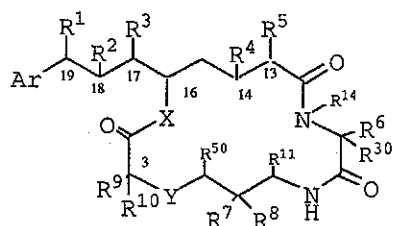


NOVEL CONJUGATES, PREPARATION THEREOF, AND THERAPEUTIC USE THEREOF

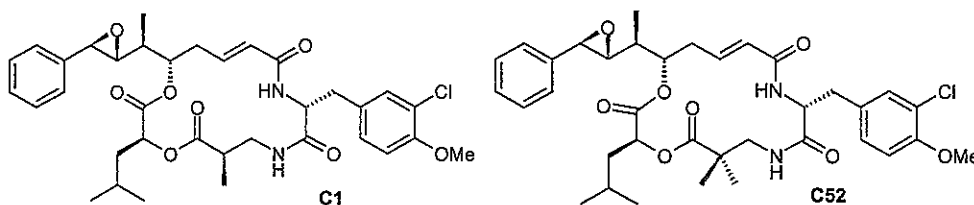
- 5 The present invention relates to cryptophycin conjugates, to compositions containing them and to their therapeutic use, especially as anticancer agents. The invention also relates to the process for preparing these compounds and to the cryptophycin derivatives themselves.

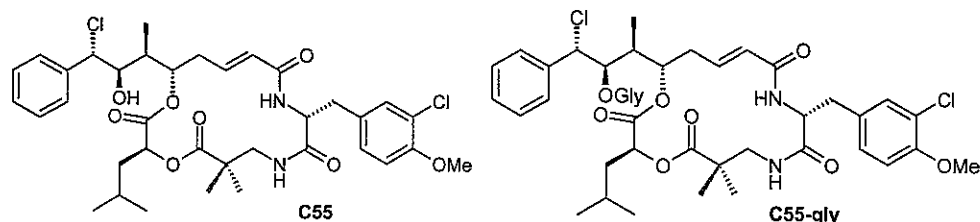
[Technical Field]

- 10 Cryptophycins are secondary metabolites belonging to the class of depsipeptide macrocycles produced by cyanobacteria of the genus *Nostoc*. Their name refers to the fact that they are highly cytotoxic towards yeasts of the genus *Cryptococcus*. The first representative of this class of molecules, cryptophycin-1 (**C-1**), was isolated in 1990 from cyanobacterium *Nostoc sp* (ATCC 53789) (see Eißler S., et al., *Synthesis* **2006**, 22, 3747-3789). The structure, the general formula and the numbering of the carbon atoms of these compounds, as described in **WO 98/08505**, are recalled below:



- Cryptophycins **C-1** and **C-52**, which are characterized by an epoxide function represented below, have anticancer properties. Phase II clinical trials in lung cancer were conducted with **C-52** (LY 355073): see Edelman M.J., et al., *Lung Cancer* **2003**, 39, 197-199; Sessa C., et al., *Eur.J.Cancer* **2002**, 38, 2388-96. Cryptophycin **C-55**, a prodrug of **C-52**, is itself characterized by a chlorohydrin function instead of the epoxide function (Bionpally R.R., et al., *Cancer Chemother Pharmacol* **2003**, 52, 25-33). **C-55** proved to be very active, but is not stable in solution. Derivatives of chlorohydrin glycinate type such as the compound **C-55 Gly** have also been described as gaining in stability (Liang J., et al., *Investigational New Drugs* **2005**, 23, 213-224).





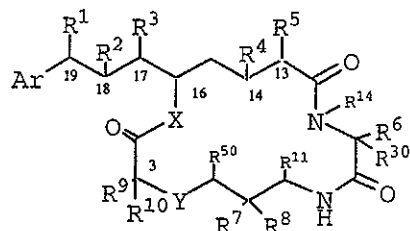
[Technical Problem]

Conjugate chemistry has been known for many years and has been applied to several families of cytotoxic agents, for instance the maytansinoids (WO 04/103 272), taxanes (WO 06/061 258), tomaymycins (WO 09/016 516), leptomycins (WO 07/144 709), CC-1065 and analogues thereof (WO 2007/102 069); see also, with regard to conjugates, Monneret C., et al., *Bulletin du Cancer*, 2000, 87(11), 829-38; Ricart A.D., et al., *Nature Clinical Practice Oncology* 2007, 4, 245-255. However, it has not been applied to cryptophycin derivatives conjugated to antibodies or to other targeting agents.

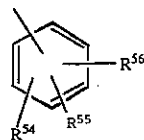
The technical problem that the present invention intends to solve is that of proposing novel conjugates based on cryptophycin derivatives, and also novel cryptophycin derivatives that are suitable for being conjugated.

[Prior Art]

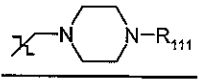
EP 0 830 136 and WO 98/08505 describe cryptophycin derivatives but do not describe cryptophycin conjugates. WO 98/08505 describes cryptophycin derivatives of formula (A):



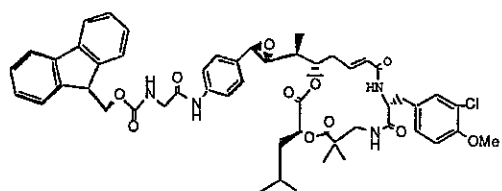
(A) in which Ar may represent a group Ar' of



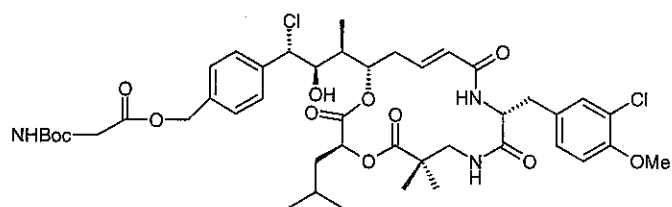
formula: in which R₅₄ represents H, a group (C₁-C₆)alkyl, (C₁-C₆)alkyl(R₅₇, R₅₇', R₅₇''), aryl, phenyl, heterocycloalkyl, a halogen atom, COOR₅₇, PO₃H, SO₃H, SO₂R₅₈, NR₅₉R₆₀,

NHOR₆₁, NHOR₆₁', CN, NO₂, OR₆₂, CH₂OR₆₂', CH₂NR₉₆R₉₆', (C₁-C₆)alkylOR₁₀₀, , SR₆₃; R₅₅ and R₅₆ represent H, a group (C₁-C₆)alkyl, C(R₅₇, R₅₇', R₅₇''), aryl, phenyl, heterocycloalkyl, a halogen atom, COOR₅₇, PO₃H, SO₃H, SO₂R₅₈, NR₅₉R₆₀, NHOR₆₁, NHCHR₆₁', CN, NO₂, OR₆₂, CH₂OR₆₂', CH₂OCOR₉₅, CH₂NR₉₆R₉₆', (C₁-C₆)alkylOR₁₀₀, (C₁-C₆)alkylNR₅₉R₆₀.

WO 98/08505 especially describes the following compounds:



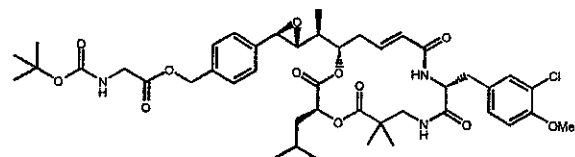
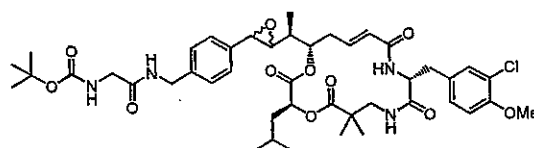
(Ex. 34)



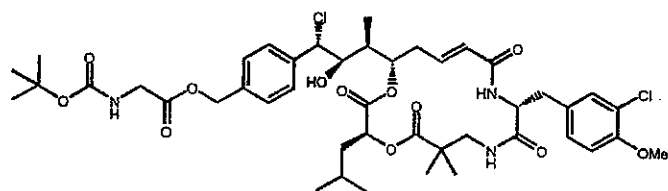
(compound 24, scheme 4)

5

(compound 33, scheme 5)

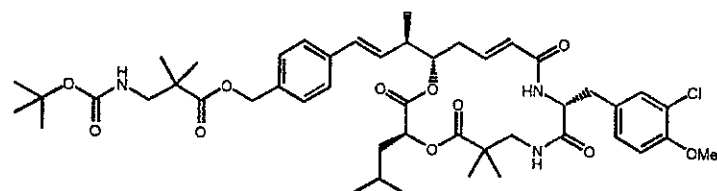


(Ex. 42)

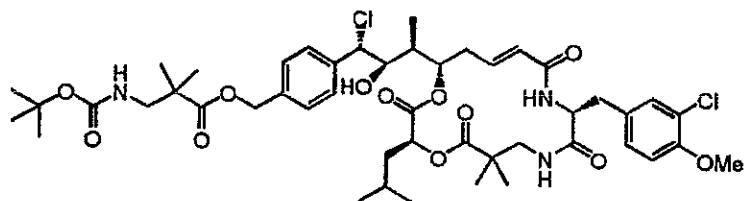


(Ex. 43)

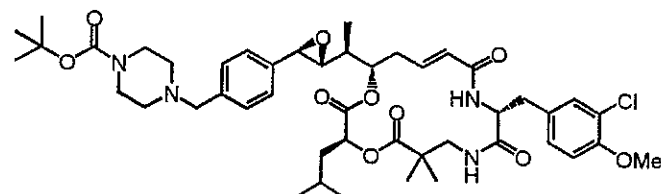
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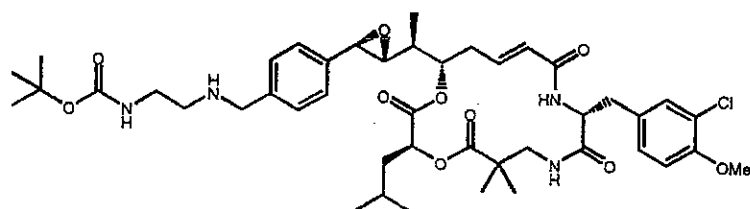
(Ex. 47)



(Ex. 48)



(Ex. 74)



(Ex. 77)

which are characterized by the end group -NHC(=O)Ot-Bu . **WO 98/08505** does not specify that these compounds are suitable for or intended to be conjugated.

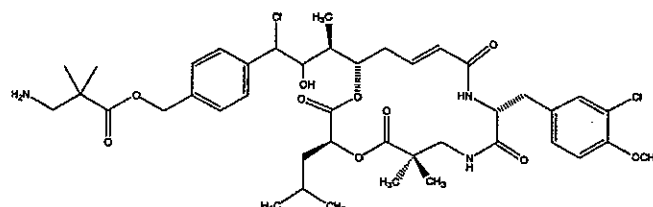
- 5 **US 2007/0 213 511** describes calicheamicin immunoconjugates. Among these, mention is made of **MYLOTARG®** (or CMA-676), which is a calicheamicin immunoconjugate used in the treatment of AML (anti-CD33-calicheamicin). See also in respect of conjugates: **WO 2006/042 240**.

- 10 Al-awar R.S., et al., *J.Med.Chem.* **2003**, 46(14), 2985-3007 and Al-awar R.S., et al., *Mol.Cancer Ther.* **2004**, 3(9), 1061-1067 describe cryptophycin derivatives, and also their *in vivo* evaluations.

- 15 **WO 08/010 101** describes an anti-EphA2 monoclonal antibody and also the corresponding conjugates comprising one or more molecules of a cytotoxic compound attached to the monoclonal antibody. **WO 08/047 242** describes an anti-CD38 monoclonal antibody and also the corresponding conjugates comprising one or more molecules of a cytotoxic compound attached to the monoclonal antibody. The cytotoxic compound may be chosen from maytansinoids, taxanes, tomaymycins, leptomycins and **CC-1065** and analogues thereof.

- 20 **WO 2009/126 934** describes anti-CD70 antibodies and their conjugates with cytotoxic compounds; cryptophycin is mentioned among the cytotoxic agents. **WO 2009/134 976** describes conjugates with an optimized degree of substitution for delivering the required amount of cytotoxic agent into the cell; cryptophycin is mentioned among the cytotoxic agents.

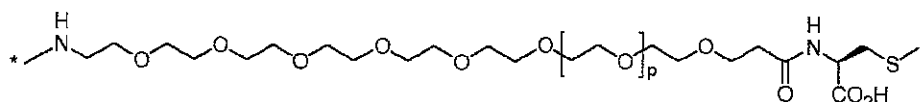
- 25 **WO 2005/116 255** describes conjugates of aptamers and of a cytotoxic agent that may be a cryptophycin (see [0037] and Table 2), the linker possibly comprising a PEG chain ([0038]). More particularly, the cryptophycin Cryp-NH₂ is described:



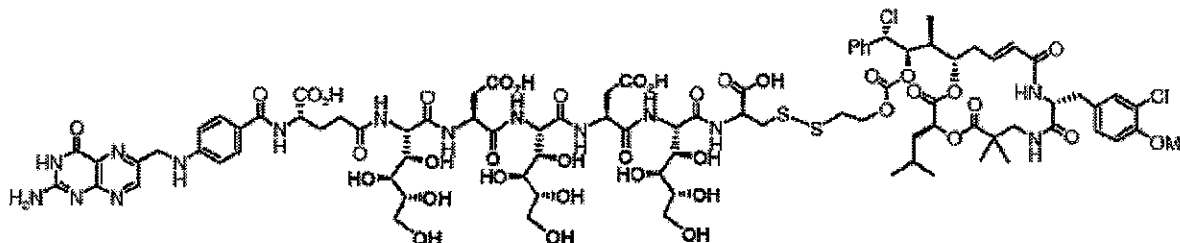
- 30 and also its conjugates of aptamers with the following linkers: NHS-PEG-erythritol, pNP-PEG-erythritol, NHS-PEG-octaPEG, pNP-PEG-octaPEG and PEG-comb (Tables 3 and 4). The nature of the sequence on the phenyl ring ($\text{-CH}_2\text{O-C(=O)-CMe}_2\text{-CH}_2\text{NH-...}$) is different from that which is envisaged in the present invention. **WO 2006/096 754** also describes conjugates of aptamers and

of a cytotoxic agent that may also be a cryptophycin.

WO 2009/002 993 describes cytotoxic conjugates of formula B-L-A comprising hydrophilic linkers, for example the linker of formula:



The cytotoxic agent may be a cryptophycin (page 46), but the point of attachment of the linker is not specified. An example of a conjugate is EC0262:



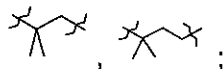
whose linker and point of attachment are different from those envisaged in the present invention.

[Definitions]

The following definitions apply:

- conjugate: a cell binding agent to which is covalently attached at least one molecule of a cytotoxic compound;
- cell binding agent: a molecule with affinity for a biological target: it may be, for example, a ligand, a protein, an antibody, more particularly a monoclonal antibody, a protein or antibody fragment, a peptide, an oligonucleotide or an oligosaccharide. The function of the binding agent is to direct the biologically active compound as a cytotoxic agent towards the biological target. Preferably, the binding agent is not an aptamer;
- biological target: an antigen (or group of antigens) preferably located at the surface of cancer cells or stromal cells associated with this tumour; these antigens may be, for example, a growth factor receptor, an oncogene product or mutated "tumour suppressant" gene product, an angiogenesis-related molecule, an adhesion molecule;
- linker: a group of atoms that can covalently attach a cytotoxic compound to the binding agent;
- alkyl group: a saturated aliphatic hydrocarbon-based group obtained by removing a hydrogen atom from an alkane. The alkyl group may be linear or branched. Examples that may be mentioned include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert-butyl, pentyl, 2,2-dimethylpropyl and hexyl groups;
- cycloalkyl group: a cyclic alkyl group comprising between 3 and 8 carbon atoms engaged in the cyclic structure. Examples that may be mentioned include cyclopropyl, cyclobutyl, cyclopentyl and cyclohexyl groups;
- heterocycloalkyl group: a cycloalkyl group comprising at least one heteroatom (O, S or N)

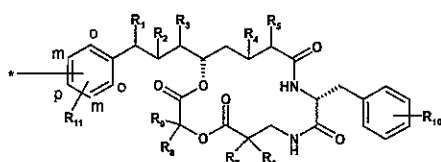
engaged in the ring and connected to the carbon atoms forming the ring;

- alkoxy group: a group -O-alkyl, in which the alkyl group is as defined above;
- alkanoyloxy group: a group -O-CO-alkyl, in which the alkyl group is as defined above;
- alkylene group: a saturated divalent group of empirical formula $-C_nH_{2n}-$, obtained by removing two hydrogen atoms from an alkane. Examples that may be mentioned include methylene ($-CH_2-$), ethylene ($-CH_2CH_2-$), propylene ($-CH_2CH_2CH_2-$), butylene ($-CH_2CH_2CH_2CH_2-$) and hexylene ($-CH_2CH_2CH_2CH_2CH_2CH_2-$) groups or the following branched groups  ;

- preferably, the alkylene group is of the formula $-(CH_2)_n-$, n representing an integer;
- in the ranges of values, the limits are included (e.g. a range of the type "n ranging from 1 to 6" or "between 1 and 6" includes limits 1 and 6).

Abbreviations used

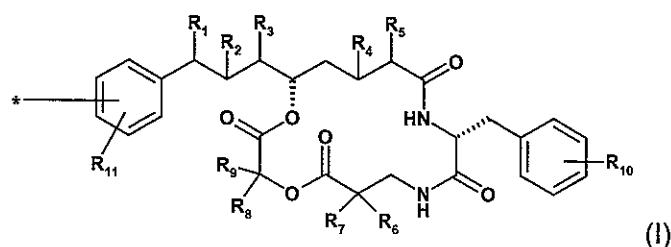
EtOAc: ethyl acetate; ALK: (C_1-C_{12}) alkylene group, more particularly (C_1-C_6) alkylene, more particularly of the form $-(CH_2)_n-$, n being an integer from 1 to 12 and preferably from 1 to 6; aq.: aqueous; TLC: thin-layer chromatography; MSC: methanesulfonyl chloride; crypto denotes the



unit of formula  , crypto especially denoting one of the D₁-D₈ cryptophycin derivatives described later or a cryptophycin derivative of an example; R_f: retention factor; DAR: degree of substitution (drug-antibody ratio); DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC: N,N'-dicyclohexylcarbodiimide; DCM: dichloromethane; DEAD: diethyl azodicarboxylate; DIC: N,N'-diisopropylcarbodiimide; DIPEA: N,N-diisopropylethylamine; DMA: dimethylacetamide; DMAP: 4-dimethylaminopyridine; DME: dimethoxyethane; DMF: dimethylformamide; DMSO: dimethyl sulfoxide; EEDQ: 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; EDCI: N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide; EDTA: ethylenediaminetetraacetic acid; eq.: equivalent; Fmoc: fluorenylmethoxycarbonyl; HOBt: 1-hydroxybenzotriazole; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mCPBA: *m*-chloroperbenzoic acid; NHS: N-hydroxysuccinimide; NMP: N-methylpyrrolidinone; AP: atmospheric pressure; PABAC: "*para*-aminobenzyl alcohol carbonate"; RP: reduced pressure; SEC: steric exclusion chromatography; SPE: solid-phase extraction; RT: room temperature; TBDMS: *tert*-butyldimethylsilyl; TCEP: tris(2-carboxyethyl)phosphine hydrochloride; TEA: triethylamine; TFA: trifluoroacetic acid; TIPS: triisopropylsilyl; THF: tetrahydrofuran; t_R: retention time.

[Detailed Description of the Invention]

The invention relates to a binding agent to which is attached at least one cryptophycin derivative of formula (I):



in which:

- **R₁** represents a halogen atom and **R₂** represents a group -OH, an acyl group derived from an amino acid **AA** or a group (C₁-C₄)alkanoyloxy;

5 or alternatively **R₁** and **R₂** form an epoxide unit;

- **AA** denotes a natural or unnatural amino acid;

- **R₃** represents a group (C₁-C₆)alkyl;

- **R₄** and **R₅** both represent H or together form a double bond CH=CH between C13 and C14;

- **R₆** and **R₇** represent, independently of each other, H or a group (C₁-C₆)alkyl;

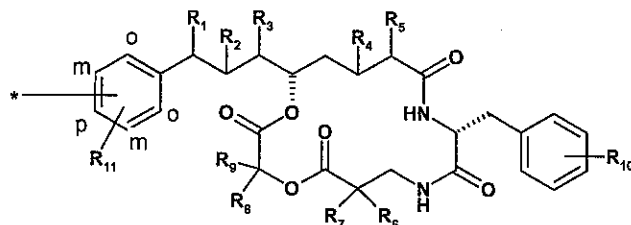
10 ▪ **R₈** and **R₉** represent, independently of each other, H or a group (C₁-C₆)alkyl;

- **R₁₀** represents at least one substituent of the phenyl nucleus chosen from: H, a group -OH, (C₁-C₄)alkoxy, a halogen atom or a group -NH₂, -NH(C₁-C₆)alkyl or -N(C₁-C₆)alkyl₂;

- **R₁₁** represents at least one substituent of the phenyl nucleus chosen from H and a group (C₁-C₄)alkyl;

15 the binding agent and the cryptophycin derivative being covalently attached, the attachment being in the ortho (o), meta (m) or para (p) position of the phenyl nucleus bearing the unit CR₁.

ortho (o), meta (m) or para (p) positions:



20 **R₁** represents a halogen atom, more particularly Cl, **R₃** represents a group (C₁-C₆)alkyl, more particularly Me, **R₆** and **R₇** represent, independently of each other, H or a group (C₁-C₆)alkyl; more particularly, they represent, independently of each other, H or a group Me, **R₈** and **R₉** represent, independently of each other, H or a group (C₁-C₆)alkyl; more particularly, **R₈** represents H and **R₉** represents isobutyl.

25

R₁₀ represents at least one substituent of the phenyl nucleus chosen from H, a group OH, (C₁-C₄)alkoxy or a halogen atom. It may also be a group -NH₂, -NH(C₁-C₆)alkyl or -N(C₁-C₆)alkyl₂ such as, for example, -NH₂ or -NMe₂, preferably in position 3 or 4 on the phenyl nucleus. More particularly, the phenyl nucleus comprises two substituents in positions 3 and 4 on the phenyl nucleus. Preferably, it is 3-Cl and 4-methoxy. **R₁₁** represents at least one substituent of the

30

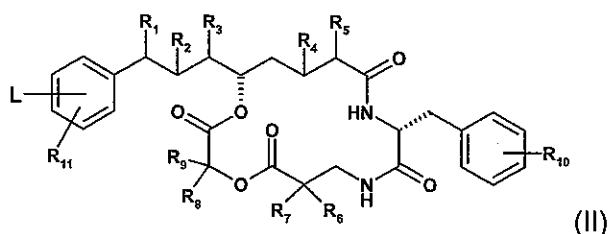
phenyl nucleus chosen from H and a group (C₁-C₄)alkyl; more particularly H.

More particularly, one of the substituents **R**₁ to **R**₁₁ may be chosen from those described in the examples. Each substituent **R**₁ to **R**₁₁ may also adopt one of the spatial configurations (e.g. R or S or alternatively Z or E) as described in the examples.

AA represents a natural or unnatural amino acid. It may be an α, β or γ amino acid. Mention may be made especially of the following amino acids: alanine (Ala), β-alanine, 2-amino-2-cyclohexylacetic acid, 2-amino-2-phenylacetic acid, arginine (Arg), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), valine (Val), γ-aminobutyric acid, α,α-dimethyl-γ-aminobutyric acid, β,β-dimethyl-γ-aminobutyric acid, ornithine (Orn), citrulline (Cit) and also the protected forms of the said amino acids (e.g. acetyl, formyl, tosyl, nitro). Preferably, it is a natural amino acid. More particularly, it is glycine.

R₁ and **R**₂ may also together form an epoxide unit.

The attachment between the cryptophycin derivative and the binding agent is produced by means of a linker **L** positioned in an ortho (o), meta (m) or para (p) position of the phenyl nucleus bearing the unit CR₁; thus, the cryptophycin derivative capable of conjugation has the formula (II):

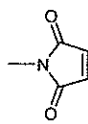


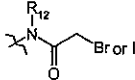
The attachment to the binding agent takes place at the other end of the linker **L** on a reactive group present on the binding agent. Thus, **L** comprises at least one reactive chemical group (RCG1) that is reactive towards a reactive chemical group (RCG2) present on the binding agent. The reaction between RCG1 and RCG2 ensures the attachment of the compound of formula (II) to the binding agent by formation of a covalent bond. Thus, the cryptophycin derivative of formula (II) is able to be conjugated to a binding agent.

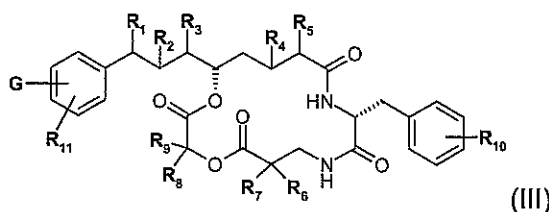
The cryptophycin derivatives of the present invention, including those that are illustrated, may exist in the form of bases or of acid-addition salts, especially of pharmaceutically acceptable acids.

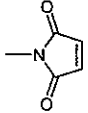
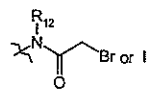
Examples of RCG1 that may be mentioned include:

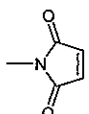
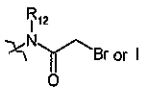
- (i) the reactive group $-SZ_a$ for which Z_a represents H or the group $-SR_a$ and R_a represents a group (C_1-C_6) alkyl, (C_3-C_7) cycloalkyl, aryl, heteroaryl or (C_3-C_7) heterocycloalkyl;
- (ii) the reactive group $-C(=O)-Z_bR_b$ for which Z_b represents a single bond, $-O-$ or $-NH-$, more particularly $-O-$, and R_b represents H or a group (C_1-C_6) alkyl, (C_3-C_7) cycloalkyl, aryl, heteroaryl or (C_3-C_7) heterocycloalkyl;

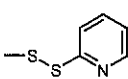
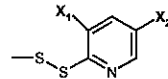
- (iii) one of the following reactive groups: $-Cl$, $-N_3$, $-OH$, $-NH_2$, the maleimido  or

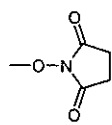
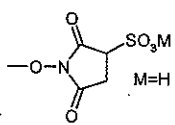
haloacetamido  reactive group with R_{12} representing H or a group (C_1-C_6) alkyl, more particularly Me in the case of the compounds of formula (III):

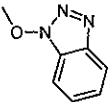
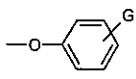
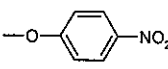


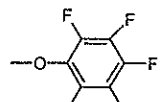
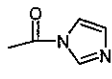
- 10 comprising the group $G = -(CH_2)_nY$ with $Y = -Cl$, $-N_3$, $-OH$, $-NH_2$,  or  with R_{12} representing H or a group (C_1-C_6) alkyl, more particularly Me;

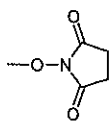
- (iv) the reactive group maleimido  or haloacetamido  with R_{12} representing H or a group (C_1-C_6) alkyl, more particularly Me.

- 15 More particularly, $-SZ_a$ may represent $-SH$ or $-SS(C_1-C_6)$ alkyl, especially $-SSMe$ or $-SS$ -heteroaryl, especially  or  (X_1 and X_2 being defined hereinbelow). More

particularly, $-Z_bR_b$ may represent $-OH$, $-OCH_3$, $-OCH_2CH=CH_2$,  or  $M=H$ or cation or

 or the group  in which Gl represents at least one electroinductive group such as $-NO_2$ or $-Hal$, especially $-F$. They may be, for example, the following groups: 

- 20 or  . Another type of group $-C(=O)Z_bR_b$ is the following:  . The reactive groups

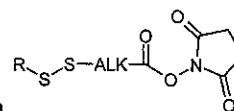


–SH and show good reactivity. More particularly, RCG1 may be chosen from one of those described in the examples.

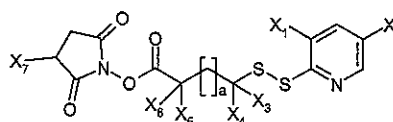
Examples of RCG2 that may be mentioned include ϵ -amino groups of lysines borne by the side chains of the lysine residues that are present at the surface of an antibody, the saccharide groups of the hinge region or the thiols of cysteines by reducing intra-chain disulfide bonds (Garnett M.C., et al., *Advanced Drug Delivery Reviews* **2001**, 53, 171-216). More recently, other approaches have been considered, for instance the introduction of cysteines by mutation (Junutula J.R., et al., *Nature Biotechnology* **2008**, 26, 925-932; **WO 09/026 274**) or the introduction of unnatural amino acids allowing other types of chemistry (de Graaf A.J., et al., *Bioconjugate Chem.* **2009**, Publication Date (Web): February 3, 2009 (Review); DOI: 10.1021/bc800294a; **WO 2006/069 246** and according to Chin J.W., et al., *JACS* **2002**, 124, 9026-9027 (technology ReCode®)). These modes of attachment used with antibodies are applicable to all of the known binding agents as a function of their structure.

It is also possible to chemically modify the binding agent so as to introduce novel reactive chemical groups RCG2. Thus, it is well known to those skilled in the art how to modify an antibody with the aid of a modifying agent (see especially **WO 2005/077 090** page 14). The modification makes it possible to improve the conjugation reaction and to use a wider variety of groups RCG1.

Modifying agents for introducing disulfide groups

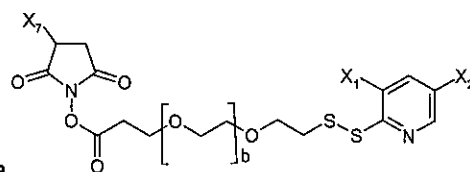


The modifying agent may be an activated ester NHS of formula in which R represents a group (C₁-C₆)alkyl, aryl, heteroaryl, (C₃-C₇)cycloalkyl, (C₃-C₇)heterocycloalkyl and ALK represents a group (C₁-C₆)alkylene; for example, it is possible to use N-succinimidyl pyridyldithiopropionate (SPDP) or N-succinimidyl pyridyldithiobutyrates (SPDB or the N-hydroxysuccinimidyl ester of 4-(2-pyridyldithio)butanoic acid) so as to introduce dithiopyridyl reactive groups RCG2 (see Bourdon M.A., et al., *Biochem. J.* **1978**, 173, 723-737 ; **US 5 208 020**) which may then react with a reactive chemical group RCG1 of the type –SH present on the linker of the cryptophycin derivative so as to form a novel –S-S– bond (see Ex. 9 for a conjugate with a disulfide bond). The N-hydroxysuccinimide group preferentially reacts on the amino groups present on the antibody so as to form amide bonds. Another example of a modifying agent is

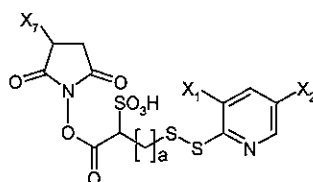


described in **WO 2004/016 801** of formula

or a pegylated



analogue of formula

described in **WO 2009/134 976** or a

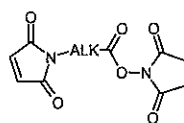
sulfonic analogue of formula

described in **WO 2009/134 977** in which:

- X_3, X_4, X_5 and X_6 represent H or a (C_1-C_6) alkyl group,
- X_1 and X_2 represent -H, $-CONX_8X_9$, $-NO_2$, $-X_8$ and $-X_9$ representing H or a group (C_1-C_6) alkyl,
- X_7 represents $-SO_3^-M^+$ or H, or alternatively a quaternary ammonium group,
- a denotes an integer from 0 to 4 and b denotes an integer ranging from 0 to 2000 and preferably between 1 and 200; a and b may take all the values between, respectively, 0 and 4 or between 0 and 2000.

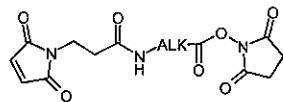
10 Modifying agents for introducing maleimido groups

Another example of a modifying agent is succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), a similar compound described in **EP 0 306 943** or a sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate). Other examples that may

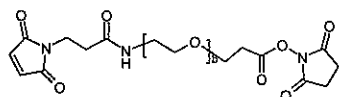


be mentioned include:

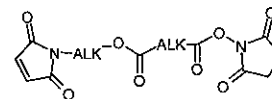
such as N-succinimidyl 3-maleimidopropanoate;



15 such as N-succinimidyl 6-(3-maleimidopropionamido)hexanoate;

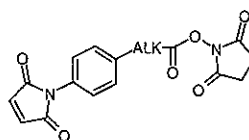


b being an integer between 0 and 2000 and preferably between 1 and 200 (b may take all the values between 0 and 2000), such as N-succinimidyl 3-(2-{2-[3-

maleimidopropionylamino]ethoxy}ethoxy)propanoate or SM(PEG)₂;

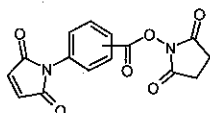
such

as maleimidoethyl N-succinimidyl succinate;



such as N-succinimidyl 4-(4-

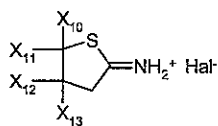
maleimidophenyl)butanoate, or



such as N-succinimidyl 3-maleimidobenzoate.

Modifying agents for introducing thiol groups

Another example of a modifying agent described in **WO 90/06774** is of the formula



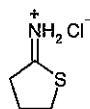
in which:

- Hal represents a halogen atom;
- 5 - X_{10} represents a halogen atom or a group COOX_{14} , nitro, unsubstituted or halogenated ($\text{C}_1\text{-C}_8$)alkyl, unsubstituted or halogenated ($\text{C}_1\text{-C}_8$)alkoxy, unsubstituted or halogenated ($\text{C}_2\text{-C}_8$)alkenyl, unsubstituted or halogenated ($\text{C}_2\text{-C}_8$)alkynyl, unsubstituted ($\text{C}_3\text{-C}_8$)cycloalkyl, aryl that is unsubstituted or substituted with one to three substituents selected from amino, halogen atom, unsubstituted or halogenated ($\text{C}_1\text{-C}_8$)alkyl group, unsubstituted or halogenated ($\text{C}_1\text{-C}_8$)alkoxy;
- 10 - each of the X_{11} , X_{12} and X_{13} independently represents a hydrogen atom or may represent X_3 ; or X_{10} and X_{11} together form a ring ($\text{C}_2\text{-C}_5$)alkylene, which is unsubstituted or substituted with one to five groups ($\text{C}_1\text{-C}_4$)alkyl; or X_{10} and X_{11} form, together with X_{12} , a ring ($\text{C}_1\text{-C}_5$)alkylene, which is unsubstituted or substituted with one to five groups ($\text{C}_1\text{-C}_4$) alkyl;
- 15 and X_{14} is -H or a group ($\text{C}_1\text{-C}_8$)alkyl.

Preferably, Hal represents a chlorine or bromine atom. The table below presents possibilities for $X_{10}\text{-}X_{13}$:

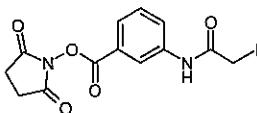
X_{10}	X_{11}	X_{12}	X_{13}	Hal
Me	H	H	H	Cl
Ph	H	H	H	Cl
t-Bu	H	H	H	Cl
Me	Me	H	H	Cl
$(\text{-CH}_2(\text{CH}_2)_3\text{CH}_2\text{-})$		H	H	Cl
H	$(\text{-CH}_2(\text{CH}_2)_3\text{CH}_2\text{-})$		H	Cl
Et	H	H	H	Br
Et	Me	H	H	Cl
$\text{-CH-CH}_2\text{-CH}$	H	H	H	Cl
Me	H	Me	H	Cl
H	H	Me	Me	Cl
Ph	Me	H	H	Cl
4-ClPh	H	H	H	Cl
3-furanyl	H	H	H	Cl
i-Pr	H	H	H	Cl
Me	Me	Me	Me	Cl
C_6H_{11}	H	H	H	Cl
CH_2Br	H	H	H	Cl
CF_3	H	H	H	Cl
CH=CH_2	H	H	H	Cl
2- NH_2Ph	H	H	H	Cl

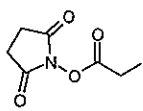
An example of a preferred iminothiolane is the following:

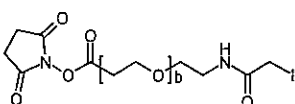


Modifying agents for introducing haloacetamido groups

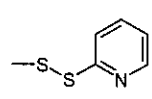
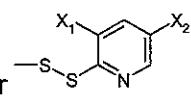
Another example of a modifying agent is succinimidyl-4-(N-iodoacetyl)aminobenzoate

5 (SIAB) , or similar compounds, among which are succinimidyl-N-iodoacetate

(SIA) , succinimidyl-N-bromoacetate (SBA), or succinimidyl-3-(N-bromoacetamido)propionate (SBAP) or a similar pegylated compound described in

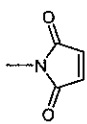
 **WO 2009/134 976**, b being as described previously. Figures 1 and 2 illustrate the modification of an amino group of a binding agent with SPDP or with the preferred iminothiolane above.

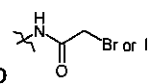
Thus, it is possible to introduce onto the binding agent disulfide groups RCG2 (–SSR), especially

of pyridyldisulfide type  or , in the case where RCG1 represents –SH. Similarly, it is possible to introduce onto the binding agent thiol groups RCG2 (–SH), for example with an iminothiolane, in the case where RCG1 represents disulfide (i.e. RCG1 = –SZ_a with Z_a ≠ H,

for example Z_a = ). In both cases, the covalent bond that forms by reaction between RCG1 and RCG2 is a cleavable disulfide bond.

It is also possible, in the case where RCG1 represents –SH, to introduce onto the surface of the

20 binding agent RCG2 groups of maleimido type () or haloacetamido type (e.g. bromo- or

iodoacetamido ). Reciprocally, it is possible to introduce onto the binding agent thiol groups RCG2 (–SH), for example with an iminothiolane, in the case where RCG1 represents

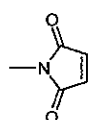
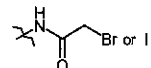
 or . In this case, the covalent bond that forms by reaction between RCG1 and RCG2 is an uncleavable sulfide bond.

Table I: examples of modifications of a binding agent when RCG1=SZ_a

modifying agent	RCG2 at the surface of the binding agent	example after reaction of an amino group, especially lysine, of an antibody noted MAb	conjugate
SPDP=			
SPDB=			
SMCC=			
sulfo-SMCC=			

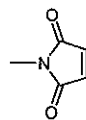
g: number of RCG2 functions on a modified binding agent; d: number of cryptophycin derivatives on the binding agent MAB

L* = fragment of a linker L comprising RCG1=SZa and such that L = -L*-SZ3

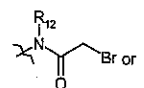
More particularly, in the case where RCG1 is of the type (iii) above, it is possible to chemically modify the binding agent using an adequate modifying agent or to introduce one or more unnatural amino acids so as to introduce the adequate functions RCG2. For example:

- with a function $-N_3$: RCG2 may be a group $-C\equiv CH$;
- with a function $-OH$ or $-NH_2$: RCG2 may be a carboxylic acid function;
- with a function $-Cl$: RCG2 may be a group $-SH$.

In the case where RCG1 represents a maleimido

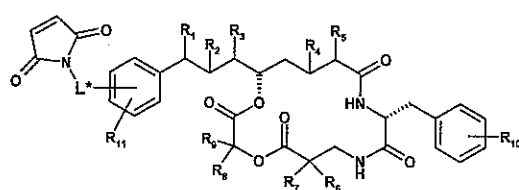


or haloacetamido

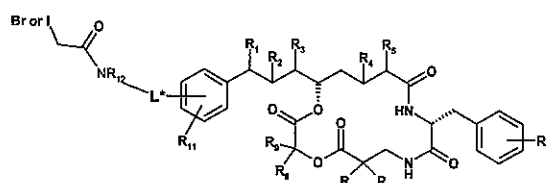


reactive

group with R_{12} representing H or (C_1-C_6) alkyl, more particularly Me, the cryptophycin derivative may be represented by the formula (IIa) or (IIb) below:



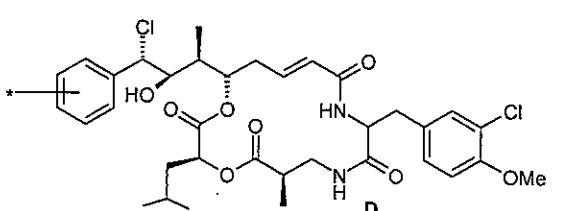
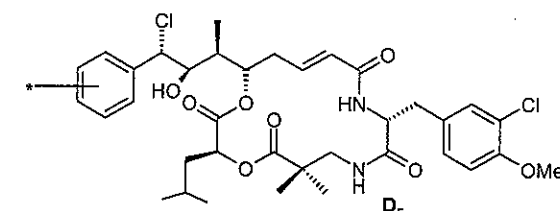
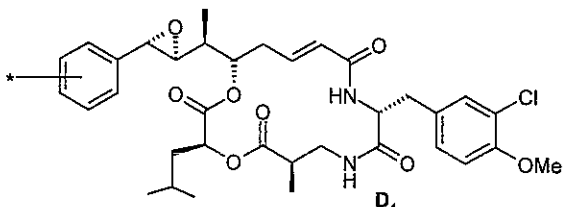
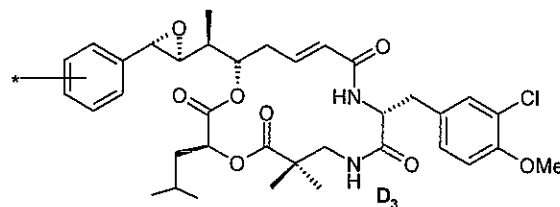
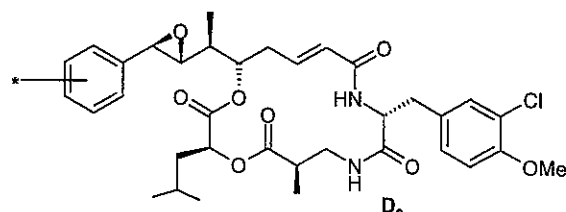
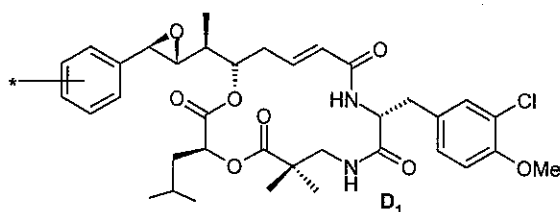
(IIa)

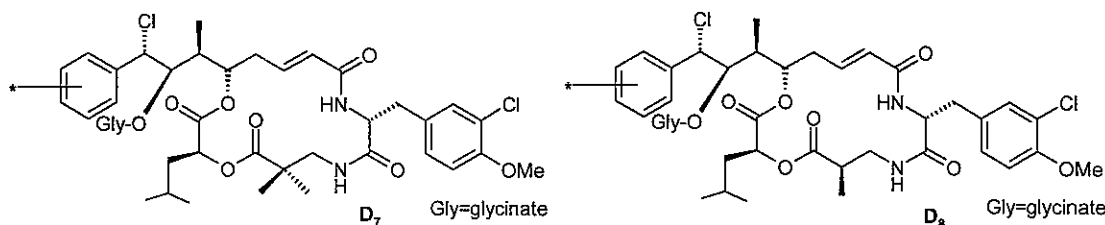


(IIb)

(L^* represents a fragment of a linker L such that $L = -L^*-\text{maleimido}$ or $L = -L^*-\text{haloacetamido}$)

The cryptophycin derivative may be, in the series **C-52** and **C-1**, one of the following D_1 - D_8 :





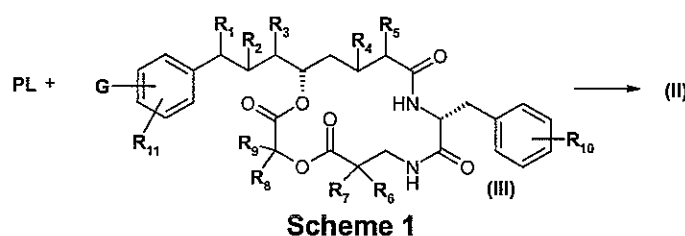
or an equivalent unit described in one of the examples.

More particularly, L is in the para position of the unit RC₁.

5

Process for preparing the cryptophycin derivatives

The compounds of formula (II) are prepared according to **Scheme 1** starting with a cryptophycin derivative of formula (III) and a linker precursor (LP):

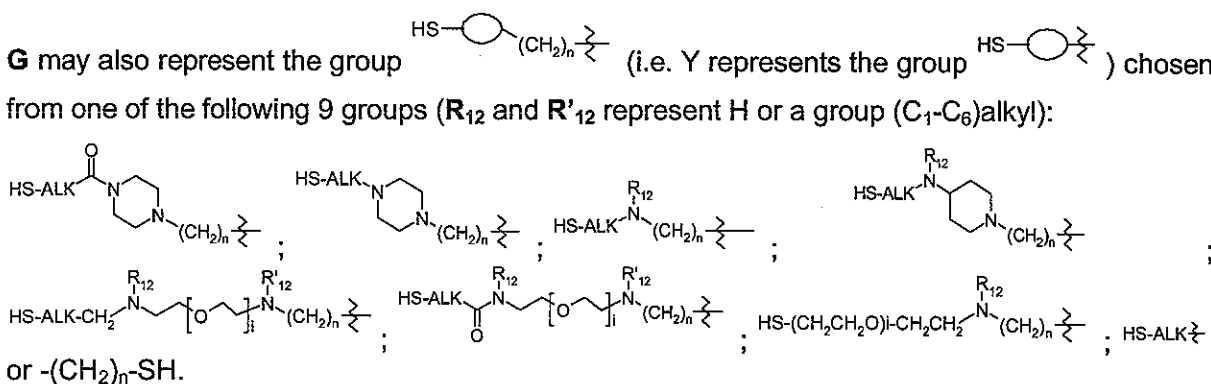


10

G represents the group $-\text{CH}=\text{CH}_2$, $-(\text{CH}_2)_n$ or $-(\text{C}_1-\text{C}_6)\text{alkylene}-\text{Y}$ with n being an integer ranging from 1 to 6 and Y representing $-\text{OH}$, $-\text{SH}$, $-\text{Cl}$, $-\text{OLG}$ in which LG denotes a leaving group, for instance a mesylate (OMs) or tosylate group, or alternatively Y represents $-\text{N}_3$, $-\text{NH}_2$, $-\text{COOH}$, $-\text{NR}_{12}-\text{CH}_2-\text{C}\equiv\text{CH}$ in which R_{12} represents H or a group $(\text{C}_1-\text{C}_6)\text{alkyl}$, more particularly a methyl group. The linker precursor **LP** has the function of introducing the linker **L** into the cryptophycin derivative after reaction between the group **G** and a chemical function present on **LP**.

15

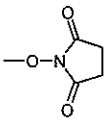
G may also represent the group $\text{HS}-\text{C}(\text{CH}_2)_n-\text{C}(\text{CH}_2)_n-\text{SH}$ (i.e. Y represents the group $\text{HS}-\text{C}(\text{CH}_2)_n-\text{C}(\text{CH}_2)_n-\text{SH}$) chosen from one of the following 9 groups (R_{12} and R'_{12} represent H or a group $(\text{C}_1-\text{C}_6)\text{alkyl}$):

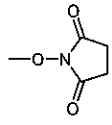


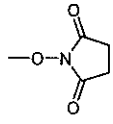
25

In **Scheme 1**, several steps and/or reactions may be necessary to arrive at the cryptophycin derivative (II) starting from the cryptophycin derivative (III). Thus, for example, in the case where $\text{Z}_a=\text{H}$, it is preferred to introduce a linker **L** for which $\text{Z}_a=-\text{S}(\text{C}_1-\text{C}_6)\text{alkyl}$ using the corresponding linker precursor, and then to reduce the disulfide function $-\text{SS}(\text{C}_1-\text{C}_6)\text{alkyl}$ to a thiol function $-\text{SH}$.

It is possible to use in order to do this, for example, TCEP: see in this respect Burns J.A., et al., *J.Org.Chem.* **1991**, 56(8), 2648-2650. This conversion $-\text{SS}(\text{C}_1\text{-C}_6)\text{alkyl} \rightarrow -\text{SH}$ may apply, for example, to the linkers **L₁₋₄** and **L₂₁₋₂₃** of **Table II**.

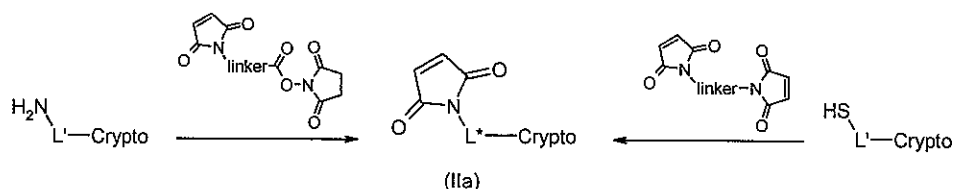
- 5 Similarly, in the case where $\text{Z}_b\text{R}_b =$ , a linker **L** for which $\text{Z}_b\text{R}_b = -\text{O-allyl}$ may be introduced using the corresponding linker precursor, followed by deprotecting the function $-\text{COOH}$ and

- 10 introducing . Deprotection may be performed by treatment with a palladium catalyst, for example $\text{Pd}(\text{PPh}_3)_4$ in the presence of a "scavenger" amine, for example morpholine; the activation may be performed with $\text{N,N'$ -disuccinimidyl carbonate in the presence of a base, for example DIPEA or with NHS in the presence of a coupling agent, for example DCC. This

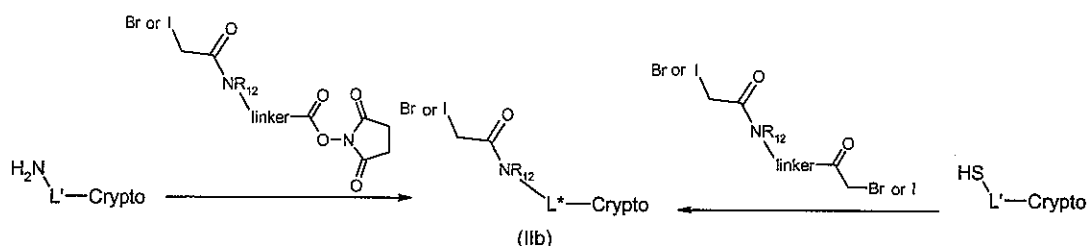
conversion of a group Z_bR_b into another group Z_bR_b (e.g. $-\text{O-allyl} \rightarrow$ ) may be applied to obtain other groups Z_bR_b , especially those described above.

- 15 In the case where R_1 represents a halogen atom and R_2 represents an acyl group, it is preferred first to prepare a compound of formula (II) for which R_2 represents a group $-\text{OH}$ (once the linker has been introduced), and then to introduce the acyl group using the corresponding acylating compound.

