10 to 18 min according to the murine Ab (Fig 10B).

5 In order to validate that the decrease of the cell surface signal was due to Ab internalization and not due to receptor shedding, cells were incubated with murine Abs for 0, 30 and 60 min à 37°C (Figure 11). Cells were then fixed and permeabilized or not in order to determine cell surface bound antibody (w/o permeabilization) and total antibody signal corresponding to cell-surface bound + internalized Ab (with
10 permeabilization). The quantity of internalized Ab (cytoplasmic) was determined as follow: MFI after permabilization – MFI w/o permeabilization. This experiment showed that the decrease of cell-surface bound Ab was due to an increase of cytoplasmic Abs demonstrating that Abs were internalized (Figure 11). In addition, the degradation of the Abs started after 1 h of incubation as indicated by the decrease of the signal after
15 permeabilization (Total).

Example 8: Study of IGF-1R internalization after binding of the generated IGF-1R antibodies by confocal analyses

To further confirm antibodies internalization, confocal microscopy was done to
20 assess the subcellular distribution of antibodies following cellular trafficking. Cells were incubated with anti-hIGF-1R Abs 37°C, fixed and permeabilized. Therefore, cells were stained using a secondary antibody Alexa-488 and with rabbit anti-Lamp-1 antibody that was revealed using a secondary anti-Rabbit IgG Alexa 555. Before incubation at 37°C, the murine 208F2 Ab was localized on the membrane of MCF-7
25 cells (Figure 12A). No colocalization with the lysosome marker, lamp-1 was noted using the colocalization highliter plug-in of the Image J software. The cell surface bound antibody decreased dramatically after 15 min of incubation at 37°C. Concomitantly to the decrease of the cell surface bound antibody, intracellular antibody was detected into vesicles. Rare colocalization with lamp-1 could be observed. After 30
30 min of incubation, the cell surface bound antibody was hardly detected. However, the colocalization of the Ab into lysosome increased. After 1 h of incubation, the intracellular Ab staining decreased as well as the number of colocalization with lamp-1.

This kinetic of cell surface bound antibody and its intracellular accumulation correlated with the kinetic of antibody surface decay measure by FACS. In addition, as already described with FACS studies, the degradation of murine Abs started after 1 h of incubation by confocal microscopy.

5 The internalization of all other hIGF-1R murine antibodies and their colocalization with Lamp-1 was also assessed (Figure 12B). After 30 min of incubation at 37°C, intracellular antibody was detected and colocalization with lamp-1 could be observed indicating that all selected anti-IGF-1R antibodies were effectively internalized into lysosomes.

10

Example 9: Inhibition of Abs degradation using lysosome inhibitor, Bafilomycin A1

In order to confirm that antibodies reached the lysosome were they are degraded, cells were treated or not with bafilomycine A1, a potent inhibitor of lysosome functions.
15 Cells were then incubated with 10 µg/ml of Ab to be tested at 4°C, washed and incubated for 2 h at 37°C. The internalized Ab was detected after cell permeabilisation using a secondary anti-mouse IgG-Alexa 488 Ab. Addition of bafilomycine A1 prevented the degradation of intracellular Ab (Figure 13) indicating that Abs were effectively internalized and degraded into lysosomes.

20

Example 10: Effect of pH on Antibody-IGF-1R binding

As antibodies were selected on the bases of their internalizing potential and shown above to co-localize with early endosomes before entering into the lysosomal compartment, an interesting approach consisted in selecting antibodies for which the
25 stability of the Ab/hIGF-1R binding was modulated regarding to pH environment and preferentially antibodies that dissociated preferentially from IGF-1R when the pH environment became acid. Indeed, the primary difference between early endosomes and lysosomes is their luminal pH: in the endosome compartment the pH is approximately 6 while in the lysosomal compartment the pH is about 4.5.

30 It is well known that once internalized after ligand binding (IGF1), hIGF-1R returns back to the cell surface through a recycling pathway.

Without being link by a theory, an hypothesis herein described is that antibodies more prone to be released from their target early at acidic pH will probably favour target recycling to the membrane and consequently could be considered as better candidates for ADC approaches. In order to investigate whether some of our antibodies display such a property and to correlate this property to cytotoxic activity, the binding of the murine anti-hIGF-1R Mabs on MCF-7 cell line was done in buffers at different pH. Increasing concentrations of murine mAbs were incubated on MCF-7 cell line for 20 min at 4°C in different pH ranging from 5 to 8. Cells were then washed 3 times and incubated with the appropriate secondary antibody coupled with Alexa 488 in FACS buffer. Cells were incubated for 20 additional minutes at 4°C in the dark and then washed 3 times in FACS buffer. The binding of anti-hIGF-1R antibodies was immediately performed on viable cells which were identified using propidium iodide that stained dead cells. The binding EC₅₀ expressed in molarity (M) was calculated using a nonlinear regression analysis (GraphPad Prims 4.0). All murine anti-IGF-1R antibodies selected showed a lower binding capacity at acidic pH as illustrated in Figure 14.

Example 11: Evaluation of a humanized form of the 208F2 Mab

The binding of the first humanized form of the c208F2 mAb was evaluated on MCF-7, COS-7 and NIH 3T3 IR⁺ cell lines. Increasing concentrations of m208F2, c208F2 or hz208F2 VH3VL3 were added on each cell line for 20 min. at 4°C. Cells were then washed and the binding of the tested mAb was revealed using the corresponding secondary antibody. In order to validate the expression of the human IR on the transfected cell line, the commercial anti-hIR antibody clone GRO5 was used and its recognition profile exemplified on (Figure15D).

Comparison of the humanized form with either murine or chimeric ones on MCF-7 (Figure15A) or monkey COS-7 (Figure15B) cells showed close profiles for the 3 tested forms. The humanisation process did not modify the specificity of recognition of the antibody that is perfectly comparable to the murine and chimeric forms regarding to the absence of cross reactivity on the human insulin receptor (Figure15C).

The calculated EC_{50s} of the first humanized form of 208F2 on the human cell line MCF-7 and the monkey cell line COS-7 were similar to the one determined with either the murine or the chimeric form of the mAb 208F2.

The capacity of the mAb hz208F2 VH3/VL3 to be internalized was assessed by flow cytometry. MCF-7 cells were incubated with 10 µg/ml of antibodies at 4°C for 20 min. Then, cells were washed and incubated at 4°C or 37°C for 4 h. The quantity of cell-surface bound antibody was determined using a secondary antibody. The ΔMFI defined as the difference between MFI measured at 4°C and MFI measured at 37°C after a 4 hour incubation time corresponded to the quantity of internalized Ab. The ΔMFI was presented in Figure 16 and Table 13. The percentage of internalization at 10 µg/ml of Ab were calculated as followed $100 * (MFI \text{ at } 4^{\circ}\text{C} - MFI \text{ at } 37^{\circ}\text{C}) / MFI \text{ at } 4^{\circ}\text{C}$ and presented in Table 15. Therefore, the humanized hz208F2 VH3/VL3 had similar binding and internalization properties as the one measured with the corresponding murine and chimeric 208F2 antibodies.

Table 15

| | ΔMFI | % internalization |
|----------------|------|-------------------|
| m208F2 | 294 | 88 |
| c208F2 | 278 | 82 |
| H208F2 VH3/VL3 | 344 | 87 |

Example 12: Definition of the dissociation constant (K_D) of the binding of five chimeric anti-IGF1R antibodies (c208F2, c213B10, c212A11, c214F8 and c219D6) and a humanized version (VH3/VL3) of the 208F2 antibody on a soluble recombinant human IGF1R

The dissociation constants (K_D) of the binding of the antibodies on a recombinant soluble human-IGF1R were defined by the ratio between the dissociation rate (k_{off}) and the association rate (k_{on}). The kinetic experiments were run on a Biacore X100 device using a CM5 sensor chip activated by a mouse anti-Tag His monoclonal antibody. Around 12000 RU of antibodies are chemically grafted on the carboxymethyl dextran matrix using the amine kit chemistry.

The experiments were carried out at 25°C with a flow rate of 30 µl/min using the HBS-EP+ buffer (GE Healthcare) as the running and sample dilution buffer.

The single cycle kinetic scheme was used to define the kinetic parameters of the binding of the anti-IGF1R antibodies on a soluble recombinant human IGF1R captured
5 by its two C-terminal 10 Histidine-tag.

1- A solution of a soluble recombinant version of the human IGF1R hetero-tetramere: 2α chains and the extracellular domains of 2β chains expressed with an additional C-terminal 10-His tag (R&D Systems catalogue number 305-GR-50) was injected during one minute on the second flowcell
10 at a concentration of 10µg/ml. A mean of 587 RU (with a standard deviation 24 RU) of the soluble receptor were captured at each of the 24 cycles realised for this study.

2- After the capture phase, either the running buffer was injected 5 times (90 s each injection) or a growing range of 5 concentrations of one of
15 the six antibodies was injected (90s each injection) on both flowcells. At the end of the fifth injection the running buffer was passed during 5 minutes in order to define the dissociation rate.

3- The surface was then regenerated with an injection of a 10mM Glycine, HCl pH 1.5 buffer during 45 s.

20 The computed signal corresponds to the difference between the response of the flowcell 2 (with captured IGF1R) and the response of the flowcell 1 (without any IGF1R molecules).

For each IGF1R the signal due to the injections the growing range of concentrations of one antibody was corrected by subtraction of the signal obtained with
25 the 5 injections of the buffer (double reference) see Figure 17.

The resulting sensorgrams were analysed by the Biaevaluation software with a 1:1 model.

Four experiences were run for each antibody using two different ranges of concentrations: 40, 20, 10, 5 and 2.5 nM for the two first experiments and: 24, 12, 6, 3
30 and 1.5 nM for the two last experiments run for each antibody.

For the 6 antibodies tested in this experiment the experimental data fitted well with an 1:1 model with significant k_{off} values when the higher concentration was defined as a constant and the other four concentrations are calculated (see figure 18).

5 The dissociation constants (K_D) calculated as the ratio: $k_{\text{off}}/k_{\text{on}}$ and the half-life of the complexes calculated as the ratio: $\text{Ln}(2)/k_{\text{off}}$ are represented in the Figures 19 and 20. They correspond to the mean of the four independent experiments run for each antibodies. The error bars correspond to the standard errors ($n=4$) of the values.

The dissociation constants are in the range of 10 to 100 pM. The c208F2 antibody presents the weaker affinity (higher dissociation constant value) for the h-
10 IGF1R (with a K_D around 75 pM) and its humanized version is at least as good as the chimeric version (with a K_D around 60 pM). The four other anti-IGF1R chimeric antibodies present a quite similar affinity for the hIGF1-R (with a K_D around 30 pM). The difference of the affinities is principally linked to the dissociation rate or the resultant half life of the complexes. With 208F2 the half-life of the complex is between
15 2 and 3 hour with the chimeric and the humanized (VH3/VL3) versions. For the four other chimeric antibodies the means half lives are between 7.0 and 9.4 h.

These very slow dissociation kinetics are clearly linked to the bivalent structure of the antibodies which are able to bind simultaneously by both of their Fab arms to two adjacent h-IGF1R molecules. In this case the level of captured IGF1R molecules may
20 have an impact on the dissociation rate. The affinities defined in this study correspond to the functional affinities (or avidities) of the antibodies for a level of captured h-IGF1R around 600 RU. The 3 fold difference of K_D observed between data shown above (table 10) and values presented in example 13 is linked to a change of the level of capture of hIGF-1R (600RU versus 160 RU in example 4).

25

Example 13: Generation of 1613F12.

To generate murine monoclonal antibodies (Mabs) against human extracellular domain (ECD) of the Axl receptor, 5 BALB/c mice were immunized 5-times s.c. with
30 15-20.10⁶ CHO-Axl cells and twice with 20 μg of the rh Axl ECD. The first immunization was performed in presence of Complete Freund Adjuvant (Sigma, St Louis, MD, USA). Incomplete Freund adjuvant (Sigma) was added for following immunizations.

Three days prior to the fusion, immunized mice were boosted with both $20 \cdot 10^6$ CHO-Axl cells and 20 μg of the rhAxl ECD with IFA.

To generate hybridomas, splenocytes and lymphocytes were prepared by perfusion of the spleen and by mincing of the proximal lymph nodes, respectively, harvested from 1 out of the 5 immunized mice (selected after sera titration) and fused to SP2/0-Ag14 myeloma cells (ATCC, Rockville, MD, USA). The fusion protocol is described by Kohler and Milstein (Nature, 256:495-497, 1975). Fused cells are then subjected to HAT selection. In general, for the preparation of monoclonal antibodies or their functional fragments, especially of murine origin, it is possible to refer to techniques which are described in particular in the manual "Antibodies" (Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor NY, pp. 726, 1988).

Approximately 10 days after the fusion, colonies of hybrid cells were screened. For the primary screen, supernatants of hybridomas were evaluated for the secretion of Mabs raised against the Axl ECD protein using an ELISA. In parallel, a FACS analysis was performed to select Mabs able to bind to the cellular form of Axl present on the cell surface using both wt CHO and Axl expressing CHO cells.

As soon as possible, selected hybridomas were cloned by limit dilution and subsequently screened for their reactivity against the Axl ECD protein. Cloned Mabs were then isotyped using an Isotyping kit (cat #5300.05, Southern Biotech, Birmingham, AL, USA). One clone obtained from each hybridoma was selected and expanded.

ELISA assays are performed as followed either using pure hybridoma supernatant or, when IgG content in supernatants was determined, titration was realized starting at $5 \mu\text{g}/\text{ml}$. Then a $\frac{1}{2}$ serial dilution was performed in the following 11 rows. Briefly, 96-well ELISA plates (Costar 3690, Corning, NY, USA) were coated $50 \mu\text{l}/\text{well}$ of the rh Axl-Fc protein (R and D Systems, cat N° 154-AL) or rhAxl ECD at $2 \mu\text{g}/\text{ml}$ in PBS overnight at 4°C . The plates were then blocked with PBS containing 0.5% gelatin (#22151, Serva Electrophoresis GmbH, Heidelberg, Germany) for 2 h at 37°C . Once the saturation buffer discarded by flicking plates, $50 \mu\text{l}$ of pure hybridoma cell supernatants or $50 \mu\text{l}$ of a $5 \mu\text{g}/\text{ml}$ solution were added to the ELISA plates and incubated for 1 h at 37°C . After three washes, $50 \mu\text{l}$ horseradish peroxidase-conjugated

polyclonal goat anti-mouse IgG (#115-035-164, Jackson Immuno-Research Laboratories, Inc., West Grove, PA, USA) was added at a 1/5000 dilution in PBS containing 0.1% gelatin and 0.05% Tween 20 (w:w) for 1 h at 37°C. Then, ELISA plates were washed 3-times and the TMB (#UP664782, Uptima, Interchim, France) substrate was added. After a 10 min incubation time at room temperature, the reaction was stopped using 1 M sulfuric acid and the optical density at 450 nm was measured.

For the selection by flow cytometry, 10^5 cells (CHO wt or CHO-Ax1) were plated in each well of a 96 well-plate in PBS containing 1% BSA and 0.01% sodium azide (FACS buffer) at 4°C. After a 2 min centrifugation at 2000 rpm, the buffer was removed and hybridoma supernatants or purified Mabs (1 µg/ml) to be tested were added. After 20 min of incubation at 4°C, cells were washed twice and an Alexa 488-conjugated goat anti-mouse antibody 1/500° diluted in FACS buffer (#A11017, Molecular Probes Inc., Eugene, USA) was added and incubated for 20 min at 4°C. After a final wash with FACS buffer, cells were analyzed by FACS (FacsCalibur, Becton-Dickinson) after addition of propidium iodide to each tube at a final concentration of 40 µg/ml. Wells containing cells alone and cells incubated with the secondary Alexa 488-conjugated antibody were included as negative controls. Isotype controls were used in each experiment (Sigma, ref M90351MG). At least 5000 cells were assessed to calculate the mean value of fluorescence intensity (MFI).

The hybridoma producing the 1613F12 was selected as a candidate.

Example 14: Humanization of 1613F12

The use of mouse antibodies (Mabs) for therapeutic applications in humans generally results in a major adverse effect, patients raise a human anti-mouse antibody (HAMA) response, thereby reducing the efficacy of the treatment and preventing continued administration. One approach to overcome this problem is to humanize mouse Mabs by replacing mouse sequences by their human counterpart but without modifying the antigen binding activity. This can be achieved in two major ways: (i) by construction of mouse/human chimeric antibodies where the mouse variable regions are joined to human constant regions (Boulianne *et al.*, 1984) and (ii) by grafting the complementarity determining regions (CDRs) from the mouse variable regions into

carefully selected human variable regions and then joining these "re-shaped human" variable regions to human constant regions (Riechmann *et al.*, 1988).

14.1 Humanization of the light chain variable domain VL

As a preliminary step, the nucleotide sequence of 1613F12 VL was compared to
5 the murine germline gene sequences part of the IMGT database (<http://www.imgt.org>).
Murine IGKV16-104*01 and IGKJ5*01 germline genes were identified. In order to
identify the best human candidate for the CDR grafting, the human germline gene
displaying the best identity with 1613F12 VL murine sequence has been searched. With
the help of the IMGT database analyses tools, a possible acceptor human V regions for
10 the murine 1613F12 VL CDRs was identified: IGKV1-27*01 and IGKJ4*02. In order
to perform the humanization to the light chain variable domain each residue which is
different between the human and mouse sequences was given a priority rank order.
These priorities (1-4) were used to create 11 different humanized variants of the light
chain variable region with up to 14 backmutations.

| | FR1-IMGT | CDR1-IMGT | FR2-IMGT | CD |
|-----------------------|-----------------------------|------------------------------|-------------------|----|
| 1613F12VL | DVQITQSPSYLATS PGETITINCRAS | KSI.....SKY | LAWYQEKPGKTNKLLIY | SG |
| Homsap IGKV1-27*01 | DIQMTQSPSSLSASVGD RVTITCRAS | QGI.....SNY | LAWYQQKPGKVPKLLIY | AA |
| Priority | V I 1 | Y A T P E T I N 3 34 4 433 2 | E T N 3 33 | |
| hz1613F12 (VL1) | DIQMTQSPSSLSASVGD RVTITCRAS | KSI.....SKY | LAWYQQKPGKVPKLLIY | SG |
| hz1613F12 (VL1I2V) | DVQMTQSPSSLSASVGD RVTITCRAS | KSI.....SKY | LAWYQQKPGKVPKLLIY | SG |
| hz1613F12 (VL1M4I) | DIQITQSPSSLSASVGD RVTITCRAS | KSI.....SKY | LAWYQQKPGKVPKLLIY | SG |
| hz1613F12 (VL2.1) | DVQITQSPSSLSASVGD RVTITCRAS | KSI.....SKY | LAWYQQKPGKVPKLLIY | SG |
| hz1613F12 (VL2.1V49T) | DVQITQSPSSLSASVGD RVTITCRAS | KSI.....SKY | LAWYQQKPGKVPKLLIY | SG |
| hz1613F12 (VL2.1P50N) | DVQITQSPSSLSASVGD RVTITCRAS | KSI.....SKY | LAWYQQKPGKVPKLLIY | SG |
| hz1613F12 (VL2.2) | DVQITQSPSSLSASVGD RVTINCRAS | KSI.....SKY | LAWYQQKPGKVPKLLIY | SG |
| hz1613F12 (VL2.2V49T) | DVQITQSPSSLSASVGD RVTINCRAS | KSI.....SKY | LAWYQQKPGKVPKLLIY | SG |
| hz1613F12 (VL2.2P50N) | DVQITQSPSSLSASVGD RVTINCRAS | KSI.....SKY | LAWYQQKPGKVPKLLIY | SG |
| hz1613F12 (VL2.3) | DVQITQSPSSLSASVGD RVTINCRAS | KSI.....SKY | LAWYQEKPGKTNKLLIY | SG |
| hz1613F12 (VL3) | DVQITQSPSYLAASVGD TITINCRAS | KSI.....SKY | LAWYQEKPGKTNKLLIY | SG |

| | R2-IMGT | FR3-IMGT |
|-----------------------|---|-----------------|
| 1613F12VL |S TLQSGVP.SRFSGSG..SGTDFTLTISSELEPEDFAMYFC | |
| Homsap IGKV1-27*01 |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYYC | |
| Priority | | E F M F 4 4 4 2 |
| hz1613F12 (VL1) |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYYC | |
| hz1613F12 (VL1I2V) |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYYC | |
| hz1613F12 (VL1M4I) |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYYC | |
| hz1613F12 (VL2.1) |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYYC | |
| hz1613F12 (VL2.1V49T) |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYYC | |
| hz1613F12 (VL2.1P50N) |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYYC | |
| hz1613F12 (VL2.2) |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYFC | |
| hz1613F12 (VL2.2V49T) |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYFC | |
| hz1613F12 (VL2.2P50N) |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYFC | |
| hz1613F12 (VL2.3) |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYFC | |
| hz1613F12 (VL3) |S TLQSGVP.SRFSGSG..SGTDFTLTISLQPEDVATYFC | |

| | CDR3-IMGT | FR4-IMGT |
|-----------------------|-----------|----------------|
| 1613F12VL | QQHHEYPLT | FGAGTELELK |
| Homsap IGKJ4*02 | LT | FGGGTKVEIK |
| Priority | | A E L L 3 33 4 |
| hz1613F12 (VL1) | QQHHEYPLT | FGGGTKVEIK |
| hz1613F12 (VL1I2V) | QQHHEYPLT | FGGGTKVEIK |
| hz1613F12 (VL1M4I) | QQHHEYPLT | FGGGTKVEIK |
| hz1613F12 (VL2.1) | QQHHEYPLT | FGGGTKVEIK |
| hz1613F12 (VL2.1V49T) | QQHHEYPLT | FGGGTKVEIK |
| hz1613F12 (VL2.1P50N) | QQHHEYPLT | FGGGTKVEIK |
| hz1613F12 (VL2.2) | QQHHEYPLT | FGGGTKVEIK |
| hz1613F12 (VL2.2V49T) | QQHHEYPLT | FGGGTKVEIK |
| hz1613F12 (VL2.2P50N) | QQHHEYPLT | FGGGTKVEIK |
| hz1613F12 (VL2.3) | QQHHEYPLT | FGGGTKVEIK |
| hz1613F12 (VL3) | QQHHEYPLT | FGAGTELELK |

14.2 Humanization of the heavy chain variable domain VH

In order to identify the best human candidate for the CDR grafting, the mouse and human germline genes displaying the best identity with 1613F12 VH were searched. The nucleotide sequence of 1613F12 VH was aligned with both mouse and human germline gene sequences by using the sequence alignment software “IMGT/V-QUEST” which is part of the IMGT database. Alignments of amino acid sequences were also performed to verify the results of the nucleotide sequence alignment using the “Align X” software of the VectorNTI package. The alignment with mouse germline genes showed that the mouse germline V-gene IGHV14-3*02 and J-gene IGHJ2*01 are the most homologue mouse germline genes. Using the IMGT database the mouse D-gene germline IGHD1-1*01 was identified as homologous sequence. In order to select an appropriate human germline for the CDR grafting, the human germline gene with the highest homology to 1613F12 VH murine sequence was identified. With the help of IMGT databases and tools, the human IGHV1-2*02 germline gene and human IGHJ5*01 J germline gene were selected as human acceptor sequences for the murine 1613F12 VH CDRs. In order to perform the humanization to the heavy chain variable domain each residue which is different between the human and mouse sequences was given a priority rank order (1-4). These priorities were used to create 20 different humanized variants of the heavy chain variable region with up to 18 backmutations,

Example 15: Axl binding specificity

In this example, the binding of 1613F12 was first studied on the rhAxl-Fc protein. Then, its binding on the two other members of the TAM family, rhDtk-Fc and rhMer-Fc, was studied.

5 Briefly, the recombinant human Axl-Fc (R and D systems, cat N° 154AL/CF), rhDtk (R and D Systems, cat N° 859-DK) or rhMer-Fc (R and D Systems, cat N° 891-MR) proteins were coated overnight at 4°C to Immulon II™ 96-well plates and, after a 1 h blocking step with a 0.5% gelatine solution, 1613F12 was added for an additional 1 h at 37°C at starting concentration of 5 µg/ml ($3.33 \cdot 10^{-8}$ M). Then ½ serial dilutions
10 were done over 12 columns. Plates were washed and a goat anti-mouse (Jackson) specific IgG-HRP was added for 1 h at 37°C. Reaction development was performed using the TMB substrate solution. The isotype control antibody mIgG1 and the commercial anti-Axl Mab 154 antibody were also used in parallel. Coating controls were performed in presence of a goat anti-human IgG Fc polyclonal serum labelled with
15 HRP (Jackson, ref 109-035-098) and/or in presence of a HRP-coupled anti-Histidine antibody (R and D Systems, ref : MAB050H).

Results are represented in Figures 24A, 24B and 24C, respectively.

This example shows that 1613F12 only binds to the rhAxl-Fc protein and does not bind on the two other members of the TAM family, rhDtk or rhMer. No cross-
20 specificity of binding of 1613F12 is observed between TAM members. No non specific binding was observed in absence of primary antibody (diluant). No binding was observed in presence of the isotype control antibody.

**Example 16: 1613F12 recognized the cellular form of Axl expressed on
25 human tumor cells.**

Cell surface Axl expression level on human tumor cells was first established using a commercial Axl antibody (R and D Systems, ref: MAB154) in parallel of calibration beads to allow the quantification of Axl expression level. Secondly, binding of the cell-surface Axl was studied using 1613F12.

30 For cell surface binding studies, two fold serial dilutions of a 10 µg/ml ($6.66 \cdot 10^{-8}$ M) primary antibody solution (1613F12, MAB154 or mIgG1 isotype control 9G4 Mab) are prepared and are applied on $2 \cdot 10^5$ cells for 20 min at 4°C. After 3 washes in

phosphate-buffered saline (PBS) supplemented with 1% BSA and 0.01% NaN₃, cells were incubated with secondary antibody Goat anti-mouse Alexa 488 (1/500° dilution) for 20 minutes at 4°C. After 3 additional washes in PBS supplemented with 1% BSA and 0.1% NaN₃, cells were analyzed by FACS (FacsCalibur, Becton-Dickinson). At least 5000 cells were assessed to calculate the mean value of fluorescence intensity.

For quantitative ABC determination using MAB154, QIFIKIT® calibration beads are used. Then, the cells are incubated, in parallel with the QIFIKIT® beads, with Polyclonal Goat Anti-Mouse Immunoglobulins/FITC, Goat F(ab')₂, at saturating concentration. The number of antigenic sites on the specimen cells is then determined by interpolation of the calibration curve (the fluorescence intensity of the individual bead populations against the number of Mab molecules on the beads).

16.1. Quantification of cell-surface Axl expression level

Axl expression level on the surface of human tumor cells was determined by flow cytometry using indirect immunofluorescence assay (QIFIKIT® method (Dako, Denmark), a quantitative flow cytometry kit for assessing cell surface antigens. A comparison of the mean fluorescence intensity (MFI) of the known antigen levels of the beads via a calibration graph permits determination of the antibody binding capacity (ABC) of the cell lines.

Table 16 presents Axl expression level detected on the surface of various human tumor cell lines (SN12C, Calu-1, MDA-MB435S, MDA-MB231, NCI-H125, MCF7, Panc1) as determined using QIFIKIT® using the MAB154 (R and D Systems). Values are given as Antigen binding complex (ABC).

Table 16

| | MCF7 | NCI-H125 | MDA-MB-435S | Panc1 | MDA-MB-231 | Calu-1 | SN12C |
|------------------|--------|----------|-------------|----------|------------|----------|-----------|
| Tumor type/organ | Breast | NSCLC | Breast | Pancreas | Breast | Lung | Renal |
| ABC (Qifikit) | 71 | 5 540 | 17 814 | 36 809 | 61 186 | >100 000 | > 100 000 |

Results obtained with MAB154 showed that Axl receptor is expressed at various levels depending of the considered human tumor cell.

16.2. Axl detection by 1613F12 on human tumor cells

5 More specifically, Axl binding was studied using 1613F12.

1613F12 dose response curves were prepared. MFIs obtained using the various human tumor cells were then analysed with Prism software. Data are presented in Figure 25.

10 Data indicate that 1613F12 binds specifically to the membrane Axl receptor as attested by the saturation curve profiles. However different intensities of labelling were observed, revealing variable levels of cell-surface Axl receptor on human tumor cells. No binding of Axl receptor was observed using MCF7 human breast tumor cell line.

Example 17: Validation of hz1613F12 vs. m1613F12

15 In order to establish whether hz1613F12 was comparable to its murine form, binding experiments were performed by ELISA using rhAxl-Fc protein assays.

In this assay, 96 well plates (Immulon II, Thermo Fisher) were coated with a 5 µg/ml of 1613F12 solution in 1x PBS, overnight at 4°C. After a saturation step, a range of rh Axl-Fc protein (R and D Systems, ref: 154-AL) is incubated for 1 hour at 37°C on
20 the coated plates. For the revelation step, a biotinylated-Axl antibody (in house product) was added at 0.85µg/ml for 1 hour at 37°C. This Axl antibody belongs to a distinct epitopic group. Then an avidin-horseradish peroxidase solution at 1/2000° in diluent buffer is added to the wells. Then the TMB substrate solution is added for 5 min. After addition of the peroxydase stop solution, the absorbance at 405 nm was measured with a
25 microplate reader.

Figure 26 shows that both murine and humanized versions of 1613F12 bind similarly the rhAxl-Fc protein.

Example 18: 1613F12 internalization study using fluorescent immunocytochemistry labelling.

30 Complementary internalization results are obtained by confocal microscopy using indirect fluorescent labelling method.

Briefly, SN12C tumor cell line was cultured in RPMI1640 with 1 % L-glutamine and 10 % of FCS for 3 days before experiment. Cells were then detached using trypsin and plated in 6-multiwell plate containing coverslide in RPMI1640 with 1 % L-glutamine and 5 % FCS. The next day, 1613F12 was added at 10 µg/ml. Cells treated with an irrelevant antibody were also included. The cells were then incubated for 1 h and 2 h at 37°C, 5% CO₂. For T 0 h, cells were incubated for 30 minutes at 4°C to determine antibody binding on cell surface. Cells were washed with PBS and fixed with paraformaldehyde for 15 minutes. Cells were rinsed and incubated with a goat anti-mouse IgG Alexa 488 antibody for 60 minutes at 4°C to identify remaining antibody on the cell surface. To follow antibody penetration into the cells, cells were fixed and permeabilized with saponin. A goat anti-mouse IgG Alexa 488 (Invitrogen) was used to stained both the membrane and the intracellular antibody. Early endosomes were identified using a rabbit polyclonal antibody against EEA1 revealed with a goat anti-rabbit IgG-Alexa 555 antibody (Invitrogen). Cells were washed three times and nuclei were stained using Draq5. After staining, cells were mounted in Prolong Gold™ mounting medium (Invitrogen) and analyzed by using a Zeiss LSM 510 confocal microscope. Photographs are presented in Figures 2727-4C.

Images were obtained by confocal microscopy. In presence of the mIgG1 isotype control (9G4), neither membrane staining nor intracellular labelling is observed (Figure 27A). A progressive loss of the membrane anti-Axl labelling is observed as soon as after 1 h incubation of the SN12C cells with 1613F12 (Figure 27B). Intracellular accumulation of 1613F12 antibody is clearly observed at 1 h and 2 h (Figure 27C). Intracellular antibody co-localizes with EEA1, an early endosome marker. These photographs confirm the internalization of 1613F12 into SN12C cells.

25

Example 19: Synthesis of the drugs of the invention

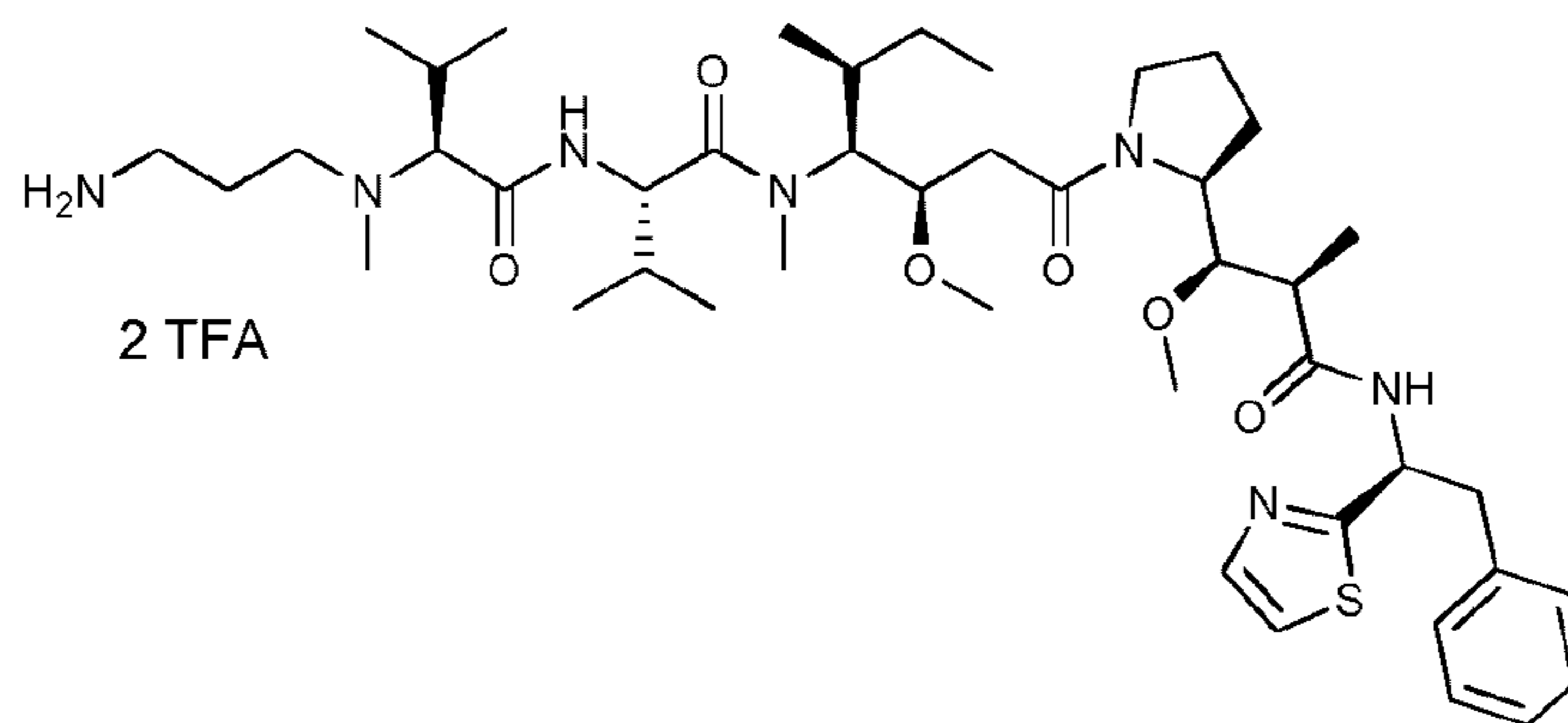
The following abbreviations are used in the following examples:

| | |
|-------|-------------------------|
| aq. | aqueous |
| ee | enantiomeric excess |
| equiv | equivalent |
| ESI | Electrospray ionisation |

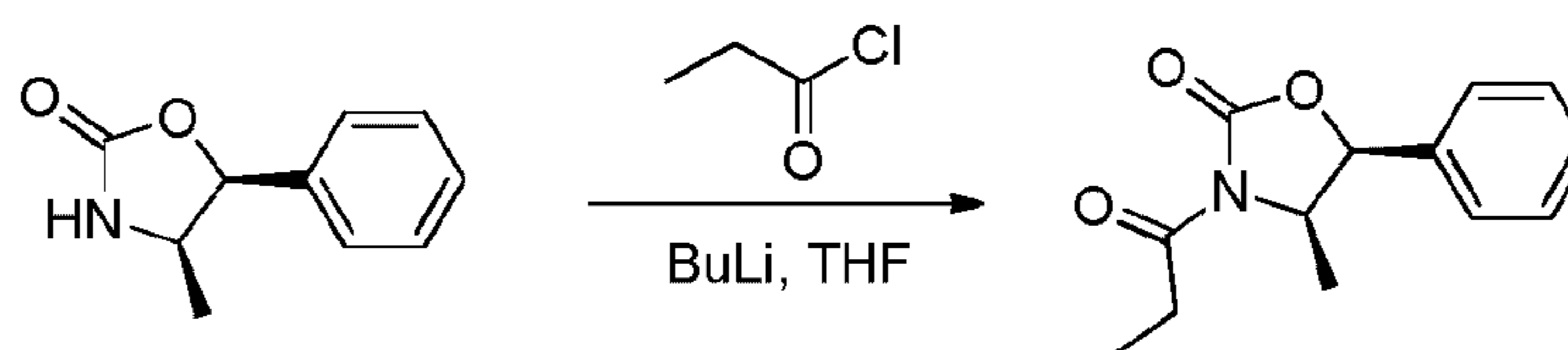
| | | |
|---|-------|--|
| | LC/MS | Liquid Chromatography coupled with Mass Spectrometry |
| | HPLC | High Performance Liquid Chromatography |
| | NMR | Nuclear Magnetic Resonance |
| | sat. | saturated |
| 5 | UV | ultraviolet |

Compound 1

(S)-2-((S)-2-((3-aminopropyl)(methyl)amino)-3-methylbutanamido)-N-
 ((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-
 10 2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-
 oxoheptan-4-yl)-N,3-dimethylbutanamide, bis trifluoroacetic acid



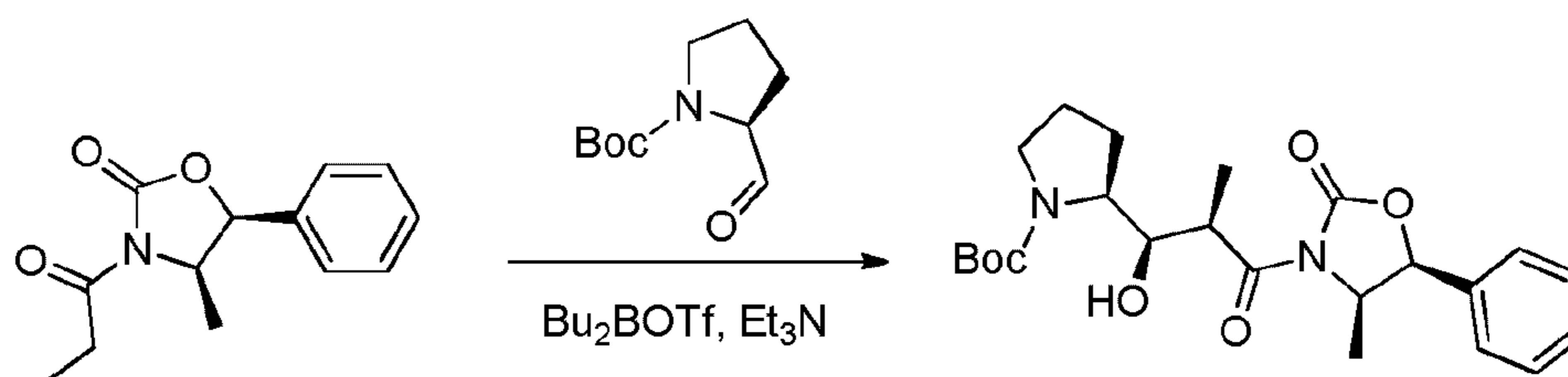
Compound 1A: (4R, 5S)-4-methyl-5-phenyl-3-propanoyl-1,3-oxazolidin-2-
 one



15 (4R, 5S)-4-methyl-5-phenyl-1,3-oxazolidin-2-one (5.8 g, 32.7 mmol, 1.00
 equiv) was dissolved in tetrahydrofuran (THF, 120 mL) in an inert atmosphere. The
 mixture was cooled to -78°C and *n*-butyllithium (14.4 mL) was added drop-wise. After
 agitation for 30 minutes at -78°C , propanoyl chloride (5.7 mL) was added. Agitation
 was continued for 30 minutes at -78°C then overnight at ambient temperature. The
 20 reaction mixture was concentrated then re-dissolved in 200 mL of water. The pH of the
 solution was adjusted to 7 with sodium bicarbonate saturated aqueous solution. This
 aqueous phase was extracted 3 times with 100 mL of ethyl acetate (EtOAc). The

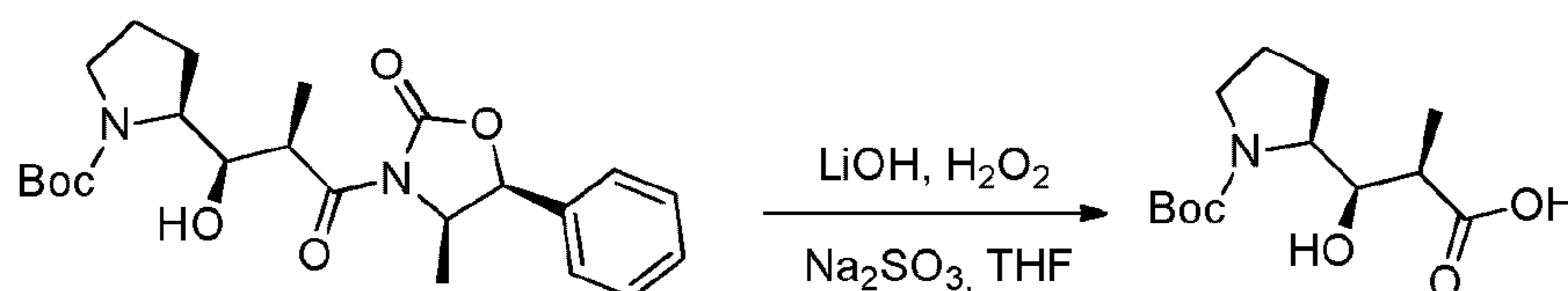
organic phases were combined, dried over sodium sulfate, filtered and concentrated to yield 6.8 g (89 %) of compound **1A** in the form of a yellow oil.

Compound 1B: *tert*-butyl (2S)-2-[(1R,2R)-1-hydroxy-2-methyl-3-[(4R,5S)-4-methyl-2-oxo-5-phenyl-1,3-oxazolidin-3-yl]-3-oxopropyl]pyrrolidine-1-carboxylate



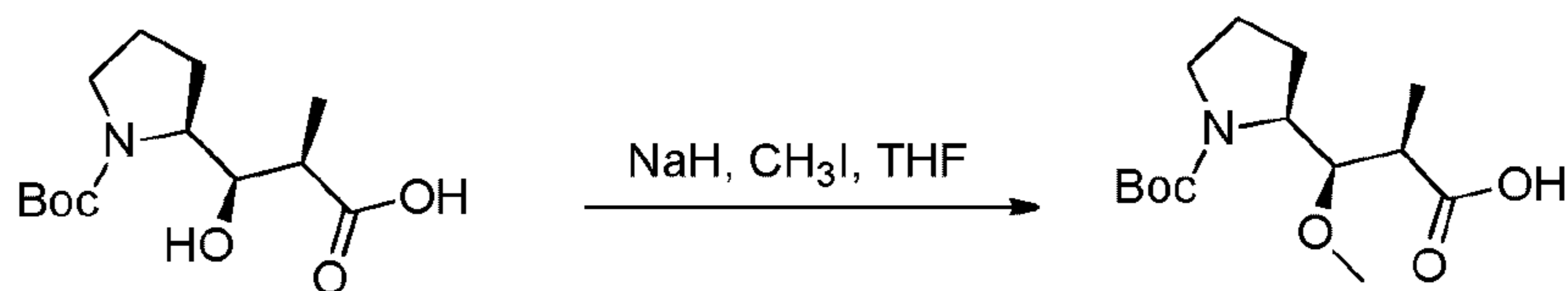
Compound **1A** (17.6 g, 75.45 mmol, 1.00 equiv) was dissolved in dichloromethane (DCM, 286 mL) in an inert atmosphere. This solution was cooled with an ice bath. Triethylamine (TEA, 12.1 mL, 1.15 equiv) and Bu_2BOTf (78.3 mL, 1.04 equiv) were added drop-wise whilst holding the temperature of the reaction mixture below 2°C . Agitation was continued at 0°C for 45 minutes, after which the reaction was cooled to -78°C . A solution of *tert*-butyl (2S)-2-formylpyrrolidine-1-carboxylate (8.5 g, 42.66 mmol, 0.57 equiv) in DCM (42 mL) was added drop-wise. Agitation was continued for 2 hours at -78°C , then for 1 hour at 0°C and finally 1 hour at ambient temperature. The reaction was neutralised with 72 mL of phosphate buffer (pH = 7.2 - 7.4) and 214 mL methanol, and cooled to 0°C . A solution of 30 % hydrogen peroxide in methanol (257 mL) was added drop-wise whilst maintaining the temperature below 10°C . Agitation was continued for 1 hour at 0°C . The reaction was neutralised with 142 mL of water, then concentrated under reduced pressure. The resulting aqueous solution was extracted 3 times with 200 mL EtOAc. The organic phases were combined, dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of EtOAc and petroleum ether (EtOAc:PE = 1:8) to yield 13.16 g (40 %) of compound **1B** in the form of a colourless oil.

Compound 1C: (2R,3R)-3-[(2S)-1-[(*tert*-butoxy)carbonyl]pyrrolidin-2-yl]-3-hydroxy-2-methylpropanoic acid

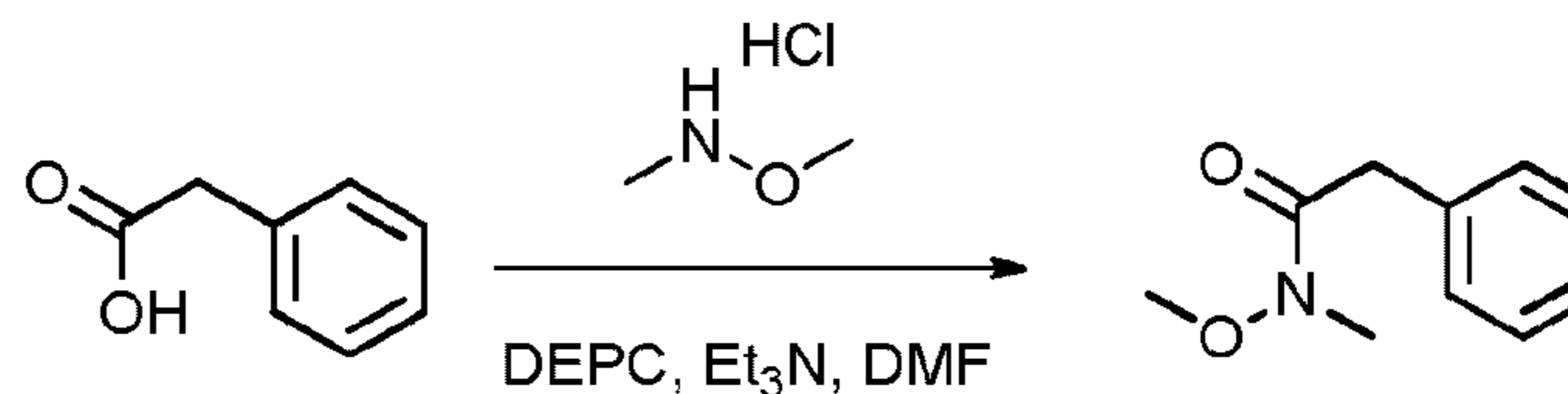


Compound **1B** (13.16 g, 30.43 mmol, 1.00 equiv) was dissolved in THF (460 mL) in the presence of hydrogen peroxide (30 % in water, 15.7 mL), then cooled with an ice bath. An aqueous solution of lithium hydroxide (0.4 mol/L, 152.1 mL) was added drop-wise whilst holding the reaction temperature below 4°C. The reaction mixture was agitated 2.5 hours at 0°C. An aqueous solution of Na₂SO₃ (1 mol/L, 167.3 mL) was added drop-wise whilst holding the temperature at 0°C. The reaction mixture was agitated 14 hours at ambient temperature, then neutralised with 150 mL of cold sodium bicarbonate saturated solution and washed 3 times with 50 mL of DCM. The pH of the aqueous solution was adjusted to 2–3 with a 1M aqueous solution of KHSO₄. This aqueous solution was extracted 3 times with 100 mL of EtOAc. The organic phases were combined, washed once with saturated NaCl solution, dried over sodium sulfate, filtered and concentrated to yield 7.31 g (88 %) of compound **1C** in the form of a colourless oil.

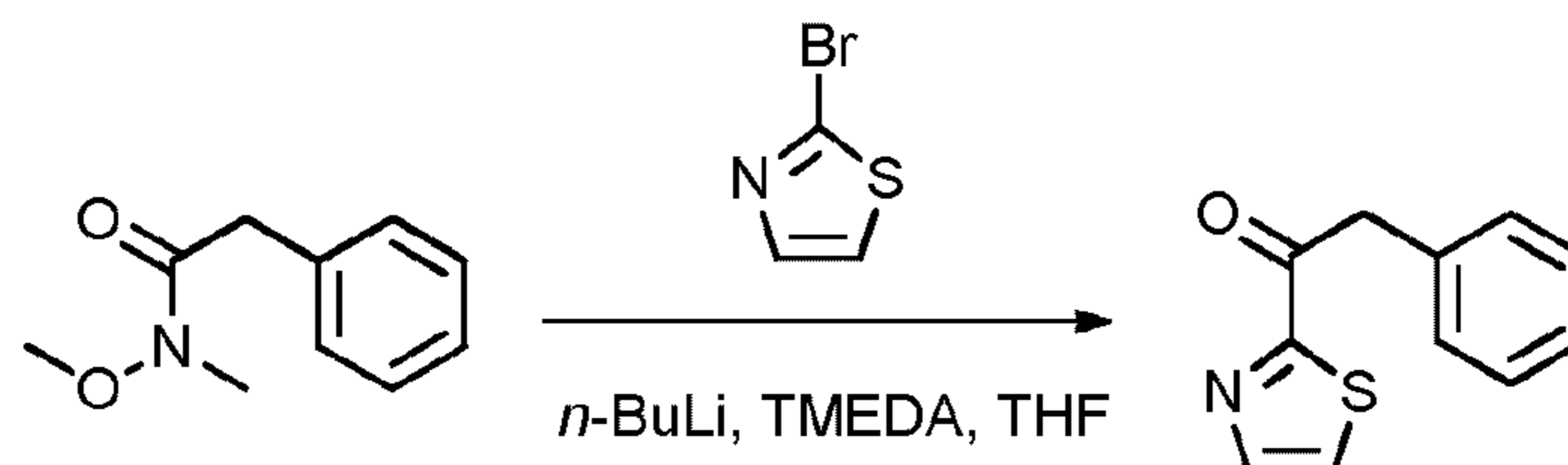
Compound 1D: (2R,3R)-3-[(2S)-1-[(*tert*-butoxy)carbonyl]pyrrolidin-2-yl]-3-methoxy-2-methylpropanoic acid



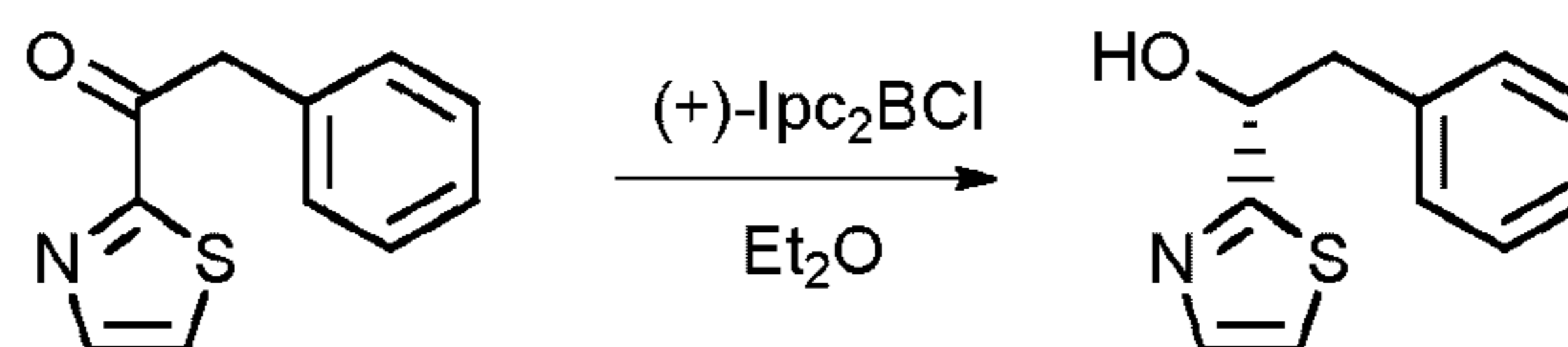
Compound **1C** (7.31 g, 26.74 mmol, 1.00 equiv) was dissolved in an inert atmosphere in THF (135 mL) in the presence of iodomethane (25.3 mL). The reaction medium was cooled with an ice bath after which NaH (60 % in oil, 4.28 g) was added in portions. The reaction was left under agitation 3 days at 0°C and then neutralised with 100 mL of sodium bicarbonate saturated aqueous solution and washed 3 times with 50 mL ether. The pH of the aqueous solution was adjusted to 3 with 1M aqueous KHSO₄ solution. This aqueous solution was extracted 3 times with 100 mL of EtOAc. The organic phases were combined, washed once with 100 mL of Na₂S₂O₃ (5 % in water), once with NaCl-saturated solution, then dried over sodium sulfate, filtered and concentrated to yield 5.5 g (72 %) of compound **1D** in the form of a colourless oil.

Compound 1E: *N*-methoxy-*N*-methyl-2-phenylacetamide

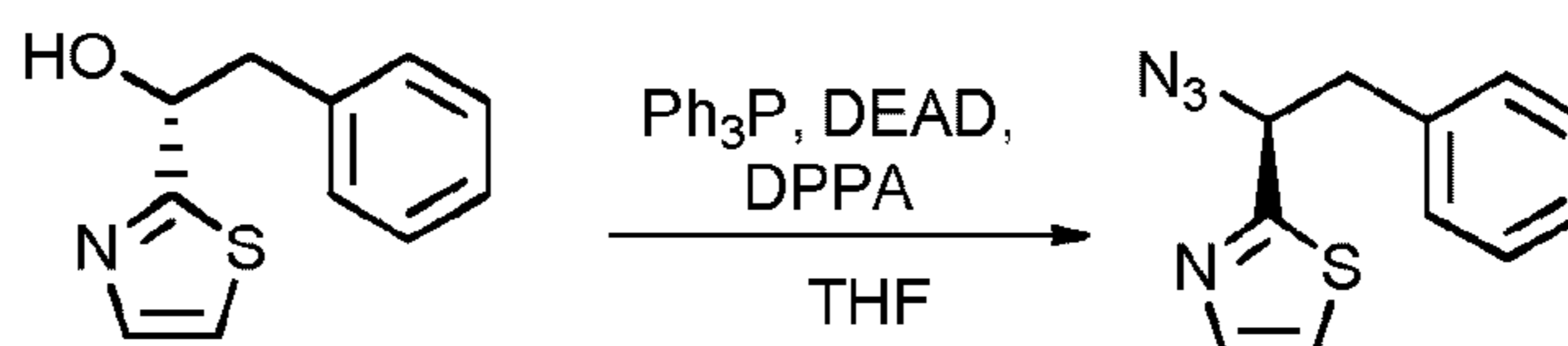
2-phenylacetic acid (16.2 g, 118.99 mmol, 1.00 equiv) was dissolved in dimethylformamide (DMF, 130 mL) then cooled to -10°C . Diethyl phosphorocyanidate (DEPC, 19.2 mL), methoxy(methyl)amine hydrochloride (12.92 g, 133.20 mmol, 1.12 equiv) and triethylamine (33.6 mL) were added. The reaction mixture was agitated 30 minutes at -10°C then 2.5 hours at ambient temperature. It was then extracted twice with 1 litre of EtOAc. The organic phases were combined, washed twice with 500 mL of NaHCO₃ (sat.), once with 400 mL of water, then dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with an EtOAc and PE mixture (1:100 to 1:3) to yield 20.2 g (95 %) of compound **1E** in the form of a yellow oil.

Compound 1F: 2-phenyl-1-(1,3-thiazol-2-yl)ethan-1-one

Tetramethylethylenediamine (TMEDA, 27.2 mL) was dissolved in THF (300 mL) in an inert atmosphere, then cooled to -78°C before the drop-wise addition of *n*-BuLi (67.6 mL, 2.5 M). 2-bromo-1,3-thiazole (15.2 mL) was added drop-wise and agitation was continued 30 minutes at -78°C . Compound **1E** (25 g, 139.50 mmol, 1.00 equiv) dissolved in THF (100 mL) was added drop-wise. Agitation was continued for 30 minutes at -78°C then 2 hours at -10°C . The reaction was neutralised with 500 mL of KHSO₄ (sat.), then extracted 3 times with 1 litre of EtOAc. The organic phases were combined, washed twice with 400 mL water and twice with 700 mL of NaCl (sat.), then dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of EtOAc and PE (1:100 to 1:10) to yield 25 g (88 %) of compound **1F** in the form of a yellow oil.

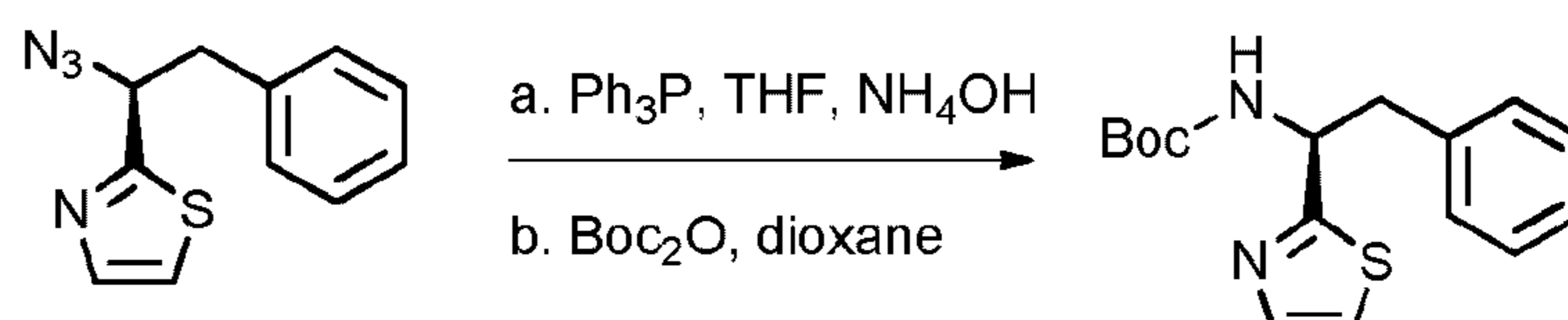
Compound 1G: (1R)-2-phenyl-1-(1,3-thiazol-2-yl)ethan-1-ol

In an inert atmosphere, a solution of compound **1F** (15 g, 73.8 mmol, 1.00 equiv.) in ether (300 mL) was added drop-wise to (+)-B-chlorodiisopinocampheylborane ((+)-Ipc₂BCl, 110.8 mL). The reaction mixture was agitated 24 hours at 0°C, then neutralised with 300 mL of a (1:1) mixture of NaOH (10 % in water) and H₂O₂ (30 % in water), and finally extracted three times with 500 mL of EtOAc. The organic phases were combined, washed twice with 300 mL of K₂CO₃ (sat.) and once with 500 mL of NaCl (sat.), then dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of EtOAc and PE (1:20 to 1:2) to yield 6.3 g (42 %) of compound **1G** in the form of a white solid.

Compound 1H: 2-[(1S)-1-azido-2-phenylethyl]-1,3-thiazole

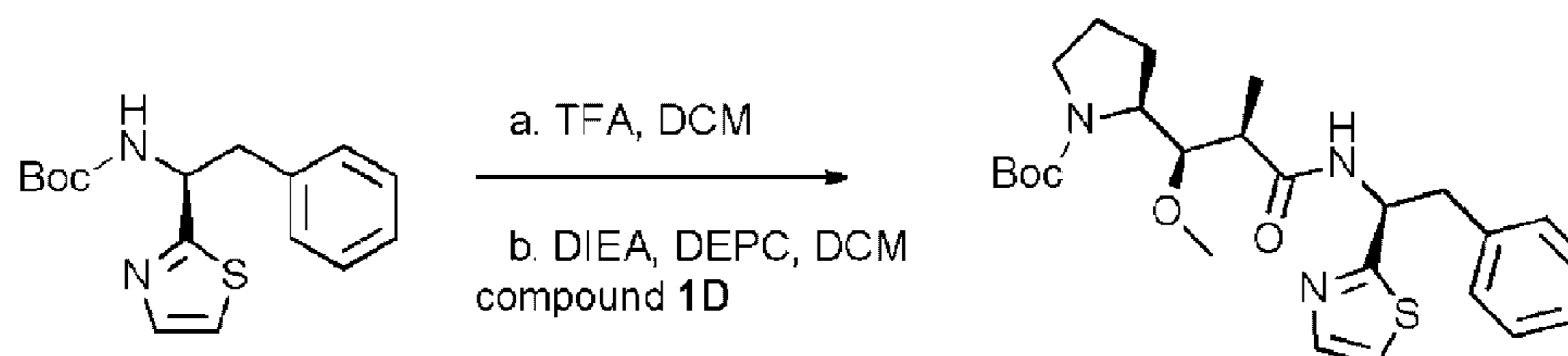
Compound **1G** (6 g, 29.23 mmol, 1.00 equiv.) was dissolved in an inert atmosphere in THF (150 mL) in the presence of triphenylphosphine (13 g, 49.56 mmol, 1.70 equiv.), then cooled to 0°C. Diethylazodicarboxylate (DEAD, 7.6 mL) was added drop-wise, followed by diphenylphosphoryl azide (DPPA, 11 mL), the cold bath was then removed and the solution was left under agitation 48 hours at ambient temperature. The medium was concentrated under reduced pressure. The residue was purified on a silica column with a mixture of EtOAc and PE (1:100 to 1:30) to yield 8 g of partly purified compound **1H** in the form of a yellow oil. Compound **1H** was used as such in the following step.

Compound 1I: *tert*-butyl *N*-[(1S)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl] carbamate.



Compound **1H** (6.5 g, 28.2 mmol, 1.00 equiv) was dissolved in an inert atmosphere in THF (100 mL) in the presence of triphenylphosphine (6.5 g, 33.9 mmol, 1.20 equiv.), and heated to 50°C for 2 hours. Ammonia (70 mL) was then added and heating was continued for 3 hours. The reaction was cooled, neutralised with 500 mL water, then extracted 3 times with 500 mL of EtOAc. The organic phases were combined and extracted twice with 500 mL of 1N HCl. The aqueous phases were combined, brought to pH 8–9 by adding a sodium hydroxide solution (10 % in water), then extracted 3 times with 500 mL of DCM. The organic phases were combined, dried over sodium sulfate, filtered and concentrated to yield 4.8 g (83 %) of (1S)-2-phenyl-1-(1,3-thiazol-2-yl)ethan-1-amine in the form of a yellow oil. This compound was then protected with a Boc group ((*tert*-butoxy)carbonyl) so that it could be purified. It was dissolved in an inert atmosphere in 1,4-dioxane (40 mL), then cooled to 0°C. (Boc)₂O (10.26 g, 47.01 mmol, 2.00 equiv) diluted in 20 mL of 1,4-dioxane was added drop-wise. The cold bath was removed and the solution left under agitation overnight at ambient temperature before being neutralised with 300 mL of water and extracted twice with 500 mL of EtOAc. The organic phases were combined, dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of EtOAc and PE (1:100 to 1:20, ee = 93 %). It was then recrystallized in a hexane/acetone mixture (~ 5–10 / 1, 1g / 10 mL) to yield 6 g (84 %) of compound **1I** in the form of a white solid (ee > 99 %).

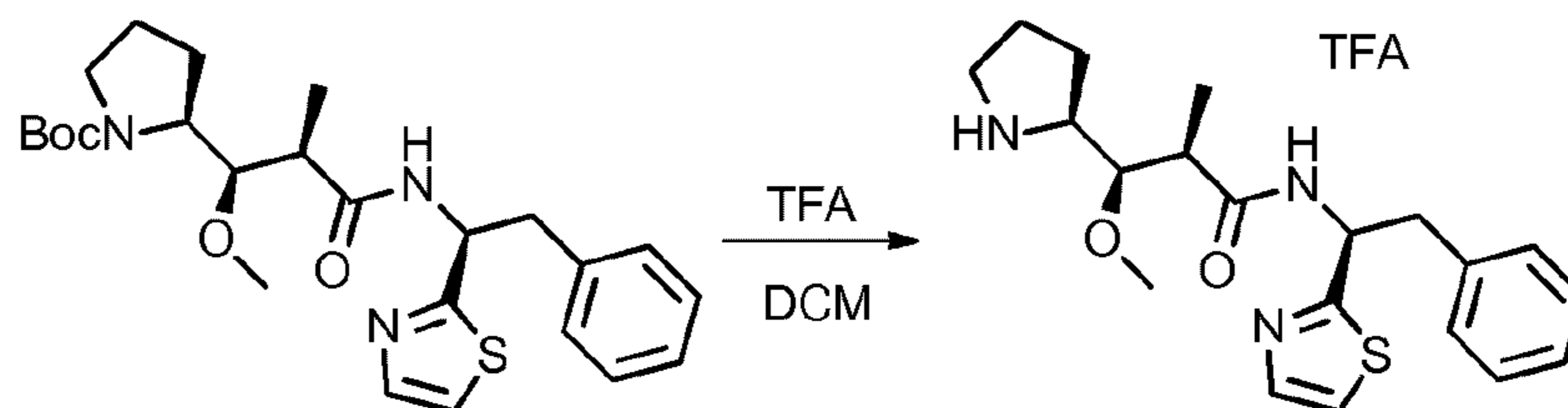
Compound 1J: *tert*-butyl (2S)-2-[(1R,2R)-1-methoxy-2-methyl-2-[[[(1S)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]carbamoyl]ethyl]pyrrolidine-1-carboxylate



Compound **1I** (3 g, 9.86 mmol, 1.00 equiv) was dissolved in an inert atmosphere in 10 mL DCM. Trifluoroacetic acid (TFA, 10 mL) was added and the solution left under agitation overnight at ambient temperature, then concentrated under reduced pressure to yield 2.0 g (64 %) of (1S)-2-phenyl-1-(1,3-thiazol-2-yl)ethan-1-amine; trifluoroacetic acid in the form of a yellow oil. This intermediate was re-dissolved in 20 mL of DCM after which compound **1D** (1.8 g, 6.26 mmol, 1.05 equiv), DEPC (1.1 g,

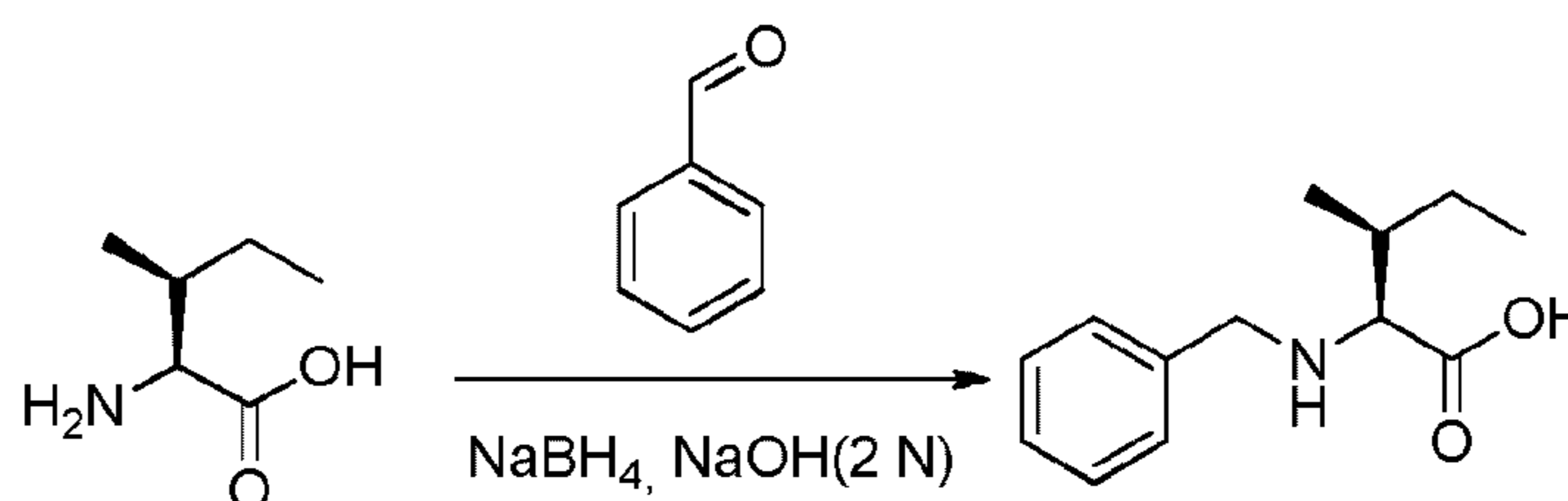
6.75 mmol, 1.13 equiv) and diisopropylethylamine (DIEA, 1.64 g, 12.71 mmol, 2.13 equiv) were added. The reaction mixture was left under agitation overnight at ambient temperature, then concentrated under reduced pressure. The residue was purified on a silica column with a mixture of EtOAc and PE (1:100 to 1:3) to yield 2.3 g (81 %) of compound **1J** in the form of a pale yellow solid.

Compound 1K: (2R,3R)-3-methoxy-2-methyl-N-[(1S)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]-3-[(2S)-pyrrolidin-2-yl]propanamide; trifluoroacetic acid

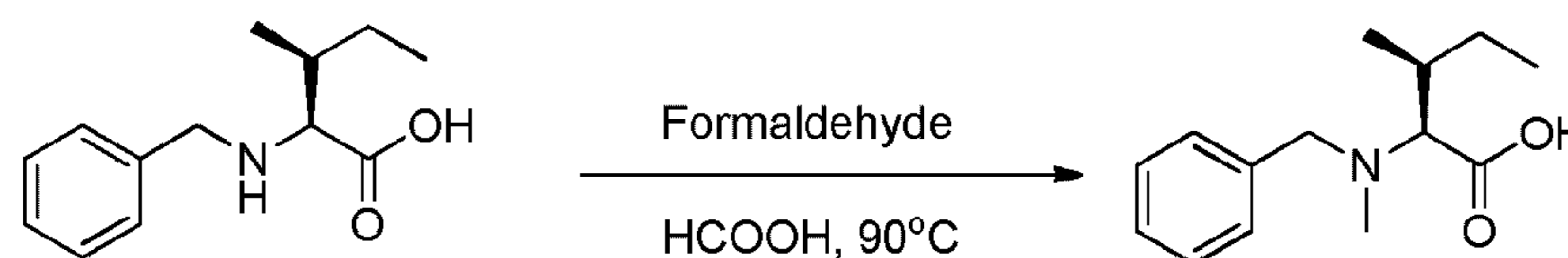


Compound **1J** (2.25 g, 4.75 mmol, 1.00 equiv) was dissolved in an inert atmosphere in 10 mL of DCM. TFA (10 mL) was added and the solution left under agitation overnight at ambient temperature, then concentrated under reduced pressure to yield 2.18 g (94 %) of compound **1K** in the form of a yellow oil.

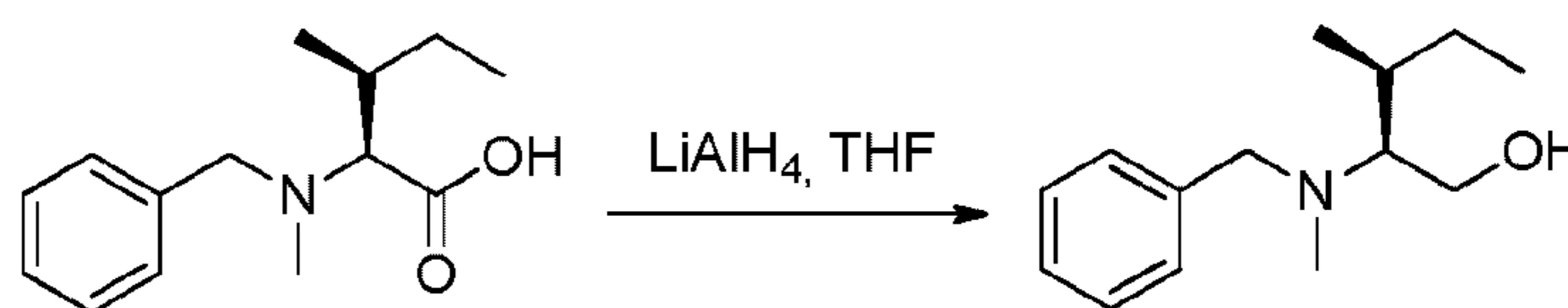
Compound 1L: (2S,3S)-2-(benzylamino)-3-methylpentanoic acid



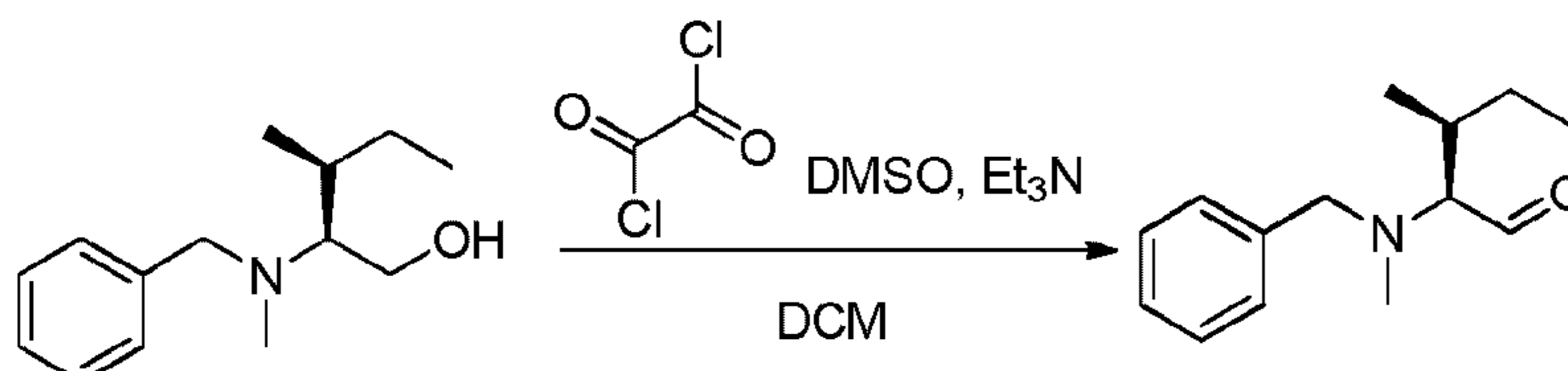
(2S,3S)-2-amino-3-methylpentanoic acid (98.4 g, 750 mmol, 1.00 equiv) was added at ambient temperature and in portions to a 2N sodium hydroxide solution (375 mL). Benzaldehyde (79.7 g, 751.02 mmol, 1.00 equiv) was quickly added and the resulting solution was agitated 30 minutes. Sodium borohydride (10.9 g, 288.17 mmol, 0.38 equiv) was added in small portions, whilst holding the temperature at between 5 and 15°C. Agitation was continued for 4 hours at ambient temperature. The reaction mixture was diluted with 200 mL of water, then washed twice with 200 mL of EtOAc. The pH of the aqueous solution was adjusted to 7 with a 2N hydrochloric acid solution. The formed precipitate was collected by filtering and gave 149.2 g (90 %) of compound **1L** in the form of a white solid.

Compound 1M: (2S,3S)-2-[benzyl(methyl)amino]-3-methylpentanoic acid

Compound **1L** (25 g, 112.97 mmol, 1.00 equiv) was dissolved in an inert atmosphere in formic acid (31.2 g) in the presence of formaldehyde (36.5 % in water, 22.3 g). The solution was agitated 3 hours at 90°C then concentrated under reduced pressure. The residue was triturated in 250 mL of acetone, then concentrated. This trituration/evaporation operation was repeated twice with 500 mL of acetone to yield 21.6 g (81 %) of compound **1M** in the form of a white solid.

Compound 1N: (2S,3S)-2-[benzyl(methyl)amino]-3-methylpentan-1-ol

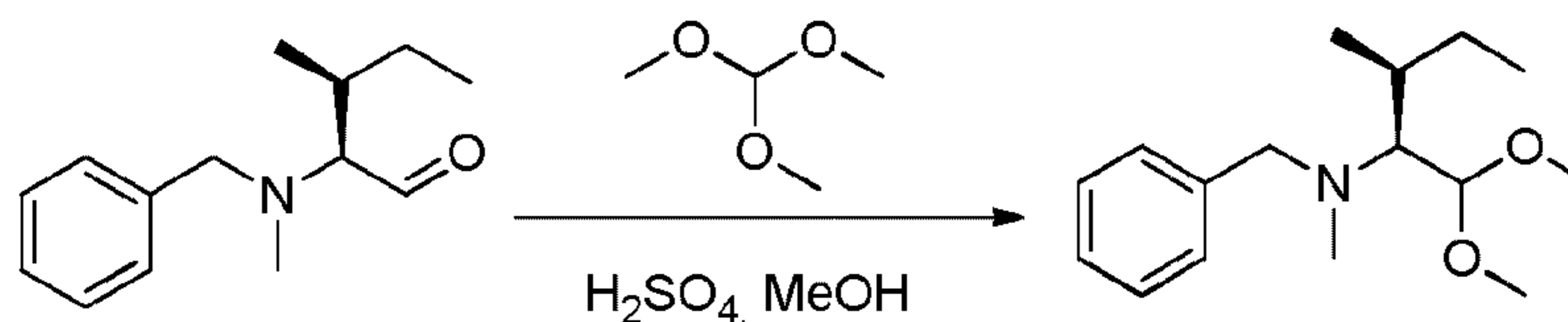
LiAlH₄ (0.36 g) was suspended in 10 mL of THF in an inert atmosphere at 0°C. Compound **1M** (1.5 g, 6.37 mmol, 1.00 equiv) was added in small portions whilst holding the temperature at between 0 and 10°C. The reaction mixture was agitated 2 hours at 65°C, then again cooled to 0°C before being neutralised with successive additions of 360 µL of water, 1 mL of 15 % sodium hydroxide and 360 µL of water. The aluminium salts which precipitated were removed by filtering. The filtrate was dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of EtOAc and PE (1:50) to yield 820 mg (58 %) of compound **1N** in the form of a pale yellow oil.

Compound 1O: (2S,3S)-2-[benzyl(methyl)amino]-3-methylpentanal

Oxalyl chloride (0.4 mL) was dissolved in DCM (15 mL) in an inert atmosphere. The solution was cooled to -70°C and a solution of dimethylsulfoxide (DMSO (0.5 mL) in DCM (10 mL) was added drop-wise for 15 minutes. The reaction mixture was agitated 30 minutes after which a solution of compound **1N** (820 mg, 3.70 mmol, 1.00

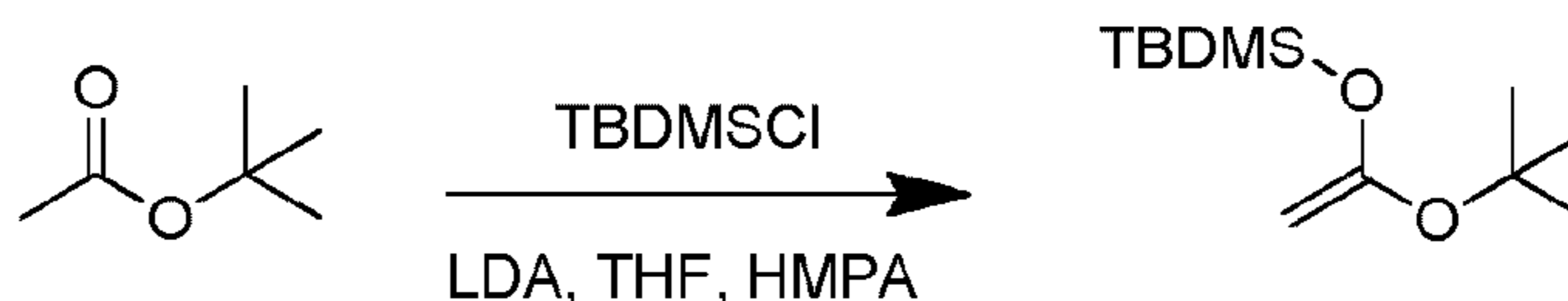
equiv) in DCM (10 mL) was added drop-wise for 15 minutes. The reaction mixture was agitated a further 30 minutes at low temperature, then triethylamine (2.5 mL) was slowly added. The reaction mixture was agitated 1 hour at -50°C , the cold bath was then removed and the reaction neutralised with 25 mL of water whilst allowing the
 5 temperature to return to normal. The solution was washed once with 30 mL of NaCl-saturated aqueous solution, then dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of EtOAc and PE (1:200) to yield 0.42 g (52 %) of compound **1O** in the form of a yellow oil.

Compound 1P: (2S,3S)-*N*-benzyl-1,1-dimethoxy-*N*,3-dimethylpentan-2-amine
 10 amine



Compound **1O** (4.7 g, 21.43 mmol, 1.00 equiv) was dissolved in 20 mL of methanol at 0°C . Concentrated sulfuric acid (4.3 mL) was added drop-wise and agitation was continued for 30 minutes at 0°C . Trimethyl orthoformate (21.4 mL) was
 15 added, the cold bath removed and the reaction medium left under agitation for 3 hours at ambient temperature. The reaction medium was diluted with 200 mL of EtOAc, successively washed with 100 mL of 10 % Na_2CO_3 and 200 mL of saturated NaCl, then dried over sodium sulfate, filtered and concentrated under reduced pressure to yield 3.4 g (60 %) of compound **1P** in the form of a pale yellow oil.

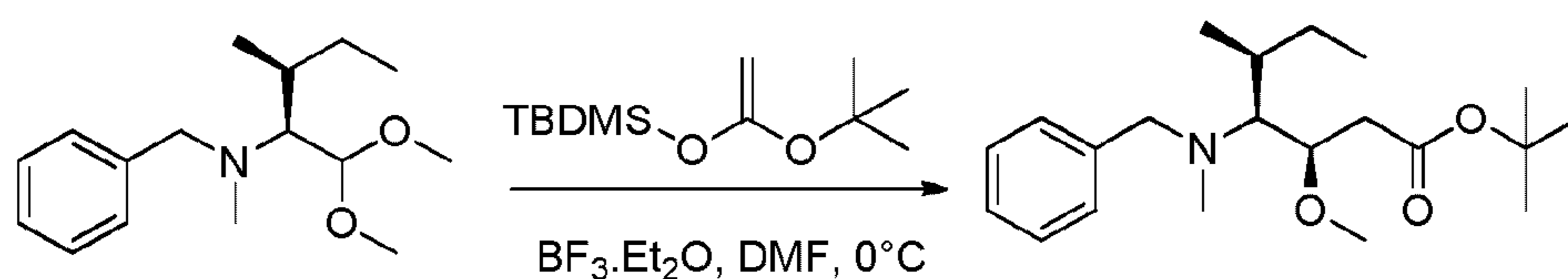
Compound 1Q: [[1-(*tert*-butoxy)ethenyl]oxy](*tert*-butyl)dimethylsilane
 20



Diisopropylamine (20 g, 186.71 mmol, 1.08 equiv) was dissolved in 170 mL of THF in an inert atmosphere and cooled to -78°C . *n*BuLi (2.4 M, 78.8 mL) was added drop-wise and the solution agitated 30 minutes at low temperature (to give LDA-lithium diisopropylamide) before adding *tert*-butyl acetate (20 g, 172.18 mmol, 1.00
 25 equiv). The reaction mixture was agitated 20 minutes at -78°C before adding hexamethylphosphoramide (HMPA, 25.8 mL) and a solution of *tert*butyldimethylchlorosilane (TBDMSCl, 28 g, 185.80 mmol, 1.08 equiv) in 35 mL of

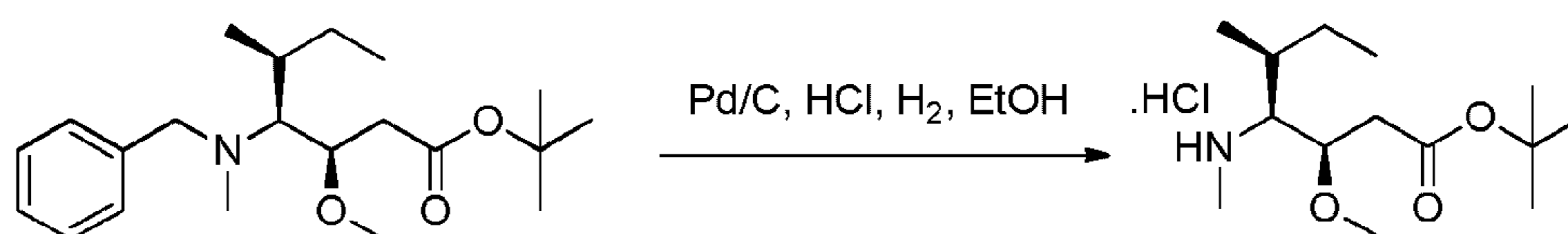
THF. Agitation was continued for 20 additional minutes at low temperature, and the cold bath was then removed. The solution was concentrated under reduced pressure. The residue was re-dissolved in 100 mL of water and extracted 3 times with 100 mL of PE. The organic phases were combined, washed once with 500 mL of NaCl-saturated aqueous solution, dried over sodium sulfate, filtered and concentrated. The residue was purified by distillation to yield 16.6 g (83 %) of compound **1Q** in the form of a colourless oil.

Compound 1R: *tert*-butyl (3R,4S,5S)-4-[benzyl(methyl)amino]-3-methoxy-5-methyl heptanoate



Compound **1P** (2.0 g, 7.54 mmol, 1.00 equiv) and compound **1Q** (2.6 g, 11.28 mmol, 1.50 equiv) were dissolved in 33 mL of DCM in an inert atmosphere. The solution was cooled to 0°C. DMF (1.2 g) was added drop-wise together with a solution of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (2.1 g) in 7.5 mL of DCM. Agitation was continued for 24 hours at 0°C. The reaction medium was washed once with 30 mL of sodium carbonate (10 %) and twice with 50 mL of NaCl-saturated aqueous solution, then dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of EtOAc and PE (1:100) to yield 1.82 g (91 %) of compound **1R** in the form of a yellow oil.

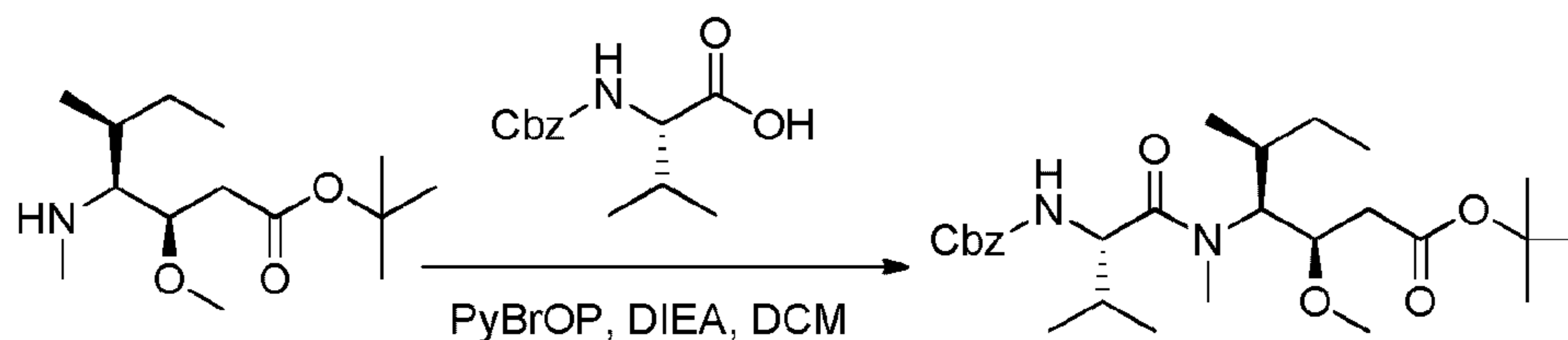
Compound 1S: (3R,4S,5S)-3-methoxy-5-methyl-4-(methylamino)heptanoate hydrochloride



Compound **1R** (2.4 g, 6.87 mmol, 1.00 equiv) was dissolved in an inert atmosphere in 35 mL of ethanol in the presence of Pd/C (0.12 g) and concentrated hydrochloric acid (0.63 mL). The nitrogen atmosphere was replaced by a hydrogen atmosphere and the reaction medium was left under agitation 18 hours at ambient temperature. The reaction medium was filtered and concentrated under reduced pressure. The residue was triturated in 50 mL of hexane and the supernatant removed

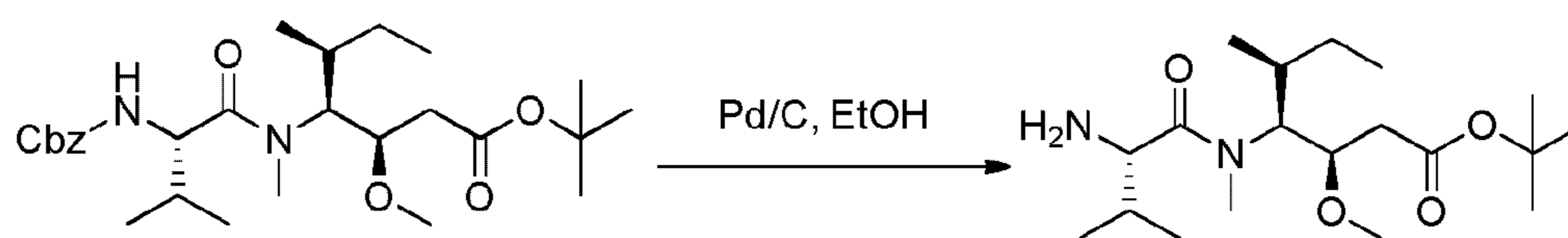
which, after drying under reduced pressure, gave 1.66 g (82 %) of compound **1S** in the form of a white solid.

Compound 1T: *tert*-butyl (3R,4S,5S)-4-[(2S)-2-[[[(benzyloxy)carbonyl]amino]-*N*,3-dimethylbutanamido]-3-methoxy-5-methylheptanoate



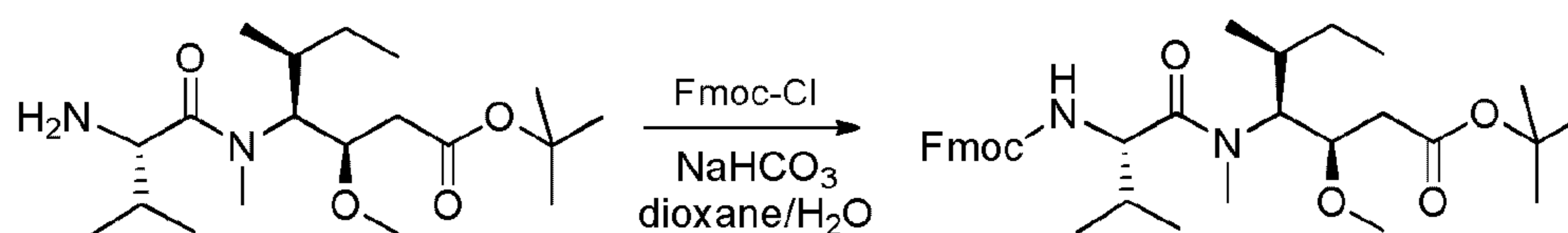
(2S)-2-[[[(benzyloxy)carbonyl]amino]-3-methylbutanoic acid (15 g, 0.40 mmol, 1.00 equiv) was dissolved in 300 mL of DCM in the presence of DIEA (38.3 mL) and bromotripyrrolidinophosphonium hexafluorophosphate (PyBrOP, 32.3g). The solution was agitated 30 minutes at ambient temperature before adding compound **1S** (15.99g, 0.42 mmol, 1.07 equiv). The reaction medium was agitated 2 hours and then concentrated. The residue was purified in reverse phase (C18) with a mixture of acetonitrile (ACN) and water (30:70 to 100:0 in 40 minutes) to yield 17 g (58 %) of compound **1T** in the form of a colourless oil.

Compound 1U: *tert*-butyl (3R,4S,5S)-4-[(2S)-2-amino-*N*,3-dimethylbutanamido]-3-methoxy-5-methylheptanoate



Compound **1T** (76 mg, 0.15 mmol, 1.00 equiv) was dissolved in an inert atmosphere in 10 mL of ethanol in the presence of Pd/C (0.05 g). The nitrogen atmosphere was replaced by a hydrogen atmosphere and the reaction agitated 2 hours at ambient temperature. The reaction medium was filtered and concentrated under reduced pressure to yield 64 mg of compound **1U** in the form of a colourless oil.

Compound 1V: (3R,4S,5S)-4-[(2S)-2-[[[(9H-fluoren-9-ylmethoxy)carbonyl]amino]-*N*,3-dimethylbutanamido]-3-methoxy-5-methylheptanoate



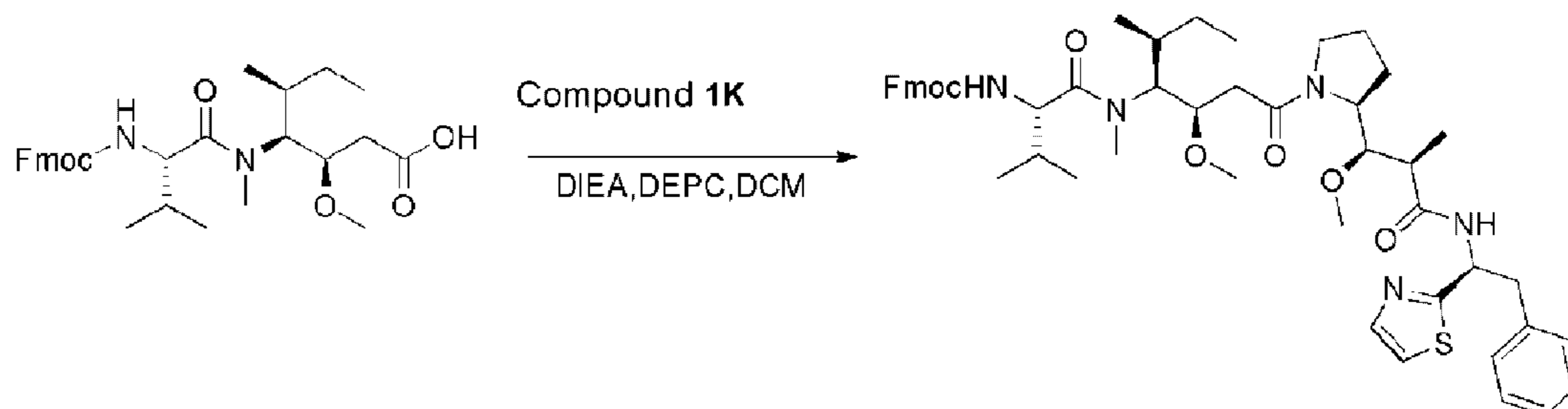
Compound **1U** (18.19 g, 50.74 mmol, 1.00 equiv) was dissolved in 400 mL of a 1,4-dioxane/water mixture (1:1) in the presence of sodium bicarbonate (12.78 g, 152 mmol, 3.00 equiv) and 9H-fluoren-9-ylmethyl chloroformate (Fmoc-Cl, 19.69 g, 76 mmol, 1.50 equiv), then agitated 2 hours at ambient temperature. The reaction medium was then diluted with 500 mL of water and extracted 3 times with 200 mL of EtOAc. The organic phases were combined, washed once with 200 mL of NaCl-saturated aqueous solution, dried over sodium sulfate, filtered and concentrated to yield 40 g of partly purified compound **1V** in the form of a pale yellow oil.

Compound 1W: (3R,4S,5S)-4-[(2S)-2-[[[(9H-fluoren-9-ylmethoxy)carbonyl]amino]-N,3-dimethylbutanamido]-3-methoxy-5-methylheptanoic acid



Compound **1V** (40 g, 68.88 mmol, 1.00 equiv) was dissolved in a neutral atmosphere in 600 mL of DCM. TFA (300 mL) was added. The solution was agitated 2 hours at ambient temperature, then concentrated under reduced pressure. The residue was purified on a silica column with a mixture of methanol and DCM (1:10) to yield 23.6 g (65 %) of compound **1W** in colourless oil form.

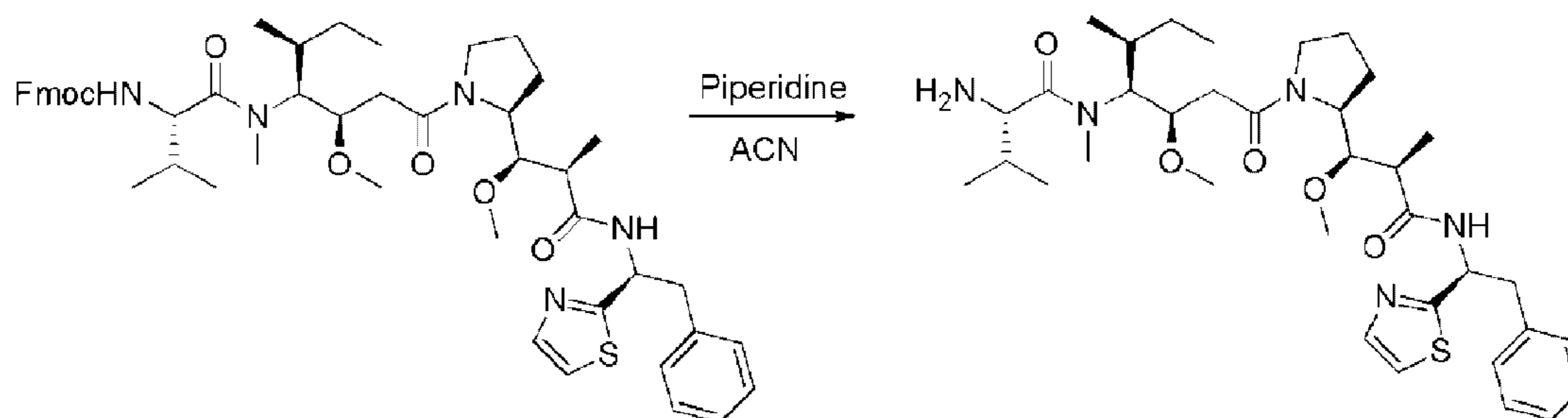
Compound 1X: 9H-fluoren-9-ylmethyl N-[(1S)-1-[[[(3R,4S,5S)-3-methoxy-1-[(2S)-2-[(1R,2R)-1-methoxy-2-methyl-2-[[[(1S)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]carbamoyl]ethyl]pyrrolidin-1-yl]-5-methyl-1-oxoheptan-4-yl](methyl)carbamoyl]-2-methylpropyl]carbamate



Compound **1W** (2.53 g, 4.82 mmol, 1.08 equiv) was dissolved in 20 mL of DCM in the presence of compound **1K** (2.18 g, 4.47 mmol, 1.00 equiv), DEPC (875 mg, 5.37 mmol, 1.20 equiv) and DIEA (1.25 g, 9.67 mmol, 2.16 equiv). The reaction mixture was left under agitation overnight at ambient temperature, then successively

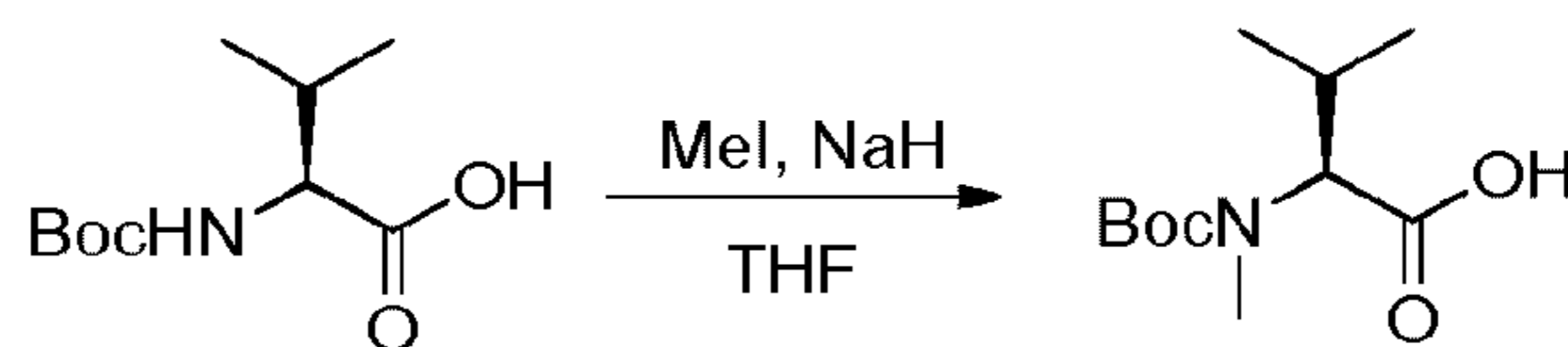
washed with 50 mL of saturated KHSO_4 and 100 mL of water, dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of methanol and DCM (1:200 to 1:40) to yield 2.8 g (71 %) of compound **1X** in the form of a pale yellow solid.

5 **Compound 1Y:** (2S)-2-amino-N-[(3R,5S)-3-methoxy-1-[(2S)-2-[(1R,2R)-1-methoxy-2-methyl-2-[[[(1S)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]carbamoyl]ethyl]pyrrolidin-1-yl]-5-methyl-1-oxoheptan-4-yl]-N,3-dimethylbutanamide



Compound **1X** (2.8 g, 3.18 mmol, 1.00 equiv) was dissolved in acetonitrile (ACN, 12 mL) in the presence of piperidine (3 mL) and left under agitation 18 hours at ambient temperature. The reaction was neutralised with 50 mL of water, then extracted twice with 100 mL of DCM. The organic phases were combined, dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of methanol and DCM (1:100 to 1:40) to yield 1.2 g (57 %) of compound **1Y** in the form of a yellow solid.

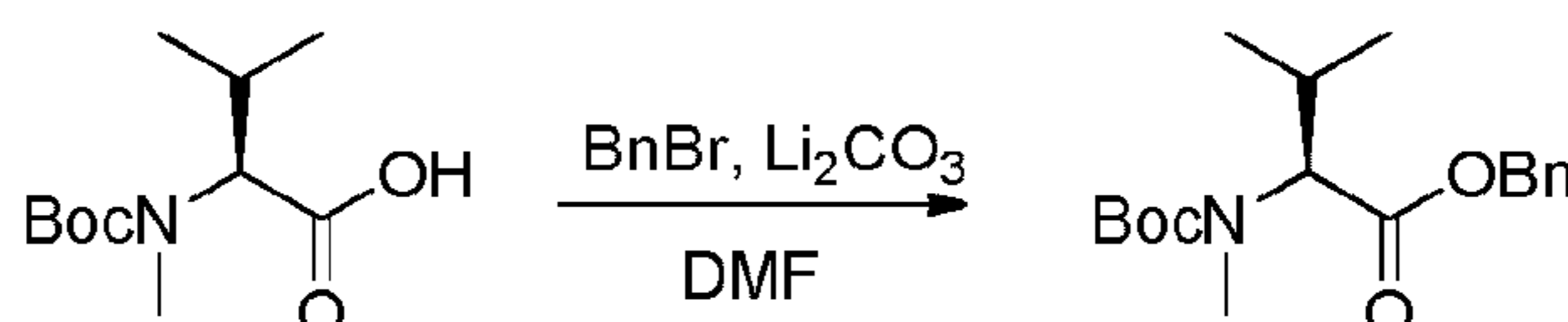
15 **Compound 1ZA:** (2S)-2-[[[(*tert*-butoxy)carbonyl](methyl)amino]-3-methylbutanoic acid



(2S)-2-[[[(*tert*-butoxy)carbonyl]amino]-3-methylbutanoic acid (63 g, 289.97 mmol, 1.00 equiv) was dissolved in an inert atmosphere in THF (1000 mL) in the presence of iodomethane (181 mL). The solution was cooled to 0°C before adding sodium hydride (116 g, 4.83 mol, 16.67 equiv) in small portions. The reaction mixture was agitated for 1.5 hours at 0°C, the cold bath was then removed and agitation continued for 18 hours. The reaction was neutralised with 200 mL of water and then concentrated under reduced pressure. The residual aqueous phase was diluted with 4 litres of water, washed once with 200 mL of EtOAc and its pH adjusted to between 3

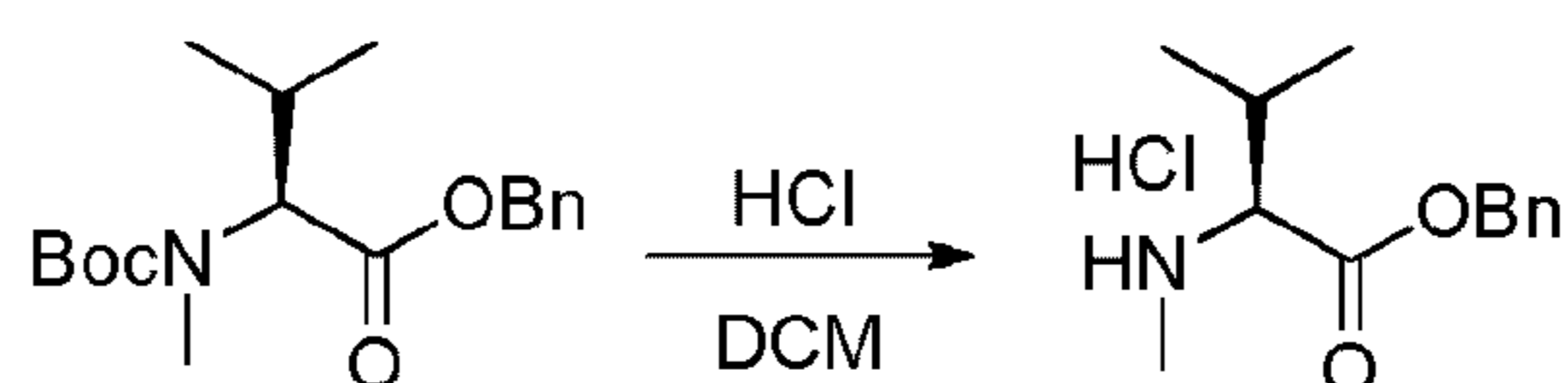
and 4 with a 1N solution of hydrochloric acid. The mixture obtained was extracted 3 times with 1.2 L of EtOAc. The organic phases were combined, dried over sodium sulfate, filtered and concentrated to yield 60 g (89 %) of compound **1ZA** in the form of a yellow oil.

5 **Compound 1ZB:** benzyl (2S)-2-[[*tert*-butoxy]carbonyl](methylamino)-3-methylbutanoate



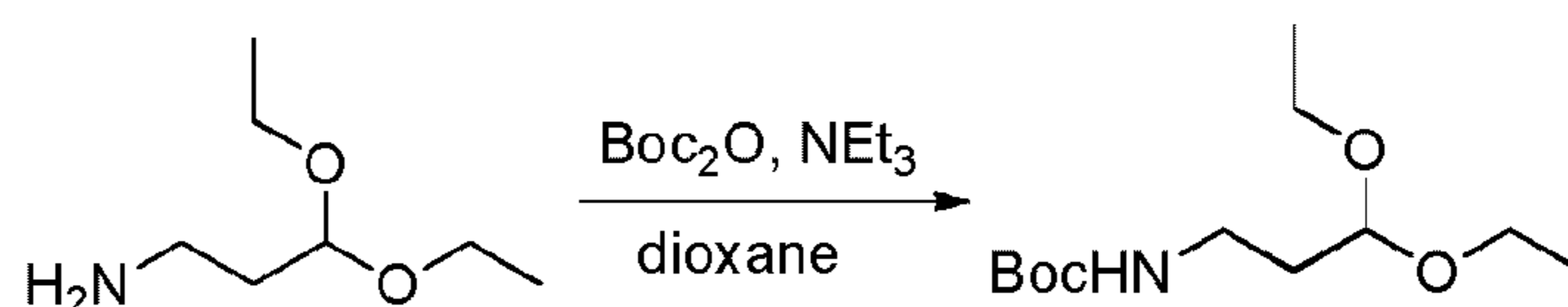
Compound **1ZA** (47 g, 203.21 mmol, 1.00 equiv) was dissolved in DMF (600 mL) in the presence of Li_2CO_3 (15.8 g, 213.83 mmol, 1.05 equiv). The solution was cooled to 0°C then benzyl bromide (BnBr 57.9 g, 338.53 mmol, 1.67 equiv) was added drop-wise. The reaction mixture was left under agitation overnight before being neutralised with 400 mL of water and filtered. The solution obtained was extracted twice with 500 mL of EtOAc. The organic phases were combined, dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of EtOAc and PE (1:100 to 1:20) to yield 22.5 g (34 %) of compound **1ZB** in the form of a yellow oil.

15 **Compound 1ZC:** benzyl (2S)-3-methyl-2-(methylamino)butanoate hydrochloride



20 Compound **1ZB** (22.5 g, 70.00 mmol, 1.00 equiv) was dissolved in 150 mL of DCM. Gaseous hydrochloric acid was bubbled. The reaction was agitated 1 hour at ambient temperature and then concentrated under reduced pressure to yield 17 g (94 %) of compound **1ZC** in the form of a yellow solid.

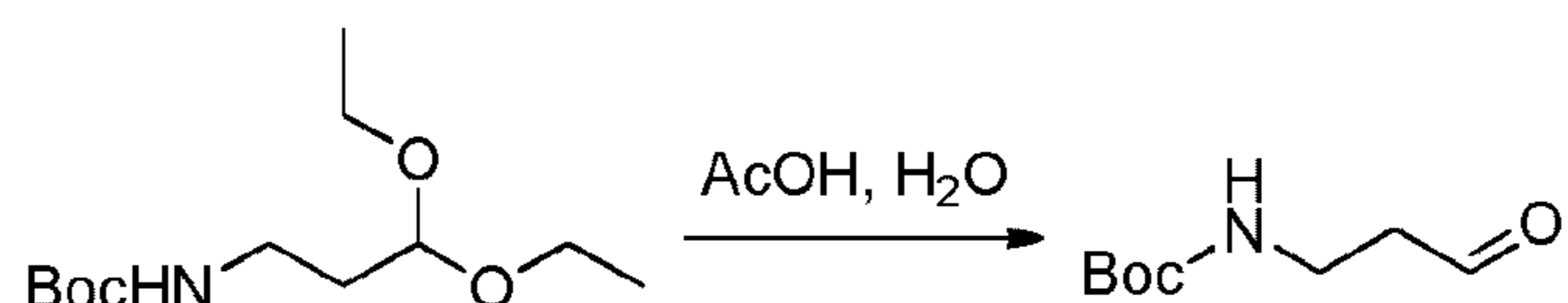
Compound 1ZD: *tert*-butyl *N*-(3,3-diethoxypropyl)carbamate



25 3,3-diethoxypropan-1-amine (6 g, 40.76 mmol, 1.00 equiv) was dissolved in 1,4-dioxane (30 mL) in the presence of TEA (4.45 g, 43.98 mmol, 1.08 equiv), then

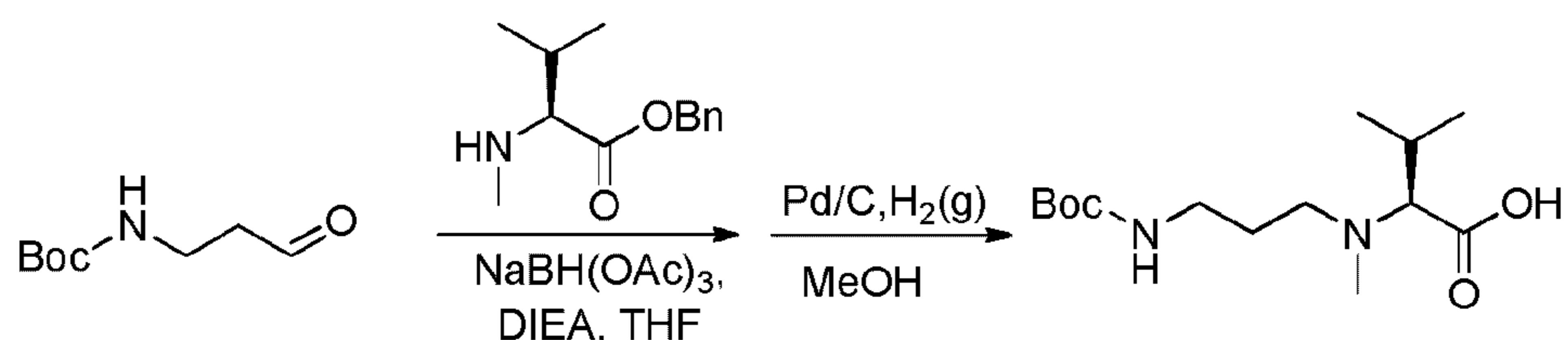
cooled to 0°C. (Boc)₂O (9.6 g, 43.99 mmol, 1.08 equiv) diluted in 20 mL of 1,4-dioxane was added drop-wise. The solution was agitated 2 hours at 0°C then overnight at ambient temperature before being neutralised with 10 mL of water. The pH was adjusted to 5 with HCl (1 %). The solution was extracted 3 times with 50 mL of EtOAc. The organic phases were combined, dried over sodium sulfate, filtered and concentrated to yield 8.21 g (81 %) of compound **1ZD** in the form of a pale yellow oil.

