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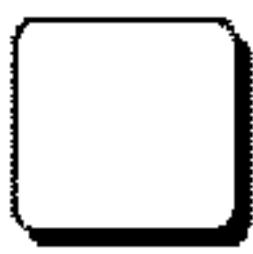
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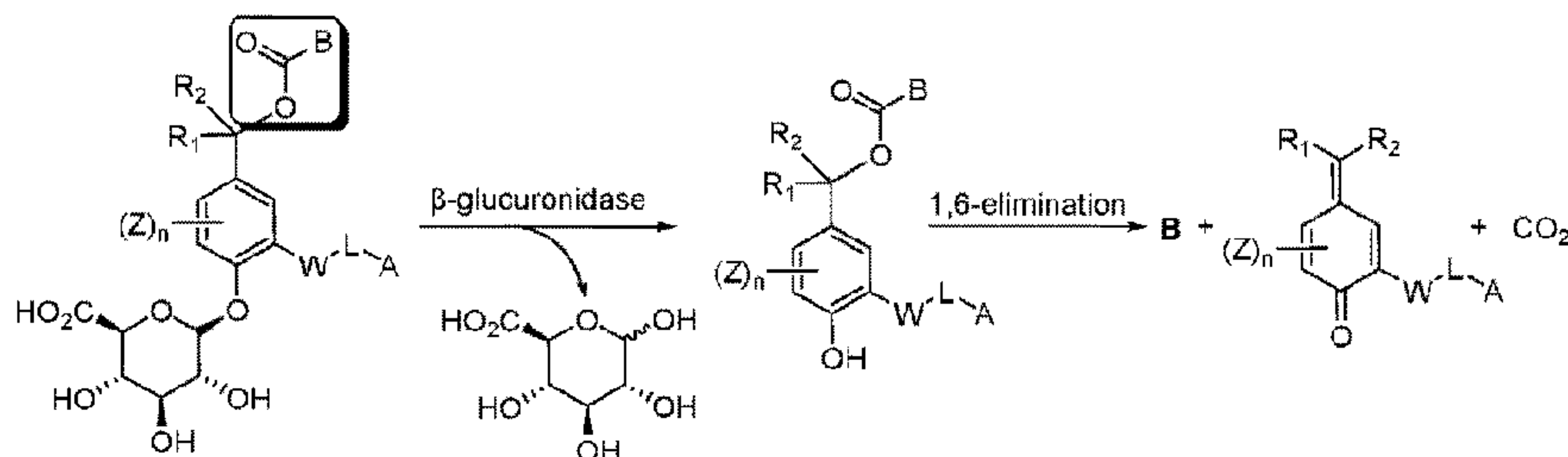
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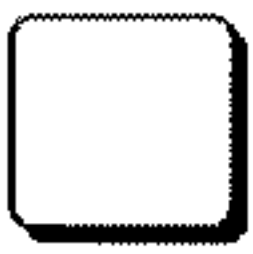
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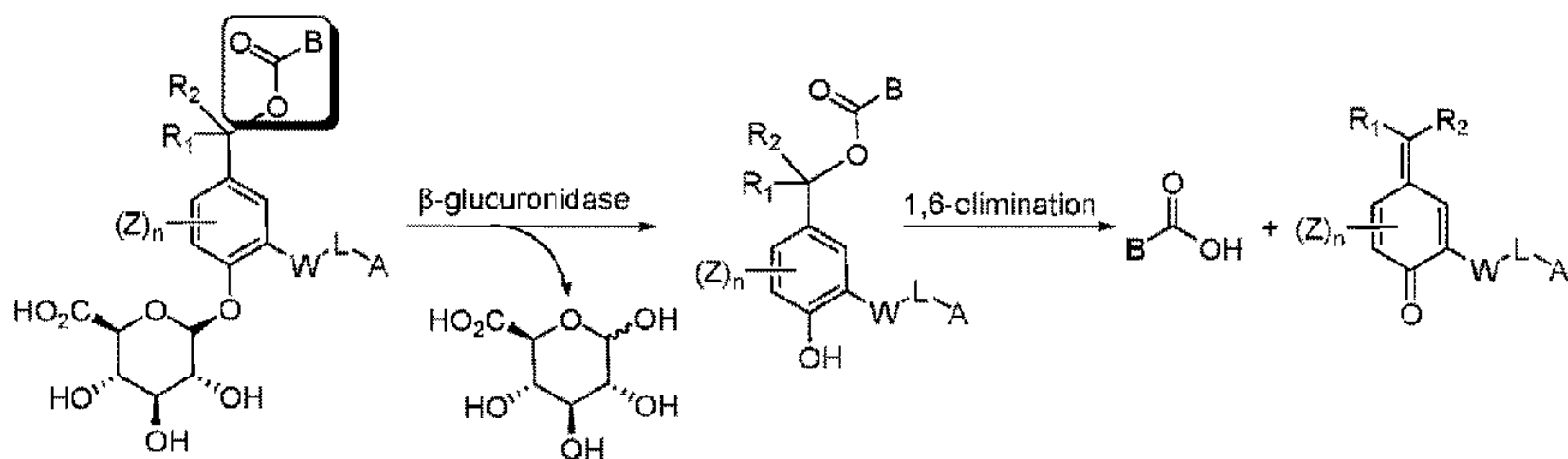
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(54) Title: CONJUGATES COMPRISING PEPTIDE GROUPS AND METHODS RELATED THERETO

**Figure 1**

When  part is carbamate,



When  part is ester,



(57) **Abrégé/Abstract:**

In some aspects, the invention relates to an antibody-drug conjugate, comprising an antibody; a linker; and at least two active agents. In preferred embodiments, the linker comprises a peptide sequence of a plurality of amino acids, and at least two of the active agents are covalently coupled to side chains of the amino acids. The antibody-drug conjugate may comprise a self-immolative group, preferably two-self-immolative groups. The linker may comprise an O-substituted oxime, e.g., wherein the oxygen atom of the oxime is substituted with a group that covalently links the oxime to the active agent; and the carbon atom of the oxime is substituted with a group that covalently links the oxime to the antibody.

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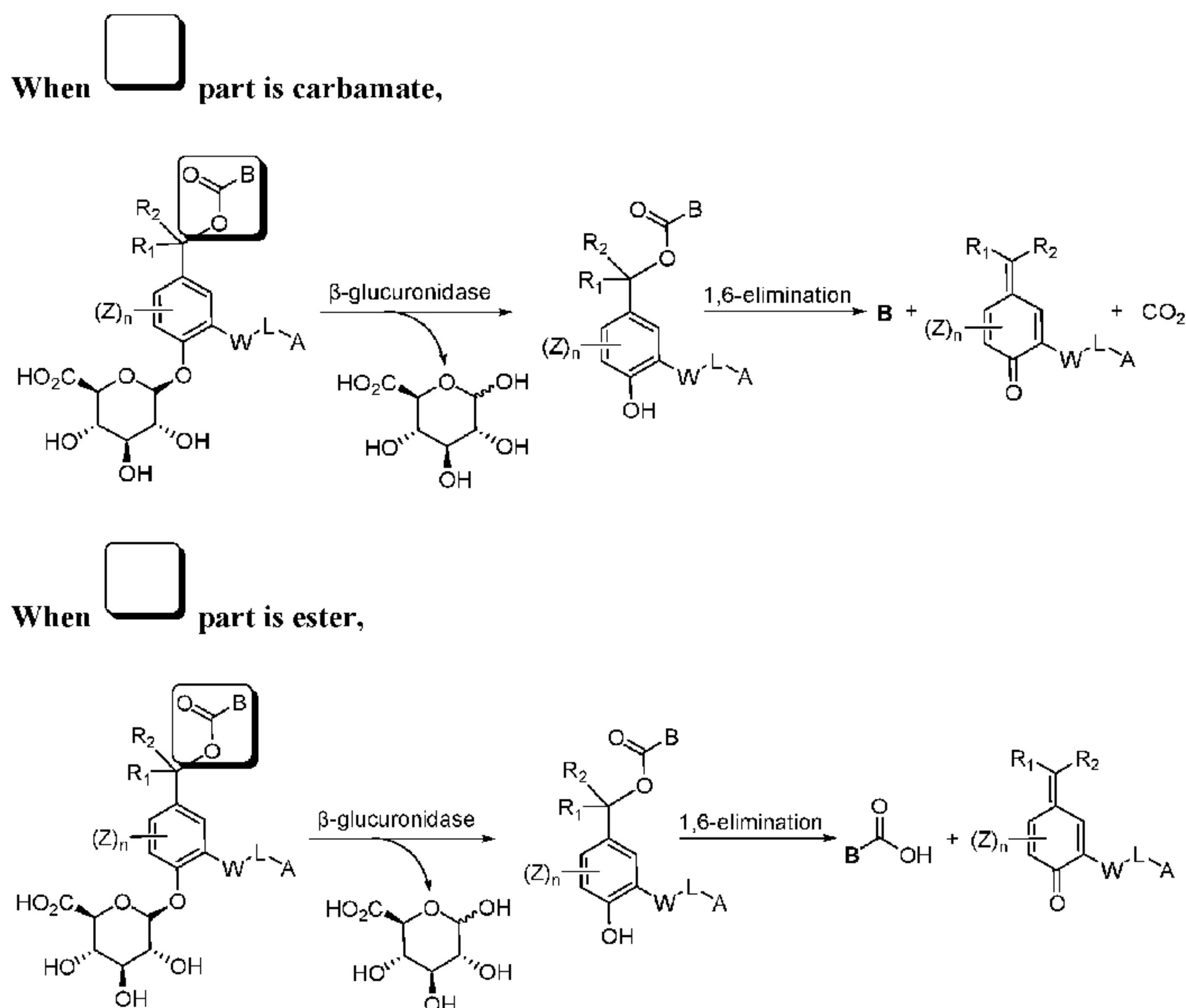
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[Continued on next page]

(54) Title: CONJUGATES COMPRISING PEPTIDE GROUPS AND METHODS RELATED THERETO

Figure 1



(57) Abstract: In some aspects, the invention relates to an antibody-drug conjugate, comprising an antibody; a linker; and at least two active agents. In preferred embodiments, the linker comprises a peptide sequence of a plurality of amino acids, and at least two of the active agents are covalently coupled to side chains of the amino acids. The antibody-drug conjugate may comprise a self-immolative group, preferably two-self-immolative groups. The linker may comprise an O-substituted oxime, e.g., wherein the oxygen atom of the oxime is substituted with a group that covalently links the oxime to the active agent; and the carbon atom of the oxime is substituted with a group that covalently links the oxime to the antibody.

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## CONJUGATES COMPRISING PEPTIDE GROUPS AND METHODS RELATED THERETO

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Application Serial No. 62/259,997, filed November 25, 2015, and U.S. Provisional Application Serial No. 62/260,006, filed November 25, 2015, each of which is hereby incorporated by reference in its entirety.

### BACKGROUND

Antibody-drug conjugate (ADC) technology is a target-oriented technology, which allows for selective apoptosis of cancer cells. Typically, ADCs function by targeting cancer cells using the antibody and then releasing a toxic material (*i.e.*, the drug) in a cell, thereby triggering cell death. Since ADC technology allows a drug to be accurately delivered to a target cancer cell and released under specific conditions, while minimizing collateral damage to healthy cells, ADC technology increases the efficacy of a therapeutic antibody and decreases the risk of an adverse reaction.

Recently, a new approach to making antibody-drug conjugates has been described, using protein prenylation of a C-terminal amino acid sequence to install a modified isoprenoid unit that allows for attachment of a drug or other active agent to the antibody in a mild and site-specific manner (*e.g.*, *see* U.S. Patent Publication No. 2012/0308584). Further refinement is possible.

Improved strategies for configuring ADCs remain desirable.

### SUMMARY

In some aspects, the invention relates to an antibody-drug conjugate, comprising an antibody; a linker; and at least two active agents. In preferred embodiments, the linker comprises a peptide sequence of a plurality of amino acids, and at least two of the active agents are covalently coupled to side chains of the amino acids. The antibody-drug conjugate may comprise a self-immolative group, preferably two-self-immolative groups. The linker may comprise an O-substituted oxime, *e.g.*, wherein the oxygen atom of the oxime is substituted with a group that covalently links the oxime to the active agents; and the carbon atom of the oxime is substituted with a group that covalently links the oxime to

the antibody.

In some aspects, the invention relates to an antibody-drug conjugate, comprising an antibody; a linker covalently coupled to the antibody; and at least two active agents covalently coupled to the linker. The linker may comprise a peptide sequence of a plurality of amino acids. At least two of the active agents may be covalently coupled to side chains of the amino acids.

At least two of the active agents may be covalently coupled to side chains of lysine, 5-hydroxylysine, 4-oxalysine, 4-thialysine, 4-selenalysine, 4-thiahomolysine, 5,5-dimethyllysine, 5,5-difluorolysine, *trans*-4-dehydrolysine, 2,6-diamino-4-hexynoic acid, *cis*-4-dehydrolysine, 6-*N*-methyllysine, diaminopimelic acid, ornithine, 3-methylornithine,  $\alpha$ -methylornithine, citrulline, and/or homocitrulline. For example, at least two of the active agents may be covalently coupled to side chains of lysine or ornithine.

The plurality of amino acids may comprise L-amino acids or D-amino acids. The plurality of amino acids may comprise  $\alpha$ -amino acids or  $\beta$ -amino acids. The plurality of amino acids may comprise alanine, aspartate, asparagine, glutamate, glutamine, glycine, histidine, lysine, ornithine, proline, serine, and/or threonine. In certain preferred embodiments, the peptide does not comprise isoleucine, methionine, leucine, phenylalanine, tryptophan, tyrosine, or valine. The peptide may comprise 2 to 20 amino acids.

In preferred embodiments, the N-terminus or the C-terminus of the peptide is substituted with a group that covalently links at least one of the active agents to the peptide. In preferred embodiments, the N-terminus or the C-terminus of the peptide is substituted with a group that covalently links the antibody to the peptide.

In some embodiments, the linker comprises an O-substituted oxime; the oxygen atom of the oxime is substituted with a group that covalently links the oxime to the peptide; and the carbon atom of the oxime is substituted with a group that covalently links the oxime to the antibody. In some embodiments, the linker comprises an O-substituted oxime; the carbon atom of the oxime is substituted with a group that covalently links the oxime to the peptide; and the oxygen atom of the oxime is substituted with a group that covalently links the oxime to the antibody.

In some embodiments, the linker comprises an alkylene having 1 to 100 carbon atoms. The alkylene may comprise at least one unsaturated bond. In preferred embodiments, the alkylene comprises at least one carbon atom of the alkylene is replaced by one or more heteroatoms selected from nitrogen (N), oxygen (O), and sulfur (S). In

certain embodiments, the alkylene is further substituted with at least one alkyl having 1 to 20 carbon atoms.

In preferred embodiments, the linker is covalently bound to the antibody by a thioether bond, and the thioether bond comprises a sulfur atom of a cysteine of the antibody.

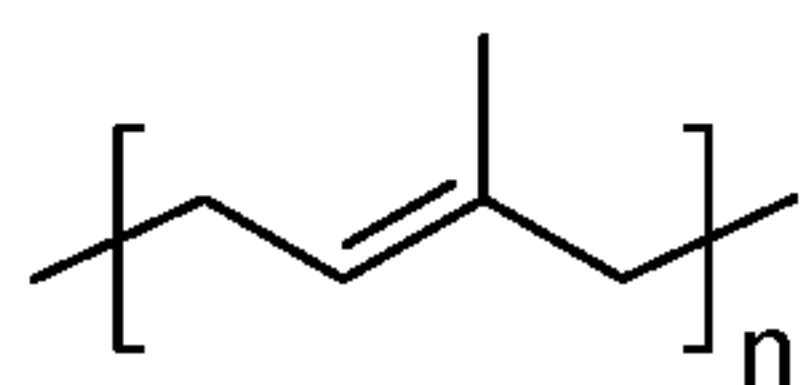
In some embodiments, the antibody comprises a C-terminal amino acid motif that is recognized by an isoprenoid transferase; and the thioether bond comprises a sulfur atom of a cysteine of the amino acid motif.

In some embodiments, the antibody comprises an amino acid motif and the amino acid motif is a sequence selected from CXX, CXC, XCXC, XXCC, and CYYX, wherein C represents cysteine; Y, independently for each occurrence, represents an aliphatic amino acid; and X, independently for each occurrence, represents glutamine, glutamate, serine, cysteine, methionine, alanine, or leucine. In certain preferred embodiments, the linker is covalently bound to the antibody by a thioether bond that comprises a sulfur atom of a cysteine of the amino acid motif. The amino acid motif may be the sequence CYYX; wherein Y, independently for each occurrence, represents alanine, isoleucine, leucine, methionine, or valine. For example, the amino acid motif may be CVIM or CVLL. In preferred embodiments, at least one of the seven amino acids preceding the amino acid motif is glycine.

In some embodiments, at least three of the seven amino acids preceding the amino acid motif are each independently selected from glycine and proline. In certain preferred embodiments, one to ten amino acids preceding the amino acid motif may be glycine, preferably at least five (e.g., GGGGG), such as where each of the five, six, seven, eight, nine, or ten amino acids preceding the amino acid motif is glycine. In certain preferred embodiments, at least three of the seven amino acids preceding the amino acid motif are each independently selected from glycine, aspartic acid, arginine, and serine.

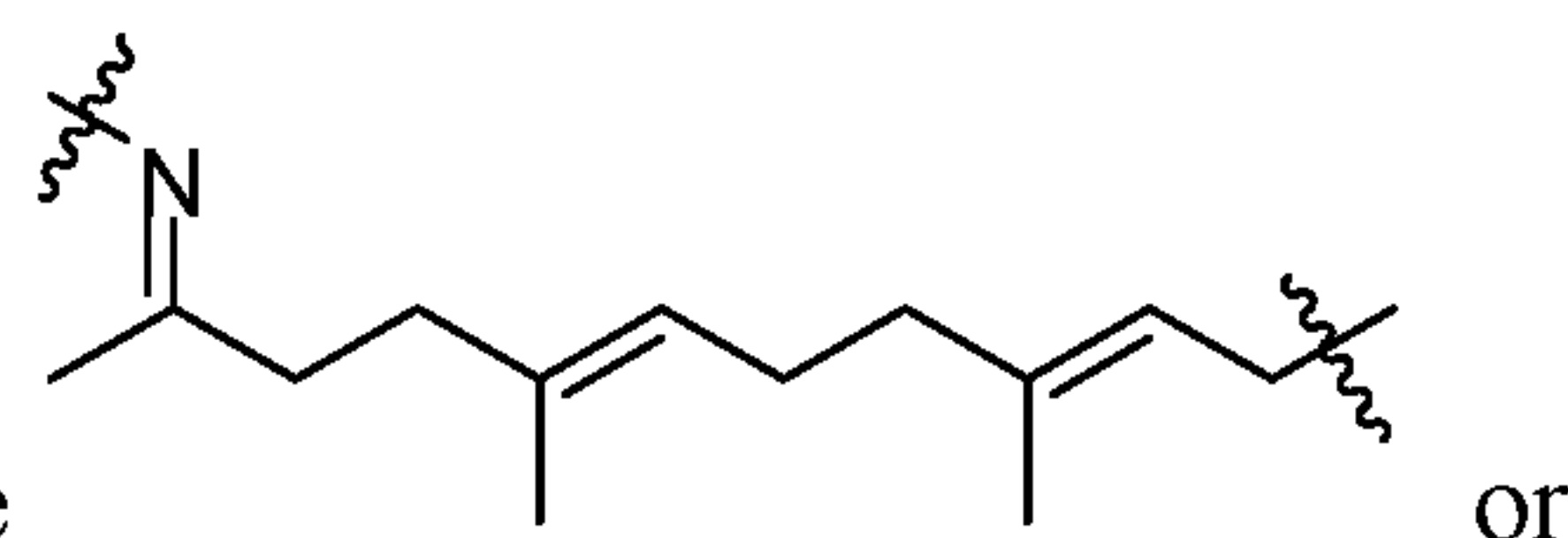
In certain preferred embodiments, a C-terminus of the antibody comprises the amino acid sequence GGGGGGGCVIM.

In some embodiments, the linker is covalently bound to the antibody by a thioether bond, and the thioether bond comprises a carbon atom of at least one isoprenyl unit,

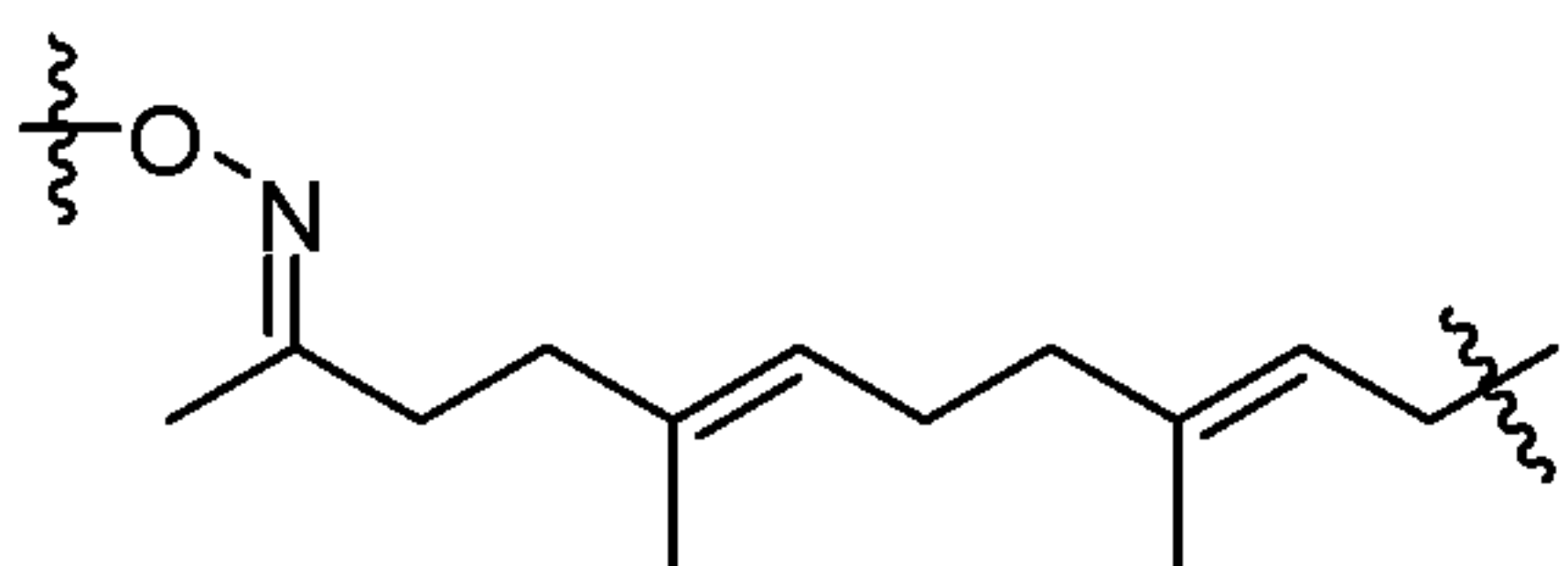


represented by  $\left[ \text{CH}_2 = \text{C}(\text{CH}_3) \text{CH}_2 \right]_n$ , e.g., wherein n is at least 2. The linker may comprise an oxime, and the at least one isoprenyl unit covalently links the oxime to the antibody.

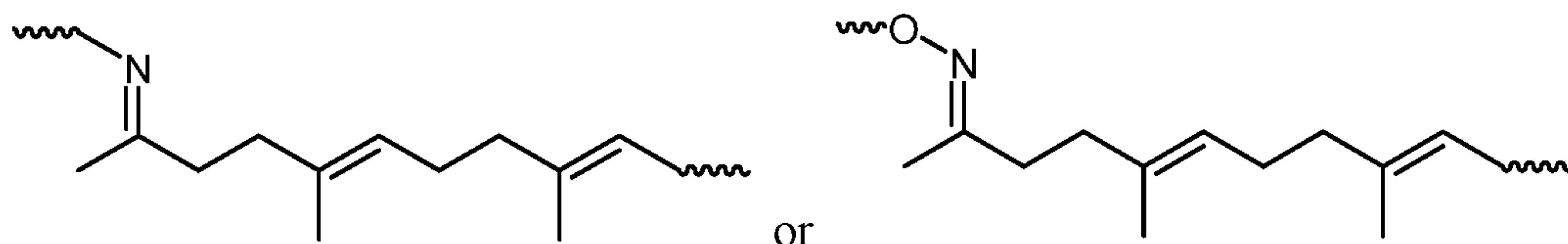
In some embodiments, the linker may comprise



or

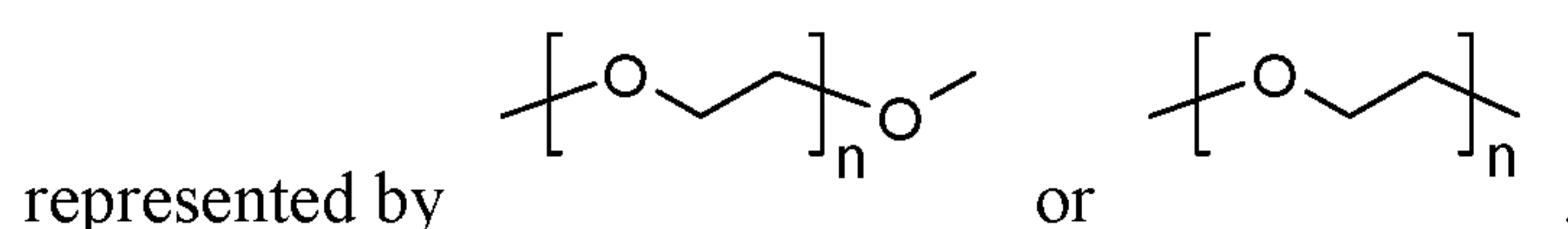


In some embodiments, the linker comprises:



or

In some embodiments, the linker comprises at least one polyethylene glycol unit,



represented by

or

In some embodiments, the linker comprises linker comprises 1 to 20  $-\text{OCH}_2\text{CH}_2-$  units. For example, the linker may comprise 4 to 20  $-\text{OCH}_2\text{CH}_2-$  units. In certain preferred embodiments, the linker may comprise 1 to 12  $-\text{OCH}_2\text{CH}_2-$  units. In other preferred embodiments, the linker may comprise 3 to 12  $-\text{OCH}_2\text{CH}_2-$  units. In some embodiments, the linker comprises an oxime, and the at least one polyethylene glycol unit covalently links the oxime to the peptide.

In some embodiments, the linker comprises a connection unit represented by  $-(\text{CH}_2)_r(\text{V}(\text{CH}_2)_p)_q-$ , wherein:

$r$  is an integer from 0 to 10, preferably 2;

$p$  is an integer from 0 to 12, preferably 2;

$q$  is an integer from 1 to 20;

$V$  is a single bond,  $-\text{O}-$ ,  $-\text{S}-$ ,  $-\text{NR}_{21}-$ ,  $-\text{C}(\text{O})\text{NR}_{22}-$ ,  $-\text{NR}_{23}\text{C}(\text{O})-$ ,  $-\text{NR}_{24}\text{SO}_2-$ , or  $-\text{SO}_2\text{NR}_{25}-$ , preferably  $-\text{O}-$ ; and

$R_{21}$  to  $R_{25}$  are each independently hydrogen,  $(\text{C}_1\text{-C}_6)\text{alkyl}$ ,  $(\text{C}_1\text{-C}_6)\text{alkyl}(\text{C}_6\text{-C}_{20})\text{aryl}$ , or  $(\text{C}_1\text{-C}_6)\text{alkyl}(\text{C}_3\text{-C}_{20})\text{heteroaryl}$ .

In some embodiments, the linker comprises a connection unit represented by  $-(\text{CH}_2)_r(\text{V}(\text{CH}_2)_p)_q-$ ,  $-((\text{CH}_2)_p\text{V})_q-$ ,  $-(\text{CH}_2)_r(\text{V}(\text{CH}_2)_p)_q\text{Y}-$ ,  $-((\text{CH}_2)_p\text{V})_q(\text{CH}_2)_r-$ ,  $-\text{Y}(((\text{CH}_2)_p\text{V})_q)-$  or  $-(\text{CH}_2)_r(\text{V}(\text{CH}_2)_p)_q\text{YCH}_2-$

wherein:

r is an integer from 0 to 10;

p is an integer from 1 to 10;

q is an integer from 1 to 20;

V and Y are each independently a single bond, -O-, -S-, -NR<sub>21</sub>-, -C(O)NR<sub>22</sub>-, -NR<sub>23</sub>C(O)-, -NR<sub>24</sub>SO<sub>2</sub>-, or -SO<sub>2</sub>NR<sub>25</sub>-; and

R<sub>21</sub> to R<sub>25</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>6</sub>-C<sub>20</sub>)aryl or (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>3</sub>-C<sub>20</sub>)heteroaryl.

In certain preferred embodiments of these linkers, q is an integer from 4 to 20. In some embodiments q is an integer from 6 to 20. In other preferred embodiments, q is an integer from 2 to 12. In some embodiments, q is 2, 5 or 11.

In some embodiments of these linkers, r is 2. In some embodiments of these linkers, p is 2. In some embodiments, V and Y are each independently -O-. In some embodiments, r is 2, p is 2, q is 2, 5, or 11, and V is -O-.

In some embodiments, the linker comprises a connection unit represented by -(CH<sub>2</sub>CH<sub>2</sub>X)<sub>w</sub>-, wherein:

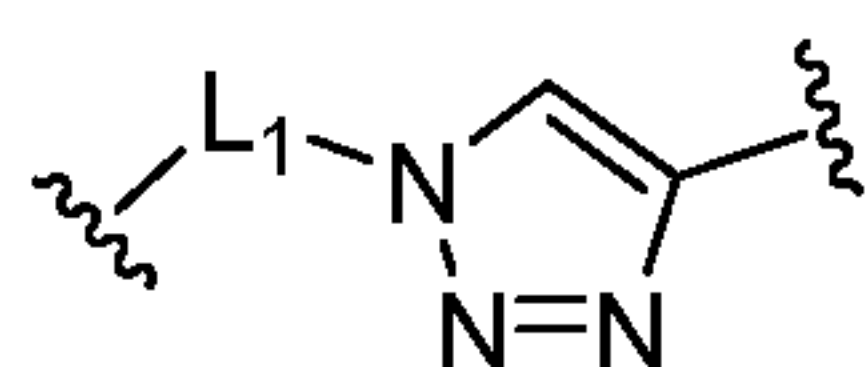
X represents -O-, (C<sub>1</sub>-C<sub>8</sub>)alkylene, or -NR<sub>21</sub>-, preferably -O-;

R<sub>21</sub> represents hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>6</sub>-C<sub>20</sub>)aryl, or (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>3</sub>-C<sub>20</sub>)heteroaryl, preferably hydrogen; and

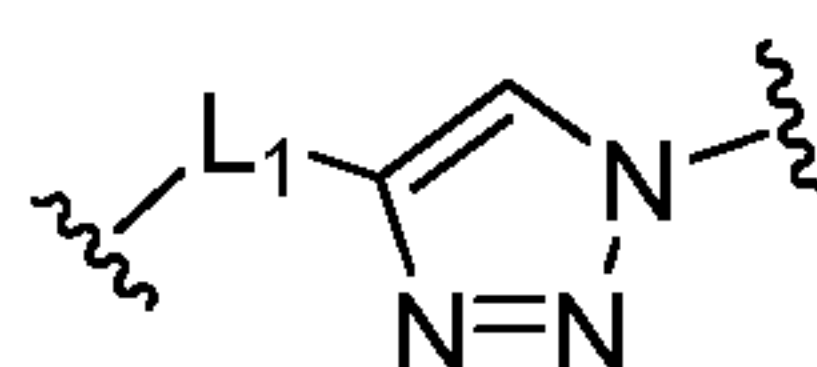
w is an integer from 1 to 20, preferably 4 to 12.

In some embodiments, the linker comprises a binding unit formed by a 1,3-dipolar cycloaddition reaction, hetero-Diels-Alder reaction, nucleophilic substitution reaction, non-aldol type carbonyl reaction, addition to carbon-carbon multiple bond, oxidation reaction, or click reaction. For example, a binding unit may be formed by a reaction between acetylene and azide, or a reaction between an aldehyde or ketone group and a hydrazine or alkoxyamine.

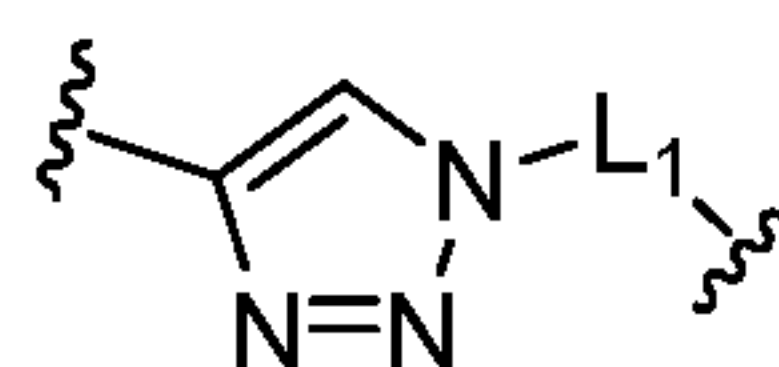
A binding unit may be represented by any one of Formulas A, B, C, or D, preferably C or D:



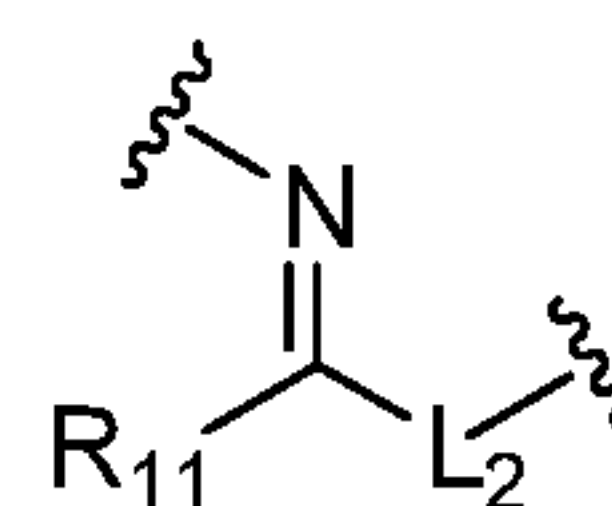
(A)



(B)



(C)



(D)

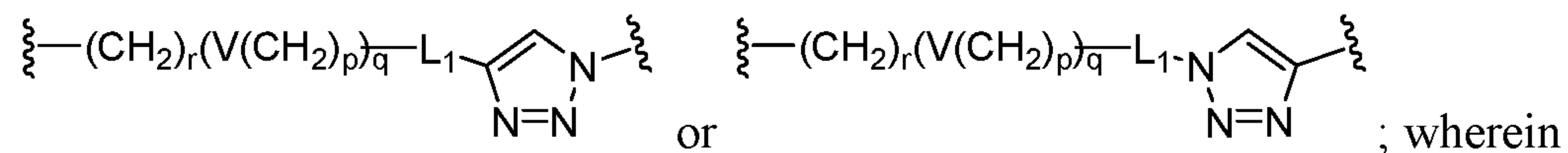
wherein:

L<sub>1</sub> is a single bond or alkylene having 1 to 30 carbon atoms, preferably 12;

$R_{11}$  is hydrogen or alkyl having 1 to 10 carbon atoms, preferably methyl; and

$L_2$  is alkylene having 1 to 30 carbon atoms, e.g., 10 or 11, preferably 11.

In some embodiments, the linker comprises



V is a single bond, -O-, -S-, -NR<sub>21</sub>-, -C(O)NR<sub>22</sub>-, -NR<sub>23</sub>C(O)-, -NR<sub>24</sub>SO<sub>2</sub>-, or -SO<sub>2</sub>NR<sub>25</sub>-, preferably -O-;

R<sub>21</sub> to R<sub>25</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>6</sub>-C<sub>20</sub>)aryl, or (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>3</sub>-C<sub>20</sub>)heteroaryl;

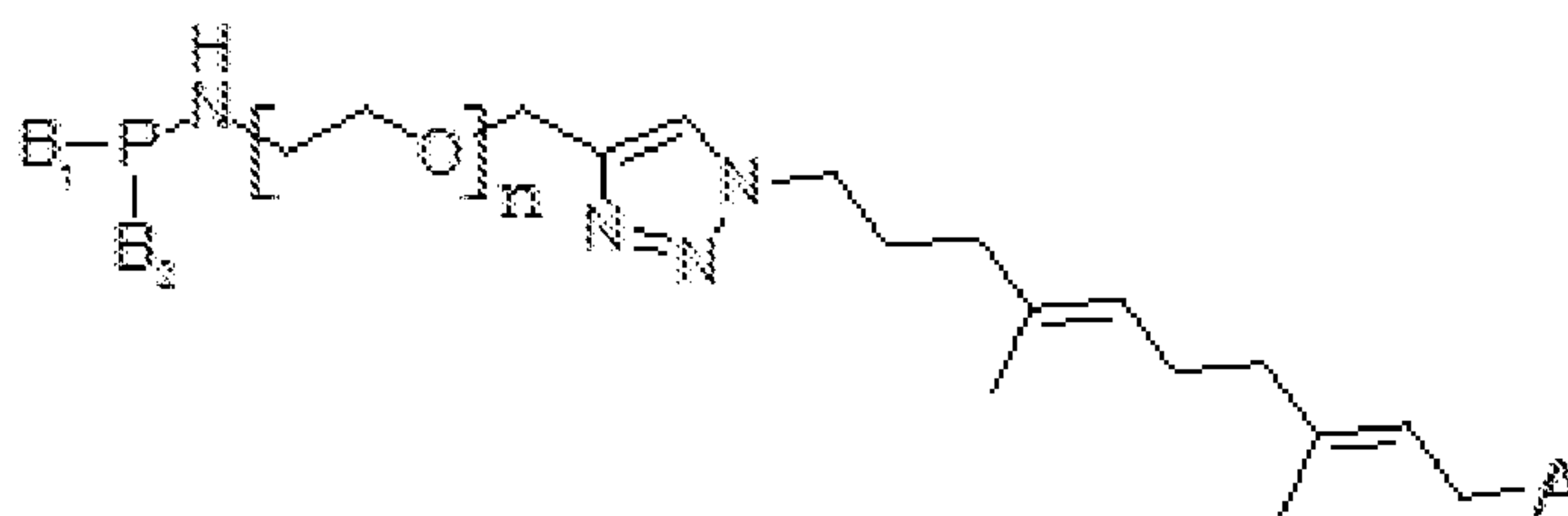
r is an integer from 1 to 10, preferably 3;

p is an integer from 0 to 10, preferably 2;

q is an integer from 1 to 20, preferably 2 to 20; and

$L_1$  is a single bond.

In some embodiments, the antibody-drug conjugate comprises the structure of Formula V:



(V)

wherein:

A represents the antibody;

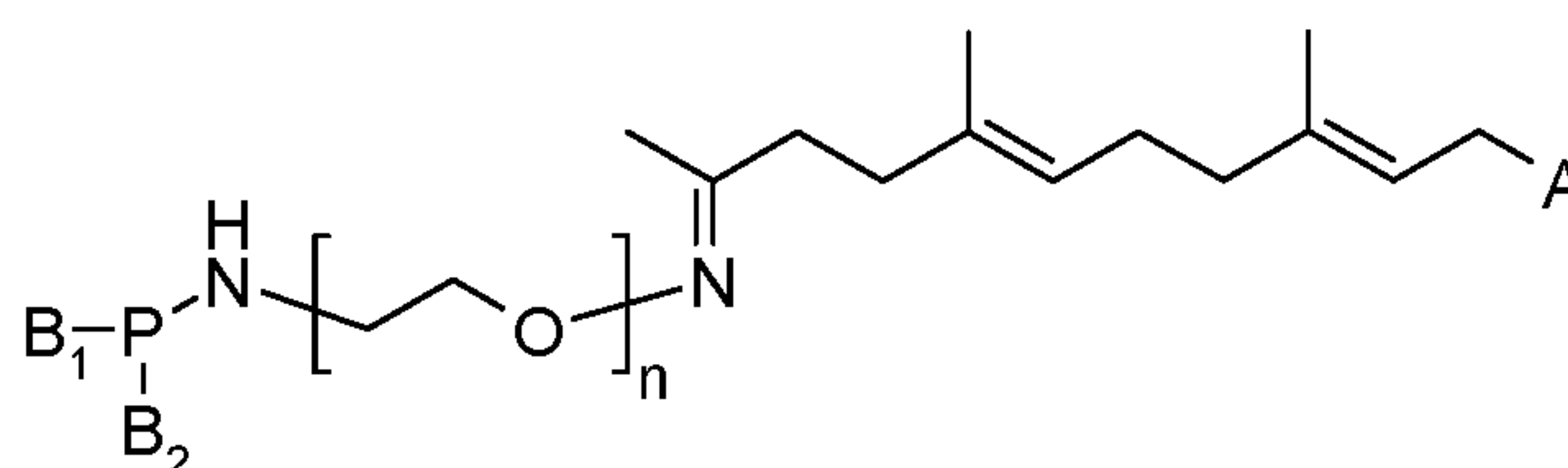
P represents the peptide

B<sub>1</sub> represents a first active agent;

B<sub>2</sub> represents a second active agent; and

n is an integer from 1 to 20.

In some embodiments, the antibody-drug conjugate comprises the structure of Formula VI:



(VI)

wherein

A represents the antibody;

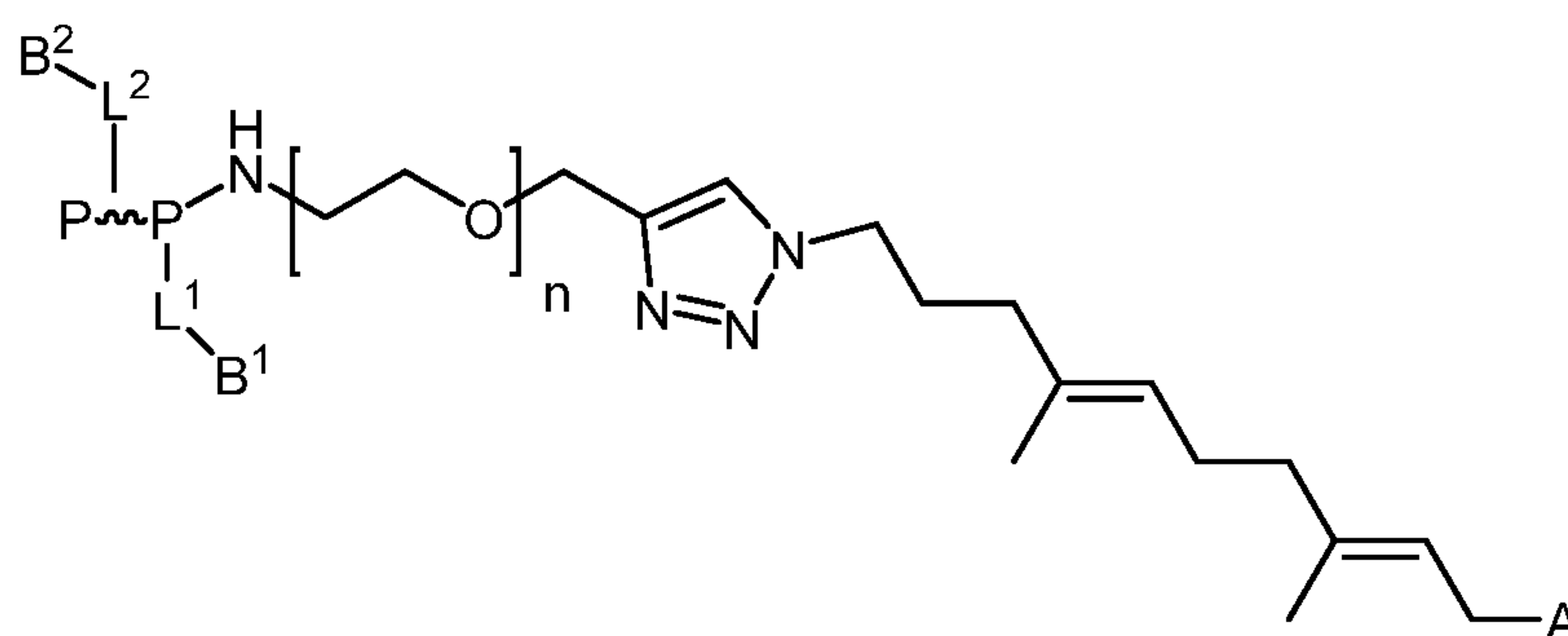
P represents the peptide;

B<sub>1</sub> represents a first active agent;

B<sub>2</sub> represents a second active agent; and

n is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

A represents a ligand;

P ~ P represents the peptide;

B<sub>1</sub> represents a first active agent;

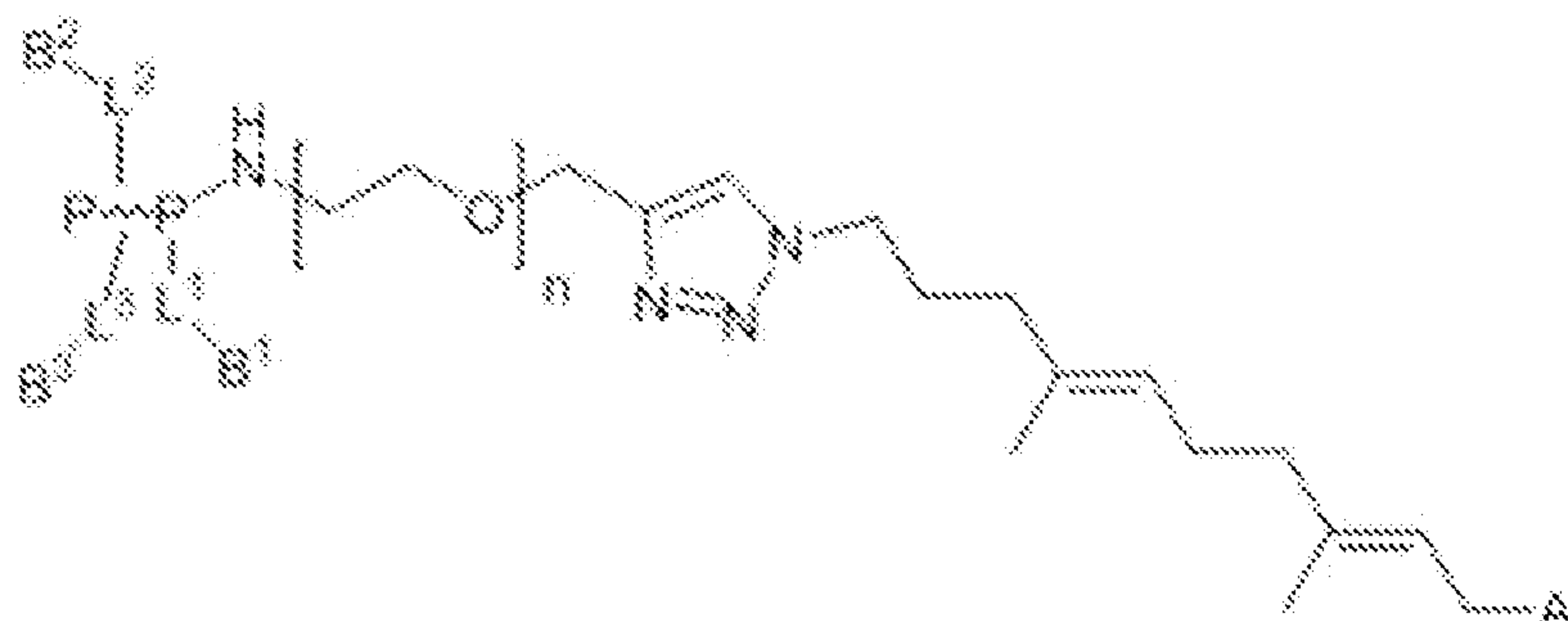
B<sub>2</sub> represents a second active agent;

L<sub>1</sub> represents a linker, optionally including a cleavage group;

L<sub>2</sub> represents a linker, optionally including a cleavage group; and

n is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

A represents a ligand;

P ~ P represents the peptide;

B<sub>1</sub> represents a first active agent;

B<sub>2</sub> represents a second active agent;

B<sub>3</sub> represents a third active agent;

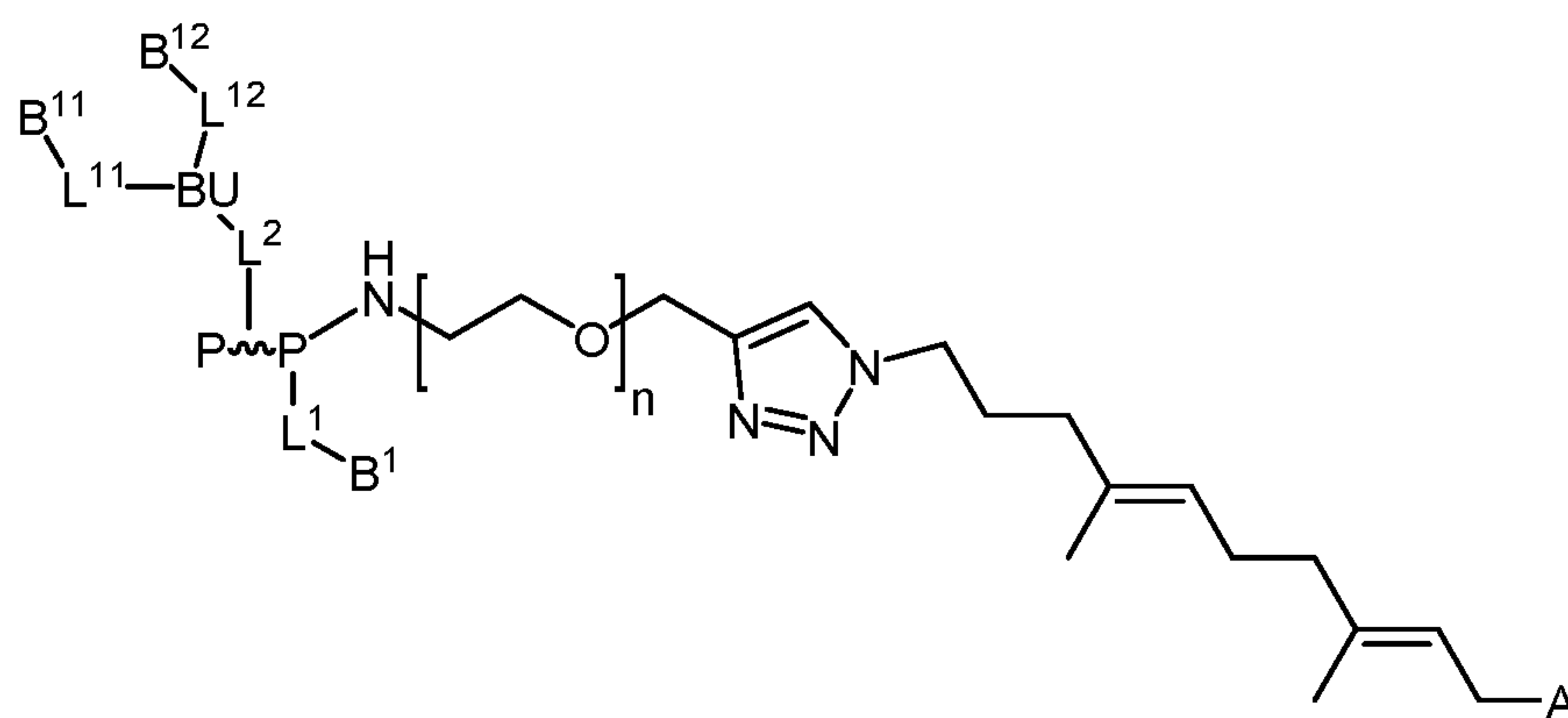
L<sub>1</sub> represents a linker, optionally including a cleavage group;

L<sub>2</sub> represents a linker, optionally including a cleavage group;

L<sub>3</sub> represents a linker, optionally including a cleavage group; and

n is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

A represents a ligand;

P ~ P represents the peptide;

B<sub>1</sub> represents a first active agent;

B<sub>11</sub> represents a second active agent;

B<sub>12</sub> represents a third active agent;

L<sub>1</sub> represents a linker;

L<sub>2</sub> represents a linker;

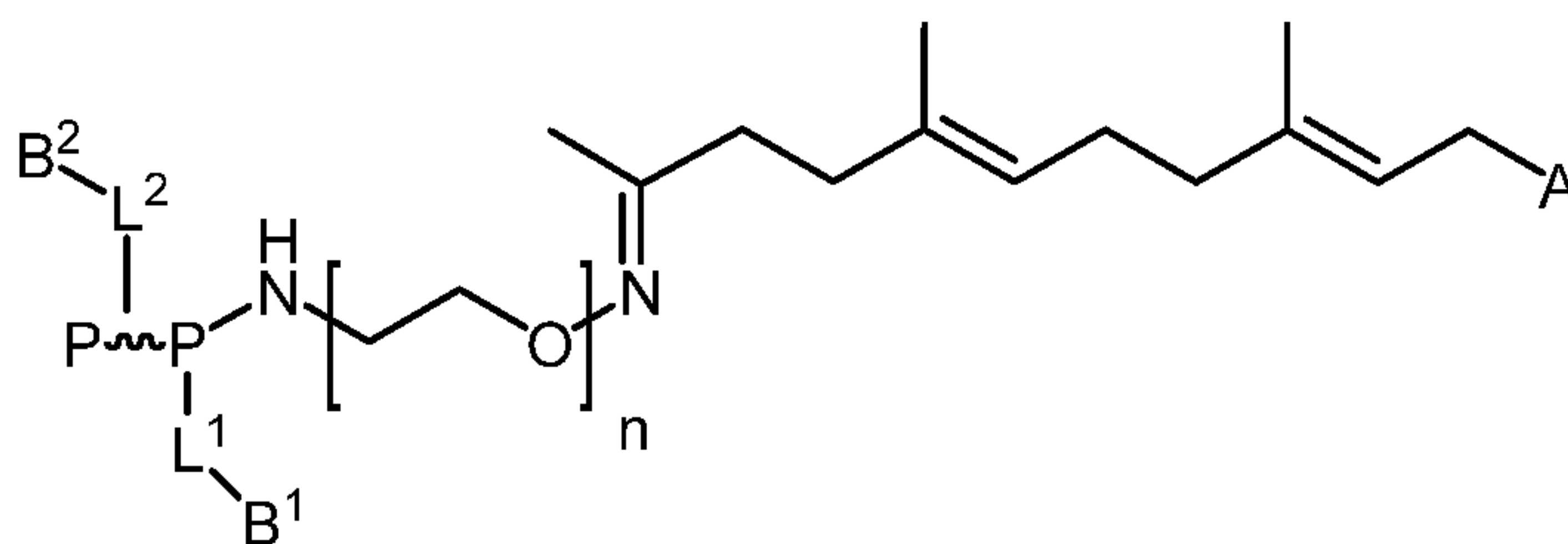
L<sub>11</sub> represents a secondary linker, optionally including a cleavage group;

L<sub>12</sub> represents a secondary linker, optionally including a cleavage group;

BU represents a branching unit; and

n is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

A represents a ligand;

P  P represents the peptide;

B<sub>1</sub> represents a first active agent;

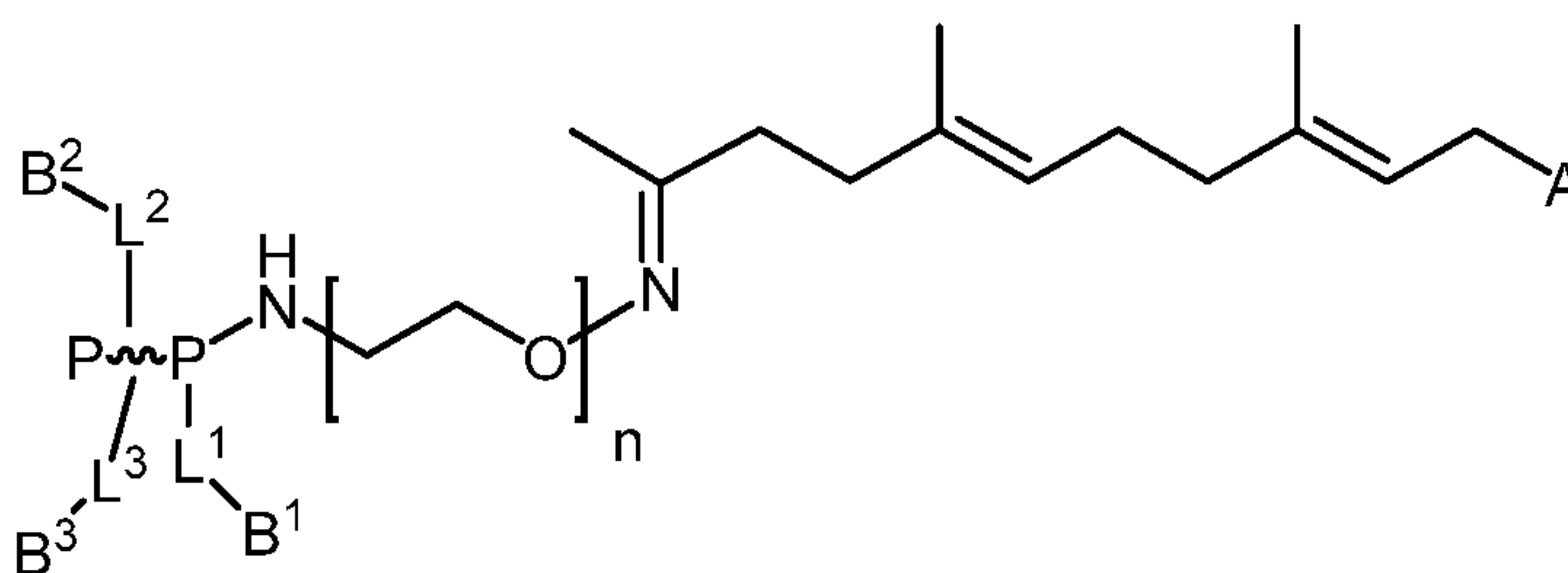
B<sub>2</sub> represents a second active agent;

L<sub>1</sub> represents a linker, optionally including a cleavage group;

L<sub>2</sub> represents a linker, optionally including a cleavage group; and

n is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

A represents a ligand;

P  P represents the peptide;

B<sub>1</sub> represents a first active agent;

B<sub>2</sub> represents a second active agent;

B<sub>3</sub> represents a third active agent;

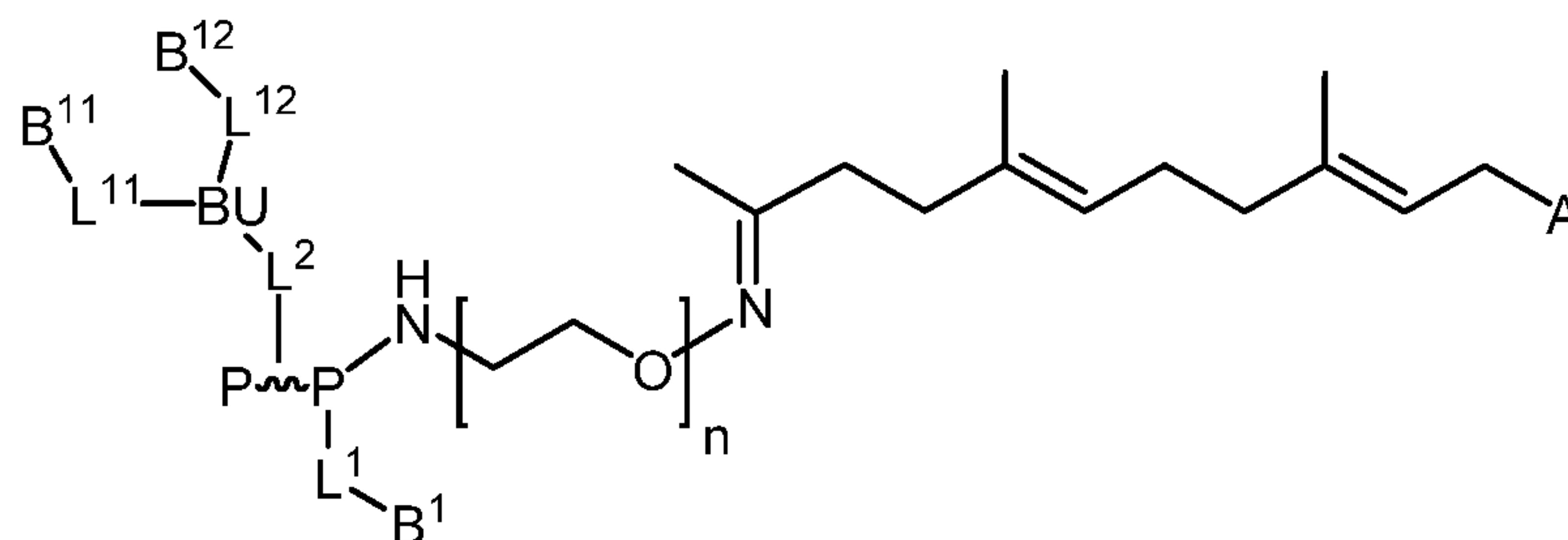
L<sub>1</sub> represents a linker, optionally including a cleavage group;

L<sub>2</sub> represents a linker, optionally including a cleavage group;

L<sub>3</sub> represents a linker, optionally including a cleavage group; and

n is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

A represents a ligand;

P  $\sim$  P represents the peptide;

B<sub>1</sub> represents a first active agent;

B<sub>11</sub> represents a second active agent;

B<sub>12</sub> represents a third active agent;

L<sub>1</sub> represents a linker;

L<sub>2</sub> represents a linker;

L<sub>11</sub> represents a secondary linker, optionally including a cleavage group;

L<sub>12</sub> represents a secondary linker, optionally including a cleavage group;

BU represents a branching unit; and

n is an integer from 1 to 20.

In certain preferred embodiments, the ligand is an antibody. The antibody may be a monoclonal antibody, polyclonal antibody, antibody fragment, Fab, Fab', Fab'-SH, F(ab')<sub>2</sub>, Fv, single chain Fv ("scFv"), diabody, linear antibody, bispecific antibody, multispecific antibody, chimeric antibody, humanized antibody, human antibody, or fusion protein comprising the antigen-binding portion of an antibody.

The antibody may be selected from muromonab-CD3 abciximab, rituximab, daclizumab, palivizumab, infliximab, trastuzumab, etanercept, basiliximab, gemtuzumab, alemtuzumab, ibritumomab, adalimumab, alefacept, omalizumab, efalizumab, tositumomab, cetuximab, ABT-806, bevacizumab, natalizumab, ranibizumab, panitumumab, eculizumab, rilonacept, certolizumab, romiplostim, AMG-531, golimumab, ustekinumab, ABT-874, belatacept, belimumab, atacicept, an anti-CD20 antibody, canakinumab, tocilizumab, atlizumab, mepolizumab, pertuzumab, HuMax CD20, tremelimumab, ticilimumab, ipilimumab, IDEC-114, inotuzumab, HuMax EGFR, aflibercept, HuMax-CD4, teplizumab, otelixizumab, catumaxomab, the anti-EpCAM antibody IGN101, adecatumomab, oregovomab, dinutuximab, girentuximab, denosumab,

bapineuzumab, motavizumab, efumgumab, raxibacumab, an anti-CD20 antibody, LY2469298, and velvuzumab.

In some embodiments, each active agent is independently selected from chemotherapeutic agents and toxins.

Each active agent may be independently selected from:

(a) erlotinib, bortezomib, fulvestrant, sunitinib, letrozole, imatinib mesylate, PTK787/ZK 222584, oxaliplatin, 5-fluorouracil, leucovorin, rapamycin, lapatinib, lonafarnib, sorafenib, gefitinib, AG1478, AG1571, thiotepa, cyclophosphamide, busulfan, improsulfan, piposulfan, benzodopa, carboquone, meturedopa, uredopa, ethylenimine, altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide, trimethylolomelamine, bullatacin, bullatacinone, camptothecin, topotecan, bryostatin, callystatin, CC-1065, adozelesin, carzelesin, bizelesin, cryptophycin 1, cryptophycin 8, dolastatin, duocarmycin, KW-2189, CB1-TM1, eleutherobin, pancratistatin, sarcodictyin, spongistatin, chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard, carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine, calicheamicin, calicheamicin gamma 1, calicheamicin omega 1, dynemicin, dynemicin A, clodronate, esperamicin, neocarzinostatin chromophore, aclacinomysins, actinomycin, antracycline, azaserine, bleomycins, cactinomycin, carubicin, carbinomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, liposomal doxorubicin, deoxydoxorubicin, epirubicin, esorubicin, marcellomycin, mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptomycin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin, 5-fluorouracil, denopterin, methotrexate, pteropterin, trimetrexate, fludarabine, 6-mercaptopurine, thiamiprine, thiguanine, ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone, aminoglutethimide, mitotane, trilostane, folinic acid, aceglatone, aldophosphamide glycoside, aminolevulinic acid, eniluracil, amsacrine, bestabucil, bisantrene, edatraxate, defofamine, demecolcine, diaziquone, elfornithine, elliptinium acetate, etoglucid, gallium nitrate, hydroxyurea, lentinan, lonidainine, maytansine, ansamitocins, mitoguazone, mitoxantrone, mopidanmol, nitraerine, pentostatin, phenamet,

pirarubicin, losoxantrone, 2-ethylhydrazide, procarbazine, polysaccharide-k, razoxane, rhizoxin, sizofiran, spirogermanium, tenuazonic acid, triaziquone, 2,2',2''-trichlorotriethylamine, T-2 toxin, verracurin A, roridin A, and anguidine, urethane, vindesine, dacarbazine, mannomustine, mitobronitol, mitolactol, pipobroman, gacytosine, arabinoside, cyclophosphamide, thiotepa, paclitaxel, albumin-engineered nanoparticle formulation of paclitaxel, doxetaxel, chlorambucil, gemcitabine, 6-thioguanine, mercaptopurine, cisplatin, carboplatin, vinblastine, platinum, etoposide, ifosfamide, mitoxantrone, vincristine, vinorelbine, novantrone, teniposide, edatrexate, daunomycin, aminopterin, xeloda, ibandronate, CPT-11, topoisomerase inhibitor RFS 2000, difluoromethylornithine, retinoic acid, capecitabine, or pharmaceutically acceptable salts, solvates or acids of any of the foregoing;

(b) monokine, a lymphokine, a traditional polypeptide hormone, parathyroid hormone, thyroxine, relaxin, prorelaxin, a glycoprotein hormone, follicle stimulating hormone, thyroid stimulating hormone, luteinizing hormone, hepatic growth factor fibroblast growth factor, prolactin, placental lactogen, tumor necrosis factor- $\alpha$ , tumor necrosis factor- $\beta$ , mullerian-inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial growth factor, thrombopoietin, erythropoietin, an osteoinductive factor, an interferon, interferon- $\alpha$ , interferon- $\beta$ , interferon- $\gamma$ , a colony stimulating factor ("CSF"), macrophage-CSF, granulocyte-macrophage-CSF, granulocyte-CSF, an interleukin ("IL"), IL-1, IL-1 $\alpha$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, a tumor necrosis factor, TNF- $\alpha$ , TNF- $\beta$ , a polypeptide factor, LIF, kit ligand, or a combination of any of the foregoing;

(c) diphtheria toxin, botulium toxin, tetanus toxin, dysentery toxin, cholera toxin, amanitin,  $\alpha$ -amanitin, amanitin derivatives, pyrrolobenzodiazepine, pyrrolobenzodiazepine derivatives, tetrodotoxin, brevetoxin, ciguatoxin, ricin, AM toxin, tubulysin, geldanamycin, maytansinoid, calicheamicin, daunomycin, doxorubicin, methotrexate, vindesine, SG2285, dolastatin, a dolastatin analog, auristatin, cryptophycin, camptothecin, camptothecin derivatives and metabolites (e.g., SN-38), rhizoxin, a rhizoxin derivative, CC-1065, a CC-1065 analogue or derivative, duocarmycin, an enediyne antibiotic, esperamicin, epothilone, azonafide, aplidine, a toxoid, or a combination of any of the foregoing;

(d) an affinity ligand, wherein the affinity ligand is a substrate, an inhibitor, a stimulating agent, a neurotransmitter, a radioisotope, or a combination of any of the foregoing;

(e) a radioactive label,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , a fluorescent dye, an electron dense reagent, an enzyme, biotin, streptavidin, dioxigenin, a hapten, an immunogenic protein, a nucleic acid molecule with a sequence complementary to a target, or a combination of any of the foregoing;

(f) an immunomodulatory compound, an anti-cancer agent, an anti-viral agent, an anti-bacterial agent, an anti-fungal agent, and an anti-parasitic agent, or a combination of any of the foregoing;

(g) tamoxifen, raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, or toremifene;

(h) 4(5)-imidazoles, aminoglutethimide, megestrol acetate, exemestane, letrozole, or anastrozole;

(i) flutamide, nilutamide, bicalutamide, leuprolide, goserelin, or troxacitabine;

(j) an aromatase inhibitor;

(k) a protein kinase inhibitor;

(l) a lipid kinase inhibitor;

(m) an antisense oligonucleotide;

(n) a ribozyme;

(o) a vaccine; and

(p) an anti-angiogenic agent.

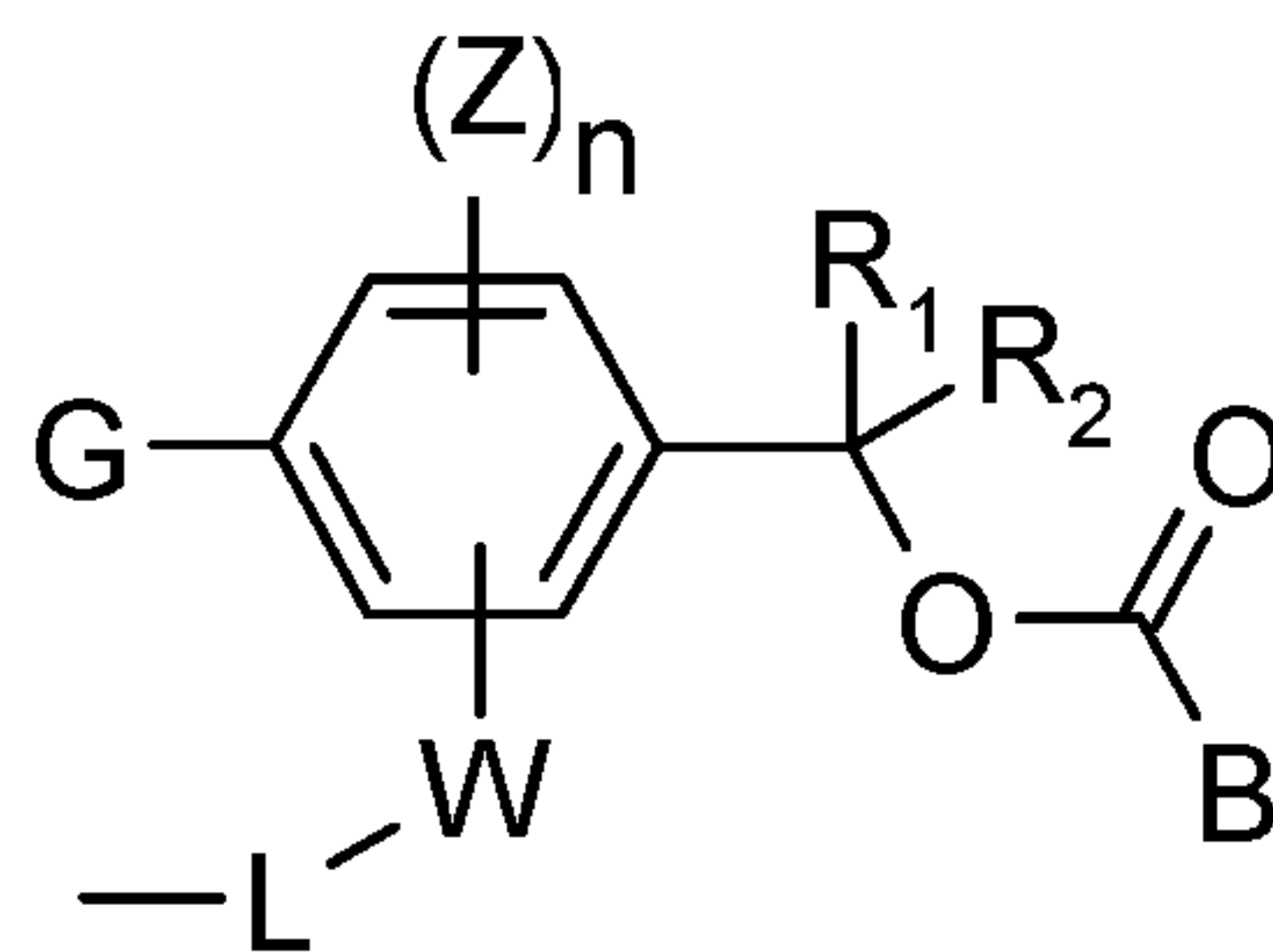
In some embodiments, the at least one active agent is talitobulin or azonafide.

In some embodiments, the conjugate comprises a moiety selected from:

In certain embodiments, each active agent is independently selected from amanitin, auristatin, calicheamicin, camptothecin, cryptophycin, daunomycin, dolastatin, doxorubicin, duocarmycin, epothilone, esperamicin, geldanamycin, maytansinoid, methotrexate, monomethyl auristatin E (“MMAE”), monomethyl auristatin F (“MMAF”), pyrrolobenzodiazepine, rhizoxin, SG2285, tubulysin, vindesine, and toxoid, or a derivative of any one of the foregoing. For example, each active agent may be independently selected from amanitin, MMAE, and MMAF, or a derivative of any one of the foregoing.

In preferred embodiments, at least one active agent is coupled to the linker through a cleavage group, *e.g.*, a group having the structure of Formula (I):

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(I)

wherein:

B represents an active agent;

G represents a sugar or sugar acid, preferably glucuronic acid;

W represents an electron-withdrawing group, preferably -C(O)NR'-, where C(O) is bonded to the phenyl ring and NR' is bonded to L;

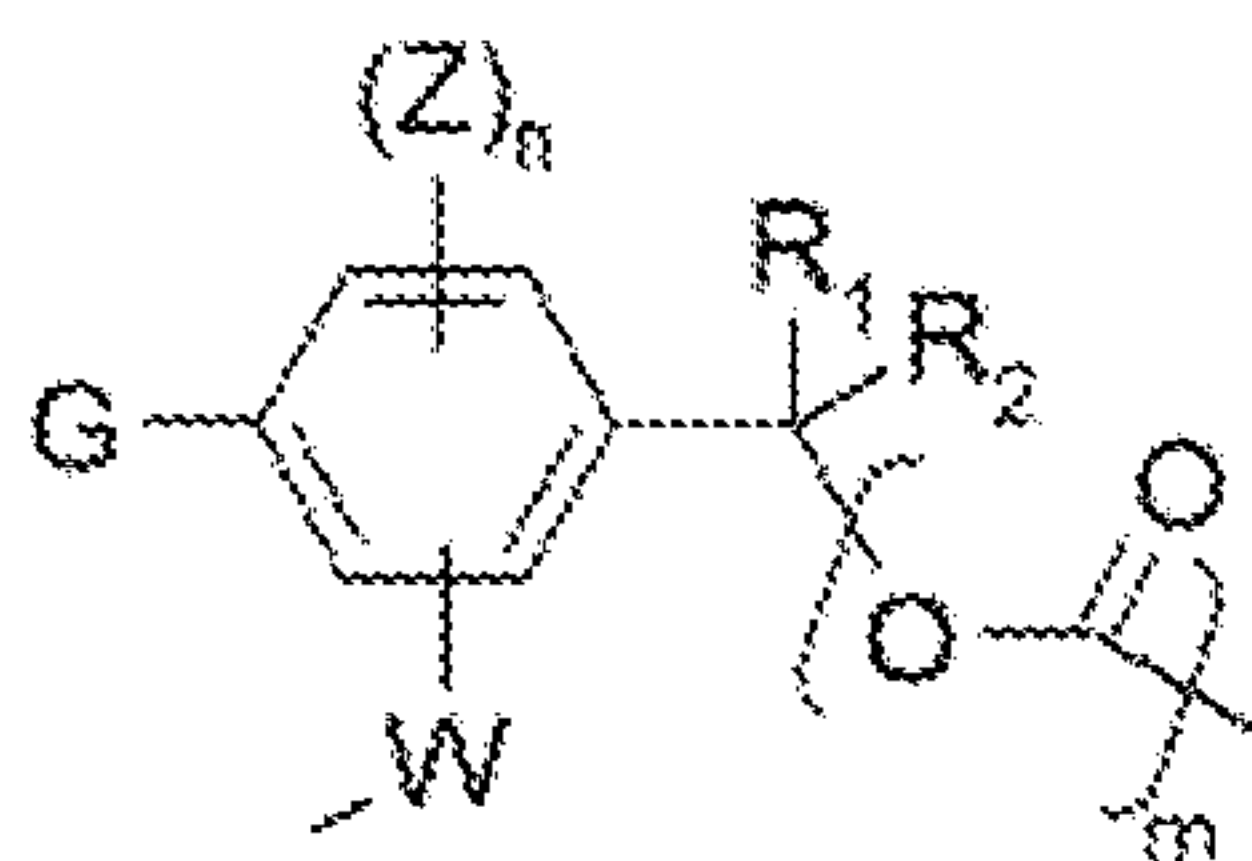
each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

R<sub>1</sub> and R<sub>2</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, preferably hydrogen, or R<sub>1</sub> and R<sub>2</sub> taken together with the carbon atom to which they are attached form a (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl ring; and

L represents a linkage to the antibody.

In some embodiments, at least one active agent is coupled to the linker through a cleavage group having the formula:



wherein:

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

W represents -C(O)-, -C(O)NR'-, -C(O)O-, -S(O)<sub>2</sub>NR'-, -P(O)R''NR'-, -S(O)NR'-, or -PO<sub>2</sub>NR'-, in each case where the C(O), S, or P is preferably directly bound to the phenyl ring, and R' and R'' are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, mono- or di-carboxyl(C<sub>1</sub>-C<sub>8</sub>)alkyl, (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>8</sub>)alkoxy, (C<sub>1</sub>-C<sub>8</sub>)alkylthio, mono- or di-(C<sub>1</sub>-C<sub>8</sub>)alkylamino, (C<sub>3</sub>-C<sub>20</sub>)heteroaryl, or (C<sub>6</sub>-C<sub>20</sub>)aryl;

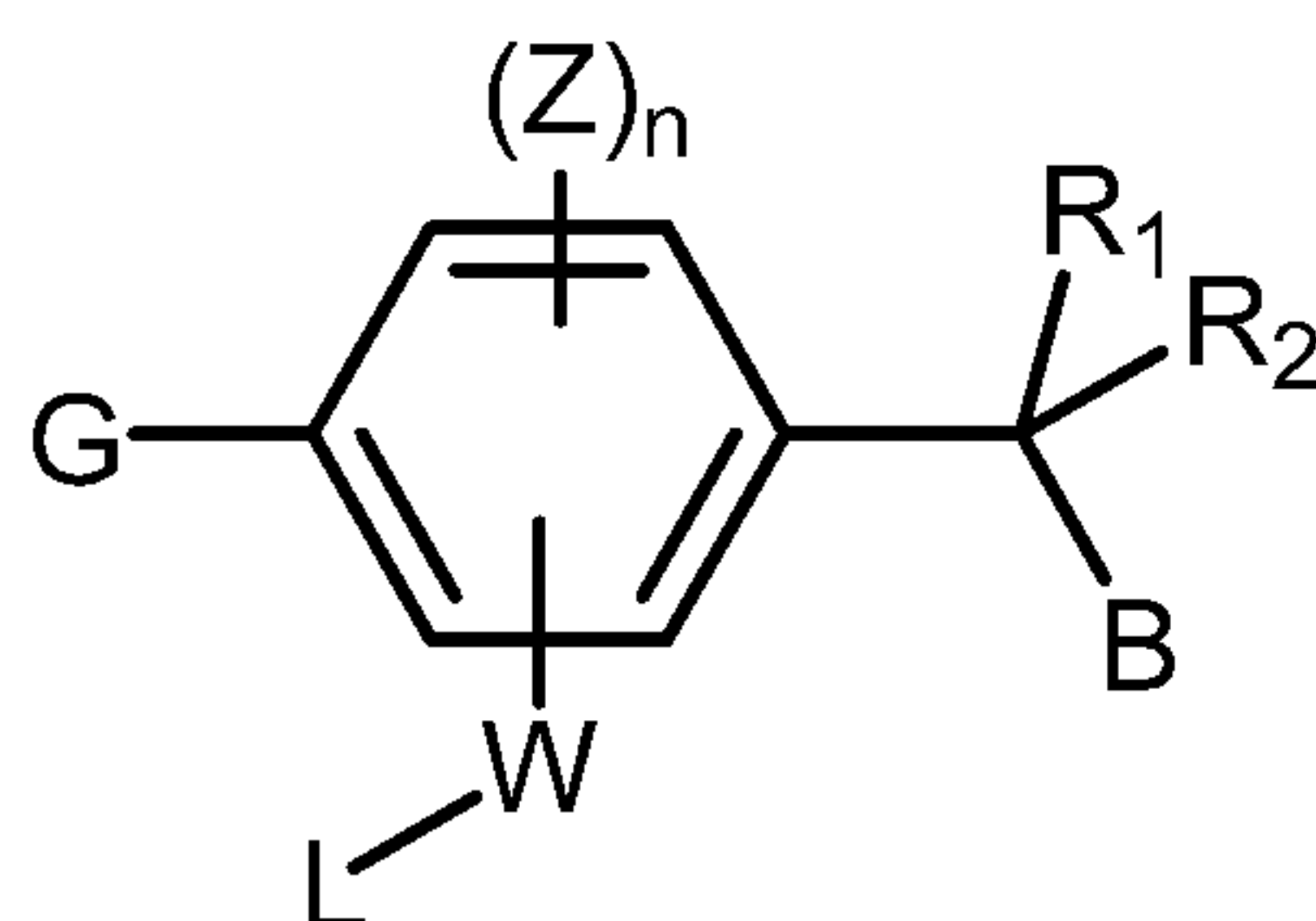
each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3;

m is 0 or 1, preferably 1; and

R<sub>1</sub> and R<sub>2</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, preferably hydrogen, or R<sub>1</sub> and R<sub>2</sub> taken together with the carbon atom to which they are attached form a (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl ring.

In some embodiments, at least one active agent is coupled to the linker through a cleavage group having the formula:



or a pharmaceutically acceptable salt thereof, wherein

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

B is a unit covalently attached to the active agent,

W represents -C(O)-, -C(O)NR'-, -C(O)O-, -S(O)<sub>2</sub>NR'-, -P(O)R''NR'-, -S(O)NR'-, or -PO<sub>2</sub>NR'-, in each case where the C(O), S, or P is directly bound to the phenyl ring, and R' and R'' are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>8</sub>)alkoxy, (C<sub>1</sub>-C<sub>8</sub>)alkylthio, mono- or di-(C<sub>1</sub>-C<sub>8</sub>)alkylamino, (C<sub>3</sub>-C<sub>20</sub>)heteroaryl, or (C<sub>6</sub>-C<sub>20</sub>)aryl;

each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro, most preferably hydrogen;

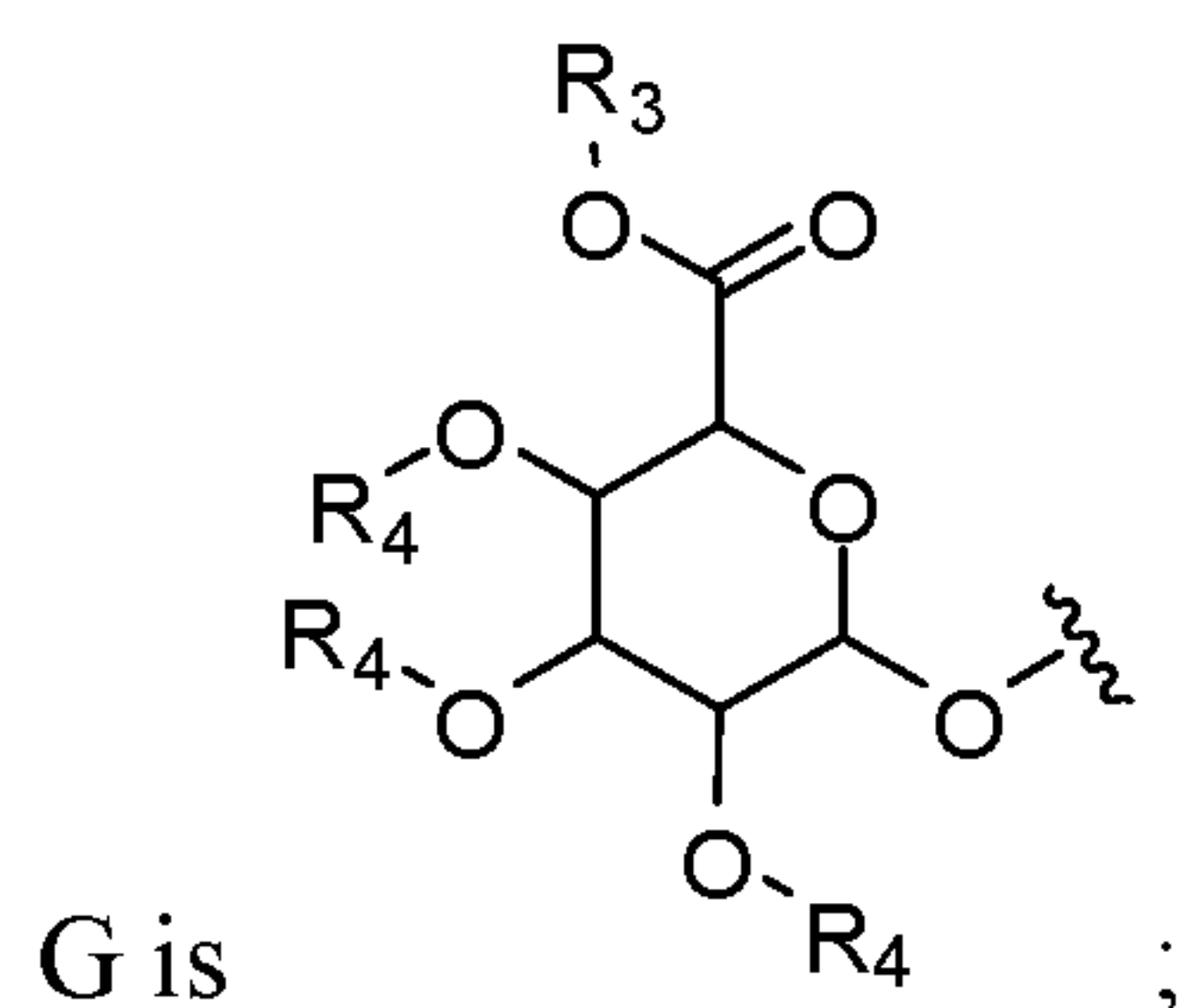
n is an integer from 1 to 3, preferably 3;

L represents a linker comprising the peptide sequence;

R<sub>1</sub> and R<sub>2</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, preferably

hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a  $(C_3-C_8)$ cycloalkyl ring.

The sugar or sugar acid may be, for example, a monosaccharide. In some embodiments,



$R_3$  is hydrogen or a carboxyl protecting group; and

each  $R_4$  is independently hydrogen or a hydroxyl protecting group.

For example,  $R_3$  may be hydrogen and each  $R_4$  may be hydrogen.

W may be  $-C(O)-$ ,  $-C(O)NR'-$ ,  $-C(O)O-$ ,  $-S(O)_2NR'-$ ,  $-P(O)R''NR'-$ ,  $-S(O)NR'-$ , or  $-PO_2NR'-$ , in each case where the C(O), S, or P is directly bound to the phenyl ring, and  $R'$  and  $R''$  are each independently hydrogen,  $(C_1-C_8)$ alkyl,  $(C_3-C_8)$ cycloalkyl,  $(C_1-C_8)$ alkoxy,  $(C_1-C_8)$ alkylthio, mono- or di- $(C_1-C_8)$ alkylamino,  $(C_3-C_{20})$ heteroaryl, or  $(C_6-C_{20})$ aryl. In preferred embodiments, W represents  $-C(O)NR'-$ , where C(O) is bonded to the phenyl ring and  $NR'$  is bonded to L.

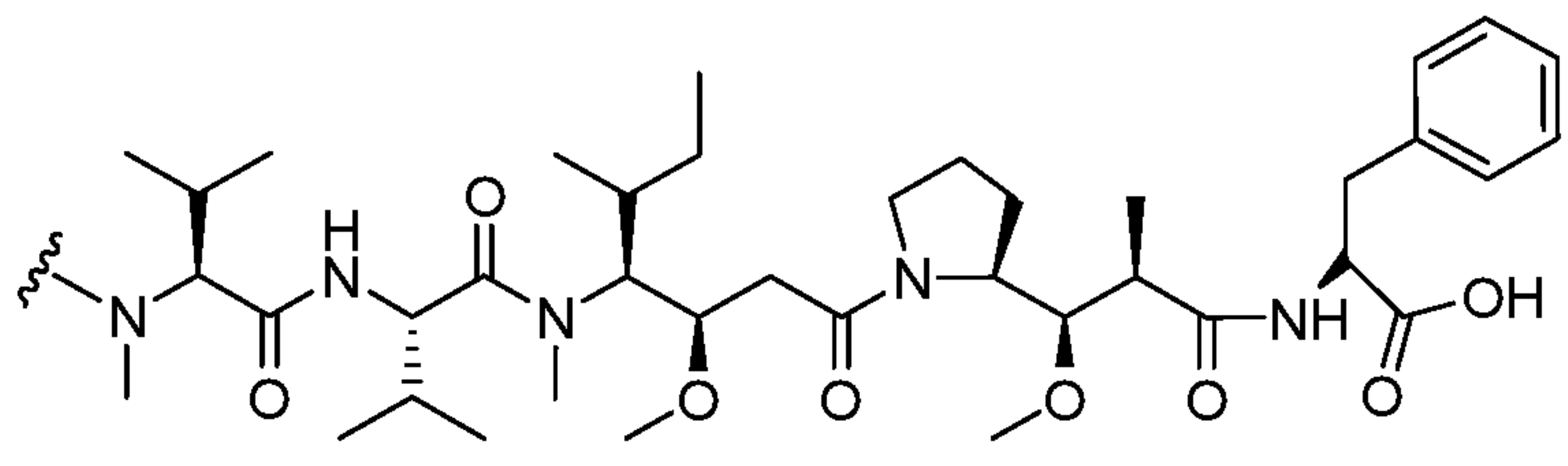
Alternative cleavage groups include valine-citrulline-p-aminobenzylcarbamate (VC-PABC).

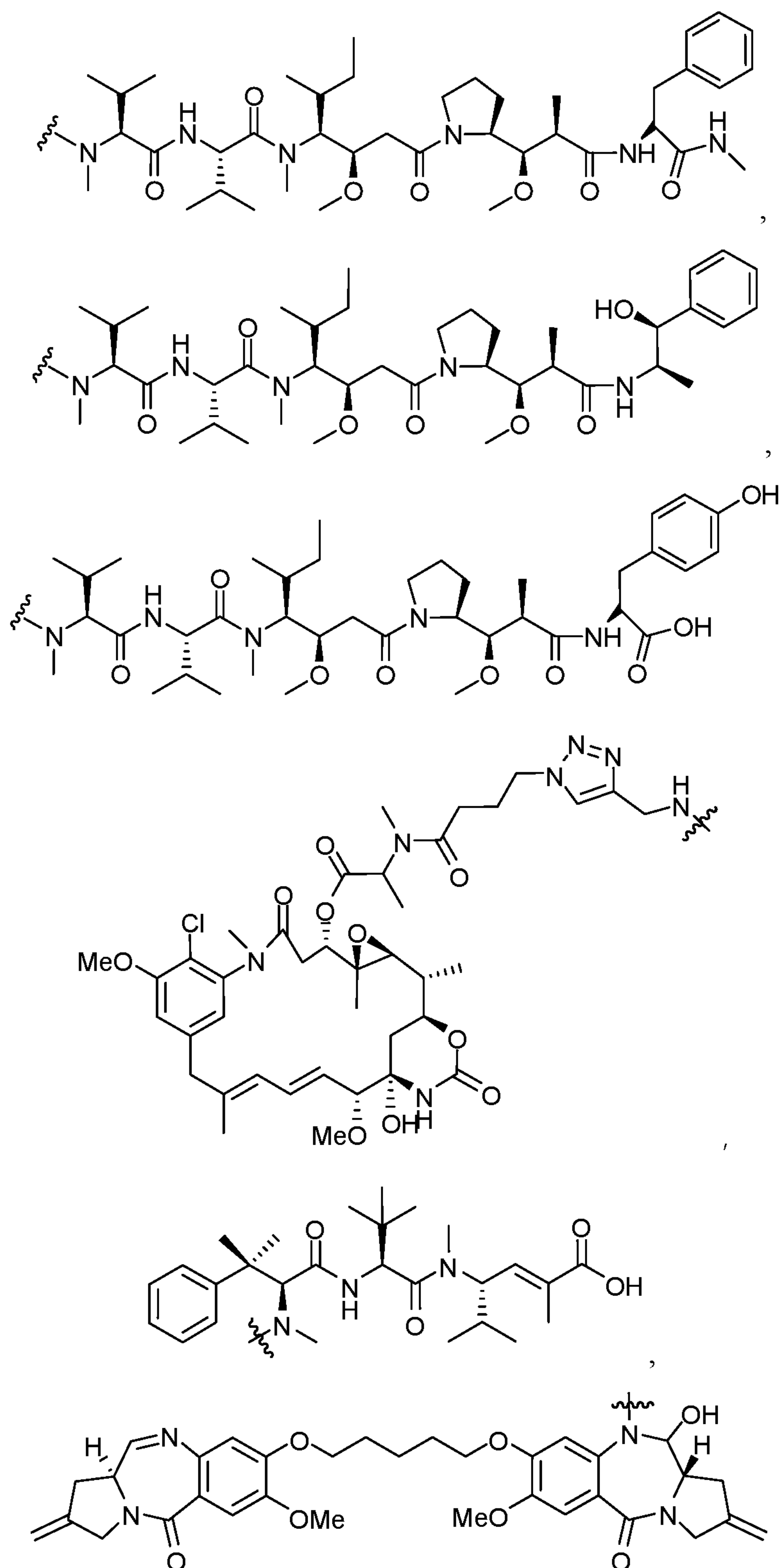
In preferred embodiments, Z represents hydrogen and n is 3.

In preferred embodiments,  $R_1$  and  $R_2$  each represent hydrogen.

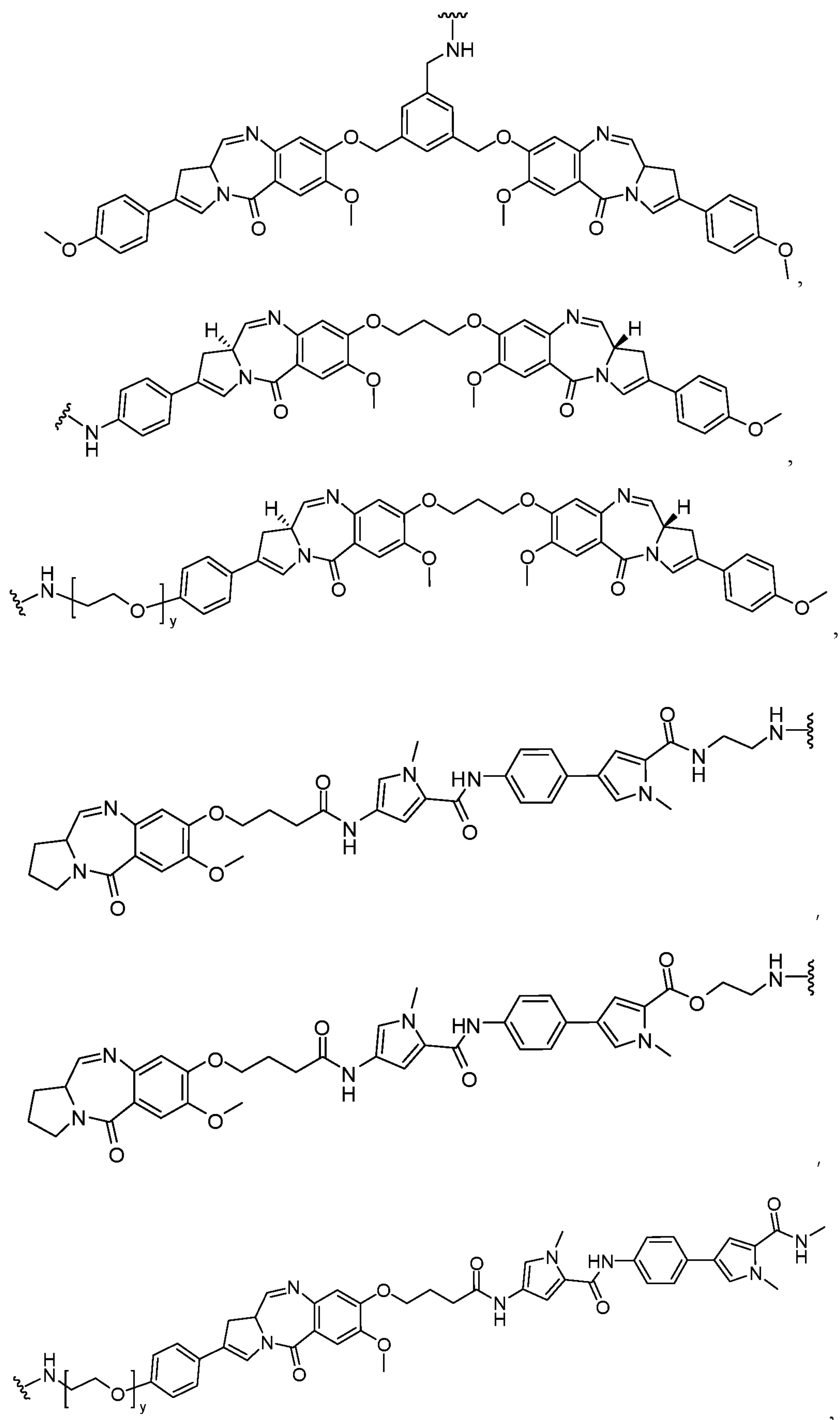
In preferred embodiments, G represents glucuronic acid; W represents  $-C(O)NR'-$ , where C(O) is bonded to the phenyl ring and  $NR'$  is bonded to L; each Z independently represents hydrogen; and  $R_1$  and  $R_2$  each represent hydrogen.

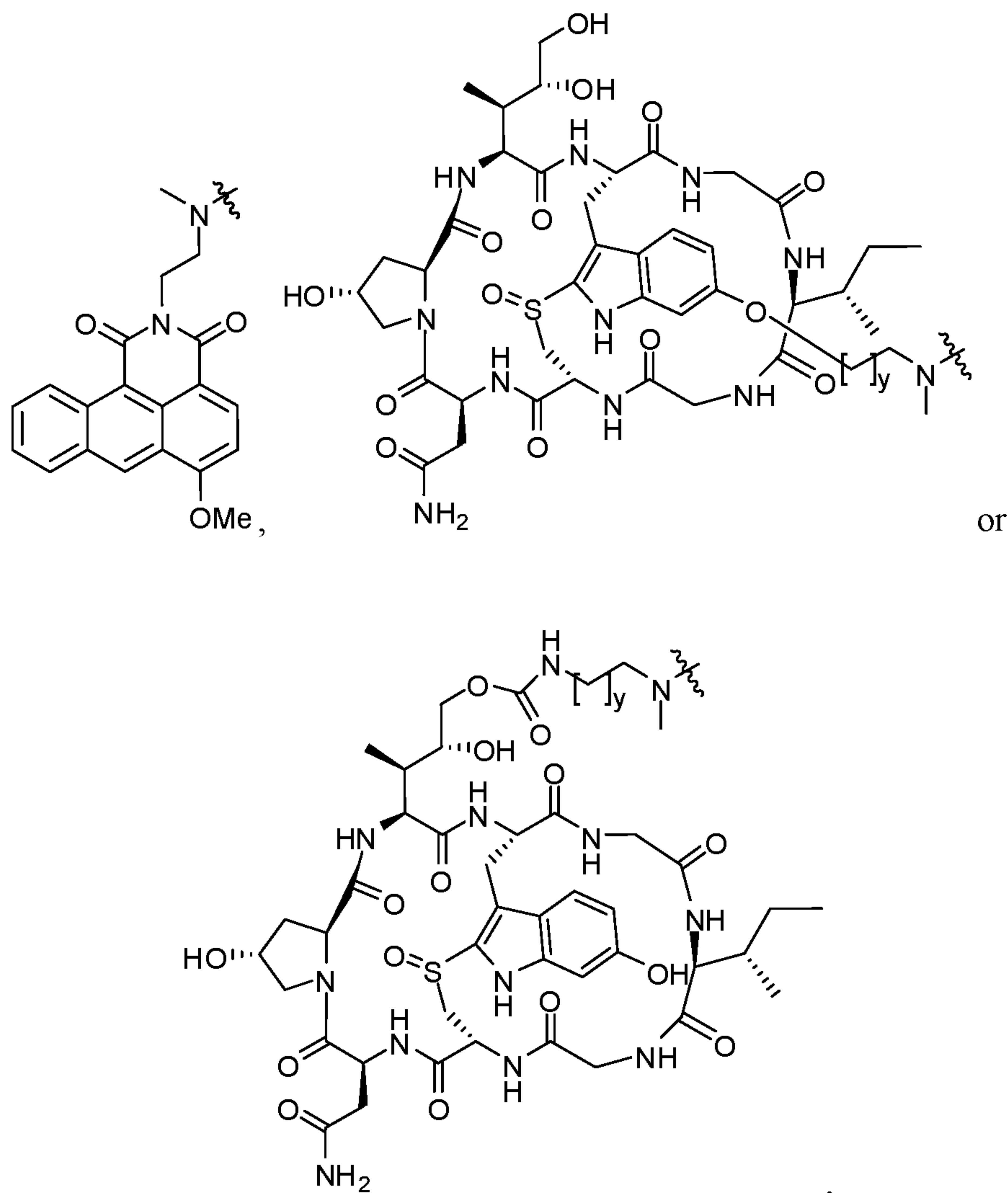
B,  $B_1$ , and/or  $B_2$  may be each independently selected from:





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wherein  $y$  is an integer from 1 to 10.

In some embodiments, an antibody-drug conjugate comprises 2 to 4 linkers as set forth in any of the preceding claims, covalently coupled to the antibody, wherein each linker preferentially comprises a peptide sequence of a plurality of amino acids, and at least two active agents are preferentially covalently coupled to side chains of the amino acids of each linker. Each linker may be coupled to a C-terminus of the antibody (*e.g.*, of the heavy and light chains of the antibody).

In various embodiments, each active agent may be the same active agent. Alternatively, an antibody-drug conjugate may comprise at least two different active agents, such as where each linker bears a different active agent from the other linkers.

Structures and components of related antibody-drug conjugates are disclosed in PCT/KR2015/005299, which is hereby incorporated by reference in its entirety, in particular for the chemical formulae and generic structures of antibody-drug conjugates, their component parts (*e.g.*, linkers, cleavage groups, etc.), and their preparation and use as

disclosed therein. In certain preferred embodiments, the various conjugates and other aspects of the present invention specifically exclude the various structures and methods disclosed in PCT/KR2015/005299.

In some aspects, the invention relates to a pharmaceutical composition comprising an antibody-drug conjugate as described herein and a pharmaceutically acceptable excipient. A pharmaceutical composition may further comprise a therapeutically effective amount of chemotherapeutic agent.

In some aspects, the invention relates to a method of treating cancer in a subject, comprising administering a pharmaceutical composition as described herein to the subject.

The subject may be a mammal. For example, the subject may be selected from rodents, lagomorphs, felines, canines, porcines, ovines, bovines, equines, and primates. In preferred embodiments, the subject is a human.

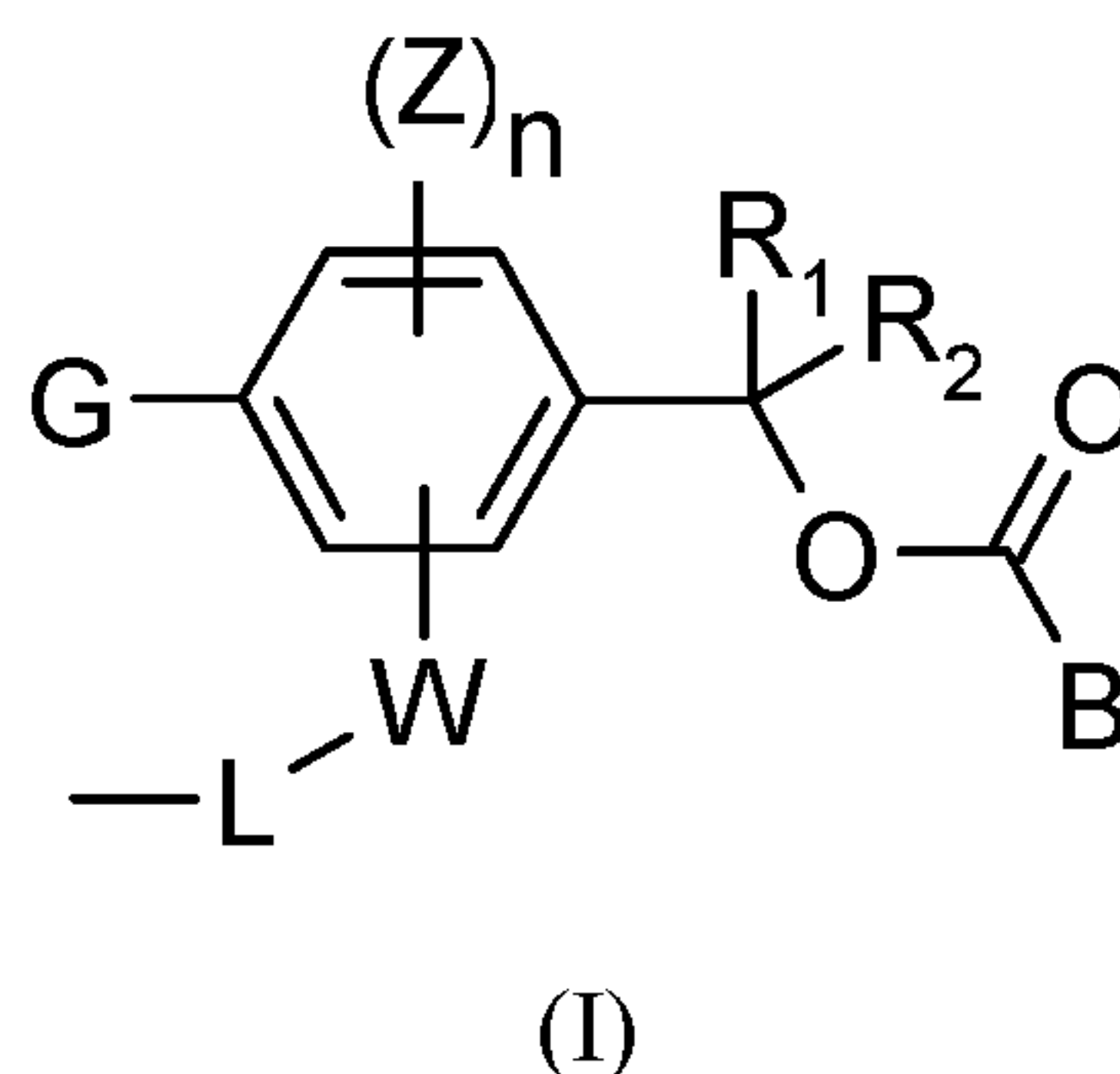
In some aspects, the invention relates to a method for making an antibody-drug conjugate as described herein, comprising reacting a biomolecule with a prodrug. The biomolecule preferentially comprises an antibody and a ketone or aldehyde. The prodrug preferentially comprises an alkoxyamine. The reaction may produce an oxime, thereby covalently linking the antibody to the prodrug.

The method may further comprise isoprenylating the antibody, thereby producing the biomolecule. For example, the antibody may comprise an isoprenylation sequence, and isoprenylating the antibody may comprise incubating the antibody with an isoprenoid transferase and an isoprenoid transferase substrate. The substrate preferentially comprises the ketone or aldehyde. The isoprenoid transferase may be, for example, farnesyltransferase or geranylgeranyltransferase.

In some embodiments, the invention relates to a method for making an antibody-drug conjugate as described herein, comprising isoprenylating an antibody. For example, the antibody may comprise an isoprenylation sequence, and isoprenylating the antibody may comprise incubating the antibody with an isoprenoid transferase and an isoprenoid transferase substrate. In preferred embodiments, the substrate comprises at least one active agent as described herein.

In some embodiments, the invention relates to a linker-active agent compound, to which a ligand (preferably an antibody) can be conjugated to result in one of the conjugates of the invention. Accordingly, in such linker-active agent compounds, i) the linker comprises a peptide sequence of a plurality of amino acids; and ii) at least two active agents

are covalently coupled to side chains of the amino acids. In some such embodiments, each active agent is coupled to the peptide sequence by a cleavage group that can be hydrolyzed to release the active agent from the ligand-active agent compound. The cleavage group may have a structure of the formula:



wherein:

B represents the active agent;

G represents a sugar or sugar acid, preferably glucuronic acid;

W represents an electron-withdrawing group, preferably  $-C(O)NR'$ -, where C(O) is bonded to the phenyl ring and  $NR'$  is bonded to L;

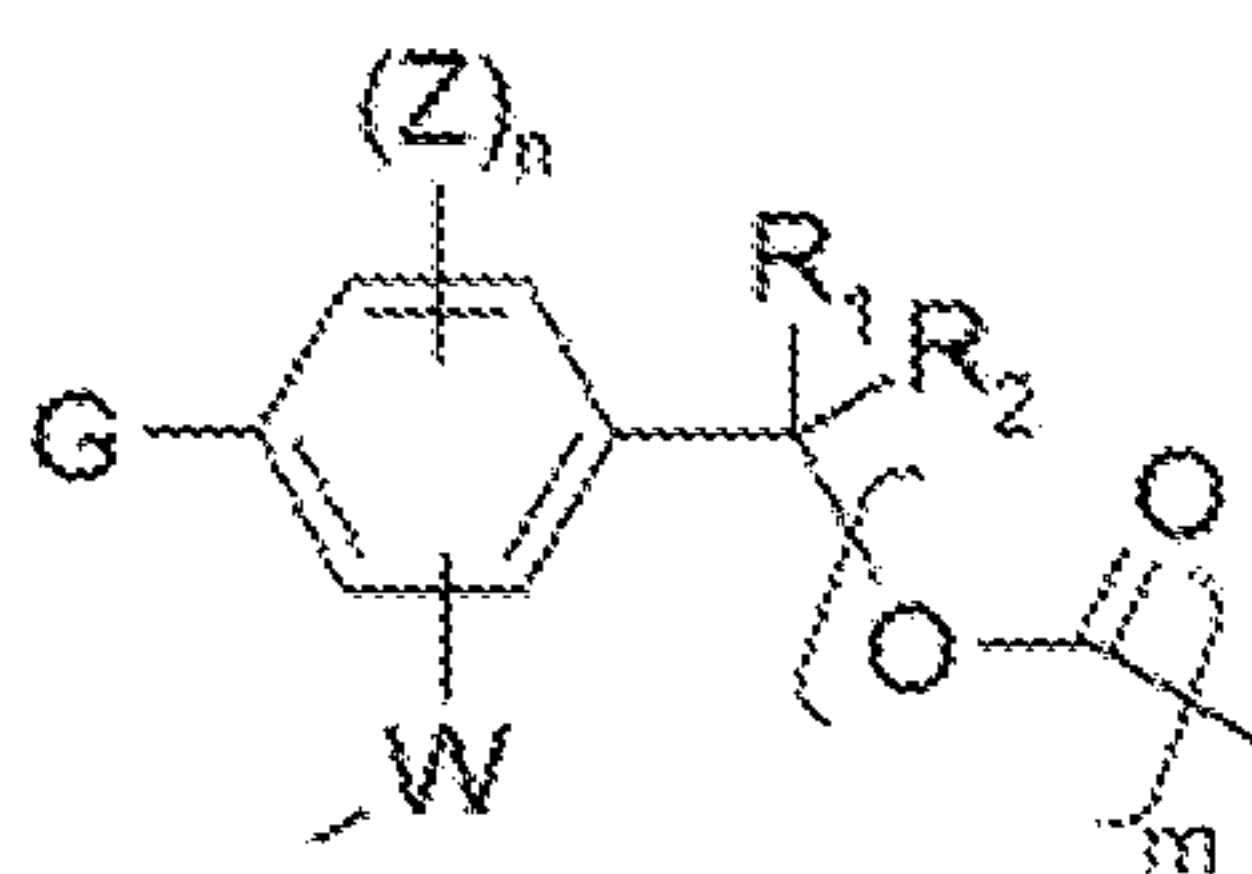
each Z independently represents hydrogen,  $(C_1-C_8)$ alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen,  $(C_1-C_8)$ alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

$R_1$  and  $R_2$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, or  $(C_3-C_8)$ cycloalkyl, preferably hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a  $(C_3-C_8)$ cycloalkyl ring; and

L represents a linkage to the peptide sequence.

In other embodiments, the cleavage group may have a structure of the formula:



wherein:

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

W represents  $-C(O)-$ ,  $-C(O)NR'-$ ,  $-C(O)O-$ ,  $-S(O)_2NR'-$ ,  $-P(O)R''NR'-$ ,  $-S(O)NR'-$ , or  $-PO_2NR'-$ , in each case where the C(O), S, or P is preferably directly bound to the

phenyl ring, and R' and R'' are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, mono- or di-carboxyl(C<sub>1</sub>-C<sub>8</sub>)alkyl, (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>8</sub>)alkoxy, (C<sub>1</sub>-C<sub>8</sub>)alkylthio, mono- or di-(C<sub>1</sub>-C<sub>8</sub>)alkylamino, (C<sub>3</sub>-C<sub>20</sub>)heteroaryl, or (C<sub>6</sub>-C<sub>20</sub>)aryl;

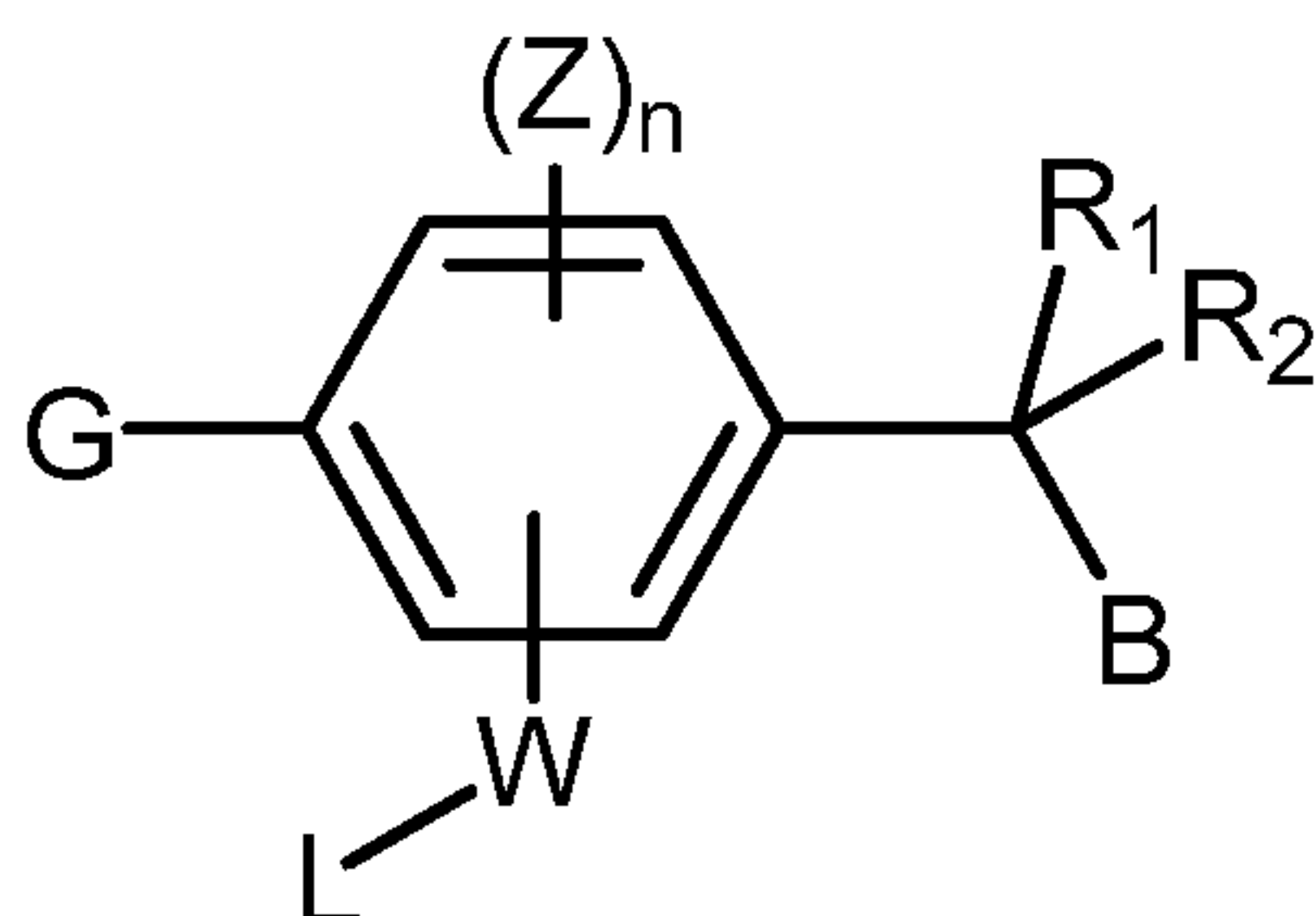
each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3;

m is 0 or 1, preferably 1; and

R<sub>1</sub> and R<sub>2</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, preferably hydrogen, or R<sub>1</sub> and R<sub>2</sub> taken together with the carbon atom to which they are attached form a (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl ring.

In yet other embodiments, the cleavage group may have a structure of the formula:



or a pharmaceutically acceptable salt thereof, wherein

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

B is a unit covalently attached to the active agent,

W represents -C(O)-, -C(O)NR'-, -C(O)O-, -S(O)<sub>2</sub>NR'-, -P(O)R''NR'-, -S(O)NR'-, or -PO<sub>2</sub>NR'-, in each case where the C(O), S, or P is directly bound to the phenyl ring, and R' and R'' are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>8</sub>)alkoxy, (C<sub>1</sub>-C<sub>8</sub>)alkylthio, mono- or di-(C<sub>1</sub>-C<sub>8</sub>)alkylamino, (C<sub>3</sub>-C<sub>20</sub>)heteroaryl, or (C<sub>6</sub>-C<sub>20</sub>)aryl;

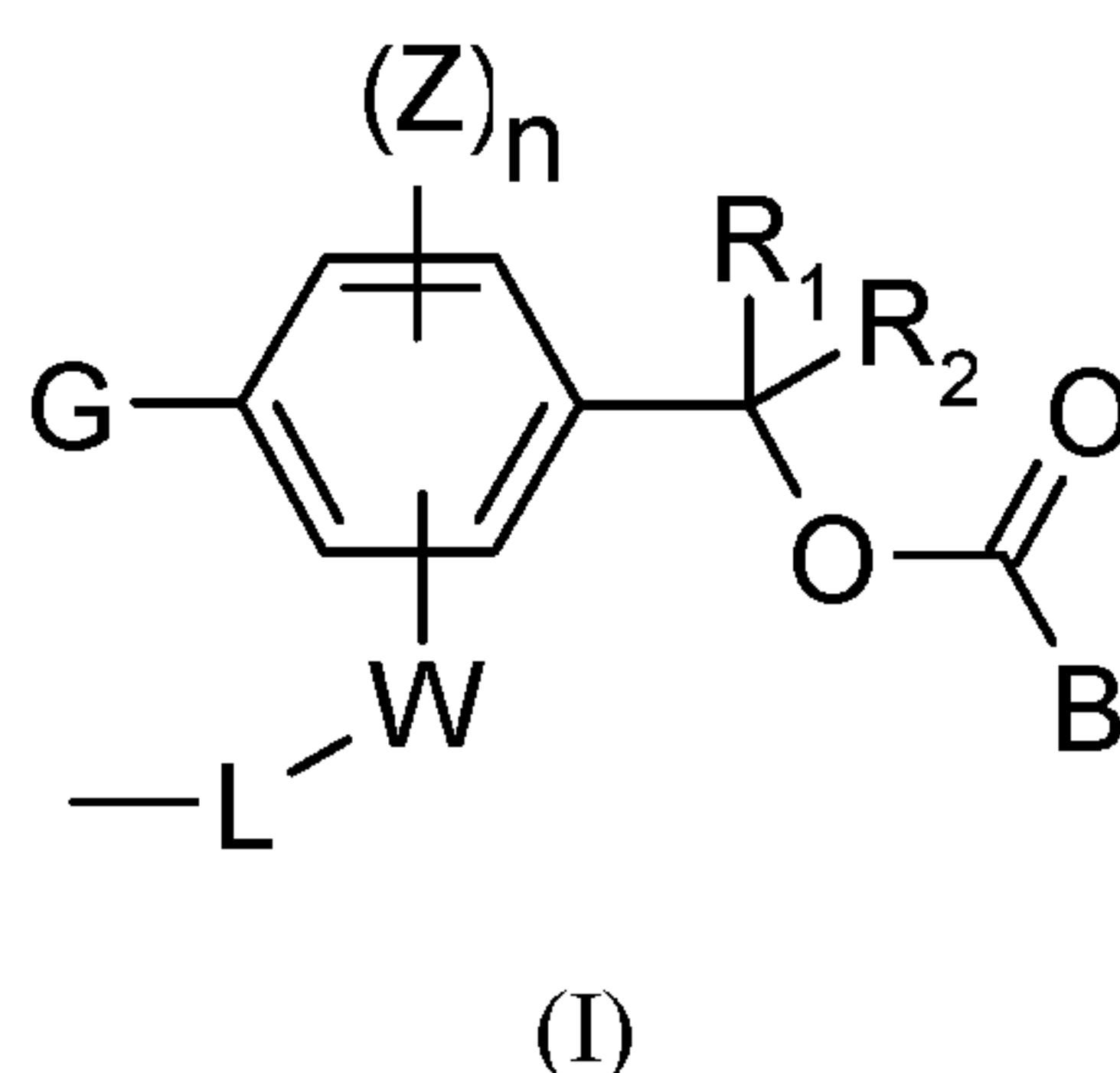
each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

L represents the a peptide sequence;

$R_1$  and  $R_2$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, or  $(C_3-C_8)$ cycloalkyl, preferably hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a  $(C_3-C_8)$ cycloalkyl ring.

In another aspect, the invention relates to a linker compound, that can be coupled to an active agent and a ligand (preferably an antibody) to result in a conjugate of the invention. Accordingly, in such a linker compound i) the linker comprises a peptide sequence of a plurality of amino acids; and ii) at least two cleavage groups are covalently coupled to side chains of the amino acids, wherein each cleavage group has a reactive moiety capable of reacting with an active agent. The cleavage group may have a structure of the formula:



wherein:

B represents a leaving group capable of being displaced an active agent, such as a halogen (esp. Cl or Br), or a unit comprising a reactive moiety capable of being coupled to an active agent, such as an isocyanate, an acid chloride, a chloroformate, etc.;

G represents a sugar or sugar acid, preferably glucuronic acid;

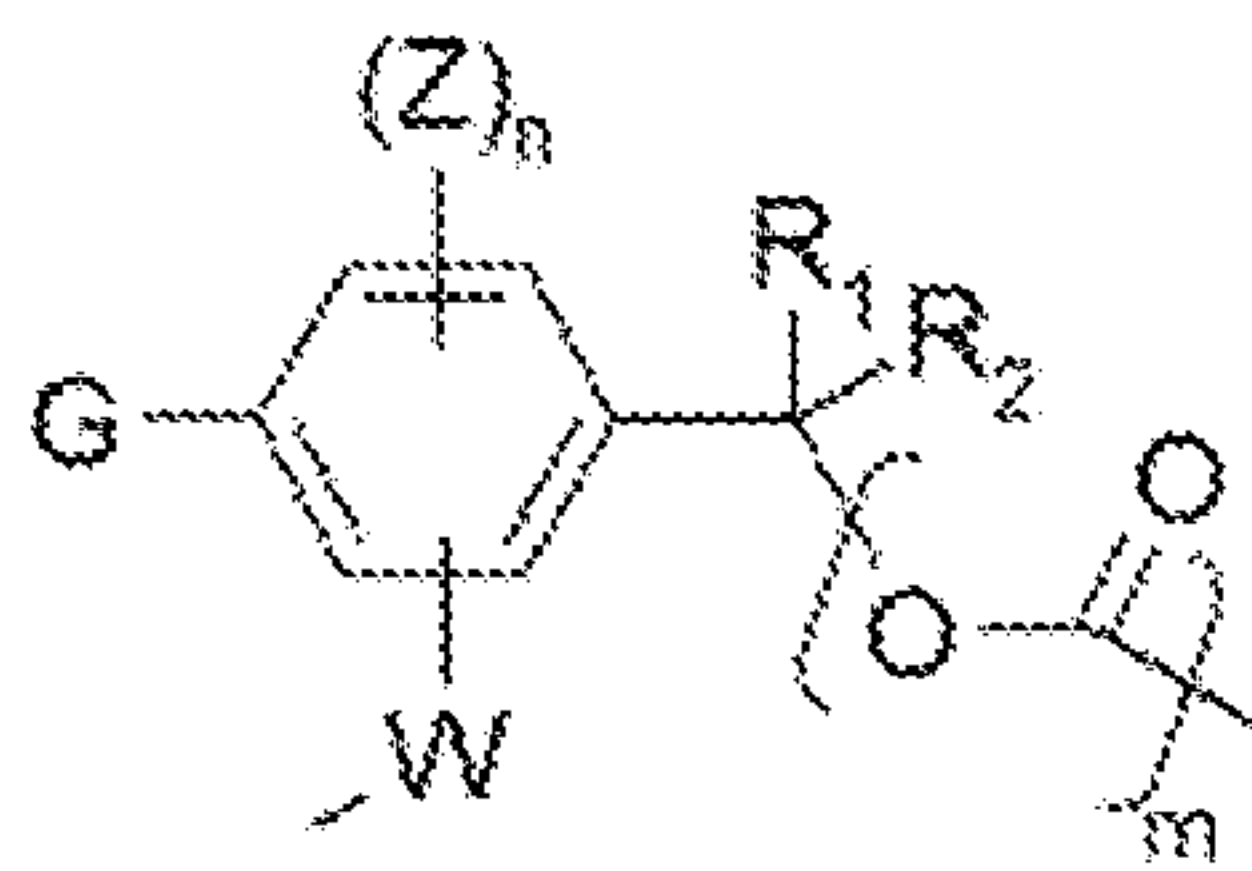
W represents an electron-withdrawing group, preferably  $-C(O)NR'$ -, where C(O) is bonded to the phenyl ring and  $NR'$  is bonded to L; each Z independently represents hydrogen,  $(C_1-C_8)$ alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen,  $(C_1-C_8)$ alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

$R_1$  and  $R_2$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, or  $(C_3-C_8)$ cycloalkyl, preferably hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a  $(C_3-C_8)$ cycloalkyl ring; and

L represents a linkage to the peptide sequence.

Alternatively, the cleavage group may have a structure of the formula:



wherein:

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

W represents  $-C(O)-$ ,  $-C(O)NR'-$ ,  $-C(O)O-$ ,  $-S(O)_2NR'-$ ,  $-P(O)R''NR'-$ ,  $-S(O)NR'-$ , or  $-PO_2NR'-$ , in each case where the C(O), S, or P is preferably directly bound to the phenyl ring, and R' and R'' are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, mono- or di-carboxyl(C<sub>1</sub>-C<sub>8</sub>)alkyl, (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>8</sub>)alkoxy, (C<sub>1</sub>-C<sub>8</sub>)alkylthio, mono- or di-(C<sub>1</sub>-C<sub>8</sub>)alkylamino, (C<sub>3</sub>-C<sub>20</sub>)heteroaryl, or (C<sub>6</sub>-C<sub>20</sub>)aryl;

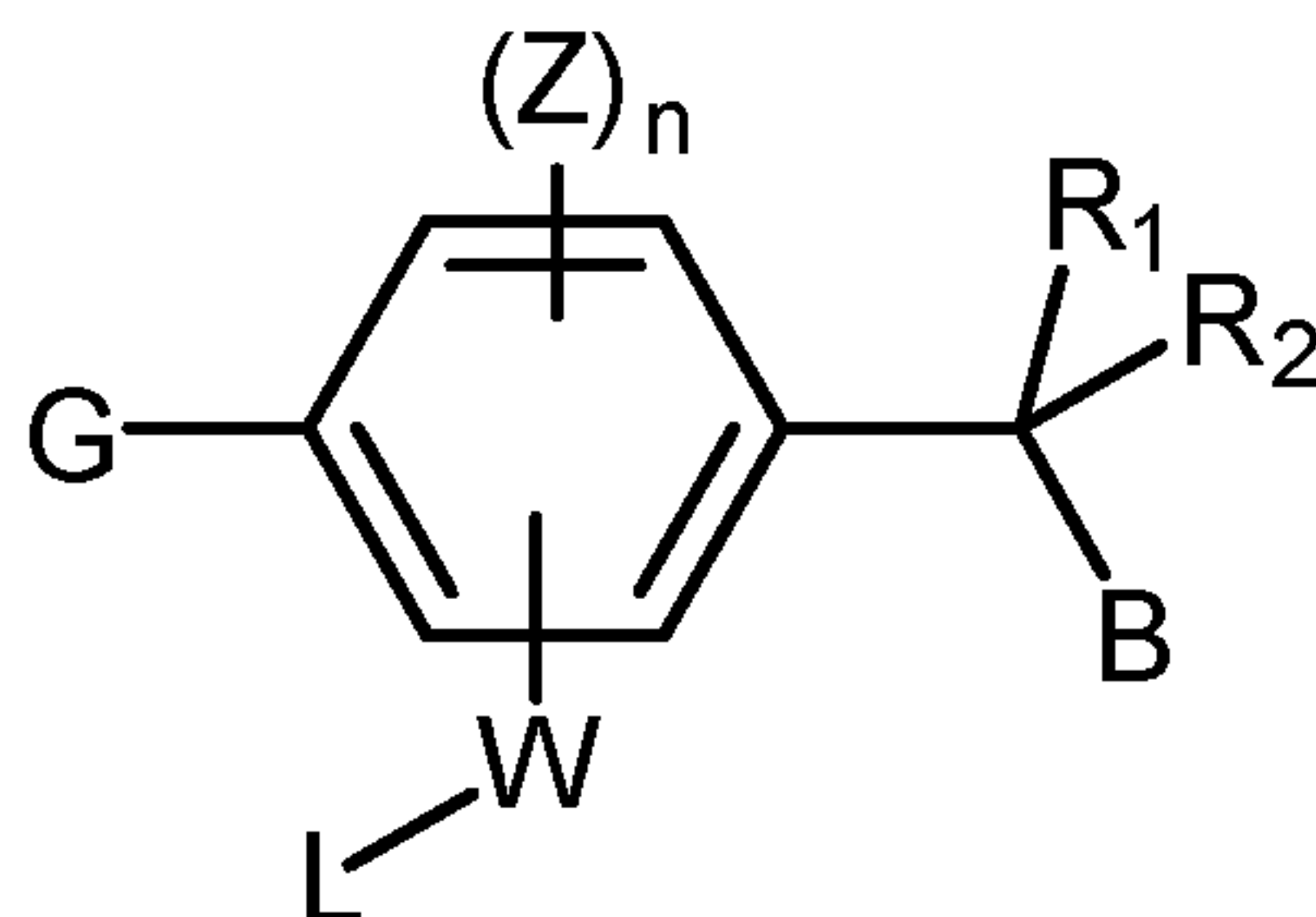
each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3;

m is 0 or 1, preferably 1; and

R<sub>1</sub> and R<sub>2</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, preferably hydrogen, or R<sub>1</sub> and R<sub>2</sub> taken together with the carbon atom to which they are attached form a (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl ring.

Alternatively, the cleavage group may have a structure of the formula:



or a pharmaceutically acceptable salt thereof, wherein

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

B is a unit comprising a reactive moiety capable of being coupled to the active agent,

W represents  $-C(O)-$ ,  $-C(O)NR'-$ ,  $-C(O)O-$ ,  $-S(O)_2NR'-$ ,  $-P(O)R''NR'-$ ,  $-S(O)NR'-$ , or  $-$

$\text{PO}_2\text{NR}'$ -, in each case where the C(O), S, or P is directly bound to the phenyl ring, and  $\text{R}'$  and  $\text{R}''$  are each independently hydrogen,  $(\text{C}_1\text{-C}_8)\text{alkyl}$ ,  $(\text{C}_3\text{-C}_8)\text{cycloalkyl}$ ,  $(\text{C}_1\text{-C}_8)\text{alkoxy}$ ,  $(\text{C}_1\text{-C}_8)\text{alkylthio}$ , mono- or di- $(\text{C}_1\text{-C}_8)\text{alkylamino}$ ,  $(\text{C}_3\text{-C}_{20})\text{heteroaryl}$ , or  $(\text{C}_6\text{-C}_{20})\text{aryl}$ ;

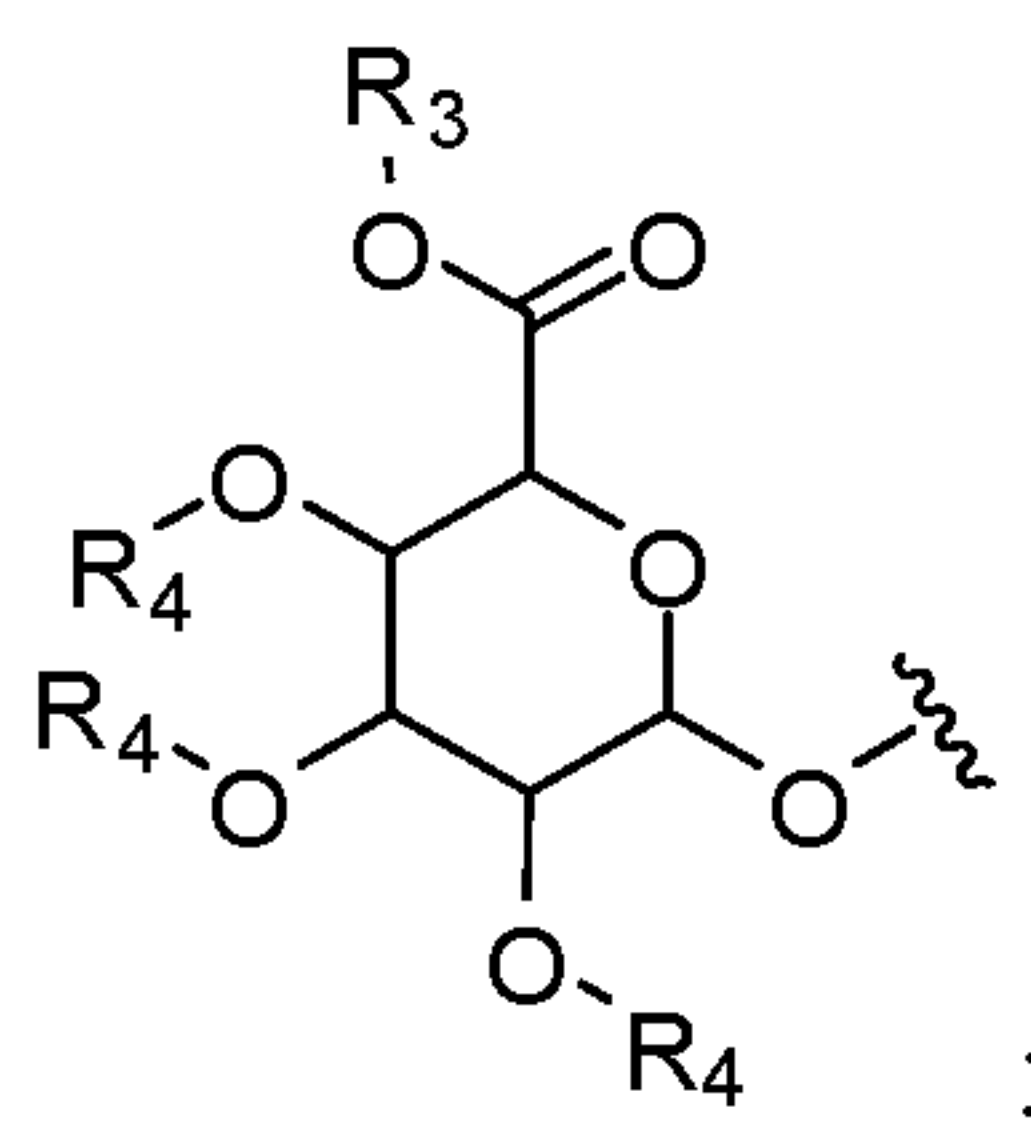
each Z independently represents hydrogen,  $(\text{C}_1\text{-C}_8)\text{alkyl}$ , or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen,  $(\text{C}_1\text{-C}_8)\text{alkyl}$ , halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

L represents a linker comprising the peptide sequence;

$\text{R}_1$  and  $\text{R}_2$  are each independently hydrogen,  $(\text{C}_1\text{-C}_8)\text{alkyl}$ , or  $(\text{C}_3\text{-C}_8)\text{cycloalkyl}$ , preferably hydrogen, or  $\text{R}_1$  and  $\text{R}_2$  taken together with the carbon atom to which they are attached form a  $(\text{C}_3\text{-C}_8)\text{cycloalkyl}$  ring.

In some such embodiments, G is



wherein  $\text{R}_3$  is hydrogen or a carboxyl protecting group; and each  $\text{R}_4$  is independently hydrogen or a hydroxyl protecting group.

In some embodiments, the linker comprises a connection unit represented by  $-(\text{CH}_2)_r(\text{V}(\text{CH}_2)_p)_{q-}$ ,  $-((\text{CH}_2)_p\text{V})_{q-}$ ,  $-(\text{CH}_2)_r(\text{V}(\text{CH}_2)_p)_q\text{Y}-$ ,  $-((\text{CH}_2)_p\text{V})_q(\text{CH}_2)_r-$ ,  $-\text{Y}(((\text{CH}_2)_p\text{V})_q)-$  or  $-(\text{CH}_2)_r(\text{V}(\text{CH}_2)_p)_q\text{YCH}_2-$

wherein:

r is an integer from 0 to 10;

p is an integer from 1 to 10;

q is an integer from 1 to 20;

V and Y are each independently a single bond,  $-\text{O}-$ ,  $-\text{S}-$ ,  $-\text{NR}_{21}-$ ,  $-\text{C}(\text{O})\text{NR}_{22}-$ ,  $-\text{NR}_{23}\text{C}(\text{O})-$ ,  $-\text{NR}_{24}\text{SO}_2-$ , or  $-\text{SO}_2\text{NR}_{25}-$ ; and

$\text{R}_{21}$  to  $\text{R}_{25}$  are each independently hydrogen,  $(\text{C}_1\text{-C}_6)\text{alkyl}$ ,  $(\text{C}_1\text{-C}_6)\text{alkyl}(\text{C}_6\text{-C}_{20})\text{aryl}$  or  $(\text{C}_1\text{-C}_6)\text{alkyl}(\text{C}_3\text{-C}_{20})\text{heteroaryl}$ .

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1** illustrates an active drug release mechanism from a  $\beta$ -glucuronide based linker.

**Figure 2** is a graph depicting the hydrolysis of a linker by  $\beta$ -glucuronidase from Experimental Example 1.

**Figure 3** is a graph depicting the plasma stability of two drug-linker conjugates from Experimental Example 2.

**Figure 4** is a graph depicting the plasma stability of compound 47a, described in Example 68.

**Figure 5** is a graph depicting the plasma stability of compound 49a, described in Example 70.

**Figure 6** is a graph depicting the plasma stability of compound 48a, described in Example 69.

**Figure 7** consists of two panels, **Figure 7A** and **Figure 7B**. **Figure 7A** displays a strategy for conjugating a drug to an antibody (DAR2). **Figure 7B** displays a strategy for conjugating a drug to an antibody (DAR4).

**Figure 8** shows relative in vitro activities of the DAR2 MMAE-conjugates, with varying PEG length in the linker, against JIMT-1 (HER2 positive) and MCF7 cells (HER2 negative).

**Figure 9** shows relative in vitro activities of the DAR4 MMAE-conjugates, which have a different PEG length in linker part, against JIMT-1 (HER2 positive) and MCF7 cells (HER2 negative).

**Figure 10** shows human beta-glucuronidase reactivity in DAR4 ADCs, which have the various linker types. 12  $\mu$ M of ADCs were incubated with 0.01  $\mu$ g of human beta-glucuronidase (R&D Systems) for 3 hours at 37°C.

**Figure 11** shows plasma stability of ADC2 and Kadcyla in mouse or human plasma.

**Figure 12** shows human plasma stability of ADC33 and ADC34 for 7 days.

**Figure 13** shows Rat PK profile of Herceptin and ADC2.

**Figure 14** shows Rat PK profile of ADC23 and ADC34.

**Figure 15** shows Rat PK profile improvement of MMAE-based ADCs by replacing linker-toxin from 2g to 1lj.

**Figure 16** shows Rat PK profile improvement by branched linker unit.

**Figure 17** shows impact of polar amino acid on Rat PK profile in MMAE ADC.

**Figure 18** shows Rat PK profile improvement by branched linker-toxin with or without polar amino acid in ADC with DAR2.

**Figure 19** shows effects of Asp in linker-toxin unit on Rat PK profile of ADC with DAR4.

**Figure 20** shows effects of Glu in linker-toxin unit on Rat PK profile of ADC with DAR4.

**Figure 21** shows in vivo efficacy of representative amine type DAR4 ADC using MMAF (ADC23) or MMAE (ADC24).

**Figure 22** shows in vivo efficacy of representative amide type DAR4 ADC using MMAF (ADC34) or MMAE (ADC33).

## DETAILED DESCRIPTION

The present invention relates to antibody-drug conjugates (ADCs) wherein a plurality of drugs are conjugated to an antibody through a linker. The linker preferably comprises a peptide sequence of a plurality of amino acids, wherein at least two of the active agents are covalently coupled to side chains of the amino acids. However, as one of skill in the art would recognize, the antibody portion of such conjugates can be replaced by any suitable ligand, and thus the invention relates in equal measure to ligand-drug conjugates. Accordingly, references to and discussions of antibody-drug conjugates herein should be understood, where not contradicted by context, as equally applicable to ligand-drug conjugates and their corresponding intermediates (e.g., ligand-linker conjugates). In all aspects related to the various ligand-drug conjugates disclosed herein, however, the ligand is preferably an antibody.

In certain such embodiments, two or more such linkers are conjugated to the antibody, e.g., 2-4 linkers, which may each be coupled to a different C-terminal cysteine of a heavy or light chain of the antibody.

An active agent may be covalently coupled to the side chain of an amino acid, termed a “linking amino acid” herein. In certain preferred embodiments, a linking amino acid, such as each linking amino acid in a linker, is lysine or ornithine. Nevertheless, many

other amino acids may be linking amino acids in various embodiments of the invention. For example, a linking amino acid may be selected from lysine, 5-hydroxylysine, 4-oxalysine, 4-thialysine, 4-selenalysine, 4-thiahomolysine, 5,5-dimethyllysine, 5,5-difluorolysine, *trans*-4-dehydrolysine, 2,6-diamino-4-hexynoic acid, *cis*-4-dehydrolysine, 6-*N*-methyllysine, diaminopimelic acid, ornithine, 3-methylornithine,  $\alpha$ -methylornithine, citrulline, and homocitrulline. A linking amino acid may be a L-amino acid or a D-amino acid. A linking amino acid may be an  $\alpha$ -amino acid or a  $\beta$ -amino acid. A linking amino acid may be a naturally-occurring amino acid or a non-naturally-occurring amino acid.

The peptide may comprise 2 to 20 amino acids. The majority of amino acids of the peptide may be independently selected from alanine, aspartate, asparagine, glutamate, glutamine, glycine, lysine, ornithine, proline, serine, and threonine. For example, each amino acid of the peptide may be independently selected from alanine, aspartate, asparagine, glutamate, glutamine, glycine, lysine, ornithine, proline, serine, and threonine.

In preferred embodiments, the peptide comprises at least one hydrophilic amino acid, *e.g.*, in addition to the linking amino acids. Hydrophilic amino acids may increase the water solubility of the antibody-drug conjugate, linker, and/or precursors of the antibody-drug conjugate. Each hydrophilic amino acid may be a naturally-occurring amino acid or a non-naturally-occurring amino acid. A hydrophilic amino acid may be an  $\alpha$ -amino acid or a  $\beta$ -amino acid. A hydrophilic amino acid may be arginine, aspartate, asparagine, glutamate, glutamine, histidine, lysine, ornithine, proline, serine, or threonine, and may be a D-amino acid or an L-amino acid. In preferred embodiments, the peptide comprises a hydrophilic amino acid selected from aspartate or glutamate, such as L-aspartate or L-glutamate. In other preferred embodiments, the peptide comprises a hydrophilic amino acid selected from lysine or arginine, such as L-lysine or L-arginine. In certain embodiments, the peptide comprises a hydrophilic amino acid with a side chain having a moiety that bears a charge at neutral pH in aqueous solution (*e.g.*, an amine, guanidine, or carboxyl moiety).

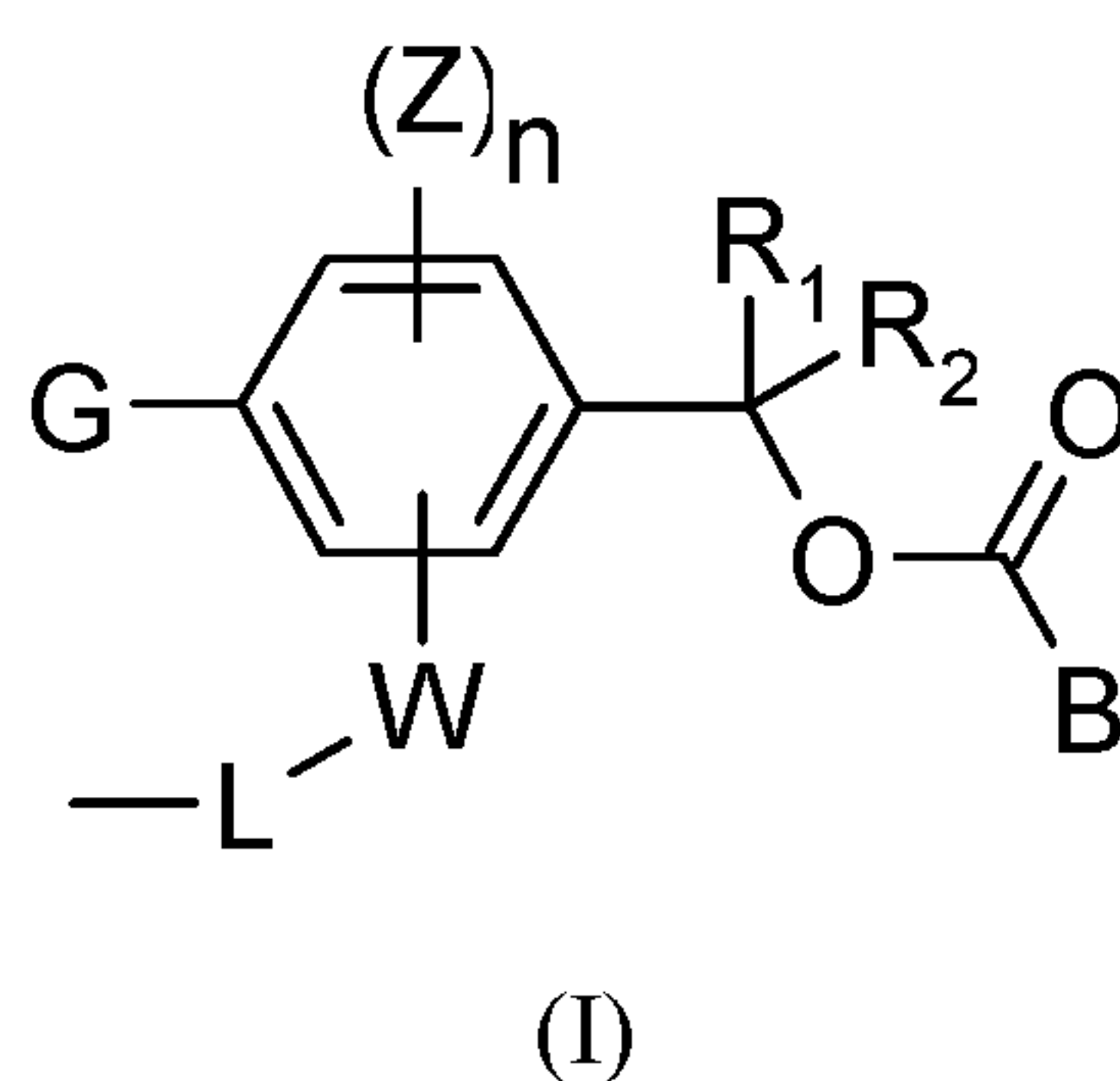
The peptide may comprise naturally-occurring amino acids and/or non-naturally-occurring amino acids. The peptide may comprise  $\alpha$ -amino acids and/or  $\beta$ -amino acids. In some embodiments, the peptide consists essentially of  $\alpha$ -amino acids. In some embodiments, the peptide consists essentially of naturally-occurring amino acids (*i.e.*, wherein the linking amino acids are naturally-occurring amino acids, such as lysine or ornithine, that are substituted with a group comprising an active agent). The peptide may comprise, consist essentially of, or even consist of amino acids selected from alanine,

aspartate, asparagine, glutamate, glutamine, glycine, lysine, ornithine, proline, serine, and threonine, any of which may be L-amino acids and/or D-amino acids. In some embodiments, the peptide consists essentially of L-amino acids. In certain embodiments, the peptide does not comprise a hydrophobic amino acid, such as an amino acid selected from isoleucine, methionine, leucine, phenylalanine, tryptophan, tyrosine, or valine; in other words, in such embodiments, the peptide is free or essentially free of these amino acids. In preferred embodiments, the peptide does not comprise any one of isoleucine, methionine, leucine, phenylalanine, tryptophan, tyrosine, and valine.

In addition to the peptide, the linker may comprise an O-substituted oxime. In preferred embodiments, the oxygen atom of the oxime is substituted with a group that covalently links the oxime to the peptide, and the carbon atom of the oxime is substituted with a group that covalently links the oxime to the antibody. In other embodiments, the carbon atom of the oxime is substituted with a group that covalently links the oxime to the peptide, and the oxygen atom of the oxime is substituted with a group that covalently links the oxime to the antibody. The oxime may covalently link the antibody to the N-terminus of the peptide or the C-terminus of the peptide, or the oxime may covalently link the antibody to a side chain of the peptide. In some embodiments, the linker does not comprise an oxime. For example, the linker may comprise a product of a cycloaddition reaction, such as a substituted triazole, instead of an oxime.

Active agents may be coupled to a linker by cleavable or non-cleavable bonds, hydrolysable or non-hydrolyzable bonds. In certain preferred embodiments, the active agents are coupled to the linker by cleavable bonds. For example, an antibody-drug conjugate may comprise a self-immolative group, preferably a self-immolative group for each active agent, *e.g.*, to release an active agent from the ADC.

For example, one or more active agents, or even each active agent, may be coupled to the linker through a cleavage group of Formula (I)



wherein

G is a sugar or sugar acid, preferably glucuronic acid or a derivative thereof;

B represents the active agent, such as a drug;

W represents an electron-withdrawing group, preferably  $-C(O)NR'$ -, where C(O) is bonded to the phenyl ring and  $NR'$  is bonded to L;

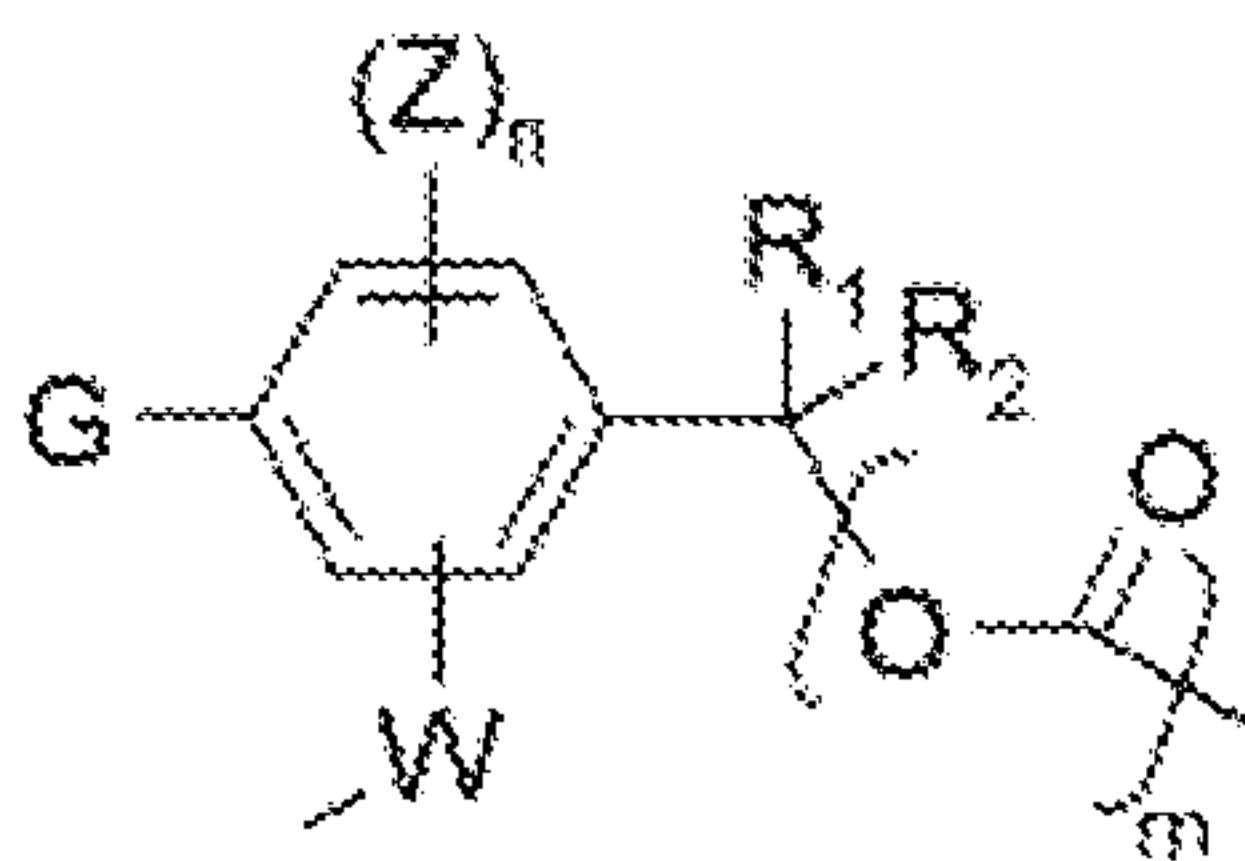
each Z independently represents hydrogen,  $(C_1-C_8)$ alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen,  $(C_1-C_8)$ alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 0 to 3, preferably 3;

L comprises a chain of 20 to 100 atoms, comprising at least one amino acid of the peptide, that covalently links the antibody to W; and

$R_1$  and  $R_2$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, or  $(C_3-C_8)$ cycloalkyl, preferably hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a  $(C_3-C_8)$ cycloalkyl ring.

Alternatively, the cleavage group may have a structure of the formula:



wherein:

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

W represents  $-C(O)-$ ,  $-C(O)NR'-$ ,  $-C(O)O-$ ,  $-S(O)_2NR'-$ ,  $-P(O)R''NR'-$ ,  $-S(O)NR'-$ , or  $-PO_2NR'-$ , in each case where the C(O), S, or P is preferably directly bound to the phenyl ring, and  $R'$  and  $R''$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, mono- or di-carboxyl $(C_1-C_8)$ alkyl,  $(C_3-C_8)$ cycloalkyl,  $(C_1-C_8)$ alkoxy,  $(C_1-C_8)$ alkylthio, mono- or di- $(C_1-C_8)$ alkylamino,  $(C_3-C_{20})$ heteroaryl, or  $(C_6-C_{20})$ aryl;

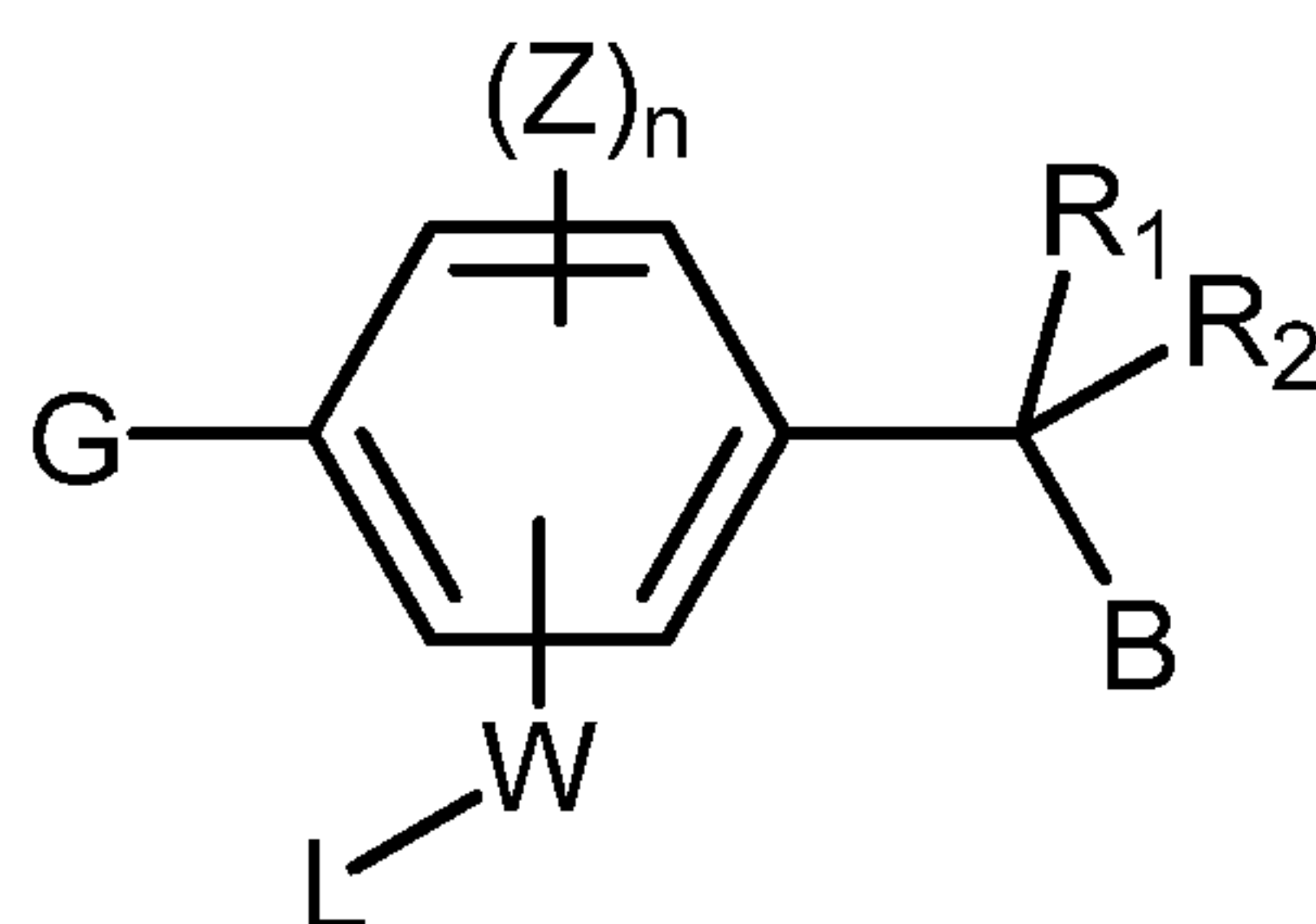
each Z independently represents hydrogen,  $(C_1-C_8)$ alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen,  $(C_1-C_8)$ alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3;

m is 0 or 1, preferably 1; and

$R_1$  and  $R_2$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, or  $(C_3-C_8)$ cycloalkyl, preferably hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a  $(C_3-C_8)$ cycloalkyl ring.

