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(54) **Tyrosine-based antibody conjugates**

(57) The present invention concerns the finding that that natural *N*-glycoprotein are not sensitive to oxidative enzymes like tyrosinase or (poly)phenol oxidase, however if the native *N*-glycan is modified such that the glycoprotein does not contain a glycan longer than two monosaccharide residues within 10 amino acids of a tyrosine residue, that tyrosine residue of the glycoprotein becomes exposed, and susceptible to oxidative enzymes, leading to the formation of *ortho*-quinone. By performing the enzymatic oxidation in the presence of a strained alkyne or alkene, the resulting *ortho*-quinone undergoes in situ [4+2] cycloaddition to form conjugates having structure Pr-[Z^1-L-(Q^2)$_x$]$_y$ (**1a**) or Pr-[Z^1-L-(D)$_x$]$_y$ (**1b**), wherein:

- Pr is an *N*-glycoprotein;
- Z^1 contains a connecting group formed by the reaction of a ortho-quinone group with a cyclic alkyne or alkene group, which is directly connected to the peptide chain of the antibody at an amino acid located within 10 amino acids of an *N*-glycosylation site, which has been modified such that the glycoprotein does not contain a glycan longer than two monosaccharide residues within 10 amino acids of the amino acid residue;
- L is a linker;
- x is an integer in the range of 1 – 4;
- y is an integer in the range of 1 – 4;
- Q^2 is a chemical handle that is reactive towards an appropriately functionalized payload;
- D is a payload.

Tyrosine-based antibody conjugates

Field of the invention

[0001] The present invention relates to the field of antibody-drug conjugates, in particular to antibody-drug conjugates prepared by tyrosinase-mediated bioconjugation, which are suitable for the treatment of cancer.

Background of the invention

[0002] Antibody-drug conjugates (ADC), considered as magic bullets in therapy, are comprised of an antibody to which is attached a pharmaceutical agent. The antibodies (also known as ligands) are generally monoclonal antibodies (mAbs) which have been selected based on their high selectivity and affinity for a given antigen, their long circulating half-lives, and little to no immunogenicity. Thus, mAbs as protein ligands for a carefully selected biological receptor provide an ideal delivery platform for selective targeting of pharmaceutical drugs. For example, a monoclonal antibody known to bind selectively with a specific cancer-associated antigen can be used for delivery of a chemically conjugated payload to the tumour, via binding, internalization, intracellular processing and finally release of active catabolite. The payload may be a small molecule toxin, a protein toxin or other formats, like oligonucleotides. As a result, the tumour cells can be selectively eradicated, while sparing normal cells which have not been targeted by the antibody. Similarly, chemical conjugation of an antibacterial drug (antibiotic) to an antibody can be applied for treatment of bacterial infections, while conjugates of anti-inflammatory drugs are under investigation for the treatment of autoimmune diseases. Finally, attachment of an oligonucleotide to an antibody selectively taken up by muscle cells is a potential promising approach for the treatment of neuromuscular diseases. Hence, the concept of targeted delivery of an active pharmaceutical drug to a specific cellular location of choice is a powerful approach for the treatment of a wide range of diseases, with many beneficial aspects versus systemic delivery of the same drug.

[0003] In the field of ADCs, a chemical linker is typically employed to attach a pharmaceutical drug to an antibody. This linker needs to possess a number of key attributes, including the requirement to be stable in plasma after drug administration for an extended period of time. A stable linker enables localization of the ADC to the projected site or cells in the body and prevents premature release of the payload in circulation, which would indiscriminately induce undesired biological response of all kinds, thereby lowering the therapeutic index of the ADC. Upon internalization, the ADC should be processed such that the payload is effectively released so it can bind to its target.

[0004] There are two families of linkers, non-cleavable and cleavable. Non-cleavable linkers consist of a chain of atoms between the antibody and the payload, which is fully stable under physiological conditions, irrespective of which organ or biological compartment the antibody-drug conjugate resides in. As a consequence, liberation of the payload from an ADC with a non-cleavable linker relies on the complete (lysosomal) degradation of the antibody after internalization of the ADC into a cell. As a consequence of this degradation, the payload will be released, still carrying the

linker, as well as a peptide fragment and/or the amino acid from the antibody the linker was originally attached to. Cleavable linkers utilize an inherent property of a cell or a cellular compartment for selective release of the payload from the ADC, which generally leaves no trace of linker after metabolic processing. For cleavable linkers, there are three commonly used mechanisms: 1) susceptibility to specific enzymes, 2) pH-sensitivity, and 3) sensitivity to redox state of a cell (or its microenvironment). The cleavable linker may also contain a self-immolative unit, for example based on a *para*-aminobenzyl alcohol group and derivatives thereof. A linker may also contain an additional, non-functional element, often referred to as spacer or stretcher unit, to connect the linker with a reactive group for reaction with the antibody.

[0005] Currently, cytotoxic payloads include for example microtubule-disrupting agents [e.g. auristatins such as monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF), maytansinoids, such as DM1 and DM4, tubulysins], DNA-damaging agents [e.g., calicheamicin, pyrrolobenzodiazepine (PBD) dimers, indolinobenzodiapine dimers, duocarmycins, anthracyclines], topoisomerase inhibitors [e.g. DXd, SN-38] or RNA polymerase II inhibitors [e.g. amanitin]. ADCs that have reached market approval include for example payloads MMAE, MMAF, DM1, calicheamicin, SN-38 and DXd, while various pivotal trials are running for ADCs based on duocarmycin, DM4 and PBD dimer. A larger variety of payloads is still under clinical evaluation or has been in clinical trials in the past, *e.g.* eribulin, indolinobenzodiazepine dimer, PNU-159,682, hemi-asterlin, doxorubicin, vinca alkaloids and others. Finally, various ADCs in late-stage preclinical stage are conjugated to novel payloads for example amanitin, KSP inhibitors, MMAD, and others.

[0006] With the exception of sacituzumab govetican (Trodelvy®), all of the clinical and marketed ADCs contain cytotoxic drugs that are not suitable as stand-alone drug. Trodelvy® is the exception because it features SN-38 as cytotoxic payload, which is also the active catabolite of irinotecan (an SN-38 prodrug). Several other payloads now used in clinical ADCs have been initially evaluated for chemotherapy as free drug, for example calicheamicin, PBD dimers and eribulin. but have failed because the extremely high potency of the cytotoxin (picomolar–low nanomolar IC_{50} values) versus the typically low micromolar potency of standard chemotherapy drugs, such as paclitaxel and doxorubicin.

[0007] Although ADCs have demonstrated clinical and preclinical activity, it has been unclear what factors determine such potency in addition to antigen expression on targeted tumour cells. For example, drug:antibody ratio (DAR), ADC-binding affinity, potency of the payload, receptor expression level, internalization rate, trafficking, multiple drug resistance (MDR) status, and other factors have all been implicated to influence the outcome of ADC treatment in vitro. In addition to the direct killing of antigen-positive tumour cells, ADCs also have the capacity to kill adjacent antigen-negative tumour cells: the so-called "bystander killing" effect, as originally reported by Sahin et al, *Cancer Res.* **1990**, *50*, 6944–6948, incorporated by reference, and for example studied by Li et al, *Cancer Res.* **2016**, *76*, 2710–2719, incorporated by reference. Generally spoken, cytotoxic payloads that are neutral will show bystander killing whereas ionic (charged) payloads do not, as a consequence of the fact that ionic species do not readily pass a cellular membrane by passive diffusion. Payloads with established bystander effect are for example MMAE and DXd. Examples

of payloads that do not show bystander killing are MMAF or the active catabolite of Kadcyla (lysine-MCC-DM1).

[0008] ADCs are prepared by chemical attachment of a reactive linker-drug to a protein, a process known as bioconjugation. Many technologies are known for bioconjugation, as summarized in G.T. Hermanson, "Bioconjugate Techniques", Elsevier, 3rd Ed. **2013**, incorporated by reference. Two main technologies can be recognized for random conjugation to antibodies, either based on acylation of lysine side chain or based on alkylation of cysteine side chain. Acylation of the ε-amino group in a lysine side-chain is typically achieved by subjecting the protein to a reagent based on an activated ester or activated carbonate derivative, for example SMCC is applied for the manufacturing of Kadcyla®. Main chemistry for the alkylation of the thiol group in cysteine side-chain is based on the use of maleimide reagents, as is for example applied in Adcetris®. Besides standard maleimide derivatives, a range of maleimide variants are also applied for more stable cysteine conjugation, as for example demonstrated by James Christie et al., *J. Contr. Rel.* **2015**, *220*, 660–670 and Lyon et al., *Nat. Biotechnol.* **2014**, *32*, 1059–1062, both incorporated by reference. Other approaches for cysteine alkylation involve for example nucleophilic substitution of haloacetamides (typically bromoacetamide or iodoacetamide), see for example Alley et al., *Bioconj. Chem.* **2008**, *19*, 759–765, incorporated by reference, or various approaches based on nucleophilic addition on unsaturated bonds, such as reaction with acrylate reagents, see for example Bernardim et al., *Nat. Commun.* **2016**, *7*, DOI: 10.1038/ncomms13128 and Ariyasu et al., *Bioconj. Chem.* **2017**, *28*, 897–902, both incorporated by reference, reaction with phosphonamidates, see for example Kasper et al., *Angew. Chem. Int. Ed.* **2019**, *58*, 11625–11630, incorporated by reference, reaction with allenamides, see for example Abbas et al., *Angew. Chem. Int. Ed.* **2014**, *53*, 7491–7494, incorporated by reference, reaction with cyanoethynyl reagents, see for example Kolodych et al., *Bioconj. Chem.* **2015**, *26*, 197–200, incorporated by reference, reaction with vinylsulfones, see for example Gil de Montes et al., *Chem. Sci.* **2019**, *10*, 4515–4522, incorporated by reference, or reaction with vinylpyridines, see for example https://iksuda.com/science/permalink/ (accessed Jan. 7th, 2020). An alternative approach to antibody conjugation via cysteine involves the addition of a payload attached to a cysteine cross-linking reagent, such as bis-sulfone reagents, see for example Balan et al., *Bioconj. Chem.* **2007**, *18*, 61–76 and Bryant et al., *Mol. Pharmaceutics* **2015**, *12*, 1872–1879, both incorporated by reference, mono- or bis-bromomaleimides, see for example Smith *et al.*, *J. Am. Chem. Soc.* **2010**, *132*, 1960–1965 and Schumacher et al., *Org. Biomol. Chem.* **2014**, *37*, 7261–7269, both incorporated by reference, bis-maleimide reagents, see for example WO2014114207, bis(phenylthio)maleimides, see for example Schumacher et al., *Org. Biomol. Chem.* **2014**, *37*, 7261–7269 and Aubrey et al., *Bioconj. Chem.* **2018**, *29*, 3516–3521, both incorporated by reference, bis-bromopyridazinediones, see for example Robinson *et al.*, *RSC Advances* **2017**, *7*, 9073–9077, incorporated by reference, bis(halomethyl)benzenes, see for example Ramos-Tomillero et al., *Bioconj. Chem.* **2018**, *29*, 1199–1208, incorporated by reference or other bis(halomethyl)aromatics, see for example WO2013173391. Typically, ADCs prepared by cross-linking of cysteines have a drug-to-antibody loading of ~4 (DAR4). Another useful technology for conjugation to a cysteine side chain is by means of disulfide bond, a bioactivatable connection

that has been utilized for reversibly connecting protein toxins, chemotherapeutic drugs, and probes to carrier molecules (see for example Pillow et al., *Chem. Sci.* **2017**, *8*, 366–370, incorporated by reference).

[0009] A frequent method for attachment of linker-drugs to azido-modified proteins is strain-promoted alkyne-azide cycloaddition (SPAAC). In a SPAAC reaction, the linker-drug is functionalized with a cyclic alkyne and the cycloaddition with azido-modified antibody is driven by relief of ring-strain. Conversely, the linker-drug is functionalized with azide and the antibody with cyclic alkyne. Various strained alkynes suitable for metal-free click chemistry are indicated in Figure 1. Besides cyclooctyne, certain cycloheptynes are also suitable for metal-free click chemistry, as reported by Weterings *et al.*, *Chem. Sci.* **2020**, doi: 10.1039/d0sc03477k, incorporated by reference. Smaller strained alkynes may also be employed, however in most cases require in situ generation of the strained alkyne due to inherent instability.

[0010] Reaction of strained alkynes with tetrazine is also a metal-free click reaction. Moreover, tetrazines also react with strained alkenes (tetrazine ligation). Both strained alkynes and strained alkenes react with tetrazines via inverse electron-demand Diels−Alder (IEDDA) reactions, exhibiting remarkably fast kinetics. For example, reaction of *trans*-cyclooctene (TCO) with tetrazine is unrivalled in its reaction speed and such rapid reaction has enabled applications in rodent models and other large organisms, settings where only minimal reaction times and reagent concentrations are tolerated. Triazine and other heteroaromatic moieties can also undergo reaction with strained alkynes or alkenes. Notably, strained alkenes typically do not undergo reaction with azides. Various strained alkenes suitable for metal-free click chemistry are indicated in Figure 2.

[0011] Besides azides, strained alkynes can also undergo reaction with a range of other functional groups, such as nitrile oxide, nitrone, *ortho*-quinone, dioxothiophene and sydnone. A list of couples of functional groups F and Q (=strained alkyne or strained alkene) for metal-free click chemistry is provided in Figure 3. A comprehensive overview of metal-free click chemistries for bioconjugation, extending also beyond proteins (*e.g.* glycans, nucleic acids), is provided by Nguyen and Prescher, *Nature rev.* **2020**, doi: 10.1038/s41570-020-0205-0, incorporated by reference.

[0012] Based on the above, a general method for the preparation of a protein conjugate, exemplified for a monoclonal antibody in Figure 4, entails the reaction of a protein containing x number of reactive moieties F with a linker-drug construct containing a single molecule Q.

[0013] Introduction of an azide or a tetrazine moiety onto a protein can be achieved by genetic encoding, by enzymatic installation or by chemical acylation. One method is based on genetic encoding of a non-natural amino acid, *e.g. p*-acetophenylalanine suitable for oxime ligation, or *p*-azidomethylphenylalanine or *p*-azidophenylalanine suitable for click chemistry conjugation, as for example demonstrated by Axup *et al. Proc. Nat. Acad. Sci.* **2012**, *109*, 16101–16106, incorporated by reference. Similarly, Zimmerman *et al.*, *Bioconj. Chem.* **2014**, *25*, 351–361, incorporated by reference have employed a cell-free protein synthesis method to introduce azidomethylphenylalanine (AzPhe) into monoclonal antibodies for conversion into ADC by means of metal-free click chemistry. Also, it has also be shown by Naim *et al.*, *Bioconj. Chem.* **2012**, *23*, 2087–2097, incorporated by reference, that a methionine analogue like azidohomoalanine (Aha)

can be introduced into protein by means of auxotrophic bacteria and further converted into protein conjugates by means of (copper-catalysed) click chemistry. Finally, genetic encoding of aliphatic azides in recombinant proteins using a pyrrolysyl-tRNA synthetase/$tRNA_{CUA}$ pair was shown by Nguyen *et al.*, *J. Am. Chem. Soc.* **2009**, *131*, 8720–8721, incorporated by reference and labelling was secured by click chemistry.

[0014] Another method is based on enzymatic installation of a non-natural functionality. For example, Dennler *at al.*, *Bioconj. Chem.* **2014**, *25*, 569–578 and Lhospice *et al.*, *Mol. Pharmaceut.* **2015**, *12*, 1863–1871, both incorporated by reference, employ the bacterial enzyme transglutaminase (BTG or TGase) for installation of an azide moiety onto an antibody. To this end, the key glutamine residue for TGase-mediated installation is first liberated by PNGase F-mediated removal of the native N-glycan, as first demonstrated by Jeger *et al.*, *Angew. Chem. Int. Ed.* **2010**, *49*,9995–9997, incorporated by reference. A genetic method based on C-terminal TGase-mediated azide introduction followed by conversion in ADC with metal-free click chemistry was reported by Cheng *et al.*, *Mol. Cancer Therap.* **2018**, *17*, 2665–2675, incorporated by reference.

[0015] It has been shown by van Geel et al., *Bioconj. Chem.* **2015**, *26*, 2233–2242 and Verkade et al., *Antibodies* **2018**, *7*, 12, all incorporated by reference, that enzymatic remodelling of the native antibody glycan at N297 also enables introduction of an azide into the antibody by means of an azido sugar, suitable for attachment of cytotoxic payload using click chemistry. Chemical approaches have also been developed for site-specific modification of antibodies without prior genetic modification, as for example highlighted by Yamada and Ito, *ChemBioChem.* **2019**, *20*, 2729–2737.

[0016] Of the functional moieties F in Figure 3, azide and nitrone can be installed onto a natural protein also by chemical modification. The resulting azide- or nitrone-containing protein can then undergo metal-free click conjugation with a suitable probe Q, providing the resulting protein conjugate in a straightforward two-stage process. For example, treatment of a natural protein with a diazo transfer reagent leads to chemical conversion of free amino groups to azide groups, as was for example reported by Schoffelen *et al.*, *Chem. Sci.* **2011**, *2*, 701–705, incorporated by reference. Moreover, careful titration of the pH in some cases leads to selective conversion of the amine with the lowest pKa (typically the amine at the N-terminus of the model proteins). The resulting azides were modified based on strain-promoted cycloaddition of functionalized cycloalkynes. Also, it was shown by Ning *et al.*, *Angew. Chem. Int. Ed.* **2010**, *49*, 3065–3068, incorporated by reference, that an N-terminal nitrone can be generated onto a natural polypeptide by periodate-mediated oxidation of an N-terminal serine or threonine, followed by treatment with excess of N-alkyl hydroxylamine. The resulting nitrone was shown to undergo rapid *in situ* cycloaddition with a strained alkyne.

[0017] Of the functional moieties F in Table 3, an *ortho*-quinone can be generated directly from a natural protein by oxidation of tyrosine side chain, as reviewed by Bruins et al., *Chem. Eur. J.* **2017**, *24*, 4749–4756, incorporated by reference. A main advantage of the generation of an *ortho*-quinone versus azide or nitrone is the fact that the *ortho*-quinone is able to undergo *in situ* follow-up chemistry to generate the protein conjugate in a one-stage process without isolation of the quinone intermediate. For example, it was reported by Wilchek and Miron, *Bioconj. Chem.* **2015**, *26*, 502,

incorporated by reference, that direct chemical conversion of phenol group in tyrosine to *ortho*-quinone can be achieved by treatment with potassium nitrosodisulfonate (PTN, also known as Fremy's salt) and used it for protein polymerization. Similarly, George *et al.*, *ChemistrySelect* **2017**, *2*, 7117–7112, incorporated by reference, showed that strain-promoted oxidation-controlled cyclooctyne-1,2-quinone cycloaddition (SPOCQ) can be employed for protein modification by generation of *ortho*-quinone with Fremy's salt followed by *in situ* reaction with bicyclononyne (BCN), as highlighted in Figure 5. However, the use of strong oxidants like Fremy's salt can result in collateral oxidation of cysteine and methionine side chains, and oxidants needs to be removed from the protein solution following the reaction. Besides, multiple tyrosine moieties may undergo oxidation to *ortho*-quinone, thereby leading to a heterogeneous mixture of protein conjugates.

[0018] One elegant solution to circumvent chemical oxidants is the use of an enzyme to generate an *ortho*-quinone. Tyrosinase- and phenol oxidase-mediated generation of *ortho*-quinones has been known for decades to mediate cross-linking between proteins in meat, whey and flour via non-selective tyrosine-tyrosine, tyrosine-cysteine, and tyrosine-lysine linkages. By performing the enzyme-mediated generation of the *ortho*-quinone in the presence of a suitable external nucleophile, the oxidized protein will readily undergo chemical conjugation, as for example demonstrated by Struck *et al.*, *J. Am. Chem. Soc.* **2016,** *138*, 3038–3045. A disadvantage of enzymatic oxidation of proteins is that the majority or all of the tyrosine moieties are typically buried in the hydrophobic interior of the protein and therefore not be accessible for a bulky enzyme like tyrosinase. On the other hand, the absence of a native tyrosine for oxidation has paved the way for selective peripheral protein oxidation by the introduction of an N- or C-terminal fusion tag with an exposed tyrosine. For example, it was shown by Bruins *et al.*, *Bioconj. Chem.* **2017**, *28*, 1189–1193, incorporated by reference, that laminarase A, a hyperstable endo-β-1,3-glucanase, could be selectively fluorophore-modified upon SPOCQ by fusion of C-terminal G_4Y-tag onto the glucanase, while the same C-terminal G_4Y-tag fused to trastuzumab light chain enabled the generation of a site-specific antibody-drug conjugate upon reaction with BCN-linker-MMAE. Bruins *et al.* have also demonstrated, *Chem. Commun.* **2018**, *54*, 7338–7341, incorporated by reference, that an antibody-drug conjugate could be generated by reaction of the C-terminal *ortho*-quinone with a linker-auristatin construct based on conformationally strained *trans*-cyclooctene (sTCO). A similar approach was most recently reported by Marmelstein *et al.*, *J. Am. Chem. Soc.* **2020**, *142*, 5078–5086, incorporated by reference, showing that a C-terminal GGY tag on trastuzumab single chain enables selective tyrosinase-mediated coupling of various tags.

[0019] The introduction of functionality F in all cases described above requires either genetic modification of the protein (genetic encoding of non-natural amino acid, introduction of specific fusion tag) or a two-stage approach where the functionality F is first introduced chemically or enzymatically. However, there currently exists no generic method for the one-step modification of native proteins based on modification of natural amino acid side-chains by means of metal-free click chemistry.

Summary of the invention

[0020] The present inventors have surprisingly found that natural *N*-glycoprotein are not sensitive to oxidative enzymes like tyrosinase or (poly)phenol oxidase, however if the native *N*-glycan is modified, e.g. (a) removed, e.g. by PNGase F hydrolysis, or (b) trimmed, e.g. by endoglycosidase, or (c) mutated to another amino acid, a nearby tyrosine residue of the glycoprotein becomes exposed, and susceptible to oxidative enzymes, leading to the formation of *ortho*-quinone (Figure 6). By performing the enzymatic oxidation in the presence of a (functionalized) strained alkyne or alkene (exemplary structures in Figure 7), the resulting *ortho*-quinone can undergo in situ [4+2] cycloaddition with a strained alkyne or strained alkene, thereby forming an glycoprotein conjugate in a one-pot process.

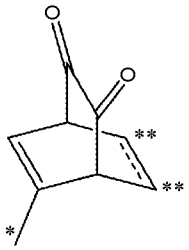
[0021] The invention first and foremost concerns conjugates having structure (**1a**) or (**1b**):

$Pr–[Z^1–L–(Q^2)_x]_y$ (**1a**)

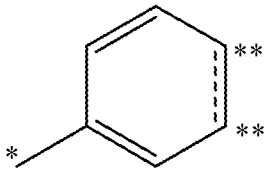
$Pr–[Z^1–L–(D)_x]_y$ (**1b**)

wherein:

- Pr is an *N*-glycoprotein;
- Z^1 comprises structure (Za) or (Zb):



(Za)



(Zb)

wherein the carbon labelled with * is directly connected to the peptide chain of the antibody at an amino acid located within 10 amino acids of an *N*-glycosylation site, which has been modified such that the glycoprotein does not contain a glycan longer than two monosaccharide residues within 10 amino acids of the amino acid residue, and both of the carbon atoms labelled with ** are connected to L, and the bond depicted as ⎯ ⎯ ⎯ is a single bond or a double bond;

- L is a linker;
- x is an integer in the range of 1 – 4;
- y is an integer in the range of 1 – 4;
- Q^2 is a chemical handle that is reactive towards an appropriately functionalized payload;
- D is a payload.

[0022] The invention further concerns a process for the synthesis of the conjugate according to the invention, the medical use of the conjugate according to the invention and a pharmaceutical composition comprising the conjugate according to the invention.

Description of the figures

[0023] Figure 1 shows cyclic alkynes suitable for metal-free click chemistry, and preferred embodiments for reactive moiety Q^1 and Q^2. The list is not comprehensive, for example alkynes can be further activated by fluorination, by substitution of the aromatic rings or by introduction of heteroatoms in the aromatic ring.

[0024] Figure 2 shows cyclic alkenes suitable for metal-free click chemistry, and preferred embodiments for reactive moiety Q^1 and Q^2. The list is not comprehensive, for example alkenes can be further activated by fluorination, by introduction of (hetero)aromatic rings, which may be further substituted.

[0025] Figure 3 shows a representative (but not comprehensive) set of functional groups (F) that can be introduced into a glycoprotein by engineering, by chemical modification, or by enzymatic means, which upon metal-free click reaction with a complementary reactive group Q lead to connecting group Z. Functional group F may be introduced into a glycoprotein at any position of choice by engineering, chemical or enzymatic modification. Various functional groups are known to react exclusively or with high preference with strained alkynes (azide, sydnone). Other functional groups F (nitrile oxide, nitrone, quinone, dioxothiophene, tetrazine, triazine) are reactive with both strained alkynes and strained alkenes. The bicyclic cycloadduct formed by reaction of *ortho*-quinone or dioxothiophene with strained alkyne may eliminate CO or SO_2, respectively, to form an aromatic ring. Similar elimination may also occur after cycloaddition with strained alkene, however will also require subsequent oxidation of the intermediate dihydrobenzene ring. The pyridine or pyridazine connecting group is the product of the rearrangement of the tetrazabicyclo[2.2.2]octane connecting group, formed upon reaction of triazine or tetrazine with alkyne (but not alkene), respectively, with loss of N_2. Similar functional groups (F) are normally present or can be introduced into a payload, for conjugation by metal-free click reaction with a complementary reactive group Q leading to connecting group Z.

[0026] Figure 4 shows the general scheme for preparation of antibody-drug conjugates by reaction of a monoclonal antibody (in most cases a symmetrical dimer) containing an x number of functionalities F. By incubation of antibody-$(F)_x$ with excess of a linker-drug construct (Q-spacer-linker-payload) a conjugate is obtained by reaction of F with Q, forming connecting group Z.

[0027] Figure 5 depicts the general concept of oxidation-mediated generation of an *ortho*-quinone on a protein, followed by *in situ* [4+2] cycloaddition with a suitable strained alkyne (for example BCN).

[0028] Figure 6 depicts the lack of reactivity of native antibodies that are N-glycosylated in the C_H2 domain (*e.g.* N297) for enzymatic oxidation (arrow to the left), however upon removal of the entire glycan (with PNGase F or other hydrolases N297 glycan is hydrolysed, leaving asparate-297) or trimming of the glycan (with endoglycosidase) the antibody becomes susceptible to tyrosinase-mediated oxidation of the neighbouring tyrosine.

[0029] Figure 7 depicts representative structures linker-payloads suitable for cycloaddition with *ortho*-quinones. For example, the linker-payload may be functionalized with a strained alkyne like BCN, may contain one or more units of a carbamoyl sulfamide, may be branched and may contain

a cleavable linker based on valine-citrulline or valine-alanine (all top structure). Alternative, the linker-payload may be functionalized with a strained alkene like sTCO and may contain non-cleavable linker (bottom structure).

[0030] Figure 8 shows the amino acid sequences of the C_H2 constant domain of human IgG1, IgG2, IgG3 and IgG4 and mouse IgG1, IgG2ab, IgG2aa, IgG2b, IgG3. Native glycosylation site (N) is underlined, tyrosine moieties (Y) that can undergo oxidation after the glycan is removed or truncated in bold italics.

[0031] Figure 9 depicts the structures of various functionalized click reagents for conjugation to *ortho*-quinone functionality (e.g. dyes, ODNs, proteins).

[0032] Figure 10 depicts the structures of BCN-linker-payloads with MMAE (**6a** and **6b**) or PBD (**7**).

[0033] Figure 11 depicts the structure of bifunctional reagent **8**, functionalized with a strained alkyne (BCN) as well as a strained alkene (TCO). Also depicted are the structures of various methyltetrazine-modified reporter molecules, *i.e.* TAMRA (**9a**), IL-2 (**9b**), UCHT1 (**9c**) and ODN1826 (**9d**).

[0034] Figure 12 shows the reducing SDS-PAGE for trastuzumab (lane I), PNGase F deglycosylated trastuzumab (lane II) and deglycosylated trastuzumab after treatment with mushroom tyrosinase in the presence BCN-lissamine **1** (lane III). Picture on the left = Coomassie staining, picture on the right = fluorescence image. A fluorescent band is apparent only for trastuzumab upon deglycosylation and treatment with tyrosinase in the presence of **1**.

[0035] Figure 13 depicts the MS data for the Fc-fragment of IdeS-treated trastuzumab (top), PNGase F deglycosylated trastuzumab (middle) and deglycosylated trastuzumab after treatment with mushroom tyrosinase in the presence BCN-lissamine **1** (bottom). Picture on the left shows full range (0–100,000 Da), picture on the right is zoom (23,000–27,000).

[0036] Figure 14 depicts the MS data for the Fc-fragment of IdeS-treated cetuximab (top), PNGase F deglycosylated cetuximab (middle) and deglycosylated cetuximab after treatment with mushroom tyrosinase in the presence BCN-lissamine **1** (bottom). Picture on the left shows full range (0–100,000 Da), picture on the right is zoom (23,000–27,000).

[0037] Figure 15 depicts the relationship between stoichiometry of BCN-lissamine (**1**) versus deglycosylation trastuzumab in the presence of mushroom tyrosinase. Clean conversion into a new product (retention time 8.7 min) is achieved with minimum of 2.5 equiv. of **1** (t = 6.4 min = LC; t = 8.3 min = HC0; t = 8.7 min = HC1).

[0038] Figure 16 shows the reducing SDS-PAGE for labelling to deglycosylated trastuzumab and cetuximab upon treatment with tyrosinase in the presence of TCO-AF568 (**3**).

[0039] Figure 17 shows the HPLC-traces for deglycosylated trastuzumab (top), after reaction with BCN-lissamine (**1**) in the presence of tyrosinase (middle) and after reaction with TCO-AF_{568} (**3**) in the presence of tyrosinase (bottom) (t = 6.6 min = LC; t = 7.9 min = HC0; t = 8.1 min = HC1 (with **3**); t = 8.6 min = HC1 (with **1**)).

[0040] Figure 18 shows the lack of fluorescence labelling for mouse IgG1 and human IgG2 before and after deglycosylation and tyrosinase in the presence of **3** treatment due to the lack of native tyrosine in the vicinity of the native glycosylation site.

[0041] Figure 19 shows the HPLC-trace for a competition experiment (bottom trace **D**) for labelling of trastuzumab-LC-G_4Y (trace **A**) in the presence of both BCN-lissamine (**1**) and TCO-AF_{568} (**3**), showing the clear formation of primarily the adduct of LC-G_4Y and **1** (t = 7.1 min) and minute amounts of LC-G_4Y adduct with **3** (t = 6.4 min). Separate experiments depicted for **1** (trace **B**) and **3** (trace **C**). In all traces shown, t = 6.2 corresponds to the unmodified LC-G_4Y (LC0) and t = 7.4 min corresponds to the unmodified HC0.

[0042] Figure 20 depicts the strategy for conversion of an antibody into a TCO-labelled antibody by (a) deglycosylation, and (b) treatment with bifunctional BCN-TCO reagent **8** in the presence of tyrosinase.

[0043] Figure 21 shows the results of treatment of trastuzumab-TCO (depicted in Figure 8, lane A) with reagents **9a–9d** (lanes B – E) by reducing SDS-PAGE (Coomassie staining and fluorescence imaging). Formation of new bands with higher molecular weight than HC is visible by Coomassie staining for reagents **9a–9c**, while a fluorescent band for HC becomes visible of **9d.**

[0044] Figure 22 shows MS data and RP-HPLC data for PNGase F-deglycosylated trastuzumab.

[0045] Figure 23 shows MS data and RP-HPLC data for PNGase F-deglycosylated B12.

[0046] Figure 24 shows SEC data, MS data and RP-HPLC data for PNGase F-deglycosylated trastuzumab, after treatment with BCN-MMAE (**6a**).

[0047] Figure 25 shows SEC data, MS data and RP-HPLC data for PNGase F-deglycosylated trastuzumab, after treatment with BCN-$MMAE_2$ (**6b**).

[0048] Figure 26 shows SEC data, MS data and RP-HPLC data for PNGase F-deglycosylated trastuzumab, after treatment with BCN-PBD (**7**). Note: *BCN-HS-PEG_2-va-PABC-PBD is not stable under the acidic conditions used in sample work-up and analysis. Therefore, some peak broadening is observed, so conversion was determined by the amount of starting material left.

[0049] Figure 27 shows SEC data, MS data and RP-HPLC data for PNGase F-deglycosylated B12, after treatment with BCN-$MMAE_2$ (**6b**).

[0050] Figure 28 shows the in vitro efficacy on HER2-positive cell line SK-BR-3 of various antibody conjugates, prepared from trastuzumab or B12 (negative control). As a positive control is included GC-ADC: BCN-MMAE **6a** conjugated to trastuzumab after enzymatic remodelling with 6-azidoGalNAc (according to WO2016170186, incorporated by reference).

[0051] Figure 29 shows the RP-HPLC analysis of the antibody conjugate obtained by endoglycosidase trimming of trastuzumab (mostly fucosylated) followed by incubation with tyrosinase and linker-payload **6a**.

[0052] Figure 30 shows mass spectrometry data of the antibody conjugate obtained by trimming of high-mannose trastuzumab (non-fucosylated) followed by incubation with tyrosinase and linker-payload **6a**.

[0053] Figure 31 shows the RP-HPLC analysis of the antibody conjugate obtained by trimming of high-mannose trastuzumab (non-fucosylated) followed by incubation with tyrosinase and linker-payload **6a**.

List of preferred embodiments

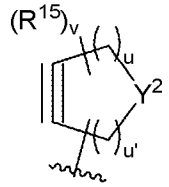
1. A process for the preparation of an glycoprotein-conjugate, comprising:
   (a) providing an *N*-glycoprotein having an exposed tyrosine residue, wherein the exposed tyrosine residue is located within 10 amino acids of an *N*-glycosylation site, but that *N*-glycosylation site has been modified such that the glycoprotein does not contain a glycan longer than two monosaccharide residues within 10 amino acids of the exposed tyrosine residue;
   (b) converting the phenol moiety of the exposed tyrosine residue into an *ortho*-quinone moiety by contacting the glycoprotein with an oxidative enzyme capable of oxidizing tyrosine;
   (c) reacting the *ortho*-quinone moiety with an alkene or alkyne compound via a [4+2] cycloaddition, wherein the compound comprises a (hetero)cycloalkene or (hetero)cycloalkyne moiety and (i) a chemical handle to further modify the compound with a payload, or (ii) a payload.
2. The process according to embodiment 1, wherein the exposed tyrosine residue is located within 5 amino acids of the *N*-glycosylation site.
3. The process according to embodiment 1 or 2, wherein the *N*-glycoprotein having an exposed tyrosine residue is provided by:
   (a1) subjecting an *N*-glycoprotein to deglycosylation by contacting it with an amidase, preferably with PNGase F, to obtain an *N*-glycoprotein from which the glycan is removed; or
   (a2) subjecting an *N*-glycoprotein to trimming by contacting it with an endoglycosidase, to form an *N*-glycoprotein having a glycan of structure –GlcNAc(Fuc)$_b$, wherein b is 0 or 1; or
   (a3) providing a mutated *N*-glycoprotein wherein the glycosylated asparagine is replaced by a non-glycosylated amino acid.
4. The process according to any one of the preceding embodiments, wherein the oxidative enzyme is tyrosinase or (poly)phenol oxidase.
5. The process according to any one of the preceding embodiments, wherein steps (b) and (c) are performed in one-pot, by contacting the *N*-glycoprotein simultaneously with the oxidative enzyme and the alkene or alkyne compound.
6. The process according to any one of the preceding embodiments, wherein the alkene or alkyne compound has the structure (**3a**) or (**3b**)

$Q^1–L–(Q^2)_x$ (**3a**) $Q^1–L–(D)_x$ (**3b**)

wherein:

- Q^1 is a (hetero)cycloalkene or (hetero)cycloalkyne moiety;
- L is a linker;
- x is an integer in the range of 1 – 4;
- Q^2 is a chemical handle that is reactive towards an appropriately functionalized payload but not towards Q^1;
- D is a payload.

7. The process according to any one of the preceding embodiments, wherein Q^1 is a (hetero)cycloalkyne according to structure (Q1):

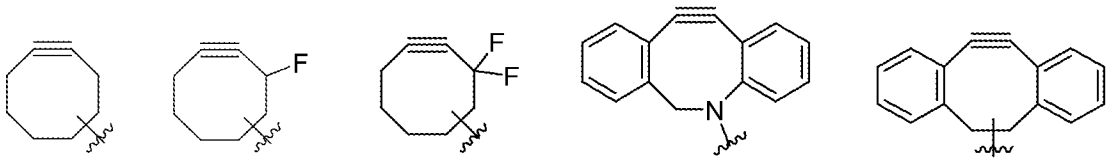


(Q1)

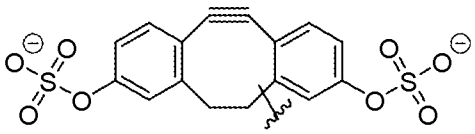
wherein:

- R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, $-NO_2$, -CN, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups and wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are optionally substituted, wherein two substituents R^{15} may be linked together to form an optionally substituted annulated cycloalkyl or an optionally substituted annulated (hetero)arene substituent, and wherein R^{16} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups;
- Y^2 is $C(R^{31})_2$, O, S, $S^{(+)}R^{31}$, $S(O)R^{31}$, $S(O)=NR^{31}$ or NR^{31}, wherein $S^{(+)}$ is a cationic sulphur atom counterbalanced by $B^{(-)}$, wherein $B^{(-)}$ is an anion, and wherein each R^{31} individually is R^{15} or a connection with Q^2 or D, connected via L;
- u is 0, 1, 2, 3, 4 or 5;
- u' is 0, 1, 2, 3, 4 or 5, wherein u + u' = 4, 5, 6, 7 or 8;
- v = an integer in the range 8 – 16.

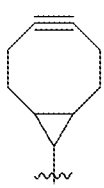
8. The process according to embodiment 7, wherein Q^1 is selected from the group consisting of (Q2) – (Q20):



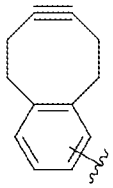
(Q2) (Q3) (Q4) (Q5) (Q6)



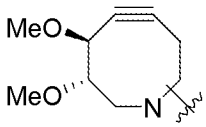
(Q7)



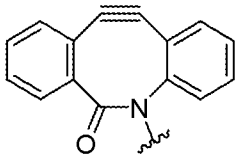
(Q8)



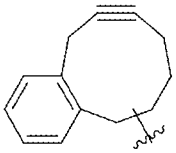
(Q9)



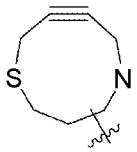
(Q10)



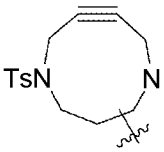
(Q11)



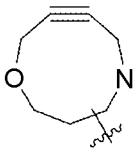
(Q12)



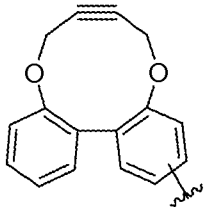
(Q13)



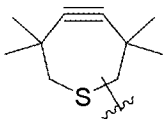
(Q14)



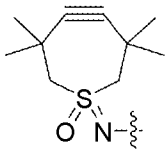
(Q15)



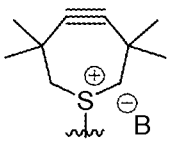
(Q16)



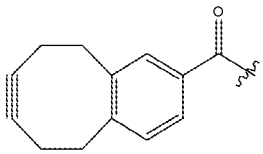
(Q17)



(Q18)



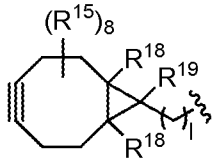
(Q19)



(Q20)

wherein $B^{(-)}$ is an anion.

9. The process according to embodiment 8, wherein Q^1 is a cyclooctyne according to structure (Q42):



(Q42)

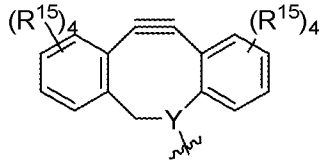
wherein:

- R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, $-NO_2$, -CN, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$,C_1 – C_{24} alkyl groups, C_5 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups and wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are optionally substituted, wherein two substituents R^{15} may be linked together to form an optionally substituted annulated cycloalkyl or an optionally substituted annulated (hetero)arene substituent, and wherein R^{16} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups;
- R^{18} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups;
- R^{19} is selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl

groups, the alkyl groups optionally being interrupted by one of more hetero-atoms selected from the group consisting of O, N and S, wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are independently optionally substituted, or R^{19} is a second occurrence of Q^1 or D connected via a spacer moiety; and

- l is an integer in the range 0 to 10;

or wherein Q^1 is a (hetero)cyclooctyne according to structure (Q43):

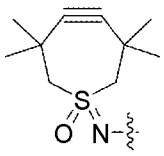


(Q43)

wherein

- R^{15} is independently selected from the group consisting of hydrogen, halogen, -OR^{16}, -NO_2, -CN, -$S(O)_2R^{16}$, -$S(O)_3^{(-)}$, C_1 – C_{24} alkyl groups, C_5 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups and wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are optionally substituted, wherein two substituents R^{15} may be linked together to form an optionally substituted annulated cycloalkyl or an optionally substituted annulated (hetero)arene substituent, and wherein R^{16} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups;
- Y is N or CR^{15};

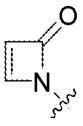
or wherein Q^1 is a heterocycloheptyne according to structure (Q37):



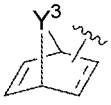
(Q37).

10. The process according to any one of embodiments 1 – 6, wherein Q^1 is a (hetero)cycloalkene selected from the group consisting of, optionally substituted, (hetero)cyclopropenyl group, (hetero)cyclobutenyl group, a norbornene group, a norbornadiene group, *trans*-(hetero)cycloheptenyl group, *trans*-(hetero)cyclooctenyl group, *trans*-(hetero)cyclononenyl group or *trans*-(hetero)cyclodecenyl group, preferably Q^1 is selected from the group consisting of (Q44) – (Q56):

(Q44)



(Q45)



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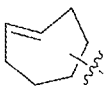

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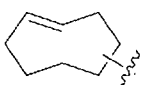
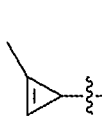
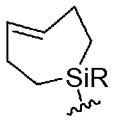


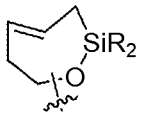
(Q48)



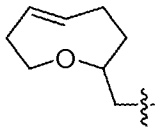
(Q49)



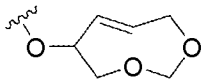
(Q50)



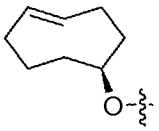
(Q51)



(Q52)



(Q53)



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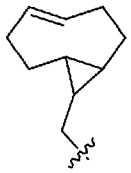
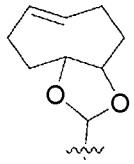


(Q55)



(Q56)

wherein Y^3 is selected from $C(R^{24})_2$, NR^{24} or O, wherein each R^{24} is individually hydrogen, C_1 – C_6 alkyl or is connected to L, optionally via a spacer, and the bond labelled ---- is a single or double bond, and the R group(s) on Si in (Q50) and (Q51) is alkyl or aryl.

