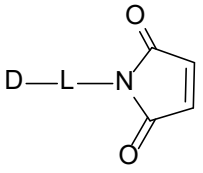
CLAIMS

1. A method of preparing a cell-binding agent-cytotoxic agent conjugate comprising a cell-binding agent (CysCBA) having one or more unpaired cysteine residues covalently linked to a cytotoxic agent, wherein the method comprises the steps of:

(a) reacting a reducing agent, at a pH between 5.0 and 8.5, with a capped cell-binding agent (cCysCBA) having one or more unpaired cysteine residues capped with a capping agent to form a reduced cell-binding agent, wherein the capping agent is removed to form a free thiol (-SH) group in the reduced cell-binding agent, wherein the reducing agent is 3-(diphenylphosphino)propylamine (DPPA) and the capping agent is cysteine, homocysteine, glutathione, or a combination thereof;

(b) reacting the reduced cell-binding agent with a selective oxidizing agent to form the CysCBA, wherein the free thiol group in the unpaired cysteine residue is not oxidized and wherein the oxidizing agent is dehydroascorbic acid (DHAA), copper sulfate, oxygen, or air; and

(c) reacting the CysCBA with a compound of formula (I):



(I)

or a pharmaceutically acceptable salt thereof, thereby forming the conjugate, wherein the reduced cell-binding agent in step (a) is used in step (b) without purification; and the CysCBA in step (b) is used in step (c) without purification, wherein:

D is a cytotoxic agent; and

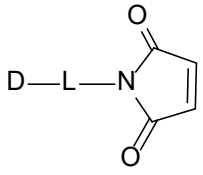
L is a linker.

2. A continuous method of preparing a cell-binding agent-cytotoxic agent conjugate (ADC) comprising a cell-binding agent (CysCBA) having one or more unpaired cysteine residues covalently linked to a cytotoxic agent, wherein the method comprises the steps of:

(a) reacting a reducing agent, at a pH between 5.0 and 8.5, with a capped cell-binding agent (cCysCBA) having one or more unpaired cysteine residues capped with a capping agent to form a reduced cell-binding agent, wherein the capping agent is removed to form a free thiol (-SH) group in the reduced cell-binding agent, wherein the reducing agent is 3-(diphenylphosphino)propylamine (DPPA) and the capping agent is cysteine, homocysteine, glutathione, or a combination thereof;

(b) reacting the reduced cell-binding agent with a selective oxidizing agent to form the CysCBA, wherein the free thiol group in the unpaired cysteine residue is not oxidized and wherein the oxidizing agent is dehydroascorbic acid (DHAA), copper sulfate, oxygen, or air; and

(c) reacting the CysCBA with a compound of formula (I):



(I)

or a pharmaceutically acceptable salt thereof, thereby forming the conjugate,

wherein the reduced cell-binding agent in step (a) is used in step (b) without purification; the CysCBA step (b) is used in step (c) without purification, and any one or more steps selected from steps (a) to (c) are performed continuously, wherein:

D is a cytotoxic agent; and

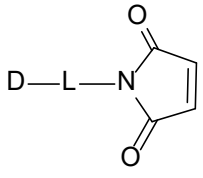
L is a linker.

3. A continuous method of preparing a cell-binding agent-cytotoxic agent conjugate (ADC) comprising a cell-binding agent (CysCBA) having one or more unpaired cysteine residues covalently linked to a cytotoxic agent, wherein the method comprises the steps of:

(a) reacting a reducing agent, at a pH between 5.0 and 8.5, with a capped cell-binding agent (cCysCBA) having one or more unpaired cysteine residues capped with a capping agent to form a reduced cell-binding agent, wherein the capping agent is removed to form a free thiol (-SH) group in the reduced cell-binding agent, wherein the reducing agent is 3-(diphenylphosphino)propylamine (DPPA) and the capping agent is cysteine, homocysteine, glutathione, or a combination thereof;

(b) reacting the reduced cell-binding agent with a selective oxidizing agent to form the CysCBA, wherein the free thiol group in the unpaired cysteine residue is not oxidized and wherein the oxidizing agent is dehydroascorbic acid (DHAA), copper sulfate, oxygen, or air; and

(c) reacting the CysCBA with a compound of formula (I):



(I)

or a pharmaceutically acceptable salt thereof, thereby forming the conjugate; and

(d) removing unconjugated compound of formula (I);

wherein the reduced cell-binding agent in step (a) is used in step (b) without purification; the CysCBA step (b) is used in step (c) without purification, any one or more steps selected from steps (a) to (d) are performed continuously; and wherein:

D is a cytotoxic agent; and

L is a linker.

~~4. The method of any one of claims 1-3, wherein the reducing agent is tris(2-carboxyethyl)phosphine hydrochloride (TCEP), trishydroxypropyl phosphine (THPP), tris (2-cyanoethyl)phosphine, dithiothreitol (DTT), dicyclohexylphosphino)benzenesulfonic acid, bis(p-sulfonatophenyl)phenylphosphine, (diphenylphosphino)benzenesulfonic acid, (diphenylphosphino)benzoic acid, 2-(diphenylphosphino)-N,N,N-trimethylbenzylammonium triflate, (diphenylphosphino)ethylamine, 2-(diisopropylphosphino)ethylamine, or 3-(diphenylphosphino)propylamine (DPPA).~~

~~5. The method of any one of claims 1-4, wherein the oxidizing agent is dehydroascorbic acid (DHAA), copper sulfate, oxygen or air.~~

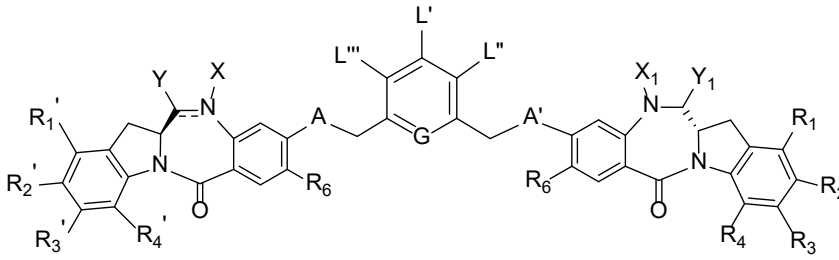
~~6. The method of any one of claims 1-3, wherein the reducing agent is TCEP and the reaction of step (a) is carried out at a pH between 6.0 and 8.0.~~

4~~7~~. The method of any one of claims 1-3, wherein the reducing agent is DPPA and the reaction of step (a) is carried out at a pH between 6.0 and 8.0.

