

Hydrophilic trans-cyclooctene (hyTCO) compounds, constructs and conjugates containing the same

FIELD OF THE INVENTION

5 The invention relates to the field of bioconjugation of functional entities (such as payloads and targeting agents) mediated by trans-cyclooctene (TCO) compounds. More particularly the present invention relates to novel trans-cyclooctene compounds, unnatural amino acids comprising such trans-cyclooctene compounds and preparation thereof. Said novel trans-cyclooctene compounds and unnatural amino acids comprising such trans-
10 cyclooctene compounds are characterized by high structural stability and hydrophilicity and thus instrumental to ease the conjugation of said functional entities.

 The invention further relates to respective trans-cyclooctene-functionalized construct obtainable by, in particular covalently, binding said novel trans-cyclooctene compounds to a targeting agent or payload molecule, said functionalized construct containing at least one
15 functionalizing residue comprising a mono-unsaturated trans-cyclooctene entity.

 The invention further relates to conjugates obtainable by covalently linking, in particular, via biorthogonal bioconjugation via a Diels-Alder-type cycloaddition, a first functionalized molecule, particularly selected from said trans-cyclooctene-functionalized constructs with a second functionalized molecule comprising a docking group (DG) capable
20 of reacting with the trans-cyclooctene-type functional group of the first functionalized molecule.

 The present invention further relates to preparation methods of said constructs and conjugates.

 The invention also relates to the use of such conjugates of the present invention for
25 use in medicine, to corresponding pharmaceutical compositions as well as to corresponding diagnostic and analytical kits.

BACKGROUND OF THE INVENTION

 The strain promoted inverse electron demand Diels-Alder reaction (SPIEDAC) is
30 among the fastest bioorthogonal reactions and broadly applied for various applications in chemical biology, biotechnology, cell biology, life science, material sciences and nuclear medicine.

 The fast reaction kinetics of this bioorthogonal click reaction enable the use in extremely low concentrations, e.g. in living systems like cells or even for pretargeting in living
35 organisms. Additionally, it enables a most efficient bioconjugation reaction for production of

bioconjugates without much influence of the utilized buffer, pH or added solvents, e.g. for production of antibody drug conjugates (ADCs) for drug delivery. Additionally, the extremely fast reaction kinetics even in comparison to other click reactions like the CuAAC or SPAAC enable the use for *in vivo* click applications like pretargeting in nuclear medicine.

5 ADCs are a fast growing class of oncology therapeutics that perceive major attention, which is reflected in the growing number of approved ADC drugs and increasing numbers of clinical trials.

 The required conjugation of the respective payload to monoclonal antibodies (mAbs) is often done via random attachment. This typically leads to inhomogeneity regarding the present species of ADCs and Drug-to-Antibody Ratios (DARs) vary from completely unmodified mAbs to unfavorable high numbers of cytotoxic molecules attached, which leads to problems with batch-to-batch variability and can cause aggregation and concomitant side effects like fast clearance, immunogenicity or hepatotoxicity.

 Incorporation of strained cyclooctyne-lysine derivative for click reactions into proteins via genetic encoding (T. Plass, S. Milles, C. Koehler, C. Schultz, E. A. Lemke, Angew. Chem. Int. Ed. 2011, 50, 3878-81) and the synthesis and genetically encoding of ncAAs that can undergo (strain-promoted) inverse-electron-demand Diels-Alder cycloadditions (IEDDA) with 1,2,4,5-tetrazines has been reported (T. Plass, S. Milles, C. Koehler, J. Szymański, R. Mueller, et al., Angew. Chem. Int. Ed. 2012, 51, 4166-70; I. Nikić, T. Plass, O. Schraidt, J. Szymański, J. A. G. Briggs, et al., Angew. Chem. Int. Ed. 2014, 53, 2245-9; E. Kozma, I. Nikić, B. R. Varga, I. V. Aramburu, J. H. Kang, et al., ChemBioChem 2016, 17, 1518-24; J.-E. Hoffmann, T. Plass, I. Nikić, I. V. Aramburu, C. Koehler, et al., Chem. Eur. J. 2015, 21, 12266-70). Extension of this GCE technology led to the site-specific introduction of such ncAAs into unglycosylated immunoglobulins produced by insect cells and subsequent modification via this click chemistry (C. Koehler, P. F. Sauter, M. Wawryszyn, G. E. Girona, K. Gupta, et al., Nat. Methods 2016, 13, 997-1000.).

 Translational incorporation of non-canonical amino acids (ncAA) into polypeptides offers the most flexible and precise basis for modification of proteins via bioconjugation of various payloads.

30 This has been used for fluorescent labeling of proteins, RICs (also pretargeting), as well as drug delivery with ADCs.

 Amongst the various candidates to be possibly selected as bioconjugation mediating moieties, trans-cyclooctene (TCO) derivatives, such as TCO based ncAA (TCO-ncAA), were shown to have a much faster reaction kinetics as compared to other candidate molecules (N. K. Devaraj, et. al 2009).

The broad success of TCO-ncAA has been hampered by the fact, that these compounds all suffer from an intrinsic instability towards isomerization from the click-reactive *trans*- to the unreactive *cis*-isomers [(Béquignat et al., 2020; Cook et al., 2016; Darko et al., 2014; Keinänen et al., 2019; Lang et al., 2012; Meyer et al., 2017; Reinkemeier et al., 2021; 5 Rondon & Degoul, 2020; Rossin et al., 2010, 2013; Ruivo et al., 2019; Zeglis et al., 2015)]. The mechanism for this phenomenon is believed to be caused by presence of thiols as well as action of metallo-proteins [(Darko et al., 2014; Rossin et al., 2013)].

This has been shown to happen not only during expression in cell media but also in circulation in the blood stream of living organism (such as mice). Additionally, previously 10 introduced ncAA, bearing the amino acid handle at the 2-position are prone to eliminating the amino acid moiety after click reaction and are therefore not suited for modification of biomolecules [(Fan et al., 2016)].

For applications in *in vivo* labeling applications very fast reaction kinetics are of utmost importance, though.

15 On top, most of the TCO or cyclooctyne compounds are lipophilic moieties, causing unspecific binding and long washout times in fluorescent labeling applications and undesirable negative impact on the hydrophilicity of bioconjugates in targeted therapy, where increased hydrophilicity has been shown to have beneficial influence on properties of ADCs for targeted drug delivery.

20 Therefore, the full potential of this technique could not be accessed by incorporation of TCO based ncAAs, yet.

This is why there is a strong demand for improved hydrophilic and especially stable, highly reactive TCO derivatives and ncAA for protein expression via genetic code expansion (GCE).

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SUMMARY OF THE INVENTION

The above-mentioned problem was surprisingly solved by the provision of novel hydrophilic hyTCO that do not undergo *trans*-to-*cis* isomerization and allow for near quantitative conjugation with diene-bearing derivatives or conjugates (such as 1,2,4,5- 30 tetrazine derivatives) and, due to their increased hydrophilicity, the conjugation of more hydrophobic payloads, and, in the case of pharmacologically active conjugates provide for a better functional profile of such conjugate in vivo.

More specifically the above mentioned problem was solved by the provision of hyTCO derivatives, bearing a geminal polar, particularly hydrophilic, substituent at the 3-position in 35 combination with a polar, in particular hydrophilic moiety at the 2-position.

The invention enables the use of such hyTCOs and hyTCO-ncAA for prolonged application in biological relevant conditions, specifically the expression of proteins incorporating such hyTCO-ncAA and the subsequent employment for bioconjugation in a highly efficient manner.

5 It also enables the use of hyTCO-modified moieties for *in vivo* click applications like pretargeting in nuclear medicine.

Additionally, it aids in optimizing the bioconjugation handle for ADCs towards hydrophilic properties, influencing pharmacokinetics, solubility and aggregation of such. The increased hydrophilicity should also aid in improved wash out properties for intracellular
10 fluorescent labeling and the reduction of unspecific binding.

Surprisingly, although the new hyTCO derivatives possess increased steric hindrance, they display very fast reaction kinetics, even outmatching that of previously known TCO-based compounds.

15 More particularly, the above-mentioned problem is solved by the provision of:

- a) novel hyTCO derivatives (represented by general formula I as referred to herein below);
- b) unnatural amino acids comprising such novel hyTCO compounds (represented by general formula II as referred to herein below);
- 20 c) novel hyTCO-functionalized construct obtainable by, in particular covalently, binding said novel hyTCO compounds to a targeting agent, or to a payload molecule (represented by general formula I' as referred to herein below); and
- d) conjugates obtainable by covalently linking said novel hyTCO-functionalized constructs with a second functionalized molecule comprising a docking group
25 (DG) capable of reacting with the hyTCO-type functional group of the first functionalized molecule.

BRIEF DESCRIPTION OF THE FIGURES

30 **Figure 1:** A) EIC of TCO-e incubated in DMEM for 2 d at 37 °C. Under these conditions more than 60% of the trans-cyclooctene is isomerized to the cis-cyclooctene; B) chemical structure of TCO-e

Figure 2: ELSD and EIC traces of compound 13 (N6-((2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)ethoxy)carbonyl)-L-lysine) incubated in various media or mouse serum for 4 days

at 37 °C, compared to EIC of compound 13 dissolved in DMEM. No change of either ELSD or EIC peaks (within normal variability) can be detected after that timeframe.

Figure 3: Comparison of ELSD and EIC traces of compound 13 incubated in DMEM for 4 d at 37 °C and the same sample 5 min after addition of tetrazine. No species with $m/z = 329$, corresponding to trans- or cis-ncAA can be detected after click reaction and only residual tetrazine and click product (shown next to mass spectrum) can be identified.

Figure 1: Calculated ClogP-values of TCO head groups presented herein and relevant conjugated species as model system. ClogP-values were calculated using ChemDraw 20.

Figure 2: A) Elution profile of Trastuzumab HC $C_{H2K16^{13}}$ -LC eluted in a linear gradient using Buffer B (0.1 M Sodium citrate, pH 3.2); B) SDS-PAGE of collected fractions of Trastuzumab HC $C_{H2K16^{13}}$ -LC; C) elution profile of size exclusion chromatography of Trastuzumab HC $C_{H2K16^{13}}$ -16-LC (Superdex S200 Increase, Cytiva, 28990944) and SDS-PAGE of collected fractions.

Figure 3: A) DAR analysis via reduced reverse phase LC of unconjugated and conjugated ADC Trastuzumab HC $C_{H2K16^{13}}$ -16-LC; B) DAR analysis of ADC Trastuzumab HC $C_{H2K16^{13}}$ -16-LC.

Figure 7: A) Absorption spectra of Trastuzumab-Cy5, showing antibody absorption at 280 nm and dye at 650 nm; B) SDS-PAGE of Trastuzumab-Cy5.

Figure 8: Plasmid map of expression plasmid pCK-HSA-Trastuzumab HC-LC modified by replacing in the heavy chain sequence position K249 the "AAG", the codon for lysine by TAG, the amber codon.

Figure 9: Trastuzumab containing ncAA 13 of the invention at different positions of heavy chain or light chain (V_{HP41} ; C_{H2K90} ; V_{LK45}) was prepared, and conjugated with payload compound 16. A cytotoxicity assay with Her2 positive SKBR-3 cells was performed. Kadcyra ® was used as positive control.

Figure 10: Structural formula of payload molecules P1 to P4 as applied in conjugation experiments

Figure 11: Results of cytotoxicity experiments performed on Her2 positive SKBR-3 cells with Trastuzumab HC V_HP41¹³-LC (conjugated in position V_HP41 with ncAA compound 13 of the present invention) and further conjugated in a molar ratio of 1:3 to the payloads P1-P4.

Figure 12: Protein sequence of Trastuzumab heavy and light chain and positioning of potential mutation sites.

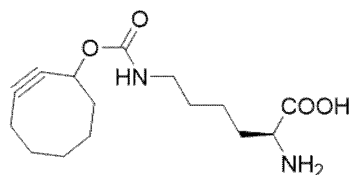
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DETAILED DESCRIPTION OF THE INVENTION

A. ABBREVIATIONS

- 15 ADC = antibody drug conjugates
aq.= aqueous
Bps = base pairs
BCN = 2-amino-6-(9-biocylo[6.1.0]non-4-ynylmethoxycarbonylamino)hexanoic acid
BOC = 2-amino-6-(tert-butoxycarbonylamino)hexanoic acid, in the examples "BOC"
20 specifically designates (2S)-2-amino-6-(tert-butoxycarbonylamino)hexanoic acid =
Boc-L-Lys-OH = *N*- α -*tert*-butyloxycarbonyl-L-lysine
conc.= concentrated
clogP = calculated logarithmic partition coefficient
DAR = Drug-to-antibody ratio
25 DOL = Degree of labelling
DCM = dichloromethane
DDQ = 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
DIPEA= *N,N*-diisopropylethylamine
DMF = dimethylformamide
30 DMSO = dimethylsulfoxide
EDC = 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EIC = Extracted Ion Count
ELSD = Evaporative Light Scattering Detector
eq.= equivalent(s)
35 EtOH = ethanol

- GCE = genetic code expansion
h= hour(s)
HATU = 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid
hexafluorophosphate, a coupling agent
5 HOBt = Hydroxybenzotriazole
IEDDA = Inverse Electron-Demand Diels–Alder Cycloaddition
kDa = kilo Dalton
min= minutes
MMAE = Monomethyl auristatin E ((S)-N-((3R,4S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-
10 hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-
3-methoxy-5-methyl-1-oxoheptan-4-yl)-N,3-dimethyl-2-((S)-3-methyl-2-
(methylamino)butanamido)butanamide), a anti-neoplastic agent
MeOH = methanol
ncAA = non-canonical amino acid
15 NES = nuclear export signal
NLS = nuclear localization signal
O-tRNA = orthogonal tRNA
O-RS = orthogonal RS
PBS = phosphate buffered saline
20 PMSF = phenylmethylsulfonylfluorid
PNP Chloroformate = 4-Nitrophenyl chloroformate
POI = polypeptide of interest,
pRS = prokaryotic RS
ptRNA = prokaryotic tRNA
25 PylRS = pyrrolysyl tRNA synthetase
PylRS^{AF} = mutant *M. mazei* pyrrolysyl tRNA synthetase comprising amino acid
substitutions Y306A and Y384F
RCF (rcf) = relative centrifugal force
RP-HPLC = reversed phase high-performance liquid chromatography
30 RS = aminoacyl tRNA synthetase
RT = room/ambient temperature (20–25°C)
SCO-Lys = 2-amino-6-(cyclooct-2-yn-1-yloxy-carbonylamino)hexanoic acid



SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPIEDAC = Strain-promoted Inverse Electron-Demand Diels–Alder cycloaddition

5 5-TAMRA = 5-Carboxytetramethylrhodamine

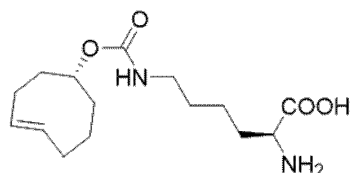
5-TAMRA-OSu = 5-Carboxytetramethylrhodamine N-succinimidyl ester, a fluorophore

TCO-Lys = N-ε-((*trans*-Cyclooct-4-en-1-yloxy)carbonyl)-L-lysine

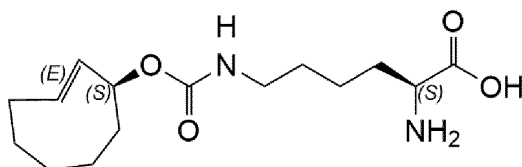
TCO*-Lys = N-ε-((*trans*-Cyclooct-2-en-1-yloxy)carbonyl)-L-lysine

TCO[#]-Lys = N-ε-((*trans*-Cyclooct-3-en-1-yloxy)carbonyl)-L-lysine

10 TCO-E-Lys = *trans*-cyclooct-4-en-L-lysine



TCO*A-Lys = *trans*-cyclooct-2-en-L-lysine



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hyTCO = hydrophilic TCO of the present invention

hyTCO-ncAA = non-canonical amino acid functionalized with an hyTCO over its side chain

TFA = trifluoroacetic acid

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THF = tetrahydrofuran

TLC = thin layer chromatography

tRNA^{Pyl} = tRNA that can be acylated with pyrrolysine by a wild-type or modified PylRS and has an anticodon that, for site-specific incorporation of the ncAA into a POI, is preferably the reverse complement of a selector codon.

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U6 promoter = promoter that normally controls expression of the U6 RNA (a small

nuclear RNA) in mammalian cells

UHPLC-MS = Ultra High Performance Liquid Chromatography/Mass Spectrometry

B. DEFINITIONS

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B.1 General Definitions

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. The meaning and scope of the terms should be clear, however, in
10 the event of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

The terms "purified", "substantially purified," and "isolated" as used herein refer to the state of being free of other, dissimilar compounds with which a compound of the invention is normally associated in its natural state, so that the "purified", "substantially purified," and
15 "isolated" subject comprises at least 0.5%, 1%, 5%, 10%, or 20%, or at least 50% or 75% of the mass, by weight, of a given sample. In one embodiment, these terms refer to the compound of the invention comprising at least 95, 96, 97, 98, 99 or 100%, of the mass, by weight, of a given sample. As used herein, the terms "purified", "substantially purified," and
20 "isolated" when referring to a nucleic acid or protein, also refers to a state of purification or concentration different than that which occurs naturally, for example in a prokaryotic or eukaryotic environment, like, for example in a bacterial or fungal cell, or in the mammalian organism, especially human body. Any degree of purification or concentration greater than that which occurs naturally, including (1) the purification from other associated structures or
25 compounds or (2) the association with structures or compounds to which it is not normally associated in said prokaryotic or eukaryotic environment, are within the meaning of "isolated". The nucleic acid or protein or classes of nucleic acids or proteins, described herein, may be isolated, or otherwise associated with structures or compounds to which they are not normally associated in nature, according to a variety of methods and processes
30 known to those of skill in the art.

In the context of the descriptions provided herein and of the appended claims, the use of "or" means "and/or" unless stated otherwise.

Similarly, "comprise," "comprises," "comprising", "include," "includes," and "including" are interchangeable and not intended to be limiting.

It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of."

5 The term "about" indicates a potential variation of $\pm 25\%$ of the stated value, in particular $\pm 15\%$, $\pm 10\%$, more particularly $\pm 5\%$, $\pm 2\%$ or $\pm 1\%$.

The term "substantially" describes a range of values of from about 80 to 100%, such as, for example, 85-99.9%, in particular 90 to 99.9%, more particularly 95 to 99.9%, or 98 to 99.9% and especially 99 to 99.9%.

10 "Predominantly" refers to a proportion in the range of above 50%, as for example in the range of 51 to 100%, particularly in the range of 75 to 99.9%; more particularly 85 to 98.5%, like 95 to 99%.

If the present disclosure refers to features, parameters and ranges thereof of different degree of preference (including general, not explicitly preferred features, parameters and
15 ranges thereof) then, unless otherwise stated, any combination of two or more of such features, parameters and ranges thereof, irrespective of their respective degree of preference, is encompassed by the disclosure of the present description.

B.2 Chemical definitions

20 The term "halogen" denotes a fluorine, bromine, chlorine or iodine radical, in particular a fluorine radical.

A "hydrocarbyl group" relates to an optionally substituted, straight-chain or branched, non-cyclic or cyclic, homo- or hetero-hydrocarbyl groups having a hydrocarbon chain comprising 1 to 50, 1 to 25, 1 to 12 or in particular 1 to 6 carbon atoms. A "hetero-
25 hydrocarbyl" group may carry within its hydrocarbon chain one or more, as for example 1, 2, 3, 4 or 5, more particular 1 or 2 identical or different heteroatoms, in particular selected from O, S and NH. Particular examples of such hydrocarbyl groups are optionally substituted, linear or branched (hetero)alkyl-, (hetero)alkenyl-, (hetero)alkinyl, (hetero)cycloalkyl- and (hetero)cycloalkenyl-groups.

30 A "hydrocarbylene group" relates to an optionally substituted, straight-chain or branched, homo- or hetero-hydrocarbylene bridging group having a hydrocarbon chain comprising 1 to 50, 1 to 25, 1 to 12 or in particular 1 to 6 carbon atoms. A "hetero-hydrocarbylene" group may carry within its hydrocarbon chain one or more, as for example 1, 2, 3, 4 or 5, more particular 1 or 2 identical or different heteroatoms, in particular selected
35 from O, S and NH. Particular examples of such hydrocarbylene groups are optionally

substituted, linear or branched (hetero)alkylene-, (hetero)alkenylene-, (hetero)alkynylene, (hetero)cycloalkylene- and (hetero)cycloalkenylene-groups.

A "bridging group" in the context of the invention is to be interpreted di- or polyvalent, in particular divalent group. A particular example of a "bridging group" are hydrocarbylene groups.

"Alkyl" or "alkanyl" refers to straight-chain or branched hydrocarbyl residues, in selected from methyl, ethyl, n-propyl, 1-methylethyl, n-butyl, 1-methylpropyl, 2-methylpropyl, 1,1-dimethylethyl, n-pentyl, 1-methylbutyl, 2-methylbutyl, 3-methylbutyl, 2,2-dimethylpropyl, 1-ethylpropyl, 1,1-dimethylpropyl, 1,2-dimethylpropyl, n-hexyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 4-methylpentyl, 1,1-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 2,2-dimethylbutyl, 2,3-dimethylbutyl, 3,3-dimethylbutyl, 1-ethylbutyl, 2-ethylbutyl, 1,1,2-trimethylpropyl, 1,2,2-trimethylpropyl, 1-ethyl-1-methylpropyl und 1-ethyl-2-methylpropyl; n-neptyl, n-octyl, n-nonyl, n-decyl, n-undecyl, n-dodecyl, n-tridecyl, n-tetradecyl, n-pentadecyl, n-hexadecyl, n-heptadecyl, n-octadecyl, n-nonadecyl, n-eicosyl, n-hencosyl, n-docosyl, n-tricosyl, n-tetracosyl, n-pentacosyl, n-hexacosyl, n-heptacosyl, n-octacosyl, n-nonacosyl, squalyl, as well as the branched isomers thereof.

"Alkenyl" relates to the mono-unsaturated analogs of the abovementioned alkyl groups comprising a single carbon-carbon double bond. As non-limiting examples there may be mentioned C₂-C₆-alkenyls, like Ethenyl, 1-Propenyl, 2-Propenyl, 1-Methylethenyl, 1-Butenyl, 2-Butenyl, 3-Butenyl, 1-Methyl-1-propenyl, 2-Methyl-1-propenyl, 1-Methyl-2-propenyl, 2-Methyl-2-propenyl, 1-Pentenyl, 2-Pentenyl, 3-Pentenyl, 4-Pentenyl, 1-Methyl-1-butenyl, 2-Methyl-1-butenyl, 3-Methyl-1-butenyl, 1-Methyl-2-butenyl, 2-Methyl-2-butenyl, 3-Methyl-2-butenyl, 1-Methyl-3-butenyl, 2-Methyl-3-butenyl, 3-Methyl-3-butenyl, 1,1-Dimethyl-2-propenyl, 1,2-Dimethyl-1-propenyl, 1,2-Dimethyl-2-propenyl, 1-Ethyl-1-propenyl, 1-Ethyl-2-propenyl, 1-Hexenyl, 2-Hexenyl, 3-Hexenyl, 4-Hexenyl, 5-Hexenyl, 1-Methyl-1-pentenyl, 2-Methyl-1-pentenyl, 3-Methyl-1-pentenyl, 4-Methyl-1-pentenyl, 1-Methyl-2-pentenyl, 2-Methyl-2-pentenyl, 3-Methyl-2-pentenyl, 4-Methyl-2-pentenyl, 1-Methyl-3-pentenyl, 2-Methyl-3-pentenyl, 3-Methyl-3-pentenyl, 4-Methyl-3-pentenyl, 1-Methyl-4-pentenyl, 2-Methyl-4-pentenyl, 3-Methyl-4-pentenyl, 4-Methyl-4-pentenyl, 1,1-Dimethyl-2-butenyl, 1,1-Dimethyl-3-butenyl, 1,2-Dimethyl-1-butenyl, 1,2-Dimethyl-2-butenyl, 1,2-Dimethyl-3-butenyl, 1,3-Dimethyl-1-butenyl, 1,3-Dimethyl-2-butenyl, 1,3-Dimethyl-3-butenyl, 2,2-Dimethyl-3-butenyl, 2,3-Dimethyl-1-butenyl, 2,3-Dimethyl-2-butenyl, 2,3-Dimethyl-3-butenyl, 3,3-Dimethyl-1-butenyl, 3,3-Dimethyl-2-butenyl, 1-Ethyl-1-butenyl, 1-Ethyl-2-butenyl, 1-Ethyl-3-butenyl, 2-Ethyl-1-butenyl, 2-Ethyl-2-butenyl, 2-Ethyl-3-butenyl, 1,1,2-Trimethyl-2-propenyl, 1-Ethyl-1-methyl-2-propenyl, 1-Ethyl-2-methyl-1-propenyl and 1-Ethyl-2-methyl-2-propenyl.

"Alkynyl" relate to the mono-unsaturated analogs of the abovementioned alkenyl groups comprising a single chemical carbon-carbon triple bond.

The term "cycloalkyl" relates to carbocyclic residues having 3 to 20 carbon atoms, and more particularly to C₃-C₁₂-cycloalkyl, residues, in particular cyclopropyl, cyclobutyl, 5 cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclononyl, cyclodecyl, cycloundecyl und cyclododecyl, cyclopropyl-methyl, cyclopropyl-ethyl, cyclobutyl-methyl, cyclobutyl-ethyl, cyclopentyl-methyl, cyclopentyl-ethyl, and cyclohexyl-methyl.

The term "cycloalkenyl" relates to mono- or polyunsaturated analogs of the above-mentioned cycloalkyl residues; as non-limiting examples there may be mentioned „mono- 10 unsaturated residues having at least 5 carbon atoms such as cyclopenten-1-yl, cyclopenten-3-yl, cyclohexen-1-yl, cyclohexen-3-yl and cyclohexen-4-yl-

The term "alkanoyl" refers to acyl analogues of the type R(C=O)- of the above alkyl groups, wherein R represents an alkyl or cycloalkyl group as defined above.

The term "heteroalkyl" or "heteroalkanyl" relates to analogues of the above-identified 15 alkyl groups carrying within its hydrocarbon chain one or more, as for example 1, 2, 3, 4 or 5, more particular 1 or 2 identical or different heteroatoms, in particular selected from O, S and NH.

The term "heteroalkenyl" relates to analogues of the above-identified alkenyl groups carrying within its hydrocarbon chain one or more, as for example 1, 2, 3, 4 or 5, more 20 particular 1 or 2 identical or different heteroatoms, in particular selected from O, S and NH.

The term "heteroalkynyl" relates to analogues of the above-identified alkynyl groups carrying within its hydrocarbon chain one or more, as for example 1, 2, 3, 4 or 5, more particular 1 or 2 identical or different heteroatoms, in particular selected from O, S and NH.

The term "heterocycloalkyl" relates to analogues of the above-identified cycloalkyl groups carrying within its hydrocarbon chain one or more, as for example 1, 2, 3, 4 or 5, 25 more particular 1 or 2 identical or different heteroatoms, in particular selected from O, S and NH.

The term "heterocycloalkenyl" relates to analogues of the above-identified cycloalkenyl groups carrying within its hydrocarbon chain one or more, as for example 1, 2, 30 3, 4 or 5, more particular 1 or 2 identical or different heteroatoms, in particular selected from O, S and NH.

The term "alkylene" refers to straight chain or branched hydrocarbon bridges having 1 to 10 or 1 to 7 carbon atoms; non-limiting examples thereof are -CH₂-, -(CH₂)₂-, -(CH₂)₃-, -(CH₂)₄-, -(CH₂)₂-CH(CH₃)-, -CH₂-CH(CH₃)-CH₂- -CH₂-CH(CH₃)-, -CH(CH₃)-CH₂-, -CH(CH₃)- 35 CH(CH₃)-, -C(CH₃)₂-CH₂-, -CH₂-C(CH₃)₂-, -CH₂-CH(CH₂CH₃)-, -CH(CH₂CH₃)-CH₂-, -(CH₂)₅-,

- $C(CH_3)_2-CH(CH_3)-$, $-CH(CH_3)-C(CH_3)_2-$, $-(CH_2)_6-$, $-(CH_2)_7-$, $-CH(CH_3)-CH_2-CH_2-CH(CH_3)-$ or $-CH(CH_3)-CH_2-CH_2-CH_2-CH(CH_3)-$; $-CH(CH_2CH_3)-CH(CH_2CH_3)-$, $-C(CH_2CH_3)_2-CH_2-$, $-CH_2-C(CH_2CH_3)_2-$, $-CH_2-CH(n-Propyl)-$, $-CH(n-Propyl)-CH_2-$, $-CH(n-Propyl)-CH(CH_3)-$, $-CH_2-CH(n-Butyl)-$, $-CH(n-Butyl)-CH_2-$, $-CH(CH_3)-CH(CH_2CH_3)-$, $-CH(CH_3)-CH(n-Propyl)-$, $-CH(CH_2CH_3)-CH(CH_3)-$, $-CH(CH_3)-CH(CH_2CH_3)-$,
- 5 $CH(CH_3)-$, $-CH(CH_3)-CH(CH_2CH_3)-$,

"Alkenylene" represent the monounsaturated analogs of the above alkylene groups

A "cycloalkenylene group" relates to the monocyclic analogues of the above alkenylene groups.

- 10 "Alkoxy" relates to a radical of the formula $R-O-$, wherein R is a straight-chain or branched alkyl group as defined above. Particular alkoxy groups have from 1 to 6, in particular 1 to 4, like 1, 2 or 3 carbon atoms as defined herein. Non-limiting examples are methoxy, ethoxy, n-propoxy, iso-propoxy, n-butoxy, 2-butoxy, iso-butoxy or tert-butoxy.

- 15 "Alkenoxy" relates to a radical of the formula $R-O-$, wherein R is a straight-chain or branched alkenyl group as defined above. Particular alkenoxy groups have from 2 to 6, in particular 2 to 4, more particularly 2 or 3 carbon atoms as defined herein.

"Aryl" relates to monovalent mono- or polycyclic aromatic moieties, in particular having 6 to 26, in particular 6 to 14 ring carbon atoms, in particular, phenyl, fluorenyl, naphthenyl and phenantrenyl.

- 20 "Heteroaryl" relates to analogues of the above-mentioned aryl groups additionally comprising at least one, like 1 to 10, more particularly 1 to 5 identical or different ring-heteroatoms, selected from O, S and N.

- 25 "Aralkyl" relates to an aryl residue linked to an alkyl group, each as defined above. In particular, such aralkyl groups comprise 7 to 24 carbon atoms. As non-limiting examples there may be mentioned phenylmethyl or phenylethyl.

"Heteroaralkyl relates to a heteroaryl residue linked to an alkyl group, each as defined above. In particular, such heteroaralkyl groups comprise 6 to 24 carbon atoms. As non-limiting examples there may be mentioned pyridyl-2-methyl or pyridyl-2-ethyl.

- 30 "Alkylaryl" relates to an alkyl residue linked to an aryl group, each as defined above. In particular, such alkylaryl groups comprise 7 to 24 carbon atoms. As non-limiting examples there may be mentioned ortho-, meta- or para-methylphenyl or ortho-, meta- or para-ethylphenyl-residues.

- 35 "Alkylheteroaryl" relates to an alkyl residue linked to a heteroaryl group, each as defined above. In particular, such alkylheteroaryl groups comprise 6 to 24 carbon atoms. As non-limiting examples there may be mentioned 2-methyl-6-pyridyl- or 2-ethyl-6-pyridyl-.

Unless indicated otherwise, the term “substituents” is selected from halogen, C₁-C₄-alkyl, CN, CF₃, hydroxyl, -O-CF₃, C₁-C₄-alkoxy, C₂-C₄-alkanoyloxy, -amino, or -N(C₁-C₄-alkyl)₂.

Unless indicated otherwise, the term “substituted” means that a radical is substituted with 1, 2 or 3, especially 1 or 2, substituent(s).

A “linkage” is formed between two neighbored structural motifs of a compound of the invention and is, unless otherwise indicated, either a chemical bond, or is selected from a ether, thioether, ester, amide, carbamate, dicarbamate, carbonate, hydrazine, urea, alkylene oxide or linear or branched polyalkylene oxide linkage in any possible orientation.

10 An “ether” linkage contains at least one group of the type: (-O-).

A “thioether” linkage contains at least one group of the type: (-S-).

An “amide” linkage contains at least one group of the type: -C(=O)N(R)- or -(R)N-C(=O)-.

15 A “carbamate” linkage contains at least one group of the type: -O-C(=O)-N(R)- or -N(R)-C(=O)-O-.

A “hydrazine” linkage contains at least one group of the type: -NH-NH-.

An “alkylene oxide” linker contains at least one group of the type: -(CH₂)_xO-, -O-(CH₂)_xO-, -(CH₂)_xO-(CH₂)_{n1}C(O)-, -O-(CH₂)_xNR-, -O-(CH₂)_xC(O)-, or -O-(CH₂)_x-, with x = 1, 2, 3 or 4, in particular 1 or 2 and n1 = 1, 2 or 3.

20 A “polyalkylene oxide” linker contains repetitive units of same or different alkylene oxides groups as defined above and may be linear or branched, in particular linear; as for example -((CH₂)_xO)_y-, -(O-(CH₂)_x)_yO-, -((CH₂)_xO)_y-(CH₂)_{n1}C(O)-, -(O-(CH₂)_x)_yNR-, -(O-(CH₂)_x)_yC(O)-, or -(O-(CH₂)_x)_y-, with n, x as defined above for “alkylene oxide” and y being an integer from 1 to 20, preferably 1 to 15, 1 to 10 or 1 to 4.

25 In the above-mentioned chemical formulae of particular linkers residues R independently of each other may represent H or C₁ to C₄-alkyl.

30 A “cleavable group” encompasses any group, which may be cleaved enzymatically or chemically, in particular under in vivo or ex vivo conditions; an enzymatic cleavage may be effected, for example, through the action of a protease; a chemical cleavage, may be effected for example through hydrolytic cleavage or reductive cleavage of S-S bonds.

Drug-to-antibody ratio (DAR) relates to the distribution of different payload, such as drugs or the cytotoxic group to an antibody-drug conjugate (ADC), or the average of different drug to antibody loads for an ADC.

35 A “tetrazine” or “tetrazinyl” group according to the present invention represents, unless otherwise defined, a residue that consists of a six-membered aromatic ring containing four

nitrogen atoms with the molecular formula $-C_2N_4-$, in particular derived from the 1,2,4,5-tetrazine or s-tetrazine isomer, and linked to neighboring groups via ring carbon positions 3 and 6.

5 A “bioorthogonal” reaction or “click reaction” refers to any chemical reaction that can occur inside of living systems without reacting with native components/substrates or interfering with native biochemical processes.

10 A “docking group” is a chemical moiety that has the ability to chemically react, in particular, via a so-called “bioorthogonal” or “click reaction”, with a trans-cyclooctene group as defined herein. In particular, such docking groups are preferably trans-cyclooctene reactive groups selected from dienes. More particularly, it is selected from dienes having the ability to react in a biological environment with the trans-cyclooctene group. As non-limiting examples there may be mentioned, groups comprising optionally substituted triazinyl or tetrazinyl residues and 1,2-quinones. Such groups are well known in the prior art [Yang et al., Angew. Chem. Int. Ed. 2012, 51, 5222–5225; Fan et al., Angew. Chem. Int. Ed. 2016, 55, 14046–14050; Mao et al., Angew. Chem. Int. Ed. 2019, 58, 1106–1109; Qu et al., Angew. Chem. Int. Ed. 2018, 57, 12057–12061; Eising et al., Bioconjugate Chem. 2018, 29, 3054–3059; Meng et al., J. Org. Chem. 2017, 82, 1676–1687; Xie et al., Angew. Chem. Int. Ed. 2020, 59, 16967–16973; Lambert et al., J. Am. Chem. Soc. 2019, 141, 17068–17074; Jemas et al., J. Am. Chem. Soc. 2022, 144, 1647–1662; Selvaraj et al., Tetrahedron Letters 2014, 55, 4795–4797; Dowling et al., J. Org. Chem. 2018, 83, 4229–4238; Battisti et al., Bioconjugate Chem. 2022, 33, 4, 608–624; Karver et al., Bioconjugate Chem. 2011, 22, 2263–2270; Bender et al., Org. Lett. 2017, 19, 5693–5696; Kamber et al., J. Am. Chem. Soc. 2015, 137, 8388–8391; Ros et al., Bioconjugate Chem. 2020, 31, 933–938; Ros et al., Chem. Commun., 2020, 56, 11086; van Onzen et al., J. Am. Chem. Soc. 2020, 142, 10955–10963; Carlson et al., J. Am. Chem. Soc. 2018, 140, 3603–3612.]

“Tetrazine ligation” refers to the reaction of a trans-cyclooctene and an s-tetrazine in an inverse-demand Diels Alder reaction followed by retro Diels Alder reaction to eliminate nitrogen (N_2). A reaction of this type proceeds with high velocity, allowing bio molecule modification at extremely low concentrations.

30 An “inverse electron-demand Diels–Alder (IEDDA) cycloaddition” is a reaction between an electron-poor diene and an electron-rich dienophile and represents only one example of different types of “bioorthogonal reactions”. The diene used may be, for example, a 1,2,4,5-tetrazine or a 1,2,4-triazine. The dienophiles encompass a variety of molecules including strained cyclic alkenes, such as *trans*-cyclooctenes (TCO, norbornenes, cyclopropenes or azetines). Of these, the reaction between a tetrazine and TCO is the fastest reported to date

and suitable for *in vivo* applications (Smeek et al, Current Opinion in Chemical Biology Volume 60, February 2021, Pages 79-88).

The term "bioorthogonal" refers to any chemical reaction that can occur inside of living systems, i.e. in aqueous environment, without interfering with native biochemical processes..

5 "Tetrazine ligation" may for example be mentioned as one type of bioorthogonal reaction. Bioorthogonal chemistry typically proceeds in two steps. First, a cellular substrate is modified with a bioorthogonal functional group (also designated chemical reporter) as for example one of the above-identified hydrophilic trans-cyclooctene compounds described herein. Cellular substrates include for example immunoglobulins, like natural or recombinant antibodies, etc.

10 The chemical reporter must not alter the structure of the substrate dramatically to avoid affecting its bioactivity. In a second step, a probe, such as a functionalized molecule containing a docking group, such as a complementary functional group, as for example a tetrazine group, is introduced to react and label the substrate. .

"Acid or base addition salts" of compounds of the invention are especially addition

15 salts with physiologically tolerated acids or bases. Physiologically tolerated acid addition salts can be formed by treatment of the base form of a compound of the invention with appropriate organic or inorganic acids. Compounds of the invention containing an acidic proton may be converted into their non-toxic metal or amine addition salt forms by treatment with appropriate organic and inorganic bases. The compounds and salts of the invention also

20 comprise the hydrates and solvent addition forms thereof, e.g. hydrates, alcoholates and the like.

"Physiologically tolerated" acids or bases are in particular those which are tolerated by the system used for the incorporation of the first and second dienophiles (e.g. a biological system such as a translation system used for preparation of polypeptides with *trans*-

25 cyclooctenyl or cyclooctynyl groups), e.g. which are substantially non-toxic to living cells.

Compounds as herein described may contain one or more asymmetric elements such as stereogenic centers, stereogenic axes and the like, e.g. asymmetric carbon atoms, so that the compounds can exist in different stereoisomeric forms. These compounds can be, for example, racemates or optically active forms. All stereoisomers, diastereomers, in purified

30 and mixture forms are included. Accordingly, when a compound is recited by specific name or a class of compounds is recited, all these forms are intended to be included. In the case of hyTCO residues of the present invention Z- and E-forms thereof are excluded.

Compounds as herein described may also exist in more than one form of structural isomers also designated as constitutional isomers or regioisomers. These are molecules that

differ only in the different sequence of their atoms or atomic groups while having the same gross formula.

Therefore, unless otherwise stated, for each of the compounds, biomolecules and conjugates as described herein, any such potential stereo- or regiosomeric form or mixture of
5 more than one stereo- and /or regiosomeric form is within the scope of the present invention.

B.3 Biochemical definitions

A "polypeptide" is any oligomer of amino acid residues (natural or unnatural, or a combination thereof), of any length, typically but not exclusively joined by covalent peptide
10 bonds. A polypeptide can be from any source, e.g., a naturally occurring polypeptide, a polypeptide produced by recombinant molecular genetic techniques, a polypeptide from a cell or translation system, or a polypeptide produced by cell-free synthetic means. A polypeptide is characterized by its amino acid sequence, e.g., the primary structure of its component amino acid residues. As used herein, the amino acid sequence of a polypeptide
15 is not limited to full-length sequences, but can be partial or complete sequences. Furthermore, it is not intended that a polypeptide be limited by possessing or not possessing any particular biological activity.

As used herein, the term "protein" is synonymous with polypeptide. The term "peptide" refers to a small polypeptide, for example but not limited to, from 2-25 amino acids in length.

20 The primary object of such targeting agent is the formation of a covalent or noncovalent linkage with a particular "target". A secondary object of the targeting agent is the targeted transport of a "payload molecule" to said target. In order to achieve said second object said targeting agent has to be combined (reversibly or irreversibly) with at least one "payload molecule".

25 Said hyTCO-ncAA can be directly incorporated into targeting polypeptides using any of a number of methods known in the art. While many embodiments utilize orthogonal translation systems as the route of direct incorporation of unnatural amino acids, other direct incorporation methods (e.g., in vitro translation systems, solid-phase synthesis, etc.) can be used alternatively. It will be appreciated that in typical embodiments herein, an unnatural
30 amino acid is preferably incorporated into target polypeptide, i.e., during construction of the polypeptide, and is not added via post-translational chemical derivatization.

