

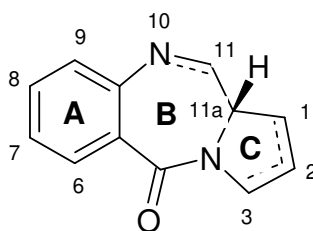
PYRROLOBENZODIAZEPINE CONJUGATES

The present invention relates to conjugates comprising pyrrolobenzodiazepines and related dimers (PBDs), and the precursor drug linkers used to make such conjugates.

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Background to the invention

Some pyrrolobenzodiazepines (PBDs) have the ability to recognise and bond to specific sequences of DNA; the preferred sequence is PuGpu. The first PBD antitumour antibiotic, anthramycin, was discovered in 1965 (Leimgruber, *et al.*, *J. Am. Chem. Soc.*, **87**, 5793-5795 (1965); Leimgruber, *et al.*, *J. Am. Chem. Soc.*, **87**, 5791-5793 (1965)). Since then, a number of naturally occurring PBDs have been reported, and over 10 synthetic routes have been developed to a variety of analogues (Thurston, *et al.*, *Chem. Rev.* **1994**, 433-465 (1994)). Family members include abbeymycin (Hochlowski, *et al.*, *J. Antibiotics*, **40**, 145-148 (1987)), chicamycin (Konishi, *et al.*, *J. Antibiotics*, **37**, 200-206 (1984)), DC-81 (Japanese Patent 58-180 487; Thurston, *et al.*, *Chem. Brit.*, **26**, 767-772 (1990); Bose, *et al.*, *Tetrahedron*, **48**, 751-758 (1992)), mazethramycin (Kuminoto, *et al.*, *J. Antibiotics*, **33**, 665-667 (1980)), neothramycins A and B (Takeuchi, *et al.*, *J. Antibiotics*, **29**, 93-96 (1976)), porothramycin (Tsunakawa, *et al.*, *J. Antibiotics*, **41**, 1366-1373 (1988)), prothracarcin (Shimizu, *et al.*, *J. Antibiotics*, **29**, 2492-2503 (1982); Langley and Thurston, *J. Org. Chem.*, **52**, 91-97 (1987)), sibanomicin (DC-102)(Hara, *et al.*, *J. Antibiotics*, **41**, 702-704 (1988); Itoh, *et al.*, *J. Antibiotics*, **41**, 1281-1284 (1988)), sibiromycin (Leber, *et al.*, *J. Am. Chem. Soc.*, **110**, 2992-2993 (1988)) and tomamycin (Arima, *et al.*, *J. Antibiotics*, **25**, 437-444 (1972)). PBDs are of the general structure:



25 They differ in the number, type and position of substituents, in both their aromatic A rings and pyrrolo C rings, and in the degree of saturation of the C ring. In the B-ring there is either an imine (N=C), a carbinolamine (NH-CH(OH)), or a carbinolamine methyl ether (NH-CH(OMe)) at the N10-C11 position which is the electrophilic centre responsible for alkylating DNA. All of the known natural products have an (S)-configuration at the chiral C11a position which provides them with a right-handed twist when viewed from the C ring towards the A ring. This gives them the appropriate three-dimensional shape for isohelicity with the minor groove of B-form DNA, leading to a snug fit at the binding site (Kohn, In

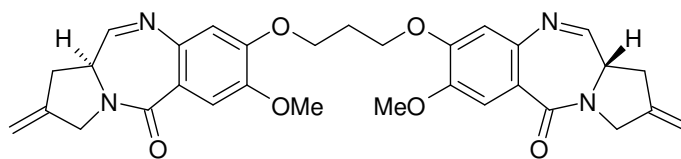
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Antibiotics III. Springer-Verlag, New York, pp. 3-11 (1975); Hurley and Needham-VanDevanter, *Acc. Chem. Res.*, **19**, 230-237 (1986)). Their ability to form an adduct in the minor groove, enables them to interfere with DNA processing, hence their use as antitumour agents.

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It has been previously disclosed that the biological activity of this molecules can be potentiated by joining two PBD units together through their C8/C'-hydroxyl functionalities via a flexible alkylene linker (Bose, D.S., *et al.*, *J. Am. Chem. Soc.*, **114**, 4939-4941 (1992); Thurston, D.E., *et al.*, *J. Org. Chem.*, **61**, 8141-8147 (1996)). The PBD dimers are thought to form sequence-selective DNA lesions such as the palindromic 5'-Pu-GATC-Py-3' interstrand cross-link (Smellie, M., *et al.*, *Biochemistry*, **42**, 8232-8239 (2003); Martin, C., *et al.*, *Biochemistry*, **44**, 4135-4147) which is thought to be mainly responsible for their biological activity.

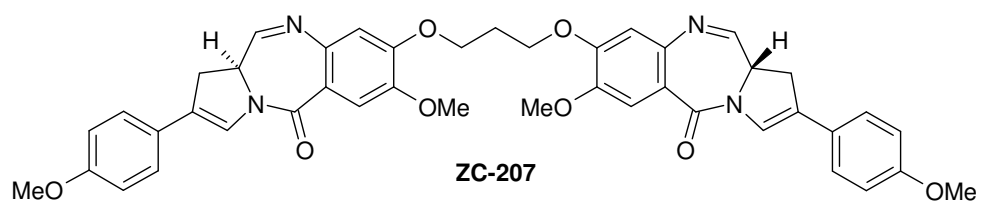
15 One example of a PBD dimer is SG2000 (SJG-136):



(Gregson, S., *et al.*, *J. Med. Chem.*, **44**, 737-748 (2001); Alley, M.C., *et al.*, *Cancer Research*, **64**, 6700-6706 (2004); Hartley, J.A., *et al.*, *Cancer Research*, **64**, 6693-6699 (2004)) which has been involved in clinical trials as a standalone agent, for example, NCT02034227 investigating its use in treating Acute Myeloid Leukemia and Chronic Lymphocytic Leukemia (see: <https://www.clinicaltrials.gov/ct2/show/NCT02034227>).

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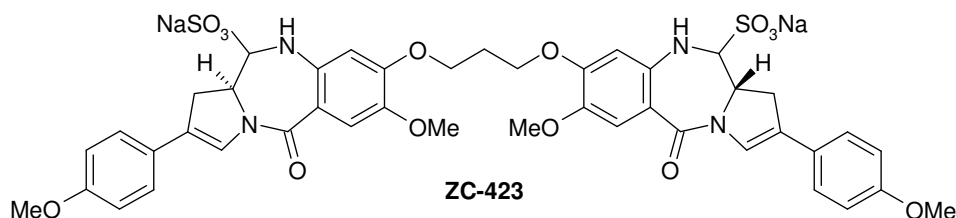
Dimeric PBD compounds bearing C2 aryl substituents, such as SG2202 (ZC-207), are disclosed in WO 2005/085251:



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and in WO2006/111759, bisulphites of such PBD compounds, for example SG2285 (ZC-423):

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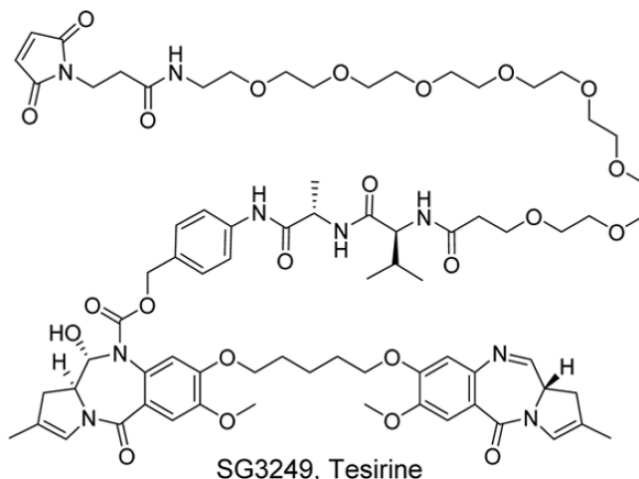
These compounds have been shown to be highly useful cytotoxic agents (Howard, P.W., *et al.*, *Bioorg. Med. Chem.* (2009), doi: 10.1016/j.bmcl.2009.09.012).

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WO 2007/085930 describes the preparation of dimer PBD compounds having linker groups for connection to a cell binding agent, such as an antibody. The linker is present in the bridge linking the monomer PBD units of the dimer.

- 10 Dimer PBD compounds having linker groups for connection to a cell binding agent, such as an antibody, are described in WO 2011/130598. The linker in these compounds is attached to one of the available N10 positions, and are generally cleaved by action of an enzyme on the linker group. If the non-bound N10 position is protected with a capping group, the capping groups exemplified have the same cleavage trigger as the linker to the
- 15 antibody.

WO 2014/057074 describes two specific PBD dimer conjugates bound via the N10 position on one monomer, the other PBD monomer being in imine form. One of the drug-linkers disclosed is SG3249, Tesirine:



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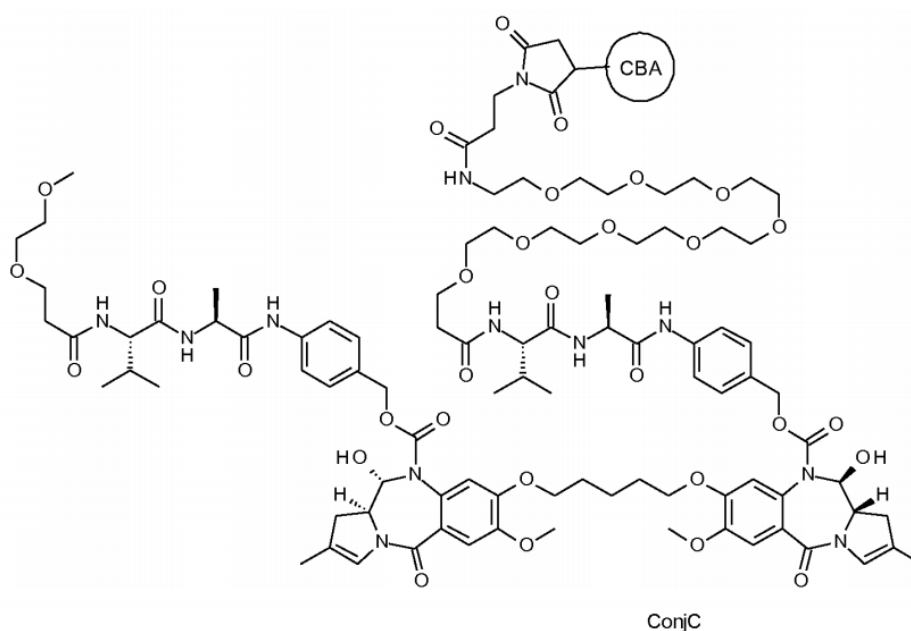
which, when conjugated to anti-DLL3 rovalpituzumab, is known as rovalpituzumab-tesirine (Rova-T), currently under evaluation for the treatment of small cell lung cancer (Tiberghien, A.C., *et al.*, *ACS Med. Chem. Lett.*, 2016, 7 (11), 983–987; DOI:

10.1021/acsmmedchemlett.6b00062). Further conjugates of this drug-linker with an engineered version of trastuzumab and a humanized antibody against human CD19 also began trials in early 2017 by ADC Therapeutics SA (Abstracts #51 and #52 in Proceedings of the American Association for Cancer Research, Volume 58, April 2017).

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WO 2015/052322 describes a specific PBD dimer conjugate bound via the N10 position on one monomer, the other PBD monomer being in imine form. It also describes a specific PBD dimer conjugate bound via the N10 position on one monomer, the other PBD monomer having a capping group with the same cleavage trigger as the linker to the antibody:

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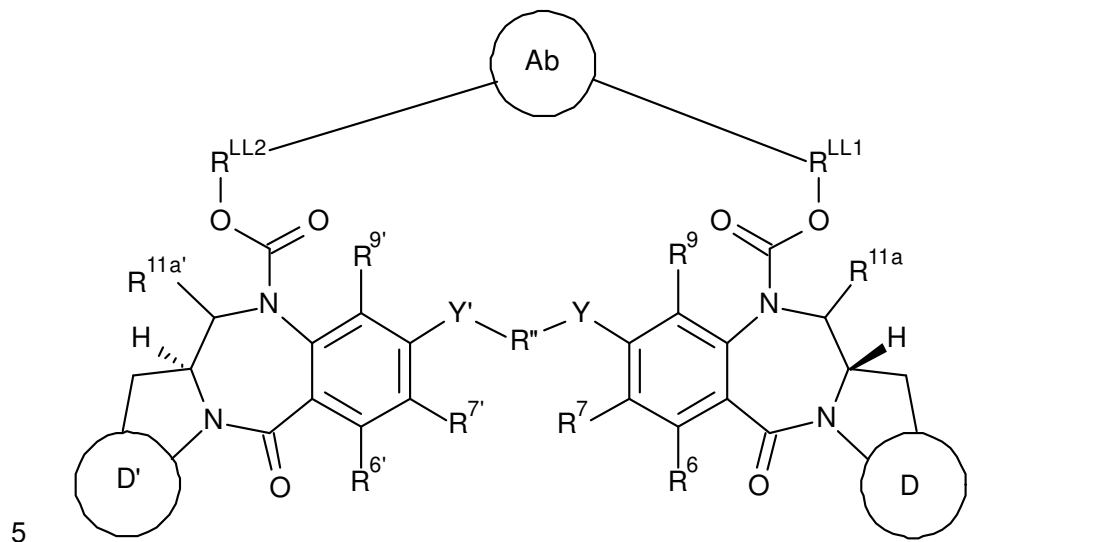
Disclosure of the invention

The present invention provides PBDs, and related PBD dimer conjugates wherein the PBDs are conjugated to antibodies that are modified so as to have at least one free conjugation site on each heavy chain, and where the conjugation is via each N10 group of the PBD via a linker.

The present inventors have found such conjugates to be surprisingly effective, despite the expectation that it was not possible to link a single PBD or related dimer to a single antibody by two linkers.

The present invention also provided PBD and related dimer drug linkers, suitable for conjugating to a modified antibodies, where both N10 groups bear linking groups.

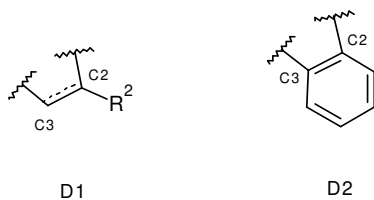
A first aspect of the present invention provides a conjugate of formula I:



Wherein

Ab is a modified antibody having at least one free conjugation site on each heavy chain;

D represents either group D1 or D2:



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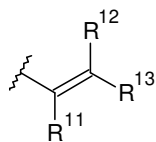
10 the dotted line indicates the optional presence of a double bond between C2 and C3;
when there is a double bond present between C2 and C3, R² is selected from the group
consisting of:

(ia) C₅₋₁₀ aryl group, optionally substituted by one or more substituents selected from the
group comprising: halo, nitro, cyano, ether, carboxy, ester, C₁₋₇ alkyl, C₃₋₇ heterocyclyl and

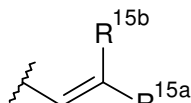
15 bis-oxy-C₁₋₃ alkylene;

(ib) C₁₋₅ saturated aliphatic alkyl;

(ic) C₃₋₆ saturated cycloalkyl;

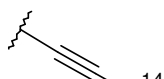


(id) , wherein each of R^{11} , R^{12} and R^{13} are independently selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl, where the total number of carbon atoms in the R^2 group is no more than 5;



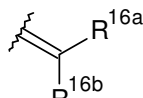
(ie) , wherein one of R^{15a} and R^{15b} is H and the other is selected from:

- 5 phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl; and



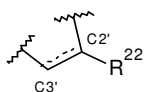
(if) , where R^{14} is selected from: H; C_{1-3} saturated alkyl; C_{2-3} alkenyl; C_{2-3} alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl;

- 10 when there is a single bond present between C2 and C3,

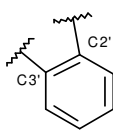


R^2 is selected from H, OH, F, diF and , where R^{16a} and R^{16b} are independently selected from H, F, C_{1-4} saturated alkyl, C_{2-3} alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C_{1-4} alkyl amido and C_{1-4} alkyl ester; or, when one of R^{16a} and R^{16b} is H, the other is selected from nitrile and a C_{1-4} alkyl ester;

- 15 D' represents either group $D'1$ or $D'2$:



D'1



D'2

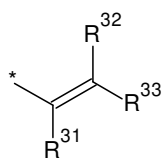
wherein the dotted line indicates the optional presence of a double bond between $C2'$ and $C3'$;

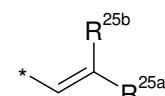
- 20 when there is a double bond present between $C2'$ and $C3'$, R^{12} is selected from the group consisting of:

(iia) C₅₋₁₀ aryl group, optionally substituted by one or more substituents selected from the group comprising: halo, nitro, cyano, ether, carboxy, ester, C₁₋₇ alkyl, C₃₋₇ heterocyclyl and bis-oxy-C₁₋₃ alkylene;

(iib) C₁₋₅ saturated aliphatic alkyl;

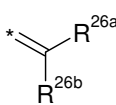
5 (iic) C₃₋₆ saturated cycloalkyl;

(iid) , wherein each of R³¹, R³² and R³³ are independently selected from H, C₁₋₃ saturated alkyl, C₂₋₃ alkenyl, C₂₋₃ alkynyl and cyclopropyl, where the total number of carbon atoms in the R¹² group is no more than 5;

(iie) , wherein one of R^{25a} and R^{25b} is H and the other is selected from:
10 phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl; and

(iif) , where R²⁴ is selected from: H; C₁₋₃ saturated alkyl; C₂₋₃ alkenyl; C₂₋₃ alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl;

15 when there is a single bond present between C2' and C3',

R¹² is selected from H, OH, F, diF and , where R^{26a} and R^{26b} are independently selected from H, F, C₁₋₄ saturated alkyl, C₂₋₃ alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C₁₋₄ alkyl amido and C₁₋₄ alkyl ester; or, when one of R^{26a} and R^{26b} is H, the other is selected from nitrile and a C₁₋₄ alkyl ester;

20 R⁶ and R⁹ are independently selected from H, R, OH, OR, SH, SR, NH₂, NHR, NRR', nitro, Me₃Sn and halo;

where R and R' are independently selected from optionally substituted C₁₋₁₂ alkyl, C₃₋₂₀ heterocyclyl and C₅₋₂₀ aryl groups;

R⁷ is selected from H, R, OH, OR, SH, SR, NH₂, NHR, NRR', nitro, Me₃Sn and halo;

25 R'' is a C₃₋₁₂ alkylene group, which chain may be interrupted by one or more heteroatoms, e.g. O, S, NR^{N2} (where R^{N2} is H or C₁₋₄ alkyl), and/or aromatic rings, e.g. benzene or pyridine;

Y and Y' are selected from O, S, or NH;

R^{11a} is selected from OH, OR^A, where R^A is C₁₋₄ alkyl;

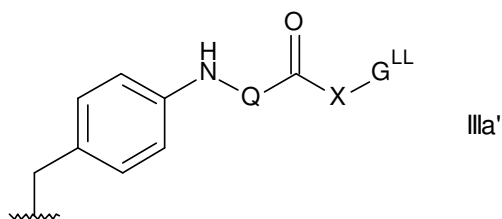
$R^{6'}$, $R^{7'}$, $R^{9'}$ and $R^{11a'}$ are selected from the same groups as R^6 , R^7 , R^9 and R^{11a} respectively;

and

R^{LL1} and R^{LL2} are linkers connected to the antibody at different sites which are

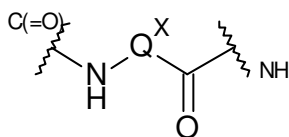
5 independently selected from:

(iii a):



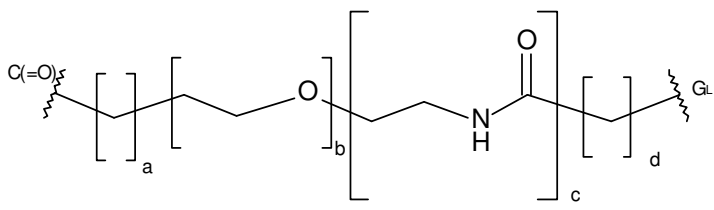
wherein

Q is:



10 , where Q^X is such that Q is an amino-acid residue, a dipeptide residue or a tripeptide residue;

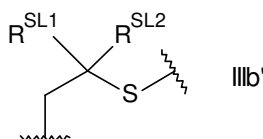
X is:



where $a = 0$ to 5, $b = 0$ to 16, $c = 0$ or 1, $d = 0$ to 5;

15 G^{LL} is a linker connected to the antibody; and

(iii b):



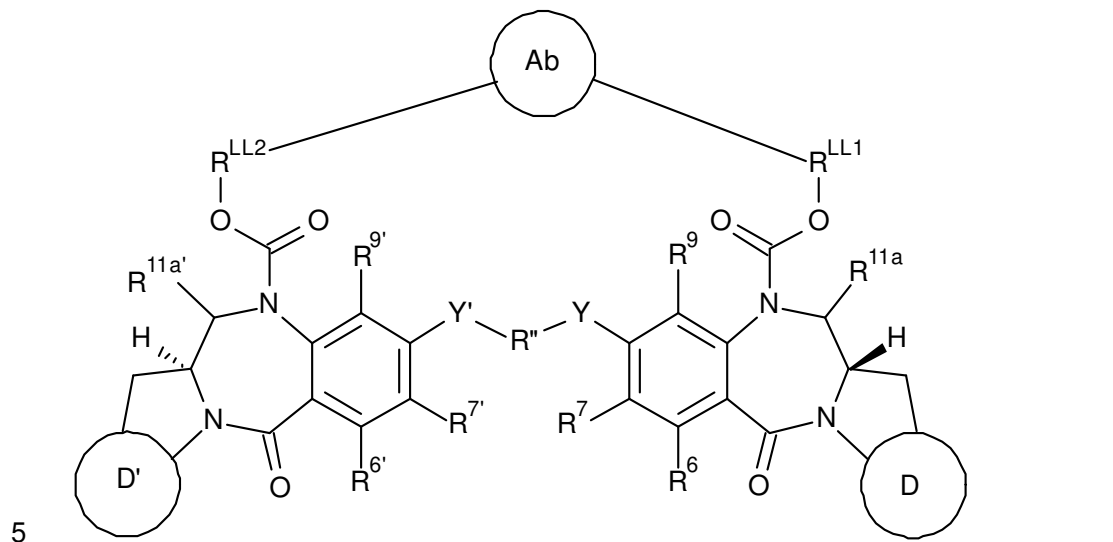
where R^{SL1} and R^{SL2} are independently selected from H and methyl, or together with the carbon atom to which they are bound form a cyclopropylene or cyclobutylene group.

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It is thought that such ADCs which effectivelly have a drug antibody ratio (DAR) of 1 could offer significant advantages including reduced off-target toxicity and an enhanced

therapeutic window by reducing the minimal effective dose requirement over ADCs consisting of heterogeneous mixtures with higher DARs.

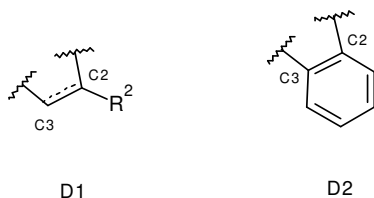
A second aspect of the present invention comprises a conjugate of formula I:



Wherein

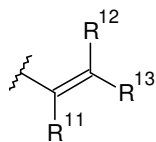
Ab is a modified antibody having at least one free conjugation site on each heavy chain

D represents either group D1 or D2:

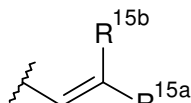


;

- 10 the dotted line indicates the optional presence of a double bond between C2 and C3;
when there is a double bond present between C2 and C3, R² is selected from the group
consisting of:
- (ia) C₅₋₁₀ aryl group, optionally substituted by one or more substituents selected from the
group comprising: halo, nitro, cyano, ether, carboxy, ester, C₁₋₇ alkyl, C₃₋₇ heterocyclyl and
15 bis-oxy-C₁₋₃ alkylene;
(ib) C₁₋₅ saturated aliphatic alkyl;
(ic) C₃₋₆ saturated cycloalkyl;

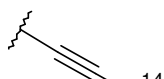


(id) , wherein each of R^{11} , R^{12} and R^{13} are independently selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl, where the total number of carbon atoms in the R^2 group is no more than 5;



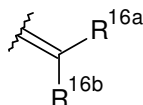
(ie) , wherein one of R^{15a} and R^{15b} is H and the other is selected from:

- 5 phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl; and



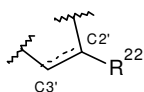
(if) , where R^{14} is selected from: H; C_{1-3} saturated alkyl; C_{2-3} alkenyl; C_{2-3} alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl;

- 10 when there is a single bond present between C2 and C3,

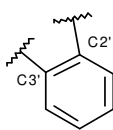


R^2 is selected from H, OH, F, diF and , where R^{16a} and R^{16b} are independently selected from H, F, C_{1-4} saturated alkyl, C_{2-3} alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C_{1-4} alkyl amido and C_{1-4} alkyl ester; or, when one of R^{16a} and R^{16b} is H, the other is selected from nitrile and a C_{1-4} alkyl ester;

- 15 D' represents either group $D'1$ or $D'2$:



D'1



D'2

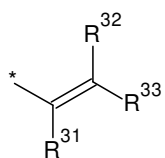
wherein the dotted line indicates the optional presence of a double bond between $C2'$ and $C3'$;

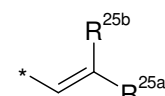
- 20 when there is a double bond present between $C2'$ and $C3'$, R^{22} is selected from the group consisting of:

(iia) C₅₋₁₀ aryl group, optionally substituted by one or more substituents selected from the group comprising: halo, nitro, cyano, ether, carboxy, ester, C₁₋₇ alkyl, C₃₋₇ heterocyclyl and bis-oxy-C₁₋₃ alkylene;

(iib) C₁₋₅ saturated aliphatic alkyl;

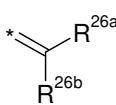
5 (iic) C₃₋₆ saturated cycloalkyl;

(iid) , wherein each of R³¹, R³² and R³³ are independently selected from H, C₁₋₃ saturated alkyl, C₂₋₃ alkenyl, C₂₋₃ alkynyl and cyclopropyl, where the total number of carbon atoms in the R²² group is no more than 5;

(iie) , wherein one of R^{25a} and R^{25b} is H and the other is selected from:
10 phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl; and

(iif) , where R²⁴ is selected from: H; C₁₋₃ saturated alkyl; C₂₋₃ alkenyl; C₂₋₃ alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl;

15 when there is a single bond present between C2' and C3',

R²² is selected from H, OH, F, diF and , where R^{26a} and R^{26b} are independently selected from H, F, C₁₋₄ saturated alkyl, C₂₋₃ alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C₁₋₄ alkyl amido and C₁₋₄ alkyl ester; or, when one of R^{26a} and R^{26b} is H, the other is selected from nitrile and a C₁₋₄ alkyl ester;

20 R⁶ and R⁹ are independently selected from H, R, OH, OR, SH, SR, NH₂, NHR, NRR', nitro, Me₃Sn and halo;

where R and R' are independently selected from optionally substituted C₁₋₁₂ alkyl, C₃₋₂₀ heterocyclyl and C₅₋₂₀ aryl groups;

R⁷ is selected from H, R, OH, OR, SH, SR, NH₂, NHR, NRR', nitro, Me₃Sn and halo;

25 R'' is a C₃₋₁₂ alkylene group, which chain may be interrupted by one or more heteroatoms, e.g. O, S, NR^{N2}, where R^{N2} is H or C₁₋₄ alkyl, and/or aromatic rings, e.g. benzene or pyridine;

Y and Y' are selected from O, S, or NH;

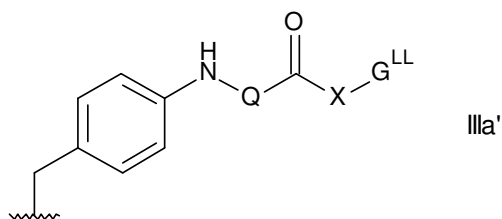
R^{11a} is selected from OH, OR^A, where R^A is C₁₋₄ alkyl;

$R^{6'}$, $R^{7'}$, $R^{9'}$ and $R^{11a'}$ are selected from the same groups as R^6 , R^7 , R^9 and R^{11a} respectively;

and

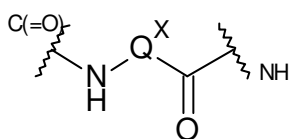
R^{LL1} and R^{LL2} are linkers connected to the antibody at different sites which are

5 independently of formula IIIa':



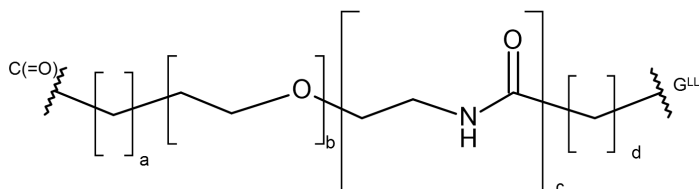
wherein

Q is:



10 , where Q^X is such that Q is an amino-acid residue, a dipeptide residue or a tripeptide residue;

X is:



where $a = 0$ to 5, $b = 0$ to 16, $c = 0$ or 1, $d = 0$ to 5;

15 G^{LL} is selected from:

(G ^{LL1-1})		(G ^{LL8-1})	
(G ^{LL1-2})		(G ^{LL8-2})	

(G ^{LL2})		(G ^{LL9-1})	
(G ^{LL3-1})		(G ^{LL9-2})	
(G ^{LL3-2})		G ^{L10}	
(G ^{LL-4})		G ^{L11}	
(G ^{LL5})		G ^{L12}	
(G ^{LL6})		G ^{L13}	
(G ^{LL7})			

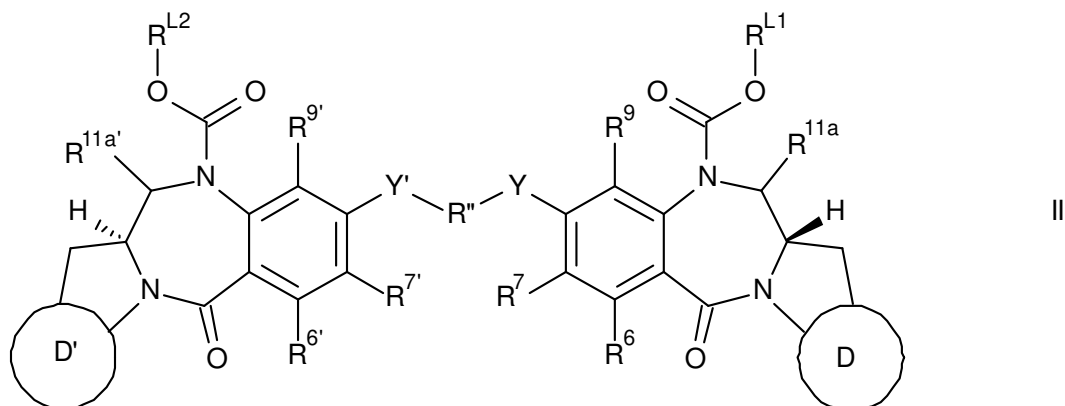
where Ar represents a C₅₋₆ arylene group, e.g. phenylene and X represents C¹⁻⁴ alkyl;

where CBA represents a cell binding agent which is a modified antibody having at least one free conjugation site on each heavy chain;

wherein the term alkyl includes the sub-classes alkenyl, alkynyl and cycloalkyl, the term

- 5 alkylene includes the sub-classes alkenylene, alkynylene, cycloalkylene, the term alkenyl pertains to an alkyl group having one or more carbon-carbon double bonds and the term alkynyl pertains to an alkyl group having one or more carbon-carbon triple bonds.

A third aspect of the present invention comprises a compound with the formula II:

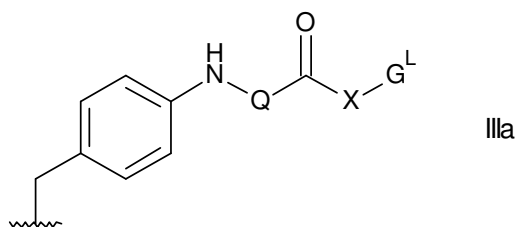


and salts and solvates thereof,

- 5 wherein D, R², R⁶, R⁷, R⁹, R^{11a}, Y, R'', Y', D', R^{6'}, R^{7'}, R^{9'}, R^{11a'} and R¹² (including the presence or absence of double bonds between C2 and C3 and C2' and C3' respectively) are as defined in the first aspect of the invention;

R^L is a linker for connecting to a cell binding agent, which is selected from:

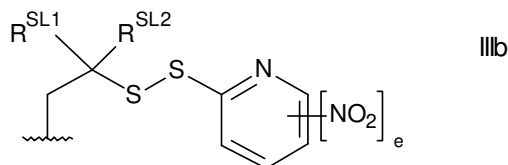
(iiiia):



10

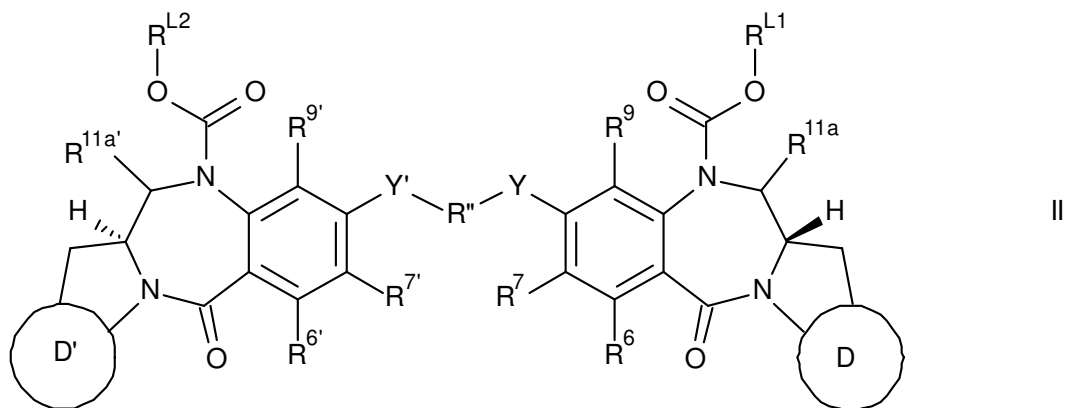
where Q and X are as defined in the first aspect and G^L is a linker for connecting to an antibody; and

(iiib):



- 15 where R^{SL1} and R^{SL2} are as defined in the first aspect and e is 0 or 1.

A fourth aspect of the present invention comprises a compound of formula II:

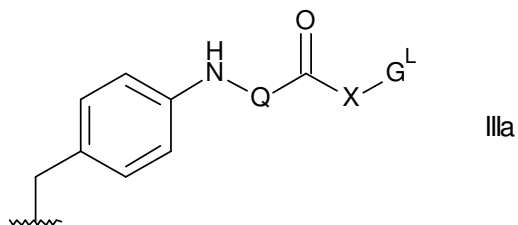


and salts and solvates thereof,

wherein D, R², R⁶, R⁷, R⁹, R^{11a}, Y, R'', Y', D', R^{6'}, R^{7'}, R^{9'}, R^{11a'} and R¹² (including the presence or absence of double bonds between C2 and C3 and C2' and C3' respectively)

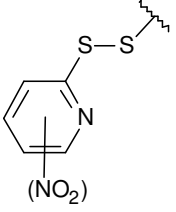
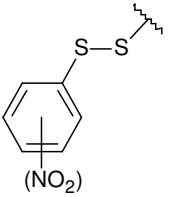
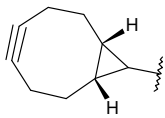
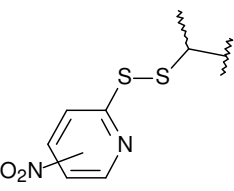
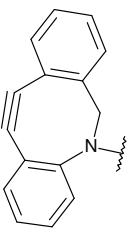
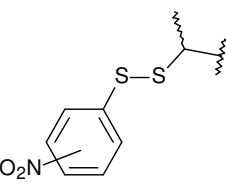
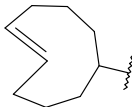
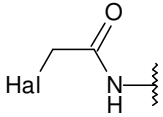
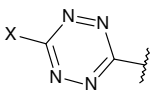
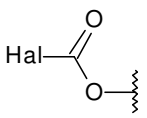
5 are as defined in the second aspect;

R^{L1} and R^{L2} are linkers for connecting to a cell binding agent, which are independently of formula IIIa:



where Q and X are as defined in the second aspect and G^L is selected from:

(G ^{L1-1})		(G ^{L6})	
(G ^{L1-2})		(G ^{L7})	
(G ^{L2})		(G ^{L8})	

(G ^{L3-1})	 where the NO₂ group is optional	(G ^{L9})	
(G ^{L3-2})	 where the NO₂ group is optional	(G ^{L10})	
(G ^{L3-3})	 where the NO₂ group is optional	(G ^{L11})	
(G ^{L3-4})	 where the NO₂ group is optional	(G ^{L12})	
(G ^{L4})	 Where Hal = I, Br, Cl	(G ^{L13})	
(G ^{L5})			

where Ar represents a C₅₋₆ arylene group, e.g. phenylene, and X represents C¹⁻⁴ alkyl.

A fifth aspect of the present invention provides the use of a conjugate of the first or second aspect of the invention in the manufacture of a medicament for treating a proliferative disease. The fifth aspect also provides a conjugate of the first or second aspect of the invention for use in the treatment of a proliferative disease. The fifth aspect also provides a

method of treating a proliferative disease comprising administering a therapeutically effective amount of a conjugate of the first or second aspect of the invention to a patient in need thereof.