- 20 **Schemes 1' and 1''** similarly illustrate the preparation of a cryptophycin derivative comprising a linker comprising, respectively, a maleimido or haloacetamido group (L^* represents a fragment of a linker such that $\text{L} = -\text{L}^*-\text{maleimido}$ or $\text{L} = -\text{L}^*-\text{haloacetamido}$).



Scheme 1'



Scheme 1''

These derivatives are obtained by reaction between a cryptophycin derivative comprising a linker L' comprising an amino or thiol group and a modifying agent for introducing, respectively, a maleimido or haloacetamido group.

5 Examples of reactions between the group G and a chemical function present on LP

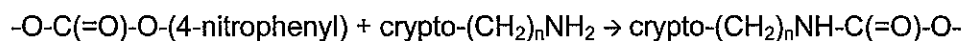
• nucleophilic substitution between a linker precursor LP bearing an amine function -NH- (an amine salt may also be suitable for use) and $G=-(CH_2)_nCl$ or $-(CH_2)_nOMs$ (see, for example, **Table II**, LP₁₋₄, LP_{7a}, LP₈₋₁₀, LP₂₁₋₂₃): this reaction may be performed in a polar aprotic solvent in the presence of a base, for instance TEA or DIPEA. See Ex. 1, compound 7 or Ex. 15, compound 48;

• acylation between a linker precursor LP bearing a carbamoyl halide function and $G=-(CH_2)_nOH$ (see, for example, **Table II**, LP₅): this reaction may be performed in a polar aprotic solvent in the presence of an amine base, for instance TEA.

According to one variant, it is also possible to react a linker precursor LP bearing an amine function -NH- and $G=-(CH_2)_nO-C(=O)-O-(4\text{-nitrophenyl})$ obtained from $G=-(CH_2)_nOH$ and p-nitrophenyl chloroformate (activation of the alcohol in the form of carbonate) according to the scheme below ($R_{12}=H$ or $(C_1-C_6)\text{alkyl}$):



• the activation of an alcohol in the form of carbonate may also be used to react a linker precursor bearing a function -OH and $G=-(CH_2)_nNH_2$ or $-(CH_2)_nOH$ to obtain, respectively, a carbamate function (-O-C(=O)-NH-) or carbonate (-O-C(=O)-O-) according to the following respective schemes:



(see, for example, **Table II**, LP_{6a-6b}, LP₂₄₋₂₅)

• esterification between a linker precursor LP bearing an acid function -COOH and $G=-(CH_2)_nOH$ (see, for example, **Table II**, LP_{14b}): this reaction may be performed in a polar aprotic solvent in the presence of an amine base, for instance DMAP and a coupling agent, for example DCC;

• amidation between a linker precursor LP bearing an acid function -COOH and $G=-(CH_2)_nNH_2$ (see, for example, **Table II**, LP_{14a}): this reaction may be performed in a polar aprotic solvent in the presence of a coupling agent, for example EDCI or HOBt;

• amidation between a linker precursor LP bearing a function -NH₂ and $G=-(CH_2)_nCOOH$ (see, for example, **Table II**, LP_{7c}): this reaction may be performed in a polar aprotic solvent in the presence of a coupling agent, for example EDCI or HOBt;

- 1,3-dipolar cycloaddition (also known as “click” chemistry) between a linker precursor LP bearing an alkyne terminal function and $G=-(CH_2)_nN_3$ or alternatively a linker precursor LP bearing an azide function and $G=-(CH_2)_nNR_{12}-CH_2C\equiv CH$ (see, for example, **Table II**, LP₁₅₋₁₈): this reaction may be performed in a polar solvent in the presence of Cu(I) as catalyst (see in this respect the Huisgen cycloaddition: Rostovtsev V.V., et al., *Angew. Chem. Int. Ed.* **2002**, 41, 2596-2599; Tornøe C.W., et al., *J. Org. Chem.* **2002**, 67, 3057-3064);
- metathesis between a linker precursor LP bearing an ethylenic end function and $G=-CH=CH_2$ (see **Table II**, LP₁₉, LP₂₀): this reaction may be performed in a polar aprotic solvent in the presence of a second-generation Grubbs catalyst (CAS No. 246047-72-3, see in this respect Poeylout-Palena A.A., et al., *J. Org. Chem.* **2008**, 73, 2024-2027).

As regards the linker L

The linker **L** may be chosen from one of the following:

- G' X (CR₁₃R₁₄)_t(OCH₂CH₂)_y(CR₁₅R₁₆)_u Q RCG₁;
 - G' X (CR₁₃R₁₄)_t(OCH₂CH₂)_y-Y'-(CR₁₅R₁₆)_u Q RCG₁;
 - G' X (CR₁₃R₁₄)_t(CR₁₇=CR₁₈)(CR₁₅R₁₆)_u(OCH₂CH₂)_y Q RCG₁;
 - G' X (CR₁₃R₁₄)_t(OCH₂CH₂)_y(CR₁₇=CR₁₈)(CR₁₅R₁₆)_u Q RCG₁;
 - G' X (CR₁₃R₁₄)_t-phenyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G' X (CR₁₃R₁₄)_t-furyl-(CR₁₅R₁₆)_u Y' Q RCG₁;
 - G' X (CR₁₃R₁₄)_t-oxazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G' X (CR₁₃R₁₄)_t-thiazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁;
 - G' X (CR₁₃R₁₄)_t-thienyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G' X (CR₁₃R₁₄)_t-imidazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁;
 - G' X (CR₁₃R₁₄)_t-piperazinyl-CO(CR₁₅R₁₆)_u Y' Q RCG₁; -G' X (CR₁₃R₁₄)_t-piperidyl-methyl-NR₁₂-CO(CR₁₅R₁₆)_u Y' Q RCG₁; -G' X (CR₁₃R₁₄)_t-piperidyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G' X (CR₁₃R₁₄)_t-piperidyl-NR₁₂-(CR₁₅R₁₆)_u Y' Q RCG₁; -G' X (CR₁₃R₁₄)_t-triazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G' X (CR₁₃R₁₄)_t-triazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁;
 - G' X (CR₁₃R₁₄)_t-phenyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-furyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-oxazolyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-thiazolyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-thienyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-imidazolyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-piperazinyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-piperidyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-piperidyl-methyl-NR₁₂-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-piperidyl-NR₁₂-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-triazolyl-(CR₁₅R₁₆)_u Q RCG₁;
- or
- G'' Y (CR₁₃R₁₄)_t(OCH₂CH₂)_y(CR₁₅R₁₆)_u Q RCG₁;
 - G'' Y (CR₁₃R₁₄)_t(OCH₂CH₂)_y-Y'-(CR₁₅R₁₆)_u Q RCG₁;
 - G'' Y (CR₁₃R₁₄)_t(CR₁₇=CR₁₈)(CR₁₅R₁₆)_u(OCH₂CH₂)_y Q RCG₁;
 - G'' Y (CR₁₃R₁₄)_t(OCH₂CH₂)_y(CR₁₇=CR₁₈)(CR₁₅R₁₆)_u Q RCG₁;
 - G'' Y (CR₁₃R₁₄)_t-phenyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-furyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-oxazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-thiazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-thienyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-imidazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁;

$-G'' Y (CR_{13}R_{14})_t \text{-piperazinyl-CO}(CR_{15}R_{16})_u Y' Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-piperidyl-methyl-NR}_{12}\text{-CO}(CR_{15}R_{16})_u Y' Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-piperidyl-(CR}_{15}R_{16})_u Y' Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-piperidyl-NR}_{12}\text{-(CR}_{15}R_{16})_u Y' Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-triazolyl-(CR}_{15}R_{16})_u Y' Q RCG_1$;
 $-G'' Y (CR_{13}R_{14})_t \text{-phenyl-(CR}_{15}R_{16})_u Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-furyl-(CR}_{15}R_{16})_u Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-oxazolyl-(CR}_{15}R_{16})_u Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-thiazolyl-(CR}_{15}R_{16})_u Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-thienyl-(CR}_{15}R_{16})_u Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-imidazolyl-(CR}_{15}R_{16})_u Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-piperazinyl-(CR}_{15}R_{16})_u Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-piperazinyl-(CR}_{15}R_{16})_u Q RCG_1$; $G'' Y (CR_{13}R_{14})_t \text{-piperidyl-(CR}_{15}R_{16})_u Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-piperidyl-methyl-NR}_{12}\text{-(CR}_{15}R_{16})_u Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-piperidyl-NR}_{12}\text{-(CR}_{15}R_{16})_u Q RCG_1$; $-G'' Y (CR_{13}R_{14})_t \text{-triazolyl-(CR}_{15}R_{16})_u Q RCG_1$;

G' represents a group $-\text{CH}=\text{CH}-$ or $-(\text{CH}_2)_n-$;

G'' represents a group $-(\text{CH}_2)_n-$;

n represents an integer ranging from 1 to 6;

X represents a single bond or a group $-\text{CO}-$, $-\text{COO}-$ or $-\text{CONR}_{12}-$, the group CO being attached to **G'**;

Y represents a group $-\text{O}-$, $-\text{OCO}-$, $-\text{OCOO}-$, $-\text{OCONR}_{12}-$, $-\text{NR}_{12}-$, $-\text{NR}_{12}\text{CO}-$, $-\text{NR}_{12}\text{CONR}'_{12}-$, $-\text{NR}_{12}\text{COO}-$ or $-\text{S(O)}_q-$, the atom O or the group NR_{12} being attached to **G''**;

Y' represents a group $-\text{O}-$, $-\text{OCO}-$, $-\text{OCOO}-$, $-\text{OCONR}_{12}-$, $-\text{NR}_{12}-$, $-\text{NR}_{12}\text{CO}-$, $-\text{NR}_{12}\text{CONR}'_{12}-$, $-\text{NR}_{12}\text{COO}-$, $-\text{S(O)}_q-$, $-\text{CO}-$, $-\text{COO}-$, or $-\text{CONR}_{12}-$;

R₁₂, **R'**₁₂, **R**₁₃, **R**₁₄, **R**₁₅ and **R**₁₆, **R**₁₇ and **R**₁₈ represent, independently of each other, H or a group $(\text{C}_1\text{-C}_6)\text{alkyl}$;

t, **u** and **y** represent integers that may range from 0 (in the case of the absent group) to 20 and such that $t+u+y$ is greater than or equal to 1;

q represents an integer that may be 0, 1 or 2;

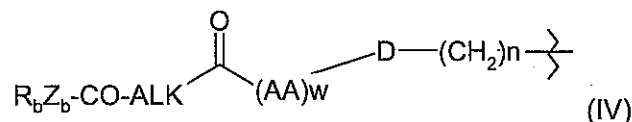
Q represents a single bond, a group $(\text{C}_1\text{-C}_{10})\text{alkylene}$ or a group $(\text{OCH}_2\text{CH}_2)_i$, **i** being an integer ranging from 1 to 20, more particularly from 1 to 10, even more particularly from 1 to 8 or from 1 to 6, and even more particularly from 2 to 5. **i** may take each of the values of these ranges, and may especially be 2, 3, 4 or 5.

In the case of the linker of formula $-G'' Y (CR_{13}R_{14})_t (\text{OCH}_2\text{CH}_2)_y -Y'-(CR_{15}R_{16})_u Q RCG_1$, if **y** is 0 (no PEG group) and if **Q** represents a single bond, then **u** cannot be 0. More particularly, linkers comprising the terminal group $-\text{NR}_{12}\text{-C(=O)-O-}$ ($Y'=\text{NR}_{12}$; $u=0$; **Q**=single bond and $\text{RCG}_1=-\text{C(=O)Z}_b\text{R}_b$) are excluded.

y represents an integer ranging from 0 to 20, more particularly from 1 to 20, even more particularly from 1 to 10, from 1 to 8 or from 1 to 6, and even more particularly from 2 to 5. **y** may take each of the values of these ranges, and may especially be 2, 3, 4 or 5.

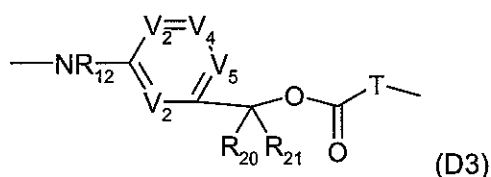
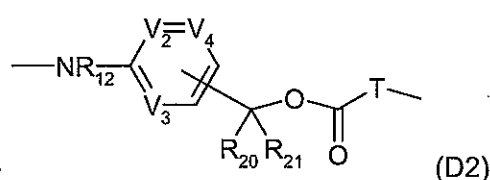
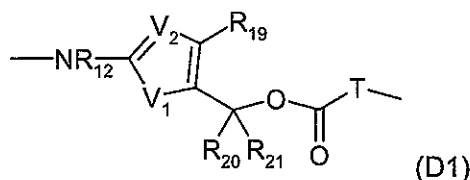
Some of these linkers have been described in patent applications **WO 07/085 930** and **WO 09/016 516**.

5 The linker **L** may be chosen from one of those of formula (IV):



in which:

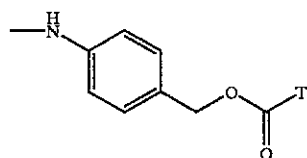
- **(AA)_w** represents a sequence of **w** amino acids **AA** connected together via peptide bonds;
- **w** represents an integer ranging from 1 to 12 and preferably from 1 to 6;
- **n** represents an integer ranging from 1 to 6;
- **D** represents one of the following units:



for which:

- 15 **R₁₂** represents H or a group (C₁-C₆)alkyl;
- R₁₉, R₂₀, R₂₁ and R₂₂** represent, independently of each other, H, a halogen atom, -OH, -CN or a group (C₁-C₄)alkyl;
- T** attached to (CH₂)_n represents NR₁₂ or O;
- V₁** represents O, S or NR₁₂;
- 20 **V₂** represents CR₂₂ or N;
- V₃, V₄ and V₅** are chosen, independently of each other, from CR₂₂ and N.

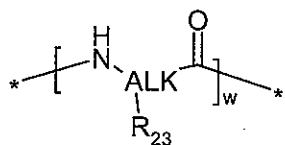
An example of D2 is as follows:



- 25 **AA** denotes a natural or unnatural amino acid, more particularly chosen from: alanine (Ala), β-alanine, 2-amino-2-cyclohexylacetic acid, 2-amino-2-phenylacetic acid, arginine (Arg), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro),

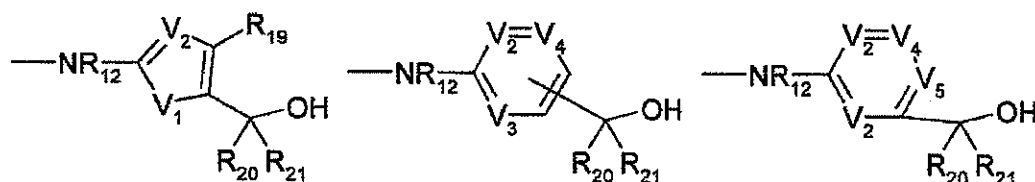
serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), valine (Val), γ -aminobutyric acid, α,α -dimethyl- γ -aminobutyric acid, β,β -dimethyl- γ -aminobutyric acid, ornithine (Orn), citrulline (Cit).

The sequence $(AA)_w$ has the formula:



5 in which R_{23} represents a residue of one of the amino acids described above. Examples of sequences are as follows: Gly-Gly, Phe-Lys, Val-Lys, Val-Cit, Phe-Phe-Lys, D-Phe-Phe-Lys, Gly-Phe-Lys, Ala-Lys, Val-Cit, Phe-Cit, Leu-Cit, Ile-Cit, Trp-Cit, Phe-Ala, Ala-Phe, Gly-Gly-Gly, Gly-Ala-Phe, Gly-Val-Cit, Gly-Phe-Leu-Cit, Gly-Phe-Leu-Gly, Ala-Leu-Ala-Leu.

10 The linker precursors are those comprising the corresponding $-\text{OH}$ units:



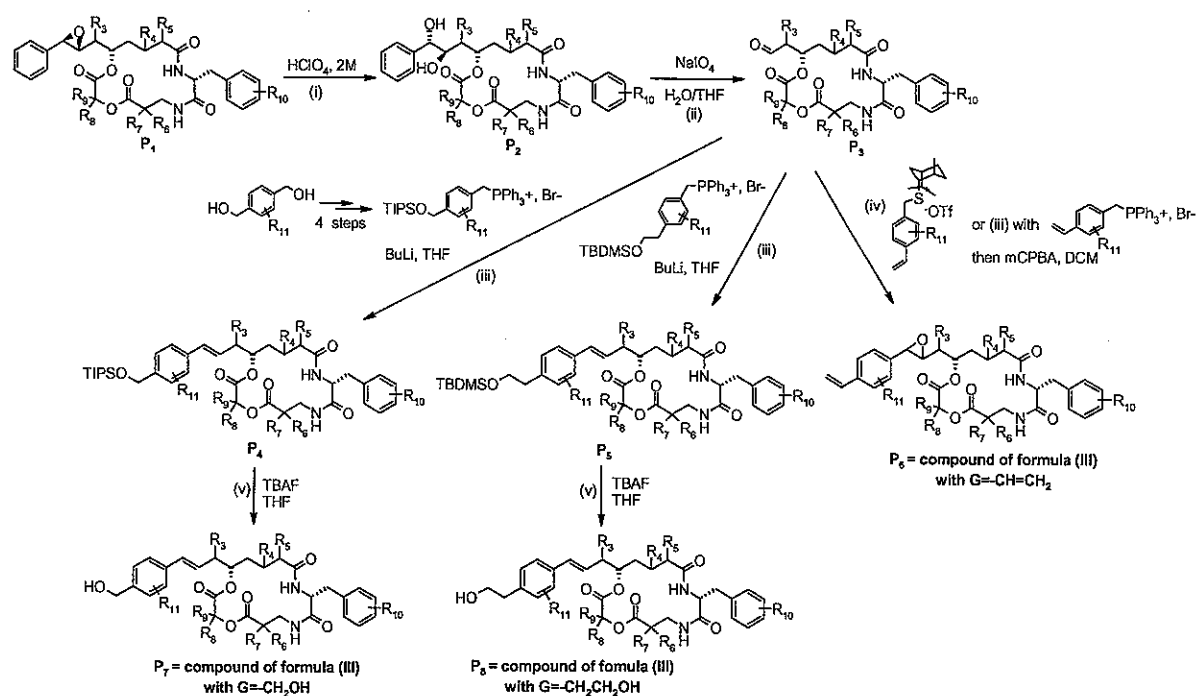
WO 2005/082 023 (see especially pages 61-64) describes how to obtain certain linker precursors.

The preparations of the linker precursors **LP25** and **LP26** described below may also be used for obtaining other similar linker precursors comprising another sequence $(AA)_w$.

15 The linker **L** may also be chosen from one of those described in **Table II** or from the illustrated compounds. In all the formulae of linkers, NR_{12} or NR'_{12} more particularly represents NH or NMe .

Preparation of the compounds of formula (III)

20 **in the case where $G=-(\text{CH}_2)_n\text{OH}$ or $-\text{CH}=\text{CH}_2$**



P₁ is prepared according to the teaching of patent applications **WO 98/08505**, **WO 00/23429** or **WO 00/34252** and also the following publications: Rej R., et al., *J. Org. Chem.* **1996**, 61, 6289-6295; Salamonczyk G.M., et al., *J. Org. Chem.* **1996**, 61, 6893-6900 or *J. Med. Chem.* **1999**, 42 (14), 2588-2603 (incorporated herein by reference). In pages 158-159 of "The isolation, characterization and development of a novel class of potent antimitotic macrocyclic depsipeptides: the cryptophycins", Chap.9, in "Anticancer agents from natural products", Taylor&Francis, CRC press book, ISBN=0-8493-1863-7 are given in the synthetic schemes for preparing the various cryptophycin fragments (A, B, C and D) and for producing **P₁**. Rej R., et al., *J. Org. Chem.* **1996**, 61, 6289-6295 describes in Schemes 1-6 the route of access to one of the cryptophycin derivatives of Figure 1, but these schemes may apply to the preparation of **P₁** using suitable starting reagents.

P₁ allows the preparation of other cryptophycin derivatives with the aid of the steps detailed hereinbelow:

Step (i): opening of the epoxide ring of **P₁** in acidic medium so as to obtain the diol function. Concentrated perchloric acid may be used, for example;

Step (ii): oxidative cleavage of the diol using, for example, sodium periodate;

Step (iii): Wittig reaction using a suitable phosphonium halide, for example a bromide, and a strong base, for instance BuLi;

Step (iv): Corey-Chaykovsky epoxidation reaction involving a chiral sulfonium salt, for example a triflate, in the presence of a base, for instance KOH;

Step (v): deprotection of the silyl ether using, for example, a tetrabutylammonium fluoride solution.

4-(Triisopropylsiloxymethyl)benzyltriphenylphosphonium bromide is obtained from 1-(bromomethyl)-4-(triisopropylsiloxymethyl)benzene (CAS No. 934667-38-6), the preparation of which starting from 1,4-benzenedimethanol (CAS No. 589-29-7, commercial product) is described by Potter R.G., et al., *Organic Letters* **2007**, 9(7), 1187-1190. The compounds for which R_{11} represents a group (C_1-C_4) alkyl are obtained in a similar manner starting with the corresponding diol, which is either a commercial product or is obtained by Friedel-Crafts C-alkylation starting with 1,4-benzenedimethanol.

Starting with 1-(bromomethyl)-4-(triisopropylsiloxymethyl)benzene (CAS No. 135408-73-0), the preparation of which is described in Scheme 4a of **EP 0 496 548** or on page 83 of the article by Nevill C.R. Jr., et al., *Bioorganic & Med. Chem. Lett.* **1991**, 1(1), 83-86, the corresponding phosphonium bromide may be obtained. The compounds for which R_{11} represents a group (C_1-C_4) alkyl are obtained in a similar manner from a compound equivalent to compound 1 described on page 83 of the article by Nevill C.R. Jr., et al., *Bioorganic & Med. Chem. Lett.* **1991**, 1(1), 83-86, which is either a commercial product or is obtained by Friedel-Crafts C-alkylation starting with *p*-tolylacetic acid.

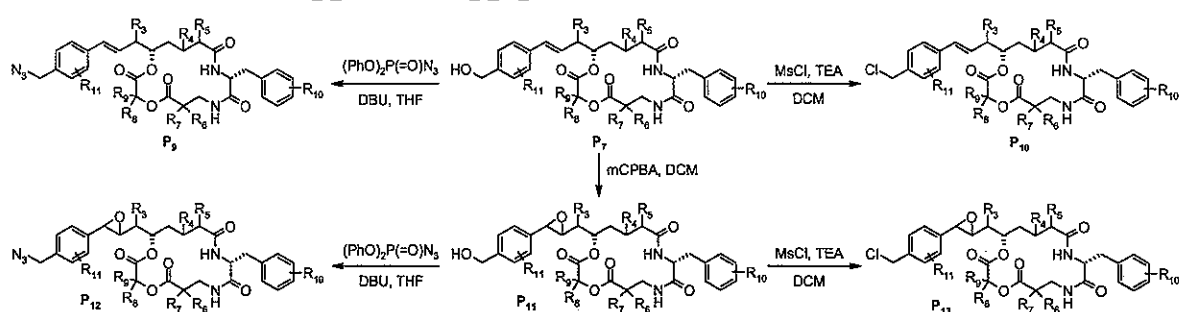
(1R,4R,5R,6R)-4,7,7-Trimethyl-6-(4-vinylbenzyl)-6-thionia-bicyclo[3.2.1]octane

trifluoromethanesulfonate used in step (iv) is obtained from (1R,4R,5R)-isothiocineole (see Aggarwal V. et al., *JACS* **2010**, 132, 1828-1830), the preparation of which from (R)-limonene (CAS No. 95327-98-3, commercial product) is described in this same reference.

Triphenyl(*p*-vinylbenzyl)phosphonium bromide (CAS No. 118766-51-1) is obtained from the corresponding bromo derivative (see Drefahl G., et al., *Chem.Ber.* **1961**, 94(8), 2002-2010), the preparation of which starting with 4-vinylbenzyl alcohol (CAS No. 1074-61-9, commercial product) is described in the article by Shimomura O., et al., *Tetrahedron* **2005**, 61, 12160-12167.

Starting with P_7 or P_8 , which are compounds of formula (III) for which $G=-(CH_2)_nOH$, other compounds of formula (III) containing other groups G may be obtained.

in the case where $G=-(CH_2)_nCl$ or $-(CH_2)_nN_3$

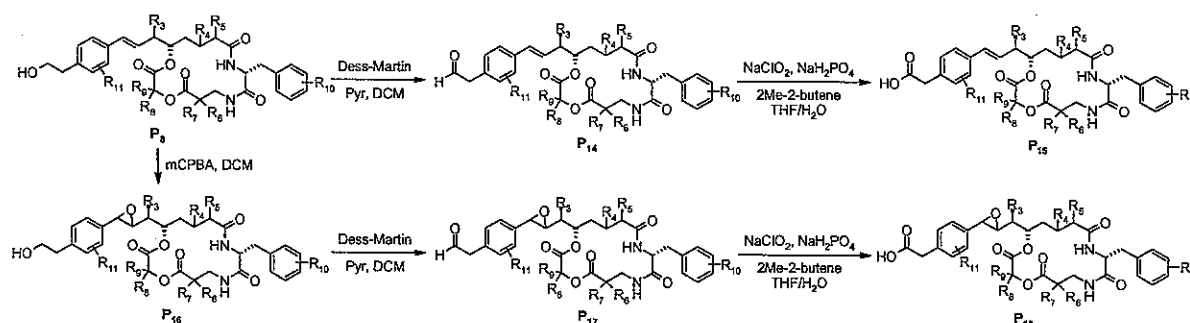


Scheme 3

Starting with the group $G = -CH_2OH$, the groups $G = -CH_2Cl$ or $-CH_2N_3$ may be obtained:

- the introduction of $-Cl$ may be performed in the presence of CMS: see Ex. 1, compound 2;
- the azidation may be performed in the presence of diphenylphosphorazide $(PhO)_2P(=O)N_3$ and a base, for example DBU. **Scheme 3** describes these reactions for the case $n=1$, but it may also apply for $n>1$.

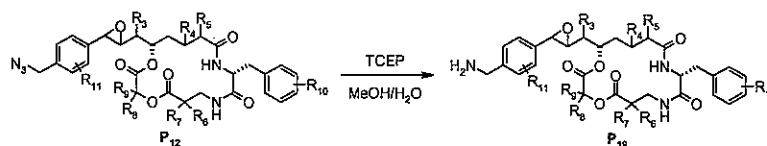
in the case where $G = -(CH_2)_nCOOH$



Scheme 4

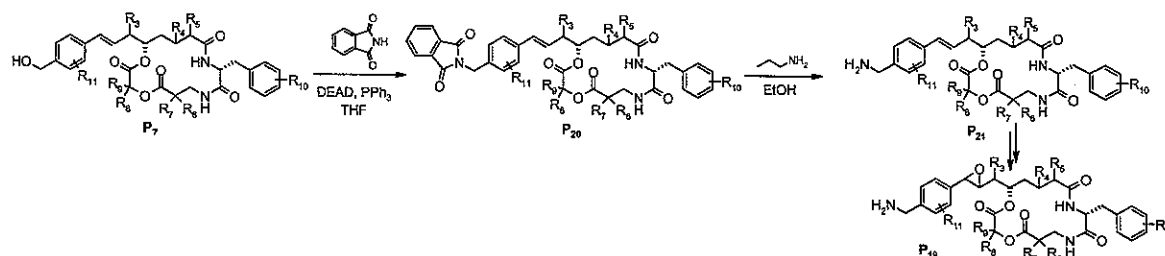
Starting with the group $G = -(CH_2)_2OH$, the group $G = -CH_2COOH$ may be obtained via an oxidation. **Scheme 4** describes a double oxidation: 1st oxidation using the Dess-Martin reagent (see "Encyclopedia of Reagents for Organic Synthesis"; Paquette L. A., Ed.; Wiley: Chichester, UK, 1995, Vol. 7, 4982-4987 or Boeckman R.K. Jr., et al., J.J. "The Dess-Martin Periodinane" *Org. Synth.* **2004**, 10, 696-702) followed by a 2nd oxidation of Pinnick type in the presence of 2-methyl-2-butene (Pinnick H.W., *Tetrahedron* **1981**, 37, 2091-2096). **Scheme 4** describes these reactions for the case of a starting compound for which $n=2$, but it may also apply for $n>2$.

in the case where $G = -(CH_2)_nNH_2$



Scheme 5

Starting with the group $G = -CH_2N_3$, the preparation of which is described in **Scheme 3**, the group $G = -CH_2NH_2$ may be obtained with the aid of a reduction reaction using a phosphine such as TCEP. In this respect, see: Faucher A.-M. et al., *Synthetic Comm.* **2003**, 33, 3503-3511:



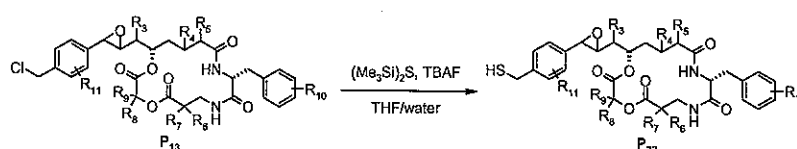
Scheme 5'

According to one variant, starting with the group $G=CH_2OH$, the group $G=CH_2NH_2$ may be obtained with the aid of a Mitsunobu reaction using triphenylphosphine and DEAD. In this respect, see: Mitsunobu O., *Synthesis* **1981**, 1-28; Hughes D.L., *Org. Reactions* **1992**, 42, 335-656; Hughes D.L., *Org. Prep.* **1996**, 28, 127-164. **Schemes 5 and 5'** describe the case $n=1$, but they may also apply for $n>1$.

in the case where $G=-(CH_2)_n-NR_{12}-CH_2C\equiv CH$

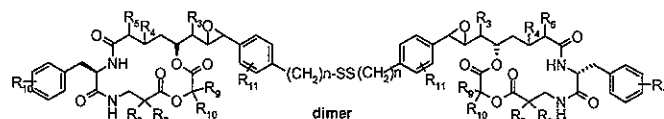
Starting with the group $G=-(CH_2)_nCl$, the group $G=-(CH_2)_n-NR_{12}-CH_2C\equiv CH$ may be obtained with the aid of a nucleophilic substitution using the compound of formula $NHR_{12}-CH_2C\equiv CH$ ($R_{12}=H$: propargylamine; $R_{12}=(C_1-C_6)alkyl$: prepared according to Mock W.L., et al., *J. Org. Chem.* **1989**, 54 (22), 5302-8).

in the case where $G=-(CH_2)_n-SH$

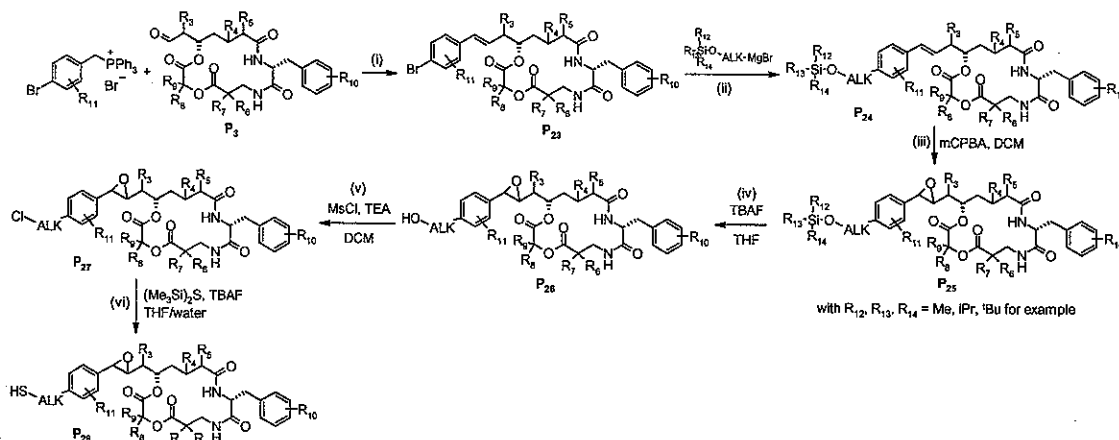


Scheme 6

Starting with the group $G=-(CH_2)_nCl$, the group $G=-(CH_2)_nSH$ may be obtained via a direct functionalization with the aid of tetrabutylammonium trimethylsilylthiolate prepared *in situ* from tetrabutylammonium fluoride and hexamethyldisilathiane according to Hu J. et al., *J. Org. Chem.* **1999**, 64, 4959-4961 (see Ex. 8). **Scheme 6** describes this reaction for the case $n=1$, but it may also apply for $n>1$. In the course of this reaction, the intermediate dimer having the formula below may be formed:



in the case where $G=ALK-SH$



Scheme 7

P₃ allows the preparation of other cryptophycin derivatives according to **Scheme 7**:

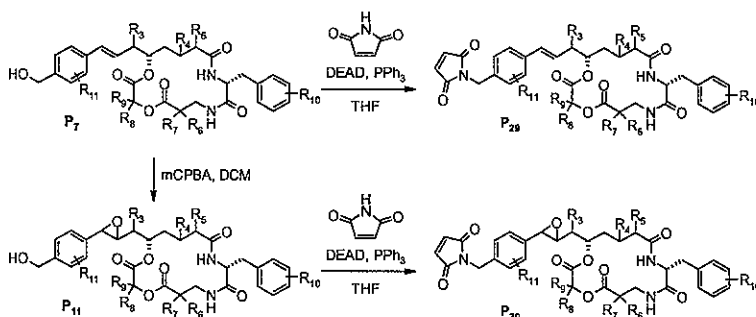
Step (i): Wittig reaction using a suitable phosphonium halide, for example a bromide, and a strong base, for instance BuLi;

- 5 **Step (ii)**: Kumada coupling using a suitable Grignard reagent, for example an alkoxymagnesium bromide protected in silyl ether form, in the presence of a palladium or nickel catalyst (see, for example, *Organic Letters* **2009**, 11, 5686-5689 or *Synthesis* **2009**, 141, 2408-2412).

Steps (iii) to (v) are described in **Scheme 2** and step (vi) is described in **Scheme 6**.

- 10 (4-Bromobenzyl)triphenylphosphonium bromide is a commercial product (CAS No. 51044-13-4). The alkoxymagnesium bromides protected in silyl ether form may be prepared from the corresponding bromo alcohols by protection of the alcohol function with the appropriate chlorosilane, followed by formation of the organomagnesium reagent in the presence of magnesium in an anhydrous polar aprotic solvent, for instance THF (see, for example, *Organic*
- 15 *Letters* **2005**, 7, 183-186). The linear or branched bromo alcohols containing 1 to 6 carbon atoms are commercially available, for instance 3-bromo-1-propanol (CAS No. 627-18-9) or 1-bromo-2-propanol (CAS No. 19686-73-8) or may be prepared from the corresponding bromo esters or bromo ketones according to methods described in the literature. The chlorosilane may be, for example, *tert*-butyldimethylchlorosilane (CAS No. 18162-48-6) or triisopropylchlorosilane (CAS
- 20 No. 13154-24-0).

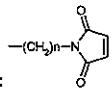
in the case where G=-(CH₂)_n-maleimido



Scheme 8

25

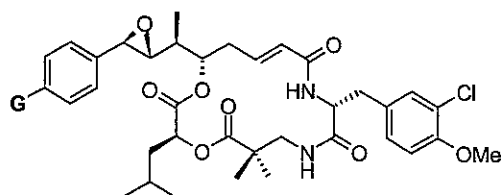
In addition to **Scheme 1'** which describes a method for preparing cryptophycin derivatives

comprising a maleimido unit, starting with the group G=CH₂OH, the group G=  may be obtained via a Mitsunobu reaction in the presence of triphenylphosphine and DEAD according to Matuszak N. et al., *J. Med. Chem.* **2009**, 52, 7410-7420. **Scheme 8** describes this reaction for the

30 case n=1, but it may also apply for n>1.

Schemes 1-8 above are given for a linker in the para position, but may identically apply for the ortho or meta positions. Similarly, they are given for a cryptophycin derivative, but may also apply to the preparation of other derivatives of formula (II), especially D₁-D₈.

- 5 More particularly, in the case of **C-52**, the following compounds may be used, the preparations of which are described in Al-awar R.S., et al., *J.Med.Chem.* **2003**, 46, 2985-3007 or in **WO 98/08505**:



G=CH₂OH : compound 31b of Scheme 5

G=CH₂CH₂OH : compound 49 of Scheme 9

G=CH₂COOH : compound 51 of Scheme 9
or Ex. 81 of **WO 98/08505**

G=C(=O)H : Ex. 80 of **WO 98/08505**

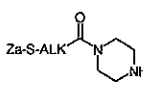
Moreover, starting with compound 31b for which G=CH₂OH, it is possible to obtain the compounds for which G=CH₂Cl or -CH₂N₃:

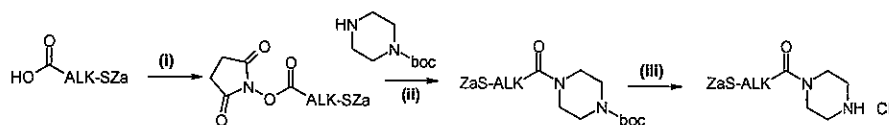
- 10 • G=CH₂Cl: see Example 1, compound 2;
• G=CH₂N₃: the conversion of -CH₂OH into -CH₂N₃ may be performed in a polar aprotic solvent in the presence of diphenylphosphorazide and a base such as DBU, see Example 19, compound 60;
15 • G=CH₂-maleimido: the conversion of -CH₂OH into -CH₂-maleimido may be performed in a polar aprotic solvent in the presence of maleimide, triphenylphosphine and DEAD.

The teaching of *J.Med.Chem.* **2003**, 46, 2985-3007 may apply to other cryptophycin derivatives comprising other substituents R₆-R₉.

Preparation of the linker precursors LP

- 20 LP may be one of the following:

LP₁  prepared according to the scheme below:

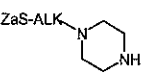


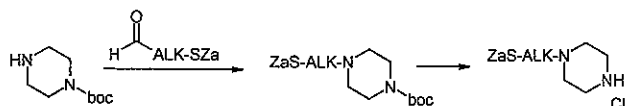
- 25 **Step (i):** activation of the acid using NHS; the activation is performed at RT in the presence of a coupling agent, for instance 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride dissolved in an anhydrous aprotic solvent such as DCM. The procedure may be based on the conditions of Example 1, compound 4.

- Step (ii):** amidation with piperidine N-Boc; the peptide coupling is performed in a polar aprotic solvent at RT in the presence of a base, which may be a tertiary amine, for instance TEA or DIPEA. The solvent may be DMF. The procedure may be based on the conditions of Example 1,
30 compound 5.

Step (iii): deprotection of the amine using an acid solution, for example hydrochloric acid (for example as a solution in dioxane). The procedure may be based on the conditions of Example 1, compound 6.

The starting acid, for example 4-methyl-4-(methyldithio)pentanoic acid, may be commercially available or prepared from a halocarboxylic acid by successive treatments with potassium thioacetate and a derivative of methanethiosulfonate type. See also **US 2 719 170**.

LP₂  prepared according to the scheme below:



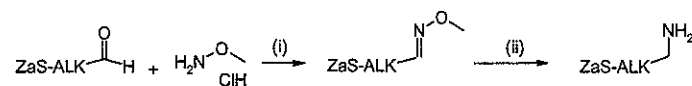
Step (i): reductive amination with an aldehyde; the reaction is performed at RT in an anhydrous polar aprotic solvent such as THF in two steps: formation of an intermediate complex in the presence of titanium isopropoxide followed by *in situ* reduction with a reducing agent, for instance sodium cyanoborohydride. The procedure may be based on the conditions of Example 5, compound 17.

Step (ii): deprotection of the amine with an acid solution, for example hydrochloric acid (for example as a solution in dioxane). The procedure may be based on the conditions of Example 5, compound 18.

The starting aldehyde, for example 2-methyl-2-(methyldithio)propanal, may be commercially available or prepared by oxidation of an alcohol bearing a disulfide unit obtained from a suitably protected halogenated alcohol (for example in silyl ether form) by successive treatments with potassium thioacetate and a derivative of methanethiosulfonate type.

LP₃ ZaS-ALK-NHR_{12} prepared according to the schemes below:

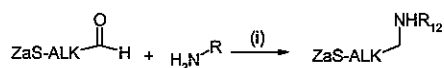
in the case where $R_{12}=\text{H}$



Step (i): formation of an oxime; the aldehyde described previously is dissolved in a polar protic solvent such as ethanol and then treated with O-methylhydroxylamine hydrochloride at reflux in the presence of a base such as sodium hydroxide. The procedure may be based on the conditions of Example 3, compound 11.

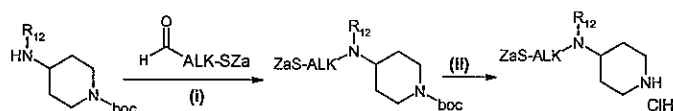
Step (ii): reduction of the oxime; the oxime is reduced via a treatment at reflux with a solution of borane-dimethyl sulfide in an anhydrous polar aprotic solvent such as THF. The procedure may be based on the conditions of Example 3, compound 12.

in the case where $R_{12}\neq\text{H}$



Step (i): reductive amination with an aldehyde; the reaction is performed at RT in an anhydrous polar aprotic solvent such as THF in the presence of a reducing agent, for instance sodium triacetoxyborohydride.

5

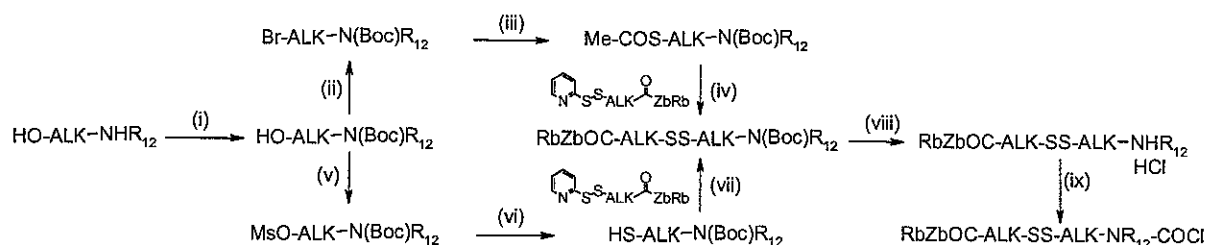


This linker is prepared in a similar manner to that presented for LP₂.

Step (i): reductive amination with an aldehyde; the reaction is performed at RT in an anhydrous polar aprotic solvent such as THF, in two steps: formation of an intermediate complex in the presence of titanium isopropoxide, followed by *in situ* reduction with a reducing agent such as sodium cyanoborohydride. The procedure may be based on the conditions of Example 5, compound 17.

Step (ii): deprotection of the amine with an acid solution, for example hydrochloric acid (for example in solution in dioxane). The procedure may be based on the conditions of Example 5, compound 18.

15



Step (i): protection of the amine; the reaction is performed at RT in a polar aprotic solvent such as DCM by treating the amine with di-tert-butyl dicarbonate in the presence of a base, for instance TEA.

Step (ii): conversion of the alcohol to a bromide; the reaction is performed at RT in a polar aprotic solvent such as THF, by treating the alcohol function with CBr₄ in the presence of a phosphine, for example triphenylphosphine; see in this respect Appel R. *Angew. Chem. Int. Ed. Engl.* **1975**, 14, 801-811 or Desmaris N., et al., *Tetrahedron Letters* **2003**, 44(41), 7589-7591.

Step (iii): substitution of the bromide with thioacetate; the reaction is performed at RT in an anhydrous polar aprotic solvent such as DMF, using potassium thioacetate as nucleophile.

Step (iv): formation of the disulfide bond; the reaction is performed in an anhydrous polar protic solvent such as methanol in the presence of a base, for instance sodium methoxide and a

30

reagent comprising a pyridyl disulfide unit.

According to one variant:

Step (v): activation of the alcohol in mesylate form; the reaction is performed in an anhydrous polar aprotic solvent such as DCM by treatment with mesyl chloride in the presence of a base, for instance TEA.

Step (vi): formation of the free thiol; the reaction is performed in a refluxing polar protic solvent such as an ethanol/water mixture, in two successive steps: displacement of the mesylate with thiourea, followed by *in situ* hydrolysis of the isothiuronium salt by adding a base such as NaOH.

Step (vii): activation of the thiol in the form of pyridyl disulfide; the reaction is performed at RT in a polar protic solvent such as ethanol, by treatment with a reagent comprising a pyridyl disulfide unit in the presence of an acid such as acetic acid.

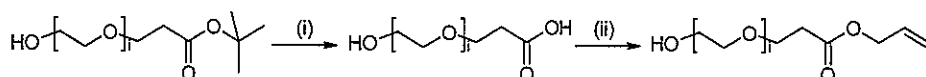
Step (viii): deprotection of the amine with an acid solution, for example hydrochloric acid (for example in solution in dioxane).

Step (ix): activation of the amine in the form of carbamoyl chloride; the reaction is performed in an anhydrous polar aprotic solvent such as DCM, by treatment with diphosgene in the presence of a base such as TEA.

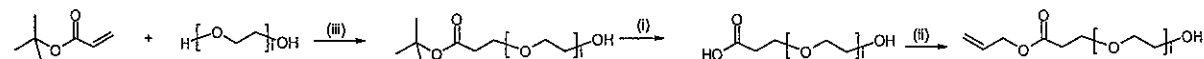
LP₆ $\text{RbZb-OC-ALK-(OCH}_2\text{CH}_2)_i\text{-OH}$ prepared according to the scheme below:

in the case where $\text{ALK}=\text{CH}_2\text{CH}_2$

Route A:



Route B:

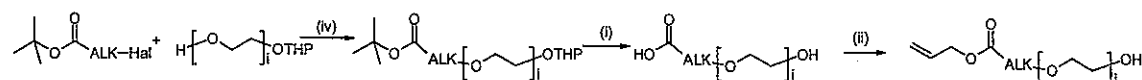


Step (i): deprotection with a solution of hydrochloric acid (for example solution in dioxane) or of trifluoroacetic acid.

Step (ii): protection of the carboxylic acid in allylic ester form; the reaction is performed at RT in a polar aprotic solvent such as DMF in the presence of allyl bromide and of a base such as potassium carbonate.

Step (iii): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF, by treatment of an unsaturated acid protected in ester form with the alkoxide generated by the action of sodium in catalytic amount.

in the case where $\text{ALK}\neq\text{CH}_2\text{CH}_2$

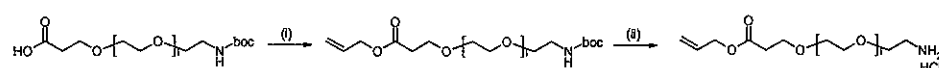


Step (iv): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF or DMF by treatment of a halogenated ester with the alkoxide of a PEG diol monoprotected in tetrahydropyran (THP) ether form. The preparation of this type of monoprotected PEG diol is well described in the literature: see, for example, Richard A. et al. *Chem. Eur. J.* **2005**, *11*, 7315-7321 or Sakellariou E.G., et al. *Tetrahedron* **2003**, *59*, 9083-9090. The PEG alcohols comprising an acid function protected in *tert*-butyl ester form are commercially available (for instance *tert*-butyl 12-hydroxy-4,7,10-trioxadodecanoate) or prepared from *tert*-butyl acrylate and a PEG diol. The starting PEG diols are commercially available for $i=3$ to 12.

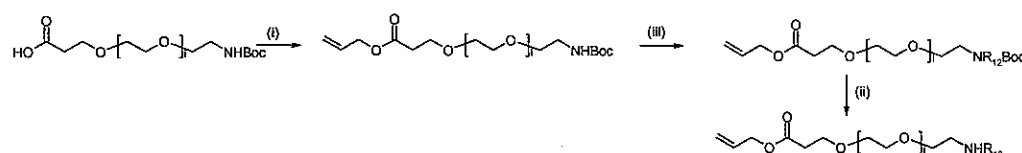
10 **LP₇** $\text{RbZbOC-ALK-(OCH}_2\text{CH}_2)_i\text{-NHR}_{12}$ prepared according to the schemes below:

in the case where $\text{ALK}=\text{CH}_2\text{CH}_2$

in the case where $\text{R}_{12}=\text{H}$



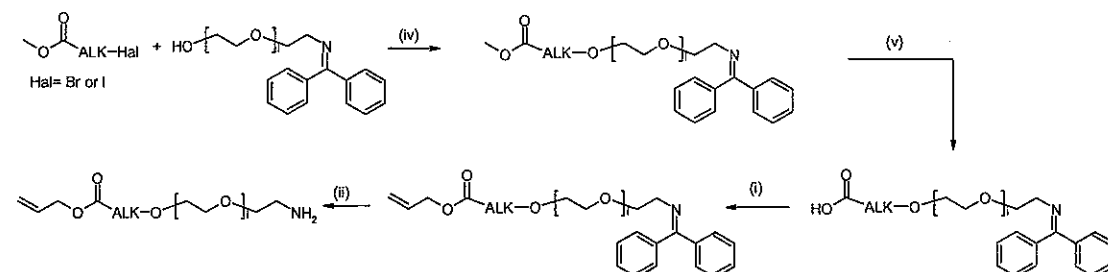
in the case where $\text{R}_{12}\neq\text{H}$



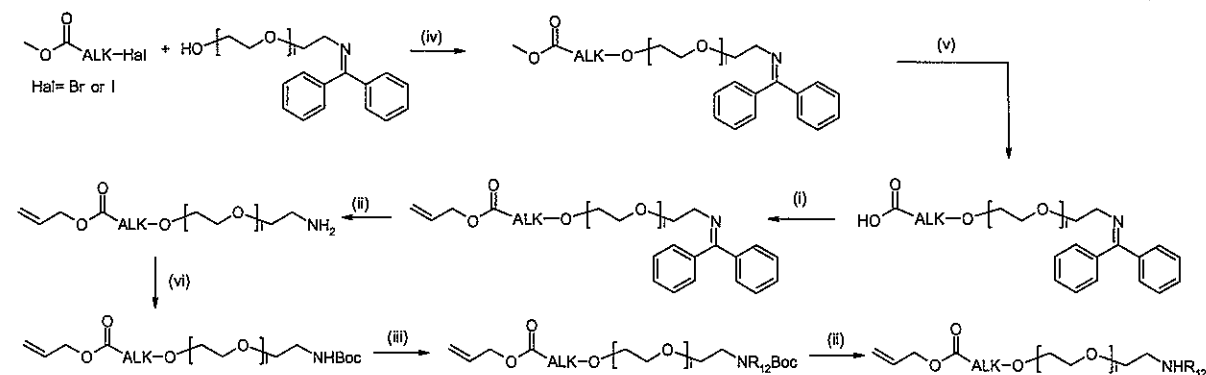
15

in the case where $\text{ALK}\neq\text{CH}_2\text{CH}_2$

in the case where $\text{R}_{12}=\text{H}$



in the case where $\text{R}_{12}\neq\text{H}$



20

Step (i): protection of the carboxylic acid in allylic ester form; the reaction is performed at RT in a polar aprotic solvent such as DCM in the presence of allyl alcohol, a coupling agent such as EDCI

and a base such as DMAP. The procedure may be based on the conditions of Example 14, compound 42.