In certain embodiments described herein, the unnatural amino acids can be site-specifically incorporated into a targeting polypeptide with high efficiency and high fidelity using "orthogonal tRNA/aminoacyl-tRNA synthetase pairs".

The term "translation system" refers to the components necessary to incorporate an amino acid in a growing polypeptide chain (protein). Components of a translation system can include, e.g., ribosomes, tRNAs, synthetases, mRNA and the like. The translation system may be an *in vivo* or an *in vitro* translation system.

5 An "*in vitro* translation system" may be a cell-free translation system. A cell-free translation system is a system for synthesizing a desired protein by obtaining protein factors required for mRNA translation, e.g., in form of a cell extract, followed by reconstituting this reaction *in vitro*. Such cell-free systems and their use for protein synthesis are known in the art. Examples include extracts of *E. coli*, wheat germ extract, or rabbit reticulocyte lysate
10 (Spirin and Swartz, Cell-free Protein Synthesis, Wiley VCH Verlag, Weinheim, Germany, 2008).

An aminoacyl tRNA synthetase (RS) is an enzyme capable of acylating a tRNA with an amino acid or amino acid analog. Expediently, the RS used in the methods of the invention is capable of acylating a tRNA with an unnatural amino acid.

15 The methods of the invention expediently utilize a "tRNA / aminoacyl tRNA synthetase (tRNA/RS) pair". Preferably, the tRNA/RS pair used in the processes of the invention is orthogonal to the translation system.

The term "orthogonal" as used herein refers to a molecule (e.g., an orthogonal tRNA (O-tRNA) and/or an orthogonal aminoacyl tRNA synthetase (O-RS)) that is used with
20 reduced efficiency by a translation system of interest (e.g., a cell). Orthogonal refers to the inability or reduced efficiency, e.g., less than 20% efficient, less than 10% efficient, less than 5% efficient, or e.g., less than 1% efficient, of an orthogonal tRNA or an orthogonal aminoacyl tRNA synthetase to function with the endogenous aminoacyl tRNA synthetases or endogenous tRNAs of the translation system of interest. For example, an orthogonal tRNA in
25 a translation system of interest is acylated by any endogenous aminoacyl tRNA synthetase of a translation system of interest with reduced or even zero efficiency, when compared to acylation of an endogenous tRNA by the endogenous aminoacyl tRNA synthetase. In another example, an orthogonal aminoacyl tRNA synthetase acylates any endogenous tRNA in the translation system of interest with reduced or even zero efficiency, as compared to
30 acylation of the endogenous tRNA by an endogenous aminoacyl tRNA synthetase.

Orthogonal tRNA/RS pairs used in processes of the invention preferably have following properties: the O-tRNA is preferentially acylated with the unnatural amino acid of the invention by the O-RS. In addition, the orthogonal pair functions in the translation system of interest, e.g., the translation system uses the unnatural amino acid acylated O-tRNA to
35 incorporate the unnatural amino acid of the invention in a polypeptide chain. Incorporation

occurs in a site specific manner, *e.g.*, the O-tRNA recognizes a selector codon, *e.g.*, an amber stop codon, in the mRNA coding for the polypeptide.

The term “preferentially acylates” refers to an efficiency of, *e.g.*, about 50% efficient, about 70% efficient, about 75% efficient, about 85% efficient, about 90% efficient, about 95% efficient, or about 99% or more efficient, at which an O-RS acylates an O-tRNA with an unnatural amino acid compared to an endogenous tRNA or amino acid of a translation system of interest. The unnatural amino acid is then incorporated in a growing polypeptide chain with high fidelity, *e.g.*, at greater than about 75% efficiency for a given selector codon, at greater than about 80% efficiency for a given selector codon, at greater than about 90% efficiency for a given selector codon, at greater than about 95% efficiency for a given selector codon, or at greater than about 99% or more efficiency for a given selector codon.

The term “selector codon” refers to codons recognized by the O-tRNA in the translation process and not recognized by an endogenous tRNA. The O-tRNA anticodon loop recognizes the selector codon on the mRNA and incorporates its amino acid, *e.g.*, an unnatural amino acid, at this site in the polypeptide. Selector codons can include, *e.g.*, nonsense codons, such as stop codons, *e.g.*, amber, ochre, and opal codons; four or more base codons; codons derived from natural or unnatural base pairs and the like. For a given system, a selector codon can also include one of the natural three base codons (*i.e.* natural triplets), wherein the endogenous system does not use said natural triplet, *e.g.*, a system that is lacking a tRNA that recognizes the natural triplet or a system wherein the natural triplet is a rare codon.

An “anticodon” has the reverse complement sequence of the corresponding codon.

An O-tRNA/O-RS pair is composed of an O-tRNA, *e.g.*, a suppressor tRNA, or the like, and an O-RS.

A “suppressor tRNA” is a tRNA that alters the reading of a messenger RNA (mRNA) in a given translation system. A suppressor tRNA can read through, *e.g.*, a stop codon, a four base codon, or a rare codon.

The O-tRNA is not acylated by endogenous synthetases and is capable of decoding a selector codon, as described herein.

The O-RS recognizes the O-tRNA, *e.g.*, with an extended anticodon loop, and preferentially acylates the O-tRNA with an unnatural amino acid.

The tRNA and the RS used in the processes of the invention can be naturally occurring or can be derived by mutation of a naturally occurring tRNA and/or RS from a variety of organisms. In various embodiments, the tRNA and RS are derived from at least one organism. In another embodiment, the tRNA is derived from a naturally occurring or mutated

naturally occurring tRNA from a first organism and the RS is derived from naturally occurring or mutated naturally occurring RS from a second organism.

A suitable tRNA/RS pair may be selected from libraries of mutant tRNA and RS, e.g. based on the results of a library screening. Alternatively, a suitable tRNA/RS pair may be a
5 heterologous tRNA/synthetase pair that is imported from a source species into the translation system. Preferably, the cell used as translation system is different from said source species.

For example a suitable orthogonal O-tRNA can be derived from an archaeobacterium, such as *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, *Halobacterium*
such as *Haloferax volcanii* and *Halobacterium species NRC-I*, *Archaeoglobus fulgidus*,
10 *Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Aeuropyrum permix*, *Methanococcus maripaludis*, *Methanopyrus kandleri*, *Methanosarcina mazei* (Mm), *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Sulfolobus solfataricus* (Ss), *Sulfolobus tokodaii*, *Thermoplasma acidophilum*, *Thermoplasma volcanium*, or the like, or a eubacterium, such as *Escherichia coli*, *Thermus thermophilus*, *Bacillus subtilis*, *Bacillus stearothermophilus*, or the like, while the
15 orthogonal O-RS can be derived from an organism or combination of organisms, e.g., an archaeobacterium, such as *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, *Halobacterium* such as *Haloferax volcanii* and *Halobacterium species NRC-J*, *Archaeoglobus fulgidus*, *Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Aeuropyrum permix*, *Methanococcus maripaludis*, *Methanopyrus kandleri*, *Methanosarcina mazei*,
20 *Methanosarcina barkeri*, *Methanosarcina hafniense*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Sulfolobus solfataricus*, *Sulfolobus tokodaii*, *Thermoplasma acidophilum*, *Thermoplasma volcanium*, or the like, or a eubacterium, such as *Escherichia coli*, *Thermus thermophilus*, *Bacillus subtilis*, *Bacillus stearothermophilus*, or the like. In one embodiment, eukaryotic sources, e.g., plants, algae, protists, fungi, yeasts, animals, e.g., mammals,
25 insects, arthropods, or the like can also be used as sources of O-tRNAs and O-RSs

Methods for evolving tRNA/RS pairs are described, e.g., in WO 02/085923 and WO 02/06075.

Preferably, the RS is a pyrrolysyl tRNA synthetase (pyIRS) capable of acylating a tRNA with the unnatural amino acid of the invention. The pyrrolysyl tRNA synthetase used in
30 methods of the invention may be a wildtype or a genetically engineered pyIRS. Examples for wildtype pyIRS include, but are not limited to pyIRS from archaeobacteria and eubacteria such as *Methanosarcina mazei*, *Methanosarcina barkeri*, *Methanococcoides burtonii*, *Methanosarcina acetivorans*, *Methanosarcina thermophila*, and *Desulfitobacterium hafniense*.

Pyrrolysyl tRNA synthetase (PylRS) is an aminoacyl tRNA synthetase (RS). RSs are enzymes capable of acylating a tRNA with an amino acid or amino acid analog. Expediently, the PylRS of the invention is enzymatically active, i.e. is capable of acylating a tRNA (tRNA^{Pyl}) with a certain amino acid or amino acid analog, preferably with an UNAA or salt thereof

The term “archaeal pyrrolysyl tRNA synthetase” (abbreviated as “archaeal PylRS”) as used herein refers to a PylRS, wherein at least a segment of the PylRS amino acid sequence, or the entire PylRS amino acid sequence, has at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at last 99%, or 100% sequence identity to the amino acid sequence of a naturally occurring PylRS from an archaeon, or to the amino acid sequence of an enzymatically active fragment of such naturally occurring PylRS.

The PylRS of the present invention may comprise a mutant archaeal PylRS, or an enzymatically active fragment thereof.

Generally, “mutant archaeal PylRSs” or “mutated archaeal PylRSs” differ from the corresponding wildtype PylRSs in comprising additions, substitutions and/or deletions of one or more than one amino acid residue. Preferably, these are modifications which improve PylRS stability, alter PylRS substrate specificity and/or enhance PylRS enzymatic activity. Particularly preferred “mutant archaeal PylRSs” or “mutated archaeal PylRSs” are described in more detail herein below.

The term “nuclear export signal” (abbreviated as “NES”) refers to an amino acid sequence which can direct a polypeptide containing it (such as a NES-containing PylRS of the invention) to be exported from the nucleus of a eukaryotic cell. Said export is believed to be mostly mediated by Crm1 (chromosomal region maintenance 1, also known as karyopherin exportin 1). NESs are known in the art. For example, the database ValidNESs (<http://validness.ym.edu.tw/>) provides sequence information of experimentally validated NES-containing proteins. Further, NES databases like, e.g., NESbase 1.0 (www.cbs.dtu.dk/databases/NESbase-1.0/; see Le Cour et al., Nucl Acids Res 31(1), 2003) as well as tools for NES prediction like NetNES (www.cbs.dtu.dk/services/NetNES/; see La Cour et al., La Cour et al., Protein Eng Des Sel 17(6):527-536, 2004), NESpredictor (NetNES, <http://www.cbs.dtu.dk/>; see Fu et al., Nucl Acids Res 41:D338-D343, 2013; La Cour et al., Protein Eng Des Sel 17(6):527-536, 2004)) and NESsential (a web interface combined with ValidNESs) are available to the public. Hydrophobic leucine-rich NESs are most common and represent the best characterized group of NESs to date. A hydrophobic leucine-rich NES is a non-conservative motif having 3 or 4 hydrophobic residues. Many of these NESs comprise the conserved amino acid sequence pattern LxxLxL or LxxxLxL, wherein each L is independently selected from leucine, isoleucine, valine, phenylalanine and

methionine amino acid residues, and each x is independently selected from any amino acid (see La Cour et al., Protein Eng Des Sel 17(6):527-536, 2004).

The term “nuclear localization signal” (abbreviated as “NLS”, also referred to in the art as “nuclear localization sequence”) refers to an amino acid sequence which can direct a polypeptide containing it (e.g., a wild-type archaeal PylRS) to be imported into the nucleus of a eukaryotic cell. Said export is believed to be mediated by binding of the NLS-containing polypeptide to importin (also known as karyopherin) so as to form a complex that moves through a nuclear pore. NLSs are known in the art. A multitude of NLS databases and tools for NLS prediction are available to the public, such as NLSdb (see Nair et al., Nucl Acids Res 31(1), 2003), cNLS Mapper (www.nls-mapper.aib.keio.ac.jp; see Kosugi et al., Proc Natl Acad Sci U S A. 106(25):10171-10176, 2009; Kosugi et al., J Biol Chem 284(1):478-485, 2009), SeqNLS (see Lin et al., PLoS One 8(10):e76864, 2013), and NucPred (www.sbc.su.se/~maccallr/nucpred/; see Branmeier et al., Bioinformatics 23(9):1159-60, 2007).

Mutant archaeal PylRSs of the invention as defined above can be further modified by removing the NLS optionally present in said naturally occurring PylRS where the mutant is derived from and/or by introducing at least one NES. The NLS in the naturally occurring PylRS can be identified using known NLS detection tools such as, e.g., cNLS Mapper.

The removal of a NLS from and/or the introduction of a NES into an archaeal PylRS or mutant thereof, can change the localization of the thus modified polypeptide when expressed in a eukaryotic cell, and in particular can avoid or reduce accumulation of the polypeptide in the nucleus of the eukaryotic cell. Thus, the localization of a PylRS mutant of the invention expressed in a eukaryotic cell can be changed compared to a PylRS or PylRS mutant, which differs from the PylRS mutant of the invention in that it (still) comprises the NLS and lacks the NES.

Where the archaeal PylRS of the invention comprises a NES but (still) comprises an NLS, the NES is preferably chosen such that the strength of the NES overrides the NLS preventing an accumulation of the PylRS in the nucleus of a eukaryotic cell.

Removal of the NLS from a wild-type or mutant PylRS and/or introduction of a NES into the wild-type or mutant PylRS so as to obtain a PylRS of the invention do not abrogate PylRS enzymatic activity. Preferably, PylRS enzymatic activity is maintained at basically the same level, i.e. the PylRS of the invention has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 91, 92, 93, 94, 95, 96, 97, 98 or 99% of the enzymatic activity of the corresponding wild-type or mutant PylRS.

The NES is expediently located within the PylRS or mutant PylRS of the invention such that the NES is functional. For example, a NES can be attached to the C-terminus (e.g.,

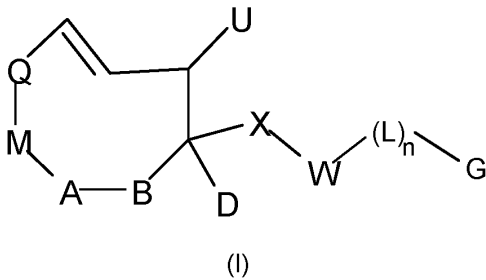
C-terminal of the last amino acid residue) or the N-terminus (e.g., in between amino acid residue 1, the N-terminal methionine, and amino acid residue 2) of a wild-type or mutant archaeal PylRS.

The disclosure of WO2018/06948 disclosing mutated PylRSs modified by the incorporation of NES and / deletion of NLS sequences is herewith explicitly referred to and incorporated by reference

C. PARTICULAR ASPECTS AND EMBODIMENTS

1. The first aspect of the present invention

According to a first aspect the invention provides a hydrophilic TCO (hyTCO) compound of general formula I



wherein

- n is 0 or an integer from 1 to 20, in particular 1 to 10, more particularly 1 to 5;
- A is $-CR^1R^2-$, $-O-$, $-S-$, $-N(R^1)-$, $>CH-OZ$, $>CH-SZ$, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$; and in particular $-C(R^1R^2)-$, $-O-$, $-S-$, $-N(R^1)-$, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$
- B is $-CR^1R^2-$, $-O-$, $-S-$, $-N(R^1)-$, $>CH-OZ$, $>CH-SZ$, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$; and in particular $-CR^1R^2-$, $-O-$, $-S-$, $-N(R^1)-$, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$;
- M is $-CR^1R^2-$, $-O-$, $-S-$, $-N(R^1)-$, $>CH-OZ$, $>CH-SZ$, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$; and in particular $-CR^1R^2-$, $-O-$, $-S-$, $-N(R^1)-$, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$;
- Q is $-CR^1R^2-$, $-O-$, $-S-$, $-N(R^1)-$, $>CH-OZ$, $>CH-SZ$, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$; and in particular $-CR^1R^2-$, $-O-$, $-S-$, $-N(R^1)-$, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$;

D is $-\text{OR}^3$, $-\text{SR}^3$, $-\text{OZ}$ or $-\text{SZ}$; and in particular $-\text{OR}^3$ or $-\text{SR}^3$; and more particularly $-\text{OR}^3$;

U is OZ , $-\text{SZ}$, $-\text{OR}^3$, $-\text{SR}^3$ or $-\text{NR}^5\text{R}^6$; and in particular $-\text{OR}^3$, $-\text{SR}^3$ or $-\text{NR}^5\text{R}^6$; and more particularly $-\text{OR}^3$;

5 wherein

residues Z independently of each other are same or different protecting groups;

10 R^1 , R^2 , R^5 and R^6 independently of each other are same or different and are selected from the group consisting of H and optionally substituted, saturated or unsaturated, linear or branched, non-cyclic or cyclic homo- or hetero-hydrocarbyl groups, such as optionally substituted, linear or branched, homo- or hetero- alkyl groups and optionally substituted linear or branched, homo- or hetero-alkanoyl groups;

15 R^3 and R^4 independently of each other are same or different and selected from the group consisting of H, optionally substituted, saturated or unsaturated, linear or branched, non-cyclic or cyclic homo- or hetero-hydrocarbyl groups, such as optionally substituted, linear or branched, homo- or hetero-alkyl groups, optionally substituted linear or branched, homo- or hetero-alkanoyl groups and $\text{C}_4\text{-C}_{24}$ homo- or hetero-aryl groups;

20 X is a bridging group;

W is a saturated or unsaturated polar group;

L is a linking group; and

G is a terminal group;

25 or a salt, like a pharmaceutically acceptable salt, or solvate thereof; each as a stereoisomer or as a mixture of at least two stereoisomers, wherein cis/trans isomers of the TCO-ring moiety are excluded.

According to a particular embodiment thereof the following applies:

30 A is $-\text{CR}^1\text{R}^2$ - if M and B independently of each other represent $-\text{O}-$, $-\text{S}-$ or $-\text{N}(\text{R}^1)-$;

or

M and B independently of each other represent $-\text{CR}^1\text{R}^2$ - if A is selected from $-\text{O}-$, $-\text{S}-$ or $-\text{N}(\text{R}^1)-$.

35 According to another particular embodiment Q is $-\text{CR}^1\text{R}^2$ -, $>\text{CH-OZ}$, $>\text{CH-SZ}$, $>\text{CH-NR}^1\text{R}^2$, $>\text{CH-OR}^3$, $>\text{CH-CN}$, $>\text{CH-NO}_2$, $>\text{CH-SO}_2\text{R}^4$ or $>\text{CH-SR}^3$; and in particular $-\text{CR}^1\text{R}^2$ -, $>\text{CH-NR}^1\text{R}^2$, $>\text{CH-OR}^3$, $>\text{CH-CN}$, $>\text{CH-NO}_2$, $>\text{CH-SO}_2\text{R}^4$ or $>\text{CH-SR}^3$.

According to a more particular embodiment Q is $-CR^1R^2$.

According to another a more particular embodiment Q is $>CH-NR^1R^2$.

According to still another a more particular embodiment Q is $>CH-OR^3$.

According to still another a more particular embodiment Q is $>CH-CN$.

5 According to still another a more particular embodiment Q is $>CH-NO_2$.

According to still another a more particular embodiment Q is $>CH-SO_2R^4$.

According to still another a more particular embodiment Q is $>CH-SR^3$.

10 According to another particular embodiment, A is $-CR^1R^2$, $-O$ -, $-S$ -, $-N(R^1)$ -, $>CH-OZ$, $>CH-SZ$, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$; and in particular $-C(R^1R^2)$ -, $-O$ -, $-S$ -, $-N(R^1)$ -, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$.

According to a more particular embodiment A is $-CR^1R^2$.

According to another a more particular embodiment A is $-O$ -.

15 According to still another a more particular embodiment A is $-S$ -.

According to still another a more particular embodiment A is $-N(R^1)$ -.

According to still another a more particular embodiment A is $>CH-NR^1R^2$.

According to still another a more particular embodiment A is, $>CH-OR^3$.

According to still another a more particular embodiment A is, $>CH-CN$.

20 According to still another a more particular embodiment A is, $>CH-NO_2$.

According to still another a more particular embodiment A is $>CH-SO_2R^4$.

According to still another a more particular embodiment A is $>CH-SR^3$.

25 According to another particular embodiment B is $-CR^1R^2$ -, $-O$ -, $-S$ -, $-N(R^1)$ -, $>CH-OZ$, $>CH-SZ$, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$; and in particular $-C(R^1R^2)$ -, $-O$ -, $-S$ -, $-N(R^1)$ -, $>CH-NR^1R^2$, $>CH-OR^3$, $>CH-CN$, $>CH-NO_2$, $>CH-SO_2R^4$ or $>CH-SR^3$.

According to a more particular embodiment B is $-CR^1R^2$ -.

According to another a more particular embodiment B is $-O$ -.

30 According to still another a more particular embodiment B is $-S$ -.

According to still another a more particular embodiment B is $-N(R^1)$ -.

According to still another a more particular embodiment B is $>CH-NR^1R^2$.

According to still another a more particular embodiment B is, $>CH-OR^3$.

According to still another a more particular embodiment B is, $>CH-CN$.

35 According to still another a more particular embodiment B is, $>CH-NO_2$.

According to still another a more particular embodiment B is $>\text{CH-SO}_2\text{R}^4$.

According to still another a more particular embodiment B is $>\text{CH-SR}^3$.

According to another particular embodiment M is $-\text{CR}^1\text{R}^2$ -, $-\text{O}$ -, $-\text{S}$ -, $-\text{N}(\text{R}^1)$ -, $>\text{CH-OZ}$,
 5 $>\text{CH-SZ}$, $>\text{CH-NR}^1\text{R}^2$, $>\text{CH-OR}^3$, $>\text{CH-CN}$, $>\text{CH-NO}_2$, $>\text{CH-SO}_2\text{R}^4$ or $>\text{CH-SR}^3$; and in particular $-\text{C}(\text{R}^1\text{R}^2)$ -, $-\text{O}$ -, $-\text{S}$ -, $-\text{N}(\text{R}^1)$ -, $>\text{CH-NR}^1\text{R}^2$, $>\text{CH-OR}^3$, $>\text{CH-CN}$, $>\text{CH-NO}_2$, $>\text{CH-SO}_2\text{R}^4$ or $>\text{CH-SR}^3$.

According to a more particular embodiment M is $-\text{CR}^1\text{R}^2$ -.

According to another a more particular embodiment M is $-\text{O}$ -.

10 According to still another a more particular embodiment M is $-\text{S}$ -.

According to still another a more particular embodiment M is $-\text{N}(\text{R}^1)$ -.

According to still another a more particular embodiment M is $>\text{CH-NR}^1\text{R}^2$.

According to still another a more particular embodiment M is, $>\text{CH-OR}^3$.

According to still another a more particular embodiment M is, $>\text{CH-CN}$.

15 According to still another a more particular embodiment M is, $>\text{CH-NO}_2$.

According to still another a more particular embodiment M is $>\text{CH-SO}_2\text{R}^4$.

According to still another a more particular embodiment M is $>\text{CH-SR}^3$.

According to a another particular embodiment compounds of general formula I are
 20 provided, wherein residues A, B, M and Q are identical and each represent $-\text{C}(\text{R}^1\text{R}^2)$ -, wherein R^1 and R^2 independently of each other represent H or an optionally substituted, linear or branched alkyl group having 1 to 10, in particular 1 to 5 and more particularly 1 or 2 carbon atoms.

According to a another particular embodiment compounds of general formula I are
 25 provided, wherein residues A, B, M and Q are identical and each represent $-\text{C}(\text{R}^1\text{R}^2)$ -, wherein R^1 and R^2 independently of each other represent H or a non-substituted linear or branched alkyl group having 1 to 10, in particular 1 to 5 and more particularly 1 or 2 carbon atoms.

According to another particular embodiment compounds of general formula I are
 30 provided, wherein residues A, B, M and Q are identical and each represent $-\text{C}(\text{R}^1\text{R}^2)$ -, wherein R^1 and R^2 represent H.

According to still another particular embodiment compounds of the general formula I are provided, wherein residues U and D each represent $-\text{OR}^3$, wherein R^3 independently of each other represents H or a linear or branched alkyl group, having one to 10, in particular 1
 35 to 5 and more particularly 1 or 2 carbon atoms.

According to another particular embodiment of the above compounds residues Z are a thiol protecting group or an alcohol protecting group, in particular a cleavable protecting group, and more particularly, independently of one another selected from the group consisting of acetyl (Ac), benzoyl (Bz), benzyl (Bn), β -methoxyethoxymethyl ether (MEM), methoxymethyl ether (MOM), methoxytrityl (MT), p-methoxybenzyl ether (PMB), p-methoxyphenyl ether (PMP), methylthiomethyl ether, pivaloyl (Piv), tetrahydropyranyl (THP), tetrahydrofuranyl (THF), trityl (triphenylmethyl, Tr), silyl ether, methyl ether, and ethoxyethyl ether (EE) residues.

10

According to another particular embodiment of the above compounds, X is selected from:

- a) linear or branched lower-alkylene, in particular $-(CH_2)_{n1}-$, wherein $n1$ is an integer from 1 to 4, in particular methylene or ethylene;
- 15 b) $-O-$, $-S-$, $>CH-OZ$, $>CH-SZ$, $>CH-OR^3$ or $>CH-SR^3$;
wherein
Z and R^3 are as defined above;
- c) linear or branched mono- or polyalkylene oxide moieties, particularly selected from linear the moieties $-((CH_2)_x-O)_y-$, $-(O-(CH_2)_x)_y-$ and $-(O-(CH_2)_x)_y-O-$, and the
20 branched or mono- or polyunsaturated analogues thereof;
wherein
x independently of each other represent an integer selected from 1, 2, 3 or 4; particularly 1 or 2; and
y independently of each other represent an integer from 1 to 20,
25 particularly 1 to 15, 1 to 10 or 1 to 4; or

According to a more particular embodiment X is as defined in a).

According to another more particular embodiment X is as defined in b).

According to another more particular embodiment X is as defined in c).

30

According to still another particular embodiment compounds of general formula I are provided, wherein X is selected from methylene or ethylene and moieties $-((CH_2)_x-O)_y-$, or $-(O-(CH_2)_x)_y-$ wherein x is 1 and 2 and y is 1 or 2.

According to another particular embodiment of the above compounds said polar group W is selected from:

a) $-\text{C}(\text{O})-$, $-\text{O}-\text{C}(\text{O})-$, $-\text{C}(\text{O})-\text{O}-$;

b) $-\text{O}-[\text{C}(\text{O})-\text{[CH}_2\text{]}_{x_1}]_{x_2}\text{C}(\text{O})-$; $-\text{O}-[\text{C}(\text{S})-\text{[CH}_2\text{]}_{y_1}]_{y_2}\text{C}(\text{O})-$

5 wherein

x_1 , x_2 , y_1 , and y_2 independently of each other represent an integer selected from 1 to 6, particularly 1 to 4, and more particularly 1 to 2;

c) $-\text{S}-\text{C}(\text{S})-$, $-\text{O}-\text{C}(\text{S})-$, $-\text{C}(\text{S})-$, $-\text{C}(\text{S})-\text{O}-$, $-\text{C}(\text{S})-\text{S}-$; $-\text{NR}^7-\text{C}(\text{O})-$, $-\text{C}(\text{O})-\text{NR}^7-$, $-\text{C}(\text{NR}^7)$, $-\text{NR}^7-\text{C}(\text{NR}^7)-$, $-\text{NR}^7-\text{C}(\text{O})\text{NR}^7-$, $-\text{NR}^7-\text{C}(\text{NR}^7)-$; or

10 d) $-\text{NR}^8-\text{[C}(\text{O})-\text{[CH}_2\text{]}_{x_1}]_{x_2}\text{C}(\text{O})-$; $\text{NR}^8-\text{[C}(\text{S})-\text{[CH}_2\text{]}_{y_1}]_{y_2}\text{C}(\text{O})-$;

 wherein

x_1 , x_2 , y_1 , and y_2 independently of each other represent an integer other selected from 1 to 6, particularly 1 to 4, and more particularly 1 to 2, and R^7 and R^8 independently of each other represent H or lower alkyl, particularly H or C_1 - C_4 -alkyl.

15

According to a more particular embodiment W is as defined in a).

According to another more particular embodiment W is as defined in b).

According to another more particular embodiment W is as defined in c).

According to another more particular embodiment W is as defined in d).

20

According to still another particular embodiment compounds of general formula I are provided, wherein W is selected from $\text{C}(\text{O})-$, $-\text{O}-\text{C}(\text{O})-$ and $-\text{C}(\text{O})-\text{O}-$.

According to another particular embodiment of the above compounds each L is independently selected from

25

a) linear or branched alkylene groups, linear or branched alkenylene groups, cycloalkylene groups, cycloalkenylene groups, or analogues thereof containing one or more heteroatoms in their carbon backbone; in particular linear or branched C_1 - C_{10} -alkylene groups, linear or branched C_2 - C_{10} -alkenylene groups, C_3 - C_8 -cycloalkylene groups, C_3 - C_8 -cycloalkenylene groups, or analogues thereof containing one or more heteroatoms in their carbon backbone; more particularly linear or branched C_1 - C_4 -alkylene groups, linear or branched C_2 - C_4 -alkenylene groups, C_3 - C_6 -cycloalkylene groups, C_3 - C_6 -cycloalkenylene groups, or analogues thereof containing one or more heteroatoms in their carbon backbone; most particularly methylene, ethylene and propylene; (Moiety La)

30

b) linear or branched mono- or polyalkylene oxide moieties, particularly selected from the linear moieties $-((\text{CH}_2)_{x3}-\text{O})_{y3}-$, $-(\text{O}(\text{CH}_2)_{x3})_{y3}-$, $-(\text{O}-(\text{CH}_2)_{x3})_{y3}-\text{O}-$, $-((\text{CH}_2)_{x3}-\text{O})_{y3}-$, $(\text{CH}_2)_{n3}\text{C}(\text{O})-$, $-(\text{O}(\text{CH}_2)_{x3})_{y3}-\text{NR}^7-$, $-(\text{O}-(\text{CH}_2)_{x3})_{y3}-\text{C}(\text{O})-$, and the branched analogues thereof;

wherein

- 5 x_3 independently of each other represent an integer selected from 1, 2, 3 or 4; particularly 1 or 2;
 y_3 independently of each other represent an integer from 1 to 20, particularly 1 to 15, 1 to 10 or 1, 2, 3 and 4;
 n_3 is an integer from 2 and 3; and
 10 R^7 represent H or lower alkyl, particularly H or C_1 - C_4 -alkyl, more particularly methyl or ethyl;

(Moiety Lb)

- 15 provided that, when two or more identical or different linker moieties La and/or Lb are present, such elements may be directly linked to each via a chemical bond or indirectly via identical or different coupling moieties selected from $-\text{C}(\text{O})-$, $-\text{O}-\text{C}(\text{O})-$, $-\text{C}(\text{O})-\text{O}-$, $-\text{S}-\text{C}(\text{S})-$, $-\text{O}-\text{C}(\text{S})-$, $-\text{C}(\text{S})-$, $-\text{C}(\text{S})-\text{O}-$, $-\text{C}(\text{S})-\text{S}-$; $-\text{NR}^7-\text{C}(\text{O})-$, $-\text{C}(\text{O})-\text{NR}^7-$, $-\text{C}(\text{NR}^7)-$, $-\text{NR}^7-\text{C}(\text{NR}^7)-$, $-\text{S}(\text{O})-$, $-\text{S}(\text{O})_2-$, $-\text{P}(\text{O})\text{OR}^7-$, $-\text{O}-\text{P}(\text{O})\text{OR}^7-$, $-\text{P}(\text{O})\text{OR}^7-\text{O}-$; wherein R^7 is as defined above.

- 20 According to a more particular embodiment L is as defined in a).
 According to another more particular embodiment Lis as defined in b).

According to another particular embodiment compounds of formula I are provided, wherein $n = 0$ or 1.

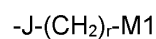
- 25 According to still another particular embodiment compounds of formula I are provided, wherein L represents a moiety La, selected from methylene, ethylene and propylene; or represents my team Lb, selected from $-((\text{CH}_2)_{x3}-\text{O})_{y3}-$ or $-(\text{O}(\text{CH}_2)_{x3})_{y3}-$, wherein x_3 and y_3 independently of each other are selected from integers 1, and 2.

- 30 According to another particular embodiment of the above compounds, G has one of the following meanings:

- a) G is H or R^7 ; and if $n \neq 0$ then G may additionally be $-\text{OR}^7$, $-\text{C}(\text{O})\text{OR}^7$, $-\text{NR}^7_2$ or $-\text{C}(\text{O})\text{NR}^7_2$; particularly R^7 represents H or C_1 - C_4 -alkyl.

- b) G is a leaving group E, selected from halogen, pentafluorophenyl (Pfp), tetrafluorophenyl (Tfp), oxazolone, succinimidyl (Su), sulfosuccinimidyl, trifluoroacetyl, azido, para-nitrophenyl (PNP), and nitro-containing aromatic groups;
- c) G is a thiol reactive moiety of the general formula

5



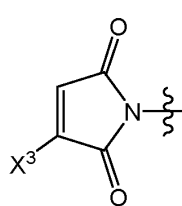
wherein

10

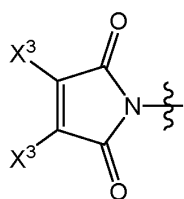
J is selected from $-NH-$, $-S-$ or $-O-$ or is missing;

r is an integer from 1 to 4 or is 0 when J is missing; and

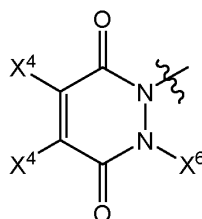
M1 is the thiol reactive portion, and more particularly selected from M2 to M16 (as for example described in WO 2022/058395):



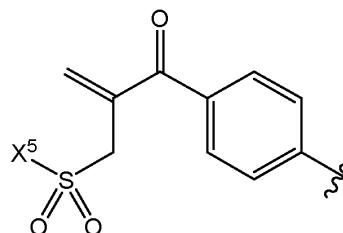
(M2)



(M3)

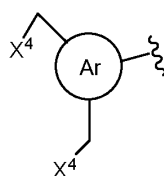


(M4)

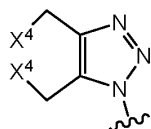


(M5)

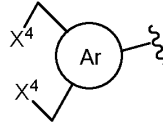
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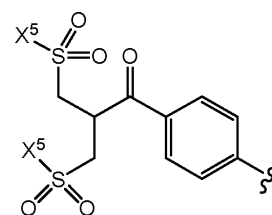
(M6)



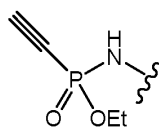
(M7)



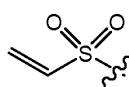
(M8)



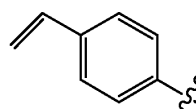
(M9)



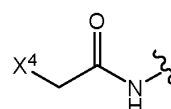
(M10)



(M11)

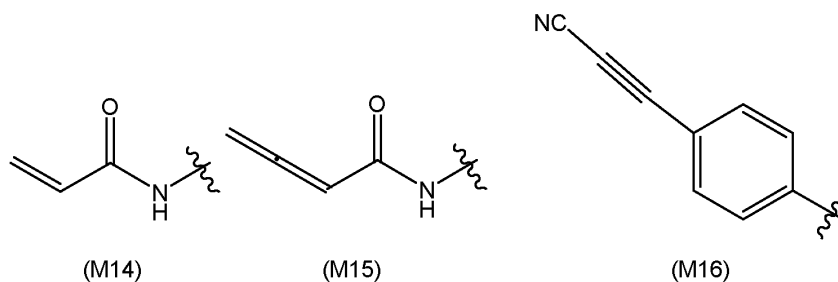


(M12)



(M13)

20



wherein:

X^3 is H, halogen, PhS, MeS;

X^4 is halogen, PhS, MeS;

5 X^6 is H or C_1 - C_{12} alkyl, preferably H or C_{1-6} alkyl;

X^5 is H, C_1 - C_{12} alkyl, C_6 - C_{12} aryl, C_7 - C_{12} alkaryl or C_7 - C_{12} aralkyl, preferably H or para-methylphenyl; and

wherein the aromatic ring of (M6) and (M8) may optionally be a heteroaromatic ring, such as a phenyl or pyridine ring;

10

d) G is $-NH-(CH_2)_2C\equiv CH$, $-NH-(CH_2)_2-N_3$, $-O$ -aryl or $-HN$ -aryl, H, $-OH$, $-NH_2$ halogen, R^9 , $-CH=C(R^9)_2$, $-C\equiv C R^9$, $-[C(R^9)_2C(R^9)_2O]_q-$ R^9 , $-CN$, $-N_3$, $-NCL$, $-LCN$, $-L R^9$, $-^+N(R^9)_2$, $-N(R^9)_3$, $-C(L)N(R^9)_2$, $C(R^9)_2L R^9$, $-C(L) R^9$, $-C(L)L R^9$, $-S(O)R^9$, $-S(O)_2R^9$, $-S(O)OR^9$, $-S(O)_2OR^9$, $-S(O)N(R^9)_2$, $-S(O)_2N(R^9)_2$, $-OS(O)R^9$, $-OS(O)_2R^9$, $-OS(O)OR^9$, $-OS(O)_2OR^9$, $-P(O)(R^9)(OR^9)$, $-P(O)(OR^9)_2$, $-OP(O)(OR^9)_2$, $-Si(R^9)_3$, $-LC(L)R^9$, $-LC(L)LR^9$, $-LC(L)N(R^9)_2$, $-N(R^9)C(L)R^9$, $-N(R^9)C(L)LR^9$ and $-N(R^9)C(L)N(R^9)_2$,

15

wherein

q is in the range of 1 to 200, in particular 1 to 20, more particularly 1 to 10 and especially 1 to 5,

20

L is oxygen or sulphur and

R^9 is independently selected from the group consisting of hydrogen, halogen, C_1 - C_{24} alkyl groups, like C_1 - C_{10} alkyl or C_1 - C_3 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;

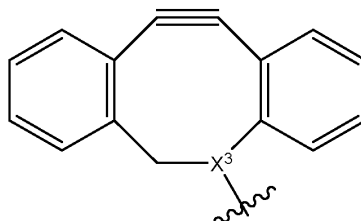
25

e) G is $-OZ$, $-SZ$, $-OR^1$, $-C(O)OR^1$, $-N(R^1)_2$, $-C(O)N(R^1)_2$, $-SR^1$, or $-R^1$,

wherein Z and R^1 are as defined above; and

f) G is a cyclic and acyclic strain promoted alkyne-azide cycloaddition (SPAAC)-reactive groups, in particular,

- i. alkynyl-terminated groups
- ii. DBCO-derived residues of the general formula



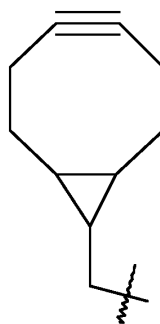
(M17)

5

wherein

 X^3 is N, $>\text{CH}-$ or $>\text{CH}-\text{O}-$; or

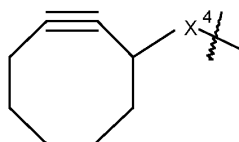
- iii. BCN derived residues of the formula



(M18)

10

- iv. cyclooctyne derived residues, in particular SCO derived residues of the general formula



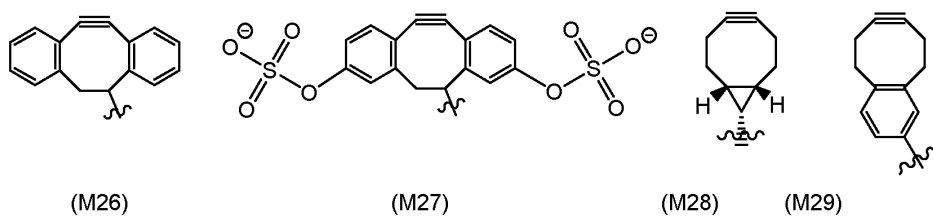
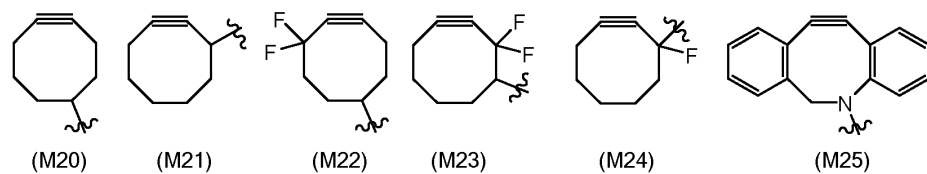
(M19)

wherein

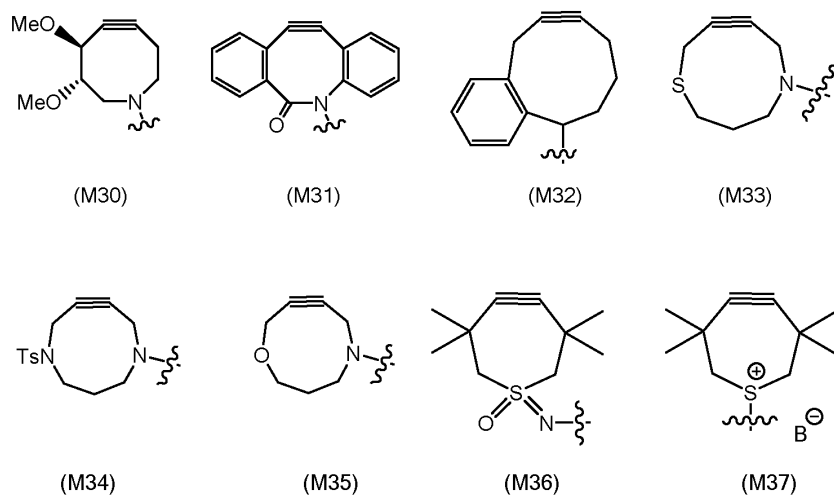
15

 X^4 is $-\text{O}-$ or $-\text{O}-(\text{CO})-$;

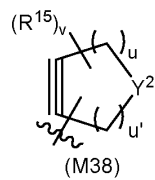
- v. or residues of the formulae M20 to M38 (as for example described in WO 2022/058395)



5



wherein

10 B⁻ is an anion or

wherein:

R¹⁵ is independently selected from the group consisting of hydrogen, halogen, -OR¹⁶, -NO₂, -CN, -S(O)₂R¹⁶, -S(O)₃⁽⁻⁾, C₁ - C₂₄ alkyl groups, C₆ - C₂₄

(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;

wherein

Y^2 is $C(R^{15})_2$, O, S or NR^{15} ;

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; and

v is an integer in the range 8 – 16.