11. The process according to any one of the preceding embodiments, wherein the compound comprises (i) a chemical handle to further modify the compound with a payload, and the process further comprises:

    (d) subjecting the chemical handle, preferably Q^2, of the glycoprotein obtained in step (c) to a conjugation reaction with a payload having structure F^2–D or F^2–L^2–$(D)_x$, wherein F^2 is reactive towards the chemical handle, L^2 is a linker and x is an integer in the range of 1 – 4.

12. The process according to any one of the preceding embodiments, wherein the payload D is selected from the group consisting of an active substance, a reporter molecule, a polymer, a solid surface, a hydrogel, a nanoparticle, a microparticle and a biomolecule.

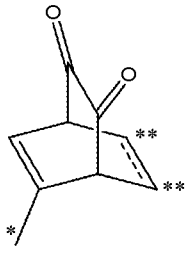
13. A glycoprotein-conjugate according to structure (**1a**) or (**1b**):

$Pr–[Z^1–L–(Q^2)_x]_y$ (**1a**)

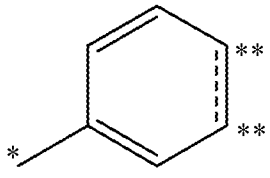
$Pr–[Z^1–L–(D)_x]_y$ (**1b**)

wherein:

- Pr is an *N*-glycoprotein;
- Z^1 comprises structure (Za) or (Zb):



(Za)

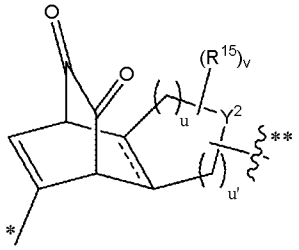


(Zb)

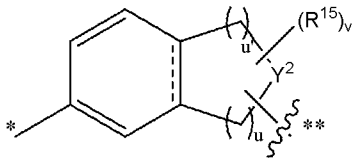
wherein the carbon labelled with * is directly connected to the peptide chain of the glycoprotein at an amino acid located within 10 amino acids of an *N*-glycosylation site, which has been modified such that the glycoprotein does not contain a glycan longer than two monosaccharide residues within 10 amino acids of the amino acid residue, and both of the carbon atoms labelled with ** are connected to L, and the bond depicted as --- is a single bond or a double bond;

- L is a linker;
- x is an integer in the range of 1 – 4;
- y is an integer in the range of 1 – 4;
- Q^2 is a chemical handle that is reactive towards an appropriately functionalized payload;
- D is a payload.

14. The glycoprotein-conjugate according to embodiment 13, wherein Z^1 has structure:



(Z1a)



(Z1b)

wherein:

- the carbon labelled with * is directly connected to the peptide chain of the glycoprotein and the bond labelled with ** is connected to L, and the bond depicted as - - - is a single bond or a double bond;
- R^{15} is independently selected from the group consisting of hydrogen, halogen, -OR^{16}, -NO_2, -CN, -$S(O)_2R^{16}$, -$S(O)_3^{(-)}$, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups and wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are optionally substituted, wherein two substituents R^{15} may be linked together to form an optionally substituted annulated cycloalkyl or an optionally substituted annulated (hetero)arene substituent, and wherein R^{16} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups;
- Y^2 is $C(R^{31})_2$, O, S, $S^{(+)}R^{31}$, $S(O)R^{31}$, $S(O)=NR^{31}$ or NR^{31}, wherein $S^{(+)}$ is a cationic sulphur atom counterbalanced by $B^{(-)}$, wherein $B^{(-)}$ is an anion, and wherein each R^{31} individually is R^{15} or a connection with Q^2 or D, connected via L;
- u is 0, 1, 2, 3, 4 or 5;
- u' is 0, 1, 2, 3, 4 or 5, wherein u + u' = 0, 1, 2, 3, 4, 5, 6, 7 or 8;
- v = an integer in the range 8 – 16.

15. The glycoprotein-conjugate according to embodiment 13 or 14, wherein Q^2 is reactive in a cycloaddition.

16. The glycoprotein-conjugate according to any one of embodiments 13 – 15, wherein the payload D is selected from an active substance, a reporter molecule, a polymer, a solid surface, a hydrogel, a nanoparticle, a microparticle and a biomolecule.

17. A process for the preparation of an glycoprotein-conjugate, comprising reacting an glycoprotein according to structure (**1a**) according to any one of embodiments 13 – 16, with a payload having structure D–F^2 or F^2–L^2–$(D)_x$, wherein F^2 is reactive towards the chemical

handle Q^2 in a conjugation reaction, preferably in a cycloaddition, L^2 is a linker and x is an integer in the range of 1 – 4.

18. Pharmaceutical composition comprising the glycoprotein-conjugate according to structure (**1b**) according to any one of embodiments 13 – 16 and a pharmaceutically acceptable carrier.

19. The glycoprotein-conjugate according to structure (**1b**) according to any one of embodiments 13 – 16 for use in the treatment of a subject in need thereof, preferably in the treatment of cancer.

20. A process for the preparation of an protein-conjugate, comprising:

(a) providing a mutant protein, which is in its native form unreactive towards oxidative enzymes capable of oxidizing tyrosine, but is rendered reactive towards such enzymes by providing a mutated form of the protein, wherein a tyrosine residue is introduced at a non-native position of the amino acid sequence of the protein where it is reactive towards oxidative enzymes capable of oxidizing tyrosine;

(b) converting the phenol moiety of the tyrosine residue into an *ortho*-quinone moiety by contacting the protein with an oxidative enzyme capable of oxidizing tyrosine;

(c) reacting the *ortho*-quinone moiety with an alkene or alkyne compound via a [4+2] cycloaddition, wherein the compound comprises a (hetero)cycloalkene or (hetero)cycloalkyne moiety and (i) a chemical handle to further modify the compound with a payload, or (ii) a payload.

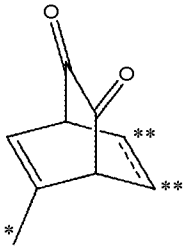
21. A protein-conjugate according to structure (**1a**) or (**1b**):

$$Pr–[Z^1–L–(Q^2)_x]_y \qquad \textbf{(1a)}$$

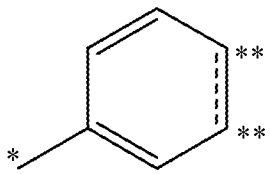
$$Pr–[Z^1–L–(D)_x]_y \qquad \textbf{(1b)}$$

wherein:

- Pr is a protein;
- Z^1 comprises structure (Za) or (Zb):



(Za)



(Zb)

wherein the carbon labelled with * is directly connected to the peptide chain of the glycoprotein at an amino acid which is in the native form of the protein not a tyrosine residue, and both of the carbon atoms labelled with ** are connected to L, and the bond depicted as - - - is a single bond or a double bond;

- L is a linker;
- x is an integer in the range of 1 – 4;
- y is an integer in the range of 1 – 4;
- Q^2 is a chemical handle that is reactive towards an appropriately functionalized payload;
- D is a payload.

21. The protein-conjugate according to embodiment 20, wherein the amino acid to which the connecting group Z^1 is connected is located at a position where a tyrosine residue is reactive towards oxidative enzymes capable of oxidizing tyrosine.
22. The protein-conjugate according to embodiment 20 or 21, wherein Pr is a mutant protein which is in its native form unreactive towards oxidative enzymes capable of oxidizing tyrosine, but is rendered reactive towards such enzyme by providing a mutated form of the protein, wherein a tyrosine residue is introduced at a non-native position in a position of the amino acid sequence of the protein where it is reactive towards oxidative enzymes capable of oxidizing tyrosine.

Detailed description of the invention

Definitions

[0054] The verb “to comprise”, and its conjugations, as used in this description and in the claims is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there is one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

[0055] The compounds disclosed in this description and in the claims may comprise one or more asymmetric centres, and different diastereomers and/or enantiomers may exist of the compounds. The description of any compound in this description and in the claims is meant to include all diastereomers, and mixtures thereof, unless stated otherwise. In addition, the description of any compound in this description and in the claims is meant to include both the individual enantiomers, as well as any mixture, racemic or otherwise, of the enantiomers, unless stated otherwise. When the structure of a compound is depicted as a specific enantiomer, it is to be understood that the invention of the present application is not limited to that specific enantiomer.

[0056] The compounds may occur in different tautomeric forms. The compounds according to the invention are meant to include all tautomeric forms, unless stated otherwise. When the structure of a compound is depicted as a specific tautomer, it is to be understood that the invention of the present application is not limited to that specific tautomer.

[0057] The compounds disclosed in this description and in the claims may further exist as *R* and *S* stereoisomers. Unless stated otherwise, the description of any compound in the description and in the claims is meant to include both the individual *R* and the individual *S* stereoisomers of a compound, as well as mixtures thereof. When the structure of a compound is depicted as a specific *S* or *R* stereoisomer, it is to be understood that the invention of the present application is not limited to that specific *S* or *R* stereoisomer.

[0058] The compounds disclosed in this description and in the claims may further exist as *R* and *S* stereoisomers. Unless stated otherwise, the description of any compound in the description and in the claims is meant to include both the individual *R* and the individual *S* stereoisomers of a compound, as well as mixtures thereof. When the structure of a compound is depicted as a specific

S or *R* stereoisomer, it is to be understood that the invention of the present application is not limited to that specific *S* or *R* stereoisomer.

[0059] The compounds disclosed in this description and in the claims may further exist as *exo* and *endo* diastereoisomers. Unless stated otherwise, the description of any compound in the description and in the claims is meant to include both the individual *exo* and the individual *endo* diastereoisomers of a compound, as well as mixtures thereof. When the structure of a compound is depicted as a specific *endo* or *exo* diastereomer, it is to be understood that the invention of the present application is not limited to that specific *endo* or *exo* diastereomer.

[0060] The compounds according to the invention may exist in salt form, which are also covered by the present invention. The salt is typically a pharmaceutically acceptable salt, containing a pharmaceutically acceptable anion. The term “salt thereof” means a compound formed when an acidic proton, typically a proton of an acid, is replaced by a cation, such as a metal cation or an organic cation and the like. Where applicable, the salt is a pharmaceutically acceptable salt, although this is not required for salts that are not intended for administration to a patient. For example, in a salt of a compound the compound may be protonated by an inorganic or organic acid to form a cation, with the conjugate base of the inorganic or organic acid as the anionic component of the salt.

[0061] The term ”pharmaceutically acceptable” salt means a salt that is acceptable for administration to a patient, such as a mammal (salts with counter ions having acceptable mammalian safety for a given dosage regime). Such salts may be derived from pharmaceutically acceptable inorganic or organic bases and from pharmaceutically acceptable inorganic or organic acids. "Pharmaceutically acceptable salt" refers to pharmaceutically acceptable salts of a compound, which salts are derived from a variety of organic and inorganic counter ions known in the art and include, for example, sodium, potassium, calcium, magnesium, ammonium, tetraalkylammonium, etc., and when the molecule contains a basic functionality, salts of organic or inorganic acids, such as hydrochloride, hydrobromide, formate, tartrate, besylate, mesylate, acetate, maleate, oxalate, etc.

[0062] The term “protein” is herein used in its normal scientific meaning. Herein, polypeptides comprising about 10 or more amino acids are considered proteins. A protein may comprise natural, but also unnatural amino acids.

[0063] The term “antibody” is herein used in its normal scientific meaning. An antibody is a protein generated by the immune system that is capable of recognizing and binding to a specific antigen. An antibody is an example of a glycoprotein. The term antibody herein is used in its broadest sense and specifically includes monoclonal antibodies, polyclonal antibodies, dimers, multimers, multi-specific antibodies (e.g. bispecific antibodies), antibody fragments, and double and single chain antibodies. The term “antibody” is herein also meant to include human antibodies, humanized antibodies, chimeric antibodies and antibodies specifically binding cancer antigen. The term “antibody” is meant to include whole immunoglobulins, but also antigen-binding fragments of an antibody. Furthermore, the term includes genetically engineered antibodies and derivatives of an

antibody. Antibodies, fragments of antibodies and genetically engineered antibodies may be obtained by methods that are known in the art.

[0064] An "antibody fragment" is herein defined as a portion of an intact antibody, comprising the antigen-binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments, diabodies, minibodies, triabodies, tetrabodies, linear antibodies, single-chain antibody molecules, scFv, scFv-Fc, multispecific antibody fragments formed from antibody fragment(s), a fragment(s) produced by a Fab expression library, or an epitope-binding fragments of any of the above which immunospecifically bind to a target antigen (e.g., a cancer cell antigen, a viral antigen or a microbial antigen).

[0065] An "antigen" is herein defined as an entity to which an antibody specifically binds.

[0066] The terms "specific binding" and "specifically binds" is herein defined as the highly selective manner in which an antibody or antibody binds with its corresponding epitope of a target antigen and not with the multitude of other antigens. Typically, the antibody or antibody derivative binds with an affinity of at least about 1×10^{-7} M, and preferably 10^{-8} M to 10^{-9} M, 10^{-10} M, 10^{-11} M, or 10^{-12} M and binds to the predetermined antigen with an affinity that is at least two-fold greater than its affinity for binding to a non-specific antigen (e.g., BSA, casein) other than the predetermined antigen or a closely-related antigen.

[0067] The term "substantial" or "substantially" is herein defined as a majority, i.e. >50% of a population, of a mixture or a sample, preferably more than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of a population.

[0068] A "linker" is herein defined as a moiety that connects two or more elements of a compound. For example in an antibody-conjugate, an antibody and a payload are covalently connected to each other via a linker. A linker may comprise one or more linkers and spacer-moieties that connect various moieties within the linker.

[0069] A "spacer" or spacer-moiety is herein defined as a moiety that spaces (i.e. provides distance between) and covalently links together two (or more) parts of a linker. The linker may be part of e.g. a linker-construct, the linker-conjugate or a bioconjugate, as defined below.

[0070] A "self-immolative group" is herein defined as a part of a linker in an antibody-drug conjugate with a function is to conditionally release free drug at the site targeted by the ligand unit. The activatable self-immolative moiety comprises an activatable group (AG) and a self-immolative spacer unit. Upon activation of the activatable group, for example by enzymatic conversion of an amide group to an amino group or by reduction of a disulfide to a free thiol group, a self-immolative reaction sequence is initiated that leads to release of free drug by one or more of various mechanisms, which may involve (temporary) 1,6-elimination of a *p*-aminobenzyl group to a *p*-quinone methide, optionally with release of carbon dioxide and/or followed by a second cyclization release mechanism. The self-immolative assembly unit can part of the chemical spacer connecting the antibody and the payload (via the functional group). Alternatively, the self-immolative group is not an inherent part of the chemical spacer, but branches off from the chemical spacer connecting the antibody and the payload.

[0071] A "conjugate" is herein defined as a compound wherein an antibody is covalently connected to a payload via a linker. A conjugate comprises one or more antibodies and/or one or more payloads.

[0072] The term "payload" refers to the moiety that is covalently attached to a targeting moiety such as an antibody, but also to the molecule that is released from the conjugate upon uptake of the protein conjugate and/or cleavage of the linker. Payload thus refers to the monovalent moiety having one open end which is covalently attached to the targeting moiety via a linker and also to the molecule that is released therefrom. In the context of the present invention, the payload is exatecan.

[0073] The terms "tyrosinase" and "(poly)phenol oxidase" refer to an enzyme that is capable of catalysing the *ortho*-hydroxylation of a monophenol moiety to an *ortho*-dihydroxybenzene (catechol) moiety, followed by further oxidation of the *ortho*-dihydroxybenzene moiety to produce an *ortho*-quinone (1,2-quinone) moiety.

[0074] The term "deglycosylation" refers to the treatment of an *N*-glycoprotein with an amidase to remove the entire glycan, *i.e.* by enzymatic hydrolysis of the amide bond between the amino acid, usually asparagine, of the protein and the first monosaccharide, usually GlcNAc, at the reducing end of the glycan.

[0075] The term "deglycosylated protein" refers to an *N*-glycoprotein that has been treated with an amidase to remove the entire glycan, *i.e.* by enzymatic hydrolysis of the amide bond between the amino acid, usually asparagine, of the protein and the first monosaccharide, usually GlcNAc, at the reducing end of the glycan.

[0076] The term "trimming" refers to the treatment of an *N*-glycoprotein with an endoglycosidase to hydrolyse the glycosidic bond between the first monosaccharide, usually GlcNAc, at the reducing end of the glycan, which is attached to an amino acid, usually asparagine, and the second monosaccharide, usually GlcNAc.

[0077] The term "trimmed protein" refers to an *N*-glycoprotein that has been treated with an endoglycosidase to hydrolyse the glycosidic bond between the first monosaccharide, usually GlcNAc, at the reducing end of the glycan, which is attached to an amino acid, usually asparagine, and the second monosaccharide, usually GlcNAc.

The Invention

[0078] The inventors have found that *N*-glycoproteins that are normally not reactive towards enzymatic oxidation of a tyrosine residue, by enzymes such as tyrosinase or (poly)phenol oxidase, can be made reactive by shortening or removing the glycan. This finding provides a new opportunity for preparing glycoprotein conjugates, as these tyrosine residues can now readily be converted into *ortho*-quinone moieties, which are in turn chemical handles that can be reacted with (hetero)cycloalkene or (hetero)cycloalkyne moieties. Thus, when the *ortho*-quinone moiety is reacted with a compound comprising a (hetero)cycloalkene or (hetero)cycloalkyne moiety, a covalent attachment is formed between the *N*-glycoprotein and that compound, and when that

compound further comprises (i) a chemical handle to further modify the compound with a payload, or (ii) a payload itself, a conjugate of the *N*-glycoprotein and a payload is readily formed.

[0079] In a first aspect, the invention concerns a process for the preparation of *N*-glycoprotein-conjugates. The process according to the invention comprises:

(a) providing an *N*-glycoprotein having an exposed tyrosine residue, wherein the exposed tyrosine residue is located within 10 amino acids of an *N*-glycosylation site, but that *N*-glycosylation site has been modified such that the glycoprotein does not contain a glycan longer than two monosaccharide residues within 10 amino acids of the exposed tyrosine residue;

(b) converting the phenol moiety of the exposed tyrosine residue into an *ortho*-quinone moiety by contacting the antibody with an oxidative enzyme capable of oxidizing tyrosine;

(c) reacting the ortho-quinone moiety with an alkene or alkyne compound via a [4+2] cycloaddition, wherein the compound comprises a (hetero)cycloalkene or (hetero)cycloalkyne moiety and (i) a chemical handle to further modify the compound with a payload, or (ii) a payload.

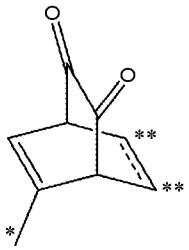
[0080] The invention further concerns conjugates obtainable by the process according to the invention. The conjugate according to the invention may also be defined as having structure (**1a**) or (**1b**):

$$Pr–[Z^1–L–(Q^2)_x]_y \qquad (\mathbf{1a})$$

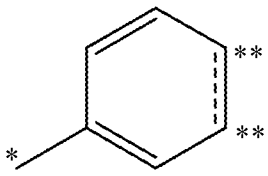
$$Pr–[Z^1–L–(D)_x]_y \qquad (\mathbf{1b})$$

wherein:

- Pr is an *N*-glycoprotein;
- Z^1 comprises structure (Za) or (Zb):



(Za)



(Zb)

wherein the carbon labelled with * is directly connected to the peptide chain of the antibody at an amino acid located within 10 amino acids of an *N*-glycosylation site, which has been modified such that the glycoprotein does not contain a glycan longer than two monosaccharide residues within 10 amino acids of the amino acid residue, and both of the carbon atoms labelled with ** are connected to L, and the bond depicted as - - - is a single bond or a double bond;

- L is a linker;
- x is an integer in the range of 1 – 4;
- y is an integer in the range of 1 – 4;
- Q^2 is a chemical handle that is reactive towards an appropriately functionalized payload;
- D is a payload.

[0081] The invention further concerns a process for the synthesis of the conjugate according to formula (**1b**) from a conjugate according to formula (**1a**), the medical use of the conjugate according

to formula (**1b**) and a pharmaceutical composition comprising the conjugate according to formula (**1b**).

The N-glycoprotein

[0082] The *N*-glycoprotein that is provided in step (a) contains an exposed tyrosine residue. A tyrosine residue is considered to be exposed in the context of the present invention as it would normally be located within 10 amino acids of an *N*-glycosylation site, but that *N*-glycosylation site has been modified such that the glycoprotein does not contain a glycan longer than two monosaccharide residues within 10 amino acids of the exposed tyrosine residue. In other words, this *N*-glycosylation site does not contain a glycan longer than two monosaccharide residues. Herein, "within X amino acids" refers to maximally X – 1 amino acids located in between the exposed tyrosine residue and the (modified) *N*-glycosylation site, such that exposed tyrosine residue is at most the X^{th} amino acids counting from the glycosylated amino acid. Thus, the exposed tyrosine residue is located within 10 amino acids of a native *N*-glycosylation site. Such a native *N*-glycosylation site is typically at a asparagine residue. Preferably, the exposed tyrosine residue is located within 8 amino acids, more preferably within 5 amino acids or even within 3 amino acids, of such an *N*-glycosylation site. The exposed tyrosine residue being located within 10 amino acids of the native *N*-glycosylation site could also refer to a tyrosine residue which is introduced, e.g. by point mutation, at the position of the *N*-glycosylated amino acid, usually an asparagine residue. By introduction of a tyrosine residue in lieu of the asparagine residue, the *N*-glycan will be absent, i.e. a glycan having no monosaccharide residues, and the introduced tyrosine residue fulfils the location requirements of being within 10 amino acids of the native *N*-glycosylation site.

[0083] *N*-glycan structures at the glycosylation site may come in various isoforms (e.g. G0, G1, G2), which have at least 5 monosaccharide residues, but typically much more such as at least 7. These large glycans block nearby tyrosine residues from being reactive towards oxidative enzymes, and these tyrosine residues are made available ("exposed") for such enzymes. The phenolic side chains of tyrosine residues are usually folded towards the interior of proteins, such that they are not reactive towards oxidative enzymes. However, the phenolic side chains of tyrosine residues nearby an *N*-glycosylation site typically point towards to outside of the protein, such that they may be reactive towards oxidative enzymes if the glycan would not be in the way. This is particularly true for antibodies, which normally have one or two tyrosine residues located nearby an *N*-glycosylation site, which are exposed for reaction with oxidative enzymes in step (b) of the process according to the invention.

[0084] The glycoprotein may not have a glycan longer than two monosaccharide residues within 10 amino acids of the exposed tyrosine residue. Preferably, such a glycan is not present within 15 amino acids or even within 20 amino acids. Most preferably, the glycoprotein does not comprise a glycan longer than two monosaccharide residues at all. Typically, this refers to the glycan at the native *N*-glycosylation site. The inventors found that glycans of at most two monosaccharide residues may be present within this range around the exposed tyrosine residue, and the reaction of step (b) will still take place, whereas such tyrosine residues would be blocked (i.e. not exposed) if

the glycan would be longer. Both partial (leaving up to two monosaccharide residues) and complete removal of the glycan is found to expose the otherwise blocked tyrosine residue and make it available for reaction as in step (b). In a preferred embodiment, the glycan is completely absent or has the structure –GlcNAc(Fuc)$_b$, wherein b is 0 or 1. Herein, the GlcNAc moiety is directly attached to a nitrogen atom of an amino acid in the peptide chain of the glycoprotein, mostly to the amide nitrogen of an asparagine residue. Such a GlcNAc moiety is referred to as a core GlcNAc moiety. The core GlcNAc moiety may be further substituted at its 6-OH by α-Fuc, in which case b = 1. Such optional fucosylation of the core GlcNAc moiety is a common feature of antibodies, and in the context of the present invention the presence of the fucosyl moiety is irrelevant.

[0085] Since the *N*-glycan(s) of the *N*-glycoprotein may be completely removed in step (a), the *N*-glycoprotein having an exposed tyrosine residue may not contain an *N*-glycan at all. Since the tyrosine residue(s) was/were originally blocked by the glycan(s), the protein that remains after removal of the glycan(s) is still referred to as an *N*-glycoprotein in the context of the present invention.

[0086] The original or native *N*-glycoprotein that is used in the process according to the invention may have more than one tyrosine residue. It is preferred that the *N*-glycoprotein only contains blocked tyrosine residues before being exposed. It is thus preferred that the *N*-glycoprotein, before the tyrosine residue(s) is/are exposed, is unreactive towards an oxidative enzyme capable of oxidizing tyrosine, such as tyrosinase or (poly)phenol oxidase. Alternatively, the *N*-glycoprotein may also contain one or more tyrosine residues that are reactive towards an oxidative enzyme capable of oxidizing tyrosine even without modification of the *N*-glycan. The process according to the invention is still beneficial for such glycoproteins, as one or more additional tyrosine residues become available as conjugation site, thus enabling the preparation of glycoprotein conjugates with higher payload loading. The *N*-glycoprotein preferably comprises 1 – 4 exposed tyrosine residues, more preferably the glycoprotein comprises 1, 2 or 4 exposed tyrosine residues, most preferably the glycoprotein comprises 2 or 4 exposed tyrosine residues. This number is also denoted as y in the definition of the conjugate. The tyrosine residue(s) that is/are exposed may be introduced by genetic modification of the *N*-glycoprotein, or preferably is/are located at the native position.

[0087] In a preferred embodiment, the *N*-glycoprotein is an antibody, preferably a recombinant antibody, generated in mammalian host systems. Antibodies normally have a conserved *N*-glycosylation site at (or around) asparagine-297 (N297), as part of the consensus sequence of *N*-glycosylation NST, see also Figure 8. Glycan structures of various isoforms (e.g. G0, G1, G2) may be present at this glycosylation site, which may have 12 to 18 monosaccharide residues. These large glycans block nearby tyrosine residue from being reactive towards oxidative enzymes. Thus, in case the *N*-glycoprotein is an antibody, it is preferred that the *N*-glycosylation site is the glycosylation site at or around position 297 of the amino acid sequence of the antibody, such as at a position in the range of 294 – 300, preferably in the range 295 – 298, most preferably at position 297. The exposed tyrosine residue is thus located within 10 amino acids of that *N*-glycosylation site, preferably within 8 amino acids, more preferably within 5 amino acids, most preferably within 3 amino acids. It is thus preferred that the exposed tyrosine residue is located at an amino acid

position in the range of 284 – 310, preferably in the range of 287 – 307, preferably in the range of 289 – 305, more preferably in the range of 292 – 302, most preferably in the range of 294 – 300 of the amino acid sequence of the antibody. More specifically, in case the *N*-glycoprotein is an antibody, it is preferred that the *N*-glycosylation site is the glycosylation site at or around N297 and the exposed tyrosine residue is located within 10 amino acids of that *N*-glycosylation site, preferably within 8 amino acids, more preferably within 5 amino acids, most preferably within 3 amino acids. Even more specifically, in case the *N*-glycoprotein is an antibody, it is preferred that the *N*-glycosylation site is the glycosylation site at N297 and the exposed tyrosine residue is located within 10 amino acids of that *N*-glycosylation site, i.e. at a position in the range of 287 – 307, preferably within 8 amino acids, i.e. at a position in the range of 289 – 305, more preferably within 5 amino acids, i.e. at a position in the range of 292 – 302, most preferably within 3 amino acids, i.e. at a position in the range of 294 – 300 of the amino acid sequence of the antibody. Preferably, the tyrosine residue at position Y296 and/or Y300 is exposed. Preferred amino acid sequences are depicted in Figure 8.

[0088] The exposed tyrosine residue may be located at a native position, i.e. at the position of a tyrosine residue in the amino acid sequence of the native *N*-glycoprotein, or at a non-native position, wherein a tyrosine residue is introduced at a position within 10 amino acids of an *N*-glycosylation site. Such point mutations wherein a specific amino acid residue is introduced at a specific site in the amino acid sequence of a protein is well-known in the art. Preferably, native tyrosine residues are used as exposed tyrosine residues in the context of the present invention.

[0089] The *N*-glycoprotein having the exposed tyrosine residue may be prepared by any means known in the art. Suitable techniques include deglycosylation, trimming, removing the glycosylated amino acid by a non-glycosylated amino acid and/or introducing a tyrosine residue at a non-native position. More specifically, the *N*-glycoprotein having the exposed tyrosine residue may be prepared by:

(a1) subjecting an *N*-glycoprotein to deglycosylation by contacting it with an amidase, preferably with PNGase F, to obtain an *N*-glycoprotein from which the glycan is removed; or

(a2) subjecting an *N*-glycoprotein to trimming by contacting it with an endoglycosidase, to form an *N*-glycoprotein having a glycan of structure –GlcNAc(Fuc)$_b$, wherein b is 0 or 1; or

(a3) providing a mutated *N*-glycoprotein wherein the *N*-glycosylated amino acid is replaced by a non-glycosylated amino acid.

[0090] Deglycosylation of step (a1) is known in the art, and can be performed in any suitable way. Typically, the *N*-glycoprotein, such as an antibody, is contacted with an amidase which removes the glycan. Thus, step (a1) affords an *N*-glycoprotein from which the glycan is completely removed, with no remaining monosaccharide moieties. Although any amidase enzyme can be used, beneficial results have been obtained with PNGase F.

[0091] Trimming of glycoproteins, as in option (a2), is known in the art, from e.g. Yamamoto, *Bitechnol. Lett.* **2013**, 35, 1733, WO 2007/133855 or WO 2014/065661, which are incorporated

herein in their entirety. The trimming of step (a2) can be performed in any suitable way. Typically, the *N*-glycoprotein, such as an antibody, is contacted with an endoglycosidase. Herein, the endoglycosidase is capable of trimming complex glycans on glycoproteins (such as antibodies) at the core GlcNAc unit, leaving only the core GlcNAc residue on the glycoprotein, which is optionally fucosylated. Depending on the nature of the glycan, a suitable endoglycosidase may be selected. The endoglycosidase is preferably selected from the group consisting of EndoS, EndoA, EndoE, EfEndo18A, EndoF, EndoM, EndoD, EndoH, EndoT and EndoSH and/or a combination thereof, the selection of which depends on the nature of the glycan. EndoSH is described in PCT/EP2017/052792, see Examples 1 – 3, and SEQ. ID No: 1, which is incorporated by reference herein.

[0092] Providing mutated glycoproteins, as in option (a3), is well-known in the art. In the context of the present invention, the glycoprotein may be mutated in any suitable way, typically, by a point mutation. Herein, the *N*-glycosylated amino acid, typically an asparagine, is replaced by any other amino acid, that is not glycosylated. Any non-glycosylated amino acid is suitable in this context, typically any amino acid except asparagine.

[0093] Preferably, a non-mutated *N*-glycoprotein is used, wherein the glycan is modified according to option (a1) or (a2), most preferably by option (a1).

[0094] In an alternative aspect of the present invention, in step (a) a mutant protein is provided, which is in its native form unreactive towards oxidative enzymes capable of oxidizing tyrosine, but is rendered reactive towards such enzyme by providing a mutated form of the protein, wherein a tyrosine residue is introduced at a non-native position in a position of the amino acid sequence of the protein where it is reactive towards oxidative enzymes capable of oxidizing tyrosine. If such a mutant protein is subjected to steps (b), (c) and optionally (d), of the process according to the present invention, it will be conjugated with one or more payloads.

[0095] Although the protein may be an *N*-glycoprotein in the context of the present aspect, it is not necessarily so, since a tyrosine residue is exposed not by modification of the glycan, but by introduction of a tyrosine residue at a specific position. The skilled person is capable of determining the position where the tyrosine residue may be introduced, for example by 3D-modeling of the mutant protein to determine the orientation of the phenolic side chain. The mutation is typically a point mutation.

Oxidation step (b)

[0096] The exposed tyrosine residue of the *N*-glycoprotein is subjected to oxidation in step (b), wherein the phenol sidechain of the tyrosine residue is converted into an *ortho*-quinone moiety. The oxidation is performed by the action of an oxidative enzyme capable of oxidizing tyrosine. Such oxidative enzymes are known in the art, and are preferably selected from tyrosinases, phenol oxidases and polyphenol oxidases. The oxidation of tyrosine residues is known in the art, but is as yet never performed on tyrosine residues that are blocked by a nearby glycan. The present

inventors have for the first time been able to subject such tyrosine residues to oxidation by exposing them.

Reaction step (c)

[0097] The *ortho*-quinone moiety that is formed during step (b) can be used as chemical handle for further functionalizing the *N*-glycoprotein. As such, payloads can be conjugated to the *N*-glycoprotein, in case the payload is functionalized with a moiety reactive towards an *ortho*-quinone moiety. In step (c), this reaction or conjugation is carried out. Thus, the *N*-glycoprotein comprising an *ortho*-quinone moiety is contacted with a compound that comprises a (hetero)cycloalkene or (hetero)cycloalkyne moiety, which is reactive towards the *ortho*-quinone moiety in a [4+2] cycloaddition, forming a covalent attachment of the glycoprotein with the compound.

[0098] The compound further comprises either (i) a chemical handle, herein also referred to as Q^2, to further modify the compound with a payload D, or (ii) a payload D. Chemical handle Q^2 can be employed to introduce a payload in a further step (d) as defined below. As such, a conjugate of the glycoprotein and the payload molecule is afforded. The compound that is covalently attached to the glycoprotein is further defined below, as well as the connecting group that is formed upon the reaction of step (c).

[0099] The use of (hetero)cycloalkenes and (hetero)cycloalkynes in metal-free click chemistry, such as the [4+2] cycloaddition of step (c), is well-known in the art (see e.g. from WO 2014/065661 and Nguyen and Prescher, *Nature rev.* **2020**, doi: 10.1038/s41570-020-0205-0, both incorporated by reference). These cycloadditions may be strain-promoted, which is also well-known in the art (e.g. a strain-promoted alkyne–azide cycloaddition, SPAAC). In a preferred embodiment, the reaction is a metal-free strain-promoted cycloaddition.

[0100] In a preferred embodiment, steps (b) and (c) are performed in a single pot, wherein the *N*-glycoprotein is contacted simultaneously with the oxidative enzyme and the alkene or alkyne compound.

Optional step (d)

[0101] In case the compound that is used in step (c) comprises chemical handle Q^2, it is preferred that the process according to the present invention includes a step (d), wherein the chemical handle obtained in step (c) is subjected to a conjugation reaction with a payload having structure F^2–D, wherein F^2 is reactive towards the chemical handle. Conjugation reactions between two compatible reactive groups, here Q^2 and F^2, are well-known in the art, and within the context of the present invention, and conjugation method can be employed.

[0102] Care should be taken that the presence of chemical handle Q^2 does not interfere with the reaction of step (c). So, it is preferred that Q^2 is not reactive towards *ortho*-quinone moieties, or the reactivity of Q^2 towards *ortho*-quinone moieties is lower than the reactivity of Q^1 towards *ortho*-quinone moieties, such that in step (c) only Q^1 will react. The product of step (c) is then a glycoprotein modified with a chemical handle Q^2, which is available for further reaction in step (d).

It is also preferred that Q^2 is not reactive towards Q^1, to avoid polymerization of the compound. In other words, Q^2 is compatible with Q^1.

[0103] In a preferred embodiment, the conjugation reaction between Q^2 and F^2 is of the same kind as the conjugation reaction between Q^1 and the *ortho*-quinone moiety. Thus, preferably the conjugation reaction between Q^2 and F^2 is a cycloaddition, preferably a 1,3-dipolar cycloaddition or a [4+2] cycloaddition. The cycloaddition of step (d) is preferably a metal-free strain-promoted cycloaddition. Preferred options for Q^2 are the same as those for Q^1 defined below, and the skilled person is capable of determining which combination of Q^1 and Q^2 is suitable such that Q^1 is more reactive then Q^2 during step (c).

[0104] A typical [4+2] cycloaddition is the (hetero)-Diels-Alder reaction, wherein Q^2 is a diene or a dienophile. As appreciated by the skilled person, the term "diene" in the context of the Diels-Alder reaction refers to 1,3-(hetero)dienes, and includes conjugated dienes ($R_2C{=}CR{-}CR{=}CR_2$), imines (e.g. $R_2C{=}CR{-}N{=}CR_2$ or $R_2C{=}CR{-}CR{=}NR$, $R_2C{=}N{-}N{=}CR_2$) and carbonyls (e.g. $R_2C{=}CR{-}CR{=}O$ or $O{=}CR{-}CR{=}O$). Hetero-Diels-Alder reactions with N- and O-containing dienes are known in the art. Any diene known in the art to be suitable for [4+2] cycloadditions may be used as reactive group Q^2. Preferred dienes include tetrazines, 1,2-quinones and triazines. Although any dienophile known in the art to be suitable for [4+2] cycloadditions may be used as reactive group Q^2, the dienophile is preferably an alkene or alkyne group as described above, most preferably an alkyne group. For conjugation via a [4+2] cycloaddition, it is preferred that Q^2 is a dienophile (and F^2 is a diene), more preferably Q^2 is or comprises an alkynyl group.

[0105] For a 1,3-dipolar cycloaddition, Q^2 is a 1,3-dipole or a dipolarophile. Any 1,3-dipole known in the art to be suitable for 1,3-dipolar cycloadditions may be used as reactive group Q^2. Preferred 1,3-dipoles include azido groups, nitrone groups, nitrile oxide groups, nitrile imine groups and diazo groups. Although any dipolarophile known in the art to be suitable for 1,3-dipolar cycloadditions may be used as reactive groups Q^2, the dipolarophile is preferably an alkene or alkyne group, most preferably an alkyne group. For conjugation via a 1,3-dipolar cycloaddition, it is preferred that Q^2 is a dipolarophile (and F^2 is a 1,3-dipole), more preferably Q^2 is or comprises an alkynyl group.

[0106] Thus, in a preferred embodiment, Q^2 is selected from dipolarophiles and dienophiles.

[0107] The skilled person also capable to determine which combination of Q^2 and F^2 is suitable for a proper conjugation reaction. Preferred options for F^2 are selected from an azide, tetrazine, triazine, nitrone, nitrile oxide, nitrile imine, diazo compound, *ortho*-quinone, dioxothiophene and sydnone, preferably F^2 is an azide moiety. Further preferred options for F^2 are provided below.

<u>The compound</u>

[0108] The compound that is reacted in step (c) comprises a (hetero)cycloalkene or (hetero)cycloalkyne moiety and (i) a chemical handle to further modify the compound with a payload, or (ii) a payload. Typically, the compound has structure (**3a**) or (**3b**):

$$Q^1{-}L{-}(Q^2)_x \qquad\qquad Q^1{-}L{-}(D)_x$$

(**3a**) (**3b**)

[0109] Herein:

- Q^1 is a (hetero)cycloalkene or (hetero)cycloalkyne moiety;

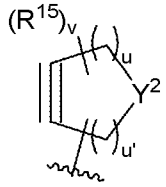
- L is a linker;
- x is an integer in the range of 1 – 4;
- Q^2 is a chemical handle that is reactive towards an appropriately functionalized payload;
- D is a payload.

Chemical handle Q^1

[0110] Q^1 serves as chemical handle for the connection to the *ortho*-quinone moiety. In other words, Q^1 is reactive towards the *ortho*-quinone moiety in a [4+2] cycloaddition. Q^1 is a cyclic (hetero)alkene or a cyclic (hetero)alkyne moiety, most preferably Q is a cyclic (hetero)alkyne moiety.

[0111] In an especially preferred embodiment, Q^1 comprises a cyclic (hetero)alkyne moiety. The alkynyl group may also be referred to as a (hetero)cycloalkynyl group, i.e. a heterocycloalkynyl group or a cycloalkynyl group, wherein the (hetero)cycloalkynyl group is optionally substituted. Preferably, the (hetero)cycloalkynyl group is a (hetero)cycloheptynyl group, a (hetero)cyclooctynyl group, a (hetero)cyclononynyl group or a (hetero)cyclodecynyl group. Herein, the (hetero)cycloalkynes may optionally be substituted. Preferably, the (hetero)cycloalkynyl group is an optionally substituted (hetero)cycloheptynyl group or an optionally substituted (hetero)cyclooctynyl group. Most preferably, the (hetero)cycloalkynyl group is a (hetero)cyclooctynyl group, wherein the (hetero)cyclooctynyl group is optionally substituted.

[0112] In an especially preferred embodiment, Q^1 comprises an (hetero)cycloalkynyl group and is according to structure (Q1):



(Q1)

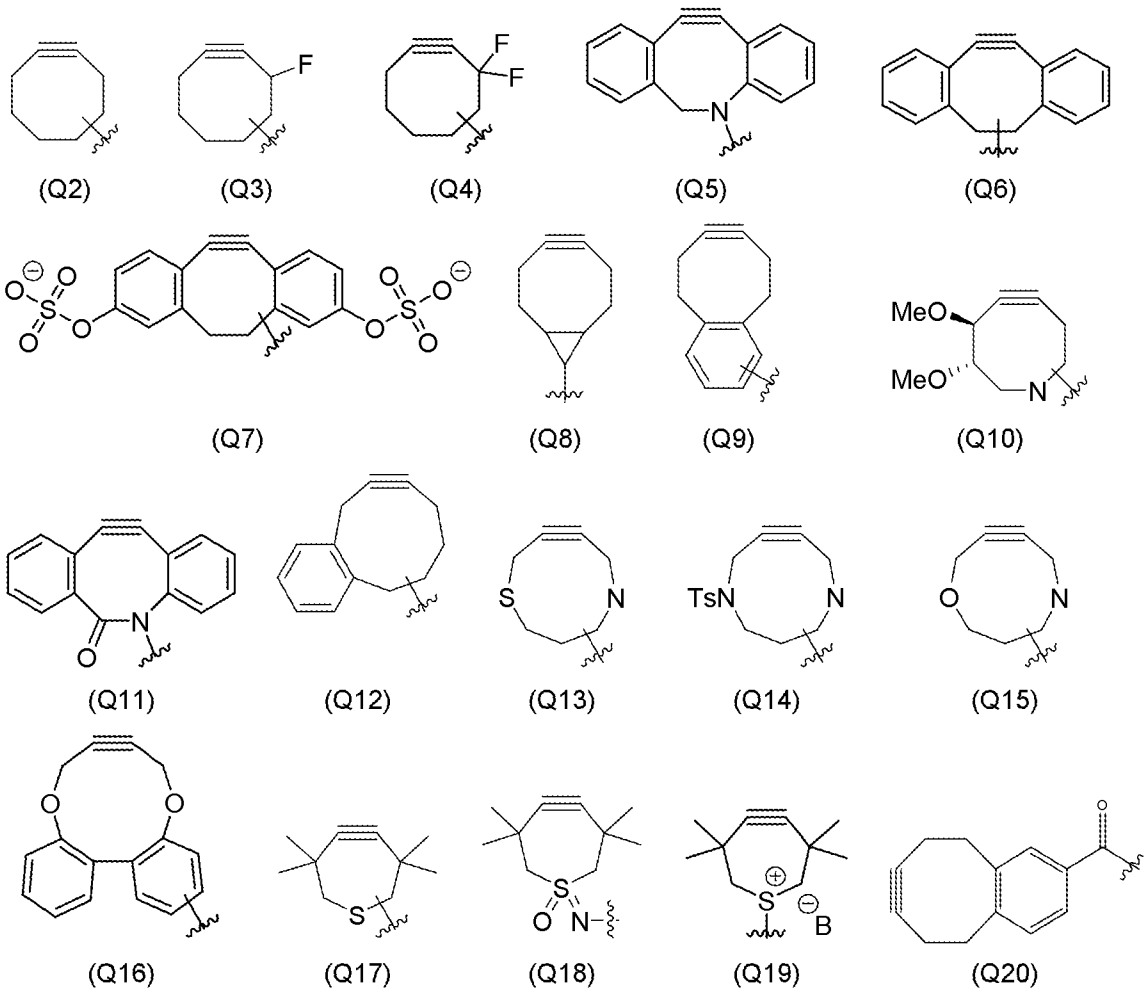
Herein:

- R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, $-NO_2$, -CN, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups and wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are optionally substituted, wherein two substituents R^{15} may be linked together to form an optionally substituted annulated cycloalkyl or an optionally substituted annulated (hetero)arene substituent, and wherein R^{16} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups;
- Y^2 is $C(R^{31})_2$, O, S, $S^{(+)}R^{31}$, $S(O)R^{31}$, $S(O)=NR^{31}$ or NR^{31}, wherein $S^{(+)}$ is a cationic sulphur atom counterbalanced by $B^{(-)}$, wherein $B^{(-)}$ is an anion, and wherein each R^{31} individually is R^{15} or a connection with Q^2 or D, connected via L;
- u is 0, 1, 2, 3, 4 or 5;

- u' is 0, 1, 2, 3, 4 or 5, wherein u + u' = 4, 5, 6, 7 or 8;
- v = an integer in the range 8 – 16.