Step (ii): deprotection using a solution of hydrochloric acid (for example solution in dioxane) or of trifluoroacetic acid. The procedure may be based on the conditions of Example 14, compound 43.

- 5 Step (iii): alkylation of the nitrogen atom; the reaction is performed in an anhydrous polar aprotic solvent such as THF by treatment with a base such as NaH in the presence of a reagent bearing a nucleofugal group such as an alkyl halide. The procedure may be based on the conditions of Example 15, compound 46.

- 10 Step (iv): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF or DMF by treatment of a halogenated ester with the alkoxide of a benzophenone-imine-PEG-alcohol generated via the action of NaH or potassium naphthalenide as described in **WO 2007/127 440**;

Step (v): saponification of the ester; the reaction is performed by reacting the ester with lithium hydroxide in the presence of water.

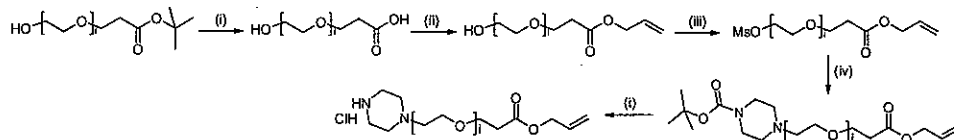
- 15 Step (vi): protection of the amine; the reaction is performed at RT in a polar aprotic solvent such as DCM by treating the amine with di-*tert*-butyl dicarbonate in the presence of a base, for instance TEA.

The amino-PEG-acids are commercially available for $i=3,5,6,10$ or may be prepared from *tert*-butyl acrylate and the corresponding amino-PEG-alcohol.

- 20 The amino-PEG-alcohols are commercially available, for example for $i=3, 4, 7, 8$ or may be prepared from the PEG diols, which are commercially available for $i=3$ to 12, according to the procedure described in **US 7 230 101**. The protection of the amine function with benzophenone may be performed by azeotropic dehydration in the presence of a Lewis acid such as BF_3 etherate.

25

$\text{RbZbOC-ALK-(OCH}_2\text{CH}_2)_i\text{N}$ prepared according to the schemes below:
 LP₈
 in the case where $\text{ALK}=\text{CH}_2\text{CH}_2$



- 30 Step (i): deprotection of the starting compound using a solution of hydrochloric acid (for example solution in dioxane) or of trifluoroacetic acid. The procedure may be based on the conditions of Example 16, compound 50.

Step (ii): protection of the carboxylic acid function in allylic ester form; the reaction is performed at RT in a polar aprotic solvent such as DMF in the presence of allyl bromide and a base such as potassium carbonate. The procedure may be based on the conditions of Example 16, compound

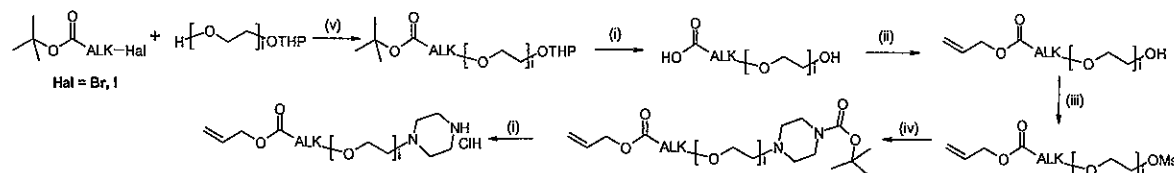
35 51.

Step (iii): activation of the alcohol in mesylate form; the reaction is performed in an anhydrous polar aprotic solvent such as DCM by treatment with mesyl chloride in the presence of a base such as TEA.

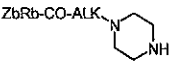
Step (iv): reaction between the mesylate function and the amine function of the compound

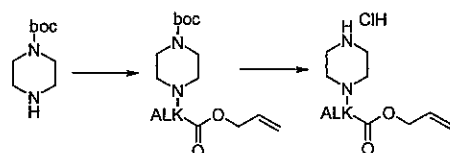
- 5  (alkylation); the reaction is performed at RT in an anhydrous polar aprotic solvent such as DCM in the presence of a base such as TEA. The procedure may be based on the conditions of Example 16, compound 52.

in the case where $ALK \neq CH_2CH_2$

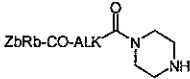


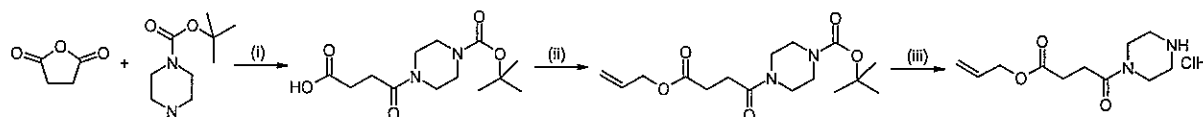
- 10 Step (v): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF or DMF by treatment of a halogenated ester with the alkoxide of a PEG diol monoprotected in tetrahydropyran (THP) ether form. The preparation of this type of monoprotected PEG diol is well described in the literature: see, for example, Richard A. et al. *Chem. Eur. J.* **2005**, *11*, 7315-7321 or Sakellariou E.G., et al. *Tetrahedron* **2003**, *59*, 9083-9090.
- 15 The PEG alcohols comprising an acid function protected in *tert*-butyl ester form are commercially available (for instance *tert*-butyl 12-hydroxy-4,7,10-trioxadodecanoate) or prepared from *tert*-butyl acrylate and a PEG diol. The starting PEG diols are commercially available for $i = 3$ to 12.

LP₉  prepared according to the scheme below:

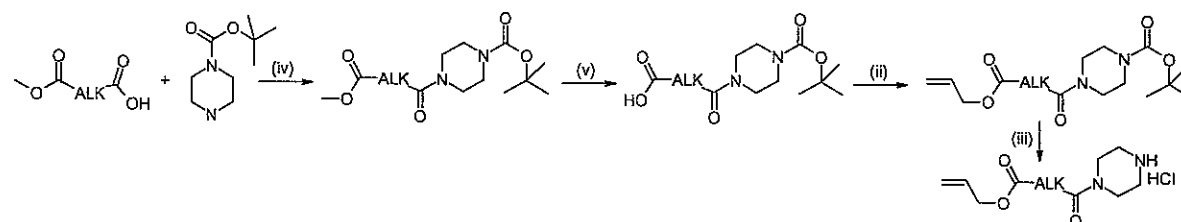


- 20 Step (i): alkylation of the amine; the reaction is performed in an anhydrous polar aprotic solvent such as acetonitrile with an allyl haloalkylcarboxylate, for instance allyl bromoacetate, in the presence of a base, for instance TEA. The procedure may be based on the conditions of Example 13, compound 38.
- 25 Step (ii): deprotection of the amine using an acid solution, for example hydrochloric acid (for example in solution in dioxane). The procedure may be based on the conditions of Example 13, compound 39.
- The allyl haloalkylcarboxylate may be obtained from allyl alcohol and the corresponding haloacyl halide and is commercially available for $ALK = -(CH_2)_{1-6}-$ (for instance bromoacetyl bromide or 4-butanoyl chloride).
- 30

LP₁₀  prepared according to the schemes below:
in the case where $ALK=CH_2CH_2$



5 in the case where $ALK \neq CH_2CH_2$



Step (i): opening of the cyclic anhydride; the reaction is performed at RT in an anhydrous polar aprotic solvent such as DCM in the presence of a base such as TEA.

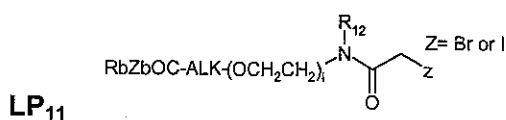
Step (ii): protection of the carboxylic acid in allylic ester form; the reaction is performed at RT in a polar aprotic solvent such as DCM in the presence of allyl alcohol, a coupling agent such as EDCI and a base such as DMAP.

Step (iii): deprotection using a solution of hydrochloric acid (for example solution in dioxane).

Step (iv): peptide coupling; the reaction between the carboxylic acid and the amine is performed at RT in a polar aprotic solvent such as DCM in the presence of a coupling agent such as the DIC/HOBt system.

Step (v): saponification of the methyl ester; the reaction is performed at RT in a mixture of polar solvents such as a THF/water mixture in the presence of lithium hydroxide.

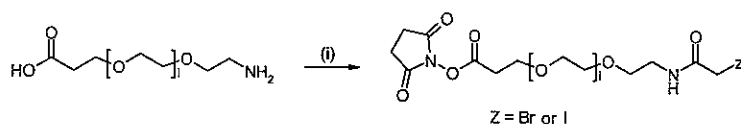
The diacids monoprotected in methyl ester form are commercially available for $ALK=-(CH_2)_{1-6}-$ (such as the monomethyl ester of 1,6-hexanedioic acid).



prepared according to the schemes below:

in the case where $ALK=CH_2CH_2$

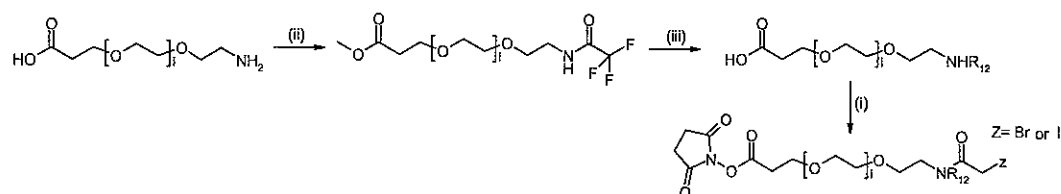
in the case where $R_{12}=H$



Step (i): formation of the amide and activation of the acid; the two steps are performed successively in a polar aprotic solvent such as DCM: reaction between the amine function and the N-hydroxysuccinimidyl haloacetate, followed by *in situ* addition of a coupling agent such as

DIC. The procedure may be based on the conditions of Example 17, compound 56.

in the case where $R_{12} \neq H$

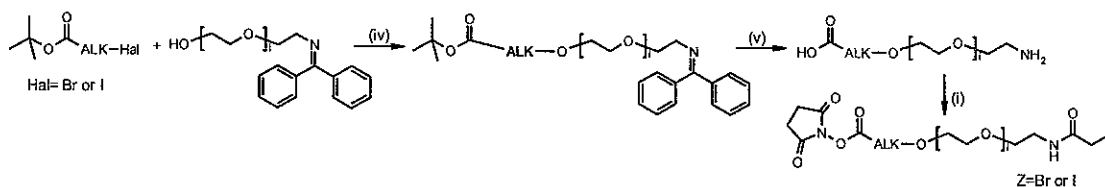


- 5 Step (ii): protections of the carboxylic acid in methyl ester form and of the amine in trifluoroacetamide form; the reaction is performed in two successive steps in a polar aprotic solvent such as DCM: protection of the acid by treatment with trimethylsilyldiazomethane in the presence of methanol, followed by protection of the amine by addition of trifluoroacetic anhydride and of a base such as TEA.
- 10 Step (iii): alkylation of the amine and saponification of the ester; the reaction is performed in two successive steps in an anhydrous polar aprotic solvent such as THF: alkylation of the amine by treatment with a base such as NaH in the presence of a reagent bearing a nucleofugal group, for instance an alkyl halide $R_{12}Hal$, followed by addition of lithium hydroxide and water.
- Step (i): following step (iii), the reactions of step (i) for the case where $R_{12} = H$ are repeated.

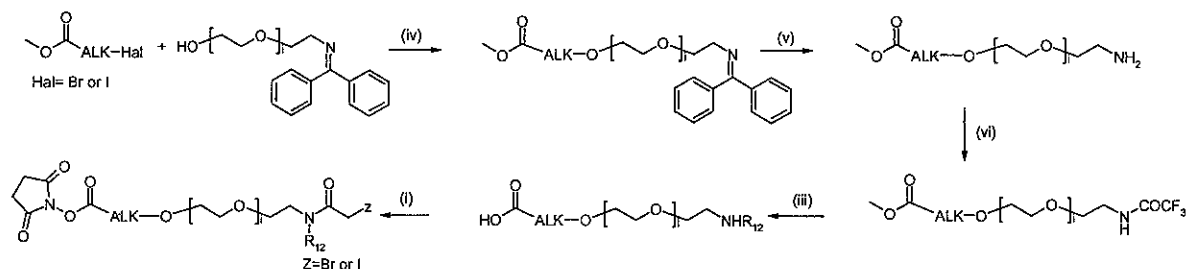
15

in the case where $ALK \neq CH_2CH_2$

in the case where $R_{12} = H$



in the case where $R_{12} \neq H$



20

Step (iv): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF or DMF by treatment of a halogenated ester with the alkoxide of a benzophenone-imine-PEG-alcohol generated via the action of NaH or potassium naphthalenide (cf. **WO 2007/127 440**);

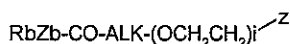
- 25 Step (v): selective cleavage of the imine by hydrogenation in the presence of palladium-on-charcoal (cf. Wessjohann, L. et al., *Synthesis* **1989**, 5, 359-63);

Step (vi): protection of the amine by addition of trifluoroacetic anhydride and of a base such as TEA.

The amino-PEG-acids are commercially available for $i=3, 5, 6, 10$ or may be prepared from *tert*-butyl acrylate and the corresponding amino-PEG-alcohol.

- 5 The amino-PEG-alcohols are commercially available, for example for $i=3, 4, 7, 8$ or may be prepared from the PEG diols, which are commercially available for $i=3$ to 12, according to the procedure described in **US 7 230 101**. The protection of the amine function with benzophenone may be performed by azeotropic dehydration in the presence of a Lewis acid such as BF_3 etherate.

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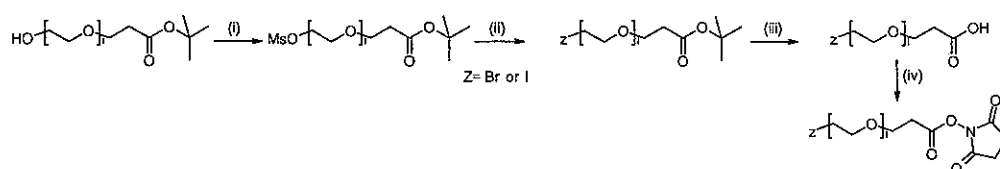
LP₁₂

Z= Br or I

prepared according to the schemes below:

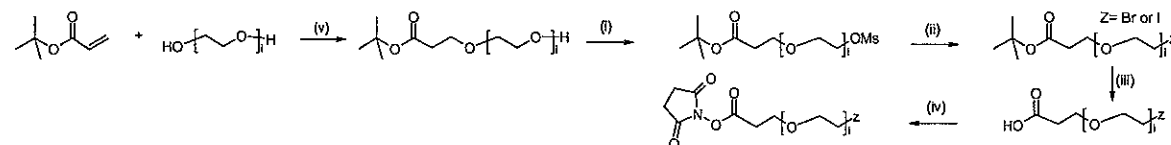
in the case where $\text{ALK}=\text{CH}_2\text{CH}_2$

Route A:

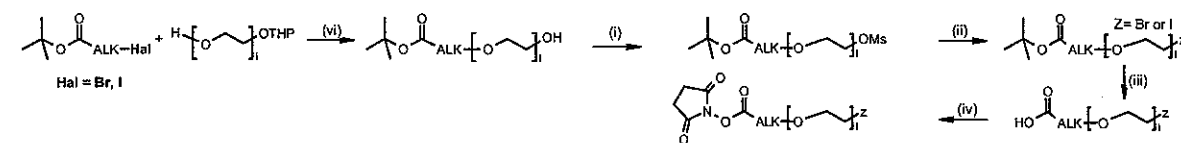


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Route B:



in the case where $\text{ALK} \neq \text{CH}_2\text{CH}_2$



20

Step (i): activation of the alcohol in mesylate form; the reaction is performed in an anhydrous polar aprotic solvent such as DCM by treatment with mesyl chloride in the presence of a base such as TEA.

- 25 Step (ii): mesylate/halogen exchange; the reaction is performed in a refluxing polar aprotic solvent such as acetone, with a sodium halide such as sodium iodide.

Step (iii): deprotection using a solution of hydrochloric acid (for example solution in dioxane) or of trifluoroacetic acid.

Step (iv): activation of the acid; the reaction is performed at RT in a polar aprotic solvent such as

DCM by treatment with NHS in the presence of a coupling agent such as DCC.

Step (v): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF by treatment of an unsaturated acid protected in ester form with the alkoxide generated by the action of sodium in catalytic amount.

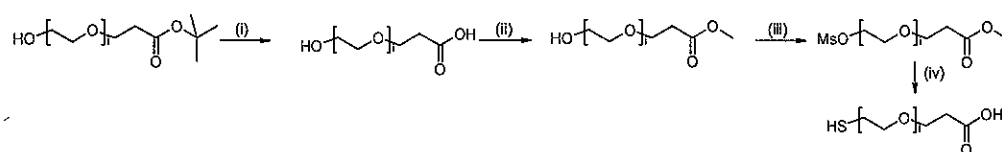
- 5 Step (vi): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF or DMF by treatment of a halogenated ester with the alkoxide of PEG diol monoprotected as the tetrahydropyran (THP) ether. The preparation of this type of monoprotected PEG diol is well described in the literature: see, for example, Richard A. et al. *Chem. Eur. J.* **2005**, *11*, 7315-7321 or Sakellariou E.G., et al. *Tetrahedron* **2003**, *59*, 9083-9090.

- 10 The intermediate formed is selectively hydrolysed at pH 5 to the hydroxy ester.

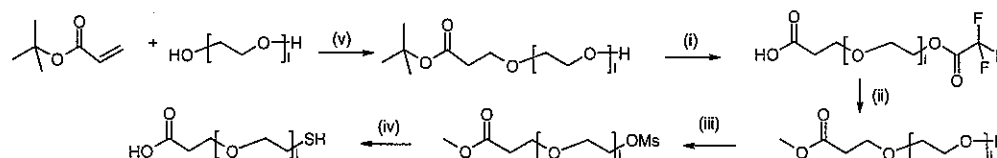
The PEG alcohols comprising an acid function protected in *tert*-butyl ester form are commercially available (for instance *tert*-butyl 12-hydroxy-4,7,10-trioxadodecanoate) or prepared from *tert*-butyl acrylate and a PEG diol. The starting PEG diols are commercially available for $i=3$ to 12.

- 15 **LP₁₃** $\text{RbZb-CO-ALK-(OCH}_2\text{CH}_2)_i\text{-SH}$ prepared according to the schemes below:
in the case where $\text{ALK}=\text{CH}_2\text{CH}_2$

Route A:

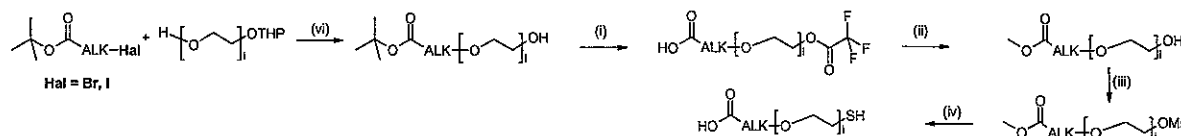


Route B:



20

in the case where $\text{ALK}\neq\text{CH}_2\text{CH}_2$



Step (i): deprotection using a solution of hydrochloric acid (e.g. solution in dioxane) or of trifluoroacetic acid. In the latter case, trifluoroacetate of the hydroxy function may be formed. It is cleaved during the following step (ii).

- 25 Step (ii): protection of the carboxylic acid in methyl ester form; the reaction is performed at RT in a polar aprotic solvent such as methanol, by treatment with trimethylsilyldiazomethane.

Step (iii): activation of the alcohol in mesylate form; the reaction is performed in an anhydrous polar aprotic solvent such as DCM by treatment with mesyl chloride in the presence of a base such as TEA.

30

Step (iv): formation of the free thiol and saponification of the methyl ester; the reaction is

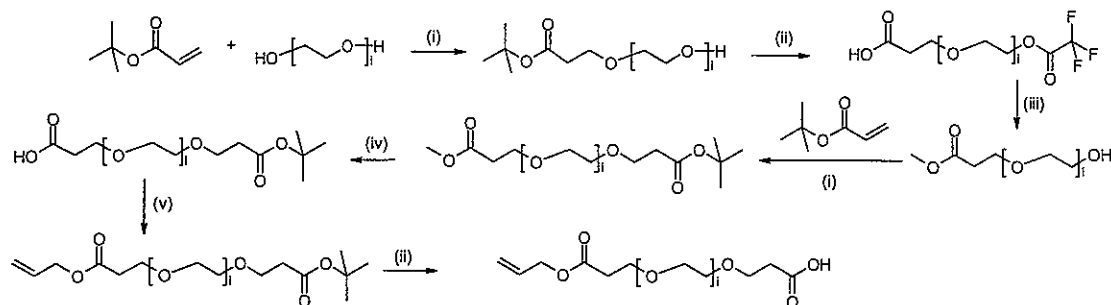
performed in a refluxing polar protic solvent, such as an ethanol/water mixture, in two successive steps: displacement of the mesylate with thiourea, followed by *in situ* hydrolysis of the isothiuronium salt by addition of a base such as sodium hydroxide.

Step (v): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF by treatment of an unsaturated acid protected in ester form with the alkoxide generated by the action of sodium in catalytic amount.

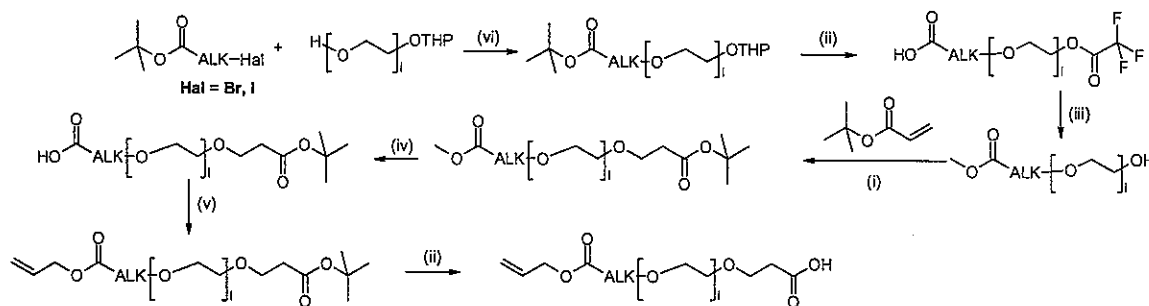
Step (vi): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF or DMF by treatment of a halogenated ester with the alkoxide of the PEG diol monoprotected as the tetrahydropyran (THP) ether. The preparation of this type of monoprotected PEG diol is well known in the literature: see, for example, Richard A. et al. *Chem. Eur. J.* **2005**, 11, 7315-7321 or Sakellariou E.G., et al. *Tetrahedron* **2003**, 59, 9083-9090.

The linker with $n = 8$ (3-[2-mercaptoethoxyhepta(ethyleneoxy)]propionic acid) is commercially available. The PEG alcohols comprising an acid function protected in *tert*-butyl ester form are commercially available (for instance *tert*-butyl 12-hydroxy-4,7,10-trioxadodecanoate) or prepared from *tert*-butyl acrylate and a PEG diol. The starting PEG diols are commercially available for $i=3$ to 12.

LP₁₄ $\text{RbZbOC-ALK-(OCH}_2\text{CH}_2\text{)}_{i+1}\text{COOH}$ prepared according to the scheme below:
in the case where $\text{ALK}=\text{CH}_2\text{CH}_2$



in the case where $\text{ALK} \neq \text{CH}_2\text{CH}_2$



Step (i): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent, such as THF, by treatment of an unsaturated acid protected in ester form with the alkoxide generated by the action of sodium in catalytic amount.

Step (ii): deprotection using a solution of hydrochloric acid (for example solution in dioxane) or of

trifluoroacetic acid. In the latter case, trifluoroacetate of the alcohol function optionally present on the structure may be formed. This trifluoroacetate is cleaved during the following step (iii).

Step (iii): protection of the carboxylic acid in methyl ester form; the reaction is performed at RT in a polar aprotic solvent such as methanol, by treatment with trimethylsilyldiazomethane.

- 5 **Step (iv):** saponification of the methyl ester; the reaction is performed at RT in a mixture of polar solvents such as a THF/water mixture in the presence of lithium hydroxide.

Step (v): protection of the carboxylic acid in allyl ester form; the reaction is performed at RT in a polar aprotic solvent such as DCM in the presence of allyl alcohol, a coupling agent such as EDCI and a base such as DMAP.

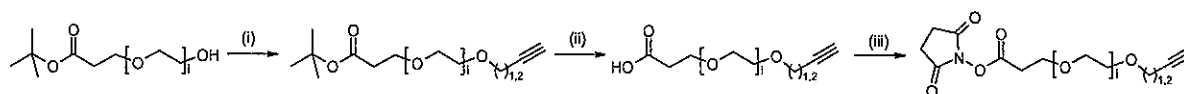
- 10 **Step (vi):** elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF or DMF by treatment of a halogenated ester with the alkoxide of the PEG diol monoprotected as the tetrahydropyran (THP) ether. The preparation of this type of monoprotected PEG diol is well described in the literature: see, for example, Richard A. et al. *Chem. Eur. J.* **2005**, 11, 7315-7321 or Sakellariou E.G., et al. *Tetrahedron* **2003**, 59, 9083-9090.

- 15 The starting PEG diols are commercially available for $i=3$ to 12.

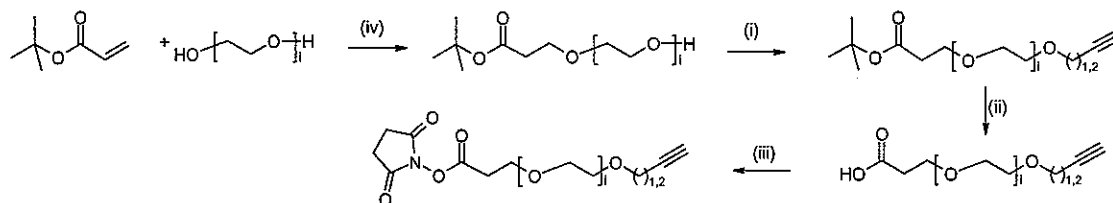
LP₁₅ $\text{RbZb-CO-ALK-(OCH}_2\text{CH}_2)_i\text{O-C}\equiv\text{C-CH}_3$ prepared according to the schemes below:

in the case where $\text{ALK}=\text{CH}_2\text{CH}_2$

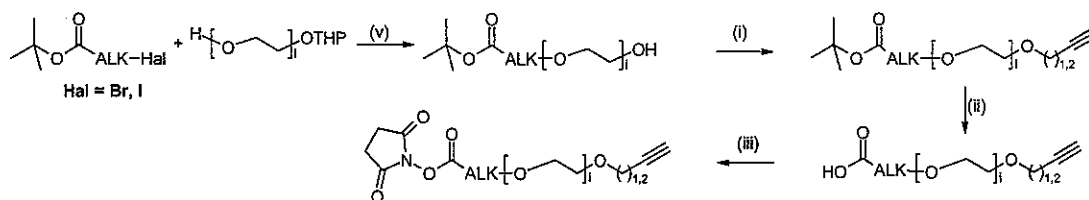
Route A:



Route B:



in the case where $\text{ALK}\neq\text{CH}_2\text{CH}_2$



Step (i): nucleophilic substitution; the reaction is performed in an anhydrous polar aprotic solvent such as THF, in the presence of a base such as NaH and of an alkynyl halide such as propargyl bromide or 4-bromo-1-butyne. The procedure may be based on the conditions of Example 20, compound 63.

- 30 **Step (ii):** deprotection using a solution of hydrochloric acid (for example solution in dioxane) or of

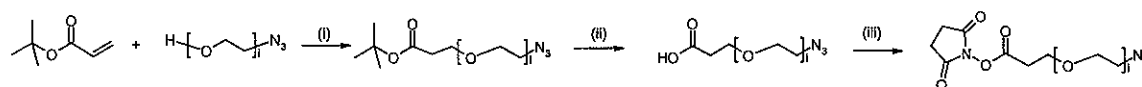
trifluoroacetic acid. The procedure may be based on the conditions of Example 20, compound 65.
Step (iii): activation of the carboxylic acid in NHS ester form; the reaction is performed at RT in a polar aprotic solvent such as DCM by treatment with NHS in the presence of a coupling agent such as supported DCC.

- 5 Step (iv): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF by treatment of an unsaturated acid protected in ester form with the alkoxide generated by the action of sodium in catalytic amount. The procedure may be based on the conditions of Example 20, compound 64.

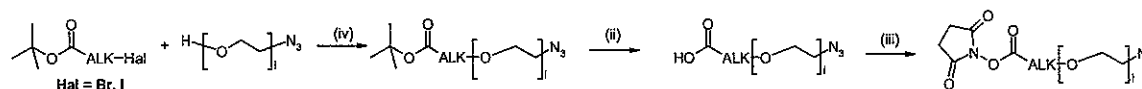
- 10 Step (v): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF or DMF by treatment of a halogenated ester with the alkoxide of the PEG diol monoprotected as the tetrahydropyran (THP) ether. The preparation of this type of monoprotected PEG diol is well described in the literature: see, for example, Richard A. et al. *Chem. Eur. J.* **2005**, 11, 7315-7321 or Sakellariou E.G., et al. *Tetrahedron* **2003**, 59, 9083-9090. The PEG alcohols comprising an acid function protected in *tert*-butyl ester form are commercially available (for instance *tert*-butyl 12-hydroxy-4,7,10-trioxadodecanoate) or prepared from *tert*-butyl acrylate and a PEG diol. The starting PEG diols are commercially available for i=3 to 12.

LP₁₆ $\text{RbZbOC-ALK-(OCH}_2\text{CH}_2)_i\text{N}_3$ prepared according to the schemes below:

in the case where $\text{ALK}=\text{CH}_2\text{CH}_2$



in the case where $\text{ALK} \neq \text{CH}_2\text{CH}_2$



- 25 Step (i): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF by treatment of an unsaturated acid protected in ester form with the alkoxide generated by the action of sodium in catalytic amount.

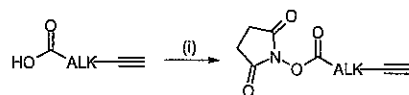
Step (ii): deprotection using a solution of hydrochloric acid (for example solution in dioxane) or of trifluoroacetic acid.

- 30 Step (iii): activation of the carboxylic acid in NHS ester form; the reaction is performed at RT in a polar aprotic solvent such as DCM by treatment with NHS in the presence of a coupling agent such as supported DCC.

Step (iv): elongation of the hydroxyazido PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF or DMF by treatment of a halogenated ester with the alkoxide of hydroxyazido PEG.

- 35 The azido PEG alcohols are commercially available or may be prepared from the corresponding PEG alcohols that are commercially available for i=3 to 12.

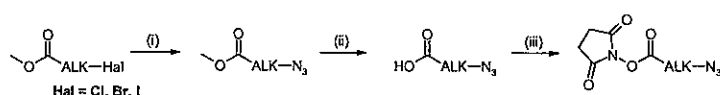
LP₁₇ $\text{RbZb-CO-ALK}\equiv$ prepared according to the scheme below:



Step (i): activation of the carboxylic acid in NHS ester form; the reaction is performed at RT in a polar aprotic solvent such as DCM by treatment with NHS in the presence of a coupling agent such as supported DCC.

The acids bearing an acetylenic group are commercially available for $\text{ALK} = (\text{CH}_2)_m-$ with $m=1$ to 10 (for instance 3-butyneic acid).

LP₁₈ RbZb-CO-ALK-N_3 prepared according to the scheme below:



Step (i): nucleophilic substitution of the halide by the azide; the reaction is performed in a polar aprotic solvent such as acetone, in the presence of sodium azide.

Step (ii): saponification of the methyl ester; the reaction is performed at RT in a mixture of polar solvents such as a THF/water mixture in the presence of lithium hydroxide.

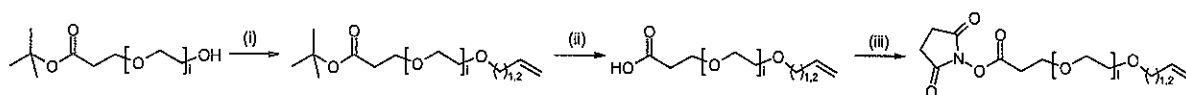
Step (iii): activation of the carboxylic acid in NHS ester form; the reaction is performed at RT in a polar aprotic solvent such as DCM by treatment with NHS in the presence of a coupling agent such as supported DCC.

The methyl esters bearing a haloalkyl unit are commercially available for $\text{ALK} = (\text{CH}_2)_m-$ with $m=1$ to 6 (for instance methyl bromoacetate).

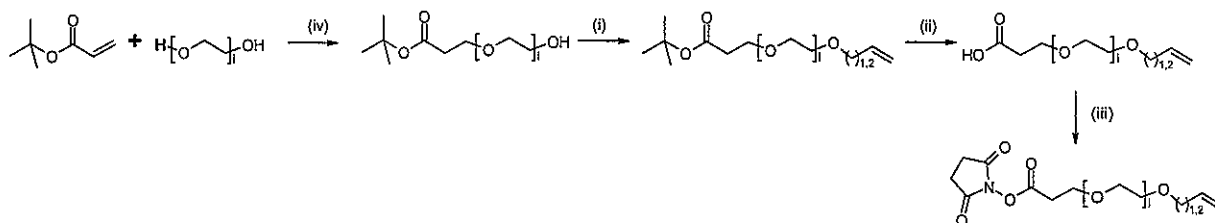
LP₁₉ $\text{RbZb-CO-ALK-(OCH}_2\text{CH}_2)_i\text{O-CH}_2\text{CH=CH}_2$ prepared according to the schemes below:

in the case where $\text{ALK} = \text{CH}_2\text{CH}_2$

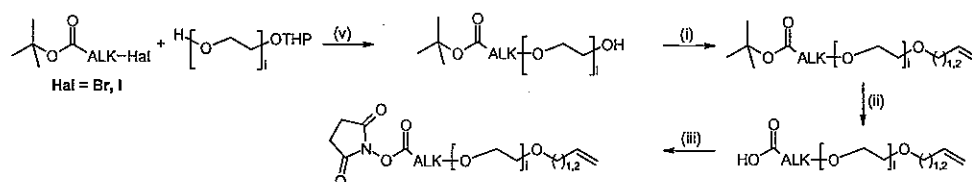
Route A:



Route B:



in the case where $\text{ALK} \neq \text{CH}_2\text{CH}_2$



Step (i): nucleophilic substitution; the reaction is performed in an anhydrous polar aprotic solvent such as THF in the presence of a base such as NaH and of an alkenyl halide such as allyl bromide or 4-bromo-1-butene.

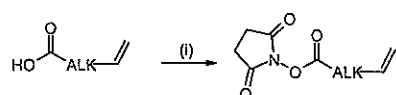
- 5 Step (ii): deprotection using a solution of hydrochloric acid (for example solution in dioxane) or of trifluoroacetic acid.

Step (iii): activation of the carboxylic acid in NHS ester form; the reaction is performed at RT in a polar aprotic solvent such as DCM by treatment with NHS in the presence of a coupling agent such as supported DCC.

- 10 Step (iv): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF by treatment of an unsaturated acid protected in ester form with the alkoxide generated by the action of sodium in catalytic amount.

- 15 Step (v): elongation of the PEG chain; the reaction is performed in an anhydrous polar aprotic solvent such as THF or DMF by treatment of a halogenated ester with the alkoxide of the PEG diol monoprotected as the tetrahydropyran (THP) ether. The preparation of this type of monoprotected PEG diol is well described in the literature: see, for example, Richard A. et al. *Chem. Eur. J.* **2005**, 11, 7315-7321 or Sakellariou E.G., et al. *Tetrahedron* **2003**, 59, 9083-9090. The PEG alcohols comprising an acid function protected in *tert*-butyl ester form are commercially available (for instance *tert*-butyl 12-hydroxy-4,7,10-trioxadodecanoate) or prepared from *tert*-butyl acrylate and a PEG diol. The starting PEG diols are commercially available for $i=3$ to 12.
- 20

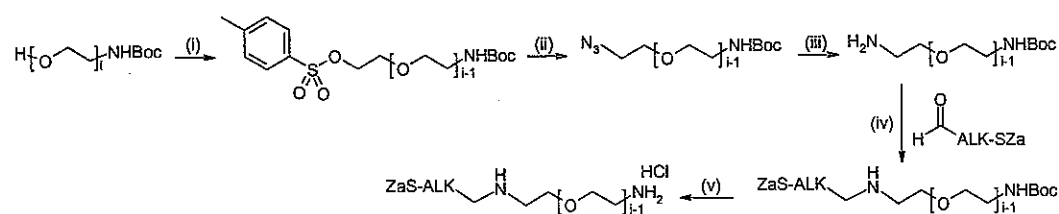
LP₂₀ $\text{RbZb-OC-ALK-CH=CH}_2$ prepared according to the scheme below:



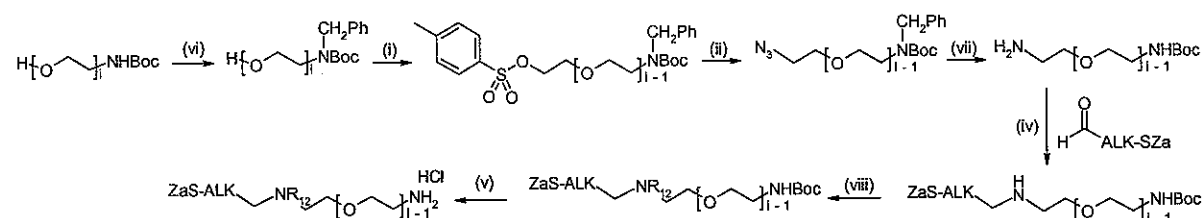
- 25 Step (i): activation of the carboxylic acid in NHS ester form; the reaction is performed at RT in a polar aprotic solvent such as DCM by treatment with NHS in the presence of a coupling agent such as supported DCC.

The acids bearing an ethylenic group are commercially available for $\text{ALK}=\text{-(CH}_2\text{)}_m\text{-}$ with $m=1$ to 10 (for instance 3-butenic acid).

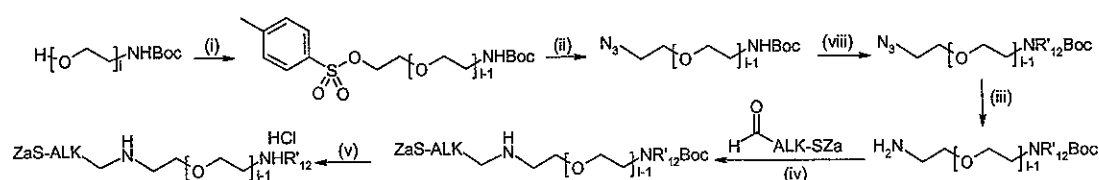
- 30 **LP₂₁** $\text{ZaS-ALK-CH}_2\text{-NR}_{12}\text{-(CH}_2\text{CH}_2\text{O)}_i\text{-CH}_2\text{CH}_2\text{-NHR}'_{12}$ prepared according to the schemes below:
in the case where R_{12} and $\text{R}'_{12}=\text{H}$



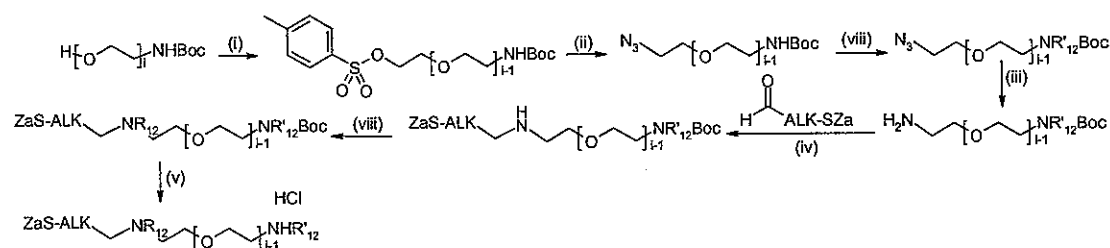
in the case where $R_{12} \neq H$ and $R'_{12} \neq H$



in the case where $R_{12} = H$ and $R'_{12} \neq H$



in the case where R_{12} and $R'_{12} \neq H$



Step (i): activation of the alcohol in tosylate form; the reaction is performed in a polar aprotic solvent such as DCM by treatment with tosyl chloride in the presence of silver oxide and potassium iodide. The procedure may be based on the conditions of Example 6, compound 23.

Step (ii): nucleophilic substitution of the tosylate; the reaction is performed in a polar aprotic solvent such as acetonitrile by treatment with sodium azide. The procedure may be based on the conditions of Example 6, compound 24.

Step (iii): reduction of the azide; the reaction is performed in a polar solvent such as a THF/water mixture in the presence of triphenylphosphine. The procedure may be based on the conditions of Example 6, compound 26.

Step (iv): reductive amination with an aldehyde; the reaction is performed at RT in an anhydrous polar aprotic solvent such as DCM in the presence of a reducing agent such as sodium triacetoxyborohydride and, if necessary, acetic acid as catalyst. The procedure may be based on the conditions of Example 6, compound 27.

Step (v): deprotection using a solution of hydrochloric acid (for example solution in dioxane) or of trifluoroacetic acid. The procedure may be based on the conditions of Example 6, compound 29.

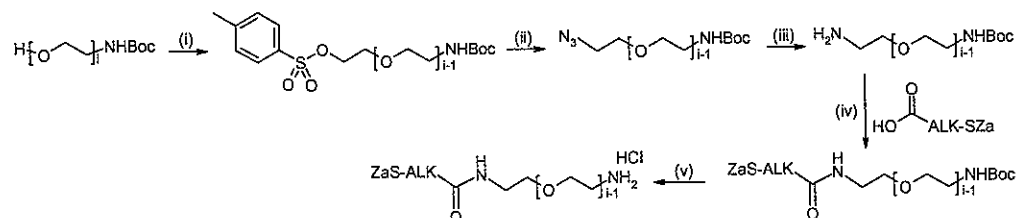
Step (vi): protection of the NHBoc function; the reaction is performed in a polar aprotic solvent such as THF or DMF by treatment with 1 equivalent of base such as sodium hydride, followed by a benzyl halide such as benzyl chloride.

Step (vii): cleavage of the benzyl group and reduction of the azido function; the reaction is performed in a protic solvent such as methanol with hydrogen in the presence of a catalyst such as palladium hydroxide.

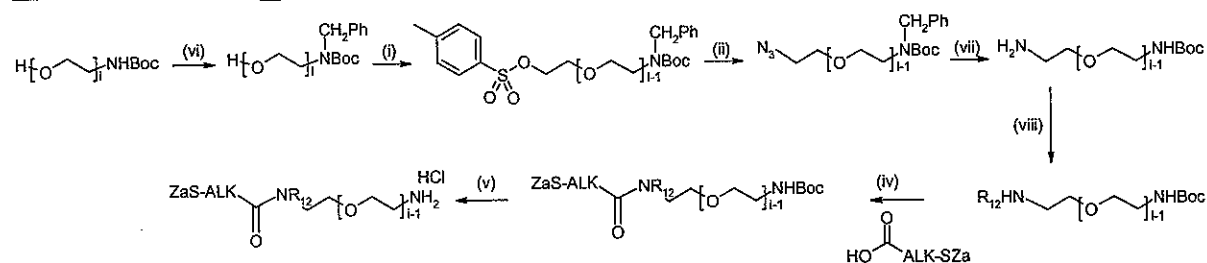
Step (viii): alkylation of the amine; the reaction is performed in an anhydrous polar aprotic solvent such as THF by treatment with a base such as sodium hydride in the presence of a reagent bearing a nucleofugal group such as an alkyl halide. The procedure may be based on the conditions of Example 6, compound 25.

The amino-PEG-alcohols optionally protected with a Boc group on the amine function are commercially available (for instance N-Boc-aminoethoxyethoxyethanol or 1-amino-3,6,9-trioxaundecanyl-11-ol) or may be prepared from PEG diols, which are commercially available for $i=3$ to 12, according to the procedure described in **US 7 230 101**. The aldehyde ZaSS-ALK-CHO, for example 2-methyl-2-(methylthio)propanal, is commercially available or may be prepared by oxidation of an alcohol bearing a disulfide unit obtained from a suitably protected halogenated alcohol (for example in silyl ether form) by successive treatments with potassium thioacetate and a derivative of methanethiosulfonate type.

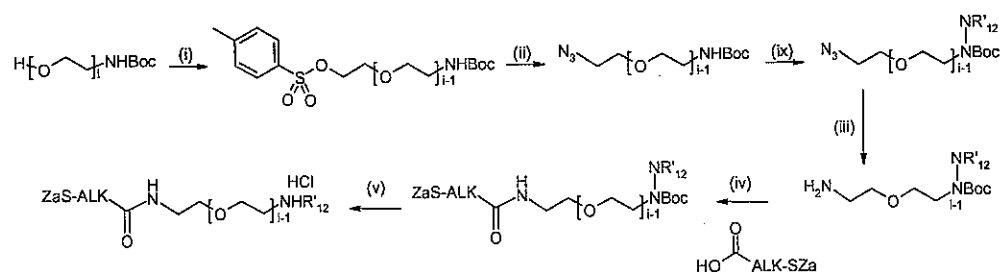
LP₂₂ $\text{ZaS-ALK-C(=O)-NR}_{12}\text{-(CH}_2\text{CH}_2\text{O)}_i\text{-CH}_2\text{CH}_2\text{-NHR}'_{12}$ prepared according to the schemes below:
in the case where R_{12} and $R'_{12}=\text{H}$



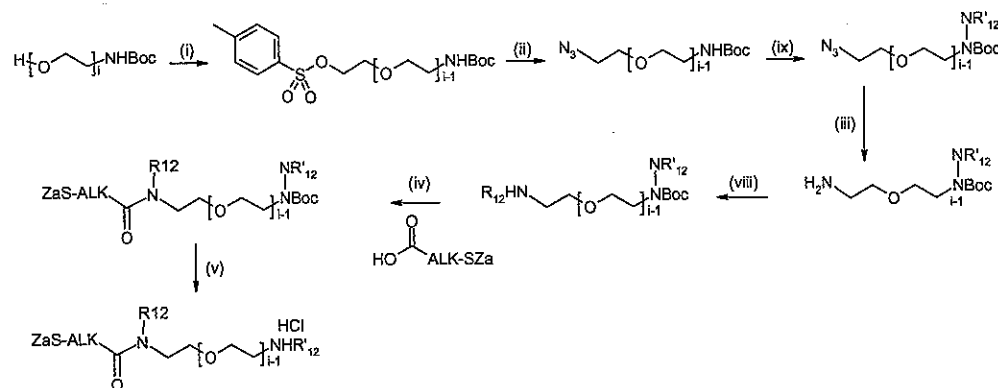
in the case where $R_{12}\neq\text{H}$ and $R'_{12}=\text{H}$



in the case where $R_{12}=\text{H}$ and $R'_{12}\neq\text{H}$



in the case where R_{12} and $R'_{12} \neq H$



Step (i): activation of the alcohol in tosylate form; the reaction is performed in a polar aprotic solvent such as DCM by treatment with tosyl chloride in the presence of silver oxide and potassium iodide. The process may be based on the conditions of Example 6, compound 23.

Step (ii): nucleophilic substitution of the tosylate; the reaction is performed in a polar aprotic solvent such as acetonitrile by treatment with sodium azide. The process may be based on the conditions of Example 6, compound 24.

Step (iii): reduction of the azide; the reaction is performed in a polar solvent such as a THF/water mixture in the presence of triphenylphosphine. The procedure may be based on the conditions of Example 6, compound 26.

Step (iv): peptide coupling; the reaction is performed in a polar aprotic solvent such as dimethylformamide in the presence of coupling agents such as the N,N'-diisopropylcarbodiimide/1-hydroxybenzotriazole system and of a base such as TEA.

Step (v): deprotection using a solution of hydrochloric acid (for example solution in dioxane) or of trifluoroacetic acid. The procedure may be based on the conditions of Example 7, compound 32.

Step (vi): protection of the function NHBoc; the reaction is performed in a polar aprotic solvent such as THF or DMF by treatment with 1 equivalent of base such as sodium hydride, followed by a benzyl halide such as benzyl chloride.

Step (vii): cleavage of the benzyl group and reduction of the azido function; the reaction is performed in a protic solvent such as methanol, with hydrogen in the presence of a catalyst such as palladium hydroxide.

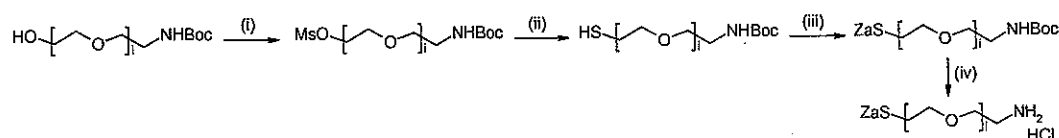
Step (viii): alkylation of the amine by reductive amination with an aldehyde; the reaction is performed at RT in an anhydrous polar aprotic solvent such as DCM in the presence of a reducing agent such as sodium triacetoxyborohydride and, if necessary, acetic acid as catalyst.

Step (ix): alkylation of the NHBoc group; the reaction is performed in an anhydrous polar aprotic solvent such as THF by treatment with a base such as sodium hydride in the presence of a reagent bearing a nucleofugal group such as an alkyl halide. The procedure may be based on the conditions of Example 6, compound 25.

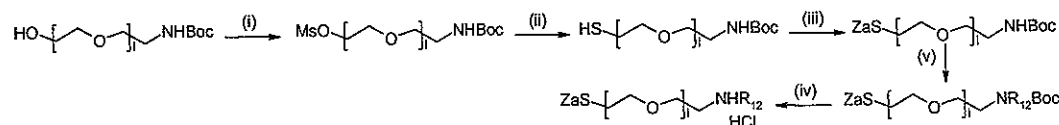
- 5 The amino-PEG-alcohols protected or otherwise with a Boc group on the amine function are commercially available (for instance N-Boc-aminoethoxyethoxyethanol or 1-amino-3,6,9-trioxaundecanyl-11-ol) or may be prepared from the PEG diols, which are commercially available for $i=3$ to 12, according to the procedure described in **US 7 230 101**. The carboxylic acid $\text{ZaS-ALK-CO}_2\text{H}$, for example 4-methyl-4-(methyldithio)pentanoic acid, may be commercially available
- 10 or prepared from a halogenated carboxylic acid via successive treatments with potassium thioacetate and a derivative of methanethiosulfonate type.

LP₂₃ $\text{ZaS-(CH}_2\text{CH}_2\text{O)}_i\text{-CH}_2\text{CH}_2\text{-NHR}_{12}$ prepared according to the schemes below:

in the case where $\text{R}_{12}=\text{H}$



in the case where $\text{R}_{12} \neq \text{H}$



Step (i): activation of the alcohol in mesylate form; the reaction is performed in an anhydrous polar aprotic solvent such as DCM by treatment with mesyl chloride in the presence of a base such as TEA.

20

Step (ii): formation of the free thiol; the reaction is performed in a refluxing polar protic solvent such as an ethanol/water mixture in two successive steps: displacement of the mesylate with thiourea, followed by *in situ* hydrolysis of the isothiuronium salt by addition of a base such as sodium hydroxide.

