SITE SPECIFIC ANTIBODY DRUG CONJUGATES AND THEIR PROCESS OF PREPARATION

Modern cancer therapeutics aim at increasing specificity for cancer cells thus reducing toxicity for healthy tissues.

Antibody drug conjugates (ADCs) represent a promising class with this potential exploiting their antigen-specificity for cancer-related targets to deliver cytotoxic compounds to cancer cells.

ADCs are highly valuable tools in cancer treatment, as they enable the targeted delivery of potent cytotoxins to cancer cells. However, the current methods used to create ADCs still have various limitations, particularly in terms of site-selectivity during payload conjugation and controlling the drug-to-antibody ratio (DAR), i.e., the number of drugs per antibody.

Considering the accelerated clearance and reduced safety profile witnessed in first generation ADCs featuring drug-to-antibody ratios (DARs) frequently reaching eight, a shift in the prevailing paradigm has occurred in favour of conjugates with lower DAR values. Particularly challenging is the generation of ADCs with site specificity for the conjugation and/or controlled degree of conjugation.

Recent endeavours have also pivoted towards disulfide re-bridging reagents specifically reacting with cysteine-thiols from (partially) reduced antibodies.

These re-bridging agents also furnish supplementary functional moieties to enable subsequent payload-linker conjugation.

Various re-bridging strategies are under investigation. They should ensure the covalent linkage of the heavy and light chains of the antibodies.

In mAbs vol.11 (3), 2019, White et al disclose a re-bridging strategy to prepare homogenous ADCs with DAR of one using an engineered antibody and a PBD dimer. However, the proposed strategy requires an engineered antibody and the use of a long linker, which can potentially impact the PK/PD parameters of the ADC.

WO 2020/164561 discloses a process for preparing antibody-drug conjugates with improved homogeneity. However, said process leads to mixtures mainly comprising ADC with a drug-to-antibody ratio of 4, in mixture with DAR of 0, 2 and 6.

There is thus still a need to provide more versatile methods, which may apply to both native and engineered antibodies, allowing efficient covalent linkage of cysteine residues with site specificity and controlled degree of conjugation.

The present invention is based *inter alia* on the provision of a versatile protein complex of coordination and a novel process for synthesizing the same. Said protein complex is a key intermediate that may conveniently lead to various drug conjugates having a site-specific conjugation.

BRIEF DESCRIPTION

According to a first object, the present invention concerns a coordination complex of protein with a divalent metal atom M, represented by formula (II):

[Ab'-M]

(II)

Wherein

Ab' is a protein or fragment thereof, herein called Protein, comprising two heavy chains and two light chains, each chain comprising one or more cysteine residue involved in inter chains disulfide bridges,

wherein each heavy chain comprises one N-proximal inter(heavy chains) cysteine residue that is located nearest to the N-terminal end of said heavy chain,

And wherein the Sulfur atom of each said N-proximal inter(heavy chains) cysteine residue is reduced, and the remaining interchain cysteine residues form interchain disulfide bridges;

M is a divalent transition metal;

Wherein M bridges together the two reduced Sulfur atoms, so as to form the following group: -S-M-S- within the coordination complex;

And the salts thereof;

Characterized in that the complexion of M is site specific.

The present invention distinctively relies on site-specificity for the coordination for e.g. monoclonal IgG antibodies.

This is based on targeting one of the two inter-heavy chain disulfide bonds in the hinge region, specifically the bond that is nearest to the N-terminal end of the Protein's heavy chains.

The invention involves the selective modification of this identified disulfide bond, distinguishing it from the second inter-heavy chain disulfide bond and the two inter-heavy-light chain disulfide bonds.

This targeted approach facilitates the creation of modified proteins, such as IgG molecules with improved specificity and potential therapeutic efficacy.

According to a further object, the present invention also concerns the process of preparation of the complex of formula (II) as defined herein, said process comprising:

Reacting a protein complex of formula (I):

$[Ab'_{Red}\text{-}M]$

(I)

With an oxidizing agent;

wherein

Ab'_{Red} is a protein or fragment thereof comprising two heavy chains and two light chains, each chain comprising one or more cysteine residue involved in inter chains disulfide bridges,

wherein each heavy chain comprises one N-proximal inter(heavy chains) cysteine residue that is located nearest to the N-terminal end of said heavy chain,

And wherein each Sulfur atom of each said N-proximal inter(heavy chains) cysteine residue is reduced and the two reduced Sulfur atoms form together with the M atom the following group: -S-M-S- within the coordination complex;

and the Sulfur atoms of the remaining interchain cysteine residues are reduced in the form of thiol –SH groups;

M is defined as in formula (II).

According to third object, the present invention also concerns a coordination complex of protein of formula (I):

$[Ab'_{Red}\text{-}M]$

(I)

Where Ab'_{Red} and M are defined herein.

According to a fourth object, the invention also concerns the process of preparation of the complex of formula (I) as defined herein, said process comprising:

- reacting Ab with a reducing agent so as to form Ab_{Red}, followed by
- reacting Ab_{Red} with 0.5 to 2.5, preferably between 1 and 2 equivalents, more preferably between 1 and 1.5, still more preferably around 1.4 equivalent of a salt of metal M so as to form the Metal coordination complexes $[Ab'_{Red}\text{-}M]$;

Wherein

Ab is a protein or fragment thereof comprising two heavy chains and two light chains, each chain comprising one or more cysteine residue involved in inter chains disulfide bridges,

Ab_{Red} refers to reduced Ab wherein all Sulfur atoms of said interchain cysteine residues are reduced and in the form of thiol –SH groups.

According to a fifth object, the invention also concerns a protein conjugate of formula (III):

$(T)_p$-Ab'-$(L-(Payload)_m)_n$

(III)

Having

- a controlled drug-to-antibody ratio (DAR) of (m x n); and
- a site specific conjugation at one or two of a pair of interchain cysteine residues of Ab' defined as the conjugated cysteine residues;

Where in formula (III):

Ab' is a protein or fragment thereof comprising two heavy chains and two light chains, each chain comprising one or more interchain cysteine residues, such that each of said conjugated cysteines are bound to either -(L-(Payload)m or optional T fragment, and the remaining interchain cysteine residues form interchain disulfide bridges;

n is 1 or 2;

m is 1, 2, 3, 4, 5 or 6;

p is 0 or 1;

T, if present, is a terminal group covalently bound to one of said two reduced Sulfur atoms of said interchain cysteine residues of Ab' that is not covalently bound to L;

each L, identical or different, is a linear or branched di-, tri-, tetra-, penta-, hexa- or hepta-valent linker moiety covalently binding Payload to said Ab', where said L is attached to Ab' through one or two of said reduced Sulfur atoms of said interchain cysteine residues and binds said Ab' with one or more Payload;

Payload is a molecule exerting a biological activity selected from the group consisting in diagnostic agents, therapeutic agents and labelling agents.

According to a sixth object, the invention also concerns a process of preparation of a protein conjugate of formula (III) as defined herein, said process comprising complexing said metal atom from said compound of formula (II) as defined herein and conjugating said de-complexed compound with 1 to n conjugating fragments $L-(Payload)_m$ as defined herein.

According to a seventh object, the invention also concerns the therapeutical use of the compounds of formula (III).

According to an eight object, the invention also concerns the use of conjugates (III) for preparing further conjugates (V) defined below.

DETAILED DESCRIPTION

The protein

The protein designated by Ab is also herein referred to as Protein.

Typically, said Protein may be a naturally occurring protein or a synthetic protein, preferably a naturally occurring antibody or a synthetic antibody, such as an engineered antibody, herein referred to as Protein.

Said Protein typically comprises one or more polypeptide chains made of amino acids, it being provided that the cysteine residues may form covalent bonds through the formation of disulphide bridges, typically between different peptide chains within Protein. These are called herein « interchain cysteine residues ».

Typically, said naturally occurring or synthetic protein comprises two heavy chains and two light chains, where preferably each heavy chain comprises three cysteine residues and each light chain comprises one cysteine residue in the hinge region.

These chains are covalently bridged together by disulfide bonds created between the sulfur atom of the cysteine residues.

As used herein, the hinge region refers to an amino acid stretch in the central part of the two heavy chains, which links these two chains by disulfide bonds, whose respective sulfur atoms belong to cysteine residues.

Typically, the heavy chains are bridged together by the two interchain disulfide bridges created between two pairs of cysteine residues (the inter(heavy chains) cysteine residues), and each heavy chain is further bridged to a light chain by a further disulfide bridge created between two cysteine residues (the inter(heavy-light chains) cysteine residues).

Typically, said Protein thus comprises two pairs of inter(heavy chains) cysteine residues and two pairs of the inter(heavy-light chains) cysteine residues.

These 8 cysteine residues involved in interchain bridges are herein referred as “interchain cysteines”.

Those interchain cysteines that are involved in the conjugation (i.e.) covalently attached to either -(L-(Payload)$_m$ or optional T fragments are herein referred to as “conjugated cysteines residues” or “cysteine residues of the conjugation site”.

More specifically, said Protein or fragment thereof comprises two heavy chains and two light chains, each chain comprising one or more cysteine residue involved in inter chains disulfide bridges, and wherein each heavy chain comprises one N-proximal inter(heavy chains) cysteine residue that is located nearest to the N-terminal end of said heavy chain.

These two N-proximal inter(heavy chains) cysteine residues (also called herein "N-proximal cysteines") are a key site for site-specific conjugation according to the invention.

Without being bound by theory, it is hypothesized that these N-proximal cysteines are adjacent to or close to histidine residues that may confer the appropriate reactivity for complex formation with a metal atom, that will then lead to a reactive position for conjugation.

Said N-proximal cysteines are often referred to as Cysteine-229. According to an embodiment, said conjugated cysteines residues are the two C-229 cysteines.

According to another embodiment, another key site for site-specific conjugation is anyone of the two pairs of inter(heavy-light chains) cysteine residues. According to an alternative embodiment, said conjugated cysteines residues are any pair of the two pairs of inter(heavy-light chains) cysteine residues.

Protein may preferably be an antibody, in particular Immunoglobulin G, or a derivative thereof, such as IgG1 or IgG4 isotype, for example, IgG1 isotype.

According to an embodiment, said synthetic or naturally occurring protein comprises one or more amino acid residues, such as peptide or a protein.
Typically, said Protein is a monoclonal antibody, such as human, humanized, mouse or chimeric antibodies. In particular, it can be a mono or bi specific antibody.
Typical antibodies include trastuzumab, bevacizumab, cetuximab, panitumumab, ipilimumab, rituximab, alemtuzumab, ofatumumab, gemtuzumab, brentuximab, ibritumomab, tositumomab, pertuzumab, adecatumumab, IGN101, INA01 labetuzumab, hua33, pemtumomab, oregovomab, minretumomab (CC49), cG250, J591, MOv-18, farletuzumab (MORAb-003), 3F8, ch14,18, KW-2871, hu3S193, IgN311, IM-2C6, CDP-791, etaracizumab, volociximab, nimotuzumab, MM-121, AMG 102, METMAB, SCH 900105, AVE1642, IMC-A12, MK-0646, R1507, CP 751871, KB004, III A4, mapatumumab, HGS-ETR2, CS-1008, denosumab, sibrotuzumab, F19, 81C6, pinatuzumab, lifastuzumab,

glembatumumab, coltuximab, lorvotuzumab, indatuximab, anti-PSMA, MLN-0264, ABT-414, milatuzumab, ramucirumab, abagovomab, abituzumab, adecatumumab, afutuzumab, altumomab pentetate, amatuximab, anatumomab, anetumab, apolizumab, arcitumomab, ascrinvacumab, atezolizumab, bavituximab, bectumomab, belimumab, bivatuzumab, brontictuzumab, cantuzumab, capromab, catumaxomab, citatuzumab, cixutumumab, clivatuzumab, codrituzumab, conatumumab, dacetuzumab, dallotuzumab, daratumumab, demcizumab, denintuzumab, depatuxizumab, derlotuximab, detumomab, dinutuximab, drozitumab, duligotumab, durvalumab, dusigitumab, ecromeximab, edrecolomab, elgemtumab, emactuzumab, enavatuzumab emibetuzumab, enfortumab, enoblituzumab, ensituximab, epratuzumab, ertumaxomab, etaracizumab, farletuzumab, ficlatuzumab, figitumumab, flanvotumab, futuximab, galiximab, ganitumab, icrucumab, igovomab, imalumab, imgatuzumab, indusatumab, inebilizumab, intetumumab, iratumumab, isatuximab, lexatuzumab, lilotomab, lintuzumab, lirilumab, lucatumumab, lumretuzumab, margetuximab, matuzumab, mirvetuximab, mitumomab, mogamulizumab, moxetumomab, nacolomab, naptumomab, narnatumab, necitumumab, nesvacumab, nimotuzumab, nivolumab, nofetumomab, obinutuzumab, ocaratuzumab, ofatumumab, olaratumab, onartuzumab, ontuxizumab, oportuzumab, oregovomab, otlertuzumab, pankomab, parsatuzumab, pasotuxizumab, patritumab, pembrolizumab, pemtumomab, pidilizumab, pintumomab, polatuzumab, pritumumab, quilizumab, racotumomab, ramucirumab, rilotumumab, robatumumab, sacituzumab, samalizumab, satumomab, seribantumab, siltuximab, sofituzumab, tacatuzumab, taplitumomab, tarextumab, tenatumomab, teprotumumab, tetulomab, ticilimumab, tigatuzumab, tositumomab, tovetumab, tremelimumab, tucotuzumab, ublituximab, ulocuplumab, urelumab, utomilumab, vadastuximab, vandortuzumab, vantictumab, vanucizumab, varlilumab, veltuzumab, vesencumab, volociximab, vorsetuzumab votumumab, zalutumumab, zatuxima, tisotumab, zilovertamab, telisotuzumab, combinations and derivatives thereof, as well as other monoclonal antibodies targeting one or more of CAI 25, CAI 5-3, CAI 9-9, L6, Lewis Y, Lewis X, alpha fetoprotein, CA 242, placental alkaline phosphatase, prostate specific antigen, prostate specific membrane antigen, prostatic acid phosphatase, epidermal growth factor, MAGE-I, MAGE-2, MAGE-3, MAGE-4, transferrin receptor, p97, MUCl, CEA, gplOO, MARTI, IL-2 receptor, CD20, CD52, CD33, CD22, human chorionic gonadotropin, CD38, CD40, mucin, P21, MPG, Neu oncogene product, HER2, Nectin-4, tissue factor, Trop-2, FRα, ROR1, EGFR, cMET, B7-H3, Ang-2, BCMA, CD3, CD19, CTLA-4, DLL3, EpCAM, Factor IXa, Factor X, Fcγ receptors, MAGE-1, MAGE-2, MAGE-3, MAGE-4, and combinations thereof.

Illustrative bi-specific antibodies include Blinatumomab (CD19 × CD3), Emicizumab (Factor IXa × Factor X), Teclistamab (BCMA × CD3), Mosunetuzumab (CD20 × CD3), Glofitamab (CD20 × CD3), Amivantamab (EGFR × MET), Zenocutuzumab (HER2 × HER3), Faricimab (VEGF-A × Ang-2), Epcoritamab (CD20 x CD3), Elranatamab (BCMA x CD3), Talquetamab (GPRC5D x CD3), Pavurutamab (BCMA × CD3), Tarlatamab (DLL3 × CD3), Catumaxomab (EpCAM × CD3), PD-1 × CTLA-4 bispecific antibodies, bispecific T-cell engager (various targets).

According to an embodiment, Protein may be an engineered antibody (or so called "mutant" antibody).

According to an embodiment, Protein may be a bispecific monoclonal antibody (BsMAb, BsAb). Bispecific monoclonal antibody is an artificial protein that can simultaneously bind to two different types of antigens or two different epitopes on the same antigen.

According to one embodiment, at least one part of the bispecific monoclonal antibody binds to a tumor-associated antigen.

A particular illustrative embodiment is an antibody comprising two heavy chains and two light chains wherein 6 out of 8 interchain cysteines, which typically form the interchain disulfide bonds, were mutated into any other amino acid, either the same or different, such as lysine, arginine, tyrosine, phenylalanine, tryptophan, histidine, serine, threonine. Typically, the resulting engineered antibody comprised a single interchain disulfide bond. Illustrative examples of such engineered antibody include trastuzumab (HC-C229S, HC-C223S, LC-C214S) mutant and J591 (HC-C220S, HC-C229S, LC-C214S) mutant.
Such representative mutated antibodies are disclosed e.g. in *Mol Cancer Ther* (2018) 17 (10): 2176–2186.

Another illustrative embodiment is an engineered antibody wherein two additional cysteine groups have been introduced into the protein sequence.
Illustrative examples of such engineered antibody include Thiomab® described in US7,521,541, as well as antibodies with cysteines introduced through following point mutations: LC-V110C, HC-A114C, HC-D265C, HC-S239C, HC-S442C, HC-N325C, HC-L328C.
Where Protein is such an engineered antibody including additional cysteine groups, said additional cysteine groups may be used for additional grafting of Payload (i.e.) in addition

to the grafting at the site-specific conjugation. Such additional grafting may occur in the same reaction as the site-specific conjugation of the process pf the invention. An example of such simultaneous additional grafting is illustrated in General Procedure D4 below.

The compounds of formula (II) and mixtures thereof

Ab' derives from said Protein in that it comprises two sulfur atoms that would otherwise form an interchain disulphide bridge that are reduced.

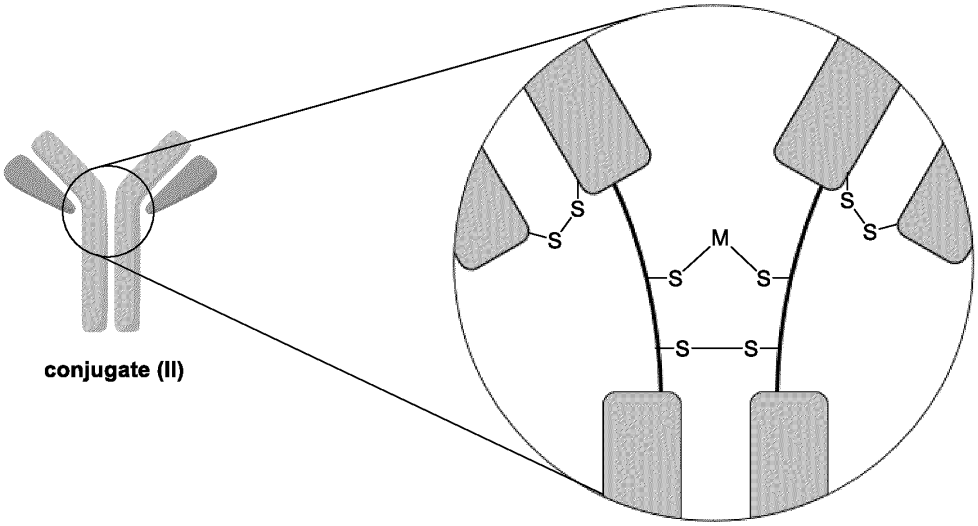
Specifically, in Ab' the Sulfur atom of each N-proximal inter(heavy chains) cysteine residue is reduced, while the remaining interchain cysteine residues are unaffected and still form interchain disulfide bridges.

In Ab', the two said reduced sulfur atoms are involved in a coordination bond with said Metal atom, thus forming a group –S-M-S- that links together the two heavy chains. Typically, the two sulfur of the two C-229 cysteines form together a –S-M-S- group.

According to an embodiment, the metal atom is a transition metal, in particular chosen from Zn, Co, Cd, Hg, radioactive isotopes of said atoms, or the combination thereof. Preferably, M is Zn.

According to an embodiment, Ab' is an antibody, in particular Immunoglobulin G, or a derivative thereof, where its two N-proximal sulfur atoms are reduced.

As an illustration, said complex of formula (II) may be schematically represented by the following formula:



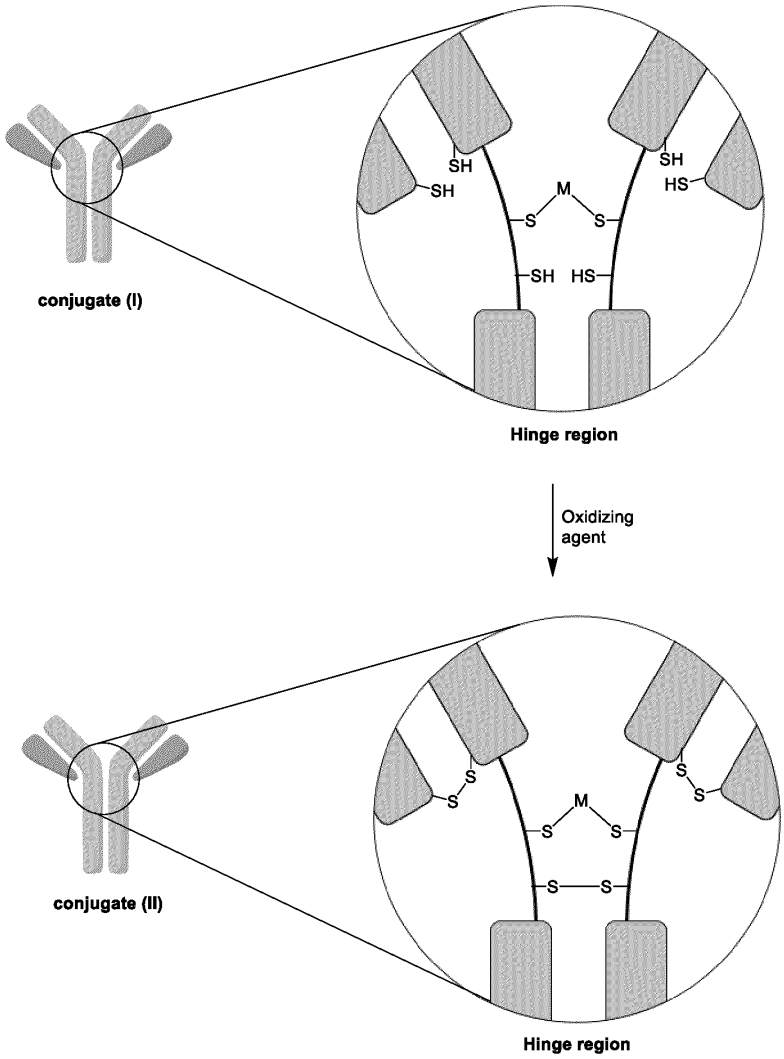
The compounds of formula (II) represent versatile key intermediates towards a diversity of antibody-drug conjugates (ADCs) of formula (III) as shown below.

The process of preparation of the compounds of formula (II) and mixtures thereof

According to an embodiment, said oxidizing agent is dehydroascorbic acid (DHAA).

According to an embodiment, the process may be carried out on an isolated compound of formula (I) or a mixture comprising the same, in particular a mixture where the compounds of formula (I) are predominantly present, as defined herein.

As an illustration, said process of preparation of said complex of formula (II) may be schematically illustrated by the following scheme:



The compounds of formula (I) and mixtures thereof

$[Ab'_{Red}\text{-}M]$

(I)

Where Ab'_{Red} differs from Ab' as defined herein in that apart from the two reduced sulfur atoms that are involved in the coordination complex with the Metal atom, the other sulfur atoms are reduced.

More specifically, Ab'_{Red} is a protein or fragment thereof comprising two heavy chains and two light chains, each chain comprising one or more cysteine residue involved in inter chains disulfide bridges,

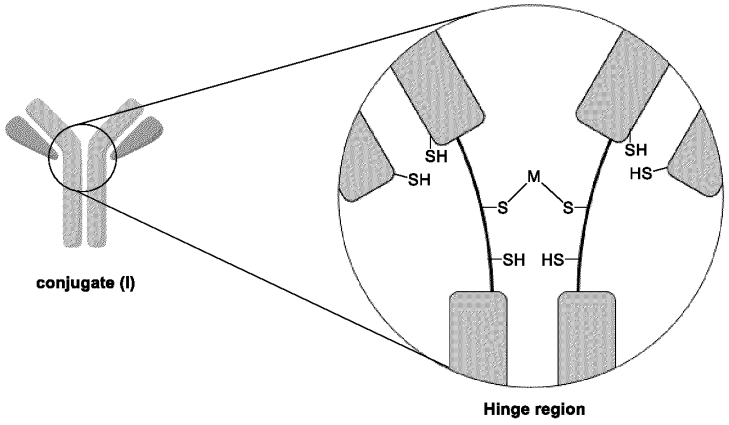
wherein each heavy chain comprises one N-proximal inter(heavy chains) cysteine residue that is located nearest to the N-terminal end of said heavy chain,

And wherein each Sulfur atom of each said N-proximal inter(heavy chains) cysteine residue is reduced and the two reduced Sulfur atoms form together with the M atom the following group: -S-M-S- within the coordination complex;

and the Sulfur atoms of the remaining interchain cysteine residues are reduced in the form of thiol –SH groups.

Typically, in compound (I), the sulfur atoms of all interchain cysteine residues are reduced in the form of thiol –SH groups, except the two sulfur atoms of the two C-229 cysteines residues that form together with the M atom the following group: -S-M-S- within the coordination complex.

As an illustration, said complex of formula (I) may be schematically represented by the following formula:



The process of preparation of the complex of formula (I) and mixtures thereof

Ab is a protein or fragment thereof comprising two heavy chains and two light chains, each chain comprising one or more cysteine residue involved in inter chains disulfide bridges. Ab may be Protein as defined above.

Ab_{Red} refers to reduced Ab, and thus differs from Ab in that all Sulfur atoms of said interchain cysteine residues are reduced and in the form of thiol –SH groups.

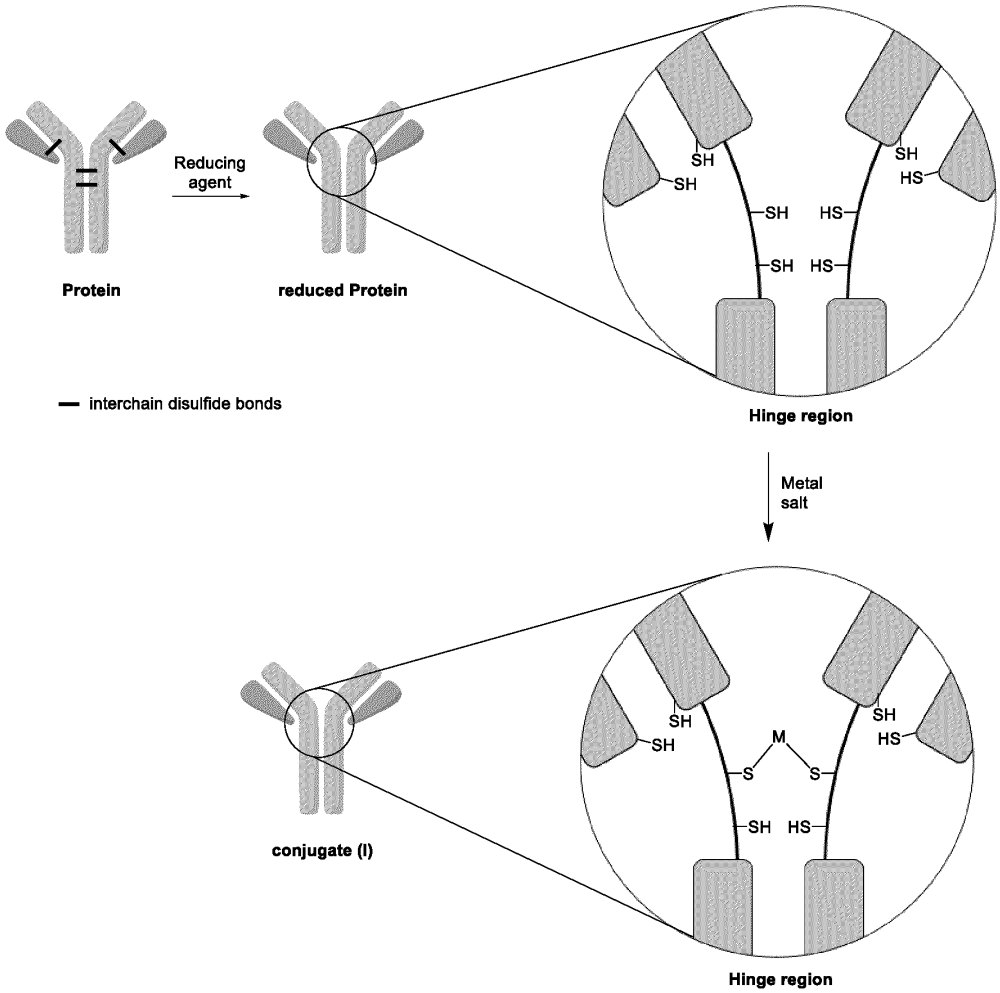
Said reducing agent may be for example Tris(2-carboxyéthyl)phosphine hydrochloride (TCEP), dithiothreitol (DTT) or 2-mercaptoethanol (BME).

For the reducing step, preferred conditions include 37 °C, 1.5 h, 5-10 equiv. TCEP.

According to an embodiment, the metal salt may be chosen from dihalogenide metal salts, such as $ZnCl_2$, or $Zn(OAc)_2$.

Typically, the step of reacting the metal salt may be conducted in aqueous buffers, such as phosphate-buffered saline, at room temperature, over 5-10 minutes.

As an illustration, said process of preparation of said complex of formula (I) may be schematically illustrated by the following scheme:



The compounds of formula (III) and mixtures thereof

Compounds of formula (III) may be referred to as « antibody-drug conjugates » (ADC).

The conjugation site of said site specific conjugation is at one or two of the interchain cysteine residues as defined above, (i.e.) the conjugated cysteine residues.

It is to be understood that conjugated cysteine residues are based on a pair of cysteine residue otherwise involved in a disulfide bridge, i.e. before reduction of the sulfur atoms.

Typically, said conjugated cysteine residues may be either N-proximal inter(heavy chains) cysteine residues or inter(heavy-light chains) cysteine residues.
It is also to be understood that when the conjugation is at one interchain cysteine residue, T is present, and when the conjugation is at two interchain cysteine residues, T is absent.

According to a first embodiment the conjugation site is at one or two of the N-proximal inter(heavy chains) cysteine residues that are located nearest to the N-terminal end of said heavy chains (i.e.) one or two of the two C-229 cysteines.

According to this first embodiment, the invention concerns a protein conjugate of formula (III'):

$$(T)_p\text{-Ab'-}(L\text{-}(Payload)_m)_n$$

(III')

Having

- a controlled drug-to-antibody ratio (DAR) of (m x n); and
- a site specific conjugation at one or two of the N-proximal inter(heavy chains) cysteine residue that is located nearest to the N-terminal end of said heavy chain;

Where in formula (III'):
Ab' is a protein or fragment thereof comprising two heavy chains and two light chains, each chain comprising one or more cysteine residue involved in inter chains disulfide bridges, wherein each heavy chain comprises one N-proximal inter(heavy chains) cysteine residue that is located nearest to the N-terminal end of said heavy chain,
And wherein each Sulfur atom of each said N-proximal inter(heavy chains) cysteine residue is reduced and covalently bound to either -(L-(Payload)$_m$ or optional T fragment, and the remaining interchain cysteine residues form interchain disulfide bridges;
n is 1 or 2;
m is 1, 2, 3, 4, 5 or 6;
p is 0 or 1;
T, if present, is a terminal group covalently bound to one of said two reduced Sulfur atoms of said N-proximal inter(heavy chains) cysteine residues of Ab' that is not covalently bound to L;
each L, identical or different, is a linear or branched di-, tri-, tetra-, penta-, hexa- or hepta-valent linker moiety covalently binding Payload to said Ab', where said L is attached to Ab'

through one or two of said reduced Sulfur atoms of said N-proximal inter(heavy chains) cysteine residue and binds said Ab' with one or more Payload;
Payload is a molecule exerting a biological activity selected from the group consisting in diagnostic agents, therapeutic agents and labelling agents.

According to a second embodiment, the conjugation site is at one or two of one pair of the inter(heavy-light chains) cysteine residues.
According to this second embodiment, the invention concerns a protein conjugate of formula (III''):

$$(T)_p\text{-Ab'-}(L\text{-}(Payload)_m)_n$$

(III'')

Having

- a controlled drug-to-antibody ratio (DAR) of (m x n); and
- a site specific conjugation at one or two of the inter(heavy-light chains) cysteine residues;

Where in formula (III''):
Ab' is a protein or fragment thereof comprising two heavy chains and two light chains, each heavy and light chain comprising one inter(heavy-light chains) cysteine residue involved as a pair in disulfide bridges bridging together a heavy chain with a light chain, each heavy chain further comprising two inter(heavy chains) cysteine residues;
wherein
each Sulfur atom of one pair of said inter(heavy-light chains) cysteine residues is reduced and covalently bound to either -(L-(Payload)$_m$ or optional T fragment, each Sulfur of the second pair of said inter(heavy-light chains) cysteine residues form inter(heavy-light chains) disulfide bridge, and the remaining inter(heavy chain) cysteine residues form inter(heavy chains) disulfide bridges;

n is 1 or 2;
m is 1, 2, 3, 4, 5 or 6;
p is 0 or 1;
T, if present, is a terminal group covalently bound to one of said two reduced Sulfur atoms of said inter(heavy-light chains) cysteine residues of Ab' that is not covalently bound to L;
each L, identical or different, is a linear or branched di-, tri-, tetra-, penta-, hexa- or hepta-valent linker moiety covalently binding Payload to said Ab', where said L is attached to Ab' through one or two of said reduced Sulfur atoms of said inter(heavy-light chains) cysteine residue and binds said Ab' with one or more Payload;

Payload is a molecule exerting a biological activity selected from the group consisting in diagnostic agents, therapeutic agents and labelling agents.