[0113] In a preferred embodiment, u + u' = 4, 5 or 6, more preferably u + u' = 5. Typically, v = (u + u') x 2 or [(u + u') x 2] – 1. In a preferred embodiment, v = 8, 9 or 10, more preferably v = 9 or 10, most preferably v = 10.

[0114] In a preferred embodiment, Q^1 is selected from the group consisting of (Q2) – (Q20) depicted here below.



(Q2) (Q3) (Q4) (Q5) (Q6)

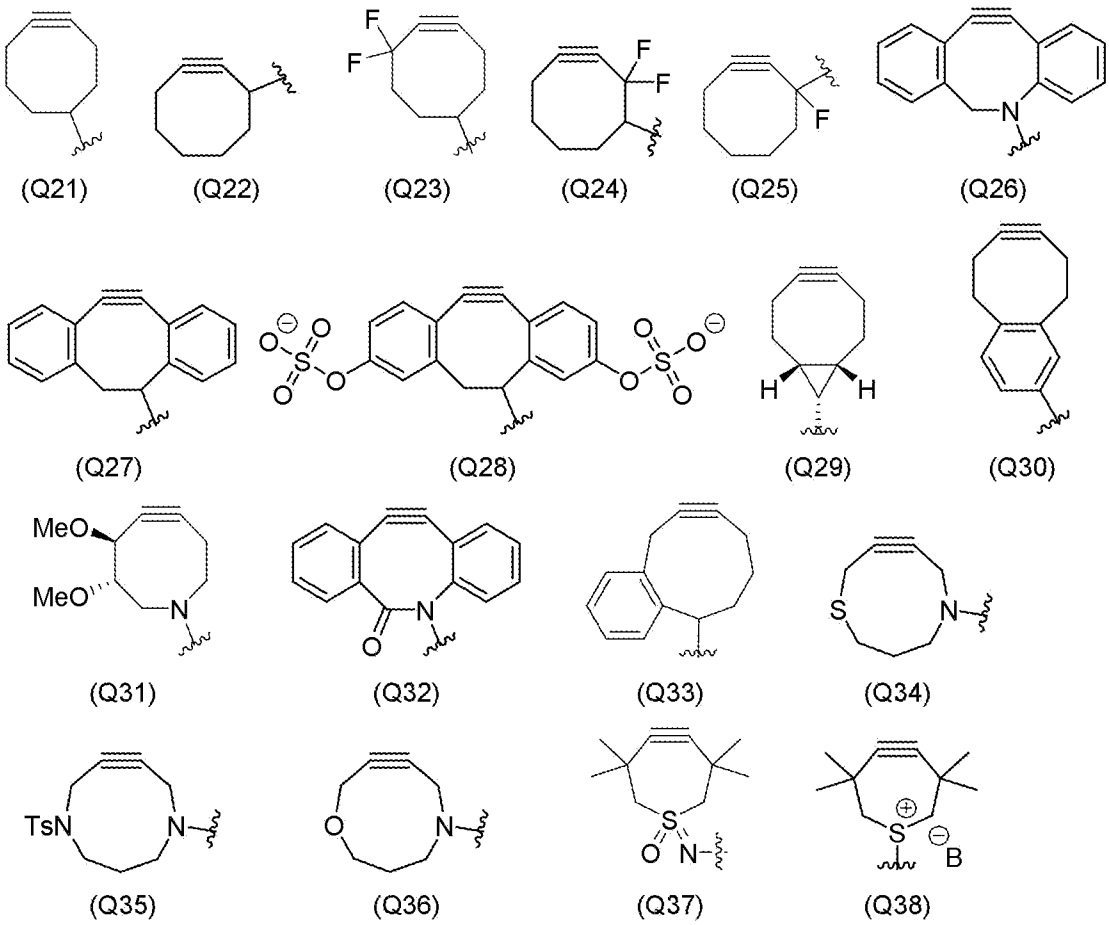
(Q7) (Q8) (Q9) (Q10)

(Q11) (Q12) (Q13) (Q14) (Q15)

(Q16) (Q17) (Q18) (Q19) (Q20)

[0115] Herein, the connection to L, depicted with the wavy bond, may be to any available carbon or nitrogen atom of Q^1. The nitrogen atom of (Q10), (Q13), (Q14) and (Q15) may bear the connection to L, or may contain a hydrogen atom or be optionally functionalized. $B^{(-)}$ is an anion, which is preferably selected from $^{(-)}OTf$, $Cl^{(-)}$, $Br^{(-)}$ or $I^{(-)}$, most preferably $B^{(-)}$ is $^{(-)}OTf$. In the conjugation reaction, $B^{(-)}$ does not need to be a pharmaceutically acceptable anion, since $B^{(-)}$ will exchange with the anions present in the reaction mixture anyway. In case (Q19) is used for Q^1, the negatively charged counter-ion is preferably pharmaceutically acceptable upon isolation of the conjugate according to the invention, such that the conjugate is readily useable as medicament.

[0116] In a further preferred embodiment, Q^1 is selected from the group consisting of (Q21) – (Q38) depicted here below.

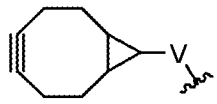


[0117] In structure (Q38), $B^{(-)}$ is an anion, which is preferably selected from $^{(-)}OTf$, $Cl^{(-)}$, $Br^{(-)}$ or $I^{(-)}$, most preferably $B^{(-)}$ is $^{(-)}OTf$.

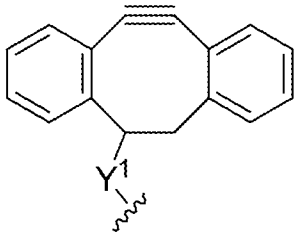
[0118] In a preferred embodiment, Q^1 comprises a (hetero)cyclooctyne moiety according to structure (Q8), more preferably according to (Q29), also referred to as a bicyclo[6.1.0]non-4-yn-9-yl] group (BCN group), which is optionally substituted. In the context of the present embodiment, Q^1 preferably is a (hetero)cyclooctyne moiety according to structure (Q39) as shown below, wherein V is $(CH_2)_l$ and l is an integer in the range of 0 to 10, preferably in the range of 0 to 6. More preferably, l is 0, 1, 2, 3 or 4, more preferably l is 0, 1 or 2 and most preferably l is 0 or 1. In the context of group (Q39), l is most preferably 1. Most preferably, Q^1 is according to structure (Q42), defined further below.

[0119] In an alternative preferred embodiment, Q^1 comprises a (hetero)cyclooctyne moiety according to structure (Q26), (Q27) or (Q28), also referred to as a DIBO, DIBAC, DBCO or ADIBO group, which are optionally substituted. In the context of the present embodiment, Q^1 preferably is a (hetero)cyclooctyne moiety according to structure (Q40) or (Q41) as shown below, wherein Y^1 is O or NR^{11}, wherein R^{11} is independently selected from the group consisting of hydrogen, a linear or branched C_1 - C_{12} alkyl group or a C_4 - C_{12} (hetero)aryl group. The aromatic rings in (Q40) are optionally O-sulfonylated at one or more positions, whereas the rings of (Q41) may be halogenated at one or more positions. Most preferably, Q^1 is according to structure (Q43), defined further below.

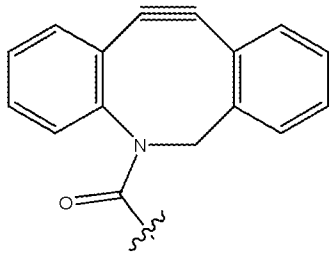
[0120] In an alternative preferred embodiment, Q^1 comprises a heterocycloheptynyl group and is according to structure (Q37).



(Q39)

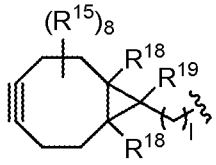


(Q40)



(Q41)

[0121] In an especially preferred embodiment, Q^1 comprises a cyclooctynyl group and is according to structure (Q42):



(Q42)

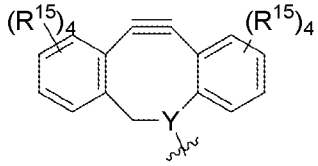
Herein:

- R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, $-NO_2$, -CN, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$, C_1 – C_{24} alkyl groups, C_5 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups and wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are optionally substituted, wherein two substituents R^{15} may be linked together to form an optionally substituted annulated cycloalkyl or an optionally substituted annulated (hetero)arene substituent, and wherein R^{16} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups;
- R^{18} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups;
- R^{19} is selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups, the alkyl groups optionally being interrupted by one of more hetero-atoms selected from the group consisting of O, N and S, wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are independently optionally substituted, or R^{19} is a second occurrence of Q^1 or D connected via a spacer moiety; and
- l is an integer in the range 0 to 10.

[0122] In a preferred embodiment of the reactive group according to structure (Q42), R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, C_1 – C_6 alkyl groups, C_5 – C_6 (hetero)aryl groups, wherein R^{16} is hydrogen or C_1 – C_6 alkyl, more preferably R^{15} is independently selected from the group consisting of hydrogen and C_1 – C_6 alkyl, most preferably all R^{15} are H. In a preferred embodiment of the reactive group according to structure (Q42), R^{18} is independently selected from the group consisting of hydrogen, C_1 – C_6 alkyl groups, most preferably

both R^{18} are H. In a preferred embodiment of the reactive group according to structure (Q42), R^{19} is H. In a preferred embodiment of the reactive group according to structure (Q42), l is 0 or 1, more preferably l is 1.

[0123] In an especially preferred embodiment, Q^1 comprises a (hetero)cyclooctynyl group and is according to structure (Q43):



(Q43)

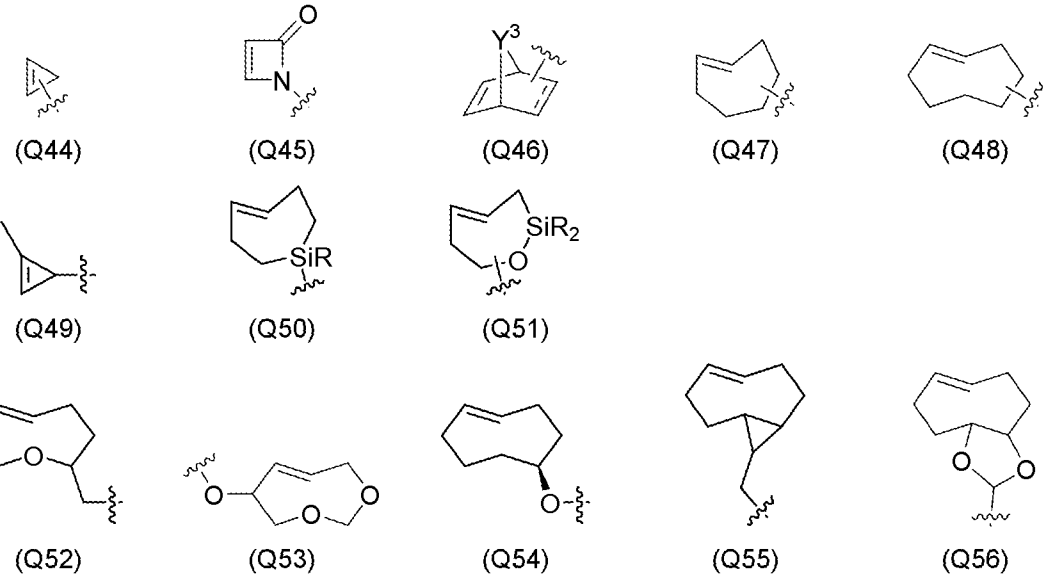
Herein:

- R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, $-NO_2$, -CN, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$, C_1 – C_{24} alkyl groups, C_5 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups and wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are optionally substituted, wherein two substituents R^{15} may be linked together to form an optionally substituted annulated cycloalkyl or an optionally substituted annulated (hetero)arene substituent, and wherein R^{16} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups;
- Y is N or CR^{15}.

[0124] In a preferred embodiment of the reactive group according to structure (Q43), R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, $-S(O)_3^{(-)}$, C_1 – C_6 alkyl groups, C_5 – C_6 (hetero)aryl groups, wherein R^{16} is hydrogen or C_1 – C_6 alkyl, more preferably R^{15} is independently selected from the group consisting of hydrogen and $-S(O)_3^{(-)}$. In a preferred embodiment of the reactive group according to structure (Q43), Y is N or CH, more preferably Y = N.

[0125] In an alternative preferred embodiment, Q^1 comprises a cyclic alkene moiety. The alkenyl group Q^1 may also be referred to as a (hetero)cycloalkenyl group, i.e. a heterocycloalkenyl group or a cycloalkenyl group, preferably a cycloalkenyl group, wherein the (hetero)cycloalkenyl group is optionally substituted. Preferably, the (hetero)cycloalkenyl group is a (hetero)cyclopropenyl group, a (hetero)cyclobutenyl group, a norbornene group, a norbornadiene group, a *trans*-(hetero)cycloheptenyl group, a *trans*-(hetero)cyclooctenyl group, a *trans*-(hetero)cyclononenyl group or a *trans*-(hetero)cyclodecenyl group, which may all optionally be substituted. Especially preferred are (hetero)cyclopropenyl groups, *trans*-(hetero)cycloheptenyl group or *trans*-(hetero)cyclooctenyl groups, wherein the (hetero)cyclopropenyl group, the *trans*-(hetero)cycloheptenyl group or the *trans*-(hetero)cyclooctenyl group is optionally substituted. Preferably, Q^1 comprises a cyclopropenyl moiety according to structure (Q44), a hetereocyclobutene moiety according to structure (Q45), a norbornene or norbornadiene group

according to structure (Q46), a *trans*-(hetero)cycloheptenyl moiety according to structure (Q47) or a *trans*-(hetero)cyclooctenyl moiety according to structure (Q48). Herein, Y^3 is selected from $C(R^{24})_2$, NR^{24} or O, wherein each R^{24} is individually hydrogen, C_1 – C_6 alkyl or is connected to L, optionally via a spacer, and the bond labelled ⎯ ⎯ ⎯ is a single or double bond. In a further preferred embodiment, the cyclopropenyl group is according to structure (Q49). In another preferred embodiment, the *trans*-(hetero)cycloheptene group is according to structure (Q50) or (Q51). In another preferred embodiment, the *trans*-(hetero)cyclooctene group is according to structure (Q52), (Q53), (Q54), (Q55) or (Q56).



(Q44) (Q45) (Q46) (Q47) (Q48)

(Q49) (Q50) (Q51)

(Q52) (Q53) (Q54) (Q55) (Q56)

[0126] Herein, the R group(s) on Si in (Q50) and (Q51) are typically alkyl or aryl, preferably C_1-C_6 alkyl.

[0127] In an alternative preferred embodiment, Q^1 is selected from the structures depicted in Figures 1 and 2.

[0128] Q^2 is a chemical handle that is reactive towards an appropriately functionalized payload. The reactivity of Q^2 is further defined above, in the context of step (d). The appropriately functionalized payload may also be referred to as F^2–D or F^2-L^2-$(D)_x$, wherein F^2 is reactive towards the chemical handle Q^2, L^2 is a linker and x is an integer in the range of 1 – 4, preferably 1 or 2. In a preferred embodiment, Q^2 is selected from the same group as Q^1, but is less reactive towards *ortho*-quinone moieties. In an especially preferred embodiment, Q^1 is a (hetero)cyclooctynyl moiety and Q^2 is a (hetero)cyclooctenyl moiety. An especially preferred combination is Q^1 being according to structure (Q42) and Q^1 being according to structure (Q48).

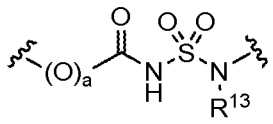
Linker L

[0129] Linkers, also referred to as linking units, are well known in the art and any suitable linker may be used. In the compound of structure (**3a**) or (**3b**), linker L connects chemical handle Q^1 with chemical handle Q^2 or payload D. After the reaction of step (c), linker L connects connecting group Z^1 with chemical handle Q^2 or payload D. Linker L^2 connects reactive moiety F^2 with payload D. The linker may be a cleavable or non-cleavable linker. The linker may contain one or more branch-

points for attachment of multiple payloads D or multiple chemical handles Q^2 to a single (hetero)cycloalkene or (hetero)cycloalkyne moiety Q^1. The further definition of the linker here below equally applies to linker L and linker L^2.

[0130] The linker may for example be selected from the group consisting of linear or branched C_1-C_{200} alkylene groups, C_2-C_{200} alkenylene groups, C_2-C_{200} alkynylene groups, C_3-C_{200} cycloalkylene groups, C_5-C_{200} cycloalkenylene groups, C_8-C_{200} cycloalkynylene groups, C_7-C_{200} alkylarylene groups, C_7-C_{200} arylalkylene groups, C_8-C_{200} arylalkenylene groups, C_9-C_{200} arylalkynylene groups. Optionally the alkylene groups, alkenylene groups, alkynylene groups, cycloalkylene groups, cycloalkenylene groups, cycloalkynylene groups, alkylarylene groups, arylalkylene groups, arylalkenylene groups and arylalkynylene groups may be substituted, and optionally said groups may be interrupted by one or more heteroatoms, preferably 1 to 100 heteroatoms, said heteroatoms preferably being selected from the group consisting of O, $S(O)_y$ and NR^{12}, wherein y is 0, 1 or 2, preferably y = 2, and R^{12} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups. The linker may contain (poly)ethylene glycoldiamines (e.g. 1,8-diamino-3,6-dioxaoctane or equivalents comprising longer ethylene glycol chains), (poly)ethylene glycol or (poly)ethylene oxide chains, (poly)propylene glycol or (poly)propylene oxide chains and 1,z-diaminoalkanes wherein z is the number of carbon atoms in the alkane, and may for example range from 2 – 25.

[0131] In a preferred embodiment, linker L comprises a sulfamide group, preferably a sulfamide group according to structure (L1):



(L1)

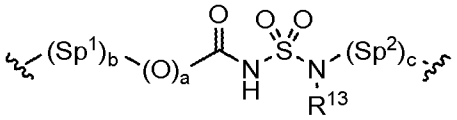
[0132] The wavy lines represent the connection to the remainder of the compound or conjugate, typically to Q^1 or Z^1 and to Q^2 or D, optionally via a spacer. Preferably, the $(O)_aC(O)$ moiety is connected to Q^1 or Z^1 and the NR^{13} moiety to Q^2 or D.

[0133] In structure (L1), a = 0 or 1, preferably a = 1, and R^{13} is selected from the group consisting of hydrogen, C_1 – C_{24} alkyl groups, C_3 – C_{24} cycloalkyl groups, C_2 – C_{24} (hetero)aryl groups, C_3 – C_{24} alkyl(hetero)aryl groups and C_3 – C_{24} (hetero)arylalkyl groups, the C_1 – C_{24} alkyl groups, C_3 – C_{24} cycloalkyl groups, C_2 – C_{24} (hetero)aryl groups, C_3 – C_{24} alkyl(hetero)aryl groups and C_3 – C_{24} (hetero)arylalkyl groups optionally substituted and optionally interrupted by one or more heteroatoms selected from O, S and NR^{14} wherein R^{14} is independently selected from the group consisting of hydrogen and C_1 – C_4 alkyl groups, or R^{13} is a second occurrence of Q^2 or D connected to N via a spacer moiety, preferably Sp^2 as defined here below.

[0134] In a preferred embodiment, R^{13} is hydrogen or a C_1 - C_{20} alkyl group, more preferably R^{13} is hydrogen or a C_1 - C_{16} alkyl group, even more preferably R^{13} is hydrogen or a C_1 - C_{10} alkyl group, wherein the alkyl group is optionally substituted and optionally interrupted by one or more heteroatoms selected from O, S and NR^{14}, preferably O, wherein R^{14} is independently selected from

the group consisting of hydrogen and C_1 – C_4 alkyl groups. In a preferred embodiment, R^{13} is hydrogen. In another preferred embodiment, R^{13} is a C_1 - C_{20} alkyl group, more preferably a C_1 - C_{16} alkyl group, even more preferably a C_1 - C_{10} alkyl group, wherein the alkyl group is optionally interrupted by one or more O-atoms, and wherein the alkyl group is optionally substituted with an -OH group, preferably a terminal -OH group. In this embodiment it is further preferred that R^{13} is a (poly)ethylene glycol chain comprising a terminal -OH group. In another preferred embodiment, R^{13} is selected from the group consisting of hydrogen, methyl, ethyl, n-propyl, i-propyl, n-butyl, s-butyl and t-butyl, more preferably from the group consisting of hydrogen, methyl, ethyl, n-propyl and i-propyl, and even more preferably from the group consisting of hydrogen, methyl and ethyl. Yet even more preferably, R^{13} is hydrogen or methyl, and most preferably R^{13} is hydrogen.

[0135] In a preferred embodiment, the linker is according to structure (L2):



(L2)

[0136] Herein, a, R^{13} and the wavy lines are as defined above, Sp^1 and Sp^2 are independently spacer moieties and b and c are independently 0 or 1. Preferably, b = 0 or 1 and c = 1, more preferably b = 0 and c = 1. In one embodiment, spacers Sp^1 and Sp^2 are independently selected from the group consisting of linear or branched C_1-C_{200} alkylene groups, C_2-C_{200} alkenylene groups, C_2-C_{200} alkynylene groups, C_3-C_{200} cycloalkylene groups, C_5-C_{200} cycloalkenylene groups, C_8-C_{200} cycloalkynylene groups, C_7-C_{200} alkylarylene groups, C_7-C_{200} arylalkylene groups, C_8-C_{200} arylalkenylene groups and C_9-C_{200} arylalkynylene groups, the alkylene groups, alkenylene groups, alkynylene groups, cycloalkylene groups, cycloalkenylene groups, cycloalkynylene groups, alkylarylene groups, arylalkylene groups, arylalkenylene groups and arylalkynylene groups being optionally substituted and optionally interrupted by one or more heteroatoms selected from the group of O, S and NR^{20}, wherein R^{20} is independently selected from the group consisting of hydrogen, C_1 – C_{24} alkyl groups, C_2 - C_{24} alkenyl groups, C_2 - C_{24} alkynyl groups and C_3 - C_{24} cycloalkyl groups, the alkyl groups, alkenyl groups, alkynyl groups and cycloalkyl groups being optionally substituted. When the alkylene groups, alkenylene groups, alkynylene groups, cycloalkylene groups, cycloalkenylene groups, cycloalkynylene groups, alkylarylene groups, arylalkylene groups, arylalkenylene groups and arylalkynylene groups are interrupted by one or more heteroatoms as defined above, it is preferred that said groups are interrupted by one or more O-atoms, and/or by one or more S-S groups.

[0137] More preferably, spacer moieties Sp^1 and Sp^2, if present, are independently selected from the group consisting of linear or branched C_1-C_{100} alkylene groups, C_2-C_{100} alkenylene groups, C_2-C_{100} alkynylene groups, C_3-C_{100} cycloalkylene groups, C_5-C_{100} cycloalkenylene groups, C_8-C_{100} cycloalkynylene groups, C_7-C_{100} alkylarylene groups, C_7-C_{100} arylalkylene groups, C_8-C_{100} arylalkenylene groups and C_9-C_{100} arylalkynylene groups, the alkylene groups, alkenylene groups, alkynylene groups, cycloalkylene groups, cycloalkenylene groups, cycloalkynylene groups, alkylarylene groups, arylalkylene groups, arylalkenylene groups and arylalkynylene groups being

optionally substituted and optionally interrupted by one or more heteroatoms selected from the group of O, S and NR^{20}, wherein R^{20} is independently selected from the group consisting of hydrogen, C_1 – C_{24} alkyl groups, C_2 - C_{24} alkenyl groups, C_2 - C_{24} alkynyl groups and C_3 - C_{24} cycloalkyl groups, the alkyl groups, alkenyl groups, alkynyl groups and cycloalkyl groups being optionally substituted.

[0138] Even more preferably, spacer moieties Sp^1 and Sp^2, if present, are independently selected from the group consisting of linear or branched C_1-C_{50} alkylene groups, C_2-C_{50} alkenylene groups, C_2-C_{50} alkynylene groups, C_3-C_{50} cycloalkylene groups, C_5-C_{50} cycloalkenylene groups, C_8-C_{50} cycloalkynylene groups, C_7-C_{50} alkylarylene groups, C_7-C_{50} arylalkylene groups, C_8-C_{50} arylalkenylene groups and C_9-C_{50} arylalkynylene groups, the alkylene groups, alkenylene groups, alkynylene groups, cycloalkylene groups, cycloalkenylene groups, cycloalkynylene groups, alkylarylene groups, arylalkylene groups, arylalkenylene groups and arylalkynylene groups being optionally substituted and optionally interrupted by one or more heteroatoms selected from the group of O, S and NR^{20}, wherein R^{20} is independently selected from the group consisting of hydrogen, C_1 – C_{24} alkyl groups, C_2 - C_{24} alkenyl groups, C_2 - C_{24} alkynyl groups and C_3 - C_{24} cycloalkyl groups, the alkyl groups, alkenyl groups, alkynyl groups and cycloalkyl groups being optionally substituted.

[0139] Yet even more preferably, spacer moieties Sp^1 and Sp^2, if present, are independently selected from the group consisting of linear or branched C_1-C_{20} alkylene groups, C_2-C_{20} alkenylene groups, C_2-C_{20} alkynylene groups, C_3-C_{20} cycloalkylene groups, C_5-C_{20} cycloalkenylene groups, C_8-C_{20} cycloalkynylene groups, C_7-C_{20} alkylarylene groups, C_7-C_{20} arylalkylene groups, C_8-C_{20} arylalkenylene groups and C_9-C_{20} arylalkynylene groups, the alkylene groups, alkenylene groups, alkynylene groups, cycloalkylene groups, cycloalkenylene groups, cycloalkynylene groups, alkylarylene groups, arylalkylene groups, arylalkenylene groups and arylalkynylene groups being optionally substituted and optionally interrupted by one or more heteroatoms selected from the group of O, S and NR^{20}, wherein R^{20} is independently selected from the group consisting of hydrogen, C_1 – C_{24} alkyl groups, C_2 - C_{24} alkenyl groups, C_2 - C_{24} alkynyl groups and C_3 - C_{24} cycloalkyl groups, the alkyl groups, alkenyl groups, alkynyl groups and cycloalkyl groups being optionally substituted.

[0140] In these preferred embodiments it is further preferred that the alkylene groups, alkenylene groups, alkynylene groups, cycloalkylene groups, cycloalkenylene groups, cycloalkynylene groups, alkylarylene groups, arylalkylene groups, arylalkenylene groups and arylalkynylene groups are unsubstituted and optionally interrupted by one or more heteroatoms selected from the group of O, S and NR^{20}, preferably O, wherein R^{20} is independently selected from the group consisting of hydrogen and C_1 – C_4 alkyl groups, preferably hydrogen or methyl.

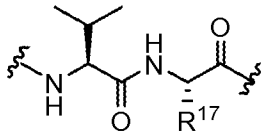
[0141] Most preferably, spacer moieties Sp^1 and Sp^2, if present, are independently selected from the group consisting of linear or branched C_1-C_{20} alkylene groups, the alkylene groups being optionally substituted and optionally interrupted by one or more heteroatoms selected from the group of O, S and NR^{20}, wherein R^{20} is independently selected from the group consisting of hydrogen, C_1 – C_{24} alkyl groups, C_2 - C_{24} alkenyl groups, C_2 - C_{24} alkynyl groups and C_3 - C_{24}

cycloalkyl groups, the alkyl groups, alkenyl groups, alkynyl groups and cycloalkyl groups being optionally substituted. In this embodiment, it is further preferred that the alkylene groups are unsubstituted and optionally interrupted by one or more heteroatoms selected from the group of O, S and NR^{20}, preferably O and/or S-S, wherein R^{20} is independently selected from the group consisting of hydrogen and C_1 – C_4 alkyl groups, preferably hydrogen or methyl.

[0142] Another class of suitable linkers comprises cleavable linkers. Cleavable linkers are well known in the art. For example Shabat *et al.*, *Soft Matter* **2012**, *6*, 1073, incorporated by reference herein, discloses cleavable linkers comprising self-immolative moieties that are released upon a biological trigger, *e.g.* an enzymatic cleavage or an oxidation event. Some examples of suitable cleavable linkers are peptide-linkers that are cleaved upon specific recognition by a protease, *e.g.* cathepsin, plasmin or metalloproteases, or glycoside-based linkers that are cleaved upon specific recognition by a glycosidase, *e.g.* glucoronidase, or nitroaromatics that are reduced in oxygen-poor, hypoxic areas.

[0143] Linker L may further contain a peptide spacer as known in the art, preferably a dipeptide or tripeptide spacer as known in the art, preferably a dipeptide spacer. Although any dipeptide or tripeptide spacer may be used, preferably the peptide spacer is selected from Val-Cit, Val-Ala, Val-Lys, Val-Arg, AcLys-Val-Cit, AcLys-Val-Ala, Phe-Cit, Phe-Ala, Phe-Lys, Phe-Arg, Ala-Lys, Leu-Cit, Ile-Cit, Trp-Cit, Ala-Ala-Asn, Ala-Asn, more preferably Val-Cit, Val-Ala, Val-Lys, Phe-Cit, Phe-Ala, Phe-Lys, Ala-Ala-Asn, more preferably Val-Cit, Val-Ala, Ala-Ala-Asn. In one embodiment, the peptide spacer is Val-Cit. In one embodiment, the peptide spacer is Val-Ala. The peptide spacer may also be attached to the payload, wherein the amino end of the peptide spacer is conveniently used as amine group in the method according to the first aspect of the invention.

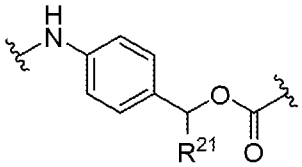
[0144] In a preferred embodiment, the peptide spacer is represented by general structure (L3):



(L3)

[0145] Herein, R^{17} = CH_3 (Val) or $CH_2CH_2CH_2NHC(O)NH_2$ (Cit). The wavy lines indicate the connection to the remainder of the molecule, preferably the peptide spacer according to structure (L3) is connected via NH to Q^1 or Z^1, typically via a spacer, and via C(O) to Q^2 or D, typically via a spacer.

[0146] Linker L may further contain a self-cleavable spacer, also referred to as self-immolative spacer. The self-cleavable spacer may also be attached to the payload. Preferably, the self-cleavable spacer is para-aminobenzyloxycarbonyl (PABC) derivative, more preferably a PABC derivative according to structure (L4).



(L4)

[0147] Herein, the wavy lines indicate the connection to the remainder of the molecule. Typically, the PABC derivative is connected via NH to Q^1 or Z^1, typically via a spacer, and via OC(O) to Q^2 or D, typically via a spacer.

[0148] R^{21} is H, R^{22} or $C(O)R^{22}$, wherein R^{22} is C_1 – C_{24} (hetero)alkyl groups, C_3 – C_{10} (hetero)cycloalkyl groups, C_2 – C_{10} (hetero)aryl groups, C_3 – C_{10} alkyl(hetero)aryl groups and C_3 – C_{10} (hetero)arylalkyl groups, which optionally substituted and optionally interrupted by one or more heteroatoms selected from O, S and NR^{23} wherein R^{23} is independently selected from the group consisting of hydrogen and C_1 – C_4 alkyl groups. Preferably, R^{22} is C_3 – C_{10} (hetero)cycloalkyl or polyalkylene glycol. The polyalkylene glycol is preferably a polyethylene glycol or a polypropylene glycol, more preferably $–(CH_2CH_2O)_sH$ or $–(CH_2CH_2CH_2O)_sH$. The polyalkylene glycol is most preferably a polyethylene glycol, preferably $–(CH_2CH_2O)_sH$, wherein s is an integer in the range 1 – 10, preferably 1 – 5, most preferably s = 1, 2, 3 or 4. More preferably, R^{21} is H or $C(O)R^{22}$, wherein R^{22} = 4-methyl-piperazine or morpholine. Most preferably, R^{21} is H.

Payload D

[0149] Linker L connects the (hetero)cycloalkane or (hetero)cycloalkyne moiety Q^1 with chemical handle Q^2 or payload D. Payload D may also be introduced in step (d). Payload molecules are well-known in the art, especially in the field of antibody-drug conjugates, as the moiety that is covalently attached to the antibody and that is released therefrom upon uptake of the conjugate and/or cleavage of the linker. In a preferred embodiment, the payload is selected from the group consisting of an active substance, a reporter molecule, a polymer, a solid surface, a hydrogel, a nanoparticle, a microparticle and a biomolecule. Especially preferred payloads are active substances and reporter molecules, in particular active substances.

[0150] The term “active substance” herein relates to a pharmacological and/or biological substance, i.e. a substance that is biologically and/or pharmaceutically active, for example a drug, a prodrug, a cytotoxin, a diagnostic agent, a protein, a peptide, a polypeptide, a peptide tag, an amino acid, a glycan, a lipid, a vitamin, a steroid, a nucleotide, a nucleoside, a polynucleotide, RNA or DNA. Examples of peptide tags include cell-penetrating peptides like human lactoferrin or polyarginine. An example of a glycan is oligomannose. An example of an amino acid is lysine.

When the payload is an active substance, the active substance is preferably selected from the group consisting of drugs and prodrugs. More preferably, the active substance is selected from the group consisting of pharmaceutically active compounds, in particular low to medium molecular weight compounds (e.g. about 200 to about 2500 Da, preferably about 300 to about 1750 Da). In a further preferred embodiment, the active substance is selected from the group consisting of cytotoxins, antiviral agents, antibacterial agents, peptides and oligonucleotides. Examples of cytotoxins include colchicine, vinca alkaloids, anthracyclines, camptothecins, doxorubicin, daunorubicin, taxanes, calicheamycins, tubulysins, irinotecans, an inhibitory peptide, amanitin, deBouganin, duocarmycins, maytansines, auristatins, enediynes, pyrrolobenzodiazepines (PBDs) or indolinobenzodiazepine dimers (IGN) or PNU159,682 and derivatives thereof. Preferred payloads are selected from MMAE, MMAF, exatecan, SN-38, DXd, maytansinoids, calicheamicin, PNU159,685 and PBD dimers.

Especially preferred payloads are PBD, SN-38, MMAE, exatecan or DXd. In one embodiment, the payload is MMAE. In one embodiment, the payload is exatecan or DXd. In one embodiment, the payload is SN-38. In one embodiment, the payload is MMAE. In one embodiment, the payload is a PDB dimer.

[0151] The term "reporter molecule" herein refers to a molecule whose presence is readily detected, for example a diagnostic agent, a dye, a fluorophore, a radioactive isotope label, a contrast agent, a magnetic resonance imaging agent or a mass label.

[0152] A wide variety of fluorophores, also referred to as fluorescent probes, is known to a person skilled in the art. Several fluorophores are described in more detail in e.g. G.T. Hermanson, *"Bioconjugate Techniques"*, Elsevier, 3rd Ed. **2013**, Chapter 10: *"Fluorescent probes"*, p. 395 - 463, incorporated by reference. Examples of a fluorophore include all kinds of Alexa Fluor (e.g. Alexa Fluor 555), cyanine dyes (e.g. Cy3 or Cy5) and cyanine dye derivatives, coumarin derivatives, fluorescein and fluorescein derivatives, rhodamine and rhodamine derivatives, boron dipyrromethene derivatives, pyrene derivatives, naphthalimide derivatives, phycobiliprotein derivatives (e.g. allophycocyanin), chromomycin, lanthanide chelates and quantum dot nanocrystals.

[0153] Examples of a radioactive isotope label include ^{99m}Tc, ^{111}In, ^{114m}In, ^{115}In, ^{18}F, ^{14}C, ^{64}Cu, ^{131}I, ^{125}I, ^{123}I, ^{212}Bi, ^{88}Y, ^{90}Y, ^{67}Cu, ^{186}Rh, ^{188}Rh, ^{66}Ga, ^{67}Ga and ^{10}B, which is optionally connected via a chelating moiety such as e.g. DTPA (diethylenetriaminepentaacetic anhydride), DOTA (1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid), NOTA (1,4,7-triazacyclononane *N,N',N''*-triacetic acid), TETA (1,4,8,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetraacetic acid), DTTA (*N*^{1}-(*p*-isothiocyanatobenzyl)-diethylenetriamine-*N*^{1},*N*^{2},*N*^{3},*N*^{3}-tetraacetic acid), deferoxamine or DFA (*N*'-[5-[[4-[[5-(acetylhydroxyamino)pentyl]amino]-1,4-dioxobutyl]hydroxyamino]pentyl]-*N*-(5-aminopentyl)-*N*-hydroxybutanediamide) or HYNIC (hydrazinonicotinamide). Isotopic labelling techniques are known to a person skilled in the art, and are described in more detail in e.g. G.T. Hermanson, *"Bioconjugate Techniques"*, Elsevier, 3rd Ed. **2013**, Chapter 12: *"Isotopic labelling techniques"*, p. 507 - 534, incorporated by reference.

[0154] Polymers suitable for use as a payload D in the compound according to the invention are known to a person skilled in the art, and several examples are described in more detail in e.g. G.T. Hermanson, *"Bioconjugate Techniques"*, Elsevier, 3rd Ed. **2013**, Chapter 18: *"PEGylation and synthetic polymer modification"*, p. 787 - 838, incorporated by reference. When payload D is a polymer, payload D is preferably independently selected from the group consisting of a poly(ethyleneglycol) (PEG), a polyethylene oxide (PEO), a polypropylene glycol (PPG), a polypropylene oxide (PPO), a 1,q-diaminoalkane polymer (wherein q is the number of carbon atoms in the alkane, and preferably q is an integer in the range of 2 to 200, preferably 2 to 10), a (poly)ethylene glycol diamine (e.g. 1,8-diamino-3,6-dioxaoctane and equivalents comprising longer ethylene glycol chains), a polysaccharide (e.g. dextran), a poly(amino acid) (e.g. a poly(L-lysine)) and a poly(vinyl alcohol).

[0155] Solid surfaces suitable for use as a payload D are known to a person skilled in the art. A solid surface is for example a functional surface (e.g. a surface of a nanomaterial, a carbon

nanotube, a fullerene or a virus capsid), a metal surface (e.g. a titanium, gold, silver, copper, nickel, tin, rhodium or zinc surface), a metal alloy surface (wherein the alloy is from e.g. aluminum, bismuth, chromium, cobalt, copper, gallium, gold, indium, iron, lead, magnesium, mercury, nickel, potassium, plutonium, rhodium, scandium, silver, sodium, titanium, tin, uranium, zinc and/or zirconium), a polymer surface (wherein the polymer is e.g. polystyrene, polyvinylchloride, polyethylene, polypropylene, poly(dimethylsiloxane) or polymethylmethacrylate, polyacrylamide), a glass surface, a silicone surface, a chromatography support surface (wherein the chromatography support is e.g. a silica support, an agarose support, a cellulose support or an alumina support), etc. When payload D is a solid surface, it is preferred that D is independently selected from the group consisting of a functional surface or a polymer surface.

[0156] Hydrogels are known to the person skilled in the art. Hydrogels are water-swollen networks, formed by cross-links between the polymeric constituents. See for example A. S. Hoffman, *Adv. Drug Delivery Rev.* **2012**, *64*, 18, incorporated by reference. When the payload is a hydrogel, it is preferred that the hydrogel is composed of poly(ethylene)glycol (PEG) as the polymeric basis.

[0157] Micro- and nanoparticles suitable for use as a payload D are known to a person skilled in the art. A variety of suitable micro- and nanoparticles is described in e.g. G.T. Hermanson, *"Bioconjugate Techniques"*, Elsevier, 3rd Ed. **2013**, Chapter 14: *"Microparticles and nanoparticles"*, p. 549 - 587, incorporated by reference. The micro- or nanoparticles may be of any shape, e.g. spheres, rods, tubes, cubes, triangles and cones. Preferably, the micro- or nanoparticles are of a spherical shape. The chemical composition of the micro- and nanoparticles may vary. When payload D is a micro- or a nanoparticle, the micro- or nanoparticle is for example a polymeric micro- or nanoparticle, a silica micro- or nanoparticle or a gold micro- or nanoparticle. When the particle is a polymeric micro- or nanoparticle, the polymer is preferably polystyrene or a copolymer of styrene (e.g. a copolymer of styrene and divinylbenzene, butadiene, acrylate and/or vinyltoluene), polymethylmethacrylate (PMMA), polyvinyltoluene, poly(hydroxyethyl methacrylate (pHEMA) or poly(ethylene glycol dimethacrylate/2-hydroxyethylmetacrylae) [poly(EDGMA/HEMA)]. Optionally, the surface of the micro- or nanoparticles is modified, e.g. with detergents, by graft polymerization of secondary polymers or by covalent attachment of another polymer or of spacer moieties, etc.

[0158] Payload D may also be a biomolecule. Biomolecules, and preferred embodiments thereof, are described in more detail below. When payload D is a biomolecule, it is preferred that the biomolecule is selected from the group consisting of proteins (including glycoproteins such as antibodies), polypeptides, peptides, glycans, lipids, nucleic acids, oligonucleotides, polysaccharides, oligosaccharides, enzymes, hormones, amino acids and monosaccharides.

[0159] In the context of the present invention, cytotoxic payloads are especially preferred. Thus, D is preferably, a cytotoxin, more preferably selected from the group consisting of colchicine, vinca alkaloids, anthracyclines, camptothecins, doxorubicin, daunorubicin, taxanes, calicheamycins, tubulysins, irinotecans, an inhibitory peptide, amanitins, amatoxins, deBouganin, duocarmycins, epothilones, mytomycins, combretastatins, maytansines, auristatins, enediynes, pyrrolobenzodiazepines (PBDs) or indolinobenzodiazepine dimers (IGN) or PNU159,682. In an especially preferred embodiment, D is MMAE or exatecan.