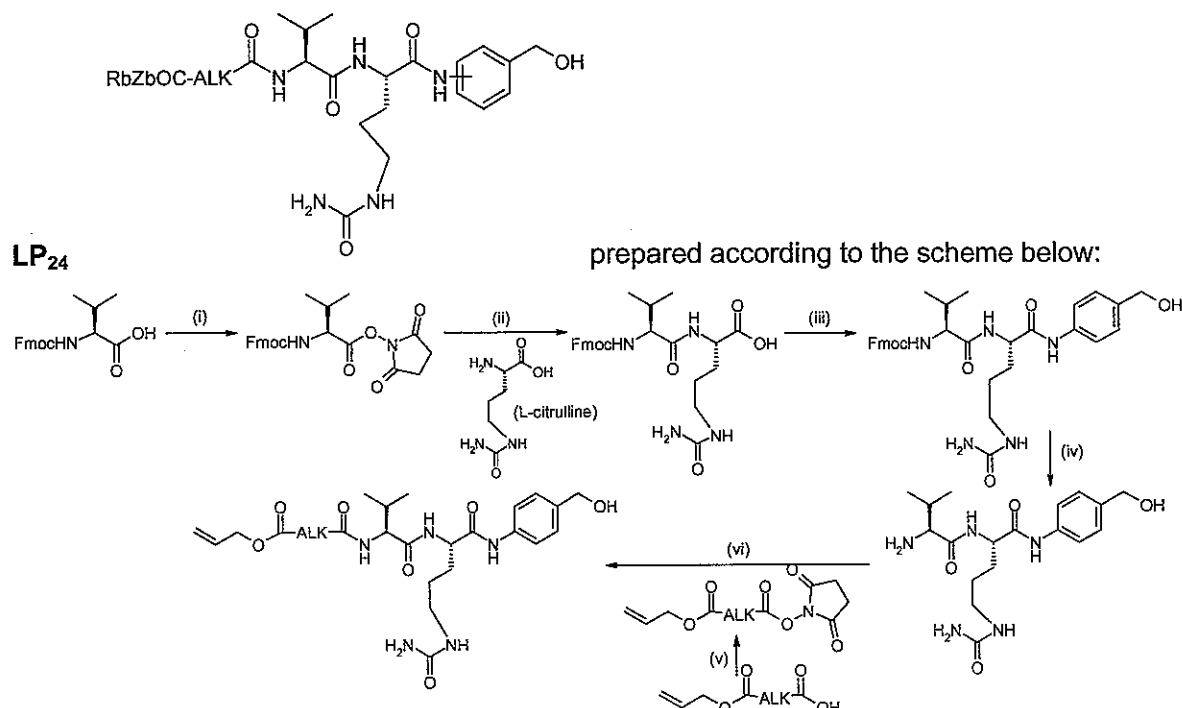
- 25 Step (iii): protection of the thiol; the reaction is performed in a mixture of polar solvents such as an ethanol/water mixture with a reagent comprising a methanethiosulfonate function, for instance methyl methanethiosulfonate, in the presence of a base such as sodium carbonate.

Step (iv): deprotection using a solution of hydrochloric acid (for example a solution in dioxane) or of trifluoroacetic acid.

- 30 Step (v): alkylation of the amine; the reaction is performed in an anhydrous polar aprotic solvent such as THF by treatment with a base such as sodium hydride in the presence of a reagent bearing a nucleofugal group, such as an alkyl halide.

The amino-PEG-alcohols protected or not protected with a Boc group on the amine function are commercially available (for instance N-Boc-aminoethoxyethoxyethanol or 1-amino-3,6,9-

trioxaundecanyl-11-ol) or may be prepared from PEG diols, which are commercially available for $i=3$ to 12, according to the procedure described in **US 7 230 101**.



5 **Step (i):** activation of the Fmoc-L-valine in the form of the NHS ester; the reaction is performed in an anhydrous polar aprotic solvent such as THF by treatment with NHS in the presence of a coupling agent such as DCC.

Step (ii): peptide coupling between Fmoc-L-valine-NHS and L-citrulline; the reaction is performed in a polar solvent such as a dimethoxyethane/THF/water mixture in the presence of a base such as sodium bicarbonate.

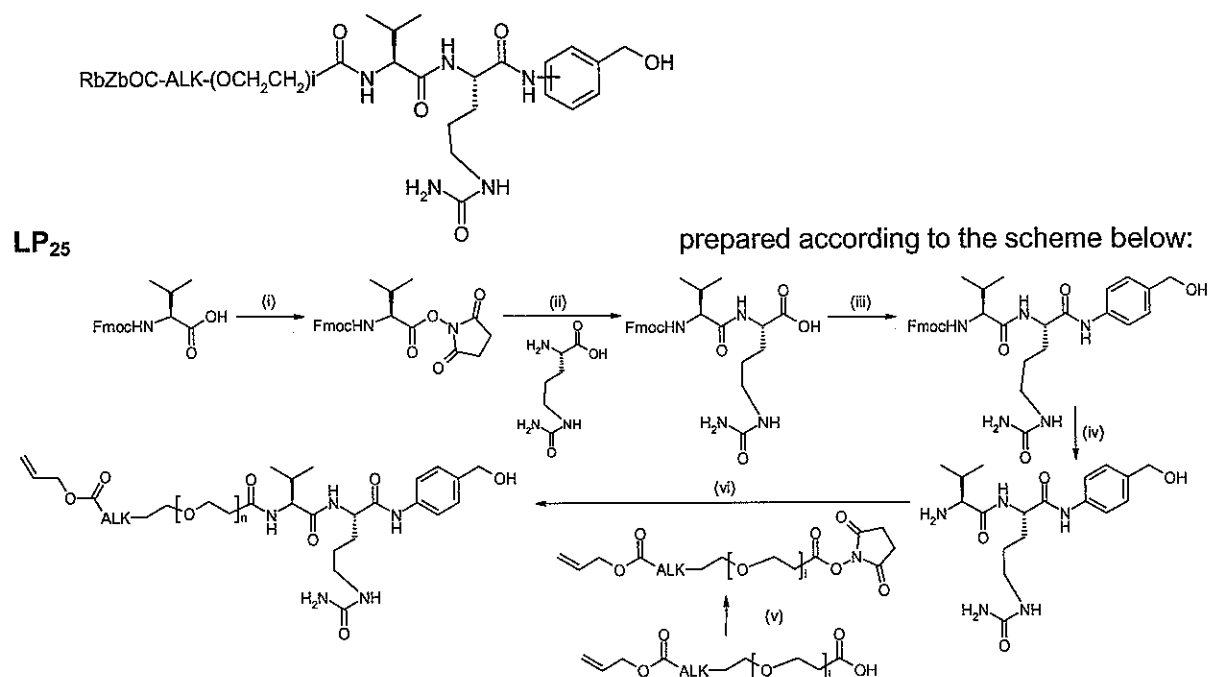
Step (iii): peptide coupling with 4-aminobenzyl alcohol; the reaction is performed in a polar solvent such as a DCM/methanol mixture in the presence of a coupling agent such as EEDQ.

Step (iv): deprotection of the amine Fmoc; the reaction is performed in a polar solvent such as a DCM/methanol mixture in the presence of a base such as diethylamine.

15 **Step (v):** activation of the carboxylic acid in NHS ester form; the reaction is performed in a polar aprotic solvent such as DCM by treatment with NHS in the presence of a coupling agent such as EDCI hydrochloride.

Step (vi): peptide coupling between the dipeptide and the NHS ester; the reaction is performed at RT in a polar aprotic solvent such as a DCM/acetonitrile mixture.

20 The diacids monoprotected in allylic ester form are commercially available for $n=2$ (monoallyl succinate) or may be prepared by transesterification of the methyl or t-butyl monoesters, which are commercially available for $n = 2$ to 6.



Step (i): activation of Fmoc-L-valine in NHS ester form; the reaction is performed in an anhydrous polar aprotic solvent such as THF by treatment with NHS in the presence of a coupling agent such as DCC.

Step (ii): peptide coupling between Fmoc-L-valine-NHS and L-citrulline; the reaction is performed in a polar solvent such as a DME/THF/water mixture in the presence of a base such as sodium bicarbonate.

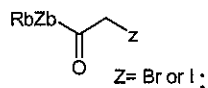
Step (iii): peptide coupling with 4-aminobenzyl alcohol; the reaction is performed in a polar solvent such as a DCM/methanol mixture in the presence of a coupling agent such as EEDQ.

Step (iv): deprotection of the amine Fmoc; the reaction is performed in a polar solvent such as a DCM/methanol mixture in the presence of a base such as diethylamine.

Step (v): activation of the carboxylic acid in NHS ester form; the reaction is performed at RT in a polar aprotic solvent such as DCM by treatment with NHS in the presence of a coupling agent such as supported DCC.

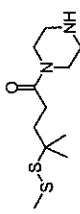
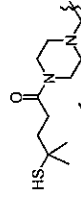
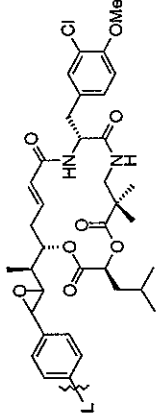
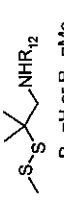
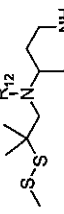
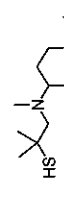
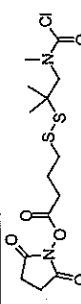
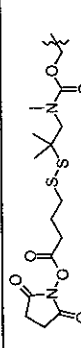
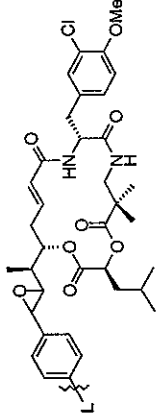
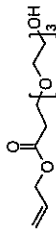
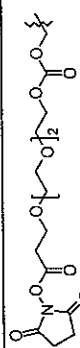
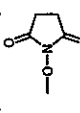
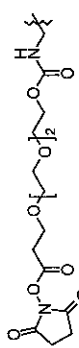
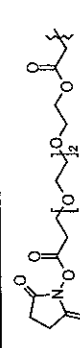
Step (vi): peptide coupling between the dipeptide and the NHS ester; the reaction is performed at RT in a polar aprotic solvent such as a DCM/acetonitrile mixture.

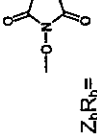
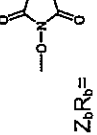
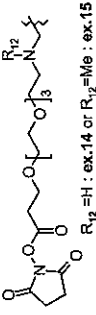
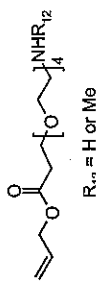
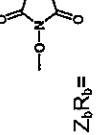
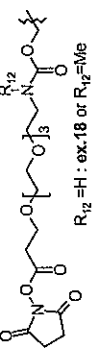
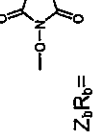
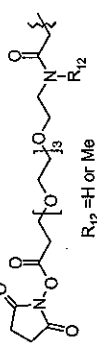
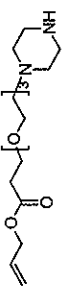
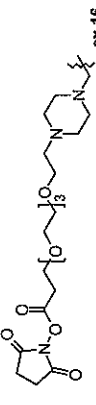
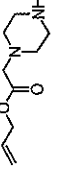
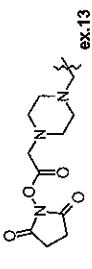
The PEG diacids monoprotected in allylic form are prepared according to the description of the preparation of the linker **L**₁₄.



2,5-Dioxopyrrolidin-1-yl bromoacetate and iodoacetate are commercial products, whose CAS numbers are, respectively, 42014-51-7 and 39028-27-8.

Table II

Linker precursors		G	Reaction(s) ¹	L	Cryptophycin derivatives of formula (II) ^{2, 5}	
LP	examples of LP				Linker family derived from the precursor LP	
LP ₁		-(CH ₂) _n Cl	Nucleophilic substitution [for Z _a =H, additional reduction step]	L ₁		with L = 
LP ₂		-(CH ₂) _n Cl		L ₂		
LP ₃		-(CH ₂) _n Cl		L ₃		
LP ₄		-(CH ₂) _n Cl		L ₄		
LP ₅		-(CH ₂) _n OH	Acylation	L ₅		
LP ₆		-(CH ₂) _n OH	activation, coupling, [optional conversion of a Z _a R _b into another Z _b R _a , for example for	L _{6a}		
		(CH ₂) _n NH ₂	Z _a R _b =  deprotection, activation with NHS]	L _{6b}		
		-(CH ₂) _n CO ₂ H	Esterification, [optional conversion of a Z _a R _b into another Z _b R _a , for example for	L _{6c}		

				 $Z_6R_6=$ deprotection, activation with NHS] nucleophilic substitution, [optionally conversion of a Z_6R_6 into another Z_6R_6, for example for					
		$-(CH_2)_nCl$	 $Z_6R_6=$ deprotection, activation with NHS] activation, coupling, [optionally conversion of a Z_6R_6 into another Z_6R_6, for example for	L7a	$RbZb-CO-ALK-(OCH_2CH_2)_j-N(R_{12})(CH_2)_n-\text{Z}$	 $R_{12} = H$: ex.14 or $R_{12} = Me$: ex.15			
LP7	 $R_{12} = H$ or Me	$-(CH_2)_nOH$	 $Z_6R_6=$ deprotection, activation with NHS] amidation, [optionally conversion of a Z_6R_6 into another Z_6R_6, for example for	L7b	$RbZb-CO-ALK-(OCH_2CH_2)_j-N(R_{12})(CH_2)_n-\text{Z}$	 $R_{12} = H$: ex.18 or $R_{12} = Me$			
		$-(CH_2)_nCO_2H$	 $Z_6R_6=$ deprotection, activation with NHS] activation, coupling, [optionally conversion of a Z_6R_6 into another Z_6R_6, for example for	L7c	$RbZb-CO-ALK-(OCH_2CH_2)_j-N(R_{12})(CH_2)_n-\text{Z}$	 $R_{12} = H$ or Me			
LP8		$-(CH_2)_nCl$	nucleophilic substitution, [optionally conversion of a Z_6R_6 into another Z_6R_6 , for example for	L8	$RbZb-CO-ALK-(OCH_2CH_2)_j-N(R_{12})(CH_2)_n-\text{Z}$	 ex.16			
LP9		$-(CH_2)_nCl$	nucleophilic substitution, [optionally conversion of a Z_6R_6 into another Z_6R_6 , for example for	L9	$RbZb-CO-ALK-(OCH_2CH_2)_j-N(R_{12})(CH_2)_n-\text{Z}$	 ex.13			

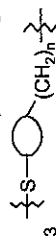
LP₁₀		$-(CH_2)_nCl$		$ZbRb =$ deprotection, activation with NHS	L₁₀		
LP₁₁		$HS-(CH_2)_n-$		nucleophilic substitution	L₁₁		
LP₁₂		$HS-(CH_2)_n-$		nucleophilic substitution	L₁₂		
LP₁₃		$-(CH_2)_nCl$		nucleophilic substitution	L₁₃		
LP₁₄		$(CH_2)_nNH_2$		amidation, deprotection, activation	L_{14a}		
		$-(CH_2)_nOH$		esterification, deprotection, activation	L_{14b}		
LP₁₅		$-(CH_2)_nN_3$		cycloaddition	L₁₅		

LP₁₆		$-(CH_2)_nNR_{12}-CH_2C\equiv CH$	cycloaddition	L₁₆	$RbZb-CO-ALK-(OCH_2CH_2)_i-N=N-N(CH_2)_n-NR_{12}-Z$	
LP₁₇		$-(CH_2)_nN_3$	cycloaddition	L₁₇	$RbZb-CO-ALK-N=N-N(CH_2)_n-Z$	
LP₁₈		$-(CH_2)_nNR_{12}-CH_2C\equiv CH$	cycloaddition	L₁₈	$RbZb-CO-ALK-N=N-N(CH_2)_n-NR_{12}-Z$	
LP₁₉		$-CH=CH_2$	metathesis	L₁₉	$RbZb-CO-ALK-(OCH_2CH_2)_i-O-(CH_2)_n-Z$	
LP₂₀		$-CH=CH_2$	metathesis	L₂₀	$RbZb-CO-ALK-Z$	
LP₂₁		$-(CH_2)_nCl$	nucleophilic substitution [for Z _a =H, additional reduction step]	L₂₁	$ZaS-ALK-CH_2-NR_{12}-N(CH_2)_n-NR_{12}-Z$	
LP₂₂		$-(CH_2)_nCl$		L₂₂	$ZaS-ALK-CH_2-NR_{12}-N(CH_2)_n-NR_{12}-Z$	
LP₂₃		$-(CH_2)_nCl$		L₂₃	$ZaS-(CH_2CH_2O)_i-CH_2CH_2-NR_{12}-N(CH_2)_n-NR_{12}-Z$	
LP₂₄		$-(CH_2)_nNH_2$	activation, coupling, deprotection, activation	L₂₄	$RbZb-CO-ALK-N=N-N(CH_2)_n-NR_{12}-Z$	

LP₂₅		$(CH_2)_nNH_2$	activation, coupling, deprotection, activation	L₂₅		
LP₂₆			nucleophilic substitution	L₂₆		

for further details, see also the "examples of reactions" section above.

the examples are given for a particular cryptophycin derivative, but may apply to any cryptophycin derivative of formula (II), especially D₁-D₈.



corresponds to a cleavable disulfide linker structure chosen from linkers L₁ to L₄ or L₂₁ to L₂₃.

⁴ R₁₂ and R'₁₂: H or group (C₁-C₆)alkyl, for example methyl; n: integer ranging from 1 to 6; i: integer ranging from 1 to 20, more particularly from 1 to 10, more particularly from 1 to 8, or from 1 to 6, and even more particularly from 2 to 5. i may take each of the values of these ranges, and may especially be 2, 3, 4 or 5.

⁵ in Table II, L is given here in the para position, but it is possible to obtain compounds in a similar manner with L in an ortho or meta position.

Process for preparing the conjugate

The conjugate is obtained via the process that consists in:

- (i) placing in contact and leaving to react an optionally buffered aqueous solution of the binding agent and a solution of the cryptophycin derivative of formula (II);
- 5 (ii) and then in optionally separating the conjugate formed in step (i) from the cryptophycin derivative and/or from the unreacted binding agent and/or from any aggregates formed.

More particularly, in step (ii) the conjugate from step (i) is separated only from the unreacted cryptophycin derivative and from any aggregates formed, and any unreacted binding agent is left
10 in the solution.

The function of the placing in contact is to react the chemical groups RCG1 and RCG2 in order to ensure attachment of the cryptophycin derivative to the binding agent by formation of a covalent bond; preferably

- 15
 - when RCG1 represents $-SZ_a$: the binding agent is modified with a modifying agent so as to introduce onto the binding agent suitable groups RCG2, especially those described in the 2nd column of the **Table I**:
 - disulfide chemical groups in the case where RCG1 represents $-SH$;
 - thiol chemical groups in the case where RCG1 represents $-SZ_a$ with $Z_a \neq H$;
 - 20 ○ maleimido or iodoacetamido chemical groups in the case where RCG1 represents $-SH$;

In the case of an antibody (MAb), the formulae of the conjugates are found in the 4th column of Table I;

- 25
 - when RCG1 represents $-C(=O)-Z_bR_b$: the reaction preferably takes place on the amino functions of the binding agent, especially the ϵ -amino groups borne by the side chains of the lysine (Lys) residues of an antibody. In the case of an antibody (MAb), a conjugate of the following formula is obtained in this case: $MAb-[NH-C(=O)-L^*-Crypto]_d$ with L^* = fragment of a linker L comprising $RCG1=-C(=O)-Z_bR_b$ and such that $L = -L^*C(=O)-Z_bR_b$;
 - 30
 - in the presence of a cryptophycin derivative of formula (III) with $G = -(CH_2)_nY$, the binding agent comprises groups $-SH$ when $Y = -Cl$, groups $-C\equiv CH$ when $Y = -N_3$ or carboxylic acid groups when $Y = -OH$ or $-NH_2$;
 - in the presence of a cryptophycin derivative comprising a reactive chemical group RCG1 of maleimido or haloacetamido type, the binding agent comprises thiol chemical groups.

35 The term "aggregates" means associations that may form between two or more binding agents, the binding agents possibly having been modified by conjugation. Aggregates are liable to form under the influence of a wide variety of parameters such as a high concentration of binding agent in the solution, the pH of the solution, high shear forces, the number of grafted dimers and their

hydrophobic nature, the temperature (see the references cited in the introduction of *J. Membrane Sci.* **2008**, 318, 311–316), the influence of some of them not, however, having been clearly elucidated. In the case of proteins or antibodies, reference may be made to *AAPS Journal*, “Protein Aggregation and Bioprocessing” **2006**, 8(3), E572-E579. The aggregate content may be determined via known techniques such as SEC (see in this respect *Analytical Biochemistry* **1993**, 212 (2), 469-480).

The aqueous solution of the binding agent may be buffered, for example with buffers such as potassium phosphate or N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES buffer) or a mixture of buffers such as buffer A described later. The buffer depends on the nature of the binding agent. The cryptophycin derivative is dissolved in a polar organic solvent, for example DMSO or DMA.

The reaction takes place at a temperature generally of between 20 and 40°C. The reaction time may vary between 1 and 24 hours. The reaction between the antibody and the cryptophycin derivative may be monitored by SEC with a refractometric and/or ultraviolet detector in order to determine its degree of progress. If the degree of substitution is insufficient, the reaction can be left for longer and/or cryptophycin derivative can be added. Reference may be made to the general method given in the examples section for further details regarding particular conditions. Particular embodiments are described in Examples 9, 10, 11, 25, 26 and 27.

A person skilled in the art has at his disposal various chromatographic techniques for the separation of step (ii): the conjugate may be purified, for example, by steric exclusion chromatography (SEC), by adsorption chromatography (for instance ion exchange, IEC), by hydrophobic interaction chromatography (HIC), by affinity chromatography, by chromatography on mixed supports such as ceramic hydroxyapatite, or by HPLC. Purification by dialysis or diafiltration may also be used.

After step (i) or (ii), the solution of the conjugate may undergo an ultrafiltration and/or diafiltration step (iii). After these steps, the conjugate in aqueous solution is thus obtained.

Antibody

The antibody (see in this respect, Janeway et al. “Immunobiology”, 5th edition, **2001**, Garland Publishing, New York) may be chosen from those described especially in **WO 04/043 344**, **WO 08/010 101**, **WO 08/047 242**, **WO 05/009 369** (anti CA6) or **WO 2010/014 812**. The antibody may optionally be modified with a modifying agent so as to promote the attachment of the cryptophycin derivative (see above). The antibody may especially be monoclonal, polyclonal or multispecific. It may also be an antibody fragment. It may also be a murine, human, humanized or

chimeric antibody.

Conjugate

A conjugate generally comprises from about 1 to 10 cryptophycin derivatives covalently attached to the binding agent (this is the degree of grafting or "drug-to-antibody ratio" or "DAR"). This number varies as a function of the nature of the binding agent and of the cryptophycin derivative, and also of the operating conditions used in the conjugation process (for example the number of equivalents of cryptophycin derivative relative to the binding agent, the reaction time, the nature of the solvent and of any cosolvent). Placing of the binding agent and the cryptophycin derivative in contact leads to a mixture comprising several conjugates that are individually distinguished from each other by different DARs; optionally the unreacted binding agent; optionally aggregates. The DAR that is determined on the final solution thus corresponds to an average DAR.

In the case where the binding agent is an antibody, UV spectroscopy may be a method used for determining the DAR. This method is based on that presented in Antony S. Dimitrov (ed), LLC, 2009, "Therapeutic Antibodies and Protocols", vol. 525, 445, Springer Science. It consists in measuring the absorbance of a solution of conjugate after the separation step (ii) at two wavelengths noted λ_1 and λ_2 . The following molar extinction coefficients of the naked antibody and of the cryptophycin derivative measured prior to conjugation are used.

The absorbances of the conjugate solution at λ_1 and λ_2 (A_{λ_1}) and (A_{λ_2}) are measured either on the corresponding peak of the SEC spectrum (this allows calculation of a "DAR(SEC)") or by using a standard UV spectrophotometer (this allows calculation of a "DAR(UV)"). The absorbances may be expressed in the form:

$$A_{\lambda_1} = (C_D \times \epsilon_{D \lambda_1}) + (C_A \times \epsilon_{A \lambda_1})$$

$$A_{\lambda_2} = (C_D \times \epsilon_{D \lambda_2}) + (C_A \times \epsilon_{A \lambda_2})$$

for which equations:

- C_D and C_A denote, respectively, the concentrations in the solution of the part of the conjugate relative to the cryptophycin derivative and the part of the conjugate relative to the antibody;
- $\epsilon_{D \lambda_1}$ and $\epsilon_{D \lambda_2}$ denote, respectively, the molar absorption coefficients for the cryptophycin derivative before conjugation at the two wavelengths λ_1 and λ_2 , these coefficients measured on the compounds of formula (II) of the type SZ_a with $Z_a = -SMe$ or of the type $-C(=O)-Z_bR_b$ with $Z_bR_b = OMe$ or $OCH_2-CH=CH_2$;
- $\epsilon_{A \lambda_1}$ and $\epsilon_{A \lambda_2}$ denote, respectively, the molar absorption coefficients of the naked antibody at the wavelengths λ_1 and λ_2 .

The term "naked antibody" means the antibody to which no cryptophycin derivative is attached, i.e. the antibody before the conjugation step.

Resolution of these two equations leads to:

$$C_D = [(\epsilon_{A\lambda_1} \times A_{\lambda_2}) - (\epsilon_{A\lambda_2} \times A_{\lambda_1})] / [(\epsilon_{D\lambda_2} \times \epsilon_{A\lambda_1}) - (\epsilon_{A\lambda_2} \times \epsilon_{D\lambda_1})]$$

$$C_A = [A_{\lambda_1} - (C_D \times \epsilon_{D\lambda_1})] / \epsilon_{A\lambda_1}$$

5

The average DAR thus corresponds to C_D/C_A . In the case of cryptophycin derivatives, the wavelength $\lambda_1 = 280$ nm may be considered, and, depending on the nature of the cryptophycin derivative, λ_2 is chosen in the specific wavelength range 246 nm - 252 nm. The DAR(UV) is preferably greater than 0.5, more particularly between 1 and 10 and even more particularly between 2 and 7.

10

The conjugate may be used as an anticancer agent. Owing to the presence of the binding agent, the conjugate is made highly selective towards tumour cells rather than healthy cells. This makes it possible to direct the cryptophycin derivative in an environment similar thereto or directly therein; (in this respect, see the following publications that describe the use of monoclonal antibody conjugates in cancer treatment: "Antibody-drug conjugates for cancer therapy" Carter P.J., et al., *Cancer J.* **2008**, 14, 154–169; "Targeted cancer therapy: conferring specificity to cytotoxic drugs" Chari R., *Acc. Chem. Res.* **2008**, 41, 98–107). It is possible to treat solid or liquid cancers. The conjugate may be used alone or in combination with at least one other anticancer agent.

20

The conjugate is formulated in the form of a buffered aqueous solution at a concentration generally of between 1 and 10 mg/ml. This solution may be injected in perfusion form per se or may be rediluted to form a perfusion solution.

25

[Examples]

Analytical methods used

High-pressure liquid chromatography – mass spectrometry (LCMS)

Method A1

The analysis is performed on a Waters ZQ machine and an XBridge C₁₈ 2.5 μ m (3×50 mm) column at 70°C with a flow rate of 0.9 ml/min, an elution gradient (7 min) of (A) water/0.1% formic acid and of (B) acetonitrile/0.1% formic acid (gradient: from 5% to 100% B over 5.3 minutes; 5.5 minutes: 100% B; 6.3 minutes: 5% B) and an electrospray ionization in positive and/or negative mode.

35

Method A2

The analysis is performed on a Waters UPLC-SQD machine and an Acquity BEH C₁₈ 1.7 μ m (2.1×50 mm) column at 50°C with a flow rate of 1 ml/min, an elution gradient (2 minutes) of (A)

water/0.1% formic acid and of (B) acetonitrile/0.1% formic acid (gradient: from 5% to 50% B over 0.8 minute; 1.2 minutes: 100% B; 1.85 minutes: 100% B; 1.95 minutes: 5% B) and electrospray ionization in positive and/or negative mode.

5 Method A3

The analysis is performed on a Waters UPLC-SQD machine and an Acquity BEH C₁₈ 1.7 µm (2.1×50 mm) column at 70°C with a flow rate of 1 ml/min, an elution gradient (2 minutes) of (A) water/0.1% formic acid and of (B) acetonitrile/0.1% formic acid (gradient: from 5% to 50% B over 1 minute; 1.3 minutes: 100% B; 1.45 minutes: 100% B; 1.75 minutes: 5% B) and electrospray ionization in positive and/or negative mode.

Method A4

The analysis is performed on a Waters ZQ machine and a Phenomenex Kinetex C₁₈ 100A 2.6 µm (3×50mm) column at 45°C with a flow rate of 1 ml/minute, an elution gradient (6 minutes) of (A) water/0.1% formic acid and of (B) acetonitrile/0.1% formic acid (gradient: 6% B: 0.8 minute; from 6% to 100% B over 4.1 minutes; 4.8 minutes: 100% B; 5.0-6.0 minutes: 6% B) and electrospray ionization in positive and/or negative mode.

Method A5

20 The analysis is performed on a Waters ZQ machine and a Phenomenex Kinetex C₁₈ 2.6 µm (3×1000mm) column at 50°C with a flow rate of 0.8 ml/minute, an elution gradient (8 minutes) of (A) water/0.1% formic acid and of (B) acetonitrile/0.1% formic acid (gradient: 4% B: 0.15 minute; from 4% to 100% B over 6.85 minutes; 7.1 minutes: 100% B; 7.4-8.2 minutes: 4% B) and electrospray ionization in positive and/or negative mode.

Mass spectrometry (MS)

The spectra were acquired by direct introduction onto a Waters GCTof machine (direct introduction without LC).

30 Steric exclusion chromatography – high resolution mass spectrometry (SEC-HRMS)

The analysis may necessitate a prior step of deglycosylation of the conjugate. This is performed by adding to the conjugate solution 2% by volume of a solution of enzyme PNGase F (prepared by making up to 100 ml a flask of 100 units of lyophilizate of N-glycanase enzyme with MilliQ water). The solution is homogenized by vortex and incubated at 37°C for 19 hours. The deglycosylated sample is ready to be analyzed by SEC-HRMS. The chromatographic analysis is performed on an Agilent HP1100 machine and a Waters Biosuite 250 HR SEC 4 µm (4.6×300 mm) column at 30°C with a flow rate of 0.4 ml/minute and an isocratic elution of (A) 25 mM ammonium formate pH=7/(B) acetonitrile 70/30 for 15 minutes. The mass spectrometry is

performed on a Waters QTOF II machine with electrospray ionization in positive mode. The mass spectra are deconvoluted with the Waters MaxEnt1 software.

Steric exclusion chromatography (SEC HPLC)

- 5 The analysis is performed on a Merck Lachrom Elite HPLC machine with an L2455 DAD spectrophotometric detector and a Tosoh Bioscience TSKgel G3000 SWXL 5 μm (7.8 $\times$ 300 mm) column with a flow rate of 0.5 ml/minute and an isocratic elution of 30 minutes with a pH 7 buffer containing 0.2 M of KCl, 0.052 M of KH_2PO_4 , 0.107 M of K_2HPO_4 and 20% by volume of isopropanol.

10

^1H nuclear magnetic resonance (NMR)

The ^1H NMR spectra were acquired on a Bruker Avance spectrometer, either of model DRX-300, DRX-400, DRX-500 or DMX-600. The chemical shifts are given in ppm.

- 15 **General method used for preparing the conjugates in the case of the cryptophycin derivatives comprising a linker L ending with -SZ_a**

Method in two successive steps

1st step

- 20 The antibody is first modified with an activated NHS ester, so as to introduce onto its surface pyridyldisulfide groups. A solution of antibody hu2H11 in an aqueous pH 6.5 buffer containing 0.05 M of potassium phosphate and 0.05 M of NaCl (referred to as buffer A) is treated with 5 to 10 eq. of the NHS activated ester dissolved in DMA such that the final antibody concentration is between 5 and 10 mg/ml and the percentage of DMA in the aqueous buffer is 5%. The reaction is continued for 2 hours at RT. The mixture is deposited onto a filtration column on gel (SephadexTM
- 25 G25 matrix, GE Healthcare) pre-equilibrated in an aqueous pH 8 buffer containing 0.05 M of HEPES, 0.05 M of NaCl and 2 mM of EDTA. The modified antibody is eluted with the pH 8 HEPES buffer, collected and then assayed by UV spectrometry so as to determine the antibody concentration of the sample and the number of pyridyldisulfide groups. A specimen of the modified antibody is treated with dithiothreitol so as to reduce the disulfide bond, and the
- 30 pyridine-2-thione released is assayed by spectrometry (extinction coefficients: $\epsilon_{343\text{ nm}}$: 8080 $\text{M}^{-1}\text{cm}^{-1}$, $\epsilon_{280\text{ nm}}$: 5100 $\text{M}^{-1}\text{cm}^{-1}$ for pyridine-2-thione, and $\epsilon_{280\text{ nm}}$: 208380 $\text{M}^{-1}\text{cm}^{-1}$ for the antibody). On average, from 3 to 6 pyridyldisulfide groups are grafted per antibody molecule.

2nd step

- 35 The modified antibody solution from the 1st step is diluted in the aqueous pH 8 buffer described above and then treated with a solution of the cryptophycin derivative (5 eq.) such that the final antibody concentration is 3 mg/ml and the percentage of DMA in the aqueous buffer is 20%; the number of equivalents of cryptophycin derivative is expressed relative to the number of pyridyldisulfide molecules introduced during the first step. The reaction is continued overnight at

30°C or with stirring at about 2000 rpm. The mixture is analyzed by SEC HPLC so as to determine the degree of grafting of the cryptophycin derivative onto the antibody. If the degree of substitution is insufficient, the mixture is treated with an additional 1 to 5 eq. of cryptophycin derivative in DMA for 3 hours at 30°C or with stirring at about 2000 rpm. The mixture is filtered through a Millex®-SV 5 µm filter (PVDF membrane, Durapore, Millipore) and then purified by gel filtration using a Superdex 200 pg matrix (HiLoad 16/60 desalting column, GE Healthcare) pre-equilibrated with an aqueous pH 6.5 buffer containing 0.01 M of phosphate, 0.14 M of NaCl and 10% to 20% of NMP. The fractions containing the conjugated antibody in monomer form are collected, pooled and concentrated on Amicon Ultra-15 (Ultracel 10k or 50k membrane, Millipore) to a concentration of between 2 and 5 mg/ml. A change of buffer is finally performed so as to remove the organic solvent from the conjugate storage buffer. The conjugate is deposited on a gel filtration column composed of a Sephadex™ G25 matrix (Nap-5, -10, PD-10, Hiprep 26/10 desalting columns, GE Healthcare) pre-equilibrated with an aqueous buffer whose composition and pH are suited to each conjugate. The final conjugate is assayed by UV spectrometry using the extinction coefficients determined for the antibody and the corresponding cryptophycin derivative so as to measure the antibody concentration and the average cytotoxic number per antibody. The degree of substitution may also be calculated from the deconvolution of the SEC-HRMS spectrum of the conjugate.

"One-pot" two-step method

The antibody is first modified by an activated NHS ester, so as to introduce onto its surface pyridyldisulfide groups. A solution of antibody hu2H11 in an aqueous pH 6.5 buffer containing 0.05 M of potassium phosphate and 0.05 M of NaCl is diluted with the pH 6.5 phosphate buffer and an aqueous 1N solution of HEPES such that the final proportion of initial pH 6.5 phosphate buffer and of HEPES is 96/4, in order to obtain a pH≈7.5-8. This antibody solution is treated with 5 to 10 eq. of the NHS activated ester dissolved in DMA such that the final antibody concentration is between 5 and 10 mg/ml and the percentage of DMA in the aqueous buffer is 5%. The reaction is continued for 2 hours at RT. The antibody solution thus modified is directly diluted with a 96/4 mixture of pH 6.5 phosphate buffer and of HEPES and then treated with a solution of the cryptophycin derivative (4 eq.) in DMA such that the final antibody concentration is 3 mg/ml and the percentage of DMA in the aqueous buffer is 20%; the number of equivalents of cryptophycin derivative is expressed relative to the number of equivalents of NHS activated ester introduced during the first step. The reaction is continued overnight at 30°C or with stirring at about 2000 rpm. The mixture is analyzed by SEC HPLC so as to determine the degree of grafting of the cryptophycin derivative onto the antibody. If the degree of substitution is insufficient, the mixture is treated with a further 1 to 5 eq. of cryptophycin derivative in DMA for 3 hours at 30°C or with stirring at about 2000 rpm. The mixture is filtered through a Millex®-SV 5 µm filter (PVDF membrane, Durapore, Millipore) and then purified by gel filtration using a Superdex 200 pg matrix

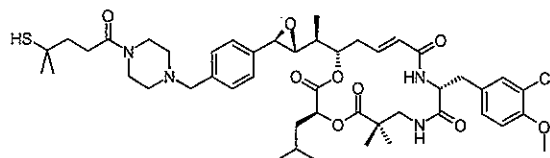
(HiLoad 16/60 desalting column, GE Healthcare) pre-equilibrated in an aqueous pH 6.5 buffer containing 0.01 M of phosphate, 0.14 M of NaCl and 10% to 20% of NMP. The fractions containing the conjugated antibody in monomer form are collected, pooled and concentrated on Amicon Ultra-15 (Ultracel 10k or 50k membrane, Millipore) to a concentration of between 2 and 5 mg/ml. A change of buffer is finally performed so as to remove the organic solvent from the conjugate storage buffer. The conjugate is deposited on a gel filtration column composed of a SephadexTM G25 matrix (Nap-5, -10, PD-10, Hiprep 26/10 desalting columns, GE Healthcare) pre-equilibrated with an aqueous buffer whose composition and pH are suited to each conjugate. The final conjugate is assayed by UV spectrometry using the extinction coefficients determined for the antibody and the corresponding cryptophycin derivative so as to measure the antibody concentration and the average cytotoxic number per antibody. The degree of substitution may also be calculated from the deconvolution of the SEC-HRMS spectrum of the conjugate.

General method used for preparing the conjugates in the case of cryptophycin derivatives comprising a linker ending with $-C(=O)Z_bR_b$

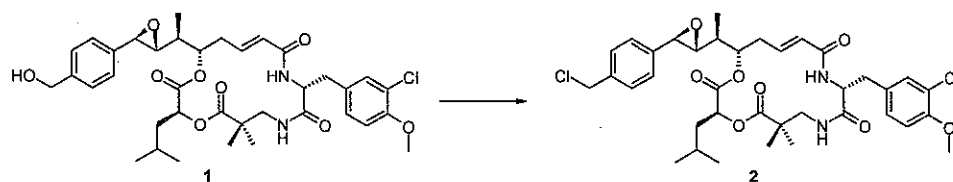
A solution of antibody hu2H11 in an aqueous pH 8 buffer containing 0.05 M of HEPES, 0.05 M of NaCl and 2 mM of EDTA or composed of a 96/4 mixture of an aqueous pH 6.5 buffer containing 0.05 M of potassium phosphate and 0.05 M of NaCl/1N HEPES is treated with an excess of a solution in DMA on the cryptophycin derivative such that the final antibody concentration is 3 mg/ml and the percentage of DMA in the aqueous buffer is 20%. The reaction is continued for 3 hours at 30°C or with stirring at about 2000 rpm. The mixture is analyzed by SEC HPLC so as to determine the degree of grafting of cytotoxic agent on the population of monomer antibodies. If the degree of substitution is insufficient, the mixture is treated with a further 1 to 5 eq. of cryptophycin derivative in DMA for 3 hours at 30°C or with stirring at about 2000 rpm. The mixture is filtered through a Millex®-SV 5 µm filter (PVDF membrane, Durapore, Millipore) and then purified by gel filtration using a Superdex 200 pg matrix (HiLoad 16/60 desalting column, GE Healthcare) pre-equilibrated in an aqueous pH 6.5 buffer containing 0.01 M of phosphate, 0.14 M of NaCl and 10% to 20% of NMP. The fractions containing the conjugated antibody in monomer form are collected, pooled and concentrated on Amicon Ultra-15 (Ultracel 10k or 50k membrane, Millipore) to a concentration of between 2 and 5 mg/ml. A change of buffer is finally performed so as to remove the organic solvent from the conjugate storage buffer. The conjugate is deposited on a gel filtration column composed of a SephadexTM G25 matrix (Nap-5, -10, PD-10 columns or Hiprep 26/10 desalting column, GE Healthcare) pre-equilibrated with an aqueous buffer whose composition and pH are suited to each conjugate. The final conjugate is assayed by UV spectrometry using the extinction coefficients determined for the antibody and the corresponding cryptophycin derivative, so as to measure the antibody concentration and the degree of grafting. The degree of substitution may also be calculated from the deconvolution of the SEC-HRMS spectrum of the conjugate.

The methods described for the case of the antibody hu2H11 may also be similarly applied to other antibodies, and also to other binding agents.

- 5 **Example 1:** (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[4-(4-mercapto-4-methylpentanoyl)piperazin-1-ylmethyl]phenyl}oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone

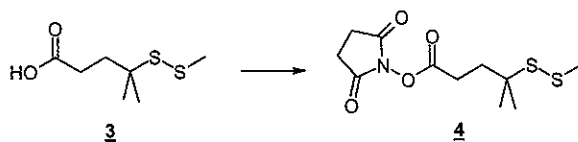


- 10 **Compound 2:** (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-16-[(S)-1-[(R)-3-(4-chloromethylphenyl)oxiranyl]ethyl]-3-isobutyl-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



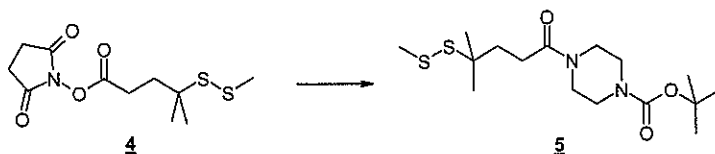
- Compound 1 (30 mg; 42.9 μ mol, prepared according to Al-awar R.S., et al., *J.Med.Chem.* **2003**, 46, 2985-3007) is dissolved in anhydrous DMF (2 ml) and the mixture is cooled to 0°C, followed by addition of TEA (107 μ mol) and then CMS (64.6 μ mol). After 15 minutes, the bath is removed and stirring is continued for 12 hours at RT. The mixture is diluted by adding EtOAc (2 ml) and the organic phase is washed with water (2x1 ml), with saturated aqueous NaHCO₃ solution (1 ml) and with saturated aqueous NaCl solution (1 ml). The organic phase is dried over MgSO₄ and, after filtering and evaporating off the solvents under reduced pressure, product 2 is obtained in the form of a colourless oil that crystallizes (25 mg; 81%). ¹H NMR (500 MHz, DMSO-*d*₆): 0.77 – 0.83 (m, 6 H); 1.02 (s, 3 H); 1.04 – 1.07 (m, 3 H); 1.14 (s, 3 H); 1.29 – 1.36 (m, 1 H); 1.54 – 1.63 (m, 2 H); 1.80 – 1.87 (m, 1 H); 2.24 – 2.33 (m, 1 H); 2.63 – 2.73 (m, 2 H); 2.96 – 3.06 (m, 3 H); 3.28 – 3.32 (m, 1 H); 3.83 (s, 3 H); 3.93 (d, *J* = 1.6 Hz, 1 H); 4.27 (ddd, *J* = 11.3, 8.0, 3.6 Hz, 1 H); 4.78 (s, 2 H); 4.93 (dd, *J* = 9.6, 3.6 Hz, 1 H); 5.13 (dd, *J* = 10.8, 5.1 Hz, 1 H); 5.81 (d, *J* = 14.8 Hz, 1 H); 6.49 (ddd, *J* = 15.0, 11.2, 3.7 Hz, 1 H); 7.07 (d, *J* = 8.5 Hz, 1 H); 7.18 (dd, *J* = 8.5, 1.9 Hz, 1 H); 7.23 (d, *J* = 9.6 Hz, 1 H); 7.29 (d, *J* = 1.9 Hz, 1 H); 7.34 (d, *J* = 8.2 Hz, 2 H); 7.47 (d, *J* = 8.2 Hz, 2 H); 8.35 (d, *J* = 8.2 Hz, 1 H). LCMS (A1): ES *m/z* = 713 [*M* + *H*]⁺; *m/z* = 715 [*M* – *H*][–]; *t*_R = 5.17 min.

- 30 **Compound 4:** 2,5-Dioxopyrrolidin-1-yl 4-methyl-4-methyldisulfanylpentanoate



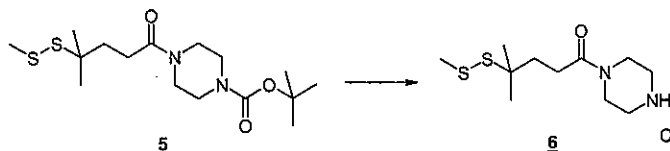
To a solution, purged with argon, of compound **3** (3.05 g, 15.7 mmol, prepared according to **WO 2007/085 930**) in DCM (30 ml) are successively added NHS (17.3 mmol) and EDCI chloride (17.3 mmol). The mixture is stirred for 3 hours at RT and then washed with pH 6 phosphate buffer (2 × 30 ml) and then with saturated NaCl solution (30 ml), dried over MgSO₄ and concentrated to dryness. The amber-coloured oil obtained, which crystallizes, is washed with a 75/25 heptane/EtOAc mixture and filtered through a sinter funnel to give compound **4** in the form of a white solid (2.08 g, 45%). The filtrate is concentrated to dryness and the crude product obtained is purified by chromatography on silica gel, eluting with a heptane/EtOAc mixture from 50/50 to 0/100. The fractions containing the expected product are concentrated to dryness and taken up in isopropyl ether (5 ml); the precipitate is filtered through a sinter funnel to give the expected compound **4** (1 g, 22%). ¹H NMR (400 MHz, DMSO-*d*₆): 1.29 (s, 6 H); 1.92 to 1.98 (m, 2 H); 2.41 (s, 3 H); 2.72 to 2.78 (m, 2 H); 2.81 (s, 4 H). LCMS (A4): EI, *m/z* = 291 [M + H]⁺.

Compound 5: *tert*-Butyl 4-(4-methyl-4-methyldisulfanylpentanoyl)piperazine-1-carboxylate



Compound **4** (200 mg, 686 μmol), 1-Boc-piperazine (686 μmol), TEA (755 μmol) and DMF (1.6 ml) are placed in a Wheatton tube. The mixture is stirred at RT overnight and then diluted with EtOAc (5 ml), washed with water (2 × 5 ml) dried over MgSO₄ and concentrated to dryness. The crude product is taken up in isopropyl ether (3 ml); the precipitate is filtered through a sinter funnel to give compound **5** (213 g, 86%). ¹H NMR (400 MHz, DMSO-*d*₆): 1.27 (s, 6 H); 1.41 (s, 9 H); 1.73 to 1.87 (m, 2 H); 2.34 to 2.41 (m, 2 H); 2.40 (s, 3 H); 3.23 to 3.36 (partially masked m, 4 H); 3.39 to 3.45 (m, 4 H). LCMS (A2): ES *m/z* = 363 [M + H]⁺; *m/z* = 307 [M + H - C₄H₈]⁺; *t*_R = 1.08 min.

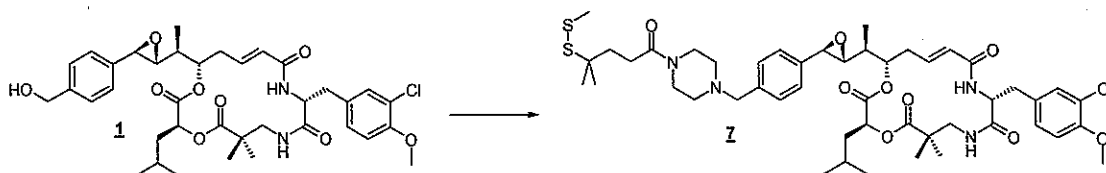
Compound 6: 4-Methyl-4-methyldisulfanyl-1-piperazin-1-ylpentan-1-one hydrochloride



To a solution of compound **5** (213 mg, 588 μmol) in dioxane (4.4 ml) is added a 4M solution of HCl in dioxane (4.4 ml). Stirring is continued for 4 hours at RT. The mixture is filtered through a sinter funnel and the solid obtained is rinsed with dioxane (2 ml) and then with isopropyl ether (2 ml) to give compound **6** (132 mg, 75%) in the form of a cream-coloured solid. ¹H NMR (500

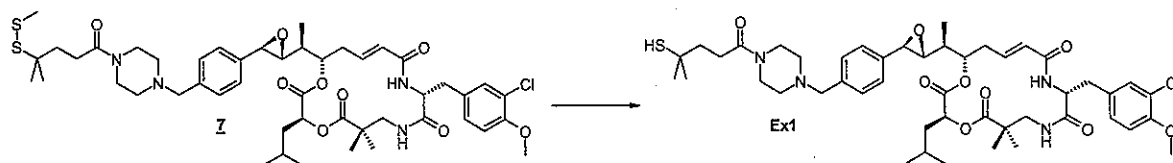
MHz, DMSO- d_6): 1.27 (s, 6 H); 1.73 to 1.85 (m, 2 H); 2.37 to 2.45 (m, 2 H); 2.40 (s, 3H); 2.98 to 3.15 (m, 4 H); 3.62 to 3.73 (m, 4H); 9.39 (broad m, 2 H). LCMS (A1): ES m/z = 263 $[M + H]^+$; t_R = 2.40 min.