It is understood that conjugates (III') and (III'') are regioisomers, and both are encompassed by formula (III).

According to an illustration of any first or second embodiment, the conjugate of formula (III) may be a monoconjugate (DAR=1). Accordingly, L is a trivalent linker covalently binding said Payload with the two sulfur atoms of the conjugated cysteine residues of Ab'.

Said protein conjugate of formula (III), (III') or (III'') may be of formula (III-A):

Ab'-L-(Payload)

(III-A)

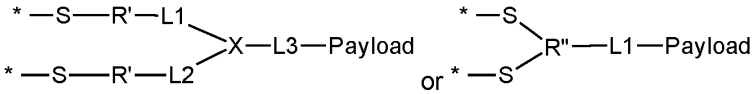
Having a controlled drug-to-antibody ratio (DAR) of 1;

Where in formula (III-A):

Ab' and Payload are defined as herein;

L is a trivalent linker moiety covalently binding together the two said reduced Sulfur atoms of Ab' with Payload.

Preferably, said conjugate of formula (III-A) comprises one of the following fragments:



Wherein each S designates the reduced Sulfur atom of the conjugated cysteine residues of Ab' to Payload;

* designates the attachment of said Sulfur atom to the rest of Ab' at each conjugated cysteine residue, typically at each heavy chain through the corresponding N-proximal inter(heavy chains) cysteine residues or at a light chain and a heavy chain through the corresponding inter(heavy-light chains) cysteine residues;

L_1, L_2 and L_3 identical or different are optional divalent Spacer moieties;

X is chosen from linear, branched or cyclic trivalent moieties, comprising 1 to 9 nitrogen atoms and from 0 to 30 carbon atoms;

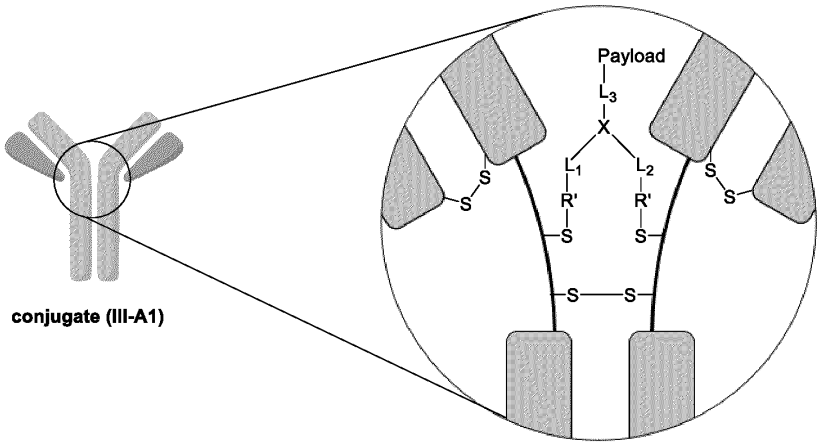
R' is a group formed as a result of a reaction between a thiol and a thiol-reactive group, such as maleimide, 3-arylpropiolonitrile, bromo-maleimide, chloroacetamide, bromoacetamide, iodoacetamide, acrylamide, vinyl sulfone, pyridyl disulfide, alpha-halo carbonyl, vinyl pyridine;

R'' is a group formed as a result of a reaction between two thiols and a bifunctional thiol-reactive group, such as 3,4-disubstituted maleimide, 3,3'-(phenylene)dipropiolonitrile, dibromopyridazinedione, bissulfone, allyl sulfone, 3-bromomaleimide or arsenous acid.

According to the first embodiment above, the compounds of formula (III-A) are of formula (III'-A) where the conjugated cysteine residues are each said N-proximal inter(heavy chains) cysteine residues.

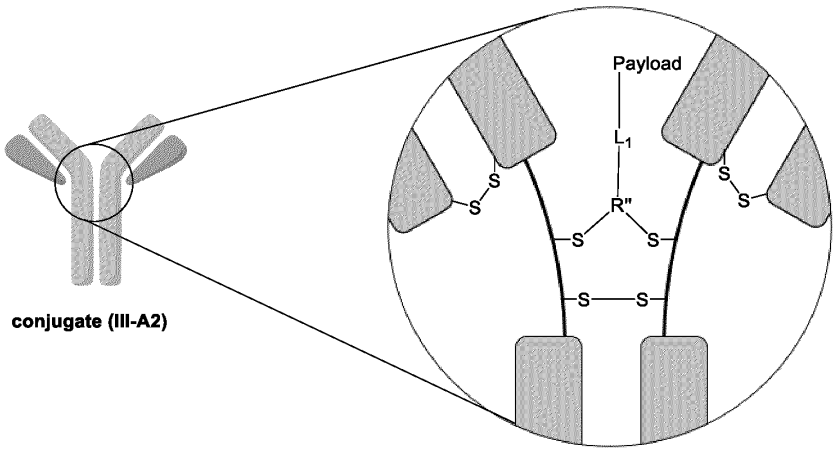
According to this embodiment, the attachment * is located at each heavy chain through the corresponding N-proximal inter(heavy chains) cysteine residue, or located at a light chain and at a heavy chain,

As an illustration, said conjugate of formula (III'-A) may be schematically represented by the following formula (III-A1):



Where Payload, X, R', L_1, L_2 and L_3 are defined as above.

As a further illustration, said conjugate of formula (III-A) may be schematically represented by the following formula (III-A2):

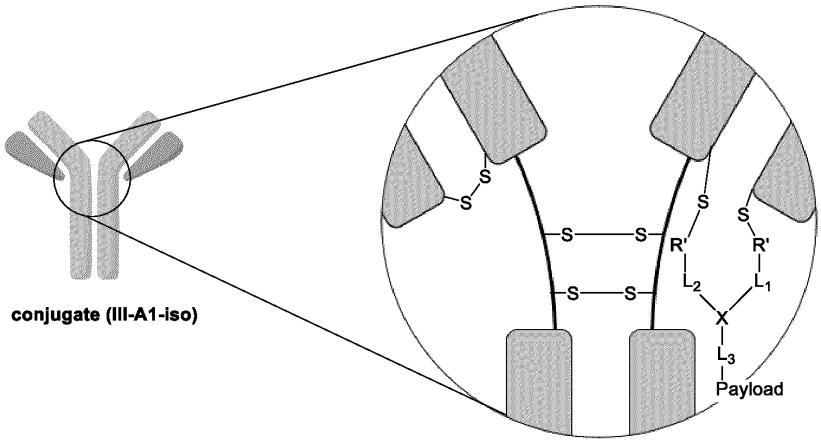


Where R'', Payload, L_1 are defined as above.

According to the second embodiment, the compounds of formula (III-A) are of formula (III''-A) where the site of conjugation is anyone of the two pairs of said inter(heavy-light chains) cysteine residue.

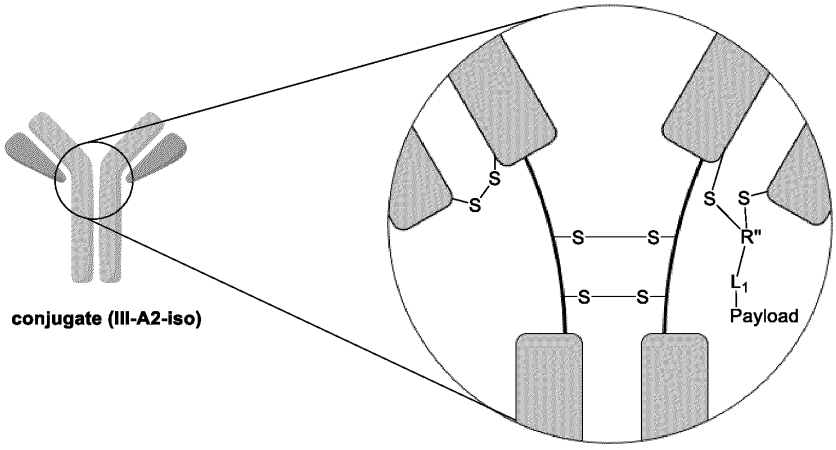
According to this embodiment, the attachment * is located at the light chain and at the heavy chain through said one inter(heavy-light chains) cysteine residue.

As an illustration, said conjugate of formula (III''-A) may be schematically represented by the following formula (III-A1-iso):



Where Payload, X, R', L_1, L_2 and L_3 are defined as above.

As a further illustration, said conjugate of formula (III''-A) may be schematically represented by the following formula (III-A2-iso):



Where Payload, R'', L_1 are defined as above.

According to another illustration of any first or second embodiment, the conjugate of formula (III) may be an even multiple conjugate (DAR=2m).

Said protein conjugate of formula (III), (III') or (III'') may be of formula (III-B):

$$Ab'\text{-}(L\text{-}(Payload)_m)_2$$

(III-B)

Having a controlled drug-to-antibody ratio (DAR) of 2m;

Where in formula (III-B):

m is as defined above, preferably 1, 2, 3, 4, 5 or 6;

Ab' and Payload are defined as herein;

Each of the two L is di-, tri-, tetra-, penta-, hexa- or hepta-valent linear or branched linker moiety covalently binding one of said reduced Sulfur atoms of said conjugated cysteine residues of Ab' with m Payload(s).

It is herein understood that when m > 1, then each Payload can be the same or different.

Preferably, said conjugate of formula (III-B) comprises two following fragments:

* —S—R'—L4—$(Payload)_m$

Wherein

m and Payload are defined as herein;

S designates the reduced Sulfur atom of each of the two said conjugated cysteine residues, respectively;

* designates the attachment of said Sulfur atom to the rest of Ab' at the conjugated cysteine residue, typically at each heavy chain through the corresponding N-proximal inter(heavy chains) cysteine residues, or at a light chain and a heavy chain through the corresponding inter(heavy-light chains) cysteine residues ;

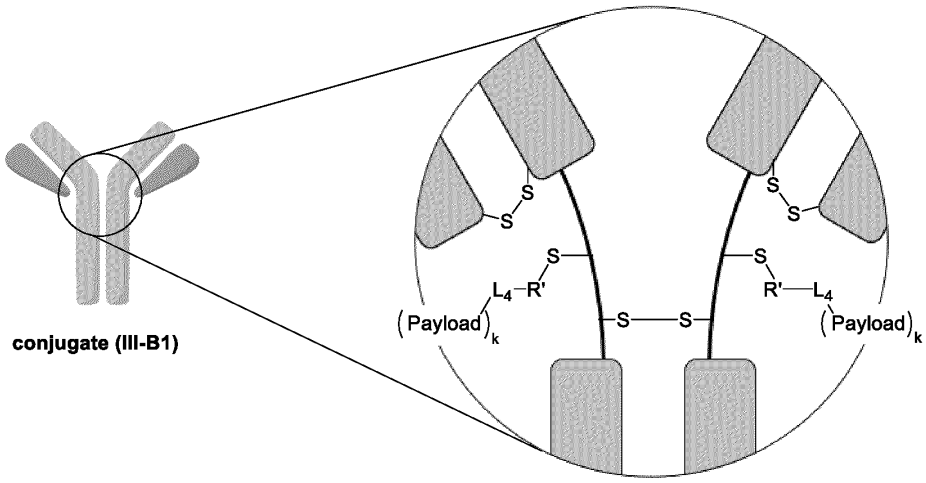
Each L_4 identical or different is an optional linear or branched Spacer moiety;

R' is a group formed as a result of a reaction between the reduced thiol group of Ab' and a thiol-reactive group, such as maleimide, 3-arylpropiolonitrile, bromo-maleimide, chloroacetamide, bromoacetamide, iodoacetamide, acrylamide, vinyl sulfone, pyridyl disulfide, alpha-halo carbonyl, vinyl pyridine.

According to the first embodiment above, the compounds of formula (III-B) are such that the conjugated cysteine residues are each of the two said N-proximal inter(heavy chains) cysteine residues (C-229 cysteine).

According to this embodiment, the attachment * is located at each heavy chain through the corresponding N-proximal inter(heavy chains) cysteine residue.

As an illustration, said conjugate of formula (III-B) may be schematically represented by the following formula (III-B1):



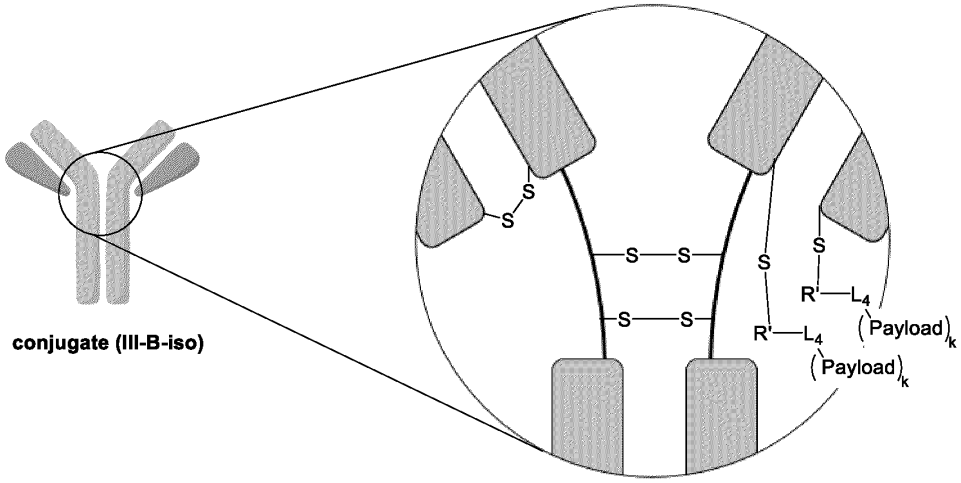
conjugate (III-B1)

Where Payload, k, R', L_4 are defined as above and k is defined as m above.

According to the second embodiment, the compounds of formula (III-B) are of formula (III''-B) where the site of conjugation is anyone of the two pairs of said inter(heavy-light chains) cysteine residues.

According to this embodiment, an attachment * is located at the light chain and the other attachment * is at the heavy chain, through said one inter(heavy-light chains) cysteine residue.

As an illustration, said conjugate of formula (III''-B) may be schematically represented by the following formula (III-B-iso):



conjugate (III-B-iso)

Where Payload, R', L_4 are defined as above and k is defined as m above.

Compounds (III-A) and (III-B) are representative compounds (III) where T is absent and there are two conjugated cysteine residues: either two N-proximal inter(heavy chains) cysteine residues are conjugated to Payload, or the two of a pair of inter(heavy-light chains) cysteine residues are conjugated to Payload.

According to another illustrative embodiment, the conjugate of formula (III) may be a multiple conjugate (DAR=m).

Said protein conjugate of formula (III) may be of formula (III-C):

$$T\text{-}Ab'\text{-}L\text{-}(Payload)_m$$

(III-C)

Having a controlled drug-to-antibody ratio (DAR) of m;

Where in formula (III-C):

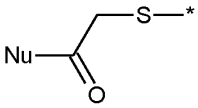
m, Ab' and Payload are as defined herein;

L is di-, tri-, tetra- or penta-valent linear or branched linker moiety covalently binding one of said reduced Sulfur atoms of the two N-proximal inter(heavy chains) cysteine residues of Ab' with m Payload(s); and

T is defined as herein and is covalently bound to Ab' through the other one of said reduced Sulfur atoms of the two N-proximal inter(heavy chains) cysteine residues of Ab'.

Preferably, said conjugate of formula (III-C) comprises each of the two following fragments:

* —S—R'—L4—$(Payload)_m$



Wherein

Payload and m are defined as herein;

Each S designates one reduced Sulfur atom of each of the two said N-proximal inter(heavy chains) cysteine residues;

Each * designates the attachment of said Sulfur atom to the rest of Ab' at each heavy chain through the corresponding N-proximal inter(heavy chains) cysteine residue;

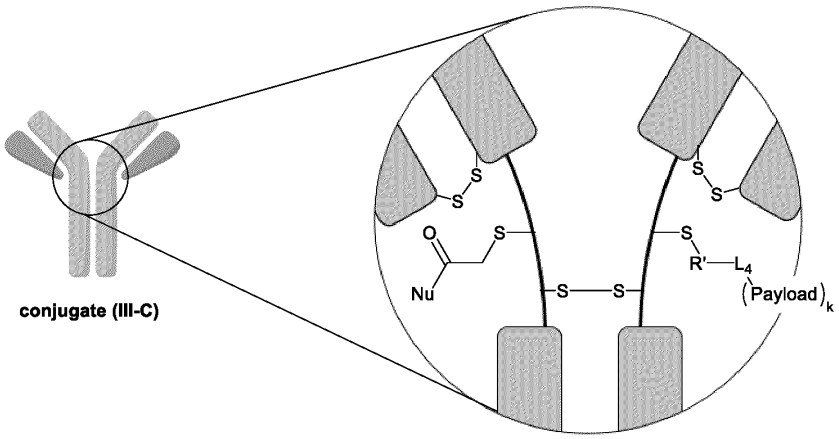
Each L_4 identical or different is an optional linear or branched Spacer moiety;

R' is a group formed as a result of a reaction between the reduced thiol group of Ab' and a thiol-reactive group, such as maleimide, 3-arylpropiolonitrile, bromo-maleimide, chloroacetamide, bromoacetamide, iodoacetamide, acrylamide, vinyl sulfone, pyridyl disulfide, alpha-halo carbonyl, vinyl pyridine;

Nu is a nucleophilic moiety chosen from OH, $-NH_2$, $-NH-NH_2$, -NH-OH, $-NH-R_1$, $-NR_1R_2$, Where R_1, R_2 and R_3 are chosen from $-CH_2-CONH_2$, $-CH_2-COOH$, C1-C12 alkyl, C6-C12 aryl, C5-C12 heteroaryl; $—(CH_2—CH_2—O—)_r—CH_3$ groups; in which r is an integer ranging from 1 to 24.

Compounds (III-C) are representative compounds (III) where only one N-proximal inter(heavy chains) cysteine residue is conjugated to Payload, and the other is covalently bound to T.

As an illustration, said conjugate of formula (III-C) may be schematically represented by the following formula:

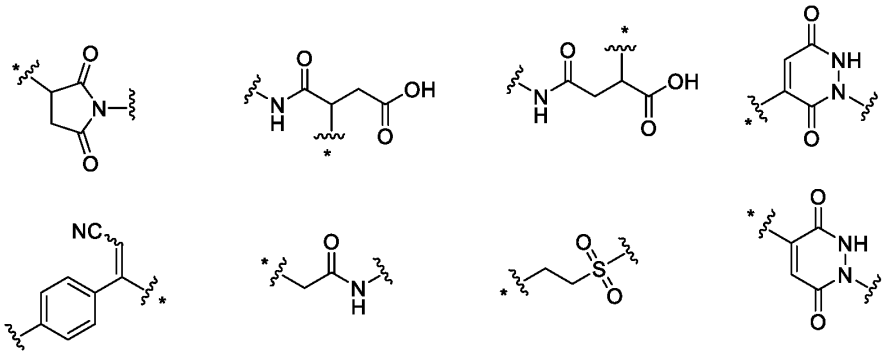


Where R', L_4 are defined as above and k is defined as m above.

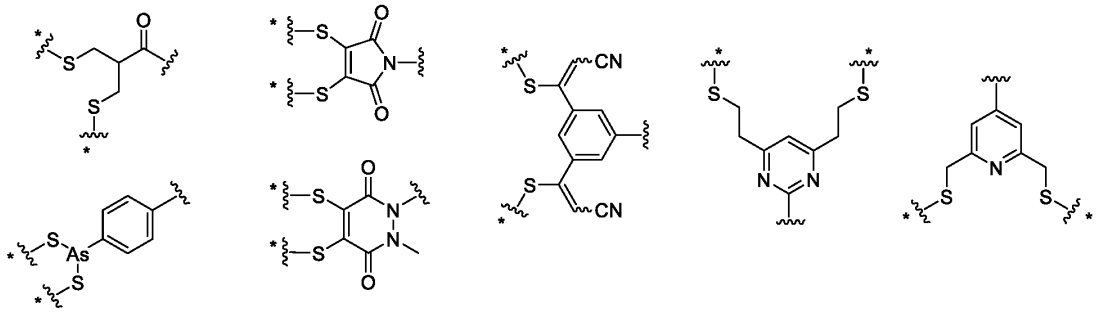
In the formulae (III), (III'), (III''), (III-A1), (III-A2), (III-A1-iso), (III-A2-iso), (III-B), (III-B1), (III-B-iso) and/or (III-C) above and below:

According to an embodiment, the optional L_1, L_2 and L_3 spacers may consist of one or multiple successive units selected from the group consisting of: a linear or branched, saturated or unsaturated, C1-C60 alkylene group optionally interrupted and/or terminated on one or both sides by one or more chemical groups selected from -O-, -S-, -S(O)-, $-SO_2-$, -O-P(O)(OH)-O-, -C(O)-, -NH-, -NMe-, -C(O)NH-, -NHC(O)-, -NH-C(O)-NH, -O-C(O)-NH-, -O-C(O)-O-; C3-C8 cycloalkylene; C3-C8 heterocyclylene; 5 to 12 membered heteroarylene; C6-C12 arylene; amino acids; polypeptides; glycosylene; $—(CH_2—CH_2—O—)_r—$ groups; in which r is an integer ranging from 1 to 24 and/or optionally substituted by one or more of OH, CN, NH_2, CF_3, $NHCONH_2$, $CONH_2$, COOH, =O, NH_2, glycosyl, C1-C12 alkyl, C3-C8 cycloalkyl, C6-C12 aryl, C5-C12 heteroaryl, $—(CH_2—CH_2—O—)_r—CH_3$;

According to an embodiment, R' may include the following groups:



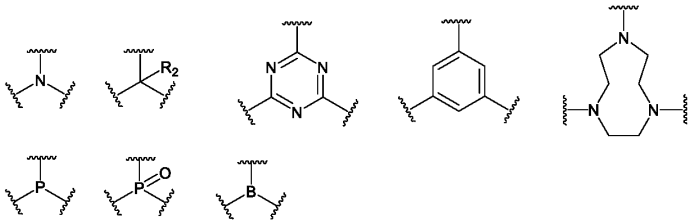
According to an embodiment, R'' may include the following groups:



where

Y may be Br, Cl, I, SPh, SCH_2CH_2OH;

According to a particular embodiment, X may be chosen from the following groups:

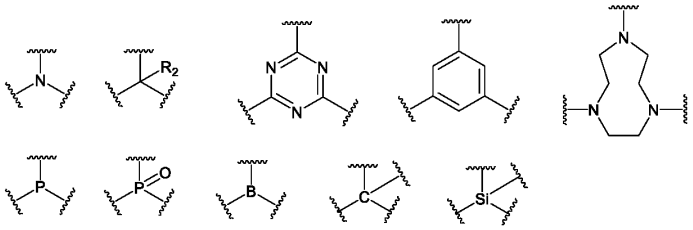


Where R_2 is chosen from H, OH, CN, NH_2, CF_3, $NHCONH_2$, $CONH_2$, COOH, =O, NH_2, C1-C12 alkyl, C6-C12 aryl, C5-C12 heteroaryl; $—(CH_2—CH_2—O—)_r—CH_3$ groups; in which r is an integer ranging from 1 to 24;

According to an embodiment, the optional L_4 spacer may consist of one or multiple linear or branched units, which links interchain cysteine residue of the antibody with one or multiple Payload molecules.

Linear units of the L_4 include: saturated or unsaturated, C1-C60 alkylene group optionally interrupted and/or terminated on one or both sides by one or more chemical groups selected from -O-, -S-, -S(O)-, $-SO_2-$, -O-P(O)(OH)-O-, -C(O)-, -NH-, -NMe-, -C(O)NH-, -NHC(O)-, -NH-C(O)-NH, -O-C(O)-NH-, -O-C(O)-O-; C3-C8 cycloalkylene; C3-C8 heterocyclylene; 5 to 12 membered heteroarylene; C6-C12 arylene; amino acids; polypeptides; glycosylene; $—(CH_2—CH_2—O—)_r—$ groups; in which r is an integer ranging from 1 to 24 and/or optionally substituted by one or more of OH, CN, NH_2, CF_3, $NHCONH_2$, $CONH_2$, COOH, =O, NH_2, glycosyl, C1-C12 alkyl, C3-C8 cycloalkyl, C6-C12 aryl, C5-C12 heteroaryl, $—(CH_2—CH_2—O—)_r—CH_3$.

Branched units of the L_4 may be chosen from branched or cyclic trivalent or tetravalent moieties, comprising 1 to 9 nitrogen atoms and from 0 to 30 carbon atoms, including the following groups:



Payload

Payload may be chosen from drug or imaging agents, oligonucleotides, chelating ligands capable of complexing with radionuclides, cytotoxic drugs and antineoplastic agents.

According to an embodiment, Payload may be chosen from drugs, such as cytotoxic drugs and antineoplastic agents.

Suitable drugs include in particular:

- dolastatins such as MMAE, MMAF, MMAD;
- maytansins such as DM1 and DM4;
- antracyclins such as doxorubicin, nemorubicin and PNU-159682;
- calicheamicins;
- duocarymycins such as CC-1065 and duocarmycin A;
- pyrrolobenzodiazepines;
- pyrrolobenzodiazepine dimers;
- indolino-benzodiazepines;
- indolino-benzodiazepine dimers;
- amanitins such as α-amanitin, β-amanitin, γ-amanitin, ε-amanitin;
- irinotecan derivatives;
- exatecan derivatives;
- exotoxins such as diphtheria toxin, shiga toxin, or subunits thereof;

in particular:

- dolastatins such as MMAE, MMAF, MMAD;
- maytansins such as DM1 and DM4;
- antracyclins such as doxorubicin, nemorubicin and PNU-159682;
- calicheamicins;
- duocarymycins such as CC-1065 and duocarmycin A;
- pyrrolobenzodiazepines;
- pyrrolobenzodiazepine dimers;

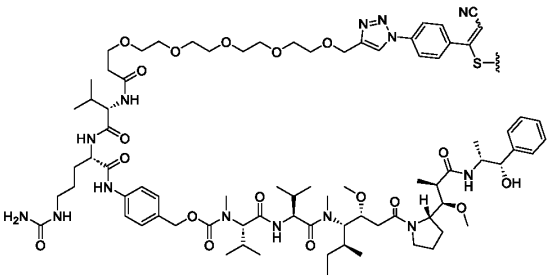
- indolino-benzodiazepines;
- indolino-benzodiazepine dimers;
- amanitins such as α-amanitin, β-amanitin, γ-amanitin, ε-amanitin; and
- irinotecan derivatives;
- exatecan derivatives.
- vincristine derivatives.

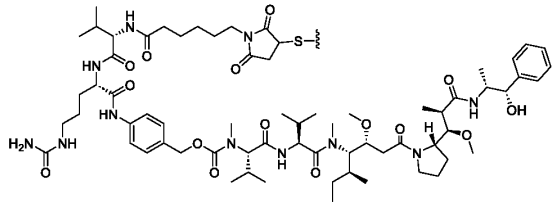
Suitable oligonucleotides include silencing RNA, microRNA, antisense oligonucleotides, DNA, or RNA oligomers. Typically they are between 10 and 5000 nucleotides in length, more particularly, between 20 and 200.

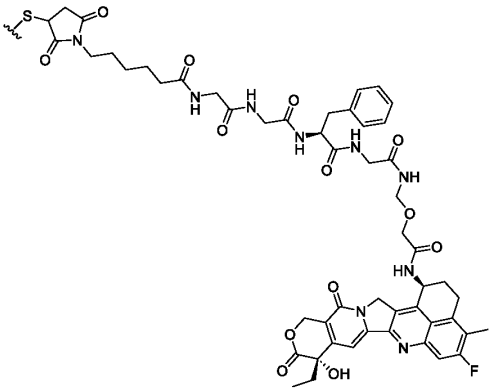
Imaging agents include detection agents, biological markers, tracing agents, such as fluorophores, dyes, radioactive tracers.

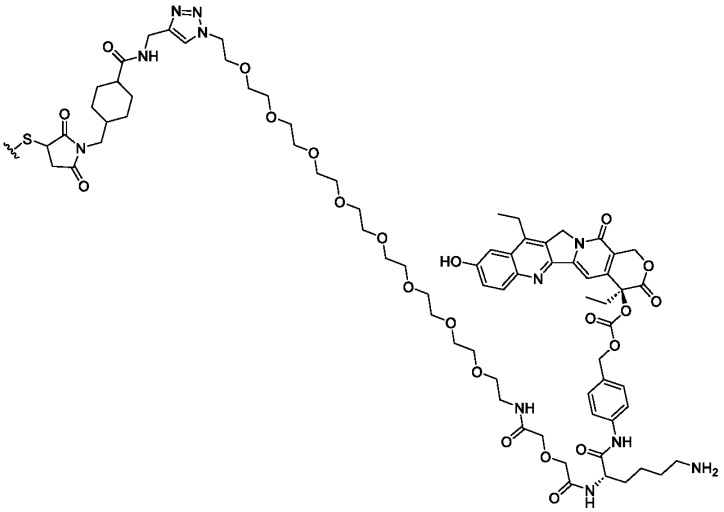
The -(L-(Payload)$_m$)$_n$ fragments present in compounds of formula (III) may be diversely chosen and are not limited according to the invention. Many such fragments have been described and developed in existing conjugates.