The conjugate

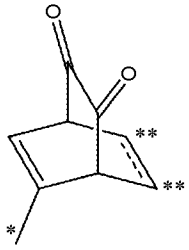
[0160] A further aspect of the invention concerns the conjugate that is obtainable by the process according to the invention. Alternatively, the conjugate according to the invention is defined as having a structure (**1a**) or (**1b**):

$Pr–[Z^1–L–(Q^2)_x]_y$ (**1a**)

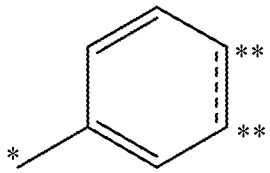
$Pr–[Z^1–L–(D)_x]_y$ (**1b**)

[0161] Herein:

- Pr is an *N*-glycoprotein;
- Z^1 is a connecting group comprising structure (Za) or (Zb):



(Za)



(Zb)

wherein the carbon labelled with * is directly connected to the peptide chain of the antibody at an amino acid located within 10 amino acids of an *N*-glycosylation site, which has been modified such that the glycoprotein does not contain a glycan longer than two monosaccharide residues within 10 amino acids of the amino acid residue, and both of the carbon atoms labelled with ** are connected to L, and the bond depicted as - - - is a single bond or a double bond;

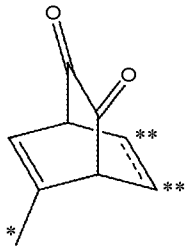
- L is a linker;
- x is an integer in the range of 1 – 4;
- y is an integer in the range of 1 – 4;
- Q^2 is a chemical handle that is reactive towards an appropriately functionalized payload;
- D is a payload.

[0162] The integer y denotes the number of tyrosine residues that are oxidized in step (b) and subsequently used as conjugation site in step (c) and optionally (d). Preferably, y = 1, 2 or 4, most preferably y = 2 or 4. The integer x denotes the number of chemical handles Q^2 or payloads D are connected to the linker. The linker may be linear, having only one occurrence of Q^2 or D connected to it, or may contain one or more branching points to connect up to 4 occurrences of Q^2 or D to the same connecting group Z^1. Preferably, x is 1 or 2. In case the compound according to structure (**3b**) is reacted in step (c), and in case the conjugate is according to structure (**1b**), it is preferred that x is 1 or 2, most preferably x = 2. In case the compound according to structure (**3a**) is reacted in step (c), and in case the conjugate is according to structure (**1a**), it is preferred that x is 1 or 2, most preferably x = 1.

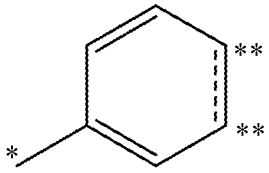
[0163] The glycoprotein Pr, linker L, payload D and chemical handle Q^2 are further defined above, which definitions equally apply to the conjugate according to the present aspect.

Connecting group Z^1

[0164] A connecting group, also referred to herein as Z^1, is formed upon reaction of step (c). Connecting group Z^1 covalently connects the glycoprotein with the compound as defined above, more in particular with chemical handle Q^2 or payload D. Connecting group Z^1 comprises structure (Za) or (Zb):



(Za)



(Zb)

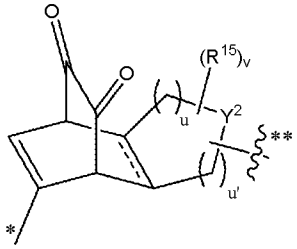
[0165] Herein, the carbon labelled with * is directly connected to the peptide chain of the antibody and both carbon atoms labelled with ** are connected to L. The bond depicted as ⎯ ⎯ ⎯ is a single bond or a double bond, and:

- L is a linker;
- x is an integer in the range of 1 – 4;
- Q^2 is a chemical handle that is reactive towards an appropriately functionalized payload;
- D is a payload

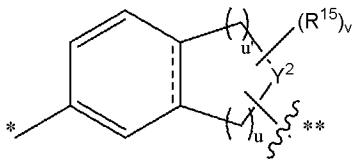
[0166] Connecting group Z^1 is formed by reaction of the *ortho*-quinone moiety with the (hetero)cycloalkene moiety, giving a single bond for ⎯ ⎯ ⎯, or the (hetero)cycloalkyne moiety, giving a double bond for ⎯ ⎯ ⎯. As the (hetero)alkene or (hetero)alkyne is present in a cyclic structure, both carbon atoms of the resulting C⎯ ⎯ ⎯C bond (labelled with **) will also be in a cyclic structure. In other words, both carbon atoms are connected to L via that cyclic structure. The carbon labelled with * originates from the exposed tyrosine residue and corresponds to the CH_2 moiety that connects the phenol moiety to the peptide main chain of the glycoprotein. In the connecting group, the CH_2 moiety labelled with * is thus directly connected to the peptide main chain.

[0167] In the [4+2] cycloaddition, the connecting group of structure (Za) is first formed. Depending on the conditions, this connecting group may eliminate two molecules of CO and *in situ* form the connecting group of structure (Zb). In the context of the present invention, the exact nature of the connecting group is irrelevant, as in any case it serves as a covalent attachment of Q^2 or D to the glycoprotein.

[0168] In one embodiment, Z^1 comprises a (hetero)cycloalkene moiety, i.e. is formed from Q^1 comprising a (hetero)cycloalkyne moiety. In an alternative embodiment, Z^1 comprises a (hetero)cycloalkane moiety, i.e. is formed from Q^1 comprising a (hetero)cycloalkene moiety. In a preferred embodiment, Z^1 has the structure (Z1a) or (Z1b):



(Z1a)



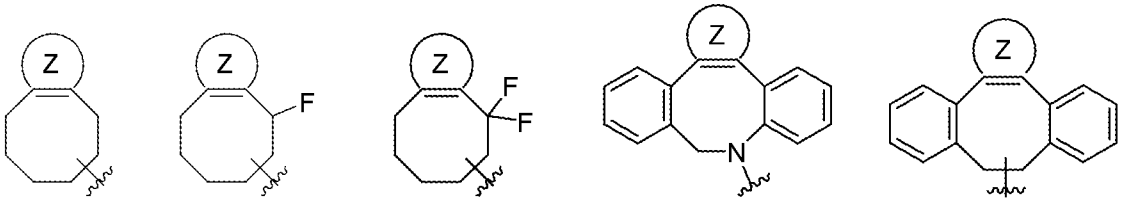
(Z1b)

[0169] Herein, the carbon labelled with * is directly connected to the peptide chain of the antibody and the bond labelled with ** is connected to L, and the bond depicted as ⎯ ⎯ ⎯ is a single bond or a double bond. Furthermore:

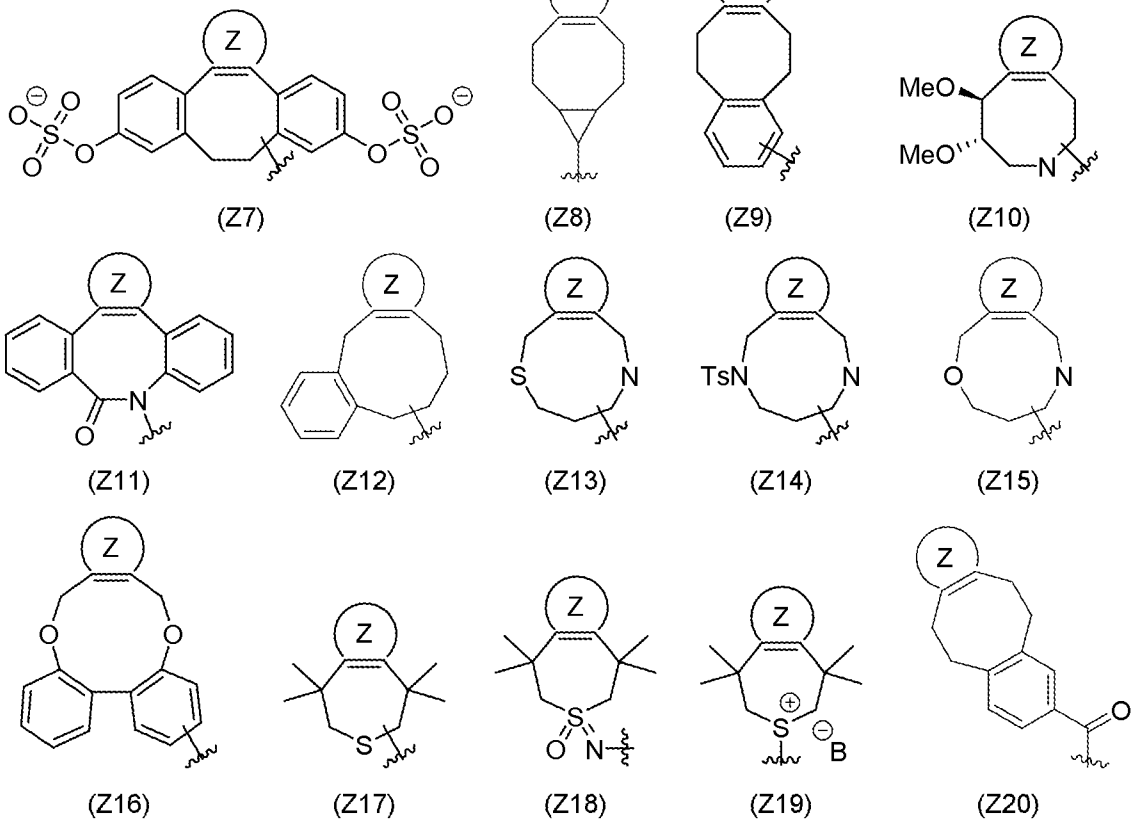
- R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, $-NO_2$, -CN, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$, $C_1 - C_{24}$ alkyl groups, $C_6 - C_{24}$ (hetero)aryl groups, $C_7 - C_{24}$ alkyl(hetero)aryl groups and $C_7 - C_{24}$ (hetero)arylalkyl groups and wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are optionally substituted, wherein two substituents R^{15} may be linked together to form an optionally substituted annulated cycloalkyl or an optionally substituted annulated (hetero)arene substituent, and wherein R^{16} is independently selected from the group consisting of hydrogen, halogen, $C_1 - C_{24}$ alkyl groups, $C_6 - C_{24}$ (hetero)aryl groups, $C_7 - C_{24}$ alkyl(hetero)aryl groups and $C_7 - C_{24}$ (hetero)arylalkyl groups;
- Y^2 is $C(R^{31})_2$, O, S, $S^{(+)}R^{31}$, $S(O)R^{31}$, $S(O)=NR^{31}$ or NR^{31}, wherein $S^{(+)}$ is a cationic sulphur atom counterbalanced by $B^{(-)}$, wherein $B^{(-)}$ is an anion, and wherein each R^{31} individually is R^{15} or a connection with Q^2 or D, connected via L;
- u is 0, 1, 2, 3, 4 or 5;
- u' is 0, 1, 2, 3, 4 or 5, wherein u + u' = 0, 1, 2, 3, 4, 5, 6, 7 or 8;
- v = an integer in the range 8 – 16.

[0170] In case the bond depicted as ⎯ ⎯ ⎯ is a double bond, it is preferred that u + u' = 4, 5, 6, 7 or 8.

[0171] It is especially preferred that Z^1 comprises a (hetero)cycloalkene moiety, i.e. the bond depicted as ⎯ ⎯ ⎯ is a double bond. In a preferred embodiment, Z^1 is selected from the structures (Z2) – (Z20), depicted here below:



(Z2) (Z3) (Z4) (Z5) (Z6)



(Z7) (Z8) (Z9) (Z10)

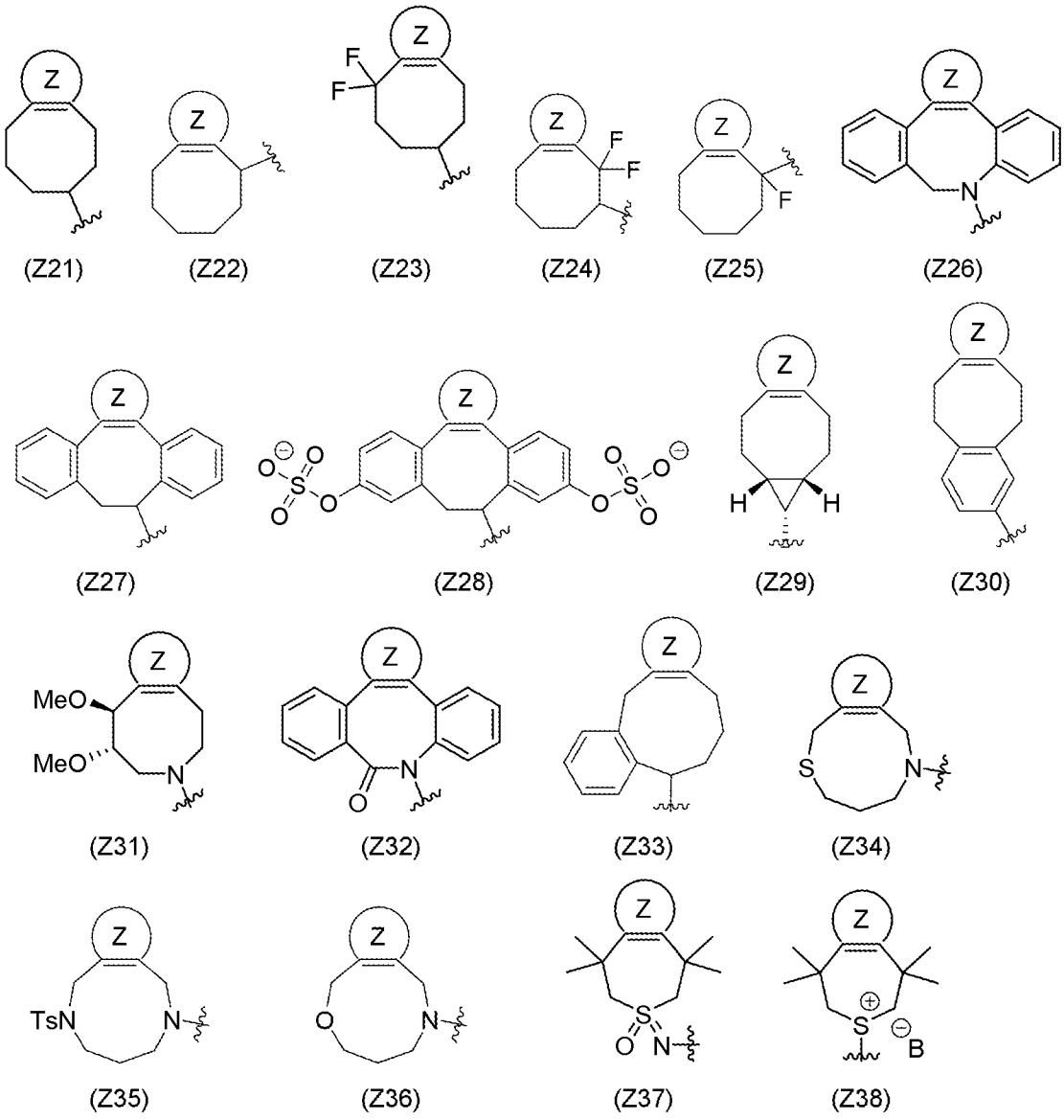
(Z11) (Z12) (Z13) (Z14) (Z15)

(Z16) (Z17) (Z18) (Z19) (Z20)

[0172] Herein, the connection to L is depicted with the wavy bond. $B^{(-)}$ is an anion, preferably a pharmaceutically acceptable anion. Ring Z is either of structure (Za) or structure (Zb), wherein the carbon atoms labelled with ** correspond to the two carbon atoms of the (hetero)cycloalkane ring of (Z2) – (Z20) to which ring Z is fused, and the carbon a carbon labelled with * is directly connected to the peptide chain of the antibody. Since the connecting group Z is formed by reaction with a (hetero)cycloalkyne in the context of the present embodiment, the bound depicted above as ⎯ ⎯ ⎯ is a double bond.

(Za) (Zb)

[0173] In a further preferred embodiment, Z^1 is selected from the structures (Z21) – (Z38), depicted here below:



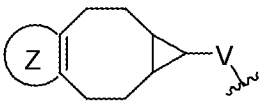
[0174] Herein, the connection to L is depicted with the wavy bond. In structure (Z38), $B^{(-)}$ is an anion, preferably a pharmaceutically acceptable anion. Ring Z is either of structure (Za) or structure (Zb), as defined above.

[0175] In a preferred embodiment, Z^1 comprises a (hetero)cyclooctene moiety according to structure (Z8), more preferably according to (Z29), which is optionally substituted. In the context of the present embodiment, Z^1 preferably comprises a (hetero)cyclooctene moiety according to structure (Z39) as shown below, wherein V is $(CH_2)_l$ and l is an integer in the range of 0 to 10, preferably in the range of 0 to 6. More preferably, l is 0, 1, 2, 3 or 4, more preferably l is 0, 1 or 2 and most preferably l is 0 or 1. In the context of group (Z39), l is most preferably 1. Most preferably, Z^1 is according to structure (Z42), defined further below.

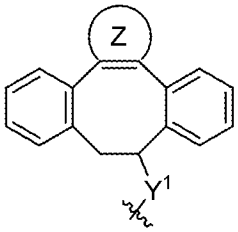
[0176] In an alternative preferred embodiment, Z^1 comprises a (hetero)cyclooctene moiety according to structure (Z26), (Z27) or (Z28), which are optionally substituted. In the context of the present embodiment, Z^1 preferably comprises a (hetero)cyclooctene moiety according to structure (Z40) or (Z41) as shown below, wherein Y^1 is O or NR^{11}, wherein R^{11} is independently selected

from the group consisting of hydrogen, a linear or branched C_1 - C_{12} alkyl group or a C_4 - C_{12} (hetero)aryl group. The aromatic rings in (Z40) are optionally O-sulfonylated at one or more positions, whereas the rings of (Z41) may be halogenated at one or more positions. Most preferably, Z^1 is according to structure (Z43), defined further below.

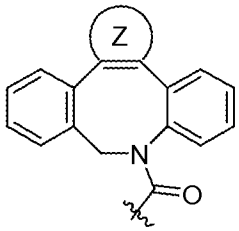
[0177] In an alternative preferred embodiment, Z^1 comprises a heterocycloheptenyl group and is according to structure (Z37).



(Z39)

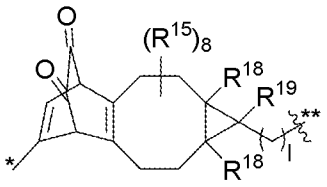


(Z40)

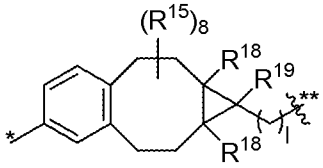


(Z41)

[0178] In an especially preferred embodiment, Z^1 comprises a cyclooctynyl group and is according to structure (Z42a) or (Z42b):



(Z42a)



(Z42b)

Herein:

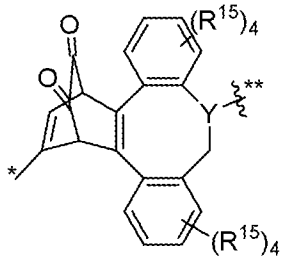
- the carbon labelled with * is directly connected to the peptide chain of the antibody and the wavy bond labelled with ** is connected to L;
- R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, $-NO_2$, -CN, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$,C_1 – C_{24} alkyl groups, C_5 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups and wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are optionally substituted, wherein two substituents R^{15} may be linked together to form an optionally substituted annulated cycloalkyl or an optionally substituted annulated (hetero)arene substituent, and wherein R^{16} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups;
- R^{18} is independently selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups;
- R^{19} is selected from the group consisting of hydrogen, halogen, C_1 – C_{24} alkyl groups, C_6 – C_{24} (hetero)aryl groups, C_7 – C_{24} alkyl(hetero)aryl groups and C_7 – C_{24} (hetero)arylalkyl groups, the alkyl groups optionally being interrupted by one of more hetero-atoms selected from the group consisting of O, N and S, wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are independently optionally substituted, or R^{19} is a second

occurrence of Q^1 or D connected via a spacer moiety; and

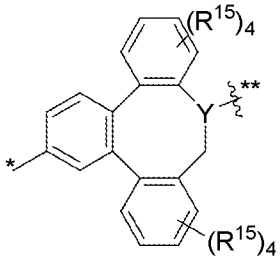
- l is an integer in the range 0 to 10.

[0179] In a preferred embodiment of the reactive group according to structure (Z42a) or (Z42b), R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, $C_1 - C_6$ alkyl groups, $C_5 - C_6$ (hetero)aryl groups, wherein R^{16} is hydrogen or $C_1 - C_6$ alkyl, more preferably R^{15} is independently selected from the group consisting of hydrogen and $C_1 - C_6$ alkyl, most preferably all R^{15} are H. In a preferred embodiment of the reactive group according to structure (Z42a) or (Z42b), R^{18} is independently selected from the group consisting of hydrogen, $C_1 - C_6$ alkyl groups, most preferably both R^{18} are H. In a preferred embodiment of the reactive group according to structure (Z42a) or (Z42b), R^{19} is H. In a preferred embodiment of the reactive group according to structure (Z42a) or (Z42b), l is 0 or 1, more preferably l is 1.

[0180] In an especially preferred embodiment, Q^1 comprises a (hetero)cyclooctynyl group and is according to structure (Z43a) or (Z43b):



(Z43a)



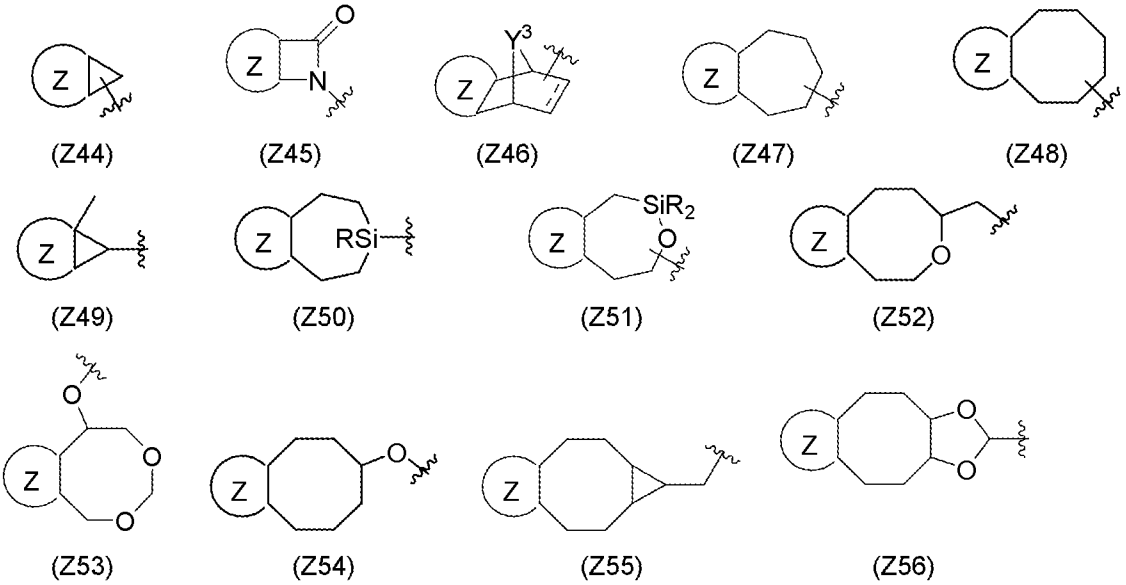
(Z43b)

Herein:

- the carbon labelled with * is directly connected to the peptide chain of the antibody and the wavy bond labelled with ** is connected to L;
- R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, $-NO_2$, $-CN$, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$, $C_1 - C_{24}$ alkyl groups, $C_5 - C_{24}$ (hetero)aryl groups, $C_7 - C_{24}$ alkyl(hetero)aryl groups and $C_7 - C_{24}$ (hetero)arylalkyl groups and wherein the alkyl groups, (hetero)aryl groups, alkyl(hetero)aryl groups and (hetero)arylalkyl groups are optionally substituted, wherein two substituents R^{15} may be linked together to form an optionally substituted annulated cycloalkyl or an optionally substituted annulated (hetero)arene substituent, and wherein R^{16} is independently selected from the group consisting of hydrogen, halogen, $C_1 - C_{24}$ alkyl groups, $C_6 - C_{24}$ (hetero)aryl groups, $C_7 - C_{24}$ alkyl(hetero)aryl groups and $C_7 - C_{24}$ (hetero)arylalkyl groups;
- Y is N or CR^{15}.

[0181] In a preferred embodiment of the reactive group according to structure (Z43a) or (Z43b), R^{15} is independently selected from the group consisting of hydrogen, halogen, $-OR^{16}$, $-S(O)_3^{(-)}$, $C_1 - C_6$ alkyl groups, $C_5 - C_6$ (hetero)aryl groups, wherein R^{16} is hydrogen or $C_1 - C_6$ alkyl, more preferably R^{15} is independently selected from the group consisting of hydrogen and $-S(O)_3^{(-)}$. In a preferred embodiment of the reactive group according to structure (Z43a) or (Z43b), Y is N or CH, more preferably Y = N.

[0182] In an alternative preferred embodiment, Z^1 comprises a (hetero)cycloalkane moiety, i.e. the bond depicted as ⎯ ⎯ ⎯ is a single bond. The (hetero)cycloalkane group may also be referred to as a heterocycloalkanyl group or a cycloalkanyl group, preferably a cycloalkanyl group, wherein the (hetero)cycloalkanyl group is optionally substituted. Preferably, the (hetero)cycloalkanyl group is a (hetero)cyclopropanyl group, a (hetero)cyclobutanyl group, a norbornane group, a norbornene group, a (hetero)cycloheptanyl group, a (hetero)cyclooctanyl group, a (hetero)cyclononnyl group or a (hetero)cyclodecanyl group, which may all optionally be substituted. Especially preferred are (hetero)cyclopropanyl groups, (hetero)cycloheptanyl group or (hetero)cyclooctanyl groups, wherein the (hetero)cyclopropanyl group, the *trans*-(hetero)cycloheptanyl group or the (hetero)cyclooctanyl group is optionally substituted. Preferably, Z^1 comprises a cyclopropanyl moiety according to structure (Z44), a hetereocyclobutane moiety according to structure (Z45), a norbornane or norbornene group according to structure (Z46), a (hetero)cycloheptanyl moiety according to structure (Z47) or a (hetero)cyclooctanyl moiety according to structure (Z48). Herein, Y^3 is selected from $C(R^{24})_2$, NR^{24} or O, wherein each R^{24} is individually hydrogen, C_1 – C_6 alkyl or is connected to L, optionally via a spacer, and the bond labelled ⎯ ⎯ ⎯ is a single or double bond. In a further preferred embodiment, the cyclopropanyl group is according to structure (Z49). In another preferred embodiment, the (hetero)cycloheptane group is according to structure (Z50) or (Z51). In another preferred embodiment, the (hetero)cyclooctane group is according to structure (Z52), (Z53), (Z54), (Z55) or (Z56).

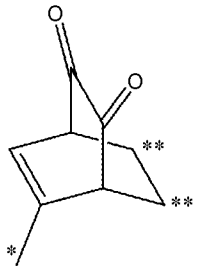


(Z44) (Z45) (Z46) (Z47) (Z48)

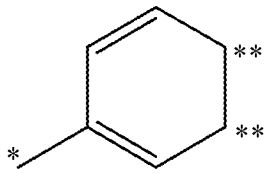
(Z49) (Z50) (Z51) (Z52)

(Z53) (Z54) (Z55) (Z56)

[0183] Herein, the R group(s) on Si in (Z50) and (Z51) are typically alkyl or aryl, preferably C_1-C_6 alkyl. Ring Z is either of structure (Za) or structure (Zb), wherein the carbon atoms labelled with ** correspond to the two carbon atoms of the (hetero)cycloalkane ring of (Z44) – (Z56) to which ring Z is fused, and the carbon a carbon labelled with * is directly connected to the peptide chain of the antibody. Since the connecting group Z is formed by reaction with a (hetero)cycloalkene in the context of the present embodiment, the bound depicted above as ⎯ ⎯ ⎯ is a single bond.



(Za)

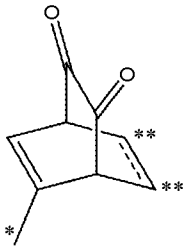


(Zb)

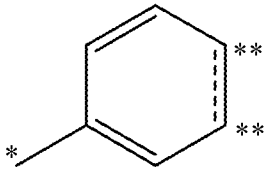
[0184] In an alternative preferred embodiment, Z^1 comprises a (hetero)cycloalkane group or a (hetero)cycloalkane group formed by conjugation reaction of the *ortho*-quinone and a chemical handle selected from the structures depicted in Figures 1 and 2.

[0185] In an alternative aspect of the present invention, the glycoprotein-conjugate has structure (**1a**) or (**1b**), wherein:

- Pr is a protein;
- Z^1 comprises structure (Za) or (Zb):



(Za)



(Zb)

wherein the carbon labelled with * is directly connected to the peptide chain of the glycoprotein at an amino acid which is in the native form of the protein not a tyrosine residue and both of the carbon atoms labelled with ** are connected to L, and the bond depicted as - - - is a single bond or a double bond.

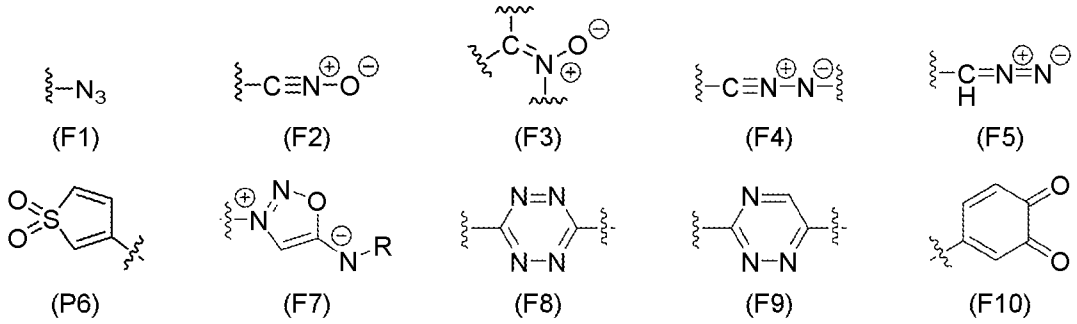
[0186] Z^1, L, x, y, Q^2 and D are as further defined elsewhere.

[0187] The protein is a mutant protein, which is in its native form unreactive towards oxidative enzymes capable of oxidizing tyrosine, but is rendered reactive towards such enzyme by providing a mutated form of the protein, wherein a tyrosine residue is introduced at a non-native position in a position of the amino acid sequence of the protein where it is reactive towards oxidative enzymes capable of oxidizing tyrosine. Thus, the amino acid to which the connecting group Z^1 is connected is located at a position where a tyrosine residue is reactive towards oxidative enzymes capable of oxidizing tyrosine. Typically, the protein has undergone a point mutation to introduce the tyrosine residue at the desired location.

[0188] Also part of the present invention is a process for preparing a conjugate according to structure (**1b**), comprising reacting a conjugate according to structure (**1a**) with a with a payload having structure D–F^2 or D–L^2–$(F^2)_x$, wherein F^2 is reactive towards Q^2 in a conjugation reaction. Herein, L^2 is a linker and x an integer in the range of 1 – 4. In the context of the present invention,

this payload may also be referred to as functionalized payload. This conjugation reaction corresponds to step (d) defined above, and everything defined for step (d) equally applies to the process according to the present aspect, and vice versa.

[0189] The functionalized payload is contacted with the conjugate according to structure (**1a**). Herein, F^2 is reactive towards Q^2 in a conjugation reaction, preferably a cycloaddition. Preferably, F^2 is reactive towards a (hetero)cycloalkene and/or a (hetero)cycloalkyne, and is typically selected from the group consisting of azide, tetrazine, triazine, nitrone, nitrile oxide, nitrile imine, diazo compound, *ortho*-quinone, dioxothiophene and sydnone. Preferred structures for the reactive group are structures (F1) – (F10) depicted here below.



[0190] Herein, the wavy bond represents the connection to the payload. For (F3), (F4), (F8) and (F9), the payload can be connected to any one of the wavy bonds. The other wavy bond may then be connected to an R group selected from hydrogen, C_1 – C_{24} alkyl groups, C_2 – C_{24} acyl groups, C_3 – C_{24} cycloalkyl groups, C_2 – C_{24} (hetero)aryl groups, C_3 – C_{24} alkyl(hetero)aryl groups, C_3 – C_{24} (hetero)arylalkyl groups and C_1 – C_{24} sulfonyl groups, each of which (except hydrogen) may optionally be substituted and optionally interrupted by one or more heteroatoms selected from O, S and NR^{32} wherein R^{32} is independently selected from the group consisting of hydrogen and C_1 – C_4 alkyl groups. The skilled person understands which R groups may be applied for each of the groups F. For example, the R group connected to the nitrogen atom of (F3) may be selected from alkyl and aryl, and the R group connected to the carbon atom of (F3) may be selected from hydrogen, alkyl, aryl, acyl and sulfonyl. Preferably, F^2 is selected from azides or tetrazines. Most preferably, F^2 is an azide.

Application

[0191] The conjugates according to structure (**1b**) are especially suitable in the treatment of cancer. By virtue of the lack of an *N*-glycan, the conjugates according to structure (**1b**) will no longer be able to bind to Fc-gamma receptors, and therefore are highly effective in the treatment of cancer. The invention thus further concerns the use of the conjugate according to structure (**1b**) in medicine, preferably in the treatment of cancer. In a further aspect, the invention also concerns a method of treating a subject in need thereof, comprising administering the conjugate according to structure (**1b**) to the subject. The method according to this aspect can also be worded as the conjugate according to structure (**1b**) for use in treatment, in particular for use in the treatment of a subject in need thereof. The method according to this aspect can also be worded as use of the conjugate

according to structure (**1b**) for the manufacture of a medicament. Herein, administration typically occurs with a therapeutically effective amount of the conjugate according to structure (**1b**).

[0192] The invention further concerns a method for the treatment of a specific disease in a subject in need thereof, comprising the administration of the conjugate according to the invention as defined above. Typically, the specific disease is cancer and the subject in need thereof is a cancer patient. The use of antibody-drug conjugates is well-known in cancer treatment, and the conjugates according to structure (**1b**) are especially suited in this respect. In the method according to this aspect, the conjugate is typically administered in a therapeutically effective amount. The present aspect of the invention can also be worded as a conjugate according to structure (**1b**) for use in the treatment of a specific disease in a subject in need thereof, preferably for the treatment of cancer. In other words, this aspect concerns the use of a conjugate according to structure (**1b**) for the preparation of a medicament or pharmaceutical composition for use in the treatment of a specific disease in a subject in need thereof, preferably for use in the treatment of cancer.

[0193] Administration in the context of the present invention refers to systemic administration. Hence, in one embodiment, the methods defined herein are for systemic administration of the conjugate. In view of the specificity of the conjugates, they can be systemically administered, and yet exert their activity in or near the tissue of interest (e.g. a tumour). Systemic administration has a great advantage over local administration, as the drug may also reach tumour metastasis not detectable with imaging techniques and it may be applicable to hematological tumours.

[0194] The invention further concerns a pharmaceutical composition comprising the conjugate according to structure (**1b**) and a pharmaceutically acceptable carrier.

Examples

[0195] The invention is illustrated by the following examples.

General reagents and analytics

[0196] Solvents were purchased from Sigma-Aldrich or Fisher Scientific and used as received. Thin layer chromatography was performed on silica gel-coated plates (Kieselgel 60 F254, Merck, Germany) with the indicated solvent mixture, spots were detected by KMnO4 staining (1.5 g $KMnO_4$, 10 g K_2CO_3, 2.5 mL 5% NaOH-solution, 150 mL H_2O), p-anisaldehyde staining (9.2 mL p-anisaldehyde, 321 mL EtOH, 17 mL H2O, 3.75 mL AcOH, 12.7 mL H_2SO_4), and UV-detection. NMR spectra were recorded on a Bruker Biospin 400 (400 MHz) and a Bruker DMX300 (300 MHz). Protein mass spectra (HRMS) were recorded on a JEOL AccuTOF JMS-T100CS (Electrospray Ionization (ESI) time-of-flight) or a JEOL AccuTOF JMS-100GCv (Electron Ionization (EI), Chemical Ionization (CI)). Low-resolution mass spectra (LRMS) were recorded on a ThermoScientific Advantage LCQ Linear ion-trap electrospray and a Waters LCMS consisting of a 2767 Sample manager, 2525 pump, 2996 UV-detector and a Micromass ZQ with an Xbridge™ C18 3.5 µm column (ESI).

[0197] Trastuzumab (Herzuma) and cetuximab (Cerbitux) were obtained from the pharmacy. PNGase F was obtained from New England Biolabs (NEB). Compound **2** (structure in Figure 9) was

obtained from ClickChemistryTools (https://clickchemistrytools.com/product/tamra-dbco/). Compound **3** (structure in Figure 9) was obtained from ClickChemistryTools (https://clickchemistrytools.com/product/af-568-tco). Compounds **4** (structure in Figure 9) and **9d** were custom synthesized by Eurogentec (www.eurogentec.com). Compound **9a** was obtained from Broadpharm: https://broadpharm.com/web/product.php?catalog=BP-22443. Compounds **9b** and **9c** were prepared according to Bruins et al., ACS Omega **2019**, 4, 11801–11807, incorporated by reference. Human IgG2 was purchased from Abcam (https://www.abcam.com/native-human-igg2-protein-ab90284.html?productWallTab=ShowAll#top-200). Mouse IgG1 was purchased from Abcam (https://www.abcam.com/mouse-igg1-kappa-monoclonal-mopc-21-isotype-control-ab18443.html).

General procedure for reducing SDS-PAGE, Coomassie staining and fluorescence detection

[0198] 12% acrylamide gels were prepared according to BIO-RAD bulletin 6201 protocol. 5 µL 1 mg/mL antibody solution was diluted with 5 µL 2x sample buffer including 5% 2-mercaptoethanol and heated to 95 °C for 5 minutes. After loading the samples, the gel was run using a BIO-RAD Mini-PROTEAN Tetra Vertical Electrophoresis Cell at 150 volts until completion.

[0199] Fluorescently labelled proteins were analysed prior to staining using a BioRad ChemiDoc™ system. Subsequently, the gel was stained using staining solution, containing 1 g/L Coomassie Brilliant Blue R-250 in 5:4:1 (v/v/v) methanol:water:acetic acid, for 30 minutes. The gel was subsequently destained using 5:4:1 (v/v/v) methanol:water:acetic acid for 60 minutes, after which it was further destained overnight using demineralized water.

General procedure for generation of Fc/2 fragments

[0200] A solution of 20 µg of (modified) IgG was incubated for 1 hour at 37 °C with IdeS/Fabricator™ (1.25 U/µL) in PBS pH 6.6 in a total volume of 10 µL.

General procedure for analytical RP-HPLC

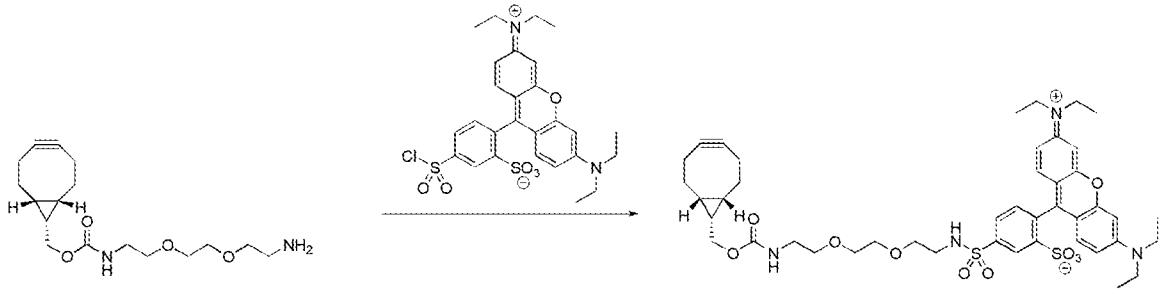
[0201] Prior to RP-HPLC analysis, IgG (10 µL, 1 mg/mL in PBS pH 7.4) was added to 12.5 mM DTT, 100 mM TrisHCl pH 8.0 (40 µL) and incubated for 15 minutes at 37 °C. The reaction was quenched by adding 49% acetonitrile, 49% water, 2% formic acid (50 µL). RP-HPLC analysis was performed on an Agilent 1100 series (Hewlett Packard). The sample (10 µL) was injected with 0.5 mL/min onto Bioresolve RP mAb 2.1*150 mm 2.7 µm (Waters) with a column temperature of 70 °C. A linear gradient was applied in 16.8 minutes from 30 to 54% acetonitrile in 0.1% TFA and water.

General procedure for analytical SEC

[0202] HPLC-SEC analysis was performed on an Agilent 1100 series (Hewlett Packard) using an Xbridge BEH200A (3.5 µM, 7.8x300 mm, PN 186007640 Waters) column. The sample was diluted to 1 mg/mL in PBS and measured with 0.86 mL/min isocratic method (0.1 M sodium phosphate buffer pH 6.9 ($NaHPO_4/Na_2PO_4$) containing 10% isopropanol) for 16 minutes.

General procedure for analytical MS analysis

[0203] Prior to mass spectral analysis, IgG was treated with IdeS, which allows analysis of the Fc/2 fragment. For analysis of the Fc/2 fragment, a solution of 20 µg (modified) IgG was incubated for 1 hour at 37 °C with IdeS/Fabricator™ (1.25 U/µL) in PBS pH 6.6 in a total volume of 10 µL. Samples were diluted to 80 µL followed by analysis electrospray ionization time-of-flight (ESI-TOF) on a JEOL AccuTOF. Deconvoluted spectra were obtained using Magtran software.



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Example 1. Synthesis of BCN-lissamine compound **1**

[0204] Compound **1** was prepared by sulfonylation of BCN-diethyleneglycol-NH_2 (prepared as described for compound **24** in WO2014065661, example 1) with commercially available sulforhodamine B acid chloride (https://www.sigmaaldrich.com/catalog/product/sigma/86186).