- 5 One of ordinary skill in the art is readily able to determine whether or not a candidate conjugate treats a proliferative condition for any particular cell type. For example, assays which may conveniently be used to assess the activity offered by a particular compound are described in the examples below.
- 10 A sixth aspect of the present invention provides the synthesis of a conjugate of the first or second aspect of the invention comprising conjugating a compound (drug linker) of the third or fourth aspect of the invention with an antibody as defined in the first or second aspect of the invention.
- 15 Brief Description of Figures
- Figure 1 shows the effect of a single dose of a conjugate of the invention compared to non-treatment in a NCI-N87 xenograft model;
- Figure 2 shows the effect of a lower single dose of the same conjugate of the invention compared to non-treatment in a NCI-N87 xenograft model.
- 20 Figure 3 shows schematic representations of (A) modified antibodies suitable for use in the present invention, (B) an antibody drug conjugate comprising a PBD of the present invention.
- Figure 4 shows a schematic representation of the conjugation of a modified antibody to Compound 10.
- 25 Figure 5 shows the heavy and light chain sequences of a Herceptin-Flexmab.
- Figure 6 shows the activity of a conjugate of the present invention compared to a conjugate not of the present invention.

Definitions

30 Substituents

The phrase "optionally substituted" as used herein, pertains to a parent group which may be unsubstituted or which may be substituted.

- Unless otherwise specified, the term "substituted" as used herein, pertains to a parent
- 35 group which bears one or more substituents. The term "substituent" is used herein in the conventional sense and refers to a chemical moiety which is covalently attached to, or if

appropriate, fused to, a parent group. A wide variety of substituents are well known, and methods for their formation and introduction into a variety of parent groups are also well known.

- 5 Examples of substituents are described in more detail below.

C₁₋₁₂ alkyl: The term "C₁₋₁₂ alkyl" as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from a carbon atom of a hydrocarbon compound having from 1 to 12 carbon atoms, which may be aliphatic or alicyclic, and which may be saturated or unsaturated (e.g. partially unsaturated, fully unsaturated). The term "C₁₋₄ alkyl" as used
10 herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from a carbon atom of a hydrocarbon compound having from 1 to 4 carbon atoms, which may be aliphatic or alicyclic, and which may be saturated or unsaturated (e.g. partially unsaturated, fully unsaturated). Thus, the term "alkyl" includes the sub-classes alkenyl, alkynyl,
15 cycloalkyl, etc., discussed below.

Examples of saturated alkyl groups include, but are not limited to, methyl (C₁), ethyl (C₂), propyl (C₃), butyl (C₄), pentyl (C₅), hexyl (C₆) and heptyl (C₇).

- 20 Examples of saturated linear alkyl groups include, but are not limited to, methyl (C₁), ethyl (C₂), n-propyl (C₃), n-butyl (C₄), n-pentyl (amyl) (C₅), n-hexyl (C₆) and n-heptyl (C₇).

Examples of saturated branched alkyl groups include iso-propyl (C₃), iso-butyl (C₄), sec-butyl (C₄), tert-butyl (C₄), iso-pentyl (C₅), and neo-pentyl (C₅).

25

C₂₋₁₂ Alkenyl: The term "C₂₋₁₂ alkenyl" as used herein, pertains to an alkyl group having one or more carbon-carbon double bonds.

- 30 Examples of unsaturated alkenyl groups include, but are not limited to, ethenyl (vinyl, -CH=CH₂), 1-propenyl (-CH=CH-CH₃), 2-propenyl (allyl, -CH-CH=CH₂), isopropenyl (1-methylvinyl, -C(CH₃)=CH₂), butenyl (C₄), pentenyl (C₅), and hexenyl (C₆).

C₂₋₁₂ alkynyl: The term "C₂₋₁₂ alkynyl" as used herein, pertains to an alkyl group having one or more carbon-carbon triple bonds.

35

Examples of unsaturated alkynyl groups include, but are not limited to, ethynyl ($-\text{C}\equiv\text{CH}$) and 2-propynyl (propargyl, $-\text{CH}_2-\text{C}\equiv\text{CH}$).

C_{3-12} cycloalkyl: The term " C_{3-12} cycloalkyl" as used herein, pertains to an alkyl group which is also a cyclyl group; that is, a monovalent moiety obtained by removing a hydrogen atom from an alicyclic ring atom of a cyclic hydrocarbon (carbocyclic) compound, which moiety has from 3 to 7 carbon atoms, including from 3 to 7 ring atoms.

Examples of cycloalkyl groups include, but are not limited to, those derived from:

10 saturated monocyclic hydrocarbon compounds:

cyclopropane (C_3), cyclobutane (C_4), cyclopentane (C_5), cyclohexane (C_6), cycloheptane (C_7), methylcyclopropane (C_4), dimethylcyclopropane (C_5), methylcyclobutane (C_5), dimethylcyclobutane (C_6), methylcyclopentane (C_6), dimethylcyclopentane (C_7) and methylcyclohexane (C_7);

15 unsaturated monocyclic hydrocarbon compounds:

cyclopropene (C_3), cyclobutene (C_4), cyclopentene (C_5), cyclohexene (C_6), methylcyclopropene (C_4), dimethylcyclopropene (C_5), methylcyclobutene (C_5), dimethylcyclobutene (C_6), methylcyclopentene (C_6), dimethylcyclopentene (C_7) and methylcyclohexene (C_7); and

20 saturated polycyclic hydrocarbon compounds:

norcarane (C_7), norpinane (C_7), norbornane (C_7).

C_{3-20} heterocyclyl: The term " C_{3-20} heterocyclyl" as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from a ring atom of a heterocyclic compound, which moiety has from 3 to 20 ring atoms, of which from 1 to 10 are ring heteroatoms. Preferably, each ring has from 3 to 7 ring atoms, of which from 1 to 4 are ring heteroatoms.

In this context, the prefixes (e.g. C_{3-20} , C_{3-7} , C_{5-6} , etc.) denote the number of ring atoms, or range of number of ring atoms, whether carbon atoms or heteroatoms. For example, the term " C_{5-6} heterocyclyl", as used herein, pertains to a heterocyclyl group having 5 or 6 ring atoms.

Examples of monocyclic heterocyclyl groups include, but are not limited to, those derived from:

- N₁: aziridine (C₃), azetidine (C₄), pyrrolidine (tetrahydropyrrole) (C₅), pyrroline (e.g., 3-pyrroline, 2,5-dihydropyrrole) (C₅), 2H-pyrrole or 3H-pyrrole (isopyrrole, isoazole) (C₅), piperidine (C₆), dihydropyridine (C₆), tetrahydropyridine (C₆), azepine (C₇);
- O₁: oxirane (C₃), oxetane (C₄), oxolane (tetrahydrofuran) (C₅), oxole (dihydrofuran) (C₅),
- 5 oxane (tetrahydropyran) (C₆), dihydropyran (C₆), pyran (C₆), oxepin (C₇);
- S₁: thiirane (C₃), thietane (C₄), thiolane (tetrahydrothiophene) (C₅), thiane (tetrahydrothiopyran) (C₆), thiepane (C₇);
- O₂: dioxolane (C₅), dioxane (C₆), and dioxepane (C₇);
- O₃: trioxane (C₆);
- 10 N₂: imidazolidine (C₅), pyrazolidine (diazolidine) (C₅), imidazoline (C₅), pyrazoline (dihydropyrazole) (C₅), piperazine (C₆);
- N₁O₁: tetrahydrooxazole (C₅), dihydrooxazole (C₅), tetrahydroisoxazole (C₅), dihydroisoxazole (C₅), morpholine (C₆), tetrahydrooxazine (C₆), dihydrooxazine (C₆), oxazine (C₆);
- 15 N₁S₁: thiazoline (C₅), thiazolidine (C₅), thiomorpholine (C₆);
- N₂O₁: oxadiazine (C₆);
- O₁S₁: oxathiole (C₅) and oxathiane (thioxane) (C₆); and,
- N₁O₁S₁: oxathiazine (C₆).
- 20 Examples of substituted monocyclic heterocyclyl groups include those derived from saccharides, in cyclic form, for example, furanoses (C₅), such as arabinofuranose, lyxofuranose, ribofuranose, and xylofuranse, and pyranoses (C₆), such as allopyranose, altropyranose, glucopyranose, mannopyranose, gulopyranose, idopyranose, galactopyranose, and talopyranose.
- 25
- C₅₋₂₀ aryl: The term “C₅₋₂₀ aryl”, as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from an aromatic ring atom of an aromatic compound, which moiety has from 3 to 20 ring atoms. The term “C₅₋₇ aryl”, as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from an aromatic ring atom of
- 30 an aromatic compound, which moiety has from 5 to 7 ring atoms and the term “C₅₋₁₀ aryl”, as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from an aromatic ring atom of an aromatic compound, which moiety has from 5 to 10 ring atoms. Preferably, each ring has from 5 to 7 ring atoms.
- 35 In this context, the prefixes (e.g. C₃₋₂₀, C₅₋₇, C₅₋₆, C₅₋₁₀, etc.) denote the number of ring atoms, or range of number of ring atoms, whether carbon atoms or heteroatoms. For

example, the term “C₅₋₆ aryl” as used herein, pertains to an aryl group having 5 or 6 ring atoms.

The ring atoms may be all carbon atoms, as in “carboaryl groups”.

- 5 Examples of carboaryl groups include, but are not limited to, those derived from benzene (i.e. phenyl) (C₆), naphthalene (C₁₀), azulene (C₁₀), anthracene (C₁₄), phenanthrene (C₁₄), naphthacene (C₁₈), and pyrene (C₁₆).

- 10 Examples of aryl groups which comprise fused rings, at least one of which is an aromatic ring, include, but are not limited to, groups derived from indane (e.g. 2,3-dihydro-1H-indene) (C₉), indene (C₉), isoindene (C₉), tetraline (1,2,3,4-tetrahydronaphthalene) (C₁₀), acenaphthene (C₁₂), fluorene (C₁₃), phenalene (C₁₃), acephenanthrene (C₁₅), and aceanthrene (C₁₆).

- 15 Alternatively, the ring atoms may include one or more heteroatoms, as in “heteroaryl groups”. Examples of monocyclic heteroaryl groups include, but are not limited to, those derived from:

N₁: pyrrole (azole) (C₅), pyridine (azine) (C₆);

O₁: furan (oxole) (C₅);

- 20 S₁: thiophene (thiole) (C₅);

N₁O₁: oxazole (C₅), isoxazole (C₅), isoxazine (C₆);

N₂O₁: oxadiazole (furazan) (C₅);

N₃O₁: oxatriazole (C₅);

N₁S₁: thiazole (C₅), isothiazole (C₅);

- 25 N₂: imidazole (1,3-diazole) (C₅), pyrazole (1,2-diazole) (C₅), pyridazine (1,2-diazine) (C₆), pyrimidine (1,3-diazine) (C₆) (e.g., cytosine, thymine, uracil), pyrazine (1,4-diazine) (C₆);

N₃: triazole (C₅), triazine (C₆); and,

N₄: tetrazole (C₅).

- 30 Examples of heteroaryl which comprise fused rings, include, but are not limited to:

C₉ (with 2 fused rings) derived from benzofuran (O₁), isobenzofuran (O₁), indole (N₁), isoindole (N₁), indolizine (N₁), indoline (N₁), isoindoline (N₁), purine (N₄) (e.g., adenine, guanine), benzimidazole (N₂), indazole (N₂), benzoxazole (N₁O₁), benzisoxazole (N₁O₁), benzodioxole (O₂), benzofurazan (N₂O₁), benzotriazole (N₃), benzothiofuran (S₁),

- 35 benzothiazole (N₁S₁), benzothiadiazoole (N₂S);

- C_{10} (with 2 fused rings) derived from chromene (O_1), isochromene (O_1), chroman (O_1), isochroman (O_1), benzodioxan (O_2), quinoline (N_1), isoquinoline (N_1), quinolizine (N_1), benzoxazine (N_1O_1), benzodiazine (N_2), pyridopyridine (N_2), quinoxaline (N_2), quinazoline (N_2), cinnoline (N_2), phthalazine (N_2), naphthyridine (N_2), pteridine (N_4);
- 5 C_{11} (with 2 fused rings) derived from benzodiazepine (N_2);
- C_{13} (with 3 fused rings) derived from carbazole (N_1), dibenzofuran (O_1), dibenzothiophene (S_1), carboline (N_2), perimidine (N_2), pyridoindole (N_2); and,
- C_{14} (with 3 fused rings) derived from acridine (N_1), xanthene (O_1), thioxanthene (S_1), oxanthrene (O_2), phenoxathiin (O_1S_1), phenazine (N_2), phenoxazine (N_1O_1), phenothiazine (N_1S_1), thianthrene (S_2), phenanthridine (N_1), phenanthroline (N_2), phenazine (N_2).
- 10

The above groups, whether alone or part of another substituent, may themselves optionally be substituted with one or more groups selected from themselves and the additional substituents listed below.

15

Halo: -F, -Cl, -Br, and -I.

Hydroxy: -OH.

- 20 Ether: -OR, wherein R is an ether substituent, for example, a C_{1-7} alkyl group (also referred to as a C_{1-7} alkoxy group, discussed below), a C_{3-20} heterocyclyl group (also referred to as a C_{3-20} heterocyclyloxy group), or a C_{5-20} aryl group (also referred to as a C_{5-20} aryloxy group), preferably a C_{1-7} alkyl group.
- 25 Alkoxy: -OR, wherein R is an alkyl group, for example, a C_{1-7} alkyl group. Examples of C_{1-7} alkoxy groups include, but are not limited to, -OMe (methoxy), -OEt (ethoxy), -O(nPr) (n-propoxy), -O(iPr) (isopropoxy), -O(nBu) (n-butoxy), -O(sBu) (sec-butoxy), -O(iBu) (isobutoxy), and -O(tBu) (tert-butoxy).
- 30 Acetal: -CH(OR¹)(OR²), wherein R¹ and R² are independently acetal substituents, for example, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably a C_{1-7} alkyl group, or, in the case of a "cyclic" acetal group, R¹ and R², taken together with the two oxygen atoms to which they are attached, and the carbon atoms to which they are attached, form a heterocyclic ring having from 4 to 8 ring atoms. Examples of acetal
- 35 groups include, but are not limited to, -CH(OMe)₂, -CH(OEt)₂, and -CH(OMe)(OEt).

Hemiacetal: $-\text{CH}(\text{OH})(\text{OR}^1)$, wherein R^1 is a hemiacetal substituent, for example, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably a C_{1-7} alkyl group. Examples of hemiacetal groups include, but are not limited to, $-\text{CH}(\text{OH})(\text{OMe})$ and $-\text{CH}(\text{OH})(\text{OEt})$.

5

Ketal: $-\text{CR}(\text{OR}^1)(\text{OR}^2)$, where R^1 and R^2 are as defined for acetals, and R is a ketal substituent other than hydrogen, for example, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably a C_{1-7} alkyl group. Examples ketal groups include, but are not limited to, $-\text{C}(\text{Me})(\text{OMe})_2$, $-\text{C}(\text{Me})(\text{OEt})_2$, $-\text{C}(\text{Me})(\text{OMe})(\text{OEt})$, $-\text{C}(\text{Et})(\text{OMe})_2$, $-\text{C}(\text{Et})(\text{OEt})_2$, and $-\text{C}(\text{Et})(\text{OMe})(\text{OEt})$.

10

Hemiketal: $-\text{CR}(\text{OH})(\text{OR}^1)$, where R^1 is as defined for hemiacetals, and R is a hemiketal substituent other than hydrogen, for example, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably a C_{1-7} alkyl group. Examples of hemiacetal groups include, but are not limited to, $-\text{C}(\text{Me})(\text{OH})(\text{OMe})$, $-\text{C}(\text{Et})(\text{OH})(\text{OMe})$, $-\text{C}(\text{Me})(\text{OH})(\text{OEt})$, and $-\text{C}(\text{Et})(\text{OH})(\text{OEt})$.

15

Oxo (keto, -one): $=\text{O}$.

20 Thione (thioketone): $=\text{S}$.

Imino (imine): $=\text{NR}$, wherein R is an imino substituent, for example, hydrogen, C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably hydrogen or a C_{1-7} alkyl group. Examples of ester groups include, but are not limited to, $=\text{NH}$, $=\text{NMe}$, $=\text{NEt}$, and $=\text{NPh}$.

25

Formyl (carbaldehyde, carboxaldehyde): $-\text{C}(=\text{O})\text{H}$.

Acyl (keto): $-\text{C}(=\text{O})\text{R}$, wherein R is an acyl substituent, for example, a C_{1-7} alkyl group (also referred to as C_{1-7} alkylacyl or C_{1-7} alkanoyl), a C_{3-20} heterocyclyl group (also referred to as C_{3-20} heterocyclylacyl), or a C_{5-20} aryl group (also referred to as C_{5-20} arylacyl), preferably a C_{1-7} alkyl group. Examples of acyl groups include, but are not limited to, $-\text{C}(=\text{O})\text{CH}_3$ (acetyl), $-\text{C}(=\text{O})\text{CH}_2\text{CH}_3$ (propionyl), $-\text{C}(=\text{O})\text{C}(\text{CH}_3)_3$ (t-butyryl), and $-\text{C}(=\text{O})\text{Ph}$ (benzoyl, phenone).

35

Carboxy (carboxylic acid): $-\text{C}(=\text{O})\text{OH}$.

Thiocarboxy (thiocarboxylic acid): -C(=S)SH .

Thiolocarboxy (thiolocarboxylic acid): -C(=O)SH .

5

Thionocarboxy (thionocarboxylic acid): -C(=S)OH .

Imidic acid: -C(=NH)OH .

10 Hydroxamic acid: -C(=NOH)OH .

Ester (carboxylate, carboxylic acid ester, oxycarbonyl): -C(=O)OR , wherein R is an ester substituent, for example, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably a C_{1-7} alkyl group. Examples of ester groups include, but are not limited to,

15 -C(=O)OCH_3 , $\text{-C(=O)OCH}_2\text{CH}_3$, $\text{-C(=O)OC(CH}_3)_3$, and -C(=O)OPh .

Acyloxy (reverse ester): -OC(=O)R , wherein R is an acyloxy substituent, for example, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably a C_{1-7} alkyl group.

Examples of acyloxy groups include, but are not limited to, -OC(=O)CH_3 (acetoxy),

20 $\text{-OC(=O)CH}_2\text{CH}_3$, $\text{-OC(=O)C(CH}_3)_3$, -OC(=O)Ph , and $\text{-OC(=O)CH}_2\text{Ph}$.

Oxycarboxyloxy: -OC(=O)OR , wherein R is an ester substituent, for example, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably a C_{1-7} alkyl group.

Examples of ester groups include, but are not limited to, -OC(=O)OCH_3 ,

25 $\text{-OC(=O)OCH}_2\text{CH}_3$, $\text{-OC(=O)OC(CH}_3)_3$, and -OC(=O)OPh .

Amino: $\text{-NR}^1\text{R}^2$, wherein R^1 and R^2 are independently amino substituents, for example, hydrogen, a C_{1-7} alkyl group (also referred to as C_{1-7} alkylamino or di- C_{1-7} alkylamino), a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably H or a C_{1-7} alkyl group, or, in the case of a "cyclic" amino group, R^1 and R^2 , taken together with the nitrogen atom to which they are attached, form a heterocyclic ring having from 4 to 8 ring atoms. Amino groups may be primary (-NH_2), secondary (-NHR^1), or tertiary ($\text{-NHR}^1\text{R}^2$), and in cationic form, may be quaternary ($\text{-NR}^1\text{R}^2\text{R}^3$). Examples of amino groups include, but are not limited to, -NH_2 , -NHCH_3 , $\text{-NHC(CH}_3)_2$, $\text{-N(CH}_3)_2$, $\text{-N(CH}_2\text{CH}_3)_2$, and -NHPh . Examples of cyclic amino groups include, but are not limited to, aziridino, azetidino, pyrrolidino, piperidino, piperazino, morpholino, and thiomorpholino.

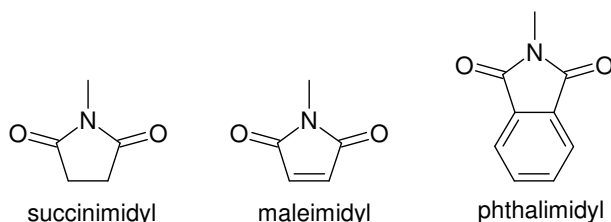
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Amido (carbamoyl, carbamyl, aminocarbonyl, carboxamide): $-C(=O)NR^1R^2$, wherein R^1 and R^2 are independently amino substituents, as defined for amino groups. Examples of amido groups include, but are not limited to, $-C(=O)NH_2$, $-C(=O)NHCH_3$, $-C(=O)N(CH_3)_2$,

- 5 $-C(=O)NHCH_2CH_3$, and $-C(=O)N(CH_2CH_3)_2$, as well as amido groups in which R^1 and R^2 , together with the nitrogen atom to which they are attached, form a heterocyclic structure as in, for example, piperidinocarbonyl, morpholinocarbonyl, thiomorpholinocarbonyl, and piperazinocarbonyl.

- 10 Thioamido (thiocarbamyl): $-C(=S)NR^1R^2$, wherein R^1 and R^2 are independently amino substituents, as defined for amino groups. Examples of amido groups include, but are not limited to, $-C(=S)NH_2$, $-C(=S)NHCH_3$, $-C(=S)N(CH_3)_2$, and $-C(=S)NHCH_2CH_3$.

- Acylamido (acylamino): $-NR^1C(=O)R^2$, wherein R^1 is an amide substituent, for example, hydrogen, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably hydrogen or a C_{1-7} alkyl group, and R^2 is an acyl substituent, for example, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably hydrogen or a C_{1-7} alkyl group. Examples of acylamide groups include, but are not limited to, $-NHC(=O)CH_3$, $-NHC(=O)CH_2CH_3$, and $-NHC(=O)Ph$. R^1 and R^2 may together form a cyclic structure, as
- 20 in, for example, succinimidyl, maleimidyl, and phthalimidyl:

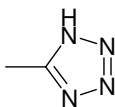


- Aminocarbonyloxy: $-OC(=O)NR^1R^2$, wherein R^1 and R^2 are independently amino substituents, as defined for amino groups. Examples of aminocarbonyloxy groups include,
- 25 but are not limited to, $-OC(=O)NH_2$, $-OC(=O)NHMe$, $-OC(=O)NMe_2$, and $-OC(=O)NEt_2$.

- Ureido: $-N(R^1)CONR^2R^3$ wherein R^2 and R^3 are independently amino substituents, as defined for amino groups, and R^1 is a ureido substituent, for example, hydrogen, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably hydrogen or a C_{1-7} alkyl
- 30 group. Examples of ureido groups include, but are not limited to, $-NHCONH_2$, $-NHCONHMe$, $-NHCONHEt$, $-NHCONMe_2$, $-NHCONEt_2$, $-NMeCONH_2$, $-NMeCONHMe$, $-NMeCONHEt$, $-NMeCONMe_2$, and $-NMeCONEt_2$.

Guanidino: -NH-C(=NH)NH_2 .

Tetrazolyl: a five membered aromatic ring having four nitrogen atoms and one carbon atom,



Imino: =NR , wherein R is an imino substituent, for example, hydrogen, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably H or a C_{1-7} alkyl group. Examples of imino groups include, but are not limited to, =NH , =NMe , and =NEt .

Amidine (amidino): -C(=NR)NR_2 , wherein each R is an amidine substituent, for example, hydrogen, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably H or a C_{1-7} alkyl group. Examples of amidine groups include, but are not limited to, -C(=NH)NH_2 , -C(=NH)NMe_2 , and -C(=NMe)NMe_2 .

Nitro: -NO_2 .

Nitroso: -NO .

Azido: -N_3 .

Cyano (nitrile, carbonitrile): -CN .

Isocyano: -NC .

Cyanato: -OCN .

Isocyanato: -NCO .

Thiocyano (thiocyanato): -SCN .

Isothiocyano (isothiocyanato): -NCS .

Sulfhydryl (thiol, mercapto): -SH .

Thioether (sulfide): -SR, wherein R is a thioether substituent, for example, a C₁₋₇ alkyl group (also referred to as a C₁₋₇alkylthio group), a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably a C₁₋₇ alkyl group. Examples of C₁₋₇ alkylthio groups include, but are not limited to, -SCH₃ and -SCH₂CH₃.

Disulfide: -SS-R, wherein R is a disulfide substituent, for example, a C₁₋₇ alkyl group, a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably a C₁₋₇ alkyl group (also referred to herein as C₁₋₇ alkyl disulfide). Examples of C₁₋₇ alkyl disulfide groups include, but are not limited to, -SSCH₃ and -SSCH₂CH₃.

Sulfine (sulfinyl, sulfoxide): -S(=O)R, wherein R is a sulfine substituent, for example, a C₁₋₇ alkyl group, a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably a C₁₋₇ alkyl group. Examples of sulfine groups include, but are not limited to, -S(=O)CH₃ and -S(=O)CH₂CH₃.

Sulfone (sulfonyl): -S(=O)₂R, wherein R is a sulfone substituent, for example, a C₁₋₇ alkyl group, a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably a C₁₋₇ alkyl group, including, for example, a fluorinated or perfluorinated C₁₋₇ alkyl group. Examples of sulfone groups include, but are not limited to, -S(=O)₂CH₃ (methanesulfonyl, mesyl), -S(=O)₂CF₃ (triflyl), -S(=O)₂CH₂CH₃ (esyl), -S(=O)₂C₄F₉ (nonafllyl), -S(=O)₂CH₂CF₃ (tresyl), -S(=O)₂CH₂CH₂NH₂ (tauryl), -S(=O)₂Ph (phenylsulfonyl, besyl), 4-methylphenylsulfonyl (tosyl), 4-chlorophenylsulfonyl (closyl), 4-bromophenylsulfonyl (brosyl), 4-nitrophenyl (nosyl), 2-naphthalenesulfonate (napsyl), and 5-dimethylamino-naphthalen-1-ylsulfonate (dansyl).

Sulfinic acid (sulfinio): -S(=O)OH, -SO₂H.

Sulfonic acid (sulfo): -S(=O)₂OH, -SO₃H.

Sulfinate (sulfinic acid ester): -S(=O)OR; wherein R is a sulfinate substituent, for example, a C₁₋₇ alkyl group, a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably a C₁₋₇ alkyl group. Examples of sulfinate groups include, but are not limited to, -S(=O)OCH₃ (methoxysulfinyl; methyl sulfinate) and -S(=O)OCH₂CH₃ (ethoxysulfinyl; ethyl sulfinate).

Sulfonate (sulfonic acid ester): -S(=O)₂OR, wherein R is a sulfonate substituent, for example, a C₁₋₇ alkyl group, a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably a

C₁₋₇ alkyl group. Examples of sulfonate groups include, but are not limited to, -S(=O)₂OCH₃ (methoxysulfonyl; methyl sulfonate) and -S(=O)₂OCH₂CH₃ (ethoxysulfonyl; ethyl sulfonate).

Sulfinyloxy: -OS(=O)R, wherein R is a sulfinyloxy substituent, for example, a C₁₋₇ alkyl group, a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably a C₁₋₇ alkyl group. Examples of sulfinyloxy groups include, but are not limited to, -OS(=O)CH₃ and -OS(=O)CH₂CH₃.

Sulfonyloxy: -OS(=O)₂R, wherein R is a sulfonyloxy substituent, for example, a C₁₋₇ alkyl group, a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably a C₁₋₇ alkyl group. Examples of sulfonyloxy groups include, but are not limited to, -OS(=O)₂CH₃ (mesylate) and -OS(=O)₂CH₂CH₃ (esylate).

Sulfate: -OS(=O)₂OR; wherein R is a sulfate substituent, for example, a C₁₋₇ alkyl group, a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably a C₁₋₇ alkyl group. Examples of sulfate groups include, but are not limited to, -OS(=O)₂OCH₃ and -SO(=O)₂OCH₂CH₃.

Sulfamyl (sulfamoyl; sulfinic acid amide; sulfonamide): -S(=O)NR¹R², wherein R¹ and R² are independently amino substituents, as defined for amino groups. Examples of sulfamyl groups include, but are not limited to, -S(=O)NH₂, -S(=O)NH(CH₃), -S(=O)N(CH₃)₂, -S(=O)NH(CH₂CH₃), -S(=O)N(CH₂CH₃)₂, and -S(=O)NHPh.

Sulfonamido (sulfinamoyl; sulfonic acid amide; sulfonamide): -S(=O)₂NR¹R², wherein R¹ and R² are independently amino substituents, as defined for amino groups. Examples of sulfonamido groups include, but are not limited to, -S(=O)₂NH₂, -S(=O)₂NH(CH₃), -S(=O)₂N(CH₃)₂, -S(=O)₂NH(CH₂CH₃), -S(=O)₂N(CH₂CH₃)₂, and -S(=O)₂NHPh.

Sulfamino: -NR¹S(=O)₂OH, wherein R¹ is an amino substituent, as defined for amino groups. Examples of sulfamino groups include, but are not limited to, -NHS(=O)₂OH and -N(CH₃)S(=O)₂OH.

Sulfonamino: -NR¹S(=O)₂R, wherein R¹ is an amino substituent, as defined for amino groups, and R is a sulfonamino substituent, for example, a C₁₋₇ alkyl group, a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably a C₁₋₇ alkyl group. Examples of sulfonamino groups include, but are not limited to, -NHS(=O)₂CH₃ and -N(CH₃)S(=O)₂C₆H₅.

Sulfinamino: $-NR^1S(=O)R$, wherein R^1 is an amino substituent, as defined for amino groups, and R is a sulfinamino substituent, for example, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably a C_{1-7} alkyl group. Examples of sulfinamino groups include, but are not limited to, $-NHS(=O)CH_3$ and $-N(CH_3)S(=O)C_6H_5$.

5

Phosphino (phosphine): $-PR_2$, wherein R is a phosphino substituent, for example, $-H$, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably $-H$, a C_{1-7} alkyl group, or a C_{5-20} aryl group. Examples of phosphino groups include, but are not limited to, $-PH_2$, $-P(CH_3)_2$, $-P(CH_2CH_3)_2$, $-P(t-Bu)_2$, and $-P(Ph)_2$.

10

Phospho: $-P(=O)_2$.

Phosphinyl (phosphine oxide): $-P(=O)R_2$, wherein R is a phosphinyl substituent, for example, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably a C_{1-7} alkyl group or a C_{5-20} aryl group. Examples of phosphinyl groups include, but are not limited to, $-P(=O)(CH_3)_2$, $-P(=O)(CH_2CH_3)_2$, $-P(=O)(t-Bu)_2$, and $-P(=O)(Ph)_2$.

15

Phosphonic acid (phosphono): $-P(=O)(OH)_2$.

20 Phosphonate (phosphono ester): $-P(=O)(OR)_2$, where R is a phosphonate substituent, for example, $-H$, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably $-H$, a C_{1-7} alkyl group, or a C_{5-20} aryl group. Examples of phosphonate groups include, but are not limited to, $-P(=O)(OCH_3)_2$, $-P(=O)(OCH_2CH_3)_2$, $-P(=O)(O-t-Bu)_2$, and $-P(=O)(OPh)_2$.

25 Phosphoric acid (phosphonooxy): $-OP(=O)(OH)_2$.

Phosphate (phosphonooxy ester): $-OP(=O)(OR)_2$, where R is a phosphate substituent, for example, $-H$, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably $-H$, a C_{1-7} alkyl group, or a C_{5-20} aryl group. Examples of phosphate groups include, but are not limited to, $-OP(=O)(OCH_3)_2$, $-OP(=O)(OCH_2CH_3)_2$, $-OP(=O)(O-t-Bu)_2$, and $-OP(=O)(OPh)_2$.

30

Phosphorous acid: $-OP(OH)_2$.

35 Phosphite: $-OP(OR)_2$, where R is a phosphite substituent, for example, $-H$, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group, preferably $-H$, a C_{1-7} alkyl group, or a

C₅₋₂₀ aryl group. Examples of phosphite groups include, but are not limited to, -OP(OCH₃)₂, -OP(OCH₂CH₃)₂, -OP(O-t-Bu)₂, and -OP(OPh)₂.

- 5 Phosphoramidite: -OP(OR¹)-NR²₂, where R¹ and R² are phosphoramidite substituents, for example, -H, a (optionally substituted) C₁₋₇ alkyl group, a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably -H, a C₁₋₇ alkyl group, or a C₅₋₂₀ aryl group. Examples of phosphoramidite groups include, but are not limited to, -OP(OCH₂CH₃)-N(CH₃)₂, -OP(OCH₂CH₃)-N(i-Pr)₂, and -OP(OCH₂CH₂CN)-N(i-Pr)₂.
- 10 Phosphoramidate: -OP(=O)(OR¹)-NR²₂, where R¹ and R² are phosphoramidate substituents, for example, -H, a (optionally substituted) C₁₋₇ alkyl group, a C₃₋₂₀ heterocyclyl group, or a C₅₋₂₀ aryl group, preferably -H, a C₁₋₇ alkyl group, or a C₅₋₂₀ aryl group. Examples of phosphoramidate groups include, but are not limited to, -OP(=O)(OCH₂CH₃)-N(CH₃)₂, -OP(=O)(OCH₂CH₃)-N(i-Pr)₂, and -OP(=O)(OCH₂CH₂CN)-N(i-Pr)₂.

15

Alkylene

- C₃₋₁₂ alkylene: The term "C₃₋₁₂ alkylene", as used herein, pertains to a bidentate moiety obtained by removing two hydrogen atoms, either both from the same carbon atom, or one from each of two different carbon atoms, of a hydrocarbon compound having from 3 to 12 carbon atoms (unless otherwise specified), which may be aliphatic or alicyclic, and which may be saturated, partially unsaturated, or fully unsaturated. Thus, the term "alkylene" includes the sub-classes alkenylene, alkynylene, cycloalkylene, etc., discussed below.
- 20

- Examples of linear saturated C₃₋₁₂ alkylene groups include, but are not limited to, -(CH₂)_n- where n is an integer from 3 to 12, for example, -CH₂CH₂CH₂- (propylene), -CH₂CH₂CH₂CH₂- (butylene), -CH₂CH₂CH₂CH₂CH₂- (pentylene) and -CH₂CH₂CH₂CH₂CH₂CH₂- (heptylene).

- Examples of branched saturated C₃₋₁₂ alkylene groups include, but are not limited to, -CH(CH₃)CH₂-, -CH(CH₃)CH₂CH₂-, -CH(CH₃)CH₂CH₂CH₂-, -CH₂CH(CH₃)CH₂-, -CH₂CH(CH₃)CH₂CH₂-, -CH(CH₂CH₃)-, -CH(CH₂CH₃)CH₂-, and -CH₂CH(CH₂CH₃)CH₂-.
- 30

- Examples of linear partially unsaturated C₃₋₁₂ alkylene groups (C₃₋₁₂ alkenylene, and alkynylene groups) include, but are not limited to, -CH=CH-CH₂-, -CH₂-CH=CH₂-, -CH=CH-CH₂-CH₂-, -CH=CH-CH₂-CH₂-CH₂-, -CH=CH-CH=CH-, -CH=CH-CH=CH-CH₂-, -
- 35

$\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-$, $-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$, $-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}=\text{CH}-$, and $-\text{CH}_2-\text{C}\equiv\text{C}-\text{CH}_2-$.

Examples of branched partially unsaturated C_{3-12} alkylene groups (C_{3-12} alkenylene and
 5 alkynylene groups) include, but are not limited to, $-\text{C}(\text{CH}_3)=\text{CH}-$, $-\text{C}(\text{CH}_3)=\text{CH}-\text{CH}_2-$,
 $-\text{CH}=\text{CH}-\text{CH}(\text{CH}_3)-$ and $-\text{C}\equiv\text{C}-\text{CH}(\text{CH}_3)-$.

Examples of alicyclic saturated C_{3-12} alkylene groups (C_{3-12} cycloalkylenes) include, but are
 not limited to, cyclopentylene (e.g. cyclopent-1,3-ylene), and cyclohexylene
 10 (e.g. cyclohex-1,4-ylene).

Examples of alicyclic partially unsaturated C_{3-12} alkylene groups (C_{3-12} cycloalkylenes)
 include, but are not limited to, cyclopentenylene (e.g. 4-cyclopenten-1,3-ylene),
 cyclohexenylene (e.g. 2-cyclohexen-1,4-ylene; 3-cyclohexen-1,2-ylene; 2,5-cyclohexadien-
 15 1,4-ylene).

Ligand Unit

The Ligand Units for use in the present invention are Cell Binding Agents, more specifically
 modified antibodies, or antigen binding fragments thereof, having at least one conjugation
 20 site on each heavy chain. Examples of particular modified antibodies suitable for use
 according to the present invention are disclosed in WO 2012/064733 (filed as
 PCT/US2011/059775), which is incorporated herein by reference.