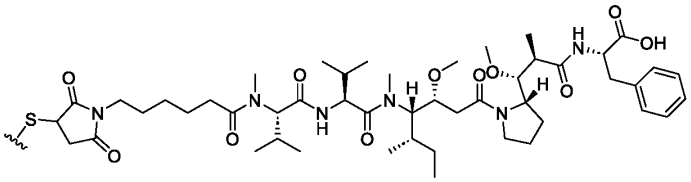
Representative known fragments for -S-L-(Payload) include:

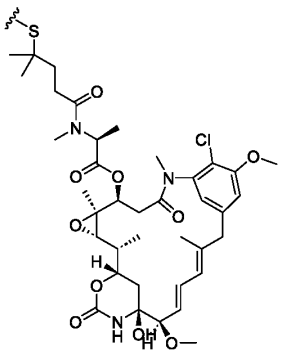


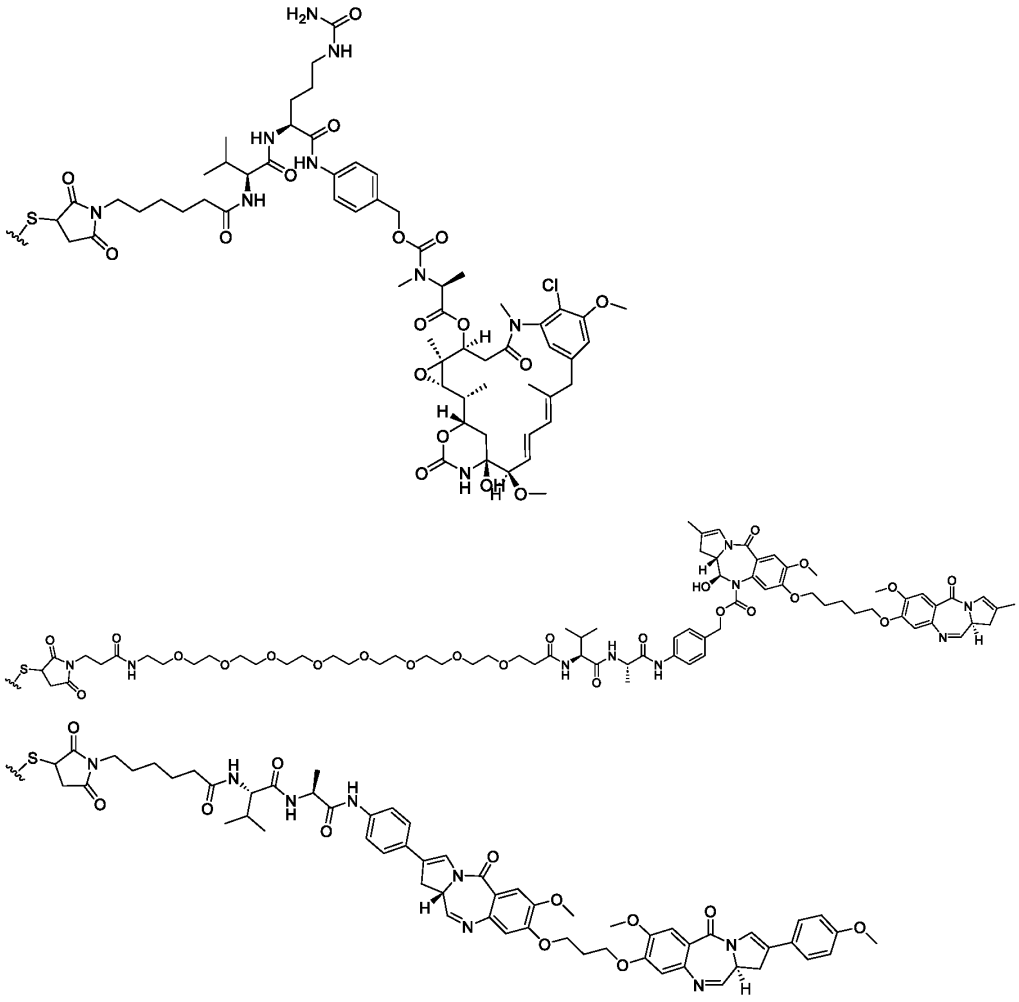


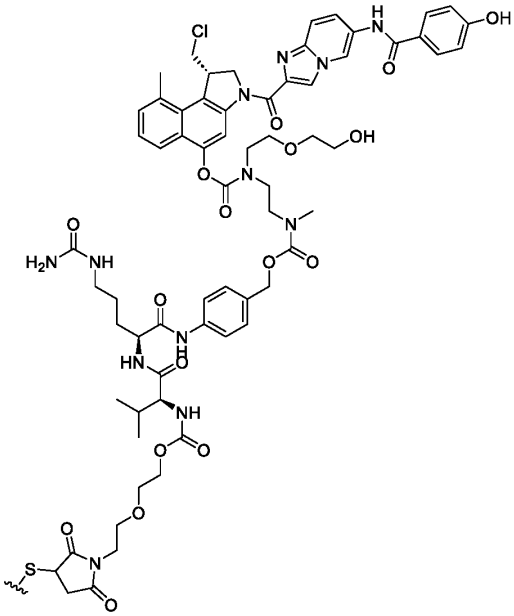


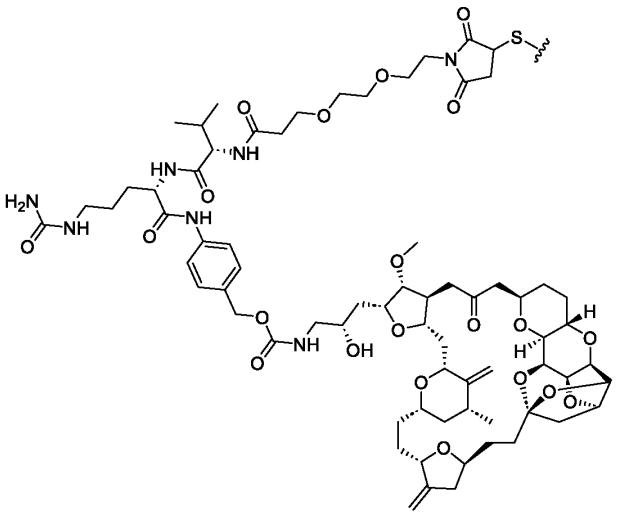


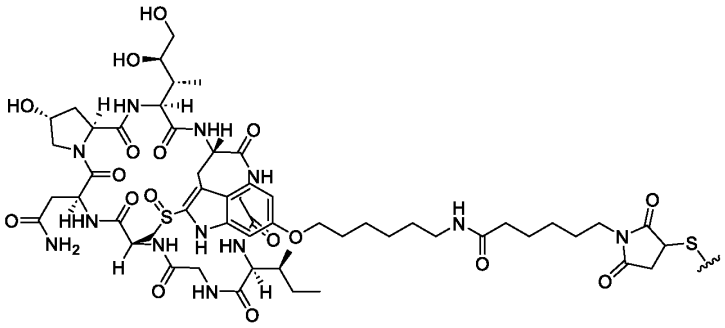


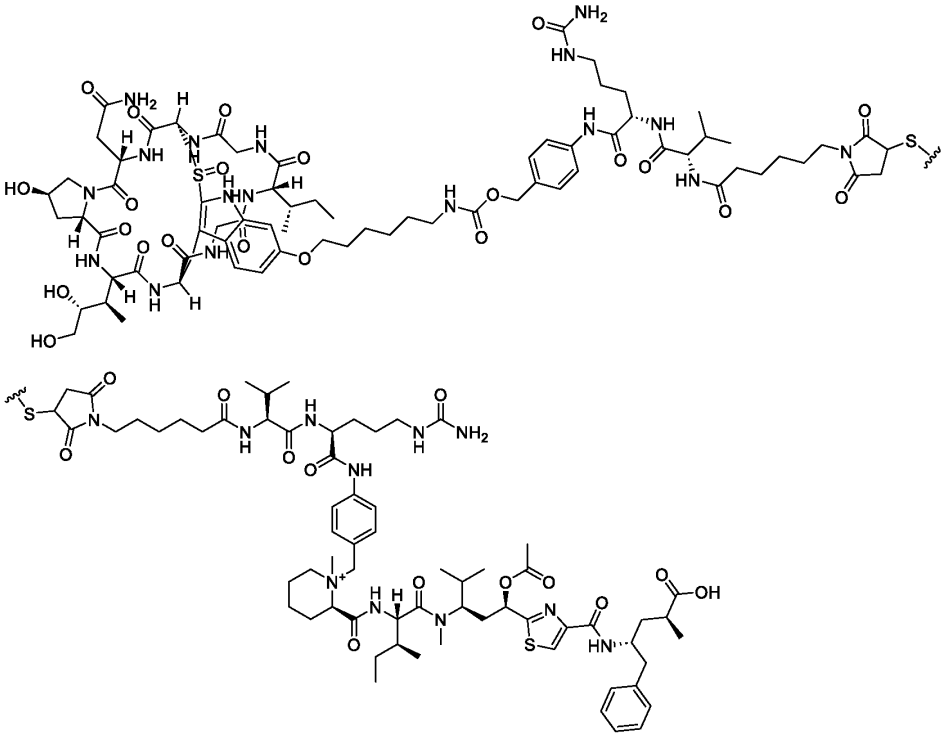


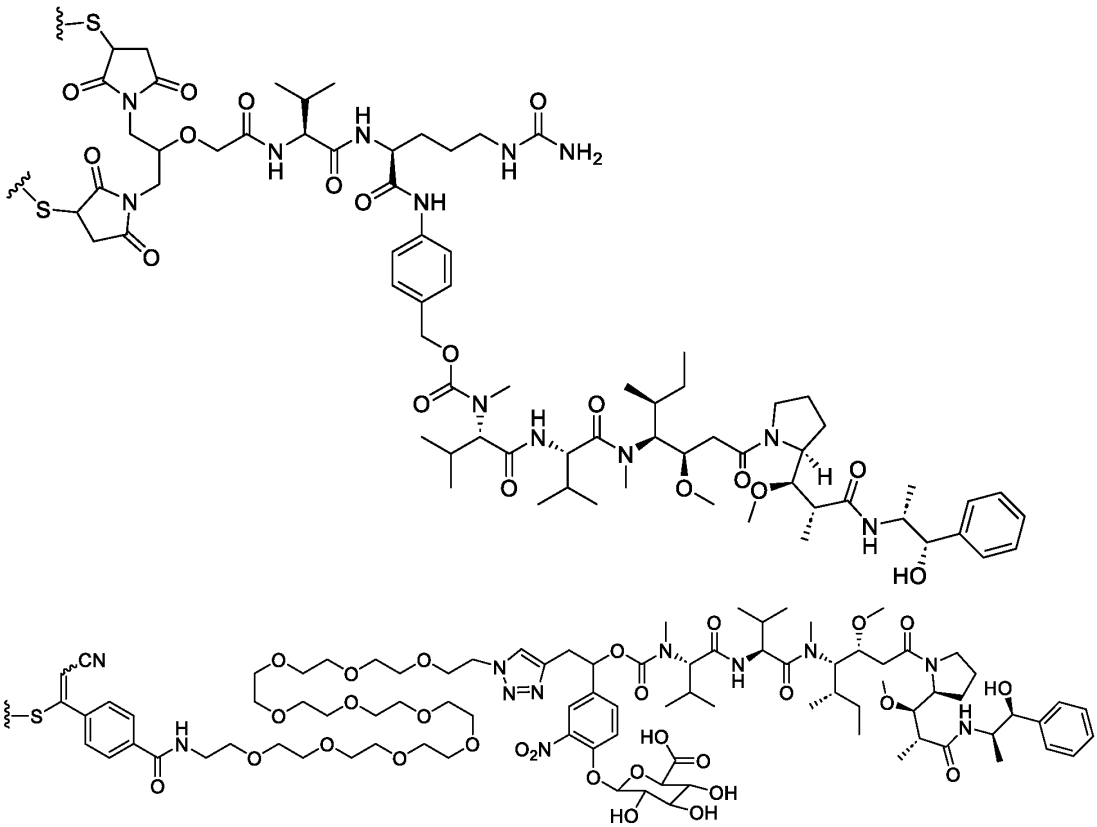


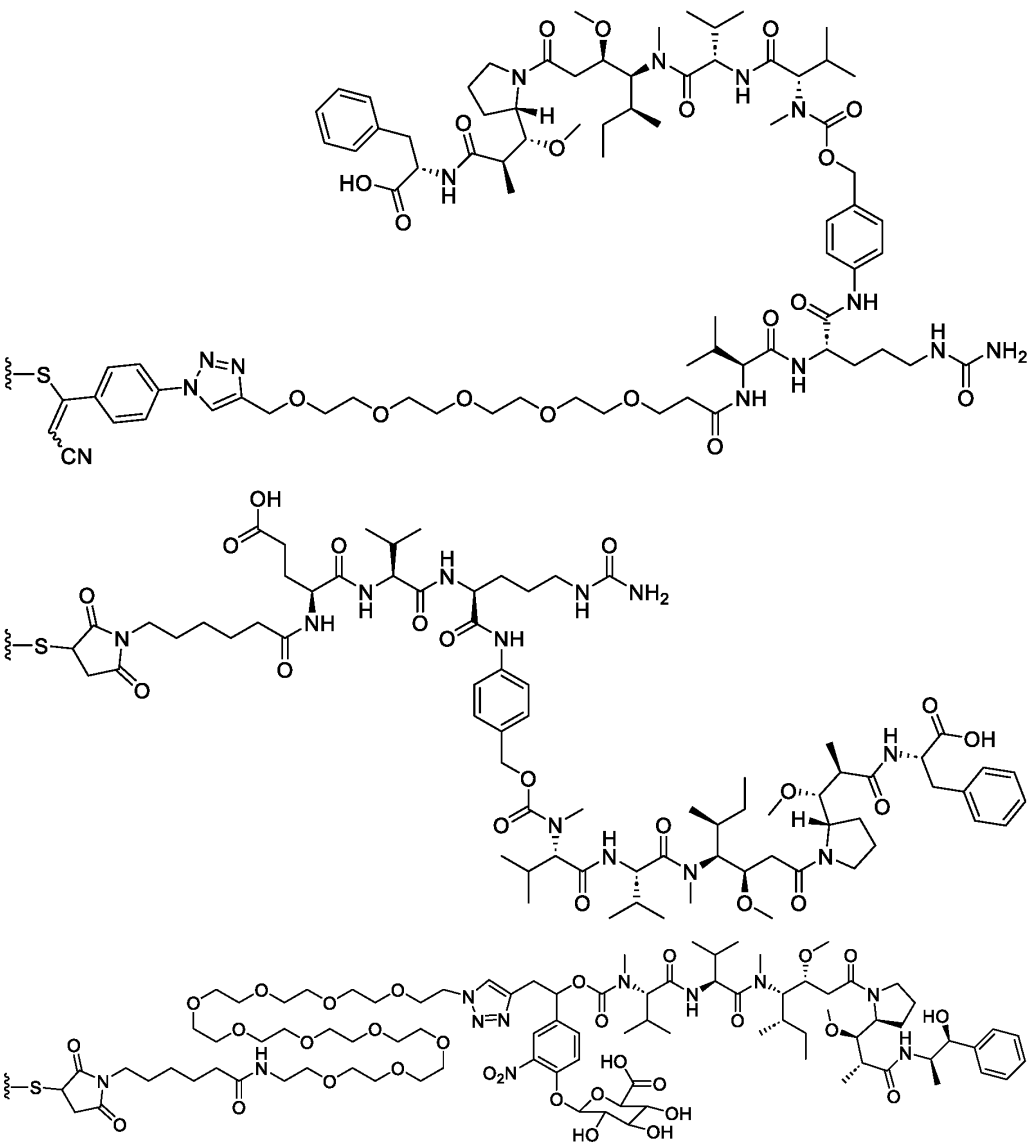


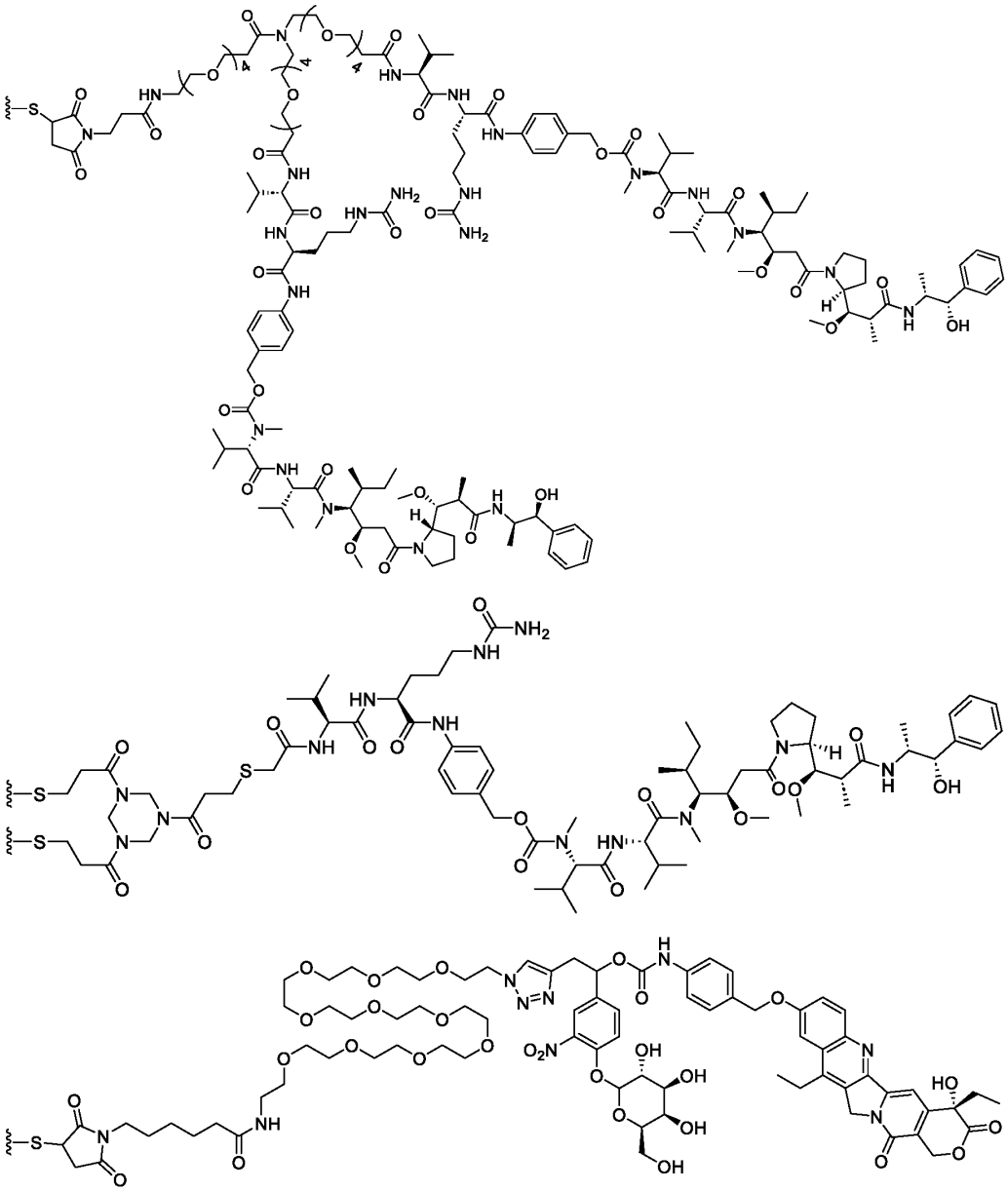


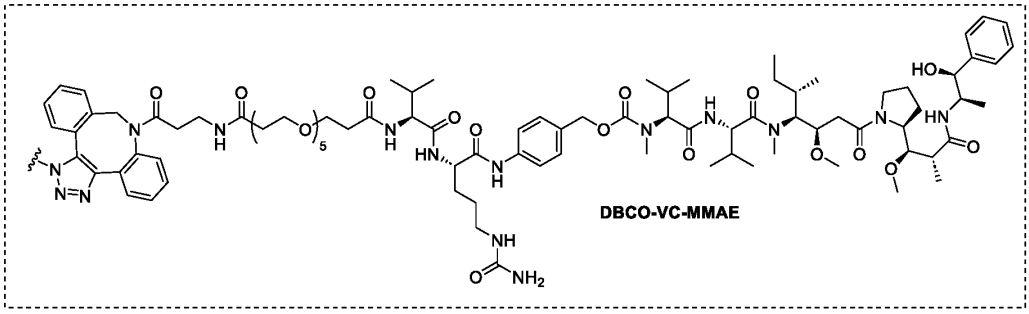


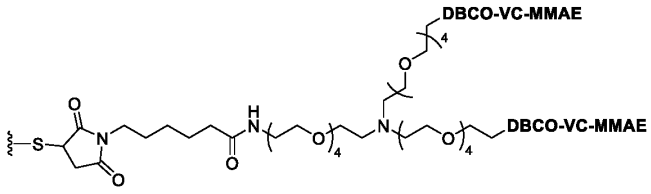


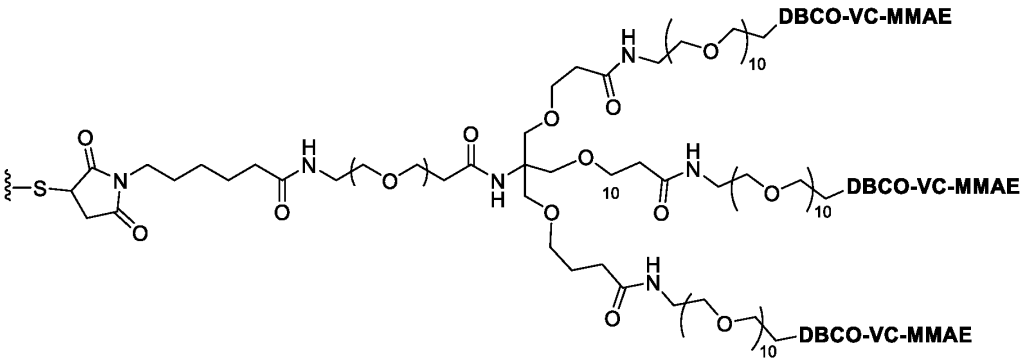


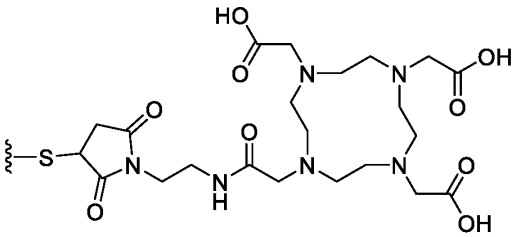


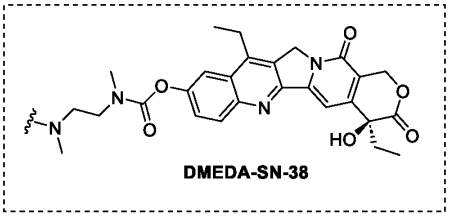


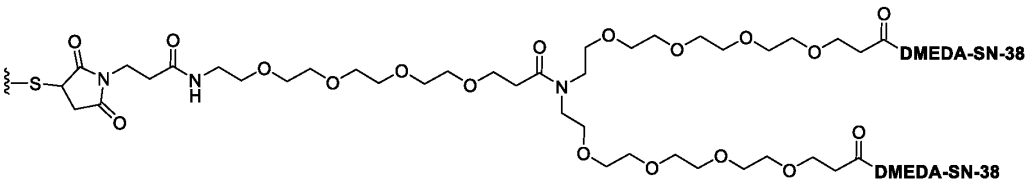

DBCO-VC-MMAE

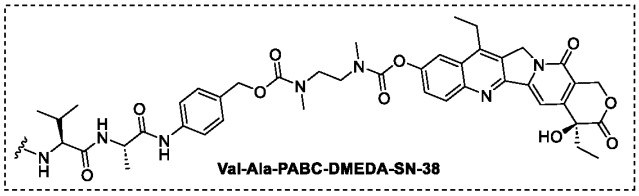

DBCO-VC-MMAE
DBCO-VC-MMAE

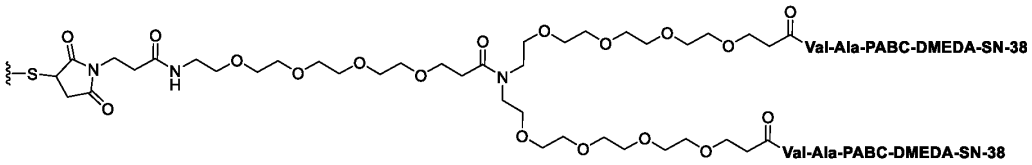

DBCO-VC-MMAE
DBCO-VC-MMAE
DBCO-VC-MMAE

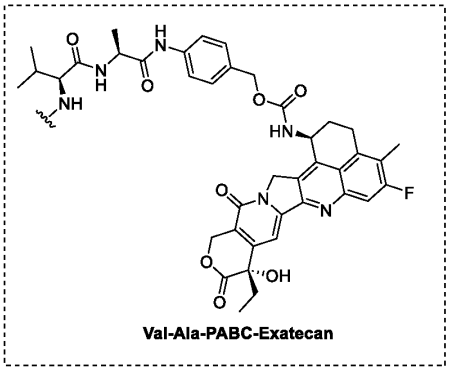


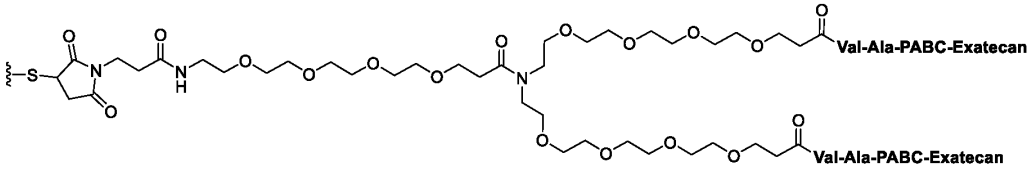

DMEDA-SN-38

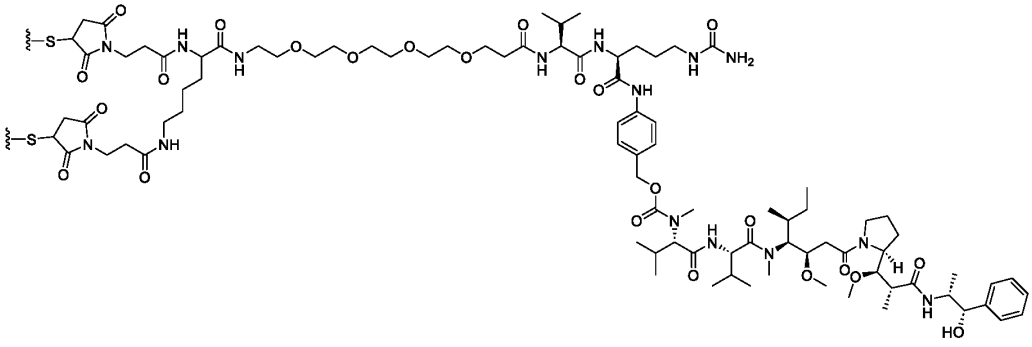

DMEDA-SN-38
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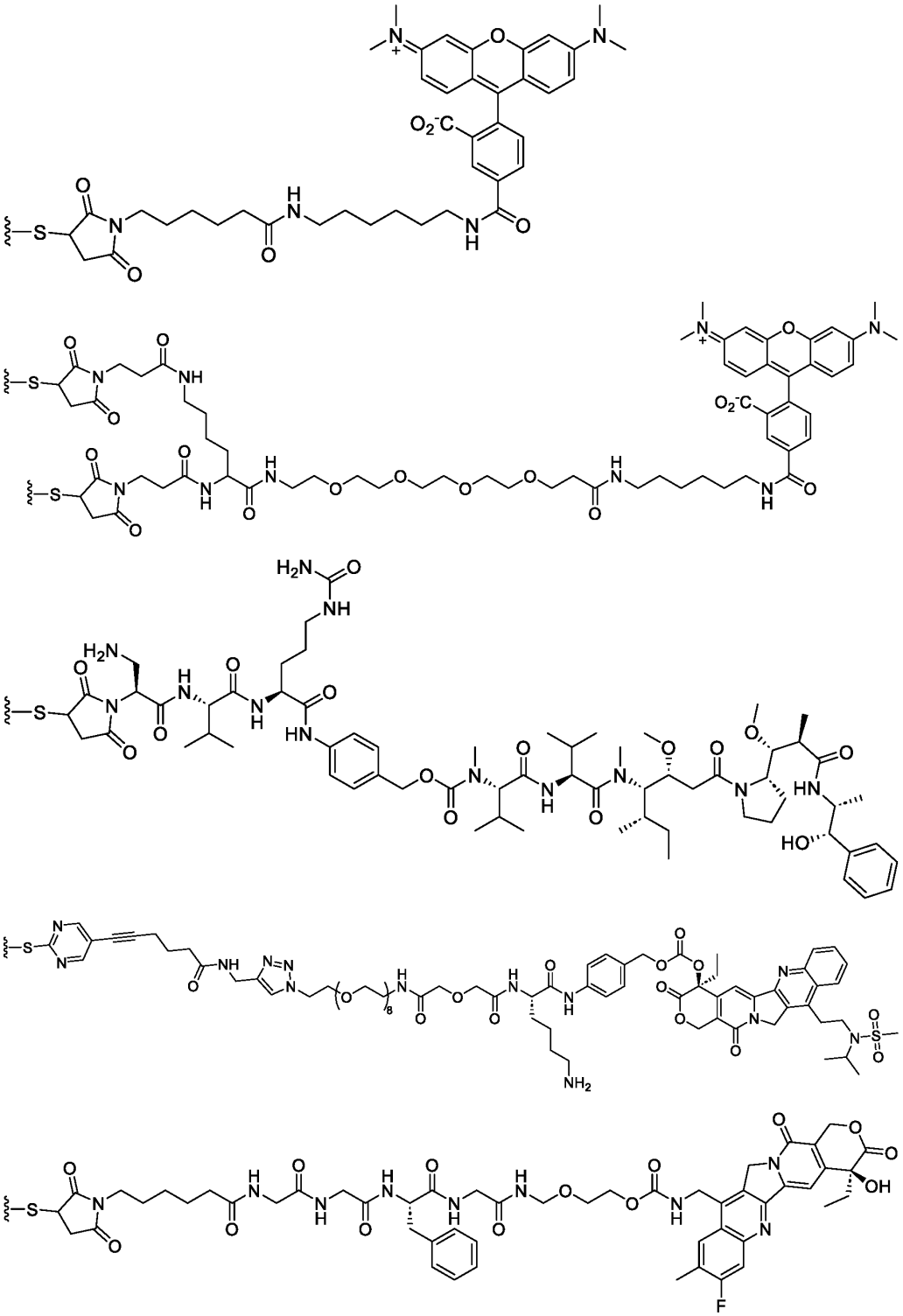

Val-Ala-PABC-DMEDA-SN-38

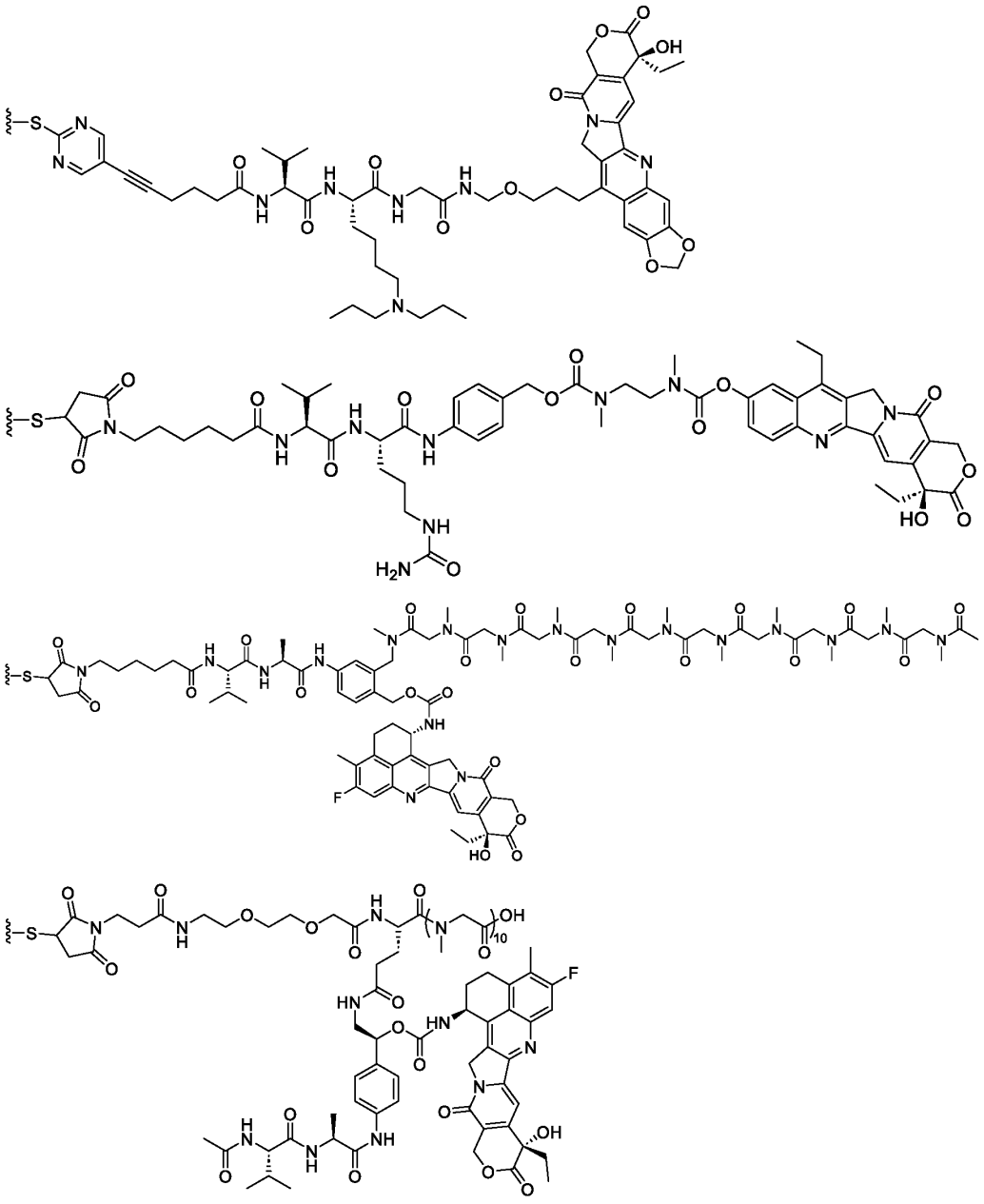

Val-Ala-PABC-DMEDA-SN-38
Val-Ala-PABC-DMEDA-SN-38


Val-Ala-PABC-Exatecan

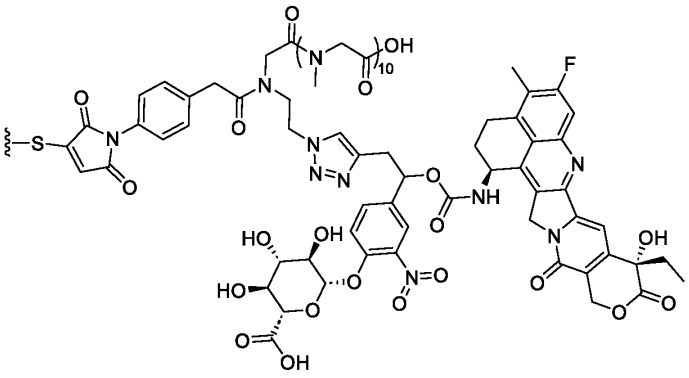

Val-Ala-PABC-Exatecan
Val-Ala-PABC-Exatecan

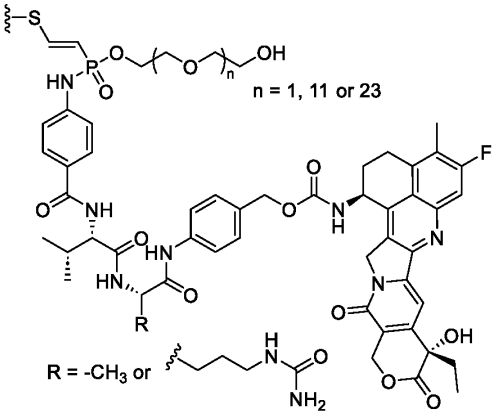


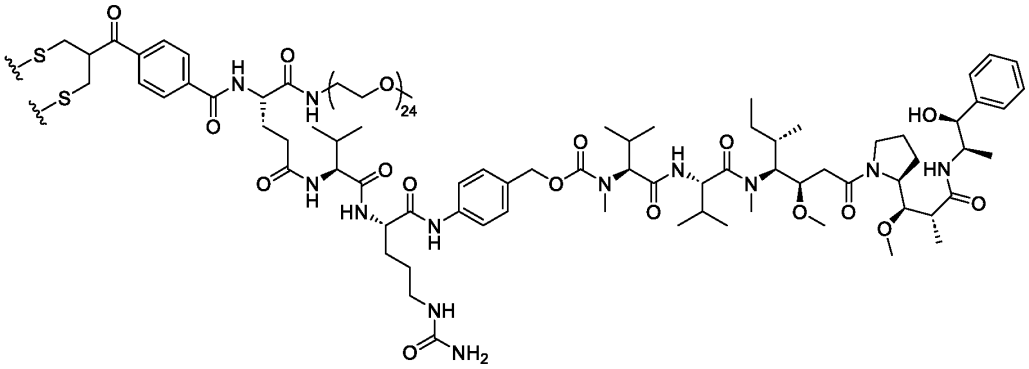


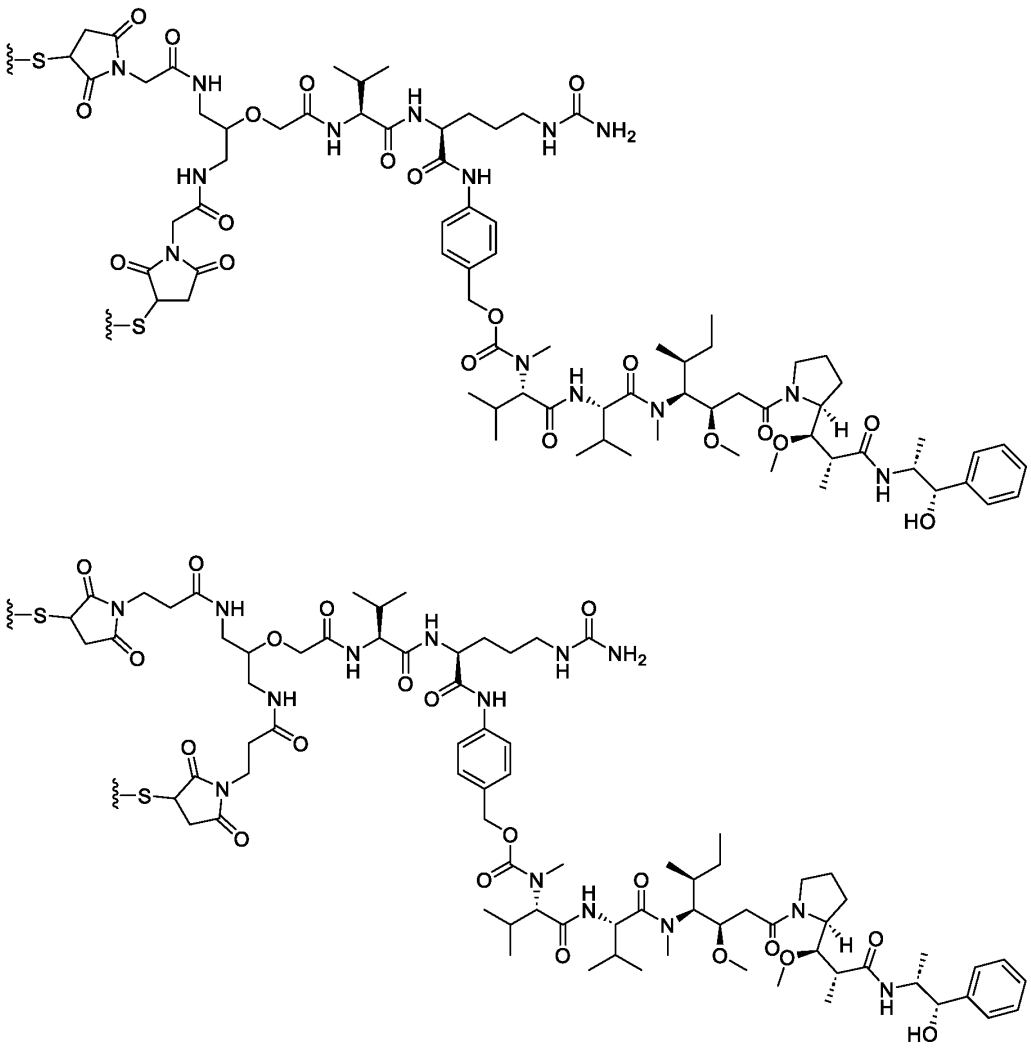


5




n = 1, 11 or 23
R = -CH3 or





According to the invention, the conjugates of formula (III) comprise -(L-(Payload)$_m$)$_n$ covalently linked at one or two of the site specific interchain cysteine residues as defined above, (i.e.) the conjugated cysteine residues.