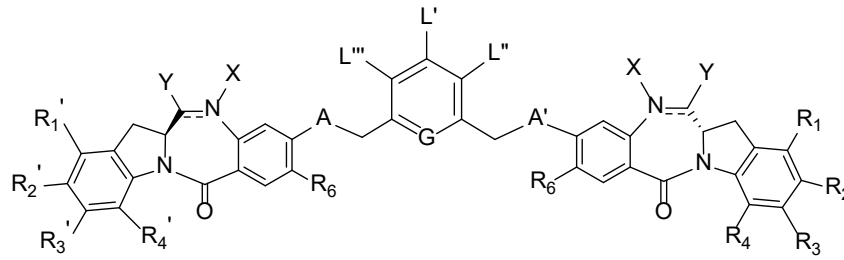
5~~8~~. The method of any one of claims 1-4~~7~~, wherein the molar ratio of the reducing agent in step (a) to the oxidizing agent in step (b) is between 2:1 and 1:2.

6~~9~~. The method of any one of claims 1-5~~8~~, wherein D is a benzodiazepine compound or a maytansinoid compound.

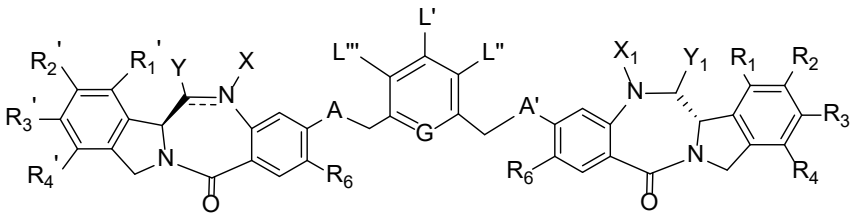
7~~10~~. The method of any one of claims 1-5~~8~~, wherein D is represented by the following formula:

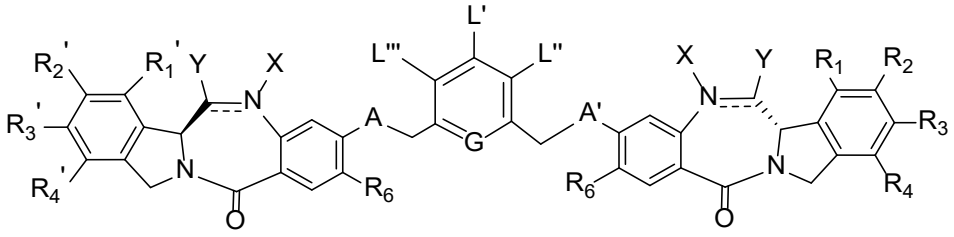


(D-IA);



(D-IB);


(D-IC); or


(D-ID);

or a pharmaceutically acceptable salt thereof, wherein:

the double line == between N and C represents a single bond or a double bond, provided that when it is a double bond, X is absent and Y is -H or a (C_1-C_4)alkyl; and when it is a single bond, X is -H or an amine protecting moiety, Y is -OH or -SO_3H or a pharmaceutically acceptable salt thereof;

one of L', L'', and L''' is represented by the following formula:

$$-Z_1\text{-}P_1\text{-}Z_2\text{-}R_{x1}\text{-}C(=O)- \quad \text{(A')}, \text{ or}$$

$$-N(R^e)\text{-}R_{x1}\text{-}C(=O)- \quad \text{(D')};$$

and the other two are each independently selected from -H, an optionally substituted linear, branched or cyclic alkyl, alkenyl or alkynyl, a polyethylene glycol unit -$(CH_2CH_2O)_n$-R^c, halogen, guanidinium [-NH(C=NH)NH_2], -OR, -NR'R'', -NO_2, -NR'COR'', -SR, -SOR', -SO_2R', -SO_3H, -OSO_3H, -SO_2NR'R'', cyano, an azido, -COR', -OCOR', and -OCONR'R'';

one of the Z_1 and Z_2 is -C(=O)-, and the other is -NR_5-;

P_1 is an amino acid residue or a peptide containing between 2 to 20 amino acid residues;

R_{x1} is an optionally substituted linear, branched or cyclic alkyl, alkenyl or alkynyl;

R^e is -H, a linear, branched or cyclic alkyl, alkenyl or alkynyl or -$(CH_2$-CH_2-$O)_n$-R^k, wherein R^k is a -H, a linear, branched cyclic alkyl having 1 to 6 carbon atoms, optionally bearing ~~a secondary amino (*e.g.*, -NHR^{101}) or tertiary amino (-NR^{101}R^{102}) group~~ or a 5- or 6-membered nitrogen containing heterocycle, wherein R^{101} and R^{102} are each independently a linear, branched, or cyclic alkyl, alkenyl or alkynyl;

R, for each occurrence, is independently selected from the group consisting of -H, an optionally substituted linear, branched or cyclic alkyl, alkenyl or alkynyl, a polyethylene

glycol unit -$(CH_2CH_2O)_n$-R^c, an optionally substituted aryl having 6 to 18 carbon atoms, an optionally substituted 5- to 18-membered heteroaryl ring containing one or more heteroatoms independently selected from nitrogen, oxygen, and sulfur, or an optionally substituted 3- to 18-membered heterocyclic ring containing 1 to 6 heteroatoms independently selected from O, S, N and P;

R' and R'' are each independently selected from -H, -OH, -OR, -NHR, -NR_2, -COR, an optionally substituted linear, branched or cyclic alkyl, alkenyl or alkynyl, a polyethylene glycol unit -$(CH_2CH_2O)_n$-R^c, and an optionally substituted 3- to 18-membered heterocyclic ring having 1 to 6 heteroatoms independently selected from O, S, N and P;

R^c is -H or an optionally substituted linear or branched alkyl having 1 to 4 carbon atoms;

n is an integer from 1 to 24;

X_1 is selected from -H, an amine-protecting group, an optionally substituted linear, branched or cyclic alkyl, alkenyl or alkynyl, a polyethylene glycol unit -$(CH_2CH_2O)_n$-R^c, an optionally substituted aryl having 6 to 18 carbon atoms, an optionally substituted 5- to 18-membered heteroaryl ring containing one or more heteroatoms independently selected from nitrogen, oxygen, and sulfur, and an optionally substituted 3- to 18-membered heterocyclic ring containing 1 to 6 heteroatoms independently selected from O, S, N and P;

Y_1 is selected from -H, an oxo group, an optionally substituted linear, branched or cyclic alkyl, alkenyl or alkynyl, an optionally substituted 6- to 18-membered aryl, an optionally substituted 5- to 18-membered heteroaryl ring containing one or more heteroatoms independently selected from nitrogen, oxygen, and sulfur, an optionally substituted 3- to 18-membered heterocyclic ring having 1 to 6 heteroatoms;