- 5 **Compound 7:** (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-16-[(S)-1-((2R,3R)-3-{4-[4-(4-methyl-4-methyldisulfanyl)pentanoyl]piperazin-1-ylmethyl}phenyl)oxiranyl)-ethyl]-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



- Compound 1 (20.3 mg; 29.0 μ mol) is dissolved in anhydrous DMF (0.77 ml) and TEA (72.6 μ mol), and then CMS (43.6 μ mol) is added. After 12 hours at RT, the product 2 formed is not isolated, and TEA (58.0 μ mol) and then 4-methyl-4-methyldisulfanyl-1-piperazin-1-ylpentan-1-one hydrochloride 6 (34.8 μ mol) are added. The mixture is stirred for a further 72 hours at RT and then diluted with EtOAc (10 ml). The organic phase is washed with water (2x2 ml), with saturated aqueous NaHCO_3 solution (2 ml) and with saturated aqueous NaCl solution (2 ml). After drying over MgSO_4 and filtering, the solvents are evaporated off under reduced pressure. The crude reaction product is purified by chromatography on silica gel, eluting with a 99/1 to 98/2 DCM/methanol mixture. A white solid, 7, is obtained (5.7 mg; 21%). TLC (DCM 90/MeOH 10): R_f =0.6; ^1H NMR (400 MHz, DMSO- d_6): 0.75 – 0.81 (m, 6 H); 1.01 (s, 3 H); 1.05 (d, J =6.8 Hz, 3 H); 1.12 (s, 3 H); 1.26 (s, 6 H); 1.28 – 1.33 (m, 1 H); 1.52 – 1.61 (m, 2 H); 1.76 – 1.83 (m, 2 H); 2.27 – 2.38 (m, 4 H); 2.39 (s, 3 H); 2.64 – 2.74 (m, 2 H); 2.95 – 3.05 (m, 2 H); 3.24 – 3.34 (m, 6 H); 3.44 (br. s., 4 H); 3.49 (s, 2 H); 3.81 (s, 3 H); 3.88 (d, J =1.7 Hz, 1 H); 4.22 – 4.29 (m, 1 H); 4.92 (dd, J =9.9, 3.5 Hz, 1 H); 5.08 – 5.15 (m, 1 H); 5.81 (d, J =14.2 Hz, 1 H); 6.48 (ddd, J =15.2, 11.3, 3.5 Hz, 1 H); 7.05 (d, J =8.6 Hz, 1 H); 7.17 (dd, J =8.4, 2.3 Hz, 1 H); 7.22 (d, J =9.3 Hz, 1 H); 7.25 – 7.34 (m, 5 H); 8.34 (d, J =8.1 Hz, 1 H); LCMS (A1): ES m/z = 943 $[M + H]^+$; m/z = 941 $[M - H]^-$; t_R = 4.03 min.

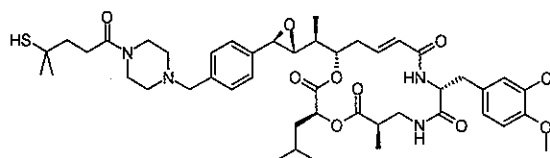
Example 1: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-16-[(S)-1-((2R,3R)-3-{4-[4-(4-mercapto-4-methylpentanoyl)piperazin-1-ylmethyl]phenyl}oxiranyl)ethyl]-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



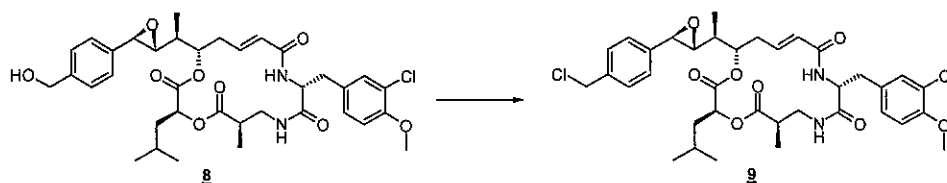
Product 7 (9.6 mg; 10.2 μ mol) is dissolved in a mixture of ethanol (1.2 ml)/water (1 ml) and the mixture becomes cloudy. TCEP (25.4 μ mol) is then added and the mixture is stirred for 5 hours at

RT. The mixture is diluted by adding EtOAc and the organic phase is washed with a 1/1 mixture in water and saturated aqueous NH_4Cl solution (1 ml). After drying the organic phase over MgSO_4 , filtering and evaporating off the solvents under reduced pressure, the final product, **Ex. 1**, is obtained in the form of a white solid (6.5 mg; 71%). TLC (DCM 90/MeOH 10): R_f = 0.56; ^1H NMR (500 MHz, $\text{DMSO}-d_6$): 0.76 – 0.81 (m, 6 H); 1.01 (s, 3 H); 1.06 (d, J = 6.8 Hz, 3 H); 1.13 (s, 3 H); 1.24 (s, 6 H); 1.27 – 1.31 (m, 1 H); 1.56 – 1.64 (m, 2 H); 1.73 – 1.85 (m, 3 H); 2.26 – 2.33 (m, 3 H); 2.36 – 2.45 (m, 4 H); 2.63 – 2.75 (m, 2 H); 2.95 – 3.06 (m, 3 H); 3.34 – 3.36 (m, 1 H); 3.42 – 3.51 (m, 6 H); 3.82 (s, 3 H); 3.89 (s, 1 H); 4.22 – 4.29 (m, 1 H); 4.92 (dd, J = 9.8, 3.4 Hz, 1 H); 5.12 (dd, J = 10.8, 4.9 Hz, 1 H); 5.81 (d, J = 15.2 Hz, 1 H); 6.48 (ddd, J = 15.0, 11.4, 3.4 Hz, 1 H); 7.06 (d, J = 8.3 Hz, 1 H); 7.18 (dd, J = 8.3, 1.5 Hz, 1 H); 7.24 (d, J = 9.8 Hz, 1 H); 7.26 – 7.36 (m, 5 H); 8.37 (d, J = 7.8 Hz, 1 H); LCMS (A2): ES m/z = 897 $[\text{M} + \text{H}]^+$; m/z = 895 $[\text{M} - \text{H}]^-$; t_R = 0.97 min.

Example 2: (E)-(3S,6R,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[4-(4-mercapto-4-methylpentanoyl)piperazin-1-ylmethyl]phenyl}oxiranyl)-ethyl]-6-methyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



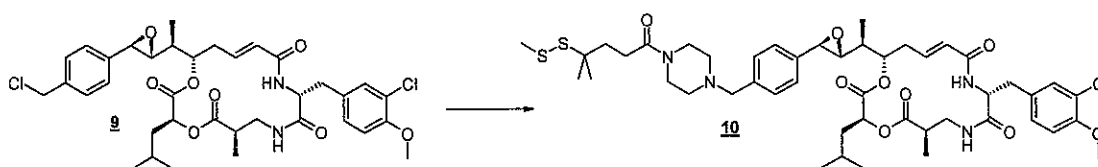
Compound 9: (E)-(3S,6R,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-16-[(S)-1-[(R)-3-(4-chloromethylphenyl)oxiranyl]ethyl]-3-isobutyl-6-methyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



Compound **8** (49 mg; 71.4 μmol , which may be prepared according to Al-awar R.S., et al., *J. Med. Chem.* **2003**, 46, 2985-3007) is dissolved in anhydrous DCM (5 ml) and the mixture is cooled to 0°C , followed by addition of DIPEA (428 μmol) and then CMS (214 μmol). The mixture is allowed to return to RT and stirring is continued for 40 hours at RT. The mixture is hydrolyzed with 5 ml of water and the aqueous phase is extracted with DCM (3 $\times$ 5 ml). The organic phases are combined, washed with saturated aqueous NaHCO_3 solution (10 ml) and with saturated aqueous NaCl solution (10 ml) and dried over MgSO_4 . After filtering and evaporating off the solvents under reduced pressure, the crude product is purified by chromatography on silica gel, eluting with a 100/0 to 90/10 DCM/MeOH mixture. Compound **9** is obtained in the form of a white solid (37 mg; 79%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$): 0.78 (d, J = 6.6 Hz, 3 H); 0.80 (d, J = 6.6 Hz, 3 H); 1.01 (d, J = 6.9 Hz, 3 H); 1.05 (d, J = 6.9 Hz, 3 H); 1.30 (m, 1 H); 1.54 (m, 1 H); 1.60 (m, 1 H); 1.82 (m, 1 H); 2.27 (m, 1 H); 2.60 to 2.78 (m, 3 H); 2.97 to 3.05 (m, 2 H); 3.15 (m, 1 H); 3.41

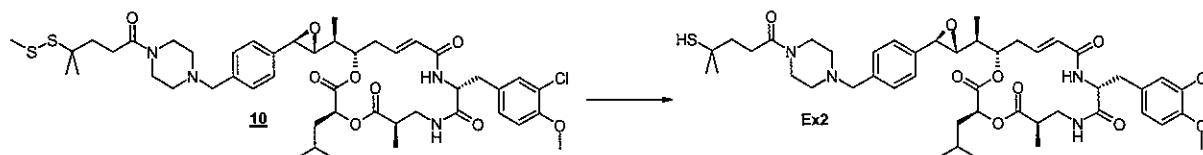
(m, 1 H); 3.81 (s, 3 H); 3.92 (d, $J = 1.6$ Hz, 1 H); 4.26 (ddd, $J = 3.8$ and 8.2 and 11.5 Hz, 1 H); 4.77 (s, 2 H); 4.88 (dd, $J = 3.8$ and 9.6 Hz, 1 H); 5.12 (ddd, $J = 1.5$ and 5.3 and 11.2 Hz, 1 H); 5.80 (dd, $J = 1.5$ and 15.0 Hz, 1 H); 6.47 (ddd, $J = 3.7$ and 11.2 and 15.0 Hz, 1 H); 7.05 (d, $J = 8.5$ Hz, 1 H); 7.17 (dd, $J = 2.2$ and 8.5 Hz, 1 H); 7.23 (dd, $J = 2.7$ and 9.1 Hz, 1 H); 7.29 (d, $J = 2.2$ Hz, 1 H); 7.32 (d, $J = 8.2$ Hz, 2 H); 7.45 (d, $J = 8.2$ Hz, 2 H); 8.34 (d, $J = 8.2$ Hz, 1 H). LCMS (A2): ES $m/z = 703$ $[M + H]^+$; $m/z = 701$ $[M - H]^-$; $m/z = 747$ $[M - H + HCO_2H]^-$ base peak; $t_R = 1.17$ min.

Compound 10: (E)-(3S,6R,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-6-methyl-16-[(S)-1-((2R,3R)-3-{4-[4-(4-methyl-4-methyldisulfanyl)pentanoyl]piperazin-1-ylmethyl}phenyl)oxiranyl)-ethyl]-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



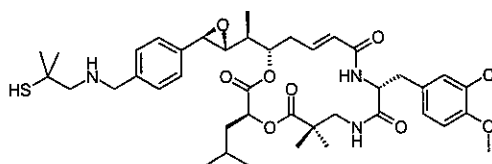
Compound **9** (36.6 mg; 52 μ mol) is dissolved in anhydrous acetonitrile (3 ml), followed by successive addition of DIPEA (260 μ mol) and Compound **6** (156 μ mol). The reaction medium is stirred for 20 hours at RT and then hydrolyzed by addition of 4 ml of water. The aqueous phase is extracted with EtOAc (3x4 ml) and the organic phases are combined, washed with saturated aqueous $NaHCO_3$ solution (5 ml) and with saturated aqueous $NaCl$ solution (5 ml). After drying over $MgSO_4$ and filtering, the solvents are evaporated off under reduced pressure. The crude reaction product is purified by chromatography on silica gel, eluting with a 100/0 to 95/5 DCM/methanol mixture. Compound **10** is obtained in the form of a white solid (39 mg; 80%). 1H NMR (500 MHz, $DMSO-d_6$): 0.78 (d, $J = 6.6$ Hz, 3 H); 0.80 (d, $J = 6.6$ Hz, 3 H); 1.01 (d, $J = 6.9$ Hz, 3 H); 1.05 (d, $J = 6.9$ Hz, 3 H); 1.26 (s, 6 H); 1.30 (m, 1 H); 1.53 (m, 1 H); 1.60 (m, 1 H); 1.75 to 1.84 (m, 3 H); 2.26 to 2.37 (m, 7 H); 2.39 (s, 3 H); 2.61 to 2.77 (m, 3 H); 2.96 to 3.05 (m, 2 H); 3.15 (m, 1 H); 3.38 (partially masked m, 1 H); 3.44 (m, 4 H); 3.48 (s, 2 H); 3.81 (s, 3 H); 3.88 (d, $J = 1.6$ Hz, 1 H); 4.27 (ddd, $J = 4.0$ and 8.1 and 11.5 Hz, 1 H); 4.88 (dd, $J = 3.8$ and 9.6 Hz, 1 H); 5.12 (ddd, $J = 1.6$ and 5.3 and 11.5 Hz, 1 H); 5.80 (dd, $J = 1.6$ and 15.1 Hz, 1 H); 6.47 (ddd, $J = 3.6$ and 11.5 and 15.1 Hz, 1 H); 7.05 (d, $J = 8.5$ Hz, 1 H); 7.17 (dd, $J = 2.2$ and 8.5 Hz, 1 H); 7.23 (dd, $J = 2.6$ and 9.2 Hz, 1 H); 7.27 (d, $J = 8.4$ Hz, 2 H); 7.29 (d, $J = 2.2$ Hz, 1 H); 7.32 (d, $J = 8.4$ Hz, 2 H); 8.35 (d, $J = 8.1$ Hz, 1 H). LCMS (A2): ES $m/z = 929$ $[M + H]^+$; $m/z = 927$ $[M - H]^-$; $m/z = 973$ $[M - H + HCO_2H]^-$ base peak; $t_R = 0.99$ min.

Example 2: (E)-(3S,6R,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-6-methyl-16-[(S)-1-((2R,3R)-3-{4-[4-(4-mercapto-4-methylpentanoyl]piperazin-1-ylmethyl}phenyl)oxiranyl)ethyl]-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone

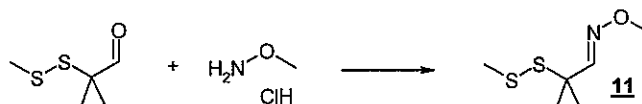


Product **10** (34 mg; 36.6 μmol) is dissolved in a mixture of ethanol (4.3 ml)/water (3.6 ml) and the mixture turns cloudy. TCEP (146 μmol) is then added, the mixture becomes colourless, and is stirred for 2 hours at RT. The mixture is diluted by adding EtOAc (20 ml) and the organic phase is washed with a 1/1 mixture of water and saturated aqueous NH_4Cl solution (20 ml). The aqueous phase is extracted with 2x20 ml of EtOAc and the organic phases are combined and washed with saturated NaCl solution (20 ml). After drying over MgSO_4 , filtering and evaporating off the solvents under reduced pressure, the crude product is purified by chromatography on silica gel, eluting with a 98/2 to 90/10 DCM/MeOH mixture. The compound **Ex. 2** is obtained in the form of a white solid (28.2 mg; 87%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$): 0.77 (d, $J = 6.6$ Hz, 3 H); 0.79 (d, $J = 6.6$ Hz, 3 H); 1.01 (d, $J = 6.9$ Hz, 3 H); 1.04 (d, $J = 6.9$ Hz, 3 H); 1.27 (m, 1 H); 1.31 (s, 6 H); 1.53 (m, 1 H); 1.59 (m, 1 H); 1.77 (m, 2 H); 1.82 (m, 1 H); 2.24 to 2.32 (m, 3 H); 2.34 to 2.44 (m, 4 H); 2.62 to 2.76 (m, 4 H); 2.98 to 3.04 (m, 2 H); 3.15 (m, 1 H); 3.41 (m, 1 H); 3.46 (m, 4 H); 3.48 (s, 2 H); 3.81 (s, 3 H); 3.88 (d, $J = 2.0$ Hz, 1 H); 4.26 (ddd, $J = 3.4$ and 8.3 and 11.5 Hz, 1 H); 4.88 (dd, $J = 3.7$ and 10.0 Hz, 1 H); 5.12 (ddd, $J = 2.0$ and 5.3 and 11.1 Hz, 1 H); 5.80 (dd, $J = 2.0$ and 15.0 Hz, 1 H); 6.47 (ddd, $J = 3.7$ and 11.1 and 15.0 Hz, 1 H); 7.05 (d, $J = 8.3$ Hz, 1 H); 7.17 (dd, $J = 2.0$ and 8.3 Hz, 1 H); 7.23 (dd, $J = 2.9$ and 9.3 Hz, 1 H); 7.27 (d, $J = 8.5$ Hz, 2 H); 7.29 (d, $J = 2.0$ Hz, 1 H); 7.32 (d, $J = 8.5$ Hz, 2 H); 8.35 (d, $J = 8.3$ Hz, 1 H). LCMS (A2): ES $m/z = 883$ [$\text{M} + \text{H}$] $^+$; $m/z = 881$ [$\text{M} - \text{H}$] $^-$; $t_R = 0.92$ min.

Example 3: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[(2-mercapto-2-methylpropylamino)methyl]phenyl}oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



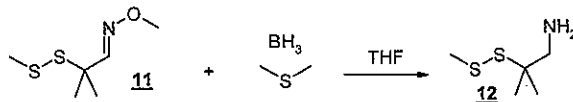
Compound 11: 2-Methyl-2-methyldisulfanylpropionaldehyde O-methyloxime



5 g (33.3 mmol) of 2-(methylthio)isobutyraldehyde are dissolved in 50 ml of ethanol, under an inert atmosphere. A suspension of 5.56 g (66.54 mmol) of O-methylhydroxylamine chloride in 50 ml of ethanol and then 6.65 ml (66.54 mmol) of NaOH are successively added. The cloudy white mixture is refluxed overnight. After cooling to RT, the mixture is poured into 500 ml of water. The aqueous phase is extracted with EtOAc (3x175 ml), and the combined organic phases are

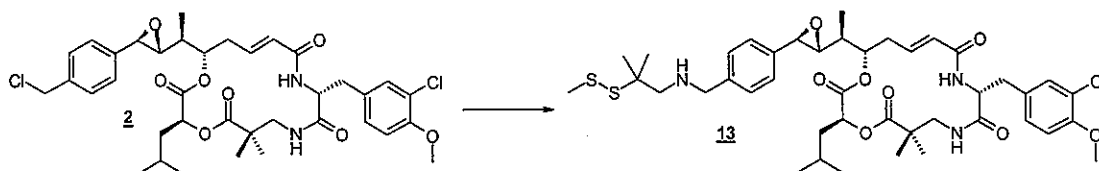
washed with saturated NaCl solution (200 ml) and dried over MgSO_4 . After filtering and concentrating under reduced pressure, compound **11** is obtained in the form of a colourless oil (5.89 g, 32.9 mmol).

5 Compound 12: 2-Methyl-2-methyldisulfanylpropylamine



1.78 g (9.9 mmol) of oxime ether **11**, 19 ml of anhydrous THF and 99.5 ml of a 2M solution of borane-methyl sulfide in THF are successively introduced, at RT, into a round-bottomed flask purged with argon. The mixture is refluxed for 16 hours. The reaction is then stopped and the mixture is allowed to cool to RT, followed by cautious dropwise addition of 100 ml of MeOH at 0°C while the foam forms, and then at RT. The mixture is evaporated under reduced pressure to give about 2.8 g of a yellow oil, which is taken up in 30 ml of a 5 to 6N solution of HCl in isopropanol. The mixture is refluxed for 1 hour and then left at RT overnight. After evaporating under reduced pressure, the residue obtained is taken up in 80 ml of 1N HCl and then extracted with diethyl ether (3×20 ml). The aqueous phase is treated with aqueous 30% ammonia until a pH of 12.5-13 is obtained, i.e. 12 ml (pale purple aqueous phase) and then extracted again with 3×20 ml of ether. The organic phases are combined, dried over MgSO_4 , filtered and evaporated to dryness under reduced pressure to give 760 mg of crude product. This residue is finally purified by chromatography on silica gel, eluting with a 100/0 to 90/10 DCM/methanol mixture. Compound **12** is obtained in the form of a pale yellow oil (301 mg, 20%). LCMS (A2) : ES m/z = 152 $[\text{M} + \text{H}]^+$; t_R = 0.27 min.

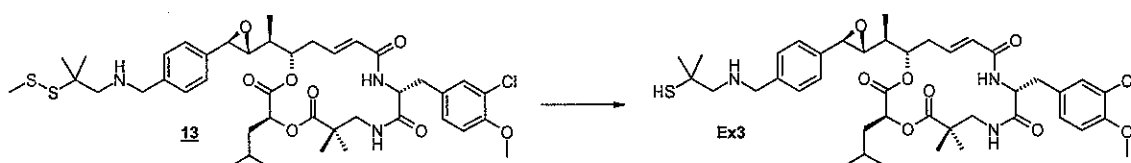
25 Compound 13: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R, 3R)-3-{4-[(2-methyl-2-methyldisulfanylpropylamino)methyl]phenyl}oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



To a solution, purged with argon, of 25 mg (35 μmol) of compound **2** in 3.1 ml of anhydrous DMF are successively added, at RT, 9.8 μl (70 μmol) of TEA and 6.3 mg (42 μmol) of compound **12**. Stirring is continued at 40°C. After reaction for 72 hours, some starting compound **2** remains, so a further 9.8 μl (70 μmol) of TEA and 6.3 mg (42 μmol) of the amine **12** are added. After a further 72 hours at 40°C, some compound **2** still remains: 6.3 mg (42 μmol) of the amine **12** are added and stirring is continued at 40°C. One day later, the reaction is complete. The mixture is diluted in 10 ml of EtOAc, washed with 2×10 ml of water, with 10 ml of saturated NaHCO_3 solution and 10 ml of saturated NaCl solution. After drying the organic phase over MgSO_4 , it is filtered and

then evaporated under reduced pressure to give 30 mg of crude product. This residue is purified by chromatography on silica gel, eluting with a 98/2 to 93/7 DCM/methanol mixture. Compound **13** is obtained in the form of a white powder (23.3 mg, 81%). TLC (DCM 90/ MeOH 10): R_f=0.55; ¹H NMR (500 MHz, DMSO-*d*₆): 0.76 to 0.81 (m, 6 H); 1.02 (s, 3 H); 1.07 (d, J = 6.9 Hz, 3 H); 1.13 (s, 3 H); 1.27 to 1.33 (m, 7 H); 1.52 to 1.63 (m, 2 H); 1.82 (sxt, J = 6.8 Hz, 1 H); 2.06 (broad s, 1 H); 2.25 to 2.34 (m, 1 H); 2.37 (s, 3 H); 2.57 (s, 2 H); 2.66 to 2.76 (m, 2 H); 2.97 to 3.06 (m, 3 H); 3.33 to 3.38 (m, 1 H); 3.75 (s, 2 H); 3.83 (s, 3 H); 3.88 (d, J = 1.6 Hz, 1 H); 4.27 (ddd, J = 3.7 and 8.0 and 11.4 Hz, 1 H); 4.93 (dd, J = 3.7 and 9.7 Hz, 1 H); 5.12 (dd, J = 5.8 and 11.3 Hz, 1 H); 5.82 (dd, J = 1.5 and 15.2 Hz, 1 H); 6.49 (ddd, J = 3.8 and 11.3 and 15.2 Hz, 1 H); 7.07 (d, J = 8.5 Hz, 1 H); 7.18 (dd, J = 1.9 and 8.5 Hz, 1 H); 7.23 (dd, J = 2.6 and 9.5 Hz, 1 H); 7.26 (d, J = 8.2 Hz, 2 H); 7.30 (d, J = 1.9 Hz, 1 H); 7.35 (d, J = 8.2 Hz, 2 H); 8.35 (d, J = 8.0 Hz, 1 H). LCMS (A2): ES m/z = 832 [M + H]⁺; m/z = 830 [M - H]⁻; t_R = 0.96 min.

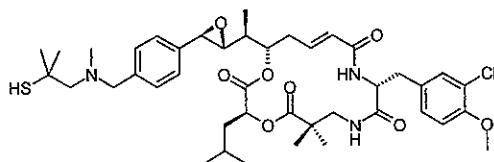
Example 3: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[(2-mercapto-2-methylpropylamino)methyl]phenyl}oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



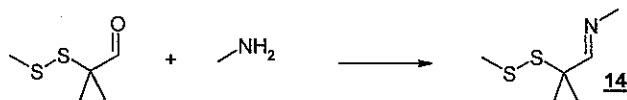
Product **13** (10.6 mg; 12.8 μmol) is dissolved in a mixture of ethanol (1.5 ml)/water (1.26 ml). TCEP (9.1 mg, 31.9 μmol) is then added and the mixture is stirred for 2 hours 30 minutes at RT. The mixture is diluted in 15 ml of EtOAc and the organic phase is washed with a 1/1 mixture of water and saturated aqueous NH₄Cl solution (5 ml). After drying the organic phase over MgSO₄, filtering and evaporating off the solvents under reduced pressure, the crude product obtained is finally purified by chromatography on silica gel, eluting with a 98/2 to 92/8 DCM/methanol mixture. The compound **Ex. 3** is obtained in the form of a white solid (6.4 mg; 63%). TLC (DCM 90/MeOH 10): R_f=0.47; ¹H NMR (500 MHz, DMSO-*d*₆): 0.80 to 0.86 (m, 6 H); 1.06 (s, 3 H); 1.11 (d, J = 6.9 Hz, 3 H); 1.18 (s, 3 H); 1.31 to 1.38 (m, 7 H); 1.57 to 1.67 (m, 2 H); 1.87 (sxt, J = 6.8 Hz, 1 H); 2.29 to 2.38 (m, 1 H); 2.56 (s, 2 H); 2.70 to 2.80 (m, 2 H); 3.00 to 3.11 (m, 3 H); 3.39 to 3.43 (m, 1 H); 3.82 (s, 2 H); 3.87 (s, 3 H); 3.92 (d, J = 1.9 Hz, 1 H); 4.31 (ddd, J = 3.6 and 8.0 and 11.5 Hz, 1 H); 4.97 (dd, J = 3.7 and 9.7 Hz, 1 H); 5.17 (dd, J = 5.8 and 11.3 Hz, 1 H); 5.86 (dd, J = 1.4 and 15.1 Hz, 1 H); 6.54 (ddd, J = 3.8 and 11.3 and 15.1 Hz, 1 H); 7.11 (d, J = 8.7 Hz, 1 H); 7.23 (dd, J = 1.9 and 8.7 Hz, 1 H); 7.27 (dd, J = 2.7 and 9.6 Hz, 1 H); 7.31 (d, J = 8.0 Hz, 2 H); 7.34 (d, J = 1.9 Hz, 1 H); 7.42 (d, J = 8.0 Hz, 2 H); 8.40 (d, J = 8.0 Hz, 1 H). LCMS (A2): ES m/z = 786 [M + H]⁺; m/z = 784 [M - H]⁻; t_R = 0.92 min.

Example 4: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-

{4-[(2-mercapto-2-methylpropyl)methylamino)methyl]phenyl}oxiranyl]ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone

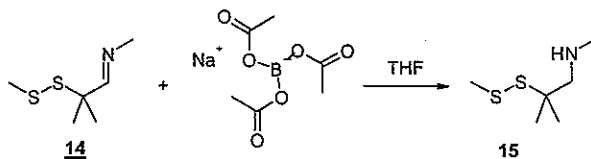


Compound 14: Methyl[2-methyl-2-methyldisulfany]prop-(E)-ylidene]amine



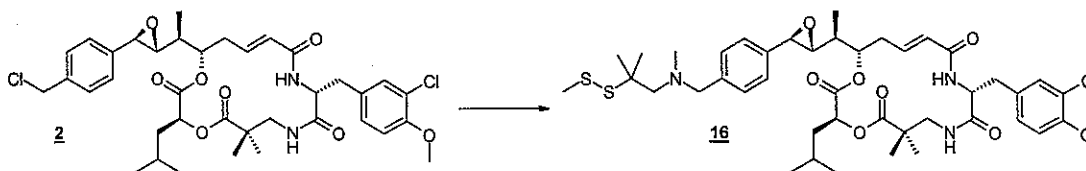
1 g (6.66 mmol) of 2-(methylthio)isobutyraldehyde are dissolved in 10 ml of anhydrous THF, under an inert atmosphere. A 2M solution of methylamine in THF (33.3 ml, 66.6 mmol) is added and the mixture is then stirred for 5 hours at RT. It is diluted in 50 ml of EtOAc, washed with water (30 ml) and with saturated NaCl solution (30 ml) and dried over MgSO₄. After filtering and concentrating under reduced pressure, compound **14** is obtained in the form of a pale yellow oil (1.01 g, 93%). ¹H NMR (400 MHz, chloroform-*d*): 1.46 (s, 6 H); 2.36 (s, 3 H); 3.32 (s, 3 H); 7.52 (s, 1 H).

Compound 15: 2-Methyl-2-methyldisulfany]propylamine



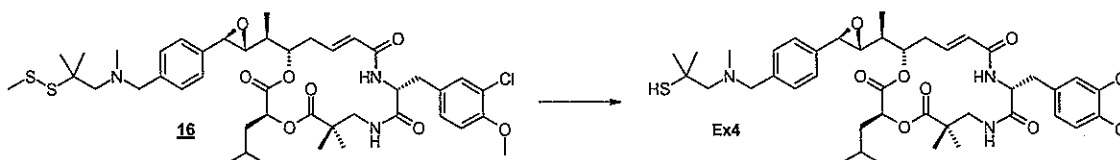
A solution of compound **14** (1.01 g, 6.185 mmol) in 30 ml of THF is purged with argon and cooled to 0°C, followed by addition, at 0°C, of 1.44 g (6.80 mmol) of sodium triacetoxyborohydride. Stirring is continued for 15 hours at RT: some starting imine remains; 1.44 g (6.80 mmol) of sodium triacetoxyborohydride and 354 µl (6.185 mmol) of acetic acid are added at 0°C and stirring is then continued for 3 hours at RT. The mixture is diluted in 50 ml of EtOAc and washed with water (50 ml). The pH of the aqueous phase is adjusted to about 12 by adding 14 ml of aqueous 1N sodium hydroxide solution, and it is then extracted with diethyl ether (3×50 ml). The organic phases are combined, washed with saturated NaCl solution (50 ml) and dried over MgSO₄. After filtering and concentrating under reduced pressure, compound **15** is obtained in the form of a colourless oil (695 mg, 68%). ¹H NMR (400 MHz, DMSO-*d*₆): 1.25 (s, 6 H); 1.47 (broad m, 1 H); 2.31 (s, 3 H); 2.38 (s, 3 H); 2.53 (m, 2 H). LCMS (A2): ES *m/z* = 166 [M + H]⁺; *t*_R = 0.28 min.

Compound 16: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-[(2R,3R)-3-(4-[(2-methyl-2-methyldisulfany]propyl)methylamino)methyl]phenyl)oxiranyl]ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



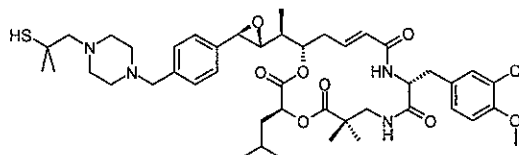
Compound **16** may be obtained by nucleophilic substitution of the chloro group of the derivative **2** with the amine **15** by applying the method described for the preparation of compound **30**.

- 5 **Example 4:** (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-[(2R,3R)-3-(4-[(2-mercapto-2-methylpropyl)methylamino]methyl)phenyl]oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone

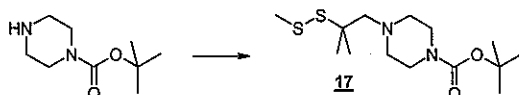


Example **4** may be obtained by applying the method described for the preparation of Example **6** to compound **16**.

Example 5: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-[(2R,3R)-3-(4-[4-(2-mercapto-2-methylpropyl)piperazin-1-ylmethyl]phenyl)oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



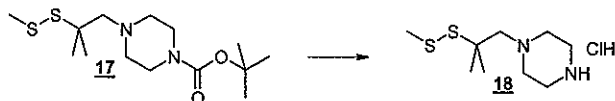
Compound 17: *tert*-Butyl 4-(2-methyl-2-methyldisulfanylpropyl)piperazine-1-carboxylate



To a solution, purged with argon, of 1-Boc-piperazine (1.0 g, 5.37 mmol) in anhydrous THF (20 ml) are added 2-(methyldithio)isobutyraldehyde (5.37 mmol) and titanium(IV) isopropoxide (6.71 mmol). The mixture is stirred for 20 minutes at RT, and a further addition of 2-(methyldithio)-isobutyraldehyde (5.37 mmol) and of titanium(IV) isopropoxide (6.71 mmol) is performed. Stirring is continued for 2 hours, and then 12 ml of ethanol are added and stirring is continued for 5 minutes. Sodium cyanoborohydride (5.37 mmol) is added and the mixture is stirred for 1 hour, followed by addition of a further 5.37 mmol of sodium cyanoborohydride. Stirring is continued for 1 hour. The mixture is concentrated to dryness and then taken up in EtOAc. Water is added, causing the formation of a precipitate, which is filtered off on a sinter funnel and rinsed with EtOAc and water. The solid obtained is dissolved in aqueous 1N HCl solution (50 ml) and the medium is neutralized with aqueous 5N NaOH solution and then extracted with DCM. The

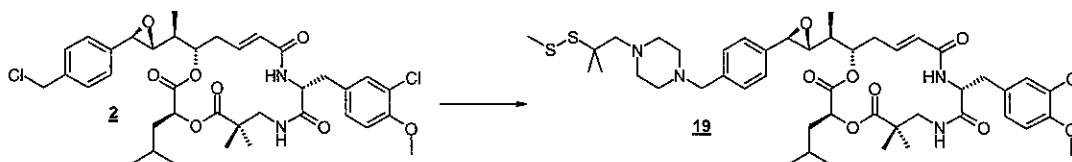
organic phases are combined, washed with saturated NaCl solution, dried over MgSO₄, filtered and concentrated under reduced pressure. The crude product is purified by chromatography on silica gel, eluting with a 100/0 to 97/3 DCM/methanol mixture. Compound **17** is obtained in the form of a pale yellow oil (650 mg; 40%). ¹H NMR (400 MHz, DMSO-*d*₆): 1.26 (s, 6 H); 1.39 (s, 9 H); 2.39 (s, 3 H); 2.42 (s, 2H); 2.44 to 2.48 (m, 4 H); 3.27 (partially masked m, 4 H). LCMS (A1) : ES m/z = 321 [M + H]⁺; m/z = 265 base peak; t_R = 3.02 min.

Compound 18: 1-(2-Methyl-2-methyldisulfanylpropyl)piperazine hydrochloride



To a solution of compound **17** (322 mg, 1.01 mmol) in dioxane (13 ml) is added a 4M solution of HCl in dioxane (5 ml). Stirring is continued for 16 hours at RT. The mixture is filtered through a sinter funnel and the solid obtained is rinsed with dioxane to give compound **18** (231 mg, 90%) in the form of a white powder. ¹H NMR (400 MHz, DMSO-*d*₆): 1.31 (broad s, 6 H); 2.42 (s, 3 H); 2.59 to 3.38 (very broad m, 10 H); 8.78 (broad m, 2 H). LCMS (A2) : ES m/z = 221 [M + H]⁺; t_R = 0.46 min.

Compound 19: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-16-[(S)-1-((2R,3R)-3-{4-[4-(2-methyl-2-methyldisulfanylpropyl)piperazin-1-ylmethyl]phenyl}oxiranyl)ethyl]-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone

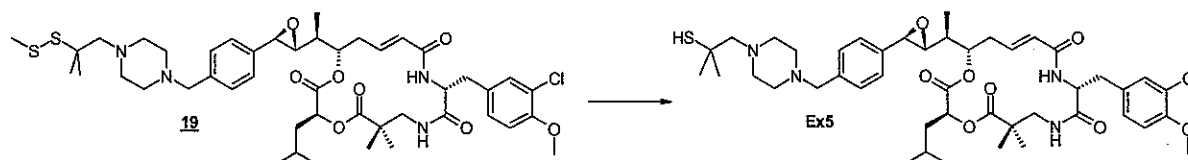


To a solution, purged with argon, of compound **2** (15.02 mg, 20.93 μmol) in anhydrous DMF (0.9 ml) is added a solution of compound **18** (25.12 μmol) and TEA (52.3 μmol) in DMF (1 ml). The mixture is stirred at 40°C under argon. After 24 hours, 25.12 μmol of compound **18** and 31.4 μmol of TEA are added. After a further 24 hours at 40°C, the reaction is complete. The mixture is diluted with EtOAc (6 ml) and washed with water (2x6 ml), with saturated NaHCO₃ solution (6 ml) and with saturated NaCl solution (6 ml). After drying the organic phase over MgSO₄, it is filtered and then evaporated under reduced pressure to give 46 mg of crude product. The residue is purified by chromatography on silica gel, eluting with a 100/0 to 95/5 DCM/methanol mixture. Compound **19** is obtained in the form of a white powder (5.2 mg, 28%). ¹H NMR (500 MHz, DMSO-*d*₆): 0.76 (d, J = 6.4 Hz, 3 H); 0.78 (d, J = 6.4 Hz, 3 H); 1.01 (s, 3 H); 1.06 (d, J = 6.8 Hz, 3 H); 1.13 (s, 3 H); 1.25 (s, 6 H); 1.27 to 1.33 (m, 1 H); 1.51 to 1.63 (m, 2 H); 1.76 to 1.85 (m, 1 H); 2.23 to 2.32 (m, 1 H); 2.37 (m, 4 H); 2.39 (s, 3 H); 2.41 (s, 2 H); 2.53 to 2.56 (m, 4 H); 2.63 to 2.76 (m, 2 H); 2.94 to 3.06 (m, 3 H); 3.34 (partially masked m, 1 H); 3.40 to 3.48 (m, 2 H); 3.82 (s, 3 H); 3.87 (d, J = 2.0 Hz, 1 H); 4.26 (ddd, J = 3.8 and 7.8 and 11.3 Hz, 1

H); 4.92 (dd, $J = 3.8$ and 9.8 Hz, 1 H); 5.07 to 5.14 (m, 1 H); 5.81 (d, $J = 15.2$ Hz, 1 H); 6.48 (ddd, $J = 3.8$ and 11.3 and 15.2 Hz, 1 H); 7.06 (d, $J = 8.5$ Hz, 1 H); 7.18 (dd, $J = 2.2$ and 8.5 Hz, 1 H); 7.21 to 7.32 (m, 6 H); 8.35 (d, $J = 8.3$ Hz, 1 H). LCMS (A1): ES $m/z = 901$ $[M + H]^+$; $m/z = 451$ base peak; $t_R = 4.33$ min.

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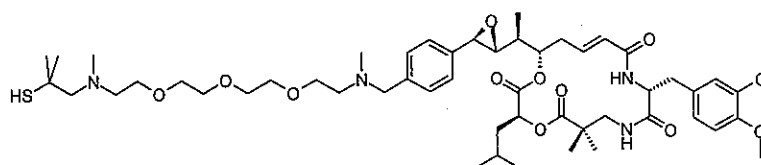
Example 5: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[4-(2-mercapto-2-methylpropyl)piperazin-1-ylmethyl]phenyl}oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



Product **19** (5.7 mg; 6.3 μ mol) is dissolved in a mixture of ethanol (0.8 ml)/water (0.5 ml). TCEP (4.54 mg, 15.83 μ mol) is then added and the mixture is stirred for 1 hour at RT. The mixture is diluted in 7 ml of EtOAc and the organic phase is washed with a 1/1 mixture of water and saturated aqueous NH_4Cl solution (7 ml). After drying the organic phase over $MgSO_4$, filtering and evaporating off the solvents under reduced pressure, the crude product obtained is purified by chromatography on silica gel, eluting with a 99/1 to 95/5 DCM/methanol mixture. The compound **Ex. 5** is obtained in the form of a white solid (2.95 mg; 55%). 1H NMR (500 MHz, $DMSO-d_6$): 0.77 (d, $J = 6.3$ Hz, 3 H); 0.78 (d, $J = 6.3$ Hz, 3 H); 1.02 (s, 3 H); 1.06 (d, $J = 6.9$ Hz, 3 H); 1.13 (s, 3 H); 1.27 (s, 6 H); 1.29 to 1.34 (m, 1 H); 1.52 to 1.63 (m, 2 H); 1.82 (m, 1 H); 2.29 (m, 1 H); 2.37 (s, 2 H); 2.39 to 2.41 (m, 4 H); 2.57 to 2.62 (m, 4 H); 2.65 to 2.76 (m, 2 H); 2.96 to 3.06 (m, 3 H); 3.34 (partially masked m, 1 H); 3.46 (s, 2 H); 3.83 (s, 3 H); 3.88 (d, $J = 2.2$ Hz, 1 H); 4.27 (ddd, $J = 3.7$ and 8.0 and 11.5 Hz, 1 H); 4.93 (dd, $J = 3.7$ and 9.7 Hz, 1 H); 5.12 (m, 1 H); 5.82 (d, $J = 15.2$ Hz, 1 H); 6.49 (ddd, $J = 3.7$ and 11.5 and 15.2 Hz, 1 H); 7.07 (d, $J = 8.5$ Hz, 1 H); 7.18 (dd, $J = 2.2$ and 8.5 Hz, 1 H); 7.21 to 7.33 (m, 6 H); 8.36 (d, $J = 8.0$ Hz, 1 H). LCMS (A1): ES $m/z = 855$ $[M + H]^+$; $m/z = 428$ $[M + 2H]^{2+}$ base peak; $t_R = 4.13$ min.

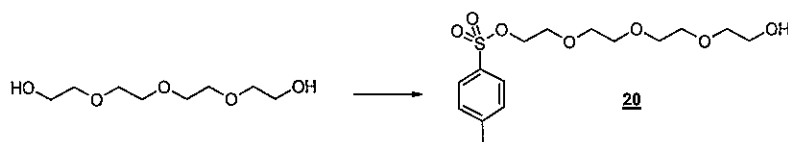
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Example 6: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[4-({2-[2-(2-{2-methyl[2-mercapto-2-methylpropyl]aminoethoxy}ethoxy)ethoxy]ethyl)-methylamino)methyl]phenyl}oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



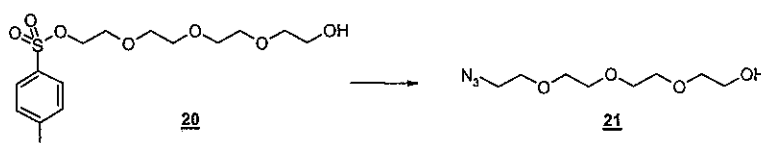
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Compound 20: 2-{2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy}ethyl 4-toluenesulfonate



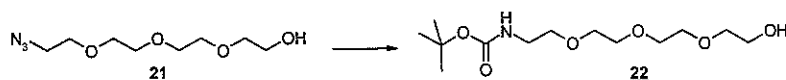
To a solution, purged under argon and cooled to 0°C, of 5 g (25.74 mmol) of tetraethylene glycol in 68.7 ml of DCM are successively added portionwise, so as to maintain suitable stirring, 8.95 g (38.61 mmol) of silver oxide and 5.40 g (28.31 mmol) of tosyl chloride. 855 mg (5.15 mmol) of KI are added portionwise so as to maintain the temperature of the mixture below 5°C. Stirring is continued for 1 hour while keeping the temperature below 5°C. After warming to RT, the mixture is filtered through Clarcel, the residue is rinsed with DCM and the filtrate is then concentrated to dryness under reduced pressure. The crude product is purified by chromatography on silica gel, using a 99/1 to 95/5 DCM/methanol mixture as eluent. Compound **20** is obtained in the form of a colourless oil (5.4 g, 60%). ¹H NMR (400 MHz, DMSO-*d*₆): 2.43 (s, 3 H); 3.40 (m, 2 H); 3.44 to 3.52 (m, 10 H); 3.58 (m, 2 H); 4.11 (m, 2 H); 4.53 (t, *J* = 5.4 Hz, 1 H); 7.48 (d, *J* = 8.3 Hz, 2 H); 7.78 (d, *J* = 8.3 Hz, 2 H). LCMS (A2): ES *m/z* = 349 [M + H]⁺; *m/z* = 371 [M + Na]⁺; *t*_R = 0.69 min.

Compound 21: 1-Azido-3,6-9-trioxaundecan-11-ol



To a solution of 5.4 g (15.51 mmol) of compound **20** in 40.6 ml of anhydrous acetonitrile are added 1.34 g (20.63 mmol) of NaN₃ and the mixture is then refluxed for 6 hours 30 minutes. After cooling to RT, the mixture is filtered through Clarcel and concentrated under reduced pressure. Some starting compound **20** remains: the crude product is taken up in 25 ml of anhydrous acetonitrile and 230 mg (3.5 mmol) of NaN₃ are added. The mixture is refluxed for 5 hours. After cooling to RT, it is filtered through Clarcel and concentrated to dryness under reduced pressure to give compound **21** in the form of a pale yellow oil (3.37 g, 99%). ¹H NMR (400 MHz, DMSO-*d*₆): 3.37 to 3.43 (m, 4 H); 3.45 to 3.58 (m, 10 H); 3.60 (m, 2 H); 4.54 (t, *J* = 5.1 Hz, 1 H). LCMS (A2): ES *m/z* = 220 [M + H]⁺; *m/z* = 242 [M + Na]⁺ base peak; *t*_R = 0.39 min.

Compound 22: N-Boc-aminoethoxyethoxyethoxyethanol

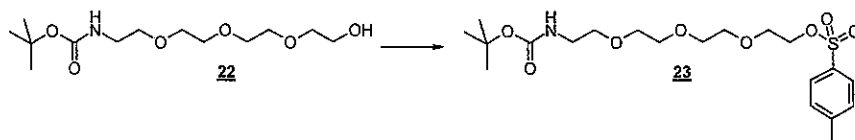


To a solution, rendered inert under argon, of 320 mg of 10% palladium-on-charcoal in 5 ml of EtOAc is added a solution of 3.25 g (14.8 mmol) of compound **21**, 6.46 g (29.6 mmol) of *tert*-butyl dicarbonate and 4.13 ml (29.6 mmol) of TEA in 45 ml of EtOAc. The reaction is performed for 17 hours at 30°C under a hydrogen pressure of 2 bar. After cooling to RT and atmospheric pressure, the mixture is filtered through Clarcel and concentrated under reduced pressure. The crude product is purified by chromatography on silica gel, using a 98/2 to 90/10 DCM/methanol mixture

as eluent. Compound **22** is obtained in the form of a colourless oil (2.92 g, 67%). ¹H NMR (400 MHz, DMSO-*d*₆): 1.37 (s, 9 H); 3.06 (q, *J* = 6.1 Hz, 2 H); 3.37 (t, *J* = 6.1 Hz, 2 H); 3.42 (m, 2 H); 3.46 to 3.53 (m, 10 H); 4.54 (broad t, *J* = 5.1 Hz, 1 H); 6.71 (broad t, *J* = 6.1 Hz, 1 H). LCMS (A1): ES *m/z* = 316 [M + Na]⁺; *m/z* = 194 base peak; *t_R* = 2.81 min.

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Compound 23: 2-{2-[2-(2-*tert*-Butoxycarbonylaminoethoxy)ethoxy]ethoxy}ethyl 4-toluenesulfonate



To a solution, purged with argon, of 3.06 g (10.42 mmol) of compound **22** in 20 ml of DCM are added 2.53 ml (31.26 mmol) of pyridine. The mixture is cooled to 0°C, followed by dropwise addition of a solution of 2.98 g (15.63 mmol) of tosyl chloride in 10 ml of DCM. Stirring is continued for 15 hours at RT. The mixture is diluted in 20 ml of DCM, washed with saturated NaHCO₃ solution (30 ml), with water (2×30 ml) and with saturated NaCl solution (30 ml) and dried over MgSO₄. After filtering and concentrating under reduced pressure, the crude product is purified by chromatography on silica gel, using a 100/0 to 90/10 DCM/methanol mixture as eluent.

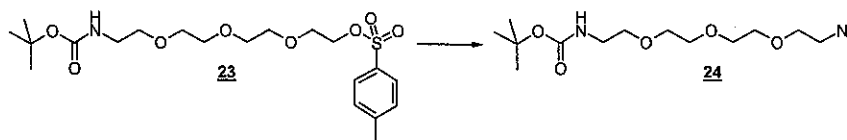
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Compound **23** is obtained in the form of a pale yellow oil (4.38 g, 94%). ¹H NMR (400 MHz, DMSO-*d*₆): 1.37 (s, 9 H); 2.42 (s, 3 H); 3.05 (q, *J* = 6.1 Hz, 2 H); 3.36 (t, *J* = 6.1 Hz, 2 H); 3.46 (m, 8 H); 3.58 (m, 2 H); 4.10 (m, 2 H); 6.71 (broad t, *J* = 6.1 Hz, 1 H); 7.48 (d, *J* = 7.8 Hz, 2 H); 7.78 (d, *J* = 7.8 Hz, 2 H). LCMS (A2): ES *m/z* = 448 [M + H]⁺; *m/z* = 470 [M + Na]⁺; *m/z* = 348 base peak; *m/z* = 492 [M - H + HCO₂H]⁺; *t_R* = 1.00 min.

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Compound 24: *tert*-Butyl (2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethyl)carbamate

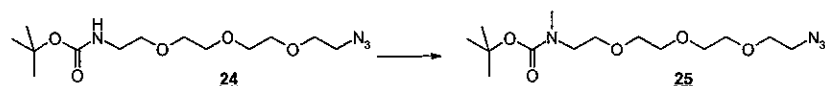


To a solution of 4.38 g (9.79 mmol) of compound **23** in 25 ml of acetonitrile are added 846 mg (13.02 mmol) of sodium azide and the mixture is then refluxed for 6 hours. A large proportion of starting compound remains: after cooling to RT, 1.7 g (26.13 mmol) are added to the mixture. Stirring is continued at reflux for 24 hours. After cooling to RT, the mixture is filtered through Clarcel and concentrated under reduced pressure. The crude product is purified by chromatography on silica gel, using a 98/2 to 90/10 DCM/methanol mixture as eluent. Compound **24** is obtained in the form of a pale yellow oil (2.16 g, 69%). ¹H NMR (400 MHz, DMSO-*d*₆): 1.37 (s, 9 H); 3.06 (q, *J* = 6.1 Hz, 2 H); 3.34 to 3.43 (m, 4 H); 3.46 to 3.58 (m, 8 H); 3.60 (m, 2 H); 6.70 (broad t, *J* = 6.1 Hz, 1 H). LCMS (A2): ES *m/z* = 341 [M + Na]⁺; *t_R* = 0.81 min.

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30

Compound 25: *tert*-Butyl (2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethyl)methylcarbamate



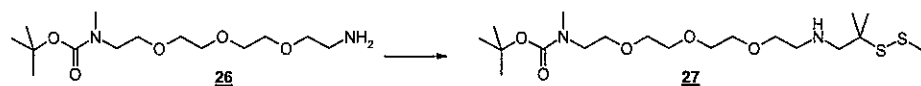
To a solution, purged with argon and cooled to 0°C, of 2.06 g (6.47 mmol) of compound **24** in 25 ml of anhydrous THF are successively added, at 0°C, 854 mg (21.35 mmol) of 60% NaH as a dispersion in mineral oil (portionwise) and 886 µl (14.23 mmol) of methyl iodide. Stirring is continued for 1 hour at 0°C and then for 16 hours at RT. The mixture is filtered through Clarcel, washed with THF and concentrated under reduced pressure. The crude product is purified by chromatography on silica gel, using a 99/1 to 90/10 DCM/methanol mixture as eluent. Compound **25** is obtained in the form of a colourless oil (1.67 g, 78%). ¹H NMR (400 MHz, DMSO-*d*₆): 1.39 (s, 9 H); 2.80 (broad s, 3 H); 3.29 (partially masked t, *J* = 5.6 Hz, 2 H); 3.38 (m, 2 H); 3.46 to 3.56 (m, 10 H); 3.60 (t, *J* = 5.4 Hz, 2 H).