Compound 1ZE: *tert*-butyl *N*-(3-oxopropyl) carbamate



Compound **1ZD** (8.20 g, 33.15 mmol, 1.00 equiv) was dissolved in 18.75 mL of acetic acid and left under agitation overnight at ambient temperature. The reaction medium was then extracted 3 times with 30 mL of EtOAc. The organic phases were combined, washed 3 times with 30 mL of saturated NaCl solution, dried over sodium sulfate, filtered and concentrated to yield 5 g (87 %) of compound **1ZE** in the form of a dark red oil.

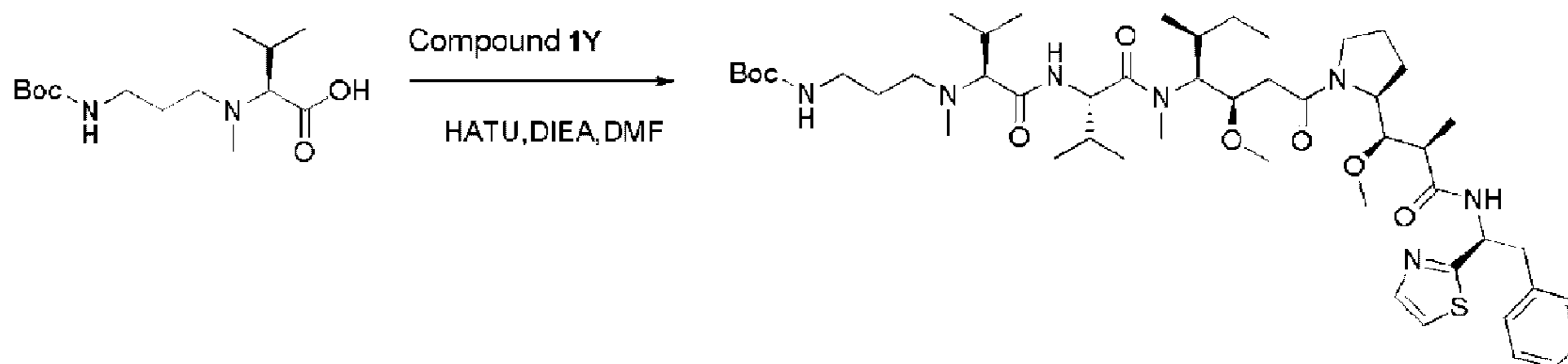
Compound 1ZF: (2*S*)-2-[(3-[[*tert*-butoxy]carbonyl]amino)propyl](methyl)amino]-3-methylbutanoic acid



Compound **1ZE** (2.4 g, 13.86 mmol, 1.00 equiv) was dissolved in 50 mL of THF in the presence of compound **1ZC** (3.56 g, 13.81 mmol, 1.00 equiv) and DIEA (9.16 mL, 4.00 equiv). The reaction mixture was agitated 30 minutes at ambient temperature before adding sodium triacetoxyborohydride (5.87 g, 27.70 mmol, 2.00 equiv). Agitation was continued overnight, then the reaction was neutralised with 100 mL of water and extracted 3 times with 50 mL of EtOAc. The organic phases were combined, dried over sodium sulfate, filtered and concentrated. The residue was partly purified on a silica column with a mixture of EtOAc and PE (1:4). The crude product obtained was re-dissolved in 20 mL of methanol in the presence of Pd/C (1.2 g) and hydrogenated for 20 minutes at normal temperature and pressure. The reaction medium

was filtered and concentrated under reduced pressure to yield 200 mg (5 %) of compound **1ZF** in the form of a white solid.

Compound 1ZG: *tert*-butyl *N*-(3-[[[(1*S*)-1-[[[(1*S*)-1-[[[(3*R*,4*S*,5*S*)-3-methoxy-1-[(2*S*)-2-[(1*R*,2*R*)-1-methoxy-2-methyl-2-[[[(1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]carbamoyl]thyl]pyrrolidin-1-yl]-5-methyl-1-oxoheptan-4yl](methyl) carbamoyl]-2-methylpropyl]carbamoyl]-2-methylpropyl](methyl)amino]propyl) carbamate



Compound **1Y** (50 mg, 0.08 mmol, 1.00 equiv) was dissolved in 2 mL of DMF in the presence of compound **1ZF** (26.2 mg, 0.09 mmol, 1.20 equiv), DIEA (37.7 mL) and *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU, 43.3 mg, 0.11 mmol, 1.50 equiv). The reaction was left under agitation overnight at ambient temperature, then diluted with 10 mL of water and extracted 3 times with 5 mL of EtOAc. The organic phases were combined, dried over sodium sulfate, filtered and concentrated to yield 100 mg of compound **1ZG** in the form of a partly purified colourless oil.

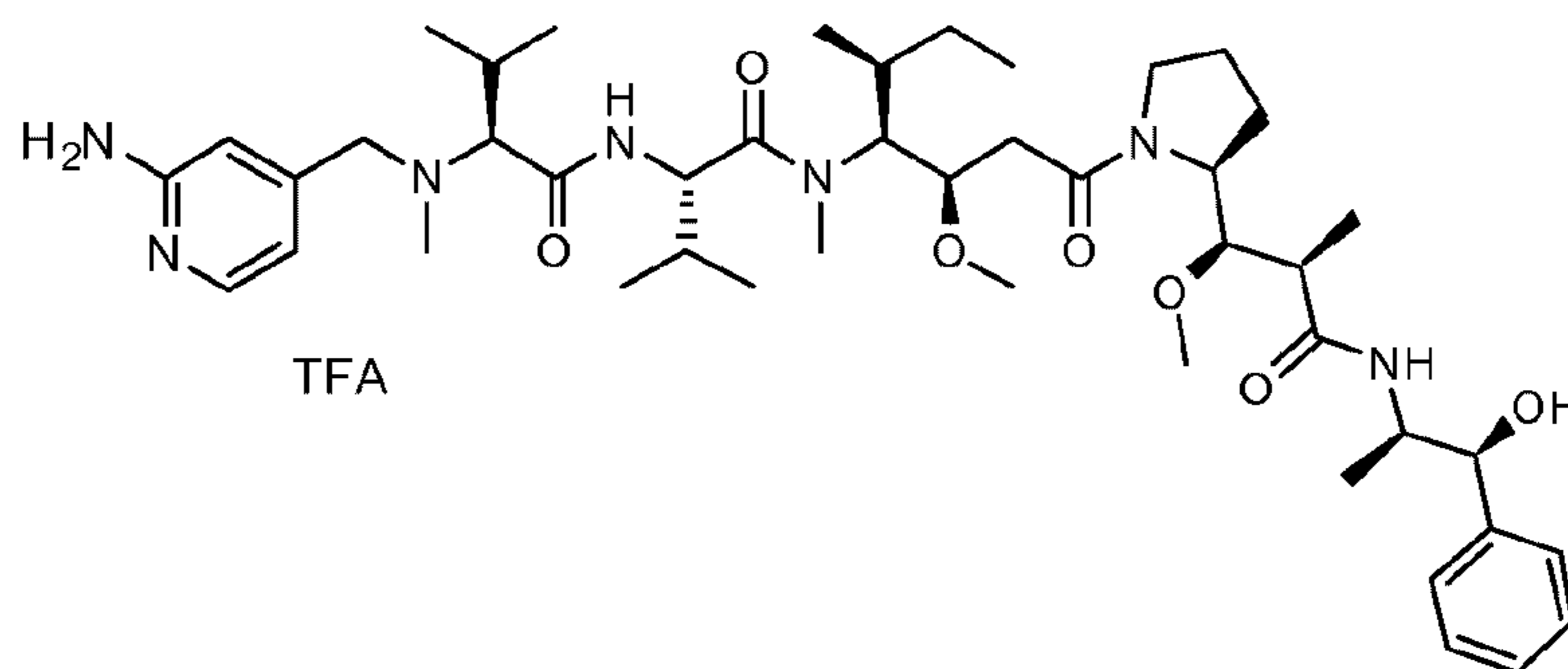
Compound **1ZG** (90 mg, 0.10 mmol, 1.00 equiv) was dissolved in a neutral atmosphere in 2 mL of DCM and the solution was cooled with an ice bath. TFA (1 mL) was added and the reaction agitated for 2 hours at ambient temperature, then concentrated under reduced pressure. The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % of TFA; Gradient of 18 % to 31 % ACN in 7 minutes then 31 % to 100 % ACN in 2 minutes; Waters 2489 UV Detector at 254 nm and 220 nm). Compound **1** was obtained with a yield of 25 % (23 mg) in the form of a white solid.

LC/MS/UV (Atlantis T3 column, 3 μm , 4.6 x 100 mm; 35°C; 1 mL / min, 30 % to 60 % ACN in water (20 mM ammonium acetate in 6 minutes); ESI ($\text{C}_{44}\text{H}_{73}\text{N}_7\text{O}_6\text{S}$, exact masse 827.53) m/z : 829 (MH^+), 5.84 min (93.7 %, 254 nm).

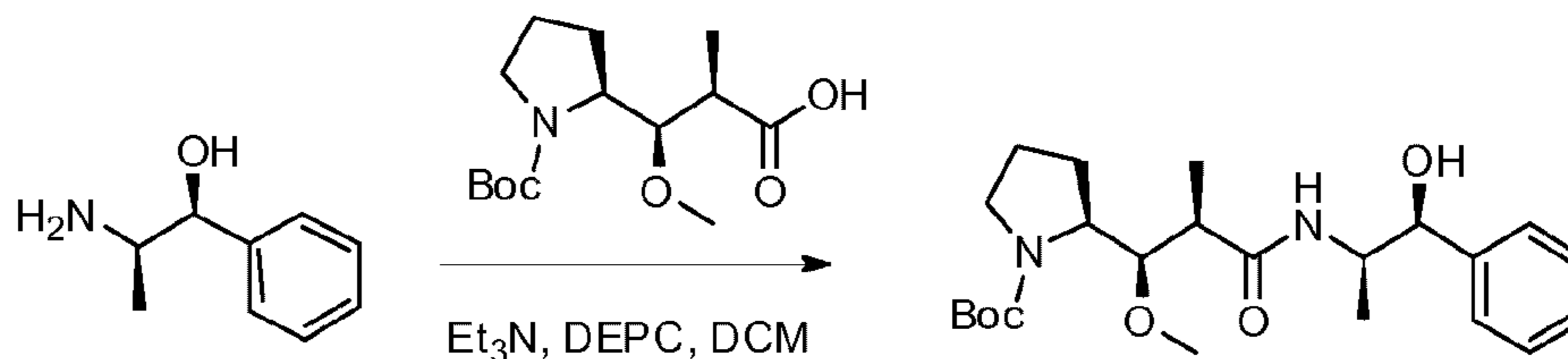
^1H NMR (300MHz, CD_3OD , ppm): δ (Presence of rotamers) 7.85 - 7.80 (m, 1H); 7.69 - 7.66 (m, 1H), 7.40 - 7.10 (m, 5H), 5.80 - 5.63 (m, 1H), 4.80 - 4.65 (m, 2H), 4.22 - 4.00 (m, 1H), 3.89 - 0.74 (m, 58H).

Compound 2

(S)-2-((S)-2-(((2-aminopyridin-4-yl)methyl)(methyl)amino)-3-methylbutanamido)-N-((3R,4S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide, trifluoroacetic acid



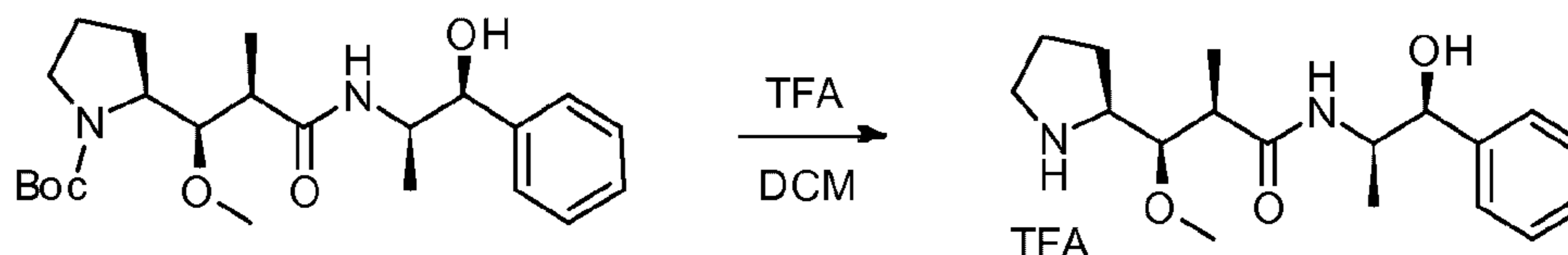
15 Compound 2A: *tert*-butyl (S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidine-1-carboxylate



20 Compound **1D** (2.5 g, 8.70 mmol, 1.00 equiv) and (1S,2R)-2-amino-1-phenylpropan-1-ol (1.315 g, 8.70 mmol, 1.00 equiv) were dissolved in an inert atmosphere in DMF (35 mL). The solution was cooled to 0 °C then DEPC (1.39 mL) and TEA (1.82 mL) were added drop-wise. The reaction mixture was agitated 2 hours at 0 °C then 4 hours at ambient temperature. The reaction mixture was diluted with 200

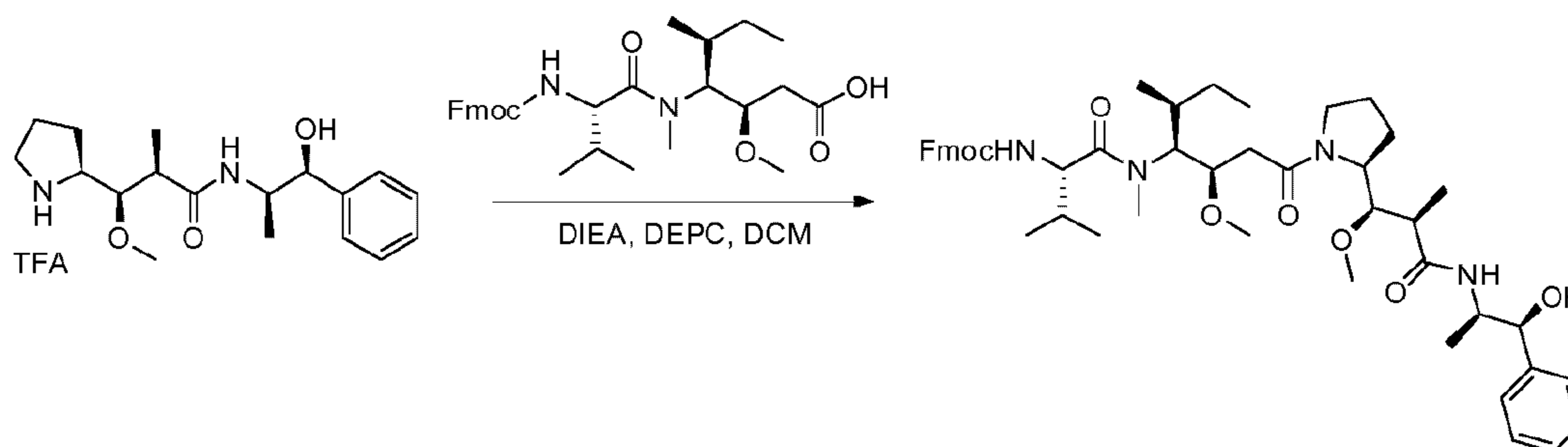
mL of water and extracted three times with 50 mL of EtOAc. The organic phases were combined, washed once with 50 mL of KHSO₄ (1 mol/L), once with 50 mL of NaHCO₃ (sat.), once with 50 mL of NaCl (sat.), then dried over sodium sulfate, filtered and concentrated under reduced pressure to yield 3.6 g (98 %) of compound **2A** in the form of a yellow solid.

Compound 2B: (2R,3R)-N-((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)-3-methoxy-2-methyl-3-((S)-pyrrolidin-2-yl)propanamide 2,2,2-trifluoroacetate



Compound **2A** (2.7 g, 6.42 mmol, 1.00 equiv) was dissolved in an inert atmosphere in DCM (40 mL) then cooled to 0 °C. TFA (25 mL) was added and the solution agitated for 2 hours at 0 °C. The reaction mixture was concentrated under reduced pressure to yield 4.4 g of compound **2B** in the form of a yellow oil.

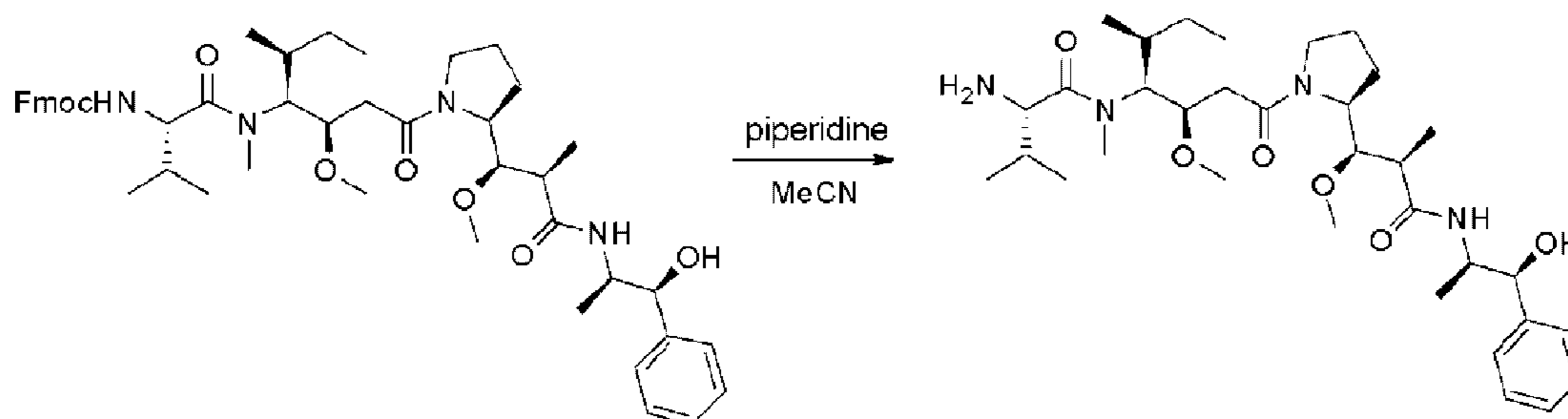
Compound 2C: (9H-fluoren-9-yl)methyl ((S)-1-(((3R,4S,5S)-1-((S)-2-(((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)(methyl)amino)-3-methyl-1-oxobutan-2-yl)carbamate



Compounds **2B** (4.4 g, 10.13 mmol, 1.00 equiv) and **1W** (5.31 g, 10.12 mmol, 1.00 equiv) were dissolved in an inert atmosphere in DCM (45 mL). The solution was cooled to 0 °C then DEPC (1.62 mL) and DIEA (8.4 mL) were added drop-wise. The reaction mixture was agitated for 2 hours at 0 °C then at ambient temperature overnight. The reaction mixture was diluted with 100 mL of water and extracted three times with 50 mL of DCM. The organic phases were combined, washed once with 50 mL of KHSO₄ (1 mol/L), once with 50 mL of NaHCO₃ (sat.), once with 50 mL of NaCl (sat.),

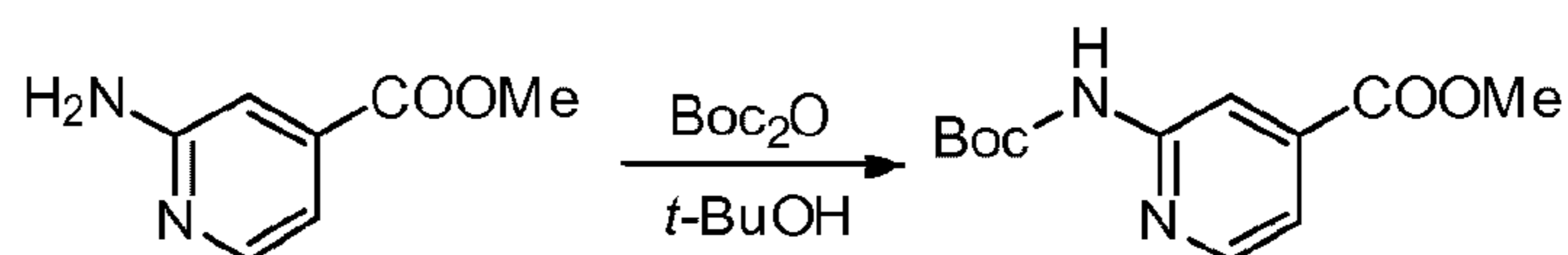
then dried over sodium sulfate, filtered and concentrated under pressure to yield 3.3 g (39 %) of compound **2C** in the form of a yellow solid.

Compound 2D: (S)-2-amino-N-((3R,4S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide



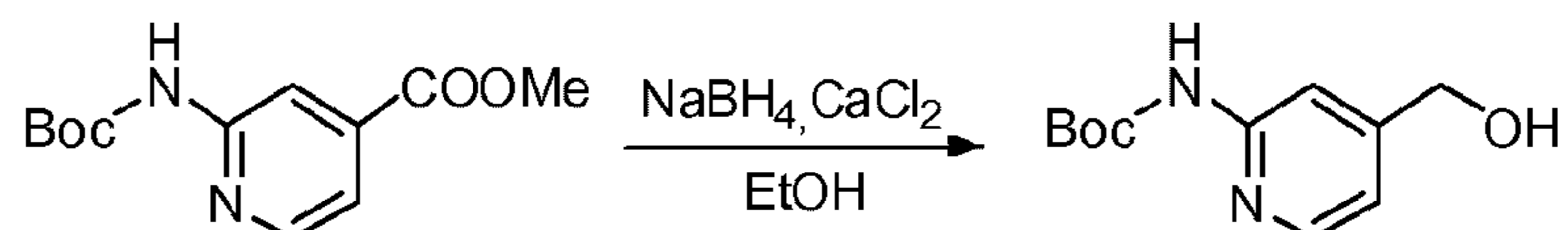
Compound **2C** (300 mg, 0.36 mmol, 1.00 eq.) was dissolved in an inert atmosphere in ACN (2 mL) and piperidine (0.5 mL). The solution was left under agitation at ambient temperature overnight then evaporated to dryness under reduced pressure. The residue was purified on a silica column with a mixture of DCM and MeOH (1:100) to yield 150 mg (68 %) of compound **2D** in the form of a white solid.

Compound 2E: methyl 2-((*tert*-butoxycarbonyl)amino)isonicotinate



Methyl 2-aminopyridine-4-carboxylate (2 g, 13.14 mmol, 1.00 equiv) was dissolved in *tert*-butanol (20 mL) after which di-*tert*-butyl dicarbonate (4.02 g, 18.42 mmol, 1.40 equiv) was added. The reaction mixture was agitated at 60°C overnight then the reaction was halted through the addition of an aqueous 1M NaHCO₃ solution (50 mL). The solid was recovered by filtration, washed with 50 mL of EtOH then dried *in vacuo* to yield 2.5 g (75 %) of compound **2E** in the form of a white solid.

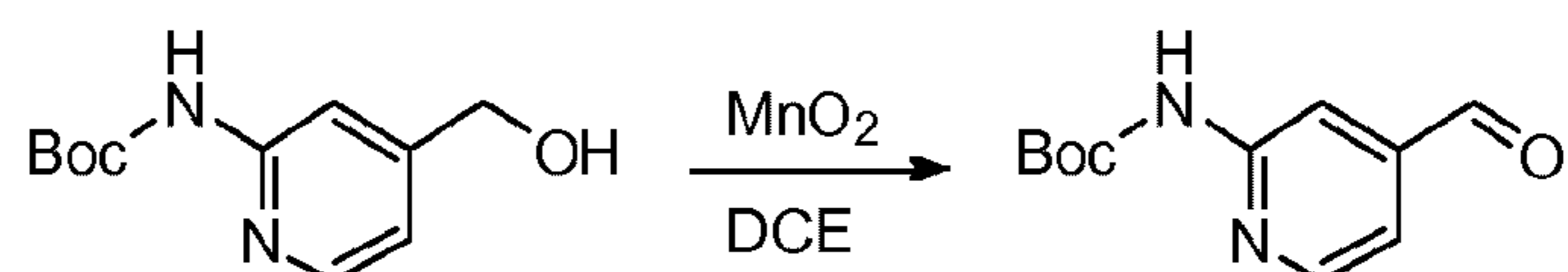
Compound 2F: *tert*-butyl (4-(hydroxymethyl)pyridin-2-yl)carbamate



Compound **2E** (2.5 g, 9.91 mmol, 1.00 equiv) and CaCl₂ (1.65 g) were dissolved in EtOH (30 mL). The solution was cooled to 0°C then NaBH₄ (1.13 g, 29.87 mmol,

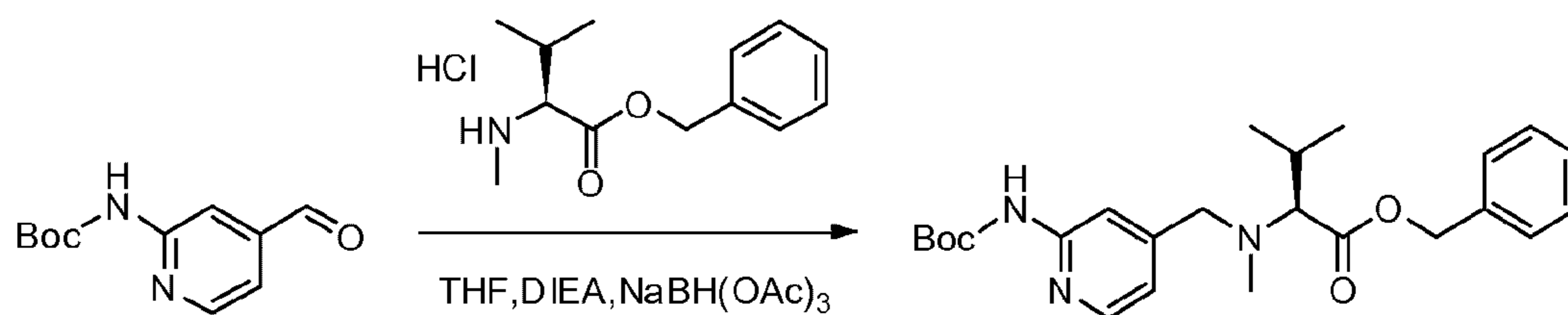
3.01 equiv) was gradually added. The solution was left under agitation overnight at ambient temperature then the reaction was halted with the addition of water (50 mL). The mixture was extracted three times with 20 mL of EtOAc. The organic phases were combined, washed twice with 20 mL of NaCl (sat.) then dried over sodium sulfate, filtered and concentrated under reduced pressure to yield 2.0 g (90 %) of compound **2F** in the form of a colourless solid.

Compound 2G: *tert*-butyl (4-formylpyridin-2-yl)carbamate



Compound **2F** (2.5 g, 11.15 mmol, 1.00 equiv) was dissolved in DCE (25 mL) then 19.4 g (223.14 mmol, 20.02 equiv) of MnO₂ were added. The mixture was left under agitation overnight at 70 °C then the solids were removed by filtering. The filtrate was evaporated to dryness to yield 1.4 g (57 %) of compound **2G** in the form of a white solid.

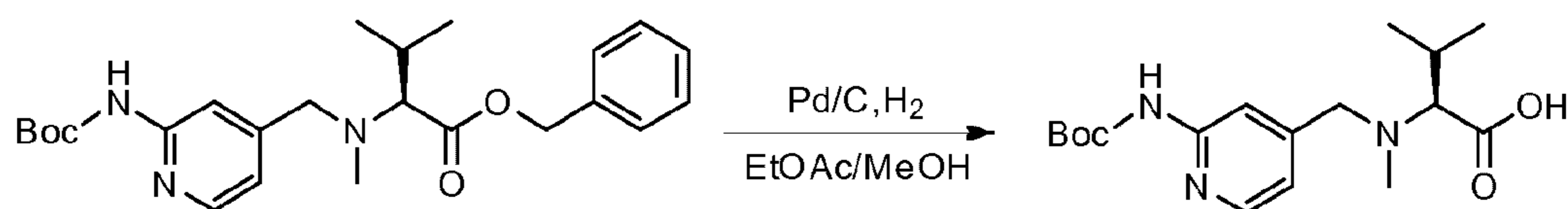
Compound 2H: benzyl (S)-2-(((2-((*tert*-butoxycarbonyl)amino)pyridin-4-yl)methyl) (methyl)amino)-3-methylbutanoate



Compound **2G** (2.3 g, 10.35 mmol, 1.00 equiv) was dissolved in 25 mL of THF in the presence of compound **1ZC** (2.93 g, 11.37 mmol, 1.10 equiv), DIEA (5.39 g, 41.71 mmol, 4.03 equiv) and NaBH(OAc)₃ (4.39 g, 20.71 mmol, 2.00 equiv). The reaction mixture was agitated for 6 hours at ambient temperature then neutralised with 60 mL of NaHCO₃ (sat.) and extracted 3 times with 20 mL of AcOEt. The organic phases were combined, washed twice with 20 mL of NaCl (sat.), dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of EtOAc and PE (1:15) to yield 2.7 g (61 %) of compound **2H** in the form of a white solid.

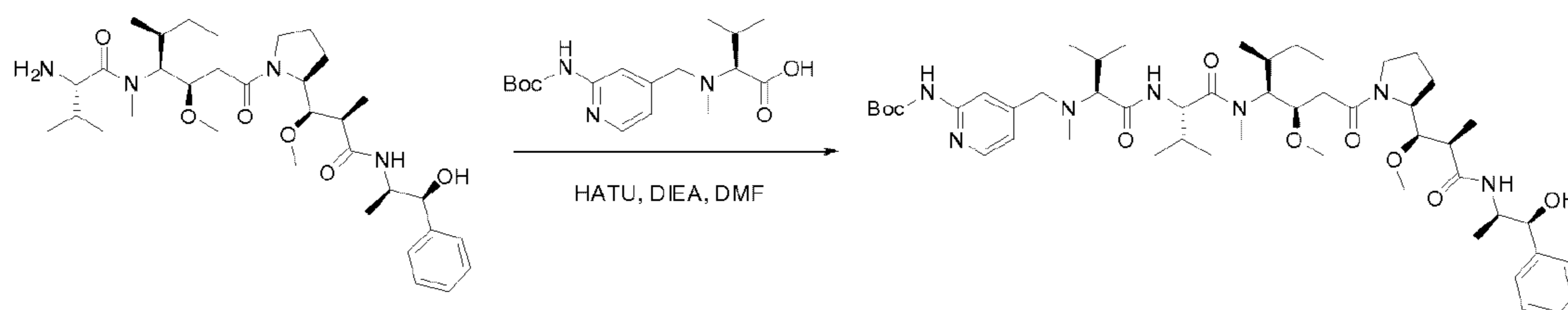
Compound 2I: (S)-2-(((2-((*tert*-butoxycarbonyl)amino)pyridin-4-yl)methyl) (methyl)amino)-3-methylbutanoic acid

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Compound **2H** (500 mg, 1.17 mmol, 1.00 equiv) was dissolved in 10 mL of AcOEt and 2 mL of methanol in the presence of Pd/C (250 mg), and hydrogenated for 3 hours at ambient temperature and atmospheric pressure. The reaction medium was
 5 filtered and concentrated under reduced pressure to yield 254 mg (64 %) of compound **2I** in the form of a colourless solid

Compound 2J: *tert*-butyl (4-((3*S*,6*S*,9*S*,10*R*)-9-((*S*)-*sec*-butyl)-10-(2-((*S*)-2-((1*R*,2*R*)-3-(((1*S*,2*R*)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-2-oxoethyl)-3,6-diisopropyl-2,8-dimethyl-
 10 4,7-dioxo-11-oxa-2,5,8-triazadodecyl)pyridin-2-yl) carbamate



Compound **2J** was prepared in similar manner to compound **1ZG** from the amine **2D** (85.2 mg, 0.14 mmol, 1.50 equiv), the acid **2I** (31.7 mg, 0.09 mmol, 1.00
 15 equiv), HATU (42.9 mg, 0.11 mmol, 1.20 equiv) and DIEA (36.7 mg, 0.28 mmol, 3.02 equiv) in DMF (3 mL). After evaporation to dryness, 100 mg of crude product were obtained in the form of a white solid.

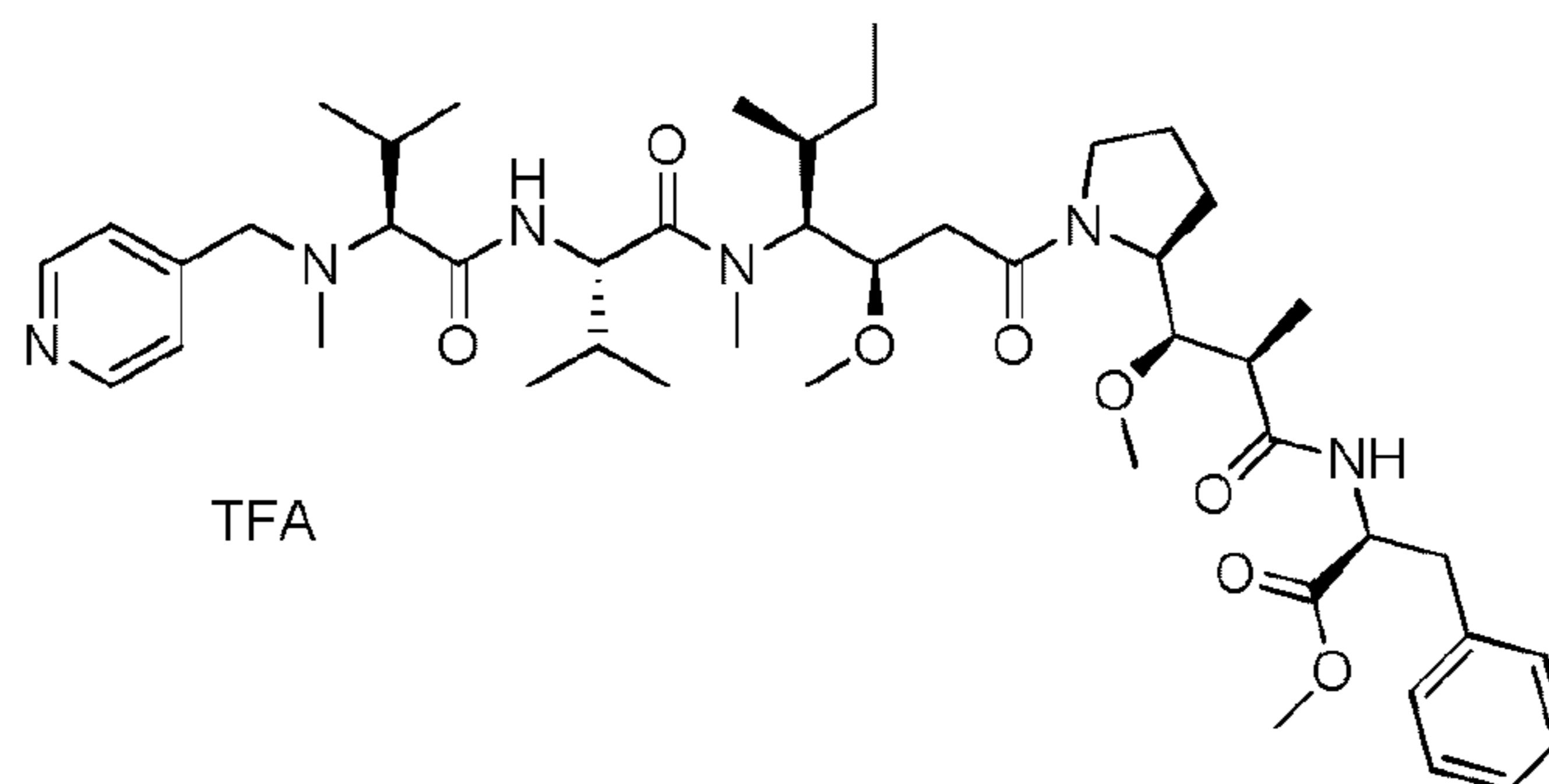
Compound **2J** (100 mg, 0.11 mmol, 1.00 equiv) was dissolved in 2 mL of DCM
 20 and 1 mL of TFA. The reaction was agitated for 1 hour at ambient temperature, then concentrated under reduced pressure. The residue (80 mg) was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2489 UV Detector
 25 at 254 nm and 220 nm). Compound **2** was obtained with a yield of 6 % (6.3 mg) in the form of a white solid.

LC/MS/UV (Ascentis Express C18 column, 2.7 μm , 4.6 x 100 mm; 40°C; 1.8 mL/min, from 10 % to 95 % ACN in water (0.05 % TFA) in 6 minutes); ESI ($\text{C}_{45}\text{H}_{73}\text{N}_7\text{O}_7$, exact mass 823.56) m/z : 824.5 (MH^+) and 412.9 ($\text{M}_2\text{H}^+/2$, 100 %), 3.21 min (99.2 %, 210 nm)

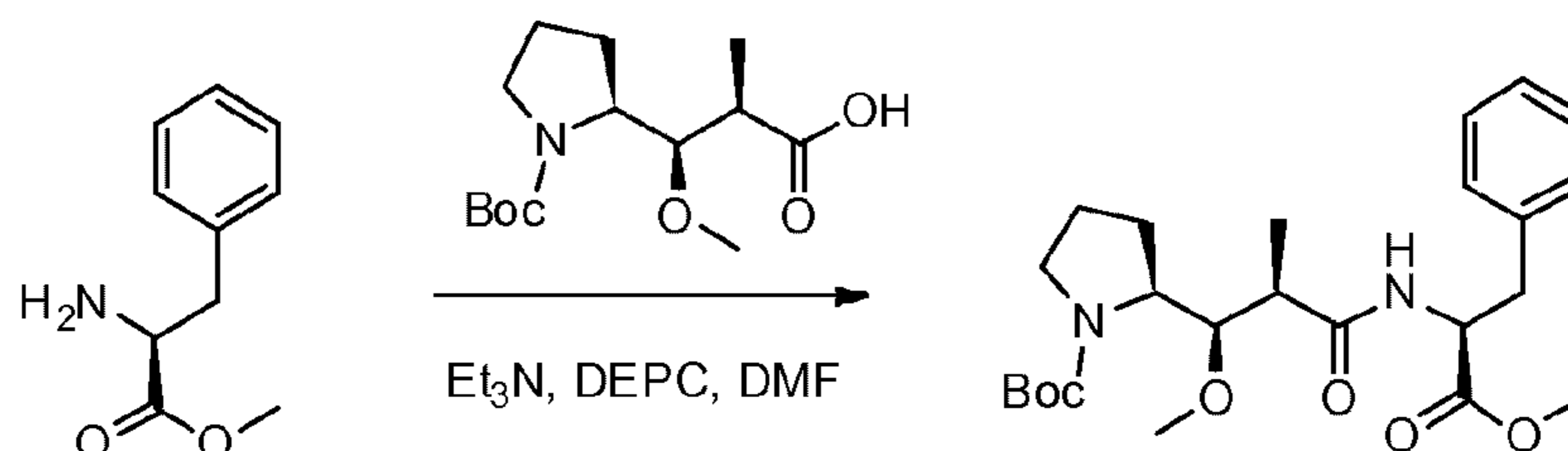
5 ^1H NMR (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 7.81 - 7.79 (m, 1H); 7.39 - 7.29 (m, 5H); 6.61 - 6.59 (m, 2H); 4.84 - 4.52 (m, 1H); 4.32 - 4.02 (m, 1H); 3.90 - 2.98 (m, 10H); 2.90 - 2.78 (m, 1H); 2.55 - 0.81 (m, 39H).

Reference Compound 3

10 methyl ((S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(pyridin-4-ylmethyl)amino)butanamido)butanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, trifluoroacetic acid



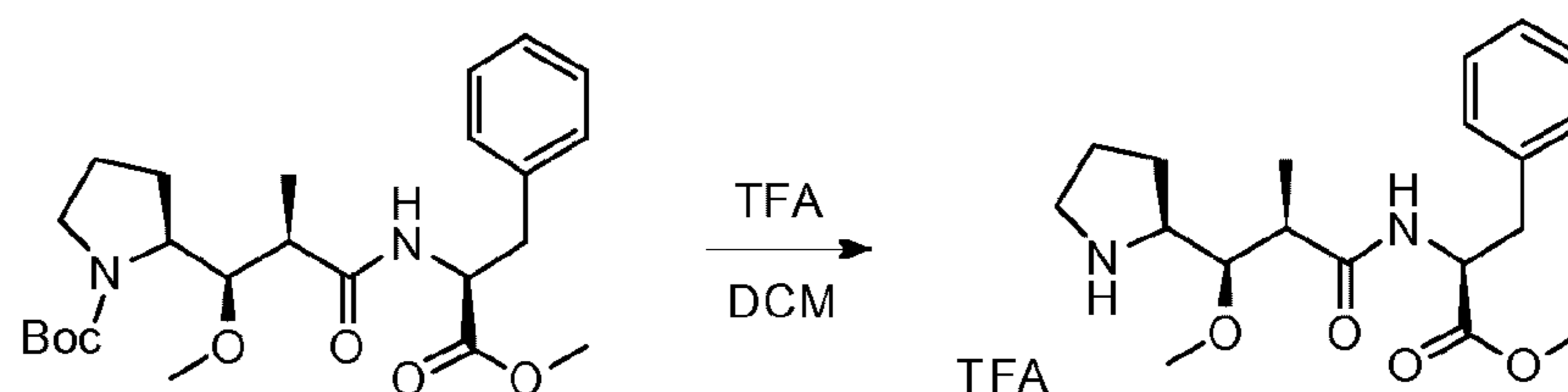
15 **Compound 3A:** *tert*-butyl (S)-2-((1R,2R)-1-methoxy-3-(((S)-1-methoxy-1-oxo-3-phenylpropan-2-yl)amino)-2-methyl-3-oxopropyl)pyrrolidine-1-carboxylate



20 Compound **1D** (3 g, 10.44 mmol, 1.00 equiv) and methyl (S)-2-amino-3-phenylpropanoate (2.25 g, 12.55 mmol, 1.20 equiv) were dissolved in an inert atmosphere in DMF (40 mL). The solution was cooled to 0 °C then DEPC (1.67 mL, 1.05 equiv) and TEA (3.64 mL, 2.50 equiv) were added drop-wise. The reaction mixture was agitated 2 hours at 0 °C then at ambient temperature overnight. The reaction mixture was diluted with 100 mL of water and extracted three times with 50

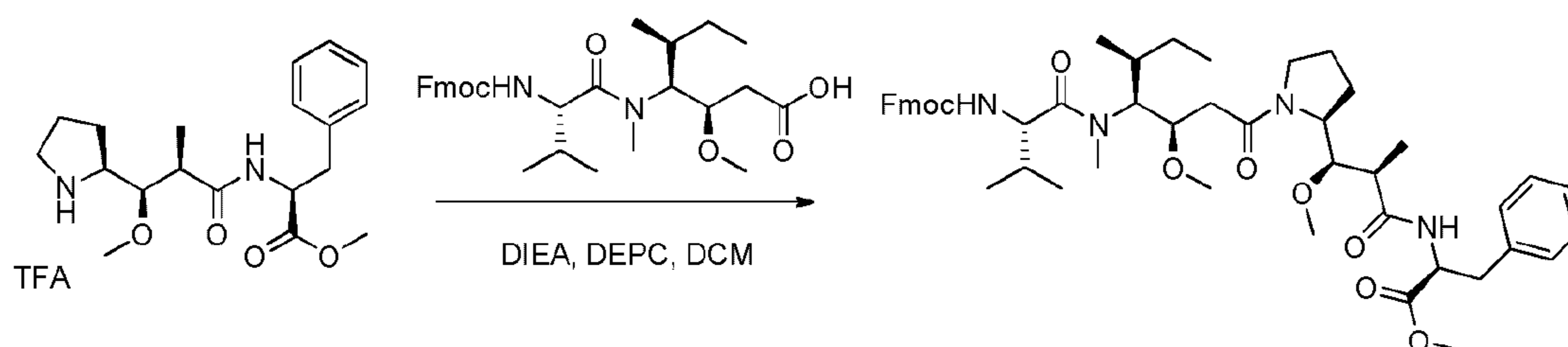
mL EtOAc. The organic phases were combined, washed once with 100 mL of KHSO_4 (1 mol/L), once with 100 mL of NaHCO_3 (sat.), once with 100 mL of NaCl (sat.), then dried over sodium sulfate, filtered and concentrated under pressure to yield 4 g (85 %) of compound **3A** in the form of a colourless oil.

5 **Compound 3B:** 2,2,2-trifluoroacetate of methyl (S)-2-((2R,3R)-3-methoxy-2-methyl-3-((S)-pyrrolidin-2-yl)propanamido)-3-phenylpropanoate



Compound **3A** (5 g, 11.15 mmol, 1.00 equiv) was dissolved in an inert atmosphere in DCM (40 mL). TFA (25 mL) was added and the solution agitated for 2 hours. The reaction mixture was concentrated under reduced pressure to yield 8 g of compound **3B** in the form of a yellow oil.

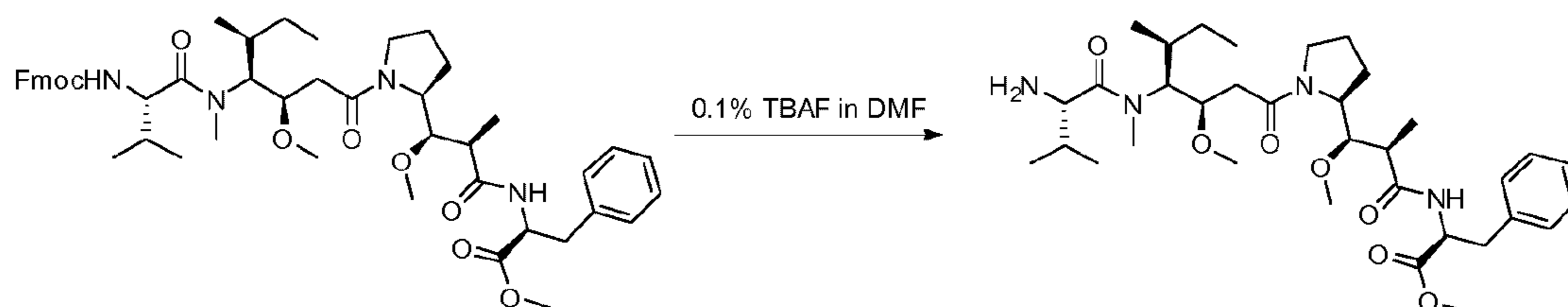
15 **Compound 3C:** methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate



Compounds **3B** (8.03 g, 17.36 mmol, 1.00 equiv) and **1W** (9.1 g, 17.34 mmol, 1.00 equiv) were dissolved in an inert atmosphere in DCM (80 mL). The solution was cooled to 0 °C then DEPC (2.8 mL) and DIEA (12 mL) were added drop-wise. The reaction mixture was agitated for 2 hours at 0 °C then at ambient temperature overnight. The reaction mixture was diluted with 200 mL of water and extracted three times with 50 mL of DCM. The organic phases were combined, washed once with 50 mL of KHSO_4 (1 mol/L), once with 50 mL of NaHCO_3 (sat.), once with 50 mL of NaCl (sat.),

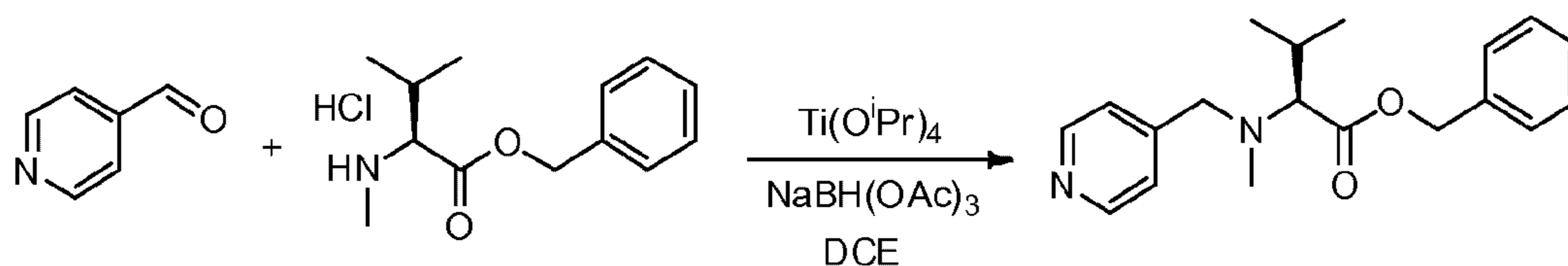
then dried over sodium sulfate, filtered and concentrated under reduced pressure to yield 5 g (34 %) of compound **3C** in the form of a yellow solid.

Compound 3D: methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-amino-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate



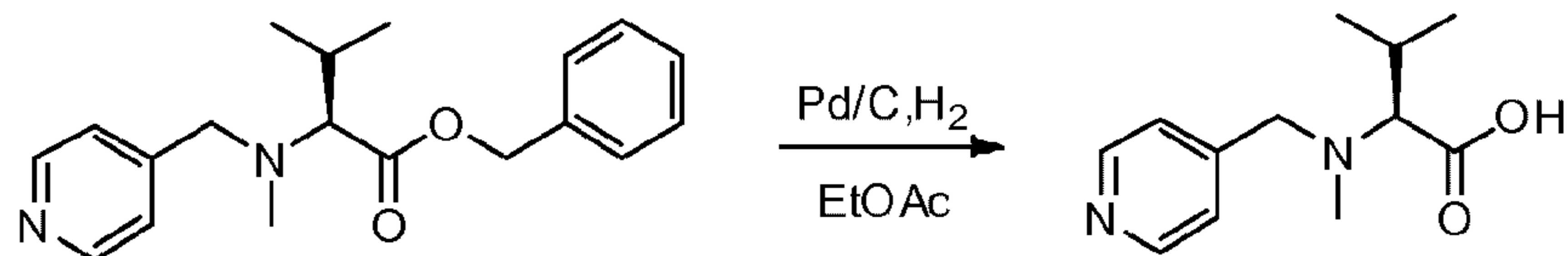
Compound **3C** (5.5 g, 6.43 mmol, 1.00 equiv) was dissolved in an inert atmosphere in a solution of tetrabutylammonium fluoride (TBAF, 2.61 g, 9.98 mmol, 1.55 equiv) in DMF (100 mL). The solution was agitated at ambient temperature for 2 hours then diluted with 100 mL of water and extracted three times with 50 mL of EtOAc. The organic phases were combined then dried over sodium sulfate, filtered and concentrated under reduced pressure to yield 3.3 g (81 %) of compound **3D** in the form of a yellow solid.

Compound 3E: benzyl (S)-3-methyl-2-(methyl(pyridin-4-ylmethyl)amino)butanoate



Pyridine-4-carbaldehyde (1 g, 9.34 mmol, 1.00 equiv) was dissolved in 10 mL of 1,2-dichloroethane (DCE) in the presence of compound **1ZC** (2.9 g, 11.25 mmol, 1.21 equiv) and titanium isopropoxide (IV) (4.19 mL, 1.40 equiv). The mixture was agitated at ambient temperature for 30 minutes then 2.77 g of NaBH(OAc)₃ (13.07 mmol, 1.40 equiv) were added. The reaction medium was left under agitation overnight then neutralised with 100 mL of water and the mixture extracted 3 times with 50 mL of AcOEt. The organic phases were combined and evaporated to dryness. The residue was purified on a silica column with a mixture of EtOAc and PE (1:20) to yield 1.3 g (45 %) of compound **3E** in the form of a colourless oil.

Compound 3F: (S)-3-methyl-2-(methyl(pyridin-4-ylmethyl)amino)butanoic acid



Compound **3E** (800 mg, 2.56 mmol, 1.00 equiv) was dissolved in 30 mL of AcOEt in the presence of Pd/C (300 mg) and hydrogenated for 3 hours at ambient temperature and atmospheric pressure. The reaction medium was filtered and concentrated under reduced pressure. The residue was purified on a silica column with a mixture of DCM and MeOH (100:1 to 5:1) to yield 100 mg (18 %) of compound **3F** in the form of a white solid.

10

Compounds **3D** (50 mg, 0.08 mmol, 1.00 equiv) and **3F** (26.34 mg, 0.12 mmol, 1.50 equiv) were dissolved in 3 mL of DCM. The solution was cooled to 0 °C then 0.018 mL of DEPC and 0.0392 mL of DIEA were added. The reaction was agitated at 0°C for 2 hours then at ambient temperature overnight. The reaction medium was concentrated under reduced pressure and the residue (70 mg) was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μm, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % of TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **3** was obtained with a yield of 27 % (20 mg) in the form of a white solid.

20

LC/MS/UV (Ascentis Express C18 column, 2.7 μm, 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % ACN in water (0.05 % TFA) in 8 minutes); ESI (C₄₆H₇₂N₆O₈, exact mass 836.5) *m/z*: 837.5 (MH⁺) and 419.4 (M.2H⁺/2 (100 %)), 7.04 min (90.0 %, 210 nm)

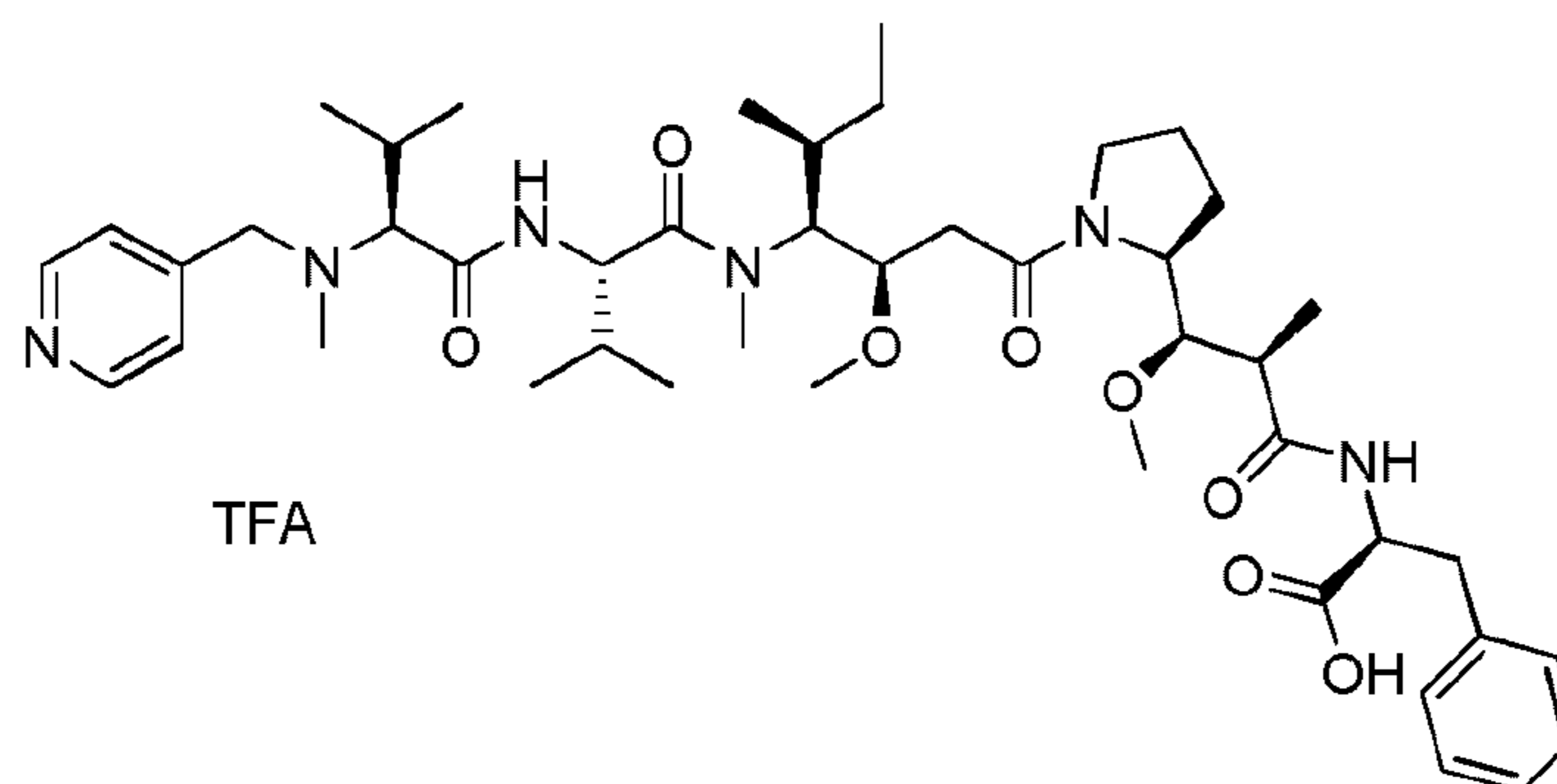
25

¹H NMR (400MHz, CD₃OD, *ppm*): δ (Presence of rotamers) 8.76 - 8.74 (m, 2H); 8.53 - 8.48 (m, 0.4H, NHCO incomplete exchange); 8.29 - 8.15 (m, 0.8H, NHCO incomplete exchange); 8.01 (s, 2H), 7.31 - 7.22 (m, 5H), 4.88 - 4.68 (m, 3H); 4.31 - 4.07 (m, 2H); 3.94 - 2.90 (m, 18H); 2.55 - 0.86 (m, 38H).

30

Reference Compound 4

(S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(pyridin-4-ylmethyl)amino)butanamido)butanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoic acid, trifluoroacetic acid



Compound **3** (100 mg, 0.11 mmol, 1.00 equiv) was dissolved in a mixture of water (5 mL), ACN (5 mL) and piperidine (2.5 mL). The reaction mixture was left under agitation overnight then concentrated under reduced pressure. The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm), to yield 20 mg (20 %) of compound **4** in the form of a white solid.

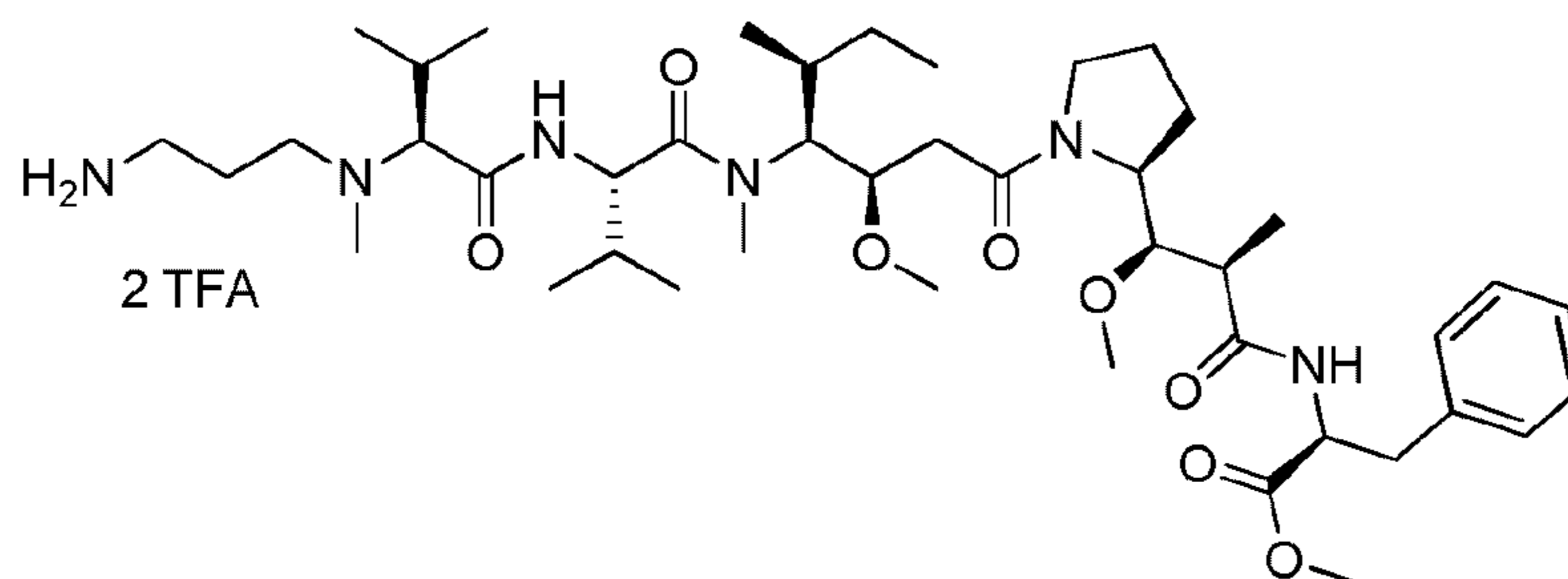
LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % ACN in water (0.05 % TFA) in 8 minutes); ESI (C₄₅H₇₀N₆O₈, exact mass 822.5) *m/z*: 823.5 (MH⁺) and 412.4 (M.2H⁺/2, 100 %), 6.84 min (89.1 %, 210 nm).

¹H NMR (400MHz, CD₃OD, ppm): δ (Presence of rotamers) 8.79 - 8.78 (m, 2H); 8.09 (m, 2H); 7.30 - 7.21 (m, 5H); 4.80 - 4.80 (m, 1H), 4.36 - 0.87 (m, 58H).

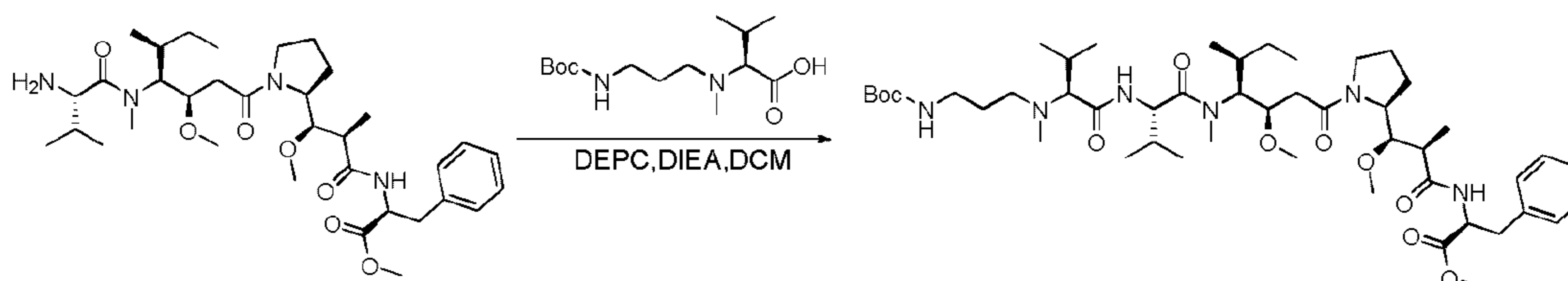
Compound 6

methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((3-aminopropyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, bis trifluoroacetic acid

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Compound 6A: methyl (2S)-2-[(2R)-2-[(R)-[(2S)-1-[(3R,4S,5S)-4-[(2S)-2-[(2S)-2-[(3-[[*tert*-butoxy]carbonyl]amino]propyl)(methyl)amino]-3-methylbutanamido]-*N*,3-dimethylbutanamido]-3-methoxy-5-methylheptanoyl]pyrrolidin-2-yl](methoxy)methyl]propanamido]-3-phenylpropanoate



Compound **3D** (157.5 mg, 0.25 mmol, 1.00 equiv) was dissolved at 0°C in an inert atmosphere in 3 mL of DCM in the presence of carboxylic acid **1ZF** (78.7 mg, 0.27 mmol, 1.10 equiv), DEPC (46 µl) and DIEA (124 µl). The reaction mixture was agitated 2 hours at low temperature and the cold bath was then removed and agitation continued for 4 hours. It was then concentrated under reduced pressure to yield 200 mg of compound **6A** in the form of a crude yellow oil. It was used as such in the following step.

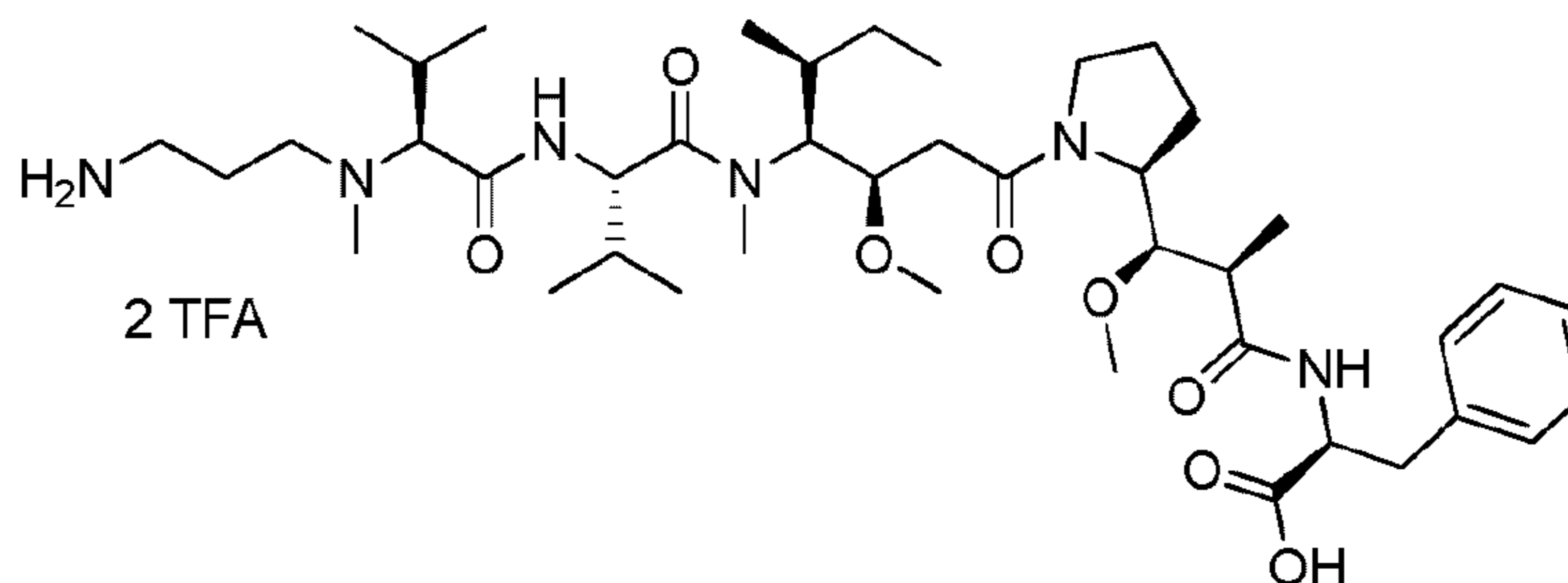
Compound **6A** (200 mg, 0.22 mmol, 1.00 equiv) was dissolved in an inert atmosphere at 0°C in 2 mL of DCM. TFA (1 mL) was added drop-wise and the cold bath removed. The reaction mixture was agitated 1 hour at ambient temperature then concentrated under reduced pressure. The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 µm, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2489 UV Detector at 254 nm and 220 nm), to yield 60 mg (26 %, yield in 2 steps) of compound **6** in the form of a white solid.

LC/MS/UV (Zorbax Eclipse Plus C8, 3.5 μm , 4.6 x 150 mm; 1 mL/min, 40°C, 30 to 80 % methanol in water (0.1 % H_3PO_4) in 18 minutes); ESI ($\text{C}_{43}\text{H}_{74}\text{N}_6\text{O}_8$, exact mass 802.56) m/z : 804 (MH^+); 11.50 min (91.5 %, 210 nm).

^1H NMR (300MHz, CD_3OD , ppm): δ (Presence of rotamers) 8.52 (d, 0.3H, NHCO incomplete exchange); 8.25 (d, 0.5H, NHCO incomplete exchange); 7.30–7.22 (m, 5H); 4.9–4.6 (m, 3H); 4.2–4.0 (m, 1H); 4.0–0.86 (m, 61H).

Compound 7

(S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((3-aminopropyl) (methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl) pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoic acid, bis trifluoroacetic acid



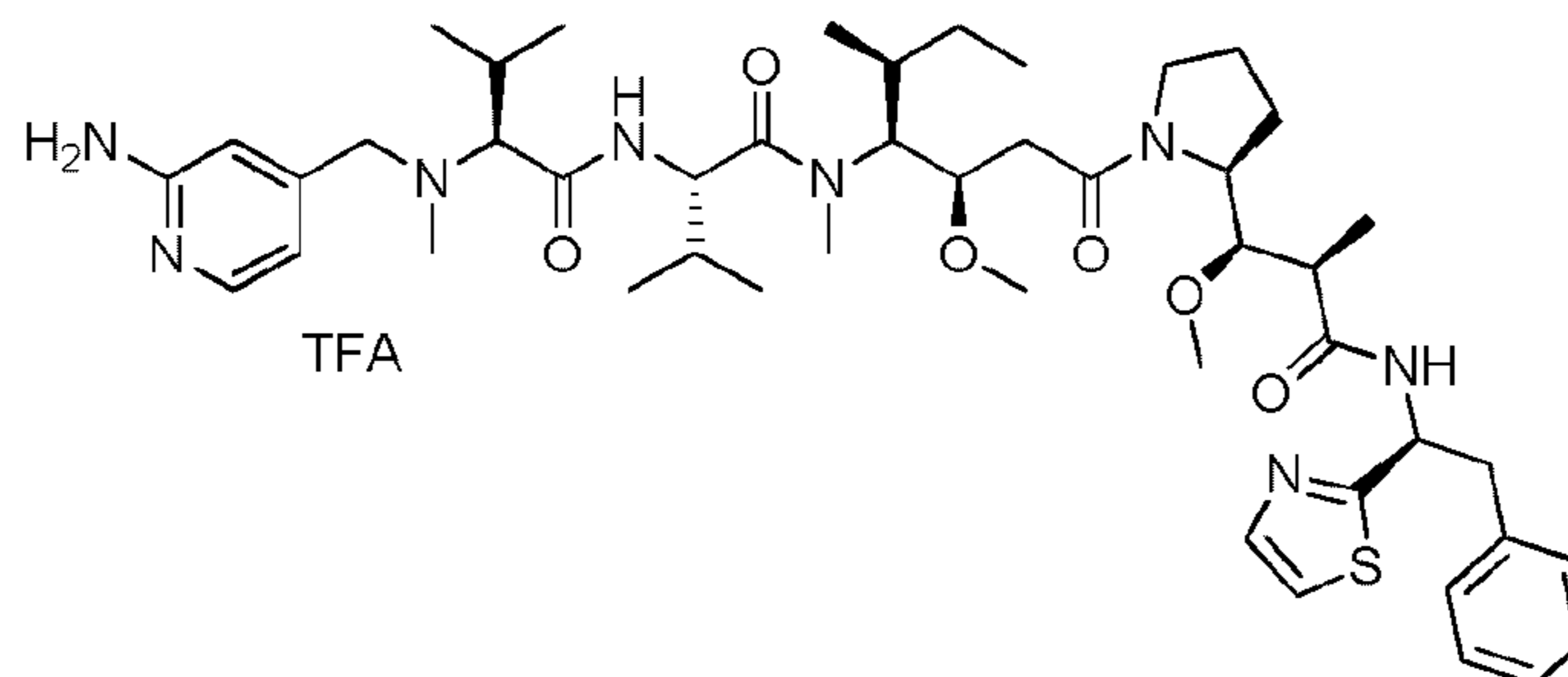
Compound 6 (70 mg, 0.08 mmol, 1.00 equiv) was dissolved in a mixture of water (5 mL), ACN (2.5 mL) and piperidine (5 mL). The reaction mixture was left under agitation overnight at ambient temperature, then concentrated under reduced pressure. The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μm , 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; UV Waters 2489 UV Detector at 254 nm and 220 nm), to yield 14.6 mg (21 %) of compound 7 in the form of a white solid.

LC/MS/UV (Ascentis Express C18, 2.7 μm , 4.6 x 100 mm; 1.5 mL/min, 40°C, 0 to 80 % methanol in water (0.05 % TFA) in 8 minutes); ESI ($\text{C}_{42}\text{H}_{72}\text{N}_6\text{O}_8$, exact mass 788.54) m/z : 790 (MH^+), 5.71 min (96.83 %, 210 nm).

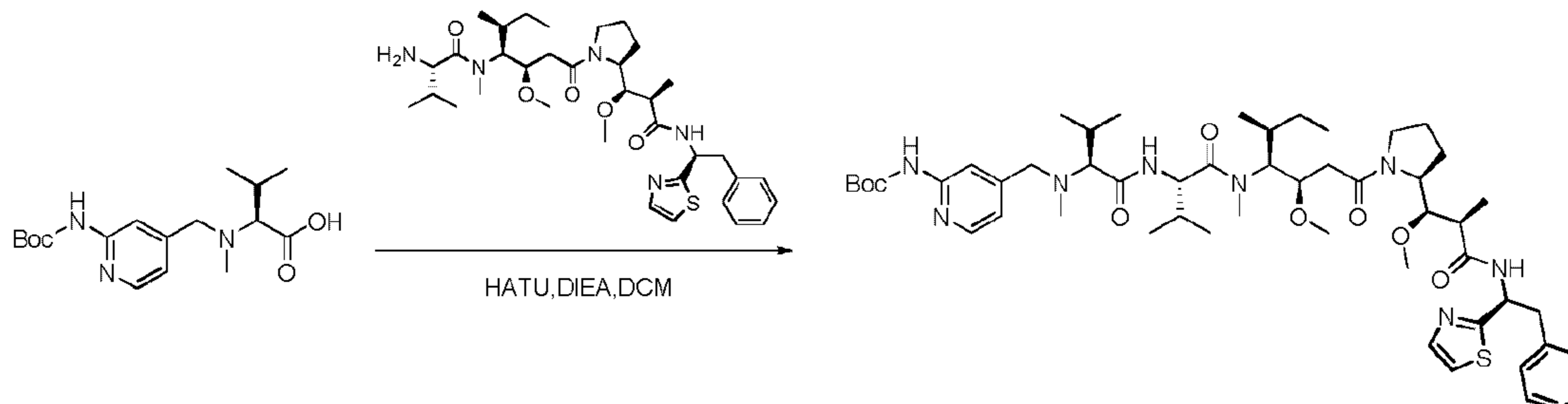
^1H NMR (300MHz, CD_3OD , ppm): δ (Presence of rotamers) 8.42 (d, 0.3H, NHCO incomplete exchange); 8.15 (d, 0.2H, NHCO incomplete exchange); 7.31–7.21 (m, 5H); 4.9–4.6 (m, 3H); 4.25–4.0 (m, 1H); 4.0–0.86 (m, 59H).

Compound 8

(S)-2-((S)-2-(((2-aminopyridin-4-yl)methyl)(methyl)amino)-3-methylbutanamido)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide, trifluoroacetic acid



Compound 8A: *tert*-butyl (4-((3S,6S,9S,10R)-9-((S)-*sec*-butyl)-3,6-diisopropyl-10-(2-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-2,8-dimethyl-4,7-dioxo-11-oxa-2,5,8-triazadodecyl)pyridin-2-yl) carbamate



Compound **8A** was synthesised in the same manner as for compound **2J** from the amine **1Y** (39 mg, 0.06 mmol, 1.00 equiv), the acid **2I** (20 mg, 0.06 mmol, 1.00 equiv), HATU (27 mg, 0.07 mmol, 1.20 equiv) and DIEA (23.2 mg, 0.18 mmol, 3.01 equiv) in DCM (3 mL). The crude product was not purified.

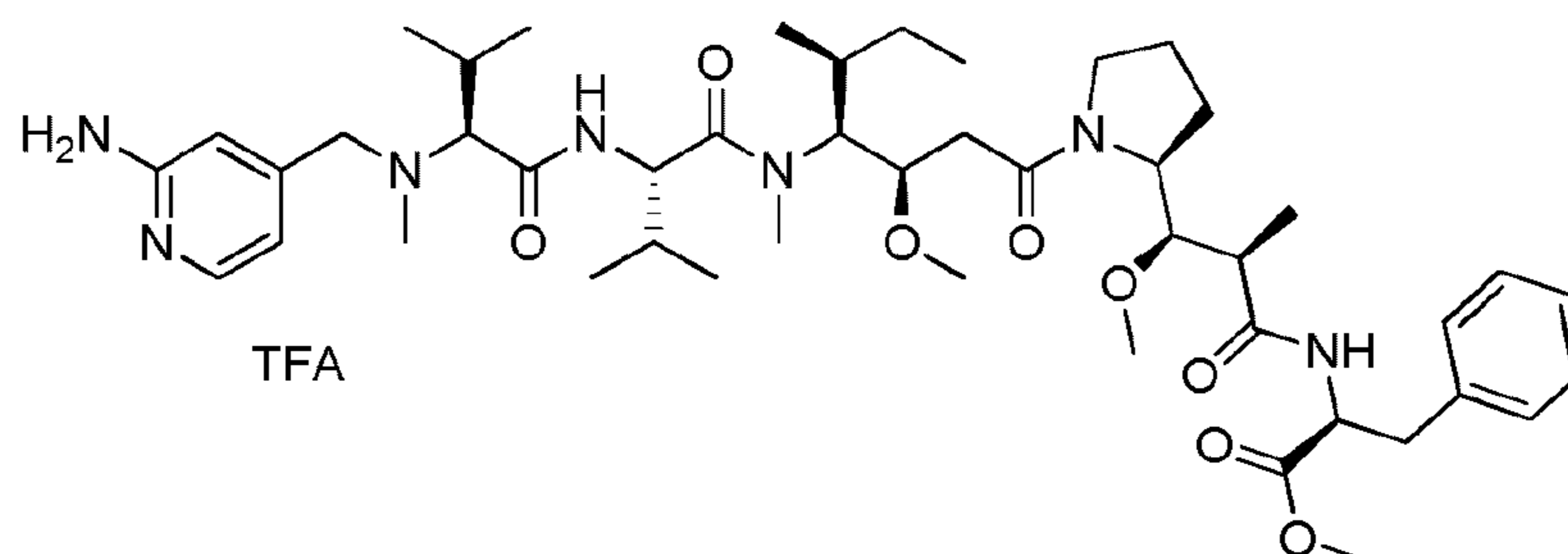
Compound 8: Compound **8** was synthesised in similar manner to compound **2** from the intermediate **8A** (100 mg, 0.10 mmol, 1.00 equiv). The crude product (100 mg) was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 18 % to 31 % ACN in 7 minutes then 31 % to 100 % ACN in 2 minutes; Waters 2489 UV Detector at 254 nm and 220 nm). Compound **8** was obtained with a yield of 8 % (8 mg) in the form of a white solid.

LC/MS/UV (Atlantis T3 column, 3 μm , 4.6 x 100 mm; 35°C; 1.8 mL / min, 25 % to 80 % ACN in water (0.05 % TFA) in 7 minutes); ESI ($\text{C}_{47}\text{H}_{72}\text{N}_8\text{O}_6\text{S}$, exact mass 876.5) m/z : 877.5 (MH^+) and 439.5 ($\text{M}.2\text{H}^+/2$, 100 %), 4.87 min (95.1 %, 254 nm).

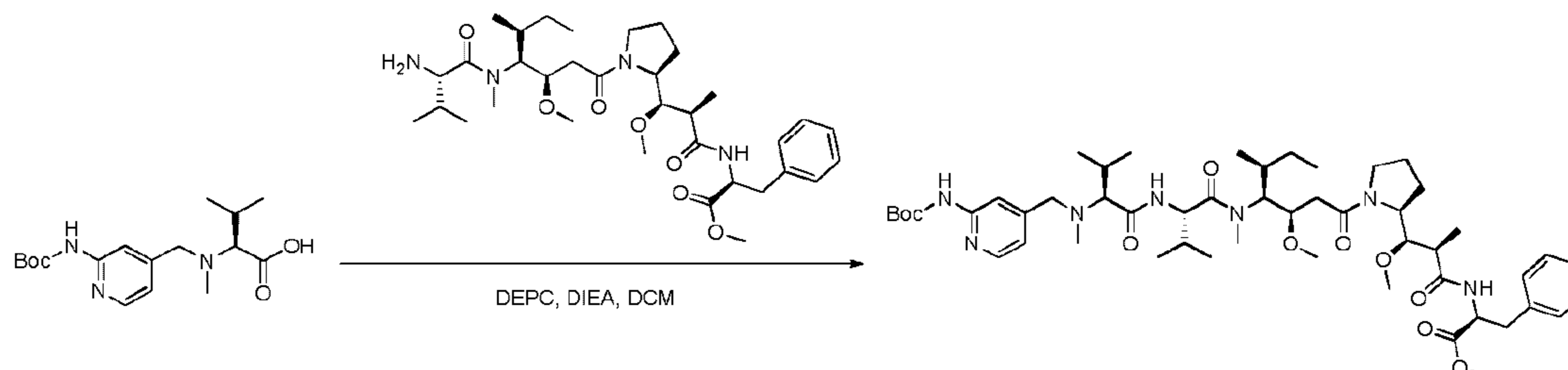
¹H NMR (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 7.83 - 7.78 (m, 2H); 7.56 - 7.52 (m, 1H); 7.34 - 7.17 (m, 5H); 6.64 - 6.62 (m, 2H); 5.77 - 5.61 (m, 1H); 4.86 - 4.68 (m, 2H); 4.25 - 4.05 (m, 1H); 3.87 - 2.83 (m, 17H); 2.56 - 0.84 (m, 37H).

Compound 9

10 **methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-(((2-aminopyridin-4-yl)methyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, trifluoroacetic acid**



15 **Compound 9A:** methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-(((2-((tert-butoxycarbonyl)amino)pyridin-4-yl)methyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate



20 Compound **9A** was synthesised in the same manner as for compound **3** from the amine **3D** (170 mg, 0.27 mmol, 1.00 equiv), the acid **2I** (99.7 mg, 0.30 mmol, 1.10 equiv), DEPC (0.049 mL, 1.05 equiv) and DIEA (0.133 mL, 3.00 equiv) in DCM (5 mL). The crude product was purified on a silica column with a mixture of EtOAc and PE (1:1) to yield 200 mg (78 %) of compound **9A** in the form of a pale yellow solid.

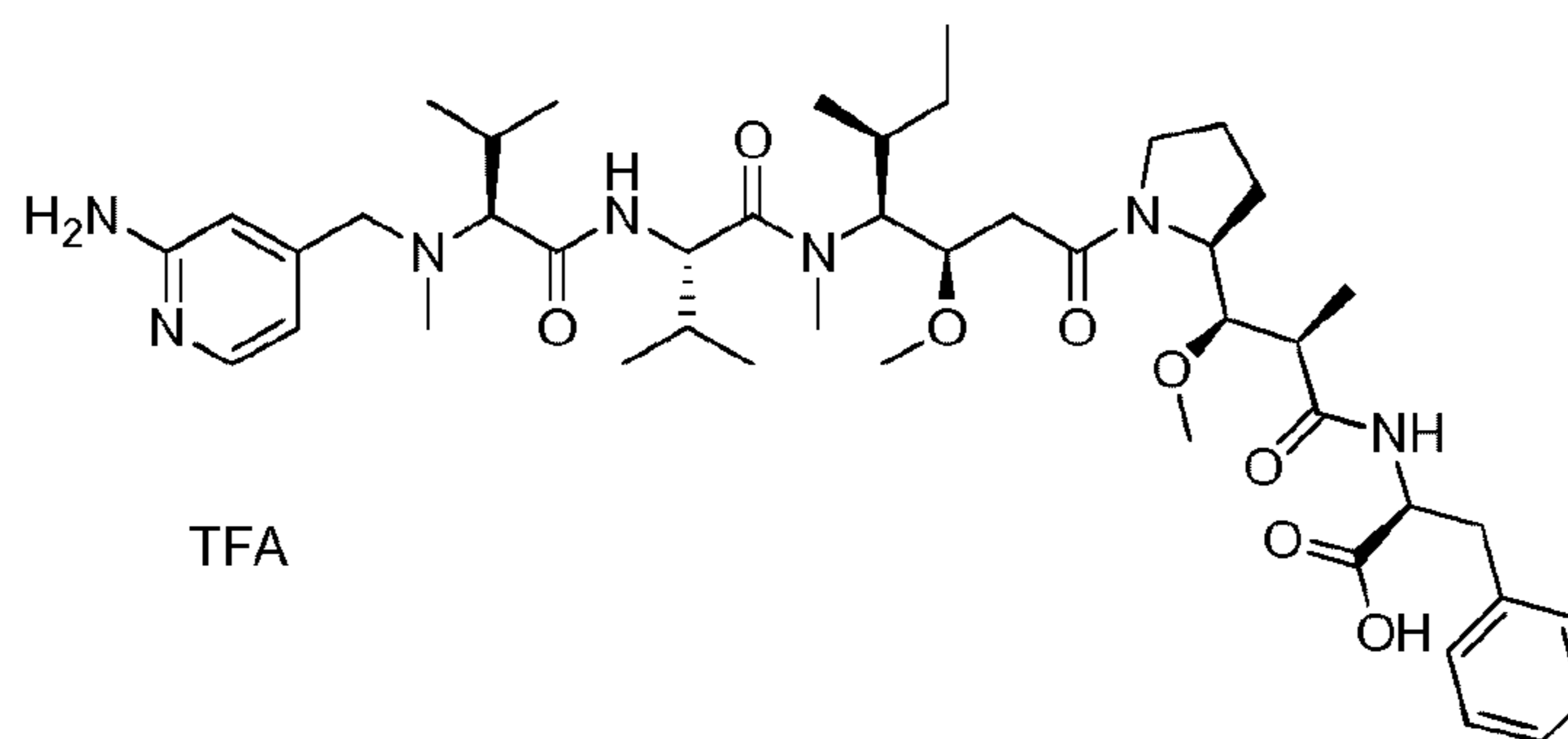
Compound 9: Compound 9 was synthesised in the same manner as for compound 2 from the intermediate 9A (200 mg, 0.21 mmol, 1.00 equiv) in DCM (4 mL) and TFA (2 mL). The crude product was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound 9 was obtained with a yield of 10 % (20 mg) in the form of a white solid.

LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI (C₄₆H₇₃N₇O₈, exact mass 851.6) *m/z*: 852.5 (MH⁺) and 426.9 (M.2H⁺/2, 100 %), 6.92 min (92.7 %, 254 nm).

¹H NMR (400MHz, CD₃OD, *ppm*): δ (Presence of rotamers) 8.51 - 8.45 (m, 0.5H, NH incomplete exchange); 8.30 - 8.24 (m, 0.3H, NH incomplete exchange); 8.17 - 8.07 (m, 0.8H, NH incomplete exchange); 7.79 - 7.77 (m, 1H); 7.36 - 7.18 (m, 5H); 7.21 - 7.16 (m, 1H); 6.94 - 6.89 (m, 1H); 4.85 - 4.65 (m, 3H); 4.20 - 3.10 (m, 20H); 3.00 - 2.85 (m, 2H); 2.55 - 0.80 (m, 36H).

Compound 10

(S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-(((2-aminopyridin-4-yl)methyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoic acid, trifluoroacetic acid



Compound 10: Compound 9 (100 mg, 0.11 mmol, 1.00 equiv) was dissolved in a mixture of water (5 mL), ACN (5 mL) and piperidine (2.5 mL). The reaction mixture was left under agitation overnight at ambient temperature and then concentrated under

reduced pressure. The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and
 5 220 nm), to yield 32.2 mg (33 %) of compound **10** in the form of a white solid.

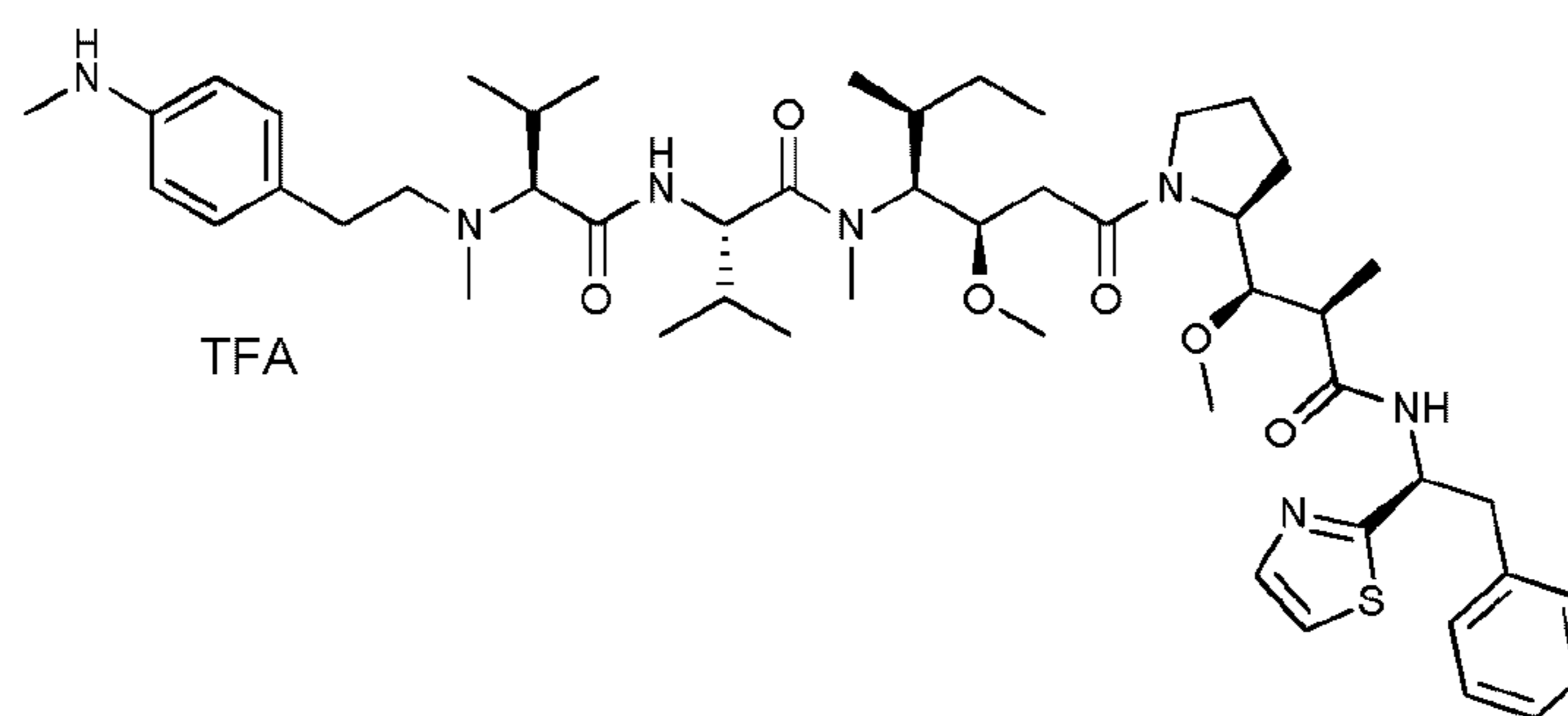
LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI (C₄₅H₇₁N₇O₆, exact mass 837.5) *m/z*: 838.5 (MH⁺) and 419.9 (M.2H⁺/2, 100 %), 6.81 min (97.7 %, 220 nm).

10 ¹H NMR (400MHz, CD₃OD, *ppm*): δ (Presence of rotamers) 8.41 - 8.32 (m, 0.3H, NH incomplete exchange); 8.20 - 8.07 (m, 0.8H, NH incomplete exchange); 7.82 - 7.75 (m, 1H); 7.36 - 7.158 (m, 5H); 7.12 - 7.03 (m, 1H); 6.94 - 6.88 (m, 1H); 4.85 - 4.66 (m, 3H); 4.20 - 3.10 (m, 16H); 3.00 - 2.85 (m, 2H); 2.57 - 0.80 (m, 37H).

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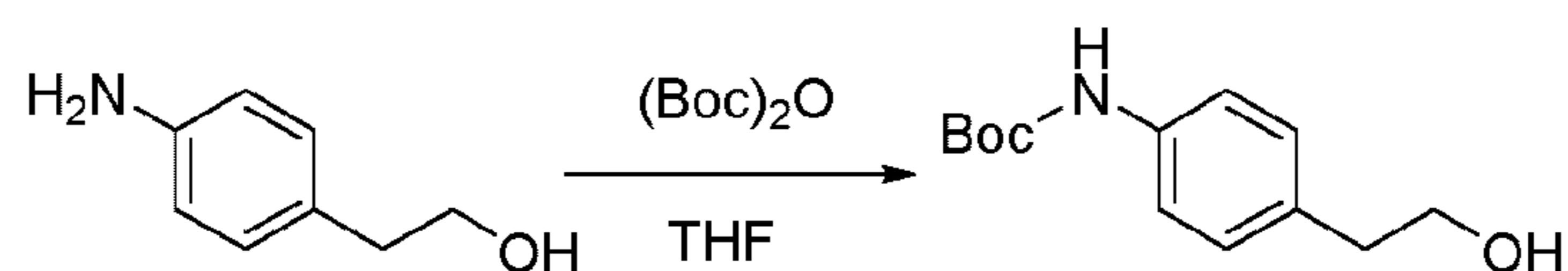
Compound 11

(S)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(4-(methylamino)phenethyl)amino) butanamido)butanamide, trifluoroacetic acid



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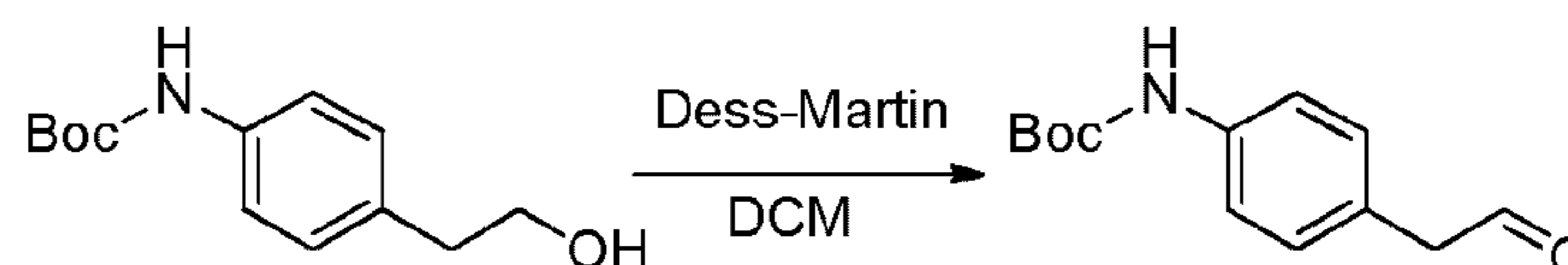
Compound 11A: *tert*-butyl *N*-[4-(2-hydroxyethyl)phenyl]carbamate



Di-*tert*-butyl dicarbonate (16.7 g, 77 mmol, 1.05 eq.) was added to a solution of 2-(4-aminophenyl)ethanol (10 g, 72.9 mmol, 1 eq.) in THF (200 mL), and the reaction stirred overnight at ambient temperature. The mixture was diluted with EtOAc (200

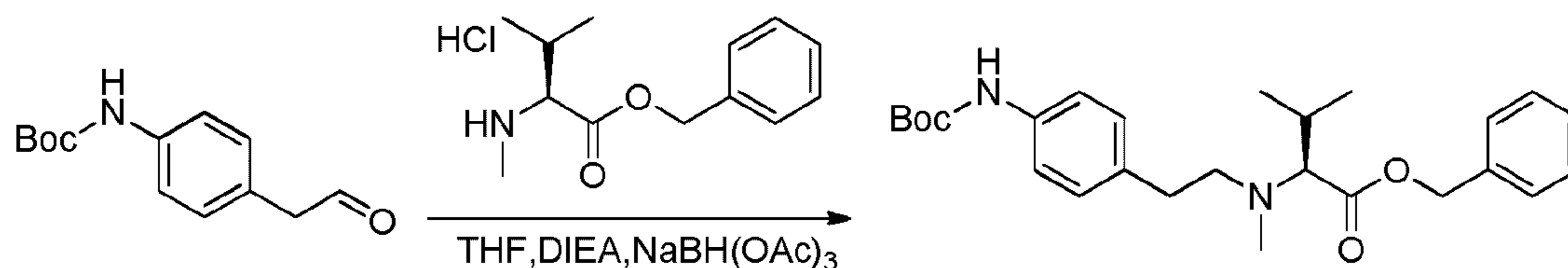
mL), washed with water (200 mL), then HCl 1M (100 mL), then saturated aqueous NaHCO₃ solution (100 mL) then brine (100 mL). The organic phase was dried over MgSO₄ then evaporated to dryness under reduced pressure. The crude product was triturated twice with heptane (150 mL) and dried under vacuum to furnish compound **11A** as a white solid (14.7 g, 84 %).

Compound 11B: *tert*-butyl *N*-[4-(2-oxoethyl)phenyl]carbamate



Compound **11A** (2.5 g, 10.5 mmol, 1.00 equiv) was dissolved in 25 mL of DCM then cooled to -78°C. A Dess-Martin Periodinane solution (DMP, 6.71 g, 15.8 mmol, 1.5 equiv) in DCM (10 mL) was added drop-wise. The cold bath was removed and agitation continued for 1 hour at ambient temperature. The reaction was neutralised with 60 mL of a 50/50 mixture of sodium bicarbonate-saturated aqueous solution and Na₂S₂O₃-saturated aqueous solution. The resulting solution was extracted 3 times with 30 mL of EtOAc. The organic phases were combined, washed twice with NaCl-saturated aqueous solution, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified on silica gel (EtOAc/PE 1/15) to yield 1.0 g (40 %) of compound **11B** in the form of a pale yellow solid.

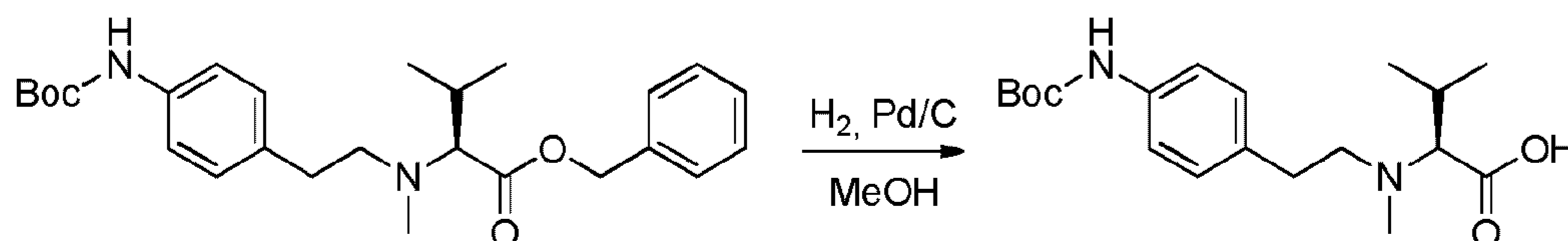
Compound 11C: benzyl (2*S*)-2-[[2-(4-[[*tert*-butoxy)carbonyl]amino]phenyl)ethyl](methyl)amino]-3-methylbutanoate.



Compound **11C** (3.5 g, 13.6 mmol, 1.1 equiv) was dissolved in THF (30 mL) in the presence of DIEA (6.4 g, 49.7 mmol, 4.0 equiv), aldehyde **11B** (2.9 g, 12.3 mmol, 1.0 equiv) and sodium triacetoxyborohydride (5.23 g, 49.7 mmol, 2.0 equiv). The reaction mixture was left under agitation overnight at ambient temperature, then neutralised with 60 mL of sodium bicarbonate-saturated solution. The resulting solution was extracted 3 times with 30 mL EtOAc. The organic phases were combined, washed twice with NaCl-saturated aqueous solution, dried over anhydrous sodium sulfate,

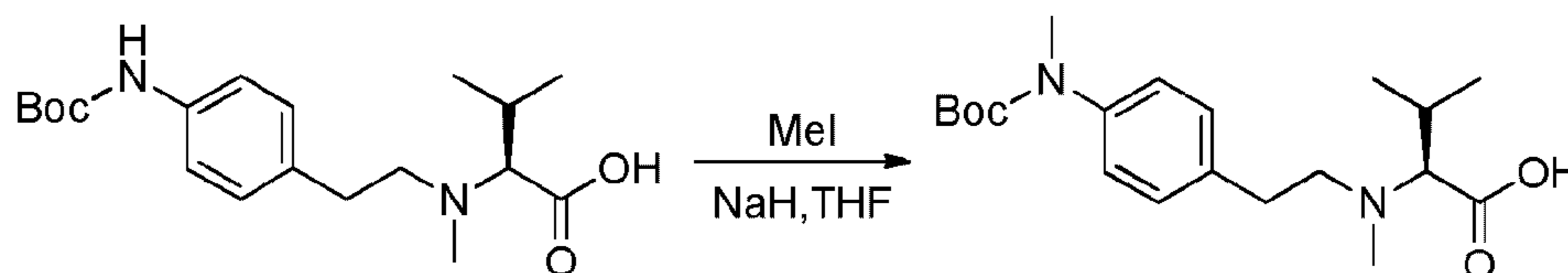
filtered and concentrated under reduced pressure. The residue was purified on silica gel (EtOAc/PE 1:20) to yield 3.7 g (68 %) of compound **11C** in the form of a yellow oil.

Compound 11D: (2S)-2-[[2-(4-[[*tert*-butoxy)carbonyl]amino]phenyl)ethyl](methyl)amino]-3-methylbutanoic acid



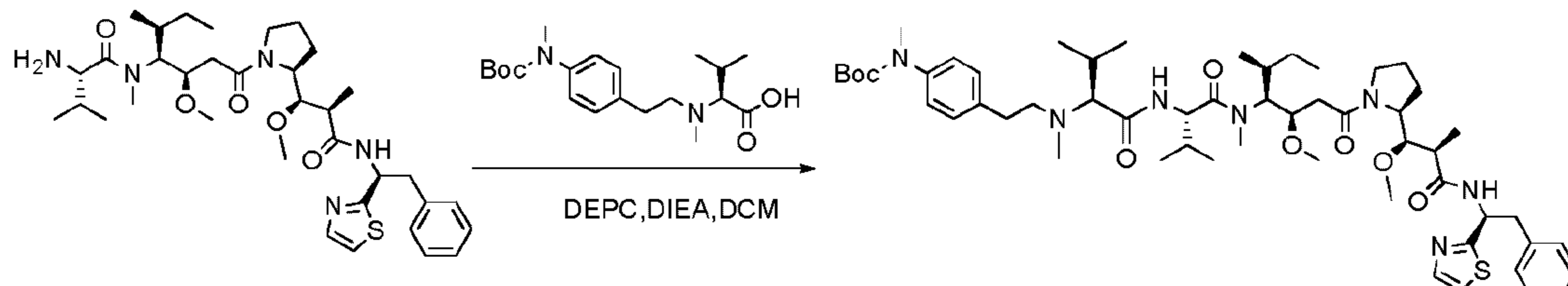
5 Compound **11C** (2 g, 4.5 mmol, 1 equiv) was dissolved in 10 mL of methanol in the presence of Pd/C (2 g) and hydrogenated for 2 hours at normal temperature and pressure. The reaction medium was filtered and concentrated under reduced pressure to yield 1.2 g (75 %) of compound **11D** in the form of a yellow oil.

Compound 11E: (2S)-2-[[2-(4-[[*tert*-butoxy)carbonyl](methyl)amino]phenyl)ethyl](methyl)amino]-3-methylbutanoic acid



15 Compound **11D** (1.2 g, 3.4 mmol, 1.00 equiv) was dissolved in an inert atmosphere in THF (20 mL). The reaction medium was cooled with an ice bath after which NaH (60 % in oil, 549 mg, 13.7 mmol, 4.0 equiv) was added in portions, followed by iodomethane (4.9 g, 34 mmol, 10 equiv). The reaction was left under agitation overnight at ambient temperature, then neutralised with water and washed with 100 mL of EtOAc. The pH of the aqueous solution was adjusted to 6-7 with 1N HCl. This aqueous solution was extracted 3 times with 100 mL of EtOAc. The organic phases were combined, dried over sodium sulfate, filtered and concentrated to yield 800 mg
20 (64 %) of compound **11E** in the form of a yellow solid.

Compound 11F: *tert*-butyl *N*-[4-(2-[[[(1S)-1-[[[(1S)-1-[[[(3R,4S,5S)-3-methoxy-1-[(2S)-2-[(1R,2R)-1-methoxy-2-methyl-2-[[[(1S)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]carbonyl]ethyl]pyrrolidin-1-yl]-5-methyl-1-oxoheptan-4yl](methyl)carbonyl]-2-methylpropyl]carbonyl]-2-methylpropyl](methyl)amino]ethyl]phenyl]-*N*-methylcarbamate



Compound **11F** was prepared in similar manner to compound **6A** from the amine **1Y** (150 mg, 0.22 mmol, 1.2 equiv) and the acid **11E** (70 mg, 0.19 mmol, 1.0 equiv). After purification on silica gel (EtOAc/PE 1:1) 100 mg (52 %) of desired product were obtained in the form of a pale yellow solid.

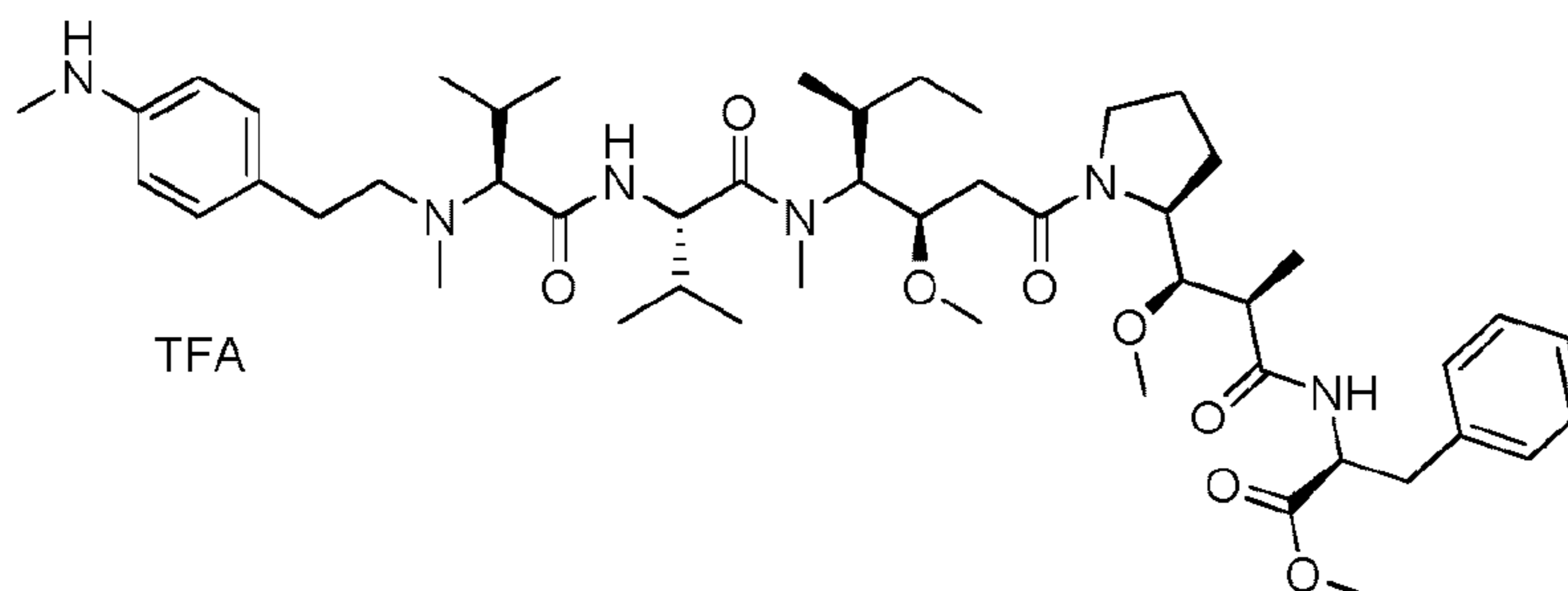
Compound **11** was prepared in the same manner as for compound **1** from the intermediate **11F** (100 mg, 0.1 mmol). The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2489 UV Detector at 254 nm and 220 nm). Compound **11** was obtained with a yield of 39 % (39.7 mg) in the form of a white solid.

LC/MS/UV (Eclipse Plus C8, 3.5 μ m, 4.6 x 150 mm; 1 mL/min, 40°C, 50 to 95 % methanol in water (0.05 % TFA) in 18 minutes); ESI ($C_{50}H_{77}N_7O_6S$, exact mass 903.57) m/z : 904.5 (MH^+), 7.53 min (93.68 %, 254 nm).

1H NMR (300MHz, CD_3OD , ppm): δ (Presence of rotamers) 8.84 (d, 0.5H, NHCO incomplete exchange); 8.7–8.5 (m, 0.9H, NHCO incomplete exchange); 7.76–7.73 (m, 1H); 7.55 - 7.4 (m, 1H); 7.28–7.22 (m, 7H); 7.08–7.05 (m, 2H); 5.51–5.72 (m, 1H); 4.9–4.80 (m, 2H); 4.3–0.7 (m, 60H).

Compound 12

methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(4-(methylamino)phenethyl)amino)butanamido)butanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, trifluoroacetic acid



In the same manner as for the final phases in the synthesis of compound **1**, compound **12** was prepared in two steps from the amine **3D** (118 mg, 0.19 mmol) and the acid **11E** (82 mg, 0.22 mmol). The final residue was purified by preparative HPLC
 5 (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2489 UV Detector at 254 nm and 220 nm). Compound **12** was obtained with a yield of 7 % (13.7 mg) in the form of a white solid.

10 LC/MS/UV (Eclipse Plus C8, 3.5 μ m, 4.6 x 150 mm; 1 mL/min, 40°C, 40 to 95 % methanol in water (0.05 % TFA) in 18 minutes); ESI ($C_{49}H_{78}N_6O_8$, exact mass 878.59) m/z : 879.7 (MH^+), 10.07 min (90.6 %, 254 nm).

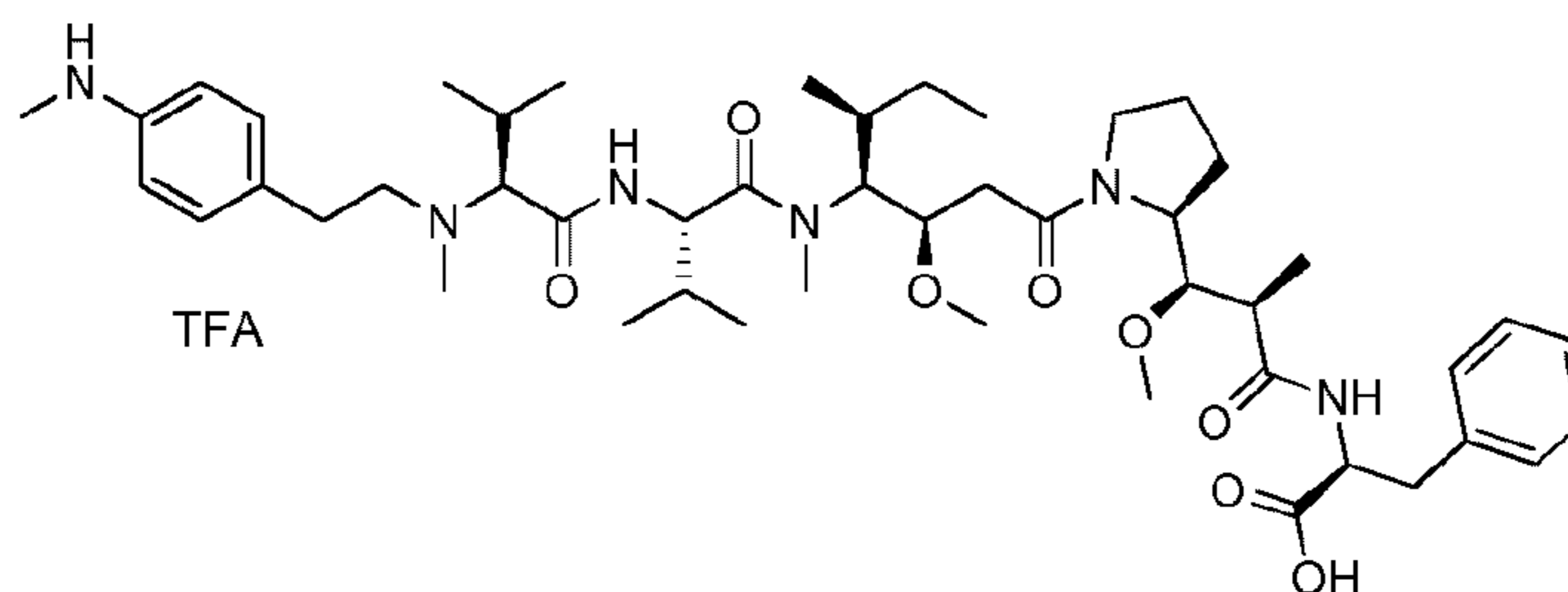
1H :NMR (300MHz, CD_3OD , ppm): δ (Presence of rotamers) 7.40 (se, 2H); 7.38–7.22 (m, 7H); 4.95–4.7 (m, 3H); 4.2–4.0 (m, 1H); 3.9–0.86 (m, 62H).