R_1, R_2, R_3, R_4, R_1', R_2', R_3' and R_4' are each independently selected from the group consisting of -H, an optionally substituted linear, branched or cyclic alkyl, alkenyl or alkynyl, a polyethylene glycol unit -$(CH_2CH_2O)_n$-R^c, halogen, guanidinium [-NH(C=NH)NH_2], -OR, -NR'R'', -NO_2, -NCO, -NR'COR'', -SR, -SOR', -SO_2R', -SO_3^-H, -OSO_3H, -SO_2NR'R'', cyano, an azido, -COR', -OCOR', and -OCONR'R'';

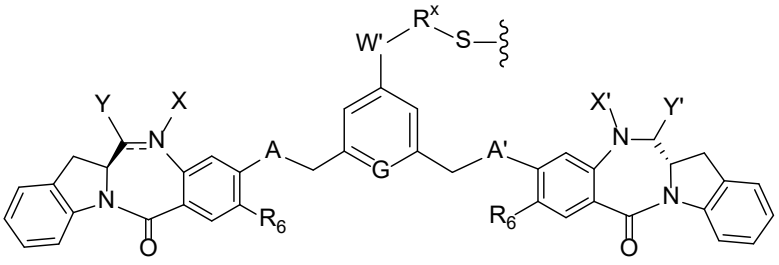
R_6 is -H, -R, -OR, -SR, -NR'R'', -NO_2, or halogen;

G is -CH- or –N-;

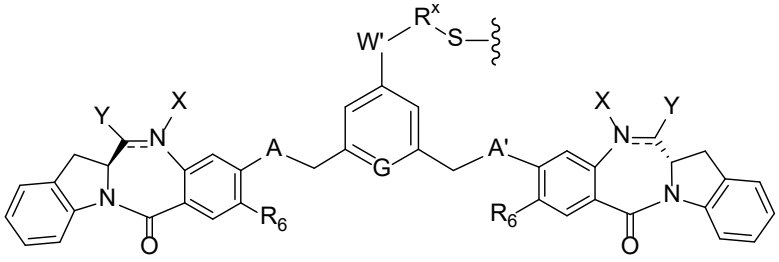
A and A' are the same or different, and are independently selected from -O-, oxo (-C(=O)-), -CRR'O-, -CRR'-, -S-, -CRR'S-, -NR_5 and -CRR'N(R_5)-; and

R_5 for each occurrence is independently -H or an optionally substituted linear or branched alkyl.

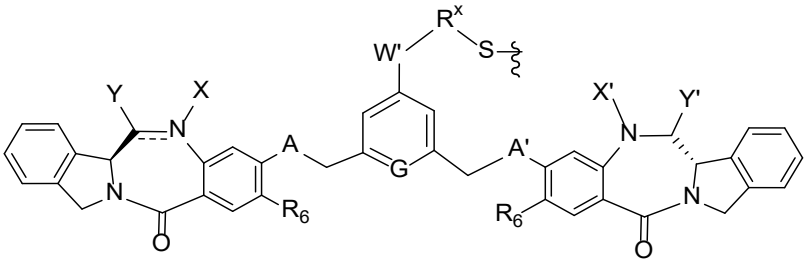
8~~11~~. The method of any one of claims 1-5~~8~~, wherein D is represented by the following formula:



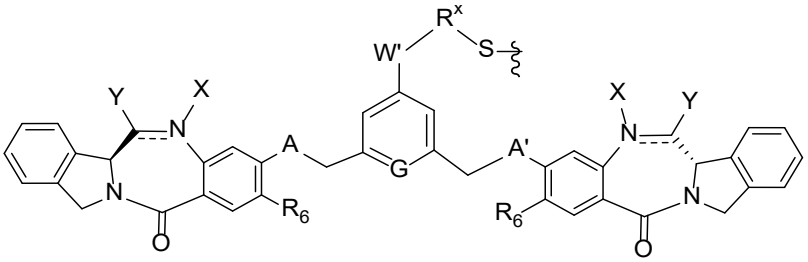
(D-IIA);



(D-IIB);



(D-IIC); or



(D-IID),

or a pharmaceutically acceptable salt thereof, wherein:

the double line == between N and C represents a single bond or a double bond, provided that when it is a double bond, X is absent and Y is -H or a (C_1-C_4)alkyl; and when it is a single bond, X is -H or an amine protecting moiety, Y is -OH or -SO_3H or a pharmaceutically acceptable salt thereof;

X' is selected from the group consisting of -H, -OH, a substituted or unsubstituted linear, branched or cyclic alkyl, alkenyl or alkynyl, phenyl, and an amine-protecting group;

Y' is selected from the group consisting of -H, an oxo group, a substituted or unsubstituted linear, branched or cyclic alkyl, alkenyl or alkynyl;

A and A’ are selected from -O- and -S-;

W’ is absent, or selected from -O-, -N(R^e)-, -N(R^e)-C(=O)-, -N(C(=O)R^e)-, -S- or -CH_2-S-, -CH_2NR^e-;

R^x is absent or selected from a linear, branched or cyclic alkyl;

R^e is -H, a linear, branched or cyclic alkyl, alkenyl or alkynyl or -$(CH_2$-CH_2-$O)_n$-R^k, wherein R^k is a -H, a linear, branched cyclic alkyl having 1 to 6 carbon atoms, optionally bearing ~~a secondary amino (*e.g.*, -NHR^{101})~~ or ~~tertiary amino (-$NR^{101}R^{102}$) group~~ or a 5- or 6-membered nitrogen containing heterocycle, wherein R^{101} and R^{102} are each independently a linear, branched, or cyclic alkyl, alkenyl or alkynyl;

n is an integer from 1 to 24;

G is selected from -CH- or -N-;

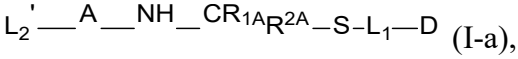
R_6 is -H, -R, -OR, -SR, -NR’R’’, -NO_2, or halogen; and

R, for each occurrence, is independently selected from the group consisting of -H, an optionally substituted linear, branched or cyclic alkyl, alkenyl or alkynyl, a polyethylene glycol unit -$(CH_2CH_2O)_n$-R^c, an optionally substituted aryl having 6 to 18 carbon atoms, an optionally substituted 5- to 18-membered heteroaryl ring containing one or more heteroatoms independently selected from nitrogen, oxygen, and sulfur, or an optionally substituted 3- to 18-membered heterocyclic ring containing 1 to 6 heteroatoms independently selected from O, S, N and P;

R’ and R’’ are each independently selected from -H, -OH, -OR, -NHR, -NR_2, -COR, an optionally substituted linear, branched or cyclic alkyl, alkenyl or alkynyl, a polyethylene glycol unit -$(CH_2CH_2O)_n$-R^c, and an optionally substituted 3- to 18-membered heterocyclic ring having 1 to 6 heteroatoms independently selected from O, S, N and P;

R^c is -H or an optionally substituted linear or branched alkyl having 1 to 4 carbon atoms.