Alternatively, the cleavage group may have a structure of the formula:



or a pharmaceutically acceptable salt thereof, wherein

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

B is a unit comprising a reactive moiety capable of being coupled to the active agent,

W represents  $-C(O)-$ ,  $-C(O)NR'-$ ,  $-C(O)O-$ ,  $-S(O)_2NR'-$ ,  $-P(O)R''NR'-$ ,  $-S(O)NR'-$ , or  $-PO_2NR'-$ , in each case where the C(O), S, or P is directly bound to the phenyl ring, and  $R'$  and  $R''$  are each independently hydrogen,  $(C_1-C_8)$ alkyl,  $(C_3-C_8)$ cycloalkyl,  $(C_1-C_8)$ alkoxy,  $(C_1-C_8)$ alkylthio, mono- or di- $(C_1-C_8)$ alkylamino,  $(C_3-C_{20})$ heteroaryl, or  $(C_6-C_{20})$ aryl;

each Z independently represents hydrogen,  $(C_1-C_8)$ alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen,  $(C_1-C_8)$ alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

L represents a linker comprising the peptide sequence;

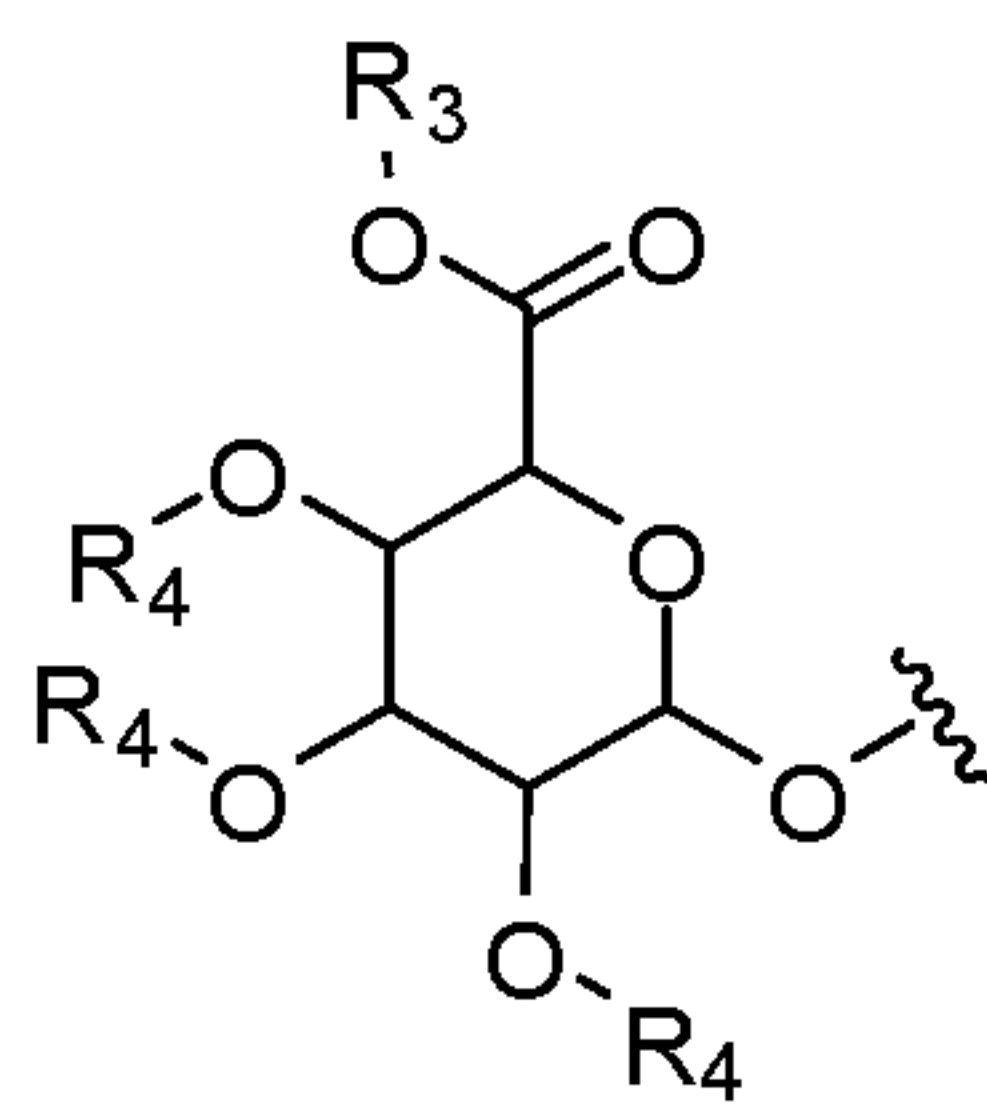
$R_1$  and  $R_2$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, or  $(C_3-C_8)$ cycloalkyl, preferably hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a  $(C_3-C_8)$ cycloalkyl ring.

The electron withdrawing group W may be  $-C(O)-$ ,  $-C(O)NR'-$ ,  $-C(O)O-$ ,  $-SO_2NR'-$ ,  $-P(O)R''NR'-$ ,  $-SONR'-$ , or  $-PO_2NR'-$ , preferably  $-C(O)NR'-$ , and  $R'$  and  $R''$  may be each independently hydrogen,  $(C_1-C_8)$ alkyl,  $(C_3-C_8)$ cycloalkyl,  $(C_1-C_8)$ alkoxy,  $(C_1-C_8)$ alkylthio, mono- or di- $(C_1-C_8)$ alkylamino,  $(C_3-C_{20})$ heteroaryl, or  $(C_6-C_{20})$ aryl, preferably hydrogen. In such embodiments, W is preferably oriented such that the carbonyl, phosphoryl,

sulphonyl, or sulphinyl group is directly bound to the phenyl ring. Where Z represents an electron-withdrawing group, Z may represent any of the moieties described in this paragraph for W.

W may represent  $-C(O)NR'$ -, and the nitrogen of W may be a nitrogen atom of the side chain of a linking amino acid, such as lysine or ornithine. Similarly, W may represent  $-C(O)NR'$ -, and the nitrogen of W may be a nitrogen atom of the N-terminal amino acid in the peptide, *e.g.*, the amine nitrogen (backbone nitrogen) of the N-terminal amino acid.

The sugar or sugar acid of the cleavage group (*i.e.*, G) is preferably linked to the phenyl ring, *e.g.*, by a bond susceptible to enzymatic cleavage, such as a glycosidic bond to an oxygen substituent on the phenyl ring. The sugar or sugar acid is preferably a monosaccharide, such as glucuronic acid or a derivative thereof, which is capable of being cleaved from the ADC by an enzyme, such as a  $\beta$ -glucuronidase, *e.g.*, an enzyme present in cells to be targeted by the conjugate. Glucuronic acid and derivatives thereof may be represented by Formula (II):



(II)

wherein R<sub>3</sub> is hydrogen or a carboxyl protecting group, preferably hydrogen, and each R<sub>4</sub> is independently hydrogen or a hydroxyl protecting group, preferably hydrogen.

A carboxyl protecting group may be any suitable protecting group for masking a carboxylic acid, *e.g.*, in organic synthesis, such as methyl, methoxymethyl, methylthiomethyl, tetrahydropyranyl, benzyloxymethyl, phenacyl, N-phthalimidomethyl, 2,2,2-trichloroethyl, 2-haloethyl, 2-(p-toluenesulfonyl)ethyl, t-butyl, cinnamyl, benzyl, triphenylmethyl, bis(o-nitrophenyl)methyl, 9-anthrylmethyl, 2-(9,10-dioxo)anthrylmethyl, piperonyl, 2-trimethylsilylethyl, trimethylsilyl, or t-butyldimethylsilyl. In some embodiments, the entire moiety R<sub>3</sub>-OC(=O)- is replaced by a carboxyl-masking moiety such as 2-alkyl-1,3-oxazoliny.

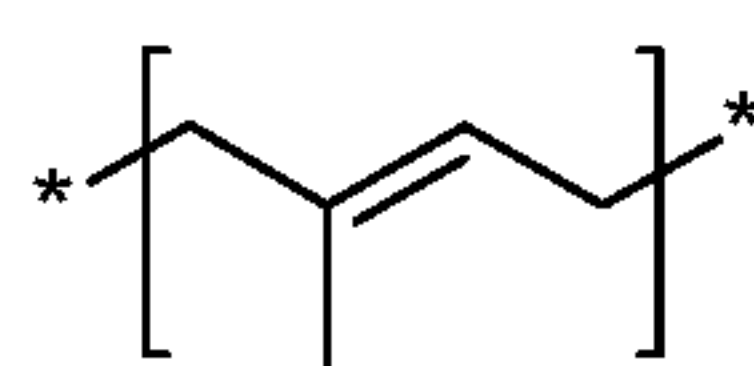
A hydroxyl protecting group may be any suitable protecting group suitable for masking a hydroxyl group, *e.g.*, in organic synthesis, such as acetyl, methyl, ethoxyethyl, benzoyl, benzyl, 4-methoxybenzyl, 3,4-dimethoxybenzyl, tetrahydropyranyl (THP),

tetrahydrofuranyl (THF), tert-butyldimethylsilyl (TBDMS), trimethylsilyl (TMS), triethylsilyl (TES), triisopropylsilyl (TIPS), tert-butyldiphenylsilyl (TBDPS), triisopropylsilyloxymethyl (TOM),  $\beta$ -methoxyethoxymethyl (MEM), methoxymethyl (MOM), allyl, or trityl.

L and/or the linker may comprise a substituted or unsubstituted alkylene having 1 to 100 carbon atoms, preferably 20 to 80 carbon atoms, and satisfy at least one, preferably at least two, of the following (i) to (iv):

- (i) the alkylene includes at least one unsaturated bond, preferably 3 or 4 double bonds and no triple bonds,
- (ii) the alkylene includes at least one heteroarylene,
- (iii) at least one carbon atom of the alkylene is replaced by one or more heteroatoms selected from nitrogen (N), oxygen (O), and sulfur (S), preferably at least one nitrogen and at least one oxygen (*e.g.*, as in an oxime), and
- (iv) the alkylene is substituted with one or more alkyls having 1 to 20 carbon atoms, preferably 2 or 3 methyls.

In preferred embodiments, a cysteine of the antibody, preferably at a C-terminus of a heavy or light chain of the antibody, forms a thioether bond with a carbon atom of an isoprenyl unit, thereby covalently linking the antibody to the linker. Thus, in some embodiments, L and/or the linker may comprise at least one isoprenyl unit, preferably two isoprenyl units, each represented by Formula (III), which is preferably recognizable by an isoprenoid transferase, *e.g.*, as part of a product or substrate of the isoprenoid transferase.



(III)

In certain such preferred embodiments, the antibody comprises an amino acid motif capable of being recognized by an isoprenoid transferase. For example, at least one C-terminus of the antibody may comprise an amino acid motif capable of being recognized by an isoprenoid transferase (*e.g.*, as a substrate, for example, prior to forming the antibody-drug conjugate, or as a product of an isoprenoid transferase, for example, after forming the antibody-drug conjugate). The antibody may further comprise a spacer, such as an amino acid or a stretch of amino acids that links a peptide chain of the antibody to the amino acid motif. The spacer may consist of 1 to 20 consecutive amino acids, preferably 7 or more amino acids. In some embodiments, glycine and proline are preferred amino acids for the

spacer, and may be used in any combination, such as a series of about 7 glycines. In other embodiments, the amino acids of the spacer are each independently selected from glycine, aspartic acid, arginine, and serine. The antibody may comprise an addition or deletion at a carboxy terminus, *e.g.*, relative to a form of the antibody not included in an ADC.

Examples of isoprenoid transferases include farnesyl protein transferase (FTase) and geranylgeranyl transferase (GGTase), which can catalyze the transfer of a farnesyl or geranyl-geranyl group to at least one C-terminal cysteine of a target protein. A GGTase may be classified as either GGTase I or GGTase II. FTase and GGTase I may recognize a CAAX motif, and GGTase II may recognize a XXCC, XCXC, or CXX motif, wherein C represents cysteine, A represents an aliphatic amino acid (*e.g.*, isoleucine, valine, methionine, leucine), and each X independently represents, for example, glutamine, glutamate, serine, cysteine, methionine, alanine, or leucine (*see* Nature Rev. Cancer, 5(5):405-12 (2005); Nature Chemical Biology 17:498-506 (2010); Lane KT, Bees LS, J. Lipid Research, 47:681-699 (2006); Kasey PJ, Seabra MC, J. Biological Chemistry, 271(10):5289-5292 (1996), each of which is hereby incorporated by reference in its entirety).

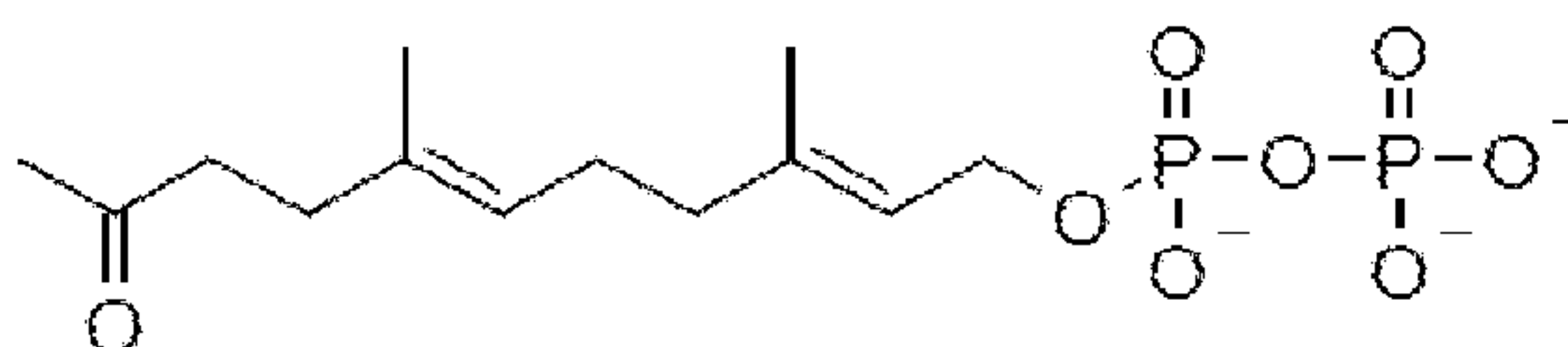
The antibody-drug conjugates according to the present invention may comprise an amino acid motif, such as CYYX, XXCC, XCXC, or CXX, preferably CYYX (wherein, C represents cysteine, Y represents an aliphatic amino acid, such as leucine, isoleucine, valine, and/or methionine, and X represents an amino acid that determines a substrate specificity of the isoprenoid transferase, such as glutamine, glutamate, serine, cysteine, methionine, alanine, and/or leucine).

Isoprenoid transferases from various sources may be used. For example, the isoprenoid transferase may be obtained from a human, animal, plant, bacteria, virus, or other source. In some embodiments, a naturally occurring isoprenoid transferase is used. In some embodiments, a naturally-modified or artificially-modified isoprenoid transferase may be used. For example, the isoprenoid transferase may comprise one or more amino acid substitutions, additions, and/or deletions, and/or the isoprenoid transferase may be modified by the addition of at least one of Histidine-tag, GST, GFP, MBP, CBP, Isopeptag, BCCP, Myc-tag, Calmodulin-tag, FLAG-tag, HA-tag, Maltose binding protein-tag, Nus-tag, Glutathione-S-transferase-tag, Green fluorescent protein-tag, Thioredoxin-tag, S-tag, Softag 1, Softag 3, Strep-tag, SBP-tag, Ty-tag, and the like.

Isoprenoid transferases recognize an isosubstrate and/or a substrate. The term

isosubstrate refers to a substrate analog comprising a chemical modification. Isoprenoid transferases can alkylate a specific amino acid motif (for example, a CAAX motif) at the C-terminus of an antibody (*see, e.g.*, Duckworth, BP et al., ChemBioChem, 8:98 (2007); Uyen TT et al., ChemBioChem, 8:408 (2007); Labadie, GR et al., J. Org. Chem., 72(24):9291 (2007); Wollack, JW et al., ChemBioChem, 10:2934 (2009), each of which is hereby incorporated by reference). A functionalized antibody may be produced using an isoprenoid transferase and an isosubstrate, which may alkylate a C-terminal cysteine.

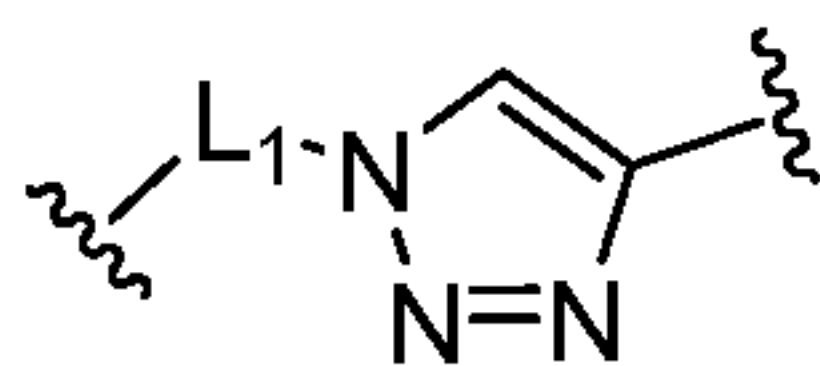
The isosubstrate may be, for example, the compound of Formula IV.



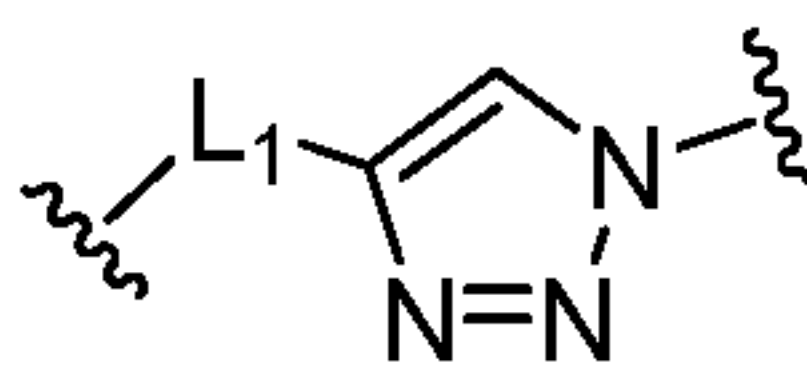
(IV)

The cysteine of a C-terminal CAAX motif may be bound to an isosubstrate using an isoprenoid transferase. In some embodiments, part of the motif, *e.g.*, AAX, may subsequently be removed by a protease, *e.g.*, leaving only the cysteine to which the isoprenoid is bound. The cysteine may optionally be methylated at the carboxyl terminus, *e.g.*, by an enzyme (*see, e.g.*, Bell, IM, J. Med. Chem., 47(8):1869 (2004), which is hereby incorporated by reference).

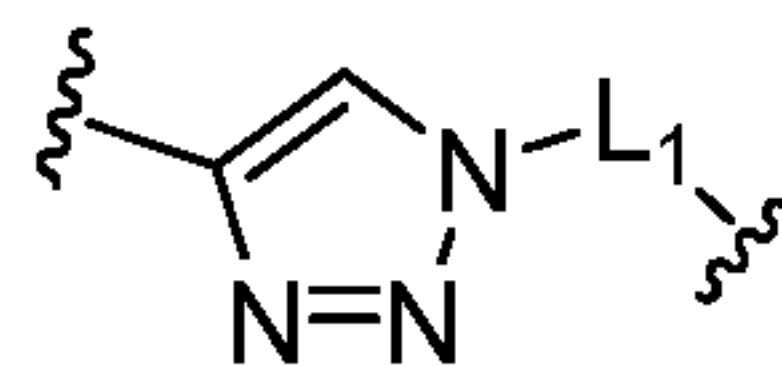
L and/or the linker may comprise a binding unit formed by a 1,3-dipolar cycloaddition reaction, hetero-Diels-Alder reaction, nucleophilic substitution reaction, non-aldol type carbonyl reaction, addition to a carbon-carbon multiple bond, oxidation reaction, or click reaction. A binding unit may be formed by a reaction between an acetylene and azide, or a non-aldol type carbonyl reaction, such as a reaction between an aldehyde or ketone group and hydrazine or alkoxyamine; such binding units may be represented by Formula (A), (B), (C), or (D).



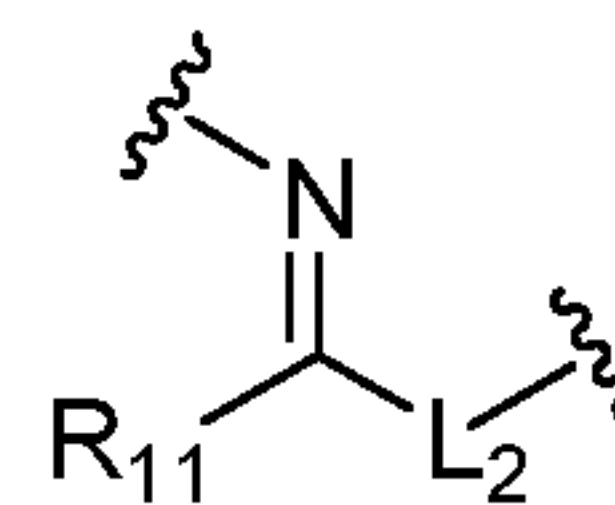
(A)



(B)



(C)



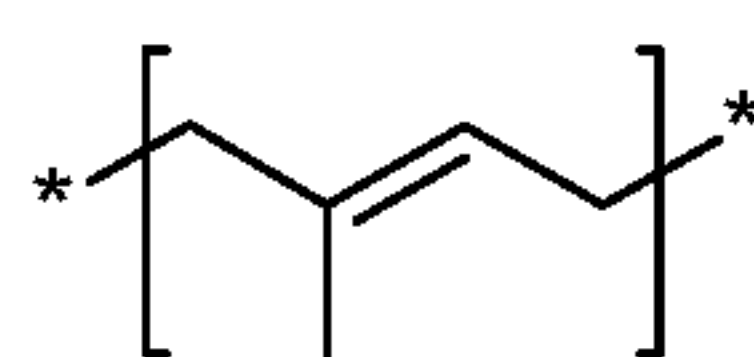
(D)

L<sub>1</sub> is a single bond or alkylene having 1 to 30 carbon atoms, preferably 10, 11, 12, 13, 14, 15, or 16 carbon atoms;

R<sub>11</sub> is hydrogen or an alkyl having 1 to 10 carbon atoms, preferably methyl; and

L<sub>2</sub> is an alkylene having 1 to 30 carbon atoms, preferably 10, 11, 12, 13, 14, 15, or 16 carbon atoms.

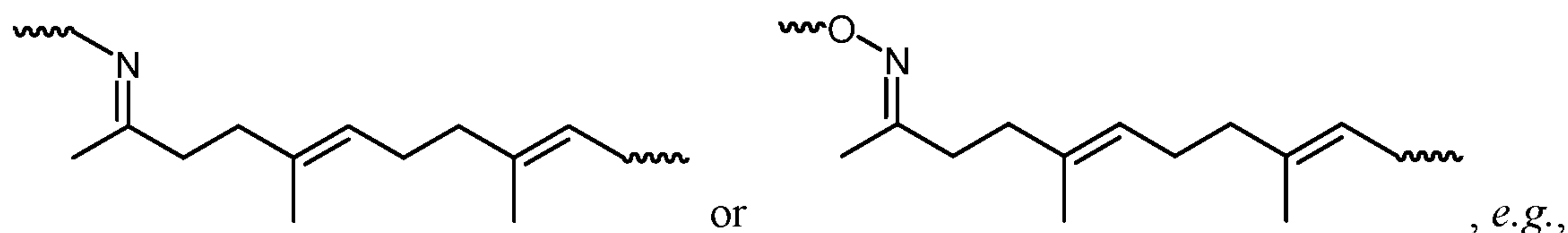
In some embodiments,  $L_1$  and/or  $L_2$  may comprise at least one isoprenyl unit, represented by Formula (III), preferably two isoprenyl units.  $L_2$  may consist of at least one isoprenyl unit, represented by Formula (III), preferably two isoprenyl units. In preferred embodiments, a carbon atom of an isoprenyl unit forms a thioether bond with the sulfur atom of a cysteine of the antibody, most preferably at a C-terminus of a heavy or light chain, thereby covalently linking the antibody and the linker.



(III)

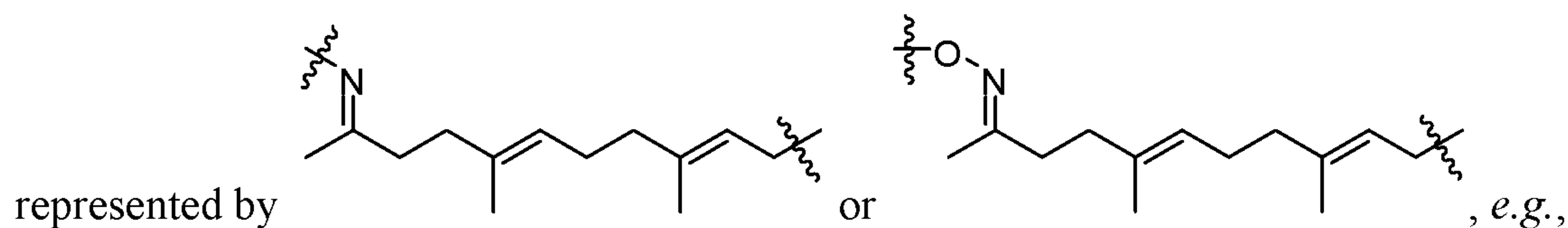
An antibody-drug conjugate may comprise the binding unit represented by Formula (D) *supra*, wherein  $L_2$  consists of at least one isoprenyl unit, preferably two isoprenyl units. The binding unit may be an O-substituted oxime, *i.e.*, the nitrogen of the binding unit may be covalently bound to a substituted oxygen. A carbon atom of an isoprenyl unit may form a thioether bond with the sulfur atom of a cysteine of the antibody, most preferably at a C-terminus of a heavy or light chain, thereby covalently linking the binding unit and the antibody.

$L$  and/or the linker may comprise an isoprenyl group represented by



wherein a carbon atom of the isoprenyl group forms a thioether bond with a sulfur atom of a cysteine of the antibody, thereby covalently linking the isoprenyl group and the antibody. The nitrogen of the isoprenyl group may covalently link the isoprenyl group to a polyethylene glycol unit of  $L$  and/or the linker.

In some embodiments,  $L$  and/or the linker may comprise an isoprenyl group



wherein a carbon atom of the isoprenyl group forms a thioether bond with a sulfur atom of a cysteine of the antibody, thereby covalently linking the isoprenyl group and the antibody. The nitrogen of the isoprenyl group may covalently link the isoprenyl group to a polyethylene glycol unit of  $L$  and/or the linker.

Click chemistry reactions may be carried out under mild conditions, which can be

performed in the presence of an antibody without denaturing the antibody. A click chemistry reaction shows high reaction specificity. Therefore, even though antibodies have various functional groups (for example, amines, carboxyls, carboxamides, and guanidiniums), a click chemistry reaction may be performed, for example, without affecting the amino acid side chains of the antibody. A click chemistry reaction between an azide group and an acetylene group, for example, may occur in the presence of an antibody without modifying the amino acid side chain functional groups of the antibody. Further, a click chemistry reaction may precisely target a specific functional group, such as functional groups rarely found in nature, regardless of the nature of the reactants. In some cases, the reactants are selected to improve overall reaction efficiency. For example, an azide-acetylene click chemistry reaction may produce triazole with a high yield (*see, e.g.*, Hia, RK et al., Chem. Rev., 109:5620 (2009); Meldal, M & Tornøe, CW, Chem Rev., 108:2952 (2008); Kolb, HC et al., Angew. Chemie Int. Ed. Engl., 40:2004 (2001), each of which is hereby incorporated by reference).

Azide and acetylene functional groups do not exist in natural proteins. Thus, none of the amino acid side chains, N-terminal amines, or C-terminal carboxyls should be affected by a click chemistry reaction that utilizes these functional groups.

The L moiety of Formula I and/or the linker may further include a connection unit represented by  $-(CH_2)_r(V(CH_2)_p)_q-$  or  $-(CH_2CH_2X)_w-$ , wherein

V is a single bond, -O-, -S-, -NR<sub>21</sub>-, -C(O)NR<sub>22</sub>-, -NR<sub>23</sub>C(O)-, -NR<sub>24</sub>SO<sub>2</sub>-, or -SO<sub>2</sub>NR<sub>25</sub>-, preferably -O-;

X is -O-, (C<sub>1</sub>-C<sub>8</sub>)alkylene, or -NR<sub>21</sub>-, preferably -O-;

R<sub>21</sub> to R<sub>25</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>6</sub>-C<sub>20</sub>)aryl, or (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>3</sub>-C<sub>20</sub>)heteroaryl, preferably hydrogen;

r is an integer from 1 to 10, preferably 2 or 3;

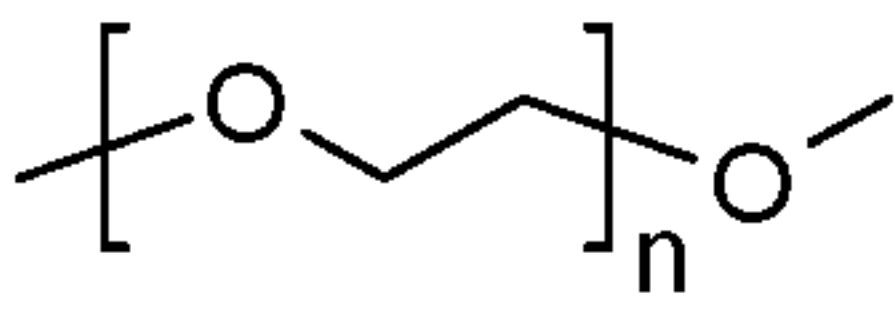
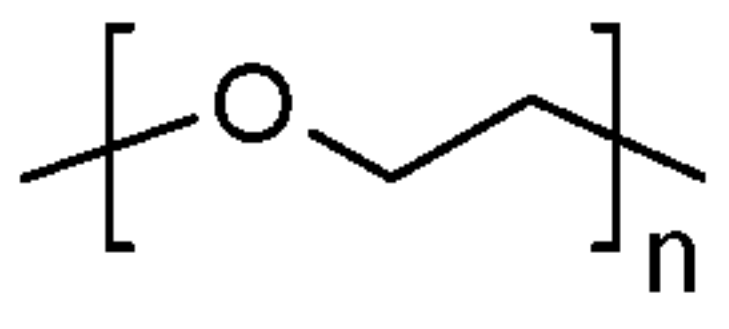
p is an integer from 0 to 12, preferably 1 or 2;

q is an integer from 1 to 20, preferably 4 to 20; and

w is an integer from 1 to 20, preferably 4 to 20.

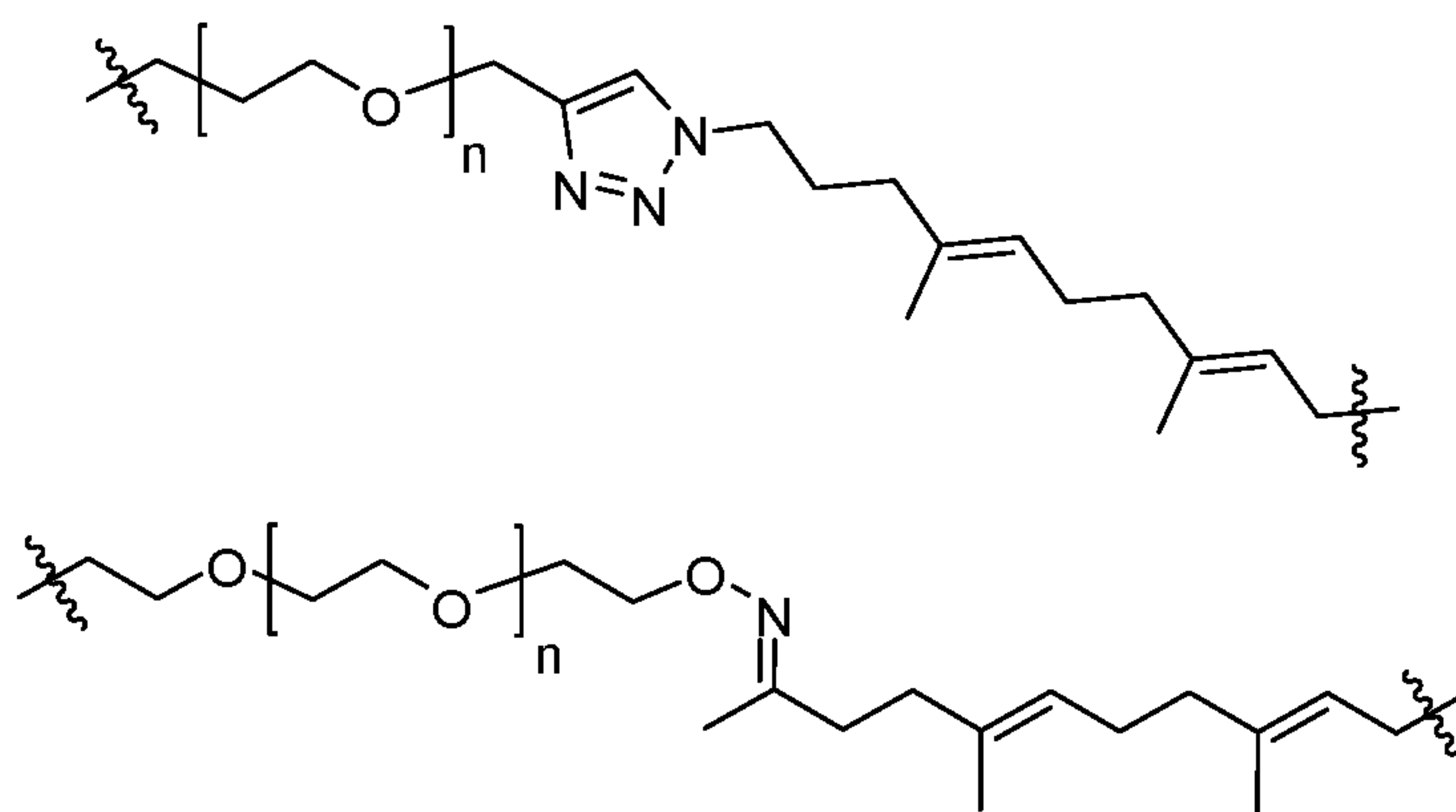
L and/or the linker preferably comprise the binding unit represented by Formula (A), (B), (C), or (D) and the connection unit represented by  $-(CH_2)_r(V(CH_2)_p)_q-$  or  $-(CH_2CH_2X)_w-$ .

In preferred embodiments, L and/or the linker comprise at least one polyethylene

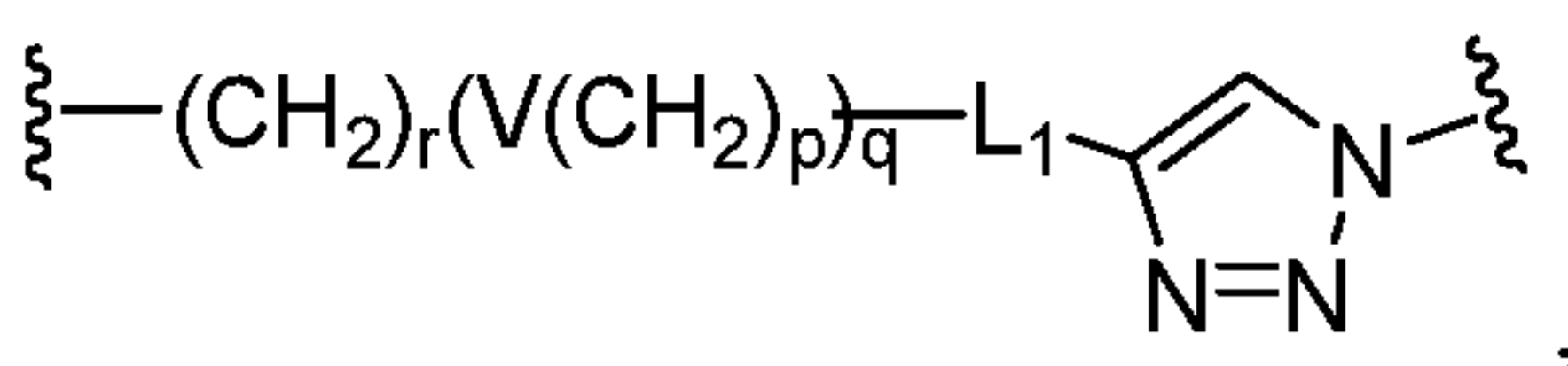
glycol unit represented by either  or . The antibody-drug conjugate may comprise from 1 to 20 -OCH<sub>2</sub>CH<sub>2</sub>- units, such as 1 to 12 -OCH<sub>2</sub>CH<sub>2</sub>- units, 5 to 12 -OCH<sub>2</sub>CH<sub>2</sub>- units, 6 to 12 -OCH<sub>2</sub>CH<sub>2</sub>- units, 5 to 20 -OCH<sub>2</sub>CH<sub>2</sub>- units, or 6 to 20 -OCH<sub>2</sub>CH<sub>2</sub>- units. In embodiments wherein L and/or the linker comprises an oxime, a polyethylene glycol unit preferentially covalently links the oxime to the peptide, *e.g.*, the N-terminus of the peptide, the C-terminus of the peptide, or a side chain of the peptide.

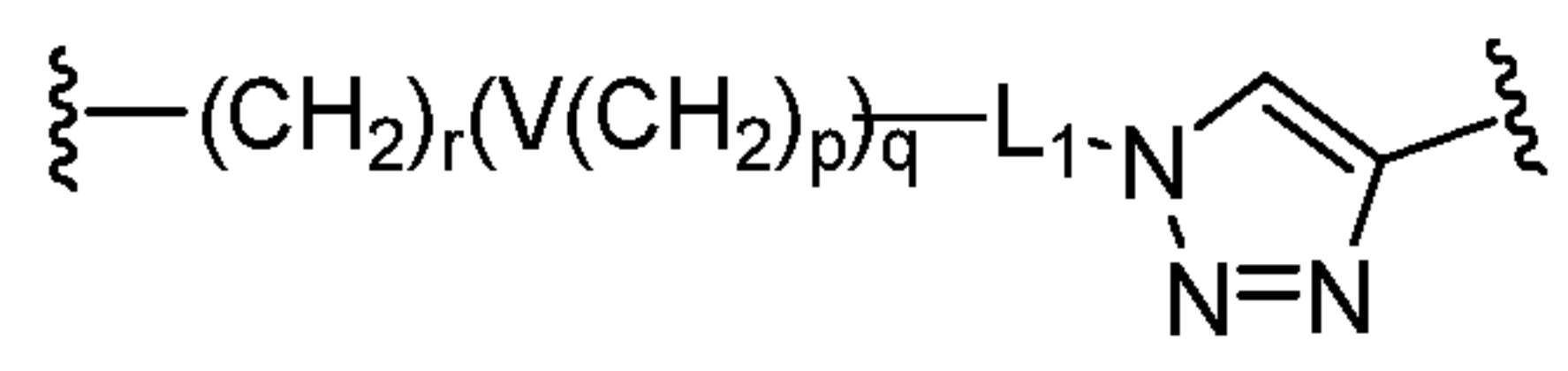
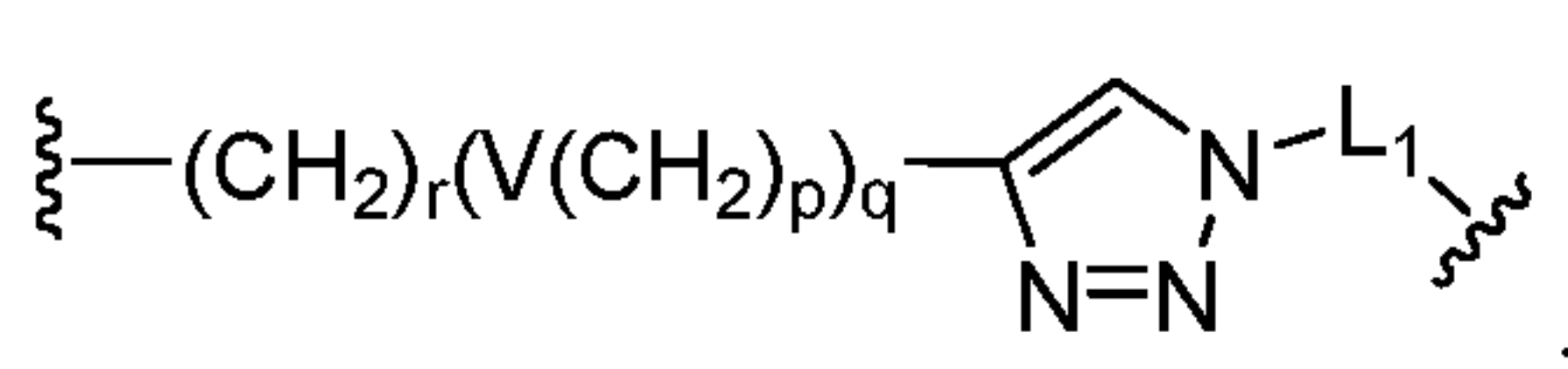
L and/or the linker preferably comprises a polyethylene glycol group represented by -(CH<sub>2</sub>CH<sub>2</sub>O)<sub>n</sub>-, wherein n is 1 to 20, such as 1 to 12, 5 to 12, 6 to 12, 5 to 20, or 6 to 20. In embodiments wherein L and/or the linker comprises an oxime, a polyethylene glycol group preferentially covalently links the oxime to the peptide.

In some embodiments, L and/or the linker preferably comprises one of the following two structures:



wherein n is an integer from 1 to 20, such as 4 to 20.

L and/or the linker may comprise ,

, or , wherein

V represents a single bond, -O-, -S-, -NR<sub>21</sub>-, -C(O)NR<sub>22</sub>-, -NR<sub>23</sub>C(O)-, -NR<sub>24</sub>SO<sub>2</sub>-, or -SO<sub>2</sub>NR<sub>25</sub>-, preferably -O-;

R<sub>21</sub> to R<sub>25</sub> represents each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>6</sub>-C<sub>20</sub>)aryl, or (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>3</sub>-C<sub>20</sub>)heteroaryl;

r is an integer from 1 to 10, preferably 2 or 3;

p is an integer from 0 to 10, preferably 1 or 2;

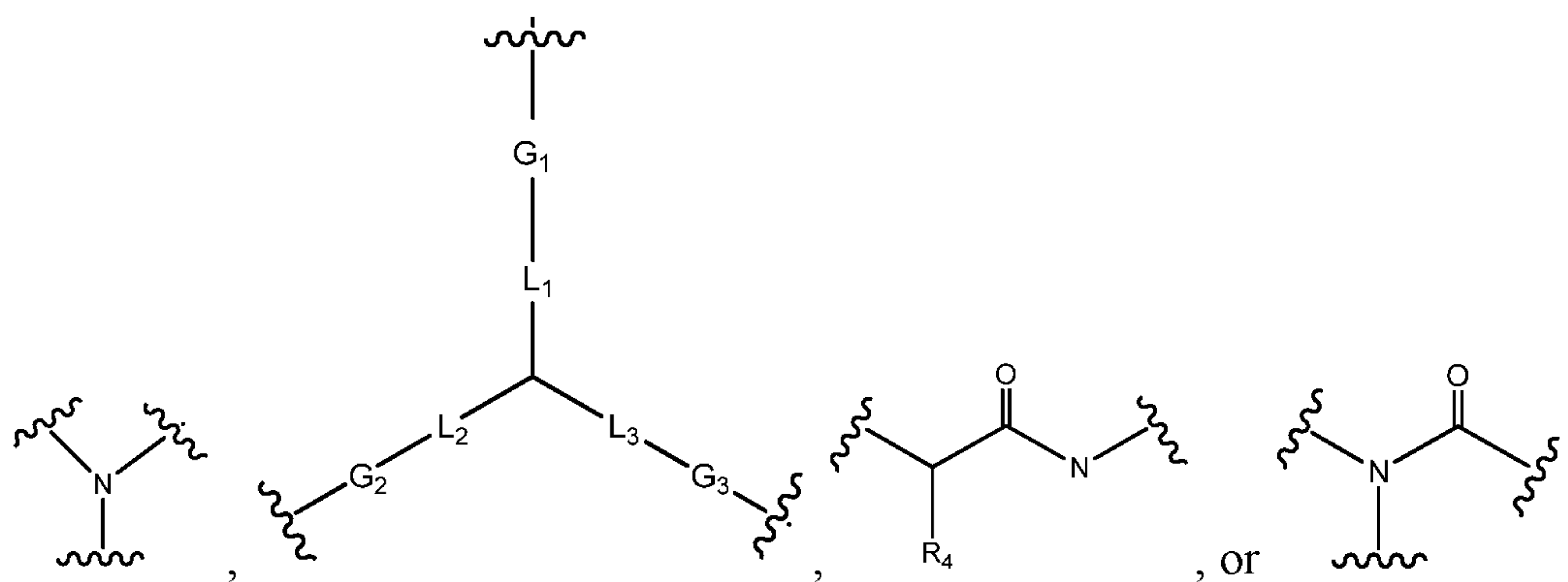
q is an integer from 1 to 20, preferably 1 to 6; and

$L_1$  is a single bond.

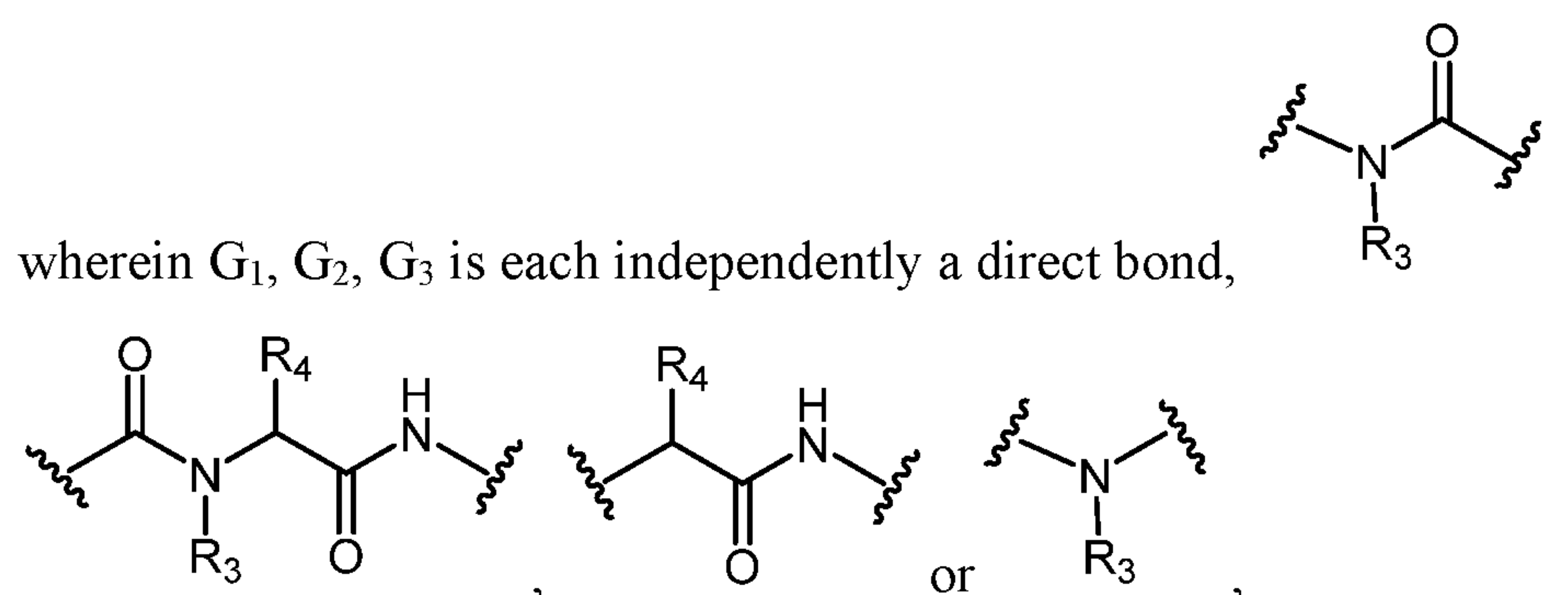
In some embodiments, at least two active agents are covalently coupled to side chains of the amino acids through one or more linkers, optionally including a cleavage group, as disclosed herein, e.g., that is capable of releasing the active agent from the conjugate in a desired location. Any or all of the linkers may be a branched linker, e.g., wherein each branch of the linker terminates in an active agent (e.g., linked through a cleavage group), or wherein one or more branches of the linker terminate in an active agent (e.g., linked through a cleavage group) and one or more others comprise a polyethylene glycol moiety and do not include an active agent. In some such embodiments, each branched linker is coupled to at least two active agents, which may be the same or different. In some embodiments, at least two, three, or four branched linkers are covalently coupled to the peptide. In other embodiments, exactly one branched linker is coupled to the peptide.

A branched linker may comprise a branching unit, such that each active agent is coupled to the branching unit through a secondary linker and the branching unit is coupled to the peptide by a primary linker.

A branching unit can have any suitable structure, such as:



wherein  $L_1$ ,  $L_2$ ,  $L_3$  is each independently a direct bond or  $-C_nH_{2n}-$  where  $n$  is a integer of 1 to 30,

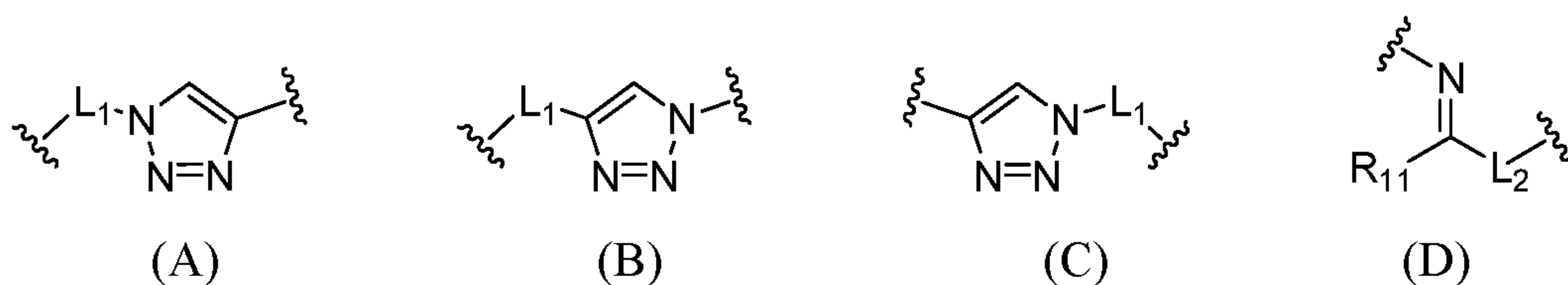


wherein  $G_1$ ,  $G_2$ ,  $G_3$  is each independently a direct bond,

wherein  $R_3$  is hydrogen or  $C_1$ - $C_{30}$  alkyl;

wherein  $R_4$  is hydrogen or  $-L_4-COOR_5$ , wherein  $L_4$  is a direct bond or  $-C_nH_{2n}-$  wherein  $n$  is a integer of 1 to 10, and  $R_5$  is hydrogen or  $C_1-C_{30}$  alkyl.

A secondary linker (e.g., linking an active agent to the peptide) may comprise a binding unit formed by a 1,3-dipolar cycloaddition reaction, hetero-Diels-Alder reaction, nucleophilic substitution reaction, non-aldol type carbonyl reaction, addition to a carbon-carbon multiple bond, oxidation reaction, or click reaction. A binding unit may be formed by a reaction between an acetylene and azide, or a non-aldol type carbonyl reaction, such as a reaction between an aldehyde or ketone group and hydrazine or alkoxyamine, reactions which allow for mild coupling of active agents and/or cleavage groups to the peptide sequence. Such binding units may be represented by Formula (A), (B), (C), or (D).

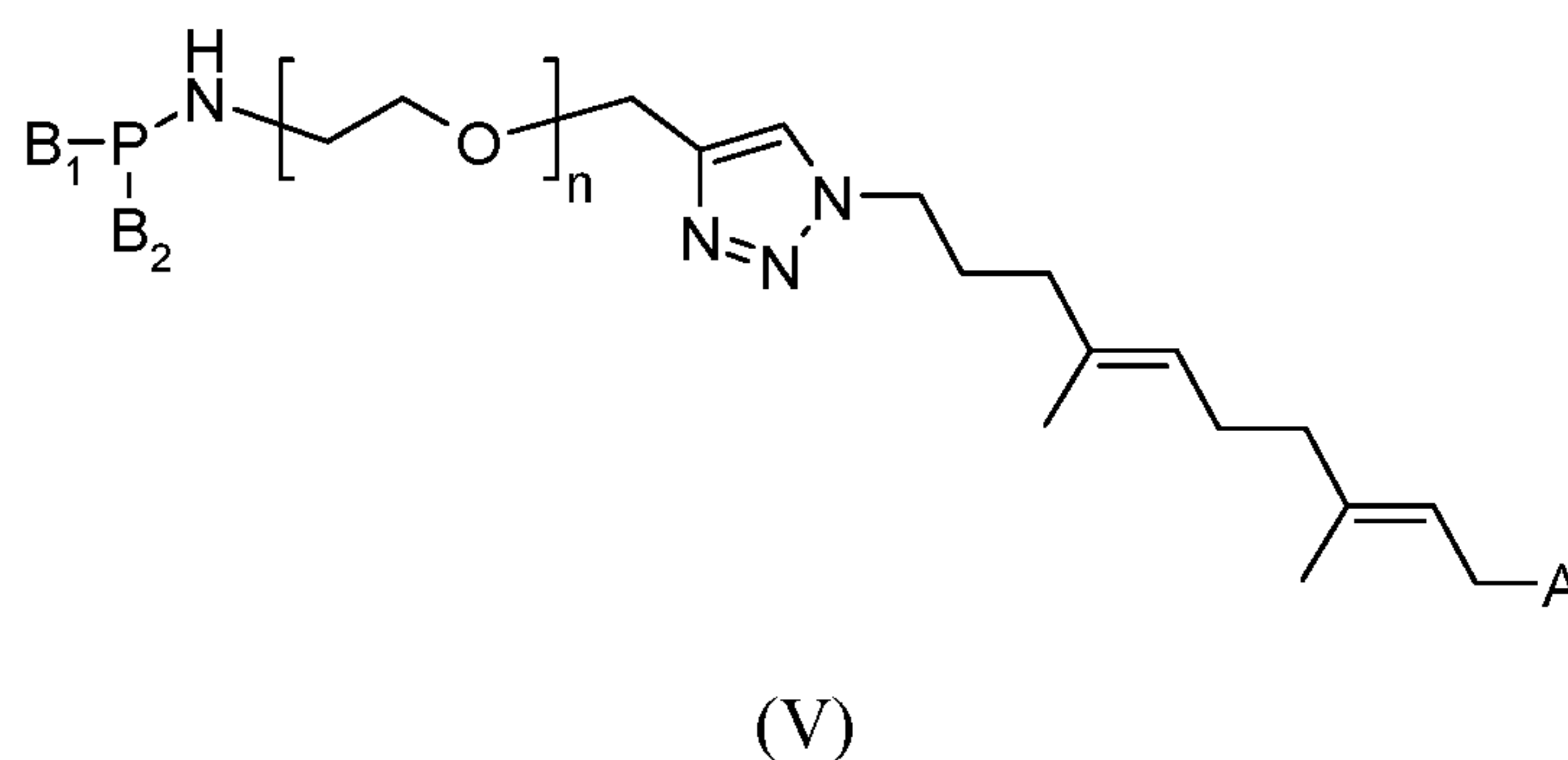


$L_1$  is a single bond or alkylene having 1 to 30 carbon atoms, preferably 10, 11, 12, 13, 14, 15, or 16 carbon atoms;

$R_{11}$  is hydrogen or an alkyl having 1 to 10 carbon atoms, preferably methyl; and

$L_2$  is an alkylene having 1 to 30 carbon atoms, preferably 10, 11, 12, 13, 14, 15, or 16 carbon atoms.

In some embodiments, the antibody-drug conjugate comprises the structure of Formula (V):



wherein:

A represents the antibody;

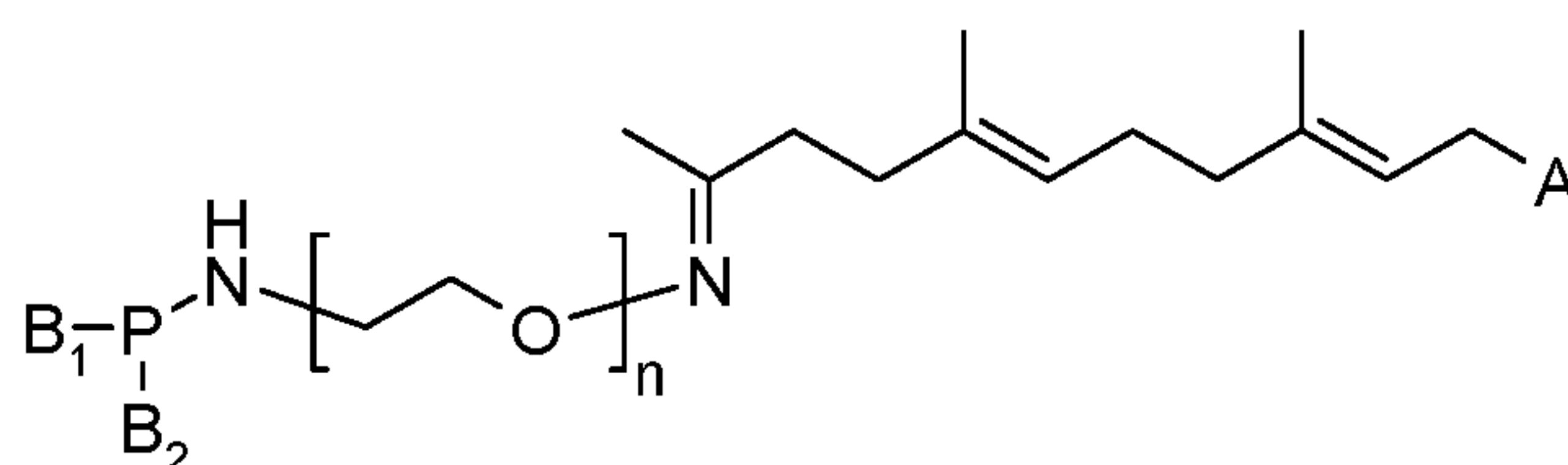
P represents the peptide;

$B_1$  represents a first active agent;

$B_2$  represents a second active agent; and

n is an integer from 1 to 20.

In some embodiments, the antibody-drug conjugate comprises the structure of Formula (VI):



(VI)

wherein:

A represents the antibody;

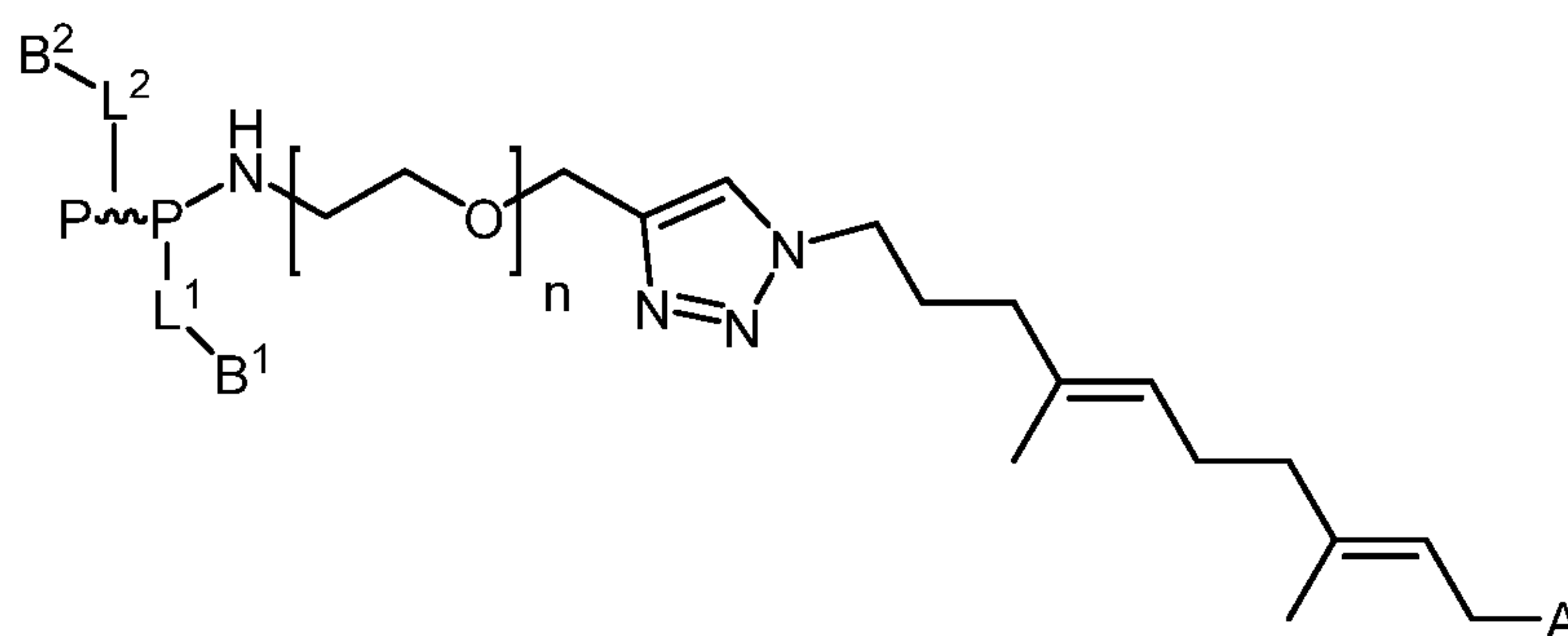
P represents the peptide;

B<sub>1</sub> represents a first active agent;

B<sub>2</sub> represents a second active agent; and

n is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

A represents the ligand;

P represents the peptide;

B<sub>1</sub> represents a first active agent;

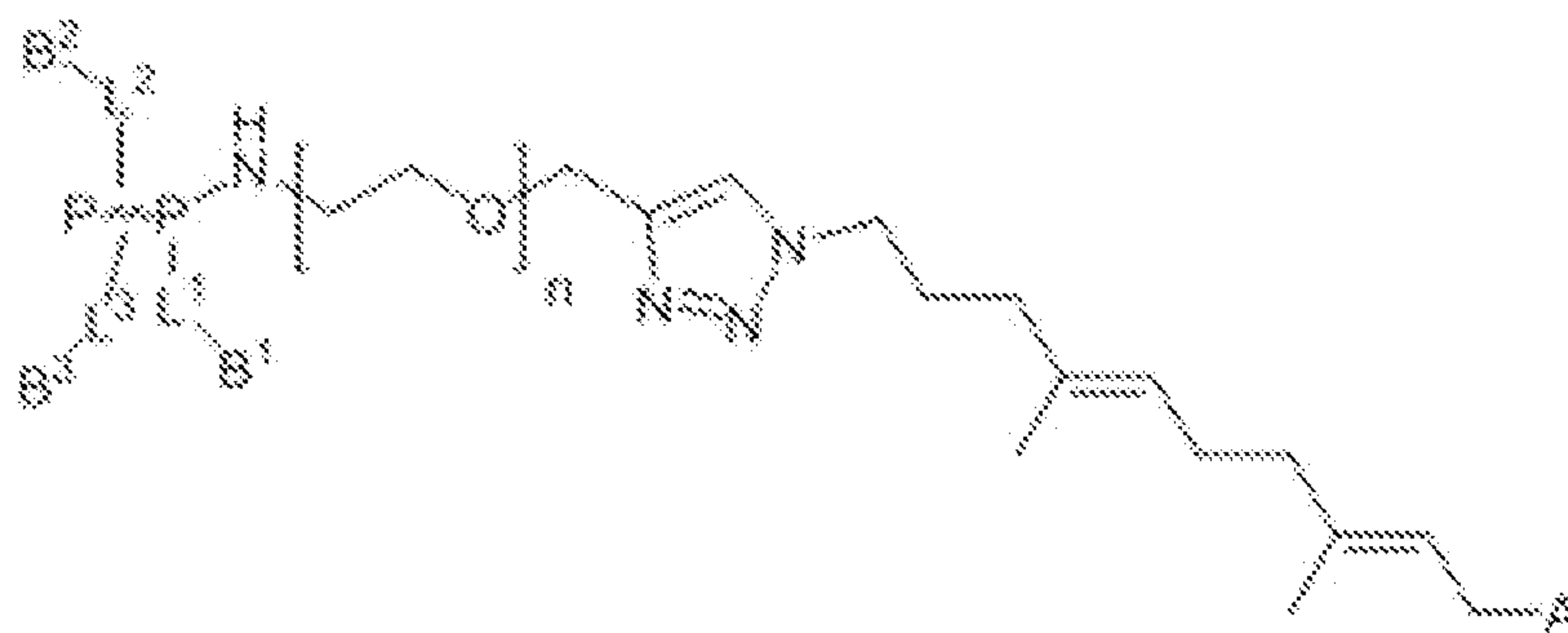
B<sub>2</sub> represents a second active agent;

L<sub>1</sub> represents a linker, optionally including a cleavage group;

L<sub>2</sub> represents a linker, optionally including a cleavage group; and

n is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

A represents the ligand;

P represents the peptide;

B<sub>1</sub> represents a first active agent;

B<sub>2</sub> represents a second active agent;

B<sub>3</sub> represents a third active agent;

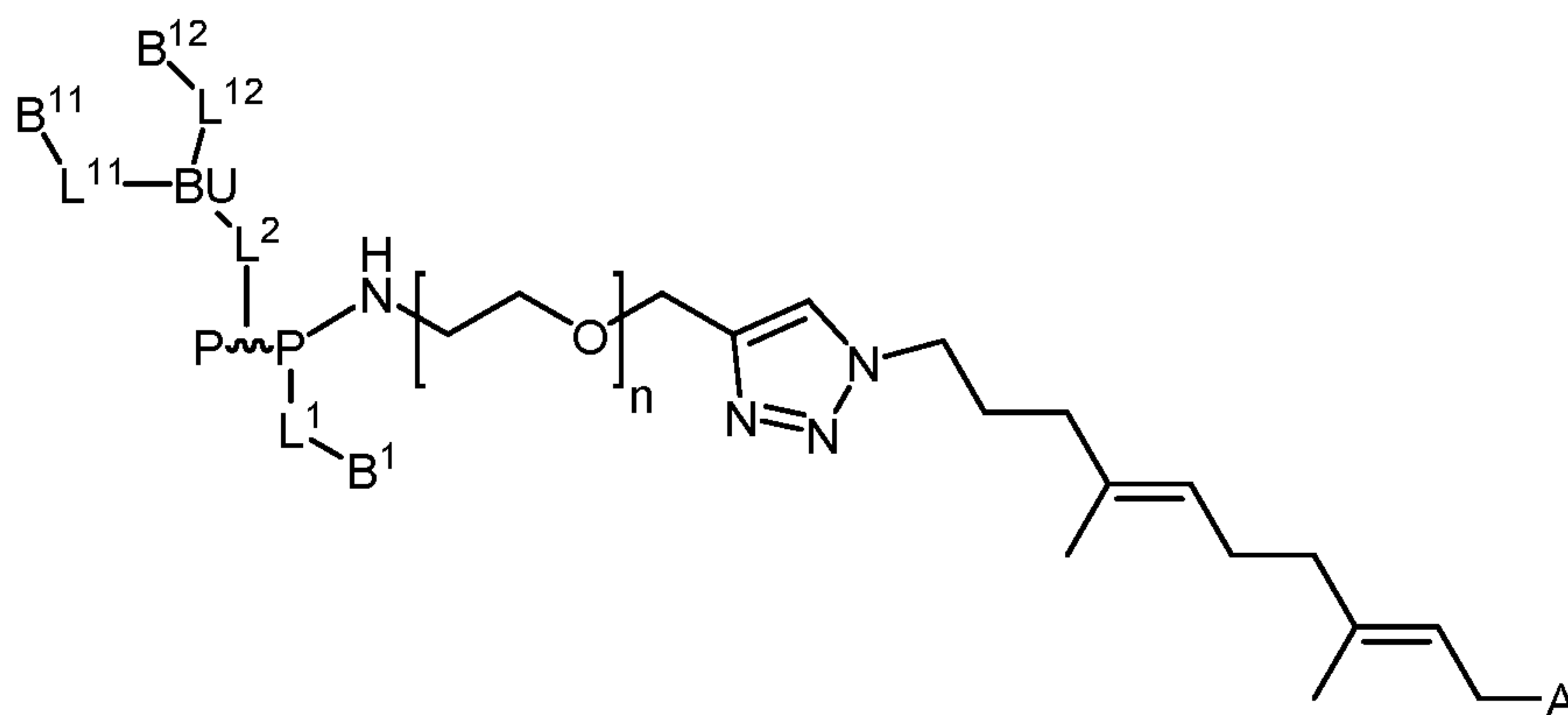
L<sub>1</sub> represents a linker, optionally including a cleavage group;

L<sub>2</sub> represents a linker, optionally including a cleavage group;

L<sub>3</sub> represents a linker, optionally including a cleavage group; and

n is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

A represents the ligand;

P represents the peptide;

B<sub>1</sub> represents a first active agent;

B<sub>11</sub> represents a second active agent;

B<sub>12</sub> represents a third active agent;

L<sub>1</sub> represents a linker;

$L_2$  represents a linker;

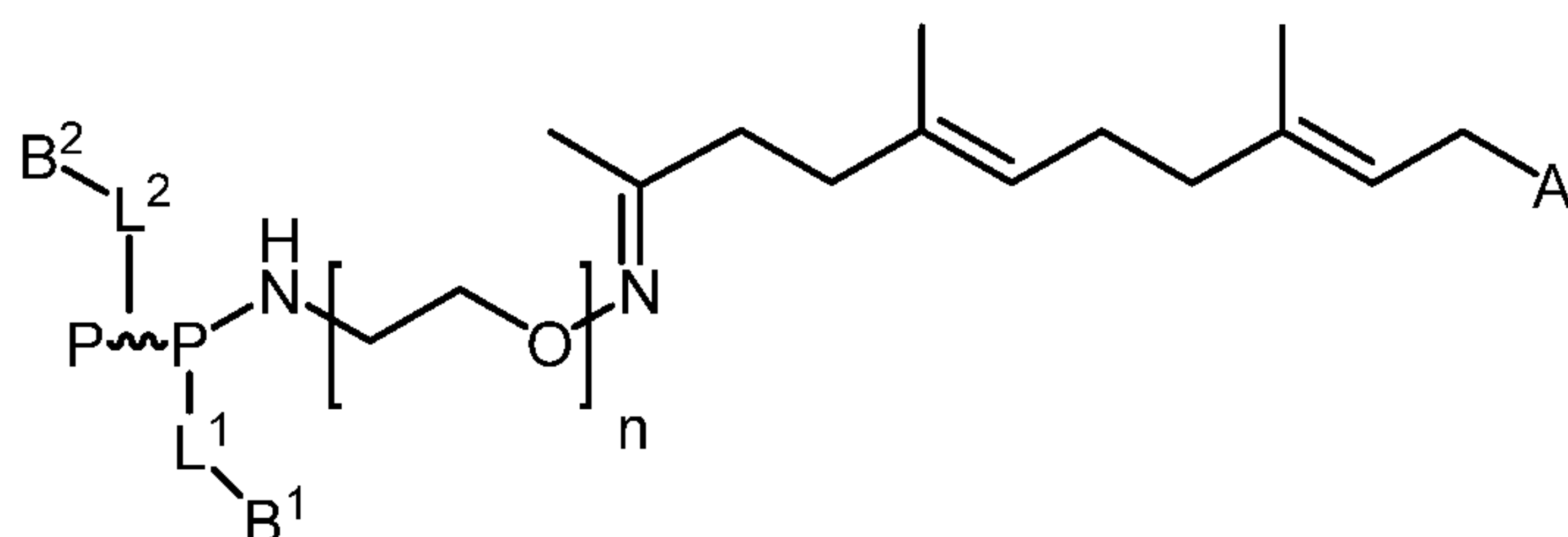
$L_{11}$  represents a secondary linker, optionally including a cleavage group;

$L_{12}$  represents a secondary linker, optionally including a cleavage group;

BU represents a branching unit; and

$n$  is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

$A$  represents the ligand;

$P \sim P$  represents the peptide;

$B_1$  represents a first active agent;

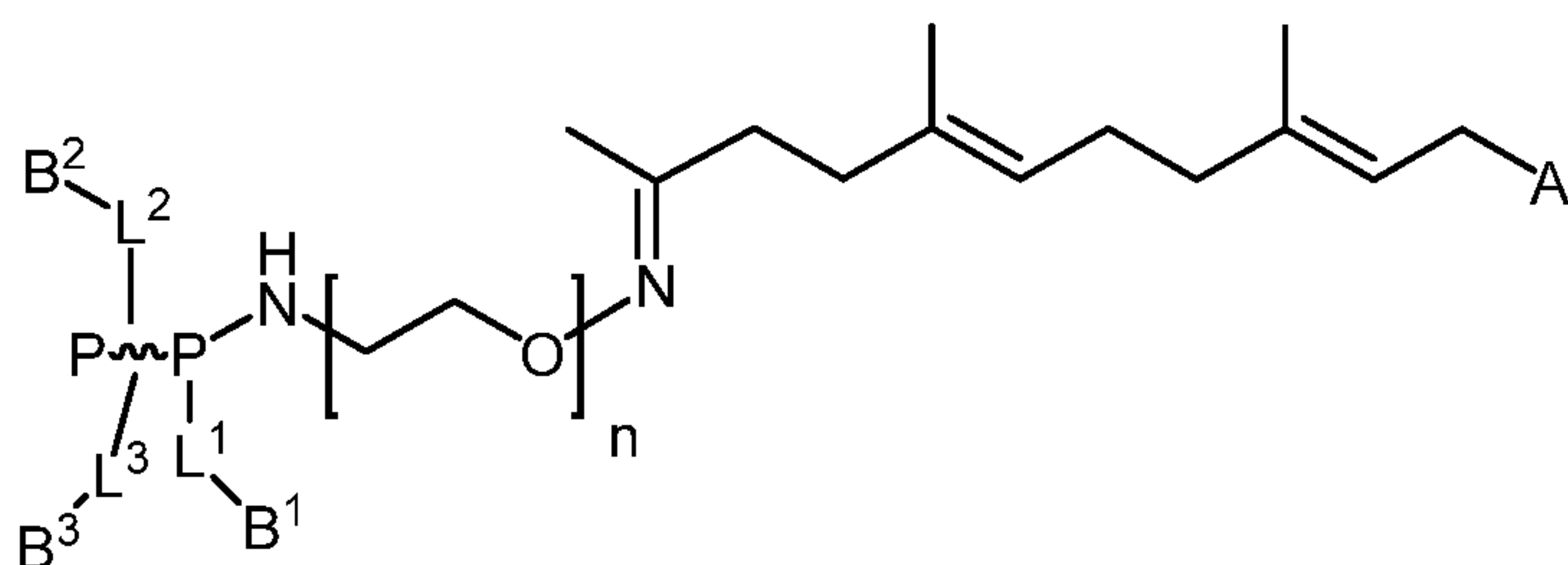
$B_2$  represents a second active agent;

$L_1$  represents a linker, optionally including a cleavage group;

$L_2$  represents a linker, optionally including a cleavage group; and

$n$  is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

$A$  represents the ligand;

$P \sim P$  represents the peptide;

$B_1$  represents a first active agent;

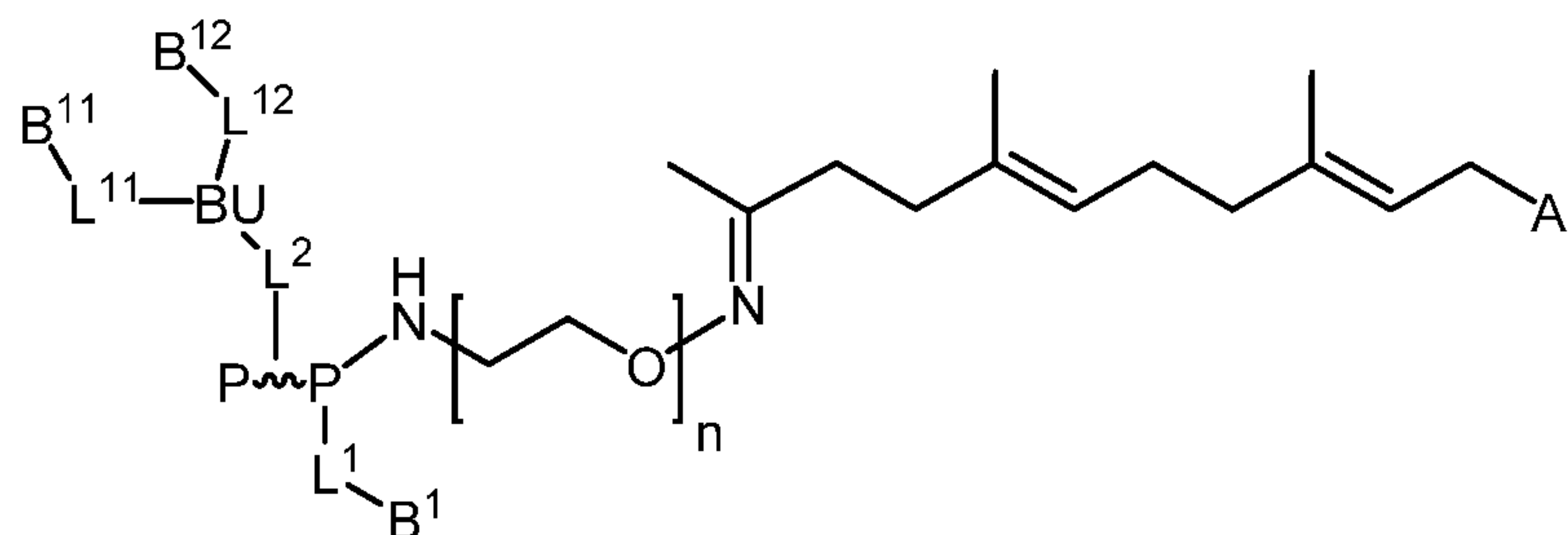
$B_2$  represents a second active agent;

$B_3$  represents a third active agent;

$L_1$  represents a linker, optionally including a cleavage group;

$L_2$  represents a linker, optionally including a cleavage group;  
 $L_3$  represents a linker, optionally including a cleavage group; and  
 $n$  is an integer from 1 to 20.

In some embodiments, the conjugate comprises a structure of the formula:



wherein

$A$  represents the ligand;

$P \sim P$  represents the peptide;

$B_1$  represents a first active agent;

$B_{11}$  represents a second active agent;

$B_{12}$  represents a third active agent;

$L_1$  represents a linker;

$L_2$  represents a linker;

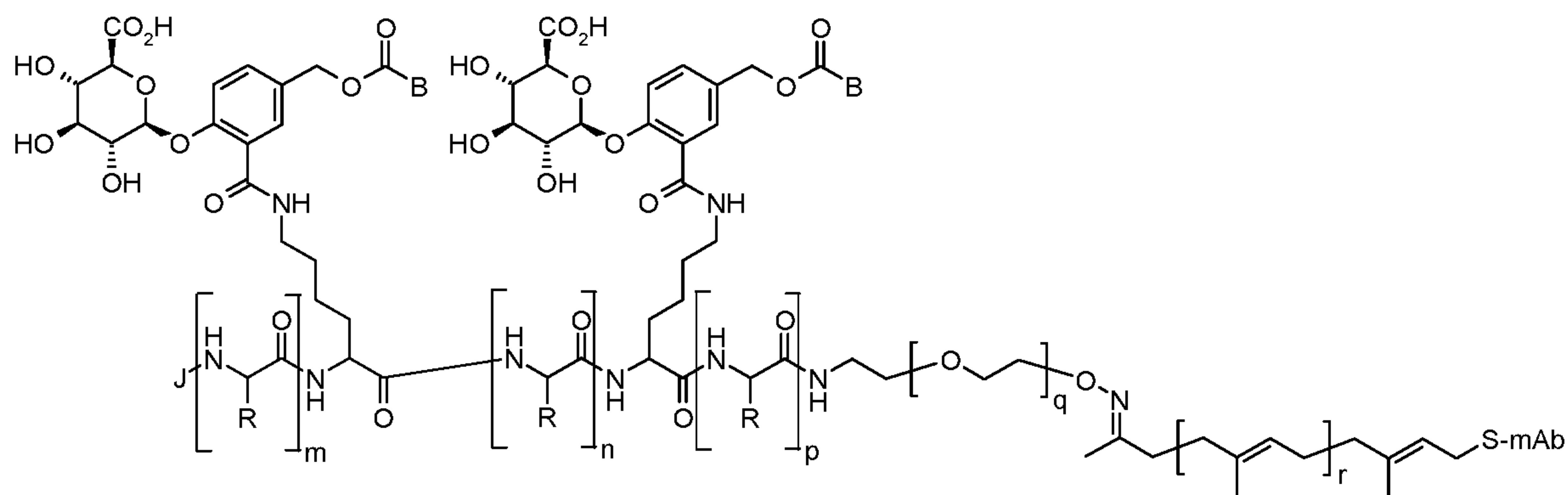
$L_{11}$  represents a secondary linker, optionally including a cleavage group;

$L_{12}$  represents a secondary linker, optionally including a cleavage group;

$BU$  represents a branching unit; and

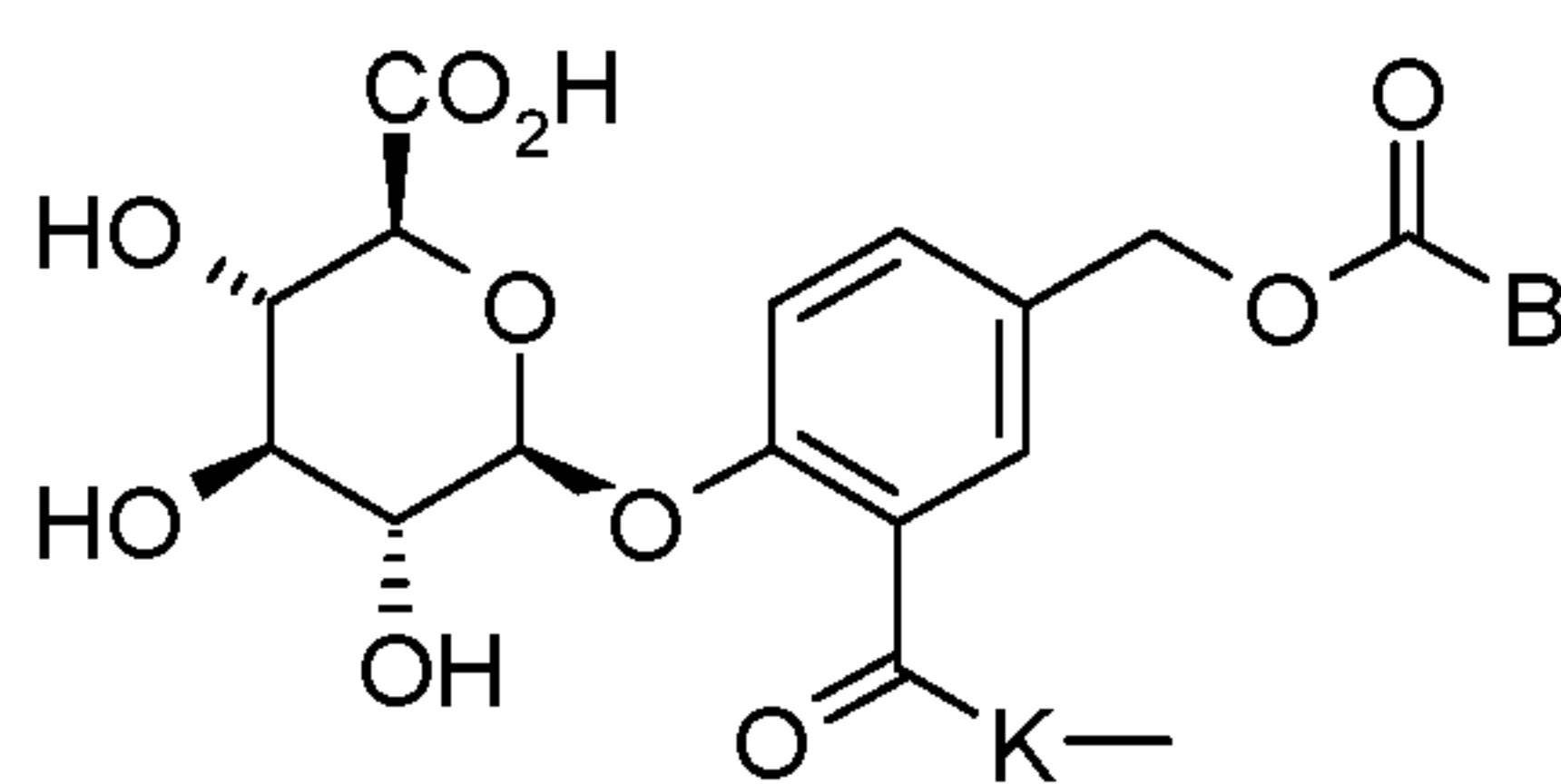
$n$  is an integer from 1 to 20.

In some embodiments, the antibody-drug conjugate has a structure of Formula (VII) or a pharmaceutically acceptable salt thereof:



(VII)

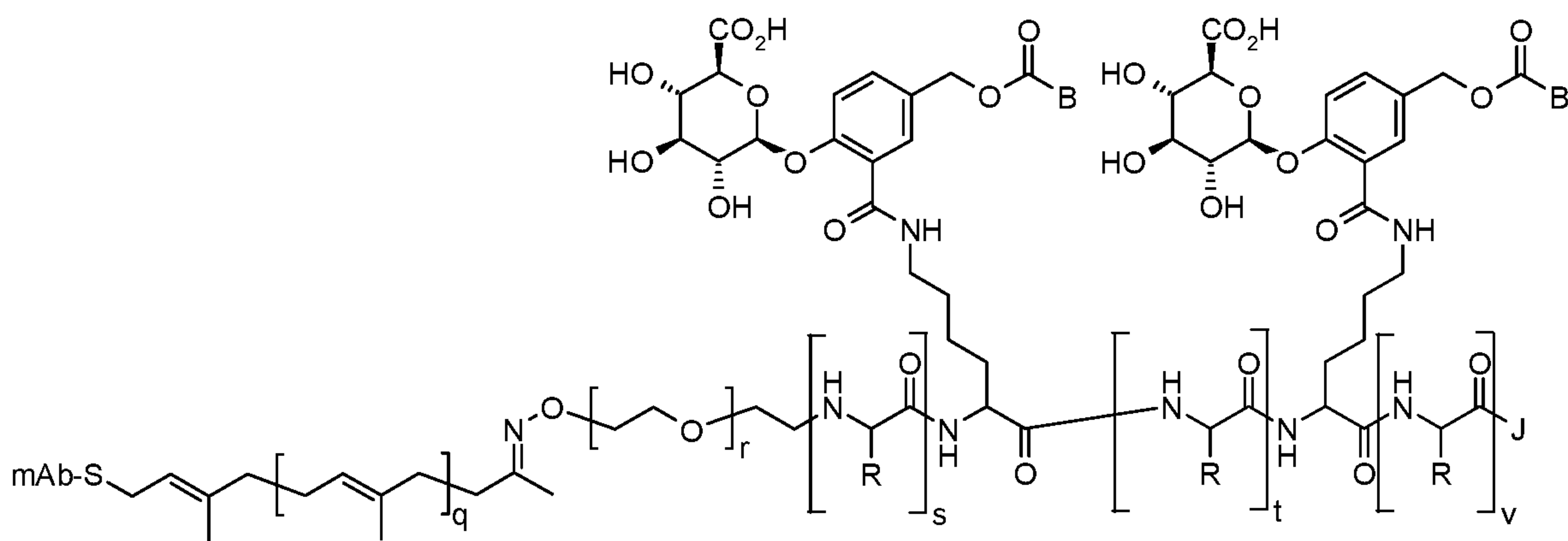
wherein B represents an active agent; mAb represents the antibody; R represents an amino acid side chain; J represents a proton, an acyl group, or a group comprising an active agent; m is an integer from 0 to 10; n is an integer from 0 to 10; p is an integer from 0 to 10; q is an integer from 1 to 20, preferably 4 to 20; and r is an integer from 0 to 10, preferably 1. Each B may be the same active agent or each B may be a different active agent. Similarly, each R may be the same amino acid side chain or each R may be a different amino acid side chain. One or more R's may covalently link one or more additional active agents to the peptide. Each R group may be the R group of an L-amino acid or a D-amino acid. Formula (VII) depicts that each amino acid of the peptide is an  $\alpha$ -amino acid. Nevertheless, the peptide may comprise one or more  $\beta$ -amino acids. The structure shows that each linking amino acid is an N-substituted lysine. Nevertheless, an antibody-drug conjugate may comprise other linking amino acids instead of or in addition to lysine. In some embodiments, J comprises a moiety with a structure of Formula (VIII):



(VIII)

wherein K may be a single bond that links the structure of Formula (VIII) to the N-terminal amide of the peptide, or K is  $-N(H)(CH_2CH_2O)_k-$ , wherein k is an integer from 1 to 20, preferably from 1 to 10.

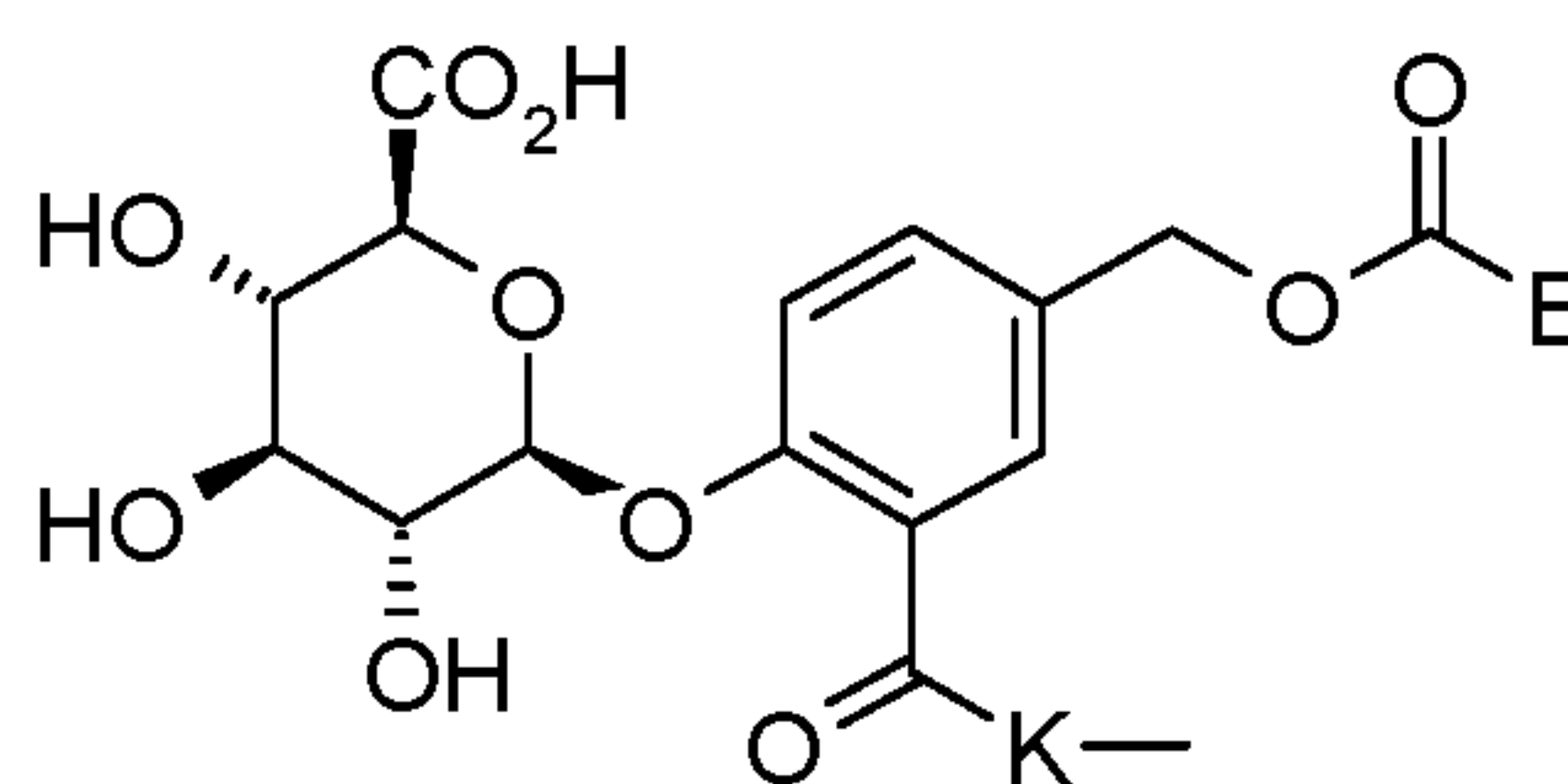
In some embodiments, the antibody-drug conjugate has a structure of Formula (IX):



(IX)

wherein B represents an active agent; mAb represents the antibody; R represents an amino

acid side chain; J represents a hydroxyl, an amide group, an amine, or a group comprising an active agent; q is an integer from 0 to 10, preferably 1; r is an integer from 0 to 20, preferably 3-20; s is an integer from 0 to 10; t is an integer from 0 to 10; and v is an integer from 0 to 10. Each B may be the same active agent or each B may be a different active agent. Similarly, each R may be the same amino acid side chain or each R may be a different amino acid side chain. One or more R's may covalently link one or more additional active agents to the peptide. Each R may be the R group of an L-amino acid or a D-amino acid. Formula (IX) depicts that each amino acid of the peptide is an  $\alpha$ -amino acid. Nevertheless, the peptide may comprise one or more  $\beta$ -amino acids. The structure shows that each linking amino acid is an N-substituted lysine. Nevertheless, an antibody-drug conjugate may comprise other linking amino acids instead of or in addition to lysine. In some embodiments, J comprises a moiety with a structure of Formula (VIII):



(VIII)

wherein K may be  $-\text{N}(\text{H})(\text{CH}_2\text{CH}_2\text{O})_k(\text{CH}_2)_m\text{N}(\text{H})-$ , wherein k is an integer from 1 to 20 and m is an integer from 0 to 5.

The antibody-drug conjugates of the invention may be prepared using any method known in the art, including molecular biology and cell biology methods. For example, transient or stable transfection methods may be used. Genetic sequences encoding a specific amino acid motif capable of being recognized by an isoprenoid transferase may be inserted into a known plasmid vector using standard PCR and/or ligation technologies so as to express an antibody having the specific amino acid motif at a C-terminus thereof. An antibody having at least one amino acid motif capable of being recognized by the isoprenoid transferase may thus be expressed in a suitable host, *e.g.*, a CHO cell or in *E. coli*.

The term “antibody” refers to an immunoglobulin molecule that recognizes and specifically binds to a different molecule through at least one antigen recognition site within a variable region of the immunoglobulin molecule. As used herein, the term “antibody” includes intact polyclonal antibodies, intact monoclonal antibodies, antibody fragments (for example, Fab, Fab', F(ab')<sub>2</sub>, Fd, and Fv fragments), single chain Fv (scFv)

mutants, multispecific antibodies such as bispecific antibodies generated from two or more intact antibodies, chimeric antibodies, humanized antibodies, human antibodies, fusion proteins including an antigen determination portion of an antibody, and any other modified immunoglobulin molecule including an antigen recognition site. The antibody may be any of the five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, or subclasses (isotypes) thereof (for example, IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2), based on the identity of its heavy chain constant domains, referred to as alpha, delta, epsilon, gamma, and mu, respectively. The different classes of immunoglobulins have different and well-known subunit structures and three-dimensional configurations. The term “antibody” does not refer to molecules that do not share homology with an immunoglobulin sequence. For example, the term “antibody” as used herein does not include “repebodies”.

The term “antibody fragment” refers to a portion of an intact antibody and refers to antigenic determining variable regions of an intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')<sub>2</sub>, Fd, and Fv fragments, linear antibodies, single chain antibodies, and multispecific antibodies formed from antibody fragments.

The term “monoclonal antibody” refers to a homogeneous antibody population involved in the highly specific recognition and binding of a single antigenic determinant or epitope. This contrasts with polyclonal antibodies that typically include different antibodies directed against a variety of different antigenic determinants. The term “monoclonal antibody” includes antibody fragments (such as Fab, Fab', F(ab')<sub>2</sub>, Fd, Fv), single chain (scFv) mutants, fusion proteins including an antibody portion, and any other modified immunoglobulin molecule including an antigen recognition site as well as both intact and full-length monoclonal antibodies, but are not limited thereto. Additionally, “monoclonal antibody” refers to such antibodies made in any number of methods, including but not limited to hybridoma, phage selection, recombinant expression, and transgenic animals.

The term “humanized antibody” refers to forms of non-human (*e.g.*, murine) antibodies that are specific immunoglobulin chains, chimeric immunoglobulins, or fragments thereof that contain minimal non-human (*e.g.*, murine) sequences. In general, humanized antibodies are human immunoglobulins in which residues from complementary determining region (CDR) are replaced by residues from CDR of a non-human species (*e.g.*, mouse, rat, rabbit, and hamster) having the desired specificity, affinity, and capability (*see, e.g.*, Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 (1988)). In some instances, Fv

framework region (FR) residues of a human immunoglobulin are replaced with the corresponding residues in an antibody from a non-human species having a desired specificity, affinity, and/or binding capability. The humanized antibody may be further modified by the substitution of additional residues either in the Fv framework region and/or within the replaced non-human residues to refine and optimize antibody specificity, affinity, and/or binding capability. In general, a humanized antibody includes substantially all of at least one, and typically two or three, variable domains containing all or substantially all of the CDRs that correspond to the non-human immunoglobulin whereas all or substantially all of the framework regions (FRs) have those of a human immunoglobulin consensus sequence. The humanized antibody may also include at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Examples of methods used to generate humanized antibodies are described in U.S. Patent No. 5,225,539, hereby incorporated by reference.

The term “human antibody” as used herein refers to an antibody encoded by a human nucleotide sequence or an antibody having an amino acid sequence corresponding to an antibody produced by a human using any technique known in the art. This definition of the human antibody includes intact full-length antibodies and/or fragments thereof.

The term “chimeric antibody” refers to an antibody wherein an amino acid sequence of an immunoglobulin molecule is derived from two or more species, one of which is preferably human. In general, variable regions of both light and heavy chains correspond to variable regions of antibodies derived from one species of mammals (*e.g.*, mouse, rat, rabbit, etc.) with the desired specificity, affinity, and capability, while constant regions are homologous to the sequences in antibodies derived from another species (usually human), *e.g.*, to avoid eliciting an immune response in that species.

The terms “epitope” and “antigenic determinant” are used interchangeably herein and refer to that portion of an antigen capable of being recognized and specifically bound by a particular antibody. When the antigen is or comprises a polypeptide or protein, epitopes may be formed from contiguous and/or non-contiguous amino acids, *e.g.*, juxtaposed by secondary, tertiary, and/or quaternary folding of a protein. Epitopes formed from contiguous amino acids are typically retained upon protein denaturing, whereas epitopes formed by tertiary folding may be lost upon protein denaturing. An epitope typically includes 3 or more, 5 or more, or 8 to 10 or more amino acids in a unique spatial conformation.

An antibody “specifically binds” to an epitope or antigenic molecule, which means that the antibody interacts or associates more frequently, more rapidly, with greater duration, with greater affinity, or with some combination of the foregoing to an epitope or antigenic molecule than alternative substances, including unrelated proteins. In specific embodiments, “specifically binds” means, for instance, that an antibody binds to a protein with a  $K_D$  of about 0.1 mM or less, but more usually, less than about 1  $\mu$ M. In specific embodiments, “specifically binds” means that an antibody binds to a protein at times with a  $K_D$  of about 0.1  $\mu$ M or less, and at other times, with a  $K_D$  of about 0.01  $\mu$ M or less. Because of the sequence identity between homologous proteins in different species, specific binding may include an antibody recognizing a particular protein in more than one species. It is understood that an antibody or binding residue that specifically binds to a first target may or may not specifically bind to a second target. As described above, “specific binding” does not necessarily require (although it may include) exclusive binding, that is, binding to a single target. Generally, but not necessarily, the term binding used herein means specific binding.

The antibodies, including fragments/derivatives thereof and monoclonal antibodies, may be obtained using methods known in the art (*see, e.g.*, McCafferty et al., *Nature* 348:552-554 (1990); Clackson et al., *Nature* 352:624-628; Marks et al., *J. Mol. Biol.* 222:581-597 (1991); Marks et al., *Bio/Technology* 10:779-783 (1992); Waterhouse et al., *Nucleic Acids Res.* 21:2265-2266 (1993); Morimoto et al., *J Biochemical & Biophysical Methods* 24:107-117 (1992); Brennan et al., *Science* 229:81(1985); Carter et al., *Bio/Technology* 10:163-167 (1992); Kohler et al., *Nature* 256:495 (1975); Kilpatrick et al., *Hybridoma* 16(4):381-389 (1997); Wring et al., *J. Pharm. Biomed. Anal.* 19(5):695-707 (1999); Bynum et al., *Hybridoma* 18(5):407-411 (1999); Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggemann et al., *Year Immuno.* 7:33 (1993); Barbas et al., *Proc. Nat. Acad. Sci. USA* 91:3809-3813 (1994); Schier et al., *Gene* 169:147-155 (1995); Yelton et al., *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); Hawkins et al., *J. Mol. Biol.* 226:889-896 (1992), U.S. Pat. Nos. 4,816,567, 5,514,548, 5,545,806, 5,569,825, 5,591,669, 5,545,807; PCT Patent Application Publication No. WO 97/17852, each of which is hereby incorporated by reference in its entirety).

The antibody may be muromonab-CD3 abciximab, rituximab, daclizumab, palivizumab, infliximab, trastuzumab, etanercept, basiliximab, gemtuzumab, alemtuzumab,

ibritumomab, adalimumab, alefacept, omalizumab, efalizumab, tositumomab, cetuximab, ABT-806, bevacizumab, natalizumab, ranibizumab, panitumumab, eculizumab, rilonacept, certolizumab, romiplostim, AMG-531, golimumab, ustekinumab, ABT-874, belatacept, belimumab, atacicept, an anti-CD20 antibody, canakinumab, tocilizumab, atlizumab, mepolizumab, pertuzumab, HuMax CD20, tremelimumab, ticilimumab, ipilimumab, IDEC-114, inotuzumab, HuMax EGFR, aflibercept, HuMax-CD4, teplizumab, orelizumab, catumaxomab, the anti-EpCAM antibody IGN101, adecatumomab, oregovomab, dinutuximab, girentuximab, denosumab, bapineuzumab, motavizumab, efumgumab, raxibacumab, an anti-CD20 antibody, LY2469298, or veltuzumab.

When the antibody comprises at least one light chain and at least one heavy chain, at least one light chain of the antibody, or at least one heavy chain of the antibody, or both may comprise an amino acid region having an amino acid motif capable of being recognized by an isoprenoid transferase. As an antibody may comprise four polypeptide chains (*e.g.*, two heavy chains and two light chains), an antibody may comprise four amino acid motifs, each of which can be used to conjugate an active agent to the antibody via a linker. Thus, an antibody-drug conjugate may comprise 4 linkers, each conjugated to at least one active agent. Accordingly, an antibody-drug conjugate may comprise at least one linker and at least two active agents. An antibody-drug conjugate may comprise at least two linkers, and an antibody-drug conjugate may comprise at least three active agents. An antibody-drug conjugate may comprise 1, 2, 3, or 4 linkers. An antibody-drug conjugate may comprise 1, 2, 3, or 4 peptides. An antibody-drug conjugate may comprise 2 to 100 active agents, such as 2 to 50 active agents, 2 to 20 active agents, 2 to 16 active agents, 4 to 16 active agents, or 4 to 8 active agents.

The active agent may be a drug, toxin, affinity ligand, detection probe, or combination of any of the foregoing.

The active agent may be selected from erlotinib; bortezomib; fulvestrant; sunitinib; letrozole; imatinib mesylate; PTK787/ZK 222584; oxaliplatin; 5-fluorouracil; leucovorin; rapamycin (Sirolimus); lapatinib; lonafarnib; sorafenib; gefitinib; AG1478; AG1571; alkylating agents (for example, thiotepa or cyclophosphamide); alkyl sulfonate (for example, busulfan, improsulfan, or piposulfan); aziridine (for example, benzodopa, carboquone, meturedopa, or uredopa); ethyleneimine, methylmelamine, altretamine, triethylenemelamine, triethylenephosphoramidate, triethylenethiophosphoramidate, trimethylolmelamine; acetogenins (for example, bullatacin or bullatacinone); camptothecin;

topotecan; bryostatin; callistatin; CC-1065 (including its adozelesin, carzelesin, or bizelesin synthetic analogs); cryptophycins (for example, cryptophycin 1 or cryptophycin 8); dolastatin; duocarmycin (including synthetic analogs, *e.g.*, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; sarcodictyin; spongistatin; nitrogen mustard (for example, chlorambucil, chlornaphazine, chlorophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, or uracil mustard); nitrousurea (for example, carmustine, chlorozotocin, fotemustine, lomustine, nimustine, or ranimustine); antibiotics (for example, enediyne antibiotics such as calicheamicin selected from calicheamicin gamma 1I and calicheamicin omega 1I, or dynemicin including dynemicin A); bisphosphonate (for example, clodronate; esperamicin, neocarzinostatin chromophore, or related chromoprotein enediyne antibiotic chromophores, aclacinomysins, actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, carabycin, carinomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (for example, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, liposomal doxorubicin, or deoxydoxorubicin), epirubicin, esorubicin, marcellomycin, mitomycins (for example, mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptomigrin, streptozocin, tubercidin, ubenimex, zinostatin, or zorubicin); anti-metabolites (for example, 5-fluorouracil); folic acid analogs (for example, denopterin, methotrexate, pteropterin, or trimetrexate); purine analogs (for example, fludarabine, 6-mercaptopurine, thiamiprine, or thiguanine); pyrimidine analogs (for example, ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, or floxuridine); androgens (for example, calusterone, dromostanolone propionate, epitiostanol, mepitiothane), or testolactone); anti-adrenals (for example, aminoglutethimide, mitotane, or trilostane); folic acid replenisher (for example, folinic acid); aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids (for example, maytansine or ansamitocins); trichothecenes (particularly T-2 toxin, verracurin A, roridin A, or anguidine); mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; polysaccharide K complex; razoxane; rhizoxin; sizofiran; spirogermanium;

tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (particularly, T-2 toxin, verracurin A, roridin A, and anguidine); urethane; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside; cyclophosphamide; thiotepa; taxoids (for example, paclitaxel), ABRAXANE<sup>TM</sup> cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel, doxetaxel; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; platinum analog (for example, cisplatin or carboplatin); vinblastine; platinum; etoposide, ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor (RFS 2000); difluoromethylornithine; retinoid (for example, retinoic acid); capecitabine, and pharmaceutically acceptable salts, solvates, acids, or derivatives thereof, but is not necessarily limited thereto.

The active agent may be selected from (i) anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators, including, for example, tamoxifen, raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene; (ii) aromatase inhibitors that inhibit aromatase enzyme, which regulates estrogen production in the adrenal glands, for example, 4(5)-imidazoles, aminoglutethimide, megestrol acetate, exemestane, letrozole, and anastrozole; (iii) anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); (iv) aromatase inhibitors; (v) protein kinase inhibitors; (vi) lipid kinase inhibitors; (vii) antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in adherent cells, for example, PKC-alpha, Raf, H-Ras; (viii) ribozyme, for example, VEGF inhibitor such as ribozyme and HER2 expression inhibitors; (ix) vaccines such as a gene therapy vaccine; ALLOVECTIN® vaccine, LEUVECTIN vaccine, VAXID vaccine; PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; (x) an anti-angiogenic agent such as Bevacizumab; and (xi) pharmaceutically acceptable salts, solvates, acids, or derivatives thereof.

In addition, cytokines may be used as the active agent. Cytokines are small cell-signaling protein molecules that are secreted by numerous cells and are a category of signaling molecules used extensively in intercellular communication. The cytokines include monokines, lymphokines, traditional polypeptide hormones, and the like. Examples of the cytokines include growth hormone (for example, human growth hormone, N-

methionyl human growth hormone, or bovine growth hormone); parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormone (for example, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), or luteinizing hormone (LH)); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor- $\alpha$ , tumor necrosis factor- $\beta$ ; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin, thrombopoietin (TPO); nerve growth factor (for example, NGF- $\beta$ ); platelet-growth factor; transforming growth factor (TGF) (for example, TGF- $\alpha$  or TGF- $\beta$ ); insulin-like growth factor-I, insulin-like growth factor-II; erythropoietin (EPO); osteoinductive factor; interferon (for example, interferon- $\alpha$ , interferon- $\beta$ , or interferon- $\gamma$ ); colony stimulating factor (CSF) (for example, macrophage-CSF (M-CSF), granulocyte-macrophage-CSF (GM-CSF), or granulocyte-CSF (G-CSF)); interleukin (IL) (for example, IL-1, IL-1 $\alpha$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, or IL-12); tumor necrosis factor (TNF) (for example, TNF- $\alpha$  or TNF- $\beta$ ); and polypeptide factor (for example, LIF or kit ligand), but are not limited thereto. Further, the term “cytokine” also includes cytokines from natural sources or recombinant cell cultures and biologically active equivalents of the native sequence cytokines.

The term “toxin” refers substances that are poisonous to living cells or organisms. Toxins may be small molecules, peptides or proteins capable of causing cell dysfunction or cell death after contact with or absorption by body tissue, *e.g.*, through an interaction with one or more biological macromolecules such as enzymes or cell receptors. Toxins include plant toxins and animal toxins. Examples of animal toxins include diphtheria toxin, botulinum toxin, tetanus toxin, dysentery toxin, cholera toxin, tetrodotoxin, brevetoxin, and ciguatoxin, but are not limited thereto. Examples of plant toxins include ricin and AM-toxin, but are not limited thereto.

Examples of small molecule toxins include auristatin, tubulysin, geldanamycin (Kerr et al., 1997, Bioconjugate Chem. 8(6):781-784), maytansinoid (EP 1391213, ACR 2008, 41, 98-107), calicheamicin (U.S. Patent Publication No. 2009/0105461, Cancer Res. 1993, 53, 3336-3342), daunomycin, doxorubicin, methotrexate, vindesine, SG2285 (Cancer Res. 2010, 70(17), 6849-6858), dolastatin, dolastatin analogs auristatin (U.S. Patent No. 5,635,483), cryptophycin, camptothecin, rhizoxin derivative, CC-1065 analog or derivative, duocarmycin, enediyne antibiotic, esperamicin, epothilone, pyrrolobenzodiazepine (PBD) derivatives,  $\alpha$ -amanitin, and toxoid, but are not limited thereto. Toxins may exhibit

cytotoxicity and cell growth-inhibiting activity by tubulin binding, DNA binding, topoisomerase suppression, and the like.

The term “ligand” refers to a molecule capable of forming a complex with a target biomolecule. An example of the ligand is a molecule bound to a predetermined position of a target protein to transmit a signal. The ligand may be a substrate, an inhibitor, a stimulating agent, a neurotransmitter, or a radioisotope.

“Detectable moiety” or a “label” refers to a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, radioactive, or chemical means. For example, useful labels include  $^{32}\text{P}$ ,  $^{35}\text{S}$ , fluorescent dyes, electron-dense reagents, enzymes (for example, enzymes commonly used in an ELISA), biotin-streptavidin, dioxigenin, haptens, and proteins for which antisera or monoclonal antibodies are available, or nucleic acid molecules with a sequence complementary to a target. The detectable moiety often generates a measurable signal, such as a radioactive, chromogenic, or fluorescent signal, that may be used to quantify the amount of bound detectable moiety in a sample. Quantitation of the signal may be achieved, for example, by scintillation counting, densitometry, flow cytometry, ELISA, or direct analysis by mass spectrometry of intact or subsequently digested peptides (one or more peptide may be assessed).

The term “probe” as used herein refers to a material that may (i) provide a detectable signal, (ii) interact a first probe or a second probe to modify a detectable signal provided by the first or second probe, such as fluorescence resonance energy transfer (FRET), (iii) stabilize an interaction with an antigen or a ligand or increase binding affinity; (iv) affect electrophoresis mobility or cell-intruding activity by a physical parameter such as charge, hydrophobicity, etc., or (v) control ligand affinity, antigen-antibody binding, or ionic complex formation.

The active agent may be an immunomodulatory compound, an anticancer agent, an antiviral agent, an antibacterial agent, an antifungal agent, an antiparasitic agent, or a combination thereof.

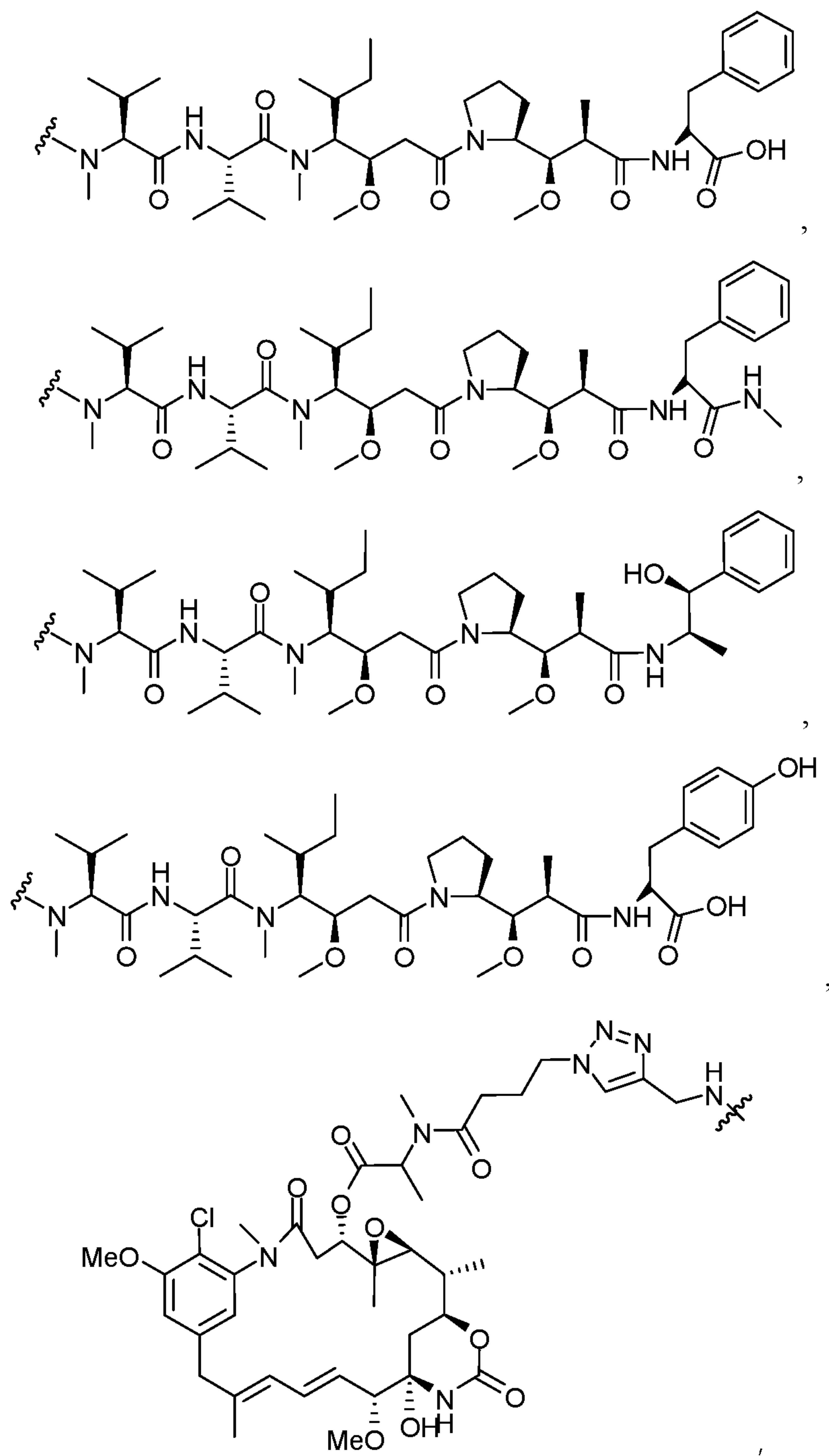
An immunomodulatory compound may be selected from aminocaproic acid, azathioprine, bromocriptine, chlorambucil, chloroquine, cyclophosphamide, cyclosporine, cyclosporine A, danazol, dehydroepiandrosterone, dexamethasone, etanercept, hydrocortisone, hydroxychloroquine, infliximab, meloxicam, methotrexate, mycophenylate mofetil, prednisone, sirolimus, and tacrolimus. An anticancer agent may be selected from 1-methyl-4-phenylpyridinium ion, 5-ethynyl-1-beta-D-ribofuranosylimidazole-4-

carboxamide (EICAR), 5-fluorouracil, 9-aminocamptothecin, actinomycin D, asparaginase, bicalutamide, bis-chloroethylnitrosourea (BCNU), bleomycin, bleomycin A2, bleomycin B2, busulfan, camptothecin, carboplatin, carmustine, CB1093, chlorambucil, cisplatin, crisnatol, cyclophosphamide, cytarabine, cytosine arabinoside, cytoxan, dacarbazine, dactinomycin, daunorubicin, decarbazine, deferoxamine, demethoxy-hypocrellin A, docetaxel, doxifluridine, doxorubicin, EB1089, epirubicin, etoposide, floxuridine, fludarabine, flutamide, gemcitabine, goserelin, hydroxyurea, idarubicin, ifosfamide, interferon- $\alpha$ , interferon- $\gamma$ , irinotecan, KH1060, leuprolide acetate, lomustine, lovastatin, megestrol, melphalan, mercaptopurine, methotrexate, mitomycin, mitomycin C, mitoxantrone, mycophenolic acid, nitrogen mustard, nitrosourea, paclitaxel, peplomycin, photosensitizer Pe4, phthalocyanine, pirarubicin, plicamycin, procarbazine, raloxifene, raltitrexed, revlimid, ribavirin, staurosporine, tamoxifen, teniposide, thalomid, thapsigargin, thioguanine, tiazofurin, topotecan, treosulfan, trimetrexate, tumor necrosis factor, velcade, verapamil, verteporfin, vinblastine, vincristine, vinorelbine, and zorubicin. An antiviral agent may be selected from penciclovir, valacyclovir, ganciclovir, foscarnet, ribavirin, idoxuridine, vidarabine, trifluridine, acyclovir, famciclovir, amantadine, rimantadine, cidofovir, antisense oligonucleotide, immunoglobulin, and interferon. An antibacterial agent may be selected from chloramphenicol, vancomycin, metronidazole, trimethoprim, sulfamethazole, quinupristin, dalfopristin, rifampin, spectinomycin, and nitrofurantoin. The antifungal agent may be selected from amphotericin B, candicidin, filipin, hamycin, natamycin, nystatin, rimocidin, bifonazole, butoconazole, clotrimazole, econazole, fenticonazole, isoconazole, ketoconazole, luliconazole, miconazole, omoconazole, oxiconazole, sertaconazole, sulconazole, tioconazole, albaconazole, fluconazole, isavuconazole, itraconazole, posaconazole, ravuconazole, terconazole, voriconazole, abafungin, amorolfine, butenafine, naftifine, terbinafine, anidulafungin, caspofungin, micafungin, benzoic acid, ciclopirox, flucytosine, griseofulvin, haloprogin, tolnaftate, undecylenic acid, crystal violet, balsam of peru, ciclopirox olamine, piroctone olamine, zinc pyrithione, and selenium sulfide. An antiparasitic agent may be selected from mebendazole, pyrantel pamoate, thiabendazole, diethylcarbamazine, ivermectin, niclosamide, praziquantel, albendazole, rifampin, amphotericin B, melarsoprol, eflornithine, metronidazole, tinidazole, and miltefosine.

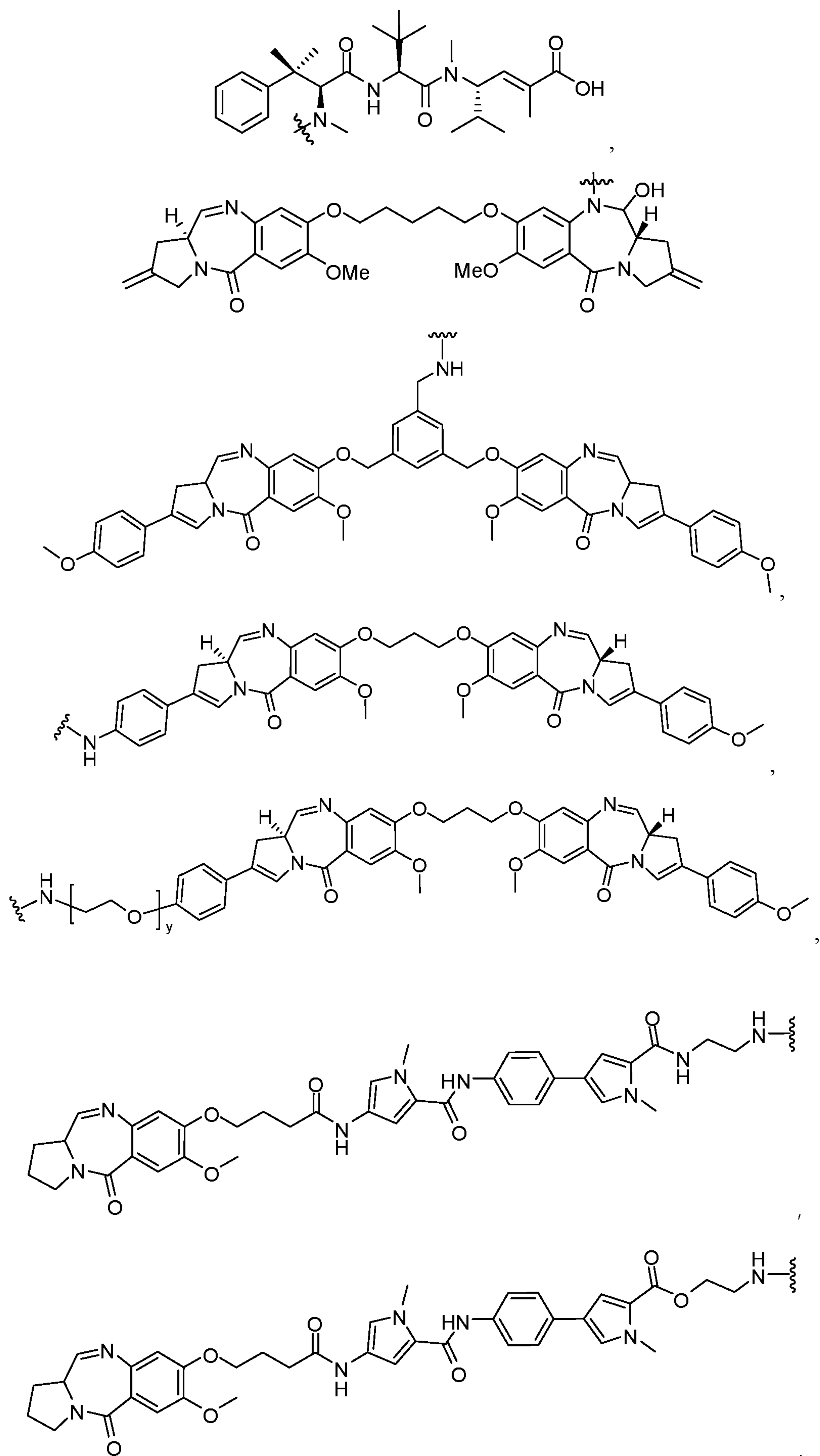
The antibody may comprise an amino acid motif selected from Ab-HC-(G)<sub>z</sub>CVIM, Ab-HC-(G)<sub>z</sub>CVLL, Ab-LC-(G)<sub>z</sub>CVIM, and Ab-LC-(G)<sub>z</sub>CVLL, wherein Ab represents an

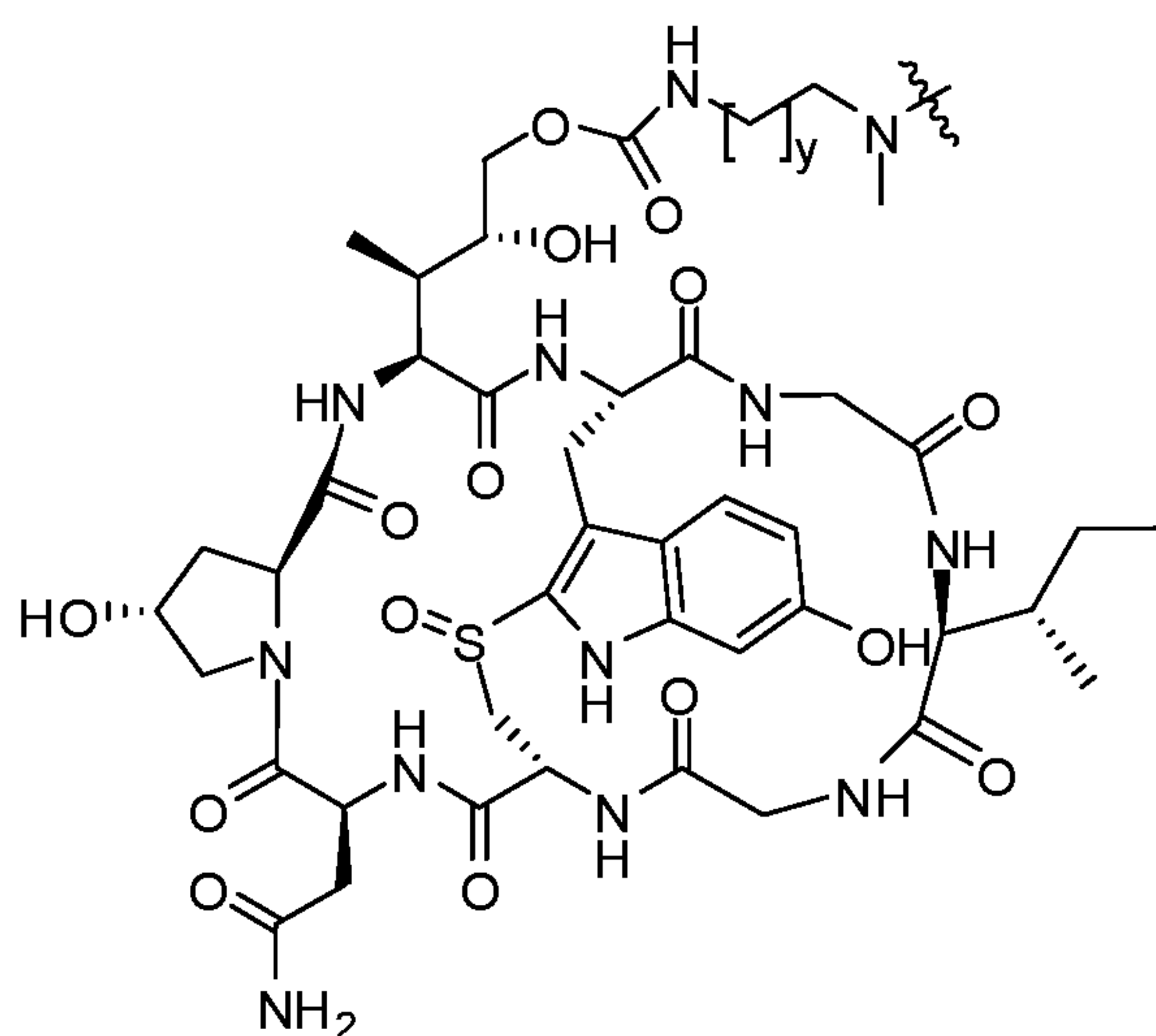
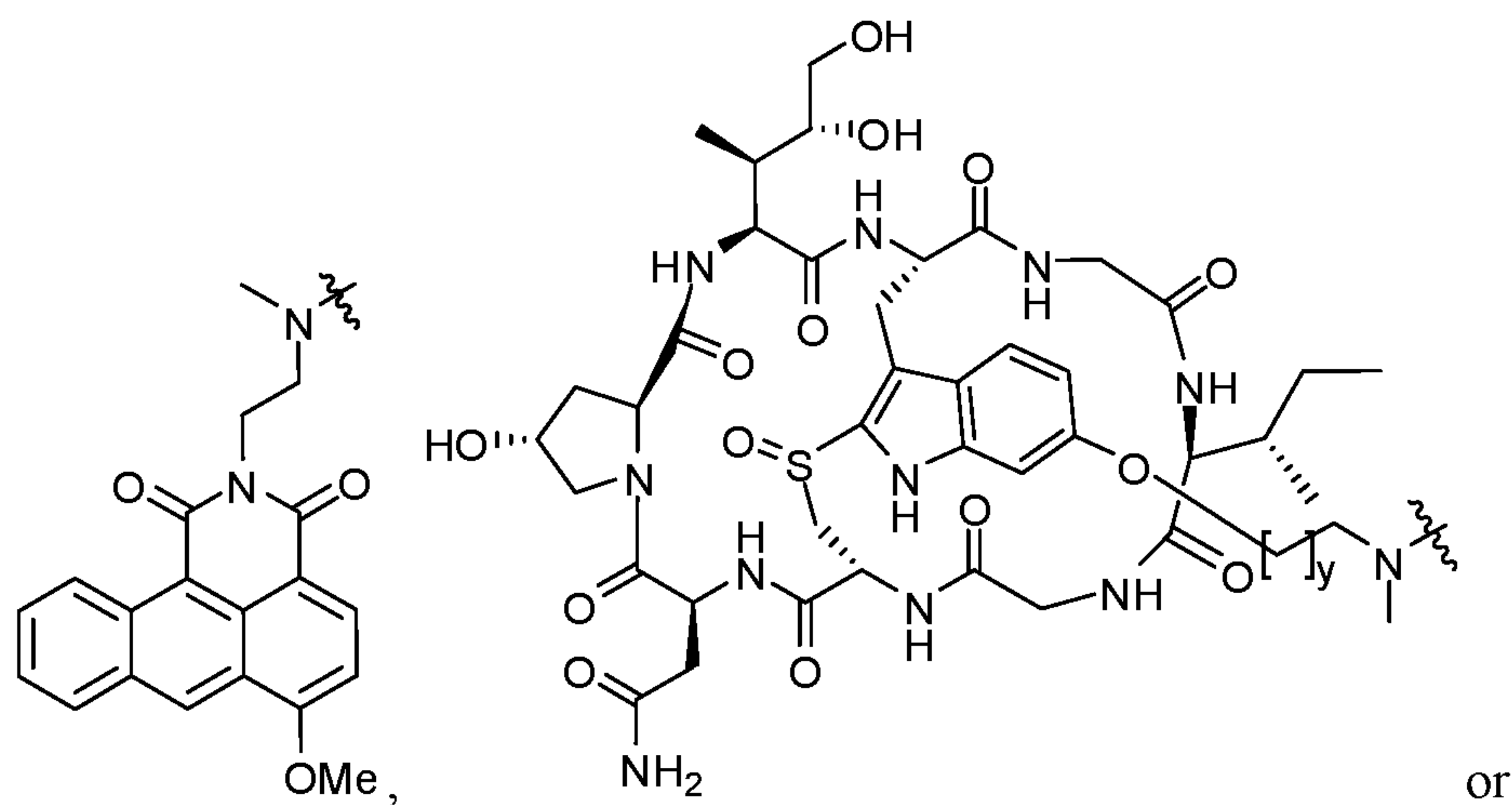
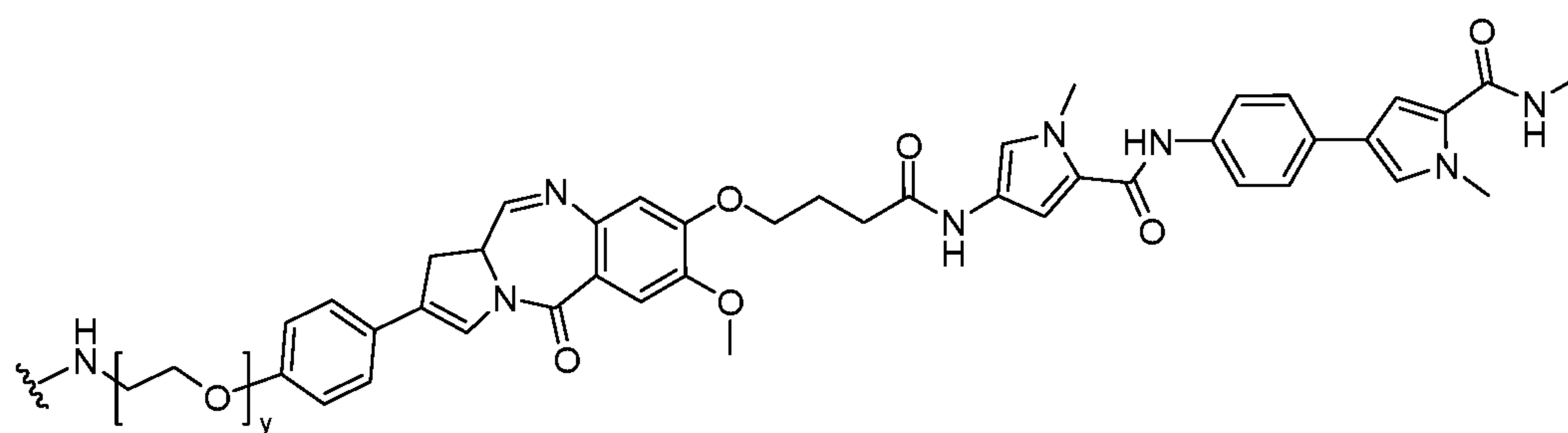
antibody, -HC- represents a heavy chain, -LC- represents a light chain, G represents a glycine, C represents cysteine, V represents valine, I represents isoleucine, M represents methionine, L represents leucine, and z is an integer from 0 to 20.

B, B<sub>1</sub>, and/or B<sub>2</sub> (e.g., of Formulas (I) and (V)-(IX)) may each independently be selected from any one of the following structures:



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wherein  $y$  is an integer from 1 to 10.

The antibody-drug conjugate may be used to transfer the active agent to a target cell of a subject to treat the subject using a method of preparing a composition known to those skilled in the art. In some aspects, the invention relates to a composition (*e.g.*, a pharmaceutical composition) comprising an antibody-drug conjugate as described herein.

Compositions may be prepared in an injectable form, either as a liquid solution or as a suspension. Solid forms suitable for injection may also be prepared, *e.g.*, as emulsions, or

with the antibody-drug conjugate encapsulated in liposomes. Antibody-drug conjugates may be combined with a pharmaceutically acceptable carrier, which includes any carrier that does not induce the production of antibodies harmful to the subject receiving the carrier. Suitable carriers typically comprise large macromolecules that are slowly metabolized, for example, proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, and the like.

The compositions may also contain diluents, for example, water, saline, glycerol, and ethanol. Auxiliary substances, for example, wetting or emulsifying agents, pH buffering substances, and the like may also be present therein. The compositions may be parenterally administered by injection, wherein such injection may be either subcutaneous or intramuscular injection. In some embodiments, a composition may be administered into a tumor. The composition may be inserted (*e.g.*, injected) into a tumor. Additional formulations are suitable for other forms of administration, such as suppository or oral administration. Oral compositions may be administered as a solution, suspension, tablet, pill, capsule, or sustained release formulation.

The compositions may be administered in a manner compatible with a dose and a formulation. The composition preferably comprises a therapeutically effective amount of the antibody-drug conjugate. The term “therapeutically effective amount” means a single dose or a composition administered in a multiple dose schedule that is effective for the treatment or prevention of a disease or disorder. A dose may vary, depending on the subject to be treated, the subject's health and physical conditions, a degree of protection to be desired, and other relevant factors. The exact amount of an active ingredient (*e.g.*, the antibody-drug conjugate) may depend on the judgment of a doctor. For example, a therapeutically effective amount of the antibody-drug conjugate or composition containing the same may be administered to a patient suffering from a cancer or tumor to treat the cancer or tumor.

The antibody-drug conjugate according to the present invention or the composition containing the same may be administered in the form of a pharmaceutically acceptable salt or solvate thereof. In some embodiments, the antibody-drug conjugate according to the present invention or the composition containing the same may be administered with a pharmaceutically acceptable carrier, a pharmaceutically acceptable excipient, and/or a pharmaceutically acceptable additive. The effective amount and the type of the pharmaceutically acceptable salt or solvate, excipient and additive may be measured using

standard methods (*see, e.g.*, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, 18th Edition, 1990).

The term “therapeutically effective amount” with regard to cancer or tumor means an amount that may decrease the number of cancer cells; decrease a size of cancer cells; inhibit cancer cells from intruding into peripheral systems or decrease the intrusion; inhibit cancer cells from spreading to other systems or decrease the spreading; inhibit cancer cells from growing; and/or ameliorate at least one symptom related to the cancer. In the treatment of cancer, the effectiveness of a drug may be assessed by time to tumor progression (TTP) and/or response rate (RR).

The term “pharmaceutically acceptable salts” used herein includes organic salts and inorganic salts. Examples thereof include hydrochloride, hydrobromide, hydroiodide, sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acidic phosphate, isonicotinate, lactate, salicylate, acidic citrate, tartrate, oleate, tannate, pantonate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, methane sulfonate, ethane sulfonate, benzene sulfonate, p-toluene sulfonate, and pamoate (that is, 1,1'-methylenebis-(2-hydroxy-3-naphthoate)). The pharmaceutically acceptable salt may include another molecule (for example, acetate ions, succinate ions, and/or other counter ions).

Exemplary solvates that may be used for pharmaceutically acceptable solvates of the antibody-drug conjugates described herein include water, isopropanol, ethanol, methanol, dimethyl sulfoxide, ethyl acetate, acetic acid, and ethanol amine. The term “acyl” is art-recognized and refers to a group represented by the general formula hydrocarbylC(O)-, preferably alkylC(O)-.

The term “acylamino” is art-recognized and refers to an amino group substituted with an acyl group and may be represented, for example, by the formula hydrocarbylC(O)NH-.

The term “acyloxy” is art-recognized and refers to a group represented by the general formula hydrocarbylC(O)O-, preferably alkylC(O)O-.

The term “alkoxy” refers to an alkyl group, preferably a lower alkyl group, having an oxygen attached thereto. Representative alkoxy groups include methoxy, ethoxy, propoxy, tert-butoxy and the like.

The term “alkoxyalkyl” refers to an alkyl group substituted with an alkoxy group and may be represented by the general formula alkyl-O-alkyl.

The term “alkenyl”, as used herein, refers to an aliphatic group containing at least one double bond and is intended to include both "unsubstituted alkenyls" and "substituted alkenyls", the latter of which refers to alkenyl moieties having substituents replacing a hydrogen on one or more carbons of the alkenyl group. Such substituents may occur on one or more carbons that are included or not included in one or more double bonds. Moreover, such substituents include all those contemplated for alkyl groups, as discussed below, except where stability is prohibitive. For example, substitution of alkenyl groups by one or more alkyl, carbocyclyl, aryl, heterocyclyl, or heteroaryl groups is contemplated.

An “alkyl” group or “alkane” is a straight chained or branched non-aromatic hydrocarbon which is completely saturated. Typically, a straight chained or branched alkyl group has from 1 to about 20 carbon atoms, preferably from 1 to about 10 unless otherwise defined. Examples of straight chained and branched alkyl groups include methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl, tert-butyl, pentyl, hexyl, heptyl and octyl. A C<sub>1</sub>-C<sub>6</sub> straight chained or branched alkyl group is also referred to as a "lower alkyl" group.

Moreover, the term "alkyl" (or "lower alkyl") as used throughout the specification, examples, and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents, if not otherwise specified, can include, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an alkoxycarbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxy, a phosphoryl, a phosphate, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of amino, azido, imino, amido, phosphoryl (including phosphonate and phosphinate), sulfonyl (including sulfate, sulfonamido, sulfamoyl and sulfonate), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), -CF<sub>3</sub>, -CN and the like. Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxy, alkylthios, aminoalkyls, carbonyl-substituted alkyls, -CF<sub>3</sub>, -CN, and the like.

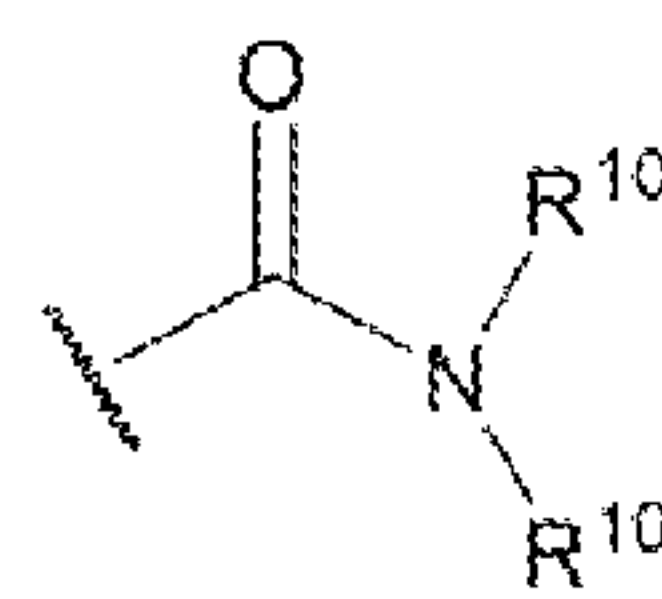
The term “C<sub>x-y</sub>” when used in conjunction with a chemical moiety, such as, acyl,

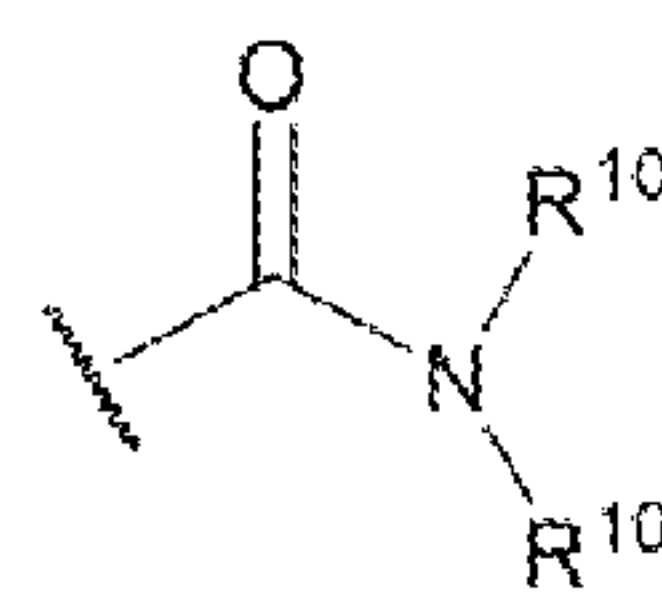
acyloxy, alkyl, alkenyl, alkynyl, or alkoxy is meant to include groups that contain from x to y carbons in the chain. For example, the term “C<sub>x-y</sub>alkyl” refers to substituted or unsubstituted saturated hydrocarbon groups, including straight-chain alkyl and branched-chain alkyl groups that contain from x to y carbons in the chain, including haloalkyl groups such as trifluoromethyl and 2,2,2-trifluoroethyl, etc. C<sub>0</sub> alkyl indicates a hydrogen where the group is in a terminal position, a bond if internal. The terms “C<sub>2-y</sub>alkenyl” and “C<sub>2-y</sub>alkynyl” refer to substituted or unsubstituted unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

The term “alkylamino”, as used herein, refers to an amino group substituted with at least one alkyl group.

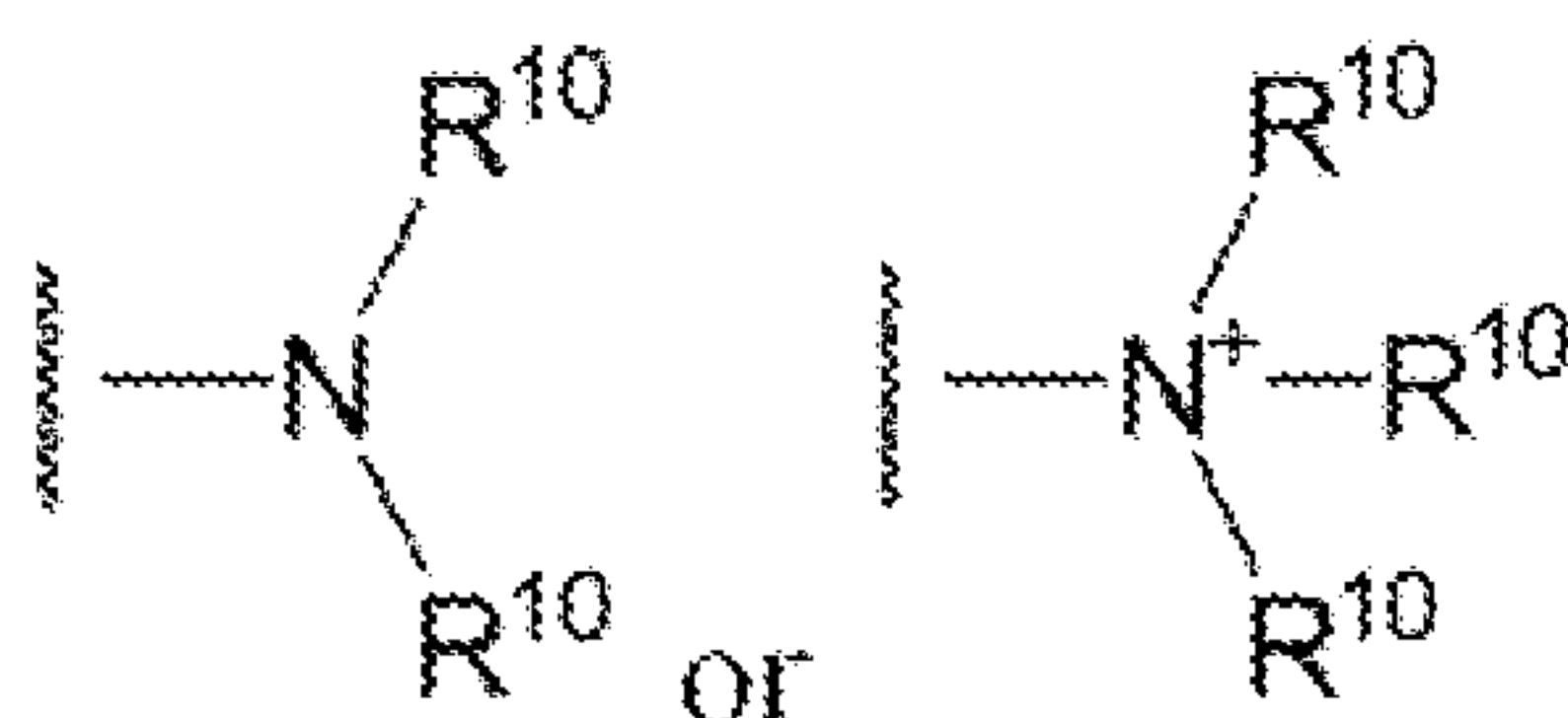
The term “alkylthio”, as used herein, refers to a thiol group substituted with an alkyl group and may be represented by the general formula alkylS-.

The term “alkynyl”, as used herein, refers to an aliphatic group containing at least one triple bond and is intended to include both "unsubstituted alkynyls" and "substituted alkynyls", the latter of which refers to alkynyl moieties having substituents replacing a hydrogen on one or more carbons of the alkynyl group. Such substituents may occur on one or more carbons that are included or not included in one or more triple bonds. Moreover, such substituents include all those contemplated for alkyl groups, as discussed above, except where stability is prohibitive. For example, substitution of alkynyl groups by one or more alkyl, carbocyclyl, aryl, heterocyclyl, or heteroaryl groups is contemplated.



The term “amide”, as used herein, refers to a group  wherein each R<sup>10</sup> independently represent a hydrogen or hydrocarbyl group, or two R<sup>10</sup> are taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure.

The terms “amine” and “amino” are art-recognized and refer to both unsubstituted and substituted amines and salts thereof, *e.g.*, a moiety that can be represented by



wherein each R<sup>10</sup> independently represents a hydrogen or a hydrocarbyl group, or two R<sup>10</sup>

are taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure.

The term “aminoalkyl”, as used herein, refers to an alkyl group substituted with an amino group.

The term “carboxy”, as used herein, refers to a group represented by the formula  $\text{-CO}_2\text{H}$ .

The terms “hetaralkyl” and “heteroaralkyl”, as used herein, refers to an alkyl group substituted with a hetaryl group.

The term "heteroalkyl", as used herein, refers to a saturated or unsaturated chain of carbon atoms and at least one heteroatom, wherein no two heteroatoms are adjacent.

The terms “heteroaryl” and “hetaryl” include substituted or unsubstituted aromatic single ring structures, preferably 5- to 7-membered rings, more preferably 5- to 6-membered rings, whose ring structures include at least one heteroatom, preferably one to four heteroatoms, more preferably one or two heteroatoms. The terms “heteroaryl” and “hetaryl” also include polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is heteroaromatic, *e.g.*, the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Heteroaryl groups include, for example, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, pyrazole, pyridine, pyrazine, pyridazine, and pyrimidine, and the like.

The term “heteroatom” as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are nitrogen, oxygen, and sulfur.

The terms “heterocyclyl”, “heterocycle”, and “heterocyclic” refer to substituted or unsubstituted non-aromatic ring structures, preferably 3- to 10-membered rings, more preferably 3- to 7-membered rings, whose ring structures include at least one heteroatom, preferably one to four heteroatoms, more preferably one or two heteroatoms. The terms “heterocyclyl” and “heterocyclic” also include polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is heterocyclic, *e.g.*, the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Heterocyclyl groups include, for example, piperidine, piperazine, pyrrolidine, morpholine, lactones, lactams, and the like. Heterocyclyl groups can also be substituted by oxo groups. For example, “heterocyclyl” encompasses both pyrrolidine and pyrrolidinone.

The term “hydroxyalkyl”, as used herein, refers to an alkyl group substituted with a hydroxy group.

The term “substituted” refers to moieties having substituents replacing a hydrogen on one or more carbons of the backbone. It will be understood that “substitution” or “substituted with” includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, *e.g.*, which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc. As used herein, the term “substituted” is contemplated to include all permissible substituents of organic compounds.

In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and non-aromatic substituents of organic compounds. The permissible substituents can be one or more and the same or different for appropriate organic compounds. For purposes of this invention, the heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valences of the heteroatoms. Substituents can include any substituents described herein, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an alkoxycarbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxy, a phosphoryl, a phosphate, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that substituents can themselves be substituted, if appropriate. Unless specifically stated as “unsubstituted,” references to chemical moieties herein are understood to include substituted variants. For example, reference to an “aryl” group or moiety implicitly includes both substituted and unsubstituted variants.

The term “thioalkyl”, as used herein, refers to an alkyl group substituted with a thiol group.

The term “thioester”, as used herein, refers to a group  $-C(O)SR^{10}$  or  $-SC(O)R^{10}$  wherein  $R^{10}$  represents a hydrocarbyl.

The term “thioether”, as used herein, is equivalent to an ether, wherein the oxygen is replaced with a sulfur.

“Protecting group” refers to a group of atoms that, when attached to a reactive

functional group in a molecule, mask, reduce or prevent the reactivity of the functional group. Typically, a protecting group may be selectively removed as desired during the course of a synthesis. Examples of protecting groups can be found in Greene and Wuts, *Protective Groups in Organic Chemistry*, 3<sup>rd</sup> Ed., 1999, John Wiley & Sons, NY and Harrison et al., *Compendium of Synthetic Organic Methods*, Vols. 1-8, 1971-1996, John Wiley & Sons, NY. Representative nitrogen protecting groups include, but are not limited to, formyl, acetyl, trifluoroacetyl, benzyl, benzyloxycarbonyl (“CBZ”), tert-butoxycarbonyl (“Boc”), trimethylsilyl (“TMS”), 2-trimethylsilyl-ethanesulfonyl (“TES”), trityl and substituted trityl groups, allyloxycarbonyl, 9-fluorenylmethyloxycarbonyl (“Fmoc”), nitro-veratryloxycarbonyl (“NVOC”) and the like. Representative hydroxylprotecting groups include, but are not limited to, those where the hydroxyl group is either acylated (esterified) or alkylated such as benzyl and trityl ethers, as well as alkyl ethers, tetrahydropyranyl ethers, trialkylsilyl ethers (*e.g.*, TMS or TIPS groups), glycol ethers, such as ethylene glycol and propylene glycol derivatives and allyl ethers.

“Covalently coupled” includes both direct bonding and indirect bonding (*e.g.*, through an intervening series of atoms) of two chemical species. For example, an amino acid may be covalently coupled to polyethylene glycol directly, *e.g.*, by forming an ester between the carboxyl of the amino acid and a hydroxyl of the polyethylene glycol, or indirectly, *e.g.*, by reacting the polyethylene glycol with epichlorohydrin to form an epoxypropyl ether and reacting the resulting epoxide with the amino group of the amino acid, thereby covalently linking the amino acid and the polyethylene glycol through a 2-hydroxypropyl linker. Various moieties and reactions for coupling diverse moieties directly or indirectly are well known in the art. In certain preferred embodiments, unless otherwise indicated by the context, an indirect bonding involves only 1-10 intervening atoms (*e.g.*, a methylene, a dibutyl ether, a tripeptide, etc.), most preferably 1-6 intervening atoms.

As used herein, a therapeutic that “prevents” a disorder or condition refers to a compound that, in a statistical sample, reduces the occurrence of the disorder or condition in the treated sample relative to an untreated control sample, or delays the onset or reduces the severity of one or more symptoms of the disorder or condition relative to the untreated control sample.

The term “treating” includes prophylactic and/or therapeutic treatments. The term “prophylactic or therapeutic” treatment is art-recognized and includes administration to the host of one or more of the subject compositions. If it is administered prior to clinical

manifestation of the unwanted condition (*e.g.*, disease or other unwanted state of the host animal) then the treatment is prophylactic (*i.e.*, it protects the host against developing the unwanted condition), whereas if it is administered after manifestation of the unwanted condition, the treatment is therapeutic, (*i.e.*, it is intended to diminish, ameliorate, or stabilize the existing unwanted condition or side effects thereof).

The term “prodrug” is intended to encompass compounds which, under physiologic conditions, are converted into the therapeutically active agents. A common method for making a prodrug is to include one or more selected moieties which are hydrolyzed under physiologic conditions to reveal the desired molecule. In other embodiments, the prodrug is converted by an enzymatic activity of the host animal. For example, esters or carbonates (*e.g.*, esters or carbonates of alcohols or carboxylic acids) are preferred prodrugs.

In some embodiments, the invention relates to a method of treating a disease, such as cancer, in a subject, comprising administering a pharmaceutical composition comprising an antibody-drug conjugate as described herein to the subject. A pharmaceutical composition may further comprise a therapeutically effective amount of chemotherapeutic agent. In preferred embodiments, the subject is a mammal. For example, the subject may be selected from rodents, lagomorphs, felines, canines, porcines, ovines, bovines, equines, and primates. In certain preferred embodiments, the subject is a human.

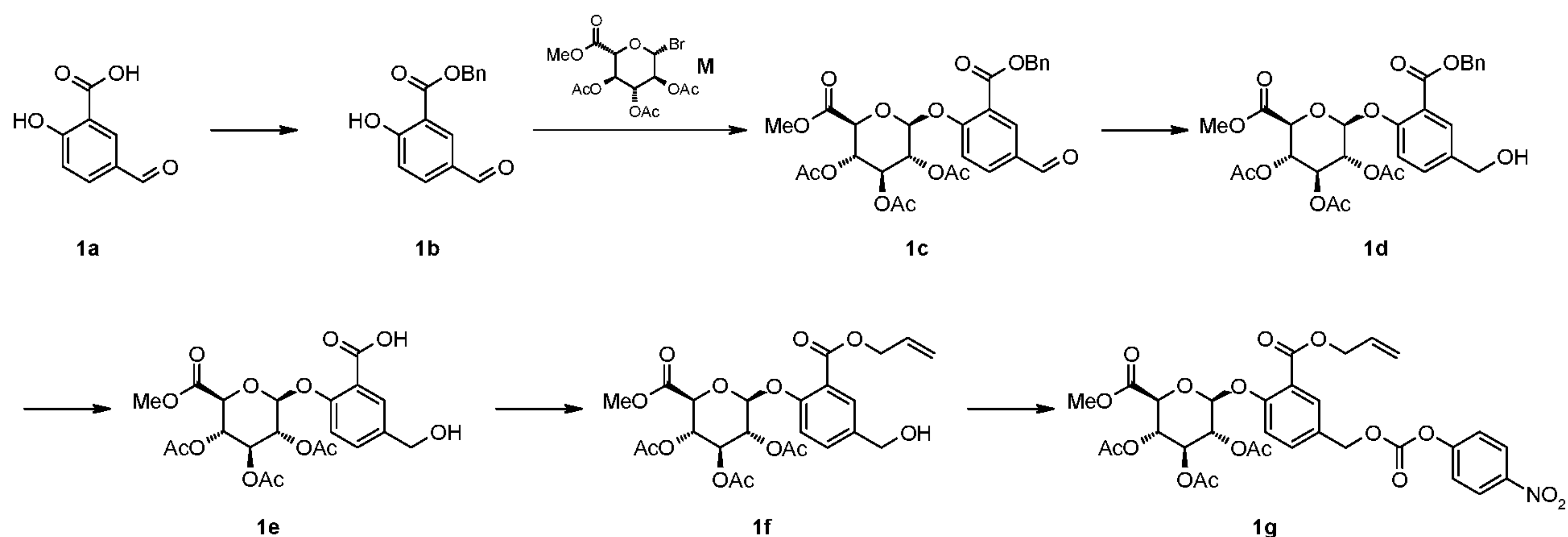
Hereinafter, configurations of the present invention will be described in detail through Examples, but the following Examples are only to assist in understanding of the present invention. The scope of the present invention is not limited thereto.