According to an embodiment, the conjugates of formula (III) may further comprise one or more identical or different -(L-(Payload)$_m$)$_n$ covalently linked at one or more additional sites of conjugation. Such conjugates comprising additionally grafted -(L-(Payload)$_m$) are encompassed by Formula (III) and are herein referred to as “further conjugated conjugates”.”

According to an embodiment, said additional sites of conjugation include cysteine residues of Protein, such as interchain cystein residues different from the conjugated cysteine residues as defined above.

According to an embodiment, said additional sites of conjugation include cysteine residues that have been introduced into Ab. A representative example is engineered antibodies such as Thiomab® described in US 7,521,541.

According to an embodiment, said additionally grafted -(L-(Payload)$_m$) are identical to the identical or different -(L-(Payload)$_m$) fragments as defined in Formula (III).

According to an embodiment, further conjugated conjugates are of formula (IV):

$$(T)_p\text{-Ab'-(L-(Payload)}_m)_{n'} \quad \text{(IV)}$$

Where in formula (IV):

Ab', L, Payload, T, p and m are defined as in Formula (III)

and

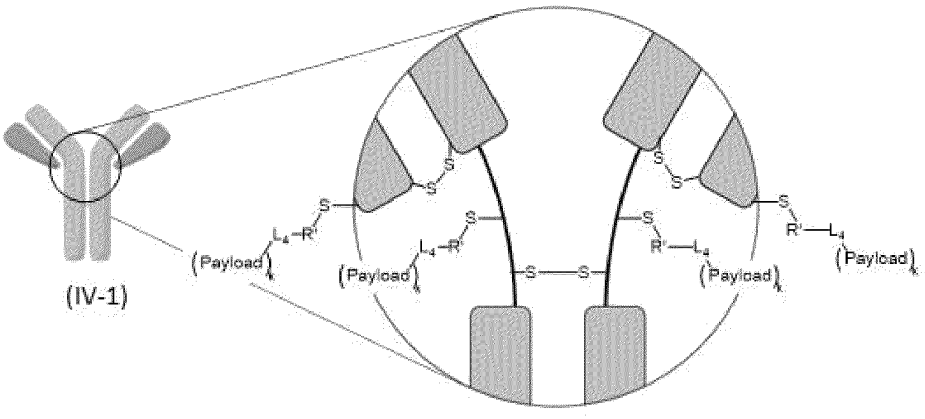
n'=n+n_a where n_a is the number of the additional cysteine residues introduced into Ab and $n_a \geq 1$;

and where

n -L-(Payload)$_m$ fragments are conjugated at two conjugated cysteine residues as defined above; the remaining interchain cysteine residues forming interchain disulfide bridges; and n_a -L-(Payload)$_m$ fragments are conjugated at additional sites of conjugation.

Typically, where Ab is Thiomab, n_a=2 and n'=4.

A representative conjugate of formula (IV) is conjugate (IV-1) depicted below:



Where R', L_4, Payload an k are as defined above, and

-R'-L_4-$(Payload)_k$ fragments are covalently bound to the Sulfur atoms of the introduced cysteine residues of a Thiomab® antibody (the additionally grafted fragments), in addition to the fragments covalently bond to the two N-proximal inter(heavy chains) cysteine residues.

According to an embodiment, said additionally grafted -(L-$(Payload)_m$) are identical or different from the -(L-$(Payload)_m$) fragments as defined in Formula (III).

The compounds of formula (III) may also be used as substrates for further conjugation of payload fragments.

According to a further object, the invention concerns conjugates of formula (V):

$$((Payload')_q\text{-}L')_r\text{-}Ab'\text{-}(L\text{-}(Payload)_m)_{n''}$$

(V)

Where T, p, Ab', L, Payload, m are defined as in Formula (III),

n'' is n as defined in Formula (III) or n=n' as defined in Formula (IV),

Payload' is a payload as defined in Formula (III) identical or different from Payload,

L' is a linker as defined in Formula (III) identical or different from L,

q is 1, 2, 3, 4, 5 or 6,

r =(8-n)+n_a where n is 1 or 2, and $n_a \geq 0$ is the number of any additional cysteine residues that have been optionally introduced into Ab;

n -L-$(Payload)_m$ fragments are conjugated at two conjugated cysteine residues as defined above; and

n_a -L-$(Payload)_m$ fragments are optionally conjugated at additional cysteine residues that have been optionally introduced into Ab;

r -L'-$(Payload')_q$ fragments are conjugated at one or more of further cysteine residues.

Typically, said further cysteine residues are interchain cysteine residues. Preferably all cysteine residues not already conjugated to L-$(Payload)_m$ fragments (i.e. the conjugated cysteine residues as defined above) are conjugated to the -L'-$(Payload')_q$ fragments.

According to an embodiment, in Conjugates of formula (V), all available interchain cysteine residues are covalently bound to either a –L'-$(Payload')_q$ fragment- or -L-$(Payload)_m$ fragment, it being understood that -L-$(Payload)_m$ fragments are conjugated at the conjugated cysteine residues are defined above.

According to an embodiment, said additional sites of conjugation are interchain cysteine residues of Ab' different from the conjugated cysteine residues as defined above.

The process of preparation of the compounds of formula (III) or mixtures thereof

The invention also concerns a process of preparation of a protein conjugate of formula (III) as defined herein, said process comprising complexing said metal atom from said compound of formula (II) as defined herein and conjugating said de-complexed compound with 1 to n conjugating fragments L-(Payload)$_m$ as defined herein.

The process may also comprise the additional step of purifying the intermediate of formula (II), prior to preparing compound (III).

The process may typically comprises reacting a compound of formula (II) with a metal complexing agent so as to form a de-complexed compound.

Said metal complexing agent may typically be chosen from ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), nitrilotriacetic acid (NTA).

The process also comprises the coupling of said decomplexed compound with a conjugation agent.

Said de-complexing step and conjugating step may be conducted separately in sequence or as a one pot reaction.

In particular, the one pot reaction may comprise preparing a reactional mixture comprising said compound of formula (II), said metal complexing agent and said conjugation agent.

According to a first alternative, the preparation of said reactional mixture may comprise adding said conjugation agent to compound (II), followed by adding said complexing agent.

According to a second alternative, the preparation of said reactional mixture may comprise adding said complexing agent to compound (II), followed by adding said conjugation agent.

Without being bound by theory, it has been observed that in either the first or second alternative, the de-complexing reaction typically occurs before the conjugation step, irrespective of the order of introduction of the complexing agent and conjugation agent.

Therefore, the preparation of conjugate (III) is herein referred as "1.complexing agent, 2.conjugation agent". This sequence is used to illustrate the order in which the reagents

react (or in other words the order in which the de-complexing and conjugation reactions occur) and not necessarily the order of introduction of the reagents.

According to an embodiment, the process of preparation of the conjugates of formula (III') comprises preparing a mixture comprising a conjugation agent, a complexing agent and compound (II). Preferably it comprises adding said conjugation agent to compound (II) followed by adding said complexing agent to the mixture.

According to another embodiment, the process of preparation of the conjugates of formula (III") comprises purifying compound of formula (II), and preparing a mixture comprising conjugation agent, complexing agent and compound (II). Preferably it comprises adding said complexing agent to compound (II) followed by adding conjugation agent to the mixture.

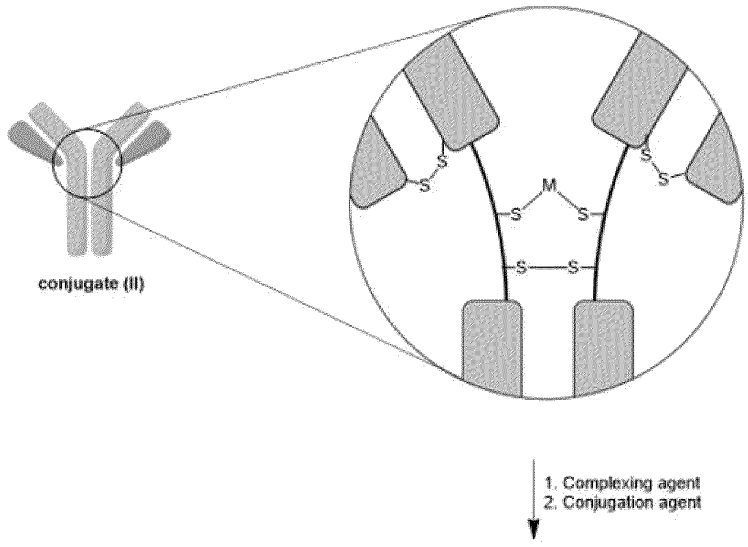
According to an embodiment, all steps are preferably conducted in a buffered reacting medium, selected from the group comprising Hepes, Histidine buffer, PBS, or MES, having a pH value comprised between 5.5 and 8, and at a temperature of about -10°C to 37°C, for example, at about 0°C to 20°C.

Typically, reactions may be conducted in conditions compatible with Protein, such as pH comprised between 7 and 9, preferably between 7 and 8, at a temperature comprised between 20 and 40°C, preferably at room temperature.
Typically, the reaction may be conducted in a buffered medium such as TBS, PPB, PBS, HEPES medium. Reaction time may be comprised between 1 and 10 hours, preferably between 1 and 5 hours.

The concentration of Protein in the reactional mixture may be adjusted; advantageously it may be comprised between 6.0 and 8.0 g/L.

The nature of the reagents and experimental conditions of the process may depend on the degree of conjugation that is desired.

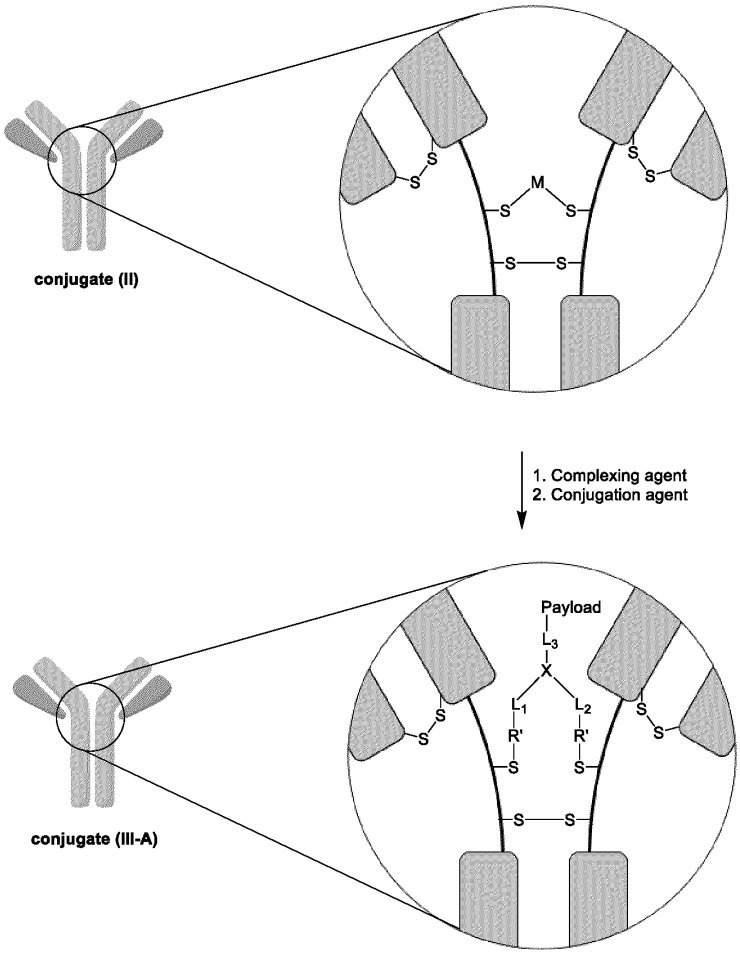
As an illustration:
The process of preparation of a conjugate of formula (III-A) may be schematically represented by the following scheme:



Ab'-L-(Payload) (III-A)

Where Ab', L, M and Payload are defined as above.

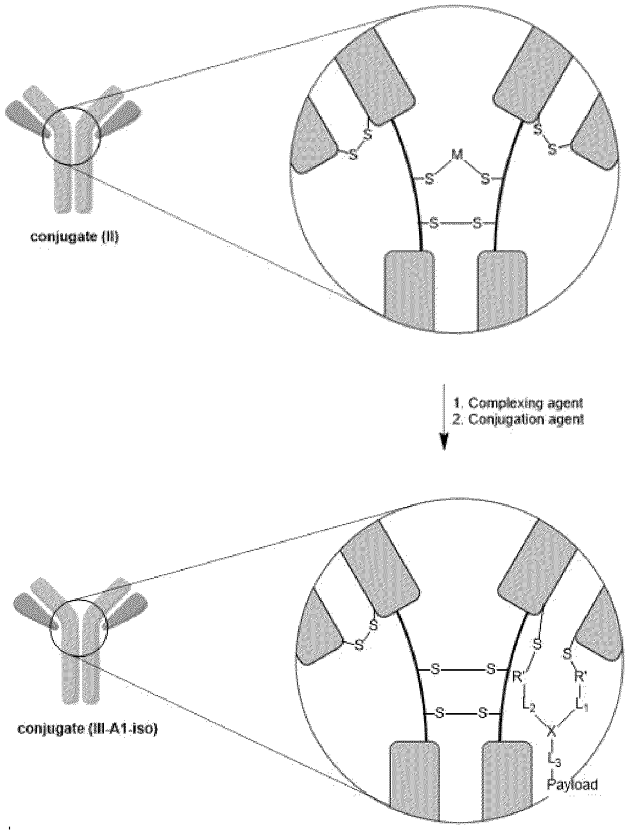
As an illustration, the process of preparation of a conjugate of formula (III-A), typically (III-A1) may be schematically represented by the following scheme:



Where L_1, L_2, L_3, R', X and Payload are defined as above.

The formation of a compound (III-A1) may be favored by adding first the conjugation agent to the compound of formula (II), followed by adding the complexing agent.

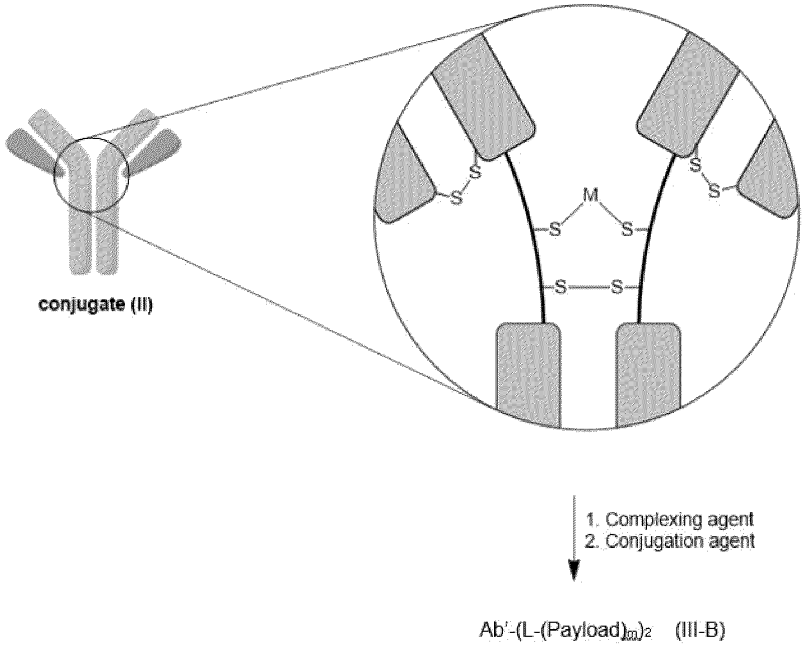
As an illustration, the process of preparation of a conjugate of formula (III-A), typically (III-A1-iso) may be schematically represented by the following scheme:



Where L_1, L_2, L_3, R', X and Payload are defined as above.

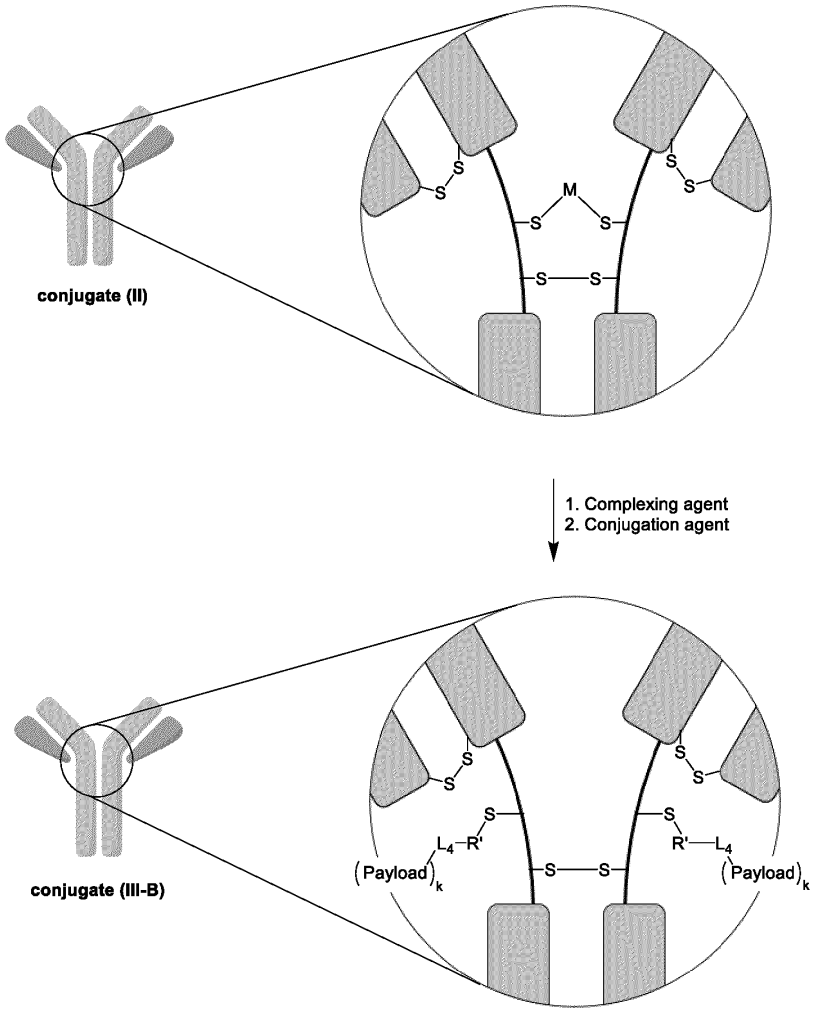
The formation of a compound (III-A1-iso) may be favored by adding first the complexing agent to the compound of formula (II), followed by adding the conjugation agent.

The process of preparation of a conjugate of formula (III-B) may be schematically represented by the following scheme:



Where Ab', L, M and Payload are defined as above.

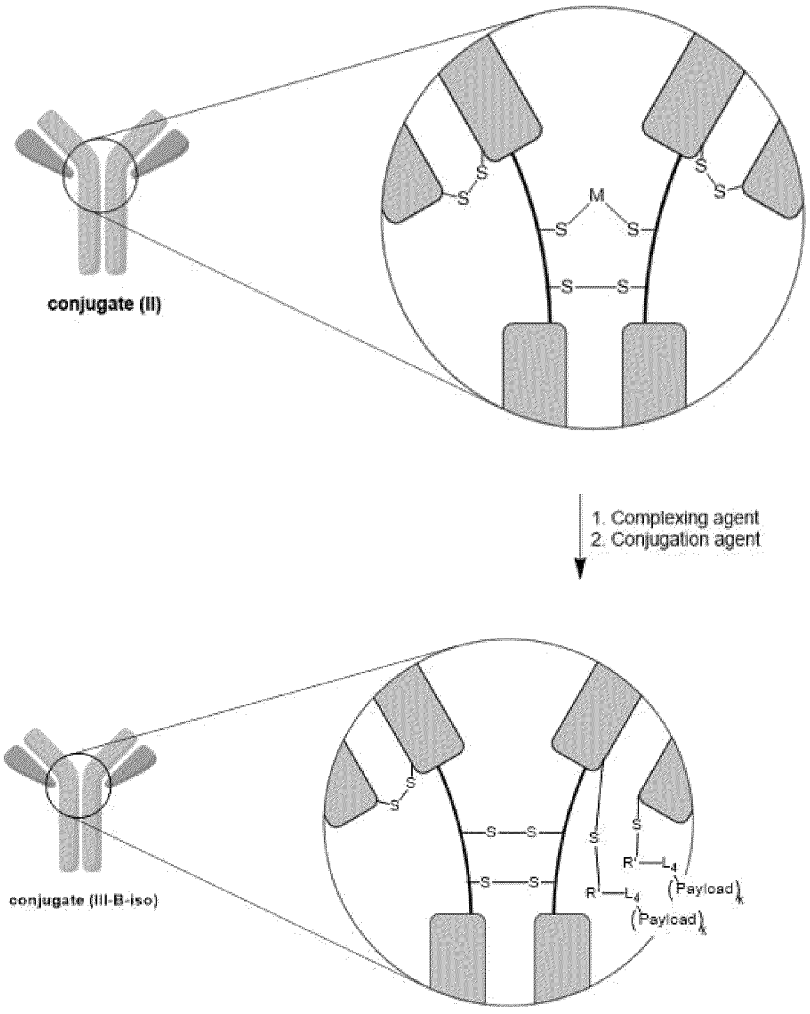
As an illustration, the process of preparation of a conjugate of formula (III-B) typically (III-B1) may be schematically represented by the following scheme:



Where k is defined as m above, and Payload and R' are defined as above.

The formation of a compound (III-B) may be favored by adding first the conjugation agent to the compound of formula (II), followed by adding the complexing agent

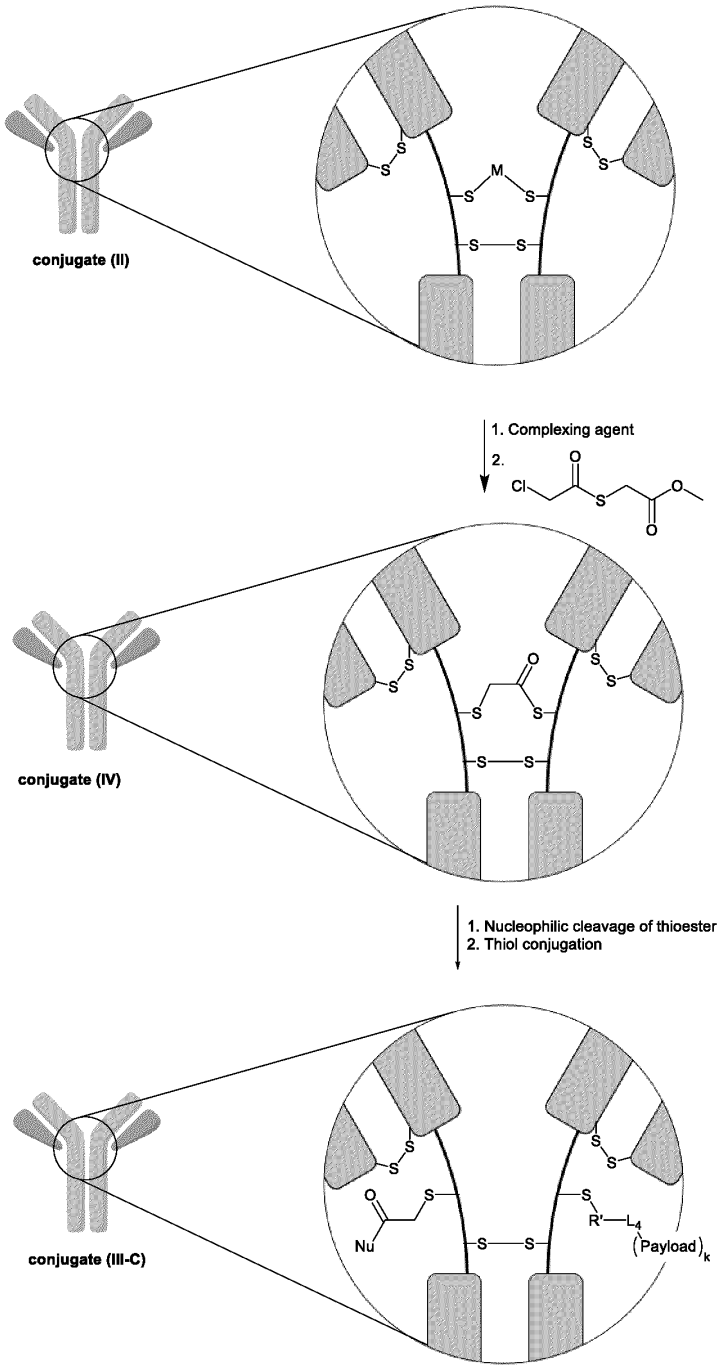
As an illustration, the process of preparation of a conjugate of formula (III-B), typically (III-B1-iso) may be schematically represented by the following scheme:



Where k is defined as m above, and Payload, L_4 and R' are defined as above.

The formation of a compound (III-B-iso) may be favored by adding first the complexing agent to the compound of formula (II), followed by adding the conjugation agent.

The process of preparation of a conjugate of formula (III-C) may be schematically represented by the following scheme:



Where k is defined as m above, and Payload, Nu and R' are defined as above.

The process of preparation of the conjugates of formula (III) also includes the process of preparation of conjugates of formula (IV) as defined above.

According to this embodiment, the process of preparation of conjugates (IV) typically includes complexing said metal atom from said compound of formula (II) and conjugating said de-complexed compound with 1 to n' conjugating fragments L-(Payload)$_m$ as defined herein,

where

in formula (II) Ab' is an engineered antibody in which n_a additional cysteine residues have been introduced, as detailed above, and

$n'=n+n_a$.

According to a further object, the invention also concerns the process of preparation of conjugates of formula (V) as defined above from the conjugates of formula (III).

The process of preparation of conjugates (V) typically comprises the step of further conjugating a compound of formula (III) as defined above with r –L'-(Payload')$_q$ fragments.

Said further conjugating is may be conducted by application or adaptation of known procedures. It may be typically unspecific in that it comprises the conjugation through the sulfur atom of all cysteine residues that are present in Ab'. These cysteine residues include those interchain cysteine residues involved in disulfur bridges, and additional cysteine residues that may have been introduced into Protein as detailed above.

The process of preparation of conjugates of formula (V) typically includes reacting a compound of formula (III) with a reducing agent, as defined above, followed by conjugating the reduced conjugate with 1 to r conjugating fragments L-(Payload)$_q$.

The conjugation step may comprise reacting a conjugation agent as defined herein.

In particular, conjugates of formula (V) may be prepared by further conjugating a conjugate of formula (IV).

The conjugation agent

The term conjugation agent refers to any compound bearing at least one L-(Payload)$_m$ fragment and one or more reactive function capable of reacting with reduced sulfur atom(s) present in Protein, so as to lead to a –S-L-(Payload)$_m$ fragment as defined above, ensuring the covalent binding to one or two of the conjugated cysteine residues.

According to an embodiment, said conjugation agent may be terminated by a thiol-reactive function, such as a maleimide or APN group.

The conjugation agent may be chosen from thiol-reactive compounds, such as compounds bearing a maleimide or APN ((hetero)arylpropiolonitrile) terminal group.

The mixtures

The above compounds of formula (I), (II) or (III) may be obtained or present in a mixture.
In said mixtures, the compounds of formula (I), (II) or (III), respectively, are present in major amounts.
According to an embodiment, said mixture comprises at least 50% in number, preferably at least 60%, more preferably at least 70%, still more preferably at least 80% of compounds of formula (I), (II) or (III), respectively.

In said mixtures, the compounds of formula (I), (II) or (III) are present alongside other compounds, such as side products, including different regioisomers where the coordination and/or conjugations has occurred at different locations.

Regioisomers may be formed because Protein generally comprises a number of cysteine residues that are available for reduction, coordination and subsequent conjugation. Nevertheless, it has been demonstrated that these regioisomers are formed in minor amounts as the conjugated cysteine residues as defined above are predominantly coordinated according to the invention. Therefore, the compounds of formula (I), (II) or (III) are thus site specifically conjugated at the conjugated cysteine residues and as such differ from the mixtures typically obtained by state-of-the-art conjugation processes.

In the sense of the invention, the term “regioisomer” thus refers to isomeric coordination complexes or conjugates, where the site of reduction of sulfur atoms with cysteine residues may vary on the backbone of Protein.
The mixtures comprising the compound of formula (III) may also comprise conjugates having different -(L-(Payload)$_m$)$_n$ fragments, including conjugates having different drug-antibody degree.

As discussed above, the conjugates of the invention may thus be “heterogeneous” in some extent, although enriched in one site-specific conjugate.

Therefore, in the sense of the invention, the mixtures comprising the conjugates of the invention alongside other compounds are also encompassed by the present invention.

According to an object, the invention concerns the mixtures comprising the conjugates of the invention.

According to an embodiment, the invention concerns a mixture comprising the protein conjugate of formula (III) as defined in anyone of claims 8 to 14, in at least 50% in number, preferably at least 60%, more preferably at least 70%, still more preferably at least 80%.

According to an embodiment, the mixtures of the invention comprise more than 50%, typically more than 60%, preferably more than 70%, more preferably more than 80%, still more preferably more than 90%, most preferably more than 95% of a conjugate of the invention, the percentage being expressed on a molar basis with respect to the total protein. As used herein the total protein species refer to the conjugated protein and unconjugated protein species that are present in the mixture.

If desired, the desired compounds of formula (I), (II) or (III) may be isolated by known techniques, such as hydrophobic interaction chromatography (HIC).