According to a more particular embodiment G is as defined in a).

According to another more particular embodiment G is as defined in b).

According to another more particular embodiment G is as defined in c).

According to another more particular embodiment G is as defined in d).

According to another more particular embodiment G is as defined in e).

According to another more particular embodiment G is as defined in f).

According to another more particular embodiment G is as defined in f) i).

According to another more particular embodiment G is as defined in f) ii).

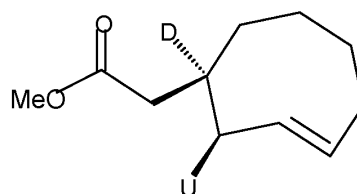
According to another more particular embodiment G is as defined in f) iii).

According to another more particular embodiment G is as defined in f) iv).

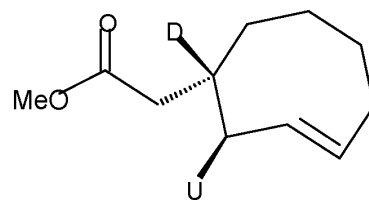
According to another more particular embodiment G is as defined in f) v).

According to another particular embodiment the compound is of the Formula I.1 to

I.12

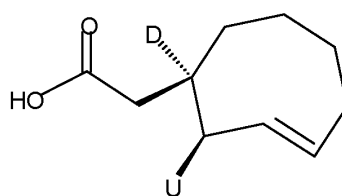


(I.1)

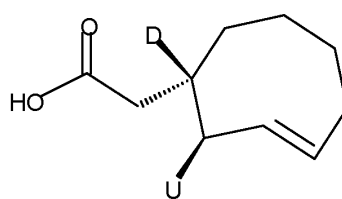


(I.2)

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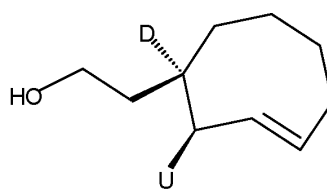


(I.3)



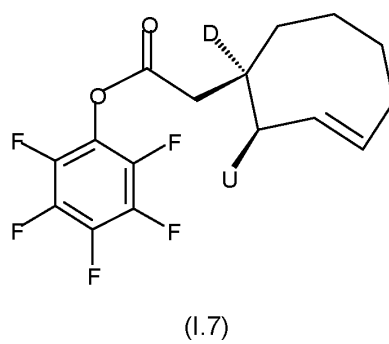
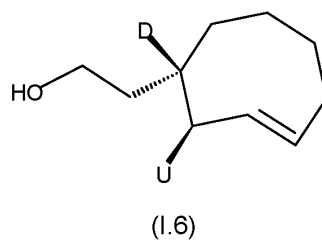
(I.4)

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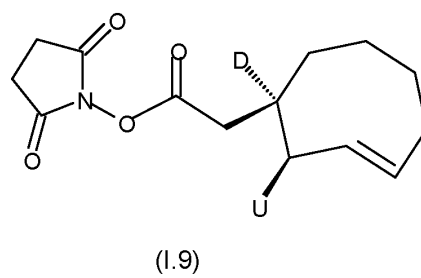
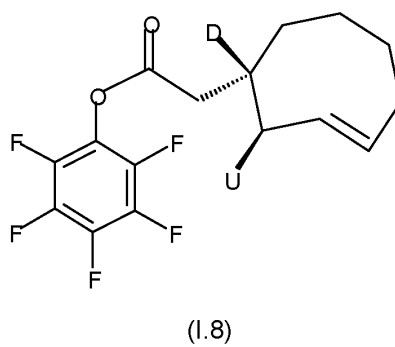


(I.5)

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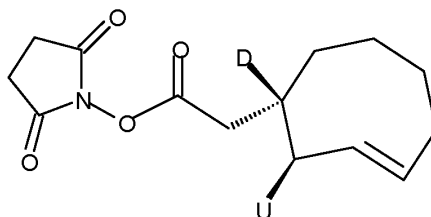


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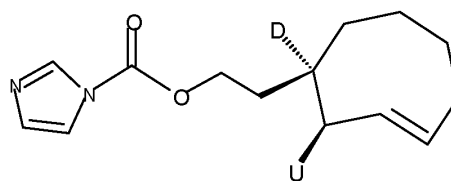


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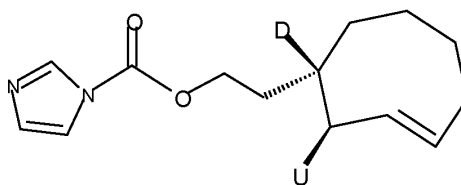
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(I.10)



(I.11)



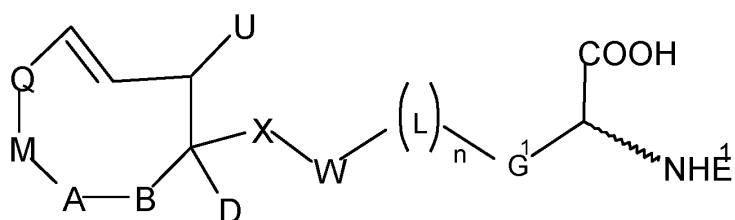
(I.12)

wherein D and U are as defined above; or a salt, like a pharmaceutically acceptable salt or a solvate thereof; each as a stereoisomer or as a mixture of at least two stereoisomers, wherein cis/trans isomers of the TCO-ring moiety are excluded.

According to still another particular embodiment of the above compounds D is –OH and U is –OAc or D is –OH and U is –OH.

2. The second aspect of the present invention

According to a second aspect of the present invention nCAA derivatives of the general formula II are provided



(II)

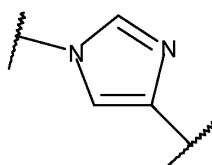
wherein

n, A, B, M, Q, D, U, X, W, L are as defined above for compounds of general formula I

E¹ is H or an amine protecting group; and

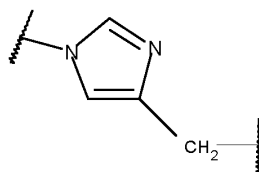
G¹ is selected from -NH-, -NH-C(=NH)NH-, -S-, -O-, -O-Aryl-, -NH-(CH₂)_m-O-Aryl-, wherein m is an integer from 1 to 10;

-HN-Aryl- or a moiety of the formula



or

is selected from any side chain residue of a natural or non-natural amino acid which allows conjugation to moiety W, optionally via moiety (L)_n of said compound of formula II, and in particular selected from -S-CH₂-, -O-CH₂-, -O-p-(phenylene)-CH₂-, -O-CH(CH₃)-, -O-(C=O)-CH₂-, -; -O-(C=O)-CH₂-CH₂-, -N-(C=O)-CH₂-, -; -N-(C=O)-CH₂-CH₂-, -N-(CH₂)₄-, -N-(CH₂)₅-, or



or a salt, like a pharmaceutically acceptable salt, or a solvate thereof; each as a stereoisomer or as a mixture of at least two stereoisomers, wherein cis/trans isomers of the TCO-ring moiety are excluded.

According to a first particular embodiment compounds of general formula II are provided, wherein residues A, B, M and Q are identical and each represent $-C(R^1R^2)-$, wherein R^1 and R^2 independently of each other represent an optionally substituted, linear or branched alkyl group having 1 to 10, in particular 1 to 5 and more particularly 1 or 2 carbon atoms.

According to another particular embodiment compounds of general formula II are provided, wherein residues A, B, M and Q are identical and each represent $-C(R^1R^2)-$, wherein R^1 and R^2 represent H.

According to still another particular embodiment compounds of the general formula II are provided, wherein residues U and D each represent $-OR^3$, wherein R^3 independently of each other represents H or a linear or branched alkyl group, having one to 10, in particular 1 to 5 and more particularly 1 or 2 carbon atoms.

According to still another particular embodiment compounds of general formula II are provided, wherein X is selected from methylene or ethylene and moieties $-((CH_2)_xO)_y-$, or $-(O-(CH_2)_x)_y-$ wherein x is 1 and 2 and y is 1 or 2.

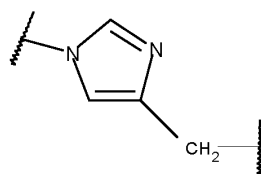
According to still another particular embodiment compounds of general formula II are provided, wherein W is selected from $C(O)-$, $-O-C(O)-$ and $-C(O)-O-$.

According to another particular embodiment compounds of formula II are provided, wherein $n = 1$.

According to still another particular embodiment compounds of formula II are provided, wherein L represents a moiety La, selected from methylene, ethylene and propylene; or represents moiety Lb, selected from $-((CH_2)_{x3}O)_{y3}-$ or $-(O(CH_2)_{x3})_{y3}-$, wherein x_3 and y_3 independently of each other are selected from integers 1, and 2.

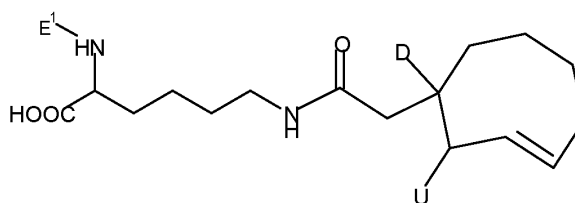
According to still another particular embodiment compounds of the general formula II are provided, wherein residue E^1 is H,

According to still another particular embodiment compounds of the general formula II are provided, wherein residue G^1 is $-S-CH_2-$, $-O-CH_2-$, $-O-p-(phenylene)-CH_2-$, $-O-CH(CH_3)-$, $-O-(C=O)-CH_2-$, $-O-(C=O)-CH_2-CH_2-$, $-N-(C=O)-CH_2-$, $-N-(C=O)-CH_2-CH_2-$, $-N-(CH_2)_4-$, $-N-(CH_2)_5-$, or

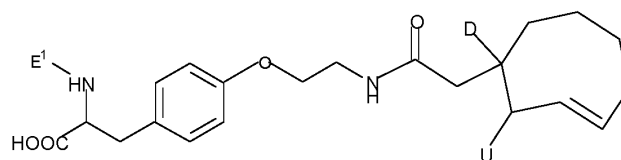


According to a particular embodiment of the above compounds of formula II, residues E¹ is H or an amine protecting group selected from the group consisting of 9-fluorenylmethyloxycarbonyl (Fmoc), carbobenzyloxy (Cbz), p-methoxybenzyl carbonyl (Moz or MeOZ), tert.-butoxyloxycarbonyl (BOC), acetyl (Ac), benzyl (Bn), carbamate, p-methoxybenzyl (PMB), 3,4-dimethoxybenzyl (DMPM), p-methoxyphenyl (PMP), or tosyl (Ts) group.

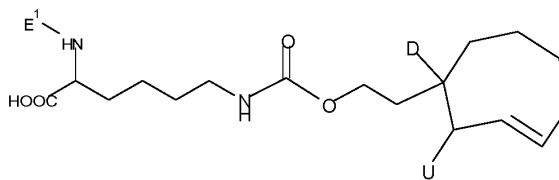
According to another particular embodiment said compound is of anyone of the formulae II.1 to II.3



(II.1)

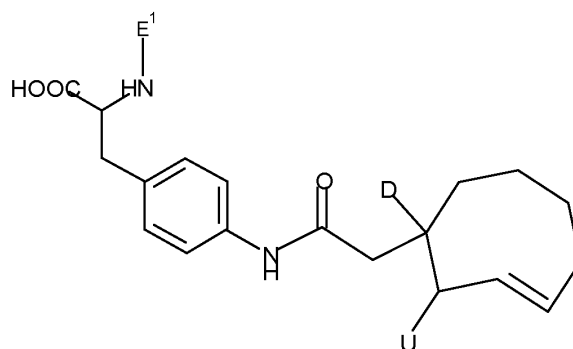


(II.2)



(II.3)

41



(II.4)

5

wherein

E¹ is H or an amine protecting group as defined above;

or a salt, like a pharmaceutically acceptable salt or a solvate thereof; each as a stereoisomer or as a mixture of at least two stereoisomers; wherein cis/trans isomers of the TCO-ring moiety are excluded.

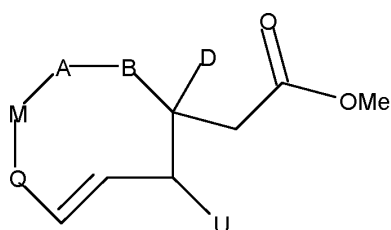
10

According to a particular embodiment of compound of formula II, D is –OH and U is –OAc or D is –OH and U is –OH.

3. The third aspect of the present invention

15

According to a third aspect of the invention a method for the preparation of a compound of formula VIII is provided



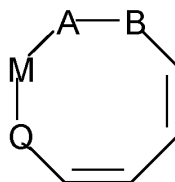
(VIII)

20

wherein M, A, B, D, Q and U are as defined above;

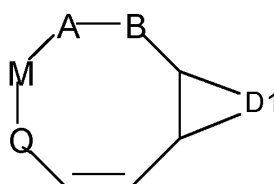
which method comprises comprising the steps of:

- a) providing a compound of formula III



(III)

- 5 b) converting a compound of formula III to compound of formula IV



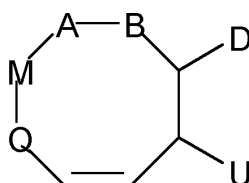
(IV)

wherein D1 is O, S or N; and in particular, if D1 is oxygen, epoxidizing a compound of formula III with an organic peroxyacid, in particular 3-chloroperbenzoic acid;

10

- c) reacting compound of formula IV with a nucleophile U-H, in particular a low molecular aliphatic carboxylic acid, like acetic or propionic acid and in the presence of an organometallic catalyst, like in particular tetrakis(triphenylphosphine)palladium(0), thereby obtaining compound of formula V

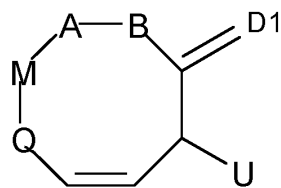
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(V)

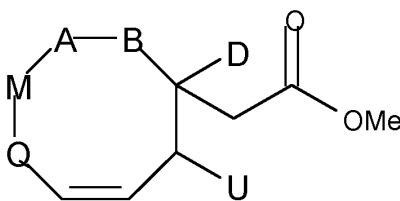
- d) oxidizing compound of formula V, in the presence of a suitable oxidant, like in particular pyridinium chlorochromate if D is -OH, thereby obtaining compound of formula VI

20



(VI)

- 5 e) reacting compound of formula VI with silyl protected enolate compound, like in particular 1-(tert-butyldimethylsilyloxy)-1-methoxyethene, in particular after activation with a BF_3 source, to thereby obtain a compound of formula VII



(VII)

and

10

- f) isomerizing, in particular photoisomerizing, said compound of formula VII, thereby obtaining compound of formula VIII.

According to a particular embodiment of said method D1 is an oxygen atom.

15

According to a another particular embodiment of said method at least one of the steps a) to f) takes place in a liquid reaction medium, in particular in an organic liquid reaction medium.

20

According to a another particular embodiment of said method step b) occurs in presence of oxidizing agent selected from the group consisting of 3-chloroperbenzoic acid, oxone, other peroxy acids, H_2O_2 , tert-butyl peroxide, other peroxides, hypochlorites, persulfates, O_2 , dimethyl dioxirane, other dioxiranes, in particular 3-chloroperbenzoic acid.

25

According to a another particular embodiment of said method step c) occurs in presence of a palladium catalyst selected from the group of

tetrakis(triphenylphosphine)palladium(0), Pd(dppe)₂, Pd₂(dba)₃, Pd(COD)Cl₂, in particular tetrakis(triphenylphosphine)palladium(0).

According to a another particular embodiment of said method wherein step d) occurs
5 in presence of oxidizing agent selected from the group consisting of pyridinium chlorochromate, pyridinium dichromate, chromic oxide, dichromates chromates, DMP, IBX, periodinanes, DMSO with oxalyl chloride, MnO₂, KMnO₄, H₂O₂, O₂, in particular pyridinium chlorochromate.

10 According to a another particular embodiment of said method, step e) occurs in presence of

- a. a lewis acid selected from the group consisting of BF₃·OEt₂, TiCl₄, Ti(OR)₄, AgOTf, TMSOTf, Cu(OTf)₂, SiCl₄, transition metal salts, in particular BF₃·OEt₂; and
- b. a silyl protected enolate ester selected from the group consisting of silylketene
15 acetals, N,O-silylketene acetals, silyl enol ethers, in particular (tert-butyl)dimethylsilyloxy)-1-methoxyethene.

According to a another particular embodiment of said method step f) occurs in presence of UV light without or together with a photosensitizer capable of absorbing UV light
20 and producing a physicochemical change in a neighboring molecule such as a substituted aryl groups selected from methyl benzoate, benzene, toluene, xylene, benzophenone, acetophenone, cumene, phthalimide, phthalates, terephthalates, benzamide, benzonitrile.

According to a another particular embodiment of said method anyone of steps a) to f)
25 takes place for a total reaction time of 15 min to 48 h, or 10 to 24h, or of 24h or of 12h, in particular of 180 min.

According to a another particular embodiment of said method anyone of steps a) to f)
takes place at a temperature of about -100 °C to about 100 °C or about -80 °C and about
30 70°C.

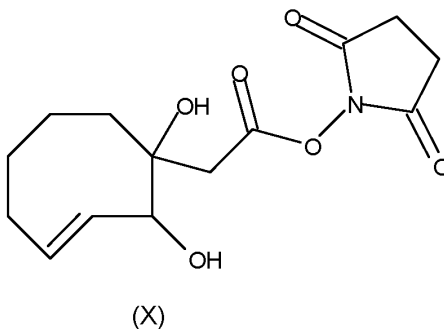
According to a another particular embodiment of said method

- a) M, A and B are each -CH₂-;
- b) in compounds of formula IV and VI D1 represents O;
- 35 c) D represents -OH; and

d) U represent OAc or OH.

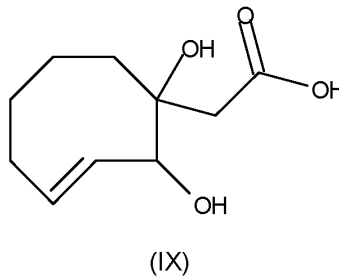
4. The fourth aspect of the present invention

- 5 According to a fourth aspect a method for the preparation of a compound of formula X is provided



comprising

- 10 a) converting compound of above formula VIII wherein M, A, B and Q each are $-\text{CH}_2-$ and D and U each are $-\text{OH}$ to compound of formula IX by removing the ester group, and



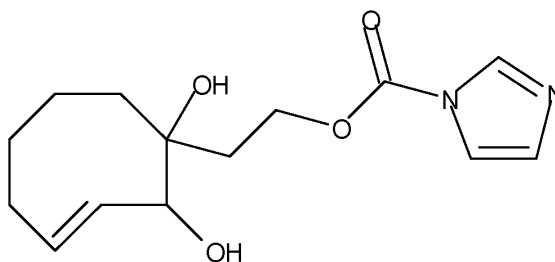
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- b) converting compound of formula IX in the presence of N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate to compound of formula X.

5. The fifth aspect of the present invention

20

According to the fifth aspect of the present invention a method for the preparation of a compound of formula XII is provided,

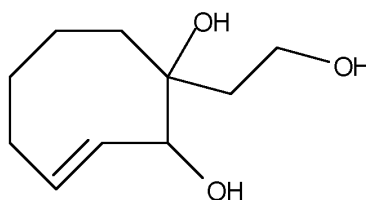


(XII)

comprising the steps of:

- 5 a) converting compound of above formula VIII
 wherein M, A, B and Q each are -CH₂- and D and U each are -OH

to compound of formula XI



(XI)

and

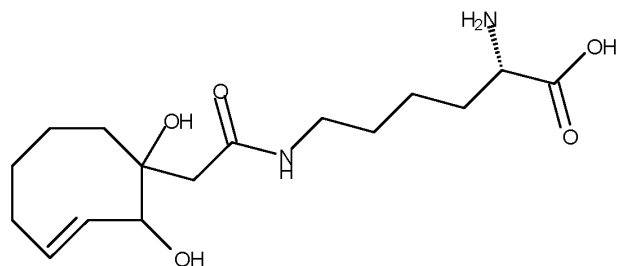
- 15 b) converting said compound of formula XI, particularly under temperature control, at
 a temperature in the range of -10 to +20°C with 1,1'-carbonyldiimidazole, to a
 compound of formula XII.

6. The sixth aspect of the present invention

20

According to the sixth aspect of the invention a method for the preparation of a compound of formula XIV is provided

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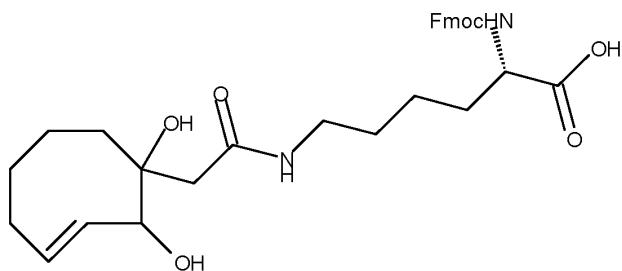


(XIV)

comprising the steps of:

5

- a) converting compound of above formula VIII, in particular in the presence of Fmoc Lys-OH and an organic nucleophilic base, like in particular DIPEA, to compound of formula XIII



(XIII)

and

10

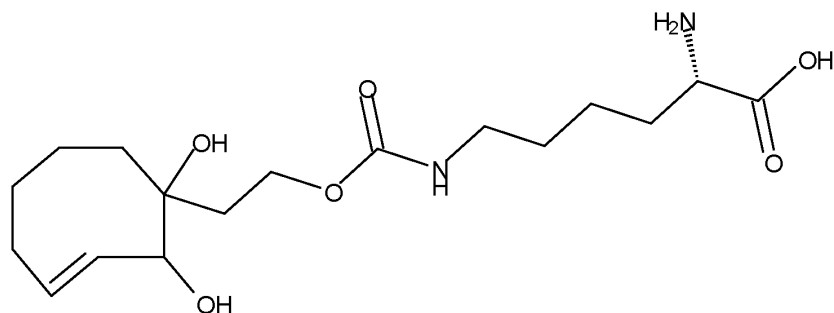
- b) converting said compound of formula XIII to compound of formula XIV, particularly by deprotecting in the presence of a secondary base, like piperidine.

15

7. The seventh aspect of the present invention

According to the seventh aspect of the invention a method for the preparation of a compound of formula XVI is provided

20

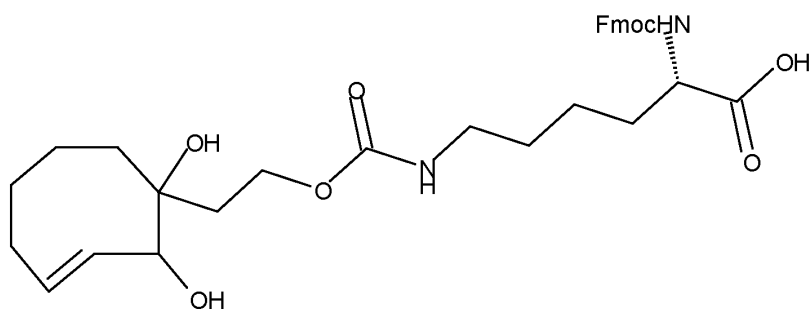


(XVI)

comprising the steps of:

5

- a) converting compound of above formula XII, in particular in the presence of Fmoc Lys-OH and an organic nucleophilic base, like in particular DIPEA, to compound of formula XV



(XV)

10

and

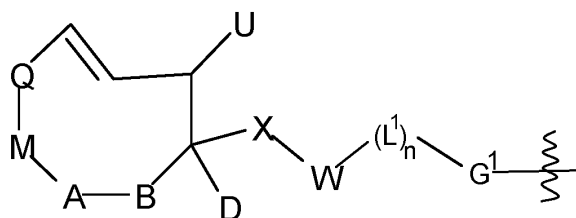
- b) converting said compound of formula XV to compound of formula XVI, particularly by deprotecting in the presence of a secondary base, like piperidine.

15 8. The eighth aspect of the present invention - hyTCO-functionalized construct

According to the eighth aspect of the invention a hydrophilic trans-cyclooctene (hyTCO)-functionalized construct is provided, obtainable by, in particular covalently, binding a compound according to anyone of first or second aspect of the invention to a targeting agent (TA), or to a payload molecule (PM), wherein said functionalized construct contains at

20

least one functionalizing residue (FR) comprising a mono-unsaturated trans-cyclooctene entity of the general formula (II')



(II')

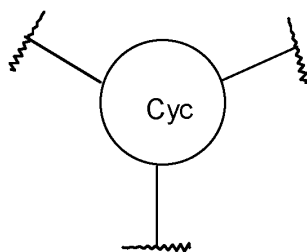
wherein

n, A, B, D, U, M, Q, X, and W are as defined herein above;

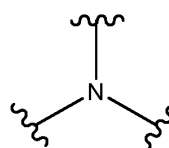
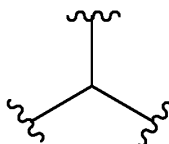
G¹ is as defined above or is missing; and

L¹ has the meaning of linker moiety L as defined above or represents a branching moiety.

A "branching moiety" represents an at least trivalent group, which allows the functionalization with more than one, as for example 2 of 3 hyTCO moieties in a single step. Non-limiting examples of such branching groups are trivalent cycloaliphatic or cycloaromatic residues of the general formula



or trivalent, non-cyclic, aliphatic groups or tertiary amino groups of the following formulae



According to a particular embodiment, said functionalized construct is a functionalized targeting agent (functionalized TA), wherein the targeting agent is selected from viruses, whole cells, phages, liposomes, biomolecules and low- or high-molecular weight chemical

compounds, such as in particular immunoglobulins, like antibodies, antibody derivatives, antibody fragments, fusion molecules comprising at least one antibody or antibody fragment, enzymes, proteins, peptides, peptidomimetics (peptoids), carbohydrates, monosaccharides, polysaccharides, oligo- or polynucleotides, in particular DNA, RNA, PNA and LNA molecules, aptamers, drugs, glycoproteins, glycans, lipids, polymers, chemotherapeutic agents, receptor agonists and receptor antagonists, cytokines, hormones, steroids, toxins and derivatives thereof.

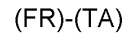
According to another particular embodiment said functionalized construct is a functionalized payload molecule (functionalized PM), wherein the payload molecule is selected from bio-active compounds, labeling agents, protein degraders, in particular payloads applicable in proteolysis targeting chimeras (PROTACs), photosensitizers, and chelators.

According to a more particular embodiment of said functionalized construct

- 15 a) the bioactive compound is selected from biomolecules and low- or high-molecular weight chemical compounds, such as in particular immunoglobulins, like antibodies, antibody derivatives, antibody fragments, fusion molecules comprising at least one antibody or antibody fragment, enzymes, proteins, peptides, peptidomimetics (peptoids), carbohydrates, monosaccharides, polysaccharides, oligo- or polynucleotides, in particular DNA, RNA, PNA and LNA molecules, aptamers, drugs, glycoproteins, glycans, lipids, polymers, chemotherapeutic agents, receptor agonists and receptor antagonists, cytokines, hormones, steroids, toxins and derivatives thereof, and more particularly selected from small organic, inorganic or organometallic molecule
20 drugs, steroids, lipids, proteins, aptamers, oligopeptides, oligonucleotides, oligosaccharides, as well as polypeptides, peptoids, amino acids, nucleotides, oligo- or polynucleotides, nucleosides, DNA, RNA, toxins, glycans and immunoglobulins;
- 25 b) the labeling agents are selected from dyes, radiolabels and fluorophores, MRI-sensitive spin labels, affinity tags, polyethylene glycol groups, photocrosslinkers, NMR probes, X-ray probes, pH probes, IR probes, resins, and solid supports;
- 30

- c) the chelators are selected from acetyl acetone (ACAC), ethylene diamine (EN), 2-(2-aminoethylamino)ethanol (AEEA), diethylene triamine (DIEN), iminodiacetate (IDA), triethylene tetramine (TRIEN), triaminotriethylamine, nitrilotriacetate (NTA) and its salts like Na₃NTA or FeNTA, ethylenediaminotriacetate (TED), ethylenediamine tetraacetate (EDTA) and its salts like Na₂EDTA and CaNa₂EDTA, diethylene triaminopentaacetate (DTPA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (DOTA), 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), oxalate (OX), tartrate (TART), citrate (CIT), dimethylglyoxime (DMG), 8-hydroxyquinoline, 2,2'-bipyridine (BPY), 1,10-phenanthroline (PHEN), dimercapto succinic acid (DMSA), 1,2-bis(diphenylphosphino)ethane (DPPE), sodium salicylate, methoxy salicylates, British anti-Lewisite or 2,3-dimercaprol (BAL), meso-2,3-dimercaptosuccinic acid (DMSA); Siderophores secreted by microorganisms, as for example desferrioxamine or deferoxamine B, also known as Deferral (Novartis), produced by *Streptomyces spp.*; deferoxamine (DFO), a trihydroxamic acid secreted by *Streptomyces pilosus*; phytochemicals like curcuminoids and derivatives of mugineic acid, like 3-hydroxy-mugineic acid and 2'-deoxy-mugineic acid; synthetically produced chelators, like Ibuprofen; derivatives of catechol, hydroxamate and hydroxypyridinone, like hydroxamate desferal and hydroxypyridinone deferiprone; deferiprone (L1 or 1,2-dimethyl-3-hydroxypyrid-4-one); D-penicillamine (DPA or D-PEN) which is β - β -dimethylcysteine or 3-mercapto-D-valine; tetraethylenetetraamine (TETA) or trientine and its two major metabolites N₁-acetyltriethylenetetraamine (MAT) and N₁,N₁₀-diacetyltriethylenetetraamine (DAT); hydroxyquinolines; clioquinol, which is a halogenated derivative of 8-hydroxyquinoline; and 5,7-dichloro-2-[(dimethylamino)methyl]quinolin-8-ol (PBT2);
- d) the photosensitizer/protein degraders are selected from payloads applicable in proteolysis targeting chimeras (PROTACs).

According to another particular embodiments of said functionalized construct the functionalizing residue (FR) of formula II' is attached (directly or via a cleavable or non-cleavable moiety) to the targeting agent (TA) or payload molecule (PM), in particular to an amino acid residue of said TA or PM, thereby forming a functionalized TA of the general formula (XX.1)



(XX.1)

wherein

5 TA is a targeting moiety as defined herein above; and
 FR is a functionalizing residue of above formula II'

or a functionalized PM of the general formula (XX.2)



(XX.2)

wherein

15

a is 0 or represents an integer selected from 1 and 2,
b is 0 or represents an integer selected from 1 and 2,
c represents an integer selected of at least 1, particularly from 1 and 2,
Y¹ represents a cleavable moiety,
20 Y² represents a self-immolative moiety,

PM is a payload molecule as defined herein above and
FR is a functionalizing residue of above formula II', wherein, when
c represents an integer of more than 1, then L¹ represents a branching
25 moiety, n is 1 and G1 is missing.

More particularly

Y¹ represents an enzymatically or chemically cleavable linker group selected from

a) a peptidyl group, in particular di-, tri- or tetra-peptidyl group;

b) a disulfide group of the formula $-(CR^7R^{8'})_n-S-S-(CR^7R^8)_n-X_5$ -

wherein

n' is 1 to 4

residues R⁷ and R⁸ independently of each other are selected from H or lower alkyl, in particular methyl; or two residues R⁷ and R⁸

together with the carbon atom which they are attached to form a cyclic C₄ -to C₈-alkyl group; and

moiety X₅ is selected from -C(O)- and -O- ;

- c) hydrazone groups selected from >C=N-N(R^{9'})- and -N(R^{9'})-N=C<

wherein

R^{9'} is H or lower alkyl; and

- d) beta-glucuronidase-sensitive cleavable linker groups, in particular carrying a beta-glucuronic acid derived trigger residue

and/or

Y² represents a self-immolative moiety, selected from

- a) p-amino-benzyl alcohol derived groups of the formula
-NH-p-phenylene-CH₂-O- or -O-CH₂-p-phenylene-NH- or
-NH-p-phenylene-CH₂-N⁺R₂-

- b) -O-C(O)-O-;

- c) -O-C(O)-NR^{10'}-(CR^{12'}R^{13'})_z-NR^{11'}-C(O)-O- or
-X^{1'}-C(O)-NR^{10'}-(CR^{12'}R^{13'})_z-NR^{11'}-C(O)-X^{2'}-

wherein

R₂ represents lower alkyl, in particular, C₁ to C₄-alkyl

Z' represents an integer selected from 1 to 6, in particular 1 to 4;

R^{10'} and R^{11'}, independently of each other, represent H or lower alkyl group

R^{12'} and R^{13'}, independently of each other, represent H, methyl or ethyl, in particular H or methyl, especially H; and

X^{1'} and X^{2'} independently of each other represent O, S or NR¹⁰

- d) methylene alkoxy carbamates (MAC) type linkages of the formula

-OC(O)-NR^{13'}-C(R^{14'}R^{15'})-(O)-

-OC(O)-NR^{13'}-C(R^{14'}R^{15'})-(S)-

-OC(O)-NR^{13'}-C(R^{14'}R^{15'})-(NR^{16'})- or

-OC(O)-NR^{13'}-C(R^{14'}R^{15'})-(NR^{16'}-C(O)O)-

wherein

R^{13'}, R^{14'}, R^{15'}, and R^{16'}, independently of each other represent H or lower alkyl, in particular, C₁ to C₄-alkyl.

According to another particular embodiment said functionalized construct is a functionalized TA of formula XX.1,

(FR)-(TA)

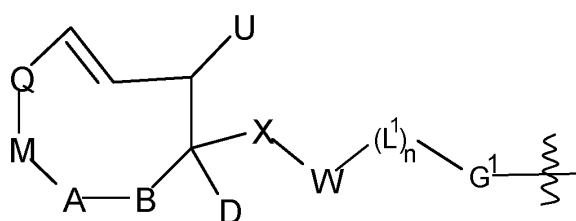
5

(XX.1)

wherein

TA is a targeting agent as defined herein above;

FR is a functionalizing residue of formula II'



10

(II')

wherein

A, B, M and Q are $-CR^1R^2-$, wherein R^1 and R^2 are as defined herein above;

15

D and U are $-OR^3$, wherein R^3 is as defined above; and

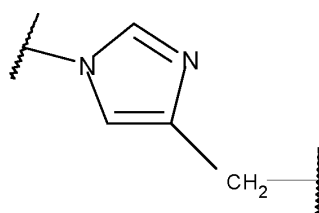
n , L^1 , X and W are as defined above;

and

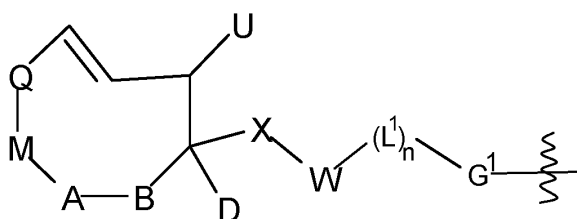
G^1 is defined above, and in particular is selected from any side chain residue of a natural or non-natural amino acid which allows conjugation to moiety W, optionally via moiety $(L^1)_n$ of said compound of formula II, and in particular selected from $-S-$, $-CH_2-$, $-O-CH_2-$, $-O-p-(\text{phenylene})-CH_2-$, $-O-CH(CH_3)-$, $-O-(C=O)-CH_2-$, $-O-(C=O)-CH_2-CH_2-$, $-N-(C=O)-CH_2-$, $-N-(C=O)-CH_2-CH_2-$, $-N-(CH_2)_4-$, $-N-(CH_2)_5-$, or

25

55



More particularly, said functionalized construct is of general formula XXI



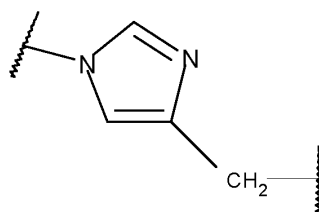
5

(XXI)

wherein

- X is methylene or ethylene;
 W is $-C(O)-$, $-C(O)-O-$ or $-O-C(O)-$;
 G¹ is selected from $-S-CH_2-$, $-O-CH_2-$, $-O-p-(\text{phenylene})-CH_2-$, $-O-CH(CH_3)-$, $-O-(C=O)-CH_2-$, $-O-(C=O)-CH_2-CH_2-$, $-N-(C=O)-CH_2-$, $-N-(C=O)-CH_2-CH_2-$, $-N-(CH_2)_4-$, $-N-(CH_2)_5-$, or

10

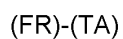


15

particularly $-O-p-(\text{phenylene})-CH_2-$, and $-N-(CH_2)_4-$,
 L¹ and n are as defined above; and TA is a targeting moiety as defined herein above.

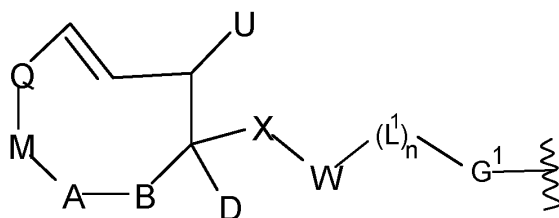
According to a very particular embodiment of said functionalized construct TA is an immunoglobulin moiety.

According to another particular embodiment of said functionalized construct, said
5 functionalized TA is of the formula XX.1



wherein

10 TA is a targeting moiety as defined herein above
 FR is a functionalizing residue of formula

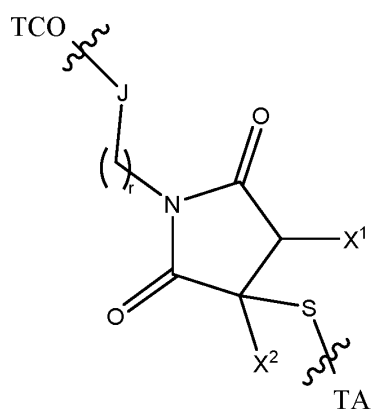


15

wherein

 L¹ and n are as defined above,
 A, B, M and Q are -CR¹R²-,
 D and U are -OR³,
20 X and W are as defined herein above;
 G¹ is missing or represents a moiety as formed by a
 conventional coupling reaction, in particular click reaction....,
 like in particular

57

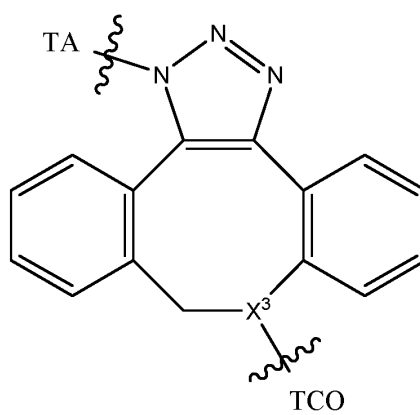


wherein

J is selected from $-\text{NH}-$, $-\text{S}-$ or $-\text{O}-$ or is missing;

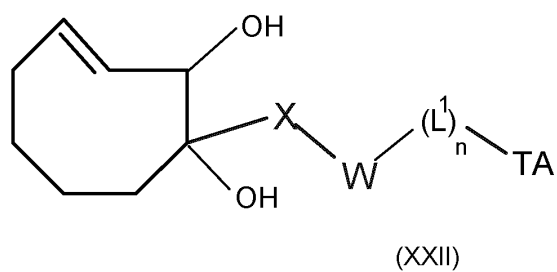
5

or



More particularly, said functionalized construct is of general formula XXII

10



wherein

L^1 and n are as defined above,

X is methylene or ethylene;

W is $-C(O)-$, $-C(O)-O-$ or $-O-C(O)-$;

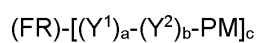
TA is a targeting moiety.

5

According to a very particular embodiment of said functionalized construct TA is an antibody moiety.

According to another particular embodiment said functionalized construct is a functionalized PM of formula XX.2,

10



(XX.2)

15

wherein

a is 0 or represents an integer selected from 1 and 2

b is 0 or represents an integer selected from 1 and 2

c represents an integer selected of at least 1, particularly from 1 and 2,

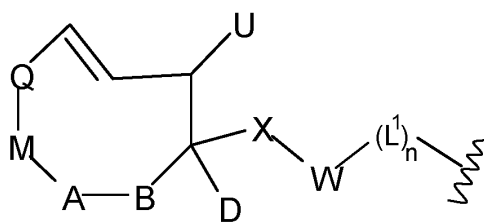
Y^1 represents a cleavable moiety as defined herein above; and

20

Y^2 represents a self-immolative moiety as defined herein above

PM is a payload moiety as defined herein above

FR is a functionalizing residue of formula II'



(I')

25

wherein

A , B , M and Q are $-CR^1R^2-$, wherein R^1 and R^2 are as defined above;

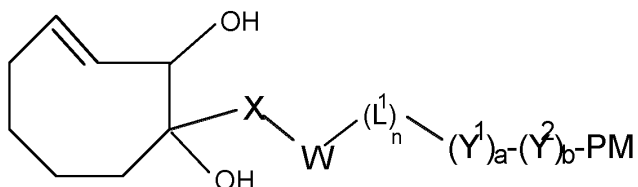
D and U are $-OR^3$, wherein R^3 is as defined above; and

n , L^1 , X and W are as defined above;

with the proviso,

that when c represents an integer of more than 1, then L^1 represents a branching moiety and n is 1.