## EXEMPLIFICATION

The table below lists the abbreviations used throughout the following Examples:

| Abbreviation | Reference                                  |
|--------------|--------------------------------------------|
| Ac           | acetyl                                     |
| AcOH         | acetic acid                                |
| aq.          | aqueous                                    |
| Bn           | benzyl                                     |
| brine        | saturated aqueous sodium chloride solution |
| Boc          | <i>t</i> -butoxycarbonyl                   |
| Cbz          | benzyloxycarbonyl                          |
| DBU          | 1,8-diazabicyclo[5.4.0]undec-7-ene         |

|                   |                                                                                    |
|-------------------|------------------------------------------------------------------------------------|
| DCM               | dichloromethane                                                                    |
| DIC               | <i>N,N'</i> -diisopropylcarbodiimide                                               |
| DIPEA             | <i>N,N</i> -diisopropylethylamine                                                  |
| DMAP              | 4-(dimethylamino)pyridine                                                          |
| DMF               | <i>N,N</i> -dimethylformamide                                                      |
| DMSO              | dimethyl sulfoxide                                                                 |
| EDC               | <i>N</i> -(3-dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide                    |
| Et                | ethyl                                                                              |
| Et <sub>2</sub> O | diethyl ether                                                                      |
| EtOAc             | ethyl acetate                                                                      |
| EtOH              | ethanol                                                                            |
| HBTU              | O-(benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium<br>hexafluorophosphate |
| Hex               | n-hexane                                                                           |
| HOBt              | 1-hydroxybenzotriazole                                                             |
| HPLC              | high performance liquid chromatography                                             |
| Me                | Methyl                                                                             |
| MeCN              | acetonitrile                                                                       |
| MeOH              | methanol                                                                           |
| MMAE              | monomethyl auristatin E                                                            |
| MMAF              | monomethyl auristatin F                                                            |
| MMAF-<br>OMe      | monomethyl auristatin F methyl ester                                               |
| <i>i</i> -PrOH    | isopropanol                                                                        |
| PyBOP             | (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate                |
| TBAF              | tetrabutylammonium fluoride                                                        |
| TBS               | <i>t</i> -butyldimethylsilyl                                                       |
| THF               | tetrahydrofuran                                                                    |
| TFA               | trifluoroacetic acid                                                               |
| Ts                | <i>p</i> -toluenesulfonyl                                                          |
| wt                | weight                                                                             |

*Example 1. Preparation of Compound 1i*Preparation of Compound 1b

To a suspension of 5-formylsalicylic acid **1a** (10.0 g, 60.1 mmol) in THF (30 mL) was added DIPEA (29.8 mL, 180 mmol) and benzyl bromide (7.15 mL, 60.1 mmol) at room temperature. Then the reaction mixture was heated under reflux. After 18 hours under reflux, the reaction mixture was diluted with 2 N aq. HCl (100 mL). The resulting mixture was extracted with EtOAc (2 x 100 mL). The combined organic layers were dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The residue was purified by column chromatography to produce the compound **1b** (12.9 g, 83 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 11.38 (s, 1H), 9.86 (s, 1H), 8.40 (s, 1H), 8.01 (d, *J* = 8.8 Hz, 1H), 7.44 (m, 5H), 7.12 (d, *J* = 8.0 Hz, 1H), 5.42 (s, 2H).

Preparation of Compound 1c

To a solution of compound **1b** (5.0 g, 19.5 mmol) and compound **M** (8.5 g, 21.4 mmol, see Example 66) in MeCN (100 mL) were added 4 Å molecular sieve (10 g) and Ag<sub>2</sub>O (18.0 g, 78.0 mmol). After stirring at room temperature for 12 hours under N<sub>2</sub>, the reaction mixture was concentrated. Then the concentrated reaction mixture was diluted with H<sub>2</sub>O (100 mL) and extracted with EtOAc (2 x 200 mL). The combined organic layers were dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The residue was purified by column chromatography to produce the compound **1c** (8.63 g, 77 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 9.94 (s, 1H), 8.28 (s, 1H), 8.02 (d, *J* = 8.8 Hz, 1H), 7.46-7.28 (m, 6H), 5.41-5.32 (m, 6H), 4.27 (d, *J* = 9.2 Hz, 1H), 3.71 (s, 3H), 2.05 (m, 9H).

Preparation of Compound 1d

To a solution of compound **1c** (3.10 g, 5.41 mmol) in *i*-PrOH/CHCl<sub>3</sub> (9 mL/45 mL)

was added silica gel (3 g) and NaBH<sub>4</sub> (0.41 g, 10.82 mmol) at 0 °C. After stirring at 0 °C for 2 hours under N<sub>2</sub>, the reaction mixture was quenched with H<sub>2</sub>O (100 mL) and extracted with EtOAc (200 mL). The organic layer was dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **1d** (2.73 g, 87 %) as white solid. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.74 (s, 1H), 7.48-7.34 (m, 6H), 7.16 (d, *J* = 8.8 Hz, 1H), 5.35-5.26 (m, 5H), 5.15 (m, 1H), 4.17 (m, 1H), 3.73 (s, 3H), 2.04 (s, 9H), 1.73 (t, 1H).

#### Preparation of Compound 1e

To a solution of compound **1d** (2.40 g, 4.17 mmol) in EtOH (150 mL) Pd/C (10 wt. %, 240 mg) was added. The reaction mixture was stirred at room temperature for 10 minutes under hydrogen. Then the reaction mixture was filtered through a celite pad and washed with EtOH (100 mL). The filtrate was concentrated to provide the crude product **1e** as white solid (2.10 g), which was used without further purification. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 8.06 (s, 1H) 7.61 (dd, *J* = 8.8 Hz, 1H), 7.23 (d, *J* = 8.0 Hz 1H), 5.43-5.29 (m, 5H), 4.17 (s, 2H), 4.32 (d, *J* = 8.4 Hz, 1H) 3.69 (s, 3H), 2.11-2.08 (t, 9H), 1.24 (t, 1H).

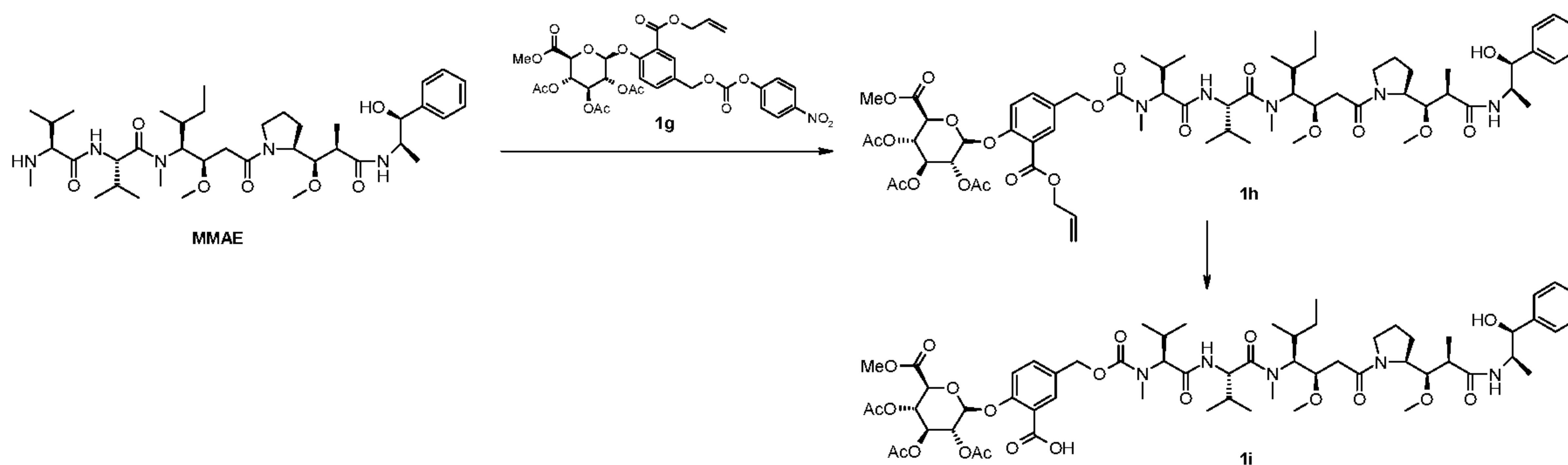
#### Preparation of Compound 1f

To a solution of the crude compound **1e** (2.10 g, 4.33 mmol) in DMF (50 mL) were added K<sub>2</sub>CO<sub>3</sub> (1.79 g, 13.01 mmol) and allyl bromide (0.41 mL, 4.76 mmol) at room temperature. After stirring at room temperature for 3 hours, the reaction mixture was diluted with 2 N aq. HCl (100 mL). The resulting mixture was extracted with EtOAc (200 mL). The organic layer was dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The residue was purified by column chromatography to produce the compound **1f** (1.55 g, 70 % for 2 steps). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.74 (s, 1H), 7.45 (dd, *J* = 8.0 Hz, 2.0 Hz, 1H), 7.16 (d, *J* = 8.8 Hz, 1H), 6.02 (m, 1H), 5.40-5.26 (m, 5H), 5.16 (m, 1H), 4.76 (m, 2H), 4.66 (s, 2H), 4.19 (m, 1H), 3.73 (s, 3H), 2.07-2.05 (m, 9H), 1.68 (t, 1H).

#### Preparation of Compound 1g

To a solution of compound **1f** (2.50 g, 4.77 mmol) in DMF (20 mL) were added bis(4-nitrophenyl)carbonate (1.30 g, 4.29 mmol) and DIPEA (0.80 mL 4.77 mmol) at 0 °C under N<sub>2</sub>. The reaction mixture was stirred at 0 °C for 30 min and allowed to warm to room temperature for 1 hour. The reaction mixture was diluted with H<sub>2</sub>O (100 mL) and extracted

with EtOAc (200 mL). The organic layer was washed with brine (100 mL) and dried over anhydrous  $\text{MgSO}_4$ . After filtration and concentration under reduced pressure, the resulting crude product was purified by column chromatography to produce the compound **1g** (2.80 g, 85 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.28 (d,  $J = 15.2$  Hz, 2H), 7.85 (d,  $J = 2.4$  Hz, 1H), 7.55 (dd,  $J = 3.2$  Hz, 2.4 Hz, 1H), 7.38 (d,  $J = 15.2$  Hz, 2H), 7.20 (d,  $J = 8.8$  Hz, 1H) 6.03 (m, 1H), 5.42-5.19 (m, 8H), 4.78 (d,  $J = 5.2$  Hz, 2H), 4.12 (d,  $J = 7.2$  Hz, 1H), 3.74 (s, 3H).



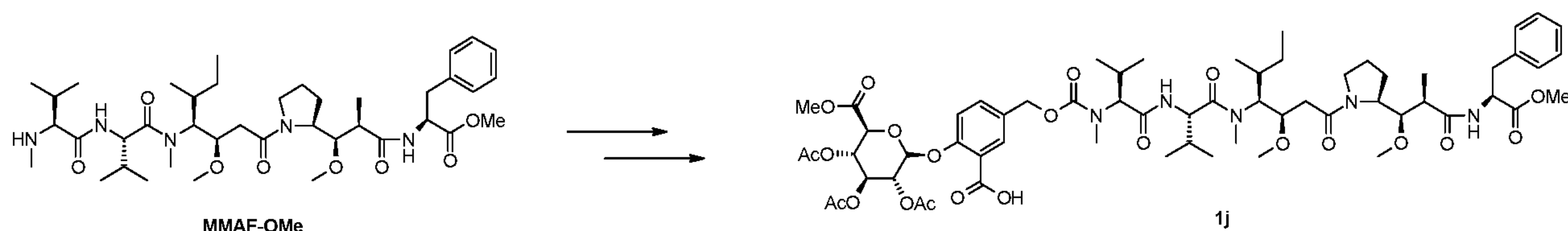
### Preparation of Compound **1h**

Compound **1g** (528 mg, 0.77 mmol), MMAE (500 mg, 0.7 mmol) and anhydrous HOBt (19 mg, 0.14 mmol) were dissolved in DMF (3 mL) at 0 °C. Then pyridine (0.7 mL) and DIPEA (0.24 mL, 1.39 mmol) were added. After stirring at room temperature for 24 hours under  $\text{N}_2$ , the reaction mixture was diluted with  $\text{H}_2\text{O}$ /saturated aqueous  $\text{NH}_4\text{Cl}$  solution (100 mL/50 mL) and extracted with EtOAc (2 x 100 mL). The combined organic layers were dried over anhydrous  $\text{MgSO}_4$ , filtered, and concentrated. The residue was purified by column chromatography to produce the compound **1h** (600 mg, 67 %). EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  1269.5,  $[\text{M}+\text{Na}]^+$  1291.5.

### Preparation of Compound **1i**

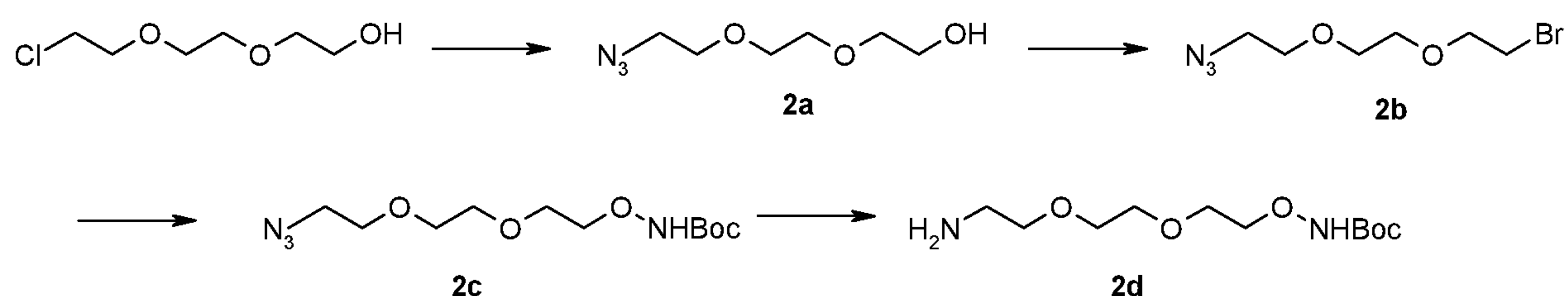
To a solution of compound **1h** (600 mg, 0.47 mmol) and triphenylphosphine (31 mg, 0.12 mmol) in DCM (10 mL) were added pyrrolidine (0.047 mL, 0.57 mmol) and  $\text{Pd}(\text{PPh}_3)_4$  (27 mg, 0.02 mmol) at room temperature. After stirring for 2 hours, the reaction mixture was diluted with  $\text{H}_2\text{O}$ /1 N aq. HCl (50 mL/50 mL) and extracted with EtOAc (3 x 50 mL). The combined organic layers were dried over anhydrous  $\text{MgSO}_4$ , filtered, and concentrated. The residue was purified by column chromatography (Hex/EtOAc 1/1 to EtOAc) to produce the compound **1i** (480 mg, 82 %) as a white solid. EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  1228.4,  $[\text{M}+\text{Na}]^+$  1250.4.

### Example 2. Preparation of Compound 1j



Compound **1j** was prepared from MMAF-OMe by a similar method of preparing compound **1i** in Example 1.

*Example 3. Preparation of Compound 2g*



### Preparation of Compound 2a

2-(2-(2-Chloroethoxy)ethoxy)ethanol (10 g, 59.3 mmol) was dissolved in DMF (90 mL) at room temperature under nitrogen, and then NaN<sub>3</sub> (5.78 g, 88.9 mmol) was added thereto. After stirring at 100 °C for 13 hours, chloroform (200 mL) and distilled water (300 mL) were added thereto to extract an organic layer, and the extracted organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The residue was subjected to column chromatography, which produced the compound **2a** (10.3 g, 99 %). <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) δ 3.75-3.73 (m, 2H), 3.70-3.68 (m, 6H), 3.63-3.61 (m, 2H), 3.40 (t, *J* = 5.4 Hz, 2H), 2.20 (t, *J* = 6.0 Hz, 1H).

### Preparation of Compound 2b

$\text{CBr}_4$  (21.4 g, 64.6 mmol) was dissolved in DCM (100 mL) at 0°C under nitrogen, and then triphenylphosphine (16.9 g, 64.6 mmol) in DCM (100 mL) and compound **2a** (10.3 g, 58.7 mmol) were added thereto, and the mixture was stirred at room temperature for 13 hours. After the reaction was completed, DCM (300 mL) and distilled water (300 mL) were added thereto to extract an organic layer, and the extracted organic layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated under reduced pressure. The residue was subjected to column chromatography, which produced the compound **2b** (12 g, 85 %).  $^1\text{H}$ -

NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  3.83 (t,  $J$  = 6.4 Hz, 2H), 3.72-3.67 (m, 6H), 3.48 (t,  $J$  = 6.0 Hz, 2H), 3.40 (t,  $J$  = 4.8 Hz, 2H)

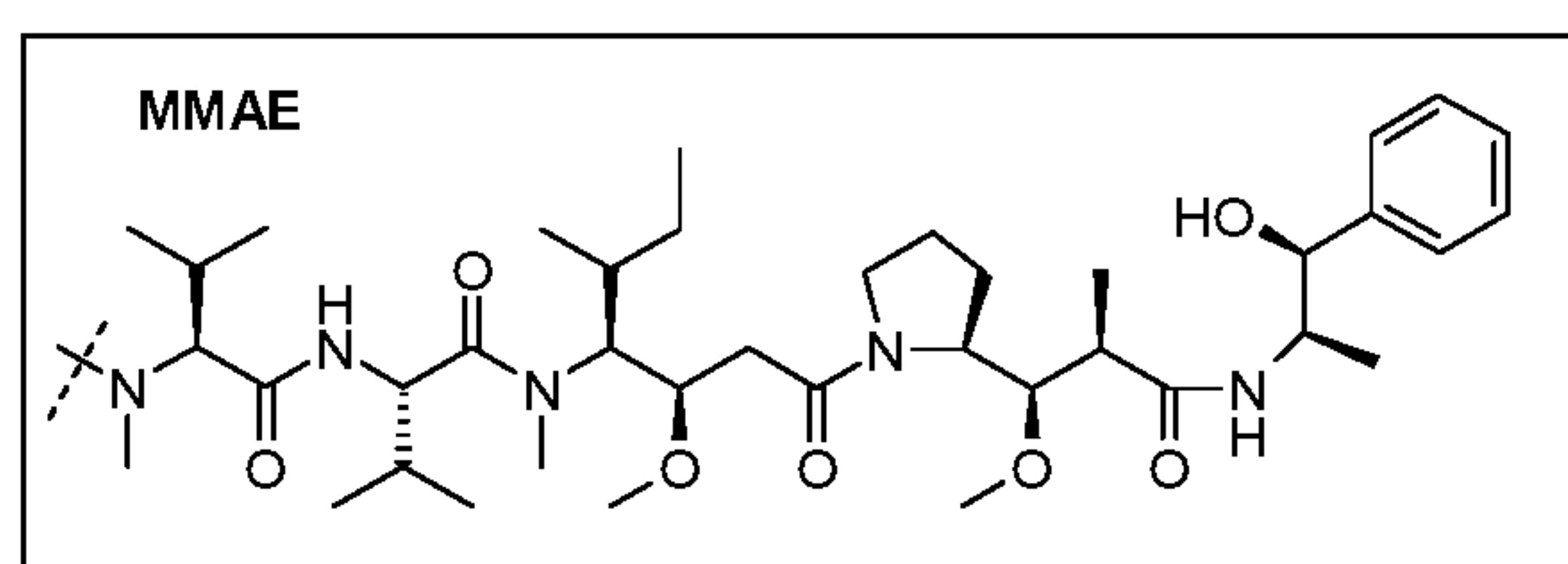
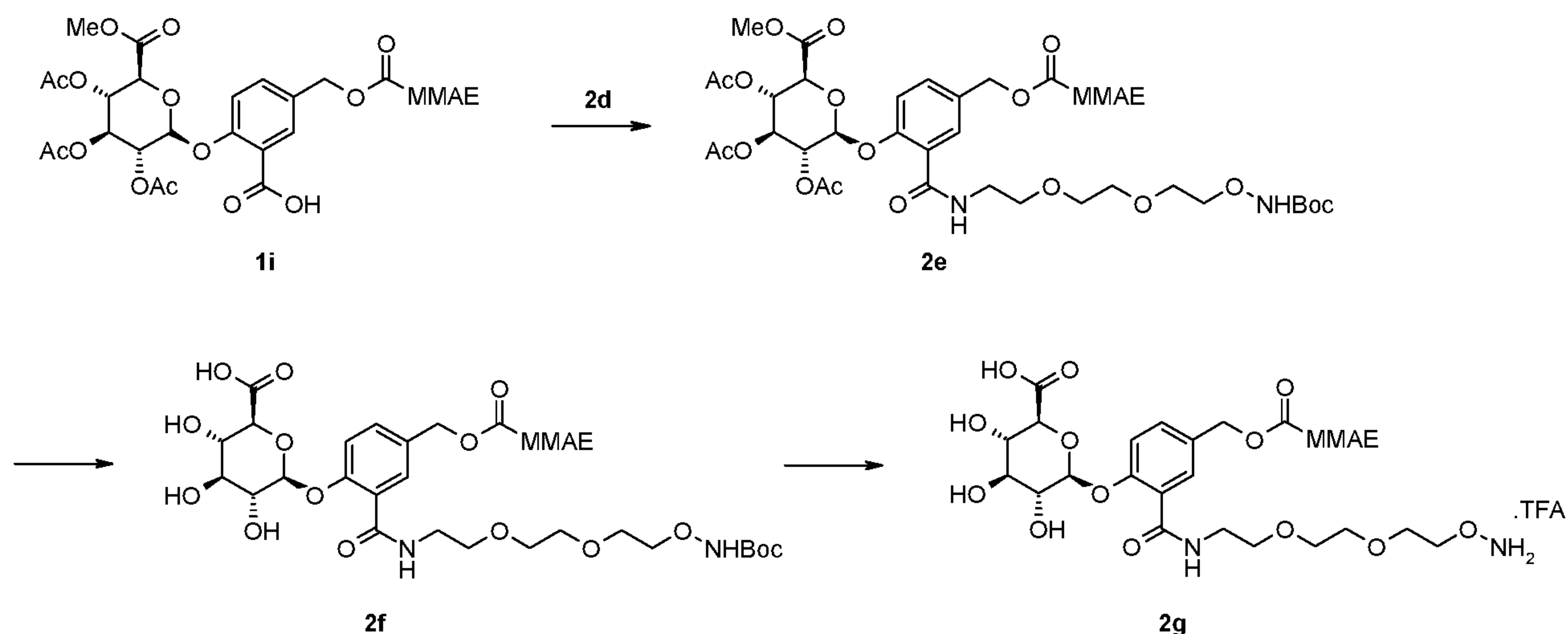
#### Preparation of Compound 2c

Compound **2b** (1g, 4.20 mmol) was dissolved in MeCN at room temperature under nitrogen, and then *N*-Boc-hydroxylamine (643 mg, 4.82 mmol) and DBU (0.66 mL, 4.41mmol) were added thereto. After stirring at 60 °C for 13 hours, DCM (300 mL) and distilled water (300 mL) were added thereto to extract an organic layer, and the extracted organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The residue was subjected to column chromatography, which produced the compound **2c** (748 mg, 70 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.55 (s, 1H), 4.05-4.03 (m, 2H), 3.76-3.74 (m, 2H), 3.74-3.69 (m, 6H), 3.42 (t,  $J$  = 4.8 Hz, 2H), 1.49 (s, 9H).

#### Preparation of Compound 2d

Compound **2c** (200 mg, 0.688 mmol) was dissolved in MeOH (5 mL), and then Pd/C (10% wt., 70 mg) was added thereto and stirred under hydrogen for 3 hours. After the reaction was completed, the reaction mixture was celite-filtered and concentrated under reduced pressure, which produced the compound **2d** (180 mg, 98 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  4.04-4.01 (m, 2H), 3.74-3.62 (m, 7H), 3.55 (t,  $J$  = 5.2 Hz, 1H), 2.88 (t,  $J$  = 5.2 Hz, 1H), 2.81 (t,  $J$  = 5.2 Hz, 1H), 1.64 (s, 2H), 1.48 (s, 9H).

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#### Preparation of Compound **2e**

DIPEA (0.042 mL, 0.32 mmol) and PyBOP (126 mg, 0.24 mmol) were added to a stirred mixture of compound **1i** (200 mg, 0.16 mmol) and compound **2d** (51 mg, 0.19 mmol) in DMF (4 mL). After stirring at room temperature for 4 hours under N<sub>2</sub>, the reaction mixture was diluted with H<sub>2</sub>O (100 mL) and extracted with EtOAc (2 x 100 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The resulting residue was purified by column chromatography to yield the compound **2e** (142 mg, 60 %). EI-MS m/z: [M+H]<sup>+</sup> 1474.7.

#### Preparation of Compound **2f**

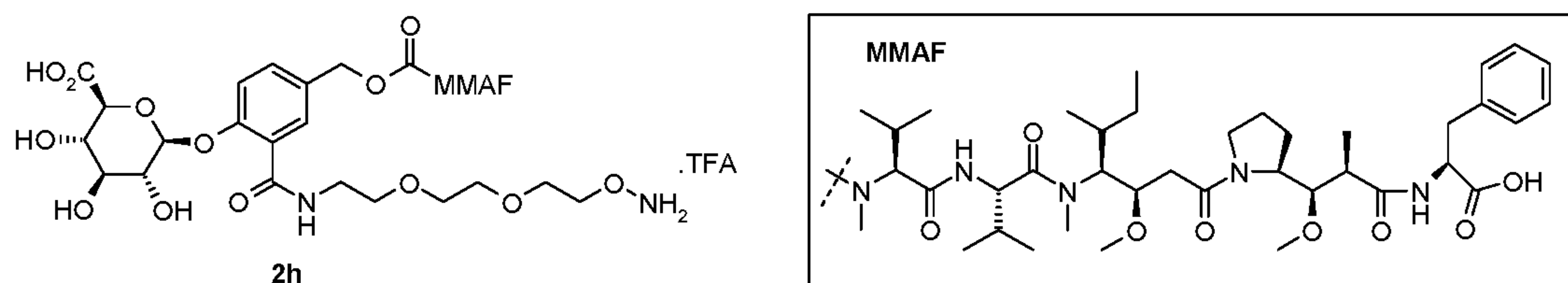
To a solution of compound **2e** (142 mg, 0.096 mmol) in MeOH (2 mL) was added LiOH monohydrate (36 mg, 0.86 mmol) in H<sub>2</sub>O (2 mL) at -20 °C. After stirred at 0 °C for 1 hour, the reaction mixture was diluted with H<sub>2</sub>O/2 N aq. HCl solution (50 mL/2 mL) and extracted with CHCl<sub>3</sub> (2 x 100 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated to yield the crude compound **2f** (128 mg), which was used without further purification. EI-MS m/z: [M+H]<sup>+</sup> 1334.5.

#### Preparation of Compound **2g**

To a solution of crude compound **2f** (105 mg, 0.08 mmol) in DCM (3 mL) HCl (4 M in 1,4-dioxane, 1 mL) was added at 0 °C. After 1 hour, the solvent and excess HCl were removed

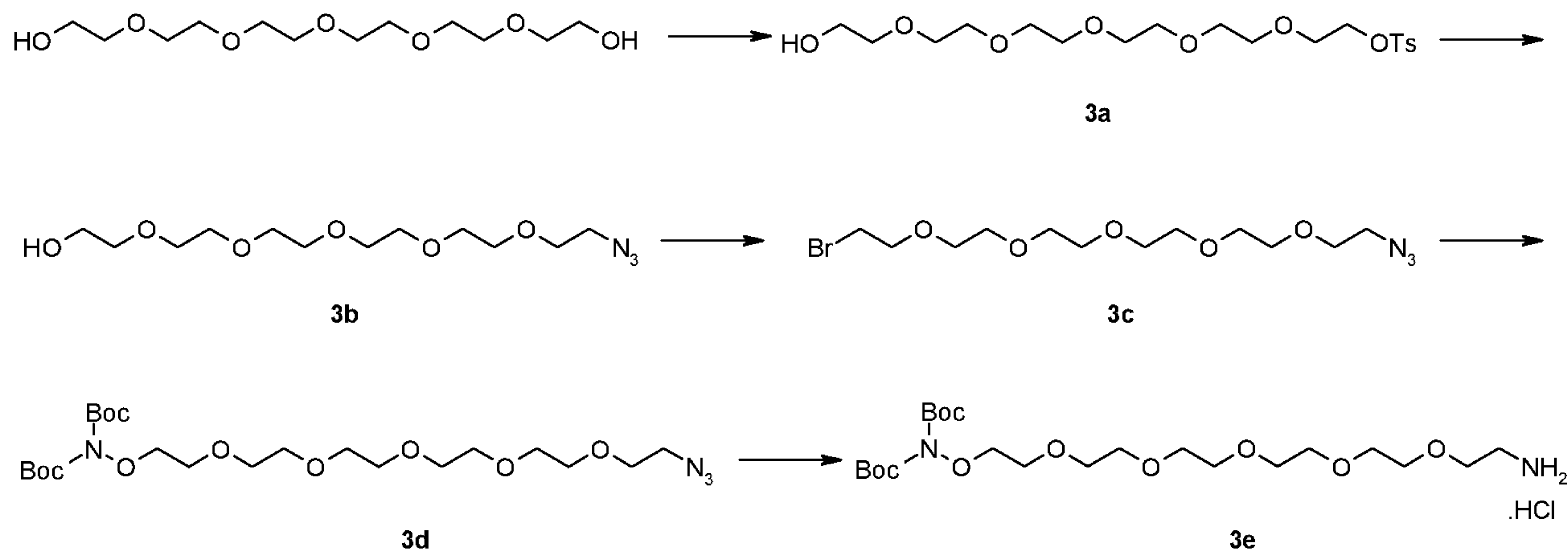
by N<sub>2</sub> flow and then the residue was purified by HPLC, which produced the compound **2g** (47 mg, 46 %) as white solid. EI-MS m/z: [M+H]<sup>+</sup> 1234.4.

*Example 4. Preparation of Compound 2h*



Compound **2h** was prepared from compound **1j** and compound **2d** by a similar method of preparing compound **2g** in Example 3. EI-MS m/z: [M+H]<sup>+</sup> 1248.9.

*Example 5. Preparation of Compound 3f*



Preparation of Compound 3a

A mixture of hexaethylene glycol (1.0 g, 3.54 mmol), Ag<sub>2</sub>O (1.23 g, 5.31 mmol) and KI (117 mg, 0.71 mmol) in DCM (10 mL) was sonicated for 15 min. The suspension was cooled to -30 °C and a solution of *p*-toluenesulfonyl chloride (688 mg, 3.61 mmol) in DCM (13 mL) was added dropwise. The mixture was then gradually warmed up to 0 °C and kept for 15 minutes at this temperature. Then the reaction mixture was dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated to produce the syrupy residue. Then, the syrupy residue was purified by column chromatography (EtOAc to EtOAc/MeOH 10/1). The pure fractions were evaporated *in vacuo* to yield the compound **3a** (1.18 g, 77 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.80 (d, *J* = 8.4 Hz, 2H), 7.34 (d, *J* = 8.4 Hz, 2H), 4.16 (m, 2H), 3.72-3.58 (m, 22H), 2.97 (br, 1H), 2.45 (s, 3H).

### Preparation of Compound 3b

Compound **3a** (1.18 g, 2.71 mmol) and NaN<sub>3</sub> (264 mg, 4.07 mmol) were dissolved in DMF (3 mL). And then the reaction mixture was heated at 100 °C. After 15 hours at 100 °C, the reaction mixture was filtered and concentrated. The residue was purified by column chromatography (EtOAc to EtOAc/MeOH 10/1) to yield the compound **3b** (728 mg, 87 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.75-3.70 (m, 2H), 3.69-3.63 (m, 18H), 3.62-3.60 (m, 2H), 3.39 (d, *J* = 5.2 Hz, 2H), 3.07 (br, 1H).

### Preparation of Compound 3c

To a stirred solution of compound **3b** (728 mg, 2.36 mmol) in THF (10 mL) at 0 °C were added triethylamine (0.73 mL, 5.21 mmol) and methanesulfonic anhydride (619 mg, 3.55 mmol). After 2 hours, LiBr (1.03 g, 11.8 mmol) was added to a stirred solution and the resulting reaction mixture was refluxed for 5 hours. After cooling to room temperature, the reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc to EtOAc/ MeOH 10/1) to yield the compound **3c** (810 mg, 92 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.81 (t, *J* = 6.4 Hz, 2H), 3.69-3.65 (m, 18H), 3.47 (t, *J* = 6.4 Hz, 2H), 3.39 (t, *J* = 5.2 Hz, 2H).

### Preparation of Compound 3d

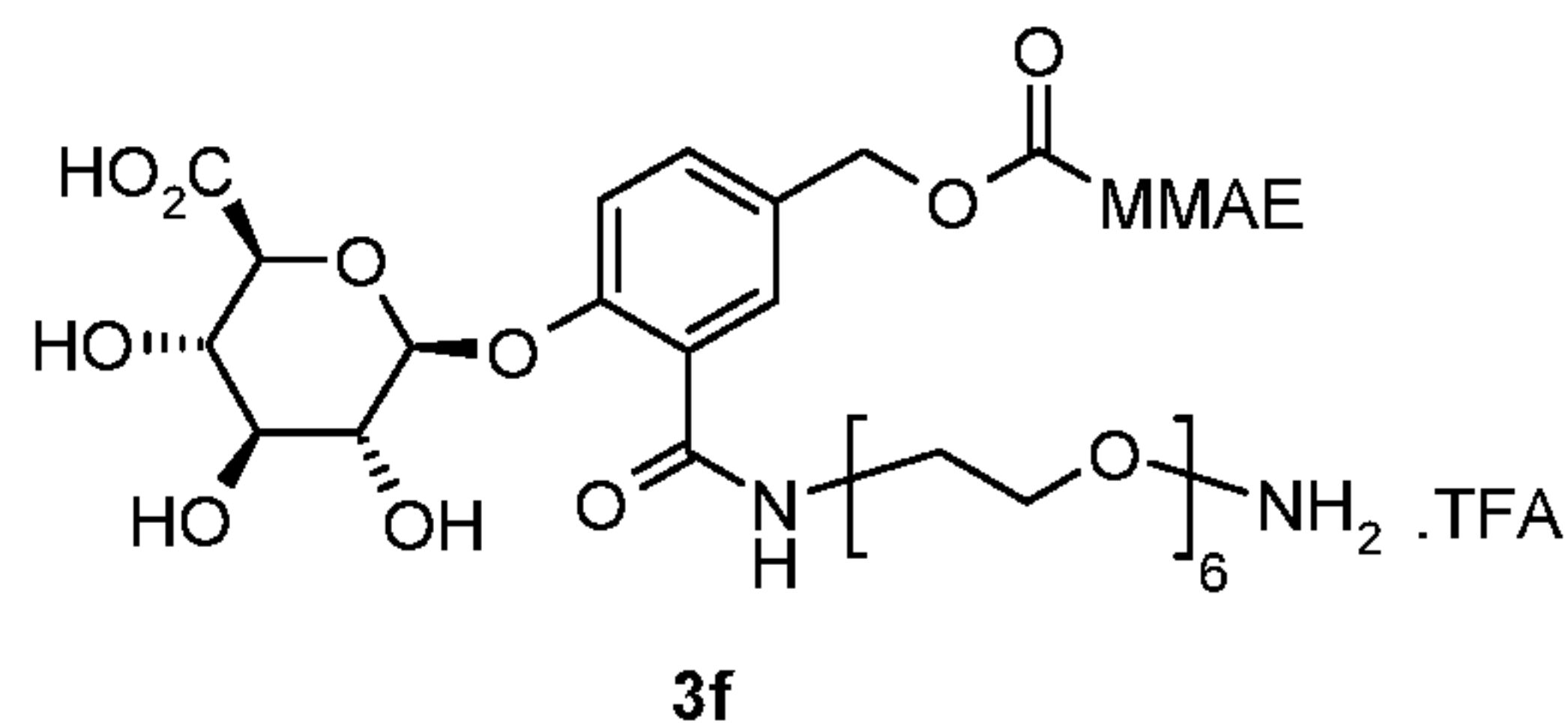
NaH (60 % in oil, 564 mg, 12.9 mmol) was added to a stirred mixture of compound **3c** (3.42 g, 9.24 mmol) and *N,N*-diBoc-hydroxylamine (2.80 g, 12.0 mmol, synthesized by the procedures in PCT publication No. WO2004/018466A2, hereby incorporated by reference) in DMF (20 mL) at 0 °C. The reaction mixture was warmed to room temperature and kept for 2 hours at this temperature. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (EtOAc/Hex 1/20 to 1/5), which produced the compound **3d** (3.51 g, 73 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.08 (t, *J* = 4.8 Hz, 2H), 3.73 (t, *J* = 4.8 Hz, 2H), 3.69-3.62 (m, 18H), 3.39 (t, *J* = 5.6 Hz, 2H), 1.53 (s, 18H).

### Preparation of Compound 3e

To a stirred mixture of compound **3d** (123 mg, 0.23 mmol), and Pd/C (10 wt. %, 25 mg) in MeOH (5 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.05 mL, 0.21 mmol).

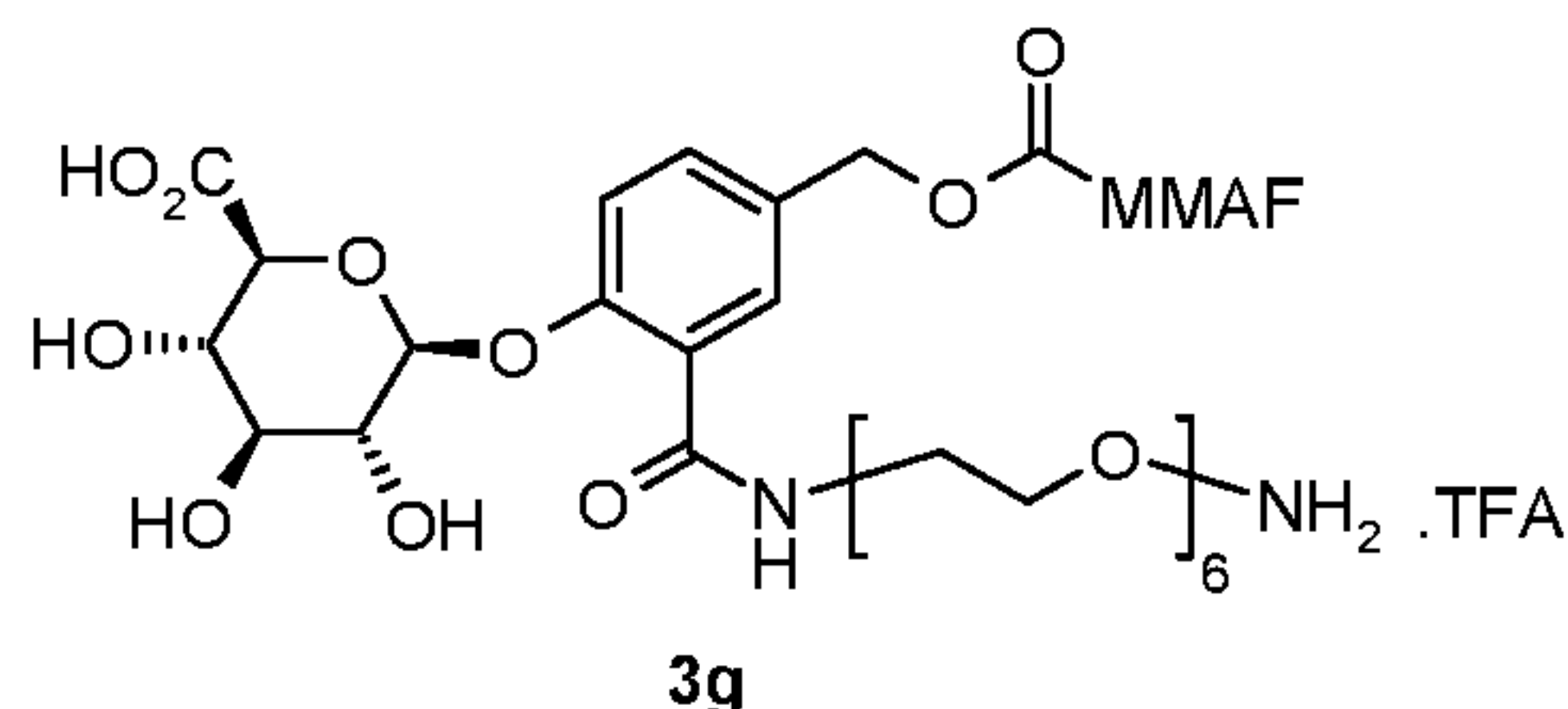
After stirring at room temperature for 5 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (100 mL). The filtrate was concentrated to produce the compound **3e** (118 mg, 95 %) as colorless oil, which was used without further purification.  $^1\text{H-NMR}$  (400 MHz, DMSO- $d_6$ )  $\delta$  3.98 (t,  $J$  = 4.4 Hz, 2H), 3.61-3.51 (m, 22H), 2.95 (br, 3H), 1.46 (s, 18H). EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  497.6.

### Preparation of Compound **3f**



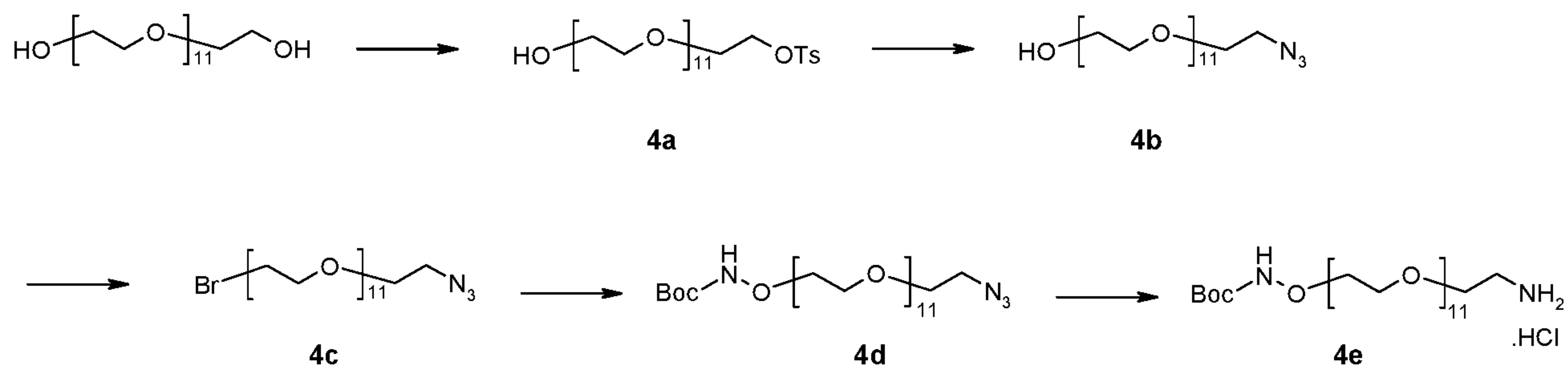
Compound **3f** was prepared from compound **1i** and compound **3e** by a similar method of preparing compound **2g** in Example 3. EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  1366.6,  $[\text{M}+\text{Na}]^+$  1389.6.

### Example 6. Preparation of Compound **3g**



Compound **3g** was prepared from compound **1j** and compound **3e** by a similar method of preparing compound **2g** in Example 3. EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  1380.6,  $[\text{M}+\text{Na}]^+$  1403.6.

### Example 7. Preparation of Compound **4f**



#### Preparation of Compound 4a

To a stirred solution of dodecaethylene glycol (1.8 g, 3.2 mmol) in DCM (18 mL) was added *p*-toluenesulfonyl chloride (656 mg, 3.4 mmol), Ag<sub>2</sub>O (1.13 g, 4.9 mmol) and KI (108 mg, 0.65 mmol). After stirring at room temperature for 30 minutes, the reaction mixture was filtered through a celite pad and washed with DCM (50 mL). The filtrate was concentrated. The resulting residue was purified by column chromatography to produce the compound **4a** (490 mg, 21 %) as light yellowish oil. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.81 (d, 2H), 7.35 (d, 2H), 4.16 (t, 2H), 3.72-3.58 (m, 46H), 2.82 (br s, 1H), 2.45 (s, 3H).

#### Preparation of Compound 4b

Compound **4a** (490 mg, 0.69 mmol) and NaN<sub>3</sub> (68 mg, 1.04 mmol) were dissolved in DMF (16 mL) and the reaction mixture was heated at 100 °C for 3 hours. The reaction mixture was filtered and concentrated. The crude product was purified by column chromatography to yield the compound **4b** (267 mg, 67 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.72-3.60 (m, 46H), 3.39 (t, 2H), 2.84 (t, 1H), 3.40 (m, 2H).

#### Preparation of Compound 4c

To a stirred solution of compound **4b** (265 mg, 0.46 mmol) in THF (10 mL) at 0 °C were added 4-methylmorpholine (0.066 mL, 0.60 mmol) and methanesulfonic anhydride (121 mg, 0.69 mmol). After 2 hours, LiBr (120 mg, 1.38 mmol) was added to a stirred solution and the resulting reaction mixture was refluxed for 6 hours. After cooling to room temperature, the reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc to EtOAc/MeOH 10/1) to yield the compound **4c** (178 mg, 60 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.81 (t, 2H), 3.65 (m, 42H), 3.47 (t, 2H), 3.39 (t, 2H).

#### Preparation of Compound 4d

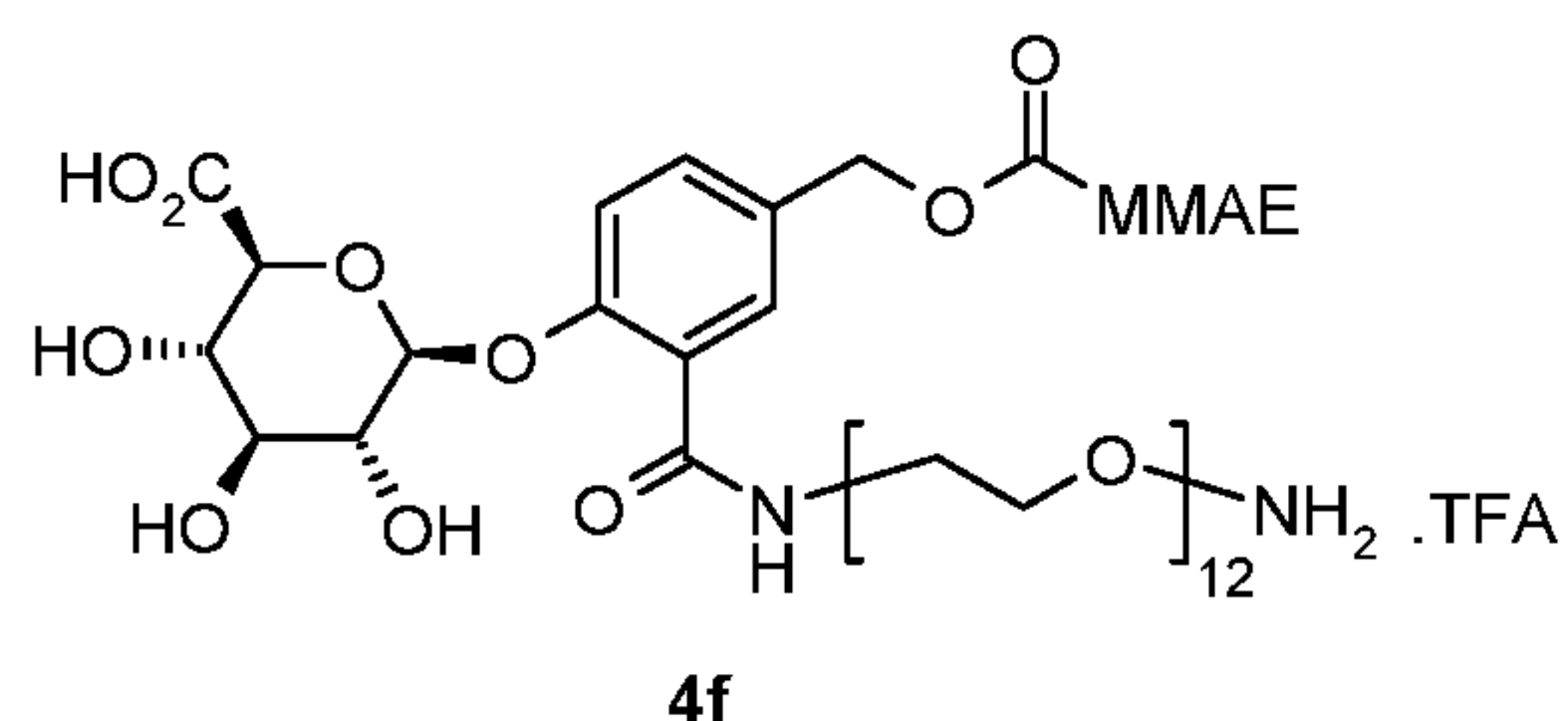
NaH (60 % in oil, 14 mg, 0.33 mmol) was added to a stirred mixture of compound **4c** (175 mg, 0.27 mmol), and *N*-Boc-hydroxylamine (47 mg, 0.35 mmol) in DMF (5 mL) at 0 °C. The reaction mixture was warmed up to room temperature and kept for 12 hours at this temperature. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (MeOH/CHCl<sub>3</sub> 1/20 to 1.5/20), which produced the compound **4d** (148 mg, 78 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.00 (t, 2H), 3.66 (m, 44H),

3.39 (t, 2H), 1.47 (d, 9H).

### Preparation of Compound 4e

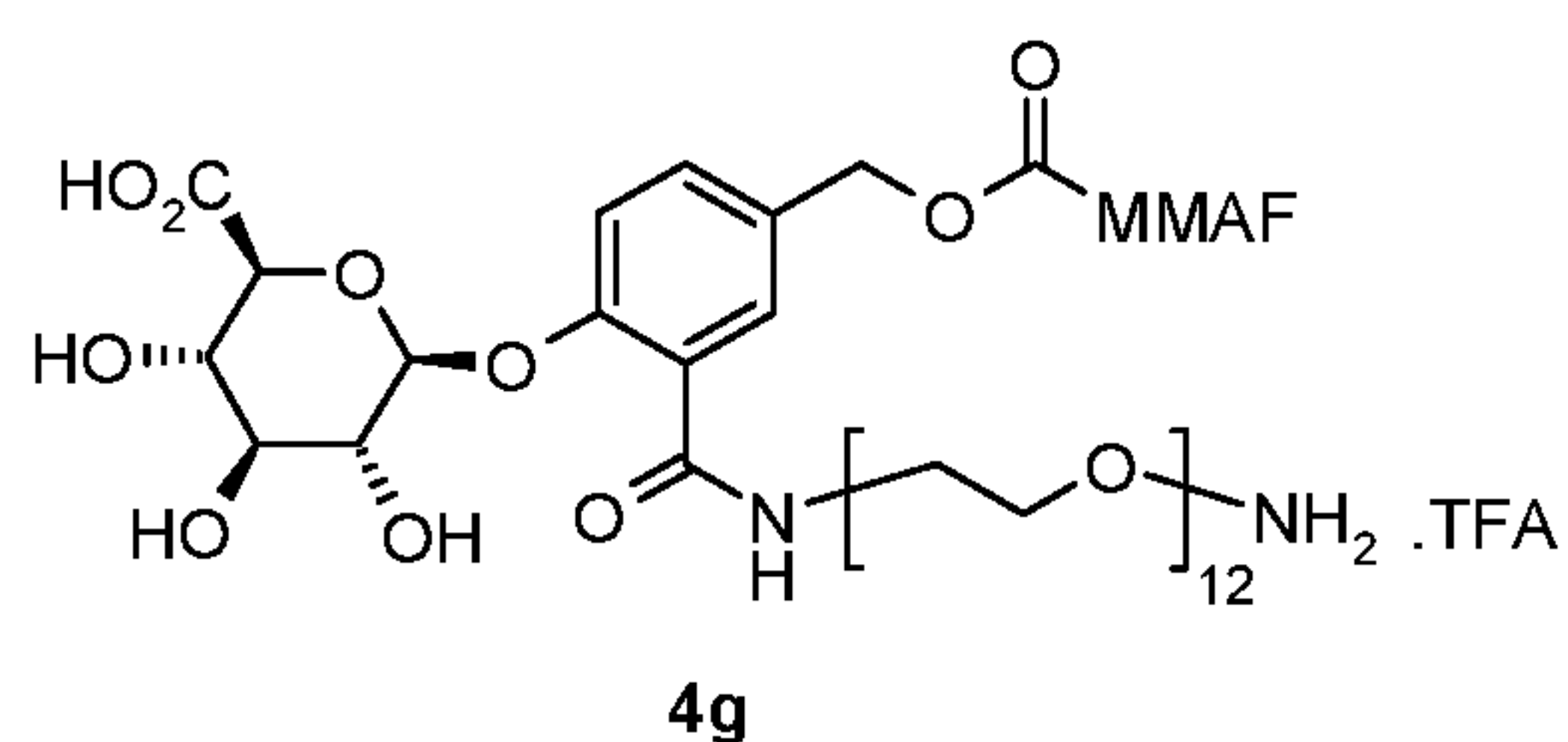
To a stirred mixture of compound **4d** (148 mg, 0.21 mmol), and Pd/C (10 wt. %, 28 mg) in MeOH (5 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.053 mL, 0.21 mmol). After stirring at room temperature for 30 minutes under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (30 mL). The filtrate was concentrated to produce the compound **4e** (142 mg, 96 %) as colorless oil, which was used without further purification. <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 4.00 (t, 2H), 3.92 (t, 2H), 3.76-3.64 (m, 42H), 3.18 (t, 2H) 1.47 (s, 9H).

### Preparation of Compound 4f



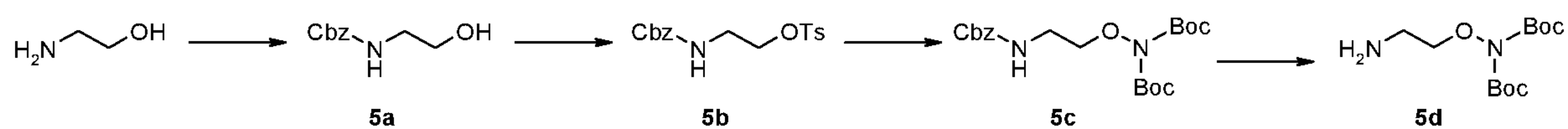
Compound **4f** was prepared from compound **1i** and compound **4e** by a similar method of preparing compound **2g** in Example 3. EI-MS m/z: [M+H]<sup>+</sup> 1631.9.

### Example 8. Preparation of Compound 4g



Compound **4g** was prepared from compound **1j** and compound **4e** by a similar method of preparing compound **2g** in Example 3. EI-MS m/z: EI-MS m/z [M+H]<sup>+</sup> 1645.3.

### Example 9. Preparation of Compound 5e



### Preparation of Compound 5a

To a solution of 2-aminoethanol (10 g, 164 mmol) in DCM (70 mL) were added triethylamine (3.9 mL, 28.1 mmol) and benzyl chloroformate (30 mL, 213 mmol) in DCM (30 mL) at 0 °C under N<sub>2</sub>. After 24 hours, the reaction mixture was concentrated. The resulting residue was diluted with H<sub>2</sub>O (50 mL) and extracted with EtOAc (3 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **5a** (17 g, 53 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.40-7.27 (m, 5H), 5.11 (s, 2H), 3.72 (s, 2H), 3.56 (s, 2H), 2.13 (br s, 1H).

### Preparation of Compound 5b

To a solution of compound **5a** (5.0 g, 25.6 mmol) in DCM (70 mL) triethylamine (3.9 mL, 28.1 mmol) were added DMAP (100 mg, 5.12 mmol) and *p*-toluenesulfonyl chloride (5.4 g, 28.1 mmol) in DCM (30 mL) at 0 °C under N<sub>2</sub>. After 15 hours at 0 °C, the reaction mixture was diluted with saturated aq. NH<sub>4</sub>Cl (100 mL) and extracted with DCM (2 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **5b** (8.29 g, 92 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.77 (d, *J* = 7.6 Hz, 2H), 7.40-7.28 (m, 7H), 5.07 (s, 3H), 4.09 (s, 2H), 3.45 (s, 2H), 2.43 (s, 3H).

### Preparation of Compound 5c

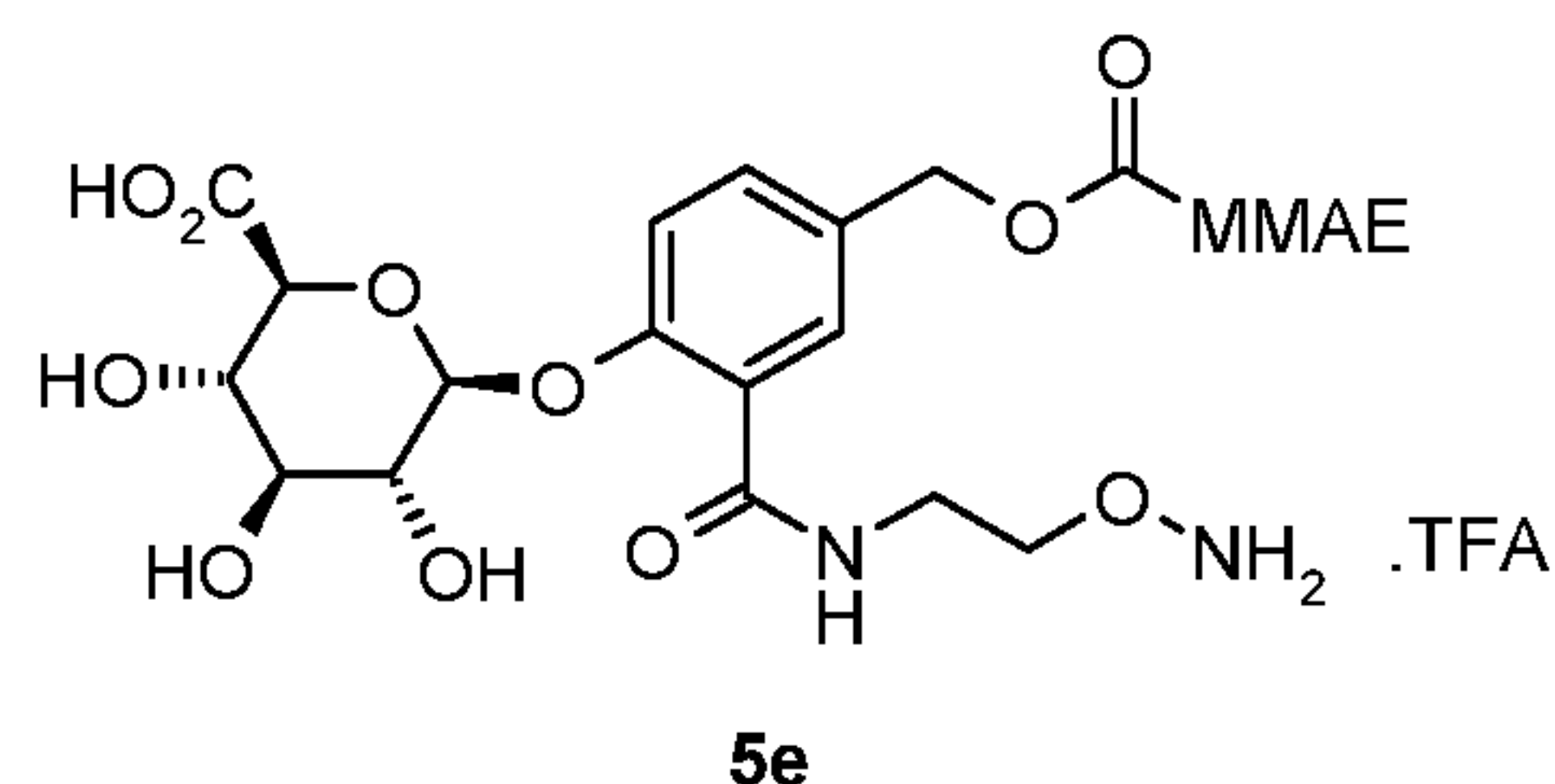
To a solution of compound **5b** (2.0 g, 7.23 mmol) in THF (20 mL) was added *N,N*-diBoc-hydroxylamine (1.7 g, 7.44 mmol) and NaH (300 mg, 6.86 mmol) at 0 °C under N<sub>2</sub>. After stirring at room temperature for 17 hours, the reaction mixture was diluted with saturated aqueous NH<sub>4</sub>Cl (50 mL) and extracted with EtOAc (3 x 50 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **5c** (375 mg, 16 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.45-7.27 (m, 5H), 5.11 (s, 2H), 4.01 (br s, 2H), 3.44 (d, *J* = 4.8 Hz, 2H), 1.52 (s, 18H). EI-MS *m/z*: [M+H]<sup>+</sup> 410.7.

### Preparation of Compound 5d

To a solution of compound **5c** (187 mg, 0.45 mmol) in MeOH (20 mL) Pd/C (10 % wt. %, 20 mg) was added and then the reaction mixture was stirred at room temperature for

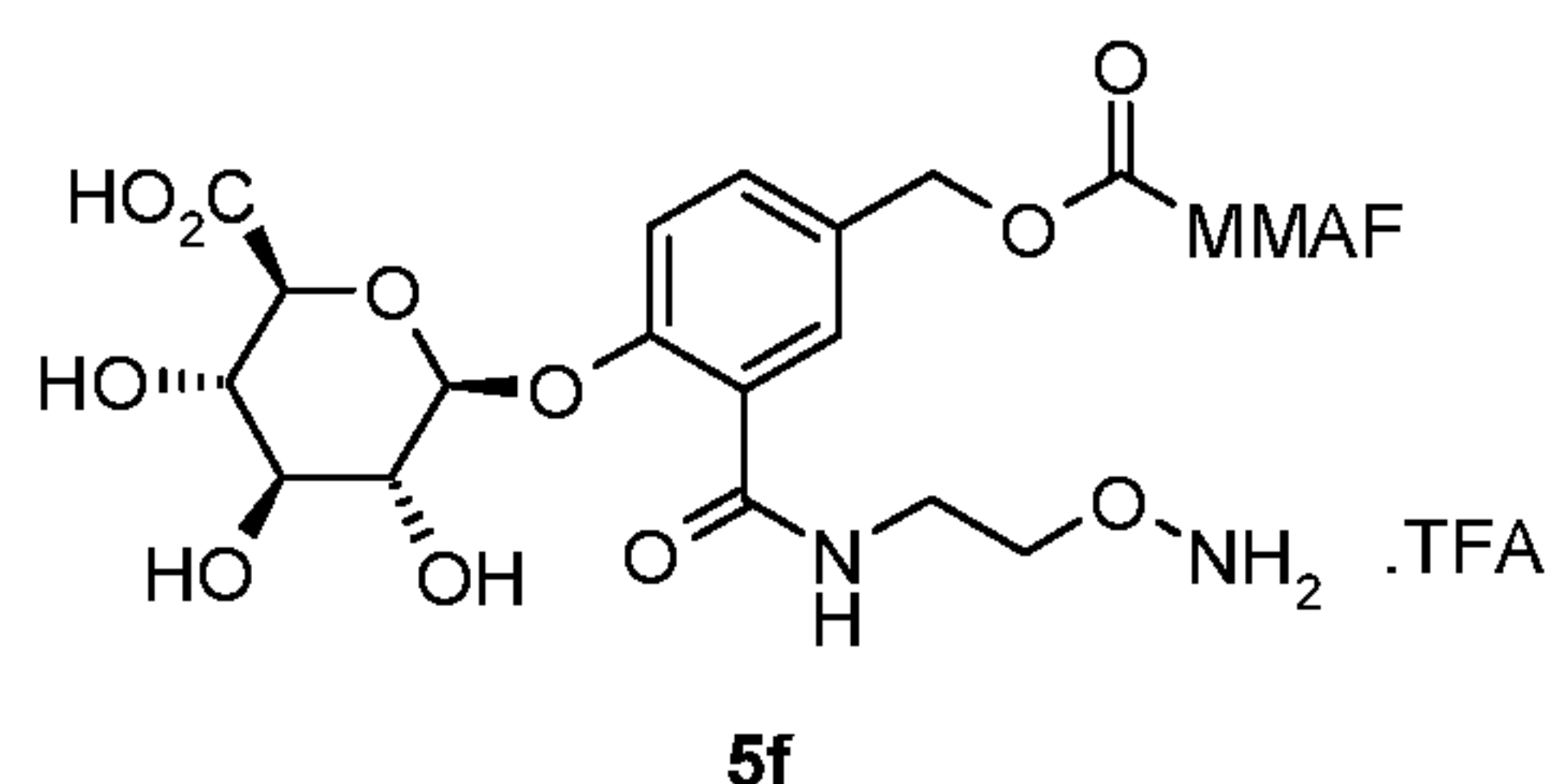
4 hours under hydrogen. The reaction mixture was filtered through a celite pad and washed with MeOH (20 mL). The filtrate was concentrated to produce the compound **5d** (120 mg) as colorless oil, which was used without further purification.

#### Preparation of Compound **5e**



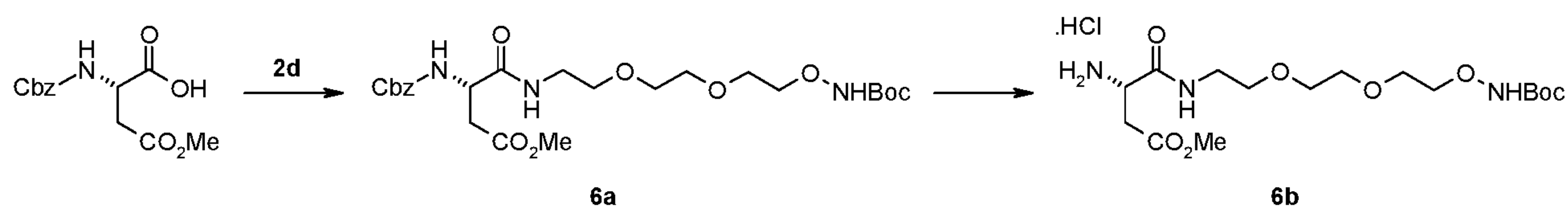
Compound **5e** was prepared from compound **1i** and compound **5d** by a similar method of preparing compound **2g** in Example 3. EI-MS  $m/z$ :  $[M+H]^+$  1146.4.

#### Example 10. Preparation of Compound **5f**



Compound **5f** was prepared from compound **1j** and compound **5d** by a similar method of preparing compound **2g** in Example 3. EI-MS  $m/z$ :  $[M+H]^+$  1160.3.

#### Example 11. Preparation of Compound **6e**



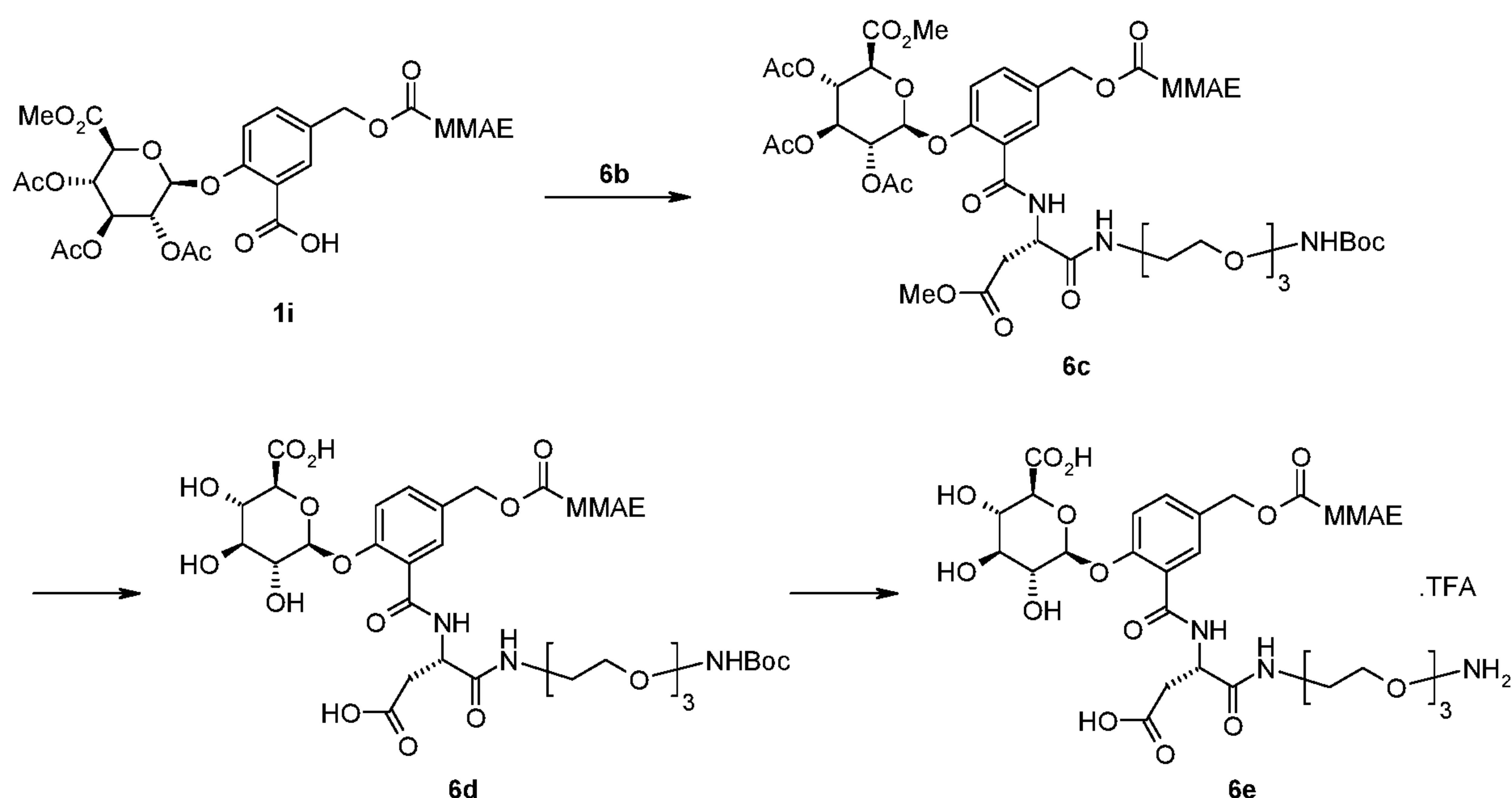
#### Preparation of Compound **6a**

DIPEA (1.2 mL, 9.96 mmol) and HBTU (1.69 g, 6.22 mmol) were added to a stirred mixture of Z-Asp(OMe)-OH (500 mg, 1.78 mmol) and compound **2d** (642 mg, 2.98 mmol) in DMF (5 mL). The reaction mixture was stirred at room temperature for 22 hours under  $N_2$ . The reaction mixture was diluted with  $H_2O$  (100 mL) and extracted with EtOAc (2 x 100 mL). The combined organic layers were dried over anhydrous  $Na_2SO_4$ , filtered and

concentrated. The resulting residue was purified by column chromatography to yield the compound **6a** (368 mg, 40 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.85-7.70 (m, 1 H), 7.45-7.28 (m, 5H), 7.04 (s, 1H), 6.02 (d,  $J = 8.4$  Hz, 1H), 5.11 (s, 2H), 4.65-4.50 (m, 1H), 4.00 (d,  $J = 3.6$  Hz, 2H), 3.72-3.30 (m, 10H), 2.80 (dd,  $J = 5.6$  Hz, 2H), 1.46 (s, 9H).

### Preparation of Compound **6b**

To a stirred mixture of compound **6a** (150 mg, 0.28 mmol) and Pd/C (10 wt. %, 20 mg) in MeOH (5 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.07 mL, 0.28 mmol). After stirring at room temperature for 2 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (20 mL). The filtrate was concentrated to produce the compound **6b** (169 mg) as colorless oil, which was used without further purification. EI-MS  $m/z$ :  $[\text{M}+1]^+$  393.7.



### Preparation of Compound **6c**

DIPEA (0.022 mL, 0.12 mmol) and HBTU (20 mg, 0.05 mmol) were added to a stirred mixture of compound **1i** (50 mg, 0.04 mmol) and compound **6b** (22 mg, 0.05 mmol) in DMF (1 mL). The reaction mixture was stirred at room temperature for 14 hours under  $\text{N}_2$ . Then, the reaction mixture was diluted with  $\text{H}_2\text{O}$  (10 mL) and extracted with EtOAc (2 x 20 mL). The combined organic layers were dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and concentrated. The resulting residue was purified by column chromatography to yield the compound **6c** (38 mg, 60 %). EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  1604.5.

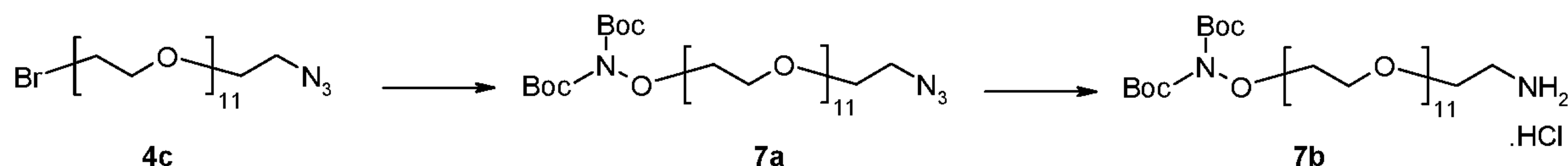
Preparation of Compound 6d

To a solution of compound **6c** (38 mg, 0.023 mmol) in MeOH (1 mL) was added LiOH monohydrate (5 mg, 0.118 mmol) in H<sub>2</sub>O (1 mL) at 0 °C. After 2 hours at 0 °C, the pH of the solution was adjusted with AcOH to 4~5 and concentrated under reduced pressure. The residue was dissolved in DMSO (1.5 mL) and purified by HPLC to produce the compound **6d** (26 mg, 78 %).

EI-MS m/z: [M+H]<sup>+</sup> 1450.5.

Preparation of Compound 6e

TFA (0.3 mL) was added to a stirred solution of compound **6d** (26 mg, 0.018 mmol) in DCM (1.5 mL). After stirring at 0 °C for 2 hours, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in DMSO (1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **6e** (19.5 mg, 80 %) as white solid. EI-MS m/z: [M+H]<sup>+</sup> 1350.6.

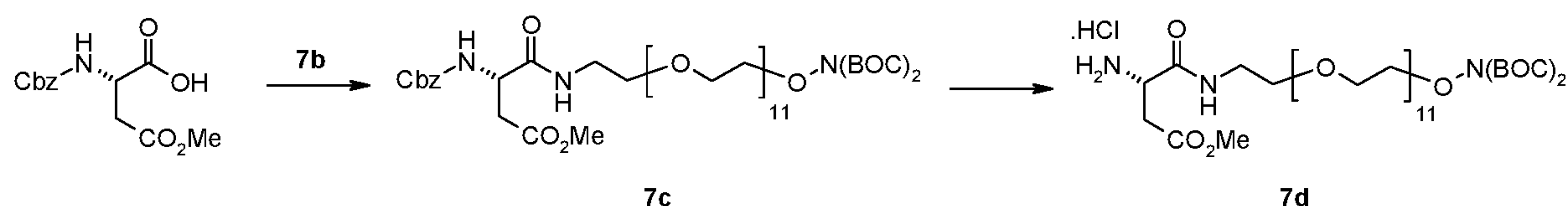
*Example 12. Preparation of Compound 7e*Preparation of Compound 7a

NaH (60 wt. %, 500 mg, 12.49 mmol) was added to a stirred mixture of compound **4c** (6.10 g, 9.61 mmol) and *N,N*-diBoc-hydroxylamine (2.69 g, 11.53 mmol) in DMF (90 mL) at 0 °C. The reaction mixture was heated up to room temperature and kept for 12 hours at this temperature. The reaction mixture was evaporated under reduced pressure and the resulting residue was purified by column chromatography. Pure fractions were evaporated *in vacuo* to yield the compound **7a** (5.70 g, 75 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.05 (t, 2H), 3.71 (t, 2H), 3.64 (m, 42H), 3.37 (t, 2H), 1.51 (d, 18H).

Preparation of Compound 7b

To a stirred mixture of compound **7a** (5.70 g, 7.21 mmol), and Pd/C (10 wt. %, 570 mg) in MeOH (100 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 1.9 mL, 7.2 mmol). After stirring at room temperature for 30 minutes under hydrogen, the reaction mixture was

filtered through a celite pad and washed with MeOH (30 mL). The filtrate was concentrated to produce the compound **7b** (5.10 g, 87 %) as colorless oil, which was used without further purification.  $^1\text{H-NMR}$  (400 MHz, DMSO- $d_6$ )  $\delta$  4.21 (t, 2H), 4.07 (s, 2H), 3.95-3.78 (m, 42H), 3.32 (s, 2H) 1.63 (s, 18H).



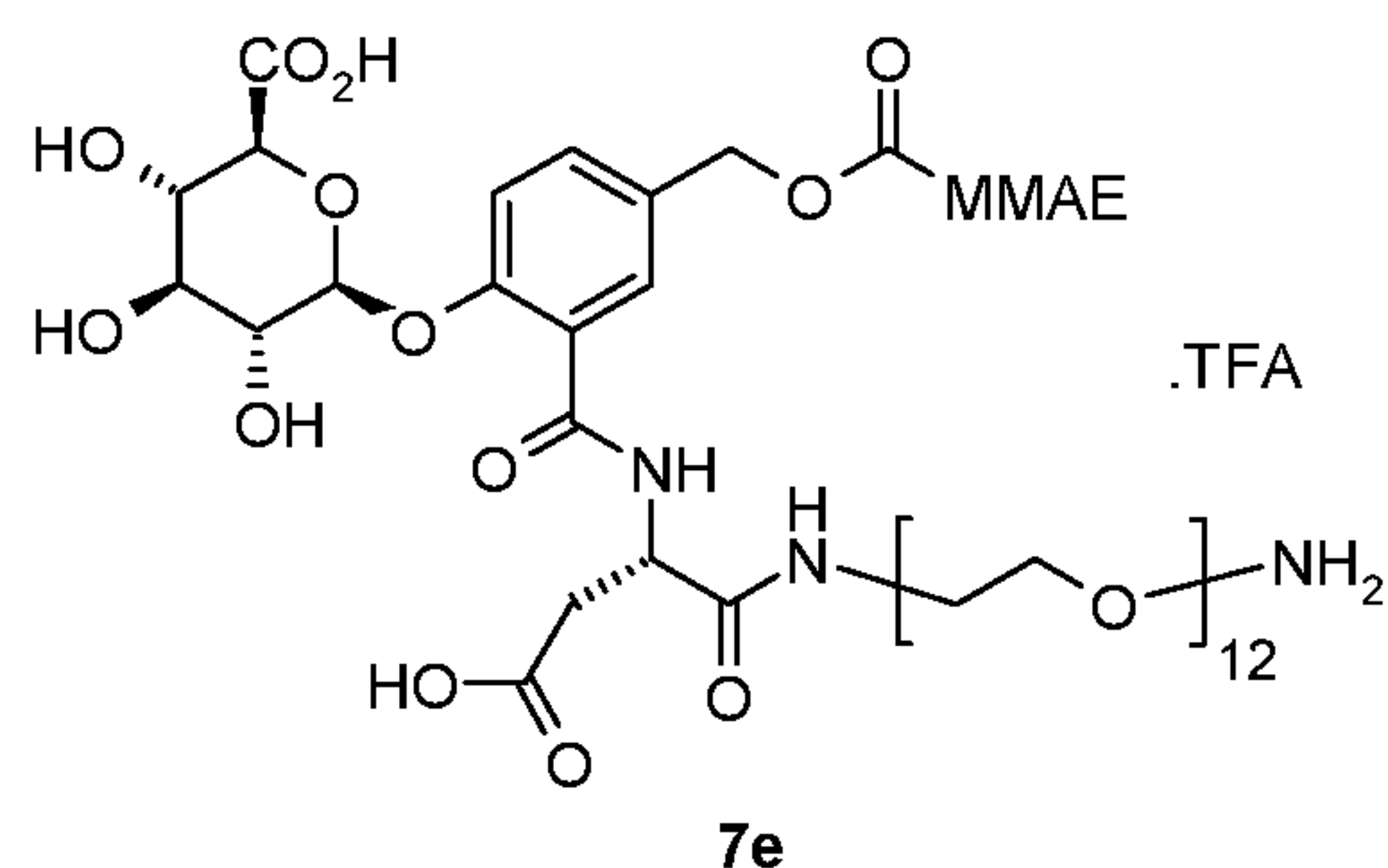
### Preparation of Compound 7c

DIPEA (0.25 mL, 1.42 mmol) and HBTU (337g, 0.89mmol) were added to a stirred mixture of Z-Asp(OMe)-OH (100 mg, 0.36 mmol) and compound **7b** (340 mg, 0.43 mmol) in DMF (10 mL). The reaction mixture was stirred at room temperature for 20 hours under  $\text{N}_2$ . Then, the reaction mixture was diluted with  $\text{H}_2\text{O}$  (100 mL) and extracted with EtOAc (2 x 100 mL). The combined organic layers were dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and concentrated. The resulting residue was purified by column chromatography to yield the compound **7c** (123 mg, 58 %). EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  1024.2.

### Preparation of Compound 7d

To a stirred mixture of compound **7c** (120 mg, 0.12 mmol) and Pd/C (10 wt. %, 20 mg) in MeOH (5 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.03 mL, 0.12 mmol). After stirring at room temperature for 2 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (20 mL). The filtrate was concentrated to produce the compound **7d** (120 mg) as colorless oil, which was used without further purification.

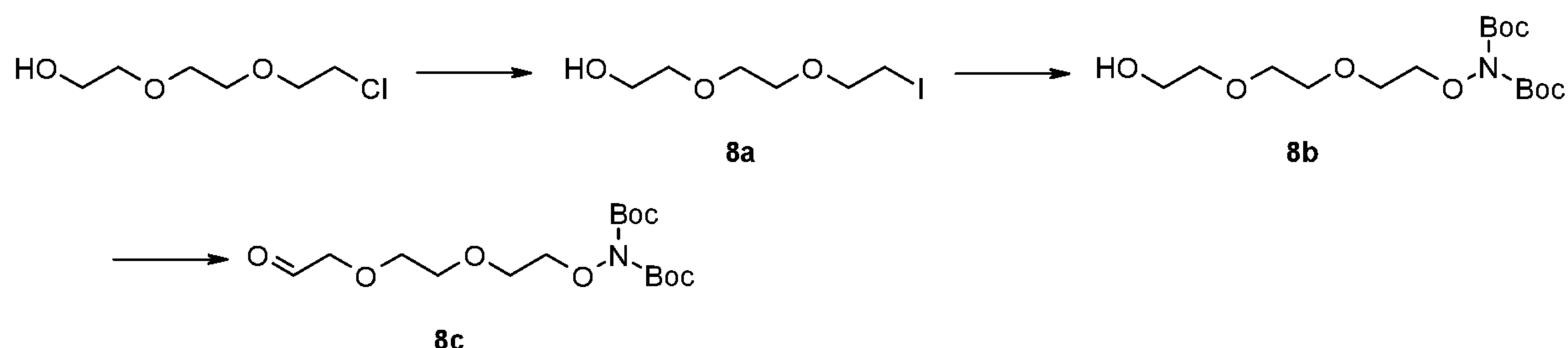
### Preparation of Compound 7e



Compound **7e** was prepared from compound **1i** and compound **7d** by a similar

method of preparing compound **6e** in Example 11. EI-MS m/z: [M+H]<sup>+</sup> 1747.1.

*Example 13. Preparation of Compound 8f*



Preparation of Compound **8a**

To a solution of 2-(2-(2-chloroethoxy)ethoxy)ethanol (5.0 g, 29.6 mmol) in acetone (30 mL) was added NaI (13.3 g, 88.9 mmol). The reaction mixture was refluxed for 12 hours. After the reaction was completed, the reaction mixture was filtered and concentrated. The crude product was purified by column chromatography to produce the compound **8a** (7.0 g, 91 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.80-3.73 (m, 4H), 3.72-3.65 (m, 4H), 3.63-3.61 (m, 2H), 3.27 (t, *J* = 6.4 Hz, 2H).

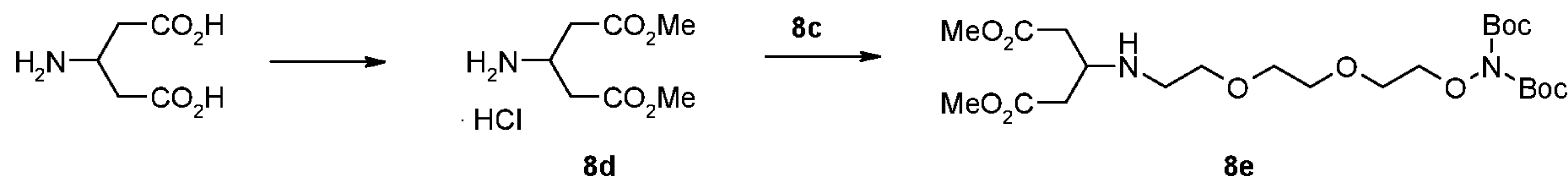
Preparation of Compound **8b**

NaH (500 mg, 12.49 mmol) was added to a stirred mixture of compound **8a** (2.0 g, 7.69 mmol), and *N,N*-diBoc-hydroxylamine (2.33 g, 10.00 mmol) in DMF (20 mL) at 0 °C under N<sub>2</sub>. After stirring at room temperature for 17 hours, the reaction mixture was diluted with saturated aq. NH<sub>4</sub>Cl (50 mL) and extracted with EtOAc (3 x 50 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **8b** (1.54 g, 54 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.45-7.27 (m, 5H), 5.11 (s, 2H), 4.01 (br s, 2H), 3.44 (d, *J* = 4.8 Hz, 2H), 1.52 (s, 18H). EI-MS m/z: [M+H]<sup>+</sup> 410.7.

Preparation of Compound **8c**

To a stirred solution of the compound **8b** (123 mg, 0.242 mmol) in DMSO (2 mL) and DCM (2 mL) were added SO<sub>3</sub>.pyridine complex (116 mg, 0.726 mmol) and triethylamine (0.17 mL, 1.21 mmol) at 0 °C under N<sub>2</sub>. After 1 hour, the reaction mixture was diluted with saturated aq. NH<sub>4</sub>Cl (10 mL) and extracted with DCM (2 x 10 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, and filtered. Concentration

under reduced pressure provided the compound **8c** (88 mg), which was used without further purification.  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  9.74 (s, 1H), 4.19 (s, 2H), 3.77-3.69 (m, 6H), 3.42 (m, 2H).



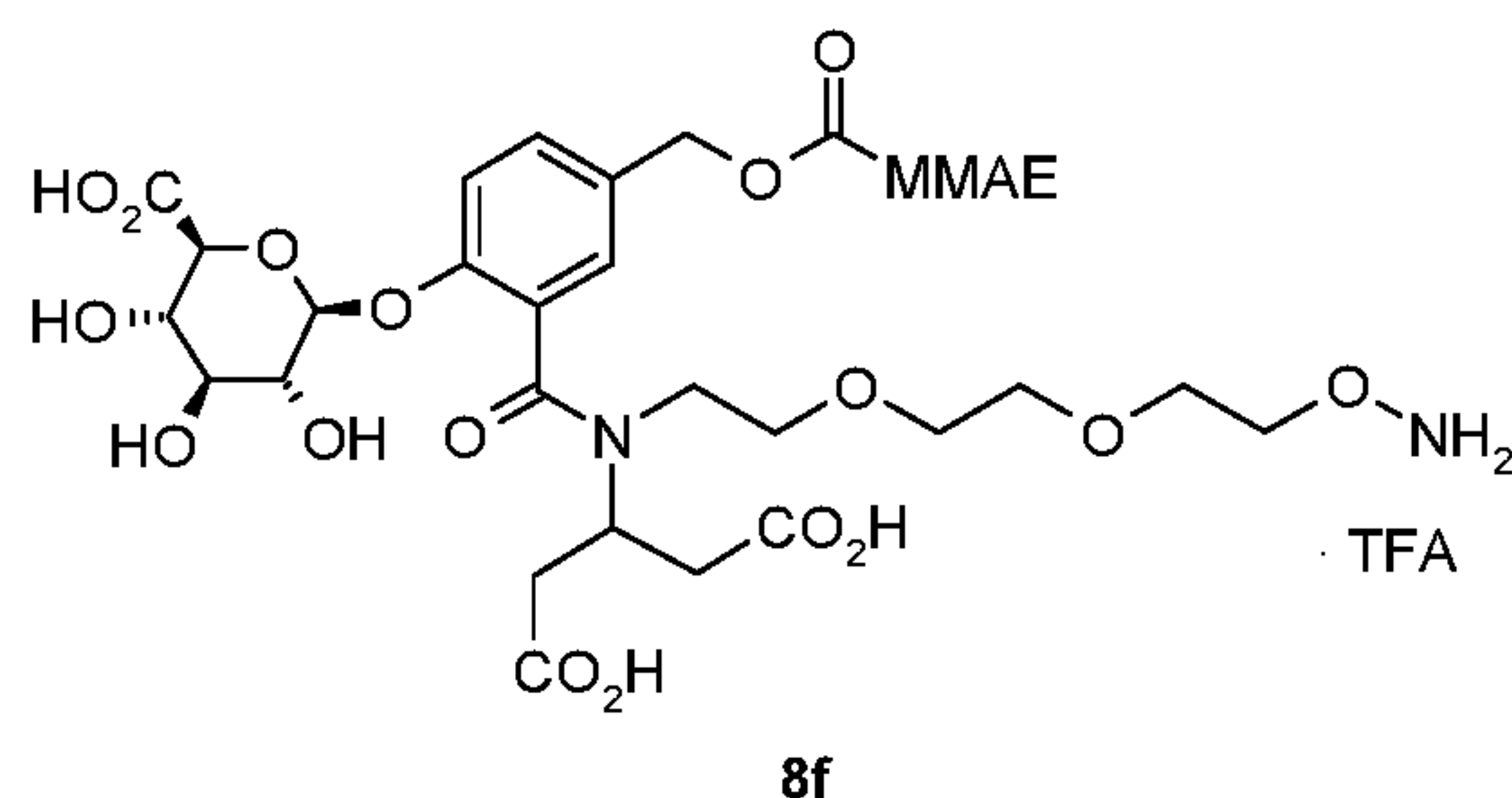
### Preparation of Compound **8d**

To a solution of  $\beta$ -glutamic acid (500 mg, 0.339 mmol) in MeOH (10 mL) was added thionyl chloride (0.148 mL, 2.04 mmol) at 0 °C under  $\text{N}_2$ . After 24 hours, the reaction mixture was concentrated to produce the compound **8d** (697 mg), which was used without further purification.  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.40-7.27 (m, 5H), 5.11 (s, 2H), 3.72 (s, 2H), 3.56 (s, 2H), 2.13 (br s, 1H).

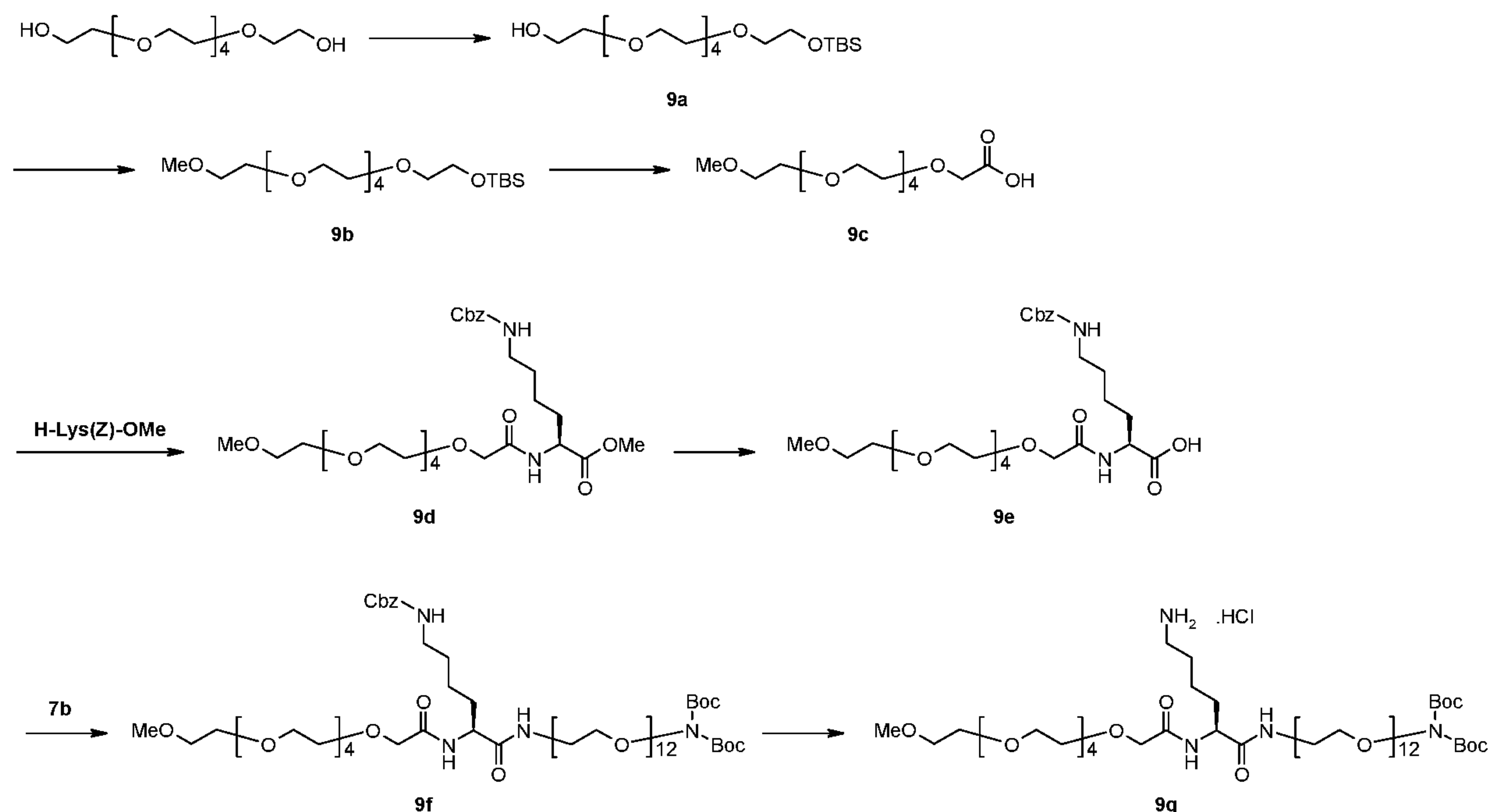
### Preparation of Compound **8e**

To a solution of compound **8d** (34 mg, 0.16 mmol) and compound **8c** (88 mg, 0.24 mmol) in MeOH (5 mL) was added  $\text{NaCNBH}_3$  (10 mg, 0.16 mmol) at room temperature under  $\text{N}_2$ . After 3 hours, the reaction mixture was filtered and concentrated. The crude product was purified by column chromatography to produce the compound **8e** (53 mg, 63 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.45-7.25 (m, 10H), 5.60 (br s, 2H), 5.03 (s, 4H), 3.80-3.25 (m, 20H), 2.81 (s, 4H).

### Preparation of Compound **8f**



Compound **8f** was prepared from compound **1i** and compound **8e** by a similar method of preparing compound **6e** in Example 11. EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  1365.0.

*Example 14. Preparation of Compound 9j*Preparation of Compound 9a

To a solution of hexaethylene glycol (10.48 g, 37.12 mmol) in DCM (400 mL) was added imidazole (3.20 g, 44.54 mmol) at 0 °C under N<sub>2</sub>. After 5 minutes, the reaction mixture was added dropwise to the solution of TBSCl (5.60 g, 37.12 mmol) in DCM (50 mL) at the same temperature under N<sub>2</sub> atmosphere. The reaction mixture was stirred at 0 °C and warmed to room temperature for 21 hours under N<sub>2</sub>. After the reaction was completed, the reaction mixture was diluted with water (200 mL) and extracted with DCM (2 x 100 mL). The organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The resulting residue was purified by column chromatography. The pure fractions were evaporated *in vacuo* to yield the compound **9a** (6.70 g, 46 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.77-3.71 (m, 4H), 3.66-3.60 (m, 18H), 3.56-3.54 (t, 2H), 0.89 (s, 9H), 0.06 (s, 6H).

Preparation of Compound 9b

To a solution of compound **9a** (3.32 g, 8.37 mmol) in dry THF (40 mL) was added NaH (55 % in oil, 438 mg, 10.05 mmol) at 0 °C under N<sub>2</sub>. After 30 minutes, MeI (0.78 mL, 12.56 mmol) was added to the reaction mixture at the same temperature under N<sub>2</sub>. The reaction mixture was stirred and warmed to room temperature for 18 hours under N<sub>2</sub>. After the reaction was completed, quenched with H<sub>2</sub>O (10 mL) and extracted with EA (3 x 10

mL). The organic layers were combined, washed with saturated aq.  $\text{NH}_4\text{Cl}$  (5 mL) and brine (10 mL), dried over anhydrous  $\text{Na}_2\text{SO}_4$  and evaporated under reduced pressure. The resulting residue was purified by column chromatography. Pure fractions were evaporated *in vacuo* to yield the compound **9b** (3.16 g, 92 %).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.78-3.75 (t, 2H), 3.65 (s, 20H), 3.57-3.54 (t, 4H), 3.38 (s, 3H), 0.89 (s, 9H), 0.06 (s, 6H).

#### Preparation of Compound 9c

To a solution of compound **9b** (3.16 g, 7.69 mmol) in acetone (100 mL) was added Jones reagent (10 mL) at 0 °C under  $\text{N}_2$ . The reaction mixture was stirred and warmed to room temperature for 17 hours under  $\text{N}_2$ . After the reaction was completed, the reaction mixture was filtered and evaporated under reduced pressure. The residue was diluted with  $\text{H}_2\text{O}$  (100 mL) and extracted with  $\text{CHCl}_3$  (3 x 50 mL). The organic layers were combined, dried over anhydrous  $\text{Na}_2\text{SO}_4$  and evaporated under reduced pressure. The resulting crude compound **9c** (2.28 g, 95 %) was used without further purification.  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  4.16 (s, 2H), 3.76-3.75 (t, 2H), 3.69-3.67 (m, 16H), 3.57-3.55 (t, 2H), 3.38 (s, 3H).

#### Preparation of Compound 9d

DIPEA (3.8 mL, 22.03 mmol), HOBt (1.29 g, 9.55 mmol) and EDC·HCl (1.83 g, 9.55 mmol) were added to a stirred mixture of compound **9c** (2.28 g, 7.34 mmol) and H-Lys(Z)-OMe hydrochloride (2.91 g, 8.81 mmol) in DMF (30 mL). After stirring at room temperature for 14 hours under  $\text{N}_2$ , the reaction mixture was concentrated. Purification by column chromatography gave the compound **9d** (1.23 g, 72 %).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.38-7.31 (m, 5H), 5.10 (s, 2H), 5.00 (s, 1H) 4.68-4.62 (m, 1H), 4.03 (s, 2H), 3.75 (s, 3H), 3.68-3.64 (m, 16H), 3.56 (t, 2H), 3.39 (s, 3H), 3.20 (m, 2H), 1.89 (m, 1H), 1.74 (m, 1H), 1.55(m, 1H), 1.40 (m, 1H). EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  586.8,  $[\text{M}+\text{Na}]^+$  608.9.

#### Preparation of Compound 9e

To a solution of compound **9d** (2.16 g, 3.68 mmol) in THF/MeOH/ $\text{H}_2\text{O}$  (18 mL/6 mL/6 mL) was added LiOH monohydrate (307 mg, 7.31 mmol) at 0 °C under  $\text{N}_2$ . The reaction mixture was stirred for 1 hour at room temperature. Then the pH of the solution was adjusted to 2~3 with 1 N aq. HCl. The reaction mixture was poured into  $\text{H}_2\text{O}$  (20 mL) and extracted with DCM (3 x 50 mL). The organic layers were combined, dried over

Na<sub>2</sub>SO<sub>4</sub>. Filtration and concentration produced the compound **9e** (2.28 g, 99 %), which was used without further purification. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.34-7.30 (m, 5H), 5.08 (s, 2H), 4.66-4.60 (q, 1H), 4.01 (s, 2H), 3.67-3.55 (m, 18H), 3.37 (s, 3H), 3.20 (m, 2H), 1.87 (m, 1H), 1.72 (m, 1H), 1.53(m, 1H), 1.38 (m, 1H).

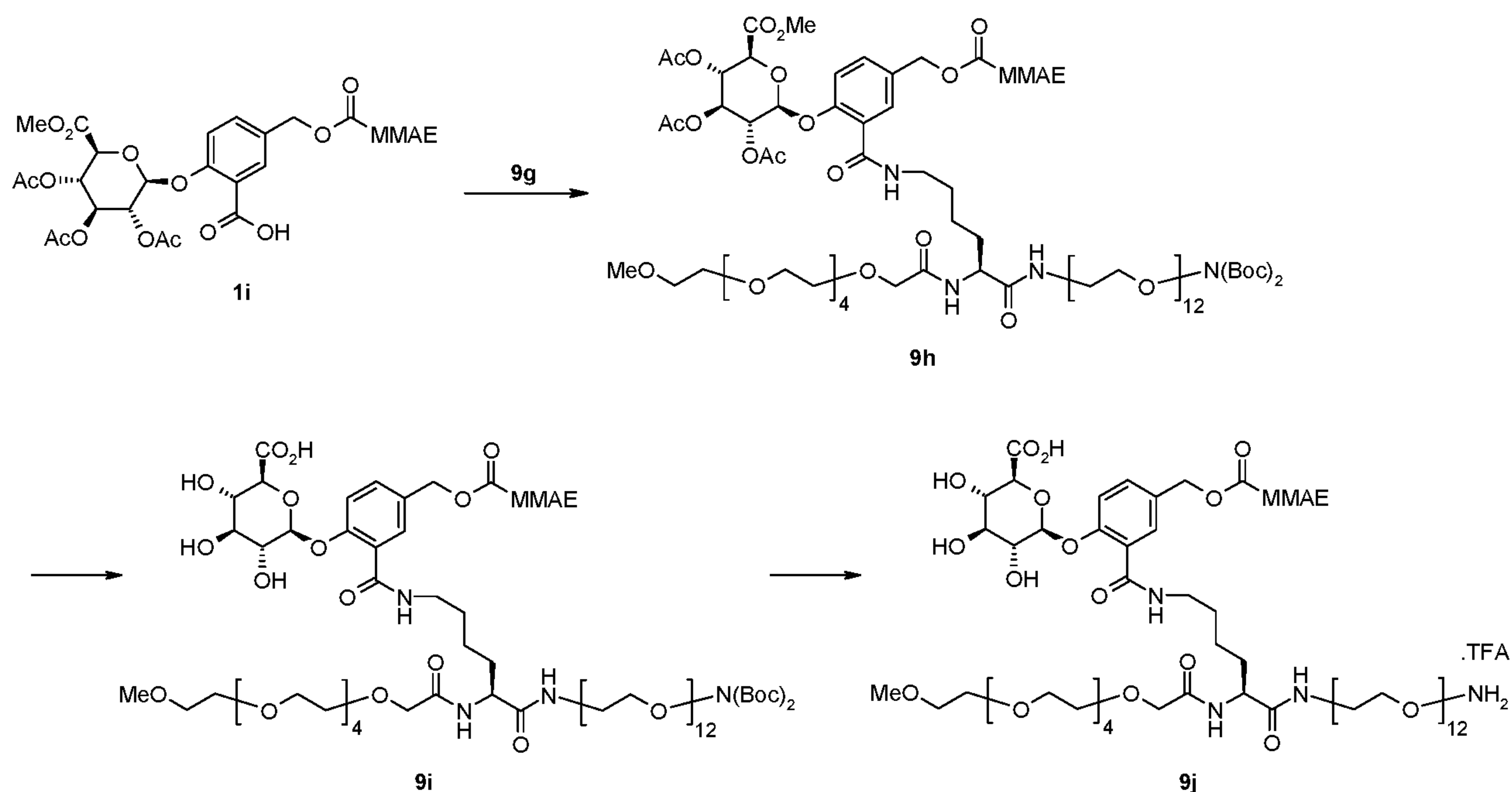
#### Preparation of Compound 9f

DIPEA (0.45 mL, 2.63 mmol), HOBt (154 mg, 0.11 mmol) and EDC·HCl (218 mg, 0.11 mmol) were added to a stirred mixture of compound **9e** (502 mg, 0.88 mmol) and compound **7b** (700 mg, 0.88 mmol) in DMF (8 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (20 mL) and extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with aq. NaHCO<sub>3</sub> (20 mL) and brine (20 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting residue was purified by column chromatography to yield the compound **9f** (499 mg, 43 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.35-7.30 (m, 5H), 6.83 (s, 1H), 5.15 (s, 1H), 5.08 (s, 2H), 4.43 (q, 1H) 4.07 (t, 1H), 3.65-3.60 (m, 54H), 3.55-3.53 (m, 4H), 3.37 (s, 3H), 3.16 (m, 2H), 1.85 (m, 1H), 1.53-1.52 (d, 19H), 1.38(m, 2H). EI-MS m/z: [M+H]<sup>+</sup> 1337.5.

#### Preparation of Compound 9g

To a stirred mixture of compound **9f** (499 mg, 0.37 mmol) and Pd/C (10 wt. %, 50 mg) in MeOH (20 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.1 mL, 0.37 mmol). After stirring at room temperature for 90 minutes under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (10 mL). The filtrate was concentrated to produce the compound **9g** (458 mg, 98 %) as colorless oil, which was used without further purification. EI-MS m/z: [M+H]<sup>+</sup> 1218.6.

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### Preparation of Compound 9h

DIPEA (0.019 mL, 0.11 mmol) and HBTU (18 mg, 0.05 mmol) were added to a stirred mixture of compound **1i** (45 mg, 0.04 mmol) and compound **9g** (57 mg, 0.05 mmol) in DMF (0.5 mL). The reaction mixture was stirred at room temperature for 14 hours under N<sub>2</sub>. The reaction mixture was diluted with H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC, which produced compound **9h** (65 mg, 57 %). EI-MS *m/z*: 1/2[M+H]<sup>+</sup> 1181.7.

### Preparation of Compound 9i

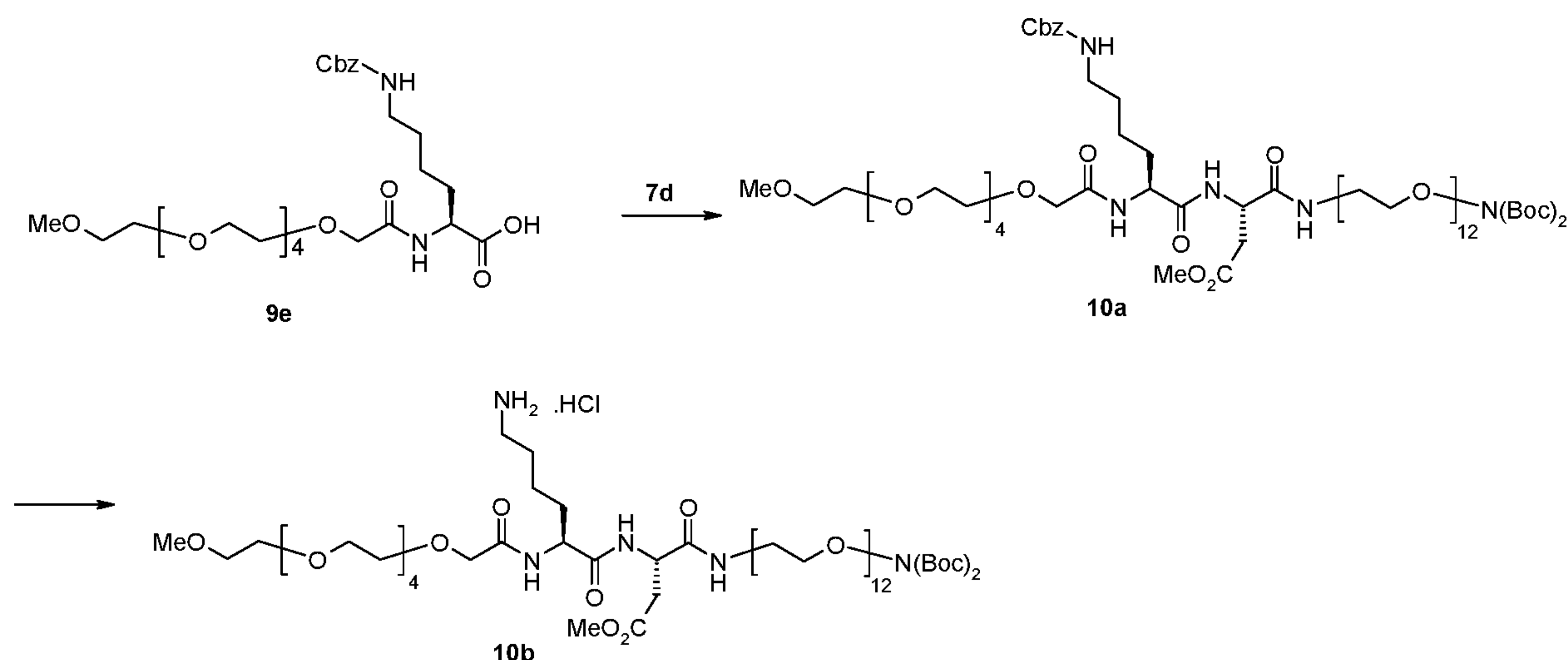
To a solution of compound **9h** (65 mg, 0.03 mmol) in MeOH (1.5 mL) was added LiOH monohydrate (10 mg, 0.24 mmol) in H<sub>2</sub>O (1.5 mL) at 0 °C. After 1 hour at 0 °C, the pH of the solution was adjusted with AcOH to 4~5 and concentrated under reduced pressure. Then the reaction mixture was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC, which produced compound **9i** (45 mg, 55 %). EI-MS *m/z*: 1/2[M+Na]<sup>+</sup> 1098.7.

### Preparation of Compound 9j

TFA (0.2 mL) was added to a stirred solution of compound **9i** (45 mg, 0.02 mmol) in DCM (1 mL). After stirring at 0 °C for 30 minutes, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in H<sub>2</sub>O/DMSO (1 mL/1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **9j** (14 mg, 32 %) as white solid. EI-MS *m/z*:

$1/2[M+H]^+$  1026.3.

*Example 15. Preparation of Compound 10c*

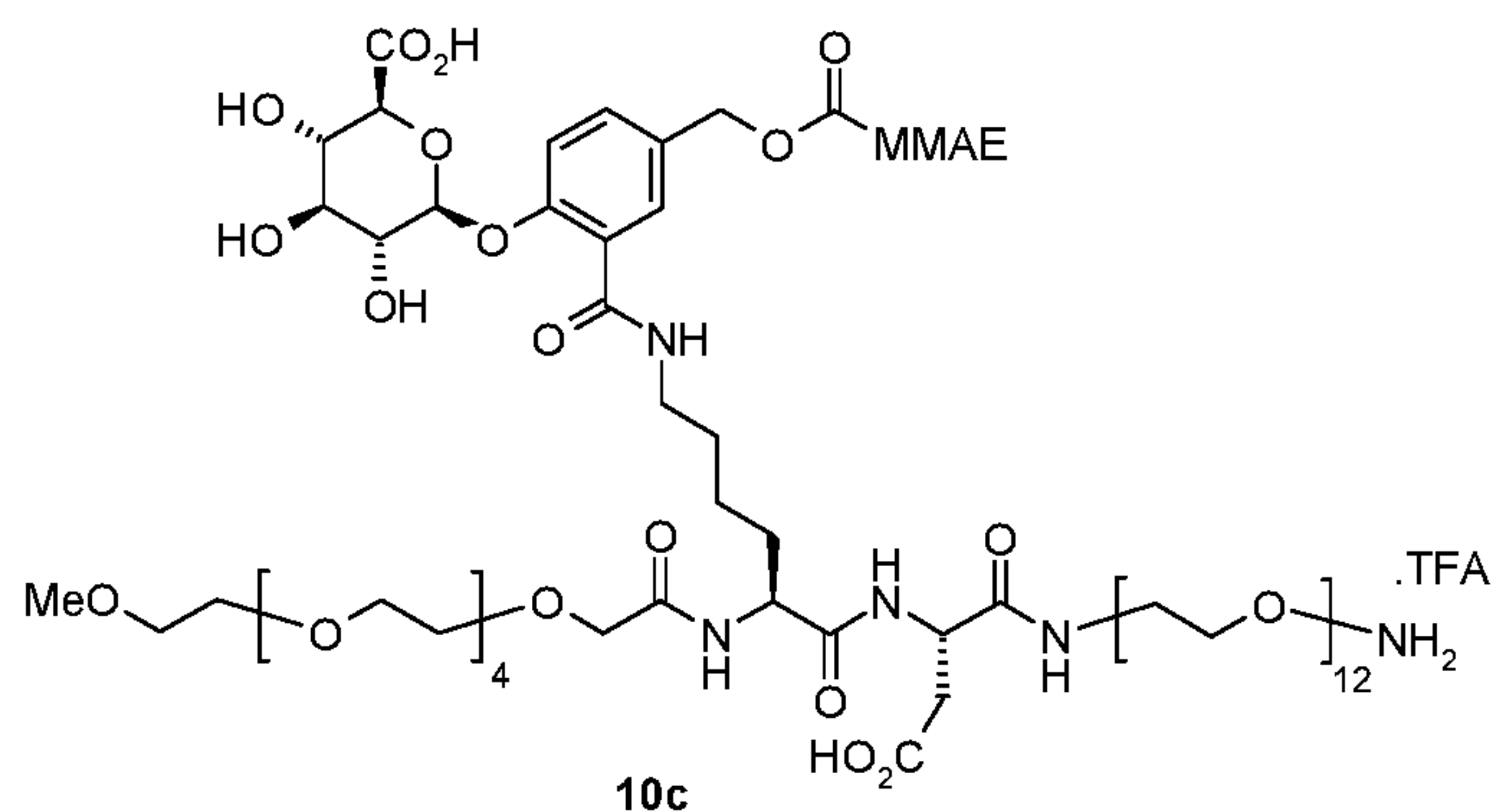


Preparation of Compound 10a

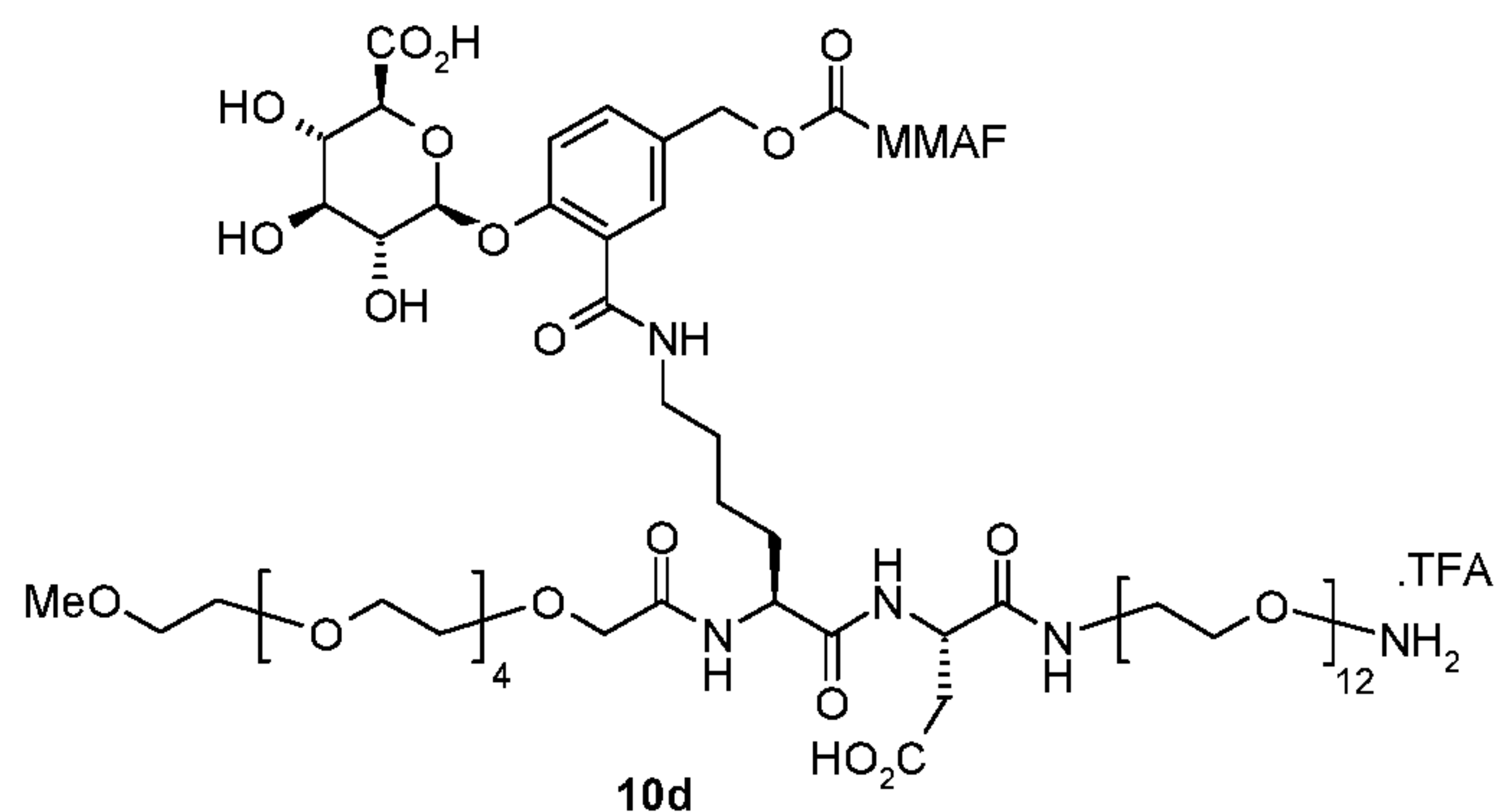
DIPEA (0.03 mL, 0.17 mmol), HOBt (10 mg, 0.075 mmol) and EDC·HCl (14 mg, 0.075 mmol) were added to a stirred mixture of compound **9e** (33 mg, 0.058 mmol) and compound **7d** (54 mg, 0.058 mmol) in DMF (3 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (10 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with 1 N aq. HCl (8 mL), saturated aq. NaHCO<sub>3</sub> (8 mL) and brine (8 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **10a** (61 mg, 73 %). EI-MS m/z:  $[M+H]^+$  1445.0,  $[M+H-Boc]^+$  1344.9.

Preparation of Compound 10b

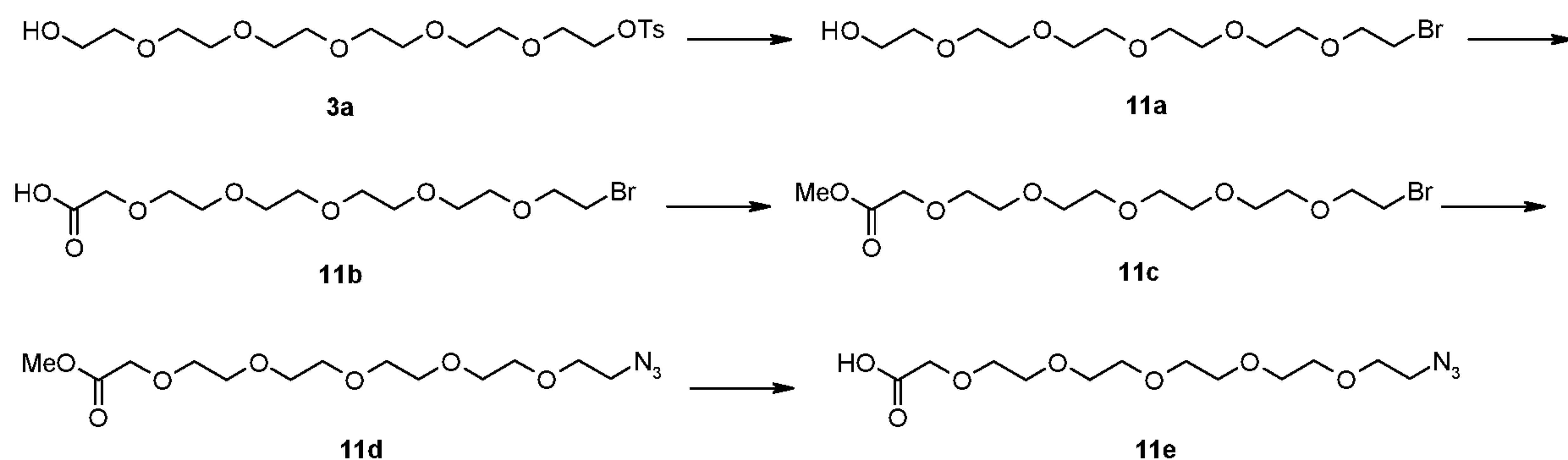
To a stirred mixture of compound **10a** (60 mg, 0.04 mmol), and Pd/C (10 wt. %, 30 mg) in MeOH (10 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.01 mL, 0.01 mmol). After stirring at room temperature for 3 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (40 mL). The filtrate was concentrated to produce the compound **10b** (56 mg, 100 %) as colorless oil, which was used without further purification. EI-MS m/z:  $[M+H]^+$  1311.0,  $[M+Na]^+$  1332.9.

Preparation of Compound 10c

Compound **10c** was prepared from compound **1i** and compound **10b** by a similar method of preparing compound **9j** in Example 14. EI-MS  $m/z$ :  $1/2[M+H]^+$  1083.8.

*Example 16. Preparation of Compound 10d*

Compound **10d** was prepared from compound **1j** and compound **10b** by a similar method of preparing compound **9j** in Example 14. EI-MS  $m/z$ :  $[M+H]^+$  2181.3,  $1/2[M+H]^+$  1091.3.

*Example 17. Preparation of Compound 11j*

### Preparation of Compound 11a

To a solution of compound **3a** (8.0 g, 18.3 mmol) in THF (50 mL) was added LiBr (7.9 g, 91.6 mmol) at room temperature. After stirring for 17 hours under reflux, the reaction mixture was filtered and concentrated. The crude product was purified by column chromatography to produce the compound **11a** (3.2 g, 50 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.95-3.50 (m, 24H).

### Preparation of Compound 11b

To a solution of compound **11a** (3.2 g, 12.3 mmol) in acetone (20 mL) at 0 °C was added Jones reagent (20 mL). After 15 hours at 0 °C, the reaction mixture was filtered and concentrated. The residue was diluted with H<sub>2</sub>O (50 mL) and extracted with EtOAc (2 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **11b** (3.2 g, 72 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.16 (s, 2H), 3.95-3.30 (m, 20H).

### Preparation of Compound 11c

To a solution of compound **11b** (3.2 g, 8.90 mmol) in MeOH (30 mL) was added oxalyl chloride (1.15 mL, 13.3 mmol) at 0 °C under N<sub>2</sub>. After 16 hours, the reaction mixture was concentrated and purified by column chromatography, which produced the compound **11c** (2.7 g, 81 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.17 (s, 2H), 3.80-3.60 (m, 21H), 3.47 (t, *J* = 6.4 Hz, 2H).

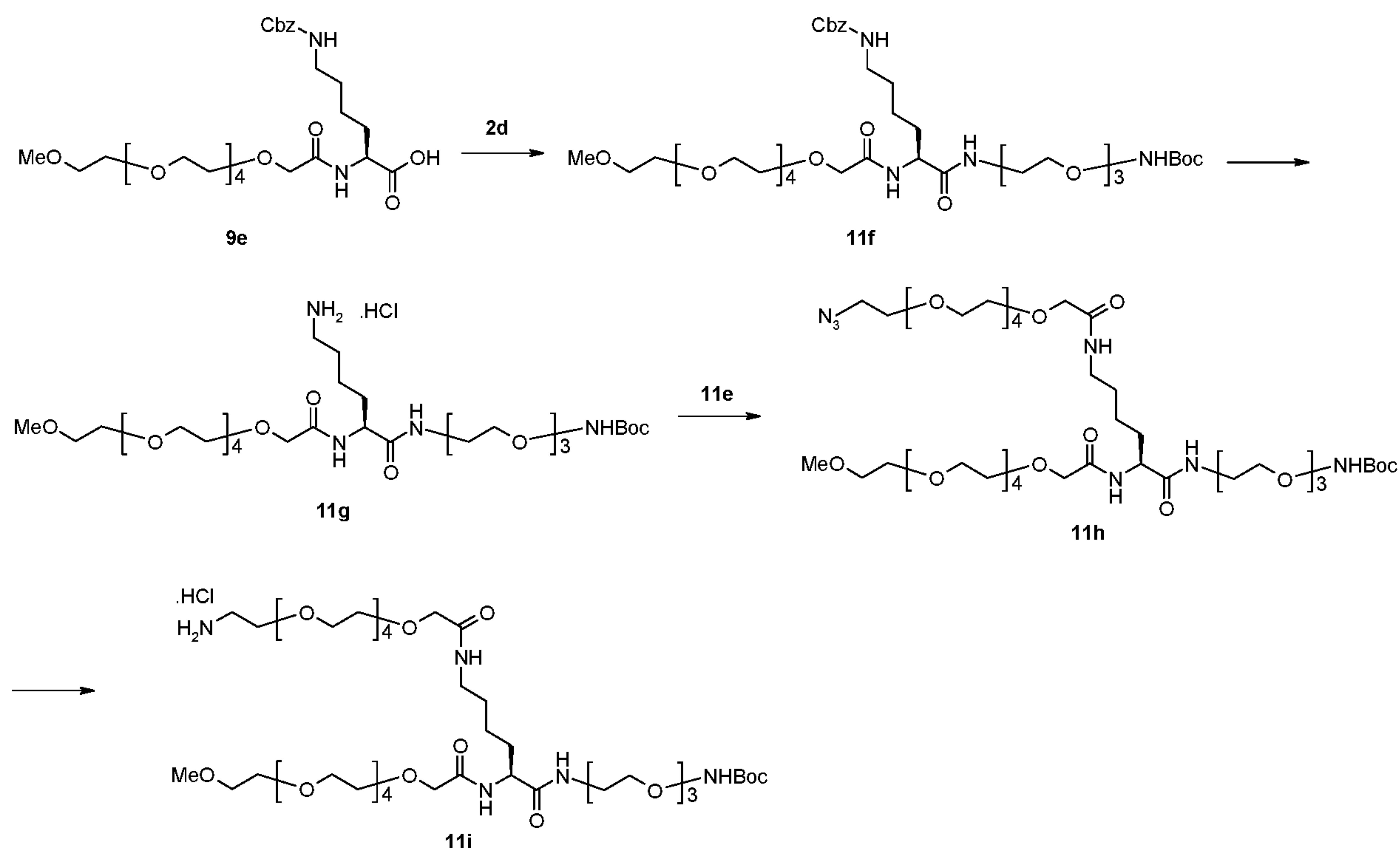
### Preparation of Compound 11d

Compound **11c** (1.0 g, 2.67 mmol) and NaN<sub>3</sub> (261 mg, 4.01 mmol) were dissolved in DMF (3 mL). The reaction mixture was heated at 100 °C for 5 hours. After the reaction was completed, the reaction mixture was filtered and concentrated. The residue was purified by column chromatography (EtOAc to EtOAc/MeOH 10/1), which produced the compound **11d** (854 mg, 95 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.17 (s, 2H), 3.76-3.64 (m, 21H), 3.39 (t, *J* = 5.2 Hz, 2H).

### Preparation of Compound 11e

To a stirred solution of compound **11d** (854 mg, 2.54 mmol) in MeOH (25 mL) at 0

°C was added 2 M aq. NaOH (6.3 mL, 12.64 mmol). The reaction mixture was stirred at room temperature for 3 hours. The solution was then concentrated under reduced pressure. The resulting suspension was acidified with aqueous 2 N HCl while cooling at 0 °C. The residue was extracted by CHCl<sub>3</sub> (8 x 50 mL). The organic layers were combined, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated to produce the compound **11e** (783 mg, 96 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.16 (s, 2H), 3.76-3.65 (m, 18H), 3.40 (t, *J* = 5.2 Hz, 2H).



### Preparation of Compound **11f**

DIPEA (0.47 mL, 2.72 mmol), HOBt (160 mg, 1.18 mmol) and EDC·HCl (226 mg, 1.18 mmol) were added to a stirred mixture of compound **9e** (520 mg, 0.91 mmol) and compound **2d** (270 mg, 0.91 mmol) in DMF (5 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (20 mL) and extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with 1 N aq. HCl (15 mL), saturated aq. NaHCO<sub>3</sub> (15 mL) and brine (15 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **11f** (631 mg, 85 %).

EI-MS *m/z*: [M+H]<sup>+</sup> 819.1, [M+H-Boc]<sup>+</sup> 719.1 [M+Na]<sup>+</sup> 841.1.

### Preparation of Compound **11g**

To a stirred mixture of compound **11f** (300 mg, 0.36 mmol), and Pd/C (10 wt. %, 70

mg) in MeOH (20 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.08 mL, 0.08 mmol). After stirring at room temperature for 3 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (40 mL). The filtrate was concentrated to produce the compound **11g** (200 mg, 99 %) as colorless oil, which was used without further purification. EI-MS m/z:  $[M+H]^+$  685.1,  $[M+Na]^+$  707.1.

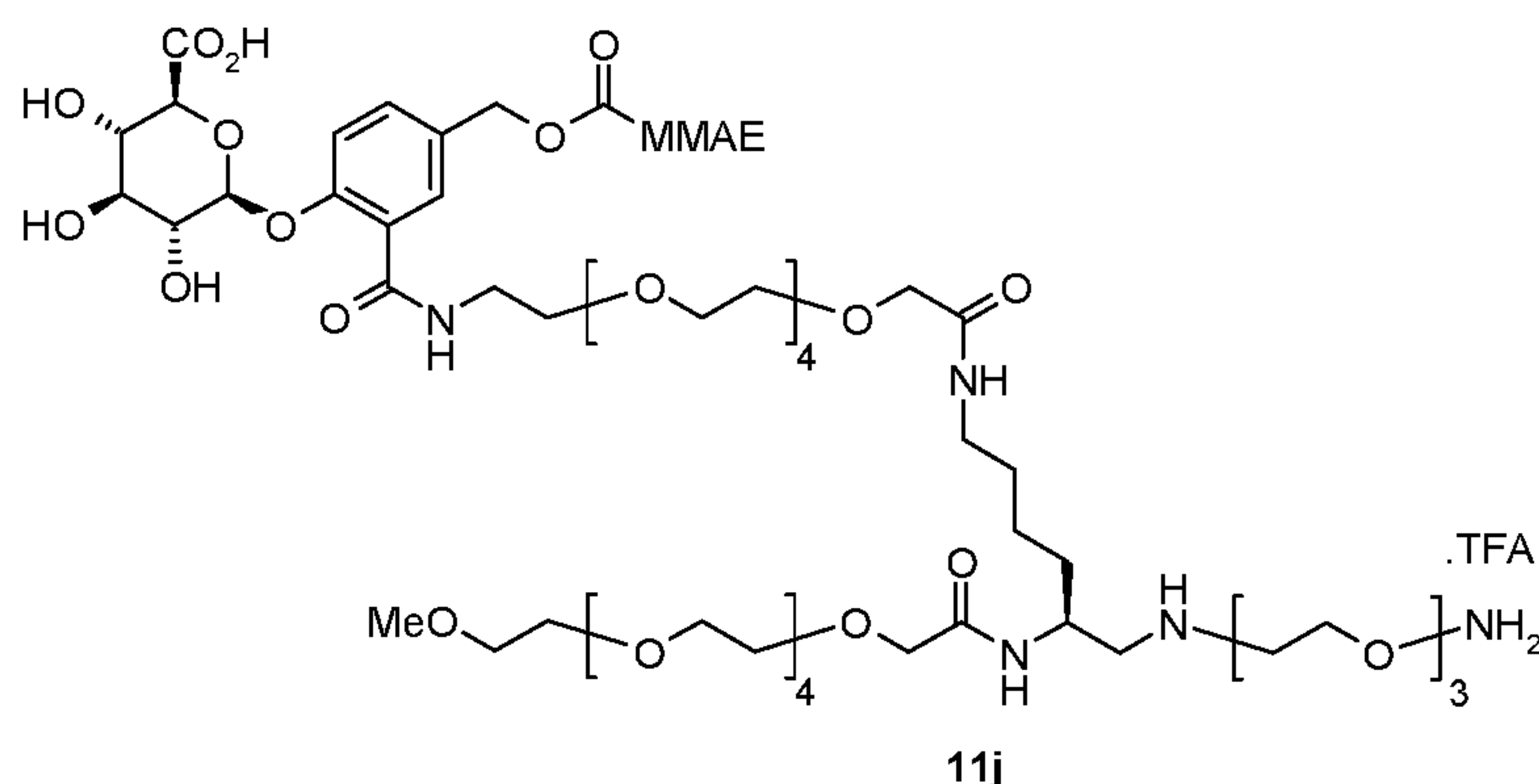
#### Preparation of Compound 11h

DIPEA (0.024 mL, 0.41 mmol), HOBt (24 mg, 0.18 mmol) and EDC·HCl (34 mg, 0.18 mmol) were added to a stirred mixture of compound **11g** (100 mg, 0.14 mmol) and compound **11e** (44 mg, 0.14 mmol) in DMF (5 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (10 mL) and extracted with DCM (3 x 10 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **11h** (73 mg, 53 %). EI-MS m/z:  $[M+H]^+$  988.4,  $[M+Na-Boc]^+$  888.2,  $[M+Na]^+$  1010.4.

#### Preparation of Compound 11i

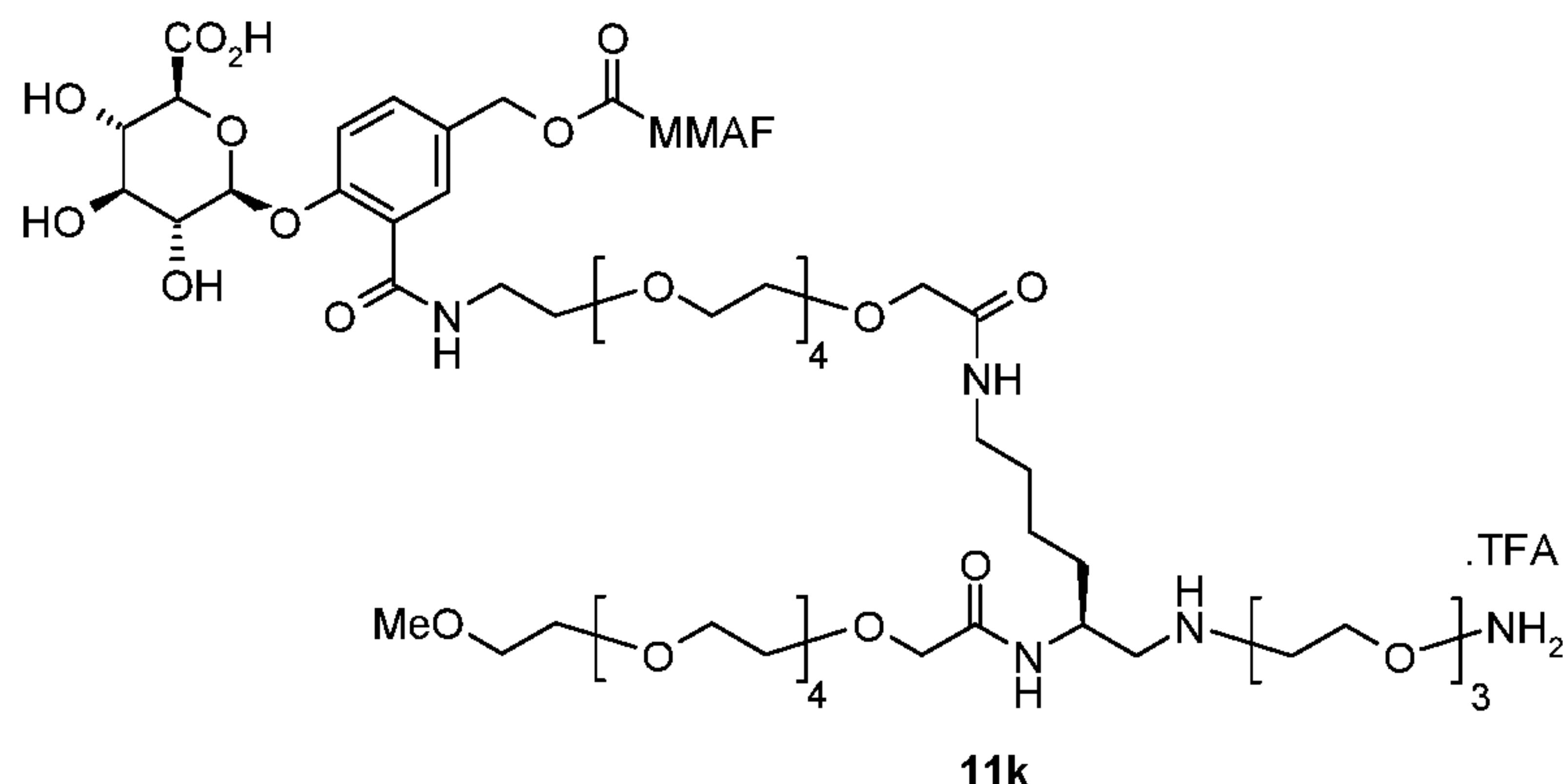
To a stirred mixture of compound **11h** (73 mg, 0.07 mmol), and Pd/C (10 wt. %, 10 mg) in MeOH (7 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.018 mL, 0.018 mmol). After stirring at room temperature for 2 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (30 mL). The filtrate was concentrated to produce the compound **11i** (72 mg, 99 %) as colorless oil, which was used without further purification. EI-MS m/z:  $[M+H]^+$  962.4,  $[M+Na]^+$  984.4.

#### Preparation of Compound 11j



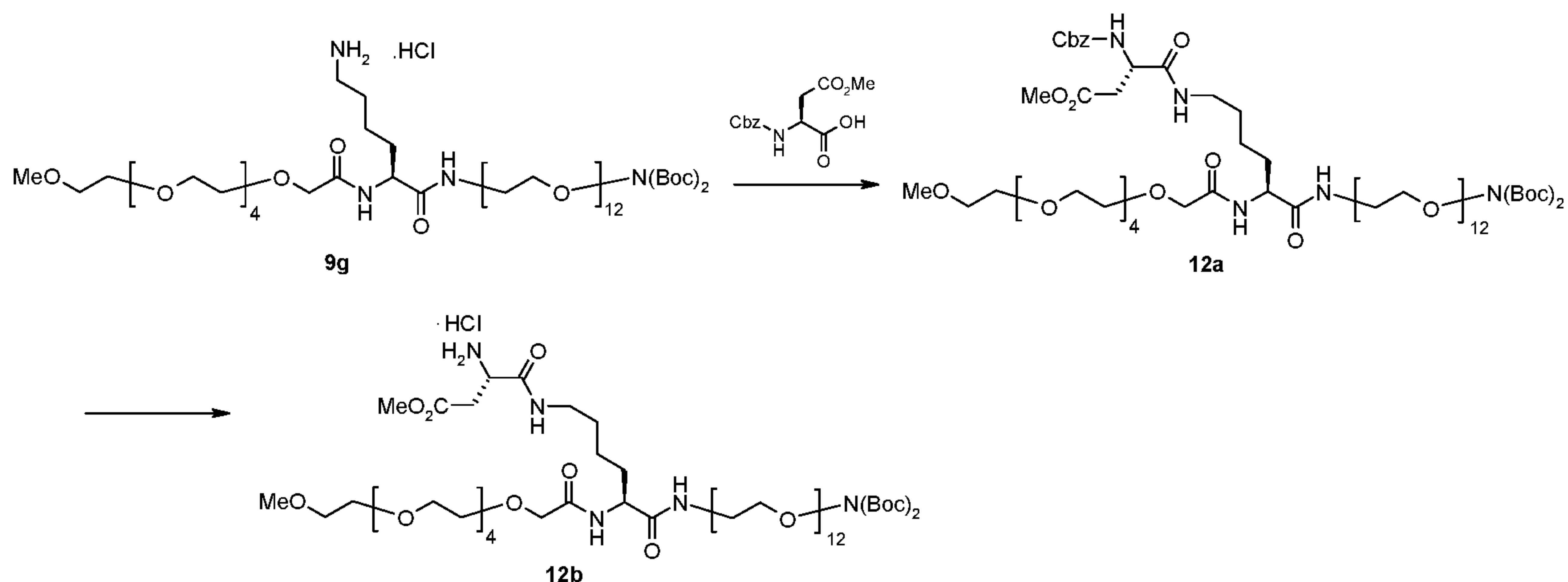
Compound **11j** was prepared from compound **1i** and compound **11i** by a similar method of preparing compound **9j** in Example 14. EI-MS m/z:  $[M+H]^+$  1932.5.

*Example 18. Preparation of Compound 11k*



Compound **11k** was prepared from compound **1j** and compound **11i** by a similar method of preparing compound **9j** in Example 14. EI-MS m/z:  $[M+H]^+$  1947.1.

*Example 19. Preparation of Compound 12c*



Preparation of Compound 12a

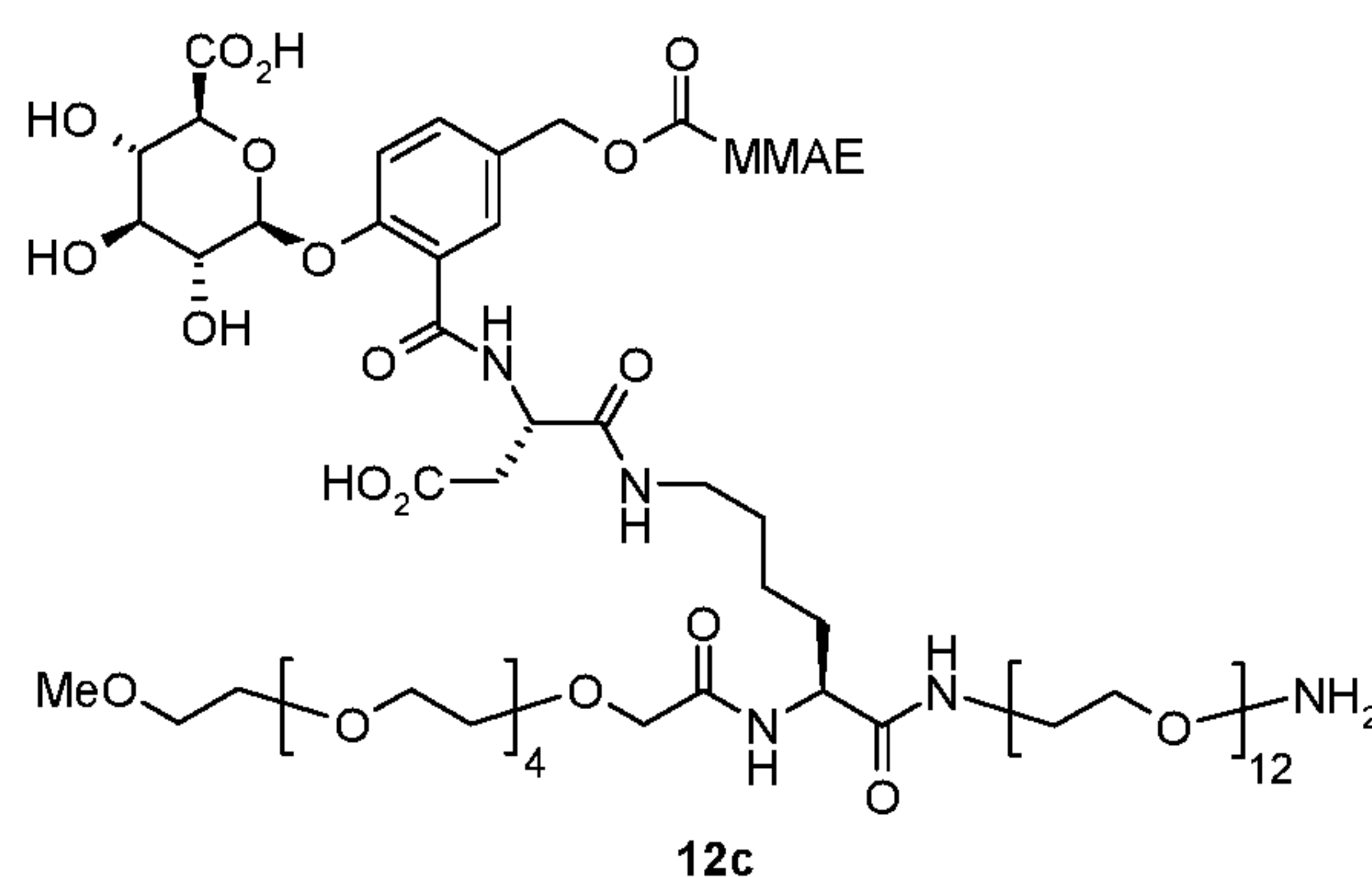
DIPEA (0.13 mL, 0.77 mmol) and HBTU (110 mg, 0.35 mmol) were added to a stirred mixture of compound **9g** (235 mg, 0.1929 mmol) and Z-Asp(OMe)-OH (54 mg, 0.212 mmol) in DMF (5 mL). After stirring at room temperature for 14 hours under  $N_2$ , the reaction mixture was poured into  $H_2O$  (20 mL) and extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with 1 N aq. HCl (7 mL), saturated aq.  $NaHCO_3$  (7 mL) and brine (7 mL), and dried over anhydrous  $Na_2SO_4$ . After filtration and concentration, the residue was purified by column chromatography, which produced the compound **12a** (260 mg, 93 %).  $^1H$ -NMR (400 MHz,  $CDCl_3$ )  $\delta$  8.07 (t, 1H), 7.62 (t, 1H), 7.54-7.52 (m, 1H), 5.73 (s, 2H), 4.27-4.25 (q, 1H), 3.96 (t, 2H), 3.88 (s, 2H), 3.82 (s, 2H), 3.58-3.48 (m,

52H), 3.19-3.18 (m, 3H), 3.04-3.03 (m, 3H), 1.44 (s, 18H), 1.39-1.37 (m, 3H), 1.21-1.19 (m, 3H). EI-MS m/z:  $[M+H-2Boc]^+$  1031.6.

### Preparation of Compound 12b

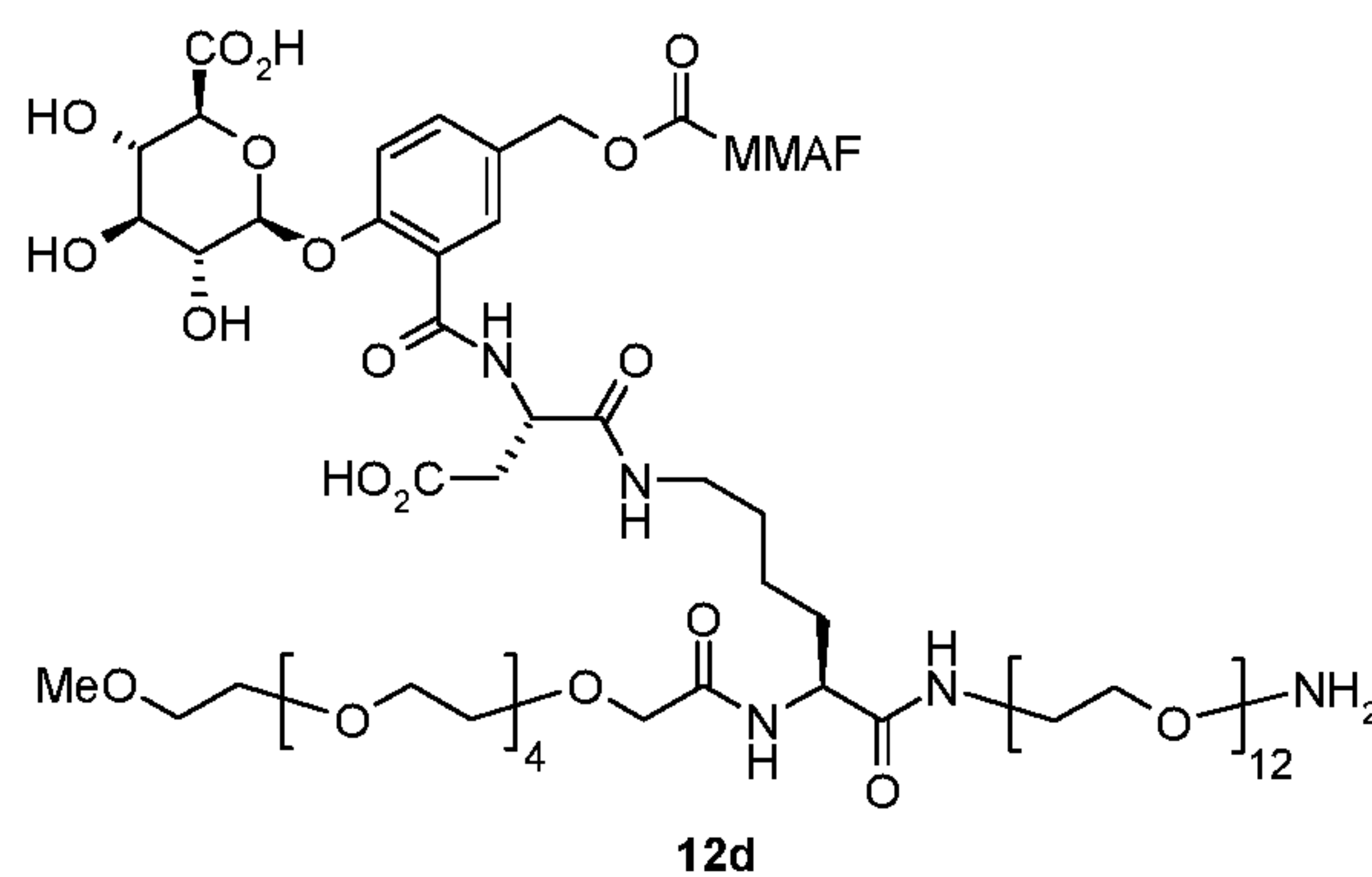
To a stirred mixture of compound **12a** (260 mg, 0.179 mmol), and Pd/C (10 wt. %, 72 mg) in MeOH (20 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.040 mL, 0.179 mmol). After stirring at room temperature for 3 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (40 mL). The filtrate was concentrated to produce the compound **12b** (242 mg, 100 %) as colorless oil, which was used without further purification. EI-MS m/z: [M+H]<sup>+</sup> 625.0, [M+H-Boc]<sup>+</sup> 525.0, [M+H-2Boc]<sup>+</sup> 424.9.

### Preparation of Compound 12c

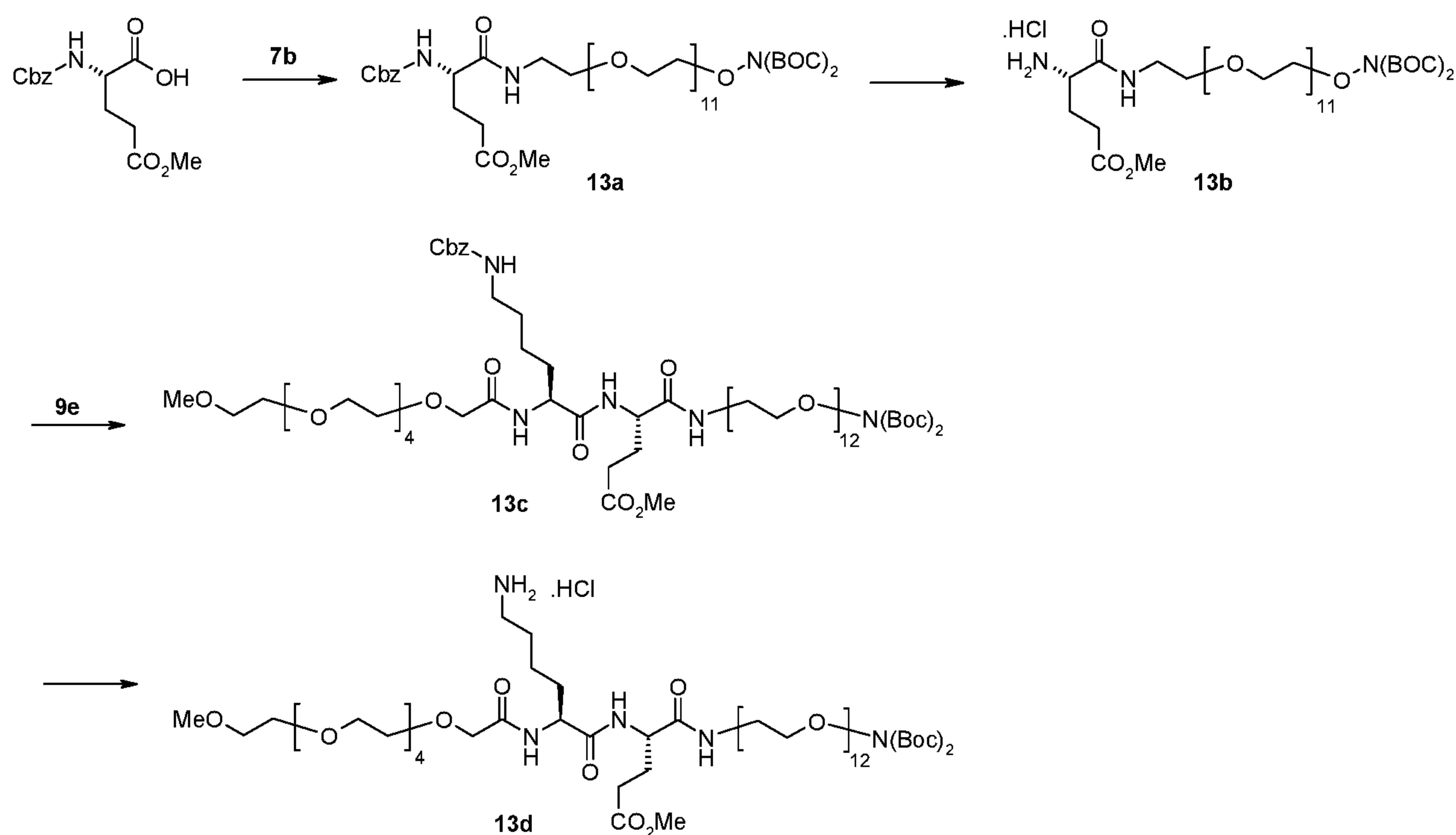


Compound **12c** was prepared from compound **1i** and compound **12b** by a similar method of preparing compound **9j** in Example 14. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1083.5.

*Example 20. Preparation of Compound 12d*



Compound **12d** was prepared from compound **1j** and compound **12b** by a similar method of preparing compound **9j** in Example 14. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1090.5.

*Example 21. Preparation of Compound 13e*Preparation of Compound 13a

DIPEA (0.22 mL, 1.25 mmol) and HBTU (356 mg, 0.94 mmol) were added to a stirred mixture of Z-Glu(OMe)-OH (222 mg, 0.75 mmol) and compound 7b (500 mg, 0.62 mmol) in DMF (5.0 mL). The reaction mixture was stirred at room temperature for 14 hours under N<sub>2</sub>. The reaction mixture was diluted with water (200 mL) and extracted with EA (3 x 100 mL). The organic layers were dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated under reduced pressure. The resulting residue was purified by column chromatography to yield the compound 13a (370 mg, 57 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.34 (br, 5H), 6.73 (br, 1H), 5.72 (d, J=7.6Hz, 1H), 5.06 (br, 2H), 4.28-4.18 (m, 1H), 4.07 (t, J=4.4Hz, 2H), 3.76-3.71 (m, 2H), 3.70-3.50 (m, 45H), 3.48-3.42 (m, 2H), 2.53-2.36 (m, 2H), 2.20-2.08 (m, 1H), 2.00-1.88 (m, 1H), 1.53 (s, 18H). EI-MS m/z: [M+Na]<sup>+</sup> 1061.2.

Preparation of Compound 13b

4N HCl in 1,4-dioxane (0.08 mL, 0.32 mmol) was added to a stirred mixture of the compound 13a (370 mg, 0.35 mmol), and Pd/C (38 mg) in MeOH (8 mL) at 0 °C. After stirring at room temperature for 20 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (400 mL). The filtrate was concentrated, producing compound 13b (301 mg, 90 %) as yellow liquid, which was used without further

purification.  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.41 (br, 1H), 8.09 (br, 3H), 4.13 (br, 1H), 3.85-3.56 (m, 51H), 2.55 (br, 2H), 2.38-2.18 (m, 2H), 1.53 (s, 18H). EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  905.0.