Antibodies

25 The term "antibody" herein is used in the broadest sense and specifically covers
 monoclonal antibodies, polyclonal antibodies, dimers, multimers, multispecific antibodies
 (e.g., bispecific antibodies), and antibody fragments, so long as they exhibit the desired
 biological activity (Miller *et al* (2003) *Jour. of Immunology* 170:4854-4861). Antibodies may
 be murine, human, humanized, chimeric, or derived from other species. An antibody is a
 30 protein generated by the immune system that is capable of recognizing and binding to a
 specific antigen. (Janeway, C., Travers, P., Walport, M., Shlomchik (2001) *Immuno*
Biology, 5th Ed., Garland Publishing, New York). A target antigen generally has numerous
 binding sites, also called epitopes, recognized by CDRs on multiple antibodies. Each
 antibody that specifically binds to a different epitope has a different structure. Thus, one
 35 antigen may have more than one corresponding antibody. An antibody includes a full-
 length immunoglobulin molecule or an immunologically active portion of a full-length

immunoglobulin molecule, *i.e.*, a molecule that contains an antigen binding site that immunospecifically binds an antigen of a target of interest or part thereof, such targets including but not limited to, cancer cell or cells that produce autoimmune antibodies associated with an autoimmune disease. The immunoglobulin can be of any type (e.g. IgG, IgE, IgM, IgD, and IgA), class (e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The immunoglobulins can be derived from any species, including human, murine, or rabbit origin.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include F(ab')₂, and scFv fragments, and dimeric epitope-binding fragments of any of the above which immunospecifically bind to cancer cell antigens, viral antigens or microbial antigens, single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

Modified antibodies suitable for use in the present invention include those wherein the native interchain cysteine residues have been substituted for amino acid residues lacking thiol groups. The antibodies may comprise at least one additional substitutions in each heavy chain of an amino acid residue comprising a reactive group suitable for conjugation to a linker. The additionally substituted amino acid may be a cysteine or a non-natural amino acid. The position that is substituted may be selected from those set forth below:

Antibody	Isotype	IgG1	IgG2	IgG3	IgG4
Position (Kabat EU) and Corresponding Amino Acid	239	Ser	Ser	Ser	Ser
	282	Val	Val	Val	Val
	289	Thr	Thr	Thr	Thr
	297	Asn	Asn	Asn	Asn
	312	Asp	Asp	Asp	Asp
	324	Ser	Ser	Ser	Ser
	330	Ala	Ala	Ala	Ser
	335	Thr	Thr	Thr	Thr
	337	Ser	Ser	Ser	Ser
	339	Ala	Thr	Thr	Ala
	356	Glu	Glu	Glu	Glu
	359	Thr	Thr	Thr	Thr
	361	Asn	Asn	Asn	Asn
	383	Ser	Ser	Ser	Ser

	384	Asn	Asn	Ser	Asn
	398	Leu	Leu	Leu	Leu
	400	Ser	Ser	Ser	Ser
	422	Val	Val	Ile	Val
	440	Ser	Ser	Ser	Ser
	442	Ser	Ser	Ser	Ser

Examples of modified antibodies suitable for use in the present invention include the Flexmab structures disclosed in WO 2012/064733, which is incorporated herein. Such Flexmabs have cysteines with free thiol groups in the hinge region of the antibody that may be used as conjugation sites for linking through the N10 groups of the PBDs of the present invention.

Other examples of modified antibodies suitable for use in the present invention include those where cysteines have been inserted in selected sites in antibodies. These are described in Dimasi, N., et al., Molecular Pharmaceutics, 2017, 14, 1501-1516 (DOI: 10.1021/acs.molpharmaceut.6b00995) and WO2015/157595. In particular, antibodies which have been modified by insertion of a cysteine after the S239 position (ie. between positions 239 and 240) are of use.

Reference is made to the listed on pages 60 to 62 of WO 2012/064733, which is incorporated herein. In some embodiments, the antibody may be to a tumour-associated antigen, for example: HER2 (ErbB2); EPHA2 (EPH receptor A2); CD19; IL2RA (Interleukin 2 receptor, alpha).

Tumour-associate antigens and cognate antibodies for use in embodiments of the present invention are listed below, and are described in more detail on pages 14 to 86 of WO 2017/186894, which is incorporated herein.

(1) BMPR1B (bone morphogenetic protein receptor-type IB)

(2) E16 (LAT1, SLC7A5)

(3) STEAP1 (six transmembrane epithelial antigen of prostate)

(4) 0772P (CA125, MUC16)

(5) MPF (MPF, MSLN, SMR, megakaryocyte potentiating factor, mesothelin)

(6) Napi3b (NAPI-3B, NPTIIB, SLC34A2, solute carrier family 34 (sodium phosphate), member 2, type II sodium-dependent phosphate transporter 3b)

(7) Sema 5b (FLJ10372, KIAA1445, Mm.42015, SEMA5B, SEMAG, Semaphorin 5b Hlog,

- 25 sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B)
- (8) PSCA hlg (2700050C12Rik, C530008O16Rik, RIKEN cDNA 2700050C12, RIKEN cDNA 2700050C12 gene)
- 5 (9) ETBR (Endothelin type B receptor)
- (10) MSG783 (RNF124, hypothetical protein FLJ20315)
- (11) STEAP2 (HGNC_8639, IPCA-1, PCANAP1, STAMP1, STEAP2, STMP, prostate cancer associated gene 1, prostate cancer associated protein 1, six transmembrane epithelial antigen of prostate 2, six transmembrane prostate protein)
- 10 (12) TrpM4 (BR22450, FLJ20041, TRPM4, TRPM4B, transient receptor potential cation 5 channel, subfamily M, member 4)
- (13) CRIPTO (CR, CR1, CRGF, CRIPTO, TDGF1, teratocarcinoma-derived growth factor)
- (14) CD21 (CR2 (Complement receptor 2) or C3DR (C3d/Epstein Barr virus receptor) or Hs.73792)
- 15 (15) CD79b (CD79B, CD79 β , Ig β (immunoglobulin-associated beta), B29)
- (16) FcRH2 (IFGP4, IRTA4, SPAP1A (SH2 domain containing phosphatase anchor protein 1a), SPAP1B, SPAP1C)
- (17) HER2 (ErbB2)
- (18) NCA (CEACAM6)
- 20 (19) MDP (DPEP1)
- (20) IL20R-alpha (IL20Ra, ZCYTOR7)
- (21) Brevican (BCAN, BEHAB)
- (22) EphB2R (DRT, ERK, Hek5, EPHT3, Tyro5)
- (23) ASLG659 (B7h)
- 25 (24) PSCA (Prostate stem cell antigen precursor)
- (25) GEDA
- (26) BAFF-R (B cell -activating factor receptor, BLyS receptor 3, BR3)
- (27) CD22 (B-cell receptor CD22-B isoform, BL-CAM, Lyb-8, Lyb8, SIGLEC-2, FLJ22814)
- (27a) CD22 (CD22 molecule)
- 30 (28) CD79a (CD79A, CD79alpha), immunoglobulin-associated alpha, a B cell-specific protein that covalently interacts with Ig beta (CD79B) and forms a complex on the surface with Ig M molecules, transduces a signal involved in B-cell differentiation), pI: 4.84, MW: 25028 TM: 2 [P] Gene Chromosome: 19q13.2).
- (29) CXCR5 (Burkitt's lymphoma receptor 1, a G protein-coupled receptor that is activated
- 35 by the CXCL13 chemokine, functions in lymphocyte migration and humoral defense, plays a role in HIV-2 infection and perhaps development of AIDS, lymphoma, myeloma, and

- leukemia); 372 aa, pl: 8.54 MW: 41959 TM: 7 [P] Gene Chromosome: 11q23.3,
- (30) HLA-DOB (Beta subunit of MHC class II molecule (Ia antigen) that binds peptides and 20 presents them to CD4+ T lymphocytes); 273 aa, pl: 6.56, MW: 30820.TM: 1 [P] Gene Chromosome: 6p21.3)
- 5 (31) P2X5 (Purinergic receptor P2X ligand-gated ion channel 5, an ion channel gated by extracellular ATP, may be involved in synaptic transmission and neurogenesis, deficiency may contribute to the pathophysiology of idiopathic detrusor instability); 422 aa, pl: 7.63, MW: 47206 TM: 1 [P] Gene Chromosome: 17p13.3).
- (32) CD72 (B-cell differentiation antigen CD72, Lyb-2); 359 aa, pl: 8.66, MW: 40225, TM: 1 10 5 [P] Gene Chromosome: 9p13.3).
- (33) LY64 (Lymphocyte antigen 64 (RP105), type I membrane protein of the leucine rich repeat (LRR) family, regulates B-cell activation and apoptosis, loss of function is associated with increased disease activity in patients with systemic lupus erythematosus); 661 aa, pl: 6.20, MW: 74147 TM: 1 [P] Gene Chromosome: 5q12).
- 15 (34) FcRH1 (Fc receptor-like protein 1, a putative receptor for the immunoglobulin Fc domain that contains C2 type Ig-like and ITAM domains, may have a role in B-lymphocyte differentiation); 429 aa, pl: 5.28, MW: 46925 TM: 1 [P] Gene Chromosome: 1q21-1q22)
- (35) IRTA2 (Immunoglobulin superfamily receptor translocation associated 2, a putative immunoreceptor with possible roles in B cell development and lymphomagenesis; 20 deregulation of the gene by translocation occurs in some B cell malignancies); 977 aa, pl: 6.88, MW: 106468, TM: 1 [P] Gene Chromosome: 1q21)
- (36) TENB2 (TMEFF2, tomoregulin, TPEF, HPP1, TR, putative transmembrane proteoglycan, related to the EGF/heregulin family of growth factors and follistatin); 374 aa)
- (37) PSMA – FOLH1 (Folate hydrolase (prostate-specific membrane antigen) 1)
- 25 (38) SST (Somatostatin Receptor; note that there are 5 subtypes)
- (38.1) SSTR2 (Somatostatin receptor 2)
- (38.2) SSTR5 (Somatostatin receptor 5)
- (38.3) SSTR1
- (38.4) SSTR3
- 30 (38.5) SSTR4
- AvB6 – Both subunits (39+40)**
- (39) ITGAV (Integrin, alpha V)
- (40) ITGB6 (Integrin, beta 6)
- (41) CEACAM5 (Carcinoembryonic antigen-related cell adhesion molecule 5)
- 35 (42) MET (met proto-oncogene; hepatocyte growth factor receptor)
- (43) MUC1 (Mucin 1, cell surface associated)

- (44) CA9 (Carbonic anhydrase IX)
- (45) EGFRvIII (Epidermal growth factor receptor (EGFR), transcript variant 3,
- (46) CD33 (CD33 molecule)
- (47) CD19 (CD19 molecule)
- 5 (48) IL2RA (Interleukin 2 receptor, alpha); NCBI Reference Sequence: NM_000417.2);
- (49) AXL (AXL receptor tyrosine kinase)
- (50) CD30 - TNFRSF8 (Tumor necrosis factor receptor superfamily, member 8)
- (51) BCMA (B-cell maturation antigen) - TNFRSF17 (Tumor necrosis factor receptor superfamily, member 17)
- 10 (52) CT Ags – CTA (Cancer Testis Antigens)
- (53) CD174 (Lewis Y) - FUT3 (fucosyltransferase 3 (galactoside 3(4)-L-fucosyltransferase, Lewis blood group)
- (54) CLEC14A (C-type lectin domain family 14, member A; Genbank accession no. NM175060)
- 15 (55) GRP78 – HSPA5 (heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)
- (56) CD70 (CD70 molecule) L08096
- (57) Stem Cell specific antigens. For example:
- 5T4 (see entry (63) below)
 - CD25 (see entry (48) above)
- 20 • CD32
- LGR5/GPR49
 - Prominin/CD133
- (58) ASG-5
- (59) ENPP3 (Ectonucleotide pyrophosphatase/phosphodiesterase 3)
- 25 (60) PRR4 (Proline rich 4 (lacrimal))
- (61) GCC – GUCY2C (guanylate cyclase 2C (heat stable enterotoxin receptor)
- (62) Liv-1 – SLC39A6 (Solute carrier family 39 (zinc transporter), member 6)
- (63) 5T4, Trophoblast glycoprotein, TPBG – TPBG (trophoblast glycoprotein)
- (64) CD56 – NCMA1 (Neural cell adhesion molecule 1)
- 30 (65) CanAg (Tumor associated antigen CA242)
- (66) FOLR1 (Folate Receptor 1)
- (67) GPNMB (Glycoprotein (transmembrane) nmb)
- (68) TIM-1 – HAVCR1 (Hepatitis A virus cellular receptor 1)
- (69) RG-1/Prostate tumor target Mindin – Mindin/RG-1
- 35 (70) B7-H4 – VTCN1 (V-set domain containing T cell activation inhibitor 1)
- (71) PTK7 (PTK7 protein tyrosine kinase 7)

- (72) CD37 (CD37 molecule)
- (73) CD138 – SDC1 (syndecan 1)
- (74) CD74 (CD74 molecule, major histocompatibility complex, class II invariant chain)
- (75) Claudins – CLs (Claudins)
- 5 (76) EGFR (Epidermal growth factor receptor)
- (77) Her3 (ErbB3) – ERBB3 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian))
- (78) RON - MST1R (macrophage stimulating 1 receptor (c-met-related tyrosine kinase))
- (79) EPHA2 (EPH receptor A2)
- 10 (80) CD20 – MS4A1 (membrane-spanning 4-domains, subfamily A, member 1)
- (81) Tenascin C – TNC (Tenascin C)
- (82) FAP (Fibroblast activation protein, alpha)
- (83) DKK-1 (Dickkopf 1 homolog (*Xenopus laevis*))
- (84) CD52 (CD52 molecule)
- 15 (85) CS1 - SLAMF7 (SLAM family member 7)
- (86) Endoglin – ENG (Endoglin)
- (87) Annexin A1 – ANXA1 (Annexin A1)
- (88) V-CAM (CD106) - VCAM1 (Vascular cell adhesion molecule 1)

20 *Connection of Linker unit to Ligand unit*

The Ligand unit may be connected to the Linker unit through a disulfide bond.

- In one embodiment, the connection between the Ligand unit and the Drug Linker is formed between a thiol group of a cysteine residue of the Ligand unit and a maleimide group of the
- 25 Drug Linker unit. Other possible groups for linking, and the resulting linking groups, are shown below.

- The cysteine residues of the Ligand unit may be available for reaction with the functional group of the Linker unit to form a connection. In other embodiments, for example where
- 30 the Ligand unit is an antibody, the thiol groups of the antibody may participate in interchain disulfide bonds. These interchain bonds may be converted to free thiol groups by e.g. treatment of the antibody with DTT prior to reaction with the functional group of the Linker unit.

In some embodiments, the cysteine residue is introduced into the heavy or light chain of an antibody. Positions for cysteine insertion by substitution in antibody heavy or light chains include those described in Published U.S. Application No. 2007-0092940 and International Patent Publication WO2008/070593, which are incorporated herein.

5

Methods of Treatment

The compounds of the present invention may be used in a method of therapy. Also provided is a method of treatment, comprising administering to a subject in need of treatment a therapeutically-effective amount of a conjugate of formula I. The term
10 “therapeutically effective amount” is an amount sufficient to show benefit to a patient. Such benefit may be at least amelioration of at least one symptom. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage, is within the responsibility of general practitioners and other medical doctors.

15

A conjugate may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated. Examples of treatments and therapies include, but are not limited to, chemotherapy (the administration of active agents, including, e.g. drugs; surgery; and radiation therapy.

20

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to the active ingredient, i.e. a conjugate of formula I, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic
25 and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous, or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or
30 liquid form. A tablet may comprise a solid carrier or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included. A capsule may comprise a solid carrier such as a gelatin.

35

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as

5 Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

The Conjugates can be used to treat proliferative disease and autoimmune disease. The term "proliferative disease" pertains to an unwanted or uncontrolled cellular proliferation of

10 excessive or abnormal cells which is undesired, such as, neoplastic or hyperplastic growth, whether *in vitro* or *in vivo*.

Examples of proliferative conditions include, but are not limited to, benign, pre-malignant, and malignant cellular proliferation, including but not limited to, neoplasms and tumours

15 (e.g., histiocytoma, glioma, astrocyoma, osteoma), cancers (e.g. lung cancer, small cell lung cancer, gastrointestinal cancer, bowel cancer, colon cancer, breast carcinoma, ovarian carcinoma, prostate cancer, testicular cancer, liver cancer, kidney cancer, bladder cancer, pancreatic cancer, brain cancer, sarcoma, osteosarcoma, Kaposi's sarcoma, melanoma), leukemias, psoriasis, bone diseases, fibroproliferative disorders (e.g. of connective

20 tissues), and atherosclerosis. Other cancers of interest include, but are not limited to, haematological; malignancies such as leukemias and lymphomas, such as non-Hodgkin lymphoma, and subtypes such as DLBCL, marginal zone, mantle zone, and follicular, Hodgkin lymphoma, AML, and other cancers of B or T cell origin.

25 Examples of autoimmune disease include the following: rheumatoid arthritis, autoimmune demyelinating diseases (e.g., multiple sclerosis, allergic encephalomyelitis), psoriatic arthritis, endocrine ophthalmopathy, uveoretinitis, systemic lupus erythematosus, myasthenia gravis, Graves' disease, glomerulonephritis, autoimmune hepatological disorder, inflammatory bowel disease (e.g., Crohn's disease), anaphylaxis, allergic

30 reaction, Sjögren's syndrome, type I diabetes mellitus, primary biliary cirrhosis, Wegener's granulomatosis, fibromyalgia, polymyositis, dermatomyositis, multiple endocrine failure, Schmidt's syndrome, autoimmune uveitis, Addison's disease, adrenalitis, thyroiditis, Hashimoto's thyroiditis, autoimmune thyroid disease, pernicious anemia, gastric atrophy, chronic hepatitis, lupoid hepatitis, atherosclerosis, subacute cutaneous lupus

35 erythematosus, hypoparathyroidism, Dressler's syndrome, autoimmune thrombocytopenia, idiopathic thrombocytopenic purpura, hemolytic anemia, pemphigus vulgaris, pemphigus,

dermatitis herpetiformis, alopecia arcata, pemphigoid, scleroderma, progressive systemic sclerosis, CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia), male and female autoimmune infertility, ankylosing spondylitis, ulcerative colitis, mixed connective tissue disease, polyarteritis nodosa,

5 systemic necrotizing vasculitis, atopic dermatitis, atopic rhinitis, Goodpasture's syndrome, Chagas' disease, sarcoidosis, rheumatic fever, asthma, recurrent abortion, anti-phospholipid syndrome, farmer's lung, erythema multiforme, post cardiectomy syndrome, Cushing's syndrome, autoimmune chronic active hepatitis, bird-fancier's lung, toxic epidermal necrolysis, Alport's syndrome, alveolitis, allergic alveolitis, fibrosing alveolitis,

10 interstitial lung disease, erythema nodosum, pyoderma gangrenosum, transfusion reaction, Takayasu's arteritis, polymyalgia rheumatica, temporal arteritis, schistosomiasis, giant cell arteritis, ascariasis, aspergillosis, Sampter's syndrome, eczema, lymphomatoid granulomatosis, Behcet's disease, Caplan's syndrome, Kawasaki's disease, dengue, encephalomyelitis, endocarditis, endomyocardial fibrosis, endophthalmitis, erythema

15 elevatum et diutinum, psoriasis, erythroblastosis fetalis, eosinophilic fasciitis, Shulman's syndrome, Felty's syndrome, filariasis, cyclitis, chronic cyclitis, heterochronic cyclitis, Fuch's cyclitis, IgA nephropathy, Henoch-Schönlein purpura, graft versus host disease, transplantation rejection, cardiomyopathy, Eaton-Lambert syndrome, relapsing polychondritis, cryoglobulinemia, Waldenström's macroglobulemia, Evan's syndrome, and

20 autoimmune gonadal failure.

In some embodiments, the autoimmune disease is a disorder of B lymphocytes (e.g., systemic lupus erythematosus, Goodpasture's syndrome, rheumatoid arthritis, and type I diabetes), Th1-lymphocytes (e.g., rheumatoid arthritis, multiple sclerosis, psoriasis,

25 Sjögren's syndrome, Hashimoto's thyroiditis, Graves' disease, primary biliary cirrhosis, Wegener's granulomatosis, tuberculosis, or graft versus host disease), or Th2-lymphocytes (e.g., atopic dermatitis, systemic lupus erythematosus, atopic asthma, rhinoconjunctivitis, allergic rhinitis, Omenn's syndrome, systemic sclerosis, or chronic graft versus host disease). Generally, disorders involving dendritic cells involve disorders of Th1-

30 lymphocytes or Th2-lymphocytes. In some embodiments, the autoimmune disorder is a T cell-mediated immunological disorder.

In some embodiments, the amount of the Conjugate administered ranges from about 0.01 to about 10 mg/kg per dose. In some embodiments, the amount of the Conjugate

35 administered ranges from about 0.01 to about 5 mg/kg per dose. In some embodiments, the amount of the Conjugate administered ranges from about 0.05 to about 5 mg/kg per

dose. In some embodiments, the amount of the Conjugate administered ranges from about 0.1 to about 5 mg/kg per dose. In some embodiments, the amount of the Conjugate administered ranges from about 0.1 to about 4 mg/kg per dose. In some embodiments, the amount of the Conjugate administered ranges from about 0.05 to about 3 mg/kg per dose.

- 5 In some embodiments, the amount of the Conjugate administered ranges from about 0.1 to about 3 mg/kg per dose. In some embodiments, the amount of the Conjugate administered ranges from about 0.1 to about 2 mg/kg per dose.

Drug loading

- 10 The drug loading (p) is the average number of PBD drugs per cell binding agent, e.g. antibody. In the present invention, this is always 1. However, any composition may comprise antibodies where a PBD is conjugated and antibodies where a PBD is not conjugated. Thus for a composition, the drug loading (or DAR) may be less than 1, for example 0.75 and higher, 0.80 and higher, 0.85 and higher, 0.90 and higher or 0.95 or higher.
- 15 higher.

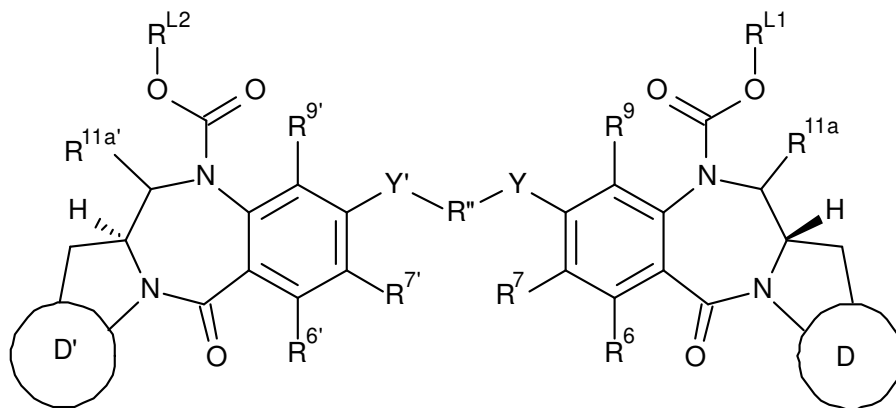
General synthetic routes

The synthesis of PBD compounds is extensively discussed in the following references, which discussions are incorporated herein by reference:

- 20 a) WO 00/12508 (pages 14 to 30);
 b) WO 2005/023814 (pages 3 to 10);
 c) WO 2004/043963 (pages 28 to 29); and
 d) WO 2005/085251 (pages 30 to 39).

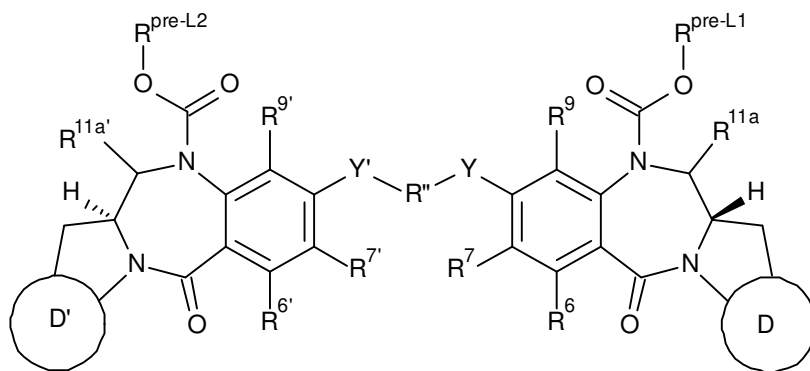
25 *Synthesis route*

Compounds of the present invention of formula I:



II

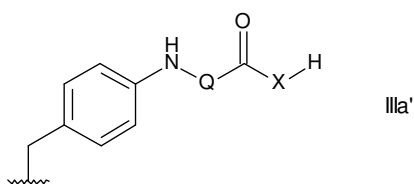
can be synthesised from a compound of Formula 2:



Formula 2

where R^2 , R^6 , R^7 , R^9 , R^{11a} , $R^{6'}$, $R^{7'}$, $R^{9'}$, $R^{11a'}$, Y , Y' and R'' are as defined for compounds of formula I, R^{pre-L1} is a precursor of R^{L1} and R^{pre-L2} is a precursor of R^{L2} – this method is

- 5 particularly applicable to compounds of formula I where R^{L1} and R^{L2} are of formula IIIa. For these compounds, R^{pre-L1} and R^{pre-L2} will typically be portions of R^{L1} and R^{L2} , such as a group of formula IIIa':

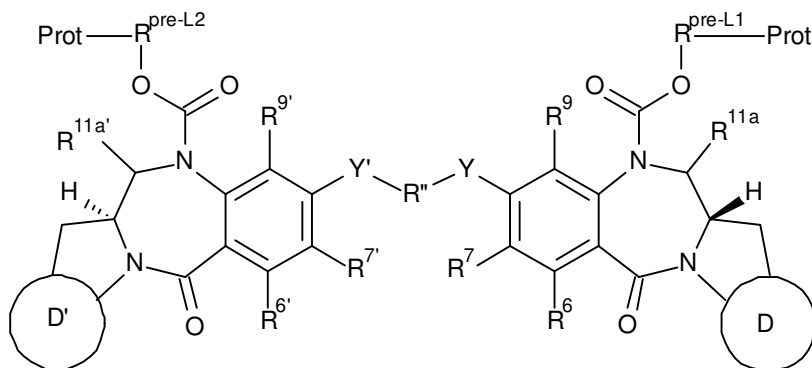


IIIa'

. In such as case, the reaction involves adding the group G^L .

10

The compounds of Formula 2 may be made by deprotecting compounds of Formula 3:

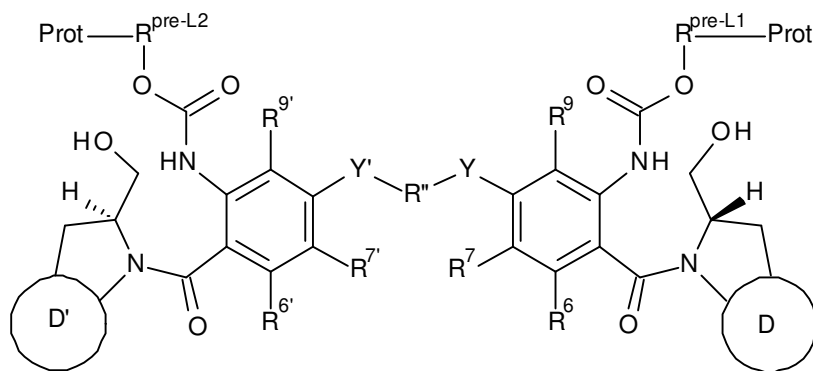


Formula 3

where R^2 , R^6 , R^7 , R^9 , R^{11a} , $R^{6'}$, $R^{7'}$, $R^{9'}$, $R^{11a'}$, Y , Y' and R'' are as defined for compounds of formula I, $R^{pre-L1Prot}$ is a protected version of R^{pre-L1} , $R^{pre-L2Prot}$ is a protected version of R^{pre-L2} and the Prot represents an appropriate carboxy/hydroxy protecting group.

15

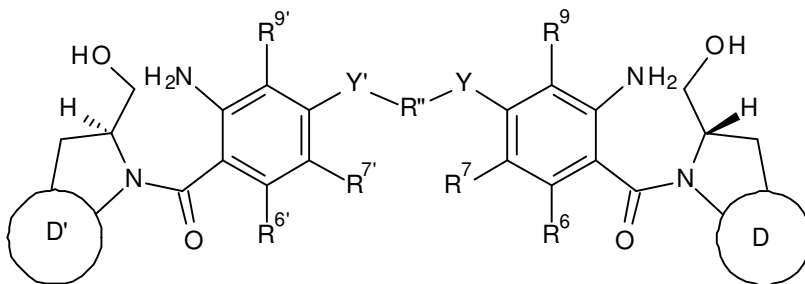
Compounds of formula 3 may be made by ring-closure of compounds of Formula 4:



Formula 4

where the ring closure is carried out by oxidation, e.g. Swern.

- 5 Compounds of formula 4 can be synthesised from compounds of formula 5:



Formula 5

- by addition of the two amino protecting groups. If the groups are different, step-wise addition can be achieved by simple protection of one amino group (e.g. by Fmoc), followed by installation of a desired protecting group at the other amino group. This can be followed by removal of the simple protecting group, and then installation of the other desired amino protecting group.

- Compounds of formula I where R^{L1} and R^{L2} are of formula IIIb, may be synthesised in a similar manner, although the complete R^{L1} and/or R^{L2} group may be installed starting from a compound of Formula 5, rather than with the use of a protected precursor.

Compounds of Formula 5 can be synthesised by known methods, such as those disclosed in WO 2011/130598.

20

Synthesis of Drug Conjugates

Antibodies can be conjugated to the Drug Linker compound generally as described in Doronina et al., Nature Biotechnology, 2003, 21, 778-784). Briefly, antibodies (4-5 mg/mL)

in PBS containing 50 mM sodium borate at pH 7.4 are reduced with tris(carboxyethyl)phosphine hydrochloride (TCEP) at 37 °C. The progress of the reaction, which reduces interchain disulfides, is monitored by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) and allowed to proceed until the desired level of thiols/mAb is achieved.

- 5 The reduced antibody is then cooled to 0°C and alkylated with 3 equivalents of drug-linker per antibody. After 1 hour, the reaction is quenched by the addition of 5 equivalents of N-acetyl cysteine. Quenched drug-linker is removed by gel filtration over a PD-10 column. The ADC is then sterile-filtered through a 0.22 µm syringe filter. Protein concentration can be determined by spectral analysis at 280 nm and 329 nm, respectively, with correction for
- 10 the contribution of drug absorbance at 280 nm. Size exclusion chromatography can be used to determine the extent of antibody aggregation, and RP-HPLC can be used to determine the levels of remaining NAC-quenched drug-linker.

Further Preferences

- 15 The following preferences may apply to all aspects of the invention as described above, or may relate to a single aspect. The preferences may be combined together in any combination.

- 20 $R^{6'}$, $R^{7'}$, $R^{9'}$, $R^{11a'}$ and Y' are selected from the same groups as R^6 , R^7 , R^9 , R^{11a} and Y respectively. In some embodiments, $R^{6'}$, $R^{7'}$, $R^{9'}$, $R^{11a'}$ and Y' are the same as R^6 , R^7 , R^9 , R^{11a} and Y respectively.

In some embodiments, R^{12} is the same as R^2 .

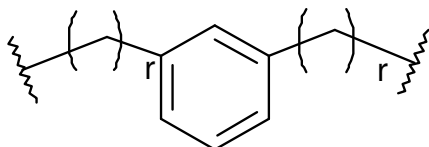
25 *Dimer link*

In some embodiments, Y and Y' are both O.

In some embodiments, R'' is a C_{3-7} alkylene group with no substituents. In some of these embodiments, R'' is a C_3 , C_5 or C_7 alkylene. In particular, R'' may be a C_3 or C_5 alkylene.

30

In other embodiments, R'' is a group of formula:



R⁶ to R⁹

In some embodiments, R⁹ is H.

- 5 In some embodiments, R⁶ is selected from H, OH, OR, SH, NH₂, nitro and halo, and may be selected from H or halo. In some of these embodiments R⁶ is H.

- In some embodiments, R⁷ is selected from H, OH, OR, SH, SR, NH₂, NHR, NRR', and halo. In some of these embodiments R⁷ is selected from H, OH and OR, where R is selected from optionally substituted C₁₋₇ alkyl, C₃₋₁₀ heterocyclyl and C₅₋₁₀ aryl groups. R may be more preferably a C₁₋₄ alkyl group, which may or may not be substituted. A substituent of interest is a C₅₋₆ aryl group (e.g. phenyl). Particularly preferred substituents at the 7- positions are OMe and OCH₂Ph. Other substituents of particular interest are dimethylamino (i.e. -NMe₂); -(OC₂H₄)_qOMe, where q is from 0 to 2; nitrogen-containing C₆ heterocyclyls, including morpholino, piperidinyl and N-methyl-piperazinyl.
- 10
15

These embodiments and preferences apply to R⁹, R⁶ and R⁷ respectively.

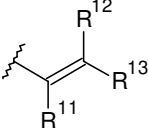
D and D'

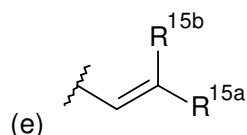
- 20 In some embodiments, D and D' are D1 and D'1 respectively.

In some embodiments, D and D' are D2 and D'2 respectively.

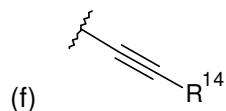
R²

- 25 When there is a double bond present between C2 and C3, R² is selected from:
- (a) C₅₋₁₀ aryl group, optionally substituted by one or more substituents selected from the group comprising: halo, nitro, cyano, ether, C₁₋₇ alkyl, C₃₋₇ heterocyclyl and bis-oxy-C₁₋₃ alkylene;
- (b) C₁₋₅ saturated aliphatic alkyl;
- 30 (c) C₃₋₆ saturated cycloalkyl;

- (d) , wherein each of R¹¹, R¹² and R¹³ are independently selected from H, C₁₋₃ saturated alkyl, C₂₋₃ alkenyl, C₂₋₃ alkynyl and cyclopropyl, where the total number of carbon atoms in the R² group is no more than 5;



phenyl, which phenyl is optionally substituted by a group selected from halo methyl, methoxy; pyridyl; and thiophenyl; and



5 alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo methyl, methoxy; pyridyl; and thiophenyl.

When R² is a C₅₋₁₀ aryl group, it may be a C₅₋₇ aryl group. A C₅₋₇ aryl group may be a phenyl group or a C₅₋₇ heteroaryl group, for example furanyl, thiophenyl and pyridyl. In
10 some embodiments, R² is preferably phenyl. In other embodiments, R² is preferably thiophenyl, for example, thiophen-2-yl and thiophen-3-yl.

When R² is a C₅₋₁₀ aryl group, it may be a C₈₋₁₀ aryl, for example a quinolinyl or isoquinolinyl group. The quinolinyl or isoquinolinyl group may be bound to the PBD core through any
15 available ring position. For example, the quinolinyl may be quinolin-2-yl, quinolin-3-yl, quinolin-4-yl, quinolin-5-yl, quinolin-6-yl, quinolin-7-yl and quinolin-8-yl. Of these quinolin-3-yl and quinolin-6-yl may be preferred. The isoquinolinyl may be isoquinolin-1-yl, isoquinolin-3-yl, isoquinolin-4-yl, isoquinolin-5-yl, isoquinolin-6-yl, isoquinolin-7-yl and isoquinolin-8-yl. Of these isoquinolin-3-yl and isoquinolin-6-yl may be preferred.

20

When R² is a C₅₋₁₀ aryl group, it may bear any number of substituent groups. It preferably bears from 1 to 3 substituent groups, with 1 and 2 being more preferred, and singly substituted groups being most preferred. The substituents may be any position.

25 Where R² is C₅₋₇ aryl group, a single substituent is preferably on a ring atom that is not adjacent the bond to the remainder of the compound, i.e. it is preferably β or γ to the bond to the remainder of the compound. Therefore, where the C₅₋₇ aryl group is phenyl, the substituent is preferably in the meta- or para- positions, and more preferably is in the para-position.

30

Where R² is a C₈₋₁₀ aryl group, for example quinolinyl or isoquinolinyl, it may bear any number of substituents at any position of the quinoline or isoquinoline rings. In some

embodiments, it bears one, two or three substituents, and these may be on either the proximal and distal rings or both (if more than one substituent).

R² substituents, when R² is a C₅₋₁₀ aryl group

- 5 If a substituent on R² when R² is a C₅₋₁₀ aryl group is halo, it is preferably F or Cl, more preferably Cl.

- 10 If a substituent on R² when R² is a C₅₋₁₀ aryl group is ether, it may in some embodiments be an alkoxy group, for example, a C₁₋₇ alkoxy group (e.g. methoxy, ethoxy) or it may in some embodiments be a C₅₋₇ aryloxy group (e.g. phenoxy, pyridyloxy, furanyloxy). The alkoxy group may itself be further substituted, for example by an amino group (e.g. dimethylamino).

- 15 If a substituent on R² when R² is a C₅₋₁₀ aryl group is C₁₋₇ alkyl, it may preferably be a C₁₋₄ alkyl group (e.g. methyl, ethyl, propyl, butyl).

- 20 If a substituent on R² when R² is a C₅₋₁₀ aryl group is C₃₋₇ heterocyclyl, it may in some embodiments be C₆ nitrogen containing heterocyclyl group, e.g. morpholino, thiomorpholino, piperidinyl, piperazinyl. These groups may be bound to the rest of the PBD moiety via the nitrogen atom. These groups may be further substituted, for example, by C₁₋₄ alkyl groups. If the C₆ nitrogen containing heterocyclyl group is piperazinyl, the said further substituent may be on the second nitrogen ring atom.

- 25 If a substituent on R² when R² is a C₅₋₁₀ aryl group is bis-oxy-C₁₋₃ alkylene, this is preferably bis-oxy-methylene or bis-oxy-ethylene.

- If a substituent on R² when R² is a C₅₋₁₀ aryl group is ester, this is preferably methyl ester or ethyl ester.

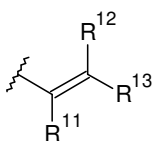
- 30 Particularly preferred substituents when R² is a C₅₋₁₀ aryl group include methoxy, ethoxy, fluoro, chloro, cyano, bis-oxy-methylene, methyl-piperazinyl, morpholino and methyl-thiophenyl. Other particularly preferred substituents for R² are dimethylaminopropoxy and carboxy.

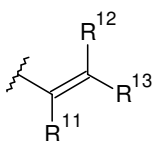
- 35 Particularly preferred substituted R² groups when R² is a C₅₋₁₀ aryl group include, but are not limited to, 4-methoxy-phenyl, 3-methoxyphenyl, 4-ethoxy-phenyl, 3-ethoxy-phenyl, 4-

fluoro-phenyl, 4-chloro-phenyl, 3,4-bisoxymethylene-phenyl, 4-methylthiophenyl, 4-cyanophenyl, 4-phenoxyphenyl, quinolin-3-yl and quinolin-6-yl, isoquinolin-3-yl and isoquinolin-6-yl, 2-thienyl, 2-furanyl, methoxynaphthyl, and naphthyl. Another possible substituted R^{12} group is 4-nitrophenyl. R^{12} groups of particular interest include 4-(4-methylpiperazin-1-yl)phenyl and 3,4-bisoxymethylene-phenyl.