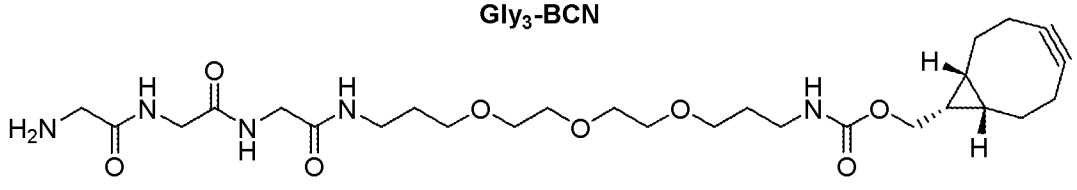
Example 2. Preparation of BCN-scFv conjugate **5**

[0205] BCN-UCHT1 conjugate was prepared according to Bartels *et al.*, *Methods* **2019**, *154*, 93–101, incorporated by reference. Thus, 1 eq. of UCHT1-G_4SLPETGGH$_6$ (see sequence below) was incubated with 1 eq. sortase A and 30 eq of Gly_3-BCN tag (Figure). Typical conditions: To 100 µL 1.86 mg/mL UCHT1-G_4SLPETGGH$_6$ in TBS pH 8.0 was added 10 µL 17 mg/mL sortase A in TBS pH 8.0 (1 eq.), 13.6 µL 100 mM $CaCl_2$ in TBS pH 8.0, Gly_3-BCN in DMSO (4 µL 50 mM, 30 eq.), and 9.6 µL DMSO (10% final concentration), incubation overnight at 37 °C. Unreacted UCHT1-G_4SLPETGGH$_6$ was removed by Ni-NTA column, and subsequent SEC-column yielded pure conjugate.

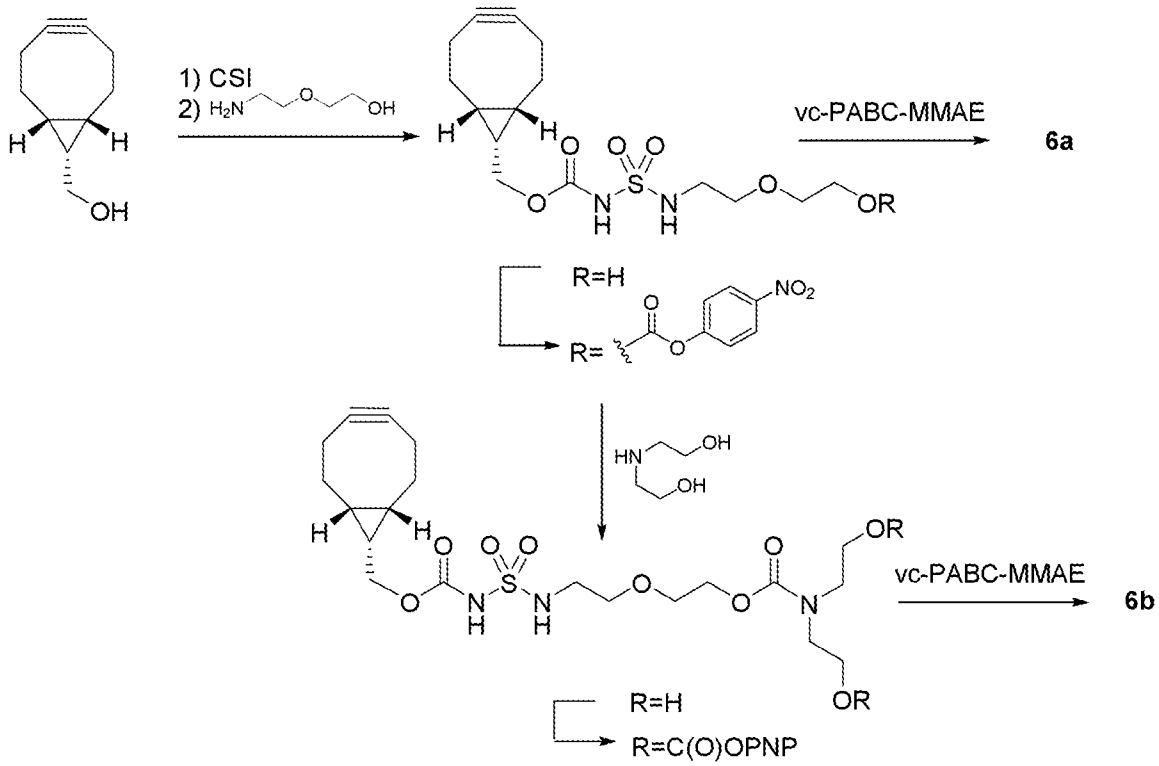
[0206] UCHT1 sequence:

VQLQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQSHGKNLEWMGLINPYKGVSTYNQKF
KDKATLTVDKSSSTAYMELLSLTSEDSAVYYCARSGYYGDSDWYFDVWGAGTTVTVSSGGGSG
GGSGGGSGGGSDIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKPDGTVKLLIYYTSR
LHSGVPSKFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPWTFAGGTKLEIKRAGGGGSLPET
GGHHHHHH

Gly_3-BCN



Molecular Weight: 567,68



[0207] Chemical structures of **6a**, **6b** and **7** are depicted in Figure 10.

Example 3a. Synthesis of BCN-MMAE compound **6a**

[0208] Compound **6a** (prepared according to procedure described by Verkade *et al., Antibodies* **2018**, *7*, doi:10.3390/antib7010012, incorporated by reference). To a solution of BCN alcohol (1.5 g, 10 mmol) in DCM (150 mL), under a N_2 atmosphere, was added CSI (0.87 mL, 1.4 g, 10 mmol), Et_3N (2.8 mL, 2.0 g, 20 mmol) and 2-(2-aminoethoxy)ethanol (1.2 mL, 1.26 g, 12 mmol). The mixture was stirred for 10 min and quenched through addition of aqueous NH_4Cl (sat., 150 mL). After separation, the aqueous layers was extracted with DCM (150 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. The residue was purified with column chromatography. The product alcohol was obtained as slightly yellow thick oil (2.06 g, 5.72 mmol, 57%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 6.0 (bs, 1H), 4.28 (d, *J* = 8.2 Hz, 2H), 3.78–3.73 (m, 2H), 3.66–3.61 (m, 2H), 3.61–3.55 (m, 2H), 3.34 (t, J = 4.9 Hz, 2H), 2.37–2.15 (m, 6H), 1.64–1.48 (m, 2H), 1.40 (quintet, *J* = 8.7 Hz, 1H), 1.05–0.92 (m, 2H).

[0209] To a solution of the alcohol prepared above (229 mg, 0.64 mmol) in DCM (20 mL) were added *p*-nitrophenyl chloroformate (128 mg, 0.64 mmol) and Et_3N (268 µL, 194 mg, 1.92 mmol). The mixture was stirred overnight at rt and subsequently concentrated under reduced pressure. The residue was purified via gradient column chromatography (20 → 70% EtOAc in heptane (1% AcOH) to afford the PNP carbonate derivative as a white solid (206 mg, 0.39 mmol, 61%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 8.31–8.26 (m, 2H), 7.45–7.40 (m, 2H), 5.56 (t, *J* = 6.0 Hz, 1H), 4.48–4.40 (m, 2H), 4.27 (d, *J* = 8.2 Hz, 2H), 3.81–3.75 (m, 2H), 3.68 (t, *J* = 5.0 Hz, 2H), 3.38–3.30 (m, 2H), 2.36–2.14 (m, 6H), 1.61–1.45 (m, 2H), 1.38 (quintet, *J* = 8.7 Hz, 1H), 1.04–0.94 (m, 2H).

[0210] To a solution of the PNP carbonate prepared above (4.7 mg, 9.0 µmol) in DMF (200 µL) was added solid H-Val-Cit-PABC-MMAE (vc-PABC-MMAE, 10 mg, 8.1 µmol) followed by addition

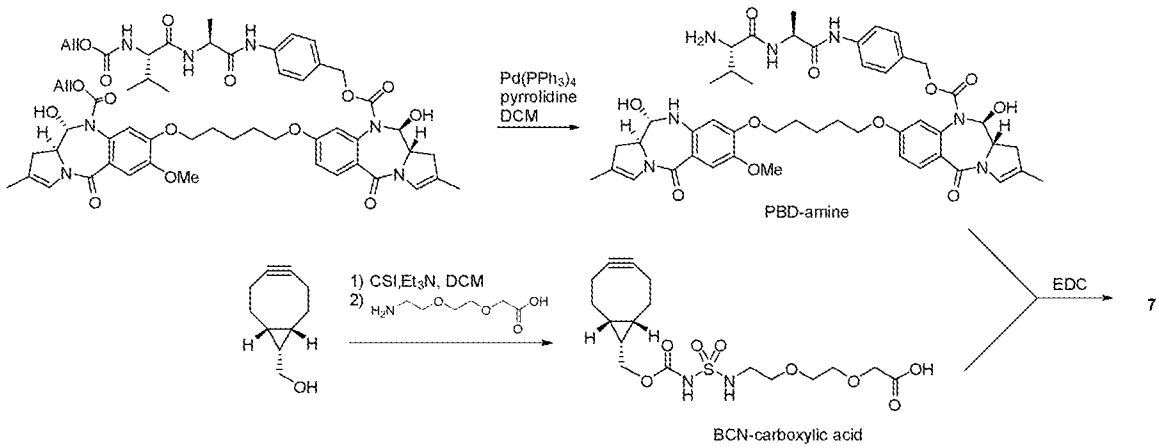
of Et_3N (3.7 µL, 2.7 mg, 27 µmol). After 23 h, 2'-(ethylenedioxy)bis(ethylamine) (1.3 µL, 1.3 mg, 8.9 µmol) in DMF was added (13 µL of 10% solution in DMF). The mixture was left for 4h and purified *via* reversed phase (C18) HPLC chromatography (30→90% MeCN (1% AcOH) in H_2O (1 % AcOH). The product **6a** was obtained as a colourless film (10.7 mg, 7.1 µmol, 87%) LCMS (ESI^+) calculated for $C_{74}H_{117}N_{12}O_{19}S^+$ ($M+H^+$) 1509.83 found 1510.59.

Example 3b. Synthesis of BCN-MMAE compound **6b**

[0211] Compound **6b** (prepared according to procedure for compound **7** described by Verkade *et al., Antibodies* **2018**, *7*, doi:10.3390/antib7010012, incorporated by reference). To solution of the PNP carbonate prepared in the synthesis of **6a** (0.39 g; 0.734 mmol) in DCM (30 mL) were added a solution of diethanolamine (DEA, 107 mg; 1.02 mmol) in DMF (2 mL) and Et_3N (305 µL; 221 mg; 2.19 mmol). The resulting mixture was stirred at rt for 17 h and washed with a saturated aqueous solution of NH_4Cl (30 mL). The aqueous phase was extracted with DCM (30 mL) and the combined organic layers were dried (Na_2SO_4) and concentrated. The residue was purified by flash column chromatography (DCM → MeOH/DCM 1/9). The product diol was obtained as a colourless film (163 mg; 0.33 mmol; 45%). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 6.29 (bs, 1H), 4.33–4.29 (m, 2H), 4.28 (d, *J* = 8.2 Hz, 2H), 3.90–3.80 (m, 4H), 3.69–3.64 (m, 2H), 3.61 (t, *J* = 4.8 Hz, 2H), 3.52 (t, *J* = 5.0 Hz, 4H), 3.32 (t, *J* = 5.1 Hz, 2H), 2.37–2.18 (m, 6H), 1.60–1.55 (m, 2H), 1.39 (quintet, J = 8.7 Hz, 1H), 1.05–0.94 (m, 2H).

[0212] To a solution of the diol prepared above (163 mg, 0.33 mmol) and 4-nitrophenyl chloroformate (134 mg, 0.66 mmol) in DCM (10 mL) was added Et_3N (230 µL; 167 mg; 1.65 mmol). The reaction mixture was stirred for 17 h and concentrated. The residue was purified by flash column chromatography (50% EtOAc in heptane → 100% EtOAc). The product was obtained as a colourless oil (69 mg; 0.084 mmol; 25%). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 8.29–8.23 (m, 4H), 7.42–7.35 (m, 4H), 5.81–5.71 (m, 1H), 4.53–4.43 (m, 4H), 4.36–4.30 (m, 2H), 4.25 (d, J = 8.2 Hz, 2H), 3.81–3.70 (m, 4H), 3.70–3.65 (m, 2H), 3.62–3.56 (m, 2H), 3.32–3.24 (m, 2H), 2.34–2.14 (m, 6H), 1.60–1.45 (m, 2H), 1.35 (quintet, *J* = 8.7 Hz, 1H), 1.02–0.91 (m, 2H).

[0213] To a solution of bis PNP-carbonate (27 mg, 33 µmol) in DMF (400 µL) were added triethylamine (22 µl; 16 mg; 158 µmol) and a solution of vc-PABC-MMAE.TFA (96 mg; 78 µmol) in DMF (1.0 mL). The mixture was left standing for 19 h and 2,2'-(ethylenedioxy)bis(ethylamine) (37 µL, 38 mg, 253 µmol) was added. After 2 h, the reaction mixture was diluted with DMF (100 µL) and purified by RP HPLC (C18, 30% → 90% MeCN (1% AcOH) in water (1% AcOH). The desired product **6b** was obtained as a colourless film (41 mg, 14.7 µmol, 45%). LCMS (ESI^+) calculated for $C_{138}H_{219}N_{23}O_{35}S^{2+}$ ($M+2H^+$) 1395.79 found 1396.31.



Example 4. Preparation of BCN-PBD compound **7**

[0214] Compound **7** (prepared according to procedure described for compound **130** in WO2017137456, incorporated by reference).

Preparation of BCN-carboxylic acid

[0215] A solution of BCN alcohol (0.384 g, 2.55 mmole) in MeCN (25 mL) under a N_2 atmosphere was cooled to 0 °C, and chlorosulfonyl isocyanate was added (CSI) was added dropwise (0.255 mL, 415 mg, 2.93 mmole, 1.15 equiv.). After stirring for 15 minutes, Et_3N was added dropwise (1.42 mL, 1.03 g, 10.2 mmole, 4 equiv.) and stirring was continued for another 10 minutes. Next, a solution of 2-(2-(2-aminoethoxy)ethoxy)acetic acid (1.0 g, 6.1 mmole, 2.4 equiv.) in H_2O (5 mL) was added and the reaction mixture was stirred to room temperature for 2 h. After this time, $CHCl_3$ (50 mL) and H_2O (100 mL) were added, and the layers were separated. To the aqueous layer in a separatory funnel was added CH_2Cl_2 (100 mL) and the pH was adjusted to 4 with 1 N HCl, before separation of layers. The water layer was extracted twice with CH_2Cl_2 (2 × 100 mL), the organic layers were combined and dried (Na_2SO_4), filtered and concentrated. The residue was purified by flask column chromatography on silica, elution with CH_2Cl_2 to 20% MeOH in CH_2Cl_2. Yield 0.42 g (1.0 mmole, 39%) of BCN-carboxylic acid as a colorless sticky wax.

Preparation of PBD-amine

[0216] Palladium tetrakistriphenylphosphine $Pd(PPh_3)_4$ (4.8 mg, 4.15 µmol) is weighed and put under an atmosphere of N_2. A solution of pyrrolidine (5.0 µL, 4.3 mg, 60 µmol) in DCM (1 mL) is degassed by bubbling N_2 through the solution. A solution of Alloc-protected PBD amine (27 mg, 24 µmol) in DCM (6 mL) is degassed by bubbling N_2 through the solution. While N_2 is still bubbled through the solution, the degassed solution of pyrrolidine is added. The weighed $Pd(PPh_3)_4$ is dissolved in CH_2Cl_2 (1 mL) and 0.9 mL of this solution is added. After 50 min of bubbling of N_2, CH_2Cl_2 (25 mL) is added and the mixture is washed with aqueous saturated NH_4Cl (25 mL). After separation, the aqueous layer is extracted with CH_2Cl_2 (2 × 25 mL). The combined organic layers are dried (Na_2SO_4) and concentrated. The residue is purified by RP-HPLC (30–90% MeCN (0.1% formic acid) in H_2O (0.1% formic acid). The combined fractions are passed through SPE (HCO_3^-)

columns and concentrated. After addition of MeCN (50 mL) the mixture is again concentrated. The resulting residue is used in the next step.

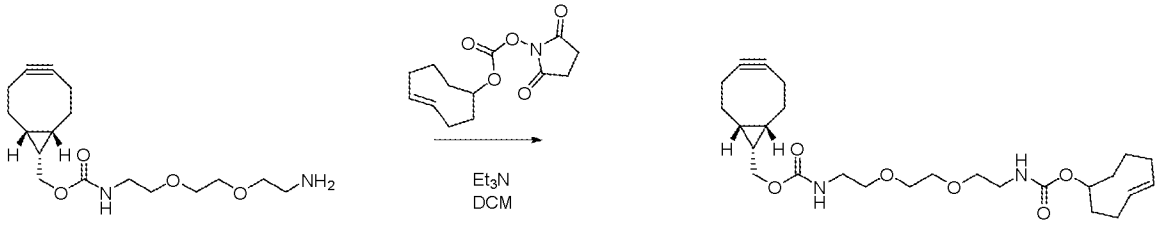
Preparation of BCN-PBD compound ***7***

[0217] To a solution of PBD-amine in $CHCl_3$ (5 mL) is added a solution of BCN-carboxylic acid (15 mg, 36 µmol) in $CHCl_3$ (0.8 mL). The resulting mixture is added to solid EDC.HCl (4.7 mg, 25 µmol), $CHCl_3$ (5 mL) was added and the mixture was left standing for 16 h. DCM (30 mL) is added and the resulting mixture is washed with water (30 mL). After separation, the aqueous phase is extracted with DCM (30 mL). The combined organic layers are dried (Na_2SO_4) and concentrated. The residue is purified by RP-HPLC (30–90% MeCN (no acid) in H_2O (0.01% formic acid). The HPLC collection tubes are filled with 5% aqueous $(NH_4)HCO_3$ before collection. The combined HPLC fractions are extracted with DCM (3 × 20 mL). The combined organic layers are dried (Na_2SO_4) and concentrated. The product **7** is obtained as slightly yellow oil (21 mg, 16 µmol, mw 1323 g/mole, 67% over two steps from Alloc-protected PDB amine).

Example 5. Synthesis of TCO-OSu

[0218] The starting TCO-OH (prepared as described by Blackman *et al.*, *J. Am. Chem. Soc.* **2008**, 130, 41, 13518–13519, incorporated by reference) (120 mg, 0.953 mmol, 1 eq.) was dissolved in 5 mL dry DCM under nitrogen. Triethylamine (193 mg, 1.91 mmol, 2 eq.) and N,N'-disuccinimidyl carbonate (269 mg, 1.05 mmol, 1.1 eq.) were added and stirred until TLC indicated completion (16 h at rt).The sample was concentrated under vacuo and purified by flash column chromatography (20-30% EtOAc in n-heptane), yielding TCO-Osu (192 mg, 0.720 mmol, 76% yield).

Example 6. Synthesis of bifunctional BCN-TCO compound **8**



[0219] BCN-diethyleneglycol-NH_2 (prepared as described in WO2014065661, example 1) (20.1 mg, 0.0620 mmol, 1 eq) was dissolved in 2 mL dry DCM under nitrogen. Triethylamine (12.5 mg, 0.124 mmol, 2 eq) was added. TCO-OSu (19.9 mg, 0.0743 mmol, 1.2 eq) was added. The reaction was stirred until TLC indicated completion (2 h at RT). The sample was concentrated under vacuo and purified by flash column chromatography (5% MeOH in DCM).

[0220] Chemical structures of **8**, **9a–d** are depicted in Figure 11.

Example 7. Preparation of methyltetrazine-IL-2 compound **9b**

[0221] MeTz-IL-2 conjugate **9b** was prepared according to Bartels *et al.*, *Methods* **2019**, *154*, 93–101, incorporated by reference. Thus, 1 eq. of IL-2-G_4SG_4SLPETGGH$_6$ (see sequence below) was

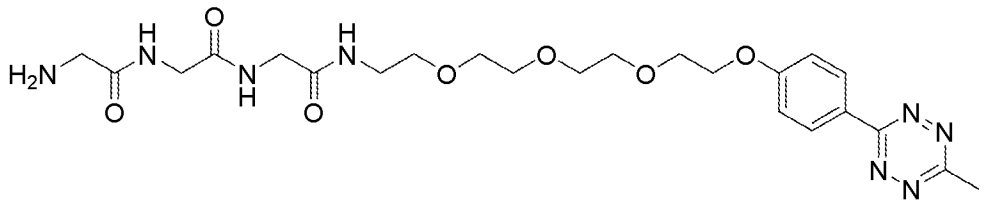
incubated with 1 eq. sortase A and 30 eq of Gly_3-MeTz tag (Figure). Typical conditions: To 100 µL 1.2 mg/mL IL-2-G_4SGG$_4$SLPETGGH$_6$ in TBS pH 8.0 was added 10 µL 17 mg/mL sortase A in TBS pH 8.0 (1 eq.), 13.6 µL 100 mM $CaCl_2$ in TBS pH 8.0, Gly_3-MeTz in DMSO (4 µL 50 mM, 30 eq.), and 9.6 µL DMSO (10% final concentration), incubate overnight at 37 °C overnight. Unreacted IL-2-G_4SGG$_4$SLPETGGH$_6$ was removed by Ni-NTA column, and subsequent SEC-column yielded pure conjugate.

[0222] IL-2 sequence:
APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTAMLTKKFYMPKKATELKHLQCLEEELKPL
EEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFSQSIISTLTG
GGGSGGGGSLPETGGHHHHHH

Example 8. Preparation of methyltetrazine-UCHT1 compound **9c**

[0223] MeTz-UCHT1 conjugate was prepared according to Bartels *et al.*, *Methods* **2019**, *154*, 93–101, incorporated by reference. Thus, 1 eq. of UCHT1-G_4SLPETGGH$_6$ (see sequence above) was incubated with 1 eq. sortase A and 30 eq of Gly_3-MeTz. Typical conditions: To 100 µL 2 mg/mL UCHT1-G_4SLPETGGH$_6$ in TBS pH 8.0 was added 10 µL 17 mg/mL sortase A in TBS pH 8.0 (1 eq.), 13.6 µL 100 mM $CaCl_2$ in TBS pH 8.0, Gly_3-MeTz in DMSO (4 µL 50 mM 30 eq.), and 9.6 µL DMSO (10% final concentration), incubation at 37 °C overnight. Unreacted UCHT1-G_4SLPETGGH$_6$ was removed by Ni-NTA column, and subsequent SEC-column yielded pure conjugate.

Gly_3-MeTz



Molecular Weight: 534.57

Example 9. Enzymatic deglycosylation of trastuzumab or cetuximab with PNGase F

[0224] Trastuzumab (Herzuma) (12 mg, 18.4 mg/mL in PBS pH 7.4) was incubated with PNGase F (15 µL, 7500 units) at 37 °C. After overnight incubation the antibody was dialyzed (3 times to PBS pH 5.5) and concentrated to 15.3 mg/mL. Mass spectral analysis of a sample after IdeS treatment showed one major Fc/2 product (observed mass 23787 Da) corresponding to the expected product.

[0225] Cetuximab (Cerbitux) was deglycosylated similarly. Mass spectral analysis of a sample after IdeS treatment showed one major Fc/2 product (observed mass 23,787 Da) corresponding to the expected product. HPLC-profiles for deglycosylated trastuzumab and cetuximab are depicted in Figures 22 and 23, respectively.

Example 10. Conjugation of deglycosylated trastuzumab with **1**

[0226] Deglycosylated trastuzumab (8.0 µL, 5.0 mg/mL, 40 µg in PBS pH 5.5) was diluted with 4.8 µL PBS pH 5.5 and incubated with **1** (1.6 µL, 2.0 mg/mL, 2.3 mM in DMF or DMSO) and mushroom

tyrosinase (1.6 µL, 10 mg/mL in phosphate buffer pH 6.0) for 16 h at 4 °C. After completion, the product was purified using protein A purification. SDS-PAGE was performed as described above, this showed the formation of a fluorescent band on the heavy chain of trastuzumab (Figure 12). Mass spectral analysis of a sample after IdeS treatment showed one major Fc/2 product (observed mass 24666 Da) corresponding to the expected product (Figure 13). HPLC analysis was performed as described above and indicated clean conversion (Figure 17).

Example 11. Conjugation of deglycosylated cetuximab with **1**

[0227] Deglycosylated cetuximab (8.0 µL, 5.0 mg/mL, 40 µg in PBS pH 5.5) was diluted with 4.8 µL PBS pH 5.5 and incubated with **1** (1.6 µL, 2.0 mg/mL, 2.3 mM in DMF or DMSO) and mushroom tyrosinase (1.6 µL, 10 mg/mL in phosphate buffer pH 6.0) for 16 h at 4 °C. After completion, the product was purified using protein A purification. Mass spectral analysis of a sample after IdeS treatment showed one major Fc/2 product (observed mass 24667 Da) corresponding to the expected product (Figure 14).

Example 12. Evaluation of stoichiometry of BCN-lissamine **1** for labeling of cetuximab

[0228] To evaluate the effect of stoichiometric ratio of **1** versus deglycosylated cetuximab, various concentrations of **1** were incubated with deglycosylated cetuximab in presence of mushroom tyrosinase. 1.39 µL 7.2 mg/mL deglycosylated cetuximab in PBS pH 5.5 was diluted with 3 uL PBS pH 5.5. To the mixture was added 0.5 µL 10 mg/mL mushroom tyrosinase in phosphate buffer pH 6.0 and 0.5 µL BCN-lissamine **1** in DMSO with varying concentrations for each sample (see table). The samples were reacted for 24 h at 4 °C, after which conversion was determined by HPLC (Figure 15, and table below).

Sample #	Concentration **1** (mg/mL)	**1** (eq. per tyrosine)	Conversion (%)
1	0.145	0.625	46
2	0.289	1.25	69
3	0.578	2.5	93
4	0.867	3.75	95
5	1.445	6.25	93

Example 13. Evaluation of labeling of trastuzumab and cetuximab with TCO-AF_{568} (**3**)

[0229] Deglycosylated cetuximab (8.0 µL, 5.0 mg/mL, 40 µg in PBS pH 5.5) was diluted with 4.8 µL PBS pH 5.5 and incubated with **3** (1.6 µL, 4.0 mg/mL, 4.3 mM in DMF or DMSO) and mushroom tyrosinase (1.6 µL, 10 mg/mL in phosphate buffer pH 6.0) for 16 h at 4 °C. After completion, the product was purified using protein A purification. SDS-PAGE was performed as described above, this showed the formation of a fluorescent band on the heavy chain of trastuzumab and cetuximab (Figure 16). HPLC analysis was performed as describe above and indicated clean conversion (Figure 17).

Example 14: Attempted labelling of intact mouse IgG1 with **3**.

[0230] Mouse IgG1 in PBS pH 7.1 (10 µL, 0.5 mg/mL) was incubated with TCO-AF568 **3** (1.0 µL, 4.0 mg/mL in DMSO, 65 eq.) and mushroom tyrosinase (1.0 µL, 10 mg/mL in phosphate buffer pH 6.5) for 48 h at 4 °C. SDS-PAGE analysis was performed as described above (Figure 18). No oxidation of the antibody was observed.

Example 15: Attempted labelling of deglycosylated mouse IgG1 with **3**

[0231] Mouse IgG1 in PBS pH 7.1 (200 µL, 0.5 mg/mL) was incubated with PNGase F (10 µL 0.1 mg/mL) for 16 hours at 37 °C. The reaction was rebuffered to PBS pH 7.1 with spin-filtration (MWCO 100 kDa), which removed PNGase F. The deglycosylated mouse IgG1 (1.1 µL, 4.5 mg/mL) was diluted with 6.9 µL PBS pH 7.1, and incubated with TCO-AF568 **3** (1.0 µL, 4.0 mg/mL in DMSO, 65 eq.) and mushroom tyrosinase (1.0 µL, 10 mg/mL in phosphate buffer pH 6.5) for 48 h at 4 °C. SDS-PAGE analysis was performed as described above (Figure 18). No oxidation of the antibody was observed.

Example 16: Attempted labelling of intact human IgG2 with **3**.

[0232] Human IgG2 in PBS pH 7.1 (2.5 µL, 2.1 mg/mL) was diluted with 5.5 µL PBS pH 7.1, and incubated with TCO-AF568 **3** (1.0 µL, 4.0 mg/mL in DMSO, 62 eq.) and mushroom tyrosinase (1.0 µL, 10 mg/mL in phosphate buffer pH 6.5) for 48 h at 4 °C. SDS-PAGE analysis was performed as described above (Figure 18). No oxidation of the antibody was observed.

Example 17: Attempted labelling of deglycosylated human IgG2 with **3**

[0233] Human IgG2 in PBS pH 7.1 (50 µL, 2.1 mg/mL) was incubated with PNGase F (10 µL 0.1 mg/mL) for 16 hours at 37 °C. The reaction was rebuffered to PBS pH 7.1 with spin-filtration (MWCO 100 kDa), which removed PNGase F. The deglycosylated human IgG2 (1 µL, 4.8 mg/mL) was diluted with 7.0 µL PBS pH 7.1, and incubated with TCO-AF568 **3** (1.0 µL, 4.0 mg/mL in DMSO, 67 eq.) and mushroom tyrosinase (1.0 µL, 10 mg/mL in phosphate buffer pH 6.5) for 48 h at 4 °C. SDS-PAGE analysis was performed as described above (Figure 18). No oxidation of the antibody was observed.

Example 19. Competition experiment for labelling of trastuzumab LC-G_4Y between BCN-reagent **1** and TCO-reagent **3**.

[0234] (**A**) Tras[LC]$G_4SG_4SG_4Y$ in PBS pH 5.5 (1,73 µL, 28.9 mg/mL, 50 µg) was diluted with 8.27 µL PBS and incubated 16 hours at 4 °C. HPLC analysis was performed as described above, and showed a clean light and heavy chain trace for Tras[LC]$G_4SG_4SG_4Y$. The HPLC-trace is depicted in Figure 19A.

[0235] (**B**) Tras[LC]$G_4SG_4SG_4Y$ in PBS pH 5.5 (1,73 µL, 28.9 mg/mL, 50 µg) was diluted with 6.78 µL PBS pH 5.5. To the solution was added BCN-lissamine **1** (0.5 µL, 5.0 mg/mL, 2.5 µg, 4.3 eq. per tyrosine tag). After homogenizing the sample, mushroom tyrosinase (1.0 µL, 10 mg/mL in phosphate buffer pH 6.0) was added. The mixture was reacted for 16 h at 4 °C. HPLC analysis was

performed as described above, and showed a clean conversion on the light chain of Tras[LC]$G_4SG_4SG_4Y$ with a 1 minute shift in retention time. The HPLC-trace is depicted in Figure 19B.

[0236] (**C**) Tras[LC]$G_4SG_4SG_4Y$ in PBS pH 5.5 (1,73 µL, 28.9 mg/mL, 50 µg) was diluted with 6.65 µL PBS pH 5.5. To which was added TCO-AF568 **3** (0.625 µL 4.0 mg/mL, 2.5 µg, 4.03 eq. per tyrosine tag). After homogenizing the sample mushroom tyrosinase (1.0 µL, 10 mg/mL in phosphate buffer pH 6.0) was added. The mixture was reacted for 16 h at 4 °C. HPLC analysis was performed as described above, and showed a clean conversion on the light chain of Tras[LC]$G_4SG_4SG_4Y$ with a 0.2 minute shift in retention time. The HPLC-trace is depicted in Figure 19C.

[0237] (**D**) Tras[LC]$G_4SG_4SG_4Y$ in PBS pH 5.5 (1,73 µL, 28.9 mg/mL, 50 µg) was diluted with 6.15 µL PBS pH 5.5. To which was added BCN-lissamine **1** (0.5 µL, 5.0 mg/mL, 2.5 µg, 4.3 eq. per tyrosine tag) and TCO-AF568 **3** (0.625 µL 4.0 mg/mL, 2.5 µg, 4.03 eq. per tyrosine tag). After homogenizing the sample mushroom tyrosinase (1.0 µL, 10 mg/mL in phosphate buffer pH 6.0) was added. HPLC analysis was performed as described above, and showed a clean conversion on the light chain of Tras[LC]$G_4SG_4SG_4Y$ with a 1 minute shift in retention time, indicating the formation of primarily BCN-conjugate. The HPLC-trace is depicted in Figure 19D.

Example 20. Conjugation of deglycosylated cetuximab with bifunctional BCN-TCO reagent **8** leading to cetuximab-TCO (conceptually depicted in Figure 20).

[0238] Deglycosylated cetuximab (11.0 µL, 9.0 mg/mL in PBS pH 5.5) was diluted with 33.0 µL PBS pH 5.5. To which was added BCN-TCO **8** (5.5 µL, 5.0 mg/mL, 27.5 µg, 44 eq. per tyrosine tag, in DMSO) and subsequently was added mushroom tyrosinase (5.5 µL, 10 mg/mL in phosphate buffer pH 6.0). The mixture was reacted for 16 h at 4 °C. The reaction was rebuffered to PBS pH 7.1 with spin-filtration (MWCO 100 kDa), which removed unreacted BCN-TCO **8**. TCO-modified cetuximab final concentration was 5.2 mg/mL.

Example 21. Reaction of cetuximab-TCO with methyltetrazine reagents **9a–9d**

[0239] TCO-modified cetuximab (1.0 µL, 5.2 mg/mL) was diluted with 3.5 µL PBS pH 7.1 and subsequently incubated with MeTz-TAMRA **9a** (0.5 µL, 1.0 mg/mL, 9.3 eq. per TCO) in DMSO. The sample was incubated at 4 °C for 30 minutes. SDS-PAGE analysis was performed as described above, this showed formation of a fluorescent band at the heavy chain (Figure 21E).

[0240] TCO-modified cetuximab (1.0 µL, 5.2 mg/mL) was diluted with 3.5 µL PBS pH 7.1 and subsequently incubated with MeTz-IL2 **9b** (0.5 µL, 7.4 mg/mL, 3.0 eq. per TCO) in PBS pH 7.1. The sample was incubated at 4 °C for 30 minutes. SDS-PAGE analysis was performed as described above, this showed formation of a fluorescent band at the heavy chain (Figure 21B).

[0241] TCO-modified cetuximab (1.0 µL, 5.2 mg/mL) was diluted with 3.33 µL PBS pH 7.1 and subsequently incubated with MeTz-UCHT1 **9c** (0.67 µL, 9.1 mg/mL, 3.1 eq. per TCO) in PBS pH 7.1. The sample was incubated at 4 °C for 30 minutes. SDS-PAGE analysis was performed as described above, this showed formation of a fluorescent band at the heavy chain (Figure 21C).

[0242] TCO-modified cetuximab (1.0 µL, 5.2 mg/mL) was diluted with 2.0 µL PBS pH 7.1 and subsequently incubated with MeTz-ODN1826 **9D** (2.0 µL, 100 µM, 2.8 eq. per TCO) in MilliQ. The sample was incubated at 4 °C for 30 minutes. SDS-PAGE analysis was performed as described above, this showed formation of a fluorescent band at the heavy chain (Figure 21D).

Example 22. Transient expression and purification of B12

[0243] B12 was transiently expressed in CHO K1 cells by Evitria (Zurich, Switzerland) at 1 L scale. The supernatant was purified using a protein A column (25 mL, CaptivA PriMAB). The supernatant was loaded onto the column followed by washing with at least 10 column volumes of 25 mM Tris pH 7.5, 150 mM NaCl (TBS). Retained protein was eluted with 0.1 M NaOAc pH 3.5. The eluted product was immediately neutralized with 2.5 M Tris-HCl pH 7.2 and dialyzed against TBS. Next, the IgG was concentrated (15–20 mg/mL) using a Vivaspin Turbo 4 ultrafiltration unit (Sartorius).

Example 23. Enzymatic deglycosylation of B12 with PNGase F

[0244] B12 (6 mg, 10 mg/mL in PBS pH 7.4) was incubated with PNGase F (6 µL, 3000 units, NEB) at 37 °C. After overnight incubation the antibody was dialyzed (3 times to PBS pH 5.5) and concentrated to 23.6 mg/mL. Mass spectral analysis of a sample after IdeS treatment showed one major Fc/2 product (observed mass 23756 Da, approximately 70% of total Fc/2) corresponding to the expected product and a minor product (observed mass 23885 Da, approximately 25% of total Fc/2) for the expected product + lysine.

Example 24. Conjugation of deglycosylated trastuzumab with BCN-HS-PEG_2-vc-PABC-MMAE (**6a**)

[0245] Deglycosylated trastuzumab (196 µL, 3 mg, 15.3 mg/mL in PBS 5.5) was incubated with BCN-HS-PEG_2-vc-PABC-MMAE **6a** (40 µL, 5 mM in DMF) and mushroom tyrosinase (60 µL, 10 mg/mL in phosphate buffer pH 6.0, Sigma Aldrich T3824) for 16h. Subsequently, the reaction was diluted with 300 µL PBS and centrifuged for 2 min at 13.000 rpm. The liquid was purified on a Superdex200 Increase 10/300 GL (GE Healthcare) column on an AKTA Purifier-10 (GE Healthcare). Mass spectral analysis of the IdeS-digested sample showed one major product (observed mass 25311 Da, approximately 90% of total Fc/2 fragment), corresponding to the conjugated Fc/2 fragment. SEC, MS and HPLC profiles of the conjugate depicted in Figure 24.

Example 25. Conjugation of deglycosylated trastuzumab with BCN-HS-PEG_2-$(vc\text{-}PABC\text{-}MMAE)_2$ (**6b**)

[0246] Deglycosylated trastuzumab (196 µL, 3 mg, 15.3 mg/mL in PBS 5.5) was incubated with BCN-HS-PEG_2-$(vc\text{-}PABC\text{-}MMAE)_2$ **6b** (40 µL, 5 mM in DMF) and mushroom tyrosinase (60 µL, 10 mg/mL in phosphate buffer pH 6.0, Sigma Aldrich T3824) for 16h. Subsequently, the reaction was diluted with 300 µL PBS and centrifuged for 2 min at 13.000 rpm. The liquid was purified on a Superdex200 Increase 10/300 GL (GE Healthcare) column on an AKTA Purifier-10 (GE Healthcare). Mass spectral analysis of the IdeS-digested sample showed one major product

(observed mass 26591 Da, approximately 90% of total Fc/2 fragment), corresponding to the conjugated Fc/2 fragment. SEC, MS and HPLC profiles of the conjugate depicted in Figure 25.

Example 26. Conjugation of deglycosylated trastuzumab with BCN-HS-PEG_2-va-PABC-PBD (**7**)

[0247] Deglycosylated trastuzumab (196 µL, 3 mg, 15.3 mg/mL in PBS 5.5) was incubated with BCN-HS-PEG_2-va-PABC-PBD **7** (40 µL, 5 mM in DMF) and mushroom tyrosinase (60 µL, 10 mg/mL in phosphate buffer pH 6.0, Sigma Aldrich T3824) for 16h. Subsequently, the reaction was diluted with 300 µL PBS and centrifuged for 2 min at 13.000 rpm. The liquid was purified on a Superdex200 Increase 10/300 GL (GE Healthcare) column on an AKTA Purifier-10 (GE Healthcare). Mass spectral analysis of the IdeS-digested sample showed one major product (observed mass 25122 Da, approximately 90% of total Fc/2 fragment), corresponding to the conjugated Fc/2 fragment. SEC, MS and HPLC profiles of the conjugate depicted in Figure 26.

Example 27. Conjugation of deglycosylated B12 with BCN-HS-PEG_2-(vc-PABC-MMAE)$_2$ (**6b**)

[0248] Deglycosylated B12 (127 µL, 3 mg, 23.6 mg/mL in PBS 5.5) was incubated with BCN-HS-PEG_2-(vc-PABC-MMAE)$_2$ **6b** (40 µL, 5 mM in DMF) and mushroom tyrosinase (60 µL, 10 mg/mL in phosphate buffer pH 6.0, Sigma Aldrich T3824) and PBS (73 µL, pH 5.5) for 16h. Subsequently, the reaction was diluted with 300 µL PBS and centrifuged for 2 min at 13.000 rpm. The liquid was purified on a Superdex200 Increase 10/300 GL (GE Healthcare) column on an AKTA Purifier-10 (GE Healthcare). Mass spectral analysis of the IdeS-digested sample showed one major product (observed mass 26599 Da, approximately 70% of total Fc/2 fragment), corresponding to the conjugated Fc/2 fragment, and one minor product (observed mass 26687 Da, approximately 20% of total Fc/2 fragment), corresponding to the conjugated Fc/2 fragment with C-terminal lysine. SEC, MS and HPLC profiles of the conjugate of B12 with **6b** depicted in Figure 27.

Summary table:

Conjugate	**%Conversion**	**% monomer**	**% Yield**	**DAR**
T-6a (BCN-MMAE)	90.0	98.2	88	1.8
T-6b (BCN-MMAE)$_2$	91.6	97.5	50	3.67
T-7 (BCN-PBD)	97.0	94.5	84	3.88
B12-6b (BCN-MMAE)$_2$	90.3	98.5	67	3.61

Example 28. In vitro analysis

[0249] SK-BR-3 (Her2 3+) and MCF-7(Her2 -) cells were plated in 96-well plates (5000 cells/well) in RPMI 1640 (Merck, R7388) supplemented with 10% fetal bovine serum (FBS) (ATCC® 30-2020™) and incubated overnight in a humidified atmosphere at 37°C and 5% CO_2. Compound T-6a/b, T-7 and B-6b were added in quadruplo in a three-fold dilution series to obtain a final concentration ranging from 2 pM to 21 nM. The cells were incubated for 5 days in a humidified atmosphere at 37°C and 5% CO_2. The culture medium was replaced by 0.01 mg/mL resazurin (Sigma Aldrich) in RPMI 1640 (Merck, R7388) supplemented with 10% fetal bovine serum (FBS)

(ATCC® 302020™). After approximately 3 to 4 hours in a humidified atmosphere at 37°C and 5% CO_2 the fluorescence was detected with a fluorescence plate reader (Envision multipabel plate reader) at 531 nm excitation and 590 nm emission. The relative fluorescent units (RFU) were normalized to cell viability percentage by setting wells without cells at 0% viability and wells with untreated cells at 100% viability. Cell killing potential for the various constructs at different concentrations is plotted in Figure 28.

Example 29: Enzymatic trimming of trastuzumab by fusion protein EndoSH

[0250] Trastuzumab (Herzuma) (1 mg, 10 mg/mL in PBS pH 7.4) was incubated with EndoSH (2 µL, 4.2 mg/mL) at 37 °C. After overnight incubation the antibody was dialyzed (3 times to PBS pH 5.5) and concentrated to 11 mg/mL. Mass spectral analysis of a sample after IdeS treatment showed one major Fc/2 product (observed mass 24134 Da,) corresponding to the expected product.

Example 30: Enzymatic trimming of high-mannose trastuzumab by fusion protein EndoSH

[0251] Trastuzumab having high-mannose glycans (obtained via transient expression in CHO K1 cells in the presence of kifunensin performed by Evitria (Zurich, Switzerland) (1.4 mg, 11.4 mg/mL in PBS pH 7.4) was incubated with EndoSH (2.7 µL, 4.2 mg/mL) at 37 °C. After incubation for 6 h the antibody was dialyzed (3 times to PBS pH 5.5) and concentrated to 16 mg/mL. Mass spectral analysis of a sample after IdeS treatment showed one major Fc/2 product (observed mass 23990 Da,) corresponding to the expected product.

Example 31. Conjugation of trimmed trastuzumab with BCN-HS-PEG_2-vc-PABC-MMAE (**6a**)

[0252] Trimmed trastuzumab (20 µL, 0.2 mg, 10 mg/mL in PBS 5.5) was incubated with BCN-HS-PEG_2-vc-PABC-MMAE **6a** (4 µL, 3.33 mM in DMF) and mushroom tyrosinase (4 µL, 10 mg/mL in phosphate buffer pH 6.0, Sigma Aldrich T3824) for 16h. RP-HPLC analysis after DTT reduction showed about 10 % conversion via a shift for the heavy chain peak corresponding to the conjugated product (Figure 29).