#### Preparation of Compound 13c

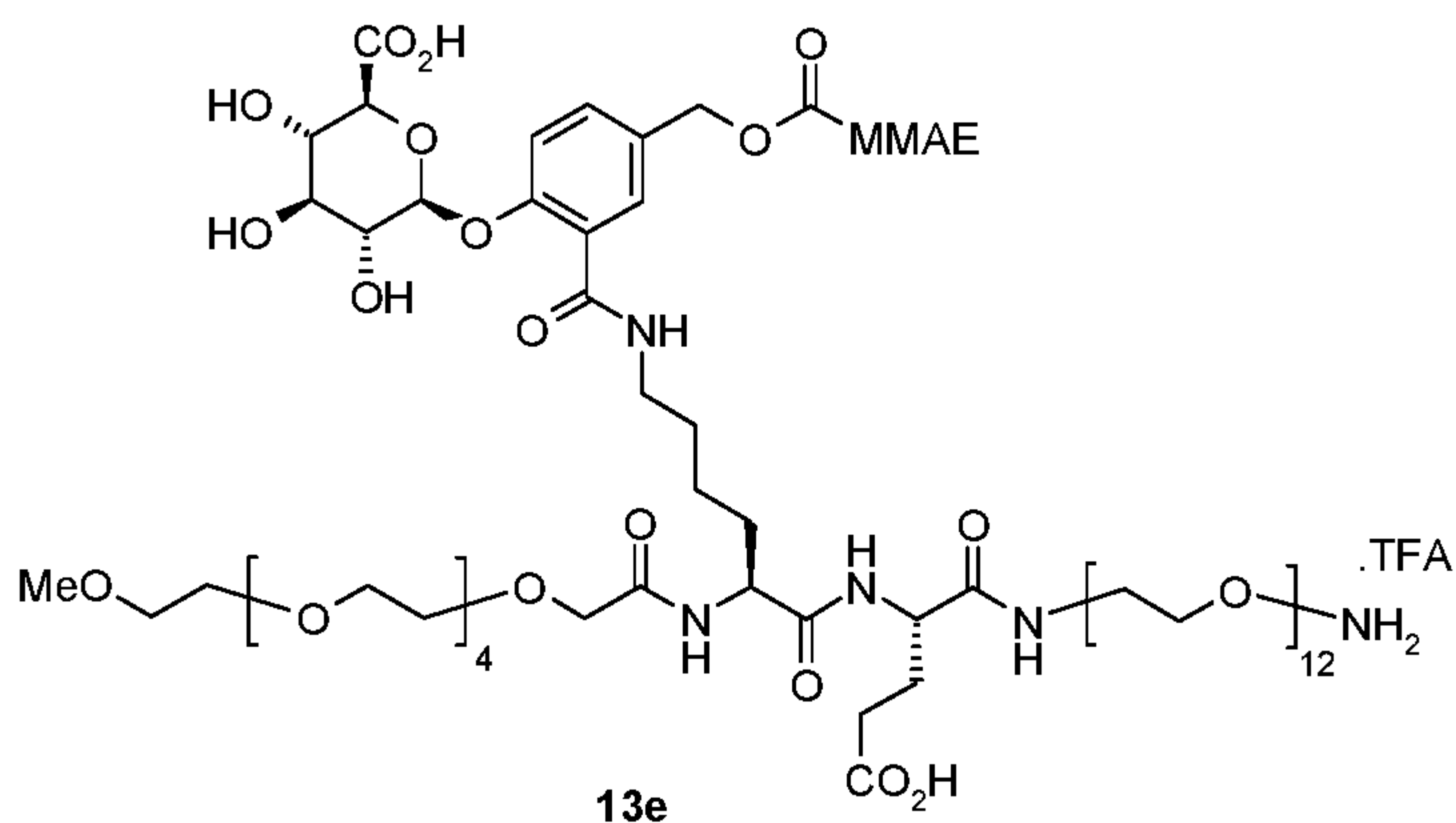
DIPEA (0.165 mL, 0.96 mmol) and HBTU (279 mg, 0.74 mmol) were added to a stirred mixture compound **13b** (300 mg, 0.32 mmol) and compound **9e** (366 mg, 0.64 mmol) in DMF (5.0 mL). The reaction mixture was stirred at room temperature for 14 hours under  $\text{N}_2$ . The reaction mixture was diluted with water (200 mL) and extracted with EtOAc (3 x 100 mL). The organic layers were dried over anhydrous  $\text{MgSO}_4$ , filtered and concentrated under reduced pressure. The resulting residue was purified by column chromatography to yield the compound **13c** (290 mg, 62 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.40-7.32 (m, 7H), 7.00 (br, 1H), 6.73 (br, 1H), 5.07 (br, 2H), 4.44-4.36 (m, 2H), 4.07 (t,  $J=4.8\text{Hz}$ , 2H), 4.02 (br, 2H), 3.73 (t,  $J=5.2\text{Hz}$ , 2H), 3.71-3.52 (m, 68H), 3.24-3.14 (m, 2H), 2.52-2.34 (m, 3H), 2.18-2.06 (m, 2H), 1.98-1.82 (m, 4H), 1.76-1.64 (m, 3H), 1.53 (s, 18H). EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  1459.7.

#### Preparation of Compound 13d

$\text{Pd/C}$  (21 mg) was added to a stirred mixture of compound **13c** (290 mg, 0.19 mmol) in MeOH (5 mL) at  $0^\circ\text{C}$ . After stirring at room temperature for 20 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (400mL). The filtrate was concentrated, producing compound **13c** (247 mg, 94 %) as yellow liquid, which was used without further purification.  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.20 (d,  $J=8.4\text{Hz}$ , 1H), 7.74 (br, 1H), 7.30 (br, 1H), 4.66-4.48 (m, 2H), 4.07 (t,  $J=5.2\text{Hz}$ , 2H), 4.01 (br, 2H), 3.74-3.62 (m, 70H), 3.57-3.53 (m, 2H), 3.04-2.98 (m, 2H), 2.24-2.15 (m, 2H), 2.14-2.06 (m, 2H), 1.99-1.86 (m, 4H), 1.84-1.74 (m, 2H), 1.53 (s, 18H). EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  1325.5.

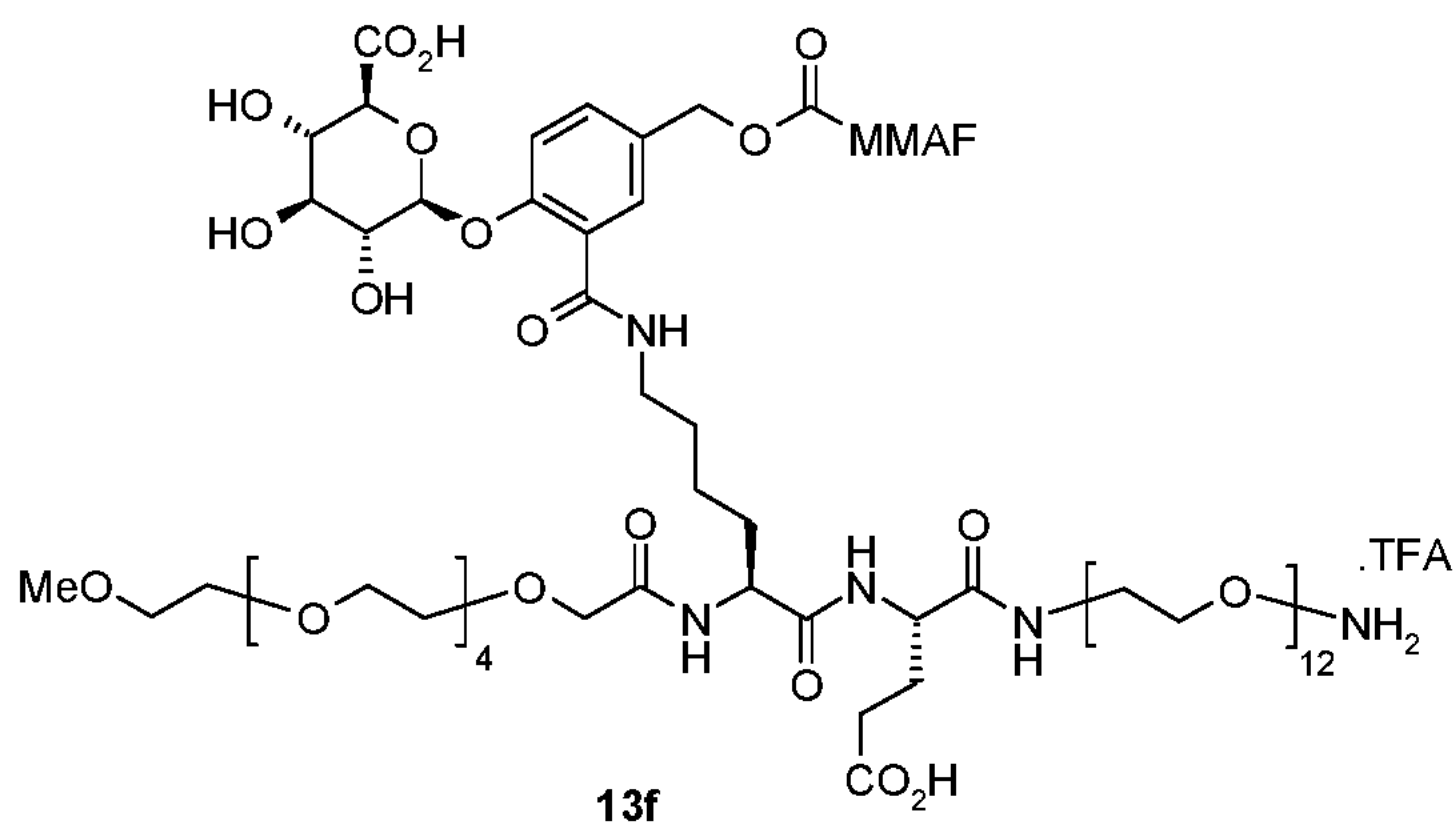
#### Preparation of Compound 13e

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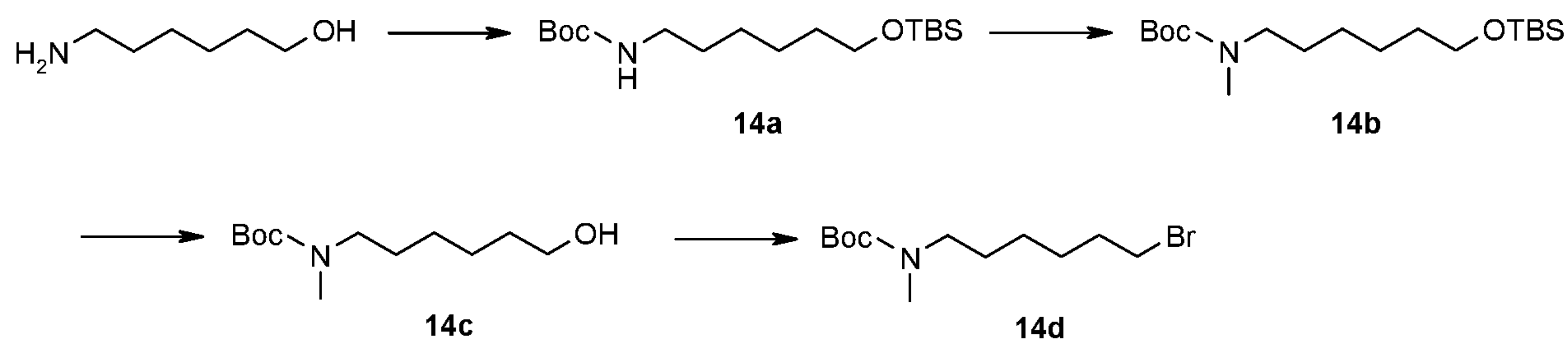
Compound **13e** was prepared from compound **1i** and compound **13d** by a similar method of preparing compound **9j** in Example 14. EI-MS  $m/z$ :  $[M+H]^+$  2181.5.

*Example 22. Preparation of Compound 13f*



Compound **13f** was prepared from compound **1j** and compound **13d** by a similar method of preparing compound **9j** in Example 14. EI-MS  $m/z$ :  $[M+H]^+$  2195.5.

*Example 23. Preparation of Compound 14m*



Preparation of Compound 14a

To a solution of 6-amino-1-hexanol (5.0 g, 42.6 mmol) in DCM (30 mL) was added di-*tert*-butyl dicarbonate (9.3 g, 42.6 mmol) at room temperature. After stirring for 18 hours, triethylamine (8.7 mL, 63.9 mmol) and *t*-butyldimethylsilyl chloride (7.7 g, 51.2

mmol) were added to the reaction mixture at 0 °C. After 24 hours at room temperature, the reaction mixture diluted with saturated aq. NH<sub>4</sub>Cl (200 mL). The resulting mixture was extracted with EtOAc (100 mL). The organic layer was washed with brine (100 mL) and dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The residue was purified by column chromatography to produce the compound **14a** (12 g, 84 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.50 (br s, 1H), 3.58 (t, *J* = 6.8 Hz, 2H), 3.10 (d, *J* = 6.4 Hz, 2H), 1.72-1.20 (m, 17H), 0.88 (s, 9H), 0.04 (s, 6H).

#### Preparation of Compound 14b

To a solution of compound **14a** (6.0 g, 18.1 mmol) in THF (30 mL) were added NaH (60 % in oil, 2.4 g, 54.2 mmol) and methyl iodide (3.4 mL, 54.2 mmol) at 0 °C under N<sub>2</sub>. After 14 hours, the reaction mixture was diluted with H<sub>2</sub>O (50 mL) and extracted with EtOAc (2 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **14b** (4.3 g, 69 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.59 (t, *J* = 6.4 Hz, 2H), 3.17 (br s, 2H), 2.82 (s, 3H), 1.62-1.21 (m, 17H), 0.88 (s, 9H), 0.04 (s, 6H).

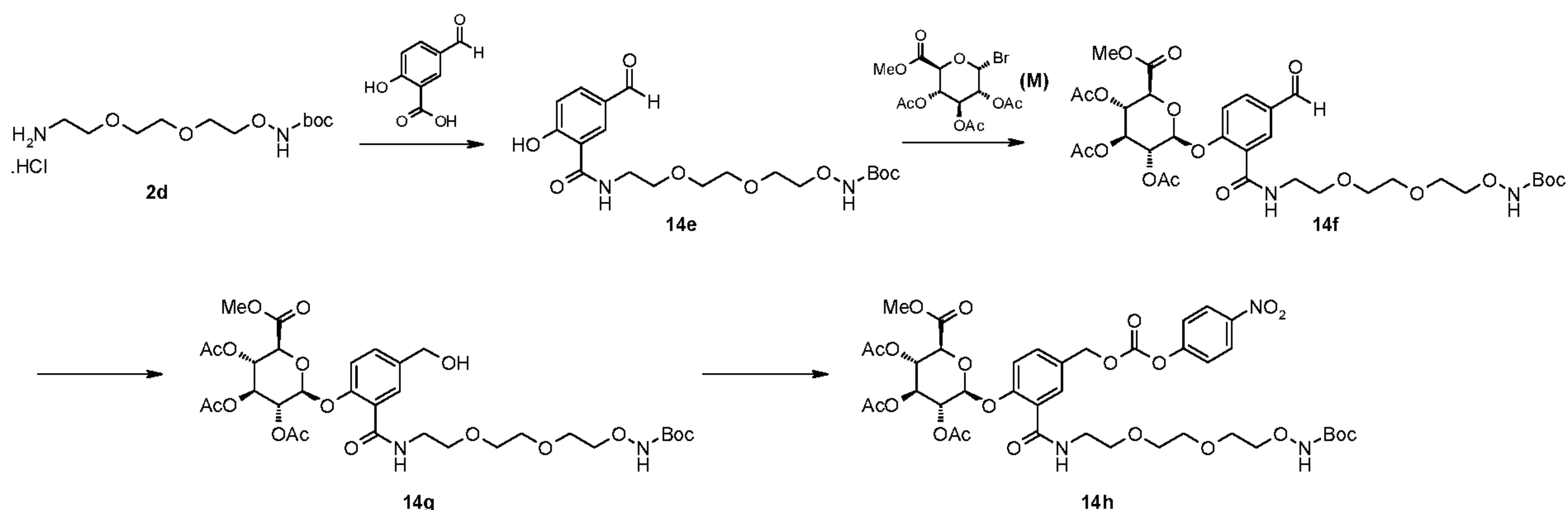
#### Preparation of Compound 14c

To a solution of compound **14b** (4.3 g, 12.4 mmol) in THF (15 mL) was added TBAF (1 M in THF, 15 mL, 14.9 mmol) at 0 °C under N<sub>2</sub>. After 5 hours, the reaction mixture was diluted with H<sub>2</sub>O (50 mL) and extracted with diethyl ether (2 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **14c** (3.0 g, 98 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.63 (br s, 2H), 3.20 (br s, 2H), 2.82 (s, 3H), 1.65-1.23 (m, 17H).

#### Preparation of Compound 14d

To a solution of compound **14c** (3.0 g, 12.9 mmol) in THF (30 mL) was added carbon tetrabromide (6.4 g, 19.4 mmol) and triphenylphosphine (5.1 g, 19.4 mmol) at 0 °C under N<sub>2</sub>. After 2 hours, the reaction mixture was filtered through silica gel and washed diethyl ether (100 mL). The filtrate was concentrated and purified by column chromatography to produce the compound **14d** (3.3 g, 86 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.40 (t, *J* = 6.8 Hz, 2H), 3.19 (br s, 2H), 2.83 (s, 3H), 1.90-1.70 (m, 2H), 1.65-1.40 (m,

13H), 1.38-1.25 (m, 2H).



### Preparation of Compound 14e

DIPEA (53.0 mL, 302.5 mmol) and EDC.HCl (35.7 g, 186.2 mmol) were added to a stirred mixture of compound **2d** (35.0 g, 116.4 mmol) and 5-formylsalicylic acid (21.3 g, 128.0 mmol) in DCM (1.6 L) at 0 °C. The reaction mixture was stirred at room temperature for 20 hours under N<sub>2</sub>. The reaction mixture was diluted with saturated aq. NH<sub>4</sub>Cl solution (1.5 L) and extracted DCM (2 x 1.5 L). The combined organic layers washed with brine (1.5 L) and dried anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **14e** (28.2 g, 59 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 13.37 (br s, 1H), 9.86 (s, 1H), 8.20 (s, 1H), 8.07 (br s, 2H), 7.90 (d, *J* = 8.4 Hz, 1H), 7.07 (d, *J* = 8.4 Hz, 1H), 4.06-4.01 (m, 2H), 3.79-3.66 (m, 10H), 1.47 (s, 9H).

### Preparation of Compound 14f

To a solution of compound **14e** (28.0 g, 67.9 mmol) in MeCN (500 mL) were added compound **M** (29.7 g, 74.7 mmol), 4 Å molecular sieve (30 g) and Ag<sub>2</sub>O (62.9 g, 272 mmol). After stirring at room temperature for 12 hours under N<sub>2</sub>, the reaction mixture was concentrated, diluted with H<sub>2</sub>O (800 mL) and extracted with EtOAc (1 L). The combined organic layers were dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The residue was purified by column chromatography to produce the compound **14f** (30.1 g, 61 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 9.99 (s, 1H), 8.54 (s, 1H), 7.99 (d, *J* = 8.8 Hz, 1H), 7.68 (s, 1H), 7.44 (br s, 1H), 7.18 (d, *J* = 8.8 Hz, 1H), 5.45-5.30 (m, 4H), 4.26 (d, *J* = 9.2 Hz, 1H), 4.02-3.97 (m, 2H), 3.80-3.55 (m, 13H), 2.06 (s, 9H), 1.46 (s, 9H).

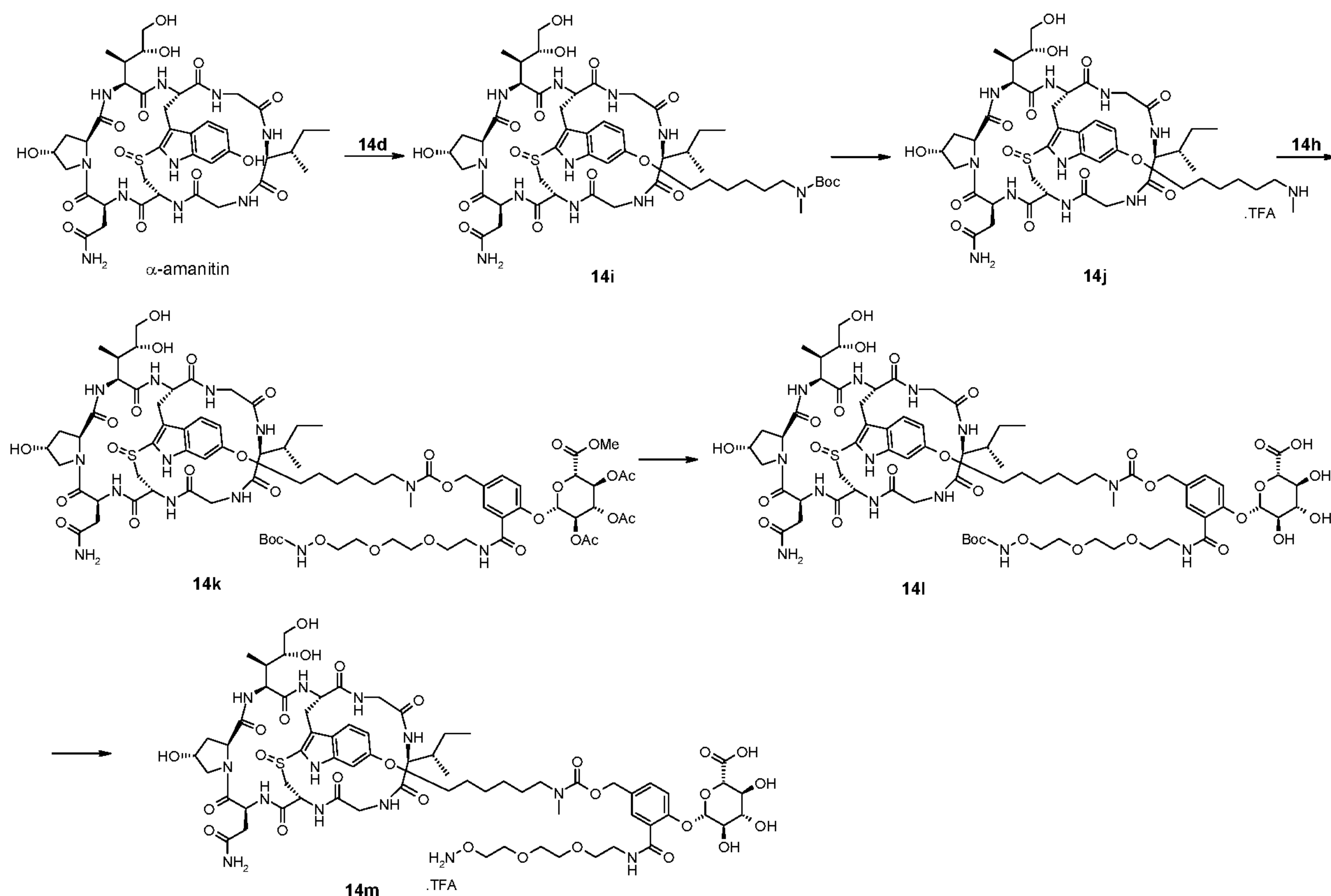
### Preparation of Compound 14g

To a solution of compound **14f** (29.0 g, 39.8 mmol) in *i*-PrOH/CHCl<sub>3</sub> (90 mL/450 mL) was added silica gel (16.7 g) and NaBH<sub>4</sub> (3.70 g, 99.5 mmol) at 0 °C. After stirring at 0 °C for 2 hours under N<sub>2</sub>, the reaction mixture was quenched with H<sub>2</sub>O (500 mL) and extracted with EtOAc (1 L). The organic layer was dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **14g** (24.1 g, 83 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.98 (s, 1H), 7.72 (s, 1H), 7.46 (d, *J* = 8.8 Hz, 1H), 7.41 (br, 1H), 7.04 (d, *J* = 8.8 Hz, 1H), 5.41-5.24 (m, 4H), 4.67 (d, *J* = 6.6 Hz, 2H), 4.19 (d, *J* = 8.8 Hz, 1H), 3.99-3.93 (m, 2H), 3.79-3.65 (m, 12H), 3.59-3.50 (m, 1H), 2.08-2.00 (m, 10H), 1.46 (s, 9H).

### Preparation of Compound 14h

To a solution of compound **14g** (23.7 g, 31.5 mmol) in DMF (50 mL) were added bis(4-nitrophenyl)carbonate (8.9 g, 29.3 mmol) and DIPEA (5.65 mL, 31.5 mmol) at 0 °C under N<sub>2</sub>. The reaction mixture was stirred at 0 °C for 30 minutes and allowed to warm to room temperature for 1 hour. The reaction mixture was diluted with H<sub>2</sub>O (500 mL) and extracted with EtOAc (500 mL). The organic layer was washed with brine (2 x 200 mL), dried over anhydrous MgSO<sub>4</sub>, filtered, and concentrated. The crude product was purified by column chromatography to produce the compound **14h** (22.4 g, 77 %) as white foam. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 8.28 (d, *J* = 7.2 Hz, 2H), 8.13 (s, 1H), 7.68 (br s, 1H), 7.52 (d, *J* = 8.8 Hz, 1H), 7.47 (br, 1H), 7.38 (d, *J* = 7.2 Hz, 2H), 7.08 (d, *J* = 8.8 Hz, 1H), 5.44-5.24 (m, 6H), 4.21 (d, *J* = 9.6 Hz, 1H), 4.00 (br s, 2H), 3.80-3.64 (m, 12 H), 3.64-3.54 (m, 1H), 2.06 (s, 9H), 1.47 (s, 9H).

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### Preparation of Compound **14i**

$\alpha$ -Amanitin (60.0 mg, 0.065 mmol) was dissolved in DMSO (2 mL) and compound **14d** (114 mg, 0.39 mmol) and potassium tert-butoxide (0.065 mL, 0.065 mmol) were added at 0 °C under N<sub>2</sub>. After 4 hours at 0 °C, the pH of the solution was adjusted to 4~5 with acetic acid. The residue was dissolved in DMSO (1 mL) and purified by HPLC, which produced the compound **14i** (29 mg, 39 %) as white solid. EI-MS m/z: [M-Boc]<sup>+</sup> 1032.4.

### Preparation of Compound **14j**

To a solution of compound **14i** (29 mg, 0.026 mmol) in DCM (3 mL) was added TFA (0.5 mL) at 0 °C. After 2 hours at 0 °C, the solvent and excess TFA were removed by N<sub>2</sub> flow and the resulting residue was purified by HPLC, which produced the compound **14j** (26 mg, 99 %) as white solid. EI-MS m/z: [M+H]<sup>+</sup> 1032.3, [M+Na]<sup>+</sup> 1054.3.

### Preparation of Compound **14k**

Compound **14j** (13 mg, 0.011 mmol), compound **14h** (10 mg, 0.011 mmol) and anhydrous HOBt (0.3 mg, 0.002 mmol) were dissolved in DMF (0.5 mL) at 0 °C. Then pyridine (0.2 mL) and DIPEA (0.004 mL, 0.023 mmol) were added. After stirring at room temperature for 24 hours under N<sub>2</sub>, the reaction mixture was dissolved in DMSO (1 mL)

and purified by HPLC, which produced the compound **14k** (11 mg, 54 %). EI-MS m/z:  $[M+H]^+$  1788.1.

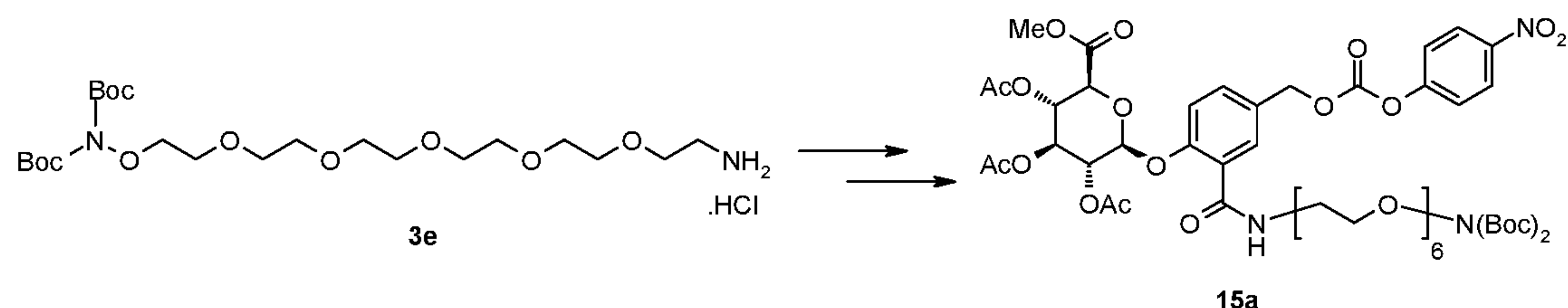
#### Preparation of Compound 14l

To a solution of compound **14k** (11 mg, 0.006 mmol) in MeOH (0.2 mL) was added LiOH monohydrate (1.3 mg, 0.03 mmol) in H<sub>2</sub>O (0.2 mL) at -20 °C. After 1 hour at 0 °C, the pH of the solution was adjusted to 4~5 with acetic acid. The resulting solution was dissolved in DMSO (1 mL) and purified by HPLC, which produced the compound **14l** (7.5 mg, 75 %) as white solid. EI-MS m/z:  $[M+H]^+$  1648.6.

#### Preparation of Compound 14m

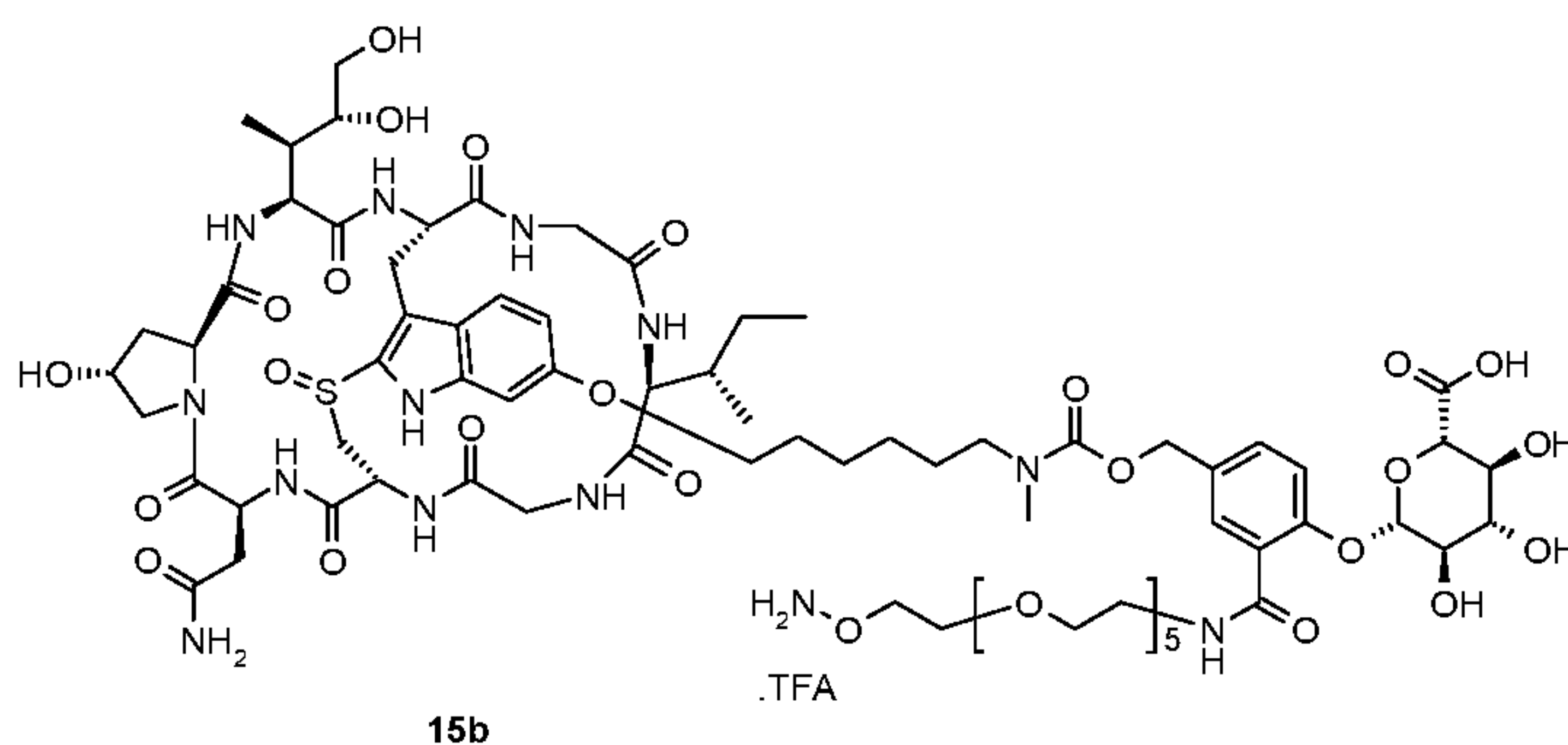
To a solution of compound **14l** (7.5 mg, 0.0045 mmol) in DCM (3 mL) was added TFA (0.5 mL) at 0 °C. After 2 hours at 0 °C, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was purified by HPLC, which produced the compound **14m** (6.2 mg, 85 %) as white solid. EI-MS m/z:  $[M+H]^+$  : 1548.5.

#### *Example 24. Preparation of Compound 15b*



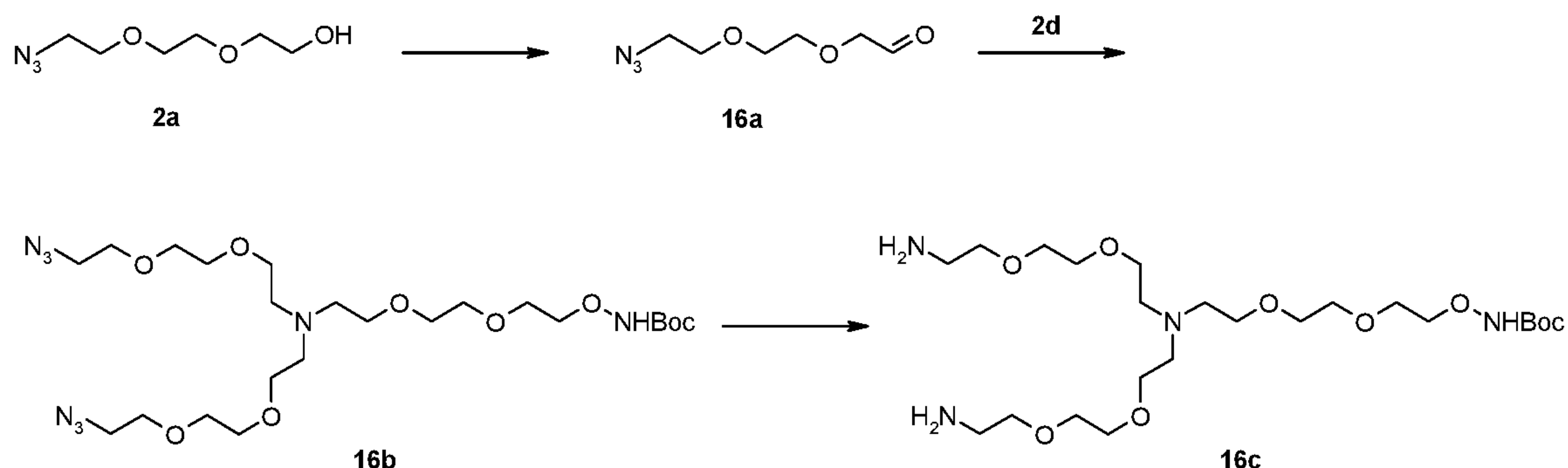
#### Preparation of Compound 15a

Compound **15a** was prepared from compound **3e** by a method similar to method of preparing compound **14h** of Example 23. EI-MS m/z:  $[M+H]^+$  1128.3,  $[M+H-Boc]^+$  1028.3,  $[M+H-2Boc]^+$  928.2.



Preparation of Compound 15b

Compound **15b** was prepared from compound **14j** and compound **15a** by a method similar to method of preparing compound **14m** of Example 23. EI-MS  $m/z$ :  $[M+H]^+$  1681.6.

*Example 25. Preparation of Compound 16f*Preparation of Compound 16a

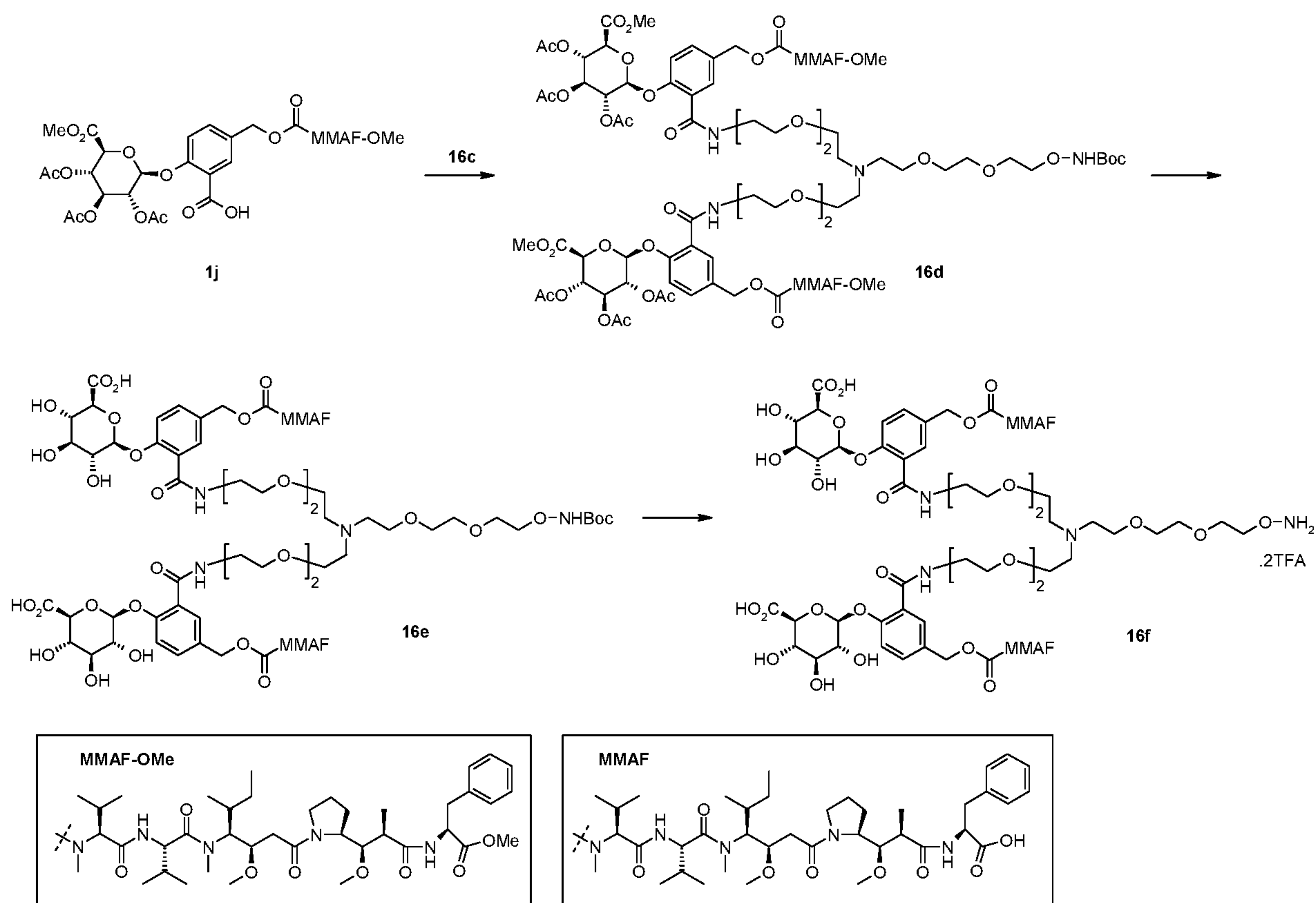
To a stirred solution of oxalyl chloride (2.8 mL, 32.5 mmol) in DCM (5 mL) DMSO (3.08 mL, 43.4 mmol) was added in DCM (15 mL) and then the reaction mixture was stirred at -78 °C for 30 minutes. To this solution was added compound **2a** (3.8 g, 21.7 mmol) at -78 °C and stirred for 1 hour. Triethylamine (15.1 mL, 108 mmol) in DCM (20 mL) was added and then the reaction mixture was allowed to warm to room temperature, diluted with H<sub>2</sub>O (100 mL) and extracted with DCM (2 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The residue was purified by column chromatography to produce the compound **16a** (1.8 g, 48 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 9.74 (s, 1H), 4.19 (s, 2H), 3.77-3.69 (m, 6H), 3.42 (m, 2H).

Preparation of Compound 16b

To a solution of compound **16a** (1.0 g, 3.32 mmol) and compound **2d** (1.72 g, 9.96 mmol) in MeOH (15 mL) AcOH (0.19 mL, 3.32 mmol) was added at 0 °C. After stirring for 30 minutes at 0 °C, NaCNBH<sub>3</sub> (658 mg, 9.96 mmol) was added and allowed to warm to room temperature over 2 hours. After the reaction was completed, the reaction mixture was diluted with H<sub>2</sub>O (50 mL) and then extracted with DCM (3 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The residue was purified by column chromatography to produce the compound **16b** (800 mg, 41 %) as light yellowish oil. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.78 (brs, 1H), 4.01 (m, 2H), 3.69-3.65 (m, 24H), 3.39 (m, 4H), 3.04 (m, 6H), 1.47 (s, 9H).

Preparation of Compound 16c

To a solution of compound **16b** (350 mg, 0.60 mmol) in MeOH (10 mL) Pd/C (10 wt. %, 300 mg) was added. After stirring at room temperature for 8 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (100 mL). Concentration provided compound **16c** as colorless oil (300 mg, 94 %), which was used without further purification.  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  4.02 (m, 2H), 3.71 (m, 2H), 3.65-3.55 (m, 22H), 2.92 (m, 4H), 2.76 (t,  $J = 5.2$  Hz, 6H), 1.47 (s, 9H). EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  527.6.

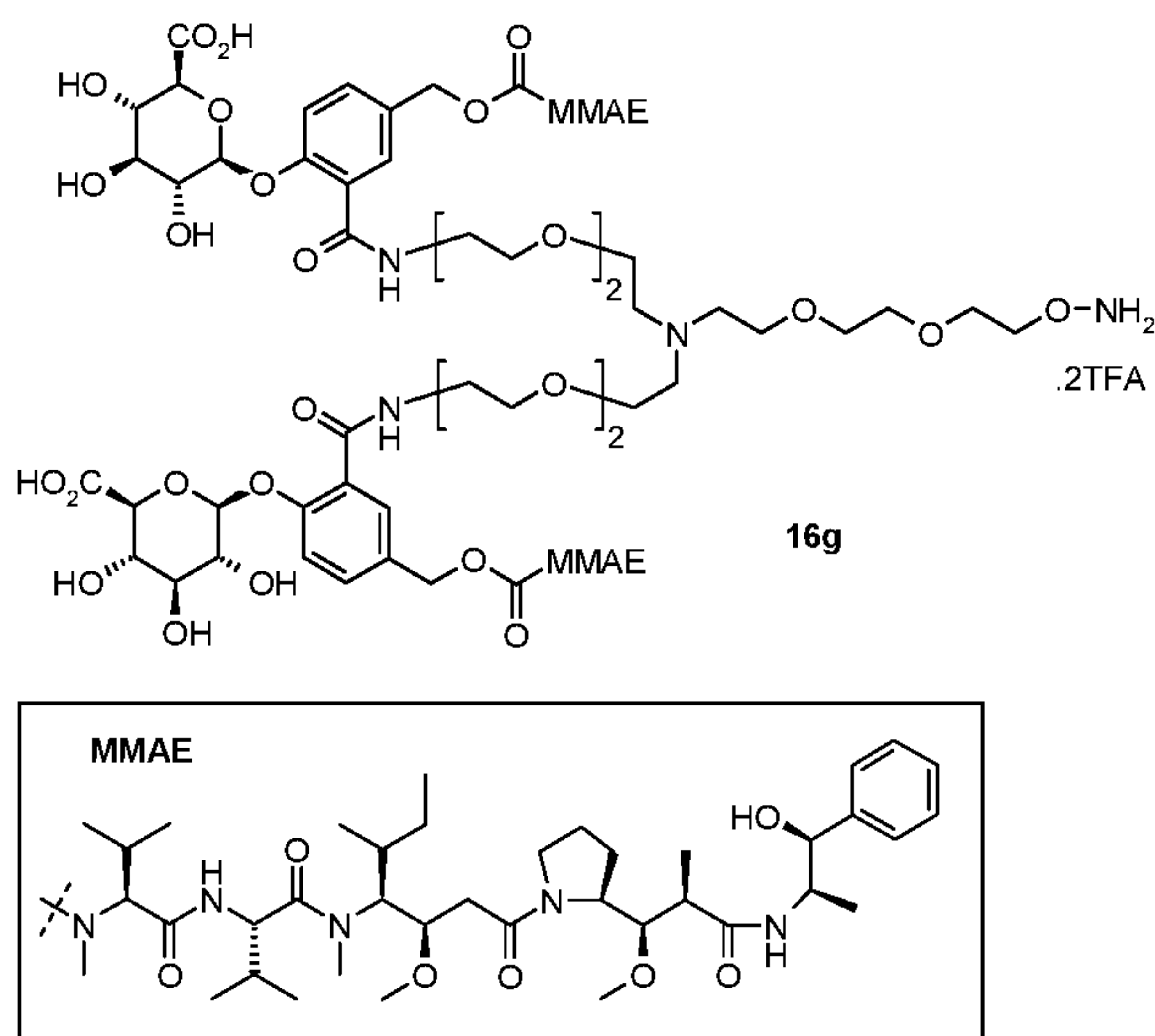
Preparation of Compound 16d

DIPEA (0.40 mL, 2.24 mmol) and PyBOP (711 mg, 1.34 mmol) were added to a stirred mixture of compound **1j** (1.57 g, 1.23 mmol) and compound **16c** (300 mg, 0.56 mmol) in DMF (15 mL). After stirring at room temperature for 4 hours under  $\text{N}_2$ , the reaction mixture was diluted  $\text{H}_2\text{O}$  (200 mL) and extracted with EtOAc (3 x 100 mL). The combined organic layers were dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under reduced pressure. The residue was dissolved in  $\text{H}_2\text{O}/\text{DMSO}$  (5 mL/5 mL) and purified by HPLC produced the compound **16d** (1.57 g, 91.8 %). EI-MS  $m/z$ :  $1/2[\text{M}+\text{H}]^+$

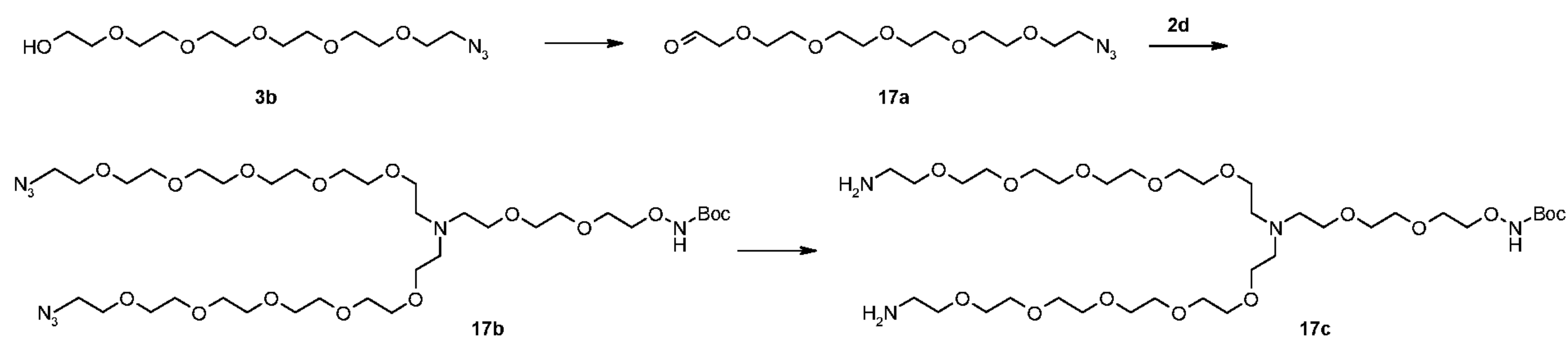
1502.7.

**Preparation of Compound 16f**

To a solution of compound **16d** (1.10 g, 0.36 mmol) in MeOH/THF (5 mL/10 mL) NaOH (175 mg, 4.32 mmol) was added dropwise in H<sub>2</sub>O (3 mL) at 0 °C. After 3 hours at 0 °C, the pH of the solution was adjusted to pH 4 using 2 N aq. HCl and concentrated. The residue was diluted with DCM (12 mL) and TFA (3 mL) at 0 °C. After 2 hours at 0 °C, the solvent and excess TFA were removed by N<sub>2</sub> flow. The residue was dissolved in H<sub>2</sub>O/MeCN (7.5 mL/7.5 mL) and purified by HPLC produced the compound **16f** (432 mg, 46 %) as white solid. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1298.5.

*Example 26. Preparation of Compound 16g*

Compound **16g** was prepared from compound **1i** and compound **16c** by a similar method of preparing compound **16f** in Example 25. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1284.5.

*Example 27. Preparation of Compound 17d*

### Preparation of Compound 17a

To a stirred solution of oxalyl chloride (0.62 mL, 7.3 mmol) in DCM (4 mL) DMSO (1.04 mL, 14.6 mmol) was added in DCM (10 mL) and then the reaction mixture was stirred at -78 °C for 30 minutes. To this solution was added compound **3b** (1.5 g, 4.88 mmol) at -78 °C and stirred for 1 hour. Triethylamine (2.72 mL, 19.50 mmol) in DCM (7 mL) was added and then the reaction mixture was allowed to warm to room temperature. After concentration under reduced pressure, the residue was purified by column chromatography (EtOAc to EtOAc/MeOH 10/1), which produced the compound **17a** (1.23 g, 82 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 9.73 (s, 1H), 4.16 (s, 2H), 3.75-3.61 (m, 18H), 3.39 (t, *J* = 5.2 Hz, 2H).

### Preparation of Compound 17b

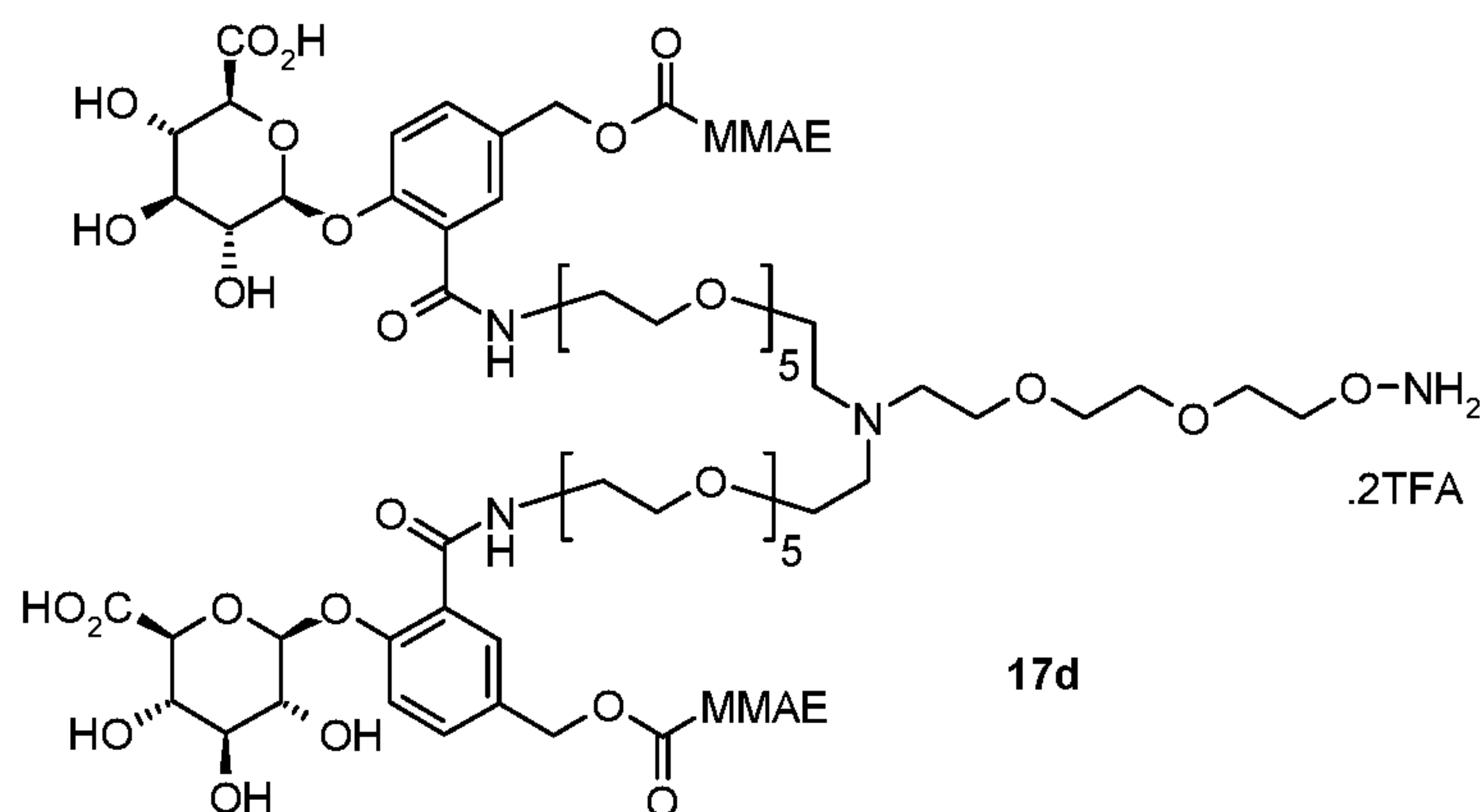
NaCNBH<sub>3</sub> (257 mg, 4.09 mmol) was added to a stirred mixture of compound **17a** (1.30 g, 4.25 mmol) and compound **2d** (492 mg, 1.63 mmol) in MeOH (5 mL) at 0 °C. The reaction mixture was then gradually heated up to room temperature over 2 hours. After the reaction was completed, the reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc to EtOAc/ MeOH 10/1), which produced the compound **17b** (620 mg, 45 %).

<sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 9.96 (br, 1H), 3.79 (t, *J* = 4.8 Hz, 2H) 3.59 (t, *J* = 4.8 Hz, 4H), 3.56-3.46 (m, 38H), 3.44-3.37 (m, 10H), 2.66-2.56 (m, 6H), 1.39 (s, 9H).

### Preparation of Compound 17c

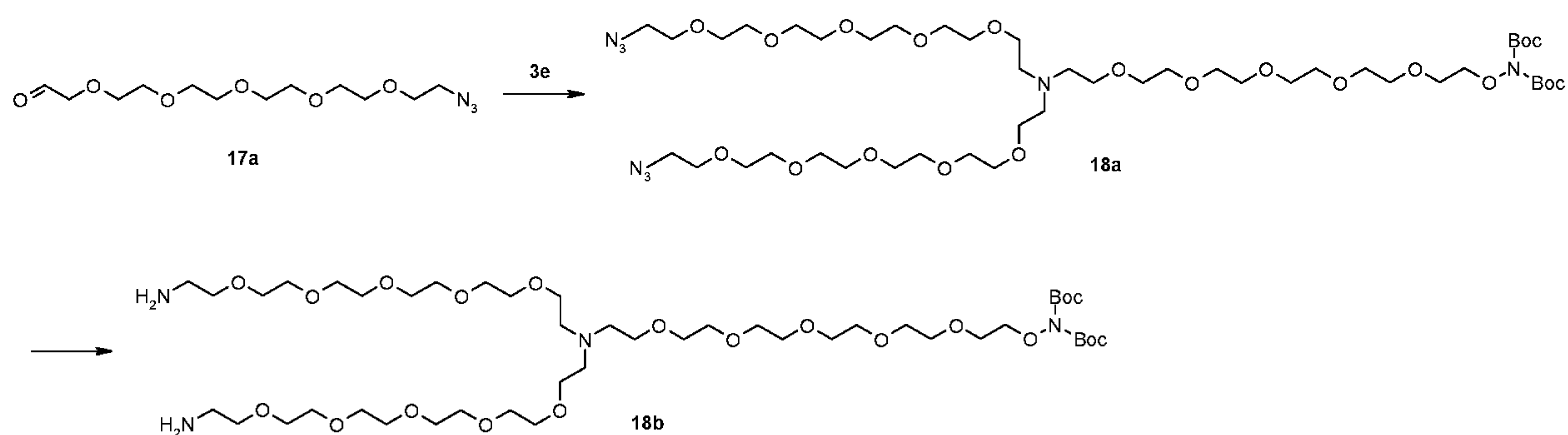
To a solution of compound **17b** (300 mg, 0.35 mmol) in MeOH (7 mL) was added Pd/C (10 wt. %, 38 mg). After stirring at room temperature for 4 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (400 mL). Concentration provided compound **17c** as colorless oil (253 mg, 90 %), which was used without further purification. <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 3.79 (t, *J*=4.4Hz, 2H), 3.55-3.45 (m, 38H), 3.42 (t, *J* = 6.0 Hz, 10H), 2.66-2.56 (m, 10H), 1.39 (s, 9H). EI-MS *m/z*: [M+H]<sup>+</sup> 791.0.

### Preparation of Compound 17d



Compound **17d** was prepared from compound **1i** and compound **17c** by a similar method of preparing compound **16f** in Example 25. EI-MS  $m/z$ :  $1/2[M+H]^+$  1415.6.

### Example 28. Preparation of Compound **18c**



### Preparation of Compound **18a**

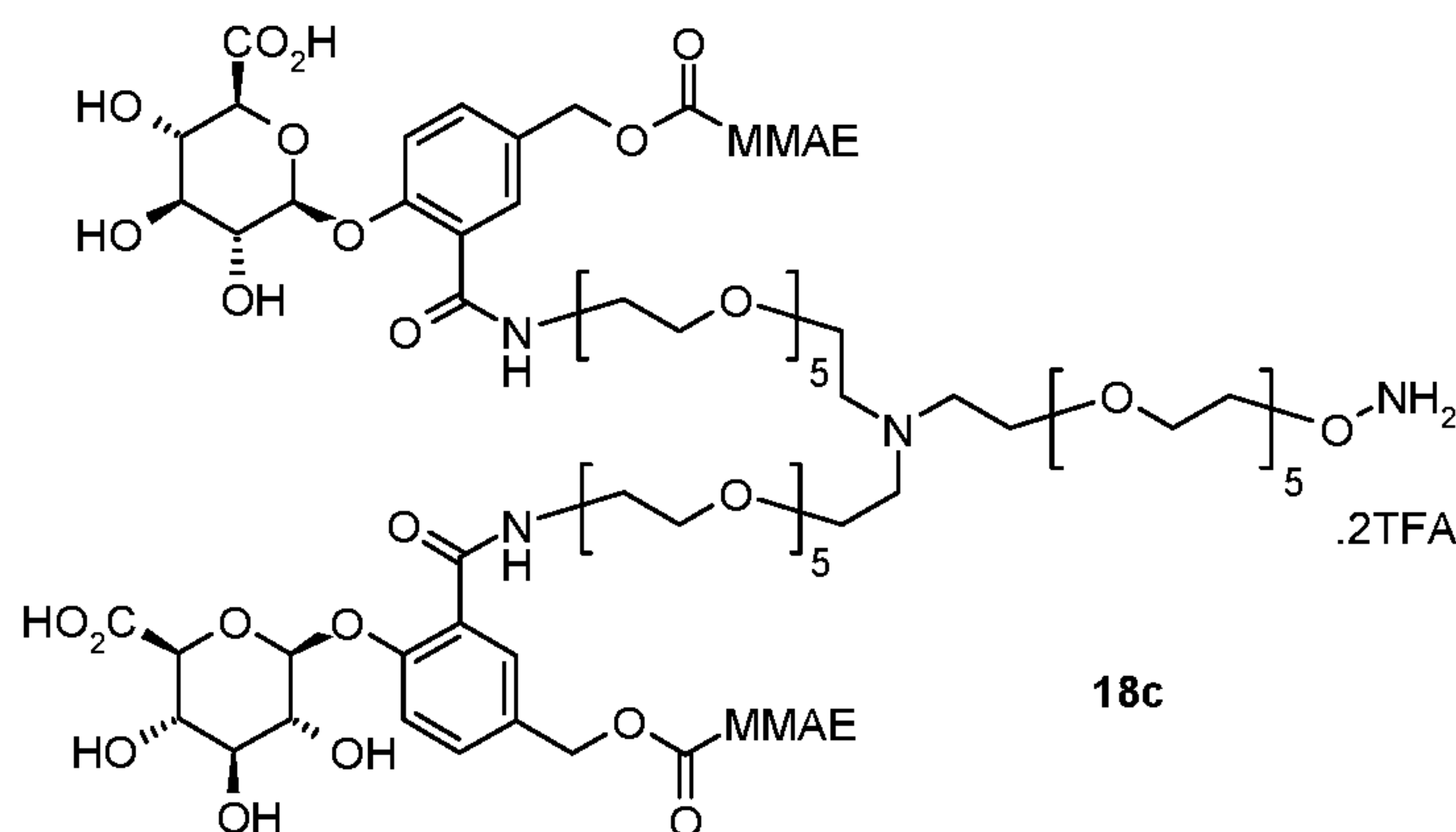
NaCNBH<sub>3</sub> (197 mg, 3.14 mmol) was added to a stirred mixture of compound **17a** (998 mg, 3.26 mmol) and compound **3e** (670 mg, 1.25 mmol) in MeOH (4 mL) at 0 °C. The reaction mixture was then gradually heated up to room temperature over 2 hours. After the reaction was completed, the reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc to EtOAc/MeOH 10/1), which produced the compound **18a** (668 mg, 49%). <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 3.97 (m, 2H) 3.63-3.57 (m, 6H), 3.56-3.44 (m, 46H), 3.44-3.36 (m, 12H), 2.66-2.61 (m, 6H), 1.45 (s, 18H).

### Preparation of Compound **18b**

To a solution of compound **18a** (60 mg, 0.055 mmol) in MeOH (1.2 mL) Pd/C (10 wt. %, 6 mg) was added. After stirring at room temperature for 4 hours under hydrogen, the

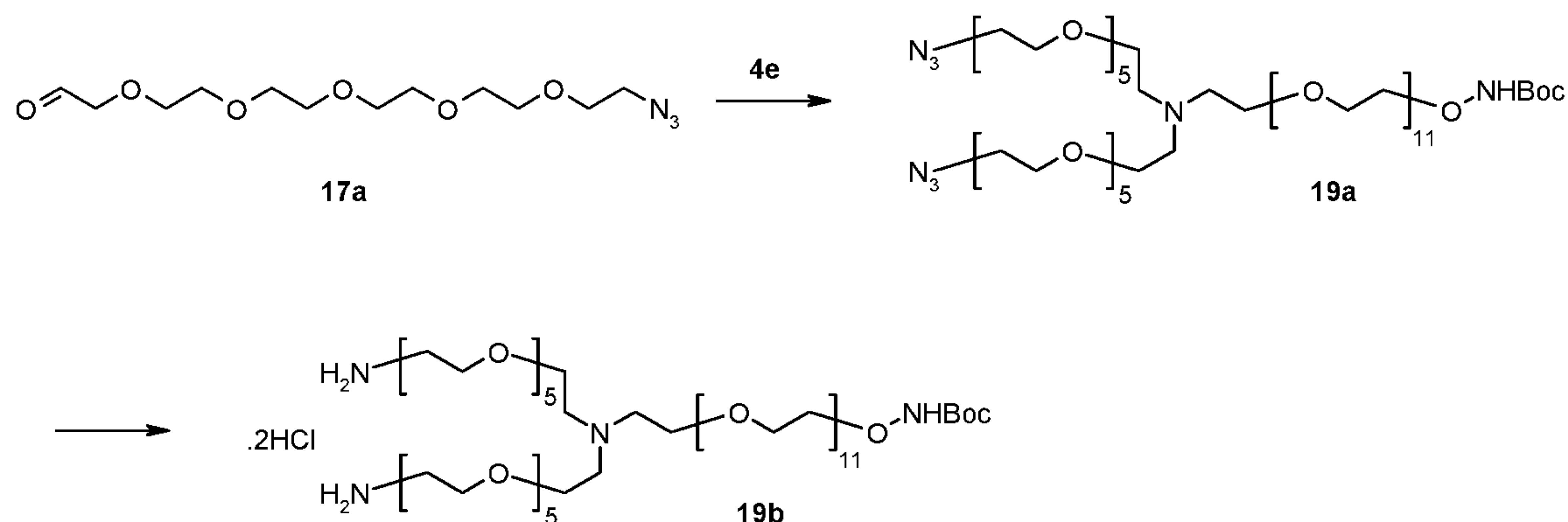
reaction mixture was filtered through a celite pad and washed with MeOH (400 mL).

Concentration provided compound **18b** (55 mg, 96 %) as colorless oil, which was used without further purification.  $^1\text{H-NMR}$  (400 MHz, DMSO- $d_6$ )  $\delta$  3.97 (m, 2H), 3.62-3.57 (m, 4H), 3.54-3.45 (m, 50H), 3.45-3.39 (m, 10H), 2.66-2.61 (m, 10H), 1.46 (s, 18H). EI-MS  $m/z$ :  $1/2[\text{M}+\text{H}]^+$  1023.3.



Compound **18c** was prepared from compound **1i** and compound **18b** by a similar method of preparing compound **16f** in Example 25. EI-MS  $m/z$ :  $1/2[\text{M}+\text{H}]^+$  1481.7.

#### Example 29. Preparation of Compound **19c**



#### Preparation of Compound **19a**

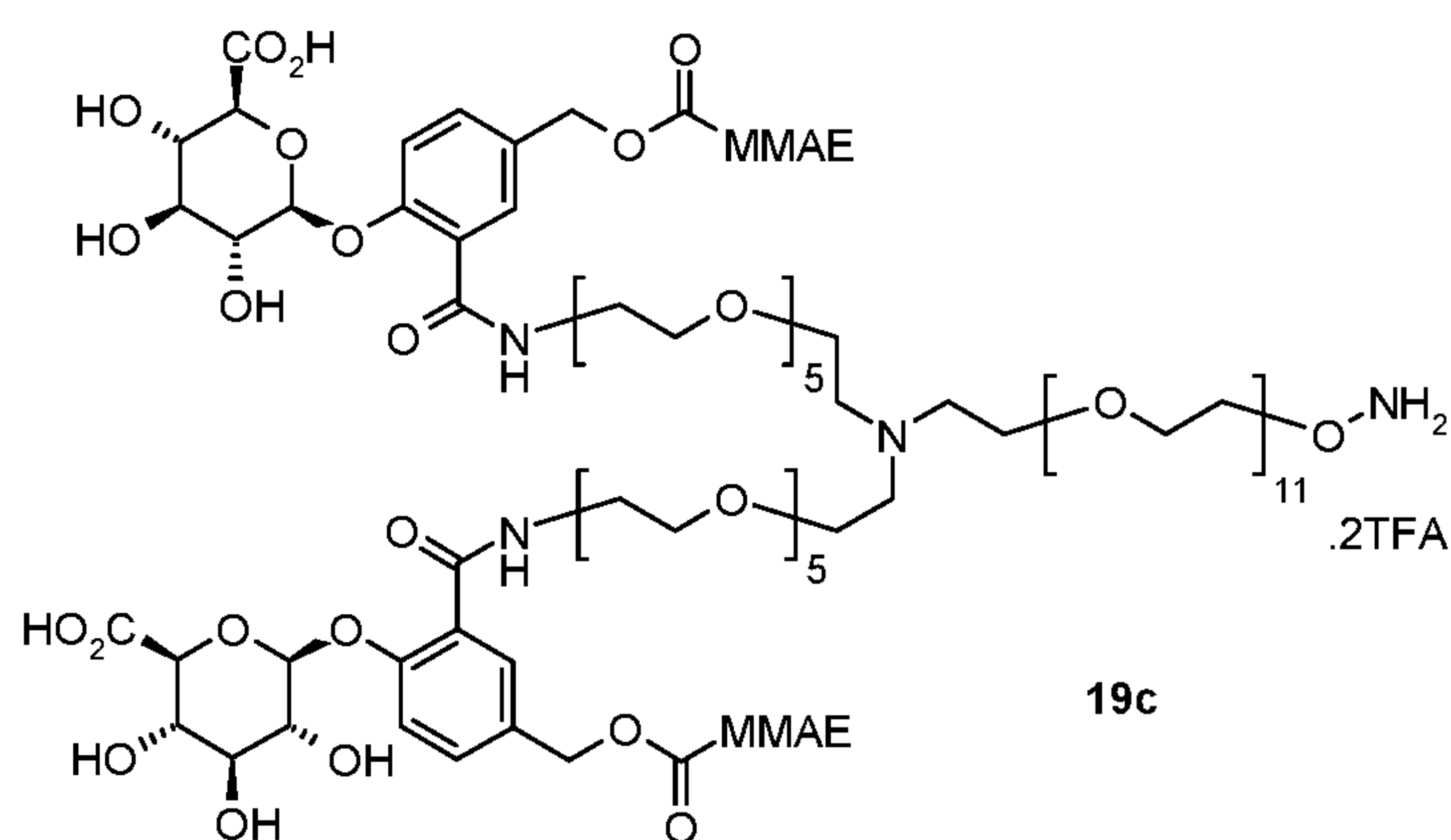
$\text{NaCNBH}_3$  (197 mg, 3.14 mmol) was added to a stirred mixture of compound **17a** (118 mg, 0.16 mmol) and compound **4e** (232 mg, 0.76 mmol) in MeOH (1 mL) at 0 °C. The reaction mixture was then gradually heated up to room temperature over 2 hours. After the reaction was completed, the reaction mixture was evaporated under reduced pressure. The residue was purified by column chromatography (EtOAc to EtOAc/ MeOH 10/1), which produced the compound **19a** (135 mg, 68 %).  $^1\text{H-NMR}$  (400 MHz, DMSO- $d_6$ )  $\delta$  7.72 (br s, 1H) 4.02 (t, 2H), 3.72-3.53 (m, 86H), 3.39 (t, 4H), 2.77 (bs, 4H), 1.47 (s, 9H). EI-MS  $m/z$ :

$[M+H]^+$  1239.6.

### Preparation of Compound 19b

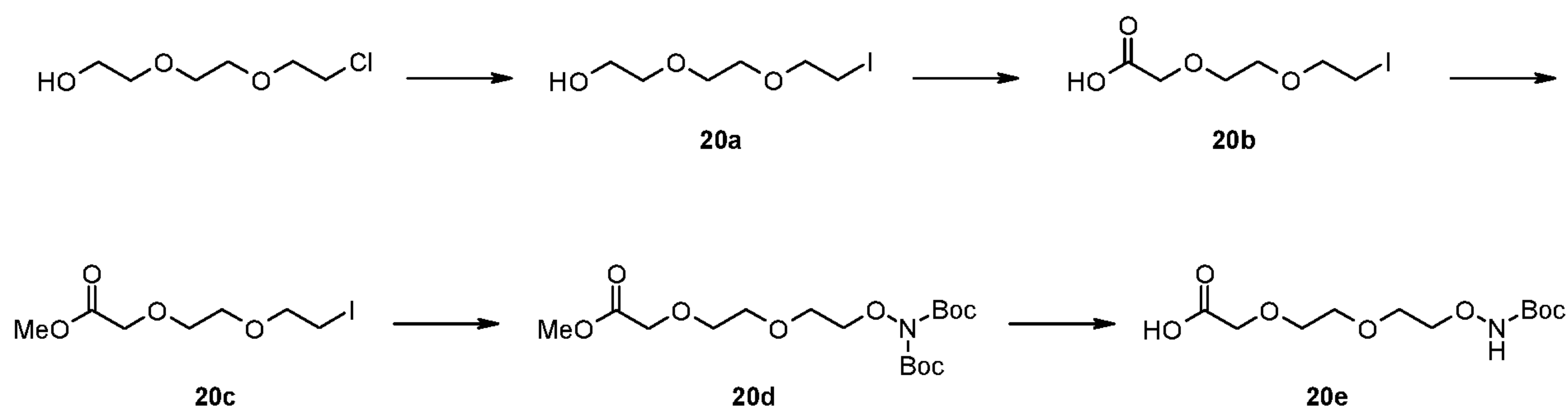
To a solution of compound **19a** (133 mg, 0.107 mmol) in MeOH (2 mL) Pd/C (10 wt. %, 26 mg) was added and HCl (4 N in 1,4-dioxane, 0.054 mL, 0.21 mmol) at 0 °C. After stirring at room temperature for 40 minutes under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (40 mL). Concentration provided compound **19b** (132 mg, 97 %) as colorless oil, which was used without further purification.  $^1\text{H-NMR}$  (400 MHz, DMSO- $d_6$ )  $\delta$  7.79 (s, 1H), 4.06-4.02 (m, 8H), 3.88 (m, 2H), 3.73-3.64 (m, 80H), 3.22 (s, 4H), 1.47 (s, 9H). EI-MS  $m/z$ :  $[M+H]^+$  : 1187.5.

### Preparation of Compound 19c



Compound **19c** was prepared from compound **1i** and compound **19b** by a similar method of preparing compound **16f** in Example 25. EI-MS  $m/z$ :  $1/2[M+H]^+$  1614.5.

### *Example 30. Preparation of Compound 20q*



### Preparation of Compound 20a

To a solution of 2-(2-(2-chloroethoxy)ethoxy)ethanol (5.0 g, 29.6 mmol) in acetone

(30 mL) was added NaI (13.3 g, 88.9 mmol). The reaction mixture was refluxed for 12 hours. After the reaction was completed, the reaction mixture was filtered and concentrated. The crude product was purified by column chromatography to produce the compound **20a** (7.0 g, 91 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.80-3.73 (m, 4H), 3.72-3.65 (m, 4H), 3.63-3.61 (m, 2H), 3.27 (t, *J* = 6.4 Hz, 2H).

#### Preparation of Compound 20b

To a solution of compound **20a** (7.0 g, 26.9 mmol) in acetone (200 mL) at 0 °C Jones reagent (20 mL) was added. After 15 hours at 0 °C, the reaction mixture was filtered and concentrated. The residue was diluted with H<sub>2</sub>O (150 mL) and extracted with EtOAc (2 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **20b** (7.0 g, 94 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.22 (s, 2H), 3.85-3.70 (m, 6H), 3.35-3.25 (m, 2H).

#### Preparation of Compound 20c

To a solution of compound **20b** (7.0 g, 25.5 mmol) in MeOH (70 mL) oxalyl chloride (3.2 mL, 38.3 mmol) was added at 0 °C under N<sub>2</sub>. After 16 hours, the reaction mixture was concentrated and purified by column chromatography, which produced the compound **20c** (5.7 g, 77 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.19 (s, 2H), 3.80-3.67 (m, 9H), 3.27 (t, *J* = 6.8 Hz, 2H).

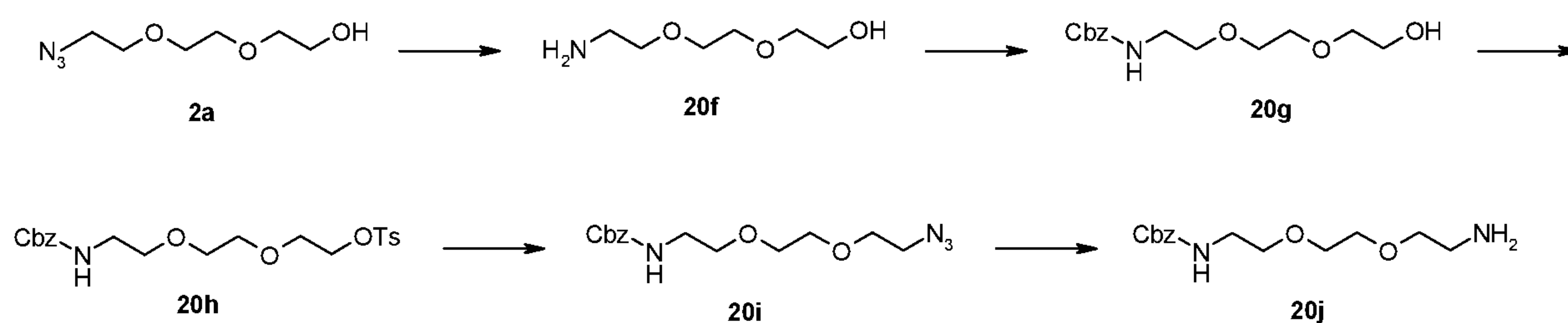
#### Preparation of Compound 20d

To a solution of compound **20c** (2.5 g, 8.67 mmol) and *N,N*-diBoc-hydroxylamine (2.6 g, 11.2 mmol) in DMF (30 mL) was added NaH (60 % in oil, 454 mg, 10.4 mmol) at 0 °C under N<sub>2</sub>. After 15 hours, the reaction mixture was diluted with H<sub>2</sub>O (50 mL) and extracted with EtOAc (3 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **20d** (1.87 g, 73 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.17 (s, 2H), 4.08 (m, 2H), 3.78-3.65 (m, 9H), 1.53 (s, 18H).

#### Preparation of Compound 20e

To a solution of compound **20d** (1.87 g, 6.38 mmol) in THF/MeOH/H<sub>2</sub>O (45 mL/15

mL/15 mL) NaOH (600 mg, 15.9 mmol) was added at 0 °C under N<sub>2</sub>. The reaction mixture was stirred for 3 hours at room temperature. Then the pH of the solution was adjusted to 4~5 with 1 N aqueous HCl. The reaction mixture was poured into H<sub>2</sub>O (100 mL) and extracted with EtOAc (2 x 100 mL). The organic layers were combined, dried over MgSO<sub>4</sub>, filtered and concentrated. The compound **20e** (1.6 g, 90 %) was produced as colorless oil, and it was used without further purification. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.17 (s, 2H), 4.08-4.02 (m, 2H), 3.80-3.67 (m, 6H), 1.48 (s, 9H).



#### Preparation of Compound 20f

Pd/C (10 wt. %, 1.0 g) was added to a solution of compound **2a** (6.7 g, 38.2 mmol) in MeOH (38 mL). After stirring at room temperature for 8 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (100 mL). Concentration provided compound **20f** (5.6 g, 99 %) as colorless oil, which was used without further purification. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.95-3.25 (m, 12H), 2.90 (s, 2H).

#### Preparation of Compound 20g

Benzyl chloroformate (6 mL, 42.2 mmol) were slowly added to a solution of compound **20f** (5.6 g, 38.2 mmol) and triethylamine (8 mL, 57.6 mmol) in THF (200 mL) at 0 °C for 30 minutes under N<sub>2</sub>. After stirring for 1 hour at 0 °C, the reaction mixture was concentrated and the crude product was purified by column chromatography, which produced the compound **20g** (5.7 g, 53 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.45-7.20 (m, 5H), 5.61 (br s, 1H), 5.07 (s, 2H), 3.85-3.20 (m, 12H).

#### Preparation of Compound 20h

To a solution of compound **20g** (2.7 g, 9.53 mM) in DCM (30 mL) were added triethylamine (1.9 mL, 12.3 mmol) and *p*-toluenesulfonyl chloride (2.3 g, 10.4 mmol) at room temperature under N<sub>2</sub>. After 8 hours, the reaction mixture was diluted with H<sub>2</sub>O (50

mL) and extracted with DCM (3 x 100 mL). The organic layers were combined, dried over anhydrous  $\text{MgSO}_4$ , filtered and concentrated. The crude product was purified by column chromatography to produce the compound **20h** (3.51 g, 84 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.78 (d,  $J = 7.2$  Hz, 2H), 7.45-7.25 (m, 7H), 5.20 (br s, 1H), 5.09 (s, 2H), 4.20-4.05 (m, 2H), 3.75-3.25 (m, 10H), 2.43 (s, 3H).

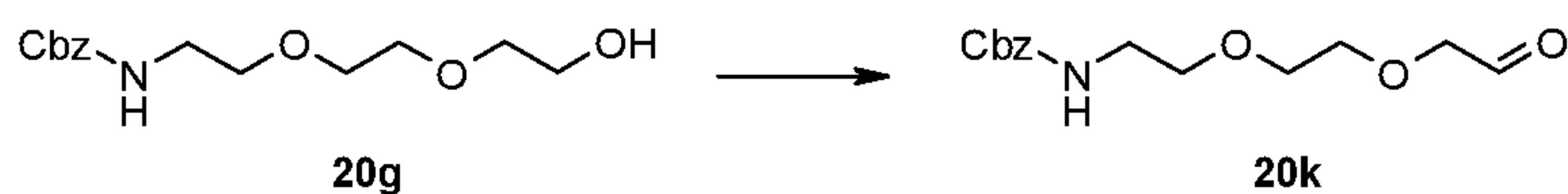
#### Preparation of Compound 20i

A solution of compound **20h** (3.51 g, 8.02 mmol) and  $\text{NaN}_3$  (3.8 g, 57.6 mmol) in DMF (27 mL) was heated at  $100^\circ\text{C}$  for 15 hours. After the reaction was completed, the reaction mixture was filtered and concentrated. The residue was diluted with  $\text{H}_2\text{O}$  (50 mL) and extracted with EtOAc (2 x 100 mL). The organic layers were combined, dried over anhydrous  $\text{MgSO}_4$ , filtered and concentrated. The crude product was purified by column chromatography to produce the compound **20i** (2.05 g, 83 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.45-7.25 (m, 5H), 5.20 (br s, 1H), 5.10 (s, 2H), 3.80-3.25 (m, 12H).

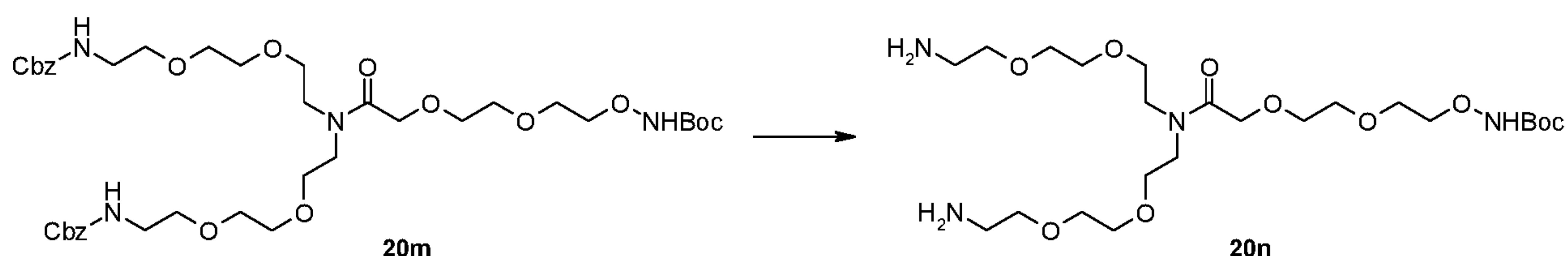
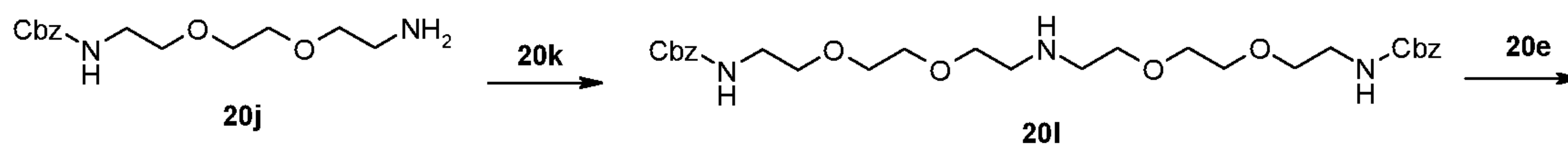
#### Preparation of Compound 20j

Triphenylphosphine (2.09 g, 7.97 mmol) was added to a solution of compound **20i** (2.05 g, 6.64 mmol) in THF (27 mL) at room temperature. After stirring for 2 hours under  $\text{N}_2$ ,  $\text{H}_2\text{O}$  (0.6 mL, 33.2 mmol) was added and the reaction mixture was refluxed for 3 hours. Then the reaction mixture was concentrated and purified by column chromatography, which produced the compound **20j** (1.78 g, 95 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.45-7.25 (m, 5H), 5.63 (br s, 1H), 5.10 (s, 2H), 3.80-3.25 (m, 10H), 2.88 (s, 2H).

1)



2)



### Preparation of Compound 20k

To a stirred solution of oxalyl chloride (1.4 mL, 15.9 mmol) in DCM (14 mL) was added DMSO (2.3 mL, 31.9 mmol) in DCM (28 mL) and then the reaction mixture was stirred at -78 °C for 30 minutes. To this solution was added compound **20g** (3.01 g, 10.6 mmol) at -78 °C. After stirring for 1 hour at -78 at 0 °C, triethylamine (7.4 mL, 53.1 mmol) was added and the reaction was allowed to warm to room temperature. The reaction mixture was poured into H<sub>2</sub>O (100 mL) and extracted with EtOAc (2 x 100 mL). The organic layers were combined, dried over MgSO<sub>4</sub>. Filtration and concentration produced the compound **20k** (2.6 g), which was used without further purification. <sup>1</sup>H-NMR (400MHz, CDCl<sub>3</sub>) δ 9.70 (s, 1H), 7.45-7.25 (m, 5 H), 5.25 (br s, 1 H), 5.10 (s, 2 H), 3.80-3.25 (m, 10 H).

### Preparation of Compound 20l

To a solution of compound **20j** (1.78 g, 6.30 mmol) and compound **20k** (2.13 g, 7.56 mmol) in MeOH (63 mL) was added NaCNBH<sub>3</sub> (674 mg, 10.7 mmol) at room temperature under N<sub>2</sub>. After 3 hours, the reaction mixture was filtered and concentrated. The crude product was purified by column chromatography to produce the compound **20l** (2.01 g, 58 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.45-7.25 (m, 10H), 5.60 (br s, 2H), 5.03 (s, 4H), 3.80-3.25 (m, 20H), 2.81 (s, 4H).

### Preparation of Compound 20m

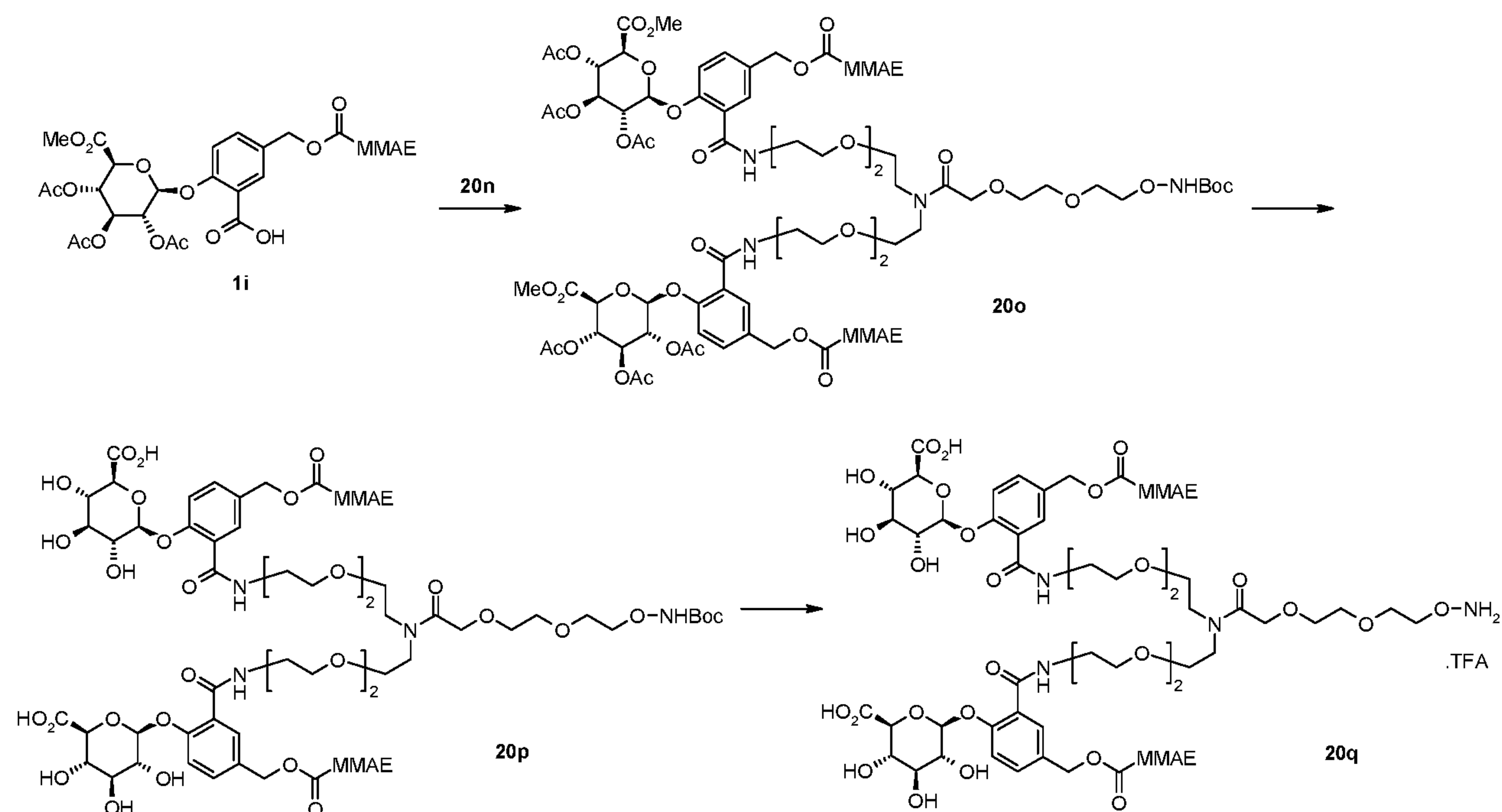
DIPEA (0.4 mL, 2.28 mmol) and PyBOP (713 mg, 1.36 mmol) were added to a stirred solution of compound **20l** (500 mg, 0.91 mmol) and compound **20e** (306 mg, 1.09 mmol) in DMF (10 mL). After stirring at room temperature for 6 hours under N<sub>2</sub>, the reaction mixture was diluted water (100 mL) and extracted with EtOAc (3 x 100 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography to produce the compound **20m** (318 mg, 43 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.45-7.25 (m, 10H), 5.47 (br s, 1H), 5.37 (br s, 1H), 5.09 (s, 4H), 3.80-3.25 (m, 34H), 1.46 (s, 9H). EI-MS m/z: [M+H]<sup>+</sup> 808.9.

### Preparation of Compound 20n

To a solution of compound **20m** (318 mg, 0.39 mmol) in MeOH (30 mL) was added

Pd/C (10 wt. %, 1.0 g). After stirring at room temperature for 3 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (100 mL).

Concentration provided compound **20n** (180 mg) as colorless oil, which was used without further purification. EI-MS  $m/z$ :  $[M+H]^+$  541.2.



### Preparation of Compound **20o**

DIPEA (0.034 mL, 0.19 mmol) and PyBOP (63 mg, 0.12 mmol) were added to a stirred solution of compound **1i** (130 mg, 0.10 mmol) and compound **20n** (26 mg, 0.04 mmol) in DMF (3 mL) at 0 °C. After stirring at 0 °C for 30 minutes, the reaction was allowed to warm to room temperature over 20 hours under N<sub>2</sub>. The reaction mixture was poured into H<sub>2</sub>O (50 mL) and extracted with EtOAc (3 x 50 mL). The organic layers were combined, filtered and concentrated under reduced pressure. The residue was dissolved in DMSO (1 mL) and purified by HPLC, which produced the compound **20o** (28 mg, 10 %) as white solid. EI-MS  $m/z$ :  $1/2[M+H]^+$  1481.5,  $1/2[M+Na]^+$  1503.8.

### Preparation of Compound **20p**

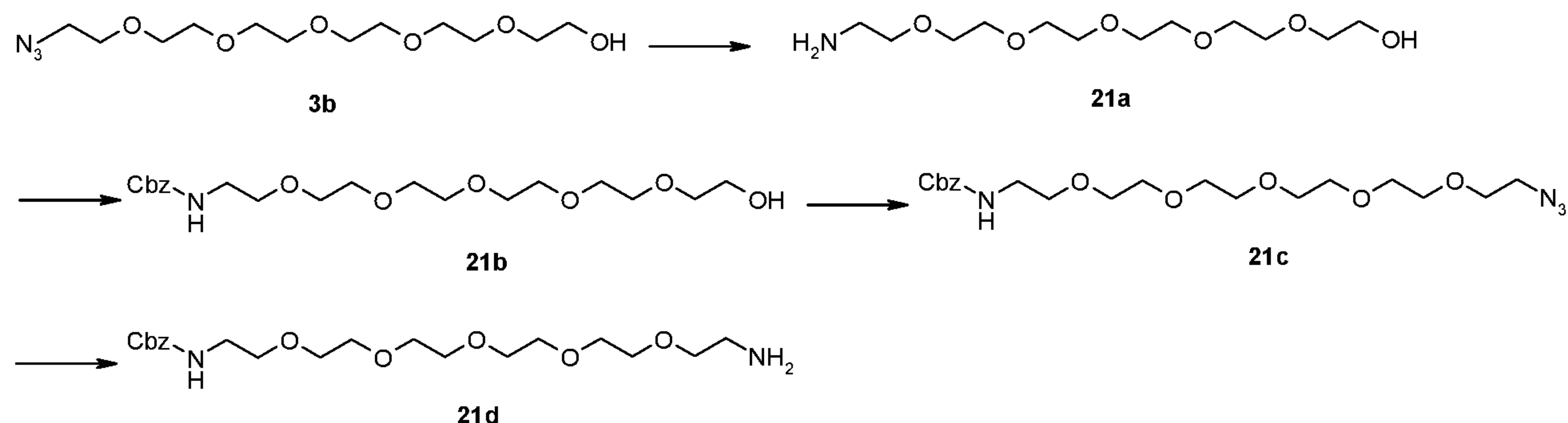
To a solution of compound **20o** (28 mg, 0.009 mmol) in MeOH (1 mL) was added LiOH monohydrate (2 mg, 0.047 mmol) in H<sub>2</sub>O (1 mL) at -5 °C. The reaction mixture was stirred at -5 °C for 1 hour. After the reaction was completed, the pH of the solution was adjusted to 4~5 with acetic acid. The residue was dissolved in DMSO (1 mL) and purified

by HPLC, which produced the compound **20p** (16 mg, 67 %) as white solid. EI-MS  $m/z$ :  $1/2[M+H]^+$ : 1341.4.

#### Preparation of Compound 20q

To a solution of compound **20p** (16 mg, 0.0059 mmol) in DCM (2 mL) was added TFA (0.2 mL) at 0 °C. After 2 hours at 0 °C, the solvent and excess TFA were removed by N<sub>2</sub> flow. The residue was dissolved in DMSO (1 mL) and purified by HPLC, which produced the compound **20q** (8.5 mg, 56 %) as white solid. EI-MS  $m/z$ :  $1/2[M+H]^+$ : 1291.3.

#### *Example 31. Preparation of Compound 21i*



#### Preparation of Compound 21a

To a solution of compound **3b** (9.0 g, 29.2 mmol) in MeOH (146 mL) was added Pd/C (10 wt. %, 3.0 g) and the reaction mixture was stirred at room temperature for 5 hours under hydrogen. Then the reaction mixture was filtered through a celite pad and washed with MeOH (100 mL). Concentration provided compound **21a** (8.2 g, 100 %) as colorless oil, which was used without further purification. <sup>1</sup>H-NMR (400MHz, CDCl<sub>3</sub>) δ 3.80-3.60 (m, 24H), 3.01 (t,  $J$  = 4.8 Hz, 2H).

#### Preparation of Compound 21b

To a solution of compound **21a** (8.24 g, 29.2 mmol) in THF (190 mL) was added triethylamine (6.1 mL, 43.9 mmol) and benzyl chloroformate (4.6 mL 32.2 mmol) at 0 °C under N<sub>2</sub>. The reaction mixture was concentrated and the crude product was purified by column chromatography to produce the compound **21b** (5.59 g, 46 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.45-7.20 (m, 5H), 5.61 (br s, 1H), 5.09 (s, 2H), 3.85-3.50 (m, 22H), 3.39 (m,

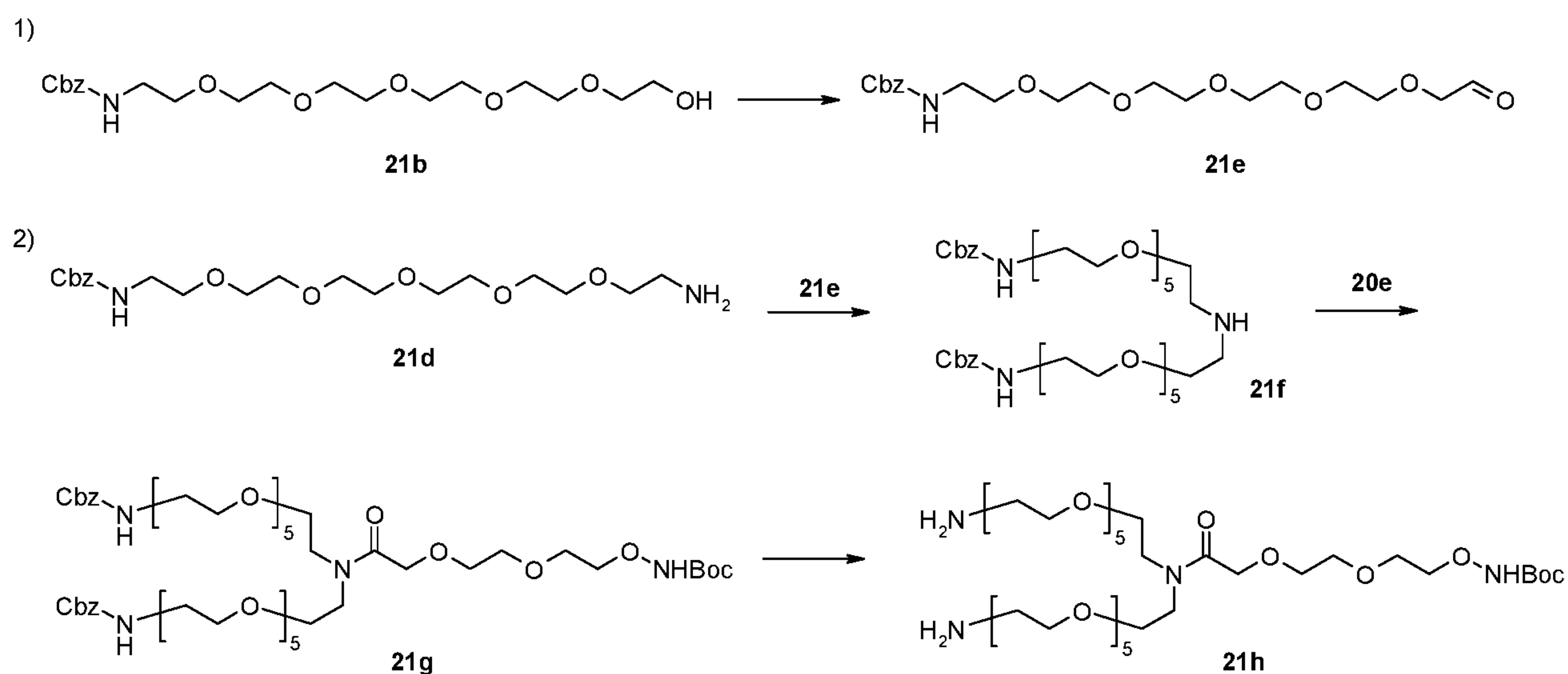
2H).

### Preparation of Compound **21c**

To a solution of compound **21b** (3.09 g, 7.43 mmol) in THF (75 mL) were added 4-methylmorpholine (1.1 mL, 9.66 mmol) and methanesulfonic anhydride (1.43 g, 8.18 mmol) at 0 °C under N<sub>2</sub>. After 5 hours at 0 °C, NaN<sub>3</sub> (969 mg, 14.9 mmol) and DMF (20 mL) were added. After 16 hours under reflux, the reaction mixture was filtered and concentrated. The residue was diluted with H<sub>2</sub>O (50 mL) and extracted with EtOAc (2 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **21c** (2.62 g, 80 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.45-7.20 (m, 5 H), 5.45 (br s, 1 H), 5.09 (s, 2 H), 3.85-3.25 (m, 24 H).

### Preparation of Compound **21d**

Triphenylphosphine (1.87 g, 7.13 mmol) was added to a solution of compound **21c** (2.62 g, 5.94 mmol) in THF (30 mL) at room temperature. After stirring for 2 hours under N<sub>2</sub>, H<sub>2</sub>O (0.54 mL, 29.7 mmol) was added and the reaction mixture was refluxed for 3 hours. The reaction mixture was concentrated and purified by column chromatography, which produced the compound **21d** (2.47 g, 95 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.45-7.25 (m, 5 H), 5.63 (br s, 1 H), 5.09 (s, 2 H), 3.80-3.25 (m, 22 H), 3.00-2.80 (m, 2 H).



### Preparation of Compound **21e**

To a stirred solution of oxalyl chloride (0.78 mL, 9.02 mmol) in DCM (14 mL) was

added DMSO (1.3 mL, 18.1 mmol) in DCM (6 mL) and then the reaction mixture was stirred at -78 °C for 30 minutes. To this solution was added compound **21b** (2.5 g, 6.01 mmol) at -78 °C. After stirred for 1 hour at -78 °C, triethylamine (4.2 mL, 30.1 mmol) was added and the reaction was allowed to warm to room temperature. The reaction mixture was poured into H<sub>2</sub>O (100 mL) and extracted with EtOAc (2 x 100 mL). The organic layers were combined, dried over MgSO<sub>4</sub>. Filtration and concentration produced the compound **21e** (2.29 g), which was used without further purification. <sup>1</sup>H-NMR (400MHz, CDCl<sub>3</sub>) δ 9.70 (s, 1H), 7.45-7.25 (m, 5H), 5.25 (br s, 1H), 5.10 (s, 2H), 3.80-3.25 (m, 24H).

#### Preparation of Compound 21f

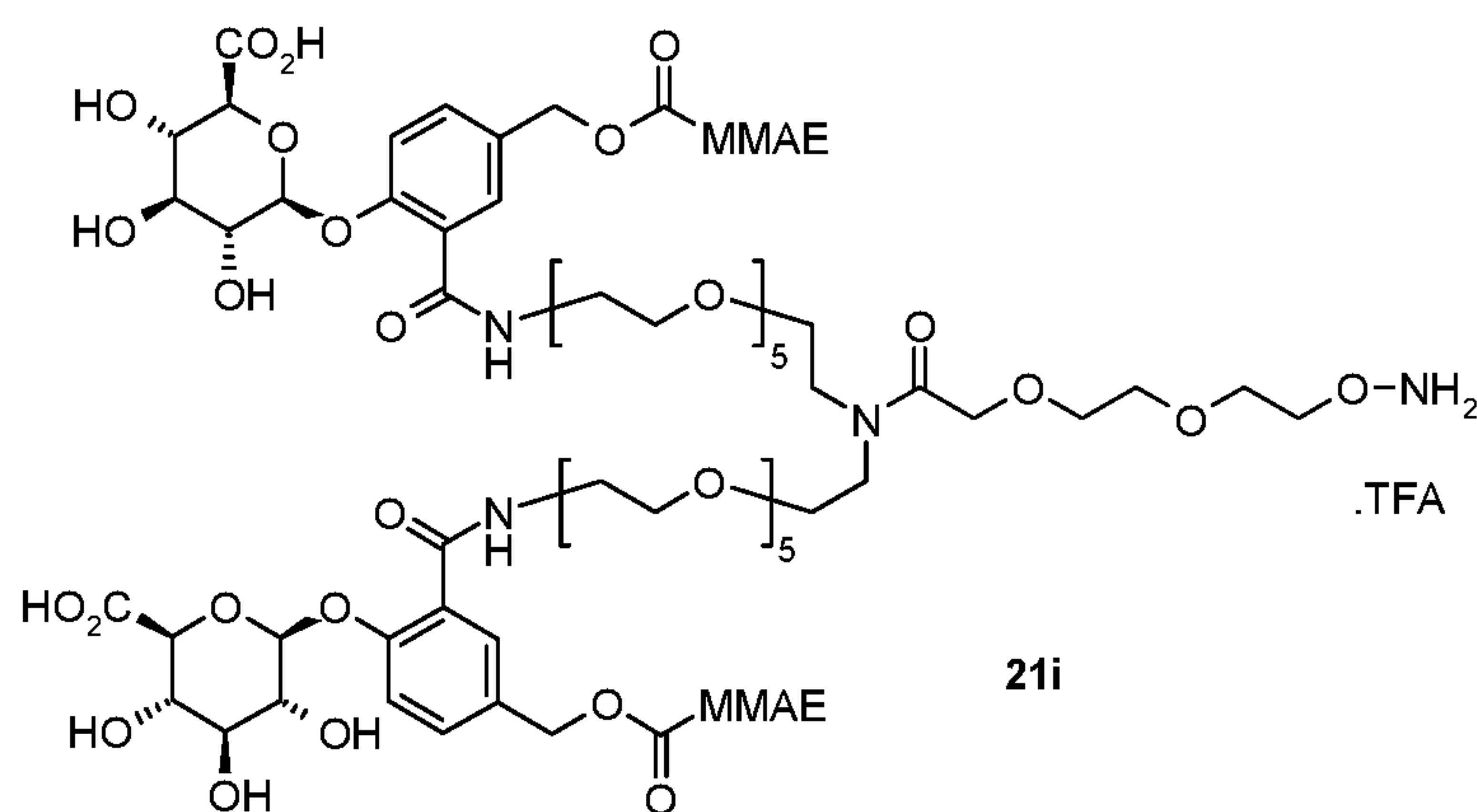
To a solution of compound **21d** (2.47 g, 5.95 mmol) and compound **21e** (2.29 g, 5.52 mmol) in MeOH (50 mL) was added NaCNBH<sub>3</sub> (530 mg, 8.44 mmol) at room temperature under N<sub>2</sub>. After 3 hours, the reaction mixture was filtered and concentrated. The crude product was purified by column chromatography to produce the compound **21f** (2.05 g, 51 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.45-7.25 (m, 10H), 5.47 (br s, 1H), 5.37 (br s, 1H), 5.09 (s, 4H), 3.80-3.25 (m, 48H).

#### Preparation of Compound 21g

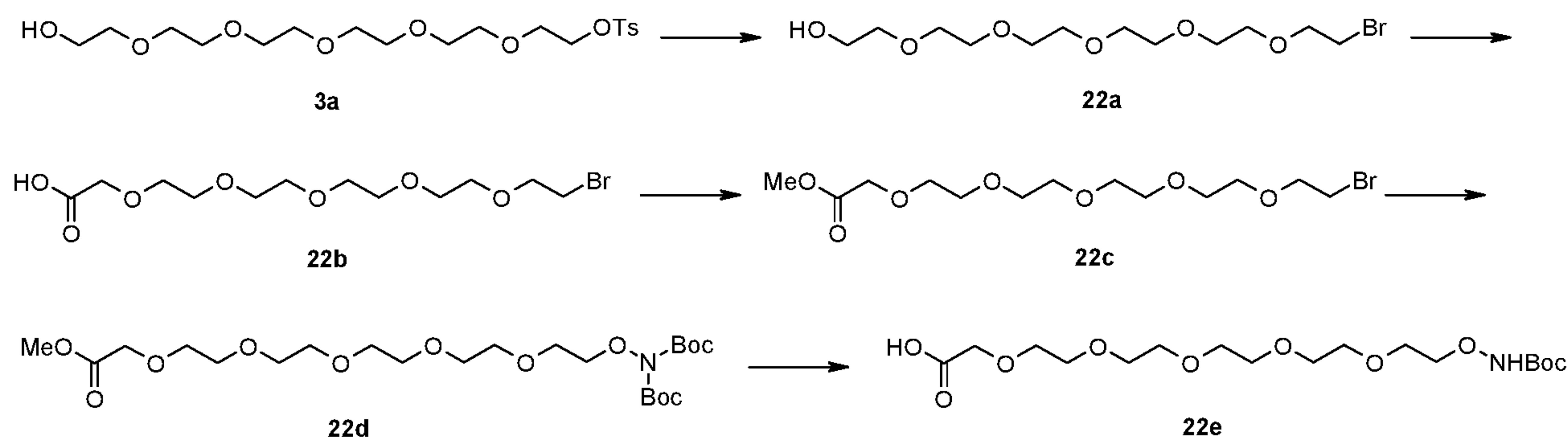
DIPEA (0.27 mL, 1.53 mmol) and HBTU (350 mg, 0.92 mmol) were added to a stirred solution of compound **21f** (380 mg, 0.61 mmol) and compound **20e** (206 mg, 0.73 mmol) in DMF (6 mL). After stirring at room temperature for 6 hours under N<sub>2</sub>, the reaction mixture was diluted water (100 mL) and extracted with EtOAc (3 x 100 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography to produce the compound **21g** (210 mg, 42 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.45-7.25 (m, 10 H), 5.47 (br s, 1 H), 5.37 (br s, 1 H), 5.09 (s, 4 H), 3.80-3.25 (m, 34 H), 1.46 (s, 9 H).

#### Preparation of Compound 21h

To a solution of compound **21g** (210 mg, 0.19 mmol) in MeOH (30 mL) was added Pd/C (10 wt. %, 1.0 g) and then the reaction mixture was stirred at room temperature for 4 hours under hydrogen. The reaction mixture was filtered through a celite pad and washed with MeOH (50 mL). Concentration provided compound **21h** (30 mg) as colorless oil, which was used without further purification. EI-MS m/z: [M+H]<sup>+</sup> 805.2, [M+Na]<sup>+</sup> 827.2.

Preparation of Compound 21i

Compound **21i** was prepared from compound **1i** and compound **21h** by a similar method of preparing compound **20q** in Example 30. EI-MS  $m/z$ :  $1/2[M+H]^+$  1423.7,  $1/2[M+Na]^+$  1445.2.

*Example 32. Preparation of Compound 22h*Preparation of Compound 22a

To a solution of compound **3a** (8.0 g, 18.3 mmol) in THF (50 mL) was added LiBr (7.9 g, 91.6 mmol) at room temperature. After stirring for 17 hours under reflux, the reaction mixture was filtered and concentrated. The crude product was purified by column chromatography to produce the compound **22a** (3.2 g, 50 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.95-3.50 (m, 24H).

Preparation of Compound 22b

To a solution of compound **22a** (3.2 g, 12.3 mmol) in acetone (20 mL) at 0 °C was added Jones reagent (20 mL). After 15 hours at 0 °C, the reaction mixture was filtered and concentrated. The residue was diluted with  $\text{H}_2\text{O}$  (50 mL) and extracted with EtOAc (2 x 100 mL). The organic layers were combined, dried over anhydrous  $\text{MgSO}_4$ , filtered and concentrated. The crude product was purified by column chromatography to produce the

compound **22b** (3.2 g, 72 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.16 (s, 2H), 3.95-3.30 (m, 20H).

#### Preparation of Compound 22c

To a solution of compound **22b** (3.2 g, 8.90 mmol) in MeOH (30 mL) was added oxalyl chloride (1.15 mL, 13.3 mmol) at 0 °C under N<sub>2</sub>. After 16 hours, the reaction mixture was concentrated and purified by column chromatography, which produced the compound **22c** (2.7 g, 81 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.17 (s, 2H), 3.80-3.60 (m, 21H), 3.47 (t, *J* = 6.4 Hz, 2H).

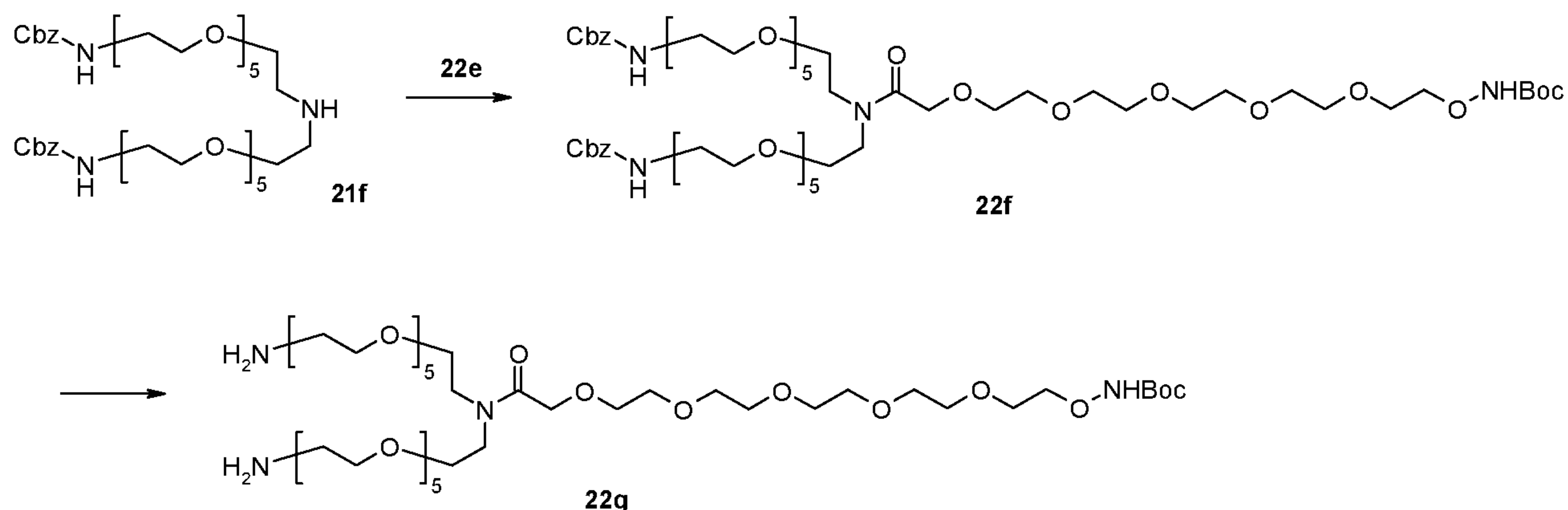
#### Preparation of Compound 22d

NaH (60 % in oil, 378 mg, 8.63 mmol) was added to a solution of compound **22c** (2.7 g, 7.23 mmol) and *N,N*-diBoc-hydroxylamine (2.2 g, 9.4 mmol) in DMF (30 mL) at 0 °C under N<sub>2</sub>. After 17 hours, the reaction mixture was concentrated. The residue was diluted with H<sub>2</sub>O (50 mL) and extracted with EtOAc (3 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **22d** (2.1 g, 55 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.17 (s, 2H), 4.08 (t, *J* = 5.2 Hz, 2H), 3.78-3.60 (m, 21H), 1.53 (s, 18H).

#### Preparation of Compound 22e

To a solution of compound **22d** (2.1 g, 3.99 mmol) in THF/MeOH/H<sub>2</sub>O (30 mL/10 mL/10 mL) was added NaOH (400 mg, 9.98 mmol) at 0 °C under N<sub>2</sub>. The reaction mixture was stirred for 3 hours at room temperature. Then the pH of the solution was adjusted to 4~5 with 1 N aqueous HCl. The reaction mixture was poured into H<sub>2</sub>O (50 mL) and extracted with EtOAc (2 x 100 mL). The organic layers were combined, dried over MgSO<sub>4</sub>. Filtration and concentration produced the compound **22e** (1.6 g) as colorless oil, which was used without further purification. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.90 (s, 1H), 4.15 (s, 2H), 4.03 (br s, 2H), 3.80-3.60 (m, 18H), 1.47 (s, 9H).

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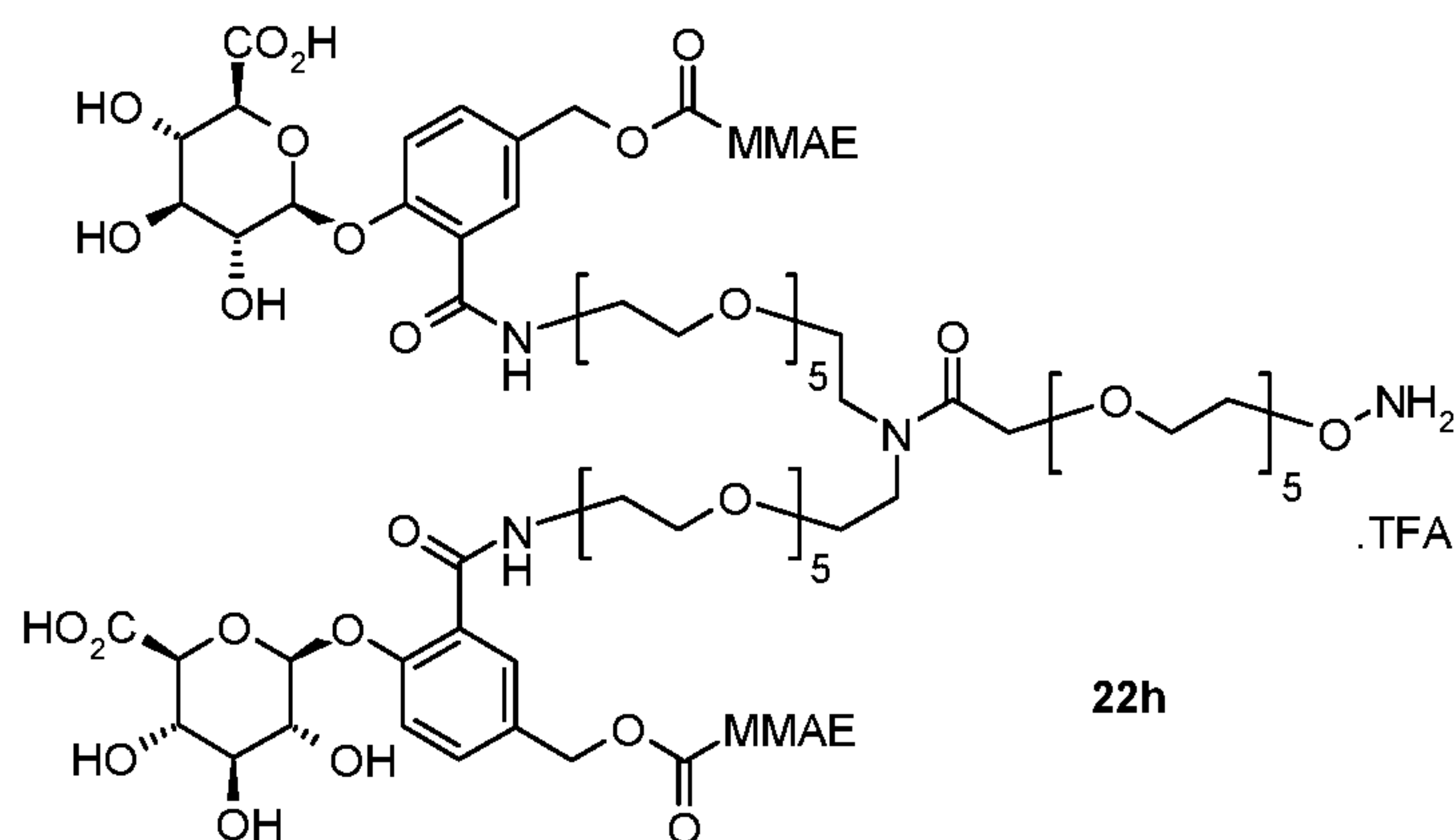
### Preparation of Compound **22f**

DIPEA (0.13 mL, 0.73 mmol) and HBTU (187 mg, 0.49 mmol) were added to a stirred solution of compound **21f** (200 mg, 0.24 mmol) and compound **22e** (152 mg, 0.36 mmol) in DMF (5 mL). The reaction mixture was stirred at room temperature for 6 hours under N<sub>2</sub>. The reaction mixture was diluted H<sub>2</sub>O (100 mL) and extracted with EtOAc (3 x 100 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography to produce the compound **22f** (100 mg, 34 %). EI-MS *m/z*: 1/2[M+H]<sup>+</sup> 1205.6.

### Preparation of Compound **22g**

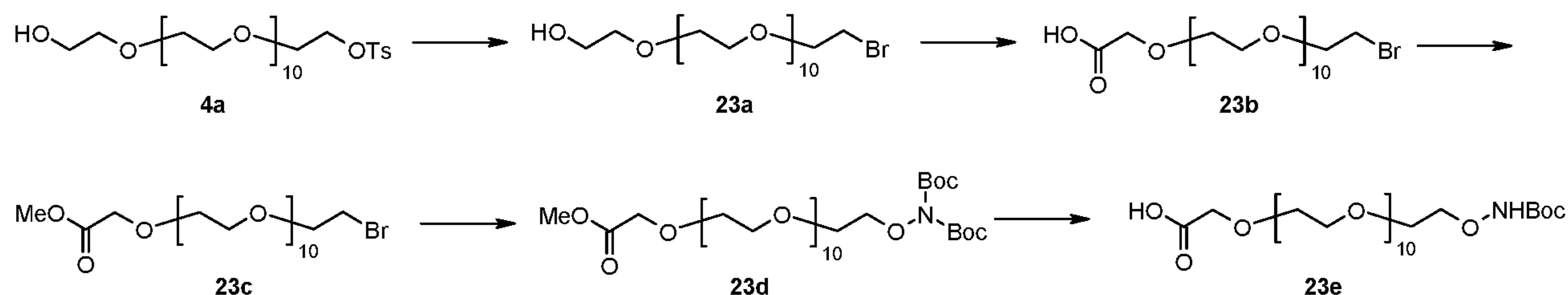
To a solution of compound **22f** (100 mg, 0.08 mmol) in MeOH (20 mL) was added Pd/C (10 wt. %, 20 mg) and then the reaction mixture was stirred at room temperature for 4 hours under hydrogen. The reaction mixture was filtered through a celite pad and washed with MeOH (20 mL). Concentration provided compound **22g** as colorless oil (70 mg), which was used without further purification. EI-MS *m/z*: [M+H]<sup>+</sup> 937.4, [M+Na]<sup>+</sup> 959.3.

### Preparation of Compound **22h**



Compound **22h** was prepared from compound **1i** and compound **22g** by a similar method of preparing compound **20q** in Example 30. EI-MS  $m/z$ :  $1/2[M+H]^+$  1489.4.

*Example 33. Preparation of Compound 23h*



Preparation of Compound 23a

To a solution of compound **4a** (483 mg, 0.69 mmol) in THF (10 mL) was added LiBr (180 mg, 2.06 mmol). The reaction mixture refluxed for 12 hours under  $N_2$ . Then the reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography to produce the compound **23a** (330 mg, 78 %).  $^1H$ -NMR (400 MHz,  $CDCl_3$ )  $\delta$  3.81 (t,  $J$  = 6.4 Hz, 2H), 3.72-3.59 (m, 44H), 3.47 (t,  $J$  = 6.4 Hz, 2H).

Preparation of Compound 23b

To a solution of compound **23a** (330 mg, 0.54 mmol) in acetone (2 mL) at 0 °C was added Jones reagent (2 mL). After 15 hours at 0 °C, the reaction mixture was filtered and concentrated. The residue was diluted with  $H_2O$  (15 mL) and extracted with EtOAc (2 x 20 mL). The organic layers were combined, dried over anhydrous  $MgSO_4$ , filtered and concentrated. The resulting crude compound **23b** was used without further purification.

Preparation of Compound 23c

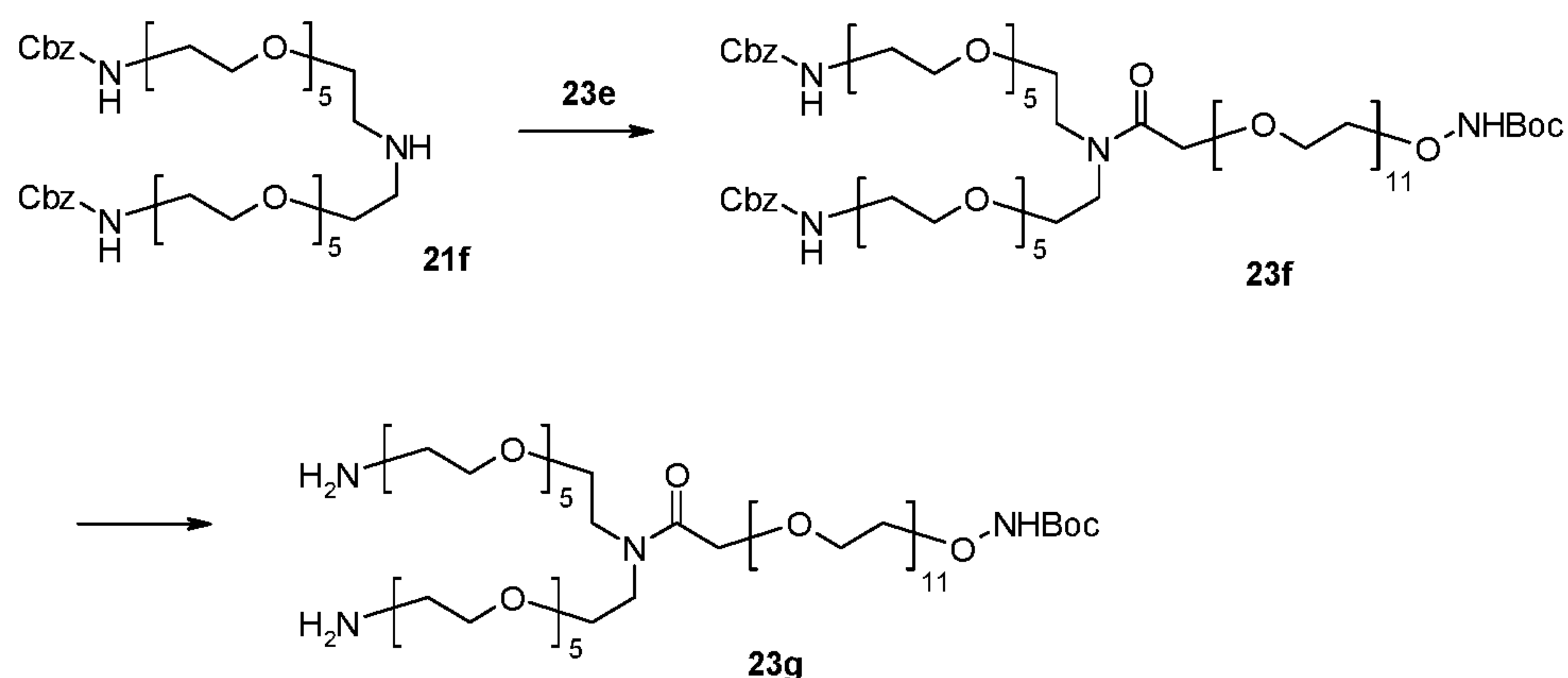
To a solution of crude compound **23b** (266 mg, 0.43 mmol) in MeOH (5 mL) was added oxalyl chloride (0.054 mL, 0.64 mmol) at 0 °C under  $N_2$ . After 16 hours, the reaction mixture was concentrated and purified by column chromatography, which produced the compound **23c** (200 mg, 58 % for 2 steps).  $^1H$ -NMR (400 MHz,  $CDCl_3$ )  $\delta$  4.17 (s, 2H), 3.81 (t,  $J$  = 6.4 Hz, 2H), 3.79-3.64 (m, 43H), 3.48 (t,  $J$  = 6.4 Hz, 2H).

Preparation of Compound **23d**

To a solution of compound **23c** (200 mg, 0.31 mmol) in DMF (3 mL) were added *N,N*-diBoc-hydroxylamine (95 mg, 0.40 mmol) and NaH (60 % in oil, 16 mg, 0.37 mmol) at 0 °C under N<sub>2</sub>. After 17 hours, the reaction mixture was concentrated. The residue was diluted with H<sub>2</sub>O (5 mL) and extracted with EtOAc (3 x 10 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **23d** (120 mg, 49 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.17 (s, 2H), 4.13 (t, *J* = 8.0 Hz, 2H), 3.75-3.64 (m, 45H), 1.53 (s, 18H).

Preparation of Compound **23e**

To a solution of compound **23d** (120 mg, 0.15 mmol) in THF/MeOH/H<sub>2</sub>O (3 mL/1 mL/1 mL) was added NaOH (15 mg, 0.38 mmol) at 0 °C under N<sub>2</sub>. The reaction mixture was stirred for 1 hour at room temperature. Then the pH of the solution was adjusted to 4~5 with 1 N aqueous HCl. The reaction mixture was poured into H<sub>2</sub>O (10 mL) and extracted with CHCl<sub>3</sub> (2 x 20 mL). The organic layers were combined, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Filtration and concentration produced the compound **23e** (100 mg), which was used without further purification. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.23 (t, *J* = 8.0 Hz, 2H), 4.15 (s, 2H), 4.08 (t, *J* = 4.0 Hz, 1H), 4.01 (t, *J* = 4.0 Hz, 1H), 3.74-3.64 (m, 40H), 1.53 (s, 9H).

Preparation of Compound **23f**

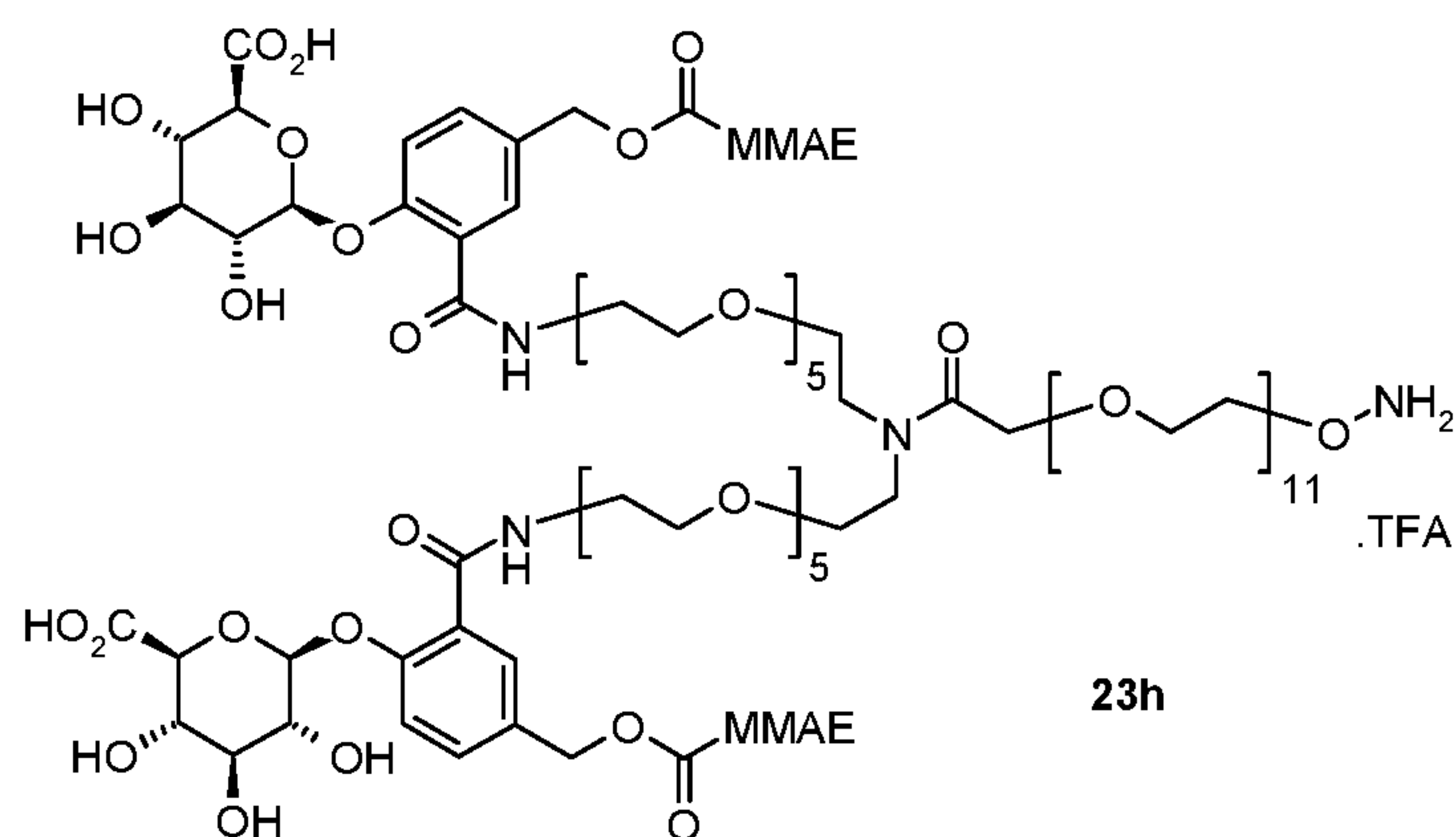
DIPEA (0.052 mL, 0.29 mmol) and HBTU (75 mg, 0.20 mmol) were added to a stirred solution of compound **21f** (80 mg, 0.09 mmol) and compound **23e** (100 mg, 0.15 mmol) in DMF (3 mL). After stirring at room temperature for 6 hours under N<sub>2</sub>, the

reaction mixture was diluted with H<sub>2</sub>O (50 mL) and extracted with EtOAc (3 x 50 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography to produce the compound **23f** (140 mg, 97 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.38-7.31 (m, 10 H), 5.44 (br, 2 H), 5.09 (s, 4 H), 4.34 (s, 2 H), 4.26-4.17 (m, 4 H), 4.09-4.08 (m, 1 H), 4.07 (br, 1 H), 3.73-3.47 (m, 76 H), 3.39-3.38 (m, 4 H), 1.53 (s, 9 H). EI-MS m/z: [M+Na]<sup>+</sup> 1491.6, [M+H-Boc]<sup>+</sup> : 1369.6.

#### Preparation of Compound **23g**

To a solution of compound **23f** (140 mg, 0.09 mmol) in MeOH (20 mL) was added Pd/C (10 wt. %, 20 mg) and then the reaction mixture was stirred at room temperature for 4 hours under hydrogen. The reaction mixture was filtered through a celite pad and washed with MeOH (20 mL). Concentration provided compound **23g** as colorless oil (120 mg), which was used without further purification. EI-MS m/z: [M+H]<sup>+</sup> 1201.7, [M+Na]<sup>+</sup> 1223.7.

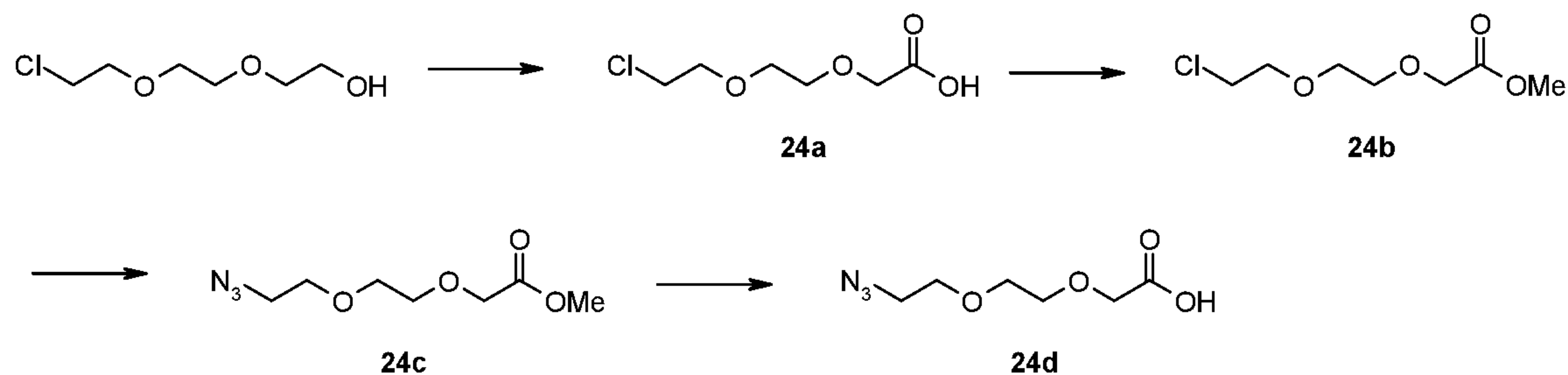
#### Preparation of Compound **23h**



Compound **23h** was prepared from compound **1i** and compound **23g** by a similar method of preparing compound **20q** in Example 30. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1620.3, 1/2[M+Na]<sup>+</sup> 1632.1.

#### *Example 34. Preparation of Compound **24l***

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#### Preparation of Compound 24a

Jones reagent (90 mL) was slowly added to a solution of compound 2-[2-(2-chloroethoxy)ethoxy]ethanol (15.0 g, 88.9 mmol) in acetone (600 mL) at 0 °C. After 15 hours at 0 °C, the reaction mixture was filtered and concentrated. The residue was diluted with H<sub>2</sub>O (200 mL) and extracted with CHCl<sub>3</sub> (5 x 300 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>. Concentration provided compound **24a** (20.0 g), which was used without further purification. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.18 (s, 2H), 3.81-3.64 (m, 8H).

#### Preparation of Compound 24b

To a solution of compound **24a** (20.0 g, 88.9 mmol) in MeOH (500 mL) was added oxalyl chloride (11.5 mL, 133.4 mmol) at 0 °C for 30 minutes under N<sub>2</sub>. After 16 hours, the reaction mixture was concentrated and purified by column chromatography, which produced the compound **24b** (13.0 g, 75 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.18 (s, 2H), 3.78-3.67 (m, 9H), 3.65 (t, *J* = 5.6 Hz, 2H).

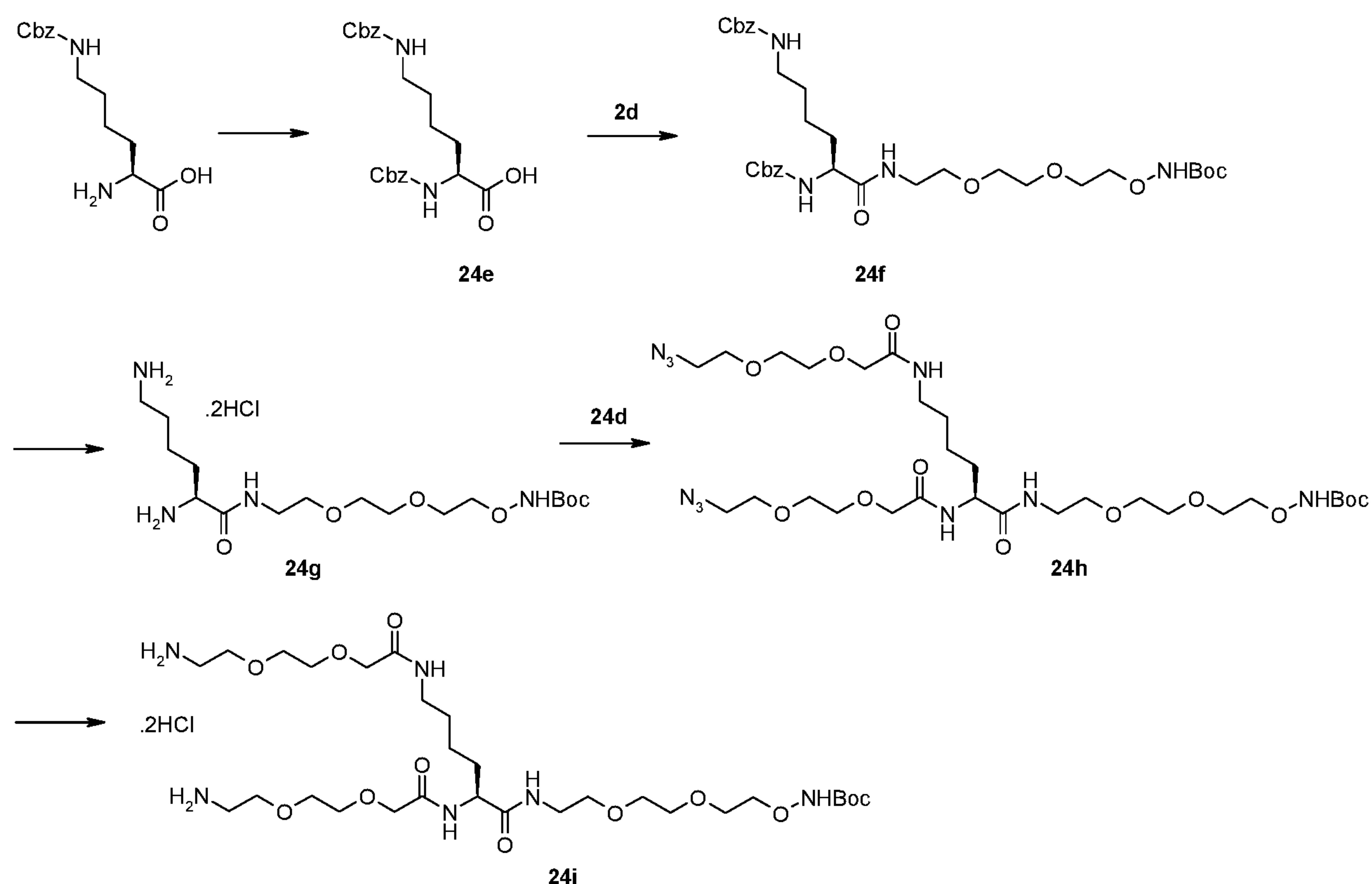
#### Preparation of Compound 24c

Compound **24b** (13.0 g, 66.1 mmol) and NaN<sub>3</sub> (6.4 g, 99.2 mmol) were dissolved in DMF (130 mL). After stirring at 100 °C for 2 hours, the reaction mixture was diluted with brine (200 mL) and extracted with CHCl<sub>3</sub> (2 x 100 mL). The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>. Concentration provided compound **24c** (11.7 g, 87 %), which was used without further purification. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.18 (s, 2H), 3.76-3.67 (m, 9H), 3.41 (t, *J* = 5.6 Hz, 2H).

#### Preparation of Compound 24d

To a solution of compound **24c** (11.5 g, 56.6 mmol) in THF/MeOH/H<sub>2</sub>O (300 mL/100 mL/100 mL) was added NaOH (4.53 g, 113.2 mmol) at 0 °C. After 2 hours at 0 °C under N<sub>2</sub>, the pH of the solution was adjusted to 2 with 4 M aqueous HCl. The reaction

mixture was poured into H<sub>2</sub>O (100 mL) and extracted with CHCl<sub>3</sub> (3 x 500 mL). The organic layers were combined, dried over MgSO<sub>4</sub>. Filtration and concentration produced the compound **24d** (10.7 g, 99 %) as colorless oil, which was used without further purification. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.19 (s, 2H), 3.79-3.77 (m, 2H), 3.72-3.70 (m, 4H), 3.44 (t, *J* = 5.2 Hz, 2H).



#### Preparation of Compound 24e

A three-necked flask was loaded consecutively with H<sub>2</sub>O (40 mL), 1,4-dioxane (70 mL) and H-Lys(Z)-OH (10 g, 35.7 mmol). The mixture was stirred until complete dissolution. The pH was adjusted to about 10.5 by adding of 2 M aqueous Na<sub>2</sub>CO<sub>3</sub>. Benzyl chloroformate (6.69 g, 39.2 mmol) was added while maintaining the pH at about 10-11 by adding at the same time 2 M aqueous Na<sub>2</sub>CO<sub>3</sub>. After completing addition, the reaction mixture was stirred at 20 °C for 1 hour. Then EtOAc (50 mL) was added and pH of the resulting mixture was adjusted to 2~3 with c-HCl. The organic layer was separated and the aqueous layer was extracted with EtOAc (50 mL). The combined organic layers were washed with brine (50 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Filtration and concentration under reduced pressure yielded the compound **24e** as yellowish oil (14.7g, 99 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.33-7.27 (m, 10H), 5.07-5.04 (d, 4H), 4.08 (m, 1H), 3.09 (t, 2H), 1.51 (br s, 1H), 1.49 (bs, 1H), 1.47-1.40 (m, 4H).

### Preparation of Compound 24f

DIPEA (0.40 mL, 2.37 mmol), HOBt (143 mg, 1.06 mmol) and EDC·HCl (240 mg, 1.25 mmol) were added to a stirred mixture of compound **24e** (400 mg, 0.96 mmol) and compound **2d** (261 mg, 0.86 mmol) in DMF (3 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (50 mL), extracted with EtOAc (3 x 50 mL), washed with aq NaHCO<sub>3</sub> (50 mL) and brine (50 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting residue was purified by column chromatography to yield the compound **24f** (380 mg, 59 %). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.16 (s, 1H), 7.34-7.28 (m, 10H), 7.49 (s, 1H) 5.08-5.07 (m, 5H), 4.17 (m, 1H), 3.99 (t, 2H), 3.68-3.16 (m, 10H), 3.17 (d, 2H), 1.66 (m, 1H), 1.51-1.27 (m, 14H). EI-MS m/z: [M+H]<sup>+</sup> 661.0.

### Preparation of Compound 24g

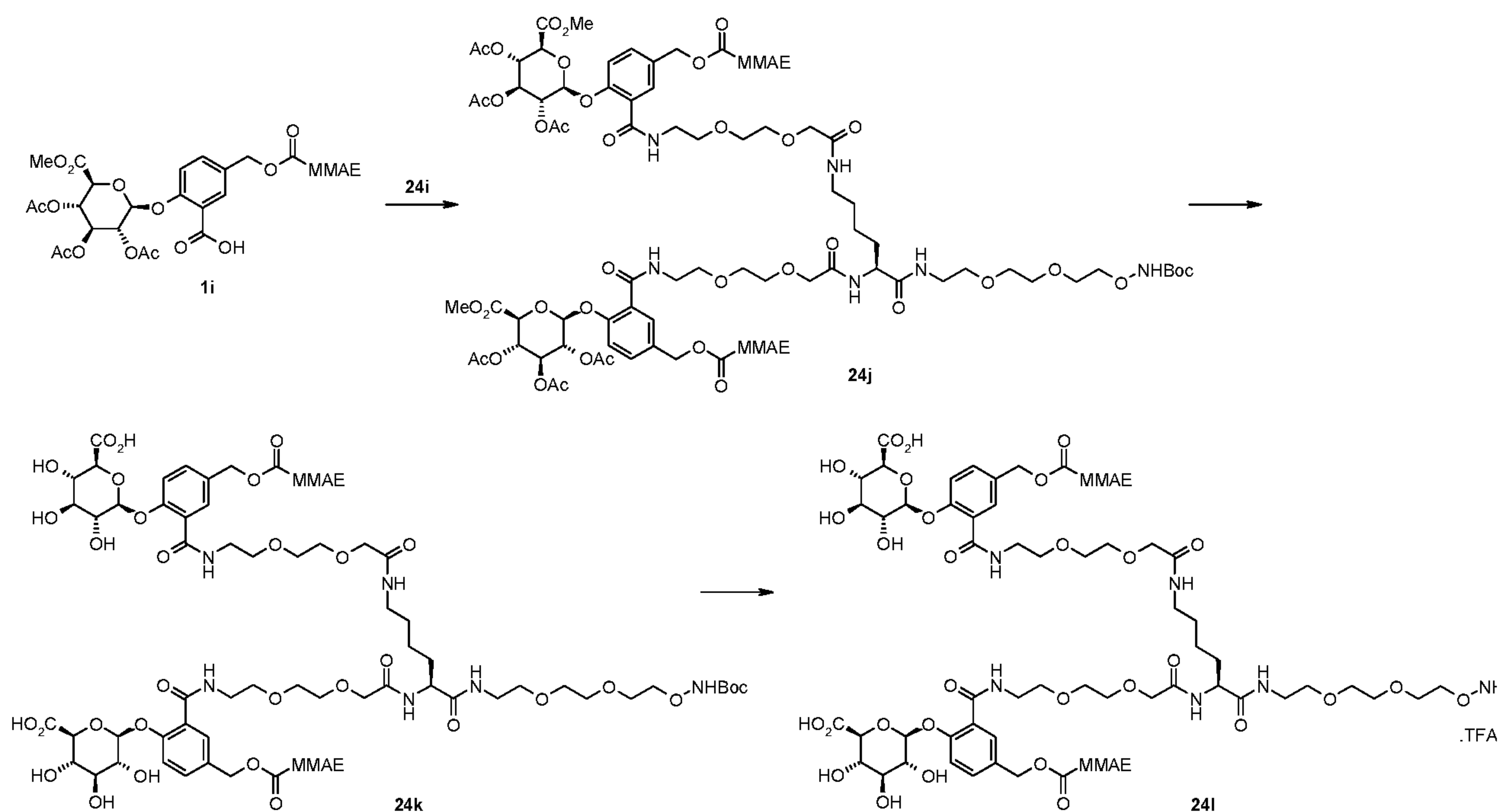
To a stirred mixture of compound **24f** (370 mg, 0.55 mmol) and Pd/C (10 wt. %, 74 mg) in MeOH (10 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.27 mL, 1.1 mmol). After stirring at room temperature for 2 h under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (40 mL). The filtrate was concentrated to produce the compound **24g** (223 mg, 87 %) as colorless oil, which was used without further purification. <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 10.02 (s, 1H), 8.62 (s, 1H), 8.22 (br, 2H), 7.90 (br, 2H), 3.81 (t, 2H), 3.56 (m, 4H), 3.46 (t, 2H), 3.39-3.27 (m, 26H), 2.75(m, 2H), 1.73 (q, 2H), 1.55 (p, 2H), 1.40-1.33 (m, 14H).

### Preparation of Compound 24h

DIPEA (1.6 mL, 9.45 mmol), HOBt (746 mg, 5.52 mmol) and EDC·HCl (1.19 g, 6.42 mmol) were added to a stirred mixture of compound **24g** (1.0 g, 5.29 mmol) and compound **24d** (1.1 g, 2.35 mmol) in DMF (15 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (20 mL), extracted with DCM (3 x 50 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting residue was purified by column chromatography to yield the compound **24h** (1.25 g, 70 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 8.36 (s, 1H), 7.30 (d, 1H), 7.08 (s, 1H), 7.68 (t, 1H), 4.46 (q, 1H), 4.07-3.98-4.01 (m, 4H), 3.98 (s, 2H), 3.75-3.663 (m, H) 3.57(t, 2H), 3.44 (m, 6H), 3.28 (m, 2H), 1.87(m, 1H), 1.66 (m, 1H), 1.59-1.52 (p,2H), 1.48 (s, 9H), 1.41-1.33 (m, 2H). EI-MS m/z: [M+H]<sup>+</sup> 735.0.

### Preparation of Compound **24i**

To a stirred mixture of compound **24h** (1.2 g, 0.163 mmol), and Pd/C (10 wt. %, 250 mg) in MeOH (30 mL) at 0 °C, 4 N HCl (1,4-dioxane, 0.81 mL, 3.26 mmol) was added. After stirring at room temperature for 1.5 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (100 mL). The filtrate was concentrated to produce the compound **24i** (1.39 g, 99 %) as colorless oil, which was used without further purification. <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 9.99 (s, 1H), 8.22 (t, 1H), 7.74 (t, 1H), 7.61 (d, 1H), 4.31, (q, 1H), 3.93 (s, 2H), 3.86 (s, 2H), 3.79 (t, 2H), 3.60-3.50 (m, 18H), 3.06 (q, 2H), 2.97 (p, 4H), 1.60-1.49 (m, 2H), 1.39 (m, 11H), 1.20 (m, 2H). EI-MS m/z: [M+H]<sup>+</sup> 683.



### Preparation of Compound **24j**

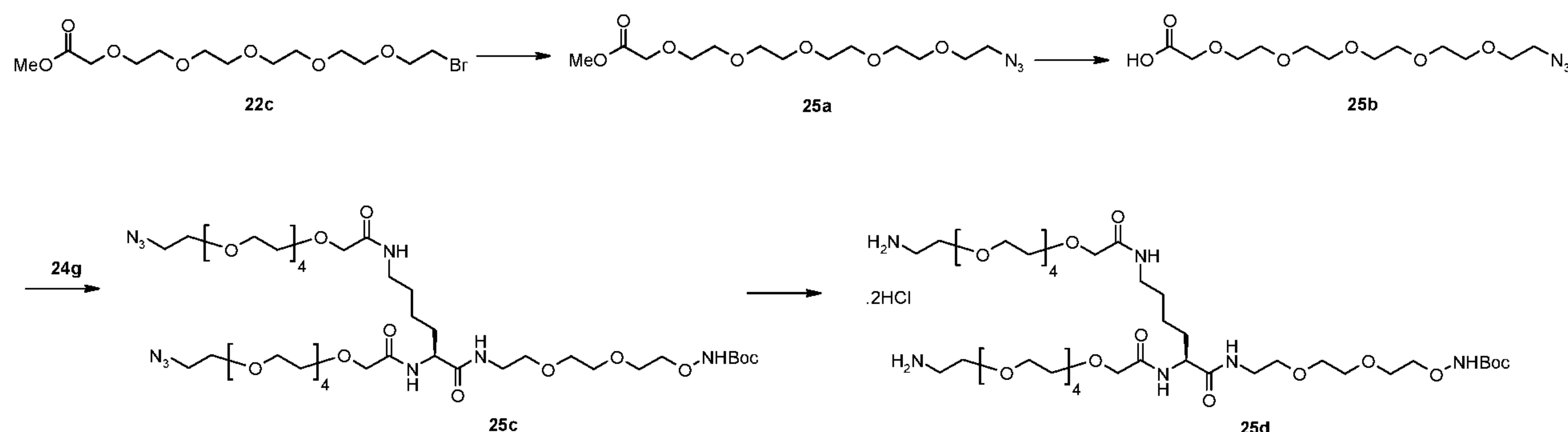
DIPEA (0.021 mL, 0.125 mmol) and HBTU (29 mg, 0.078 mmol) were added to a stirred mixture of compound **1i** (85 mg, 0.069 mmol) and compound **24i** (23 mg, 0.031 mmol) in DMF (0.7 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC. Pure fractions with the same retention time were combined and concentrated to produce the compound **24j** (67 mg, 68 %). EI-MS m/z: 1/2[M+H]<sup>+</sup> 1552.5.

Preparation of Compound **24k**

To a solution of compound **24j** (67 mg, 0.021 mmol) in MeOH (1.7 mL) was added LiOH monohydrate (16 mg, 0.388 mmol) in H<sub>2</sub>O (1.7 mL) at 0 °C. After stirring for 2 hours at 0 °C, the reaction mixture was neutralized using acetic acid (0.018 mL) and concentrated under reduced pressure. The reaction mixture was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC. Pure fractions with the same retention time were combined and concentrated to produce the compound **24k** (37 mg, 62 %). EI-MS *m/z*: 1/2[M+H]<sup>+</sup> 1412.3.

Preparation of Compound **24l**

TFA (0.4 mL) was added to a stirred solution of compound **24k** (37 mg, 0.013 mmol) in DCM (2.0 mL). After stirring at 0 °C for 2 hours, the solvent and excess TFA were blown off with N<sub>2</sub>. The residue was dissolved in H<sub>2</sub>O/acetonitrile (1 mL/1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **24l** (19.8 mg, 53 %) as white solid. EI-MS *m/z*: 1/2[M+H]<sup>+</sup> 1362.3.

*Example 35. Preparation of Compound **25e***Preparation of Compound **25a**

Compound **22c** (1.0 g, 2.67 mmol) and NaN<sub>3</sub> (261 mg, 4.01 mmol) were dissolved in DMF (3 mL). The reaction mixture was heated at 100 °C for 5 hours. After the reaction was completed, the reaction mixture was filtered and concentrated. The residue was purified by column chromatography (EtOAc to EtOAc/MeOH 10/1), which produced the compound **25a** (854 mg, 95 %).

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.17 (s, 2H), 3.76-3.64 (m, 21H), 3.39 (t, *J* = 5.2 Hz, 2H).

### Preparation of Compound 25b

To a stirred solution of compound **25a** (854 mg, 2.54 mmol) in MeOH (25 mL) at 0 °C, 2 M aq. NaOH (6.3 mL, 12.64 mmol) was added. The reaction mixture was stirred at room temperature for 3 hours. The solution was then concentrated under reduced pressure. The resulting suspension was acidified with aqueous 2 N HCl while cooling at 0 °C. The residue was extracted by CHCl<sub>3</sub> (8 x 500 mL). The organic layers were combined, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated to produce the compound **25b** (783 mg, 96 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.16 (s, 2H), 3.76-3.65 (m, 18H), 3.40 (t, *J* = 5.2 Hz, 2H).

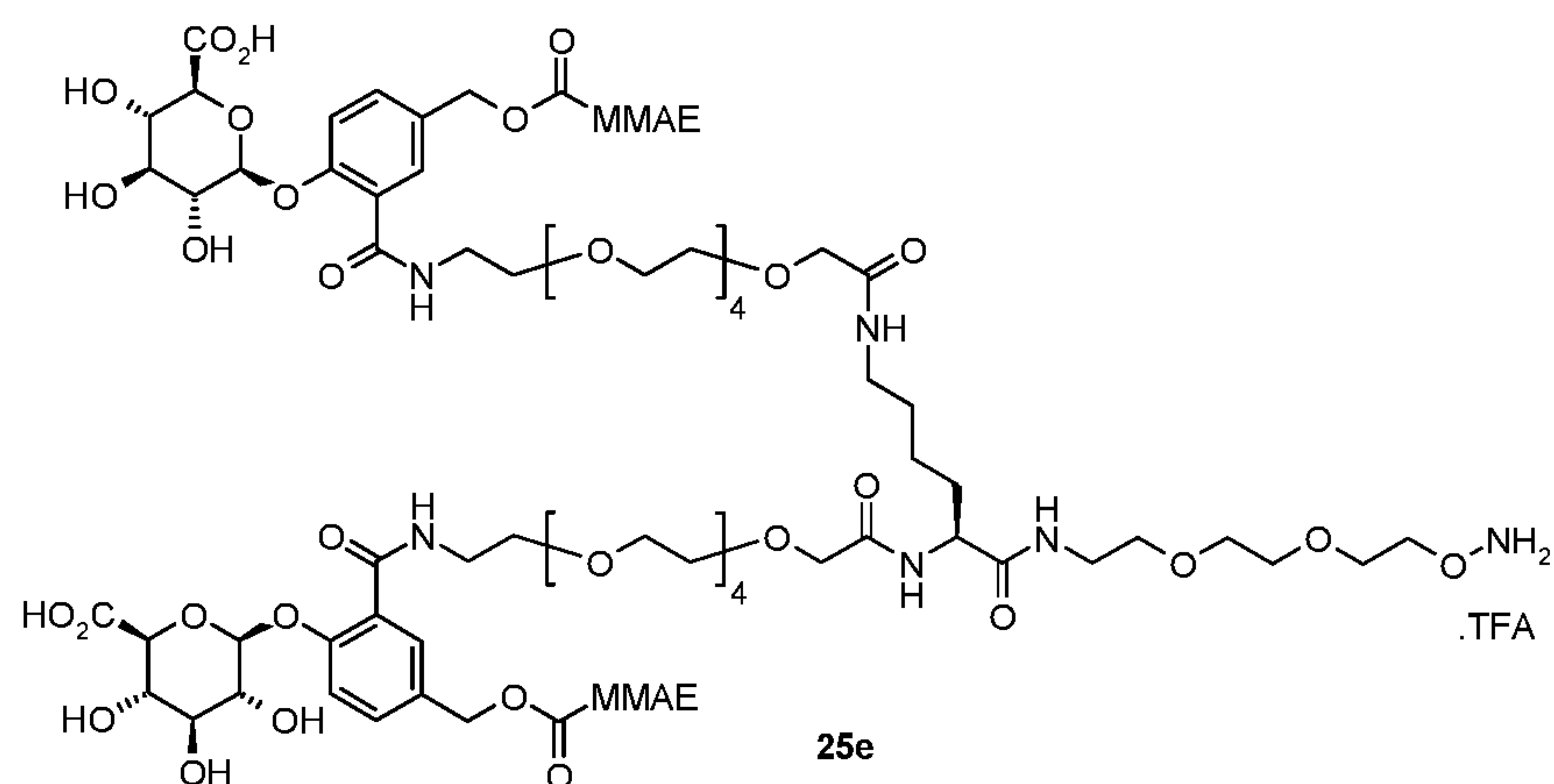
### Preparation of Compound 25c

DIPEA (0.30 mL, 1.70 mmol) and HBTU (483 mg, 1.27 mmol) were added to a stirred mixture of compound **25b** (337 mg, 1.05 mmol) and compound **24g** (198 mg, 0.42 mmol) in DMF (3 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was concentrated and purified by column chromatography (EtOAc to EtOAc/ MeOH 10/1), which produced the compound **25c** (358 mg, 84 %). <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 9.98 (s, 1H), 8.09 (t, *J* = 5.2 Hz, 1H), 7.63 (t, *J* = 5.2 Hz, 1H), 7.55 (d, *J* = 8.4 Hz, 1H), 4.31-4.25 (m, 1H), 3.90 (s, 2H), 3.84 (s, 2H), 3.80 (m, 2H), 3.62-3.46 (m, 34H), 3.42-3.36 (m, 6H), 3.25-3.17 (m, 2H), 3.08-3.03 (m, 2H), 1.61-1.51 (m, 2H), 1.39 (s, 9H), 1.26-1.10 (m, 7H). EI-MS *m/z*: [M+H]<sup>+</sup> 999.1.