When R^2 is C_{1-5} saturated aliphatic alkyl, it may be methyl, ethyl, propyl, butyl or pentyl. In some embodiments, it may be methyl, ethyl or propyl (n-pentyl or isopropyl). In some of these embodiments, it may be methyl. In other embodiments, it may be butyl or pentyl, which may be linear or branched.

When R^2 is C_{3-6} saturated cycloalkyl, it may be cyclopropyl, cyclobutyl, cyclopentyl or cyclohexyl. In some embodiments, it may be cyclopropyl.



When R^2 is , each of R^{11} , R^{12} and R^{13} are independently selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl, where the total number of carbon atoms in the R^2 group is no more than 5. In some embodiments, the total number of carbon atoms in the R^2 group is no more than 4 or no more than 3.

In some embodiments, one of R^{11} , R^{12} and R^{13} is H, with the other two groups being selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl.

In other embodiments, two of R^{11} , R^{12} and R^{13} are H, with the other group being selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl.

In some embodiments, the groups that are not H are selected from methyl and ethyl. In some of these embodiments, the groups that are not H are methyl.

In some embodiments, R^{11} is H.

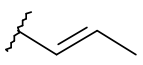
In some embodiments, R^{12} is H.

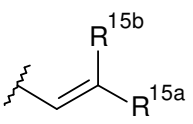
In some embodiments, R^{13} is H.

In some embodiments, R^{11} and R^{12} are H.

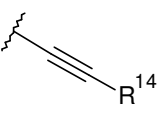
In some embodiments, R^{11} and R^{13} are H.

- 5 In some embodiments, R^{12} and R^{13} are H.

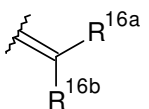
An R^2 group of particular interest is: .

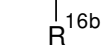
When R^2 is , one of R^{15a} and R^{15b} is H and the other is selected from:

- 10 phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl. In some embodiments, the group which is not H is optionally substituted phenyl. If the phenyl optional substituent is halo, it is preferably fluoro. In some embodiment, the phenyl group is unsubstituted.

- 15 When R^2 is , R^{14} is selected from: H; C_{1-3} saturated alkyl; C_{2-3} alkenyl; C_{2-3} alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo methyl, methoxy; pyridyl; and thiophenyl. If the phenyl optional substituent is halo, it is preferably fluoro. In some embodiment, the phenyl group is unsubstituted.
- In some embodiments, R^{14} is selected from H, methyl, ethyl, ethenyl and ethynyl. In some
- 20 of these embodiments, R^{14} is selected from H and methyl.

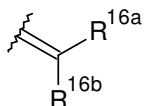
When there is a single bond present between C2 and C3,



R^2 is H or , where R^{16a} and R^{16b} are independently selected from H, F, C_{1-4} saturated alkyl, C_{2-3} alkenyl, which alkyl and alkenyl groups are optionally substituted by a

25 group selected from C_{1-4} alkyl amido and C_{1-4} alkyl ester; or, when one of R^{16a} and R^{16b} is H, the other is selected from nitrile and a C_{1-4} alkyl ester.

In some embodiments, R^2 is H.



In some embodiments, R^2 is

In some embodiments, it is preferred that R^{16a} and R^{16b} are both H.

- 5 In other embodiments, it is preferred that R^{16a} and R^{16b} are both methyl.

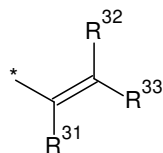
In further embodiments, it is preferred that one of R^{16a} and R^{16b} is H, and the other is selected from C_{1-4} saturated alkyl, C_{2-3} alkenyl, which alkyl and alkenyl groups are optionally substituted. In these further embodiment, it may be further preferred that the group which is not H is selected from methyl and ethyl.

10

R^{22}

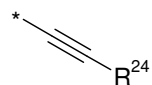
When there is a double bond present between $C2'$ and $C3'$, R^{22} is selected from:

- (a) C_{5-10} aryl group, optionally substituted by one or more substituents selected from the group comprising: halo, nitro, cyano, ether, C_{1-7} alkyl, C_{3-7} heterocyclyl and bis-oxy- C_{1-3} alkylene;
- 15 (b) C_{1-5} saturated aliphatic alkyl;
- (c) C_{3-6} saturated cycloalkyl;



- (d) , wherein each of R^{31} , R^{32} and R^{33} are independently selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl, where the total number of carbon atoms in the R^{22} group is no more than 5;
- 20

- (e) , wherein one of R^{25a} and R^{25b} is H and the other is selected from: phenyl, which phenyl is optionally substituted by a group selected from halo methyl, methoxy; pyridyl; and thiophenyl; and



- (f) , where R^{24} is selected from: H; C_{1-3} saturated alkyl; C_{2-3} alkenyl; C_{2-3} alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo methyl, methoxy; pyridyl; and thiophenyl.
- 25

When R^{22} is a C_{5-10} aryl group, it may be a C_{5-7} aryl group. A C_{5-7} aryl group may be a phenyl group or a C_{5-7} heteroaryl group, for example furanyl, thiophenyl and pyridyl. In some embodiments, R^{22} is preferably phenyl. In other embodiments, R^{22} is preferably thiophenyl, for example, thiophen-2-yl and thiophen-3-yl.

5

When R^{22} is a C_{5-10} aryl group, it may be a C_{8-10} aryl, for example a quinolinyl or isoquinolinyl group. The quinolinyl or isoquinolinyl group may be bound to the PBD core through any available ring position. For example, the quinolinyl may be quinolin-2-yl, quinolin-3-yl, quinolin-4-yl, quinolin-5-yl, quinolin-6-yl, quinolin-7-yl and quinolin-8-yl. Of these quinolin-3-yl and quinolin-6-yl may be preferred. The isoquinolinyl may be isoquinolin-1-yl, isoquinolin-3-yl, isoquinolin-4-yl, isoquinolin-5-yl, isoquinolin-6-yl, isoquinolin-7-yl and isoquinolin-8-yl. Of these isoquinolin-3-yl and isoquinolin-6-yl may be preferred.

10

15 When R^{22} is a C_{5-10} aryl group, it may bear any number of substituent groups. It preferably bears from 1 to 3 substituent groups, with 1 and 2 being more preferred, and singly substituted groups being most preferred. The substituents may be any position.

20

Where R^{22} is C_{5-7} aryl group, a single substituent is preferably on a ring atom that is not adjacent the bond to the remainder of the compound, i.e. it is preferably β or γ to the bond to the remainder of the compound. Therefore, where the C_{5-7} aryl group is phenyl, the substituent is preferably in the meta- or para- positions, and more preferably is in the para-position.

25

Where R^{22} is a C_{8-10} aryl group, for example quinolinyl or isoquinolinyl, it may bear any number of substituents at any position of the quinoline or isoquinoline rings. In some embodiments, it bears one, two or three substituents, and these may be on either the proximal and distal rings or both (if more than one substituent).

30 *R^{22} substituents, when R^{22} is a C_{5-10} aryl group*

If a substituent on R^{22} when R^{22} is a C_{5-10} aryl group is halo, it is preferably F or Cl, more preferably Cl.

35

If a substituent on R^{22} when R^{22} is a C_{5-10} aryl group is ether, it may in some embodiments be an alkoxy group, for example, a C_{1-7} alkoxy group (e.g. methoxy, ethoxy) or it may in some embodiments be a C_{5-7} aryloxy group (e.g. phenoxy, pyridyloxy, furanyloxy). The

alkoxy group may itself be further substituted, for example by an amino group (e.g. dimethylamino).

5 If a substituent on R^{22} when R^{22} is a C_{5-10} aryl group is C_{1-7} alkyl, it may preferably be a C_{1-4} alkyl group (e.g. methyl, ethyl, propyl, butyl).

10 If a substituent on R^{22} when R^{22} is a C_{5-10} aryl group is C_{3-7} heterocyclyl, it may in some embodiments be C_6 nitrogen containing heterocyclyl group, e.g. morpholino, thiomorpholino, piperidinyl, piperazinyl. These groups may be bound to the rest of the PBD moiety via the nitrogen atom. These groups may be further substituted, for example, by C_{1-4} alkyl groups. If the C_6 nitrogen containing heterocyclyl group is piperazinyl, the said further substituent may be on the second nitrogen ring atom.

15 If a substituent on R^{22} when R^{22} is a C_{5-10} aryl group is bis-oxy- C_{1-3} alkylene, this is preferably bis-oxy-methylene or bis-oxy-ethylene.

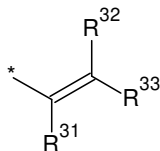
If a substituent on R^{22} when R^{22} is a C_{5-10} aryl group is ester, this is preferably methyl ester or ethyl ester.

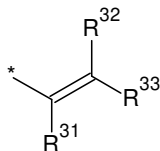
20 Particularly preferred substituents when R^{22} is a C_{5-10} aryl group include methoxy, ethoxy, fluoro, chloro, cyano, bis-oxy-methylene, methyl-piperazinyl, morpholino and methyl-thiophenyl. Other particularly preferred substituents for R^{22} are dimethylaminopropoxy and carboxy.

25 Particularly preferred substituted R^{22} groups when R^{22} is a C_{5-10} aryl group include, but are not limited to, 4-methoxy-phenyl, 3-methoxyphenyl, 4-ethoxy-phenyl, 3-ethoxy-phenyl, 4-fluoro-phenyl, 4-chloro-phenyl, 3,4-bisoxymethylene-phenyl, 4-methylthiophenyl, 4-cyanophenyl, 4-phenoxyphenyl, quinolin-3-yl and quinolin-6-yl, isoquinolin-3-yl and isoquinolin-6-yl, 2-thienyl, 2-furanyl, methoxynaphthyl, and naphthyl. Another possible
30 substituted R^{22} group is 4-nitrophenyl. R^{22} groups of particular interest include 4-(4-methylpiperazin-1-yl)phenyl and 3,4-bisoxymethylene-phenyl.

35 When R^{22} is C_{1-5} saturated aliphatic alkyl, it may be methyl, ethyl, propyl, butyl or pentyl. In some embodiments, it may be methyl, ethyl or propyl (n-pentyl or isopropyl). In some of these embodiments, it may be methyl. In other embodiments, it may be butyl or pentyl, which may be linear or branched.

When R^{22} is C_{3-6} saturated cycloalkyl, it may be cyclopropyl, cyclobutyl, cyclopentyl or cyclohexyl. In some embodiments, it may be cyclopropyl.



- 5 When R^{22} is , each of R^{31} , R^{32} and R^{33} are independently selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl, where the total number of carbon atoms in the R^{22} group is no more than 5. In some embodiments, the total number of carbon atoms in the R^{22} group is no more than 4 or no more than 3.

- 10 In some embodiments, one of R^{31} , R^{32} and R^{33} is H, with the other two groups being selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl.

In other embodiments, two of R^{31} , R^{32} and R^{33} are H, with the other group being selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl.

15

In some embodiments, the groups that are not H are selected from methyl and ethyl. In some of these embodiments, the groups that are not H are methyl.

In some embodiments, R^{31} is H.

20

In some embodiments, R^{32} is H.

In some embodiments, R^{33} is H.

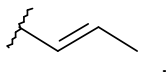
- 25 In some embodiments, R^{31} and R^{32} are H.

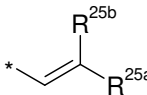
In some embodiments, R^{31} and R^{33} are H.

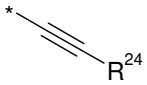
In some embodiments, R^{32} and R^{33} are H.

30

An R^{22} group of particular interest is:

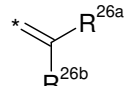


When R^{22} is , one of R^{25a} and R^{25b} is H and the other is selected from: phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl. In some embodiments, the group which is not H is optionally substituted phenyl. If the phenyl optional substituent is halo, it is preferably fluoro. In some embodiment, the phenyl group is unsubstituted.

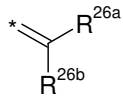
When R^{22} is , R^{24} is selected from: H; C_{1-3} saturated alkyl; C_{2-3} alkenyl; C_{2-3} alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo methyl, methoxy; pyridyl; and thiophenyl. If the phenyl optional substituent is halo, it is preferably fluoro. In some embodiment, the phenyl group is unsubstituted.

In some embodiments, R^{24} is selected from H, methyl, ethyl, ethenyl and ethynyl. In some of these embodiments, R^{24} is selected from H and methyl.

When there is a single bond present between $C2'$ and $C3'$,

R^{22} is H or , where R^{26a} and R^{26b} are independently selected from H, F, C_{1-4} saturated alkyl, C_{2-3} alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C_{1-4} alkyl amido and C_{1-4} alkyl ester; or, when one of R^{26a} and R^{26b} is H, the other is selected from nitrile and a C_{1-4} alkyl ester.

In some embodiments, R^{22} is H.

In some embodiments, R^{22} is .

In some embodiments, it is preferred that R^{26a} and R^{26b} are both H.

In other embodiments, it is preferred that R^{26a} and R^{26b} are both methyl.

In further embodiments, it is preferred that one of R^{26a} and R^{26b} is H, and the other is selected from C_{1-4} saturated alkyl, C_{2-3} alkenyl, which alkyl and alkenyl groups are

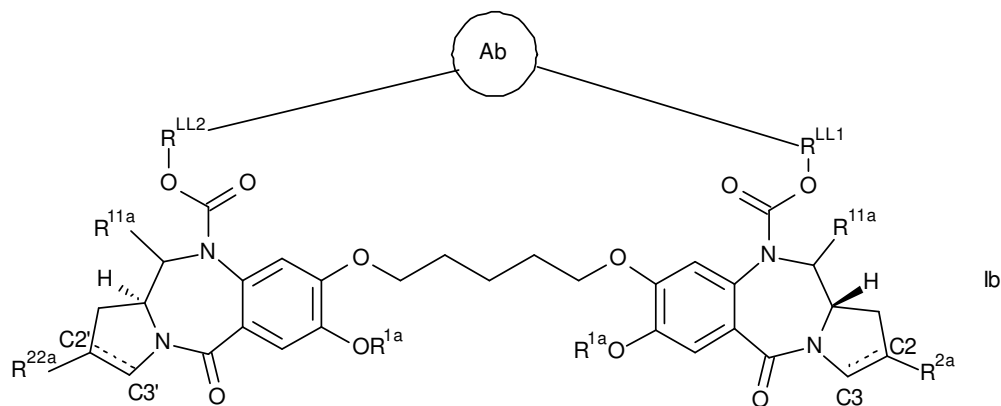
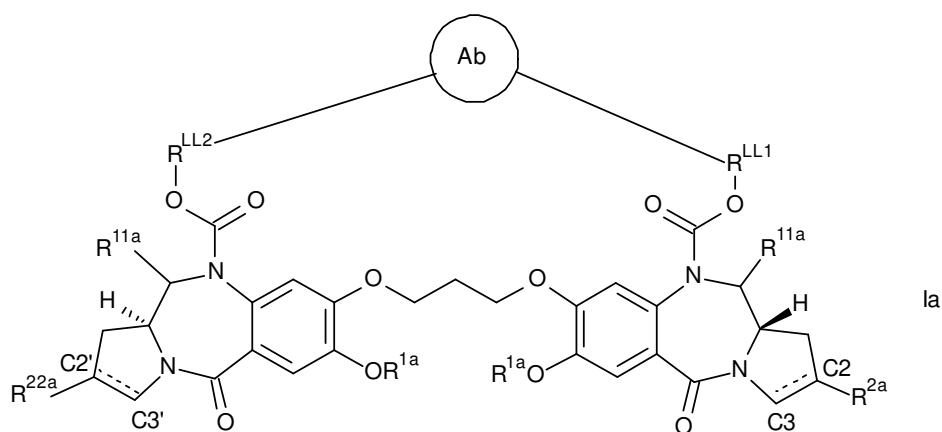
optionally substituted. In these further embodiment, it may be further preferred that the group which is not H is selected from methyl and ethyl.

R^{11}

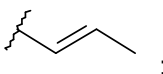
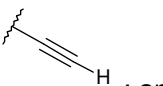
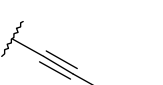
- 5 In some embodiments, R^{11a} is OH.

In some embodiments, R^{11a} is OR^A , where R^A is C_{1-4} alkyl. In some of these embodiments, R^A is methyl.

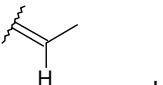
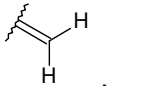
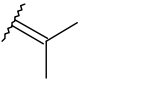
- 10 In some embodiments of the first aspect of the present invention are of formula Ia-1, Ia-2 or Ia-3:



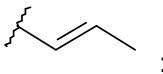
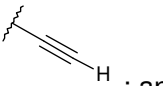
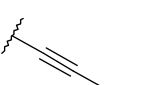
57

- (i) Methyl; (v)  ;
- (ii) Ethyl; (vi)  H ; and
- (iii) Propyl; (vi)  ;
- (iv) Cyclopropyl;

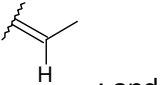
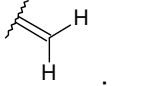
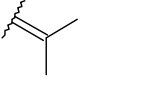
Thus in these embodiments where there is no double bond present between C2 and C3, R² may be selected from:

- (i) H; (iii)  ; and
- (ii)  ;
- (iv)  .

- 5 Thus in these embodiments where there is a double bond present between C2' and C3', R²² may be selected from:

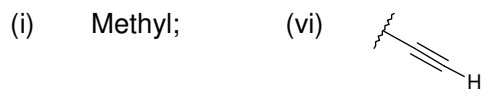
- (i) Methyl; (v)  ;
- (ii) Ethyl; (vi)  H ; and
- (iii) Propyl; (vi)  ;
- (iv) Cyclopropyl;

Thus in these embodiments where there is no double bond present between C2' and C3', R²² may be selected from:

- (i) H; (iii)  ; and
- (ii)  ;
- (iv)  .

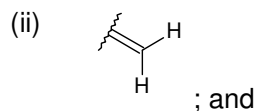
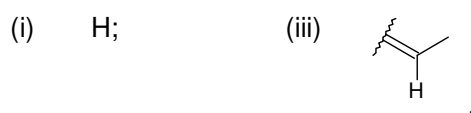
In some of these embodiments both R^2 and R^{22} comprise no more than 2 carbon atoms.

Thus in these embodiments where there is a double bond present between C2 and C3, R^2 may be selected from:



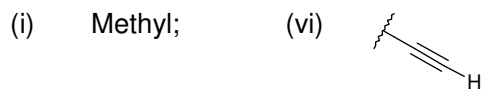
5

Thus in these embodiments where there is no double bond present between C2 and C3, R^2 may be selected from:

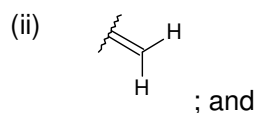


Thus in these embodiments where there is a double bond present between C2' and C3',

10 R^{22} may be selected from:



Thus in these embodiments where there is no double bond present between C2' and C3', R^{22} may be selected from:



15 In further of these embodiments both R^2 and R^{22} comprise no more than 1 carbon atom.

Thus in these embodiments where there is a double bond present between C2 and C3, R^2 may be methyl. Thus in these embodiments where there is no double bond present between C2 and C3, R^2 may be selected from:



Thus in these embodiments where there is a double bond present between C2' and C3', R²² may be methyl. Thus in these embodiments where there is no double bond present between C2' and C3', R²² may be selected from:



5

Without wishing to be bound by theory, where the substituent at the C2 position of the PBD dimers are small, the use of the glucuronide capping unit in these drug linkers is believed to be particularly advantageous, as it significantly increases the hydrophilicity of the drug linker, making the drug linkers easier to conjugate to a ligand unit.

10

These embodiments and preferences also apply to the second aspect of the invention.

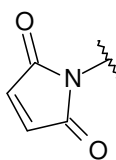
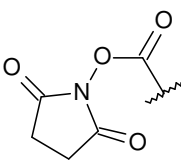
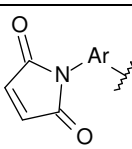
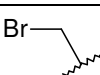
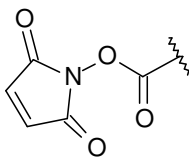
Linker (R^L/R^{LL})

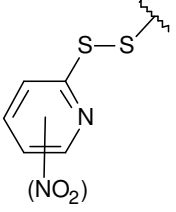
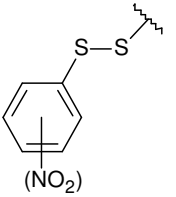
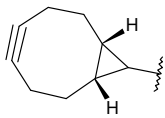
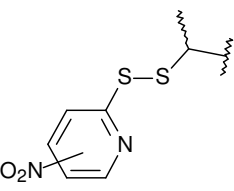
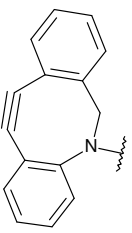
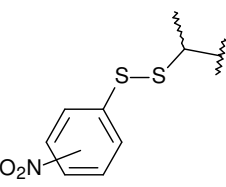
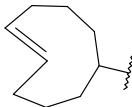
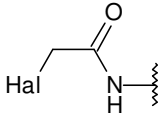
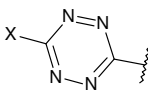
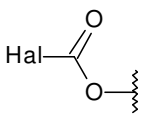
In some embodiments, R^{LL1} and R^{LL2} are of formula IIIa'.

15 In some embodiments, R^{L1} and R^{L2} are of formula IIIa.

G^L

G^L may be selected from

(G ^{L1-1})		(G ^{L6})	
(G ^{L1-2})		(G ^{L7})	
(G ^{L2})		(G ^{L8})	

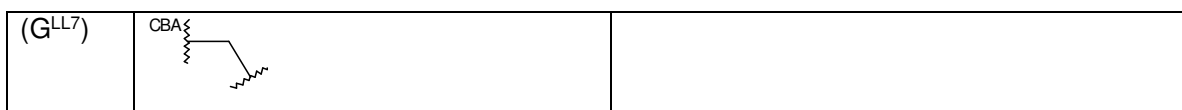
(G ^{L3-1})	 where the NO₂ group is optional	(G ^{L9})	
(G ^{L3-2})	 where the NO₂ group is optional	(G ^{L10})	
(G ^{L3-3})	 where the NO₂ group is optional	(G ^{L11})	
(G ^{L3-4})	 where the NO₂ group is optional	(G ^{L12})	
(G ^{L4})	 Where Hal = I, Br, Cl	(G ^{L13})	
(G ^{L5})			

where Ar represents a C₅₋₆ arylene group, e.g. phenylene, and X represents C¹⁻⁴ alkyl

In some embodiments, G^L is selected from G^{L1-1} and G^{L1-2}. In some of these embodiments, G^L is G^{L1-1}.

G^{LL} may be selected from:

(G ^{LL1-1})		(G ^{LL8-1})	
(G ^{LL1-2})		(G ^{LL8-2})	
(G ^{LL2})		(G ^{LL9-1})	
(G ^{LL3-1})		(G ^{LL9-2})	
(G ^{LL3-2})		G ^{L10}	
(G ^{LL-4})		G ^{L11}	
(G ^{LL5})		G ^{L12}	
(G ^{LL6})		G ^{L13}	



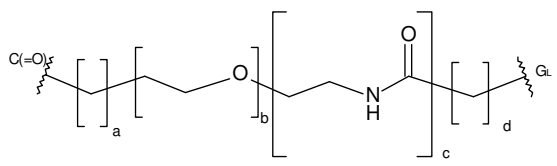
where Ar represents a C_{5-6} arylene group, e.g. phenylene and X represents C^{1-4} alkyl.

In some embodiments, G^{LL} is selected from G^{LL1-1} and G^{LL1-2} . In some of these embodiments, G^{LL} is G^{LL1-1} .

5

X

X is:



where $a = 0$ to 5 , $b = 0$ to 16 , $c = 0$ or 1 , $d = 0$ to 5 .

10

a may be 0 , 1 , 2 , 3 , 4 or 5 . In some embodiments, a is 0 to 3 . In some of these embodiments, a is 0 or 1 . In further embodiments, a is 0 .

b may be 0 , 1 , 2 , 3 , 4 , 5 , 6 , 7 , 8 , 9 , 10 , 11 , 12 , 13 , 14 , 15 or 16 . In some embodiments, b is 0 to 12 . In some of these embodiments, b is 0 to 8 , and may be 0 , 2 , 4 or 8 .

15

c may be 0 or 1 .

d may be 0 , 1 , 2 , 3 , 4 or 5 . In some embodiments, d is 0 to 3 . In some of these embodiments, d is 1 or 2 . In further embodiments, d is 2 .

20

In some embodiments of X, a is 0 , c is 1 and d is 2 , and b may be from 0 to 8 . In some of these embodiments, b is 0 , 4 or 8 .

25 Q^X

In one embodiment, Q^X is an amino acid residue. The amino acid may be a natural amino acid or a non-natural amino acid.

In one embodiment, Q^X is selected from: Phe, Lys, Val, Ala, Cit, Leu, Ile, Arg, and Trp, where Cit is citrulline.

30

In one embodiment, Q^x comprises a dipeptide residue. The amino acids in the dipeptide may be any combination of natural amino acids and non-natural amino acids. In some embodiments, the dipeptide comprises natural amino acids. Where the linker is a cathepsin labile linker, the dipeptide is the site of action for cathepsin-mediated cleavage.

5 The dipeptide then is a recognition site for cathepsin.

In one embodiment, Q^x is selected from:

CO-Phe-Lys-NH,
CO-Val-Ala-NH,
10 CO-Val-Lys-NH,
CO-Ala-Lys-NH,
CO-Val-Cit-NH,
CO-Phe-Cit-NH,
CO-Leu-Cit-NH,
15 CO-Ile-Cit-NH,
CO-Phe-Arg-NH, and
CO-Trp-Cit-NH;

where Cit is citrulline.

20 Preferably, Q^x is selected from:

CO-Phe-Lys-NH,
CO-Val-Ala-NH,
CO-Val-Lys-NH,
CO-Ala-Lys-NH,
25 CO-Val-Cit-NH.

Most preferably, Q^x is selected from CO-Phe-Lys-NH, CO-Val-Cit-NH and CO-Val-Ala-NH.

Other dipeptide combinations of interest include:

30 CO-Gly-Gly-NH,
CO-Pro-Pro-NH, and
CO-Val-Glu-NH.

Other dipeptide combinations may be used, including those described by Dubowchik et al.,
35 *Bioconjugate Chemistry*, 2002, 13,855-869, which is incorporated herein by reference.

In some embodiments, Q^x is a tripeptide residue. The amino acids in the tripeptide may be any combination of natural amino acids and non-natural amino acids. In some embodiments, the tripeptide comprises natural amino acids. Where the linker is a cathepsin labile linker, the tripeptide is the site of action for cathepsin-mediated cleavage.

5 The tripeptide then is a recognition site for cathepsin.

In one embodiment, the amino acid side chain is chemically protected, where appropriate. The side chain protecting group may be a group as discussed below. Protected amino acid sequences are cleavable by enzymes. For example, a dipeptide sequence comprising

10 a Boc side chain-protected Lys residue is cleavable by cathepsin.

Protecting groups for the side chains of amino acids are well known in the art and are described in the Novabiochem Catalog, and as described above.

15 In some embodiments, R^{LL1} and R^{LL2} are of formula IIIb'.
In some embodiments, R^{L1} and R^{L2} are of formula IIIb.

R^{SL1} and R^{SL2} are independently selected from H and methyl, or together with the carbon atom to which they are bound form a cyclopropylene or cyclobutylene group.

20

In some embodiments, both R^{SL1} and R^{SL2} are H.

In some embodiments, R^{SL1} is H and R^{SL2} is methyl.

25 In some embodiments, both R^{SL1} and R^{SL2} are methyl.

In some embodiments, R^{SL1} and R^{L2} together with the carbon atom to which they are bound form a cyclopropylene group.

30 In some embodiments, R^{SL1} and R^{SL2} together with the carbon atom to which they are bound form a cyclobutylene group.

In the group IIIb, in some embodiments, e is 0. In other embodiments, e is 1 and the nitro group may be in any available position of the ring. In some of these embodiments, it is in

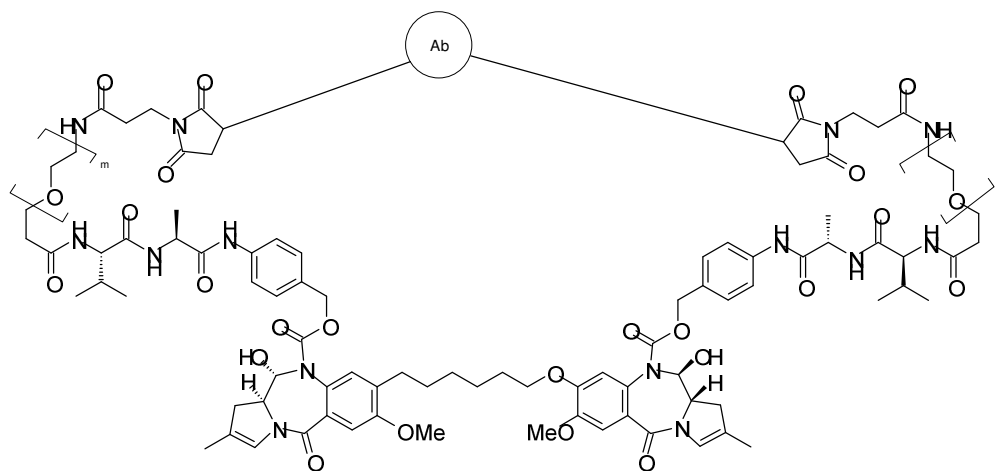
35 the ortho position. In others of these embodiments, it is in the para position.

In some embodiments, R^{L1} and R^{L2} are the same.

In some embodiments, R^{LL1} and R^{LL2} are the same.

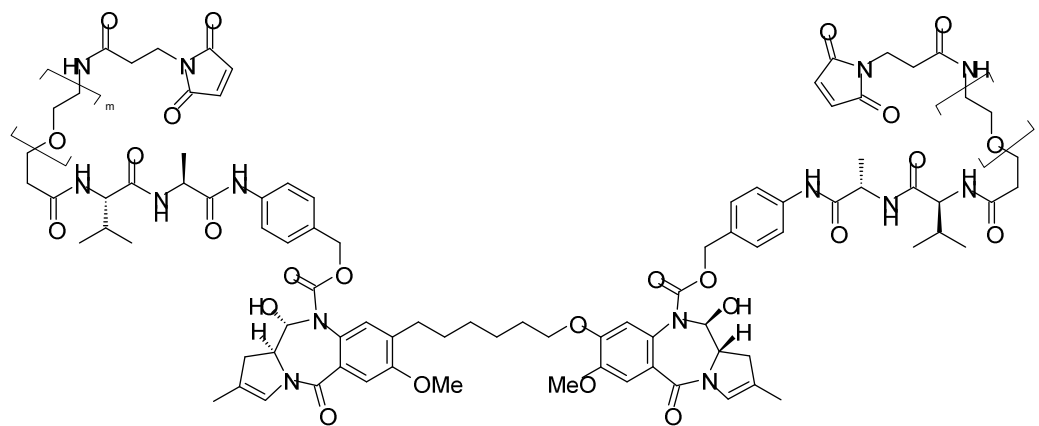
In one particular embodiment, the first aspect of the invention comprises a conjugate of

5 formula Id:



where m is an integer from 2 to 8.

10 In one particular embodiment, the second aspect of the invention, the Drug linker (D^L) is of formula (Id'):



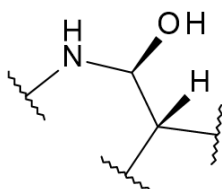
where m is an integer from 2 to 8.

15 In some embodiments, R^{L1} and R^{L2} are different.

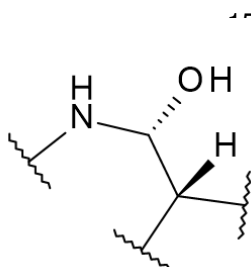
In some embodiments, R^{LL1} and R^{LL2} are different.

In particular, in embodiments where the linking groups are different, differences may only be in the G groups, such that the remainder of the linking groups are the same (so that the cleavage triggers are the same).

- 5 In some embodiments of the present invention, the C11 substituent may be in the following stereochemical arrangement relative to neighbouring groups:



In other embodiments, the C11 substituent may be in the following stereochemical arrangement relative to neighbouring groups:



Compounds of particular interest include those of the examples.

Examples

- 25 Flash chromatography was performed using silica gel under pressure. Fractions were checked for purity using thin-layer chromatography (TLC) using Merck Kieselgel 60 F254 silica gel, with fluorescent indicator on aluminium plates. Visualisation of TLC was achieved with UV light or iodine vapour unless otherwise stated. Extraction and chromatography solvents were bought and used without further purification from VWR U.K. All fine
- 30 chemicals were purchased from Sigma-Aldrich unless otherwise stated. Pegylated reagents were obtained from Quanta biodesign US *via* Stratech UK or from Pierce Scientific *via* Thermo Fisher

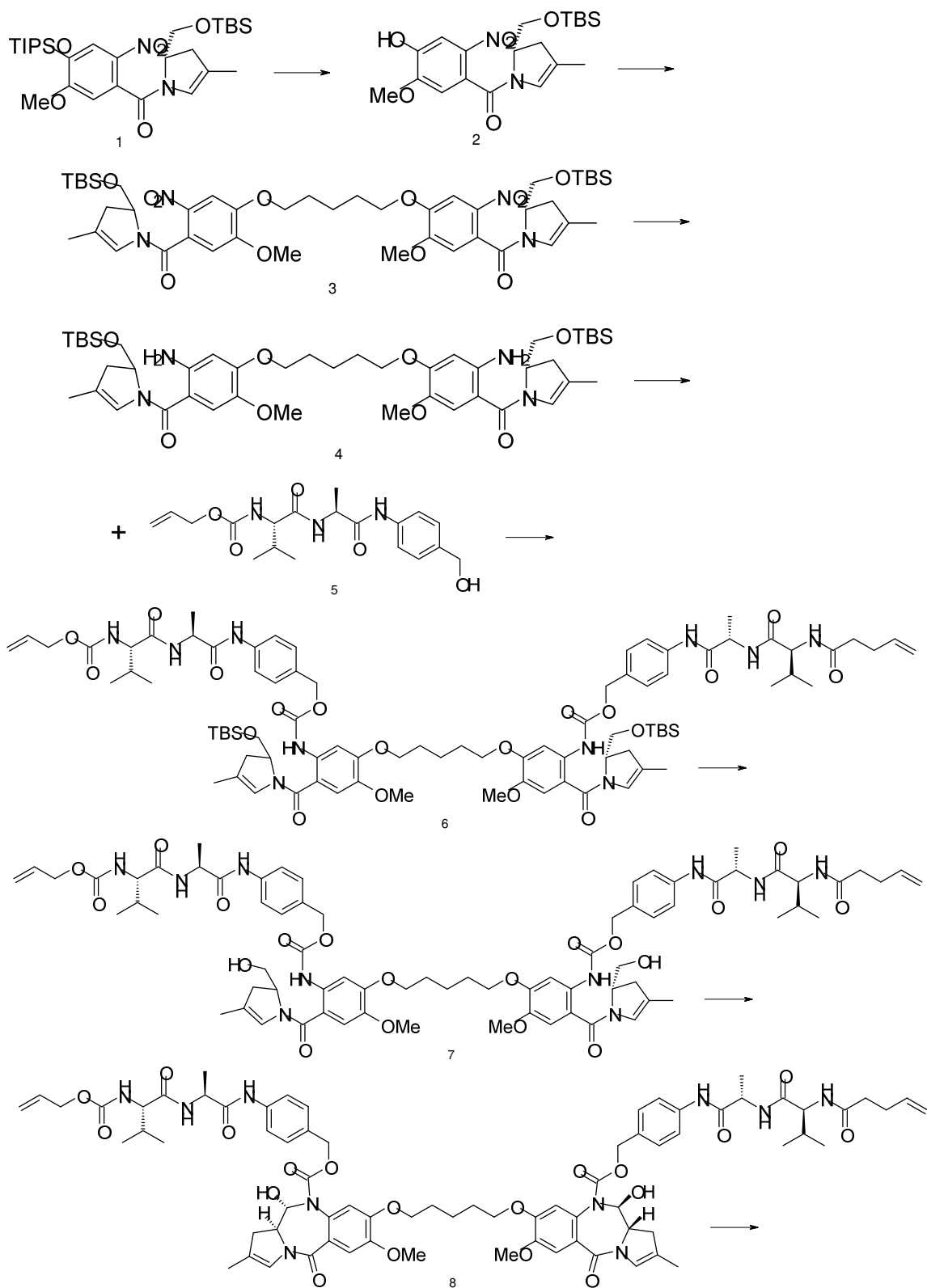
- ¹H and ¹³C NMR spectra were obtained on a Bruker Avance® 400 spectrometer. Coupling
- 35 constants are quoted in hertz (Hz). Chemical shifts are recorded in parts per million (ppm)

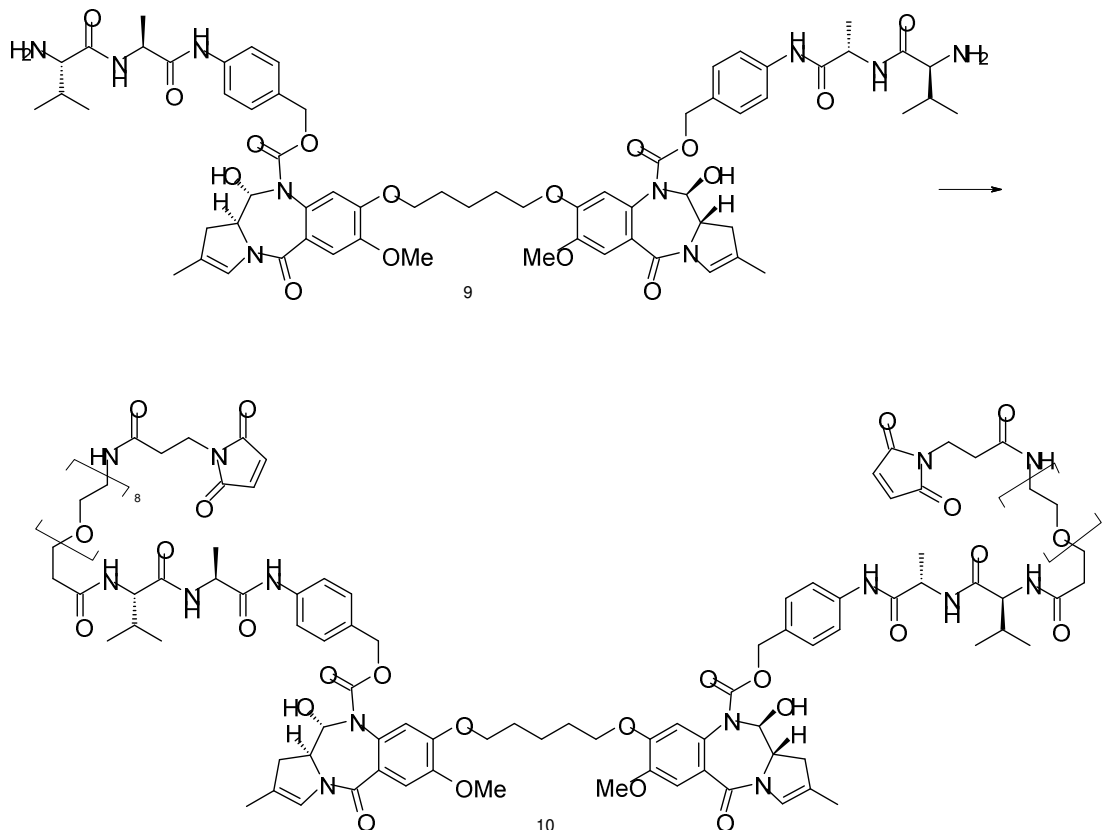
downfield from tetramethylsilane. Spin multiplicities are described as s (singlet), bs (broad singlet), d (doublet), t (triplet), and m (multiplet).