Compound 26: *tert*-Butyl (2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}ethyl)methylcarbamate



To a solution, purged with argon, of 1.66 g (4.99 mmol) of compound **25** in 20 ml of THF are successively added 1.31 g (4.994 mmol) of triphenylphosphine and 108 µl (5.99 mmol) of water. Stirring is continued for 25 hours 30 minutes and the mixture is then concentrated to dryness and purified by SPE filtration on an SCX cartridge (Varian) conditioned and washed with methanol and then eluted with a 0.5 N solution of aqueous ammonia in methanol. Compound **26** is obtained in the form of a colourless oil (1.23 g, 80%). ¹H NMR (400 MHz, DMSO-*d*₆): 1.39 (s, 9 H); 2.65 (t, *J* = 5.9 Hz, 2 H); 2.80 (broad s, 3 H); 3.29 (t, *J* = 5.9 Hz, 2 H); 3.36 (t, *J* = 5.7 Hz, 2 H); 3.45 to 3.56 (m, 10 H). LCMS (A2) : ES *m/z* = 307 [*M* + *H*]⁺; *t*_R = 0.49 min.

Compound 27: *tert*-Butyl methyl[2-(2-{2-[2-(2-methyl-2-methyldisulfany)propylamino)ethoxy]ethoxy}ethoxy)ethyl]carbamate



To a solution, purged with argon, of 131 mg (426 µmol) of compound **26** in 3 ml of DCM are successively added 64 mg (426 µmol) of 2-methyldithioisobutyraldehyde, 126 mg (596 µmol) of sodium triacetoxyborohydride and 24.4 µl (426 µmol) of acetic acid. Stirring is continued for 6 hours at RT under argon, the reaction is quenched by addition of 1 ml of aqueous 1N sodium hydroxide solution and then the mixture is extracted with 10 ml of diethyl ether. After concentrating under reduced pressure, the crude product is purified by chromatography on silica gel, using a 90/10 DCM/isopropanol mixture as eluent, which does not make it possible to separate out the expected product from the residual starting amine. The product obtained is again purified by RP18 reverse-phase chromatography using a 95/5 to 5/95 water/acetonitrile mixture as eluent. Compound **27** is obtained in the form of a colourless oil (67 mg, 36%). ¹H NMR

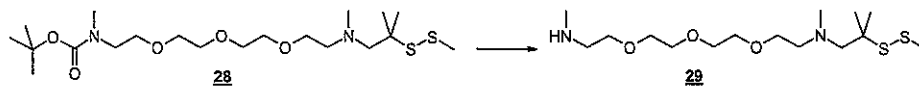
(400 MHz, DMSO- d_6): 1.25 (s, 6 H); 1.39 (s, 9 H); 1.58 (broad m, 1 H); 2.38 (s, 3 H); 2.59 (s, 2 H); 2.68 (t, $J = 5.6$ Hz, 2 H); 2.80 (broad s, 3 H); 3.46 (t, $J = 5.6$ Hz, 2 H); 3.48 to 3.54 (m, 12 H). LCMS (A1) : ES $m/z = 441$ $[M + H]^+$; $t_R = 3.29$ min.

- 5 **Compound 28:** *tert*-Butyl methyl{2-[2-(2-[2-[methyl(2-methyl-2-methyldisulfanyl)propyl)amino]-ethoxy]ethoxy]ethoxy]ethyl}carbamate



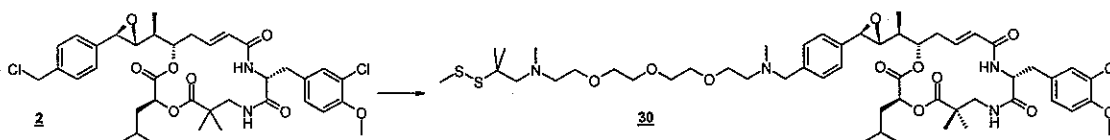
- To a solution, purged with argon and cooled to 0°C, of 65 mg (148 μ mol) of compound **27** in 1 ml of DCM are successively added 19.5 mg (487 μ mol) of NaH as a 60% dispersion in a mineral oil and 20 μ l (325 μ mol) of methyl iodide. Stirring is continued for 45 minutes at 0°C and then for 72 hours at RT. The mixture is filtered through Clarcel and then concentrated under reduced pressure. The crude product is purified by chromatography on silica gel, using a 99/1 to 90/10 DCM/methanol mixture as eluent. Compound **28** is obtained in the form of a colourless oil (43 mg, 64%). ^1H NMR (400 MHz, DMSO- d_6): 1.25 (s, 6 H); 1.39 (s, 9 H); 2.32 (s, 3 H); 2.39 (s, 3 H); 2.54 (m, 2 H); 2.61 (t, $J = 6.1$ Hz, 2 H); 2.80 (broad s, 3 H); 3.42 to 3.54 (m, 14 H). LCMS (A2): ES $m/z = 455$ $[M + H]^+$; $t_R = 0.77$ min.

Compound 29: Methyl(2-[2-[2-(2-methylaminoethoxy)ethoxy]ethoxy]ethyl)(2-methyl-2-methyl-disulfanylpropyl)amine



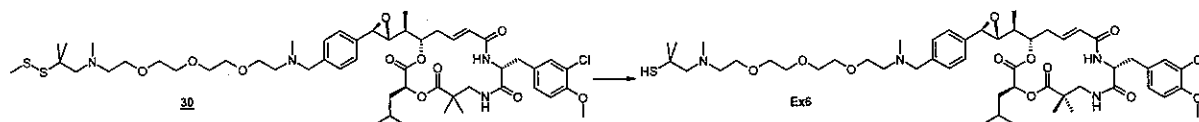
- To a solution of 43 mg (95 μ mol) of compound **28** in 1.5 ml of DCM are added 351 μ l of TFA. Stirring is continued for 5 hours at RT and the mixture is then concentrated to dryness. The crude product is purified by SPE filtration on an SCX cartridge (Varian) conditioned and washed with methanol, and then eluted with a 0.5 N solution of aqueous ammonia in methanol. Compound **29** is obtained in the form of a colourless oil (25 mg, 75%). ^1H NMR (400 MHz, DMSO- d_6): 1.25 (s, 6 H); 1.75 (broad m, 1 H); 2.27 (s, 3 H); 2.32 (s, 3 H); 2.39 (s, 3 H); 2.53 (m, 2 H); 2.57 to 2.64 (m, 4 H); 3.44 (t, $J = 5.7$ Hz, 2 H); 3.46 to 3.55 (m, 10 H). LCMS (A2) : ES $m/z = 355$ $[M + H]^+$; $t_R = 0.30$ min.

- 30 **Compound 30:** (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[4-({2-[2-(2-[2-methyl[2-methyl-2-methyldisulfanyl]propyl]aminoethoxy)ethoxy]ethoxy]ethyl)methylamino)methyl]phenyl]oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



To a solution, purged with argon, of 15.3 mg (21.3 μ mol) of compound **2** in 1 ml of anhydrous acetonitrile are successively added 18.6 μ l (106.6 μ mol) of DIPEA and a solution of 22.7 mg (64 μ mol) of compound **29** in 1 ml of anhydrous acetonitrile. Stirring is continued for 24 hours under argon at 40°C. After cooling to RT, the mixture is diluted with 7 ml of EtOAc, washed with water (2 $\times$ 3 ml), with saturated NaHCO₃ solution (3 ml) and with saturated NaCl solution (3 ml) and dried over MgSO₄. After filtering and concentrating under reduced pressure, the crude product is purified by chromatography on silica gel, using a 90/10 DCM/methanol mixture as eluent. Compound **30** is obtained in the form of a colourless solid (14.6 mg, 66%). ¹H NMR (500 MHz, DMSO-*d*₆): 0.76 (d, *J* = 6.1 Hz, 3 H); 0.76 (d, *J* = 6.1 Hz, 3 H); 1.00 (s, 3 H); 1.04 (d, *J* = 6.8 Hz, 3 H); 1.12 (s, 3 H); 1.24 (s, 6 H); 1.29 (m, 1 H); 1.50 to 1.62 (m, 2 H); 1.81 (m, 1 H); 2.15 (s, 3 H); 2.27 (m, 1 H); 2.31 (s, 3 H); 2.38 (s, 3 H); 2.45 to 2.54 (partially masked m, 4 H); 2.60 (t, *J* = 6.1 Hz, 2 H); 2.63 to 2.75 (m, 2 H); 2.92 to 3.06 (m, 3 H); 3.25 to 3.35 (partially masked m, 1 H); 3.44 to 3.54 (m, 14 H); 3.81 (s, 3 H); 3.87 (d, *J* = 2.0 Hz, 1 H); 4.25 (ddd, *J* = 3.9 and 8.3 and 11.7 Hz, 1 H); 4.91 (dd, *J* = 3.4 and 9.8 Hz, 1 H); 5.11 (ddd, *J* = 1.5 and 5.7 and 11.5 Hz, 1 H); 5.80 (dd, *J* = 1.5 and 15.5 Hz, 1 H); 6.47 (ddd, *J* = 3.5 and 11.5 and 15.5 Hz, 1 H); 7.05 (d, *J* = 8.8 Hz, 1 H); 7.17 (dd, *J* = 2.0 and 8.8 Hz, 1 H); 7.20 to 7.26 (m, 3 H); 7.27 to 7.33 (m, 3 H); 8.35 (d, *J* = 8.3 Hz, 1 H). LCMS (A2): ES *m/z* = 1035 [*M* + *H*]⁺; *m/z* = 518 [*M* + 2*H*]²⁺ base peak; *t_R* = 0.86 min.

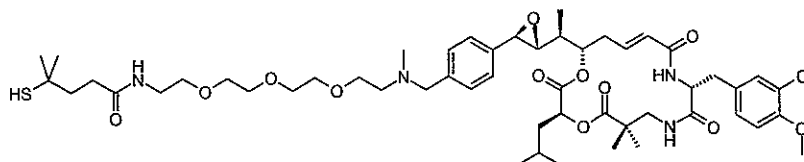
Example 6: (E)(3*S*,10*R*,16*S*)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(*S*)-1-((2*R*,3*R*)-3-{4-[4-({2-[2-(2-{2-methyl[2-mercapto-2-methylpropyl]aminoethoxy}ethoxy)ethoxy]ethyl)methylamino)-methyl]phenyl}oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



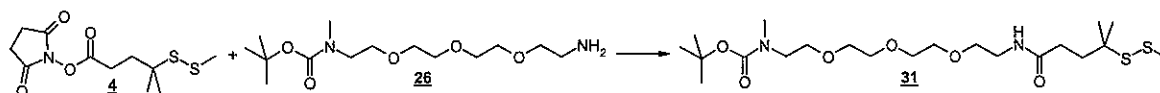
To a solution of 10.9 mg (10.5 μ mol) of compound **30** in 1.24 ml of ethanol is added a solution of 12.06 mg (42.1 μ mol) of TCEP in 1.04 ml of water. Stirring is continued for 4 hours at RT. The mixture is diluted with 6 ml of EtOAc, washed with a 1/1 mixture of water/NH₄Cl_{sat} (6 ml) and with saturated NaCl solution (6 ml), and dried over MgSO₄. After filtering and concentrating under reduced pressure, the crude product is purified by chromatography on silica gel, using a 99/1 to 90/10 DCM/methanol mixture. Compound **Ex. 6** is obtained in the form of a white solid (7.36 mg, 71%). ¹H NMR (500 MHz, DMSO-*d*₆): 0.76 (d, *J* = 5.9 Hz, 3 H); 0.78 (d, *J* = 5.9 Hz, 3 H); 1.00 (s, 3 H); 1.04 (d, *J* = 6.8 Hz, 3 H); 1.12 (s, 3 H); 1.25 (s, 6 H); 1.28 (m, 1 H); 1.51 to 1.62 (m, 2 H); 1.80 (m, 1 H); 2.15 (s, 3 H); 2.27 (m, 1 H); 2.34 (s, 3 H); 2.44 (s, 2 H); 2.51 (t, *J* = 6.4 Hz, 2 H); 2.60 (s, 1 H); 2.63 (t, *J* = 6.4 Hz, 2 H); 2.66 to 2.74 (m, 2 H); 2.93 to 3.04 (m, 3 H); 3.25 to 3.37 (partially masked m, 1 H); 3.45 to 3.55 (m, 14 H); 3.81 (s, 3 H); 3.87 (d, *J* = 1.5 Hz, 1 H); 4.25 (ddd, *J* = 3.7 and 8.0 and 11.5 Hz, 1 H); 4.91 (dd, *J* = 3.4 and 9.8 Hz, 1 H); 5.11 (ddd, *J* = 1.5 and 5.6 and 11.5 Hz, 1 H); 5.80 (dd, *J* = 1.5 and 15.2 Hz, 1 H); 6.47 (ddd, *J* = 3.9 and 11.5 and 15.2

Hz, 1 H); 7.05 (d, $J = 8.8$ Hz, 1 H); 7.17 (dd, $J = 2.2$ and 8.8 Hz, 1 H); 7.20 to 7.26 (m, 3 H); 7.27 to 7.32 (m, 3 H); 8.35 (d, $J = 8.0$ Hz, 1 H), LCMS (A1): ES $m/z = 989$ $[M + H]^+$; $m/z = 495$ $[M + 2H]^{2+}$ base peak; $t_R = 3.24$ min.

- 5 **Example 7** : (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[4-({2-[2-(2-{4-methyl-4-methyldisulfanyl}pentanoylaminoethoxy)ethoxy]ethyl}methylethyl)phenyl]oxiranyl}ethyl)-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone

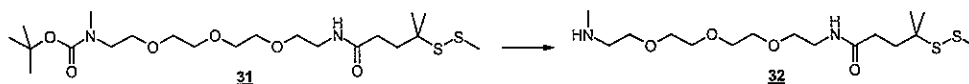


- 10 **Compound 31**: *tert*-Butyl methyl[2-(2-{2-[2-(4-methyl-4-methyldisulfanyl)pentanoylamino]ethoxy]ethoxy]ethyl]carbamate



- 270 mg (649 μ mol) of compound 4, 199 mg (649 μ mol) of compound 26 are dissolved in 1.5 ml of DMF, and 100 μ l (714 μ mol) of TEA are then added to the mixture. Stirring is continued for 16 hours at RT. The mixture is diluted with 10 ml of EtOAc, washed with water (2x5 ml) and with saturated NaCl solution (5 ml) and dried over $MgSO_4$. After filtering and concentrating under reduced pressure, the crude product is purified by chromatography on silica gel, using a 98/2 to 90/10 DCM/methanol mixture as eluent. Compound 31 is obtained in the form of a colourless oil (251 mg, 80%). 1H NMR (400 MHz, $DMSO-d_6$): 1.24 (s, 6 H); 1.39 (s, 9 H); 1.79 (m, 2 H); 2.16 (m, 2 H); 2.40 (s, 3 H); 2.80 (broad s, 3 H); 3.18 (q, $J = 5.9$ Hz, 2 H); 3.39 (t, $J = 5.9$ Hz, 2 H); 3.45 to 3.54 (m, 12 H); 7.88 (broad t, $J = 5.9$ Hz, 1 H). LCMS (A2) : ES $m/z = 483$ $[M + H]^+$; $m/z = 505$ $[M + Na]^+$; $m/z = 527$ $[M - H + HCO_2H]^+$; $t_R = 1.04$ min.

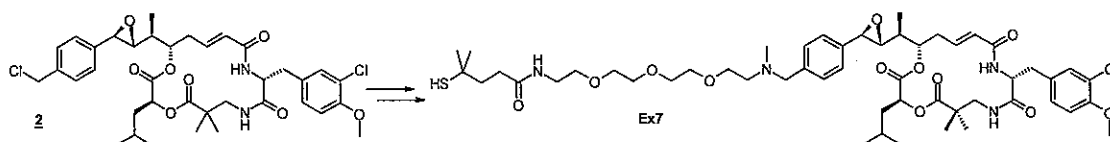
- 25 **Compound 32** : N-(2-{2-[2-(2-Methylaminoethoxy)ethoxy]ethoxy}ethyl)-4-methyl-4-methyldisulfanylpentamide



- To a solution of 251 mg (520 μ mol) of compound 31 in 8 ml of DCM are added 1.93 ml (26 mmol) of TFA. Stirring is continued for 3 hours at RT and the mixture is then concentrated to dryness. The crude product is dissolved in a minimum amount of DCM and then entrained several times with toluene. The crude product is purified by SPE filtration on an SCX cartridge (Varian) conditioned and washed with methanol and then eluted with a 0.5 N solution of aqueous ammonia in methanol. Compound 32 is obtained in the form of a colourless oil (159 mg, 80%). 1H NMR (400 MHz, $DMSO-d_6$): 1.24 (s, 6 H); 1.79 (m, 2 H); 2.15 (m, 2 H); 2.27 (s, 3 H); 2.40 (s, 3

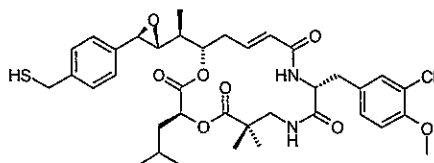
H); 2.58 (t, J = 5.7 Hz, 2 H); 3.18 (q, J = 5.7 Hz, 2 H); 3.39 (t, J = 5.7 Hz, 2 H); 3.44 (t, J = 5.7 Hz, 2 H); 3.47 to 3.54 (m, 8 H); 7.89 (broad t, J = 5.7 Hz, 1 H), LCMS (A1): ES m/z = 383 [M + H]⁺; t_R = 2.68 min.

- 5 **Example 7:** (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[4-((2-[2-(2-{4-methyl-4-methyldisulfanyl}pentanoylaminoethoxy)ethoxy)ethoxy]ethyl)methylamino)methyl]phenyl]oxiranyl]ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone

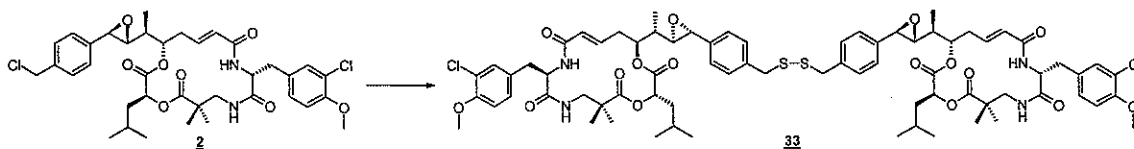


- 10 Example 7 may be obtained by nucleophilic substitution of the chloro group of derivative 2 with the amine 32 by applying the method described for the preparation of compound 30, followed by reduction of the disulfide by applying the method described for the preparation of Example 6.

- 15 **Example 8:** (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-(4-mercaptomethylphenyl)oxiranyl]ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



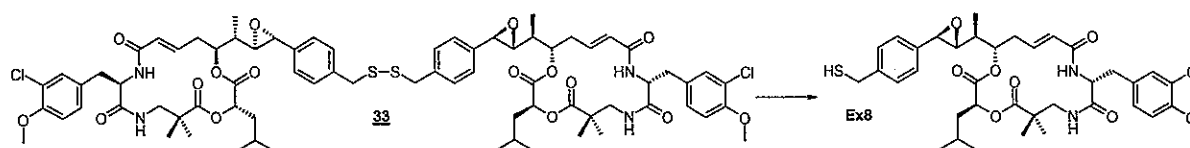
Compound 33: dimer of Example 8



- 20 Compound 2 (24.5 mg; 34.1 μmol) is dissolved in anhydrous THF (2.5 ml) and the mixture is cooled to -10°C, followed by addition of hexamethyldisilathiane (44.3 μmol) and then a 1M solution of tetrabutylammonium fluoride in THF (40.9 μmol). The mixture is cooled to room temperature and stirring is continued for 1 hour 30 minutes. The mixture is diluted by adding EtOAc (5 ml) and the organic phase is washed with saturated aqueous NH₄Cl solution (5 ml). The
- 25 aqueous phase is extracted with EtOAc (2×5 ml). The organic phases are combined and washed with saturated aqueous NaCl solution (5 ml). After drying over MgSO₄ and filtering, the solvents are evaporated off under reduced pressure. The crude reaction product is purified by chromatography on silica gel, eluting with a 100/0 to 97/3 DCM/methanol mixture as eluent. A white solid, compound 33, is obtained (19 mg; 78%). ¹H NMR (400 MHz, DMSO-*d*₆): 0.78 (m, 12 H); 1.00 (s, 6 H); 1.04 (d, J = 6.8 Hz, 6 H); 1.11 (s, 6 H); 1.30 (m, 2 H); 1.49 – 1.63 (m, 4 H); 1.82
- 30 (m, 2 H); 2.26 (m, 2 H); 2.63 – 2.72 (m, 4 H); 2.93 – 3.05 (m, 6 H); 3.25 – 3.37 (partially masked

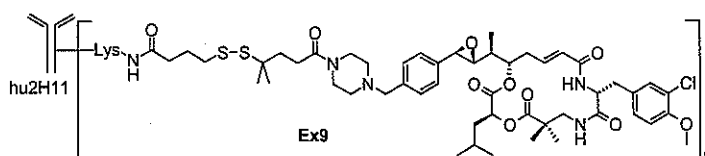
m, 2 H); 3.81 (s, 6 H); 3.82 (s, 4 H); 3.89 (d, $J = 2.0$ Hz, 2 H); 4.25 (ddd, $J = 3.7, 8.0$ and 11.5 Hz, 2 H); 4.91 (dd, $J = 3.7, 9.6$ Hz, 2 H); 5.10 (ddd, $J = 1.3, 5.3$ and 10.8 Hz, 2 H); 5.79 (dd, $J = 1.3, 15.3$ Hz, 2 H); 6.47 (ddd, $J = 3.7, 10.8$ and 15.3 Hz, 2 H); 7.05 (d, $J = 8.6$ Hz, 2 H); 7.17 (dd, $J = 2.0, 8.6$ Hz, 2 H); 7.22 (dd, $J = 2.5, 9.3$ Hz, 2 H); 7.26 – 7.32 (m, 10 H); 8.34 (d, $J = 8.0$ Hz, 2 H),
 5 LCMS (A2) : ES $m/z = 1427$ $[M + H]^+$; $t_R = 1.31$ min.

Example 8: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-[(2R,3R)-3-(4-mercaptomethylphenyl)oxiranyl]ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



Compound **33** (11 mg; $7.7 \mu\text{mol}$) is dissolved in methanol (8.8 ml). TCEP ($76.7 \mu\text{mol}$) dissolved in 2.2 ml of water is then added and the mixture is stirred for 2 hours at RT. The mixture is diluted in 20 ml of EtOAc and the organic phase is washed with a 1/1 mixture of water and of saturated aqueous NH_4Cl solution (20 ml). The aqueous phase is extracted with EtOAc (2×15 ml). The organic phases are combined, washed with saturated aqueous NaCl solution (15 ml). After drying over MgSO_4 and filtering, the solvents are evaporated off under reduced pressure. The crude reaction product is purified by chromatography on silica gel, eluting with a 100/0 to 95/5 DCM/methanol mixture. A white solid, **Ex. 8**, is obtained (4.7 mg; 31%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 0.79 (d, $J = 6.4$ Hz, 6 H); 1.00 (s, 3 H); 1.05 (d, $J = 6.9$ Hz, 3 H); 1.12 (s, 3 H); 1.30 (m, 1 H); 1.50 – 1.63 (m, 2 H); 1.80 (m, 1 H); 2.27 (m, 1 H); 2.62 – 2.75 (m, 2 H); 2.84 (broad t, $J = 6.5$ Hz, 1 H); 2.93 – 3.06 (m, 3 H); 3.34 (partially masked m, 1 H); 3.73 (broad d, $J = 6.5$ Hz, 2 H); 3.81 (s, 3 H); 3.87 (d, $J = 1.6$ Hz, 1 H); 4.25 (ddd, $J = 3.7, 8.0$ and 11.4 Hz, 1 H); 4.91 (dd, $J = 3.6, 9.6$ Hz, 1 H); 5.11 (ddd, $J = 1.3, 5.3$ and 11.4 Hz, 1 H); 5.79 (dd, $J = 1.3, 15.2$ Hz, 1 H); 6.47 (ddd, $J = 3.4, 11.3$ and 15.2 Hz, 1 H); 7.05 (d, $J = 8.5$ Hz, 1 H); 7.17 (dd, $J = 1.9, 8.5$ Hz, 1 H); 7.22 (dd, $J = 2.3, 9.5$ Hz, 1 H); 7.25 (d, $J = 8.3$ Hz, 2 H); 7.28 (d, $J = 1.9$ Hz, 2 H); 7.35 (d, $J = 8.3$ Hz, 2 H); 8.34 (d, $J = 8.0$ Hz, 2 H). LCMS (A2): ES $m/z = 715$ $[M + H]^+$; $m/z = 713$ $[M - H]^-$; $t_R = 1.18$ min.

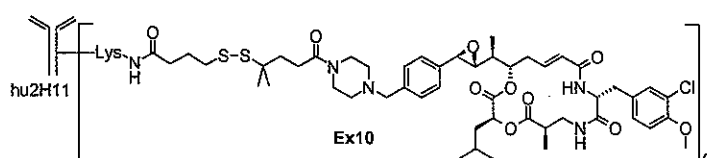
Example 9: hu2H11-SPDB-Ex1



According to the general two-step method, 10.4 mg ($0.071 \mu\text{mol}$, 1.174 ml) of naked antibody hu2H11 with an initial concentration of 8.86 mg/ml are treated with 7 eq. of the N-hydroxy-succinimidyl ester of 4-(2-pyridyldithio)butanoic acid (0.16 mg, $0.496 \mu\text{mol}$) dissolved in 34.2 μl of

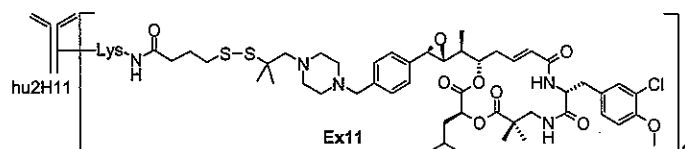
DMA such that the final antibody concentration is 8 mg/ml in the mixture. After purification, 2.2 ml of modified antibody hu2H11 at a concentration of 4.28 mg/ml (9.42 mg, 91%) are obtained with, on average, 4.68 pyridyldisulfide molecules per antibody. 1.68 ml (7.2 mg, 0.049 μ mol) of modified antibody hu2H11 are treated with 1.03 mg of (E)-(3S,10R,16S)-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[4-(4-mercapto-4-methylpentanoyl)piperazin-1-ylmethyl]phenyl}oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone (compound **Ex. 1**, 1.148 μ mol) dissolved in 101.2 μ l of DMA. After purification on Superdex in the presence of 10% NMP and concentration on Amicon Ultra-15, the final change of buffer is performed in an aqueous pH 6.5 buffer containing 0.01 M of phosphate and 0.14 M of NaCl. 1.5 ml of conjugate **Ex. 9** are thus obtained at a concentration of 1.1 mg/ml with, on average, 3 cryptophycin derivatives per antibody (HRMS) and a monomer purity of 99.9%.

Example 10: hu2H11-SPDB-Ex2



According to the one-step general method, 13.5 mg (0.092 μ mol, 1.318 ml) of naked antibody hu2H11 at an initial concentration of 10.24 mg/ml are treated with 6 eq. of the N-hydroxy-succinimidyl ester of 4-(2-pyridyldithio)butanoic acid (0.18 mg, 0.551 μ mol) dissolved in 38.4 μ l of DMA such that the final antibody concentration is 9 mg/ml in the mixture. After stirring for 2 hours at about 2000 rpm at RT, 1.333 ml (12.0 mg, 0.081 μ mol) of the mixture of modified antibody hu2H11, 1.760 ml of pH 7.5-8 buffer, 543 μ l of DMA and then 1.73 mg of (E)-(3S,6R,10R,16S)-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[4-(4-mercapto-4-methylpentanoyl)piperazin-1-ylmethyl]phenyl}oxiranyl)ethyl]-6-methyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone (compound **Ex. 2**, 1.958 μ mol) dissolved in 182 μ l of DMA are successively added. After purification on Superdex in the presence of 20% of NMP and concentration on Amicon Ultra-15, the final buffer change is performed in an aqueous pH 6.5 buffer containing 0.01 M of histidine, 10% of sucrose (w/v) and 5% of NMP (v/v). 1.5 ml of conjugate **Ex. 10** are thus obtained at a concentration of 2.83 mg/ml with, on average, 3.7 cryptophycin derivatives per antibody and a monomeric purity of 98.8%.

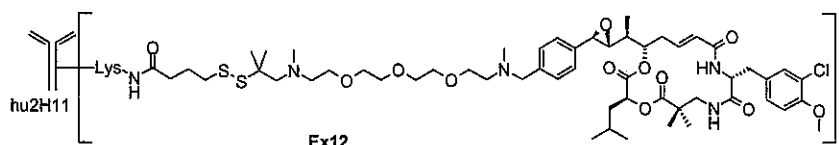
Example 11: hu2H11-SPDB-Ex5



According to the one-step general method, 9.45 mg (0.064 μ mol, 0.923 ml) of naked antibody hu2H11 at an initial concentration of 10.24 mg/ml are treated with 6 eq. of the N-hydroxy-

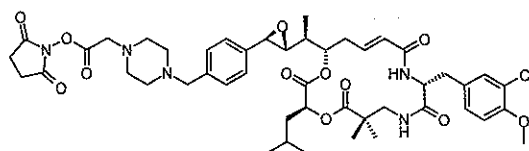
succinimidyl ester of 4-(2-pyridyldithio)butanoic acid (0.13 mg, 0.398 μmol) dissolved in 26.88 μl of DMA, such that the final antibody concentration is 9 mg/ml in the mixture. After stirring for 2 hours at about 2000 rpm at RT, 1.0 ml (9.0 mg, 0.061 μmol) of the reaction medium of modified antibody hu2H11, 1.45 ml of pH $\approx$ 7.5-8 buffer, 265 μl of DMA and then 1.26 mg of (E)-(3S,10R,16S)-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[4-(2-mercapto-2-methylpropyl)piperazin-1-ylmethyl]phenyl}oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone (compound **Ex. 5**, 1.472 μmol) dissolved in 285 μl of DMA are successively added. After purification on Superdex in the presence of 20% of NMP and concentration on Amicon Ultra-15, the final buffer change is formed in an aqueous pH 6.5 buffer containing 0.01 M of histidine, 10% of sucrose (w/v) and 5% of NMP (v/v). 2.5 ml of conjugate **Ex. 12** are thus obtained at a concentration of 1.70 mg/ml with, on average, 3.7/3.1 cryptophycin derivatives (UV/HRMS) per antibody and a monomer purity of 98.0%.

Example 12: hu2H11-SPDB-Ex6

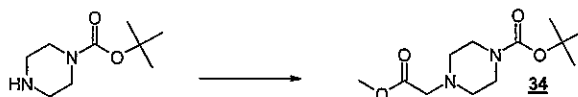


According to the one-step general method, 10.31 mg (0.070 μmol , 1.006 ml) of naked antibody hu2H11 at an initial concentration of 10.24 mg/ml are treated with 6 eq. of the N-hydroxy-succinimidyl ester of 4-(2-pyridyldithio)butanoic acid (0.14 mg, 0.429 μmol) dissolved in 29.32 μl of DMA, such that the final antibody concentration is 9 mg/ml in the mixture. After stirring for 2 hours at about 2000 rpm at RT, 1.595 ml of pH $\approx$ 7.5-8 buffer, 291 μl of DMA and then 1.60 mg of (E)-(3S,10R,16S)-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-16-[(S)-1-((2R,3R)-3-{4-[4-(2-{2-methyl[2-mercapto-2-methylpropyl]aminoethoxy}ethoxy)ethoxy]ethyl}methylamino)methyl]phenyl}oxiranyl)ethyl]-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone (compound **Ex. 6**, 1.617 μmol) dissolved in 314 μl of DMA are successively added to 1.1 ml (9.9 mg, 0.067 μmol) of the mixture of modified antibody hu2H11. After purification on Superdex in the presence of 20% of NMP and concentrating on Amicon Ultra-15, the final buffer change is performed in an aqueous pH 6.5 buffer containing 0.01 M of histidine, 10% of sucrose (w/v) and 5% of NMP (v/v). 3 ml of conjugate **Ex. 12** at a concentration of 1.97 mg/ml are thus obtained with, on average, 3.4 cryptophycin derivatives per antibody (HRMS) and a monomer purity of 99.8%.

Example 13: 2,5-Dioxopyrrolidin-1-yl (4-{4-[(2R,3R)-3-[(S)-1-[(E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl]oxiranyl]benzyl}piperazin-1-yl)acetate



Compound 34: *tert*-Butyl 4-methoxycarbonylmethylpiperazine-1-carboxylate



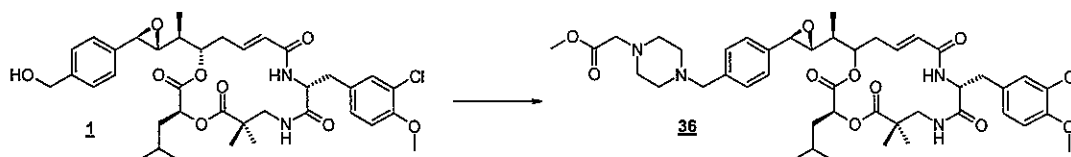
200 mg (1.07 mmol) of *tert*-butyl piperazine-1-carboxylate are dissolved in anhydrous acetonitrile (10 ml). TEA (1.07 mmol) and then methyl bromoacetate (1.61 mmol) are then added. The white suspension is stirred for 48 hours at RT, followed by addition of saturated aqueous NaHCO₃ solution (10 ml). The aqueous phase is extracted with DCM (3×10 ml) and the organic phases are combined, washed with saturated aqueous NaCl solution and dried over MgSO₄. After filtering and evaporating off the solvents under reduced pressure, the crude product is obtained. This crude product is purified by chromatography on silica gel, eluting with a 98/2 DCM/methanol mixture. The expected product **34**, a colourless oil, is thus obtained (174.8 mg; 63%). TLC (DCM 90 /MeOH 10): R_f = 0.66; ¹H NMR (300 MHz, DMSO-*d*₆): 1.39 (s, 9 H); 2.40 to 2.48 (m, 4 H); 3.25 (s, 2 H); 3.28 to 3.33 (partially masked m, 4 H); 3.61 (s, 3 H).

Compound 35: Methyl piperazin-1-ylacetate hydrochloride



Compound **34** (174 mg; 0.67 mmol) is dissolved in anhydrous dioxane (5 ml) and a 4 M solution of HCl in dioxane (0.02 mmol) is added. The mixture is stirred for 5 hours at RT and the suspension is then filtered on a sinter funnel. The solid thus obtained is washed with dioxane (2 ml) and then with isopropyl ether (2 ml), and then dried under vacuum. A beige-coloured solid, **35**, is obtained (131 mg; 100%). ¹H NMR (300 MHz, DMSO-*d*₆): 2.88 to 2.97 (m, 4 H); 3.09 to 3.19 (m, 4 H); 3.53 to 3.59 (m, 2 H); 3.65 (s, 3 H); 8.65 to 9.22 (broad m, 2 H).

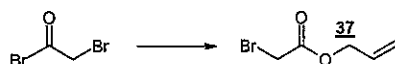
Compound 36: Methyl (4-{4-[(2R,3R)-3-[(S)-1-[(E)-(3S,10R,16S)-10-[3-Chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl]oxiranyl]benzyl]piperazin-1-yl)acetate



The derivative **1** (20 mg; 28.6 μmol) is dissolved in anhydrous DCM (1 ml), and TEA (71.5 μmol) and then CMS (45.8 μmol) are added. After 12 hours at RT, the product **2** formed is not isolated. TEA (85.7 μmol) and then methyl piperazin-1-ylacetate hydrochloride **35** (42.8 μmol) are added.

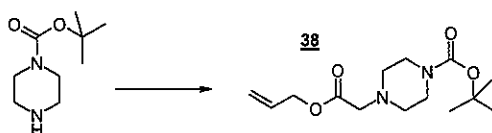
The mixture is stirred for a further 72 hours at RT, followed by addition of anhydrous DMF (1 ml) and NaI (30 μ mol). The mixture is stirred for 48 hours at 45°C, followed by dilution with EtOAc (5 ml). The organic phase is washed with water (2 $\times$ 2 ml), with saturated aqueous NaHCO₃ solution (2 ml) and with saturated aqueous NaCl solution (2 ml). After drying over MgSO₄ and filtering, the solvents are evaporated off under reduced pressure. The crude reaction product is purified by chromatography on silica gel, eluting with a 100/0 to 98/2 DCM/methanol mixture. Compound **36** is obtained in the form of a white solid (8.2 mg; 34%). TLC (DCM 90/MeOH 10): R_f = 0.45; ¹H NMR (400 MHz, DMSO-*d*₆): 0.77 (d, J = 6.1 Hz, 3 H); 0.78 (d, J = 6.1 Hz, 3 H); 1.00 (s, 3 H); 1.05 (d, J = 7.1 Hz, 3 H); 1.12 (s, 3 H); 1.27 to 1.32 (m, 1 H); 1.50 to 1.60 (m, 2 H); 1.74 to 1.84 (m, 1 H); 2.21 to 2.30 (m, 1 H); 2.37 (broad s, 4 H); 2.64 to 2.74 (m, 2 H); 2.94 to 3.06 (m, 3 H); 3.18 to 3.52 (partially masked m, 9 H); 3.60 (s, 3 H); 3.81 (s, 3 H); 3.87 (d, J = 2.0 Hz, 1 H); 4.20 to 4.31 (m, 1 H); 4.91 (dd, J = 3.8 and 9.9 Hz, 1 H); 5.11 (m, 1 H); 5.80 (d, J = 15.2 Hz, 1 H); 6.48 (ddd, J = 3.8 and 11.2 and 15.2 Hz, 1 H); 7.05 (d, J = 8.3 Hz, 1 H); 7.17 (dd, J = 2.2 and 8.3 Hz, 1 H); 7.20 to 7.33 (m, 6 H); 8.34 (d, J = 8.1 Hz, 1 H). LCMS (A1) : ES m/z = 839 [M + H]⁺; m/z = 420 [M + 2H]²⁺ (base peak); m/z = 837 [M - H]⁻; m/z = 883 [M + HCO₂H - H]⁻ (base peak); t_R = 3.57 min.

Compound 37: Allyl bromoacetate



288 mg (4.95 mmol) of allyl alcohol are dissolved in 25 ml of DCM. The solution is cooled to 0°C and then 760 μ l (5.45 mmol) of TEA and 3.03 mg (24.8 μ mol) of DMAP are added. The mixture is stirred at 0°C and 1.0 g (4.95 mmol) of bromoacetyl bromide is then added. The reaction is continued overnight at RT. Water is added to the mixture; the aqueous phase is washed with DCM. The organic fractions are combined, washed with water and with saturated NaCl solution and dried over MgSO₄. After filtering and evaporating off the solvents under reduced pressure, product **37** is obtained (747 mg, 84%). ¹H NMR (400 MHz, DMSO-*d*₆): 4.18 (s, 2 H); 4.64 (td, J = 1.5 and 5.4 Hz, 2 H); 5.25 (qd, J = 1.5 and 10.5 Hz, 1 H); 5.35 (qd, J = 1.5 and 17.2 Hz, 1 H); 5.92 (tdd, J = 5.4 and 10.5 and 17.2 Hz, 1 H).

Compound 38: *tert*-Butyl 4-allyloxycarbonylmethylpiperazine-1-carboxylate



To a solution of 200 mg (1.07 mmol) of 1-Boc-piperazine in 8 ml of acetonitrile are added 150 μ l (1.07 mmol) of TEA and 250 mg (1.40 mmol) of allyl bromoacetate **37**. The mixture is stirred at RT overnight. The reaction is stopped by adding saturated NaHCO₃ solution. The mixture is extracted with EtOAc (3 times); the organic phases are combined, washed with saturated NaCl

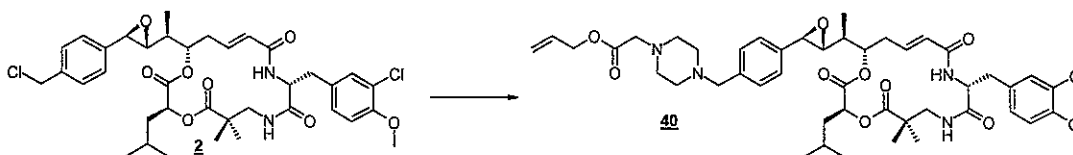
solution and dried over MgSO_4 . After filtering and evaporating off the solvent under reduced pressure, the crude product is purified by chromatography on silica gel, eluting with a 100/0 to 95/5 DCM/methanol mixture. The expected product **38** is obtained in the form of a yellow oil (314 mg; 100%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 1.39 (s, 9 H); 2.45 to 2.48 (m, 4 H); 3.25 to 3.34 (partially masked m, 6 H); 4.57 (td, $J = 1.5$ and 5.6 Hz, 2 H); 5.22 (qd, $J = 1.5$ and 10.3 Hz, 1 H); 5.30 (qd, $J = 1.5$ and 17.1 Hz, 1 H); 5.83 to 5.98 (m, 1 H). LCMS (A2): ES $m/z = 285$ $[\text{M} + \text{H}]^+$; $m/z = 229$ base peak; $t_R = 0.51$ min.

Compound 39: Allyl piperazin-1-ylacetate hydrochloride



314 mg (1.10 mmol) of compound **38** are dissolved in 12.6 ml of dioxane, followed by addition of 5.5 ml (22.0 mmol) of a 4M solution of HCl in dioxane. Stirring is continued overnight at RT. The mixture is concentrated to dryness to give the expected compound **39** in the form of a yellow oil (260 mg; 100%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 2.79 to 2.88 (m, 4 H); 3.07 to 3.15 (m, 4 H); 3.48 to 3.51 (m, 2 H); 4.60 (td, $J = 1.5$ and 5.6 Hz, 2 H); 5.23 (qd, $J = 1.5$ and 10.3 Hz, 1 H); 5.32 (qd, $J = 1.5$ and 7.4 Hz, 1 H); 5.86 to 5.98 (m, 1 H); 8.74 (broad s, 2 H). LCMS (A2): ES $m/z = 185$ $[\text{M} + \text{H}]^+$; $t_R = 0.19$ min.

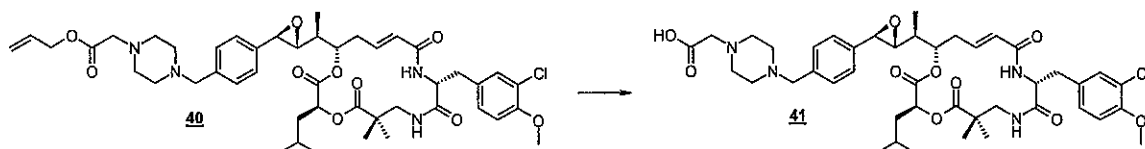
Compound 40: Allyl (4-{4-[(2R,3R)-3-((S)-1-((E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl)-oxiranyl]benzyl}piperazin-1-yl)acetate



To a solution, purged with argon, of compound **2** (28.3 mg, 39.5 μmol) in anhydrous acetonitrile (2.5 ml) is added a solution of compound **39** (118.5 μmol) and TEA (198 μmol) in anhydrous acetonitrile (1 ml). Stirring is continued for 24 hours at 40°C. The mixture is diluted in EtOAc (10 ml). The organic phase is washed with water (10 ml), with saturated aqueous NaHCO_3 solution (10 ml) and saturated aqueous NaCl solution (10 ml). After drying over MgSO_4 and filtering, the solvents are evaporated off under reduced pressure. The crude reaction product is purified by chromatography on silica gel, eluting with a 98/2 DCM/methanol mixture. Compound **40** is obtained in the form of a white solid (19.2 mg; 56%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$): 0.75 (d, $J = 6.3$ Hz, 3 H); 0.77 (d, $J = 6.3$ Hz, 3 H); 1.00 (s, 3 H); 1.05 (d, $J = 6.9$ Hz, 3 H); 1.12 (s, 3 H); 1.25 to 1.33 (m, 1 H); 1.50 to 1.61 (m, 2 H); 1.74 to 1.84 (m, 1 H); 2.27 (dt, $J = 11.3$ and 14.2 Hz, 1 H); 2.32 to 2.42 (m, 4 H); 2.52 (partially masked m, 4 H); 2.64 to 2.74 (m, 2 H); 2.94 to 3.05 (m, 3 H); 3.25 (s, 2 H); 3.32 to 3.35 (partially masked m, 1 H); 3.40 to 3.48 (m, 2 H); 3.81 (s, 3 H);

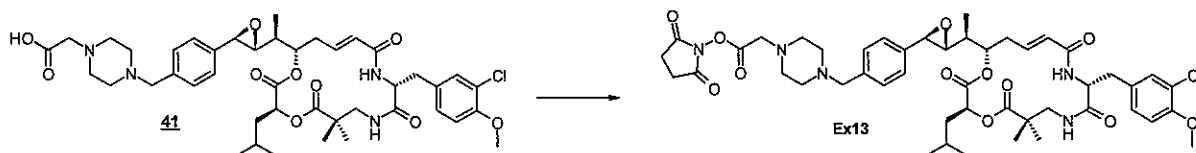
3.87 (d, $J = 1.6$ Hz, 1 H); 4.25 (ddd, $J = 3.7$ and 8.0 and 11.6 Hz, 1 H); 4.56 (td, $J = 1.5$ and 5.5 Hz, 2 H); 4.91 (dd, $J = 3.7$ and 9.7 Hz, 1 H); 5.11 (m, 1 H); 5.21 (qd, $J = 1.5$ and 10.5 Hz, 1 H); 5.30 (qd, $J = 1.5$ and 17.3 Hz, 1 H); 5.80 (d, $J = 16.2$ Hz, 1 H); 5.86 to 5.95 (m, 1 H); 6.47 (ddd, $J = 3.8$ and 11.2 and 15.2 Hz, 1 H); 7.05 (d, $J = 8.5$ Hz, 1 H); 7.17 (dd, $J = 2.2$ and 8.5 Hz, 1 H); 7.19 to 7.32 (m, 6 H); 8.34 (d, $J = 8.0$ Hz, 1 H). LCMS (A2) : ES $m/z = 865$ $[M + H]^+$; $m/z = 433.5$ $[M + 2H]^{2+}$ (base peak); $m/z = 863$ $[M - H]^-$; $m/z = 909$ $[M + HCO_2H - H]^-$; $t_R = 0.92$ min.

Compound 41: (4-{4-[(2R,3R)-3-[(S)-1-[(E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl)-oxiranyl]benzyl]piperazin-1-yl)acetic acid



To a solution under argon of compound **40** (12.8 mg, 14.8 μ mol) in anhydrous THF are added tetrakis(triphenylphosphine)palladium (1.48 μ mol) and morpholine (148 μ mol). After reaction for 3 hours, the mixture is concentrated to dryness and taken up in 5 ml of DCM. The organic phase is washed with 1 ml of 0.1N HCl solution in 3 ml of water (pH $\approx$ 5), dried over $MgSO_4$, filtered and evaporated to give compound **41** (6.7 mg, 55%). 1H NMR (500 MHz, $DMSO-d_6$): 0.76 (d, $J = 6.0$ Hz, 3 H); 0.78 (d, $J = 6.0$ Hz, 3 H); 1.00 (s, 3 H); 1.05 (d, $J = 6.9$ Hz, 3 H); 1.12 (s, 3 H); 1.28 (m, 1 H); 1.52 to 1.59 (m, 2 H); 1.76 to 1.84 (m, 1 H); 2.21 to 2.32 (m, 1 H); 2.42 (d, $J = 6.6$ Hz, 4 H); 2.58 to 2.76 (m, 6 H); 2.97 to 3.09 (m, 3 H); 3.14 (broad s, 2 H); 3.33 (masked m, 1 H); 3.46 (broad s, 2 H); 3.81 (s, 3 H); 3.87 (d, $J = 1.8$ Hz, 1 H); 4.20 to 4.29 (m, 1 H); 4.89 to 4.93 (m, 1 H); 5.06 to 5.14 (m, 1 H); 5.80 (d, $J = 15.2$ Hz, 1 H); 6.43 to 6.50 (m, 1 H); 7.05 (d, $J = 8.5$ Hz, 1 H); 7.17 (dd, $J = 2.2$ and 8.5 Hz, 1 H); 7.20 to 7.32 (m, 6 H); 8.34 (d, $J = 8.0$ Hz, 1 H). LCMS (A2): ES $m/z = 825$ $[M + H]^+$; $m/z = 413$ $[M + 2H]^{2+}$ (base peak); $m/z = 823$ $[M - H]^-$; $t_R = 0.86$ min.

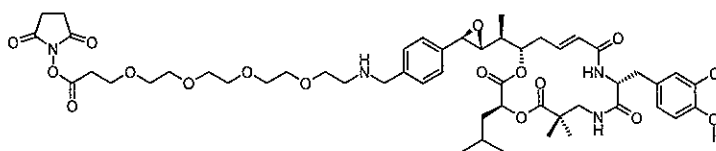
Example 13: 2,5-Dioxopyrrolidin-1-yl (4-{4-[(2R,3R)-3-[(S)-1-[(E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl)oxiranyl]benzyl]piperazin-1-yl)acetate



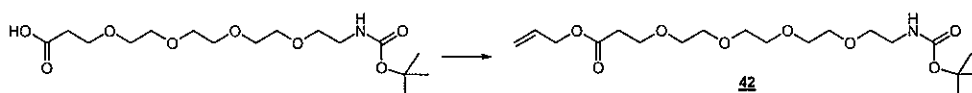
Example **13** may be obtained by activating the acid **41** according to the method described for Example **18**.

Example 14: 2,5-Dioxopyrrolidin-1-yl (2-{2-[2-(2-{4-[(2R,3R)-3-[(S)-1-[(E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diaza-

cyclohexadec-13-en-16-yl}ethyl)oxiranyl]benzylamino}ethoxy)ethoxy}ethoxy)ethoxy)-propanoate

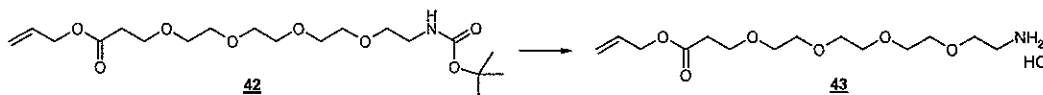


Compound 42: Allyl 3-(2-[2-[2-(2-*tert*-butoxycarbonylaminoethoxy)ethoxy]ethoxy]ethoxy)ethoxy)-propanoate



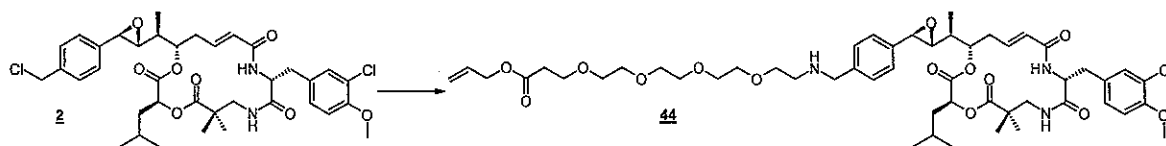
To a solution of Boc-15-amino-4,7,10,13-tetraoxapentadecanoic acid (50 mg, 137 μ mol) in 1.25 ml of DCM are successively added EDCI hydrochloride (164.2 μ mol), DMAP (13.7 μ mol) and allyl alcohol (164.2 μ mol). The mixture is stirred at RT for 16 hours and then evaporated to dryness. The crude product is purified by chromatography on silica gel, eluting with a 100/0 to 95/5 DCM/methanol mixture. Compound **42** is obtained in the form of a colourless oil (37.5 mg; 67%). ^1H NMR (400 MHz, DMSO- d_6): 1.37 (s, 9 H); 2.57 (t, J = 6.1 Hz, 2 H); 3.06 (q, J = 6.1 Hz, 2 H); 3.37 (t, J = 6.1 Hz, 2 H); 3.44 to 3.53 (m, 12 H); 3.64 (t, J = 6.1 Hz, 2 H); 4.55 (td, J = 1.6 and 5.4 Hz, 2 H); 5.20 (qd, J = 1.6 and 10.5 Hz, 1 H); 5.30 (qd, J = 1.6 and 17.3 Hz, 1 H); 5.90 (tdd, J = 5.4 and 10.5 and 17.3 Hz, 1 H); 6.70 (broad t, J = 6.1 Hz, 1 H). LCMS (A2) ES m/z = 406 [M + H] $^+$; m/z = 428 [M + Na] $^+$; m/z = 306 base peak; t_R = 0.91 min.