The therapeutical use of the compounds of formula (III)

According to a particular object, the present invention concerns a pharmaceutical composition comprising a conjugate of formula (III) or mixture thereof as defined above and one or more pharmaceutically excipient.

According to a still further object, the present invention also concerns a conjugate of formula (III) or the mixture thereof as defined above, where Payload is a cytotoxic drug or antineoplastic agent, or an imaging agent for use for treating, preventing and/or diagnosing a condition or disorder chosen from the group consisting in tumor, cancer, autoimmune disease, or infectious disease in a subject.

According to a further object, the present invention also concerns a method of treating, preventing and/or diagnosing a condition or disorder chosen from the group consisting in tumor, cancer, autoimmune disease, or infectious disease in a patient in the need thereof, comprising administering a conjugate of formula (III) as defined above to said patient.

The identification of those subjects who are in need of treatment of herein-described diseases and conditions is well within the ability and knowledge of one skilled in the art. A clinician skilled in the art can readily identify, by the use of clinical tests, physical examination, genetic tests and medical/family history, those subjects who are in need of such treatment.

The therapeutically effective amount of conjugates of formula (III) can be readily determined by the attending diagnostician, as one skilled in the art, by the use of conventional techniques and by observing results obtained under analogous circumstances. In determining the therapeutically effective amount, a number of factors are considered by the attending diagnostician, including, but not limited to: the species of subject; its size, age, and general health; the specific disease involved; the degree of involvement or the severity of the disease; the response of the individual subject; the particular compound administered; the mode of administration; the bioavailability characteristic of the preparation administered; the dose regimen selected; the use of concomitant medication; and other relevant circumstances. The amount of conjugates of formula (II) which is required to achieve the desired biological effect, will vary depending upon a number of factors, including the dosage of the conjugates to be administered, the chemical characteristics (e.g. hydrophobicity) of the compounds employed, the potency of the compounds, the type of disease, the diseased state of the patient and the route of administration.

"Pharmaceutically" or "pharmaceutically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate.

As used herein, "pharmaceutically acceptable excipient" includes any diluents, adjuvants, excipients, or vehicles, such as preserving agents, fillers, disintegrating agents, wetting agents, emulsifying agents, suspending agents, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

In the context of the invention, the term "treating" or "treatment", as used herein, means reversing, alleviating, inhibiting the progress of, or preventing the disorder or condition to which such term applies, or one or more symptoms of such disorder or condition.

"Therapeutically effective amount" means an amount of a compound/medicament according to the present invention effective in producing the desired therapeutic effect.

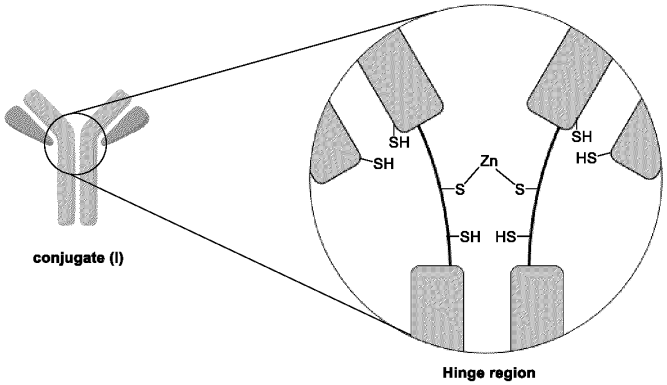
According to the invention, the term "patient", or "patient in need thereof", is intended for a human or non-human mammal affected or likely to be affected with a neuropsychological disorder. Preferably, the patient is a human.

The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well known in the pharmaceutical art, for example, as described in

Remington: The Science and Practice of Pharmacy, 20th ed.; Gennaro, A. R., Ed.; Lippincott Williams & Wilkins: Philadelphia, PA, 2000. Pharmaceutically compatible binding agents and/or adjuvant materials can be included as part of the composition. Oral compositions will generally include an inert diluent carrier or an edible carrier.

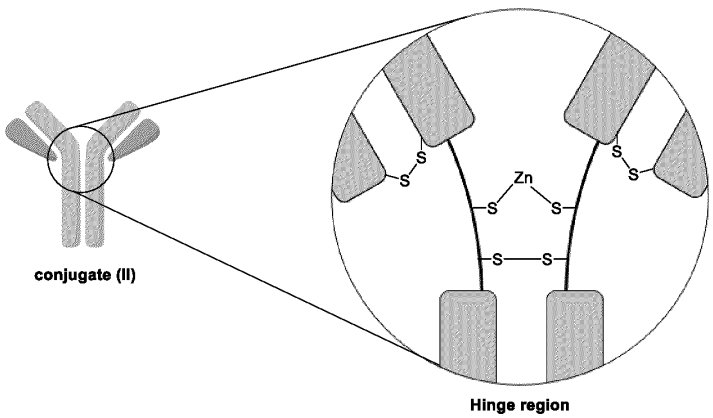
EXAMPLES

General procedure A for the preparation of conjugate (I) with Zn as M



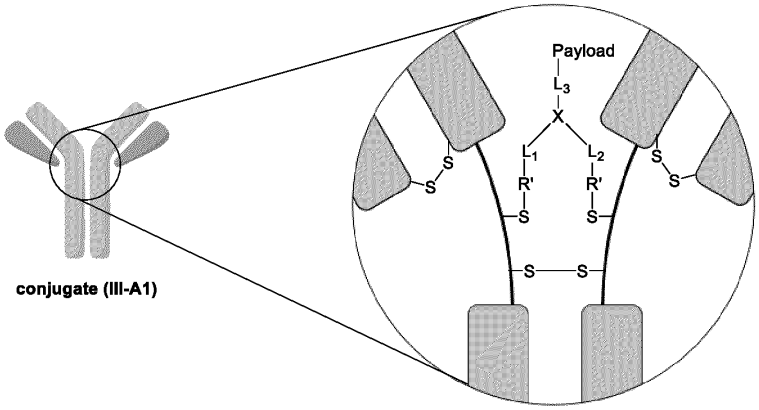
To a solution of Protein (100 µL, 40 µM in PBS pH 7.4) were added TCEP·HCl (10 mM water, 2.4 µL, 6 equiv.). The mixture was incubated at 37 °C for 2 hours, followed by cooling to 20°C. Subsequently, a solution of $Zn(OAc)_2$ (4 mM in water, 1.4 µL, 1.4 equiv.) was added to the mixture and the mixture was incubated at 20°C for 5 minutes. The resulting conjugate (I) was purified using Bio-Spin® P-30Gel Columns (Bio-Rad) which had been equilibrated with PBS buffer (1X, pH 7.4).

General procedure B for the preparation of conjugate (II) with Zn as M



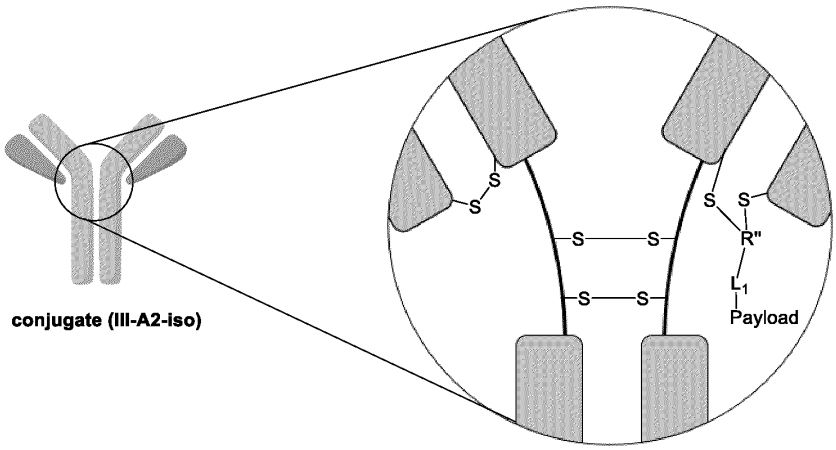
To a solution of Protein (100 µL, 40 µM in PBS pH 7.4) were added TCEP·HCl (10 mM water, 2.4 µL, 6 equiv.). The mixture was incubated at 37 °C for 2 hours, followed by cooling to 20°C. Subsequently, a solution of $Zn(OAc)_2$ (4 mM in water, 1.4 µL, 1.4 equiv.) was added to the mixture and the mixture was incubated at 20°C for 5 minutes to allow for complexation of Zn^{2+} with the protein. To the resulting mixture was added dehydroascorbic acid (DHAA, 10 mM in DMSO, 6 µL, 15 equiv.) and the mixture was incubated at 20°C for 1 hour. The resulting conjugate (II) was purified using Bio-Spin® P-30Gel Columns (Bio-Rad) which had been equilibrated with PBS buffer (1X, pH 7.4).

General procedure C for the preparation of conjugate (III-A1)



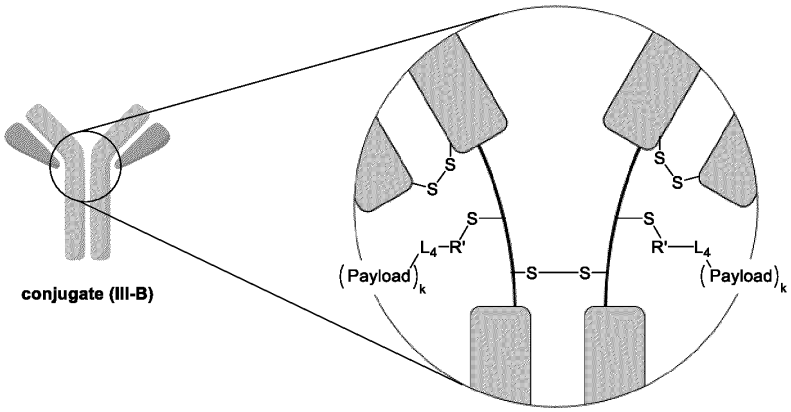
To a solution of Protein (100 µL, 40 µM in PBS pH 7.4) were added TCEP·HCl (10 mM in water, 2.4 µL, 6 equiv.). The mixture was incubated at 37 °C for 2 hours, followed by cooling to 20°C. Subsequently, a solution of $Zn(OAc)_2$ (4 mM in water, 1.4 µL, 1.4 equiv.) was added to the mixture, followed by the addition of dehydroascorbic acid (DHAA, 10 mM in DMSO, 6 µL, 15 equiv.). The resulting mixture was incubated at 20°C for 1 hour, then purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4) and cooled to 4°C. To the resulting mixture was added the solution of Payload (10 mM in DMSO, 1.2 µL, 3 equiv.) followed by EDTA (0.5 M in water, 0.5 µL, pH 8.0). The mixture was incubated until completion of the conjugation reaction (e.g. 1.5 hours at 4°C for maleimide-based payloads, 16h at 20°C for APN-based payloads). The resulting conjugate (III-A1) was purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4).

General procedure C2 for the preparation of conjugate (III-A2-iso)



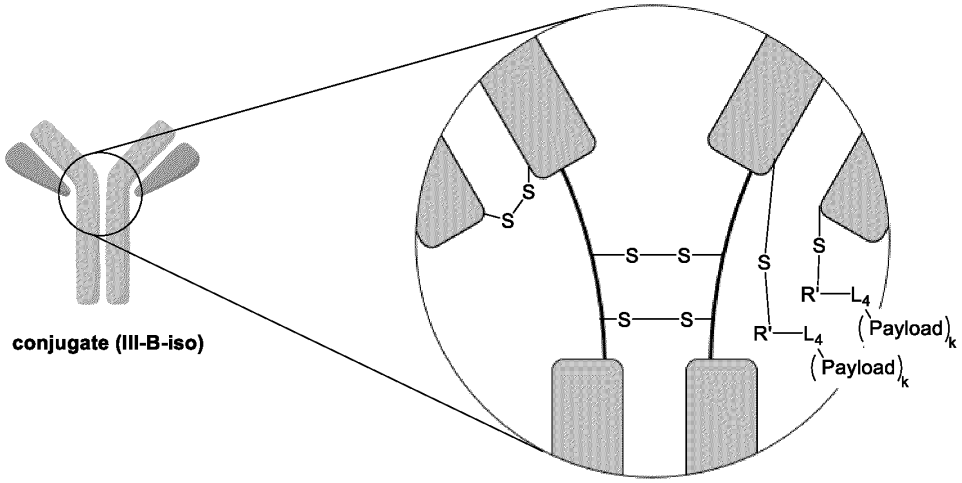
To a solution of Protein (100 µL, 40 µM in PBS pH 7.4) were added TCEP·HCl (10 mM in water, 2.4 µL, 6 equiv.). The mixture was incubated at 37 °C for 2 hours, followed by cooling to 20°C. Subsequently, a solution of $Zn(OAc)_2$ (4 mM in water, 1.4 µL, 1.4 equiv.) was added to the mixture, followed by the addition of dehydroascorbic acid (DHAA, 10 mM in DMSO, 6 µL, 15 equiv.). The resulting mixture was incubated at 20°C for 1 hour, then purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4) and cooled to 4°C. To the resulting mixture was added EDTA (0.5 M in water, 0.5 µL, pH 8.0), followed by the solution of Payload (10 mM in DMSO, 1.2 µL, 3 equiv.). The mixture was incubated until completion of the conjugation reaction (e.g. 1.5 hours at 4°C for maleimide-based payloads, 16h at 20°C for APN-based payloads). The resulting conjugate (III-A2-iso) was purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4).

General procedure D for the preparation of conjugate (III-B)



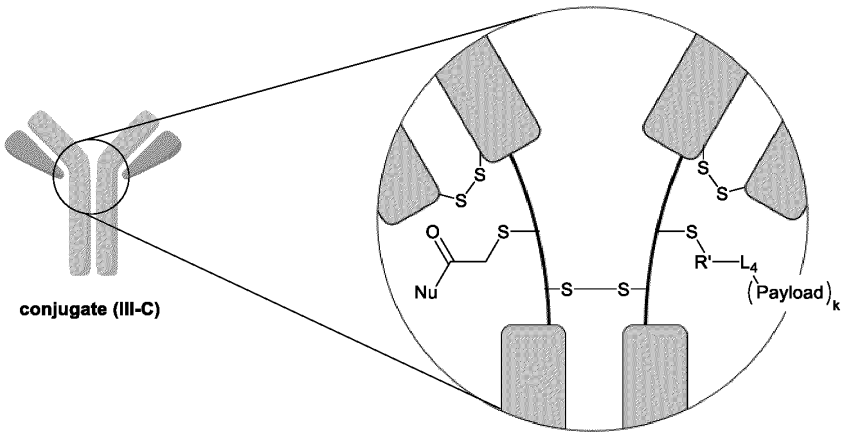
To a solution of Protein (100 µL, 40 µM in PBS pH 7.4) were added TCEP·HCl (10 mM in water, 2.4 µL, 6 equiv.). The mixture was incubated at 37 °C for 2 hours, followed by cooling to 20°C. Subsequently, a solution of $Zn(OAc)_2$ (4 mM in water, 1.4 µL, 1.4 equiv.) was added to the mixture, followed by the addition of dehydroascorbic acid (DHAA, 10 mM in DMSO, 6 µL, 15 equiv.). The resulting mixture was incubated at 20°C for 1 hour, then purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4) and cooled to 4°C. To the resulting mixture was added the solution of Payload (10 mM in DMSO, 4.0 µL, 10 equiv.) followed by EDTA (0.5 M in water, 0.5 µL, pH 8.0). The mixture was incubated until completion of the conjugation reaction (e.g. 1.5 hours at 4°C for maleimide-based payloads, 16h at 20°C for APN-based payloads). The resulting conjugate (III-B) was purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4).

General procedure D2 for the preparation of conjugate (III-B-iso)



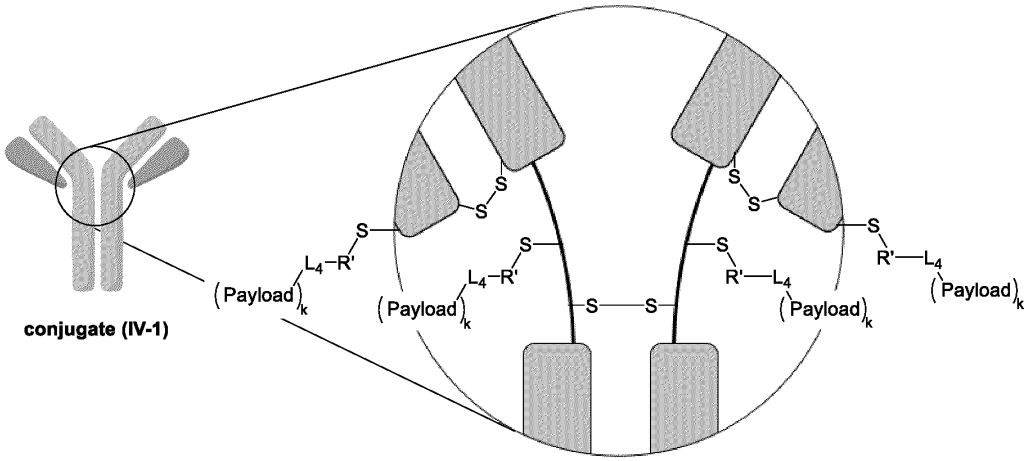
To a solution of Protein (100 µL, 40 µM in PBS pH 7.4) were added TCEP·HCl (10 mM in water, 2.4 µL, 6 equiv.). The mixture was incubated at 37 °C for 2 hours, followed by cooling to 20°C. Subsequently, a solution of $Zn(OAc)_2$ (4 mM in water, 1.4 µL, 1.4 equiv.) was added to the mixture, followed by the addition of dehydroascorbic acid (DHAA, 10 mM in DMSO, 6 µL, 15 equiv.). The resulting mixture was incubated at 20°C for 1 hour, then purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4) and cooled to 4°C. To the resulting mixture was added EDTA (0.5 M in water, 0.5 µL, pH 8.0), followed by the solution of Payload (10 mM in DMSO, 4.0 µL, 10 equiv.). The mixture was incubated until completion of the conjugation reaction (e.g. 1.5 hours at 4°C for maleimide-based payloads, 16h at 20°C for APN-based payloads). The resulting conjugate (III-B) was purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4).

General procedure D3 for the preparation of conjugates (III-C)



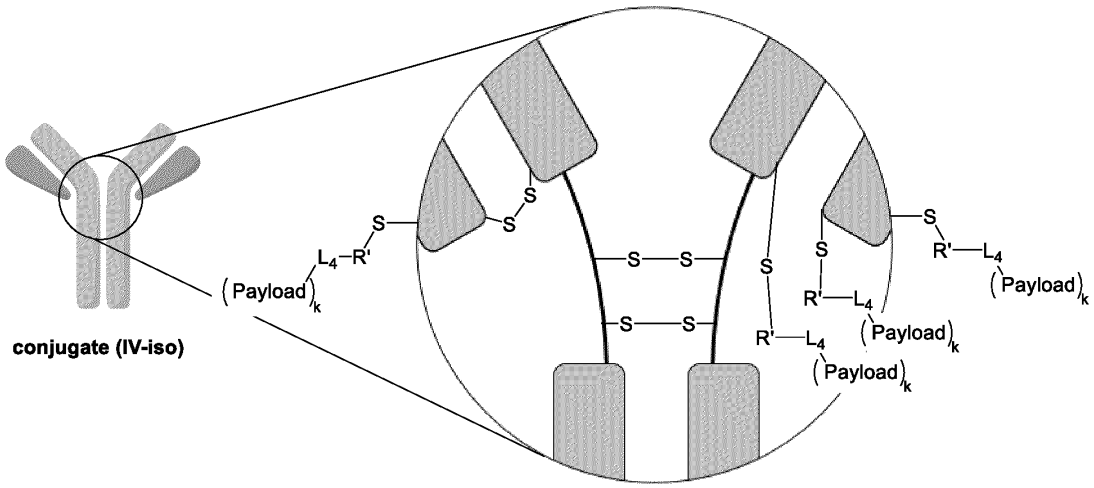
To a solution of Protein (100 µL, 40 µM in PBS pH 7.4) were added TCEP·HCl (10 mM in water, 2.4 µL, 6 equiv.). The mixture was incubated at 37 °C for 1.5 hours, followed by cooling to 20°C. Subsequently, a solution of $Zn(OAc)_2$ (4 mM in water, 1.4 µL, 1.4 equiv.) was added to the mixture, followed by the addition of dehydroascorbic acid (DHAA, 10 mM in DMSO, 6 µL, 15 equiv.). The resulting mixture was incubated at 20°C for 1 hour, then purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4). To the resulting mixture was added methyl 2-((2-chloroacetyl)thio)acetate (1 mM in DMSO, 4.4 µL, 1.1 eq.), EDTA (0.5 M in water, pH 8.0, 0.13 µL, 16 equiv.) and HEPES (50 mM in water, pH 8.5, 0.94 µL) and the mixture was incubated at 20°C for 40 min. To the resulting mixture was added hydrazine (10 mM in water, 16 µL, 40 equiv.) and the mixture was incubated at 37°C for 4 hours and then cooled to 4°C To the resulting mixture was added the solution of Payload (10 mM in DMSO, 9.6 µL, 24 equiv.). The mixture was incubated until completion of the conjugation reaction (e.g. 1.5 hours at 4°C for maleimide-based payloads, 16h at 20°C for APN-based payloads). The resulting conjugate (III-C) was purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4).

General procedure D4 for the preparation of conjugates (IV), such as (IV-1)



To a solution of Protein (typically an engineered antibody such as disclosed in US 7,521,541) (100 µL, 40 µM in PBS pH 7.4) were added TCEP·HCl (10 mM in water, 3.2 µL, 8 equiv.). The mixture was incubated at 37 °C for 2 hours, followed by cooling to 20°C. Subsequently, a solution of $Zn(OAc)_2$ (4 mM in water, 1.4 µL, 1.4 equiv.) was added to the mixture, followed by the addition of dehydroascorbic acid (DHAA, 10 mM in DMSO, 6 µL, 15 equiv.). The resulting mixture was incubated at 20°C for 1 hour, then purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4) and cooled to 4°C. To the resulting mixture was added the solution of Payload (10 mM in DMSO, 4.0 µL, 10 equiv.) followed by EDTA (0.5 M in water, 0.5 µL, pH 8.0). The mixture was incubated until completion of the conjugation reaction (e.g. 1.5 hours at 4°C for maleimide-based payloads, 16h at 20°C for APN-based payloads). The resulting conjugate (IV-1) was purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4).

General procedure D5 for the preparation of conjugate (IV-iso)



To a solution of Protein (100 µL, 40 µM in PBS pH 7.4) were added TCEP·HCl (10 mM in water, 3.2 µL, 8 equiv.). The mixture was incubated at 37 °C for 2 hours, followed by cooling to 20°C. Subsequently, a solution of $Zn(OAc)_2$ (4 mM in water, 1.4 µL, 1.4 equiv.) was added to the mixture, followed by the addition of dehydroascorbic acid (DHAA, 10 mM in DMSO, 6 µL, 15 equiv.). The resulting mixture was incubated at 20°C for 1 hour, then purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4) and cooled to 4°C. To the resulting mixture was added EDTA (0.5 M in water, 0.5 µL, pH 8.0), followed by the solution of Payload (10 mM in DMSO, 4.0 µL, 10 equiv.). The mixture was incubated until completion of the conjugation reaction (e.g. 1.5 hours at 4°C for maleimide-based payloads, 16h at 20°C for APN-based payloads). The resulting conjugate (III-B) was purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4).

General procedure D6 for the preparation of conjugates (V)

$$Ab'\text{-}(L\text{-}(Payload)_m)_n \rightarrow ((Payload2)_q\text{-}L)_r\text{-}Ab'\text{-}(L\text{-}(Payload)_m)_n$$

(IV) (V)

To a solution of Substrate (conjugate of formula (III) such as a conjugate of formula (IV), 100 µL, 40 µM in PBS pH 7.4) were added EDTA (0.5 M in water, 1 µL, pH 8.0) and TCEP·HCl (10 mM in water, 2 µL, 5 equiv.). The mixture was incubated at 37 °C for 2 hours, followed by cooling to 4°C. To the resulting mixture was added the solution of Payload (10 mM in DMSO, 4 µL, 10 equiv.). The mixture was incubated until completion of the conjugation reaction. The resulting conjugate (V) was purified using Bio-Spin® P-30Gel Column (Bio-Rad) equilibrated with PBS (1X, pH 7.4).

General procedure E for hydrophobic interaction chromatography (HIC)

HIC was performed using a liquid chromatography system (Alliance HPLC, Waters, Manchester, UK) coupled to a 2487 UV/visible detector (Waters, Manchester, U.K.). Analyses were conducted on a TSKButyl-NPR, 3.5 × 4.6 mm column (Tosoh Bioscience, Tokyo, Japan) set at 20°C. The mobile phase A consisted of 1.5 M ammonium sulfate and 25 mM potassium phosphate at pH 7.0, while mobile phase B was a mixture of 25 mM potassium phosphate and 25% isopropanol at pH 7. Separation was achieved with a linear gradient of 0%–100% B over 12 min at a flow rate of 0.8 mL/min. Analyses were performed by injecting 20 µg of sample diluted to 1 mg/mL with mobile phase A and integrating the UV area at 210 nm for each species.

General procedure F for reverse-phase high performance liquid chromatography – mass spectrometry (RP-HPLC MS) characterization of the conjugates

RP-HPLC MS analyses were conducted using a ThermoFisher Ultimate 3000 HPLC system (Thermo Fisher Scientific, Germering, Germany), coupled to an Exactive™ Plus Extended Mass Range (EMR, Orbitrap analyzer, Thermo Fisher Scientific, Bremen, Germany). This setup was equipped with an HESI-II probe source operating in positive ion mode. Instrument control and data acquisition were handled using the Thermo Xcalibur™ mass spectrometry data system. For the analysis, an Agilent PLRP-S, 1000 Å, 2.1 × 50 mm, 5 µm column (P/N: PL1912-1502) was utilized and maintained at 40 °C. Mobile phase A was 0.1% formic acid in DI water, mobile phase B was 0.1% formic acid in acetonitrile. The separation process involved a flow rate of 0.600 mL/min and employed the following gradient: 0-4 min, isocratic 20% B; 4-5 min, 20% to 40% B; 5-10 min, 40% to 70% B; 10-11 min, 70% to 90% B. Full MS spectra were acquired in positive polarity with a scan range of 800–4,000 m/z. The resolution was set at 70,000, with an AGC target of 1 × 106 ions. Sheath gas was set to 35 AU and auxiliary gas was set to 10 AU. The spray voltage was 2.2 kV, the capillary temperature was 250 °C. Prior to analysis, all protein samples were deglycosylated using Endo S enzyme (New England Biolabs, P/N: P0741S) following manufacturer's instructions. For each analysis, 1 µg of the protein conjugate was injected. All MS data of the conjugates were deconvoluted and analyzed using UniDec software (version 5.2.1, Marty et al. Anal. Chem. 2015. DOI: 10.1021/acs.analchem.5b00140).

General procedure G for peptide mapping

50 µg of ADC were solubilized 80 µL of 50 mM NH_4HCO_3, 0.1% RapiGest (Waters) at pH 7.8. IdeS digestion was performed by adding 50 units of FabRICATOR (Genovis) at 37°C for 30 min. Disulfide reduction was performed by incubating with 5 mM TCEP for 30 min at 57°C. Alkylation of cysteins was performed in 10 mM IAM in the dark at 20°C for 40 min.

Trypsin (Promega, V5111) was prepared by suspending 20 µg in 100 µL of H_2O. Digestion was performed by adding 5 µL of trypsin solution, which corresponds to a 1:50 enzyme:substrate ratio. Samples were incubated overnight at 20°C. The reaction was stopped by adding 1 µL of TFA. RapiGest was eliminated by incubation at 37°C for 30 min and centrifugation at 13,000 g for 5 min.

NanoLC-MS/MS analysis was performed using a nanoACQUITY Ultra-Performance-LC (Waters Corporation, Milford, USA) coupled to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The nanoLC system was composed of ACQUITY UPLC® CSH130 C18 column (250 mm x 75 µm with a 1.7 µm particle size, Waters Corporation, Milford, USA) and a Symmetry C18 precolumn (20 mm × 180 µm with a 5 µm particle size, Waters Corporation, Milford, USA). The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Sample was loaded into the enrichment column during 3 min at 5 µL/min with 99% of solvent A and 1% of solvent B. Elution of the peptides was performed at a flow rate of 350 nL/min with a 2-44% linear gradient of solvent B in 38 minutes, followed by a 44-64% linear gradient in 30 minutes.

The Q-Exactive Plus was operated in data-dependent acquisition mode by automatically switching between full MS and consecutive MS/MS acquisitions. Full-scan MS spectra were collected from 300 - 1,800 m/z at a resolution of 70,000 at 200 m/z with an automatic gain control target fixed at 3 x 106 ions and a maximum injection time of 50 ms. The top 10 precursor ions with an intensity exceeding 5 x 104 ions and charge states ≥ 2 were selected from each MS spectrum for fragmentation by higher-energy collisional dissociation. Spectra were collected at a resolution of 17,500 at 200 m/z with a fixed first mass of 100 m/z, an automatic gain control target fixed at 1 x 105 ions and a maximum injection time of 100 ms. Dynamic exclusion time was set to 3 s.

Peptides containing drug linker were identified by using Byos® Desktop Software (Protein Metrics) or searched manually in raw data.

General procedure H for middle analysis

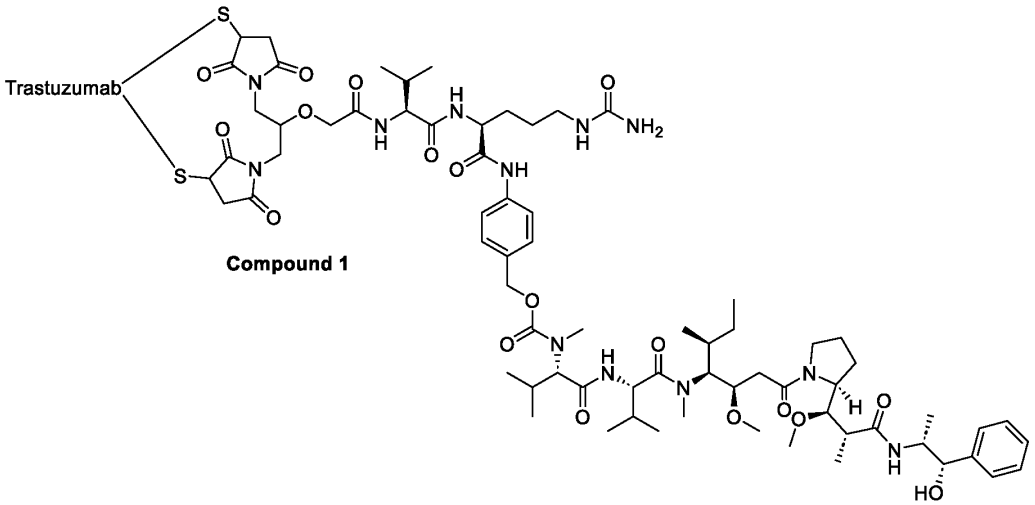
50 µg of ADC were solubilized in 50 µL of 20 mM Tris-HCl at pH 7.5. IdeS digestion was performed by adding 50 units of FabRICATOR (Genovis) at 37°C for 30 min. In order to perform a strong denaturation, 25 mg of guanidine HCl was directly added, followed by reduction with 60 mM TCEP. After 60 min incubation at 57 °C, the reaction was quenched by adding 1% TFA.

rpLC-MS analyses on large antibody fragments were also performed using the BioAccord system. A volume equivalent to 3 µg of sample preparation, was injected on a BioResolve

RP mAb Polyphenyl Column, 450Å, 2.7 µm, 2.1 mm X 50 mm (Waters, Manchester, UK, ref. 186008944) set at 80 °C.

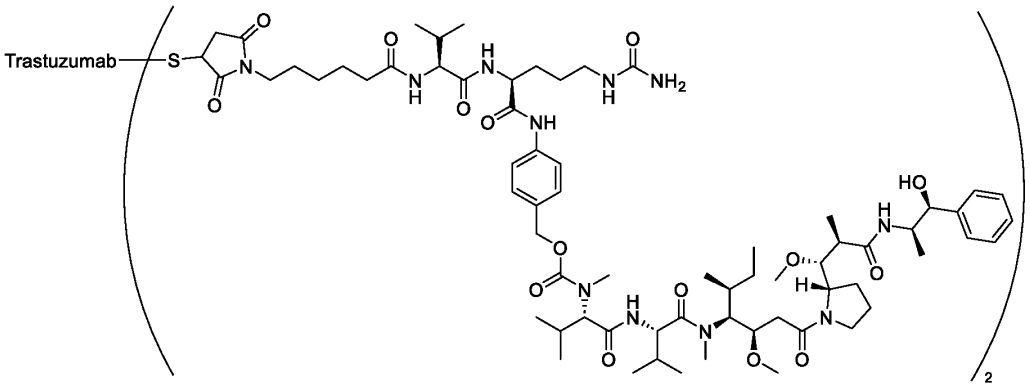
Ultrapure water for mobile phase A (0.1% FA) and acetonitrile for mobile phase B (0.1% FA) were used to perform the chromatographic separation. The gradient was generated at a flow rate of 300 µL/min and the elution program started at 20% mobile phase B from initial conditions to reach 40% in 10 minutes. The temperature column was maintained at 80 °C. The ACQUITY RDa was operated in positive mode with a capillary voltage of 1.5 kV while sample cone and desolvation temperature were set to 50 V and 550 °C, respectively. Acquisitions were performed under full scan mode and high mass range (400–7,000 m/z) with 2 Hz scan rate. The mass spectrometer was calibrated using the ACQUITY RDa Detector Calibrant and Wash Kit (Waters, Manchester, UK, ref. 186009013). The ACQUITY RDa Waters connect LockMass Kit (Waters, Manchester, UK, ref. 186009298) was used before and after each injection in order to compensate for mass variations due to temperature changes in the laboratory. UNIFI Scientific Information System (Waters, Manchester, UK) was used as a single solution to encompass data acquisition and processing.