The analytical LC/MS conditions (for reaction monitoring and purity determination) were as follows: Positive mode electrospray mass spectrometry was performed using a Shimadzu Nexera®/Prominence® LCMS-2020. Mobile phases used were solvent A (H₂O with 0.1% formic acid) and solvent B (CH₃CN with 0.1% formic acid). Gradient for routine 3-minute run: Initial composition 5% B held over 25 seconds, then increased from 5% B to 100% B over a 1 minute 35 seconds' period. The composition was held for 50 seconds at 100% B, then returned to 5% B in 5 seconds and held there for 5 seconds. The total duration of the gradient run was 3.0 minutes. Gradient for 15-minute run: Initial composition 5% B held over 1.25 minutes, then increased from 5% B to 100% B over an 8.75 minute period. The composition was held for 2.5 minutes at 100% B, then returned to 5% B in 30 seconds and held there for 2 minutes. The total duration of the gradient run was 15.0 minutes. Flow rate was 0.8 mL/minute (for 3-minute run) and 0.5 mL/minute (for 15-minute run). Detection was at 254 nm. Columns: Waters Acquity UPLC® BEH Shield RP18 1.7µm 2.1 x 50 mm at 50 °C fitted with Waters Acquity UPLC® BEH Shield RP18 VanGuard Pre-column, 130A, 1.7µm, 2.1 mm x 5 mm (routine 3-minute run); and Waters Acquity UPLC CSH C18, 1.7µm, 2.1 x 100mm fitted with Waters Acquity UPLC® BEH Shield RP18 VanGuard Pre-column, 130A, 1.7µm, 2.1 mm x 5 mm (15 minute run).

The preparative HPLC conditions were as follows: Reverse-phase ultra-fast high-performance liquid chromatography (UFLC) was carried out on a Shimadzu Prominence® machine using a Phenomenex® Gemini NX 5µ C18 column (at 50 °C) 150 x 21.2 mm. Eluents used were solvent A (H₂O with 0.05% formic acid) and solvent B (CH₃CN with 0.05% formic acid). All UFLC experiments were performed with gradient conditions: Initial composition 13% B, the composition was then increased to 100% B over a total of 17 minutes at a gradient suitable to effect the desired separation, then held for 1 minute at 100% B, then returned to 13% B in 0.1 minute and held there for 1.9 minutes. The total duration of the gradient run was 20.0 minutes. Flow rate was 20.0 mL/minute and detection was at 254 and 280 nm.

Example 1





(a) **(S)**-2-(((*tert*-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrol-1-yl(4-hydroxy-5-methoxy-2-nitrophenyl)methanone (**2**)

Lithium acetate dihydrate (3.52 g, 34.5 mmol, 1.0 eq.) was added to a stirred solution of
 5 TIPS ether (**1**) (19.96 g, 34.5 mmol, 1.0 eq.) in DMF/H₂O (300 mL/4 mL). The resultant red solution was stirred at room temperature for 3.5h. The reaction mixture was diluted with EtOAc (600 mL) and washed with 1M citric acid solution (2 x 250 mL), H₂O (2 x 250 mL), saturated brine (300 mL) and dried (MgSO₄). The solvent was evaporated under reduced pressure to afford the product as a yellow solid (14.57 g, 100%). The product was used
 10 without further purification. Analytical Data: LC/MS, RT 1.74 min; MS (ES⁺) *m/z* (relative intensity) 423 ([*M* + H]⁺, 100); 445 ([*M* + Na]⁺, 75).

(b) ((Pentane-1,5-diylbis(oxy))bis(5-methoxy-2-nitro-4,1-phenylene))bis(((S)-2-(((*tert*-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrol-1-yl)methanone) (**3**)

Potassium carbonate (5.03 g, 36.44 mmol, 1.1 eq.) was added to a stirred solution of phenol (**2**) (14 g, 33.13 mmol, 1.0 eq.) and 1,5 diiodopentane (21.46 g, 9.86 mL, 66.26 mmol, 2.0 eq.) in DMF (250 mL). The solution was heated at 70°C for 3.5h. The solution was poured into a mixture of ice/water (800 mL) and extracted with EtOAc (4 x 500 mL). The combined extracts were washed with H₂O (2 x 250 mL), saturated brine (400 mL),
 20 dried (MgSO₄) and evaporated under reduced pressure to give a brown oil. Purification by

flash column chromatography [*n*-heptane/EtOAc 40% to 80% in 10% increments] gave the product as a yellow foam (12.7 g, 85%). Analytical Data: LC/MS, RT 2.16 min; MS (ES⁺) *m/z* (relative intensity) 913 ([*M* + H]⁺, 100); 935 ([*M* + Na]⁺, 100).

- 5 (c) ((*Pentane-1,5-diylbis(oxy)*))*bis*(2-amino-5-methoxy-4,1-phenylene))*bis*(((**S**)-2-(((*tert*-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1*H*-pyrrol-1-yl)methanone) (**4**)
- Zinc dust (19.9 g, 304 mmol, 40 eq.) was treated with 1M HCl (100 mL) and stirred for 10 minutes at room temperature. The mixture was then sonicated for 10 minutes and the activated Zinc collected by vacuum filtration then washed with 1M HCl (50 mL), H₂O (to pH
- 10 6 to 7), MeOH and dried *in vacuo* on the filter pad. The activated zinc was added to a vigorously stirred solution of the *bis* nitro compound (**3**) (6.94 g, 7.6 mmol, 1.0 eq.) in EtOH/H₂O/EtOAc (60 mL/4 mL/60 mL) at room temperature. The reaction mixture was treated drop-wise with a solution of 5% *v/v* HCO₂H in MeOH (76 mL). A colour change from green to metallic grey and an exotherm to 42 °C were observed. Once the exotherm had
- 15 subsided to 30°C LC/MS indicated that the reaction was not complete. A further portion of 5% *v/v* HCO₂H in MeOH (20 mL) was added and a further exotherm was observed (34°C) The reaction mixture was allowed to cool to room temperature at which point analysis by LC/MS revealed complete conversion to desired product. The mixture was filtered through celite® and the pad washed with EtOAc. The filtrate was washed with saturated aqueous
- 20 NaHCO₃ (2 x 300 mL), water (300 mL), saturated brine (300 mL), dried (MgSO₄), filtered and evaporated *in vacuo* to provide the *bis*-aniline as a yellow foam (6.22g, 96%). The product was used without further purification. Analytical Data: LC/MS, RT 2.12 min; MS (ES⁺) *m/z* (relative intensity) 853 ([*M* + H]⁺, 15).
- 25 (d) *Bis*(4-(((**S**)-2-(((**S**)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl)) ((*pentane-1,5-diylbis(oxy)*))*bis*(6-(((**S**)-2-(((*tert*-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1*H*-pyrrole-1-carbonyl)-4-methoxy-3,1-phenylene))dicarbamate (**6**)
- Triethylamine (0.171 g, 235 µL, 1.69 mmol, 4.4 eq.) was added *via* syringe to a stirred
- 30 solution of *bis* aniline (**4**) (0.33 g, 0.38 mmol, 1.0 eq.) and triphosgene (0.082 g, 0.28 mmol, 0.72 eq.) in dry THF under an argon atmosphere. The resultant suspension was heated to 40°C and after 5 min sampled in MeOH for LC/MS as the *bis* methyl carbamate (MS (ES⁺) *m/z* (relative intensity) 969 ([*M* + H]⁺, 80); 992 ([*M* + Na]⁺, 100). Dibutyltin dilaurate (0.024 g, 23 µL, 38 µmol, 0.1 eq.) then solid linker (**5**) (0.319 g, 0.85 mmol, 2.2 eq.) and
- 35 trimethylamine (0.085 g, 118 µL, 0.85 mmol, 2.2 eq.) were added and the mixture heated at 40°C with stirring under an argon atmosphere for 5h. The reaction mixture was allowed to

cool, filtered and the THF evaporated under reduced pressure. The residue was purified by flash column chromatography [$\text{CHCl}_3/\text{MeOH}$ 0%, 1%, 1.5%, 2%, gradient elution] to give the product as a yellow foam (0.42 g, 66%). Analytical Data: LC/MS, RT 2.16 min; MS (ES^+) m/z (relative intensity) 1660 ($[M + \text{H}]^+$, 60); 1682 ($[M + \text{Na}]^+$, 65).

5

(e) *Bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl) ((pentane-1,5-diylbis(oxy))bis(6-((S)-2-(hydroxymethyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-3,1-phenylene))dicarbamate (7)*

- 10 *p*-Toluenesulfonic acid (0.296 g, 1.7 mmol, 2.2 eq.) was added to a stirred solution of *bis-tert*-butyldimethylsilyl ether (**6**) (1.26 g, 0.76 mmol, 1.0 eq.) in 10% v/v H_2O in THF. The solution was stirred at room temperature for 18h. The reaction mixture was diluted with EtOAc (100 mL) and washed with saturated NaHCO_3 solution (2 x 100 mL), H_2O (100 mL), saturated brine (100 mL), dried (MgSO_4) and evaporated under reduced pressure. The
- 15 residue was purified by flash column chromatography [$\text{CHCl}_3/\text{MeOH}$ 0% to 5% in 1% increments] to give the product as a white foam (0.896 g, 92%). Analytical Data: LC/MS, RT 1.61 min; MS (ES^+) m/z (relative intensity) 1432 ($[M + \text{H}]^+$, 5); 1454 ($[M + \text{Na}]^+$, 5).

(f) *Bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-*

- 20 *methylbutanamido)propanamido)benzyl) 8,8'-(pentane-1,5-diylbis(oxy))(11S,11aS,11'S,11a'S)-bis(11-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate) (8)*
- Dess-Martin periodinane (0.24 g, 0.57 mmol, 2.0 eq.) was added to a stirred solution of *bis*-alcohol (**7**) in dry DCM (20 mL). The resultant white suspension was stirred at room
- 25 temperature for 24h. The reaction mixture was diluted with DCM (100 mL) and extracted with saturated NaHCO_3 solution (2 x 100 mL), water (100 mL), saturated brine (100 mL), dried (MgSO_4) and evaporated under reduced pressure. Purification by flash column chromatography [$\text{CHCl}_3/\text{MeOH}$ 0% to 3% in 0.5% increments] gave the product as a white foam (0.28 g, 69%). Analytical Data: LC/MS, RT 1.58 min; MS (ES^+) m/z (relative intensity)
- 30 1428 ($[M + \text{H}]^+$, 20); 1450 ($[M + \text{Na}]^+$, 30).

(g) *Bis(4-((S)-2-((S)-2-amino-3-methylbutanamido)propanamido)benzyl) 8,8'-(pentane-1,5-diylbis(oxy))(11S,11aS,11'S,11a'S)-bis(11-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate) (9)*

- 35 $\text{Pd}(\text{PPh}_3)_4$ (8 mg, 7 μmol , 0.04 eq.) was added to a stirred solution of *bis*-alloc derivative (**8**) (0.25 g, 0.176 mmol 1.0 eq.) and pyrrolidine (31 mg, 36 μL 0.44 mmol, 2.5 eq.) in dry DCM

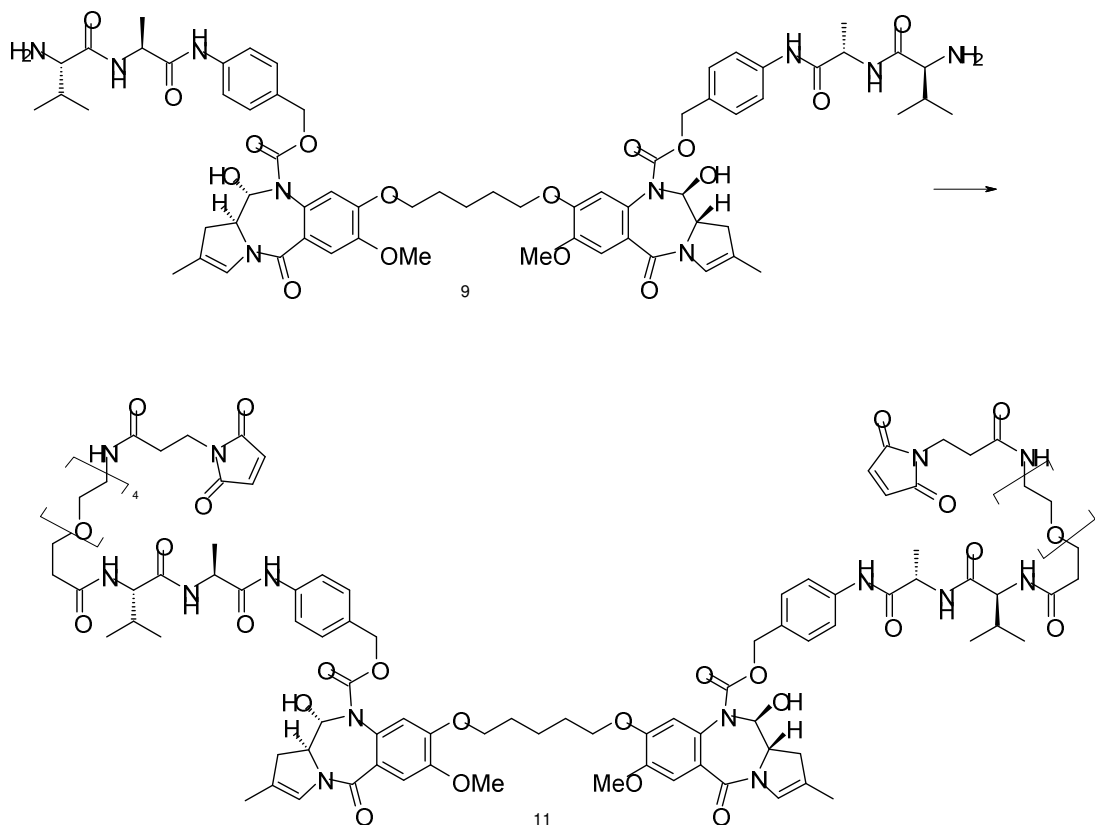
(10 mL). The solution was stirred at room temperature for 2h. The reaction mixture was partitioned between saturated NH₄Cl solution (50 mL) and DCM (50 mL). The DCM was separated and washed with saturated brine (100 mL), dried (MgSO₄) and evaporated under reduced pressure. The solid residue was triturated/sonicated with Et₂O (3 x 15 mL) and dried under vacuum to give the product as a white solid (0.207 g, 93%). The product was used without further purification. Analytical Data: LC/MS, RT 1.06 min; MS (ES⁺) *m/z* (relative intensity) 630 ([*M* + 2H]⁺, 100).

(h) *Bis(4-((2S,5S)-37-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-isopropyl-2-methyl-4,7,35-trioxo-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl) 8,8'-(pentane-1,5-diylbis(oxy))(11S,11aS,11'S,11a'S)-bis(11-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate) (10)*

EDCI.HCl (56 mg, 0.29 mmol, 3 eq.) was added to a stirred solution of *bis*-amine (**9**) (0.123 g, 98 μmol, 1.0 eq.) and MaldPEG®OH (0.128 g, 0.22 mmol, 2.2 eq.) in CHCl₃ (15 mL).

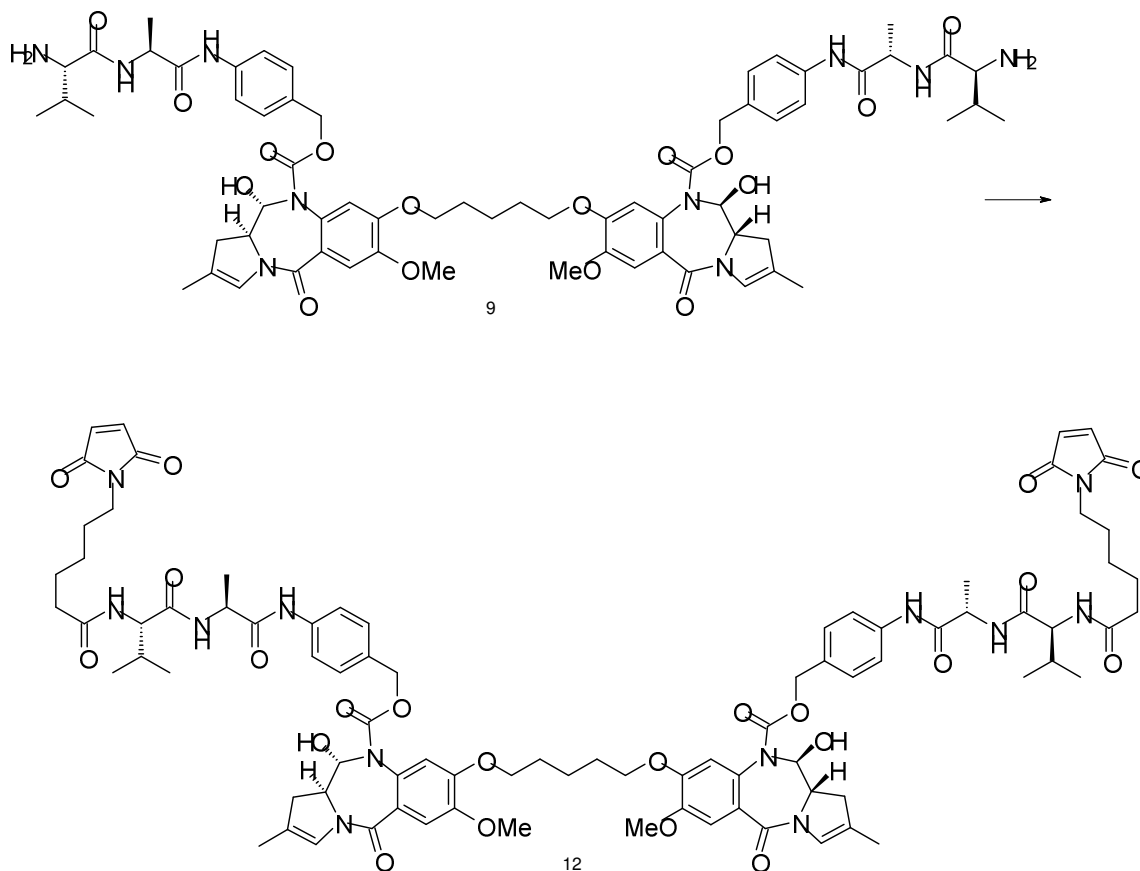
The reaction mixture was stirred at room temperature for 30 min then diluted with CHCl₃ (50 mL) washed with H₂O (100 mL), saturated brine (100 mL), dried (MgSO₄) and evaporated under reduced pressure. Purification by preparative HPLC followed by lyophilisation gave the product as a white foam (0.047 g, 20%). Analytical Data: LC/MS, RT 6.61 min; MS (ES⁺) *m/z* (relative intensity) 1205 ([*M* + 2H]⁺, 55).

Example 2



Bis(4-((2S,5S)-25-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-isopropyl-2-methyl-4,7,23-trioxo-10,13,16,19-tetraoxa-3,6,22-triazapentacosanamido)benzyl) 8,8'-(pentane-1,5-
diylbis(oxy))((11S,11aS,11'S,11a'S)-bis(11-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-
dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate) (11)

DIPEA (30 mg, 42 μ L, 0.23 mmol, 3 eq.) was added to a stirred solution of *bis*-amine (**9**) (98 mg, 78 μ mol, 1.0 eq.) and MalPEG₄OSu (88 mg, 0.17 mmol, 2.2 eq.) in CHCl₃ (10 mL). The reaction mixture was stirred at room temperature for 72h then diluted with CHCl₃ (50 mL) washed with H₂O (100 mL), saturated brine (100 mL), dried (MgSO₄) and evaporated under reduced pressure. Purification by preparative HPLC followed by lyophilisation gave the product as a white foam (0.043 g, 25%). Analytical Data: LC/MS, RT 6.11 min; MS (ES⁺) *m/z* (relative intensity) 1028 ([*M* + 2H]⁺, 80).

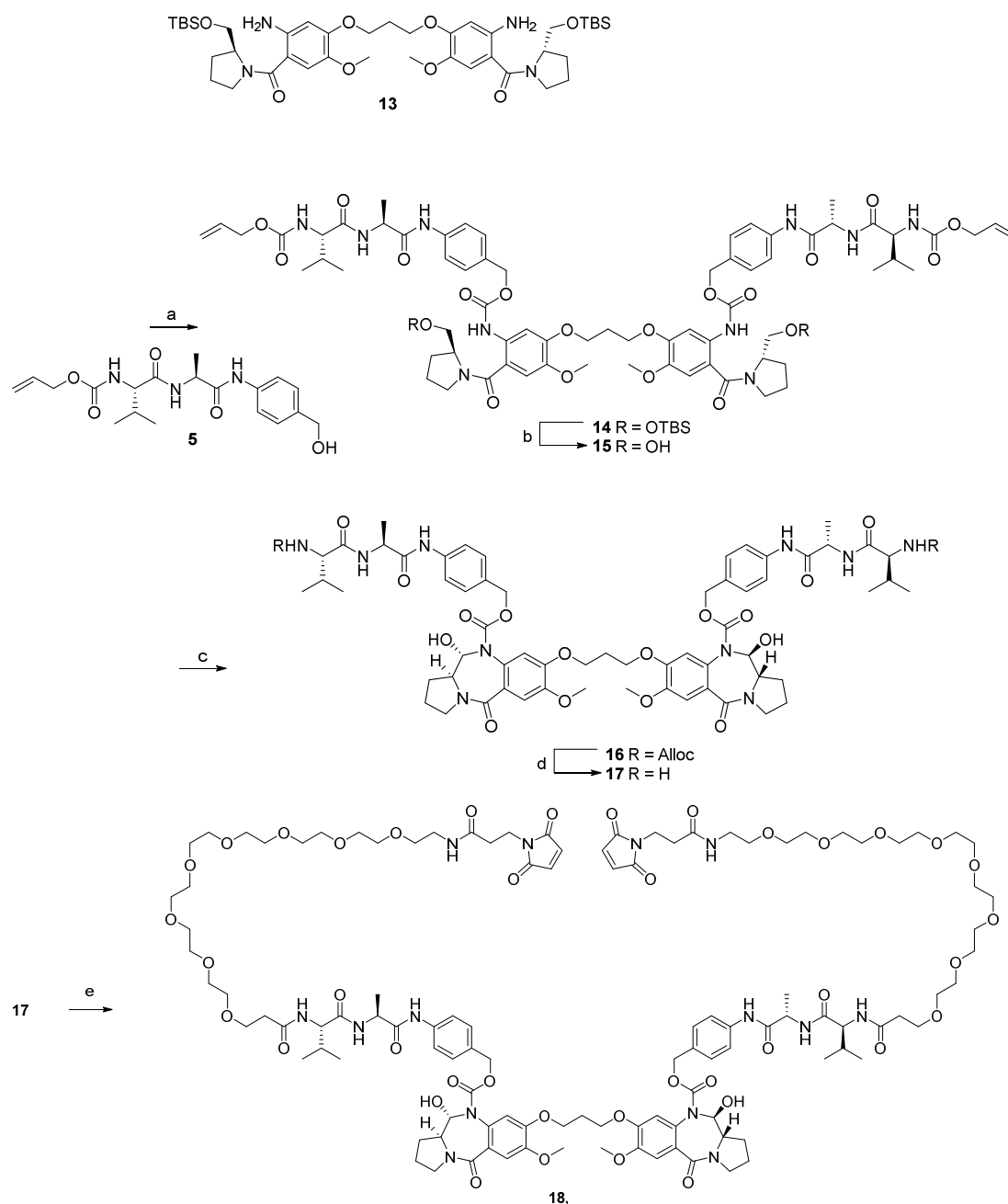
Example 3

bis(4-((S)-2-((S)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-

diylbis(oxy)))(11S,11aS,11'S,11a'S)-bis(11-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-
 5 *dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate) (12)*

EDCI.HCl (50 mg, 0.26 mmol, 3 eq.) was added to a stirred solution of *bis*-amine (**9**) (0.109
 10 g, 86.5 μ mol, 1.0 eq.) and MCOSu (40 mg, 0.19 mmol, 2.2 eq.) in CHCl_3 (10 mL). The
 reaction mixture was stirred at room temperature for 30 min then diluted with CHCl_3 (50
 mL) washed with H_2O (100 mL), saturated brine (100 mL), dried (MgSO_4) and evaporated
 under reduced pressure. Purification by preparative HPLC followed by lyophilisation gave
 the product as a white foam (0.045 g, 32%). Analytical Data: LC/MS, RT 6.82 min; MS
 (ES^+) m/z (relative intensity) 1646 ($[\text{M} + \text{H}]^+$, 20); 1667 ($[\text{M} + \text{Na}]^+$, 30).

Example 4



(a) bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-

- 5 methylbutanamido)propanamido)benzyl) ((propane-1,3-diylbis(oxy))bis(6-((S)-2-(((tert-butyl)dimethylsilyl)oxy)methyl)pyrrolidine-1-carbonyl)-4-methoxy-3,1-phenylene))dicarbamate (14)

Triphosgene (472 mg, 1.59 mmol, 0.72 eq) was added in one portion to a mixture of 13 (1.77 g, 2.21 mmol) and triethylamine (1.35 mL, 9.69 mmol, 4.38 eq) in dichloromethane (3.6 mL). After 10 min, 5 (1.83 g, 4.85 mmol, 2.19 eq) was added in one portion as a fine powder, followed by triethylamine (0.68 mL, 4.9 mmol, 2.2 eq) and dibutyltin dilaurate (132

μL, 0.221 mmol, 0.1 eq) The reaction mixture was allowed to stir at 37 °C for 4h, followed by stirring at room temperature overnight. The organic phase was washed with water and decanted in a filtration cartridge. The DCM was removed by evaporation, and the residue was dry loaded on silica gel, followed by chromatography with a 50g ultra biotage cartridge (gradient DCM / DCM:MeOH 90:10, from 5% up to 32%, elution at 32%). The pure fractions were combined to yield the product **14** (2.35 g, 1.46 mmol, 66.2% Yield). Analytical Data: LC/MS, 3 min lipophilic method, RT 2.24 min; MS (ES⁺) *m/z* (relative intensity) 1608.9 ([*M* + H]⁺, 100);

(b) *bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl) ((propane-1,3-diylbis(oxy))bis(6-((S)-2-(hydroxymethyl)pyrrolidine-1-carbonyl)-4-methoxy-3,1-phenylene))dicarbamate (15)*
 Paratoluenesulfonic acid hydrate (277 mg, 1.46 mmol, 1 eq) was added in one portion to a mixture of **14** (2.34 g, 1.46 mmol) in tetrahydrofuran (53.0 mL) and water (5.00 mL) at 0 °C (ice/water bath). The reaction mixture was allowed to stir at 20 °C for 7 h until completion as monitored by LCMS. The reaction mixture was partitioned between ethyl acetate and water, and washed with NaHCO₃, then brine. The organics were dried over magnesium sulfate and concentrated under vacuum. The residue was purified by chromatography (50 g ultra, dry loaded on a samplet, DCM versus DCM:MeOH 90:10, gradient from 20% to 64%, elution around 64%). Pure fractions were combined and concentrated under vacuum to give the product **15** (1.60 g, 1.16 mmol, 79.7% Yield) as a white solid. Analytical Data: LC/MS, 3 min lipophilic method, RT 1.50 min; MS (ES⁺) *m/z* (relative intensity) 1380.9 ([*M* + H]⁺, 100);

(c) *bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl) 8,8'-(propane-1,3-diylbis(oxy))((11S,11aS,11'S,11a'S)-bis(11-hydroxy-7-methoxy-5-oxo-2,3,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate) (16)*
 Stahl Tempo 0.2M Solution (2.10 mL, 0.420 mmol, 0.44 eq) followed by tetrakisacetonitrile copper(I) triflate (160 mg, 0.425 mmol, 0.44 eq) was added to a solution of **15** (1.32 g, 0.957 mmol) in DMF (4.00 mL) in a 500 mL flask. The reaction mixture was stirred rapidly and heated at 40 °C for 5h, then 35 °C for 18h under an air balloon, at which point completion was observed by LCMS. The solvents were removed by evaporation. Traces of DMF were removed by a second evaporation with butanone, followed by hard vacuum. The residue was dry loaded on a samplet (10g) with acetone, followed by chromatography with a 50g ultra column on a biotage isolera system. Gradient with 10% MeOH in DCM / DCM,

from 20% up to 63% in 8 CV. Elution and hold around 60%. The impure front fractions were repurified using the same system on a 25g column. All pure fractions were pooled. The residue was dissolved in acetone. Addition of heptane caused the precipitation of a white product. The volatiles were evaporated to leave the product **16** as a white powder after hard vacuum. (892 mg, 0.648 mmol, 67.8% Yield) Analytical Data: LC/MS, 3 min lipophilic method, RT 1.42 min; MS (ES⁺) *m/z* (relative intensity) 1376.6 ([*M* + H]⁺, 100);

(d) *bis(4-((S)-2-((S)-2-amino-3-methylbutanamido)propanamido)benzyl) 8,8'-(propane-1,3-diylbis(oxy))(11S,11aS,11'S,11a'S)-bis(11-hydroxy-7-methoxy-5-oxo-2,3,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate) (17)*

Tetrakis(triphenylphosphine)palladium(0) (10.0 mg, 0.00865 mmol, 0.034 eq) was added to a mixture of **16** (350 mg, 0.254 mmol) and pyrrolidine (65.0 µL, 0.780 mmol, 3.07 eq) in dichloromethane (7.50 mL) and methanol (0.5 mL). The reaction mixture was stirred under argon at room temperature for 1h30 minutes and was found complete by LCMS

Ammonium chloride in water (30 mL, 34.3 mmol, 6 mass%) was added and the mixture was stirred vigorously. The mixture was then decanted in a biotage phase separation cartridge. The DCM layer was evaporated to dryness under vacuum. The residue was dissolved in chloroform (20 mL) and the solvent removed by evaporation under vacuum at 35°C. This cycle was repeated a second time, followed by drying under hard vacuum (3 mbar) to give the crude product **17** (307 mg, 0.254 mmol, 100%) as a white solid which was used directly in the next step without further purification. Analytical Data: LC/MS, 3 min lipophilic method, 2 peaks, RT 0.22 min; MS (ES⁺) *m/z* (relative intensity) 604.9 ([*M* + 2H]²⁺, 100); 1208.2 ([*M* + H]⁺, 10);

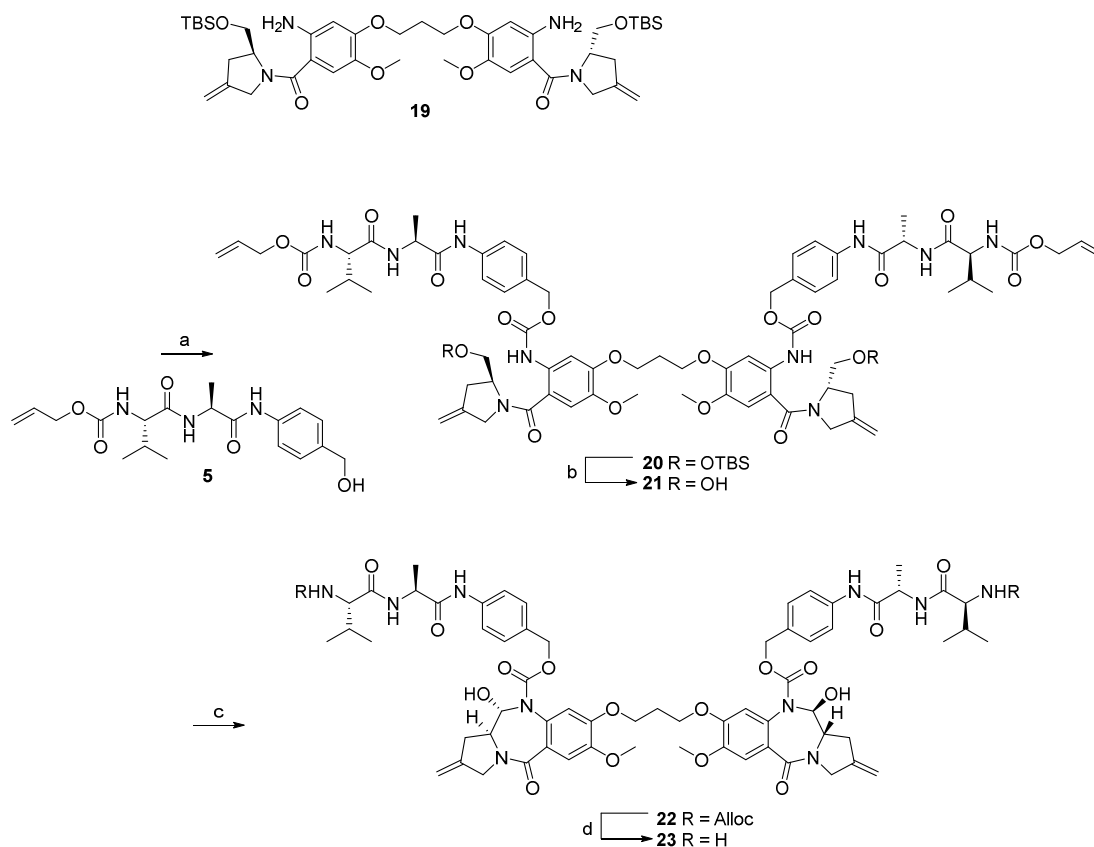
(e) *bis(4-((2S,5S)-37-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-isopropyl-2-methyl-4,7,35-trioxo-10,13,16,19,22,25,28,31-octa-3,6,34-triazaheptatriacontanamido)benzyl) 8,8'-(propane-1,3-diylbis(oxy))(11S,11aS,11'S,11a'S)-bis(11-hydroxy-7-methoxy-5-oxo-2,3,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate) (18)*

Chloroform (10.00 mL) and methanol (0.4 mL) were added to crude **17** (307 mg, 0.254 mmol) followed by mal-amido-peg8-acid (339 mg, 0.561 mmol, 2.2 eq) and EDCI (107 mg, 0.558 mmol, 2.19 eq). The reaction was allowed to proceed at room temperature for 45 min when completion was observed by LCMS. Ammonium chloride in water (30 mL, 6 mass%) was added and the mixture was stirred vigorously. The mixture was decanted in a biotage phase separation cartridge. The DCM layer was evaporated to dryness under vacuum.

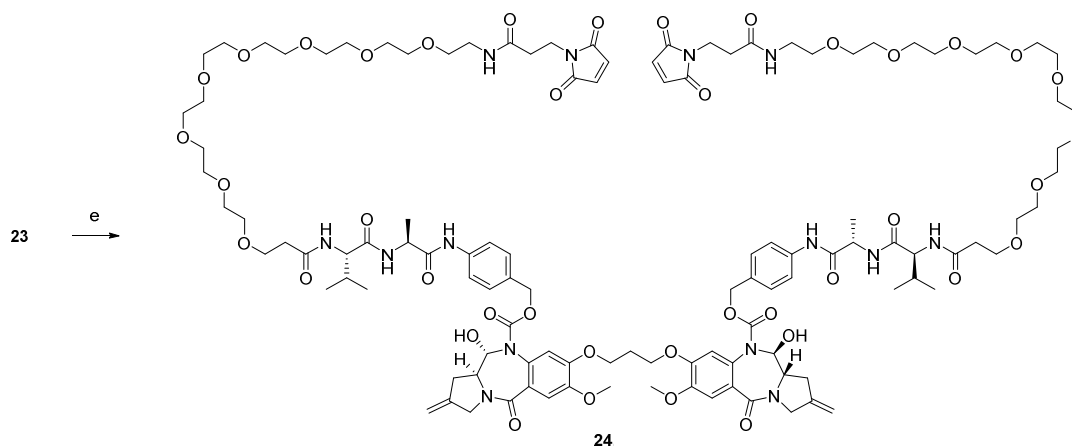
The volatiles were removed by rotoevaporation and the crude residue was purified by chromatography (50g Ultra, Biotage, gradient 30/70 to 100/0 of 16% MeOH in DCM / DCM

in 10CV; Elution at more than 10% of MeOH). All fractions were analysed by TLC (10% MeOH in DCM). The pure fractions were pooled. The solvent was removed by evaporation to give **18** (200 mg). LCMS analysis showed traces of mal-peg8-acid, and the material was purified further by preparative HPLC, freeze-dried, aliquoted in dichloromethane, and dried under high vacuum to give **18** as a white solid. The purity was 99.45%. (B, 110 mg, 0.0467 mmol, 18.3% Yield). Analytical Data: LC/MS, 15 min method, RT 5.90 min; MS (ES⁺) *m/z* (relative intensity) 1179.5 ([*M* + 2H]²⁺, 100); ¹H NMR (400 MHz, DMSO-d₆) δ 9.92 (s, 2H), 8.16 (d, *J* = 6.9 Hz, 2H), 7.99 (t, *J* = 5.7 Hz, 2H), 7.86 (d, *J* = 8.7 Hz, 2H), 7.55 (s, 4H), 7.18 (s, 4H), 7.07 (s, 2H), 7.00 (s, 4H), 6.79 (s, 2H), 6.50 (s, 2H), 5.48 (s, 2H), 5.23 – 4.77 (m, 4H), 4.39 (t, *J* = 7.0 Hz, 2H), 4.22 (dd, *J* = 8.7, 6.6 Hz, 2H), 4.10 (s, 4H), 3.77 (s, 6H), 3.64 – 3.55 (m, 8H), 3.55 – 3.42 (m, 56H), 3.37 (t, *J* = 5.9 Hz, 6H), 3.28 (t, *J* = 8.3 Hz, 2H), 3.15 (q, *J* = 5.8 Hz, 4H), 2.49 – 2.37 (m, 4H), 2.37 – 2.30 (m, 4H), 2.17 (s, 2H), 2.09 – 1.73 (m, 10H), 1.30 (d, *J* = 7.0 Hz, 6H), 0.85 (dd, *J* = 15.3, 6.7 Hz, 12H).