15

Compound 13

(S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(4-(methylamino)phenethyl)amino)butanamido)butanamido)-3-methoxy-5-methyl heptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoic acid, trifluoroacetic acid

20



Compound **13** was prepared in the same manner as for compound **7** from compound **12** (100 mg, 0.10 mmol). The residue was purified by preparative HPLC

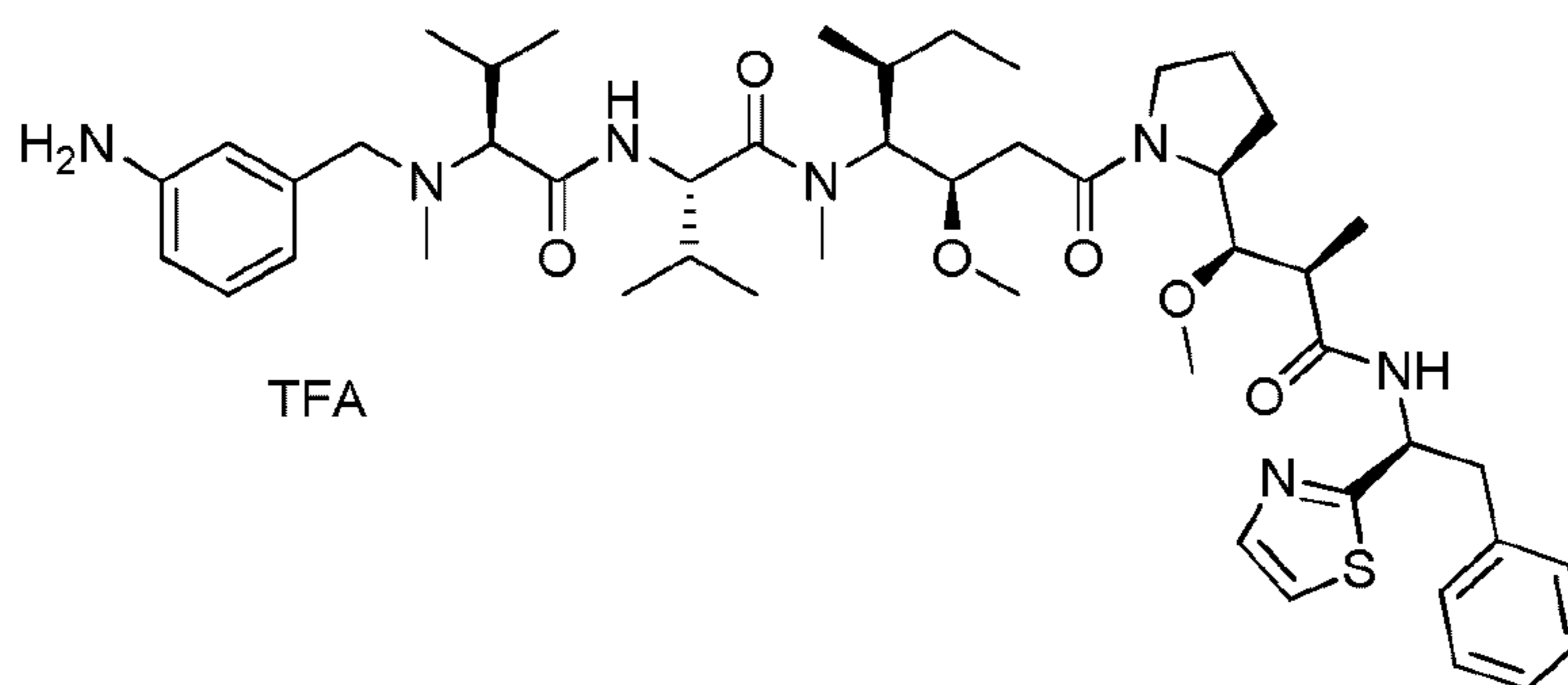
(Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2489 UV Detector at 254 nm and 220 nm). Compound **13** was obtained with a yield of 20 % (20 mg) in the form of a white solid.

LC/MS/UV (Ascentis Express C18, 2.7 μ m, 4.6 x 100 mm; 1.5 mL/min, 40°C, 10 to 95 % methanol in water (0.05 % TFA) in 8 minutes); ESI ($C_{48}H_{76}N_6O_8$, exact mass 864.57) m/z : 865.6 (MH^+), 6.05 min (90.9 %, 210 nm).

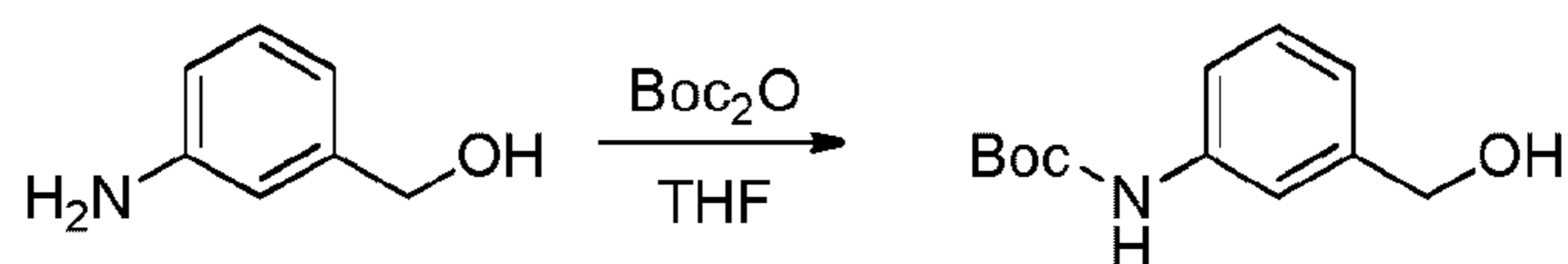
1H NMR: (300MHz, CD_3OD , ppm): δ (Presence of rotamers) 7.32–7.19 (m, 9H); 4.9–4.65 (m, 3H); 4.2–4.0 (m, 1H); 3.9–0.86 (m, 59H).

Compound 14

(S)-2-((S)-2-((3-aminobenzyl)(methyl)amino)-3-methylbutanamido)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide, trifluoroacetic acid



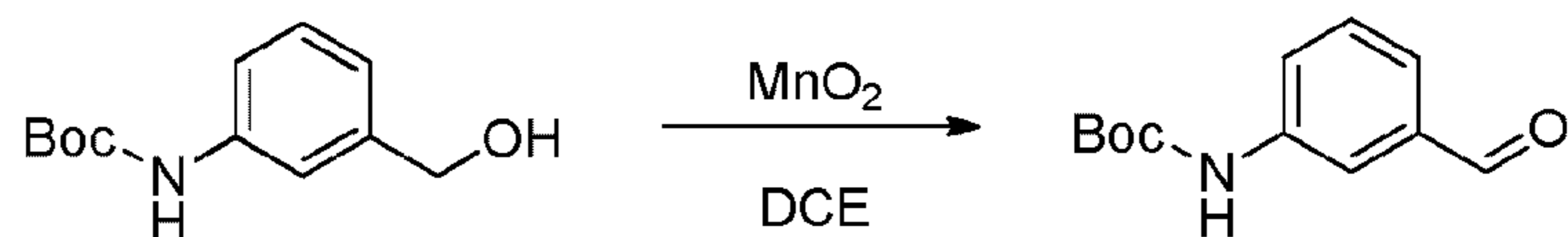
Compound 14A: *tert*-butyl (3-(hydroxymethyl)phenyl) carbamate



(3-aminophenyl)methanol (3 g, 24.36 mmol, 1.00 equiv) was dissolved in THF (60 mL) after which di-*tert*-butyl dicarbonate (6.38 g, 29.23 mmol, 1.20 equiv) was then added. The reaction mixture was left under agitation overnight at ambient temperature and the reaction was then diluted by adding 200 mL of water. The product was extracted 3 times with 100 mL of AcOEt and the organic phases were then

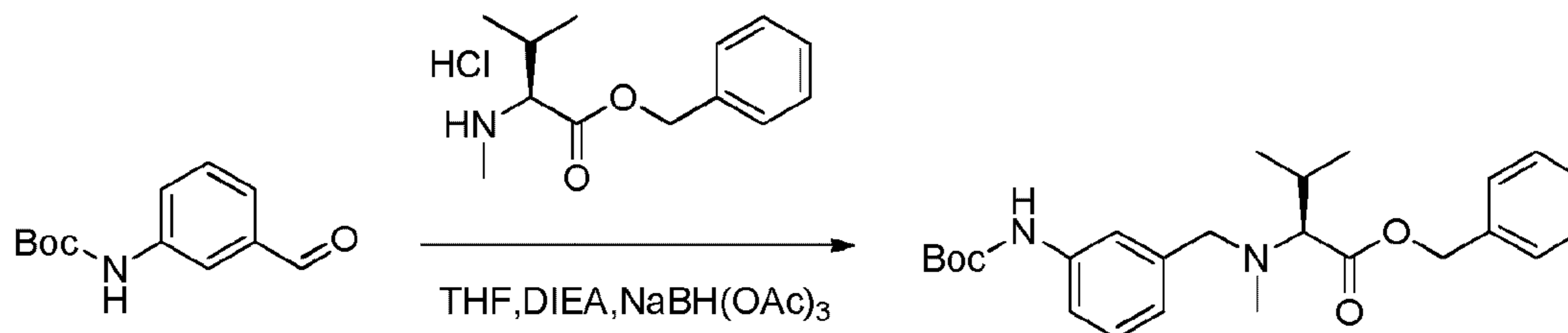
recombined, dried over sodium sulfate, filtered and concentrated under reduced pressure to yield the crude product (13.85 g of compound **14A**) in the form of a yellow oil.

Compound 14B: *tert*-butyl (3-formylphenyl)carbamate



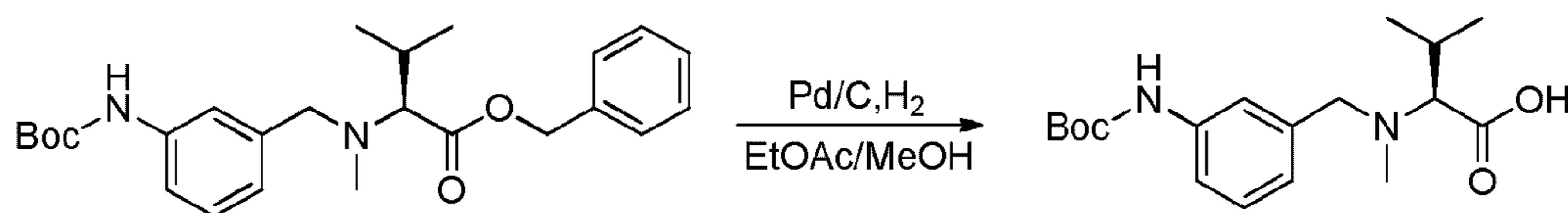
5 Compound **14A** (13.8 g, 61.81 mmol, 1.00 equiv) was dissolved in DCE (400 mL) and MnO₂ (54 g, 621.14 mmol, 10.05 equiv) was then added. The mixture was left under agitation at ambient temperature for 3 days after which the solids were removed by filtering. The filtrate was evaporated to dryness and the residue was purified on a silica column with a mixture of EtOAc and PE (1:30) to yield 3 g (22 %) of compound **14B** in the form of a white solid.

Compound 14C: benzyl (S)-2-((3-((*tert*-butoxycarbonyl)amino)benzyl)(methyl)amino)-3-methylbutanoate



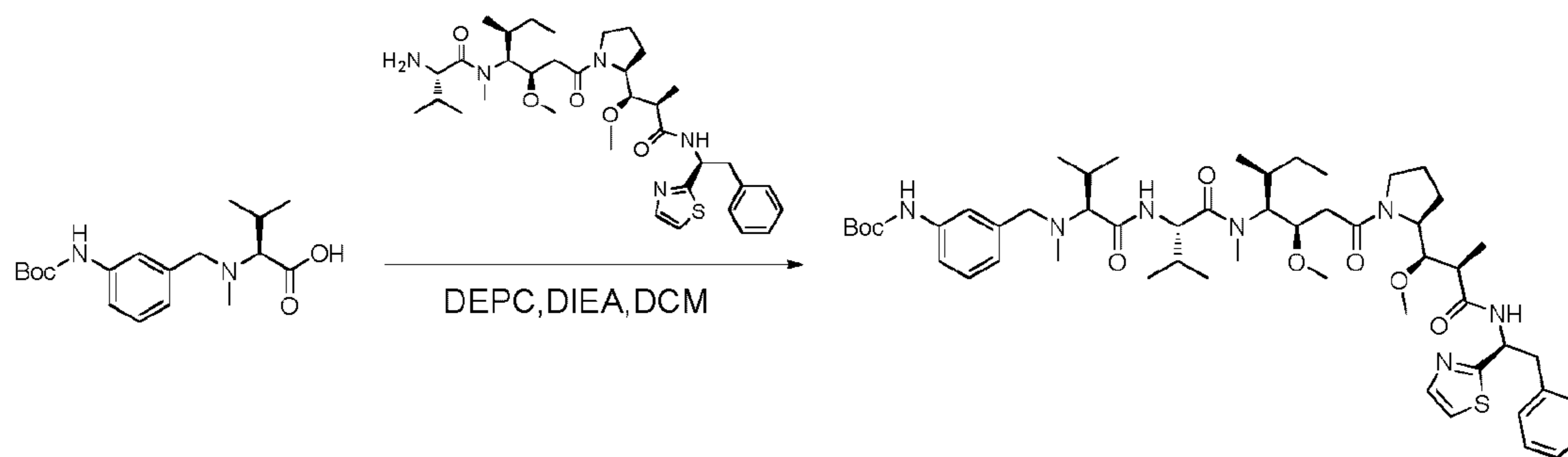
15 Compound **14B** (1 g, 4.52 mmol, 1.00 equiv) was dissolved in 20 mL of THF in the presence of compound **14C** (1.16 g, 4.50 mmol, 1.00 equiv), DIEA (3 mL) and NaBH(OAc)₃ (1.92 g, 9.06 mmol, 2.01 equiv). The reaction mixture was left under agitation overnight at ambient temperature and then neutralised with 100 mL of water and extracted 3 times with 50 mL of AcOEt. The organic phases were combined, dried over sodium sulfate, filtered and concentrated. The residue was purified on a silica column with a mixture of EtOAc and PE (1:50) to yield 1.9 g (99 %) of compound **14C** in the form of a white solid.

Compound 14D: (S)-2-((3-((*tert*-butoxycarbonyl)amino)benzyl)(methyl)amino)-3-methylbutanoic acid



Compound **14C** (1 g, 2.34 mmol, 1.00 equiv) was dissolved in 30 mL of AcOEt and 4 mL of methanol in the presence of Pd/C (400 mg) and hydrogenated for 1 hour at ambient temperature and atmospheric pressure. The reaction medium was filtered and concentrated under reduced pressure to yield 680 mg (86 %) of compound **14D** in the form of a white solid.

Compound 14E: *tert*-butyl (3-(((3*S*,6*S*,9*S*,10*R*)-9-((*S*)-*sec*-butyl)-3,6-diisopropyl-10-(2-((*S*)-2-((1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-(((*S*)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-2,8-dimethyl-4,7-dioxo-11-oxa-2,5,8-triazadodecyl)phenyl) carbamate



Compound **14E** was synthesised in the same manner as for compound **3** from the amine **1Y** (100 mg, 0.15 mmol, 1.00 equiv), the acid **14D** (102.27 mg, 0.30 mmol, 2.00 equiv), DEPC (0.053 mL) and DIEA (0.046 mL) in DCM (3 mL). The crude product (80 mg) was purified on a silica column with a mixture of EtOAc and PE (1:1) to yield 100 mg (67 %) of compound **14E** in the form of a pale yellow solid.

Compound **14** was synthesised in the same manner as for compound **2** from the intermediate **14E** (100 mg, 0.10 mmol, 1.00 equiv). The crude product (80 mg) was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **14** was obtained with a yield of 10 % (10 mg) in the form of a white solid.

LC/MS/UV (Eclipse plus C8 column, 3.5 μ m, 4.6 x 150 mm; 40°C; 1.0 mL / min, 40 % to 95 % MeOH in water (0.05 % TFA) in 18 minutes); ESI ($C_{48}H_{73}N_7O_6S$, exact mass 875.5) m/z : 876.5 (MH^+) and 438.9 ($M.2H^+/2$, 100 %), 11.35 min (95.6 %, 210 nm).

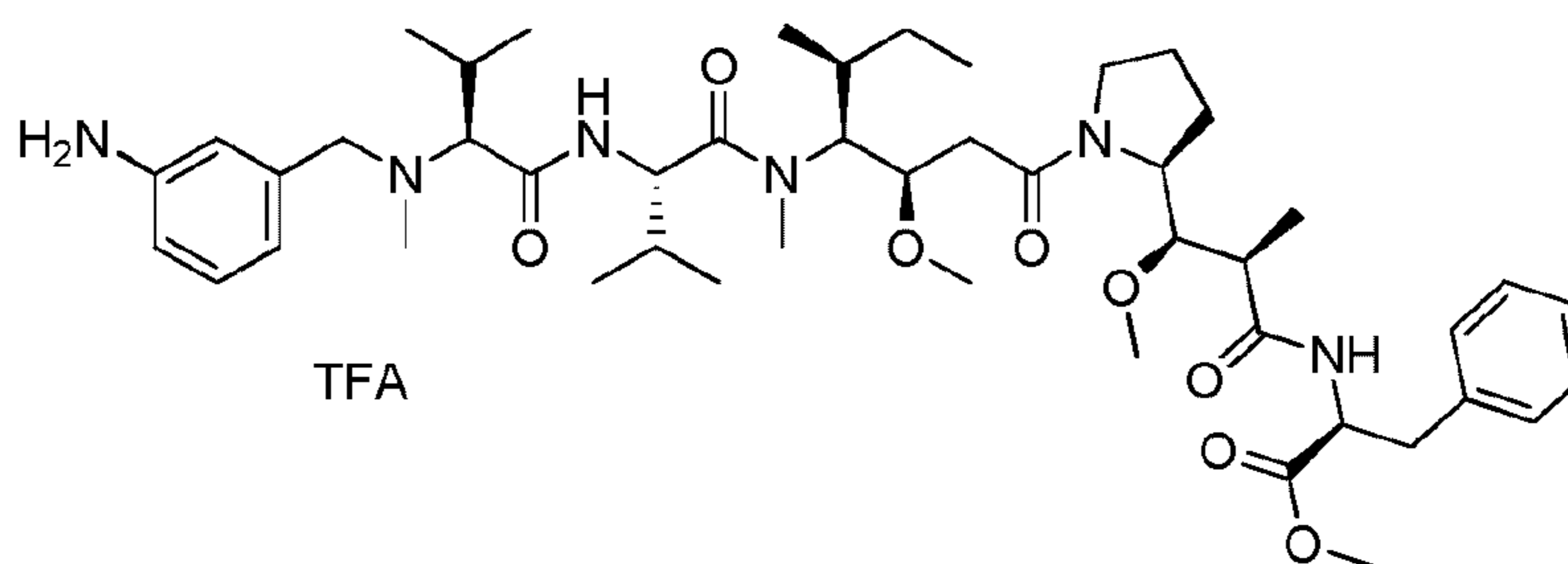
¹H NMR (400MHz, CD₃OD, ppm): δ (Presence of rotamers) 8.92 - 8.86 (m, 0.4H, NH incomplete exchange); 8.70 - 8.54 (m, 0.6H, NH incomplete exchange); 7.88 - 7.78 (m, 1H); 7.60 - 7.50 (m, 1H); 7.45 - 6.97 (m, 9H); 5.80 - 5.65 (m, 1H); 4.85 - 4.70 (m, 1H); 4.40 - 0.80 (m, 56H).

5

Compound 15

methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((3-aminobenzyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, trifluoroacetic acid

10

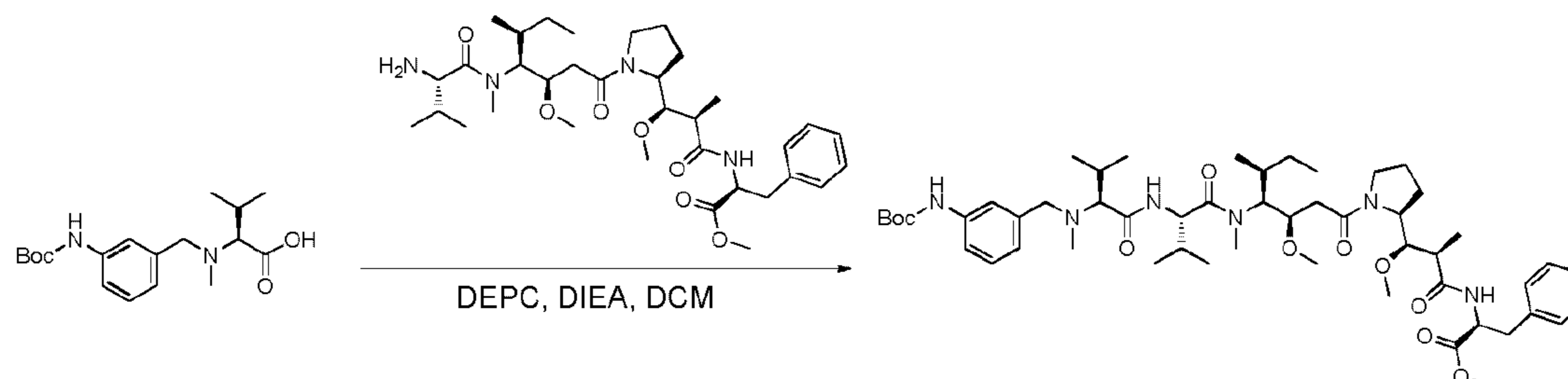


Compound 15A: methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-

((S)-2-((3-((tert-butoxycarbonyl)amino)benzyl)(methyl)amino)-3-

methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-

15 methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate



Compound **15A** was synthesised in the same manner as for compound **3** from the amine **3D** (200 mg, 0.32 mmol, 1.00 equiv), the acid **14D** (212.6 mg, 0.63 mmol, 2.00 equiv), DEPC (0.1103 mL) and DIEA (0.157 mL, 3.00 equiv) in DCM (5 mL). The crude product was purified on a silica column with a mixture of EtOAc and PE (1:1) to yield 200 mg (67 %) of compound **15A** in the form of a yellow solid.

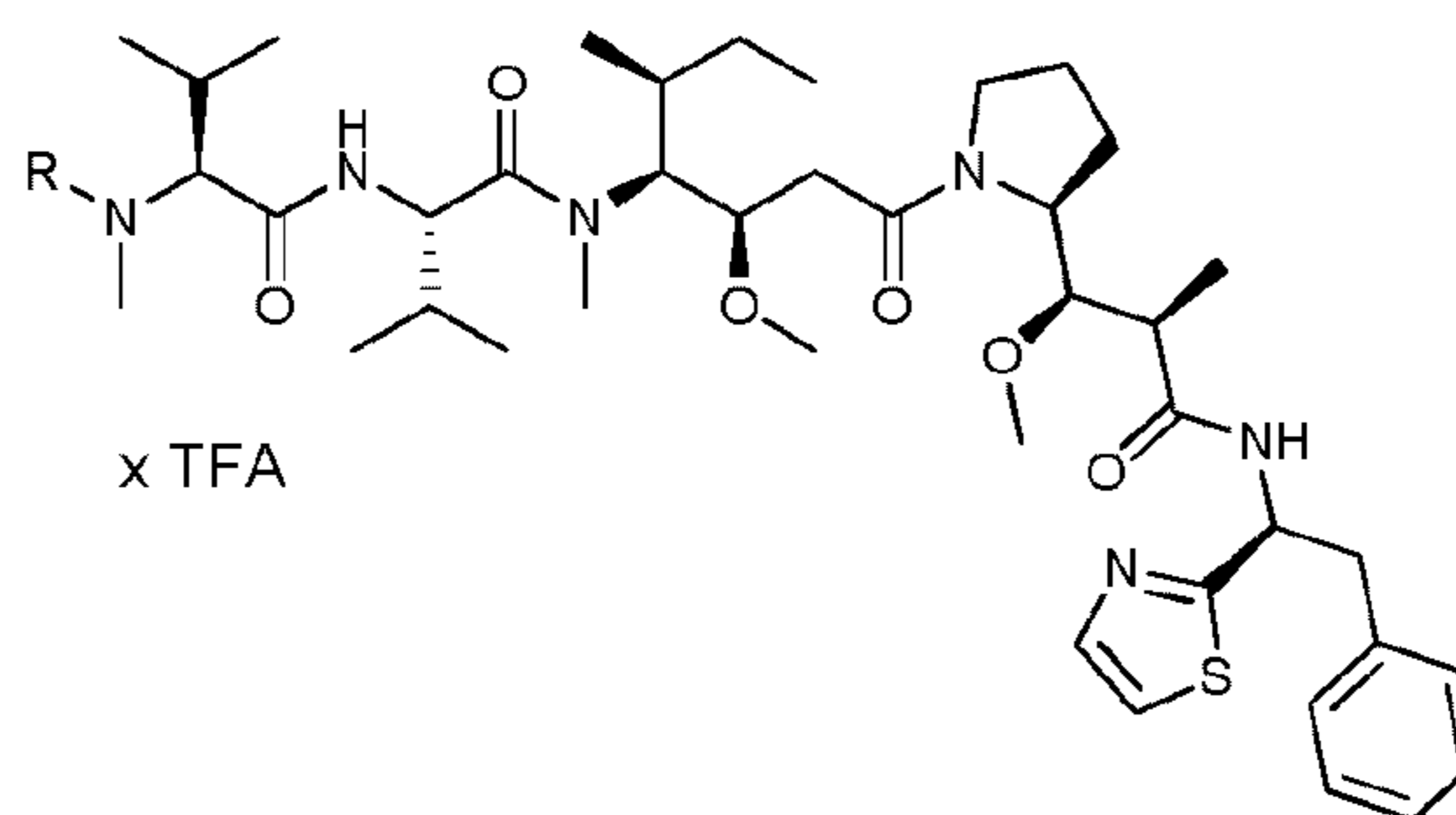
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Compound **15** was synthesised in the same manner as for compound **2** from the intermediate **15A** (200 mg, 0.21 mmol, 1.00 equiv). The crude product was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters UV Detector 2545 at 254 nm and 220 nm). Compound **15** was obtained with a yield of 19 % (38.6 mg) in the form of a white solid.

LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI ($C_{47}H_{74}N_6O_8$, exact mass 850.5) m/z : 851.5 (MH^+) and 426.4 ($M.2H^+/2$, 100 %), 6.61 min (91.1 %, 210 nm).

1H NMR (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 7.53 - 7.42 (m, 1H); 7.35 - 7.18 (m, 8H); 4.88 - 4.79 (m, 2H); 4.42 - 4.00 (m, 3H); 3.93 - 2.71 (m, 22H); 2.61 - 0.81 (m, 33H).

Compounds 16 to 20



Compounds **16** to **20** were prepared in the same manner as for compound **1**, from the amines **1Y** and **1ZC** and corresponding aldehydes.

The *tert*-butyl (4-oxobutyl)carbamate, involved in the preparation of compound **16**, was prepared as for compound **1ZE** in 2 steps from 4,4-diethoxybutan-1-amine.

The *tert*-butyl-*N*-methyl-*N*-(2-oxoethyl)carbamate involved in the preparation of compound **17** was commercial.

The 2-(2-((*tert*-butyldimethylsilyl)oxy)ethoxy)acetaldehyde, involved in the preparation of compound **18**, was prepared in 2 steps as follows:

2-(2-Hydroxyethoxy)ethan-1-ol (7 g, 66 mmol, 9.9 equiv) was dissolved in an inert atmosphere in pyridine (10 mL) in the presence of 4-dimethylaminopyridine

(DMAP, 80 mg, 0.65 mmol, 0.1 equiv). The solution was cooled to 0°C then TBDMSCl (1 g, 6.6 mmol, 1.0 equiv) was added in portions. The reaction mixture was left under agitation overnight at ambient temperature, diluted with 100 mL of EtOAc and successively washed twice with 100 mL of 1N HCl and twice with NaCl-saturated aqueous solution. The organic phase was dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure to yield 1.3 g (88 %) of 2-[2-[(*tert*-butyldimethylsilyl)oxy]ethoxy]ethan-1-ol in the form of a colourless oil.

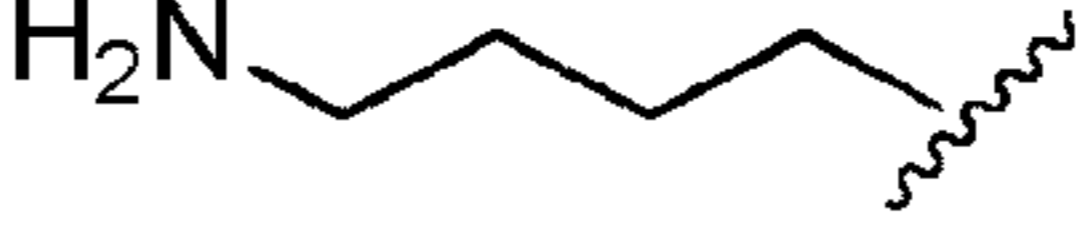
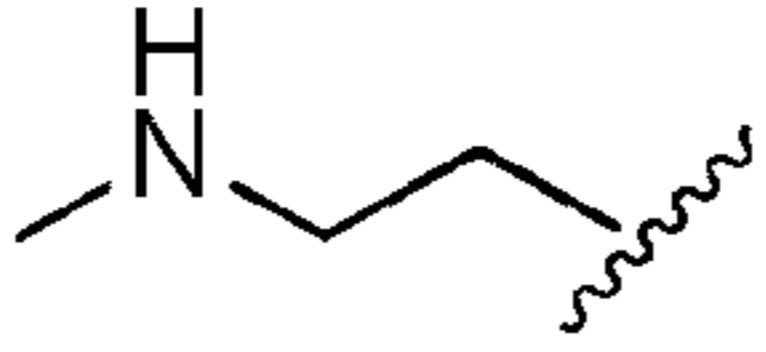
The oxalyl chloride (760 mg, 6 mmol, 1.3 equiv) was dissolved in an inert atmosphere in DCM (40 mL) and cooled to -78°C. Dimethylsulfoxide (DMSO, 1.07 g, 13.7 mmol, 3 equiv) diluted in DCM (5 mL) was added drop-wise. After an agitation time of 30 minutes at low temperature, 2-[2-[(*tert*-butyldimethylsilyl)oxy]ethoxy]ethan-1-ol (1 g, 4.5 mmol, 1.0 equiv) dissolved in 5 mL of DCM was added. Agitation was continued for 1 hour at low temperature before adding TEA (2.78 g, 27 mmol, 6 equiv). The reaction mixture was agitated 15 minutes at -78°C and overnight at ambient temperature before being neutralised with 100 mL of water. It was then extracted 3 times with 100 mL of DCM. The organic phases were combined, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified on silica gel (EtOAc/PE 1:20) and yielded 0.8 g (80 %) of 2-[2-[(*tert*-butyldimethylsilyl)oxy]ethoxy]acetaldehyde in the form of a colourless oil.

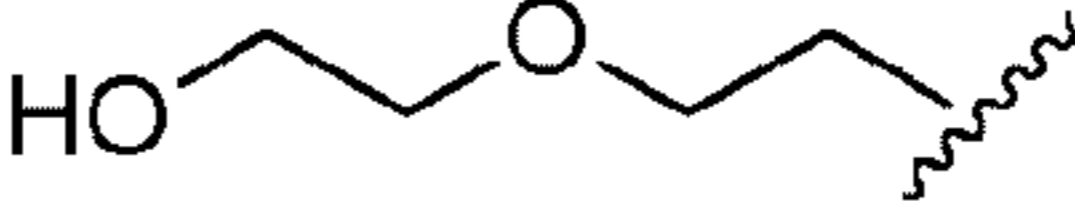
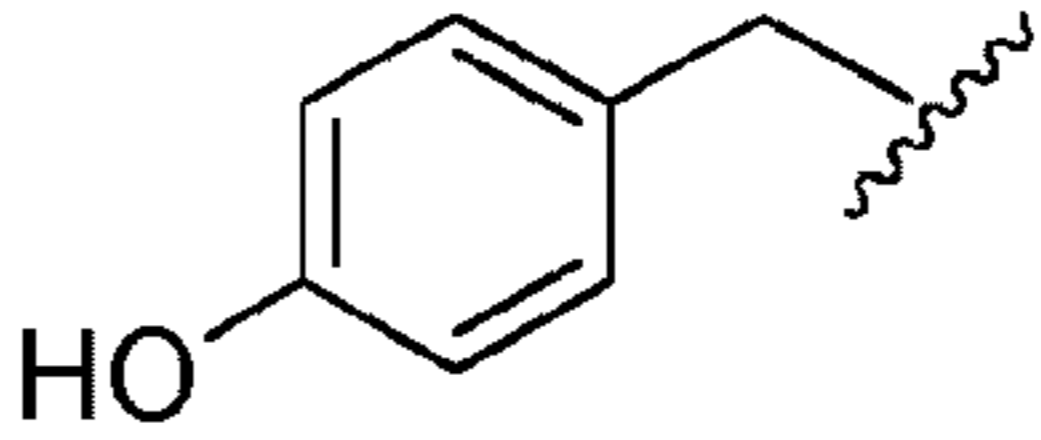
The *tert*-butyl 4-formylphenyl carbonate involved in the preparation of compound **19** was prepared in a single step as follows: 4-hydroxybenzaldehyde (2.5 g, 20.5 mmol, 1.0 equiv) was dissolved in an inert atmosphere in THF (20 mL) in the presence of 18-crown-6 (0.25 g) and potassium carbonate (5 g). The reaction mixture was cooled to 0°C and the di-*tert*-butyl dicarbonate (5.8 g, 26.58 mmol, 1.30 equiv) was then added. Agitation was continued for 1 hour at low temperature after which the reaction was neutralised with 30 mL of water. The resulting solution was extracted three times with 200 mL of EtOAc. The organic phases were combined, dried over anhydrous sodium sulfate filtered and concentrated under reduced pressure. The residue was purified on silica gel (EtOAc/PE 1:10) and yielded 4.2 g (92 %) of *tert*-butyl 4-formylphenyl carbonate in the form of a pale yellow solid.

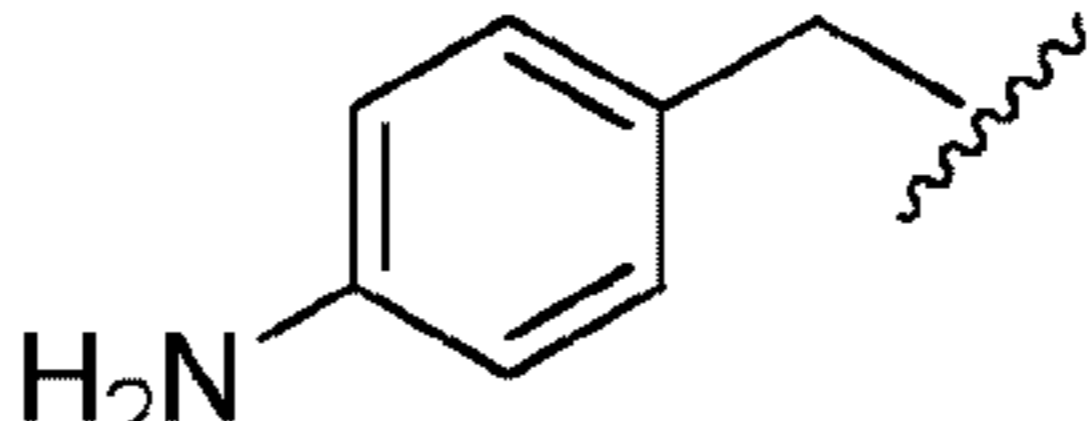
The 4-nitrobenzaldehyde involved in the preparation of compound **20** was commercial.

The synthesis of compound **18** was completed by deprotection of the silylated alcohol. This was performed as follows: (S)-N-(((S)-1-(((3R,4S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)(methyl)amino)-3-methyl-1-oxobutan-2-yl)-11-isopropyl-2,2,3,3,10-pentamethyl-4,7-dioxa-10-aza-3-siladodecan-12-amide (40 mg, 0.04 mmol, 1.0 equiv) was dissolved in an inert atmosphere in THF (10 mL) in the presence of TBAF (2 mg, 0.09 mmol, 2 equiv) and agitated 4 hours at ambient temperature. The reaction was neutralised with 50 mL of water then extracted three times with 50 mL of EtOAc. The organic phases were combined, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure to yield compound **18** in the crude state.

The synthesis of compound **20** was completed by reducing the nitro group. This was performed as follows: (2S)-N-[(3R,4S,5S)-1-[(2S)-2-[(1R,2R)-2-[[1-(1S,2R)-1-hydroxy-1-phenylpropan-2-yl]carbamoyl]-1-methoxy-2-methylethyl]pyrrolidin-1-yl]-3-methoxy-5-methyl-1-oxoheptan-4-yl]-N,3-dimethyl-2-[(2S)-3-methyl-2-[methyl[(4-nitrophenyl)methyl]amino]butanamido]butanamide (40 mg, 0.05 mmol, 1.0 equiv) was dissolved in 15 mL of ethanol. Dihydrated tin chloride (II) (317 mg, 1.4 mmol, 30 equiv) was added and the solution left under agitation for 3 days at ambient temperature. The reaction was neutralised with 50 mL of water, then extracted three times with 50 mL of EtOAc. The organic phases were combined, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure to yield compound **20** in the crude state.

| N° | Name | x | R | Purity* | Quantity |
|----|--|---|---|---------|----------|
| 16 | (S)-2-((S)-2-((4-aminobutyl)(methyl)amino)-3-methylbutanamido)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide, bis trifluoroacetic acid | 2 |  | 94.9 % | 11.5 mg |
| 17 | (S)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(2-(methylamino)ethyl)amino)butanamido)butanamide, bis trifluoroacetic acid | 2 |  | 99.6 % | 65.5 mg |

| N° | Name | x | R | Purity* | Quantity |
|----|---|---|---|---------|----------|
| 18 | (S)-2-((S)-2-((2-(2-hydroxyethoxy)ethyl)(methylamino)-3-methylbutanamido)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide, trifluoroacetic acid | 1 |  | 94.5 % | 46.4 mg |
| 19 | (S)-2-((S)-2-((4-hydroxybenzyl)(methylamino)-3-methylbutanamido)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide, trifluoroacetic acid | 1 |  | 93.2 % | 21.6 mg |

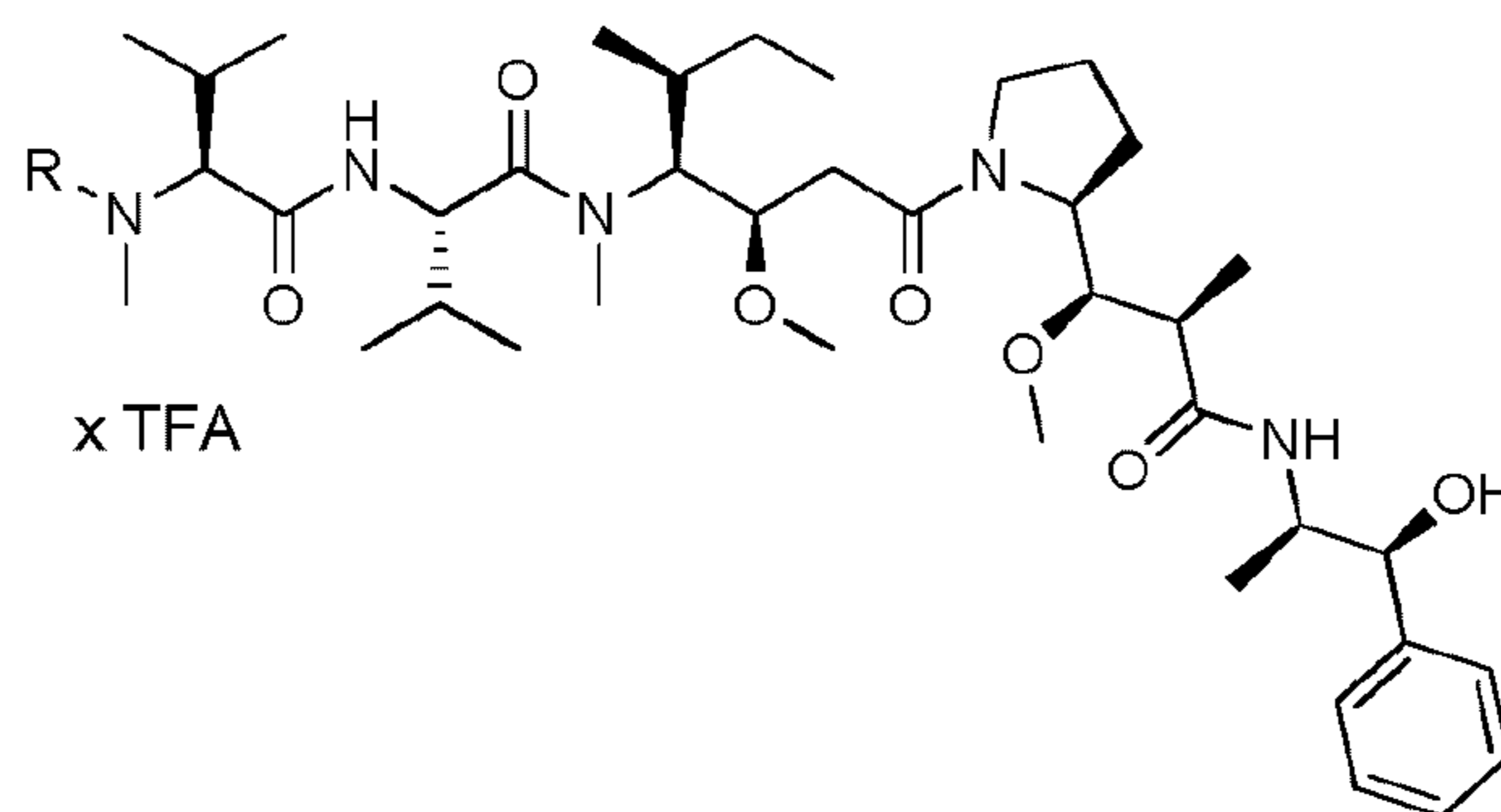
| N° | Name | x | R | Purity* | Quantity |
|----|---|---|---|---------|----------|
| 20 | (S)-2-((S)-2-((4-aminobenzyl)(methyl)amino)-3-methylbutanamido)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide, trifluoroacetic acid | 1 |  | 96.7 % | 21.1 mg |

* The compounds were purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2489 UV Detector at 254 nm and 220 nm), to give the corresponding TFA salts in the form of white solids.

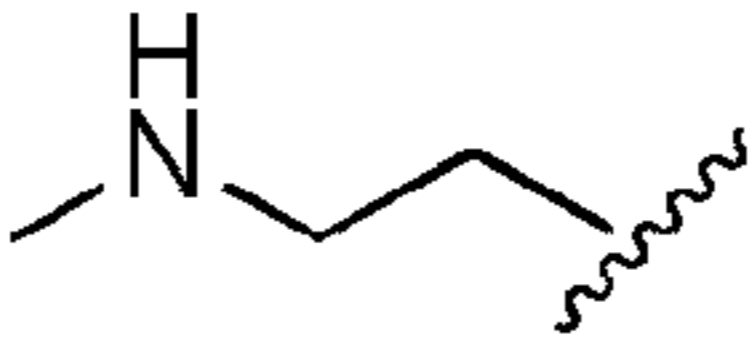
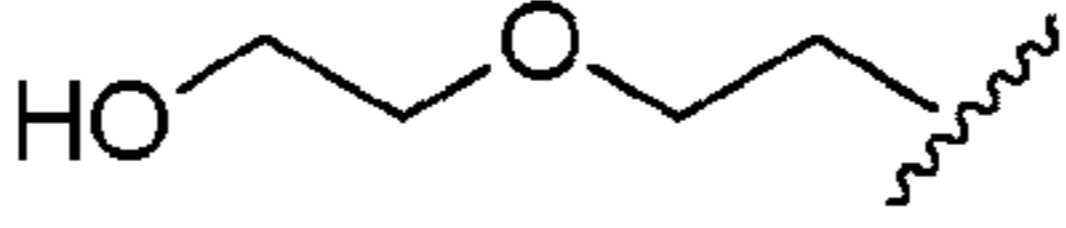
Characterization of the end products: Compound **16** LC/MS/UV (Eclipse Plus C8, 3.5 μ m, 4.6 x 150 mm; 1 mL/min, 40°C, 5 to 95 % methanol in water (0.05 % TFA) in 18 minutes); ESI: (C₄₅H₇₅N₇O₆S, exact mass 841.55) *m/z* 842.5 (MH⁺), 421.9 (100 %, (M.2H⁺)/2); UV: 14.02 min (94.9 %, 210 nm). ¹H NMR (300MHz, CD₃OD, ppm): δ (Presence of rotamers) 8.55–8.2 (m, 0.8H, NHCO incomplete exchange); 8.0 (0.55H, NHCO incomplete exchange); 7.70 (d, 1H); 7.44 (d, 1H); 7.21–7.15 (m, 5H); 5.65–5.45 (m, 1H); 4.8–4.5 (m, 2H); 4.15–3.9 (m, 2H); 3.8–0.6 (m, 59H). Compound **17** LC/MS/UV ESI: (C₄₄H₇₃N₇O₆S, exact mass 827.53) *m/z* 828 (MH⁺), 415 [100 %, (M.2H⁺)/2]; UV: RT = 6.72 min (99.6 %, 254 nm) ¹H NMR: (400MHz, CD₃OD, ppm): δ (Presence of rotamers) 7.82–7.80 (m, 1H); 7.56–7.54 (m, 1H); 7.35–7.20 (m, 5H); 5.8–5.55 (m, 1H); 4.85–4.6 (m, 1H); 4.25–4.05 (m, 1H); 3.95–0.8 (m, 60H). Compound **18** LC/MS/UV (Atlantis T3, 3 μ m, 4.6 x 100 mm; 1.2 mL/min, 40°C, 5 to 95 % methanol in water (0.05 % TFA) in 7 minutes) i; ESI: (C₄₅H₇₄N₆O₈S, exact mass 858.53) *m/z* 859 (MH⁺), 881 (MNa⁺), 430 (100 %, (M.2H⁺)/2); UV: 4.85 min (96.8 %, 220 nm). ¹H NMR: (400MHz, CD₃OD, ppm): δ (Presence of rotamers) 8.75–8.55 (m, 0.5H, NHCO incomplete exchange); 7.85–7.80 (m, 1H); 7.6–7.5 (m, 1H); 7.40–7.15 (m,

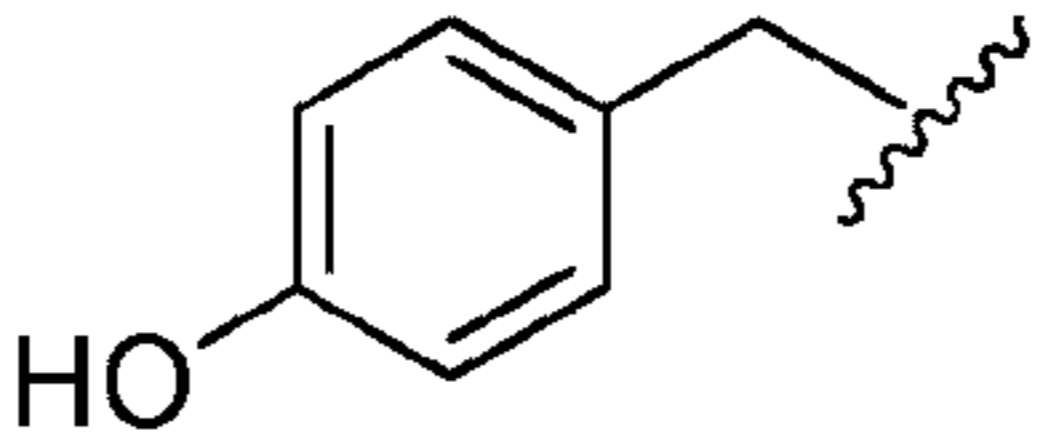
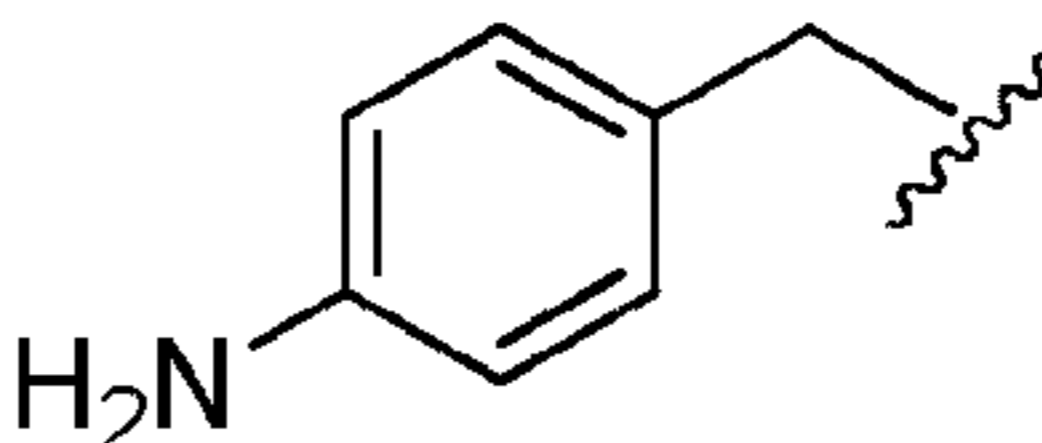
5H); 5.8–5.6 (m, 1H); 4.8–4.55 (m, 2H); 4.15–4.0 (m, 1H); 4.0–0.8 (m, 60H).
 Compound **19** LC/MS/UV ESI: (C₄₈H₇₂N₆O₇S, exact mass 876.52) *m/z* 877 (MH⁺), 439
 [100 %, (M.2H⁺)/2]; UV: RT = 1.76 min (93.2 %, 220 nm). Compound **20** ¹H NMR:
 (400MHz, CD₃OD, *ppm*): δ (Presence of rotamers) 7.85–7.80 (m, 1H); 7.6–7.5 (m, 1H);
 5 7.4–7.15 (m, 5H); 7.1–7.05 (m, 2H); 6.73–6.70 (m, 2H); 5.8–5.55 (m, 1H); 5.0–4.7 (m,
 2H); 4.25–4.05 (m, 1H); 4.0–0.8 (m, 54H). LC/MS/UV ESI: (C₄₈H₇₃N₇O₇S, exact mass
 875.53) *m/z* 876 (MH⁺), 439 [75 %, (M.2H⁺)/2]; UV: RT = 4.83 min (96.8 %, 254 nm).
¹H NMR (400MHz, CD₃OD, *ppm*): δ (Presence of rotamers) 7.85–7.80 (m, 1H); 7.6–
 7.5 (m, 1H); 7.4–7.1 (m, 7H); 6.76–6.72 (m, 2H); 5.8–5.55 (m, 1H); 4.9–4.65 (m, 2H);
 10 4.25–4.05 (m, 1H); 4.0–0.8 (m, 54H).

Compounds 21 to 24



15 Compounds **21** to **24** were prepared in the same manner as for compounds **17** to
20, replacing the amine **1Y** by the amine **2D**.

| N° | Name | x | R | Purity* | Quantity |
|----|---|---|---|---------|----------|
| 21 | (S)-N-((3R,4S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(2-(methylamino)ethyl)amino)butanamido)butanamide, bis trifluoroacetic acid | 2 |  | 97.5 % | 24.4 mg |
| 22 | (S)-N-((3R,4S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)-2-((S)-2-((2-(2-hydroxyethoxy)ethyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamide, trifluoroacetic acid | 1 |  | 95.5 % | 26.1 mg |

| N° | Name | x | R | Purity* | Quantity |
|----|--|---|---|---------|----------|
| 23 | (S)-N-((3R,4S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)-2-((S)-2-((4-hydroxybenzyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamide, trifluoroacetic acid | 1 |  | 98.5 % | 5.8 mg |
| 24 | (S)-2-((S)-2-((4-aminobenzyl)(methyl)amino)-3-methylbutanamido)-N-((3R,4S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide, trifluoroacetic acid | 1 |  | 99.1 % | 6.9 mg |

* The compounds were purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2489 UV Detector at 254 nm and 220 nm), to give the corresponding TFA salts in the form of white solids.

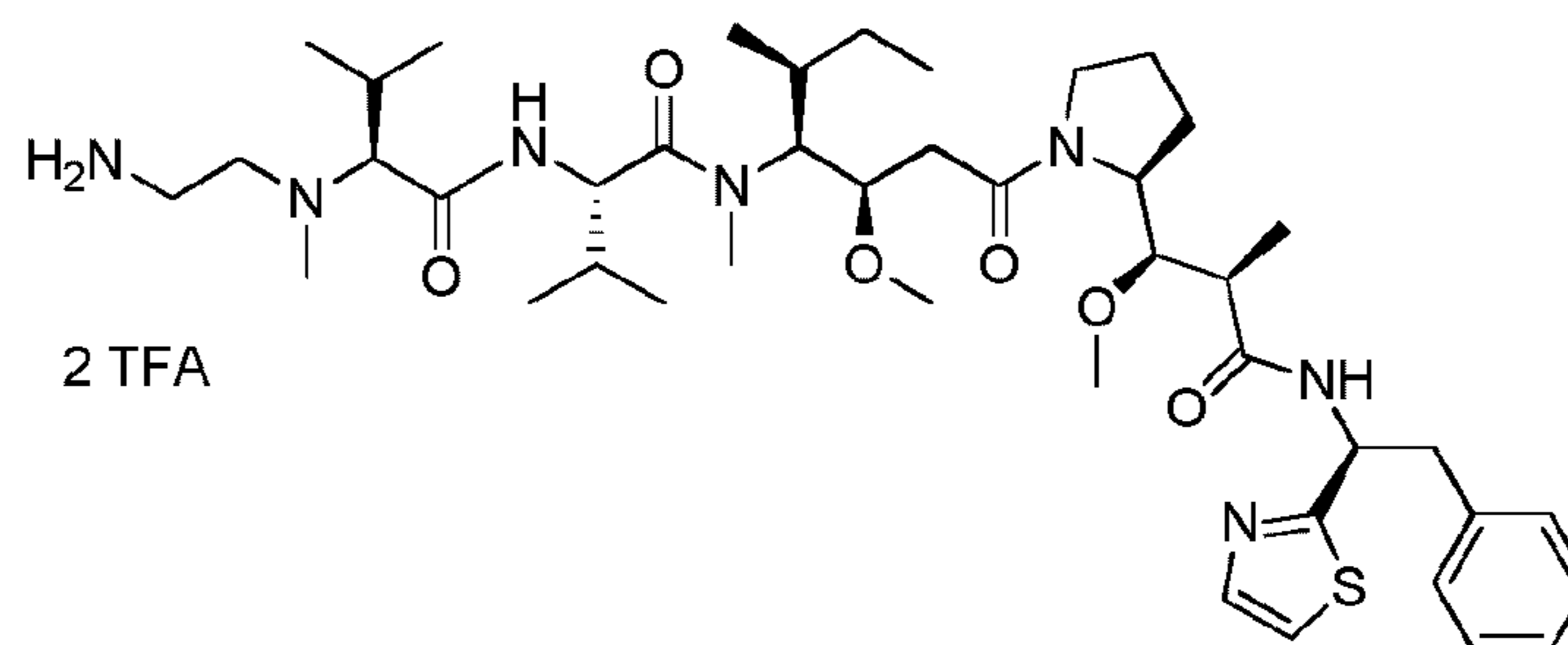
Characterization of the end products: Compound **21** LC/MS/UV (ESI) ($C_{42}H_{74}N_6O_7$, exact mass 774.56) m/z 775 (MH^+), 797 (MNa^+), 388 (100 %, ($M.2H^+$)/2); UV: 3.14 min (97.6 %, 215 nm). 1H NMR (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 8.05–7.7 (m, 0.8H, NHCO incomplete exchange); 7.45–7.15 (m,

5H); 4.9–4.45 (m, 2H); 4.35–4.00 (m, 2H); 3.95–0.8 (m, 61H). Compound **22**
 LC/MS/UV (ESI) (C₄₃H₇₅N₅O₉, exact mass 805.56) *m/z* 806 (MH⁺), 828 (MNa⁺), 404
 (100 %, (M.2H⁺)/2); UV: 4.47 min (95.6 %, 215 nm). ¹H NMR: (400MHz, CD₃OD,
 ppm): δ (Presence of rotamers) 8.1–7.7 (m, 0.4H, NHCO incomplete exchange); 7.45–
 5 7.15 (m, 5H); 4.9–4.5 (m, 3H); 4.4–4.05 (m, 2H); 4.05–0.8 (m, 61H). Compound **23**
 LC/MS/UV (ESI) (C₄₆H₇₃N₅O₈, exact mass 823.55) *m/z* 824 (MH⁺), 846 (MNa⁺), 413
 (100 %, (M.2H⁺)/2); UV: 4.76 min (98.5 %, 215 nm). ¹H NMR (400MHz, CDCl₃,
 ppm): δ (Presence of rotamers) 7.5–7.2 (m, 5H); 7.9–7.75 (m, 2H); 5.5–5.3 (m, 1H);
 4.9–4.6 (m, 2H); 4.55–4.15 (m, 4H); 4.0–0.8 (m, 55H). Compound **24**
 10 LC/MS/UV (ESI) (C₄₆H₇₄N₆O₇, exact mass 822.56) *m/z* 823 (MH⁺), 845 (MNa⁺), 861
 (MK⁺); UV: 3.68 min (99.15 %, 254 nm). ¹H NMR (400MHz, CD₃OD, ppm): δ
 (Presence of rotamers) 8.0–7.7 (m, 0.5H, NHCO incomplete exchange); 7.5–7.0 (m,
 7H); 6.75–6.65 (m, 2H); 4.85–4.5 (m, 2H); 4.4–4.05 (m, 2H); 4.0–0.8 (m, 56H).

15

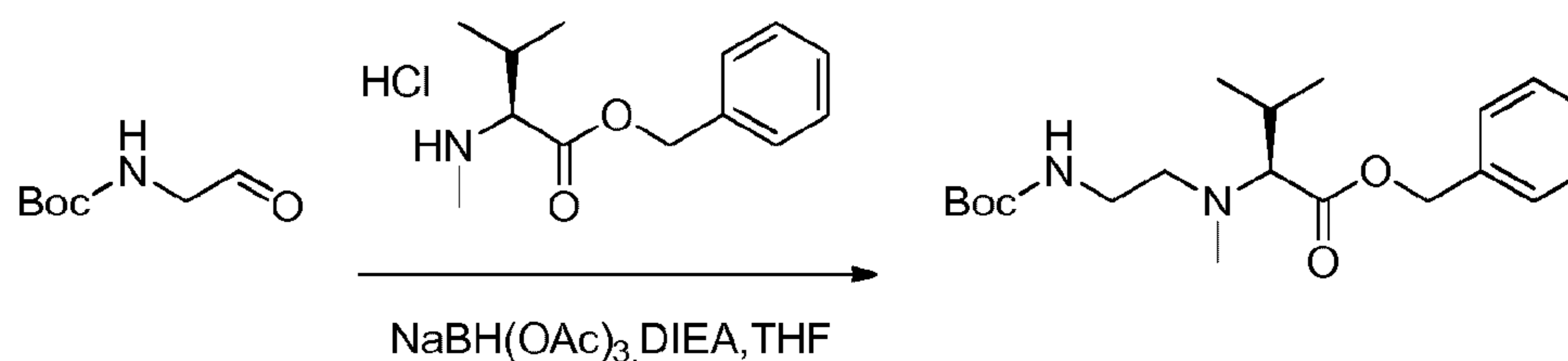
Compound 26

(S)-2-((S)-2-((2-aminoethyl)(methyl)amino)-3-methylbutanamido)-N-
 ((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-
 2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-
 oxoheptan-4-yl)-N,3-dimethylbutanamide, bis trifluoroacetic acid



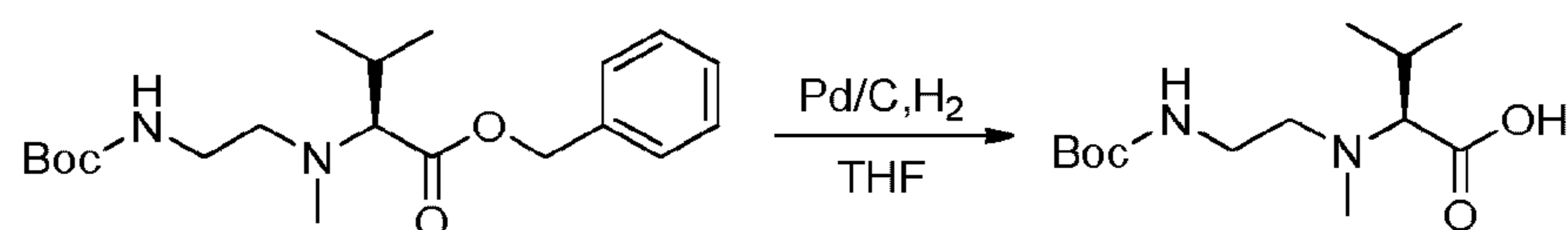
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Compound 26A: benzyl (S)-2-((2-((*tert*-butoxycarbonyl)amino)ethyl)(methyl)
 amino)-3-methylbutanoate



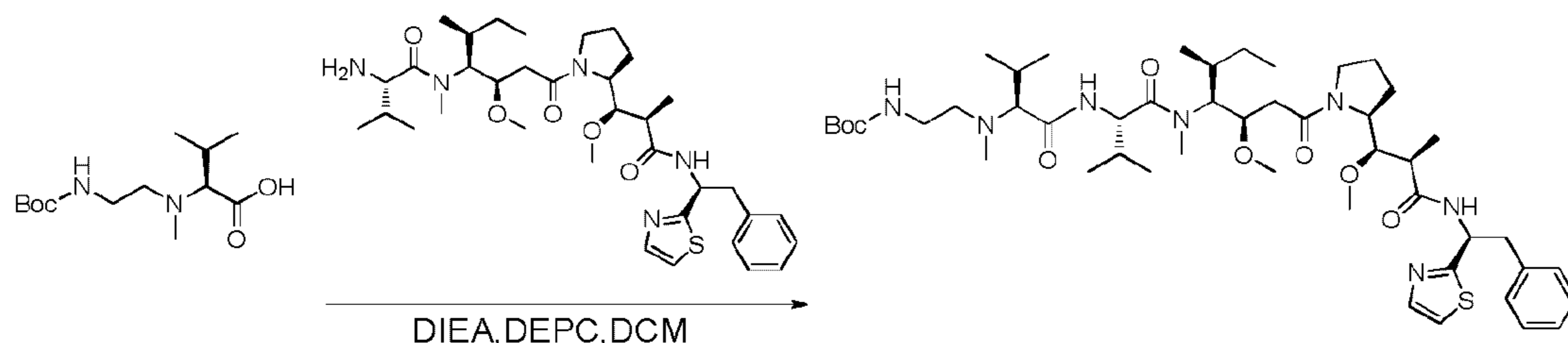
Compound **26A** was prepared in the same manner as for compound **2H** from the amine **1ZC** (1.3 g, 5.04 mmol, 1.00 equiv), *tert*-butyl (2-oxoethyl)carbamate (800 mg, 5.03 mmol, 1.00 equiv), DIEA (3.52 g, 27.24 mmol, 5.42 equiv) and NaBH(OAc)₃ (2.25 g, 10.62 mmol, 2.11 equiv) in THF (25 mL). The mixture was left under agitation overnight and neutralised with 50 mL of water. The residue was purified on a silica column with a mixture of EtOAc and PE (10:1) to yield 0.6 g (33 %) of compound **26A** in the form of a colourless oil.

Compound 26B: (S)-2-((2-((*tert*-butoxycarbonyl)amino)ethyl)(methyl)amino)-3-methylbutanoic acid



Compound **26A** (600 mg, 1.65 mmol, 1.00 equiv) was dissolved in 40 mL of THF in the presence of Pd/C (300 mg) and hydrogenated for 1 hour at ambient temperature and atmospheric pressure. The reaction medium was filtered and concentrated under reduced pressure. The residue was purified on a silica column with a mixture of EtOAc and MeOH to yield 0.4 g (89 %) of compound **26B** in the form of a colourless oil.

Compound 26C: *tert*-butyl ((3R,4S,7S,10S)-4-((S)-*sec*-butyl)-7,10-diisopropyl-3-(2-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-5,11-dimethyl-6,9-dioxo-2-oxa-5,8,11-triazatridecan-13-yl) carbamate



Compound **26C** was prepared in the same manner as for compound **3** from the amine **1Y** (70 mg, 0.11 mmol, 1.00 equiv), the acid **26B** (58.4 mg, 0.21 mmol, 2.00 equiv), DEPC (0.032 mL) and DIEA (0.053 mL) in DCM (3 mL). After evaporation to dryness, compound **26C** was obtained in the form of a yellow oil (100 mg).

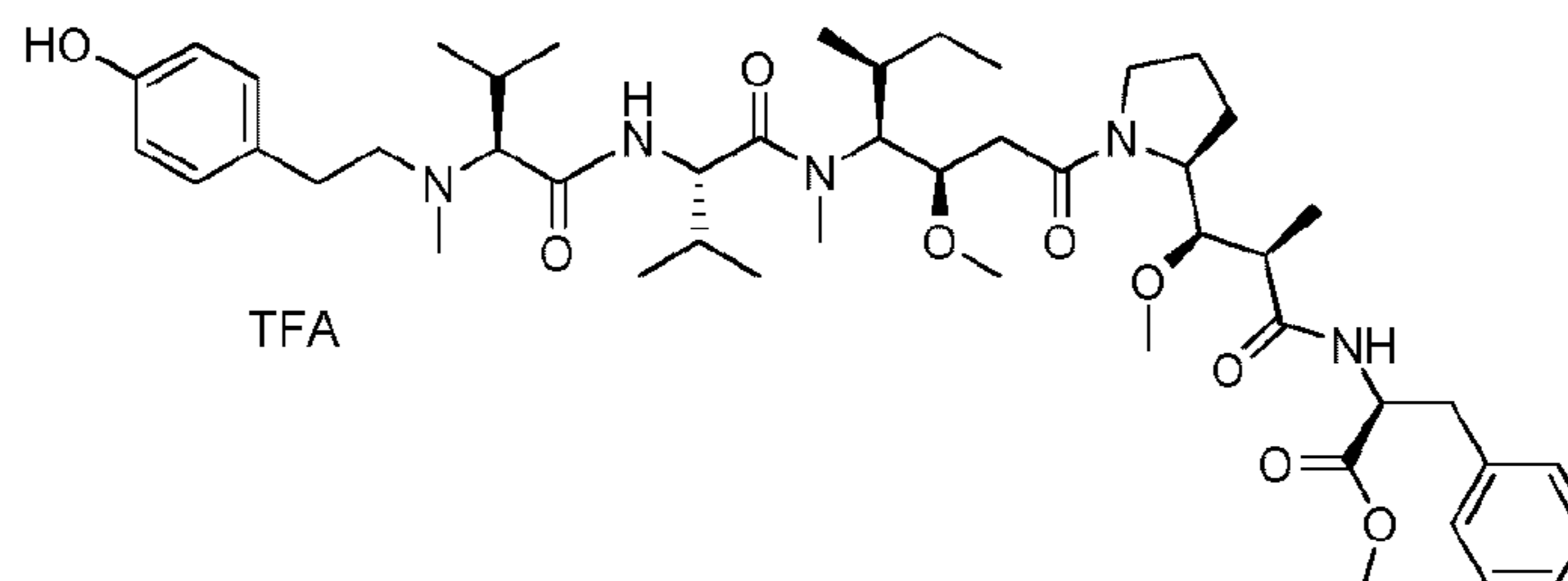
Compound 26: Compound **26** was synthesised in the same manner as for compound **2** from the intermediate **26C** (100 mg, 0.11 mmol, 1.00 equiv) in DCM (3 mL) and TFA (1.5 mL). The crude product was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 45 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **26** was obtained with a yield of 38 % (38.1 mg) in the form of a white solid.

LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C; 1.0 mL/min, 5 % to 95 % MeOH in water (0.05 % TFA) on 18 minutes); ESI (C₄₃H₇₁N₇O₆S, exact mass 813.52) *m/z*: 814.5 (MH⁺) and 407.9 (M.2H⁺/2, 100 %), 15.78 min (91.2 %, 210 nm).

¹H NMR: (400MHz, CD₃OD, ppm): δ (Presence of rotamers) 8.90 - 8.82 (m, 0.5 H, NH incomplete exchange); 8.71 - 8.65 (m, 0.3H, NH incomplete exchange); (7.85 - 7.77 (m, 1H); 7.60 - 7.49 (m, 1H); 7.37 - 7.15 (m, 5H); 5.78 - 5.55 (m, 1H); 4.82 - 4.62 (m, 1.6H); 4.32 - 3.83 (m, 3.6H); 3.75 - 3.35 (m, 7.4H); 3.30 - 2.60 (m, 13H); 2.58 - 0.80 (m, 42H).

Compound 27

methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((4-hydroxyphenethyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, trifluoroacetic acid



Compound 27: Compound **27** was prepared in the same manner as for compound **3** from the amine **3D** (70 mg, 0.11 mmol, 1.00 equiv), the acid **49C** (55.5 mg, 0.22 mmol, 2.00 equiv), DEPC (0.034 mL, 2.00 equiv) and DIEA (0.055 mL, 3.00 equiv) in DCM (3 mL). The crude product was purified by preparative HPLC (Pre-

HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 45 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **27** was obtained with a yield of 3 % (2.9 mg) in the form of a white solid.

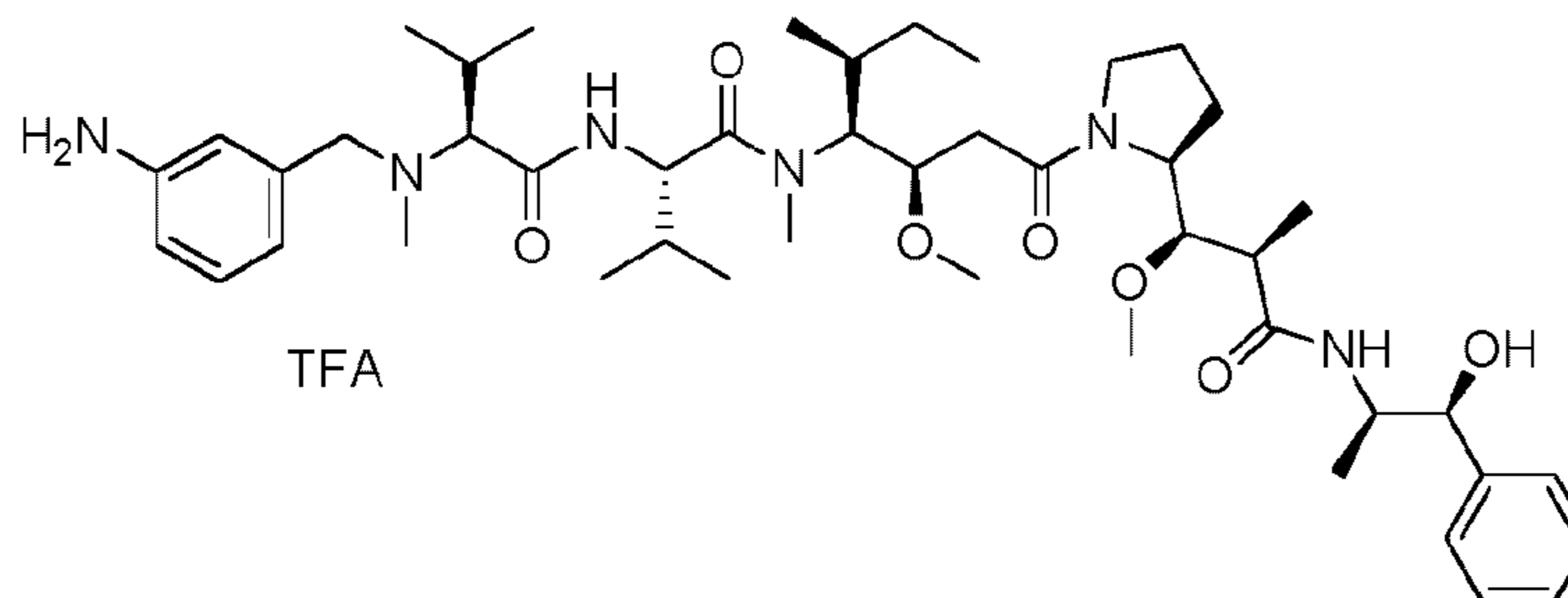
LC/MS/UV (Eclipse Plus C8 column, 3.5 μ m, 4.6 x 150 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI ($C_{48}H_{75}N_5O_9$, exact mass 866.56) m/z : 866.5 (MH^+) and 433.9 ($M.2H^+/2$, 100 %), 6.61 min (89.1 %, 210 nm).

1H NMR (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 8.70 - 8.49 (m, 0.9 H, NH/OH incomplete exchange); 8.30 - 8.22 (m, 0.3H, NH incomplete exchange); 7.36 - 7.02 (m, 7H); 6.86 - 6.62 (m, 2H); 4.82 - 4.69 (m, 2H); 4.20 - 4.03 (m, 1H); 3.91 - 3.33 (m, 12H); 3.30 - 2.90 (m, 17H); 2.55 - 0.80 (m, 35H).

15

Compound 28

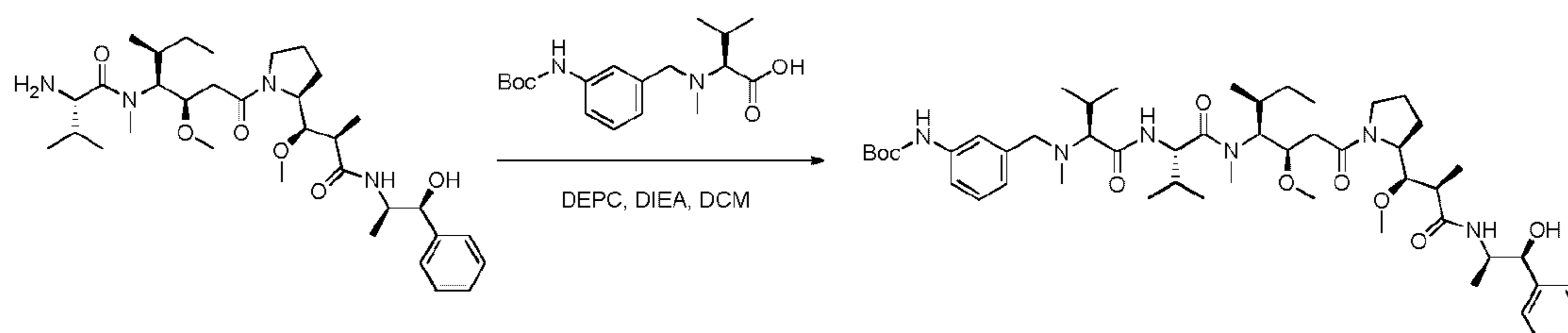
(S)-2-((S)-2-((3-aminobenzyl)(methyl)amino)-3-methylbutanamido)-N-((3R,4S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide, trifluoroacetic acid



20

Compound 28A: *tert*-butyl (3-((3S,6S,9S,10R)-9-((S)-*sec*-butyl)-10-(2-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-2-oxoethyl)-3,6-diisopropyl-2,8-dimethyl-4,7-dioxo-11-oxa-2,5,8-triazadodecyl)phenyl)carbamate

176



Compound **28A** was prepared in the same manner as for compound **3** from the amine **2D** (100 mg, 0.17 mmol, 1.00 equiv), the acid **14D** (111.25 mg, 0.33 mmol, 2.00 equiv), DEPC (0.058 mL) and DIEA (0.05 mL) in DCM (3 mL). The residue was
 5 purified on a silica column with a mixture of EtOAc and hexane (1:1) to yield 100 mg (66 %) of compound **28A** in the form of a white solid.

Compound 28: Compound **28** was synthesised in the same manner as for compound **2** from the intermediate **28A** (100 mg, 0.11 mmol, 1.00 equiv). The crude product (80 mg) was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU,
 10 SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **28** was obtained with a yield of 20 % (20 mg) in the form of a white solid.

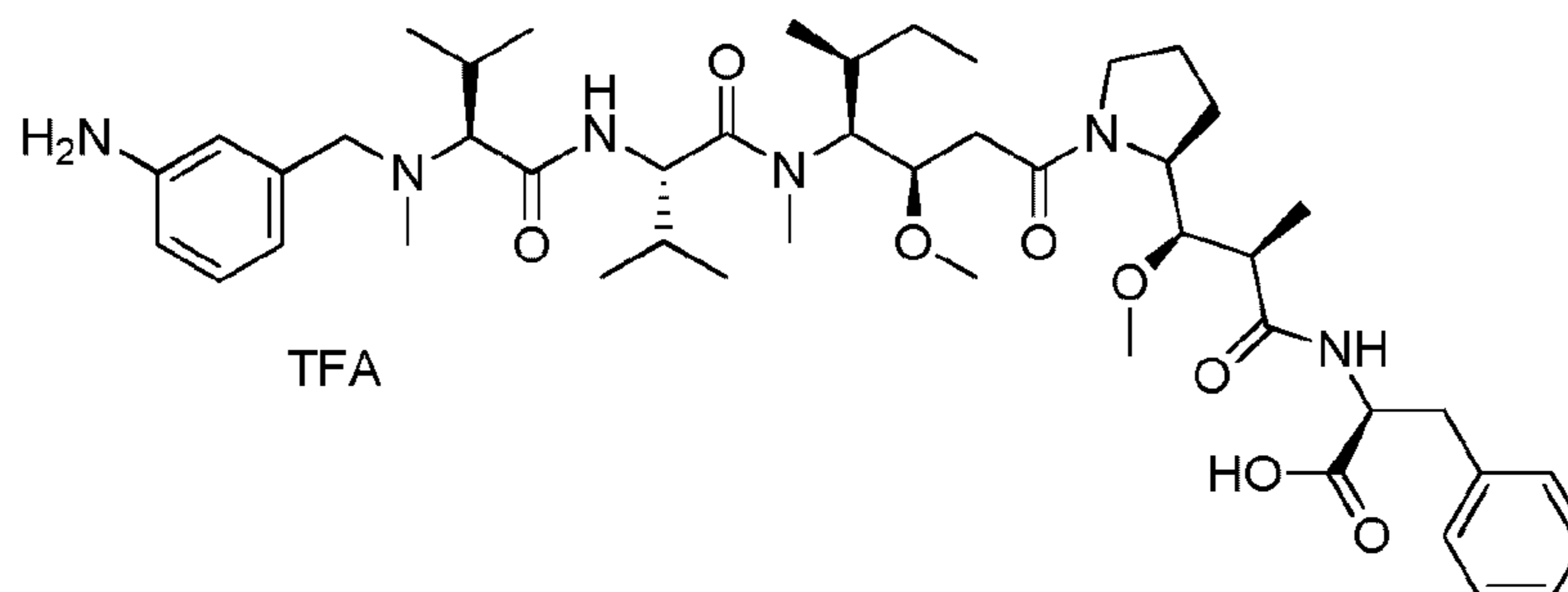
LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C;
 15 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI (C₄₆H₇₄N₆O₇, exact mass 822.56) *m/z*: 823.5 (MH⁺) and 412.4 (M.2H⁺/2, 100 %), 12.45 min (87.2 %, 210 nm).

¹H NMR: (400MHz, CD₃OD, ppm): δ (Presence of rotamers) 7.47 - 7.20 (m, 5H); 7.10 - 7.01 (m, 1H); 6.80 - 6.56 (m, 3H); 4.82 - 4.52 (m, 3H); 4.33 - 4.03 (m, 2H);
 20 3.91 - 3.82 (m, 0.5H); 3.75 - 3.35 (m, 9.5H); 3.28 - 3.10 (m, 2H); 2.79 - 2.90 (m, 1H); 2.60 - 2.40 (m, 2H); 2.30 - 0.80 (m, 40H).

Compound 29

(S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((3-aminobenzyl)
 25 (methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoic acid, trifluoroacetic acid

177



Compound **15** (100 mg, 0.10 mmol, 1.00 equiv) was dissolved in a mixture of water (5 mL), ACN (5 mL) and piperidine (2.5 mL). The reaction mixture was left under agitation overnight at ambient temperature and then concentrated under reduced pressure. The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm), to yield 20 mg (20 %) of compound **29** in the form of a white solid.

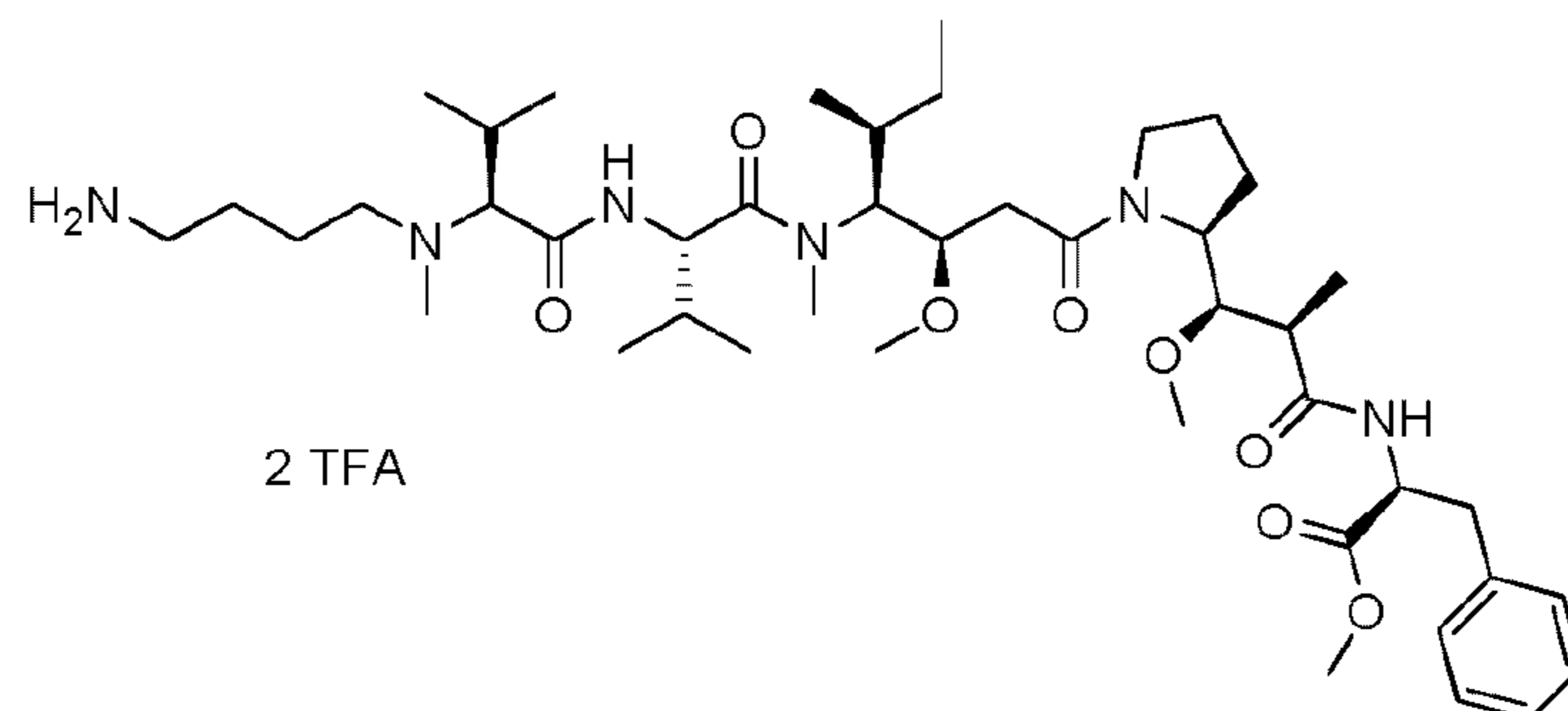
LC/MS/UV (Eclipse Plus C8 column, 3.5 μ m, 4.6 x 150 mm; 40°C; 1.0 mL/min, 40 % to 95 % MeOH in water (0.05 % TFA) in 18 minutes); ESI ($C_{46}H_{72}N_6O_8$, exact mass 836.54) m/z : 837.5 (MH^+) and 419.4 ($M.2H^+/2$, 100 %), 10.61 min (92.5 %, 210 nm).

1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 7.38 - 7.15 (m, 6H); 7.00 - 6.99 (m, 3H); 4.85 - 4.68 (m, 2H); 4.37 - 3.38 (m, 11H); 3.31 - 2.70 (m, 8H); 2.60 - 0.82 (m, 35H).

Compound 30

methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((4-aminobutyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, bis trifluoroacetic acid

178



Compound **30** was prepared in the same manner as for compound **16**, from the amine **3D** and the corresponding carboxylic acid.

LC/MS/UV (Ascentis Express C18, 2.7 μm , 4.6 x 100 mm; 1.5 mL/min, 40°C, 10 to 95 % methanol in water (0.05 % TFA) in 15 minutes); ESI ($\text{C}_{44}\text{H}_{76}\text{N}_6\text{O}_8$, exact mass 816.57) m/z : 817.6 (MH^+), 409.4 ($\text{M} \cdot 2\text{H}^+/2$); 12.0 min (90 %, 210 nm).

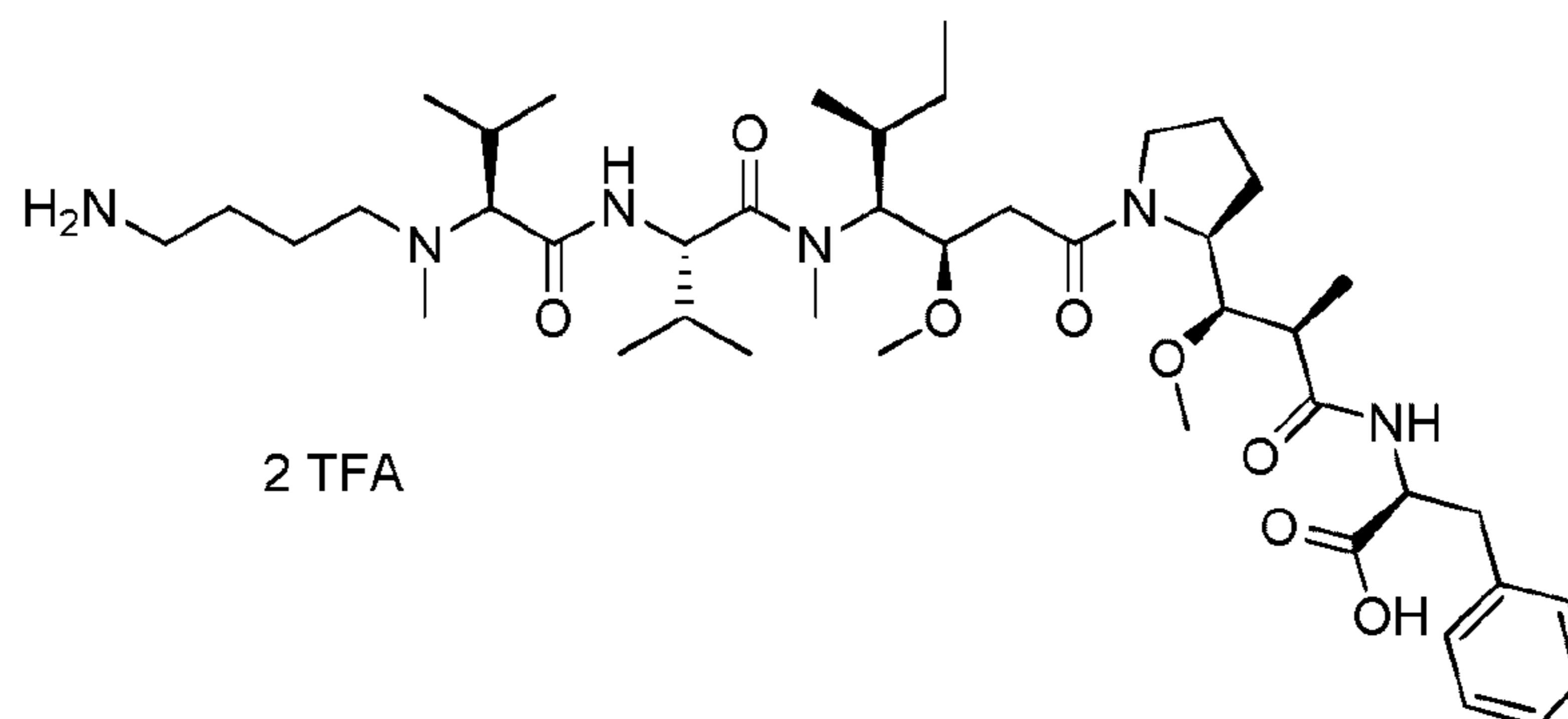
^1H NMR (300MHz, CD_3OD , *ppm*): δ (Presence of rotamers) 8.7–8.2 (m, 1H, NHCOs, incomplete exchange); 7.4–7.1 (m, 5H); 4.95–4.7 (m, 3H); 4.2–4.0 (m, 1H); 3.9–0.8 (m, 63H).

10

Compound 31

(S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((4-aminobutyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoic acid, bis trifluoroacetic acid

15



Compound **31** was prepared in the same manner as for compound **4**, from the methyl ester **30**.

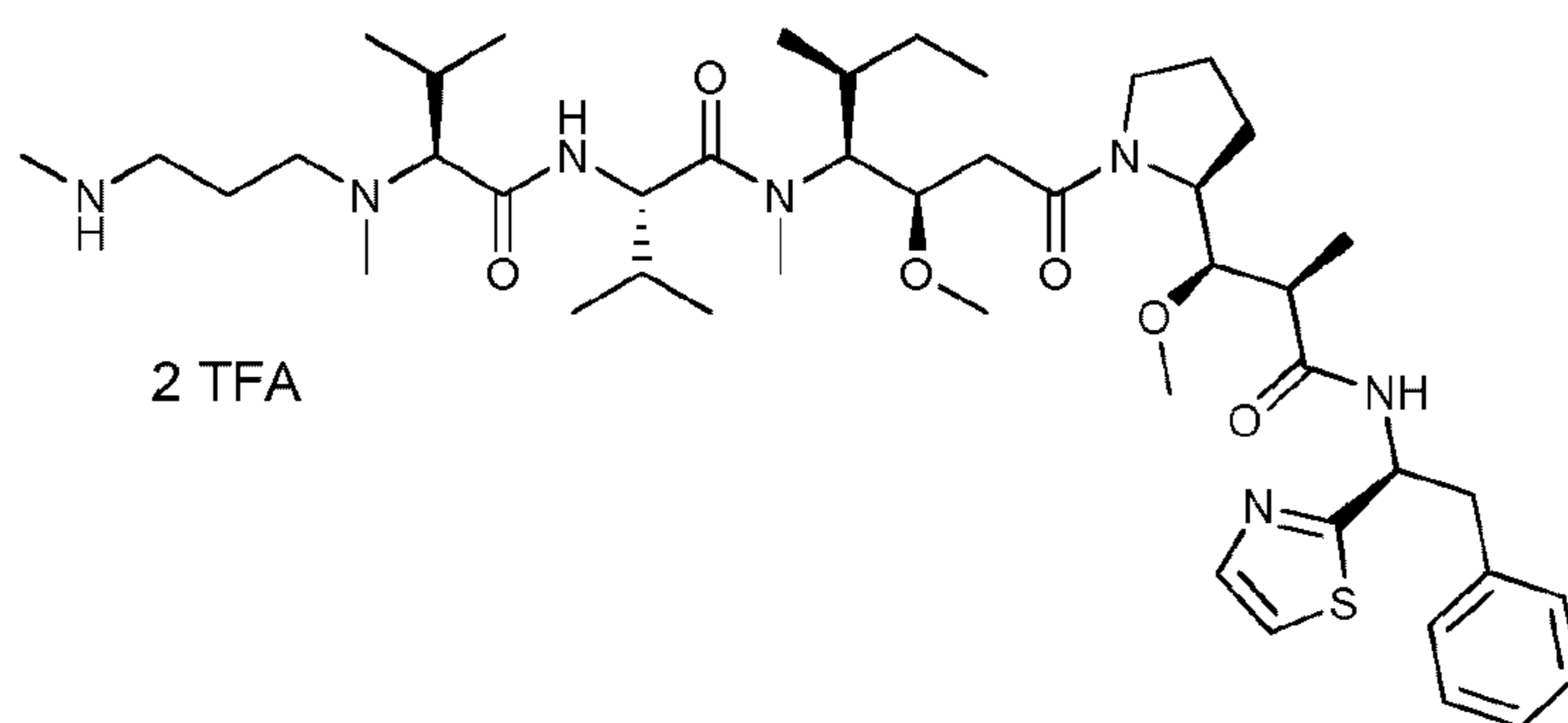
LC/MS/UV (Ascentis Express C18, 2.7 μm , 4.6 x 100 mm; 1.5 mL/min, 40°C, 10 to 95 % methanol in water (0.05 % TFA) in 18 minutes); ESI ($\text{C}_{43}\text{H}_{74}\text{N}_6\text{O}_8$, exact mass 802.56) m/z : 803.6 (MH^+), 402.4 ($\text{M} \cdot 2\text{H}^+/2$); 13.68 min (98.9 %, 210 nm).

20

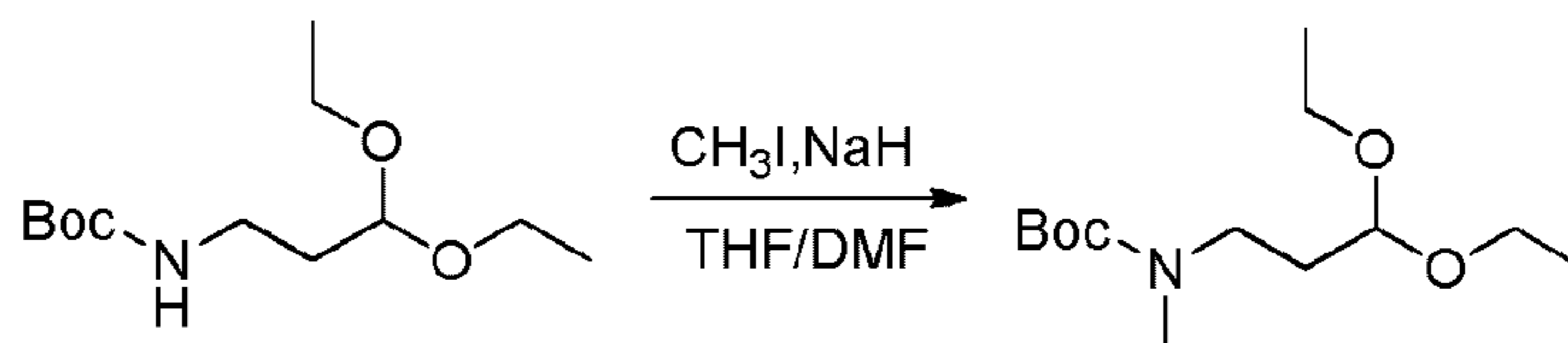
¹H NMR (300MHz, CD₃OD, ppm): δ (Presence of rotamers) 7.4–7.1 (m, 5H); 4.95–4.7 (m, 3H); 4.2–4.0 (m, 1H); 3.9–0.8 (m, 61H).

Compound 32

5 **(S)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(3-(methylamino)propyl)amino) butanamido)butanamide, bis trifluoroacetic acid**



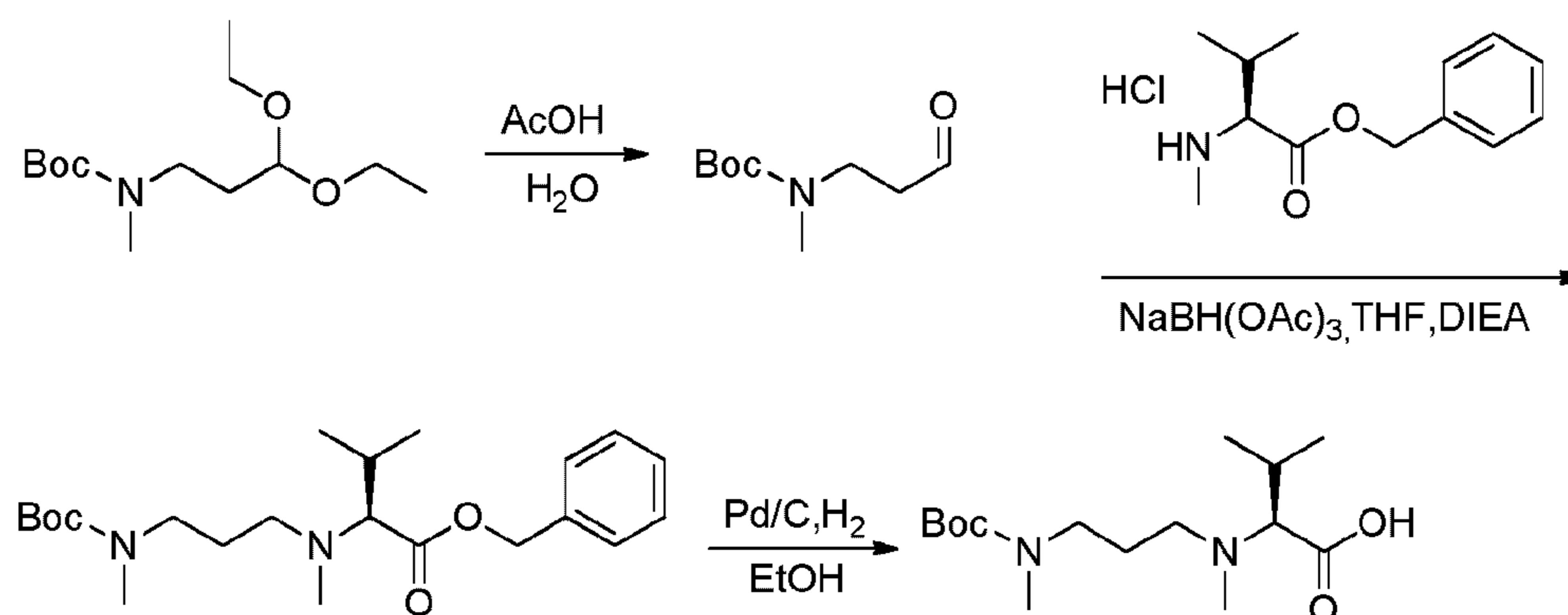
10 **Compound 32A: tert-butyl (3,3-diethoxypropyl)(methyl)carbamate**



Compound **1ZD** (247 mg, 1 mmol, 1.00 equiv) was dissolved in an inert atmosphere in 30 mL of a 1:1 mixture of THF and DMF. The reaction medium was cooled over an ice bath after which the NaH (60 % in oil, 60 mg, 1.5 equiv) was added in portions, followed by the MeI (0.28 mL) drop-wise. The reaction was left under agitation for 2 days at ambient temperature, then neutralised with 5 mL of NH₄Cl-saturated aqueous solution and extracted twice with 15 mL of EtOAc. The organic phases were combined, dried over sodium sulfate, filtered and concentrated under reduced pressure to yield 200 mg (77 %) of compound **32A** in the form of a yellow solid.

20 **Compound 32B: (S)-2-((3-((tert-butoxycarbonyl)(methyl)amino)propyl)(methyl)amino)-3-methylbutanoic acid**

180



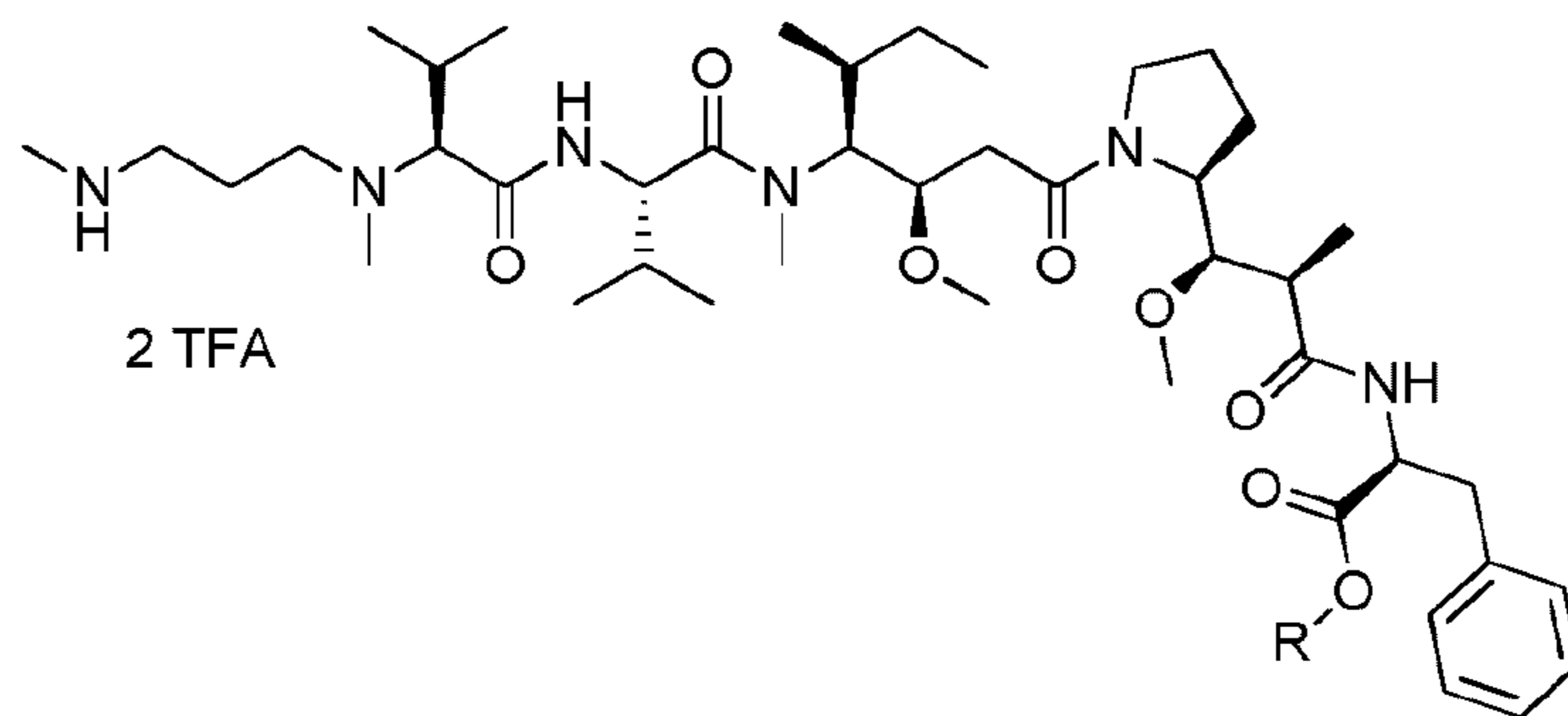
Compound **32B** was prepared following the same protocol described for the preparation of compound **1ZF**, replacing compound **1ZD** by compound **32A**.

Compound 32: Compound **32** was prepared in two steps, following the same protocol described for the preparation of compound **1**, from the amine **1Y** and the carboxylic acid **32B**.

LC/MS/UV (Zorbax SB-Aq, 1.8 μm , 4.6 x 100 mm, 40°C, 10 to 95 % methanol in water (0.05 % TFA) in 13 minutes); ESI ($\text{C}_{45}\text{H}_{75}\text{N}_7\text{O}_6\text{S}$, exact mass 842.19) m/z : 843 (MH^+), 421.9 ($\text{M}.2\text{H}^+/2$); 11.91 min (88 %, 210 nm).

^1H NMR: (300MHz, CD_3OD , ppm): δ (Presence of rotamers) 8.5 - 9.0 (m, 0.5H, incomplete exchange NHCOs), 7.85 - 7.80 (m, 1H); 7.60 - 7.50 (m, 1H), 7.35 - 7.15 (m, 5H), 5.80 - 5.63 (m, 1H), 4.80 - 4.65 (m, 2H), 4.30 - 4.00 (m, 1H), 3.95 - 0.80 (m, 61H).

Compounds 33 and 34



Compounds **33** and **34** were prepared in the same manner as for compounds **6** and **7**, replacing the carboxylic acid **1ZF** by compound **32B**.

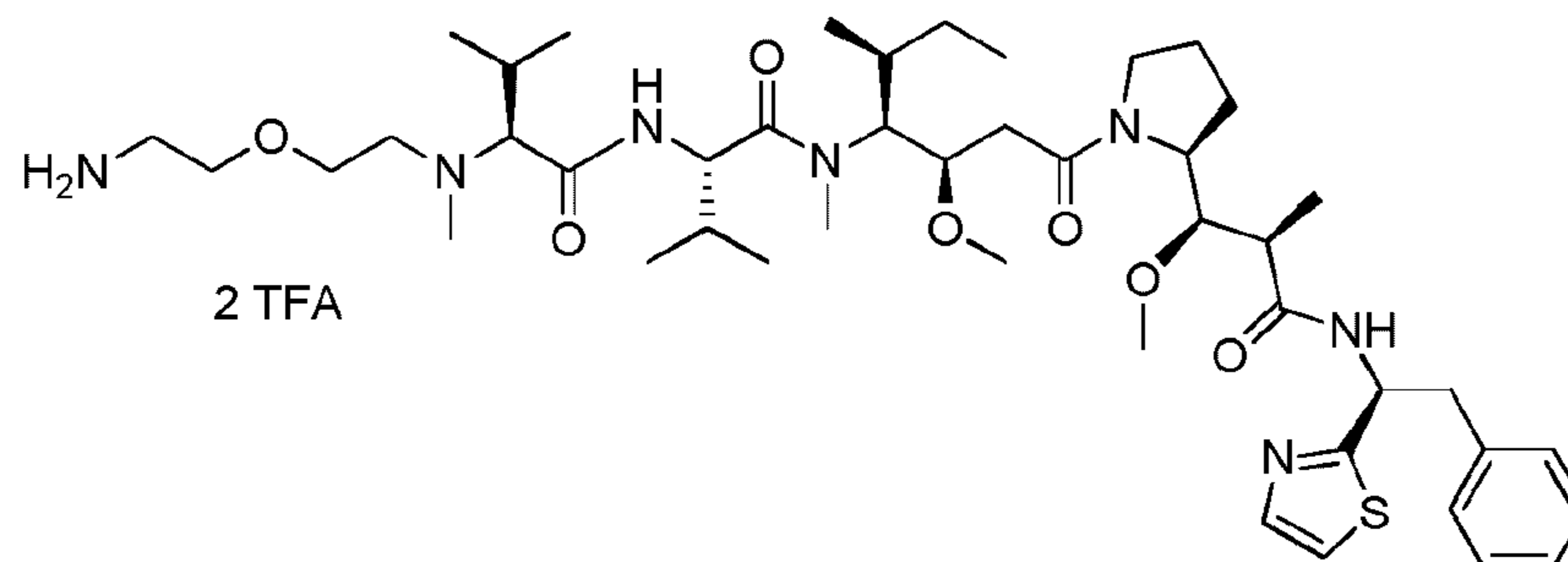
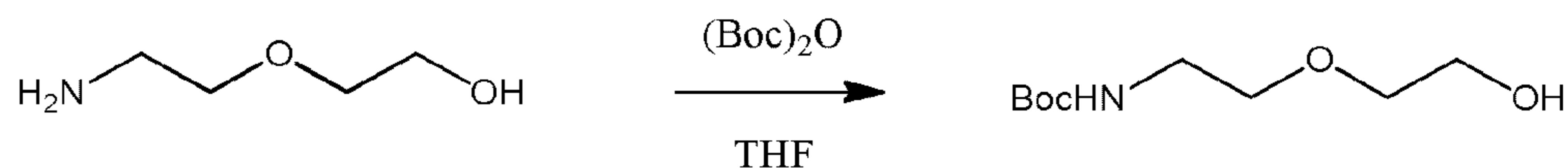
| N° | Name | R | Purity* | Quantity |
|----|--|----|---------|----------|
| 33 | methyl (S)-2-((2R,3R)-3-((S)-1-((7S,10S,13S,14R)-13-((S)- <i>sec</i> -butyl)-7,10-diisopropyl-14-methoxy-6,12-dimethyl-8,11-dioxo-2,6,9,12-tetraazahexadecan-16-oyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, bis trifluoroacetic acid | Me | 95 % | 32 mg |
| 34 | (S)-2-((2R,3R)-3-((S)-1-((7S,10S,13S,14R)-13-((S)- <i>sec</i> -butyl)-7,10-diisopropyl-14-methoxy-6,12-dimethyl-8,11-dioxo-2,6,9,12-tetraazahexadecan-16-oyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoic acid, bis trifluoroacetic acid | H | 98 % | 18 mg |

* The compounds were purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2489 UV Detector at 254 nm and 220 nm), to yield the corresponding TFA salts in the form of white solids.

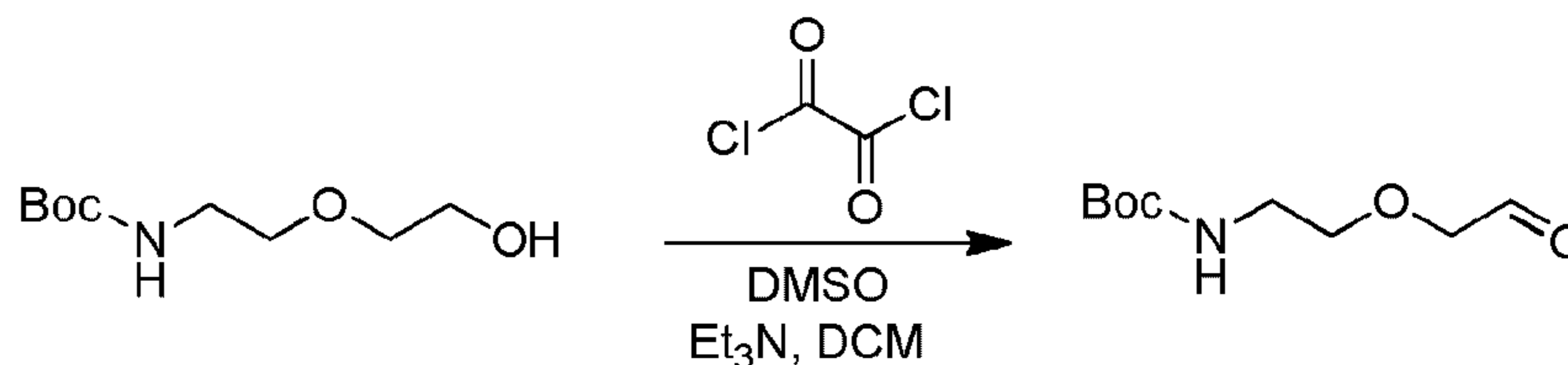
Characterization of the end products: Compound **33** LC/MS/UV (ESI) ($C_{44}H_{76}N_6O_8$, exact mass 816.57) m/z 817.6 (MH^+), 409.4 ($M.2H^+/2$); UV: 5.94 min (95 %, 210 nm). 1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers) δ 8.6 - 8.2 (m, 0.8H, NHCO incomplete exchange) 7.30 - 7.22 (m, 5H), 4.80 (m, 2H), 4.23 - 0.86 (m, 66H). Compound **34** LC/MS/UV (ESI) ($C_{43}H_{74}N_6O_8$, exact mass 802.56) m/z 803.6 (MH^+), 402.4 ($M.2H^+/2$); UV: 5.94 min (97.5 %, 210 nm). 1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers) δ 7.30 - 7.20 (m, 5H), 4.80 (m, 2H), 4.25 - 0.86 (m, 63H).