9~~12~~. The method of any one of claims 1-5~~8~~, wherein the compound of formula (I) is presented by the following formula:



(I-a),

or a pharmaceutically acceptable salt thereof, wherein:

L_2’ is a spacer bearing a maleimide moiety;

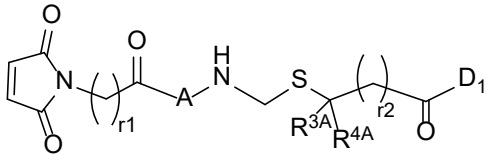
A is an amino acid or a peptide comprising 2 to 20 amino acids;

R^{1A} and R^{2A} are each independently H or a $C_{1\text{-}3}$alkyl;

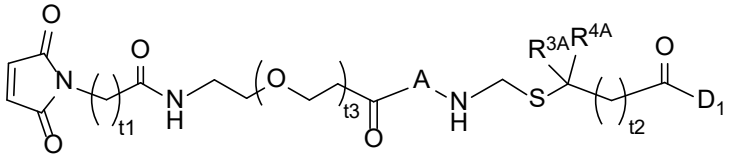
L_1 is a spacer; and

D-L_1-SH is a cytotoxic agent.

10~~13~~. The method of claim 9~~12~~, wherein the compound of formula (I) is represented by the following formula:



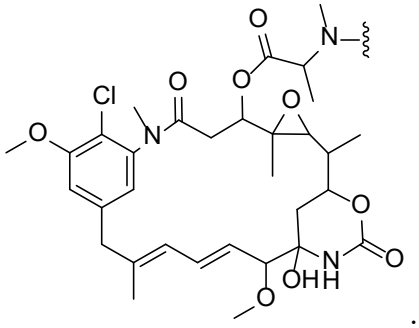
(I-b); or



(I-c);

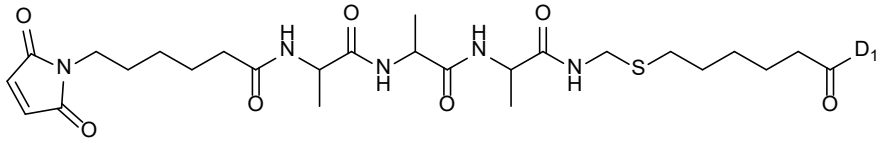
or a pharmaceutically acceptable salt thereof, wherein:

R^{3A} and R^{4A} are each independently H or Me;
r1 and t1 are each independently an integer from 1 to 6;
r2 and t2 are each independently an integer from 1 to 7;
t3 is an integer from 1 to 12;
D_1 is represented by the following formula:

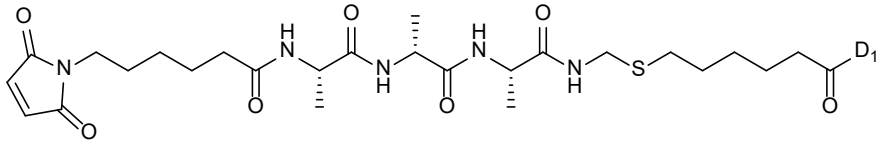


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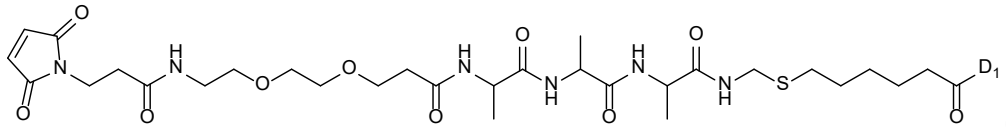
11~~14~~. The method of claim 10~~13~~, wherein the compound is represented by the following formula:

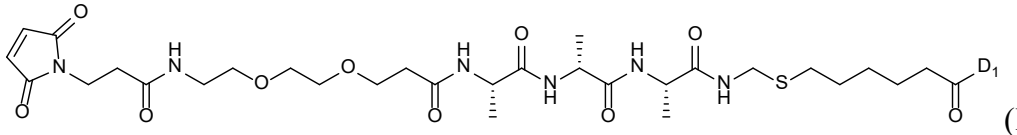


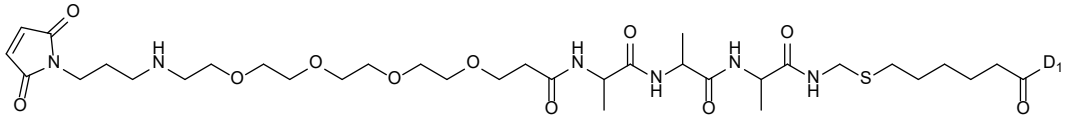
(I10);

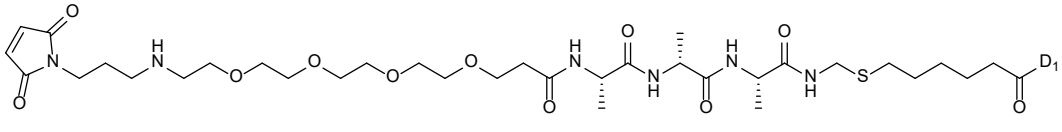


(I11);

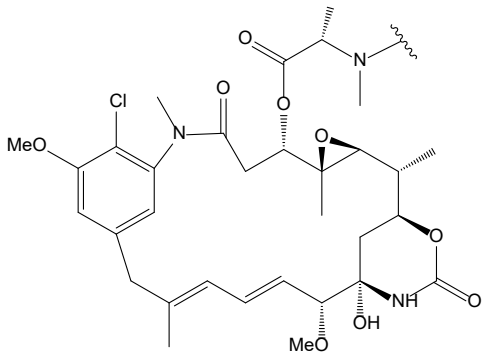

(I12);


(I13);

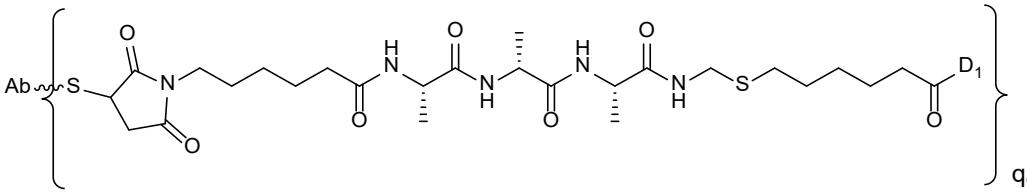

(I14); or


(I15),

wherein D_1 is represented by the following formula:

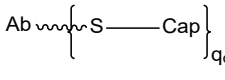

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12~~15~~. A method of preparing an antibody-cytotoxic agent conjugate (ADC) represented by the following formula:


,

comprising the steps of:

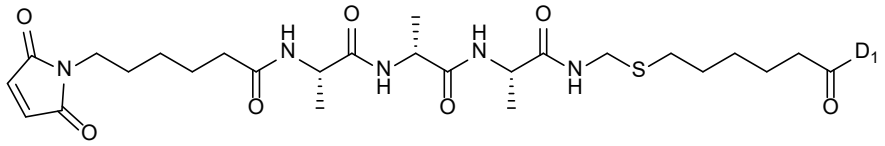
(a) reacting a reducing agent, at a pH between 5.0 and 8.5, with a capped cysteine-engineered antibody (cCysAb) represented by the following formula:


,

to form a reduced antibody or antigen-binding fragment thereof, wherein a ~~the~~ capping agent ~~(Cap)~~ is removed to form a free thiol (-SH) group in the reduced antibody or antigen-binding fragment thereof, wherein the reducing agent is 3-(diphenylphosphino)propylamine (DPPA) and the capping agent is cysteine, homocysteine, glutathione, or a combination thereof;

(b) reacting the reduced antibody or antigen-binding fragment thereof with a selective oxidizing agent to form the CysAb, wherein the free thiol group in the engineered cysteine residue is not oxidized and wherein the oxidizing agent is dehydroascorbic acid (DHAA), copper sulfate, oxygen, or air; and

(c) reacting the CysAb with a compound represented by the following formula:



(I11),

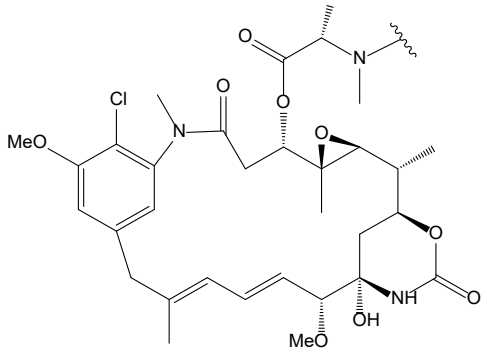
or a pharmaceutically acceptable salt thereof, thereby forming the ADC, wherein the reduced antibody or antigen-binding fragment thereof in step (a) is used in step (b) without purification, and the CysAb or antigen-binding fragment thereof of step (b) is used in step (c) without purification,

wherein:

Ab〰S— is a cysteine-engineered antibody (CysAb) or antigen-binding fragment thereof covalently linked to the cytotoxic agent through the thiol group located on the engineered cysteine residue; and wherein the CysAb or antigen-binding fragment thereof is a humanized anti-ADAM9 antibody or ADAM9-binding fragment thereof comprising a CDR_H1 domain, a CDR_H2 domain, and a CDR_H3 domain and a CDR_L1 domain, a CDR_L2 domain, and a CDR_L3 domain having the sequences of SEQ ID NOs: 24, 25, and 26 and SEQ ID NOs: 21, 22, 23, respectively;

q_c is 1 or 2;

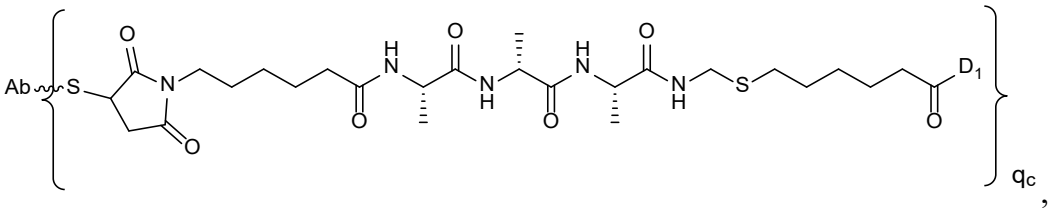
D_1 is represented by the following formula:



, and

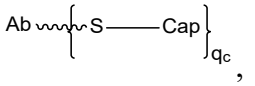
Cap is the ~~a~~ capping agent.

13~~16~~. A continuous method of preparing an antibody-cytotoxic agent conjugate (ADC) represented by the following formula:



comprising the steps of:

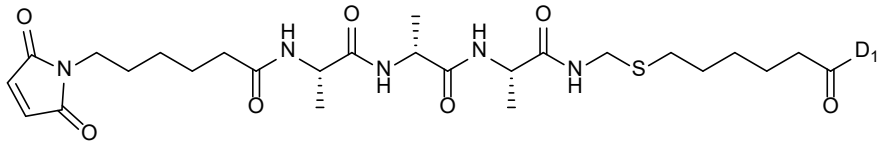
(a) reacting a reducing agent, at a pH between 5.0 and 8.5, with a capped cysteine-engineered antibody (cCysAb) represented by the following formula:



to form a reduced antibody or antigen-binding fragment thereof, wherein a ~~the~~ capping agent ~~(Cap)~~ is removed to form a free thiol (-SH) group in the reduced antibody or antigen-binding fragment thereof, wherein the reducing agent is 3-(diphenylphosphino)propylamine (DPPA) and the capping agent is cysteine, homocysteine, glutathione, or a combination thereof;

(b) reacting the reduced antibody or antigen-binding fragment thereof with a selective oxidizing agent to form the CysAb, wherein the free thiol group in the engineered cysteine residue is not oxidized and wherein the oxidizing agent is dehydroascorbic acid (DHAA), copper sulfate, oxygen, or air; and

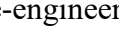
(c) reacting the CysAb with a compound represented by the following formula:



(I11),

or a pharmaceutically acceptable salt thereof, thereby forming the ADC, wherein the reduced antibody or antigen-binding fragment thereof in step (a) is used in step (b) without purification, and the CysAb or antigen-binding fragment thereof of step (b) is used in step (c) without purification, steps (a) to (c) are performed continuously, and

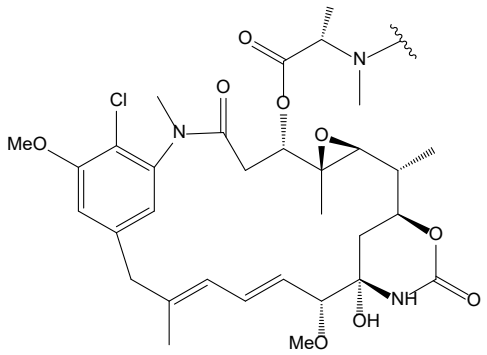
wherein:

 is a cysteine-engineered antibody (CysAb) or antigen-binding fragment thereof covalently linked to the cytotoxic agent through the thiol group located on the engineered cysteine residue; and wherein the CysAb or antigen-binding fragment thereof is a humanized

anti-ADAM9 antibody or ADAM9-binding fragment thereof comprising a CDR_H1 domain, a CDR_H2 domain, and a CDR_H3 domain and a CDR_L1 domain, a CDR_L2 domain, and a CDR_L3 domain having the sequences of SEQ ID NOs: 24, 25, and 26 and SEQ ID NOs: 21, 22, 23, respectively;

q_c is 1 or 2;