Compound 1



Compound 1 was prepared following General procedure C, using Trastuzumab as protein and Bi-MC-VC-MMAE (CAS 1620837-70-8) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 147 279 Da; observed mass 147 295 Da.

Compound 2

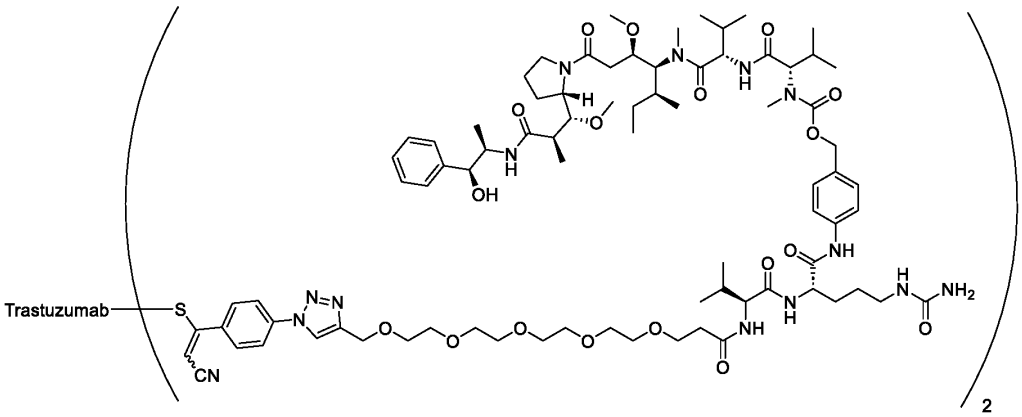


Compound 2

Compound 2 was prepared following General procedure D, using Trastuzumab as protein and MC-VC-MMAE (CAS 646502-53-6) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 148 497 Da; observed mass 148 509 Da.

Compound 2 was characterized by peptide mapping following General Procedure G and by middle analysis following General Procedure H. Both analyses confirmed the predominant conjugation site for the payload at C229 on the heavy chain of trastuzumab, as numbered in IMGT/2Dstructure-DB (INN number 7637).

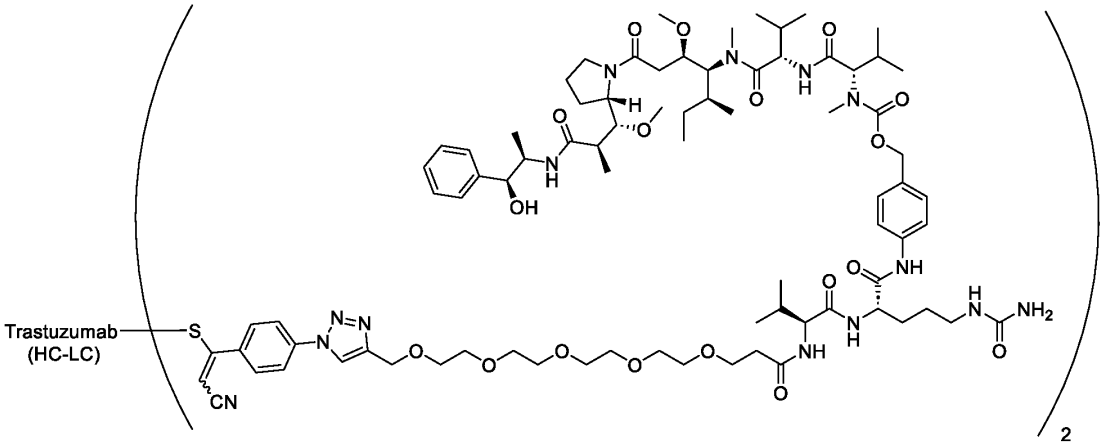
Compound 3



Compound 3

Compound 3 was prepared following General procedure D, using Trastuzumab as protein and APN-VC-MMAE (CAS 2252392-31-5) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 149 019 Da; observed mass 149 028 Da.

Compound 3-iso

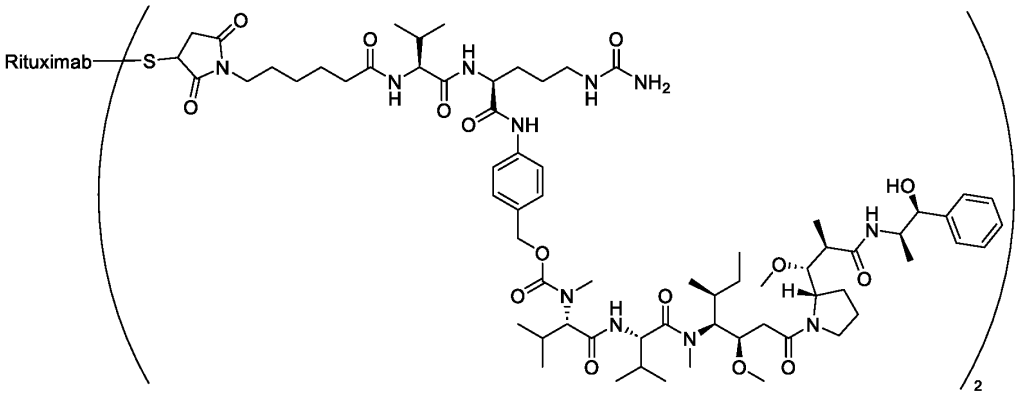


Compound 3-iso

Compound 3-iso was prepared following General procedure D2, using Trastuzumab as protein and APN-VC-MMAE (CAS 2252392-31-5) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 149 019 Da; observed mass 149 023 Da.

Compound 3-iso was characterized by peptide mapping following General Procedure G and by middle analysis following General Procedure H. Both analyses confirmed the predominant conjugation site for the payload at C223 of the heavy chain and C214 of the light chain of trastuzumab, as numbered in IMGT/2Dstructure-DB (INN number 7637).

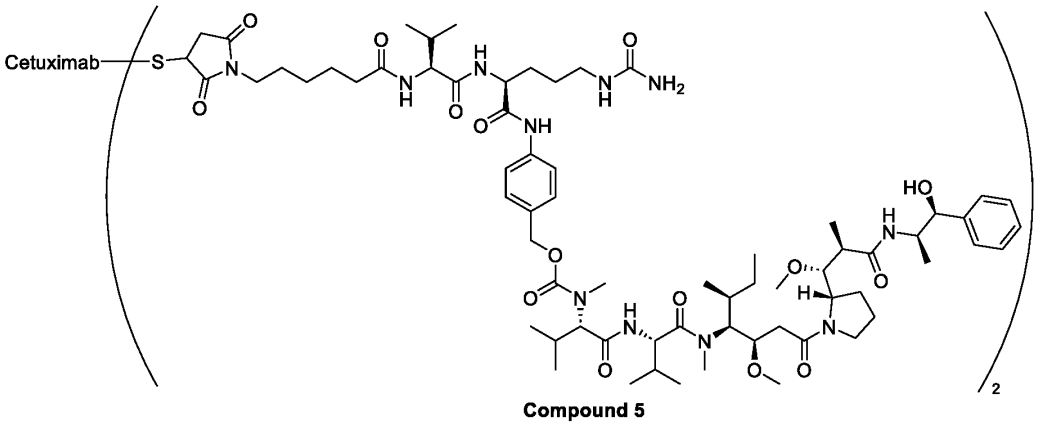
Compound 4



Compound 4

Compound 4 was prepared following General procedure D, using Rituximab as protein and MC-VC-MMAE (CAS 646502-53-6) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 147 529 Da; observed mass 147 524 Da.

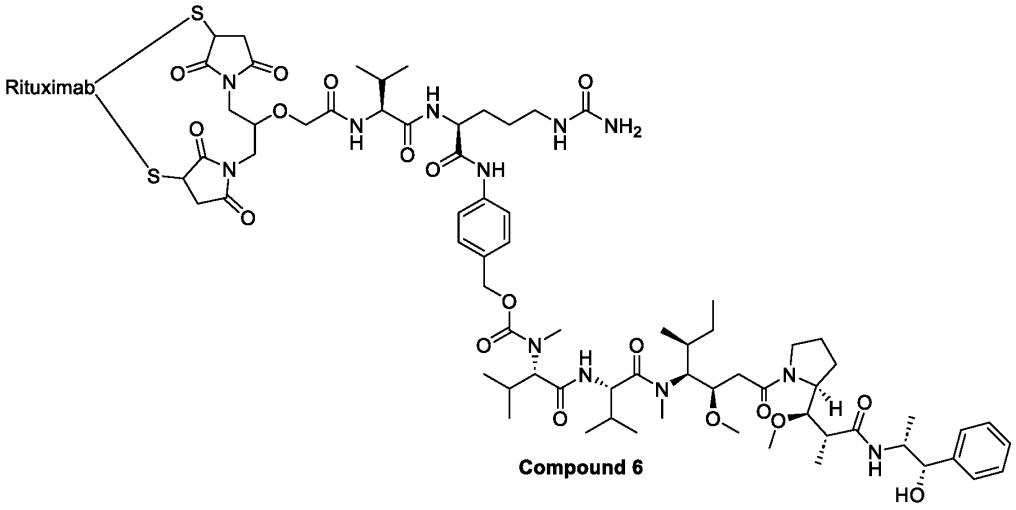
Compound 5



Compound 5

Compound 5 was prepared following General procedure D, using Cetuximab as protein and MC-VC-MMAE (CAS 646502-53-6) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 152 937 Da; observed mass 152 936 Da.

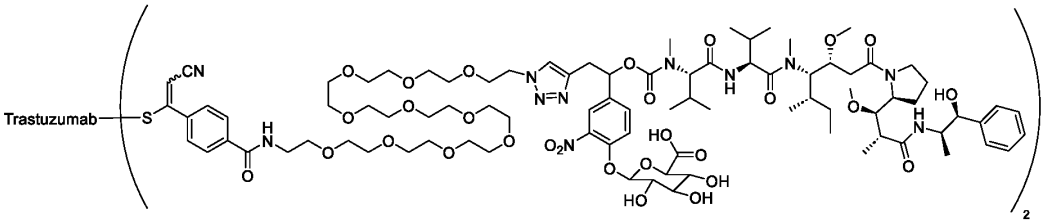
Compound 6



Compound 6

Compound 6 was prepared following General procedure C, using Rituximab as protein and Bi-MC-VC-MMAE (CAS 1620837-70-8) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 146 311 Da; observed mass 146 315 Da.

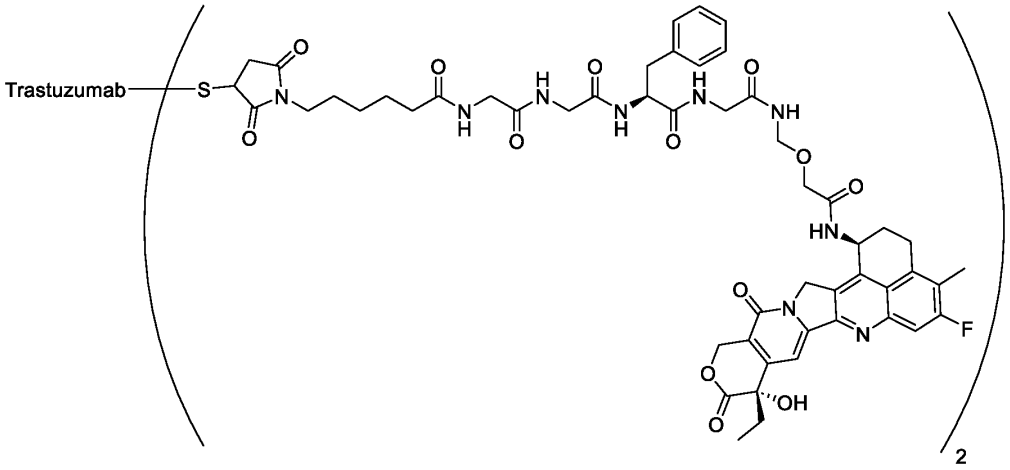
Compound 7



Compound 7

Compound 7 was prepared following General procedure D, using Trastuzumab as protein and APN-Glu-MMAE (CAS 2252392-28-0) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 149 477 Da; observed mass 149 477 Da.

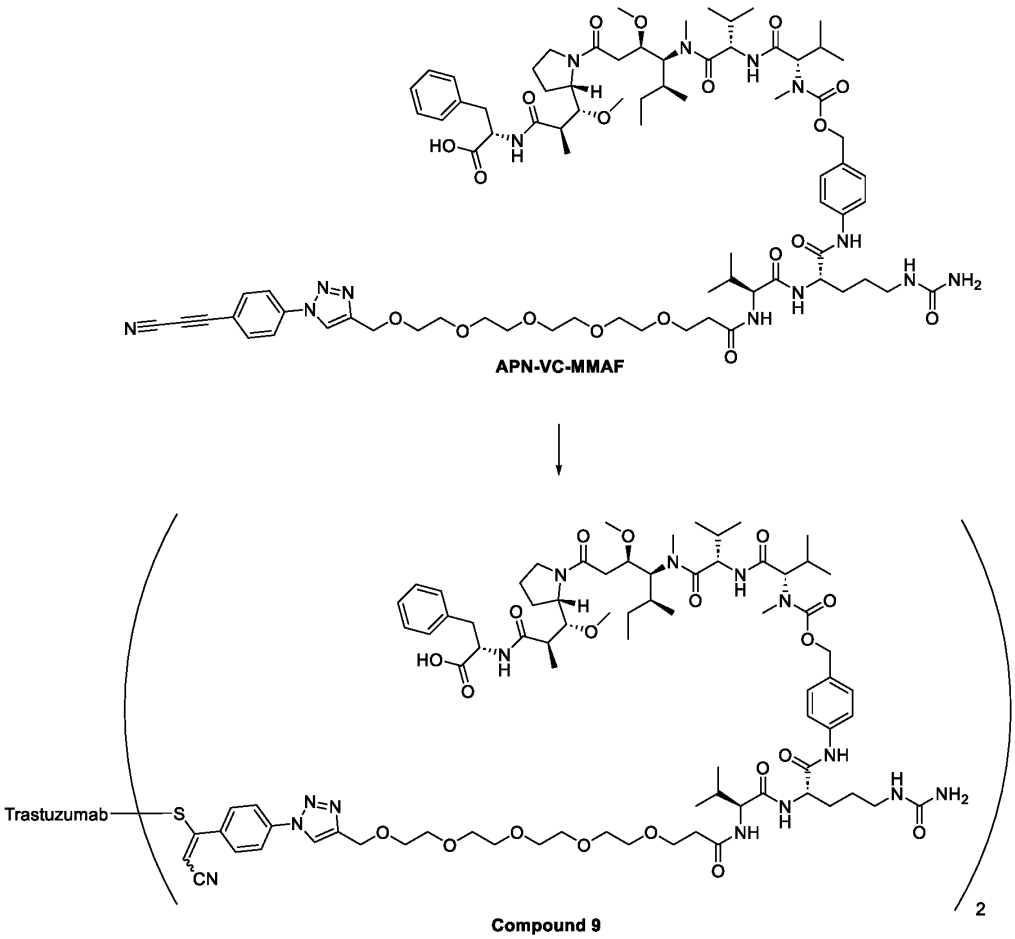
Compound 8



Compound 8

Compound 8 was prepared following General procedure D, using Trastuzumab as protein and Deruxtecan (CAS 1599440-13-7) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 147 931 Da; observed mass 147 946 Da.

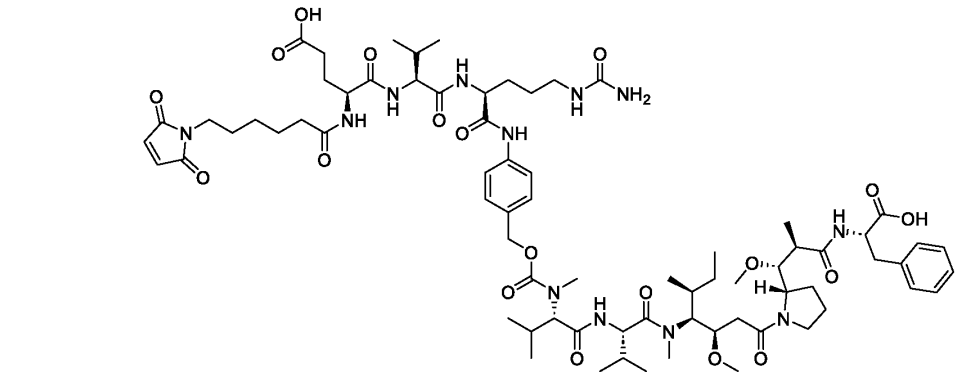
Compound 9



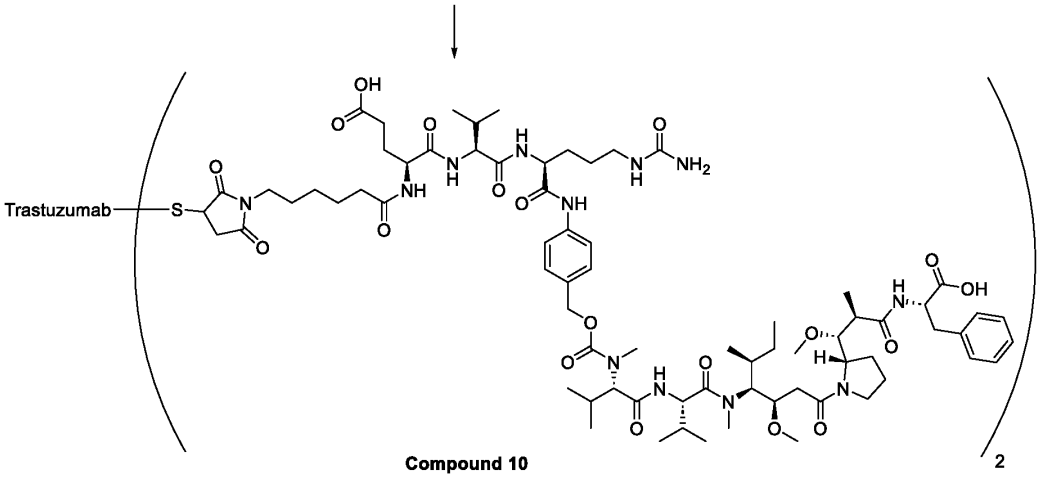
Compound 9 was prepared following General procedure D, using Trastuzumab as protein and APN-VC-MMAF as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 149 049 Da; observed mass 149 057 Da.

Compound 10



MC-EVC-MMAF

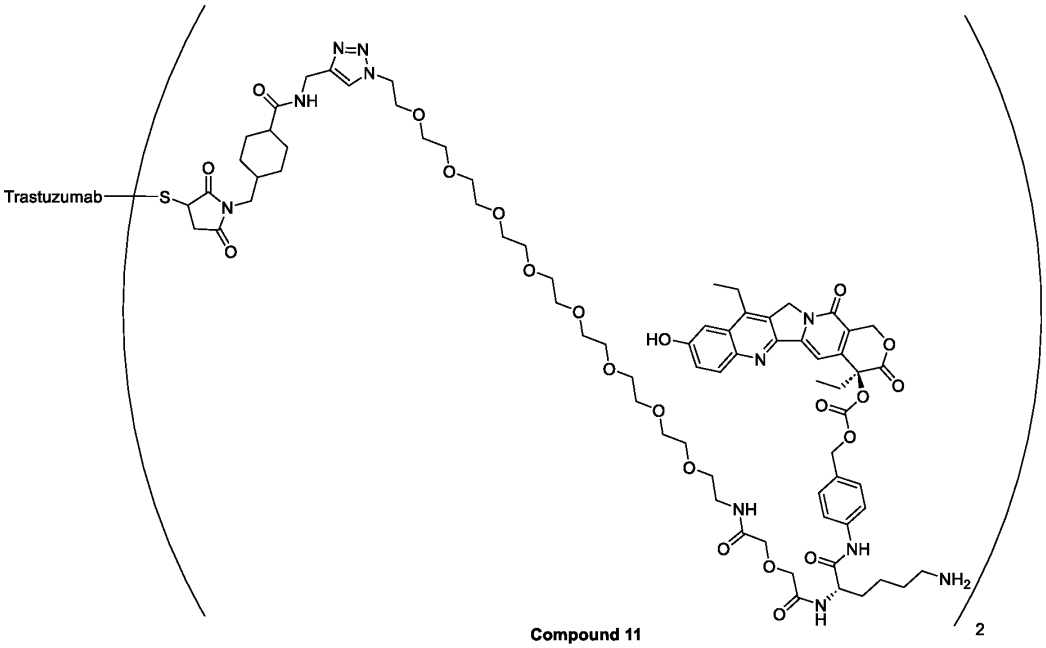


Compound 10

Compound 10 was prepared following General procedure D, using Trastuzumab as protein and MC-EVC-MMAF as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 148 783 Da; observed mass 148 794 Da.

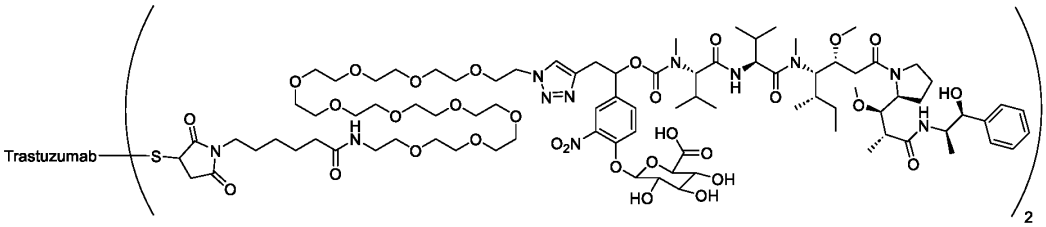
Compound 11



Compound 11

Compound 11 was prepared following General procedure D, using Trastuzumab as protein and CL2A-SN-38 (CAS 1279680-68-0) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 148 825 Da; observed mass 148 838 Da.

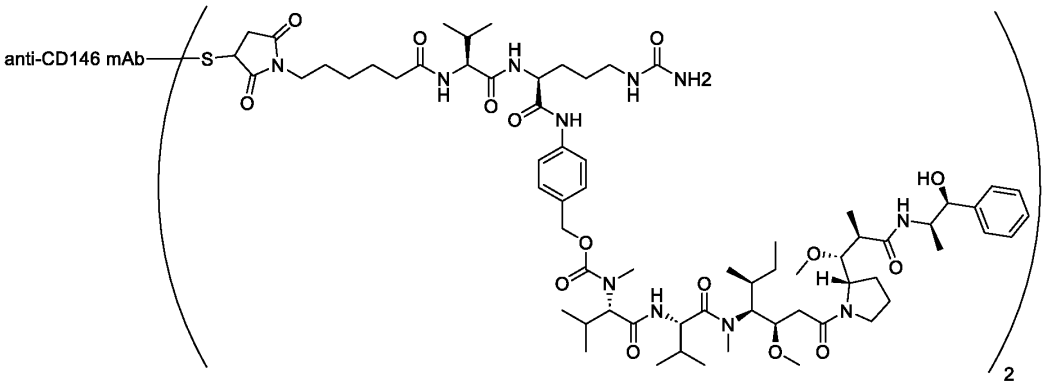
Compound 12



Compound 12

Compound 12 was prepared following General procedure D, using Trastuzumab as protein and MC-Glu-MMAE (CAS 1803182-85-5) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 149 557 Da; observed mass 149 572 Da.

Compound 13

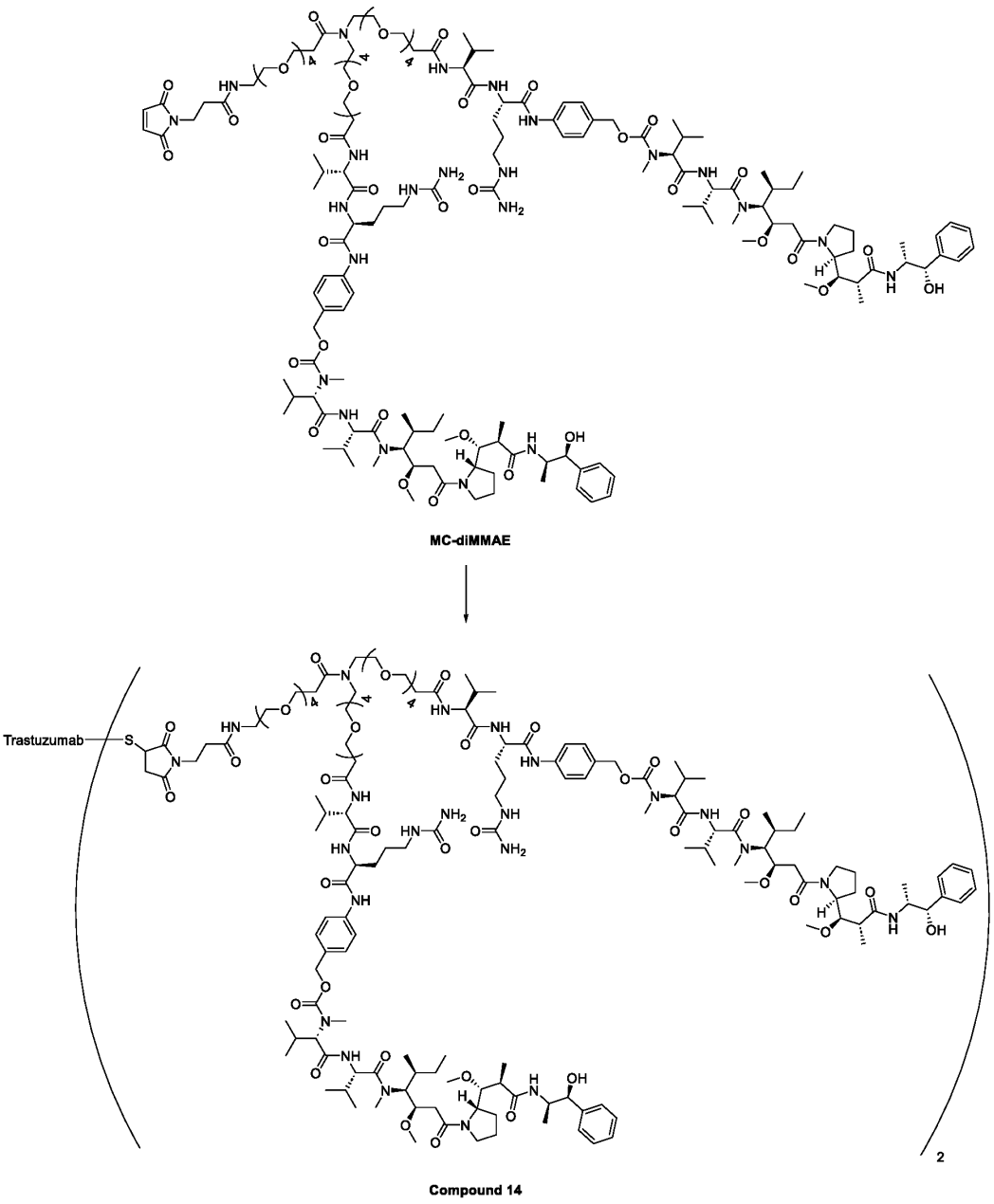


Compound 13

Compound 13 was prepared following General procedure D, using anti-CD146 mAb (described in WO2019068842A1) as protein and MC-VC-MMAE (CAS 646502-53-6) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 148 185 Da; observed mass 148 182 Da.

Compound 14

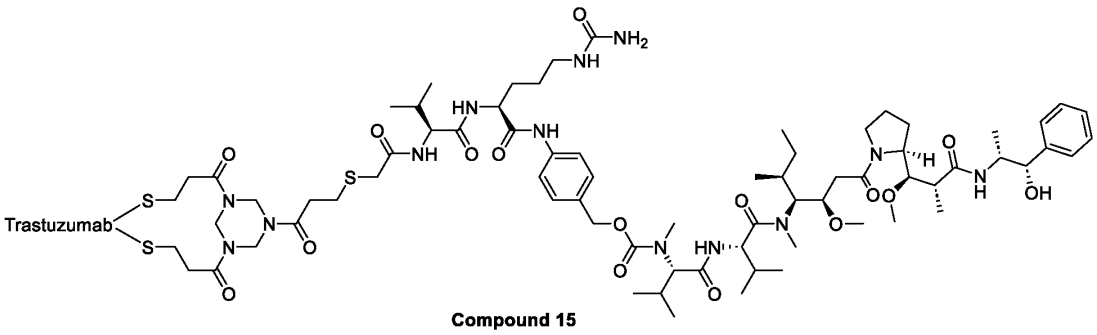


Compound 14

Compound 14 was prepared following General procedure D, using Trastuzumab as protein and MC-diMMAE as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 152 111 Da; observed mass 152 123 Da.

Compound 15

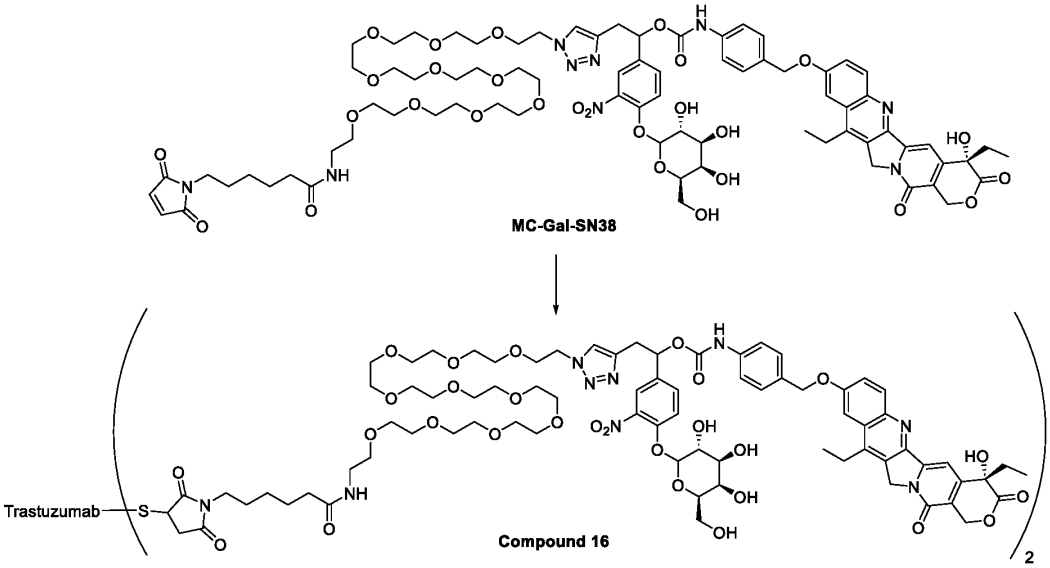


Compound 15

Compound 15 was prepared following General procedure C, using Trastuzumab as protein and Py-MAA-Val-Cit-PAB-MMAE (CAS 2247398-68-9) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 147 312 Da; observed mass 147 319 Da.

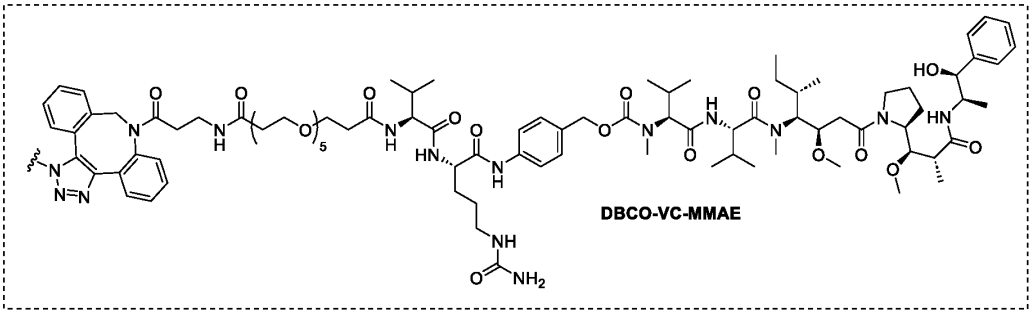
Compound 16

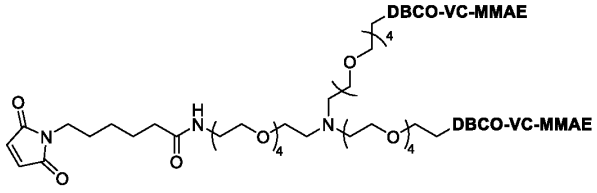


Compound 16 was prepared following General procedure D, using Trastuzumab as protein and MC-Gal-SN38 as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

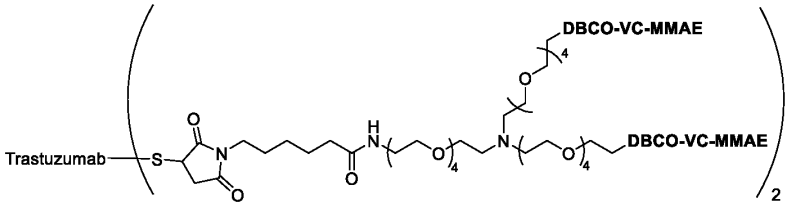
Expected mass 149 135 Da; observed mass 149 144 Da.