Compound 35

(S)-2-((S)-2-((2-(2-aminoethoxy)ethyl)(methyl)amino)-3-methylbutanamido)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide, bis trifluoroacetic acid

**Compound 35A:** *tert*-butyl (2-(2-hydroxyethoxy)ethyl)carbamate

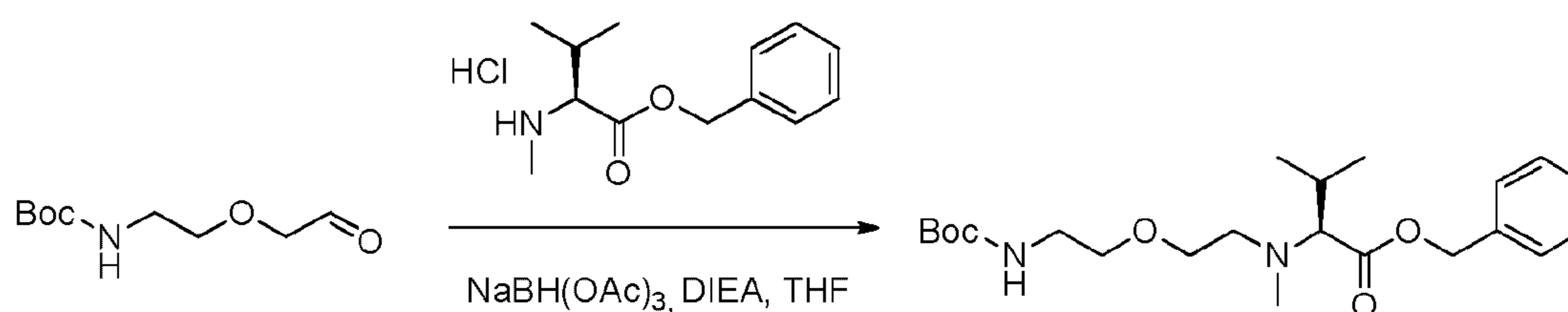
2-(2-aminoethoxy)ethanol (5 g, 47.56 mmol, 1.00 equiv) was dissolved in THF (100 mL) at 0°C and sodium hydroxide (2 g, 50.00 mmol, 1.05 equiv) was then added (solution in 25 mL of water). A solution of di-*tert*-butyl dicarbonate (10.38 g, 47.56 mmol, 1.00 equiv) in THF (20 mL) was added drop-wise and the reaction was then left under agitation overnight at ambient temperature. The reaction was diluted by adding 50 mL of water and the product was extracted with 3 times 75 mL of AcOEt. The organic phases were combined, washed once with 100 mL of NaCl (sat.), then dried over sodium sulfate, filtered and concentrated under reduced pressure to yield 9 g (92 %) of compound **35A** in the form of a yellow oil.

Compound 35B: *tert*-butyl (2-(2-oxoethoxy)ethyl)carbamate

A solution of DMSO (3.46 mL, 5.00 equiv) in DCM (20 mL) was added drop-wise to a solution of oxalyl chloride (1.9 mL, 2.30 equiv) in DCM (20 mL) at -78 °C under nitrogen. After the addition (30 min), the solution was agitated for 30 minutes and

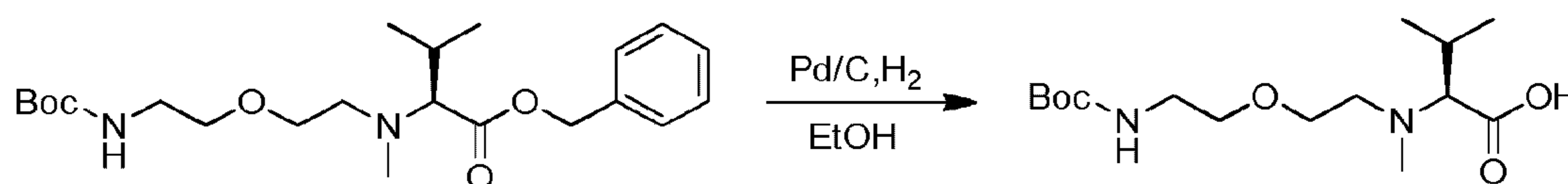
a solution of compound **35A** (2 g, 9.74 mmol, 1.00 equiv) in 20 mL DCM was then added. After the addition of TEA (12.2 mL), the reaction was agitated at -78°C for 30 minutes and then at ambient temperature overnight. The reaction was diluted by adding 100 mL of water and the product was extracted 3 times with 50 mL of AcOEt. The organic phases were combined, dried over sodium sulfate, filtered and concentrated under reduced pressure to yield 1.9 g of compound **35B** in the form of a yellow oil.

Compound 35C: benzyl (S)-12-isopropyl-2,2,11-trimethyl-4-oxo-3,8-dioxo-5,11-diazatridecan-13-oate



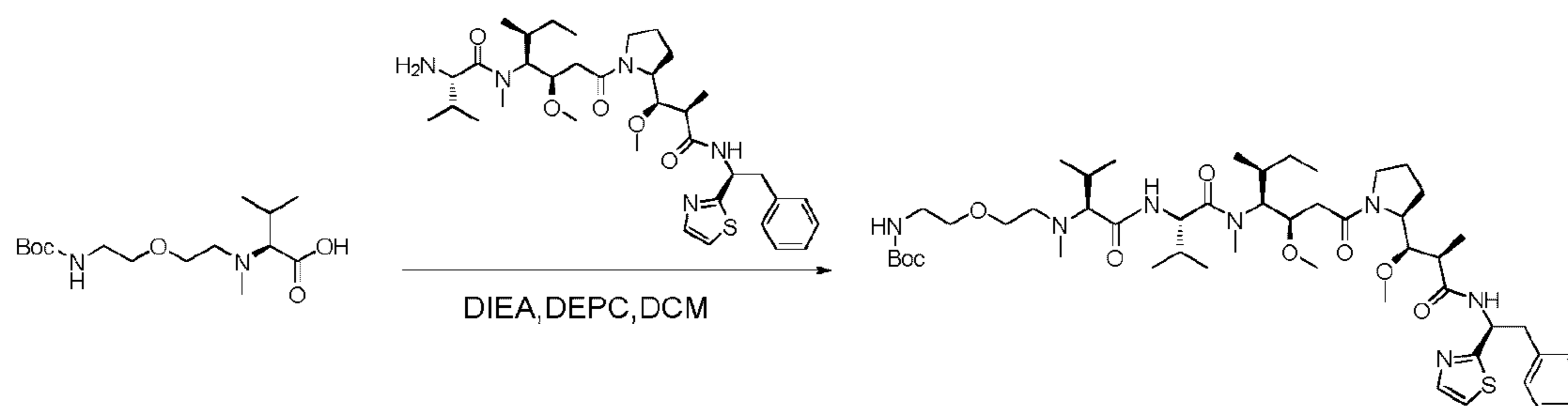
Compound **35C** was synthesised in the same manner as for compound 14C from the amine **1ZC** (2.4 g, 9.31 mmol, 1.00 equiv), the aldehyde **35B** (1.9 g, 9.35 mmol, 1.00 equiv), $\text{NaBH}(\text{OAc})_3$ (3.96 g, 18.68 mmol, 2.00 equiv) and DIEA (6.2 mL) in THF (40 mL). The reaction mixture was neutralised with 200 mL of water and extracted 3 times with 100 mL of AcOEt. The organic phases were combined, dried over sodium sulfate, filtered and concentrated to yield 2.3 g of compound **35C** in the form of a yellow oil.

Compound 35D: (S)-12-isopropyl-2,2,11-trimethyl-4-oxo-3,8-dioxo-5,11-diazatridecan-13-oic acid



Compound **35C** (200 mg, 0.49 mmol, 1.00 equiv) was dissolved in 10 mL of EtOH in the presence of Pd/C (200 mg) and hydrogenated overnight. The reaction medium was filtered and concentrated under reduced pressure to yield 150 mg (96 %) of compound **35D** in the form of a white solid.

Compound 35E: *tert*-butyl ((3R,4S,7S,10S)-4-((S)-*sec*-butyl)-7,10-diisopropyl-3-(2-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-5,11-dimethyl-6,9-dioxo-2,14-dioxo-5,8,11-triazahexadecan-16-yl) carbamate



Compound **35E** was synthesised in the same manner as for compound **3** from the amine **1Y** (70 mg, 0.11 mmol, 1.00equiv), the acid **35D** (40.6 mg, 0.13 mmol, 1.20 equiv), DEPC (0.0324 mL) and DIEA (0.0527 mL) in DCM (3 mL). The crude product (100 mg, 98 %) was isolated in the form of a yellow oil and subsequently used as such.

Compound 35: Compound **35** was synthesised in the same manner as for compound **2** from the intermediate **35E** (100 mg, 0.10 mmol, 1.00 equiv). The crude product was purified by preparative HPLC (Pre-HPLC-010), SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **35** was obtained with a yield of 23 % (22.9 mg) in the form of a white solid.

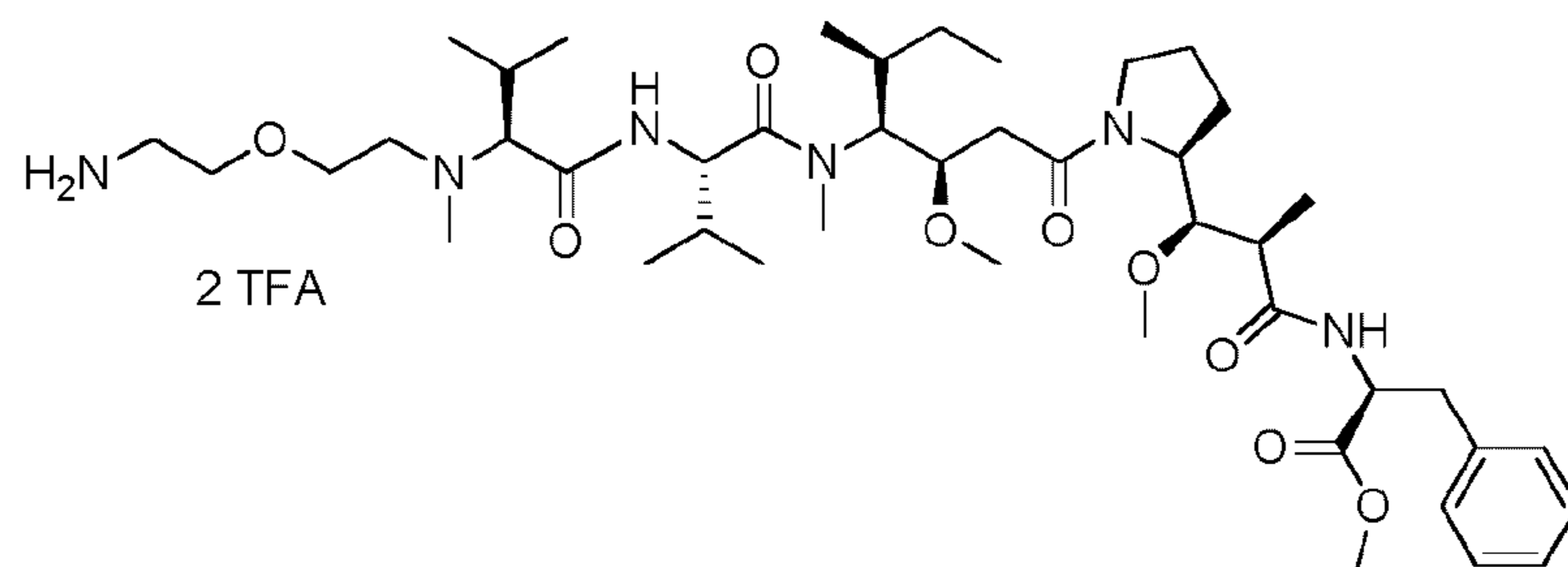
LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI (C₄₅H₇₅N₇O₇S, exact mass 857.54) *m/z*: 858.5 (MH⁺) and 429.9 (M.2H⁺/2, 100 %), 5.89 min (89.7 %, 210 nm).

¹H NMR: (400MHz, CD₃OD, ppm): δ (Presence of rotamers) δ 8.9 - 8.5 (m, 0.5H, NHCO incomplete exchange), 7.8 - 7.7 (m, 1H), 7.55 - 7.45 (m, 1H), 7.35 - 7.1 (m, 5H), 5.45 - 5.5 (m, 1H), 4.9 - 4.6 (m, 1H), 4.3 - 0.75 (m, 62H).

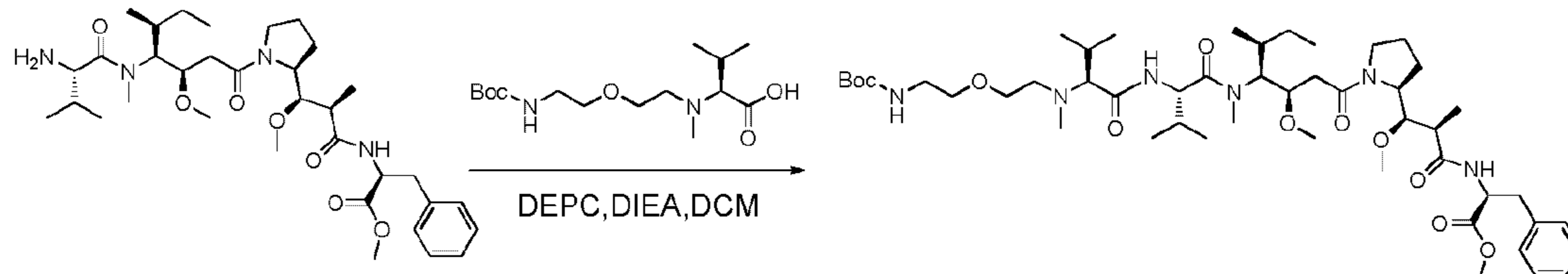
Compound 36

methyl (S)-2-((2R,3R)-3-((S)-1-((7S,10S,13S,14R)-1-amino-13-((S)-*sec*-butyl)-7,10-diisopropyl-14-methoxy-6,12-dimethyl-8,11-dioxo-3-oxa-6,9,12-triazahexadecan-16-oyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, bis trifluoroacetic acid

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Compound 36A: methyl (S)-2-((2R,3R)-3-((S)-1-((12S,15S,18S,19R)-18-
 ((S)-*sec*-butyl)-12,15-diisopropyl-19-methoxy-2,2,11,17-tetramethyl-4,13,16-
 trioxo-3,8-dioxo-5,11,14,17-tetraazahenicosan-21-oyl)pyrrolidin-2-yl)-3-methoxy-
 2-methylpropanamido)-3-phenylpropanoate



Compound **36A** was synthesised in the same manner as for compound **3** from
 the amine **3D** (50 mg, 0.08 mmol, 1.00 equiv), the acid **35D** (25 mg, 0.11 mmol, 1.48
 10 equiv), DEPC (0.0337 mL) and DIEA (0.0548 mL) in DCM (3 mL). The crude product
 (100 mg) was isolated in the form of a yellow oil and subsequently used as such.

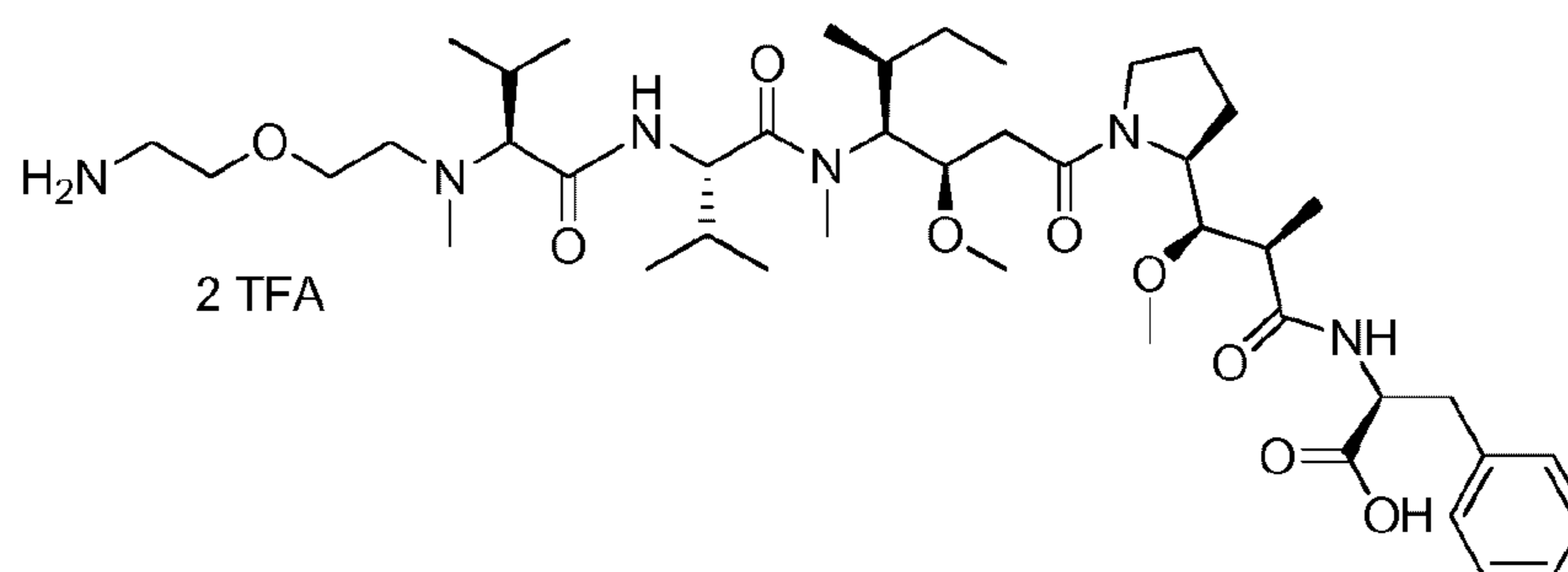
Compound 36: Compound **36** was synthesised in the same manner as for
 compound **2** from the intermediate **36A** (100 mg, 0.11 mmol, 1.00 equiv). The crude
 product was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep
 15 C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / ACN buffered with
 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in
 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **36** was
 obtained with a yield of 13 % (12.7 mg) in the form of a white solid.

LC/MS/UV (Agilent ZORBAX SB-Aq column, 1.8 μ m, 4.6 x 100 mm; 40°C;
 20 1.5 mL/min, 2 % MeOH in water (0.05 % TFA) for 1 minute then 2 % to 95 % MeOH
 in water in 13 minutes, then 95 % MeOH in water for 2 minutes); ESI ($C_{44}H_{76}N_6O_9$,
 exact mass 832.57) m/z : 833.5 (MH^+) and 417.4 ($M.2H^+/2$, 100 %), 11.58 min (98.5 %, 210 nm).

1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 8.1 - 8.5 (m, 0.8H,
 25 NHCO incomplete exchange), 7.30 - 7.1 (m, 5H), 4.9 - 4.6 (m, 3H), 4.2 - 0.8 (m, 64H).

Compound 37

(S)-2-((2R,3R)-3-((S)-1-((7S,10S,13S,14R)-1-amino-13-((S)-*sec*-butyl)-7,10-
 diisopropyl-14-methoxy-6,12-dimethyl-8,11-dioxo-3-oxa-6,9,12-
 5 triazahexadecan-16-oyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-
 phenylpropanoic acid, bis trifluoroacetic acid



Compound **37** was prepared in the same manner as for compound **4**, from
 compound **36** (100 mg, 0.11 mmol, 1.00 equiv). The residue was purified by preparative
 10 HPLC (Pre-HPLC-001 SHIMADZU, Atlantis Prep OBD T3 column, 5 μ m, 19 x 150
 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 %
 ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector
 at 254 nm and 220 nm), to yield 18.4 mg (19 %) of compound **37** in the form of a white
 solid.

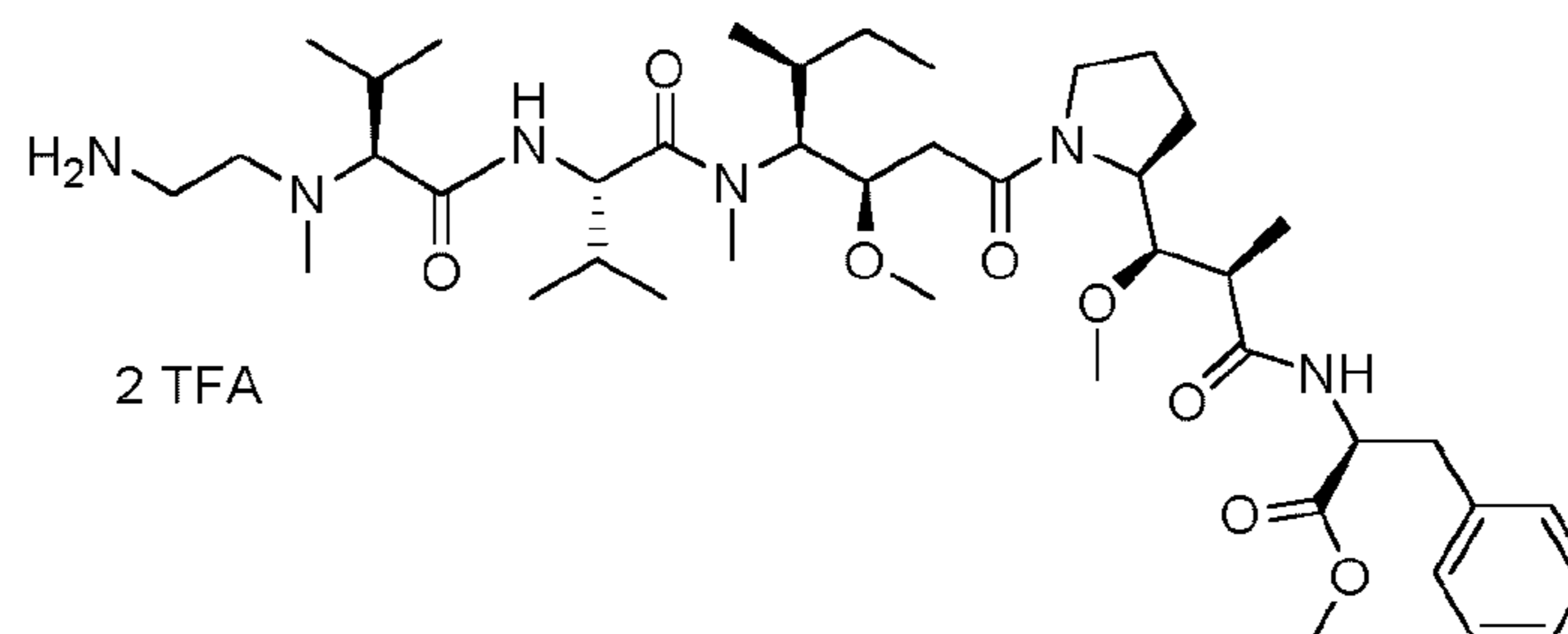
15 LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C;
 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes);
 ESI (C₄₃H₇₄N₆O₉, exact mass 818.6) *m/z*: 819.6 (MH⁺) and 410.4 (M.2H⁺/2, 100 %),
 5.48 min (96.7 %, 210 nm).

¹H NMR: (400MHz, CD₃OD, *ppm*): δ (Presence of rotamers) 7.35 - 7.2 (m, 5H),
 20 5.0 - 4.65 (m, 3H), 4.3 - 0.8 (m, 61H).

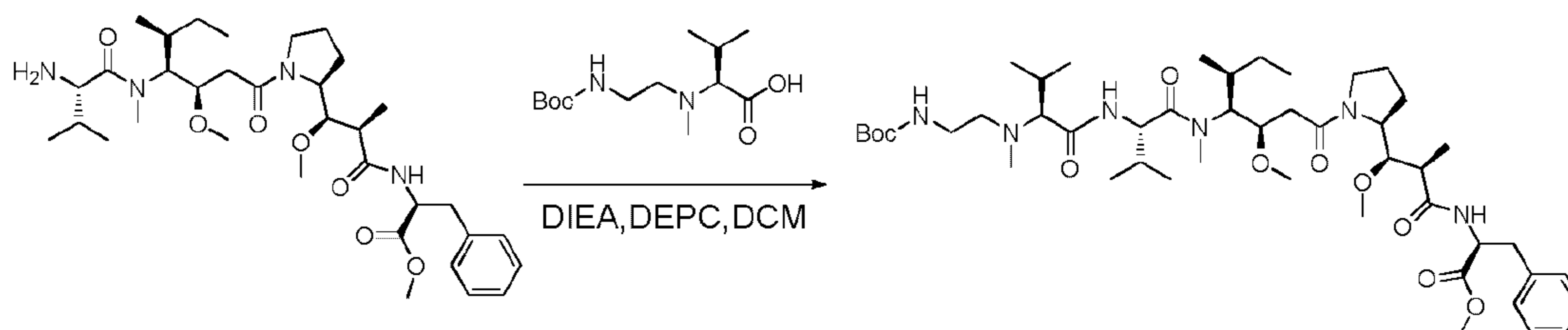
Compound 38

methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((2-
 aminoethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-
 25 methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-
 methylpropanamido)-3-phenylpropanoate, bis trifluoroacetic acid

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Compound 38A: methyl (S)-2-((2R,3R)-3-((S)-1-((9S,12S,15S,16R)-15-
 ((S)-*sec*-butyl)-9,12-diisopropyl-16-methoxy-2,2,8,14-tetramethyl-4,10,13-trioxo-
 3-oxa-5,8,11,14-tetrazaoctadecan-18-oyl)pyrrolidin-2-yl)-3-methoxy-2-
 5 methylpropanamido)-3-phenylpropanoate



Compound **38A** was synthesised in the same manner as for compound **3** from
 the amine **3D** (70 mg, 0.11 mmol, 1.00 equiv), the acid **26B** (60.7 mg, 0.22 mmol, 2.00
 equiv), DEPC (0.0337 mL) and DIEA (0.0548 mL) in DCM (3 mL). The crude product
 10 (100 mg) was isolated in the form of a yellow oil.

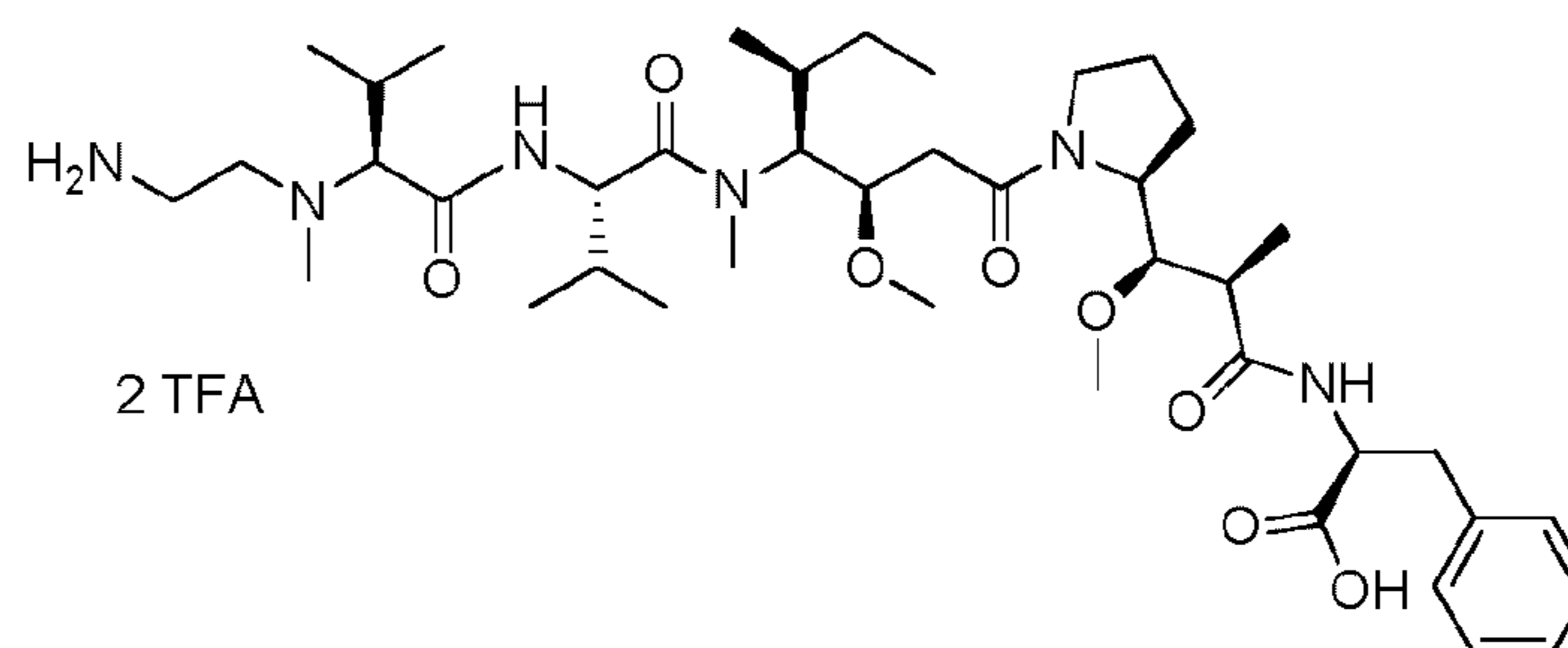
Compound 38: Compound **38** was synthesised in the same manner as for
 compound **2** from the intermediate **38A** (100 mg, 0.11 mmol, 1.00 equiv). The crude
 product was purified by preparative HPLCPre-HPLC-001 SHIMADZU, SunFire Prep
 C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with
 15 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in
 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **38** was
 obtained with a yield of 10 % (10.3 mg) in the form of a white solid.

LC/MS/UV (Agilent ZORBAX SB-Aq column, 1.8 μ m, 4.6 x 100 mm; 40°C;
 1.5 mL/min, 2 % MeOH in water (0.05 % TFA) for 1 minute then 2 % to 95 % MeOH
 20 in water in 13 minutes then 95 % MeOH in water for 2 minutes); ESI ($C_{42}H_{72}N_6O_8$,
 exact mass 788.5) m/z : 789.5 (MH^+) and 395.4 ($M.2H^+/2$, 100 %), 12.97 min (91.0 %, 210 nm).

1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 7.30 - 7.1 (m, 5H),
 4.9 - 4.6 (m, 3H), 4.2 - 0.8 (m, 60H).

Compound 39

(S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((2-aminoethyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoic acid, bis trifluoroacetic acid



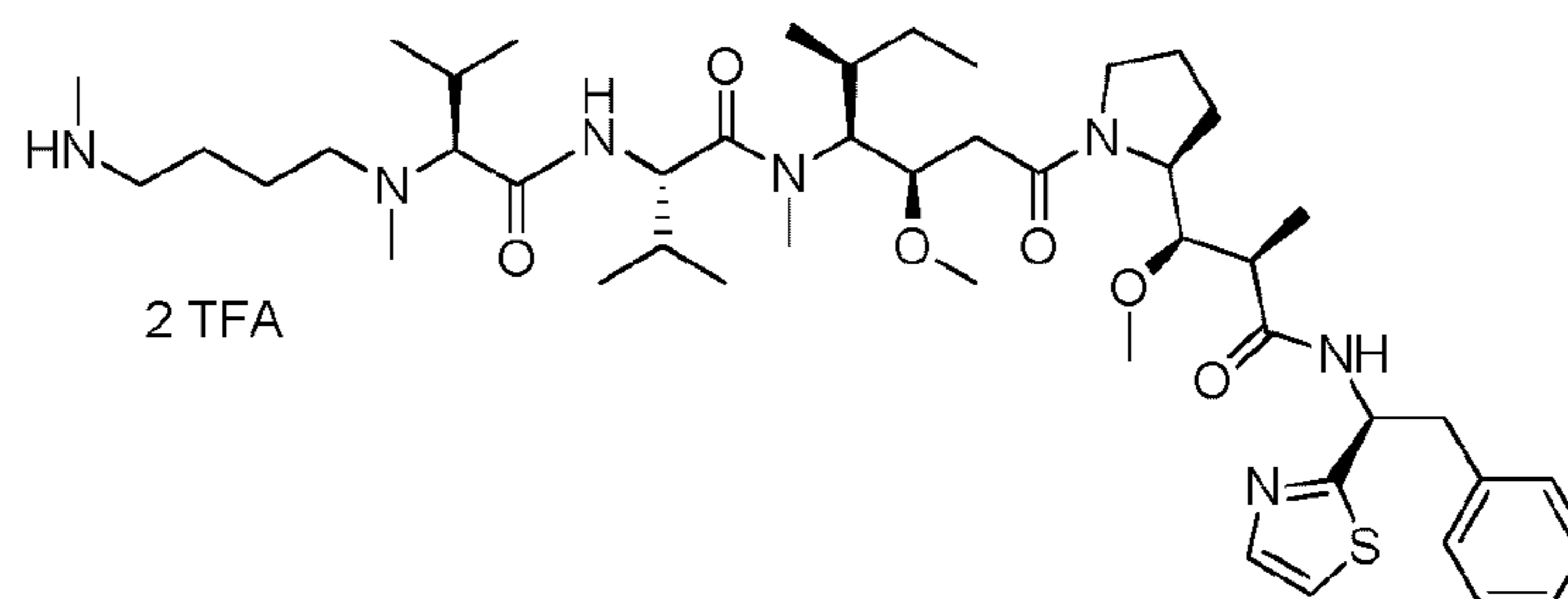
Compound **39** was prepared in the same manner as for compound **4**, from compound **38** (100 mg, 0.11 mmol, 1.00 equiv). The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm), to yield 8.2 mg (8 %) of compound **39** in the form of a white solid.

LC/MS/UV (Eclipse Plus C18 column, 3.5 μ m, 4.6 x 150 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI (C₄₁H₇₀N₆O₈, exact mass 774.5) *m/z*: 775.5 (MH⁺) and 388.4 (M.2H⁺/2, 100 %), 6.47 min (93.6 %, 210 nm).

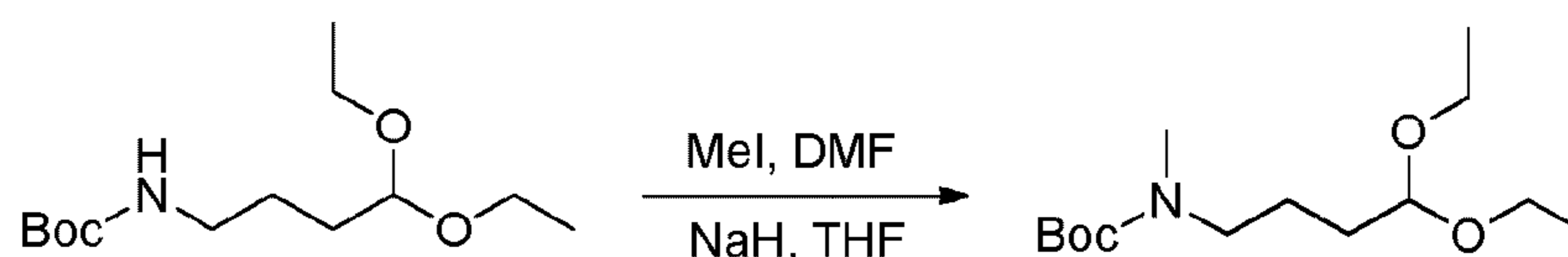
¹H NMR: (400MHz, CD₃OD, *ppm*): δ (Presence of rotamers) 7.35 - 7.15 (m, 5H), 4.9 - 4.6 (m, 3H), 4.2 - 0.8 (m, 57H).

Compound 40

(S)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(4-(methylamino)butyl)amino)butanamido)butanamide, bis trifluoroacetic acid

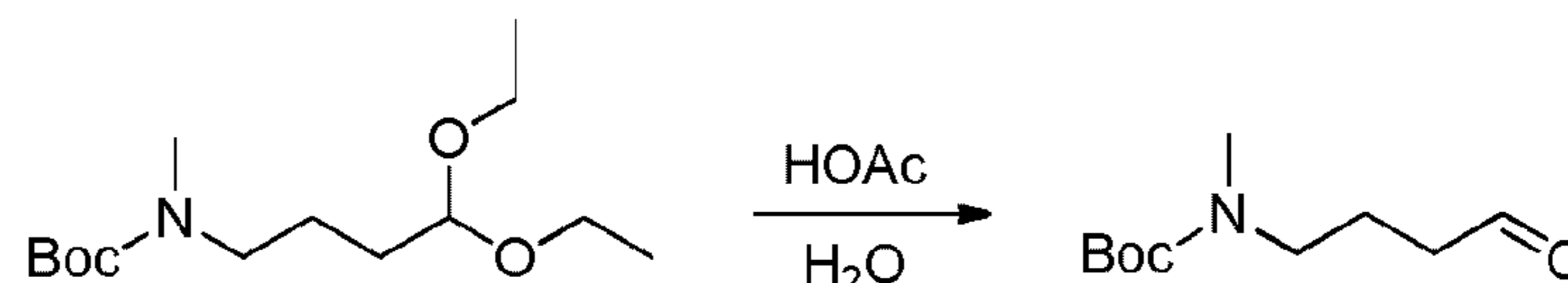


Compound 40A: *tert*-butyl (4,4-diethoxybutyl)(methyl)carbamate



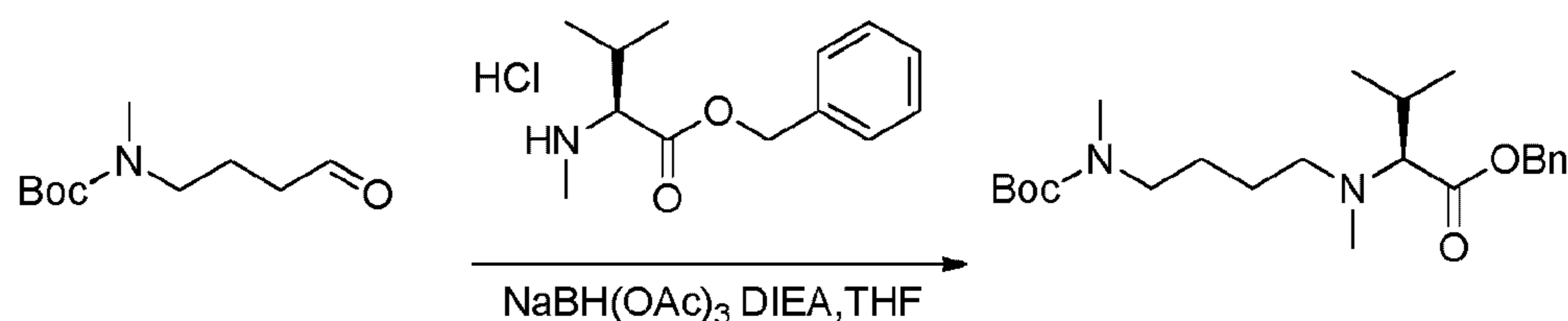
Compound **40A** was prepared in the same manner as for compound **11E**, from
 5 *tert*-butyl(4,4-diethoxybutyl)(methyl)carbamate (5.5 g, 19.97 mmol, 1.00 equiv), NaH
 (60 % in oil, 3.2 g, 80.00 mmol, 4.01 equiv) and iodomethane (14 mL) in THF/DMF
 (40/20 mL). The reaction was neutralised with 50 mL of NH₄Cl(aq). Compound **40A**
 was isolated in the form of a yellow oil, 5.5 g (95 %).

Compound 40B: *tert*-butyl methyl(4-oxobutyl)carbamate



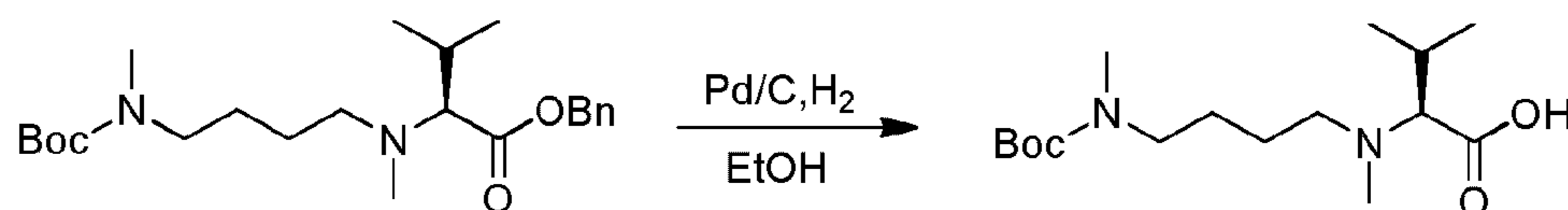
10 Compound **40A** (3 g, 10.89 mmol, 1.00 equiv) was dissolved in a mixture of
 AcOH and water (15/4 mL) and the solution was left under agitation overnight. The pH
 was brought to 7–8 with NaHCO₃-saturated aqueous solution and then extracted twice
 with 50 mL of EtOAc. The organic phases were combined, washed twice with 50 mL of
 NaCl-saturated aqueous solution, dried over anhydrous sodium sulfate, filtered and
 15 concentrated under reduced pressure. Compound **40B** was isolated in the form of yellow
 oil, 2.1 g (96 %).

Compound 40C: benzyl (S)-2-((4-((*tert*-butoxycarbonyl)(methyl)amino)butyl)
 (methyl)amino)-3-methylbutanoate



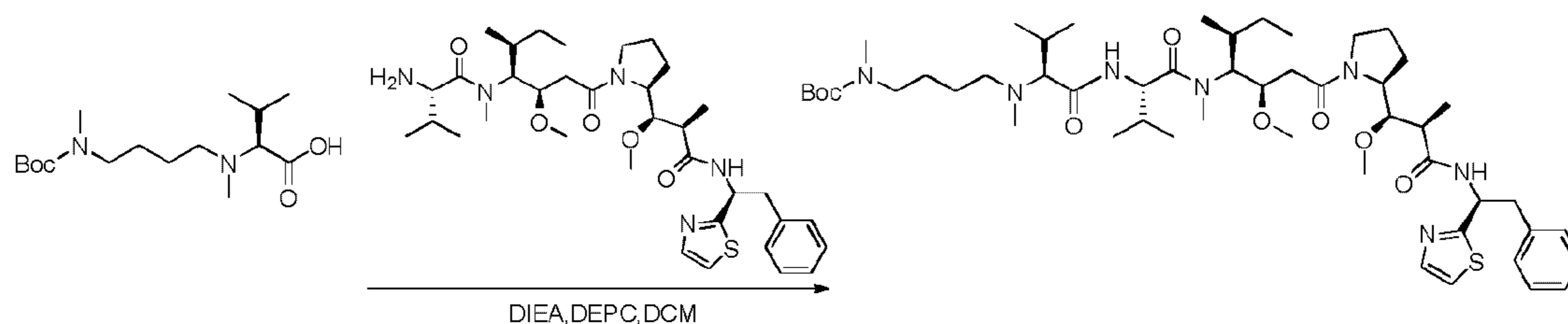
Compound **40C** was synthesised in the same manner as for compound **14C** from the amine **1ZC** (2.45 g, 9.53 mmol, 0.80 equiv), the aldehyde **40B** (2.4 g, 11.92 mmol, 1.00 equiv), NaBH(OAc)₃ (5.06 g, 23.87 mmol, 2.00 equiv) and DIEA (6.16 g, 47.66 mmol, 4.00 equiv) in THF (15 mL). The reaction mixture was neutralised with 100 mL of water and extracted twice with 100 mL of AcOEt. The organic phases were combined, dried over sodium sulfate, filtered and concentrated. The residue was purified on silica gel (EtOAc/PE (1:100–1:20)) to yield 1.2 g (25 %) of compound **40C** in the form of a yellow oil.

Compound 40D: (S)-2-((4-((*tert*-butoxycarbonyl)(methyl)amino)butyl)(methyl) amino)-3-methylbutanoic acid



Compound **40C** (500 mg, 1.23 mmol, 1.00 equiv) was dissolved in 20 mL of EtOH in the presence of Pd/C (550 mg) and hydrogenated for 1 hour at ambient temperature and atmospheric pressure. The reaction medium was filtered and concentrated under reduced pressure to yield 350 mg (90 %) of compound **40D** in the form of a colourless oil.

Compound 40E: *tert*-butyl ((3R,4S,7S,10S)-4-((S)-*sec*-butyl)-7,10-diisopropyl-3-(2-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-5,11-dimethyl-6,9-dioxo-2-oxa-5,8,11-triazapentadecan-15-yl)(methyl)carbamate



Compound **40E** was synthesised in the same manner as for compound **3** from the amine **1Y** (60 mg, 0.09 mmol, 1.00 equiv), the acid **40D** (57.8 mg, 0.18 mmol, 2.00 equiv), DEPC (0.0278 mL) and DIEA (0.0452 mL) in DCM (3 mL). The crude product (100 mg) was subsequently used as such.

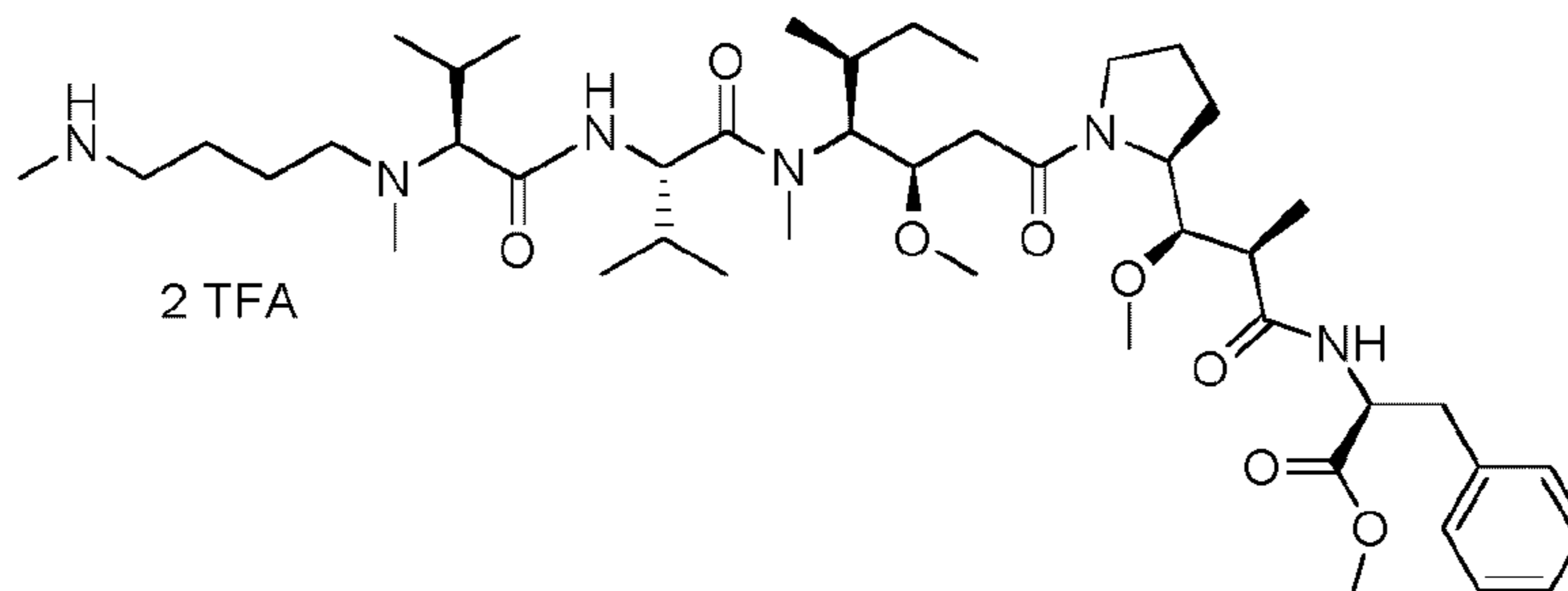
Compound 40: Compound 40 was synthesised in the same manner as for compound 2 from the intermediate 40E (100 mg, 0.10 mmol, 1.00 equiv). The crude product was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 95 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound 40 was obtained with a yield of 24 % (24.6 mg) in the form of a white solid.

LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI (C₄₆H₇₇N₇O₆S, exact mass 855.6) *m/z*: 856.6 (MH⁺) and 428.8 (M.2H⁺/2, 100 %), 5.89 min (97.0 %, 210 nm).

¹H NMR: (400MHz, CD₃OD, ppm): δ (Presence of rotamers) 8.9 - 8.5 (0.7H, NHCO incomplete exchange), 7.8 - 7.7 (m, 1H), 7.55 - 4.45 (m, 1H), 7.35 - 7.1 (m, 5H), 5.5 - 5.75 (m, 1H), 4.9 - 4.6 (m, 2H), 4.2 - 0.8 (m, 64H).

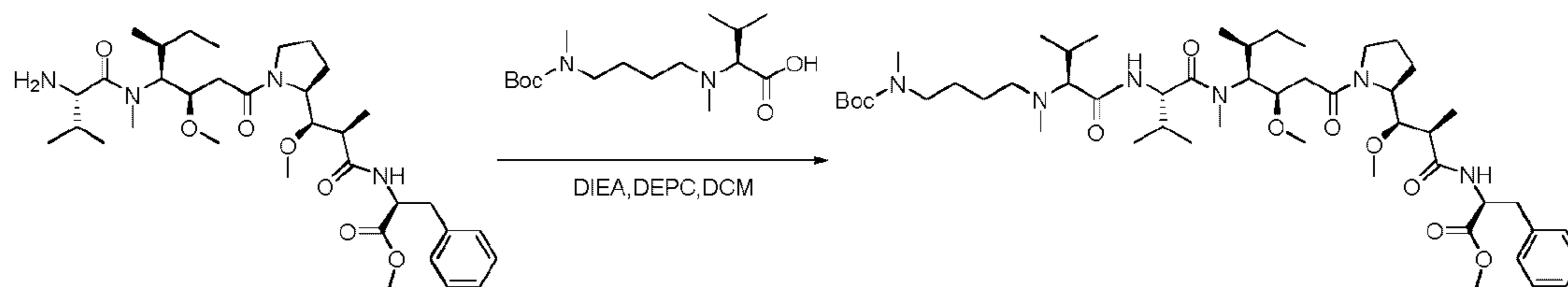
Compound 41

methyl (S)-2-((2R,3R)-3-((S)-1-((8S,11S,14S,15R)-14-((S)-*sec*-butyl)-8,11-diisopropyl-15-methoxy-7,13-dimethyl-9,12-dioxo-2,7,10,13-tetraazaheptadecan-17-oyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, bis trifluoroacetic acid



Compound 41A: methyl (S)-2-((2R,3R)-3-((S)-1-((11S,14S,17S,18R)-17-((S)-*sec*-butyl)-11,14-diisopropyl-18-methoxy-2,2,5,10,16-pentamethyl-4,12,15-trioxo-3-oxa-5,10,13,16-tetrazaicosan-20-oyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate

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Compound **41A** was synthesised in the same manner as for compound **3** from the amine **3D** (170 mg, 0.27 mmol, 1.00 equiv), the acid **40D** (170 mg, 0.54 mmol, 2.09 equiv), DEPC (0.0819 mL) and DIEA (0.133 mL) in DCM (5 mL). The crude product (200 mg) was subsequently used as such.

Compound 41: Compound **41** was synthesised in the same manner as for compound **2** from the intermediate **41A** (100 mg, 0.11 mmol, 1.00 equiv). The crude product was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 95 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **41** was obtained with a yield of 25 % (25 mg) in the form of a white solid.

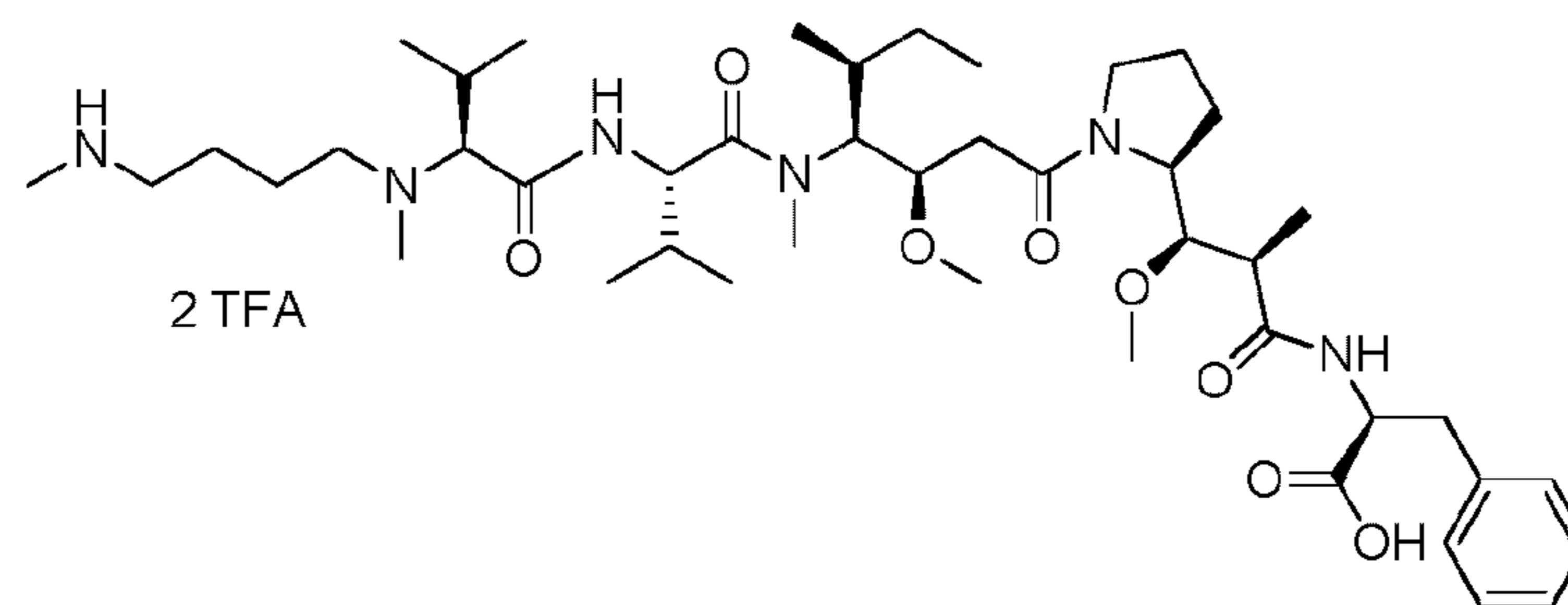
LC/MS/UV (Agilent Zorbax SB-Aq column, 1.8 μ m, 4.6 x 100 mm; 40°C; 1.5 mL/min, 2 % MeOH in water (0.05 % TFA) for 1 minute then 2 % to 95 % MeOH in water in 13 minutes, then 95 % MeOH in water for 2 minutes); ESI ($C_{45}H_{78}N_6O_8$, exact mass 830.6) m/z : 831.6 (MH^+) and 416.4 ($M.2H^+/2$, 100 %), 11.58 min (97.2 %, 210 nm).

1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 8.55 - 8.15 (0.75H, NHCO incomplete exchange), 7.30 - 7.1 (m, 5H), 4.9 - 4.6 (m, 3H), 4.2 - 0.8 (m, 67H).

Compound 42

(S)-2-((2R,3R)-3-((S)-1-((8S,11S,14S,15R)-14-((S)-*sec*-butyl)-8,11-diisopropyl-15-methoxy-7,13-dimethyl-9,12-dioxo-2,7,10,13-tetraazaheptadecan-17-oyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoic acid, bis trifluoroacetic acid

193



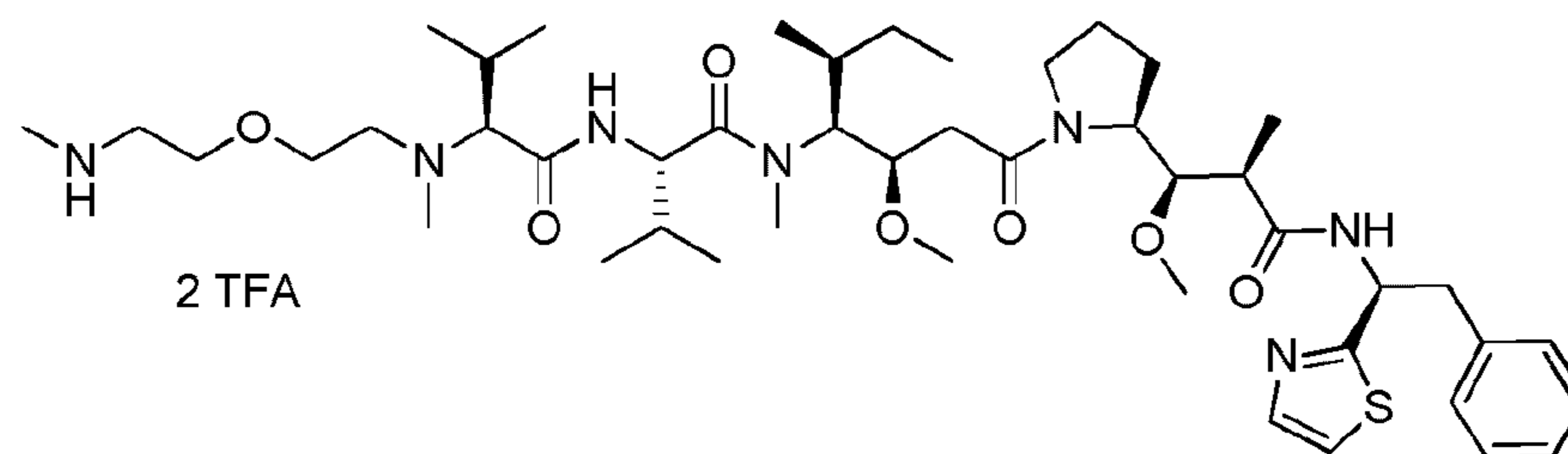
Compound **42** was prepared in the same manner as for compound **4**, from compound **41** (100 mg, 0.11 mmol, 1.00 equiv). The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, Atlantis Prep OBD T3 column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm), to yield 30.6 mg (31 %) of compound **42** in the form of a white solid.

LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C; 1.5 mL/min, 0 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI (C₄₄H₇₆N₆O₈, exact mass 816.6) *m/z*: 817.6 (MH⁺) and 409.4 (M.2H⁺/2, 100 %), 5.75 min (100 %, 210 nm).

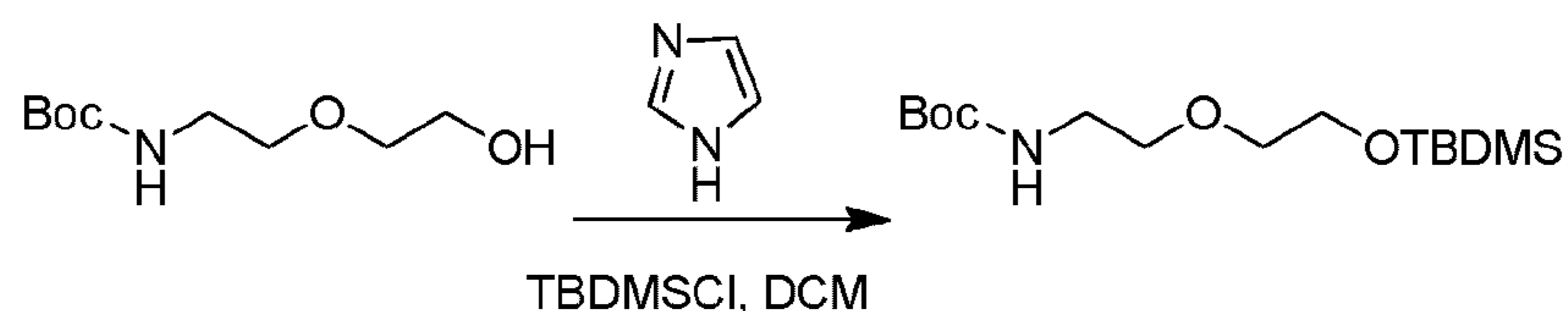
¹H NMR: (400MHz, CD₃OD, *ppm*): δ (Presence of rotamers) 8.5 - 8.1 (0.3H, NHCO incomplete exchange), 7.30 - 7.1 (m, 5H), 4.9 - 4.6 (m, 3H), 4.2 - 0.8 (m, 64H).

Compound 43

(S)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethyl-2-((R)-3-methyl-2-(methyl(2-(2-(methylamino)ethoxy)ethyl)amino)butanamido)butanamide, bis trifluoroacetic acid

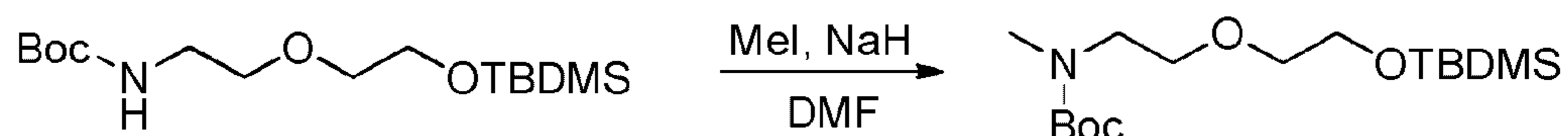


Compound 43A: *tert*-butyl (2-(2-((*tert*butyldimethylsilyl)oxy)ethoxy)ethyl) carbamate



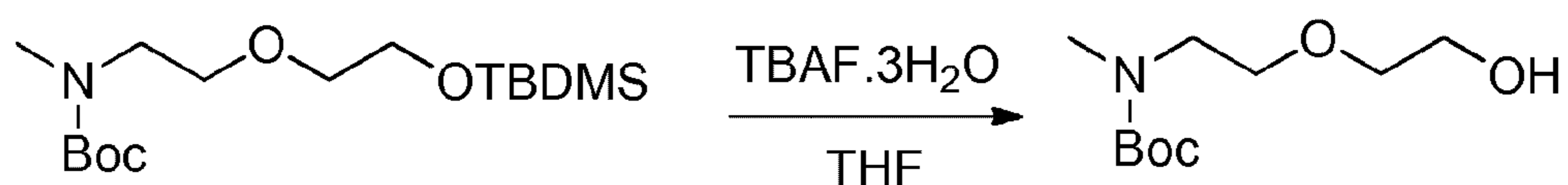
Compound **35A** (*tert*-butyl ((2-(2-hydroxyethoxy)ethyl)carbamate) (8.21 g, 40.00 mmol, 1.00 equiv) and imidazole (6 g, 88.14 mmol, 2.20 equiv) were dissolved in an inert atmosphere in DCM (200 mL). Tertbutyldimethylsilane chloride (TBDMSCl, 6.62 g, 43.92 mmol, 1.10 equiv) was added drop-wise and the reaction medium was left under agitation overnight at ambient temperature. The reaction mixture was diluted with 100 mL of DCM then washed twice with 200 mL of 0.5 M HCl, twice with 200 mL of NaHCO₃ (sat.), then 300 mL of NaCl (sat.). The organic phase was dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified on silica gel (EtOAc/PE (1:3) to yield 10 g (78 %) of compound **43A** in the form of a white solid.

Compound 43B: *tert*-butyl (2-(2-((*tert*-butyldimethylsilyl)oxy)ethoxy)ethyl)(methyl)carbamate



Compound **43B** was prepared in the same manner as for compound **11E**, from compound **43A** (10 g, 31.30 mmol, 1.00 equiv), NaH (60 % in oil, 5 g, 208.33 mmol, 4.00 equiv) and iodomethane (22 g, 5.00 equiv) in DMF (200 mL). The reaction medium was neutralised with 200 mL of water and washed 3 times with 100 mL of AcOEt then 300 mL of NaCl (sat.). The organic phase was dried over sodium sulfate, filtered and concentrated under reduced pressure to yield 10 g (96 %) of compound **43B** in the form of a white solid.

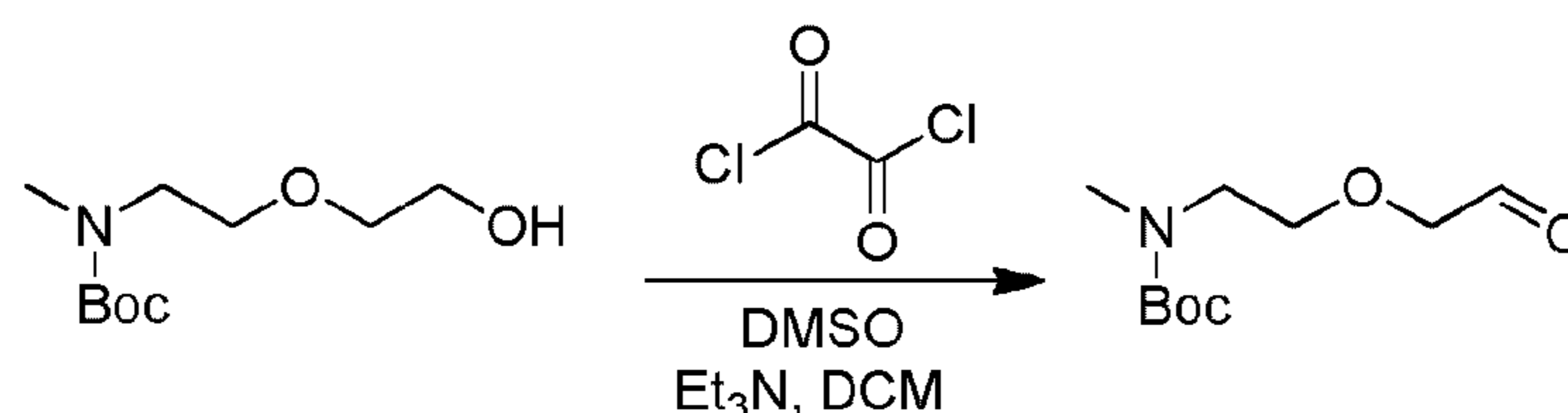
Compound 43C: *tert*-butyl (2-(2-hydroxyethoxy)ethyl)(methyl)carbamate



Compound **43B** (10 g, 29.89 mmol, 1.00 equiv) and TBAF.3H₂O (20.8 g, 65.93 mmol, 2.20 equiv) were dissolved in THF (200 mL). The mixture was agitated at ambient temperature for 2 hours then extracted 3 times with 100 mL of AcOEt. The organic phases were recombined, washed twice with 300 mL of water, then twice with

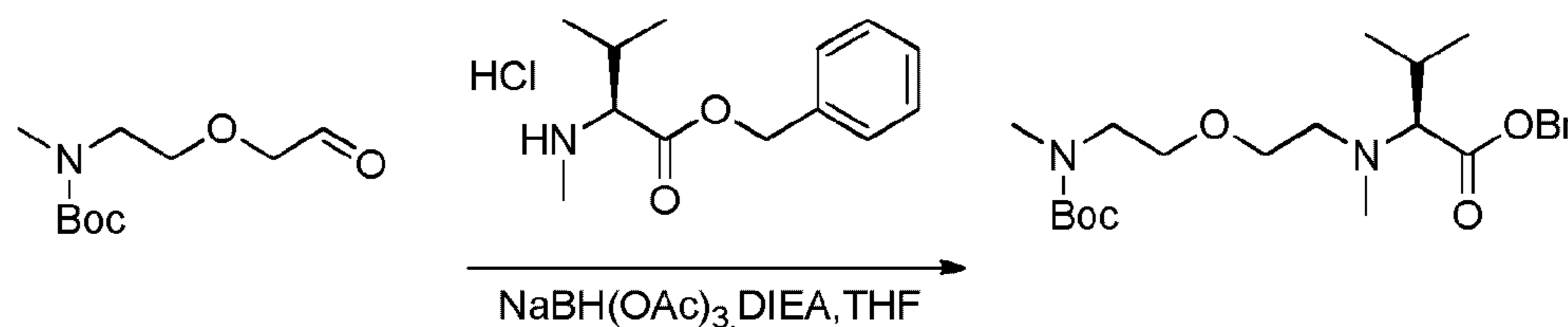
300 mL of NaCl (sat.), dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified on silica gel (EtOAc/PE (1:3 - 1:1)) to yield 6.6 g of compound **43C** in the form of colourless oil.

Compound 43D: *tert*-butyl methyl(2-(2-oxoethoxy)ethyl)carbamate



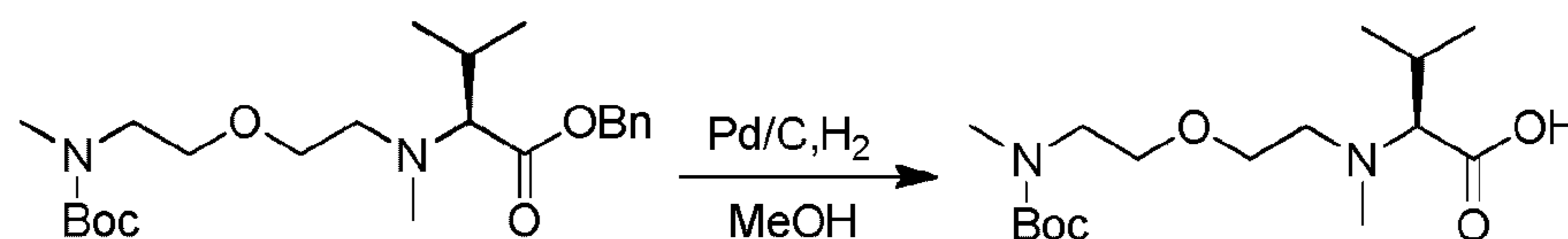
5 Compound **43D** was prepared in the same manner as for compound **35B**, from compound **43C** (2 g, 9.12 mmol, 1.00 equiv), oxalyl chloride (1.9 mL), TEA (11.3 mL) and DMSO (3.3 mL). Compound **43D** (2 g) was isolated in the form of yellow oil.

Compound 43E: benzyl (S)-12-isopropyl-2,2,5,11-tetramethyl-4-oxo-3,8-dioxa-5,11-diazatridecan-13-oate



10 Compound **43E** was synthesised in the same manner as for compound **14C** from the amine **1ZC** (2.4 g, 9.31 mmol, 1.00 equiv), the aldehyde **43D** (2 g, 9.16 mmol, 1.00 equiv), NaBH(OAc)₃ (4 g, 18.87 mmol, 2.06 equiv) and DIEA (6 mL) in THF (100 mL). The reaction mixture was neutralised with 100 mL of water and extracted 3 times with 100 mL of AcOEt. The organic phases were combined, dried over sodium sulfate,
15 filtered and concentrated. The residue was purified on silica gel (EtOAc/PE (4:1)) to yield 1 g (37 %) of compound **43E** in the form of a white solid.

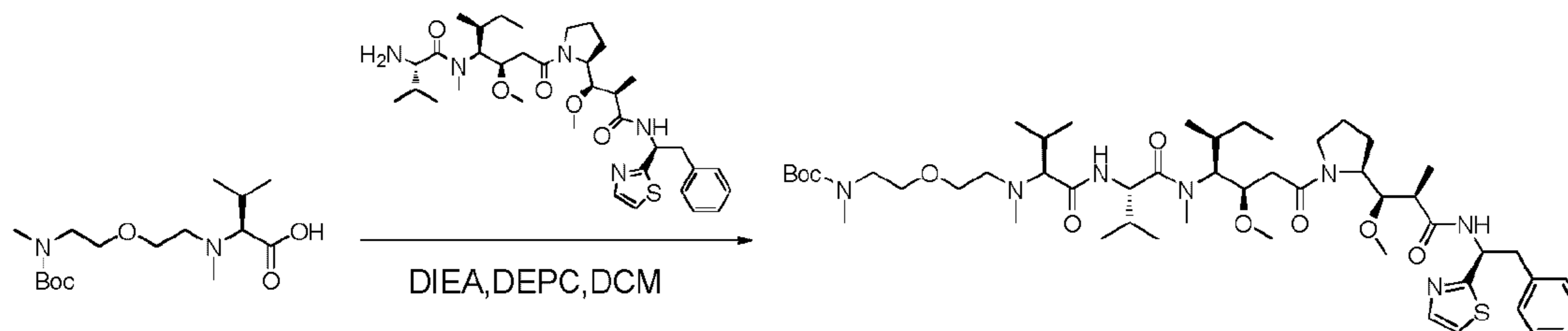
Compound 43F: (S)-12-isopropyl-2,2,5,11-tetramethyl-4-oxo-3,8-dioxa-5,11-diazatridecan-13-oic acid



20 Compound **43E** (1 g, 2.37 mmol, 1.00 equiv) was dissolved in 40 mL of MeOH in the presence of Pd/C (1 g) and hydrogenated for 1 hour at ambient temperature and

atmospheric pressure. The reaction medium was filtered and concentrated under reduced pressure to yield 600 mg (76 %) of compound **43F** in the form of a white solid.

Compound 43G: *tert*-butyl ((3R,4S,7S,10R)-4-((S)-*sec*-butyl)-7,10-diisopropyl-3-(2-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-5,11-dimethyl-6,9-dioxo-2,14-dioxo-5,8,11-triazahexadecan-16-yl)(methyl)carbamate



Compound **43G** was synthesised in the same manner as for compound **3** from the amine **1Y** (50 mg, 0.08 mmol, 1.00 equiv), the acid **43F** (50 mg, 0.08 mmol, 1.00 equiv), DEPC (24.79 mg, 0.15 mmol, 2.00 equiv) and DIEA (29.46 mg, 0.23 mmol, 3.00 equiv) in DCM (1 mL). The crude product (59 mg) was subsequently used as such.

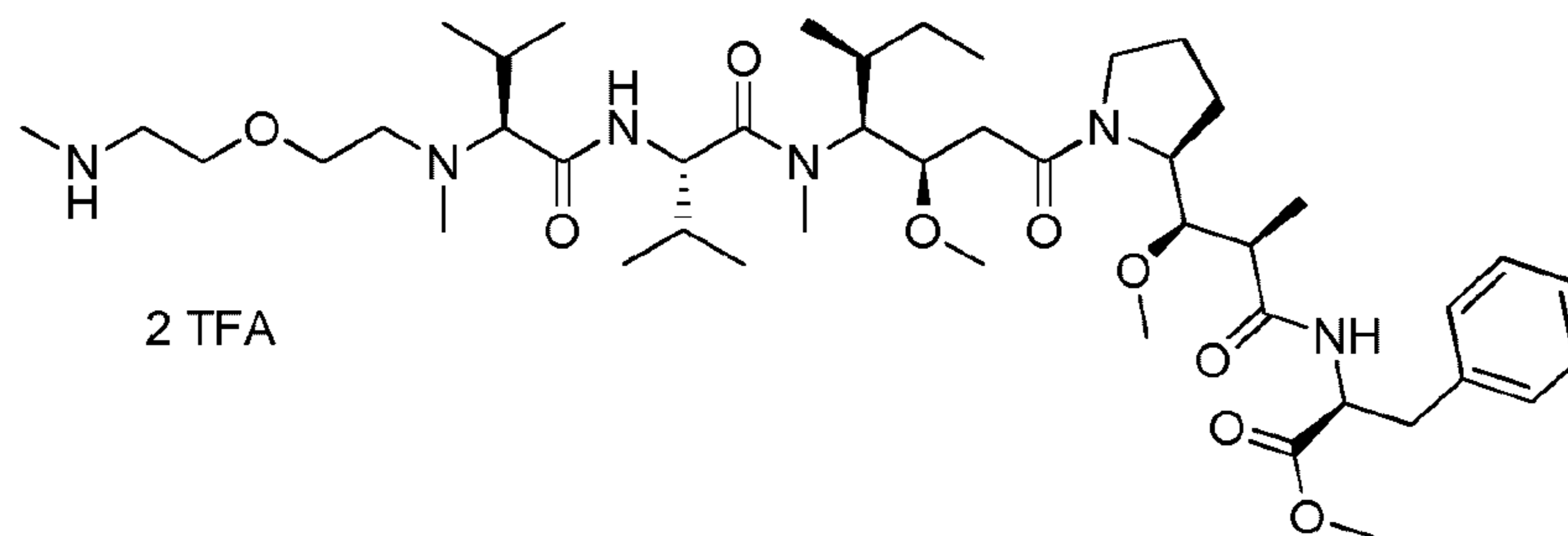
Compound 43: Compound **43** was synthesised in the same manner as for compound **2** from the intermediate **43G** (81 mg, 0.08 mmol, 1.00 equiv). The crude product was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 95 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **43** was obtained with a yield of 64 % (52.6 mg) in the form of a white solid.

LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeCN in water (0.05 % TFA) for 8 minutes then 95 % MeCN in water for 2 minutes); ESI (C₄₈H₇₇N₇O₇S, exact mass 871.6) *m/z*: 872.5 (MH⁺) and 436.9 (M.2H⁺/2, 100 %), 3.90 min (100 %, 210 nm).

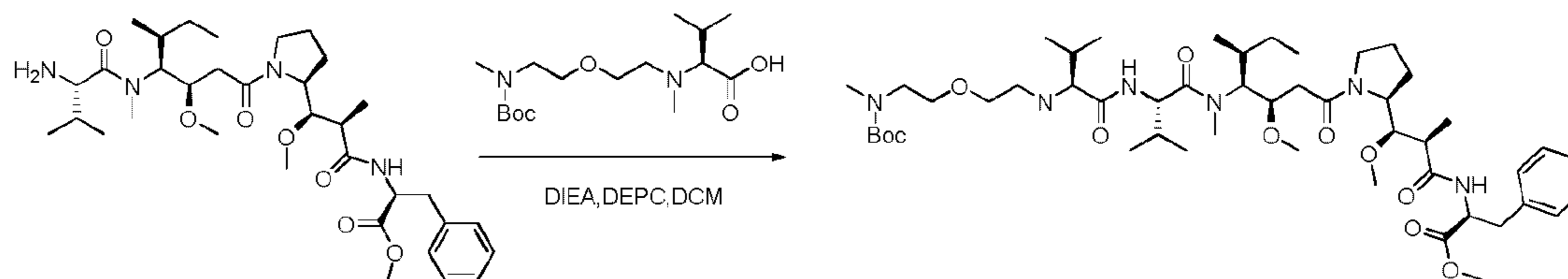
¹H NMR: (400MHz, CD₃OD, ppm): δ (Presence of rotamers) 7.8 - 7.7 (m, 1H), 7.55 - 4.45 (m, 1H), 7.35 - 7.1 (m, 5H), 5.5 - 5.75 (m, 1H), 4.9 - 4.6 (m, 2H), 4.2 - 0.8 (m, 64H).

Compound 44

methyl (S)-2-((2R,3R)-3-((S)-1-((9S,12S,15S,16R)-15-((S)-*sec*-butyl)-9,12-diisopropyl-16-methoxy-8,14-dimethyl-10,13-dioxo-5-oxa-2,8,11,14-tetrazaoctadecan-18-oyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, bis trifluoroacetic acid



Compound 44A: methyl (S)-2-((2R,3R)-3-((S)-1-((12S,15S,18S,19R)-18-((S)-*sec*-butyl)-12,15-diisopropyl-19-methoxy-2,2,5,11,17-pentamethyl-4,13,16-trioxo-3,8-dioxa-5,11,14,17-tetraazahenicosan-21-oyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate



Compound **44A** was synthesised in the same manner as for compound **3** from the amine **3D** (60 mg, 0.09 mmol, 1.00 equiv), the acid **43F** (47 mg, 0.14 mmol, 1.50 equiv), DEPC (31 mg, 0.19 mmol, 2.00 equiv) and DIEA (37 mg, 0.28 mmol, 3.00 equiv) in DCM (1.5 mL). The crude product (58 mg) was subsequently used as such.

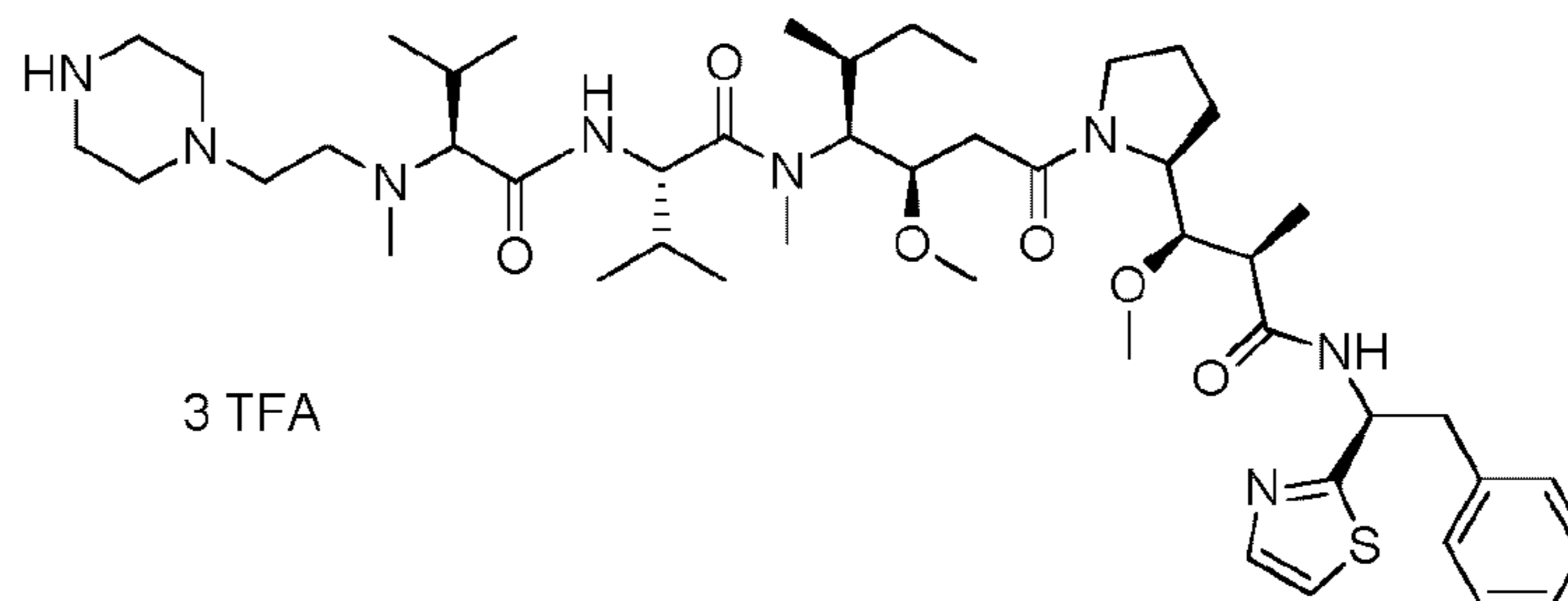
Compound 44: Compound **44** was synthesised in the same manner as for compound **2** from the intermediate **44A** (58 mg, 0.06 mmol, 1.00 equiv). The crude product was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 95 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **44** was obtained with a yield of 40 % (23.7 mg) in the form of a white solid.

LC/MS/UV (Ascentis Express C18 column, 2.7 μm , 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeCN in water (0.05 % TFA) for 8 minutes then 95 % MeCN in water for 2 minutes); ESI ($\text{C}_{45}\text{H}_{78}\text{N}_6\text{O}_9$, exact mass 846.6) m/z : 847.6 (MH^+) and 424.4 ($\text{M.2H}^+/2$, 100 %), 3.20 min (100 %, 210 nm).

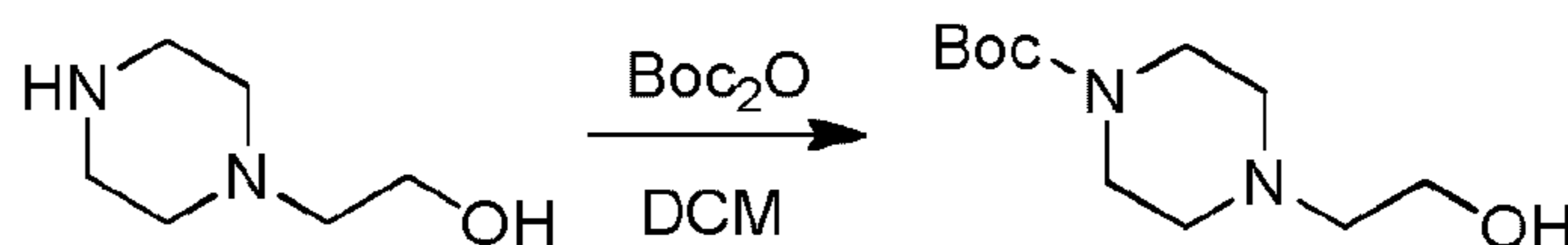
5 ^1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 7.3 - 7.1 (m, 5H), 4.9 - 4.6 (m, 3H), 4.2 - 0.8 (m, 67H).

Compound 45

10 **(S)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(2-(piperazin-1-yl)ethyl)amino)butanamido)butanamide, tris trifluoroacetic acid**



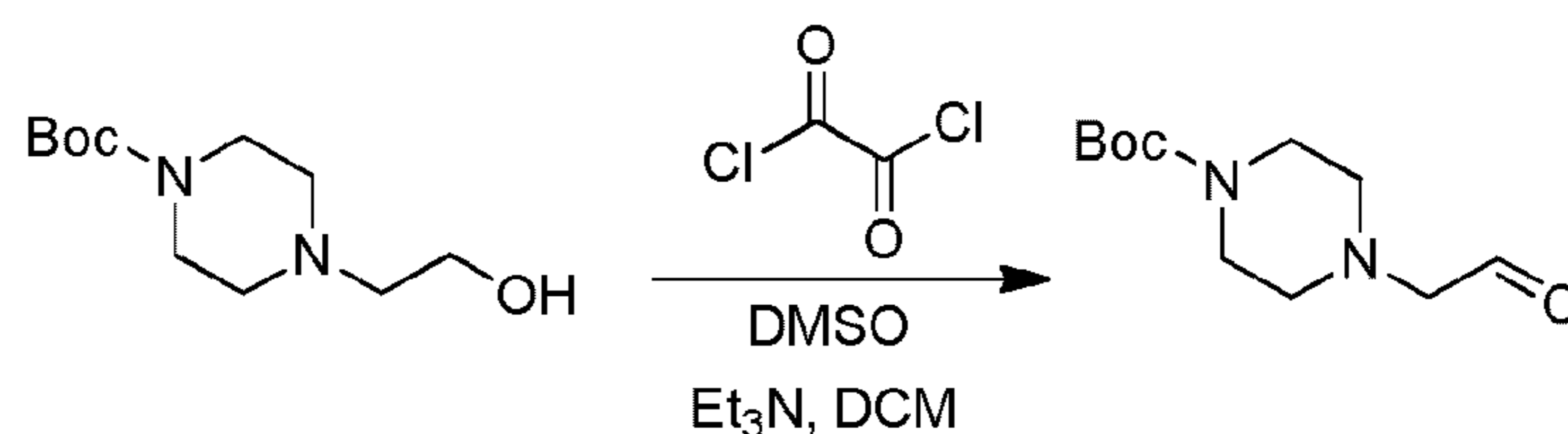
Compound 45A: *tert*-butyl 4-(2-hydroxyethyl)piperazine-1-carboxylate



15 2-(piperazin-1-yl)ethan-1-ol (5 g, 38.41 mmol, 1.00 equiv) was dissolved in DCM (100 mL), and a solution of di-*tert*-butyl dicarbonate (8.38 g, 38.40 mmol, 1.00 equiv) in DCM (20 mL) was added drop-wise. The reaction was left under agitation overnight at ambient temperature. The reaction was evaporated to dryness and the residue dissolved in 200 mL of AcOEt, washed 5 times with NaCl
20 (sat.), dried over sodium sulfate, filtered and concentrated under reduced pressure to yield 8.5 g (96 %) of compound **45A** in the form of a white solid.

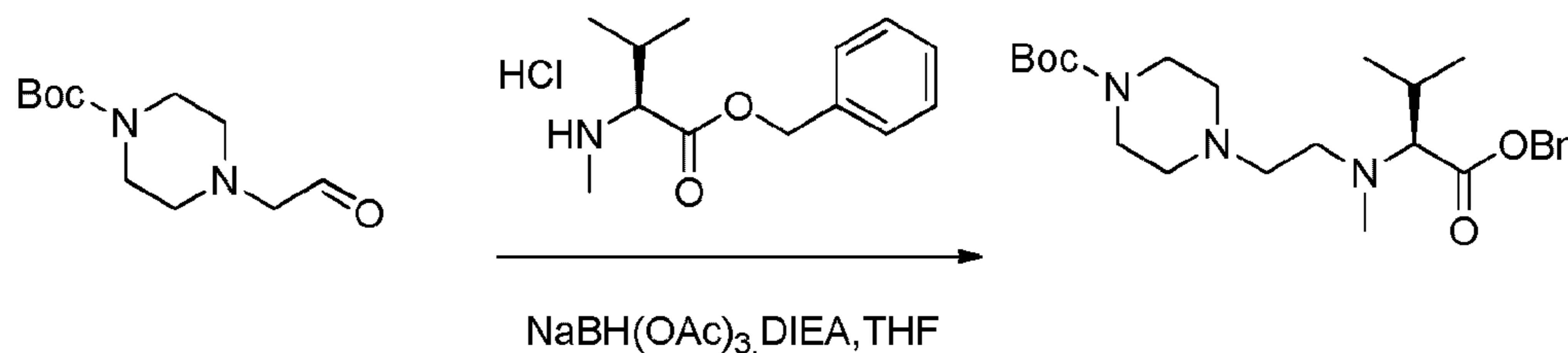
Compound 45B: *tert*-butyl 4-(2-oxoethyl)piperazine-1-carboxylate

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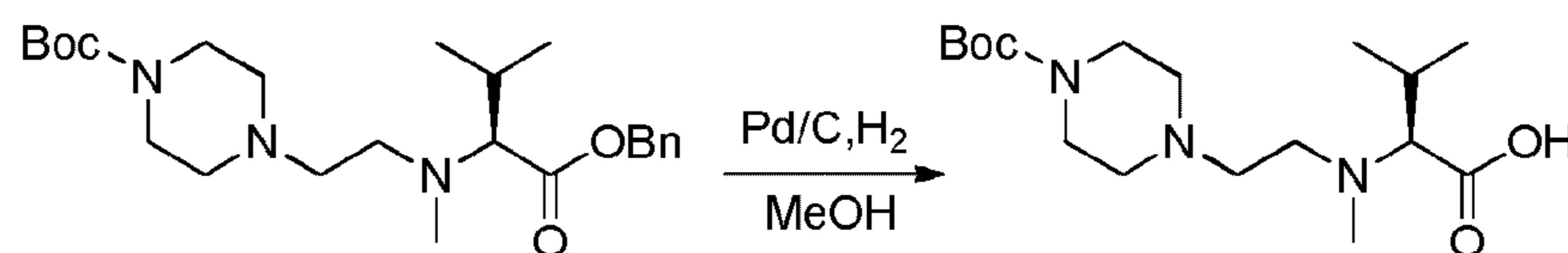
Compound **45B** was prepared in the same manner as for compound **35B**, from compound **45A** (1 g, 4.34 mmol, 1.00 equiv), oxalyl chloride (610 mg, 4.80 mmol, 1.12 equiv), TEA (2.13 g, 21.09 mmol, 4.90 equiv) and DMSO (0.82 g, 2.40 equiv).
 5 Compound **45B** (0.8 g, 81 %) was isolated in the form of a colourless oil.

Compound 45C: *tert*-butyl (S)-4-(2-((1-(benzyloxy)-3-methyl-1-oxobutan-2-yl)(methyl)amino)ethyl)piperazine-1-carboxylate



Compound **45C** was synthesised in the same manner as for compound **14C** from
 10 the amine **1ZC** (720 mg, 2.79 mmol, 0.80 equiv), the aldehyde **45B** (800 mg, 3.50 mmol, 1.00 equiv), NaBH(OAc)₃ (1.6 g, 7.55 mmol, 2.15 equiv) and DIEA (2.5 mL) in THF (50 mL). The reaction mixture was neutralised with 5 mL of water and extracted 3 times with 5 mL of AcOEt. The organic phases were combined, dried over sodium sulfate, filtered and concentrated. The residue was purified on silica gel
 15 (EtOAc/PE (3:1) to yield 400 mg (33 %) of compound **45C** in the form of a colourless oil.

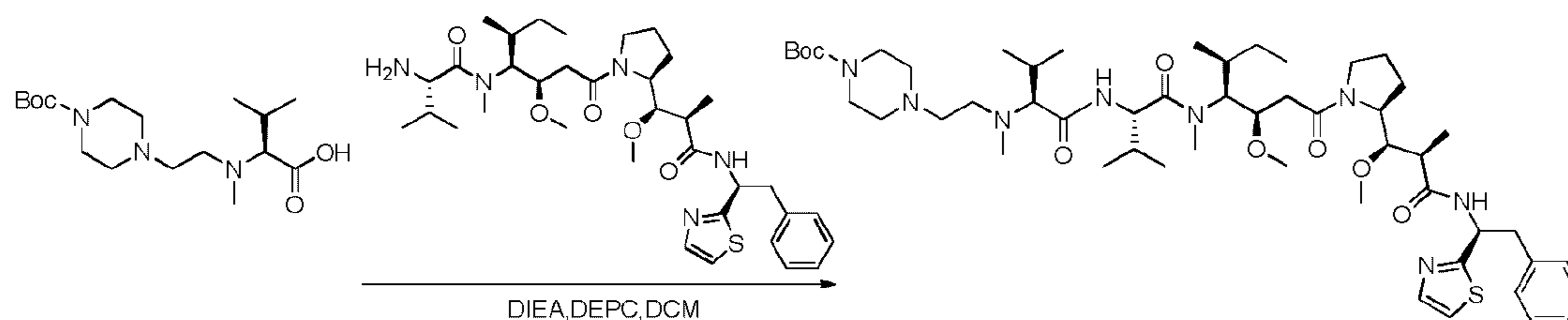
Compound 45D: (S)-2-((2-(4-(*tert*-butoxycarbonyl)piperazin-1-yl)ethyl)(methyl)amino)-3-methylbutanoic acid



20 Compound **45C** (400 mg, 0.92 mmol, 1.00 equiv) was dissolved in 30 mL of MeOH in the presence of Pd/C (400 mg) and hydrogenated for 1 hour at ambient temperature and atmospheric pressure. The reaction medium was filtered and

concentrated under reduced pressure to yield 300 mg (95 %) of compound **45D** in the form of a white solid.

Compound 45E: *tert*-butyl 4-((3R,4S,7S,10S)-4-((S)-*sec*-butyl)-7,10-diisopropyl-3-(2-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-5,11-dimethyl-6,9-dioxo-2-oxa-5,8,11-triazatridecan-13-yl)piperazine-1-carboxylate



Compound **45E** was synthesised in the same manner as for compound **3** from the amine **1Y** (60 mg, 0.09 mmol, 1.00 equiv), the acid **45D** (62.7 mg, 0.18 mmol, 2.00 equiv), DEPC (0.0278 mL) and DIEA (0.0452 mL) in DCM (3 mL). The crude product (100 mg) was subsequently used as such.

Compound 45: Compound **45** was synthesised in the same manner as for compound **2** from the intermediate **45E** (100 mg, 0.10 mmol, 1.00 equiv). The crude product was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 95 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **45** was obtained with a yield of 19 % (19.4 mg) in the form of a white solid.

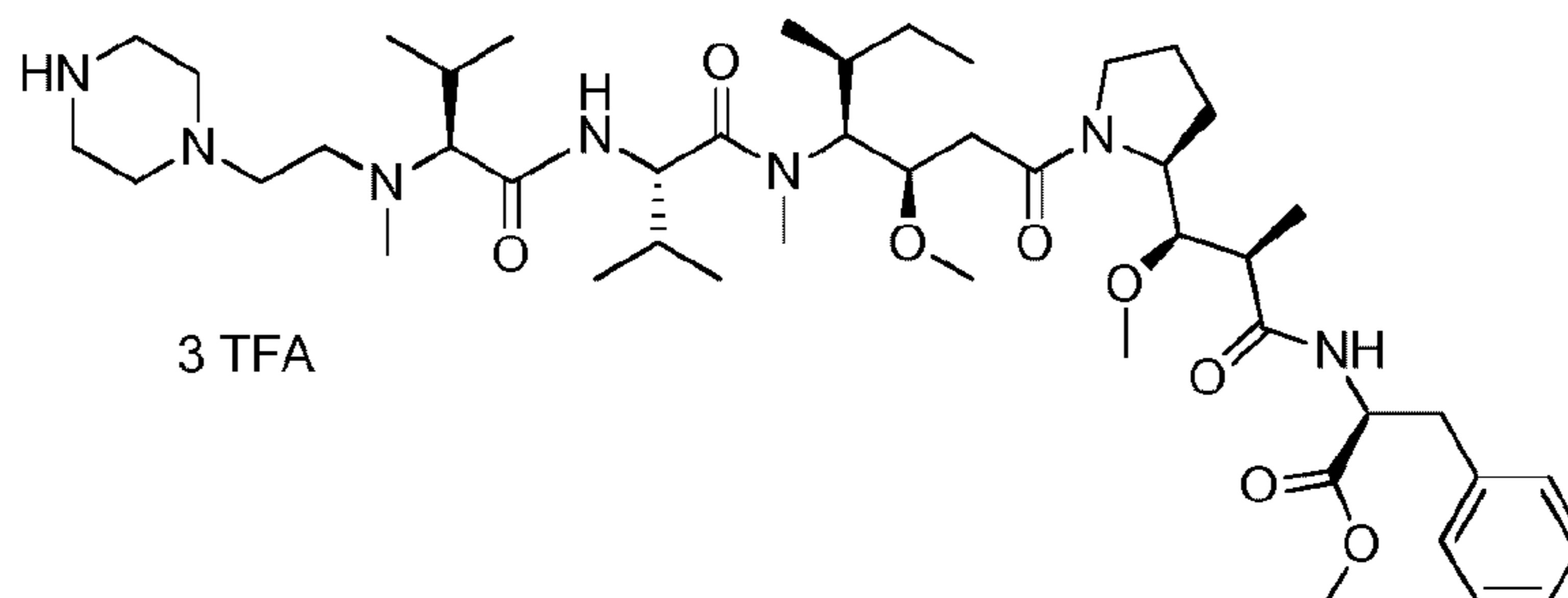
LC/MS/UV (Agilent ZORBAX SB-Aq column, 1.8 μ m, 4.6 x 100 mm; 40°C; 1.0 mL/min, 2 % MeOH in water (0.05 % TFA) for 1 minute then 2 % to 95 % MeOH in water in 13 minutes then 95 % MeOH in water for 2 minutes); ESI ($C_{47}H_{78}N_8O_6S$, exact mass 882.6) m/z : 883.5 (MH^+) and 442.4 ($M.2H^+/2$, 100 %), 10.95 min (98.8 %, 210 nm).

1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers), 7.80 - 7.70 (m, 1H), 7.52 - 7.43 (m, 1H), 7.31 - 7.09 (m, 5H), 5.70 - 5.51 (m, 1H), 4.80 - 4.60 (m, 1H), 4.20 - 0.75 (m, 66H).

Compound 46

methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(2-(piperazin-1-yl)ethyl)amino)butanamido)butanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, tris trifluoroacetic acid

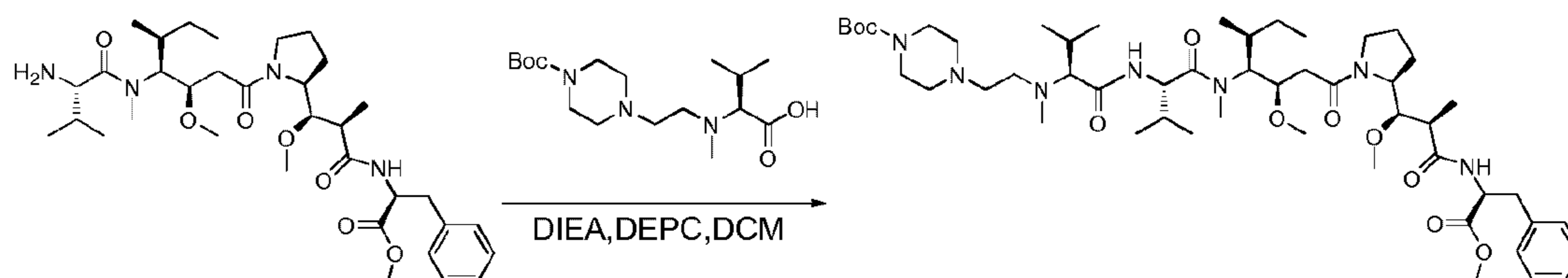
5



Compound 46A: *tert*-butyl 4-((3R,4S,7S,10S)-4-((S)-*sec*-butyl)-7,10-

diisopropyl-3-(2-((S)-2-((1R,2R)-1-methoxy-3-(((S)-1-methoxy-1-oxo-3-phenylpropan-2-yl)amino)-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-2-oxoethyl)-5,11-dimethyl-6,9-dioxo-2-oxa-5,8,11-triazatridecan-13-yl)piperazine-1-carboxylate

10



Compound **46A** was synthesised in the same manner as for compound **3** from the amine **3D** (170 mg, 0.27 mmol, 1.00 equiv) the acid **45D** (184.6 mg, 0.54 mmol, 2.00 equiv), DEPC (0.0819 mL) and DIEA (0.133 mL) in DCM (5 mL). The crude product (200 mg) was subsequently used as such.

Compound 46: Compound **46** was synthesised in the same manner as for compound **2** from the intermediate **46A** (100 mg, 0.10 mmol, 1.00 equiv). The crude product was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 95 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm). Compound **46** was obtained with a yield of 19 % (19.1 mg) in the form of a white solid.

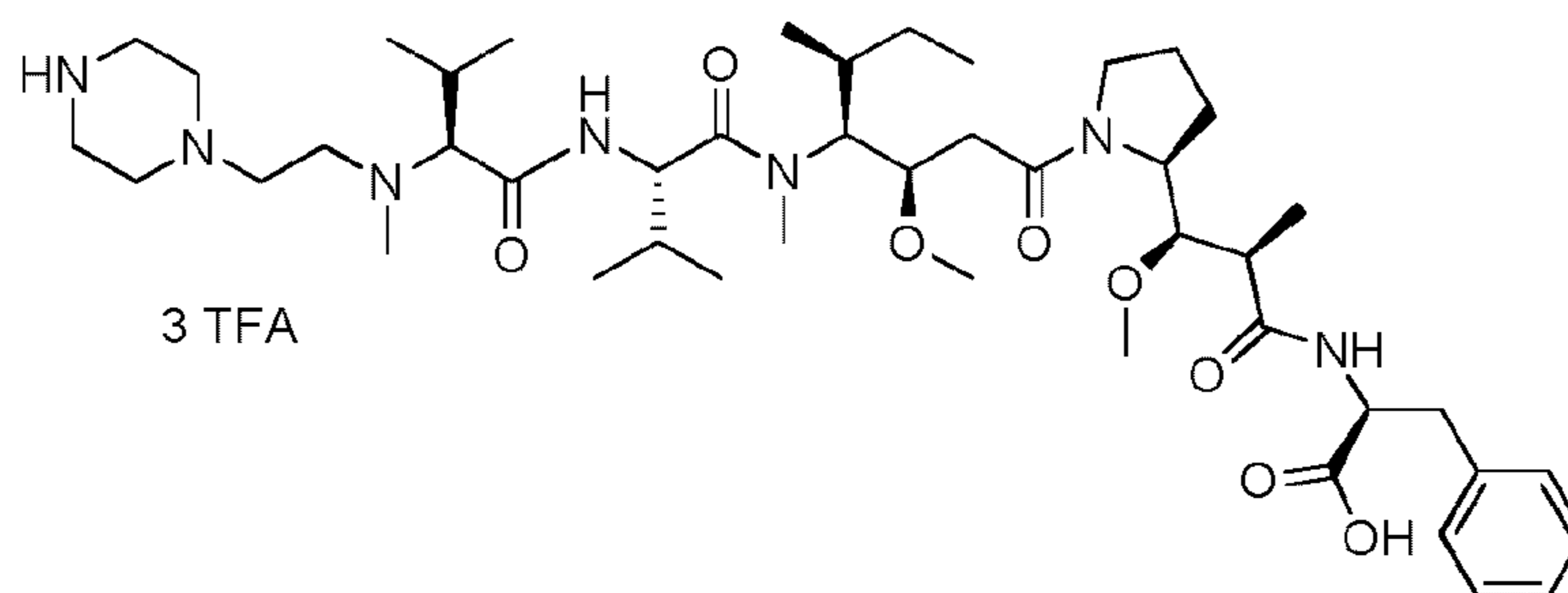
20

LC/MS/UV (Ascentis Express C18 column, 2.7 μm , 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeCN in water (0.05 % TFA) for 8 minutes then 95 % MeCN in water for 2 minutes); ESI ($\text{C}_{46}\text{H}_{79}\text{N}_7\text{O}_8$, exact mass 857.6) m/z : 858.6 (MH^+) and 429.9 ($\text{M}_2\text{H}^+/2$, 100 %), 5.93 min (100 %, 210 nm).

5 ^1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 8.58 - 8.50 (m, 0.5 H, NHCO, incomplete exchange), 8.29 - 8.22 (m, 0.4 H, NHCO, incomplete exchange), 7.35 - 7.15 (m, 5H), 4.87 - 4.69 (m, 3H), 4.22 - 0.82 (m, 68H).

Compound 47

10 **(S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-N,3-dimethyl-2-((S)-3-methyl-2-(methyl(2-(piperazin-1-yl)ethyl)amino)butanamido)butanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoic acid, tris trifluoroacetic acid**



15 Compound 47 was prepared in the same manner as for compound 4, from compound 46 (100 mg, 0.10 mmol, 1.00 equiv). The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, Atlantis Prep OBD T3 column, 5 μm , 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector
20 at 254 nm and 220 nm), to yield 32.6 mg (33 %) of compound 47 in the form of a white solid.

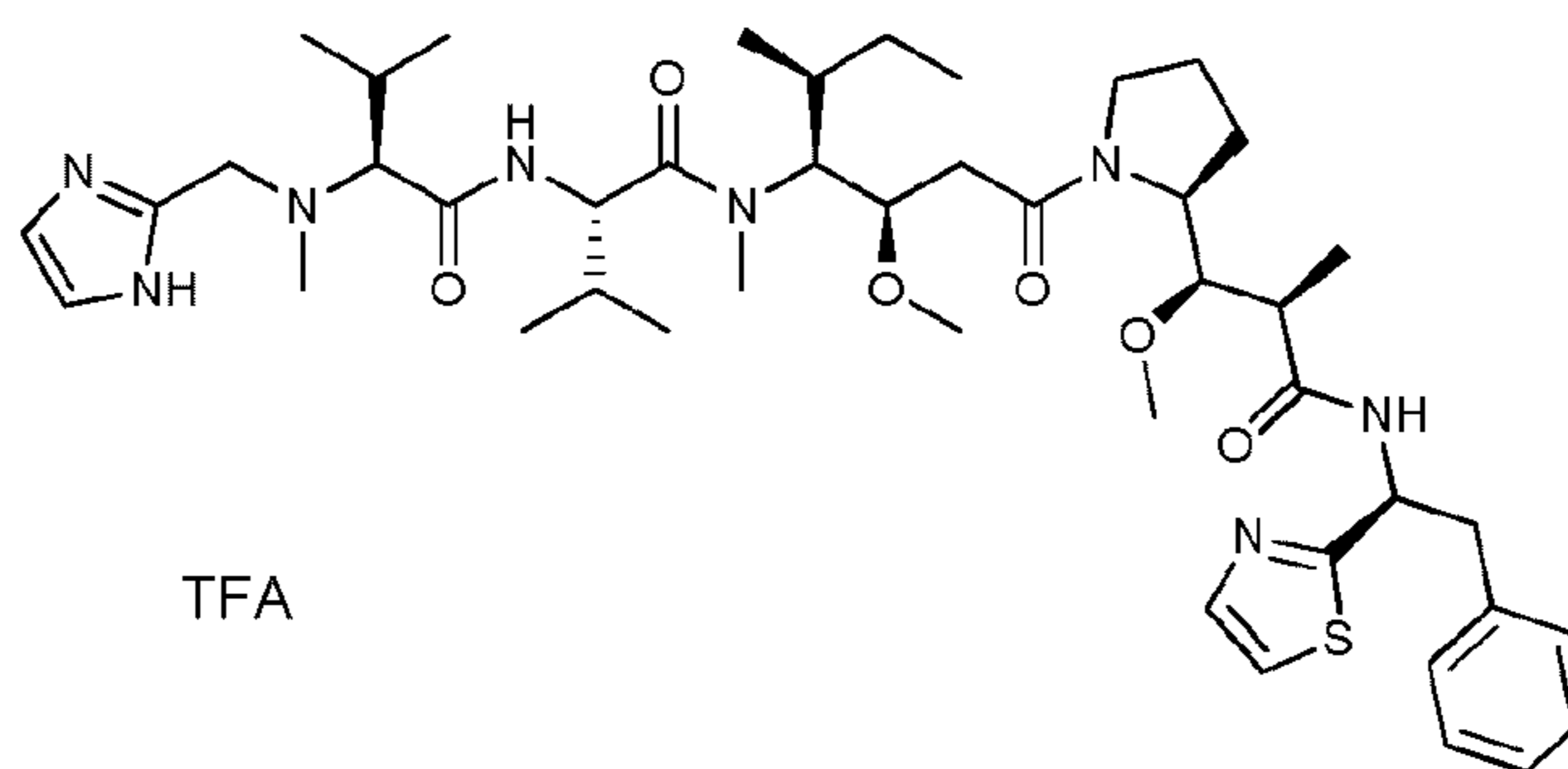
LC/MS/UV (Ascentis Express C18 column, 2.7 μm , 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI ($\text{C}_{46}\text{H}_{77}\text{N}_7\text{O}_8$, exact mass 843.6) m/z : 844.6 (MH^+) and 422.9 ($\text{M}_2\text{H}^+/2$, 100 %), 5.73 min (100 %, 210 nm).
25

^1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 8.66 - 8.57 (m, 0.3 H, NHCO, incomplete exchange), 8.41 - 8.32 (m, 0.3 H, NHCO, incomplete exchange),

8.13 - 8.06 (m, 0.2 H, NHCO, incomplete exchange), 7.30 - 7.10 (m, 5H), 4.80 - 4.61 (m, 3H), 4.19 - 0.78 (m, 65H).

Compound 48

- 5 **(S)-2-((S)-2-(((1H-imidazol-2-yl)methyl)(methyl)amino)-3-methylbutanamido)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide, trifluoroacetic acid**



- 10 Compound **48** was prepared in the same manner as for compound **1**, from the amines **1Y** and **1ZC** and *1H*-imidazole-2-carbaldehyde. The end product was purified by preparative HPLC under the following conditions: SunFire Prep C18 OBD column, 5 μm , 19x150 mm, mobile phases buffered with 0.05 % TFA, gradient of 15.0 to 30 % ACN in water in 10 minutes then up to 95.0 % ACN in 2 minutes, UV Detection UV
15 220 nm.