According to another particular embodiment said functionalized construct is a
5 functionalized PM of formula XXIII



(XXIII)

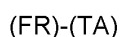
wherein

10 X, W, n, a, b, L^1 , Y^1 and Y^2 are as defined herein above.

9. The ninth aspect of the present invention - Conjugates (TA-PM)

According to a ninth aspect of the present invention a conjugate is provided
15 obtainable by covalently linking a first functionalized molecule, selected from the hydrophilic trans-cyclooctene (hyTCO)-functionalized construct as defined above with a second functionalized molecule comprising a docking group (DG) capable of reacting with said trans-hyTCO-functional group of the first molecule; in particular obtainable via biorthogonal bioconjugation via a Diels-Alder-type cycloaddition reaction of said two functionalized
20 molecules; more particularly, wherein said DG selected from an optionally substituted triazinyl or optionally substituted tetrazinyl group, capable of covalently reacting in a copper-free strain promoted inverse-electron-demand Diels-Alder cycloaddition (SPIEDAC) with said trans-cyclooctene group.

25 According to a particular embodiment of said conjugate said hydrophilic trans-cyclooctene (hyTCO)-functionalized construct is selected from a functionalized targeting agent TA of the general formula (XX.1)



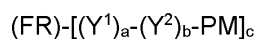
(XX.1)

30

wherein

FR is a functionalizing residue of formula II' and
 TA is a targeting moiety as defined herein above;

5 or a functionalized PM of the general formula (XX.2)



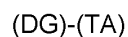
(XX.2)

10 wherein

a is 0 or represents an integer selected from 1 and 2,
 b is 0 or represents an integer selected from 1 and 2,
 c represents an integer selected of at least 1, particularly from 1 and 2,
 Y¹ represents a cleavable moiety,
 15 Y² represents a self-immolative moiety,
 FR is a functionalizing residue of formula II' wherein, when
 c represents an integer of more than 1, then L1 represents a branching
 moiety, n is 1 and G1 is missing
 and
 20 PM is a payload molecule as defined herein above.

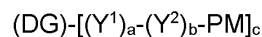
According to another particular embodiment of said conjugate said second
 functionalized molecule comprising a docking group (DG) is selected from
 a functionalized targeting agent TA of the general formula XX.3

25



(XX.3)

30 or a functionalized payload molecule PM of the general formula (XX.4)



(XX.4)

35

wherein

a, b and c, as well as Y¹, and Y², are as defined above;

TA is a targeting moiety as defined herein above and

PM is a payload molecule as defined herein above, and

DG is a docking group as defined above.

In said compound of formula XX.3 said DG may be linked to said TA via suitable amino acid side chains chemically in a manner known per se, for example by reacting a DG carrying an active ester group to a lysine side chain or by reacting a DG carrying a thiol reactive group, like an maleimide group, with a cysteine side chain. Conventional enzymatic reactions, like glycan remodelling are also suitable.

According to another particular embodiment, said conjugate is obtainable

i) by reacting (performing a click-reaction of) a functionalized targeting agent TA

$$(FR)-(TA)$$

$$(XX.1)$$

wherein

FR is a functionalizing residue of formula II', and

TA is a targeting moiety as defined herein above ;

and a functionalized payload molecule PM of the general formula XX.4

$$(DG)-[(Y^1)_a-(Y^2)_b-PM]_c$$

$$(XX.4)$$

wherein

a, b and c, as well as Y¹ and Y², are as defined above;

PM is a payload molecule as defined herein above, and

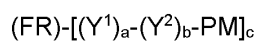
DG is a docking group as defined above.

and optionally isolating the obtained conjugate

or

- ii) by reacting (performing a click-reaction of) a functionalized payload molecule PM of the general formula (XX.2)

5



(XX.2)

wherein

10

a, b and c, as well as Y¹ and Y², are as defined above;

FR is a functionalizing residue of formula II', wherein, when

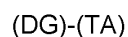
c represents an integer of more than 1, then L1 represents a branching moiety, n is 1 and G1 is missing

TA is a targeting moiety as defined herein above and

15

PM is a payload molecule as defined herein above;

and a functionalized targeting agent TA of the general formula XX.2



20

(XX.3)

wherein

TA is a targeting moiety as defined herein above, and

25

DG is a docking group as defined above.

30 According to still another particular embodiment of said conjugate said DG is a diene comprising group selected from tetrazines or triazines capable of covalently reacting in a copper-free strain promoted inverse-electron-demand Diels-Alder cycloaddition (SPIEDAC) with said trans cyclooctene group of above formula II'.

According to still another particular embodiment of said conjugate said TA is an immunoglobulin molecule, in particular monoclonal antibody, derivative or fragment thereof, carrying at least one non-canonical amino acid residue (ncAA) in a polypeptide chain,

wherein said ncAA carries a hyTCO-type side chain as defined in anyone of the first and second aspect as defined above

According to still another particular embodiment of said conjugate said PM is defined as defined above.

10. The tenth aspect of the present invention

According to a tenth aspect of the invention a pharmaceutical composition comprising in a pharmaceutically acceptable carrier at least one conjugate as defined above, or a diagnostic composition comprising in a diagnostically applicable carrier at least one conjugate as defined above are provided.

11. The eleventh aspect of the present invention

According to an eleventh aspect of the invention a conjugate, in particular ADC as defined above for use in medicine, as for example in diagnosis and therapy is provided

More particularly an antibody payload conjugate (APC), in particular ADC as defined above for use in the diagnosis or treatment of cancers, like breast cancer, gastric cancer or other Her2 overexpressing tumors, as for example tumors of ovary, endometrium, bladder, lung, colon, and head and neck is provided.

12. The twelfth aspect of the present invention

According to a twelfth aspect a diagnostic or analytical kit comprising at least one hyTCO-type compound or conjugate as defined above is provided

D. FURTHER EMBODIMENTS

1. Payload molecules (PM)

1.1 Bioactive compounds

Bioactive compounds include, but are not limited to, the following:

Bioactive compounds applicable according to the present invention include but are not limited to: small organic molecule drugs, steroids, lipids, proteins, aptamers, oligopeptides, oligonucleotides, oligosaccharides, as well as peptides, peptoids, amino acids,

nucleotides, oligo- or polynucleotides, nucleosides, DNA, RNA, toxins, glycans and immunoglobulins.

Exemplary classes of bioactive compounds that can be used in the practice of the present invention include but are not limited to hormones, cytotoxins, antiproliferative/antitumor agents, antiviral agents, antibiotics, cytokines, anti-inflammatory agents, antihypertensive agents, chemosensitizing, photosensitizing and radiosensitizing agents, anti-AIDS substances, anti-viral agents, immunosuppressants, immunostimulants, enzyme inhibitors, anti-Parkinson agents, neurotoxins, channel blockers, modulators of cell-extracellular matrix interactions including cell growth inhibitors and anti-adhesion molecules, inhibitors of DNA, RNA or protein synthesis, steroidal and non-steroidal anti-inflammatory agents, anti-angiogenic factors, anti-Alzheimer agents.

In some embodiments, the bioactive compound is a low to medium molecular weight compound (e.g. about 200 to 5000 Da, about 200 to about 1500 Da, preferably about 300 to about 1000 Da).

Exemplary cytotoxic drugs are particularly those which are used for cancer therapy. Such drugs include, in general, DNA damaging agents, anti-metabolites, natural products and their analogs, enzyme inhibitors such as dihydro folate reductase inhibitors and thymidylate synthase inhibitors, DNA binders, DNA alkylators, radiation sensitizers, DNA intercalators, DNA cleavers, microtubule stabilizing and destabilizing agents, topoisomerases inhibitors. Examples include but are not limited to platinum-based drugs, the anthracycline family of drugs, the vinca drugs, the mitomycins, the bleomycins, the cytotoxic nucleosides, taxanes, lexitropsins, the pteridine family of drugs, diynenes, the podophyllotoxins, dolastatins, maytansinoids, differentiation inducers, and taxols. Particularly useful members of those classes include, for example, auristatins, maytansines, maytansinoids, calicheamicins, dactinomycines, duocarmycins, CC1065 and its analogs, camptothecin and its analogs, SN-38 and its analogs; DXd, tubulysin M, cryptophycins, pyrrolobenzodiazepines and pyrrolobenzodiazepine dimers (PBDs), pyridinobenzodiazepines (PDDs) and indolinobenzodiazepines (IBDs) (cf. US20210206763A1), methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, DNA minor groove binders, 6-mercaptopurine, cytosine arabinoside, melphalan, leurosine, leurosideine, actinomycin, anthracyclines (doxorubicin, epirubicin, idarubicin, daunorubicin, PNU-159682 (cf. US 10,288,745 B2.) and its analogs, mitomycin C, mitomycin A, caminomycin, aminopterin, tallysomycin, podophyllotoxin and podophyllotoxin derivatives such as etoposide or etoposide phosphate, vinblastine, vincristine, vindesine, taxol, taxotere retinoic acid, butyric acid, N8-acetyl spermidine,

staurosporin, colchicine, camptothecin, esperamicin, ene-diynes, and their analogues, hemiasterlin and its analogues.

Other exemplary drug classes are angiogenesis inhibitors, cell cycle progression inhibitors, P13K/m-TOR/AKT pathway inhibitors, MAPK signaling pathway inhibitors, kinase
5 inhibitors, protein chaperones inhibitors, HDAC inhibitors, PARP inhibitors, Wnt/Hedgehog signaling pathway inhibitors, RNA polymerase inhibitors, and protein degraders (cf. <https://pubs.acs.org/doi/10.1021/acscchembio.0c00285>).

Examples of auristatins include dolastatin 10, monomethyl auristatin E (MMAE), auristatin F, monomethyl auristatin F (MMAF), auristatin F hydroxypropylamide (AF HPA),
10 auristatin F phenylene diamine (AFP), monomethyl auristatin D (MMAD), auristatin PE, auristatin EB, auristatin EFP, auristatin TP and auristatin AQ. Suitable auristatins are also described in U.S. ;Publication Nos. 2003/0083263, 2011/0020343, and 2011/0070248; PCT Application ;Publication Nos. WO09/117531, WO2005/081711, WO04/010957; WO02/088172 and WO01/24763, and U.S. Patent Nos. 7,498,298; 6,884,869; 6,323,315;
15 6,239,104; 6,124,431; ;6,034,065; 5,780,588; 5,767,237; 5,665,860; 5,663,149; 5,635,483; 5,599,902; 5,554,725; ;5,530,097; 5,521,284; 5,504,191; 5,410,024; 5,138,036; 5,076,973; 4,986,988; 4,978,744; ;4,879,278; 4,879,278; 4,816,444; and 4,486,414, the disclosures of which are incorporated herein by reference in their entirety.

Exemplary drugs include the dolastatins and analogues thereof including: dolastatin A
20 (U.S. Pat No. 4,486,414), dolastatin B (U.S. Pat No. 4,486,414), dolastatin 10 (U.S. Pat No. 4,486,444, 5,410,024, 5,504,191, 5,521,284, 5,530,097, 5,599,902, 5,635,483, 5,663,149, 5,665,860, 5,780,588, 6,034,065, 6,323,315), dolastatin 13 (U.S. Pat No. 4,986,988), dolastatin 14 (U.S. Pat No. 5,138,036), dolastatin 15 (U.S. Pat No. 4,879,278), dolastatin 16 (U.S. Pat No. 6,239,104), dolastatin 17 (U.S. Pat No. . 6,239,104), and dolastatin 18 (U.S.
25 Pat No. . 6,239,104), each patent incorporated herein by reference in their entirety.

Exemplary maytansines, maytansinoids, such as DM-1 and DM-4, or maytansinoid analogs, including maytansinol and maytansinol analogs, are described in U.S. Patent Nos.
4,424,219; 4,256,746; 4,294,757; 4,307,016; 4,313,946; 4,315,929; 4,331,598; 4,361,650; 4,362,663; 4,364,866; 4,450,254; 4,322,348; 4,371,533; 5,208,020; 5,416,064; 5,475,092;
30 5,585,499; 5,846,545; 6,333,410; 6,441,163; 6,716,821 and 7,276,497.

Other examples include mertansine and ansamitocin. ;Pyrrolobenzodiazepines (PBDs), which expressly include dimers and analogs, include but are not limited to those described in [Denny, Exp. Opin. Ther. Patents, 10(4):459-474 (2000)], [Hartley et al., Expert Opin Investig Drugs. 2011, 20(6):733-44], Antonow et al., Chem Rev. 2011, 111(4), 2815-
35 64].

Calicheamicins include, e.g. enediyne, esperamicin, and those described in U.S. Patent Nos. 5,714,586 and 5,739,116.

Examples of duocarmycins and analogs include CC1065, duocarmycin SA, duocarmycin A, duocarmycin B I, duocarmycin B2, duocarmycin CI, duocarmycin C2, 5 duocarmycin D, DU- 86, KW-2189, adozelesin, bizelesin, carzelesin, seco- adozelesin. Other examples include those described in, for example, US Patent No. 5,070,092; 5,101,092; 5,187,186; 5,475,092; 5,595,499; 5,846,545; 6,534,660; 6,548,530; 6,586,618; 6,660,742; 6,756,397; 7,049,316; 7,553,816; 8,815,226; US20150104407; 61/988,011 filed May 2, 2014 and 62/010,972 filed June 11, 2014; the disclosure of each of which is incorporated herein in 10 its entirety.

Exemplary vinca alkaloids include vincristine, vinblastine, vindesine, and navelbine, and those disclosed in U.S. Publication Nos. 2002/0103136 and 2010/0305149, and in U.S. Patent No. 7,303,749, the disclosures of which are incorporated herein by reference in their entirety.

15 Exemplary epothilone compounds include epothilone A, B, C, D, E, and F, and derivatives thereof. Suitable epothilone compounds and derivatives thereof are described, for example, in U.S. Patent Nos. 6,956,036; 6,989,450; 6,121,029; 6,117,659; 6,096,757; 6,043,372; ; 5,969,145; and 5,886,026; and WO97/19086; WO98/08849; WO98/22461; WO98/25929; WO98/38192; WO99/01124; WO99/02514; WO99/03848; WO99/07692; 20 WO99/27890; and WO99/28324; the disclosures of which are incorporated herein by reference in their entirety.

Exemplary cryptophycin compounds are described in U.S. Patent Nos. 6,680,311 and ;6,747,021; the disclosures of which are incorporated herein by reference in their entirety.

Exemplary platinum compounds include cisplatin, carboplatin, oxaliplatin, iproplatin, 25 ormaplatin, tetraplatin.

Exemplary DNA binding or alkylating drugs include CC-1065 and its analogs, anthracyclines, calicheamicins, dactinomycines, mitomycines, pyrrolobenzodiazepines, and the like.

Exemplary microtubule stabilizing and destabilizing agents include taxane 30 compounds, such as paclitaxel, docetaxel, tesetaxel, and carbazitaxel; maytansinoids, auristatins and analogs thereof, vinca alkaloid derivatives, epothilones and cryptophycins.

Exemplary topoisomerase inhibitors include camptothecin and camptothecin derivatives, camptothecin analogs and non-natural camptothecins, such as, for example, CPT-11, SN-38, topotecan, 9-aminocamptothecin, rubitecan, gimatecan, karenitecin, 35 silatecan, lurtotecan, exatecan, DXd, diflometotecan, belotecan, lurtotecan and S39625.

Other camptothecin compounds that can be used in the present invention include those described in, for example, J. Med. Chem., 29:2358-2363 (1986); J. Med. Chem., 23:554 (1980); J. Med. Chem., 30: 1774 (1987).

Angiogenesis inhibitors include, but are not limited to, MetAP2 inhibitors, VEGF inhibitors, PIGF inhibitors, VGFR inhibitors, PDGFR inhibitors, MetAP2 inhibitors. Exemplary VGFR and PDGFR inhibitors include sorafenib, sunitinib and vatalanib. Exemplary MetAP2 inhibitors include fumagillol analogs, meaning compounds that include the fumagillin core structure.

Exemplary cell cycle progression inhibitors include CDK inhibitors such as, for example, BMS-387032 and PD0332991; Rho-kinase inhibitors such as, for example, AZD7762; aurora kinase inhibitors such as, for example, AZD1152, MLN8054 and MLN8237; PLK inhibitors such as, for example, BI 2536, BI6727, GSK461364, ON-01910; and KSP inhibitors such as, for example, SB 743921, SB 715992, MK-0731, AZD8477, AZ3146 and ARRY-520.

Exemplary P13K/m-TOR/AKT signalling pathway inhibitors include phosphoinositide 3- kinase (P13K) inhibitors, GSK-3 inhibitors, ATM inhibitors, DNA-PK inhibitors and PDK-1 inhibitors.

Exemplary P13 kinases are disclosed in U.S. Patent No. 6,608,053, and include BEZ235, BGT226, BKM120, CAL263, demethoxyviridin, GDC-0941, GSK615, IC87114, LY294002, Palomid 529, perifosine, PF-04691502, PX-866, SAR245408, SAR245409, SF1126, Wortmannin, XL147 and XL765.

Exemplary AKT inhibitors include, but are not limited to AT7867.

Exemplary MAPK signaling pathway inhibitors include MEK, Ras, JNK, B-Raf and p38 MAPK inhibitors.

Exemplary MEK inhibitors are disclosed in U.S. Patent No. 7,517,944 and include GDC-0973, GSKI 120212, MSC1936369B, AS703026, R05126766 and R04987655, PD0325901, AZD6244, AZD8330 and GDC-0973.

Exemplary B-raf inhibitors include CDC-0879, PLX-4032, and SB590885.

Exemplary B p38 MAPK inhibitors include BIRB 796, LY2228820 and SB 202190.

Exemplary receptor tyrosine kinases inhibitors include but are not limited to AEE788 (NVP-AEE 788), BIBW2992 (Afatinib), Lapatinib, Erlotinib (Tarceva), Gefitinib (Iressa), AP24534 (Ponatinib), ABT-869 (linifanib), AZD2171, CHR-258 (Dovitinib), Sunitinib (Sutent), Sorafenib (Nexavar), and Vatalinib.

Exemplary protein chaperon inhibitors include HSP90 inhibitors. Exemplary inhibitors include 17AAG derivatives, BIIB021, BIIB028, SNX-5422, NVP-AUY-922 and KW-2478.

Exemplary HDAC inhibitors include Belinostat (PXD101), CUDC-101, Droxinostat, ITF2357 (Givinostat, Gavinostat), JNJ-26481585, LAQ824 (NVP-LAQ824, Dacinostat), LBH-589 (Panobinostat), MC1568, MGCD0103 (Mocetinostat), MS-275 (Entinostat), PCI- 24781, Pyroxamide (NSC 696085), SB939, Trichostatin A and Vorinostat (SAHA). Exemplary PARP
5 inhibitors include iniparib (BSI 201), olaparib (AZD-2281), ABT-888 (Veliparib), AG014699, CEP9722, MK 4827, KU-0059436 (AZD2281), LT-673, 3- aminobenzamide, A-966492, and AZD2461.

Exemplary Wnt/Hedgehog signalling pathway inhibitors include vismodegib, cyclopamine and XAV-939.

10 Exemplary RNA polymerase inhibitors include amatoxins. Exemplary amatoxins include alpha-amanitins, beta amanitins, gamma amanitins, eta amanitins, amanullin, amanullic acid, amanisamide, amanon, and proamanullin.

Exemplary cytokines include IL-2, IL-7, IL-10, IL-12, IL-15, IL-21, TNF.

As non-limiting examples of particular drugs there may be mentioned Auristatins,
15 Maytansinoids, PBDs, topoisomerase inhibitors, anthracyclines

In another embodiment, a combination of two or more different drugs as described above are used.

According to another embodiment, the bioactive compound may be selected from any synthetic or naturally occurring compounds comprising one or more natural and/or non-
20 natural, proteinogenic and/or non-proteinogenic amino acid residues, such as in particular oligo- or polypeptides or proteins.

A particular group of such compounds comprises immunoglobulin molecules as for example antibodies, antibody derivatives, antibody fragments, antibody (fragment) fusions (e.g. bi-specific and tri-specific mAb fragments or derivatives), polyclonal or monoclonal
25 antibodies, such as human, humanized, mouse or chimeric antibodies.

Typical non-limiting examples of antibodies for use in the present invention are selected from biologically, in particular pharmacologically active antibody molecules. Non-limiting examples are selected from the following group: trastuzumab, bevacizumab, cetuximab, panitumumab, ipilimumab, rituximab, alemtuzumab, ofatumumab, gemtuzumab, brentuximab, ibritumomab, tositumomab, pertuzumab, adecatumumab, IGN101 , INA01
30 labetuzumab, hua33, pemtumomab, oregovomab, minretumomab (CC49), cG250, J591 , MOv-18, farletuzumab (MORAb-003), 3F8, ch14,18, KW-2871 , hu3S193, IgN31 1 , IM- 2C6, CDP-791 , etaracizumab, volociximab, nimotuzumab, MM-121 , AMG 102, METMAB, SCH 900105, AVE1642, IMC-A12, MK-0646, R1507, CP 751871 , KB004, III A4, mapatumumab,
35 HGS-ETR2, CS-1008, denosumab, sibrotuzumab, F19, 81 C6, pinatuzumab, lifastuzumab,

glembatumumab, coltuximab, lorvotuzumab, indatuximab, anti-PSMA, MLN-0264, ABT-414, milatuzumab, ramucirumab, abagovomab, abituzumab, adecatumumab, afutuzumab, altumomab pentetate, amatuximab, anatumomab, anetumab, apolizumab, arcitumomab, ascrinvacumab, atezolizumab, bavituximab, bectumomab, belimumab, bivatuzumab, 5 brontictuzumab, cantuzumab, capromab, catumaxomab, citatuzumab, cixutumumab, clivatuzumab, codrituzumab, conatumumab, dacetuzumab, dallotuzumab, daratumumab, demcizumab, denintuzumab, depatuxizumab, derlotuximab, detumomab, dinutuximab, drozitumab, duligotumab, durvalumab, dusigitumab, ecromeximab, edrecolomab, elgemtumab, emactuzumab, enavatuzumab emibetuzumab, enfortumab, enoblituzumab, 10 ensituximab, epratuzumab, ertumaxomab, etaracizumab, farletuzumab, ficlatuzumab, figitumumab, flinvotumab, futuximab, galiximab, ganitumab, icrucumab, igovomab, imalumab, imgatuzumab, indusatumab, inebilizumab, intetumumab, iratumumab, isatuximab, lexatuzumab, lilotomab, lintuzumab, lirilumab, lucatumumab, lumretuzumab, margetuximab, matuzumab, mirvetuximab, mitumomab, mogamulizumab, moxetumomab, nacolomab, 15 naptumomab, narnatumab, necitumumab, nesvacumab, nimotuzumab, nivolumab, nofetumomab, obinutuzumab, ocaratuzumab, ofatumumab, olaratumab, onartuzumab, ontuxizumab, oportuzumab, oregovomab, otlertuzumab, pankomab, parsatuzumab, pasotuxizumab, patritumab, pembrolizumab, pentumomab, pidilizumab, pintumomab, polatuzumab, primumab, quilizumab, racotumomab, ramucirumab, rilatumumab, 20 robatumumab, sacituzumab, samalizumab, satumomab, seribantumab, siltuximab, sofituzumab, tacatuzumab, taplitumomab, tarextumab, tenatumomab, teprotumumab, tetulomab, ticilimumab, tigatuzumab, tositumomab, tovetumab, tremelimumab, tucotuzumab, ublituximab, ulocuplumab, urelumab, utomilumab, vadastuximab, vandortuzumab, vantictumab, vanucizumab, varlilumab, veltuzumab, vesencumab, volociximab, 25 vorsetuzumab votumumab, zalutumumab, zatuxima, combination and derivatives thereof, as well as other monoclonal antibodies targeting CAI 25, CAI 5-3, CAI 9-9, L6, Lewis Y, Lewis X, alpha fetoprotein, CA 242, placental alkaline phosphatase, prostate specific antigen, prostate specific membrane antigen, prostatic acid phosphatase, epidermal growth factor, MAGE- 1, MAGE-2, MAGE-3, MAGE-4, transferrin receptor, p97, MUCI, CEA, gp100, 30 MARTI, IL-2 receptor, CD20, CD52, CD33, CD22, human chorionic gonadotropin, CD38, CD40, mucin, P21 , MPG, and Neu oncogene product.

1.2 Labeling Agents

Labeling agents which may be used according to the invention can comprise any type of label known in the art which does not inhibitor negatively affect reactivity of the tetrazine moiety.

Labels of the invention include, but are not limited to, dyes (e.g. fluorescent, luminescent, or phosphorescent dyes, such as dansyl, coumarin, fluorescein, acridine, rhodamine, silicon-rhodamine, BODIPY, or cyanine dyes), chromophores (e.g., phytochrome, phycobilin, bilirubin, etc.), radiolabels (e.g. radioactive forms of hydrogen, fluorine, carbon, phosphorous, sulphur, or iodine, such as tritium, fluorine-18, carbon-11, carbon-14, phosphorous-32, phosphorous-33, sulphur-33, sulphur-35, iodine-123, or iodine-125), MRI-sensitive spin labels, affinity tags (e.g. biotin, His-tag, Flag-tag, strep-tag, sugars, lipids, sterols, PEG-linkers, benzylguanines, benzylcytosines, or co-factors), polyethylene glycol groups (e.g., a branched PEG, a linear PEG, PEGs of different molecular weights, etc.), photocrosslinkers (such as p-azidoiodoacetanilide), NMR probes, X-ray probes, pH probes, IR probes, resins, solid supports .

In some embodiments, exemplary dyes can include an NIR contrast agent that fluoresces in the near infrared region of the spectrum. Exemplary near-infrared fluorophores can include dyes and other fluorophores with emission wavelengths (e.g., peak emission wavelengths) between about 630 and 1000 nm, e.g., between about 630 and 800 nm, between about 800 and 900 nm, between about 900 and 1000 nm, between about 680 and 750 nm, between about 750 and 800 nm, between about 800 and 850 nm, between about 850 and 900 nm, between about 900 and 950 nm, or between about 950 and 1000 nm. Fluorophores with emission wavelengths (e.g., peak emission wavelengths) greater than 1000 nm can also be used in the methods described herein.

In some embodiments, exemplary fluorophores include 7-amino-4-methylcoumarin-3-acetic acid (AMCA), TEXAS RED™ (Molecular Probes, Inc., Eugene, Oreg.), 5-(and -6)-carboxy-X-rhodamine, lissamine rhodamine B, 5-(and -6)-carboxyfluorescein, fluorescein-5-isothiocyanate (FITC), 7-diethylaminocoumarin-3-carboxylic acid, tetramethylrhodamine-5-(and -6)-isothiocyanate, 5 -(and -6)-carboxytetramethylrhodamine, 7-hydroxycoumarin-3-carboxylic acid, 6-[fluorescein 5-(and -6)-carboxamido]hexanoic acid, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a diaza-3-indacenepropionic acid, eosin-5-isothiocyanate, erythrosin-5-isothiocyanate, and CASCADE™ blue acetylazide (Molecular Probes, Inc., Eugene, Oreg.) and ATTO dyes.

Other suitable fluorophores are for example described in EP3572468A1. Further labelling agents are 177-Lutetium, 89-Zirkonium, 131-Iod, 68-Gallium, 99m-Technecium,

225-Actinium, 213-Bismut, 90-Yttrium, 212-Plumbum, 111-Indium, 64-Copper, 67-Copper, 124-Iodine, 227-Thorium and 188-Rhenium.

1.3 Chelators

- 5 Lists of typically applicable chelators and their short names are given below; Corresponding salts thereof are also applicable.:

 Acetyl acetone (ACAC), ethylene diamine (EN), 2-(2-aminoethylamino)ethanol (AEEA), diethylene triamine (DIEN), iminodiacetate (IDA), triethylene tetramine (TRIEN), triaminotriethylamine, nitrilotriacetate (NTA) and its saltslike Na₃NTA or FeNTA, 10 ethylenediaminotriacetate (TED), ethylenediamine tetraacetate (EDTA) and its salts like Na₂EDTA and CaNa₂EDTA, diethylene triaminopentaacetate (DTPA), 1,4,7,10-ztetraazacyclododecane-1,4,7,10-tetraacetate (DOTA), 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), Oxalate (OX), tartrate (TART), citrate (CIT), dimethylglyoxime (DMG), 8-hydroxyquinoline, 2,2'-bipyridine (BPY), 1,10-phenanthroline (PHEN), dimercapto succinic 15 acid (DMSA), 1,2-bis(diphenylphosphino)ethane (DPPE), sodium salicylate, methoxy salicylates, British anti-Lewisite or 2,3-dimercaprol (BAL), meso-2,3-dimercaptosuccinic acid (DMSA); Siderophores secreted by microorganisms, as for example desferrioxamine or deferoxamine B, also known as Deferral (Novartis), produced by *Streptomyces spp.*; deferoxamine (DFO) , a trihydroxamic acid secreted by *Streptomyces pilosus*; 20 phytochemicals like curcuminoids and derivatives of mugineic acid, like 3-hydroxy-mugineic acid and 2'-deoxy-mugineic acid; synthetically produced chelators, like Ibuprofen; derivatives of catechol, hydroxamate and hydroxypyridinone, like hydroxamate desferal and hydroxypyridinone deferiprone; deferiprone (L1 or 1,2-dimethyl-3-hydroxypyrid-4-one); D-penicillamine (DPA or D-PEN) which is β - β -dimethylcysteine or 3-mercapto-D-valine; 25 tetraethylenetetraamine (TETA) or trientine and its two major metabolites N₁ - acetyltriethylenetetramine (MAT) and N₁,N₁₀ -diacetyltriethylenetetramine (DAT); hydroxyquinolines; clioquinol, which is a halogenated derivative of 8-hydroxyquinoline; and 5,7-dichloro-2-[(dimethylamino)methyl]quinolin-8-ol (PBT2).

30 1.4 Protein degraders

 As non-limiting examples there may be mentioned PROTACs in general, but there is a plethora of different E3 ligase binding molecules in combination with specific targeted proteins to degrade (vgl WO2017201449A1). (Maneiro, M. et al ACS Chem. Biol. 2020, 15, 6, 1306–1312)

As protein degrades there should be mentioned compounds that are suitable in targeted protein degradation (TPD). A major class of molecules is known as proteolysis-targeting chimeras (PROTACs). Reference can be made to Békés, M., Langley, D.R. & Crews, C.M. PROTAC targeted protein degraders: the past is prologue. *Nat Rev Drug Discov* **21**, 181–200 (2022). These chimeras are heterobifunctional small molecules consisting of two ligands joined by a linker. One ligand recruits and binds a protein of interest (POI) while the other recruits and binds an E3 ubiquitin ligase. Simultaneous binding of the POI and ligase by the PROTAC induces ubiquitylation of the POI and its subsequent degradation by the ubiquitin-proteasome system, after which the PROTAC is recycled to target another copy of the POI.

2. Targeting agents (TA) and their Targets

The primary object of such **targeting agent** is the formation of a covalent or noncovalent linkage with a particular “**target**”. A secondary object of the **targeting agent** is the targeted transport of a “**payload molecule**” to said target. In order to achieve said second object the POI has to be combined (reversibly or irreversibly) with at least one payload molecule. For this purpose said POI has to be functionalised by introducing said at least one ncAA. The functionalized POI carrying said at least one ncAA may then be linked to said at least one payload molecule through bioconjugation via said ncAA residue. Said ncAA is reactive with a payload molecule which in turn carries a corresponding moiety reactive with said at least one ncAA residue of the POI. The thus obtained bioconjugate, i.e. the targeting agent, allows the transfer of the payload molecule to the intended target.

For example, a “target” can be any molecule, which is present in and/or on an organism, tissue or cell. Such targets may be nonspecific or specific for a particular organism, tissue or cell. Targets include cell surface targets, e.g. receptors, glycoproteins, glycans, carbohydrates; structural proteins, e.g. amyloid plaques; abundant extracellular targets such as in stroma, extracellular matrix targets such as growth factors, and proteases; intracellular targets, e.g. surfaces of Golgi bodies, surfaces of mitochondria, RNA, DNA, enzymes, components of cell signaling pathways; and/or foreign bodies, e.g. pathogens such as viruses, bacteria, fungi, yeast or parts thereof.

Examples of targets include compounds such as proteins of which the presence or expression level is correlated with a certain tissue or cell type or of which the expression level is up-regulated or down-regulated in a certain disorder.

In particular, such target is a protein such as a (internalizing or non-internalizing) receptor.

Targets can be selected from any suitable targets within the human or animal body or on a pathogen or parasite.

Non-limiting examples of **suitable targets** include but are not limited to a group comprising cellular components such as cell membranes and cell walls, receptors such as

5 cell membrane receptors, intracellular structures such as Golgi bodies or mitochondria, enzymes, receptors, DNA, RNA, viruses or viral particles, macrophages, tumor-associated macrophages, antibodies, proteins, carbohydrates, monosaccharides, polysaccharides, cytokines, hormones, steroids, somatostatin receptor, monoamine oxidase, muscarinic receptors, myocardial sympathetic nerve system, leukotriene receptors, e.g. on leukocytes,

10 urokinase plasminogen activator receptor (uPAR), folate receptor, apoptosis marker, (anti-) angiogenesis marker, gastrin receptor, dopaminergic system, serotonergic system, GABAergic system, adrenergic system, cholinergic system, opioid receptors, GPIIb/IIIa receptor and other thrombus related receptors, fibrin, calcitonin receptor, tuftsin receptor, P-glycoprotein, neurotensin receptors, neuropeptide receptors, substance P receptors, NK

15 receptor, CCK receptors, sigma receptors, interleukin receptors, herpes simplex virus tyrosine kinase, human tyrosine kinase, integrin receptor, fibronectin targets, AOC3, ALK, AXL, C242, CA-125, CCL11, CCR5, CD2, CD3, CD4, CD5, CD15, CA15-3, CD18, CD19, CA19-9, CD20, CD21, CD22, CD23, CD25, CD28, CD30, CD31, CD33, CD37, CD38, CD40, CD41, CD44v6, CD45, CD51, CD52, CD54, CD56, CD62E, CD62P, CD62L, CD70, CD72,

20 CD74, CD79-B, CD80, CD105, CD125, CD138, CD141, CD147, CD152, CD154, CD174, CD227, CD326, CD340, VEGF/EGF and VEGF/EGF receptors, VEGF-A, VEGFR2, VEGFR1, TAG72, CEA, MUC1, MUC16, GPNMB, PSMA, Cripto, Tenascin C, Melanocortin-1 receptor, G250, HLA DR, ED-B, TMEFF2, EphB2, EphB4, EphA2, FAP, Mesothelin, GD2, GD3, CAIX, 5T4, clumping factor, CTLA-4, CXCR2, FGFR1, FGFR2, FGFR3, FGFR4,

25 NaPi2b, NOTCH1, NOTCH2, NOTCH3, NOTCH4, ErbB2, ErbB3, EpCAM, FLT3, HGF, HER2, HER3, HMI24, ICAM, ICOS-L, IGF-1 receptor, TRPV1, CFTR, gdNMB, CA9, c-KIT, c-MET, ACE, APP, adrenergic receptor beta2, Claudine 3, RON, RORI, PD-L1, PD-L2, B7-H3, B7-H4, IL-2 receptor, IL-4 receptor, IL-13 receptor, integrins, IFN-alpha, IFN-gamma, IgE, IGF-1 receptor, IL-1, IL-4, IL-5, IL-6, IL-12, IL-13, IL-22, IL-23, interferon receptor, ITGB2

30 (CD18), LFA-1 (CD11a), L-selectin, P-selectin, E-selectin, mucin, myostatin, NCA-90, NGF, PDGFR alpha, prostatic carcinoma cells, Pseudomonas aeruginosa, rabies, RANKL, respiratory syncytial virus, Rhesus factor, SLAMF7, sphingosine-1-phosphate, TGF-1, TGFbeta2, TGFbeta, TNFalpha, TRAIL-R1, TRAIL-R2, CTAA 16.88, vimentin, matrix metalloproteinases (MMP) such as MMP2, MMP9, MMP14, LDL receptor, endoglins,

35 polysialic acids and their corresponding lectins. An example of fibronectin targets are the alternatively spliced extra-domain-A (ED-A) and extra-domain-B (ED-B) of fibronectin. Non-

limiting examples of targets in stroma can be found in V. Hofmeister, D. Schrama, J. C. Becker, Cancer Immun. ;Immunother. 2008, 57, 1, the contents of which are hereby incorporated by reference.

More particularly, in order to allow a (specific) targeting of the above-listed targets,
5 the **targeting agent** can comprise compounds comprising an ncAA-functionalized peptide sequence. Such compounds include but are not limited to antibodies, antibody derivatives, antibody fragments, antibody (fragment) fusions (e.g. bi-specific and tri-specific mAb fragments or derivatives), proteins, peptides, e.g. octreotide and derivatives, VIP, MSH, LHRH, chemotactic peptides, bombesin, elastin, peptide mimetics, receptor agonists and
10 antagonists, cytokines, hormones, steroids, toxins.

According to a particular aspect, the target is a receptor and a **targeting agent** is employed, which is capable of specific binding to the target. Suitable targeting agents include but are not limited to, the ligand of such a receptor or a part thereof, which still binds to the receptor, e.g. a receptor binding peptide in the case of receptor binding protein ligands.

15 Other examples of targeting agents of protein nature include insulin, transferrin, fibrinogen-gamma fragment, thrombospondin, claudin, apolipoprotein E, Affibody molecules such as for example ABY-025, Ankyrin repeat proteins, ankyrin-like repeat proteins, interferons, e.g. alpha, beta, and gamma interferon, interleukins, lymphokines, colony stimulating factors and protein growth factor, such as tumor growth factor, e.g. alpha, beta
20 tumor growth factor, platelet-derived growth factor (PDGF), uPAR targeting protein, apolipoprotein, LDL, annexin V, endostatin, and angiostatin.

Examples of peptides molecules, like antibody, as used in targeting agents include LHRH receptor targeting peptides, EC-1 peptide, RGD peptides, HER2-targeting peptides, PSMA targeting peptides, somatostatin-targeting peptides, bombesin. Other examples of
25 targeting agents include lipocalins, such as anticalins.

One particular embodiment uses Affibodies™ and multimers and derivatives.

In one particular embodiment, antibodies are used to form a **targeting agent**. While antibodies or immunoglobulins derived from IgG antibodies are particularly well-suited for use in this invention, immunoglobulins from any of the classes or subclasses may be
30 selected, e.g. IgG, IgA, IgM, IgD and IgE. Suitably, the immunoglobulin is of the class IgG including but not limited to IgG subclasses (IgG1, 2, 3 and 4) or the class IgM which is able to specifically bind to a specific epitope on an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies may exist in a variety of forms
35 including, for example, polyclonal antibodies, monoclonal antibodies, camelized single domain antibodies, recombinant antibodies, anti-idiotypic antibodies, multispecific antibodies,

antibody fragments, such as, Fv, VHH, Fab, F(ab)₂, Fab', Fab'-SH, F(ab')₂, single chain variable fragment antibodies (scFv), tandem/bis-scFv, Fc, pFc', scFv-Fc, disulfide Fv (dsFv), bispecific antibodies (bc-scFv) such as BiTE antibodies, trispecific antibody derivatives such as tribodies, camelid antibodies, minibodies, nanobodies, resurfaced antibodies, humanized antibodies, fully human antibodies, single domain antibodies (sdAb, also known as Nanobody™), chimeric antibodies, chimeric antibodies comprising at least one human constant region, dual-affinity antibodies such as dual-affinity retargeting proteins (DART™), and multimers and derivatives thereof, such as divalent or multivalent single-chain variable fragments (e.g. di-scFvs, tri-scFvs) including but not limited to minibodies, diabodies, triabodies, tribodies, tetrabodies, and the like, and multivalent antibodies. Reference is made to [Trends in Biotechnology 2015, 33, 2, 65], [Trends Biotechnol. 2012, 30, 575-582], and [Cane. Gen. Prot. 2013 10, 1-18], and [BioDrugs 2014, 28, 331-343], the contents of which are hereby incorporated by reference.

"Antibody fragment" refers to at least a portion of the variable region of the immunoglobulin that binds to its target, i.e. the antigen-binding region.

Other embodiments use antibody mimetics as targeting agents, such as but not limited to Affimers, Anticalins, Avimers, Alphabodies, Affibodies, DARPins, and multimers and derivatives thereof; reference is made to [Trends in Biotechnology 2015, 33, 2, 65], the contents of which is hereby incorporated by reference.

For the avoidance of doubt, in the context of this invention the term "antibody" is meant to encompass all of the antibody variations, fragments, derivatives, fusions, analogs and mimetics outlined in this paragraph, unless specified otherwise.

In a preferred embodiment the targeting agent is selected from agents derived from antibodies and antibody derivatives such as antibody fragments, fragment fusions, proteins, peptides, peptide mimetics.

In another preferred embodiment the targeting agent is selected from agents derived from antibody fragments, fragment fusions, and other antibody derivatives that do not contain a Fc domain.