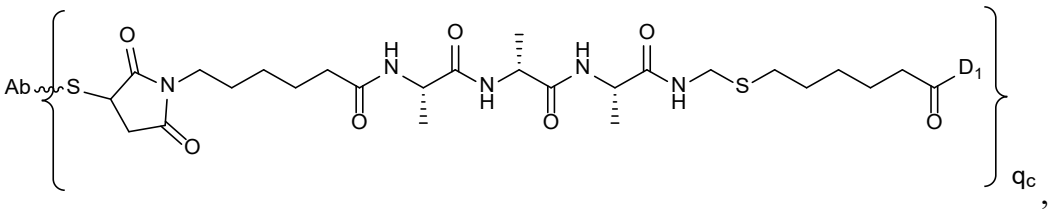
D_1 is represented by the following formula:



, and

Cap is <u>the</u> ~~a~~ capping agent.

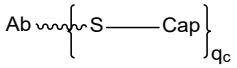
<u>14</u>~~17~~. A continuous method of preparing an antibody-cytotoxic agent conjugate (ADC) represented by the following formula:



,

comprising the steps of:

(a) reacting a reducing agent<u>, at a pH between 5.0 and 8.5,</u> with a capped cysteine-engineered antibody (cCysAb) represented by the following formula:

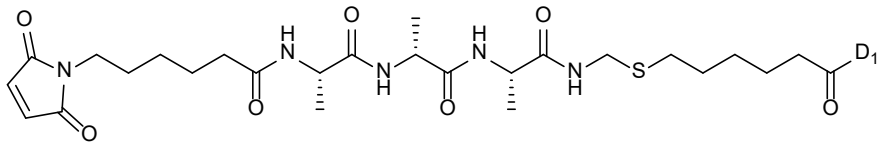


,

to form a reduced antibody or antigen-binding fragment thereof, wherein <u>a</u> ~~the~~ capping agent ~~(Cap)~~ is removed to form a free thiol (-SH) group in the reduced antibody or antigen-binding fragment thereof<u>, wherein the reducing agent is 3-(diphenylphosphino)propylamine (DPPA) and the capping agent is cysteine, homocysteine, glutathione, or a combination thereof</u>;

(b) reacting the reduced antibody or antigen-binding fragment thereof with a selective oxidizing agent to form the CysAb, wherein the free thiol group in the engineered cysteine residue is not oxidized and wherein the oxidizing agent is dehydroascorbic acid (DHAA), copper sulfate, oxygen, or air;

(c) reacting the CysAb with a compound represented by the following formula:



(I11),

or a pharmaceutically acceptable salt thereof, thereby forming the ADC; and

(d) removing unconjugated compound of formula (I11);

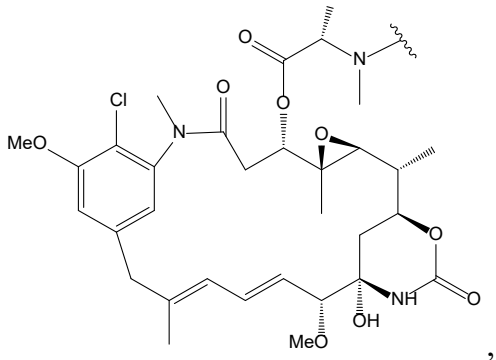
wherein the reduced antibody or antigen-binding fragment thereof in step (a) is used in step (b) without purification, and the CysAb or antigen-binding fragment thereof of step (b) is used in step (c) without purification, steps (a) to (d) are performed continuously, and

wherein:

Ab∼S— is a cysteine-engineered antibody (CysAb) or antigen-binding fragment thereof covalently linked to the cytotoxic agent through the thiol group located on the engineered cysteine residue; and wherein the CysAb or antigen-binding fragment thereof is a humanized anti-ADAM9 antibody or ADAM9-binding fragment thereof comprising a CDR_H1 domain, a CDR_H2 domain, and a CDR_H3 domain and a CDR_L1 domain, a CDR_L2 domain, and a CDR_L3 domain having the sequences of SEQ ID NOs: 24, 25, and 26 and SEQ ID NOs: 21, 22, 23, respectively;

q_c is 1 or 2;

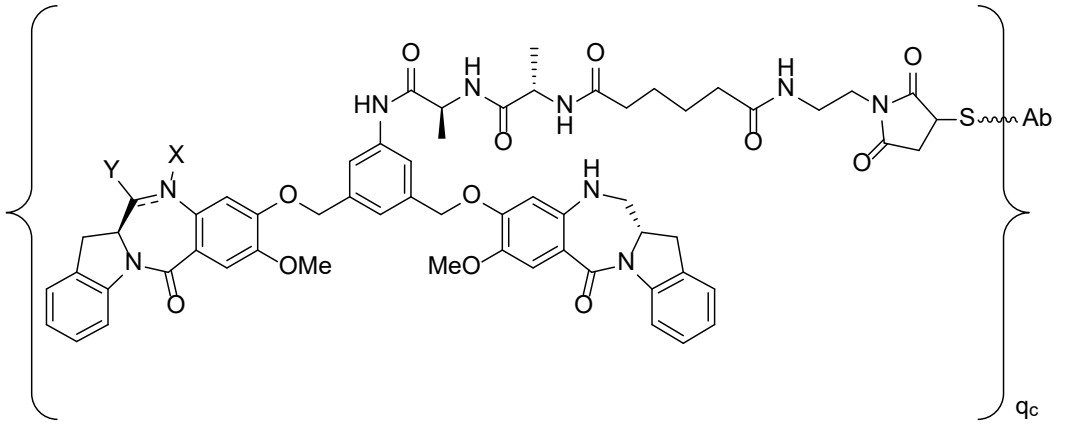
D_1 is represented by the following formula:



, and

Cap is the ~~a~~ capping agent.