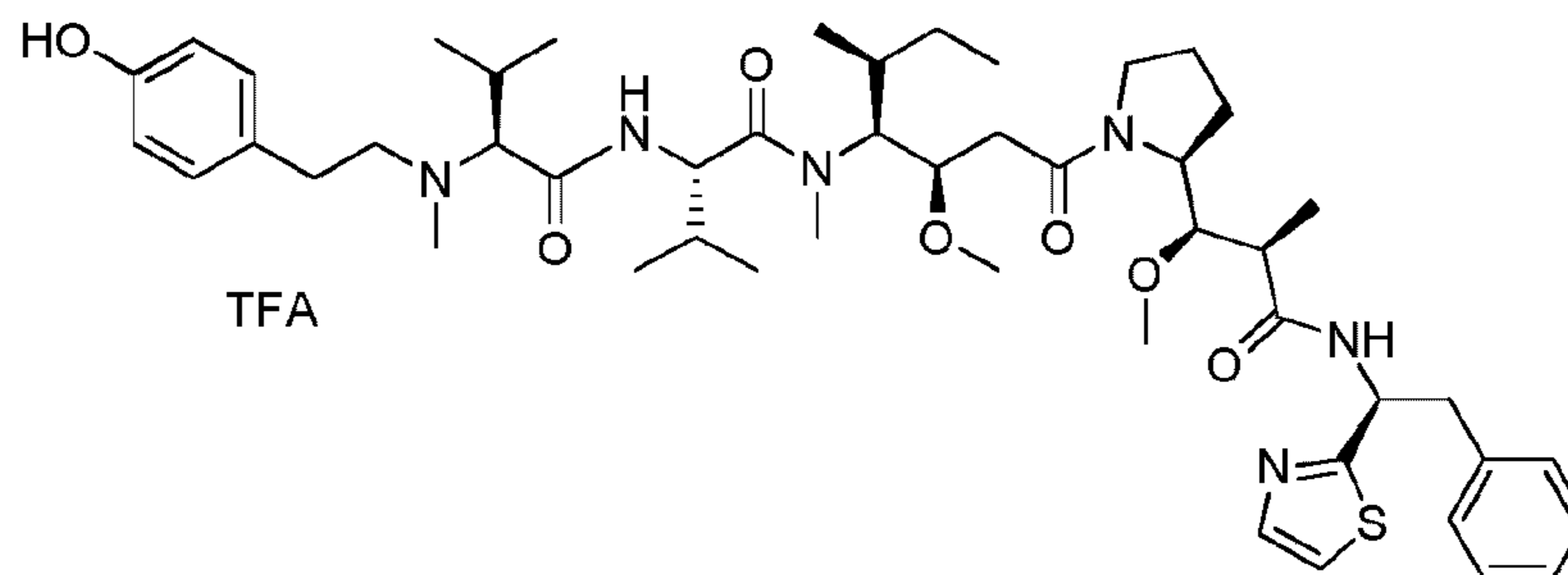
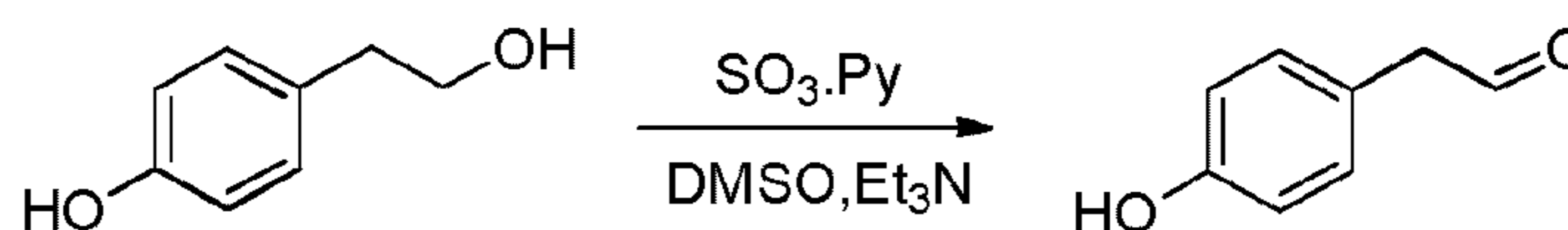
LC/MS/UV (Zorbax Eclipse Plus C8, 1.8 μm , 4.6 x 100 mm; 1 mL/min, 40°C, 2 % methanol in water (eluting phases buffered with 0.05 % TFA) for 1 minute, then 2 % to 95 % methanol for 12 minutes; ESI ($\text{C}_{45}\text{H}_{70}\text{N}_8\text{O}_6\text{S}$, exact mass 850.51) m/z : 851.2 (MH^+), 873.5 (MNa^+), 426.3 ($\text{M.2H}^+/2$); 12.75 min (90.5 %, 210 nm).

- 20 ^1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 7.83 - 7.81 (m, 1H), 7.80 - 7.53 (m, 3H), 7.53 - 7.22 (m, 5H), 5.6 - 5.8 (m, 1H), 5.0 - 4.6 (m, 2H); 4.6 - 0.85 (m, 55H).

Compound 49

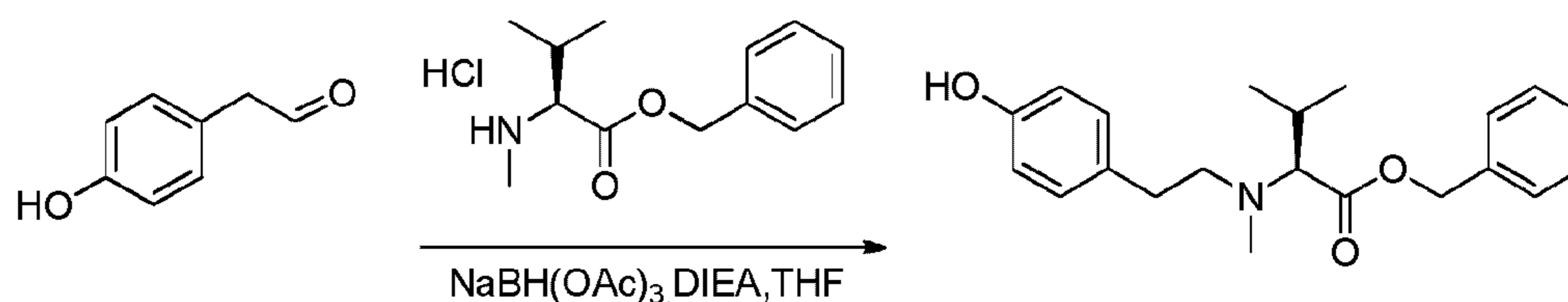
(S)-2-((S)-2-((4-hydroxyphenethyl)(methyl)amino)-3-methylbutanamido)-N-
 ((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-
 phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-
 oxoheptan-4-yl)-N,3-dimethylbutanamide, trifluoroacetic acid

5

**Compound 49A: 2-(4-hydroxyphenyl)acetaldehyde**

10 4-(2-hydroxyethyl)phenol (4 g, 28.95 mmol, 1.00 equiv) was dissolved in
 DMSO (32 mL) and TEA (8.8 mL, 2.20 equiv) was then added dropwise. A solution of
 SO₃.Py (10 g, 2.20 equiv) in DMSO (36 mL) was added and the mixture was left under
 agitation overnight at ambient temperature. The reaction mixture was neutralised with
 250 mL of water and extracted 3 times with 100 mL of AcOEt. The organic phases were
 15 combined, washed 5 times with water (100 mL) then twice with 150 mL of NaCl (sat.),
 dried over sodium sulfate, filtered and concentrated. The residue was purified on silica
 gel (EtOAc/PE (1:10) to yield 1 g (25 %) of compound **49A** in the form of a colourless
 oil.

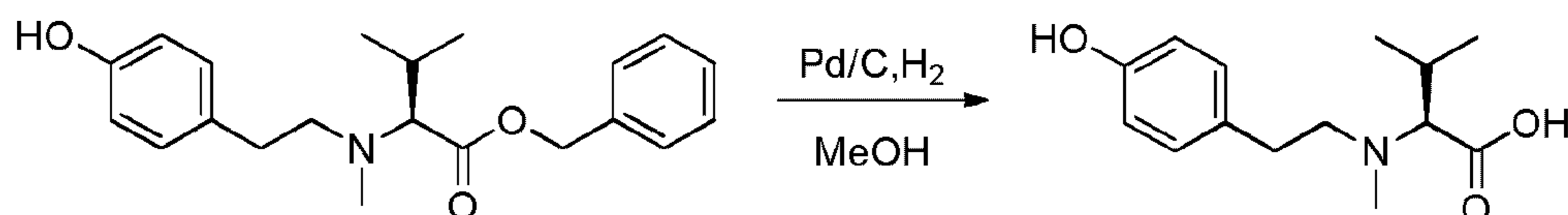
20 **Compound 49B:** benzyl (S)-2-((4-hydroxyphenethyl)(methyl)amino)-3-
 methylbutanoate



Compound **49B** was synthesised in the same manner as for compound **14C** from
 the amine **1ZC** (1.5 g, 5.82 mmol, 0.99 equiv), the aldehyde **49A** (800 mg, 5.88 mmol,

1.00 equiv), NaBH(OAc)₃ (2.7 g, 12.74 mmol, 2.17 equiv) and DIEA (4.23 mL) in THF (25 mL). The reaction mixture was neutralised with 50 mL of water and extracted 3 times with 50 mL of AcOEt. The organic phases were combined, dried over sodium sulfate, filtered and concentrated. The residue was purified on silica gel (EtOAc/PE
5 (1:10) to yield 600 mg (37 %) of compound **49B** in the form of a white solid.

Compound 49C: (S)-2-((4-hydroxyphenethyl)(methyl)amino)-3-methylbutanoic acid



Compound **49B** (0.5 g, 1.46 mmol, 1.00 equiv) was dissolved in 40 mL of
10 MeOH in the presence of Pd/C (250 mg) and hydrogenated for 3 hours at ambient temperature and atmospheric pressure. The reaction medium was filtered and concentrated under reduced pressure to yield 0.4 g of compound **49C** in the form of a white solid.

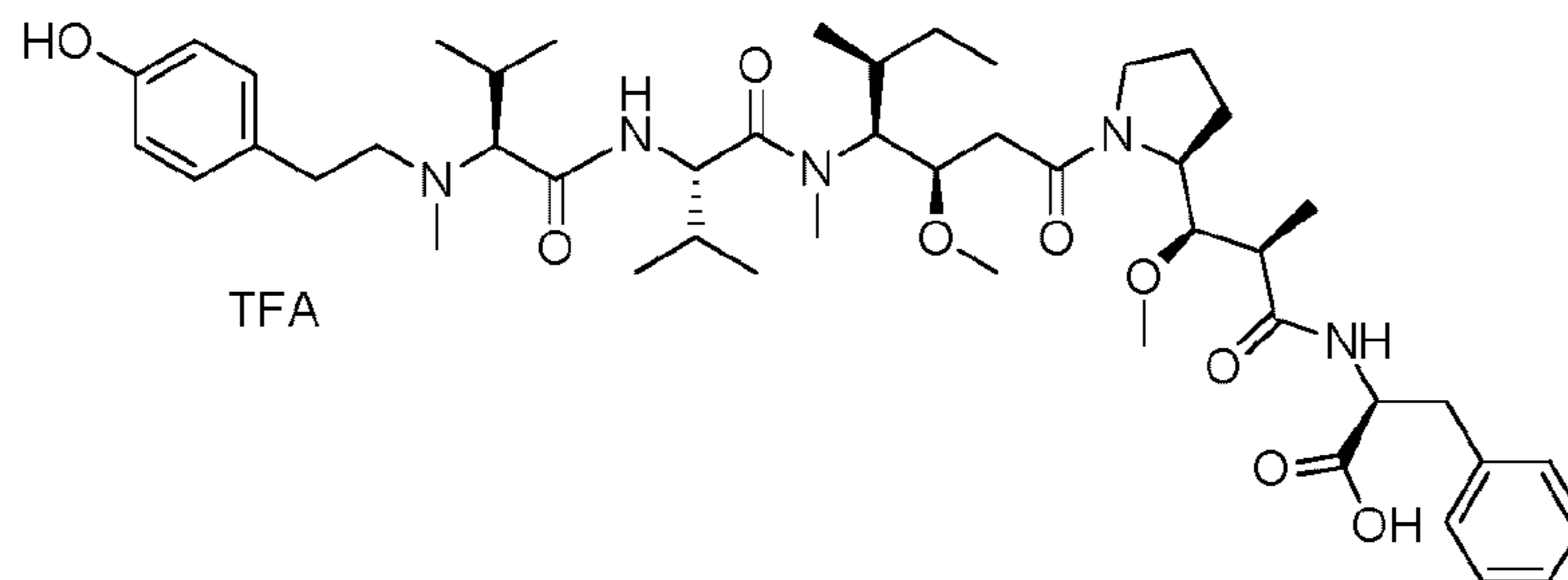
Compound 49: Compound **49** was synthesised in the same manner as for
15 compound **3** from the amine **1Y** (53.4 mg, 0.08 mmol, 2.00 equiv), the acid **49C** (70 mg, 0.28 mmol, 1.00 equiv), DEPC (0.032 mL, 2.00 equiv) and DIEA (0.053 mL, 3.00 equiv) in DCM (3 mL). The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, Atlantis Prep OBD T3 column, 5 μm, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 45 % ACN in 10 minutes then
20 45 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm), to yield 3 mg (1 %) of compound **49** in the form of a white solid.

LC/MS/UV (Ascentis Express C18 column, 2.7 μm, 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI (C₄₉H₇₄N₆O₇S, exact mass 890.5) *m/z*: 891.5 (MH⁺) and 446.4 (M.2H⁺/2, 100 %), 6.69 min (100 %, 25 210 nm).

¹H NMR: (400MHz, CD₃OD, ppm): δ (Presence of rotamers) 8.92 - 8.87 (m, 0.5 H, NHCO, incomplete exchange), 8.70 - 8.63 (m, 0.4 H, NHCO, incomplete exchange), 8.85 - 8.77 (m, 1H), 7.59 - 7.51 (m, 1H), 7.35 - 7.03 (m, 7H), 6.82 - 6.71 (m, 2H), 5.77 - 5.58 (m, 1H), 5.81 - 5.70 (m, 1H), 4.21 - 0.80 (m, 58H).

Compound 50

(S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((4-hydroxyphenethyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoic acid, trifluoroacetic acid



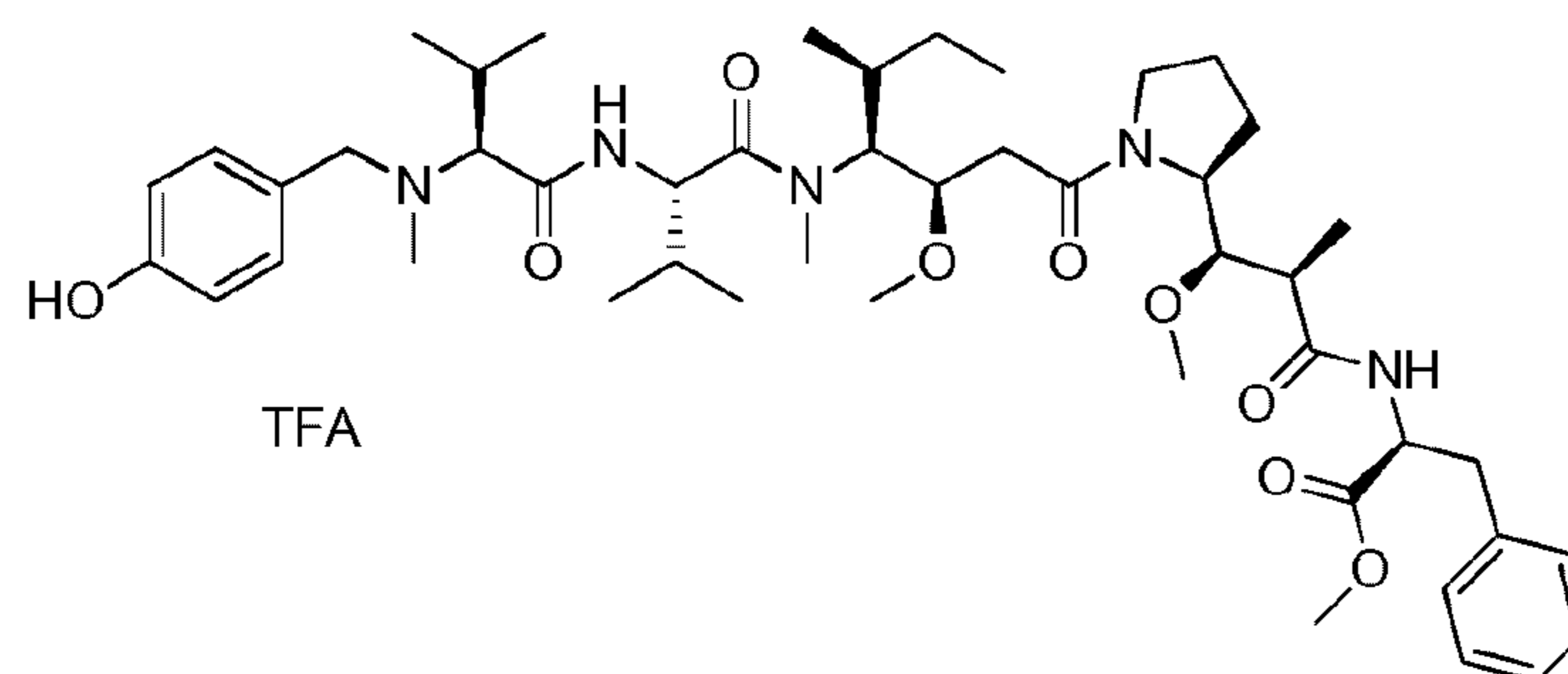
Compound **50** was prepared in the same manner as for compound **4**, from compound **27** (100 mg, 0.10 mmol, 1.00 equiv). The residue was purified by preparative HPLC (Pre-HPLC-001 SHIMADZU, Atlantis Prep OBD T3 column, 5 μ m, 19 x 150 mm; Eluting phase: water / ACN buffered with 0.05 % TFA; Gradient of 20 % to 40 % ACN in 10 minutes then 40 % to 100 % ACN in 2 minutes; Waters 2545 UV Detector at 254 nm and 220 nm), to yield 10.7 mg (11 %) of compound **50** in the form of a white solid.

LC/MS/UV (Ascentis Express C18 column, 2.7 μ m, 4.6 x 100 mm; 40°C; 1.5 mL/min, 10 % to 95 % MeOH in water (0.05 % TFA) in 8 minutes); ESI ($C_{47}H_{73}N_5O_9$, exact mass 851.5) m/z : 852.5 (MH^+) and 426.8 ($M.2H^+/2$, 100 %), 6.46 min (91.7 %, 210 nm).

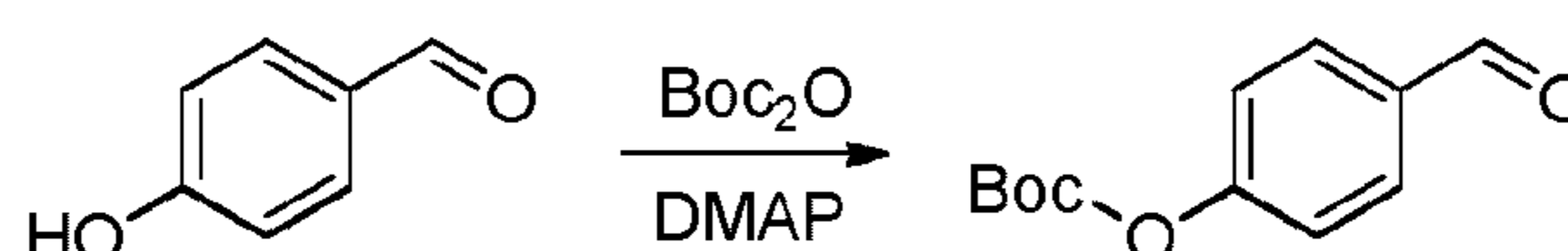
1H NMR: (400MHz, CD_3OD , ppm): δ (Presence of rotamers) 7.34 - 7.15 (m, 5H); 7.15 - 7.04 (se, 2H), 6.82 - 6.83 (m, 2H), 4.83 - 4.70 (m, 1H), 4.21 - 4.00 (m, 1H), 3.90 - 3.80 (m, 1H), 3.74 - 3.62 (m, 1H), 3.57 - 2.86 (m, 20H), 2.56 - 0.80 (m, 36H).

Compound 51

methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((4-hydroxybenzyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate, trifluoroacetic acid

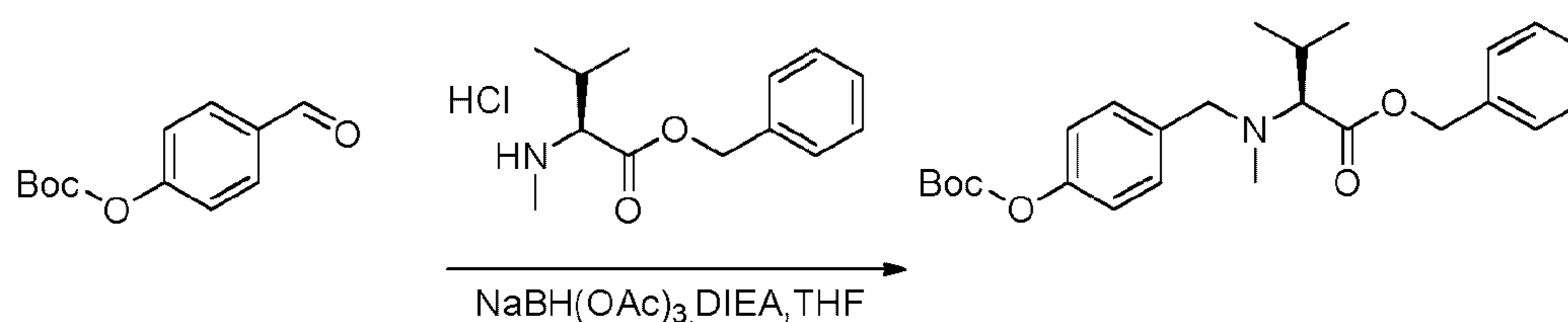


Compound 51A: *tert*-butyl (4-formylphenyl)carbonate



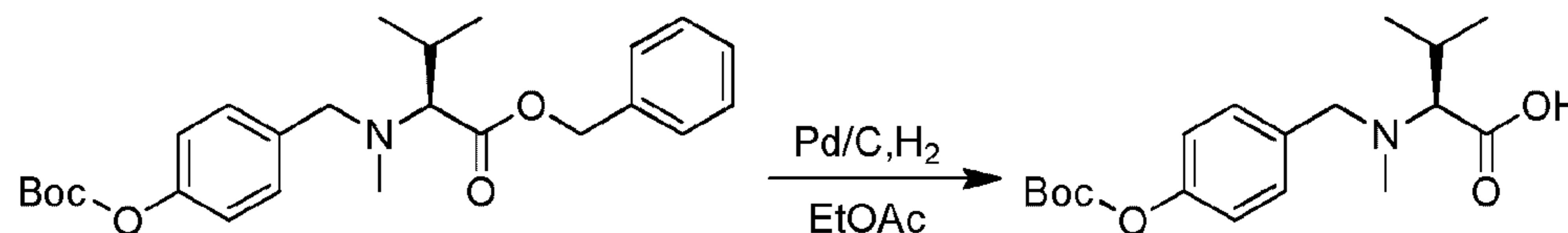
4-hydroxybenzaldehyde (3.0 g, 24 mmol) was dissolved in 30 mL of DCM in the presence of 4-DMAP (300 mg, 2.46 mmol, 0.1 equiv.) and di-*tert*-butyl dicarbonate (5.35 g, 24 mmol, 1.0 equiv.) and agitated 1 hour at ambient temperature. The solution was then diluted with 200 mL of water and extracted 3 times with 100 mL of DCM. The organic phases were combined, dried over sodium sulfate, filtered and concentrated under reduced pressure to yield 5 g (92 %) of compound **51A** in the form of a white solid.

Compound 51B: benzyl (S)-2-((4-((*tert*-butoxycarbonyl)oxy)benzyl)(methyl)amino)-3-methylbutanoate



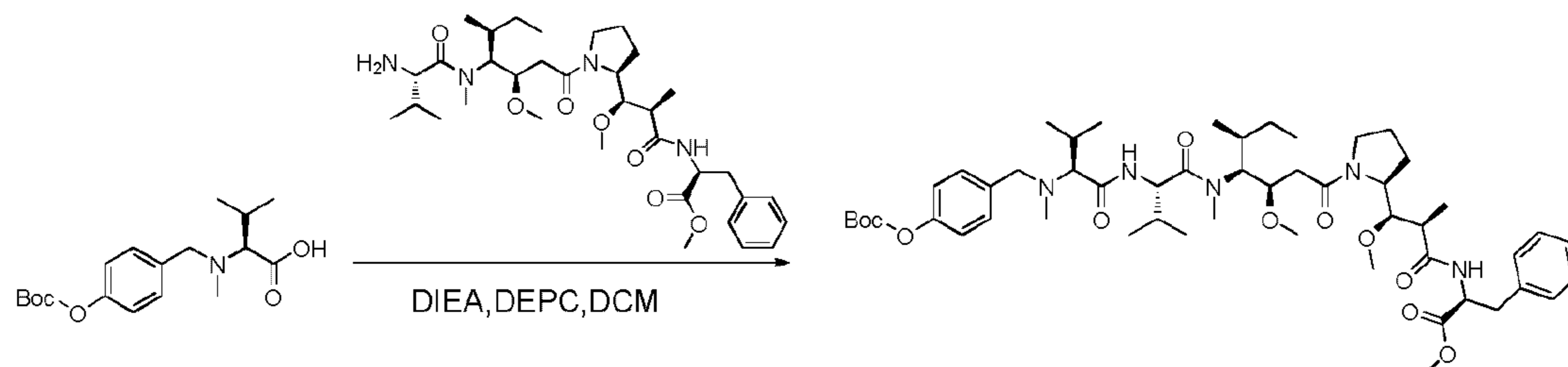
Compound **51A** (220 mg, 0.99 mmol) was dissolved in 5 mL of THF in the presence of compound **1ZC** (255 mg, 0.99 mmol, 1.0 equiv.), NaBH(OAc)₃ (420 mg, 2 mmol, 2.0 equiv.) and DIEA (654 μl) and agitated overnight at ambient temperature. The solution was then diluted with 100 mL of water and extracted 3 times with 50 mL of EtOAc. The organic phases were combined, dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified on a silica column with a mixture of EtOAc and PE (1:100) to yield 200 mg (47 %) of compound **51B** in the form of a white solid.

Compound 51C: (S)-2-((4-((*tert*-butoxycarbonyl)oxy)benzyl)(methyl)amino)-3-methyl butanoic acid



Compound **51C** was prepared by hydrogenation of compound **51B** (200 mg),
5 following the protocol used for the preparation of compound **3F**.

Compound 51D: methyl (S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((4-((*tert*-butoxycarbonyl)oxy)benzyl)(methyl)amino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanoate



10

Compound **51D** was prepared by coupling compound **51C** with amine **3D**,
following the protocol used for the preparation of compound **3** to obtain the desired
product in the form of yellow oil with a yield of 60 %.

Compound 51: Compound **51D** (80 mg, 0.08 mmol) was dissolved in 1 mL of
15 DCM in the presence of 0.5 mL TFA, agitated 2 hours at ambient temperature and then
concentrated under reduced pressure. The residue was purified by preparative HPLC
(Pre-HPLC-010, SunFire Prep C18 OBD column, 5 μ m, 19 x 150 mm; Eluting phase:
water / ACN buffered with 0.05 % TFA; Gradient of 23 % to 40 % ACN in 10 minutes
then 40 % to 95 % ACN in 2 minutes; Waters 2489 UV Detector at 254 nm and 220
20 nm). Compound **51** was obtained with a yield of 24 % (20 mg) in the form of a white
solid.

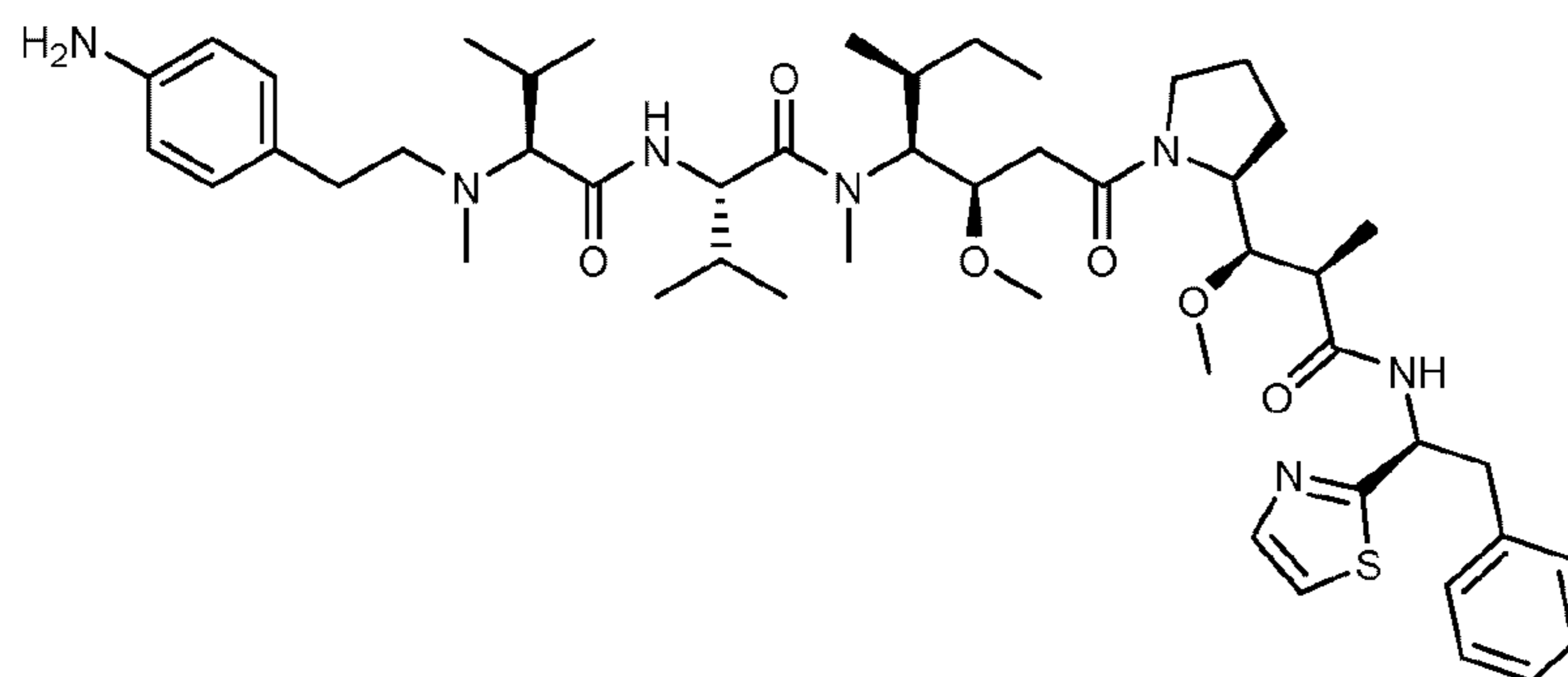
LC/MS/UV (Zorbax SB-Aq, 1.8 μ m, 4.6 x 100 mm; 2 % MeOH in water
(0.05 % TFA) for 1 minute then 2 % to 95 % MeOH in 13 minutes); ESI ($C_{47}H_{73}N_5O_9$,
exact mass 851.54) m/z : 874.5 (MNa^+), 426.9 ($M.2H^+/2$); 12.48 min (96 %, 210 nm).

¹H NMR: (300MHz, CD₃OD, ppm): δ (Presence of rotamers) 8.1 - 8.6 (m, 0.9H, NHCO incomplete exchange); 7.29 - 7.27 (m, 2H), 7.25 - 6.86 (m, 5H), 6.84 - 6.83 (m, 2H), 4.83 - 4.72 (m, 3H), 4.26 - 0.82 (m, 58H).

5

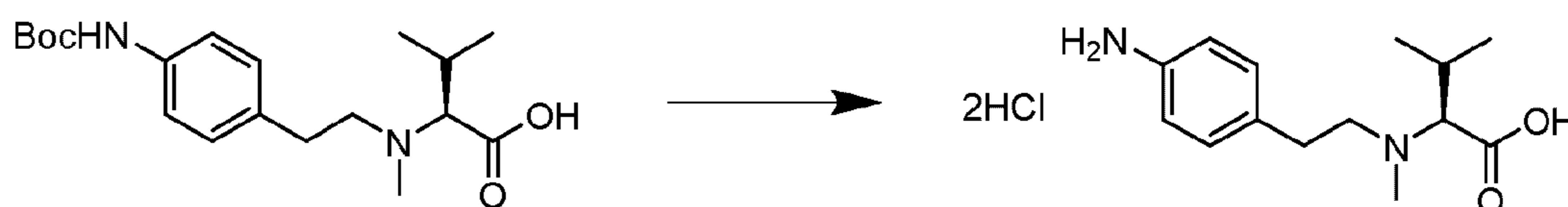
Compound 61

(S)-2-((S)-2-((4-aminophenethyl)(methyl)amino)-3-methylbutanamido)-N-((3R,4S,5S)-3-methoxy-1-((S)-2-((1R,2R)-1-methoxy-2-methyl-3-oxo-3-(((S)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-5-methyl-1-oxoheptan-4-yl)-N,3-dimethylbutanamide



10

Compound 61A: *N*-(4-aminophenethyl)-*N*-methyl-*L*-valine dihydrochloride



15

Compound **11D** (962 mg, 2.75 mmol) was dissolved in 10 ml of a commercially available solution of HCl in propan-2-ol (5 - 6 M), and stirred at room temperature for 2 hours. TLC analysis indicated complete consumption of starting material. The solvent was evaporated under reduced pressure, and the resulting yellow solid triturated with Et₂O (2 x 10 ml). The product was dried under vacuum to furnish compound **61A** as a yellow solid (322 mg, 47 %).

20

Compound 61: Carboxylic acid **61A** (73 mg, 0.23 mmol, 1 eq.) and amine **1Y** (150 mg, 0.23 mmol, 1 eq.) were dissolved in dry DMF (2 ml). DIEA (158 μl, 0.90 mmol, 4 eq.) and DECP (51 μl, 0.34 mmol, 1.5 eq.) were added and the reaction stirred for 4 hours at room temperature. Analysis by LC-MS showed complete consumption of the starting material. The solvent was evaporated under reduced pressure, and the

residue purified by flash chromatography on silica gel (DCM/MeOH) to furnish compound **61** as a light yellow solid (83 mg, 40 %).

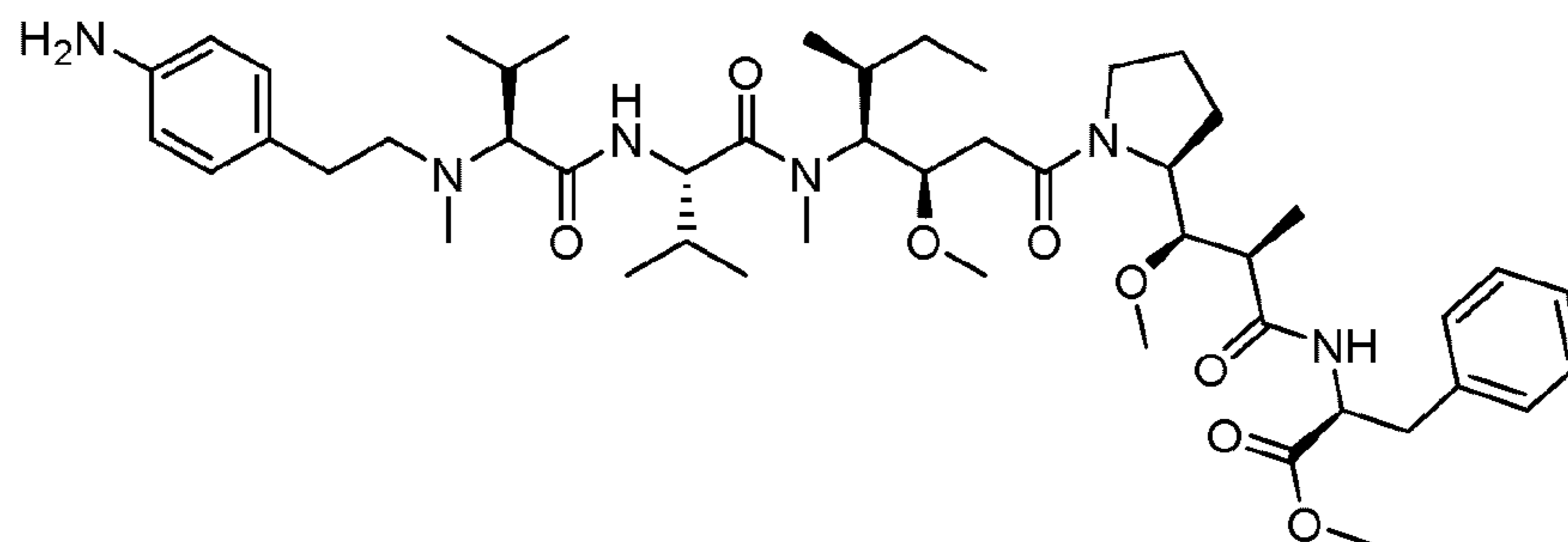
¹H NMR: (500MHz, DMSO-*d*₆, ppm): δ (Presence of rotamers), 8.86 (d, 0.5H, NHCO); 8.65 (d, 0.5H, NHCO), 8.11-8.05 (m, 1H, NHCO), 7.80 (d, 0.5H, thiazole),
 5 7.78 (d, 0.5H, thiazole), 7.65 (d, 0.5H, thiazole), 7.63 (d, 0.5H, thiazole), 7.32 – 7.12 (m, 5H), 6.83 (d, *J*=8.3 Hz, 2H), 6.45 (d, *J*=8.3 Hz, 2H), 5.56 – 5.49 (m, 0.5 H), 5.42 – 5.35 (m, 0.5H), 4.78 (s, 2H, NH₂), 4.74 – 4.46 (m, 2H), 4.01 – 0.66 (m, 57H).

HPLC (Xbridge Shield C18, 3.5 μm, 4.6 x 50 mm ; 3.5 ml/min, 40°C, 0 to 95 % MeCN in water (0.1 % TFA) in 2.25 minutes then 95 % MeCN for 0.5 minutes, Tr =
 10 1.31 min (96.5 %, 220 nm).

m/z (Q-TOF ESI⁺) 890.5558 (2%, MH⁺, C₄₉H₇₆N₇O₆S requires 890.5572), 445.7834 (100 %, (MH₂)²⁺, C₄₉H₇₇N₇O₆S requires 445.7823).

Compound 62

15 **Methyl ((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((4-aminophenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalaninate**



20 Compound **62** was prepared in the same manner as for compound **61**, using carboxylic acid **61A** (69 mg, 0.21 mmol, 1 eq.), amine **3D** (135 mg, 0.21 mmol, 1 eq.), DIEA (75 μl, 0.43 mmol, 2 eq.) and DECP (49 μl, 0.32 mmol, 1.5 eq.). The crude product was purified by flash chromatography on silica gel (DCM/MeOH) to furnish compound **62** as a yellowish solid (82 mg, 45 %).

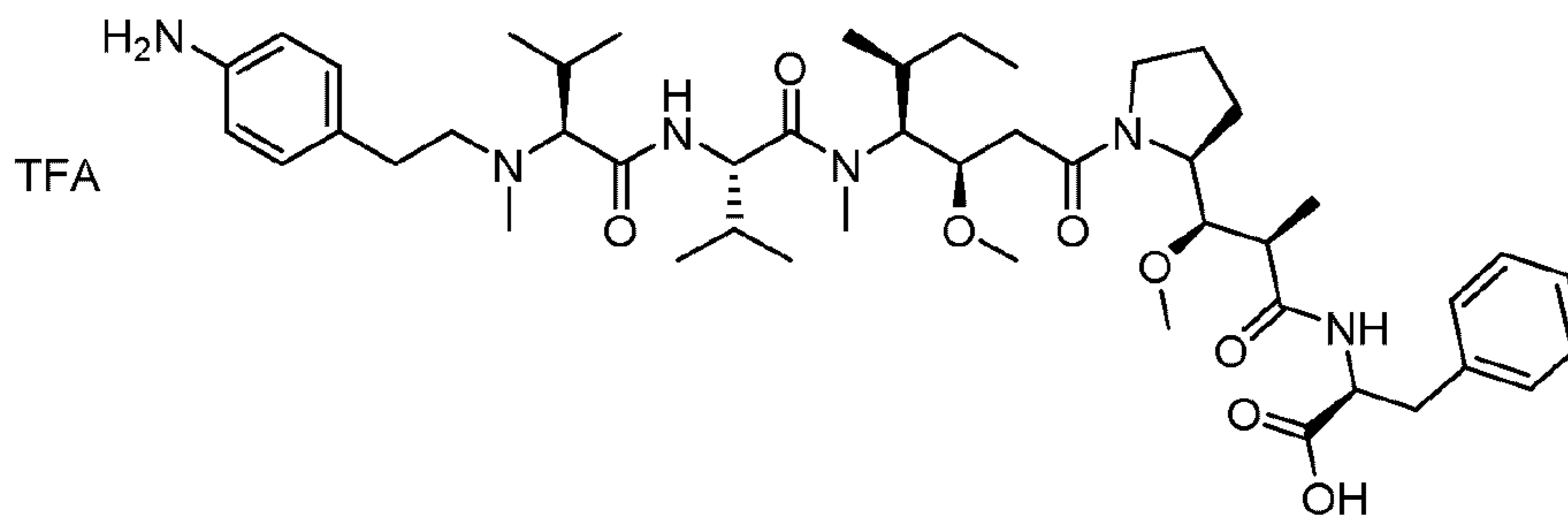
¹H NMR: (500MHz, DMSO-*d*₆, ppm): δ (Presence of rotamers), 8.50 (d, *J*=8.3, 0.5H, NHCO); 8.27 (d, *J*=8.0, 0.5H, NHCO), 8.15-8.04 (m, 1H, NHCO), 7.27 – 7.13 (m, 5H), 6.86 – 6.79 (m, 2H), 6.48 – 6.42 (m, 2H), 4.78 (s, 2H, NH₂), 4.74 – 4.44 (m, 3H), 4.01 – 3.72 (m, 1.5H), 3.66 (s, 1.5H, CO₂Me), 3.63 (s, 1.5H, CO₂Me), 3.57 - 0.65 (m, 55.5H).

HPLC (Xbridge Shield C18, 3.5 μm, 4.6 x 50 mm ; 3.5 ml/min, 40°C, 0 to 95 % MeCN in water (0.1 % TFA) in 2.25 minutes then 95 % MeCN for 0.5 minutes, Tr = 1.29 min (95.3 %, 220 nm).

m/z (Q-TOF ESI⁺) 865.5800 (2%, MH⁺, C₄₈H₇₇N₆O₈ requires 865.5797),
433.2937 (100 %, (MH₂)²⁺, C₄₈H₇₈N₆O₈ requires 433.2935).

Compound 63

((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-((4-aminophenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalanine 2,2,2-trifluoroacetate



Compound **62** (23 mg, 0.03 mmol) was dissolved in a mixture of water (1 ml) and acetonitrile (1 ml). Piperidine (0.75 ml) was added and the mixture stirred at room temperature for 5 hours. TLC analysis indicated complete consumption of the starting material. The solvent was evaporated under reduced pressure, and the residue purified by preparative HPLC (SunFire Prep column C18 OBD, 5 μm, 19 x 150 mm; Mobile phase: water/MeCN buffered with 0.1 % TFA; Gradient of 20 % to 40 % MeCN in 10 minutes, then from 40 % to 100 % MeCN in 2 minutes; Detector UV Waters 2545 at 254 nm et 220 nm). Compound **63** was obtained as a white solid (14 mg, 66 %).

¹H NMR: (500MHz, DMSO-*d*₆, ppm): δ (Presence of rotamers), 12.7 (s(br), 1H, CO₂H), 9.58 (m(br), 1H); 9.04 – 8.89 (m, 1H), 8.41 (d, 0.6H, NHCO), 8.15 (d, 0.4H, NHCO), 7.27 – 7.13 (m, 5H), 7.13 – 6.99 (m(br), 2H), 6.90 – 6.64 (s(br), 2H), 4.77 – 3.40 (m, 10H), 3.34 – 2.75 (m, 20H), 2.34 – 1.94 (m, 4H), 1.90 – 0.7 (m, 25H).

5 HPLC (Xbridge Shield C18, 3.5 μm, 4.6 x 50 mm ; 3.5 ml/min, 40°C, 0 to 95 % MeCN in water (0.1 % TFA) in 2.25 minutes then 95 % MeCN for 0.5 minutes, Tr = 1.24 min (100 %, 220 nm).

m/z (Q-TOF ESI⁺) 851.5641 (6%, MH⁺, C₄₇H₇₅N₆O₈ requires 851.5641), 426.2854 (100 %, (MH₂)²⁺, C₄₇H₇₆N₆O₈ requires 426.2857).

Example 20: Antiproliferative activity of the Drugs

10 **Method:**

Cell culture. A549 (Non Small Cell Lung Cancer - ATCC CCL-185) and MDA-MB-231 (breast adenocarcinoma – ATCC HTB-26) cells were cultured in Minimum Essential Medium Eagle (MEM) with 5% fetal calf serum (FCS) and Dulbecco's modified Eagle Medium (DMEM) with 10% FCS respectively. MCF7 (breast ductal carcinoma – ATCC HTB-22) and SN-12C (kidney carcinoma – ATCC) cells were
15 maintained in RPMI1640 medium (without phenol red for MCF7 cells) containing 10% FCS. All the media were supplemented with fungizone (1.25 μg/mL) and penicillin-streptomycin (100 U / 100 μg/mL). Cells were cultured under standard conditions in an incubator at 37°C, 5% CO₂ and 95% atmospheric humidity.

20 **Antiproliferative activity on 4 tumor cell lines.** Selected drugs were investigated for their antiproliferative activity using an ATPlite proliferation assay (Perkin Elmer, Villebon sur Yvette, France) on a comprehensive panel of 4 cell lines. Cells were seeded in 96 well plates (10³ cells/well for A549, 2.10³ for MCF7, MDA-MB-231 and SN12C) at day 0 at a concentration to ensure cells remained in logarithmic
25 cell growth phase throughout the 72 h drug treatment period. After a 24h incubation period, all the cells were treated with serial dilutions of the tested compounds (11μL of a 10X solution in 1% DMSO – 6 wells/ condition). To avoid adherence of the compounds onto the tips, tips were changed between two consecutive dilutions. Cells were then placed in 37°C, 5% CO₂ incubator. On day 4, cell viability was evaluated by
30 dosing the ATP released by viable cells. The number of viable cells was analyzed in

comparison with the number of solvent treated cells. The EC₅₀ values were determined with curve fitting analysis (non linear regression model with a sigmoidal dose response, variable hill slope coefficient), performed with the algorithm provided by the GraphPad Software (GraphPad Software Inc., CA, USA).

5

Results:

Various drugs:

Various drugs were tested to determine their antiproliferative activity on the MDA-MB-231 cell line following the above-described method. The measured activities gave values of EC₅₀ < 0.1 μM.

10

The few following examples chosen from among the above exemplified drugs illustrate their fully remarkable antiproliferative properties:

Example 12: EC₅₀ = 5.80x10⁻¹⁰ M; Example 13: EC₅₀ = 7.95x10⁻⁸ M; Example 15: EC₅₀ = 1.70x10⁻¹⁰ M; Example 27: EC₅₀ = 1.20x10⁻¹⁰ M.

15

Various cell lines:

Compound 15 was tested on different cell lines (A549, MDA-MB-231, MCF-7, SN12C) following the above-described method. The measured activities gave values of EC₅₀ < 0.1 μM on all the tested cell lines.

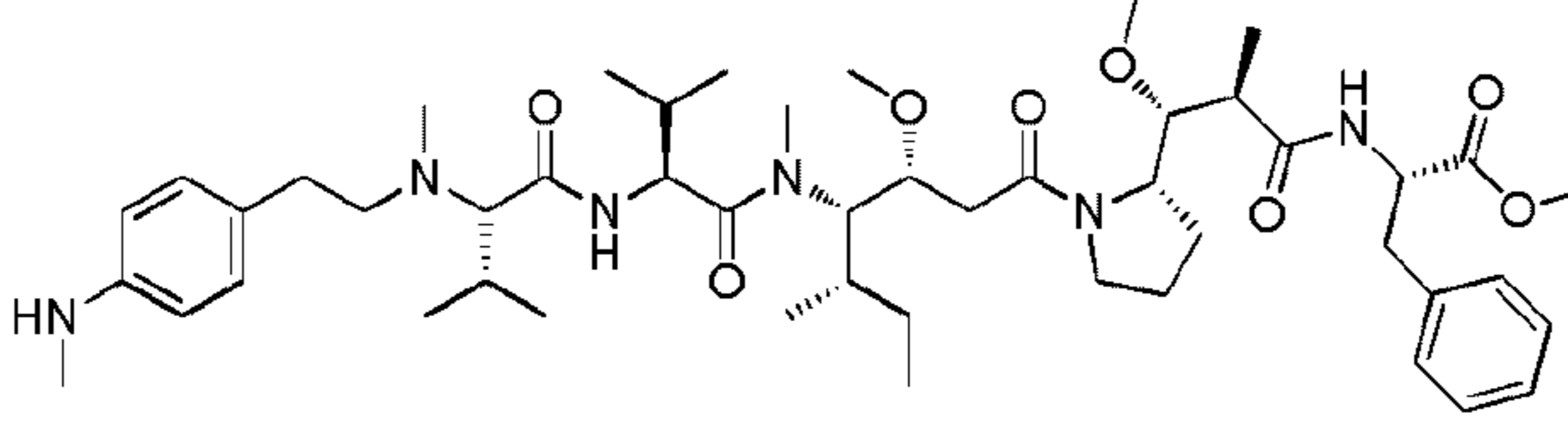
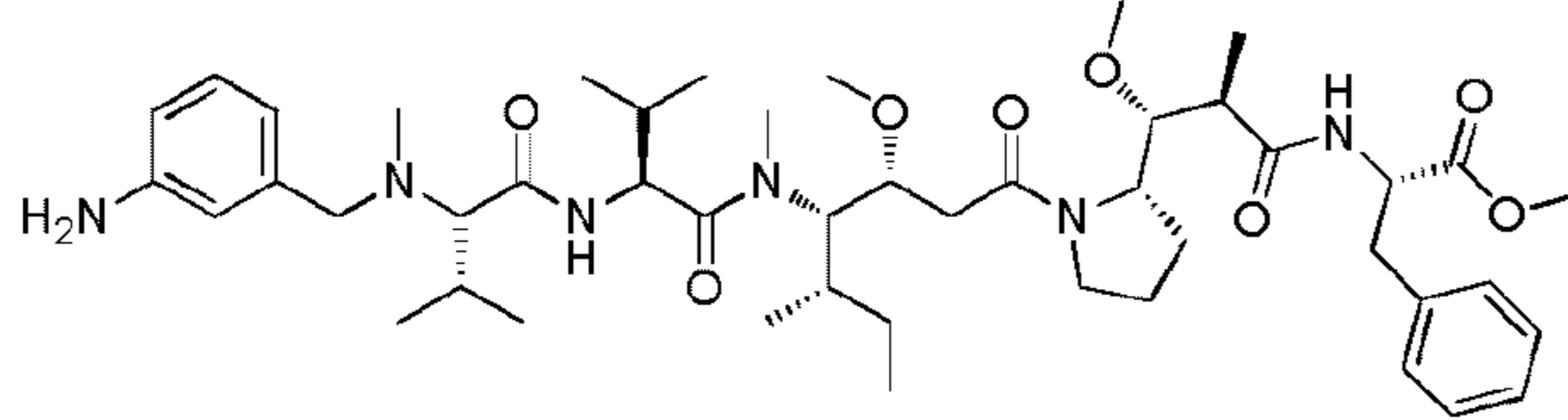
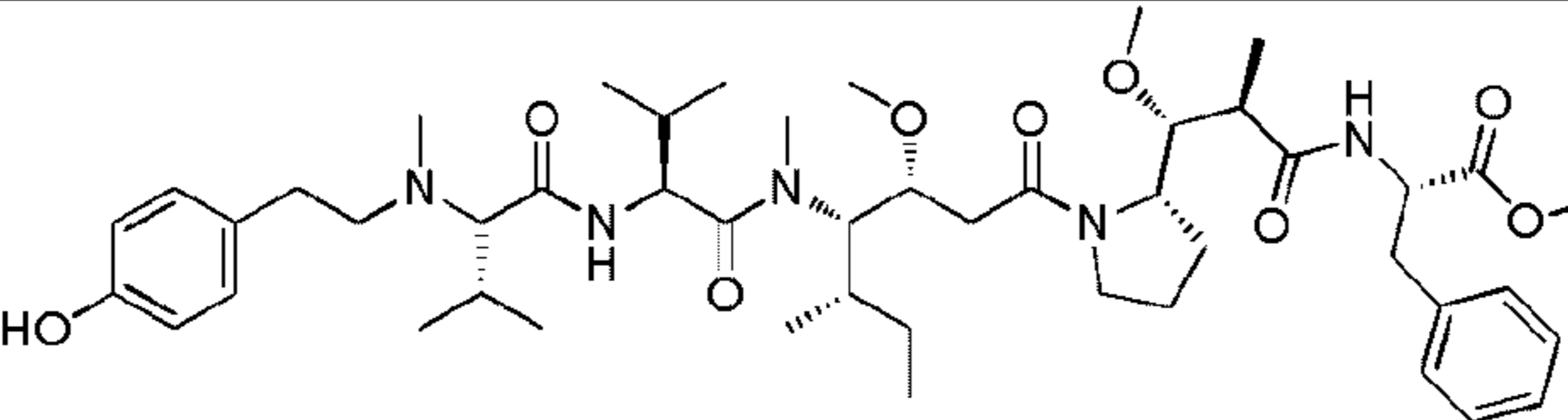
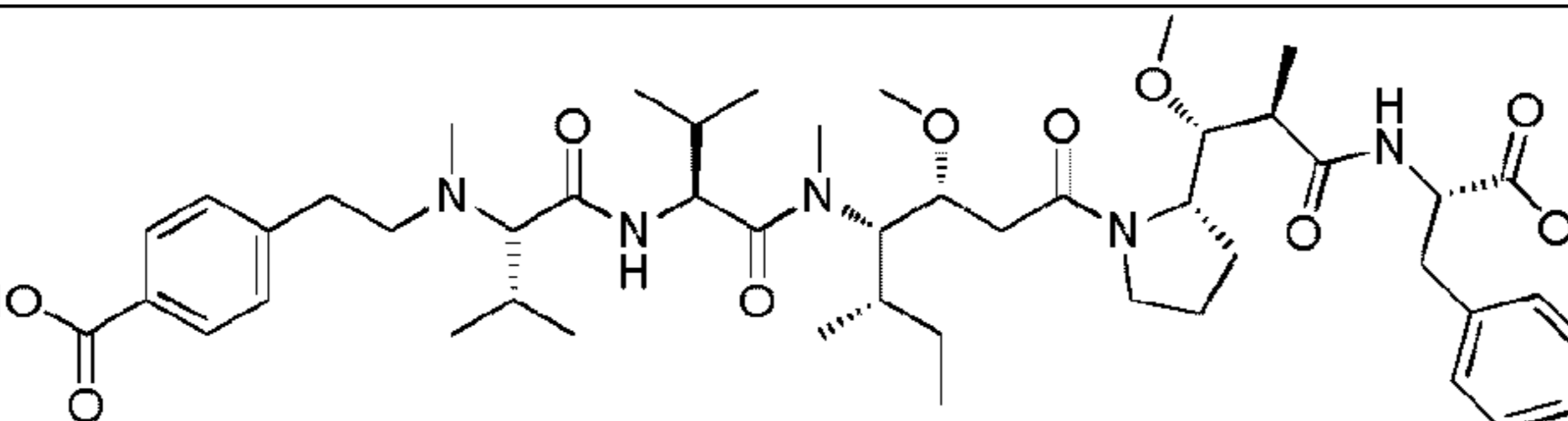
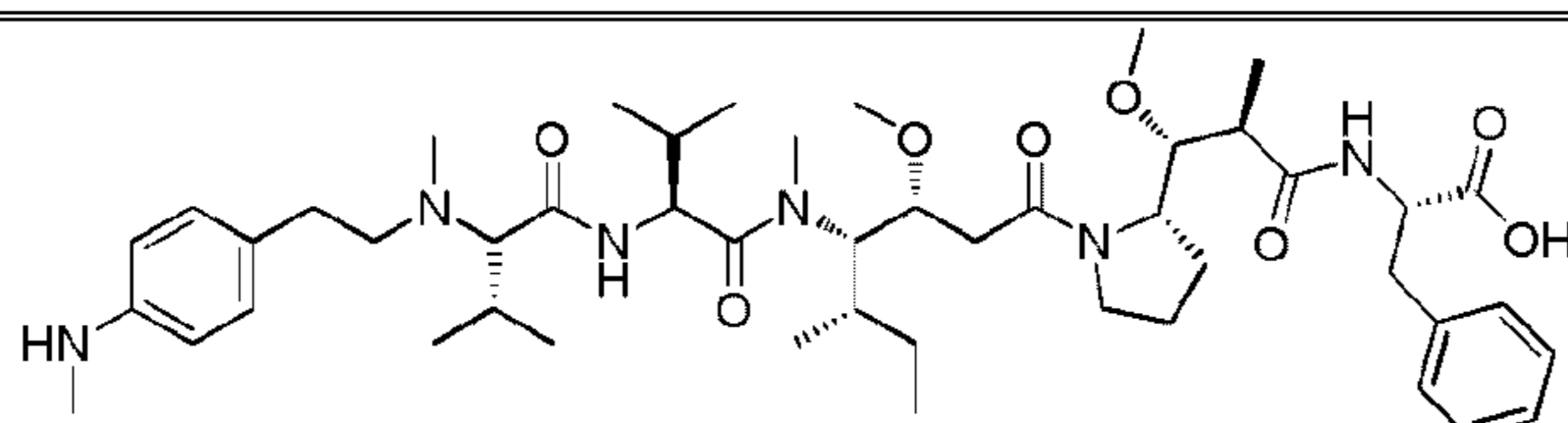
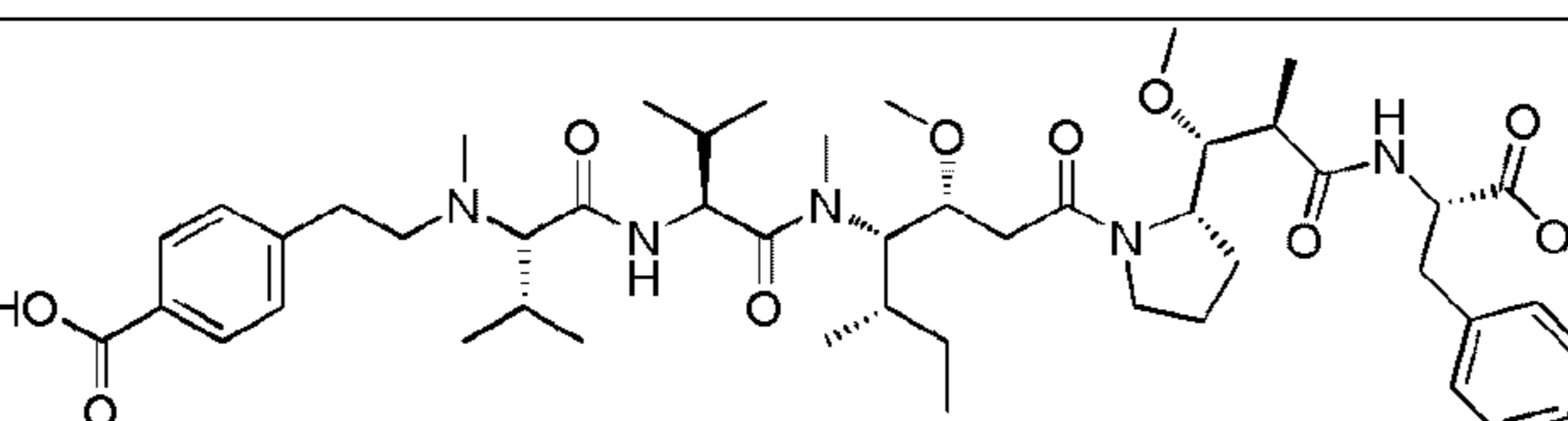
| EC₅₀ (M) | A549 | MDA-MB-231 | MCF-7 | SN12C |
|----------------------------|------------------------|------------------------|------------------------|------------------------|
| Compound 15 | 1.45x10 ⁻¹⁰ | 1.70x10 ⁻¹⁰ | 7.15x10 ⁻¹⁰ | 2.18x10 ⁻¹⁰ |

20

Comparative examples:

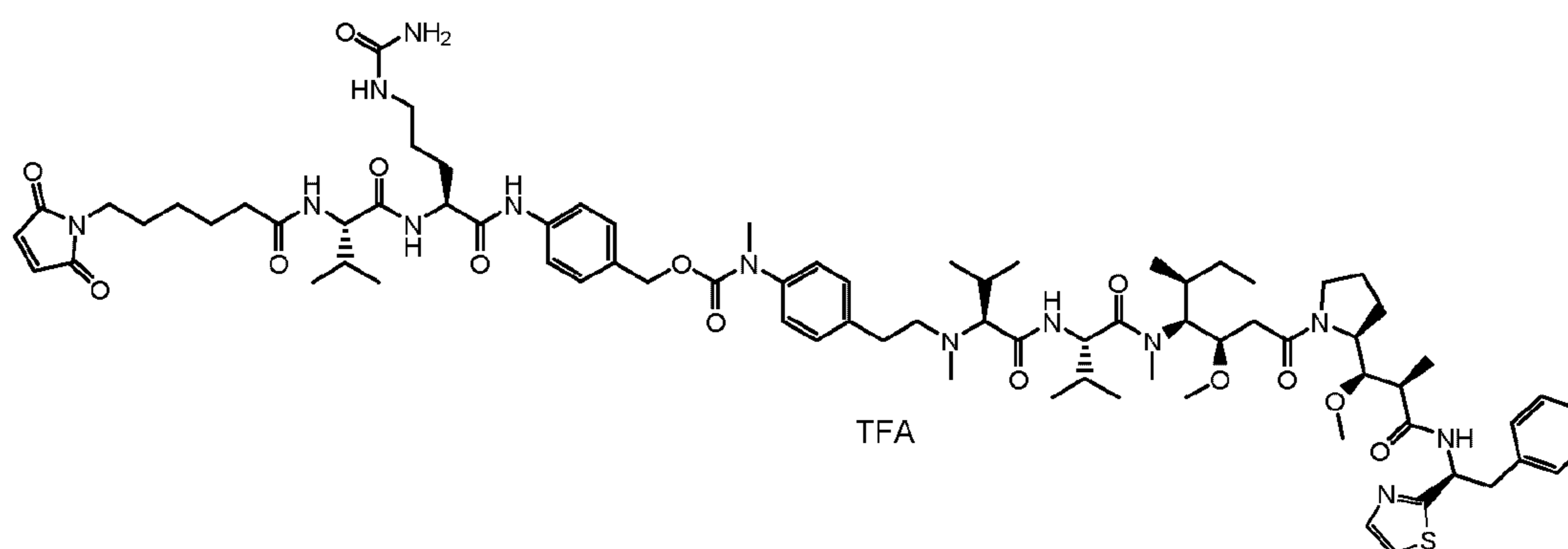
The substitution on the phenyl ring (amino/hydroxyl v. carboxyl) was studied in the comparative examples below showing the improved antiproliferative activity of the drugs according to the invention comprising an amino or hydroxyl substituent.

25

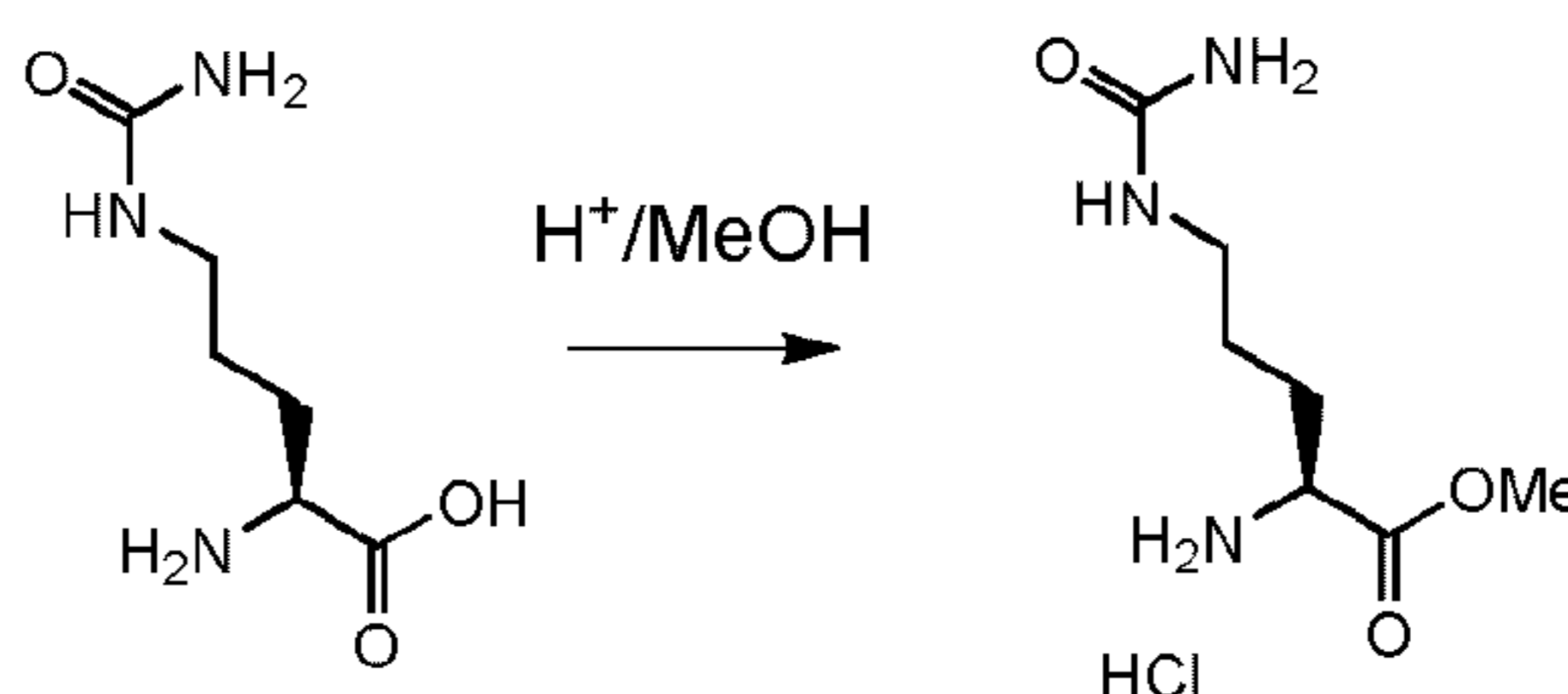
| N° | Structure | EC ₅₀ (M) | |
|---------------------|--|------------------------|------------------------|
| | | A549 | MDA-MB-231 |
| 12 |  | 1.48x10 ⁻¹⁰ | 5.80x10 ⁻¹⁰ |
| 15 |  | 1.45x10 ⁻¹⁰ | 1.70x10 ⁻¹⁰ |
| 27 |  | 8.60x10 ⁻¹¹ | 1.20x10 ⁻¹⁰ |
| Comparative example |  | 3.76x10 ⁻⁹ | 2.29x10 ⁻⁹ |
| 13 |  | 2.71x10 ⁻⁸ | 7.95x10 ⁻⁸ |
| Comprative example |  | 4.03x10 ⁻⁷ | 9.75x10 ⁻⁷ |

Example 21: Synthesis of the Drug-Linker moiety**Compound E-11**

4-((*S*)-2-((*S*)-2-(6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl (4-((3*R*,4*S*,7*S*,10*S*)-4-((*S*)-*sec*-butyl)-7,10-diisopropyl-3-(2-((*S*)-2-((1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-(((*S*)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-5,11-dimethyl-6,9-dioxo-2-oxa-5,8,11-triazatridecan-13-yl)phenyl)(methyl)carbamate
2,2,2-trifluoroacetate



10

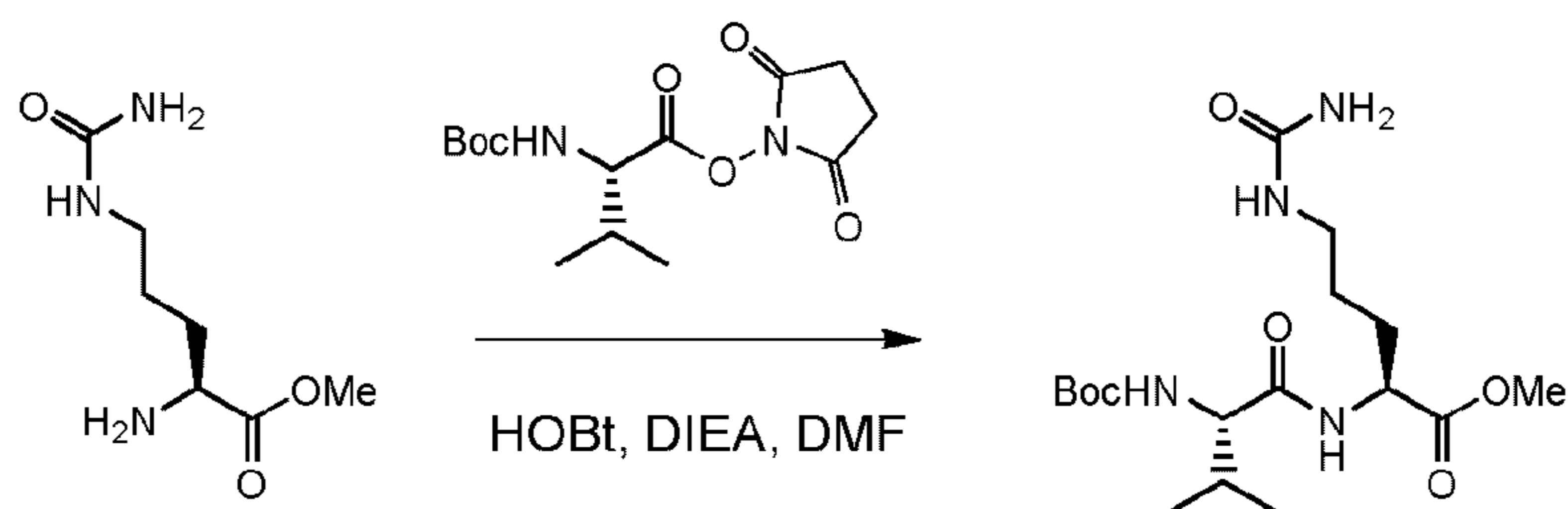
Compound E-11-1: methyl (*S*)-2-amino-5-ureidopentanoate hydrochloride

15

Acetyl chloride (10 mL) was added dropwise to MeOH (120 mL) at 0°C with stirring. After 20 minutes, L-Citrulline (10 g, 57 mmol, 1.00 eq.) was added and the mixture heated at reflux overnight. The solvent was evaporated under reduced pressure to yield 15 g (116 %) of compound **E-11-1** as a white solid. The product was used in the next step without further drying.

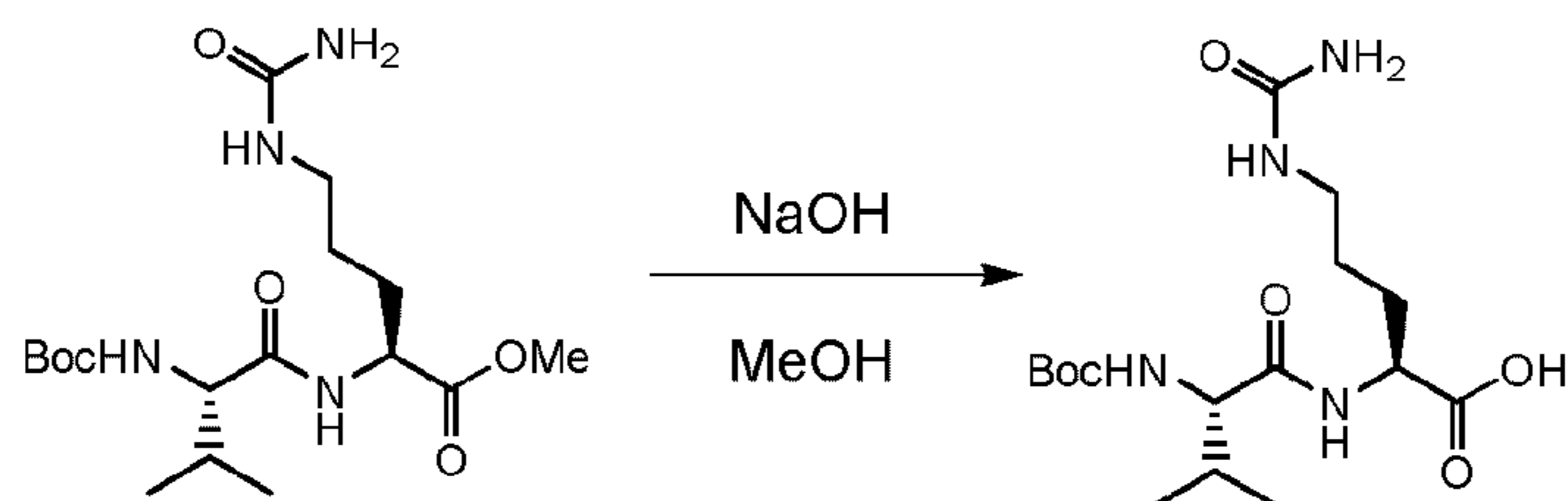
Compound E-11-2: methyl (*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-5-ureidopentanoate

216



Compound **E-11-1** (13 g, 57.6 mmol, 1.1 eq.) was dissolved in DMF (140 mL) at 0°C under an inert atmosphere. DIEA (30 mL, 173 mmol, 3.0 eq.), hydroxybenzotriazole (HOBt - 10.59 g, 69.1 mmol, 1.2 eq.) and Boc-L-valine hydroxysuccinimide ester (Boc-Val-OSu - 18.1 g, 57.6 mmol, 1.0 eq.) were added. The reaction mixture was agitated overnight at ambient temperature, then the solvent was evaporated under reduced pressure. The residue was dissolved in water (100 mL) and extracted twice with DCM (150 mL). The organic phases were combined, dried over Na₂SO₄ and concentrated under reduced pressure. The residue was purified on silica gel (DCM/MeOH) to yield 18.8 g (84 %) of compound **E-11-2** as a white solid.

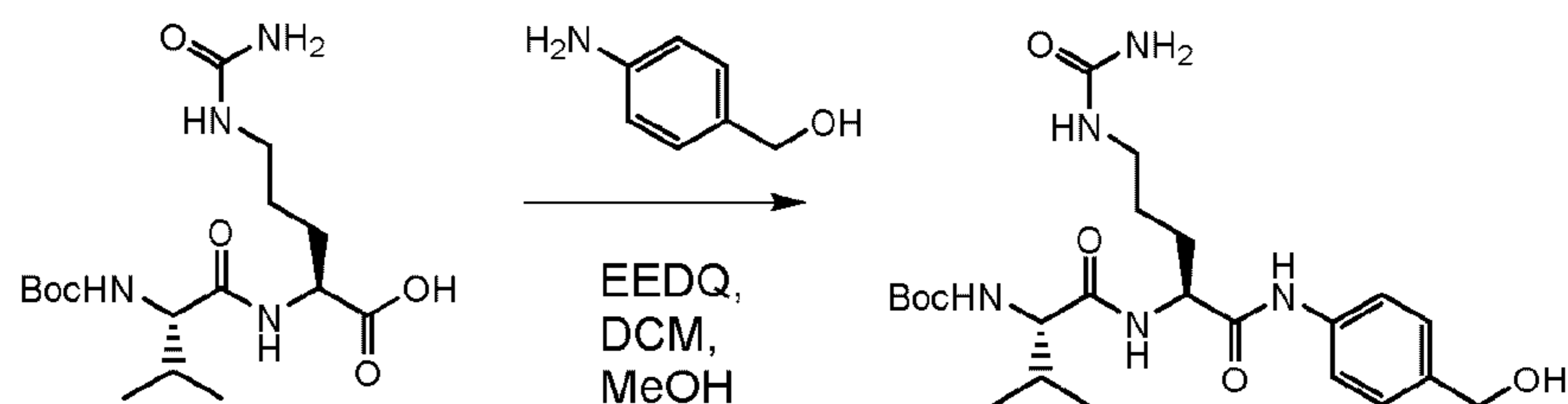
Compound E-11-3: (*S*)-2-(((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-5-ureidopentanoic acid



Compound **E-11-2** (18.8 g, 48.4 mmol, 1 eq.) was dissolved in MeOH (200 mL) at 0°C. A solution of NaOH 1M (72 mL, 72 mmol, 1.5 eq.) was added and the mixture stirred for 2 hours at room temperature. The MeOH was removed under reduced pressure and the remaining aqueous solution acidified with HCl 1M. The aqueous phase was evaporated to dryness and the residue purified on silica gel (DCM/MeOH) to yield 18 g (99 %) of compound **E-11-3** as a white solid.

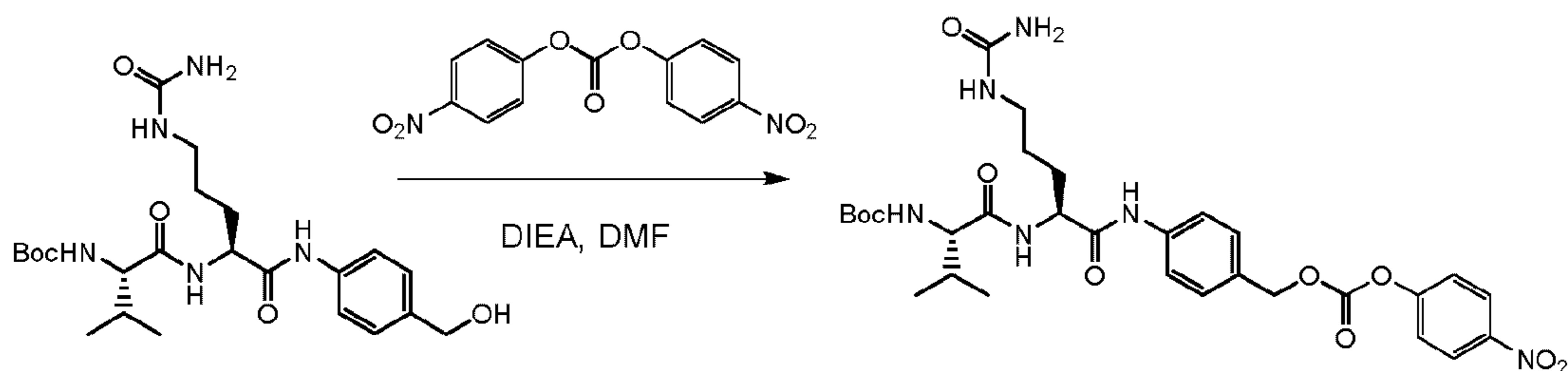
Compound E-11-4: *tert*-butyl ((*S*)-1-(((*S*)-1-((4-(hydroxymethyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate

217



Compound **E-11-3** (5g, 13.4 mmol, 1 eq.) was dissolved in a mixture of dry DCM (65 ml) and dry MeOH (35 ml). (4-aminophenyl)methanol (1.81 g, 14.7 mmol, 1.1 eq.) and *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ - 6.60 g, 26.7 mmol, 2 eq.) were added and the mixture stirred in the dark overnight. The solvents were evaporated under reduced pressure, and the residue purified on silica gel (DCM/MeOH) to yield 5.2 g (73 %) of compound **E-11-4** as an off-white solid.

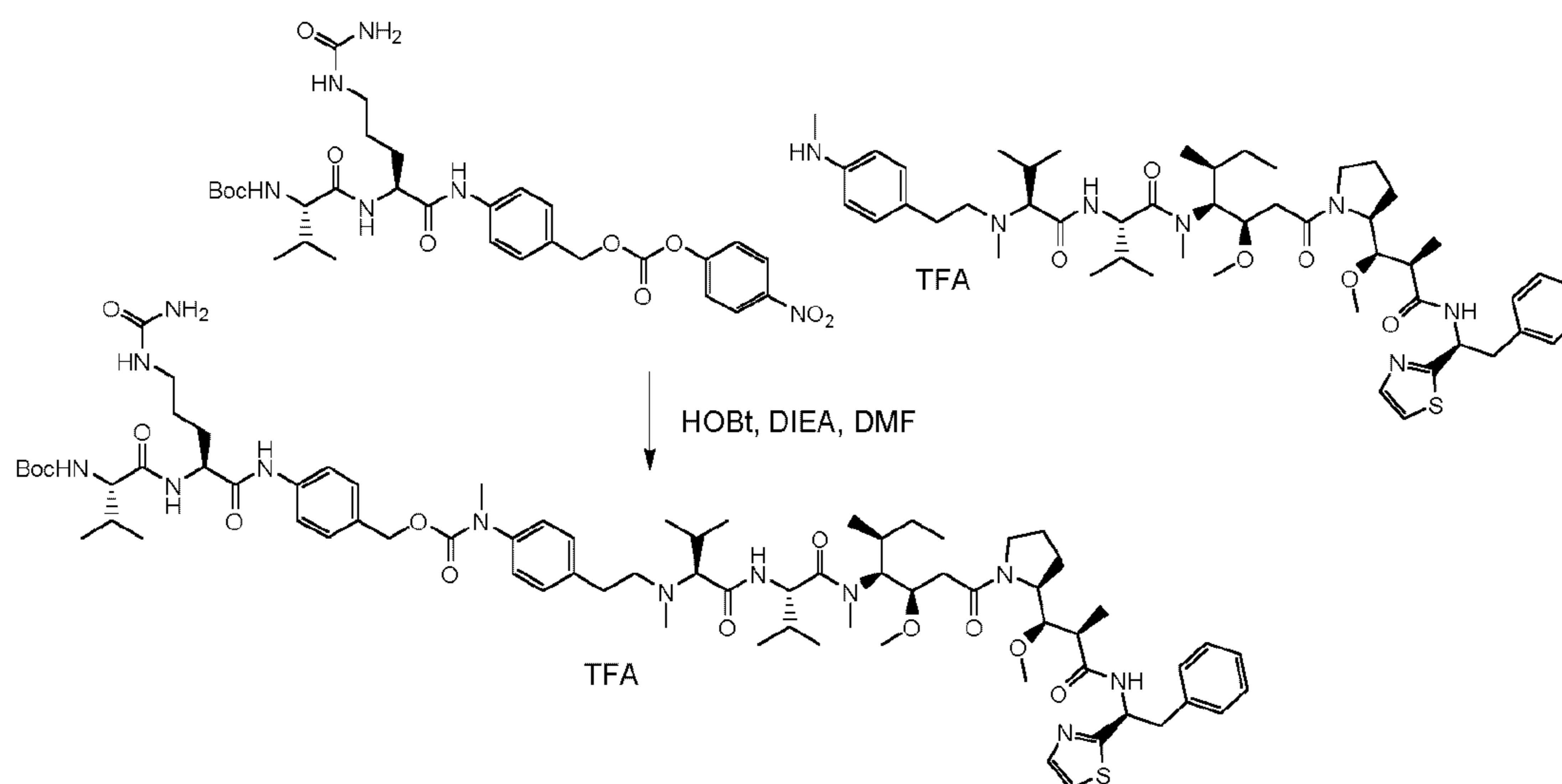
Compound E-11-5: *tert*-butyl ((*S*)-3-methyl-1-(((*S*)-1-((4-(((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-1-oxobutan-2-yl)carbamate



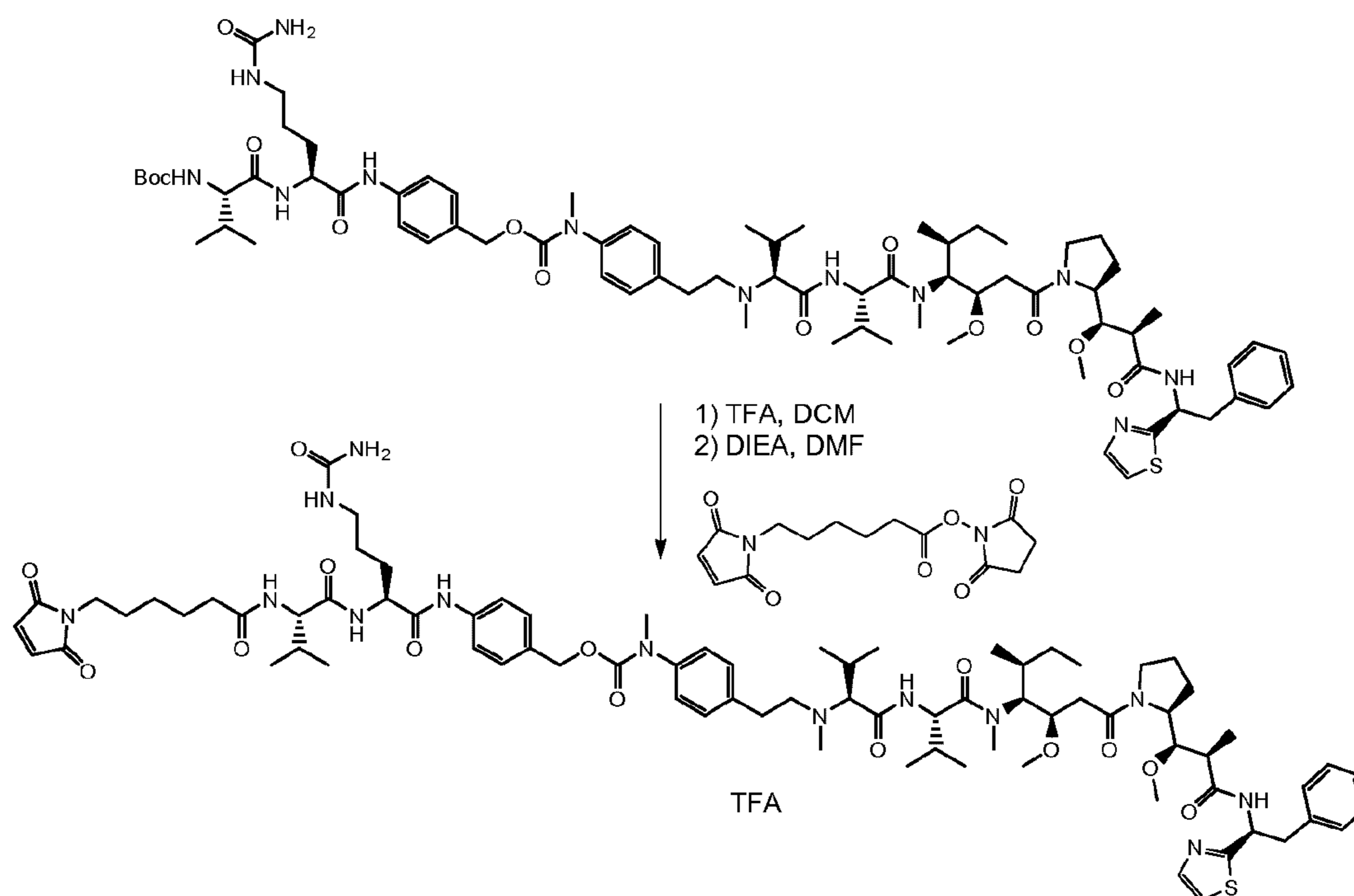
Compound **E-11-4** (1.1 g, 2.29 mmol, 1 eq.) was dissolved in dry DMF (5 ml) at ambient temperature under an inert atmosphere. Bis(4-nitrophenyl) carbonate (1.40 g, 4.59 mmol, 2 eq.) was added, followed by DIEA (600 μ l, 3.44 mmol, 1.5 eq.), and the resulting yellow solution stirred overnight. The DMF was evaporated under reduced pressure, and the residue purified on silica gel (DCM/MeOH) to yield 1.27 g (84 %) of compound **E-11-5** as an off-white solid.

Compound E-11-6: 4-(((*S*)-2-(((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl (4-(((3*R*,4*S*,7*S*,10*S*)-4-(((*S*)-*sec*-butyl)-7,10-diisopropyl-3-(2-(((*S*)-2-((1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-(((*S*)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-5,11-dimethyl-6,9-dioxo-2-oxa-5,8,11-triazatridecan-13-yl)phenyl)(methyl)carbamate 2,2,2-trifluoroacetate

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Carbonate **E-11-5** (114 mg, 0.177 mmol, 1.2 eq.) and aniline **11F** (150 mg, 0.147 mmol, 1 eq.) were dissolved in dry DMF (4 mL). HOBt (38 mg, 0.295 mmol, 2 eq.) and DIEA (54 μ L, 0.295 mmol, 2 eq.) were added and the mixture stirred for the weekend at room temperature. The DMF was evaporated under reduced pressure and the residue purified by flash chromatography on silica, eluting with DCM. The product was repurified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **E-11-6** as a white solid (89 mg, 39 %).

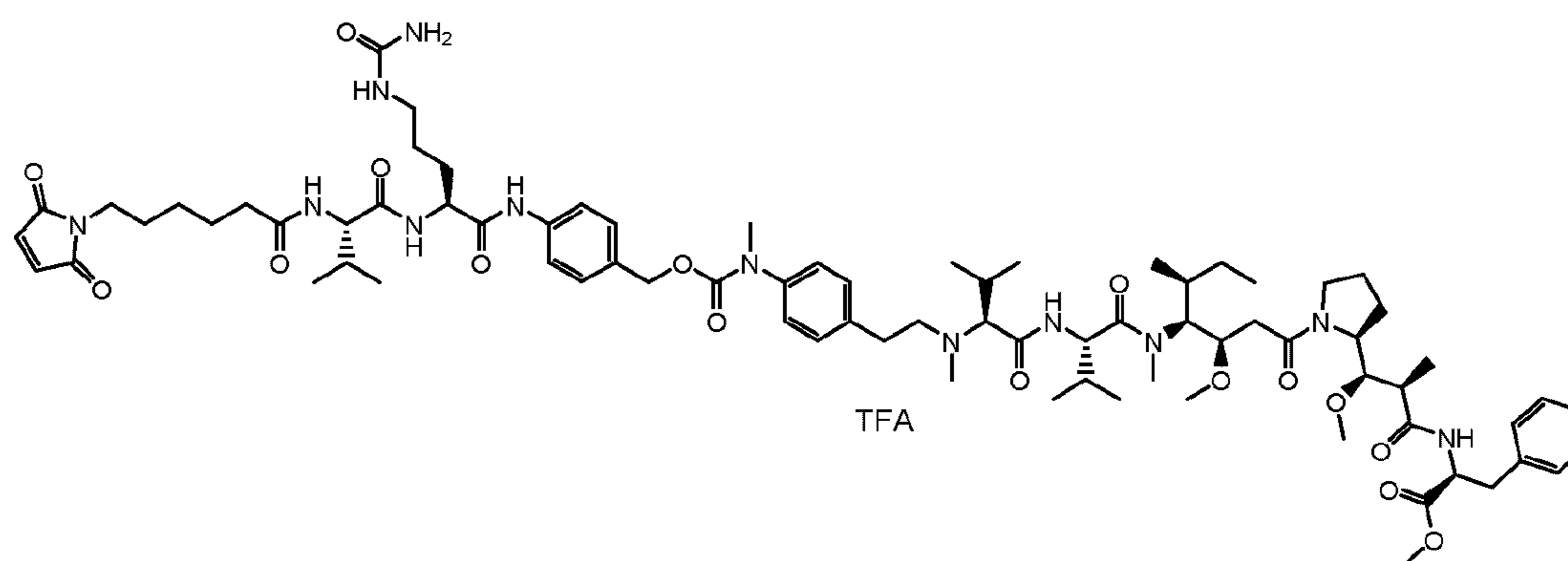
Compound E-11:

Compound **E-11-6** (21 mg, 0.014 mmol, 1.0 eq.) was dissolved in DCM (0.25 mL) and TFA (40 μ L) was added. The solution was stirred for 2 hours at room temperature, after which, LC-MS analysis indicated complete consumption of starting material. The mixture was briefly cooled (bath of liquid nitrogen) whilst simultaneously adding DMF (0.5 mL) then DIEA (100 μ L) in order to neutralise the TFA. The cooling bath was then removed and 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (4 mg, 0.012 mmol, 1 eq.) was added. The mixture was stirred at room temperature for 48 hours and the product purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **E-11** as a white solid (11 mg, 54 %).

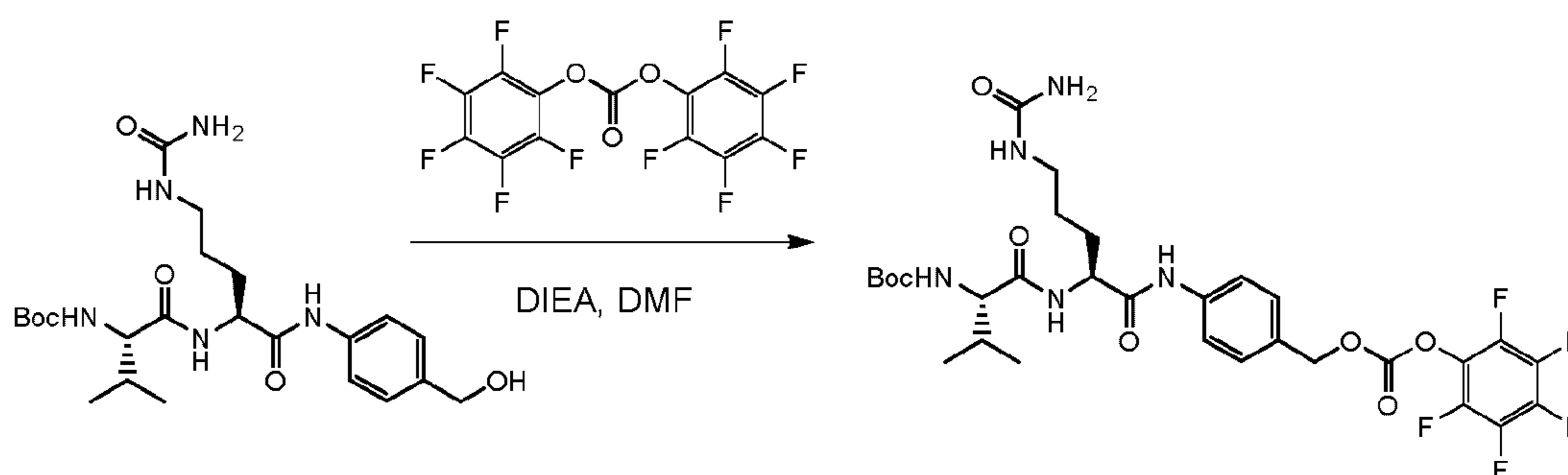
m/z (Q-TOF MS ESI+) 1524.8282 (2 %, MNa^+ , $C_{79}H_{115}N_{13}NaO_{14}S$ requires 1524.8299), 751.9283 (100 %, $(MH_2)^{2+}$, $C_{79}H_{117}N_{13}O_{14}S$ requires 751.9276).

Compound E-12

methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-(((4-((*S*)-2-((*S*)-2-(6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy)carbonyl)(methyl)amino)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalaninate 2,2,2-trifluoroacetate

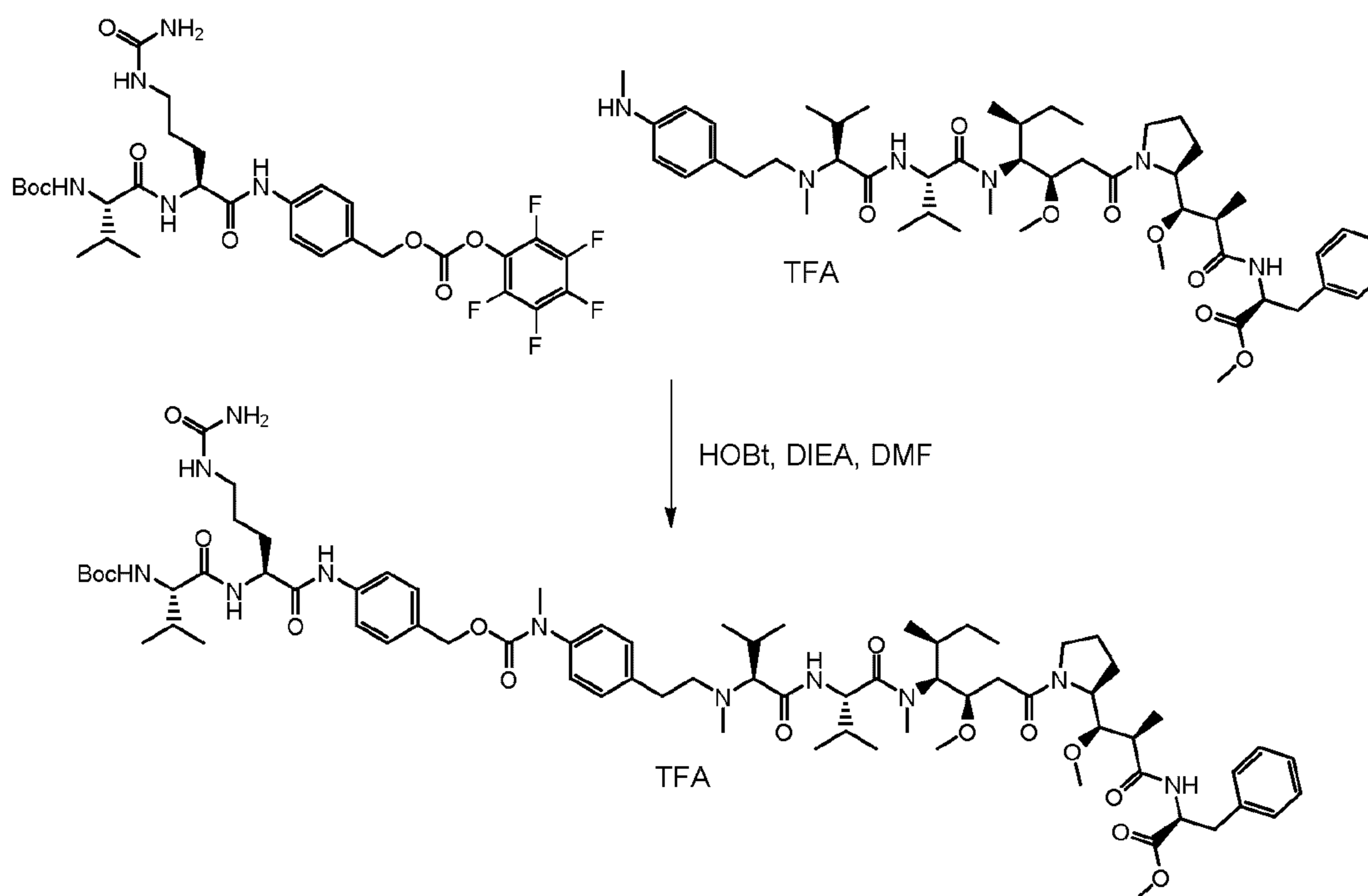


Compound E-12-1: *tert*-butyl ((*S*)-3-methyl-1-oxo-1-(((*S*)-1-oxo-1-((4-(((perfluorophenoxy)carbonyl)oxy)methyl)phenyl)amino)-5-ureidopentan-2-yl)amino)butan-2-yl)carbamate



Compound E-11-4 (670 mg, 1.26 mmol, 1 eq.) was dissolved in dry DMF (6 ml) at 0°C under an inert atmosphere. Bis(perfluorophenyl) carbonate (991 mg, 2.51 mmol, 2 eq.) was added, followed by DIEA (329 μ l, 1.89 mmol, 1.5 eq.), and the resulting colourless solution stirred for 30 minutes at room temperature. The DMF was evaporated under reduced pressure, and the residue purified on silica gel (DCM/MeOH) to yield 836 mg (96 %) of compound E-12-1 as an off-white solid.

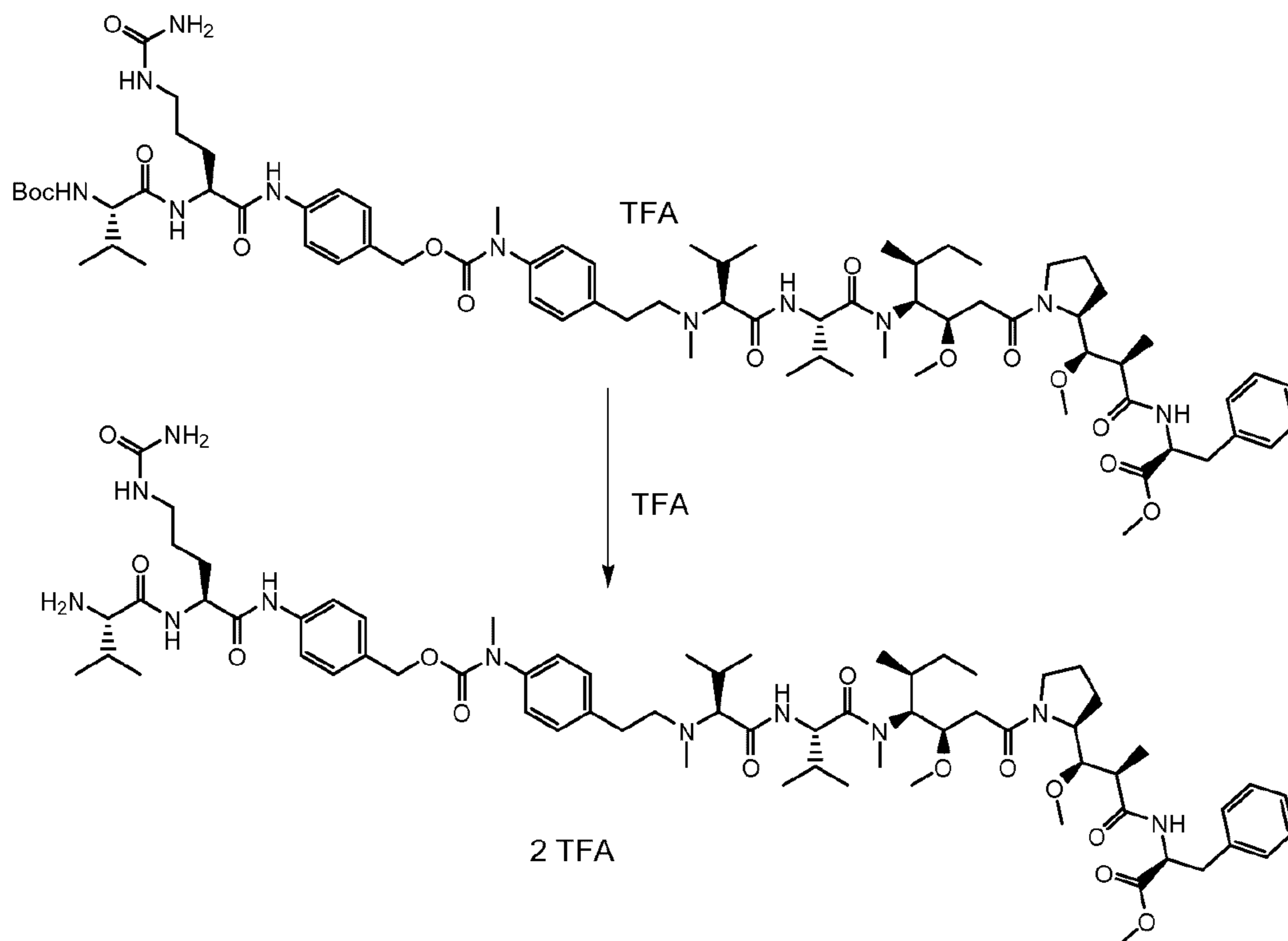
Compound E-12-2: methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-(((4-((*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy)carbonyl)(methyl)amino)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalaninate 2,2,2-trifluoroacetate



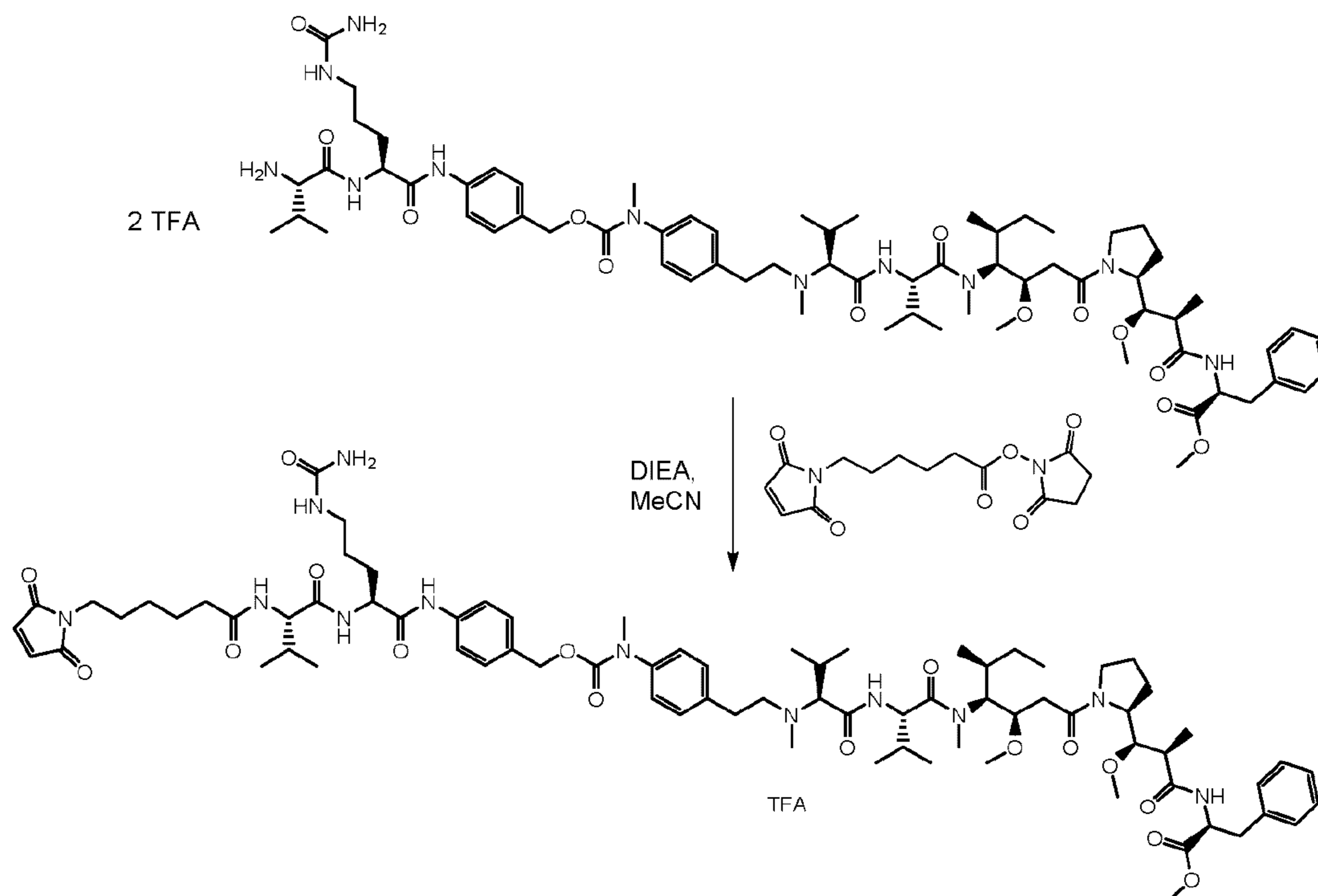
Aniline **12** (165 mg, 0.189 mmol, 1.0 eq.) was dissolved in DMF (5 mL) at 0°C under an inert atmosphere. Carbonate **E-12-1** (194 mg, 0.282 mmol, 1.5 eq.), HOBt (51 mg, 0.375 mmol, 2 eq.) and DIEA (66 μ L, 0.375 mmol, 2 eq.) were added and the mixture stirred at room temperature for 8 hours. The solvent was evaporated under reduced pressure and the residue purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **E12-7** as a white solid (247 mg, 77 %).

Compound E-12-3: methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-(((4-((*S*)-2-((*S*)-2-amino-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy)carbonyl)(methyl)amino)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-

dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalaninate bis(2,2,2-trifluoroacetate)



- Compound **E-12-2** (5.6 mg, 4.04 μmol , 1.0 eq.) was dissolved TFA (100 μL).
- 5 After 5 minutes, 2 ml of water was added and the mixture lyophilised overnight to yield compound **E-12-3** as an off-white solid (5.6 mg, 98 %).

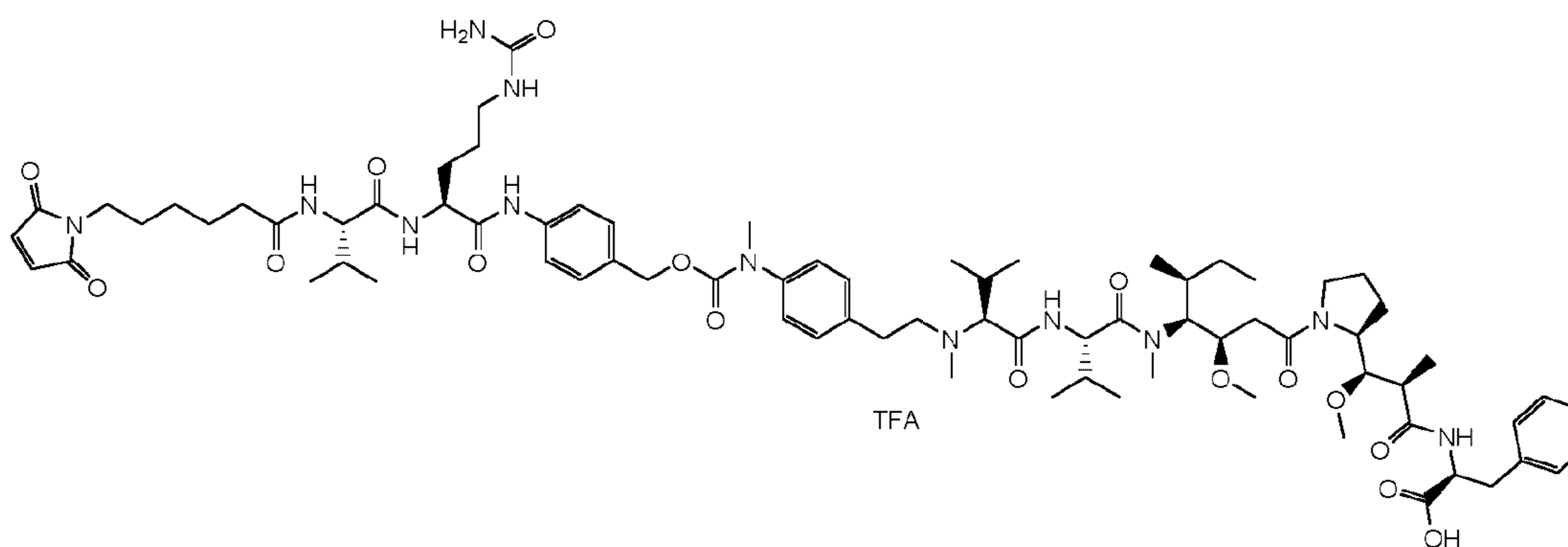
Compound E-12:

Compound **E-12-3** (5.6 mg, 4 μmol , 1.0 eq.) was dissolved in acetonitrile (0.5 mL), and DIEA (5 μL , 7 eq.) was added, followed by 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (2.5 mg, 8 μmol , 2 eq.). The mixture was stirred for 6 hours at room temperature. After controlling the reaction by LC-MS, 200 μL of water was added, and the resulting solution purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μm , 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **E-12** as a white solid (4.6 mg, 70 %).

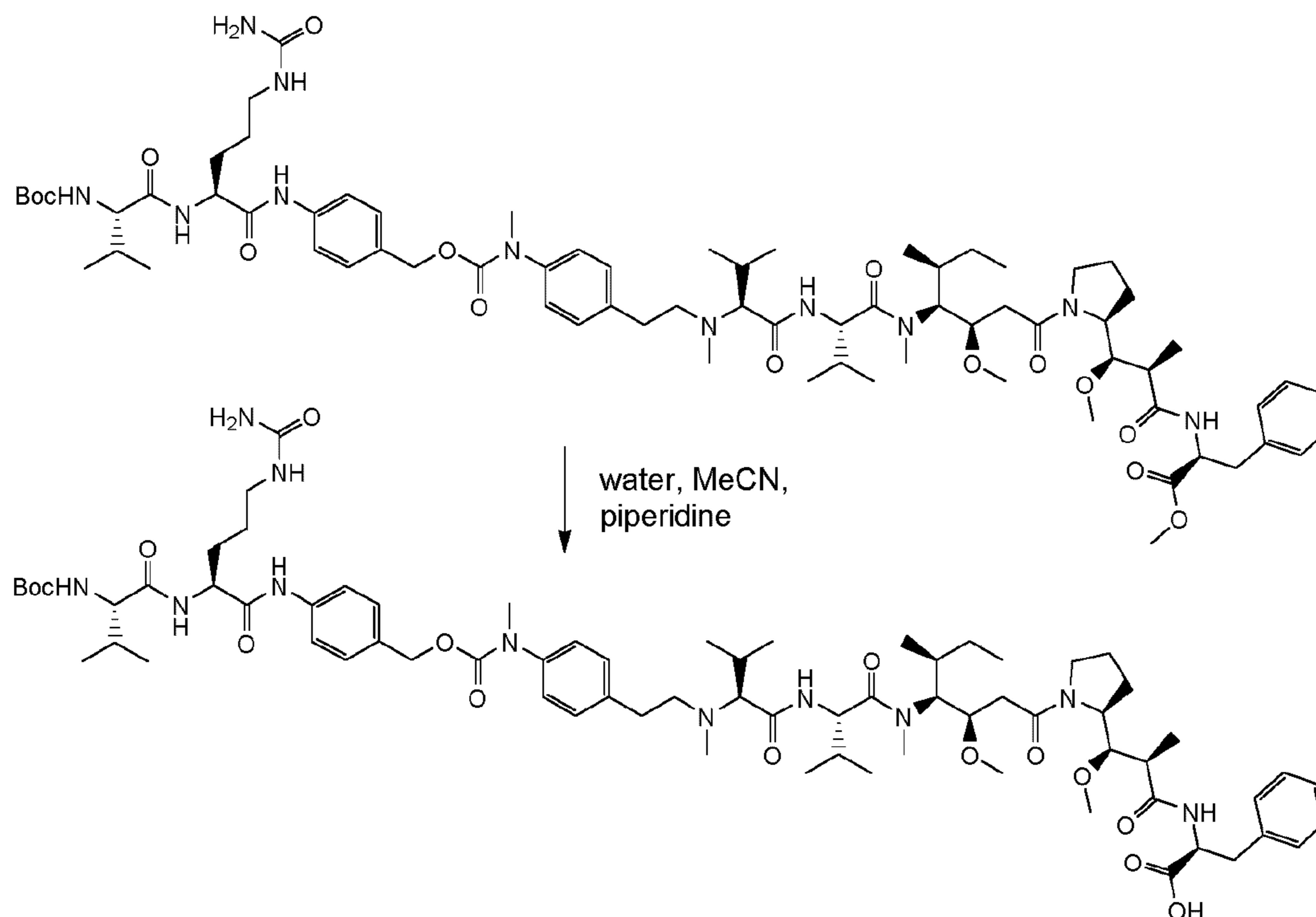
m/z (Q-TOF MS ESI+) 739.4389 (100 %, $(\text{MH}_2)^{2+}$, $\text{C}_{78}\text{H}_{118}\text{N}_{12}\text{O}_{16}$ requires 739.4389).