15 Example 5



79



(a) *bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl) ((propane-1,3-diylbis(oxy))bis(6-((S)-2-(((tert-butyl)dimethylsilyl)oxy)methyl)-4-methylenepyrrolidine-1-carbonyl)-4-methoxy-3,1-phenylene))dicarbamate (20)*

Triphosgene (816 mg, 2.75 mmol, 0.72 eq) was added in one portion to a mixture of **19** (3.15 g, 3.82 mmol) and triethylamine (2.34 mL, 16.8 mmol, 4.4 eq) in dichloromethane (75 mL) at 0°C. The ice batch was removed, and after 15 min, alcohol **5** (3.17 g, 8.40 mmol, 2.2 eq) was added in one portion as a fine powder, followed by triethylamine (1.17 mL, 8.39 mmol, 2.2 eq) and dibutyltin dilaurate (229 µL, 0.383 mmol, 0.1 eq). The reaction mixture was allowed to stir at 37 °C for 1h, followed by stirring at room temperature overnight. The organic phase was diluted with DCM (100 mL) and washed with water (200 mL), saturated ammonium chloride (100 mL), and brine (50 mL), followed by drying over magnesium sulfate. The volatiles were removed by evaporation under reduced pressure.

The crude product was dry-loaded on silica gel and eluted on a 340g Ultra, with a gradient of ethyl acetate - acetone, from 20% up to 100% in 7CV. Rapid elution in 2CV at around 30% acetone gave pure fractions which were dried under vacuum to give **20** (4.00 g, 2.45 mmol, 100 mass%, 64.2% Yield). Analytical Data: LC/MS, 3 min lipophilic method, RT 2.34 min; MS (ES⁺) *m/z* (relative intensity) 1661.1 ([*M* + *H*]⁺, 100);

(b) *bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl) ((propane-1,3-diylbis(oxy))bis(6-((S)-2-((hydroxymethyl)-4-methylenepyrrolidine-1-carbonyl)-4-methoxy-3,1-phenylene))dicarbamate (21)*

Bis-TBS ether **20** (4.00 g, 2.45 mmol) and paratoluenesulfonic acid hydrate (300 mg, 1.58 mmol) were dissolved in a mixture of 2-methyltetrahydrofuran (25.0 mL, 249 mmol, 100 mass%), acetic acid (4.00 mL, 69.8 mmol, 100 mass%) and water (4.00 mL, 222 mmol, 100 mass%). The mixture was heated at 40°C. After 2h, completion was observed by

LCMS. The reaction mixture was partitioned between ethyl acetate (150 mL) and water (200 mL), then washed with saturated NaHCO₃ (150 mL), and brine (100 mL). The organics were dried over magnesium sulfate and concentrated under vacuum. The residue was purified by chromatography (100 g ultra, dry loaded on 10g sample, ethyl acetate / acetone, gradient from 85/15 to 0/100, elution around 80% acetone. The pure fractions were combined and concentrated under vacuum to give the pure product **21** (960 mg, 0.684 mmol, 27.9% Yield) as a white solid. Analytical Data: LC/MS, 3 min lipophilic method, RT 1.54 min; MS (ES⁺) *m/z* (relative intensity) 1402.3 ([*M* + H]⁺, 100);

(c) *bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl) 8,8'-(propane-1,3-diylbis(oxy))*(11*S*, 11*aS*, 11'*S*, 11*a'S*)-*bis(11-hydroxy-7-methoxy-2-methylene-5-oxo-2,3,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate)* (**22**)
 Stahl Tempo 0.2M Solution (1.34 mL, 0.268 mmol, 0.4 eq) followed by Tetrakisacetonitrile copper(I) triflate (190 mg, 0.504 mmol, 0.75 eq) was added to a solution of alcohol **21** (940 mg, 0.670 mmol) in DMF (3.00 mL) and DCM (13.0 mL) in a 500 mL flask. The reaction mixture was stirred rapidly and heated at 37 °C for 5h (almost complete), followed by -20 °C for 96h, at which point the reaction mixture was diluted with dichloromethane (60 mL) and water (60 mL) and stirred for 5 min. The reaction mixture was decanted in a phase separator and the DCM phase was dried under reduce pressure. MEK (60 mL) was added and the residual DMF was removed by azeotroping with MEK under reduce pressure (2 times) to give the crude product as a solid. This was redissolved in DCM/isopropanol 80/20 (5 to 10 mL) and loaded onto a Biotage samplet (10g), dried and loaded on a 100g Ultra column. Gradient from 88/12 DCM/20% MeOH in DCM up to 70/30 in 10 CV. The pure fractions were combined to give pure **22** (602 mg, 0.430 mmol, 64.2% Yield) as a white product. Analytical Data: LC/MS, 3 min lipophilic method, RT 1.51 min; MS (ES⁺) *m/z* (relative intensity) 1401.5 ([*M* + H]⁺, 100);

(d) *bis(4-((S)-2-((S)-2-amino-3-methylbutanamido)propanamido)benzyl) 8,8'-(propane-1,3-diylbis(oxy))*(11*S*, 11*aS*, 11'*S*, 11*a'S*)-*bis(11-hydroxy-7-methoxy-2-methylene-5-oxo-2,3,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate)* (**23**)
 Tetrakis(triphenylphosphine)palladium(0) (8.2 mg, 0.0071 mmol, 100 mass%) was added to a mixture of **22** (250 mg, 0.179 mmol) and pyrrolidine (37.0 µL, 0.444 mmol, 2.49 eq) in DCM (7.50 mL) and methanol (0.5 mL). The reaction mixture was stirred under argon at room temperature for 1h30 minutes and was found complete by LCMS.

Ammonium chloride in water (30 mL, 6 mass%) was added and the mixture was stirred vigorously. The mixture was then decanted in a biotage phase separation cartridge. The DCM layer was evaporated to dryness under vacuum. The residue was dissolved in chloroform (20 mL) and the solvent removed by rotoevaporation under vacuum at 35°C.

- 5 This cycle was repeated a second time, followed by drying under hard vacuum (3 mbar, on rotoevaporator) to give the crude product **23** (220 mg, 0.179 mmol, 100%) as a white solid which was used directly in the next step without further purification. Analytical Data: LC/MS, 3 min method, 2 peaks, RT 1.15 min; MS (ES⁺) *m/z* (relative intensity) 616.9 ([*M* + 2H]²⁺, 100); 1232.1 ([*M* + H]⁺, 10).

10

(*e*) bis(4-((2*S*,5*S*)-37-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-5-isopropyl-2-methyl-4,7,35-trioxo-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl) 8,8'-(propane-1,3-diylbis(oxy))(11*S*,11*aS*,11'*S*,11'*aS*)-bis(11-hydroxy-7-methoxy-2-methylene-5-oxo-2,3,11,11*a*-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepine-10(5*H*)-carboxylate)

15 (**24**)

Chloroform (4.1 mL) and methanol (0.2 mL) were added to **23**, followed by mal-amido-peg8-acid (238 mg, 0.394 mmol, 2.2 eq) and EDCI (85.0 mg, 0.443 mmol, 2.48 eq). The reaction was allowed to proceed at room temperature for 45 min when completion was observed by LCMS. The reaction mixture was concentrated (2 mL), loaded on a 3g biotage silica samplet and dried under vacuum. The samplet was loaded on a 25g Ultra Biotage column, and eluted (gradient 10/90 to 58/42 of 20% MeOH in DCM / DCM in 12CV; Elution at around 55% of 20% MeOH). All fractions were analysed by TLC (10% MeOH in DCM).

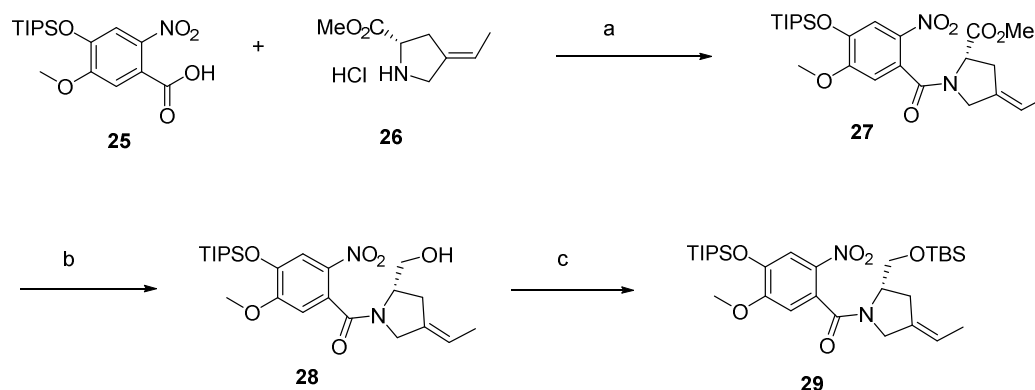
20

The pure fractions were pooled. The solvent was removed by evaporation to give **24** (250 mg, 0.105 mmol, 58.8% Yield). Analytical Data: LC/MS, 15 min method, RT 6.20 min; MS (ES⁺) *m/z* (relative intensity) 1191.5 ([*M* + 2H]²⁺, 100); ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.92 (s, 2H), 8.16 (d, *J* = 6.9 Hz, 2H), 7.99 (t, *J* = 5.5 Hz, 2H), 7.86 (d, *J* = 8.6 Hz, 2H), 7.68 – 7.42 (m, 4H), 7.39 – 7.11 (m, 4H), 7.07 (s, 2H), 7.00 (s, 4H), 6.81 (s, 2H), 6.60 (s, 2H), 5.46 – 5.30 (m, 2H), 5.21 – 4.79 (m, 8H), 4.39 (t, *J* = 7.0 Hz, 2H), 4.22 (dd, *J* = 8.7, 6.7 Hz, 2H), 4.15 – 3.88 (m, 8H), 3.77 (s, 6H), 3.65 – 3.55 (m, 8H), 3.54 – 3.40 (m, 58H), 3.37 (t, *J* = 5.9 Hz, 4H), 3.15 (q, *J* = 5.8 Hz, 4H), 2.95 – 2.79 (m, 2H), 2.57 – 2.52 (m, 2H), 2.49 – 2.37 (m, 4H), 2.37 – 2.29 (m, 4H), 2.22 – 2.10 (m, 2H), 2.03 – 1.88 (m, 2H), 1.30 (d, *J* = 7.0 Hz, 6H), 0.85 (dd, *J* = 15.3, 6.7 Hz, 12H).

30

Example 6

(i) Synthesis of (S,E)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-ethylidenepyrrolidin-1-yl)-(5-methoxy-2-nitro-4-((triisopropylsilyl)oxy)phenyl)methanone (**29**)



- 5 Compound **25** is described in Tiberghien et al, ACS Med. Chem. Lett., 2016, 7 (11), pp 983–987. Compound **26** is described in Smits and Zemribo, Org. Lett., 2013, 15 (17), pp 4406–4409.

(a) methyl(S,E)-4-ethylidene-1-(5-methoxy-2-nitro-4-

- 10 ((triisopropylsilyl)oxy)benzoyl)pyrrolidine-2-carboxylate (**27**)

25 (325g, 1.2 eq) and **26** (1.0 eq.) were dissolved in DCM (3.25 L) and cooled to -40°C.

T3P (2 eq) was added portionwise at -40°C, followed by DIEA (6.0eq). The mixture was stirred for 1h at -40°C. Reaction completion was observed by LCMS. Aqueous acetic acid (10%, 3.25 L) was added at 0°C. The organic phase was separated and washed a second

- 15 time with aqueous acetic acid (10%, 3.25 L), followed by brine (3.25 L). The volatiles were removed under vacuum to leave the crude product **27** as a brown oil, which was purified by silica gel chromatography (petroleum ether/EtOAc, gradient from 100/1 to 10/1, collection from 20/1. (591 g, purity 87.6% by LC, 70% by NMR, yield = 60%). RT: 6.374 min.

- 20 Analytical method used for compound 27

Column :Agilent Poroshell 120 EC- C18 4.6*100mm,2.7um

Mobile phase A: 0.05% TFA in Water

Mobile phase B: 0.05% TFA in ACN

Diluent: ACN

- 25 Flow rate: 1.0 mL/min

Injection volume: 1 µL

Column temperature: 40 °C

Detector: 220 nm

Run Time: 8.1 minutes

Post time: 2 minutes

Gradient Table

Time (min)	0.0	4.0	8.0	8.1
% Mobile Phase A	80	0	0	95
% Mobile Phase B	20	100	100	5

5

(b) *(S,E)*-(4-ethylidene-2-(hydroxymethyl)pyrrolidin-1-yl)(5-methoxy-2-nitro-4-((triisopropylsilyl)oxy)phenyl)methanone (**28**)

27 (591g, 1 eq) was dissolved to DCM and cooled to 0°C. Lithium borohydride (2.0 eq) was added portionwise. The reaction mixture was stirred at 0°C for 6h. Reaction completion was observed by LCMS. Aqueous acetic acid (10%, 5.9 L) was added at 0°C. The organic phase was separated and washed a second time with aqueous acetic acid (10%, 5.9 L), followed by brine (5.9 L). The volatiles were removed under vacuum to leave a residue which was purified by flash chromatography (petroleum ether/EtOAc, gradient from 50/1 to 1/1. Collection from 5/1) to give **28** as an off white solid (250 g, 64% yield). RT: 7.922 min.

15

(c) *(S,E)*-(2-(((tert-butyl dimethylsilyl)oxy)methyl)-4-ethylidenepyrrolidin-1-yl)(5-methoxy-2-nitro-4-((triisopropylsilyl)oxy)phenyl)methanone (**29**)

28 (250g, 1 eq) and imidazole (2 eq) were dissolved in DCM (1.5 L, 6 V) at room temperature. TBSCl (1.5 eq) was added portionwise whilst keeping the temperature below 30°C. The reaction mixture was allowed to stir at 25°C for 1 hour, when disappearance of starting material was observed by HPLC. The mixture was filtered through cotton wool. The filter cake was washed with DCM (500 mL). The filtrate was washed with aqueous acetic acid (10%, 2.5 L) at 10°C, followed by brine (2.5 L). The organic phase was dried with anhydrous sodium sulphate and concentrated under vacuum to give the product **29** as a yellow oil which was found sufficiently pure to be used in the next step (285 g, 92.2% yield). RT: 11.002 min. MS (ES⁺) *m/z* (relative intensity) 663.4 ([*M* + *H*]⁺, 100);

25

Analytical method used for compound 28 and 29

Column: Agilent Poroshell 120 EC- C18 4.6*100mm,2.7um

30 Mobile phase A: 0.05% TFA in Water

Mobile phase B: 0.05% TFA in ACN

Diluent: ACN

Flow rate: 1.0 mL/min

Injection volume: 2 µL

Column temperature: 40 °C

5 Detector: 220 nm

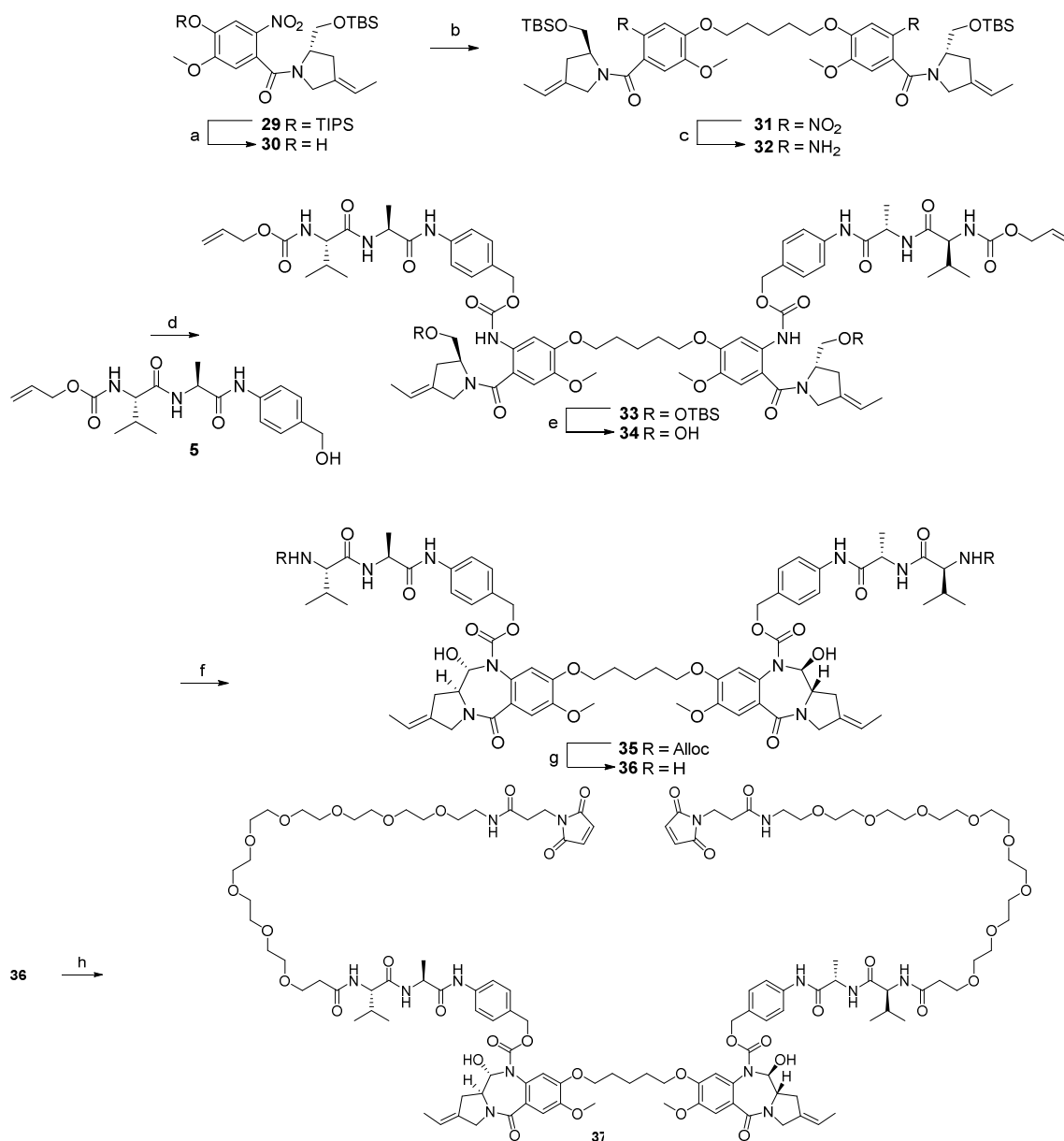
Run Time: 12.1 minutes

Post time: 2 minutes

Gradient Table

Time (min)	0.0	6.0	12.0	12.1
% Mobile Phase A	80	0	0	80
% Mobile Phase B	20	100	100	20

- (ii) Synthesis of bis(4-((2S,5S)-37-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-isopropyl-2-methyl-4,7,35-trioxo-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl) 8,8'-(pentane-1,5-diylbis(oxy))((2E,2'E,11S,11aS,11'S,11a'S)-bis(2-ethylidene-11-hydroxy-7-methoxy-5-oxo-2,3,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate) (**37**)



(a) *(S,E)*-2-(((*tert*-butyldimethylsilyl)oxy)methyl)-4-ethylidenepyrrolidin-1-yl)(4-hydroxy-5-methoxy-2-nitrophenyl)methanone (**30**)

TIPS protected phenol **29** (10.0 g, 16.9 mmol) was dissolved in a mixture of ethyl acetate (20.0 mL) and DMF (20.0 mL) at 40°C. A solution of lithium acetate (0.668 g, 10.1 mmol, 5 0.6 eq) in water (3.0 mL) was added. The reaction was allowed to proceed at 40°C for 4h at which point completion was observed by LCMS. The reaction mixture was partitioned between 2-MeTHF (200 mL) and 2% citric acid in water (200 mL). The organic phase was washed with brine (70 mL) and dried over magnesium sulfate. The volatiles were removed under vacuum. The solid residue was precipitated by addition of diethyl ether (50 mL) and 10 hexane (200 mL). The product was collected by filtration, washed with a little diethyl ether and dried overnight under vacuum to give **30** as a pale yellow solid. (5.8 g, 13 mmol, 79% Yield). Analytical Data: LC/MS, 3 min lipophilic method, RT 1.82 min; MS (ES⁺) *m/z* (relative intensity) 437.8 ($[M + H]^+$, 100);

15 (b) *((pentane-1,5-diylbis(oxy))bis(5-methoxy-2-nitro-4,1-phenylene))bis(((S,E)*-2-(((*tert*-butyldimethylsilyl)oxy)methyl)-4-ethylidenepyrrolidin-1-yl)methanone) (**31**)
1,5-dibromopentane (0.986 g, 4.29 mmol, 0.5 eq) followed by potassium carbonate (1.30 g, 9.41 mmol, 1.1 eq) was added to a solution of **30** (3.74 g, 8.57 mmol) and tetrabutylammonium iodide (0.63 g, 1.7 mmol, 0.2 eq) in acetone (20.0 mL) in a 100 mL 20 round-bottomed flask. The reaction mixture was stirred rapidly and heated at 60 °C for 2h, and then allowed to stir at 45 °C overnight. The reaction was found complete by LCMS. The mixture was partitioned in ethyl acetate (150 mL) and water (200 mL, then washed with brine (100 mL), dried over magnesium sulfate. The volatiles were removed under vacuum to give the product **31** (4.04 g, 4.29 mmol, 100% Yield), which was used in the 25 next step without further purification. Analytical Data: LC/MS, 3 min lipophilic method, RT 2.39 min; MS (ES⁺) *m/z* (relative intensity) 942.3 ($[M + H]^+$, 100);

(c) *((pentane-1,5-diylbis(oxy))bis(2-amino-5-methoxy-4,1-phenylene))bis(((S,E)*-2-(((*tert*-butyldimethylsilyl)oxy)methyl)-4-ethylidenepyrrolidin-1-yl)methanone) (**32**)

30 Zinc (20.6 g, 315 mmol, 74 eq) was added to a mixture of ethanol (64.0 mL), water (4.00 mL), and formic acid (4.00 mL, 106 mmol, 25 eq) at 10 °C (ice bath), and stirred vigorously. To this mixture, a solution of **31** (4.00 g, 4.25 mmol) in ethanol (16.0 mL) was added dropwise with a pipette, whilst keeping the temperature below 35 °C. The zinc mass was occasionally stirred manually. The reaction was allowed to proceed further for 30 min at 35 room temperature, when completion was reached. The mixture was diluted with ethyl acetate (200 mL). The solids were removed by filtration over celite. The sinter was rinsed

with ethyl acetate (200 mL). The filtrate was washed with water (300 mL), saturated sodium bicarbonate (150 mL), brine (100 mL), and dried over magnesium sulphate. The volatiles were removed by evaporation and the residue was purified by automated flash chromatography (100 g ultra, biotage, ethyl acetate / hexane gradient from 30% up 80% in 8 CV, elution from 58% from 10 CV, to give **32** (1.94 g, 2.20 mmol, 51.8% Yield) as a pale yellow foam. Analytical Data: LC/MS, 3 min lipophilic method, RT 2.29 min; MS (ES⁺) *m/z* (relative intensity) 882.4 ($[M + H]^+$, 100);

(d) *bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-*

10 *methylbutanamido)propanamido)benzyl) ((pentane-1,5-diylbis(oxy))bis(6-((S,E)-2-(((tert-butyl)dimethylsilyl)oxy)methyl)-4-ethylidenepyrrolidine-1-carbonyl)-4-methoxy-3,1-phenylene))dicarbamate (**33**)*

Triphosgene (0.461 g, 1.55 mmol, 0.72 eq) was added in one portion to a mixture of **32** (1.90 g, 2.16 mmol) and triethylamine (1.32 g, 13.0 mmol, 6 eq) in DCM (45 mL) at 0°C.

15 The ice batch was removed, and after 15 min, **5** (1.79 g, 4.74 mmol, 2.2 eq) was added in one portion as a fine powder, followed by triethylamine (0.661 g, 6.53 mmol, 3 eq) and dibutyltin dilaurate (0.129 mL, 0.215 mmol, 0.1 eq). The reaction mixture was allowed to stir at 37 °C for 4h, followed by stirring at room temperature overnight. The organic phase was diluted with DCM (100 mL) and washed with water (200 mL), saturated ammonium chloride (100 mL), and brine (50 mL), followed by drying over magnesium sulfate. The volatiles were removed by evaporation under reduced pressure to give **3** (3.00 g, 1.78 mmol, 82% Yield). The crude product was reacted directly in the next step.

20 Analytical Data: LC/MS, 3 min lipophilic method, RT 2.31 min; MS (ES⁺) *m/z* (relative intensity) 1689.6 ($[M + H]^+$, 100);

25

(e) *bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-*

*methylbutanamido)propanamido)benzyl) ((pentane-1,5-diylbis(oxy))bis(6-((S,E)-4-ethylidene-2-(hydroxymethyl)pyrrolidine-1-carbonyl)-4-methoxy-3,1-phenylene))dicarbamate (**34**)*

30 Bis-TBS ether **33** (3.00 g, 1.78 mmol) was dissolved in a mixture of 2-methyltetrahydrofuran (9 mL), acetic acid (9 mL) and water (1.5 mL). The mixture was heated at 40 °C for 2h. LCMS monitoring indicated an unsatisfactory rate of reaction (40 %) completion. Paratoluenesulfonic acid hydrate (203 mg, 1.07 mmol, 0.6 eq) was added, which accelerated the reaction. Completion was observed in 30 min.

35 The reaction mixture was partitioned between ethyl acetate (150 mL) and water (200 mL), then washed with saturated NaHCO₃ (150 mL), and brine (100 mL). The organics were

dried over magnesium sulfate and concentrated under vacuum. The residue was purified by chromatography (50 g ultra, dry loaded on loose silica gel, ethyl acetate / acetone, gradient from 85/15 to 0/100, elution around 55% acetone. Pure fractions were combined and concentrated under vacuum to give the pure product **34** (2.20 g, 1.51 mmol, 84.8% Yield) as a white solid. Analytical Data: LC/MS, 3 min lipophilic method, RT 1.67 min; MS (ES⁺) *m/z* (relative intensity) 1461.6 ([*M* + *H*]⁺, 100);

(f) *bis*(4-((*S*)-2-((*S*)-2-((allyloxy)carbonyl)amino)-3-

methylbutanamido)propanamido)benzyl) 8,8'-(pentane-1,5-

diylbis(oxy))(2*E*,2'*E*,11*S*,11*aS*,11'*S*,11*a'S*)-*bis*(2-ethylidene-11-hydroxy-7-methoxy-5-oxo-2,3,11,11*a*-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepine-10(5*H*)-carboxylate) (**35**)

Stahl Tempo 0.2M Solution (3.50 mL, 0.700 mmol, 0.47 eq) followed by tetrakisacetonitrile copper(I) triflate (290 mg, 0.770 mmol, 0.52 eq) was added to a solution of **34** (2.17 g, 1.49 mmol) in DMF (3.00 mL) in a 500 mL flask. The reaction mixture was stirred rapidly and heated at 40 °C for 5h (completion), followed by 30 °C for 18h under an air balloon, at which point the reaction mixture was diluted with dichloromethane (60 mL) and water (60 mL) and stirred for 5 min. The reaction mixture was decanted in a phase separator and the DCM phase was dried under reduce pressure. MEK (60 mL) was added and the residue DMF was removed by azeotrope under reduce pressure (2 times) to give the crude product as a solid. This was redissolved in DCM (5 to 10 mL) and loaded on a 100g Ultra column. Gradient from 75/25 DCM/10% MeOH in DCM up to 40/60 (elution around 50/50). The pure fractions were combined to give **35** (1.35 g, 0.927 mmol, 62.4% Yield) as a white product. Analytical Data: LC/MS, 3 min lipophilic method, RT 1.63 min; MS (ES⁺) *m/z* (relative intensity) 1457.3 ([*M* + *H*]⁺, 100); 15 min method, RT 7.52 min; MS (ES⁺) *m/z* (relative intensity) 1456.6 ([*M* + *H*]⁺, 100);

(g) *bis*(4-((*S*)-2-((*S*)-2-amino-3-methylbutanamido)propanamido)benzyl) 8,8'-(pentane-1,5-diylbis(oxy))(2*E*,2'*E*,11*S*,11*aS*,11'*S*,11*a'S*)-*bis*(2-ethylidene-11-hydroxy-7-methoxy-5-oxo-2,3,11,11*a*-tetrahydro-1*H*-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepine-10(5*H*)-carboxylate) (**36**)

Tetrakis(triphenylphosphine)palladium(0) (10.0 mg, 0.0086 mmol, 0.01 eq) was added to a mixture of **35** (1.33 g, 0.914 mmol) and pyrrolidine (190 µL, 2.28 mmol, 2.5 eq) in DCM (7.50 mL) and methanol (0.5 mL). The reaction mixture was stirred under argon at room temperature for 1h30 minutes and was found complete by LCMS. Ammonium chloride in water (30 mL, 6 mass%) was added and the mixture was stirred vigorously. The mixture was then decanted in a biotage phase separation cartridge. The DCM layer was evaporated to dryness under vacuum. The residue was dissolved in chloroform (20 mL)

and the solvent removed by evaporation under vacuum at 35°C. This cycle was repeated a second time, followed by drying under hard vacuum (3 mbar) to give crude **36** (1.17 g, 0.914 mmol, 100% Yield) as white solid. Analytical Data: LC/MS, 3 min method, RT 1.23 min, 2 peaks; MS (ES⁺) *m/z* (relative intensity) 645.0 ([*M* + 2H]²⁺, 100); 1288.8 ([*M* + H]⁺, 10).

(h) *bis(4-((2S,5S)-37-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-isopropyl-2-methyl-4,7,35-trioxo-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl) 8,8'-(pentane-1,5-diylbis(oxy))(2E,2'E,11S,11aS,11'S,11a'S)-bis(2-ethylidene-11-hydroxy-7-methoxy-5-oxo-2,3,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate) (37)*

DCM (10.00 mL) and methanol (0.4 mL) were added to **36** (393 mg, 0.305 mmol), followed by mal-amido-peg8-acid (380 mg, 0.628 mmol, 2.06 eq) and EDCI (128 mg, 0.668 mmol, 2.2 eq). The reaction was allowed to proceed at room temperature for 4h when completion was observed by LCMS. Ammonium chloride in water (30 mL, 6 mass%) was added and the mixture was stirred vigorously. The mixture was decanted in a biotage phase separation cartridge. The DCM layer was evaporated to dryness under vacuum and the crude residue was purified by chromatography (25g Ultra gradient 15/85 to 100/0 of 20% MeOH in DCM / DCM in 12CV; hold at elution around 48%). The fractions were analysed by TLC (10% MeOH in DCM). The pure fractions were pooled. The solvent was removed by evaporation. The residue was purified further by reverse phase preparative HPLC (gradient 15 to 75% water/acetonitrile + 0.01% formic acid) followed by freeze-drying and aliquoted from DCM to give **37** (516 mg, 0.212 mmol, 69.4% Yield) as a white foam. The purity was 97.65%. Analytical Data: LC/MS, 15 min method, RT 6.61 min; MS (ES⁺) *m/z* (relative intensity) 1219.7 ([*M* + 2H]²⁺, 100); ¹H NMR (400 MHz, DMSO-d₆) δ 9.92 (s, 2H), 8.17 (d, *J* = 6.9 Hz, 2H), 8.01 (t, *J* = 5.6 Hz, 2H), 7.87 (d, *J* = 8.7 Hz, 2H), 7.72 – 7.44 (m, 4H), 7.39 – 7.10 (m, 4H), 7.05 (s, 2H), 7.00 (s, 4H), 6.76 (s, 2H), 6.66 – 6.46 (m, 2H), 5.56 (d, *J* = 7.1 Hz, 2H), 5.34 (dd, *J* = 9.7, 5.9 Hz, 2H), 5.21 – 4.70 (m, 4H), 4.39 (t, *J* = 7.0 Hz, 2H), 4.22 (dd, *J* = 8.7, 6.7 Hz, 2H), 4.15 – 4.01 (m, 2H), 3.94 (d, *J* = 15.3 Hz, 4H), 3.86 – 3.72 (m, 8H), 3.60 (t, *J* = 7.3 Hz, 8H), 3.55 – 3.42 (m, 58H), 3.37 (t, *J* = 5.9 Hz, 4H), 3.15 (q, *J* = 5.8 Hz, 4H), 2.76 – 2.56 (m, 4H), 2.46 (t, *J* = 6.8 Hz, 2H), 2.40 (t, *J* = 6.5 Hz, 2H), 2.36 – 2.29 (m, 4H), 1.96 (q, *J* = 6.7 Hz, 2H), 1.78 (s, 4H), 1.66 (d, *J* = 6.6 Hz, 6H), 1.57 (d, *J* = 8.6 Hz, 2H), 1.30 (d, *J* = 7.0 Hz, 6H), 0.85 (dd, *J* = 15.2, 6.7 Hz, 12H).

Production of Herceptin-Flexmab and NIP228-Flexmab antibodies

General

Cell lines SKBR-3 (HER2⁺, 1.5×10⁶ receptors/cell), MDA-MB-453 (HER2⁺, 7.7×10⁴ receptors/cell), and MCF-7 (HER2⁻) were obtained from ATCC and maintained in T175 tissue culture flasks (Corning) using the manufacturer's recommended media (SKBR-3: 5 McCoy's 5A + 10% FBS, MDA-MB-453: DMEM + 10% FBS, and MCF-7: DMEM + 10% FBS). 293F cells (Invitrogen) used for transfection were maintained in 293F Freestyle media (Invitrogen). SKBR-3, MDA-MB-453, and MCF-7 cells were cultured in a 37°C incubator with 5% CO₂. 293F cells were cultured in shake flasks (2 L, Corning) at 37°C with 8% CO₂ and rotation at 120 rpm. All reagents were purchased from Sigma Aldrich, 10 VWR, or JT Baker unless otherwise specified and used without additional purification.

Design and construction of Herceptin-Flexmab and NIP228-Flexmab antibodies

The Herceptin wild-type antibody was used as the template to engineer the Herceptin-Flexmab. The light chain of the Herceptin-Flexmab consists of two mutations, F118C and 15 C214V, whereas the heavy chain contains three mutations, L124C, C216V, and C225V (see Figures 3 and 5 – in figure 5, C represent engineered cysteines, V represent cysteine to valine mutations and C represents cysteine used for conjugation/rebridging). The F118C mutation in the light chain forms a disulfide bond with the L124C mutation in the 20 heavy chain. This engineered disulfide is not solvent exposed, but serves to preserve the covalent linkage between the light and heavy chains. The C222 hinge cysteine was left unmodified and served as the location for site-specific conjugation with the pBD-based drug linker. The light chain and heavy chain sequences for the Herceptin-Flexmab were codon-optimized for mammalian expression and procured from GeneArt (Life 25 Technologies). The optimized Herceptin-Flexmab construct was subcloned with standard molecular biology techniques using the BssHII/NheI sites (light chain) and the Sall/NotI sites (heavy chain) into a MedImmune proprietary mammalian expression vector which contains an IgG light chain signal peptide for secretion and cytomegalovirus promoters for recombinant expression. The completed mammalian expression plasmid, pOE-Herceptin-Flexmab was confirmed by DNA sequencing. The negative control NIP228-Flexmab 30 antibody was generated as described for the Herceptin-Flexmab while using the wild-type NIP228 antibody (MedImmune proprietary) as a template.

Expression and purification of Herceptin-Flexmab and NIP228-Flexmab antibodies

35 Expression and purification of Herceptin-Flexmab and NIP228-Flexmab antibodies was conducted according to previously published methods (Dimasi, N., *et al.*, *Journal of*

Molecular Biology, 2009, **393**, 672-692; DOI: 10.1016/j.jmb.2009.08.032). Following transient 293F expression and protein-A purification, the antibodies were formulated into conjugation buffer (1X PBS, 0.1 mM EDTA, pH 7.2) using Slide-A-Lyzer dialysis cassettes at 4°C (10 kDa MWCO, Thermo) and concentrated to 8.0 mg/mL (Herceptin-Flexmab) and 5.52 mg/mL (NIP228-Flexmab) using Vivaspin concentrators (10 kDa MWCO, GE Healthcare). Final concentrations were determined using a Nanodrop spectrophotometer (A₂₈₀, Thermo). Transient expression yields after 6 days were 500 mg/L and 150 mg/L for Herceptin-Flexmab and NIP228-Flexmab, respectively.