### Preparation of Compound 25d

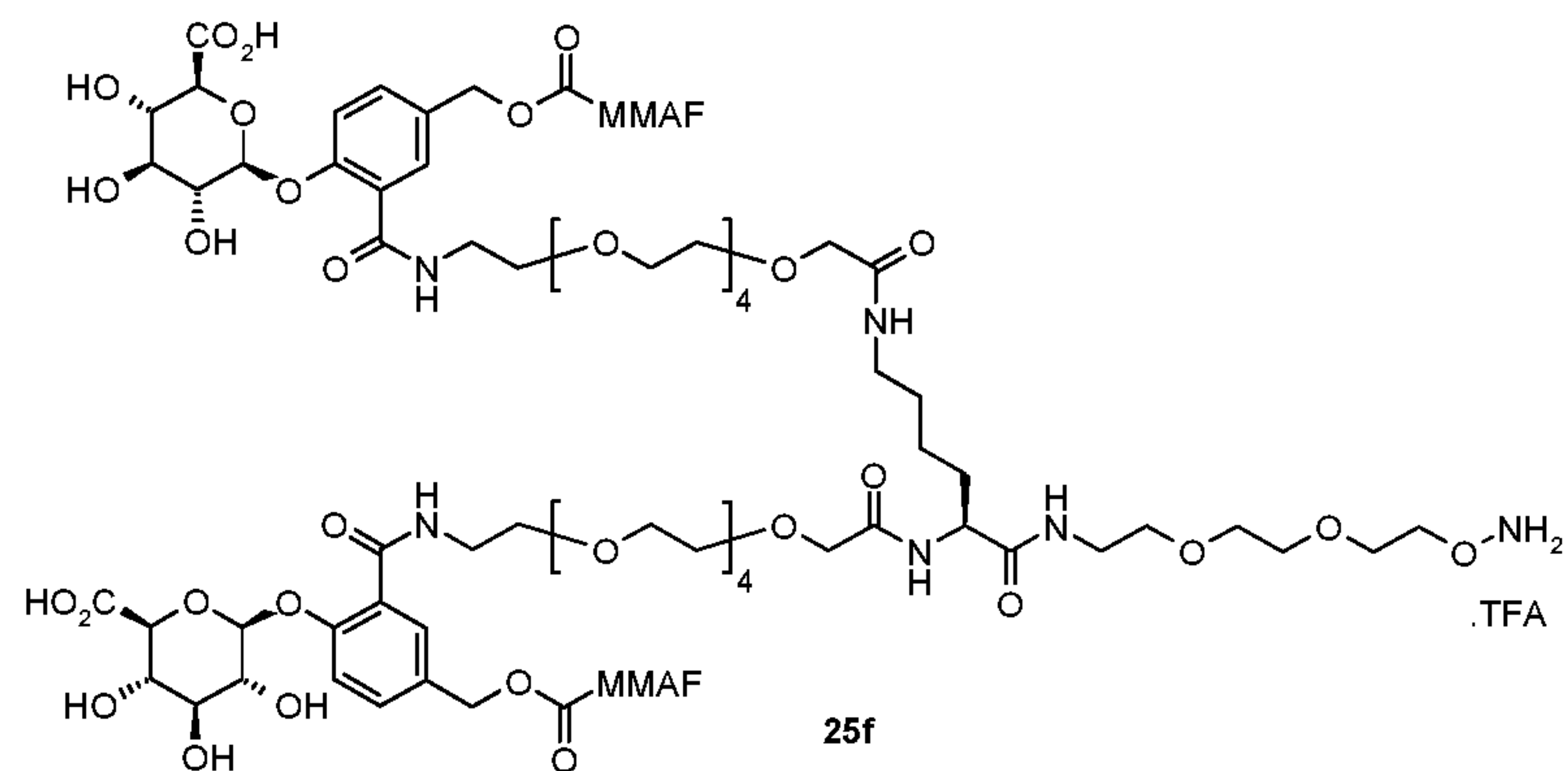
To a solution of compound **25c** (358 mg, 0.35 mmol) in MeOH (7 mL) was added Pd/C (10 wt. %, 38 mg) and HCl (4 N in 1,4-dioxane, 0.18 mL, 0.72 mmol). After stirring at room temperature for 5 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (400 mL). The filtrate was concentrated to produce the compound **25d** (314 mg, 93 %) as colorless oil, which was used without further purification. <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 9.98 (s, 1H), 8.10 (m, 1H), 7.68 (m, 1H), 7.57 (m, 1H), 4.31-4.25 (m, 1H), 3.90 (s, 2H), 3.84 (s, 2H), 3.80 (m, 2H), 3.62-3.46 (m, 30H), 3.42-3.36 (m, 10H), 3.45-3.16 (m, 4H), 3.08-3.03 (m, 3H), 2.72-2.66 (m, 3H), 1.61-1.51 (m, 2H), 1.39 (s, 9H), 1.26-1.10 (m, 6H). EI-MS *m/z*: [M+H]<sup>+</sup> 947.1.

### Preparation of Compound 25e



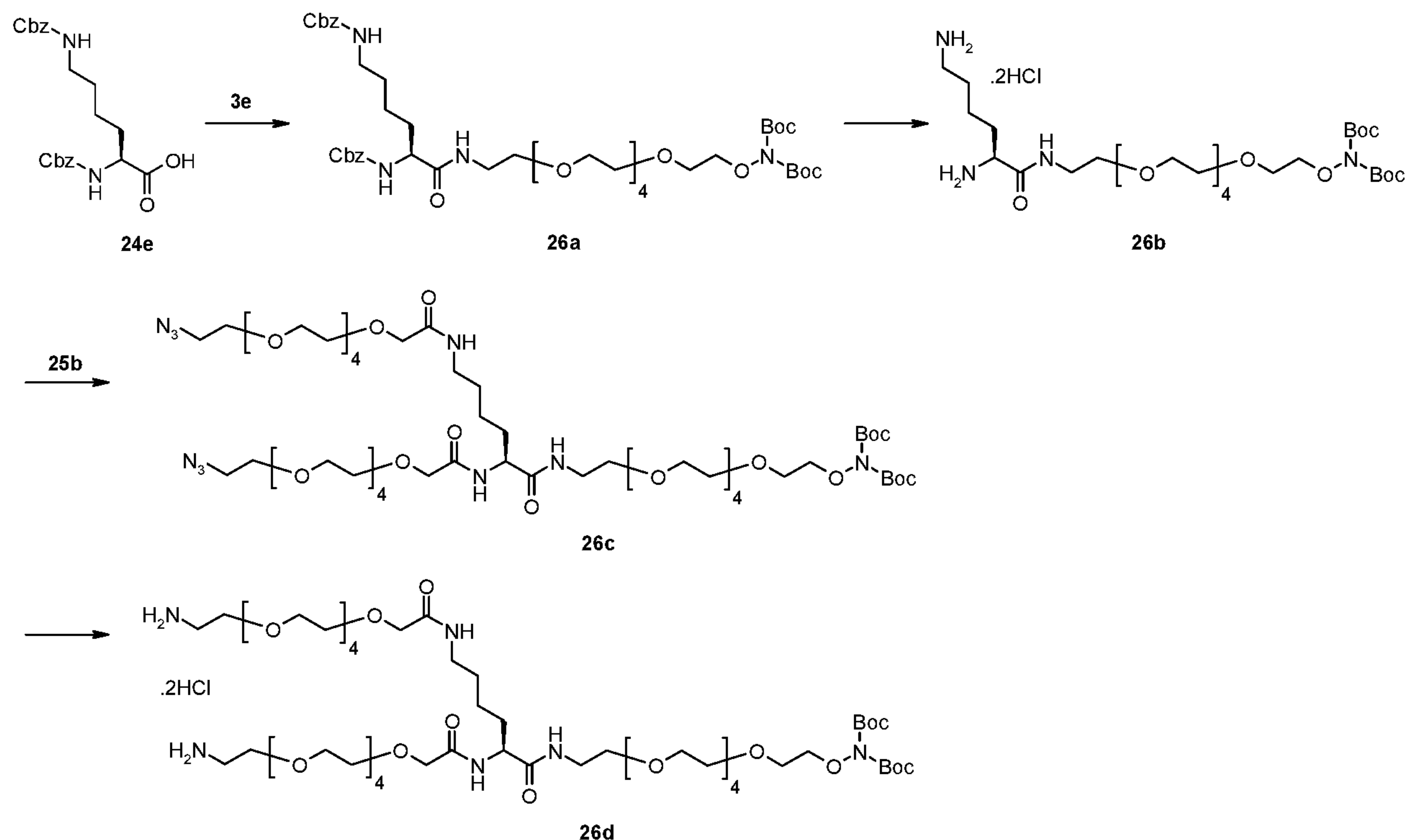
Compound **25e** was prepared from compound **1i** and compound **25d** by a similar method of preparing compound **24i** in Example 34. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1493.7.

*Example 36. Preparation of Compound 25f*



Compound **25f** was prepared from compound **1j** and compound **25d** by a similar method of preparing compound **24i** in Example 34. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1508.2.

*Example 37. Preparation of Compound 26e*



#### Preparation of Compound **26a**

DIPEA (0.65 mL, 0.004 mmol), HOBt (218 mg, 1.61 mmol) and EDC·HCl (364 mg, 1.9 mmol) were added to a stirred mixture of compound **24e** (1.0 g, 2.43 mmol) and compound **3e** (810 mg, 1.52 mmol) in DMF (10 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (50 mL) and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with 1 N aq. HCl (30 mL), saturated aq. NaHCO<sub>3</sub> (30 mL), and brine (30 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **26a** (988 mg, 73 %).

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.33-7.26 (m, 8 H), 6.85 (s, 1H), 5.63 (s, 1H), 5.08-5.02 (s, 4 H), 4.16-4.11 (m, 1 H), 4.09-4.05 (m, 2 H), 3.72-3.70 (m, 2 H), 3.62-3.59 (m, 14H), 3.53 (s, 2H), 3.44-3.43 (m, 2H), 3.18-3.16 (m, 2H), 1.82 (m, 1H), 1.72 (s, 7H), 1.66 (m, 1H), 1.52 (s, 18H), 1.38-1.36 (m, 2H), 1.24-1.27 (s, 1H). EI-MS m/z: [M+H-2Boc]<sup>+</sup> 693.1.

#### Preparation of Compound **26b**

To a stirred mixture of compound **26a** (988 mg, 1.1 mmol) and Pd/C (10 wt. %, 196 mg) in MeOH (6 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.55 mL, 2.2 mmol). After stirring at room temperature for 1.5 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (40 mL). The filtrate was concentrated to produce the compound **26b** (767 mg, 99 %) as a yellow form, which was used without

further purification. EI-MS  $m/z$ :  $[M+H]^+$  625.0,  $[M+H-Boc]^+$  525.0,  $[M+H-2Boc]^+$  424.9.

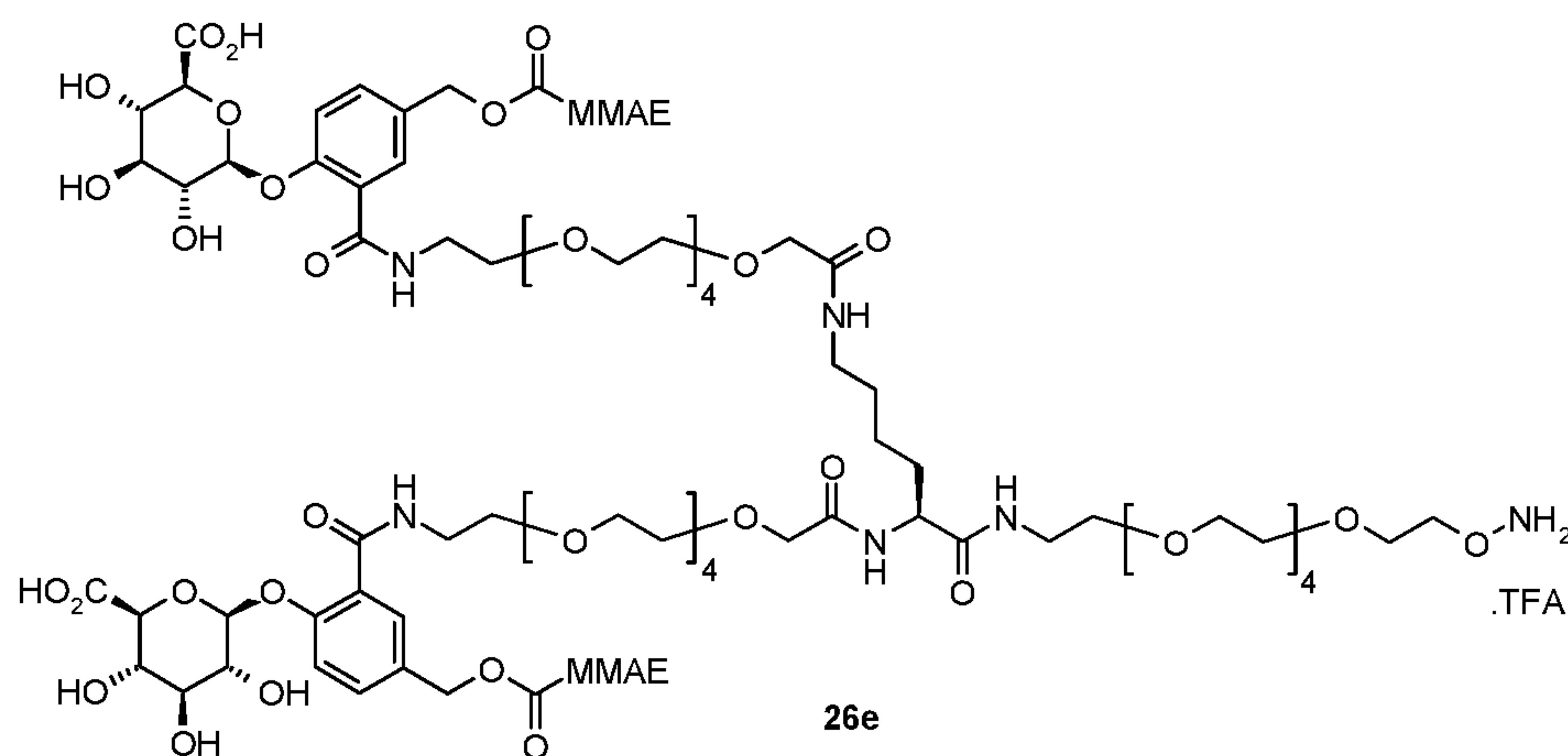
#### Preparation of Compound 26c

DIPEA (0.2 mL, 1.14 mmol), HOBt (89 mg, 0.66 mmol) and EDC·HCl (142 mg, 0.74 mmol) were added to a stirred mixture of compound **26b** (200 mg, 0.29 mmol) and compound **25b** (202 mg, 0.63 mmol) in DMF (5 mL). After stirring at room temperature for 14 hours under  $N_2$ , the reaction mixture was poured into  $H_2O$  (10 mL) and extracted with DCM (3 x 10 mL). The combined organic layers were dried over anhydrous  $Na_2SO_4$ . After filtration and concentration, the residue was purified by column chromatography, which produced the compound **26c** (270 mg, 77 %).  $^1H$ -NMR (400 MHz,  $CDCl_3$ )  $\delta$  8.07 (t, 1H), 7.62 (t, 1H), 7.54-7.52 (m, 1H), 5.73 (s, 2H), 4.27-4.25 (q, 1H), 3.96 (t, 2H), 3.88 (s, 2H), 3.82 (s, 2H), 3.58-3.48 (m, 52H), 3.19-3.18 (m, 3H), 3.04-3.03 (m, 3H), 1.44 (s, 18H), 1.39-1.37 (m, 3H), 1.21-1.19 (m, 3H). EI-MS  $m/z$ :  $[M+H-2Boc]^+$  1031.6.

#### Preparation of Compound 26d

To a stirred mixture of compound **26c** (160 mg, 0.13 mmol) and Pd/C (10 wt. %, 28 mg) in MeOH (20 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.07 mL, 0.28 mmol). After stirring at room temperature for 30 minutes under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (30 mL). The filtrate was concentrated to produce the compound **26d** (140 mg, 91 %) as colorless oil, which was used without further purification. EI-MS  $m/z$ :  $[M+H]^+$  1179.7.

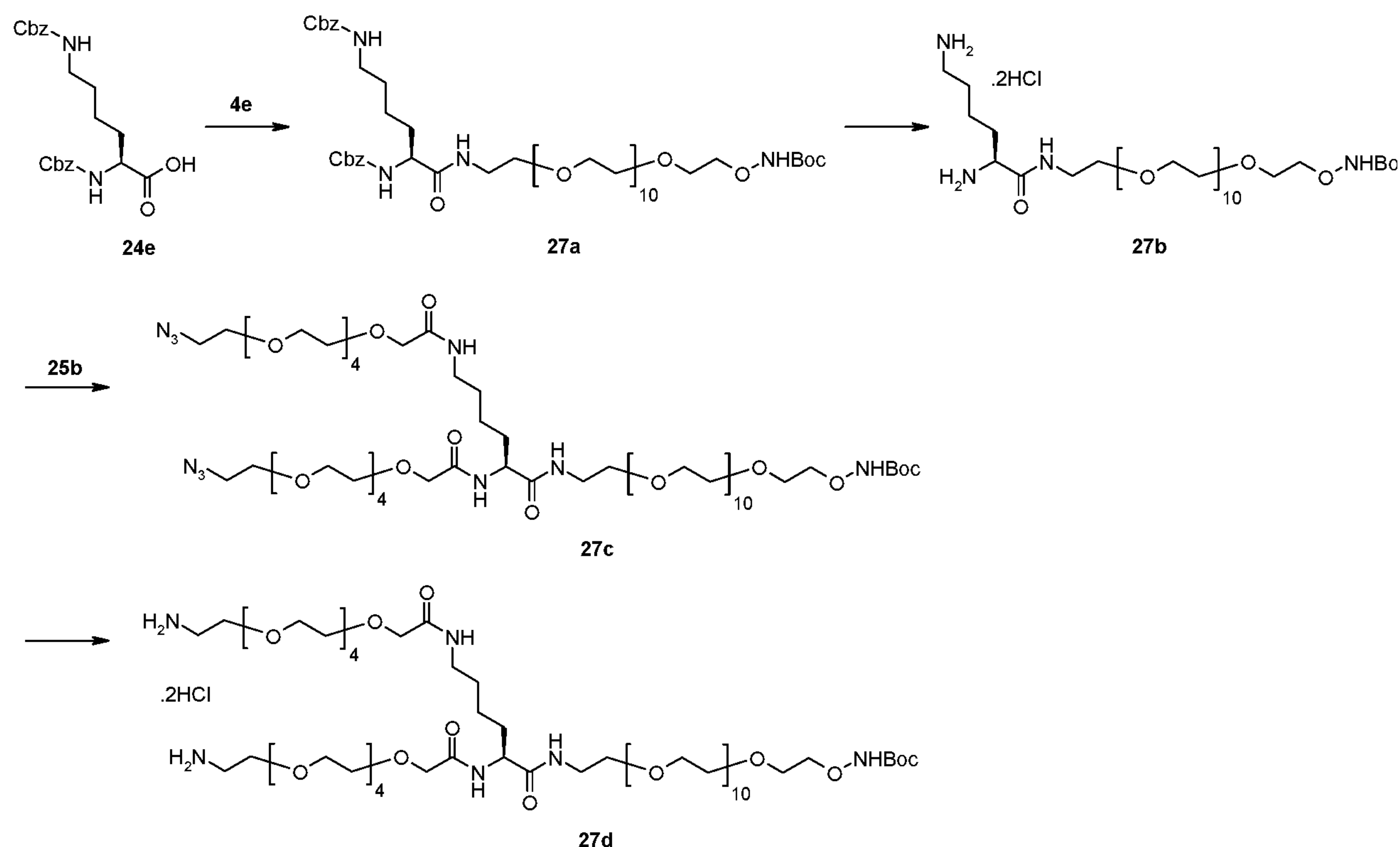
#### Preparation of Compound 26e



Compound **26e** was prepared from compound **1i** and compound **26d** by a similar method of preparing compound **24i** in Example 34. EI-MS  $m/z$ :  $1/2[M+H]^+$  1560.6,

$1/3[M+H]^+$  1040.7.

*Example 38. Preparation of Compound 27e*



Preparation of Compound 27a

DIPEA (0.19 mL, 1.1 mmol), HOBt (64 mg, 0.47 mmol), and EDC·HCl (91 mg, 0.47 mmol) were added to a stirred mixture of compound **24e** (228 mg, 0.55 mmol) and compound **4e** (256 mg, 0.36 mmol) in DMF (4 mL). After stirring at room temperature for 4 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (10 mL) and extracted with EtOAc (3 x 15 mL). The combined organic layers were washed with 1 N aq. HCl (20 mL), saturated aq. NaHCO<sub>3</sub> (10 mL), and brine (10 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **27a** (327 mg, 85 %).

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.73 (s, 1H), 7.33-7.26 (m, 11 H), 6.91 (s, 1H), 5.67 (br, 1H), 5.08-5.07 (m, 5 H), 4.15 (m, 1 H), 4.02 (t, 2 H), 3.72-3.44 (m, 46H), 3.16 (d, 2H), 1.82 (m, 1H), 1.63 (m, 1H), 1.55-1.36 (m, 13H).

Preparation of Compound 27b

To a stirred mixture of compound **27a** (327 mg, 0.309 mmol) and Pd/C (10 wt. %, 65 mg) in MeOH (6 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.15 mL, 0.618

mmol). After stirring at room temperature for 1.5 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (40 mL). The filtrate was concentrated to produce the compound **27b** (244 mg, 91 %) as colorless oil, which was used without further purification. EI-MS  $m/z$ :  $[M+H]^+$  789.2.

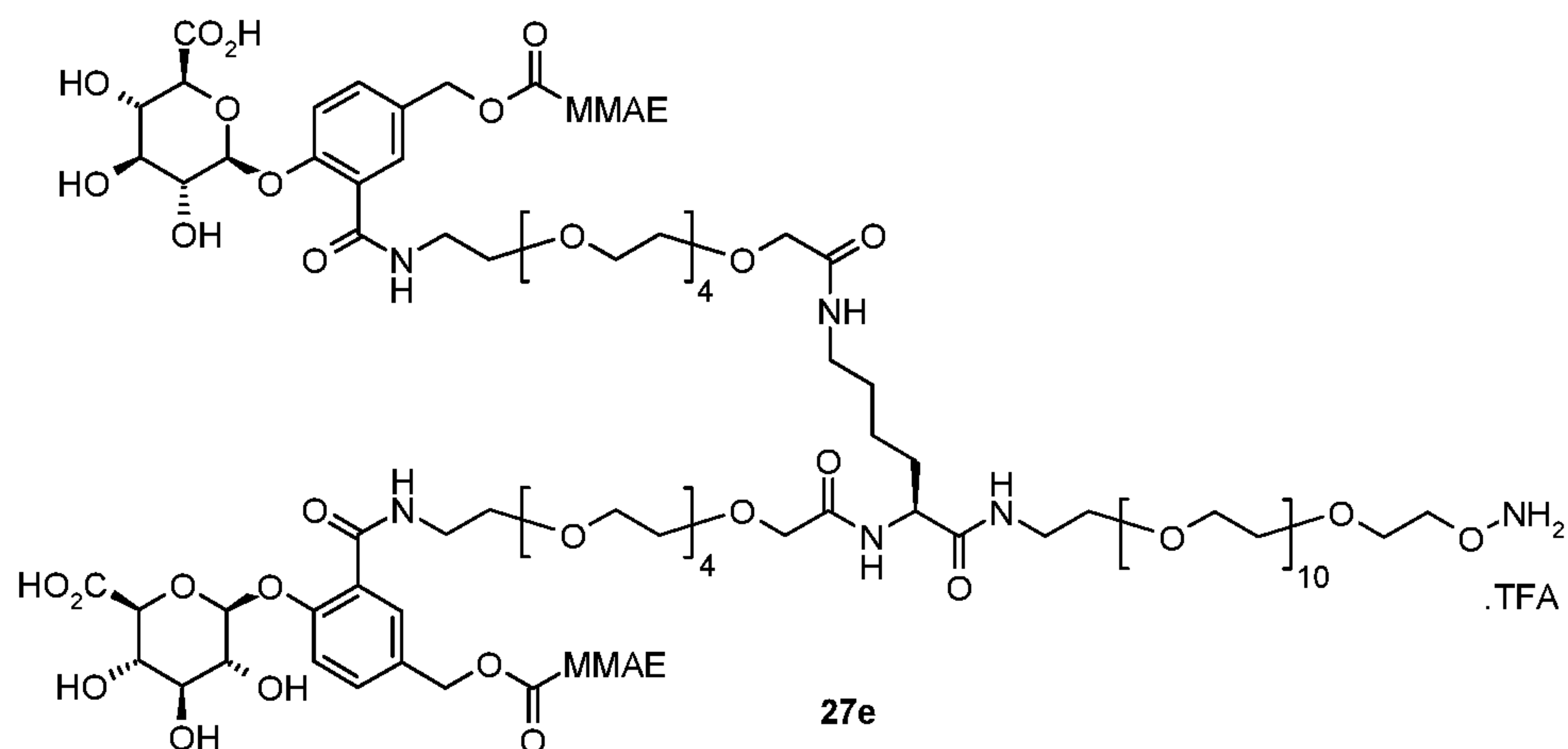
#### Preparation of Compound 27c

DIPEA (0.19 ml, 1.13 mmol), HOBt (95 mg, 0.707 mmol), and EDC·HCl (135 mg, 0.707 mmol) were added to a stirred mixture of compound **25b** (227 mg, 0.707 mmol) and compound **27b** (244 mg, 0.283 mmol) in DMF (6 mL). After stirring at room temperature for 3 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (5 mL) and extracted with DCM (3 x 10 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **27c** (339 mg, 85 %). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.69 (s, 1H), 7.29 (d, 1H), 6.99 (s, 1H), 6.82 (s, 1H), 4.39 (q, 1H), 3.99-3.94 (m, 6H), 3.69-3.58 (m, 80H), 3.51 (t, 2H), 3.44-3.34 (m, 8H), 3.25 (m, 2H), 1.68-1.64 (m, 1H), 1.53-1.48 (m, 2H), 1.44 (s, 9H), 1.33 (m, 2H). EI-MS  $m/z$ :  $[M+H]^+$  1395.6.

#### Preparation of Compound 27d

To a stirred mixture of compound **27c** (339 mg, 0.242 mmol), and Pd/C (10 wt. %, 67 mg) in MeOH (6 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.12 mL, 0.484 mmol). After stirring at room temperature for 30 minutes under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (30 mL). The filtrate was concentrated to produce the compound **27d** (300 mg, 87 %) as colorless oil, which was used without further purification. EI-MS  $m/z$ :  $[M+H]^+$  1343.5.

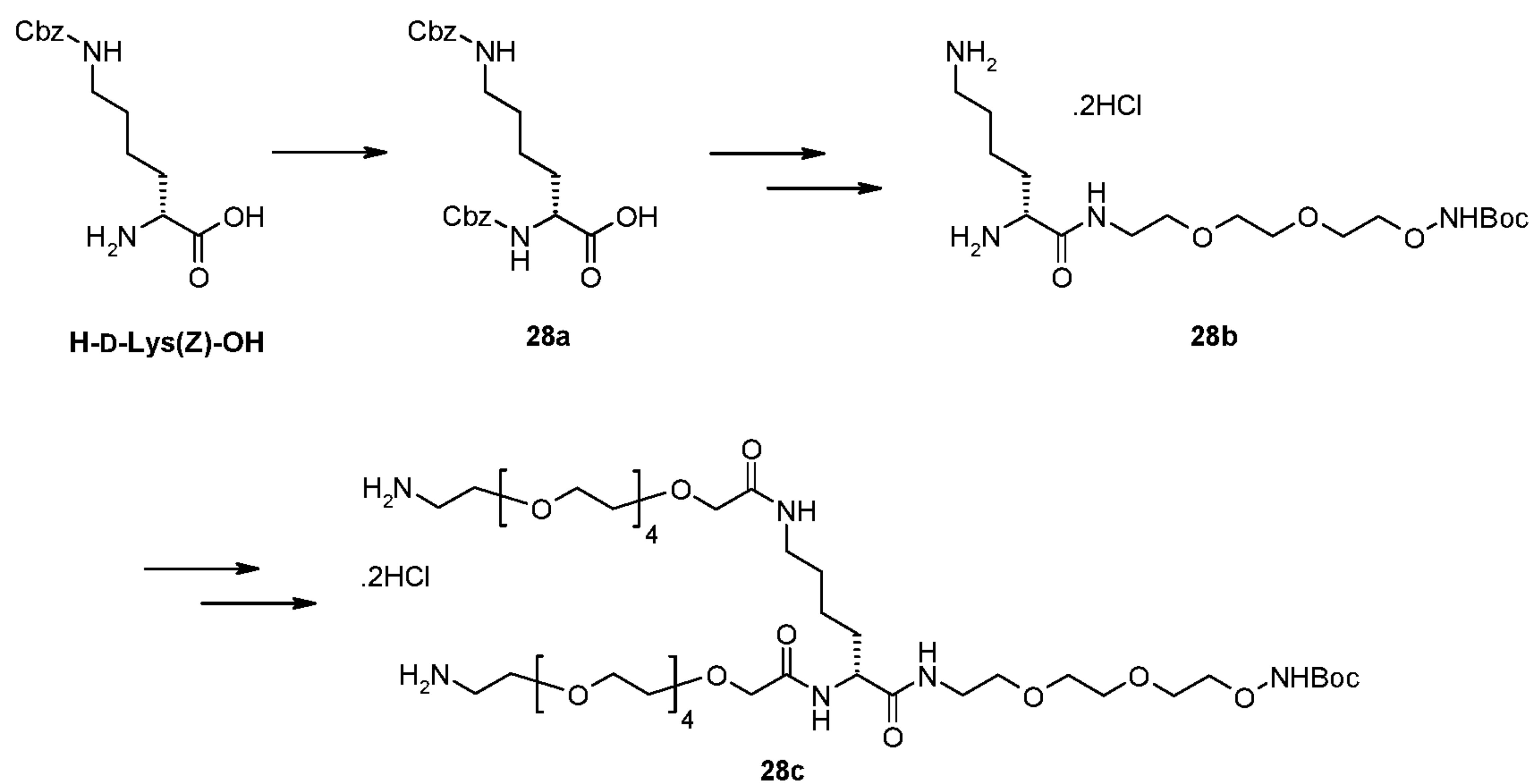
#### Preparation of Compound 27e



Compound **27e** was prepared from compound **1i** and compound **27d** by a similar method of preparing compound **24i** in Example 34. EI-MS  $m/z$ :  $1/2[M+H]^+$  1692.5.

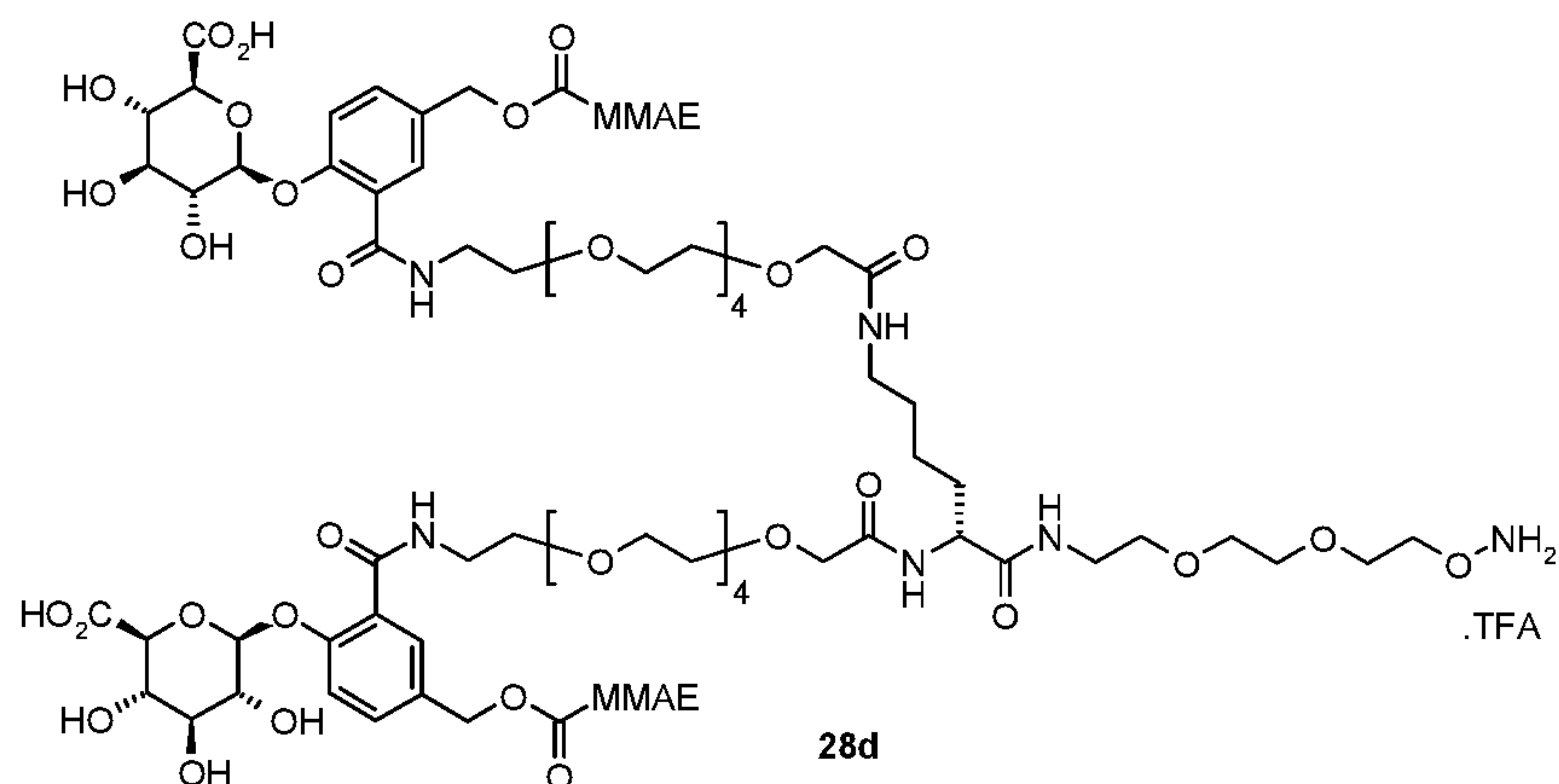
### Example 39. Preparation of Compound **28d**

#### Preparation of Compound **28c**



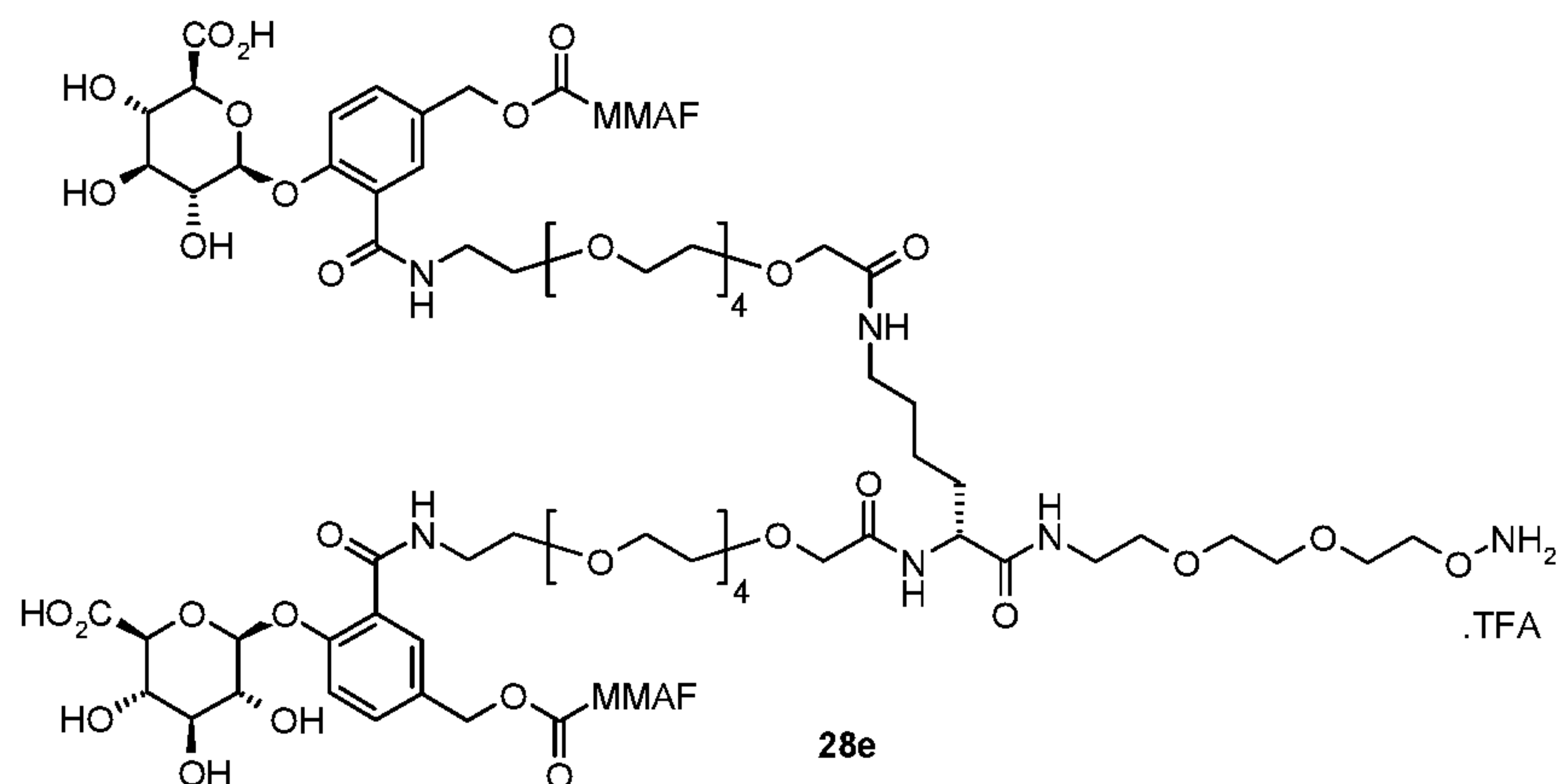
Compound **28c** was prepared from H-D-Lys(Z)-OH by a similar method of preparing compound **25d** in Example 35.

#### Preparation of Compound **28d**



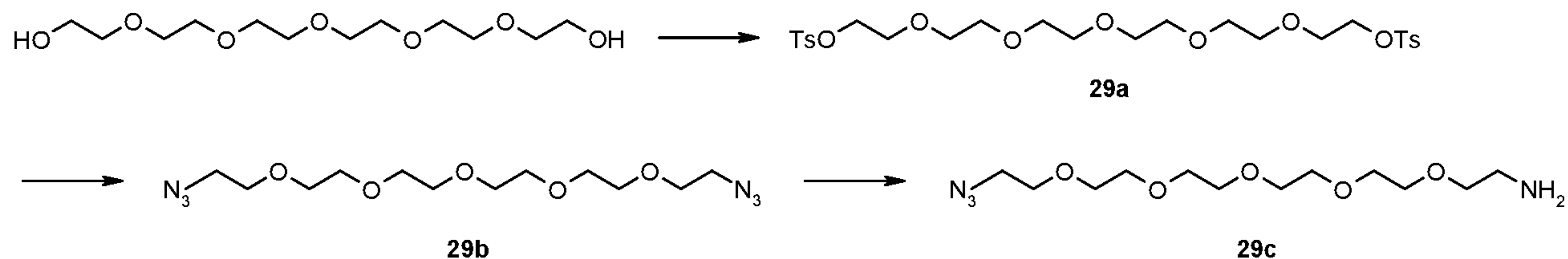
Compound **28d** was prepared from compound **1i** and compound **28c** by a similar method of preparing compound **25e** in Example 35. EI-MS  $m/z$ :  $1/2[M+H]^+$  1494.9.

*Example 40. Preparation of Compound 28e*



Compound **28e** was prepared from compound **1j** and compound **28c** by a similar method of preparing compound **25e** in Example 35. EI-MS  $m/z$ :  $1/2[M+H]^+$  1509.2.

*Example 41. Preparation of Compound 29j*



Preparation of Compound 29a

To a solution of hexaethylene glycol (25.0 g, 88.5 mmol) in DCM (100 mL) were added triethylamine (61.7 mL, 443 mmol) and *p*-toluenesulfonyl chloride (50.6 g, 266 mmol) at 0 °C under N<sub>2</sub>. After 5 hours at 0 °C, the reaction mixture was poured into 1 N aq.

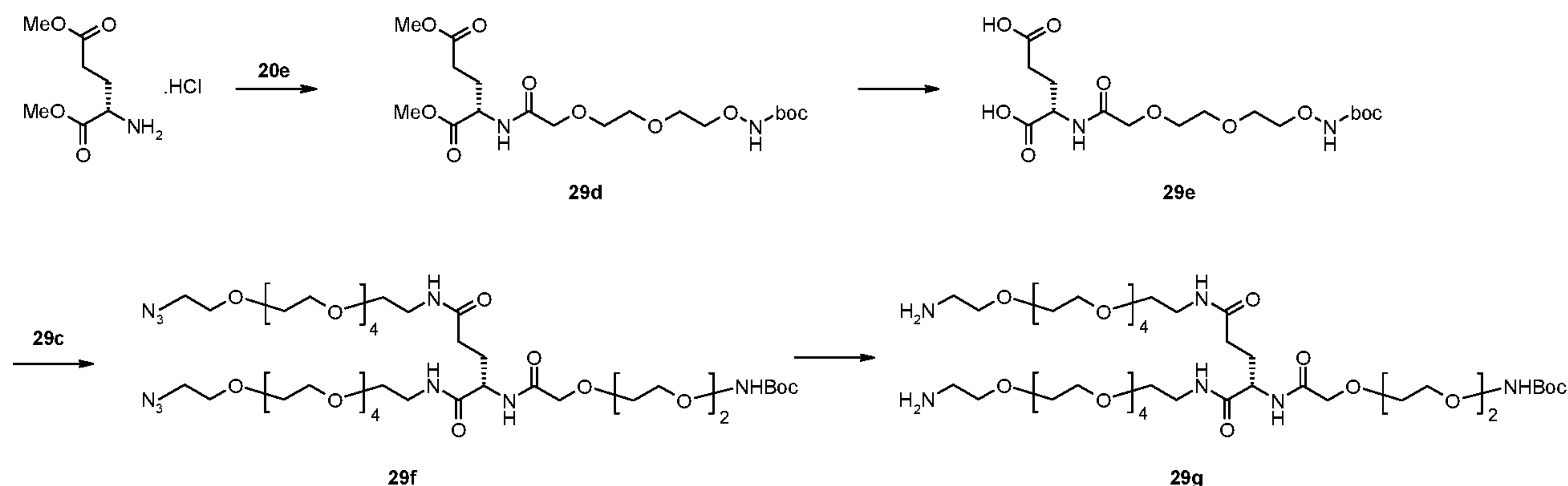
HCl (200 mL) and extracted with DCM (2 x 200 mL). The combined organic layers were washed with saturated aq. NaHCO<sub>3</sub> (100 mL) and brine (100 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **29a** (45.0 g, 87 %) as brown oil. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 7.79 (d, *J* = 7.6 Hz, 4H), 7.34 (d, *J* = 7.6 Hz, 4H), 4.16-4.14 (m, 4H), 3.69-3.67 (m, 4H), 3.64-3.56 (m, 16H), 2.44 (s, 6H).

#### Preparation of Compound **29b**

To a solution of compound **29a** (17.6 g, 29.7 mmol) in DMF (100 mL) were added NaN<sub>3</sub> (9.65 g, 148 mmol) and tetrabutylammonium iodide (550 mg, 1.49 mmol). The reaction mixture was heated up to 80 °C. After stirring for 16 hours at 80 °C, the reaction mixture was allowed to cool to room temperature. The reaction mixture was filtered through a celite pad and washed with DCM (100 mL). After concentration, the residue was purified by column chromatography, which produced the compound **29b** (9.4 g, 94 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.68 (m, 20H), 3.39 (t, 4H).

#### Preparation of Compound **29c**

To a solution of **29b** (8.4 g, 24.9 mmol) in DCM (24 mL) and toluene (24 mL) were added 1 N aq. HCl (40.3 mL) and triphenylphosphine (6.9 g, 23.6 mmol). The reaction mixture was stirred at room temperature under N<sub>2</sub> for 16 hours. After removal of the solvent under reduced pressure, H<sub>2</sub>O (20 mL) was added into the reaction mixture, and the aqueous layer was extracted with EtOAc (20 mL). Then the pH of the aqueous phase was adjusted to 13. The resulting aqueous phase was extracted with DCM (3 x 30 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure to produce the compound **29c** (6.6 g, 84 %) as colorless oil. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 3.67 (m, 20H), 3.52 (t, 2H), 3.39(t, 2H), 2.86(t, 2H). EI-MS *m/z*: [M+H]<sup>+</sup> 306.9.



### Preparation of Compound 29d

DIPEA (2.67 mL, 15.4 mmol) and HBTU (3.49 g, 9.21 mmol) were added to a stirred mixture of L-glutamic acid dimethyl ester hydrochloride (1.3 g, 6.14 mmol) and compound **20e** (1.72 g, 6.14 mmol) in DMF (15 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 minutes and allowed to warm to room temperature over 16 hours under N<sub>2</sub>. The reaction mixture was poured into water (50 mL) and extracted with DCM (3 x 50 mL). The combined organic layers were washed with 0.5 N HCl (50 mL), saturated aq. NaHCO<sub>3</sub> (50 mL) and brine (50 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting crude product was purified by column chromatography to produce the compound **29d** (2.18 g, 81 %). <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 10.00 (s, 1H), 8.04 (d, 1H), 4.34 (m, 1H), 3.93 (s, 1H), 3.77 (s, 1H), 3.63 (s, 3H), 3.58 (s, 9H), 3.38-3.34 (t, 2H), 2.14(m, H), 1.90 (m, 1H), 1.39 (s, 9H). EI-MS m/z: [M+H]<sup>+</sup> 437.35.

### Preparation of Compound 29e

To a solution of compound **29d** (2.18 g, 4.99 mmol) in THF:MeOH:H<sub>2</sub>O (12 mL:4 mL:4 mL) was added NaOH (499 mg, 12.5 mmol) at room temperature under N<sub>2</sub>. After 3 hours, the pH of the reaction mixture was adjusted to 4 and concentrated. Then the residue was extracted with DCM/MeOH (80 mL/20 mL). Concentration provided compound **29e** (1.0 g, 49 %) as yellow oil, which was used without further purification. EI-MS m/z: [M+H-Boc]<sup>+</sup> 309.20.

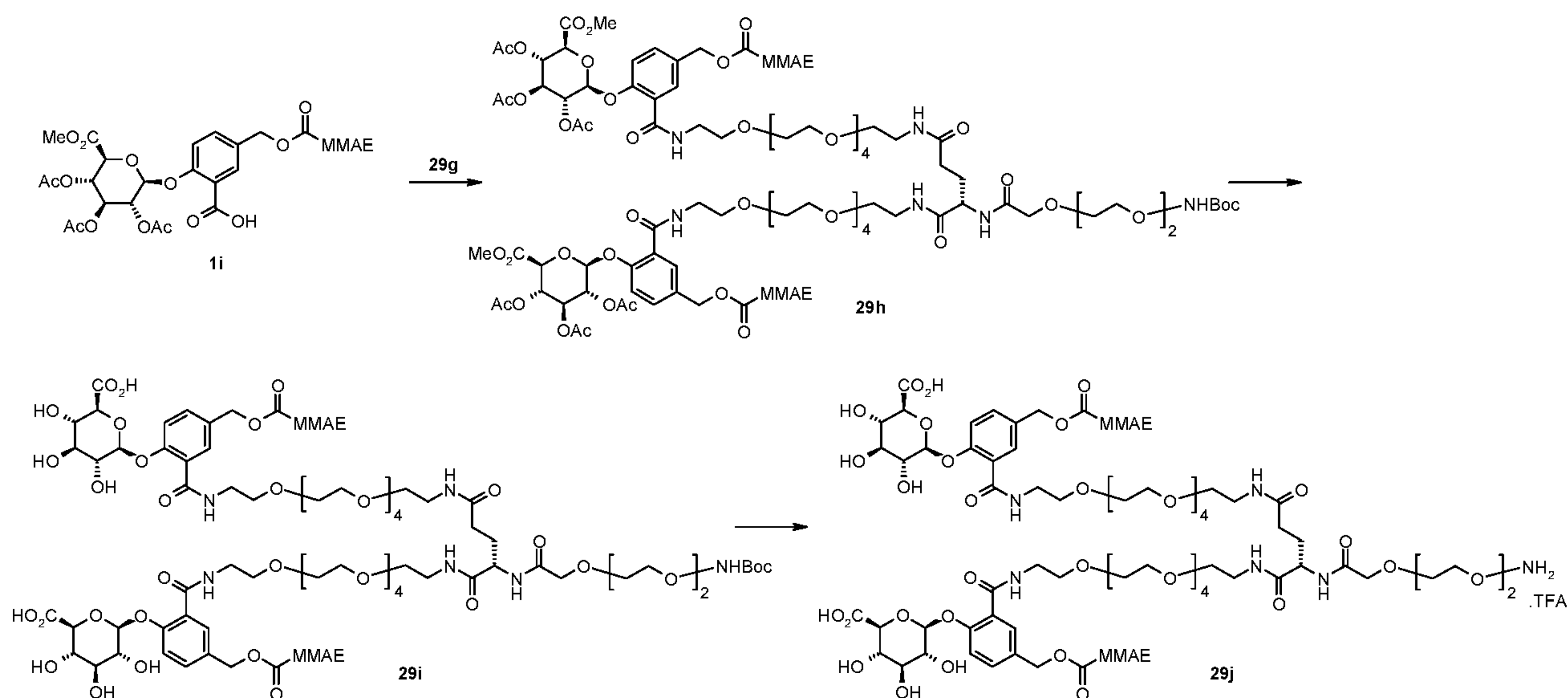
### Preparation of Compound 29f

DIPEA (1.7 mL, 9.79 mmol) and HBTU (2.79 g, 7.35 mmol) were added to a stirred mixture of compound **29e** (1.0 g, 2.45 mmol) and compound **29c** (2.25 g, 7.35 mmol) in

DMF (10 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 minutes and allowed to warm to room temperature over 16 hours under N<sub>2</sub>. The reaction mixture was poured into water (50 mL) and extracted with DCM (3 x 50 mL). The combined organic layers were washed with 0.5 N aq. HCl (50 mL), saturated aq. NaHCO<sub>3</sub> (50 mL) and brine (50 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting crude product was purified by column chromatography to produce the compound **29f** (611 mg, 25 %). <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 9.97 (s, 1H), 8.08 (t, 1H), 7.85 (t, 1H), 7.64 (d, 1H), 4.27 (m, 1H), 3.83 (s, 2H), 3.82-3.61 (m, 2H), 3.61-3.50 (m, 42H), 3.42-3.37 (m, 8H), 3.28-3.15 (m, 4H), 2.90 (s, H), 2.08-2.04 (m, 2H), 1.88 (m, 1H), 1.75 (m, 1H), 1.39 (s, 9H). EI-MS m/z: [M+H]<sup>+</sup> 986.73.

### Preparation of Compound **29g**

To a stirred mixture of compound **29f** (611 mg, 0.62 mmol) in MeOH (50 mL) was added Pd/C (10 wt. %, 132 mg 0.62 mmol). After stirring at room temperature for 2 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (40 mL). The filtrate was concentrated to produce the compound **29g** as colorless oil (518 mg, crude), which was used without further purification. EI-MS m/z: [M+H]<sup>+</sup> 933.85.



### Preparation of Compound **29h**

DIPEA (0.026 mL, 0.150 mmol) and HBTU (40 mg, 0.105 mmol) were added to a stirred mixture of compound **29g** (35 mg, 0.037 mmol) and compound **1i** (106 mg, 0.086 mmol) in DMF (3 mL). After stirring at room temperature for 16 hours under N<sub>2</sub>, the

reaction mixture was diluted with water (20 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with 0.5 N HCl (20 mL), saturated aq. NaHCO<sub>3</sub> (20 mL) and brine (20 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting crude product was purified by column chromatography to produce the compound **29h** (81.4 mg, 65 %). EI-MS m/z: 1/2[M+H]<sup>+</sup> 1677.94, 1/3[M+H]<sup>+</sup> 1119.03.

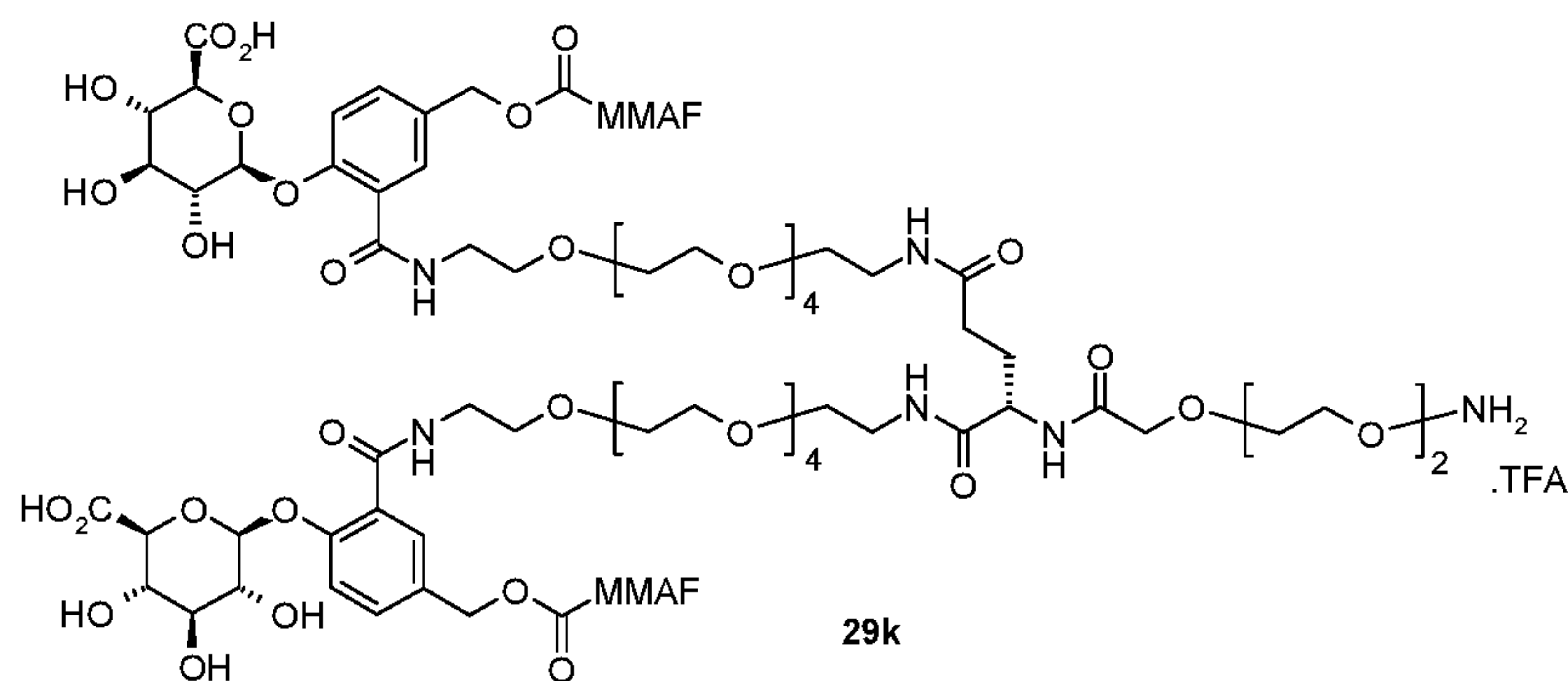
#### *Preparation of Compound 29i*

To a solution of compound **29h** (81 mg, 0.024 mmol) in MeOH (1 mL) was added LiOH monohydrate (8.1 mg, 0.19 mmol) in H<sub>2</sub>O (1 mL) at -10 °C. After stirring for 2 hours at -10 °C, the reaction mixture was neutralized using acetic acid and concentrated under reduced pressure. Then the reaction mixture was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC, which produced the compound **29i** (53 mg, 72 %) as white solid. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1537.86, 1/3[M+H]<sup>+</sup> 1025.66.

#### *Preparation of Compound 29j*

TFA (0.3 mL) was added to a stirred solution of compound **29i** (53 mg, 0.017 mmol) in DCM (1.0 mL) at 0 °C. After stirring for 1 hour, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in H<sub>2</sub>O/MeCN (1 mL/1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **29j** (23.1 mg, 43 %) as white solid. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1487.99, 1/3[M+H]<sup>+</sup> 992.40.

#### *Example 42. Preparation of Compound 29k*

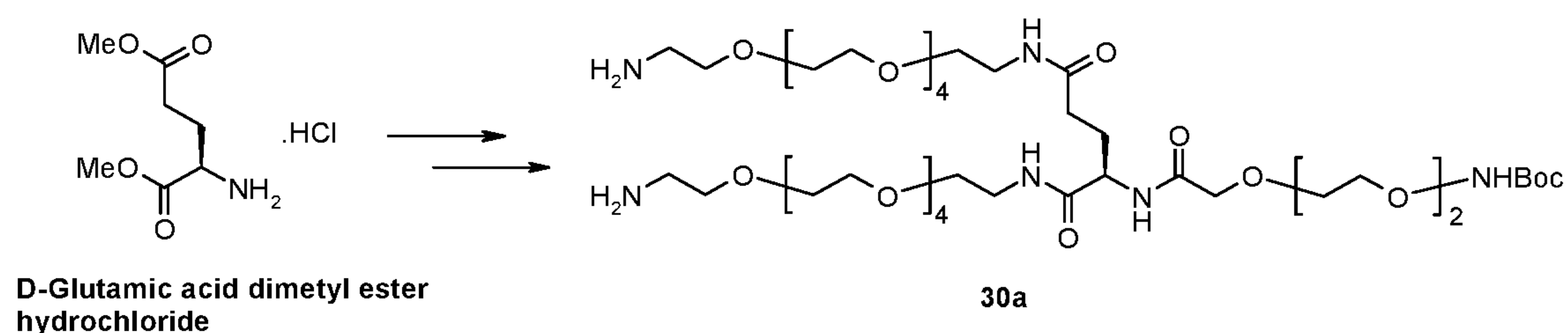


Compound **29k** was prepared from compound **1j** and compound **29g** by a similar method of

preparing compound **29j** in Example 41. EI-MS m/z:  $1/2[M+H]^+$  1501.93,  $1/3[M+H]^+$  1001.69.

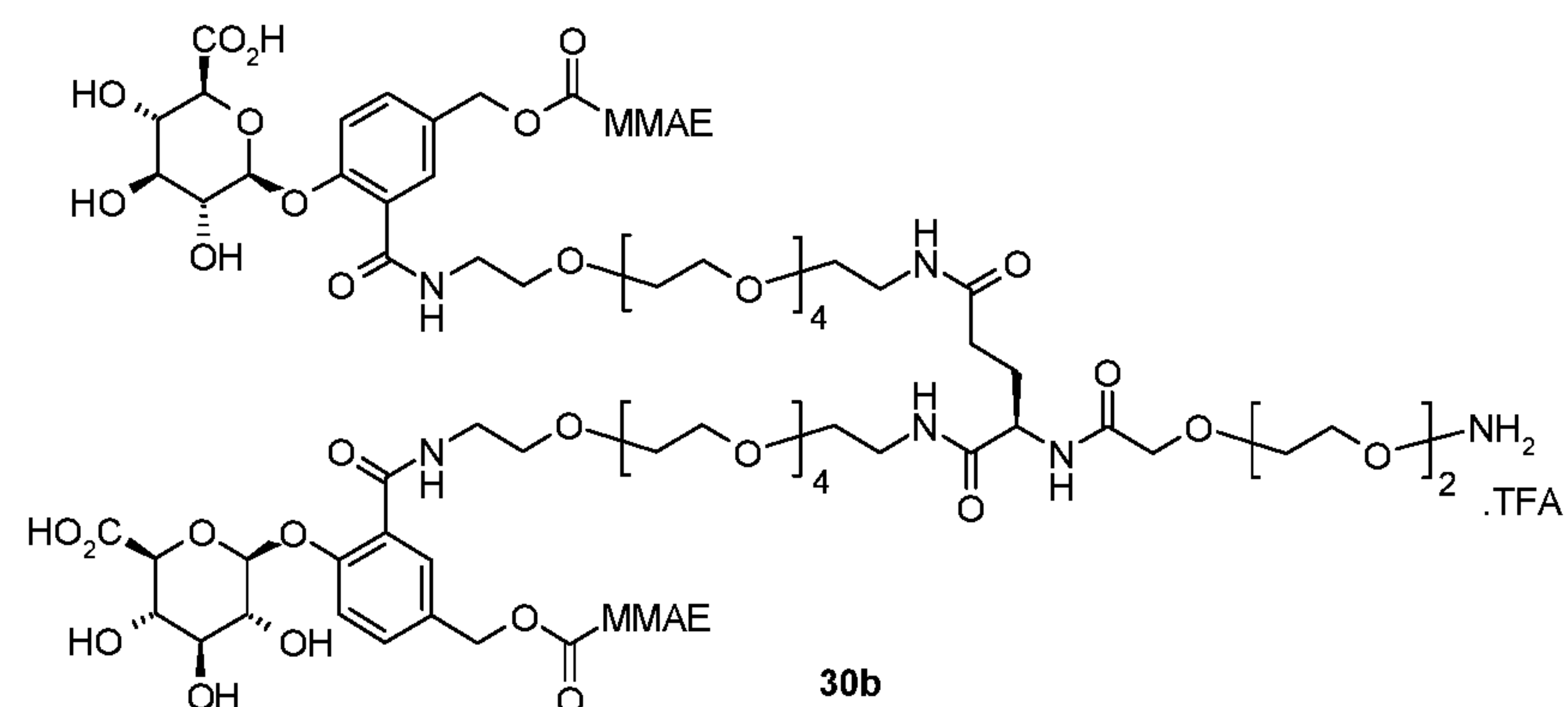
*Example 43. Preparation of Compound 30b*

Preparation of Compound 30a



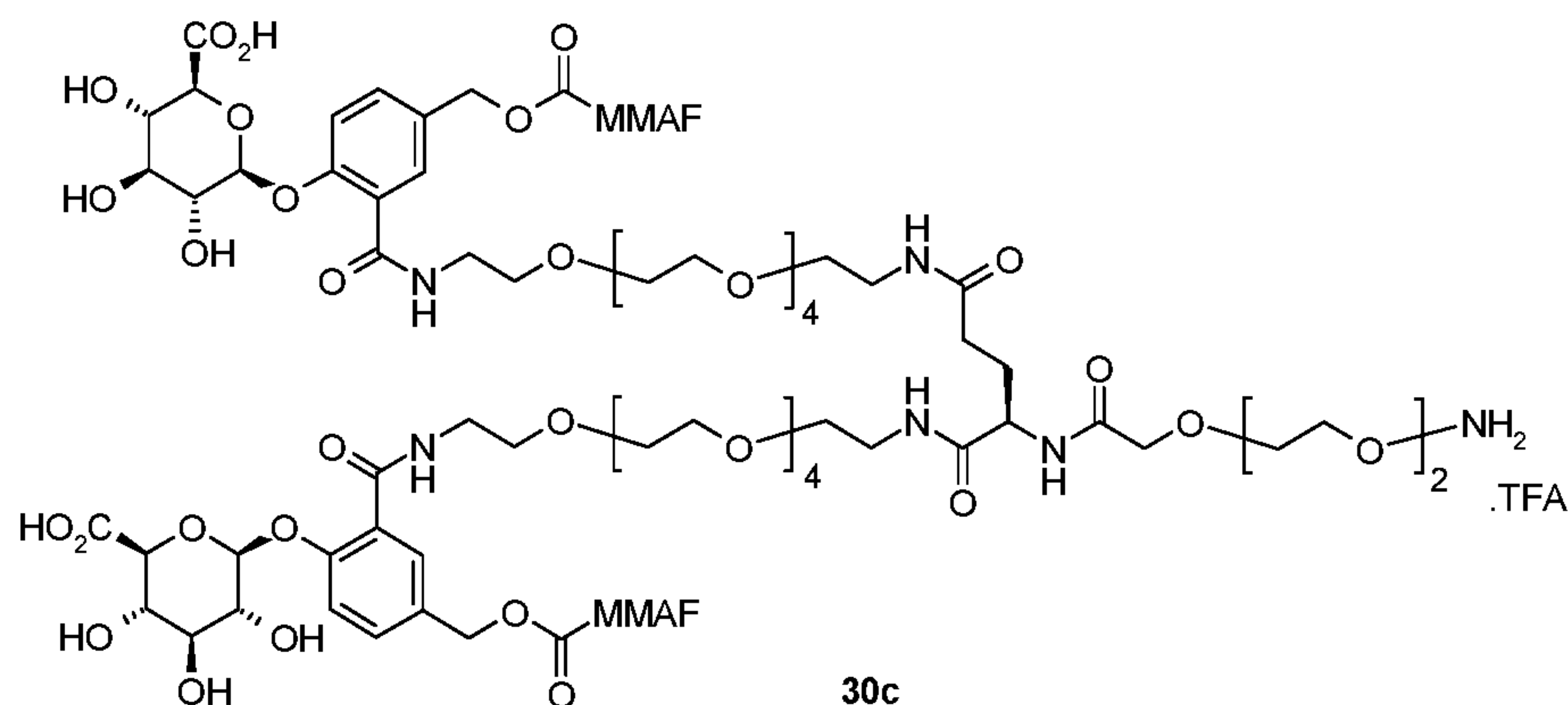
Compound **30a** was prepared from D-glutamic acid dimethyl ester hydrochloride by a similar method of preparing compound **29g** in Example 41. EI-MS m/z:  $[M+H]^+$  933.89.

Preparation of Compound 30b



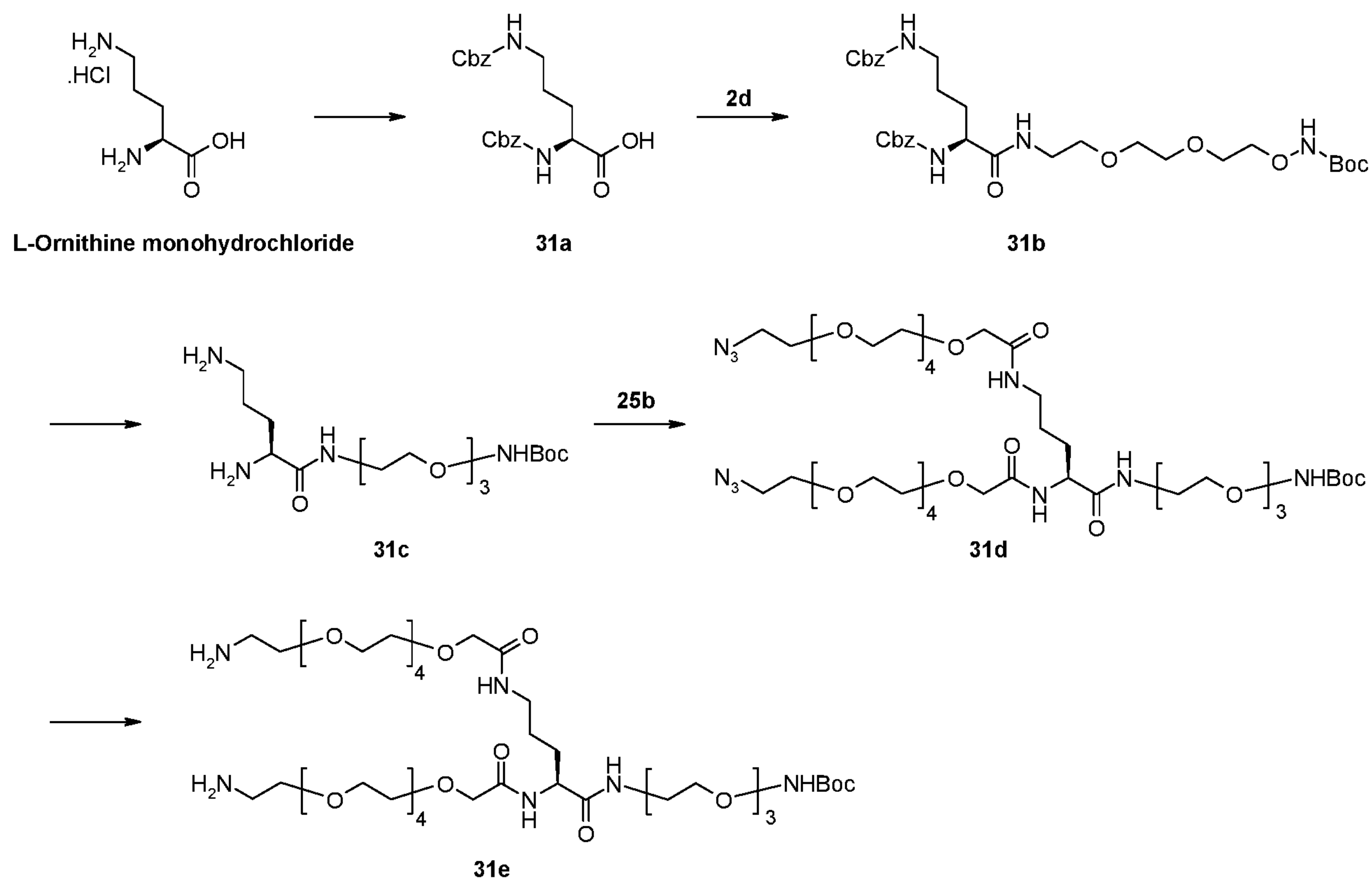
Compound **30b** was prepared from compound **1i** and compound **30a** by a similar method of preparing compound **29j** in Example 41. EI-MS m/z:  $1/2[M+H]^+$  1488.07,  $1/3[M+H]^+$  992.40.

*Example 44. Preparation of Compound 30c*



Compound **30c** was prepared from compound **1j** and compound **30a** by a similar method of preparing compound **29j** in Example 41. EI-MS  $m/z$ :  $1/2[M+H]^+$  1501.93,  $1/3[M+H]^+$  1001.69.

*Example 45. Preparation of Compound 31f*



Preparation of Compound 31a

A three-necked flask was loaded consecutively with H<sub>2</sub>O (18 mL), 1,4-dioxane (30 mL) and L-ornithine monohydrochloride (3.0 g, 17.8 mmol). The mixture was stirred until complete dissolution. The pH was adjusted to about 10.5 by addition of 2 M aq. Na<sub>2</sub>CO<sub>3</sub>. Benzyl chloroformate (6.37g, 37.4 mmol) was added while maintaining the pH at about 10-

11 by adding at the same time 2 M aq.  $\text{Na}_2\text{CO}_3$ . After the end of the addition, the reaction mixture was stirred at 20 °C for 1 hour. Then EtOAc (50 mL) was added and pH of the resulting mixture was adjusted to 2~3 with c-HCl. The organic layer was separated and the aqueous layer was extracted with EtOAc (50 mL). The combined organic layers were washed with brine (50 mL), and dried over  $\text{Na}_2\text{SO}_4$ . Filtration and concentration under reduced pressure provided compound **31a** (7.1 g).  $^1\text{H}$ -NMR (400 MHz,  $\text{DMSO-d}_6$ )  $\delta$  12.54 (s, 1H), 7.54 (s, 1H), 7.44-7.29 (m, 10H), 7.24-7.22 (m, 1H), 5.16-5.00 (d, 4H), 3.95-3.89 (m, 1H), 3.00-2.96 (m, 2H), 1.98-1.57 (m, 1H), 1.56-1.46(m, 3H).

#### Preparation of Compound 31b

DIPEA (1.41 mL, 8.12 mmol) and HBTU (1.85 g, 4.87 mmol) were added to a stirred mixture of compound **31a** (1.30 g, 3.25 mmol) and compound **2d** (891 mg, 3.57 mmol) in DMF (10 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 minutes and allowed to warm to room temperature over 16 hours under  $\text{N}_2$ . The reaction mixture was poured into water (50 mL) and extracted with DCM (3 x 50 mL). The combined organic layers were washed with 0.5 N aq. HCl (50 mL), saturated aq.  $\text{NaHCO}_3$  (50 mL) and brine (50 mL) sequentially, and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . After filtration and concentration under reduced pressure, the resulting crude product was purified by column chromatography to produce the compound **31b** (1.2 g, 57 %). EI-MS m/z:  $[\text{M}+\text{H}]^+$  647.54,  $[\text{M}+\text{H}-\text{Boc}]^+$  547.47

#### Preparation of Compound 31c

To a stirred mixture of compound **31b** (1.2 g, 1.86 mmol) in MeOH (50 mL) was added Pd/C (10 wt. %, 59 mg 5.57 mmol). After stirring at room temperature for 2 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (40 mL). The filtrate was concentrated to produce the compound **31c** (717 mg), which was used without further purification.  $^1\text{H}$ -NMR (400 MHz,  $\text{DMSO-d}_6$ )  $\delta$  7.93 (s, 1H), 3.81 (t, 2H), 3.55 (t, 2H), 3.51 (s, 5H), 3.42-3.22 (m, 13H), 1.37 (s, 9H).

#### Preparation of Compound 31d

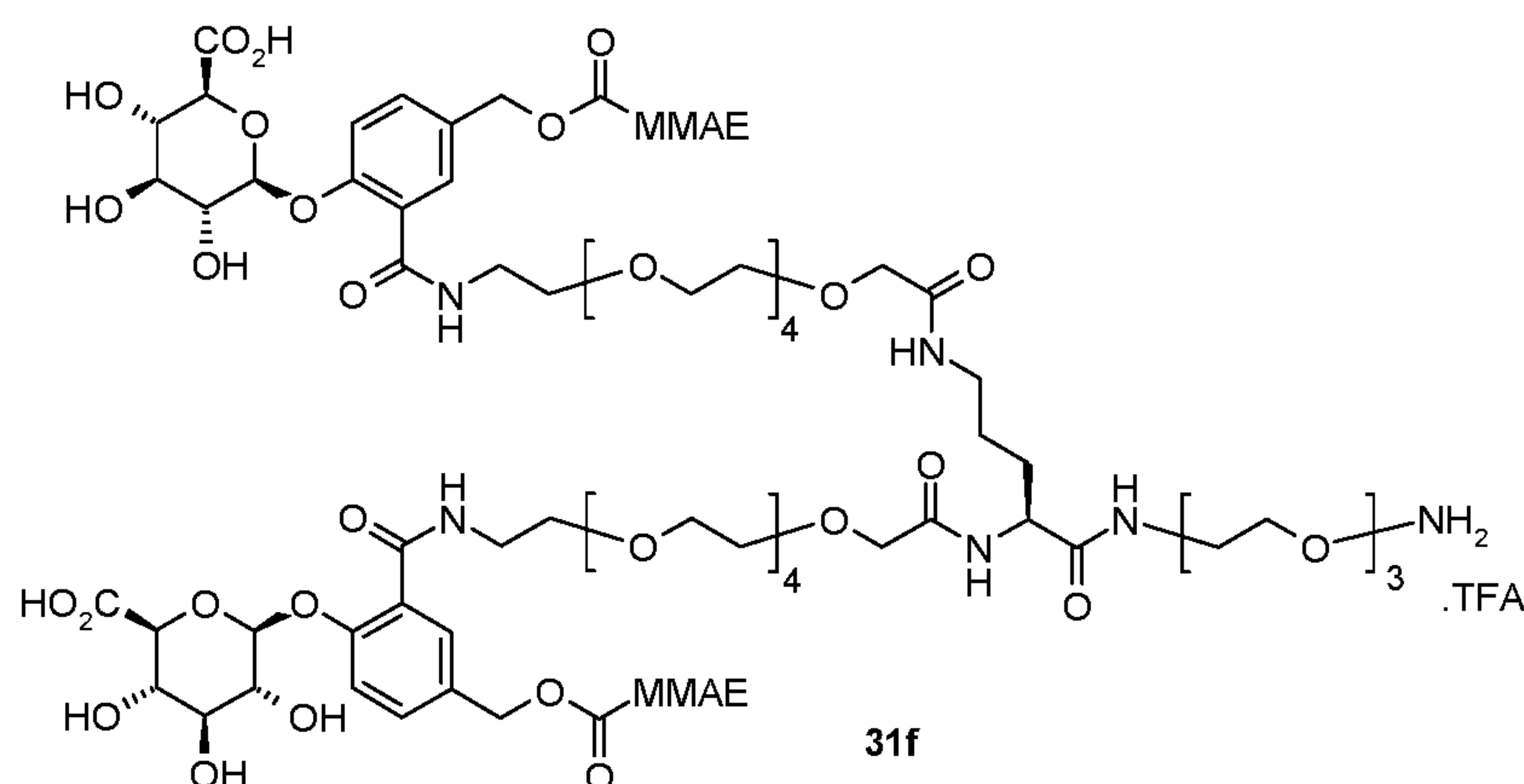
DIPEA (0.55 mL, 3.17 mmol) and HBTU (902 mg, 2.38 mmol) were added to a stirred mixture of compound **31c** (300 mg, 0.79 mmol) and compound **25b** (637 mg, 1.98 mmol) in DMF (5 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 minutes and

allowed to warm to room temperature over 16 hours under N<sub>2</sub>. The reaction mixture was poured into water (30 mL) and extracted with DCM (3 x 30 mL). The combined organic layers were washed with 0.5 N aq. HCl (30 mL), saturated aq. NaHCO<sub>3</sub> (30 mL) and brine (30 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting crude product was purified by column chromatography to produce the compound **31d** (551 mg, 71%). EI-MS m/z: [M+H]<sup>+</sup> 985.87.

#### Preparation of Compound 31e

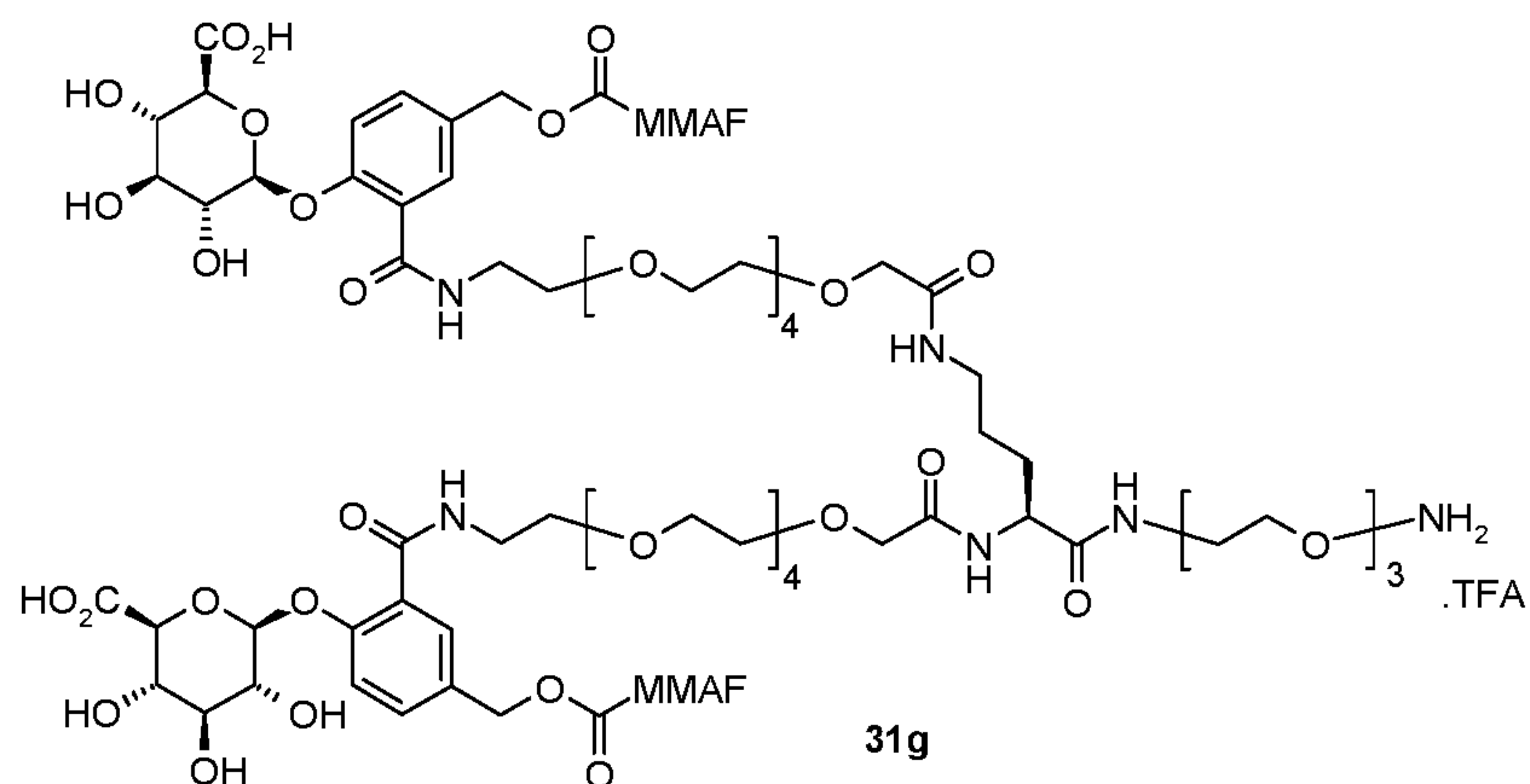
To a stirred mixture of compound **31d** (491 mg, 0.50 mmol) in MeOH (30 mL) was added Pd/C (10 wt. %, 106 mg 1.00 mmol). After stirring at room temperature for 2 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (40 mL). The filtrate was concentrated to produce the compound **31e** (452 mg), which was used without further purification. EI-MS m/z: [M+H]<sup>+</sup> 933.94.

#### Preparation of Compound 31f



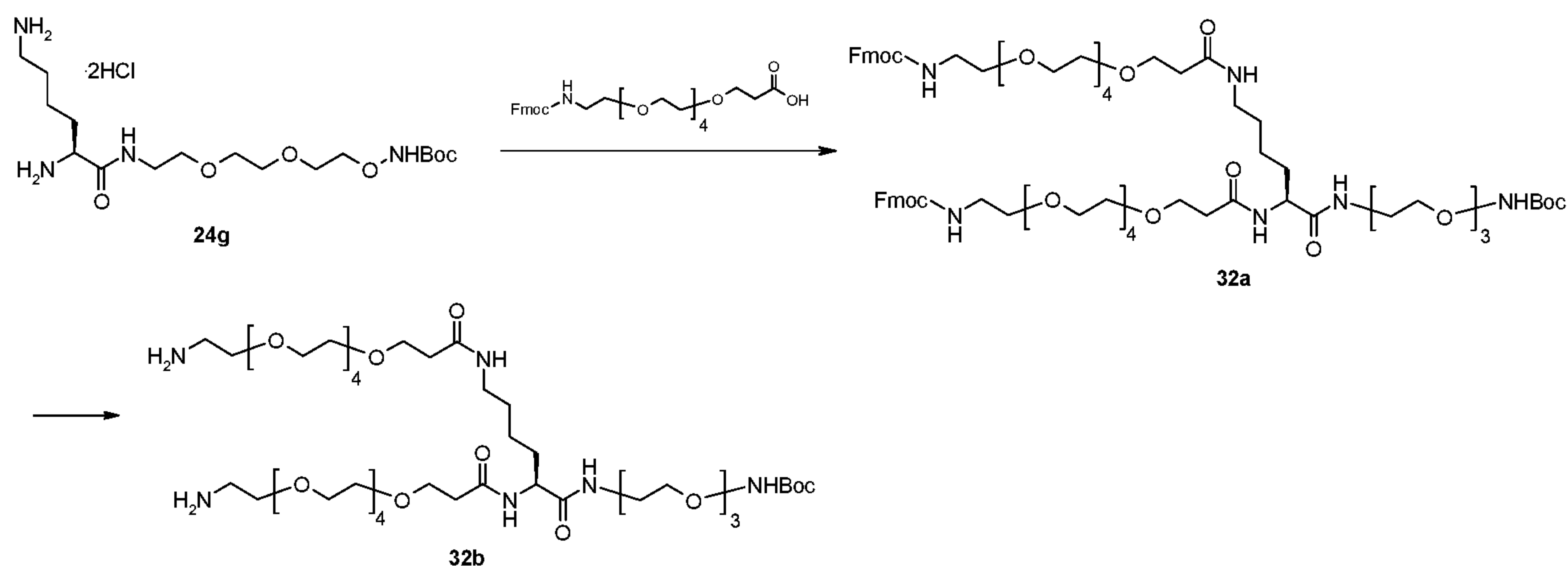
Compound **31f** was prepared from compound **1i** and compound **31e** by a similar method of preparing compound **25e** in Example 35. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1488.20, 1/3[M+H]<sup>+</sup> 992.54.

#### *Example 46. Preparation of Compound 31g*



Compound **31g** was prepared from compound **1j** and compound **31e** by a similar method of preparing compound **25e** in Example 35. EI-MS  $m/z$ :  $1/2[M+H]^+$  1502.23,  $1/3[M+H]^+$  1001.86.

*Example 47. Preparation of Compound 32c*



*Preparation of Compound 32a*

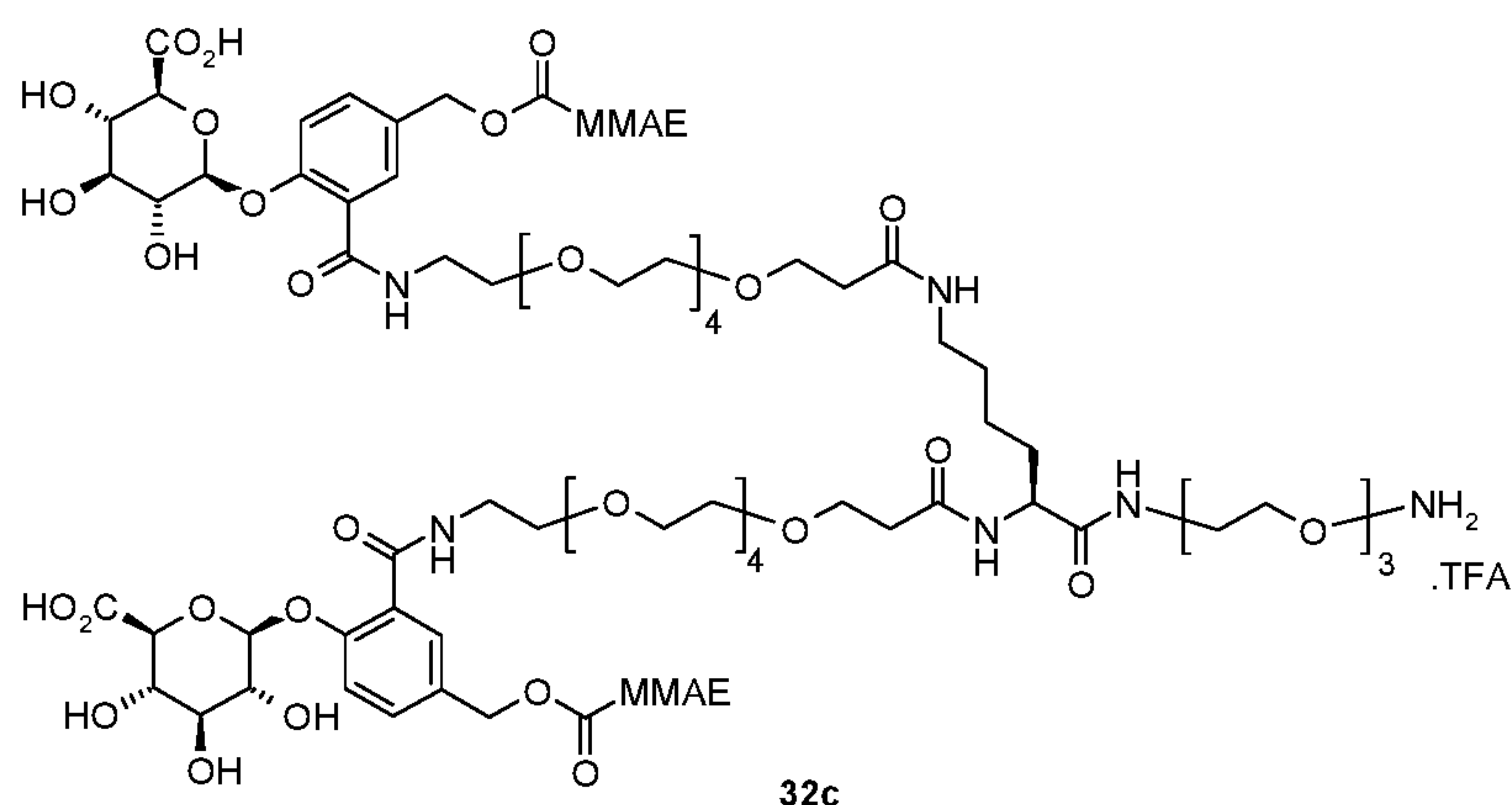
DIPEA (0.6 mL, 7.07 mmol) and HBTU (972 mg, 5.30 mmol) were added to a stirring mixture of compound **24g** (483 mg, 0.855 mmol) and Fmoc-NH-PEG5-CH<sub>2</sub>CH<sub>2</sub>COOH (1.0 g, 3.89 mmol) in DMF (10 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (30 mL) and extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with 1 N aq. HCl (10 mL), saturated aq. NaHCO<sub>3</sub> (10 mL) and brine (10 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the resulting residue was purified by column chromatography, which produced the compound **32a** (1.16 g, 90 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.77 (d, 4H), 7.60(d, 4H), 7.39 (t, 4H), 7.31 (t, 4H), 4.39 (d, 4H), 4.33 (m, 1H), 4.22 (m, 2H), 4.09 (m, 2H), 3.71-3.39 (m, 52H), 3.19 (m, 2H), 2.51 (m, 4H), 1.50 (m, 1H),

1.46 (m, 1H), 1.43 (s, 9H), 1.25 (m, 2H). EI-MS  $m/z$ :  $[M+H]^+$  1520.0.

### Preparation of Compound **32b**

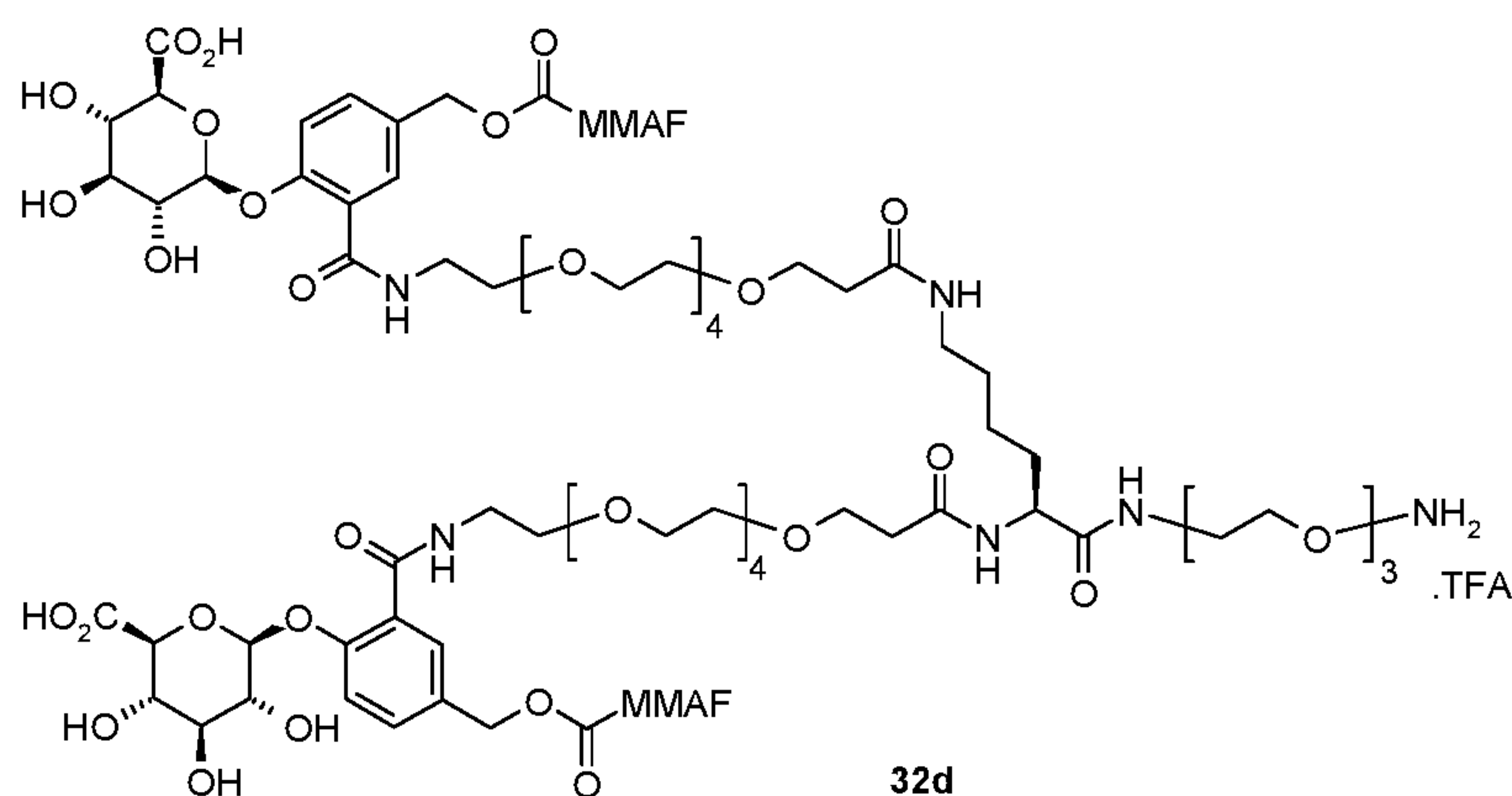
To a solution of compound **32a** (500 mg, 0.328 mmol) in THF (8 mL) was added piperidine (2 mL) at room temperature. After stirring for 20 minutes, the reaction mixture was concentrated under reduced pressure. The resulting residue was purified by column chromatography to produce the compound **32b** (175 mg, 50 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  4.41 (m, 1H), 4.01 (m, 2H), 3.75-3.56 (m, 43H), 3.54 (m, 2H), 3.24 (m, 2H), 2.89 (m, 3H), 2.52 (m, 4H), 1.83 (m, 1H), 1.80 (m, 1H), 1.53 (s, 9H), 1.39 (m, 2H). EI-MS  $m/z$ :  $[M+H]^+$  975.5.

### Preparation of Compound **32c**



Compound **32c** was prepared from compound **1i** and compound **32b** by a similar method of preparing compound **25e** in Example 35. EI-MS  $m/z$ :  $1/2[M+H]^+$  1508.8.

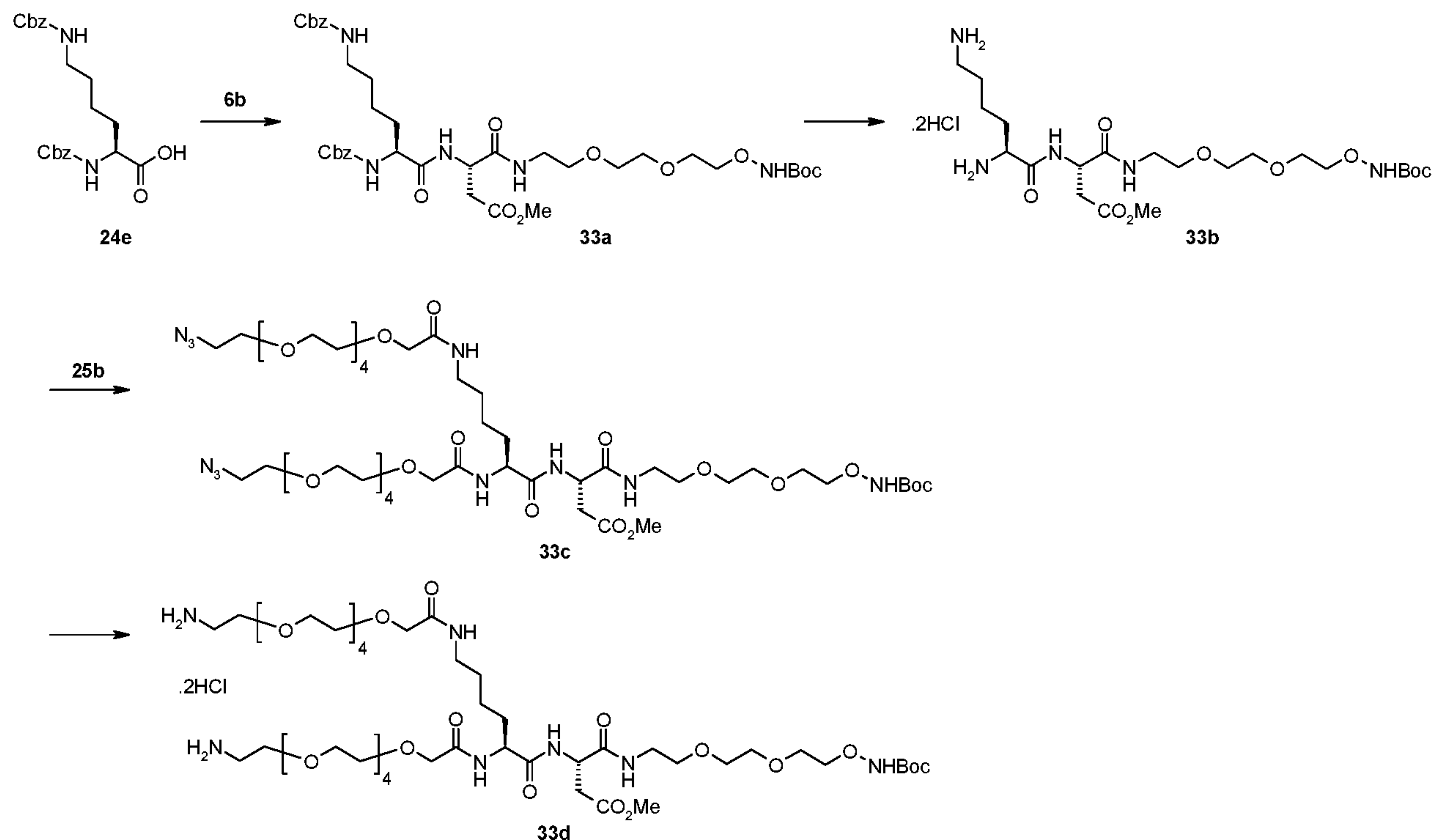
### Example 48. Preparation of Compound **32d**



Compound **32d** was prepared from compound **1j** and compound **32b** by a similar

method of preparing compound **25e** in Example 35. EI-MS  $m/z$ :  $1/2[M+H]^+$  1522.8.

*Example 49. Preparation of Compound 33e*



Preparation of Compound 33a

DIPEA (1.98 mL, 11.37 mmol) and HBTU (2.15 g, 5.68 mmol) were added to a stirred mixture of compound **24e** (1.57 g, 3.79 mmol) and compound **6b** (1.30 g, 3.15 mmol) in DMF (37 mL). After stirring at room temperature for 14 hours under  $N_2$ , the reaction mixture was poured into  $H_2O$  (40 mL) and extracted with EtOAc (3 x 40 mL). The combined organic layers were washed with 1 N aq. HCl (40 mL), saturated aq.  $NaHCO_3$  (40 mL) and brine (40 mL) sequentially, and dried over anhydrous  $Na_2SO_4$ . After filtration and concentration, the residue was purified by column chromatography, which produced the compound **33a** (2.2 g, 88 %).  $^1H$ -NMR (400 MHz,  $DMSO-d_6$ )  $\delta$  9.99 (s, 1H), 8.19 (d, 1H), 7.79 (t, 1H), 7.47 (d, 1H), 7.34-7.31 (m, 5H), 7.24 (t, 1H), 5.01 (d, 4H), 4.55 (q, 1H), 3.91 (q, 1H), 3.79 (t, 2H), 3.55-3.48 (m, 9H), 3.24-3.11 (m, 2H), 2.75-2.54 (m, 2H), 1.57-1.49 (m, 2H), 1.38 (s, 9H), 1.25 (m, 2H). EI-MS  $m/z$ :  $[M+H]^+$  790.47,  $[M+Na]^+$  812.4.

Preparation of Compound 33b

To a stirred mixture of compound **33a** (2.2 g, 2.78 mmol) and Pd/C (10 wt. %, 400

mg) in MeOH (60 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 1.39 mL, 5.56 mmol). After stirring at room temperature for 3 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (40 mL). The filtrate was concentrated to produce the compound **33b** (1.67 g, 99 %), which was used without further purification. <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 9.98 (s, 1H), 8.87 (d, 1H), 8.28 (bs, 3H), 8.12 (1H), 7.96 (bs, 3H), 4.51 (q, 1H), 3.77 (t, 2H), 3.72 (bs, 1H), 3.57 (s, 3H), 3.52-3.47 (m, 7H), 3.12 (s, 3H), 2.76-2.61 (m, 4H) 1.71 (q, 2H), 1.55 (q, 2H) 1.36 (s, 9H). EI-MS m/z: [M+H]<sup>+</sup> 522.4, [M+Na]<sup>+</sup> 544.3.

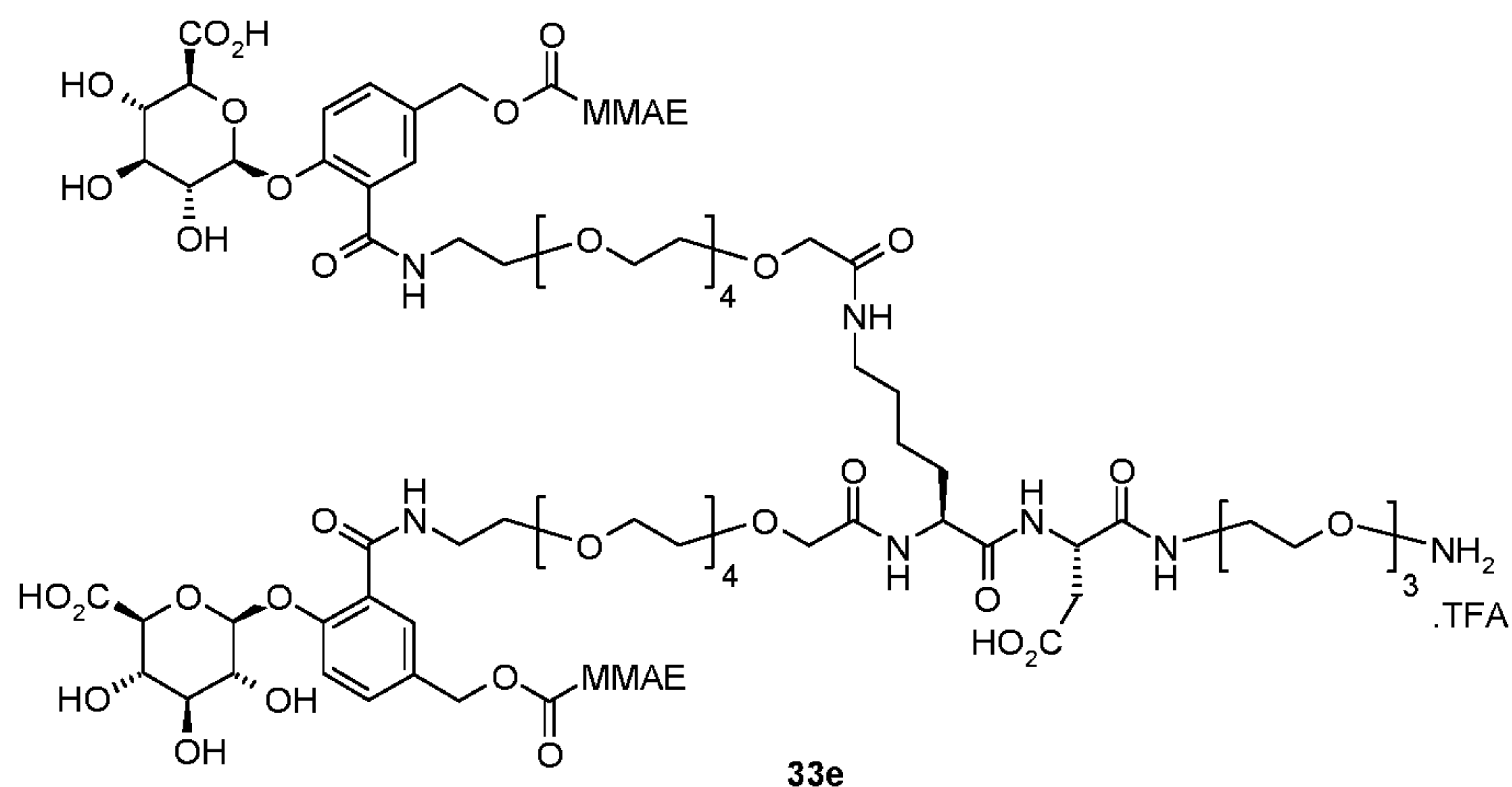
#### Preparation of Compound 33c

DIPEA (1.95 mL, 11.23 mmol) and HBTU (3.19 g, 8.42 mmol) were added to a stirred mixture of compound **25b** (1.98 g, 6.17 mmol) and compound **33b** (1.67 g, 2.80 mmol) in DMF (20 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was concentrated and purified by column chromatography, which produced the compound **33c** (2 g, 63 %). <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 9.97 (s, 1H), 8.28 (d, 1H), 7.82 (t, 1H), 7.65 (s, 1H), 7.64 (s, 1H), 4.54 (q, 1H), 4.25 (q, 1H), 3.91 (s, 2H), 3.84 (s, 2H), 3.80 (t, 2H), 3.60-3.49 (m, 48H), 3.26-3.12 (m, 3H), 3.07 (q, 2H), 2.75-2.54 (m, 2H), 1.65-1.55 (m, 2H), 1.39 (s, 10H), 1.21 (m, 3H). EI-MS m/z: [M+H]<sup>+</sup> 1128.8, [M+Na]<sup>+</sup> 1150.7.

#### Preparation of Compound 33d

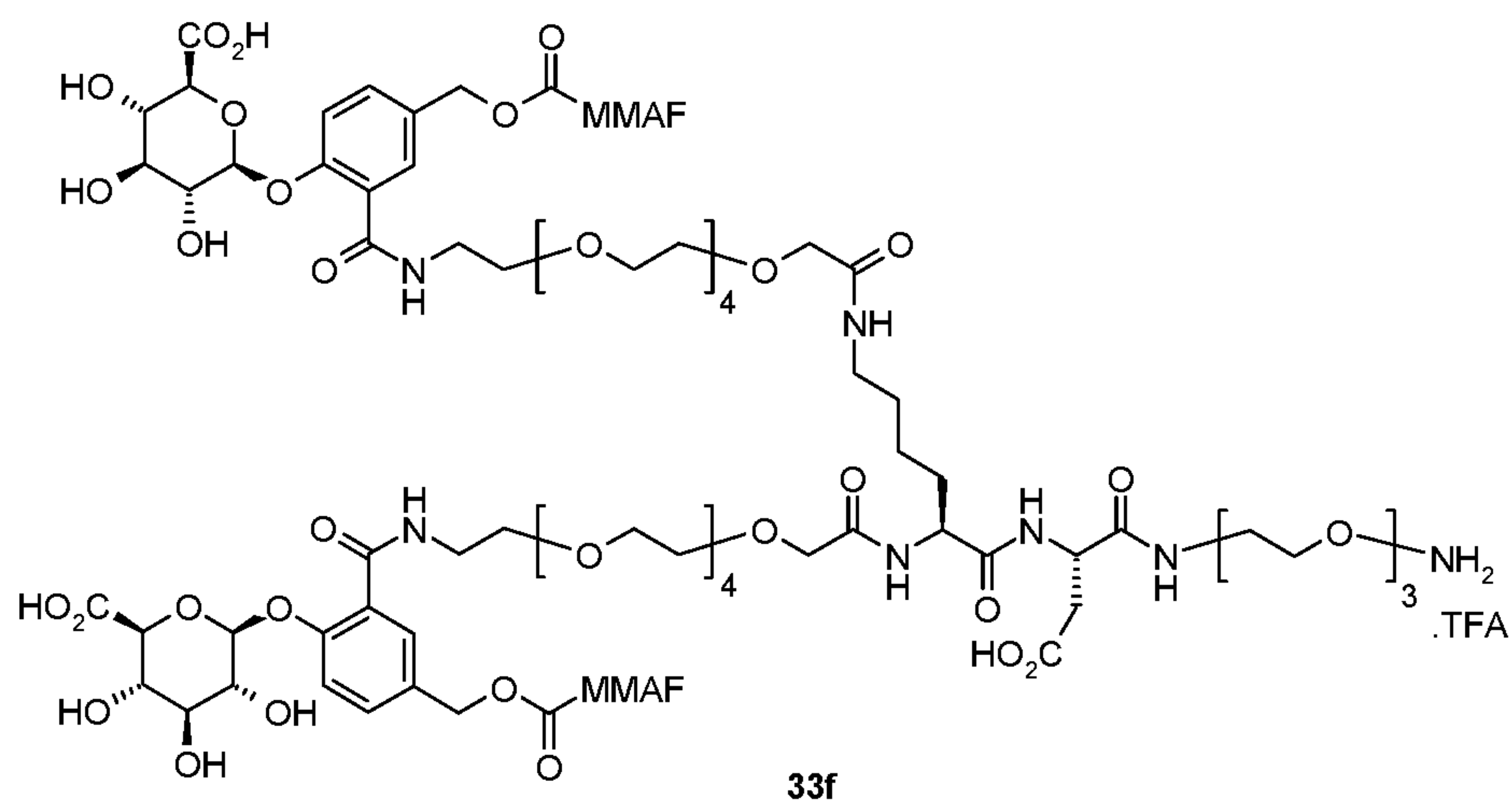
To a stirred mixture of compound **33c** (1 g, 0.88 mmol) and Pd/C (10 wt. %, 200 mg) in MeOH (20 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.44 mL, 0.88 mmol). After stirring at room temperature for 3 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (20 mL). The filtrate was concentrated to produce the compound **33d** (936 mg, 92 %), which was used without further purification. <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 9.98 (s 1H), 8.30 (d, 1H), 7.70 (t, 2H), 4.54 (q, 1H), 4.26 (q, 1H), 3.93 (s, 2H), 3.85 (s, 2H), 3.80 (t, 2H), 3.61-3.49 (m, 46H), 3.22-3.12 (m, 4H), 3.06 (q, 2H), 2.97 (q, 4H), 2.76-2.54 (m, 2H), 1.64-1.55 (m, 2H), 1.39 (s, 10H), 1.26 (m, 3H). EI-MS m/z: [M+H]<sup>+</sup> 1076.8.

#### Preparation of Compound 33e



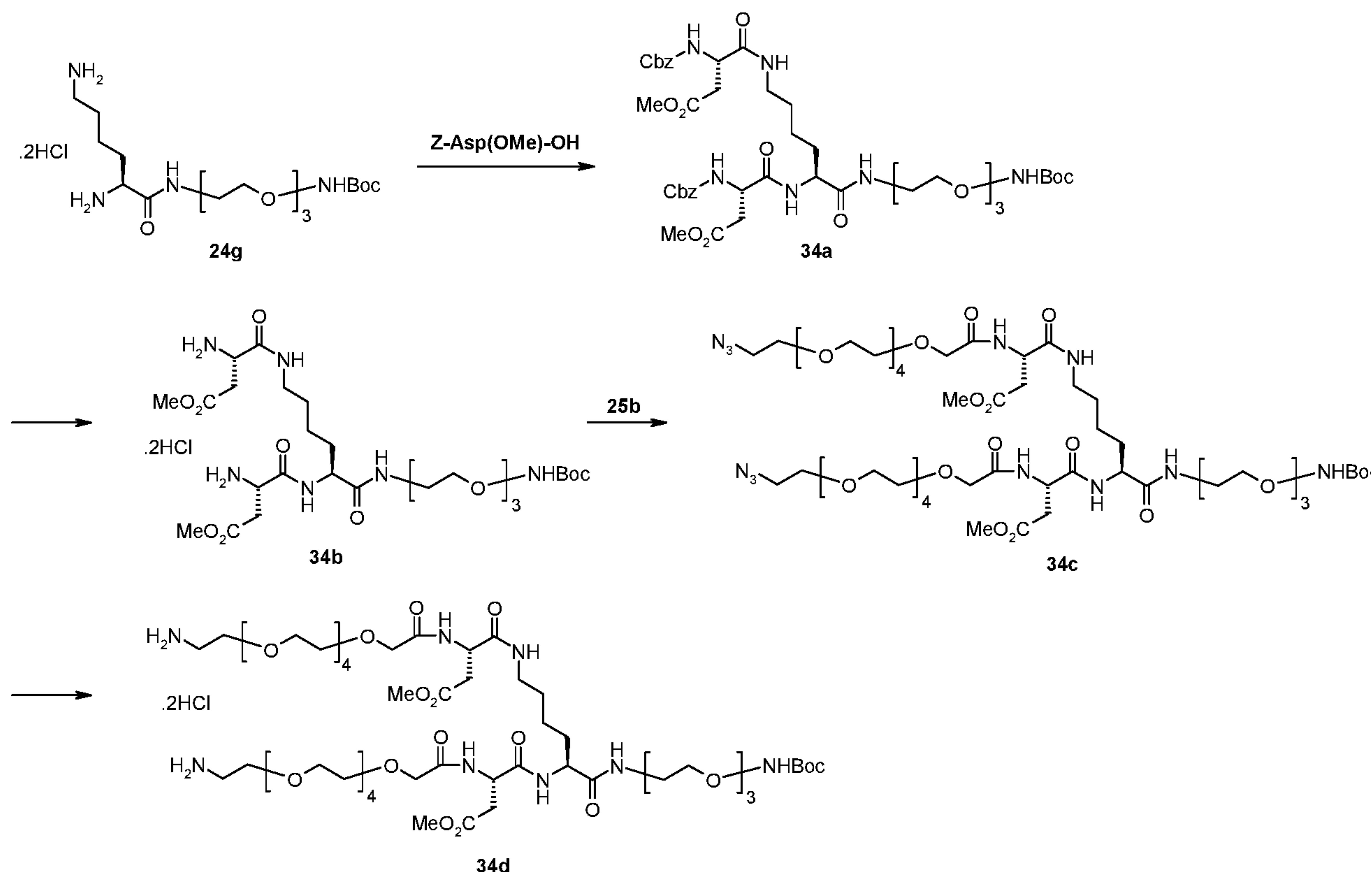
Compound **33e** was prepared from compound **1i** and compound **33d** by a similar method of preparing compound **25e** in Example 35. EI-MS  $m/z$ :  $1/2[M+H]^+$  1552.2.

*Example 50. Preparation of Compound 33f*



Compound **33f** was prepared from compound **1j** and compound **33d** by a similar method of preparing compound **25e** in Example 35. EI-MS  $m/z$ :  $1/2[M+H]^+$  1566.4.

*Example 51. Preparation of Compound 34e*



#### Preparation of Compound **34a**

DIPEA (0.8 mL, 4.56 mmol) and HBTU (1.3 g, 3.42 mmol) were added to a stirred mixture of compound **24g** (530 mg, 1.14 mmol) and Z-Asp(OMe)-OH (704 mg, 2.5 mmol) in DMF (5 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (50 mL) and extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with 1 N aq. HCl (40 mL), saturated aq. NaHCO<sub>3</sub> (40 mL) and brine (40 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **34a**. (713 mg, 68 %). <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) : δ 9.97 (s, 1H), 7.88 (m, 3H), 7.64 (d, 2H), 7.51 (d, 2H), 7.35 (m, 10H), 5.02 (m, 4H), 4.43-4.31 (m, 2H), 4.17 (m, 1H), 3.80 (t, 2H), 3.58-3.50 (m, 12H), 3.41-3.16 (m, 6H), 2.98 (m, 2H), 2.79-2.67 (m, 3H), 2.57 (m, 2H), 1.60-1.34 (m, 13H).

#### Preparation of Compound **34b**

To a solution of compound **34a** (530 mg, 0.58 mmol) in MeOH (5 mL) was added Pd/C (20 wt. %, 106 mg) and HCl (4 N in 1,4-dioxane, 0.29 mL, 1.16 mmol). After stirring at room temperature for 3 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (30 mL). The filtrate was concentrated to produce the

compound **34b** (420 mg, 100 %), which was used without further purification.  $^1\text{H-NMR}$  (400 MHz,  $\text{DMSO-d}_6$ )  $\delta$  9.97 (s, 1H), 8.62 (d, 1H), 8.54 (s, 1H), 8.27 (m, 4H), 7.02 (s, 1H), 4.17 (m, 2H), 4.02 (m, 1H), 3.76 (t, 2H), 3.61 (m, 4H), 3.51-3.11 (m, 12H), 3.09-2.77 (m, 8H), 1.60-1.24 (m, 13H). EI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  651.5.

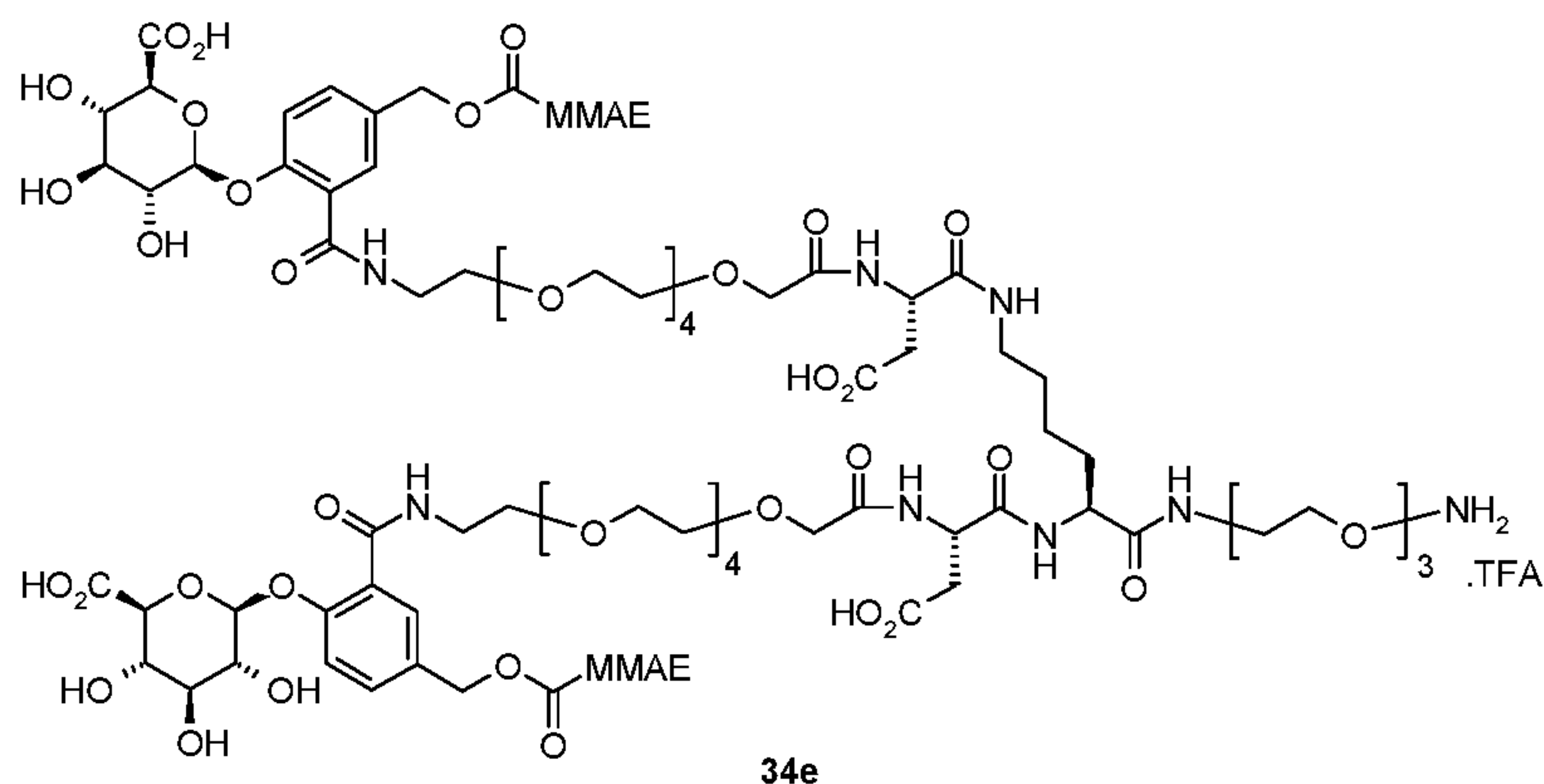
#### Preparation of Compound **34c**

DIPEA (0.4 mL, 2.32 mmol) and HBTU (660 mg, 1.74 mmol) were added to a stirred mixture of compound **34b** (420 mg, 0.58 mmol) and compound **25b** (299 mg, 0.93 mmol) in DMF (5 mL). After stirring at room temperature for 14 hours under  $\text{N}_2$ , the reaction mixture was poured into  $\text{H}_2\text{O}$  (30 mL) and extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with 1 N aq. HCl (20 mL), saturated aq.  $\text{NaHCO}_3$  (20 mL) and brine (20 mL) sequentially, and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . After filtration and concentration, the residue was purified by column chromatography, which produced the compound **34c** (466 mg, 70.8 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.28 (s, 1H), 7.78 (q, 1H), 7.31 (d, 1H), 7.71 (s, 1H), 6.94 (s, 1H), 4.85 (m, 2H), 4.35 (m, 1H), 4.07-4.03 (m, 6H), 3.75-3.41 (m, 56H), 3.23 (q, 2H), 2.92-2.84 (m, 4H), 1.91-1.32 (m, 15H). EI-MS  $m/z$ :  $[\text{M}+2\text{H}]^+$  1158.1.

#### Preparation of Compound **34d**

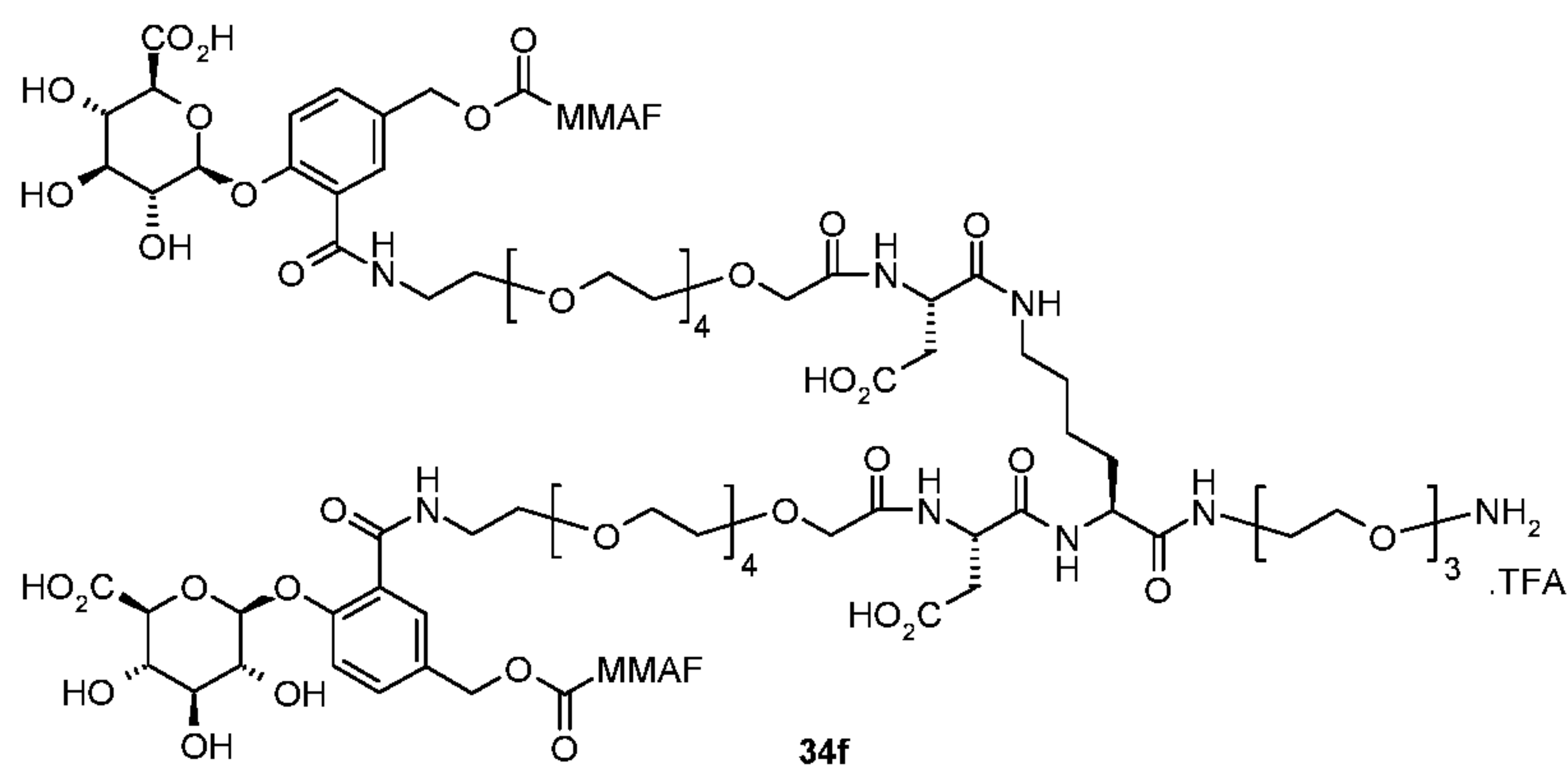
To a stirred mixture of compound **34c** (260 mg, 0.21 mmol) and Pd/C (10 wt. %, 52 mg) in MeOH (20 mL) at 0 °C was added HCl (4 N in 1,4-dioxane, 0.10 mL, 0.41 mmol). After stirring at room temperature for 2 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (50 mL). The filtrate was concentrated to produce the compound **34d** (249 mg, 100 %), which was used without further purification. EI-MS  $m/z$ :  $[\text{M}+2\text{H}]^+$  1206.1.

#### Preparation of Compound **34e**



Compound **34e** was prepared from compound **1i** and compound **34d** by a similar method of preparing compound **25e** in Example 35. EI-MS  $m/z$ :  $1/2[M+H]^+$  1610.4.

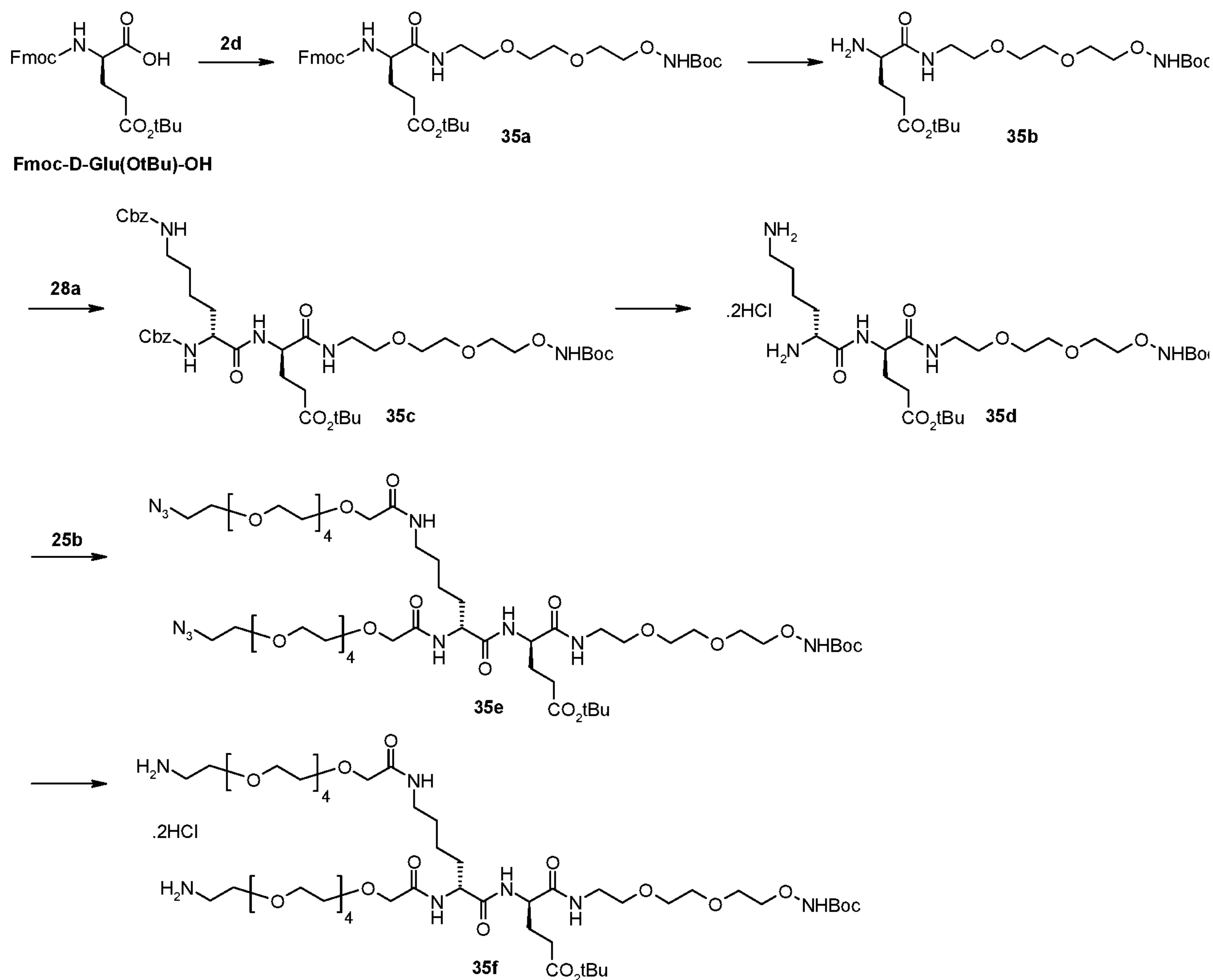
*Example 52. Preparation of Compound 34f*



Compound **34f** was prepared from compound **1j** and compound **34d** by a similar method of preparing compound **25e** in Example 35. EI-MS  $m/z$ :  $1/2[M+H]^+$  1624.3.

*Example 53. Preparation of Compound 35g*

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### Preparation of Compound **35a**

DIPEA (0.61 mL, 3.52 mmol) and HBTU (665 mg, 1.175 mmol) were added to a stirring mixture of Fmoc-D-Glu(OtBu)-OH (500 mg, 1.17 mmol) and compound **2d** (424 mg, 1.404 mmol) in DMF (10 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (30 mL) and extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with 1 N aq. HCl (20 mL), saturated aq. NaHCO<sub>3</sub> (20 mL) and brine (20 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the resulting residue was purified by column chromatography, which produced the compound **35a** (708 mg, 89 %). EI-MS m/z: [M+H]<sup>+</sup> 672.7.

### Preparation of Compound **35b**

To a solution of compound **35a** (708 mg, 1.04 mmol) in THF (8 mL) was added piperidine (2 mL) at room temperature. After stirring for 20 minutes, the reaction mixture was concentrated under reduced pressure. The resulting residue was purified by column

chromatography, which produced the compound **35b** (400 mg, 85 %). EI-MS m/z:  $[M+H]^+$  450.1.

#### Preparation of Compound 35c

DIPEA (0.19 mL, 1.1 mmol) and HBTU (253 mg, 0.66 mmol) were added to a stirring mixture of compound **28a** (203 mg, 0.484 mmol) and compound **35b** (200 mg, 0.44 mmol) in DMF (10 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (30 mL) and extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with 1 N aq. HCl (10 mL), saturated aq. NaHCO<sub>3</sub> (10 mL) and brine (10 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **35c** (235 mg, 63 %). EI-MS m/z:  $[M+H]^+$  847.0.

#### Preparation of Compound 35d

To a solution of compound **35c** (235 mg, 0.277 mmol) in MeOH (15 mL) was added Pd/C (10 wt. %, 30 mg). After stirring at room temperature for 2 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (50 mL). The filtrate was concentrated to produce the compound **35d** (160 mg, 100 %), which was used without further purification. EI-MS m/z:  $[M+H]^+$  578.7.

#### Preparation of Compound 35e

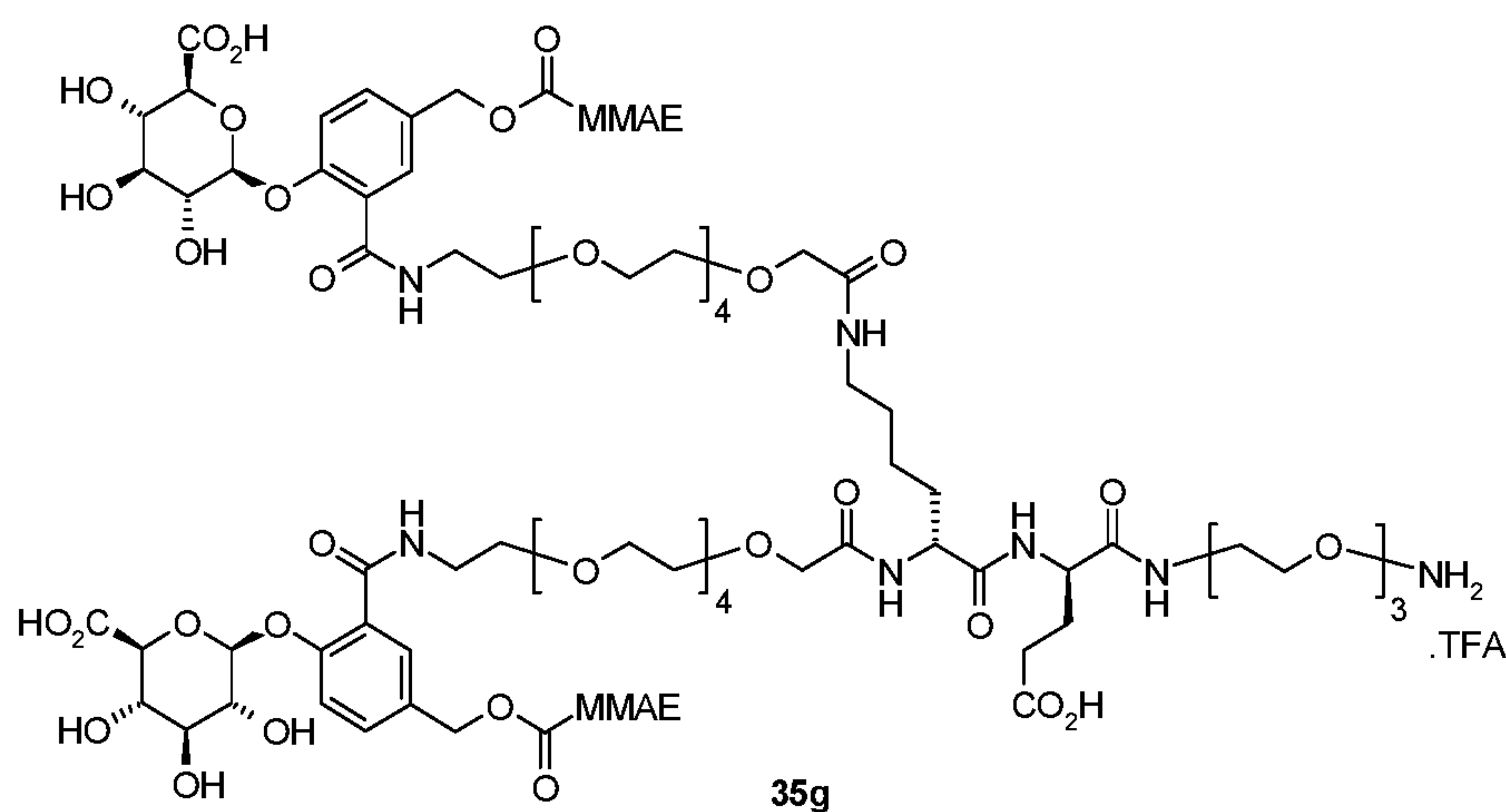
DIPEA (0.145 mL, 1.758 mmol) and HBTU (262 mg, 1.465 mmol) were added to a stirring mixture of compound **35d** (160 mg, 0.276 mmol) and compound **25b** (187 mg, 0.581 mmol) in DMF (3 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (30 mL) and extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with 1 N aq. HCl (10 mL), saturated aq. NaHCO<sub>3</sub> (10 mL) and brine (10 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **35e** (260 mg, 79 %). EI-MS m/z:  $[M+H]^+$  1185.4.

#### Preparation of Compound 35f

To a solution of compound **35e** (70 mg, 0.059 mmol) in MeOH (5 mL) was added Pd/C (10 wt. %, 15 mg). After stirring at room temperature for 90 minutes under hydrogen,

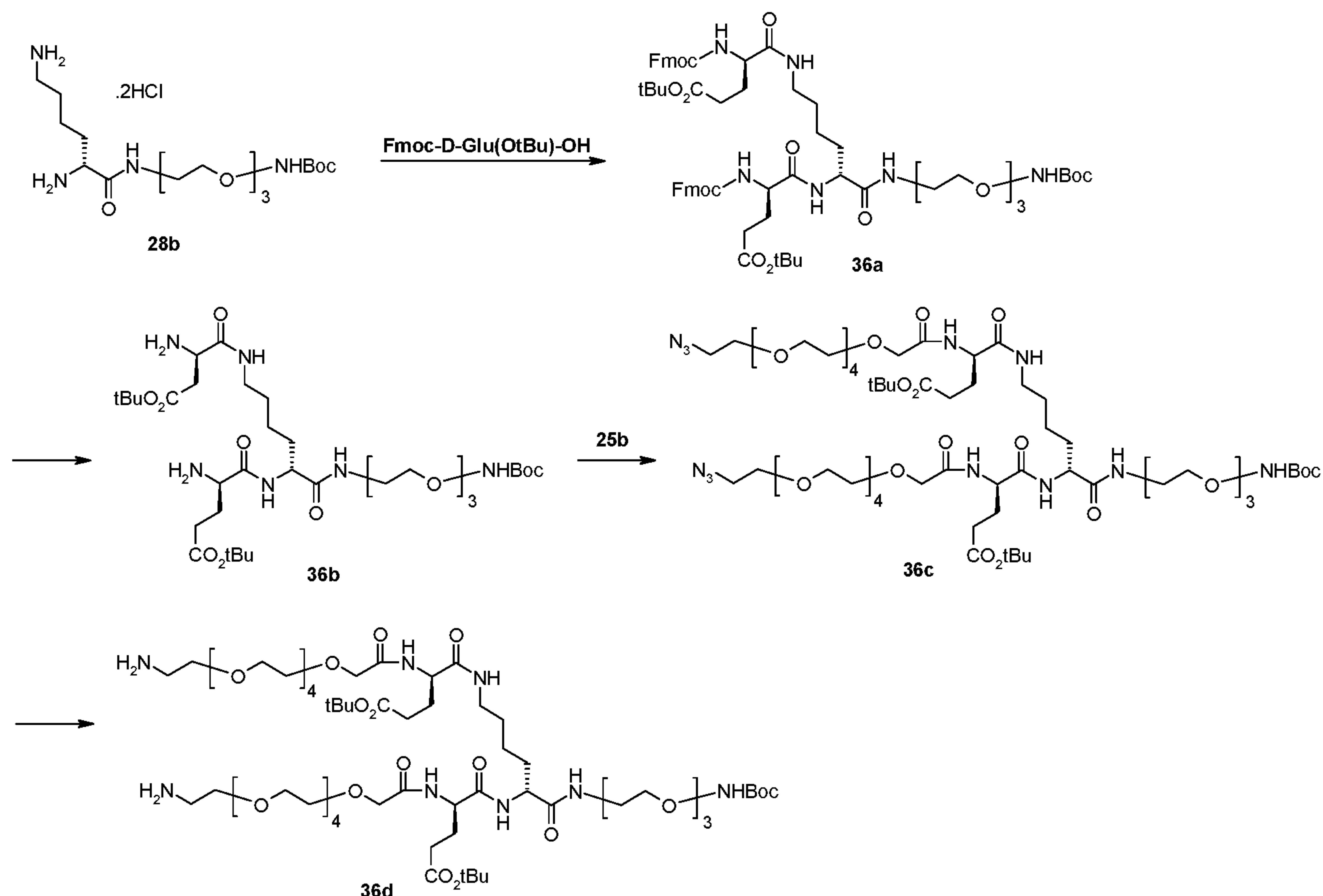
the reaction mixture was filtered through a celite pad and washed with MeOH (30 mL). The filtrate was concentrated to produce the compound **35f** (67 mg, 100 %), which was used without further purification. EI-MS  $m/z$ :  $[M+H]^+$  : 1133.3.

#### Preparation of Compound **35g**



Compound **35g** was prepared from compound **1i** and compound **35f** by a similar method of preparing compound **25e** in Example 35. EI-MS  $m/z$ :  $1/2[M+H]^+$  1559.9.

#### *Example 54. Preparation of Compound **36e***



### Preparation of Compound **36a**

DIPEA (0.3 mL, 3.10 mmol) and HBTU (474 mg, 2.275 mmol) were added to a stirring mixture of the Fmoc-D-Glu(OtBu)-OH (484 mg, 1.138 mmol) and compound **28b** (223 mg, 0.569 mmol) in DMF (7 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (30 mL) and extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with 1 N aq. HCl (20 mL), saturated aq. NaHCO<sub>3</sub> (20 mL) and brine (20 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **36a** (593 mg, 86 %). EI-MS m/z: [M+H]<sup>+</sup> 1208.3.

### Preparation of Compound **36b**

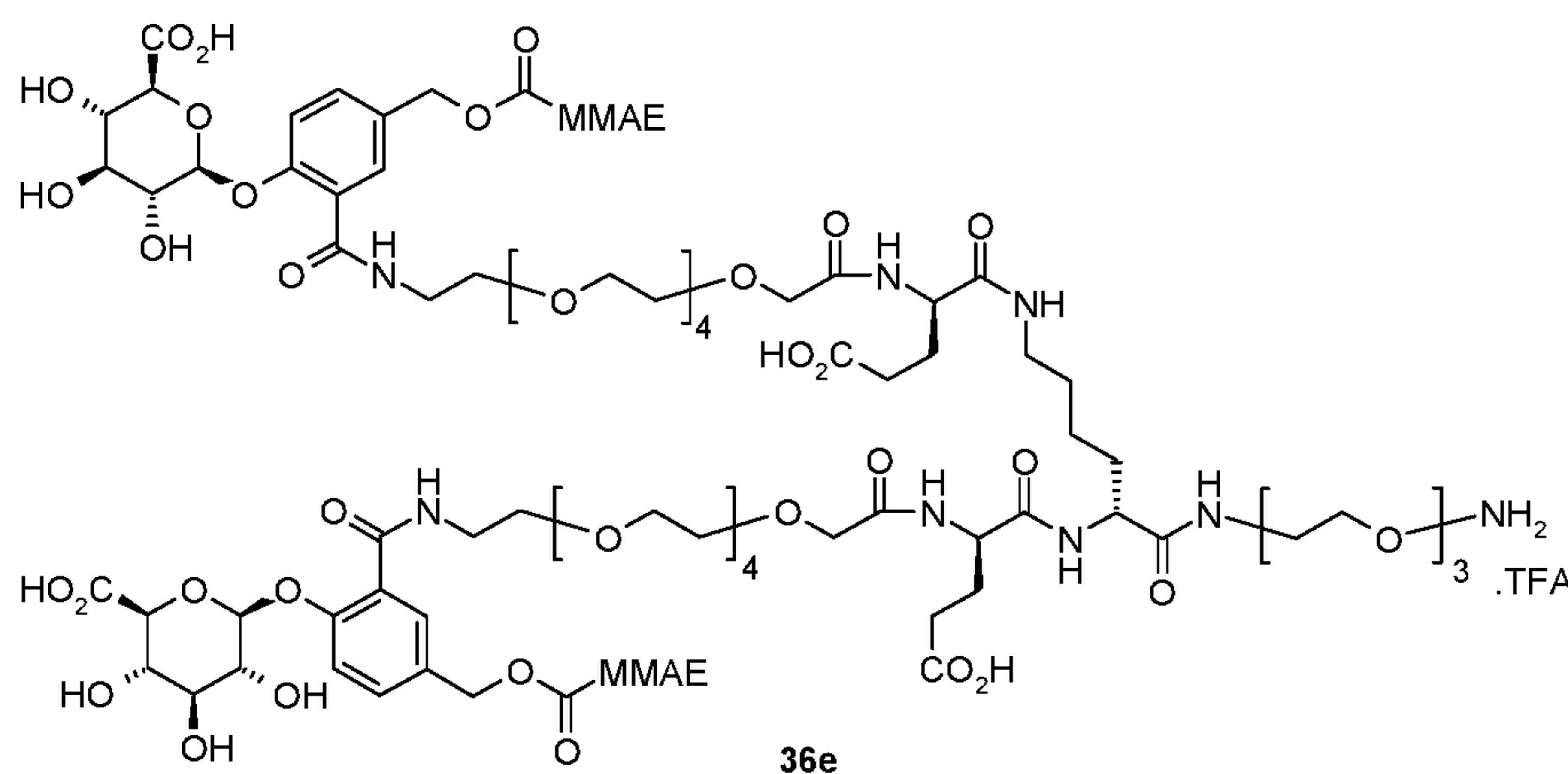
To a solution of compound **36a** (593 mg, 0.49 mmol) in THF (8 mL) was added piperidine (1 mL) at room temperature. After stirring for 20 minutes, the reaction mixture was concentrated under reduced pressure. The resulting residue was purified by column chromatography, which produced the compound **36b** (166 mg, 44 %). EI-MS m/z: [M+H]<sup>+</sup> 763.9.

Preparation of Compound 36c

DIPEA (0.15 mL, 0.84 mmol) and HBTU (247 mg, 0.63 mmol) were added to a stirred mixture of compound **36b** (166 mg, 0.21 mmol) and compound **25b** (147 mg, 0.441 mmol) in DMF (3 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (30 mL) and extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with 1 N aq. HCl (10 mL), saturated aq. NaHCO<sub>3</sub> (10 mL) and brine (10 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **36c** (195 mg, 68 %). EI-MS m/z: [M+H]<sup>+</sup> 1370.6.

Preparation of Compound 36d

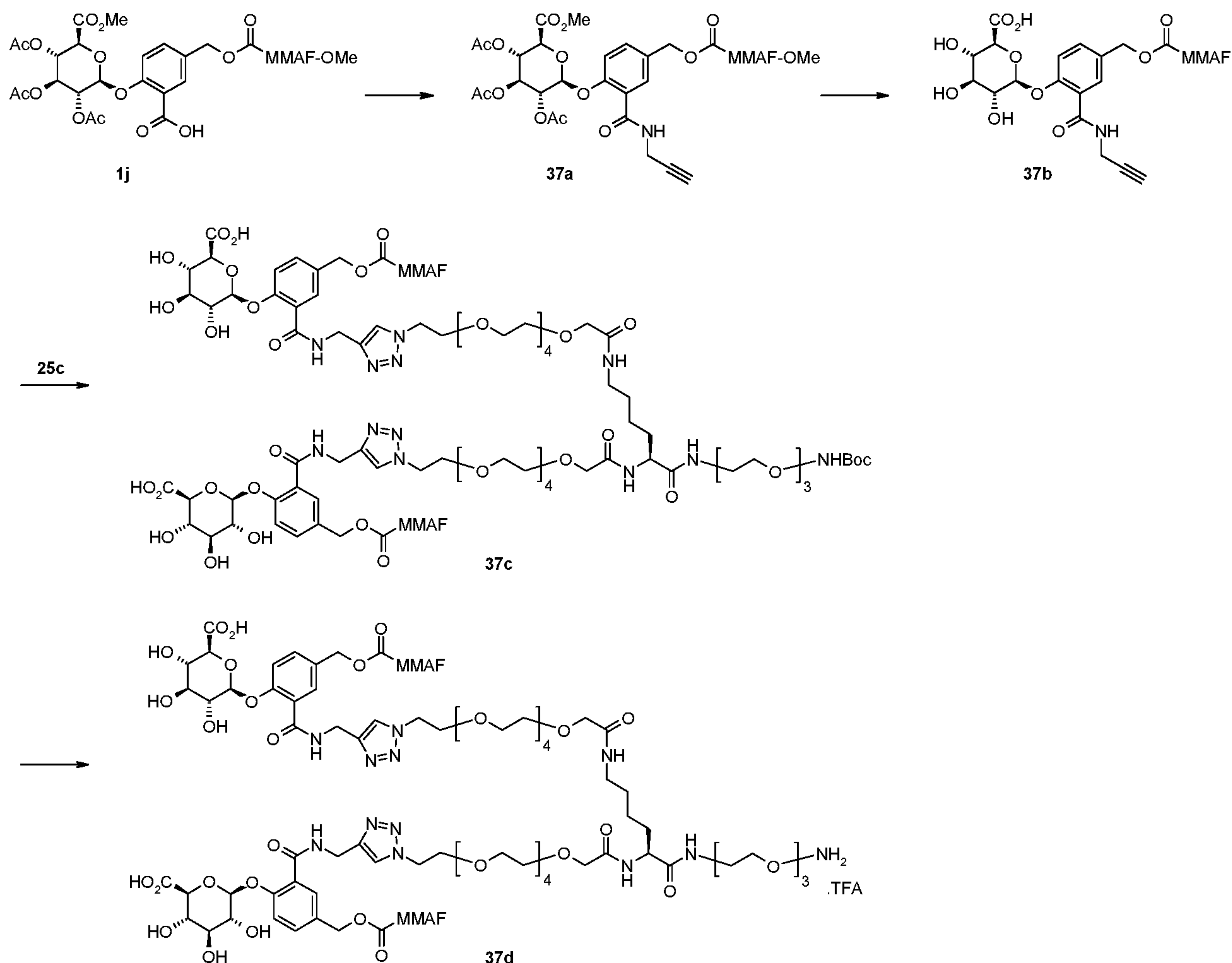
To a solution of compound **36c** (195 mg, 0.14 mmol) in MeOH (10 mL) was added Pd/C (10 wt. %, 30 mg). Then the reaction mixture was stirring at room temperature for 90 minutes under hydrogen. The reaction mixture was filtered through a celite pad and washed with MeOH (30 mL). The filtrate was concentrated to produce the compound **36d** (187 mg, 100 %), which was used without further purification. EI-MS m/z: [M+H]<sup>+</sup> 1318.6.

Preparation of Compound 36e

Compound **36e** was prepared from compound **1i** and compound **36d** by a similar method of preparing compound **25e** in Example 35. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1624.4.

*Example 55. Preparation of Compound 37d*

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### Preparation of Compound 37a

DIPEA (0.083 mL, 0.71 mmol) and HBTU (136 mg, 0.36 mmol) were added to a stirred mixture of propargyl amine (0.018 mL, 0.285 mmol) and compound **1j** (300 mg, 0.238 mmol) in DMF (3 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (10 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with 1 N aq. HCl (10 mL), saturated aq. NaHCO<sub>3</sub> (10 mL) and brine (10 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the resulting residue was purified by column chromatography, which produced the compound **37a** (300 mg, 97 %). EI-MS m/z: [M+H]<sup>+</sup> 1294.0.

### Preparation of Compound 37b

To a solution of compound **37a** (300 mg, 0.24 mmol) in THF (2 mL) and MeOH (2 mL) was added LiOH monohydrate (50 mg, 1.20 mmol) in H<sub>2</sub>O (2 mL) at 0 °C. After stirring for 2 hours at 0 °C, the reaction mixture was neutralized using acetic acid and was concentrated under reduced pressure. Then the residue was dissolved in H<sub>2</sub>O/DMSO (1.5

mL/1.5 mL) and purified by HPLC. Pure fractions with the same retention time were combined and concentrated to produce the compound **37b** (165 mg, 60 %). EI-MS m/z:  $[M+H]^+$  1140.8.

#### Preparation of Compound 37c

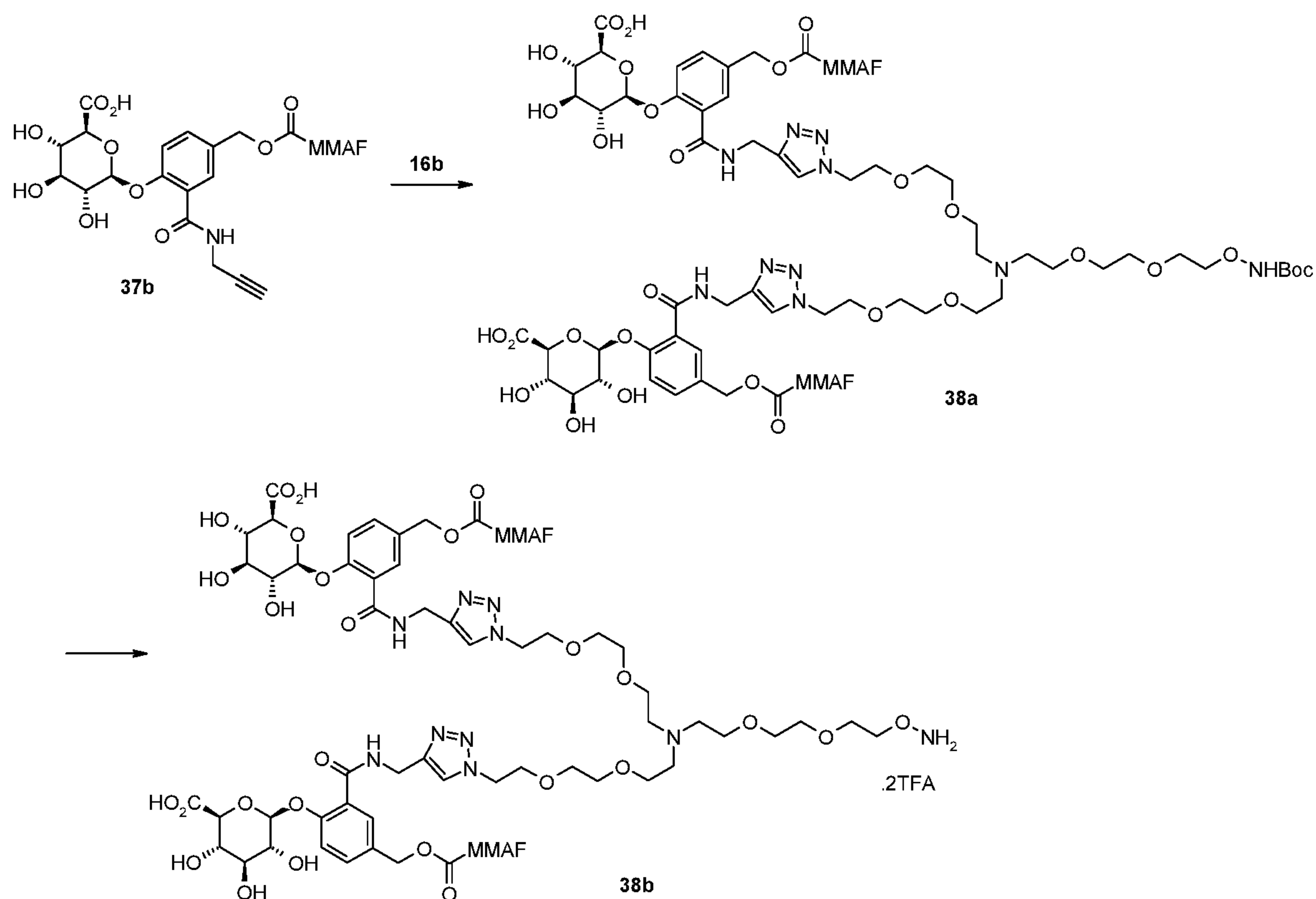
CuSO<sub>4</sub>·5H<sub>2</sub>O (1 mg) and sodium ascorbate (2 mg) were added to a stirred mixture of compound **37b** (50 mg, 0.042 mmol) and compound **25c** (23 mg, 0.02 mmol) in THF (2 mL) and H<sub>2</sub>O (2 mL). The pH was adjusted to about 7 by addition of 1 M aq. Na<sub>2</sub>CO<sub>3</sub>. After stirring at 20 °C for 1 hour, the reaction mixture was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC. Pure fractions with the same retention time were combined and concentrated to produce the compound **37c** (32.4 mg, 48 %). EI-MS m/z:  $1/2[M+H]^+$  1638.2.

#### Preparation of Compound 37d

TFA (0.4 mL) was added to a solution of compound **37c** (32.4 mg, 0.01 mmol) in DCM (2 mL). After stirring at 0 °C for 2 hours, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in H<sub>2</sub>O/MeCN (1 mL/1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **37d** (19.6 mg, 62 %) as white solid. EI-MS m/z:  $1/2[M+H]^+$  1590.2.

#### *Example 56. Preparation of Compound 38b*

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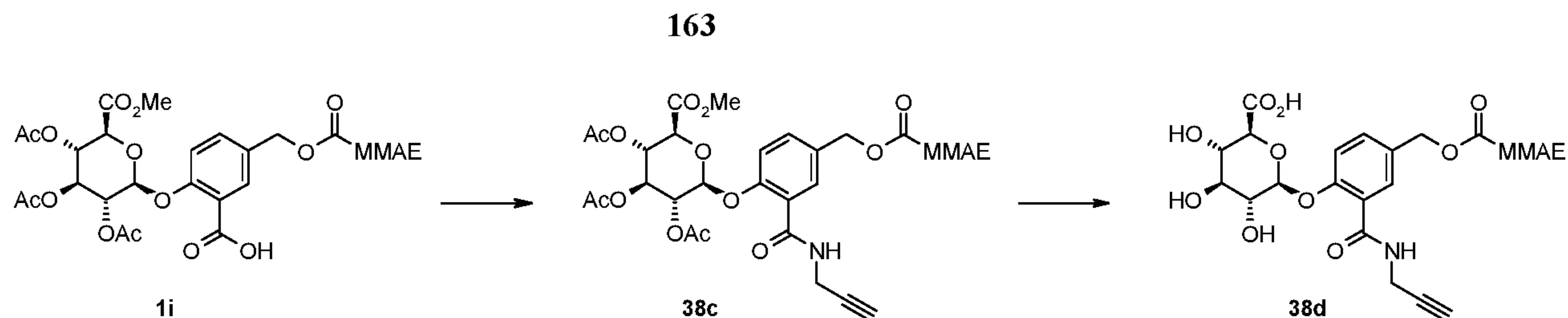
### Preparation of Compound **38a**

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (1 mg) and sodium ascorbate (2 mg) were added to a stirred mixture of compound **37b** (60 mg, 0.052 mmol) and compound **16b** (14 mg, 0.025 mmol) in THF (2 mL) and  $\text{H}_2\text{O}$  (2 mL). The pH was adjusted to about 7 by addition of 1 M aq.  $\text{Na}_2\text{CO}_3$ . After stirring at 20 °C for 1 hour, the reaction mixture was dissolved in  $\text{H}_2\text{O}/\text{DMSO}$  (1.5 mL/1.5 mL) and purified by HPLC. Pure fractions with the same retention time were combined and concentrated to produce the compound **38a** (61 mg, 82 %). EI-MS  $m/z$ :  $1/2[\text{M}+\text{H}]^+$  1430.2.