Compound 43: Allyl 3-(2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]ethoxy)propanoate hydrochloride



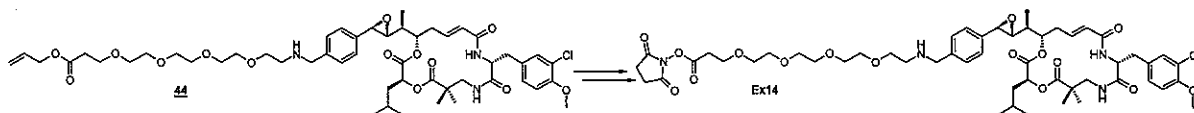
To a solution of compound **42** (37.5 mg, 92.5 μ mol) in 2 ml of dioxane are added 460 μ l (1.85 mmol) of a 4M solution of hydrogen chloride in dioxane. Stirring is continued at RT overnight and the reaction medium is then evaporated to dryness to give compound **43** in the form of a colourless oil (31 mg, quantitative). ^1H NMR (400 MHz, DMSO- d_6): 2.58 (t, J = 6.1 Hz, 2 H); 2.96 (t, J = 5.4 Hz, 2 H); 3.48 to 3.53 (m, 8 H); 3.55 to 3.58 (m, 4 H); 3.60 (t, J = 5.4 Hz, 2 H); 3.65 (t, J = 6.1 Hz, 2 H); 4.56 (td, J = 1.6 and 5.4 Hz, 2 H); 5.21 (qd, J = 1.6 and 10.5 Hz, 1 H); 5.30 (qd, J = 1.6 and 17.3 Hz, 1 H); 5.91 (tdd, J = 5.4 and 10.5 and 17.3 Hz, 1 H); 7.91 (broad m, 3 H). LCMS (A2) : ES m/z = 306 [M + H] $^+$; t_R = 0.42 min.

Compound 44: Allyl (2-[2-[2-(2-{4-[(2R,3R)-3-((S)-1-[(E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl}ethyl)oxiranyl]benzylamino}ethoxy)ethoxy]ethoxy)ethoxy)ethoxy)propanoate



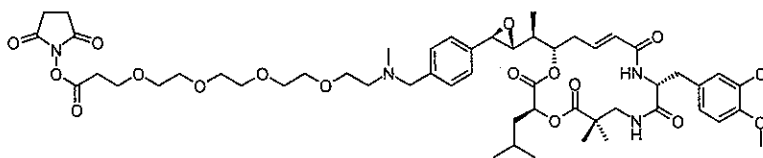
To a solution, purged with argon, of compound **2** (12.7 mg, 17.7 μmol) in 1.48 ml of anhydrous acetonitrile are successively added 22.2 μl of TEA (159 μmol) and 30.2 mg of compound **43** (88.4 μmol). Stirring is continued at 40°C for 24 hours. Some starting compound **2** remains; 22.2 μl of TEA (159 μmol) and 30.2 mg of compound **43** (88.4 μmol) are added to the mixture and stirring is continued at 40°C for a further 48 hours. 2 ml of water are added to the mixture, which is then extracted with 2x2 ml of EtOAc. The organic phases are combined, washed with saturated NaHCO_3 solution (2 ml) and with saturated sodium chloride solution (2 ml) and dried over MgSO_4 . After filtering and concentrating under reduced pressure, the crude reaction product is purified by chromatography on silica gel, eluting with a 100/0 to 95/5 DCM/methanol mixture. Compound **44** is obtained in the form of a white solid (4.8 mg; 27%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$): 0.77 (m, 6 H); 1.00 (s, 3 H); 1.05 (d, $J = 6.9$ Hz, 3 H); 1.12 (s, 3 H); 1.29 (m, 1 H); 1.49 to 1.60 (m, 2 H); 1.81 (m, 1 H); 2.26 (m, 1 H); 2.56 (t, $J = 6.1$ Hz, 2 H); 2.61 to 2.73 (m, 4 H); 2.93 to 3.04 (m, 3 H); 3.28 to 3.38 (partially masked m, 1 H); 3.45 to 3.53 (m, 14 H); 3.63 (t, $J = 6.1$ Hz, 2 H); 3.72 (s, 2 H); 3.81 (s, 3 H); 3.86 (broad s, 1 H); 4.25 (ddd, $J = 3.4$ and 8.2 and 11.4 Hz, 1 H); 4.55 (dm, $J = 5.4$ Hz, 2 H); 4.90 (dd, $J = 3.9$ and 9.8 Hz, 1 H); 5.11 (ddd, $J = 1.4$ and 5.4 and 11.2 Hz, 1 H); 5.19 (dm, $J = 10.8$ Hz, 1 H); 5.29 (dm, $J = 17.2$ Hz, 1 H); 5.79 (dd, $J = 1.4$ and 15.2 Hz, 1 H); 5.90 (m, 1 H); 6.47 (ddd, $J = 3.9$ and 11.2 and 15.2 Hz, 1 H); 7.05 (d, $J = 8.8$ Hz, 1 H); 7.17 (dd, $J = 2.0$ and 8.8 Hz, 1 H); 7.21 (m, 1 H); 7.24 (d, $J = 8.3$ Hz, 2 H); 7.28 (d, $J = 2.0$ Hz, 1 H); 7.33 (d, $J = 8.3$ Hz, 2 H); 8.35 (d, $J = 8.2$ Hz, 1 H). LCMS (A2): ES $m/z = 986$ $[\text{M} + \text{H}]^+$; $m/z = 493.5$ $[\text{M} + 2\text{H}]^{2+}$ base peak; $m/z = 984$ $[\text{M} - \text{H}]^-$; $m/z = 1030$ $[\text{M} - \text{H} + \text{HCO}_2\text{H}]^-$ base peak; $t_R = 0.95$ min.

Example 14: 2,5-Dioxopyrrolidin-1-yl (2-{2-[2-(2-{4-[(2R,3R)-3-((S)-1-[(E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl)oxiranyl]benzylamino}ethoxy)ethoxy]ethoxy}ethoxy)propanoate

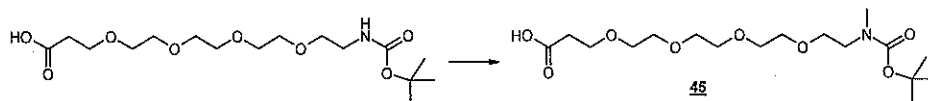


Example **14** may be obtained by deprotecting compound **44** according to the method described for compound **41** and by activating the acid obtained according to the method described for Example **18**.

Example 15: 2,5-Dioxopyrrolidin-1-yl (2-{2-[2-(2-{4-[(2R,3R)-3-((S)-1-[(E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl)oxiranyl]benzylmethanolamino}ethoxy)ethoxy]ethoxy}ethoxy)propanoate

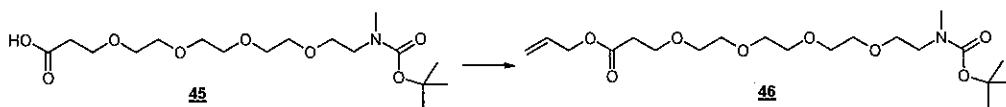


Compound 45: 3-[2-(2-[2-(2-(*tert*-Butoxycarbonylmethylamino)ethoxy]ethoxy)ethoxy)ethoxy]propanoic acid



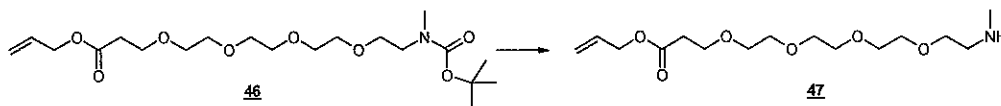
- 5 520 mg (1.423 mmol) of Boc-15-amino-4,7,10,13-tetraoxapentadecanoic acid are dissolved in 14 ml of anhydrous THF and the mixture is then cooled to 0°C, followed by addition, by spatula, of 85.4 mg (2.135 mmol) of sodium hydride. Stirring is continued for 10 minutes at 0°C, and then 150.6 µl (2.419 mmol) of methyl iodide are added at 0°C. The temperature is allowed to return to RT and stirring is continued for 2 hours. 8 ml of water are added to the mixture, the pH of which is
- 10 then acidified by addition of acetic acid to obtain pH≈4. It is extracted with 3×10 ml of EtOAc. The organic phases are combined, washed with 10 ml of saturated NaCl solution and dried over MgSO₄. After filtering and concentrating to dryness under reduced pressure, compound **45** is obtained in the form of a colourless oil (414 mg, 77%). ¹H NMR (400 MHz, DMSO-*d*₆): 1.38 (s, 9 H); 2.43 (t, *J* = 6.4 Hz, 2 H); 2.80 (broad s, 3 H); 3.29 (t, *J* = 5.9 Hz, 2 H); 3.45 to 3.52 (m, 14 H);
- 15 3.60 (t, *J* = 6.4 Hz, 2 H); 12.01 (broad m, 1 H). LCMS (A2) : ES *m/z* = 402 [M + Na]⁺; *m/z* = 378 [M - H]⁻; *m/z* = 280 base peak; *t*_R = 0.95 min.

Compound 46: Allyl 3-[2-(2-[2-(2-(*tert*-butoxycarbonylmethylamino)ethoxy]ethoxy)ethoxy)ethoxy]propanoate



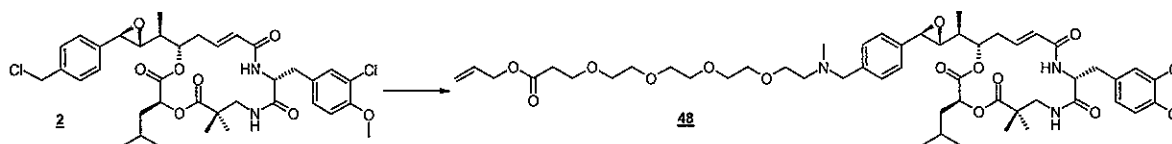
- 20 To a solution of 540 mg (1.423 mmol) of compound **45** in 15 ml of anhydrous DCM are successively added 327 mg (1.71 mmol) of EDCI, 17.4 mg (142 µmol) of DMAP and 116 µl (1.71 mmol) of allyl alcohol. Stirring is continued for 15 hours at RT and the mixture is then concentrated to dryness. The crude product obtained is purified by chromatography on silica gel, eluting with a 100/0 to 90/10 DCM/methanol mixture. Compound **46** is obtained in the form of a colourless oil (337 mg; 56%). ¹H NMR (400 MHz, DMSO-*d*₆): 1.38 (s, 9 H); 2.57 (t, *J* = 6.1 Hz, 2 H); 2.80 (broad s, 3 H); 3.22 to 3.33 (partially masked m, 2 H); 3.44 to 3.54 (m, 14 H); 3.64 (t, *J* = 6.1 Hz, 2 H); 4.55 (broad d, *J* = 4.9 Hz, 2 H); 5.20 (broad d, *J* = 10.3 Hz, 1 H); 5.30 (broad d, *J* = 17.1 Hz, 1 H); 5.90 (m, 1 H). LCMS (A2) : ES *m/z* = 442 [M + Na]⁺; *m/z* = 320 base peak; *t*_R =
- 25 0.98 min.
- 30

Compound 47: Allyl 3-(2-[2-(2-(2-methylaminoethoxy)ethoxy]ethoxy)ethoxy)propanoate



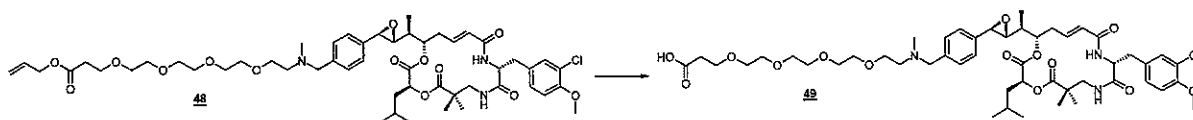
To a solution of 337 mg (0.802 mmol) of compound **46** in 20 ml of DCM are added 1.19 ml (16.04 mmol) of TFA. Stirring is continued for 3 hours at RT and the mixture is then concentrated to dryness under reduced pressure. The crude product is purified by SPE filtration on an SCX cartridge (Varian) conditioned and washed with methanol, and then eluted with a 0.5 N solution of aqueous ammonia in methanol. Compound **47** is obtained in the form of a colourless oil (208 mg, 81%). ¹H NMR (400 MHz, DMSO-*d*₆): 2.27 (s, 3 H); 2.54 to 2.61 (m, 4 H); 3.44 (t, *J* = 5.6 Hz, 2 H); 3.47 to 3.52 (m, 12 H); 3.65 (t, *J* = 6.2 Hz, 2 H); 4.56 (broad d, *J* = 5.4 Hz, 2 H); 5.20 (broad d, *J* = 10.5 Hz, 1 H); 5.30 (broad d, *J* = 17.2 Hz, 1 H); 5.90 (m, 1 H). LCMS (A2) : ES *m/z* = 320 [*M* + *H*]⁺; *t*_R = 0.42 min.

Compound 48: Allyl (2-{2-[2-(2-{4-[(2*R*,3*R*)-3-((*S*)-1-{(*E*)-(3*S*,10*R*,16*S*)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl)oxiranyl]benzylamino}ethoxy)ethoxy]ethoxy)ethoxy)propanoate



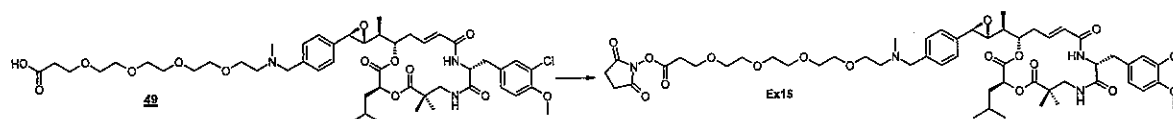
To a solution, purged with argon, of compound **2** (40 mg, 55.7 μmol) in 5 ml of anhydrous acetonitrile are successively added 48.5 μl of DIPEA (279 μmol) and 53 mg of compound **47** (167 μmol). Stirring is continued at 40°C for 15 hours; 5 ml of water are added to the mixture, which is then extracted with 4x5 ml of EtOAc. The organic phases are combined, washed with saturated NaHCO₃ solution (5 ml) and with saturated NaCl solution (5 ml) and dried over MgSO₄. After filtering and concentrating under reduced pressure, the crude reaction product is purified by chromatography on silica gel, eluting with a 100/0 to 90/10 DCM/methanol mixture. Compound **48** is obtained in the form of a colourless solid (45.3 mg; 80%). ¹H NMR (500 MHz, DMSO-*d*₆): 0.76 (d, *J* = 5.9 Hz, 3 H); 0.78 (d, *J* = 5.9 Hz, 3 H); 1.00 (s, 3 H); 1.04 (d, *J* = 6.8 Hz, 3 H); 1.12 (s, 3 H); 1.29 (m, 1 H); 1.50 to 1.61 (m, 2 H); 1.80 (m, 1 H); 2.15 (s, 3 H); 2.28 (m, 1 H); 2.52 (t, *J* = 6.4 Hz, 2 H); 2.56 (t, *J* = 6.4 Hz, 2 H); 2.63 to 2.73 (m, 2 H); 2.94 to 3.06 (m, 3 H); 3.25 to 3.35 (partially masked m, 1 H); 3.48 (s, 2 H); 3.49 to 3.51 (m, 12 H); 3.52 (t, *J* = 6.4 Hz, 2 H); 3.63 (t, *J* = 6.4 Hz, 2 H); 3.81 (s, 3 H); 3.87 (d, *J* = 1.5 Hz, 1 H); 4.25 (ddd, *J* = 3.4 and 8.3 and 11.5 Hz, 1 H); 4.55 (broad d, *J* = 5.0 Hz, 2 H); 4.91 (dd, *J* = 3.7 and 9.5 Hz, 1 H); 5.11 (ddd, *J* = 1.5 and 5.7 and 11.5 Hz, 1 H); 5.19 (dm, *J* = 10.3 Hz, 1 H); 5.29 (dm, *J* = 17.6 Hz, 1 H); 5.80 (dd, *J* = 1.5 and 15.2 Hz, 1 H); 5.90 (m, 1 H); 6.47 (ddd, *J* = 3.7 and 11.5 and 15.2 Hz, 1 H); 7.05 (d, *J* = 8.5 Hz, 1 H); 7.17 (dd, *J* = 2.0 and 8.5 Hz, 1 H); 7.22 (dd, *J* = 2.9 and 9.8 Hz, 1 H); 7.25 (d, *J* = 8.3 Hz, 2 H); 7.28 (d, *J* = 2.0 Hz, 1 H); 7.30 (d, *J* = 8.3 Hz, 2 H); 8.35 (d, *J* = 8.3 Hz, 1 H). LCMS (A2): ES *m/z* = 1000 [*M* + *H*]⁺; *m/z* = 500.5 [*M* + 2*H*]²⁺; *m/z* = 1044 [*M* - *H* + HCO₂H]⁺; *t*_R = 1.01 min.

Compound 49: (2-{2-[2-(2-{4-[(2R,3R)-3-((S)-1-((E)-(3S,10R,16S)-10-[3-Chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl)oxiranyl]benzylamino)ethoxy)ethoxy]ethoxy}ethoxy)propanoic acid



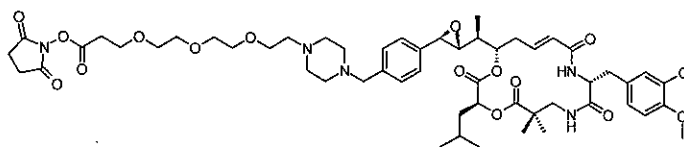
19 mg (18.9 μmol) of compound **48** are dissolved in 3.8 ml of anhydrous THF and purged with argon. 6 μl (18.9 μmol) of diethylamine are added to the mixture, which is stirred for 15 minutes at RT, followed by addition of 4.45 mg (19.8 μmol) of palladium(II) acetate and 18.89 mg of supported triphenylphosphine. Stirring is continued for 6 days at RT: some starting material remains, but the reaction no longer proceeds. The mixture is filtered, concentrated to dryness, taken up in 2 ml of anhydrous THF and treated with 10 μl (31.5 μmol) of diethylamine and 10 mg of tetrakis(triphenylphosphine)palladium for 1 hour. The mixture is hydrolyzed with 2 ml of aqueous 2M sodium hydrogensulfate solution and extracted with 3 $\times$ 2 ml of DCM. The organic phases are combined, washed with saturated NaCl solution (2 ml) and dried over MgSO_4 . After filtering and concentrating under reduced pressure, the crude product is purified by chromatography on diol-grafted silica gel, eluting with a 98/2 to 90/10 DCM/methanol mixture. Compound **49** is obtained in the form of a colourless solid (5.4 mg; 30%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$): 0.76 (t, $J = 5.9$ Hz, 3 H); 0.78 (t, $J = 5.9$ Hz, 3 H); 1.00 (s, 3 H); 1.04 (d, $J = 6.8$ Hz, 3 H); 1.12 (s, 3 H); 1.31 (m, 1 H); 1.51 to 1.62 (m, 2 H); 1.80 (m, 1 H); 2.15 (s, 3 H); 2.28 (m, 1 H); 2.42 (t, $J = 6.4$ Hz, 2 H); 2.52 (t, $J = 6.4$ Hz, 2 H); 2.64 to 2.73 (m, 2 H); 2.94 to 3.04 (m, 3 H); 3.25 to 3.44 (partially masked m, 3 H); 3.45 to 3.51 (m, 12 H); 3.53 (t, $J = 6.4$ Hz, 2 H); 3.59 (t, $J = 6.4$ Hz, 2 H); 3.81 (s, 3 H); 3.87 (d, $J = 2.0$ Hz, 1 H); 4.25 (ddd, $J = 4.2$ and 7.8 and 11.5 Hz, 1 H); 4.91 (dd, $J = 3.4$ and 9.8 Hz, 1 H); 5.11 (dd, $J = 5.4$ and 11.2 Hz, 1 H); 5.80 (d, $J = 15.2$ Hz, 1 H); 6.47 (ddd, $J = 3.7$ and 11.2 and 15.2 Hz, 1 H); 7.05 (d, $J = 8.3$ Hz, 1 H); 7.17 (dd, $J = 2.0$ and 8.3 Hz, 1 H); 7.21 to 7.27 (m, 3 H); 7.28 to 7.32 (m, 3 H); 8.38 (broad d, $J = 7.8$ Hz, 1 H); 11.22 (very broad m, 1 H). LCMS (A2): ES $m/z = 960$ $[\text{M} + \text{H}]^+$; $m/z = 480.5$ $[\text{M} + 2\text{H}]^{2+}$ base peak; $m/z = 958$ $[\text{M} - \text{H}]^-$; $t_R = 0.90$ min.

Example 15: 2,5-Dioxopyrrolidin-1-yl (2-{2-[2-(2-{4-[(2R,3R)-3-((S)-1-((E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl)oxiranyl]benzylamino)ethoxy)ethoxy]ethoxy}ethoxy)propanoate

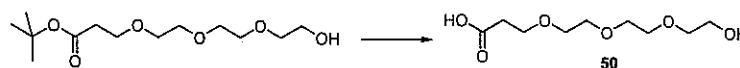


Example **15** may be prepared by activating the acid **49** according to the method described for Example **18**.

Example 16: 2,5-Dioxopyrrolidin-1-yl (2-{2-[2-(4-{(2R,3R)-3-[(S)-1-[(E)-(3S,10R,16S)-10-{3-chloro-4-methoxybenzyl}-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl]oxiranyl}benzyl)piperazin-1-yl)ethoxy]ethoxy}ethoxy)-propanoate

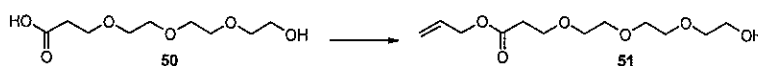


Compound 50: 3-{2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy}propanoic acid



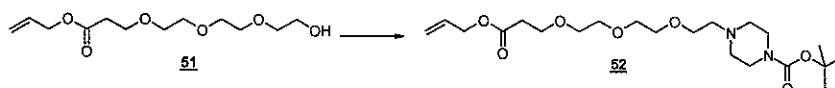
To a solution of 300 mg (1.08 mmol) of *tert*-butyl 12-hydroxy-4,7,10-trioxadodecanoate in 6 ml of DCM are added 1.6 ml (21.56 mmol) of TFA. Stirring is continued at RT for 3 hours. The mixture is concentrated to dryness, taken up in a minimum amount of DCM and entrained several times with toluene to give compound **50** in the form of a pale yellow oil (240 mg, quantitative).

Compound 51: Allyl 3-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}propanoate



To a solution of 240 mg (1.08 mmol) of compound **50** in 3 ml of DCM are successively added 248 mg (1.29 mmol) of EDCI, 13.2 mg (1.29 mmol) of DMAP and 88 μ l (1.29 mmol) of allyl alcohol. Stirring is continued at RT for 15 hours and the mixture is then concentrated to dryness under reduced pressure, and the crude product purified by chromatography on silica gel using as eluent a 98/2 to 90/10 DCM/methanol mixture. Compound **51** is obtained in the form of a colourless oil (144 mg, 51%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 2.57 (t, $J = 6.2$ Hz, 2 H); 3.41 (m, 2 H); 3.46 to 3.52 (m, 10 H); 3.65 (t, $J = 6.2$ Hz, 2 H); 4.52 (t, $J = 5.5$ Hz, 1 H); 4.56 (m, 2 H); 5.20 (dm, $J = 10.5$ Hz, 1 H); 5.50 (dm, $J = 17.4$ Hz, 1 H); 5.90 (m, 1 H).

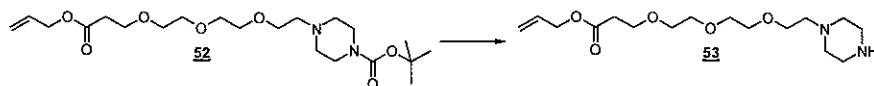
Compound 52: *tert*-Butyl 4-(2-{2-[2-(2-allyloxycarbonyloxy)ethoxy]ethoxy}ethyl)piperazine-1-carboxylate



To a solution, cooled to 0°C , of 94 mg (357 μ mol) of compound **51** in 3.7 ml of DCM are successively added 125 μ l (893 μ mol) of TEA and 30.4 μ l (393 μ mol) of mesyl chloride, and stirring is continued at RT for 1 hour. The mixture is concentrated to dryness under reduced pressure and then taken up in 5 ml of acetonitrile. 249 μ l (1.785 mmol) of TEA and 200 mg (1.071 mmol) of Boc-piperazine are added to the solution and the mixture is stirred and heated for 15 hours at 40°C . After cooling to RT, the mixture is concentrated to dryness and purified by chromatography on silica gel, using a 98/2 to 90/10 DCM/methanol mixture as eluent. Compound **52** is obtained in the form of a colourless oil (69 mg, 45%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 1.39

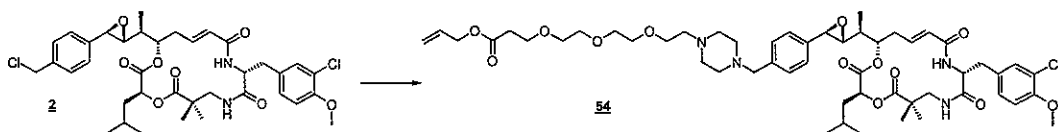
(s, 9 H); 2.33 (m, 4 H); 2.46 (t, J = 5.9 Hz, 2 H); 2.57 (t, J = 6.2 Hz, 2 H); 3.28 (partially masked m, 4 H); 3.45 to 3.53 (m, 10 H); 3.64 (t, J = 6.2 Hz, 2 H); 4.55 (m, 2 H); 5.20 (dm, J = 10.5 Hz, 1 H); 5.30 (dm, J = 17.2 Hz, 1 H); 5.90 (m, 1 H).

5 **Compound 53:** Allyl 3-{2-[2-(2-piperazin-1-ylethoxy)ethoxy]ethoxy}propanoate



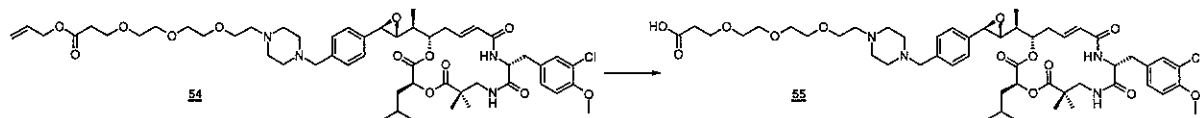
To a solution of 110 mg (256 μ mol) of compound **52** in 10 ml of DCM are added 380 μ l (5.11 mmol) of TFA. Stirring is continued for 24 hours at RT. The mixture is concentrated to dryness, taken up in a minimum amount of DCM and entrained several times with toluene. The crude product is purified by SPE filtration on an SCX cartridge (Varian) conditioned and washed with methanol and then eluted with a 0.5 N solution of aqueous ammonia in methanol. Compound **53** is obtained in the form of a colourless oil (47 mg, 55%). ^1H NMR (400 MHz, DMSO- d_6): 2.58 (t, J = 6.2 Hz, 2 H); 3.10 to 3.36 (broad m, 6 H); 3.45 to 3.78 (partially masked m, 16 H); 4.56 (m, 2 H); 5.20 (dm, J = 10.5 Hz, 1 H); 5.30 (dm, J = 17.2 Hz, 1 H); 5.90 (m, 1 H); 9.00 (broad m, 1 H).

15 **Compound 54:** Allyl (2-{2-[2-(4-{(2R,3R)-3-[(S)-1-((E)-(3S,10R,16S)-10-{3-chloro-4-methoxybenzyl}-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl]oxiranyl}benzyl)piperazin-1-yl)ethoxy]ethoxy}ethoxy)propanoate



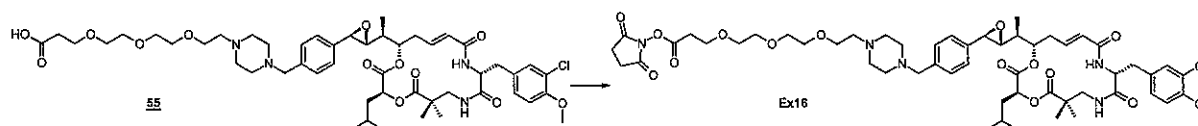
20 Compound **54** may be obtained by nucleophilic substitution of the chloro group of derivative **2** with the amine **53** by applying the method described for the preparation of compound **30**.

25 **Compound 55:** (2-{2-[2-(4-{(2R,3R)-3-[(S)-1-((E)-(3S,10R,16S)-10-{3-Chloro-4-methoxybenzyl}-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl]-oxiranyl}benzyl)piperazin-1-yl)ethoxy]ethoxy}ethoxy)propanoic acid



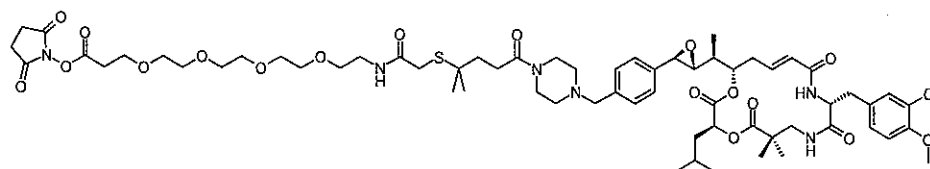
Compound **55** may be obtained according to the method described for Compound **41**.

30 **Example 16:** 2,5-Dioxopyrrolidin-1-yl (2-{2-[2-(4-{(2R,3R)-3-[(S)-1-((E)-(3S,10R,16S)-10-{3-chloro-4-methoxybenzyl}-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl]oxiranyl}benzyl)piperazin-1-yl)ethoxy]ethoxy}ethoxy)propanoate

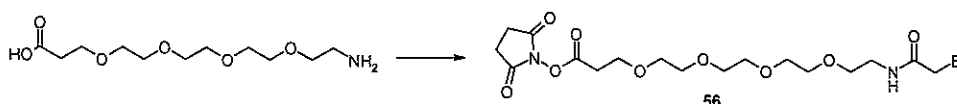


Example 16 may be obtained according to the method described for Example 18.

Example 17: 2,5-Dioxopyrrolidin-1-yl 3-(2-{2-[2-(2-{2-[4-(4-{[(2R,3R)-3-((S)-1-((E)-(10R,16S)-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl)oxiranyl]benzyl]piperazin-1-yl)-1,1-dimethyl-4-oxobutylsulfanyl]acetylamino}ethoxy)ethoxy]ethoxy)ethoxy)propanoate

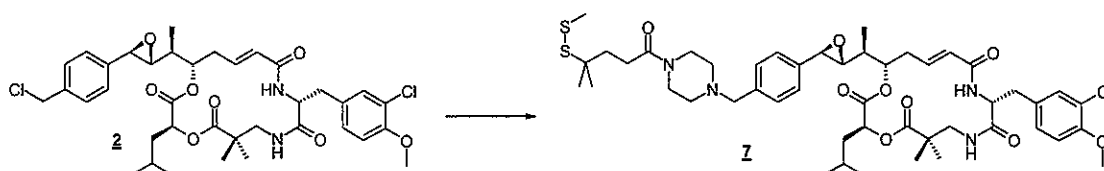


Compound 56: 2,5-Dioxopyrrolidin-1-yl 3-(2-{2-[2-(2-{2-bromoacetylamino}ethoxy)ethoxy]ethoxy)ethoxy)propanoate



To a solution of 3-(2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy)ethoxy)propionic acid (671 mg, 2.53 mmol) in DCM (10 ml) is added a solution of 2,5-dioxopyrrolidin-1-yl bromoacetate (2.53 mmol) in 4.7 ml of DCM. Stirring is continued for 15 minutes at RT and DCC is then added to the mixture. After reaction for 4 hours, the mixture is filtered through a sinter funnel and the filtrate is then evaporated and purified by chromatography on silica gel, eluting with a 99/1 to 94/6 DCM/methanol mixture. The oil obtained (800 mg) is again purified by chromatography on a cyano-grafted silica gel, eluting with a 99/1 DCM/methanol mixture. Compound 56 is obtained in the form of a colourless oil (611 mg, 50%). ¹H NMR (500 MHz, DMSO-*d*₆): 2.81 (s, 4 H); 2.92 (t, J = 5.9 Hz, 2 H); 3.23 (q, J = 5.9 Hz, 2 H); 3.43 (t, J = 5.9 Hz, 2 H); 3.48 to 3.55 (m, 12 H); 3.72 (t, J = 5.9 Hz, 2 H); 3.85 (s, 2 H); 8.30 (broad t, J = 5.9 Hz, 1 H). LCMS (A2): ES *m/z* = 483 [M + H]⁺; *m/z* = 481 [M - H]⁻; *t*_R = 0.51 min.

Compound 7: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-16-[(S)-1-((2R,3R)-3-{4-[4-(4-methyl-4-methyldisulfanyl)pentanoyl]piperazin-1-ylmethyl]phenyl)oxiranyl)-ethyl]-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



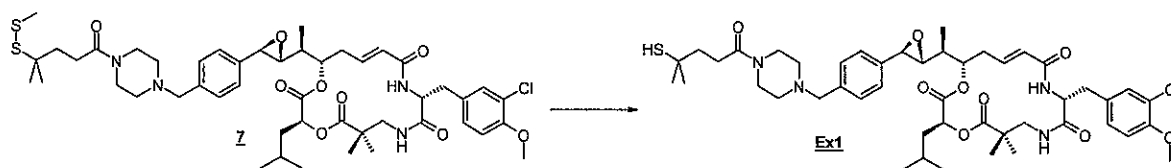
To a solution, purged with argon, of compound 2 (19.8 mg, 27.6 μmol) in anhydrous acetonitrile (2.5 ml) are successively added TEA (138 μmol) and 4-methyl-4-methyldisulfanyl-1-piperazine-1-

ylpentan-1-one hydrochloride **6** (83 μ mol). Stirring is continued at 40°C for 24 hours and the mixture is then diluted in EtOAc (10 ml). The organic phase is washed with water (2×10 ml), with saturated aqueous NaHCO₃ solution (10 ml) and with saturated aqueous NaCl solution (10 ml). After drying over MgSO₄ and filtering, the solvents are evaporated off under reduced pressure.

5 The crude reaction product is purified by chromatography on silica gel, eluting with a 99/1 to 90/10 DCM/methanol mixture. A white powder, **7**, is obtained (19.4 mg; 75%). TLC (DCM 90 / MeOH 10): R_f = 0.6; ¹H NMR (400 MHz, DMSO-*d*₆): 0.75 – 0.81 (m, 6 H); 1.01 (s, 3 H); 1.05 (d, J = 6.8 Hz, 3 H); 1.12 (s, 3 H); 1.26 (s, 6 H); 1.28 – 1.33 (m, 1 H); 1.52 – 1.61 (m, 2 H); 1.76 – 1.83 (m, 2 H); 2.27 – 2.38 (m, 4 H); 2.39 (s, 3 H); 2.64 – 2.74 (m, 2 H); 2.95 – 3.05 (m, 2 H); 3.24 – 3.34 (m, 6 H); 3.44 (br. s., 4 H); 3.49 (s, 2 H); 3.81 (s, 3 H); 3.88 (d, J = 1.7 Hz, 1 H); 4.22 – 4.29 (m, 1 H); 4.92 (dd, J = 9.9, 3.5 Hz, 1 H); 5.08 – 5.15 (m, 1 H); 5.81 (d, J = 14.2 Hz, 1 H); 6.48 (ddd, J = 15.2, 11.3, 3.5 Hz, 1 H); 7.05 (d, J = 8.6 Hz, 1 H); 7.17 (dd, J = 8.4, 2.3 Hz, 1 H); 7.22 (d, J = 9.3 Hz, 1 H); 7.25 – 7.34 (m, 5 H); 8.34 (d, J = 8.1 Hz, 1 H). LCMS (A1): ES m/z = 943 [M + H]⁺; m/z = 941 [M - H]⁻; t_R = 4.03 min.

15 Note: Compound **7** may also be prepared from G=OMs (see Example 1)

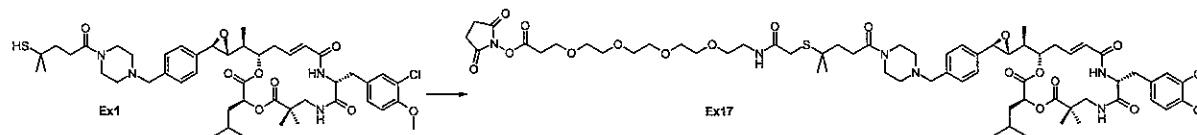
Compound Ex. 1: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-16-[(S)-1-((2R,3R)-3-{4-[4-(4-mercapto-4-methylpentanoyl)piperazin-1-ylmethyl]phenyl}oxiranyl)-ethyl]-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



20 Compound **7** (17.9 mg; 18.97 μ mol) is dissolved in a mixture of ethanol (2.2 ml)/water (1.8 ml), and the mixture turns cloudy. TCEP (47.4 μ mol) is then added and the mixture is stirred for 3 hours at RT and then diluted by adding EtOAc (20 ml) and the organic phase is washed with a 1/1 mixture of water and saturated aqueous NH₄Cl solution (20 ml) and then with 20 ml of saturated NaCl solution. After drying the organic phase over MgSO₄, filtering and evaporating off the solvents under reduced pressure, the product **Ex. 1** is obtained in the form of a white solid (15.6 mg; 92%). TLC (DCM 90 / MeOH 10): R_f = 0.56; ¹H NMR (500 MHz, DMSO-*d*₆): 0.76 – 0.81 (m, 6 H); 1.01 (s, 3 H); 1.06 (d, J = 6.8 Hz, 3 H); 1.13 (s, 3 H); 1.24 (s, 6 H); 1.27 – 1.31 (m, 1 H); 1.56 – 1.64 (m, 2 H); 1.73 – 1.85 (m, 3 H); 2.26 – 2.33 (m, 3 H); 2.36 – 2.45 (m, 4 H); 2.63 – 2.75 (m, 2 H); 2.95 – 3.06 (m, 3 H); 3.34 – 3.36 (m, 1 H); 3.42 – 3.51 (m, 6 H); 3.82 (s, 3 H); 3.89 (s, 1 H); 4.22 – 4.29 (m, 1 H); 4.92 (dd, J = 9.8, 3.4 Hz, 1 H); 5.12 (dd, J = 10.8, 4.9 Hz, 1 H); 5.81 (d, J = 15.2 Hz, 1 H); 6.48 (ddd, J = 15.0, 11.4, 3.4 Hz, 1 H); 7.06 (d, J = 8.3 Hz, 1 H); 7.18 (dd, J = 8.3, 1.5 Hz, 1 H); 7.24 (d, J = 9.8 Hz, 1 H); 7.26 – 7.36 (m, 5 H); 8.37 (d, J = 7.8 Hz, 1 H). LCMS (A2): ES m/z = 897 [M + H]⁺; m/z = 895 [M - H]⁻; t_R = 0.97 min.

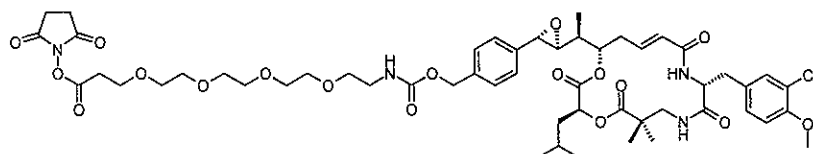
35 **Example 17:** 2,5-Dioxopyrrolidin-1-yl 3-(2-{2-[2-(2-[4-(4-{4-[(2R,3R)-3-((S)-1-((E)-(10R,16S)-10-

(3-chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl]oxiranyl]benzyl]piperazin-1-yl)-1,1-dimethyl-4-oxo-butylsulfanyl]-acetylamino}ethoxy)ethoxy]ethoxy)ethoxy)propanoate

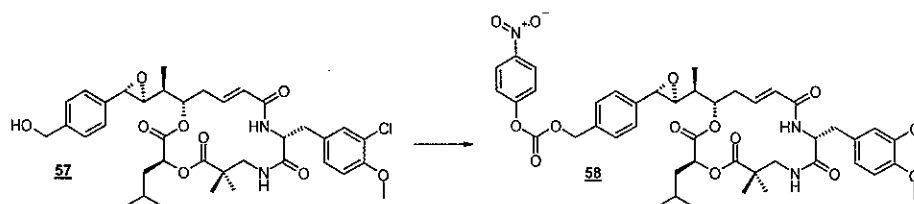


- 5 To a solution, purged with argon, of compound **Ex. 1** (15.6 mg, 17.8 μmol) in anhydrous acetonitrile (1.0 ml) are successively added DIPEA (19.12 μmol) and compound **56** (19.12 μmol). Stirring is continued at RT for 4 hours, and a further 29.5 μmol of DIPEA are then added, and stirring is continued for 16 hours. The next day, a further 29.5 μmol of compound **56** and of DIPEA are added and the mixture is heated to 50°C. After reaction for a further 24 hours,
- 10 22.6 μmol of compound **56** and 29.5 μmol of DIPEA are added and the mixture is heated at 60°C for a further 24 hours. The heating is then stopped and stirring is continued at RT for 64 hours. The mixture is diluted in 10 ml of EtOAc and the organic phase is washed with 2x10 ml of water and then with 10 ml of saturated NaCl solution. After drying the organic phase over MgSO_4 , filtering and evaporating off the solvents under reduced pressure, the crude product is purified by
- 15 chromatography on silica gel, eluting with a 99/1 to 90/10 DCM/methanol mixture. The product **Ex. 17** is obtained in the form of a colourless solid (9.2 mg; 41%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$): 0.77 to 0.82 (m, 6 H): 1.02 (s, 3 H): 1.06 (d, $J = 6.9$ Hz, 3 H): 1.13 (s, 3 H): 1.23 (s, 6 H): 1.26 to 1.35 (m, 1 H): 1.52 to 1.64 (m, 2 H): 1.68 to 1.77 (m, 2 H): 1.78 to 1.86 (m, 1 H): 2.26 to 2.33 (m, 3 H): 2.35 to 2.41 (m, 4 H): 2.68 to 2.76 (m, 2 H): 2.82 (s, 4 H): 2.93 (t, $J = 6.0$ Hz, 2 H): 2.96 to
- 20 3.07 (m, 4 H): 3.13 (s, 2 H): 3.20 (q, $J = 5.8$ Hz, 2 H): 3.33 (m, 1 H): 3.38 to 3.57 (m, 18 H): 3.73 (t, $J = 5.8$ Hz, 2 H): 3.83 (s, 3 H): 3.89 (s, 2 H): 4.27 (m, 1 H): 4.93 (dd, $J = 3.8$ and 10.0 Hz, 1 H): 5.13 (m, 1 H): 5.82 (d, $J = 15.4$ Hz, 1 H): 6.49 (ddd, $J = 3.8$ and 11.1 and 15.4 Hz, 1 H): 7.07 (d, $J = 8.8$ Hz, 1 H): 7.15 to 7.37 (m, 7 H): 8.01 (t, $J = 5.8$ Hz, 1 H): 8.35 (d, $J = 8.0$ Hz, 1 H). LCMS (A2) : ES $m/z = 1299$ $[\text{M} + \text{H}]^+$: $m/z = 650$ $[\text{M} + 2\text{H}]^{2+}$: $m/z = 1297$ $[\text{M} - \text{H}]^-$: $t_R = 0.92$ min.

25 **Example 18:** 2,5-Dioxopyrrolidin-1-yl 3-(2-{2-[2-(2-{4-[(2S,3S)-3-[(S)-1-[(E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl]oxiranyl]benzyl]oxycarbonylamino}ethoxy)ethoxy]ethoxy)-ethoxy)propanoate

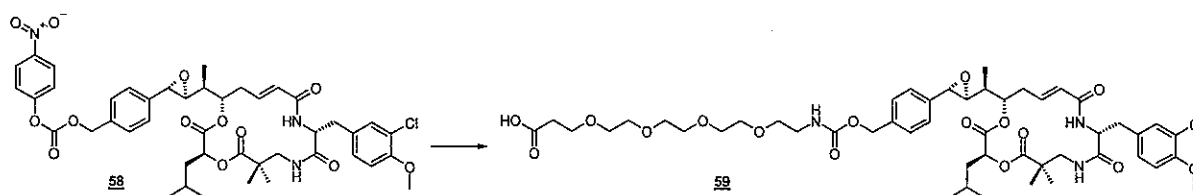


30 **Compound 58:** 4-Nitrophenyl 4-((2S,3S)-3-[(S)-1-[(E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl]oxiranyl]benzyl carbonate



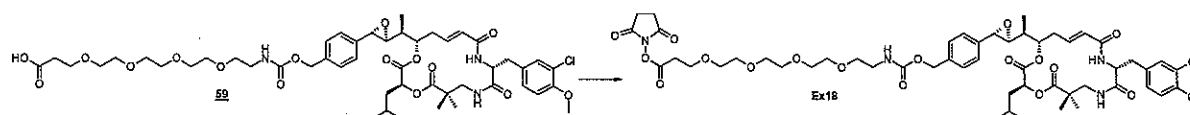
Derivative **57** (20 mg; 28.6 μ mol, prepared according to Al-awar R.S., et al., *J.Med.Chem.* **2003**, 46, 2985-3007) is dissolved in anhydrous DCM (0.3 ml) and the solution is purged with argon, followed by addition of TEA (40 μ mol) and then 4-nitrophenyl chloroformate (32.32 μ mol). After stirring for 3 hours 30 minutes at RT, the mixture is hydrolyzed and diluted in 7 ml of EtOAc. The organic phase is washed with water and then with saturated NaCl solution, dried over MgSO₄, filtered and evaporated to dryness to give compound **58** in the form of a white solid (23 mg, 93%). LCMS (A3): ES m/z = 864 [M + H]⁺; m/z = 908 [M + HCO₂H - H]⁺; t_R = 1.43 min.

10 Compound 59: 3-(2-{2-[2-(2-{4-[(2S,3S)-3-((S)-1-{(E)-(3S,10R,16S)-10-[3-Chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]-ethyl)oxiranyl]benzyloxycarbonylamino)ethoxy)ethoxy}ethoxy)ethoxy)propanoic acid



To a solution, purged with argon, of compound **58** (23 mg, 26.6 μmol) in anhydrous acetonitrile (1.6 ml) are successively added 3-(2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}ethoxy)propionic acid (39.9 μmol) and TEA (53.2 μmol). After stirring for 24 hours at RT, the mixture is diluted in 10 ml of EtOAc. The organic phase is washed with 10 ml of water containing 250 μl of 0.1N HCl (pH $\approx$ 4). The aqueous phase is extracted with 10 ml of EtOAc (2 $\times$); the organic phases are combined, washed with 10 ml of water and then with 10 ml of saturated NaCl solution, dried over MgSO_4 , filtered and evaporated to dryness to give compound **59** in the form of a very pale yellow solid (18.5 mg, 70%). LCMS (A3): ES m/z = 990 $[\text{M} + \text{H}]^+$; m/z = 988 $[\text{M} - \text{H}]^-$; t_R = 1.32 min.

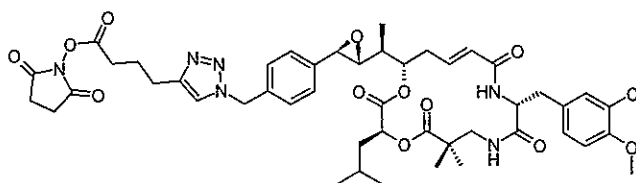
Example 18: 2,5-Dioxopyrrolidin-1-yl 3-(2-{2-[2-(2-{4-[(2S,3S)-3-[(S)-1-[(E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl)oxiranyl]benzyloxycarbonylamino}ethoxy)ethoxy]ethoxy)ethoxy)-propanoate



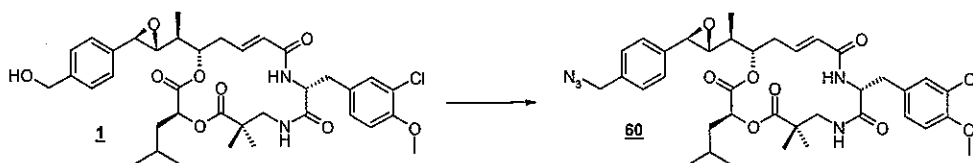
To a solution, purged with argon, of compound **59** (18.5 mg, 18.6 μmol) in THF (1.5 ml) are successively added DIPEA (55.8 μmol) and N,N'-disuccinimidyl carbonate (37.2 μmol). After

stirring for 19 hours at RT, the mixture is diluted in 10 ml of EtOAc, washed with 10 ml of water (twice) and then with 10 ml of saturated NaCl solution, dried over MgSO₄ and evaporated to dryness. The crude product is purified by chromatography on silica gel, eluting with a 100/0 to 0/100 heptane/EtOAc mixture containing 10% isopropanol. The product **Ex. 17** is obtained in the form of a white solid (6.08 mg; 30%). ¹H NMR (500 MHz, DMSO-*d*₆): 0.83 to 0.88 (m, 6 H); 0.97 (d, *J* = 7.1 Hz, 3 H); 1.02 (s, 3 H); 1.15 (s, 3 H); 1.47 to 1.56 (m, 1 H); 1.58 to 1.68 (m, 2 H); 1.82 to 1.90 (m, 1 H); 2.39 to 2.47 (m, 1 H); 2.60 to 2.66 (m, 1 H); 2.71 (dd, *J* = 11.5 and 14.4 Hz, 1 H); 2.80 (s, 4 H); 2.91 (t, *J* = 6.0 Hz, 2 H); 2.94 to 3.07 (m, 3 H); 3.14 (q, *J* = 6.0 Hz, 2 H); 3.37 to 3.56 (partially masked m, 15 H); 3.71 (t, *J* = 6.0 Hz, 2 H); 3.79 (m, 1 H); 3.81 (s, 3 H); 4.27 (ddd, *J* = 3.8 and 8.0 and 11.3 Hz, 1 H); 4.96 to 5.05 (m, 3 H); 5.11 (m, 1 H); 5.88 (d, *J* = 15.4 Hz, 1 H); 6.48 (ddd, *J* = 3.8 and 11.3 and 15.4 Hz, 1 H); 7.05 (d, *J* = 8.8 Hz, 1 H); 7.14 to 7.39 (m, 8 H); 8.40 (d, *J* = 8.0 Hz, 1 H). LCMS (A2): ES *m/z* = 1087 [*M* + *H*]⁺; *m/z* = 1085 [*M* - *H*]⁻; *t*_R = 1.09 min.