10 **Example 7 - Construction of Herceptin-Flexmab and NIP228-Flexmab ADCs**

Herceptin-Flexmab (15 mg, 100 nmoles) in conjugation buffer (1X PBS, 1 mM EDTA, pH 7.2, 3 mL) was reduced using TCEP (3 eq., 300 nmoles, Thermo) for 2 h at room temperature. Following reduction, DMSO (10% v/v, 300 µL) was added to the reduced antibody, followed by the addition of compound **10** (3 eq., 300 nmoles). The conjugation reaction was allowed to proceed at room temperature for 3 h. Excess compound **10** was quenched using *N*-acetyl cysteine (5 eq. over compound **10**, 1.5 µmoles, Sigma Aldrich) and the ADC was dialyzed against three buffer exchanges of conjugation buffer at 4°C using a Slide-A-Lyzer dialysis cassette (10 kDa MWCO, Thermo). The ADC was diluted 1:5 with DI-H₂O and loaded onto a type II ceramic hydroxyapatite column (Bio-Rad) at 5 mL/min using an AKTA Pure FPLC (GE Healthcare) and the column was washed with 20 column volumes of CHT buffer A (10 mM NaPO₃, pH 7). Elution of the ADC was performed using a linear gradient of CHT buffer B (0-2 M NaCl in 10 mM NaPO₃, pH 7) over 20 mins. The eluted ADC was dialyzed overnight at 4°C into conjugation buffer using a Slide-A-Lyzer dialysis cassette (10 kDa MWCO) and diluted 1:5 with HIC buffer A (25 mM Tris-HCl, 1.5M (NH₄)₂ SO₄, pH 8). The ADC was loaded onto a semi-preparative hydrophobic interaction chromatography (HIC) column (HiTrap Butyl-S FF, GE Healthcare) at 1 mL/min using an AKTA Pure FPLC and washed with 20 column volumes of HIC buffer A. The ADC was eluted using a linear gradient of HIC buffer B (25 mM Tris-HCl, 5% isopropyl alcohol) over 45 mins at 1 mL/min. Purified Herceptin-Flexmab-**10** was dialyzed into conjugation buffer overnight at 4°C, concentrated using a Vivaspin concentrator (10 kDa MWCO) to 2 mg/mL, and sterile filtered through a 0.2 µm syringe filter (Pall Corporation). The process is shown schematically in Figure 4. The highlighted V's represent valine mutations.

The site-specific conjugation of compound **10** to NIP228-Flexmab and subsequent purification was performed as described for Herceptin-Flexmab-**10**.

Example 8 - Analytical characterization of ADCs

SDS-PAGE

SDS-PAGE was used to confirm the molecular weights of the Herceptin-Flexmab, Herceptin-Flexmab-**10**, NIP228-Flexmab, and NIP228-Flexmab-**10** constructs. Samples (2 μ g, parental or conjugated) were mixed 1:4 with LDS Bolt sample buffer (Invitrogen), 1:10 with NuPAGE reducing buffer (Invitrogen), and heated to 70°C for 10 minutes followed by loading into a 10% Bis-tris Bolt gel (Invitrogen). Gels were electrophoresed at 150 V and stained using Simply Blue staining reagent (Invitrogen) and destained using DI-H₂O. Gels were imaged using a Gel Doc EZ Imaging system (Bio-RAD).

Reduced SDS-PAGE was used to confirm molecular weight of the purified Herceptin-Flexmab and NIP228-Flexmab antibodies and ADCs. The results demonstrates separation of light (LC) and heavy chains (HC) of the Herceptin-Flexmab with molecular weights of ~25 kDa and 50 kDa, respectively. Conjugation of the dual-maleimide compound **10** payload to the Herceptin-Flexmab resulted in very efficient bridging of the heavy chains with the presence of a 100 kDa band. Similar results were observed with the NIP228-Flexmab, with clear identification of the light and heavy chains under reducing conditions. Highly efficient disulfide bridging of the NIP228-Flexmab heavy chains with compound **10** was observed. No aggregation was observed for either antibody or ADC.

Hydrophobic interaction chromatography

Analytical hydrophobic interaction chromatography was used to assess the conjugation efficiencies of compound **10** onto the Herceptin-Flexmab and NIP228-Flexmab antibodies and to evaluate drug-to-antibody ratios (DARs) for each ADC. ADCs (500 μ g in 50 μ L) were individually loaded onto a Proteomix HIC Butyl-NP5 column (4.6 mm I.D. $\times$ 3.5 cm $\times$ 5 μ m, Sepax) using HIC buffer A (25 mM Tris-HCl, 1.5M (NH₄)₂ SO₄, pH 8) and the ADCs were eluted using a linear gradient of HIC buffer B (25 mM Tris-HCl, 5% isopropyl alcohol, pH 7, 5-100%) over 13 mins at 0.8 mL/min. Absorbance was measured at 280_{nm} and 330_{nm} and eluted peaks were manually integrated to determine conjugation efficiency of each ADC. Conjugation efficiencies and DARs were calculated based on Equation 1 and Equation 2, respectively.

Equation 1: Conjugation Efficiency

$$= \left[\frac{Area_{conjugated}}{(Area_{unconjugated} + Area_{conjugated})} \right] \times 100$$

$$\text{Equation 2: DAR} = \left[\frac{\text{Area}_{\text{DAR1}} + 2(\text{Area}_{\text{DAR2}}) + n(\text{Area}_{\text{DAR} \dots n})}{(\text{Area}_{\text{DAR0}} + \text{Area}_{\text{DAR1}} + \text{Area}_{\text{DAR2}} + \text{Area}_{\text{DAR} \dots n})} \right]$$

ADC	Area _{DAR0}	Area _{DAR1}	Area _{DAR2}	Eff _{conj}	DAR
Herceptin-Flexmab3-10	138.4	1285.9	N/A	90.3	0.90
NIP228-Flexmab3-10	118.1	1191.4	N/A	90.1	0.90

Size-exclusion chromatography

- 5 Size-exclusion chromatography HPLC (SEC-HPLC) was conducted on the parental antibodies and ADCs to analyze purity and aggregation using an Agilent 1200 series HPLC. Samples (100 µg in 100 µL conjugation buffer) were injected onto a TSK Gel column (G3000SW, 8 mm I.D. × 30 cm × 5 µm, Tosoh Bioscience) using 0.1 M NaPO₄, 0.1M NaSO₄, 10% isopropanol, pH 6.8 as the mobile phase at a flow rate of 1 mL/min.
- 10 Absorbance of eluted peaks were measured at 280_{nm} followed by manual integration to determine purity and percent aggregation of each sample.

Following protein A purification, each antibody yielded high monomeric contents in excess of 98%, and these characteristic were maintained following the conjugation of the

- 15 compound **10** payload to generate the DAR=1 ADCs. The Herceptin-Flexmab and Herceptin-Flexmab-**10** eluted with retention times (T_R) of 8.65 mins and 8.66 mins, and 9.01 mins, respectively. The NIP228-Flexmab and NIP228-Flexmab-**10** eluted with a T_R=8.52 mins and 8.54 mins respectively.

20 *Reduced reverse-phase HPLC*

- To confirm site-specific conjugation of compound **10** onto the heavy chain of the antibodies, reduced reverse-phase HPLC (rRP-HPLC) was utilized. ADCs were treated with dithiothreitol (DTT, 50 mM) for 30 minutes at room temperature. Following reduction,
- 25 the ADCs were injected onto a PLRP-S column (1000Å, 2.1 mm × 50 mm, Agilent) and eluted using a gradient mobile phase of RP-HPLC solvent A (0.1% trifluoroacetic acid in water) and RP-HPLC solvent B (0.1% trifluoroacetic acid in acetonitrile) consisting of 5% solvent B – 100% solvent B over 25 mins. Gradient elutions were conducted at 80°C using a flow rate of 1 mL/min. Absorbance was measured at 280_{nm}.

The chromatograms of the Herceptin-Flexmab and Herceptin-Flexmab-**10** ADC were overlaid. The light chains of both species co-eluted (Herceptin-Flexmab-**10** T_R = 17.57 mins; Herceptin-Flexmab T_R = 17.54 mins), however there was a marked shift in retention time for the heavy chain of the Herceptin-Flexmab-**10** (T_R = 21.31 mins) when compared to the unconjugated Herceptin-Flexmab antibody (T_R = 19.75 mins). A small amount of unconjugated heavy chain was also visible on the Herceptin-Flexmab-**10** chromatogram (T_R = 19.97 mins).

The chromatograms for the negative controls NIP228-Flexmab and NIP228-Flexmab-**10** were also overlaid for comparative analysis. The heavy chain of the NIP228-Flexmab-**10** ADC showed a shift in retention time (T_R =21.57 mins) when compared to the heavy chain of the NIP228-Flexmab (T_R =20.09 mins). A small amount of unconjugated heavy chain was visible for the NIP228-Flexmab-**10** (T_R =20.35 mins).

Mass spectrometry

Intact and reduced reverse-phase liquid chromatography mass spectrometry (LCMS) was utilized to confirm molecular weights of the Herceptin-Flexmab and NIP228-Flexmab antibodies and ADCs. Approximately 2 μ g (4 μ L) of antibody or ADC was injected onto an Agilent 1200 series HPLC connected in series to an Agilent 6520 Accurate-Mass Time-of-Flight (TOF) LC-MS. The antibody or ADC was loaded onto a Zorbax 300 Diphenyl Rapid Resolution HD column (2.1 mm $\times$ 50 mm $\times$ 1.8 μ m) and eluted using a flow rate of 0.5 mL/min consisting of a step gradient of 1-80% Solvent B (0.1% formic acid in acetonitrile) after 2 mins (Solvent A: 0.1 formic acid in water). Data was acquired and analyzed using MassHunter software (Agilent).

Purified Herceptin-Flexmab produced a peak at 147,985.36 Da (G0f calc: 147,980.8 Da). Following conjugation of the compound **10** payload (MW: 2408.67 Da), LCMS revealed the molecular weight of the Herceptin-Flexmab3-**10** as 150,396.71 Da (G0f calc: 150,394.03 Da). Analysis of the NIP228-Flexmab by LCMS revealed a peak at 146,770.36 Da (G0f calc: 146743.98 Da). Conjugation of the compound **10** payload produced a peak with MW of 149,199.75 Da (G0f calc: 149,152.65 Da).

Differential scanning calorimetry (DSC)

Antibodies and ADCs were extensively dialyzed into 25 mM Histidine pH 6 at 4°C and formulated at 0.5 mg/mL. DSC experiments were carried out using a MicroCal VP-DSC instrument (Malvern). The raw data was normalized for concentration and scan rate

(1°C/min). Data analysis and deconvolution were carried out using the Origin 7 software (Malvern). Deconvolution analysis was conducted using a non-two-state model and the best fits were obtained using 10-15 iteration cycles. The denaturation temperatures, T_m , corresponding to the maximum of the transition peaks were determined for each construct.

5

Results from DSC experiments revealed the T_m transition temperatures for the CH2 and Fab domains of the Herceptin (CH2 T_m 1=68.95°C, Fab T_m 2=81.43°C) and NIP228 (CH2 T_m 1=69.09°C, Fab T_m 2=74.22°C) wild-type antibodies (Wakankar, A.A., *et al.*, *Bioconjugate Chemistry*, 2010, **21**, 1588-1595; DOI: 10.1021/bc900434c). The NIP228 antibody displayed a third T_m transition temperature for the CH3 domain at 81.92°C. The introduction of the Flexmab technology into these antibodies caused these T_m transition temperatures to reverse, with the Fab domain having a lower T_m transition temperature compared to the CH2 domain (Herceptin-Flexmab Fab T_m 1=68.21°C, CH2 T_m 1=81.05°C; NIP228-Flexmab Fab T_m 1=66.58°C, CH2 T_m 3=81.85°C). As seen with the NIP228 wild-type antibody, we observed a third T_m transition temperature on the NIP228-Flexmab for the CH3 domain (T_m 2=76.28°C). As expected, following conjugation of compound **10** to the Herceptin-Flexmab and NIP228-Flexmab antibodies, very minimal changes were observed in the T_m transition temperatures. (Herceptin-Flexmab-**10** Fab T_m 1=67.83°C, CH2 T_m 1=81.11°C; NIP228-Flexmab-**10** Fab T_m 1=66.11°C, CH2 T_m 3=82.19°C). The third T_m transition temperature for the CH3 domain of NIP228-Flexmab-**10** (T_m 2=78.78°C) was minimally changed compared to the NIP228-Flexmab.

Example 9 - *In vitro* characterization of Herceptin-Flexmab and NIP228-Flexmab antibodies and ADCs

25 *Cell binding by flow cytometry*

Binding affinities and specificities of the Herceptin-Flexmab and NIP228-Flexmab ADCs were confirmed using flow cytometry. On the study day, SKBR-3 (HER2⁺) and MCF-7 (HER2⁻) cells were dissociated from their flask with TrypLE (Life Technologies) trypsin, and resuspended in their respective growth media. Cells were counted on a ViCell cell counter (Beckman Coulter) and brought to a concentration of 1×10^6 cells/mL. Cells were transferred in duplicate to wells (5×10^4 cells/well) of a 96-well plate (Falcon) and centrifuged at 1200 rpm at 4°C. Pelleted cells were resuspended in 180 μ L of flow cytometry buffer (PBS pH 7.2, 2% FBS, on ice) and antibody or ADC was individually added to cells (20 μ L of serial dilution: 200 μ g/mL-0.01 μ g/mL; final concentration 20 μ g/mL-0.001 μ g/mL). Antibodies and cells were incubated at 4°C for 1 hour, after which they were washed with flow cytometry buffer and pelleted by centrifugation (2x, 1200 rpm). After the final spin, cell pellets were

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resuspended in AlexaFluor 647-conjugated anti-human secondary antibody (150 μ L, 8 μ g/mL, in PBS pH 7.2, 2% FBS) and incubated at 4°C for 1 hour. Cells were washed with flow cytometry buffer and centrifuged (2x, 1200 rpm), followed by resuspension in 135 μ L of flow cytometry buffer. DAPI was added (15 μ L from 10X stock, 1 μ M final, Sigma Aldrich) to each cell suspension to act as a live/dead stain. Fluorescence data from the cells was collected using a LSRII flow cytometer (Beckton Dickson) and data was analyzed using FlowJo analysis software (Version 9, FlowJo, LLC). Binding curves were generated using GraphPad Prism (Version 6, GraphPad Software, Inc.).

- 10 The Herceptin-Flexmab-**10** ADC showed high affinity (EC_{50} =0.24 μ g/mL) and selectivity to the SKBR-3 cell line whereas no binding was observed towards the MCF-7 cell line.

Serum stability studies

- 15 Mouse serum (Jackson Immunoresearch Labs) was filtered through a 0.2 μ m syringe filter (Pall Corporation) into sterile polypropylene tubes and kept on ice. ADC (200 μ g) was added to mouse serum to a final concentration of 200 μ g/mL and samples were incubated at 37°C. PBS was used as a negative control. Aliquots of 200 μ L were taken from each sample at T=0, 24, 72, and 148 hours of incubation. The T=0 time point was placed on dry ice within the first minutes after the addition of ADC to serum. Samples were stored at
- 20 -80°C until subjected to affinity capture and analysis by LCMS. Anti-human IgG (Fc-specific) agarose (Sigma Aldrich) was used to affinity capture the ADCs from mouse serum. For each time point, 50 μ L of anti-human Fc agarose beads were mixed with 300 μ L of PBS and 100 μ L of serum sample for 30 min at room temperature under continuous rotation. The beads were washed three times with 1 $\times$ PBS to remove any unbound serum
- 25 proteins and the ADCs were eluted using 100 μ L IgG elution buffer (Thermo Scientific) and neutralized with 20 μ L 1 M Tris pH 8. Individual samples (20 μ L) were analyzed by LCMS as described above and the raw data was analyzed using Masshunter software.

- After seven days of incubation, LCMS revealed that less than 1% of the compound **10** payload was lost from Herceptin-Flexmab-**10**. Such good stability *in vitro* suggests that off-target toxicity could be reduced *in vivo*.
- 30

Cytotoxicity assays

- SKBR-3, MDA-MD-453, and MCF-7 cells were maintained as described above. On the day prior to treatment, cells were dissociated from their flask with TrypLE trypsin, and
- 35 resuspended in growth media. Cells were counted on a ViCell cell counter and brought to a

concentration of 1.0×10^5 cells/mL in their respective growth media. Cell suspensions (100 μ L, 1.0×10^4 cells/well) were transferred to wells of a white wall, clear bottom 96-well plate (Corning). Cells were allowed to adhere overnight in a 37°C incubator with 5% CO₂. On the treatment day, serial dilutions of ADCs were prepared with a range of 30 μ g/mL- 1.5 ng/mL and 50 μ L of each dilution was added to the wells in triplicate (10 μ g/mL-0.5 ng/mL final ADC concentrations, 150 μ L total volume/well). Appropriate untreated wells were also added to each plate to serve as controls. On day 5, plates were removed from the incubator and allowed to equilibrate to room temperature. Plates were centrifuged (1300 rpm, 5 mins) and supernatants were aspirated. Media (SKBR-3: McCoy's 5A, MDA-MB-453 and MCF-7: DMEM) without phenol red or FBS was added to each well (50 μ L), followed by the addition of CellTiter-Glo® reagent (50 μ L). Plates were shaken for 1 hour in the dark at room temperature and luminescence was measured using a Envision™ plate reader (PerkinElmer). Percent viability was calculated as: (Unknown/Avg controls)*100. Experimental data was plotted using GraphPad Prism to generate IC₅₀ curves.

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After 3 days of incubation on MDA-MB-453 cells (low HER2 expression; 7.7×10^4 HER2 receptors/cell), Herceptin-Flexmab-**10** showed an IC₅₀ = 1.08 nM with ~90% cell viability. After 5 days, Herceptin-Flexmab-**10** had an IC₅₀ = 0.0375 nM with ~35% cell viability.

20

After 3 days of incubation on SKBR-3 cells (high HER2 expression; 1.5×10^6 HER2 receptors/cell) Herceptin-Flexmab-**10** showed an IC₅₀ = 0.229 nM with ~30% cell viability. After 5 days, Herceptin-Flexmab-**10** had an IC₅₀ = 0.0355 nM with ~5% cell viability.

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Example 10 - *In vivo* characterization of Herceptin-Flexmab and NIP228-Flexmab antibodies and ADCs

All studies involving the use of animals were performed humanely under a protocol approved by the MedImmune Institutional Animal Care and Use Committee in a facility accredited by AAALAC International.

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Xenografts

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NCI-N87 cells (5×10^6) in 50% Matrigel were inoculated subcutaneously into 4-6 week old female athymic nude mice (Harlan). When tumors reached 200 mm³, mice were randomized assigned into groups, 5 mice per group. ADCs were administered IV at the indicated doses and dosed at day 5 post cell inoculation. Tumor volumes were measured twice weekly with calipers. The tumor volumes were calculated using the formula $\frac{1}{2} \times L \times W^2$ (L = length; W = width). Body weights were measured to assess tolerability of the

treatments. The tumor growth and body weight curves were plotted using Prism5 software (GraphPad, La Jolla, CA). Tumor volumes are expressed as mean $\pm$ SEM.

Figure 1 shows the effect of a single dose at 1.0 mg/kg (◆) compared to non-treatment (●)

5 – the dose yielded tumor regression followed by tumor stasis for 55 days.

Figure 2 shows the effect of a single dose at 0.3 mg/kg (◆) compared to non-treatment (●)

– the dose also yielded tumor regression followed by tumor stasis for 55 days.

10 Toxicity

Male Sprague Dawley rats (8-12 weeks old, 5 per group) were administered a single IV injection (Day 1) of 0.75, 1.5, 3, or 4 mg/kg of Herceptin-Flexmab-**10** and rats were evaluated for 21 days. Toxicokinetic (TK) satellite animals (3 per group) were included in each treatment arm to measure plasma concentration of total antibody and ADC. Control rats (5 per group) were administered a single IV injection of vehicle control on Day 1. All main study animals were evaluated for clinical signs, changes in body weight, clinical pathology, gross pathology with organ weights, and microscopic observations. All TK satellite animals were evaluated for clinical signs, changes in body weight, and pharmacokinetic analysis. Hematology and serum chemistry samples were collected and analyzed on Days 8 and 15. Additional samples for coagulation analysis were collected and analyzed on Day 22 only. Blood samples for pharmacokinetic analysis were collected in K₂ EDTA tubes prior to dosing and at multiple time points on Days 1, 8, 15, 22. A gross necropsy was performed on all main study animals and a standard list of organs, including brain, lung, liver, kidney, spleen, thymus, testes, heart, and bone, were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically by a board certified veterinary pathologist.

Doses as high as 4 mg/kg of Herceptin-Flexmab-**10** were well tolerated.

30 Therapeutic Index

The Therapeutic Index can be calculated by dividing the maximum tolerated single dose (MTD) of non-targeted ADC in rat, by the minimal effective single dose (MED) of the a targeted ADC. The MED is the single dose necessary to achieve tumour stasis in an *in vivo* model at 28 days (for NCI-N87 xenograft). Thus for Herceptin-Flexmab-**10** the calculated Therapeutic Index is at least 13.3.

Example 11

Herceptin and R347 antibodies engineered to have cysteine inserted between the 239 and 240 positions were produced following the methods described in Dimasi, N., et al., Molecular Pharmaceutics, 2017, 14, 1501-1516 (DOI:

5 10.1021/acs.molpharmaceut.6b00995).

HerC239i-10 ADC

DTT (100 molar equivalent/antibody, 26.7 micromole) was added to a solution of Herceptin-C239i antibody (40 mg, 266.7 nanomole) in PBS, 1 mM EDTA, pH 7.4 and the
10 final volume was made up to 8 mL. The reduction was carried out at room temperature for 4 hrs with gentle shaking, before DTT was removed by spin filtration using Amicon Ultracell 30 kDa MWCO spin filter. (L)-dehydroascorbic acid (DHAA, 20 molar equivalent/antibody, 5.3 micromole, 106.7 μ L at 50 mM in DMSO) was added to the reduced antibody (5 mg/mL, 8 mL) in PBS, 1mM EDTA, pH 7.4, and the reoxidation took place at room
15 temperature for overnight with gentle shaking. The DHAA was removed by filtration through a 0.22 μ m membrane filter, and Compound **10** was added as a DMSO solution (3 molar equivalent/antibody, 0.8 micromole, in 0.9 mL DMSO) to 8.1 mL of the reoxidised antibody (40 mg, 266.7 nanomole) in PBS, 1mM EDTA, pH 7.4, for a 10% (v/v) final DMSO concentration. The solution was left to react at room temperature for 4 hrs with gentle
20 shaking. The conjugation was quenched by the addition of *N*-acetyl cysteine (4 micromoles, 40 μ L at 100 mM), and purified by hydrophobic interaction chromatography using FPLC and HP-Butyl column (5mL) with a gradient run of 1 M (NH₄)₂SO₄, 25 mM Potassium Phosphate pH 6.0, and 25 mM Potassium Phosphate pH 6.0. Fractions containing over 95% DAR1 were pooled, concentrated, buffer exchanged to PBS, pH 7.4,
25 by spin filtration using 15 mL Amicon Ultracell 50 kDa MWCO spin filter, sterile filtered and analysed.

UHPLC analysis on a Shimadzu Prominence system using a Proteomix HIC Butyl-NP5, 5 μ m, non-porous, 4.6x35 mm (Sepax) column eluting with a gradient of 1.5M ammonium sulphate, 25 mM sodium acetate, pH 7.4 and 25 mM sodium acetate, pH 7.4 with 20% acetonitrile (v/v) on a neat sample of HerC239i-10 ADC at 214 nm shows singly
30 conjugated Compound 10 only, consistent with a drug-per-antibody ratio (DAR) of 1.00 molecules of Compound 10 per antibody.

35 UHPLC analysis on a Shimadzu Prominence system using a Tosoh Bioscience TSKgel SuperSW mAb HTP 4 μ m 4.6 x 150 mm column (with a 4 μ m 3.0 x 20 mm guard column)

eluting with 0.3 mL/minute sterile-filtered SEC buffer containing 200 mM potassium phosphate pH 6.95, 250 mM potassium chloride and 10% isopropanol (v/v) on a neat sample of HerC239i-**10** ADC at 280 nm shows a monomer purity of 99%. UHPLC SEC analysis gives a concentration of final HerC239i-**10** ADC at 1.71 mg/mL in 11.1 mL, obtained mass of HerC239i-**10** ADC is 18.9 mg (47% yield).

R347C239i-**10** ADC

DTT (100 molar equivalent/antibody, 133.3 micromole) was added to a solution of R347-Maia antibody (200 mg, 1.33 micromole) in PBS, 1 mM EDTA, pH 7.4 and the final volume was made up to 40 mL. The reduction was carried out at room temperature for 4 hrs with gentle shaking, before DTT was removed by tangential flow filtration (30kDa fiber filter). (L)-dehydroascorbic acid (DHAA, 20 molar equivalent/antibody, 26.7 micromole, 533.3 µL at 50 mM in DMSO) was added to the reduced antibody (4 mg/mL, 50 mL) in PBS, 1mM EDTA, pH 7.4, and the reoxidation took place at room temperature for overnight with gentle shaking. The DHAA was removed by filtration through a 0.22 µm membrane filter, and Compound **10** was added as a DMSO solution (2 molar equivalent/antibody, 2.67 micromole, in 5.6 mL DMSO) to 50.5 mL of the reoxidised antibody (200 mg, 1.33 micromole) in PBS, 1mM EDTA, pH 7.4, for a 10% (v/v) final DMSO concentration. The solution was left to react at room temperature for 4 hrs with gentle shaking. The conjugation was quenched by the addition of *N*-acetyl cysteine (6.7 micromoles, 66.7 µL at 100 mM), and purified by hydrophobic interaction chromatography using FPLC and HP-Butyl column (5mL) with a gradient run of 1 M (NH₄)₂SO₄, 25 mM Potassium Phosphate pH 6.0, and 25 mM Potassium Phosphate pH 6.0. Fractions containing over 95% DAR1 were pooled, concentrated, buffer exchanged to 25 mM Histidine, 200 mM Sucrose, pH 6.0, by spin filtration using 15 mL Amicon Ultracell 50 kDa MWCO spin filter, sterile filtered and analysed.

UHPLC analysis on a Shimadzu Prominence system using a Proteomix HIC Butyl-NP5, 5 µm, non-porous, 4.6x35 mm (Sepax) column eluting with a gradient of 1.5M ammonium sulphate, 25 mM sodium acetate, pH 7.4 and 25 mM sodium acetate, pH 7.4 with 20% acetonitrile (v/v) on a neat sample of R347C239i-**10** ADC at 214 nm shows singly conjugated Compound **10** only, consistent with a drug-per-antibody ratio (DAR) of 1.00 molecules of Compound **10** per antibody.

UHPLC analysis on a Shimadzu Prominence system using a Tosoh Bioscience TSKgel SuperSW mAb HTP 4 µm 4.6 x 150 mm column (with a 4 µm 3.0 x 20 mm guard column)

eluting with 0.3 mL/minute sterile-filtered SEC buffer containing 200 mM potassium phosphate pH 6.95, 250 mM potassium chloride and 10% isopropanol (v/v) on a neat sample of R347C239i-**10** ADC at 280 nm shows a monomer purity of 99%. UHPLC SEC analysis gives a concentration of final R347C239i-**10** ADC at 1.72 mg/mL in 55 mL, obtained mass of R347C239i-**10** ADC is 94.5 mg (47% yield).

1C1C239i-**10** ADC

DTT (100 molar equivalent/antibody, 3.3 micromole) was added to a solution of 1C1-Maia antibody (5 mg, 33.3 nanomole) in PBS, 1 mM EDTA, pH 7.4 and the final volume was made up to 2.5 mL. The reduction was carried out at room temperature for 5 hrs with gentle shaking, before DTT was removed by spin filtration using Amicon Ultracell 30 kDa MWCO spin filter. (L)-dehydroascorbic acid (DHAA, 20 molar equivalent/antibody, 0.67 micromole, 13.3 µL at 50 mM in DMSO) was added to the reduced antibody (2 mg/mL, 2.5 mL) in PBS, 1mM EDTA, pH 7.4, and the reoxidation took place at room temperature for overnight with gentle shaking. The DHAA was removed by filtration through a 0.22 µm membrane filter, and Compound **10** was added as a DMSO solution (3 molar equivalent/antibody, 0.1 micromole, in 0.27 mL DMSO) to 2.5 mL of the reoxidised antibody (5 mg, 33.3 nanomole) in PBS, 1mM EDTA, pH 7.4, for a 10% (v/v) final DMSO concentration. The solution was left to react at room temperature for 5 hrs with gentle shaking. The conjugation was quenched by the addition of *N*-acetyl cysteine (2 micromoles, 39.6 µL at 100 mM), and purified by preparative size exclusion chromatography using FPLC and Superdex 200 26/600 column with PBS pH 7.4 as an elution buffer. Fractions containing over 95% monomers were pooled, concentrated, buffer exchanged to 25 mM Histidine, 200 mM Sucrose, pH 6.0 by spin filtration using 15 mL Amicon Ultracell 50 kDa MWCO spin filter, sterile filtered and analysed.

UHPLC analysis on a Shimadzu Prominence system using a Proteomix HIC Butyl-NP5, 5 µm, non-porous, 4.6x35 mm (Sepax) column eluting with a gradient of 1.5M ammonium sulphate, 25 mM sodium acetate, pH 7.4 and 25 mM sodium acetate, pH 7.4 with 20% acetonitrile (v/v) on a neat sample of 1C1C239i-**10** ADC at 214 nm shows unconjugated antibody and a mixture of singly conjugated and doubly conjugated Compound **10**, consistent with a drug-per-antibody ratio (DAR) of 1.04 molecules of Compound **10** per antibody.

UHPLC analysis on a Shimadzu Prominence system using a Tosoh Bioscience TSKgel SuperSW mAb HTP 4 µm 4.6 x 150 mm column (with a 4 µm 3.0 x 20 mm guard column)

eluting with 0.3 mL/minute sterile-filtered SEC buffer containing 200 mM potassium phosphate pH 6.95, 250 mM potassium chloride and 10% isopropanol (v/v) on a neat sample of 1C1C239i-**10** ADC at 280 nm shows a monomer purity of 100%. UHPLC SEC analysis gives a concentration of final 1C1C239i-**10** ADC at 1.45 mg/mL in 2.3 mL, obtained mass of 1C1C239i-**10** ADC is 3.34 mg (67% yield).

HerC239i-**11** ADC

DTT (100 molar equivalent/antibody, 3.3 micromole) was added to a solution of Herceptin-Maia antibody (5 mg, 33.3 nanomole) in PBS, 1 mM EDTA, pH 7.4 and the final volume was made up to 2.5 mL. The reduction was carried out at room temperature for 5 hrs with gentle shaking, before DTT was removed by spin filtration using Amicon Ultracell 30 kDa MWCO spin filter. (L)-dehydroascorbic acid (DHAA, 20 molar equivalent/antibody, 0.67 micromole, 13.3 µL at 50 mM in DMSO) was added to the reduced antibody (2 mg/mL, 2.5 mL) in PBS, 1mM EDTA, pH 7.4, and the reoxidation took place at room temperature for overnight with gentle shaking. The DHAA was removed by filtration through a 0.22 µm membrane filter, and Compound **11** was added as a DMSO solution (1.5 molar equivalent/antibody, 0.05 micromole, in 0.27 mL DMSO) to 2.5 mL of the reoxidised antibody (5 mg, 33.3 nanomole) in PBS, 1mM EDTA, pH 7.4, for a 10% (v/v) final DMSO concentration. The solution was left to react at room temperature for overnight with gentle shaking. The conjugation was quenched by the addition of *N*-acetyl cysteine (2 micromoles, 39.6 µL at 100 mM), and purified by preparative size exclusion chromatography using FPLC and Superdex 200 26/600 column with PBS pH 7.4 as an elution buffer. Fractions containing over 95% monomers were pooled, concentrated, buffer exchanged to 25 mM Histidine, 200 mM Sucrose, pH 6.0 by spin filtration using 15 mL Amicon Ultracell 50 kDa MWCO spin filter, sterile filtered and analysed.

UHPLC analysis on a Shimadzu Prominence system using a Proteomix HIC Butyl-NP5, 5 µm, non-porous, 4.6x35 mm (Sepax) column eluting with a gradient of 1.5M ammonium sulphate, 25 mM sodium acetate, pH 7.4 and 25 mM sodium acetate, pH 7.4 with 20% acetonitrile (v/v) on a neat sample of HerC239i-**11** ADC at 214 nm shows unconjugated antibody and a mixture of singly conjugated and doubly conjugated Compound **11**, consistent with a drug-per-antibody ratio (DAR) of 1.10 molecules of Compound **11** per antibody.

UHPLC analysis on a Shimadzu Prominence system using a Tosoh Bioscience TSKgel SuperSW mAb HTP 4 µm 4.6 x 150 mm column (with a 4 µm 3.0 x 20 mm guard column)

eluting with 0.3 mL/minute sterile-filtered SEC buffer containing 200 mM potassium phosphate pH 6.95, 250 mM potassium chloride and 10% isopropanol (v/v) on a sample of HerC239i-11 ADC at 280 nm shows a monomer purity of 99%. UHPLC SEC analysis gives a concentration of final HerC239i-11 ADC at 1.29 mg/mL in 4.0 mL, obtained mass of
5 HerC239i-11 ADC is 3.23 mg (65% yield).

1C1C239i-11 ADC

DTT (100 molar equivalent/antibody, 3.3 micromole) was added to a solution of 1C1-Maia antibody (5 mg, 33.3 nanomole) in PBS, 1 mM EDTA, pH 7.4 and the final volume was
10 made up to 2.5 mL. The reduction was carried out at room temperature for 5 hrs with gentle shaking, before DTT was removed by spin filtration using Amicon Ultracell 30 kDa MWCO spin filter. (L)-dehydroascorbic acid (DHAA, 20 molar equivalent/antibody, 0.67 micromole, 13.3 µL at 50 mM in DMSO) was added to the reduced antibody (2 mg/mL, 2.5 mL) in PBS, 1mM EDTA, pH 7.4, and the reoxidation took place at room temperature for
15 overnight with gentle shaking. The DHAA was removed by filtration through a 0.22 µm membrane filter, and Compound 11 was added as a DMSO solution (1.5 molar equivalent/antibody, 0.05 micromole, in 0.27 mL DMSO) to 2.5 mL of the reoxidised antibody (5 mg, 33.3 nanomole) in PBS, 1mM EDTA, pH 7.4, for a 10% (v/v) final DMSO concentration. The solution was left to react at room temperature for overnight with gentle
20 shaking. The conjugation was quenched by the addition of *N*-acetyl cysteine (2 micromoles, 39.6 µL at 100 mM), and purified by preparative size exclusion chromatography using FPLC and Superdex 200 26/600 column with PBS pH 7.4 as an elution buffer. Fractions containing over 95% monomers were pooled, concentrated, buffer exchanged to 25 mM Histidine, 200 mM Sucrose, pH 6.0 by spin filtration using 15 mL
25 Amicon Ultracell 50 kDa MWCO spin filter, sterile filtered and analysed.

UHPLC analysis on a Shimadzu Prominence system using a Proteomix HIC Butyl-NP5, 5 µm, non-porous, 4.6x35 mm (Sepax) column eluting with a gradient of 1.5M ammonium sulphate, 25 mM sodium acetate, pH 7.4 and 25 mM sodium acetate, pH 7.4 with 20%
30 acetonitrile (v/v) on a neat sample of 1C1C239i-11 ADC at 214 nm shows unconjugated antibody and a mixture of singly conjugated and doubly conjugated Compound 11, consistent with a drug-per-antibody ratio (DAR) of 1.05 molecules of Compound 11 per antibody.

35 UHPLC analysis on a Shimadzu Prominence system using a Tosoh Bioscience TSKgel SuperSW mAb HTP 4 µm 4.6 x 150 mm column (with a 4 µm 3.0 x 20 mm guard column)

eluting with 0.3 mL/minute sterile-filtered SEC buffer containing 200 mM potassium phosphate pH 6.95, 250 mM potassium chloride and 10% isopropanol (v/v) on a sample of 1C1C239i-11 ADC at 280 nm shows a monomer purity of 99%. UHPLC SEC analysis gives a concentration of final 1C1C239i-11 ADC at 1.50 mg/mL in 2.2 mL, obtained mass of

5 1C1C239i-11 ADC is 3.3 mg (66% yield).

Additional conjugations were carried out to the following antibodies with Compound 10: RSV- C239i; B7H4-E02- C239i; PSMA- C239i; and CDH6-50B- C239i.

10 **Example 12**

In vitro PC3 1C1 assay

Medium from sub-confluent (80-90% confluency) PC3 cells in a T75 flask was aspirated and the flask rinsed with PBS (about 20 ml) and emptied. Trypsin-EDTA (5 ml) was added, the flask returned to the 37 °C gassed incubator for up to about 5 minutes, then rapped

15 sharply to dislodge and dissociate cells from the plastic. The cell suspension was transferred to a sterile 50 ml screw-top centrifuge tube, diluted with growth medium to a final volume of 15 ml, then centrifuged (400g for 5 min). The supernatant was aspirated and the pellet re-suspended in 10ml culture medium. Repeated pipetting may be necessary to produce monodisperse cell suspensions. The cell concentration and viability are

20 measured of trypan blue cell stained cells, using the LUNA II. Cells were diluted to 1500 cells/well, dispensed (50 µl /well) into white 96 well flat bottom plates and incubated overnight before use.