Compound 17





MC-di(DBCO-VC-MMAE)

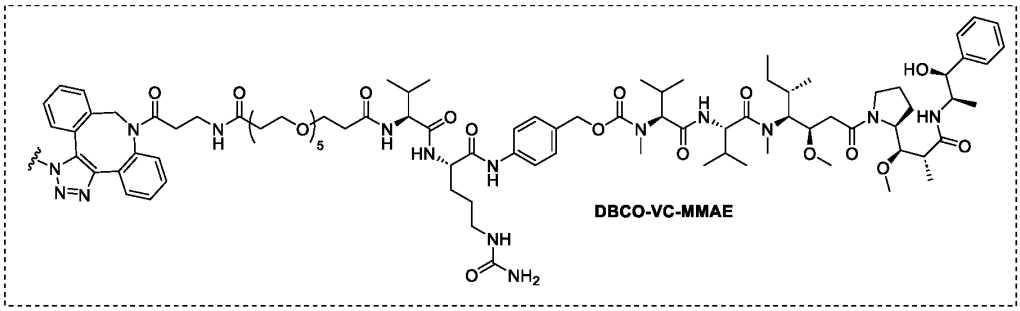


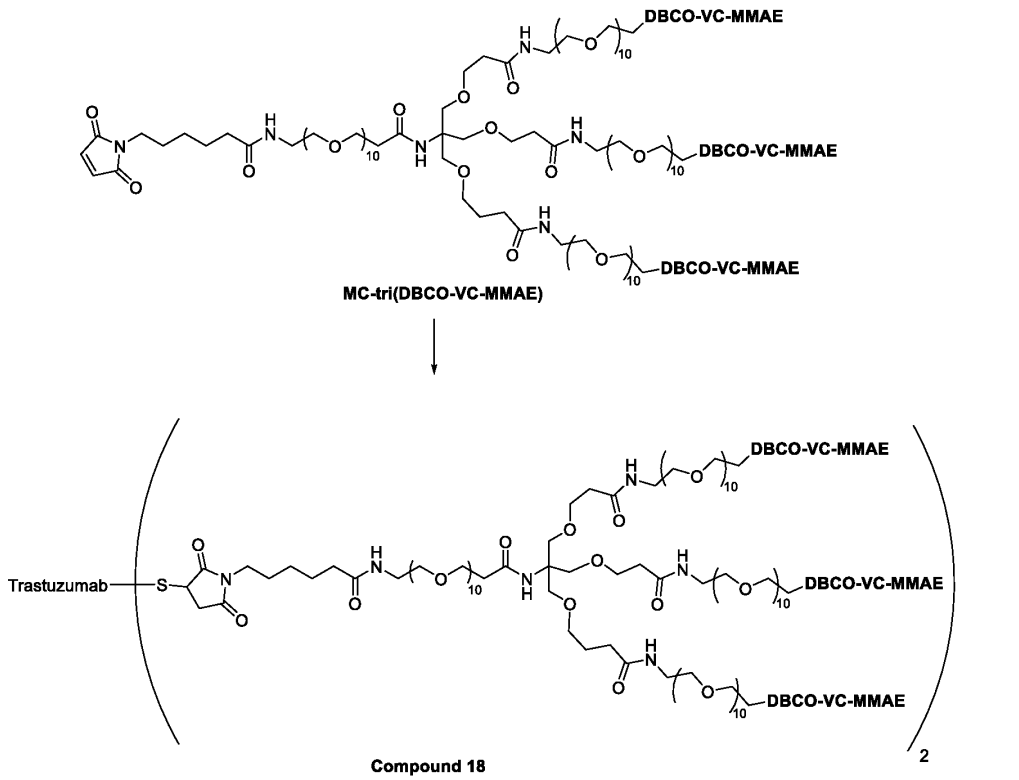
Compound 17

Compound 17 was prepared following General procedure D, using Trastuzumab as protein and MC-di(DBCO-VC-MMAE) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 154 511 Da; observed mass 154 521 Da.

Compound 18

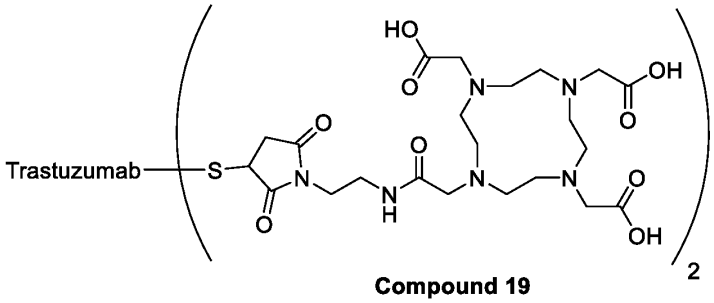




Compound 18 was prepared following General procedure D, using Trastuzumab as protein and MC-tri(DBCO-VC-MMAE) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 161 241 Da; observed mass 161 221 Da.

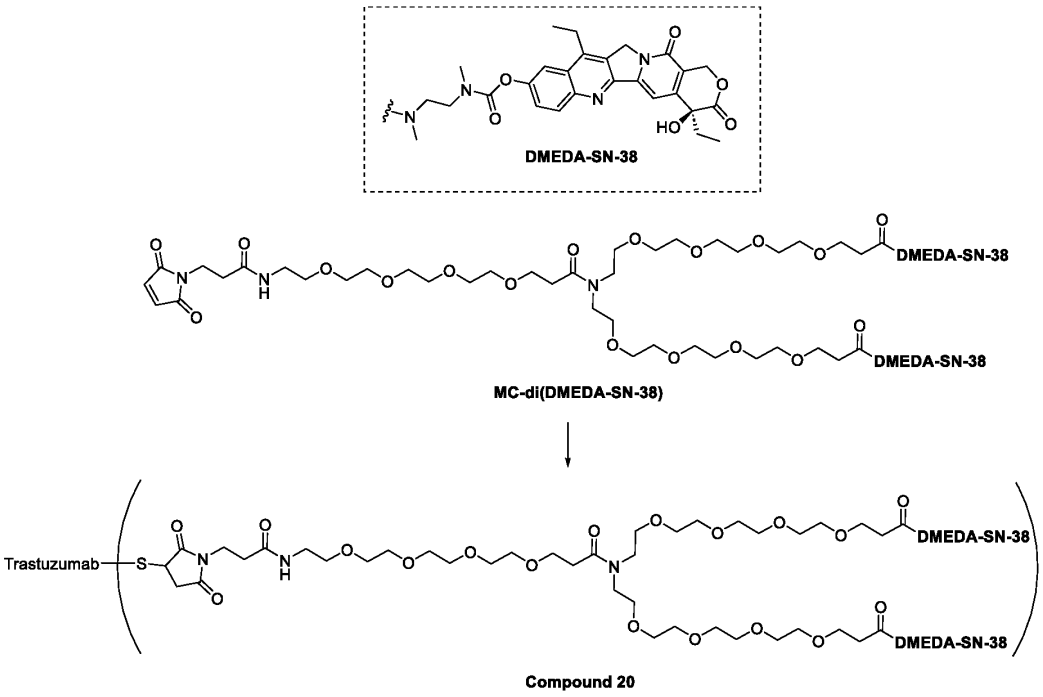
Compound 19



Compound 19

Compound 19 was prepared following General procedure D, using Trastuzumab as protein and Maleimide-DOTA (CAS 1006711-90-5) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:
Expected mass 146 917 Da; observed mass 146 920 Da.

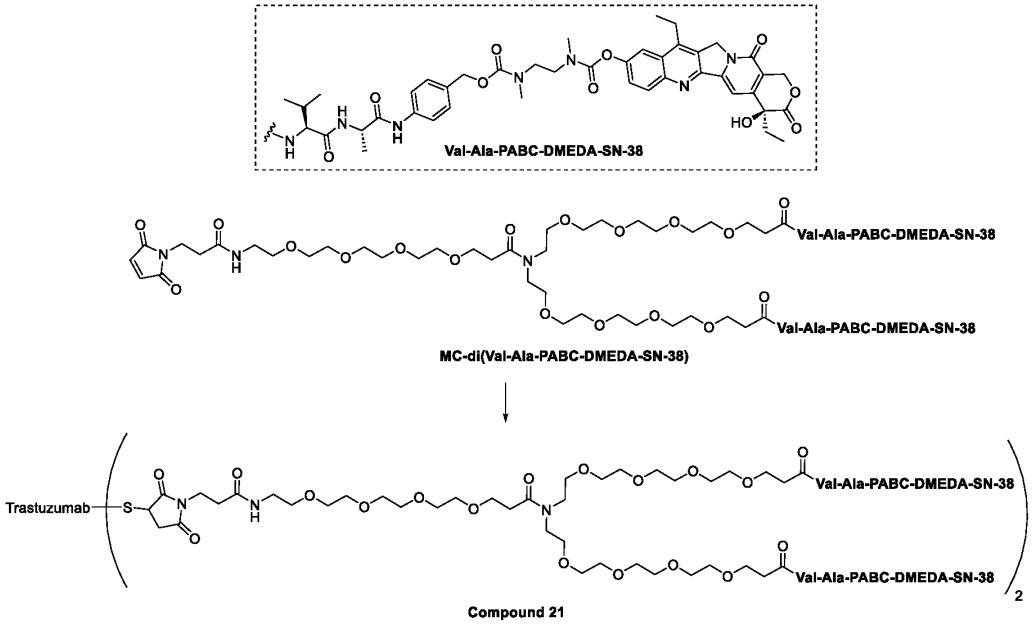
Compound 20



Compound 20

Compound 20 was prepared following General procedure D, using Trastuzumab as protein and MC-di(DMEDA-SN-38) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:
Expected mass 149 643 Da; observed mass 149 651 Da.

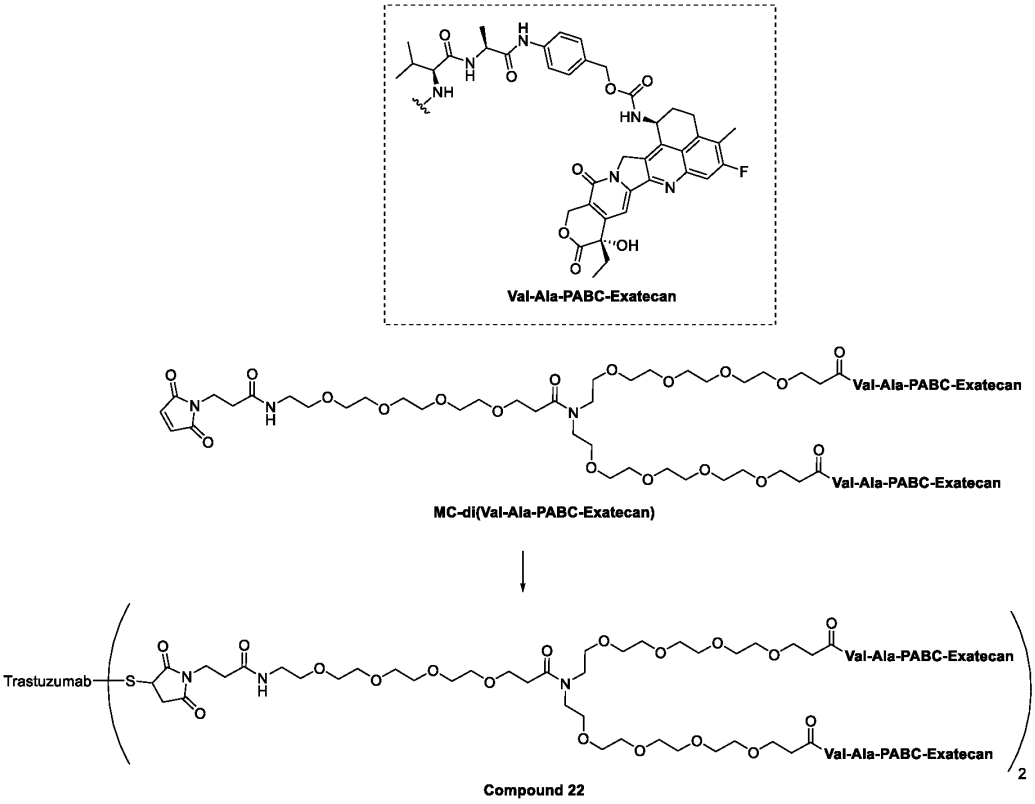
Compound 21



Compound 21

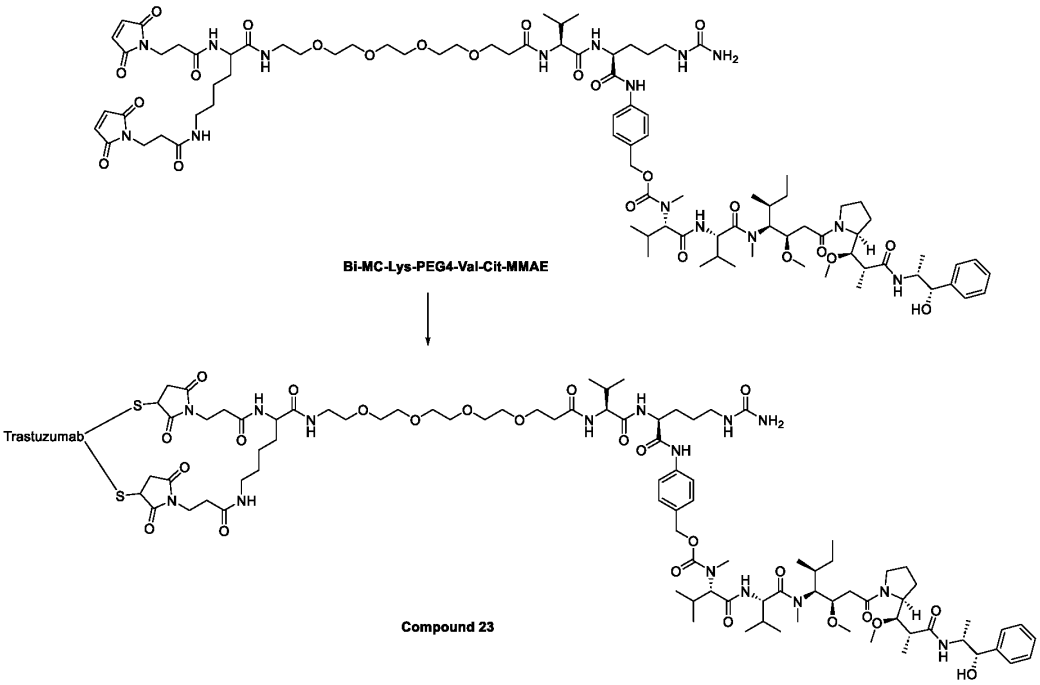
Compound 21 was prepared following General procedure D, using Trastuzumab as protein and MC-di(Val-Ala-PABC-DMEDA-SN-38) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 150 919 Da; observed mass 150 932 Da.

Compound 22



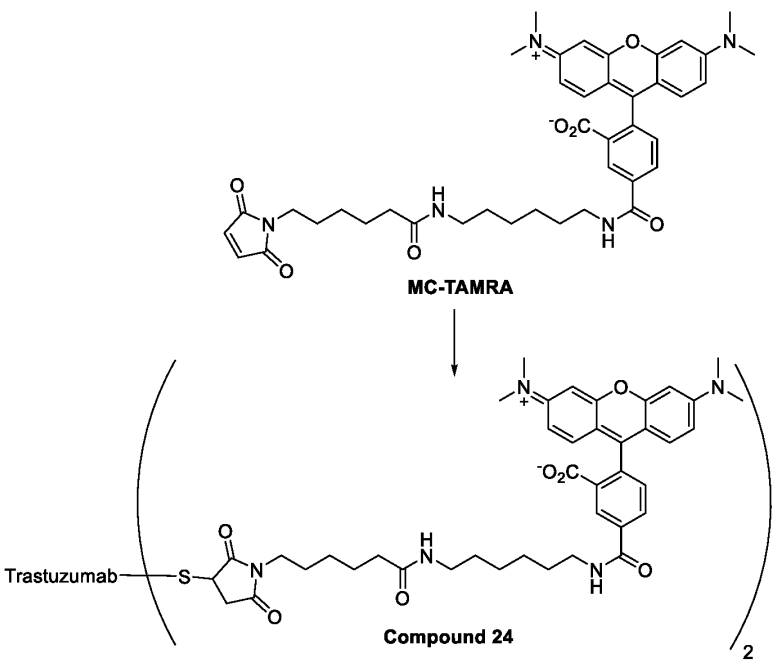
Compound 22 was prepared following General procedure D, using Trastuzumab as protein and MC-di(Val-Ala-PABC-Exatecan) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 150 635 Da; observed mass 150 642 Da.

Compound 23



Compound 23 was prepared following General procedure C, using Trastuzumab as protein and Bi-MC-Lys-PEG4-Val-Cit-MMAE as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 147 665; observed mass 147 669 Da.

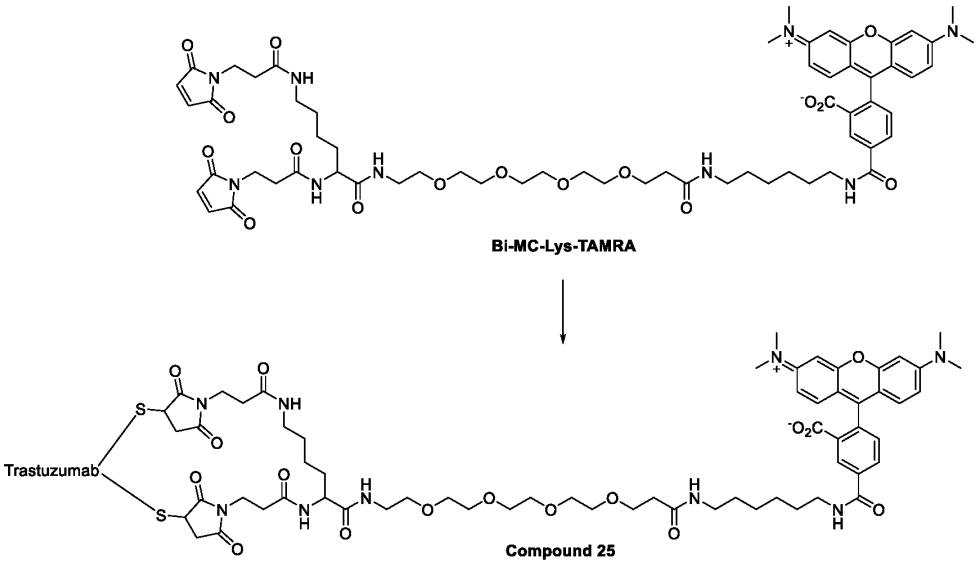
Compound 24



Compound 24 was prepared following General procedure D, using Trastuzumab as protein and MC-TAMRA as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

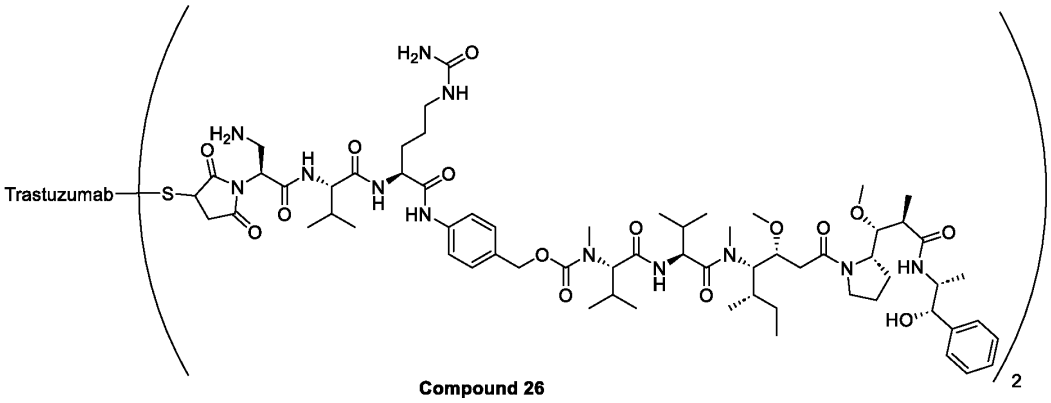
Expected mass 147 307 Da; observed mass 147 312 Da.

Compound 25



Compound 25 was prepared following General procedure C, using Trastuzumab as protein and Bi-MC-Lys-TAMRA as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:
Expected mass 147 070 Da; observed mass 147 074 Da.

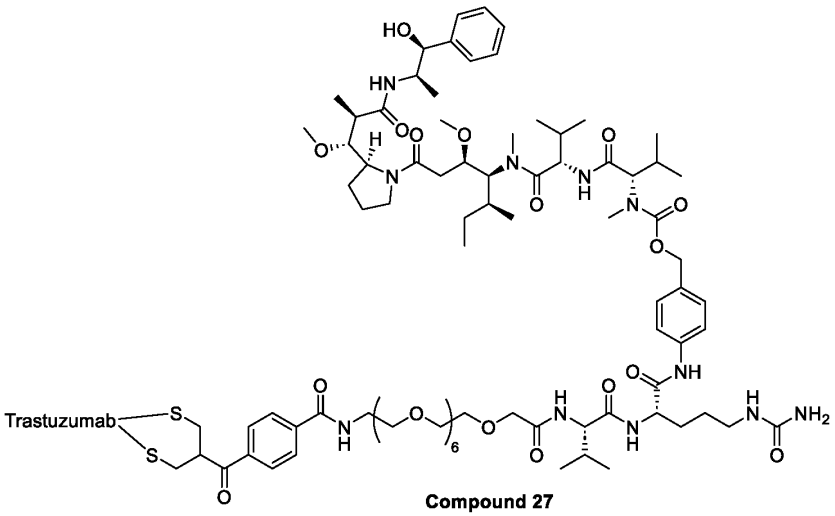
Compound 26



Compound 26

Compound 26 was prepared following General procedure C, using Trastuzumab as protein and mDPR-Val-Cit-PAB-MMAE (CAS 2185872-76-6) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:
Expected mass 148 443 Da; observed mass 148 451 Da.

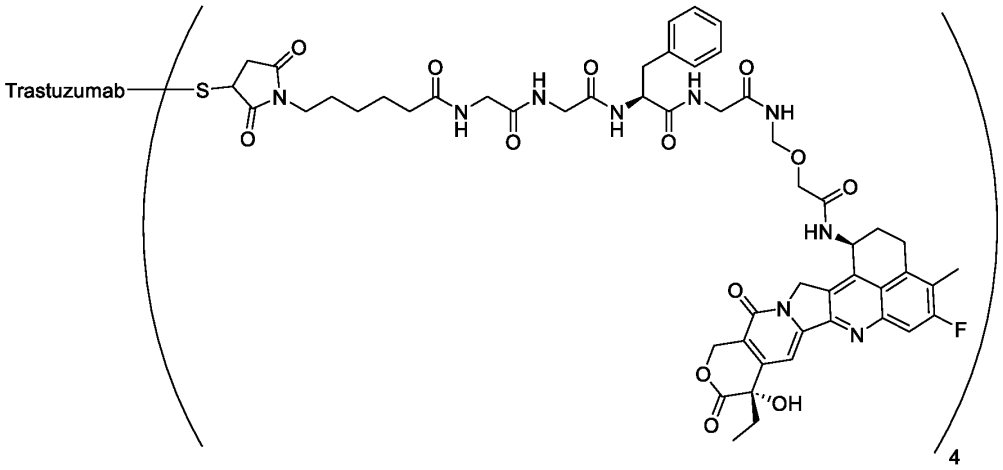
Compound 27



Compound 27

Compound 27 was prepared following General procedure C2, using Trastuzumab as protein and bisAlk-vc-MMAE (CAS 1774376-59-8) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 147 525 Da; observed mass 147 531 Da.

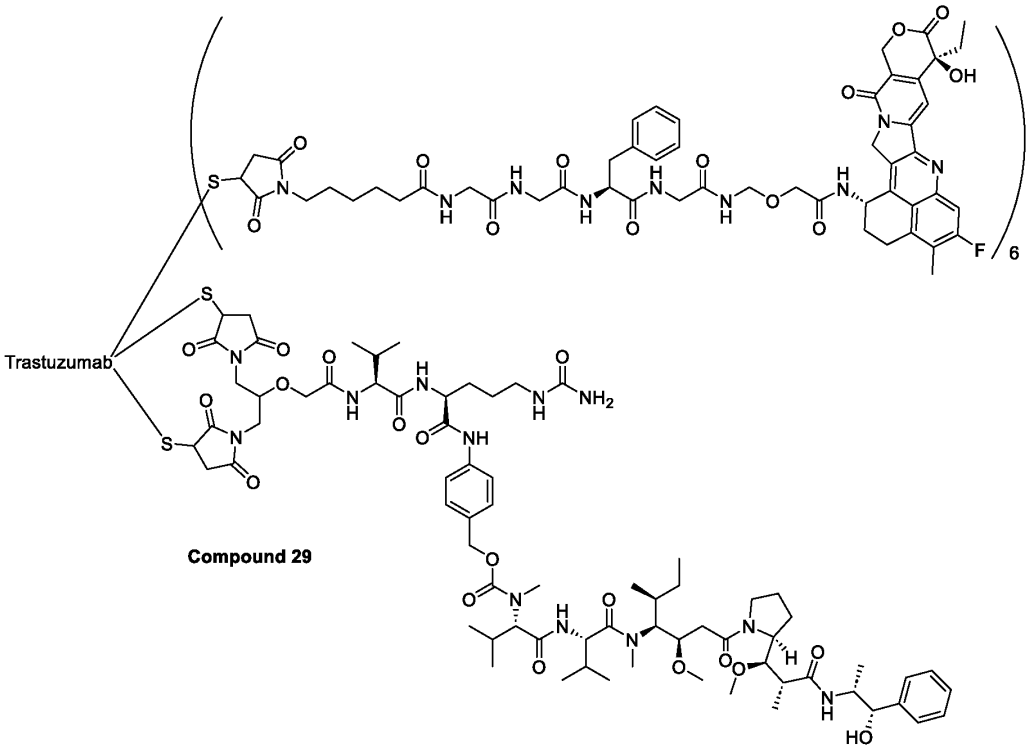
Compound 28



Compound 28

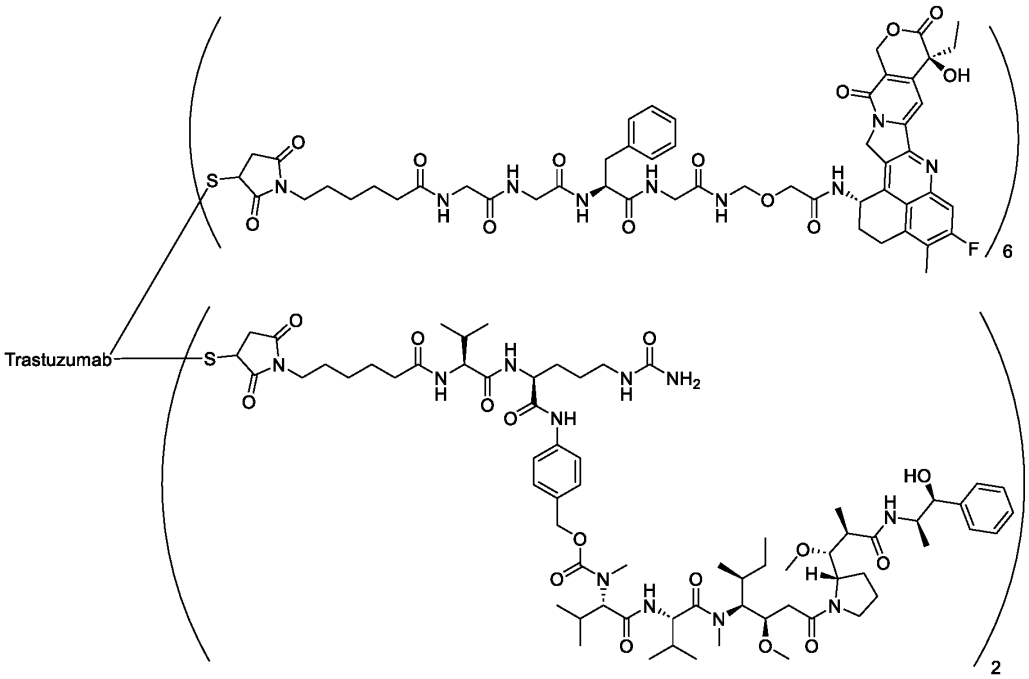
Compound 28 was prepared following General procedure D4, using mutant Trastuzumab with V110C mutation on the light chain as Protein (as disclosed in US 7,521,541) and Deruxtecan (CAS 1599440-13-7) as thiol-reactive Payload, The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:
Expected mass 149 997 Da; observed mass 149 986 Da.

Compound 29



Compound 28 was prepared following General procedure D6, using Compound 1 as Substrate and Deruxtecan (CAS 1599440-13-7) as thiol-reactive Payload. The reaction mixture was incubated at 4 °C for 5 hours to achieve complete conjugation. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 153 477 Da; observed mass 153 485 Da.

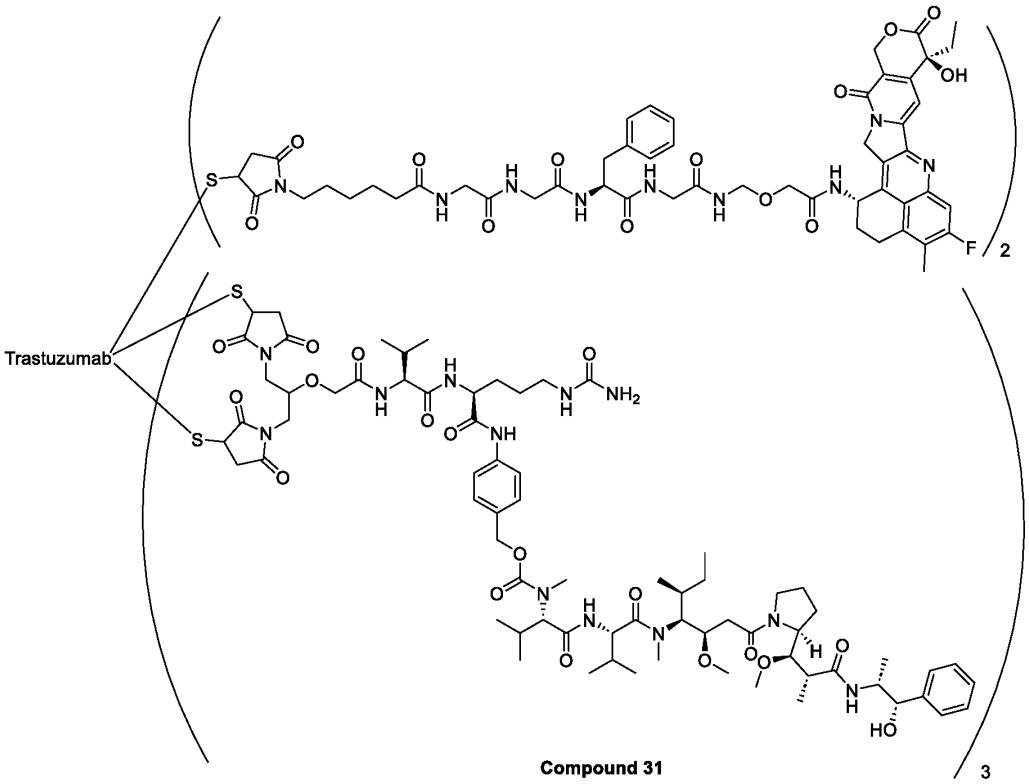
Compound 30



Compound 30

Compound 30 was prepared following General procedure D6, using Compound 2 as Substrate and Deruxtecan (CAS 1599440-13-7) as thiol-reactive Payload. The reaction mixture was incubated at 4 °C for 5 hours to achieve complete conjugation. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 154 695 Da; observed mass 154 705 Da.

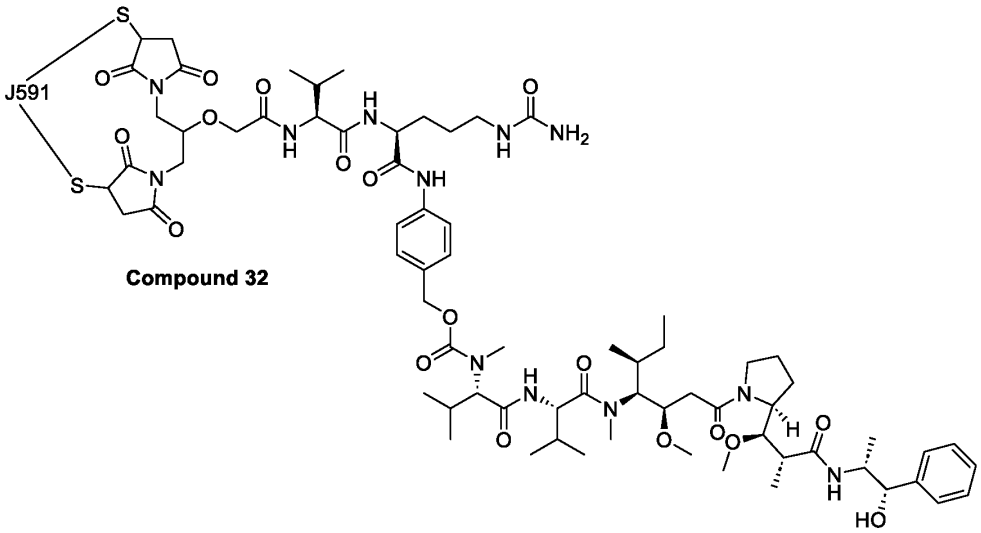
Compound 31



Compound 31 was prepared following General procedure D6, using Compound 8 as Substrate and Bi-MC-VC-MMAE (CAS 1620837-70-8) as thiol-reactive Payload. The reaction mixture was incubated at 4 °C for 2 hours to achieve complete conjugation. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 152 173 Da; observed mass 152 160 Da.