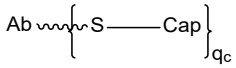
15~~18~~. A method of preparing an antibody-cytotoxic agent conjugate (ADC) represented by the following formula:



,

comprising the steps of:

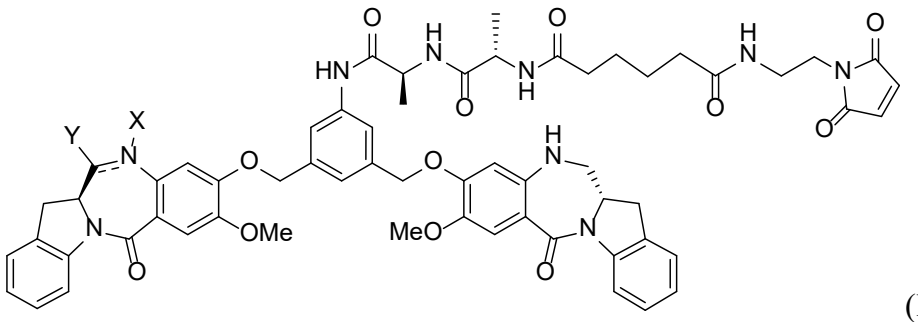
(a) reacting a reducing agent, at a pH between 5.0 and 8.5, with a capped cysteine-engineered antibody (cCysAb) represented by the following formula:



to form a reduced antibody or antigen-binding fragment thereof, wherein a ~~the~~ capping agent is removed to form a free thiol (-SH) group in the reduced antibody or antigen-binding fragment thereof, wherein the reducing agent is 3-(diphenylphosphino)propylamine (DPPA) and the capping agent is cysteine, homocysteine, glutathione, or a combination thereof;

(b) reacting the reduced antibody or antigen-binding fragment thereof with a selective oxidizing agent to form the CysAb, wherein the free thiol group in the engineered cysteine residue is not oxidized and wherein the oxidizing agent is dehydroascorbic acid (DHAA), copper sulfate, oxygen, or air; and

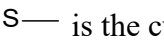
(c) reacting the CysAb with a compound represented by the following formula:



(I1a),

or a pharmaceutical acceptable salt thereof, thereby forming the ADC, wherein the reduced antibody or antigen-binding fragment thereof in step (a) is used in step (b) without purification, and the CysAb or antigen-binding fragment thereof of step (b) is used in step (c) without purification, wherein:

the double line == between N and C represents a single bond or a double bond, provided that when it is a double bond, X is absent and Y is -H, and when it is a single bond, X is -H, and Y is $-SO_3M$, and M is H^+ or a cation;

 is the cysteine-engineered antibody or antigen-binding fragment thereof covalently linked to the cytotoxic agent through the thiol group located on the engineered cysteine residue; wherein the cysteine-engineered antibody is an anti-CD123 antibody or antigen-binding fragment comprising:

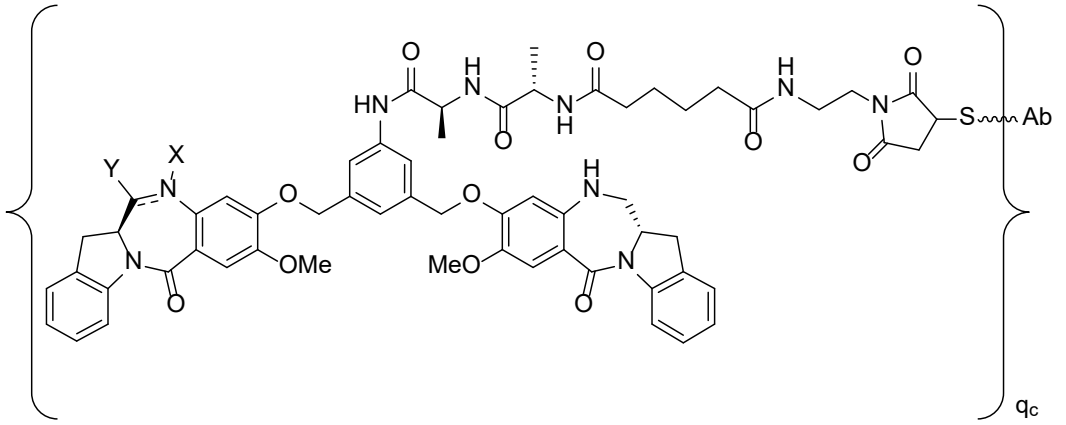
a) an immunoglobulin heavy chain variable region comprising a CDR_H1 having an amino acid sequence set forth in SEQ ID NO:4, a CDR_H2 having an amino acid sequence set forth in SEQ ID NO:5, and a CDR_H3 having an amino acid sequence set forth in SEQ ID NO:6; and

b) an immunoglobulin light chain variable region comprising a CDR_L1 having an amino acid sequence set forth in SEQ ID NO:1, a CDR_L2 having an amino acid sequence set forth in SEQ ID NO:2, and a CDR_L3 having an amino acid sequence set forth in SEQ ID NO:3;

q_c is 1 or 2; and

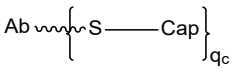
Cap is the ~~a~~ capping agent.

16~~19~~. A continuous method of preparing an antibody-cytotoxic agent conjugate (ADC) represented by the following formula:



comprising the steps of:

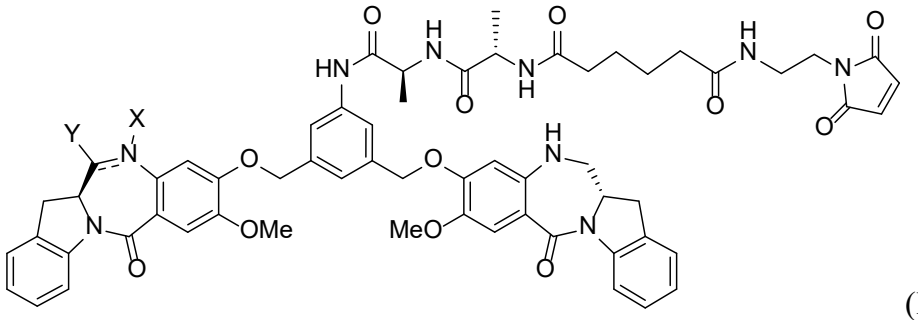
(a) reacting a reducing agent, at a pH between 5.0 and 8.5, with a capped cysteine-engineered antibody (cCysAb) represented by the following formula:



to form a reduced antibody or antigen-binding fragment thereof, wherein a ~~the~~ capping agent is removed to form a free thiol (-SH) group in the reduced antibody or antigen-binding fragment thereof, wherein the reducing agent is 3-(diphenylphosphino)propylamine (DPPA) and the capping agent is cysteine, homocysteine, glutathione, or a combination thereof;

(b) reacting the reduced antibody or antigen-binding fragment thereof with a selective oxidizing agent to form the CysAb, wherein the free thiol group in the engineered cysteine residue is not oxidized and wherein the oxidizing agent is dehydroascorbic acid (DHAA), copper sulfate, oxygen, or air; and

(c) reacting the CysAb with a compound represented by the following formula:



(I1a),

or a pharmaceutical acceptable salt thereof, thereby forming the ADC, wherein the reduced antibody or antigen-binding fragment thereof in step (a) is used in step (b) without purification, and the CysAb or antigen-binding fragment thereof of step (b) is used in step (c) without purification; and steps (a) to (c) are performed continuously,

wherein:

the double line == between N and C represents a single bond or a double bond, provided that when it is a double bond, X is absent and Y is -H, and when it is a single bond, X is -H, and Y is $-SO_3M$, and M is H^+ or a cation;

Ab〰S— is the cysteine-engineered antibody or antigen-binding fragment thereof covalently linked to the cytotoxic agent through the thiol group located on the engineered cysteine residue; wherein the cysteine-engineered antibody is an anti-CD123 antibody or antigen-binding fragment comprising:

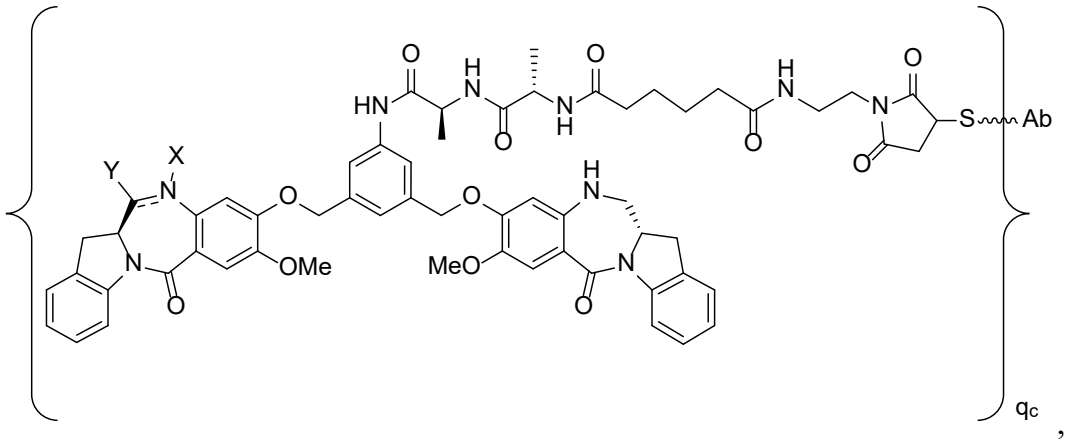
a) an immunoglobulin heavy chain variable region comprising a CDR_H1 having an amino acid sequence set forth in SEQ ID NO:4, a CDR_H2 having an amino acid sequence set forth in SEQ ID NO:5, and a CDR_H3 having an amino acid sequence set forth in SEQ ID NO:6; and

b) an immunoglobulin light chain variable region comprising a CDR_L1 having an amino acid sequence set forth in SEQ ID NO:1, a CDR_L2 having an amino acid sequence set forth in SEQ ID NO:2, and a CDR_L3 having an amino acid sequence set forth in SEQ ID NO:3;

q_c is 1 or 2; and

Cap is <u>the</u> ~~a~~ capping agent.

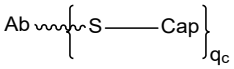
<u>17</u>~~20~~. A continuous method of preparing an antibody-cytotoxic agent conjugate (ADC) represented by the following formula:



,

comprising the steps of:

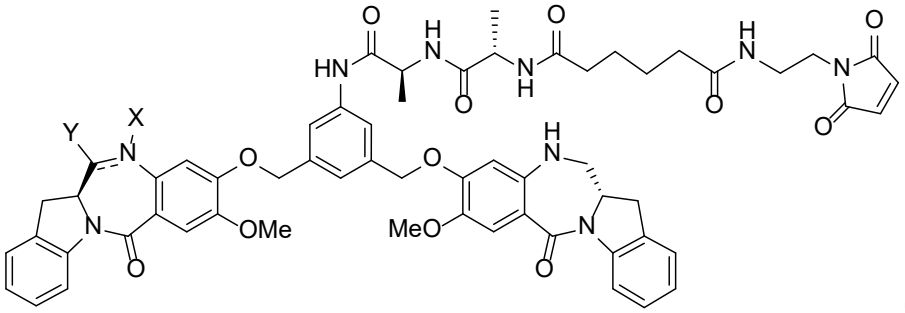
(a) reacting a reducing agent<u>, at a pH between 5.0 and 8.5,</u> with a capped cysteine-engineered antibody (cCysAb) represented by the following formula:



to form a reduced antibody or antigen-binding fragment thereof, wherein a ~~the~~ capping agent is removed to form a free thiol (-SH) group in the reduced antibody or antigen-binding fragment thereof, wherein the reducing agent is 3-(diphenylphosphino)propylamine (DPPA) and the capping agent is cysteine, homocysteine, glutathione, or a combination thereof;

(b) reacting the reduced antibody or antigen-binding fragment thereof with a selective oxidizing agent to form the CysAb, wherein the free thiol group in the engineered cysteine residue is not oxidized and wherein the oxidizing agent is dehydroascorbic acid (DHAA), copper sulfate, oxygen, or air; and

(c) reacting the CysAb with a compound represented by the following formula:



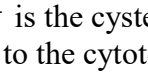
(I1a),

or a pharmaceutical acceptable salt thereof, thereby forming the ADC; and

(d) removing unconjugated compound of formula (I1a), wherein the reduced antibody or antigen-binding fragment thereof in step (a) is used in step (b) without purification, and the CysAb or antigen-binding fragment thereof of step (b) is used in step (c) without purification; and steps (a) to (d) are performed continuously,

wherein:

the double line == between N and C represents a single bond or a double bond, provided that when it is a double bond, X is absent and Y is -H, and when it is a single bond, X is -H, and Y is $-SO_3M$, and M is H^+ or a cation;

 is the cysteine-engineered antibody or antigen-binding fragment thereof covalently linked to the cytotoxic agent through the thiol group located on the engineered cysteine residue; wherein the cysteine-engineered antibody is an anti-CD123 antibody or antigen-binding fragment comprising:

a) an immunoglobulin heavy chain variable region comprising a CDR_H1 having an amino acid sequence set forth in SEQ ID NO:4, a CDR_H2 having an amino acid sequence set forth in SEQ ID NO:5, and a CDR_H3 having an amino acid sequence

set forth in SEQ ID NO:6; and

b) an immunoglobulin light chain variable region comprising a CDR_L1 having an amino acid sequence set forth in SEQ ID NO:1, a CDR_L2 having an amino acid sequence set forth in SEQ ID NO:2, and a CDR_L3 having an amino acid sequence set forth in SEQ ID NO:3;

q_c is 1 or 2; and

Cap is the ~~a~~ capping agent.