Compound E-13

5 **((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-(((4-((*S*)-2-((*S*)-2-(6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy)carbonyl)(methyl)amino)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalanine 2,2,2-trifluoroacetate**

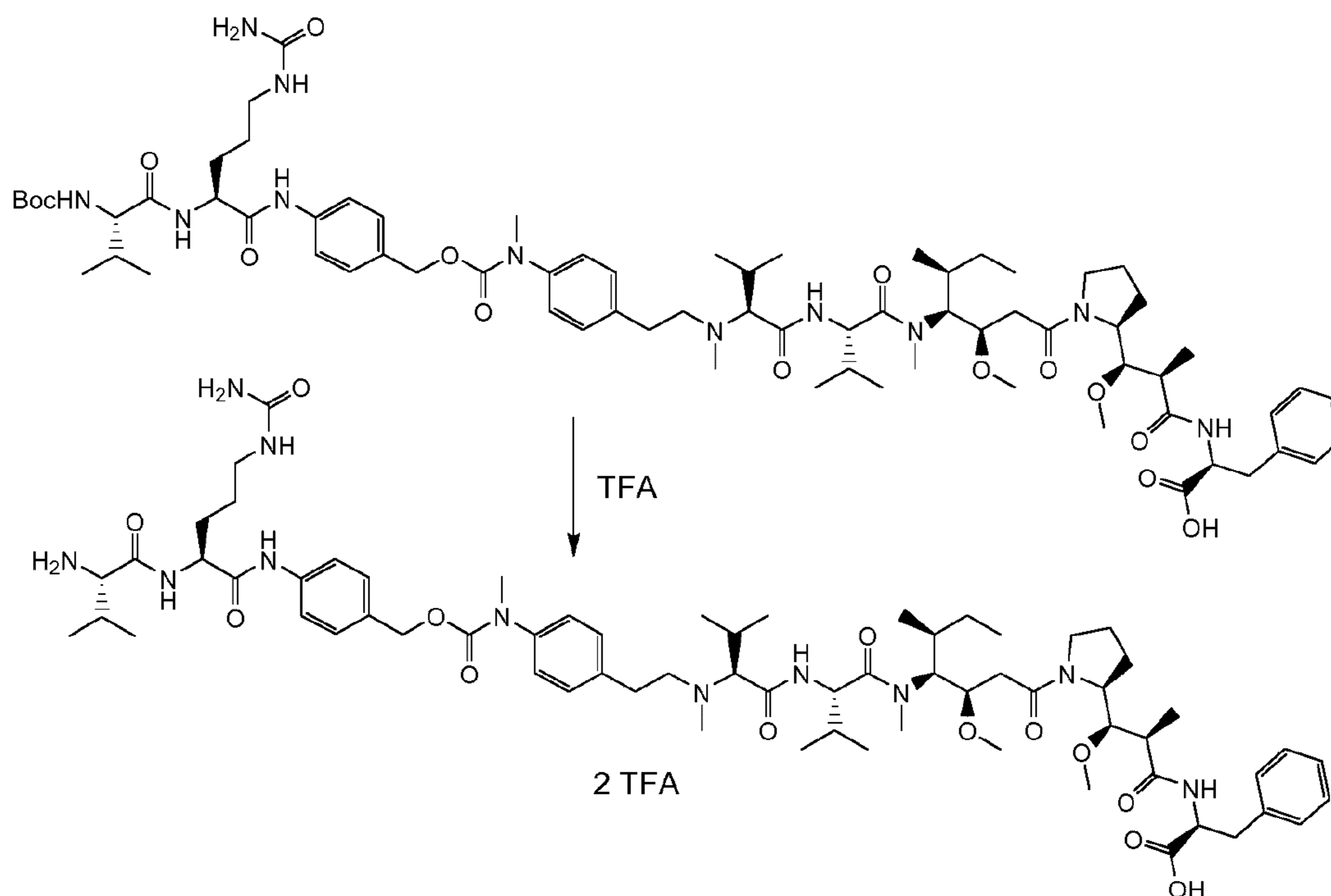


10 **Compound E-13-1: ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-(((4-((*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy)carbonyl)(methyl)amino)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalanine**



Compound **E-12-2** (185 mg, 0.123 mmol, 1.0 eq.) was dissolved in a mixture of water (5 mL) and acetonitrile (5 mL) at room temperature. Piperidine (3.67 mL, 300 eq.) was added and the mixture stirred for 6 hours at room temperature. The solvents were evaporated to dryness under reduced pressure, and the residue triturated with Et₂O (60 mL). The solid was rinsed with twice Et₂O (20 mL) and dried under vacuum to yield compound **E-13-1** as an off-white solid (175 mg, 95 %).

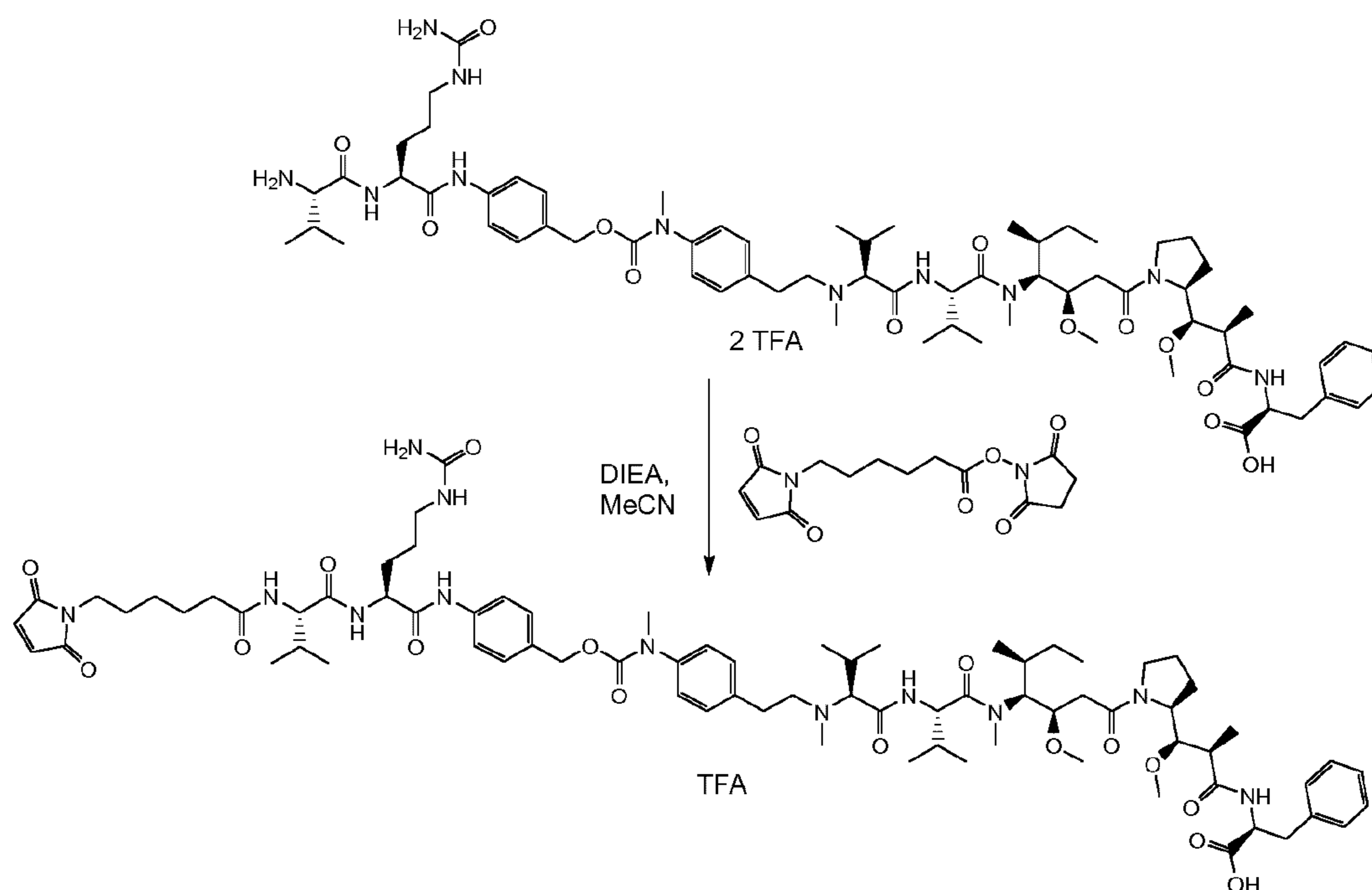
Compound E-13-2: ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-(((4-((*S*)-2-((*S*)-2-amino-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy)carbonyl)(methyl)amino)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalanine bis(2,2,2-trifluoroacetate)



Compound **E-13-1** (175 mg, 0.128 mmol, 1.0 eq.) was dissolved TFA (200 μ L). After 5 minutes, water (1 mL) and acetonitrile (1 mL) were added and the solution lyophilised overnight to yield compound **E-13-2** as an off-white solid (180 mg, 87 %).

5 **Compound E-13:** ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-(((4-((*S*)-2-((*S*)-2-(6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy)carbonyl)(methyl)amino)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalanine, 2,2,2-trifluoroacetate

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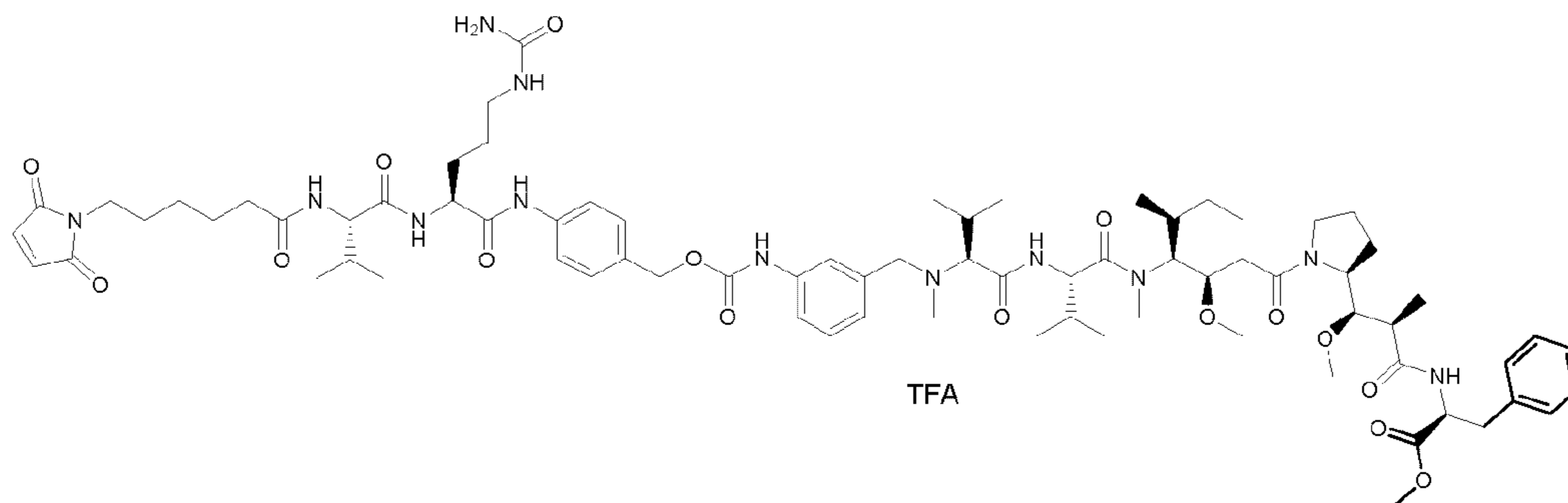
Compound **E-13-2** (80 mg, 0.058 mmol, 1.0 eq.) was dissolved in a mixture of acetonitrile (1.5 mL) and DMF (0.4 mL). DIEA (50 μ L, 0.289 mmol, 5 eq.) was added, followed by 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (36 mg, 0.116 mmol, 2 eq.). The mixture was stirred for 3 hours at room temperature. After controlling the reaction by LC-MS, the solvent was evaporated under reduced pressure and the residue purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV
 5
 10
 15
 Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **E-13** as a white solid (32 mg, 35 %).

m/z (Q-TOF MS ESI-) 1461.8336 (100 %, (M-H)⁻, C₇₇H₁₁₃N₁₂O₁₆ requires 1461.8403).
 m/z (Q-TOF MS ESI+) 1463.8565 (2 %, MH⁺, C₇₇H₁₁₅N₁₂O₁₆ requires 1463.8549),
 732.4317 (100 %, (MH₂)²⁺, C₇₇H₁₁₆N₁₂O₁₆ requires 732.4311).

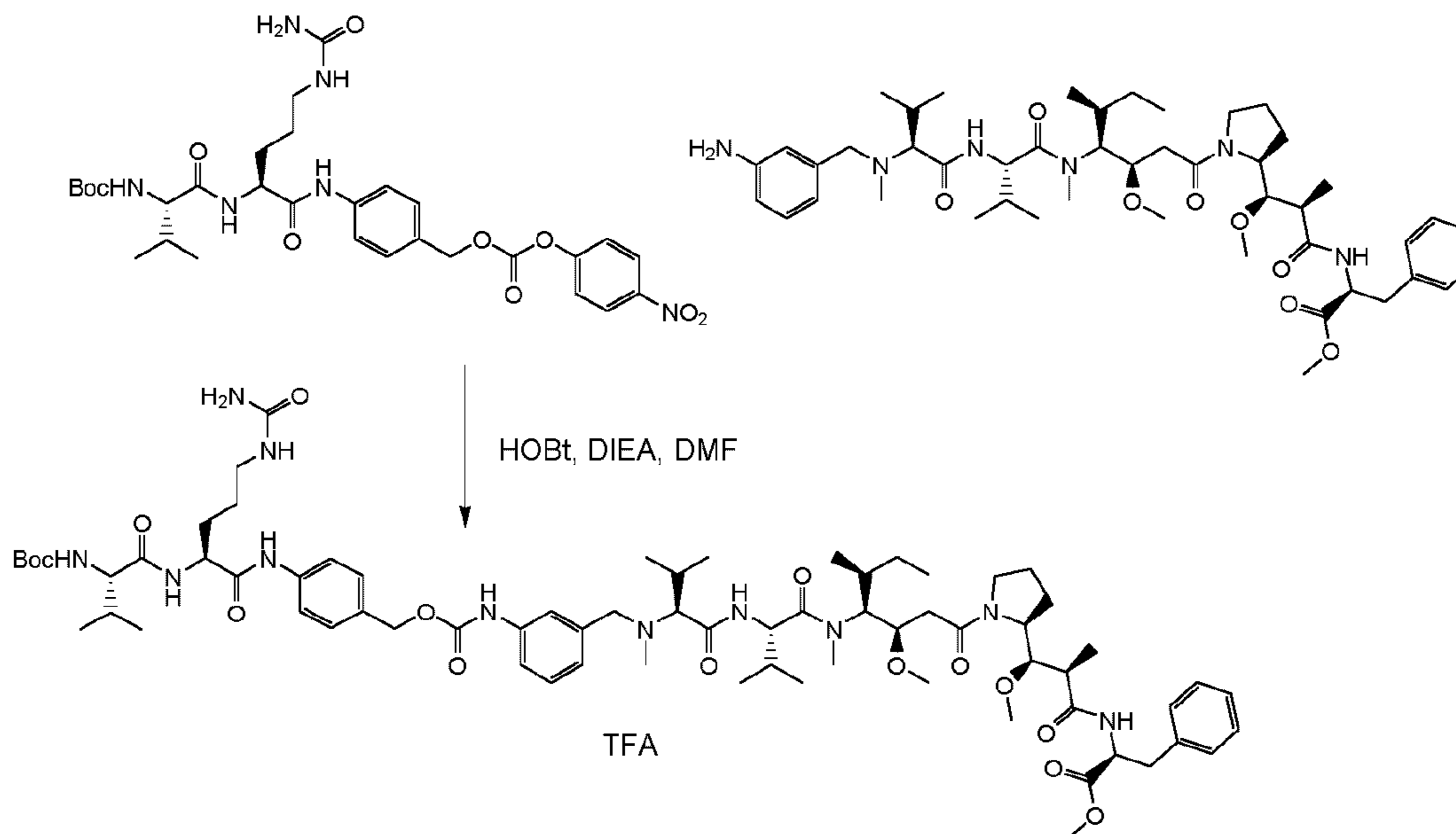
15

Compound E-15

methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((3-(((4-((*S*)-2-((*S*)-2-(6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy)carbonyl)amino)benzyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalaninate 2,2,2-trifluoroacetate

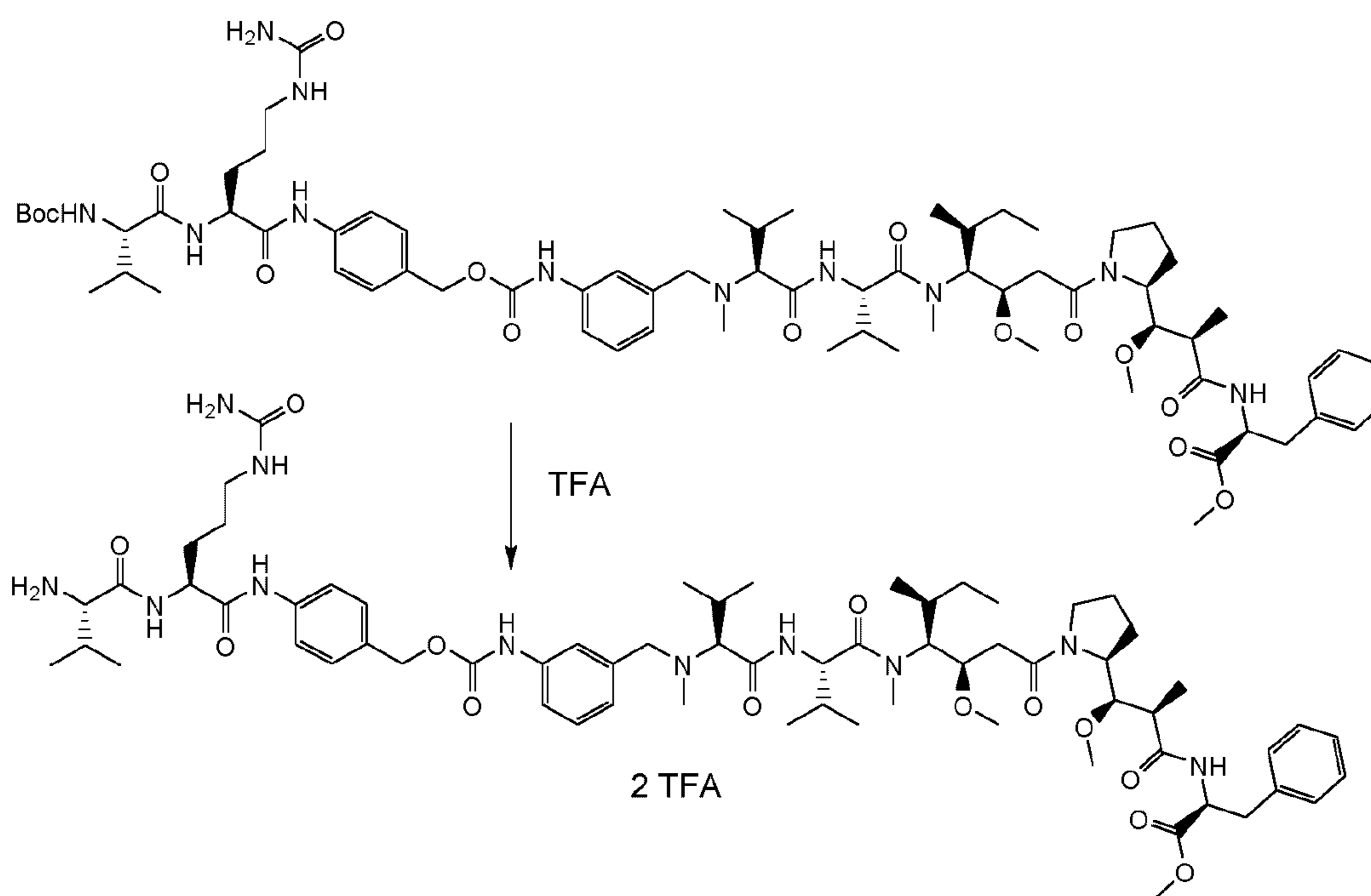


Compound E-15-1: methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((3-(((4-((*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy)carbonyl)amino)benzyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalaninate 2,2,2-trifluoroacetate



Compound **E-15-1** was prepared according to the same method as for compound **E-11-6**, using carbonate **E-11-5** (28 mg, 0.044 mmol, 1 eq.), aniline **15** (42 mg, 0.044 mmol, 1 eq.), HOBt (3 mg, 0.022 mmol, 0.5 eq.), and DIEA (15 μ L, 0.087 mmol, 2 eq.) in DMF (2 mL). Compound **E-15-1** was isolated as a white solid (8.2 mg, 13 %).

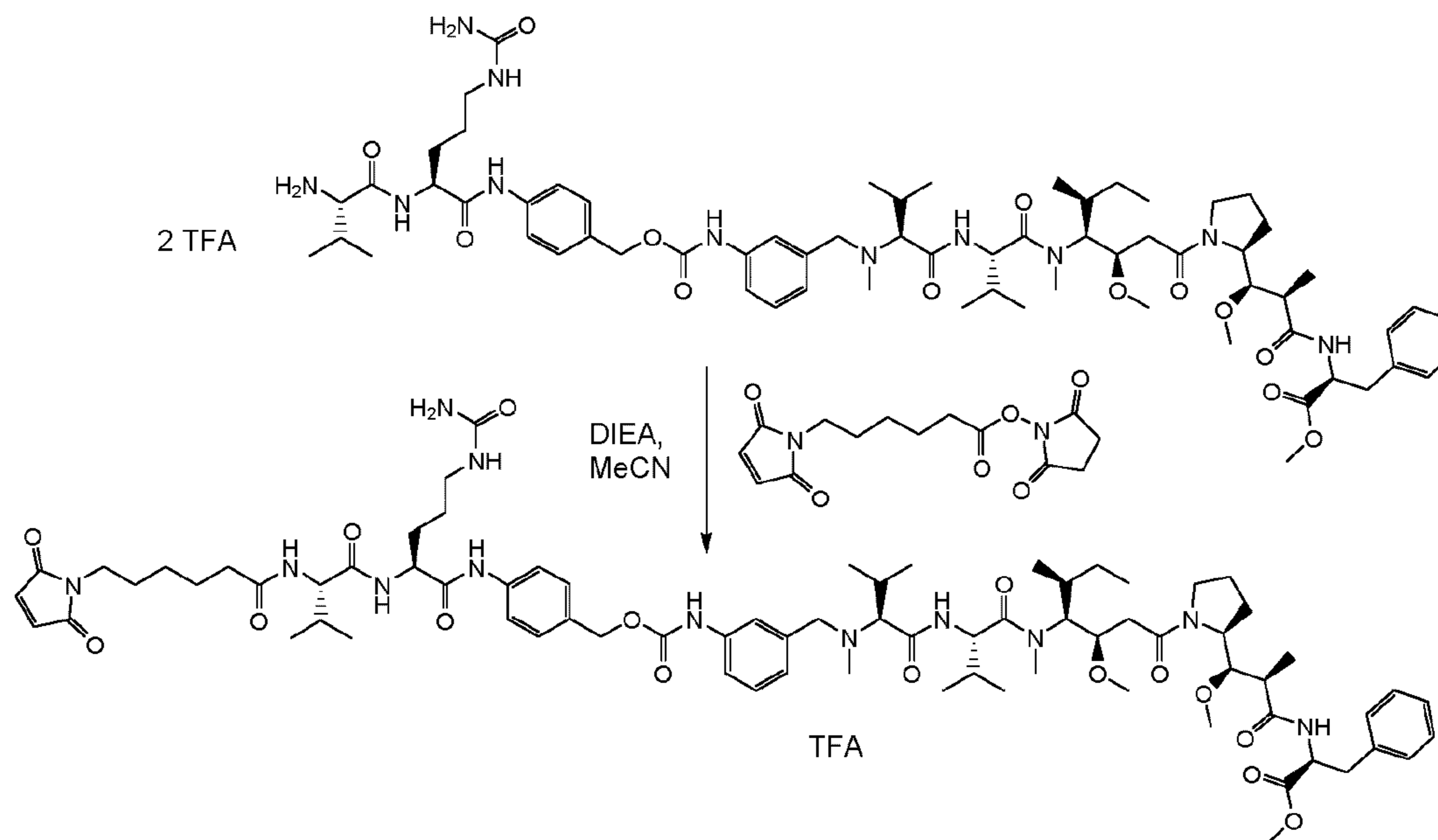
5 **Compound E-15-2:** methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((3-(((4-((*S*)-2-((*S*)-2-amino-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy carbonyl)amino)benzyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalaninate bis(2,2,2-trifluoroacetate)



Compound **E-15-1** (8.2 mg, 5.58 μ mol, 1.0 eq.) was dissolved in TFA (200 μ L). After 5 minutes, water (1 mL) was added and the solution lyophilised overnight to yield compound **E-15-8** as a white solid (7.6 mg, 99 %).

Compound E-15:

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Compound E-15 was prepared according to the same method as for compound E-12, using amine E-15-2 (7.6 mg, 5.55 μmol , 1 eq.), 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanoate (2 mg, 6.65 μmol , 1.2 eq.) and DIEA (5 μL , 0.028 mmol, 5 eq.) in acetonitrile (0.5 mL). Compound E-15 was isolated as a white solid (4.2 mg, 48 %).

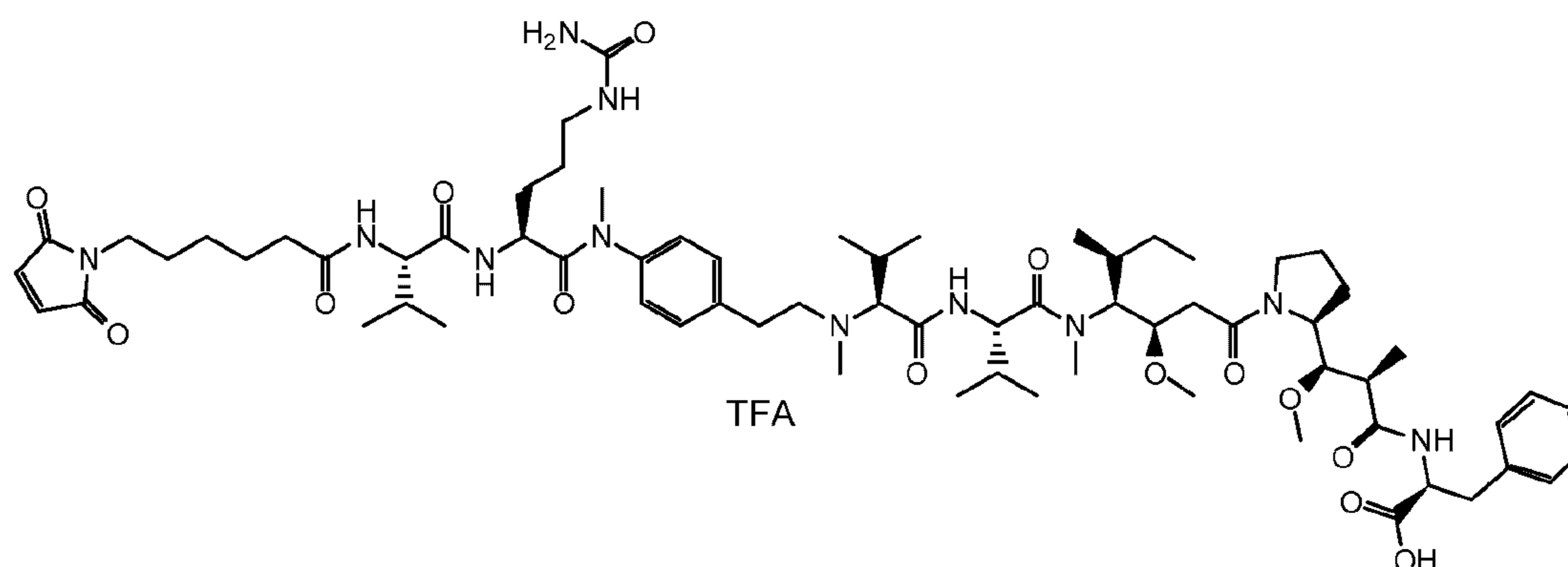
m/z (Q-TOF MS ESI+) 1471.8169 (2 %, MNa^+ , $\text{C}_{76}\text{H}_{112}\text{N}_{12}\text{NaO}_{16}$ requires 1471.8211), 725.4223 (100 %, $(\text{MH}_2)^{2+}$, $\text{C}_{76}\text{H}_{114}\text{N}_{12}\text{O}_{16}$ requires 725.4232), 483.9482 (10 %, $(\text{MH}_3)^{3+}$, $\text{C}_{76}\text{H}_{115}\text{N}_{12}\text{O}_{16}$ requires 483.9513).

10

Compound F-13

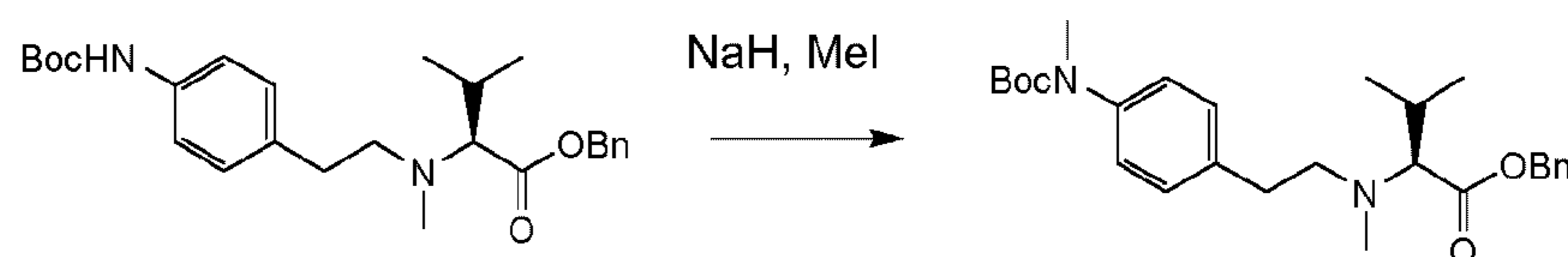
((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-((*S*)-2-((*S*)-2-(6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-*N*-methyl-5-ureidopentanamido)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalanine 2,2,2-trifluoroacetate

15



Compound F-13-1: benzyl *N*-(4-((*tert*-butoxycarbonyl)(methyl)amino)phenethyl)-*N*-methyl-*L*-valinate

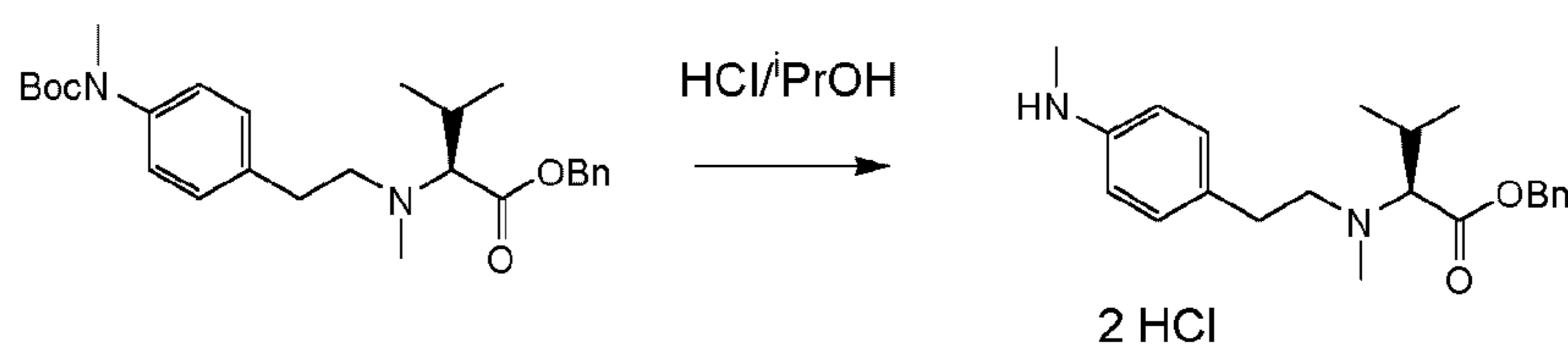
5



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Compound **11C** (250 mg, 0.567 mmol, 1 eq.) was dissolved in THF (10 ml) followed by the addition of NaH (60 % suspension in mineral oil, 68 mg, 1.702 mmol, 3 eq.). The mixture was stirred for 5 minutes before adding iodomethane (106 μ L, 1.702 mmol, 3 eq.). The reaction was stirred for 2 hours at room temperature before quenching with water and separating between EtOAc (100 mL) and water (50 mL). The organic phase was dried over MgSO₄ and evaporated to dryness to yield compound **F-13-1** as a yellow oil (250 mg, 97 %), which was used without further purification.

Compound F-13-2: benzyl *N*-methyl-*N*-(4-(methylamino)phenethyl)-*L*-valinate



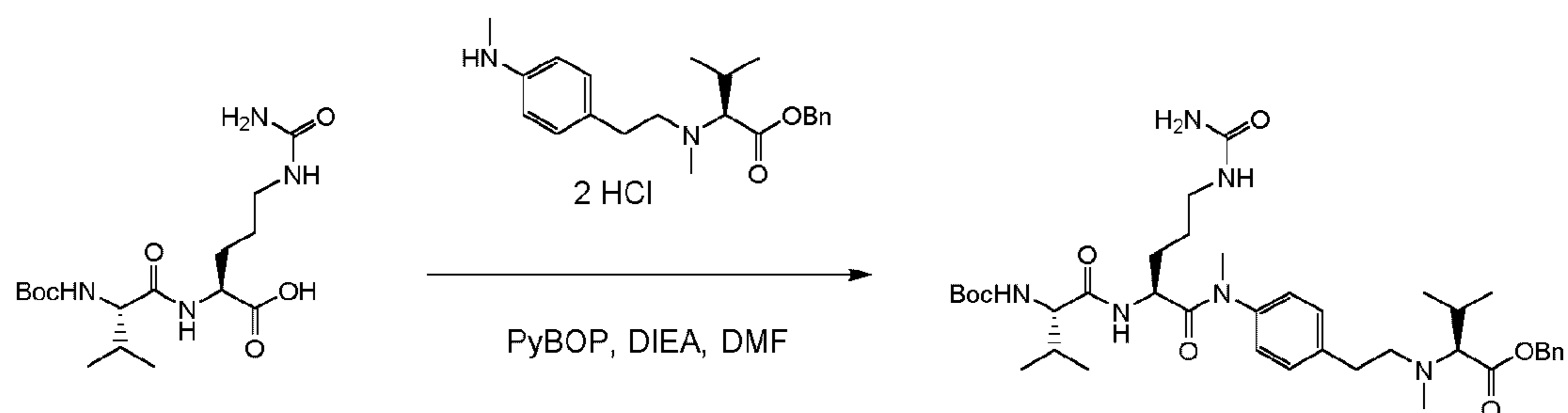
15

Boc-protected aniline **F-13-1** (250 mg, 0.550 mmol, 1 eq) was dissolved in MeOH (5 mL) followed by the addition of 1 mL of a commercially-available solution of HCl in *i*PrOH (5 – 6 M). The solution was stirred at room temperature for 2 hours before evaporating to dryness under reduced pressure. The resulting yellow oil was triturated with Et₂O to yield compound **F-13-2** as a yellow solid (202 mg, 94 %).

20

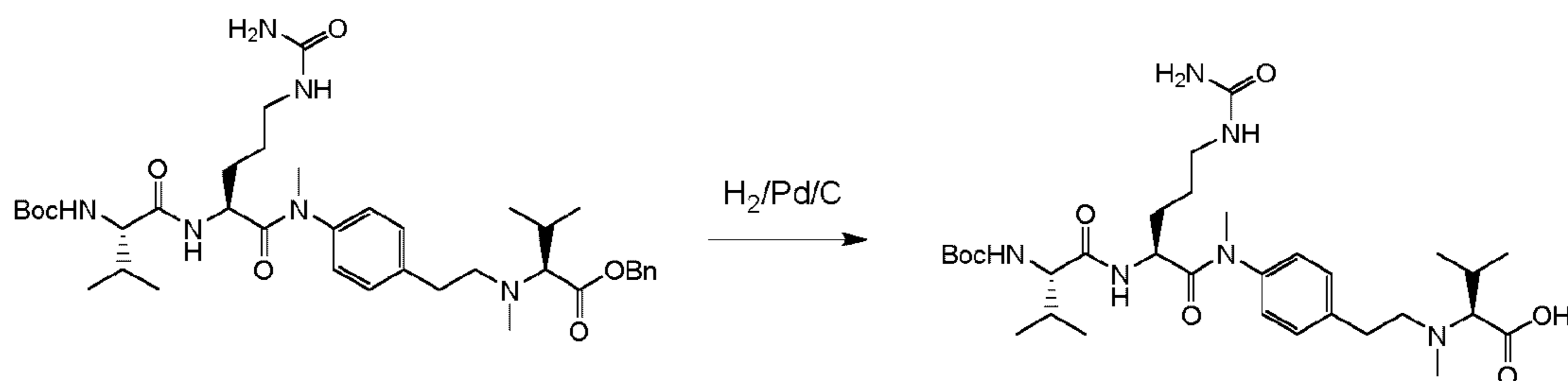
Compound F-13-3: benzyl *N*-(4-((*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-*N*-methyl-5-ureidopentanamido)phenethyl)-*N*-methyl-*L*-valinate

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Acid **F-11-3** (190 mg, 0.508 mmol, 1.5 eq.) was dissolved in dry DMF (1 ml), followed by the addition of DIEA (118 μ L, 0.677 mmol, 2 eq.), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP - 264 mg, 0.508 mmol, 1.5 eq.) and aniline **F-13-2** (120 mg, 0.339 mmol, 1 eq.). The mixture was stirred at room temperature overnight and the solvents evaporated under reduced pressure. The residue was purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **F-13-3** as a white solid (140 mg, 45 %).

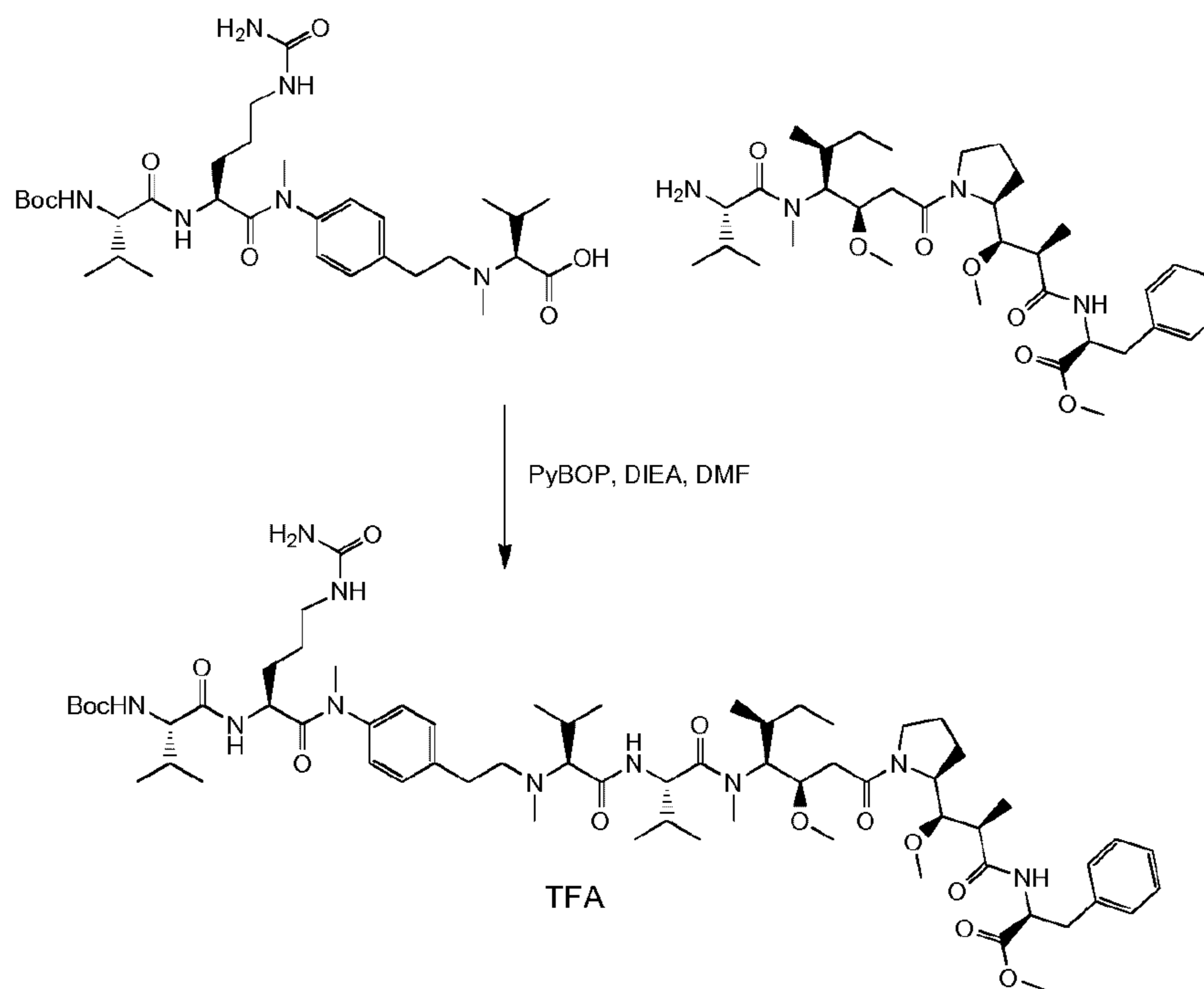
Compound F-13-4: *N*-(4-((*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-*N*-methyl-5-ureidopentanamido)phenethyl)-*N*-methyl-*L*-valine



Compound **F-13-3** (116 mg, 0.163 mmol, 1 eq.) was dissolved in MeOH (5 ml) in the presence of Pd/C 10% (30 mg) and hydrogenated for 2 hours at ambient temperature and atmospheric pressure. The reaction medium was filtered and concentrated under reduced pressure to yield 110 mg (99 %) of compound **F-13-4** as a beige solid.

Compound F-13-5: methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-((*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-*N*-methyl-5-ureidopentanamido)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-

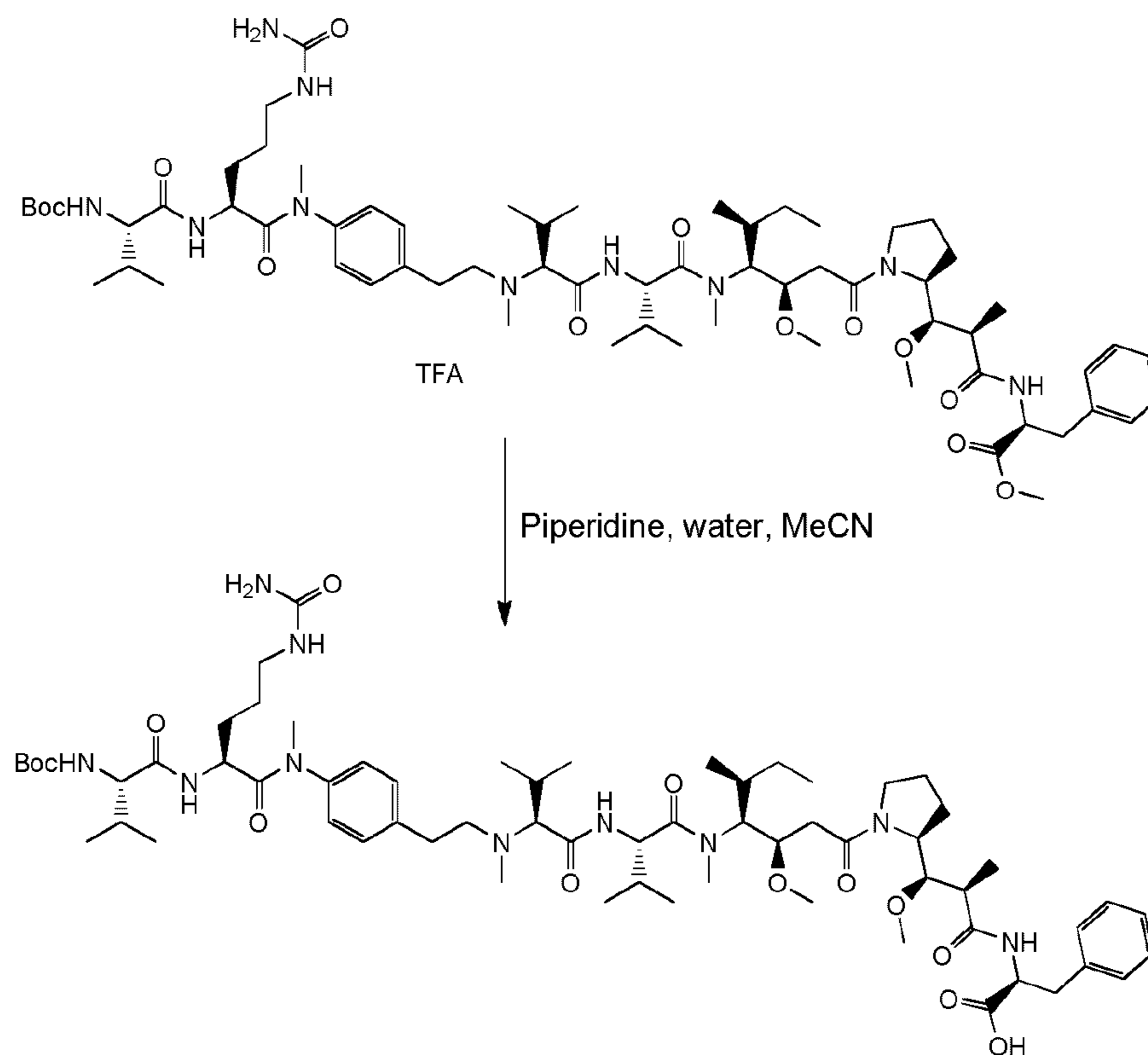
dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalaninate 2,2,2-trifluoroacetate



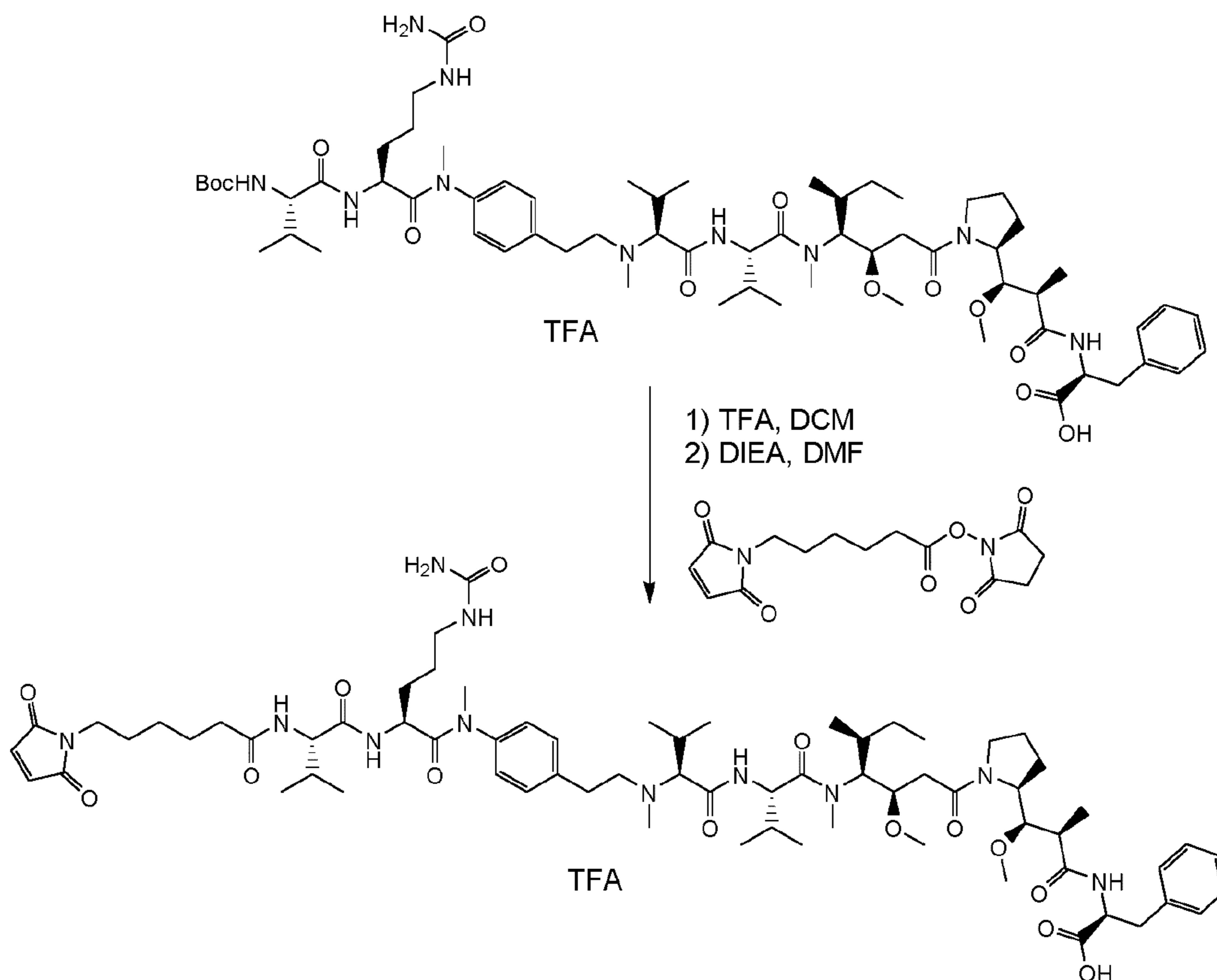
Amine **3D** (89 mg, 0.140 mmol, 1 eq.) and acid **F-13-4** (145 mg, 0.210 mmol, 1.5 eq.) were dissolved in dry DMF (4 mL), and PyBOP (109 mg, 0.210 mmol, 1.5 eq.) and DIEA (73 μ L, 0.420 mmol, 3 eq.) were added. The mixture was stirred for 1 hour at room temperature and the solvent evaporated. The residue was separated between EtOAc and water, and the organic phase dried over MgSO_4 , filtered and evaporated under reduced pressure. The crude product was purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **F-13-5** as a white solid (140 mg, 73 %).

Compound F-13-6: ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-((*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-*N*-methyl-5-ureidopentanamido)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalanine 2,2,2-trifluoroacetate

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Compound **F-13-5** (140 mg, 0.104 mmol, 1 eq.) was dissolved in a mixture of water (4 mL), acetonitrile (4 mL) and piperidine (2 mL) and stirred at room temperature for 4 hours. The solvent was evaporated under reduced pressure and the residue purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **F-13-6** as a white solid (115 mg, 83 %).

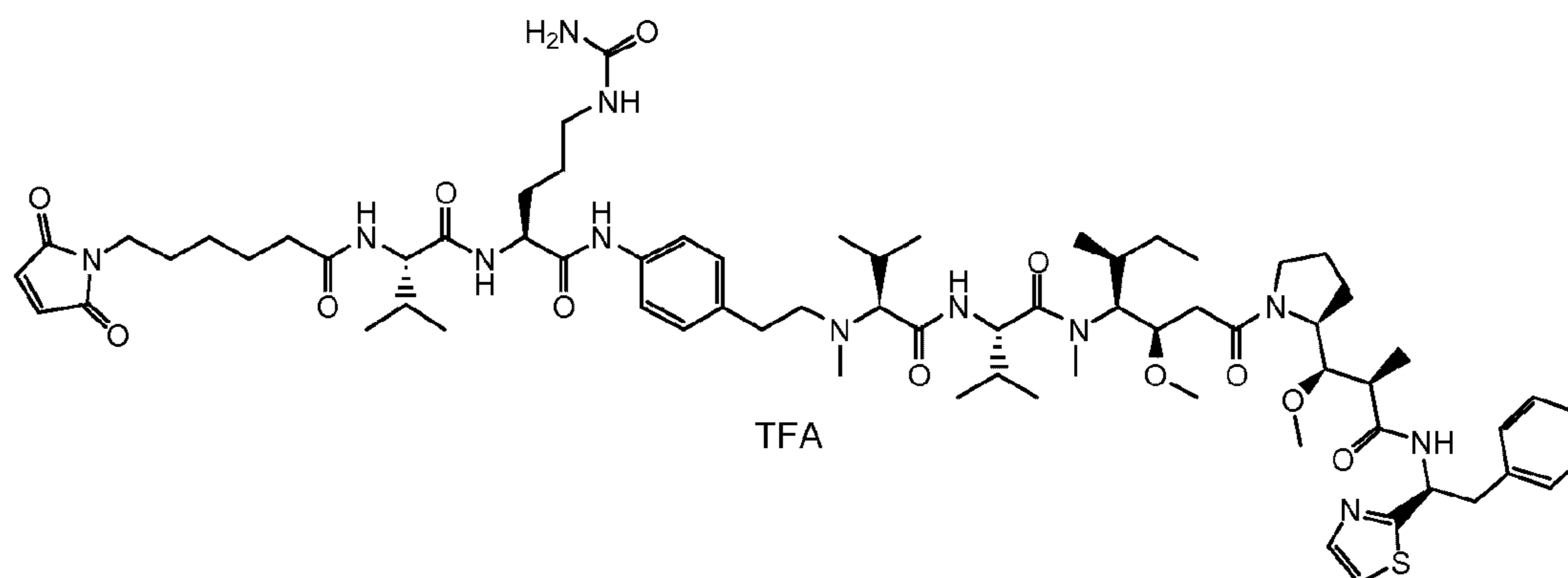
Compound F-13:

Compound **F-13** was prepared according to the same method as for compound **E-11**, using Boc-protected amine **F-13-6** (55 mg, 0.041 mmol, 1.0 eq.) in DCM (0.5 mL) and TFA (100 μ L, 30 eq.), followed by dilution with DMF (1 mL), quenching with (DIEA (320 μ L, 45 eq) then reaction with 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanoate (15 mg, 0.049 mmol, 1.2 eq.). After purification by preparative HPLC and lyophilisation, compound **F-13** was obtained as a white solid (14 mg, 24 %).

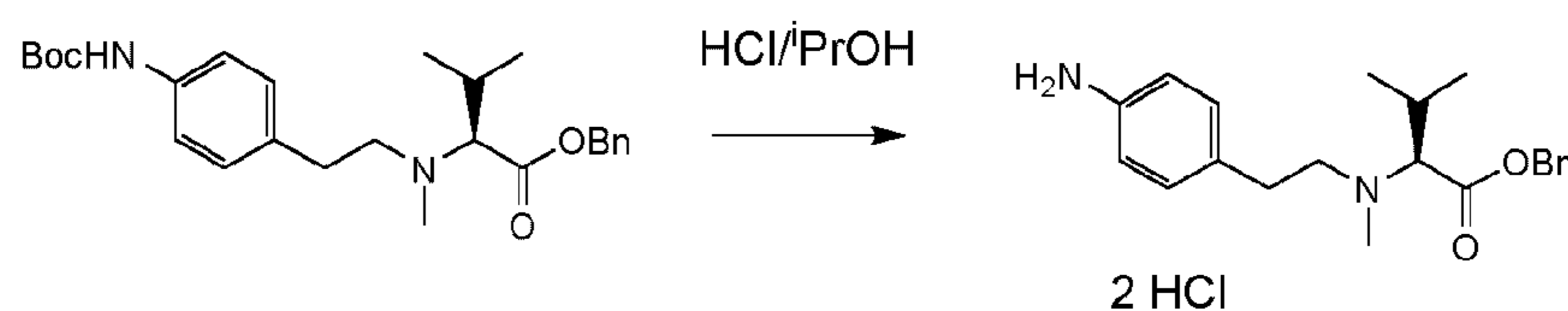
10 m/z (Q-TOF MS ESI+) 1314.8067 (2 %, MH^+ , $C_{69}H_{108}N_{11}O_{14}$ requires 1314.8072), 657.9067 (100 %, $(MH_2)^{2+}$, $C_{69}H_{109}N_{11}O_{14}$ requires 657.9072).

Compound F-61

N-((*S*)-1-(((*S*)-1-(4-((3*R*,4*S*,7*S*,10*S*)-4-((*S*)-*sec*-butyl)-7,10-diisopropyl-3-(2-((*S*)-2-
 ((1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-(((*S*)-2-phenyl-1-(thiazol-2-
 yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-5,11-dimethyl-6,9-dioxo-2-oxa-
 5,8,11-triazatridecan-13-yl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-3-
 methyl-1-oxobutan-2-yl)-6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanamide
 2,2,2-trifluoroacetate



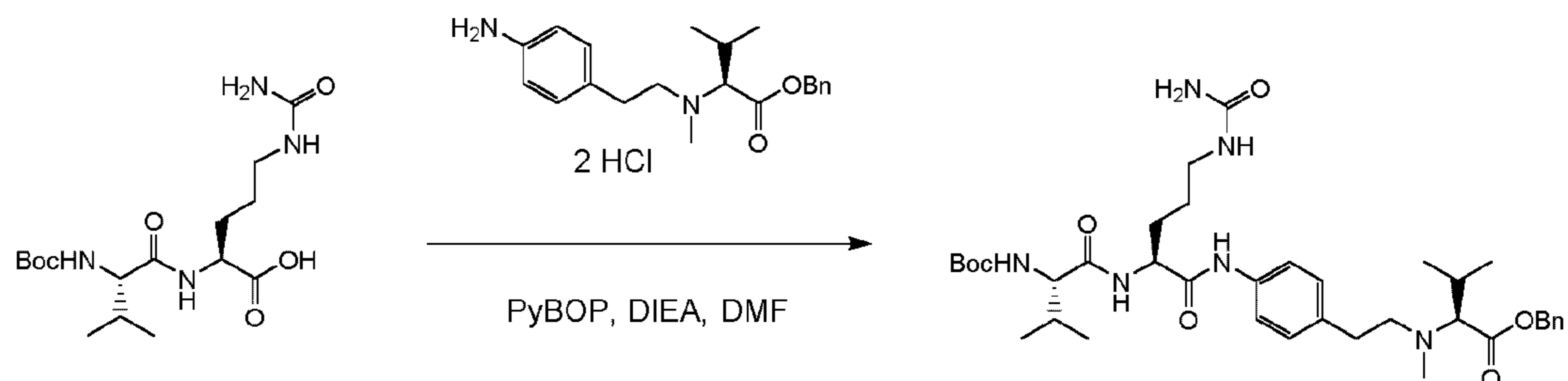
Compound F-61-1: benzyl *N*-(4-aminophenethyl)-*N*-methyl-*L*-valinate
 dihydrochloride



Compound **11C** (1.0 g, 2.27 mmol, 1 eq.) was dissolved in 8 mL of a
 commercially-available solution of HCl in *i*PrOH (5 – 6 M). The mixture was stirred for
 2 hours at room temperature before evaporating to dryness under reduced pressure. The
 residue was triturated twice with Et₂O (30 mL) and dried under vacuum to yield
 compound **F-61-1** as a white solid (916 mg, 98 %).

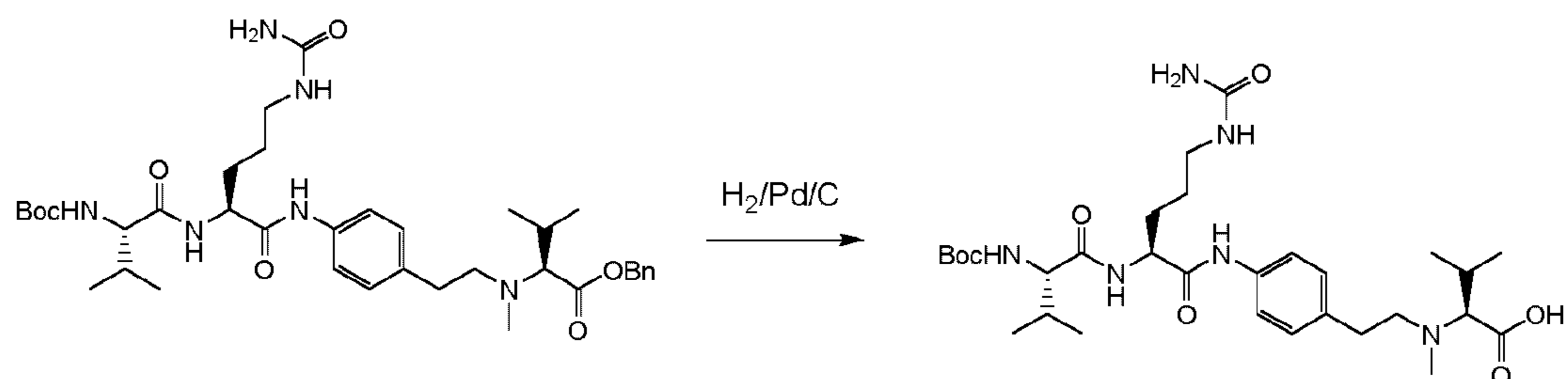
Compound F-61-2: benzyl *N*-(4-((*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-
 methylbutanamido)-5-ureidopentanamido)phenethyl)-*N*-methyl-*L*-valinate

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Acid **E-11-3** (769 mg, 2.05 mmol, 1.5 eq.) was dissolved in dry DMF (2.5 ml) followed by the addition of DIEA (957 μ L, 5.48 mmol, 4 eq.) and PyBOP (1.07 g, 2.05 mmol, 1.5 eq.). Aniline **F-61-1** (566 mg, 1.369 mmol, 1 eq.) was added and the mixture stirred at room temperature overnight. The solvents were evaporated under reduced pressure, and the residue purified on silica gel (DCM/MeOH) to yield 969 mg (102 %) of compound **F-61-2** as a white solid.

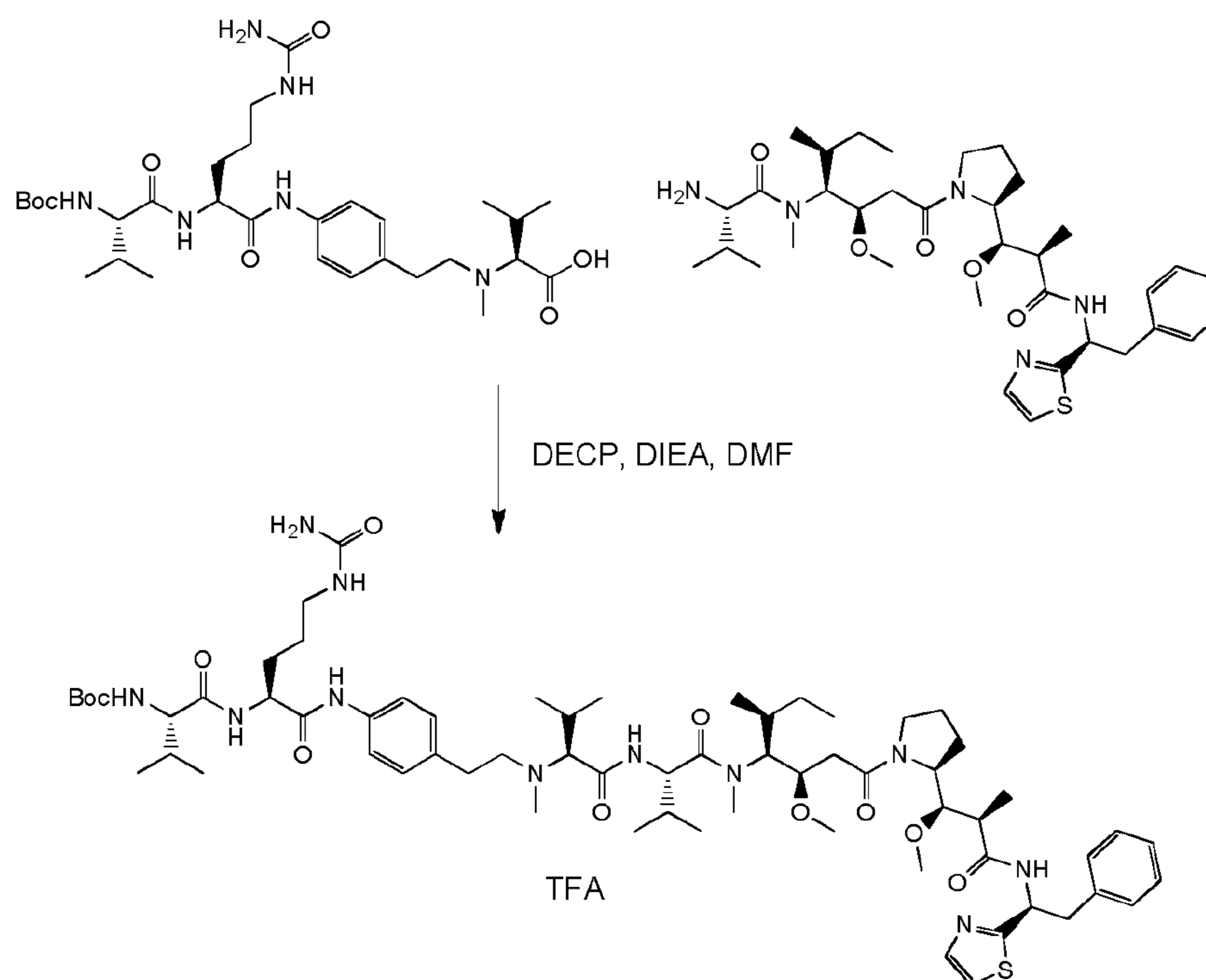
Compound F-61-3: *N*-(4-((*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)phenethyl)-*N*-methyl-*L*-valine



Compound **F-61-2** (969 mg, 1.28 mmol, 1 eq.) was dissolved in MeOH (20 ml) in the presence of Pd/C 10% (270 mg) and hydrogenated for 3 hours at ambient temperature and atmospheric pressure. The reaction medium was filtered and concentrated under reduced pressure, and the residue purified on silica gel (DCM/MeOH/AcOH) to yield 520 mg (67 %) of compound **F-61-3** as a white solid.

Compound F-61-4: *tert*-butyl ((*S*)-1-(((*S*)-1-((4-((3*R*,4*S*,7*S*,10*S*)-4-((*S*)-*sec*-butyl)-7,10-diisopropyl-3-(2-((*S*)-2-((1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-(((*S*)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-5,11-dimethyl-6,9-dioxo-2-oxa-5,8,11-triazatridecan-13-yl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate 2,2,2-trifluoroacetate

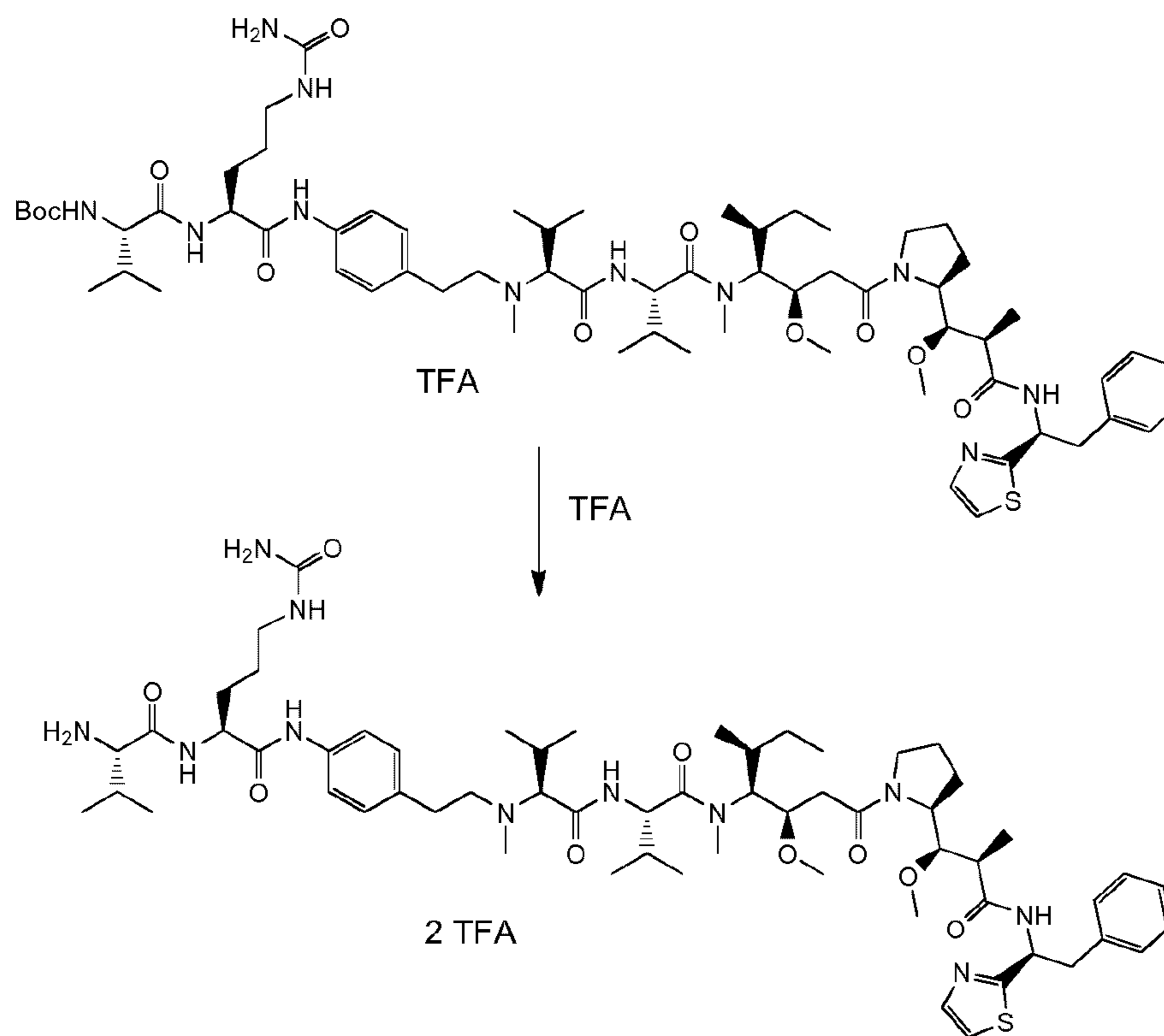
238



Acid **F-61-3** (67.5 mg, 0.111 mmol, 1.5 eq.) was dissolved in dry DMF (2 mL) and DECP (17 μ L, 0.111 mmol, 1.5 eq.) and DIEA (39 μ L, 0.223 mmol, 3 eq.) were added. After stirring for 15 minutes at room temperature, amine **1Y** (50 mg, 0.074 mmol, 1 eq.) was added and the solution stirred overnight. The solvent was evaporated under reduced pressure, and the residue purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **F61-4** as a white solid (28 mg, 28 %).

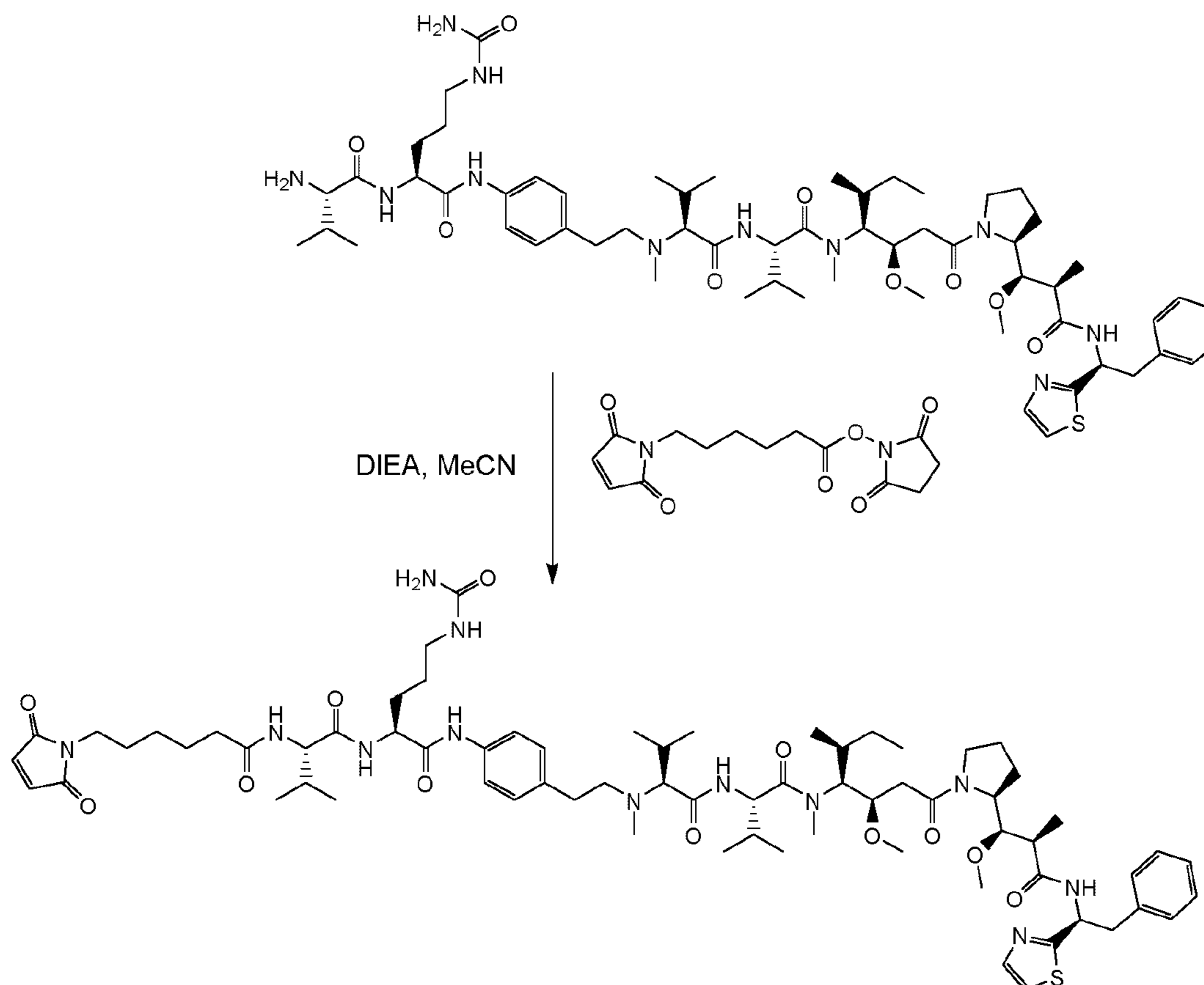
Compound F-61-5: (*S*)-2-((*S*)-2-amino-3-methylbutanamido)-*N*-(4-((*3R,4S,7S,10S*)-4-((*S*)-*sec*-butyl)-7,10-diisopropyl-3-(2-((*S*)-2-((*1R,2R*)-1-methoxy-2-methyl-3-oxo-3-(((*S*)-2-phenyl-1-(thiazol-2-yl)ethyl)amino)propyl)pyrrolidin-1-yl)-2-oxoethyl)-5,11-dimethyl-6,9-dioxo-2-oxa-5,8,11-triazatridecan-13-yl)phenyl)-5-ureidopentanamide bis(2,2,2-trifluoroacetate)

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Compound **F-61-4** (28 mg, 0.021 mmol, 1.0 eq.) was dissolved in TFA (200 μ L). After 5 minutes, water (2 mL) and acetonitrile (0.5 mL) were added and the solution lyophilised overnight to yield compound **F-61-5** as a colourless oil (38 mg, 134

5 %).

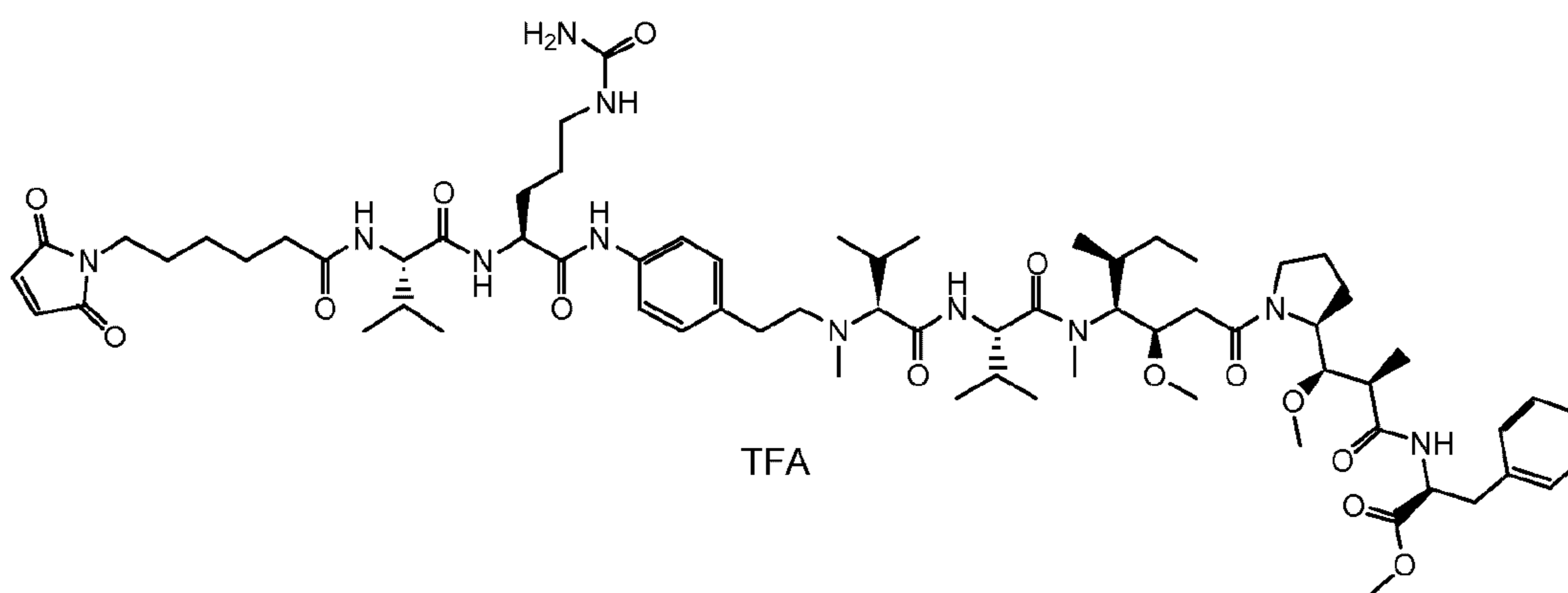
Compound F-61:

Compound **F-61-5** (28.3 mg, 0.020 mmol, 1 eq.) was dissolved in acetonitrile (0.5 mL), followed by 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (9 mg, 0.029 μmol , 1.4 eq.) and DIEA (25 μL , 0.143 mmol, 7 eq.). The mixture was stirred for 4.5 hours, after which time HPLC analysis showed the presence of starting material but complete consumption of the succinimide. Supplementary 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate was therefore added (3 mg, 0.01 μmol , 0.5 eq.) and the reaction stirred for 1.5 hours. HPLC analysis showed complete consumption of the starting material. The solvent was evaporated to dryness and the residue triturated twice with a mixture of EtOAc/Et₂O (80/20) to yield compound **F-61** as an off-white solid (19.4 mg, 70 %).

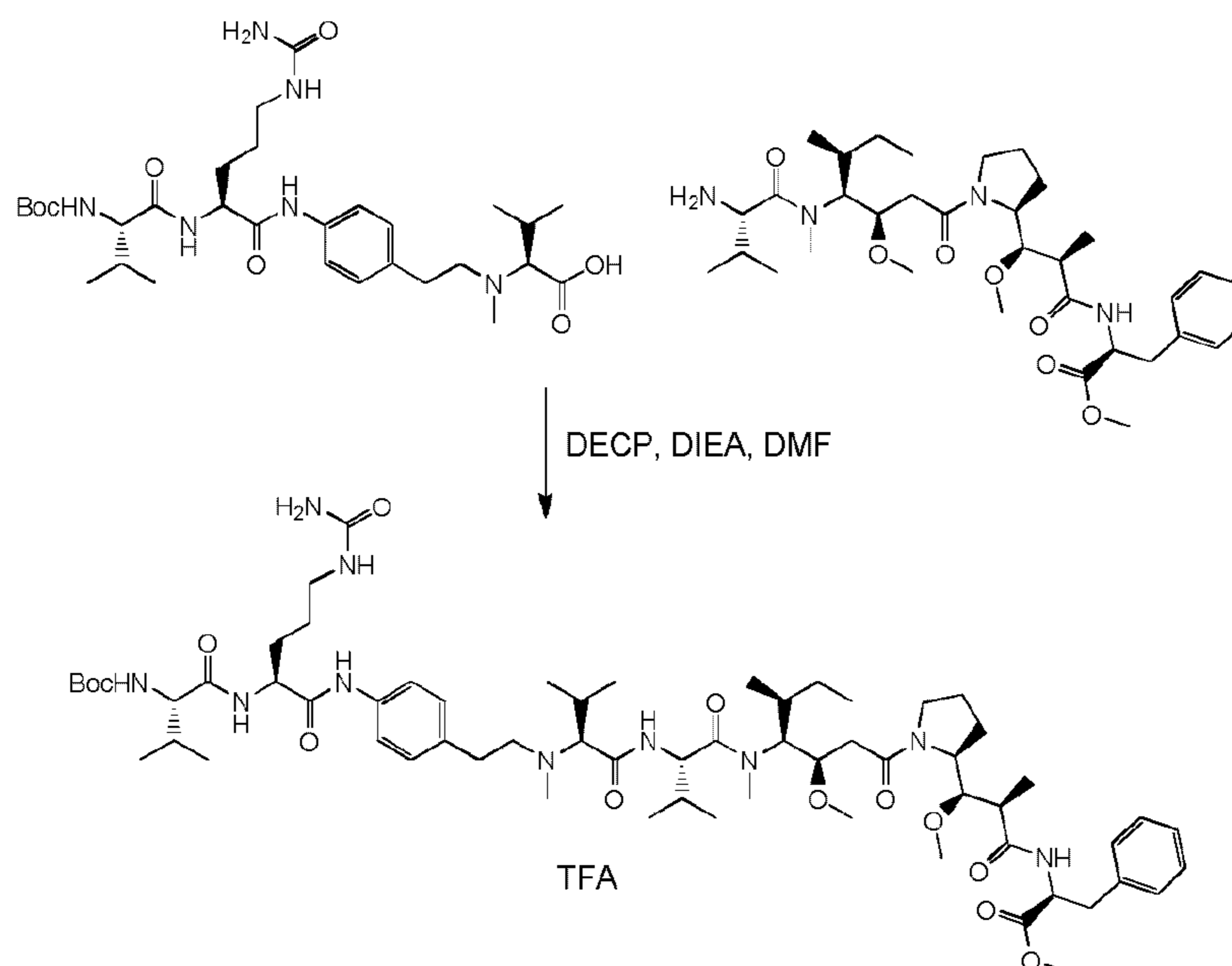
m/z (Q-TOF MS ESI+) 1361.7725 (2 %, MNa^+ , $\text{C}_{70}\text{H}_{106}\text{N}_{12}\text{NaO}_{12}\text{S}$ requires 1361.7666), 670.3961 (100 %, $(\text{MH}_2)^{2+}$, $\text{C}_{70}\text{H}_{108}\text{N}_{12}\text{O}_{12}\text{S}$ requires 670.3960).

Compound F-62:

methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-((*S*)-2-((*S*)-2-(6-(2,5-dioxo-
 2,5-dihydro-1*H*-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-
 ureidopentanamido)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-
 5 dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-
 2-methylpropanoyl)-*L*-phenylalaninate 2,2,2-trifluoroacetate

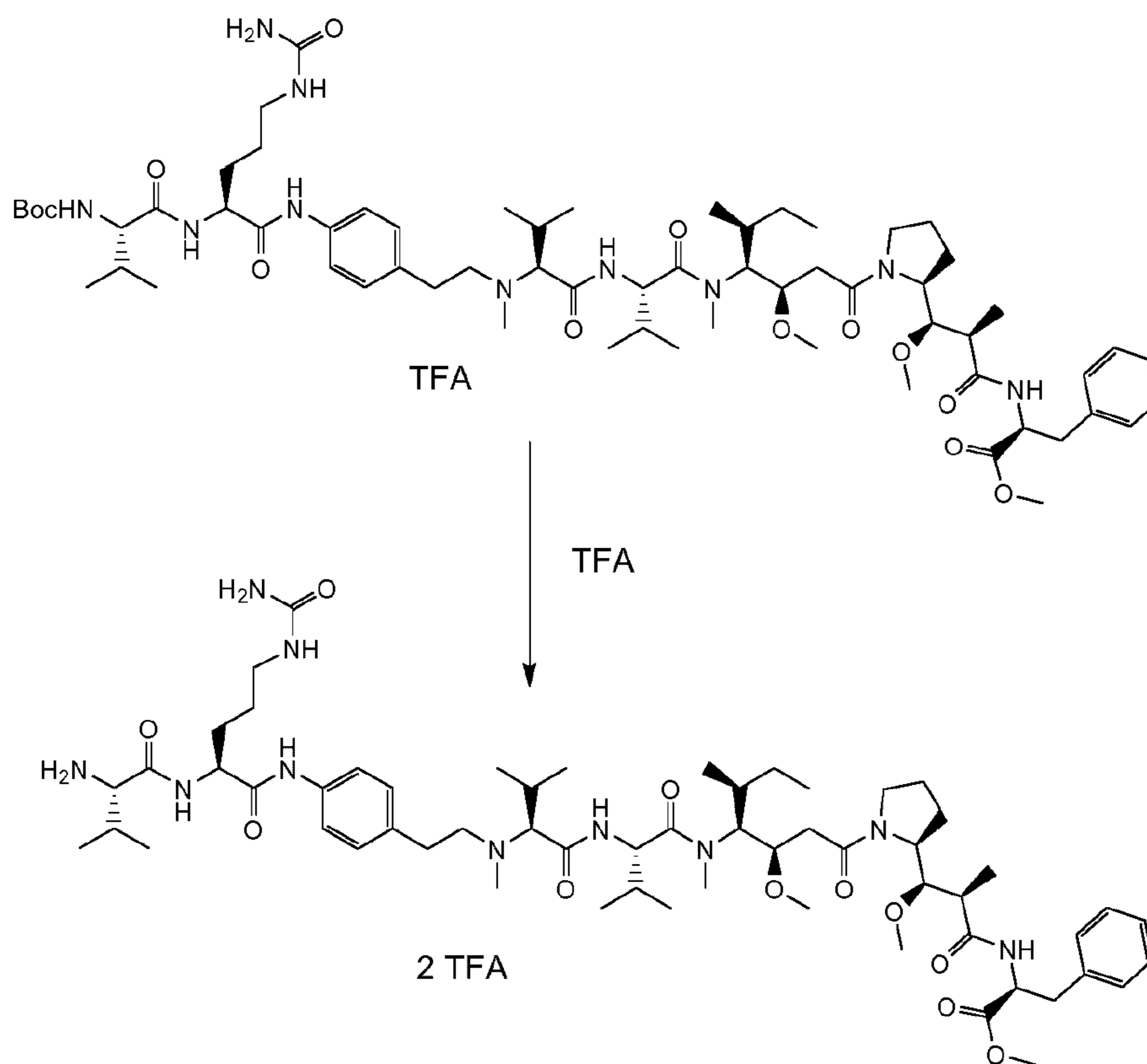
**Compound F-62-1:** methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-

10 ((*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-5-
 ureidopentanamido)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-
 dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-
 methylpropanoyl)-*L*-phenylalaninate 2,2,2-trifluoroacetate

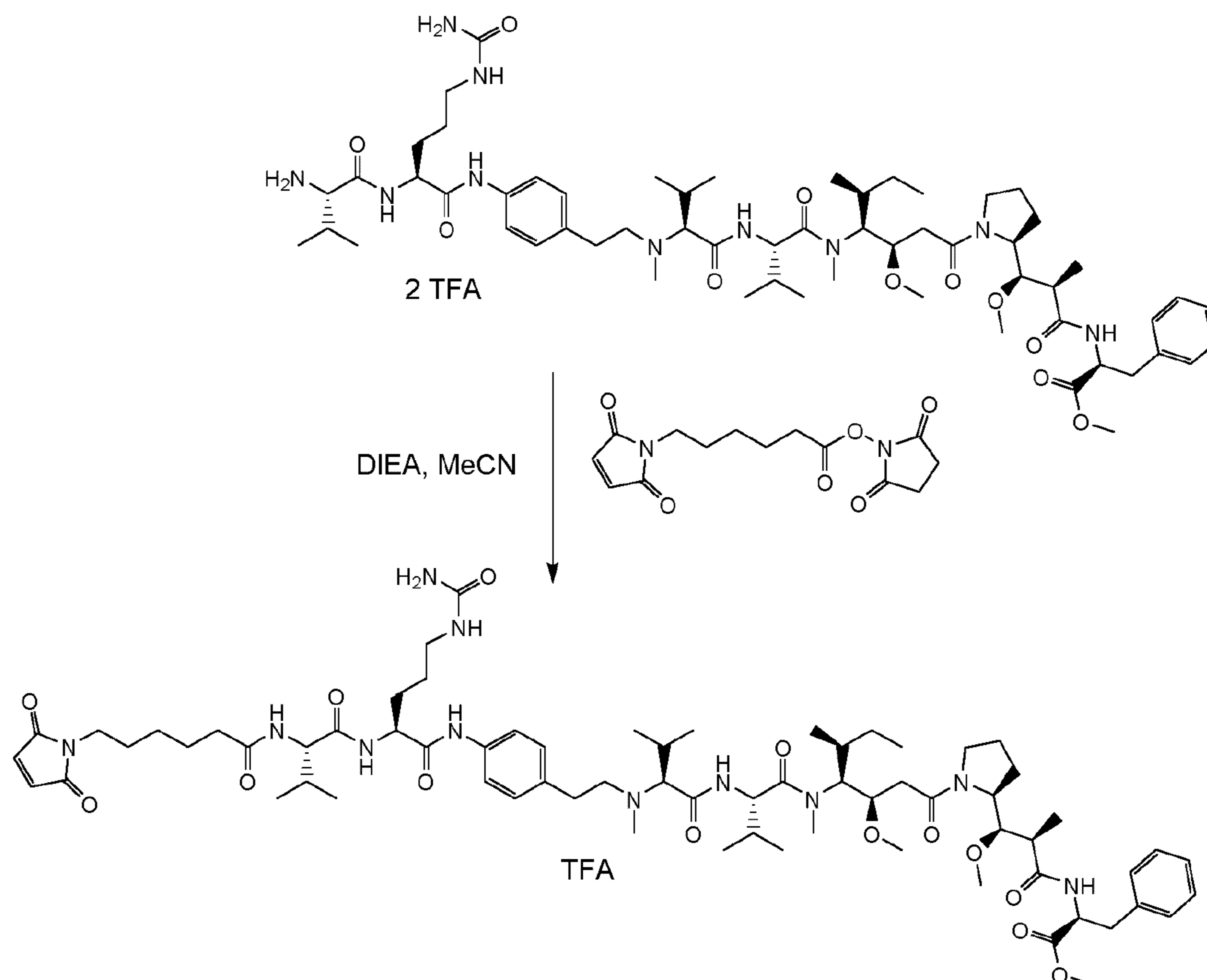


Compound **F-62-1** was prepared in similar manner to compound **F-61-4** from amine **3D** (100 mg, 0.158 mmol, 0.9 eq.), acid **F-61-3** (108 mg, 0.178 mmol, 1 eq.), DECP (41 μ L, 0.267 mmol, 1.5 eq.) and DIEA (93 μ L, 0.534 mmol, 3 eq.) in DMF (2 mL). After purification by preparative HPLC, compound **F-62-1** was obtained as a white solid (93 mg, 39 %).

Compound F-62-2: methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-((*S*)-2-((*S*)-2-amino-3-methylbutanamido)-5-ureidopentanamido)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalaninate bis(2,2,2-trifluoroacetate)



Compound **F-62-1** (35 mg, 0.026 mmol, 1.0 eq.) was dissolved in TFA (200 μ L). After 10 minutes, water (2 mL) and acetonitrile (0.5 mL) were added and the solution lyophilised overnight to yield compound **F-62-2** as a white solid (34 mg, 105 %).

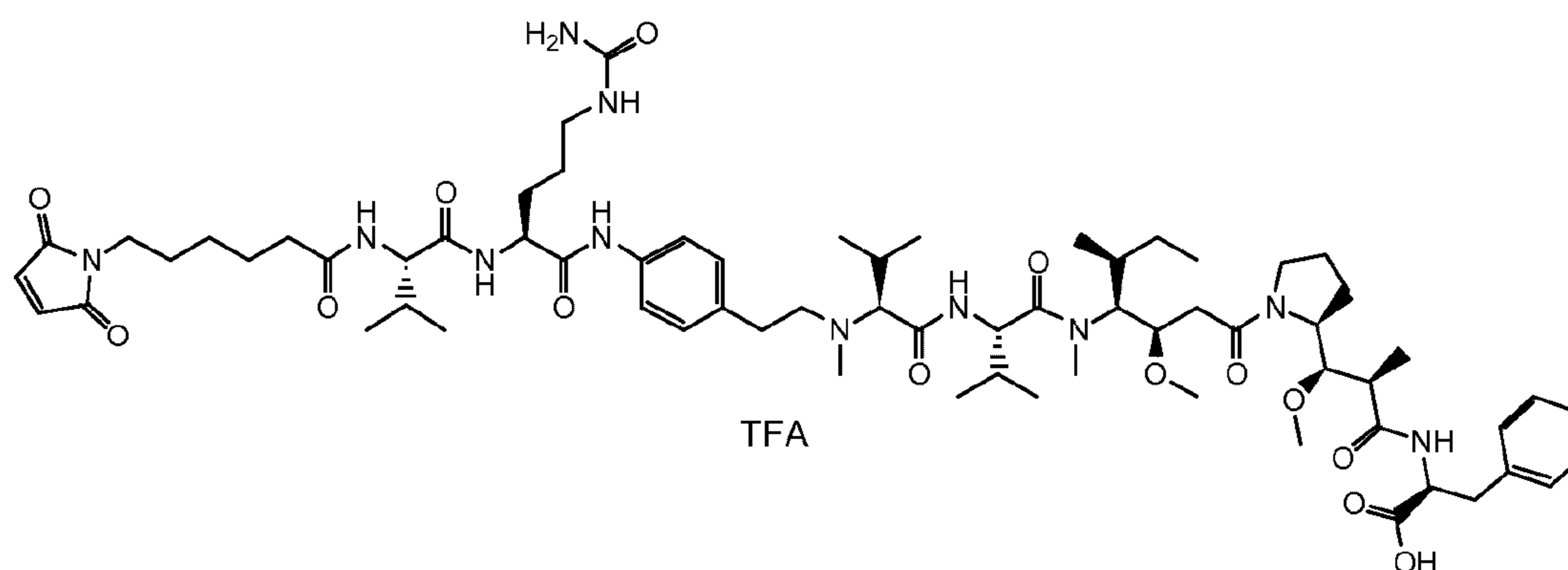
Compound F-62:

Amine **F-62-2** (34 mg, 5.55 μmol , 1 eq.) was dissolved in acetonitrile (3 mL). DIEA (5 μL , 0.028 mmol, 5 eq.) and 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (2 mg, 6.65 μmol , 1.2 eq.) were added. HPLC analysis showed complete consumption of the starting material. The solvent was evaporated to dryness and the residue triturated with a mixture of EtOAc/Et₂O (80/20). The crude product was purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μm , 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **F-62** as a white solid (5.5 mg, 13 %).

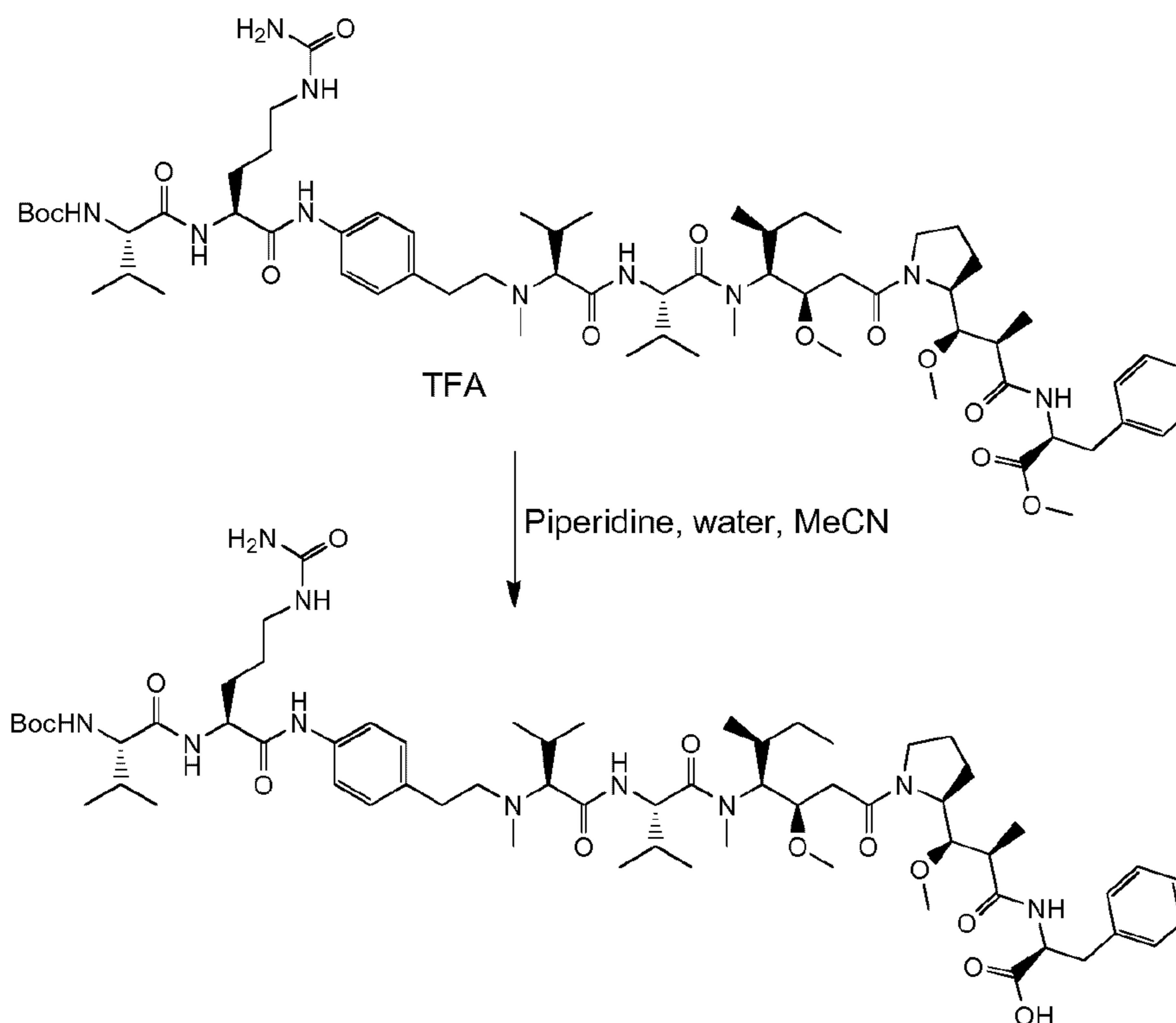
m/z (Q-TOF MS ESI⁺) 1336.7859 (2 %, MNa^+ , $\text{C}_{69}\text{H}_{107}\text{N}_{11}\text{NaO}_{14}$ requires 1336.7891), 657.9073 (100 %, $(\text{MH}_2)^{2+}$, $\text{C}_{69}\text{H}_{109}\text{N}_{11}\text{O}_{14}$ requires 657.9072).

Compound F-63:

5 **((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-((*S*)-2-((*S*)-2-(6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)phenethyl)(methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalanine 2,2,2-trifluoroacetate**



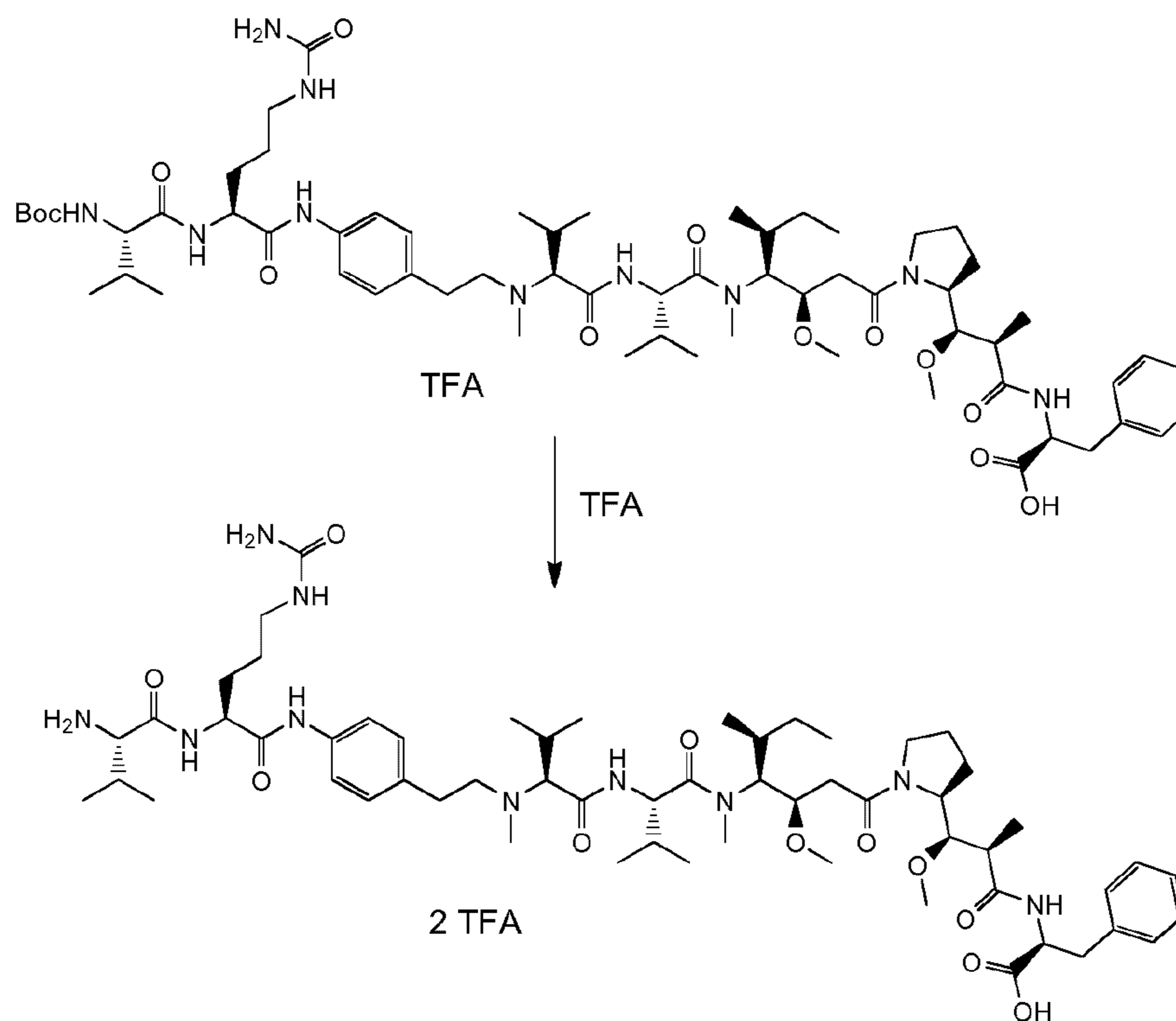
10 **Compound F-63-1:** ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-((*S*)-2-((*S*)-2-((*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)phenethyl) (methyl)amino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalanine



Compound **F-62-1** (157 mg, 0.118 mmol, 1 eq.) was dissolved in a mixture of water (4.5 mL), acetonitrile (4.5 mL) and piperidine (3.5 mL) and stirred at room temperature for 5 hours. The solvent was evaporated under reduced pressure and the residue triturated Et₂O (60 mL). The solid was collected by filtration and rinsed twice with Et₂O (10 mL) to yield compound **F-63-1** as an off-white solid (153 mg, 100 %).

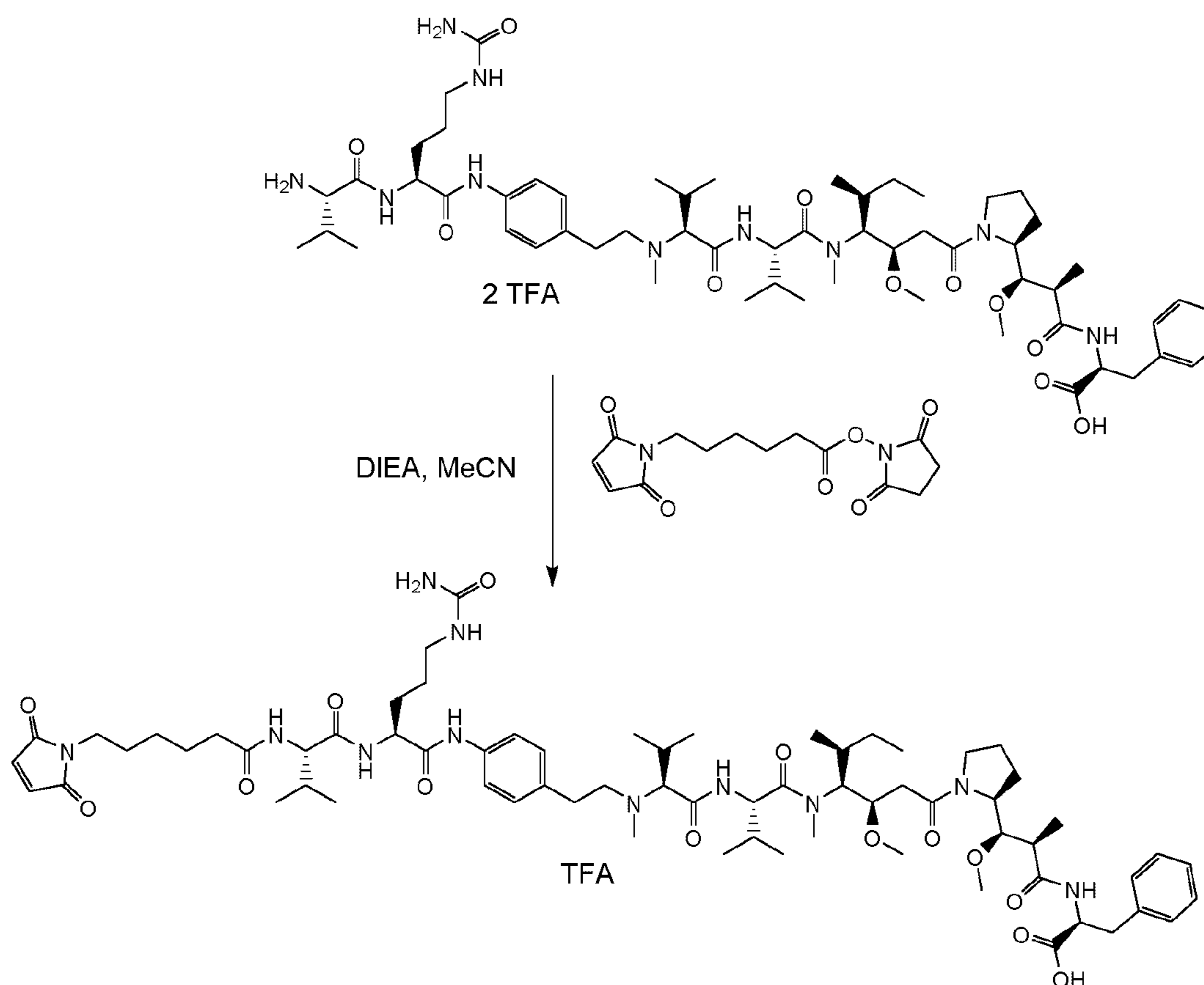
Compound F-63-2: ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-((*S*)-2-((*S*)-2-amino-3-methylbutanamido)-5-ureidopentanamido)phenethyl)(methylamino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalanine bis 2,2,2-trifluoroacetate

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Compound **F-63-1** (153 mg, 0.127 mmol, 1.0 eq.) was dissolved in TFA (200 μ L). After 10 minutes, water (2 mL) and acetonitrile (0.5 mL) were added and the solution lyophilised overnight to yield compound **F-63-2** as a white solid (34 mg, 105

5 %).

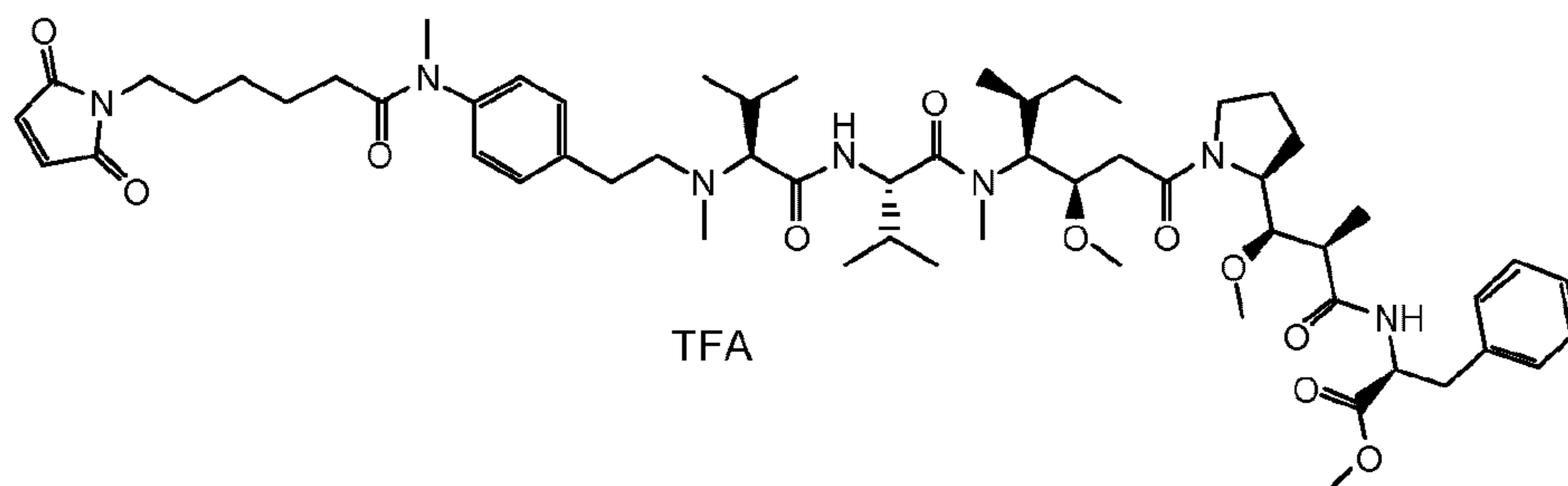
Compound F-63:

Amine **F-63-2** (100 mg, 0.082 mmol, 1 eq.) was dissolved in a mixture of acetonitrile (2 mL) and DMF (0.5 mL), and 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanoate (45 mg, 0.147 mmol, 1.8 eq.) and DIEA (71 μ L, 0.409 mmol, 5 eq.) were added. After stirring at room temperature for 4.5 hours, the solvent was evaporated under reduced pressure. The crude product was purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **F-63** as a white solid after (42 mg, 36 %).

m/z (Q-TOF MS ESI+) 1300.7901 (2 %, MH^+ , $C_{68}H_{106}N_{11}O_{14}$ requires 1300.7915), 650.8990 (100 %, $(MH_2)^{2+}$, $C_{68}H_{107}N_{11}O_{14}$ requires 650.8994).