Typical non-limiting examples of **antibody molecules to be further modified to form ncAA modified targeting agents** of the present invention are selected from biologically, in particular pharmacologically active antibody molecules. Non-limiting examples are selected from the following group: trastuzumab, bevacizumab, cetuximab, panitumumab, ipilimumab, rituximab, alemtuzumab, ofatumumab, gemtuzumab, brentuximab, ibritumomab, tositumomab, pertuzumab, adecatumumab, IGN101, INA01, labetuzumab, hua33, pentumomab, oregovomab, minretumomab (CC49), cG250, J591, MOv-18, farletuzumab (MORAb-003), 3F8, ch14,18, KW-2871, hu3S193, IgN31, IM-2C6, CDP-791,

etaracizumab, volociximab, nimotuzumab, MM-121 , AMG 102, METMAB, SCH 900105, AVE1642, IMC-A12, MK-0646, R1507, CP 751871 , KB004, III A4, mapatumumab, HGS-ETR2, CS-1008, denosumab, sibrotuzumab, F19, 81 C6, pinatuzumab, lifastuzumab, glembatumumab, coltuximab, lorvotuzumab, indatuximab, anti-PSMA, MLN-0264, ABT-414, milatuzumab, ramucirumab, abagovomab, abituzumab, adecatumumab, afutuzumab, altumomab pentetate, amatuximab, anatumomab, anetumab, apolizumab, arcitumomab, ascrinvacumab, atezolizumab, bavituximab, bectumomab, belimumab, bivatumumab, brontictuzumab, cantuzumab, capromab, catumaxomab, citatuzumab, cixutumumab, clivatuzumab, codrituzumab, conatumumab, dacetuzumab, dallotuzumab, daratumumab, demcizumab, denintuzumab, depatuxizumab, derlotuximab, detumomab, dinutuximab, drozitumab, duligotumab, durvalumab, dusigitumab, ecromeximab, edrecolomab, elgemtumab, emactuzumab, enavatuzumab emibetuzumab, enfortumab, enoblituzumab, ensituximab, epratuzumab, ertumaxomab, etaracizumab, farletuzumab, ficlatuzumab, figitumumab, flanvotumab, futuximab, galiximab, ganitumab, icrucumab, igovomab, imalumab, imgatuzumab, indusatumab, inebilizumab, intetumumab, iratumumab, isatuximab, lexatuzumab, lilotomab, lintuzumab, lirilumab, lucatumumab, lumretuzumab, margetuximab, matuzumab, mirvetuximab, mitumomab, mogamulizumab, moxetumomab, nacolomab, naptumomab, narnatumab, necitumumab, nesvacumab, nimotuzumab, nivolumab, nofetumomab, obinutuzumab, ocaratuzumab, ofatumumab, olaratumab, onartuzumab, ontuxizumab, oportuzumab, oregovomab, otlertuzumab, pankomab, parsatuzumab, pasotuxizumab, patritumab, pembrolizumab, pentumomab, pidilizumab, pintumomab, polatuzumab, primumab, quilizumab, racotumomab, ramucirumab, rilotumumab, robatumumab, sacituzumab, samalizumab, satumomab, seribantumab, siltuximab, sofituzumab, tacatuzumab, taplitumomab, tarextumab, tenatumomab, teprotumumab, tetulomab, ticilimumab, tigatuzumab, tositumomab, tovetumab, tremelimumab, tucotuzumab, ublituximab, ulocuplumab, urelumab, utomilumab, vadastuximab, vandortuzumab, vantictumab, vanucizumab, varlilumab, veltuzumab, vesencumab, volociximab, vorsetuzumab votumumab, zalutumumab, zatuxima, combination and derivatives thereof, as well as other monoclonal antibodies targeting CAI 25, CAI 5-3, CAI 9-9, L6, Lewis Y, Lewis X, alpha fetoprotein, CA 242, placental alkaline phosphatase, prostate specific antigen, prostate specific membrane antigen, prostatic acid phosphatase, epidermal growth factor, MAGE- 1, MAGE-2, MAGE-3, MAGE-4, transferrin receptor, p97, MUCI, CEA, gp100, MARTI, IL-2 receptor, CD20, CD52, CD33, CD22, human chorionic gonadotropin, CD38, CD40, mucin, P21 , MPG, and Neu oncogene product.

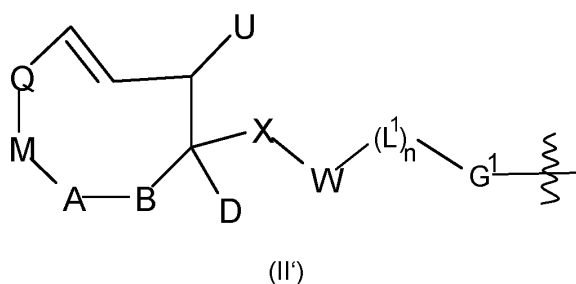
According to a further particular embodiment of the invention, the target and targeting agent are selected so as to result in the specific or increased targeting of a tissue or disease, such as cancer, an inflammation, an infection, a cardiovascular disease, e.g. thrombus, atherosclerotic lesion, hypoxic site, e.g. stroke, tumor, cardiovascular disorder, brain disorder, apoptosis, angiogenesis, an organ, and reporter gene/enzyme. This can be achieved by selecting targets with tissue-, cell- or disease- specific expression.

By way of example, the targeting agent specifically binds or complexes with a cell surface molecule, such as a cell surface receptor or antigen, for a given cell population. Following specific binding or complexing of the targeting agent with the receptor, the drug will enter the cell.

As used herein, a targeting agent that "specifically binds or complexes with" or "targets" a cell surface molecule, an extracellular matrix target, or another target, preferentially associates with the target via intermolecular forces. For example, the ligand can preferentially associate with the target with a dissociation constant (K_d or KD) of less than about 50 nM, less than about 5 nM, or less than about 500 pM.

3. hyTCO functionalized constructs

The hyTCO moieties of the present invention are, as part of a functionalizing residue of formula II',



wherein

n, A, B, D, U, M, Q, X, and W are as defined herein above;
 G¹ is as defined above or is missing; and
 L¹ has the meaning of linker moiety L as defined above or represents a branching moiety

applicable in the formation of particular hyTCO functionalized constructs. These are in particular:

a functionalized TA of the general formula (XX.1)

$$(FR)-(TA)$$

$$(XX.1)$$

5

wherein

TA is a targeting moiety as defined herein above; and

FR is a functionalizing residue of above formula II'

or a functionalized PM of the general formula (XX.2)

10

$$(FR)-[(Y^1)_a-(Y^2)_b-PM]_c$$

$$(XX.2)$$

15

wherein

a is 0 or represents an integer selected from 1 and 2,

b is 0 or represents an integer selected from 1 and 2,

c represents an integer selected of at least 1, particularly from 1 and 2,

20

Y^1 represents a cleavable moiety,

Y^2 represents a self-immolative moiety,

PM is a payload molecule as defined herein above and

FR is a functionalizing residue of above formula II', wherein, when

c represents an integer of more than 1, then L^1 represents a branching

25

moiety, n is 1 and G1 is missing.

3.1 Enzymatically or chemically cleavable moiety (Y^1)

A "cleavable moiety" encompasses any group, which may be cleaved enzymatically or chemically, in particular under *in vivo* or *ex vivo* conditions. An enzymatic cleavage may be effected, for example, through the action of a protease. A chemical cleavage, may be effected for example through hydrolytic cleavage or reductive cleavage of S-S bonds.

Suitable cleavable moieties are well known from the prior art.

Reference can be made to: Bargh et al., Chem Soc Rev 2019, 48(16), 4361-4374; Poreba, FEBS J 2020, 287(10), 1936-1969 and Salomon et al., Mol Pharmaceutics 2019 16,(12), 4817-4825.

35

According to a particular embodiment of the present invention said moiety Y^1 is an enzymatically or chemically cleavable linker group, selected from

- a) a peptidyl group, in particular di-, tri- or tetra-peptidyl group;
 b) a disulfide group of the formula $-(CR^7R^8)_{n2}-S-S-(CR^7R^8)_{n2}-X_5-$ or $X_5-(CR^7R^8)_{n2}-S-S-(CR^7R^8)_{n2}-X_5-$

wherein

$n2$ represents an integer from 1 to 4

residues R^7 and R^8 independently of each other are selected from H or lower alkyl, in particular methyl; or two residues R^7 and R^8 together with the carbon atom which they are attached to form a cyclic C_4 -to C_8 -alkyl group; and

moiety X_5 is selected from $-C(O)-$ and $-O-$;

moiety X_5 is selected from $-C(O)-$ and $-(O)C-(CH_2)-NH-$;

- c) hydrazone groups selected from $>C=N-N(R^9)-$ and $-N(R^9)-N=C<$

wherein

R^9 is H or lower alkyl; and

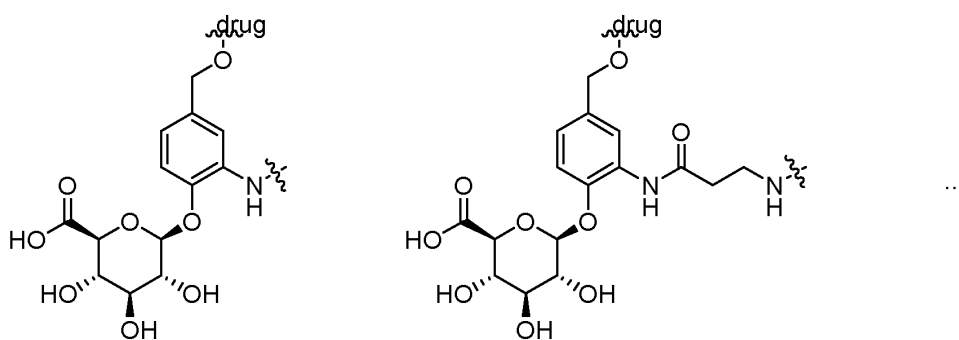
- d) beta-glucuronidase-sensitive cleavable linker groups (glucuronide-linker groups), in particular carrying a beta-glucuronic acid derived trigger residue;

According to a particular embodiment thereof, the cleavable linker is a peptidyl group according to feature a).

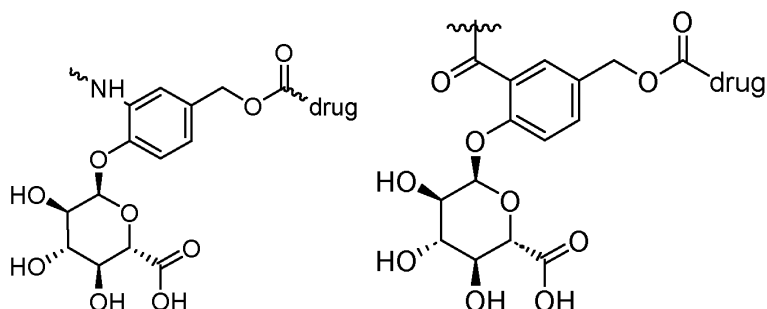
According to another particular embodiment thereof, the cleavable linker is a glucuronide-linker group according to feature d).

As further examples there may be mentioned β -glucuronide linkers, carrying a beta-glucuronic acid derived trigger residue.

Non-limiting examples thereof are:



or



3.2 Self-immolative moiety(Y²)

5 Suitable self-immolative moieties Y² are well known from the prior art. Reference can be made to: Santi et al., J Med Chem 2014, 57(6), 2303–2314; Alouane et al., Angew Chem Int Ed 2015, 54(26), 7492-7509; and Kolakowski et al., Angew Chem 2016, 128(28), 8080-8083.

10 **4. Conjugates of the present invention**

The present invention also relates to conjugates, which are synthesized by forming a covalent linkage between a first functionalized molecule, which is a hyTCO-functionalized construct as described above with a second functionalized molecule comprising a docking group (DG) capable of reacting with said hyTCO functional group of the first molecule.

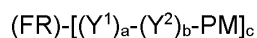
Methods of preparing such conjugates are in principle well known in the art. Biorthogonal bioconjugation via a Diels-Alder-type cycloaddition reaction of said two molecules may be performed in line with prior art teaching (see for example Oliveira et al, Chem Soc Rev, 2017, 46,4895-4950). More particularly, said docking group (DG) is selected from an optionally substituted triazinyI or optionally substituted tetrazinyI groups, capable of covalently reacting in a copper-free strain promoted inverse-electron-demand Diels-Alder cycloaddition (SPIEDAC) with said hyTCO group of the invention.

In principle, said hyTCO-functionalized construct is selected from a functionalized targeting agent TA of the general formula (XX.1)

25 (FR)-(TA)
(XX.1)

as defined above

or a functionalized PM of the general formula (XX.2)

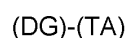


(XX.2)

5 as defined above.

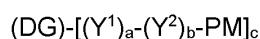
Said hyTCO-functionalized construct is conjugated with the respective DG-functionalized counterpart selected from a functionalized targeting agent TA of the general formula (XX.3)

10



(XX.3)

15 or a functionalized payload molecule PM of the general formula (XX.4)

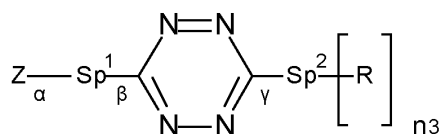


(XX.4)

20 each as defined above, in order to form a construct suitable for targeted delivery of a PM.

Suitable tri- or tetrazine docking groups are also known from the prior art. Particular examples are for example described in WO2023/10494 or different scientific articles [Yang et al., Angew. Chem. Int. Ed. 2012, 51, 5222–5225; Fan et al., Angew. Chem. Int. Ed. 2016, 55, 14046–14050; Mao et al., Angew. Chem. Int. Ed. 2019, 58, 1106–1109; Qu et al., Angew. Chem. Int. Ed. 2018, 57, 12057–12061; Eising et al., Bioconjugate Chem. 2018, 29, 3054–3059; Meng et al., J. Org. Chem. 2017, 82, 1676–1687; Xie et al., Angew. Chem. Int. Ed. 2020, 59, 16967–16973; Lambert et al., J. Am. Chem. Soc. 2019, 141, 17068–17074; Jemas et al., J. Am. Chem. Soc. 2022, 144, 1647–1662; Selvaraj et al., Tetrahedron Letters 30 2014, 55, 4795–4797; Dowling et al., J. Org. Chem. 2018, 83, 4229–4238; Battisti et al., Bioconjugate Chem. 2022, 33, 4, 608–624; Karver et al., Bioconjugate Chem. 2011, 22, 2263–2270; Bender et al., Org. Lett. 2017, 19, 5693–5696; Kamber et al., J. Am. Chem. Soc. 2015, 137, 8388–8391; Ros et al., Bioconjugate Chem. 2020, 31, 933–938; Ros et al., Chem. Commun., 2020, 56, 11086; van Onzen et al., J. Am. Chem. Soc. 2020, 142, 35 10955–10963; Carlson et al., J. Am. Chem. Soc. 2018, 140, 3603–3612].

WO2023/10494 discloses phosphonate-terminated DGs of the formula



wherein

n_3 represent an integer selected from 1 or 2

Sp^1 and Sp^2 are same or different spacer groups

5 linkages α , β , and γ are independently from each other selected from a chemical bond, or an ether, thioether, ester, amide, carbonyl (in particular keto), carbamate, dicarbamate, carbonate, hydrazine, urea, alkylene oxide or linear or branched polyalkylene oxide linkage;

10 Z represents a phosphor containing hydrophilic group, in particular $(R^1O)_2P(O)-$, $(R^{1a}O)_2P(O)-O-$, and $(R^2O)_3P-O-$;

wherein

R^1 , R^{1a} and R^2 are same or different and independently of each other represent H or lower alkyl, in particular methyl or ethyl; and even more particularly H;

15 and

R represents H or a chemical group capable of forming a chemical bond, or capable of forming an ether, thioether, ester, such as active esters like succinimidyl- or pentafluorophenyl- ester, amide, carbamate, dicarbamate, carbonate, hydrazine, urea, alkylene oxide or linear or branched polyalkylene oxide linkage.

20

As examples of suitable spacer groups there may be mentioned

a) mono- or polycyclic optionally mono- or poly-substituted aromatic moieties having 6 to 14 ring carbon atoms, in particular 1,2-phenylene 1,3-phenylene or 1,4-phenylene; wherein said one or more optional substituents are independently of each other selected from -Hal, -CHal₃, -OH, -SH, , -NR'₂, NO₂, -CN, -C(=O)R'', -C(=O)OR''', alkyl, alkenyl, alkynyl, and alkoxy;

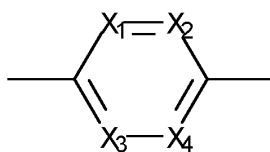
25

wherein

R' , R'' and R''' independently of each other are selected from H and C₁ -to C₄-alkyl; (Moiety M1);

30

b) heterocyclic residues of the general formula X



(X)

wherein

one, two or three of the ring moieties X_1 to X_4 represents N and the other represent $>\text{CH}$;

5 (Moiety M2);

c) linear or branched lower-alkylene, in particular $-(\text{CH}_2)_{n1}-$,

wherein $n1$ is an integer from 1 to 4; more particularly methylene; (Moiety M3);

10 d) linear or branched polyalkylene oxide moieties, in particular selected from linear the moieties $-((\text{CH}_2)_{x1}-\text{O})_{y1}-$ or $-(\text{O}-(\text{CH}_2)_{x1})_{y1}-$ and the branched analogues thereof;

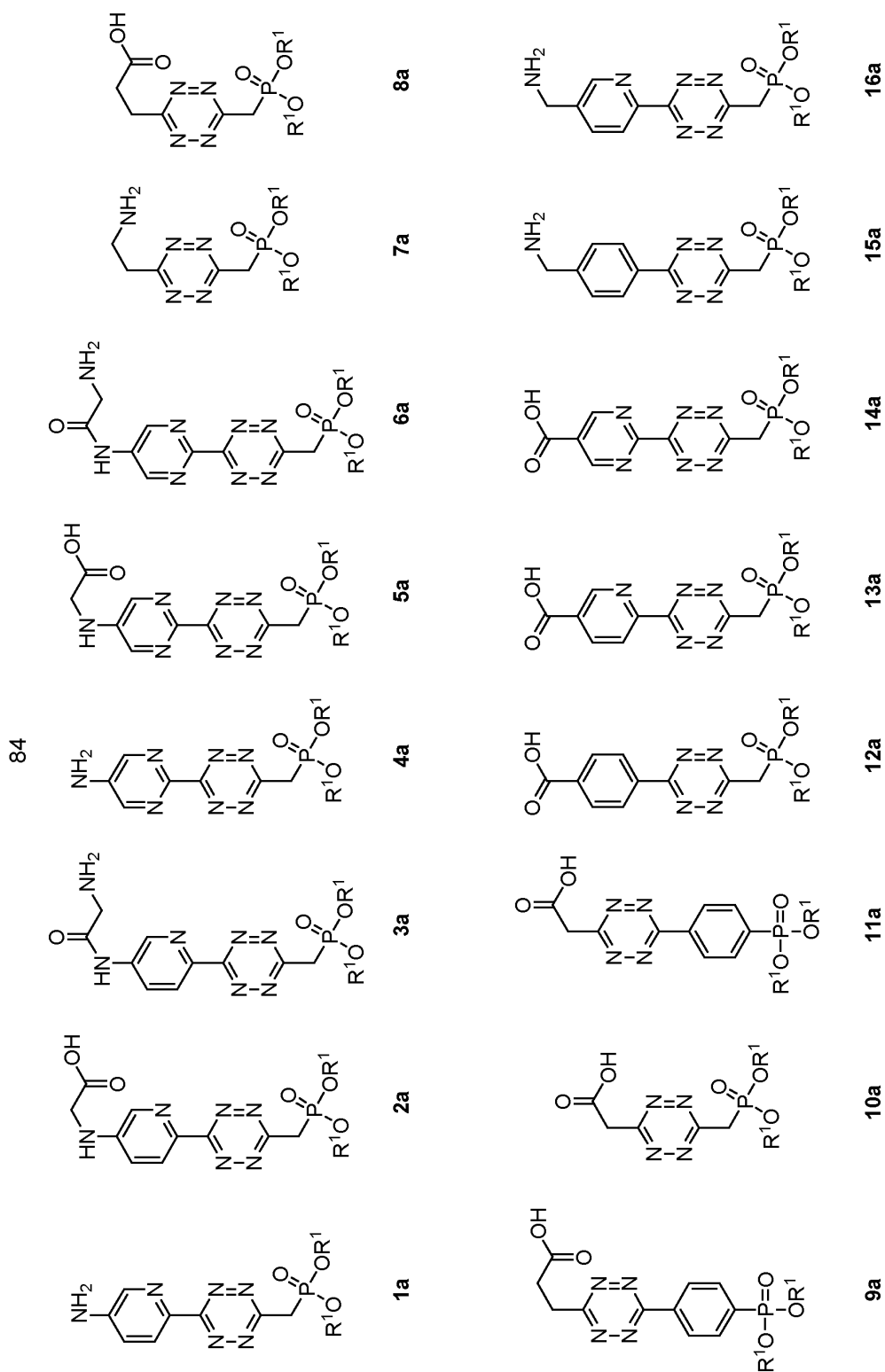
wherein

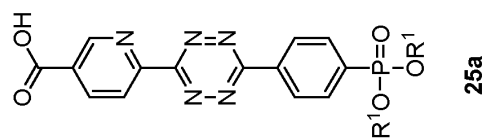
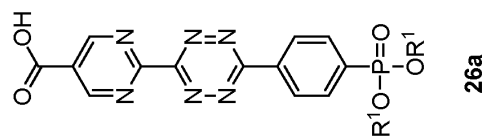
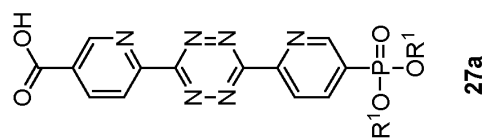
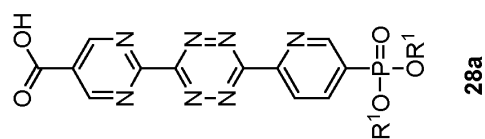
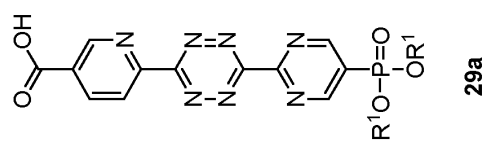
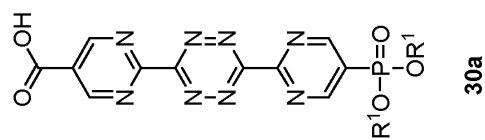
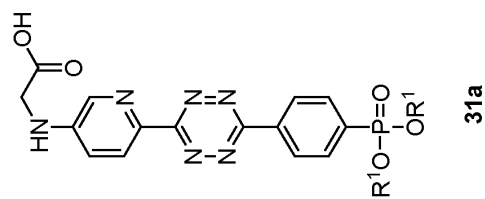
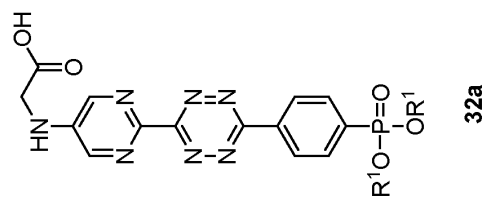
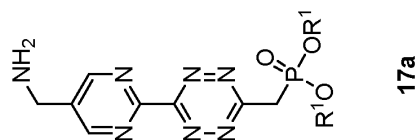
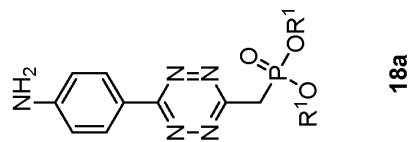
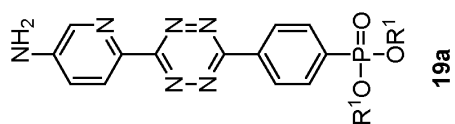
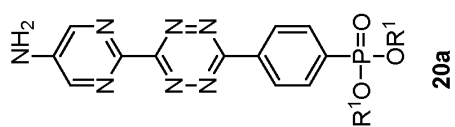
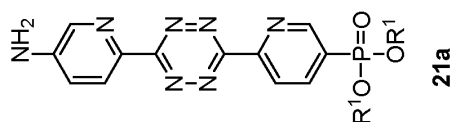
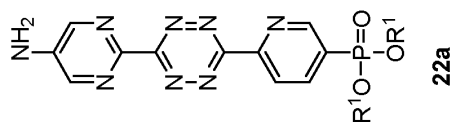
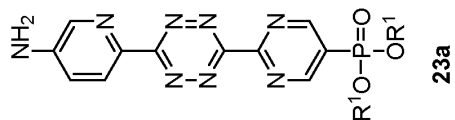
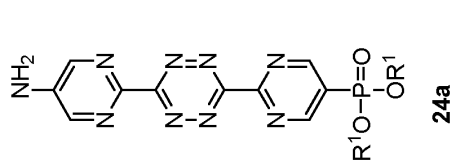
$x1$ independently of each other represent an integer selected from 1, 2, 3 or 4; in particular 1 or 2; and

15 $y1$ independently of each other represent an integer from 1 to 20, in particular 1 to 4; (Moiety M4);

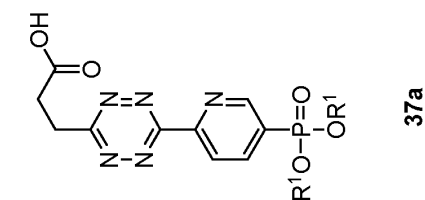
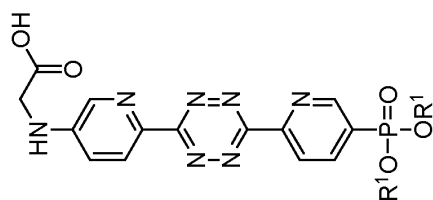
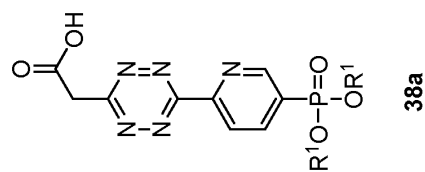
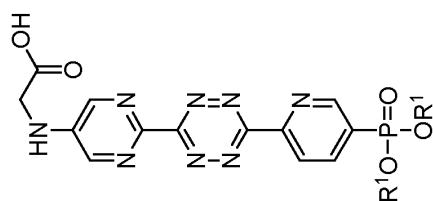
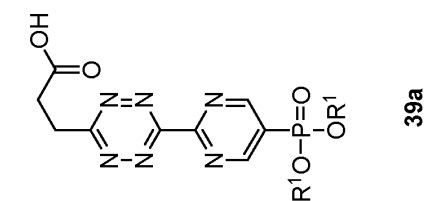
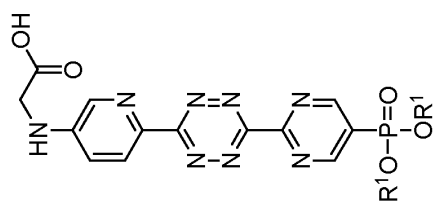
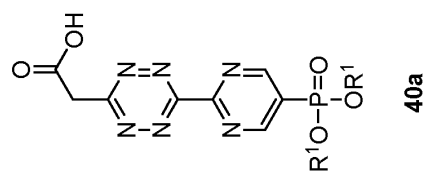
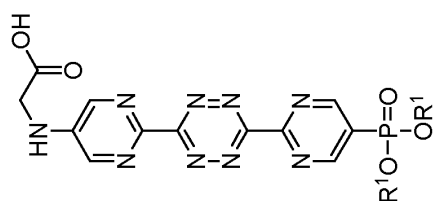
wherein Sp^1 is selected from M1, M2, M3 or combinations thereof; and Sp^2 is selected from M1 to M4 or combinations thereof.

20 In the following, particular preferred structures of compounds of general formula II are displayed. In said formulae residues R^1 independently of each other are H or lower alkyl, in particular methyl or ethyl; and even more particularly H

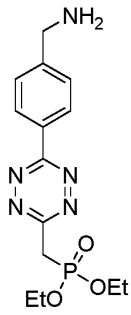
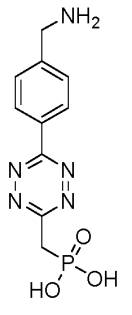
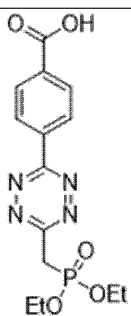
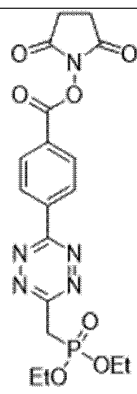
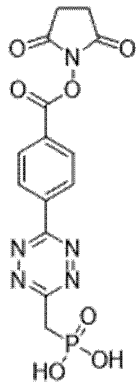
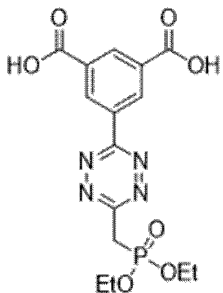


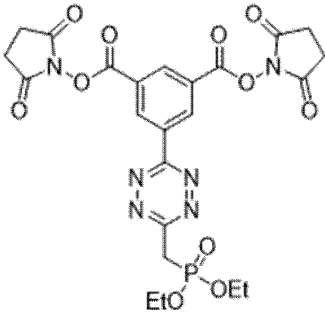
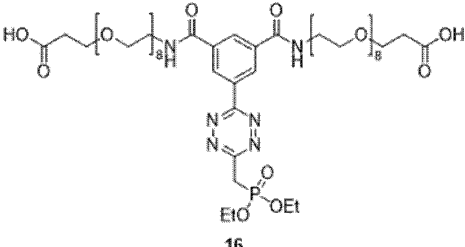
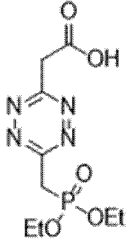
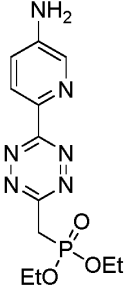
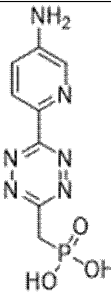
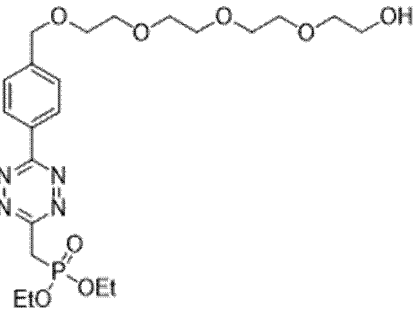


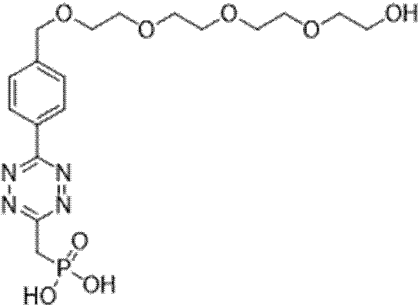
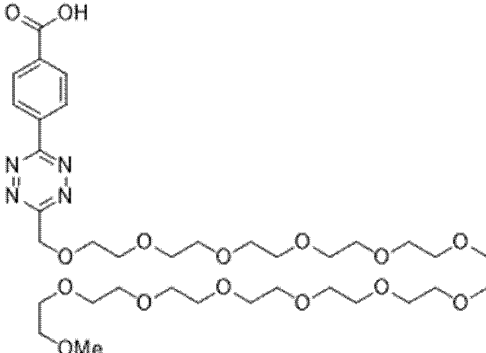
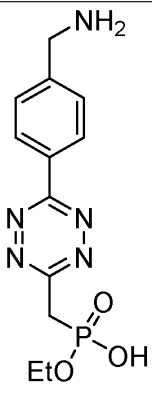
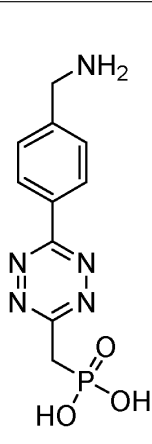
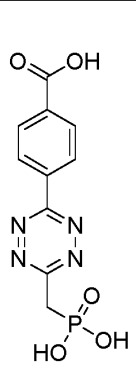
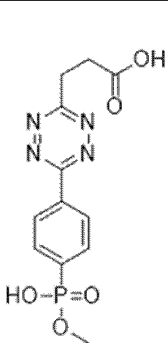
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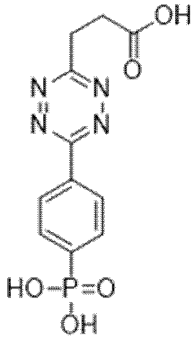
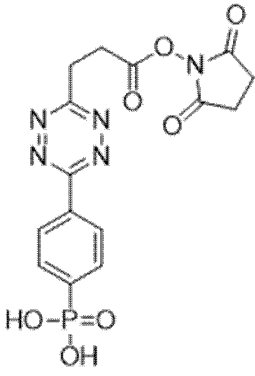
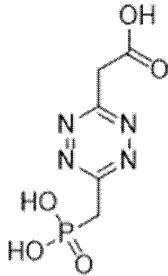
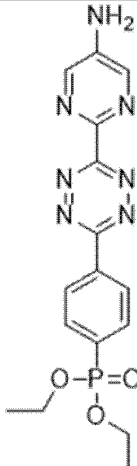


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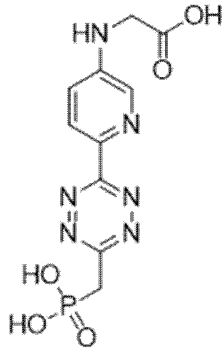
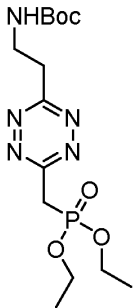
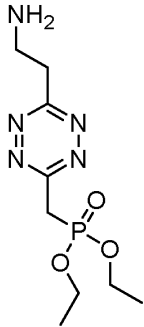
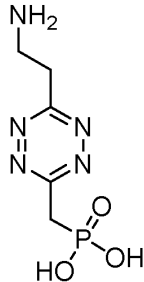
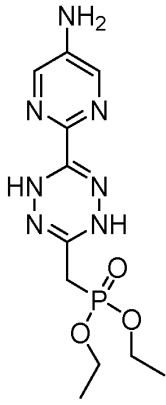
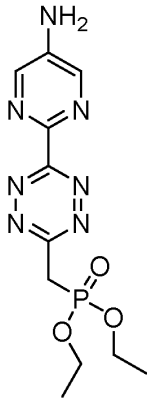
Formula	Formula
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 3	 6
 12	 14

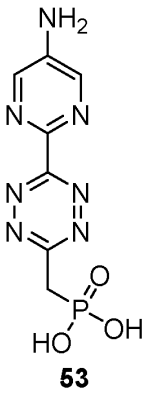
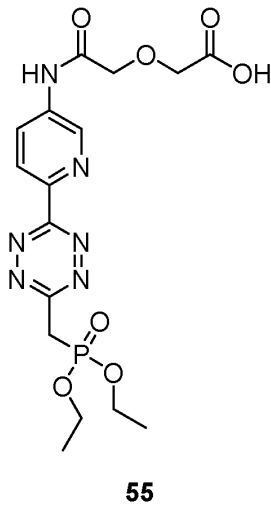
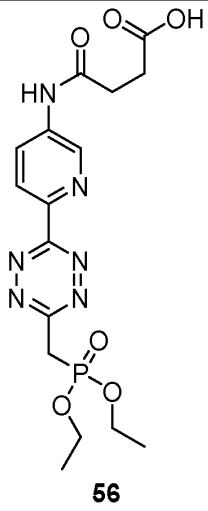
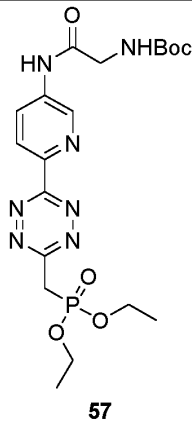
Formula	Formula
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 17	 18
 19	 20

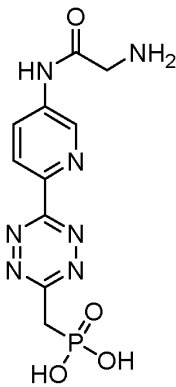
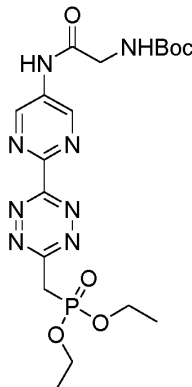
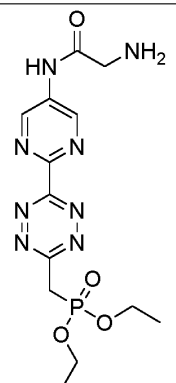
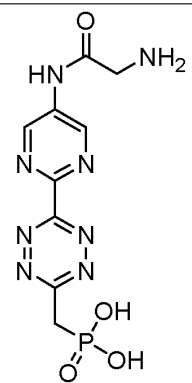
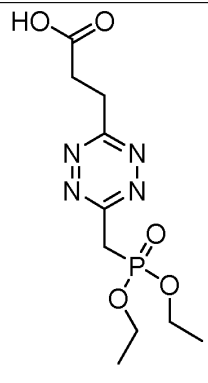
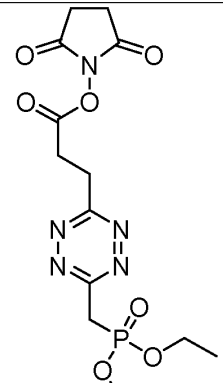
Formula	Formula
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 24	 25
 26	 30

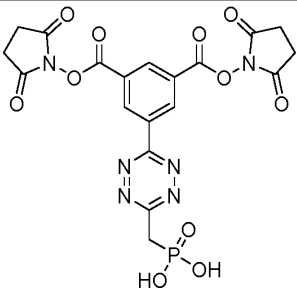
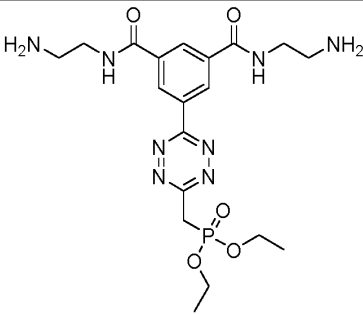
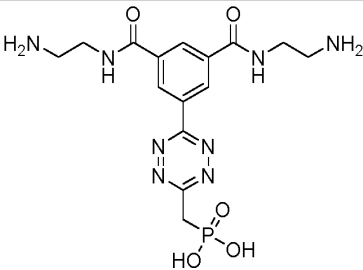
Formula	Formula
 31	 32
 33	 34

Formula	Formula
35	36
37	38

Formula	Formula
 39	 48
 49	 50
 51	 52

Formula	Formula
 53	 55
 56	 57

Formula	Formula
 58	 60
 61	 62
 68	 69

Formula	Formula
 72	 75
 76	

As further tetrazines suitable as DGs there may be mentioned analogues of the above specific compounds wherein the phosphonate group is missing and replaced by H, or where the tetrazine moiety is replaced by an 1, 2, 4-triazine moiety.

5

5. Bioorthogonal bioconjugation

A targeting agent, in particular a targeting agent comprising a polypeptide portion, comprising one or more than one UNAA residue can be prepared according to the present invention using a suitable translation system, in particular *in vivo* translation system. An *in vivo* translation system can be a cell, e.g. a prokaryotic or eukaryotic cell. The cell can be a bacterial cell, e.g. *E. coli*; a fungal cell such as a yeast cell, e.g. *S. cerevisiae* or a methylotrophic yeast; a plant cell, or an animal cell such as an insect cell or a mammalian cell, e.g. a HEK cell or a HeLa cell. Eukaryotic cells used for polypeptide expression may be single cells or parts of a multicellular organism.

10

The applied cellular system comprises (e.g., is fed with) at least one unnatural amino acid or a salt thereof corresponding to the UNAA residue(s) of the targeting agent to be prepared. The cellular system further comprises:

- 5 (i) a PylRS of the invention and a tRNA^{Pyl}, wherein the PylRS is capable of (preferably selectively) acylating the tRNA^{Pyl} with the UNAA or salt thereof; and
- (ii) a polynucleotide encoding the targeting agent, wherein any position of the targeting agent, occupied by an UNAA residue is encoded by a codon (e.g. selector codon) that is the reverse complement of the anticodon of the tRNA^{Pyl}.

10 The cellular system is cultured so as to allow translation of the targeting agent, - encoding polynucleotide (ii), thereby producing the targeting agent.

For producing a targeting agent, according to a method of the present invention, the translation in step (b) can be achieved by culturing the cellular system under suitable conditions, preferably in the presence of (e.g., in a culture medium containing) the UNAA or salt thereof, for a time suitable to allow translation at a ribosome of the cell. Depending on
15 the polynucleotide(s) encoding the targeting agent, (and optionally the PylRS, tRNA^{Pyl}), it may be required to induce expression by adding a compound inducing transcription, such as, e.g., arabinose, isopropyl β -D-thiogalactoside (IPTG) or tetracycline. mRNA that encodes the targeting agent, (and comprises one or more than codon that is the reverse complement of the anticodon comprised by the tRNA^{Pyl}) is bound by the ribosome. Then, the polypeptide is
20 formed by stepwise attachment of amino acids and UNAAs at positions encoded by codons which are recognized (bound) by respective aminoacyl tRNAs. Thus, the UNAA(s) is/are incorporated in the targeting agent, at the position(s) encoded by the codon(s) that is/are the reverse complement of the anticodon comprised by the tRNA^{Pyl}.

The cellular system may comprise a polynucleotide sequence encoding the PylRS of
25 the invention which allows for expression of the PylRS by the cell. Likewise, the tRNA^{Pyl} may be produced by the cellular system based on a tRNA^{Pyl}-encoding polynucleotide sequence comprised by the cell. The PylRS-encoding polynucleotide sequence and the tRNA^{Pyl}-encoding polynucleotide sequence can be located either on the same polynucleotide or on separate polynucleotides.