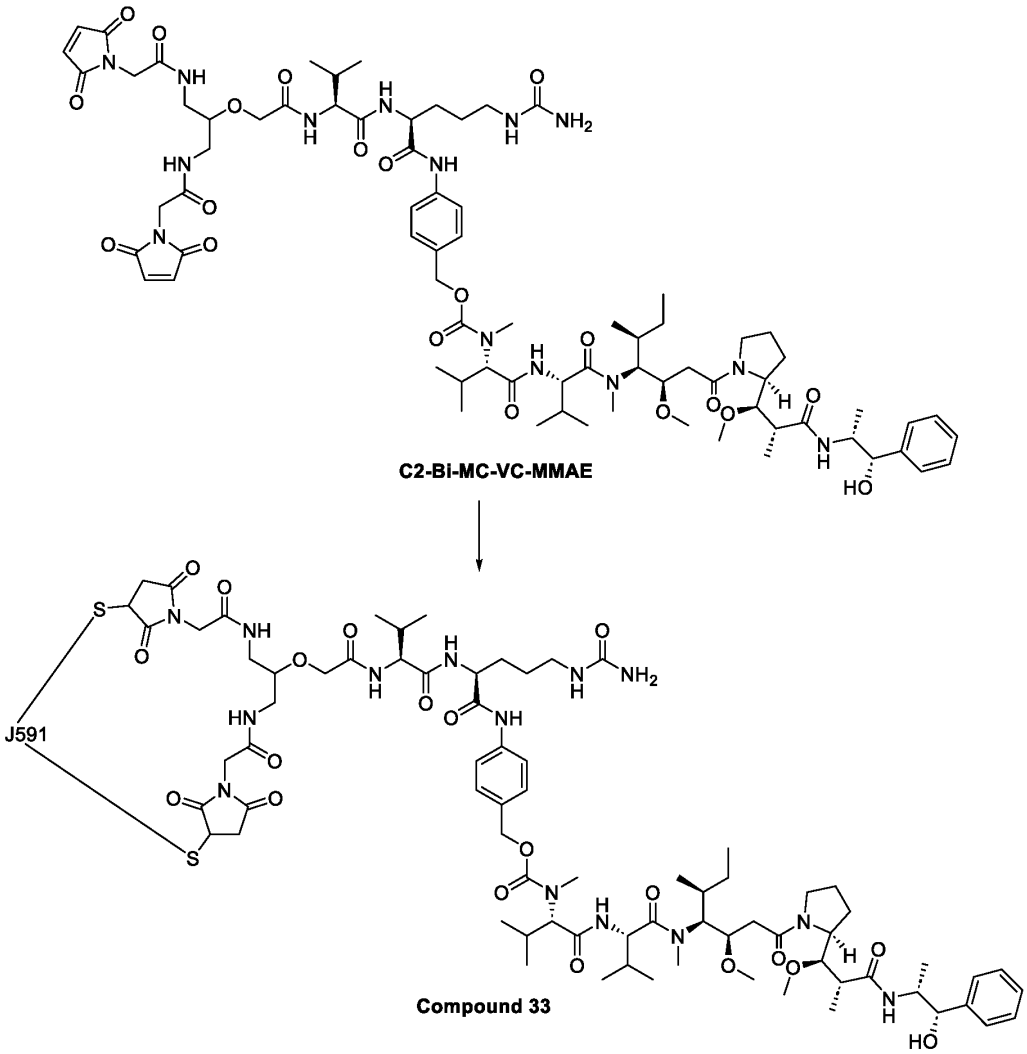
Compound 32



Compound 32

Compound 32 was prepared following General procedure C, using PSMA-targeting J591 antibody (with sequence described in IMGT/2Dstructure-DB, INN number 11334) as Protein and Bi-MC-VC-MMAE (CAS 1620837-70-8) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 146 225 Da; observed mass 146 220 Da.

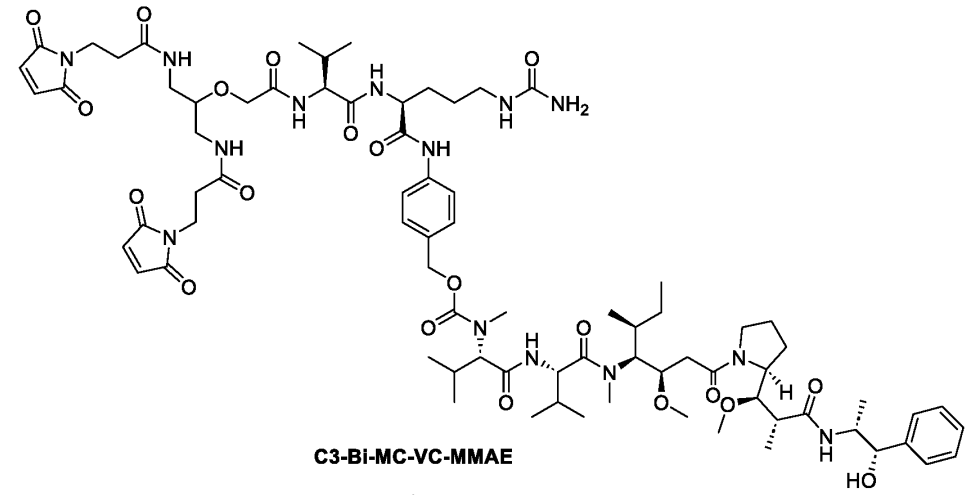
Compound 33



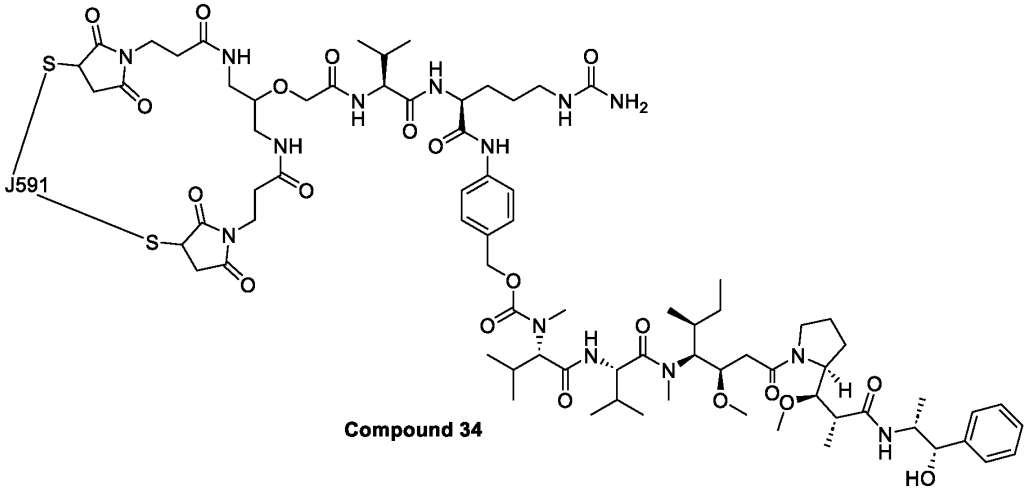
Compound 33 was prepared following General procedure C, using PSMA-targeting J591 antibody (with sequence described in IMGT/2Dstructure-DB, INN number 11334) as Protein and C2-Bi-MC-VC-MMAE as thiol-reactive Payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 146 337 Da; observed mass 146 329 Da.

Compound 34



C3-Bi-MC-VC-MMAE

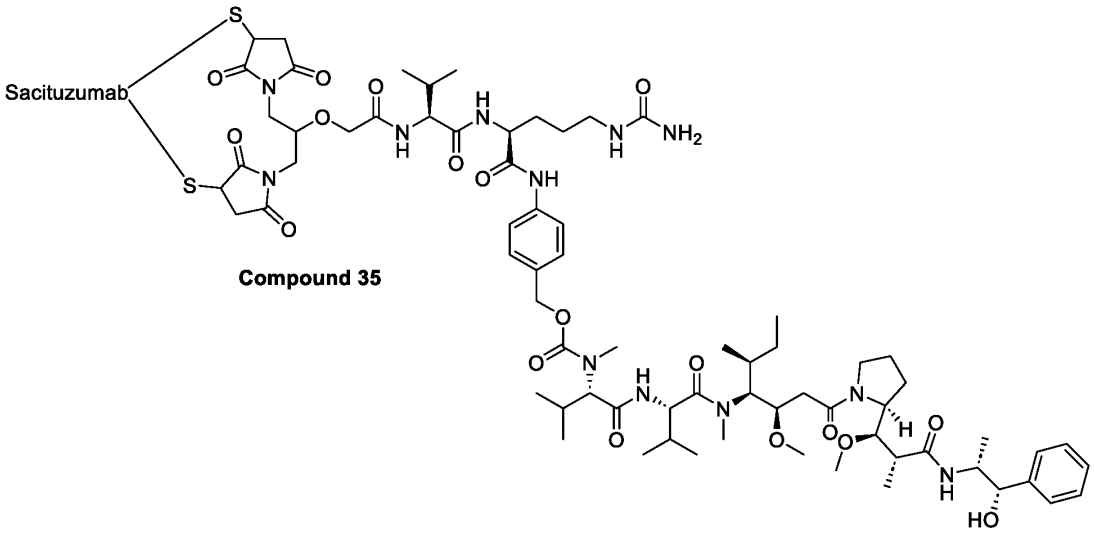


Compound 34

Compound 34 was prepared following General procedure C, using PSMA-targeting J591 antibody (with sequence described in IMGT/2Dstructure-DB, INN number 11334) as Protein and C3-Bi-MC-VC-MMAE as thiol-reactive Payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 146 365 Da; observed mass 146 362 Da.

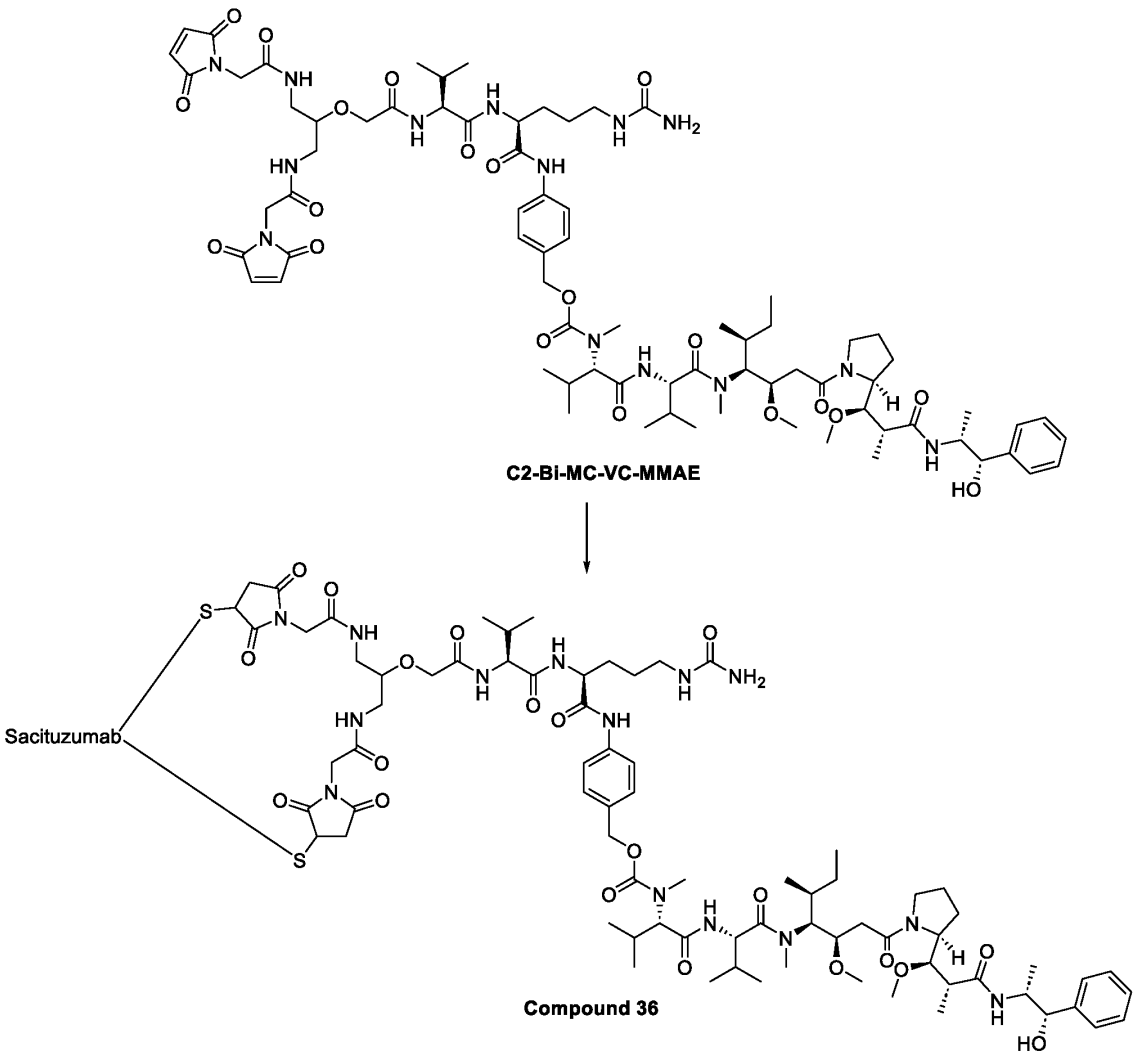
Compound 35



Compound 35

Compound 32 was prepared following General procedure C, using Sacituzumab as Protein and Bi-MC-VC-MMAE (CAS 1620837-70-8) as thiol-reactive payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results: Expected mass 147 349 Da; observed mass 147 344 Da.

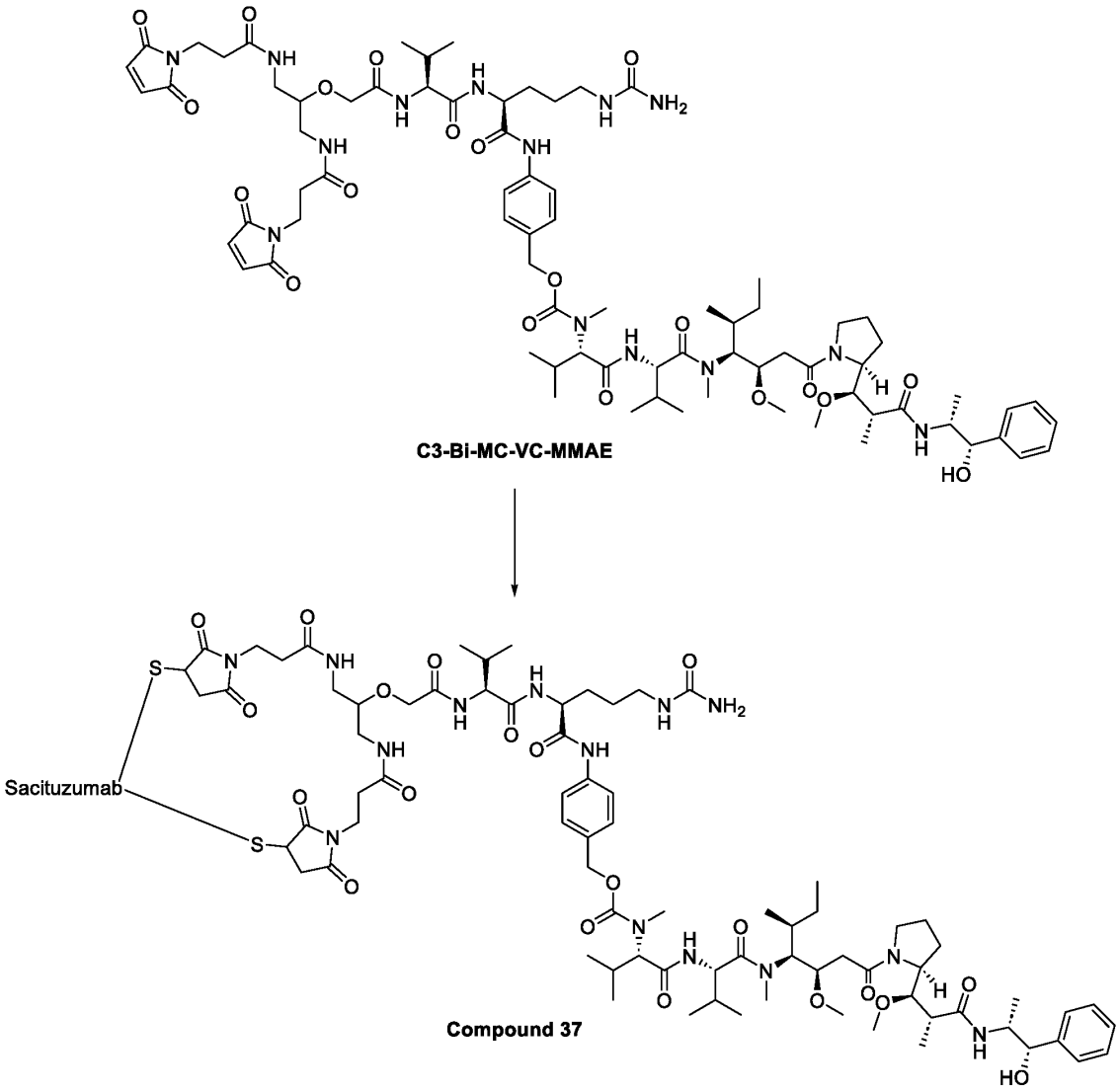
Compound 36



Compound 33 was prepared following General procedure C, using Sacituzumab as Protein and C2-Bi-MC-VC-MMAE as thiol-reactive Payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 147 461 Da; observed mass 147 453 Da.

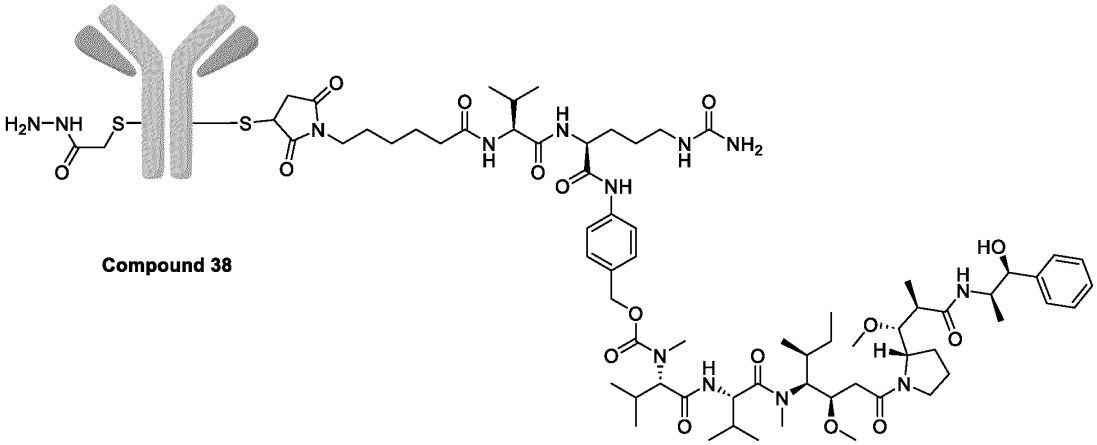
Compound 37



Compound 34 was prepared following General procedure C, using Sacituzumab as Protein and C3-Bi-MC-VC-MMAE as thiol-reactive Payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 147 489 Da; observed mass 147 486 Da.

Compound 38

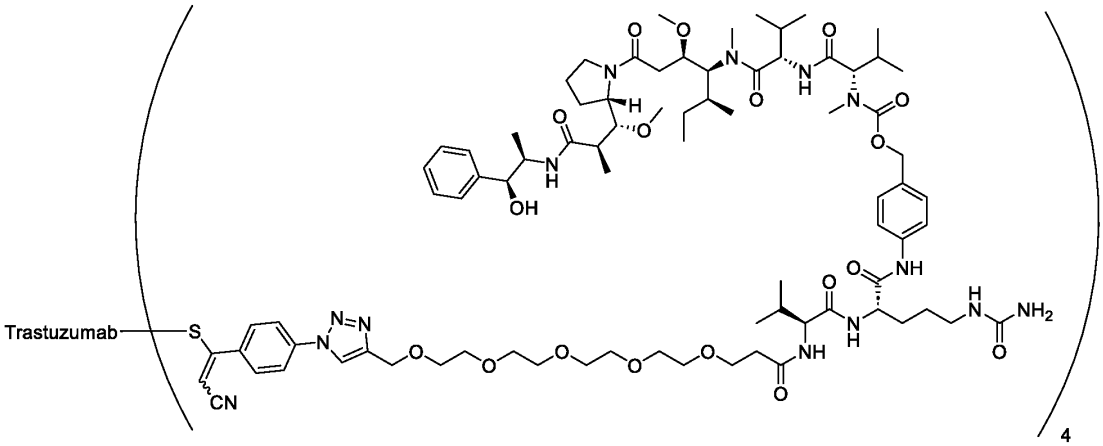


Compound 38

Compound 38 was prepared following General procedure D3, using Trastuzumab as protein and MC-VC-MMAE (CAS 646502-53-6) as Payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 147 254 Da; observed mass 147 259 Da.

Compound 39



Compound 39

Compound 39 was prepared following General procedure D5, using mutant Trastuzumab with V110C mutation on the light chain as Protein and APN-VC-MMAE (CAS 2252392-31-5) as Payload. The product was characterized by RP-HPLC MS following General procedure F. RP-HPLC MS results:

Expected mass 152 173 Da; observed mass 152 166 Da.

CLAIMS

1. A coordination complex of protein with a divalent metal atom M, represented by formula (II):

[Ab'-M]

(II)

Wherein

Ab' is a protein or fragment thereof comprising two heavy chains and two light chains, each chain comprising one or more cysteine residue involved in inter chains disulfide bridges,

wherein each heavy chain comprises one N-proximal inter(heavy chains) cysteine residue that is located nearest to the N-terminal end of said heavy chain,

And wherein the Sulfur atom of each said N-proximal inter(heavy chains) cysteine residue is reduced, and the remaining interchain cysteine residues form interchain disulfide bridges;

M is a divalent transition metal;

Wherein M bridges together the two reduced Sulfur atoms, so as to form the following group: -S-M-S- within the coordination complex;

And the salts thereof;

Characterized in that the complexion of M is site specific.

2. The complex of formula (II) according to claim 1 wherein in formula (II) M is Zn.

3. The complex of formula (II) according to claim 1 or 2 wherein in formula (II) Ab' is an antibody, in particular Immunoglobulin G, or a derivative thereof.

4. Process of preparation of the complex of formula (II), said process comprising:

Reacting a protein complex of formula (I):

$[Ab'_{Red}-M]$

(I)

With an oxidizing agent;

wherein

Ab'_{Red} is a protein or fragment thereof comprising two heavy chains and two light chains, each chain comprising one or more cysteine residue involved in inter chains disulfide bridges,

wherein each heavy chain comprises one N-proximal inter(heavy chains) cysteine residue that is located nearest to the N-terminal end of said heavy chain,

And wherein each Sulfur atom of each said N-proximal inter(heavy chains) cysteine residue is reduced and the two reduced Sulfur atoms form together with the M atom the following group: -S-M-S- within the coordination complex;
and the Sulfur atoms of the remaining interchain cysteine residues are reduced in the form of thiol –SH groups;
M is defined as in claim 1 or 2.

5. A coordination complex of protein of formula (I):

$$[Ab'_{Red}\text{-}M] \quad (I)$$

Where Ab'_{Red} and M are defined as in claim 4.

6. The process of preparation of the complex of formula (I) according to claim 5, said process comprising:
- reacting Ab with a reducing agent so as to form Ab_{Red}, followed by
- reacting Ab_{Red} with 0.5 to 2.5, preferably between 1 and 2 equivalents of a salt of metal M so as to form the Metal coordination complexes $[Ab'_{Red}\text{-}M]$;
Wherein
Ab is a protein or fragment thereof comprising two heavy chains and two light chains, each chain comprising one or more cysteine residue involved in inter chains disulfide bridges,
Ab_{Red} refers to reduced Ab wherein all Sulfur atoms of said interchain cysteine residues are reduced and in the form of thiol –SH groups.

7. A protein conjugate of formula (III):

$$(T)_p\text{-}Ab'\text{-}(L\text{-}(Payload)_m)_n \quad (III)$$

Having
- a controlled drug-to-antibody ratio (DAR) of (m x n); and
- a site specific conjugation at one or two of a pair of interchain cysteine residues of Ab' defined as the conjugated cysteine residues;

Where in formula (III):
Ab' is a protein or fragment thereof comprising two heavy chains and two light chains, each chain comprising one or more interchain cysteine residues, such that each of said conjugated cysteines are bound to either -(L-(Payload)m or optional T fragment, and the remaining interchain cysteine residues form interchain disulfide bridges;
n is 1 or 2;

m is 1, 2, 3, 4, 5 or 6;

p is 0 or 1;

T, if present, is a terminal group covalently bound to one of said two reduced Sulfur atoms of said interchain cysteine residues of Ab' that is not covalently bound to L;

each L, identical or different, is a linear or branched di-, tri-, tetra-, penta-, hexa- or hepta-valent linker moiety covalently binding Payload to said Ab', where said L is attached to Ab' through one or two of said reduced Sulfur atoms of said interchain cysteine residues and binds said Ab' with one or more Payload;

Payload is a molecule exerting a biological activity selected from the group consisting in diagnostic agents, therapeutic agents and labelling agents.

8. A protein conjugate of formula (III'):

$$(T)_p\text{-Ab'-}(L\text{-}(Payload)_m)_n$$

(III')

Having

- a controlled drug-to-antibody ratio (DAR) of (m x n); and
- a site specific conjugation at one or two of the N-proximal inter(heavy chains) cysteine residue that is located nearest to the N-terminal end of said heavy chain;

Where in formula (III'):

Ab' is a protein or fragment thereof comprising two heavy chains and two light chains, each chain comprising one or more cysteine residue involved in inter chains disulfide bridges, wherein each heavy chain comprises one N-proximal inter(heavy chains) cysteine residue that is located nearest to the N-terminal end of said heavy chain,

And wherein each Sulfur atom of each said N-proximal inter(heavy chains) cysteine residue is reduced and covalently bound to either -(L-(Payload)$_m$ or optional T fragment, and the remaining interchain cysteine residues form interchain disulfide bridges;

n is 1 or 2;

m is 1, 2, 3, 4, 5 or 6;

p is 0 or 1;

T, if present, is a terminal group covalently bound to one of said two reduced Sulfur atoms of said N-proximal inter(heavy chains) cysteine residues of Ab' that is not covalently bound to L;

each L, identical or different, is a linear or branched di-, tri-, tetra-, penta-, hexa- or hepta-valent linker moiety covalently binding Payload to said Ab', where said L is attached to Ab' through one or two of said reduced Sulfur atoms of said N-proximal inter(heavy chains) cysteine residue and binds said Ab' with one or more Payload;

Payload is a molecule exerting a biological activity selected from the group consisting in diagnostic agents, therapeutic agents and labelling agents.

9. A protein conjugate of formula (III''):

$$(T)_p\text{-Ab'-}(L\text{-}(Payload)_m)_n$$

(III'')

Having

- a controlled drug-to-antibody ratio (DAR) of (m x n); and
- a site specific conjugation at one or two of the inter(heavy-light chains) cysteine residues;

Where in formula (III''):

Ab' is a protein or fragment thereof comprising two heavy chains and two light chains, each heavy and light chain comprising one inter(heavy-light chains) cysteine residue involved as a pair in disulfide bridges bridging together a heavy chain with a light chain, each heavy chain further comprising two inter(heavy chains) cysteine residues;

wherein

each Sulfur atom of one pair of said inter(heavy-light chains) cysteine residues is reduced and covalently bound to either $-(L\text{-}(Payload)_m$ or optional T fragment, each Sulfur of the second pair of said inter(heavy-light chains) cysteine residues form inter(heavy-light chains) disulfide bridge, and the remaining inter(heavy chain) cysteine residues form inter(heavy chains) disulfide bridges;

n is 1 or 2;

m is 1, 2, 3, 4, 5 or 6;

p is 0 or 1;

T, if present, is a terminal group covalently bound to one of said two reduced Sulfur atoms of said inter(heavy-light chains) cysteine residues of Ab' that is not covalently bound to L;

each L, identical or different, is a linear or branched di-, tri-, tetra-, penta-, hexa- or hepta-valent linker moiety covalently binding Payload to said Ab', where said L is attached to Ab' through one or two of said reduced Sulfur atoms of said inter(heavy-light chains) cysteine residue and binds said Ab' with one or more Payload;

Payload is a molecule exerting a biological activity selected from the group consisting in diagnostic agents, therapeutic agents and labelling agents.

10. The protein conjugate of formula (III), (III') or (III'') according to claim 7, 8 or 9 wherein it is of formula (III-A):

Ab'-L-(Payload)

(III-A)

Having a controlled drug-to-antibody ratio (DAR) of 1;

Where in formula (III-A):

Ab' and Payload are defined as in claim 7, 8 or 9;

L is a trivalent linker moiety covalently binding together the two said reduced Sulfur atoms of Ab' with Payload.

11. The protein conjugate of formula (III), (III') or (III'') according to claim 7, 8 or 9 wherein it is of formula (III-B):

$$Ab'\text{-}(L\text{-}(Payload)_m)_2$$

(III-B)

Having a controlled drug-to-antibody ratio (DAR) of 2m;

Where in formula (III-B):

m is as defined above, preferably 1, 2, 3, 4, 5 or 6;

Ab' and Payload are defined as herein;

Each of the two L is di-, tri-, tetra-, penta-, hexa- or hepta-valent linear or branched linker moiety covalently binding one of said reduced Sulfur atoms of said conjugated cysteine residues of Ab' with m Payload(s).

12. A conjugate of formula (III), (III') or (III'') according to anyone of claims 7 to 11 which is of formula (IV):

$$(T)_p\text{-}Ab'\text{-}(L\text{-}(Payload)_m)_{n'}$$

(IV)

Where in formula (IV):

Ab', L, Payload, T, p and m are defined as in anyone of claims 7 to 11;

and

$n'=n+n_a$ where n is defined as in anyone of claims 7 to 11 and n_a is the number of the additional cysteine residues introduced into Ab and $n_a \geq 1$;

and where

n -L-$(Payload)_m$ fragments are conjugated at two conjugated cysteine residues as defined above; the remaining interchain cysteine residues forming interchain disulfide bridges; and n_a -L-$(Payload)_m$ fragments are conjugated at additional sites of conjugation.

13. A conjugate of formula (V):

$$((Payload')_q\text{-}L')_r\text{-}Ab'\text{-}(L\text{-}(Payload)_m)_{n''}$$

(V)

Where T, p, Ab', L, Payload, m are defined as in in anyone of claims 7 to 11,
n'' is n as defined in anyone of claims 1 to 16 or n=n' as defined in claim 12,
Payload' is a payload identical or different from Payload,
L' is a linker identical or different from L,
q is 1, 2, 3, 4, 5 or 6,
r =(8-n)+n_a where n is 1 or 2, and $n_a \geq 0$ is the number of any additional cysteine residues that have been optionally introduced into Ab;
n -L-$(Payload)_m$ fragments are conjugated at two conjugated cysteine residues as defined above; and
n_a -L-$(Payload)_m$ fragments are optionally conjugated at additional cysteine residues that have been optionally introduced into Ab;
r -L'-$(Payload')_q$ fragments are conjugated at one or more of further cysteine residues.

14. The process of preparation of a protein conjugate of formula (III), (III'), (III''), (IV), (V) according to anyone of claims 7 to 13, said process comprising complexing said metal atom from said compound of formula (II) as defined in anyone of claims 1 to 3 and conjugating the obtained de-complexed compound with 1 to n conjugating fragments L-$(Payload)_m$, where L, Payload, m and n are defined as in claims 7 to 11.

15. The process of preparation of a protein conjugate of formula (III) or (III') according to anyone of claims 7 to 12, said process comprising adding a conjugation agent to said compound of formula (II) as defined in anyone of claims 1 to 3 followed by adding a complexing agent.

16. A mixture comprising more than 50%, typically more than 60%, preferably more than 70%, more preferably more than 80%, still more preferably more than 90%, most preferably more than 95% of a compound according to anyone of claims 1 to 13, the percentage being expressed on a molar basis with respect to the total protein species.

17. A pharmaceutical composition comprising an effective amount of the protein conjugate of formula (III), (III'), (III''), (IV) or (V) according to anyone of claims 7 to 13 or a mixture according to claim 16, together with a pharmaceutically acceptable carrier or vehicle.

18. The protein conjugate of formula (III), (III'), (III''), (IV) or (V) according to anyone of claims 7 to 13 or the mixture according to claim 16 for use for treating a condition or disorder

chosen from the group consisting in tumor, cancer, autoimmune disease, or infectious disease in a subject.