Example 32. Conjugation of trimmed high-mannose trastuzumab with BCN-HS-PEG_2-vc-PABC-MMAE (**6a**)

[0253] Trimmed high-mannose trastuzumab (20 µL, 0.2 mg, 10 mg/mL in PBS 5.5) was incubated with BCN-HS-PEG_2-vc-PABC-MMAE **6a** (4 µL, 3.33 mM in DMF) and mushroom tyrosinase (4 µL, 10 mg/mL in phosphate buffer pH 6.0, Sigma Aldrich T3824) for 16h. Subsequently, an extra portion of mushroom tyrosinase (4 µL, 10 mg/mL in phosphate buffer pH 6.0, Sigma Aldrich T3824) was added and the reaction was incubated for an additional 24h. Mass spectral analysis of the IdeS-digested sample showed one major product (observed mass 25512 Da, approximately 40% of total Fc/2 fragment), and one fragmentation product (observed mass 24752 Da, approximately 30% of total Fc/2 fragment), both peaks correspond to the conjugated product (Figure 30). RP-HPLC analysis (Figure 31) indicates good conversion to the conjugate with a drug-to-antibody ratio of 1.50.

SEQUENCE LISTING

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Gln Tyr Asn Ser Thr Phe Arg Val Val Ser Val Leu Thr Val Leu His
65                  70                  75                  80


Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys
                85                  90                  95


Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys
            100                 105                 110


<210>  4
<211>  110
<212>  PRT
<213>  Human

<400>  4

Ala Pro Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys
1               5                   10                  15


Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val
            20                  25                  30


Val Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr
        35                  40                  45


Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu
    50                  55                  60


Gln Phe Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His
65                  70                  75                  80


Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys
                85                  90                  95


Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys
            100                 105                 110


<210>  5
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<212>  PRT
<213>  Mouse
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<400> 5

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Val Pro Glu Val Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp
1               5                   10                  15


Val Leu Thr Ile Thr Leu Thr Pro Lys Val Thr Cys Val Val Val Asp
            20                  25                  30


Ile Ser Lys Asp Asp Pro Glu Val Gln Phe Ser Trp Phe Val Asp Asp
        35                  40                  45


Val Glu Val His Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn
    50                  55                  60


Ser Thr Phe Arg Ser Val Ser Glu Leu Pro Ile Met His Gln Asp Trp
65                  70                  75                  80


Leu Asn Gly Lys Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe Pro
                85                  90                  95


Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys
            100                 105
```

<210> 6
<211> 110
<212> PRT
<213> Mouse

<400> 6

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Ala Pro Asp Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys
1               5                   10                  15


Ile Lys Asp Val Leu Met Ile Ser Leu Ser Pro Met Val Thr Cys Val
            20                  25                  30


Val Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe
        35                  40                  45


Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu
    50                  55                  60
```

```
Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His
65                  70                  75                  80


Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Arg
                85                  90                  95


Ala Leu Pro Ser Pro Ile Glu Lys Thr Ile Ser Lys Pro Arg
            100                 105                 110


<210>  7
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<213>  Mouse

<400>  7

Ala Pro Asn Leu Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys
1               5                   10                  15


Ile Lys Asp Val Leu Met Ile Ser Leu Ser Pro Ile Val Thr Cys Val
            20                  25                  30


Val Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe
        35                  40                  45


Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu
    50                  55                  60


Asp Tyr Asn Ser Thr Leu Arg Val Val Ser Ala Leu Pro Ile Gln His
65                  70                  75                  80


Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys
                85                  90                  95


Asp Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys
            100                 105                 110


<210>  8
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<212>  PRT
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<213> Mouse

<400> 8

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Ala Pro Asn Leu Glu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Asn
1               5                   10                  15


Ile Lys Asp Val Leu Met Ile Ser Leu Thr Pro Lys Val Thr Cys Val
            20                  25                  30


Val Val Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe
        35                  40                  45


Val Asn Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu
    50                  55                  60


Asp Tyr Asn Ser Thr Ile Arg Val Val Ser Thr Leu Pro Ile Gln His
65                  70                  75                  80


Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys
                85                  90                  95


Asp Leu Pro Ser Pro Ile Glu Arg Thr Ile Ser Lys Ile Lys
            100                 105                 110
```

<210> 9
<211> 110
<212> PRT
<213> Mouse

<400> 9

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Ala Gly Asn Ile Leu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Lys
1               5                   10                  15


Pro Lys Asp Ala Leu Met Ile Ser Leu Thr Pro Lys Val Thr Cys Val
            20                  25                  30


Val Val Asp Val Ser Glu Asp Asp Pro Asp Val His Val Ser Trp Phe
        35                  40                  45


Val Asp Asn Lys Glu Val His Thr Ala Trp Thr Gln Pro Arg Glu Ala
```

50 55 60

Gln Tyr Asn Ser Thr Phe Arg Val Val Ser Ala Leu Pro Ile Gln His
65 70 75 80

Gln Asp Trp Met Arg Gly Lys Glu Phe Lys Cys Lys Val Asn Asn Lys
85 90 95

Ala Leu Pro Ala Pro Ile Glu Arg Thr Ile Ser Lys Pro Lys
100 105 110

<210> 10
<211> 263
<212> PRT
<213> Artificial Sequence

<220>
<223> UCHT1

<400> 10

Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala Ser
1 5 10 15

Met Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr Gly Tyr Thr
20 25 30

Met Asn Trp Val Lys Gln Ser His Gly Lys Asn Leu Glu Trp Met Gly
35 40 45

Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr Asn Gln Lys Phe Lys
50 55 60

Asp Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr Met
65 70 75 80

Glu Leu Leu Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala
85 90 95

Arg Ser Gly Tyr Tyr Gly Asp Ser Asp Trp Tyr Phe Asp Val Trp Gly
100 105 110

```
Ala Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Ser Gly Gly Gly
        115                 120                 125


Ser Gly Gly Gly Ser Gly Gly Gly Ser Asp Ile Gln Met Thr Gln Thr
    130                 135                 140


Thr Ser Ser Leu Ser Ala Ser Leu Gly Asp Arg Val Thr Ile Ser Cys
145                 150                 155                 160


Arg Ala Ser Gln Asp Ile Arg Asn Tyr Leu Asn Trp Tyr Gln Gln Lys
                165                 170                 175


Pro Asp Gly Thr Val Lys Leu Leu Ile Tyr Tyr Thr Ser Arg Leu His
            180                 185                 190


Ser Gly Val Pro Ser Lys Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr
        195                 200                 205


Ser Leu Thr Ile Ser Asn Leu Glu Gln Glu Asp Ile Ala Thr Tyr Phe
    210                 215                 220


Cys Gln Gln Gly Asn Thr Leu Pro Trp Thr Phe Ala Gly Gly Thr Lys
225                 230                 235                 240


Leu Glu Ile Lys Arg Ala Gly Gly Gly Gly Ser Leu Pro Glu Thr Gly
                245                 250                 255


Gly His His His His His His
            260
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<210> 11
<211> 155
<212> PRT
<213> Artificial Sequence

<220>
<223> IL-2

<400> 11

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Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln Leu Glu His
```

```
1               5                   10                  15

Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Asn Asn Tyr Lys
            20                  25                  30

Asn Pro Lys Leu Thr Ala Met Leu Thr Lys Lys Phe Tyr Met Pro Lys
        35                  40                  45

Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys
    50                  55                  60

Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys Asn Phe His Leu
65                  70                  75                  80

Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile Val Leu Glu Leu
                85                  90                  95

Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala
            100                 105                 110

Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Ser Gln Ser Ile
        115                 120                 125

Ile Ser Thr Leu Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Leu
    130                 135                 140

Pro Glu Thr Gly Gly His His His His His His
145                 150                 155


<210>  12
<211>  229
<212>  PRT
<213>  Artificial Sequence

<220>
<223>   LC-GGGGY

<400>  12

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15
```

```
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn Thr Ala
            20                  25                  30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Tyr Thr Thr Pro Pro
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
            100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
        115                 120                 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
    130                 135                 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145                 150                 155                 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                165                 170                 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
            180                 185                 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
        195                 200                 205

Phe Asn Arg Gly Glu Cys Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
    210                 215                 220
```

Gly Gly Gly Gly Tyr
225

Conclusies

1. Werkwijze voor de bereiding van een glycoproteïne-conjugaat, omvattende:
   (a) het verschaffen van een *N*-glycoproteïne met een blootgesteld tyrosineresidu, waarvij het blootgestelde tyrosineresidu binnen 10 aminozuren van een *N*-glycosyleringsplek gelegen is, maar waarbij de *N*-glycosyleringsplek zodanig gemodificeerd is dat het glycoproteïne geen glycaan van langer dan twee monosaccharideresiduen binnen 10 aminozuren van het blootgestelde tyrosineresidu bevat;
   (b) het converteren van de fenolgroep van het blootgestelde tyrosineresidu in een *ortho*-chinongroep door het in contact brengen van het glycoproteïne met een oxidatief enzym dat in staat is om tyrosine te oxideren;
   (c) het laten reageren van de *ortho*-chinongroep met een alkeen- of alkynverbinding via een [4+2] cycloadditie, waarbij de verbinding een (hetero)cycloalkeen- of een (hetero)cycloalkyngroep en (i) een chemisch handvat om de verbinding verder te modificeren met een payload, of (ii) een payload.

2. De werkwijze volgens conclusie 1, waarbij het blootgestelde tyrosineresidu binnen 5 aminozuren van de *N*-glycosyleringsplek gelegen is.

3. De werkwijze volgens conclusie 1 of 2, waarbij het *N*-glycoproteïne met een blootgesteld tyrosineresidu verschaft wordt door:
   (a1) het onderwerpen van een *N*-glycoproteïne aan deglycosylering door het in contact te brengen met een amidase, bij voorkeur met PNGase F, om een *N*-glycoproteïne waarvan de glycaan verwijderd is te verkrijgen; of
   (a2) het onderwerpen van een *N*-glycoproteïne aan trimming door het in contact te brengen met een endoglycosidase, om een *N*-glycoproteïne met een glycaan met structuur –GlcNAc(Fuc)$_b$, waarbij b 0 of 1 is, te verkrijgen; of
   (a3) het verschaffen van een gemuteerd *N*-glycoproteïne waarbij de geglycosyleerde asparagine vervangen is door een niet geglycosyleerd aminozuur.

4. De werkwijze volgens één van de voorgaande conclusies, waarbij het oxidatieve enzym tyrosinase of (poly)phenoloxidase is.

5. De werkwijze volgens één van de voorgaande conclusies s, waarbij stappen (b) en (c) in one-pot worden uitgevoerd, door het gelijktijdig in contact brengen van het *N*-glycoproteïne met het oxidatieve enzym en de alkeen- of alkynverbinding.

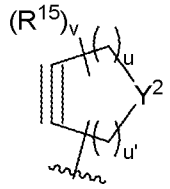
6. De werkwijze volgens één van de voorgaande conclusies, waarbij alkeen- of alkynverbinding structuur (**3a**) of (**3b**) heeft:

$Q^1–L–(Q^2)_x$ **(3a)** $Q^1–L–(D)_x$ **(3b)**

waarbij:

- Q^1 een (hetero)cycloalkeen- of een (hetero)cycloalkyngroep is;
- L een linker is;
- x een integer in bereik van 1 – 4 is;
- Q^2 een chemisch handvat is dat reactief is ten opzichte van een op geschikte wijze gefunctionaliseerde payload is, maar niet ten opzichte van Q^1;
- D een payload is.

7. De werkwijze volgens één van de voorgaande conclusies, waarbij Q^1 een (hetero)cycloalkyngroep volgens structuur (Q1) is:

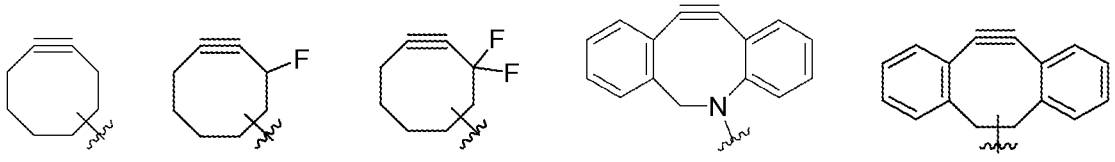


(Q1)

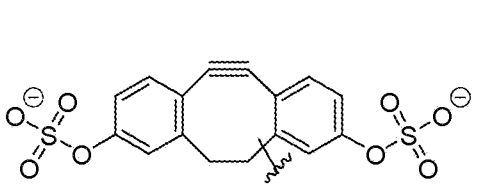
waarbij:

- R^{15} onafhankelijk gekozen wordt uit de groep bestaande uit waterstof, halogeen, $-OR^{16}$, $-NO_2$, -CN, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$, C_1 – C_{24} alkylgroepen, C_6 – C_{24} (hetero)arylgroepen, C_7 – C_{24} alkyl(hetero)arylgroepen en C_7 – C_{24} (hetero)arylalkylgroepen, en waarbij de alkylgroepen, (hetero)arylgroepen, alkyl(hetero)arylgroepen en (hetero)arylalkylgroepen eventueel gesubstitueerd zijn, waarbij twee substituenten R^{15} aan elkaar gekoppeld kunnen zijn om een eventueel gesubstitueerde geannuleerde cycloalkyl of een eventueel gesubstitueerde geannuleerde (hetero)areensubstituent te verkrijgen, en waarbij R^{16} onafhankelijk gekozen wordt uit de groep bestaande uit waterstof, halogeen, C_1 – C_{24} alkylgroepen, C_6 – C_{24} (hetero)arylgroepen, C_7 – C_{24} alkyl(hetero)arylgroepen en C_7 – C_{24} (hetero)arylalkylgroepen;
- Y^2 $C(R^{31})_2$, O, S, $S^{(+)}R^{31}$, $S(O)R^{31}$, $S(O)=NR^{31}$ of NR^{31} is, waarbij $S^{(+)}$ een cationisch zwavelatoom is dat $B^{(-)}$ als tegenion heeft, en waarbij elke R^{31} onafhankelijk R^{15} of een connectie met Q^2 of D, via L, is;
- u = 0, 1, 2, 3, 4 of 5;
- u' = 0, 1, 2, 3, 4 of 5, waarbij u + u' = 4, 5, 6, 7 of 8;
- v = een integer in bereik van 8 – 16.

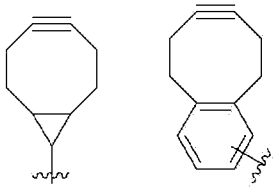
8. De werkwijze volgens conclusie 7, waarbij Q^1 gekozen wordt uit de groep bestaande uit (Q2) – (Q20):



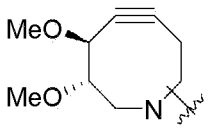
(Q2) (Q3) (Q4) (Q5) (Q6)



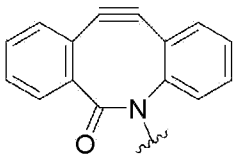
(Q7)



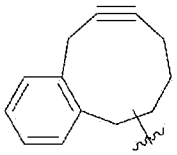
(Q8) (Q9)



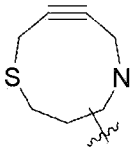
(Q10)



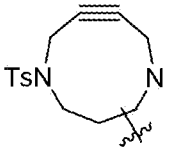
(Q11)



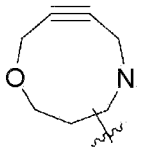
(Q12)



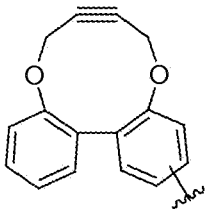
(Q13)



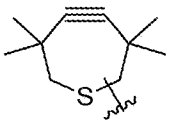
(Q14)



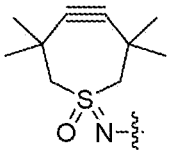
(Q15)



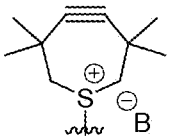
(Q16)



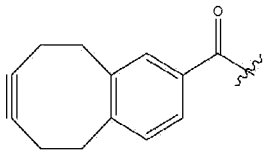
(Q17)



(Q18)



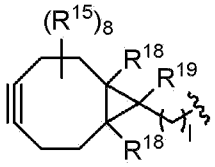
(Q19)



(Q20)

waarbij $B^{(-)}$ een anion is.

9. De werkwijze volgens conclusie 8, waarbij Q^1 een cyclooctyn volgens structuur (Q42) is:



(Q42)

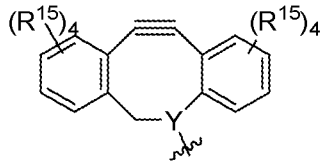
waarbij:

- R^{15} onafhankelijk gekozen wordt uit de groep bestaande uit waterstof, halogeen, $-OR^{16}$, $-NO_2$, -CN, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$, C_1 – C_{24} alkylgroepen, C_6 – C_{24} (hetero)arylgroepen, C_7 – C_{24} alkyl(hetero)arylgroepen en C_7 – C_{24} (hetero)arylalkylgroepen, en waarbij de alkylgroepen, (hetero)arylgroepen, alkyl(hetero)arylgroepen en (hetero)arylalkylgroepen eventueel gesubstitueerd zijn, waarbij twee substituenten R^{15} aan elkaar gekoppeld kunnen zijn om een eventueel gesubstitueerde geannuleerde cycloalkyl of een eventueel gesubstitueerde geannuleerde (hetero)areensubstituent te verkrijgen, en waarbij R^{16} onafhankelijk gekozen wordt uit de groep bestaande uit waterstof, halogeen, C_1 – C_{24} alkylgroepen, C_6 – C_{24} (hetero)arylgroepen, C_7 – C_{24} alkyl(hetero)arylgroepen en C_7 – C_{24} (hetero)arylalkylgroepen;
- R^{18} onafhankelijk gekozen wordt uit de groep bestaande uit waterstof, halogeen, C_1 – C_{24} alkylgroepen, C_6 – C_{24} (hetero)arylgroepen, C_7 – C_{24} alkyl(hetero)arylgroepen en C_7 – C_{24} (hetero)arylalkylgroepen;
- R^{19} onafhankelijk gekozen wordt uit de groep bestaande uit waterstof, halogeen, C_1 – C_{24} alkylgroepen, C_6 – C_{24} (hetero)arylgroepen, C_7 – C_{24} alkyl(hetero)arylgroepen en C_7 – C_{24}

(hetero)arylalkylgroepen, waarbij de alkylgroepen eventueel onderbroken kunnen zijn door één of meer heteroatomen gekozen uit de groep bestaande uit O, N en S, wherein the alkylgroepen, (hetero)arylgroepen, alkyl(hetero)arylgroepen en de (hetero)arylalkylgroepen onafhankelijk eventueel gesubstitueerd zijn, of R^{19} is een tweede Q^1 of D verbonden via een spacer; en

- l = een integer in bereik van 0 tot 10;

of waarbij Q^1 een (hetero)cyclooctyn volgens structuur (Q43) is:

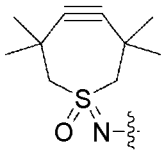


(Q43)

waarbij:

- R^{15} onafhankelijk gekozen wordt uit de groep bestaande uit waterstof, halogeen, $-OR^{16}$, $-NO_2$, -CN, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$, C_1 – C_{24} alkylgroepen, C_6 – C_{24} (hetero)arylgroepen, C_7 – C_{24} alkyl(hetero)arylgroepen en C_7 – C_{24} (hetero)arylalkylgroepen, en waarbij de alkylgroepen, (hetero)arylgroepen, alkyl(hetero)arylgroepen en (hetero)arylalkylgroepen eventueel gesubstitueerd zijn, waarbij twee substituenten R^{15} aan elkaar gekoppeld kunnen zijn om een eventueel gesubstitueerde geannuleerde cycloalkyl of een eventueel gesubstitueerde geannuleerde (hetero)areensubstituent te verkrijgen, en waarbij R^{16} onafhankelijk gekozen wordt uit de groep bestaande uit waterstof, halogeen, C_1 – C_{24} alkylgroepen, C_6 – C_{24} (hetero)arylgroepen, C_7 – C_{24} alkyl(hetero)arylgroepen en C_7 – C_{24} (hetero)arylalkylgroepen;
- Y N of CR^{15} is;

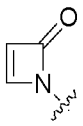
of waarbij Q^1 een (hetero)cycloheptyn volgens structuur (Q37) is:



(Q37).

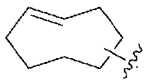
10. De werkwijze volgens één van de conclusies 1 – 6, waarbij Q^1 een (hetero)cycloalkeen is gekozen uit de groep bestaande uit, eventueel gesubstitueerde, (hetero)cyclopropenylgroep, (hetero)cyclobutenylgroep, een norborneengroep, een norbornadieengroep, *trans*-(hetero)cycloheptenylgroep, *trans*-(hetero)cyclooctenylgroep, *trans*-(hetero)cyclononenylgroep of *trans*-(hetero)cyclodecenylgroep, bij voorkeur wordt Q^1 is gekozen uit de groep bestaande uit (Q44) – (Q56):

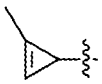

(Q44)

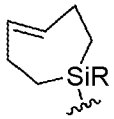

(Q45)

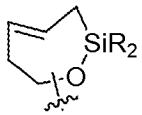

(Q46)

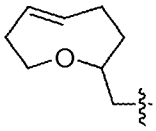

(Q47)

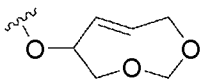

(Q48)

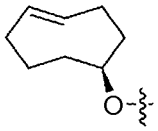

(Q49)

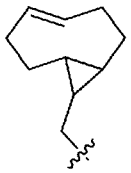

(Q50)

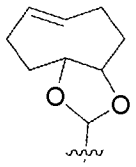

(Q51)


(Q52)


(Q53)


(Q54)


(Q55)


(Q56)

waarbij Y^3 gekozen wordt uit $C(R^{24})_2$, NR^{24} of O, waarbij elke R^{24} onafhankelijk waterstof, C_1 – C_6 alkyl of verbonden aan L is, eventueel via een spacer, en de binding gelabeld ⎯ ⎯ ⎯ is een enkele of dubbele binding, en de R-groep(en) op Si in (Q50) en (Q51) is/zijn alkyl of aryl.

11. De werkwijze volgens één van de voorgaande conclusies, waarbij de verbinding (i) een chemisch handvat omvat om de verbinding verder te modificeren met een payload, en de werkwijze verder omvat:

    (d) het onderwerpen van het chemisch handvat, bij voorkeur Q^2, van het glycoproteïne verkregen in stap (c) aan een conjugatiereactie met een payload met structuur F^2–D of F^2–L^2–$(D)_x$, waarbij F^2 reactief is ten opzichte van het chemisch handvat, L^2 een linker is en x een geheel getal in het bereik van 1 – 4.

12. De werkwijze volgens één van de voorgaande conclusies, waarbij het payload D gekozen wordt uit de groep bestaande uit een actieve verbinding, een reportermolecuul, een polymeer, een vast oppervlak, een hydrogel, een nanodeeltje, een microdeeltje en een biomolecuul.

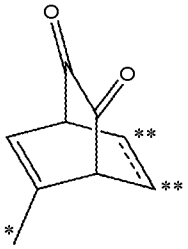
13. Een glycoproteïne-conjugaat volgens structuur (**1a**) of (**1b**):

$$Pr–[Z^1–L–(Q^2)_x]_y \qquad Pr–[Z^1–L–(D)_x]_y$$

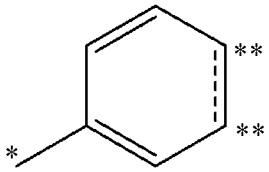
(**1a**) (**1b**)

waarbij:

- Pr een *N*-glycoproteïne is;
- Z^1 structuur (Za) of (Zb) omvat:



(Za)

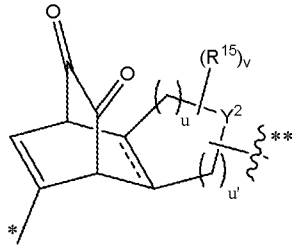


(Zb)

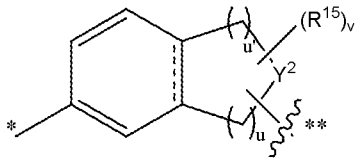
waarbij het koolstofatoom gelabeld met * direct verbonden is met de peptideketen van het glycoproteïne aan een aminozuur dat binnen 10 aminozuren van een *N*-glycosyleringsplek gelegen is, maar waarbij de *N*-glycosyleringsplek zodanig gemodificeerd is dat het glycoproteïne geen glycaan van langer dan twee monosaccharideresiduen binnen 10 aminozuren van dat aminozuurresidu bevat, en beide koolstofatomen gelabeld met ** verbonden zijn aan L, en de binding gelabeld ___ is een enkele of dubbele binding;

- L een linker is;
- x een integer in bereik van 1 – 4 is;
- y een integer in bereik van 1 – 4 is;
- Q^2 een chemisch handvat is dat reactief is ten opzichte van een op geschikte wijze gefunctionaliseerde payload is;
- D een payload is.

14. Het glycoproteïne-conjugaat volgens conclusie 13, wherein Z^1 has structure:



(Z1a)



(Z1b)

waarbij:

- waarbij het koolstofatoom gelabeld met * direct verbonden is met de peptideketen van het glycoproteïne en het koolstofatoom gelabeld met ** verbonden is met L, en de binding gelabeld ___ is een enkele of dubbele binding;
- R^{15} onafhankelijk gekozen wordt uit de groep bestaande uit waterstof, halogeen, $-OR^{16}$, $-NO_2$, -CN, $-S(O)_2R^{16}$, $-S(O)_3^{(-)}$, C_1 – C_{24} alkylgroepen, C_6 – C_{24} (hetero)arylgroepen, C_7 – C_{24} alkyl(hetero)arylgroepen en C_7 – C_{24} (hetero)arylalkylgroepen, en waarbij de alkylgroepen, (hetero)arylgroepen, alkyl(hetero)arylgroepen en (hetero)arylalkylgroepen eventueel gesubstitueerd zijn, waarbij twee substituenten R^{15} aan elkaar gekoppeld kunnen zijn om een eventueel gesubstitueerde geannuleerde cycloalkyl of een eventueel gesubstitueerde geannuleerde (hetero)areensubstituent te verkrijgen, en waarbij R^{16} onafhankelijk gekozen wordt uit de

groep bestaande uit waterstof, halogeen, C_1 – C_{24} alkylgroepen, C_6 – C_{24} (hetero)arylgroepen, C_7 – C_{24} alkyl(hetero)arylgroepen en C_7 – C_{24} (hetero)arylalkylgroepen;

- Y^2 $C(R^{31})_2$, O, S, $S^{(+)}R^{31}$, $S(O)R^{31}$, $S(O)=NR^{31}$ of NR^{31} is, waarbij $S^{(+)}$ een cationisch zwavelatoom is dat $B^{(-)}$ als tegenion heeft, en waarbij elke R^{31} onafhankelijk R^{15} of een connectie met Q^2 of D, via L, is;
- u = 0, 1, 2, 3, 4 of 5;
- u' = 0, 1, 2, 3, 4 of 5, waarbij u + u' = 0, 1, 2, 3, 4, 5, 6, 7 of 8;
- v = een integer in bereik van 8 – 16.

15. Het glycoproteïne-conjugaat volgens conclusie 13 of 14, waarbij Q^2 reactief in een cycloadditie is.

16. Het glycoproteïne-conjugaat volgens één van conclusies 13 – 15, waarbij het payload D gekozen wordt uit de groep bestaande uit een actieve verbinding, een reportermolecuul, een polymeer, een vast oppervlak, een hydrogel, een nanodeeltje, een microdeeltje en een biomolecuul.

17. Werkwijze voor de bereiding van een glycoproteïne-conjugaat, omvattende het laten reageren van een glycoproteïne volgens structuur (**1a**) volgens één van conclusies 13 – 16, met een payload met structuur D–F^2 of F^2–L^2–$(D)_x$, waarbij F^2 reactief is ten opzichte van het chemische handvat Q^2 in een conjugatiereactie, bij voorkeur in een cycloadditie, L^2 een linker is en x is een integer in bereik van 1 – 4.

18. Farmaceutische samenstelling omvattende het glycoproteïne-conjugaat volgens to structuur (**1b**) volgens één van conclusies 13 – 16 een een farmaceutisch aanvaardbare drager.

19. Het glycoproteïne-conjugaat volgens structuur (**1b**) volgens één van conclusies 13 – 16 voor toepassing in de behandeling van een subject die dat nodig heeft, bij voorkeur in de behandeling van kanker.

20. Werkwijze voor de bereiding van een glycoproteïne-conjugaat, omvattende:
    (a) het verschaffen van een mutant-proteïne die in natieve vorm niet reactief is ten opzichte van oxidatieve enzymen die in staat is om tyrosine te oxideren, maar die reactief ten opzichte van zulke enzymen gemaakt is door het verschaffen van een gemuteerde vorm van het proteïne, waarbij een tyrosineresidu geïntroduceerd is op een niet-natieve positie van de aminozuursequentie van het proteïne alwaar het reactief is ten opzichte van oxidatieve enzymen die in staat zijn om tyrosine te oxideren;

(b) het converteren van de fenolgroep van het tyrosineresidu in een *ortho*-chinongroep door het in contact brengen van het proteïne met een oxidatief enzym dat in staat is om tyrosine te oxideren;

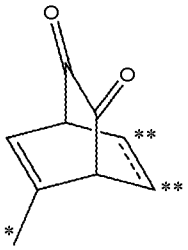
(c) het laten reageren van de *ortho*-chinongroep met een alkeen- of alkynverbinding via een [4+2] cycloadditie, waarbij de verbinding een (hetero)cycloalkeen- of een (hetero)cycloalkyngroep en (i) een chemisch handvat om de verbinding verder te modificeren met een payload, of (ii) een payload.

21. Een proteïne-conjugaat volgens structuur (**1a**) of (**1b**):

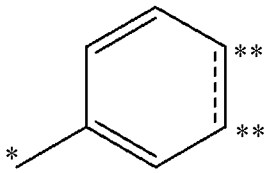
$Pr–[Z^1–L–(Q^2)_x]_y$ (**1a**) $Pr–[Z^1–L–(D)_x]_y$ (**1b**)

waarbij:

- Pr een proteïne is;
- Z^1 structuur (Za) of (Zb) omvat:



(Za)



(Zb)

waarbij het koolstofatoom gelabeld met * direct verbonden is met de peptideketen van het glycoproteïne aan een aminozuur dat in de natieve vorm van het proteïne geen tyrosineresidu is, en beide koolstofatomen gelabeld met ** verbonden zijn aan L, en de binding gelabeld ___ is een enkele of dubbele binding;

- L een linker is;
- x een integer in bereik van 1 – 4 is;
- y een integer in bereik van 1 – 4 is;
- Q^2 een chemisch handvat is dat reactief is ten opzichte van een op geschikte wijze gefunctionaliseerde payload is;
- D een payload is.

22. Het proteïne-conjugaat volgens conclusie 21, waarbij het aminozuur waaraan connectiegroep Z^1 verbonden is gelegen is op een positie waar een tyrosineresidu reactief is ten opzichte van oxidatieve enzymen die in staat zijn om tyrosine te oxideren rosine.

23. Het proteïne-conjugaat volgens conclusie 21 of 22, waarbij Pr een mutant-proteïne is, die in natieve vorm niet reactief is ten opzichte van oxidatieve enzymen die in staat is om tyrosine te oxideren, maar die reactief ten opzichte van zulke enzymen gemaakt is door het verschaffen van een gemuteerde vorm van het proteïne, waarbij een tyrosineresidu geïntroduceerd is op

een niet-natieve positie van de aminozuursequentie van het proteïne alwaar het reactief is ten opzichte van oxidatieve enzymen die in staat zijn om tyrosine te oxideren.

Fig. 1

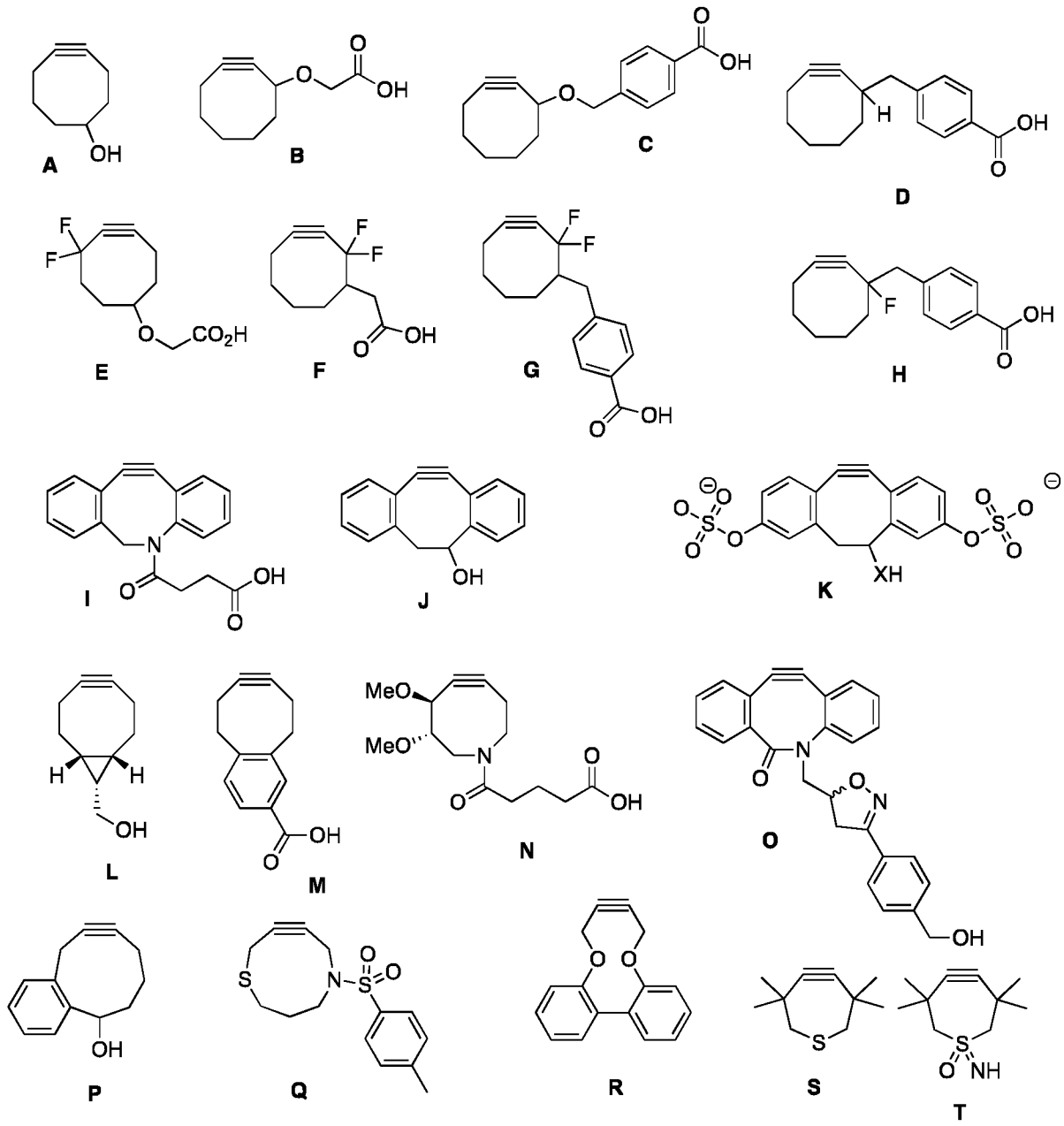


Fig. 2

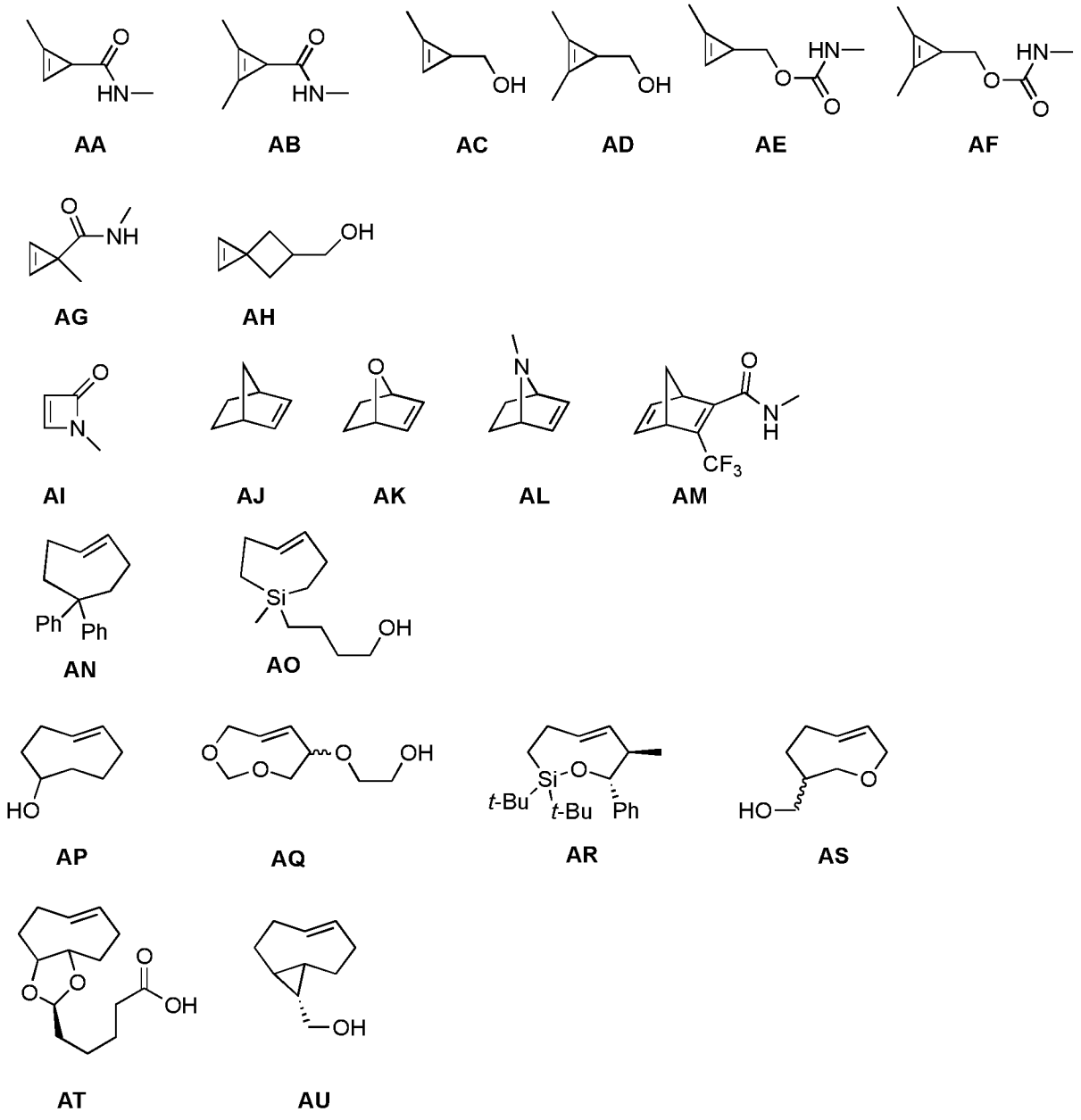


Fig. 3

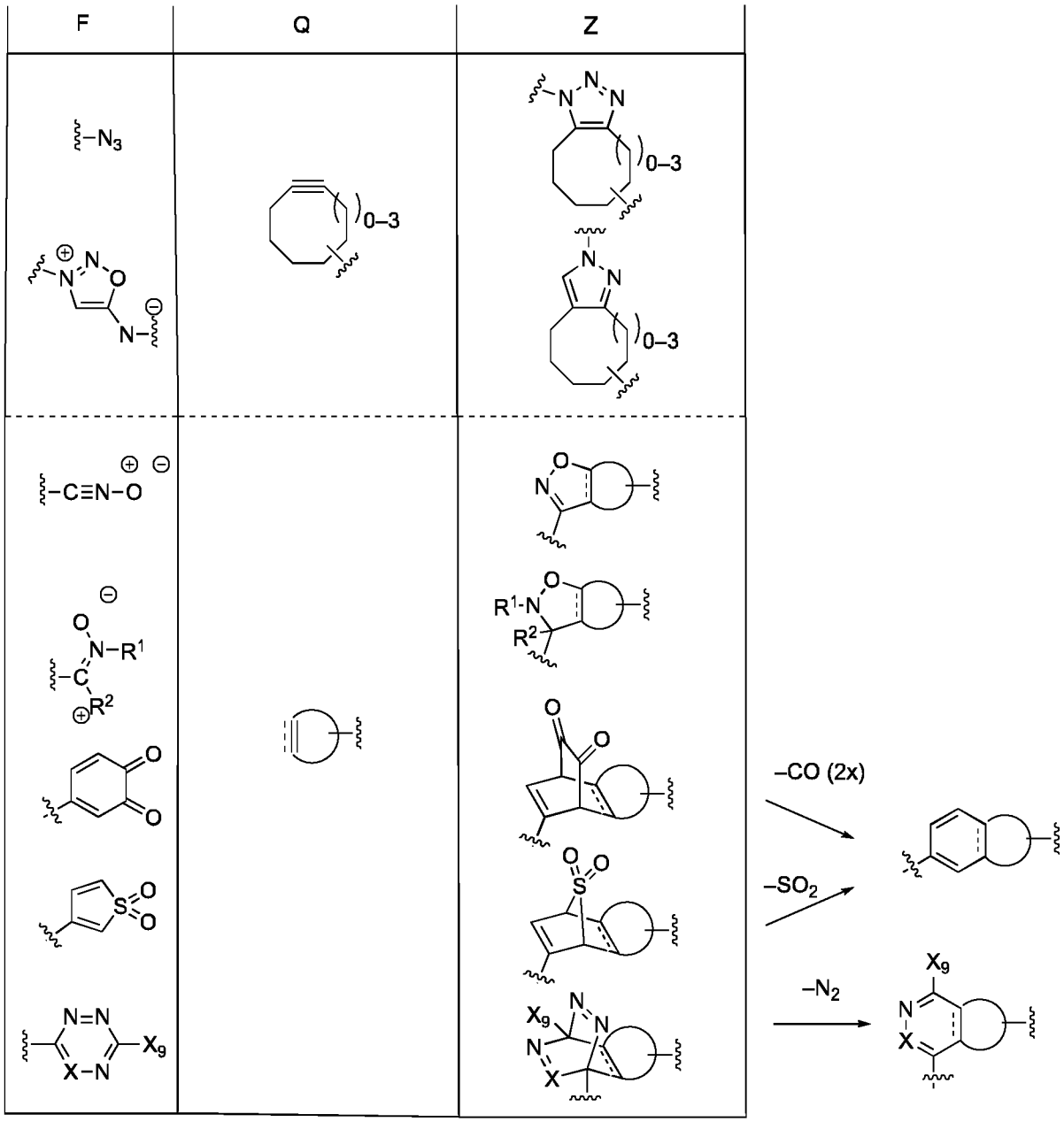


Fig. 4

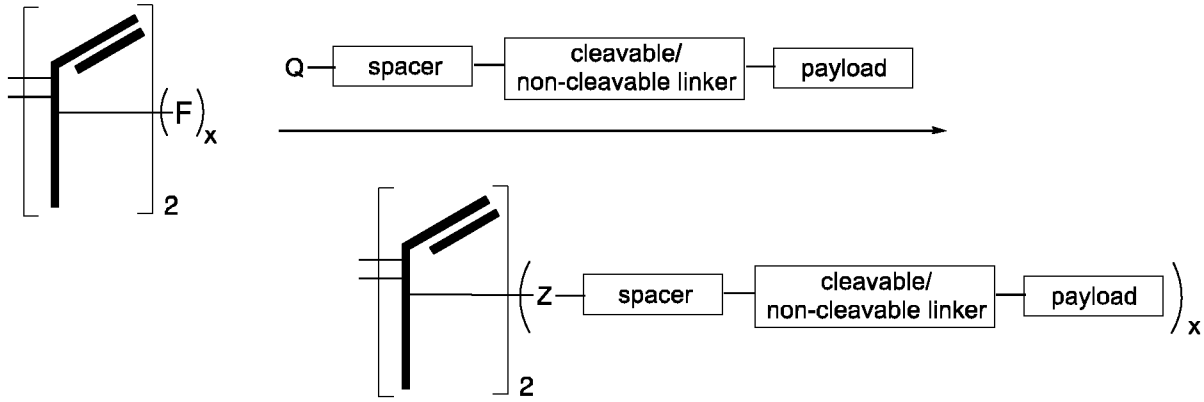

(F)x
2
Q
spacer
cleavable/
non-cleavable linker
payload
Z
spacer
cleavable/
non-cleavable linker
payload
x
2

Fig. 5

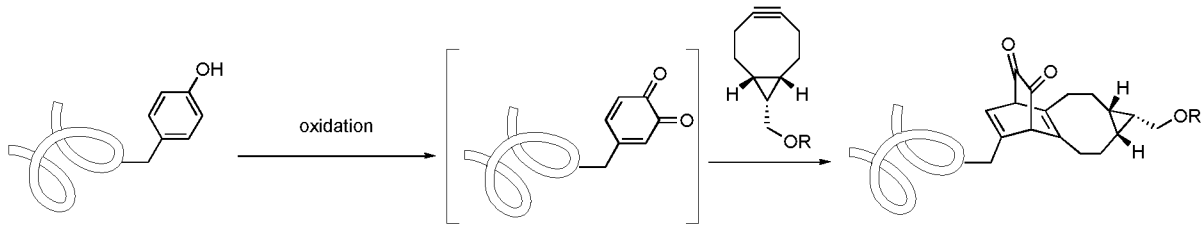

OH
oxidation
O
O
H
H
OR
O
O
H
OR
H

Fig. 6

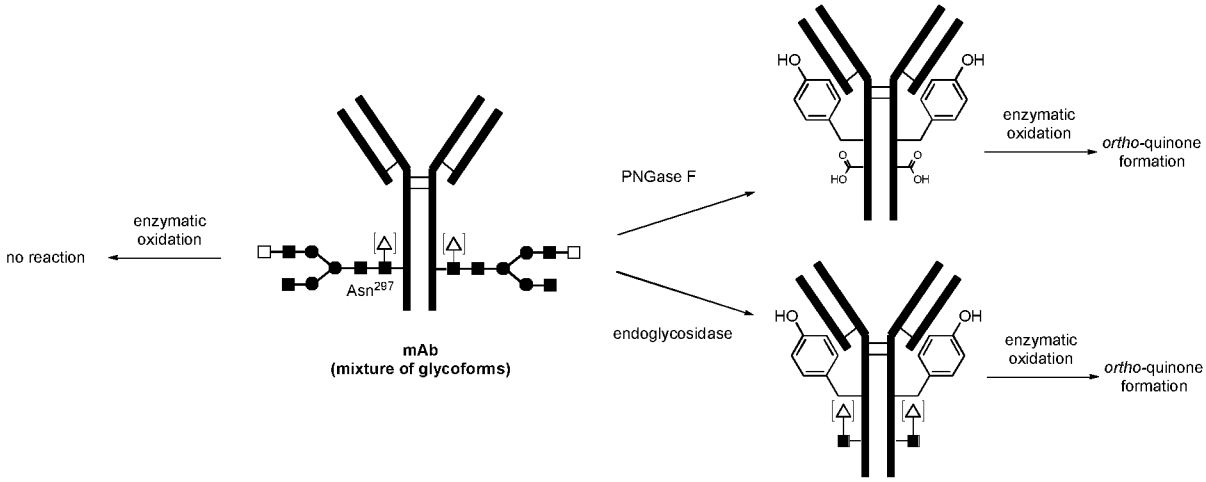

no reaction
enzymatic oxidation
Asn297
mAb
(mixture of glycoforms)
PNGase F
endoglycosidase
enzymatic oxidation
ortho-quinone formation
enzymatic oxidation
ortho-quinone formation

Fig. 7

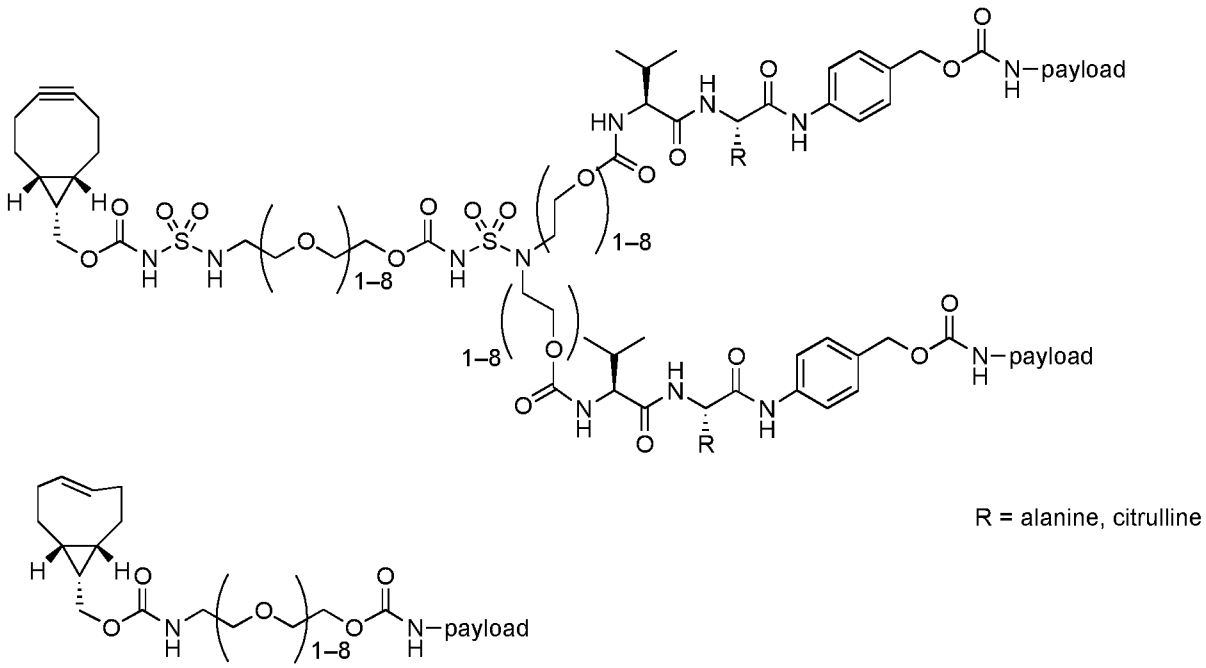

N–payload
1–8
1–8
N–payload
R = alanine, citrulline
1–8
N–payload

Fig. 8

Human

IgG1