30 Thus, in one embodiment, the present invention provides a method for producing a targeting agent, comprising one or more than one UNAA residue, wherein the method comprises the steps of:

- (a) providing a cellular system comprising polynucleotide sequences encoding:
 - 35 - at least one PylRS of the invention,
 - at least one tRNA (tRNA^{Pyl}) that can be acylated by the PylRS, and

- at least one targeting agent, wherein any position of the targeting agent, occupied by an UNAA residue is encoded by a codon that is the reverse complement of the anticodon of the tRNA^{Pyl}; and
- (b) allowing for translation of the polynucleotide sequences by the cellular system in the presence of an UNAA or a salt thereof, thereby producing the PylRS, tRNA^{Pyl} and the POI.

The cellular system used for preparing a targeting agent, comprising one or more than one unnatural amino acid residue as described herein can be prepared by introducing polynucleotide sequences encoding the PylRS, the tRNA^{Pyl} and the targeting agent, into a (host) cell. Said polynucleotide sequences can be located on the same polynucleotide or on separate polynucleotides, and can be introduced into the cell by methods known in the art (such as, e.g., using virus-mediated gene delivery, electroporation, microinjection, lipofection, or others).

After translation, the targeting agent, prepared according to the present invention may optionally be recovered and purified, either partially or substantially to homogeneity, according to procedures generally known in the art. Unless the targeting agent, is secreted into the culture medium, recovery usually requires cell disruption. Methods of cell disruption are well known in the art and include physical disruption, e.g., by (ultrasound) sonication, liquid-shear disruption (e.g., via French press), mechanical methods (such as those utilizing blenders or grinders) or freeze-thaw cycling, as well as chemical lysis using agents which disrupt lipid-lipid, protein-protein and/or protein-lipid interactions (such as detergents), and combinations of physical disruption techniques and chemical lysis. Standard procedures for purifying polypeptides from cell lysates or culture media are also well known in the art and include, e.g., ammonium sulfate or ethanol precipitation, acid or base extraction, column chromatography, affinity column chromatography, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, hydroxylapatite chromatography, lectin chromatography, gel electrophoresis and the like. Protein refolding steps can be used, as desired, in making correctly folded mature proteins. High performance liquid chromatography (HPLC), affinity chromatography or other suitable methods can be employed in final purification steps where high purity is desired. Antibodies made against the polypeptides of the invention can be used as purification reagents, *i.e.* for affinity-based purification of the polypeptides. A variety of purification/protein folding methods are well known in the art, including, e.g., those set forth in Scopes, Protein Purification, Springer, Berlin (1993); and Deutscher, Methods in Enzymology Vol. 182: Guide to Protein Purification, Academic Press (1990); and the references cited therein.

As noted, those of skill in the art will recognize that, after synthesis, expression and/or purification, polypeptides can possess a conformation different from the desired conformations of the relevant polypeptides. For example, polypeptides produced by prokaryotic systems often are optimized by exposure to chaotropic agents to achieve proper folding. During purification from, e.g., lysates derived from *E. coli*, the expressed polypeptide is optionally denatured and then renatured. This is accomplished, e.g., by solubilizing the proteins in a chaotropic agent such as guanidine HCl. In general, it is occasionally desirable to denature and reduce expressed polypeptides and then to cause the polypeptides to re-fold into the preferred conformation. For example, guanidine, urea, DTT, DTE, and/or a chaperonin can be added to a translation product of interest. Methods of reducing, denaturing and renaturing proteins are well known to those of skill in the art. Polypeptides can be refolded in a redox buffer containing, e.g., oxidized glutathione and L-arginine.

The targeting agent thus prepared may then be converted to a respective bioconjugate by reaction with a tetrazine compound of the above general formula I

15

6. Pharmaceutical compositions

The conjugates or bioconjugates of the present invention, as for example APCs, in particular ADCs (i.e. the active agents or ingredients) of this invention are generally given as "pharmaceutical compositions" comprised of a therapeutically and/or prophylactically effective amount or a diagnostically effective amount of at least one such active ingredient or its pharmaceutically acceptable salt and optionally at least one pharmaceutically acceptable excipient.

Said pharmaceutical compositions may be delivered via suitable routes of administration such as via oral, rectal, transmucosal, topical, ophthalmic, otologic, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, as the case may be.

Depending on the nature or the mode of administration and dosage form said composition said at least one additional pharmaceutical excipient may be different.

An "excipient" is a substance formulated alongside the active ingredient and is included for different purpose, as for example for long-term stabilization, bulking up solid formulations that contain potent active ingredients in small amounts (thus often referred to as "bulking agents", "fillers", or "diluent"), or to confer a therapeutic enhancement on the active ingredient in the final dosage form, such as for example facilitating drug absorption, reducing viscosity, or enhancing solubility. Excipients can also be useful in the manufacturing process

of the pharmaceutical composition, to aid in the handling of the active substance concerns such as by facilitating powder flowability or non-stick properties, in addition to aiding *in vitro* stability such as prevention of denaturation or aggregation over the expected shelf life. The selection of appropriate excipients not only depends upon the route of administration and the dosage form, but also on the particular active ingredient and other factors.

Excipients may be selected from the following classes: immunological adjuvants, antiadherents, binders, coatings, colours, disintegrant, flavours, glidants, lubricants, preservatives, sorbents, sweeteners, and vehicles.

Non limiting examples of excipients comprise diluents, preserving agents, stabilizers, emulsifying agents, like emulsifying polymers, such as polysorbates or poloxamers, antioxidants; anti-irritants, chelating agents and stabilizing salts, such as chlorides, sulfates, phosphates, diphosphates, hydrobromides and nitrates, suspending agents, antibacterial agents or antifungal agents. Further, buffering agents such as buffering systems of low molecular weight organic acids together with the respective salts, or inorganic buffering substances, such as phosphate buffers, can be used. Further suitable ingredients are also known from relevant pharmacological standard literature. Also the proportion of the various components will vary depending on the nature of the specific component used and is generally known to the person skilled in the art (Remington's Pharmaceutical science ("Handbook of Pharmaceutical Excipients", 2nd Edition, (1994), Edited by A Wade and PJ Weller or in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R Gennaro edit. 1985).

A pharmaceutical composition as used herein may be presented in the form of a "dosage form" or "unit dose" and may comprise one or more APC, in particular ADCs as described herein. Thus, a pharmaceutical composition as used herein could, for example, provide two active agents admixed together in a unit dose or provide two active agents combined in a dosage form wherein the active agents are physically separated.

Furthermore, one may administer the pharmaceutical composition in a targeted drug delivery system, for example, in a liposome coated with endothelial cell-specific antibody.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, emulsifying, encapsulating, entrapping or combinations thereof. Proper formulation is dependent upon the route of administration chosen.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of

sound medical judgment, suitable for use in contact with the tissues of patients without excessive toxicity, irritation, allergic response, or other problem or complication commensurate with a reasonable risk/benefit ratio.

The invention includes all "pharmaceutically acceptable salt forms" of the active ingredient. Pharmaceutically acceptable salts are those in which the counter ions do not contribute significantly to the physiological activity or toxicity of the compounds and as such function as pharmacological equivalents. These salts can be made according to common organic techniques employing commercially available reagents. Some anionic salt forms include acetate, acistrate, besylate, bromide, chloride, citrate, fumarate, glucuronate, hydrobromide, hydrochloride, hydroiodide, iodide, lactate, maleate, mesylate, nitrate, pamoate, phosphate, succinate, sulfate, tartrate, tosylate, and xinofoate. Some cationic salt forms include ammonium, aluminum, benzathine, bismuth, calcium, choline, diethylamine, diethanolamine, lithium, magnesium, meglumine, 4-phenylcyclohexylamine, piperazine, potassium, sodium, tromethamine, and zinc.

A "therapeutically effective amount" and/or "prophylactically effective amount" means an amount effective, when administered to a human or non-human patient, to provide any therapeutic and/or prophylactic benefit. More particularly, a "therapeutically effective amount" is an amount of an active ingredient disclosed herein or a combination of two or more such active ingredients, which inhibits, totally or partially, the progression of the condition or alleviates, at least partially, one or more symptoms of the condition.

A "diagnostically effective amount" means an amount effective to allow obtaining from the patient a diagnostically valuable information on status or progression of a disease state.

A therapeutic benefit may be an amelioration of symptoms of a diseased patient, e.g., an amount effective to decrease the symptoms of a diseased patient. In certain circumstances a patient may not present symptoms of a condition for which the patient is being treated. Thus, a prophylactically effective amount of a compound is also an amount sufficient to provide a significant positive effect on any indicia of a disease, disorder or condition e.g. an amount sufficient to significantly reduce the frequency and severity of disease symptoms to occur.

A therapeutically effective amount can also be an amount, which is prophylactically effective.

A "patient" as used herein means human or non-human, in particular human, animals.

A "dosage form" is any unit of administration ("unit dose") of one or more active agents as described herein.

The term "treating" or "treatment" refers to: (i) preventing a disease, disorder or condition from occurring in a patient which may be predisposed to the disease, disorder and/or condition but has not yet been diagnosed as having it; (ii) inhibiting the disease, disorder or condition, i.e., arresting its development; and (iii) relieving the disease, disorder or condition, i.e., causing regression of the disease, disorder and/or condition. In particular it encompasses a prophylactic or therapeutic treatment or combinations thereof.

"Frequency" of dosage may vary depending on the compound used and the particular type of infection treated. A dosage regimen of once per day is possible. Dosage regimens in which the active agent is administered for several times daily, as for example 2 to 10 times, like 2, 3, 4, 5, 6, 7, 8, 9 or 10 times may occasionally be more helpful.

It will be understood, however, that the specific dose level and frequency for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination and the severity of the particular disease in the patient undergoing therapy. Patients may generally be monitored for therapeutic or prophylactic effectiveness using assays suitable for the condition being treated or prevented, which will be familiar to those of ordinary skill in the art.

Particular examples of pharmaceutical compositions according to the present invention are liquid form preparations such as solutions, suspensions, and emulsions and comprise, a therapeutically effective amount of at least one APC, in particular ADC component as defined above, optionally together with at least one further pharmaceutically acceptable excipient as defined above and may be administered through any suitable route.

Further examples of pharmaceutical compositions according to the present invention are solid form preparations such as powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules.

The numerous possible variations that will become immediately evident to a person skilled in the art after having considered the disclosure provided herein also fall within the scope of the invention.

The following examples are illustrative only and are not intended to limit the scope of the embodiments described herein.

EXPERIMENTAL PART

A) Materials and Methods

Reagents were purchased from commercial suppliers and used without further purification. All solvents, including anhydrous solvents, were used as obtained from the commercial sources. Air and water-sensitive reagents and reactions were generally handled under argon atmosphere.

- 5 The reaction progress was monitored by TLC on Merck silica gel plates 60 F254 or via UHPLC-MS. TLC-detection was executed either via UV-light at 254 nm or with potassium permanganate staining.

Flash chromatographic purification was performed on a Biotage Isolera One purification system using silica gel (0.060-0.200 mm), KP-Sil cartridges.

- 10 Preparative HPLC purification was performed on Agilent Infinity 1260 series equipment consisting of Agilent 1260 preparative pumps, a 1260 preparative autosampler, a 1260 fraction collector and a 1260 multiple wavelength detector VL. The preparative column used was a Waters X-Bridge Prep C18 column: 5 μ m; 19x150 mm operated with a linear gradient of H₂O and acetonitrile, both containing 0.1% TFA as solvents.

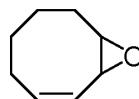
- 15 Nuclear magnetic resonance spectra were recorded on a Bruker Avance (400 MHz) NMR System at room temperature. Chemical shifts (δ) are given in parts per million (ppm), coupling constants (J) given in Hertz (Hz) and multiplicity is reported using standard abbreviations.

- 20 UHPLC-MS analyses were performed on Agilent Infinity 1290 series equipment consisting of an Agilent 1290 quaternary pump, a 1290 sampler, a 1290 thermostated column compartment and a 1290 Diode array detector VL+ equipped with a quadrupole LC/MS 6120 and an Infinity 1260 ELSD. The analytical column used was an Acquity UPLC BEH C18 column: 1.7 μ m; 2.1x50 mm operated with a linear gradient of H₂O and acetonitrile, both containing 0.1% TFA as solvents.

- 25 Photoisomerization of cis-cyclooctenes was conducted using a custom built continuous flow photoreactor consisting of a fan-ventilated light-impermeable aluminum wall UV-unit casing, housing 3 low pressure Hg-lamps (40 mm x 533 mm, 2 times HNS L 55W 2G11 and one time HNS L 96W 2G11, Osram Licht AG) and a quartz tube (OD 16 mm, ID 14 mm, QSIL GmbH) capped with tightly fitting Teflon adaptors containing an O-ring and threaded hole (made in house) for the attachment of the flanged Teflon tubing via threaded PEEK adapters; an electrical unit allowing independent switching of the low pressure Hg-lamps of variable intensity (2x55 W and 1x95 W) to control the intensity of the UV-light; HPLC pump (Model P402) purchased from Latek Labortechnik-Geräte GmbH & Co. The Teflon tubing included a connection to a male and female luer, for attachment of silica
35 cartridges (Biotage SNAP) of different sizes.

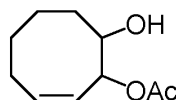
B) Synthesis of Compounds**EXAMPLE 1: Synthesis of (Z)-9-Oxabicyclo[6.1.0]non-2-ene (1)**

5

**1**

10 A solution of 3-chloroperbenzoic acid (12.7 g, 73.4 mmol, 1.00 eq) in CH₃Cl (220 mL) was added dropwise to 1,3-cyclooctadiene (11.4 mL, 9.93 g, 91.8 mmol, 1.25 eq) over 3 h with stirring. After complete addition the mixture was stirred at room temperature (rt) overnight. The formed precipitate was filtered off and the filter cake washed with CH₂Cl₂ several times. The filtrate was washed with an aqueous solution of NaSO₃ followed by a
15 saturated aqueous solution of NaHCO₃. The organic phase was dried over Na₂SO₄ and the solvent removed under reduced pressure.

Purification via flash chromatography (cyclohexane/EtOAc 20:1→10:1) yielded **1** as colorless oil (8.93 g, 98%).

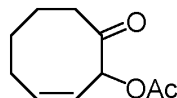
20 EXAMPLE 2: Synthesis of (Z)-8-hydroxycyclooct-2-en-1-yl acetate (2)**2**

25

To a solution of acetic acid (5.80 mL, 6.09 g, 101 mmol, 1.50 eq) and tetrakis(triphenylphosphine)palladium(0) (7.82 g, 6.76 mmol, 0.100 eq) in THF (190 mL) was added **1** (8.40 g, 67.6 mmol, 1.00 eq) dropwise at 0 °C. After complete addition the mixture was stirred at this temperature for 2 h. The reaction was quenched by addition of 5 mL H₂O₂
30 (30% in water). A saturated aqueous solution of NaHCO₃ was added and the mixture extracted with CH₂Cl₂. The organic phase was dried over Na₂SO₄ and the solvent removed under reduced pressure.

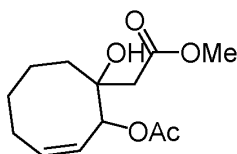
Purification via flash chromatography (cyclohexane/EtOAc 10:1→1:1) yielded **2** as colorless oil (10.9 g, 87%).

35

EXAMPLE 3: Synthesis of – (Z)-8-oxocyclooct-2-en-1-yl acetate (3)**3**

To a solution of **2** (9.96 g, 54.1 mmol, 1.00 eq) in CH₂Cl₂ (540 mL) was added pyridinium chlorochromate (17.5 g, 81.1 mmol, 1.50 eq) and the mixture was stirred at rt for 8 h. The reaction mixture was filtered through a plug of silica and the solvent was removed under reduced pressure.

Purification via flash chromatography (cyclohexane/EtOAc 20:1→5:1) yielded **3** as colorless oil (7.11 g, 72%).

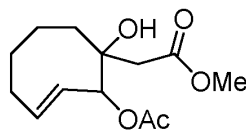
EXAMPLE 4: Synthesis of methyl (Z)-2-(2-acetoxy-1-hydroxycyclooct-3-en-1-yl)acetate (4)**4**

To a solution of **3** (7.11 g, 39.0 mmol, 1.00 eq) in CH₂Cl₂ (390 mL) was added BF₃·OEt₂ (9.63 mL, 11.1 g, 78.0 mmol, 2.00 eq) at -78 °C. Afterwards 1-(tert-Butyldimethylsilyloxy)-1-methoxyethene (10.2 mL, 8.82 g, 46.8 mmol, 1.20 eq) was added dropwise and the reaction stirred at this temperature for 1 h. The reaction was quenched by addition of saturated aqueous solution of NaHCO₃. The phases were separated, and the aqueous phase was extracted with CH₂Cl₂. The combined organic phases were dried over Na₂SO₄ and the solvent removed under reduced pressure.

Purification via flash chromatography (cyclohexane/EtOAc 20:1→1:1) yielded **4** as a mixture of anti- and syn-isomer (4:1) as colorless oil (8.16 g, 82%).

EXAMPLE 5: Synthesis of methyl (E)-2-(2-acetoxy-1-hydroxycyclooct-3-en-1-yl)acetate (5)

105



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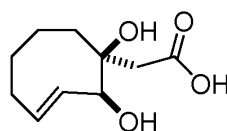
A 2-neck 500 mL flask was connected to the continuous flow photoreactor. A 100 g Biotage SNAP cartridge was equipped with 10% AgNO₃ silica gel (58.4 g, 34.4 mmol, 1.30 eq) on top of normal silica. The photoisomerization system was equilibrated at a flow of 100 mL/min with the cartridge attached with Et₂O/hexane (500 mL) for 20 min. Then **4** (6.78 g, 26.5 mmol, 1.00 eq) dissolved in a minimal amount of Et₂O and methyl benzoate (6.67 mL, 7.20 g, 52.9 mmol, 2.00 eq) were added into the 2-neck flask and the system equilibrated for another 20 min while cooling at 0 °C. Afterwards the two 55 W UV-lamps were switched on and the photoisomerization conducted under continuous flow (100 mL/min) for 30 h while cooling at 0 °C. Afterwards the system was flushed with 500 mL Et₂O and the cartridge was purged with air. The AgNO₃ silica was transferred into an Erlenmeyer flask and CH₂Cl₂ and a 25% aqueous solution of NH₄OH was added and the mixture stirred vigorously for 5 min.

The silica was filtered off and the filter cake washed with 25% NH₄OH and CH₂Cl₂ several times. The phases were separated, and the aqueous phase was extracted with CH₂Cl₂. The combined organic phases were washed with water, dried over Na₂SO₄ and the solvent removed under reduced pressure.

Purification via flash chromatography (cyclohexane/EtOAc 20:1→3:1) yielded **5** as a separable mixture of anti- and syn-isomer (**anti-5** and **syn-5**) as colorless crystalline solid (syn-isomer) and colorless oil (anti-isomer) (3.02 g, 45%).

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EXAMPLE 6: Synthesis of – (E)-2-(1,2-dihydroxycyclooct-3-en-1-yl)acetic acid (anti-6, syn-6)



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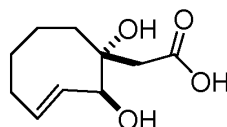
anti-6

To a solution of **anti-5** (1.17 g, 4.57 mmol, 1.00 eq) in MeOH (45 mL) was added 1 M NaOH_{aq} (45.7 mL, 45.7 mmol, 10.0 eq) and the mixture was stirred at rt for 1.5 h. It was

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acidified with 1 M HCl_{aq} and extracted with EtOAc 2 times. The combined organic phases were dried over Na₂SO₄ and the solvent removed under reduced pressure. Coevaporation with toluene was conducted to remove residual AcOH impurities to yield **anti-6** as a colorless crystalline solid (837 mg, 92%).

5

**syn-6**

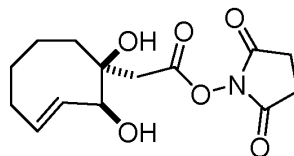
10

To a solution of **syn-5** (150 mg, 585 μmol, 1.00 eq) in MeOH (5.85 mL) was added 1 M NaOH_{aq} (5.85 mL, 5.85 mmol, 10.0 eq) and the mixture was stirred at rt for 1 h. It was acidified with 1 M HCl_{aq} and extracted with EtOAc 2 times. The combined organic phases were dried over Na₂SO₄ and the solvent removed under reduced pressure. Coevaporation with toluene was conducted to remove residual AcOH impurities to yield **syn-6** as a colorless crystalline solid (79.0 mg, 67%).

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EXAMPLE 7: Synthesis of 2,5-dioxopyrrolidin-1-yl (E)-2-(1,2-dihydroxycyclooct-3-en-1-yl)acetate (7)

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**7**

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To a solution of **anti-6** (1.20 g, 5.99 mmol, 1.00 eq) in DMF (8 mL) was added N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (2.17 g, 7.19 mmol, 1.20 eq) and DIPEA (1.3 mL, 968 mg, 7.49 mmol, 1.25 eq) and the reaction stirred at rt for 1 h. The reaction was quenched with H₂O, extracted with CH₂Cl₂. The combined organic phases were dried over Na₂SO₄ and the solvent removed under reduced pressure.

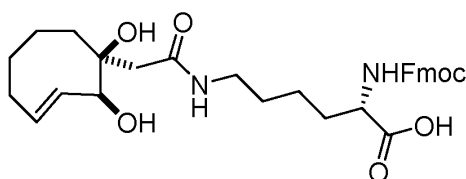
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Purification via flash chromatography (cyclohexane/EtOAc 9:1→1:1) yielded **7** as colorless solid (1.34 g, 75%).

EXAMPLE 8: Synthesis of N2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N6-(2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)acetyl)-L-lysine (8)

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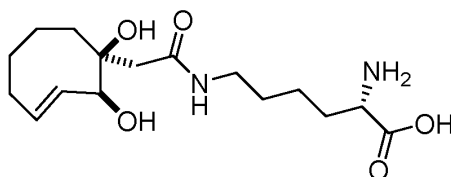
107

**8**

To a crude solution of **7** (1.24 g, 4.18 mmol, 1.00 eq) in DMF (42 mL) was added Fmoc-Lys-OH (1.85 g, 5.02 mmol, 1.20 eq) and DIPEA (910 μ L, 675 mg, 5.23 mmol, 1.25 eq) and the reaction stirred at rt for 3 h. It was acidified with 1 M HCl_{aq} and extracted with EtOAc 2 times. The combined organic phases were dried over Na₂SO₄ and the solvent removed under reduced pressure.

Purification via reverse phase flash chromatography (H₂O/MeOH 0%→100%) yielded **8** as colorless solid (2.11 g, 92% over 2 steps).

EXAMPLE 9: Synthesis of N6-(2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)acetyl)-L-lysine (9)

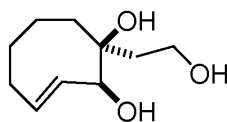
**9**

To a crude solution of **7** (2.11 g, 3.83 mmol, 1.00 eq) in DMF (38 mL) was added piperidine (9.5 mL) and the reaction stirred at rt for 15 min. The solvent removed under reduced pressure and the residue was taken up in water and filtered. The filter cake was washed with water.

Purification via reverse phase flash chromatography (H₂O/MeOH 0%→100%) yielded **9** as colorless solid (984 mg, 78%).

EXAMPLE 10: Synthesis of (E)-1-(2-hydroxyethyl)cyclooct-3-ene-1,2-diol (10)

108



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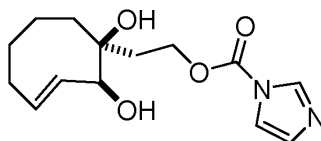
10

To a suspension of LiAlH_4 (29.6 mg, 780 μmol , 2.00 eq) in Et_2O (1 mL) was added **anti-5** (100 mg, 390 μmol , 1.00 eq) in THF (1 mL) at 0 °C and the mixture was stirred at rt for 2 h. Afterwards water was added and it was extracted with EtOAc. The combined organic phases were dried over Na_2SO_4 and the solvent removed under reduced pressure.

Purification via flash chromatography (cyclohexane/EtOAc 20:1→1:1) yielded **10** as colorless oil (35.0 mg, 48%).

EXAMPLE 11: Synthesis of 2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)ethyl 1H-imidazole-1-carboxylate (11)

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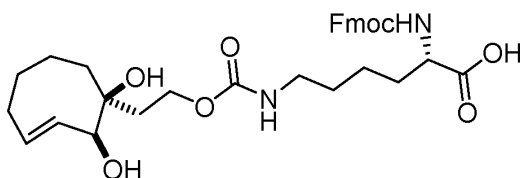
11

To a solution of **10** (79.0 mg, 424 μmol , 1.00 eq) in DMF (1 mL) was added 1,1'-carbonyldiimidazole (75.7 mg, 467 μmol , 1.10 eq) at 0 °C and the reaction stirred for 1 h. The solution containing crude **11** was directly used for the next step.

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EXAMPLE 12: Synthesis of N2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N6-((2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)ethoxy)carbonyl)-L-lysine (12)

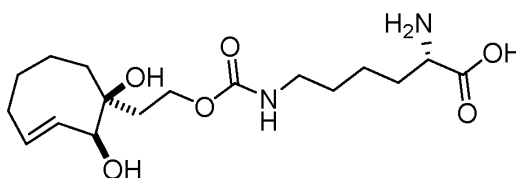
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**12**

To a crude solution of **11** (119 mg, 424 μ mol, 1.00 eq) in DMF (1 mL) was added Fmoc-Lys-OH (234 mg, 636 μ mol, 1.50 eq) and DIPEA (111 μ L, 82.2 mg, 636 μ mol, 1.50 eq) and the reaction stirred at rt for 5 h. Afterwards water was added to dissolve remaining Fmoc-Lys-OH and the mixture was stirred at 60 °C overnight. It was acidified with 1 M HCl_{aq} and extracted with EtOAc 2 times. The combined organic phases were dried over Na₂SO₄ and the solvent removed under reduced pressure.

Purification via reverse phase flash chromatography (H₂O/MeOH 0%→100%) yielded **12** as colorless solid (17.0 mg, 7% over 2 steps).

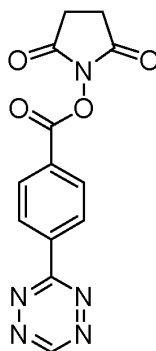
EXAMPLE 13: Synthesis of N6-((2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)ethoxy)carbonyl)-L-lysine (13)



13

To a crude solution of **12** (17.0 mg, 29.3 μ mol, 1.00 eq) in DMF (0.3 mL) was added piperidine (75.0 μ L) and the reaction stirred at rt for 15 min. The reaction mixture was directly subjected to reverse phase flash chromatography (H₂O/MeOH 0%→100%) to yield **13** as colorless solid (6.10 mg, 58%).

EXAMPLE 14: Synthesis of 2,5-dioxopyrrolidin-1-yl 4-(1,2,4,5-tetrazin-3-yl)benzoate (14)



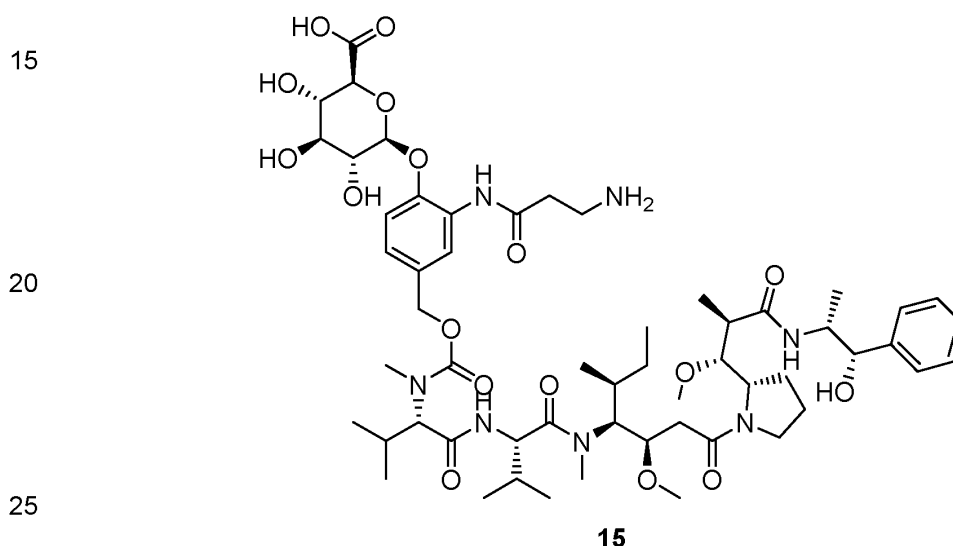
14

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To a solution of 4-(1,2,4,5-tetrazin-3-yl)benzoic acid (92.5 mg, 458 μ mol, 1.00 eq) in dichloromethane (4.6 mL) was added N-hydroxysuccinimide (80.0 mg, 686 μ mol, 1.50 eq) and EDC (132 mg, 686 μ mol, 1.50 eq) and the mixture was stirred at room temperature overnight.

- 5 Purification via flash chromatography (dichloromethane/MeOH 20:1) yielded **14** as pink solid (108 mg, 79%).

EXAMPLE 15: Synthesis of (2S,3S,4S,5R,6S)-6-(2-(3-aminopropanamido)-4-((5S,8S,11S,12R)-11-((S)-sec-butyl)-12-(2-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-2-oxoethyl)-5,8-diisopropyl-4,10-dimethyl-3,6,9-trioxo-2,13-dioxo-4,7,10-triazatetradecyl)phenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (15)

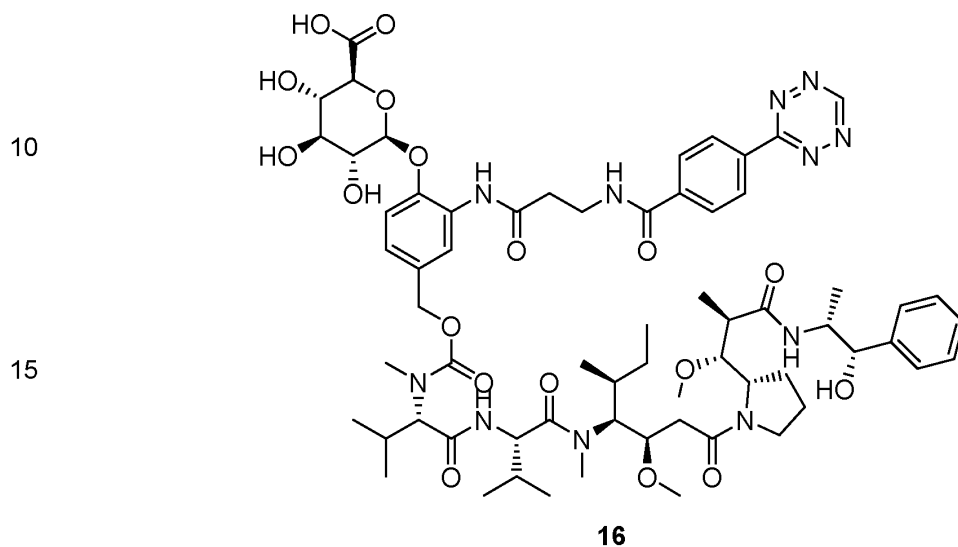


To a solution of (2S,3S,4S,5R,6S)-methyl-6-(2-(3-(((9H-fluoren-9-yl)methoxy)carbonyl-amino)propanamido)-4-(((4-nitrophenoxy)carbonyloxy)methyl)phenoxy)-3,4,5-triacetoxy-tetrahydro-2H-pyran-2-carboxylate (54.0 mg, 59.1 μ mol, 1.00 eq) and monomethyl auristatin E (42.4 mg, 59.1 μ mol, 1.00 eq) in DMF/pyridine (2:1, 0.9 mL) was added HOBt (0.904 mg, 5.91 μ mol, 0.100 eq) and DIPEA (10.3 μ L, 7.64 mg, 59.1 μ mol, 1.00 eq) and the mixture was stirred at room temperature overnight. Afterwards 1 M NaOH_{aq} (591 μ L, 591 μ mol, 10.0 eq) was added and the mixture stirred at rt for 1 h.

Purification via HPLC yielded **15** as white solid (46.7 mg, 70%).

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EXAMPLE 16: Synthesis of (2S,3S,4S,5R,6S)-6-(2-(3-(4-(1,2,4,5-tetrazin-3-yl)benzamido)propanamido)-4-((5S,8S,11S,12R)-11-((S)-sec-butyl)-12-(2-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-2-oxoethyl)-5,8-diisopropyl-4,10-dimethyl-3,6,9-trioxo-2,13-dioxo-4,7,10-triazatetradecyl)phenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (**16**)



To a solution of **15** (38.5 mg, 34.1 μ mol, 1.00 eq) and **14** (20.4 mg, 68.1 μ mol, 2.00 eq) in DMF/pyridine (4:1, 0.7 mL) was added NEt_3 (5.67 μ L, 4.14 mg, 40.9 μ mol, 1.20 eq) and the mixture was stirred at room temperature overnight. Afterwards 1 M NaOH_{aq} (591 μ L, 591 μ mol, 10.0 eq) was added and the mixture stirred at rt overnight. Purification via HPLC yielded **16** as pink solid (7.9 mg, 18%).

EXAMPLE 17: Synthesis of 2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)-N-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)acetamide (**17**)



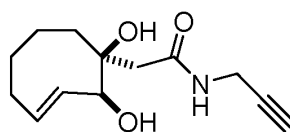
112

To a solution of **7** (43.0 mg, 145 μ mol, 1.00 eq) and 1-(2-aminoethyl)-1H-pyrrole-2,5-dione (51.1 mg, 289 μ mol, 2.00 eq) in DMF (1 mL) was added DIPEA (50.4 μ L, 289 μ mol, 2.00 eq) and the mixture stirred at rt for 2h. The reaction mixture was directly subjected to purification via HPLC.

5 Purification via HPLC yielded **17** (11.0 mg, 24%) as white powder.

EXAMPLE 18: Synthesis of 2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)-N-(prop-2-yn-1-yl)acetamide (18)

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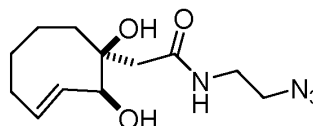
**18**

15 To a solution of **7** (20.0 mg, 67.3 μ mol, 1.00 eq) and prop-2-yn-1-amine (5.17 μ L, 80.7 μ mol, 1.20 eq) in DMF (0.5 mL) was added DIPEA (23.4 μ L, 135 μ L, 2.00 eq) and the mixture stirred at rt for 30 min. The reaction mixture was directly subjected to purification via HPLC. Purification via HPLC yielded **18** (3.7 mg, 23%).

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EXAMPLE 19: Synthesis of N-(2-azidoethyl)-2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)acetamide (19)

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**19**

30 To a solution of **7** (20.0 mg, 67.27 μ mol, 1.00 eq) and 2-azidoethanamine hydrochloride (9.89 mg, 80.7 μ mol, 1.20 eq) in DMF (0.5 mL) was added DIPEA (23.4 μ L, 135 μ L, 2.00 eq) and the mixture stirred at rt for 30 min. The reaction mixture was directly subjected to purification via HPLC.

Purification via HPLC yielded **19** (1.9 mg, 11%).

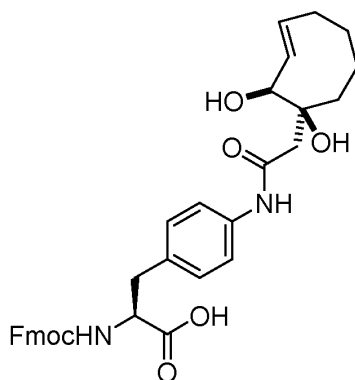
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EXAMPLE 20: Synthesis of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)acetamido)phenyl)propanoic acid (20)

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**20**

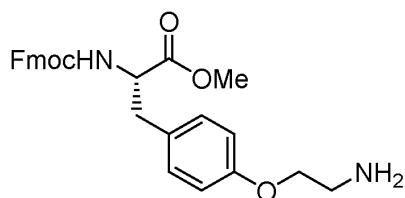
To a solution of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-aminophenyl)propanoic acid (44.7 mg, 111 μ mol, 1.10 eq) and **7** (30.0 mg, 101 μ mol, 1.00 eq) in DMF (1 mL) was added DIPEA (15.7 mg, 21.1 μ L, 121 μ mol, 1.20 eq) and the mixture stirred at rt.

Purification via HPLC yielded **20** (4.3 mg, 7%).

EXAMPLE 21: Synthesis of methyl (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(2-aminoethoxy)phenyl)propanoate (21)

20

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**21**

To a solution of Fmoc-Tyr-OMe (200 mg, 479 μ mol, 1.00 eq), tert-butyl (2-hydroxyethyl)carbamate (81.1 mg, 503 μ mol, 1.05 eq) and triphenylphosphine (132 mg, 503 μ mol, 1.05 eq) in THF (1 mL) was added DIAD (102 mg, 99.0 μ L, 503 μ mol, 1.05 eq) dropwise at 0 °C. The mixture was stirred at rt.

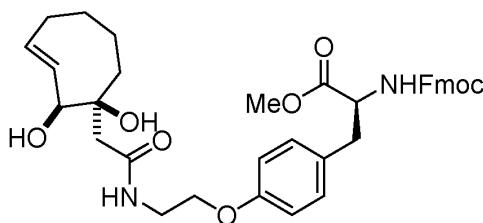
After completion of the reaction the reaction mixture was purified directly via column chromatography (cyclohexane/EtOAc 20:1→1:1) and the isolated Boc-protected intermediate deprotected in dichloromethane/TFA (3:1, 4 mL). The solvents were evaporated under reduced pressure.

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Purification via HPLC yielded **21** (47.0 mg, 17%).

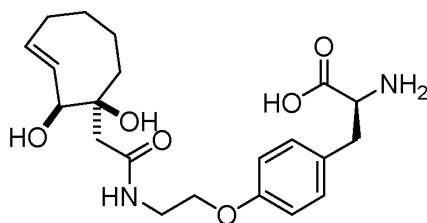
EXAMPLE 22: Synthesis of methyl (2S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(2-(2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)acetamido)ethoxy)phenyl)propanoate (22)

**22**

To a solution of **7** (24.3 mg, 81.8 μ mol, 1.00 eq) and **21** (47.0 mg, 81.8 μ mol, 1.00 eq) in DMF (0.8 mL) was added DIPEA (12.7 mg, 17.1 μ L, 98.2 μ mol, 1.20 eq) and the mixture stirred at rt.

The solvent was evaporated under reduced pressure and **22** was used crude for the next step.

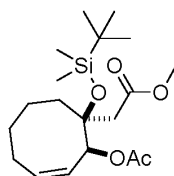
EXAMPLE 23: Synthesis of (S)-2-amino-3-(4-(2-(2-((E)-1,2-dihydroxycyclooct-3-en-1-yl)acetamido)ethoxy)phenyl)propanoic acid (23)

**23**

To a solution of **22** (51.4 mg, 80.0 μ mol, 1.00 eq) in MeOH (0.8 mL) was added 1 M NaOH_{aq} (32.0 mg, 800 μ L, 800 μ mol, 10.0 eq) and the mixture stirred at rt for 1 h. Afterwards the reaction was neutralized by addition of 1 M HCl_{aq}, and the mixture directly subjected to purification via reverse phase flash chromatography.

Purification via reverse phase flash chromatography yielded **23** (16.7 mg, 51%).

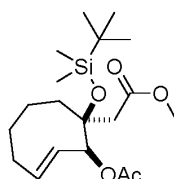
EXAMPLE 24 – Synthesis of methyl (Z)-2-(2-acetoxy-1-((tert-butyl)dimethylsilyl)oxy)cyclooct-3-en-1-yl)acetate (24):



24

To a solution of **3** (2.00 g, 1 Eq, 11.0 mmol) in diethyl ether (22.0 mL) was added 1,1,1-trifluoro-N-trifluoromethanesulfonylmethanesulfonamide (15.4 mg, 11.3 μ L, 0.005 Eq, 54.9 μ mol) at 20 °C under N₂ atmosphere. The reaction mixture was cooled to –20 °C and *tert*-butyl[(1-methoxyvinyl)oxy]dimethyl silane (2.17 g, 2.39 mL, 1.05 Eq, 11.5 mmol) was slowly added at this temperature, then left at –20 °C and stirred for 30 min. Ice was removed and the reaction left steering at 20 °C over night. The crude reaction mixture was evaporated in vacuo, then the mixture was purified by column chromatography on silica gel (eluent cyclohexane/ethyl acetate 0-10%), fractions were evaporated in vacuo to yield **24** (1.90 g, 5.13 mmol, 47%) as colorless oil.

EXAMPLE 25 – Synthesis of methyl (E)-2-(2-acetoxy-1-((tert-butyl)dimethylsilyl)oxy)cyclooct-3-en-1-yl)acetate (25):



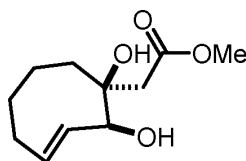
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A 2-neck flask was connected to the continuous flow photoreactor. A 100 g Biotage Sfaer SiOH cartridge was equipped with 10% AgNO₃ silica gel (35.8 g, 10% Wt, 2.6 eq, 21.0 mmol) on top of normal silica. The photoisomerization system was equilibrated at a flow of 100 mL/min with the cartridge attached with Et₂O/hexane (300 mL) for 20 min. Methyl benzoate (1.16 g, 1.07 mL, 1.05 eq, 8.50 mmol) and **24** (3.00 g, 1 eq, 8.10 mmol) in 30mL

hexane/Et₂O (6:4) were added into the 2-neck flask and the system equilibrated for another 20 min while cooling at 0 °C. Afterwards one 55 W UV-lamp was switched on and the photoisomerization conducted under continuous flow (100 mL/min) for 47 h while cooling at 0 °C. Afterwards the system was flushed with Et₂O and the cartridge was purged with air. The AgNO₃ silica was transferred into an Erlenmeyer flask and CH₂Cl₂ and a 25% aqueous solution of NH₄OH was added and the mixture stirred vigorously for 5 min. The mixture was filtered over Celite, filter cake was washed with 25ml NH₄OH (1x) and 3 times with 50ml DCM (10% MeOH).