### Preparation of Compound **38b**

TFA (0.4 mL) was added to a solution of compound **38a** (59.8 mg, 0.02 mmol) in DCM (2.0 mL). After stirring at 0 °C for 2 hours, the solvent and excess TFA were removed by  $\text{N}_2$  flow. Then the residue was dissolved in  $\text{H}_2\text{O}/\text{AN}$  (1 mL/1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **38b** (14.6 mg, 24 %) as white solid. EI-MS  $m/z$ :  $1/2[\text{M}+\text{H}]^+$  1380.1.

### Example 57. Preparation of Compound **38e**



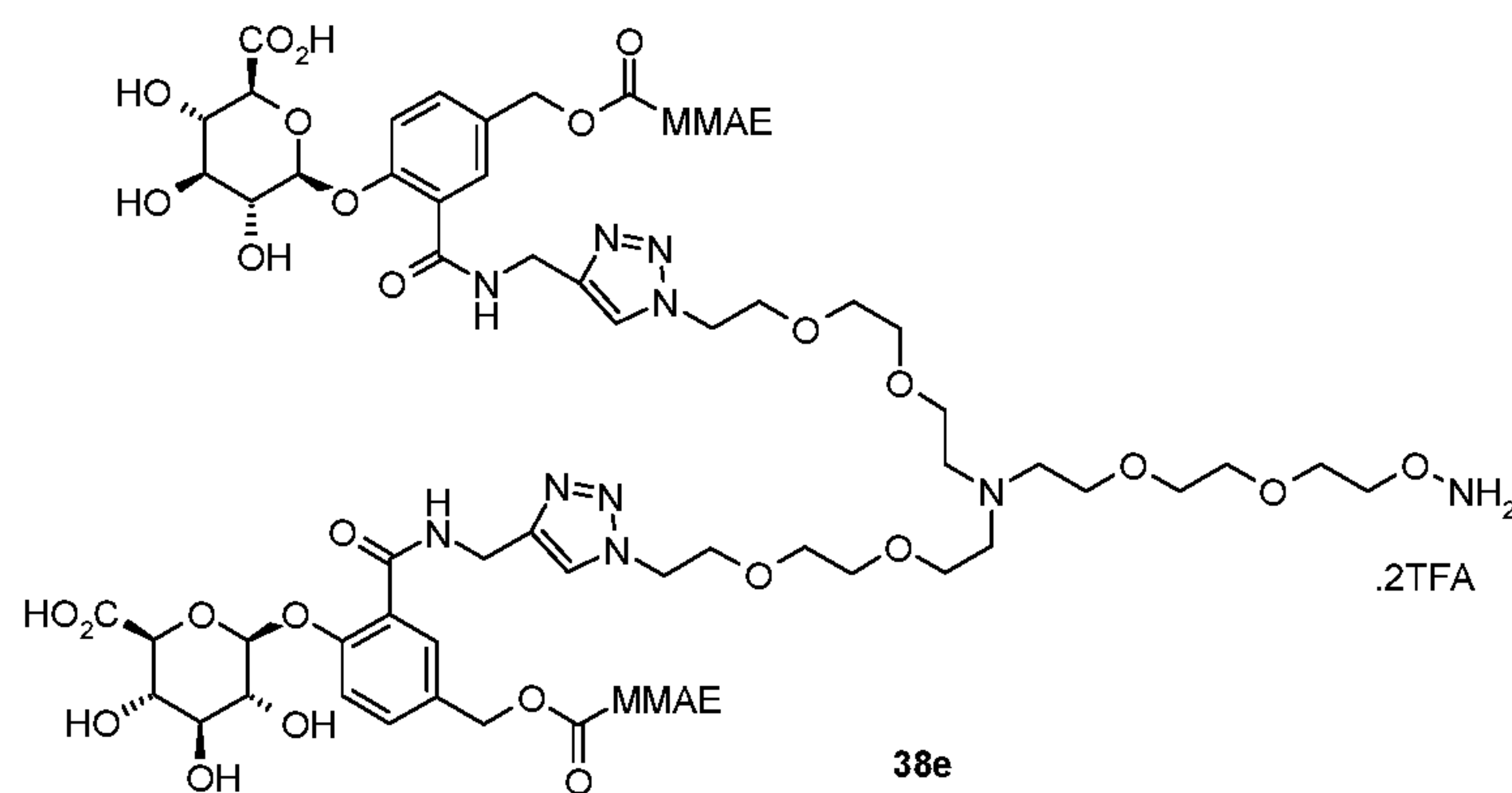
### Preparation of Compound **38c**

DIPEA (0.075 mL, 0.428 mmol) and HBTU (122 mg, 0.321 mmol) were added to a stirred mixture of propargyl amine (0.016 mL, 0.256 mmol) and compound **1i** (264 mg, 0.214 mmol) in DMF (3 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into H<sub>2</sub>O (10 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with 1 N aq. HCl (10 mL), saturated aq. NaHCO<sub>3</sub> (10 mL) and brine (10 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration, the residue was purified by column chromatography, which produced the compound **38c** (270 mg, 100 %). EI-MS m/z: [M+H]<sup>+</sup> 1266.2.

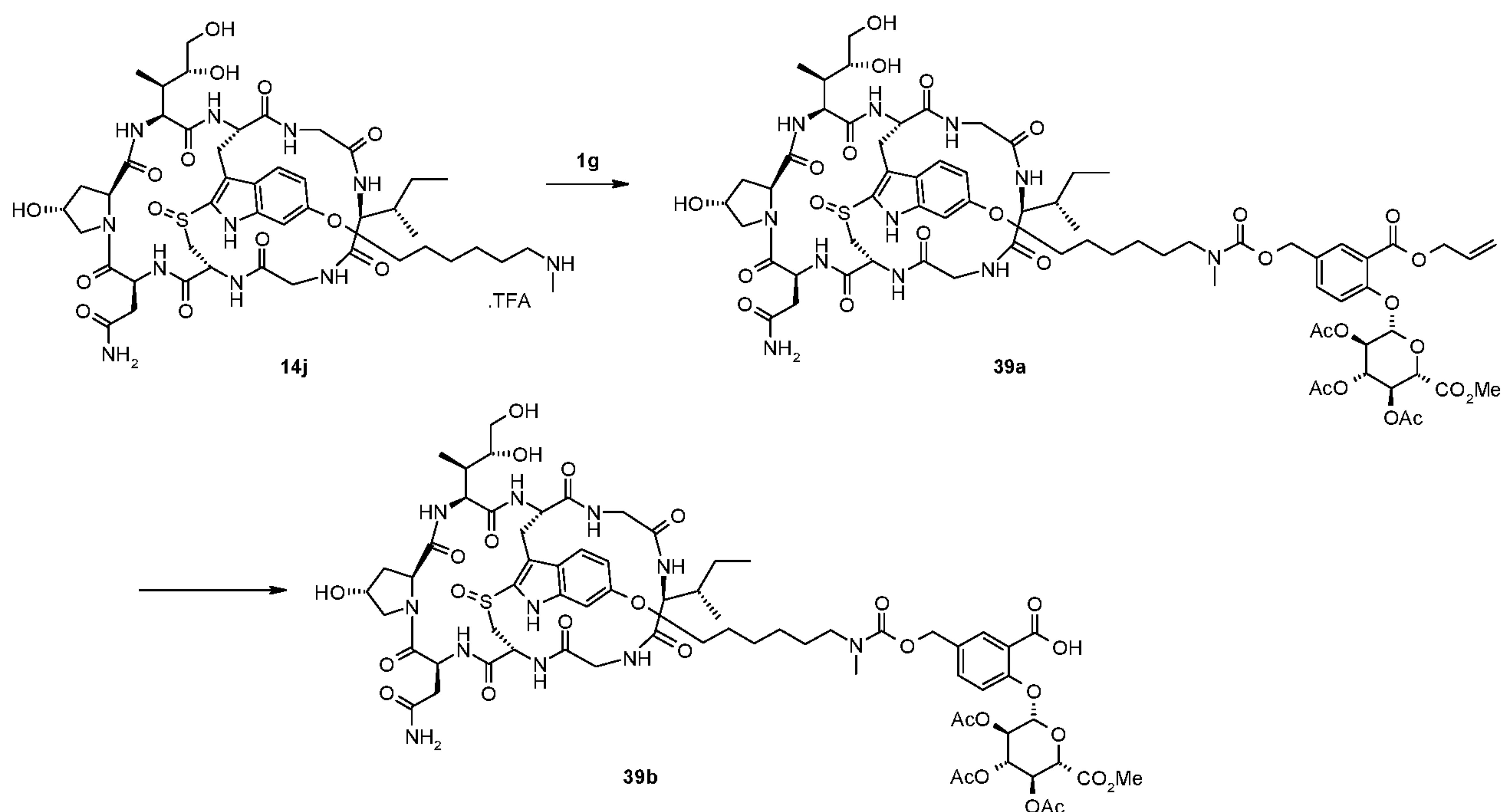
### Preparation of Compound **38d**

To a solution of compound **38c** (270 mg, 0.213 mmol) in THF (2 mL) and MeOH (2 mL) was added LiOH monohydrate (36 mg, 0.853 mmol) in H<sub>2</sub>O (2 mL) at 0 °C. After stirring for 2 hours at 0 °C, the reaction mixture was neutralized using acetic acid and was concentrated under reduced pressure. Then the residue was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC. Pure fractions with the same retention time were combined and concentrated to produce the compound **38d** (168 mg, 70 %). EI-MS m/z: [M+H]<sup>+</sup> 1126.1.

### Preparation of Compound **38e**



Compound **38e** was prepared from compound **38d** and compound **16b** by a similar method of preparing compound **38b** in Example 56. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1366.2.

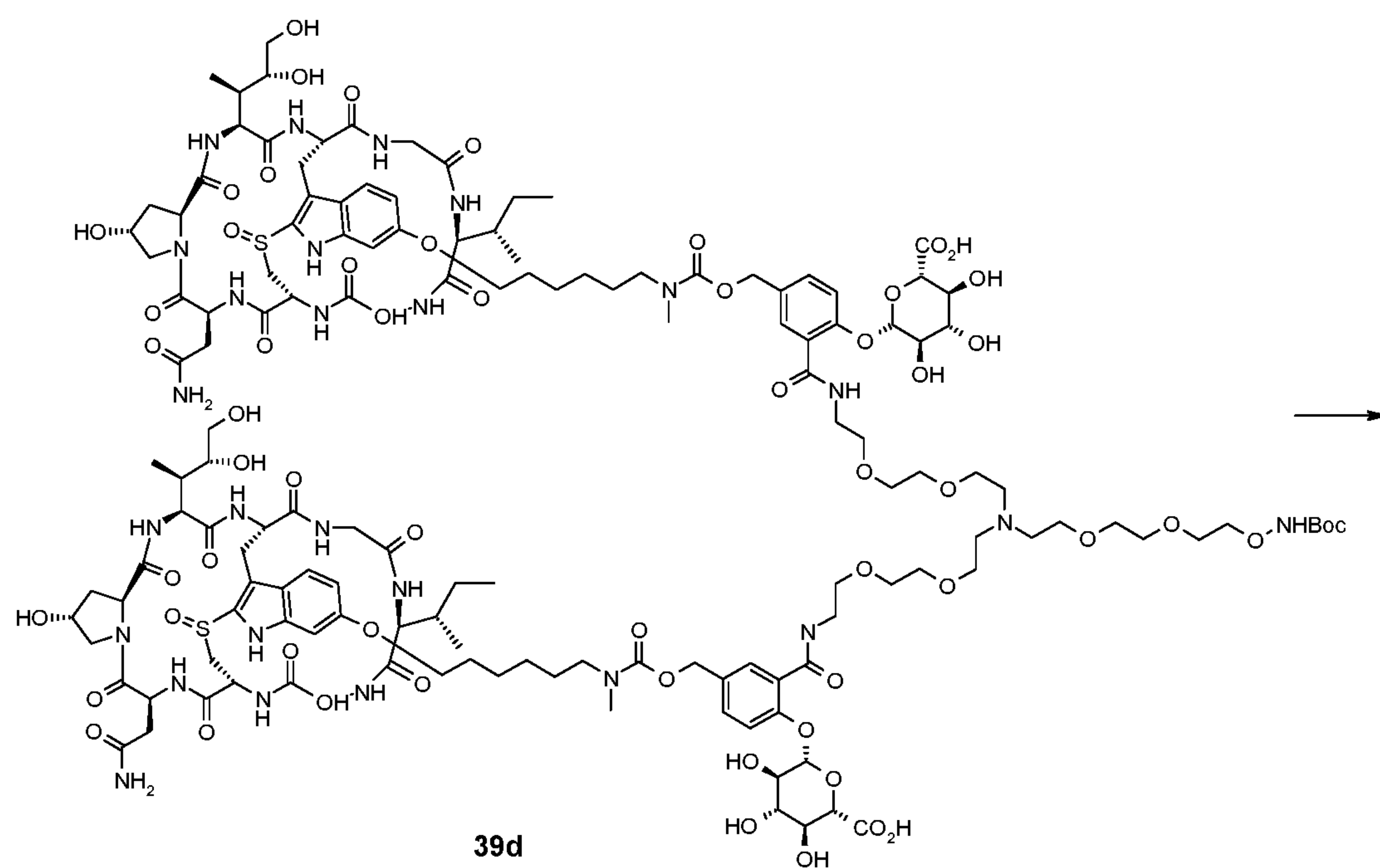
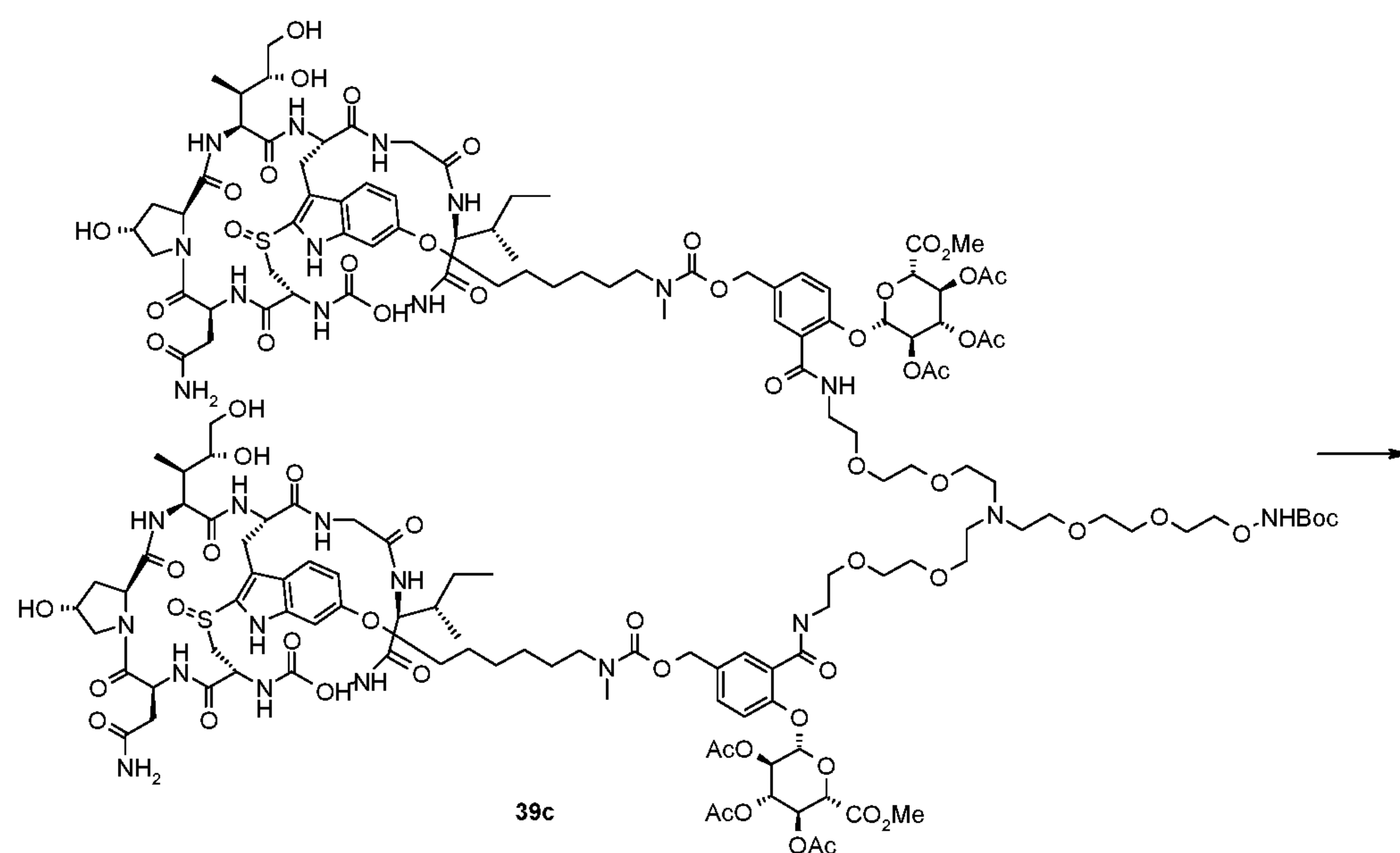
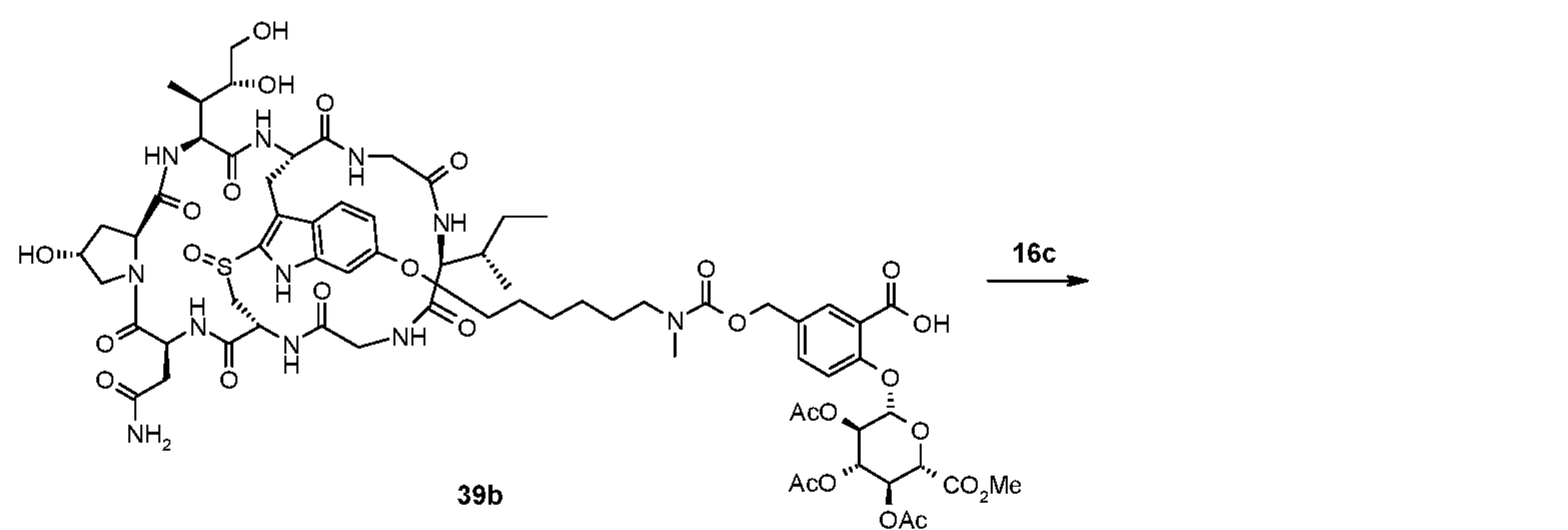
*Example 58. Preparation of Compound 39e*Preparation of compound 39a

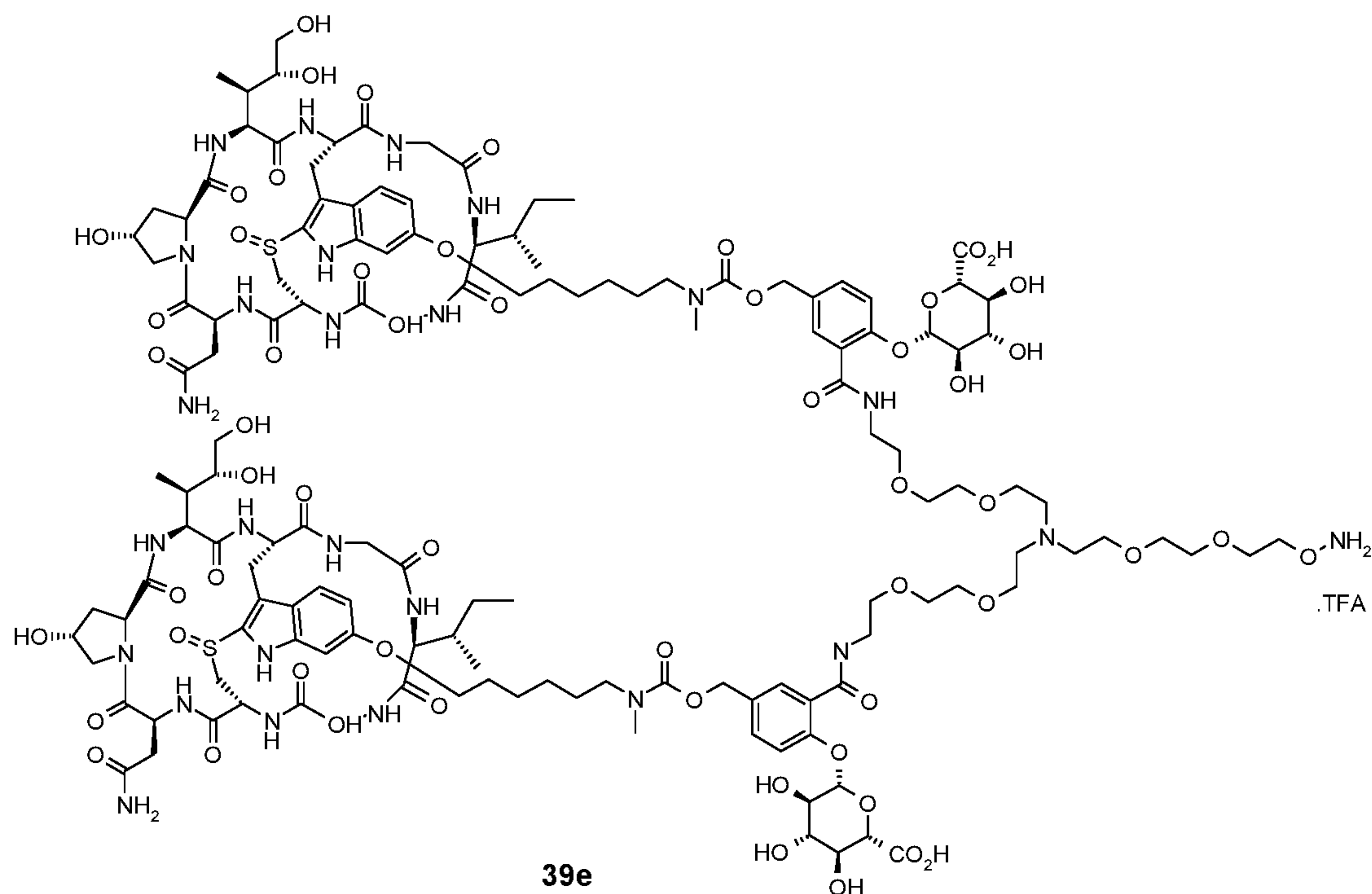
Compound **1g** (27 mg, 0.039 mmol), compound **14j** (45 mg, 0.039 mmol) and anhydrous HOBt (1 mg, 0.0078 mmol) were dissolved in DMF (2 mL) at 0 °C. Then pyridine (0.2 mL) and DIPEA (0.014 mL, 0.078 mmol) were added. After stirring at 0 °C to room temperature for 24 hours under N<sub>2</sub>, the reaction mixture was dissolved in DMSO (1 mL) and purified by HPLC, which produced the compound **39a** (36 mg, 58 %) as white solid. EI-MS *m/z*: [M+H]<sup>+</sup> 1582.9, [M+Na]<sup>+</sup> 1604.5.

Preparation of compound 39b

Compound **39a** (35 mg, 0.022 mmol) and triphenylphosphine (1.5 mg, 0.005 mmol) were dissolved in DCM (2 mL). Pyrrolidine (0.0025 mL, 0.026 mmol) and Pd(PPh<sub>3</sub>)<sub>4</sub> (1.3 mg, 0.001 mmol) were added to the reaction mixture at room temperature and then allowed to stir for 2 hours. The reaction mixture was diluted with H<sub>2</sub>O (50 mL) and extracted with *n*-butanol (2 x 50 mL). The combined organic layers were dried over anhydrous MgSO<sub>4</sub>, evaporated under reduced pressure. The resulting residue was dissolved in DMSO (1 mL) and purified by HPLC, which produced the compound **39b** (34 mg, crude) as white solid. EI-MS *m/z*: [M+H]<sup>+</sup> : 1542.7.

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#### Preparation of Compound **39c**

DIPEA (0.0026 mL, 0.039 mmol) and PyBOP (4.7 mg, 0.023 mmol) were added to a stirred mixture of compound **39b** (15 mg, 0.009 mmol) and compound **16c** (2.0 mg, 0.0038 mmol) in DMF (0.3 mL). After stirring at room temperature for 13 hours under N<sub>2</sub>, the reaction mixture was dissolved in DMSO (1.5 mL) and purified by HPLC, which produced the compound **39c** (12 mg, 35 %) as white solid. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1788.5.

#### Preparation of Compound **39d**

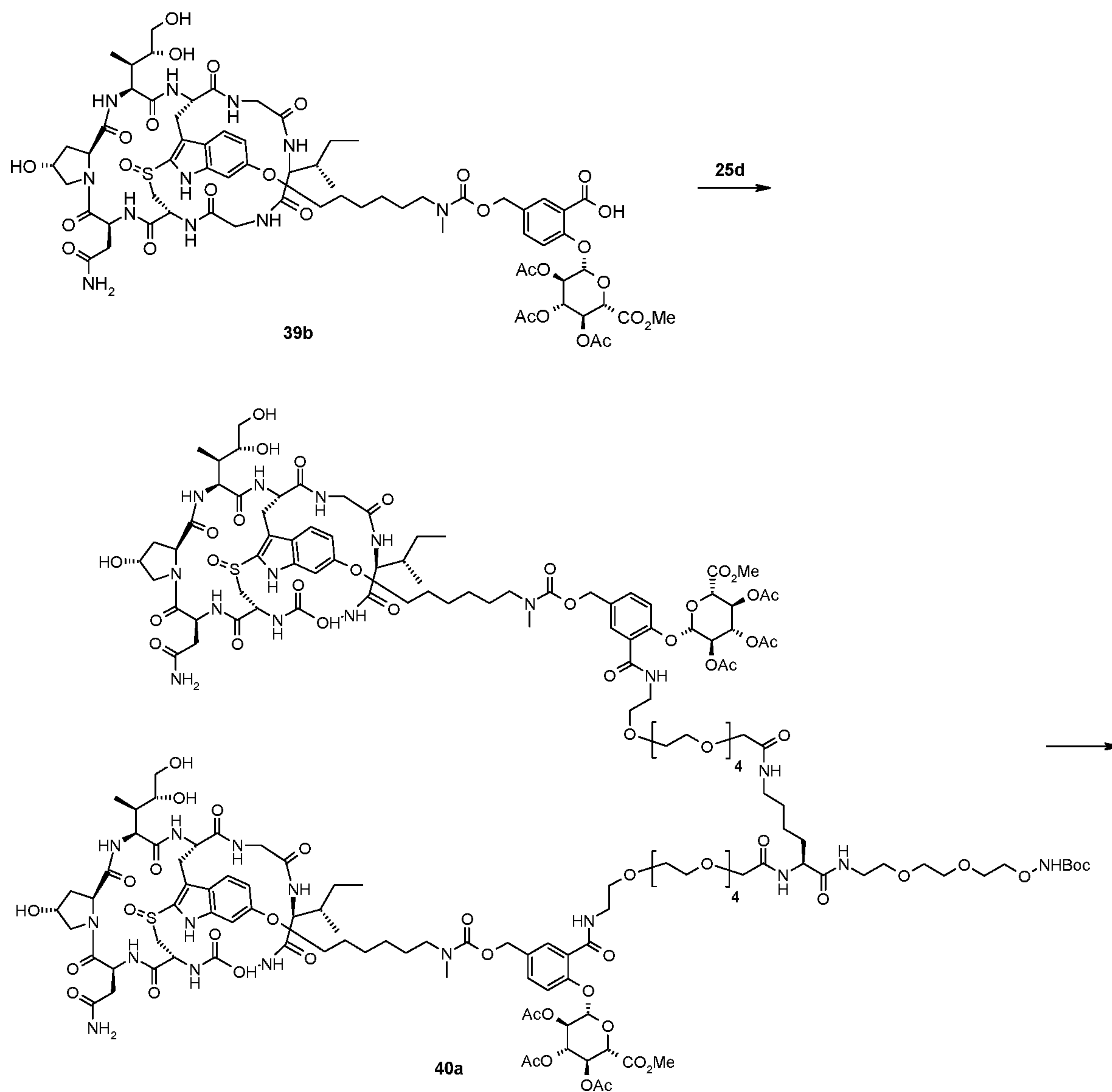
To a solution of compound **39c** (12 mg, 0.0033 mmol) in MeOH (1 mL) was added LiOH monohydrate (1.4 mg, 0.033 mmol) in H<sub>2</sub>O (1 mL) at 0 °C. After 2 hours at 0 °C, the pH of the solution was adjusted with acetic acid to 4~5, and the reaction mixture was concentrated under reduced pressure. The resulting residue was dissolved in DMSO (1.5 mL) and purified by HPLC, which produced the compound **39d** (11 mg, 98 %). EI-MS m/z: 1/2[M+H]<sup>+</sup> 1648.6.

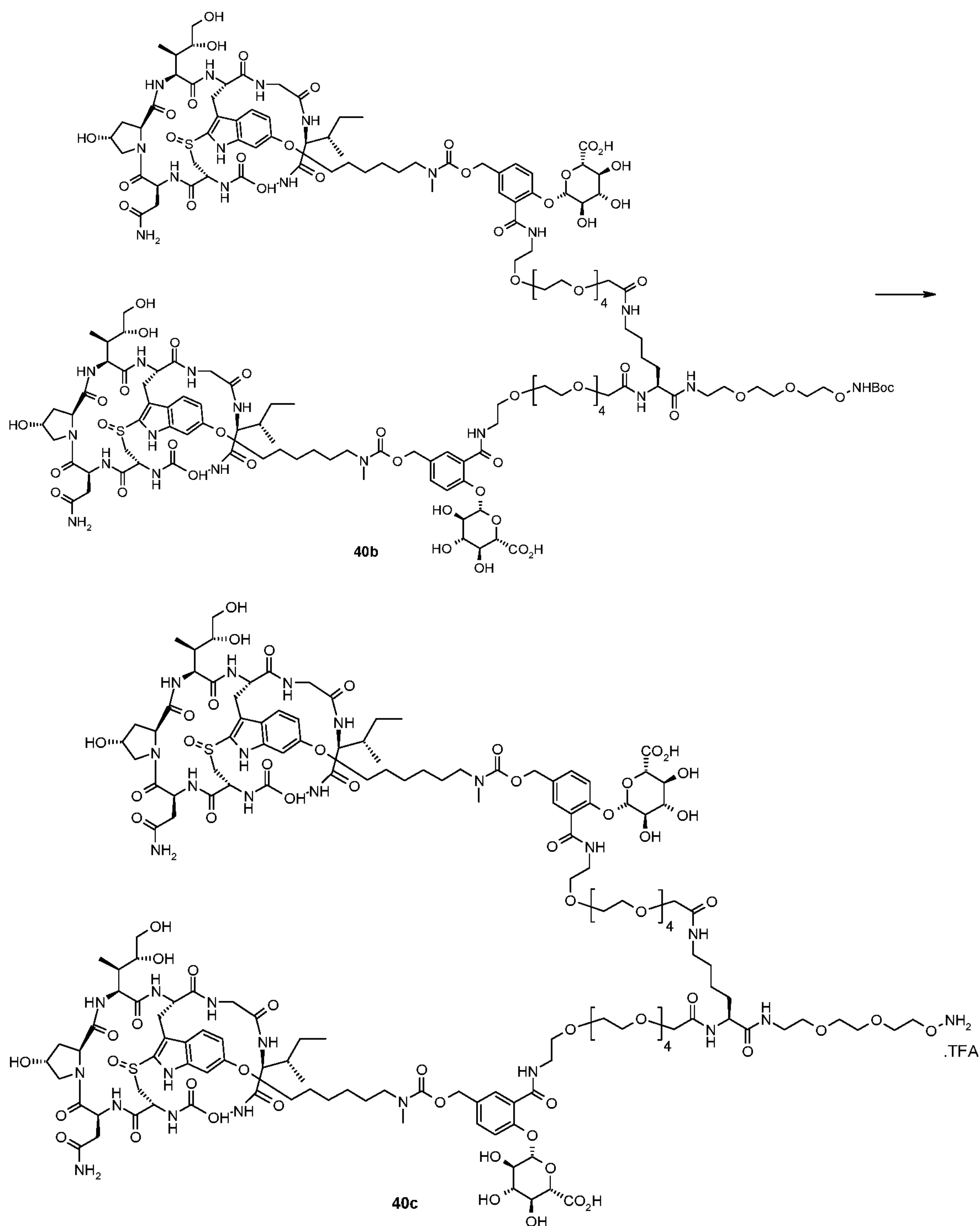
#### Preparation of Compound **39e**

TFA (0.5 mL) was added to a stirred solution of compound **39d** (11 mg, 0.003 mmol) in DCM (3.0 mL) at 0 °C. After 2 hours at 0 °C, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in DMSO (1 mL) and purified by

HPLC, which produced the compound **39e** (1.2 mg, 11 %) as white solid. EI-MS  $m/z$ :  $1/2[M+H]^+$  1598.3.

*Example 59. Preparation of Compound 40c*





### Preparation of Compound **40a**

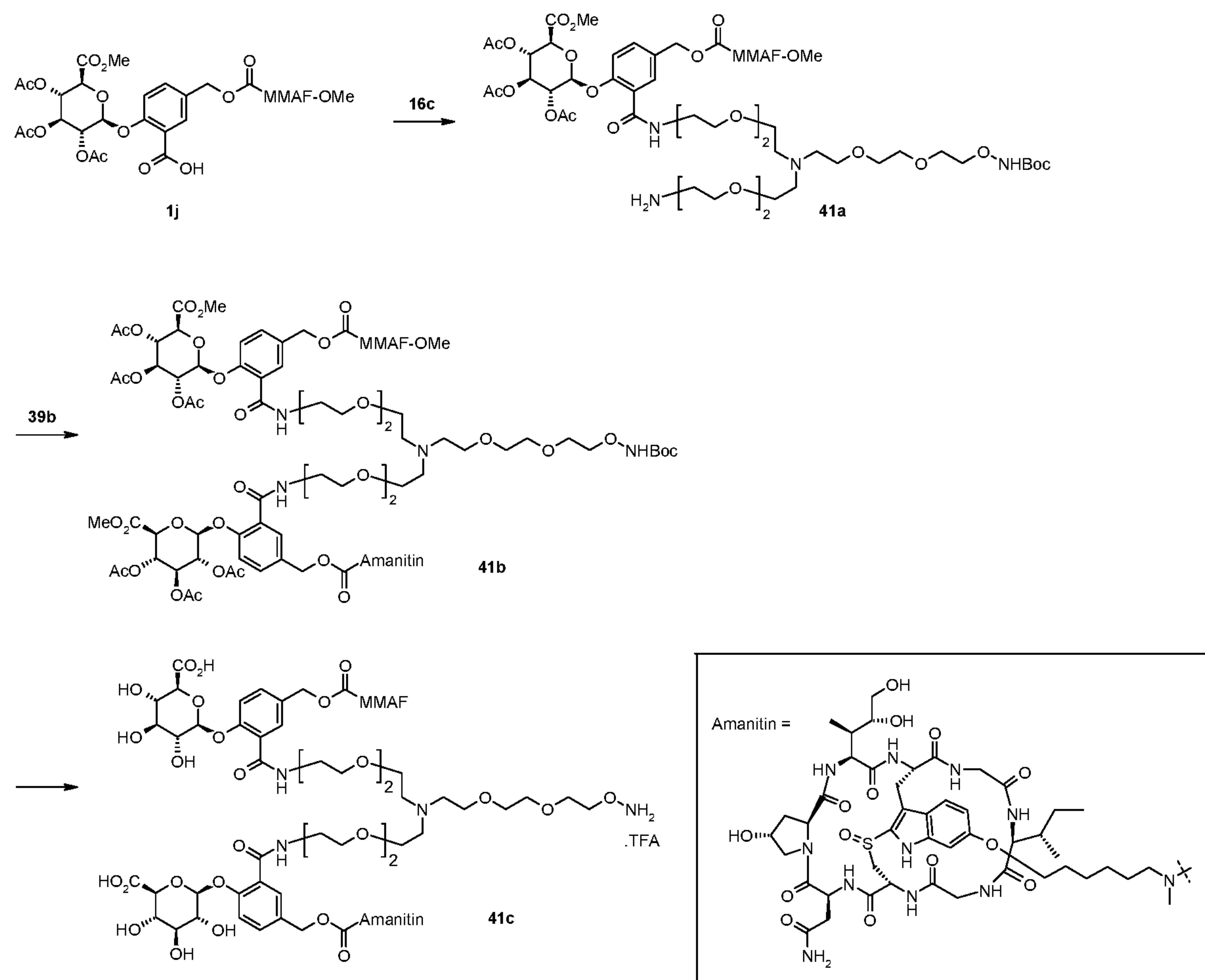
DIPEA (0.004 mL, 0.021 mmol) and HBTU (5.0 mg, 0.013 mmol) were added to a stirred mixture of compound **39b** (20 mg, 0.012 mmol) and compound **25d** (5.0 mg, 0.005 mmol) in DMF (1.5 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was dissolved in DMSO (1.0 mL) and purified by HPLC, which produced the compound **40a** (14.5 mg, 30 %). EI-MS m/z: 1/2[M+H]<sup>+</sup> 1998.8.

Preparation of Compound 40b

To a solution of compound **40a** (10 mg, 0.0025 mmol) in MeOH (1 mL) was added LiOH monohydrate (1.0 mg, 0.025 mmol) in H<sub>2</sub>O (1 mL) at 0 °C. After 2 hours at 0 °C, the reaction mixture was neutralized using acetic acid and concentrated under reduced pressure. Then the residue was dissolved in DMSO (1.5 mL) and purified by HPLC, which produced the compound **40b** (6.9 mg, 74 %). EI-MS *m/z*: 1/2[M+H]<sup>+</sup> 1858.3.

Preparation of Compound 40c

TFA (0.2 mL) was added to a stirred solution of compound **40b** (6.9 mg, 0.0018 mmol) in DCM (2.0 mL). After stirring at 0 °C for 2 hours, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in DMSO (1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **40c** (1.5 mg, 23 %) as white solid. EI-MS *m/z*: 1/2[M+H]<sup>+</sup> 1808.6.

*Example 60. Preparation of Compound 41c*

*Preparation of Compound 41a*

DIPEA (0.116 mL, 0.66 mmol) and PyBOP (127 mg, 0.24 mmol) were added to a stirred mixture of compound **16c** (280 mg, 0.22 mmol) and compound **1j** (587 mg, 1.10 mmol) in DMF (10 mL). After stirring at room temperature for 2 hours under N<sub>2</sub>, the reaction mixture was diluted with H<sub>2</sub>O (200 mL) and extracted with EtOAc (2 x 100 mL). The combined organic layers were dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The crude product was purified by column chromatography to produce the compound **41a** (250 mg, 64 %). EI-MS m/z: 1/2[M+H]<sup>+</sup> 883.2, [M+H]<sup>+</sup> 1766.

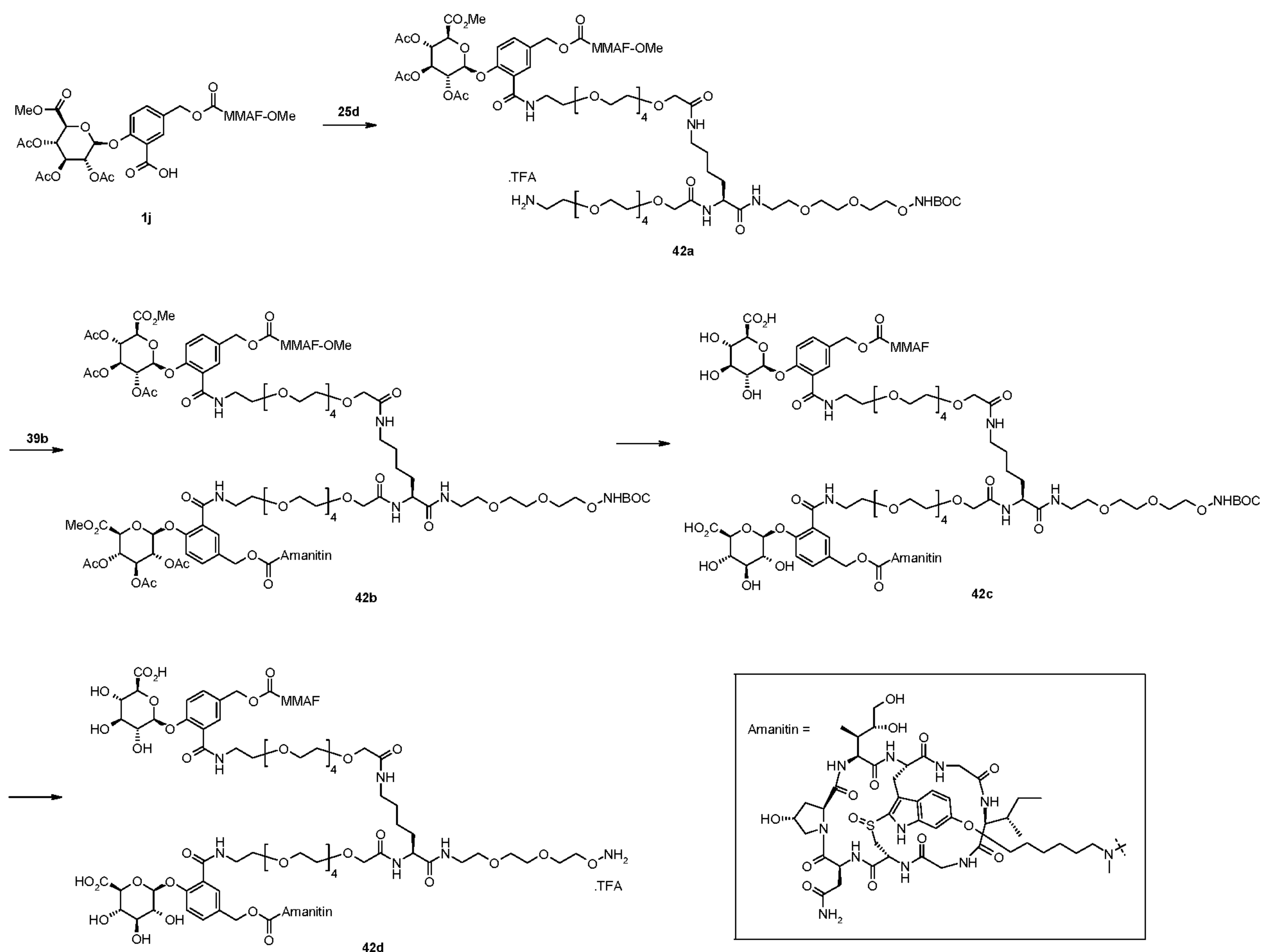
*Preparation of Compound 41b*

DIPEA (0.0017 mL, 0.0096 mmol) and PyBOP (2.0 mg, 0.0038 mmol) were added to a stirred mixture of compound **41a** (5.7 mg, 0.0032 mmol) and compound **39b** (5.0 mg, 0.0032 mmol) in DMF (0.5 mL). After stirring at room temperature for 3 hours under N<sub>2</sub>, the reaction mixture was dissolved in MeCN (1 mL) and purified by HPLC, which produced the compound **41b** (8.0 mg, 75 %). EI-MS m/z: 1/2[M+H]<sup>+</sup> 1645.

*Preparation of Compound 41c*

To a solution of compound **41b** (8.0 mg, 0.0024 mmol) in MeOH (0.5 mL) was added LiOH monohydrate (1.2 mg, 0.028 mmol) in H<sub>2</sub>O (0.1 mL) at 0 °C. After 2 hours at 0 °C, the reaction mixture was neutralized using 2 N aq. HCl solution and concentrated under reduced pressure. The resulting residue was diluted with DCM (2 mL) and H<sub>2</sub>O (3 drops). Then TFA (0.1 mL) was added at 0 °C. After 2 hours at 0 °C, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in DMSO (1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **41c** (3.1 mg, 44 %) as white solid. EI-MS m/z: 1/2[M+H]<sup>+</sup> 1448, 1/2[M+Na]<sup>+</sup> 1459.

*Example 61. Preparation of Compound 42d*



### Preparation of Compound 42a

DIPEA (0.026 mL, 0.23 mmol) and HBTU (22 mg, 0.06 mmol) were added to a stirred mixture of compound **1j** (60 mg, 0.048 mmol) and compound **25d** (214 mg, 0.19 mmol) in DMF (3 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was dissolved in DMSO (1.0 mL) and purified by HPLC, which produced the compound **42a** (64 mg, 58 %). EI-MS m/z: [M+H]<sup>+</sup> 2286.8.

### Preparation of Compound 42b

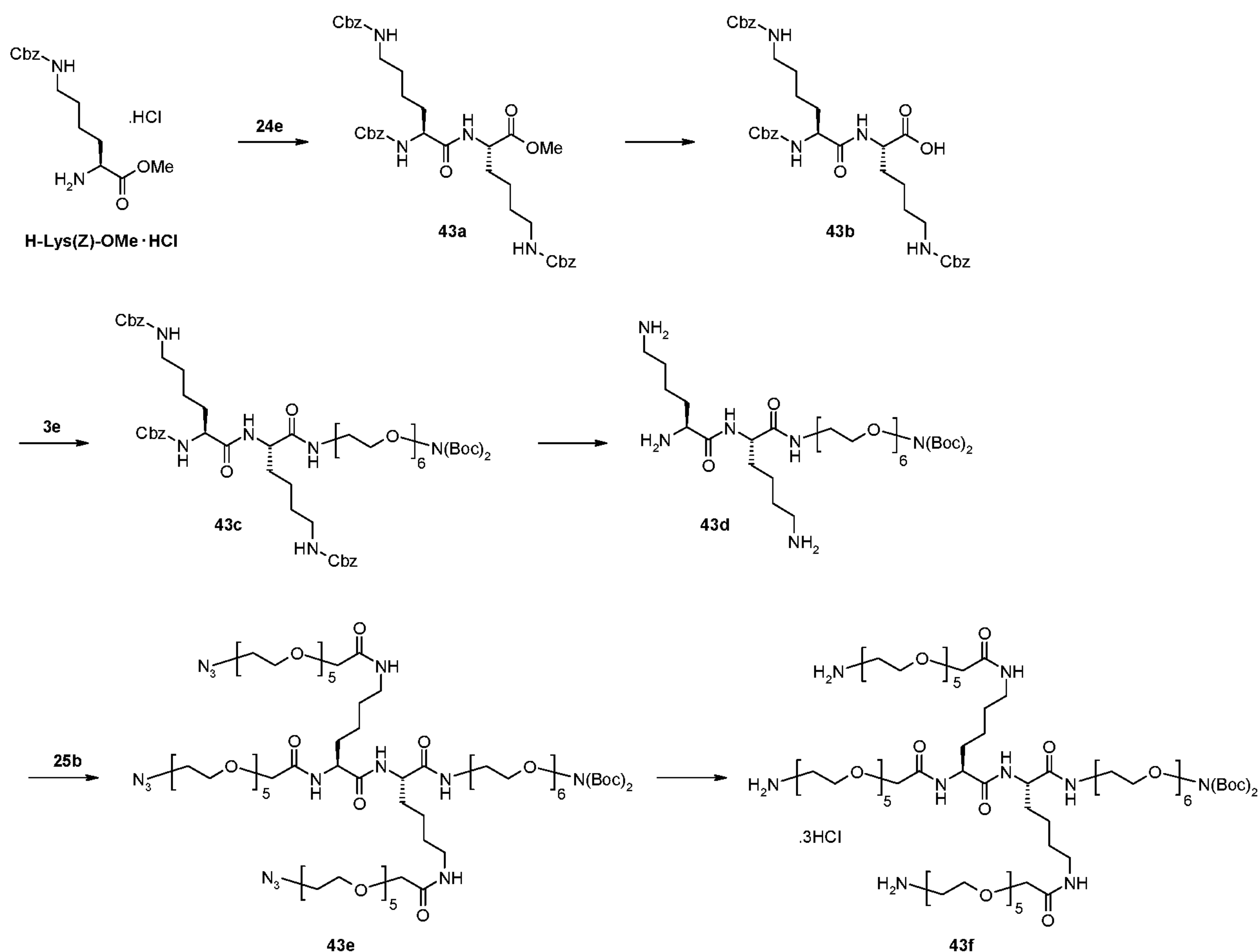
DIPEA (0.011 mL, 0.06 mmol) and HBTU (14 mg, 0.036 mmol) were added to a stirred mixture of compound **42a** (68 mg, 0.03 mmol) and compound **39b** (46 mg, 0.03 mmol) in DMF (3 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was dissolved in DMSO (1.0 mL) and purified by HPLC, which produced the compound **42b** (60 mg, 52 %). EI-MS m/z: 1/2[M+H]<sup>+</sup> 1906.3.

Preparation of Compound 42c

To a solution of compound **42b** (60 mg, 0.016 mmol) in MeOH (2 mL) was added LiOH monohydrate (5 mg, 0.126 mmol) in H<sub>2</sub>O (2 mL) at 0 °C. After 2 hours at 0 °C, the reaction mixture was neutralized using acetic acid and concentrated under reduced pressure. Then the residue was dissolved in DMSO (1 mL) and purified by prep. HPLC, which produced the compound **42c** (37 mg, 65 %). EI-MS  $m/z$ :  $1/2[M+H]^+$  1759.3.

Preparation of Compound 42d

TFA (0.3 mL) was added to a stirred solution of compound **42c** (37 mg, 0.01 mmol) in DCM (3 mL). After stirring at 0 °C for 2 hours, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in DMSO (1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **42d** (15 mg, 45 %) as white solid. EI-MS  $m/z$ :  $1/2[M+H]^+$  1659.6.

*Example 62. Preparation of Compound 43i*

### Preparation of Compound 43a

DIPEA (10.4 mL, 23.8 mmol) and HBTU (13.5 g, 35.7 mmol) were added to a stirred mixture of H-Lys(z)-OMe hydrochloride (7.0 g, 23.8 mmol) and compound **24e** (9.86 mg, 23.8 mmol) in DMF (50 mL). After stirring at room temperature for 8 hours under N<sub>2</sub>, the reaction mixture was diluted with water (100 mL) and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with 0.5 N aq. HCl (50 mL), saturated aq. NaHCO<sub>3</sub> (50 mL) and brine (50 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting crude product was purified by column chromatography to produce the compound **43a** (9.3 g, 57 %). <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 8.22 (d, 1H), 7.37-7.29 (m, 15H), 7.22 (m, 2H), 5.00 (s, 6H), 4.18 (m, 1H), 4.00 (m, 1H), 3.59 (s, 3H), 2.96 (m, 4H), 1.67-1.50 (m, 4H), 1.38-1.29 (m, 4H), 1.19-1.18 (m, 4H). EI-MS m/z: [M+Na]<sup>+</sup> 712.96.

### Preparation of Compound 43b

To a solution of compound **43a** (9.3 g, 13.5 mmol) in THF:MeOH:H<sub>2</sub>O (60 mL:30 mL:30 mL) was added LiOH monohydrate (1.13 g, 26.9 mmol) at 0 °C under N<sub>2</sub>. After 2 hours, the reaction mixture was acidified with 1 N aq. HCl until pH 4, and extracted with EtOAc (3 x 100 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Filtration and concentration under reduced pressure provided compound **43b** (9.1 g, crude), which was used without further purification. EI-MS m/z: [M+H]<sup>+</sup> 677.48, 2[M+H]<sup>+</sup> 1353.82.

### Preparation of Compound 43c

DIPEA (1.47 mL, 8.44 mmol), HOBt (484 mg, 3.58 mmol) and EDC·HCl (809 mg, 4.22 mmol) were added to a stirred mixture of compound **43b** (2.5 g, 3.71 mmol) and compound **3e** (1.8 g, 3.38 mmol) in DMF (20 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into water (50 mL) and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with 0.5 N aq. HCl (50 mL), saturated aq. NaHCO<sub>3</sub> (50 mL) and brine (50 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting residue was purified by column chromatography to produce the compound **43c** (2.3 g, 59 %). EI-MS m/z: [M+H]<sup>+</sup> 1155.92, [M+H-Boc]<sup>+</sup> 1055.83.

### Preparation of Compound 43d

To a stirred mixture of compound **43c** (2.3 g, 1.99 mmol) and Pd/C (10 wt. %, 424 mg 3.98 mmol) in MeOH (200 mL) was added HCl (4 N in 1,4-dioxane, 0.99 mL, 3.98 mmol). After stirring at room temperature for 2 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (100 mL). The filtrate was concentrated to produce the compound **43d** (1.5 g, crude), which was used without further purification. EI-MS m/z:  $[M+H]^+$  753.29.

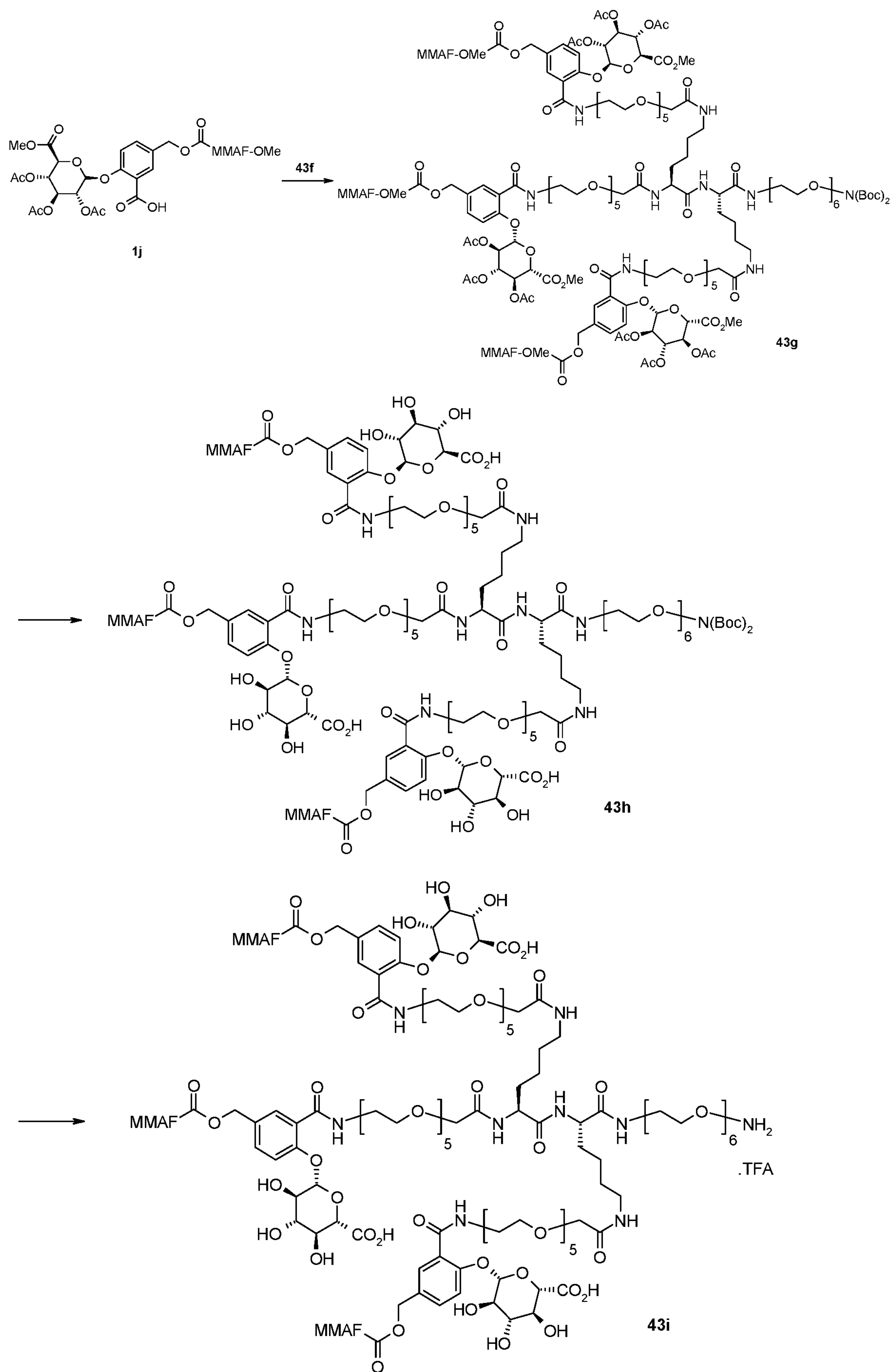
### Preparation of Compound 43e

DIPEA (0.14 mL, 0.80 mmol), HOBt (59 mg, 0.43 mmol) and EDC·HCl (102 mg, 0.53 mmol) were added to a stirred mixture of compound **43d** (2.5 g, 3.71 mmol) and compound **25b** (150 g, 0.46 mmol) in DMF (5 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into water (50 mL) and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with 1 N aq. HCl (30 mL), saturated aq. NaHCO<sub>3</sub> (30 mL) and brine (30 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting crude product was purified by column chromatography to produce the compound **43e** (100 mg, 45 %) as colorless oil. EI-MS m/z:  $[M+Na]^+$  1685.11,  $1/2[M+H-Boc]^+$  731.82.

### Preparation of Compound 43f

To a stirred mixture of compound **43e** (100 mg, 0.06 mmol) and Pd/C (10 wt. %, 20 mg 0.192 mmol) in MeOH (20 mL) was added HCl (4 N in 1,4-dioxane, 0.045 mL, 0.18 mmol). After stirring at room temperature for 2 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (30 mL). The filtrate was concentrated to produce the compound **43f** (95 mg) as brown foam, which was used without further purification. EI-MS m/z:  $[M+H]^+$  1586.30,  $1/2[M+H]^+$  793.02.

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### Preparation of Compound 43g

DIPEA (0.030 mL, 0.170 mmol) and HBTU (36 mg, 0.094 mmol) were added to a stirred mixture of compound **43f** (45 mg, 0.028 mmol) and compound **1j** (114 mg, 0.091 mmol) in DMF (3 mL). After stirring at room temperature for 16 hours under N<sub>2</sub>, the reaction mixture was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC. Pure fractions with the same retention time were combined and concentrated to produce the compound **43g** (31 mg, 21 %). EI-MS m/z: 1/3[M+H-Boc]<sup>+</sup> 1705.74, 1/4[M+H-2Boc]<sup>+</sup> 1254.79.

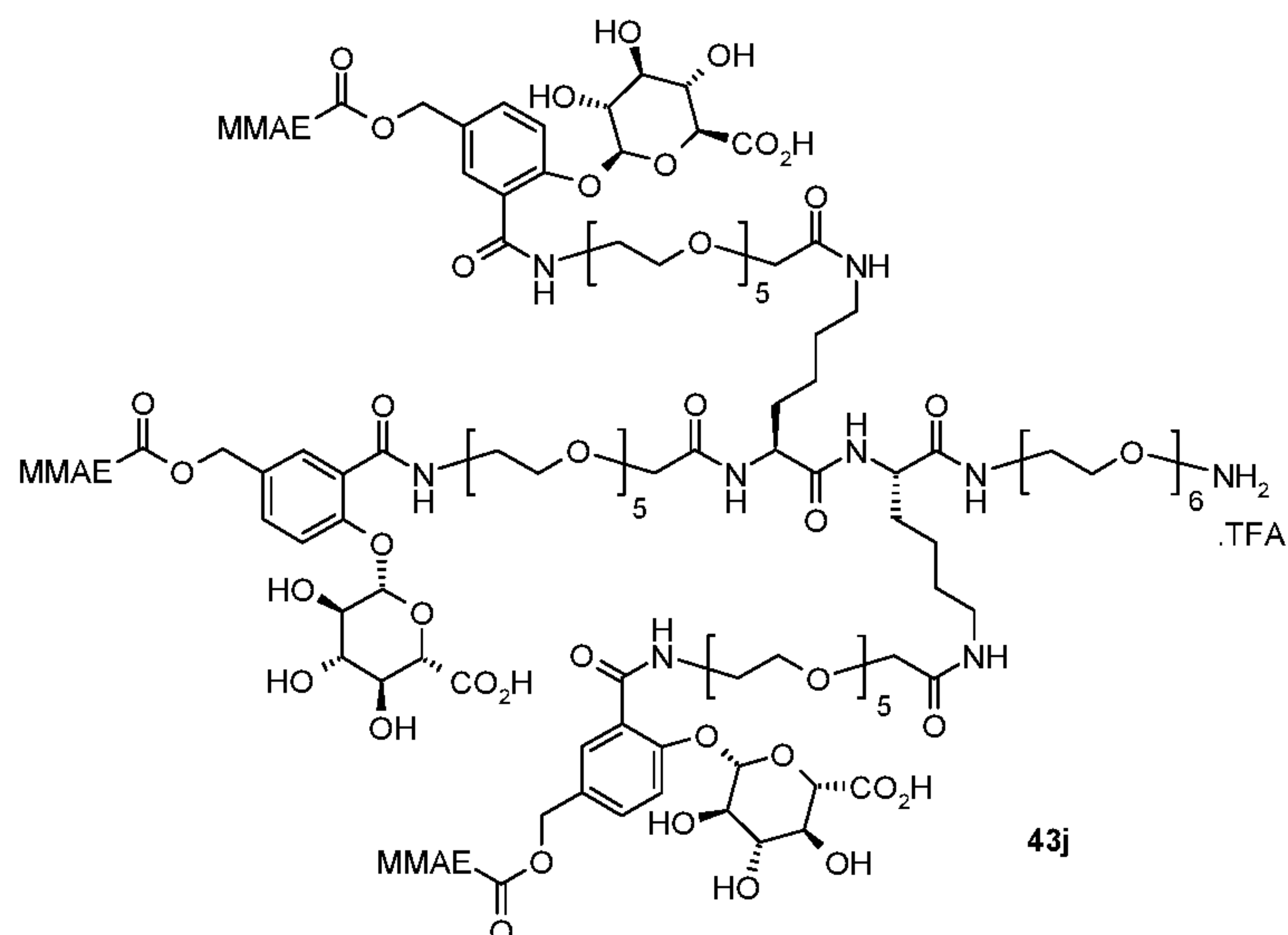
### Preparation of Compound 43h

To a solution of compound **43g** (31 mg, 0.006 mmol) in MeOH (1 mL) was added LiOH monohydrate (3.7 mg, 0.088 mmol) in H<sub>2</sub>O (1 mL) at -20 °C. After stirring for 2 hours at -20 °C, the reaction mixture was neutralized using acetic acid and concentrated under reduced pressure. Then the reaction mixture was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC, which produced the compound **43h** (18 mg, 64 %) as white solid. EI-MS m/z: 1/3[M+H-Boc]<sup>+</sup> 1735.19, 1/4[M+H]<sup>+</sup> 1301.95, 1/5[M+H-Boc]<sup>+</sup> 1021.71.

### Preparation of Compound 43i

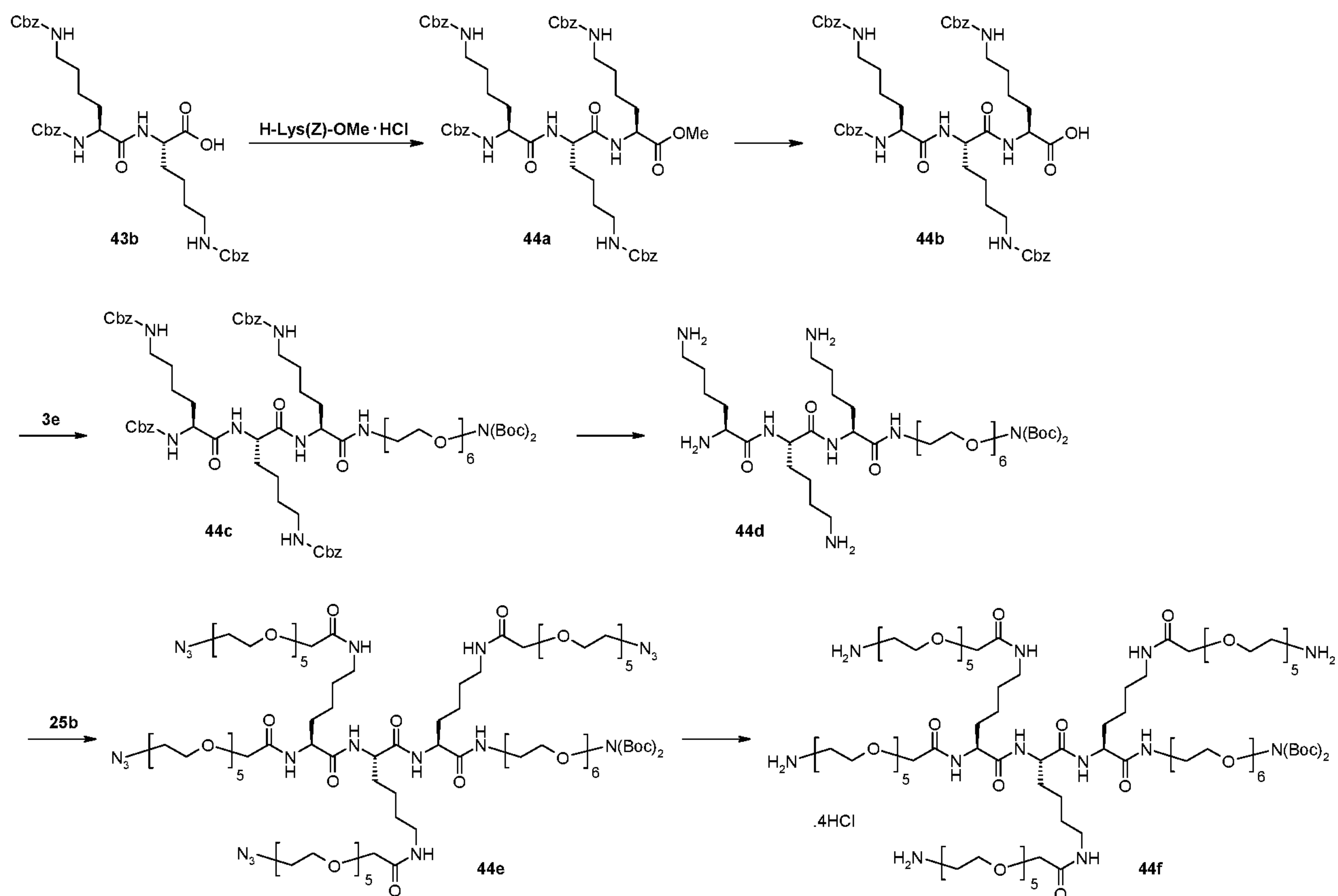
TFA (0.3 mL) was added to a stirred solution of compound **43h** (18 mg, 0.004 mmol) in DCM (1.0 mL) at 0 °C. After stirring for 1 hour, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in H<sub>2</sub>O/MeCN (1 mL/1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **43i** (6 mg, 33 %) as white solid. EI-MS m/z: 1/3[M+H]<sup>+</sup> 1547.75, 1/4[M+H]<sup>+</sup> 1161.14.

### *Example 63. Preparation of Compound 43j*



Compound **43j** was prepared from compound **1i** and compound **43f** by a similar method of preparing compound **43i** in Example 62. EI-MS  $m/z$ :  $1/3[M+H]^+$  1532.37,  $1/4[M+H]^+$  1149.69.

*Example 64. Preparation of Compound 44i*



#### Preparation of Compound 44a

DIPEA (1.9 mL, 11.0 mmol) and HBTU (2.5 g, 6.64 mmol) were added to a stirred mixture of compound H-Lys(z)-OMe hydrochloride (1.3 g, 4.43 mmol) and compound **43b** (3.0 g, 4.43 mmol) in DMF (30 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was diluted with water (100 mL) and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with 0.5 N aq. HCl (50 mL), saturated aq. NaHCO<sub>3</sub> (50 mL) and brine (50 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting residue was purified by column chromatography to produce the compound **44a** (3.9 g, 93 %). EI-MS m/z: [M+H]<sup>+</sup> 953.42.

#### Preparation of Compound 44b

To a solution of compound **44a** (2.1 g, 2.20 mmol) in THF:MeOH:H<sub>2</sub>O (24 mL:8 mL:8 mL) was added LiOH monohydrate (185 mg, 4.40 mmol) at room temperature under N<sub>2</sub>. After 2 hours, the reaction mixture was acidified with 1 N aq. HCl until pH 4, and extracted with EtOAc (3 x 50 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Filtration and concentration under reduced pressure provided compound **44b** (2.0 g), which was used without further purification. EI-MS m/z: [M+H]<sup>+</sup> 939.35, [M+Na]<sup>+</sup> 961.37.

#### Preparation of Compound 44c

DIPEA (0.93 mL, 5.33 mmol) and HBTU (1.21 g, 3.20 mmol) were added to a stirred mixture of compound **44b** (2.0 g, 2.13 mmol) and compound **3e** (1.14 g, 2.13 mmol) in DMF (20 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was poured into water (50 mL) and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with 0.5 N aq. HCl (50 mL), saturated aq. NaHCO<sub>3</sub> (50 mL) and brine (50 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting residue was purified by column chromatography to produce the compound **44c** (2.60 g, 86 %). EI-MS m/z: [M+H]<sup>+</sup> 1418.44, [M+Na]<sup>+</sup> 1440.39, [M+H-Boc]<sup>+</sup> 1318.47.

#### Preparation of Compound 44d

To a stirred mixture of compound **44c** (2.60 g, 1.83 mmol) and Pd/C (10 wt. %, 781 mg 7.34 mmol) in MeOH (50 mL) was added HCl (4 N in 1,4-dioxane, 0.9 mL, 3.67

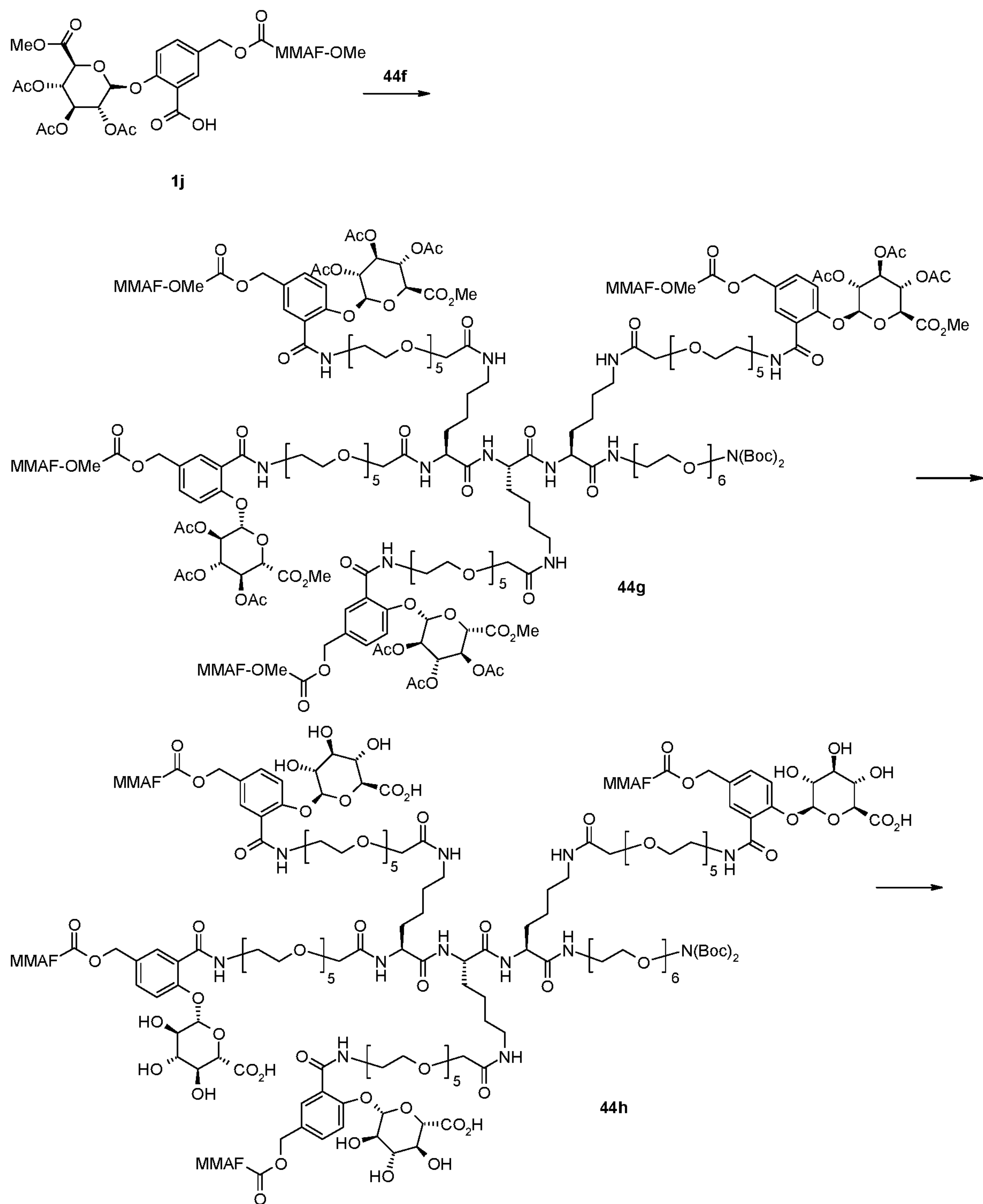
mmol). And then the reaction was stirred at room temperature for 2 hours under hydrogen. The reaction mixture was filtered through a celite pad and washed with MeOH (50 mL). The filtrate was concentrated to produce the compound **44d** (1.73 g) as yellow form, which was used without further purification. EI-MS m/z:  $[M+H]^+$  881.90.

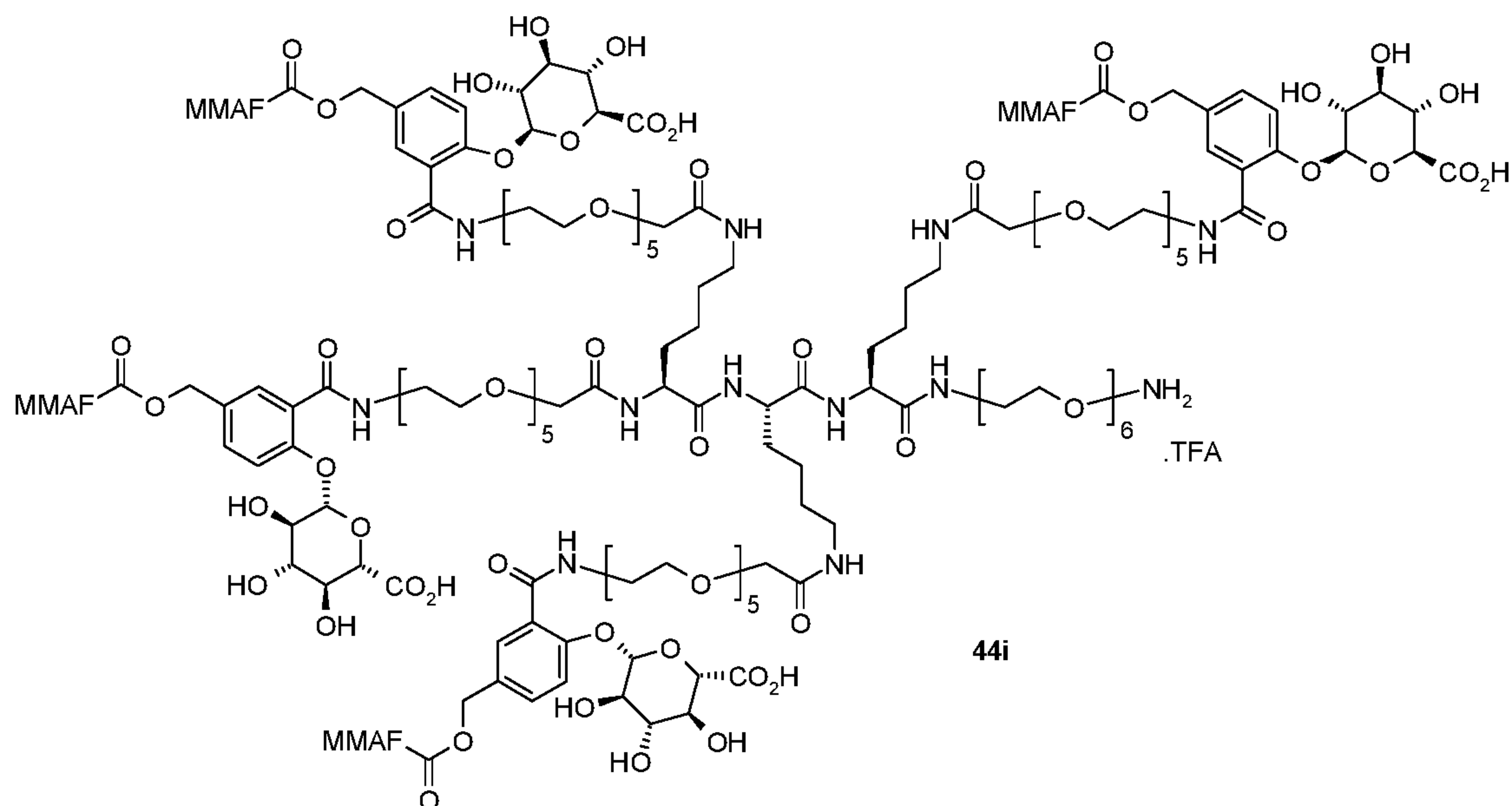
#### Preparation of Compound 44e

DIPEA (1.58 mL, 9.08 mmol) and HBTU (2.58 g, 6.81 mmol) were added to a stirred mixture of compound **44d** (1.0 g, 1.13 mmol) and compound **25b** (1.82 g, 5.67 mmol) in DMF (20 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was diluted with water (100 mL) and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with 0.5 N HCl (50 mL), saturated aq. NaHCO<sub>3</sub> (50 mL) and brine (50 mL) sequentially, and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration under reduced pressure, the resulting residue was purified by column chromatography to produce the compound **44e** (848 mg, 36 %). EI-MS m/z:  $1/2[M+H-2Boc]^+$  947.63.

#### Preparation of Compound 44f

To a stirred mixture of compound **44e** (848 mg, 0.40 mmol) and Pd/C (10 wt. %, 172 mg, 1.62 mmol) in MeOH (50 mL) was added HCl (4N in 1,4-dioxane, 0.4 mL, 1.62 mmol). After stirring at room temperature for 2 hours under hydrogen, the reaction mixture was filtered through a celite pad and washed with MeOH (50 mL). The filtrate was concentrated to produce the compound **44f** (625 mg, crude), which was used without further purification. EI-MS m/z:  $1/2[M+H]^+$  996.40,  $1/3[M+H]^+$  664.59





### Preparation of Compound 44g

DIPEA (0.067 mL, 0.386 mmol) and HBTU (110 mg, 0.289 mmol) were added to a stirred mixture of compound **44f** (96 mg, 0.048 mmol) and compound **1j** (303 mg, 0.24 mmol) in DMF (3 mL). After stirring at room temperature for 16 hours under N<sub>2</sub>, the reaction mixture was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC. Pure fractions with the same retention time were combined and concentrated to produce the compound **44g** (67 mg, 20 %). EI-MS m/z: 1/3[M+H]<sup>+</sup> 2315.93, 1/4[M+H]<sup>+</sup> 1737.60, 1/5[M+H]<sup>+</sup> 1390.37.

### Preparation of Compound 44h

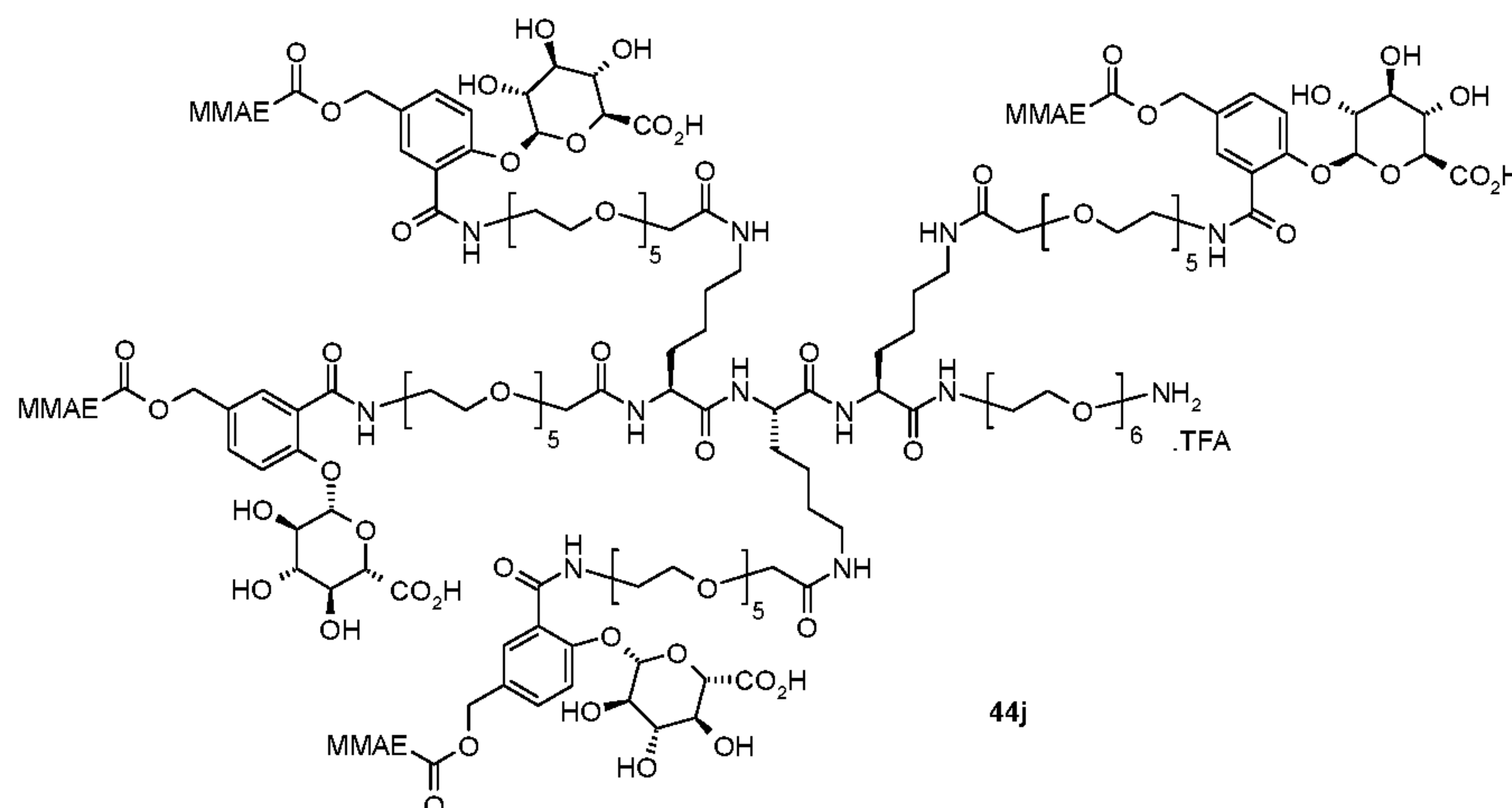
To a solution of the compound **44g** (67 mg, 0.009 mmol) in MeOH (1 mL) was added LiOH monohydrate (8.1 mg, 0.192 mmol) in H<sub>2</sub>O (1 mL) at -20 °C. After stirring for 2 hours at -20 °C, the reaction mixture was neutralized using acetic acid and concentrated under reduced pressure. Then the reaction mixture was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC, which produced the compound **44h** (27.9 mg, 45 %) as white solid. EI-MS m/z: 1/3[M+H]<sup>+</sup> 2110.24, 1/4[M+H]<sup>+</sup> 1582.97, 1/4[M+H-Boc]<sup>+</sup> 1557.91.

### Preparation of Compound 44i

TFA (0.3 mL) was added to a stirred solution of compound **44h** (27.9 mg, 0.004 mmol) in DCM (1.0 mL) at 0 °C. After stirring for 1 hour, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in H<sub>2</sub>O/MeCN (1 mL/1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and

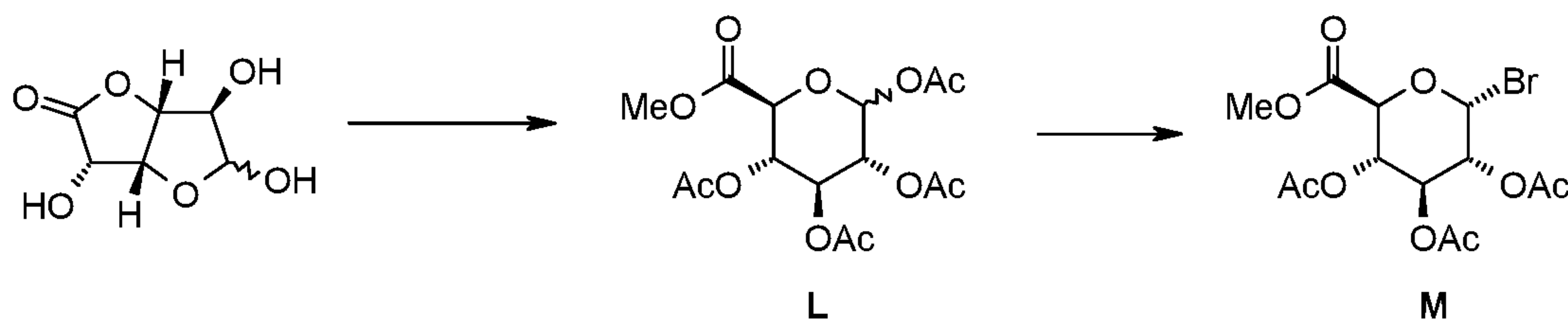
lyophilized to produce the compound **44i** (13.6 mg, 50 %) as white solid. EI-MS  $m/z$ :  $1/3[M+H]^+$  2043.49,  $1/4[M+H]^+$  1532.96,  $1/5[M+H]^+$  1226.62.

*Example 65. Preparation of Compound 44j*



Compound **44j** was prepared from compound **1i** and compound **44f** by a similar method of preparing compound **44i** in Example 64. EI-MS  $m/z$ :  $1/3[M+H]^+$  2025.37,  $1/4[M+H]^+$  1519.10,  $1/5[M+H]^+$  1215.60.

*Example 66. Preparation of Compound 45k*

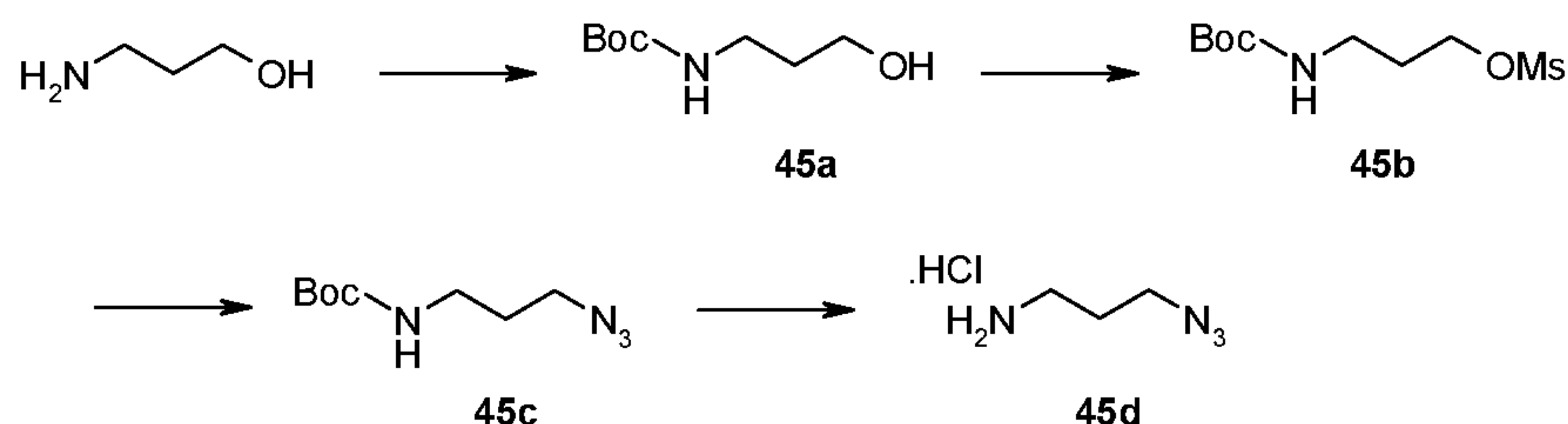


Preparation of Compound L

D-Glucurono-6,3-lactone (25.0 g, 141.9 mmol) was dissolved in MeOH (250 mL) at room temperature under nitrogen, and a solution of NaOH (141 mg) in MeOH (100 mL) was slowly added thereto. After stirring for 24 hours, the reaction mixture was concentrated under reduced pressure, and then pyridine (66 mL) and acetic anhydride (71 mL) were added below 10 °C. After stirring at room temperature for 4 hours, the reaction mixture was concentrated under reduced pressure and was subjected to column chromatography, which produced the compound **L** (41.6 g, 77 %).  $^1\text{H-NMR}$  (600 MHz,  $\text{CDCl}_3$ )  $\delta$  5.77 (d,  $J = 7.8$  Hz, 1H), 5.31 (t,  $J = 9.6$  Hz, 1H), 5.24 (t,  $J = 9.6$  Hz, 1H), 5.14 (m, 1H), 4.17 (d,  $J = 9$  Hz, 1H), 3.74 (s, 3H), 2.12 (s, 3H), 2.04 (m, 9H).

Preparation of Compound M

Compound **L** (10.0 g, 26.6 mmol) was dissolved in HBr (33% in AcOH, 20 mL) at 0 °C under nitrogen. The reaction mixture was warmed to room temperature. After stirring for 2 hours, toluene (50 mL) was added thereto, and the mixture was concentrated under reduced pressure. The resulting residue was purified by column chromatography to produce the compound **M** (10.9 g, 99 %). <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) δ 6.64 (d, *J* = 3.6 Hz, 1H), 5.61 (t, *J* = 3.6 Hz, 1H), 5.24 (t, *J* = 3.6 Hz, 1H), 4.85 (m, 1H), 4.58 (d, d, *J* = 10.2 Hz, 1H), 3.76 (s, 3H), 2.10 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H).

Preparation of Compound 45a

3-Amino-1-propanol (3.0 g, 66.57 mmol) was dissolved in DCM (150 mL) at 0 °C under nitrogen, and di-*tert*-butyl dicarbonate (16 g, 73.23 mmol) was added thereto. The obtained mixture was stirred at room temperature for 12 hours. After the reaction was completed, the solvent was concentrated under reduced pressure. The residue was subjected to column chromatography, which produced the compound **45a** (6.4 g, 92 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.78 (s, 1H), 3.65 (m, 2H), 3.30 (m, 2H), 2.90 (s, 1H), 1.68 (m, 2H), 1.48 (s, 9H).

Preparation of Compound 45b

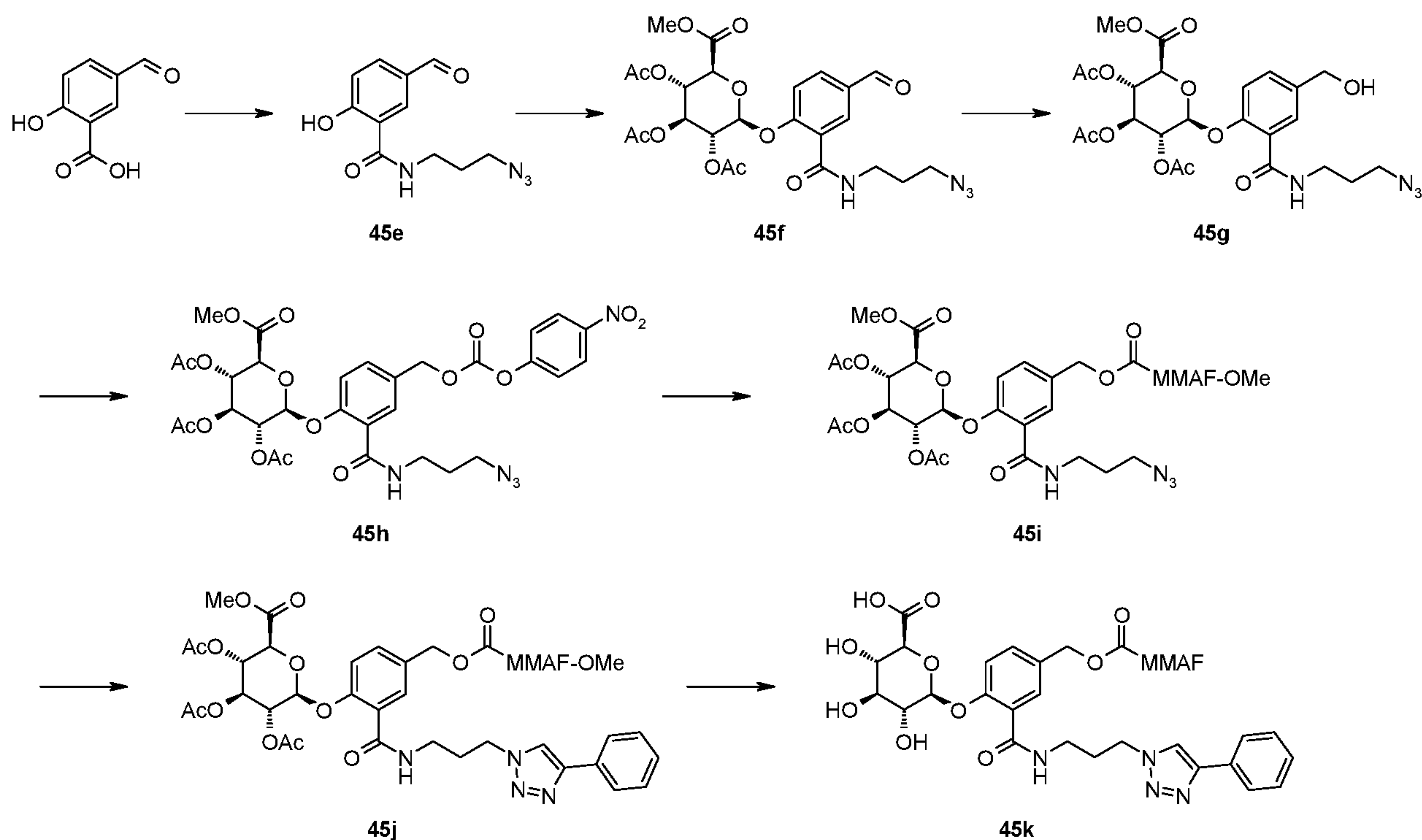
Compound **45a** (6.04 g, 34.47 mmol) and triethylamine (14.4 mL, 103.4 mmol) were dissolved in THF at 0 °C under nitrogen and then, slowly treated with methanesulfonyl anhydride (7.21 g, 41.36 mmol). The obtained mixture was stirred at room temperature under nitrogen for 12 hours. After the reaction was completed, the solvent was concentrated under reduced pressure. The residue was subjected to column chromatography, which produced the compound **45b** (9.01 g, 98 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 4.73 (s, 1H), 4.30 (t, *J* = 5.9 Hz, 2H), 3.31-3.24 (m, 2H), 3.04 (s, 3H), 1.94 (t, *J* = 6.1 Hz, 2H), 1.44 (s, 9H).

Preparation of Compound 45c

Compound **45b** (3.0 g, 11.84 mmol) was dissolved in DMF (40 mL) at room temperature under nitrogen, and then treated with NaN<sub>3</sub> (924 mg, 14.21 mmol), and the obtained mixture was stirred at 60 °C for 12 hours. After the reaction was completed, EtOAc (50 mL), distilled water (50 mL), and 1 N aq. HCl (5 mL) were added thereto. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The residue was subjected to column chromatography, which produced the compound **45c** (2.3 g, 99 %). <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) δ 4.63 (s, 1H), 3.36 (t, *J* = 6.6 Hz, 2H), 3.24-3.18 (m, 2H), 1.80-1.75 (m, 2H), 1.45 (s, 9H).

Preparation of Compound 45d

Compound **45c** (3.8 g, 18.98 mmol) was dissolved in DCM (10 mL) at 0 °C under nitrogen, and then 4 M-HCl in dioxane (10 mL) was slowly added thereto. After stirring for 12 hours, the reaction mixture was concentrated under reduced pressure, which produced the compound **45d** (2.5 g, 99 %). <sup>1</sup>H-NMR (600 MHz, DMSO-d<sub>6</sub>) δ 8.06 (s, 3H), 3.47 (t, *J* = 6.6 Hz, 2H), 2.82 (t, *J* = 7.2 Hz, 2H), 1.84-1.79 (m, 2H).

Preparation of Compound 45e

Compound **45d** (58 mg, 0.42 mmol) and 5-formylsalicylic acid (100 mg, 0.60

mmol) were dissolved in DMF (2 mL) at 0 °C under nitrogen, and then DIPEA (0.2 mL, 1.20 mmol) and PyBop (375 mg, 0.72 mmol) were added to the reaction mixture. After stirring at room temperature for 3 hours, EtOAc (30 mL) and distilled water (10 mL) were added thereto. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The residue was subjected to column chromatography, which afforded compound **45e** (82 mg, 79 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 13.39 (s, 1H), 9.87 (s, 1H), 8.29 (s, 1H), 7.89 (dd, *J* = 1.6, 7.2 Hz, 1H), 7.60 (s, 1H), 7.10 (d, *J* = 8.8 Hz, 1H), 3.63-3.57 (m, 2H), 3.48 (t, *J* = 6.4 Hz, 2H), 1.99-1.92 (m, 2H).

#### Preparation of Compound 45f

Compound **45e** (78 mg, 0.31 mmol) and compound **M** (125 mg, 0.31 mmol) were dissolved in MeCN (3 mL) at room temperature under nitrogen, and then silver oxide (291 mg, 1.26 mmol) and 4 Å molecular sieve (125 mg) were added thereto. After stirring at room temperature for 3 hours, the mixture was celite-filtered, and the filtrate was concentrated under reduced pressure. The residue was subjected to column chromatography, which produced the compound **45f** (160 mg, 90 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 10.00 (s, 1H), 8.66 (d, *J* = 2.4 Hz, 1H), 8.02 (dd, *J* = 2.0, 6.4 Hz, 1H), 7.46 (t, *J* = 6.4 Hz, 1H), 7.14 (d, *J* = 8.4 Hz, 1H), 5.48-5.33 (m, 4H), 4.28 (d, *J* = 8.8 Hz, 1H), 3.74 (s, 3H), 3.73-3.64 (m, 1H), 3.50-3.42 (m, 3H), 2.09-2.07 (m, 9H), 2.00-1.92 (m, 2H).

#### Preparation of Compound 45g

Compound **45f** (160 mg, 1.51 mmol) was dissolved in 2-propanol (0.4 mL) and chloroform (2 mL) at 0 °C under nitrogen, and then silica gel (2 g) and sodium borohydride (27 mg, 0.71 mmol) were added thereto. After stirring at 0 °C for 2 hours, the reactant was celite-filtered, and the filtrate was concentrated under reduced pressure. The residue was subjected to column chromatography, which produced the compound **45g** (115 mg, 71 %). <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) δ 8.06 (d, *J* = 2.4 Hz, 1H), 7.50-7.44 (m, 2H), 7.01 (d, *J* = 9.0 Hz, 1H), 5.45-5.31 (m, 4H), 4.38 (s, 2H), 4.22 (d, *J* = 9.0 Hz, 1H), 3.74 (s, 3H), 3.67-3.61 (m, 1H), 3.46-3.41 (m, 3H), 2.07-2.04 (m, 9H), 1.97-1.91 (m, 2H).

#### Preparation of Compound 45h

Compound **45g** (100 mg, 0.18 mmol) was dissolved in DMF (1 mL) at 0 °C under

nitrogen, and then bis(4-nitrophenyl)carbonate (110 mg, 0.35 mmol) and DIPEA (0.050 mL, 0.27 mmol) were added thereto. After stirring at room temperature for 2 hours, EtOAc (30 mL) and distilled water (10 mL) were added thereto. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The residue was subjected to column chromatography to produce the compound **45h** (75 mg, 58 %). <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) δ 8.29-8.27 (m, 2H), 8.23 (d, *J* = 2.4 Hz, 1H), 7.54 (dd, *J* = 2.4, 6.6 Hz, 1H), 7.49 (t, *J* = 6.4 Hz, 1H), 7.39-7.37 (m, 2H), 7.04 (d, *J* = 8.4 Hz, 1H), 5.45-5.29 (m, 4H), 5.28 (s, 2H), 4.23 (d, *J* = 9.0 Hz, 1H), 3.75 (s, 3H), 3.68-3.64 (m, 1H), 3.46-3.42 (m, 3H), 2.08-2.05 (m, 9H), 1.98-1.93 (m, 2H).

#### Preparation of Compound 45i

Compound **45h** (50 mg, 0.068 mmol) was dissolved in DMF (0.8 mL) at room temperature under nitrogen, and then MMAF-OMe (51 mg, 0.068 mmol) was added thereto. The resulting mixture was treated with HOBt (2 mg, 0.013 mmol), pyridine (0.24 mL), and DIPEA (0.012 mL, 0.068 mmol). After stirring at room temperature for 18 hours, EtOAc (20 mL) and distilled water (10 mL) were added thereto. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The residue was subjected to column chromatography to produce the compound **45i** (71 mg, 78 %). EI-MS *m/z*: [M+H]<sup>+</sup> 1339.

#### Preparation of Compound 45j

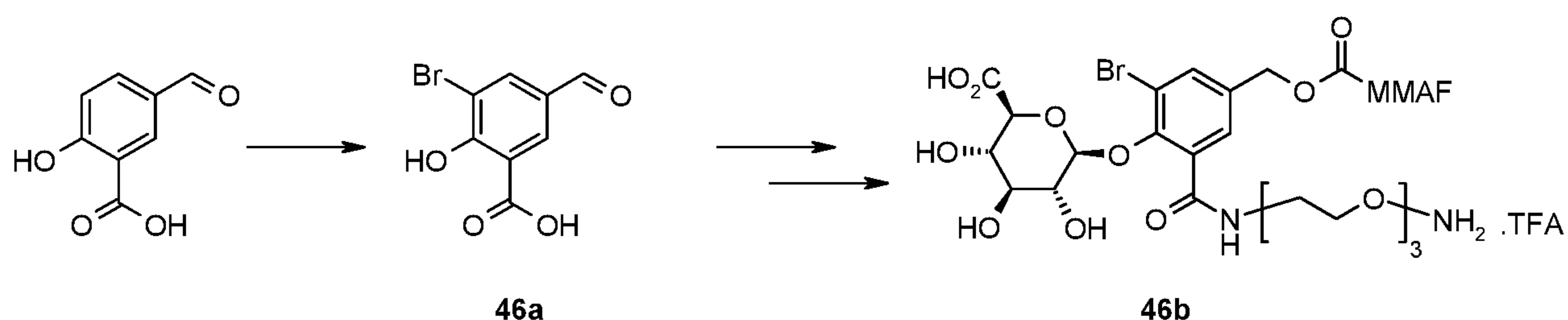
Compound **45i** (30 mg, 0.022 mmol) and phenylacetylene (3.7 μL, 0.033 mmol) were dissolved in EtOH (0.2 mL) and water (30 μL) at room temperature under nitrogen, and then 0.1 M CuSO<sub>4</sub> aq. solution (30 μL) and 1.0 M sodium ascorbate aq. solution (30 μL) were added thereto. The resulting mixture was treated with HOBt (2 mg, 0.013 mmol), pyridine (0.24 mL), and DIPEA (12 μL, 0.068 mmol). After stirring at room temperature for 5 hours, EtOAc (20 mL) and distilled water (5 mL) were added thereto. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The residue was subjected to column chromatography to produce the compound **45j** (26 mg, 81 %). EI-MS *m/z*: [M+H]<sup>+</sup> 1441.

#### Preparation of Compound 45k

Compound **45j** (20 mg, 0.013 mmol) was dissolved in MeOH (0.2 mL) at 0 °C

under nitrogen, and then LiOH·H<sub>2</sub>O (6 mg, 0.14 mmol) in water (0.2 mL) was added thereto. After stirring at room temperature for 1 hour, chloroform (10 mL), MeOH (1 mL), distilled water (10 mL), and 0.5 N aq. HCl (1 mL) were added thereto. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The residue was subjected to column chromatography to produce the compound **45k** (17 mg, 87 %). EI-MS m/z: [M+H]<sup>+</sup> 1286.

*Example 67. Preparation of Compound 46b*



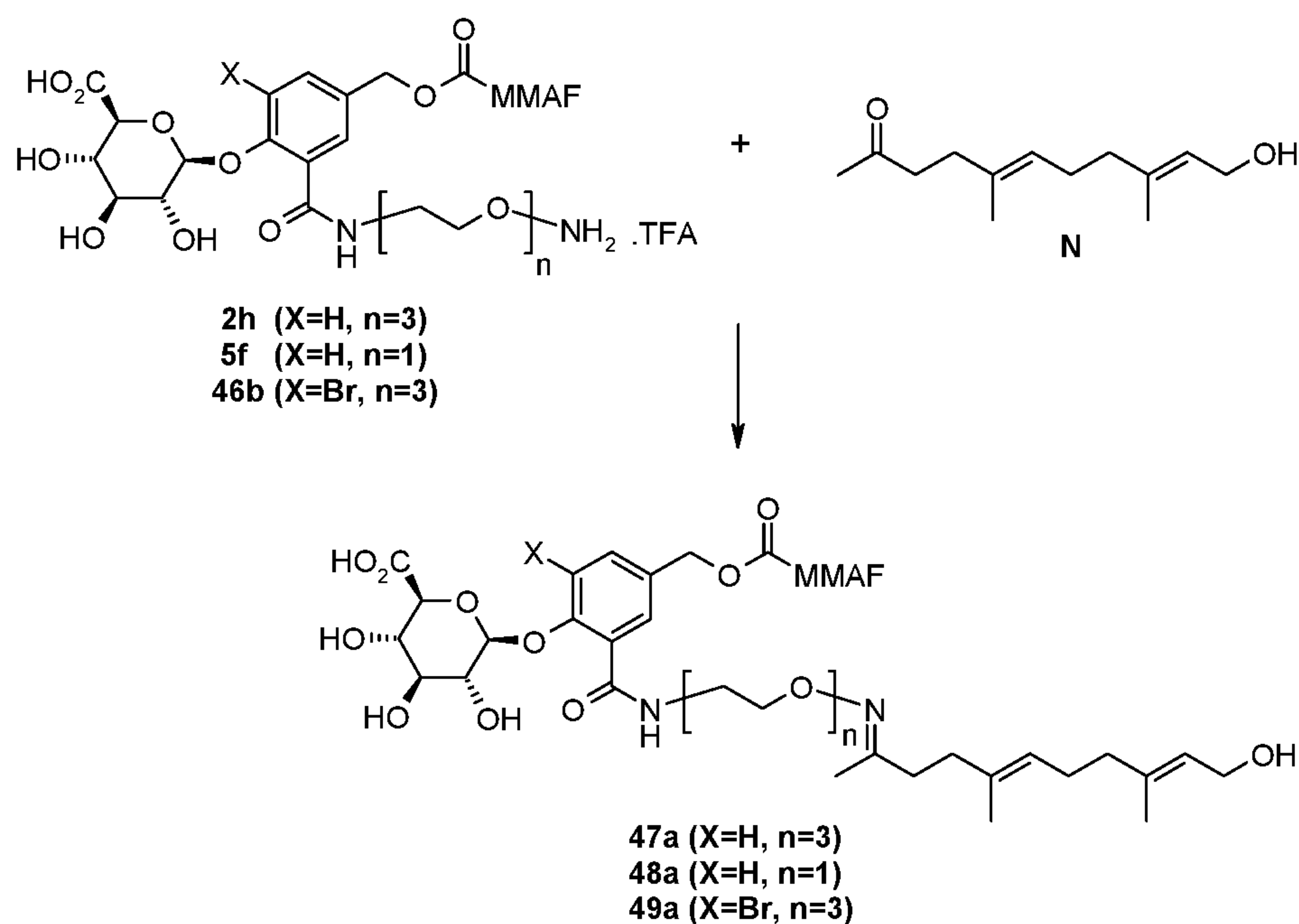
Preparation of Compound 46a

5-Formylsalicylic acid (1.0 g, 6.02 mmol) was dissolved in DMF (20 mL) at 0 °C under nitrogen, and then *N*-bromosuccinimide (1.07 g, 6.11 mmol) was added thereto and the mixture was stirred at 70 °C for 3 hours. After the reaction was completed, EtOAc (100 mL), 2 N aq. HCl solution (2 mL), and distilled water (100 mL) were added thereto. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The residue was subjected to column chromatography to produce the compound **46a** (1.2 g, 84 %). <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ 9.64 (s, 1H), 8.19 (d, *J* = 2.4 Hz, 1H), 8.00 (d, *J* = 2.0 Hz, 1H), 3.16 (s, 1H).

Preparation of Compound 46b

Compound **46b** was prepared from compound **46a** by a similar method of preparing compound **2h** in Example 4. EI-MS m/z: [M+H]<sup>+</sup> 1328.

*Examples 68 to 70. Preparation of Compound 47a, Compound 48a, and Compound 49a*



Compound **N** was prepared by a method disclosed in Korean Patent Laid-Open Publication No. 10-2014-0035393.

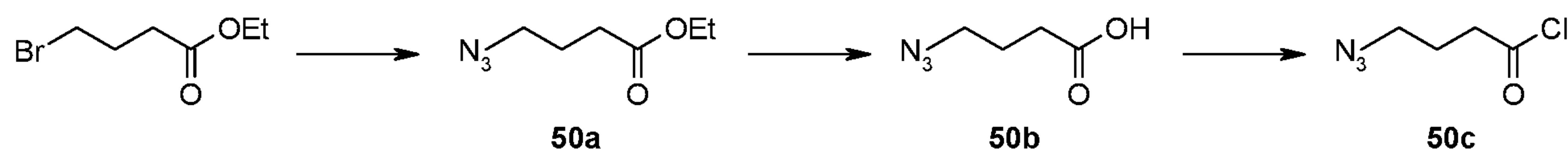
*Examples 68. Preparation of Compound 47a*

Compound **2h** (20 mg, 0.014 mmol) was dissolved in EtOH (0.7 mL) at room temperature under nitrogen, and then compound **N** (3.7 mg, 0.017 mmol) was added thereto, and the mixture was stirred at 45 °C for 2 hours. After the reaction was completed, compound **47a** (10.2 mg, 49 %) was obtained using HPLC. EI-MS  $m/z$ :  $[M+H]^+$  1441.

*Examples 69 and 70. Preparation of Compound 48a and 49a*

Compound **48a** (Example 69) and compound **49a** (Example 70) were prepared by a similar method of preparing compound **47a** in Example 68. EI-MS of compound **48a**  $m/z$ :  $[M+H]^+$  1353. EI-MS of compound **49a**  $m/z$ :  $[M+H]^+$  1520.

*Comparative Example 66. Preparation of Compound 50k*



Preparation of Compound 50a

Ethyl 4-bromobutanoate (5.0 mL, 34.6 mmol) was dissolved in MeOH (7.5 mL) at

room temperature under nitrogen, and then  $\text{NaN}_3$  (4.5 g, 69.2 mmol) in water (25 mL) was added thereto and stirred at 85 °C for 8 hours. After the reaction was completed, the solvent was concentrated under reduced pressure, and chloroform (300 mL) and distilled water (200 mL) were added thereto. The organic layer obtained as described above was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated under reduced pressure. The residue was subjected to column chromatography to produce the compound **50a** (5.1 g, 94 %).  $^1\text{H}$ -NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  4.15 (q,  $J = 7.2$  Hz, 2H), 3.36 (t,  $J = 7.2$  Hz, 2H), 2.41 (t,  $J = 7.2$  Hz, 2H), 1.94-1.89 (m, 2H), 1.28 (t,  $J = 8.4$  Hz, 3H).

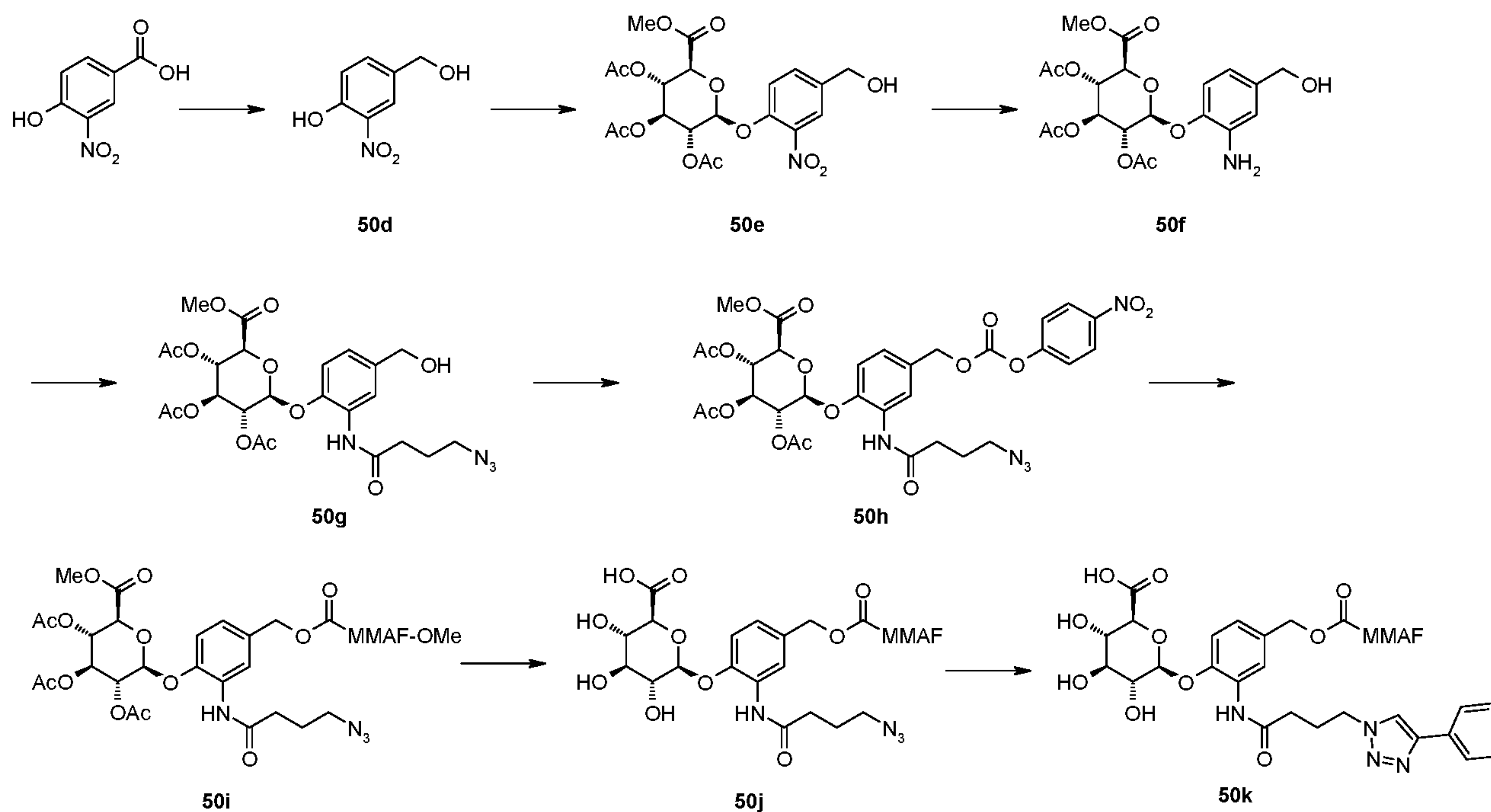
#### Preparation of Compound 50b

Compound **50a** (2.0 g, 12.7 mmol) was dissolved in MeOH (32 mL) at 0 °C under nitrogen, and then KOH (3.56 g, 63.6 mmol) in water (26 mL) was slowly added thereto. After stirring at room temperature for 6 hours, the solvent was concentrated under reduced pressure, and chloroform (300 mL), 1 N aq. HCl (100 mL), and distilled water (100 mL) were added thereto. The organic layer obtained as described above was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated under reduced pressure. The resulting residue was subjected to column chromatography to produce the compound **50b** (1.28 g, 78 %).  $^1\text{H}$ -NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  3.38 (t,  $J = 7.2$  Hz, 2H), 2.48 (t,  $J = 7.2$  Hz, 2H), 1.95-1.90 (m, 2H).

#### Preparation of Compound 50c

Compound **50b** (850 mg, 6.58 mmol) was dissolved in MeOH (10 mL) at 0 °C under nitrogen, and then oxalyl chloride (1.1 mL, 13.2 mmol) and DMF (1 drop) were added thereto and stirred at room temperature for 6 hours. After the reaction was completed, the solvent was concentrated under reduced pressure to produce the compound **50c** (965 mg), which was used without further purification.

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Preparation of Compound 50d

4-Hydroxy-3-nitrobenzoic acid (5.0 g, 27.3 mmol) was dissolved in THF (120 mL) at 0 °C under nitrogen, and then 1 M BH<sub>3</sub>-THF complex (54.6 mL, 54.6 mmol) was added thereto and stirred at room temperature for 20 hours. After the reaction was completed, EtOAc (200 mL), 0.5 N aq. HCl (20 mL), and distilled water (100 mL) were added thereto. The organic layer obtained as described above was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The resulting residue was subjected to column chromatography to produce the compound **50d** (4.2 g, 91 %). <sup>1</sup>H-NMR (600 MHz, CD<sub>3</sub>OD) δ 8.06 (d, *J* = 1.2 Hz, 1H), 7.59 (dd, *J* = 1.2, 7.8 Hz, 1H), 7.12 (d, *J* = 8.4 Hz, 1H), 4.83 (s, 2H).

Preparation of Compound 50e

Compound **50d** (937 mg, 5.54 mmol) was dissolved in MeCN (15 mL) at room temperature under nitrogen, and compound **M** (2.0 g, 5.04 mmol), silver oxide (4.66 g, 20.1 mmol), and 4 Å molecular sieve (2.0 g) were added thereto, and stirred at room temperature for 14 hours. After the reaction was completed, the mixture was celite-filtered, and the filtrate was concentrated under reduced pressure. The resulting residue was subjected to column chromatography to produce the compound **50e** (1.0 g, 40 %). <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) δ 7.81 (d, *J* = 1.8 Hz, 1H), 7.54 (dd, *J* = 1.8, 6.6 Hz, 1H), 7.37 (d, *J* = 8.4 Hz, 1H), 5.37-5.27 (m, 3H), 5.20 (d, *J* = 6.6 Hz, 1H), 4.72 (d, *J* = 6.0 Hz, 2H), 4.21 (d, *J* = 9.0 Hz,

1H), 3.75 (s, 3H), 2.12 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 2.04-2.02 (m, 1H).

#### Preparation of Compound 50f

Compound **50e** (900 mg, 6.35 mmol) was dissolved in EtOAc (100 mL), and then platinum (IV) oxide (84.2 mg, 0.370 mmol) was added thereto and stirred at room temperature under hydrogen for 3 hours. After the reaction was completed, the mixture was celite-filtered, and the filtrate was concentrated under reduced pressure to produce the compound **50f** (700 mg, 83%), which was used without further purification.

#### Preparation of Compound 50g

Compound **50f** (350mg, 0.77 mmol) was dissolved in DCM (10 mL) at 0 °C under nitrogen, and then compound **50c** (136 mg, 0.92 mmol) and DIPEA (0.27 mL, 1.54 mmol) were added thereto and stirred at room temperature for 20 minutes. After the reaction was completed, EtOAc (50 mL) and distilled water (50 mL) were added thereto. The organic layer obtained as described above was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The resulting residue was subjected to column chromatography to produce the compound **50g** (280 mg, 65 %). <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) δ 8.37 (d, *J* = 1.2 Hz, 1H), 8.00 (s, 1H), 7.07 (dd, *J* = 1.8, 6.6 Hz, 1H), 6.93 (d, *J* = 8.4Hz, 1H), 5.43-5.28 (m, 3H), 5.06 (d, *J* = 7.8 Hz, 1H), 4.63 (s, 2H), 4.19 (d, *J* = 9.6 Hz, 1H), 3.76 (s, 3H), 3.44-3.41 (m, 2H), 2.56 (t, *J* = 7.8 Hz, 2H), 2.17-2.00 (m, 12H).

#### Preparation of Compound 50h

Compound **50g** (250 mg, 0.44 mmol) was dissolved in DMF (4 mL) at 0 °C under nitrogen, and then bis(4-nitrophenyl)carbonate (270 mg, 0.88 mmol) and DIPEA (0.12 mL, 0.66 mmol) were added thereto, and stirred at room temperature for 1 hour. After the reaction was completed, EtOAc (50 mL) and distilled water (50 mL) were added thereto. The organic layer obtained as described above was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The resulting residue was subjected to column chromatography to produce the compound **50h** (290 mg, 90 %). <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) δ 8.54 (d, *J* = 1.8 Hz, 1H), 8.28-8.25 (m, 2H), 8.02 (s, 1H), 7.40-7.36 (m, 2H), 7.11 (dd, *J* = 1.8, 6.6 Hz, 1H), 6.96 (d, *J* = 8.4 Hz, 1H), 5.44-5.29 (m, 3H), 5.23 (s, 2H), 5.10 (d, *J* = 7.8 Hz, 1H), 4.21 (d, *J* = 9.6 Hz, 1H), 3.76 (s, 3H), 3.45-3.42 (m, 2H), 2.58 (t, *J* = 7.2 Hz, 2H), 2.11-2.00 (m, 12H).

### Preparation of Compound 50i

Compound **50h** (250 mg, 0.34 mmol) was dissolved in DMF (4 mL) at room temperature under nitrogen, and then MMAF-OMe (255 mg, 0.34 mmol) was added thereto. The resulting mixture was treated with HOBT (9 mg, 0.068 mmol), pyridine (1.2 mL), and DIPEA (0.060 mL, 0.34 mmol). After stirring at room temperature for 2 days, EtOAc (50 mL), 2 N aq. HCl (5 mL), and distilled water (50 mL) were added thereto. The organic layer obtained as described above was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The residue was subjected to column chromatography to produce the compound **50i** (340 mg, 74 %). EI-MS m/z: [M+H]<sup>+</sup> 1339.

### Preparation of Compound 50j

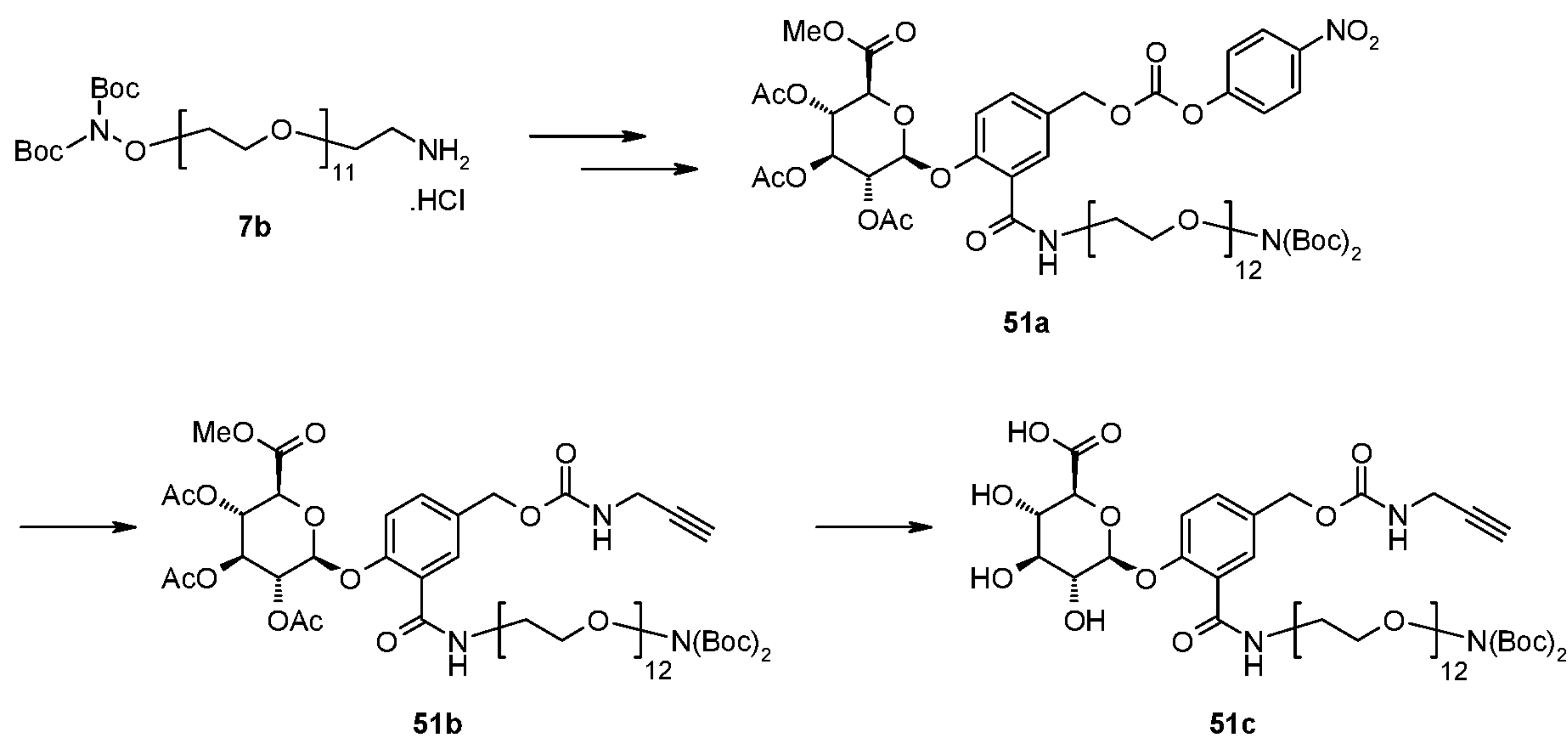
Compound **50i** (210 mg, 0.156 mmol) was dissolved in MeOH (2 mL) at 0 °C under nitrogen, and then LiOH·H<sub>2</sub>O (66 mg, 1.56 mmol) in water (2 mL) was added thereto. After stirring at room temperature for 1.5 hours, chloroform (50 mL), MeOH (5 mL), distilled water (50 mL), and 0.5 N aq. HCl (5 mL) were added thereto. The organic layer obtained as described above was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The resulting residue was subjected to column chromatography to produce the compound **50j** (107 mg, 57 %). EI-MS m/z: [M+H]<sup>+</sup> 1184.

### Preparation of Compound 50k

Compound **50j** (10 mg, 0.008 mmol) and phenylacetylene (0.92 μL, 0.008 mmol) were dissolved in EtOH (0.15 mL) and water (10 μL) at room temperature under nitrogen, and then 0.1 M CuSO<sub>4</sub> aqueous solution (10 μL) and 1.0 M sodium ascorbate aqueous solution (10 μL) were added thereto. After stirring at room temperature for 5 hours, EtOAc (10 mL) and distilled water (5 mL) were added thereto. The organic layer obtained as described above was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The resulting residue was subjected to column chromatography to produce the compound **50k** (5 mg, 46 %). EI-MS m/z: [M+H]<sup>+</sup> 1286.

### *Example 71. Preparation of Compound 51h*

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### Preparation of Compound 51a

Compound **51a** was prepared from compound **7b** by a method similar to method of preparing compound **14h** of Example 23. EI-MS  $m/z$ :  $[M+H]^+$  1392.8,  $[M+H-Boc]^+$  1292.7,  $[M+Na]^+$  1414.8.

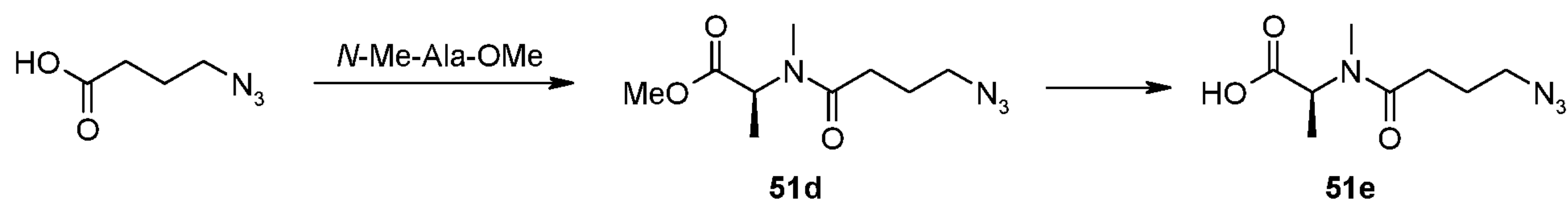
### Preparation of Compound 51b

Compound **51a** (1.8 g, 1.29 mmol), propargylamine (0.1 mL, 1.55 mmol) and anhydrous HOBt (35 mg, 0.25 mmol) were dissolved in DMF (5 mL) at 0 °C. Then pyridine (0.2 mL) and DIPEA (0.45 mL, 2.59 mmol) were added. After stirring at room temperature for 24 hours under  $N_2$ , the reaction mixture was diluted with  $H_2O$  (100 mL) and saturated aq.  $NH_4Cl$  solution (50 mL). After extraction with EtOAc (2 x 100 mL), the combined organic layers were dried over anhydrous  $MgSO_4$ , filtered, and concentrated. The residue was purified by column chromatography to produce the compound **51b** (1.15 g, 68 %).  $^1H$ -NMR (400 MHz,  $CDCl_3$ )  $\delta$  8.01 (s, 1H), 7.48-7.31 (m, 2H), 7.02 (d,  $J$  = 8.4 Hz, 1H), 5.45-5.20 (m, 4H), 5.09 (s, 2H), 4.19 (d,  $J$  = 9.2 Hz, 1H), 4.10-4.05 (m, 2H), 3.97 (s, 2H), 3.85-3.45 (m, 49H), 2.24 (s, 1H), 2.05 (s, 9H), 1.53 (s, 18H). EI-MS  $m/z$ :  $[M+Na]^+$  1330.3.

### Preparation of Compound 51c

To a solution of compound **51b** (1.15 g, 0.879 mmol) in THF/MeOH (20 mL/20 mL) was added LiOH monohydrate (151 mg, 3.603 mmol) in  $H_2O$  (20 mL) at 0 °C. After 2

hours at 0 °C, the reaction mixture was neutralized using acetic acid and concentrated under reduced pressure. The resulting residue was dissolved in DMSO (5 mL) and purified by prep. HPLC, which produced the compound **51c** (600 mg, 60 %). EI-MS  $m/z$ :  $[M+H]^+$  1169.2.

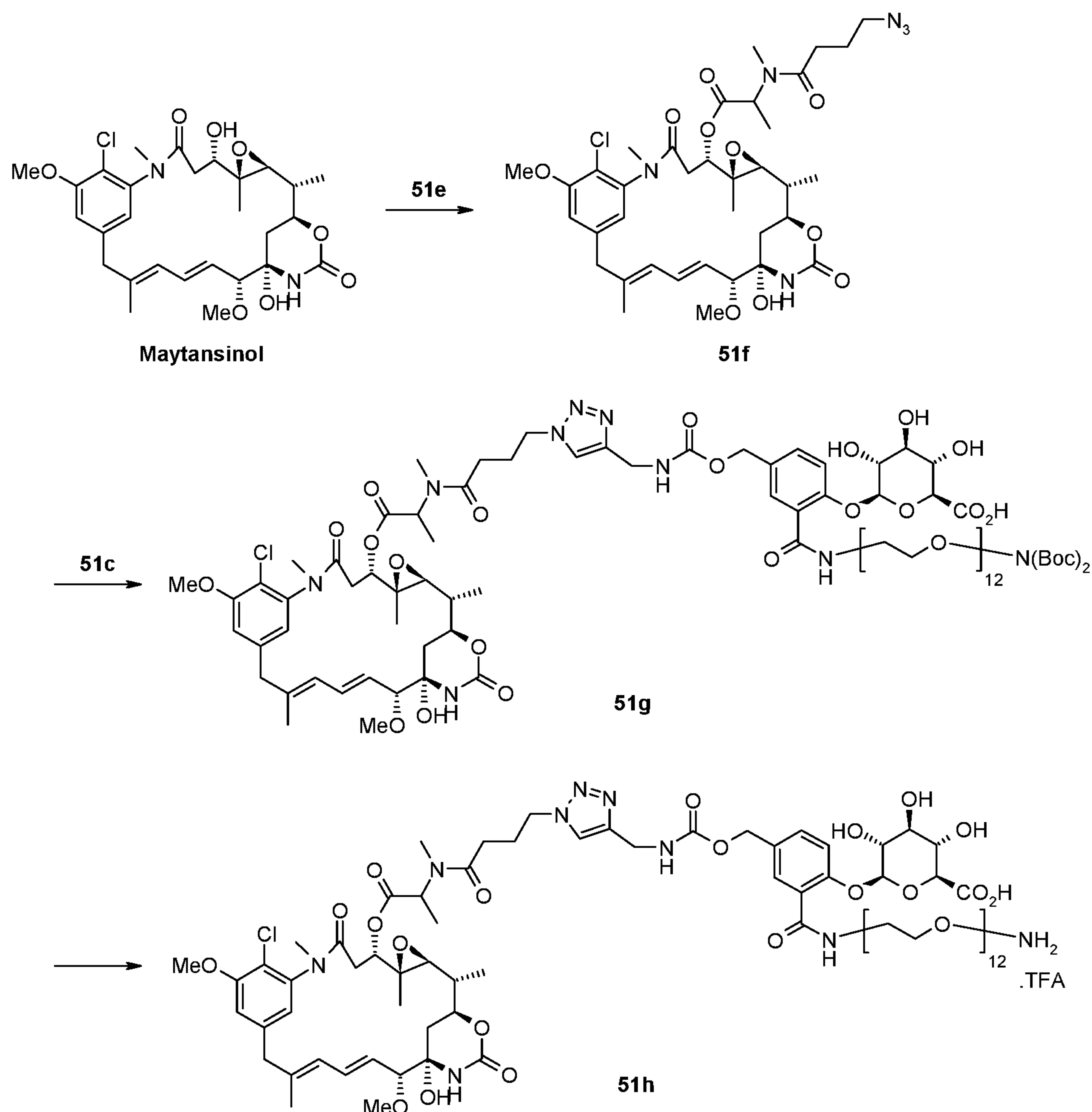


#### Preparation of Compound **51d**

DIPEA (0.92 mL, 5.30 mmol) and HBTU (1.0 g, 2.64 mmol) were added to a stirred mixture of 4-azidobutanoic acid (228 mg, 1.76 mmol) and *N*-Me-Ala-OMe (298 mg, 1.94 mmol) in DMF (10 mL). After stirring at room temperature for 14 hours under N<sub>2</sub>, the reaction mixture was diluted with H<sub>2</sub>O (50 mL) and extracted with EtOAc (2 x 50 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The resulting residue was purified by column chromatography to yield the compound **51d** (310 mg, 77 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 5.22 (q, 1H), 3.71 (s, 3H), 3.39 (t, *J* = 6.6 Hz, 2H), 2.95 (s, 3H), 2.52-2.39 (m, 2H), 1.98-1.92 (m, 2H), 1.41 (d, 3H).

#### Preparation of Compound **51e**

To a solution of compound **51d** (310 mg, 1.36 mmol) in MeOH (3 mL) was added LiOH monohydrate (114 mg, 2.72 mmol) in H<sub>2</sub>O (3 mL) at -20 °C. After stirring at 0 °C for 1 hour, the reaction mixture was diluted with H<sub>2</sub>O/2 N aq. HCl solution (50 mL/2 mL) and extracted with Et<sub>2</sub>O (2 x 30 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Filtration and concentration produced the compound **51e** (246 mg), which was used without further purification. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ 5.15 (q, 1H), 3.39 (t, *J* = 6.6 Hz, 2H), 2.98 (s, 3H), 2.49-2.45 (m, 2H), 1.98-1.92 (m, 2H), 1.41 (d, 3H).



### Preparation of Compound **51f**

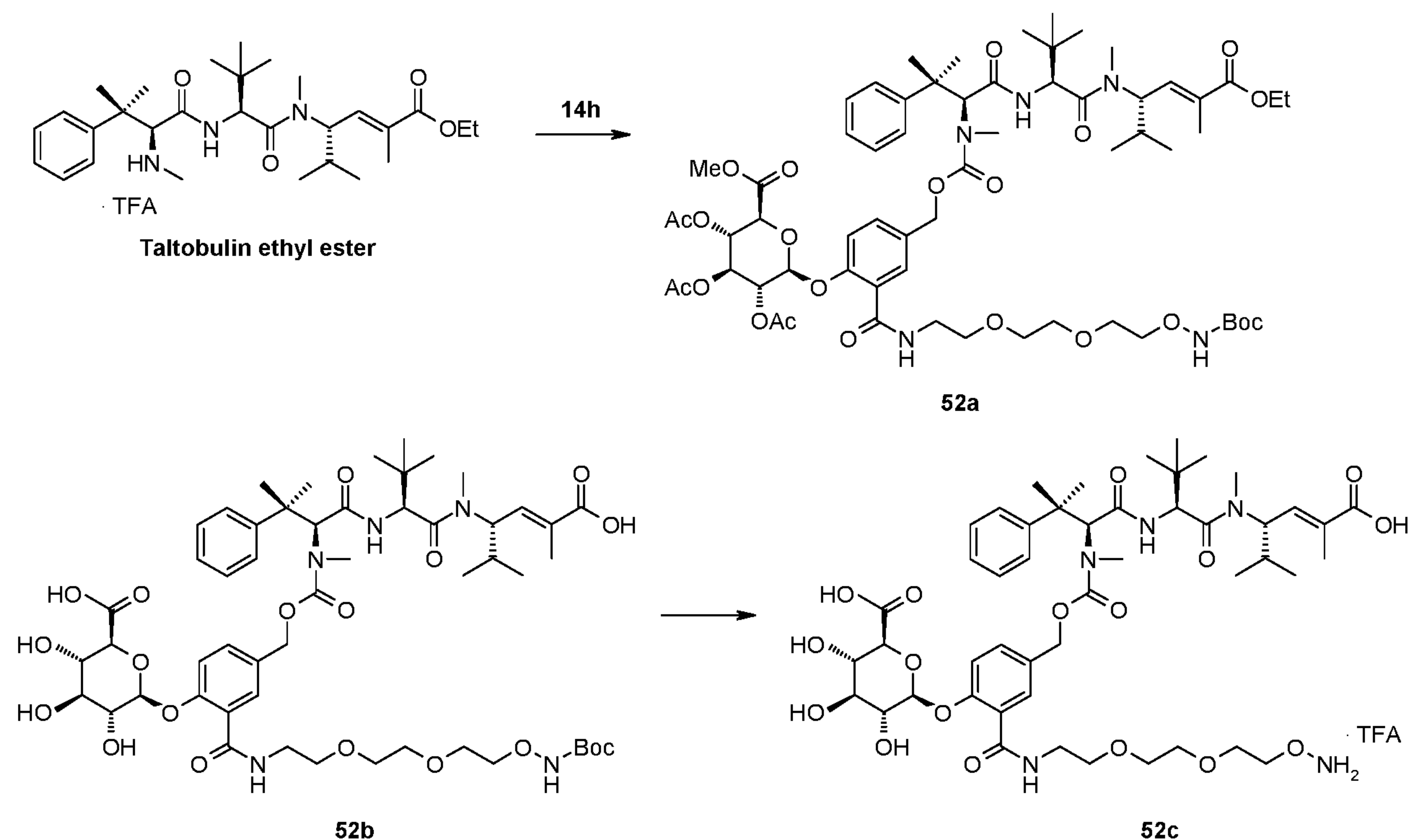
To a solution of maytansinol (50 mg, 0.088 mmol) and compound **51e** (113 mg, 0.528 mmol) in DCM (6 mL) under N<sub>2</sub> was added a solution of DIC (0.087 mL, 0.557 mmol) in DCM (1.4 mL). After 1 minute, a solution of ZnCl<sub>2</sub> (1 M in Et<sub>2</sub>O, 0.11 mL, 0.11 mmol) was added. After stirring at room temperature for 2 hours, the reaction mixture was diluted with EtOAc (10 mL). The organic layer was washed with saturated aq. NaHCO<sub>3</sub> (4 mL) and brine (2 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated under reduced pressure. The resulting residue was purified by column chromatography to yield a mixture of diastereomeric maytansinoids compound **51f** (50 mg, 74 %). EI-MS m/z: [M+H]<sup>+</sup> 761.7.

Preparation of Compound **51g**

CuSO<sub>4</sub>·5H<sub>2</sub>O (2 mg) and sodium ascorbate (10 mg) were added to a stirring mixture of compound **51f** (78 mg, 0.102 mmol) and compound **51c** (132 mg, 0.112 mmol) in DMSO (4 mL) and H<sub>2</sub>O (1 mL). The pH was adjusted to about 7 by addition of 1 M aq. Na<sub>2</sub>CO<sub>3</sub>. After stirring at 20 °C for 1 hour, the reaction mixture was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC. Pure fractions with the same retention time were combined and concentrated to produce the compound **51g** (72.1 mg, 37 %). EI-MS m/z: [M+H]<sup>+</sup> 1930.9, [M+H-Boc]<sup>+</sup> 1830.9.

Preparation of Compound **51h**

TFA (0.2 mL) was added to a stirring solution of compound **51g** (72.1 mg, 0.037 mmol) in DCM (1 mL). After stirring at 0 °C for 2 hours, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in H<sub>2</sub>O/MeCN (1 mL/1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **51h** (more polar isomer 17 mg and less polar isomer 6.0 mg, 36 %) as white solid. EI-MS m/z: [M+H]<sup>+</sup> 1730.8.

Example 72. Preparation of Compound **52c**

### Preparation of Compound 52a

Taltobulin ethyl ester (TFA salt, 80 mg, 0.029 mmol), compound **14h** (128 mg, 0.0142 mmol) and anhydrous HOBt (3.5 mg, 0.026 mmol) were dissolved in DMF (3 mL) at 0 °C. Then pyridine (0.5 mL) and DIPEA (0.045 mL, 0.26 mmol) were added. After stirring at room temperature for 24 hours under N<sub>2</sub>, the reaction mixture was diluted with H<sub>2</sub>O (10 mL) and extracted with EtOAc (2 x 10 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The resulting residue was purified by column chromatography to yield the compound **52a** (70 mg, 43 %). EI-MS m/z: [M+H]<sup>+</sup> 1258.6, [M+H-Boc]<sup>+</sup> 1158.6.

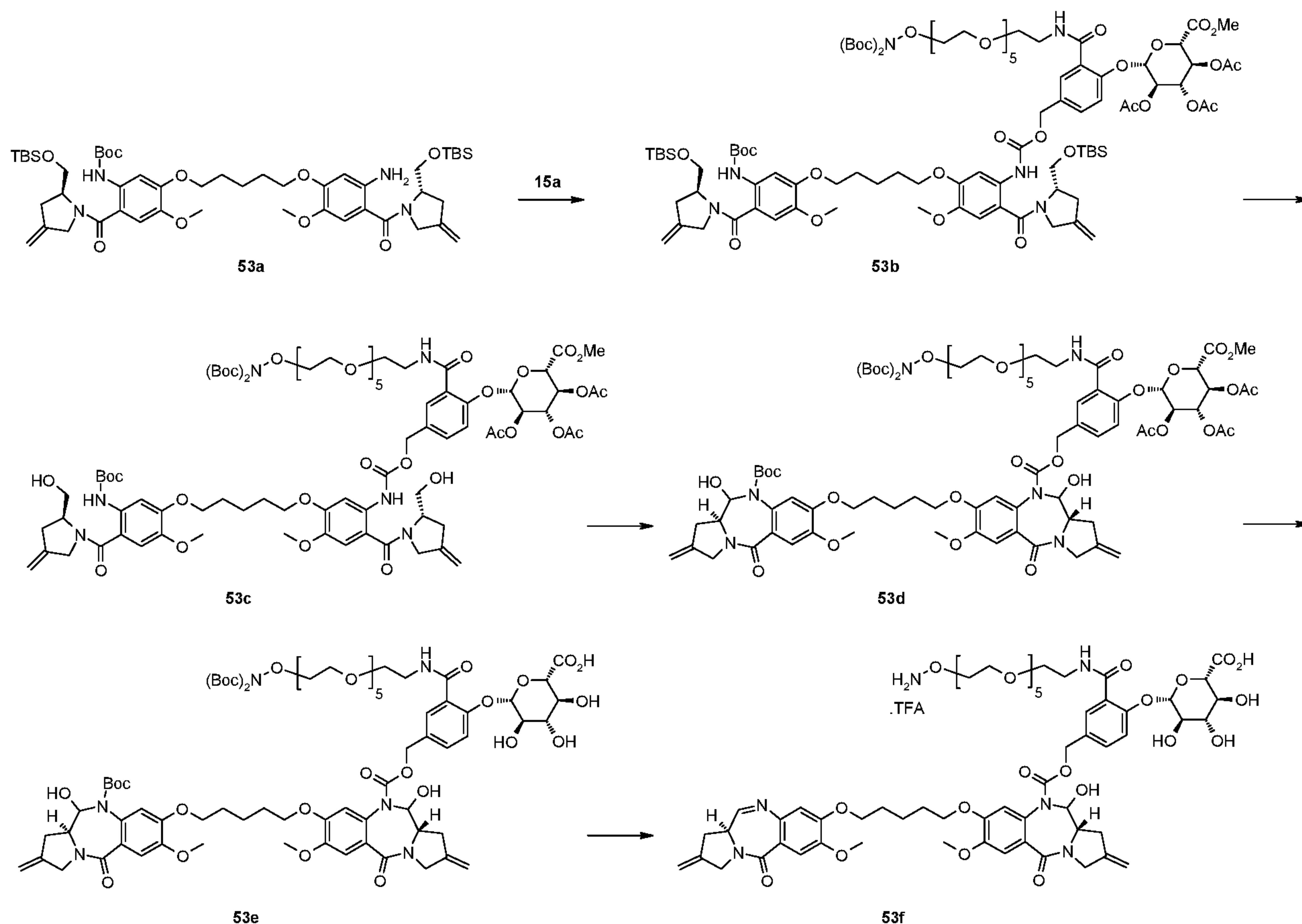
### Preparation of Compound 52b

To a solution of compound **52a** (70 mg, 0.055 mmol) in MeOH (1.4 mL) was added LiOH monohydrate (11.7 mg, 0.275 mmol) in H<sub>2</sub>O (1.4 mL) at -20 °C. After 1 hour at 0 °C, the pH of the solution was adjusted to 4~5 with acetic acid. The resulting solution was dissolved in DMSO (1 mL) and purified by HPLC, which produced the compound **52b** (4.5 mg, 8 %) as white solid. EI-MS m/z: [M+H]<sup>+</sup> 1090.4.

### Preparation of Compound 52c

To a solution of compound **52b** (4.5 mg, 0.0041 mmol) in DCM (1 mL) was added TFA (0.2 mL) at 0 °C. After 2 hours at 0 °C, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was purified by HPLC, which produced the compound **52c** (2.4 mg, 59 %) as white solid. EI-MS m/z: [M+H]<sup>+</sup> 990.4.

### Example 73. Preparation of Compound 53f



### Preparation of Compound **53b**

Compound **53a** (300 mg, 0.31 mmol, Compound **53a** was prepared by a method disclosed in patent WO2013/055987 A1), compound **15a** (355 mg, 0.31 mmol) and anhydrous HOBt (10 mg, 0.06 mmol) were dissolved in DMF (0.5 mL) at 0 °C. Then pyridine (0.3 mL) and DIPEA (0.14 mL, 0.78 mmol) were added. After stirring at room temperature for 23 hours under N<sub>2</sub>, the reaction mixture was diluted with H<sub>2</sub>O/saturated aq. NH<sub>4</sub>Cl solution (100 mL/50 mL) and extracted with EtOAc (2 x 100 mL). The combined organic layers were dried over anhydrous MgSO<sub>4</sub>, filtered, and concentrated. The residue was purified by column chromatography to produce the compound **53b** (250 mg, 41 %). EI-MS m/z: [M+H]<sup>+</sup> 1943.6, [M+Na]<sup>+</sup> 1965.6.

### Preparation of Compound **53c**

To a solution of compound **53b** (300 mg, 0.31 mmol) in THF/H<sub>2</sub>O (2 mL/1 mL) was added acetic acid (3 mL) at 0 °C under N<sub>2</sub>. After 22 hours, the reaction mixture was diluted with H<sub>2</sub>O (100 mL) and extracted with EtOAc (2 x 100 mL). The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. The resulting residue

was purified by column chromatography to yield the compound **53c** (140 mg, 68 %). EI-MS  $m/z$ :  $[M+H]^+$  1713.6.

#### Preparation of Compound 53d

To a solution of compound **53c** (120 mg, 0.07 mmol) in DCM (10 mL) were added pyridinium chlorochromate (158 mg, 0.42 mmol) and 4 Å molecular sieve (50 mg) at room temperature under  $N_2$ . After stirring for 18 hours, the reaction mixture was filtered through a celite pad and concentrated under reduced pressure. The resulting compound **53d** (95 mg, 75 %) was obtained as colorless oil, which was used without further purification. EI-MS  $m/z$ :  $[M+Na]^+$  1732.8.

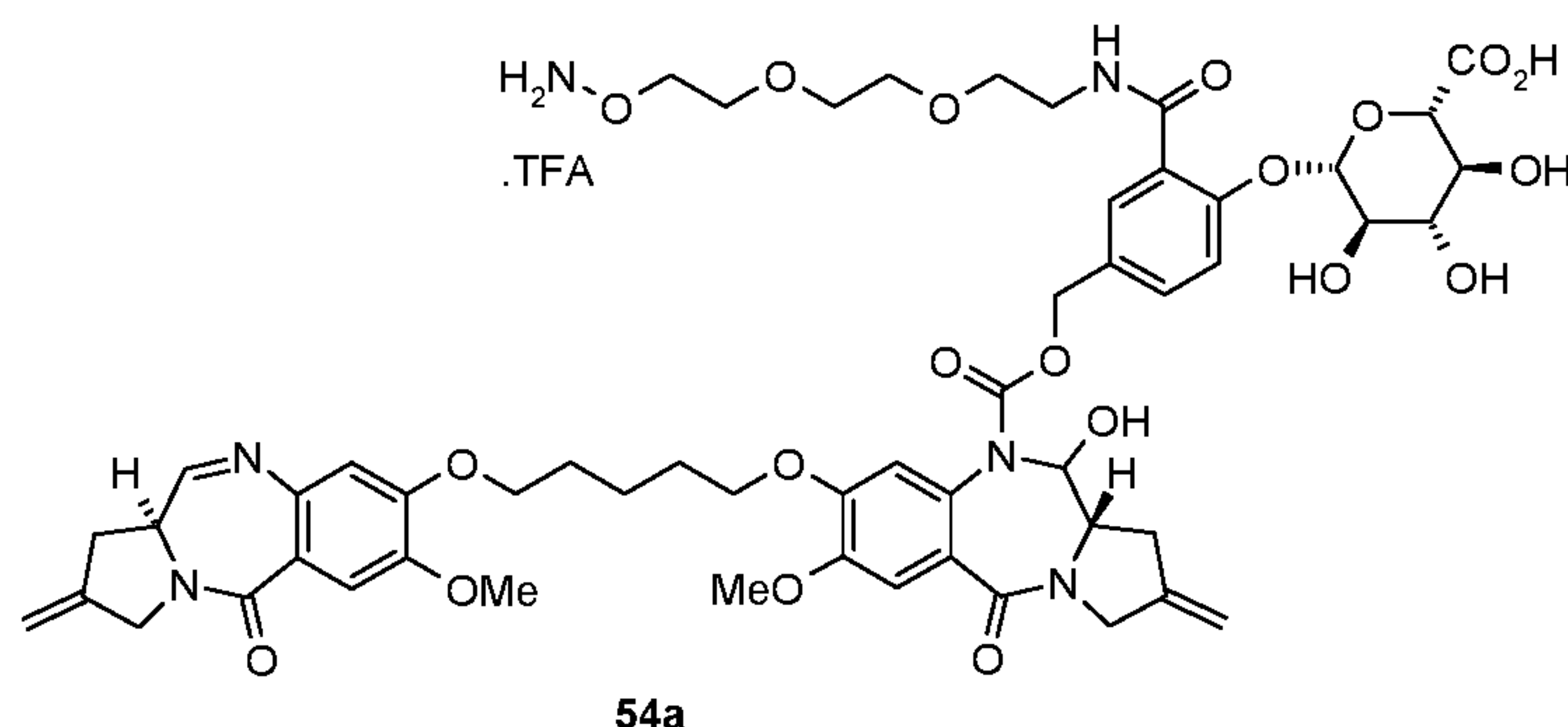
#### Preparation of Compound 53e

To a solution of compound **53d** (95 mg, 0.056 mmol) in MeOH (1 mL) was added LiOH monohydrate (12 mg, 0.278 mmol) in  $H_2O$  (1 mL) at 0 °C. After 2 hours at 0 °C, the reaction mixture was neutralized using acetic acid and concentrated under reduced pressure. The resulting residue was dissolved in DMSO (1 mL) and purified by prep. HPLC, which produced the compound **53e** (6 mg, 7 %). EI-MS  $m/z$ :  $[M+H]^+$  1569.7.

#### Preparation of Compound 53f

TFA (0.2 mL) was added to a stirred solution of compound **53e** (6 mg, 0.004 mmol) in DCM (2 mL). After stirring at 0 °C for 2 hours, the solvent and excess TFA were removed by  $N_2$  flow. Then the residue was dissolved in DMSO (1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **53f** (2.7 mg, 53 %) as white solid. EI-MS  $m/z$ :  $[M+H]^+$  1251.3.

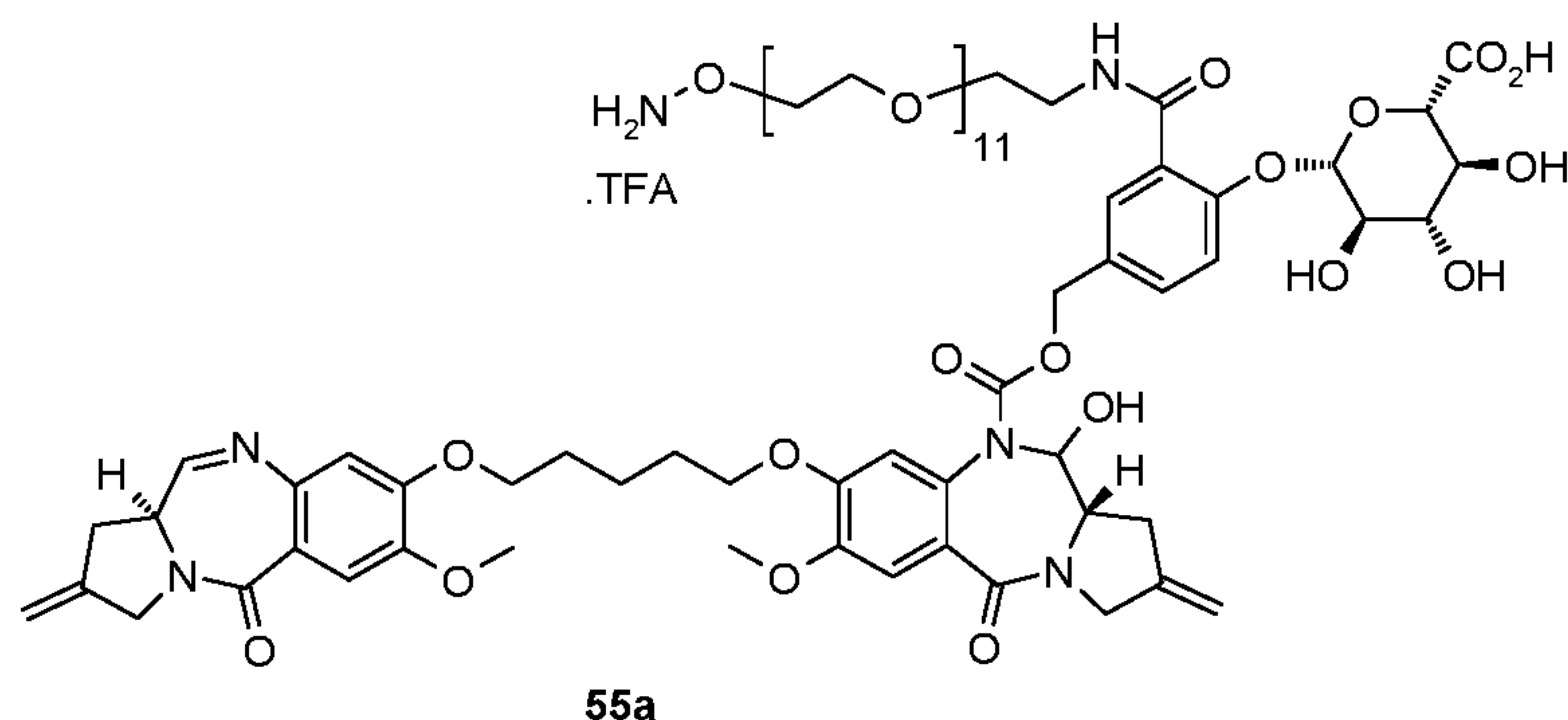
#### Example 74. Preparation of Compound 54a



Compound **54a** was prepared from compound **53a** and compound **14h** by a similar

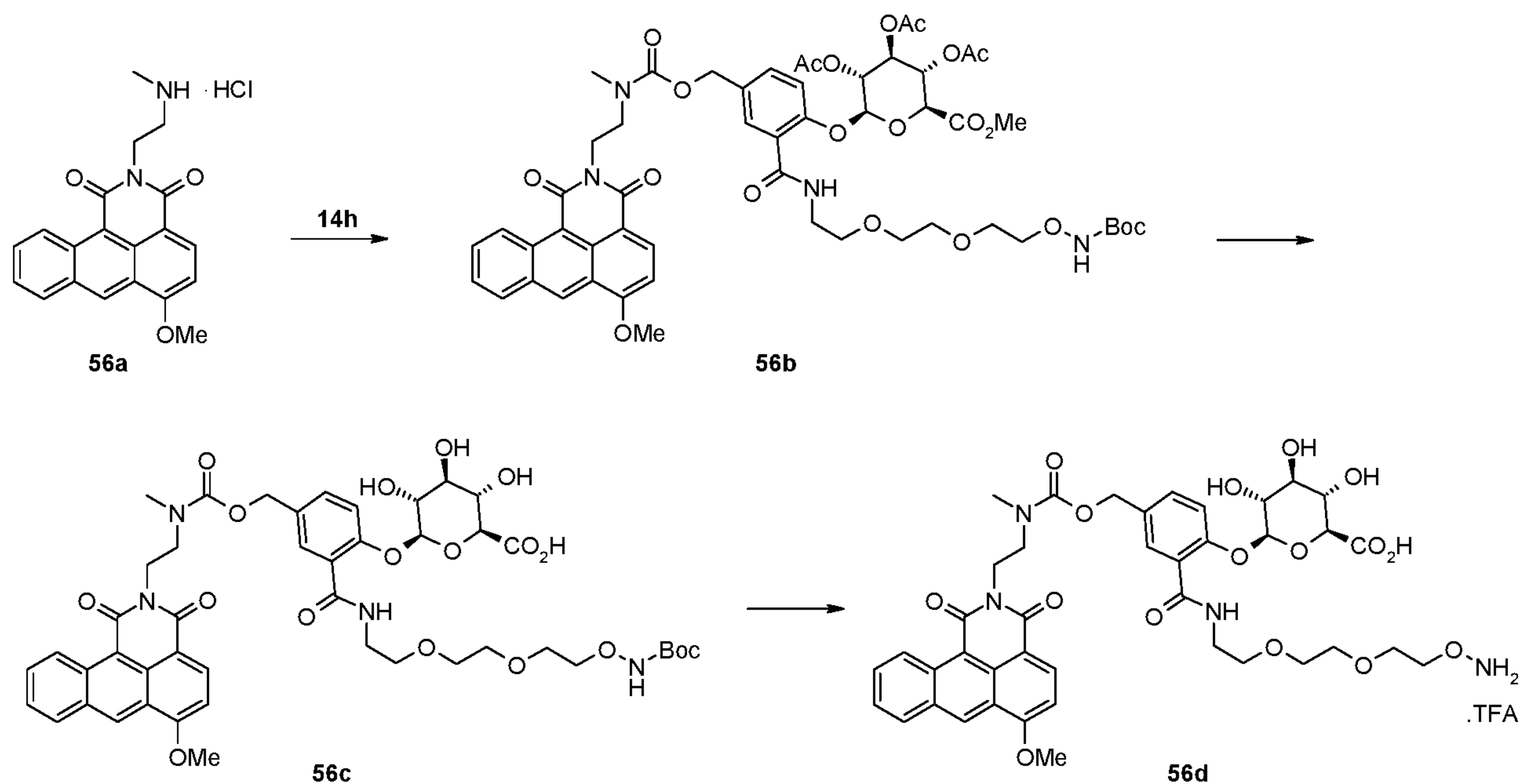
method of preparing compound **53f** in Example 73.