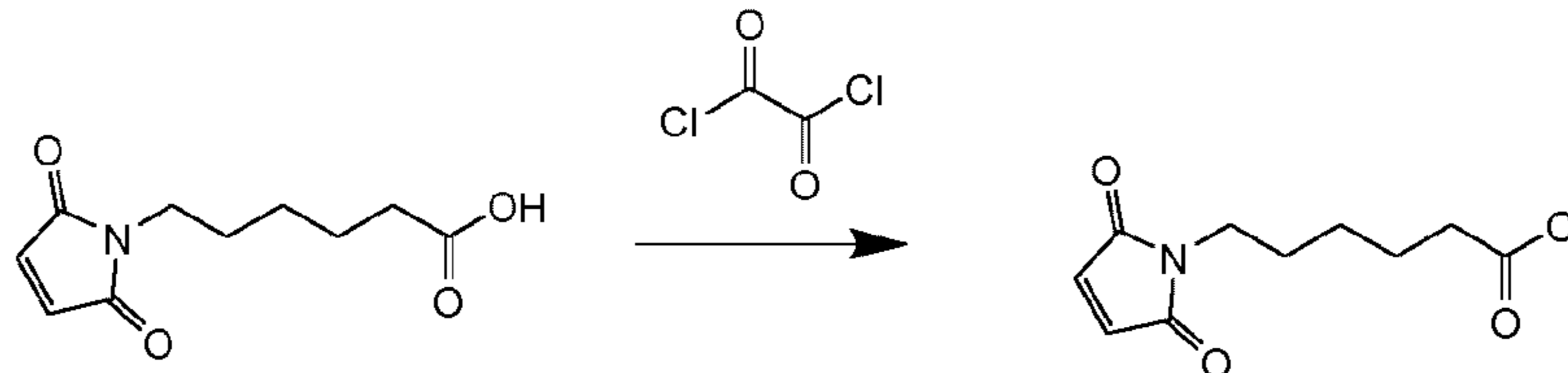
Compound G-12

methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-(6-(2,5-dioxo-2,5-dihydro-
 1*H*-pyrrol-1-yl)-*N*-methylhexanamido)phenethyl)(methylamino)-3-
 methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-
 5 methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-
 phenylalaninate 2,2,2-trifluoroacetate



Compound G-12-1: benzyl *N*-(4-aminophenethyl)-*N*-methyl-*L*-valinate

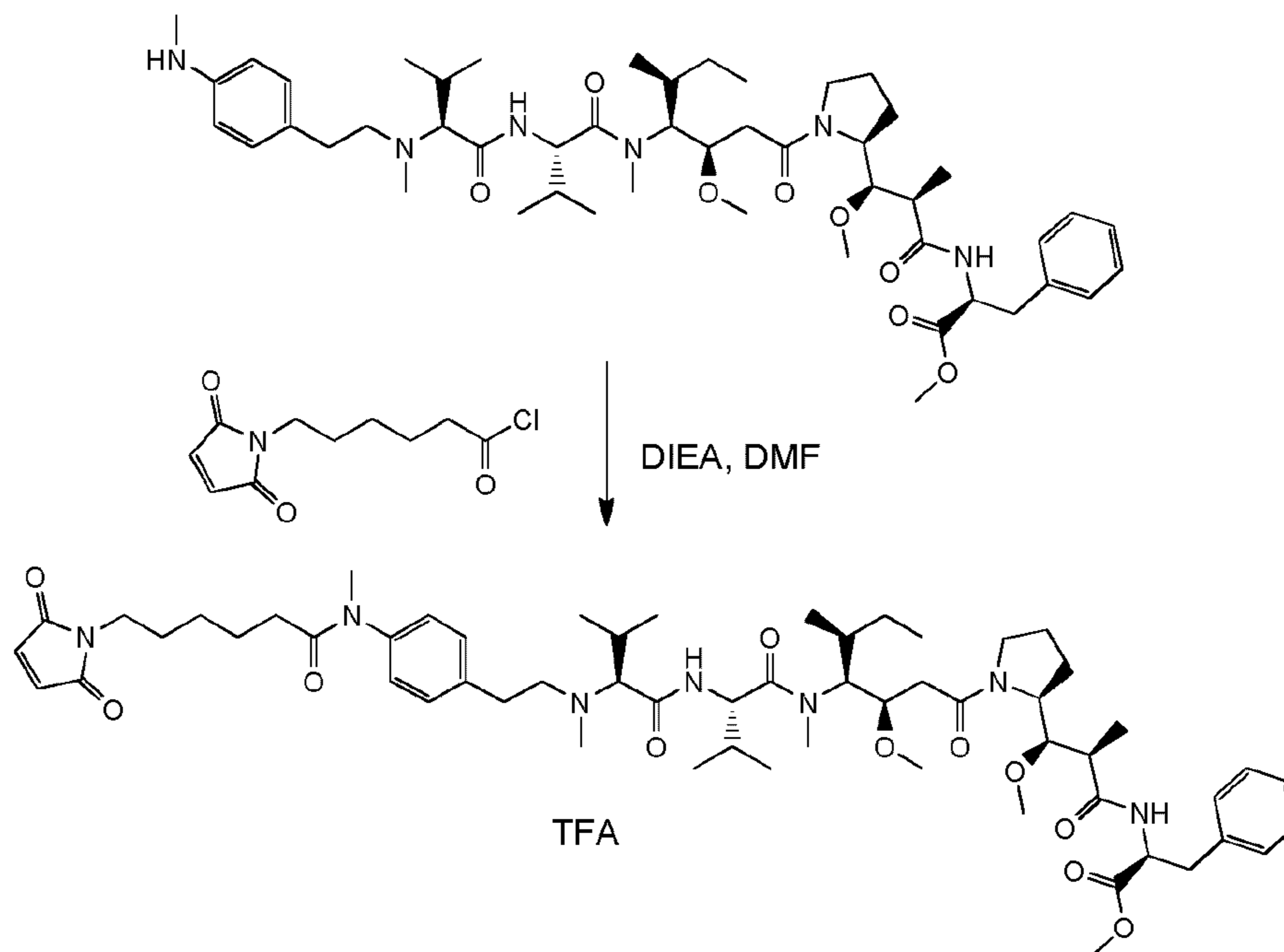
10 dihydrochloride



Into oxalyl chloride (3 mL) was dissolved 6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-
 1-yl)hexanoic acid (200 mg, 0.947 mmol, 1 eq.). The solution was stirred at room
 temperature for 5 hours before evaporating to dryness under reduced pressure.

15 Compound **G-12-1** was obtained as a beige solid (217 mg, 100 %) and used in the next
 step without purification.

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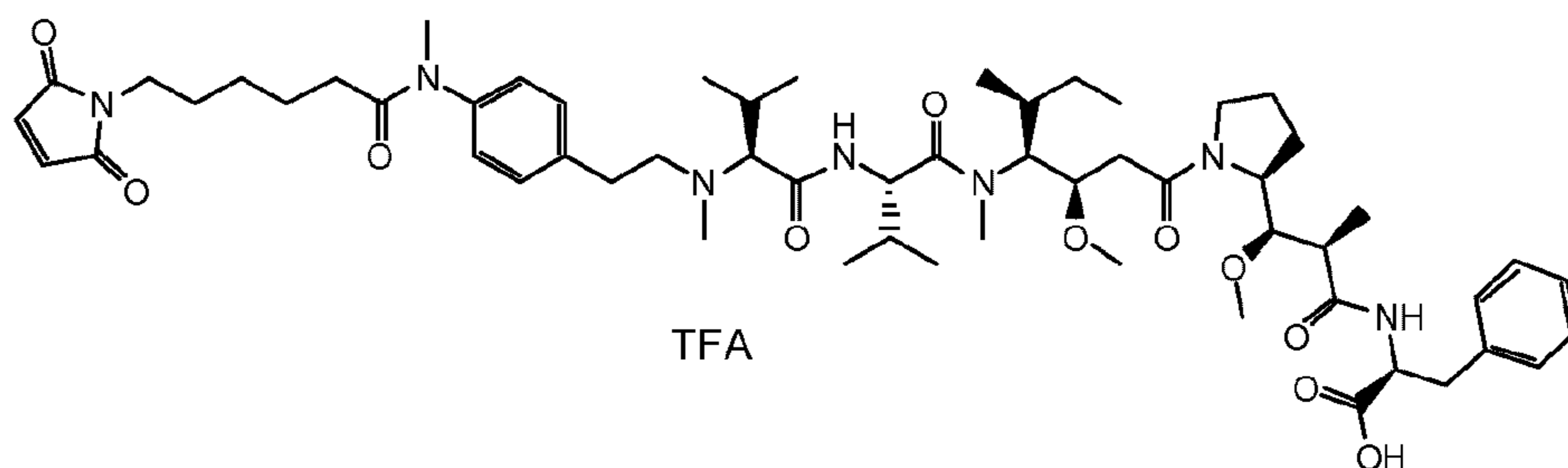
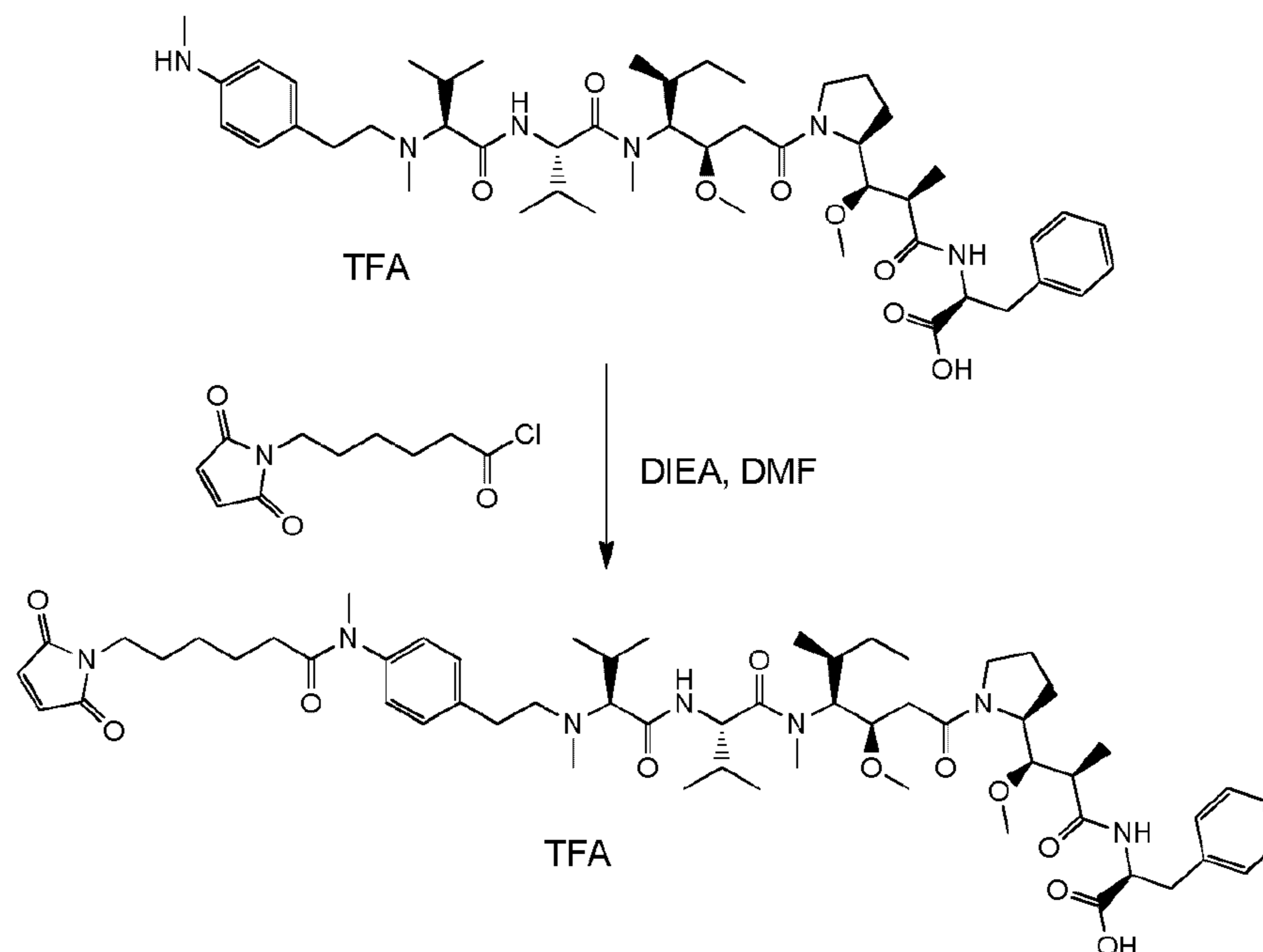
Compound G-12:

Aniline **12** (40 mg, 0.045 mmol, 1 eq.) was dissolved in dry DCM (1 mL) at 0°C and DIEA (8 μ L, 0.045 mmol, 1 eq.) was added. After stirring for 30 minutes, a solution of compound **G-12-1** (10 mg, 0.45 mmol, 1 eq.) in dry DCM (1 mL) was introduced and the reaction stirred for 1 hour at 0°C. The mixture was diluted with DCM (25 ml) and washed twice with water (20 mL), once with brine (10 mL). The organic phase was dried over Na₂SO₄, filtered and evaporated under reduced pressure to yield the crude product as a light brown solid (54 mg). This was purified by flash chromatography on silica gel (DCM/MeOH) followed by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μ m, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The isolated product was lyophilised to yield a white solid (23 mg), which was re-purified by preparative HPLC and the selected fractions combined and lyophilised to furnish compound **G-12** as a white solid (9 mg, 16 %).

m/z (Q-TOF MS ESI+) 1094.6543 (20 %, MNa⁺, C₅₉H₈₉N₇NaO₁₁ requires 1094.6512), 1072.6722 (16 %, MH⁺, C₅₉H₉₀N₇O₁₁ requires 1072.6693), 536.8358 (100 %, (MH₂)²⁺, C₅₉H₉₁N₇O₁₁ requires 536.8383).

Compound G-13

**((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((4-(6-(2,5-dioxo-2,5-dihydro-1*H*-
 pyrrol-1-yl)-*N*-methylhexanamido)phenethyl)(methylamino)-3-
 methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-
 5 methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-*L*-phenylalanine
 2,2,2-trifluoroacetate**

**Compound G-13:**

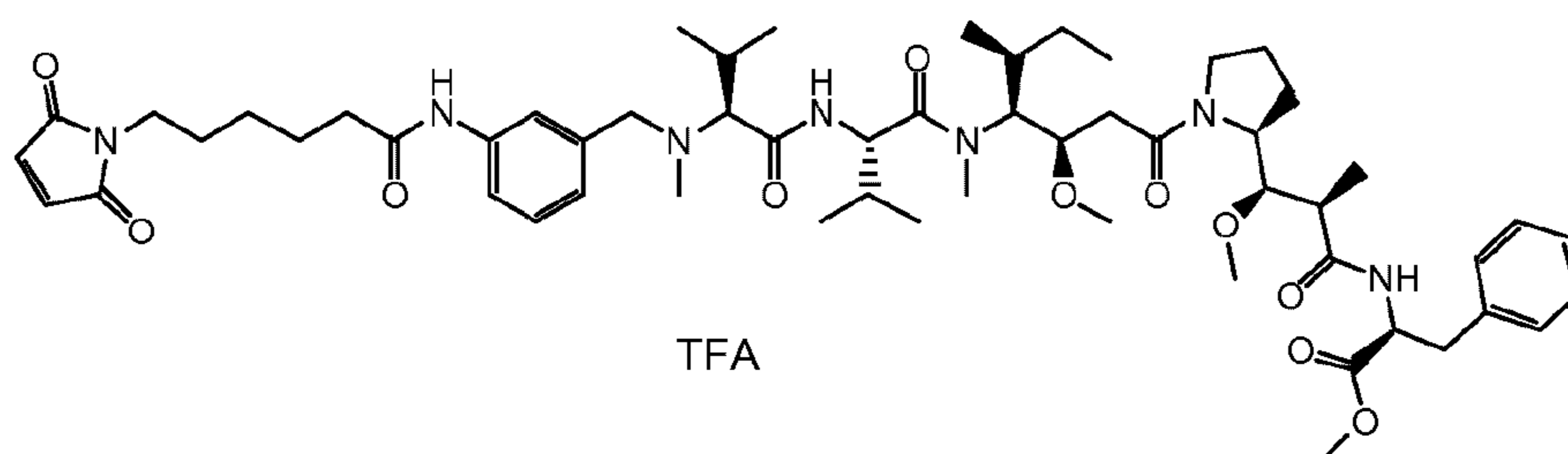
10 Aniline **13** (15 mg, 0.015 mmol, 1 eq.) was dissolved in dry DCM (1.5 mL) at
 0°C and DIEA (8 μ L, 0.046 mmol, 3 eq.) was added. A solution of compound **G-12-1**
 (3.5 mg, 0.046 mmol, 1 eq.) in dry DCM (0.5 mL) was introduced and the reaction
 stirred for 1.5 hours at 0°C. The solvent was evaporated under reduced pressure and the
 crude product purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD
 15 column, 5 μ m, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA;

Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **G-13** as a white solid (11.4 mg, 62 %).

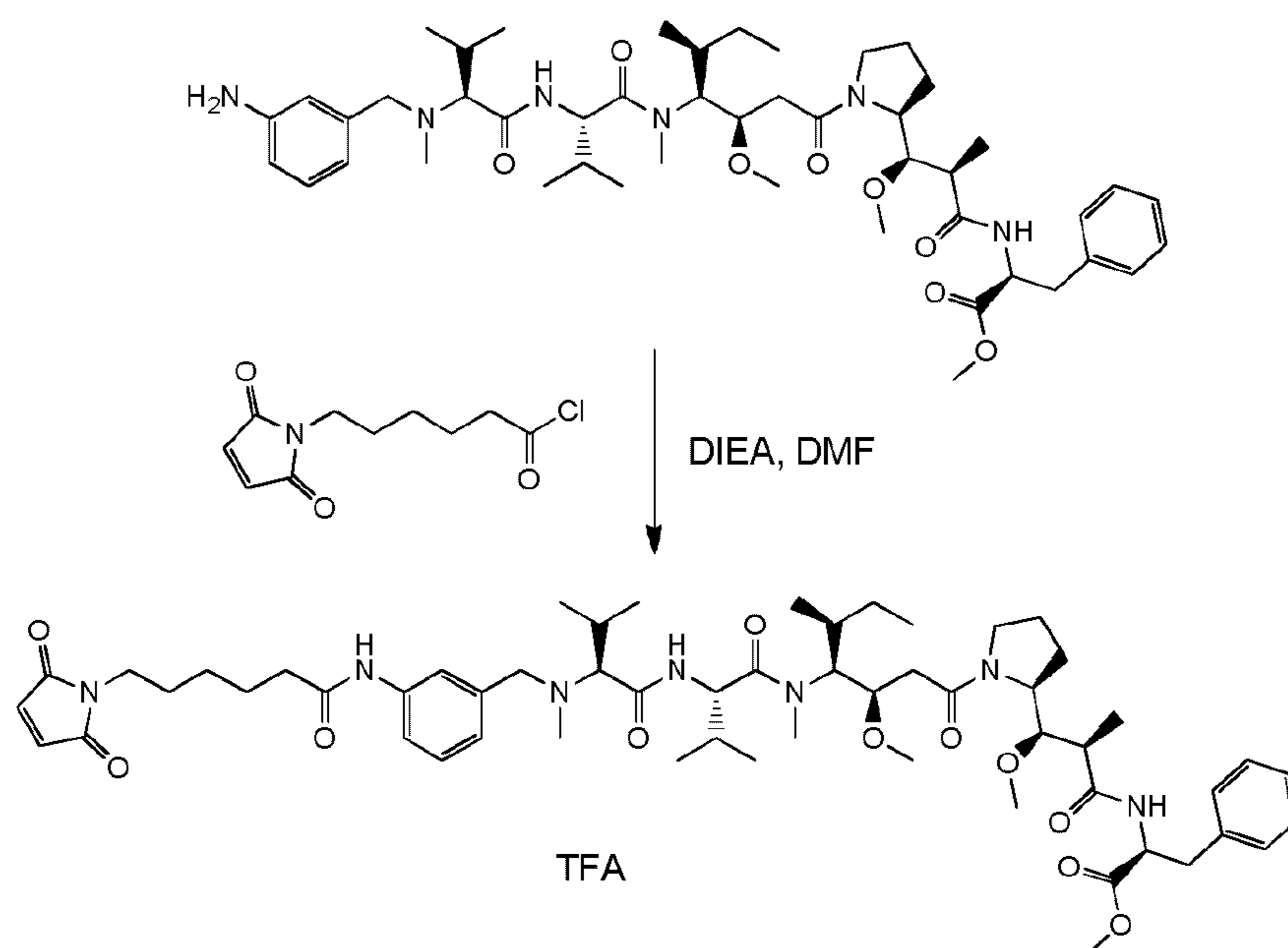
m/z (Q-TOF MS ESI+) 1058.6510 (30 %, MH⁺, C₅₈H₈₈N₇O₁₁ requires 1058.6536),
 5 529.8285 (100 %, (MH₂)²⁺, C₅₈H₈₉N₇O₁₁ requires 529.8305).

Compound G-15

methyl ((2*R*,3*R*)-3-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-((3-(6-(2,5-dioxo-2,5-dihydro-
 1*H*-pyrrol-1-yl)hexanamido)benzyl)(methylamino)-3-methylbutanamido)-*N*,3-
 10 dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-
 2-methylpropanoyl)-*L*-phenylalaninate 2,2,2-trifluoroacetate



Compound G-15:



15 Aniline **15** (40 mg, 0.047 mmol, 1 eq.) was dissolved in dry DCM (2 mL) at 0°C and DIEA (10 μL, 0.056 mmol, 1.2 eq.) was added. A solution of compound **G-12-1**

(108 mg, 0.47 mmol, 10 eq.) in dry DCM (1 mL) was introduced and the reaction stirred for 1.5 hours at 0°C. The mixture was diluted with DCM (10 ml) and washed twice with water (5 mL). The organic phase was dried over MgSO₄, filtered and evaporated under reduced pressure to yield the crude product as a beige solid. This was
5 purified by preparative HPLC (Waters 600E, SunFire Prep C18 OBD column, 5 μm, 19 x 100 mm; Eluting phase: water / MeCN buffered with 0.1 % TFA; Gradient of 5 % to 100 % MeCN in 15 minutes; Waters 2487 UV Detector at 220 nm). The selected fractions were combined and lyophilised to furnish compound **G15** as a white solid (27 mg, 50 %).

10 m/z (Q-TOF MS ESI+) 1066.6517 (2 %, MNa⁺, C₅₇H₈₅N₇NaO₁₁ requires 1066.6199), 522.8224 (100 %, (MH₂)²⁺, C₅₇H₈₇N₇O₁₁ requires 522.8226).

Example 22: ADC synthesis, purification and characterization

The procedure described below applies to chimeric and humanized IgG1 forms.
15 It must be understood that for any other forms, such as IgG2, IgG4, etc., the person skilled in the art would be capable of adapting this procedure using the general knowledge.

Antibodies (1-5 mg/ml) were partially reduced with Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in 10 mM borate buffer pH 8.4
20 containing 150 mM NaCl and 2 mM EDTA for 2 h at 37°C. Typically, 2.5-3 molar equivalents of TCEP were used to target a Drug-to-Antibody Ratios (DAR) of around 4, respectively. The partial antibody reduction was confirmed by SDS-PAGE analysis under non reducing conditions. Before Linker-Drug coupling to the released interchain cysteine residues, the reduction mixture was allowed to cool to room temperature. The
25 antibody concentration was then adjusted to 1 mg/ml with 10 mM borate buffer pH 8.4 containing 150 mM NaCl and 2 mM EDTA, and a 5 molar excess of drug to antibody was added from a 10 mM solution in dimethyl sulfoxide (DMSO). The final DMSO concentration was adjusted to 10% to maintain the solubility of the drug in the aqueous medium during coupling. The reaction was carried out for 1 h at room temperature. The
30 drug excess was quenched by addition of 1.5 moles of N-acetylcysteine per mole of drug and incubation for 1 h at room temperature. After dialysis against 25 mM His buffer pH 6.5 containing 150 mM NaCl overnight at 4°C, the antibody-drug-conjugates

were purified by using methods known to persons skilled in the art based with commercial chromatography columns and ultrafiltration units. First, the non coupled drug and the ADC aggregates were eliminated by size exclusion chromatography (SEC) on S200 (GE Life Sciences) or TSK G3000 SW (Tosoh) column. The purified ADC monomers were then concentrated to 2-3 mg/ml by ultrafiltration on 30 or 50 kDa MWCO filtration units or by affinity chromatography on Protein A. The purified ADCs were stored at 4°C after sterile filtration on 0.2 µm filter. They were further analyzed by SDS-PAGE under reducing and non reducing conditions to confirm drug conjugation and by SEC on analytical S200 or TSK G3000 SWXL columns to determine the content of monomers and aggregated forms. Protein concentrations were determined by using the bicinchoninic acid (BCA) assay with IgG as standard. The DAR was estimated for each purified ADC by HIC and LC-MS. Typically, the content of aggregated forms was lower than 5% and the DAR was comprised between 3.5 and 5.

15 **Example 23: Cytotoxicity evaluation of IGF-1R antibodies coupled with different drugs**

The five IGF-1R antibodies were shown to be rapidly internalized into lysosomes and to have a lower binding capacity into acidic environments. In that respect, those Abs had all properties to be used as ADCs. Thus, the five chimeric anti-IGF-1R antibodies were coupled with three different compounds (G-13; E-13 and F-63). The drug antibody ratio of those ADCs was about 4. In order to evaluate the non specific cytotoxicity, an irrelevant chimeric antibody c9G4 was also coupled with those compounds at the same DAR. MCF-7 cells were incubated with increasing concentrations of each ADCs at 37°C for 6 days in complete culture medium. Cell viability was assessed using a luminescent cell viability assay (CellTiter-Glo, Promega). Luminescent signal was read using a the Mithras plate reader (Berthold Technologies). The irrelevant chimeric antibody c9G4 coupled with either E-13, G-13 or F-63 showed no or modest cytotoxic activity on MCF-7 cells (Figure 21). On the contrary, addition of all other ADCs obtained after coupling anti-IGF-1R antibodies with either E-13, G-13 or F-63 decreased dramatically MCF-7 cell viability.

Example 24: *In vivo* activity of the c208F2 antibody conjugated to either E-13, G-13 or F-63 compounds in the MCF-7 xenograft model.

In order to confirm that the *in vitro* efficacy of the c208F2 coupled to G-13, E-13 or F-63 compounds could be translated *in vivo*, they have been tested in the MCF-7
5 xenograft model.

All animal procedures were performed according to the guidelines of the 2010/63/UE Directive on the protection of animals used for scientific purposes. The protocol was approved by the Animal Ethical Committee of the Pierre Fabre Institute. Five millions MCF-7 cells were injected subcutaneous into 7 weeks old Swiss/Nude
10 mice. Prior to cell injection, oestrogen pellets (Innovative Research of America) were implanted to the left flank to mice in order to release estrogens necessary to the *in vivo* growth of MCF-7 tumors.

Twenty days after MCF-7 cell implantation, when tumors reached an average size of 120-150 mm³, the animals were divided into groups of 5 mice according to
15 tumor size and aspect. The different treatments were inoculated by intraperitoneal injections. The health status of animals was monitored daily. Tumor volume was measured twice a week with an electronic calliper until study end. Tumor volume is calculated with the following formula: $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. Toxicity was evaluated following the weight of animals three times per week. Statistical analyses
20 were performed at each measure using a Mann-Whitney test. All compounds were injected intraperitoneally (i.p.). In this example, the anti-tumor activity of c208F2 mAb coupled with either E-13, F-13 or F-63 at about DAR 4 was evaluated after 2 injections of a 7 mg/kg dose at D20 and D27 (Figure 22A, 22B and 22C). In parallel the capped-drug moieties E-13, F-13 and F-63 were injected at the equivalent dose of the one
25 corresponding to 7mg/kg of c208F2-E-13, c208F2-F-13 and c208F2-F-63 DAR about 4.

Injection of either c208-E-13 (Figure 22A), c208F2-G-13 (Figure 22B) or c208F2-F-63 (Figure 22C) significantly inhibited and even induced a complete tumor growth regression ($p < 0.05$ vs corresponding capped-drug). No statistical activity difference between c208-E-13, c208F2-G-13 and c208F2-F-63 could be noted. Capped
30 drugs had no effect on MCF-7 tumor growth ($p > 0.05$ vs control group)

A second set of experiments was performed with c208F2 coupled with either E-13 or G-13 and with the irrelevant antibody c9G4 coupled with either E-13 or G-13 in

MCF-7 xenograft models as described previously. Mice were injected i.p. with 7 mg/kg of each ADCs at D20 and D27 (Figures 23A and 23B).

Injection of both c9G4-E-13 and c9G4-F-13 affected moderately and transiently the growth of MCF-7 xenograft tumors. However, this second experiment confirmed that injections of either c208-E-13 or c208F2-G-13 induced complete tumor regression since D43 showing the high anti-tumor activity of those ADCs.

Example 25: Potent cytotoxicity *in vitro* of Axl ADCs coupled with different drugs

The cytotoxic activity of ADCs for inhibition of tumor cell growth was tested in a cell proliferation assay using SN12C (Axl⁺ human renal cell carcinoma) and MCF-7 (Axl⁻ human breast adenocarcinoma). Briefly, cells were seeded into 96 well multi-well plates the day before drug treatment at 2500 cells per well. ADCs and controls were serially diluted and then added to the mw-96 plates. Cells were then incubated for 6 days at 37°C and 5% CO₂. The cell viability was quantified by measuring the level of ATP in the wells using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega catalog #G7571). The percentage of cell viability was calculated considering untreated cells as 100%. Using a nonlinear regression analysis (GraphPad Prism 4.0), the IC₅₀, the concentration of compound needed to yield a 50% reduction in viability compared with untreated cells (control = 100%), was determined and expressed in molarity (Figure 28).

Data showed in Figures 28A and 28B that hz1613F12 conjugated to either E-13 or G-13 give IC₅₀ values of 1.048 10⁻¹⁰ M, 1.413 10⁻¹⁰ M, respectively. Thus E-13 and G-13 can trigger a strong cytotoxicity when coupled to an Axl antibody such as 1613F12. No cytotoxicity is observed on MCF7 cells (Axl).

Example 26: *In vivo* activity of the trastuzumab antibody conjugated to either E-13 or G-13 compounds in the Calu-3 xenograft model.

In order to confirm the *in vivo* efficacy of antibodies coupled to G-13 or E-13 compounds, they have been coupled to Trastuzumab and tested in the HER2 sensitive xenograft model Calu-3 known for its HER2 amplification and (3⁺) expression. The antibody Trastuzumab was purchased from Euromedex, 24 Rue des Tuileries 67460 SOUFFELWEYERSHEIM / France.

All animal procedures were performed according to the guidelines of the 2010/63/UE Directive on the protection of animals used for scientific purposes. The protocol was approved by the Animal Ethical Committee of the Pierre Fabre Institute. Seven millions Calu-3 cells were injected subcutaneous into 7 weeks old SCID mice.

5 Six days after Calu-3 cell implantation, when tumors reached an average size of 250-260 mm³, the animals were divided into groups of 6 mice according to tumor size and aspect. The different treatments were inoculated by intraperitoneal injections. The health status of animals was monitored daily. Tumor volume was measured twice a week with an electronic calliper until study end. Tumor volume is calculated with the
10 following formula: (length x width²)/2. Toxicity was evaluated following the weight of animals three times per week. Statistical analyses were performed at each measure using a Mann-Whitney test. All compounds were injected intraperitoneally (i.p.). In this example, the anti-tumor activity of Trastuzumab mAb coupled with either E-13 or G-13 at about DAR 4 was evaluated after 1 injections of a 3 mg/kg dose at D6. In parallel
15 Trastuzumab alone was injected at the equivalent dose of the one corresponding to 3mg/kg of naked antibody.

Injection of either Trastuzumab-E-13 (Figure 29A) or Trastuzumab-G-13 (Figure 29B) significantly inhibited the tumor growth and even induced a complete tumor growth regression in all treated mice (p <0.05 vs corresponding naked antibody).
20 No statistical activity difference was observed between Trastuzumab-E-13, and Trastuzumab-G-13 groups. Compared to published data (Cretella et al. Molecular Cancer 2014, 13:143) on this Calu-3 model, TDM-1 did not induced complete regression even if its dosing was higher (15 mg/kg every 6 days vs 3 mg/kg, one injection respectively) than the one used for either Trastuzumab-E-13 or Trastuzumab-
25 G-13.

Example 27: *In vivo* activity of the trastuzumab antibody conjugated to either E-13 or G-13 compounds in the JIMT-1 xenograft model.

In order to know whether Trastuzumab antibody conjugates display also an
30 activity on a model known to be resistant to Trastuzumab, the JIMT-1 xenograft model that highly expressed HER2 but that was resistant to Trastuzumab therapy was evaluated. All animal procedures were performed according to the guidelines of the

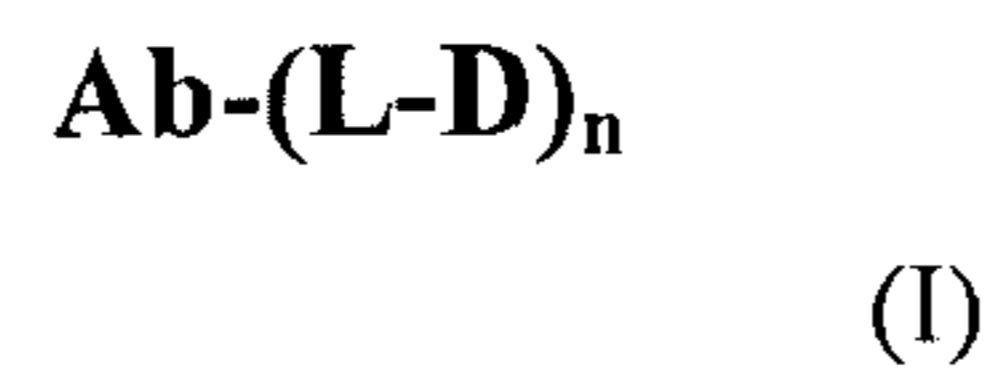
2010/63/UE Directive on the protection of animals used for scientific purposes. The protocol was approved by the Animal Ethical Committee of the Pierre Fabre Institute. Seven millions JIMT-1 cells were injected subcutaneous into 7 weeks old SCID mice.

5 Fourteen days after JIMT-1 cell implantation, when tumors reached an average size of 220-230 mm³, the animals were divided into groups of 5 mice according to tumor size and aspect. The different treatments were inoculated by intraperitoneal injections. The health status of animals was monitored daily. Tumor volume was measured twice a week with an electronic calliper until study end. Tumor volume is calculated with the following formula: (length x width²)/2. Toxicity was evaluated
10 following the weight of animals three times per week. Statistical analyses were performed at each measure using a Mann-Whitney test. All compounds were injected intraperitoneally (i.p.). In this example, the anti-tumor activity of Trastuzumab mAb coupled with either E-13 or G-13 at about DAR 4 was evaluated after 1 injections of a 3 mg/kg dose at D6 (Figures 30A and 30B). In a first experiment, we have showed that
15 Trastuzumab alone did not have any anti-tumoral effect (Figure 30C). This result is in agreement with published data.

Injection of either Trastuzumab-E-13 (Figure 30A) or Trastuzumab-G-13 (Figure 30B) significantly inhibited the tumor growth traduced by respectively 73 and 70% of growth inhibition at day 34. No statistical activity difference could be noted
20 between Trastuzumab-E-13, and Trastuzumab-G-13. As already observed in the Calu-3 model and compared to published data (Barok et al. Breast Cancer Research 2011, 13:R46) on this JIMT-1 model, TDM-1 seems to be less potent even if its dosing (15 mg/kg every 6 days vs 3 mg/kg, one injection respectively) was higher than the one used for either Trastuzumab-E-13 or Trastuzumab-G-13.

CLAIMS

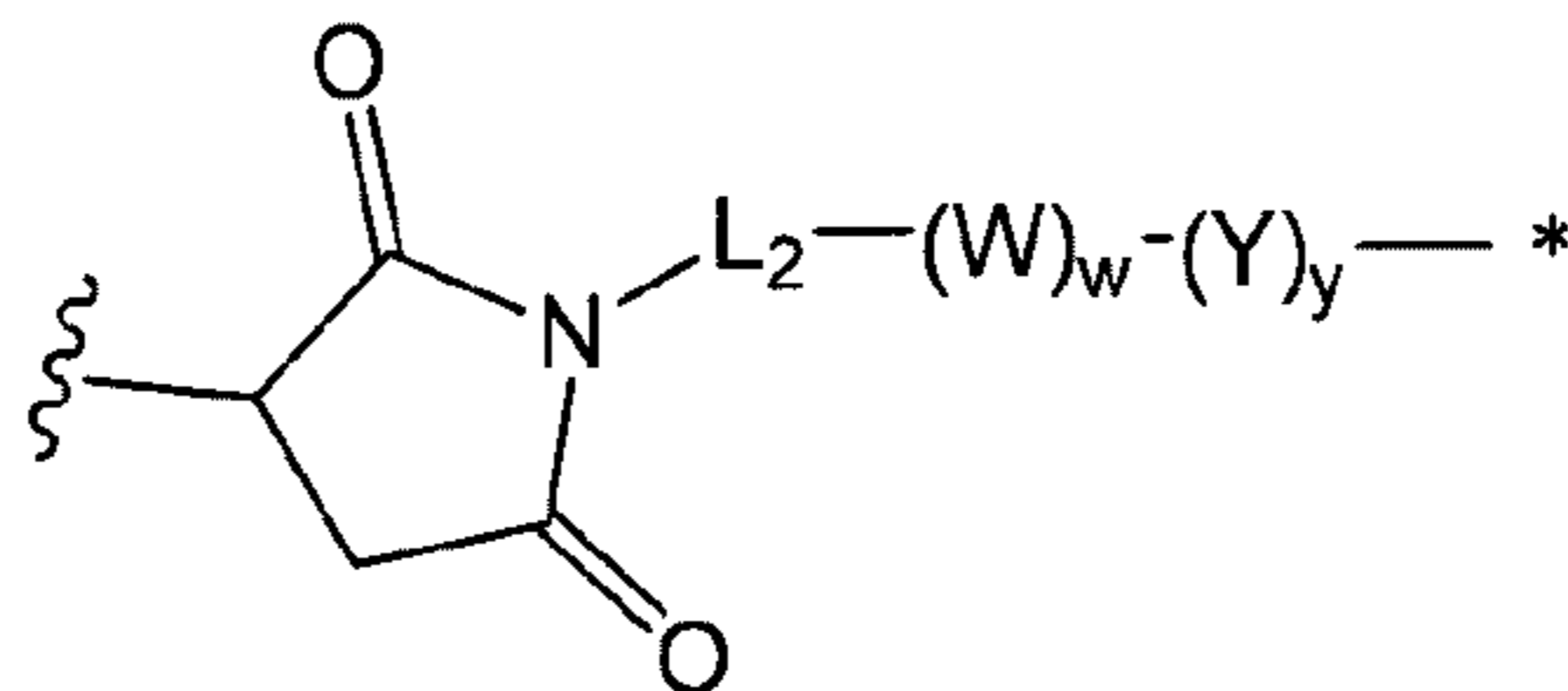
1. An antibody-drug-conjugate of the following formula (I):



5 or a pharmaceutically acceptable salt thereof,
wherein

Ab is a [Target] antibody, or a [Target] binding fragment thereof;

L is a linker of the following formula (III):

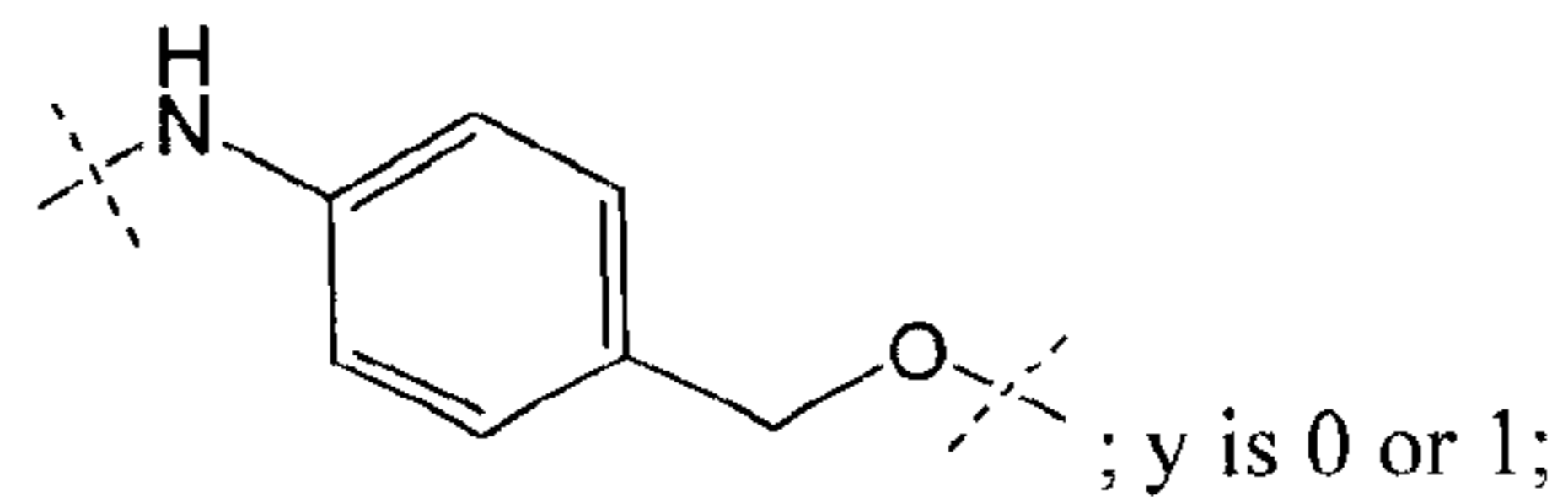


10 (III)

wherein

L_2 is (C_4-C_{10}) cycloalkyl-carbonyl, (C_2-C_6) alkyl, or (C_2-C_6) alkyl-carbonyl,

W is an amino acid unit; w is an integer comprised between 0 and 5;

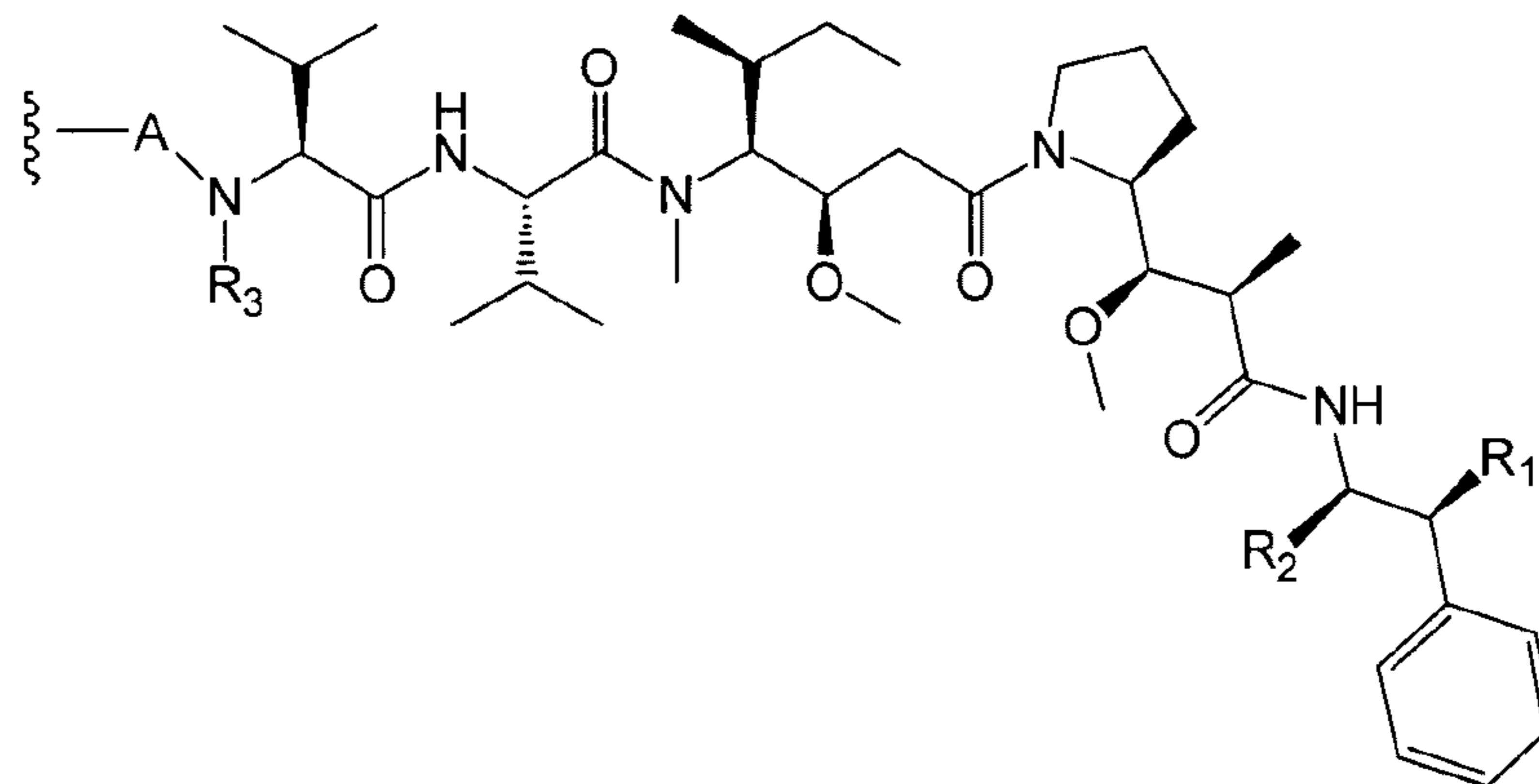


Y is PAB-carbonyl with PAB being

15 the asterisk indicates the point of attachment to **D**;

the wavy line indicates the point of attachment to **Ab**;

D is a drug moiety of the following formula (II):



(II)

wherein:

R_1 is H or OH;

R_2 is a group: (C₁-C₆)alkyl, COOH, COO-((C₁-C₆)alkyl) or thiazolyl;

R_3 is H or a (C₁-C₆)alkyl group;

5

A is:

– a group of formula -Het-Alk- wherein Alk is a (C₁-C₈)alkanediyl group and is linked to NR₃, and Het is a heterocycle optionally substituted by a (C₁-C₆)alkyl group and containing at least one nitrogen atom, said nitrogen atom being linked to **L**, or

10

– a group of formula -A_a-A_b- wherein A_a is linked to **L** and is O or NR₉ with R₉ being H or (C₁-C₆)alkyl and A_b is linked to NR₃ and is:

15

- a (C₁-C₈)alkanediyl group,
- a -(CH₂CH₂X₁)_{a1}(CH₂CH₂X₂)_{a2}(CH₂CH₂X₃)_{a3}(CH₂CH₂X₄)_{a4}CH₂CH₂- group with X₁, X₂, X₃ and X₄ each independently of one another representing O or NR₈; a₁, a₂, a₃ and a₄ each independently of one another representing 0 or 1; and R₈ representing H or a (C₁-C₆)alkyl group, or

20

- an aryl-(C₁-C₈)alkanediyl or heterocycle-(C₁-C₈)alkanediyl group, said group being optionally substituted by a (C₁-C₆)alkyl group, the aryl or heterocycle moiety being linked to A_a and the (C₁-C₈)alkanediyl moiety being linked to NR₃;

the wavy line indicates the point of attachment to **L**; and

n is 1 to 12.

2. The antibody-drug-conjugate of claim 1, wherein the [Target] of said [Target] antibody, or a [Target] binding fragment thereof, is selected from CD19, CD20, CD22, CD25, CD30, CD33, CD40, CD56, CD64, CD70, CD74, CD79, CD105, CD138, CD174, CD205, CD227, CD326, CD340, MUC16, GPNMB, PSMA, Cripto, ED-B, TMEFF2, EphB2, EphA2, FAP, av integrin, Mesothelin, EGFR, TAG-72, GD2, CAIX, 5T4, HER1, HER3, HER2, IGF-1R, and Axl, or is an extra cellular membrane (ECD) fragment of CD19, CD20, CD22, CD25, CD30, CD33, CD40, CD56, CD64, CD70, CD74, CD79, CD105, CD138, CD174, CD205, CD227, CD326, CD340, MUC16, GPNMB, PSMA, Cripto, ED-B, TMEFF2, EphB2, EphA2, FAP, av integrin, Mesothelin, EGFR, TAG-72, GD2, CAIX, 5T4, HER1, HER3, HER2, IGF-1R, or Axl.

3. The antibody-drug-conjugate of claim 1, wherein said [Target] of said [Target] antibody, or a [Target] binding fragment thereof, is selected from the HER2, the IGF-1R and the protein Axl, or is an extra cellular membrane (ECD) fragment of the HER2, the IGF-1R or the protein Axl.

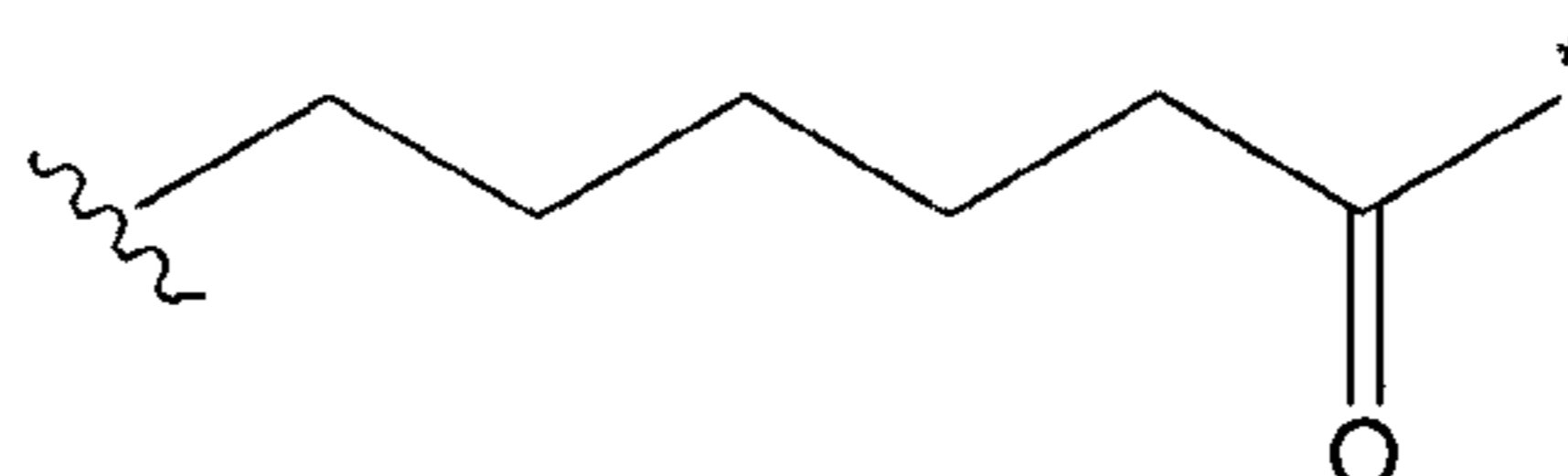
4. The antibody-drug-conjugate of claim 3, wherein said [Target] of said [Target] antibody, or a [Target] binding fragment thereof, is selected from the human HER2, the human IGF-1R and the human protein Axl, or is an extra cellular membrane (ECD) fragment of the human HER2, the human IGF-1R or the human protein Axl.

5. The antibody-drug-conjugate of claim 1, wherein said **Ab** is an antibody, or a [Target] binding fragment thereof, capable of binding to the human protein Axl selected from:

- i) an antibody which comprises the three heavy chain CDRs of sequence SEQ ID No. 59, 60 and 61 and the three light chain CDRs of sequence SEQ ID No. 56, 57 and 58;
- ii) an antibody which competes for binding to Axl with the antibody of i); and
- iii) an antibody which binds to the same epitope of Axl as the antibody of i).

6. The antibody-drug-conjugate of claim 1, wherein said **Ab** is an antibody capable of binding to the human HER2 consisting of Trastuzumab, or a [Target] binding fragment thereof.

5 7. The antibody-drug-conjugate of any one of claims 1 to 6, wherein L_2 is of the following formula:

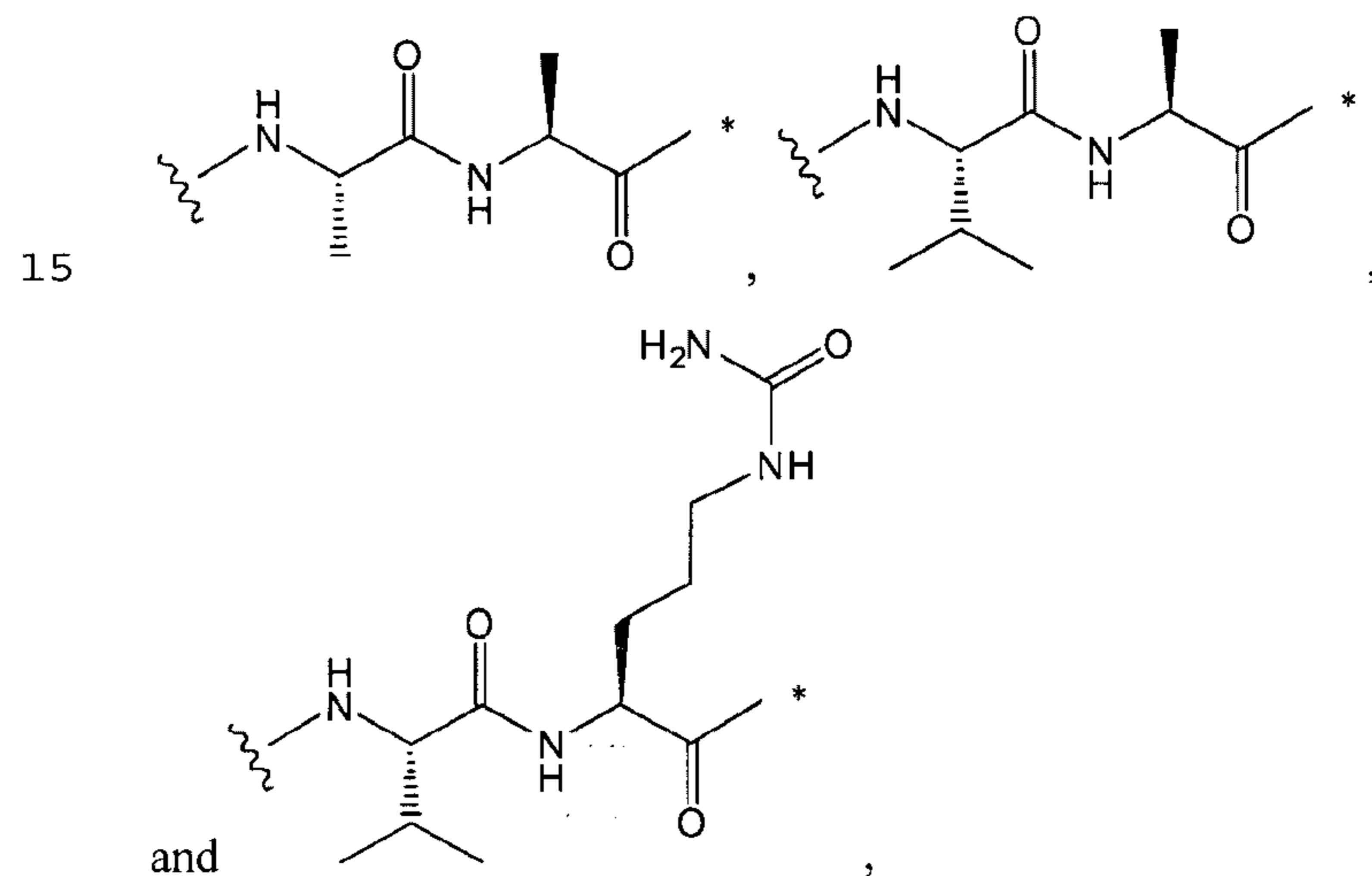


wherein

the asterisk indicates the point of attachment to $(W)_w$; and

10 the wavy line indicates the point of attachment to the nitrogen atom of the maleimide moiety.

8. The antibody-drug-conjugate of any one of claims 1 to 6, wherein $w = 0$; or $w = 2$ and $(W)_w$ is selected from:



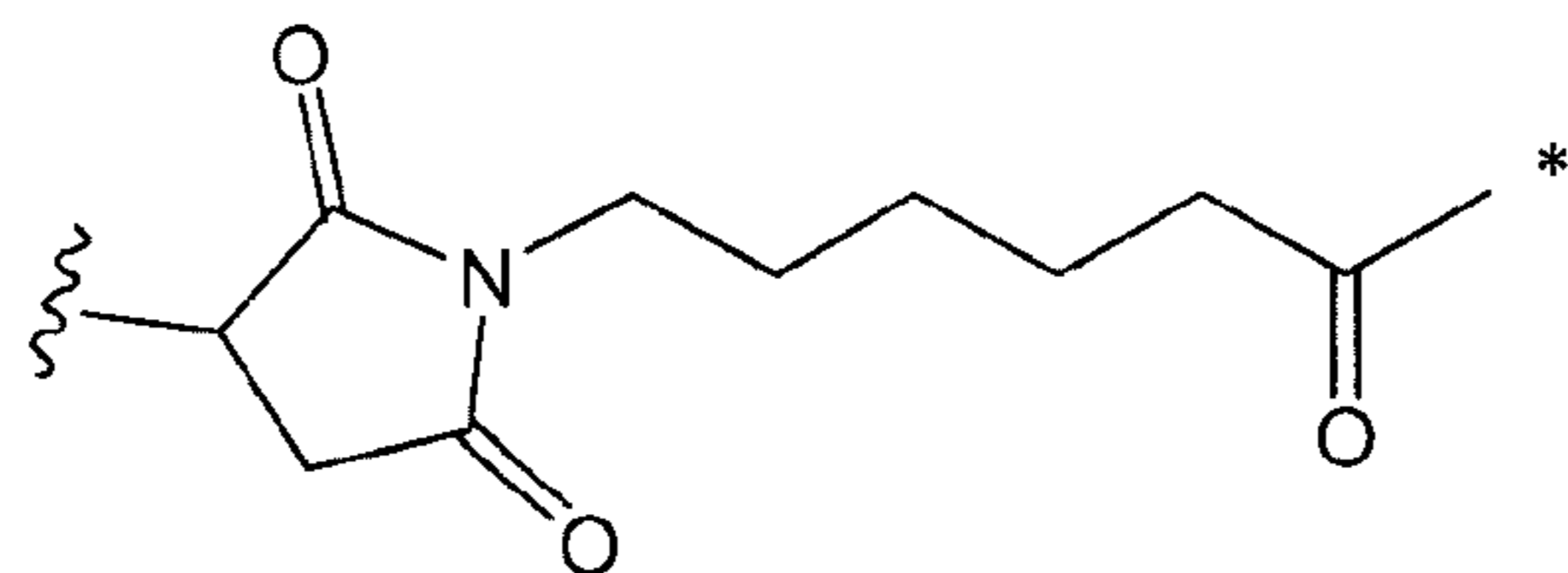
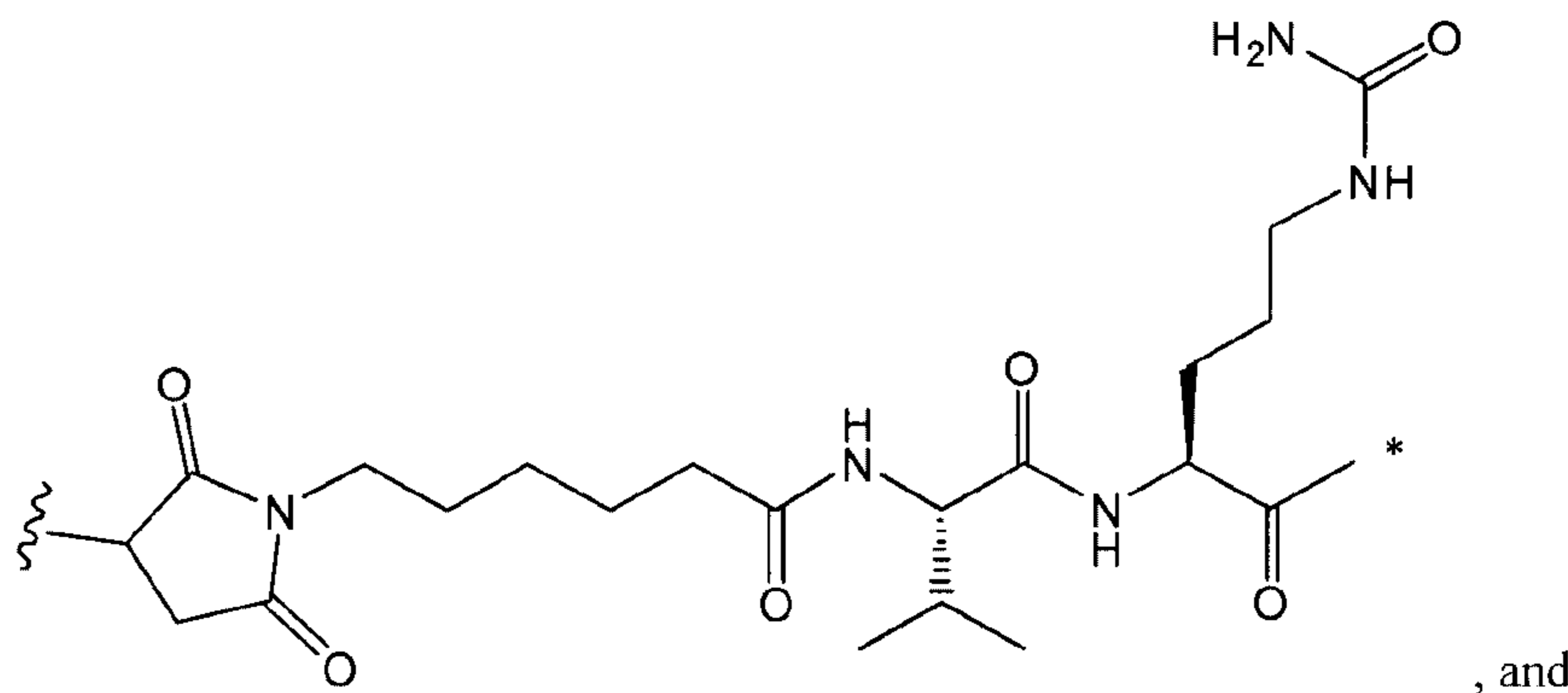
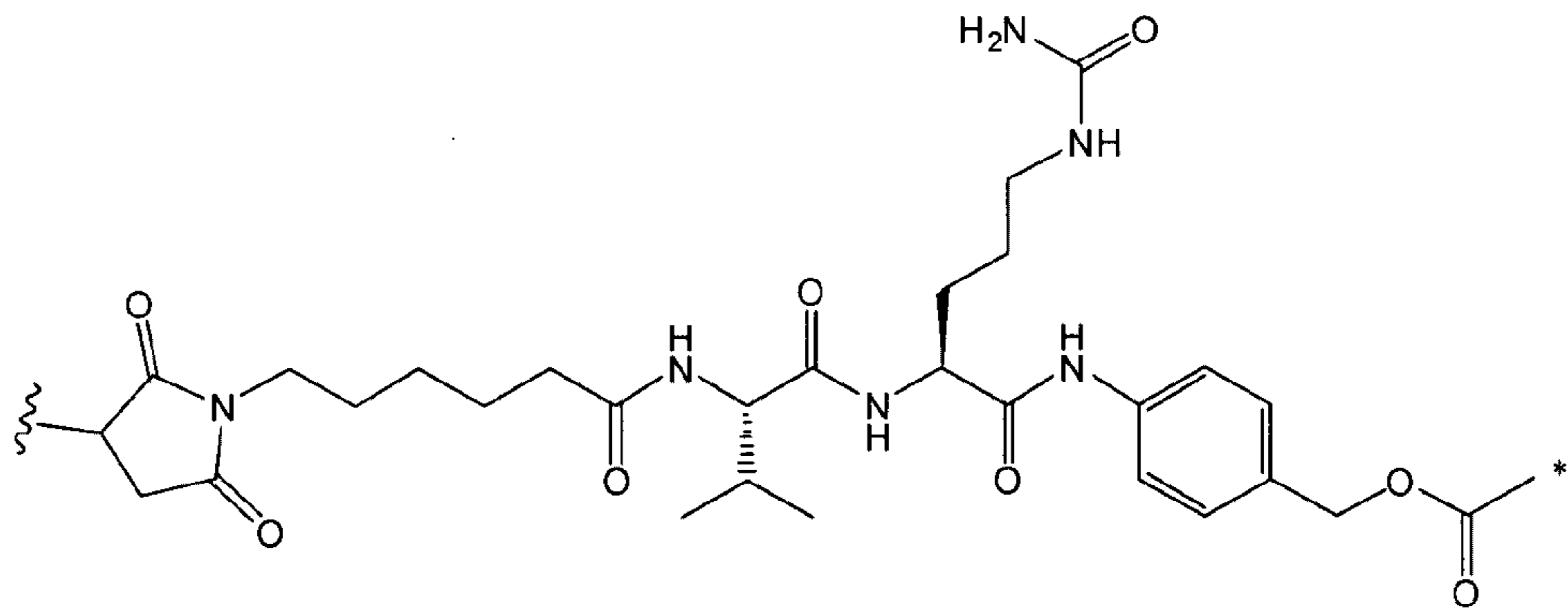
wherein

the asterisk indicates the point of attachment to $(Y)_y$; and

the wavy line indicates the point of attachment to L_2 .

20

9. The antibody-drug-conjugate of any one of claims 1 to 6, wherein **L** is selected from:



5

wherein the asterisk indicates the point of attachment to **D**, and the wavy line indicates the point of attachment to **Ab**.

10. The antibody-drug-conjugate of any one of claims 1 to 9, wherein **A** is a group of formula $-A_a-A_b-$ in which A_a is as defined in claim 1 and A_b is a group:

- phenyl-(C_1-C_2)alkanediyl, or
- heterocycle-(C_1-C_2)alkanediyl optionally substituted by a (C_1-C_6)alkyl group, the heterocycle being a saturated, unsaturated or aromatic ring with 5 or 6 members comprising 1 or 2 nitrogen atoms.

11. The antibody-drug-conjugate of claim 10, wherein the heterocycle is chosen from among pyridine, piperidine and imidazole.

5 12. The antibody-drug-conjugate of claim 10, wherein the heterocycle is a pyridine.

13. The antibody-drug-conjugate of claim 1, wherein said **Ab** is an antibody, or a [Target] binding fragment thereof, and is selected from:

10 a) an antibody, or a [Target] binding fragment thereof, capable of binding to the human IGF-1R selected from:

 i) the antibodies 208F2, 212A11, 214F8, 219D6 and 213B10;

 ii) the antibodies which compete for binding to IGF-1R with the antibodies of i); and

 iii) the antibodies which bind to the same epitope of IGF-1R as the antibodies of i),

15 b) an antibody, or a [Target] binding fragment thereof, capable of binding to the human IGF-1R selected from:

 i) an antibody which comprises the three heavy chain CDRs of sequence SEQ ID No. 1, 2 and 3 and the three light chain CDRs of sequence SEQ ID No. 4, 5 and 6;

 ii) an antibody which competes for binding to IGF-1R with the antibody of i); and

20 iii) an antibody which binds to the same epitope of IGF-1R as the antibody of i),

c) an antibody which comprises:

 i) a heavy chain variable domain (VH) of sequence SEQ ID No. 33 wherein said sequence SEQ ID No. 33 comprises at least 1 back-mutation selected from the residues 20, 34, 35, 38, 48, 50, 59, 61, 62, 70, 72, 74, 76, 77, 79, 82 and 95; and

25 ii) a light chain variable domain (VL) of sequence SEQ ID No. 35, wherein said sequence SEQ ID No. 35 comprises at least 1 back-mutation selected from the residues 22, 53, 55, 65, 71, 72, 77 and 87,

d) an antibody, or a [Target] binding fragment thereof, capable of binding to the human protein Axl selected from:

30 i) an antibody which comprises the three heavy chain CDRs of sequence SEQ ID No. 59, 60 and 61 and the three light chain CDRs of sequence SEQ ID No. 56, 57 and 58;

 ii) an antibody which competes for binding to Axl with the antibody of i); and

iii) an antibody which binds to the same epitope of Axl as the antibody of i), and
 e) an antibody capable of binding to the human HER2 consisting of Trastuzumab or
 Pertuzumab, or a [Target] binding fragment thereof.

5 14. The antibody-drug-conjugate of any one of claims 1 to 13, wherein **n** is 2.

15. The antibody-drug-conjugate of any one of claims 1 to 13, wherein **n** is 4.

16. The antibody-drug-conjugate of any one of claims 1 to 15 for use as a
 10 medicament in the treatment of cancer.

17. A composition comprising at least one antibody-drug-conjugate of any one
 of claims 1 to 15 with at least an excipient and/or a pharmaceutical acceptable vehicle.

15 18. The composition of claim 17 for use in the treatment of a [Target]-
 expressing cancer.

19. The composition of claim 18, wherein the [Target] is selected from CD19,
 CD20, CD22, CD25, CD30, CD33, CD40, CD56, CD64, CD70, CD74, CD79, CD105,
 20 CD138, CD174, CD205, CD227, CD326, CD340, MUC16, GPNMB, PSMA, Cripto,
 ED-B, TMEFF2, EphB2, EphA2, FAP, av integrin, Mesothelin, EGFR, TAG-72, GD2,
 CAIX, 5T4, HER2, IGF-1R, and Axl, or is an extra cellular membrane (ECD) fragment
 of CD19, CD20, CD22, CD25, CD30, CD33, CD40, CD56, CD64, CD70, CD74,
 CD79, CD105, CD138, CD174, CD205, CD227, CD326, CD340, MUC16, GPNMB,
 25 PSMA, Cripto, ED-B, TMEFF2, EphB2, EphA2, FAP, av integrin, Mesothelin, EGFR,
 TAG-72, GD2, CAIX, 5T4, HER2, IGF-1R, or Axl.

20. The composition of any one of claims 17 to 19 for use in the treatment of
 an HER2-, IGF-1R- or Axl-expressing cancer.

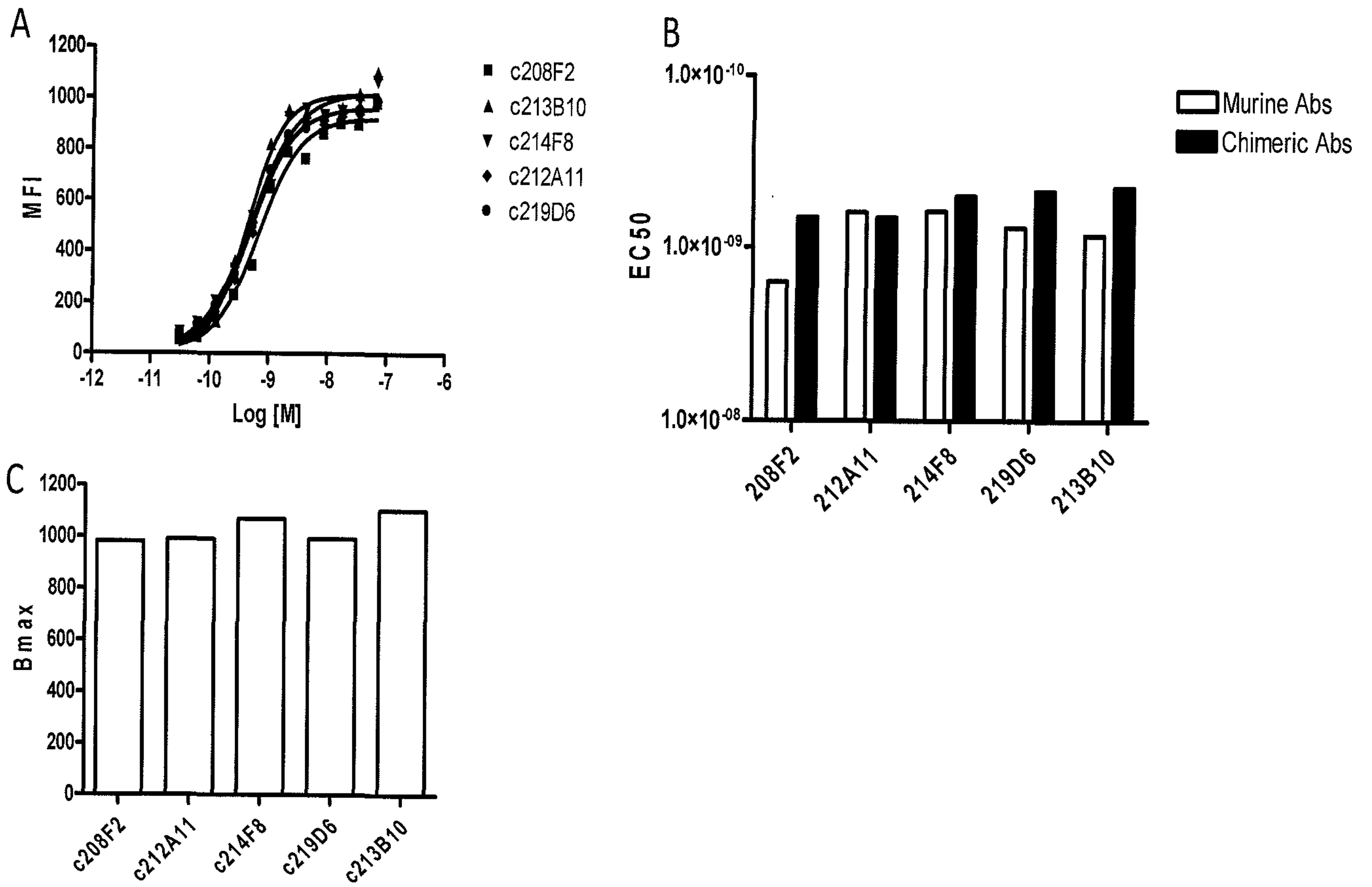
30

21. The composition of claim 18 or 19, wherein said [Target]-expressing cancer
 is a cancer chosen from breast, colon, esophageal carcinoma, hepatocellular, gastric,

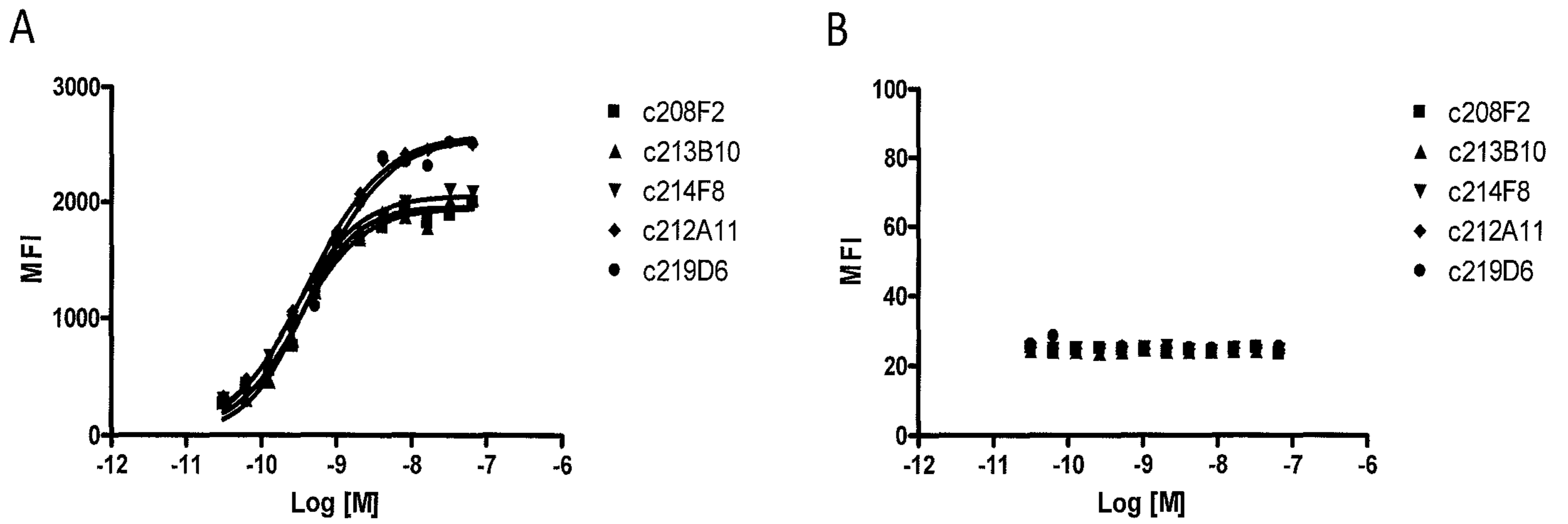
glioma, lung, melanoma, osteosarcoma, ovarian, prostate, rhabdomyosarcoma, renal, thyroid, uterine endometrial cancer, mesothelioma, oral squamous carcinoma, Kaposi sarcoma, acute leukemia, colorectal carcinoma, melanoma, pancreatic ductal adenocarcinoma and any drug resistant cancer.

5

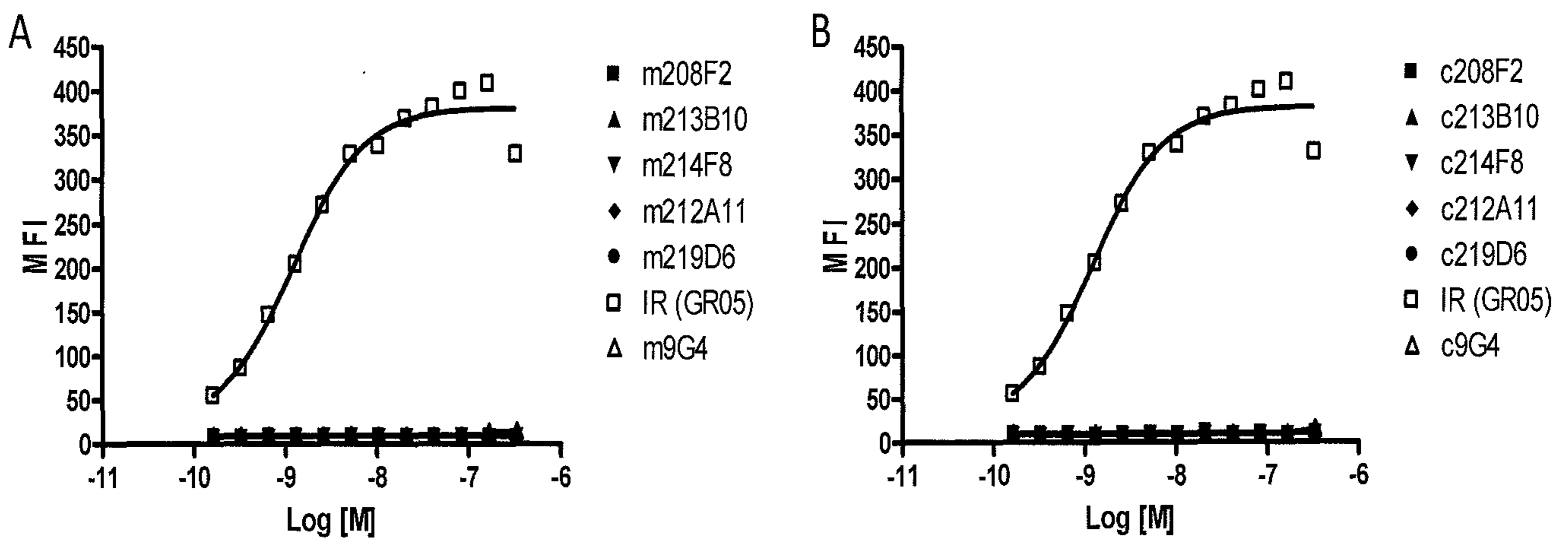
22. Use of the antibody-drug-conjugate of any one of claims 1 to 15 or of the composition of claim 17 for producing a medicinal product intended for the treatment of cancer.



FIGURES 1A-1C



FIGURES 2A-2B



FIGURES 3A-3C

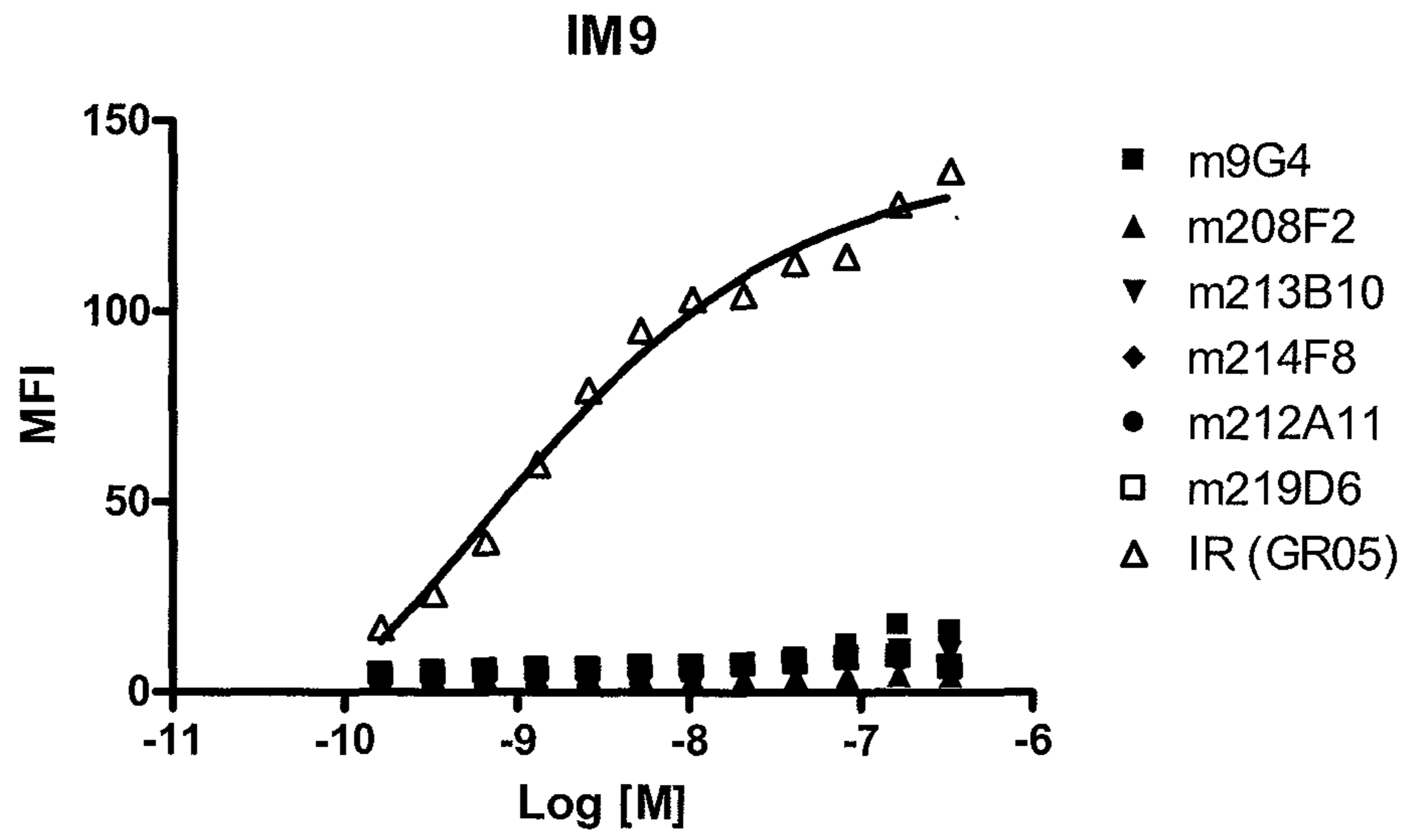
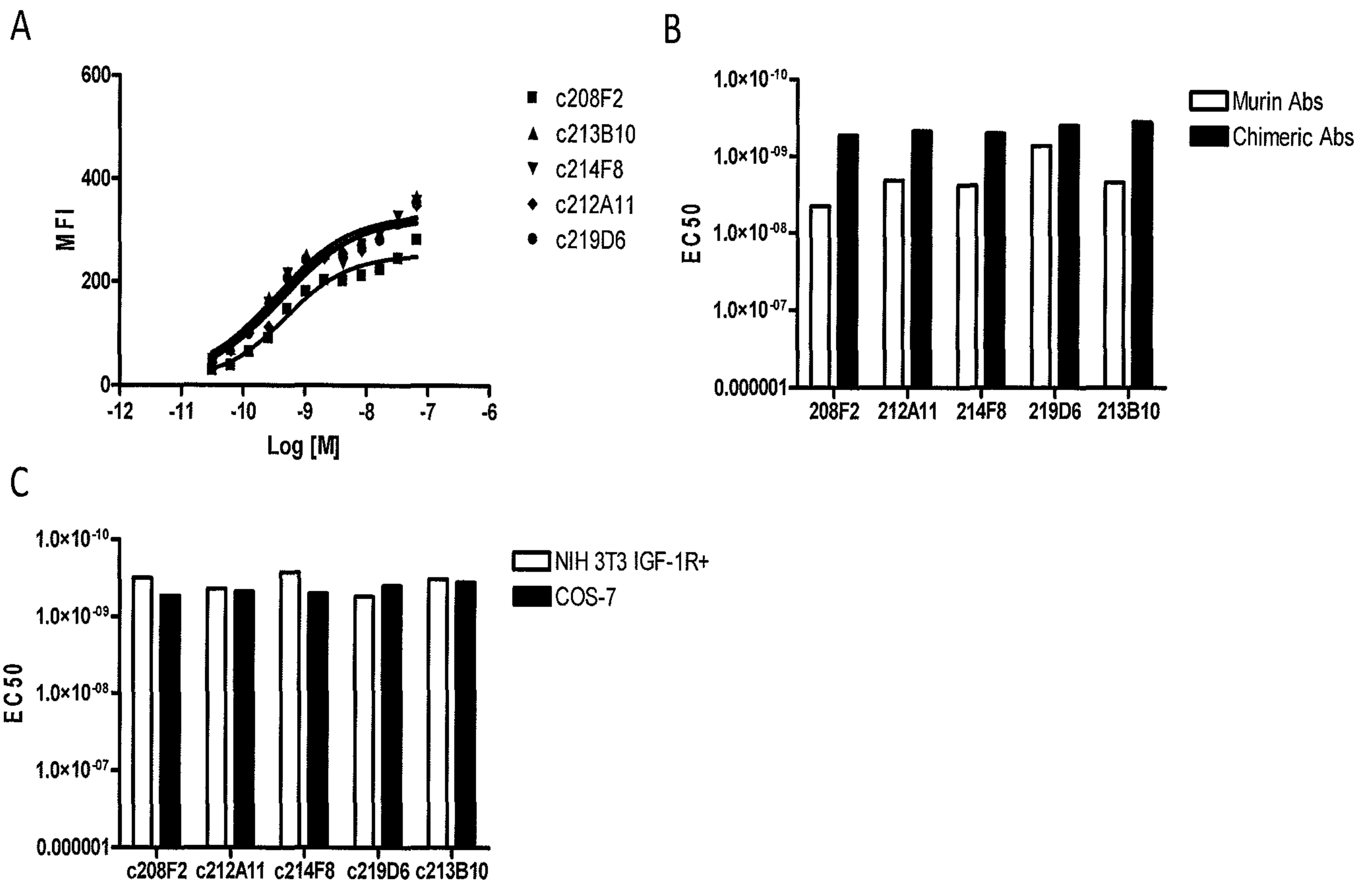


FIGURE 4



FIGURES 5A-5C

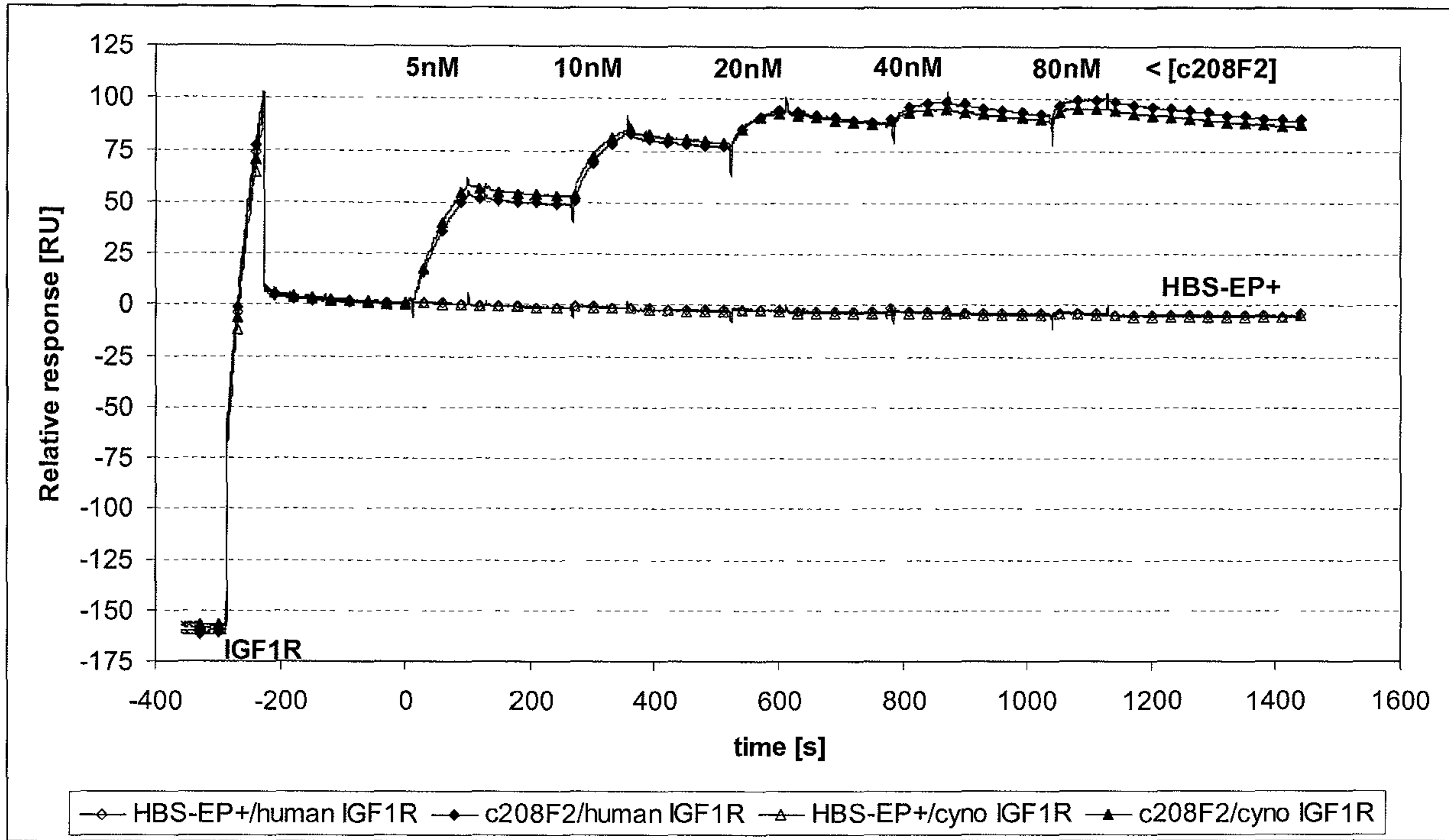


FIGURE 6

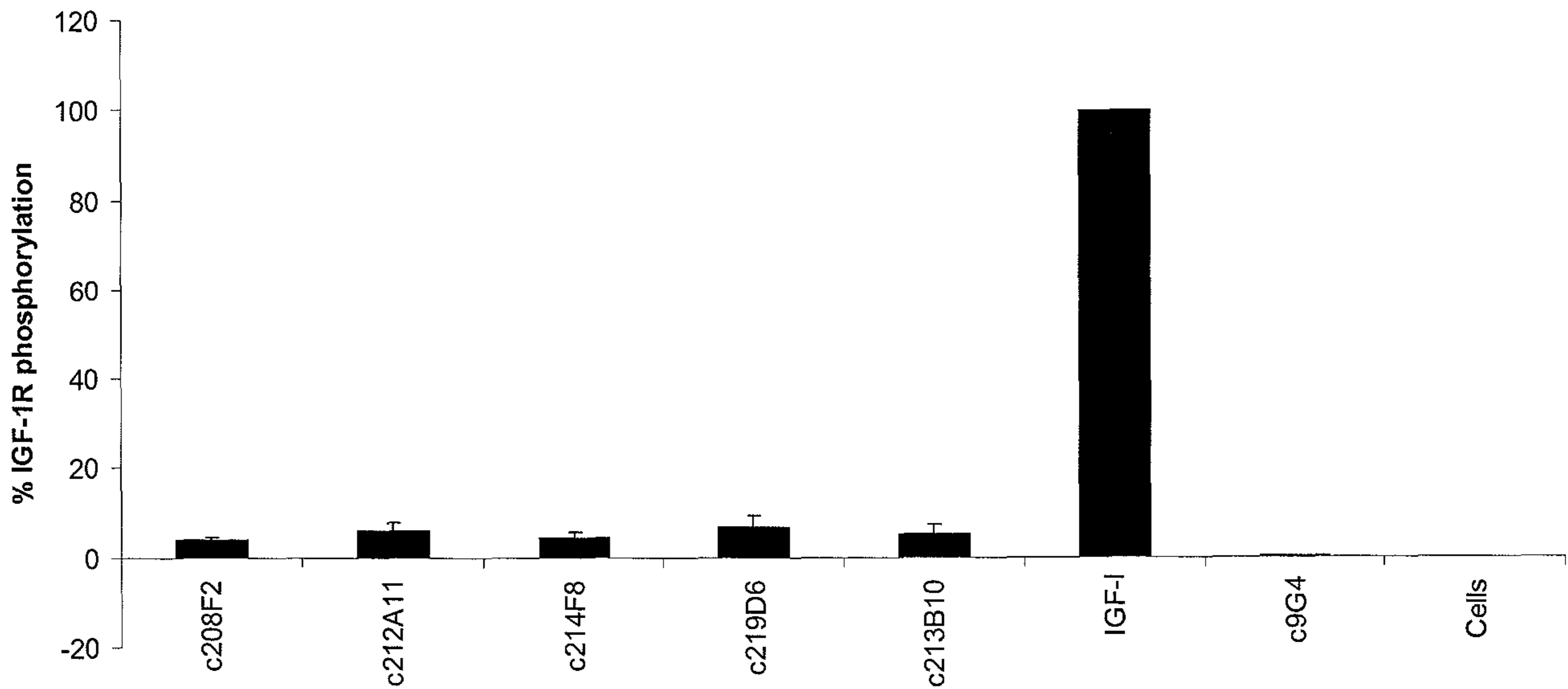


FIGURE 7

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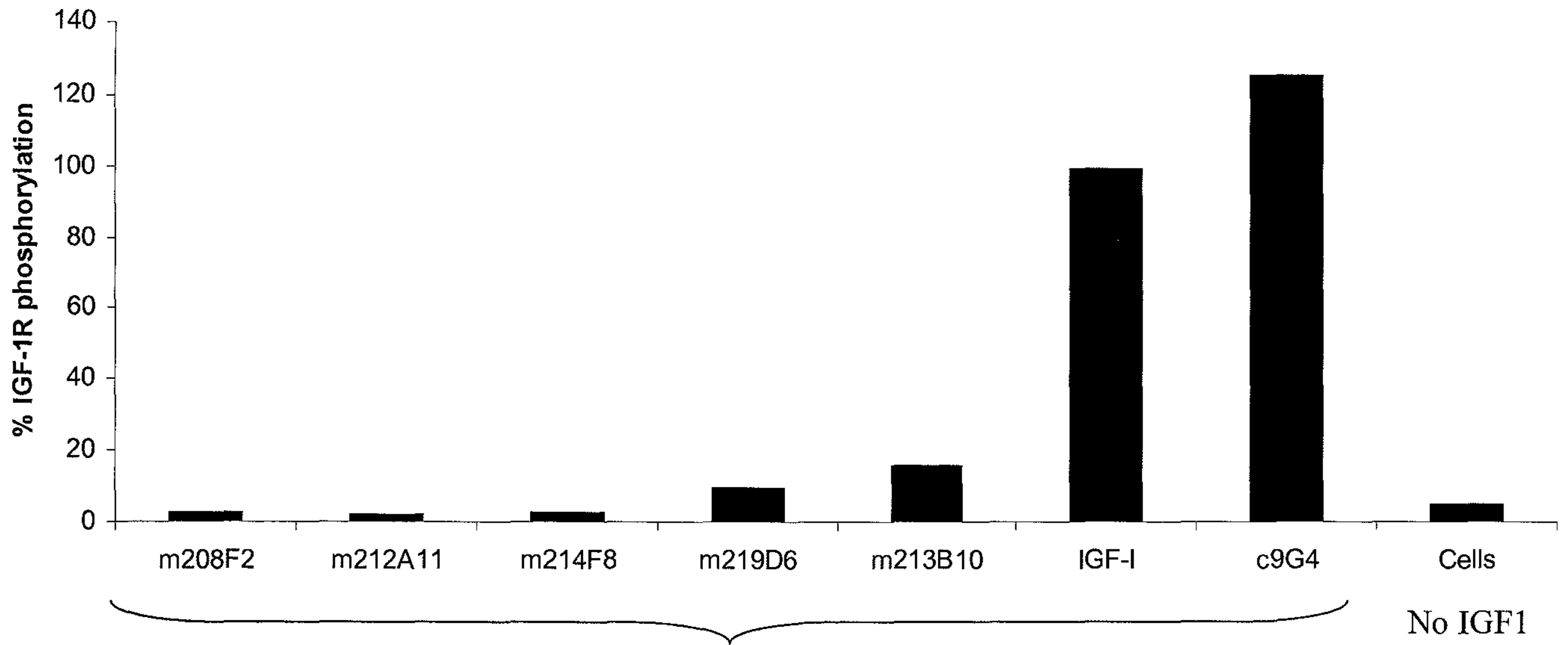


FIGURE 8

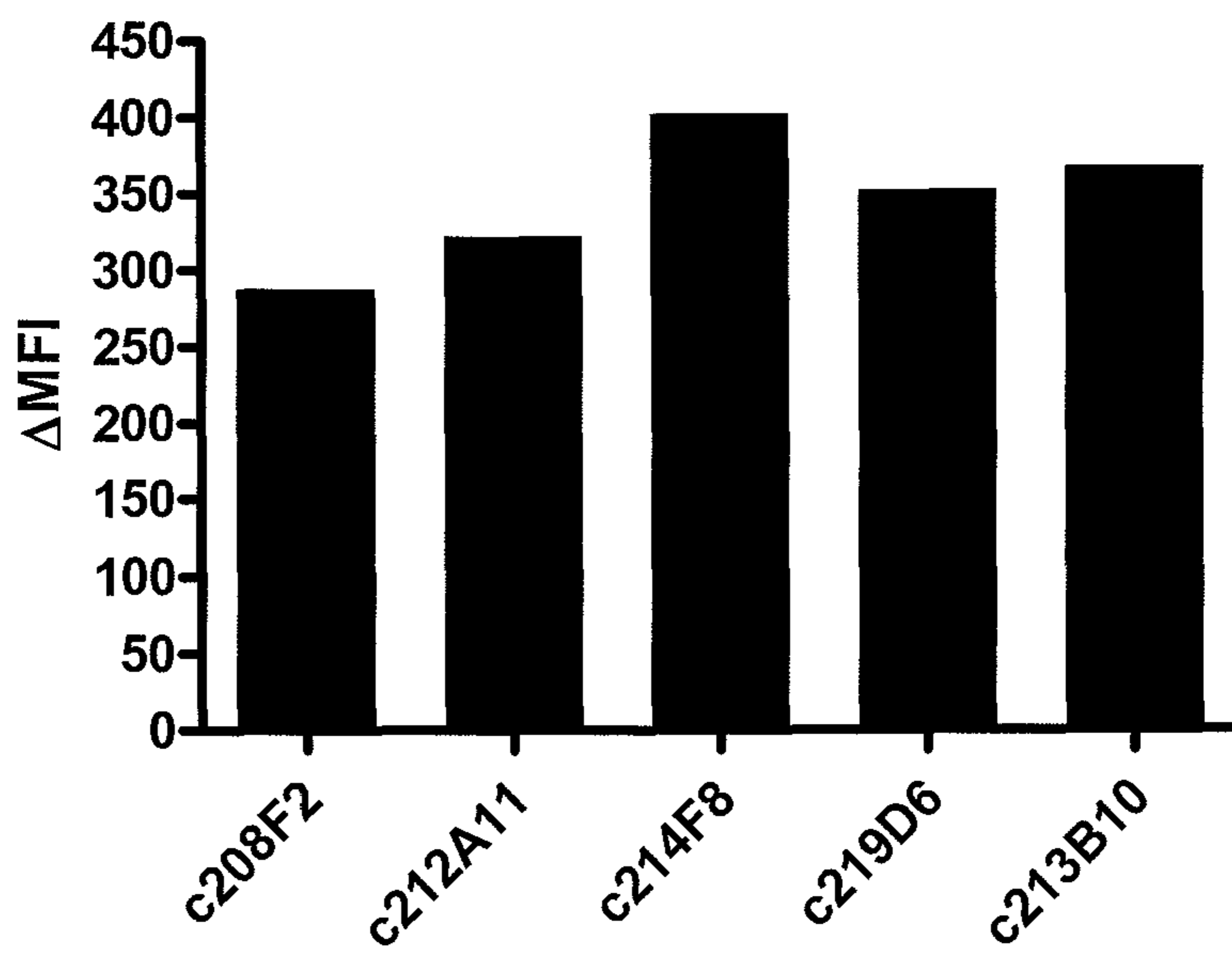
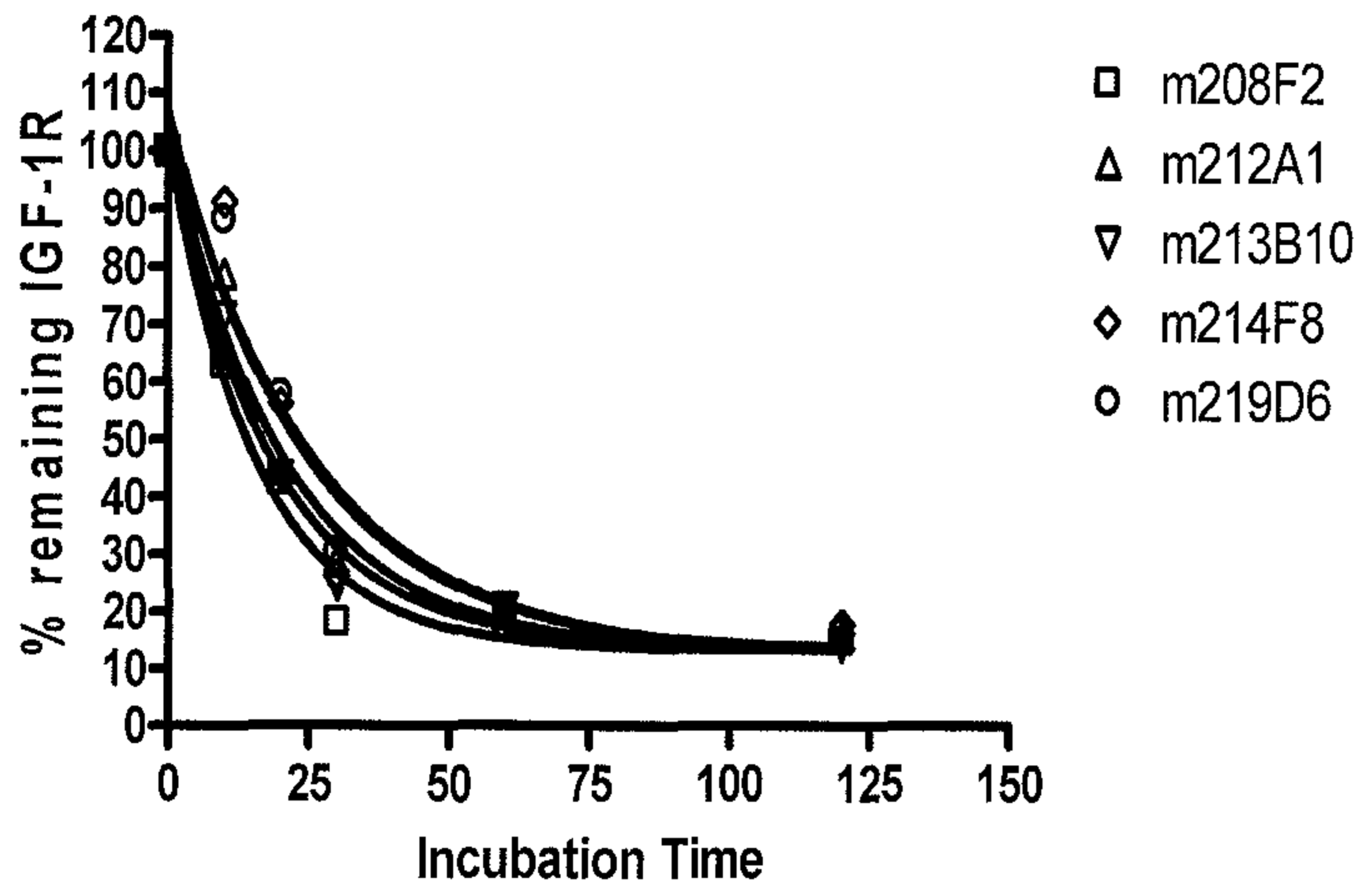


FIGURE 9

A



B

| Abs | HalfLife (t1/2) |
|---------|-----------------|
| m208F2 | 11.11 |
| m212A11 | 14.3 |
| m214F8 | 16.81 |
| m219D6 | 17.85 |
| m213B10 | 12.83 |

FIGURES 10A-10B

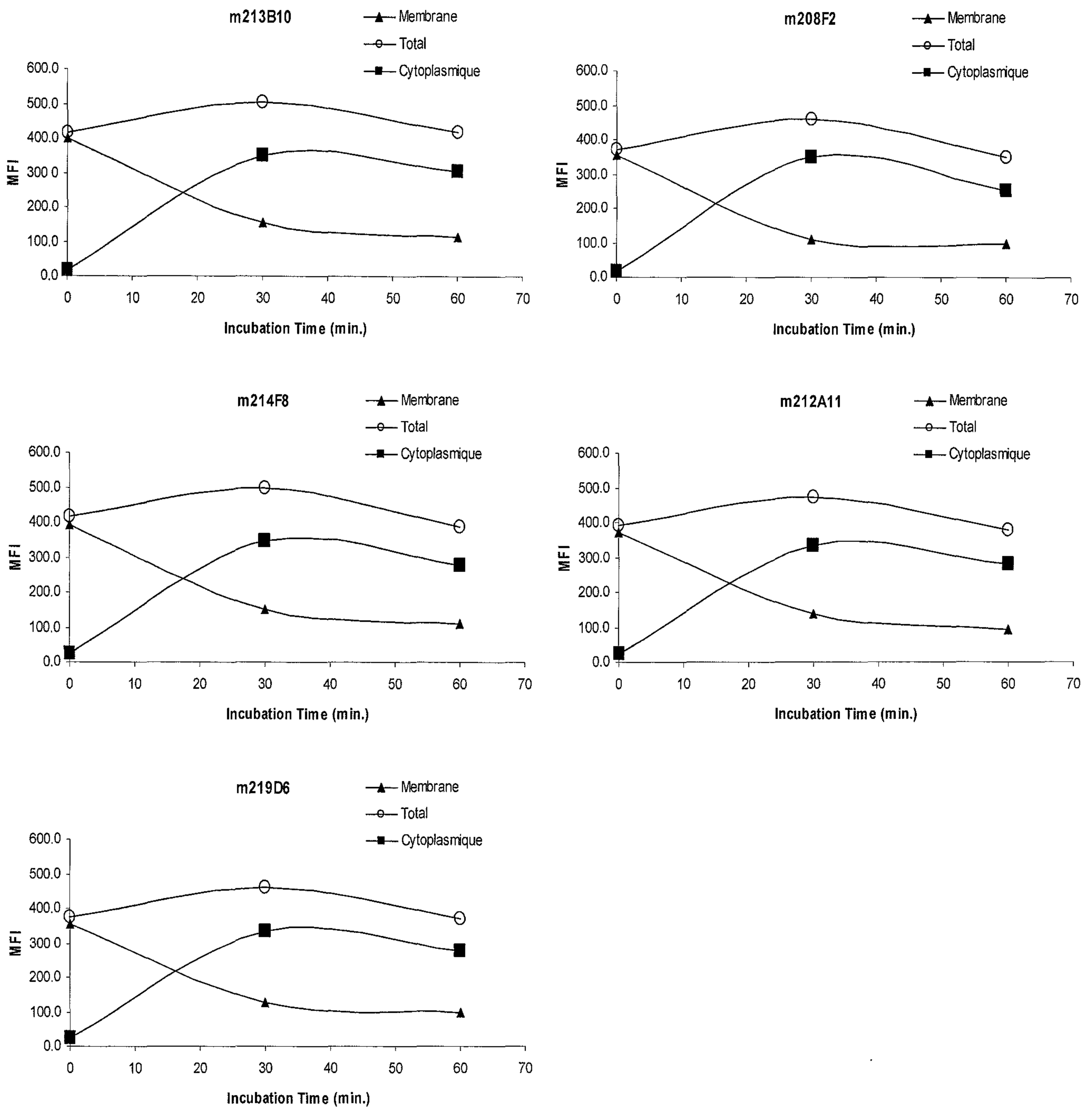


FIGURE 11

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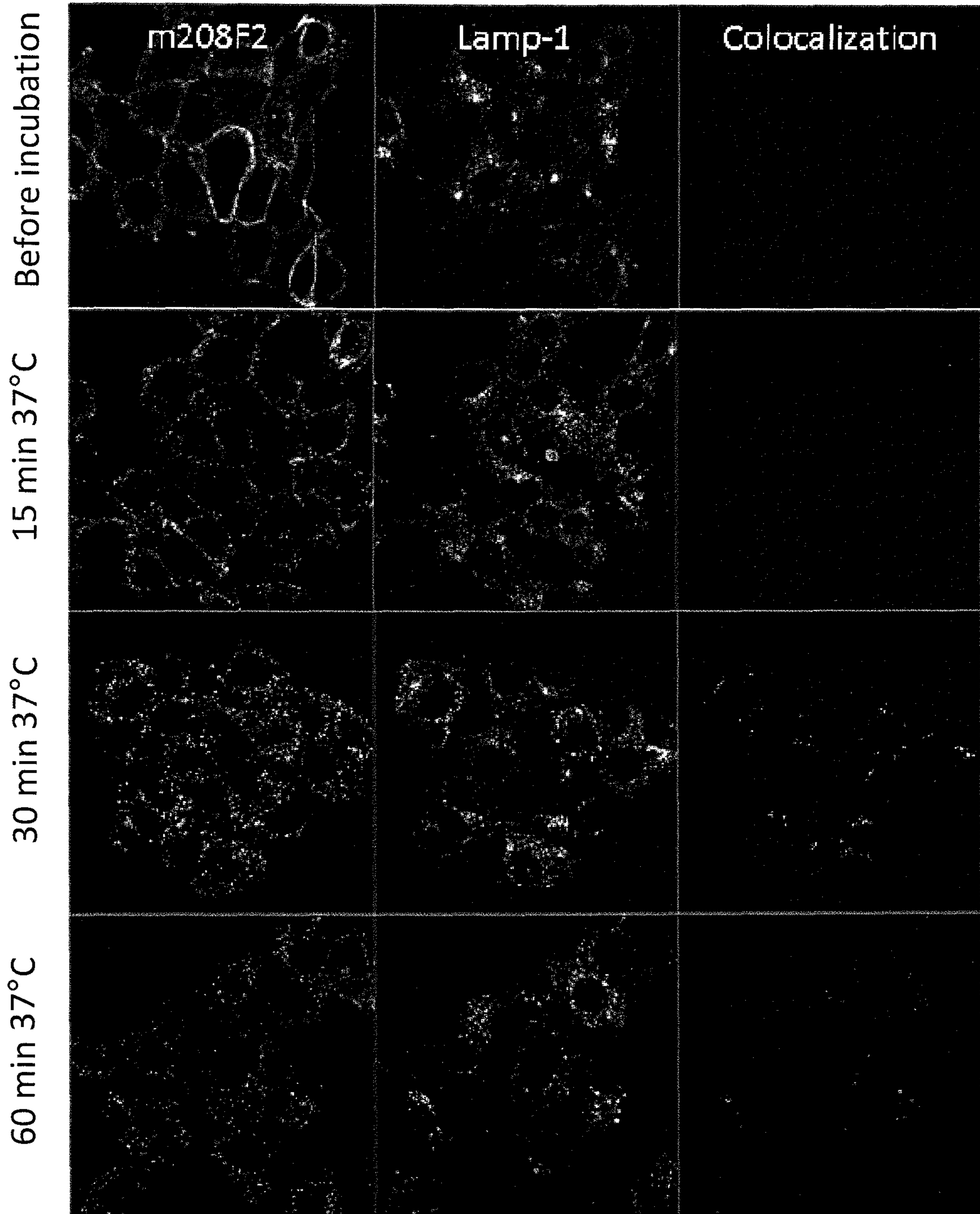