The layers were separated, extracted 2x with 100ml DCM and the combined DCM layer was dried with Na₂SO₄, filtered, and evaporated. The crude was purified by column chromatography on silica gel (eluent cyclohexane/Ethyl acetate 0-10%), fractions were evaporated in vacuo to yield **25** (260 mg, 702 μmol, 9%) as white solid.

EXAMPLE 26 – Synthesis of methyl 2-((1S,E)-1,2-dihydroxycyclooct-3-en-1-yl)acetate (26):

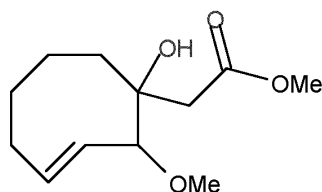


26

To a solution of compound **5** prepared according to Example 5, above, (4.78 g, 1.0 eq, 18.7 mmol) in methanol (15.0 mL) was added sodium methanolate (806 mg, 853 μL, 0.200 eq, 3.7 mmol) at 25 °C and the reaction mixture was stirred for 1 hour. The reaction mixture was diluted with CH₂Cl₂ and washed with brine. The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure to yield **26** (3.88 g, 18.1 mmol, 97%) as yellow oil.

EXAMPLE 27 – Synthesis of methyl (E)-2-(1-hydroxy-2-methoxycyclooct-3-en-1-yl)acetate (27):

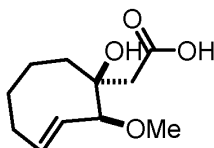
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27

To a solution of **26** (1.52 g, 1.0 eq, 7.1 mmol) in DMF (5.0 mL) was added iodomethane (5.02 g, 5.0 eq, 35.4 mmol) under N₂ atmosphere. The reaction mixture was cooled to 0 °C. A suspension of sodium hydride (311 mg, 1.1 eq, 7.8 mmol) in DMF (50.0 mL) was slowly added to the reaction mixture and left stirring at room temperature for 3 h. The reaction mixture was quenched with sat. NH₄Cl. The organic layer was extracted with ethyl acetate, dried over Na₂SO₄ and concentrated under reduced pressure. The crude was purified by column chromatography on silica gel (ethyl acetate in cyclohexane: 2-20%). Fractions of product were combined and concentrated to yield **27** (0.77 g, 3.39 mmol, 48%) as colorless oil.

EXAMPLE 28 – Synthesis of (E)-2-(1-hydroxy-2-methoxycyclooct-3-en-1-yl)acetic acid (28):



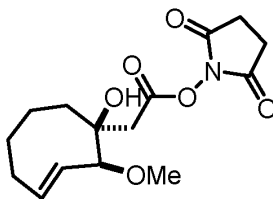
28

To a solution of **27** (0.77 g, 1.0 eq, 3.4 mmol) in methanol (10.0 mL) was added 1 M NaOH_{aq} (1.36 g, 10.0 eq, 33.9 mL, 33.9 mmol) and the reaction mixture was left stirring at room temperature for 2 h. Afterwards 1 M HCl solution was added until the mixture became acidic. The organic layer was extracted with ethyl acetate, dried over Na₂SO₄ and concentrated under reduced pressure to yield **28** (0.72 g, 3.35 mmol, 99%) as colorless oil.

EXAMPLE 29 – Synthesis of 2,5-dioxopyrrolidin-1-yl (E)-2-(1-hydroxy-2-methoxycyclooct-3-en-1-yl)acetate (29):

118

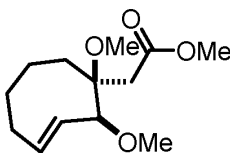
5

**29**

To a solution of **28** (0.72 g, 1.0 eq, 3.3 mmol) and 2-(2,5-dioxopyrrolidin-1-yl)-1,1,3,3-tetramethylisouronium tetrafluoroborate (1.21 g, 1.2 eq, 4.0 mmol) in DMF (8.0 mL) was added DIPEA (541 mg, 1.3 eq, 720 μ L, 4.2 mmol) and the reaction mixture was left stirring at room temperature for 2 h.

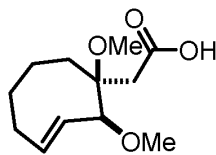
The reaction mixture was diluted with ethyl acetate and washed with brine. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude was purified by column chromatography on silica gel (ethyl acetate in cyclohexane: 5-100%). Fractions of product were combined, concentrated and triturated with diethyl ether to get rid of remaining impurities. Product was dried in vacuo to yield **29** (0.88 g, 2.83 mmol, 85%) as white powder.

EXAMPLE 30 – Synthesis of methyl (E)-2-(1,2-dimethoxycyclooct-3-en-1-yl)acetate (30):

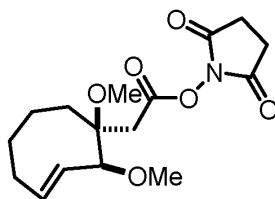
**30**

To a solution of **28** (100 mg, 1.0 eq, 471 μ mol) in DMF (2.0 mL) was added iodomethane (335 mg, 5.0 eq, 147 μ L, 2.4 mmol) under N_2 atmosphere. This mixture was added dropwise to a cooled suspension of sodium hydride (226 mg, 12.0 eq, 5.7 mmol) in DMF (3.0 mL) at 0 $^\circ\text{C}$ under N_2 atmosphere. The reaction mixture was left stirring at room temperature for 3 h.

The reaction mixture was quenched with sat. NH_4Cl . The organic layer was extracted with diethyl ether, dried over Na_2SO_4 and concentrated under reduced pressure. The crude was purified by column chromatography on silica gel (ethyl acetate in cyclohexane: 5-60%). Fractions of product were combined and concentrated to yield **30** (5.3 mg, 218 μ mol, 46%) as colorless oil.

EXAMPLE 31 – Synthesis of (E)-2-(1,2-dimethoxycyclooct-3-en-1-yl)acetic acid (31):**31**

To a solution of **30** (70.0 mg, 1.0 eq, 289 μ mol) in methanol (3.0 mL) was added 1 M NaOH_{aq} (116 mg, 10.0 eq, 2.89 mL, 2.89 mmol) and the reaction mixture was left stirring at room temperature for 2 h. Afterwards 1 M HCl solution was added until the mixture became acidic. The organic layer was extracted with ethyl acetate, dried over Na₂SO₄ and concentrated under reduced pressure to yield **31** (49.2 mg, 216 μ mol, 75%) as colorless oil.

EXAMPLE 32 – Synthesis of 2,5-dioxopyrrolidin-1-yl (E)-2-(1,2-dimethoxycyclooct-3-en-1-yl)acetate (32):**32**

To a solution of **31** (48.0 mg, 1.0 eq, 0.2 mmol) and 2-(2,5-dioxopyrrolidin-1-yl)-1,1,3,3-tetramethylisouronium tetrafluoroborate (76.0 mg, 1.2 eq, 0.3 mmol) in DMF (2.0 mL) was added DIPEA (34.0 mg, 1.3 eq, 45.0 μ L, 0.3 mmol) and the reaction mixture was left stirring at room temperature for 3 h. The reaction mixture was diluted with ethyl acetate and washed with brine. The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The crude was purified by column chromatography on silica gel (ethyl acetate in cyclohexane: 5-100%). Fractions of product were combined, concentrated and triturated with diethyl ether to get rid of remaining impurities. Product was dried in vacuo to yield **32** (34.2 mg, 0.1 mmol, 50%) as white powder.

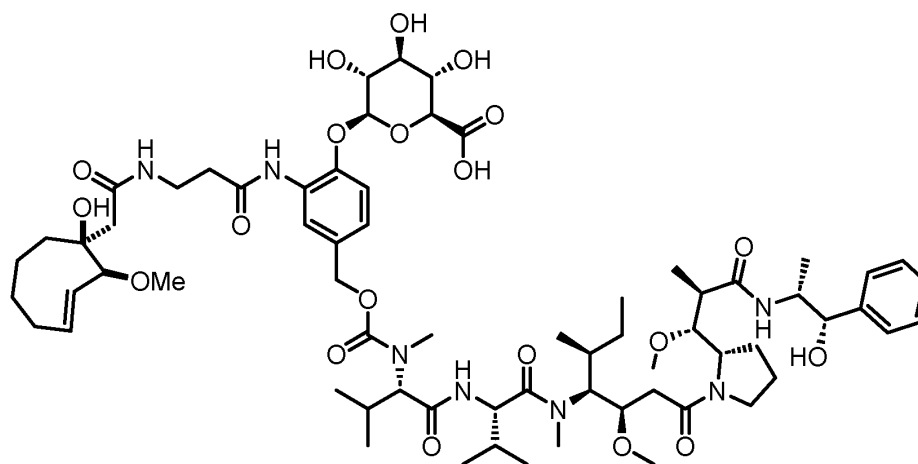
EXAMPLE 33 – Synthesis of (2S,3S,4S,5R,6S)-6-(4-((5S,8S,11S,12R)-11-((S)-sec-butyl)-12-(2-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-2-oxoethyl)-5,8-diisopropyl-4,10-dimethyl-3,6,9-

trioxo-2,13-dioxo-4,7,10-triazatetradecyl)-2-(3-(2-((1S,2S,E)-1-hydroxy-2-methoxycyclooct-3-en-1-yl)acetamido)propanamido)phenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (**33**):

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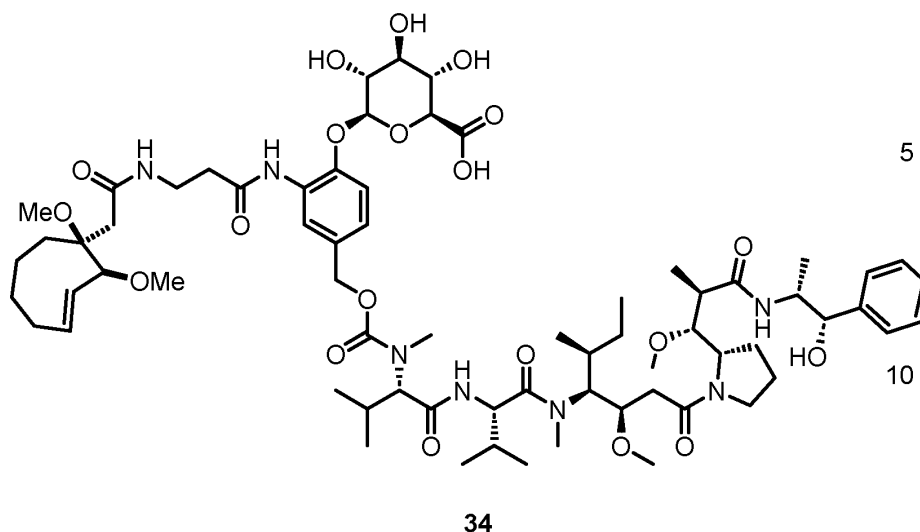
**33**

To a solution of **29** (10.0 mg, 1.0 eq, 32.1 μ mol) in DMF (0.1 mL) was added a solution of 6-(2-(3-aminopropanamido)-4-((5S,8S,11S,12R)-11-((S)-sec-butyl)-12-(2-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-2-oxoethyl)-5,8-diisopropyl-4,10-dimethyl-3,6,9-trioxo-2,13-dioxo-4,7,10-triazatetradecyl)phenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (39.9 mg, 1.1 eq, 35.3 μ mol) in DMF (0.3 mL). Then triethylamine (6.5 mg, 2.0 eq, 8.9 μ L, 64.2 μ mol) was added and reaction mixture was stirred at room temperature for 1 h. The crude reaction mixture was purified via preparative HPLC. The fractions were combined and lyophilized to yield **33** (25.8 mg, 19.4 μ mol, 61%) as white powder.

EXAMPLE 34 – Synthesis of (2S,3S,4S,5R,6S)-6-(4-((5S,8S,11S,12R)-11-((S)-sec-butyl)-12-(2-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-2-oxoethyl)-5,8-diisopropyl-4,10-dimethyl-3,6,9-trioxo-2,13-dioxo-4,7,10-triazatetradecyl)-2-(3-(2-((1S,2S,E)-1,2-dimethoxycyclooct-3-en-1-yl)acetamido)propanamido)phenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-

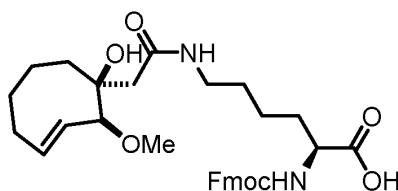
121

carboxylic acid (34):



15 To a solution of **32** (10.0 mg, 1.0 eq, 30.7 μmol) in DMF (0.1 mL) was added a solution of 6-(2-(3-aminopropanamido)-4-((5S,8S,11S,12R)-11-((S)-sec-butyl)-12-(2-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-2-oxoethyl)-5,8-diisopropyl-4,10-dimethyl-3,6,9-trioxo-2,13-dioxo-4,7,10-triazatetradecyl)phenoxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid (38.2 mg, 1.1 eq, 33.8 μmol) in DMF (0.3 mL). Then triethylamine (24.9 mg, 8.0 eq, 34.3 μL , 246 μmol) was added and the reaction mixture was stirred at room temperature for 4 h. The crude reaction mixture was purified via preparative HPLC. The fractions were combined and lyophilized to yield **34** (23.3 mg, 17.4 μmol , 56%) as white powder.

25 **EXAMPLE 35 – Synthesis of N2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N6-(2-((E)-1-hydroxy-2-methoxycyclooct-3-en-1-yl)acetyl)-L-lysine (35):**



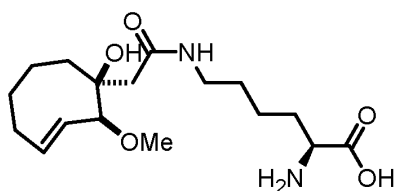
35

To a solution of **29** (48.5 mg, 1.0 eq, 156 μmol) in DMF (3.0 mL) was added (((9H-fluoren-9-

122

yl)methoxy)carbonyl)-L-lysine (68.9 mg, 1.2 eq, 187.0 μ mol) and triethylamine (18.9 mg, 1.2 eq, 26.1 μ L, 187 μ mol) and the reaction mixture was stirred at room temperature for 4 h. Afterwards 1 M HCl solution was added until the mixture became acidic. The organic layer was extracted with ethyl acetate, dried over Na₂SO₄ and concentrated under reduced pressure. Crude mixture was purified via RP flash chromatography (MeCN in water: 10-100%). Fractions were combined and evaporated in vacuo to yield **35** (66.0 mg, 117 μ mol, 75%) as colorless oil.

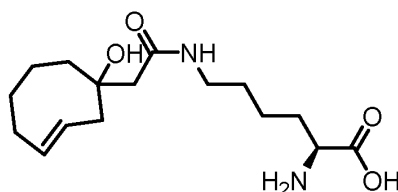
EXAMPLE 36 – Synthesis of N2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N6-(2-((E)-1-hydroxy-2-methoxycyclooct-3-en-1-yl)acetyl)-L-lysine (36):

**36**

To a solution of **35** (66.0 mg, 1.0 eq, 117.0 μ mol) in DMF (0.6 mL) was added piperidine (124.0 mg, 12.5 eq, 144.0 μ L, 1.5 mmol) and the reaction mixture was stirred for 20 min. The reaction mixture was diluted with water and filtered. The filtrate was evaporated in vacuo. The crude product was dissolved in water and purified via RP flash chromatography (MeCN in water: 10-100%). Fractions were combined and evaporated in vacuo to yield **36** (22.8 mg, 66.6 μ mol, 57%) as white solid.

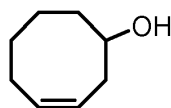
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COMPARATIVE EXAMPLE 37 – Synthesis of N6-(2-((E)-1-hydroxycyclooct-3-en-1-yl)acetyl)-L-lysine (44) – an analogue to a compound described in WO2022/133225:

**44**

Step a) – Synthesis of (Z)-cyclooct-3-en-1-ol (37):

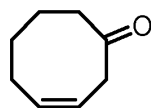
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**37**

To a solution of **1** (2.58 g, 1.0 eq, 20.8 mmol) in dry THF (20.0 mL) was added lithium aluminum hydride solution (1 M in THF) (394 mg, 0.5 eq, 10.4 mL, 10.4 mmol) dropwise at 0 °C and the reaction mixture was stirred at room temperature overnight. The reaction mixture was quenched carefully with water (3 mL) and filtered. The filtrate was dried over Na₂SO₄ and concentrated under reduced pressure. The crude was purified by column chromatography on silica gel (ethyl acetate in cyclohexane: 2-20%). Fractions of product were combined and evaporated in vacuo to yield **37** (1.52 g, 12.0 mmol, 58%) as colorless oil.

Step b) – (Z)-cyclooct-3-en-1-one (38):

20

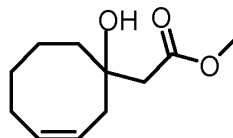
**38**

To a flask (Z)-cyclooct-3-en-1-ol (1.37 g, 1.0 eq, 10.8 mmol) in dry DCM (20.0 mL) was added. 3-oxo-1lambda5-benzo[d][1,2]iodaoxole-1,1,1(3H)-triyl triacetate (5.15 g, 1.1 eq, 12.15 mmol) was added and the reaction mixture was stirred at room temperature over night. Reaction mixture was diluted with diethyl ether and washed with sat. NaHCO₃. Organic layer was extracted, dried over Na₂SO₄ and celite and filtered. The crude product was concentrated under reduced pressure and purified by column chromatography on silica gel (ethyl acetate in cyclohexane: 2% for 1CV, 2-10% for 15CV. Fractions of product were combined and evaporated on vacuo to yield **38** (1.06 g, 8.5 mmol, 79%) as colorless oil.

Step c) – methyl (Z)-2-(1-hydroxycyclooct-3-en-yl) acetate (39):

124

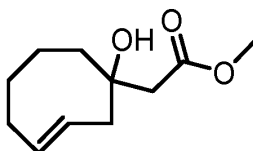
5

**39**

To a solution of **38** (0.90 g, 1.0 eq, 7.2 mmol) in dry DCM (120 mL) was added boron trifluoride etherate (2.10 g, 2.0 eq, 1.80 mL, 14.0 mmol) at -78 °C followed by dropwise addition of *tert*-butyl((1-methoxyvinyl)oxy)dimethylsilane (1.6 g, 1.2 eq, 1.8 mL, 8.7 mmol). The reaction mixture was left stirring at room temperature overnight and then quenched with sat. NaHCO₃. The organic layer was extracted with DCM and dried over Na₂SO₄. The crude product was concentrated under reduced pressure and purified by column chromatography on silica gel (ethyl acetate in cyclohexane: 3-30%). Fractions of product were combined and evaporated in vacuo to yield **39** (0.86 g, 4.4 mmol, 60%) as colorless oil.

Step d) – Synthesis of methyl (E)-2-(1-hydroxycyclooct-3-en-yl) acetate (40):

20

**40**

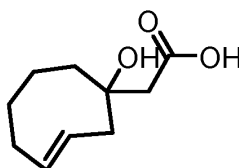
A 2-neck flask was connected to the continuous flow photoreactor. A 25 g Biotage Sfaer SiOH cartridge was equipped with 10% AgNO₃ silica gel (9.2 g, 10% Wt, 1.3 eq, 5.4 mmol) on top of normal silica. The photoisomerization system was equilibrated at a flow of 100 mL/min with the cartridge attached with Et₂O/hexane (300 mL) for 20 min. Methyl benzoate (567 mg, 0.52 mL, 1.0 eq, 4.16 mmol) and **39** (0.83 g, 1.00 eq, 4.16 mmol) in 100 mL hexane/Et₂O (1:1) were added into the 2-neck flask and the system equilibrated for another 20 min while cooling at 0 °C. Afterwards both 55 W UV-lamp were switched on and the photoisomerization conducted under continuous flow (100 mL/min) for 20 h while cooling at 0 °C. Afterwards the system was flushed with Et₂O and the cartridge was purged with air. The AgNO₃ silica was transferred into an Erlenmeyer flask and CH₂Cl₂ and a 25% aqueous solution of NH₄OH was added and the mixture stirred vigorously for 5 min. The mixture was filtered over Celite, filter cake was washed with 25ml NH₄OH (1x) and 3 times with 50ml

125

DCM (10% MeOH). The layers were separated, extracted 2x with 100 ml DCM and the combined organic layers were dried over Na₂SO₄, filtered, and evaporated. The crude brown oil was purified via column chromatography on silica gel (ethyl acetate in cyclohexane: 10-40%). Fractions of product were combined and evaporated in vacuo to yield **40** (0.12 g, 595
5 μmol, 14%) as white solid.

Step e) – Synthesis of (E)-2-(1-hydroxy-cyclooct-3-en-1-yl)acetic acid (41**):**

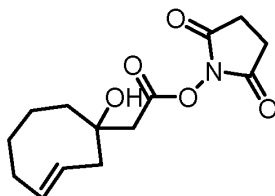
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**41**

To a solution of **40** (54.6 mg, 1.0 eq, 0.28 mmol) in methanol (2.0 mL) was added 1 M NaOH_{aq} (54.6 mg, 10.0 eq, 2.8 mL, 2.8 mmol) and the reaction mixture was left stirring at
15 room temperature for 2 h. Afterwards 1 M HCl solution was added until the mixture became acidic. The organic layer was extracted with ethyl acetate, dried over Na₂SO₄ and concentrated under reduced pressure to yield **41** (20.8 mg, 0.11 mmol, 41%) as colorless oil.

Step f) – Synthesis of 2,5-dioxopyrrolidin-1-yl (E)-2-(1-hydroxycyclooct-3-en-1-yl)acetate (42**):**

25

**42**

To a solution of **41** (20.8 mg, 1.0 eq, 0.11 mmol) and 2-(2,5-dioxopyrrolidin-1-yl)-1,1,3,3-tetramethylisouronium tetrafluoroborate (40.8 mg, 1.2 eq, 0.14 mmol) in DMF (1.0 mL) was
30 added DIPEA (18.2 mg, 1.3 eq, 24.3 μL, 0.14 mmol) and the reaction mixture was left stirring at room temperature for 3.5 h. The reaction mixture was diluted with ethyl acetate and washed with brine. The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The crude was purified by column chromatography on silica gel (ethyl acetate in cyclohexane: 5-100%). Fractions of product were combined, concentrated and
35 triturated with diethyl ether. The product was dried in vacuo to yield **42** (29.2 mg, 0.11 mmol,

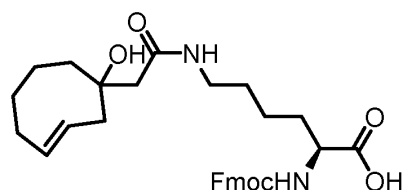
126

92%) as white powder.

Step g) – Synthesis of N2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N6-(2-((E)-1-hydroxycyclooct-3-en-1-yl)acetyl)-L-lysine (43):

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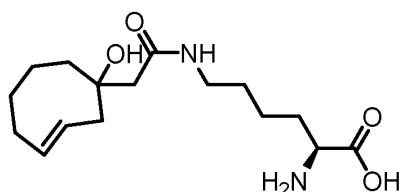
**43**

To a solution of **42** (29.2 mg, 1.0 eq, 0.10 μ mol) in DMF (3.0 mL) was added (((9H-fluoren-9-yl)methoxy)carbonyl)-L-lysine (45.9 mg, 1.2 eq, 125 μ mol) and triethylamine (12.6 mg, 1.2 eq, 17.4 μ L, 125 μ mol) and the reaction mixture was stirred at room temperature overnight. Afterwards 1 M HCl solution was added until the mixture became acidic. The organic layer was extracted with ethyl acetate, dried over Na₂SO₄ and concentrated under reduced pressure. The crude mixture was purified via RP flash chromatography (MeCN in water: 10-100%). Fractions were combined and evaporated in vacuo to yield **43** (12.1 mg, 22.6 μ mol, 22%) as colorless oil.

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Step h) – Synthesis of N6-(2-((E)-1-hydroxycyclooct-3-en-1-yl)acetyl)-L-lysine (44):

25

**44**

To a solution of **43** (12.0 mg, 1.0 eq, 22.4 μ mol) in DMF (1.0 mL) was added piperidine (23.9 mg, 12.5 eq, 27.7 μ L, 0.28 mmol) and the reaction mixture was stirred for 20 min, then diluted with water and filtered. The filtrate was evaporated in vacuo. The crude product was dissolved in water and purified via RP flash chromatography (MeCN in water: 10-100%). Fractions were combined and evaporated in vacuo to yield **44** (3.50 mg, 11.2 μ mol, 50%) as

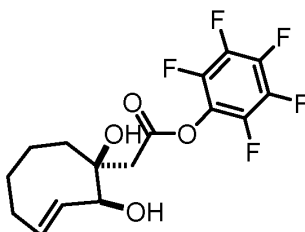
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127

white solid.

EXAMPLE 38 – Synthesis of perfluorophenyl (E)-2-(1,2-dihydroxycyclooct-3-en-1-yl)acetate (45) (active ester):

5



10

45

To a solution of **anti-6** (prepared according to Example 6 above) (197 mg, 1.0 eq, 0.98 mmol) and 2,3,4,5,6-pentafluorophenol (217 mg, 1.2 eq, 1.18 mmol) in DCM (10.0 mL) were added. 3-(((ethylimino)methylene)amino)-N,N-dimethylpropan-1-amine hydrochloride (283 mg, 1.5 eq, 1.5 mmol) and N,N-dimethylpyridin-4-amine (24.0 mg, 0.2 eq, 0.2 mmol) and the reaction mixture stirred for 2 h.

The reaction mixture was quenched with sat. NH_4Cl and extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude mixture was purified via flash chromatography (ethyl acetate in cyclohexane: 2-35%). Fractions were combined and evaporated in vacuo to yield **45** (223 mg, 608 μmol , 62%) as colorless oil.

25 C) Test Results 1

1. Stability Experiments

For assessment of the stability of the ncAA of the present invention under physiological conditions compound **13** (obtained according to Example 13 above) was dissolved into various cell media (LB, DMEM, FreeStyle HEK, FreeStyle CHO) or mouse serum into a final concentration of 2 mmol/L and the solution incubated for 4 d at 37 °C.

LC-MS analysis was conducted via determining the extracted ion count (EIC) of $m/z = 329$, which corresponds to the mass of **13**. No noticeable change of detected species with $m/z = 329$ could be detected (cf. Figure 2), opposed to previously described ncAAs (Reinkemeier et al., Chem. Eur. J. 2021, 27, 6094; cf. Figure 1).

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Therefore, **13** is completely stable for at least 4 days under physiological conditions. This is also confirmed by the DAR analysis of ADCs incorporating **13** (Figure 6).

To confirm that no unreactive species is formed with the same retention time during the course of the experiment, a slight excess of diethyl ((6-(5-aminopyridin-2-yl)-1,2,4,5-tetrazin-3-yl)methyl)phosphonate was added and after 5 min at room temperature the same LC-MS analysis with determination of the EIC of $m/z = 329$ was conducted. For all examined samples the species with $m/z = 329$ disappeared and only the corresponding click-product could be identified (representative data for **13** incubated in DMEM shown in Figure 3).

2. Hydrophilicity experiments

Comparison of clogP-hydrophilicity values of different TCO compounds of the invention shows the improved hydrophilicity of these derivatives (cf. Figure 4). Furthermore, the head groups as well as the final ncAAs are soluble in pure water, opposed to most other reported TCOs.

3. Genetic code expansion experiments

3.1 Expression of Trastuzumab HC C_H2K16¹³-LC

Trastuzumab HC C_H2K16¹³-LC is a Trastuzumab variant carrying integrated in its heavy chain (c_H2 domain) in position K249 the ncAA as described in Example 13, above. The preparation of different Trastuzumab variants, of suitable cloning and expression constructs is described in WO2023/094525 of the present Applicant, which is herewith incorporated by reference.

Freestyle™ 293-F cells (Thermo Fisher scientific, R79007) were cultured in a shaking incubator at 37°C at 8% CO₂ shaking at 120 rpm using Freestyle™ 293 Expression Medium (Thermo Fisher Scientific, 12338026). One day prior transfections, the required volume of cells were split to a density of 0.5×10^6 cells/ml and incubated for another 24 hours in the incubator. At the day of transfection, the cells were harvested at 100 rcf for 10-20 minutes and resuspended in fresh medium to receive a density of 1×10^6 cells/ml. 1 µg DNA/ml expression culture (cf. Figure 8; plasmid map of expression plasmid pCK-HSA-Trastuzumab HC-LC modified by replacing in the heavy chain sequence position K249 the "AAG", the codon for lysine by TAG, the amber codon) was mixed with 40 µl/ml expression culture of medium. PEI Max (Polysciences, 49553-93-7) was added in a 1:4 ratio (DNA: PEI Max).

The mixture was vortexed 3 x 5 seconds, incubated for 15 minutes in the hood and added dropwise to the cells. Compound **13** was dissolved in water, sterile filtered and added to the cells to gain a final concentration of 500 µM in the expression culture.

5 3.2 Purification of Trastuzumab HC C_H2K16¹³-LC

The expression culture was harvested at 4500 rpm for 30 minutes and the supernatant was filtered through a 0.45 µm filter. The volume of the cleared supernatant was measured and 10 x Buffer A (0.2M Na₂PO₄, 1.5 M NaCl, pH 7.2) was added to obtain a final concentration of 1 x Buffer A (1/10 of the total volume). This mixture was loaded on HiTrap Fibro™ Prisma column (Cytiva, 17549855) equilibrated in Buffer A (0.02M Na₂PO₄, 0.15 M NaCl, pH 7.2). After washing out unbound material, the antibody was eluted in a linear gradient using Buffer B (0.1 M Sodium citrate, pH 3.2)(Figure 5, A). Fractions were collected throughout the gradient and analysed on SDS-PAGE (Figure 5, B). The antibody containing fractions were pooled, concentrated and buffer exchanged to 1 x PBS using a 30 kDa cut-off amicon filter device (Millipore, UFC903008). Concentration was determined using a DeNovix DS-11 spectrometer.

4. Conjugation and stability experiments

20 4.1 Conjugation of Trastuzumab HC C_H2K16¹³-LC to the drug compound 16

0.243 nmol of Trastuzumab HC C_H2K16¹³-LC were mixed with 973 nmol of compound **16** (cf. Example 16) in a total volume of 36 µl and incubated for 4 hours at RT. The reaction was purified via size exclusion chromatography (Superdex S200 Increase, Cytiva, 28990944) and the peaks was collected in fraction, analysed on SDS-PAGE and the desired fractions concentrated (Figure 5, C).

4.2 DAR analysis of Trastuzumab HC C_H2K16¹³-16-LC

The final ADC was deglycosylated using Immobilized PNGase F (Genovis, G1-PF6-010) following the standard protocol described in the manual of the product. Drug-to-antibody ratio (DAR) was analyzed via reduced reverse phase liquid chromatography (LC). Therefore, the deglycosylated antibody was reduced via incubation with DTT in PBS (50 mmol/L) at 37 °C for 30 min and afterwards subjected to LC analysis using an AdvanceBio RP-mAb C4 column (Agilent) at 80 °C column temperature. DAR is calculated via integrals of conjugated (I_{con}) and unconjugated (I_{uncon}) species:

130

$$DAR = \frac{I_{con}}{I_{con} + I_{uncon}} \times 2$$

The DAR of Trastuzumab HC CH2K16¹³-**16**-LC was determined to be 1.7-1.8 based on the chromatogram shown in Figure 6, A. Comparison to a corresponding DAR analysis of an ADC Trastuzumab HC CH2K16TCO*a-**16**-LC incorporating TCO*A-Lys instead of Compound **13** illustrates the highly increased stability of the latter during expression of the protein. Due to isomerization of TCO*a during expression a major portion of antibody remains unconjugated (Figure 6, B).

4.3 Conjugation of active ester **7** to Trastuzumab

1.5 mg Trastuzumab (0.01 µmol) was dissolved in PBS (1.50 mL) and the pH was adjusted by addition of 0.5 mL 0.1 M NaHCO₃ solution to pH = 8. To the antibody solution was added 10 µL of a 25 mmol/L solution of compound **7** in DMF/H₂O (1:1) and the mixture incubated at RT for 1 h while shaking.

The mixture was diluted to a final volume of 2.5 mL with PBS and purified via PD-10 cartridge (Sephadex G25-M) eluting with PBS. The 2.5 mL flowthrough was discarded and the elution collected in fractions of 0.5 mL. Fractions were examined via SDS-page and fractions containing antibody were pooled and concentrated (fractions 2-8) to yield 1.03 mg of Trastuzumab-**7**.

4.4 Conjugation of Trastuzumab-**7** to Cy5-Tetrazine and determination of degree of labelling (DOL)

0.15 mg of Trastuzumab-**7** (1 nmol) were dissolved in 150 µL PBS and afterwards 2 µL of a 10 mmol/L solution of Cy5-Tetrazine (also designated sulfo-Cy5-Tetraazine, which is a fluorophore derivative with a tetrazine group for TCO-linked based labeling; CAS-Nr.: 1801695-57-7, Click Chemistry Tools) in DMSO/H₂O (20 nmol) were added and the mixture incubated for 20 min at RT.

The mixture was diluted to a final volume of 2.5 mL with PBS and purified via PD-10 cartridge (Sephadex G25-M) eluting with PBS. The 2.5 mL flowthrough was discarded and the elution collected in fractions of 0.5 mL. Visibly blue fractions were pooled and concentrated (fractions 2-7) to isolate Trastuzumab-Cy5.

DOL was assessed via UV/Vis spectrometry of fraction 5 on a DS-11+ spectrophotometer, assuming quantitative conversion. The DOL was determined to be 3.8.

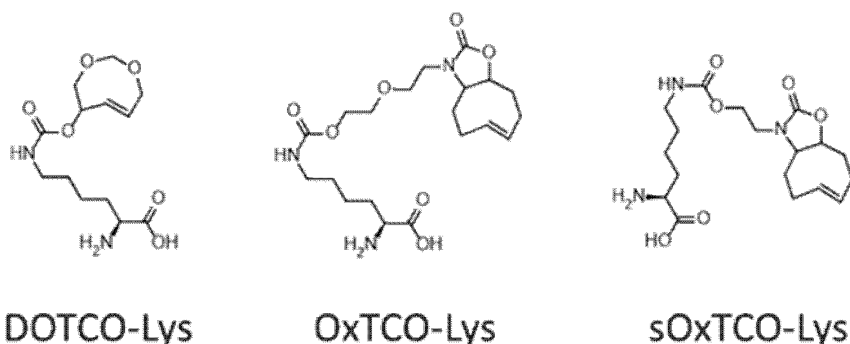
The results are shown in Figure 7.

D) Test Results 2

5 1. Further Stability Experiments

For further assessment of the stability of the ncAAs of the present invention under physiological conditions compound **36** (obtained according to Example 36 above) was dissolved into LB media into a final concentration of 2 mmol/L and the solution incubated at 37 °C.

10 Prior art ncAAs DOTCO-Lys, OxTCO-Lys, sOxTCO-Lys of the following formulae



and comparative ncAA **44** as prepared above were also dissolved into LB media and
15 incubated at 37 °C. For DOTCO-Lys, OxTCO-Lys, sOxTCO-Lys the respective TCO structure as disclosed in the prior art (cf. Kozma, E., et al Chembiochem (2016) 17, No. 16 1518-1524; or WO 2016/025480) was linked to the amino acid lysine for allow a comparison with the ncAAs of the present invention,

LC-MS analysis was conducted via determining the extracted ion count (EIC) of the
20 respective masses of the compounds. The prior TCO-based ncAAs were already significantly instable after only 2 d incubation (DOTCO-Lys = 0% trans, OxTCO-Lys = 55% trans, sOxTCO-Lys = 45% trans remaining) and are completely isomerized after 6d. Comparative ncAA **44** was also completely isomerized after 6 d while ncAA **36** remained >85% trans even after 6 d at 37 °C in LB medium. This highlights the required combination of the geminal 3
25 substitution in combination with a 2-substituent to enable the increased stability of these derivatives.

2. Genetic code expansion experiments and ADC preparation

2.1 Expression of Trastuzumab mutants containing ncAA 13 at different positions

Expression of different Trastuzumab containing ncAA 13 (see Example 13 above) at different positions of heavy or light chain (V_HP41; C_H2K90; V_LK45) was done using genetic code expansion technology in Expi293F suspension cells (Thermo Fisher). Briefly, cells have been transfected with the Trastuzumab expression plasmid comprising an amber STOP codon at the desired position, e.g. in the variable heavy chain (VH), constant heavy chain (CH) or variable light chain (VL). In addition, the plasmid encoding *Methanosarcina mazei* PylRS and tRNA^{Pyl} was used for the transfection to ensure the incorporation of HTCO into the Trastuzumab antibody throughout the expression. After 7 days of expression, Trastuzumab was purified out of the supernatant of the expression culture via Protein A.

2.2 Conjugation of Trastuzumab mutants to payload 16

Said Trastuzumab mutants were mixed with compound 16 (cf. Example 16 above) in PBS and incubated for 4 hours at RT. The reaction was purified via size exclusion chromatography (Superdex S200 Increase, Cytiva, 28990944) and the peaks were collected in fraction, analysed on SDS-PAGE and the desired fractions concentrated.

2.3 Cytotoxicity assay of conjugated Trastuzumab mutants

SKBR-3 (Her2 positive cells) were seeded at a density of 5000 cells/ml in a 96-well plate and the ADC was added in different concentrations. As a control, Kadcyra was used. After 5 days of incubation, CellTiter-Glo® 2.0 Cell Viability Assay (Promega) was added to the cells and the luminescence was measured using a plate reader. The data was analysed using Graph Pad Prism. The results are shown in Figure 9.

The data illustrate the isomerization stability of nCAAs of the invention independent of conjugation position within the Ab.

2.4 Conjugation of Trastuzumab HC V_HP41¹³-LC to different payloads and cytotoxicity assay

Trastuzumab HC V_HP41¹³-LC (conjugated in position V_HP41 with compound 13) was conjugated in a molar ratio of 1:3 to the payloads P1-P4 (see Figure 10). After incubation at 37 °C overnight the ADC was purified via size exclusion chromatography. For the cytotoxicity assay, SKBR-3 (Her2 positive cells) were seeded at a density of 5000 cells/ml in a 96-well plate and the ADC was added in different concentrations. After 5 days of incubation, CellTiter-Glo® 2.0 Cell Viability Assay (Promega) was added to the cells and the

luminescence was measured using a plate reader. The data was analysed using Graph Pad Prism. The results are shown in Figure 11.

2.5 Expression of Trastuzumab HC V_HP41³⁶-LC

5 Expi293-F cells (Thermo Fisher scientific, R79007) were cultured in a shaking incubator at 37°C at 8% CO₂ shaking at 120 rpm using Expi™ 293 Expression Medium (Thermo Fisher Scientific, A1435101). One day prior transfections, the required volume of cells were split to a density of 3-4 x 10⁶ cells/ml and incubated for another 24 hours in the incubator. At the day of transfection, the cells were split 3 x 10⁶ cells/ml. 1 µg DNA/ml
10 expression culture was mixed with 1000 µl/ml expression culture of OptiPro SFM medium. PEI Max (Polysciences, 49553-93-7) was added in a 1:4 ratio (DNA: PEI Max) for transfection. The mixture was vortexed 3 x 5 seconds, incubated for 15 minutes in the hood and added dropwise to the cells. The respective amount of compound **36** to gain a final concentration of 1 mM in the expression culture, was dissolved in H₂O, sterile filtered and
15 added to the cells.

The expression culture was harvested at 4500 rpm for 30 minutes and the supernatant was filtered through a 0.22 µm filter. The volume of the cleared supernatant was measured and 10 x Buffer A (PBS pH 7.4) was added to obtain a final concentration of 1 x Buffer A (1/10 of the total volume). This mixture was loaded on HiTrap Fibro™ Prisma
20 column (Cytiva, 17549855) equilibrated in Buffer A (1xPBS, pH 7.4). After washing out unbound material, the antibody (Trastuzumab HC V_HP41³⁶-LC incorporating compound **36** in position V_HP41) was eluted in a linear gradient using Buffer B (0.1 M Sodium citrate, pH 3.4). Fractions were collected throughout the gradient, neutralized with 10M Tris pH10 and analysed on SDS-PAGE. The antibody containing fractions were pooled, concentrated and
25 buffer exchanged to 1 x PBS using a 30 kDa cut-off amicon filter device (Millipore, UFC903008). Concentration was determined using a DeNovix DS-11 spectrometer.

2.6 Conjugation of Trastuzumab HC V_HP41³⁶-LC to payload **16**

1.713 nmol of Trastuzumab HC V_HP41³⁶-LC were mixed with 5.14 nmol of payload **16**
30 (see Example 16 above) in a total volume of 50 µl and incubated overnight at 37 °C, shaking at 300 rpm. The reaction was purified via size exclusion chromatography (Superdex S200 Increase, Cytiva, 28990944) and the peaks were collected in fractions, analysed on SDS-PAGE and the desired fractions concentrated. DAR was determined to be 1.8.

This further illustrates the isomerization stability of ncAAs of the invention.

35 With reference to SEQ ID NO:1 (Trastuzumab heavy chain) and SEQ ID NO:2 (Trastuzumab light chain) the above term

C_H2K16 refers to position K249 of SEQ ID NO:1

C_H2K90 refers to position K323 of SEQ ID NO:1

V_HP41 refers to position P41 of SEQ ID NO:1 and

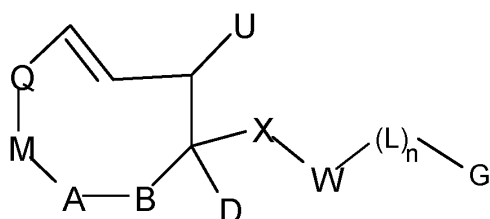
V_LK45 refers to position K45 of SEQ ID NO:2.