A stock solution (1 ml) of antibody drug conjugate (ADC) (20µg/ml) was made by dilution of

25 filter-sterilised ADC into cell culture medium. A set of 8x 10-fold dilutions of stock ADC were made in a 24 well plate by serial transfer of 100 µl onto 900 µl of cell culture medium. ADC dilution was dispensed (50 µl/ well) into 4 replicate wells of the 96-well plate, containing 50 µl cell suspension seeded the previous day. Control wells received 50 µl cell culture medium. The 96-well plate containing cells and ADCs was incubated at 37 °C in a

30 CO₂-gassed incubator for 6 days. At the end of the incubation period, plates were equilibrated to room temperature for 30min before CellTiter-Glo (Promega) was dispensed (100 µl per well) into each well. Plates were placed on an orbital shaker for 2 min before stabilisation at room temperature for 10 min. Well luminescence was measured and percentage cell survival was calculated from the mean luminescence in the 4 ADC-treated

35 wells compared to the mean luminescence in the 4 control untreated wells (100%). IC₅₀ was determined from the dose-response data using GraphPad Prism using the non-linear

curve fit algorithm: sigmoidal dose response, X is log(concentration). Cell growth medium for PC3 was: F12K with glutamine, 10% (v/v) HyClone™ Fetal Bovine Serum.

ADC	EC50 (µg/ml)
1C1C239i- 10 ADC	0.002247
1C1C239i- 11 ADC	0.003987

5 *In vitro* MTS assay

The in vitro activity of ADCs was measured in the Her2-expressing cell line NCI-N87 and the Her2 negative cell line MDA-MB-468.

10 The concentration and viability of cells from a sub-confluent (80-90% confluency) T75 flask are measured by trypan blue staining, and counted using the LUNA-II™ Automated Cell Counter. Cells were diluted to 2×10^5 /ml, dispensed (50 µl per well) into 96-well flat-bottom plates.

15 A stock solution (1 ml) of antibody drug conjugate (ADC) (20 µg/ml) was made by dilution of filter-sterilised ADC into cell culture medium. A set of 8x 10-fold dilutions of stock ADC were made in a 24-well plate by serial transfer of 100 µl into 900 µl of cell culture medium. ADC dilution was dispensed (50 µl per well) into 4 replicate wells of the 96-well plate, containing 50 µl cell suspension seeded the previously. Control wells received 50 µl cell culture medium. The 96-well plate containing cells and ADCs was incubated at 37C in a CO₂-gassed incubator for the exposure time.

20 At the end of the incubation period, cell viability was measured by MTS assay. MTS (Promega) was dispensed (20 µl per well) into each well and incubated for 4 hours at 37C in the CO₂-gassed incubator. Well absorbance was measured at 490 nm. Percentage cell survival was calculated from the mean absorbance in the 4 ADC-treated wells compared to the mean absorbance in the 4 control untreated wells (100%). IC₅₀ was determined from
25 the dose-response data using GraphPad Prism using the non-linear curve fit algorithm: sigmoidal dose-response curve with variable slope.

ADC incubation times were 4 days with MDA-MB-468 and 7 days for NCI-N87. MDA-MB-468 and NCI-N87 were cultured in RPMI 1640 with Glutamax + 10% (v/v) HyClone™ Fetal Bovine Serum.

EC50 (µg/ml)	NCI-N87	MDA-MB-468
HerC239i-10 ADC	0.0002893	14.9
HerC239i-11 ADC	0.0005823	11.4

Example 13*Mice*

Female severe combined immune-deficient mice (Fox Chase SCID®, C.B-17/lcr-Prkdcscid, Charles River) were ten weeks old with a body weight (BW) range of 16.2 to 21.9 grams on Day 1 of the study. The animals were fed ad libitum water (reverse osmosis, 1 ppm Cl), and NIH 31 Modified and Irradiated Lab Diet® consisting of 18.0% crude protein, 5.0% crude fat, and 5.0% crude fibre. The mice were housed on irradiated Enricho'cobs™ Laboratory Animal Bedding in static micro-isolators on a 12-hour light cycle at 20–22°C (68–72°F) and 40–60% humidity. CR Discovery Services specifically complies with the recommendations of the Guide for Care and Use of Laboratory Animals with respect to restraint, husbandry, surgical procedures, feed and fluid regulation, and veterinary care. The animal care and use program at CR Discovery Services is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), which assures compliance with accepted standards for the care and use of laboratory animals.

JIMT-1 Xenografts*Tumour Cell Culture*

JIMT-1 human breast carcinoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G sodium, 100 µg/mL streptomycin sulfate, and 25 µg/mL gentamicin. The cells were cultured in tissue culture flasks in a humidified incubator at 37 °C, in an atmosphere of 5% CO₂ and 95% air.

25

In Vivo Implantation and Tumour Growth

On the day of implant, JIMT-1 cells were harvested during log phase growth and resuspended in phosphate buffered saline (PBS) at a concentration of 1×10^8 cells/ml in 50% Matrigel™ (BD Biosciences). Xenografts were initiated by subcutaneously implanting 1×10^7 JIMT-1 cells (0.1 ml suspension) into the right flank of each test animal. Tumours were monitored as their volumes approached the target range of 100 to 150 mm³ and were

30

measured in two dimensions using calipers. Tumour volume was calculated using the formula:

$$\text{Tumour Volume (mm}^3\text{)} = \frac{w^2 \times l}{2}$$

- where w = width and l = length, in mm, of the tumour. Tumour weight may be estimated with the assumption that 1 mg is equivalent to 1 mm³ of tumour volume.

Treatment

- Fourteen days after tumour implantation, designated as Day 1 of the study, the animals were sorted into groups ($n=10$) with individual tumour volumes of 75 to 162 mm³ and group mean tumour volumes of 115 to 117 mm³.

HerC239i-**10** ADC and HerC239i-**SG3249** ADC were administered intravenously in a single dose of 0.3 mg/kg on Day 1. A vehicle-treated group served as tumour growth controls. Tumours were measured twice per week.

15

HerC239i-**SG3249** is a conjugate made from SG32349, as described, for example, in Dimasi, N., et al., Molecular Pharmaceutics, 2017, 14, 1501-1516 (DOI: 10.1021/acs.molpharmaceut.6b00995) and has a DAR of ???

- 20 The effect on tumour volume is shown in Figure 6 where:

Vehicle	●
HerC239i- SG3249	◆
HerC239i- 10 ADC	▲

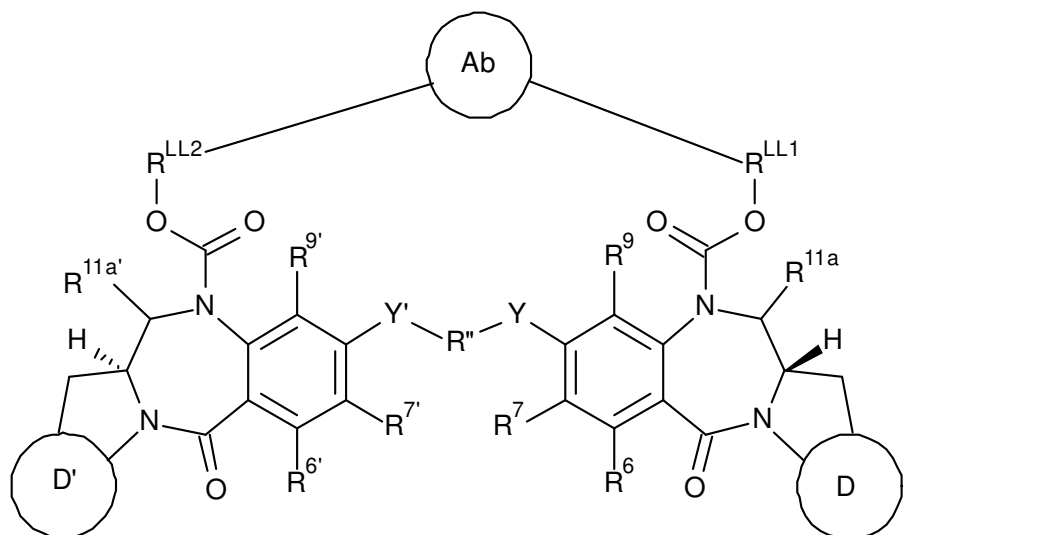
HerC239i-**10** ADC demonstrates an equivalent activity to HerC239i-**SG3249** ADC, despite only have half as many PBD dimer warheads.

25

All documents and other references mentioned above are herein incorporated by reference.

CLAIMS

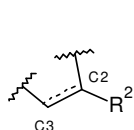
1. A conjugate of formula I:



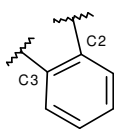
- 5 Wherein

Ab is a modified antibody having at least one free conjugation site on each heavy chain

D represents either group D1 or D2:



D1



D2

;

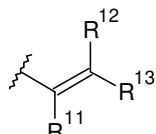
the dotted line indicates the optional presence of a double bond between C2 and C3;

- 10 when there is a double bond present between C2 and C3, R² is selected from the group consisting of:

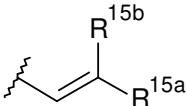
(ia) C₅₋₁₀ aryl group, optionally substituted by one or more substituents selected from the group comprising: halo, nitro, cyano, ether, carboxy, ester, C₁₋₇ alkyl, C₃₋₇ heterocyclyl and bis-oxy-C₁₋₃ alkylene;

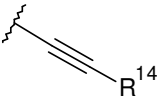
- 15 (ib) C₁₋₅ saturated aliphatic alkyl;

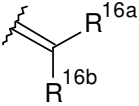
(ic) C₃₋₆ saturated cycloalkyl;



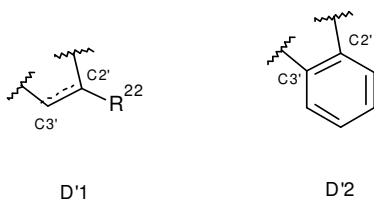
- (id) , wherein each of R¹¹, R¹² and R¹³ are independently selected from H, C₁₋₃ saturated alkyl, C₂₋₃ alkenyl, C₂₋₃ alkynyl and cyclopropyl, where the total number of carbon atoms in the R² group is no more than 5;

(ie)  , wherein one of R^{15a} and R^{15b} is H and the other is selected from: phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl; and

(if)  , where R¹⁴ is selected from: H; C₁₋₃ saturated alkyl; C₂₋₃ alkenyl; C₂₋₃ alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl; when there is a single bond present between C2 and C3,

R² is selected from H, OH, F, diF and  , where R^{16a} and R^{16b} are independently selected from H, F, C₁₋₄ saturated alkyl, C₂₋₃ alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C₁₋₄ alkyl amido and C₁₋₄ alkyl ester; or, when one of R^{16a} and R^{16b} is H, the other is selected from nitrile and a C₁₋₄ alkyl ester;

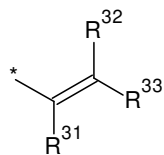
D' represents either group D'1 or D'2:



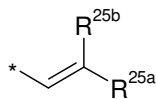
wherein the dotted line indicates the optional presence of a double bond between C2' and C3';

when there is a double bond present between C2' and C3', R²² is selected from the group consisting of:

- (iia) C₅₋₁₀ aryl group, optionally substituted by one or more substituents selected from the group comprising: halo, nitro, cyano, ether, carboxy, ester, C₁₋₇ alkyl, C₃₋₇ heterocyclyl and bis-oxy-C₁₋₃ alkylene;
- (iib) C₁₋₅ saturated aliphatic alkyl;
- (iic) C₃₋₆ saturated cycloalkyl;

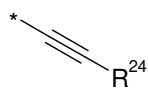


(iid) , wherein each of R^{31} , R^{32} and R^{33} are independently selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl, where the total number of carbon atoms in the R^{22} group is no more than 5;



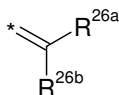
(iie) , wherein one of R^{25a} and R^{25b} is H and the other is selected from:

- 5 phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl; and



(iif) , where R^{24} is selected from: H; C_{1-3} saturated alkyl; C_{2-3} alkenyl; C_{2-3} alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl;

- 10 when there is a single bond present between $C2'$ and $C3'$,



R^{22} is selected from H, OH, F, diF and , where R^{26a} and R^{26b} are independently selected from H, F, C_{1-4} saturated alkyl, C_{2-3} alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C_{1-4} alkyl amido and C_{1-4} alkyl ester; or, when one of R^{26a} and R^{26b} is H, the other is selected from nitrile and a C_{1-4} alkyl ester;

- 15 R^6 and R^9 are independently selected from H, R, OH, OR, SH, SR, NH_2 , NHR, NRR' , nitro, Me_3Sn and halo;

where R and R' are independently selected from optionally substituted C_{1-12} alkyl, C_{3-20} heterocyclyl and C_{5-20} aryl groups;

R^7 is selected from H, R, OH, OR, SH, SR, NH_2 , NHR, NRR' , nitro, Me_3Sn and halo;

- 20 R'' is a C_{3-12} alkylene group, which chain may be interrupted by one or more heteroatoms, e.g. O, S, NR^{N2} , where R^{N2} is H or C_{1-4} alkyl, and/or aromatic rings, e.g. benzene or pyridine;

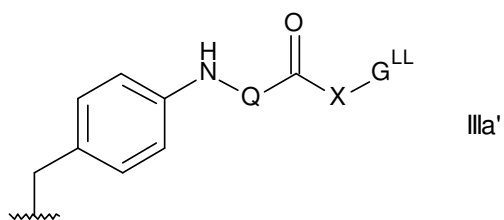
Y and Y' are selected from O, S, or NH;

R^{11a} is selected from OH, OR^A , where R^A is C_{1-4} alkyl;

- 25 R^6 , R^7 , R^9 and $R^{11a'}$ are selected from the same groups as R^6 , R^7 , R^9 and R^{11a} respectively;

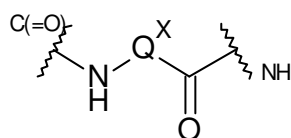
and

R^{LL1} and R^{LL2} are linkers connected to the antibody at different sites which are independently of formula IIIa':



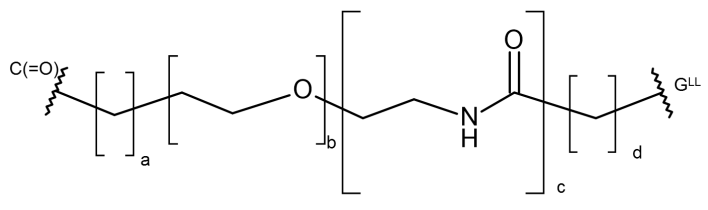
wherein

Q is:



5 , where Q^X is such that Q is an amino-acid residue, a dipeptide residue or a tripeptide residue;

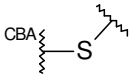
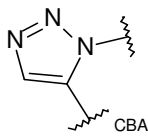
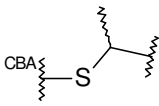
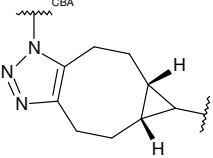
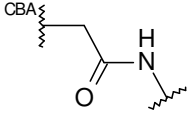
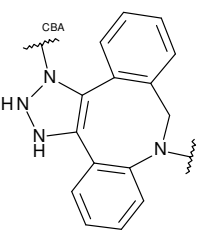
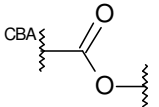
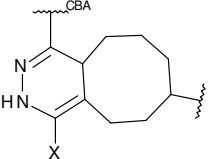
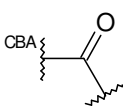
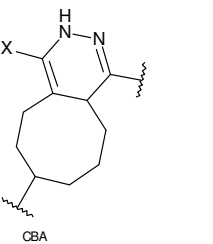
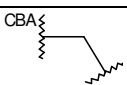
X is:



where $a = 0$ to 5 , $b = 0$ to 16 , $c = 0$ or 1 , $d = 0$ to 5 ;

10 G^{LL} is selected from:

(G^{LL1-1})		(G^{LL8-1})	
(G^{LL1-2})		(G^{LL8-2})	
(G^{LL2})		(G^{LL9-1})	

(G ^{LL3-1})		(G ^{LL9-2})	
(G ^{LL3-2})		G ^{L10}	
(G ^{LL-4})		G ^{L11}	
(G ^{LL5})		G ^{L12}	
(G ^{LL6})		G ^{L13}	
(G ^{LL7})			

where Ar represents a C₅₋₆ arylene group, e.g. phenylene and X represents C¹⁻⁴ alkyl;

where CBA represents a cell binding agent which is a modified antibody having at least one free conjugation site on each heavy chain;

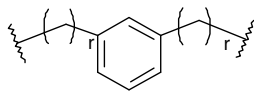
wherein the term alkyl includes the sub-classes alkenyl, alkynyl and cycloalkyl, the term

- 5 alkylene includes the sub-classes alkenylene, alkynylene, cycloalkylene, the term alkenyl pertains to an alkyl group having one or more carbon-carbon double bonds and the term alkynyl pertains to an alkyl group having one or more carbon-carbon triple bonds.

2. A conjugate according to claim 1, wherein:

- 10 a) both Y and Y' are O; and/or

- b) R'' is C₃₋₇ alkylene or
a group of formula:



where r is 1 or 2; and/or

5

- c) R⁹ is H; and/or

- d) R⁶ is H; and/or

- 10 e) R⁷ is selected from H, OH, OR or C₁₋₄ alkyloxy group.

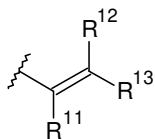
3. A conjugate according to either claim 1 or 2, wherein D is D1, there is a double bond between C2 and C3, and R² is:

- 15 a) a C₅₋₇ aryl group wherein R² optionally bears one to three substituent groups selected from methoxy, ethoxy, fluoro, chloro, cyano, bis-oxy-methylene, methyl-piperazinyl, morpholino and methyl-thiophenyl; or

- 20 b) a C₈₋₁₀ aryl group wherein R² optionally bears one to three substituent groups selected from methoxy, ethoxy, fluoro, chloro, cyano, bis-oxy-methylene, methyl-piperazinyl, morpholino and methyl-thiophenyl; or

- c) a C₁₋₅ saturated aliphatic alkyl group, optionally wherein R² is methyl, ethyl or propyl; or

- d) a group of formula:

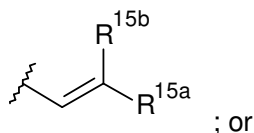


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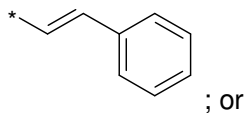
, optionally wherein:

- i) the total number of carbon atoms in the R² group is no more than 4;
ii) one of R¹¹, R¹² and R¹³ is H, with the other two groups being selected from H, C₁₋₃ saturated alkyl, C₂₋₃ alkenyl, C₂₋₃ alkynyl and cyclopropyl; or
iii) two of R¹¹, R¹² and R¹³ are H, with the other group being selected from H, C₁₋₃ saturated
30 alkyl, C₂₋₃ alkenyl, C₂₋₃ alkynyl and cyclopropyl; or

- e) a group of formula:

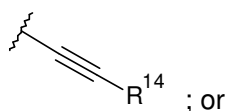


f) the group:



5

g) a group of formula:

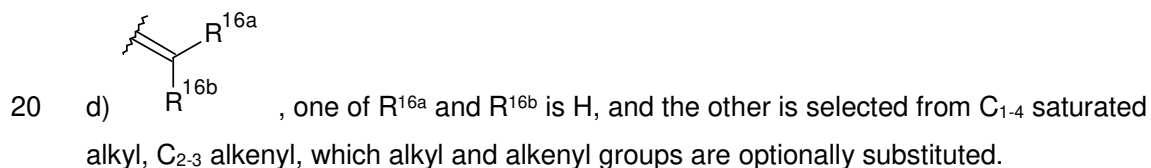
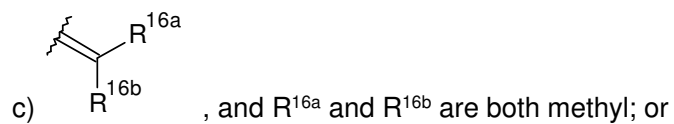
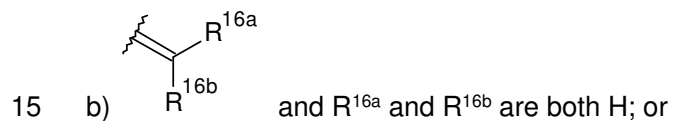


h) C₃₋₆ saturated cycloalkyl group, optionally wherein R² is cyclopropyl.

10

4. A conjugate according to either claim 1 or 2, wherein D is D1, there is a single bond between C2 and C3, and R² is:

a) H; or,



5. A conjugate according to any one of claims 1 to 4, wherein D' is D'1, there is a double bond between C2' and C3', and R²² is:

a) a C₅₋₇ aryl group wherein wherein R²² optionally bears one to three substituent groups selected from methoxy, ethoxy, fluoro, chloro, cyano, bis-oxy-methylene, methyl-piperazinyl, morpholino and methyl-thiophenyl; or

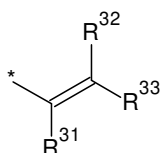
5 b) a C₈₋₁₀ aryl group wherein wherein R²² optionally bears one to three substituent groups selected from methoxy, ethoxy, fluoro, chloro, cyano, bis-oxy-methylene, methyl-piperazinyl, morpholino and methyl-thiophenyl; or

c) a C₁₋₅ saturated aliphatic alkyl group; or

10

d) a C₃₋₆ saturated cycloalkyl group; or

e) a group of formula:



wherein:

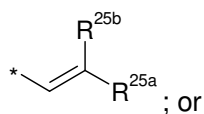
15 i) the total number of carbon atoms in the R²² group is no more than 4; or

ii) one of R³¹, R³² and R³³ is H, with the other two groups being selected from H, C₁₋₃ saturated alkyl, C₂₋₃ alkenyl, C₂₋₃ alkynyl and cyclopropyl; or

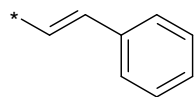
iii) two of R³¹, R³² and R³³ are H, with the other group being selected from H, C₁₋₃ saturated alkyl, C₂₋₃ alkenyl, C₂₋₃ alkynyl and cyclopropyl; or

20

f) a group of formula:



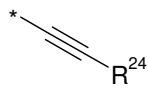
g) the group:



25

; or

h) a group of formula:

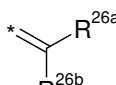


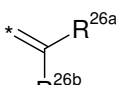
wherein R²⁴ is selected from H, methyl, ethyl, ethenyl and ethynyl.

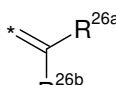
6. A conjugate according to any one of claims 1 to 4, wherein D' is D'1, there is a single bond between C2' and C3', and R²² is:

a) H; or

5

b)  and R^{26a} and R^{26b} are both H; or

c) , and R^{26a} and R^{26b} are both methyl; or

10 d) , one of R^{26a} and R^{26b} is H, and the other is selected from C₁₋₄ saturated alkyl, C₂₋₃ alkenyl, which alkyl and alkenyl groups are optionally substituted.

7. A conjugate according to any one of claims 1 to 6, wherein R^{11a} is:

a) OH; or

15 b) OR^A, where R^A is C₁₋₄ alkyl.

8. A conjugate according to any one of claims 1 to 7, wherein:

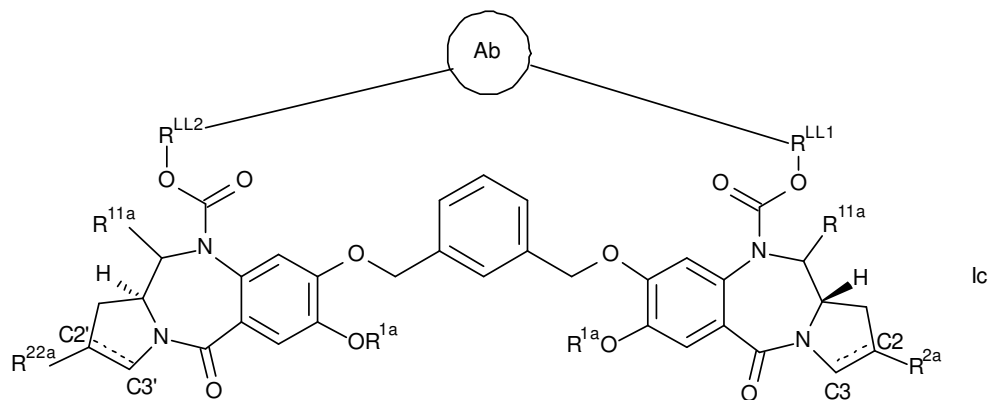
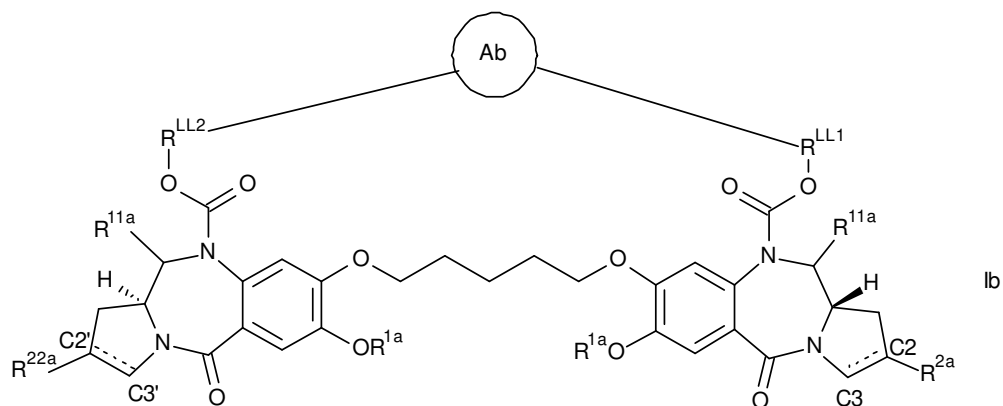
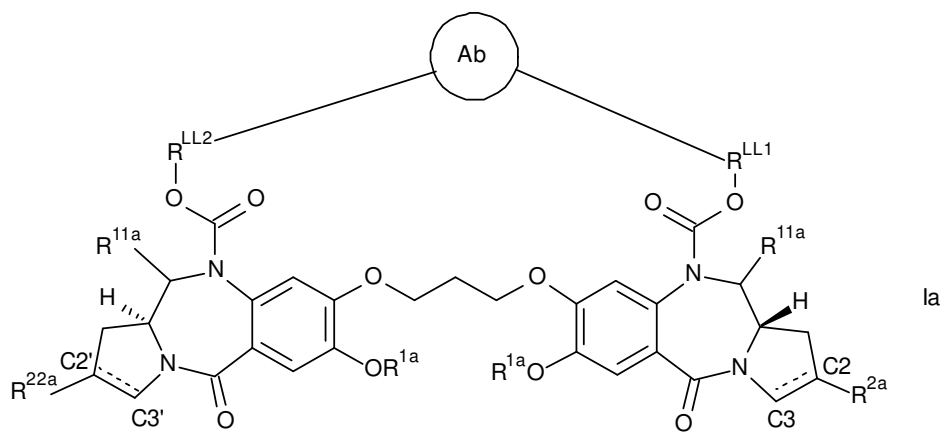
a) R^{6'} is selected from the same groups as R⁶, R^{7'} is selected from the same groups as R⁷, R^{9'} is selected from the same groups as R⁹, R^{11a'} is selected from the same groups as R^{11a} and Y' is selected from the same groups as Y; and/or

20

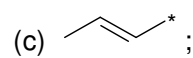
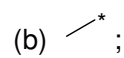
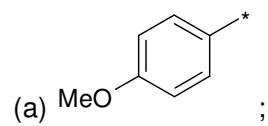
b) R^{6'} is the same group as R⁶, R^{7'} is the same group as R⁷, R^{9'} is the same group as R⁹, R^{11a'} is the same group as R^{11a} and Y' is the same group as Y; and/or

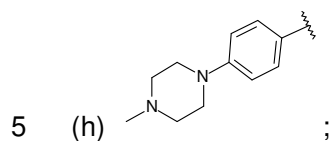
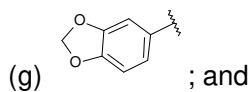
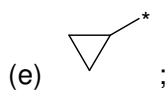
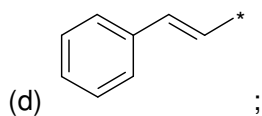
25 c) R²² is the same group as R².

9. A conjugate according to claim 1, which is of formula Ia-1, Ia-2 or Ia-3:



where R^{2a} and R^{22a} are the same and are selected from:





R^{1a} is selected from methyl and benzyl;

R^{LL1} , R^{LL2} and R^{11a} are as defined in claim 1.

10. A conjugate according to any one of claims 1 to 9, wherein R^{LL1} is of formula IIIa', and Q^x is:

a) an amino acid residue selected from Phe, Lys, Val, Ala, Cit, Leu, Ile, Arg, and Trp; or

b) a dipeptide residue selected from:

15 CO-Phe-Lys-NH,
CO-Val-Ala-NH,
CO-Val-Lys-NH,
CO-Ala-Lys-NH,
CO-Val-Cit-NH,
CO-Phe-Cit-NH,
20 CO-Leu-Cit-NH,
CO-Ile-Cit-NH,
CO-Phe-Arg-NH, and
CO-Trp-Cit-NH; or

25 c) a tripeptide residue.

11. A conjugate according to any one of claims 1 to 10, wherein R^{LL1} is of formula IIIa' and:

a) a is 0 to 3; and/or

b) b is 0 to 12; and/or

c) d is 0 to 3.

5

12. A conjugate according to any one of claims 1 to 11, wherein R^{LL1} is of formula IIIa' and a is 0, c is 1 and d is 2, and b is from 0 to 8 optionally wherein b is 0, 4 or 8.

13. A conjugate according to any one of claims 1 to 9, wherein R^{LL2} is of formula IIIa', and Q^x is:

10

a) an amino acid residue selected from Phe, Lys, Val, Ala, Cit, Leu, Ile, Arg, and Trp; or

b) a dipeptide residue selected from:

15

CO-Phe-Lys-NH,

CO-Val-Ala-NH,

CO-Val-Lys-NH,

CO-Ala-Lys-NH,

CO-Val-Cit-NH,

CO-Phe-Cit-NH,

20

CO-Leu-Cit-NH,

CO-Ile-Cit-NH,

CO-Phe-Arg-NH, and

CO-Trp-Cit-NH; or

25 c) a tripeptide residue.

14. A conjugate according to any one of claims 1 to 13, wherein R^{LL2} is of formula IIIa' and:

a) a is 0 to 3; and/or

30

b) b is 0 to 12; and/or

c) d is 0 to 3.

15. A conjugate according to any one of claims 1 to 13, wherein R^{LL2} is of formula IIIa' and a is 0, c is 1 and d is 2, and b is from 0 to 8.

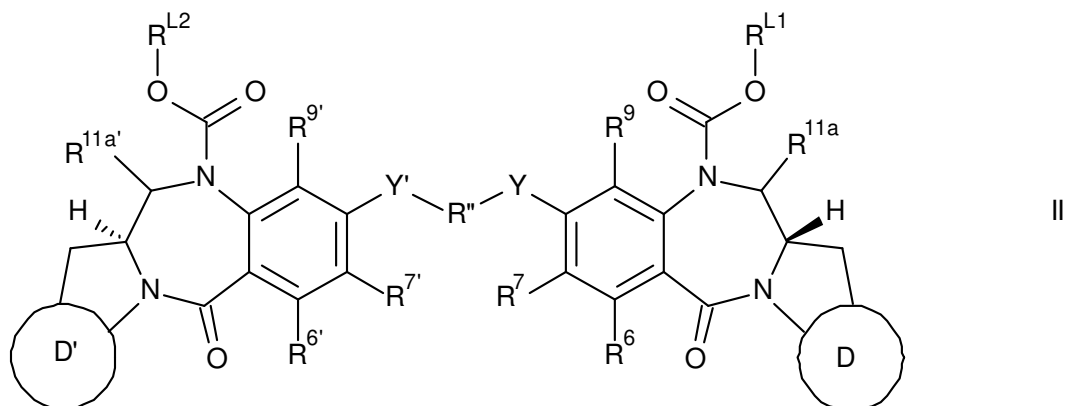
35

25 substitution in each heavy chain of an amino acid wherein the position that is substituted is selected from those set forth below:

Antibody	Isotype	IgG1	IgG2	IgG3	IgG4
Position (Kabat EU) and Corresponding Amino Acid	239	Ser	Ser	Ser	Ser
	282	Val	Val	Val	Val
	289	Thr	Thr	Thr	Thr
	297	Asn	Asn	Asn	Asn
	312	Asp	Asp	Asp	Asp
	324	Ser	Ser	Ser	Ser
	330	Ala	Ala	Ala	Ser
	335	Thr	Thr	Thr	Thr
	337	Ser	Ser	Ser	Ser
	339	Ala	Thr	Thr	Ala
	356	Glu	Glu	Glu	Glu
	359	Thr	Thr	Thr	Thr
	361	Asn	Asn	Asn	Asn
	383	Ser	Ser	Ser	Ser
	384	Asn	Asn	Ser	Asn
	398	Leu	Leu	Leu	Leu
	400	Ser	Ser	Ser	Ser
	422	Val	Val	Ile	Val
	440	Ser	Ser	Ser	Ser
	442	Ser	Ser	Ser	Ser
SEQ ID NO:		1	2	3	4

19. The conjugate according to any one of claims 1 to 18, for use in therapy.
- 5 20. A pharmaceutical composition comprising the conjugate of any one of claims 1 to 18 a pharmaceutically acceptable diluent, carrier or excipient.
21. The conjugate according to any one of claims 1 to 18 or the pharmaceutical composition according to claim 20, for use in the treatment of a proliferative disease in a
- 10 subject, optionally wherein the disease treated is cancer.
22. Use of the conjugate according to any one of claims 1 to 18 in the manufacture of a medicament for the treatment of a proliferative disease.

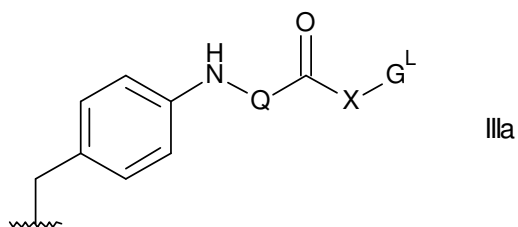
23. A compound of formula II:



and salts and solvates thereof,

- 5 wherein D, R², R⁶, R⁷, R⁹, R^{11a}, Y, R'', Y', D', R^{6'}, R^{7'}, R^{9'}, R^{11a'} and R¹² (including the presence or absence of double bonds between C2 and C3 and C2' and C3' respectively) are as defined in any one of claims 1 to 9;

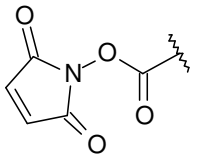
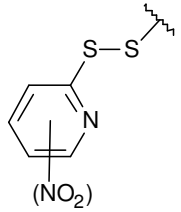
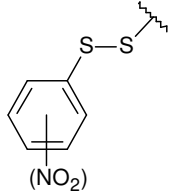
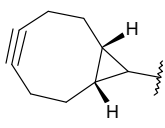
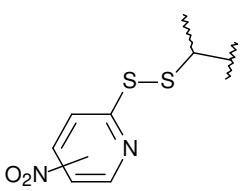
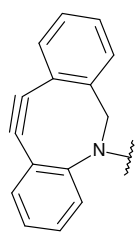
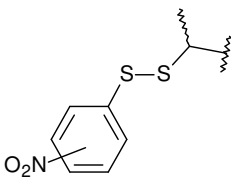
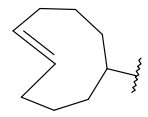
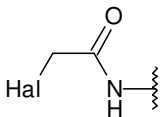
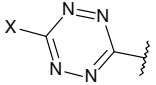
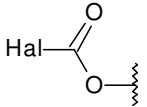
R^{L1} and R^{L2} are linkers for connecting to a cell binding agent, which are independently of formula IIIa:



10

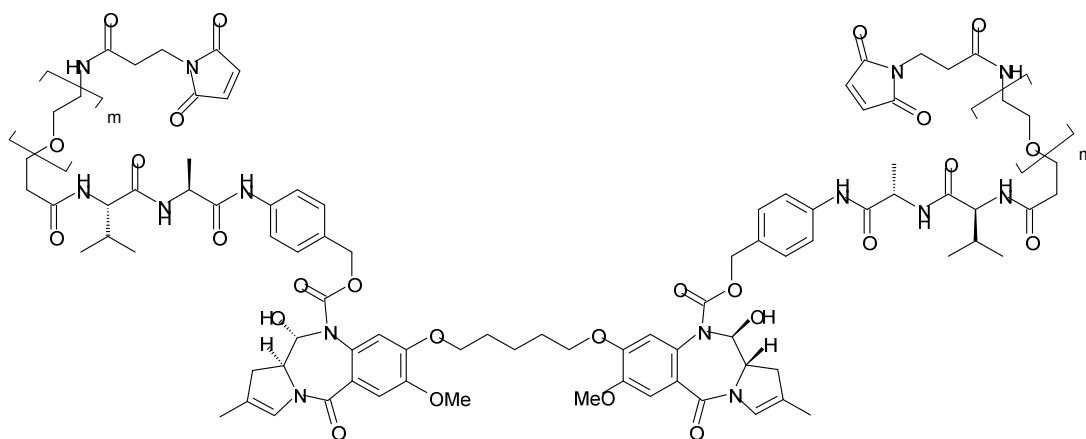
where Q and X are as defined in any one of claims 1, 10 to 12 and 13 to 15 and G^L is selected from:

(G ^{L1-1})		(G ^{L6})	
(G ^{L1-2})		(G ^{L7})	

(G ^{L2})		(G ^{L8})	
(G ^{L3-1})	 where the NO ₂ group is optional	(G ^{L9})	
(G ^{L3-2})	 where the NO ₂ group is optional	(G ^{L10})	
(G ^{L3-3})	 where the NO ₂ group is optional	(G ^{L11})	
(G ^{L3-4})	 where the NO ₂ group is optional	(G ^{L12})	
(G ^{L4})	 Where Hal = I, Br, Cl	(G ^{L13})	
(G ^{L5})			

where Ar represents a C₅₋₆ arylene group, e.g. phenylene, and X represents C¹⁻⁴ alkyl.

24. A compound according to claim 23, wherein the compound is of formula Id':



where m is an integer from 2 to 8.