```
231         241         251         261         271         281
APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK
291         301         311         321         331
PREEQ**Y**NST**Y** RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK
```

IgG2

```
APP_VAGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVQFNWYVD GVEVHNAKTK
PREEQFNSTF RVVSVLTVVH QDWLNGKEYK CKVSNKGLPA PIEKTISKTK
```

IgG3

```
APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVQFKWYVD GVEVHNAKTK
PREEQ**Y**NSTF RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKTK
```

IgG4

```
APEFLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSQED PEVQFNWYVD GVEVHNAKTK
PREEQFNST**Y** RVVSVLTVLH QDWLNGKEYK CKVSNKGLPS SIEKTISKAK
```

Mouse

IgG1

```
VPEV___SSV FIFPPKPKDV LTITLTPKVT CVVVDISKDD PEVQFSWFVD DVEVHTAQTQ
PREEQFNSTF RSVSELPIMH QDWLNGKEFK CRVNSAAFPA PIEKTISKTK
```

IgG2ab

```
APDLLGGPSV FIFPPKIKDV LMISLSPMVT CVVVDVSEDD PDVQISWFVN NVEVHTAQTQ
THRED**Y**NSTL RVVSALPIQH QDWMSGKEFK CKVNNRALPS PIEKTISKPR
```

IgG2aa

```
APNLLGGPSV FIFPPKIKDV LMISLSPIVT CVVVDVSEDD PDVQISWFVN NVEVHTAQTQ
THRED**Y**NSTL RVVSALPIQH QDWMSGKEFK CKVNNKDLPA PIERTISKPK
```

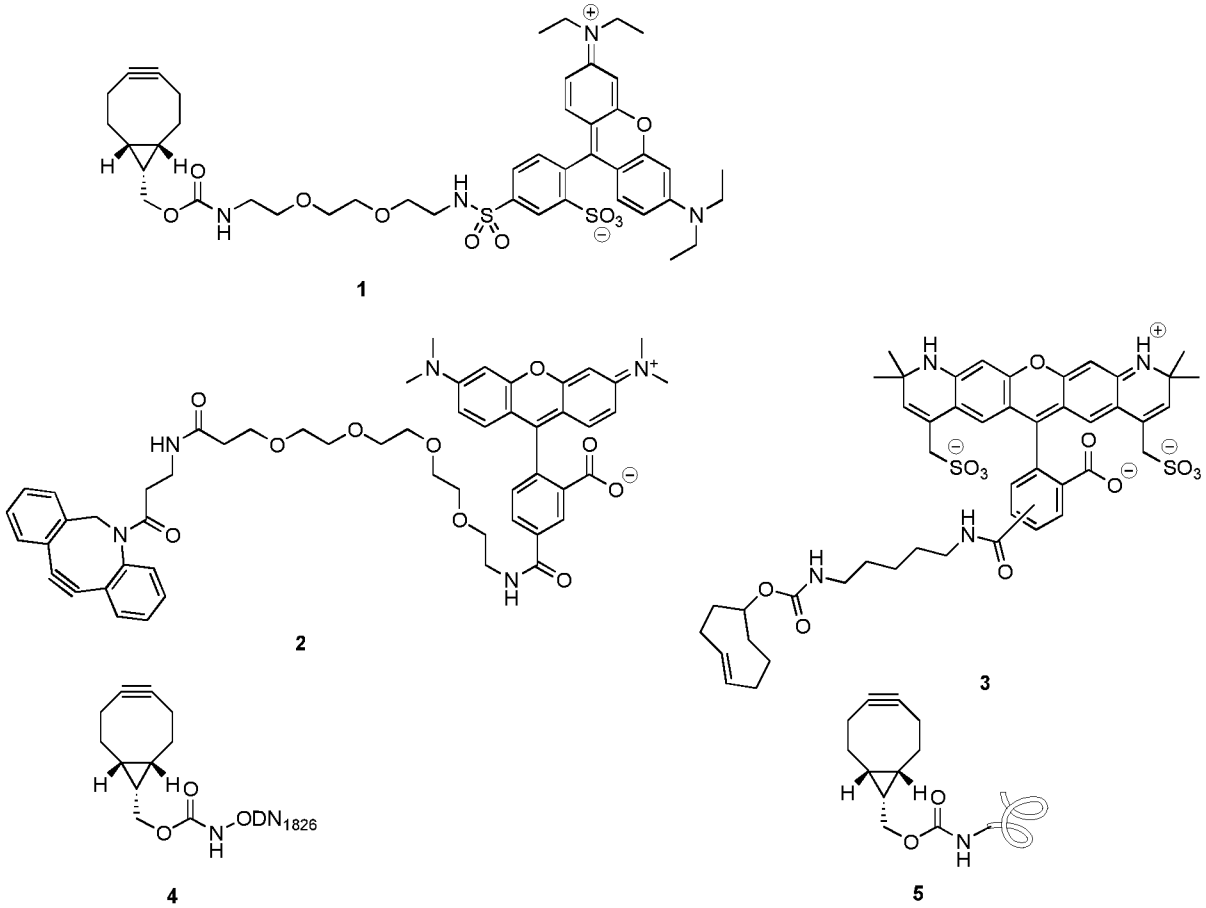
IgG2b

```
APNLEGGPSV FIFPPNIKDV LMISLTPKVT CVVVDVSEDD PDVQISWFVN NVEVHTAQTQ
THRED**Y**NSTI RVVSTLPIQH QDWMSGKEFK CKVNNKDLPS PIERTISKIK
```

IgG3