5

The disclosure of any document as cited herein is herewith incorporated by reference.

CLAIMS

1. A compound of general formula I



(I)

wherein

n is 0 or an integer from 1 to 20, in particular 1 to 10, more particularly 1 to 5;

A is $-\text{CR}^1\text{R}^2-$, $-\text{O}-$, $-\text{S}-$, $-\text{N}(\text{R}^1)-$, $>\text{CH}-\text{OZ}$, $>\text{CH}-\text{SZ}$, $>\text{CH}-\text{NR}^1\text{R}^2$, $>\text{CH}-\text{OR}^3$, $>\text{CH}-\text{CN}$, $>\text{CH}-\text{NO}_2$, $>\text{CH}-\text{SO}_2\text{R}^4$ or $>\text{CH}-\text{SR}^3$;

B is $-\text{CR}^1\text{R}^2-$, $-\text{O}-$, $-\text{S}-$, $-\text{N}(\text{R}^1)-$, $>\text{CH}-\text{OZ}$, $>\text{CH}-\text{SZ}$, $>\text{CH}-\text{NR}^1\text{R}^2$, $>\text{CH}-\text{OR}^3$, $>\text{CH}-\text{CN}$, $>\text{CH}-\text{NO}_2$, $>\text{CH}-\text{SO}_2\text{R}^4$ or $>\text{CH}-\text{SR}^3$;

M is $-\text{CR}^1\text{R}^2-$, $-\text{O}-$, $-\text{S}-$, $-\text{N}(\text{R}^1)-$, $>\text{CH}-\text{OZ}$, $>\text{CH}-\text{SZ}$, $>\text{CH}-\text{NR}^1\text{R}^2$, $>\text{CH}-\text{OR}^3$, $>\text{CH}-\text{CN}$, $>\text{CH}-\text{NO}_2$, $>\text{CH}-\text{SO}_2\text{R}^4$ or $>\text{CH}-\text{SR}^3$;

Q is $-\text{CR}^1\text{R}^2-$, $>\text{CH}-\text{OZ}$, $>\text{CH}-\text{SZ}$, $>\text{CH}-\text{NR}^1\text{R}^2$, $>\text{CH}-\text{OR}^3$, $>\text{CH}-\text{CN}$, $>\text{CH}-\text{NO}_2$, $>\text{CH}-\text{SO}_2\text{R}^4$ or $>\text{CH}-\text{SR}^3$;

provided that

A is $-\text{CR}^1\text{R}^2-$ if M and B independently of each other represent $-\text{O}-$, $-\text{S}-$ or $-\text{N}(\text{R}^1)-$, or

M and B independently of each other represent $-\text{CR}^1\text{R}^2-$ if A is selected from $-\text{O}-$, $-\text{S}-$ or $-\text{N}(\text{R}^1)-$,

D is $-\text{OR}^3$, $-\text{SR}^3$, $-\text{OZ}$ or $-\text{SZ}$;

U is OZ , $-\text{SZ}$, $-\text{OR}^3$, $-\text{SR}^3$ or $-\text{NR}^5\text{R}^6$

wherein

residues Z independently of each other are same or different protecting groups;

R^1 , R^2 , R^3 , R^4 , R^5 and R^6 independently of each other are same or different and are selected from the group consisting of H and linear or branched lower alkyl groups;

X is a bridging group;

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W is a saturated or unsaturated polar group;

L is a linking group; and

G is a terminal group;

or a salt, like a pharmaceutically acceptable salt, or solvate thereof; each as a stereoisomer or as a mixture of at least two stereoisomers.

2. The compound of claim 1, wherein residues Z are a thiol protecting group or an alcohol protecting group, in particular a cleavable protecting group, and more particularly, independently of one another selected from the group consisting of acetyl (Ac), benzoyl (Bz), benzyl (Bn), β -methoxyethoxymethyl ether (MEM), methoxymethyl ether (MOM), methoxytrityl (MT), p-methoxybenzyl ether (PMB), p-methoxyphenyl ether (PMP), methylthiomethyl ether, pivaloyl (Piv), tetrahydropyranyl (THP), tetrahydrofuranyl (THF), trityl (triphenylmethyl, Tr), silyl ether, methyl ether, and ethoxyethyl ether (EE) residues.

and/or

wherein X is selected from:

- a) linear or branched lower-alkylene, in particular $-(CH_2)_{n1}-$, wherein $n1$ is an integer from 1 to 4, in particular methylene or ethylene;
- b) $-O-$, $-S-$, $>CH-OZ$, $>CH-SZ$, $>CH-OR^3$ or $>CH-SR^3$;
 wherein
 Z and R^3 are as defined above;
- c) C_1 - C_4 -alkylene, particularly C_1 - C_3 -alkylene, more particularly methylene or ethylene;
- d) linear or branched mono- or polyalkylene oxide moieties, particularly selected from linear the moieties $-((CH_2)_x-O)_y-$, $-(O-(CH_2)_x)_y-$ and $-(O-(CH_2)_x)_y-O-$, and the branched or mono- or polyunsaturated analogues thereof;
 wherein
 x independently of each other represent an integer selected from 1, 2, 3 or 4; particularly 1 or 2; and
 y independently of each other represent an integer from 1 to 20, particularly 1 to 15, 1 to 10 or 1 to 4; or

and/or

wherein said polar group W is selected from:

- a) $-C(O)-$, $-O-C(O)-$, $-C(O)-O-$;

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b) $-\text{O}-[\text{C}(\text{O})-\text{[CH}_2\text{]}_{x_1}\text{C}(\text{O})-; -\text{O}-[\text{C}(\text{S})-\text{[CH}_2\text{]}_{y_1}\text{C}(\text{O})-$

wherein

x_1 , x_2 , y_1 , and y_2 independently of each other represent an integer selected from 1 to 6, particularly 1 to 4, and more particularly 1 to 2;

5 c) $-\text{S}-\text{C}(\text{S})-, -\text{O}-\text{C}(\text{S})-, -\text{C}(\text{S})-, -\text{C}(\text{S})-\text{O}-, -\text{C}(\text{S})-\text{S}-; -\text{NR}^7-\text{C}(\text{O})-, -\text{C}(\text{O})-\text{NR}^7-, -\text{C}(\text{NR}^7), -\text{NR}^7-\text{C}(\text{NR}^7)-, -\text{NR}^7-\text{C}(\text{O})\text{NR}^7-, -\text{NR}^7-\text{C}(\text{NR}^7)-; \text{ or}$

d) $-\text{NR}^8-[\text{C}(\text{O})-\text{[CH}_2\text{]}_{x_1}\text{C}(\text{O})-; \text{NR}^8-[\text{C}(\text{S})-\text{[CH}_2\text{]}_{y_1}\text{C}(\text{O})-$

wherein

10 x_1 , x_2 , y_1 , and y_2 independently of each other represent an integer other selected from 1 to 6, particularly 1 to 4, and more particularly 1 to 2, and

R^7 and R^8 independently of each other represent H or lower alkyl, particularly H or C_1 - C_4 -alkyl.

15 and/or

wherein each L is independently selected from

a) linear or branched alkylene groups, linear or branched alkenylene groups, cycloalkylene groups, cycloalkenylene groups, or analogues thereof containing one or more heteroatoms in their carbon backbone; in particular linear or branched C_1 - C_{10} -alkylene groups, linear or branched C_2 - C_{10} -alkenylene groups, C_3 - C_8 -cycloalkylene groups, C_3 - C_8 -cycloalkenylene groups, or analogues thereof containing one or more heteroatoms in their carbon backbone; more particularly linear or branched C_1 - C_4 -alkylene groups, linear or branched C_2 - C_4 -alkenylene groups, C_3 - C_6 -cycloalkylene groups, C_3 - C_6 -cycloalkenylene groups, or analogues thereof containing one or more heteroatoms in their carbon backbone; most particularly methylene, ethylene and propylene; (Moiety La)

b) linear or branched mono- or polyalkylene oxide moieties, particularly selected from the linear moieties $-\text{((CH}_2\text{)}_{x_3}-\text{O)}_{y_3}-$, $-\text{(O(CH}_2\text{)}_{x_3}\text{)}_{y_3}-$, $-\text{(O-(CH}_2\text{)}_{x_3}\text{)}_{y_3}-\text{O}-$, $-\text{((CH}_2\text{)}_{x_3}-\text{O)}_{y_3}-\text{(CH}_2\text{)}_{n_3}\text{C(O)-}$, $-\text{(O(CH}_2\text{)}_{x_3}\text{)}_{y_3}-\text{NR}^7-$, $-\text{(O-(CH}_2\text{)}_{x_3}\text{)}_{y_3}-\text{C(O)-}$, and the branched analogues thereof;

wherein

x_3 independently of each other represent an integer selected from 1, 2, 3 or 4; particularly 1 or 2;

35 y_3 independently of each other represent an integer from 1 to 20, particularly 1 to 15, 1 to 10 or 1, 2, 3 and 4;

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n_3 is an integer from , 2 and 3; and

$R^{7'}$ represent H or lower alkyl, particularly H or C₁- C₄-alkyl, more particularly methyl or ethyl;

(Moiety Lb)

5

provided that, when two or more identical or different linker moieties La and/or Lb are present, such elements may be directly linked to each via a chemical bond or indirectly via identical or different coupling moieties selected from -C(O)-, -O-C(O)-, -C(O)-O-, -S-C(S)-, -O-C(S)-, -C(S)-, -C(S)-O-, -C(S)-S-, -NR⁷-C(O)-, -C(O)-NR⁷-, -C(NR⁷)-, -NR⁷-C(NR⁷)-, -S(O)-, -S(O)₂-, -P(O)OR⁷-, -O-P(O)OR⁷-, -P(O)OR⁷-O-; wherein R⁷ is as defined above.

10

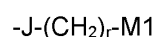
and/or

15

wherein G has one of the following meanings:

- i. G is H or R⁷; and if $n \neq 0$ then G may additionally be -OR⁷, -C(O)OR⁷, -NR⁷₂ or -C(O)NR⁷₂; wherein R⁷ is as defined above;
- ii. a leaving group E, selected from halogen, pentafluorophenyl (Pfp), tetrafluorophenyl (Tfp), oxazolone, succinimidyl (Su), sulfosuccinimidyl, trifluoroacetyl, azido, para-nitrophenyl (PNP), and nitro-containing aromatic groups;
- iii. a thiol reactive moiety of the general formula

20



25

wherein

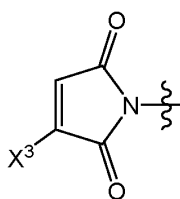
J is selected from -NH-, -S- or -O- or is missing;

r is an integer from 1 to 4 or is 0 when J is missing; and

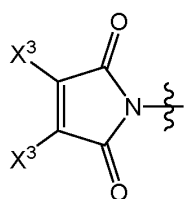
M1 is the thiol reactive portion, and more particularly selected from M2 to M16:

30

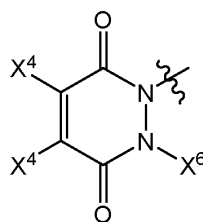
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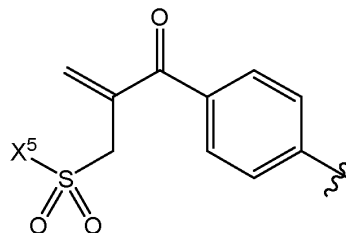
(M2)



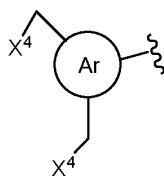
(M3)



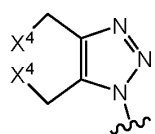
(M4)



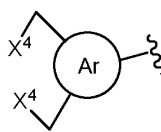
(M5)



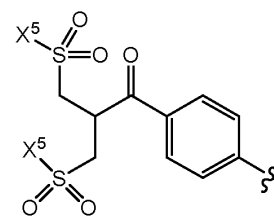
(M6)



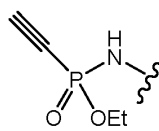
(M7)



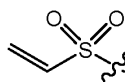
(M8)



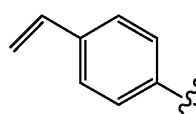
(M9)



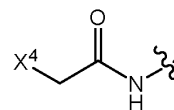
(M10)



(M11)

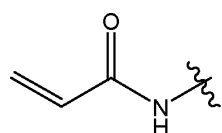


(M12)

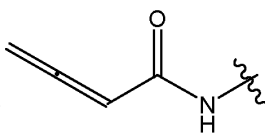


(M13)

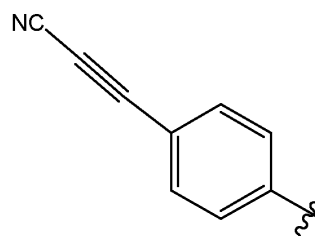
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(M14)



(M15)



(M16)

wherein:

X³ is H, halogen, PhS, MeS;

10

X⁴ is halogen, PhS, MeS;X⁶ is H or C₁-C₁₂ alkyl, preferably H or C₁₋₆ alkyl;X⁵ is H, C₁-C₁₂ alkyl, C₆-C₁₂ aryl, C₇-C₁₂ alkaryl or C₇-C₁₂ aralkyl, preferably H or para- methylphenyl; and

wherein the aromatic ring of (M6) and (M8) may optionally be a heteroaromatic ring, such as a phenyl or pyridine ring;

15

- iv. $-\text{NH}-(\text{CH}_2)_2\text{C}\equiv\text{CH}$, $-\text{NH}-(\text{CH}_2)_2\text{N}_3$, $-\text{O-Aryl}$ or $-\text{HN-Aryl}$, H , $-\text{OH}$, $-\text{NH}_2$ halo-
 gen, R^9 , $-\text{CH}=\text{C}(\text{R}^9)_2$, $-\text{C}\equiv\text{C R}^9$, $-\text{[C}(\text{R}^9)_2\text{C}(\text{R}^9)_2\text{O]}_q-$ R^9 , $-\text{CN}$, $-\text{N}_3$, $-\text{NCL}$, $-\text{LCN}$,
 5 $-\text{L R}^9$, $-\text{N}(\text{R}^9)_2$, $-\text{N}(\text{R}^9)_3$, $-\text{C}(\text{L})\text{N}(\text{R}^9)_2$, $\text{C}(\text{R}^9)_2\text{L R}^9$, $-\text{C}(\text{L})\text{R}^9$, $-\text{C}(\text{L})\text{L R}^9$, $-\text{S}(\text{O})\text{R}^9$,
 $-\text{S}(\text{O})_2\text{R}^9$, $-\text{S}(\text{O})\text{OR}^9$, $-\text{S}(\text{O})_2\text{OR}^9$, $-\text{S}(\text{O})\text{N}(\text{R}^9)_2$, $-\text{S}(\text{O})_2\text{N}(\text{R}^9)_2$, $-\text{OS}(\text{O})\text{R}^9$,
 $-\text{OS}(\text{O})_2\text{R}^9$, $-\text{OS}(\text{O})\text{OR}^9$, $-\text{OS}(\text{O})_2\text{OR}^9$, $-\text{P}(\text{O})(\text{R}^9)(\text{OR}^9)$, $-\text{P}(\text{O})(\text{OR}^9)_2$, $-\text{OP}(\text{O})(\text{OR}^9)_2$,
 $-\text{Si}(\text{R}^9)_3$, $-\text{LC}(\text{L})\text{R}^9$, $-\text{LC}(\text{L})\text{LR}^9$, $-\text{LC}(\text{L})\text{N}(\text{R}^9)_2$, $-\text{N}(\text{R}^9)\text{C}(\text{L})\text{R}^9$, $-\text{N}(\text{R}^9)\text{C}(\text{L})\text{LR}^9$ and $-\text{N}(\text{R}^9)\text{C}(\text{L})\text{N}(\text{R}^9)_2$,

wherein

10

q is in the range of 1 to 200, in particular 1 to 20, more particularly 1 to 10 and especially 1 to 5,

L is oxygen or sulphur and

15

R^9 is independently selected from the group consisting of hydrogen, halogen, C_1 - C_{24} alkyl groups, like C_1 - C_{10} alkyl or C_1 - C_3 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;

- v. $-\text{OZ}$, $-\text{SZ}$, $-\text{OR}^1$, $-\text{C}(\text{O})\text{OR}^1$, $-\text{N}(\text{R}^1)_2$, $-\text{C}(\text{O})\text{N}(\text{R}^1)_2$, $-\text{SR}^1$, or $-\text{R}^1$,

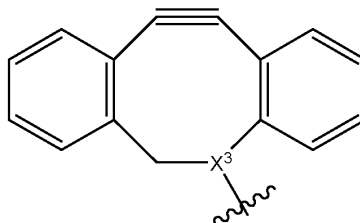
wherein Z and R^1 are as defined above; and

20

- vi. cyclic and acyclic strain promoted alkyne-azide cycloaddition (SPAAC)-reactive groups, in particular,

i. alkynyl-terminated groups

ii. DBCO-derived residues of the general formula



(M17)

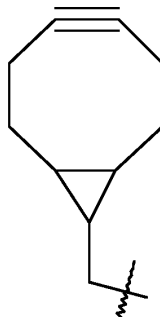
25

wherein

X^3 is N , $>\text{CH}-$ or $>\text{CH}-\text{O}-$; or

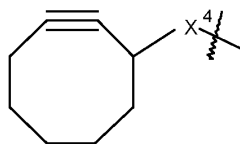
iii. BCN derived residues of the formula

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(M18)

iv. cyclooctyne derived residues, in particular SCO derived residues of the general formula



(M19)

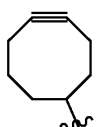
wherein

X^4 is $-O-$ or $-O-(CO)-$;

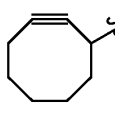
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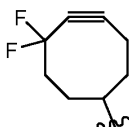
v. or residues of the formulae M20 to M38



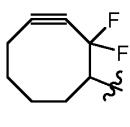
(M20)



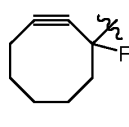
(M21)



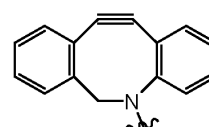
(M22)



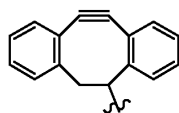
(M23)



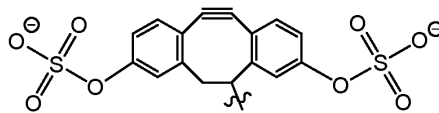
(M24)



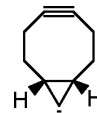
(M25)



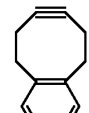
(M26)



(M27)



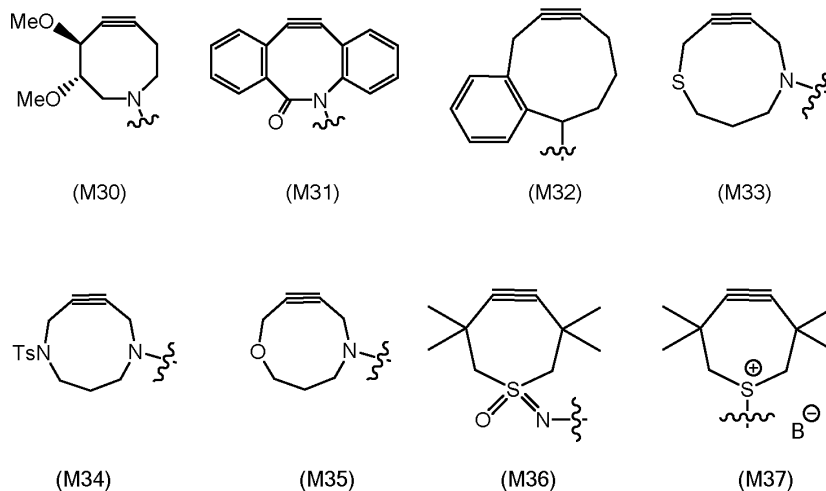
(M28)



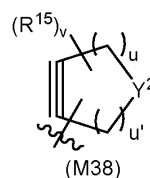
(M29)

15

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wherein

5 B⁻ is an anion or

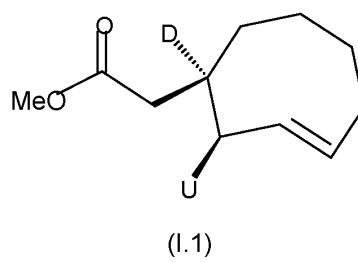
wherein:

10 R¹⁵ is independently selected from the group consisting of hydrogen, halogen, -OR¹⁶, -NO₂, -CN, -S(O)₂R¹⁶, -S(O)₃R¹⁶, C₁ - C₂₄ alkyl groups, C₆ - C₂₄ (hetero)aryl groups, C₇ - C₂₄ alkyl(hetero)aryl groups and C₇ - C₂₄ (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 R¹⁵ may be linked together to form an optionally substituted annulated cycloalkyl or an optionally substituted annulated (hetero)arene substituent, and wherein R¹⁶ is independently selected from the group consisting of hydrogen, halogen, C₁ - C₂₄ alkyl groups, C₆ - C₂₄ (hetero)aryl groups, C₇ - C₂₄ alkyl(hetero)aryl groups and C₇ - C₂₄ (hetero)arylalkyl groups;

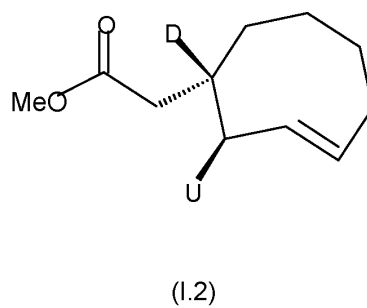
20 Y² is C(R¹⁵)₂, O, S or NR¹⁵;
 u is 0, 1, 2, 3, 4 or 5;
 u' is 0, 1, 2, 3, 4 or 5, wherein u + u' = 4, 5, 6, 7 or 8; and
 v is an integer in the range 8 - 16;

3. The compound of anyone of the preceding claims, wherein the compound is selected from anyone of the formulae I.1 to I.12

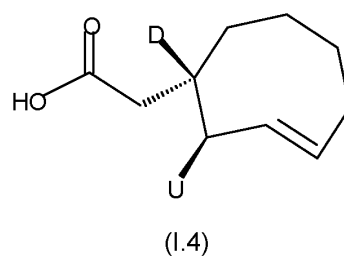
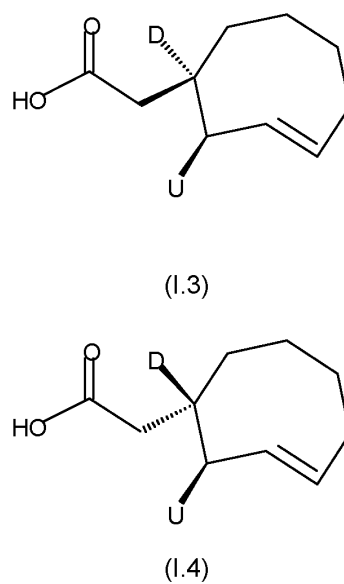
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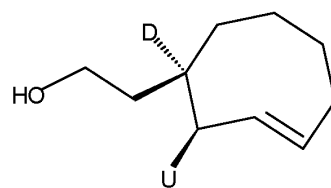
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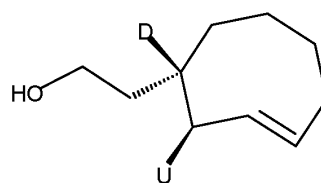
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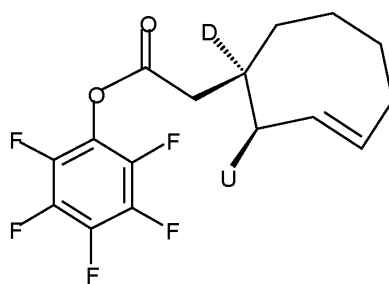


(I.5)

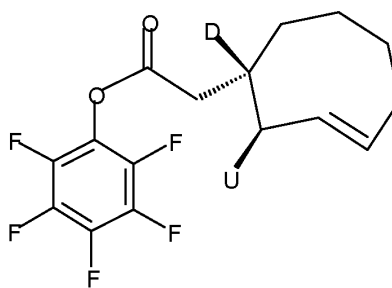


(I.6)

5



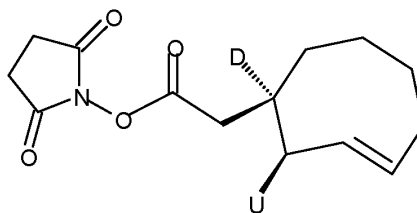
(I.7)



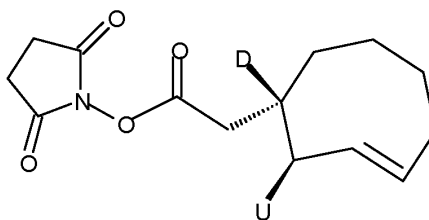
(I.8)

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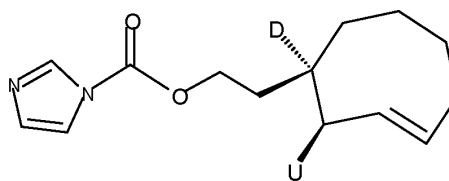
145



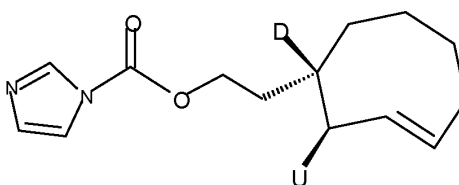
(I.9)



(I.10)



(I.11)

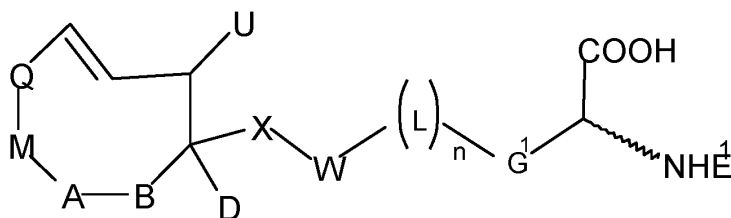


(I.12)

wherein, D and U are as defined above; or a salt, like a pharmaceutically acceptable salt or a solvate thereof; each as a stereoisomer or as a mixture of at least two stereoisomers, wherein, in particular,

- a) D is -OH and U is -OAc or
- b) D is -OH and U is -OH.

4. A compound of general formula II



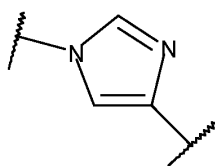
(II)

wherein

n, A, B, M, Q, D, U, X, W, L are as defined above for compounds of general formula I

E¹ is H or an amine protecting group; and

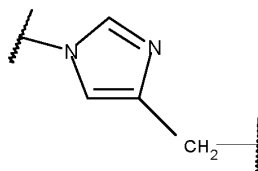
- 10 G¹ is selected from -NH-, -NH-C(=NH)NH-, -S-, -O-, -O-Aryl-, -NH-(CH₂)_m-O-Aryl-, wherein m is an integer from 1 to 10; -HN-Aryl- or a moiety of the formula



or

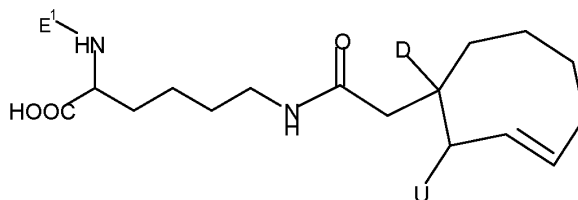
- 15 is selected from any side chain residue of a natural or non-natural amino acid which allows conjugation to moiety W, optionally via moiety (L)_n of said compound of formula II, and in particular selected from -S-CH₂-, -O-CH₂-, -O-p-(phenylene)-CH₂-, -O-CH(CH₃)-, -O-(C=O)-CH₂-, -; -O-(C=O)-CH₂-CH₂-, -N-(C=O)-CH₂-, -; -N-(C=O)-CH₂-CH₂-, -N-(CH₂)₄-, -N-(CH₂)₅-, or

20

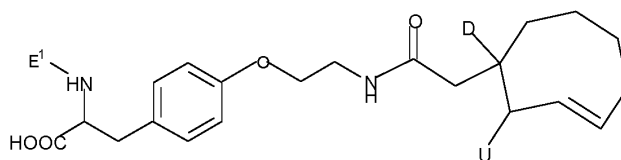


or a salt, like a pharmaceutically acceptable salt, or a solvate thereof; each as a stereoisomer or as a mixture of at least two stereoisomers.

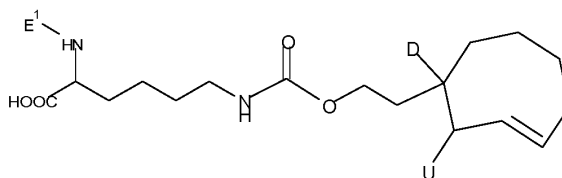
5. The compound claim 4, wherein the compound is selected from anyone of the formulae II.1 to II.4



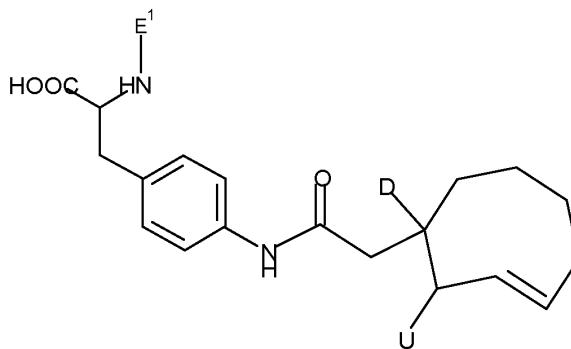
(II.1)



(II.2)



(II.3)



(II.4)

wherein

E¹ is H or an amine protecting group as defined above;

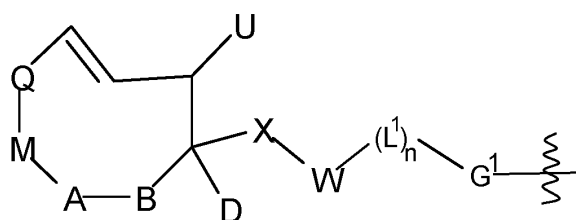
or a salt, like a pharmaceutically acceptable salt or a solvate thereof; each as a stereoisomer or as a mixture of at least two stereoisomers.; and wherein, in particular

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- a) D is —OH and U is —OAc or
 b) D is —OH and U is —OH.

- 5 6. A hydrophilic trans-cyclooctene (hyTCO)-functionalized construct obtainable by, in particular covalently, binding a compound according to anyone of claims 1 to 5 to a targeting agent (TA), or to a payload molecule (PM), wherein said functionalized construct contains at least one functionalizing residue (FR) comprising a mono-unsaturated trans-cyclooctene entity of the general formula (II')

10



(II')

wherein

n, A, B, D, U, M, Q, X, and W are as defined herein above;

15

G¹ is as defined above or is missing; and

L¹ has the meaning of linker moiety L as defined above or represents a branching moiety.

7. The functionalized construct of claim 6, which is
 20 a functionalized targeting agent (TA), wherein the targeting agent is selected from viruses, whole cells, phages, liposomes, biomolecules and low- or high-molecular weight chemical compounds, such as in particular immunoglobulins, like antibodies, antibody derivatives, antibody fragments, fusion molecules comprising at least one antibody or antibody fragment, enzymes, proteins, peptides, peptidomimetics (peptoids), carbohydrates, monosaccharides, polysaccharides, oligo- or polynucleotides, in particular DNA, RNA, PNA and LNA molecules, aptamers, drugs, glycoproteins, glycans, lipids, polymers, chemotherapeutic agents, receptor agonists and receptor antagonists, cytokines, hormones, steroids, toxins and derivatives thereof.:

30

or

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which is a functionalized payload molecule (PM), wherein the payload molecule is selected from bio-active compounds, labeling agents, protein degraders, in particular payloads applicable in proteolysis targeting chimeras (PROTACs), photosensitizers, and chelators.

5

8. The functionalized construct of anyone of the claims 6 and 7, wherein the functionalizing residue (FR) of formula II' is attached, in particular directly or via a cleavable or non-cleavable moiety, to the targeting agent (TA) or payload molecule (PM), in particular to an amino acid residue of said TA or PM, thereby forming a functionalized TA of the general formula (XX.1)

10

$$(FR)-(TA)$$

$$(XX.1)$$

15

wherein

TA is a targeting moiety as defined herein above; and

FR is a functionalizing residue of above formula II'

or a functionalized PM of the general formula (XX.2)

20

$$(FR)-[(Y^1)_a-(Y^2)_b-PM]_c$$

$$(XX.2)$$

wherein

25

a is 0 or represents an integer selected from 1 and 2,

b is 0 or represents an integer selected from 1 and 2,

c represents an integer selected of at least 1, particularly from 1 and 2,

30

Y¹ represents a cleavable moiety,

Y² represents a self-immolative moiety,

PM is a payload molecule as defined herein above and

FR is a functionalizing residue of above formula II',

wherein, when

35

c represents an integer of more than 1, then L¹ represents a branching moiety, n is 1 and G1 is missing.

150

9. The functionalized construct of claim 8, which is a functionalized TA of formula XX.1,

(FR)-(TA)

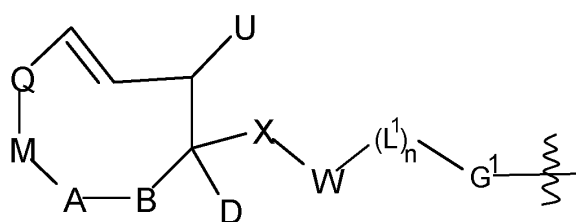
5

(XX.1)

wherein

TA is a targeting agent as defined herein above;

FR is a functionalizing residue of formula II'



10

(II')

wherein

A, B, M and Q are $-CR^1R^2-$, wherein R^1 and R^2 are as defined herein above;

15

D and U are $-OR^3$, wherein R^3 is as defined above; and

n , L^1 , X and W are as defined above;

and

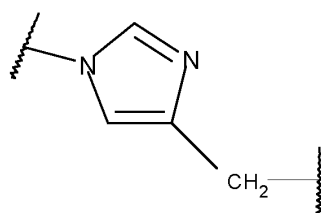
G^1 is defined above, and in particular is selected from any side chain residue of a natural or non-natural amino acid which allows conjugation to moiety W, optionally via moiety $(L^1)_n$ of said compound of formula II, and in particular selected from $-S-CH_2-$, $-O-CH_2-$, $-O-p(\text{phenylene})-CH_2-$, $-O-CH(CH_3)-$, $-O-(C=O)-CH_2-$, $-O-(C=O)-CH_2-CH_2-$, $-N-(C=O)-CH_2-$, $-N-(C=O)-CH_2-CH_2-$, $-N-(CH_2)_4-$, $-N-(CH_2)_5-$,

20

25

or

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or

which is a functionalized TA of the formula (XX)

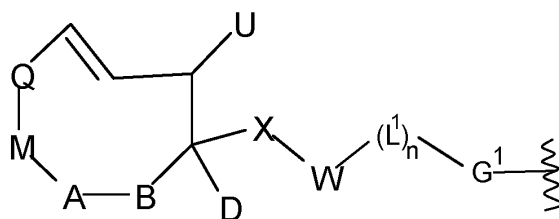
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(FR)-(TA)

(XX.1)

wherein

10 TA is a targeting moiety as defined herein above
 FR is a functionalizing residue of formula

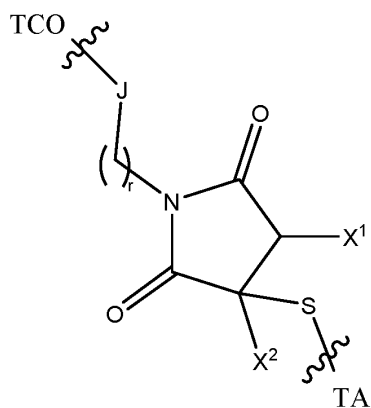


(II')

wherein

15 L^1 and n are as defined above,
 A , B , M and Q are $-CR^1R^2-$,
 D and U are $-OR^3$,
 X and W are as defined herein above;
 G^1 is missing or represents a moiety as formed by a con-
 20 ventional coupling reaction, in particular click reaction, like
 in particular

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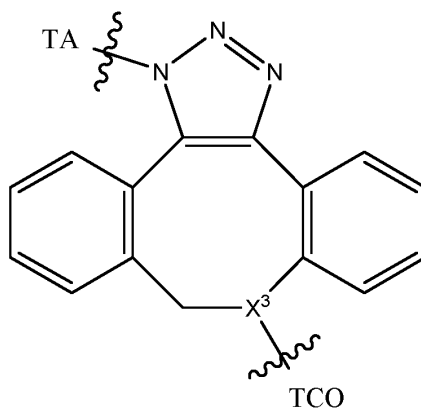


wherein

J is selected from -NH-, -S- or -O- or is missing;

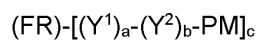
5

or



or

10 which is a functionalized PM of formula XX.2,



(XX.2)

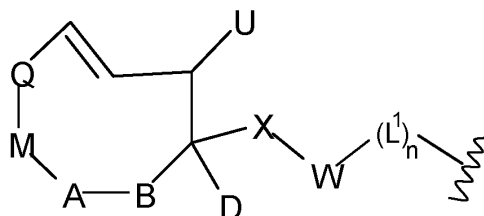
wherein

15

- a is 0 or represents an integer selected from 1 and 2
- b is 0 or represents an integer selected from 1 and 2
- c represents an integer selected of at least 1, particularly from 1 and 2,

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- Y¹ represents a cleavable moiety as defined herein above; and
 Y² represents a self-immolative moiety as defined herein above
 PM is a payload moiety as defined herein above
 FR is a functionalizing residue of formula II'



(I')

wherein

A, B, M and Q are $-CR^1R^2-$, wherein R^1 and R^2 are as defined above;

D and U are $-OR^3$, wherein R^3 is as defined above; and

n , L^1 , X and W are as defined above;

with the proviso,

that when c represents an integer of more than 1, then L^1 represents a branching moiety and n is 1.

10. A conjugate prepared by covalently linking a first functionalized molecule, selected from the hydrophilic trans-cyclooctene (hyTCO)-functionalized construct of anyone of the claims 6 to 9 with a second functionalized molecule comprising a docking group (DG) capable of reacting with the trans-cyclooctene-type functional group of the first molecule; in particular obtainable via biorthogonal bioconjugation via a Diels-Alder-type cycloaddition reaction of said two functionalized molecules; more particularly, wherein said docking group (DG) is selected from an optionally substituted triazinyl or optionally substituted tetrazinyl group, capable of covalently reacting in a copper-free strain promoted inverse-electron-demand Diels-Alder cycloaddition (SPIEDAC) with said trans-cyclooctene group.
11. The conjugate of claim 10, wherein said hyTCO-functionalized construct is selected from a functionalized targeting agent TA of the general formula (XX.1)

(FR)-(TA)

(XX.1)

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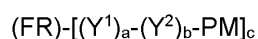
wherein

FR is a functionalizing residue of formula II' and

TA is a targeting moiety as defined herein above;

5

or a functionalized PM of the general formula (XX.2)



10

(XX.2)

wherein

a is 0 or represents an integer selected from 1 and 2,

b is 0 or represents an integer selected from 1 and 2,

c represents an integer selected of at least 1, particularly from 1 and 2,

15

Y¹ represents a cleavable moiety,Y² represents a self-immolative moiety,

FR is a functionalizing residue of formula II' wherein, when

c represents an integer of more than 1, then L1 represents a

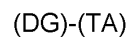
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branching moiety, n is 1 and G1 is missing;

and

PM is a payload molecule as defined herein above.

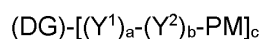
12. The conjugate of anyone of the claims 10 and 11, wherein the second functional-
 25 ized molecule comprising a docking group (DG) is selected from
 a functionalized targeting agent TA of the general formula (XX.3)



30

(XX.3)

or a functionalized payload molecule PM of the general formula (XX.4)



35

(XX.4)

wherein

a, b and c, as well as Y¹, and Y², are as defined above;

TA is a targeting moiety as defined herein above and

PM is a payload molecule as defined herein above, and

5 DG is a docking group as defined above.

13. The conjugate of anyone of claims 10 to 12, wherein said docking group (DG) group is a diene comprising group selected from tetrazines or triazines capable of covalently reacting in a copper-free strain promoted inverse-electron-demand Diels-Alder cycloaddition (SPIEDAC) with said trans cyclooctene group of above formula II'.
14. The conjugate of anyone of claims 10 to 13, wherein said TA is an immunoglobulin molecule, in particular monoclonal antibody, or a derivative or antigen binding fragment thereof, carrying at least one non-canonical amino acid residue (ncAA) in a polypeptide chain, wherein said ncAA carries a hyTCO-type side chain as defined in anyone of the claims 1 to 5.
15. A pharmaceutical composition comprising in a pharmaceutically acceptable carrier at least one conjugate as defined in anyone of the claim 10 to 14, or a diagnostic composition comprising in a diagnostically applicable carrier at least one conjugate as defined in anyone of the claim 10 to 14.
16. A conjugate as defined in anyone of the claims 10 to 14 for use in medicine, as in particular in diagnosis and therapy.
17. The conjugate as defined in anyone of the claims 10 to 14, which is an ADC for use in the diagnosis or treatment of cancers, in particular breast cancer, gastric cancer or other Her2 overexpressing tumors, as for example tumors of ovary, endometrium, bladder, lung, colon, and head and neck
18. A diagnostic or analytical kit comprising at least one hyTCO-type compound construct or conjugate as defined in anyone of the claims 1 to 14.