**Example 75. Preparation of Compound 55a**



Compound **55a** was prepared from compound **53a** and compound **51a** by a similar method of preparing compound **53f** in Example 73. EI-MS  $m/z$ :  $[M+H]^+$  1516.7,  $1/2[M+H]^+$  758.7.

**Example 76. Preparation of Compound 56d**



**Preparation of Compound 56b**

Compound **56a** (HCl salt, 100 mg, 0.27 mmol, Compound **56a** was prepared by a method disclosed in *Curr. Med. Chem.* 2009, 16, 1192-1213.), compound **14h** (242 mg, 0.27 mmol), and anhydrous HOBt (7.3 mg, 0.05 mmol) were dissolved in DMF (3 mL) at 0 °C. Then pyridine (0.4 mL) and DIPEA (0.09 mL, 0.60 mmol) were added. After stirring at room temperature for 16 hours under N<sub>2</sub>, the reaction mixture was diluted with saturated aq.

NH<sub>4</sub>Cl solution (10 mL) and extracted with EtOAc (2 x 20 mL). The combined organic layers were dried over anhydrous MgSO<sub>4</sub>, filtered and concentrated. The residue was purified by column chromatography to produce the compound **56b** (184 mg, 63 %). EI-MS m/z: [M+H]<sup>+</sup> 1091.9, [M+H-Boc]<sup>+</sup> 991.7.

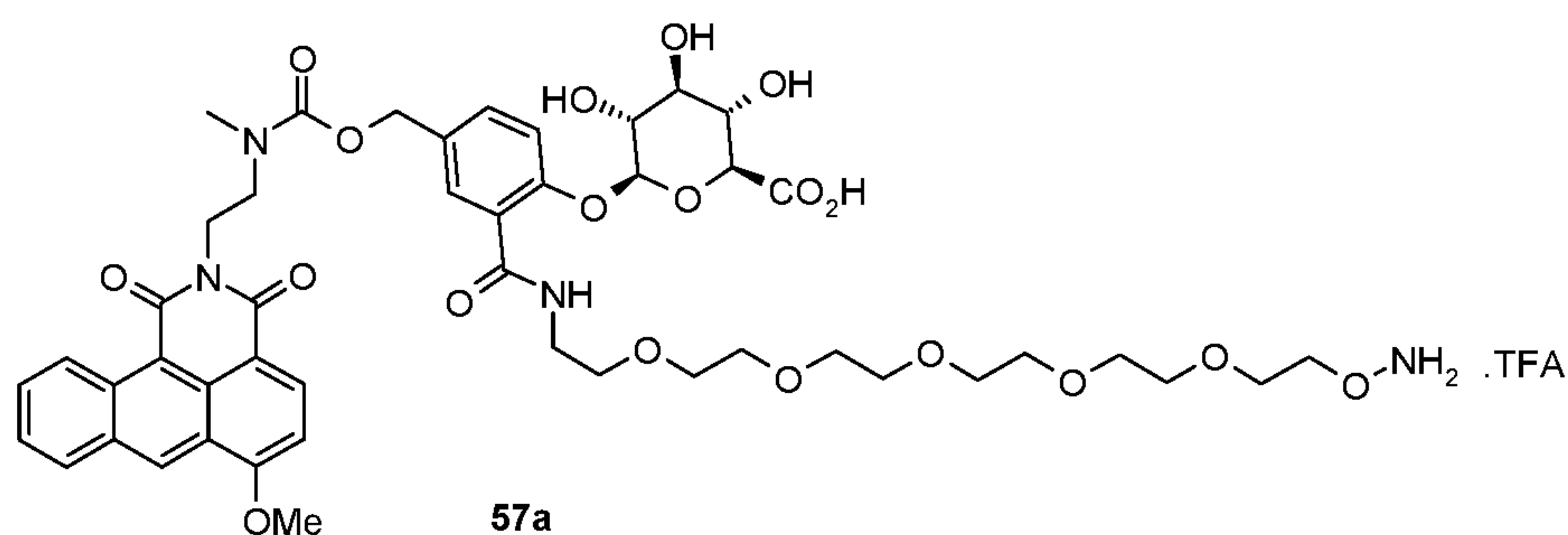
#### Preparation of Compound 56c

To a solution of compound **56b** (90 mg, 0.08 mmol) in MeOH (2 mL) was added LiOH monohydrate (17 mg, 0.41 mmol) in H<sub>2</sub>O (2 mL) at -20 °C. After stirring for 2 hours at -20 °C, the reaction mixture was neutralized using acetic acid and concentrated under reduced pressure. Then the reaction mixture was dissolved in H<sub>2</sub>O/DMSO (1.5 mL/1.5 mL) and purified by HPLC, which produced the compound **56c** (35 mg, 45 %) as yellow solid. EI-MS m/z: [M+H]<sup>+</sup> 951.7, [M+H-Boc]<sup>+</sup> 851.5.

#### Preparation of Compound 56d

TFA (0.3 mL) was added to a stirred solution of compound **56c** (35 mg, 0.04 mmol) in DCM (2.0 mL) at 0 °C. After stirring for 1 hour, the solvent and excess TFA were removed by N<sub>2</sub> flow. Then the residue was dissolved in H<sub>2</sub>O/MeCN (1 mL/1 mL) and purified by HPLC. Pure fractions with the same retention time were combined and lyophilized to produce the compound **56d** (24.9 mg, 68 %) as yellow solid. EI-MS m/z: [M+H]<sup>+</sup> 851.6.

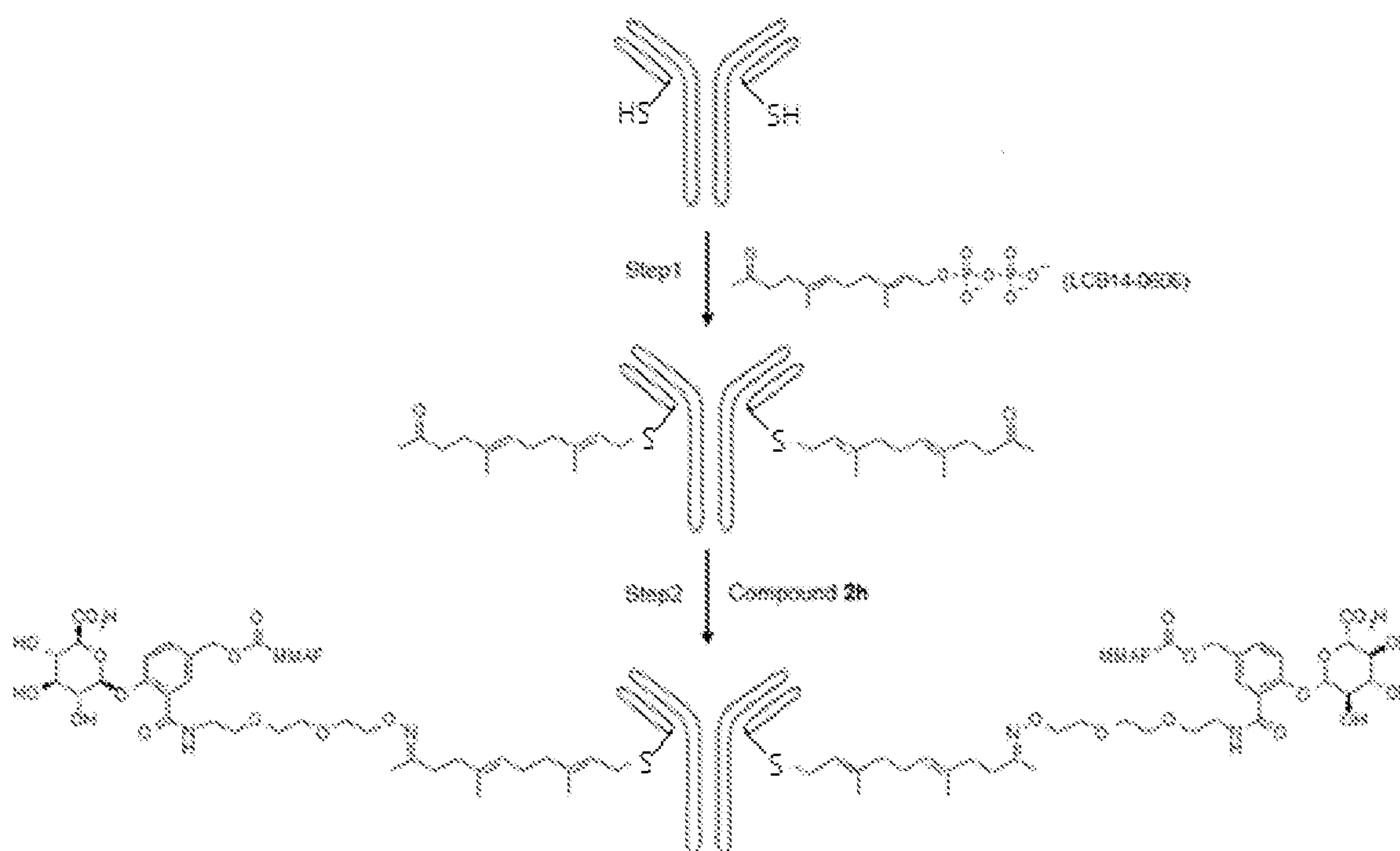
#### *Example 77. Preparation of Compound 57a*



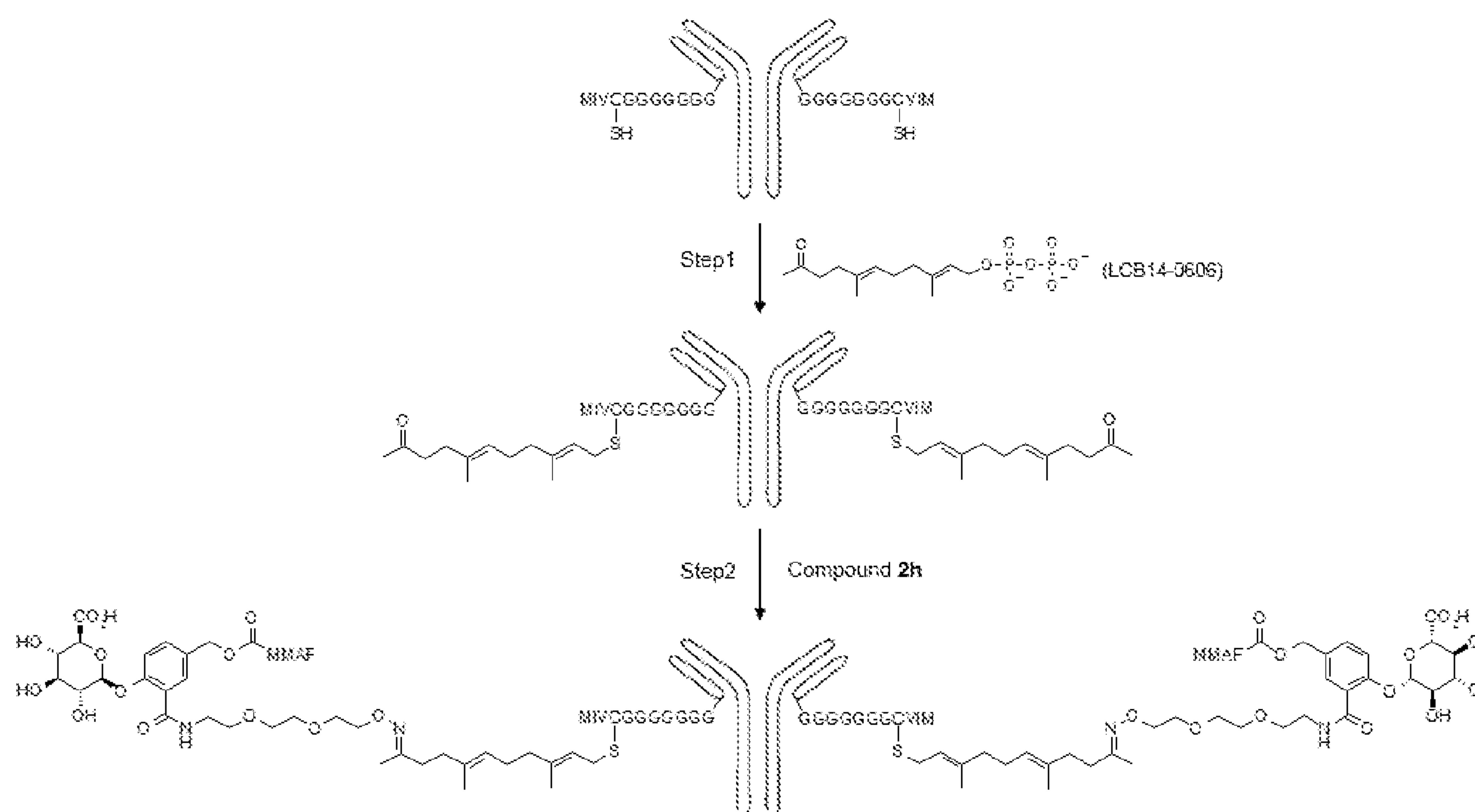
Compound **57a** was prepared from compound **56a** and compound **15a** by a similar method of preparing compound **56d** in Example 76. EI-MS m/z: [M+H]<sup>+</sup> 983.3.

#### *Example 78. ADC2 Synthesis*

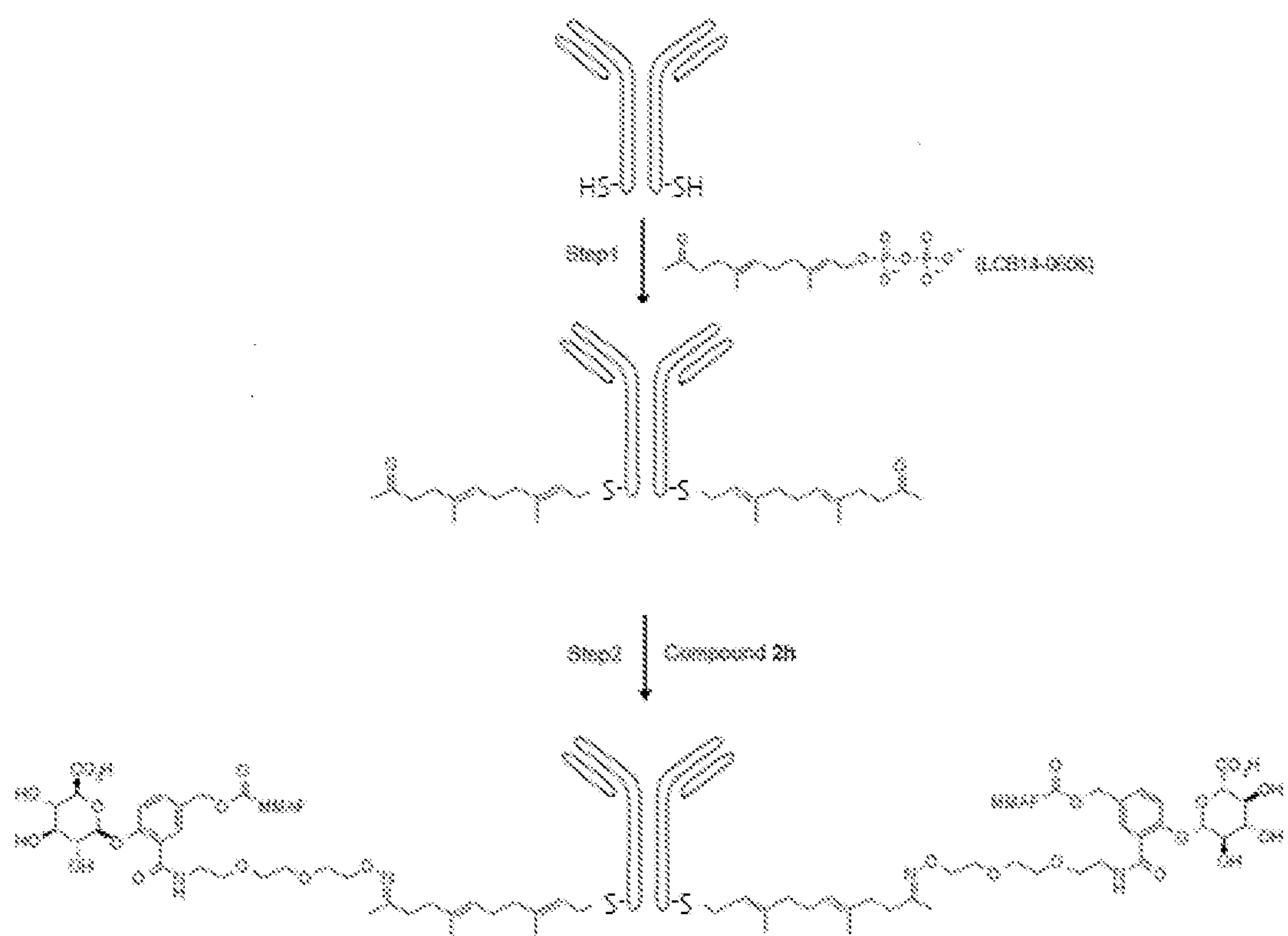
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*Example 79. ADC2 Synthesis*

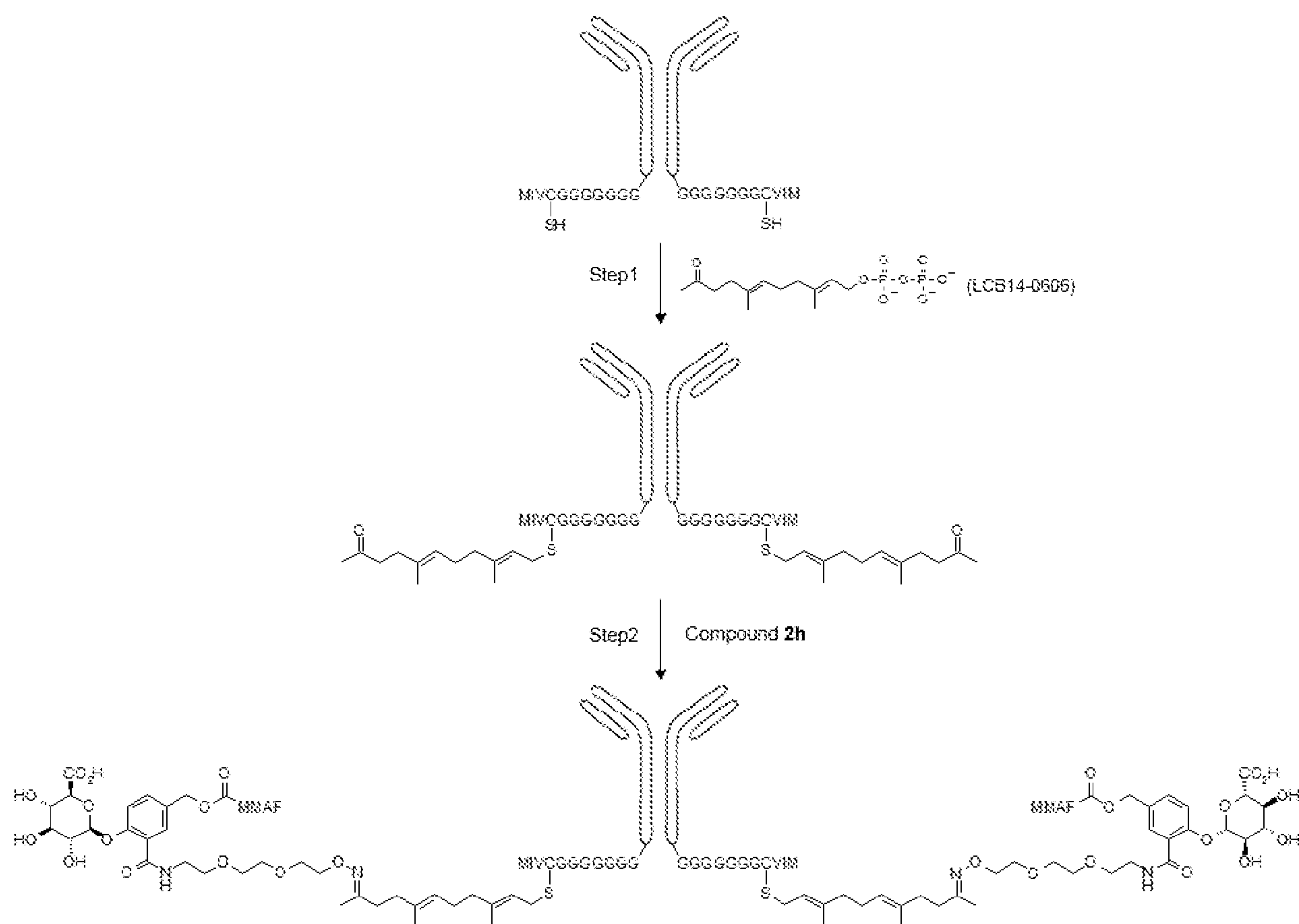


*Example 80. ADC86 Synthesis*

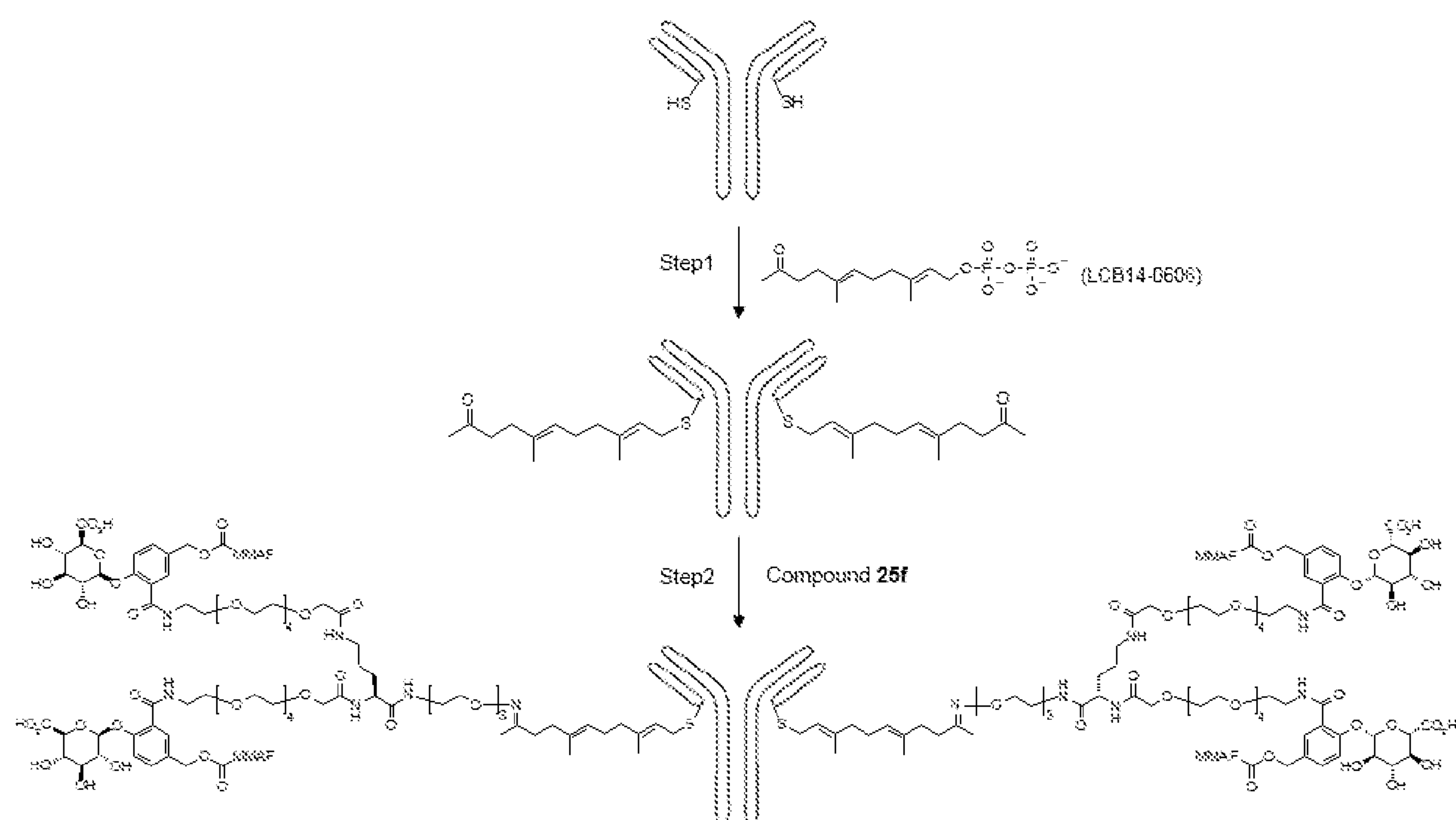


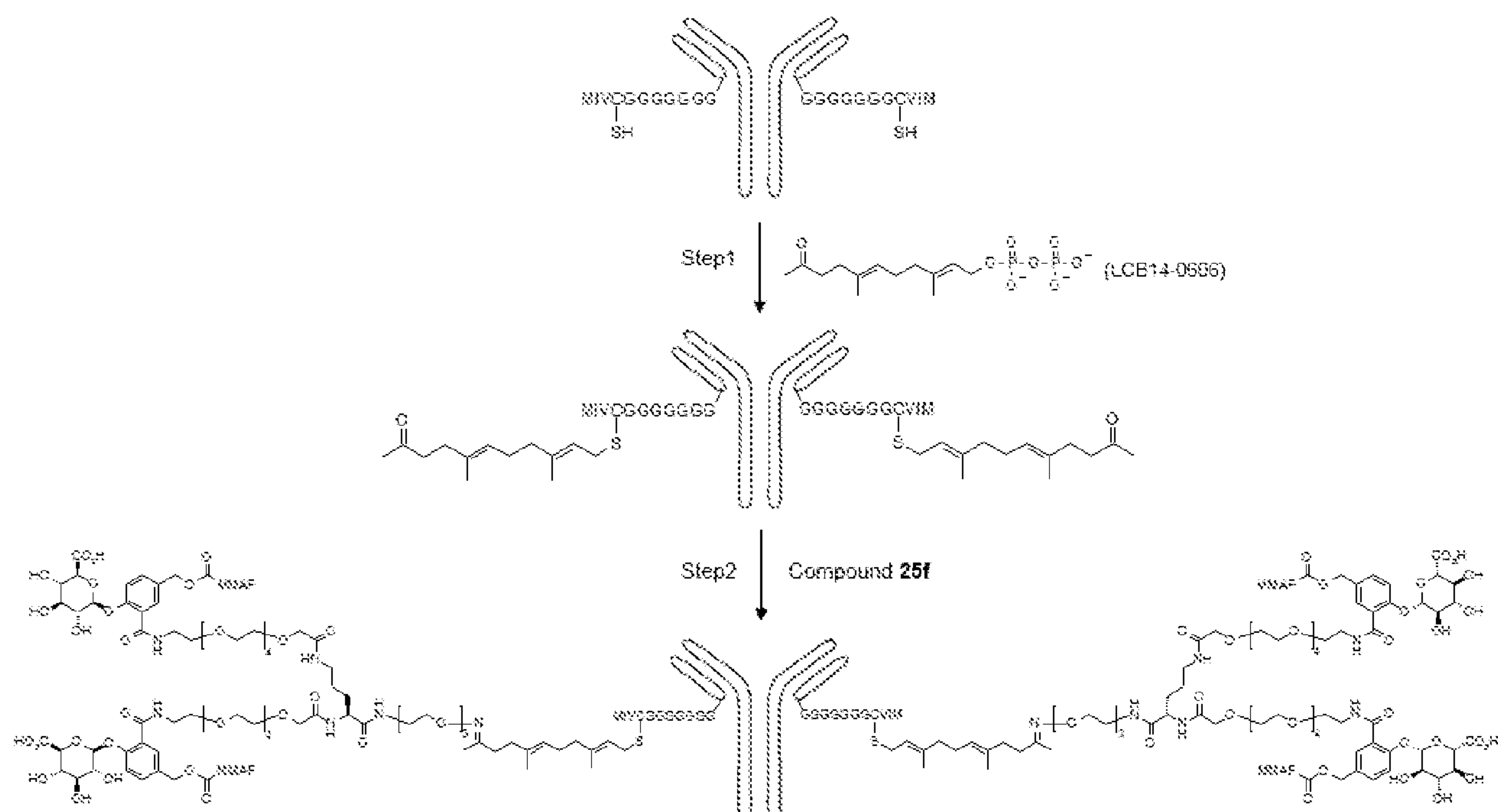
*Example 81. ADC86 Synthesis*

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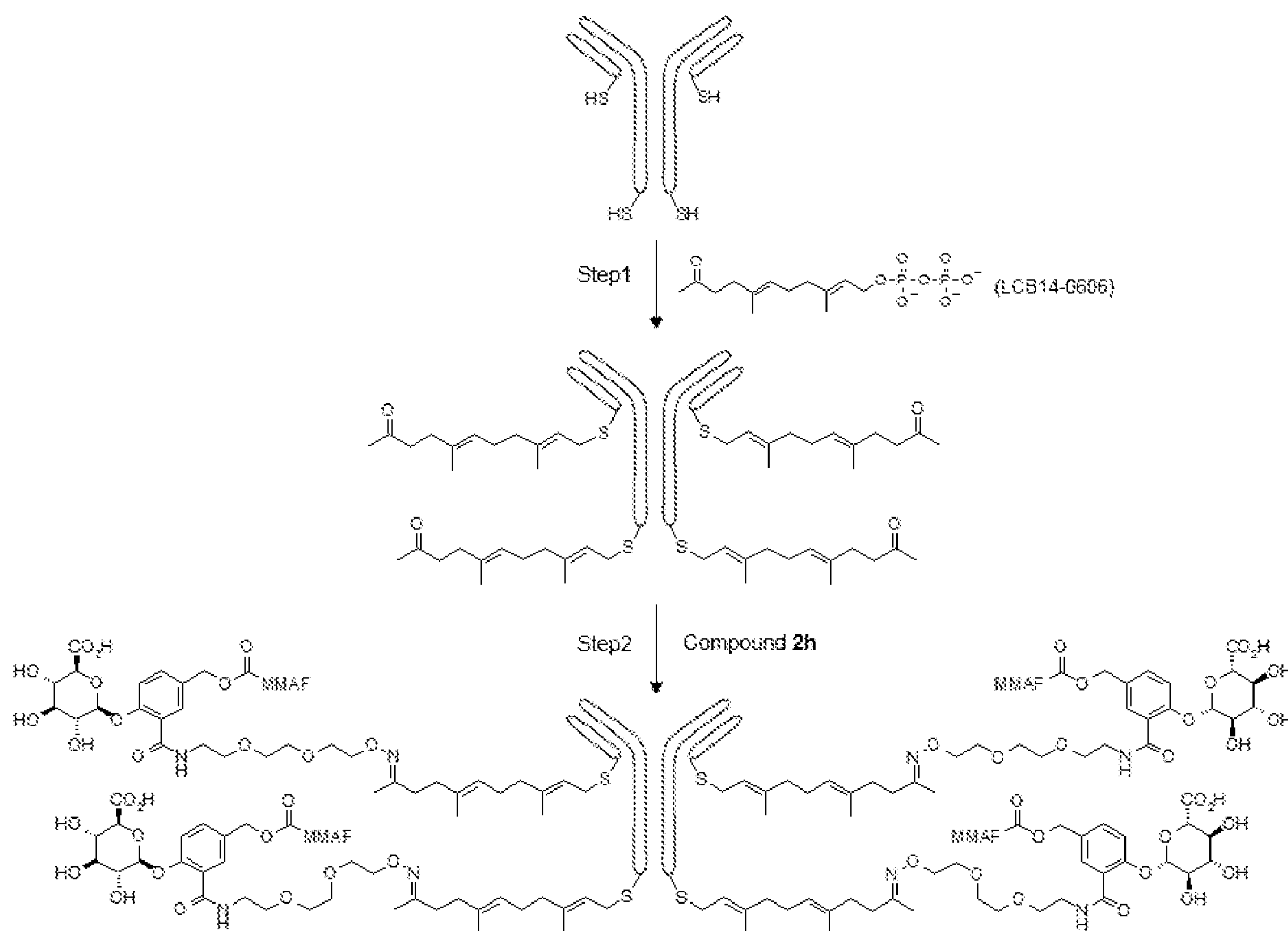


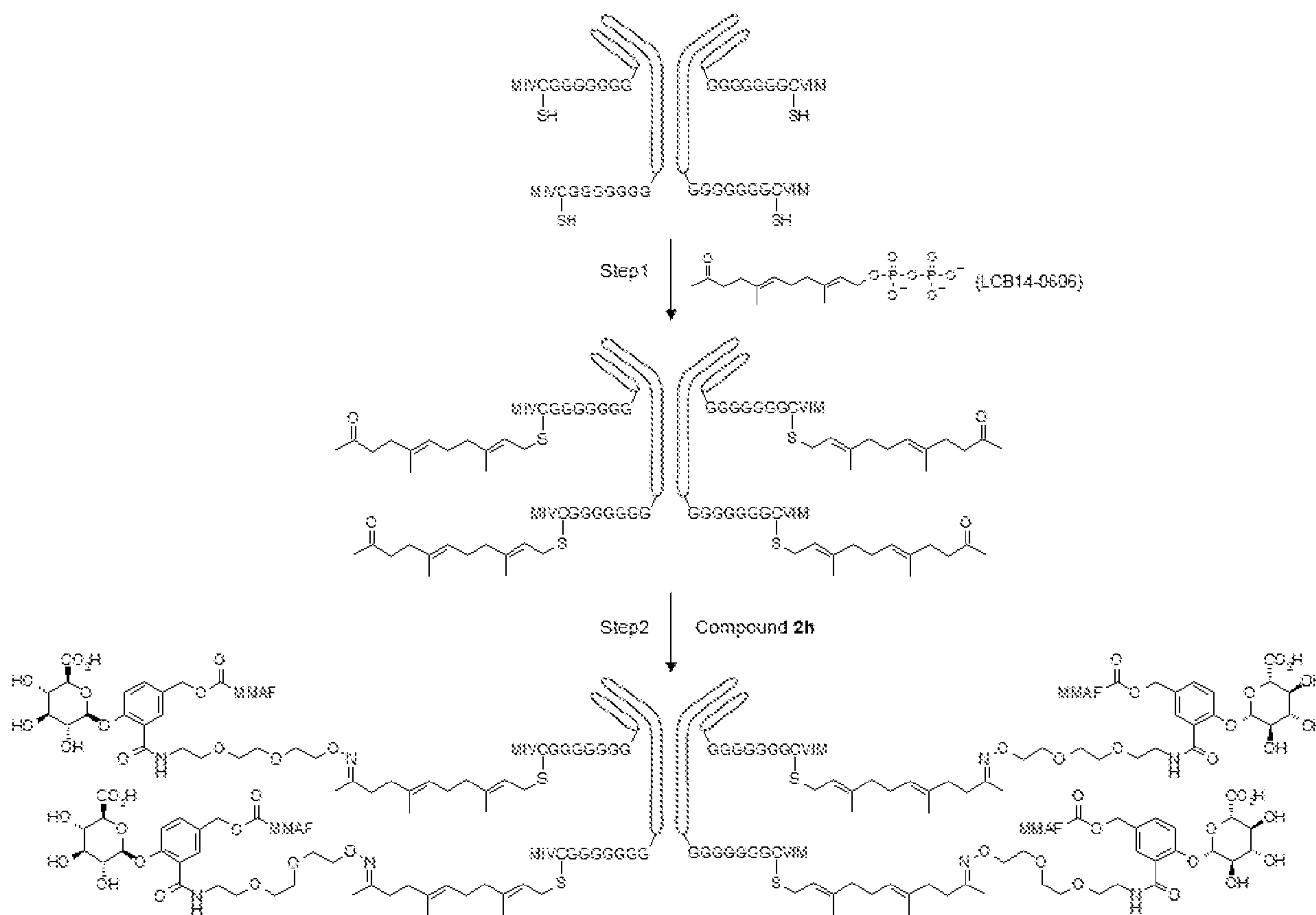
### Example 82. ADC4 Synthesis



Example 83. ADC4 SynthesisExample 84. ADC75 Synthesis

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Example 85. ADC75 Synthesis



*Experimental Example 1. Responsiveness Comparison Test with respect to  $\beta$ -Glucuronidase.*

In order to compare responsiveness of Compound 45k of Example 66 and Compound 50k of Comparative Example 66 to  $\beta$ -glucuronidase with each other, comparison test was performed as follows.

Compound 45k of Example 66 and Compound 50k of Comparative Example 66 were each prepared as 500  $\mu$ M and 50  $\mu$ M DMSO stock solutions. Reaction solutions in which 880  $\mu$ L of phosphate buffer saline (PBS) solution and 100  $\mu$ L of Compound 45k and Compound 50k stock solutions were mixed with each other, respectively, were prepared (final concentrations thereof were 50  $\mu$ M and 5  $\mu$ M, respectively). After 20  $\mu$ L of *E. coli*  $\beta$ -glucuronidase enzyme (1 mg/ml, Sigma: E.C.3.2.1.31 Type IX-A; 1 mg/mL in PBS; 3.6  $\mu$ g, 13  $\mu$ mol) was added to the reaction solutions, reactions were initiated in a constant temperature water bath at 37°C. 100  $\mu$ L of the mixed solutions were dispensed at 0 min, 25 min, 60 min, and 90 min, respectively, and 200  $\mu$ L of acetonitrile was added thereto. MMAF released from each of the supernatants obtained by performing centrifugation (4°C, 15 min, 14000 rpm) on the mixture samples was quantitatively analyzed using LC-MS/MS.

(the experiment was performed by a method similar to a method disclosed in U.S. Patent No. 8,568,728, hereby incorporated by reference).

The test results were illustrated in Figure 2, and it was confirmed from Figure 2 that MMAF was significantly rapidly released from each Compound 45k of Example 66 and Compound 50k of Comparative Example 66 through a 1,6-elimination reaction after enzyme reactions by  $\beta$ -glucuronidase (U.S. Patent No. 8,568,728, hereby incorporated by reference).

*Experimental Example 2. Plasma Stability Comparison Test linker toxin*

The plasma stability of Compound 45k of Example 66 and Compound 50k of Comparative Example 66 were compared.

10  $\mu$ L of Compound 45k or 50k was dissolved in DMSO at 5 mM, and each composition was mixed with 990  $\mu$ L of mouse plasma, thereby preparing 50  $\mu$ M samples, for assessing plasma stability. The plasma/compound solutions were incubated at 37 °C for 7 days. During the 6-day incubation, 100  $\mu$ L aliquots were taken at 0, 1, 2, and 7 days and mixed with 200  $\mu$ L of acetonitrile containing an internal standard for monitoring plasma protein precipitation. Supernatants were obtained by centrifuging the acetonitrile/plasma samples (4 °C, 15 min, 14000 rpm), and the amount of each compound and product was quantified by performing LC-MS/MS on the supernatants. (The experiment was performed using similar to those disclosed in J. Chromatography B, 780:451-457 (2002)).

Results obtained for Compound 45k of Example 66 and Compound 50k of Comparative Example 66 using LS-MS/MS are illustrated in Figure 3 and Table 1. The stability of Compound 50k of Comparative Example 66 and stability of Compound 45k of Example 66 was 14% and 80% at 1 day, respectively. Thus, the stability of Compound 45k of Example 66 in mouse plasma was superior to Compound 50k of Comparative Example 66.

Table 1. Stability of Compound 45k and Compound 50k in mouse plasma

|                                    | Compound 45k of Example 66 | Compound 50k of Comparative Example 66 |
|------------------------------------|----------------------------|----------------------------------------|
| Linker                             | Glucuronide                | Glucuronide                            |
| Plasma Stability<br>(mouse plasma) | 80% Stability<br>(@7 days) | 14% Stability<br>(@1 day)              |

|        |        |          |
|--------|--------|----------|
| Result | Stable | Unstable |
|--------|--------|----------|

The plasma stability of Compound 47a, 48a, and 49a were performed by using the method mentioned above (Figure 4-6).

### *Experimental Example 3. Preparation of Antibody-Drug Conjugate*

Step 1. Method of Prenylated Antibody (prepared according to the method of Korean Patent Laid-Open Publication No. 10-2014-0035393)

A prenylation reaction mixture of an antibody was prepared and reacted at 30°C for 16 hours. The antibodies comprising the GGGGGGGCVIM sequence (“G7CVIM”) added to the c-terminus of each light chain were used. The G7CVIM sequence was added at the C-terminus of heavy chain (ADC86-91) or both heavy and light chain (ADC75-77). The sources of sequences of antibodies used were like following Table 2.

Table 2. The used antibody list for ADC preparation

| Target (Antibody)           | References                                                                              |
|-----------------------------|-----------------------------------------------------------------------------------------|
| HER2 (Herceptin®)           | <a href="http://www.drugbank.ca/drugs/DB00002">http://www.drugbank.ca/drugs/DB00002</a> |
| EGFR (Erbix®)               | <a href="http://www.drugbank.ca/drugs/DB00002">http://www.drugbank.ca/drugs/DB00002</a> |
| CD19 (DI-B4)                | US8691952 B2                                                                            |
| CD20 (Rituxan®)             | <a href="http://www.drugbank.ca/drugs/DB00073">http://www.drugbank.ca/drugs/DB00073</a> |
| EGFR wt & EGFRvIII (ABT806) | US 2014/02555410 A9                                                                     |

The reaction mixture was composed of a buffer solution (50 mM Tris-HCl (pH7.4), 5 mM MgCl<sub>2</sub>, 10 μM ZnCl<sub>2</sub>, 0.25 mM DTT) containing 24 μM antibody, 200 nM FTase (Calbiochem #344145), and 144 μM LCB14-0606 (prepared in house according to the method of Korean Patent Laid-Open Publication No. 10-2014-0035393, hereby incorporated by reference). After the reaction was completed, a prenylated antibody was purified by FPLC.

### Step 2. Method of Preparing ADC

An oxime bond formation reaction mixture between the prenylated antibody and linker-toxin was prepared by mixing 100 mM Na-acetate buffer (pH 4.5, 10% DMSO), 12 μM prenylated antibody, and 120 μM linker-toxin (in house) and gently stirred at 30°C.

After incubating the reaction for 24 hours, the antibody-drug conjugate was purified by desalting via FPLC and hydrophobic interaction chromatography-HPLC.

Table 3. List of anti- HER2 ADCs (DAR2)

| ADC#  | Comp'd # |
|-------|----------|
| ADC1  | 2g       |
| ADC2  | 2h       |
| ADC3  | 3f       |
| ADC4  | 3g       |
| ADC5  | 4f       |
| ADC6  | 4g       |
| ADC7  | 5e       |
| ADC8  | 5f       |
| ADC9  | 6e       |
| ADC10 | 7e       |
| ADC11 | 8f       |
| ADC12 | 9j       |
| ADC13 | 10c      |
| ADC14 | 10d      |
| ADC15 | 11j      |
| ADC16 | 11k      |
| ADC17 | 12c      |
| ADC18 | 12d      |
| ADC19 | 13e      |
| ADC20 | 13f      |
| ADC86 | 2h       |
| ADC87 | 2g       |

Table 4. List of anti- HER2 ADCs (DAR4)

| ADC#  | Comp'd # |
|-------|----------|
| ADC23 | 16f      |
| ADC24 | 16g      |

|       |     |
|-------|-----|
| ADC25 | 17d |
| ADC26 | 18c |
| ADC27 | 19c |
| ADC28 | 20q |
| ADC29 | 21i |
| ADC30 | 22h |
| ADC31 | 23h |
| ADC32 | 24l |
| ADC33 | 25e |
| ADC34 | 25f |
| ADC35 | 26e |
| ADC36 | 27e |
| ADC37 | 28d |
| ADC38 | 28e |
| ADC39 | 29j |
| ADC40 | 29k |
| ADC41 | 30b |
| ADC42 | 30c |
| ADC43 | 31f |
| ADC44 | 31g |
| ADC45 | 32c |
| ADC46 | 32d |
| ADC47 | 33e |
| ADC48 | 33f |
| ADC49 | 34e |
| ADC50 | 34f |
| ADC51 | 35g |
| ADC52 | 36e |
| ADC53 | 37d |
| ADC54 | 38b |
| ADC55 | 38e |
| ADC76 | 2h  |

|       |     |
|-------|-----|
| ADC88 | 16f |
| ADC89 | 16g |
| ADC90 | 25f |
| ADC91 | 25e |

Table 5. List of anti- HER2 ADCs (DAR4<)

|        | ADC#  | Comp'd # |
|--------|-------|----------|
| <DAR6> | ADC60 | 43i      |
|        | ADC61 | 43j      |
| <DAR8> | ADC62 | 44i      |
|        | ADC63 | 44j      |
|        | ADC77 | 16f      |

Table 6. List of anti-HER2 ADCs using amanitin as a payload

| DAR 2 |          | DAR4  |          |
|-------|----------|-------|----------|
| ADC#  | Comp'd # | ADC#  | Comp'd # |
| ADC21 | 14m      | ADC56 | 39e      |
| ADC22 | 15b      | ADC57 | 40c      |
|       |          | ADC58 | 41c      |
|       |          | ADC59 | 42d      |

Table7. List of ADCs using antibodies targeting various proteins

| Target (Antibody) | ADC#  | Comp'd # |
|-------------------|-------|----------|
| EGFR (Erbitux®)   | ADC64 | 2h       |
|                   | ADC65 | 25e      |
|                   | ADC66 | 25f      |
| CD19 (DI-B4)      | ADC67 | 2h       |
|                   | ADC68 | 25e      |
|                   | ADC69 | 25f      |
| CD20 (Rituxan®)   | ADC70 | 2h       |
|                   | ADC71 | 25e      |
|                   | ADC72 | 25f      |

|                                |       |     |
|--------------------------------|-------|-----|
| EGFR wt & EGFRvIII<br>(ABT806) | ADC73 | 4g  |
|                                | ADC74 | 25e |
|                                | ADC75 | 25f |

*Experimental Example 4. Cytotoxicity of anti-HER2 ADCs*

Commercially available human breast cancer cell lines MCF-7 (HER2 negative to normal), OE-19 (HER2 positive), NCI-N87 (HER2 positive), SK-OV-3 (HER2 positive), JIMT-1 (HER2 positive), and SK-BR-3 (HER2 positive) were used. The cell lines were cultured according to recommended specifications provided with the commercially available cell lines.

Anti-proliferation activities of the antibodies, drugs, and conjugates with regard to the cancer cell lines were measured. The cells were plated in 96-well, tissue culture plates at  $1 \times 10^4$  cells per well. After 24 hour incubation, the antibodies, drugs, and conjugates were added in various concentrations. The number of viable cells after 72 hours were counted using SRB assay. Absorbance was measured at 540 nm using SpectraMax 190 (Molecular Devices, USA).

Table 8. IC<sub>50</sub> value of the different anti-HER2 ADCs (DAR2)

| Payload | ADC   | Linker-toxin | SK-BR-3 | JIMT-1 | OE-19 | NCI-N87 | SK-OV-3 | MCF-7  |
|---------|-------|--------------|---------|--------|-------|---------|---------|--------|
| MMAF    | ADC8  | 5f           | 0.10    | 0.34   | -     | -       | -       | >33.33 |
|         | ADC2  | 2h           | 0.14    | 0.16   | 0.42  | 0.75    | 1.19    | >33.33 |
|         | ADC4  | 3g           | 0.10    | 0.32   | -     | 0.97    | 1.21    | >33.33 |
|         | ADC6  | 4g           | 0.03    | 0.38   | -     | -       | -       | >33.33 |
|         | ADC16 | 11k          | 0.06    | 0.26   | -     | -       | -       | >33.33 |
|         | ADC14 | 10d          | 0.09    | 0.31   | -     | -       | -       | >33.33 |
|         | ADC18 | 12d          | 0.10    | 0.34   | -     | -       | -       | >33.33 |
|         | ADC20 | 13f          | 0.08    | 0.29   | -     | -       | -       | >33.33 |
| MMAE    | ADC7  | 5e           | 0.10    | 13.44  | -     | -       | -       | >33.33 |
|         | ADC1  | 2g           | 0.47    | 275    | -     | 0.69    | 241     | >33.33 |
|         | ADC3  | 3f           | 0.29    | 1.63   | -     | 1.04    | 1.34    | >33.33 |
|         | ADC5  | 4f           | 0.15    | 0.97   | -     | 0.40    | 1.04    | >33.33 |

|  |       |     |      |       |   |   |   |        |
|--|-------|-----|------|-------|---|---|---|--------|
|  | ADC9  | 6e  | 0.10 | >33.3 | - | - | - | >33.33 |
|  | ADC15 | 11j | 0.17 | >33.3 | - | - | - | >33.33 |
|  | ADC10 | 7e  | 0.12 | 0.64  | - | - | - | >33.33 |
|  | ADC12 | 9j  | 0.20 | 2.01  | - | - | - | >33.33 |
|  | ADC13 | 10c | 0.19 | 1.09  | - | - | - | >33.33 |
|  | ADC17 | 12c | 0.03 | 0.11  | - | - | - | >33.33 |
|  | ADC19 | 13e | 0.13 | 0.38  | - | - | - | >33.33 |

Table 9. IC<sub>50</sub> value of the different PEG length-ADCs

| Test samples |     |      |               |              | IC <sub>50</sub> (nM) |       |
|--------------|-----|------|---------------|--------------|-----------------------|-------|
| Toxin        | DAR | ADC  | # of PEG unit | Linker-toxin | JIMT-1                | MCF-7 |
| MMAE         | 2   | ADC7 | 1             | 5e           | >10.0                 | >10.0 |
|              |     | ADC1 | 3             | 2g           | >10.0                 | >10.0 |
|              |     | ADC3 | 6             | 3f           | >10.0                 | >10.0 |
|              |     | ADC5 | 12            | 4f           | 1.01                  | >10.0 |

Table 10. IC<sub>50</sub> value of MMAF ADCs with the different types of linkers

| ADC   | Linker-toxin | SK-BR-3 | JIMT-1 | NCI-N87 | SK-OV-3 | MCF-7  |
|-------|--------------|---------|--------|---------|---------|--------|
| ADC23 | 16f          | 0.05    | 0.12   | 0.35    | 0.44    | >33.33 |
| ADC34 | 25f          | 0.03    | 0.10   | -       | -       | >33.33 |
| ADC38 | 28e          | 0.03    | 0.05   | -       | -       | >33.33 |
| ADC40 | 29k          | 0.02    | 0.06   | -       | -       | >33.33 |
| ADC42 | 30c          | 0.03    | 0.04   | -       | -       | >33.33 |
| ADC44 | 31g          | 0.03    | 0.05   | -       | -       | >33.33 |
| ADC46 | 32d          | 0.04    | 0.08   | -       | -       | >33.33 |
| ADC48 | 33f          | 0.03    | 0.08   | -       | -       | >33.33 |
| ADC50 | 34f          | 0.03    | 0.05   | -       | -       | >33.33 |
| ADC53 | 37d          | 0.03    | 0.05   | -       | -       | >33.33 |
| ADC54 | 38b          | 0.05    | 0.12   | -       | -       | >33.33 |

Table 11. IC<sub>50</sub> value of MMAE ADCs with the different types of linkers

| ADC   | Linker-toxin | SK-BR-3 | JIMT-1 | NCI-N87 | SK-OV-3 | MCF-7  |
|-------|--------------|---------|--------|---------|---------|--------|
| ADC24 | 16g          | 0.14    | 0.26   | 0.17    | 0.37    | >33.33 |
| ADC25 | 17d          | 0.03    | 0.10   | 0.36    | 0.37    | >33.33 |
| ADC26 | 18c          | 0.08    | 0.14   | -       | -       | >33.33 |
| ADC27 | 19c          | 0.05    | 0.43   | 0.29    | 0.38    | >33.33 |
| ADC28 | 20q          | 0.03    | 0.12   | 0.36    | 0.36    | >33.33 |
| ADC29 | 21i          | 0.03    | 0.41   | 0.31    | 0.49    | >33.33 |
| ADC30 | 22h          | 0.05    | 0.41   | 0.30    | 0.35    | >33.33 |
| ADC31 | 23h          | 0.14    | 0.24   | -       | -       | >33.33 |
| ADC32 | 24l          | 0.03    | 0.40   | 0.32    | 0.35    | >33.33 |
| ADC33 | 25e          | 0.04    | 0.23   | 0.27    | 0.33    | >33.33 |
| ADC35 | 26e          | 0.13    | 0.19   | -       | -       | >33.33 |
| ADC36 | 27e          | 0.13    | 0.22   | -       | -       | >33.33 |
| ADC37 | 28d          | 0.04    | 0.07   | -       | -       | >33.33 |
| ADC39 | 29j          | 0.03    | 0.11   | -       | -       | >33.33 |
| ADC41 | 30b          | 0.03    | 0.10   | -       | -       | >33.33 |
| ADC43 | 31f          | 0.03    | 0.04   | -       | -       | >33.33 |
| ADC45 | 32c          | 0.03    | 0.08   | -       | -       | >33.33 |
| ADC47 | 33e          | 0.04    | 0.07   | -       | -       | >33.33 |
| ADC49 | 34e          | 0.03    | 0.05   | -       | -       | >33.33 |
| ADC51 | 35g          | 0.04    | 0.24   | 0.34    | -       | >33.33 |
| ADC52 | 36e          | 0.07    | 0.40   | 0.37    | -       | >33.33 |
| ADC55 | 38e          | 0.04    | 0.07   | -       | -       | >33.33 |

Table 12. IC<sub>50</sub> value of the hybrid ADCs

| Test samples |     |       |              | IC <sub>50</sub> (nM) |        |         |         |        |
|--------------|-----|-------|--------------|-----------------------|--------|---------|---------|--------|
| Toxin        | DAR | ADC   | Linker-toxin | SK-BR-3               | JIMT-1 | NCI-N87 | SK-OV-3 | MCF-7  |
| MMAF         | 2   | ADC2  | 2h           | 0.08                  | 0.40   | 0.84    | 0.84    | >33.33 |
|              | 4   | ADC23 | 16f          | 0.05                  | 0.10   | 0.25    | 0.36    | >33.33 |

|               |   |       |     |      |       |      |      |        |
|---------------|---|-------|-----|------|-------|------|------|--------|
| Amanitin      | 2 | ADC21 | 14m | 0.09 | >33.3 | 0.88 | 0.25 | >33.33 |
| MMAF          | 4 | ADC58 | 41c | 0.06 | 0.73  | 0.95 | 0.64 | >33.33 |
| &<br>Amanitin | 8 | ADC78 | 41c | 0.03 | 0.03  | 0.33 | 0.76 | >33.33 |
| MMAF-OMe      |   |       |     | 0.12 | 0.07  | 0.49 | 0.78 | 0.60   |

The comparison of two different toxin conjugated ADCs and same toxin conjugated ADCs

Table 13. IC<sub>50</sub> value of the various DAR-ADCs

| Test samples |          |              | IC <sub>50</sub> (nM) |        |
|--------------|----------|--------------|-----------------------|--------|
|              |          |              | JIMT-1                | MCF-7  |
| DAR#         | ADC code | Linker-toxin |                       |        |
| DAR2         | ADC2     | 2h           | 1.37                  | >33.33 |
| DAR4         | ADC23    | 16f          | 0.21                  | >33.33 |
|              | ADC34    | 25f          | 0.09                  | >33.33 |
|              | ADC76    | 2h           | 0.27                  | >33.33 |
| DAR8         | ADC62    | 44i          | 0.08                  | >33.33 |
|              | ADC77    | 16f          | 0.02                  | >33.33 |

*Experimental Example 5. Cytotoxicity of Erbitux (LC)-Glucuronide Linker-MMAF*

A431 cells, which express high levels of EGFR, and MCF-7 cells, which express low levels of EGFR, were plated at about 1000 cells per well in a 96-well plate in 100  $\mu$ L of media. HCC-827 cells, which express an intermediate level of EGFR were plated at about 5000 cells per well in a 96-well plate in 100  $\mu$ L of media. The cells were incubated at 37°C in 5% CO<sub>2</sub> for 24 hours. Then, serial dilutions of monomethyl auristatin F-OMe (MMAF-OMe), Erbitux (LC)-G7CVIM, and the antibody drug conjugate ADC64 comprising Erbitux (LC)-G7CVIM and MMAF were added to the cells at concentrations of 100 to 0.00128 nM. The cells were incubated for 72 hours and then fixed for 1 hour at 4°C after adding 100  $\mu$ L of ice-cold 10% trichloroacetic acid to each well. Viable cells were counted using SRB dye (Sulforhodamine B, Sigma S1402) and a Molecular Devices SpectraMax 190 plate reader running Softmax Pro v5, monitoring absorbance at 540 nm (Table 14)

Erbitux (LC)-G7CVIM had an IC<sub>50</sub> greater than 100 nM for each cell line (A431,

MCF-7, and HCC-827). MMAF-OMe had an IC<sub>50</sub> of 1.81 nM against MCF-7 cells, 1.99 nM against HCC-827 cells, and 1.11 nM against A431 cells. The antibody-drug conjugate ADC64, 65, and 66 had an IC<sub>50</sub> of greater than 100 nM against MCF-7 cells, 0.47, 0.17, and 0.11 nM against HCC-827 cells, respectively. ADC64 showed 1.3 nM against A431 cells, thus displaying superior specificity over MMAF-OMe and superior potency over Erbitux (LC)-G7CVIM.

Table 14. IC<sub>50</sub> value of anti-EGFR mAb, Erbitux based ADCs

| ADC Code | Cell-lines |         |        |
|----------|------------|---------|--------|
|          | A431       | HCC-827 | MCF-7  |
| ADC64    | 1.30       | 0.47    | >33.33 |
| ADC65    | -          | 0.17    | >33.33 |
| ADC66    | -          | 0.11    | >33.33 |

*Experimental Example 6. Cytotoxicity of ABT-806 (LC)-Glucuronide Linker-MMAF*

Cytotoxicity of ABT-806 based ADCs were tested against patient derived cell lines established Samsung Medical Center (Seoul, Republic of Korea). The cells were maintained in Neurobasal®-A Media (Thermo Fisher Scientific) with supplement of L-glutamine (200 nM), bFGF (20 ng/mL), EGF (20 ng/mL), N2 supplement, and B27 supplement. For the viability test, cells were aliquoted to 96-well plate (5000 cells/well) and incubated at 37°C in 5% CO<sub>2</sub> for 1 day before treatment. After ADC treatment, cells were incubated for 72 hr. 100 µL of CellTiter-Glo® Reagent (Promega) was added to each well to analyze the cell viability. After 10 minutes incubation, luminescent signal was analyzed using Luminometer.

DAR4 ADCs (ADC74, ADC75) had better potency than DAR2 ADC (ADC73) as expected. Some patient's cells showed a little different sensitive to payload. 22 & 780 cells were more sensitive to MMAF over MMAE, 464 cells vice versa.

Table 15. Cytotoxic activity of ABT-806 based ADCs against patient derived cell lines

| Test samples |       | Patient Derived Cell-lines |       |       |       |       |
|--------------|-------|----------------------------|-------|-------|-------|-------|
|              |       | vIII352T1                  | 780   | 437   | 464   | 22    |
| ABT806       | ADC73 | 0.572                      | 0.959 | 0.357 | 0.472 | 0.501 |
|              | ADC75 | 0.104                      | 0.227 | 0.241 | 0.151 | 0.282 |

|  |       |       |       |       |       |       |
|--|-------|-------|-------|-------|-------|-------|
|  | ADC74 | 0.170 | 0.425 | 0.253 | 0.069 | 0.489 |
|--|-------|-------|-------|-------|-------|-------|

#### *Experimental Example 7. Cytotoxicity of anti-CD19 ADCs*

Ramos cells, which are human Burkitt's lymphoma cells, were seeded in a 96-well plate at 20,000 cells/well in 100  $\mu$ L of growth media. The cells were incubated at 37°C in 5% CO<sub>2</sub> for 1 day. Serial dilutions of anti-CD19 antibodies DI-B4-(LC)-G7CVIM and ADCs from 33.33 nM to 5.1 pM in 100  $\mu$ L media were added to the wells, and the cells were incubated with the antibody & ADCs for 72 hours. Cell viability was assessed using WST-1 (TaKaRa MK400) and a Molecular Devices SpectraMax 190 plate reader running Softmax Pro v5, monitoring absorbance at 450 nm (Table 16).

The experiments in Ramos cells were performed in parallel with experiments on K562 cells, human myelogenous leukemia cells that do not express CD19, as a negative control to assess any non-specific cytotoxicity.

ADC68 and ADC69 displayed an IC<sub>50</sub> of 0.09 nM against Ramos cells, which was superior to unconjugated DI-B4 (Table 16). No antibody displayed cytotoxicity below 33.33 nM against the K562 control cells.

Table 16. Cytotoxic activity of anti-CD19 antibody based ADCs

| ADC Code | Cell-lines |        |
|----------|------------|--------|
|          | Ramos      | K562   |
| ADC67    | >33.33     | >33.33 |
| ADC68    | 0.09       | >33.33 |
| ADC69    | 0.09       | >33.33 |

#### *Experimental Example 8. Cytotoxicity of Rituxan based ADCs*

Ramos cells, which are human Burkitt's lymphoma cells, were seeded in a 96-well plate at 20,000 cells/well in 100  $\mu$ L of growth media. The cells were incubated at 37°C in 5% CO<sub>2</sub> for 1 day. Serial dilutions of Rituxan (LC)-G7CVIM and ADCs from 33.33 nM to 5.1 pM in 100  $\mu$ L media were added to the wells, and the cells were incubated with the antibody & ADCs for 72 hours. Cell viability was assessed using WST-1 (TaKaRa MK400) and a Molecular Devices SpectraMax 190 plate reader running Softmax Pro v5, monitoring absorbance at 450 nm (Table 17).

The experiments in Ramos cells were performed in parallel with experiments on K562 cells, human myelogenous leukemia cells that do not express CD20, as a negative control to assess any non-specific cytotoxicity.

ADC70, ADC71, and ADC72 displayed an  $IC_{50}$  of 4.56 nM, 1.47 nM, and 1.78 nM against Ramos cells respectively, which was superior to unconjugated anti-CD20 antibody (Table 17). No antibody displayed cytotoxicity below 33.33 nM against the K562 control cells.

Table 17. Cytotoxic activity of Rituxan-based ADCs

| ADC Code | Cell-lines |        |
|----------|------------|--------|
|          | Ramos      | K562   |
| ADC70    | 4.56       | >33.33 |
| ADC71    | 1.47       | >33.33 |
| ADC72    | 1.78       | >33.33 |

*Experimental Example 9. Differences in Beta glucuronidase susceptibility*

ADCs in 0.06 M Na-acetate buffer (pH5.2) were aliquoted into the 1.5 mL micro tube. The final concentration of ADC in the mixture was adjusted to 12  $\mu$ M. 0.001  $\mu$ g of human  $\beta$ -glucuronidase (R&D systems: 6144-GH-020) was added to each tube. Then, the mixtures were incubated at 37°C water bath for 3h. The reaction was terminated by the addition of cold PBS buffer (pH7.4) to the 15-fold dilution. The change of ADC-pattern by beta-glucuronidase was analyzed by HIC-HPLC. The efficacy of enzyme activity was visualized by % of remaining (Figure 10)

The attribute to susceptibility seemed to be the Branch Unit (BR) of linker-toxin part. When Lys was located in BR, the toxin release was occurred very efficiently. Amide and amine showed less susceptibility than Lys.

*Experimental Example 10. Plasma stability of ADCs*

To compare plasma stability between ADC2 (Herceptin- LBG-MMAF, DAR2) and Kadcyla, those ADCs were incubated in mouse and human plasma for 5 seconds (0h) or 96 hours (96h), followed by SRB *in vitro* cytotoxicity test using SK-BR3 cells for 72 hr. Plasma-incubated ADC2 retains potent cytotoxicity (no change in  $IC_{50}$ ; 0.06 (0h) and 0.07 nM (96h) for MP, 0.08 (0h) and 0.08 nM (96h) for HP) while plasma-incubated Kadcyla displayed decreased cytotoxicity compared to 0h Kadcyla (increase in  $IC_{50}$ ; 0.26 (0h) and

1.59 nM (96h) for MP, 0.29 (0h) and 4.21 nM (96h) for HP) (Figure 11)

To characterize the plasma stability of ADCs made of various antibody, ADCs were incubated in human plasma for 5 seconds (0h) or 168 hours (168h), followed by SRB *in vitro* cytotoxicity test using SK-BR3 cells for 72hr. (Table 18-20, and Figure 12)

Table 18. Plasma stability of Herceptin based ADCs (nM)

| Test samples |      |       | Plasma incubation time |      |
|--------------|------|-------|------------------------|------|
|              |      |       | 0h                     | 168h |
| MMAF-OMe     |      |       | 0.48                   | N.D. |
| Herceptin    | MMAF | ADC2  | 0.09                   | 0.15 |
|              |      | ADC6  | 0.07                   | 0.09 |
|              |      | ADC8  | 0.11                   | 0.18 |
|              |      | ADC14 | 0.06                   | 0.08 |
|              |      | ADC16 | 0.05                   | 0.07 |
|              |      | ADC23 | 0.04                   | 0.05 |
|              |      | ADC34 | 0.03                   | 0.04 |
|              |      | ADC40 | 0.03                   | 0.05 |
|              |      | ADC46 | 0.03                   | 0.03 |
|              |      | ADC48 | 0.03                   | 0.04 |
|              |      | ADC62 | 0.02                   | 0.02 |
|              | MMAE | ADC1  | 0.26                   | 0.41 |
|              |      | ADC3  | 0.20                   | 0.32 |
|              |      | ADC5  | 0.19                   | 0.31 |
|              |      | ADC7  | 0.17                   | 0.26 |
|              |      | ADC12 | 0.51                   | 0.79 |
|              |      | ADC13 | 0.63                   | 0.87 |
|              |      | ADC15 | 0.52                   | 0.70 |
|              |      | ADC24 | 0.08                   | 0.11 |
|              |      | ADC25 | 0.07                   | 0.12 |
|              |      | ADC26 | 0.17                   | 0.22 |
|              |      | ADC27 | 0.10                   | 0.14 |

|  |  |       |      |      |
|--|--|-------|------|------|
|  |  | ADC28 | 0.07 | 0.08 |
|  |  | ADC29 | 0.06 | 0.08 |
|  |  | ADC30 | 0.15 | 0.19 |
|  |  | ADC31 | 0.08 | 0.12 |
|  |  | ADC32 | 0.05 | 0.09 |
|  |  | ADC33 | 0.04 | 0.09 |
|  |  | ADC35 | 0.11 | 0.21 |
|  |  | ADC36 | 0.09 | 0.12 |
|  |  | ADC39 | 0.12 | 0.17 |
|  |  | ADC45 | 0.04 | 0.05 |
|  |  | ADC47 | 0.04 | 0.05 |
|  |  | ADC61 | 0.32 | 0.32 |

Table 19. Plasma stability of anti-CD19 antibody based ADCs (nM)

| Test samples |       | Plasma incubation |       |
|--------------|-------|-------------------|-------|
|              |       | 0h                | 168h  |
| MMAF-OMe     |       | 0.160             | N.D.  |
| CD19         | ADC68 | 0.036             | 0.048 |
|              | ADC69 | 0.047             | 0.135 |

Table 20. Plasma stability of anti-CD20 antibody based ADCs (nM)

| Test samples |       | Plasma incubation |       |
|--------------|-------|-------------------|-------|
|              |       | 0h                | 168h  |
| MMAF-OMe     |       | 0.160             | N.D.  |
| CD20         | ADC71 | 4.001             | 4.134 |
|              | ADC72 | 2.026             | 3.851 |

*Experimental Example 11. Pharmacokinetics of Herceptin® and ADCs*

Male Sprague Dawley rats were dosed intravenously with 3 mg/kg of antibodies or the antibody-drug conjugates. Blood samples were taken at multiple time points after dosing, chilled in ice water, and plasma was isolated. Plasma was frozen at -80°C until subsequent

## LC/MS/MS analysis.

20  $\mu\text{L}$  of each sample was mixed with 340  $\mu\text{L}$  of PBS and 60  $\mu\text{L}$  of protein A magnetic beads and incubated for 2 hours at room temperature with gentle shaking. The beads were washed three times with PBS. Then, 25  $\mu\text{L}$  of an internal standard (isotope-labeled peptides at 10  $\mu\text{g/mL}$ ), 75  $\mu\text{L}$  of RapiGest SF (Waters), and 10  $\mu\text{L}$  of dithiothreitol were added to the beads. The mixture was shaken for 1 minute and then incubated for 1 hour at 60°C. 25  $\mu\text{L}$  of iodoacetic acid was added to the mixture, the mixture was shaken for 1 minute, and then incubated for 30 minutes at room temperature. 10  $\mu\text{L}$  of sequencing grade modified trypsin (Promega) was added to the mixture, the mixture was shaken for 1 minute, and the mixture was incubated overnight at 37°C. 15  $\mu\text{L}$  of hydrochloric acid was added to the mixture, the mixture was shaken for 1 minute, and the mixture was incubated for 30 minutes at 37°C. The mixture was centrifuged at  $5000 \times g$  for 10 minutes at 4°C and the supernatant was transferred into an HPLC vial.

The liquid chromatography-mass spectrometry system consisted of two Shimadzu LC-20AD pumps, a Shimadzu CBM-20A HPLC pump controller (Shimadzu Corporation, Columbia, MD, USA), a CTC HTS PAL autosampler (CEAP Technologies, Carrboro, NC, USA) and a triple time of flight 5600 mass spectrometer (Triple TOF MS) (AB Sciex, Foster City, CA, USA). The analytical column was a Phenomenex Kinetex XB-C18 column,  $2.1 \times 30$  (2.6  $\mu\text{m}$ ). HPLC was performed with a water/acetonitrile gradient and acidified with 0.1% formic acid. Injection volumes were 10  $\mu\text{L}$ . Triple TOF MS, equipped with a Duospray<sup>TM</sup> ion source, was used to complete the high resolution experiment. The Triple TOF MS was operated in the positive ion mode. High-purity nitrogen gas was used for the nebulizer/Duospray<sup>TM</sup> and curtain gases. The source temperature was set at 500°C with a curtain gas flow of 30 L/min. The ion spray voltage was set at 5500 V, declustering potential was 145 V, and the collision energy was 38 V. The product ion mode was used as scan mode. Analyst<sup>®</sup> TF Version 1.6 (AB Sciex) operated with Windows<sup>®</sup> (Microsoft) was used for instrument control and data acquisition. Peak integrations were performed with MultiQuant<sup>®</sup> Version 2.1.1 (AB Sciex). Calculations were performed with MultiQuant<sup>®</sup> Version 2.1.1 for peak area ratios, standard curve regressions, sample concentration values, and descriptive statistics. The LC/MS/MS was calibrated using standard solutions at concentrations of 0.1, 0.4, 1, 2, 5, 10, 20, 40, 80, and 100  $\mu\text{g/mL}$ . A representative PK profile was shown in Figure 13. PK profile of ADC2 (Herceptin (LC)-MMAF, DAR2) was a very similar with that of Herceptin.

*Experimental Example 12. PEG (connecting unit) combination effect in Branched linker-toxin*

To identify critical attributes that affect PK profile of ADC, different length and structure of connecting unit (PEG number and arrangement) were tested. Experiment for PK analysis was done as described in experimental example 9. Although ADC23 (a DAR4 ADC) had more potent efficacy *in vitro* and *in vivo* than DAR2 ADCs, its PK profile was reduced in half life and AUC (Figure 14). By replacing linker-toxin from 16f to 25f (attaching additional connecting unit (3 PEGs) to after branch unit (BR), PK profile had been recovered as much as that of Herceptin (Figure 14). These effects were well reproduced in MMAE based ADCs, ADC24 & ADC33 (Figure 15). Because MMAE is more hydrophobic than MMAF, ADCs based on MMAE has worsened in PK profile as shown by ADC1 and ADC24. Adding longer PEG unit was traditional application for extending half-life and AUC. However, simple elongation of PEG unit numbers from 3 to 12 didn't show big differences, when comparing ADC1 with ADC5. In the other hand, by replacing linear linker unit(compound 2g or 4f) to branched one (compound 11j, ADC15), the PK profile was significantly improved (Figure 16), indicating that critical attribute for PK might not be a just simple length, but the structure of connecting unit.

*Experimental Example 13. Effects of hydrophilic connecting unit in PK of ADC*

Many payloads used for ADC have hydrophobic character, resulted in bad PK property. To compensate the hydrophobicity, hydrophilic compounds were tested as a part of connecting unit. Inserting hydrophilic compounds such as Asp enhanced AUC and half-life of ADCs (Figure 17, 18, 19). In cases of DAR2, ADC with connecting unit including Asp showed higher AUC than Herceptin (Figure 17, 18). The compensate effect by polar amino acid, such as Asp or Glu, can be observed in ADCs with DAR4 (Figure 19, 20). ADC49 (2 Asp) and ADC52 (2 D-Glu) were superior to ADC47 (1 Asp) and ADC51 (1D-Glu) respectively in AUC and half-life.

*Experimental Example 14. in vivo efficacy*

A frozen JIMT-1 cell stock was thawed and cultivated under the 37°C, 5% CO<sub>2</sub> condition. JIMT-1 cells of the best condition that the viability was more than 95% were used for implantation. Cells of 5×10<sup>6</sup> suspended in 50 µL cold-saline were implanted into right hind leg of balb/c-nude mouse. 5 mice per group were used for the experiments.

Tumor formation and growth were periodically monitored. Tumor volume was calculated by the formulation;  $\text{volume} = (a^2b)/2$ , “a” means short diameter and “b” means long diameter.

When the tumor volume reaches to about  $200 \text{ mm}^3$ , mice having average value were selected and grouped according to tumor volume. Then, mice were treated with PBS (vehicle control), or ADCs indicated in Figure 21 and 22. Tumor size was determined 2 times a week in 3~4 days interval during the experimental period. Tumor volumes measured from the first day of administration to the end date were plotted for tumor growth curve.

Representative ADCs were tested by single injection. In general, the ADCs with branching unit (BR) containing Lys had better efficacy than ADCs with BR containing amide.

#### INCORPORATION BY REFERENCE

Each of the patents, published patent applications, and non-patent references cited herein are hereby incorporated by reference in their entirety.

#### EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed:

1. A ligand-drug conjugate, comprising a ligand; a linker covalently coupled to the ligand; and at least two active agents covalently coupled to the linker, wherein:  
the linker comprises a peptide sequence of a plurality of amino acids; and  
at least two of the active agents are covalently coupled to side chains of the amino acids.
2. The conjugate of claim 1, wherein the ligand is an antibody.
3. The conjugate of any one of the preceding claims, wherein at least two of the active agents are covalently coupled to side chains of lysine, 5-hydroxylysine, 4-oxalysine, 4-thialysine, 4-selenalysine, 4-thiahomolysine, 5,5-dimethyllysine, 5,5-difluorolysine, *trans*-4-dehydrolysine, 2,6-diamino-4-hexynoic acid, *cis*-4-dehydrolysine, 6-*N*-methyllysine, diaminopimelic acid, ornithine, 3-methylornithine,  $\alpha$ -methylornithine, citrulline, and/or homocitrulline.
4. The conjugate of claim 3, wherein at least two of the active agents are covalently coupled to side chains of lysine or ornithine.
5. The conjugate of any one of the preceding claims, wherein the plurality of amino acids comprise L-amino acids.
6. The conjugate of any one of the preceding claims, wherein the plurality of amino acids comprise D-amino acids.
7. The conjugate of any one of the preceding claims, wherein the plurality of amino acids comprise  $\alpha$ -amino acids.
8. The conjugate of any one of the preceding claims, wherein the plurality of amino acids comprise  $\beta$ -amino acids.
9. The conjugate of any one of the preceding claims, wherein the plurality of amino acids comprises alanine, aspartate, asparagine, glutamate, glutamine, glycine, histidine,

lysine, ornithine, proline, serine, and/or threonine.

10. The conjugate of any one of the preceding claims, wherein the peptide does not comprise isoleucine, methionine, leucine, phenylalanine, tryptophan, tyrosine, or valine amino acids.

11. The conjugate of any one of the preceding claims, wherein the peptide comprises 2 to 20 amino acids.

12. The conjugate of any one of the preceding claims, wherein the N-terminus or the C-terminus of the peptide is substituted with a group that covalently links at least one of the active agents to the peptide.

13. The conjugate of any one of the preceding claims, wherein the N-terminus or the C-terminus of the peptide is substituted with a group that covalently links the ligand to the peptide.

14. The conjugate of the preceding claims, wherein:  
the linker comprises an O-substituted oxime;  
the oxygen atom of the oxime is substituted with a group that covalently links the oxime to the peptide; and  
the carbon atom of the oxime is substituted with a group that covalently links the oxime to the ligand.

15. The conjugate of the preceding claims, wherein:  
the linker comprises an O-substituted oxime;  
the carbon atom of the oxime is substituted with a group that covalently links the oxime to the peptide; and  
the oxygen atom of the oxime is substituted with a group that covalently links the oxime to the ligand.

16. The conjugate of the preceding claims, wherein:  
the linker comprises an alkylene having 1 to 100 carbon atoms;

the alkylene comprises at least one unsaturated bond;  
at least one carbon atom of the alkylene is replaced by one or more heteroatoms selected from nitrogen (N), oxygen (O), and sulfur (S); and  
the alkylene is further substituted with at least one alkyl having 1 to 20 carbon atoms.

17. The conjugate of any one of the preceding claims, wherein the linker is covalently bound to the ligand by a thioether bond, and the thioether bond comprises a sulfur atom of a cysteine of the ligand.

18. The conjugate of claim 17, wherein:  
the ligand comprises a C-terminal amino acid motif that is recognized by an isoprenoid transferase; and  
the thioether bond comprises a sulfur atom of a cysteine of the amino acid motif.

19. The conjugate of claim 18, wherein:  
the amino acid motif is a sequence CYYX;  
C represents cysteine;  
Y, independently for each occurrence, represents an aliphatic amino acid;  
X, independently for each occurrence, represents glutamine, glutamate, serine, cysteine, methionine, alanine, or leucine; and  
the thioether bond comprises a sulfur atom of a cysteine of the amino acid motif.

20. The conjugate of claim 19, wherein:  
the amino acid motif is a sequence CYYX; and  
Y, independently for each occurrence, represents alanine, isoleucine, leucine, methionine, or valine.

21. The conjugate of claim 20, wherein the amino acid motif is a sequence CVIM or CVLL.

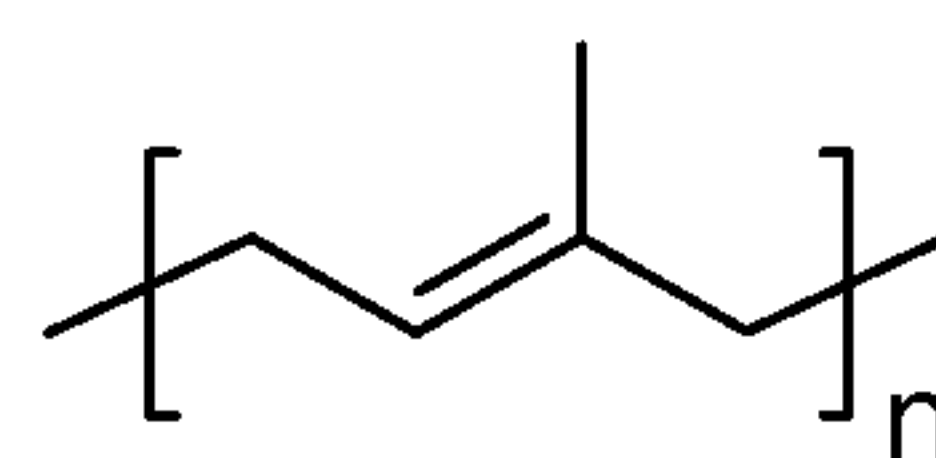
22. The conjugate of any one of claims 18 to 21, wherein at least one of the seven amino acids preceding the amino acid motif is glycine.

23. The conjugate of claim 22, wherein at least three of the seven amino acids preceding the amino acid motif are each independently selected from glycine and proline.

24. The conjugate of claim 23, wherein each of the one, two, three, four, five, six, seven, eight, nine, or ten amino acids preceding the amino acid motif is glycine.

25. The conjugate of claim 24, wherein a C-terminus of the ligand comprises the amino acid sequence GGGGGGGCVIM.

26. The conjugate of any one of claims 17 to 25, wherein the thioether bond comprises a

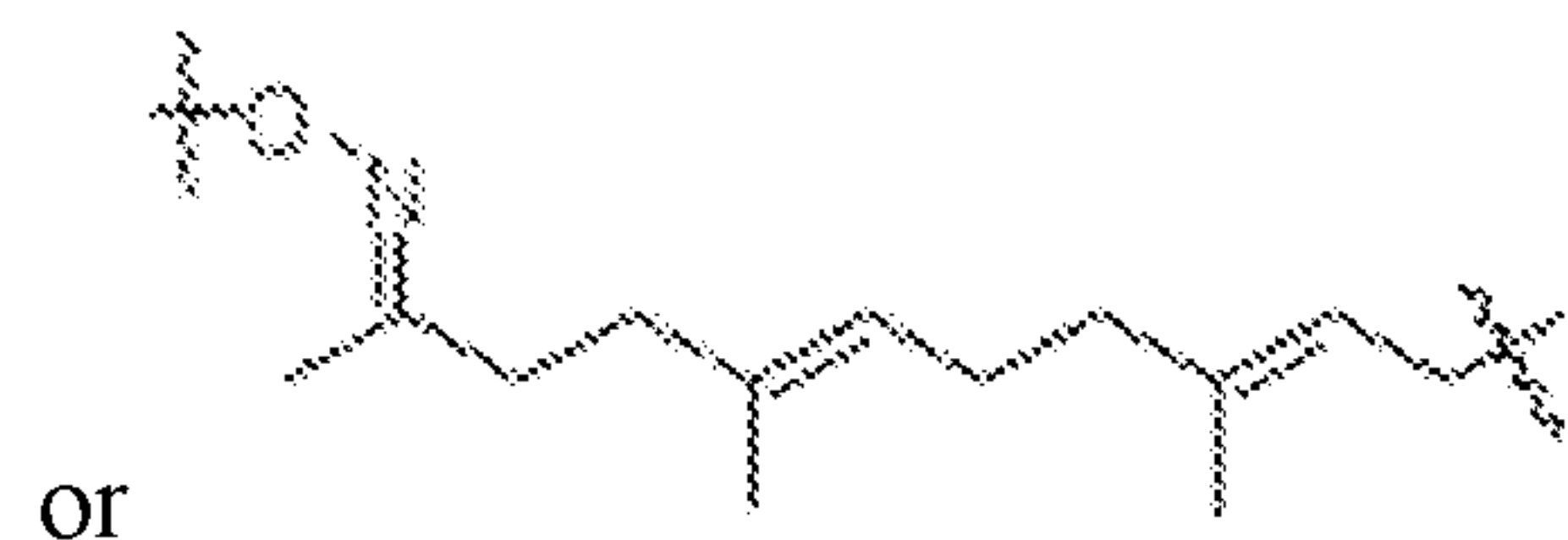
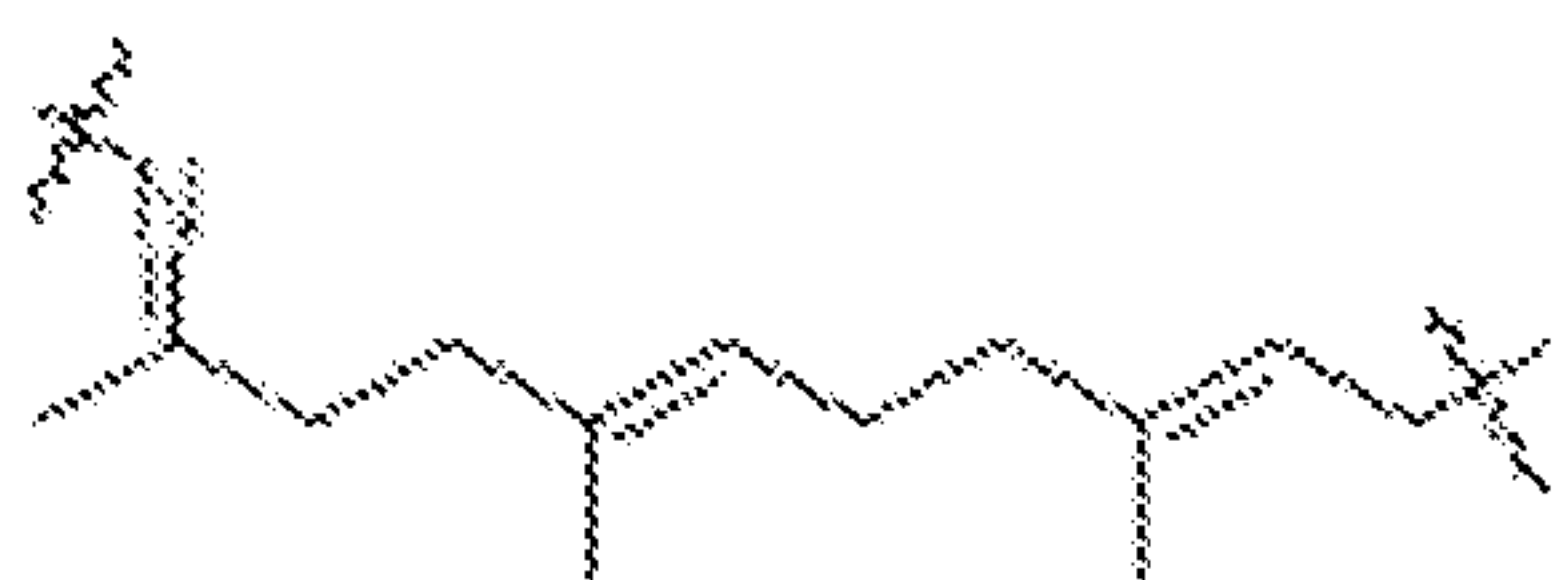


carbon atom of at least one isoprenyl unit, represented by

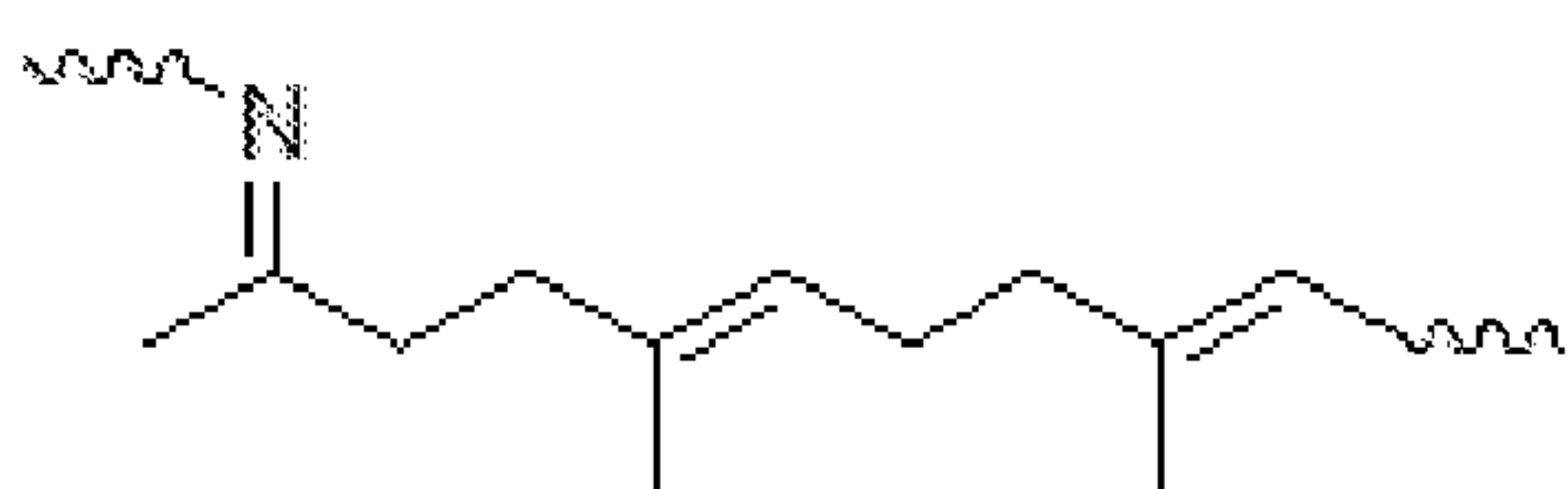
27. The conjugate of claim 26, wherein n is at least 2.

28. The conjugate of claim 26 or 27, wherein the linker comprises an oxime, and the at least one isoprenyl unit covalently links the oxime to the ligand.

29. The conjugate of claim 28, wherein the linker comprises:



30. The conjugate of claim 28, wherein the linker comprises:





$((\text{CH}_2)_p\text{V})_q(\text{CH}_2)_r-$ ,  $-\text{Y}(((\text{CH}_2)_p\text{V})_q-$  or  $-(\text{CH}_2)_r(\text{V}(\text{CH}_2)_p)_q\text{YCH}_2-$

wherein:

$r$  is an integer from 0 to 10;

$p$  is an integer from 1 to 10;

$q$  is an integer from 1 to 20;

$V$  and  $Y$  are each independently a single bond,  $-\text{O}-$ ,  $-\text{S}-$ ,  $-\text{NR}_{21}-$ ,  $-\text{C}(\text{O})\text{NR}_{22}-$ ,  $-\text{NR}_{23}\text{C}(\text{O})-$ ,  $-\text{NR}_{24}\text{SO}_2-$ , or  $-\text{SO}_2\text{NR}_{25}-$ ; and

$R_{21}$  to  $R_{25}$  are each independently hydrogen,  $(\text{C}_1-\text{C}_6)$ alkyl,  $(\text{C}_1-\text{C}_6)$ alkyl $(\text{C}_6-\text{C}_{20})$ aryl or  $(\text{C}_1-\text{C}_6)$ alkyl $(\text{C}_3-\text{C}_{20})$ heteroaryl.

40. The conjugate of claim 38 or 39, wherein  $q$  is an integer from 4 to 20.

41. The conjugate of claim 38 or 39, wherein  $q$  is an integer from 6 to 20.

42. The conjugate of claim 38 or 39, wherein  $q$  is an integer from 2 to 12.

43. The conjugate of claim 38 or 39, wherein  $q$  is 2, 5 or 11.

44. The conjugate of any one of claims 38 to 43, wherein  $r$  is 2.

45. The conjugate of any one of claims 38 to 44, wherein  $p$  is 2.

46. The conjugate of any one of claims 39-45, wherein  $V$  and  $Y$  are each independently  $-\text{O}-$ .

47. The conjugate of claim 38 or 39, wherein:

$r$  is 2;

$p$  is 2;

$q$  is 2, 5, or 11, and

$V$  is  $-\text{O}-$ .

48. The conjugate of any one of the preceding claims, wherein the linker comprises a connection unit represented by  $-(\text{CH}_2\text{CH}_2\text{X})_w-$ , wherein:

X represents -O-, (C<sub>1</sub>-C<sub>8</sub>)alkylene, or -NR<sub>21</sub>-, preferably -O-;

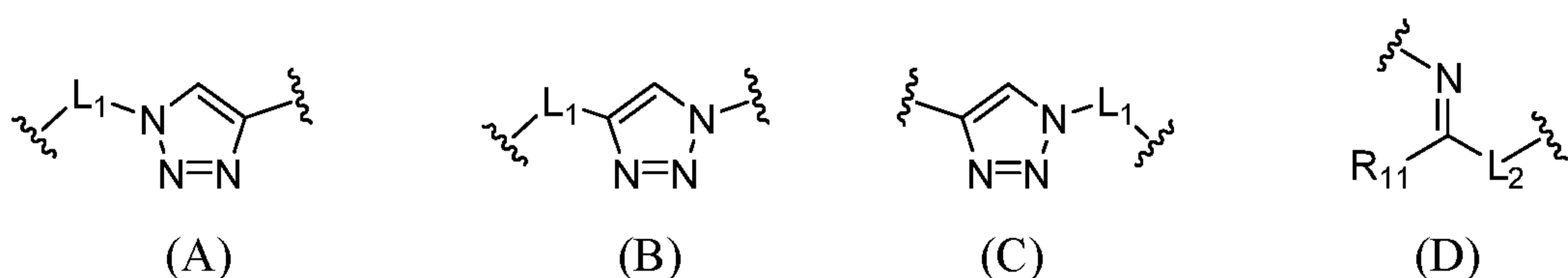
R<sub>21</sub> represents hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>6</sub>-C<sub>20</sub>)aryl, or (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>3</sub>-C<sub>20</sub>)heteroaryl, preferably hydrogen; and

w is an integer from 1 to 20, preferably 4 to 12.

49. The conjugate of any one of the preceding claims, wherein the linker comprises a binding unit formed by a 1,3-dipolar cycloaddition reaction, hetero-Diels-Alder reaction, nucleophilic substitution reaction, non-aldol type carbonyl reaction, addition to carbon-carbon multiple bond, oxidation reaction, or click reaction.

50. The conjugate of claim 49, wherein the binding unit is formed by a reaction between acetylene and azide, or a reaction between an aldehyde or ketone group and a hydrazine or alkoxyamine.

51. The conjugate of claim 50, wherein the binding unit is represented by any one of Formulas A, B, C, or D, preferably C or D:



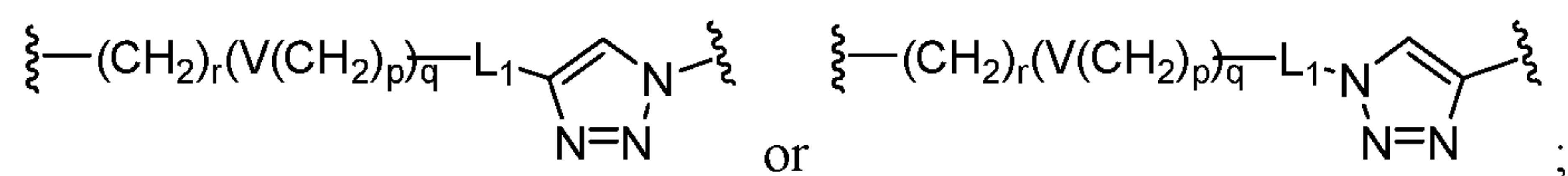
wherein:

L<sub>1</sub> is a single bond or alkylene having 1 to 30 carbon atoms, preferably 12;

R<sub>11</sub> is hydrogen or alkyl having 1 to 10 carbon atoms, preferably methyl; and

L<sub>2</sub> is alkylene having 1 to 30 carbon atoms, preferably 11.

52. The conjugate of claim 51, wherein the linker comprises:



V is a single bond, -O-, -S-, -NR<sub>21</sub>-, -C(O)NR<sub>22</sub>-, -NR<sub>23</sub>C(O)-, -NR<sub>24</sub>SO<sub>2</sub>-, or -SO<sub>2</sub>NR<sub>25</sub>-, preferably -O-;

R<sub>21</sub> to R<sub>25</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>6</sub>)alkyl, (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>6</sub>-C<sub>20</sub>)aryl, or (C<sub>1</sub>-C<sub>6</sub>)alkyl(C<sub>3</sub>-C<sub>20</sub>)heteroaryl;

r is an integer from 1 to 10, preferably 3;

p is an integer from 0 to 10, preferably 2;

q is an integer from 1 to 20, preferably 2 to 20; and  
L<sub>1</sub> is a single bond.

53. The conjugate of any one of the preceding claims, wherein each of the at least two active agents are covalently coupled to side chains of the amino acids through one or more linkers.

54. The conjugate of claim 53, wherein at least one of the linkers coupling an active agent to a side chain of an amino acid comprises a binding unit as defined in any one of claims 49-52.

55. The conjugate of claim 53 or 54, wherein at least one of the linkers is a branched linker.

56. The conjugate of claim 55, wherein at least two branched linkers are covalently coupled to the peptide.

57. The conjugate of claim 56, wherein three branched linkers are coupled to the peptide.

58. The conjugate of claim 56, wherein four branched linkers are coupled to the peptide.

59. The conjugate of claim 53 or 54, wherein exactly one branched linker is coupled to the peptide.

60. The conjugate of any one of claims 55 to 59, wherein each branched linker is coupled to at least two active agents.

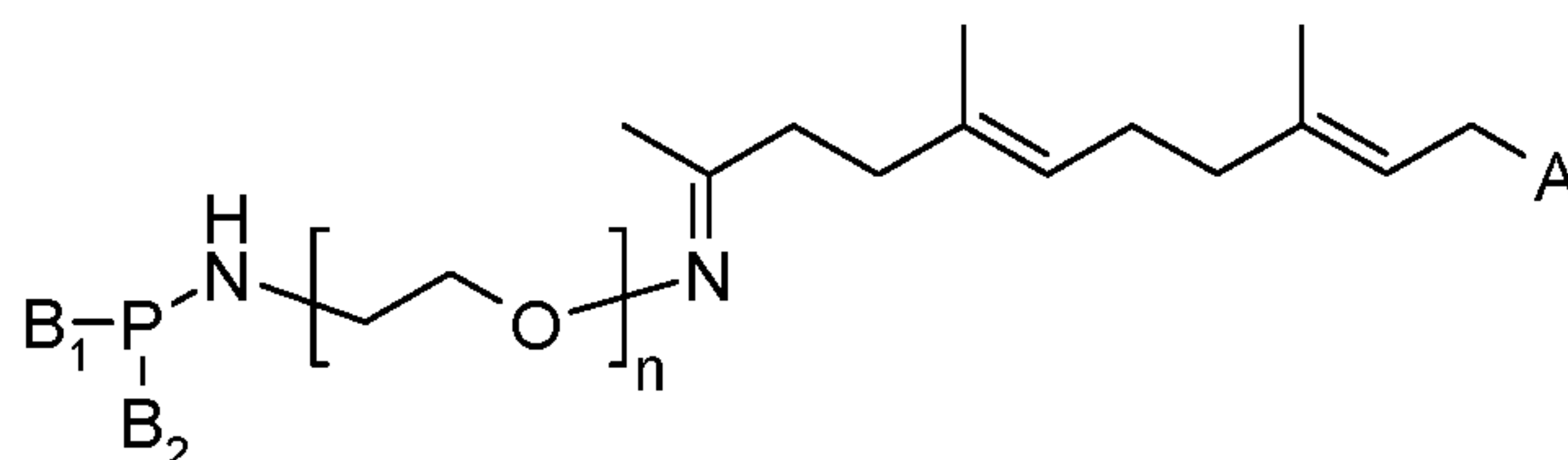
61. The conjugate of claim 60, wherein at least one branched linker is coupled to two different active agents.

62. The conjugate of any one of claims 55 to 61, wherein each branched linker comprises a branching unit, and each active agent is coupled to the branching unit through a



B<sub>2</sub> represents a second active agent; and  
n is an integer from 1 to 20.

65. The conjugate of any one of claims 1 to 63, comprising the structure of Formula VI:



(VI)

wherein

A represents the ligand;

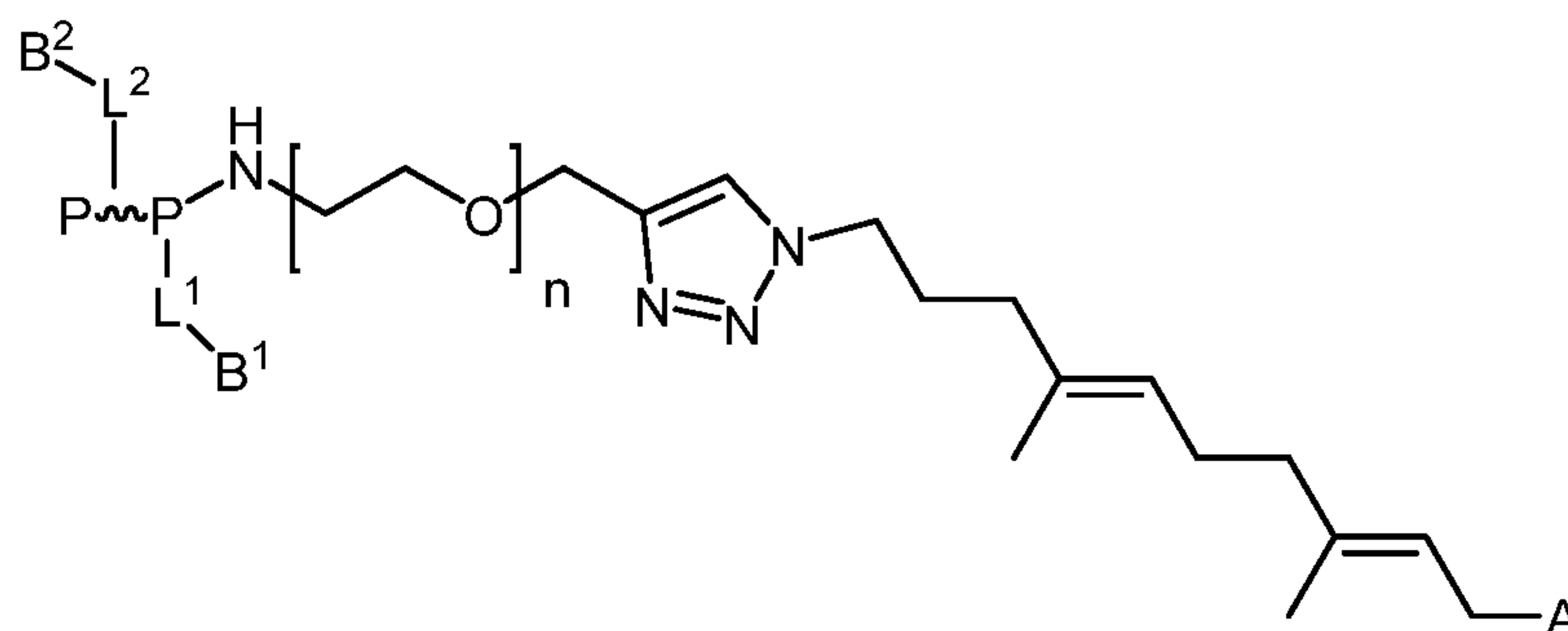
P represents the peptide;

B<sub>1</sub> represents a first active agent;

B<sub>2</sub> represents a second active agent; and

n is an integer from 1 to 20.

66. The conjugate of any one of claims 1 to 63, comprising a structure of:



wherein

A represents the ligand;

P ~ P represents the peptide;

B<sub>1</sub> represents a first active agent;

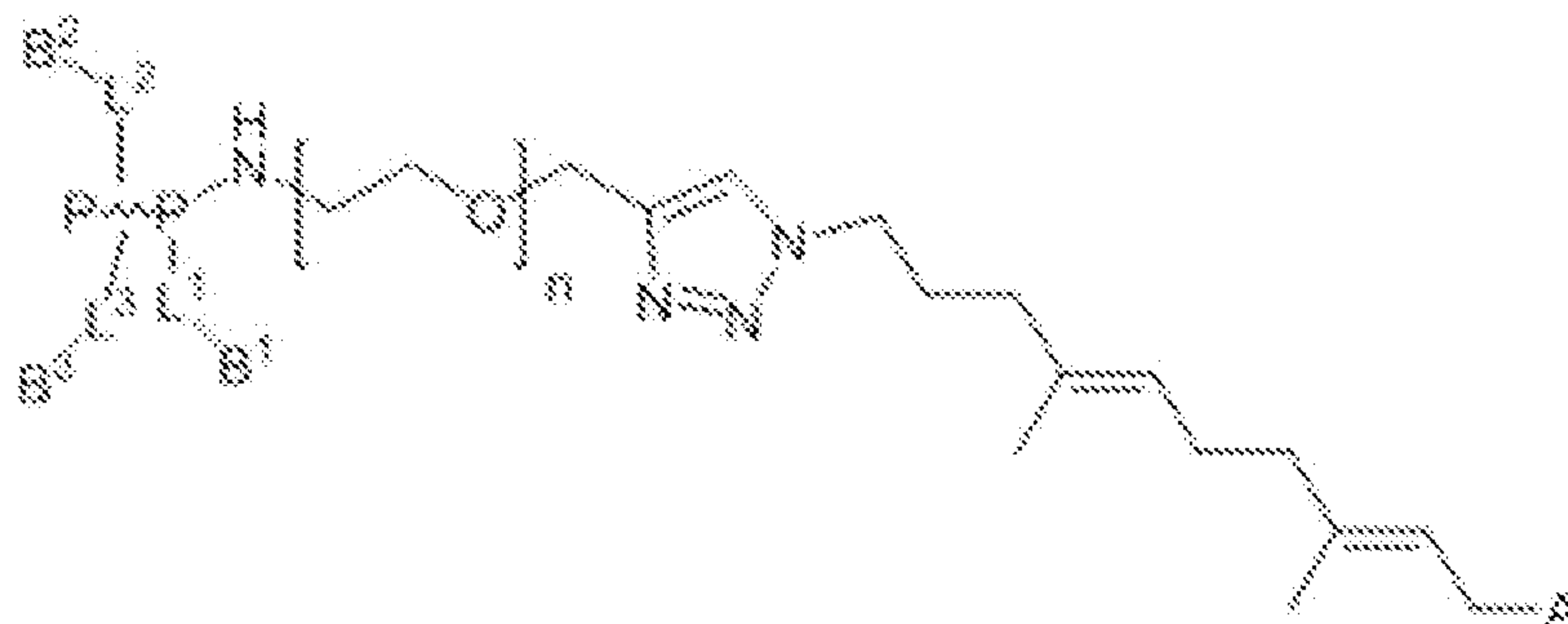
B<sub>2</sub> represents a second active agent;

L<sub>1</sub> represents a linker, optionally including a cleavage group;

L<sub>2</sub> represents a linker, optionally including a cleavage group; and

n is an integer from 1 to 20.

67. The conjugate of claim 66, comprising a structure of:



wherein

A represents the ligand;

P represents the peptide;

B<sub>1</sub> represents a first active agent;

B<sub>2</sub> represents a second active agent;

B<sub>3</sub> represents a third active agent;

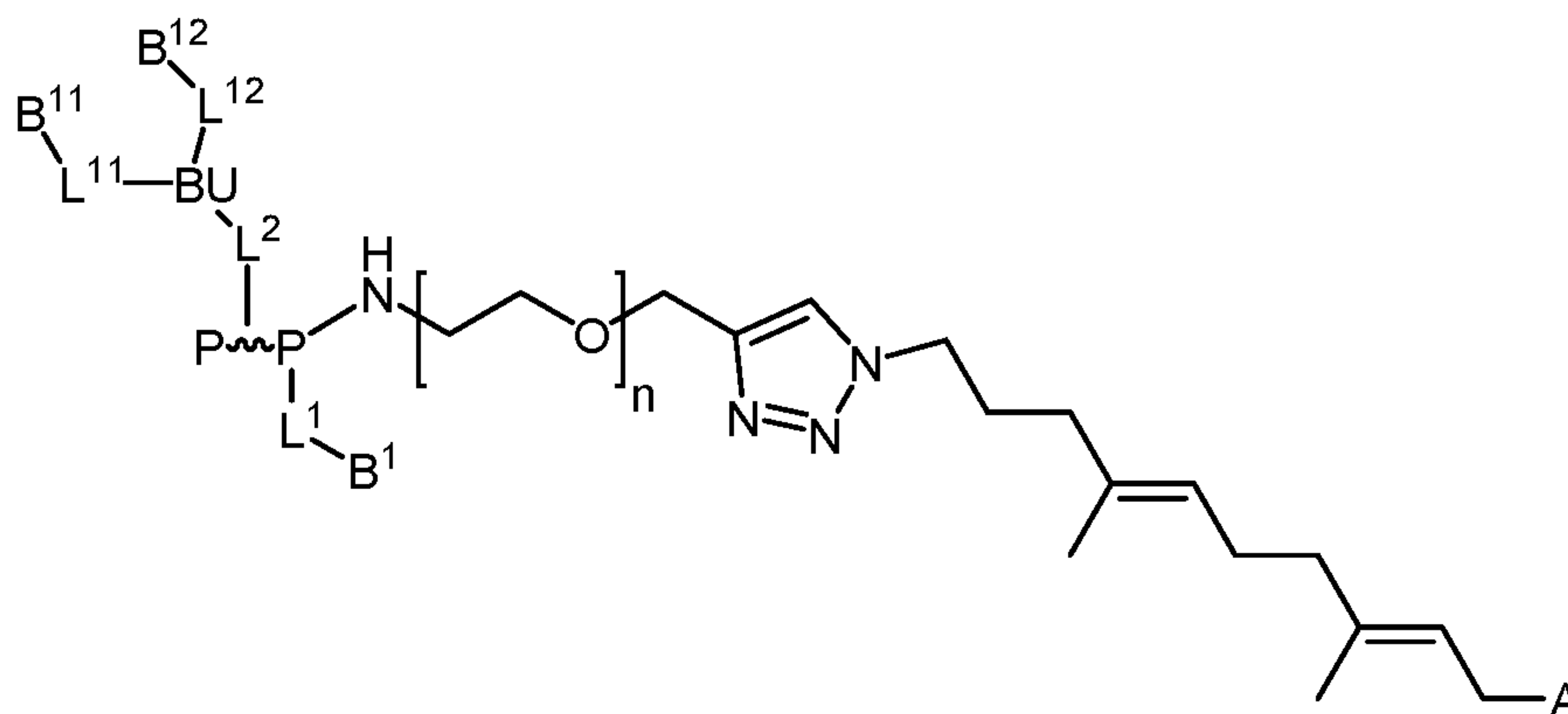
L<sub>1</sub> represents a linker, optionally including a cleavage group;

L<sub>2</sub> represents a linker, optionally including a cleavage group;

L<sub>3</sub> represents a linker, optionally including a cleavage group; and

n is an integer from 1 to 20.

68. The conjugate of claim 66, comprising a structure of:



wherein

A represents the ligand;

P represents the peptide;

B<sub>1</sub> represents a first active agent;

B<sub>11</sub> represents a second active agent;

B<sub>12</sub> represents a third active agent;

L<sub>1</sub> represents a linker;

L<sub>2</sub> represents a linker;

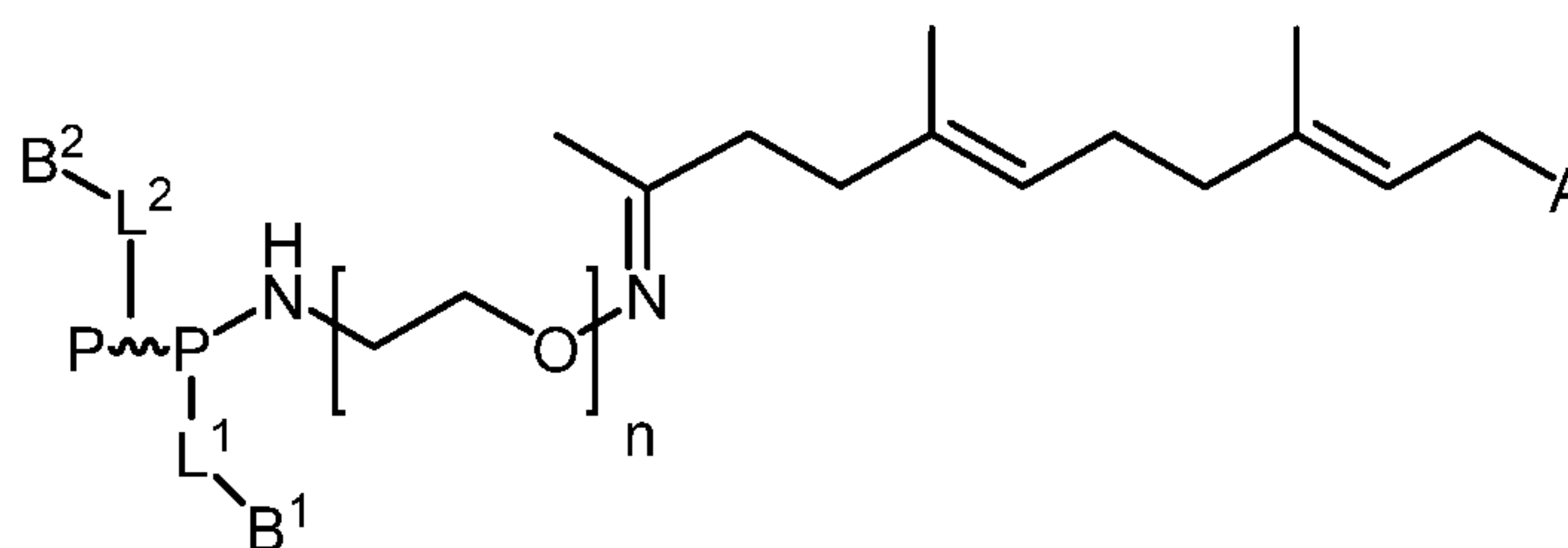
$L_{11}$  represents a secondary linker, optionally including a cleavage group;

$L_{12}$  represents a secondary linker, optionally including a cleavage group;

BU represents a branching unit; and

$n$  is an integer from 1 to 20.

69. The conjugate of any one of claims 1 to 63, comprising a structure of:



wherein

A represents the ligand;

$P \sim P$  represents the peptide;

$B_1$  represents a first active agent;

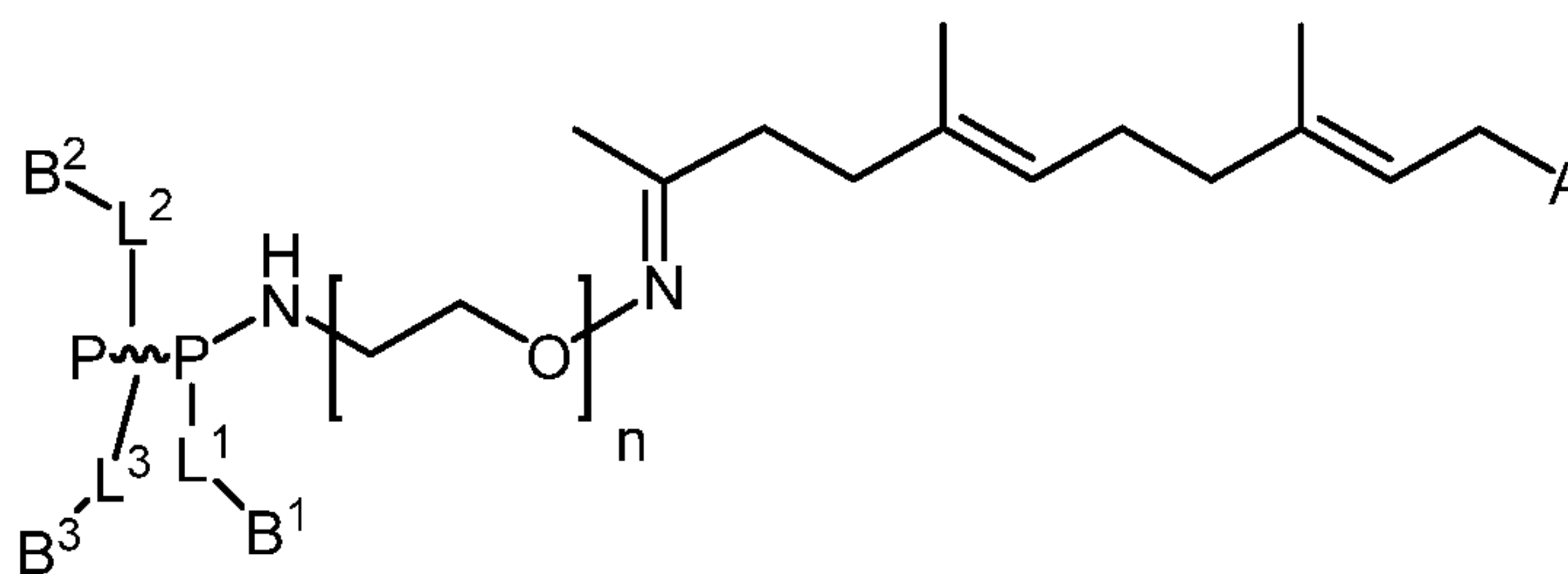
$B_2$  represents a second active agent;

$L_1$  represents a linker, optionally including a cleavage group;

$L_2$  represents a linker, optionally including a cleavage group; and

$n$  is an integer from 1 to 20.

70. The conjugate of any one of claims 1 to 63, comprising a structure of:



wherein

A represents the ligand;

$P \sim P$  represents the peptide;

$B_1$  represents a first active agent;

$B_2$  represents a second active agent;

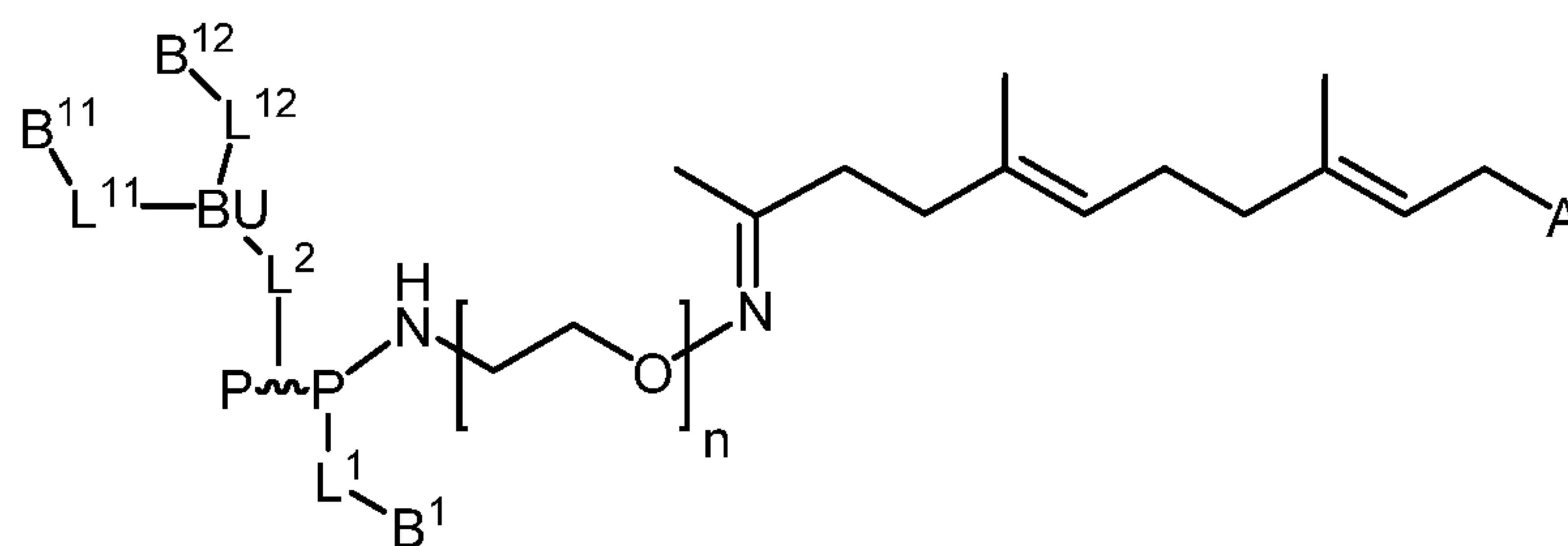
$B_3$  represents a third active agent;

$L_1$  represents a linker, optionally including a cleavage group;

$L_2$  represents a linker, optionally including a cleavage group;

$L_3$  represents a linker, optionally including a cleavage group; and  
 $n$  is an integer from 1 to 20.

71. The conjugate of any one of claims 1 to 63, comprising a structure of:



wherein

A represents the ligand;

$P \sim P$  represents the peptide;

$B_1$  represents a first active agent;

$B_{11}$  represents a second active agent;

$B_{12}$  represents a third active agent;

$L_1$  represents a linker;

$L_2$  represents a linker;

$L_{11}$  represents a secondary linker, optionally including a cleavage group;

$L_{12}$  represents a secondary linker, optionally including a cleavage group;

BU represents a branching unit; and

$n$  is an integer from 1 to 20.

72. The conjugate of any one of the preceding claims, wherein the ligand is a monoclonal antibody, polyclonal antibody, antibody fragment, Fab, Fab', Fab'-SH, F(ab')<sub>2</sub>, Fv, single chain Fv ("scFv"), diabody, linear antibody, bispecific antibody, multispecific antibody, chimeric antibody, humanized antibody, human antibody, or fusion protein comprising the antigen-binding portion of an antibody.

73. The conjugate of any one of the preceding claims, wherein the ligand is selected from muromonab-CD3 abciximab, rituximab, daclizumab, palivizumab, infliximab, trastuzumab, etanercept, basiliximab, gemtuzumab, alemtuzumab, ibritumomab, adalimumab, alefacept, omalizumab, efalizumab, tositumomab, cetuximab, ABT-806, bevacizumab, natalizumab, ranibizumab, panitumumab, eculizumab, rilonacept,

certolizumab, romiplostim, AMG-531, golimumab, ustekinumab, ABT-874, belatacept, belimumab, atacicept, an anti-CD20 antibody, canakinumab, tocilizumab, atlizumab, mepolizumab, pertuzumab, HuMax CD20, tremelimumab, ticilimumab, ipilimumab, IDEC-114, inotuzumab, HuMax EGFR, aflibercept, HuMax-CD4, teplizumab, orelizumab, catumaxomab, the anti-EpCAM antibody IGN101, adecatumomab, oregovomab, dinutuximab, girentuximab, denosumab, bapineuzumab, motavizumab, efumgumab, raxibacumab, LY2469298, and velutuzumab.

74. The conjugate of any one of the preceding claims, wherein each active agent is independently selected from chemotherapeutic agents and toxins.

75. The conjugate of any one of the preceding claims, wherein each active agent is independently selected from:

(a) erlotinib, bortezomib, fulvestrant, sunitinib, letrozole, imatinib mesylate, PTK787/ZK 222584, oxaliplatin, 5-fluorouracil, leucovorin, rapamycin, lapatinib, lonafarnib, sorafenib, gefitinib, AG1478, AG1571, thiotepa, cyclophosphamide, busulfan, improsulfan, piposulfan, benzodopa, carboquone, meturedopa, uredopa, ethylenimine, altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide, trimethylolomelamine, bullatacin, bullatacinone, camptothecin, topotecan, bryostatin, callystatin, CC-1065, adozelesin, carzelesin, bizelesin, cryptophycin 1, cryptophycin 8, dolastatin, duocarmycin, KW-2189, CB1-TM1, eleutherobin, pancratistatin, sarcodictyin, spongistatin, chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard, carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine, calicheamicin, calicheamicin gamma 1, calicheamicin omega 1, dynemicin, dynemicin A, clodronate, esperamicin, neocarzinostatin chromophore, aclacinomysins, actinomycin, antracycline, azaserine, bleomycins, cactinomycin, carbacine, carinomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, liposomal doxorubicin, deoxydoxorubicin, epirubicin, esorubicin, marcellomycin, mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptomycin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin, 5-fluorouracil, denopterin, methotrexate, pteropterin,

trimetrexate, fludarabine, 6-mercaptopurine, thiamiprine, thiguanine, ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone, aminoglutethimide, mitotane, trilostane, folinic acid, aceglatone, aldophosphamide glycoside, aminolevulinic acid, eniluracil, amsacrine, bestrabucil, bisantrene, edatraxate, defofamine, demecolcine, diaziquone, elfornithine, elliptinium acetate, etoglucid, gallium nitrate, hydroxyurea, lentinan, lonidainine, maytansine, ansamitocins, mitoguazone, mitoxantrone, mopidanmol, nitraerine, pentostatin, phenamet, pirarubicin, losoxantrone, 2-ethylhydrazide, procarbazine, polysaccharide-k, razoxane, rhizoxin, sizofiran, spirogermanium, tenuazonic acid, triaziquone, 2,2',2''-trichlorotriethylamine, T-2 toxin, verracurin A, roridin A, and anguidine, urethane, vindesine, dacarbazine, mannomustine, mitobronitol, mitolactol, pipobroman, gacytosine, arabinoside, cyclophosphamide, thiotepa, paclitaxel, albumin-engineered nanoparticle formulation of paclitaxel, doxetaxel, chlorambucil, gemcitabine, 6-thioguanine, mercaptopurine, cisplatin, carboplatin, vinblastine, platinum, etoposide, ifosfamide, mitoxantrone, vincristine, vinorelbine, novantrone, teniposide, edatrexate, daunomycin, aminopterin, xeloda, ibandronate, CPT-11, topoisomerase inhibitor RFS 2000, difluoromethylornithine, retinoic acid, capecitabine, or pharmaceutically acceptable salts, solvates or acids of any of the foregoing;

(b) monokine, a lymphokine, a traditional polypeptide hormone, parathyroid hormone, thyroxine, relaxin, prorelaxin, a glycoprotein hormone, follicle stimulating hormone, thyroid stimulating hormone, luteinizing hormone, hepatic growth factor fibroblast growth factor, prolactin, placental lactogen, tumor necrosis factor- $\alpha$ , tumor necrosis factor- $\beta$ , mullerian-inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial growth factor, thrombopoietin, erythropoietin, an osteoinductive factor, an interferon, interferon- $\alpha$ , interferon- $\beta$ , interferon- $\gamma$ , a colony stimulating factor ("CSF"), macrophage-CSF, granulocyte-macrophage-CSF, granulocyte-CSF, an interleukin ("IL"), IL-1, IL-1 $\alpha$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, a tumor necrosis factor, TNF- $\alpha$ , TNF- $\beta$ , a polypeptide factor, LIF, kit ligand, or a combination of any of the foregoing;

(c) diphtheria toxin, botulium toxin, tetanus toxin, dysentery toxin, cholera toxin, amanitin,  $\alpha$ -amanitin, amanitin derivatives, pyrrolobenzodiazepine, pyrrolobenzodiazepine derivatives, tetrodotoxin, brevetoxin, ciguatoxin, ricin, AM toxin, tubulysin, geldanamycin,

maytansinoid, calicheamicin, daunomycin, doxorubicin, methotrexate, vindesine, SG2285, dolastatin, a dolastatin analog, cryptophycin, camptothecin, camptothecin derivatives and metabolites (*e.g.*, SN-38), rhizoxin, a rhizoxin derivative, CC-1065, a CC-1065 analogue or derivative, duocarmycin, an enediyne antibiotic, esperamicin, epothilone, azonafide, aplidine, a toxoid, or a combination of any of the foregoing;

(d) an affinity ligand, wherein the affinity ligand is a substrate, an inhibitor, a stimulating agent, a neurotransmitter, a radioisotope, or a combination of any of the foregoing;

(e) a radioactive label,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , a fluorescent dye, an electron dense reagent, an enzyme, biotin, streptavidin, dioxigenin, a hapten, an immunogenic protein, a nucleic acid molecule with a sequence complementary to a target, or a combination of any of the foregoing;

(f) an immunomodulatory compound, an anti-cancer agent, an anti-viral agent, an anti-bacterial agent, an anti-fungal agent, and an anti-parasitic agent, or a combination of any of the foregoing;

(g) tamoxifen, raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, or toremifene;

(h) 4(5)-imidazoles, aminoglutethimide, megestrol acetate, exemestane, letrozole, or anastrozole;

(i) flutamide, nilutamide, bicalutamide, leuprolide, goserelin, or troxacitabine;

(j) an aromatase inhibitor;

(k) a protein kinase inhibitor;

(l) a lipid kinase inhibitor;

(m) an antisense oligonucleotide;

(n) a ribozyme;

(o) a vaccine; and

(p) an anti-angiogenic agent.

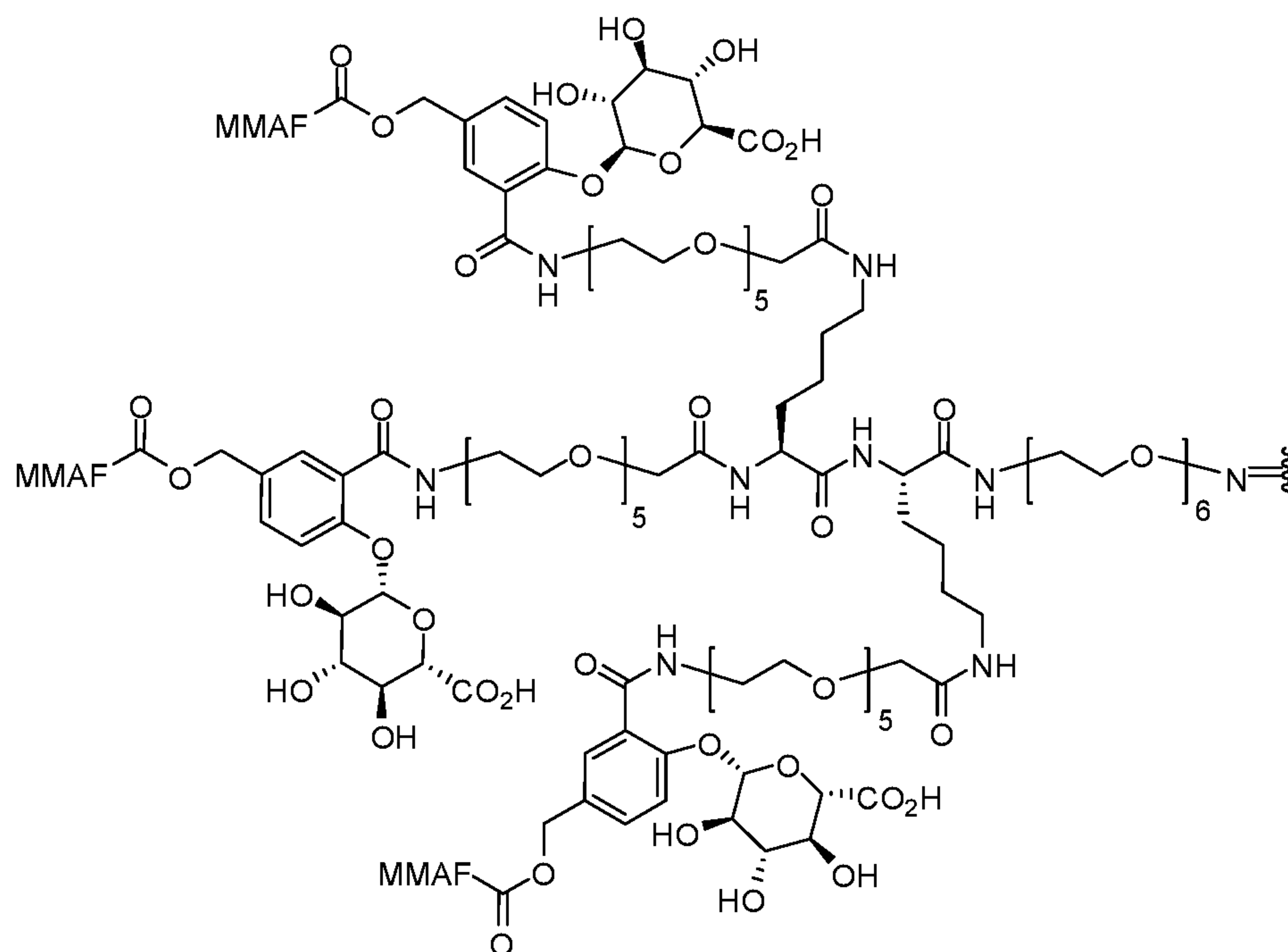
76. The conjugate of any one of the preceding claims, wherein at least one active agent is taltobulin.

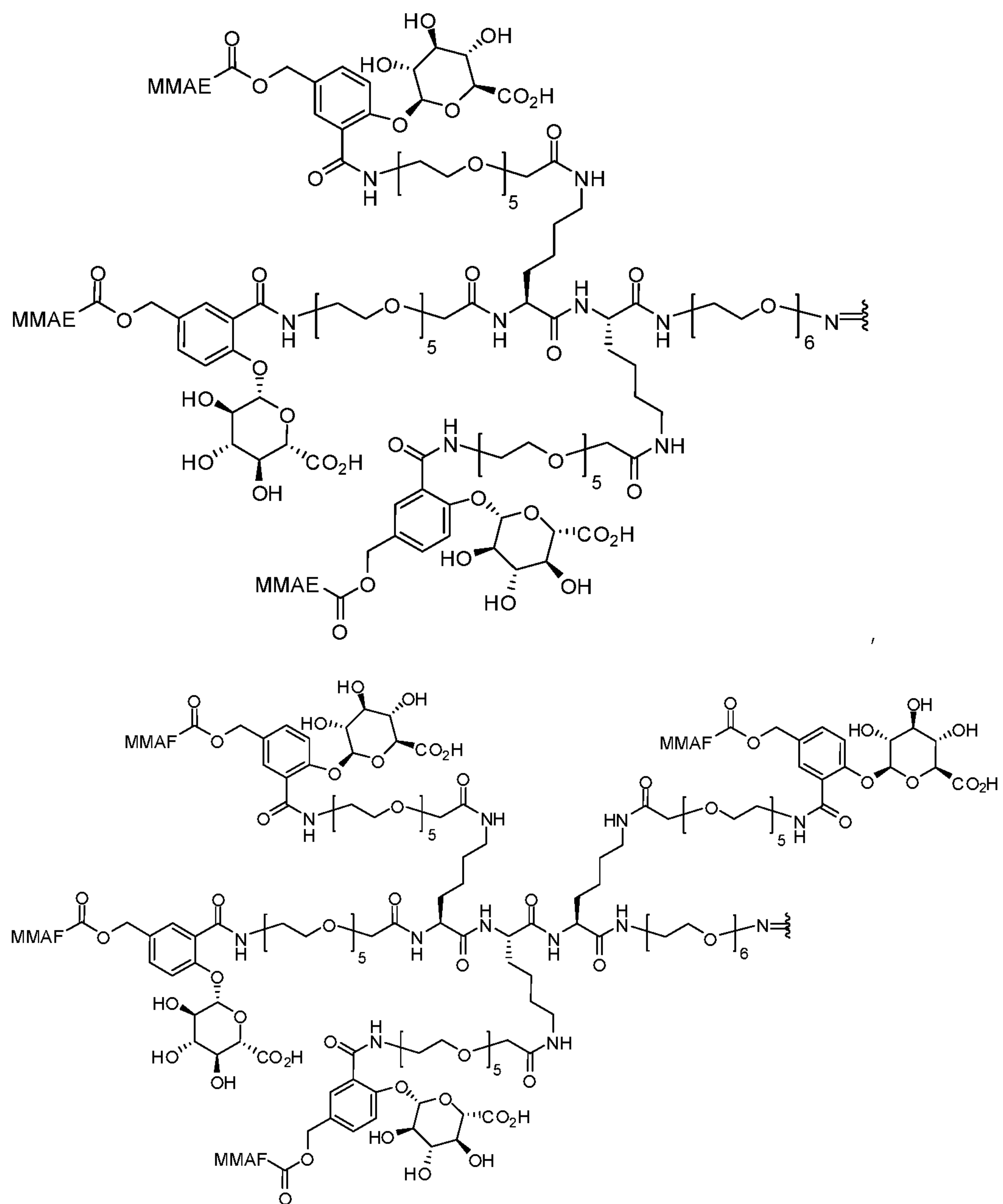
77. The conjugate of any one of the preceding claims, wherein at least one active agent is azonafide.

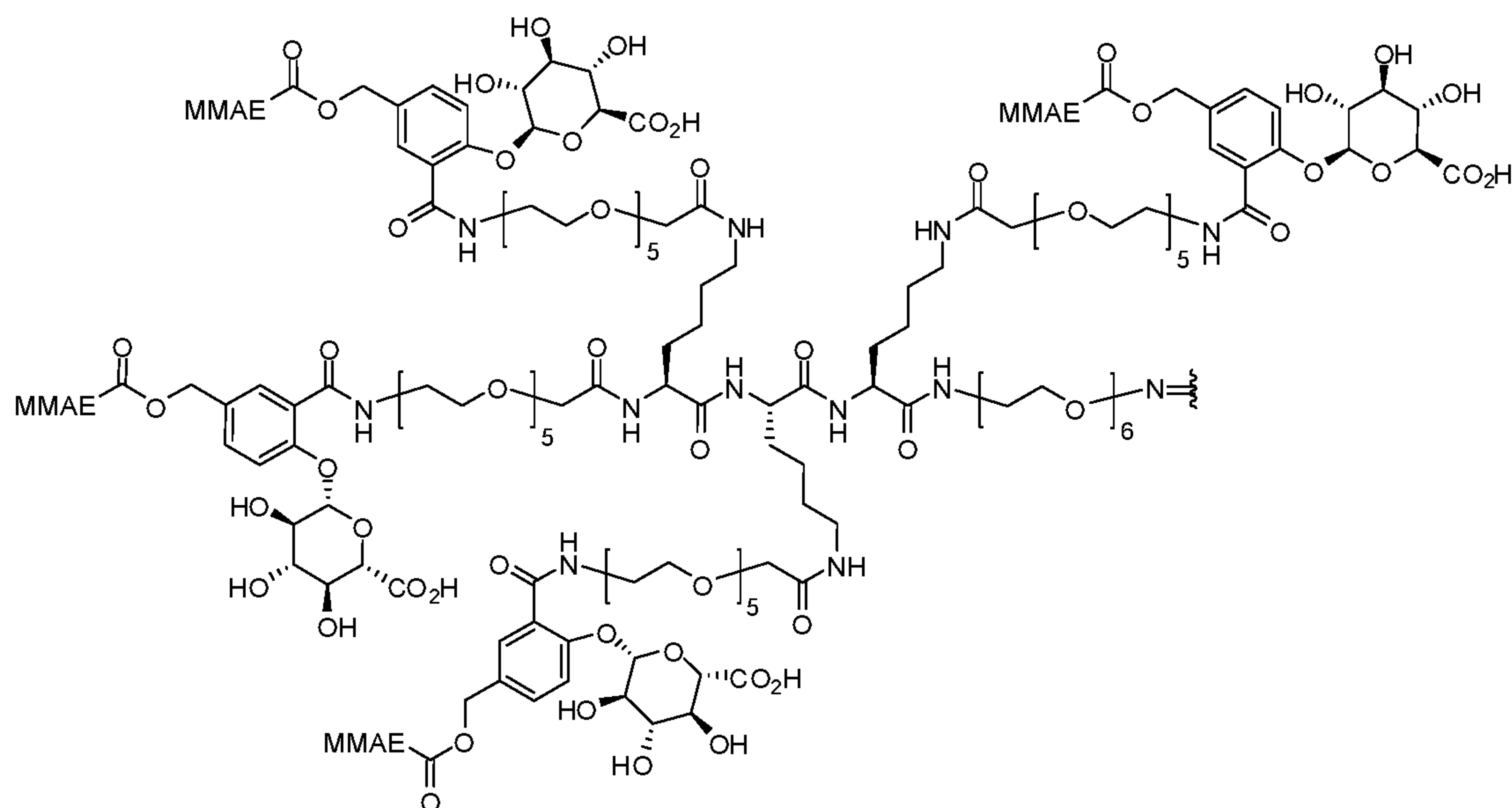
78. The conjugate of any one of the preceding claims, wherein each active agent is independently selected from amanitin, auristatin, calicheamicin, camptothecin, cryptophycin, daunomycin, dolastatin, doxorubicin, duocarmycin, epothilone, esperamicin, geldanamycin, maytansinoid, methotrexate, monomethyl auristatin E (“MMAE”), monomethyl auristatin F (“MMAF”), pyrrolobenzodiazepine, rhizoxin, SG2285, tubulysin, vindesine, and toxoid, or a derivative of any one of the foregoing.

79. The conjugate of claim 73, wherein each active agent is independently selected from amanitin, MMAE, and MMAF, or a derivative of any one of the foregoing.

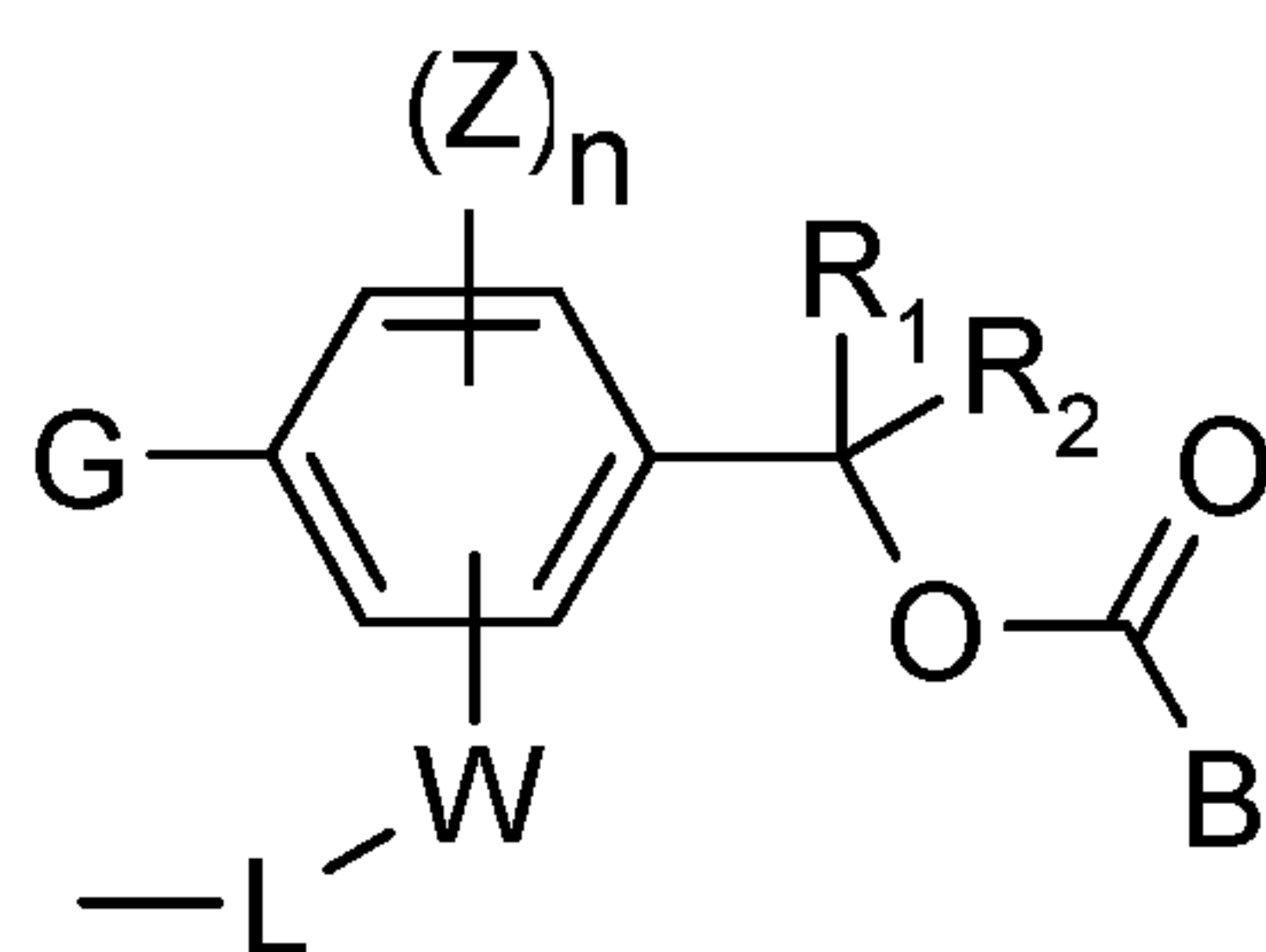
80. The conjugate of any one the preceding claims, wherein the conjugate comprises one or more branched linkers comprising a moiety selected from:







81. The conjugate of any one the preceding claims, wherein at least one active agent is coupled to the linker through a cleavage group having the structure of Formula (I):



(I)

wherein:

B represents an active agent;

G represents a sugar or sugar acid, preferably glucuronic acid;

W represents an electron-withdrawing group, preferably  $-C(O)NR'$ , where C(O) is bonded to the phenyl ring and  $NR'$  is bonded to L;

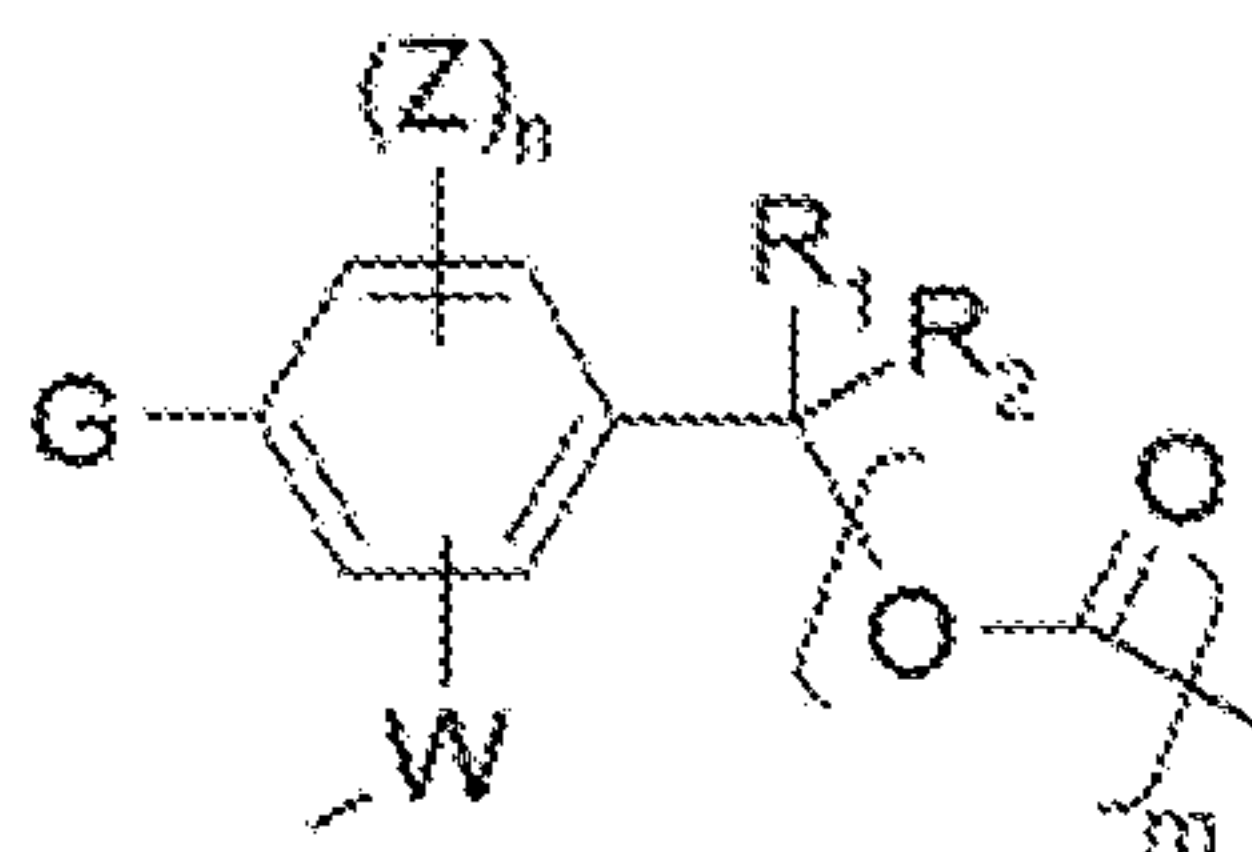
each Z independently represents hydrogen,  $(C_1-C_8)$ alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen,  $(C_1-C_8)$ alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

$R_1$  and  $R_2$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, or  $(C_3-C_8)$ cycloalkyl, preferably hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a  $(C_3-C_8)$ cycloalkyl ring; and

L represents a linkage to the ligand.

82. The conjugate of any one of claims 1-80, wherein at least one active agent is coupled to the linker through a cleavage group having the formula:



wherein:

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

W represents  $-C(O)-$ ,  $-C(O)NR'-$ ,  $-C(O)O-$ ,  $-S(O)_2NR'-$ ,  $-P(O)R''NR'-$ ,  $-S(O)NR'-$ , or  $-PO_2NR'-$ , in each case where the C(O), S, or P is preferably directly bound to the phenyl ring, and R' and R'' are each independently hydrogen,  $(C_1-C_8)$ alkyl, mono- or di-carboxyl $(C_1-C_8)$ alkyl,  $(C_3-C_8)$ cycloalkyl,  $(C_1-C_8)$ alkoxy,  $(C_1-C_8)$ alkylthio, mono- or di- $(C_1-C_8)$ alkylamino,  $(C_3-C_{20})$ heteroaryl, or  $(C_6-C_{20})$ aryl;

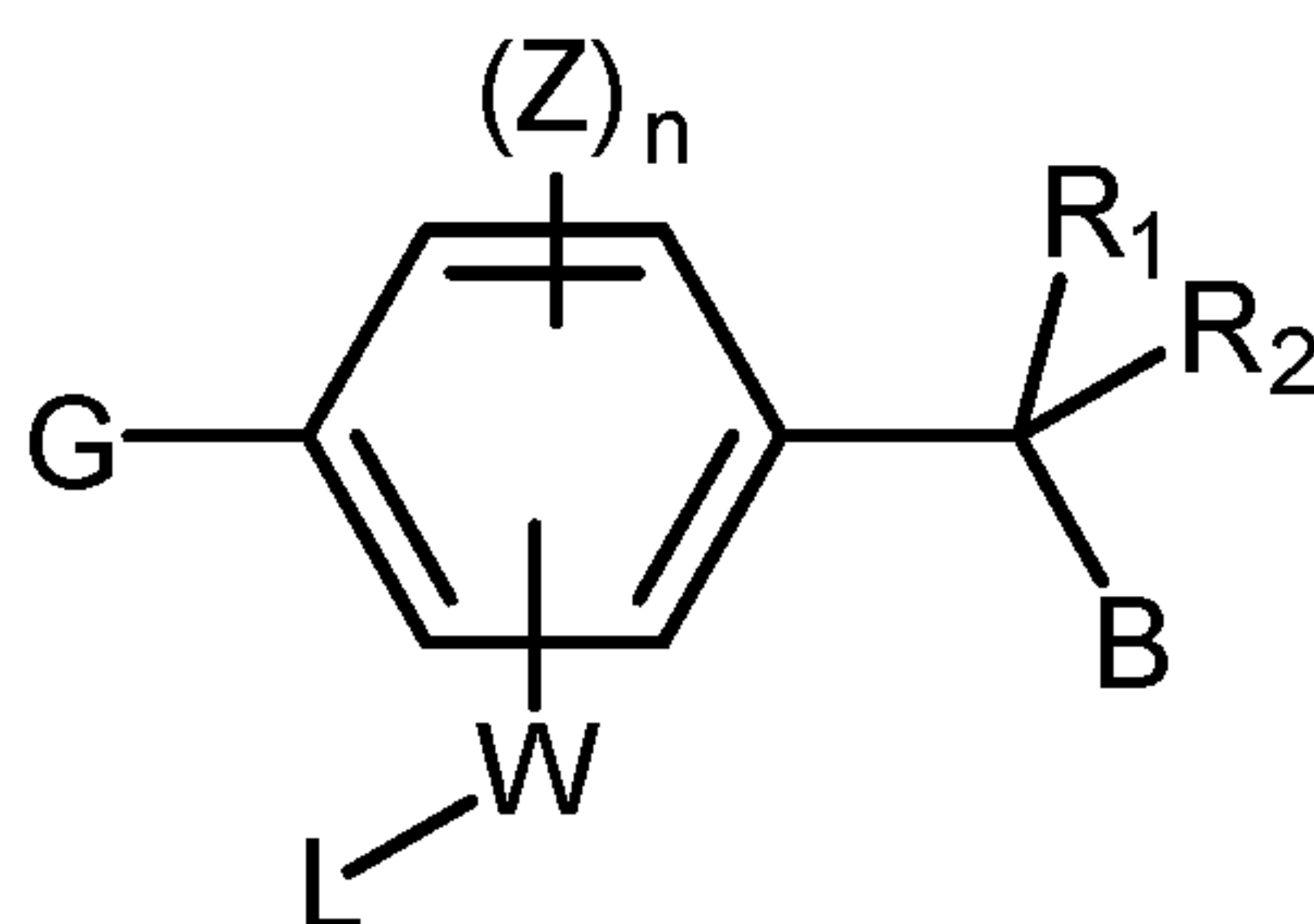
each Z independently represents hydrogen,  $(C_1-C_8)$ alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen,  $(C_1-C_8)$ alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3;

m is 0 or 1, preferably 1; and

R<sub>1</sub> and R<sub>2</sub> are each independently hydrogen,  $(C_1-C_8)$ alkyl, or  $(C_3-C_8)$ cycloalkyl, preferably hydrogen, or R<sub>1</sub> and R<sub>2</sub> taken together with the carbon atom to which they are attached form a  $(C_3-C_8)$ cycloalkyl ring.

83. The conjugate of any one of claims 1-80, wherein at least one active agent is coupled to the linker through a cleavage group having the formula:



or a pharmaceutically acceptable salt thereof, wherein

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

B is a unit covalently attached to the active agent,

W represents  $-C(O)-$ ,  $-C(O)NR'-$ ,  $-C(O)O-$ ,  $-S(O)_2NR'-$ ,  $-P(O)R''NR'-$ ,  $-S(O)NR'-$ , or  $-PO_2NR'-$ , in each case where the C(O), S, or P is directly bound to the phenyl ring, and R' and R'' are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>8</sub>)alkoxy, (C<sub>1</sub>-C<sub>8</sub>)alkylthio, mono- or di-(C<sub>1</sub>-C<sub>8</sub>)alkylamino, (C<sub>3</sub>-C<sub>20</sub>)heteroaryl, or (C<sub>6</sub>-C<sub>20</sub>)aryl

each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

L represents a linker comprising the peptide sequence;

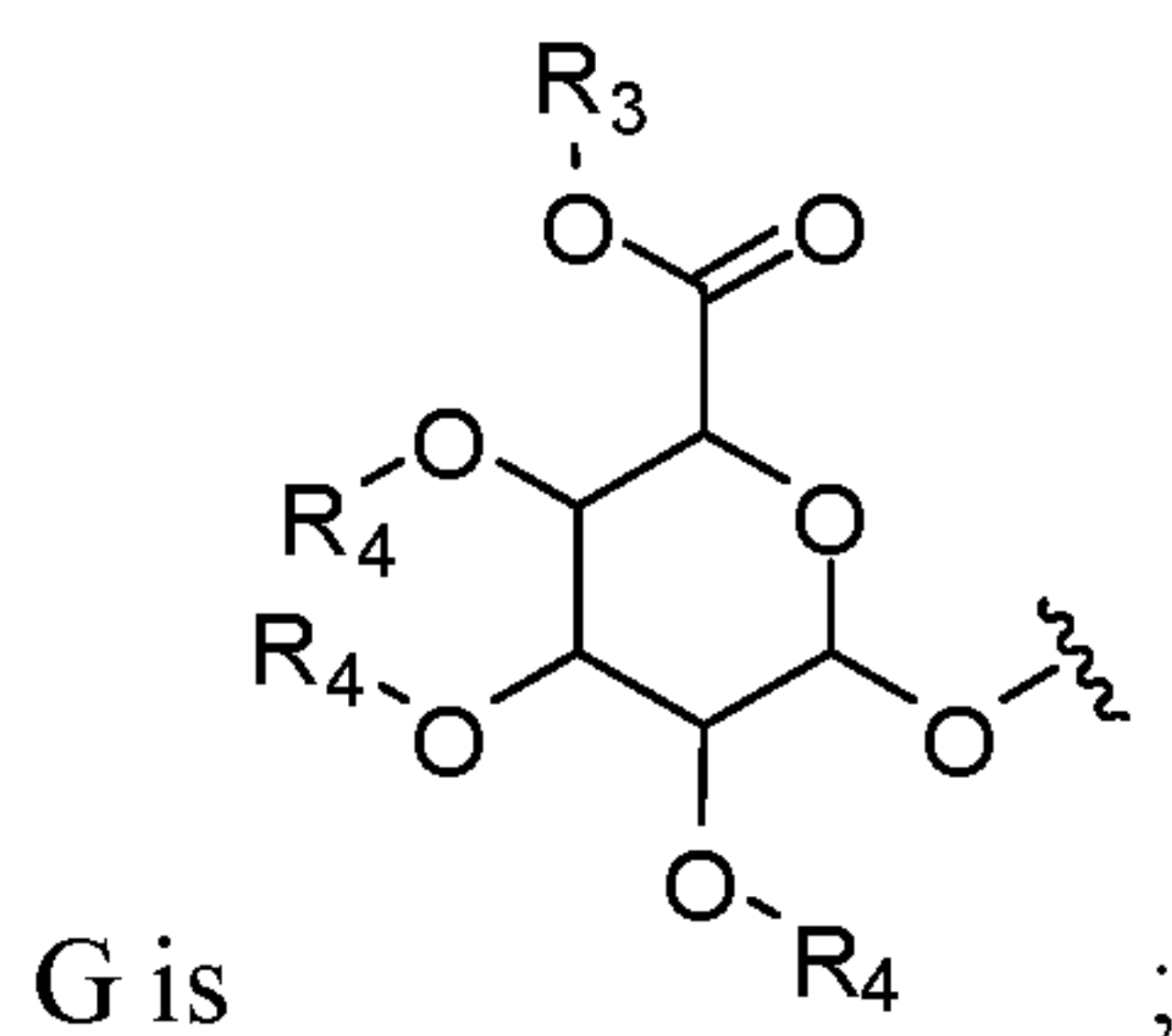
R<sub>1</sub> and R<sub>2</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, preferably hydrogen, or R<sub>1</sub> and R<sub>2</sub> taken together with the carbon atom to which they are attached form a (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl ring.