```
AGNILGGPSV FIFPPKPKDA LMISLTPKVT CVVVDVSEDD PDVHVSWFVD NKEVHTAWTQ
PREAQ**Y**NSTF RVVSALPIQH QDWMRGKEFK CKVNNKALPA PIERTISKPK
```

Fig. 9



ODN1826 = 5'-tccatgacgttcctgacgtt-3' (5'-OH, 3'-amino-modifier C6, full phosphorothioate

Fig. 10

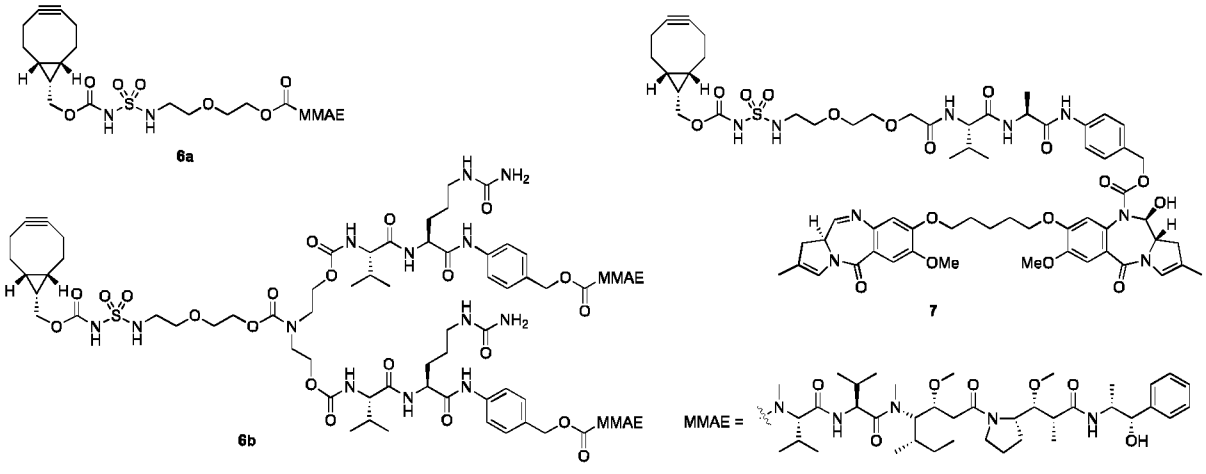


Fig. 11

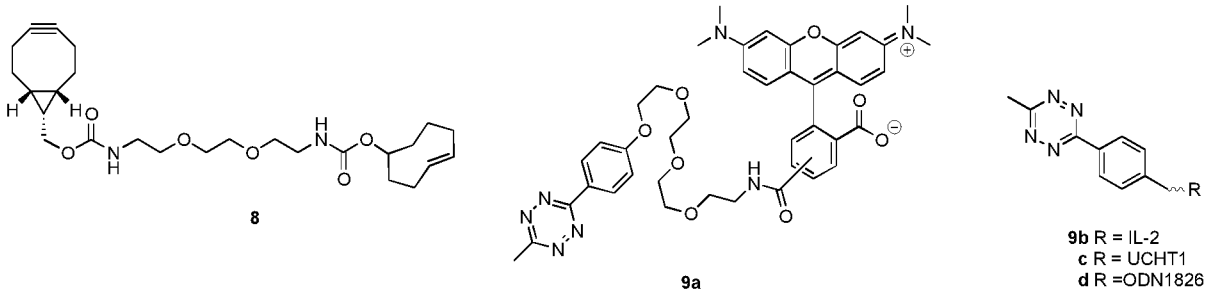

8
9a
9b R = IL-2
c R = UCHT1
d R =ODN1826

Fig. 12

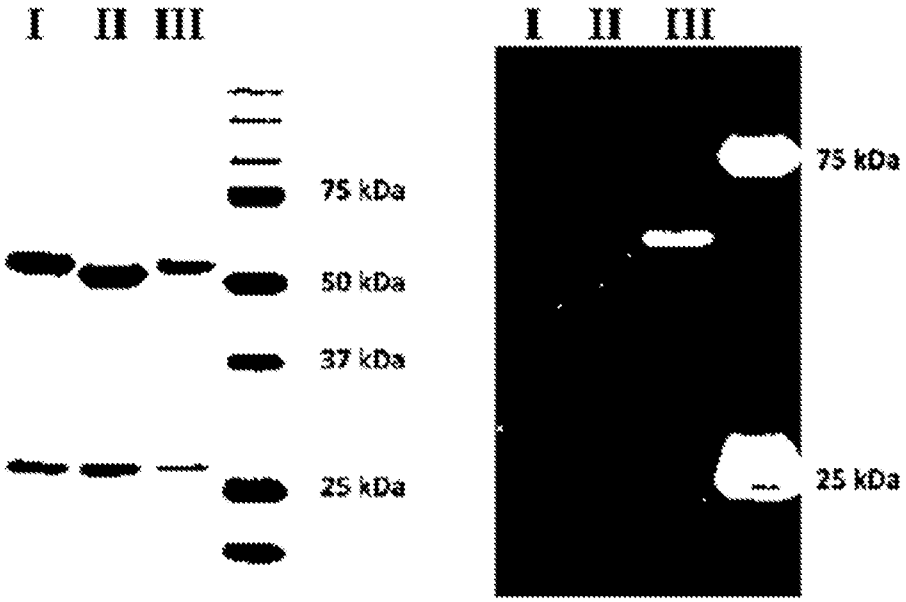

I II III
75 kDa
50 kDa
37 kDa
25 kDa
I II III
75 kDa
25 kDa

Fig. 13

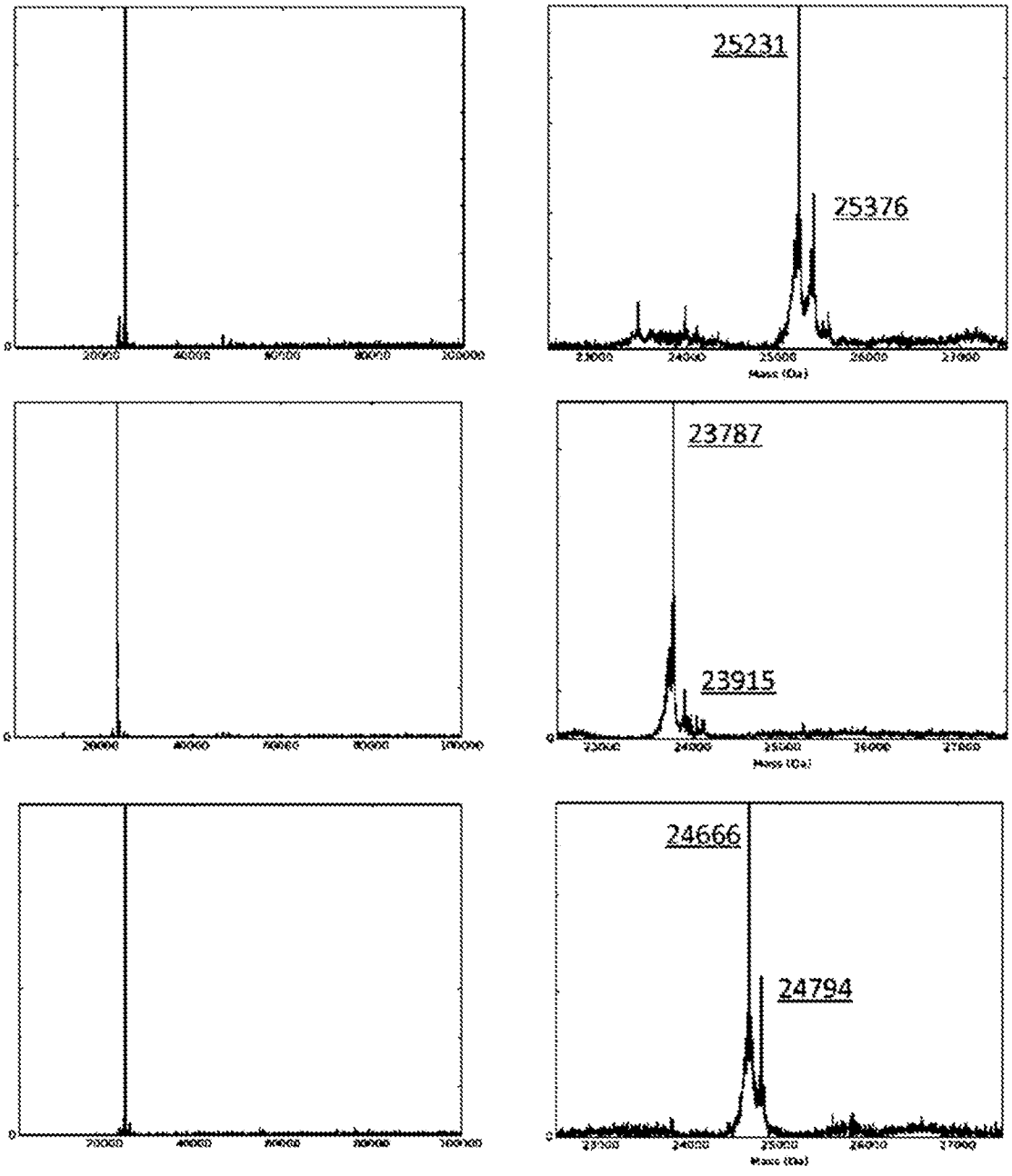


Fig. 14

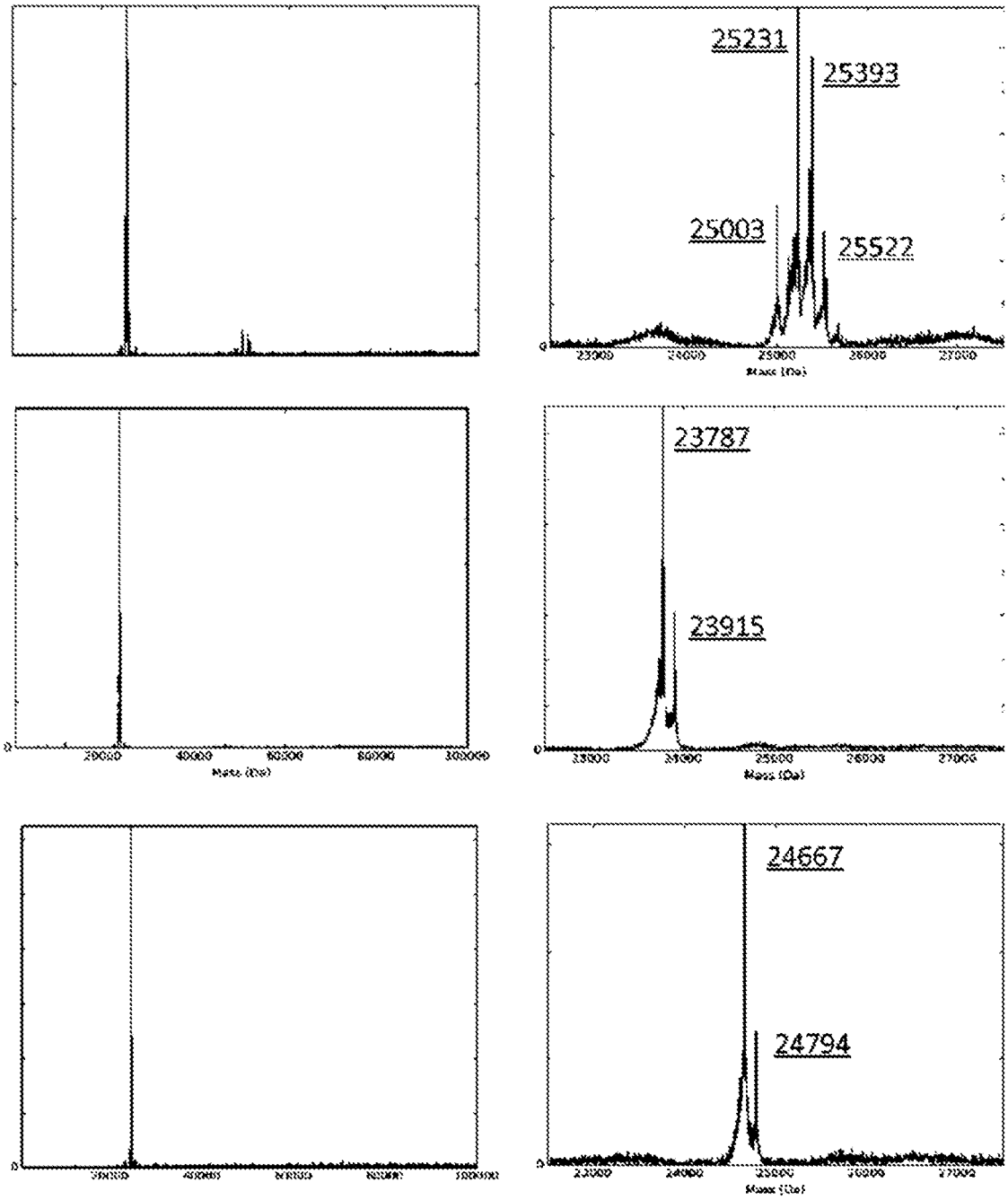


Fig. 15

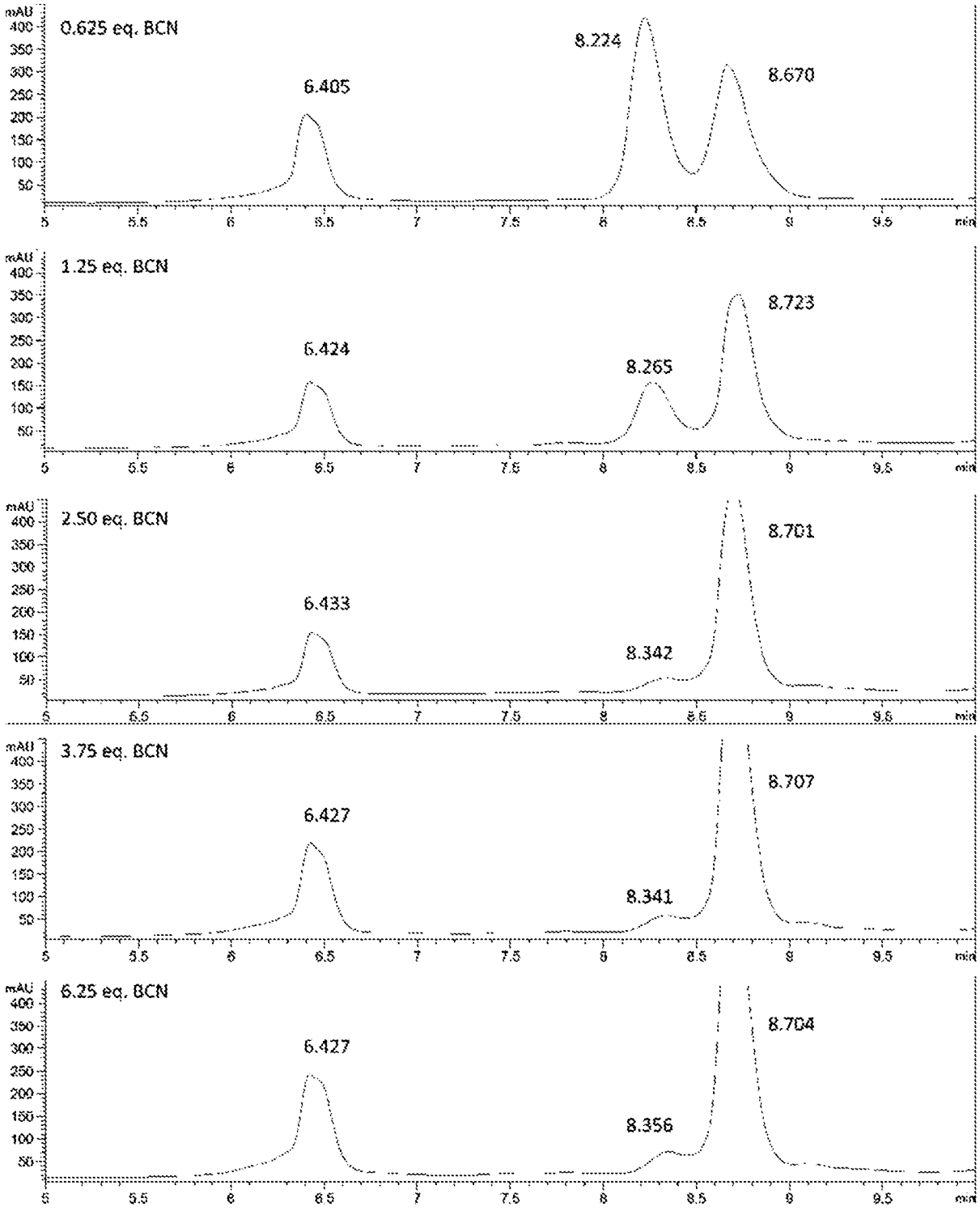

0.625 eq. BCN
6.405
8.224
8.670
1.25 eq. BCN
6.424
8.265
8.723
2.50 eq. BCN
6.433
8.342
8.701
3.75 eq. BCN
6.427
8.341
8.707
6.25 eq. BCN
6.427
8.356
8.704
mAU
min

Fig. 16

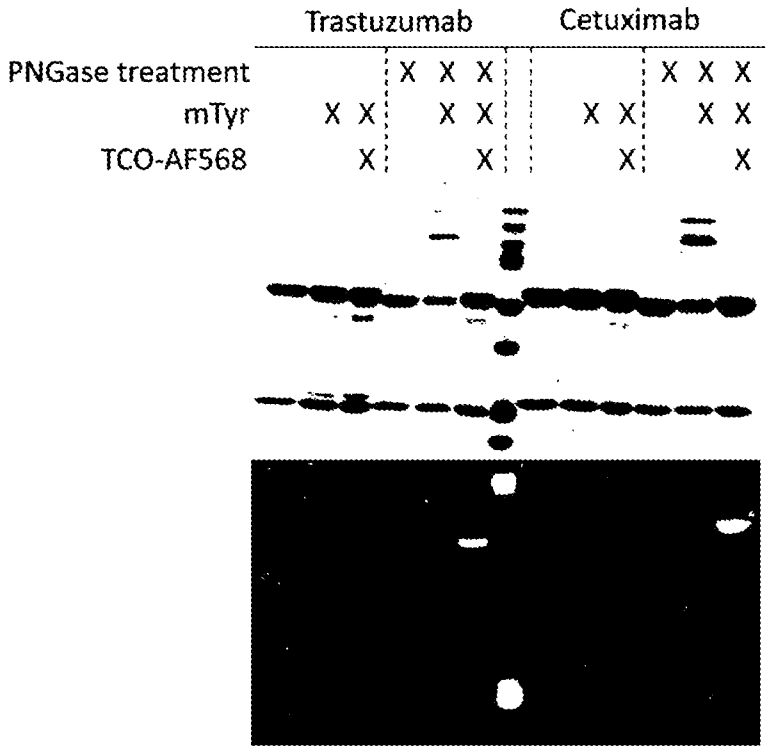

Trastuzumab
Cetuximab
PNGase treatment
mTyr
TCO-AF568
X X X X X X X X X X X X

Fig. 17

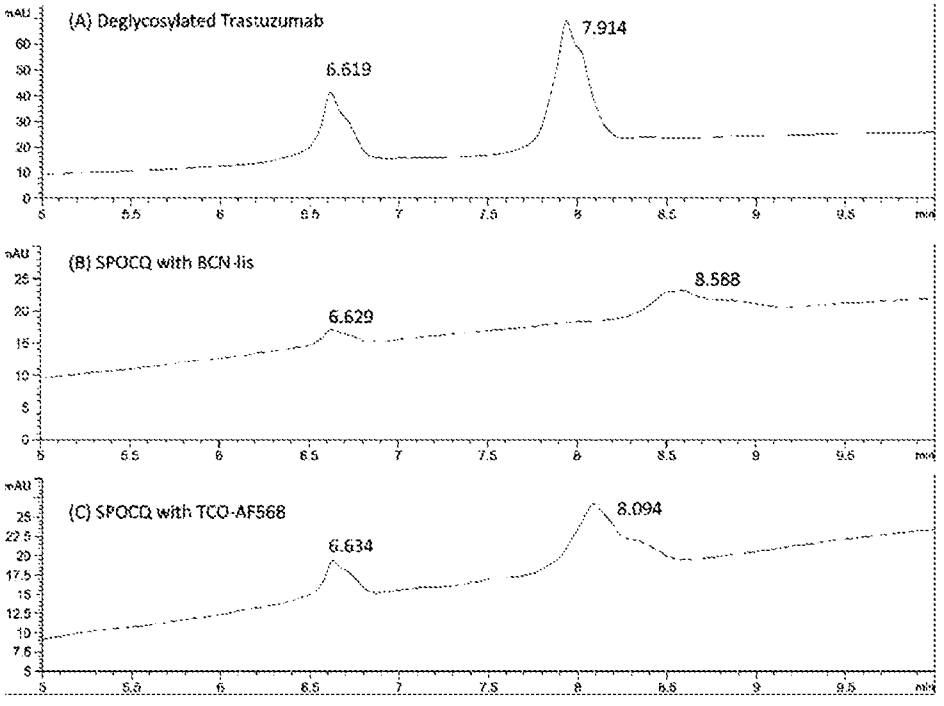

(A) Deglycosylated Trastuzumab
6.619
7.914
(B) SPOCQ with BCN-lis
6.629
8.588
(C) SPOCQ with TCO-AF568
6.634
8.094
mAU
min

Fig. 18

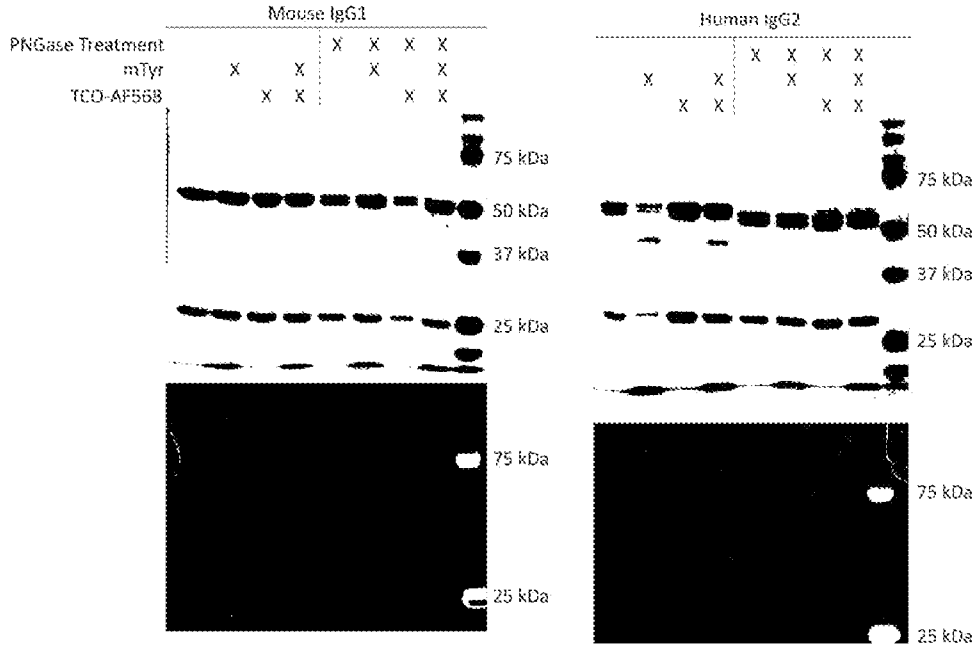

Mouse IgG1
Human IgG2
PNGase Treatment
mTyr
TCO-AF568
75 kDa
50 kDa
37 kDa
25 kDa
75 kDa
25 kDa
75 kDa
50 kDa
37 kDa
25 kDa
75 kDa
25 kDa

Fig. 19

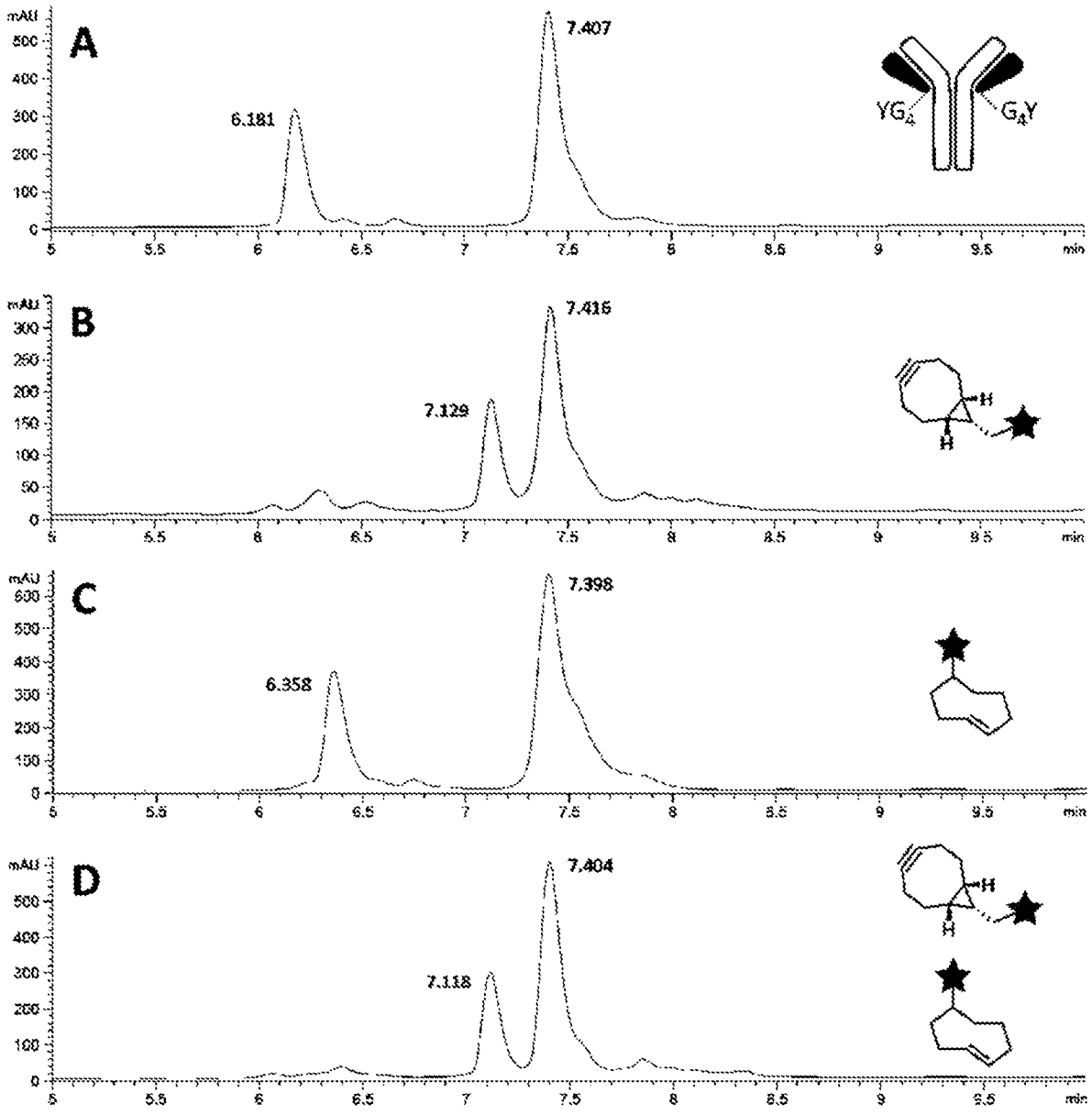


Fig. 20

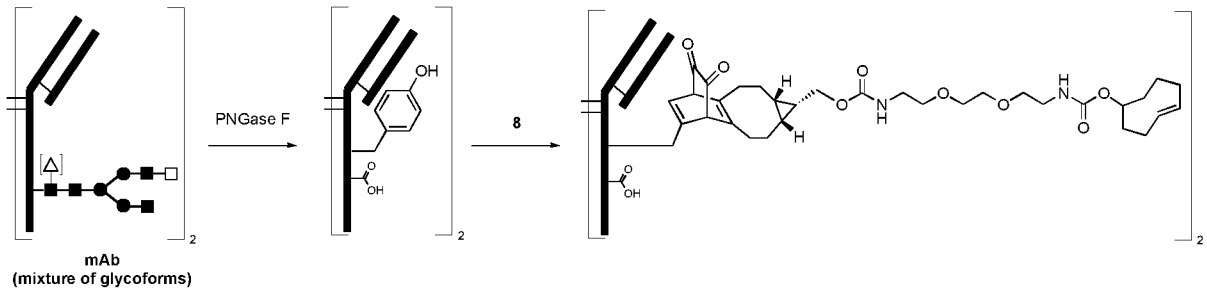

PNGase F
8
mAb
(mixture of glycoforms)

Fig. 21

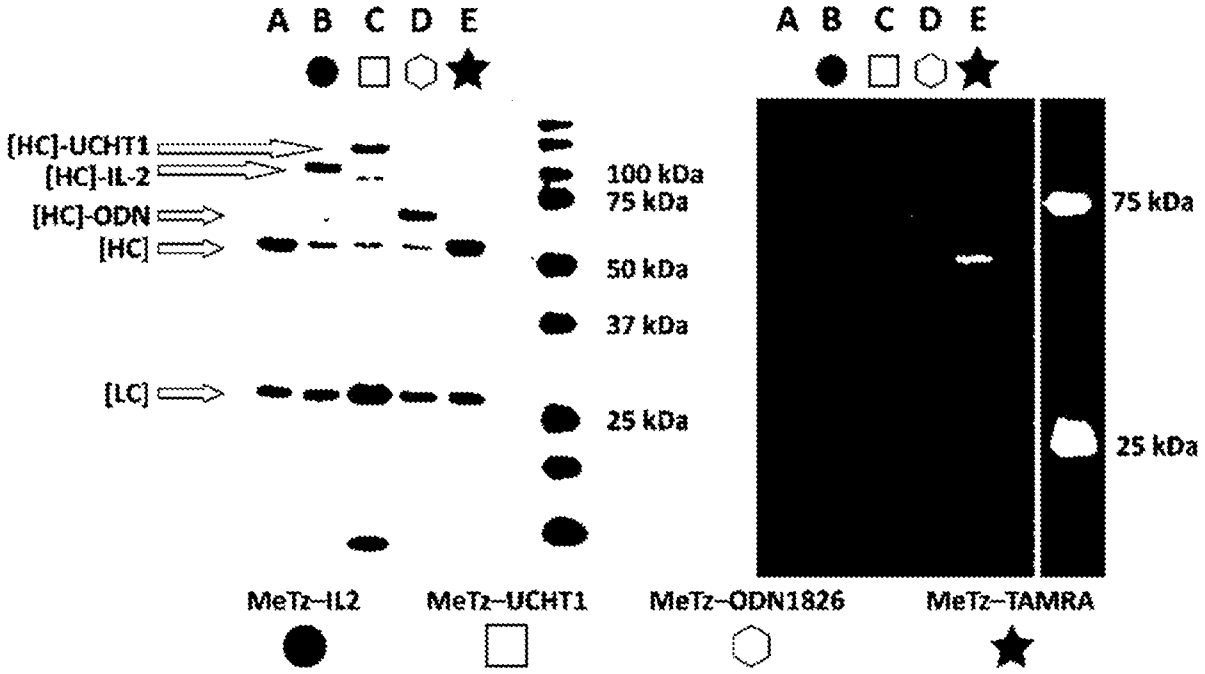

A B C D E
[HC]-UCHT1
[HC]-IL-2
[HC]-ODN
[HC]
[LC]
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
A B C D E
75 kDa
25 kDa
MeTz–IL2
MeTz–UCHT1
MeTz–ODN1826
MeTz–TAMRA

Fig. 22

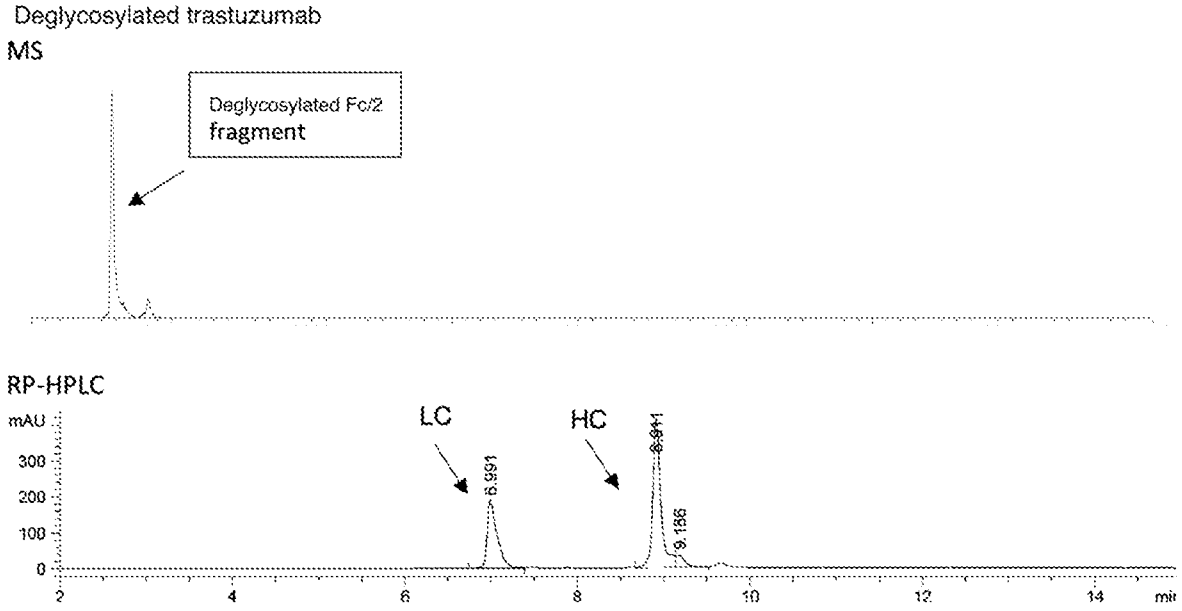

Deglycosylated trastuzumab
MS
Deglycosylated Fc/2
fragment
RP-HPLC
mAU
300
200
100
0
LC
HC
6.991
9.186
2
4
6
8
10
12
14
min

Fig. 23

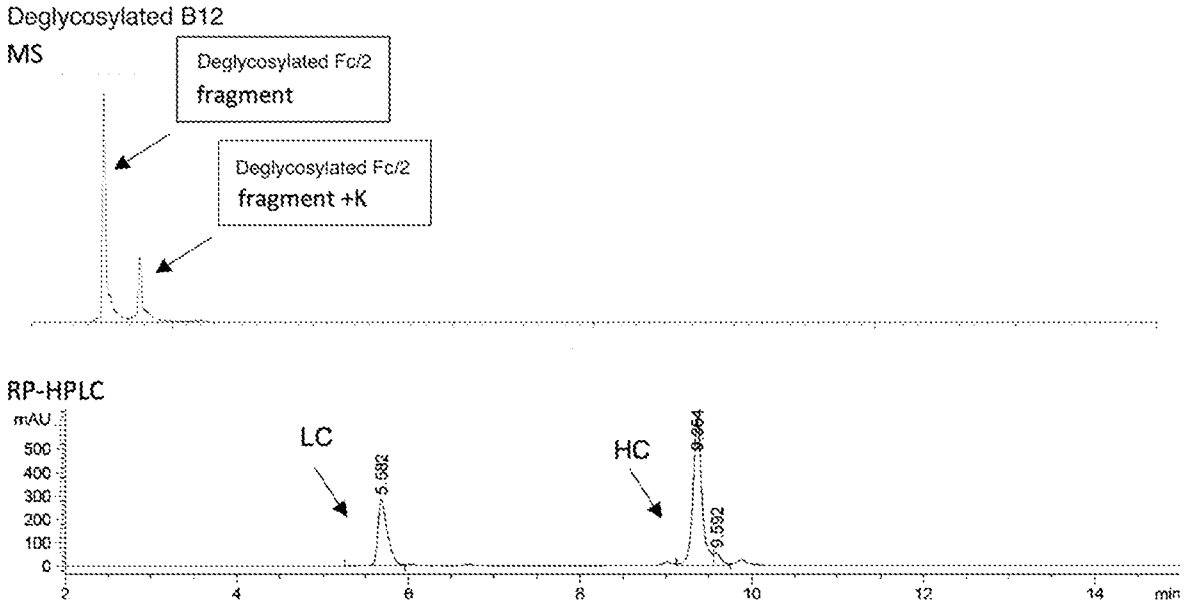

Deglycosylated B12
MS
Deglycosylated Fc/2
fragment
Deglycosylated Fc/2
fragment +K
RP-HPLC
mAU
500
400
300
200
100
0
LC
HC
5.582
9.592
2
4
6
8
10
12
14
min

Fig. 24

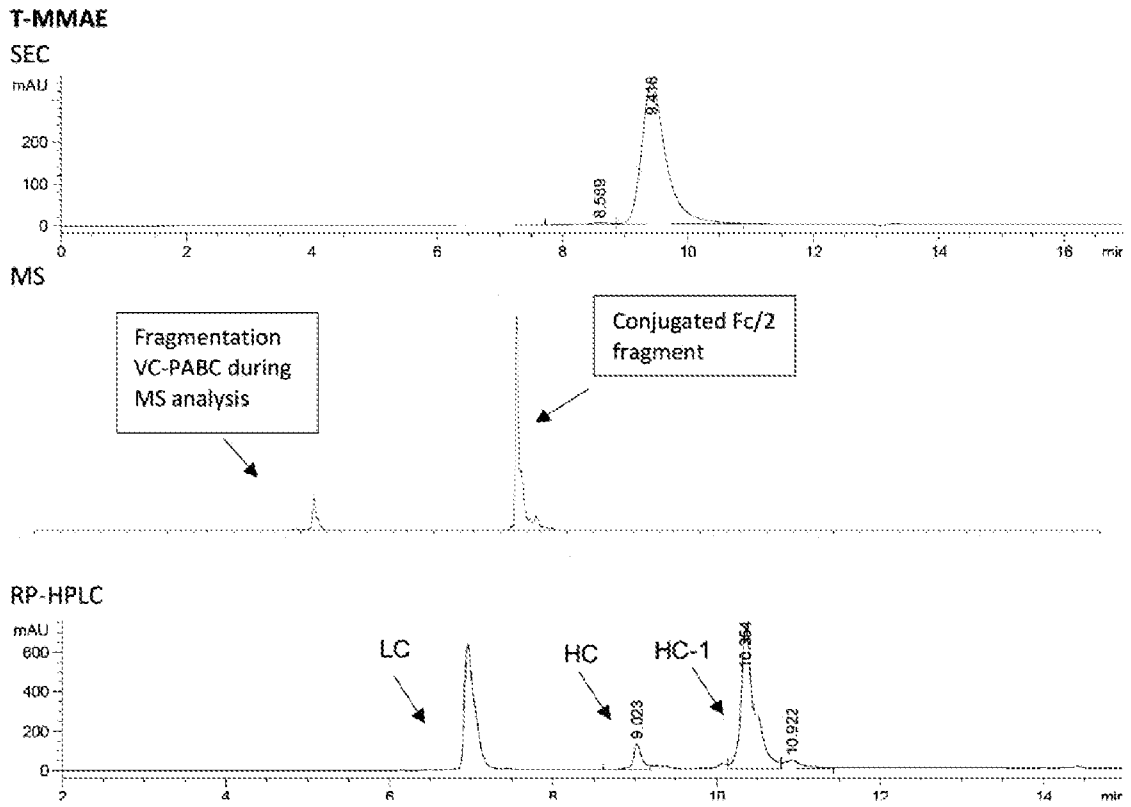

T-MMAE
SEC
mAU
8.589
9.416
MS
Fragmentation VC-PABC during MS analysis
Conjugated Fc/2 fragment
RP-HPLC
mAU
LC
HC
HC-1
9.023
10.364
10.922
min

Fig. 25

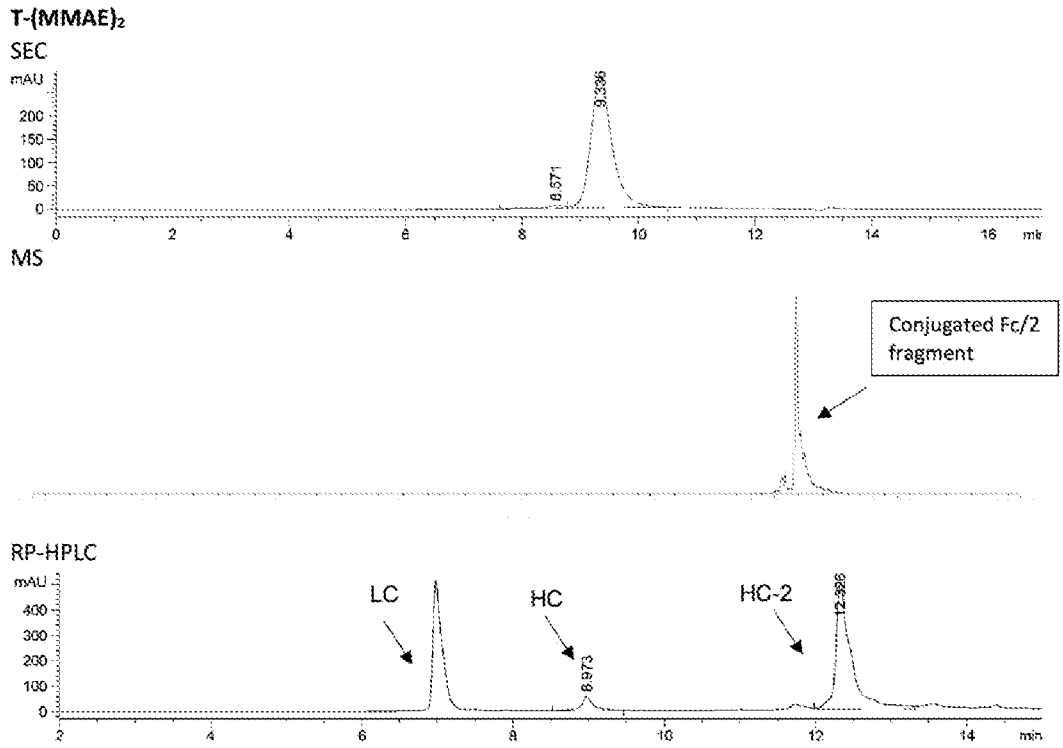

T-(MMAE)2
SEC
mAU
8.571
9.396
MS
Conjugated Fc/2 fragment
RP-HPLC
mAU
LC
HC
HC-2
8.973
12.386
min

Fig. 26

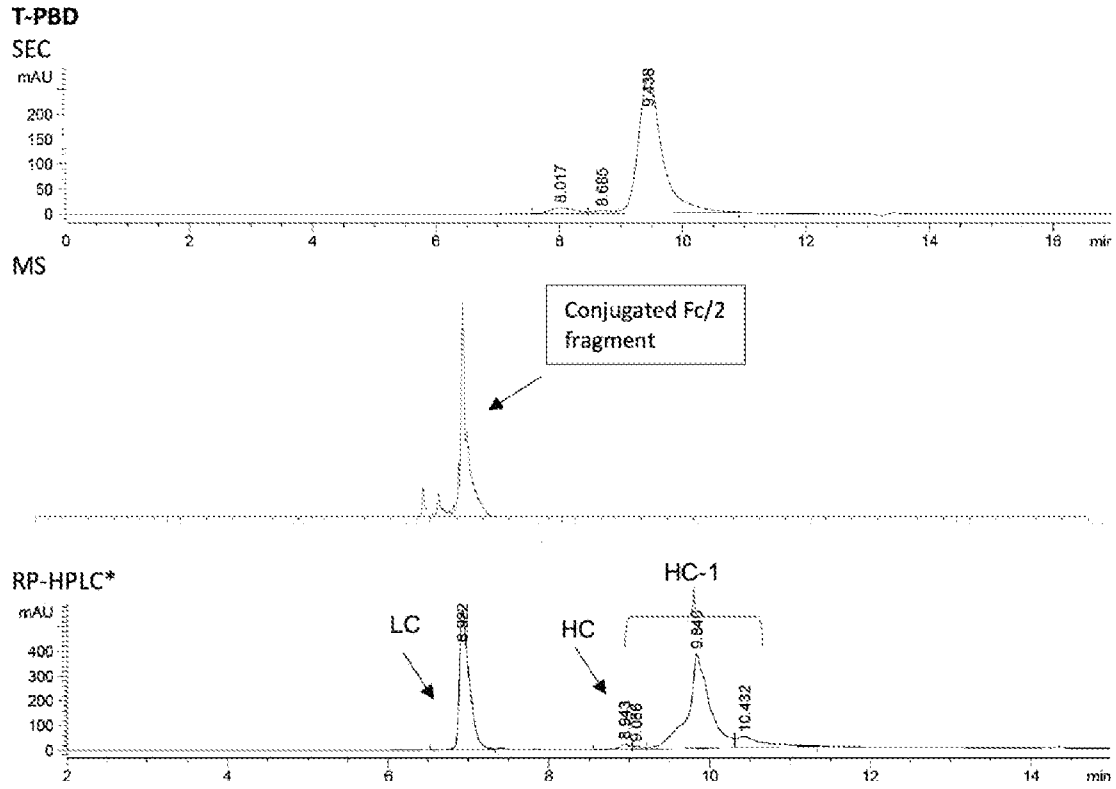

T-PBD
SEC
mAU
200
150
100
50
0
8.017
8.685
9.438
0
2
4
6
8
10
12
14
16
min
MS
Conjugated Fc/2 fragment
RP-HPLC*
mAU
400
300
200
100
0
LC
HC
HC-1
8.922
9.843
10.432
2
4
6
8
10
12
14
min

Fig. 27

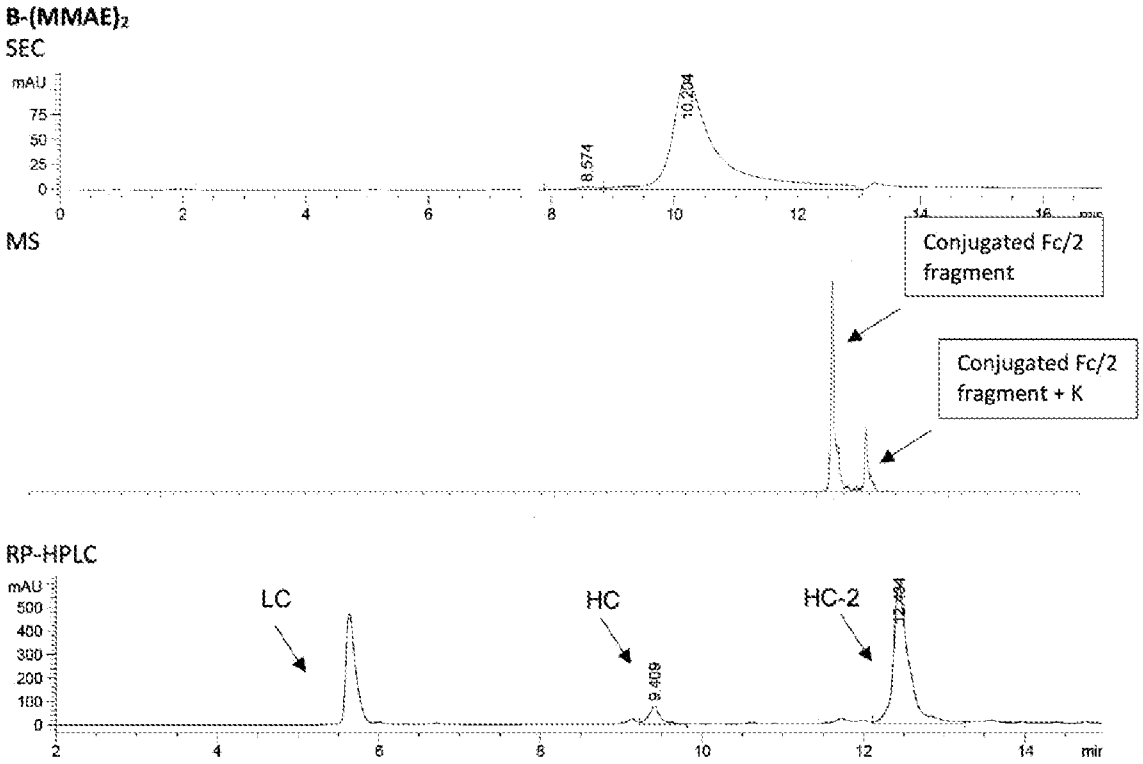

B-(MMAE)2
SEC
mAU
75
50
25
0
8.574
10.204
0
2
4
6
8
10
12
MS
Conjugated Fc/2 fragment
Conjugated Fc/2 fragment + K
RP-HPLC
mAU
500
400
300
200
100
0
LC
HC
HC-2
9.409
12.494
2
4
6
8
10
12
14
min

Fig. 28

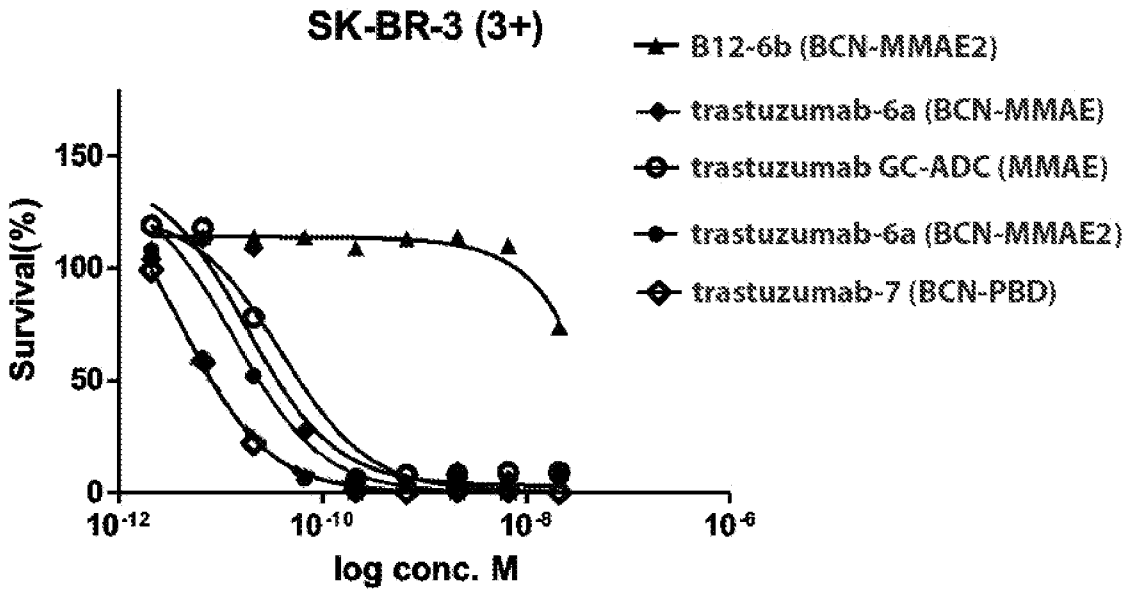

SK-BR-3 (3+)
B12-6b (BCN-MMAE2)
trastuzumab-6a (BCN-MMAE)
trastuzumab GC-ADC (MMAE)
trastuzumab-6a (BCN-MMAE2)
trastuzumab-7 (BCN-PBD)
Survival(%)
150
100
50
0
10-12
10-10
10-8
10-6
log conc. M

Fig. 29

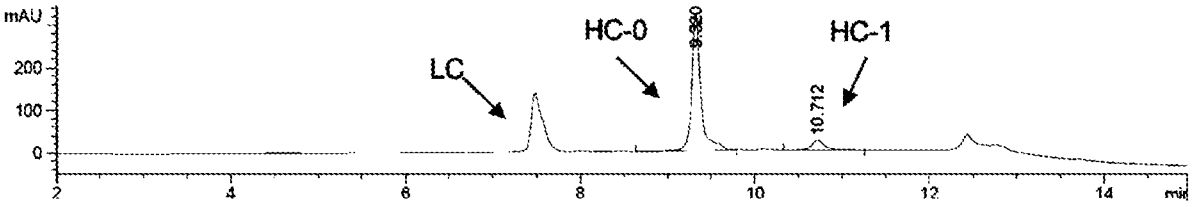

mAU
200
100
0
LC
HC-0
HC-1
10.712
2
4
6
8
10
12
14
min

Fig. 30

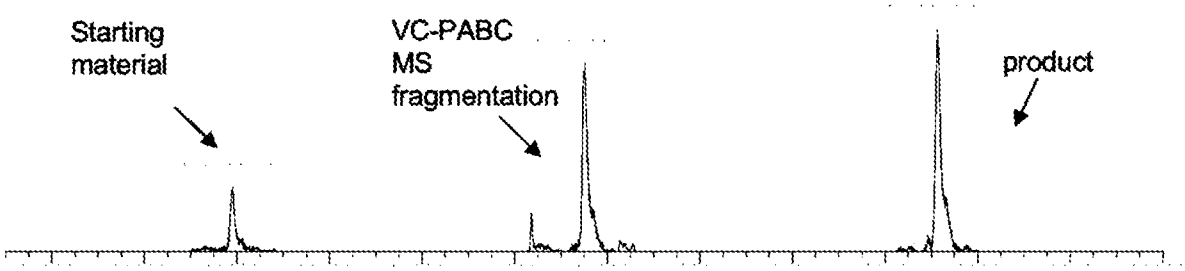
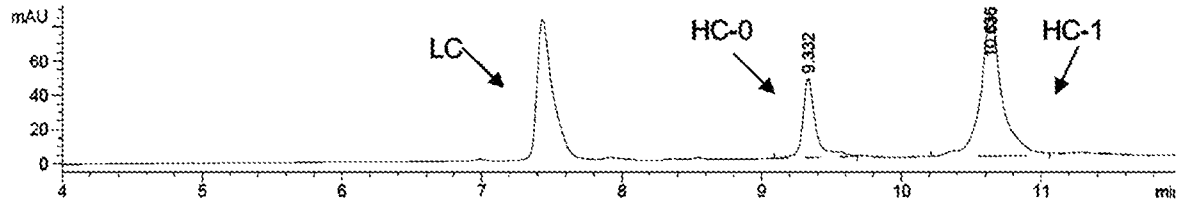

Starting material
VC-PABC MS fragmentation
product

Fig. 31


mAU
60
40
20
0
LC
HC-0
9.332
HC-1
4
5
6
7
8
9
10
11

SAMENWERKINGSVERDRAG (PCT)

RAPPORT BETREFFENDE NIEUWHEIDSONDERZOEK VAN INTERNATIONAAL TYPE

IDENTIFICATIE VAN DE NATIONALE AANVRAGE	KENMERK VAN DE AANVRAGER OF VAN DE GEMACHTIGDE
Nederlands aanvraag nr. **2026947**	Indieningsdatum **20-11-2020**
	Ingeroepen voorrangsdatum
Aanvrager (Naam) **Synaffix B.V.**	
Datum van het verzoek voor een onderzoek van internationaal type **05-06-2021**	Door de Instantie voor Internationaal Onderzoek aan het verzoek voor een onderzoek van internationaal type toegekend nr. **SN78780**

I. CLASSIFICATIE VAN HET ONDERWERP (bij toepassing van verschillende classificaties, alle classificatiesymbolen opgeven)

Volgens de internationale classificatie (IPC)

Zie onderzoeksrapport

II. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte minimumdocumentatie

Classificatiesysteem	Classificatiesymbolen
IPC	**Zie onderzoeksrapport**

Onderzochte andere documentatie dan de minimum documentatie, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

III.	☐	**GEEN ONDERZOEK MOGELIJK VOOR BEPAALDE CONCLUSIES**	(opmerkingen op aanvullingsblad)
IV.	☐	**GEBREK AAN EENHEID VAN UITVINDING**	(opmerkingen op aanvullingsblad)

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2026947

A. CLASSIFICATIE VAN HET ONDERWERP
INV. A61K47/68 A61P35/00
ADD.

Volgens de Internationale Classificatie van octrooien (IPC) of zowel volgens de nationale classificatie als volgens de IPC.

B. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte miminum documentatie (classificatie gevolgd door classificatiesymbolen)
A61K A61P

Onderzochte andere documentatie dan de mimimum documentatie, voor dergelijke documenten, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

Tijdens het onderzoek geraadpleegde elektronische gegevensbestanden (naam van de gegevensbestanden en, waar uitvoerbaar, gebruikte trefwoorden)
EPO-Internal, BIOSIS, EMBASE, CHEM ABS Data

C. VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X	BRUINS JORICK J. ET AL: "Inducible, Site-Specific Protein Labeling by Tyrosine Oxidation-Strain-Promoted (4 + 2) Cycloaddition", BIOCONJUGATE CHEMISTRY, deel 28, nr. 4, 19 april 2017 (2017-04-19) , bladzijden 1189-1193, XP055824731, ISSN: 1043-1802, DOI: 10.1021/acs.bioconjchem.7b00046 Gevonden op het Internet: URL:https://pubs.acs.org/doi/pdf/10.1021/acs.bioconjchem.7b00046>	13-16, 18,19, 21-23
Y	* samenvatting * * figuren 1,3 *	1-12,17, 20
	----- -/--	

[X] Verdere documenten worden vermeld in het vervolg van vak C.

[X] Leden van dezelfde octrooifamilie zijn vermeld in een bijlage

° Speciale categorieën van aangehaalde documenten

"A" niet tot de categorie X of Y behorende literatuur die de stand van de techniek beschrijft

"D" in de octrooiaanvrage vermeld

"E" eerdere octrooi(aanvrage), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven

"L" om andere redenen vermelde literatuur

"O" niet-schriftelijke stand van de techniek

"P" tussen de voorrangsdatum en de indieningsdatum gepubliceerde literatuur

"T" na de indieningsdatum of de voorrangsdatum gepubliceerde literatuur die niet bezwarend is voor de octrooiaanvrage, maar wordt vermeld ter verheldering van de theorie of het principe dat ten grondslag ligt aan de uitvinding

"X" de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur

"Y" de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht

"&" lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

Datum waarop het onderzoek naar de stand van de techniek van internationaal type werd voltooid	Verzenddatum van het rapport van het onderzoek naar de stand van de techniek van internationaal type
12 oktober 2021	

Naam en adres van de instantie	De bevoegde ambtenaar
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Dullaart, Anwyn

1

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2026947

C.(Vervolg). VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X	Bruins J: "Inducible, Selective Labeling of Proteins via Enzymatic Oxidation of Tyrosine" In: "Enzyme-Mediated Ligation Methods", 4 juni 2019 (2019-06-04), Humana, US, XP055824726, ISBN: 978-1-4939-9546-2 deel 2012, bladzijden 357-368, DOI: 10.1007/978-1-4939-9546-2_18, Gevonden op het Internet: URL:https://link.springer.com/protocol/10.1007%2F978-1-4939-9546-2_18>	13-16, 18,19, 21-23
Y	* samenvatting * * figuren *	1-12,17, 20

X	BRUINS JORICK J. ET AL: "ortho -Quinones and Analogues Thereof: Highly Reactive Intermediates for Fast and Selective Biofunctionalization", CHEMISTRY - A EUROPEAN JOURNAL, deel 24, nr. 19, 19 december 2017 (2017-12-19), bladzijden 4749-4756, XP055824730, ISSN: 0947-6539, DOI: 10.1002/chem.201703919 Gevonden op het Internet: URL:https://api.wiley.com/onlinelibrary/tdm/v1/articles/10.1002%2Fchem.201703919>	13-16, 18,19, 21-23
Y	* samenvatting * * bladzijde 4750 - bladzijde 4752 * * Schemes *	1-12,17, 20

X	JORICK J. BRUINS ET AL: "Orthogonal, dual protein labelling by tandem cycloaddition of strained alkenes and alkynes to ortho -quinones and azides", CHEMICAL COMMUNICATIONS, deel 54, nr. 53, 1 januari 2018 (2018-01-01), bladzijden 7338-7341, XP055629136, ISSN: 1359-7345, DOI: 10.1039/C8CC02638F	13-16, 18,19, 21-23
Y	* bladzijde 7338 * * figuren *	1-12,17, 20

Y	WO 2018/146188 A1 (MEDIMMUNE LTD [GB]) 16 augustus 2018 (2018-08-16) * bladzijde 124 - bladzijde 126 *	1-23

	-/--	

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2026947

C.(Vervolg). VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
Y	TANG FENG ET AL: "Chemoenzymatic synthesis of glycoengineered IgG antibodies and glycosite-specific antibody-drug conjugates", NATURE PROTOCOLS, deel 12, nr. 8, 27 juli 2017 (2017-07-27), bladzijden 1702-1721, XP037551079, ISSN: 1754-2189, DOI: 10.1038/NPROT.2017.058 [gevonden op 2017-07-27] * samenvatting * * figuren * * bladzijde 1705 - bladzijde 1708 *	1-23
Y	US 2019/218291 A1 (VAN BERKEL PATRICIUS HENDRIKUS CORNELIS [CH] ET AL) 18 juli 2019 (2019-07-18) * bladzijde 13 - bladzijde 14 * * voorbeelden *	1-23
Y	WO 2017/137458 A1 (SYNAFFIX BV [NL]) 17 augustus 2017 (2017-08-17) * voorbeeld 7 *	1-23
Y	WO 2017/137457 A1 (SYNAFFIX BV [NL]) 17 augustus 2017 (2017-08-17) * voorbeelden *	1-23
Y	WO 2017/137423 A1 (SYNAFFIX BV [NL]) 17 augustus 2017 (2017-08-17) * voorbeeld 13 *	1-23
Y,D	WO 2017/137456 A1 (SYNAFFIX BV [NL]) 17 augustus 2017 (2017-08-17) in de aanvraag genoemd * voorbeeld 20 *	1-23
Y	KRISTENSEN LOTTE K. ET AL: "Site-specifically labeled 89 Zr-DFO-trastuzumab improves immuno-reactivity and tumor uptake for immuno-PET in a subcutaneous HER2-positive xenograft mouse model", THERANOSTICS, deel 9, nr. 15, 9 juni 2019 (2019-06-09), bladzijden 4409-4420, XP055849054, ISSN: 1838-7640, DOI: 10.7150/thno.32883 * samenvatting * * bladzijde 4411 * * figuren; tabellen *	1-23

-/--

1

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2026947

C.(Vervolg). VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
Y	AGNEW BRIAN: "A novel highly-efficient, site-specific, and directional antibody crosslinking method for the rapid production and screening of bispecific antibodies", MOLECULAR IMAGING AND BIOLOGY, deel 17, nr. 1, suppl.1, 26 oktober 2017 (2017-10-26), bladzijden S465-S465, XP055849065, & 2017 World Molecular Imaging Congress, WMIC 2017 20170913 to 20170916 Philadelphia, PA Gevonden op het Internet: URL:https://link.springer.com/content/pdf/10.1007/s11307-017-1138-y.pdf> * samenvatting * -----	1-23

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Informatie over leden van dezelfde octrooifamilie

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2026947

In het rapport genoemd octrooigeschrift	Datum van publicatie	Overeenkomend(e) geschrift(en)	Datum van publicatie
WO 2018146188	A1 16-08-2018	CN 110461366 A	15-11-2019
		EP 3579882 A1	18-12-2019
		JP 2020506951 A	05-03-2020
		US 2019358342 A1	28-11-2019
		WO 2018146188 A1	16-08-2018
US 2019218291	A1 18-07-2019	AU 2018253948 A1	19-09-2019
		BR 112019021880 A2	02-06-2020
		CA 3057748 A1	25-10-2018
		CN 110536703 A	03-12-2019
		EP 3612234 A1	26-02-2020
		JP 2020517652 A	18-06-2020
		KR 20190141666 A	24-12-2019
		US 2019218291 A1	18-07-2019
		US 2020129637 A1	30-04-2020
		WO 2018193102 A1	25-10-2018
WO 2017137458	A1 17-08-2017	CN 108883191 A	23-11-2018
		EP 3413916 A1	19-12-2018
		WO 2017137458 A1	17-08-2017
WO 2017137457	A1 17-08-2017	GEEN	
WO 2017137423	A1 17-08-2017	EP 3413922 A1	19-12-2018
		US 2019038765 A1	07-02-2019
		WO 2017137423 A1	17-08-2017
WO 2017137456	A1 17-08-2017	CN 109152844 A	04-01-2019
		EP 3413920 A1	19-12-2018
		JP 2019507741 A	22-03-2019
		US 2019262467 A1	29-08-2019
		WO 2017137456 A1	17-08-2017

WRITTEN OPINION

File No.	Filing date *(day/month/year)*	Priority date *(day/month/year)*	Application No.
SN78780	20.11.2020		NL2026947

International Patent Classification (IPC)
INV. A61K47/68 A61P35/00

Applicant
Synaffix B.V.

This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the application
- ☐ Box No. VIII Certain observations on the application

	Examiner
	Dullaart, Anwyn

Application number

WRITTEN OPINION

NL2026947

Box No. I Basis of this opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ on paper

   ☒ in electronic form

   c. time of filing/furnishing:

   ☐ contained in the application as filed.

   ☐ filed together with the application in electronic form.

   ☒ furnished subsequently for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes:	Claims	1-12, 17, 20
	No:	Claims	13-16, 18, 19, 21-23
Inventive step	Yes:	Claims	
	No:	Claims	1-23
Industrial applicability	Yes:	Claims	1-23
	No:	Claims	

2. Citations and explanations

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1 BRUINS JORICK J. ET AL: "Inducible, Site-Specific Protein Labeling by Tyrosine Oxidation-Strain-Promoted (4 + 2) Cycloaddition", BIOCONJUGATE CHEMISTRY, deel 28, nr. 4, 19 april 2017 (2017-04-19), bladzijden 1189-1193, XP055824731, ISSN: 1043-1802, DOI: 10.1021/acs.bioconjchem.7b00046, Gevonden op het Internet: URL:https://pubs.acs.org/doi/pdf/10.1021/acs.bioconjchem.7b00046

D2 Bruins J: "Inducible, Selective Labeling of Proteins via Enzymatic Oxidation of Tyrosine"
In: "Enzyme-Mediated Ligation Methods", 4 juni 2019 (2019-06-04), Humana, US, XP055824726, ISBN: 978-1-4939-9546-2, deel 2012, bladzijden 357-368, DOI: 10.1007/978-1-4939-9546-2_18, Gevonden op het Internet: URL:https://link.springer.com/protocol/10.1007%2F978-1-4939-9546-2_18

D3 BRUINS JORICK J. ET AL: "ortho -Quinones and Analogues Thereof: Highly Reactive Intermediates for Fast and Selective Biofunctionalization", CHEMISTRY - A EUROPEAN JOURNAL, deel 24, nr. 19, 19 december 2017 (2017-12-19), bladzijden 4749-4756, XP055824730, ISSN: 0947-6539, DOI: 10.1002/chem.201703919; Gevonden op het Internet: URL:https://api.wiley.com/onlinelibrary/tdm/v1/articles/10.1002%2Fchem.201703919

D4 JORICK J. BRUINS ET AL: "Orthogonal, dual protein labelling by tandem cycloaddition of strained alkenes and alkynes to ortho -quinones and azides", CHEMICAL COMMUNICATIONS, deel 54, nr. 53, 1 januari 2018 (2018-01-01), bladzijden 7338-7341, XP055629136, ISSN: 1359-7345, DOI: 10.1039/C8CC02638F

D5 WO 2018/146188 A1 (MEDIMMUNE LTD [GB]) 16 augustus 2018 (2018-08-16)

D6 TANG FENG ET AL: "Chemoenzymatic synthesis of glycoengineered IgG antibodies and glycosite-specific antibody-drug conjugates", NATURE PROTOCOLS, deel 12, nr. 8, 27 juli 2017 (2017-07-27), bladzijden 1702-1721, XP037551079, ISSN: 1754-2189, DOI: 10.1038/NPROT.2017.058

D7 US 2019/218291 A1 (VAN BERKEL PATRICIUS HENDRIKUS CORNELIS [CH] ET AL) 18 juli 2019 (2019-07-18)

D8 WO 2017/137458 A1 (SYNAFFIX BV [NL]) 17 augustus 2017 (2017-08-17)

D9 WO 2017/137457 A1 (SYNAFFIX BV [NL]) 17 augustus 2017 (2017-08-17)

D10 WO 2017/137423 A1 (SYNAFFIX BV [NL]) 17 augustus 2017 (2017-08-17)

D11 WO 2017/137456 A1 (SYNAFFIX BV [NL]) 17 augustus 2017 (2017-08-17) in de aanvraag genoemd

D12 KRISTENSEN LOTTE K. ET AL: "Site-specifically labeled 89 Zr-DFO-trastuzumab improves immuno-reactivity and tumor uptake for immuno-PET in a subcutaneous HER2-positive xenograft mouse model", THERANOSTICS, deel 9, nr. 15, 9 juni 2019 (2019-06-09), bladzijden 4409-4420, XP055849054, ISSN: 1838-7640, DOI: 10.7150/thno.32883

D13 AGNEW BRIAN: "A novel highly-efficient, site-specific, and directional antibody crosslinking method for the rapid production and screening of bispecific antibodies", MOLECULAR IMAGING AND BIOLOGY, deel 17, nr. 1, suppl.1, 26 oktober 2017 (2017-10-26), bladzijden S465-S465, XP055849065, & 2017 World Molecular Imaging Congress, WMIC 2017 20170913 to 20170916 Philadelphia, PA
Gevonden op het Internet:
URL:https://link.springer.com/content/pdf/10.1007/s11307-017-1138-y.pdf

Present claims 1-12, 17 and 20 define a method for preparing a protein conjugate, wherein the protein is first "trimmed" to reduce the length of the N-glycosylation spots, followed by the oxidation of a tyrosine residue to an ortho-quinone moiety, which is subsequently used for conjugation through a [4+2] cycloaddition.

Claims 13-16 and 21-23 define the conjugate thus prepared.

Claim 18 defines a pharmaceutical composition containing the conjugate, and claim 19 defines its use in medicine, preferably in the treatment of cancer.

Conjugates in which an active agent is attached to the tyrosine residue of an antibody have, however, already been prepared in the cited prior art.

D1 describes the conjugation to the tyrosine of an antibody by cycloaddition of the resulting 1,2-quinone, after oxidation of tyrosine with mushroom tyrosinase.

According to D2, strain-promoted oxidation-controlled ortho-quinone cycloaddition (SPOCQ) is a reaction based on the facile (4 + 2) cycloaddition of an ortho-quinone (also known as 1,2-quinone) with a strained alkyne or strained alkene (see e.g. Figure 1). The enzyme mushroom tyrosinase generates a quinone by oxidizing the tyrosine, which in turn can perform strain-promoted oxidation-controlled ortho-quinone cycloaddition (SPOCQ) with strained alkynes and alkenes, generating a stable conjugation product.

In D3, ortho-quinones are formed by stepwise oxidation of phenols to 1,2-catechols to quinones by for example, mushroom tyrosinase. The central feature of these molecules is a cyclic 3,5-dien-1-one system that is conjugated in an exocyclic manner to either an additional ketone (ortho-quinone), an imine (ortho-iminoquinone) or an alkene (ortho-quinone methide) functionality (Figure 1).

D4 demonstrated that strain-promoted oxidation-controlled ortho-quinone cycloaddition (SPOCQ) with cyclooctyne can be successfully applied for the site-specific conjugation of a cyclooctyne-functionalized fluorophore or a toxic payload to tyrosine-engineered proteins, including the monoclonal antibody trastuzumab.

The conjugates disclosed in these documents are used for the treatment of cancer.

Thus, in view of these documents, present claims 13-16, 18, 19 and 21-23 do not meet the requirements for novelty.

The process for preparing the conjugates as defined in claims 1-12, 17 and 20 can be distinguished from the disclosure of D1-D4 by the fact that the glycoprotein is first "trimmed", to reduce the length of the saccharide chains.

The closest prior art for the process of preparation is found in any of D1 to D4, which each disclosed the conjugation by [4+2] cycloaddition to the quinone formed from the tyrosine residue by oxidation. The presently claimed process has not been compared with the process as described in D1 to D4. Thus, the objective problem to be solved by the distinguishing technical feature is the provision of an alternative process for preparing antibody-drug conjugates.

Preparing an antibody for conjugation by previous trimming of the saccharide chains is, however, a well known technique, as is shown by D5 to D13.

D5 discloses antibody modification by a one-pot glycan remodelling reaction, using EndoSH.

D6 describes the preparation of glycosite-specific antibody-drug conjugates using endoglycosidases.

D7 discloses the preparation of antibody conjugates using endoglycosidase for trimming of all glycan isoforms.

In D8, example 7 describes the preparation of trimmed cAC10 by means of fusion protein EndoSH.

The preparation of trimmed trastuzumab by means of fusion protein EndoSH is described in D9 (example 6), D10 (example 13) and D11 (example 20).

D12 describes the site-specific conjugation to trastuzumab, using β-galactosidase or endoglycosidase S2 for enzymatically modifying glycans.

In D13, Fc domain antibody glycans are cleaved with endoglycosidase S2 (EndoS2) leaving single core GlcNAc residues at the core mAb glycosylation sites, for conjugation by click chemistry.

The skilled person, starting from any of D1-D4 as closest prior art, and wishing to provide an alternative method for preparing antibody-drug conjugates, would turn to any of D5-D13, which are also in the field of preparing antibody-drug conjugates, and apply its teachings, thus arriving at the presently claimed process without an efforts that could justify an inventive step.