FIGURE 12A

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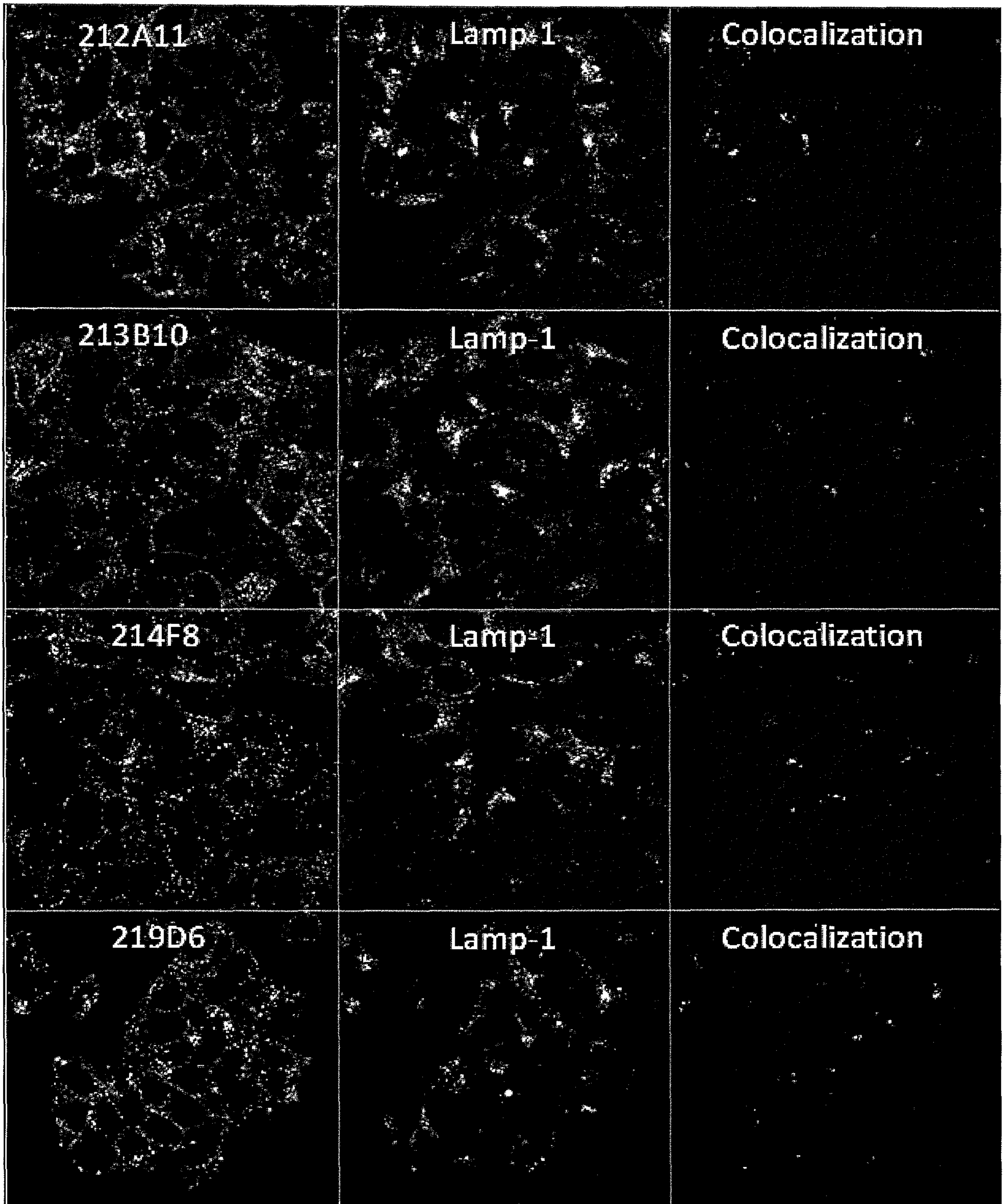


FIGURE 12B

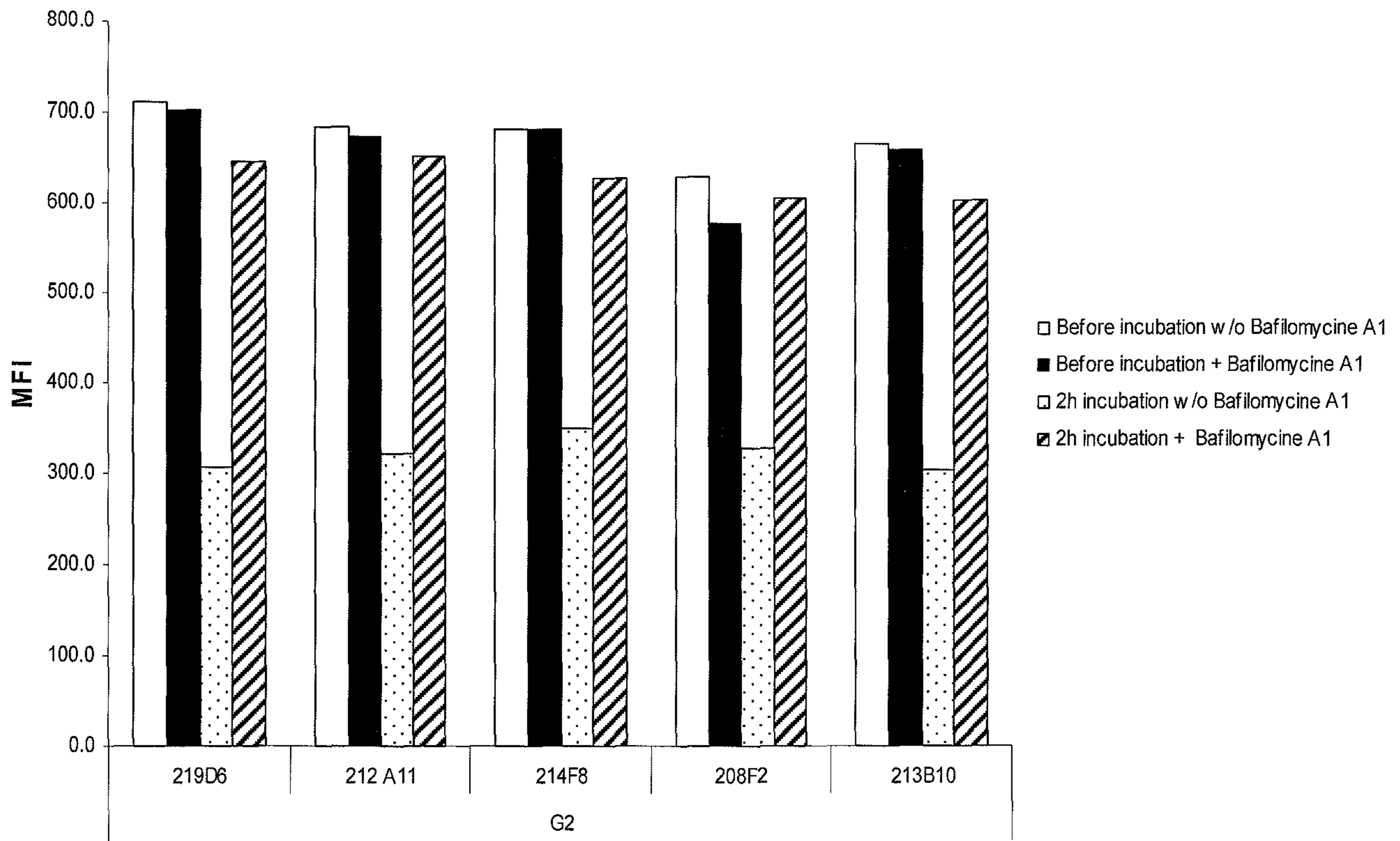


FIGURE 13

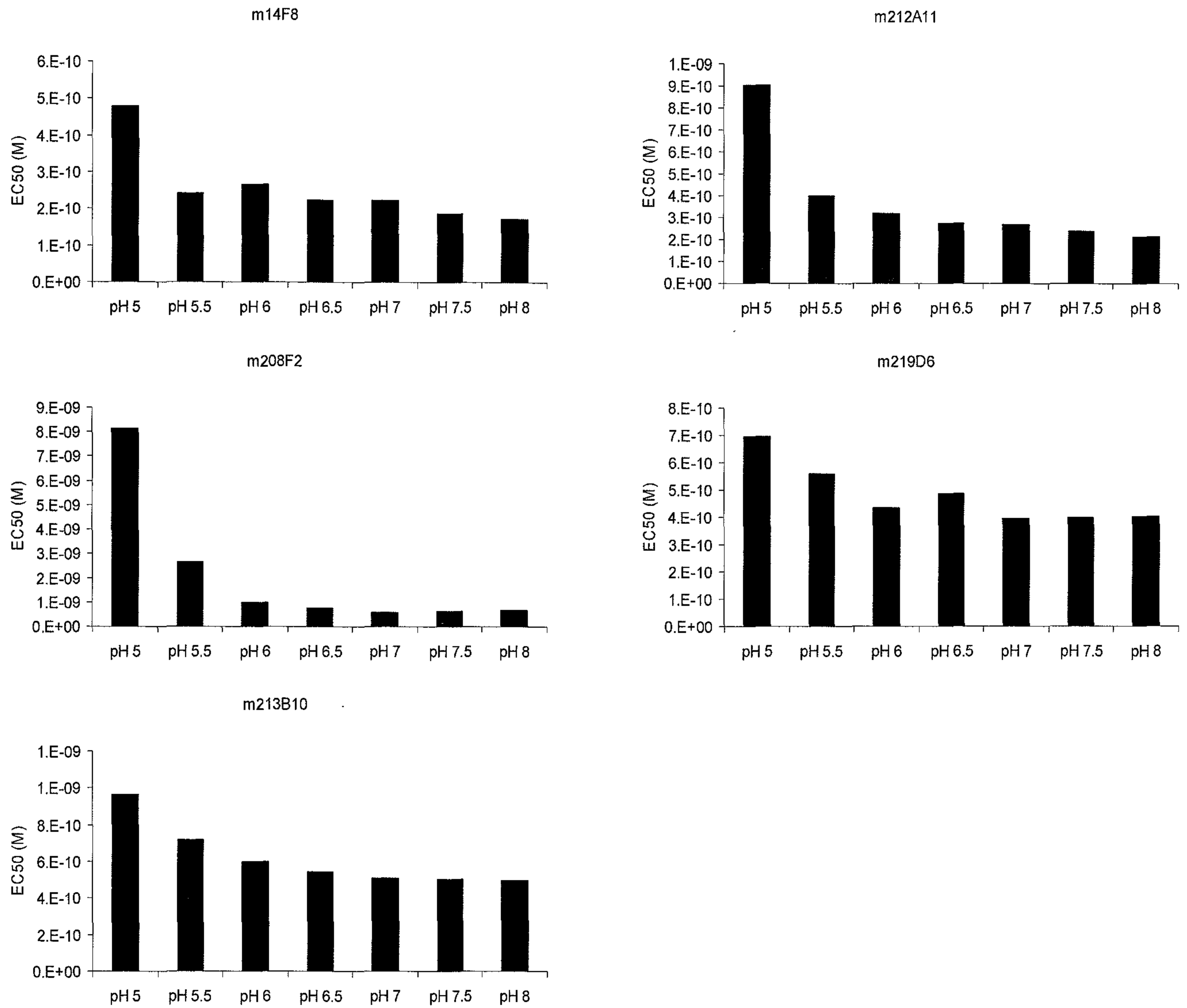
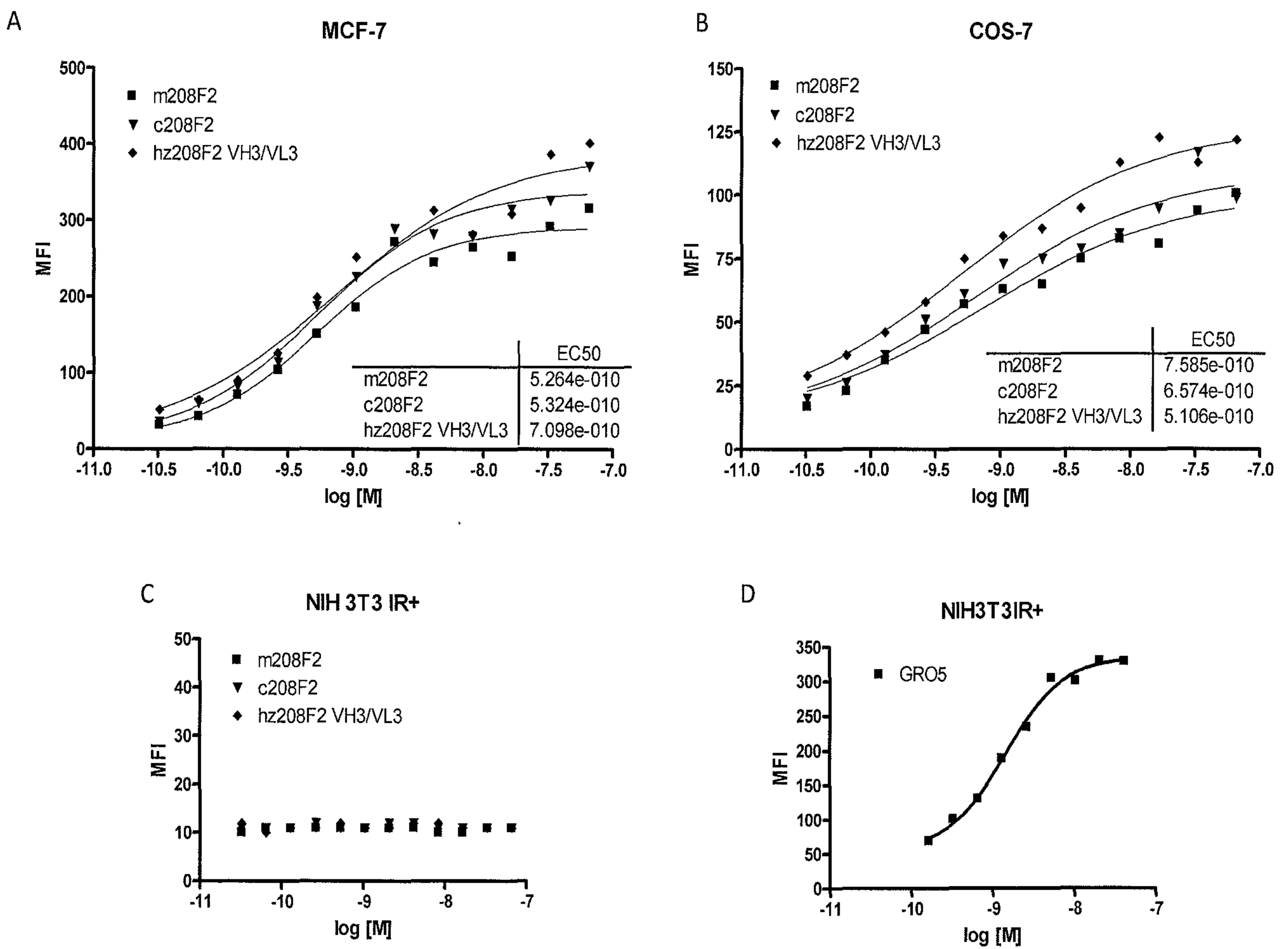


FIGURE 14



FIGURES 15A-15D

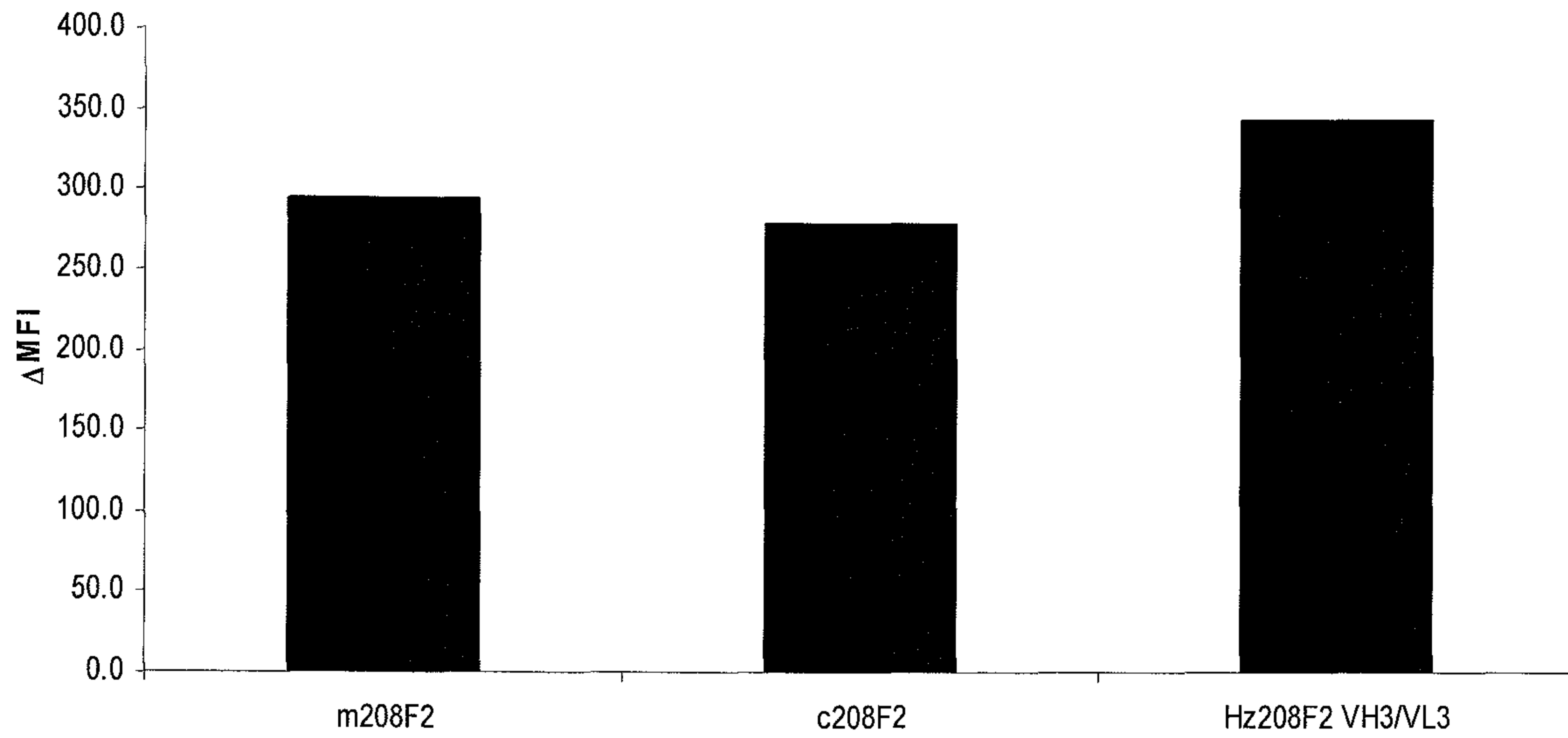


FIGURE 16

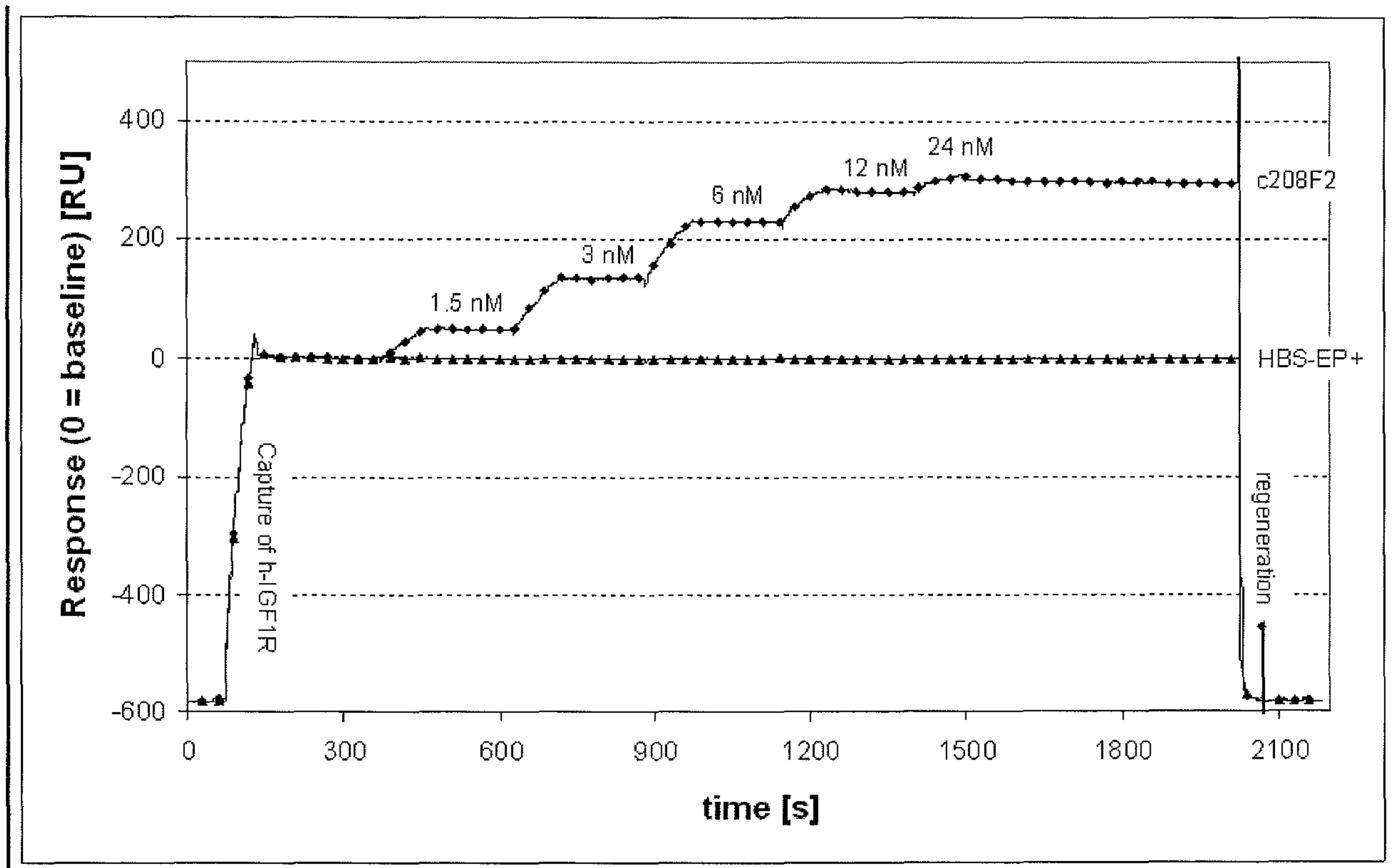


FIGURE 17

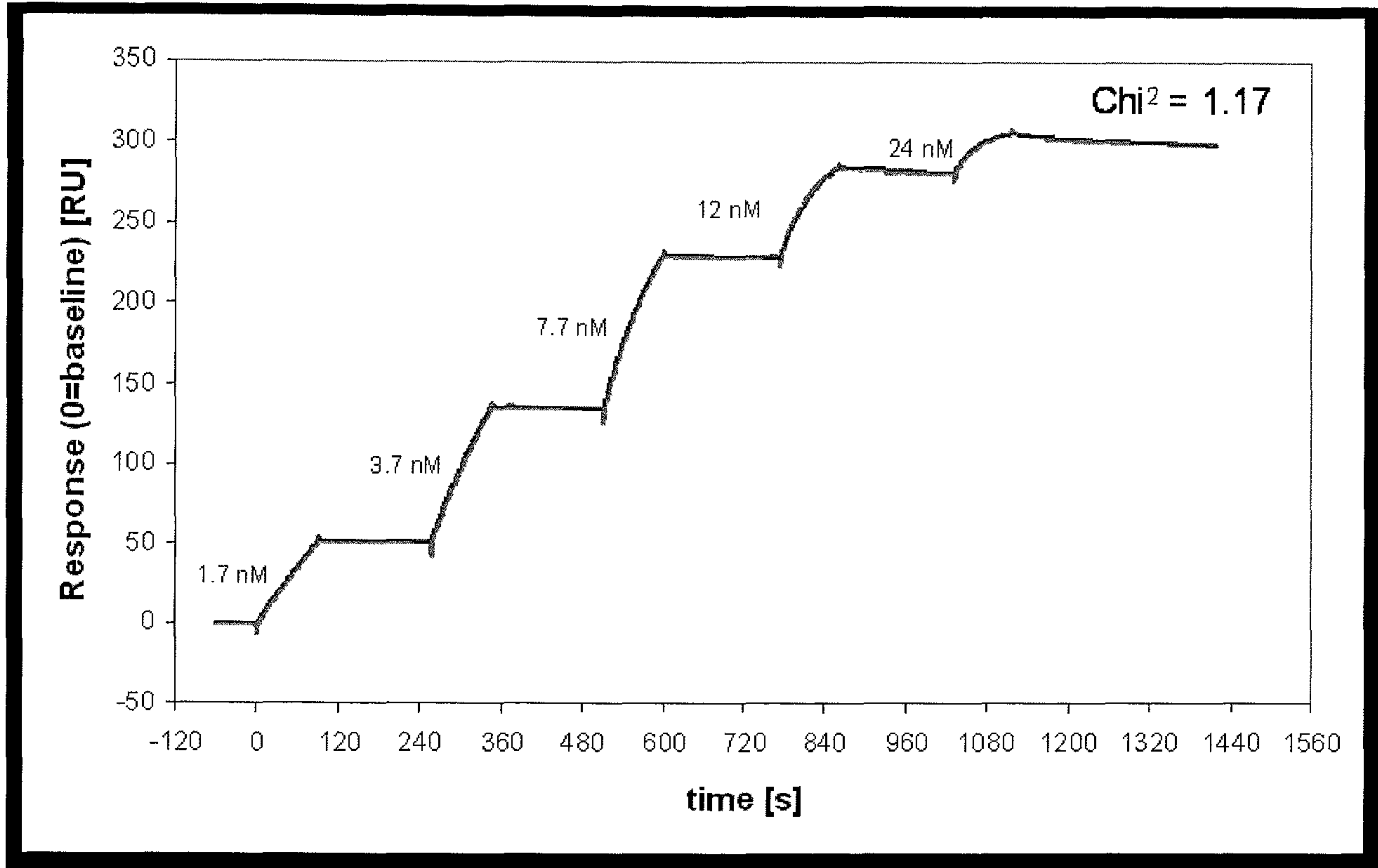


FIGURE 18

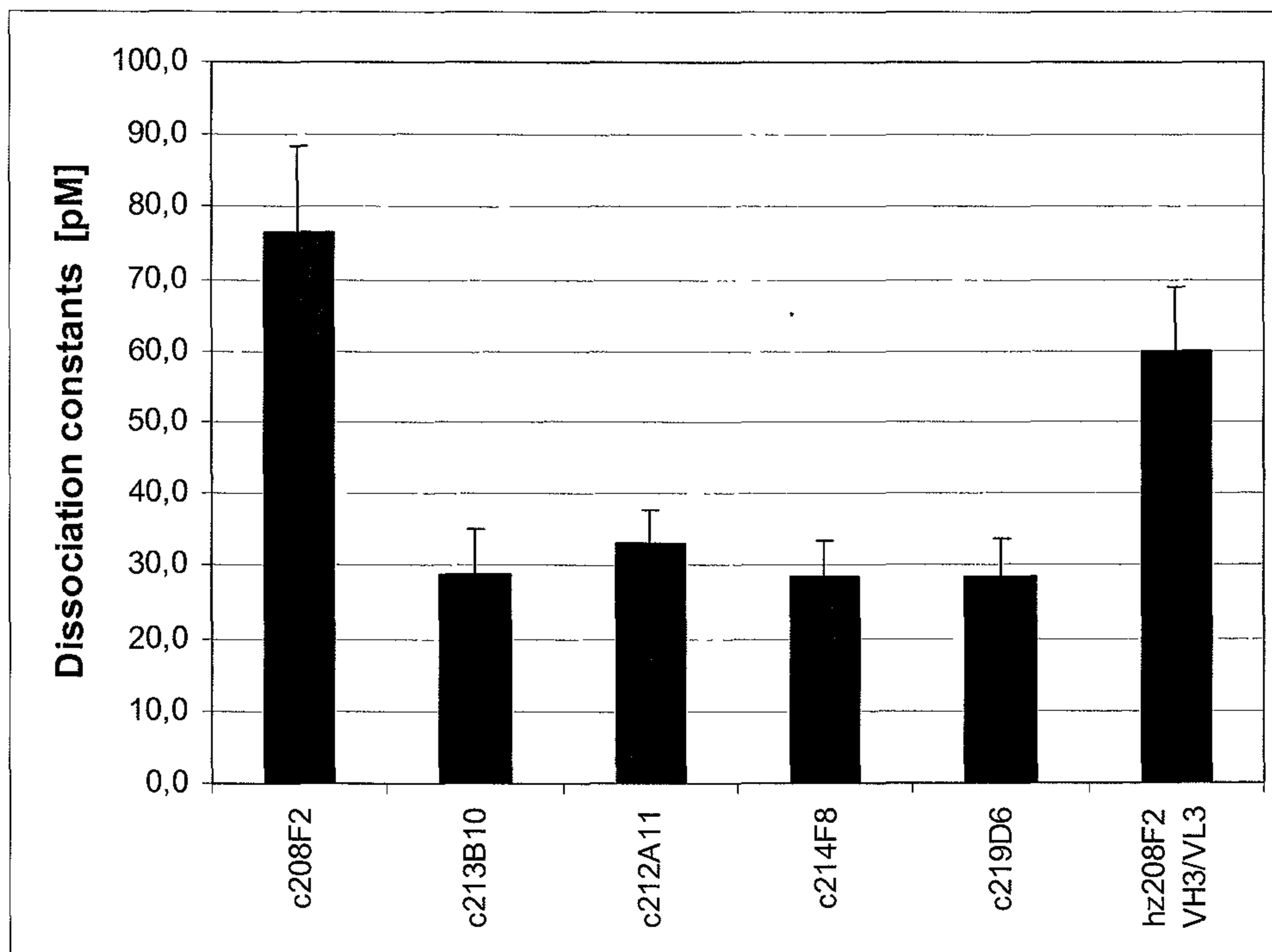


FIGURE 19

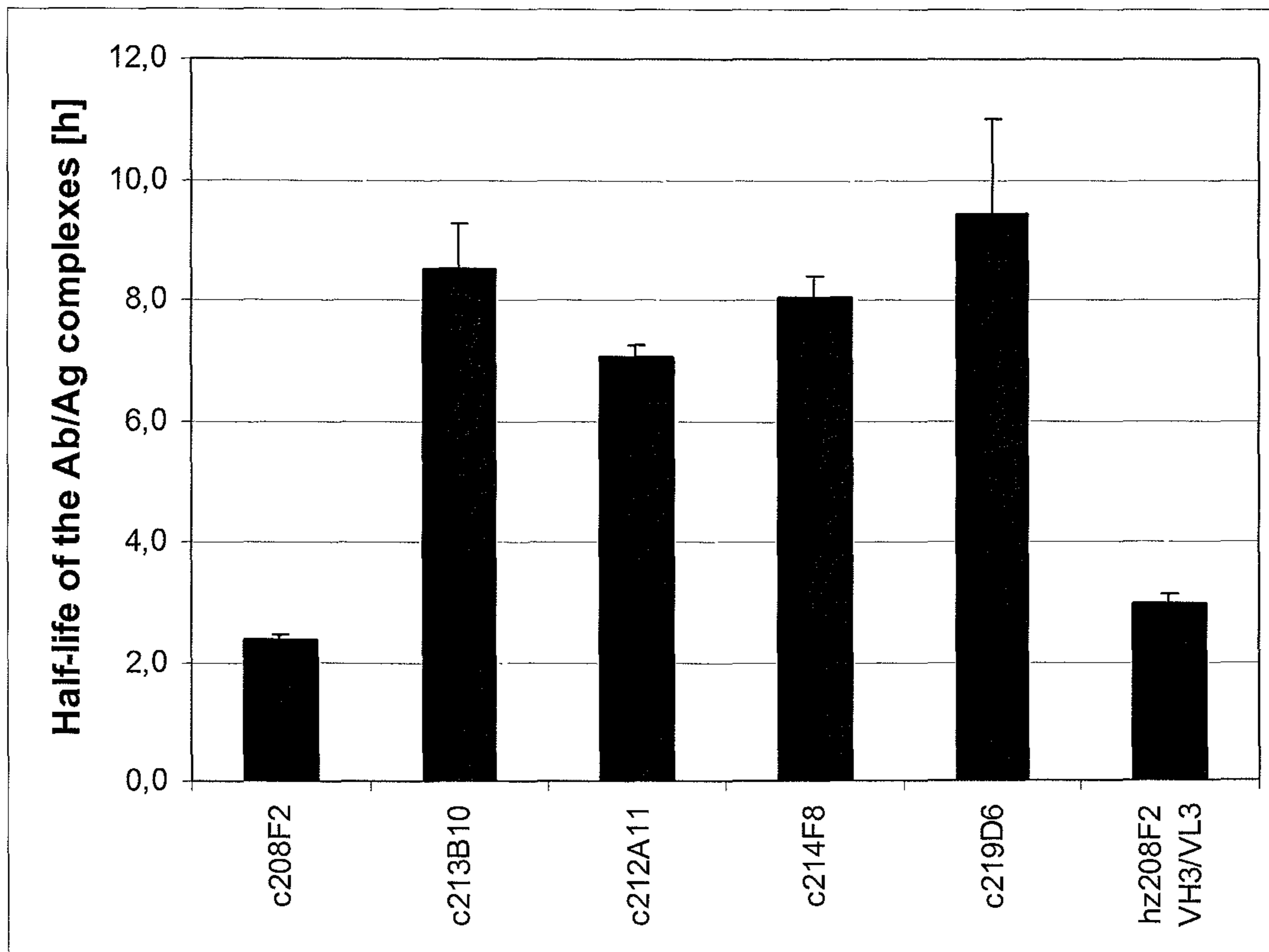


FIGURE 20

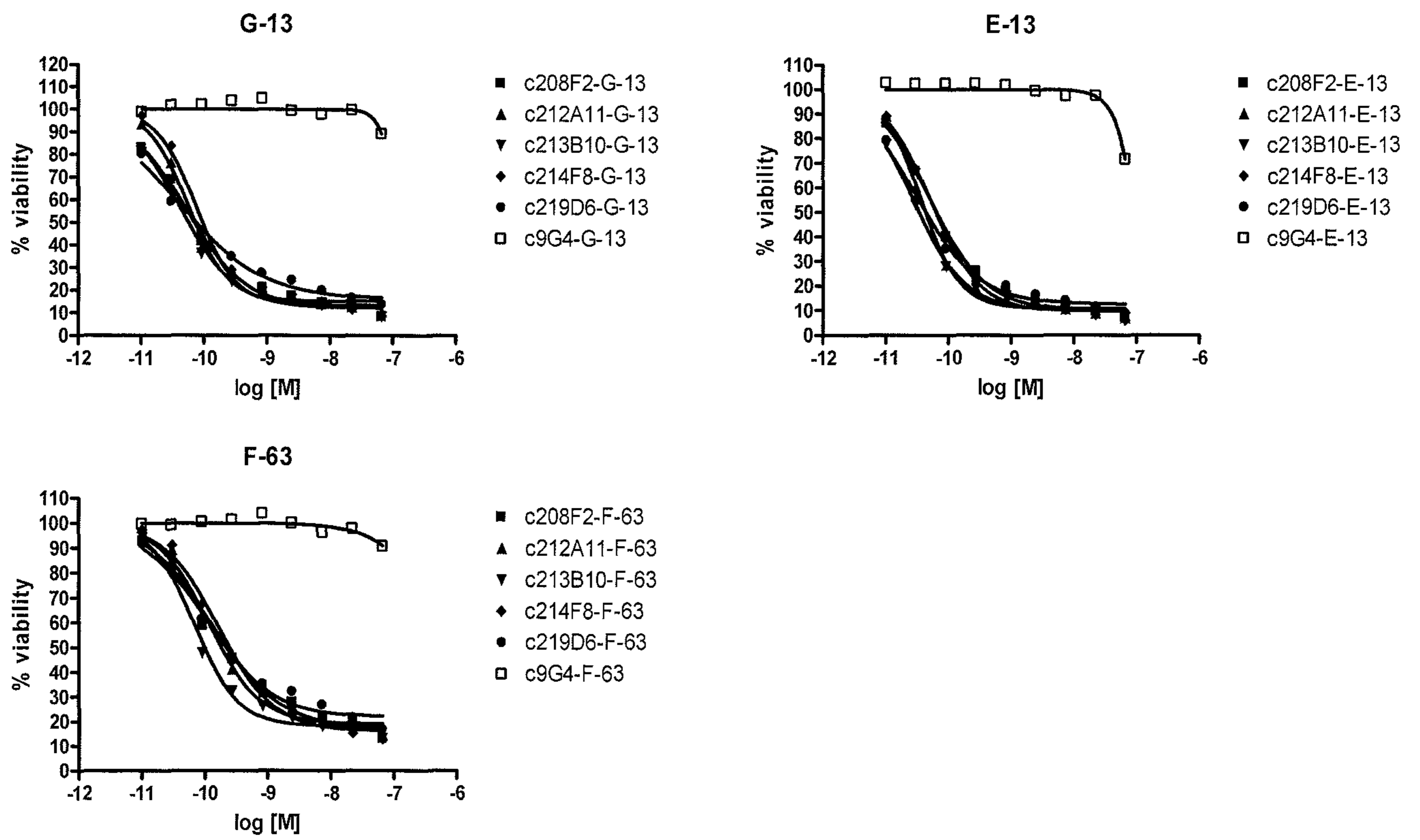


FIGURE 21

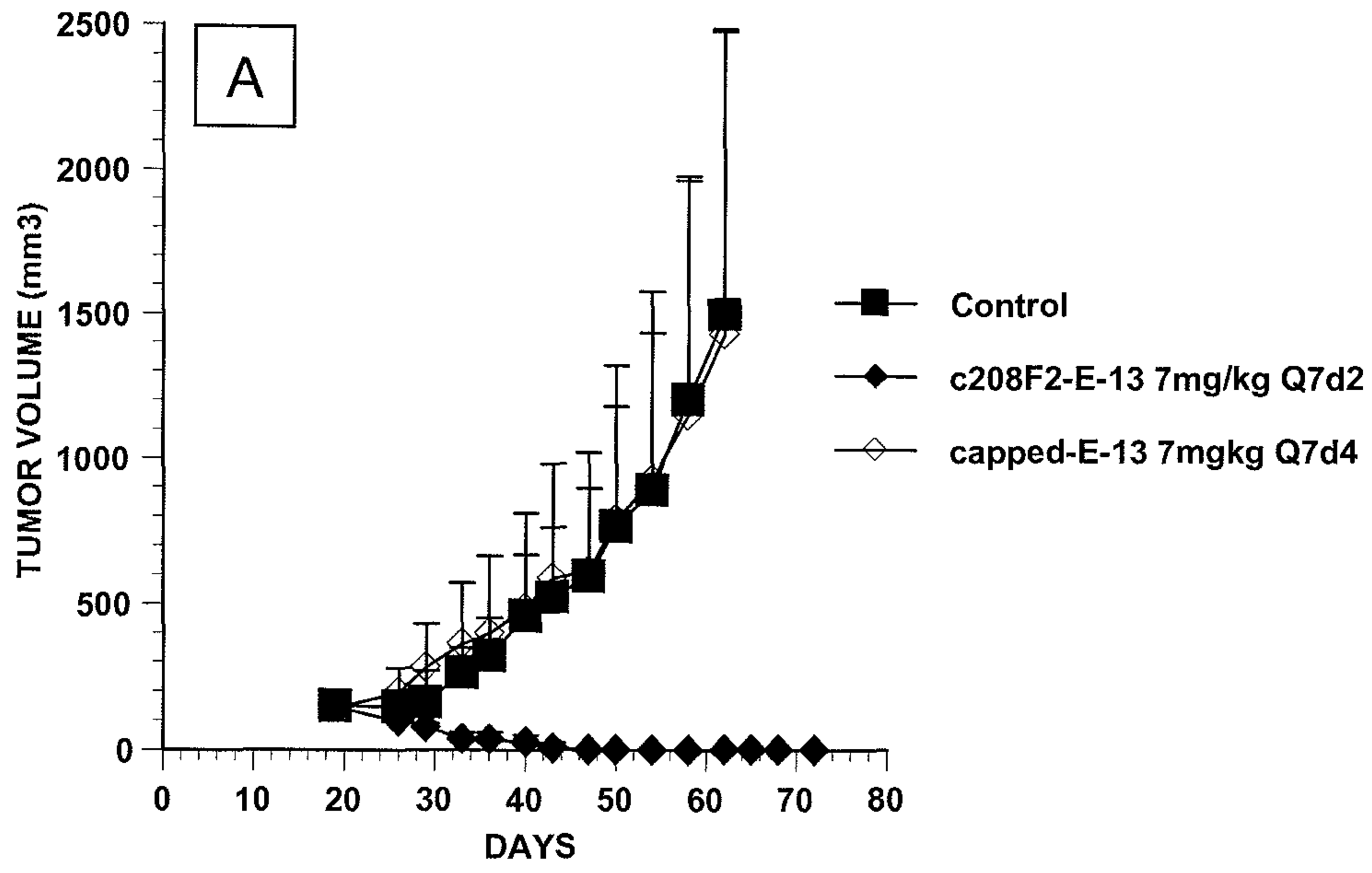


FIGURE 22A

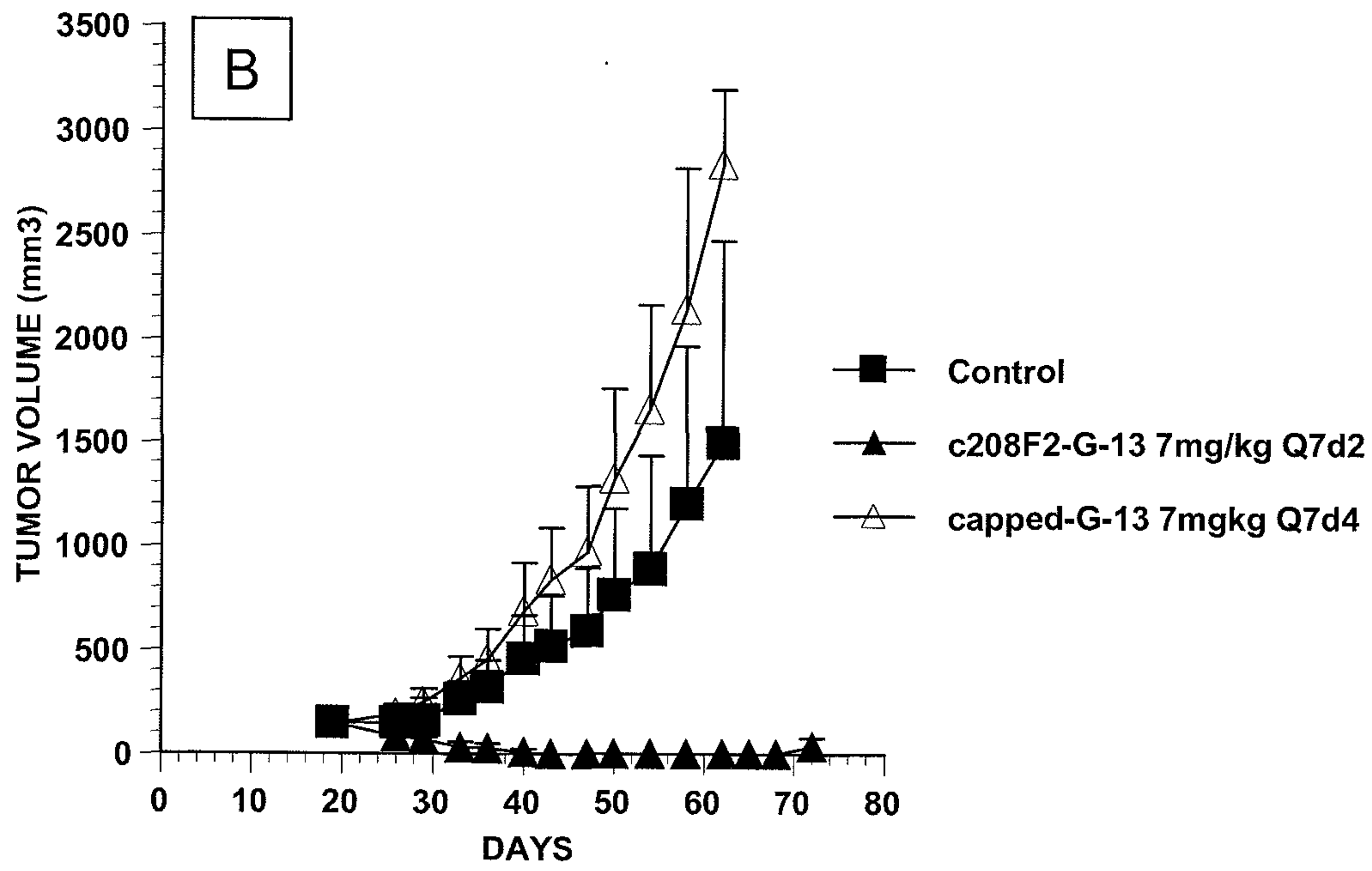


FIGURE 22B

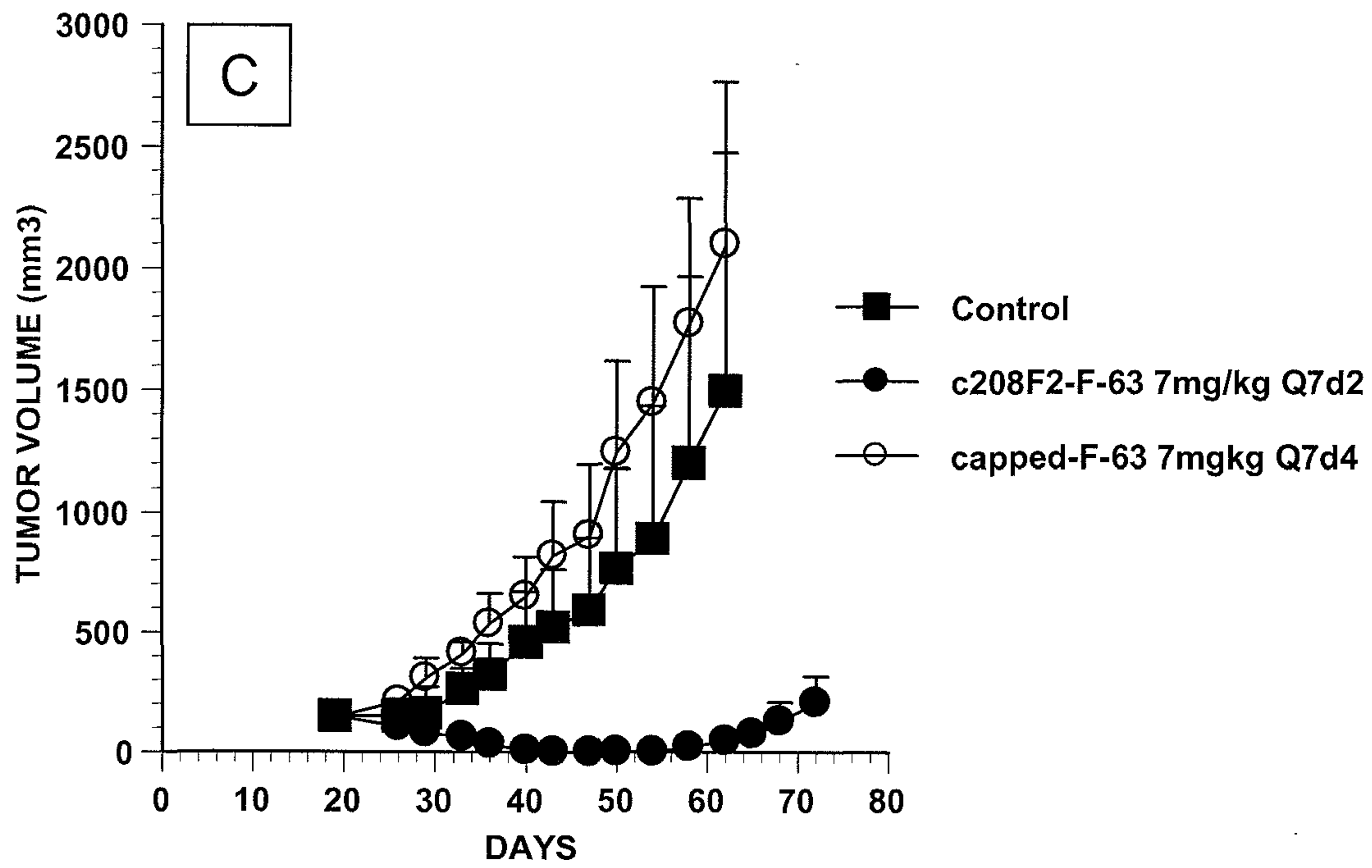


FIGURE 22C

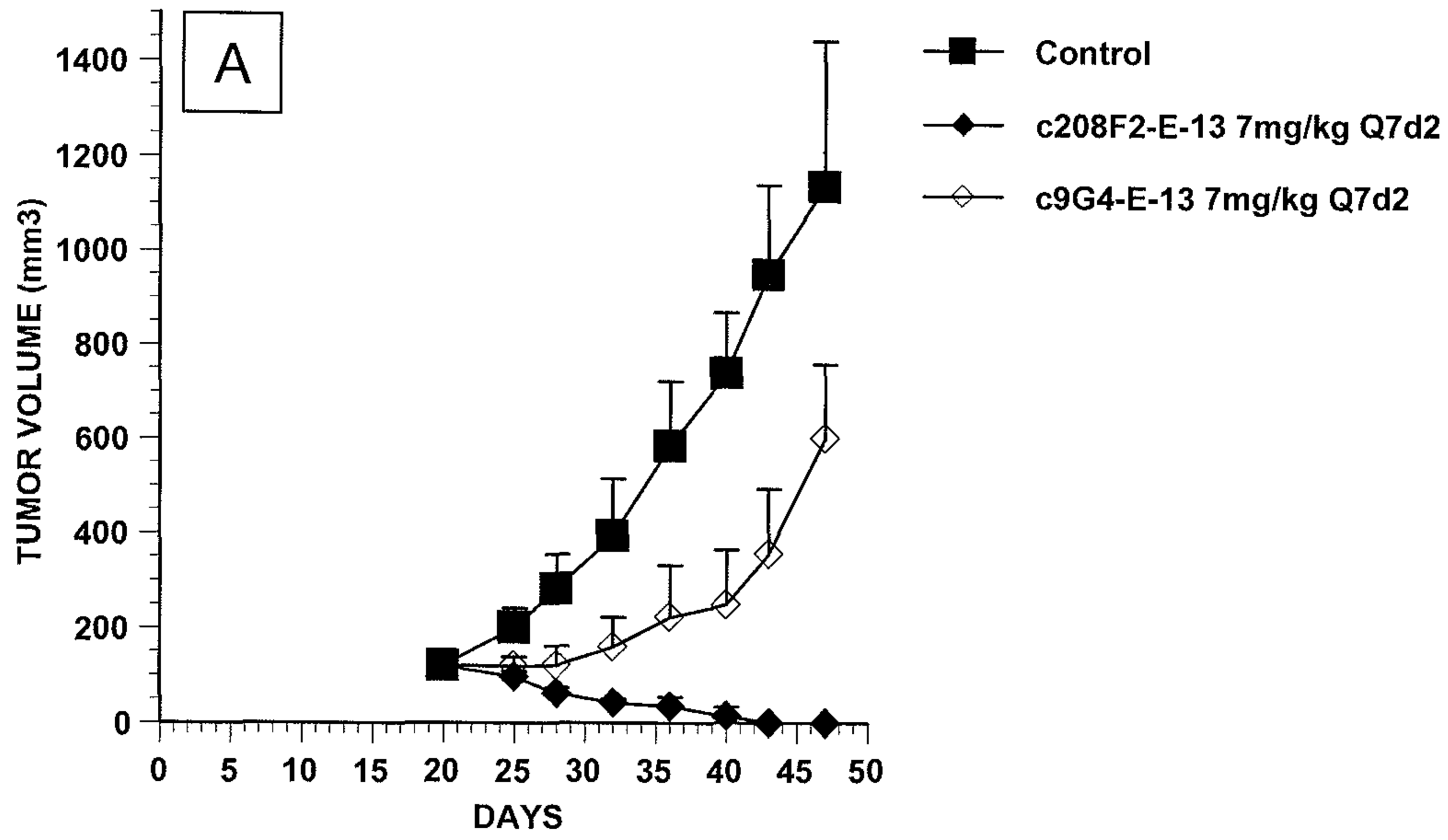


FIGURE 23A

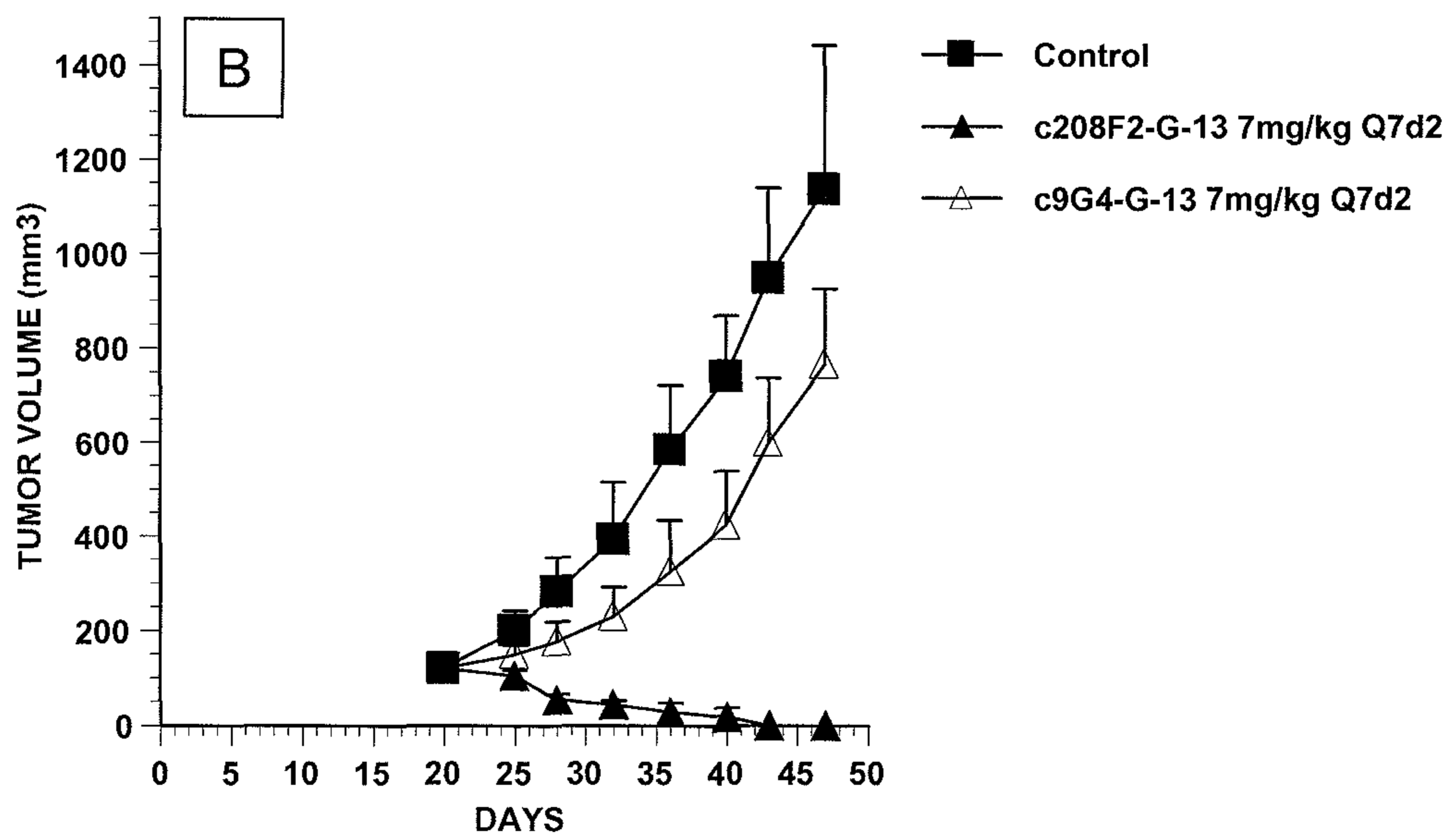


FIGURE 23B

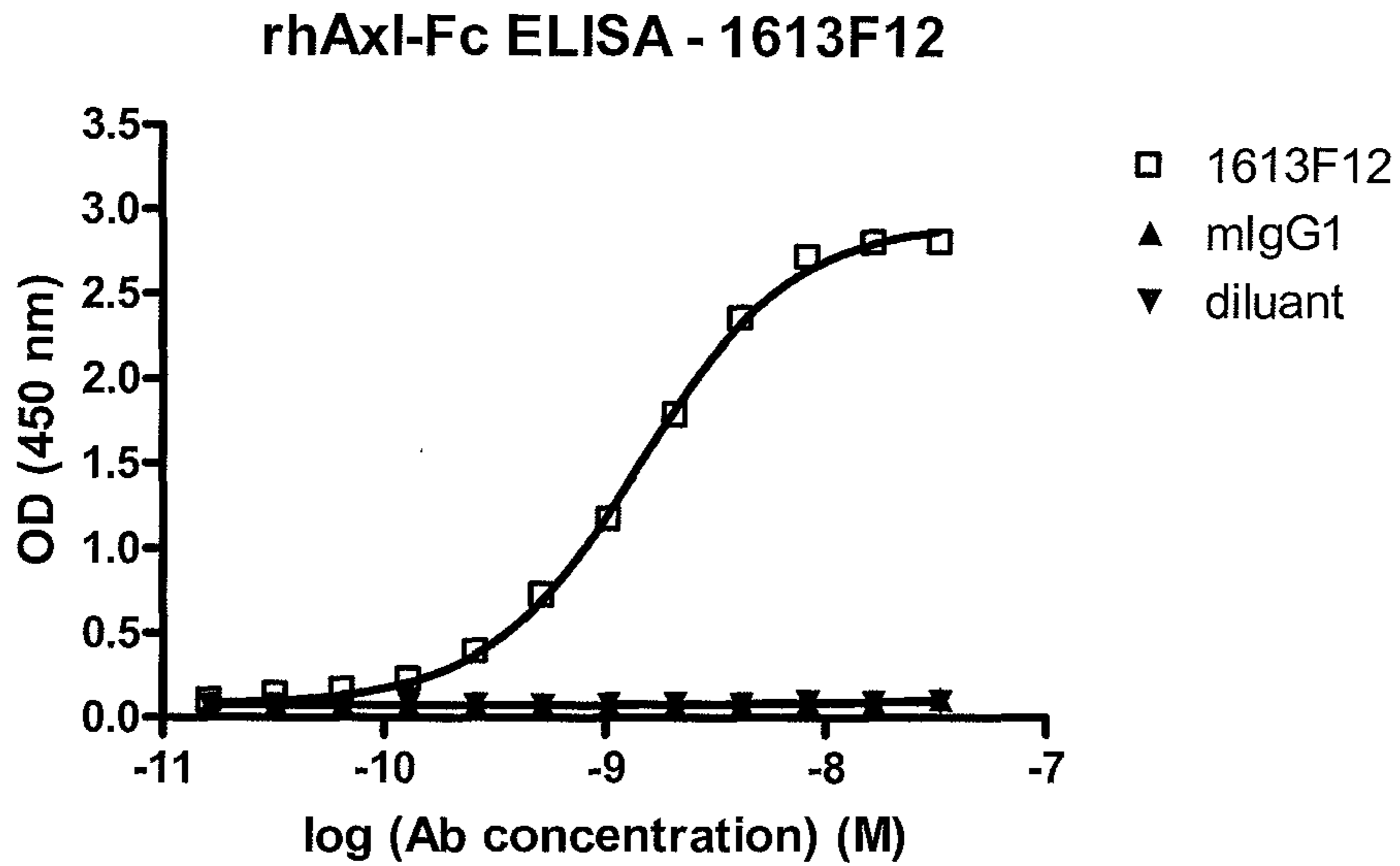


FIGURE 24A

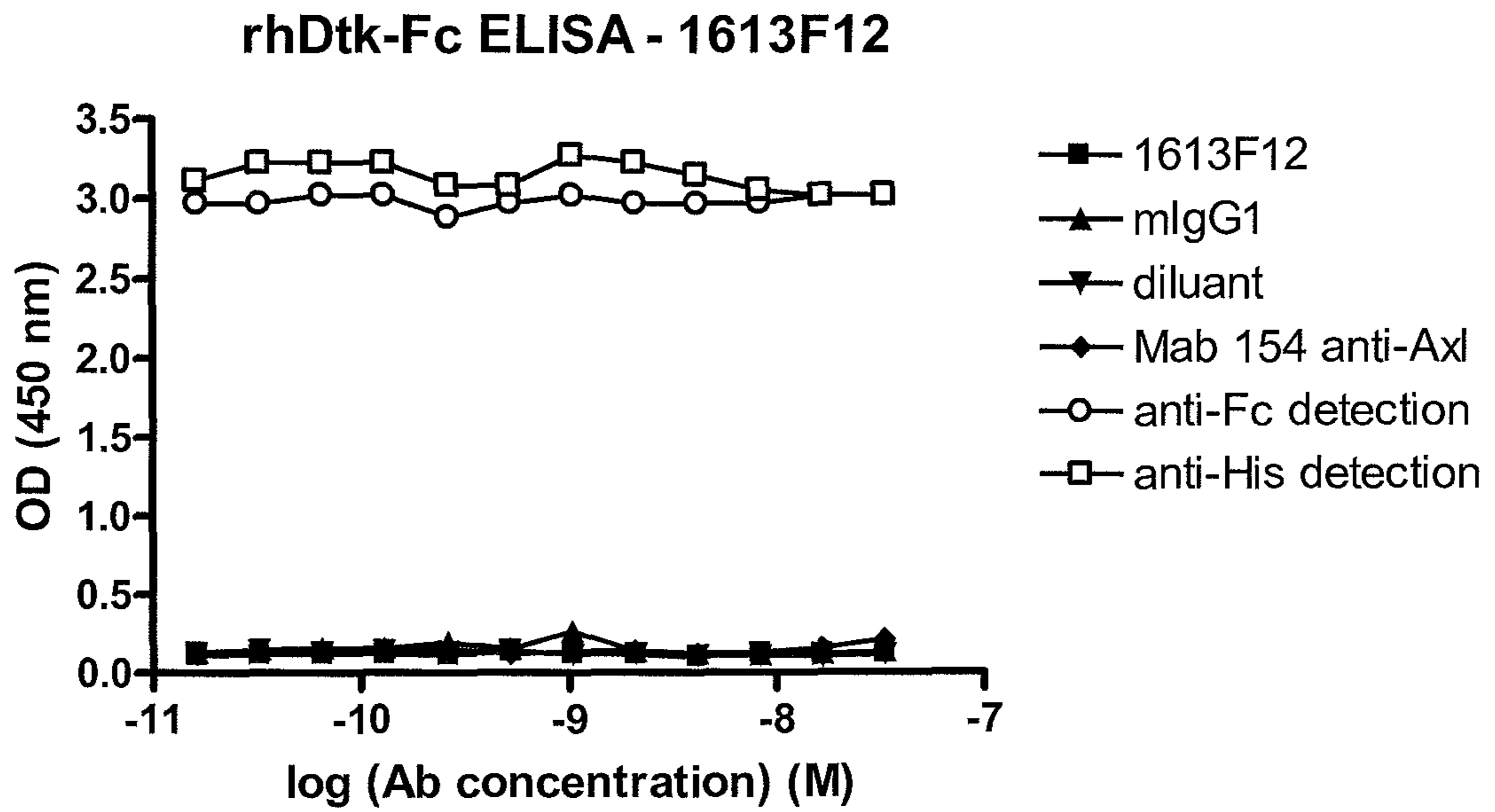


FIGURE 24B

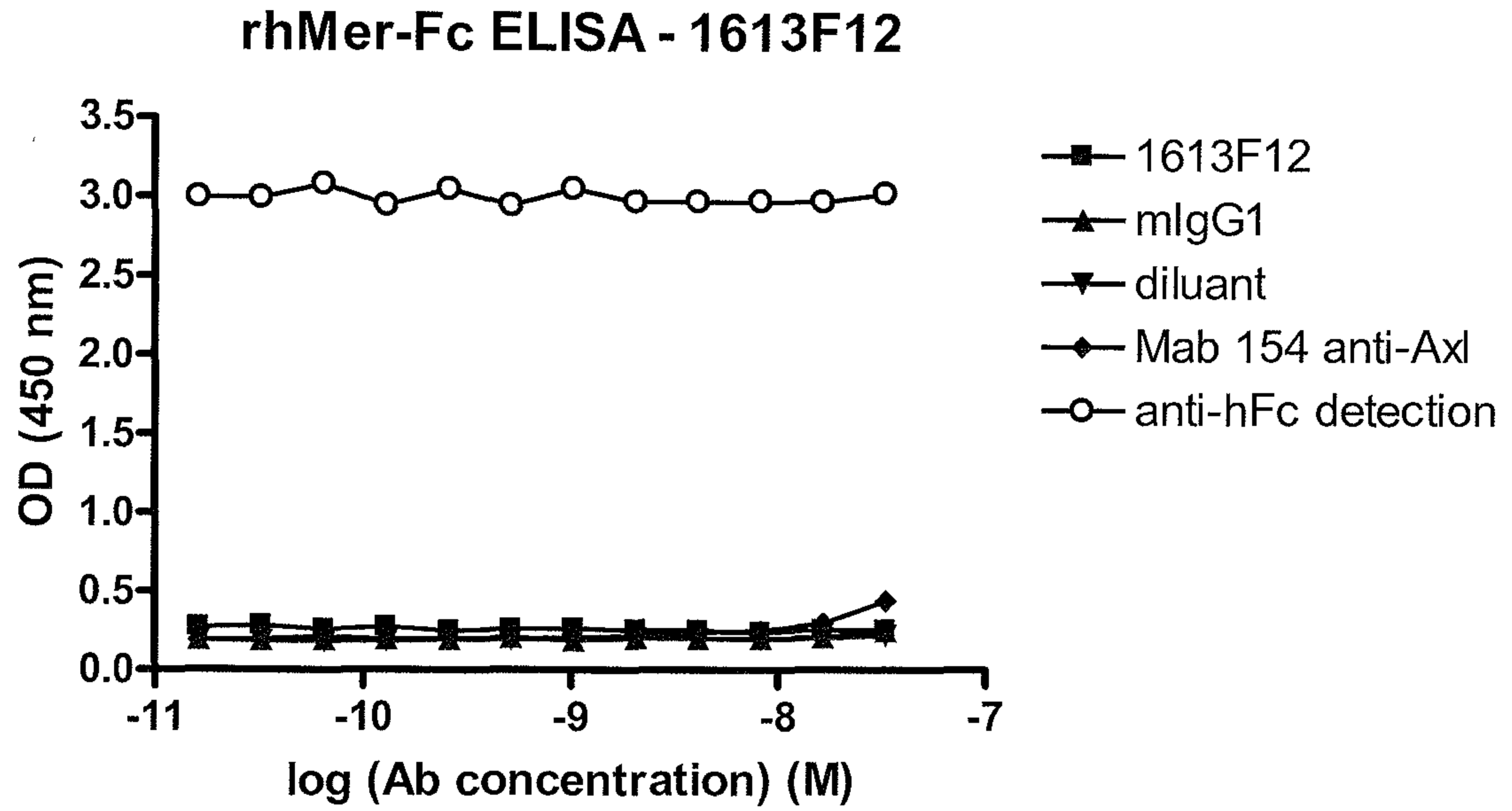


FIGURE 24C

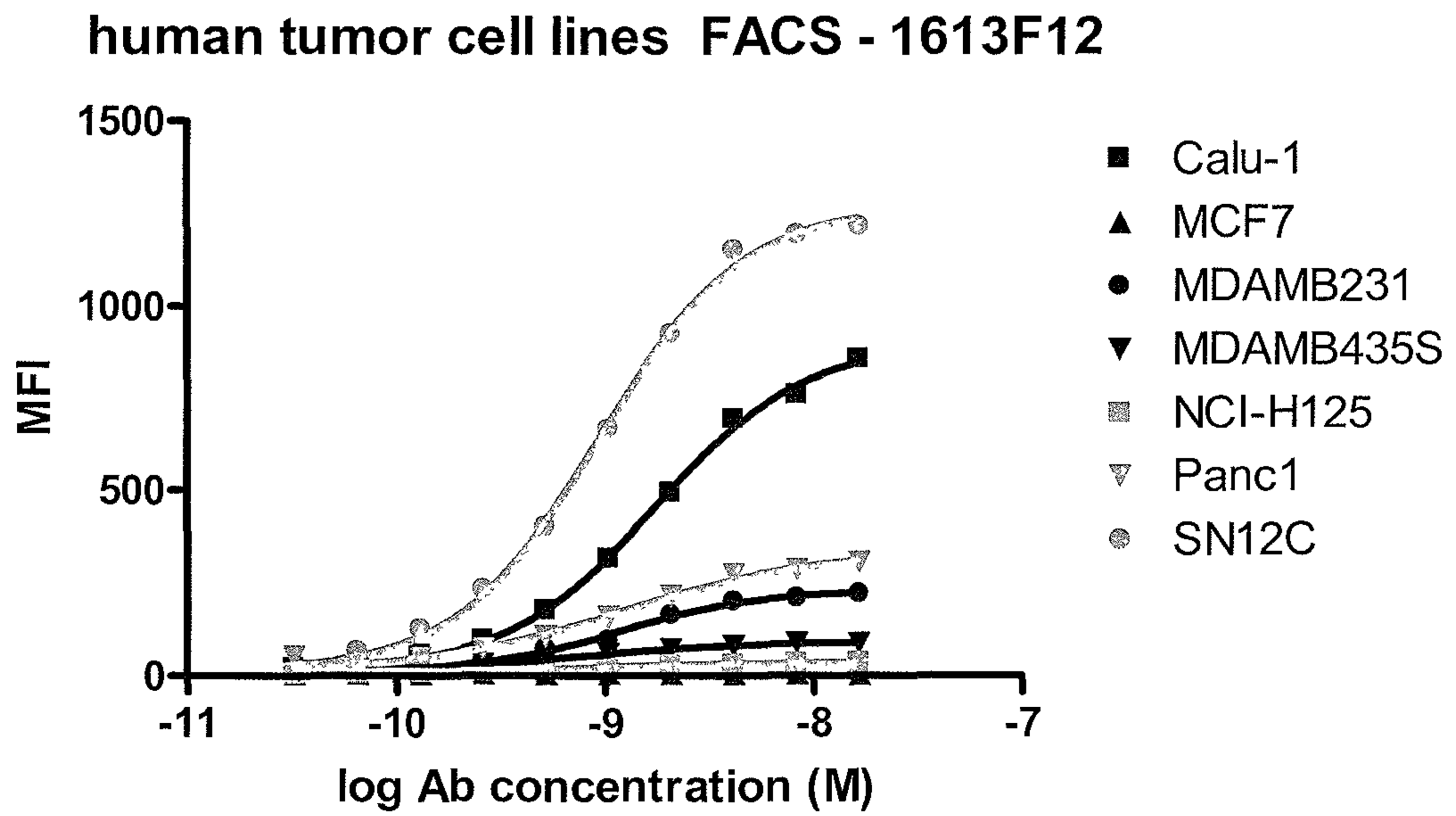


FIGURE 25

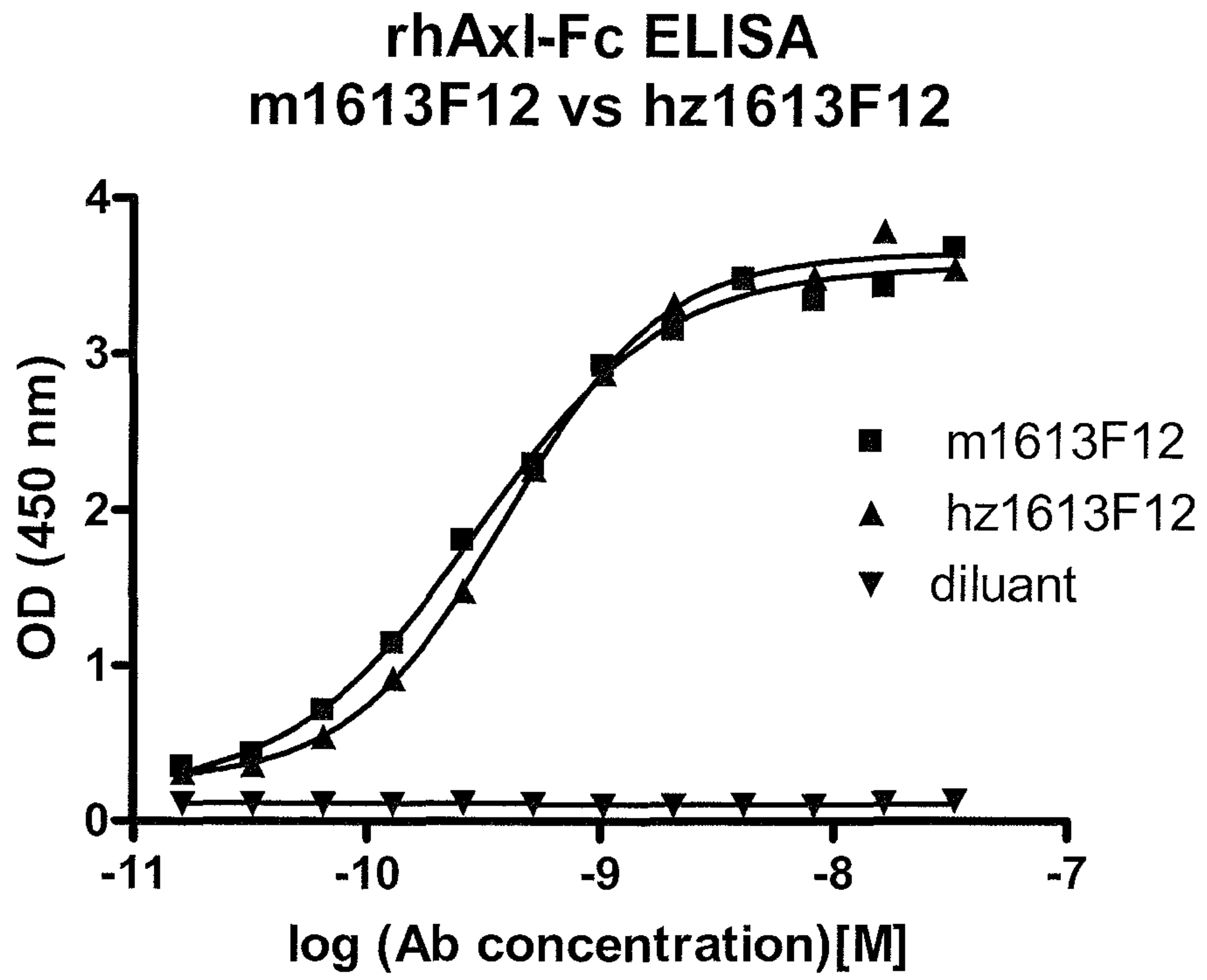


FIGURE 26

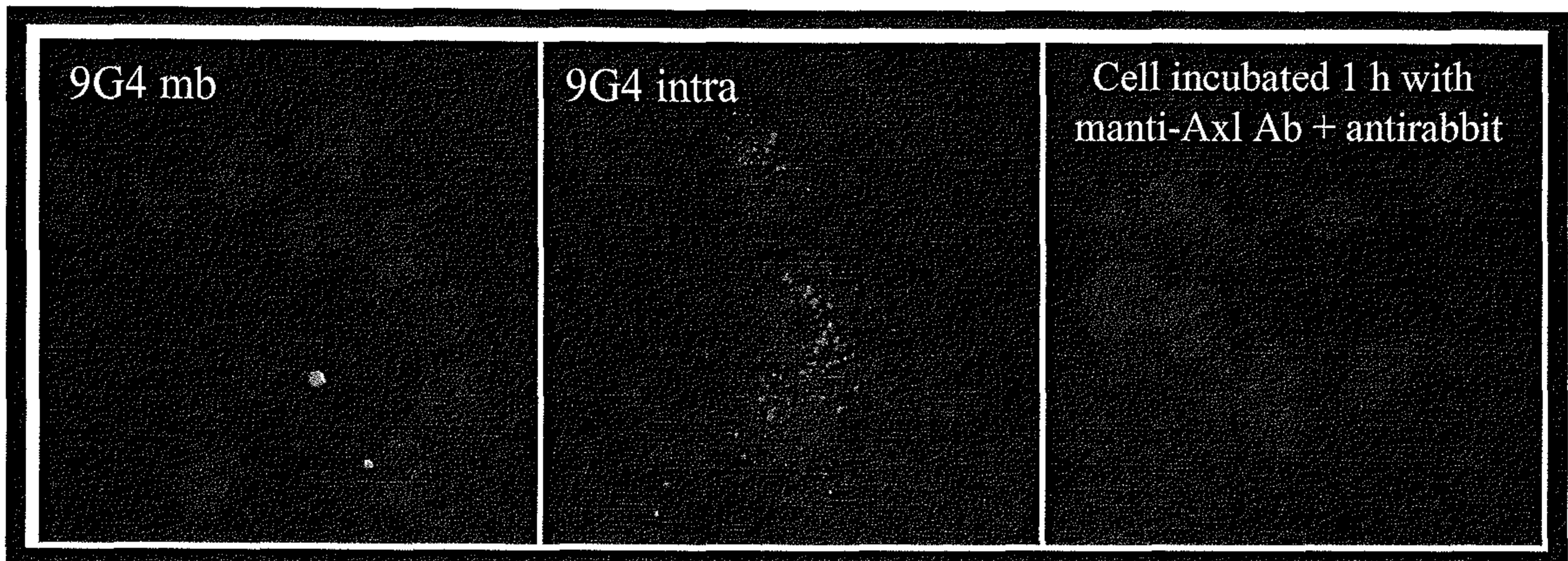


FIGURE 27A

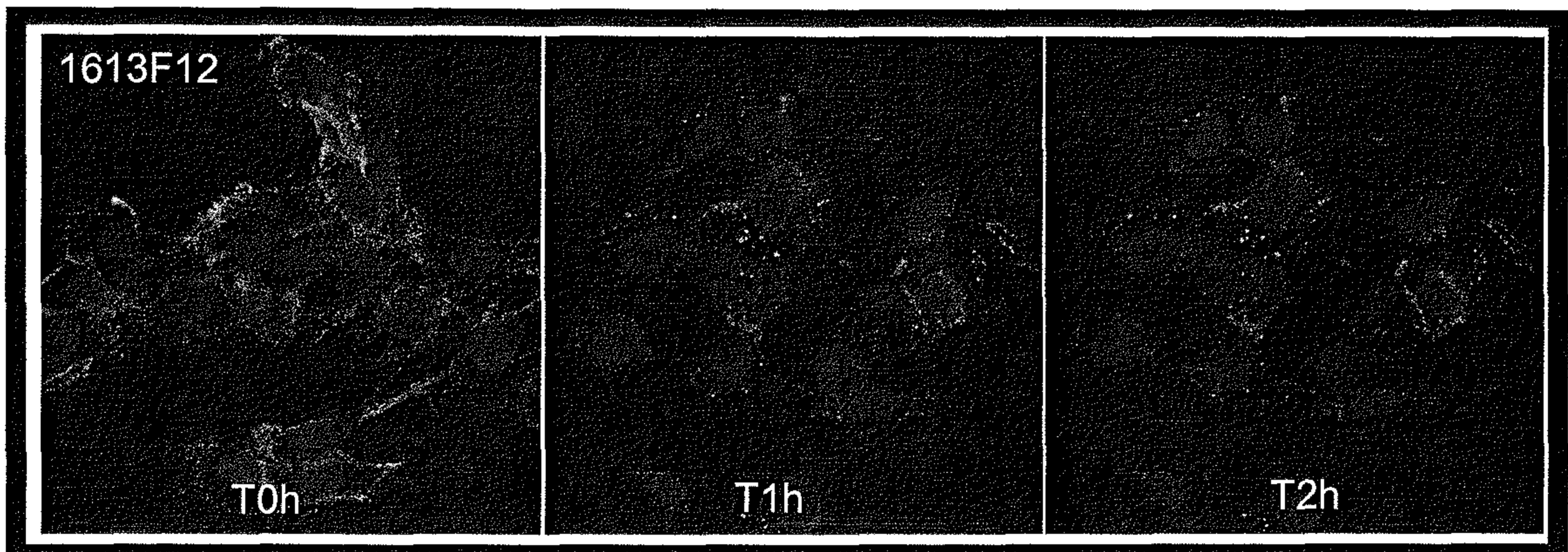


FIGURE 27B

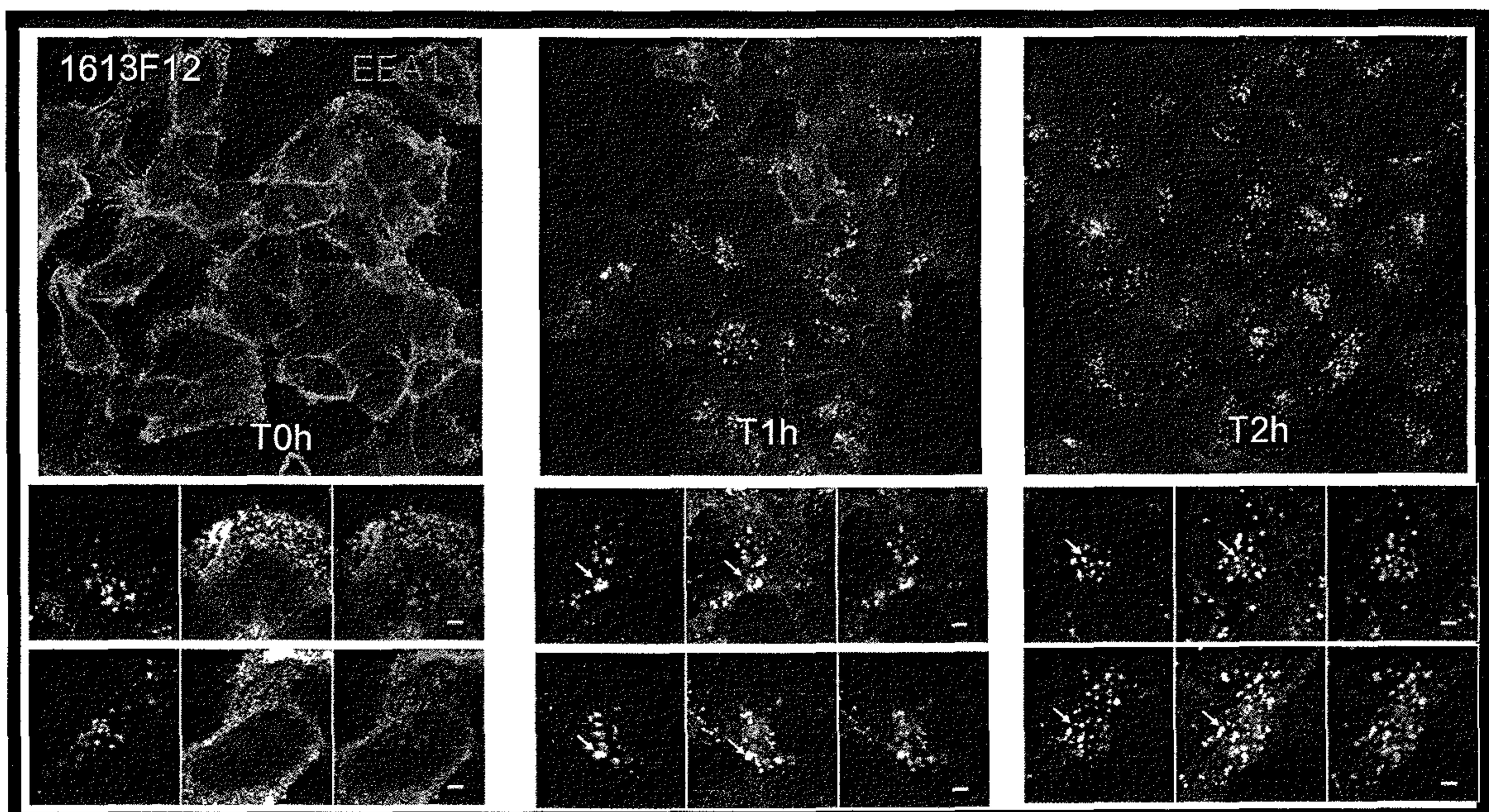
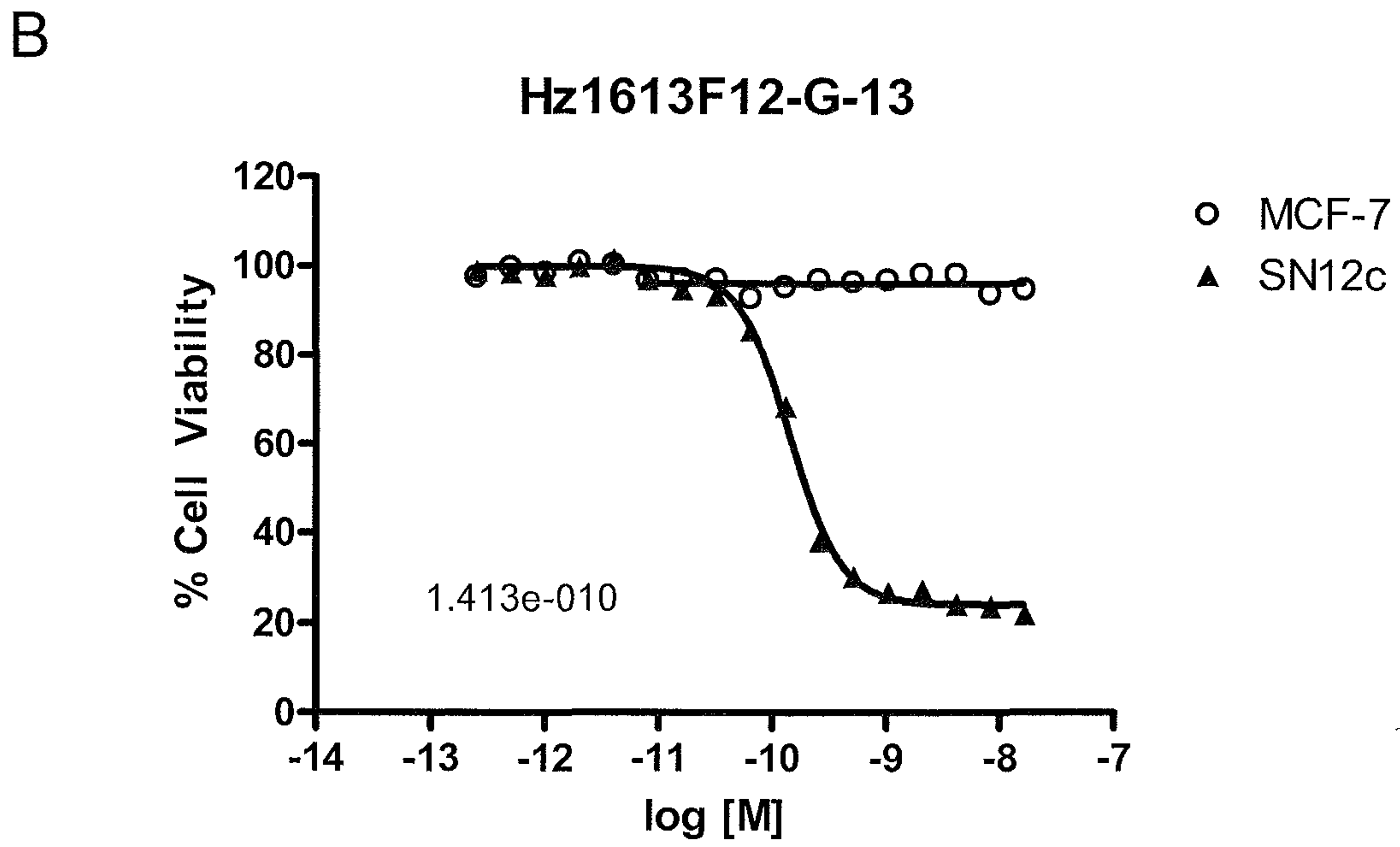
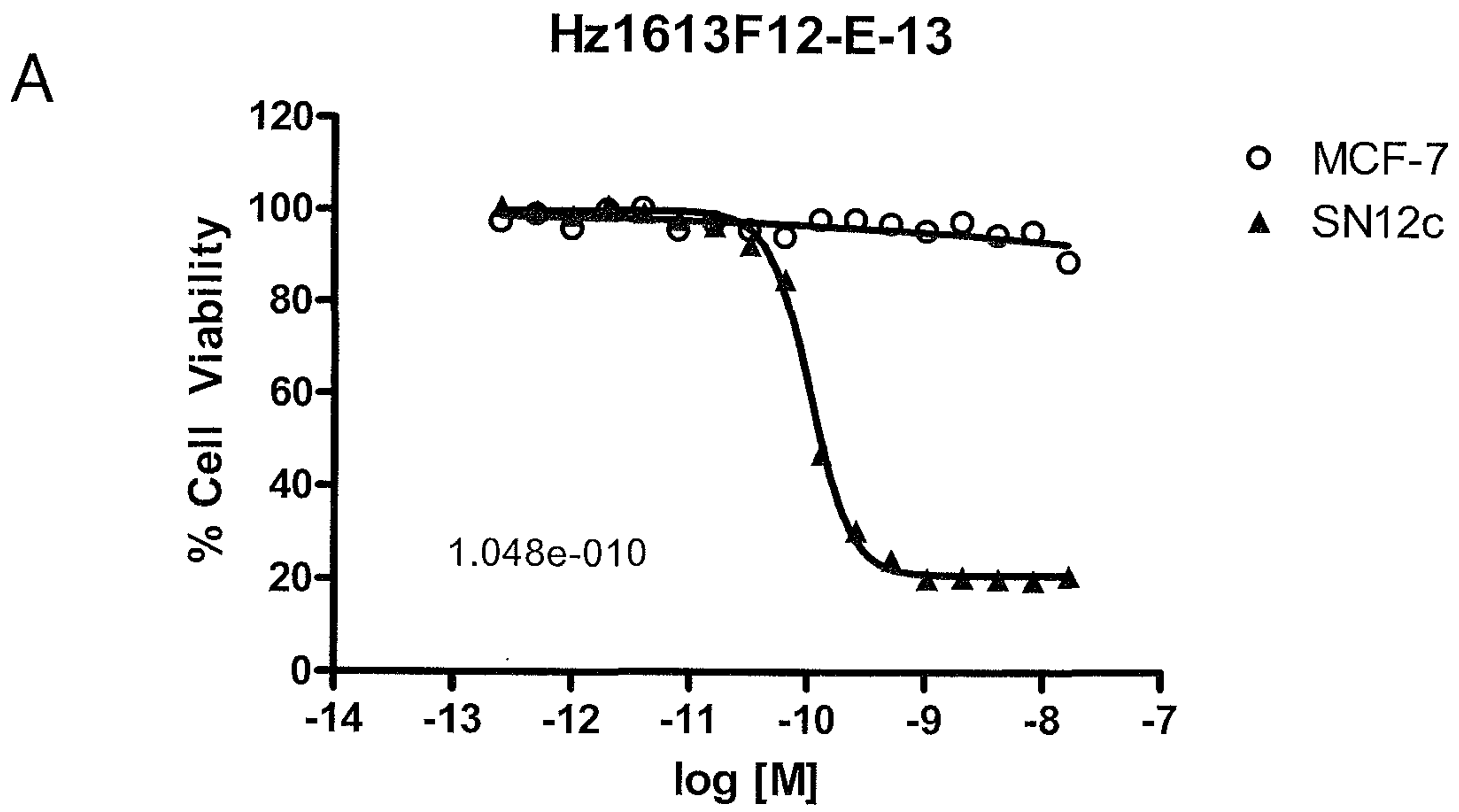


FIGURE 27C



FIGURES 28A-28B

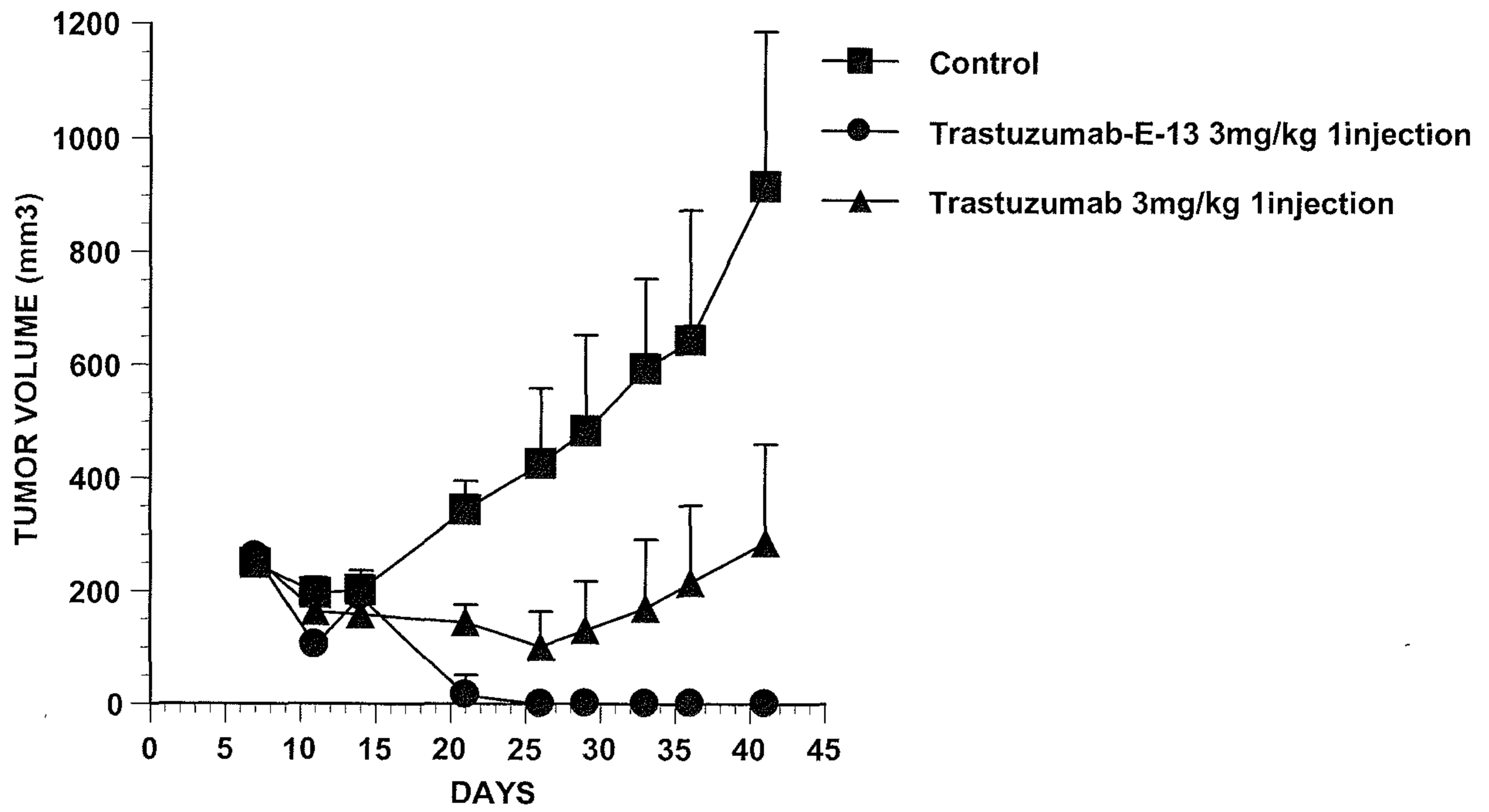


FIGURE 29A

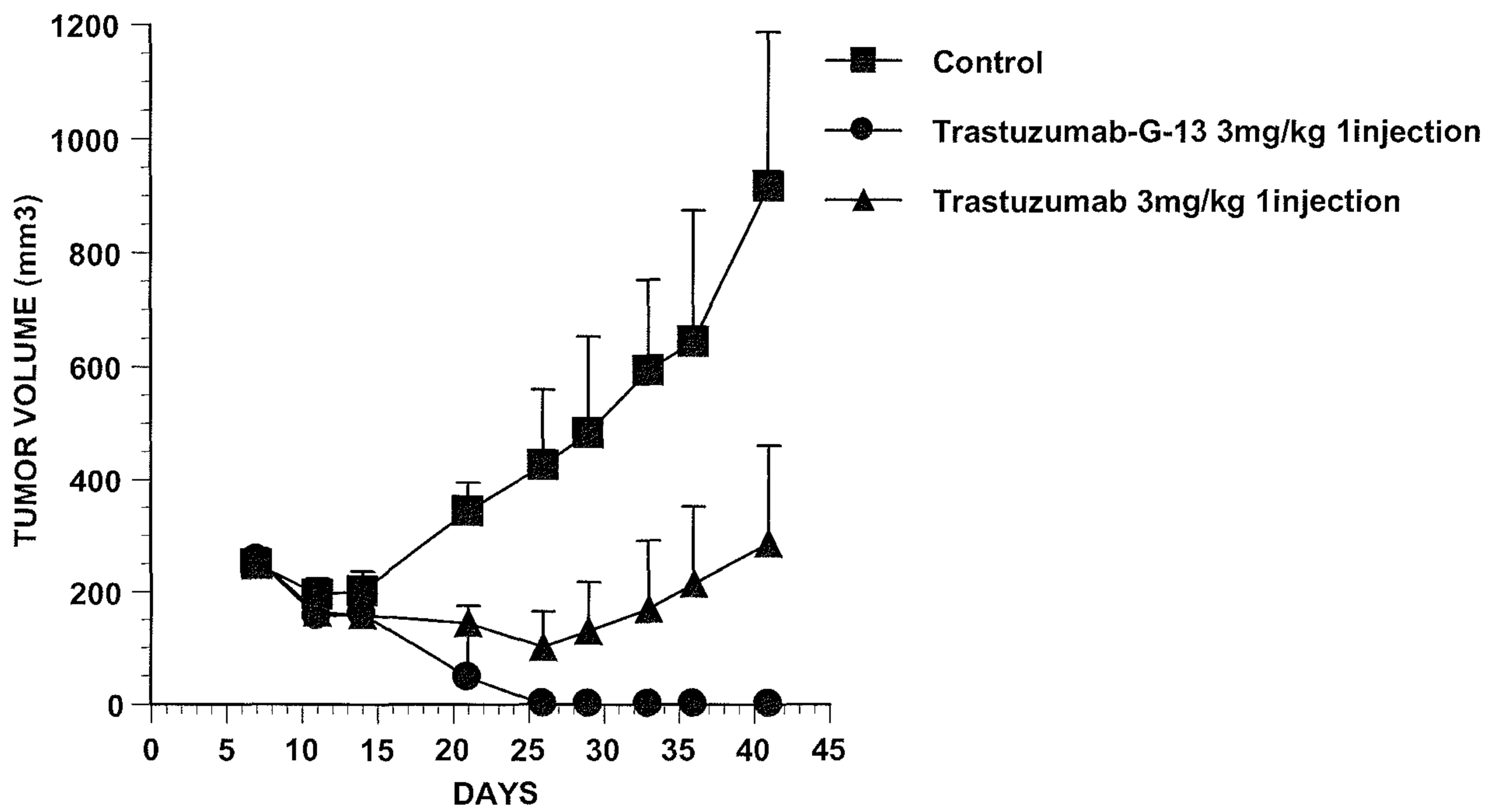


FIGURE 29B

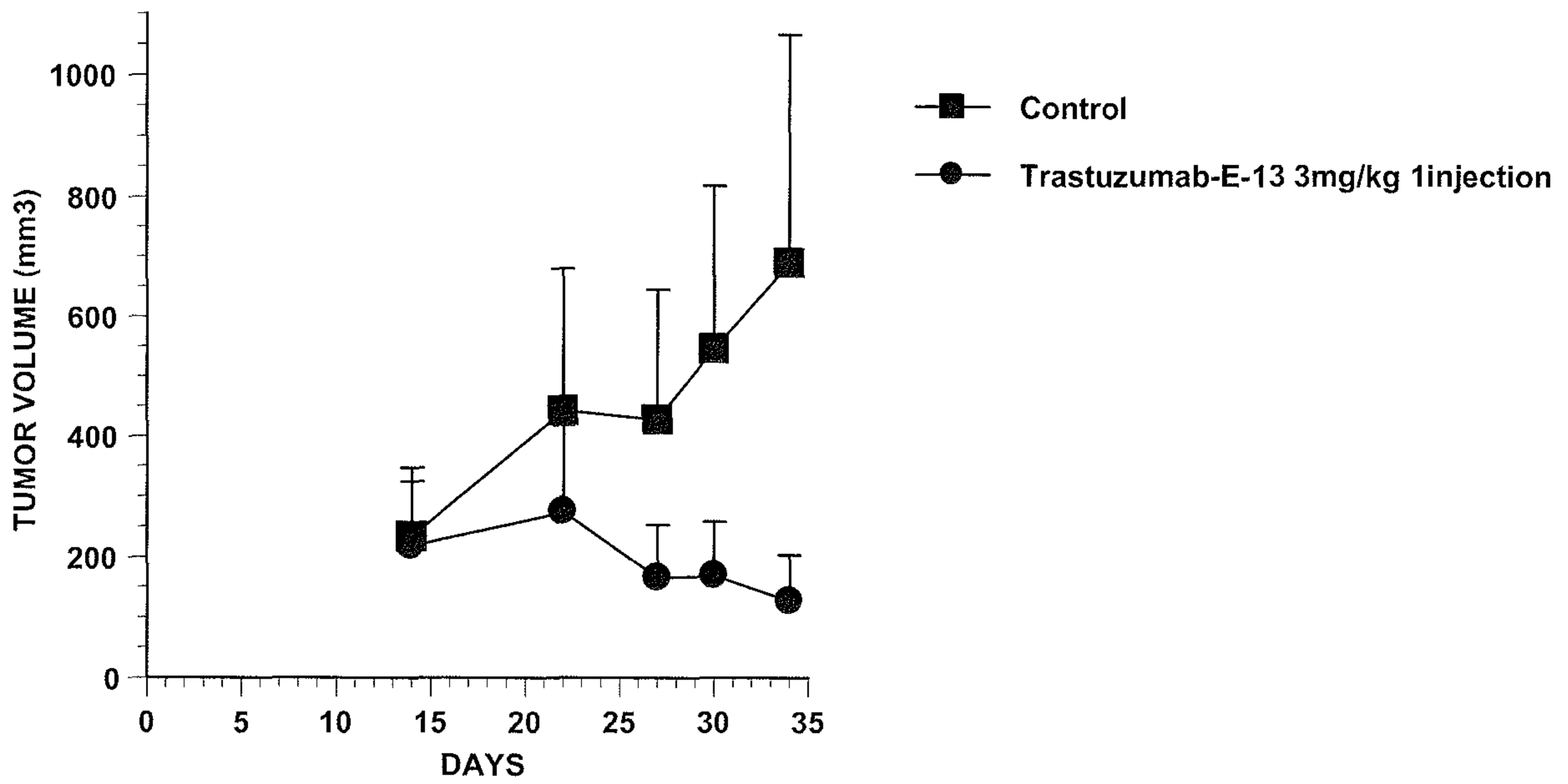


FIGURE 30A

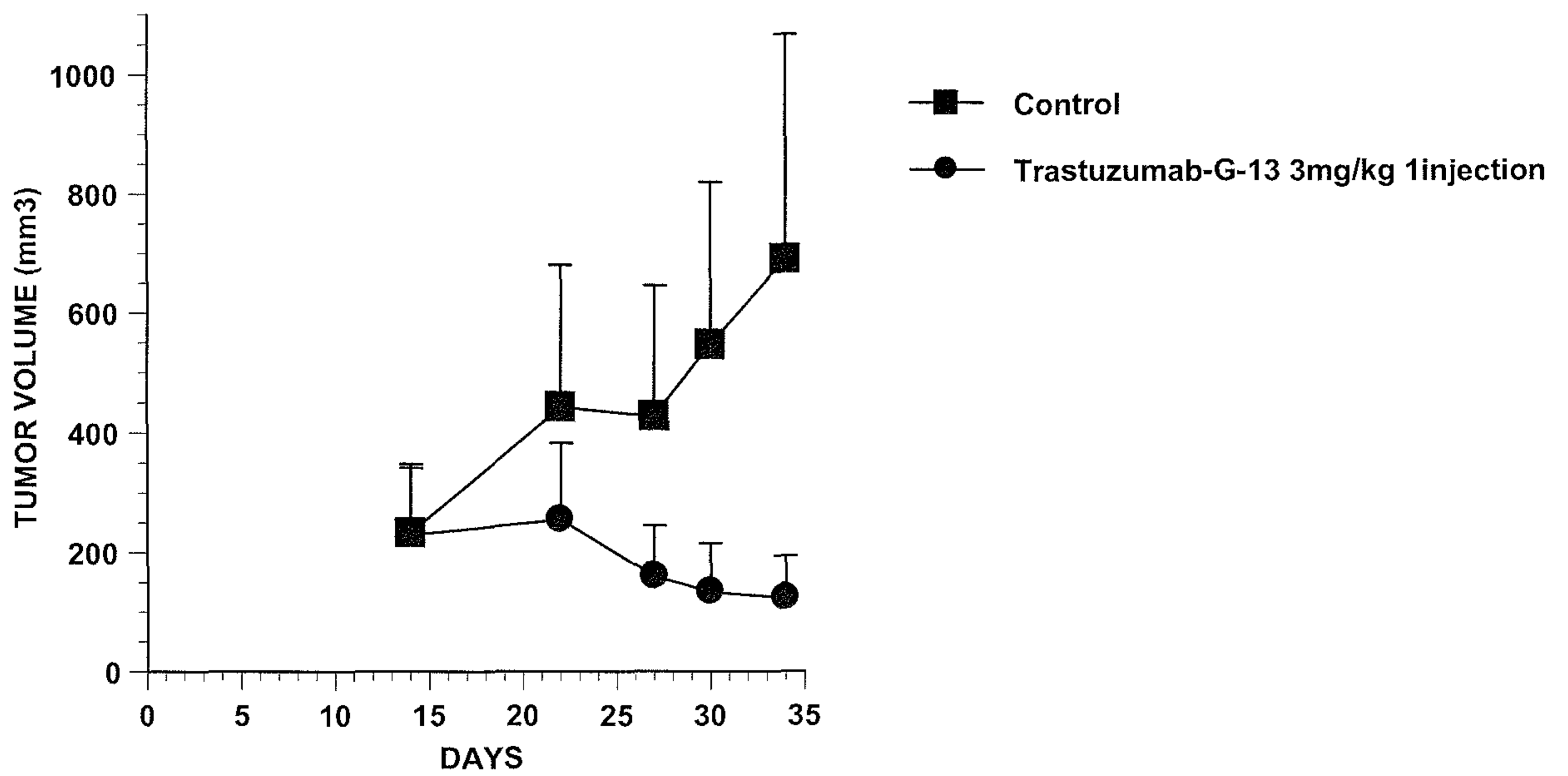


FIGURE 30B

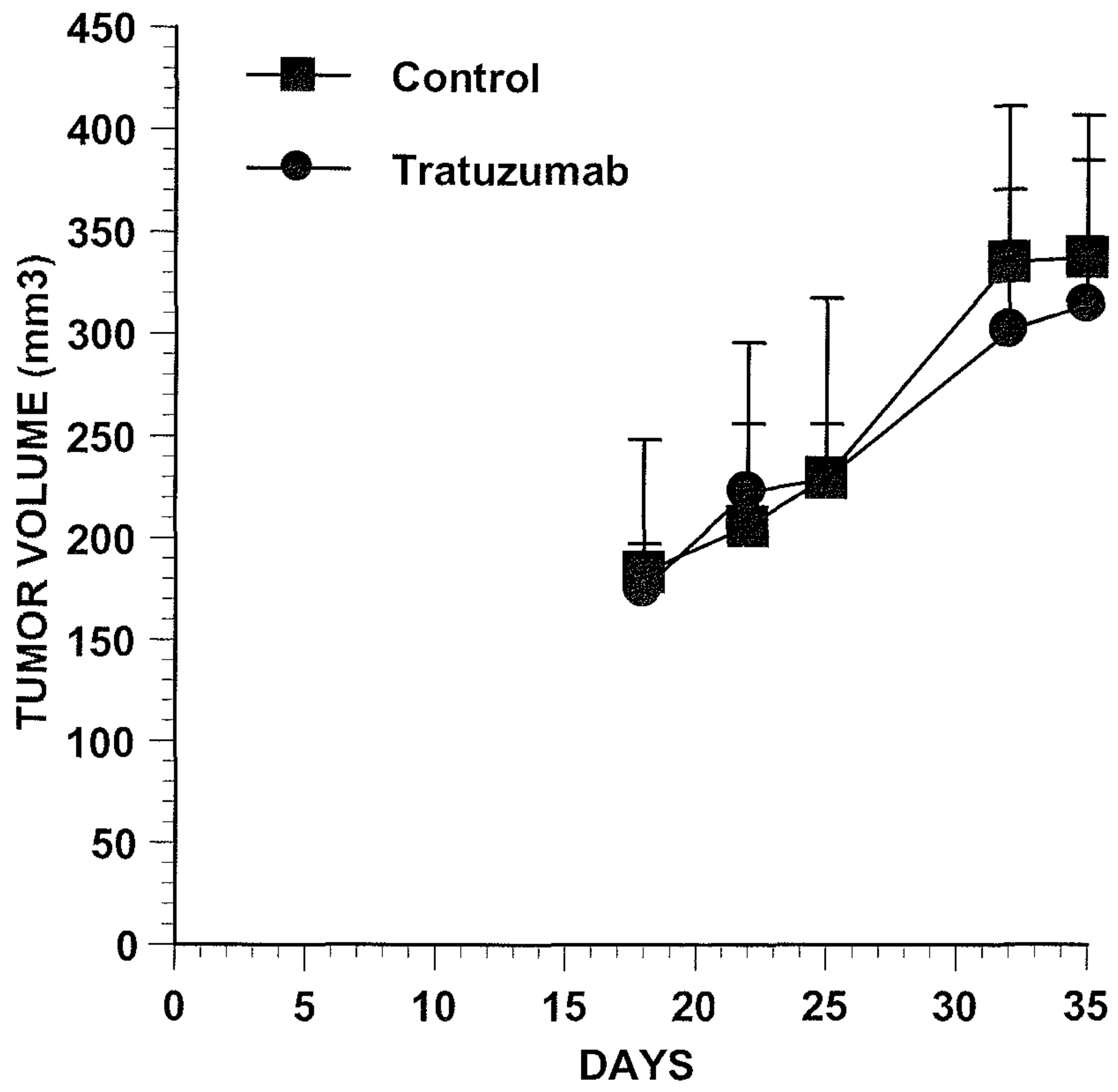


FIGURE 30C