84. The conjugate of any one of claims 81-83, wherein each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro.

85. The conjugate of claim 84, wherein each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro.

86. The conjugate of any one of claims 81-85, wherein the sugar or sugar acid is a monosaccharide.

87. The conjugate of claim 86, wherein:



$R_3$  is hydrogen or a carboxyl protecting group; and  
each  $R_4$  is independently hydrogen or a hydroxyl protecting group.

88. The conjugate of claim 87, wherein  $R_3$  is hydrogen and each  $R_4$  is hydrogen.

89. The conjugate of any one of claims 81-88, wherein W is  $-C(O)-$ ,  $-C(O)NR'-$ ,  $-C(O)O-$ ,  $-S(O)_2NR'-$ ,  $-P(O)R''NR'-$ ,  $-S(O)NR'-$ , or  $-PO_2NR'-$ , in each case where the C(O), S, or P is directly bound to the phenyl ring, and  $R'$  and  $R''$  are each independently hydrogen,  $(C_1-C_8)$ alkyl,  $(C_3-C_8)$ cycloalkyl,  $(C_1-C_8)$ alkoxy,  $(C_1-C_8)$ alkylthio, mono- or di- $(C_1-C_8)$ alkylamino,  $(C_3-C_{20})$ heteroaryl, or  $(C_6-C_{20})$ aryl.

90. The conjugate of claim 89, wherein W represents  $-C(O)NR'-$ , where C(O) is bonded to the phenyl ring and  $NR'$  is bonded to L.

91. The conjugate of any one of claims 81 to 90, wherein each Z represents hydrogen and n is 3.

92. The conjugate of any one of claims 81 to 91, wherein  $R_1$  and  $R_2$  each represent hydrogen.

93. The conjugate of any one of claims 81 to 92, wherein:

G represents glucuronic acid;

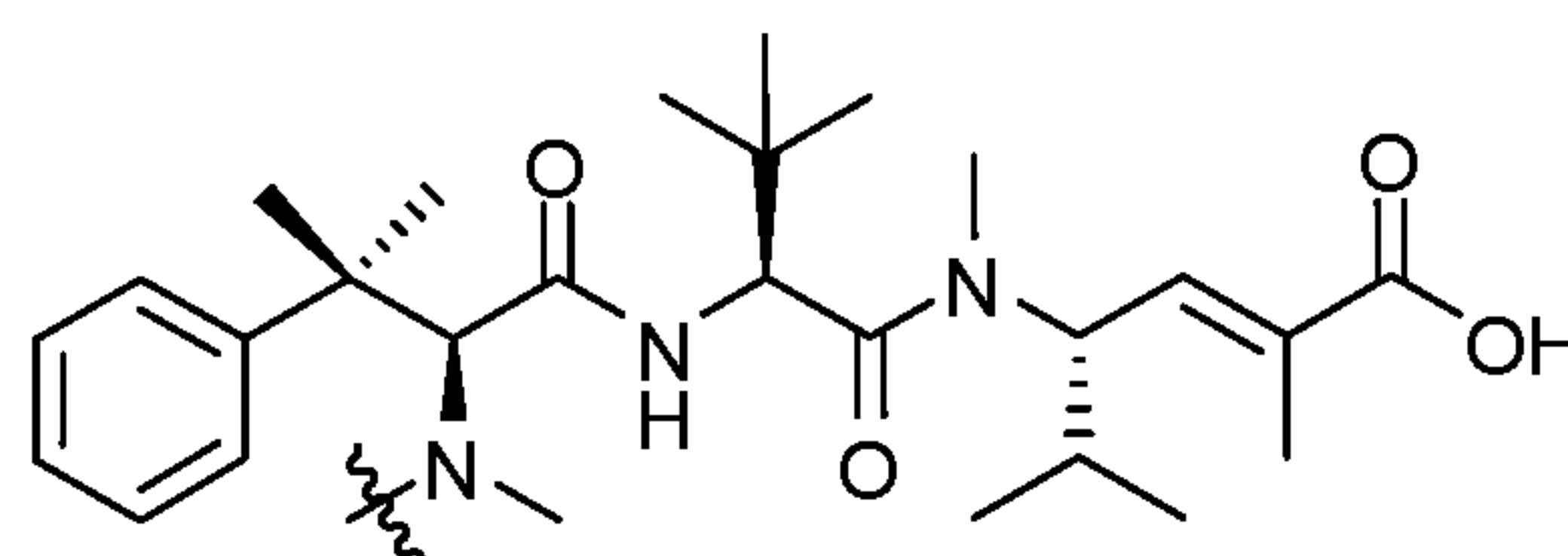
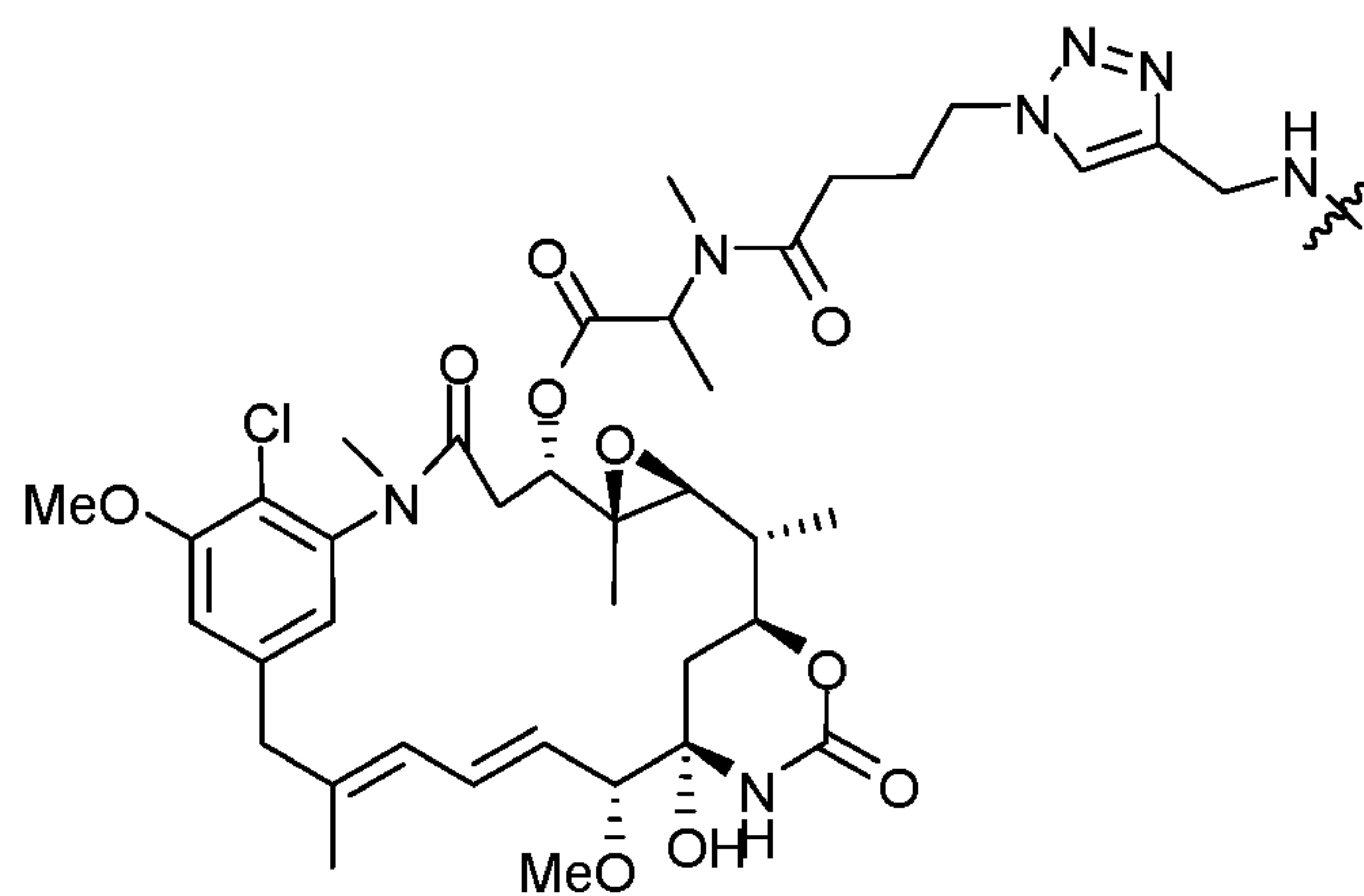
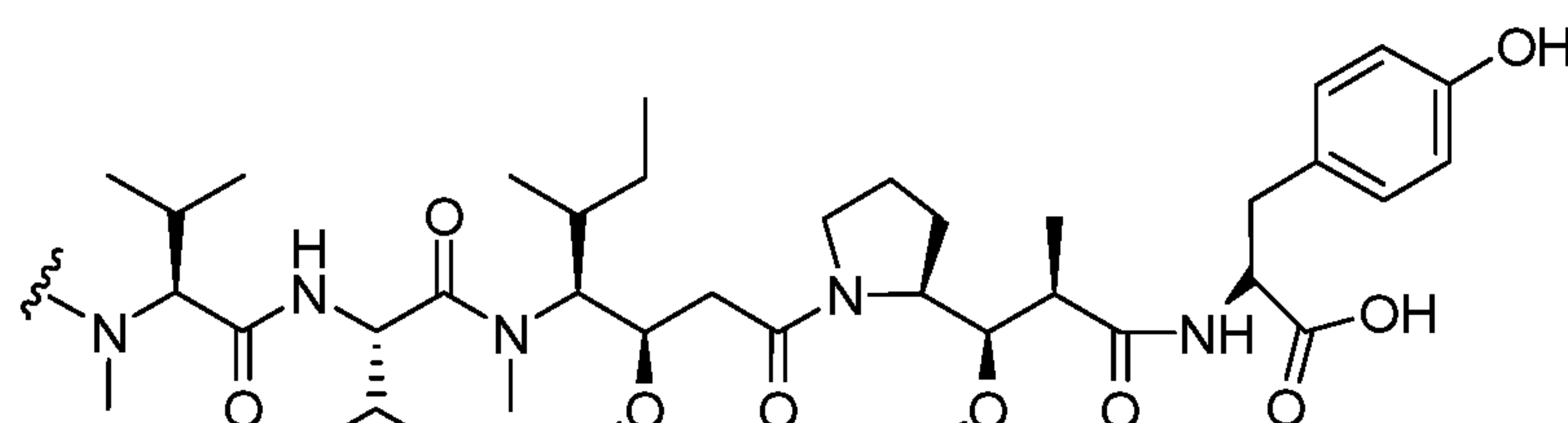
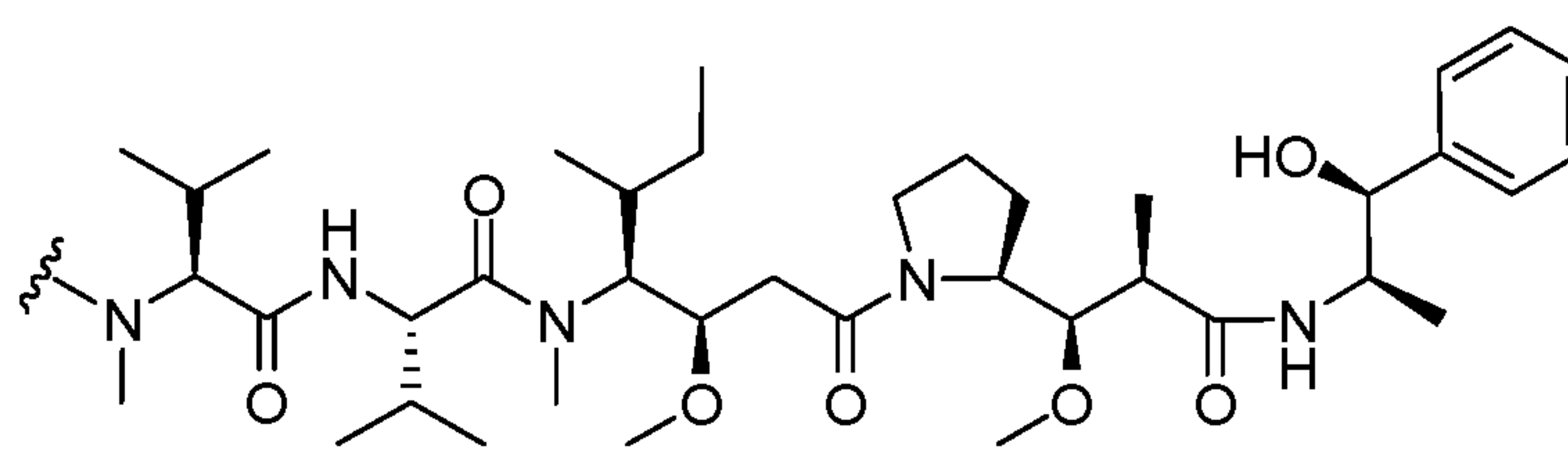
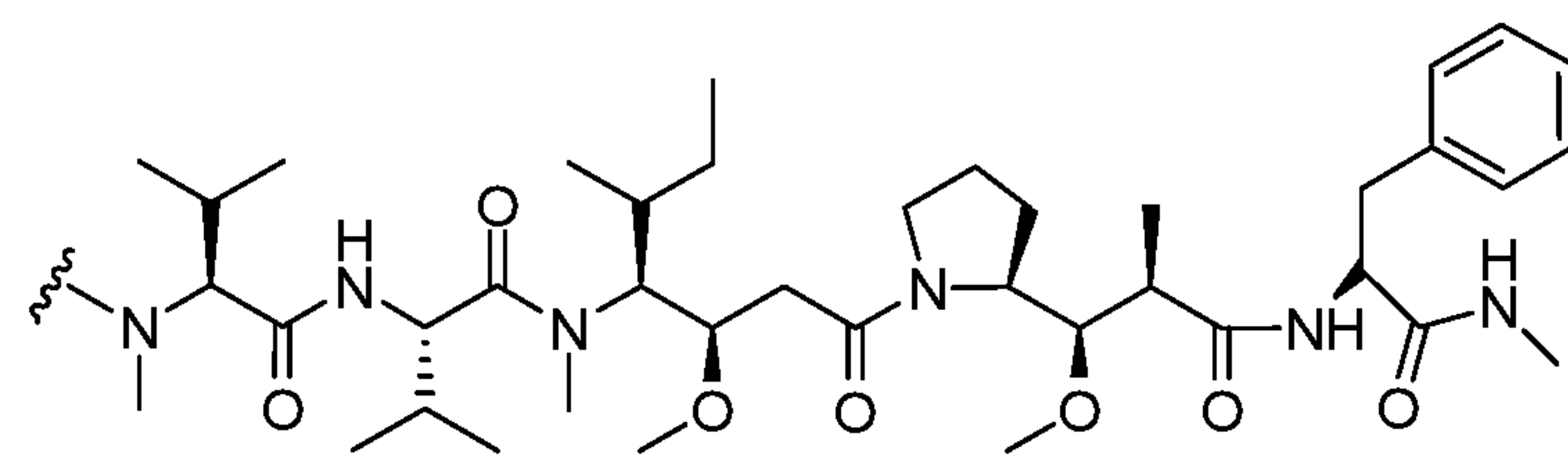
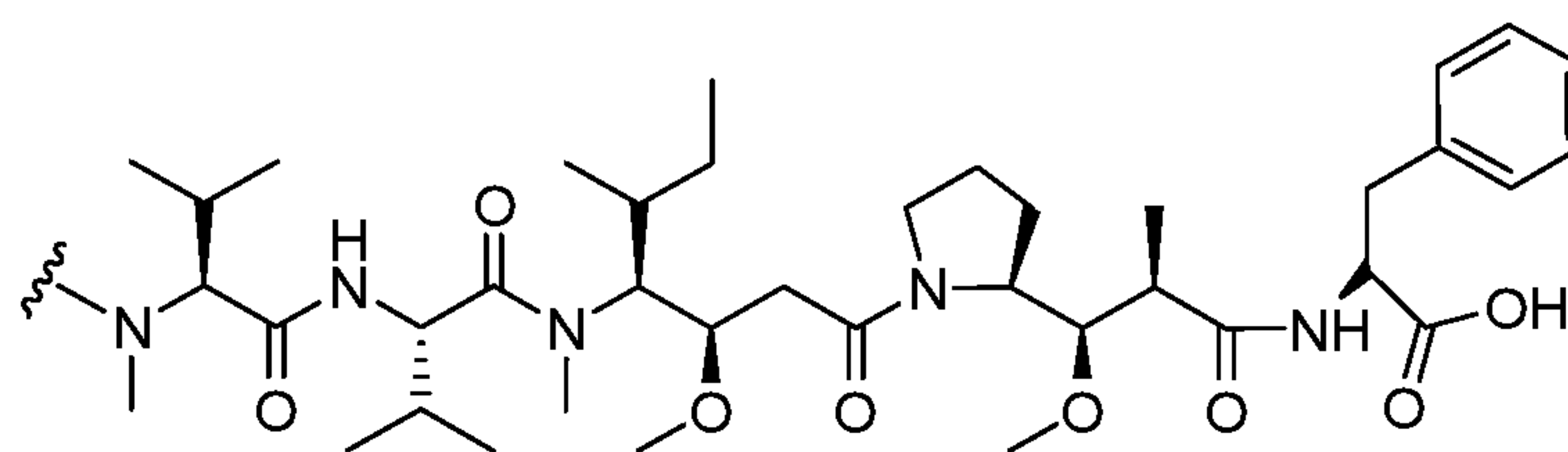
W represents  $-C(O)NR'-$ , where C(O) is bonded to the phenyl ring and  $NR'$  is bonded to L;

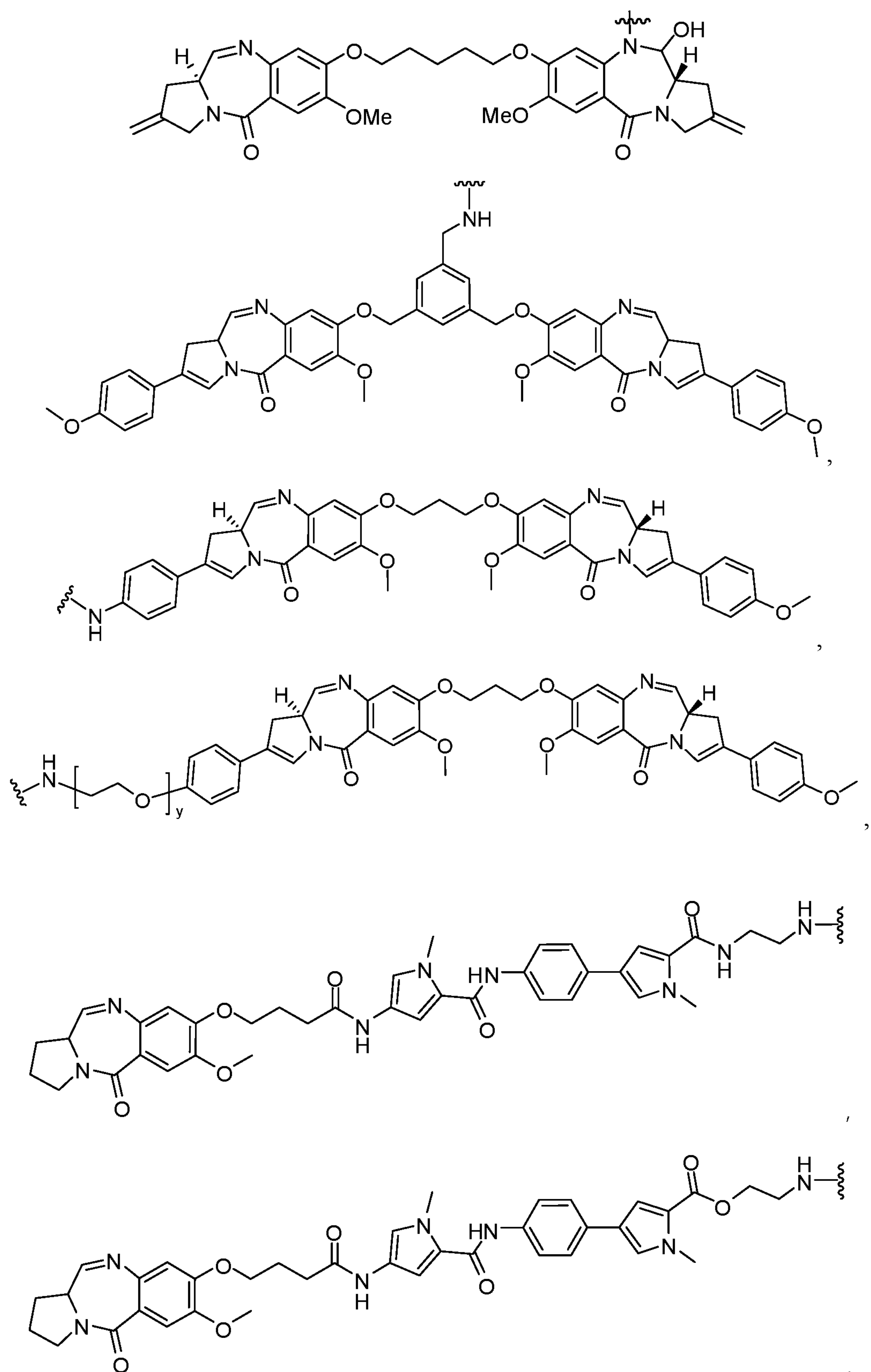
each Z independently represents hydrogen; and

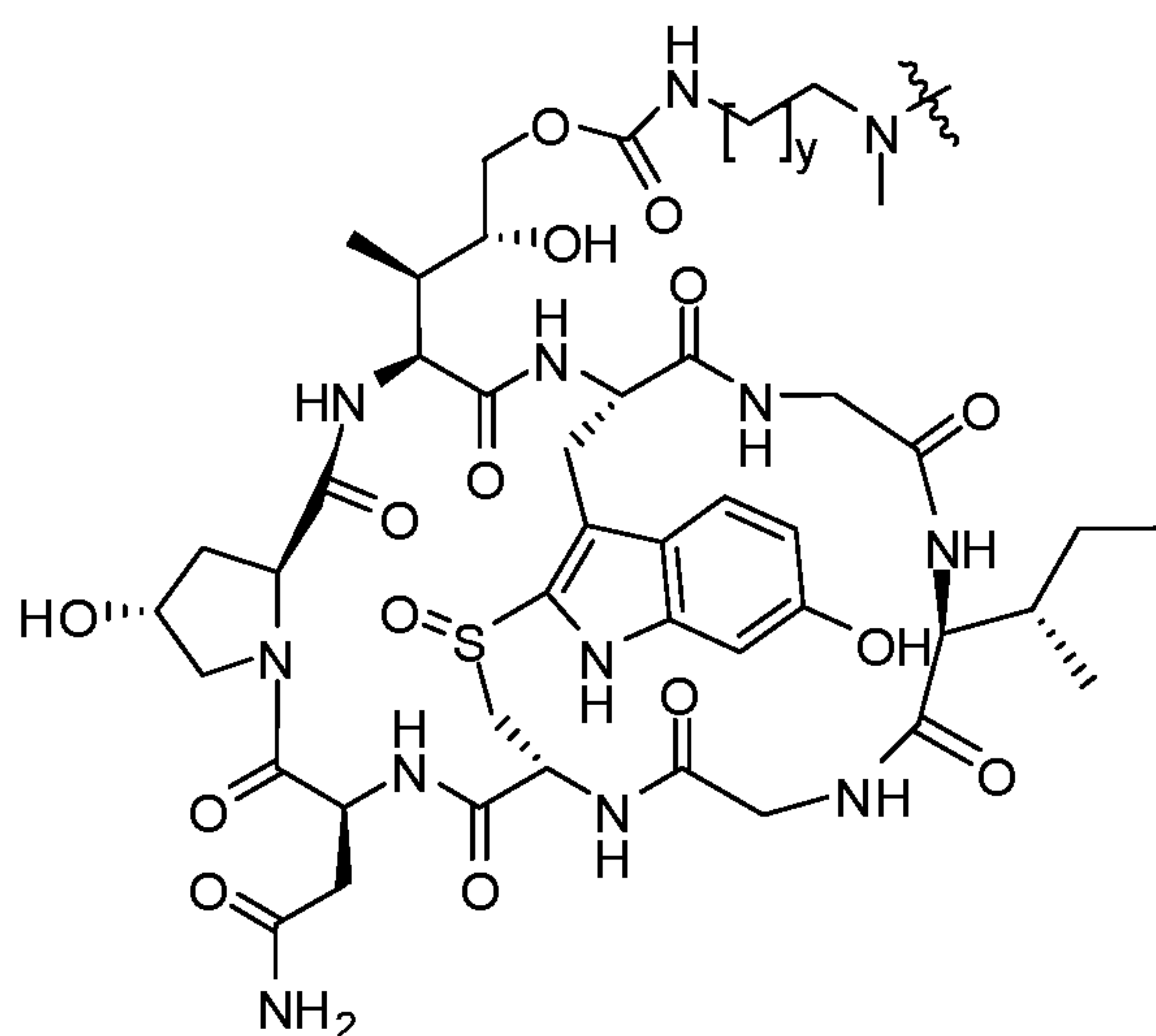
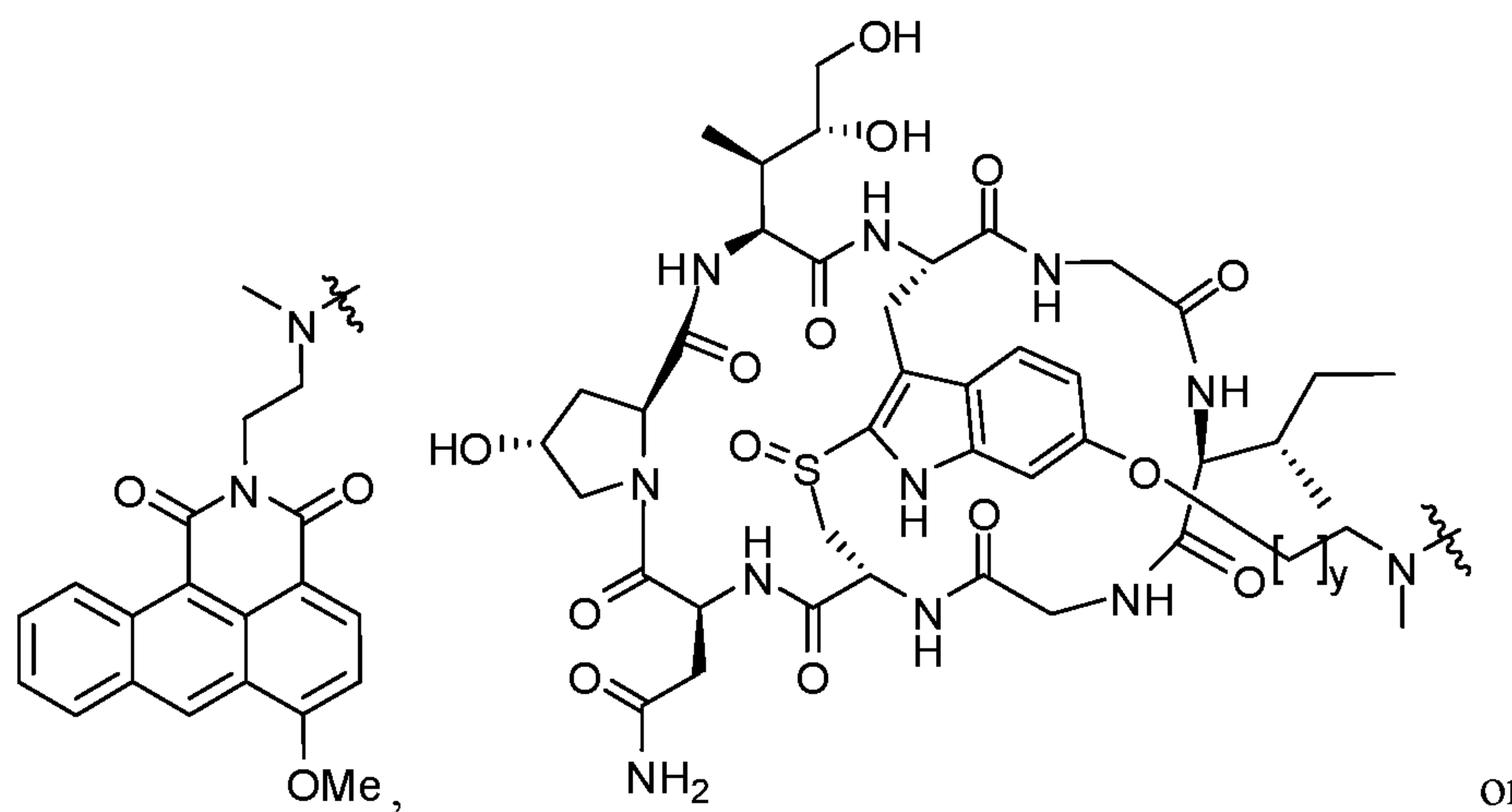
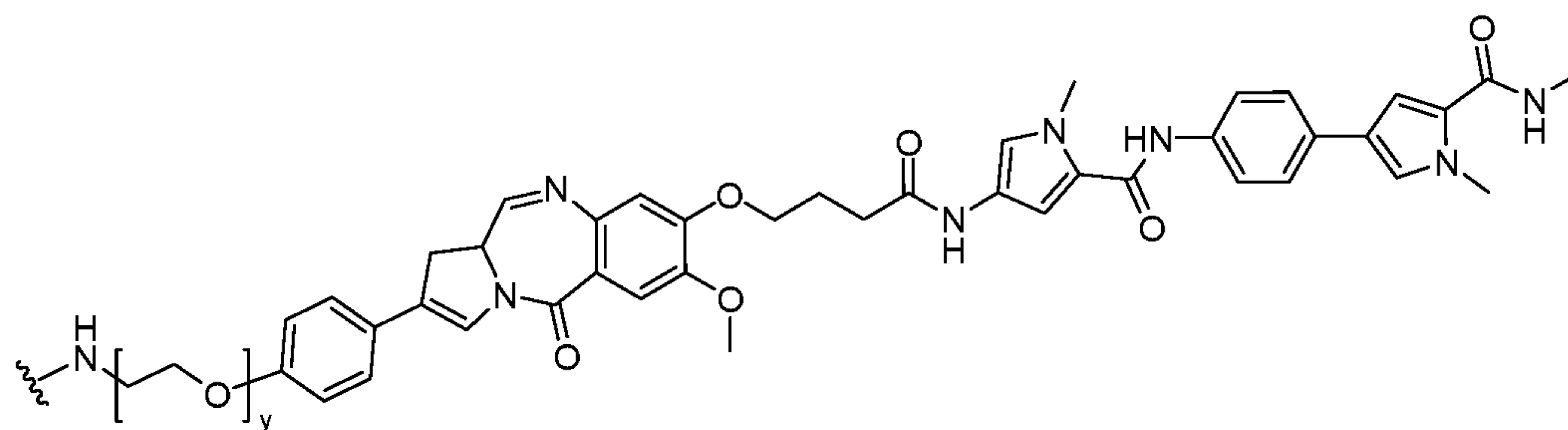
$R_1$  and  $R_2$  each represent hydrogen.

94. The conjugate of any one of claims 64 to 93, wherein B,  $B_1$ , and/or  $B_2$  are each

independently selected from:







wherein  $y$  is an integer from 1 to 10.

95. A conjugate of any one of the preceding claims, comprising from 2 to 4 linkers as set forth in any of the preceding claims covalently coupled to the ligand, wherein:
- each linker comprises a peptide sequence of a plurality of amino acids; and
  - at least two active agents are covalently coupled to side chains of the amino acids of each linker.

96. The conjugate of claim 95, wherein each linker is coupled to a C-terminus of the ligand.

97. The conjugate of claim 96, wherein the ligand is an antibody and each linker is coupled to a C-terminus of the antibody (*e.g.*, of the heavy and light chains of the antibody).

98. The conjugate of any preceding claim, wherein each active agent is the same active agent.

99. The conjugate of any one of claims 1-97, wherein the active agents comprise at least two different active agents.

100. A pharmaceutical composition comprising the conjugate of any one of the preceding claims and a pharmaceutically acceptable excipient.

101. The pharmaceutical composition of claim 100, further comprising a therapeutically effective amount of chemotherapeutic agent.

102. A method of treating cancer in a subject, comprising administering the pharmaceutical composition of claim 100 or 101 to the subject.

103. The method of claim 102, wherein the subject is a mammal.

104. The method of claim 103, wherein the subject is selected from rodents, lagomorphs, felines, canines, porcines, ovines, bovines, equines, and primates.

105. The method of claim 103, wherein the subject is a human.

106. A method for making a ligand-drug conjugate of any one of claims 1 to 99, comprising reacting a biomolecule with a prodrug, wherein:

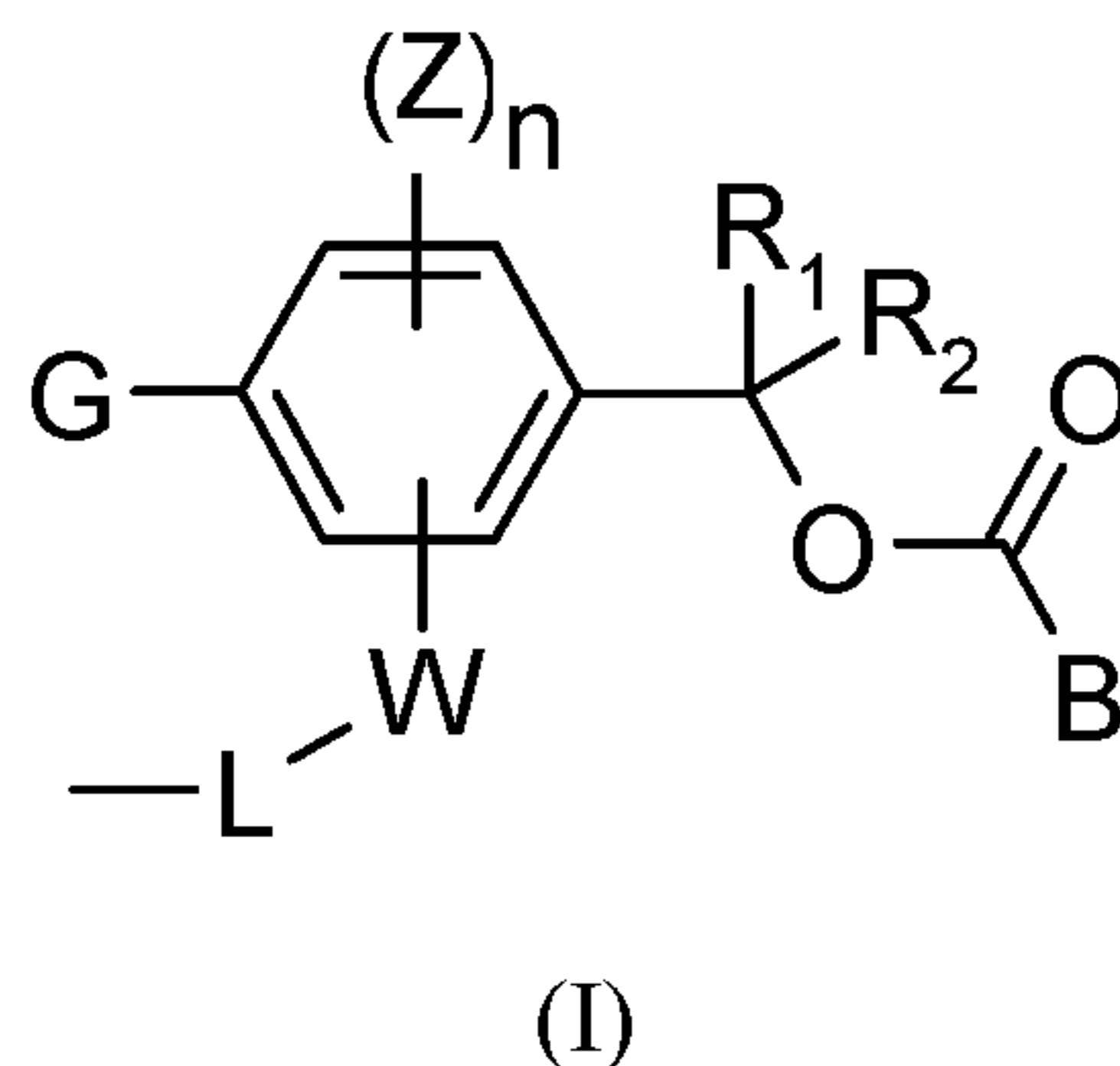
the biomolecule comprises a ligand and a ketone or aldehyde;

the prodrug comprises an alkoxyamine; and

the reaction produces an oxime, thereby covalently linking the ligand to the prodrug.

107. The method of claim 106, wherein the ligand is an antibody.
108. The method of claim 106 or 107, further comprising isoprenylating the ligand, thereby producing the biomolecule, wherein:
- the ligand comprises an isoprenylation sequence;
  - isoprenylating the ligand comprises incubating the ligand with an isoprenoid transferase and an isoprenoid transferase substrate; and
  - the substrate comprises the ketone or aldehyde.
109. The method of claim 108, wherein the ligand is an antibody.
110. The method of claim 108 or 109, wherein the isoprenoid transferase is farnesyltransferase or geranylgeranyltransferase.
111. A method for making a ligand-drug conjugate of any one of claims 1 to 99, comprising isoprenylating a ligand, wherein:
- the ligand comprises an isoprenylation sequence;
  - isoprenylating the ligand comprises incubating the ligand with an isoprenoid transferase and an isoprenoid transferase substrate; and
  - the substrate comprises the active agent.
112. The method of claim 111, wherein the ligand is an antibody.
113. A linker-active agent compound, wherein
- i) the linker comprises a peptide sequence of a plurality of amino acids; and
  - ii) at least two active agents are covalently coupled to side chains of the amino acids.
114. The linker-active agent compound of claim 113, wherein each active agent is coupled to the peptide sequence by a cleavage group that can be hydrolyzed to release the active agent from the ligand-active agent compound.

115. The linker-active agent compound of claim 114, wherein the cleavage group is represented by the structure having the formula:



wherein:

B represents an active agent;

G represents a sugar or sugar acid, preferably glucuronic acid;

W represents an electron-withdrawing group, preferably  $-C(O)NR'$ -, where C(O) is bonded to the phenyl ring and  $NR'$  is bonded to L;

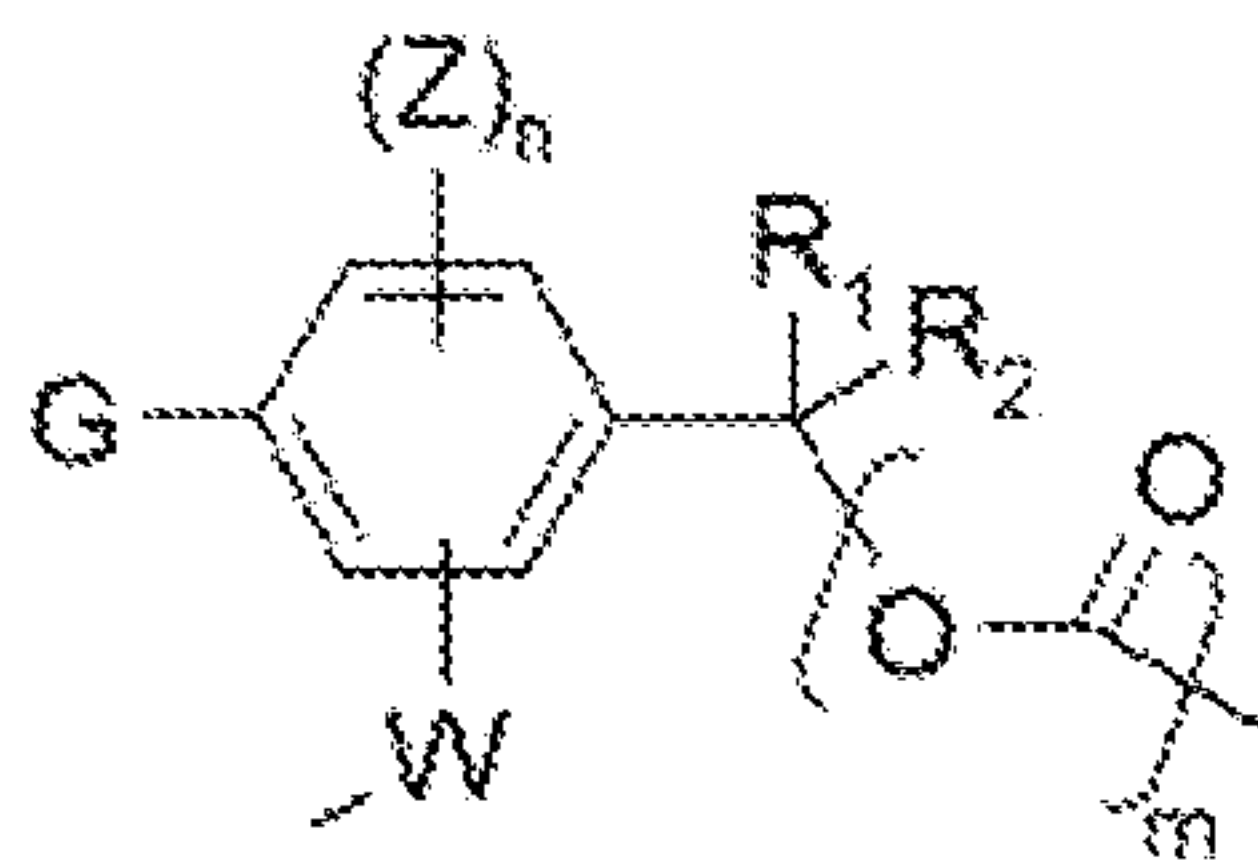
each Z independently represents hydrogen,  $(C_1-C_8)$ alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen,  $(C_1-C_8)$ alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

$R_1$  and  $R_2$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, or  $(C_3-C_8)$ cycloalkyl, preferably hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a  $(C_3-C_8)$ cycloalkyl ring; and

L represents a linkage to the peptide sequence.

116. The linker-active agent compound of claim 114, wherein the cleavage group is represented by the structure having the formula:



wherein:

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

W represents  $-C(O)-$ ,  $-C(O)NR'$ -,  $-C(O)O-$ ,  $-S(O)_2NR'$ -,  $-P(O)R''NR'$ -,  $-S(O)NR'$ -, or -

$\text{PO}_2\text{NR}'$ -, in each case where the C(O), S, or P is preferably directly bound to the phenyl ring, and R' and R'' are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, mono- or di-carboxyl(C<sub>1</sub>-C<sub>8</sub>)alkyl, (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>8</sub>)alkoxy, (C<sub>1</sub>-C<sub>8</sub>)alkylthio, mono- or di-(C<sub>1</sub>-C<sub>8</sub>)alkylamino, (C<sub>3</sub>-C<sub>20</sub>)heteroaryl, or (C<sub>6</sub>-C<sub>20</sub>)aryl;

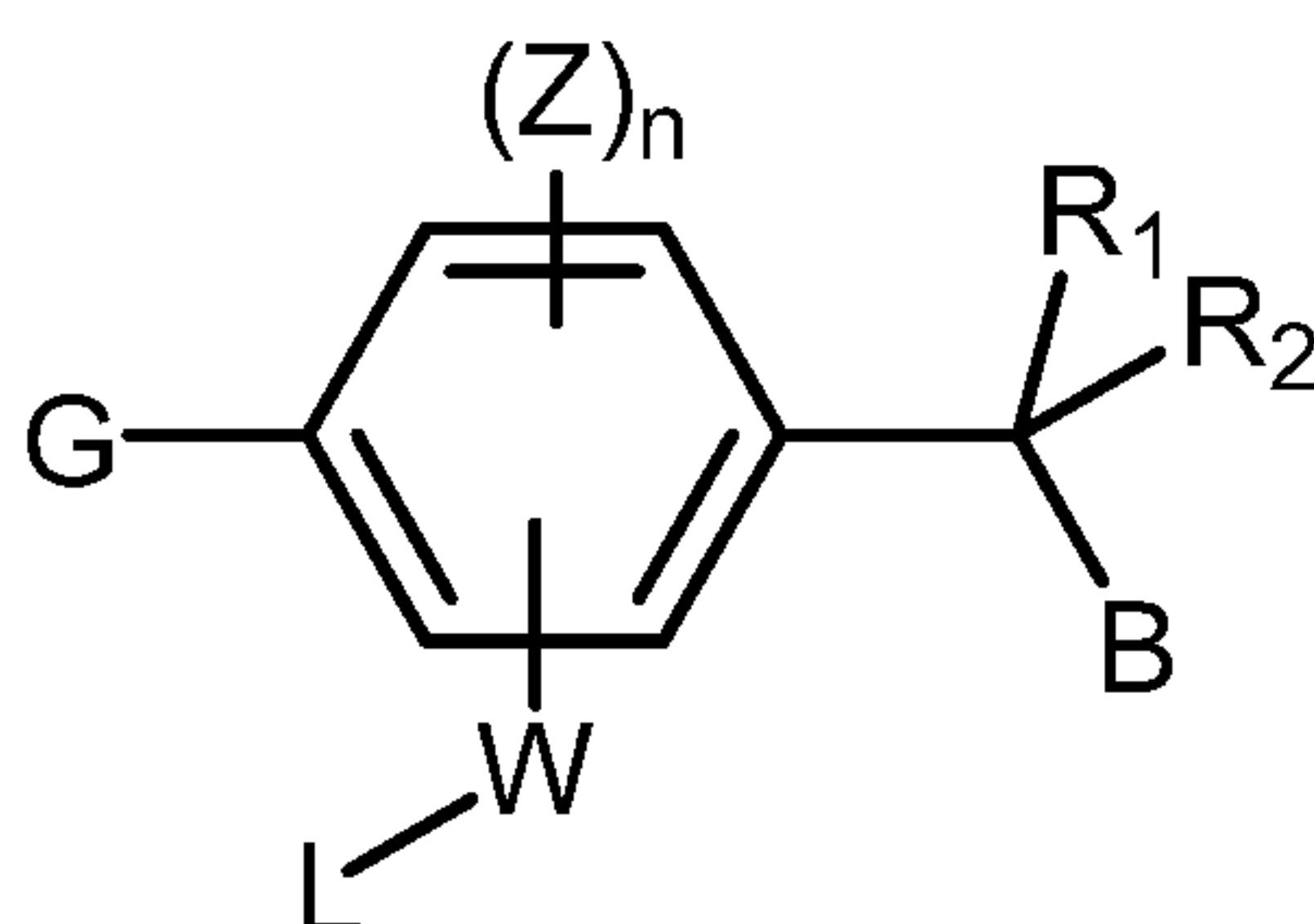
each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3;

m is 0 or 1, preferably 1; and

R<sub>1</sub> and R<sub>2</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, preferably hydrogen, or R<sub>1</sub> and R<sub>2</sub> taken together with the carbon atom to which they are attached form a (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl ring.

117. The linker-active agent compound of claim 114, wherein the cleavage group is represented by the structure having the formula:



or a pharmaceutically acceptable salt thereof, wherein

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

B is a unit covalently attached to the active agent,

W represents  $-\text{C}(\text{O})-$ ,  $-\text{C}(\text{O})\text{NR}'$ -,  $-\text{C}(\text{O})\text{O}-$ ,  $-\text{S}(\text{O})_2\text{NR}'$ -,  $-\text{P}(\text{O})\text{R}''\text{NR}'$ -,  $-\text{S}(\text{O})\text{NR}'$ -, or  $-\text{PO}_2\text{NR}'$ -, in each case where the C(O), S, or P is directly bound to the phenyl ring, and R' and R'' are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>8</sub>)alkoxy, (C<sub>1</sub>-C<sub>8</sub>)alkylthio, mono- or di-(C<sub>1</sub>-C<sub>8</sub>)alkylamino, (C<sub>3</sub>-C<sub>20</sub>)heteroaryl, or (C<sub>6</sub>-C<sub>20</sub>)aryl;

each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro),

preferably a hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

L represents a linkage to the peptide sequence;

R<sub>1</sub> and R<sub>2</sub> are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, preferably hydrogen, or R<sub>1</sub> and R<sub>2</sub> taken together with the carbon atom to which they are attached form a (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl ring.

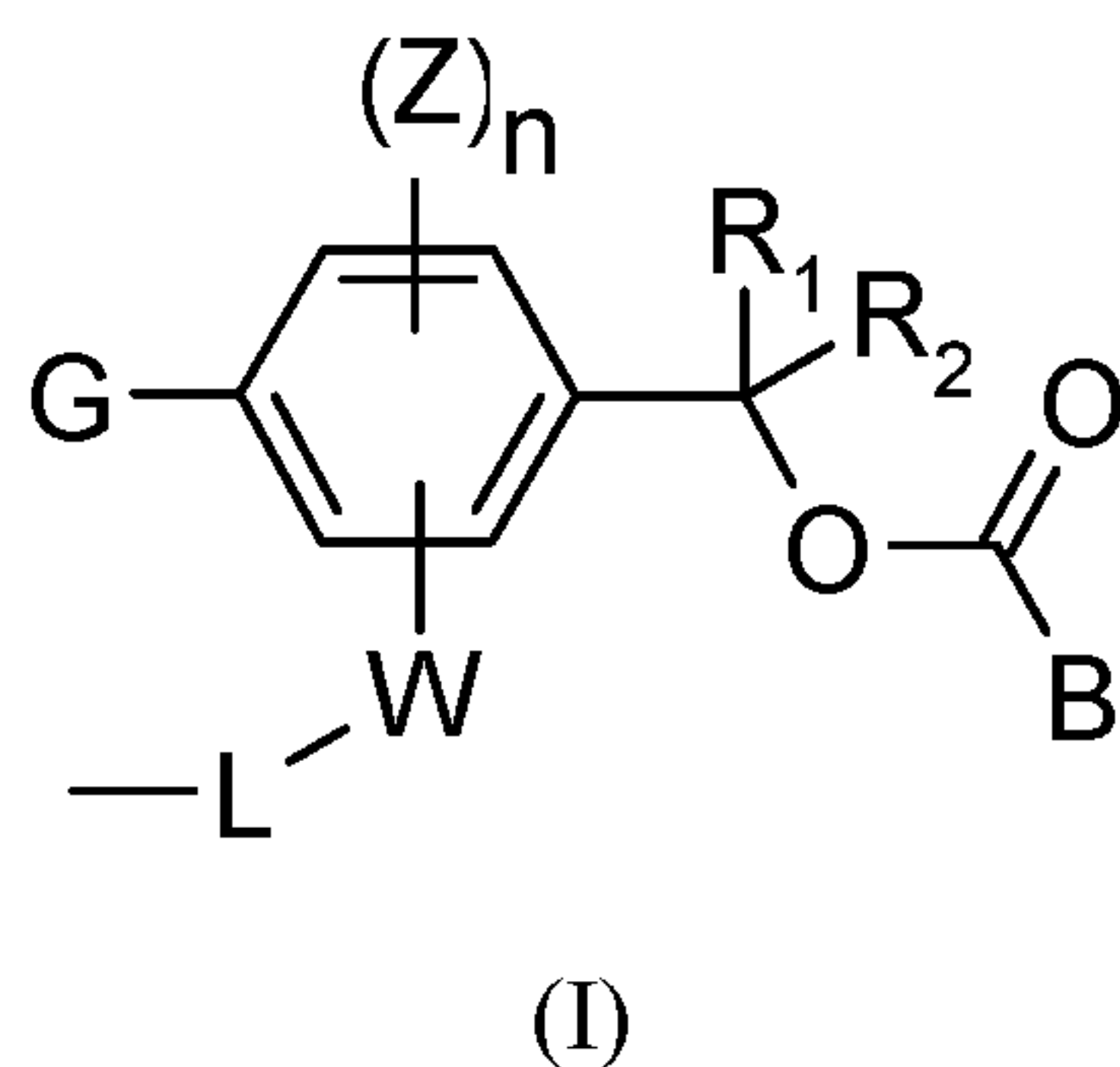
118. The linker-active agent compound of any one of claims 115-117, wherein each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro.

119. The linker-active agent compound of claim 118, wherein each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro.

120. A linker compound, wherein

- i) the linker comprises a peptide sequence of a plurality of amino acids; and
- ii) at least two cleavage groups are covalently coupled to side chains of the amino acids, wherein each cleavage group has a reactive moiety capable of reacting with an active agent.

121. The linker compound of claim 120, wherein the cleavage group is represented by the structure having the formula:



wherein:

B represents a leaving group capable of being displaced an active agent, such as a halogen (esp. Cl or Br), or a unit comprising a reactive moiety capable of being coupled to an active agent, such as an isocyanate, an acid chloride, a chloroformate, etc.;

G represents a sugar or sugar acid, preferably glucuronic acid;

W represents an electron-withdrawing group, preferably  $-C(O)NR'$ -, where  $C(O)$  is bonded to the phenyl ring and  $NR'$  is bonded to L;

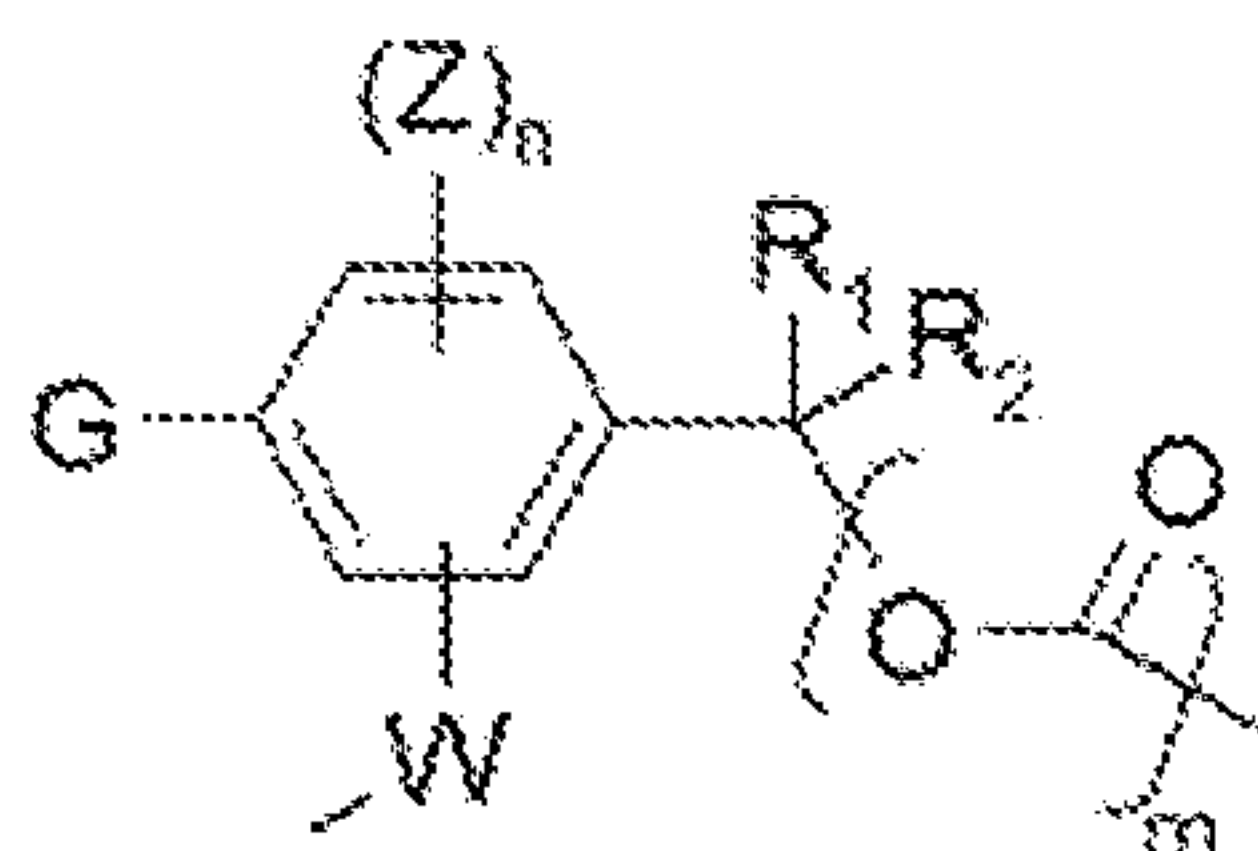
each Z independently represents hydrogen,  $(C_1-C_8)$ alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen,  $(C_1-C_8)$ alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

$R_1$  and  $R_2$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, or  $(C_3-C_8)$ cycloalkyl, preferably hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a  $(C_3-C_8)$ cycloalkyl ring; and

L represents a linkage to the peptide sequence.

122. The linker compound of claim 120, wherein the cleavage group is represented by the structure having the formula:



wherein:

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

W represents  $-C(O)-$ ,  $-C(O)NR'$ -,  $-C(O)O-$ ,  $-S(O)_2NR'$ -,  $-P(O)R''NR'$ -,  $-S(O)NR'$ -, or  $-PO_2NR'$ -, in each case where the  $C(O)$ , S, or P is preferably directly bound to the phenyl ring, and  $R'$  and  $R''$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, mono- or di-carboxyl $(C_1-C_8)$ alkyl,  $(C_3-C_8)$ cycloalkyl,  $(C_1-C_8)$ alkoxy,  $(C_1-C_8)$ alkylthio, mono- or di- $(C_1-C_8)$ alkylamino,  $(C_3-C_{20})$ heteroaryl, or  $(C_6-C_{20})$ aryl;

each Z independently represents hydrogen,  $(C_1-C_8)$ alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen,  $(C_1-C_8)$ alkyl, halogen, cyano, or nitro, most preferably hydrogen;

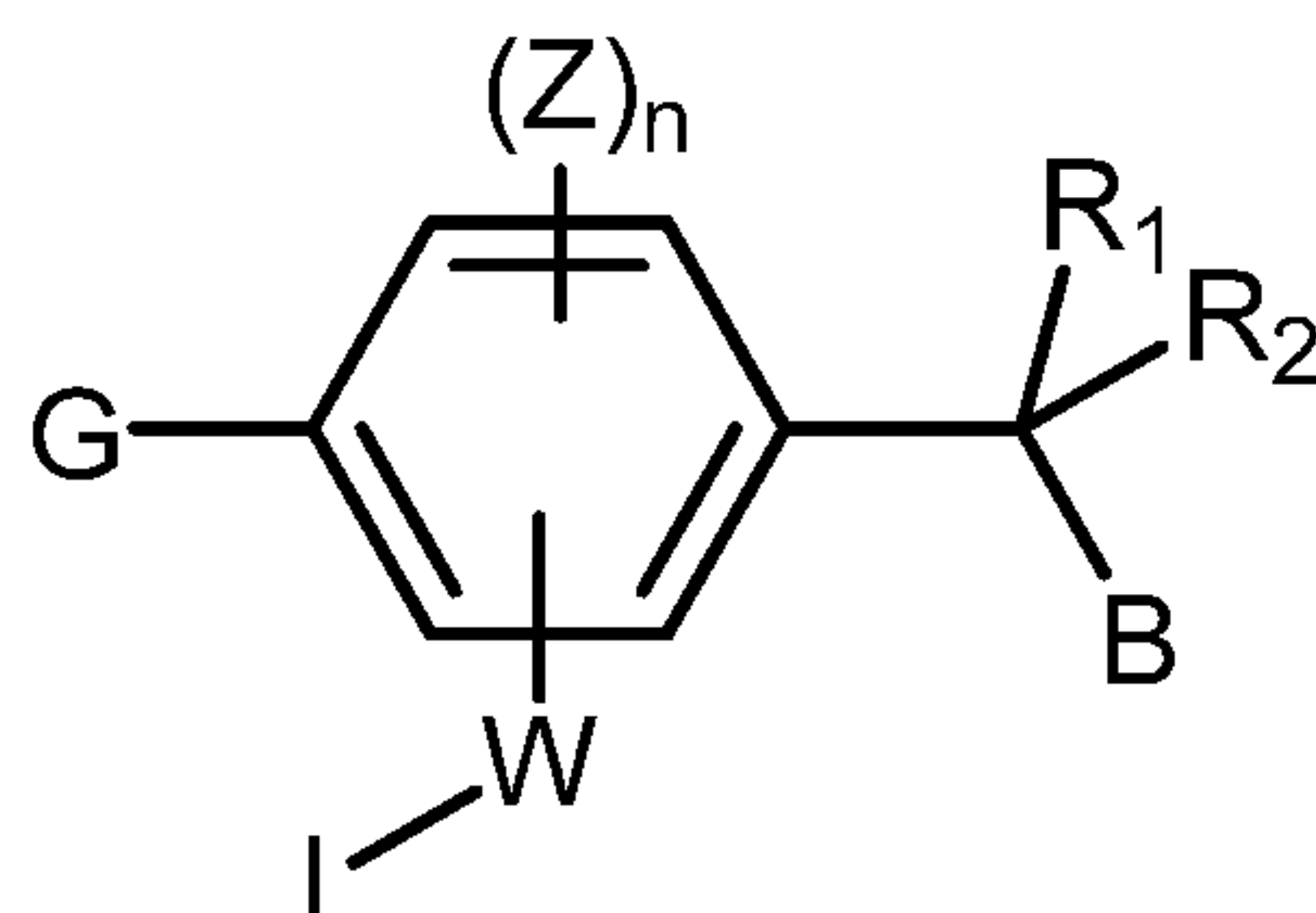
n is an integer from 1 to 3;

m is 0 or 1, preferably 1; and

$R_1$  and  $R_2$  are each independently hydrogen,  $(C_1-C_8)$ alkyl, or  $(C_3-C_8)$ cycloalkyl, preferably

hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl ring.

123. The linker compound of claim 120, wherein the cleavage group is represented by the structure having the formula:



or a pharmaceutically acceptable salt thereof, wherein

G represents a sugar, sugar acid, or modified sugar, preferably a sugar or sugar acid, most preferably glucuronic acid;

B is a unit comprising a reactive moiety capable of being coupled to the active agent,

W represents -C(O)-, -C(O)NR'-, -C(O)O-, -S(O)<sub>2</sub>NR'-, -P(O)R''NR'-, -S(O)NR'-, or -PO<sub>2</sub>NR'-, in each case where the C(O), S, or P is directly bound to the phenyl ring, and R' and R'' are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, (C<sub>1</sub>-C<sub>8</sub>)alkoxy, (C<sub>1</sub>-C<sub>8</sub>)alkylthio, mono- or di-(C<sub>1</sub>-C<sub>8</sub>)alkylamino, (C<sub>3</sub>-C<sub>20</sub>)heteroaryl, or (C<sub>6</sub>-C<sub>20</sub>)aryl

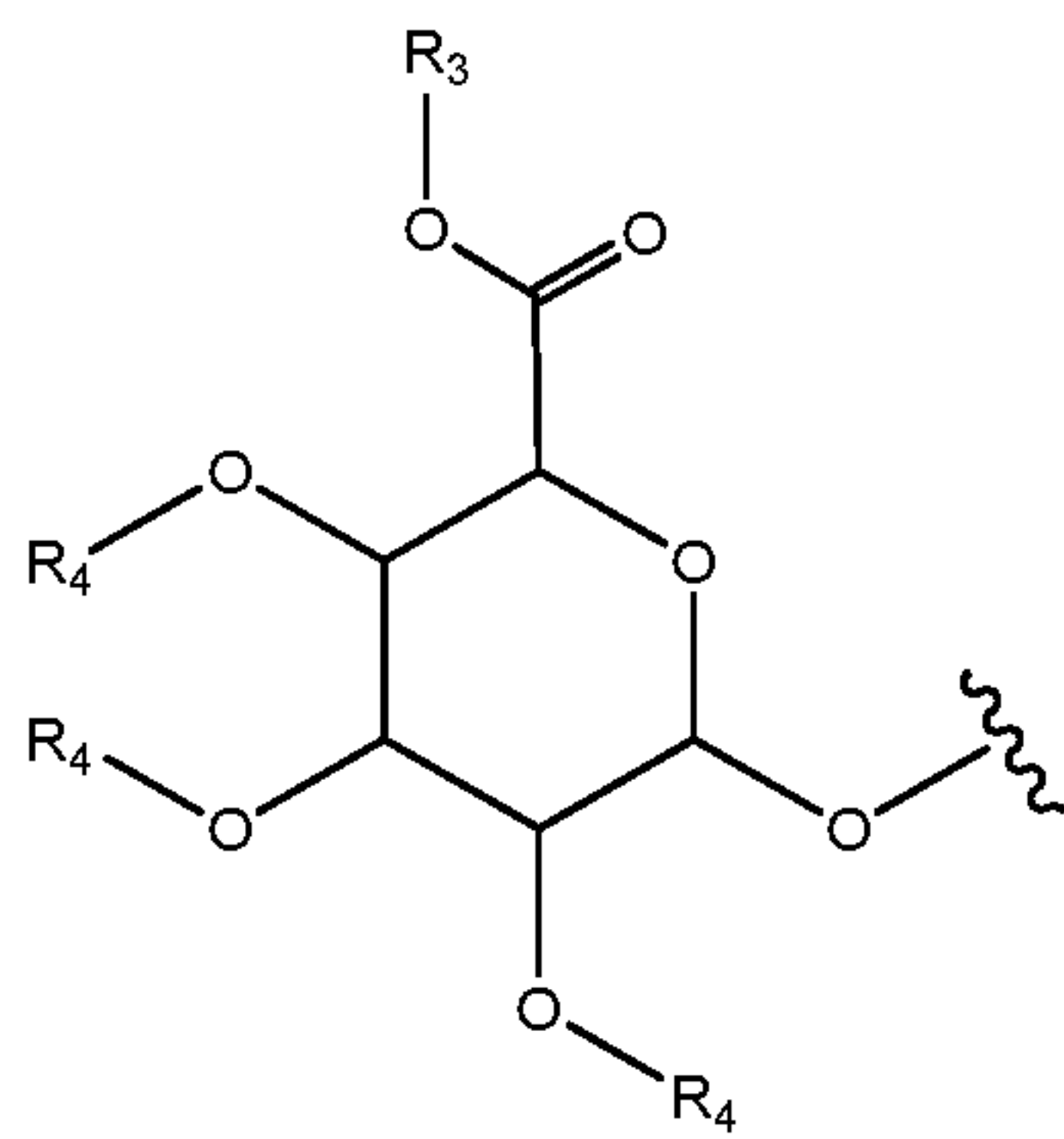
each Z independently represents hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or an electron-withdrawing group (such as an amide, carboxylic acid, carboxylic acid ester, halogen, cyano, or nitro), preferably a hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, halogen, cyano, or nitro, most preferably hydrogen;

n is an integer from 1 to 3, preferably 3;

L represents a linker comprising the peptide sequence;

$R_1$  and  $R_2$  are each independently hydrogen, (C<sub>1</sub>-C<sub>8</sub>)alkyl, or (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl, preferably hydrogen, or  $R_1$  and  $R_2$  taken together with the carbon atom to which they are attached form a (C<sub>3</sub>-C<sub>8</sub>)cycloalkyl ring.

124. The linker compound of any one of claims 121 to 122, wherein:



G is

;

$R_3$  is hydrogen or a carboxyl protecting group; and

each  $R_4$  is independently hydrogen or a hydroxyl protecting group.

125. The linker compound of any one of claims 121 to 124, wherein the linker comprises a connection unit represented by  $-(CH_2)_r(V(CH_2)_p)_{q-}$ ,  $-((CH_2)_pV)_{q-}$ ,  $-(CH_2)_r(V(CH_2)_p)_qY-$ ,  $-((CH_2)_pV)_q(CH_2)_r-$ ,  $-Y(((CH_2)_pV)_{q-})$  or  $-(CH_2)_r(V(CH_2)_p)_qYCH_2-$  wherein:

$r$  is an integer from 0 to 10;

$p$  is an integer from 1 to 10;

$q$  is an integer from 1 to 20;

$V$  and  $Y$  are each independently a single bond,  $-O-$ ,  $-S-$ ,  $-NR_{21}-$ ,  $-C(O)NR_{22}-$ ,  $-NR_{23}C(O)-$ ,  $-NR_{24}SO_2-$ , or  $-SO_2NR_{25}-$ ; and

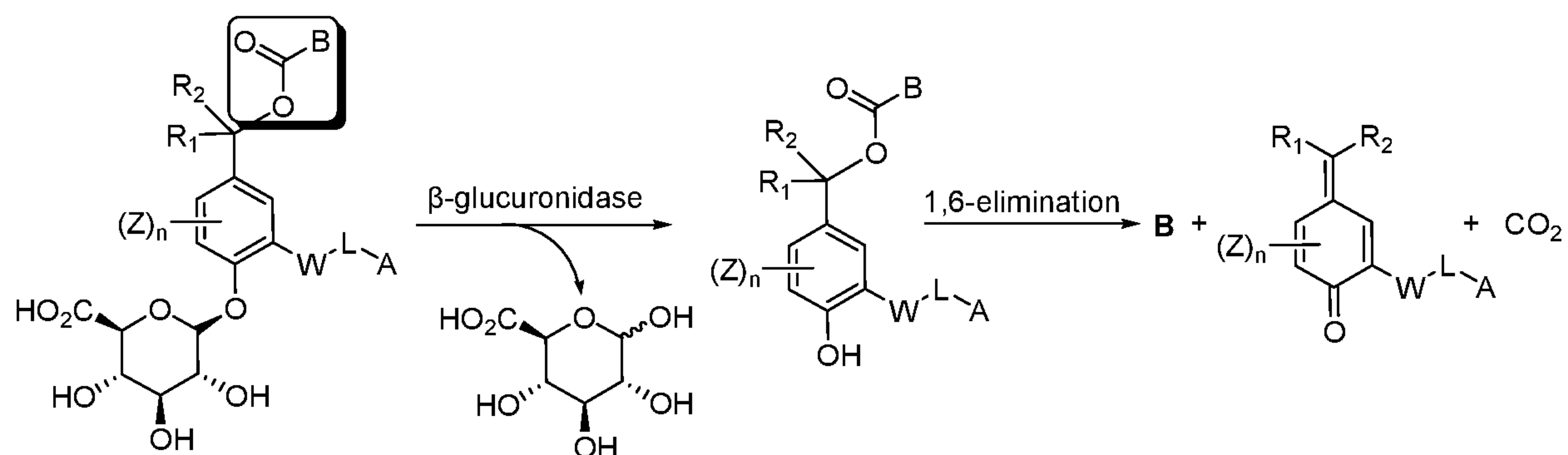
$R_{21}$  to  $R_{25}$  are each independently hydrogen,  $(C_1-C_6)$ alkyl,  $(C_1-C_6)$ alkyl $(C_6-C_{20})$ aryl or  $(C_1-C_6)$ alkyl $(C_3-C_{20})$ heteroaryl.

126. The conjugate of any one of the preceding claims, wherein the cleavage group is capable of cleaving within a target cell.

127. The conjugate of any one of the preceding claims, wherein the cleavage group is capable of releasing one or more active agents.

Figure 1

When  part is carbamate,



When  part is ester,

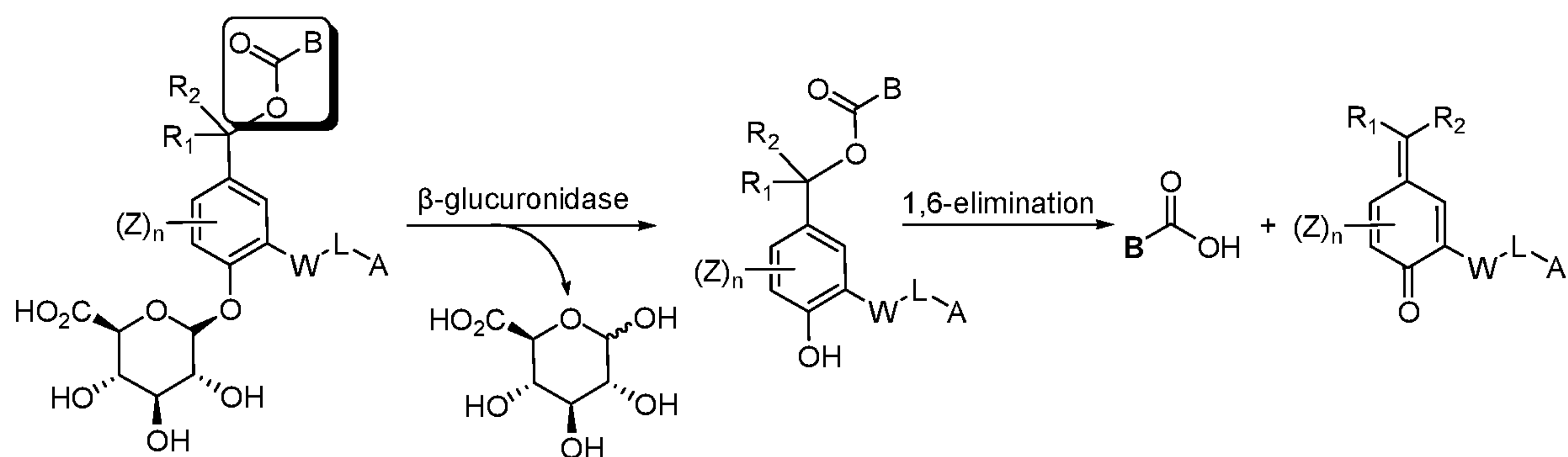


Figure 2

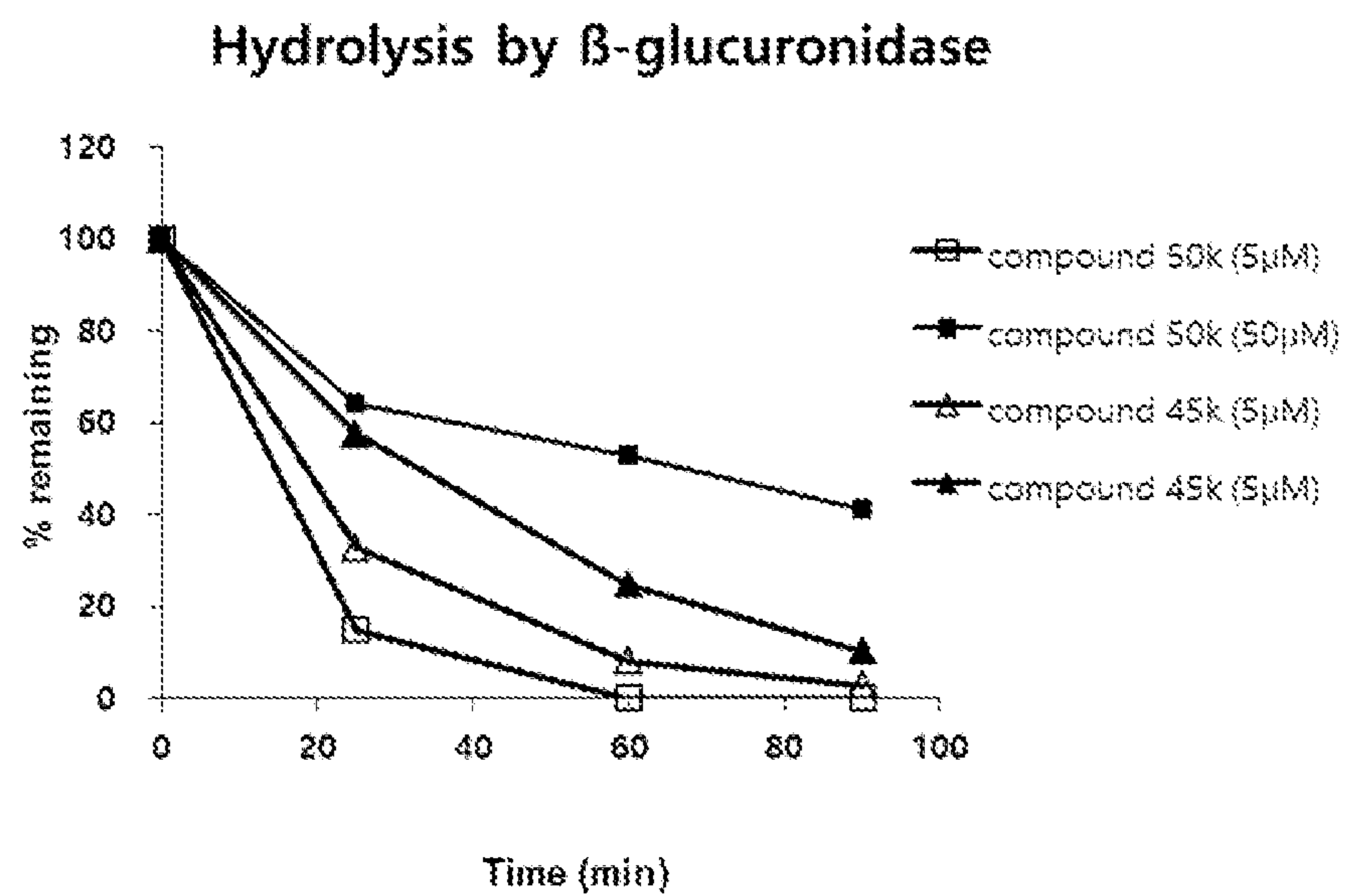


Figure 3

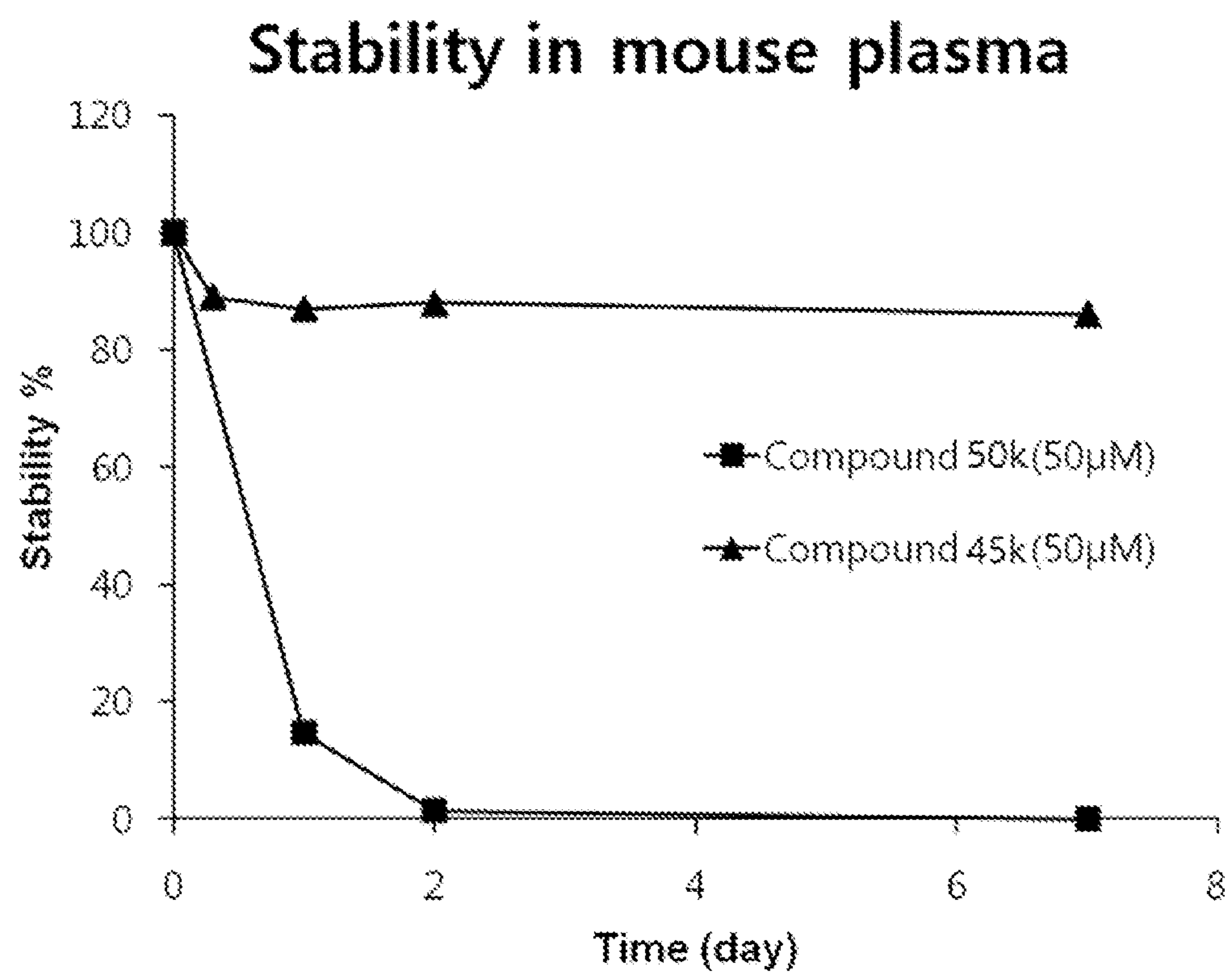


Figure 4

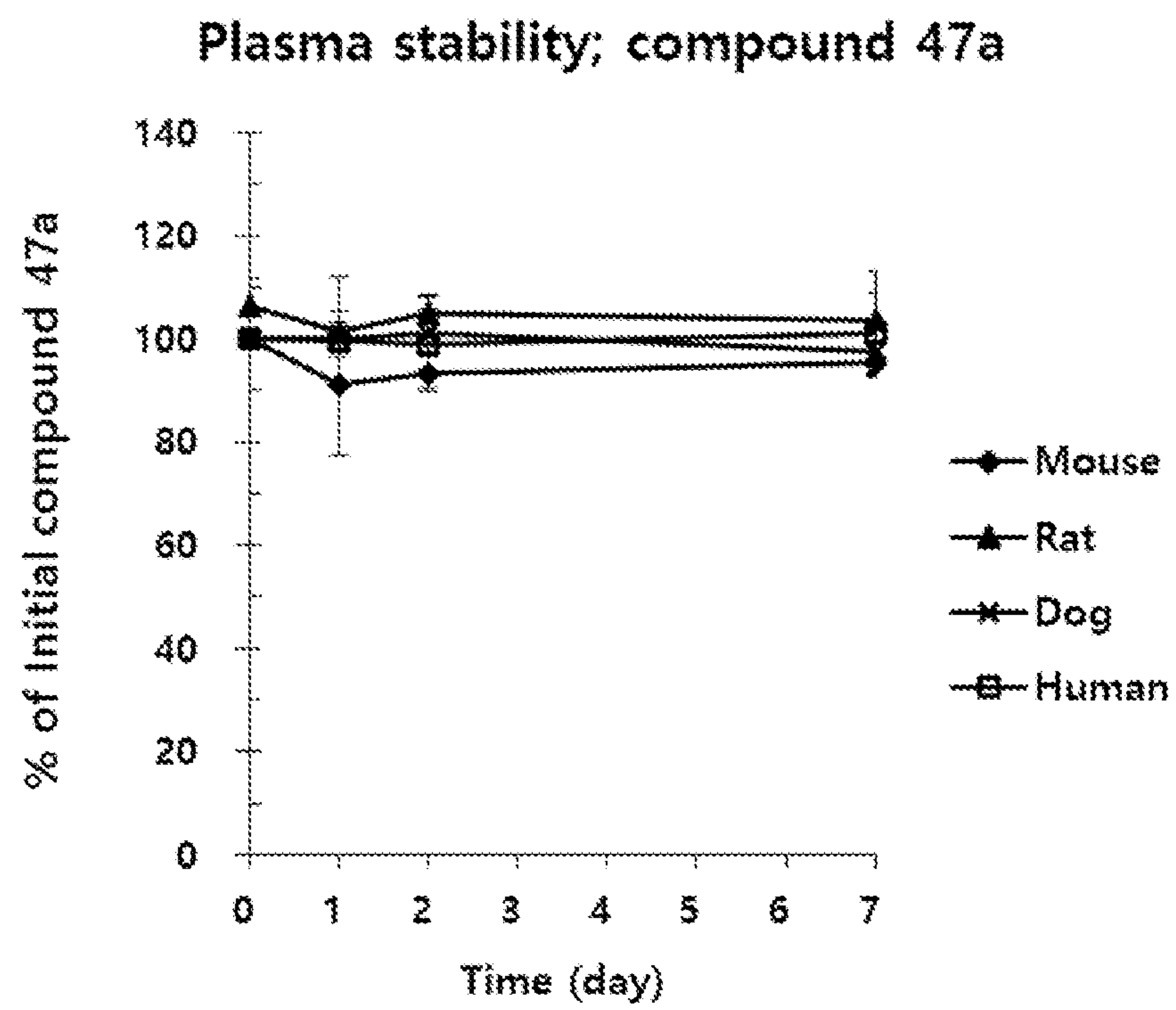


Figure 5

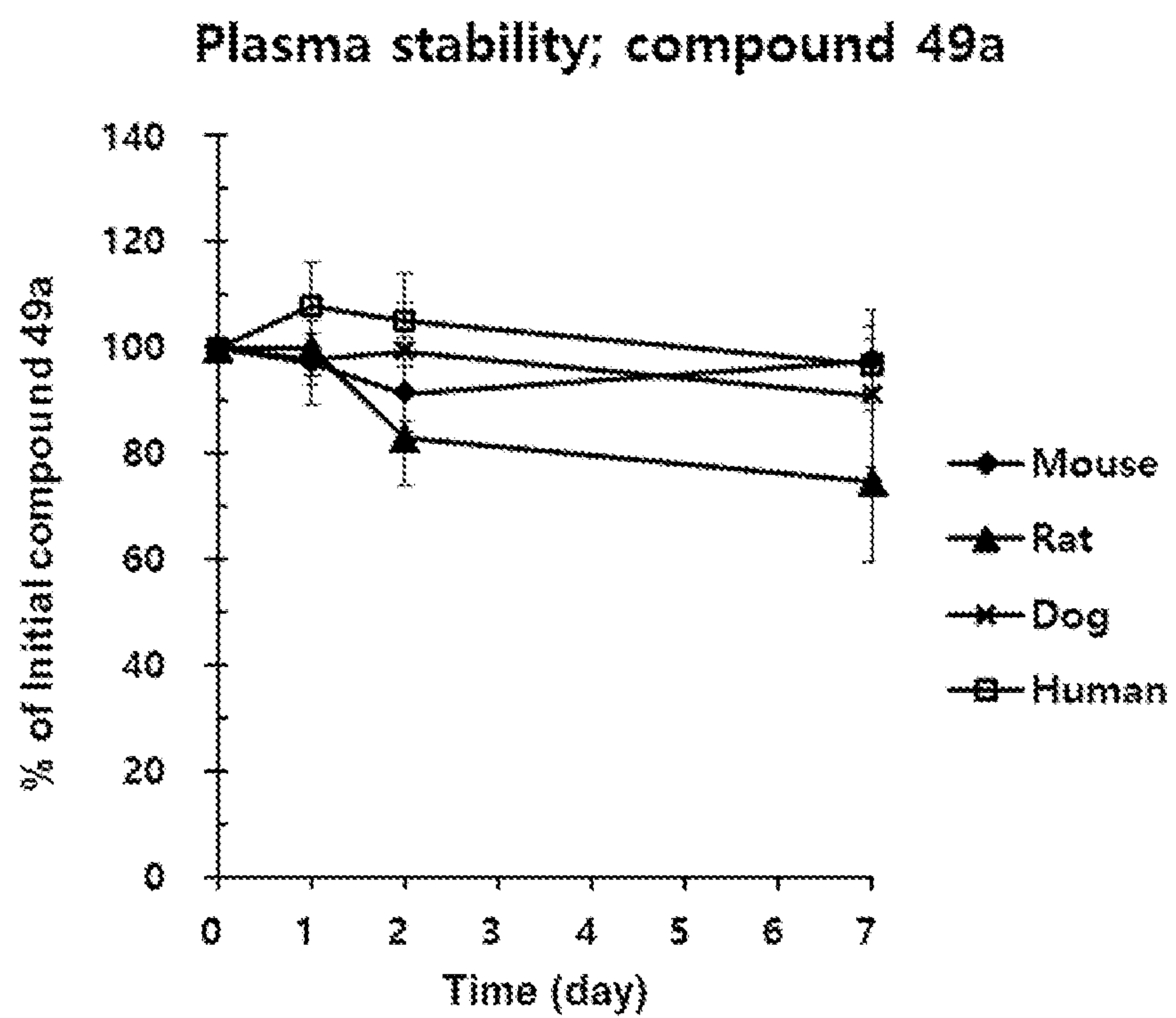


Figure 6

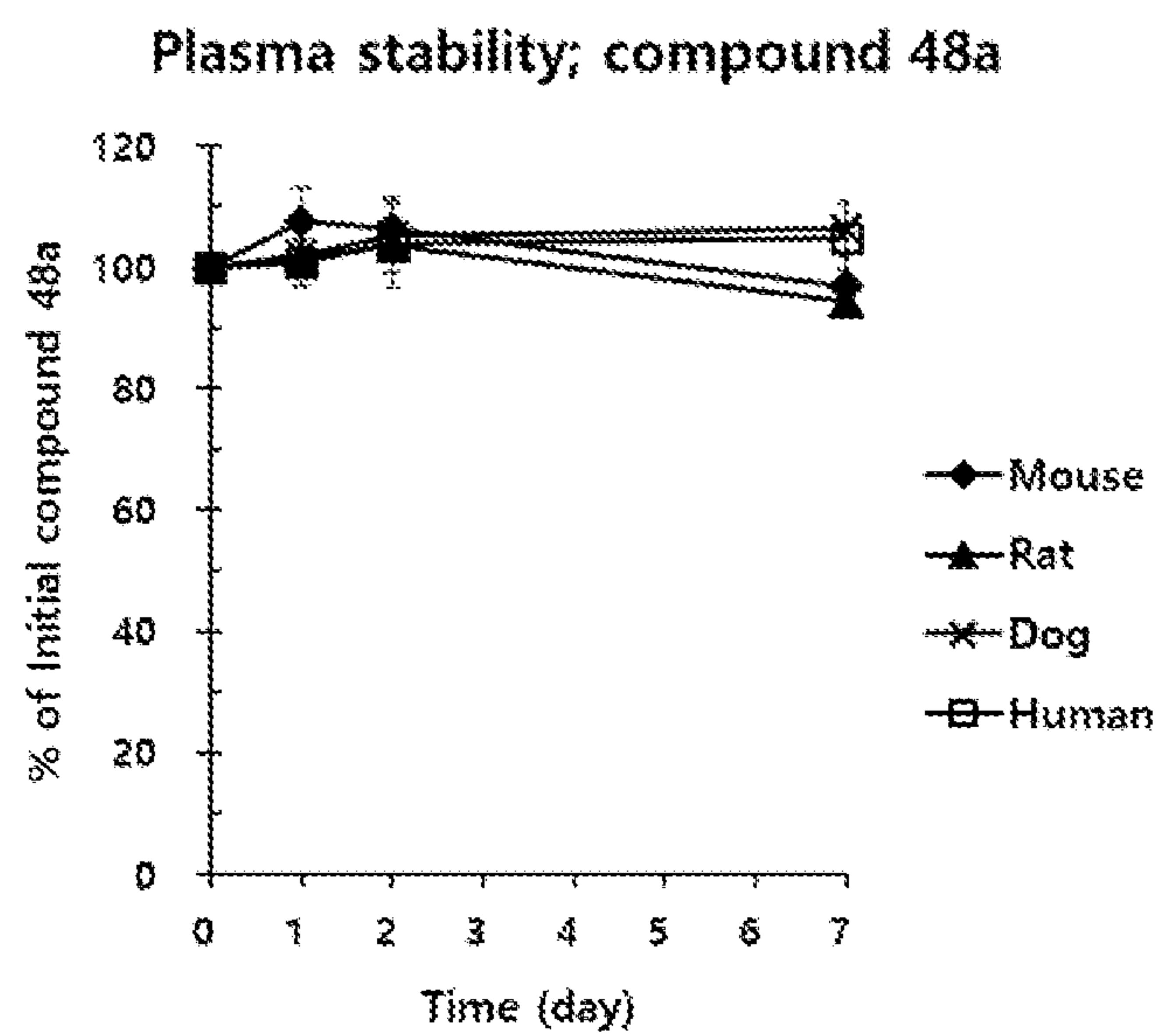


**Figure 7A**

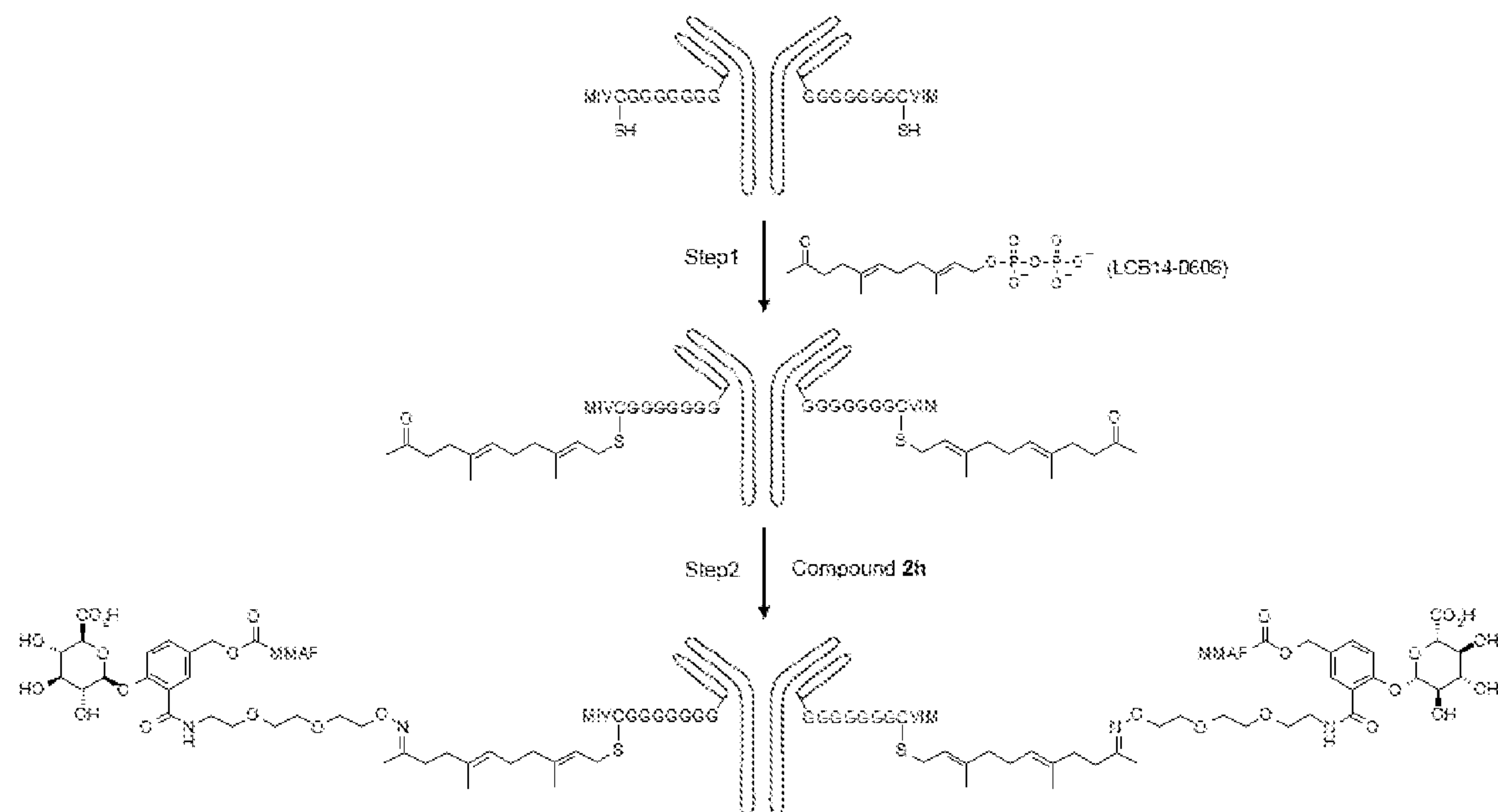


Figure 7B

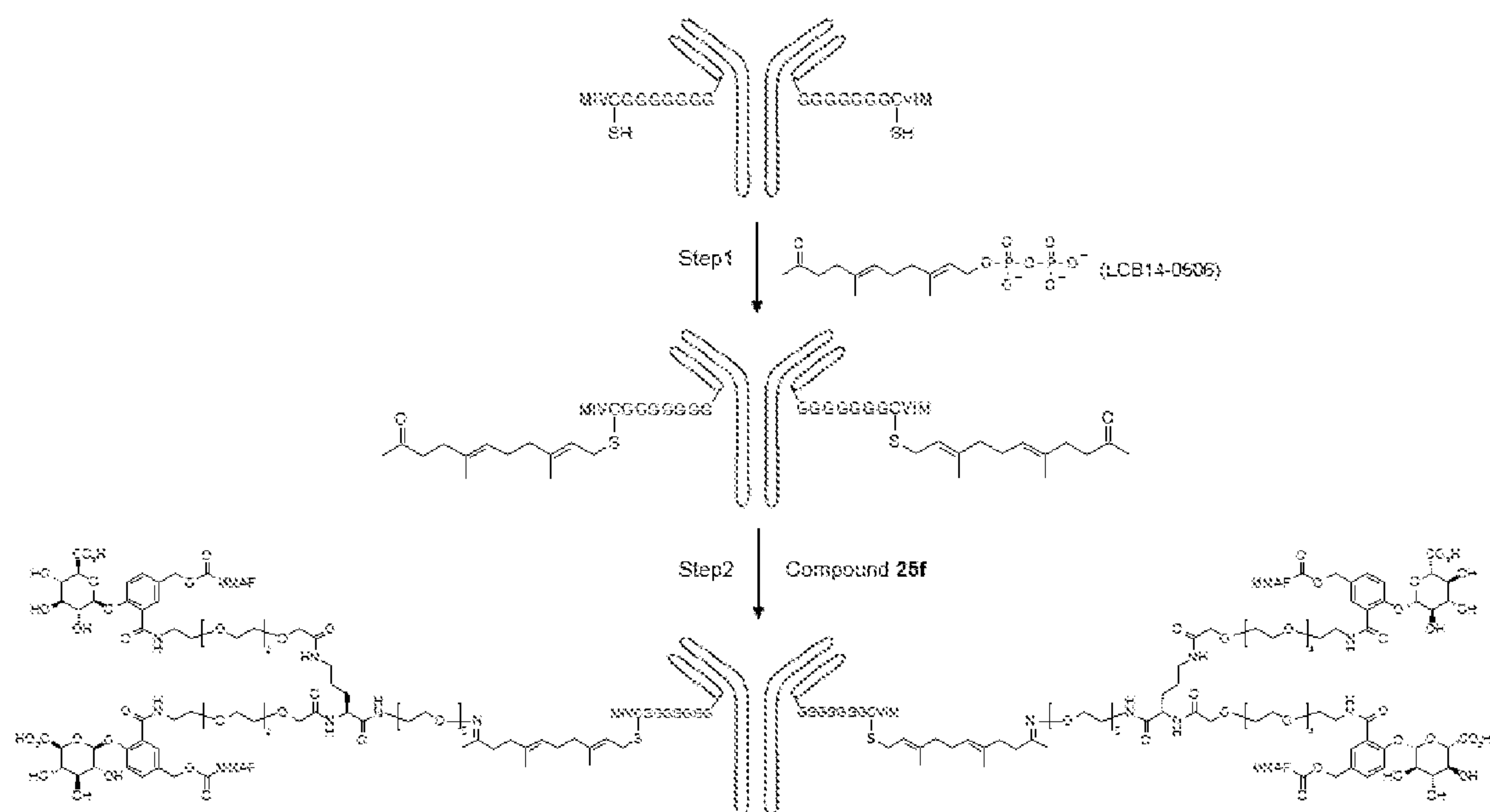


Figure 8

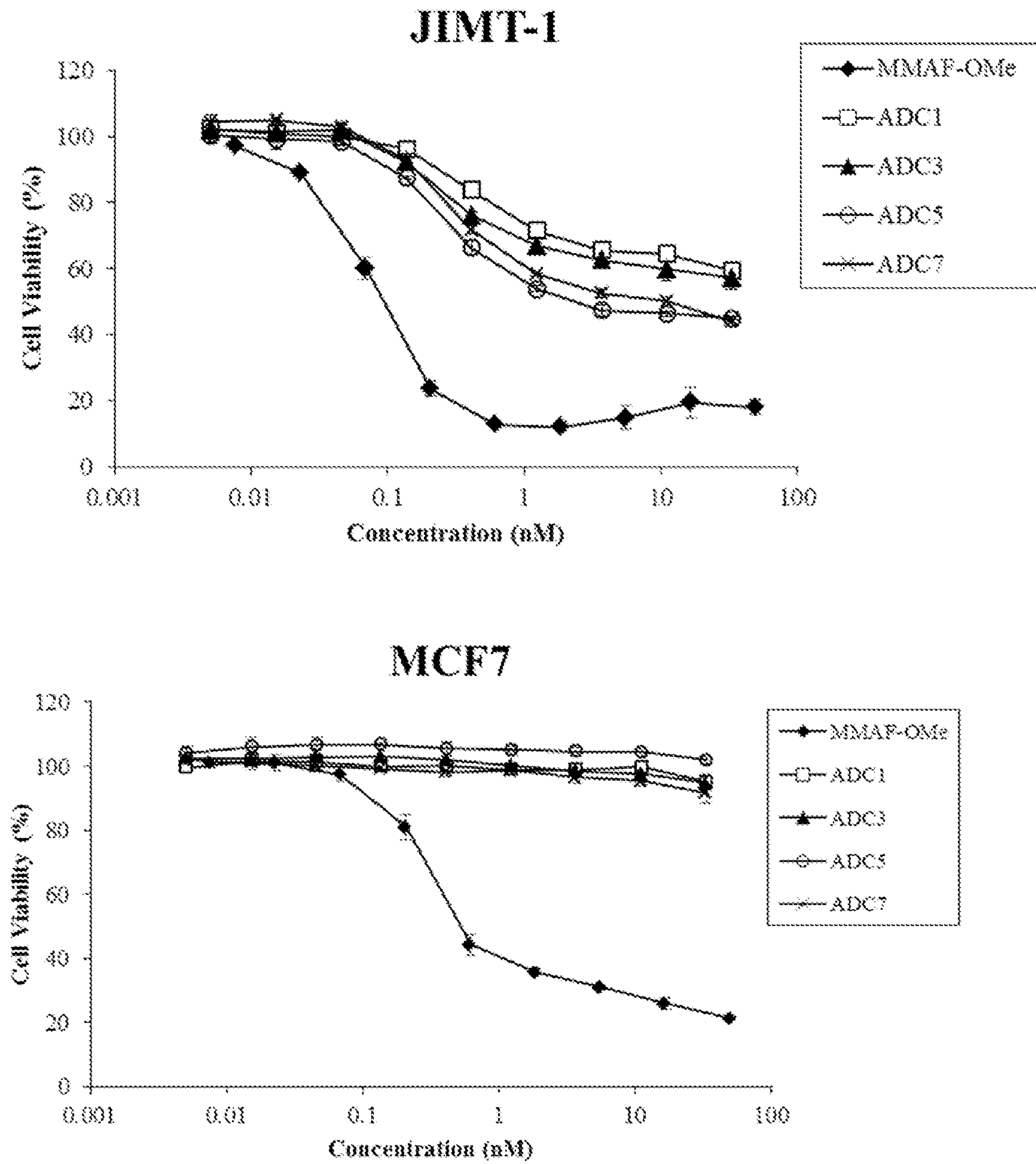


Figure 9

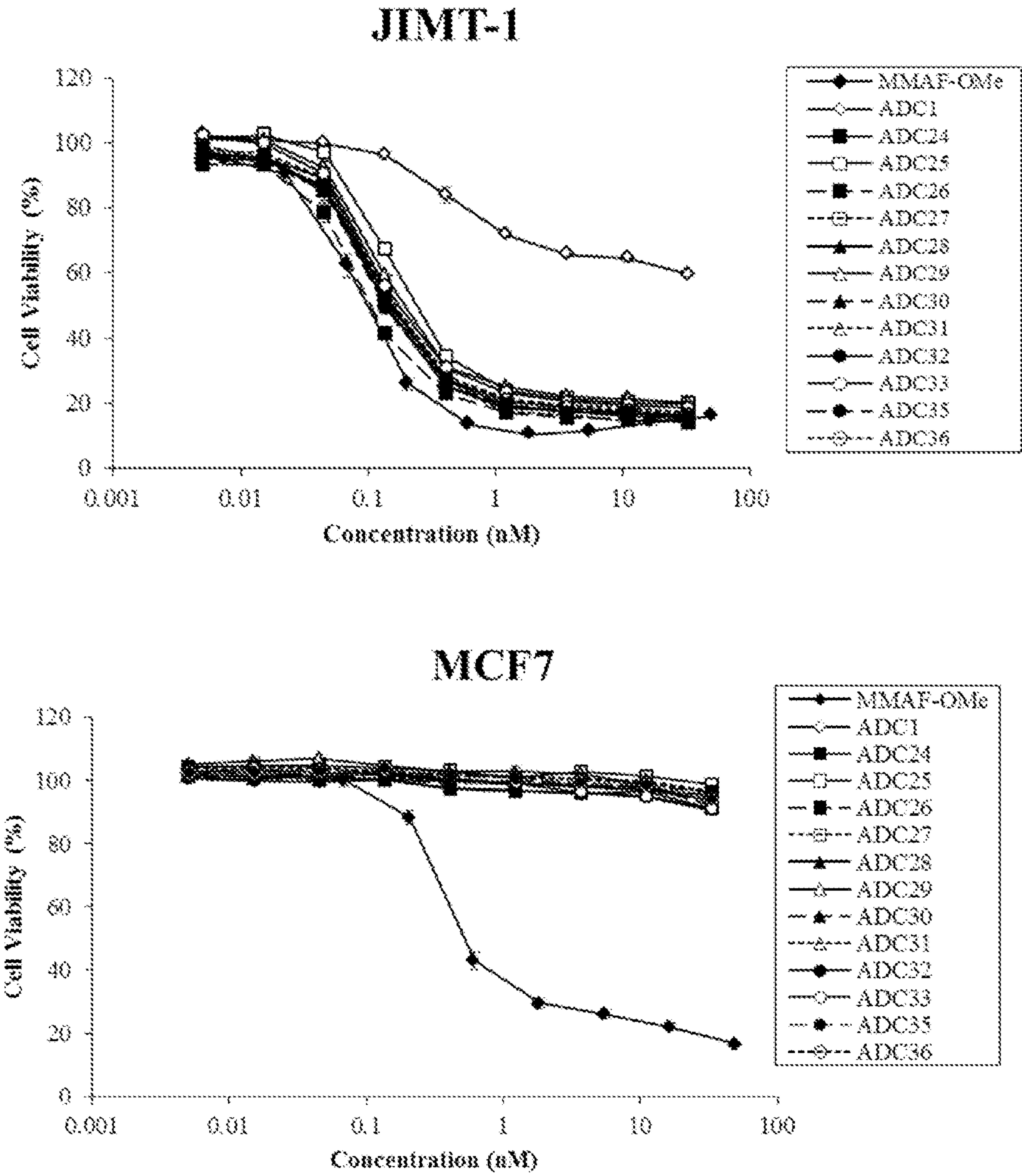


Figure 10

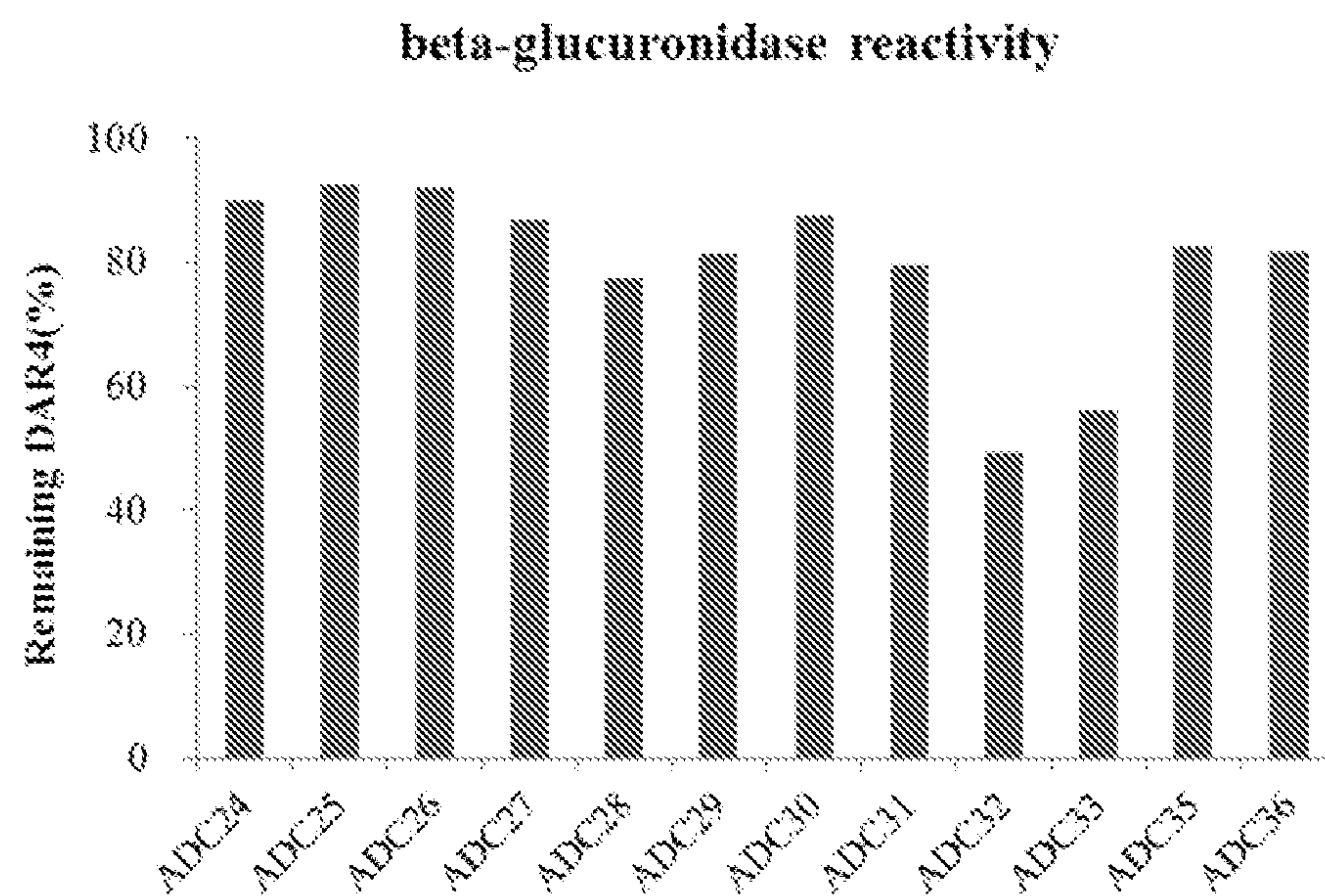


Figure 11

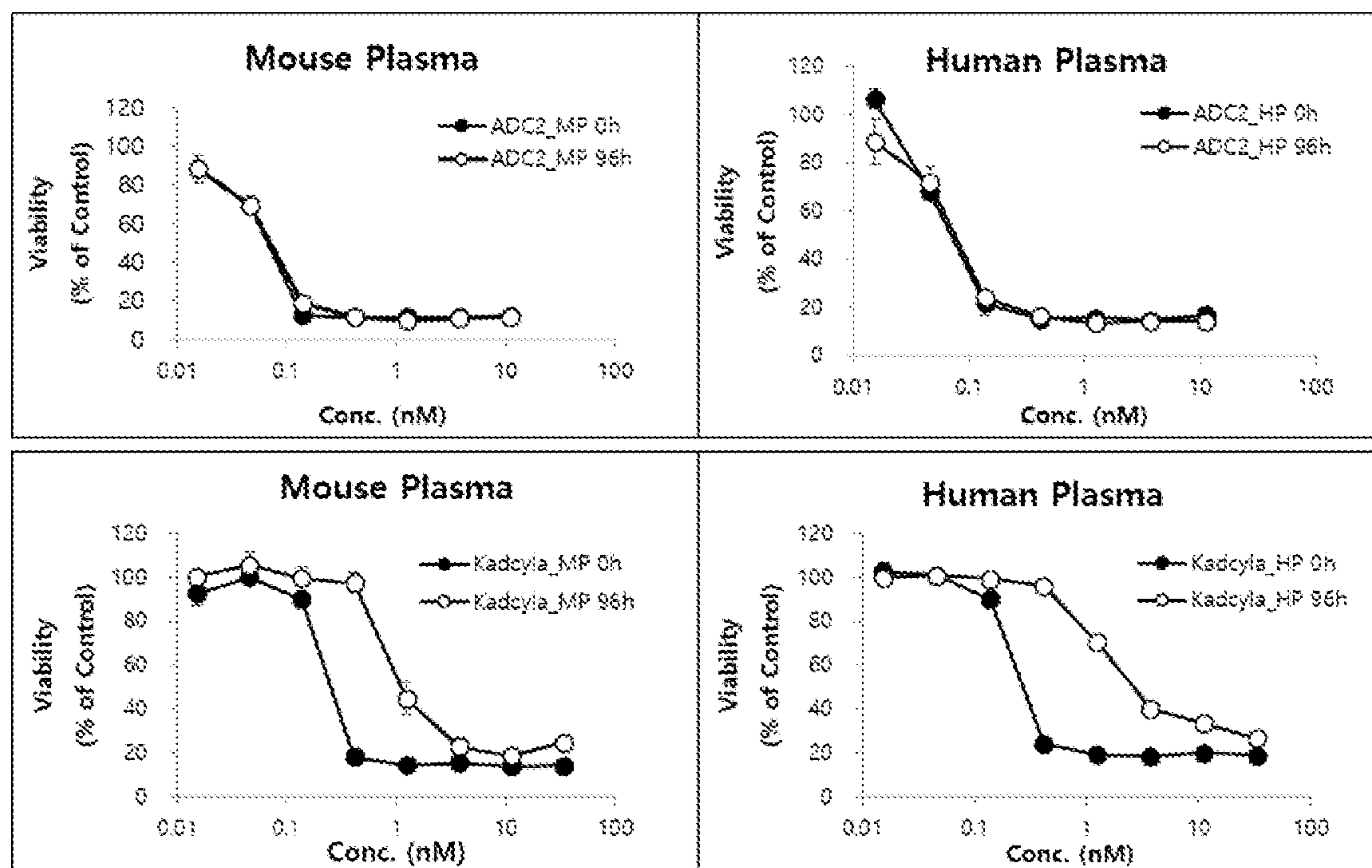


Figure 12

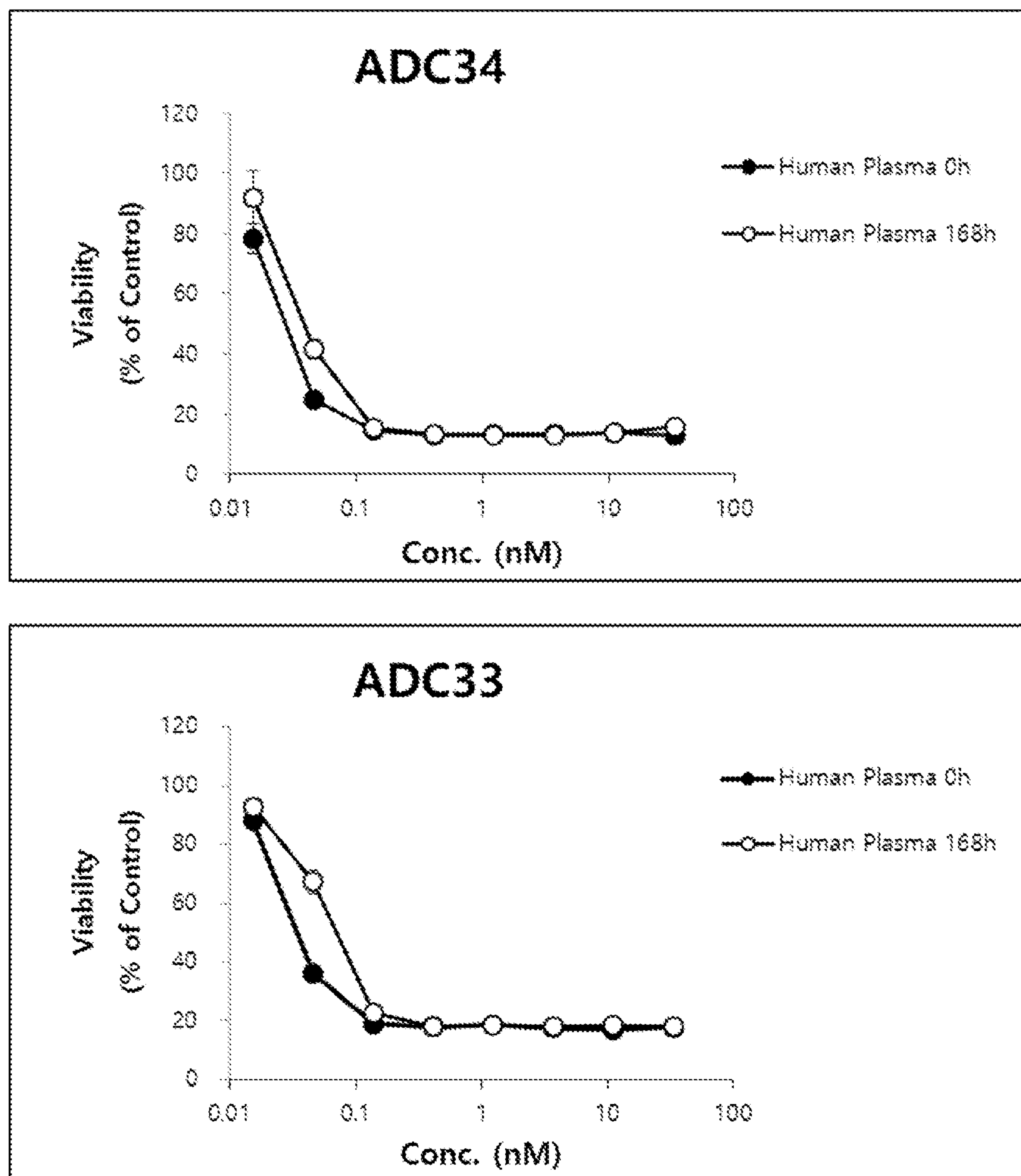


Figure 13

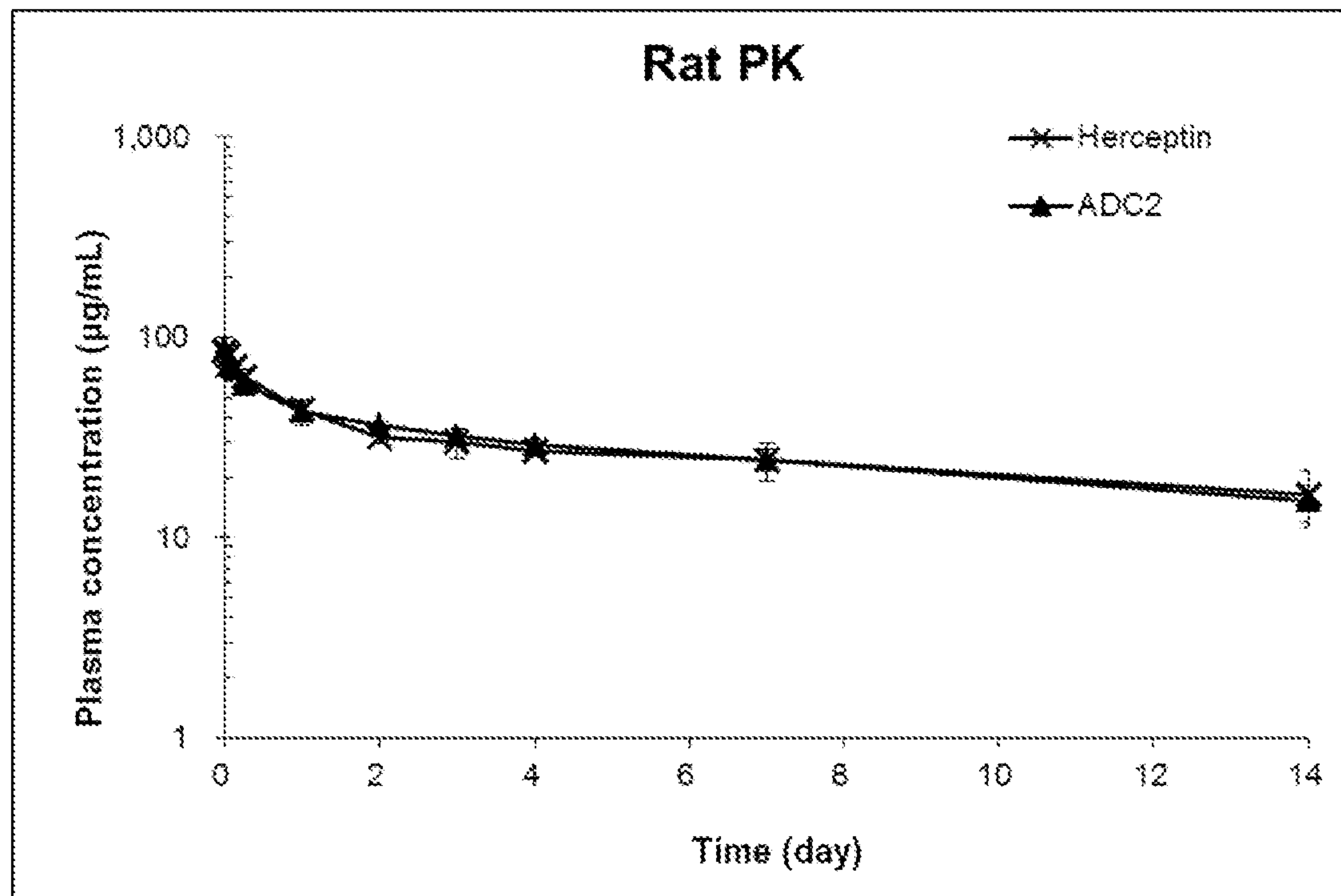


Figure 14

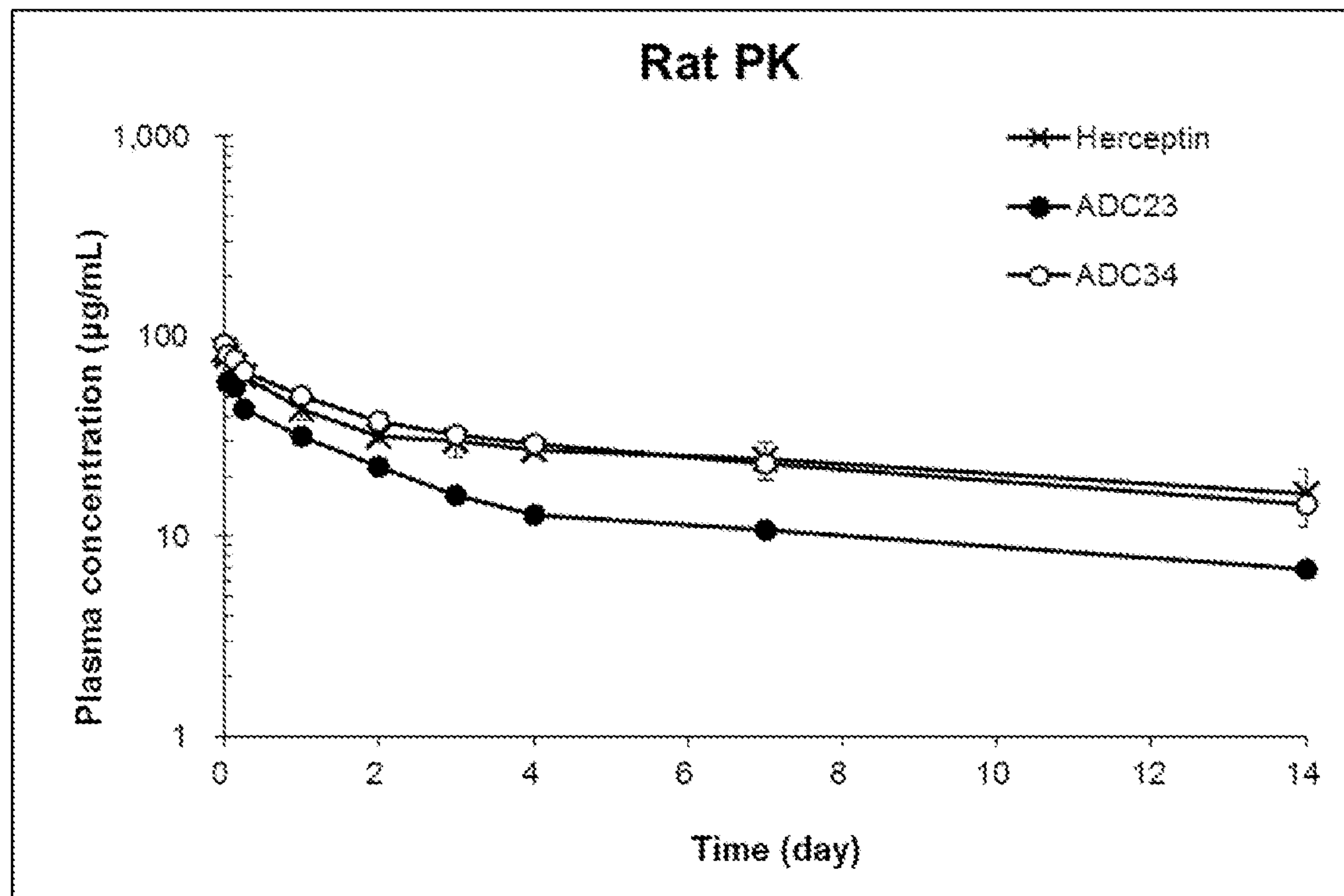


Figure 15

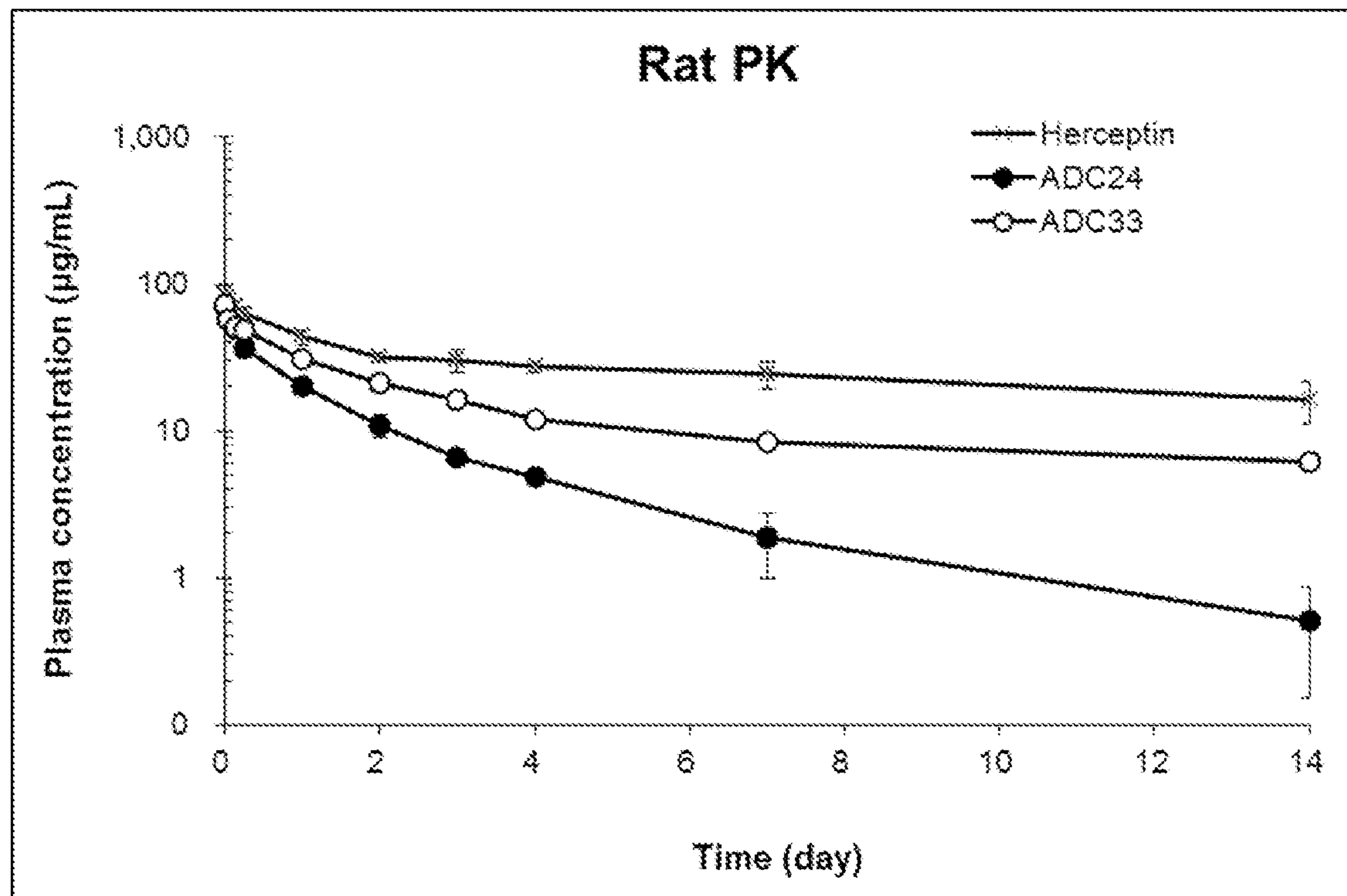


Figure 16

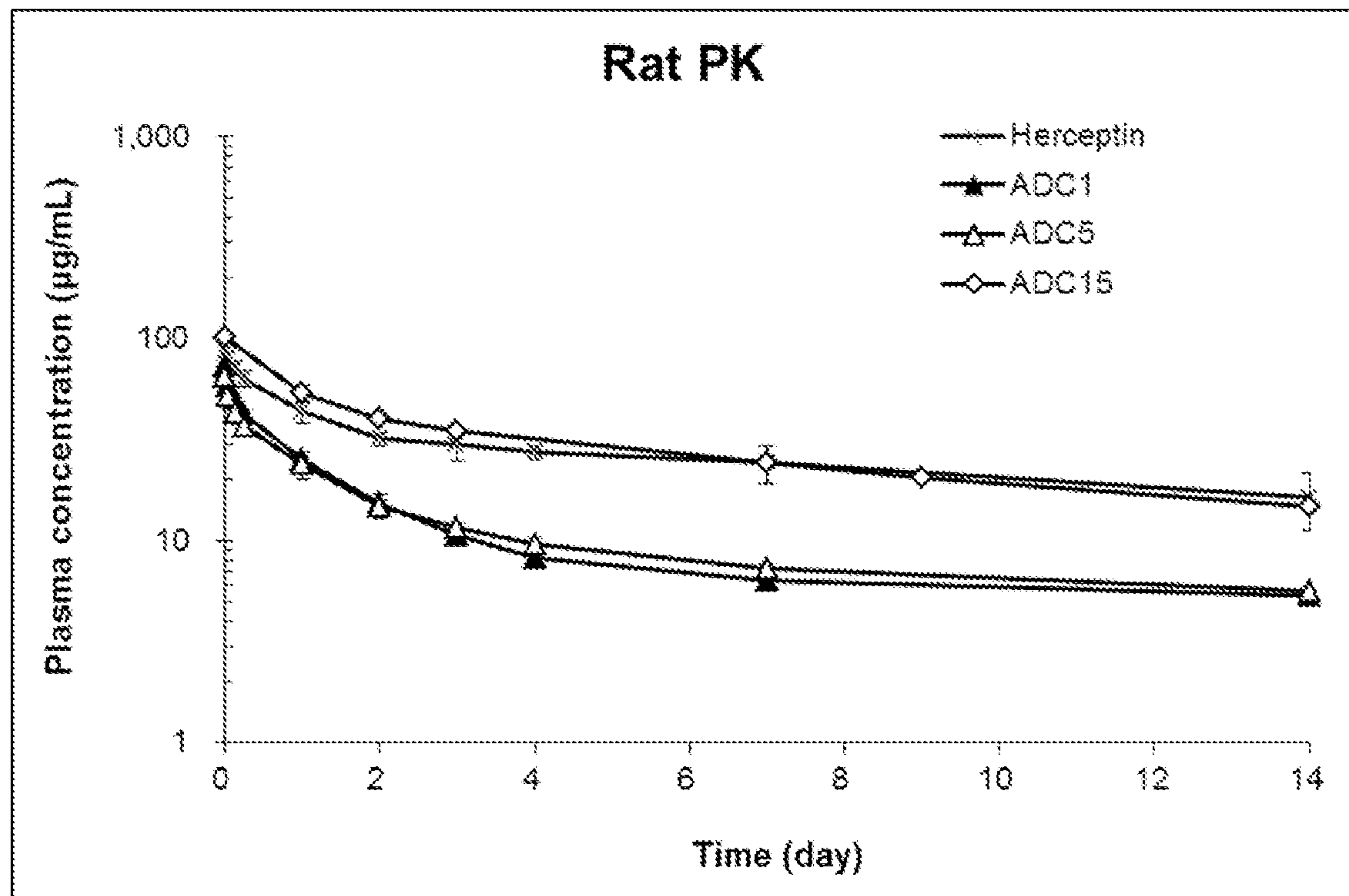


Figure 17

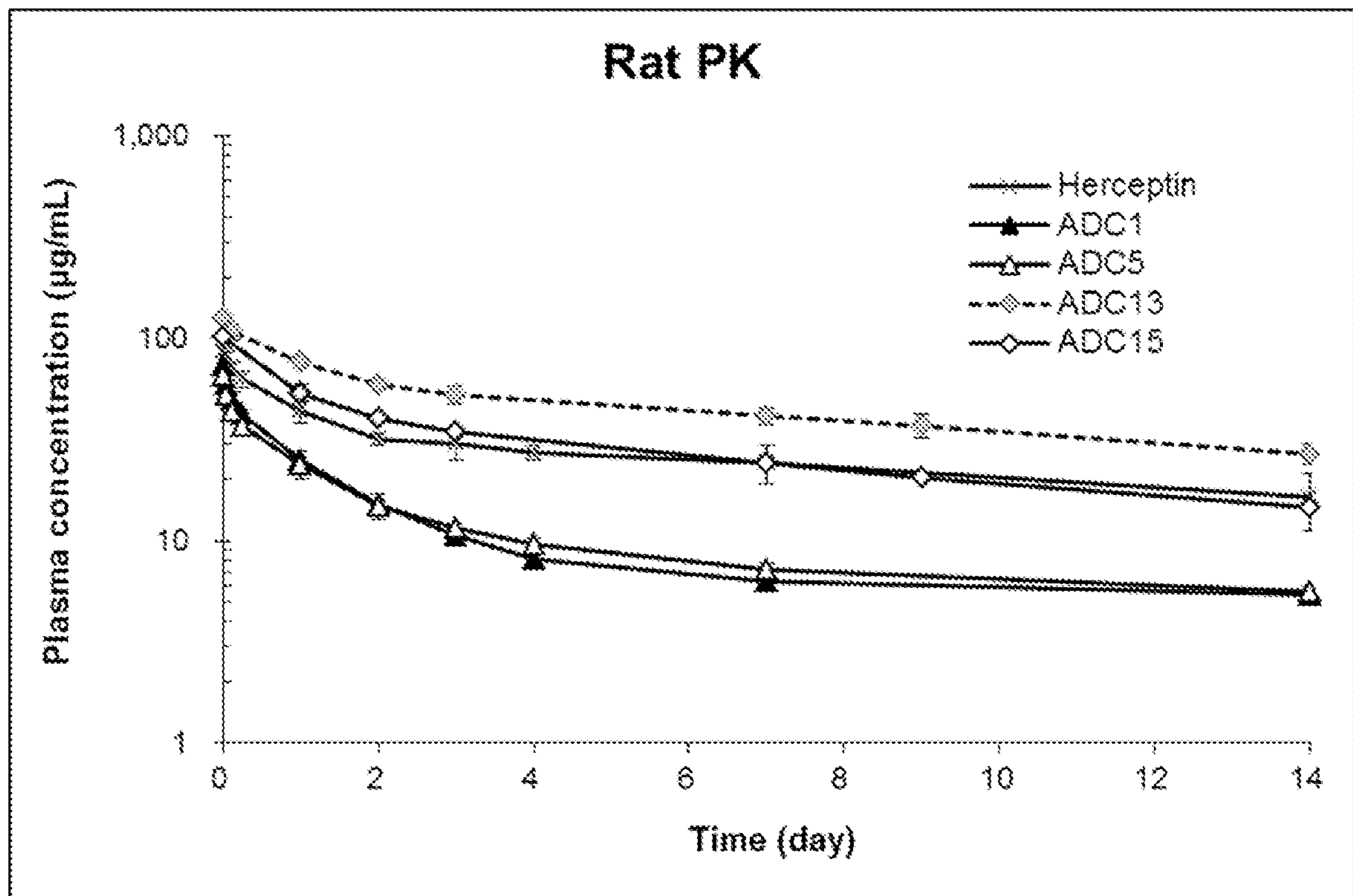


Figure 18

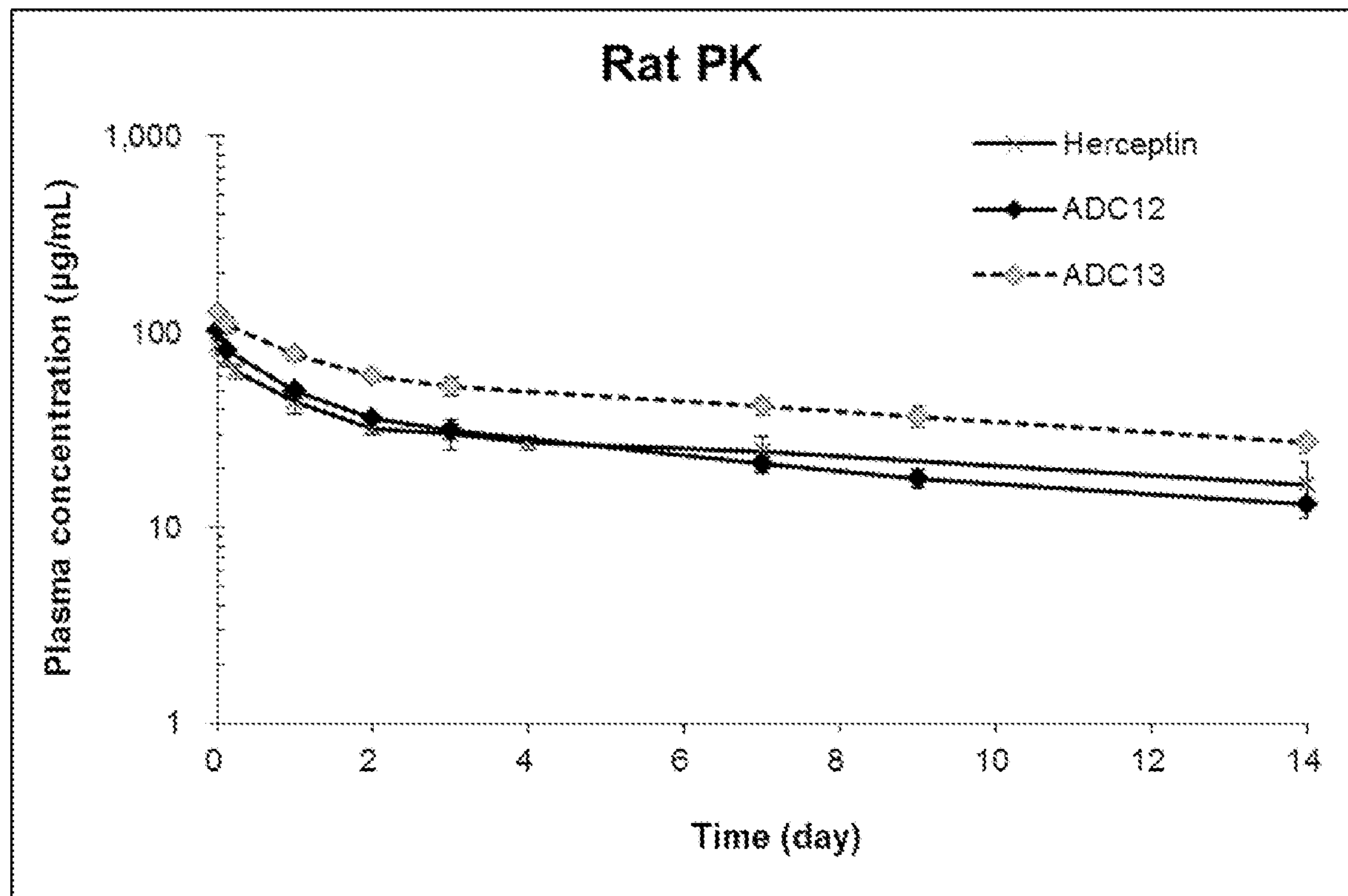


Figure 19

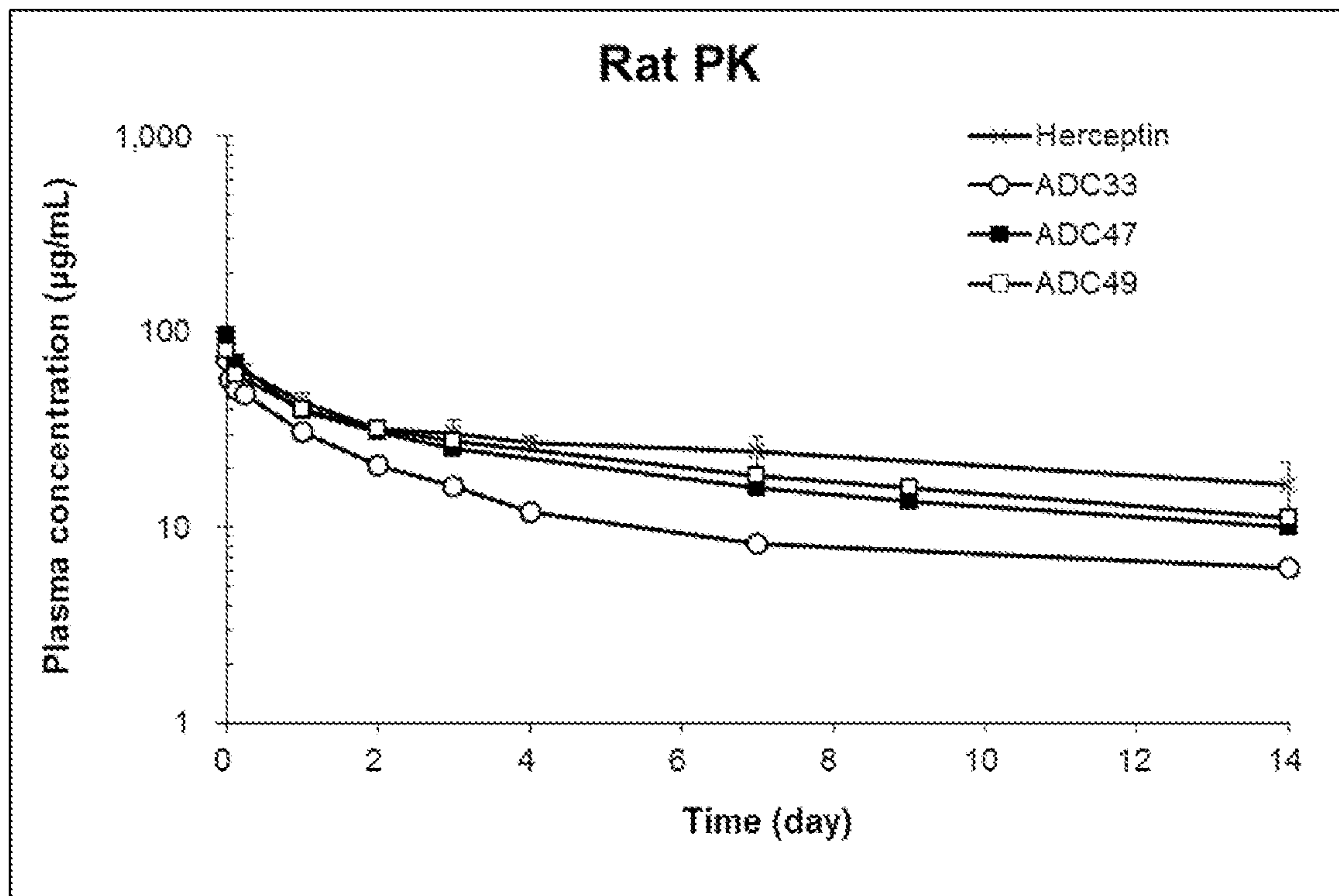


Figure 20

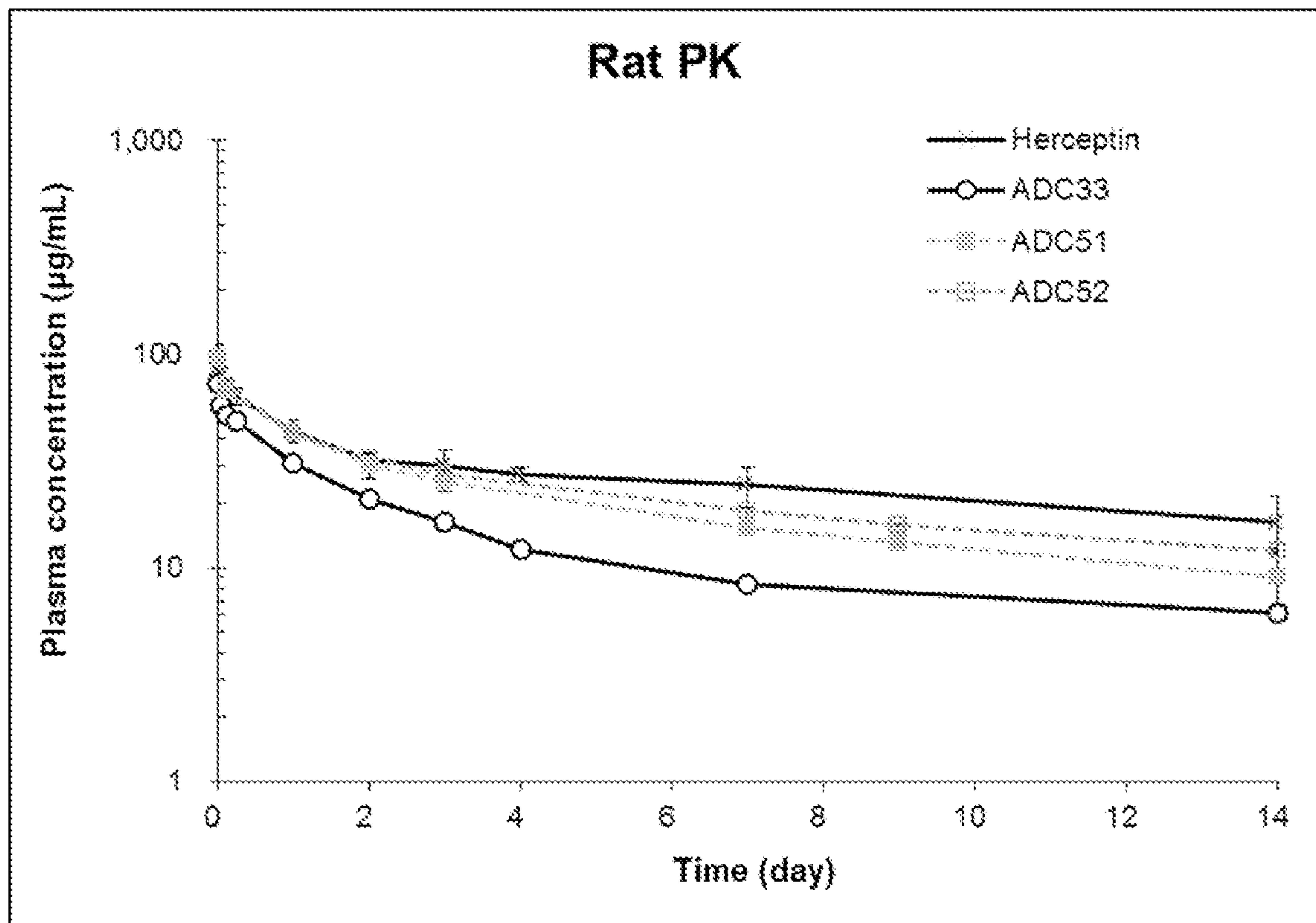


Figure 21

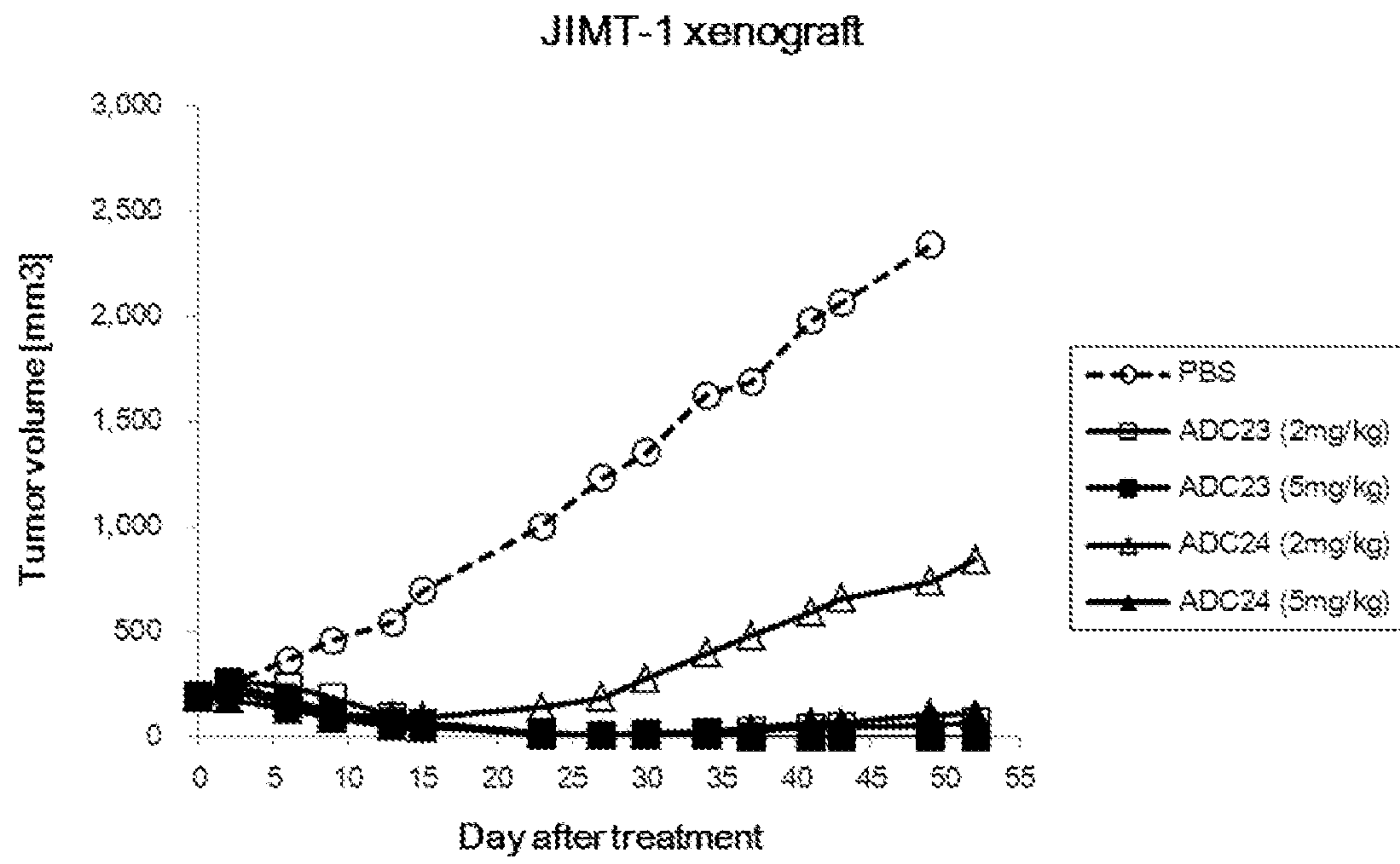


Figure 22

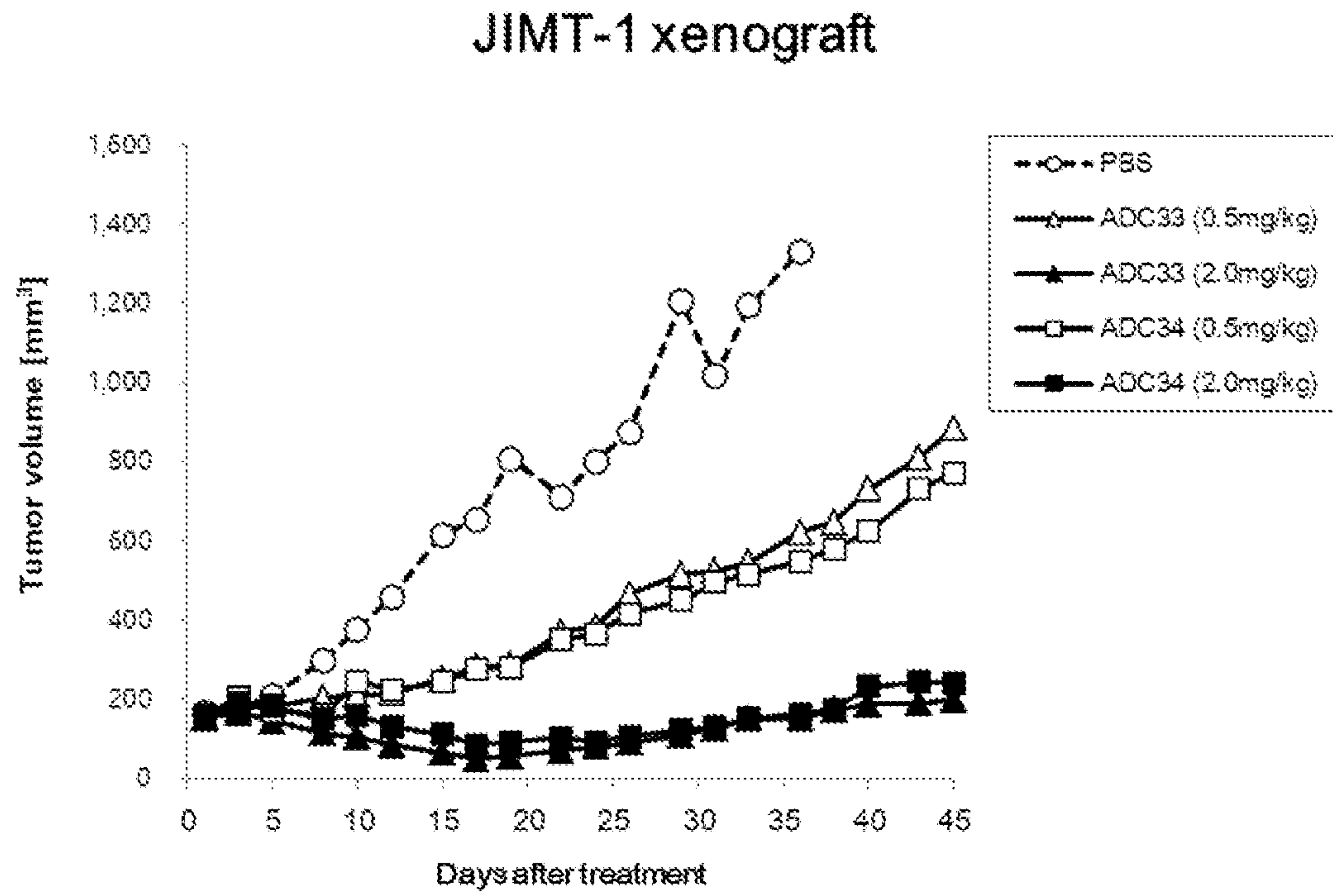
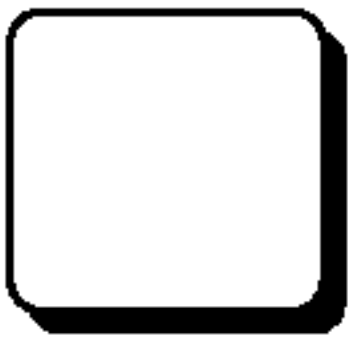
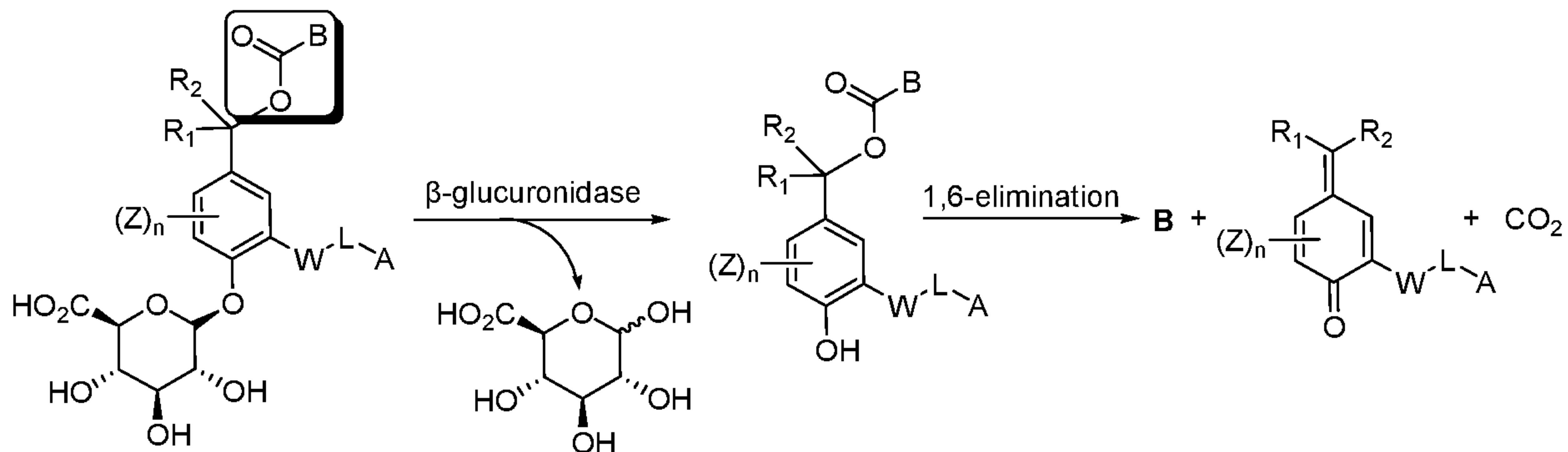
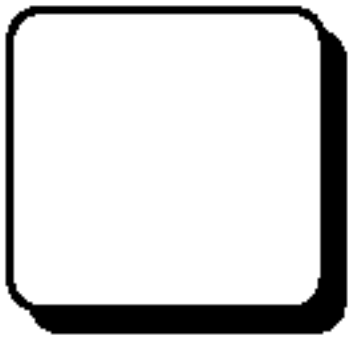


**Figure 1**

When  part is carbamate,



When  part is ester,