Example 19: 2,5-Dioxopyrrolidin-1-yl (1-{4-[(2R,3R)-3-[(S)-1-[(E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl]oxiranyl]benzyl}-1H-1,2,3-triazol-4-yl)butanoate



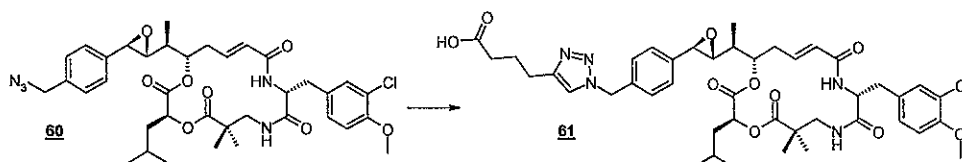
Compound 60: (E)-(3S,10R,16S)-16-{(S)-1-[(2R,3R)-3-(4-Azidomethylphenyl)oxiranyl]ethyl}-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



To a solution, purged with argon, of 60 mg (85.8 μmol) of compound **1** in 9 ml of anhydrous THF are added 25.9 μl (120 μmol) of diphenylphosphorazide. The mixture is stirred at RT for 10 minutes and then cooled to 0°C, followed by addition of 18.0 μl (120 μmol) of DBU. Stirring is continued at RT for 15 hours. The reaction is incomplete: 25.9 μl (120 μmol) of diphenylphosphorazide and 18.0 μl (120 μmol) of DBU are added and stirring is continued for 24 hours. Some starting compound **1** remains: 25.9 μl (120 μmol) of diphenylphosphorazide and 18.0 μl (120 μmol) of DBU are added and stirring is continued for 24 hours. The reaction mixture is hydrolyzed by addition of 6 ml of water and is then extracted with DCM (3x6 ml). The organic phases are combined, washed with saturated NaCl solution (8 ml) and dried over MgSO₄. After filtering and concentrating under reduced pressure, the crude product is purified by chromatography on silica gel, using a 100/0 to 90/10 DCM/methanol mixture as eluent.

Compound **60** is obtained in the form of a white solid (46 mg, 74%). ^1H NMR (500 MHz, DMSO- d_6): 0.76 (d, J = 6.3 Hz, 3 H); 0.78 (d, J = 6.3 Hz, 3 H); 1.00 (s, 3 H); 1.05 (d, J = 6.9 Hz, 3 H); 1.12 (s, 3 H); 1.30 (m, 1 H); 1.49 to 1.63 (m, 2 H); 1.82 (m, 1 H); 2.27 (m, 1 H); 2.63 to 2.74 (m, 2 H); 2.95 to 3.05 (m, 3 H); 3.25 to 3.38 (partially masked m, 1 H); 3.81 (s, 3 H); 3.92 (d, J = 1.9 Hz, 1 H); 4.25 (ddd, J = 3.6 and 8.0 and 11.5 Hz, 1 H); 4.46 (s, 2 H); 4.92 (dd, J = 3.6 and 9.6 Hz, 1 H); 5.11 (ddd, J = 1.5 and 5.7 and 11.3 Hz, 1 H); 5.80 (dd, J = 1.5 and 15.1 Hz, 1 H); 6.48 (ddd, J = 3.8 and 11.3 and 15.1 Hz, 1 H); 7.05 (d, J = 8.6 Hz, 1 H); 7.17 (dd, J = 2.2 and 8.6 Hz, 1 H); 7.22 (dd, J = 2.6 and 9.5 Hz, 1 H); 7.28 (d, J = 2.2 Hz, 1 H); 7.34 (d, J = 8.3 Hz, 2 H); 7.38 (d, J = 8.3 Hz, 2 H); 8.34 (d, J = 8.0 Hz, 1 H). LCMS (A2) : ES m/z = 724 $[\text{M} + \text{H}]^+$; m/z = 722 $[\text{M} - \text{H}]^-$; m/z = 768 $[\text{M} - \text{H} + \text{HCO}_2\text{H}]^-$ base peak; t_R = 1.18 min.

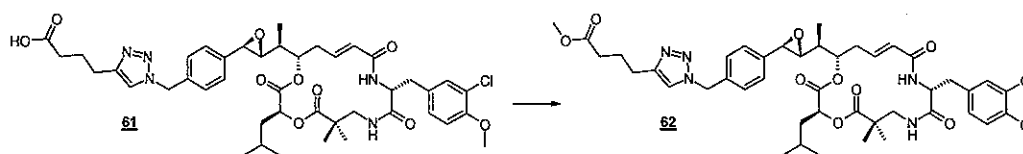
Compound 61: (1-{4-[(2R,3R)-3-((S)-1-((E)-(3S,10R,16S)-10-[3-Chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl)-oxiranyl]benzyl}-1H-1,2,3-triazol-4-yl)butanoic acid



To a suspension of 22 mg (30.4 μmol) of compound **60** in 500 μl of water is added a solution of 6.8 mg (60.8 μmol) of 5-hexynoic acid in 500 μl of THF. 122 μl (12.2 μmol) of aqueous 0.1M copper sulfate solution and 122 μl (24.3 μmol) of aqueous 0.2M sodium ascorbate solution are then added. Stirring is continued for 2 hours at RT. The mixture is hydrolyzed by adding 2 ml of water and is then extracted with EtOAc (3 $\times$ 2 ml). The organic phases are combined, washed with saturated NaCl solution (3 ml) and dried over MgSO_4 . After filtering and concentrating under reduced pressure, the crude product is purified by chromatography on silica gel, using a 98/2 to 90/10 DCM/methanol mixture as eluent. Compound **61** is obtained in the form of a white solid (19.4 mg, 76%). ^1H NMR (500 MHz, DMSO- d_6): 0.73 (d, J = 6.4 Hz, 3 H); 0.75 (d, J = 6.4 Hz, 3 H); 1.00 (s, 3 H); 1.04 (d, J = 6.9 Hz, 3 H); 1.11 (s, 3 H); 1.29 (m, 1 H); 1.50 to 1.59 (m, 2 H); 1.76 to 1.85 (m, 3 H); 2.20 to 2.30 (m, 3 H); 2.62 (t, J = 7.7 Hz, 2 H); 2.65 to 2.72 (m, 2 H); 2.94 to 3.04 (m, 3 H); 3.28 to 3.38 (partially masked m, 1 H); 3.81 (s, 3 H); 3.89 (d, J = 1.6 Hz, 1 H); 4.25 (ddd, J = 3.6 and 8.0 and 11.5 Hz, 1 H); 4.90 (dd, J = 3.6 and 9.6 Hz, 1 H); 5.10 (ddd, J = 1.6 and 5.2 and 11.2 Hz, 1 H); 5.53 (s, 2 H); 5.78 (dd, J = 1.6 and 15.0 Hz, 1 H); 6.46 (ddd, J = 3.7 and 11.2 and 15.0 Hz, 1 H); 7.05 (d, J = 8.5 Hz, 1 H); 7.17 (dd, J = 1.9 and 8.5 Hz, 1 H); 7.22 (dd, J = 2.3 and 9.5 Hz, 1 H); 7.28 (d, J = 1.9 Hz, 1 H); 7.31 (s, 4 H); 7.92 (s, 1 H); 8.37 (broad d, J = 8.0 Hz, 1 H); 12.03 (very broad m, 1 H). LCMS (A2) : ES m/z = 836 $[\text{M} + \text{H}]^+$; m/z = 418.5 $[\text{M} + 2\text{H}]^{2+}$ base peak; m/z = 834 $[\text{M} - \text{H}]^-$; t_R = 1.04 min.

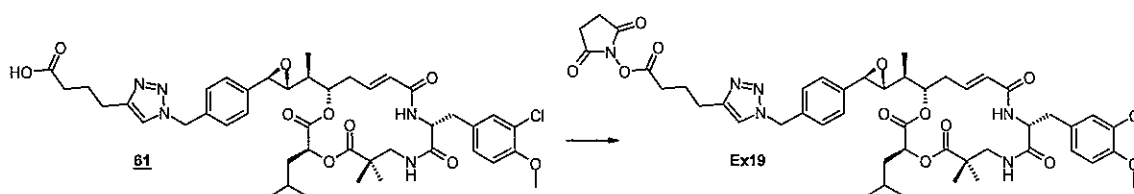
Compound 62: Methyl (1-{4-[(2R,3R)-3-((S)-1-((E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-

3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl}ethyl)-oxiranyl]benzyl}-1H-1,2,3-triazol-4-yl)butanoate



To a solution of 5 mg (6 μ mol) of compound **61** in 500 μ l of DCM and 200 μ l of methanol are added 4.5 μ l (9.0 μ mol) of a 2M solution of trimethylsilyldiazomethane in hexane. Stirring is continued for 30 minutes. The mixture is concentrated under reduced pressure and purified by filtration on silica gel, eluting with a 98/2 DCM/methanol mixture. Compound **62** is obtained in the form of a white solid (5 mg, 98%). ^1H NMR (500 MHz, DMSO- d_6): 0.72 (d, J = 6.4 Hz, 3 H); 0.75 (d, J = 6.4 Hz, 3 H); 0.99 (s, 3 H); 1.04 (d, J = 6.8 Hz, 3 H); 1.11 (s, 3 H); 1.28 (m, 1 H); 1.50 to 1.59 (m, 2 H); 1.79 (m, 1 H); 1.83 (m, 2 H); 2.25 (m, 1 H); 2.35 (t, J = 7.6 Hz, 2 H); 2.62 (t, J = 7.6 Hz, 2 H); 2.65 to 2.72 (m, 2 H); 2.94 to 3.05 (m, 3 H); 3.25 to 3.38 (partially masked m, 1 H); 3.57 (s, 3 H); 3.81 (s, 3 H); 3.89 (d, J = 1.5 Hz, 1 H); 4.25 (ddd, J = 3.7 and 8.3 and 11.4 Hz, 1 H); 4.89 (dd, J = 3.4 and 9.8 Hz, 1 H); 5.09 (ddd, J = 1.5 and 5.4 and 11.4 Hz, 1 H); 5.53 (s, 2 H); 5.78 (dd, J = 1.5 and 15.2 Hz, 1 H); 6.46 (ddd, J = 3.4 and 11.4 and 15.2 Hz, 1 H); 7.05 (d, J = 8.8 Hz, 1 H); 7.17 (dd, J = 2.0 and 8.8 Hz, 1 H); 7.22 (dd, J = 2.5 and 9.8 Hz, 1 H); 7.28 (d, J = 2.0 Hz, 1 H); 7.31 (s, 4 H); 7.92 (s, 1 H); 8.34 (d, J = 8.3 Hz, 1 H). LCMS (A2) : ES m/z = 850 $[\text{M} + \text{H}]^+$; m/z = 425.5 $[\text{M} + 2\text{H}]^{2+}$ base peak; m/z = 848 $[\text{M} - \text{H}]^-$; m/z = 894 $[\text{M} - \text{H} + \text{HCO}_2\text{H}]^-$ base peak; t_R = 1.11 min.

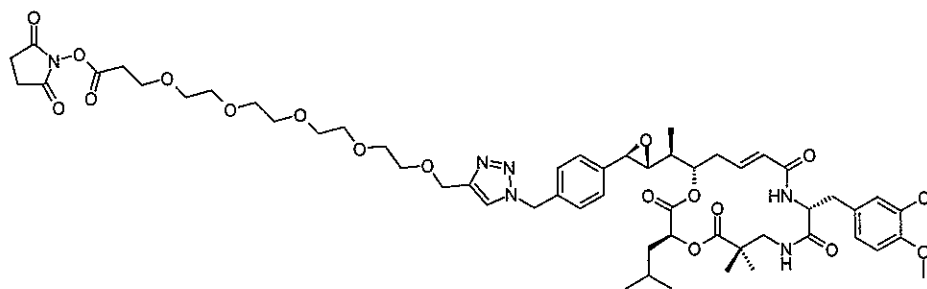
Example 19: 2,5-Dioxopyrrolidin-1-yl (1-{4-[(2R,3R)-3-((S)-1-{(E)-(3S,10R,16S)-10-[3-chloro-4-methoxybenzyl]-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl}ethyl)oxiranyl]benzyl}-1H-1,2,3-triazol-4-yl)butanoate



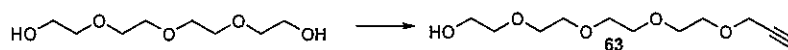
To a solution, purged with argon, of 13.4 mg (16 μ mol) of compound **61** in 1.2 ml of THF are successively added 8.4 μ l (48.1 μ mol) of DIPEA and 8.21 mg (32.04 μ mol) of N,N'-disuccinimidyl carbonate. Stirring is continued at RT for 26 hours. The mixture is hydrolyzed by addition of 2 ml of water and is then extracted with 2x2 ml of EtOAc. The organic phases are combined and dried over MgSO_4 . After filtering and concentrating under reduced pressure, the crude product is purified by filtration on silica gel, eluting with a 98/2 DCM/methanol mixture. The product obtained contains 0.15 mol of NHS. It is taken up in 2 ml of DCM and washed with water (2x3 ml) and then dried over MgSO_4 . After filtering and concentrating to dryness, compound **Ex. 19** is obtained in the form of a white solid (12.56 mg, 84%). ^1H NMR (500 MHz, DMSO- d_6): 0.73 (d, J = 6.3 Hz, 3

H); 0.75 (d, J = 6.6 Hz, 3 H); 1.00 (s, 3 H); 1.04 (d, J = 6.9 Hz, 3 H); 1.11 (s, 3 H); 1.30 (m, 1 H); 1.49 to 1.60 (m, 2 H); 1.79 (m, 1 H); 1.95 (quin, J = 7.5 Hz, 2 H); 2.26 (m, 1 H); 2.65 to 2.77 (m, 6 H); 2.81 (broad s, 4 H); 2.92 to 3.05 (m, 3 H); 3.34 (partially masked m, 1 H); 3.81 (s, 3 H); 3.89 (d, J = 1.9 Hz, 1 H); 4.25 (ddd, J = 3.7 and 8.1 and 11.5 Hz, 1 H); 4.90 (dd, J = 3.6 and 9.6 Hz, 1 H); 5.09 (ddd, J = 1.4 and 5.5 and 11.3 Hz, 1 H); 5.54 (s, 2 H); 5.79 (dd, J = 1.4 and 15.1 Hz, 1 H); 6.46 (ddd, J = 3.8 and 11.3 and 15.1 Hz, 1 H); 7.05 (d, J = 8.5 Hz, 1 H); 7.17 (dd, J = 2.2 and 8.5 Hz, 1 H); 7.21 (dd, J = 2.5 and 9.6 Hz, 1 H); 7.28 (d, J = 2.2 Hz, 1 H); 7.31 (s, 4 H); 7.95 (s, 1 H); 8.33 (d, J = 8.1 Hz, 1 H). LCMS (A2): ES m/z = 933 [M + H]⁺; m/z = 467 [M + 2H]²⁺ base peak; m/z = 977 [M - H + HCO₂H]⁻ base peak; t_R = 1.08 min.

Example 20: 2,5-Dioxapyrrolidin-1-yl 3-(2-{2-[2-(2-{1-[1-(4-((2R,3R)-3-[(S)-1-((E)-(10R,16S)-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl]oxiranyl}benzyl)-1,2,3-triazol-1-yl]methoxy}ethoxy)-ethoxy]ethoxy}ethoxy)propanoate

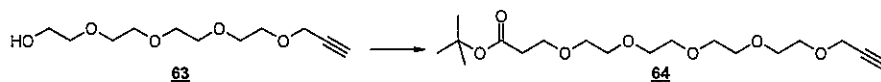


Compound **63**: 2-{2-[2-(2-Prop-2-ynloxyethoxy)ethoxy]ethoxy}ethanol



To a solution, purged with argon and cooled to 0°C, of 1 g (5.15 mmol) of tetraethylene glycol in 25 ml of anhydrous THF are added 144 mg (3.60 mmol) of NaH as a 60% dispersion in a mineral oil. Stirring is continued for 30 minutes at 0°C, and 194 µl (2.58 mmol) of propargyl bromide are then added. Stirring is continued at RT for 15 hours. The mixture is concentrated under reduced pressure and the crude product is then purified by chromatography on silica gel, using a 98/2 to 90/10 DCM/methanol mixture as eluent. Compound **63** is obtained in the form of a colourless oil (789 mg, 65%). ¹H NMR (400 MHz, DMSO-*d*₆): 3.40 (t, *J* = 2.4 Hz, 1 H); 3.42 (t, *J* = 5.5 Hz, 2 H); 3.48 (q, *J* = 5.5 Hz, 2 H); 3.51 to 3.58 (m, 12 H); 4.14 (d, *J* = 2.4 Hz, 2 H); 4.53 (t, *J* = 5.5 Hz, 1 H). LCMS (A2) : ES *m/z* = 233 [*M* + *H*]⁺; *t*_R = 0.38 min.

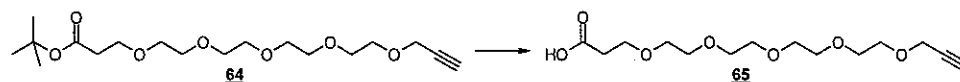
Compound 64: *tert*-Butyl 3-(2-{2-[2-(2-prop-2-ynyloxyethoxy)ethoxy]ethoxy}ethoxy)propanoate



To a solution, purged with argon, of 790 mg (3.40 mmol) of compound **63** in 8.8 ml of anhydrous THF are added 4.4 mg (190 μ mol) of sodium. The mixture is heated at 40°C for 2 hours to fully dissolve it; after cooling to RT, 740 μ l (5.09 mmol) of *tert*-butyl acrylate are added to the mixture.

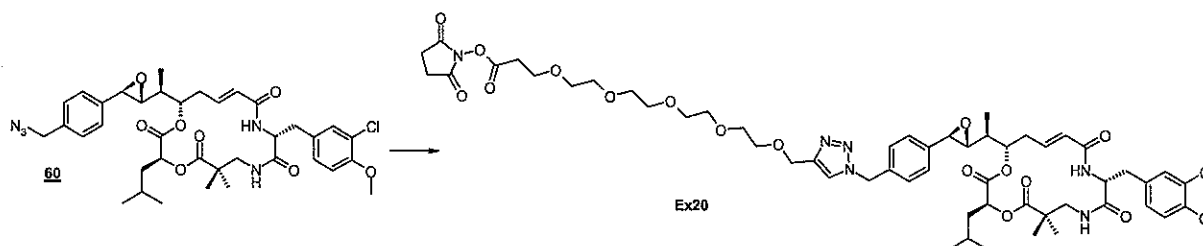
Stirring is continued for 15 hours at RT and the mixture is then concentrated under reduced pressure and the crude product is purified by chromatography on silica gel, using a 98/2 to 90/10 DCM/methanol mixture as eluent. Compound **64** is obtained in the form of a colourless oil (944 mg, 77%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 1.44 (s, 9 H); 2.41 (t, $J = 6.2$ Hz, 2 H); 3.38 (t, $J = 2.4$ Hz, 1 H); 3.46 to 3.62 (m, 16 H); 3.59 (t, $J = 6.2$ Hz, 2 H); 4.14 (d, $J = 2.4$ Hz, 2 H). LCMS (A1): ES $m/z = 361$ $[\text{M} + \text{H}]^+$; $m/z = 383$ $[\text{M} + \text{Na}]^+$; $t_R = 3.79$ min.

Compound 65: 3-(2-{2-[2-(2-prop-2-ynyloxyethoxy)ethoxy]ethoxy}ethoxy)propanoic acid



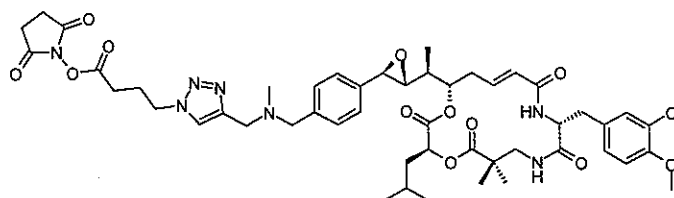
To a solution of 944 mg (2.62 mmol) of compound **64** are added 2 ml (52.4 mmol) of TFA. The mixture is stirred for 6 hours at RT and then concentrated to dryness, taken up in a minimum amount of DCM and entrained several times with toluene. Compound **65** is obtained in the form of a colourless oil (722 mg, 91%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 2.44 (t, $J = 6.4$ Hz, 2 H); 3.39 (t, $J = 2.4$ Hz, 1 H); 3.46 to 3.56 (m, 16 H); 3.60 (t, $J = 6.4$ Hz, 2 H); 4.14 (d, $J = 2.4$ Hz, 2 H); 7.44 to 9.73 (very broad m, 1 H). LCMS (A2): ES $m/z = 305$ $[\text{M} + \text{H}]^+$; $m/z = 303$ $[\text{M} - \text{H}]^-$; $t_R = 0.48$ min.

Example 20: 2,5-Dioxapyrrolidin-1-yl 3-(2-{2-[2-(2-{1-[1-(4-{(2R,3R)-3-[(S)-1-((E)-(10R,16S)-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl]oxiranyl}benzyl)-1,2,3-triazol-1-yl]methoxy}ethoxy)ethoxy]ethoxy}ethoxy)propanoate

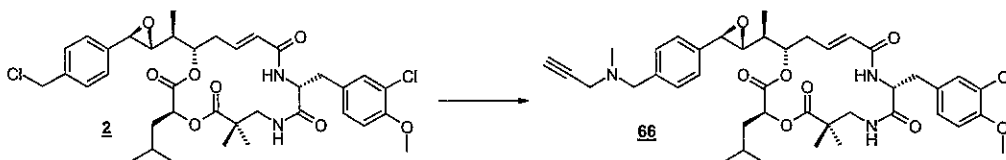


Example **20** may be obtained from compounds **60** and **65** according to the method described for compound **61**, followed by activating the acid according to the method described for Example **19**.

Example 21: 2,5-Dioxapyrrolidin-1-yl (4-{1-[4-{(2R,3R)-3-[(S)-1-((E)-(3S,10R,16S)-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl]oxiranyl}benzyl)methylamino]methyl}-1,2,3-triazol-1-yl)-butanoate



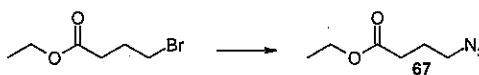
Compound 66: (E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-16-[(S)-1-((2R,3R)-3-{4-[(methylprop-2-ynylamino)methyl]phenyl}oxiranyl)ethyl]-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



- 5 To a solution, purged with argon, of 40 mg (55.7 μmol) of compound **2** in 5 ml of anhydrous acetonitrile are successively added 48.5 μl (279 μmol) of DIPEA and 13.9 μl (167 μmol) of N-methylpropargylamine. The mixture is heated at 40°C for 15 hours. Some starting compound **2** remains: a further 48.5 μl (279 μmol) of DIPEA and 13.9 μl (167 μmol) of N-methylpropargylamine are added. After stirring for 24 hours at 40°C, the mixture is diluted with
- 10 5 ml of EtOAc and then washed with 5 ml of water. The aqueous phase is extracted with 3x5 ml of EtOAc and the organic phases are combined, washed with saturated NaHCO_3 solution (10 ml) and with saturated NaCl solution (10 ml) and dried over MgSO_4 . After filtering and concentrating under reduced pressure, the crude product is purified by chromatography on silica gel, using a 98/2 to 90/10 DCM/methanol mixture as eluent. Compound **66** is obtained in the form of a white solid (45 mg, quant.). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 0.78 (m, 6 H); 1.00 (s, 3 H); 1.05 (d, $J = 6.8$ Hz, 3 H); 1.12 (s, 3 H); 1.30 (m, 1 H); 1.49 to 1.61 (m, 2 H); 1.81 (m, 1 H); 2.20 (s, 3 H); 2.28 (m, 1 H); 2.63 to 2.76 (m, 2 H); 2.92 to 3.07 (m, 3 H); 3.17 (t, $J = 2.4$ Hz, 1 H); 3.27 (d, $J = 2.4$ Hz, 2 H); 3.33 (partially masked m, 1 H); 3.50 (s, 2 H); 3.81 (s, 3 H); 3.88 (d, $J = 1.7$ Hz, 1 H); 4.25 (ddd, $J = 3.4$ and 7.8 and 11.4 Hz, 1 H); 4.92 (dd, $J = 3.5$ and 9.7 Hz, 1 H); 5.11 (ddd, $J = 1.5$ and
- 15 5.5 and 11.2 Hz, 1 H); 5.80 (dd, $J = 1.5$ and 15.0 Hz, 1 H); 6.47 (ddd, $J = 3.9$ and 11.2 and 15.0 Hz, 1 H); 7.05 (d, $J = 8.6$ Hz, 1 H); 7.17 (dd, $J = 2.1$ and 8.6 Hz, 1 H); 7.22 (dd, $J = 2.1$ and 9.4 Hz, 1 H); 7.25 to 7.33 (m, 5 H); 8.33 (d, $J = 7.8$ Hz, 1 H). LCMS (A2) : ES $m/z = 750$ $[\text{M} + \text{H}]^+$; ES $m/z = 375.5$ $[\text{M} + 2\text{H}]^{2+}$ base peak; ES $m/z = 748$ $[\text{M} - \text{H}]^-$; $m/z = 794$ $[\text{M} - \text{H} + \text{HCO}_2\text{H}]^-$; $t_R = 0.91$ min.

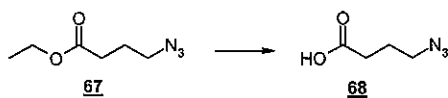
25

Compound 67: Ethyl 4-azidobutanoate



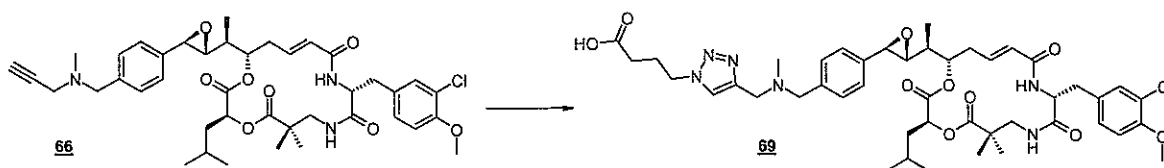
- 2.86 ml (20 mmol) of ethyl 4-bromobutanoate and 2.6 g (40 mmol) of NaN_3 are dissolved in 30 ml of a 2/1 acetone/water mixture. The mixture is refluxed for 7 hours. After cooling to RT and
- 30 concentrating under reduced pressure, the residue is taken up in 50 ml of water. The aqueous phase is extracted with 3x30 ml of DCM. The organic phases are combined, dried over MgSO_4 , filtered and concentrated under reduced pressure to give compound **67** in the form of a colourless oil (3 g, 95%). ^1H NMR (400 MHz, chloroform- d): 1.27 (t, $J = 7.2$ Hz, 3 H); 1.92 (m, 2 H); 2.41 (t, $J = 7.2$ Hz, 2 H); 3.36 (t, $J = 6.7$ Hz, 2 H); 4.15 (q, $J = 7.2$ Hz, 2 H).

35

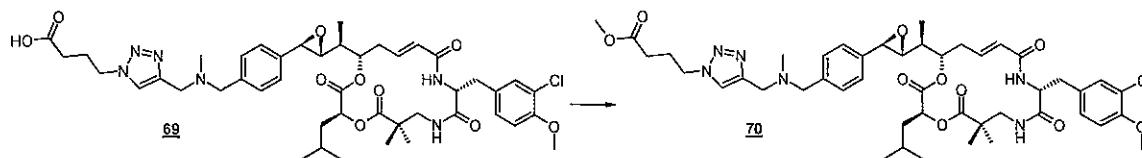
Compound 68: 4-Azidobutanoic acid

To a solution of 3 g (19.9 mmol) of compound **67** in 47 ml of methanol and 37 ml of water are added portionwise 5.58 g (99.5 mmol) of potassium hydroxide. The mixture is stirred at RT for 6 hours and then concentrated under reduced pressure to about 28 ml. The residue is diluted with 25 ml of water and extracted with 2×20 ml of DCM. The aqueous phase is acidified to pH≈1 by adding concentrated HCl and is then extracted with 3×25 ml of diethyl ether. The organic phases are combined, washed with saturated NaCl solution (25 ml) and dried over MgSO₄. After filtering and concentrating to dryness under reduced pressure, compound **68** is obtained in the form of a colourless oil (2.05 g, 80%). ¹H NMR (400 MHz, chloroform-*d*): 1.93 (m, 2 H); 2.49 (t, J = 7.1 Hz, 2 H); 3.39 (t, J = 6.7 Hz, 2 H); 11.24 (broad m, 1 H).

Compound 69: (4-{1-[(4-{[(2R,3R)-3-[(S)-1-[(E)-(3S,10R,16S)-10-{3-Chloro-4-methoxybenzyl}-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl]-oxiranyl)benzyl)methylamino]methyl}-1,2,3-triazol-1-yl)butanoic acid

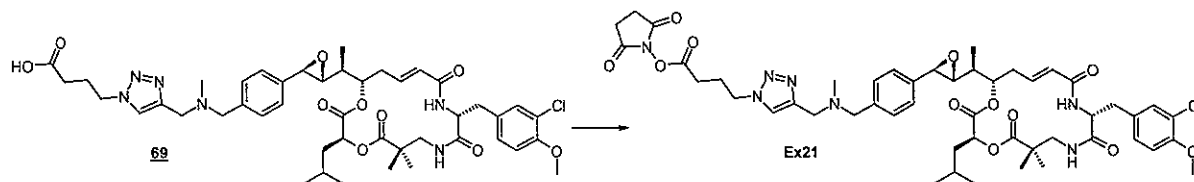


Compound 70: Methyl (4-{1-[(4-{(2R,3R)-3-[(S)-1-((E)-(3S,10R,16S)-10-{3-chloro-4-methoxybenzyl}-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl]oxiranyl}benzyl)methylamino]methyl}-1,2,3-triazol-1-yl)butanoate



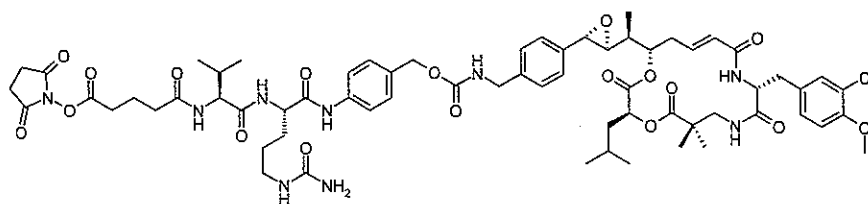
To a solution, purged with argon, of 5.5 mg (6.2 μ mol) of compound **69** in 0.5 ml of DCM and 0.2 ml of methanol are added 4.7 μ l (9.3 μ mol) of trimethylsilyldiazomethane. The mixture is stirred for 45 minutes at RT and then concentrated to dryness. The crude product is purified by chromatography on silica gel, using a 98/2 to 95/5 DCM/methanol mixture as eluent. Compound **70** is obtained in the form of a white solid (3.4 mg, 62%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$): 0.75 (d, $J = 6.6$ Hz, 3 H); 0.77 (d, $J = 6.6$ Hz, 3 H); 1.00 (s, 3 H); 1.05 (d, $J = 6.9$ Hz, 3 H); 1.11 (s, 3 H); 1.28 (m, 1 H); 1.49 to 1.61 (m, 2 H); 1.80 (m, 1 H); 2.07 (m, 2 H); 2.10 (m, 3 H); 2.26 (m, 1 H); 2.31 (t, $J = 7.0$ Hz, 2 H); 2.62 to 2.74 (m, 2 H); 2.93 to 3.04 (m, 3 H); 3.27 to 3.37 (partially masked m, 1 H); 3.48 (s, 2 H); 3.58 (s, 3 H); 3.61 (s, 2 H); 3.81 (s, 3 H); 3.87 (d, $J = 2.0$ Hz, 1 H); 4.25 (ddd, $J = 3.7$ and 8.2 and 11.6 Hz, 1 H); 4.38 (t, $J = 7.0$ Hz, 2 H); 4.91 (dd, $J = 3.9$ and 9.8 Hz, 1 H); 5.11 (ddd, $J = 1.5$ and 5.3 and 11.2 Hz, 1 H); 5.79 (dd, $J = 1.5$ and 15.2 Hz, 1 H); 6.47 (ddd, $J = 3.9$ and 11.2 and 15.2 Hz, 1 H); 7.05 (d, $J = 8.6$ Hz, 1 H); 7.16 (dd, $J = 2.2$ and 8.6 Hz, 1 H); 7.22 (dd, $J = 2.4$ and 9.8 Hz, 1 H); 7.26 (d, $J = 8.6$ Hz, 2 H); 7.28 (d, $J = 2.2$ Hz, 1 H); 7.33 (d, $J = 8.6$ Hz, 2 H); 8.03 (s, 1 H); 8.34 (d, $J = 8.2$ Hz, 1 H). LCMS (A2): ES $m/z = 893$ $[\text{M} + \text{H}]^+$; $m/z = 447$ $[\text{M} + 2\text{H}]^{2+}$ base peak; $m/z = 891$ $[\text{M} - \text{H}]$; $m/z = 937$ $[\text{M} - \text{H} + \text{HCO}_2\text{H}]$ base peak; $t_R = 0.90$ min.

Example 21: 2,5-Dioxopyrrolidin-1-yl (4-{1-[(4-{(2R,3R)-3-[(S)-1-((E)-(3S,10R,16S)-10-{3-chloro-4-methoxybenzyl}-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl)ethyl]oxiranyl}benzyl)methylamino]methyl}-1,2,3-triazol-1-yl)butanoate

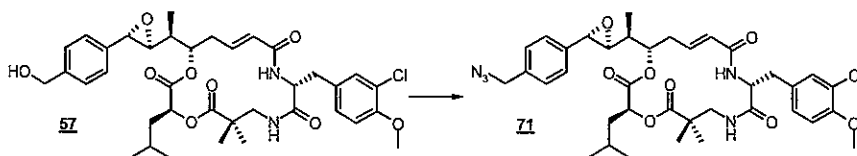


Example **21** may be obtained by activating the acid **69** according to the method described for Example **19**.

Example 22

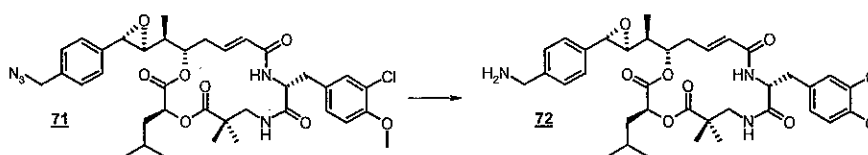


Compound 71: (E)-(3S,10R,16S)-16-((S)-1-((2S,3S)-3-(4-Azidomethylphenyl)oxiranyl)ethyl)-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



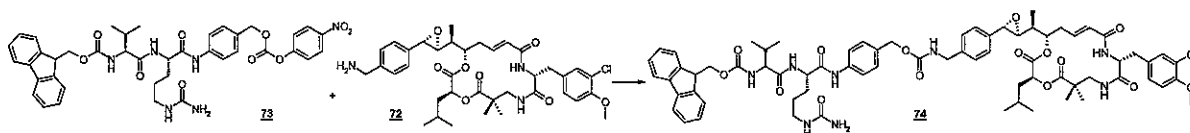
The alcohol **57** (36 mg; 51.48 μmol , prepared according to Al-awar R.S., et al., *J. Med. Chem.* **2003**, 46, 2985-3007) is dissolved in anhydrous THF (2 ml). The solution is purged with argon and cooled in a bath of ice-cold water, followed by addition of DPPA (74 μmol) and then DBU (80 μmol). The mixture is allowed to warm to RT and stirring is continued overnight. The next day, the solution is again cooled in a bath of ice-cold water, followed by addition of a further 74 μmol of DPPA and 80 μmol of DBU. After reaction for 2 hours at 0°C and 2 hours at RT, the mixture is hydrolyzed with 5 ml of water and then extracted 3 times with DCM. The combined organic phases are dried over Na_2SO_4 , filtered and evaporated under reduced pressure. The crude product is then purified by chromatography on silica gel, eluting with a 100/0 to 95/5 DCM/methanol mixture. Compound **57** is obtained in the form of a colourless solid (24 mg; 64%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ (ppm): 0.84 (d, J = 6.3 Hz, 3 H); 0.86 (d, J = 6.3 Hz, 3 H); 0.98 (d, J = 7.1 Hz, 3 H); 1.03 (s, 3 H); 1.15 (s, 3 H); 1.48 to 1.67 (m, 3 H); 1.88 (m, 1 H); 2.45 (m, 1 H); 2.63 (m, 1 H); 2.71 (dd, J = 11.5 and 14.0 Hz, 1 H); 2.97 to 3.08 (m, 3 H); 3.36 (partially masked m, 1 H); 3.82 (broad s, 4 H); 4.28 (ddd, J = 3.6 and 8.0 and 11.5 Hz, 1 H); 4.44 (s, 2 H); 4.99 (dd, J = 3.7 and 9.5 Hz, 1H); 5.12 (ddd, J = 1.4 and 5.7 and 11.4 Hz, 1 H); 5.88 (dd, J = 1.4 and 15.2 Hz, 1 H); 6.49 (ddd, J = 3.8 and 11.4 and 15.2 Hz, 1 H); 7.05 (d, J = 8.5 Hz, 1 H); 7.18 (dd, J = 2.2 and 8.5 Hz, 1 H); 7.26 to 7.32 (m, 4 H); 7.36 (d, J = 8.5 Hz, 2 H); 8.40 (d, J = 8.0 Hz, 1 H). LCMS (A2): ES m/z = 722 $[\text{M} - \text{H}]^-$; m/z = 724 $[\text{M} + \text{H}]^+$; m/z = 768 $[\text{M} + \text{HCO}_2\text{H} - \text{H}]^-$; t_R = 1.19 min.

Compound 72: (E)-(3S,10R,16S)-16-((S)-1-((2S,3S)-3-(4-Aminomethylphenyl)oxiranyl)ethyl)-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



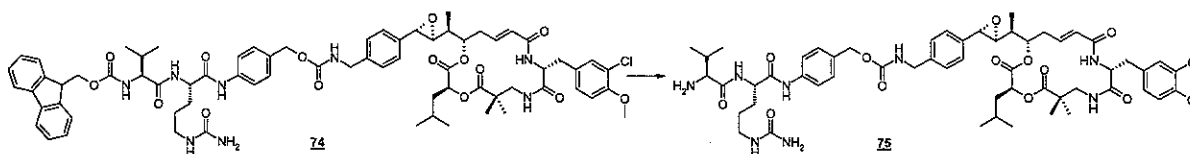
Compound **71** (22 mg, 30.38 μmol) is dissolved in a mixture of methanol (1 ml)/water (0.2 ml), followed by addition of TCEP (34.89 μmol). The solution obtained is stirred overnight at RT and then concentrated under reduced pressure. The residue is then taken up in saturated aqueous NaHCO_3 solution and extracted 3 times with DCM. The combined organic phases are dried over Na_2SO_4 , filtered and concentrated under reduced pressure. The intermediate **72** is thus obtained in the form of a colourless solid (21 mg, 99%), which is used in crude form in the following step. LCMS (A5): ES $m/z = 696$ $[\text{M} - \text{H}]^-$; $m/z = 698$ $[\text{M} + \text{H}]^+$; $m/z = 742$ $[\text{M} + \text{HCO}_2\text{H} - \text{H}]^-$; $t_R = 3.19$ min.

Compound 74: FmocVal-Cit-PABAC- α -aminocryptophycin 72



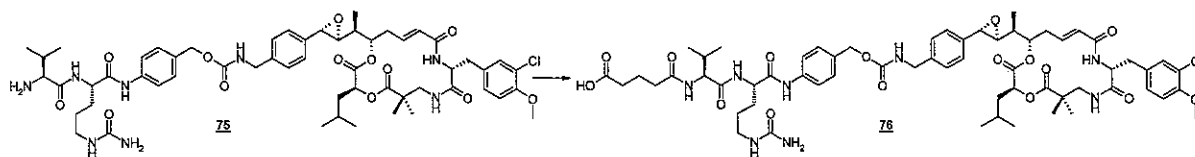
Compound **72** (21 mg, 30.08 μmol) is dissolved in a mixture of acetonitrile (2 ml)/anhydrous DMF (0.5 ml), followed by addition of a solution of compound **73** (23 mg, 30.1 μmol ; prepared according to **WO 2006/110 476**) in acetonitrile (2 ml). The mixture is stirred for 22 hours at RT and then concentrated under reduced pressure. The residue is taken up in DCM and washed with saturated aqueous NaHCO_3 solution and then dried over Na_2SO_4 , filtered and concentrated under reduced pressure. The crude product is then purified by chromatography on silica gel, eluting with a 100/0 to 90/10 DCM/methanol mixture. Compound **74** is obtained in the form of a white solid (22 mg), which is used in crude form in the following step. LCMS (A5): ES $m/z = 1325$ $[\text{M} + \text{H}]^+$; $m/z = 1369$ $[\text{M} + \text{HCO}_2\text{H} - \text{H}]^-$; $t_R = 4.30$ min.

Compound 75: Val-Cit-PABAC- α -aminocryptophycin 72



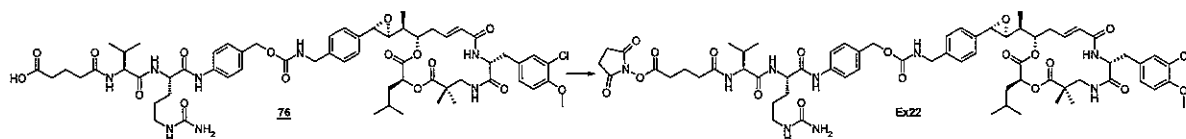
Compound **74** (22 mg, 16.59 μmol) is dissolved in DMF (3 ml), followed by addition of piperidine (150 μl , 1.51 mmol) and the mixture is stirred for 30 minutes at RT. The mixture is concentrated under reduced pressure; the residue is dissolved in a minimum amount of methanol and precipitated from ether. Compound **75** is thus obtained in the form of a white solid (15 mg, 82%). LCMS (A5): ES $m/z = 1103$ $[\text{M} + \text{H}]^+$; $m/z = 1101$ $[\text{M} - \text{H}]^-$; $m/z = 1148$ $[\text{M} + \text{HCO}_2\text{H} - \text{H}]^-$; $t_R = 3.38$ min.

Compound 76: Glutaric acid-Val-Cit-PABAC- α -aminocryptophycin 72



A solution of glutaric anhydride (8 mg, 70 μmol) in anhydrous DMF (200 μl) is added to compound **75** (15 mg, 13.59 μmol) conditioned under argon. The solution obtained is stirred overnight at RT, followed by addition of saturated aqueous NH_4Cl solution and extraction 3 times with DCM. The combined organic phases are dried over Na_2SO_4 , filtered and concentrated under reduced pressure. The residue obtained is taken up in DCM and precipitated with ether. After filtering through a sinter funnel and washing with ether, compound **76** is obtained in the form of a beige-coloured solid (8 mg, 48%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ (ppm): 0.80 to 0.92 (m, 12 H); 0.96 (d, $J = 7.1$ Hz, 3 H); 1.02 (s, 3 H); 1.15 (s, 3 H); 1.30 to 1.72 (m, 9 H); 1.86 (m, 1 H); 2.00 (m, 1 H); 2.11 to 2.26 (m, 4 H); 2.42 (m, 1 H); 2.60 to 2.74 (m, 2 H); 2.91 to 3.11 (m, 5 H); 3.38 (partially masked m, 1 H); 3.76 (d, $J = 1.8$ Hz, 1 H); 3.81 (s, 3 H); 4.17 (m, 3 H); 4.27 (m, 1 H); 4.37 (m, 1 H); 4.90 to 5.04 (m, 3 H); 5.11 (m, 1 H); 5.50 (broad m, 2 H); 5.88 (broad d, $J = 15.2$ Hz, 1 H); 6.20 (broad m, 1 H); 6.48 (m, 1 H); 7.06 (d, $J = 8.3$ Hz, 1 H); 7.11 to 7.32 (m, 9 H); 7.60 (m, 2 H); 7.75 (m, 1 H); 7.89 (m, 1 H); 8.28 (broad m, 1 H); 8.41 (d, $J = 7.8$ Hz, 1 H); 9.92 (broad s, 1 H); 12.06 (broad m, 1 H). LCMS (A2): ES $m/z = 1217$ $[\text{M} + \text{H}]^+$; $m/z = 1215$ $[\text{M} - \text{H}]^-$; $t_R = 1.05$ min.

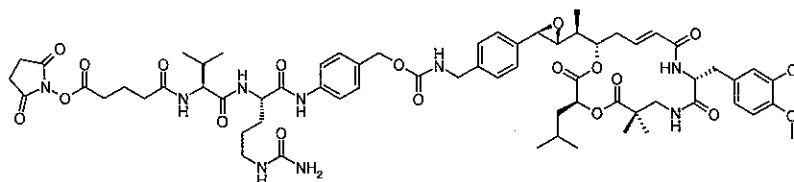
Example 22



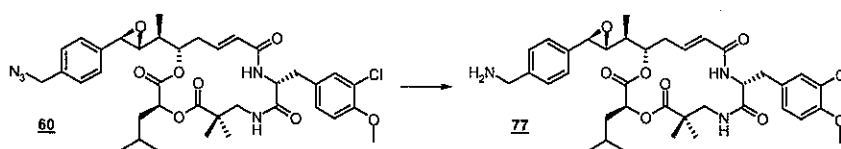
Compound **76** (6 mg, 4.93 μmol) is dissolved in a mixture of DCM (0.5 ml) and DMF (0.1 ml), followed by successive addition of N,N' -disuccinimidyl carbonate (6 mg, 23.42 μmol) and DIPEA (4 μl , 22.96 μmol). After reaction for 3 hours at RT, saturated aqueous NH_4Cl solution is added and the mixture is extracted 3 times with DCM. The combined organic phases are dried over Na_2SO_4 , filtered and concentrated under reduced pressure. The crude product is purified by chromatography on silica gel, eluting with a 100/0 to 90/10 DCM/methanol mixture. Compound **Ex. 22** is obtained in the form of a white solid (4 mg; 61%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ (ppm): 0.80 to 0.90 (m, 12 H); 0.96 (d, $J = 7.3$ Hz, 3 H); 1.03 (s, 3 H); 1.15 (s, 3 H); 1.32 to 1.76 (m, 7 H); 1.80 to 1.89 (m, 3 H); 1.97 (m, 1 H); 2.29 (m, 2 H); 2.44 (m, 1 H); 2.60 to 2.75 (m, 4 H); 2.81 (s, 4 H); 2.89 to 3.08 (m, 5 H); 3.41 (partially masked m, 1 H); 3.77 (d, $J = 2.0$ Hz, 1 H); 3.81 (s, 3 H); 4.14 to 4.24 (m, 3 H); 4.27 (ddd, $J = 3.9$ and 8.3 and 11.5 Hz, 1 H); 4.38 (m, 1 H); 4.93 to 5.03 (m, 3 H); 5.11 (m, 1 H); 5.39 (broad s, 2 H); 5.88 (broad d, $J = 15.2$ Hz, 1 H); 5.96 (t, $J = 5.9$ Hz, 1 H); 6.48 (ddd, $J = 3.7$ and 11.1 and 15.2 Hz, 1 H); 7.05 (d, $J = 8.3$ Hz, 1 H); 7.16 to 7.36 (m, 9 H); 7.59 (d, $J = 7.8$ Hz, 2 H); 7.76 (t, $J = 6.1$ Hz, 1 H); 7.88 (d, $J = 8.8$ Hz, 1 H); 8.10 (d, $J = 7.3$

Hz, 1 H); 8.41 (d, $J = 8.3$ Hz, 1 H); 9.97 (broad s, 1 H). LCMS (A2): ES $m/z = 1314$ $[M + H]^+$; $m/z = 1358$ $[M + HCO_2H - H]^+$; $t_R = 1.06$ min.

Example 23

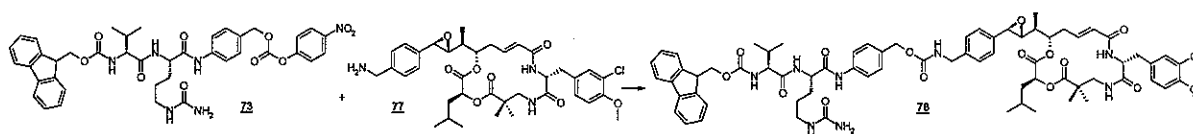


Compound 77: (E)-(3S,10R,16S)-16-[(S)-1-[(2R,3R)-3-(4-Aminomethylphenyl)oxiranyl]ethyl]-10-(3-chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-1,4-dioxo-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetraone



Compound **60** (23 mg, 31.76 μmol) is dissolved in a mixture of methanol (1 ml) and water (0.2 ml), followed by addition of TCEP (34.90 μmol) and DCM (an amount sufficient to dissolve it). The solution obtained is stirred overnight at RT and is then concentrated under reduced pressure. The residue is then taken up in saturated aqueous NaHCO_3 solution and extracted 3 times with DCM. The combined organic phases are dried over Na_2SO_4 , filtered and concentrated under reduced pressure. The crude product is finally purified by chromatography on silica gel, eluting with a 100/0 to 90/10 DCM/methanol mixture. Compound **77** is thus obtained in the form of a white solid (12 mg, 59%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ (ppm): 0.78 (d, $J = 6.4$ Hz, 6 H); 1.00 (s, 3 H); 1.05 (d, $J = 6.9$ Hz, 3 H); 1.12 (s, 3 H); 1.30 (m, 1 H); 1.47 to 1.62 (m, 2 H); 1.79 (m, 1 H); 2.22 to 2.32 (m, 3 H); 2.63 to 2.73 (m, 2 H); 2.92 to 3.05 (m, 3 H); 3.35 (partially masked m, 1 H); 3.71 (s, 2 H); 3.81 (s, 3 H); 3.86 (d, $J = 1.6$ Hz, 1 H); 4.25 (ddd, $J = 3.6$ and 8.0 and 11.3 Hz, 1 H); 4.90 (dd, $J = 3.6$ and 9.6 Hz, 1 H); 5.11 (ddd, $J = 1.5$ and 5.2 and 11.3 Hz, 1 H); 5.79 (dd, $J = 1.5$ and 15.0 Hz, 1 H); 6.47 (ddd, $J = 3.6$ and 11.3 and 15.0 Hz, 1 H); 7.05 (d, $J = 8.5$ Hz, 1 H); 7.17 (dd, $J = 1.9$ and 8.5 Hz, 1 H); 7.19 to 7.26 (m, 3 H); 7.28 (d, $J = 1.9$ Hz, 1 H); 7.34 (d, $J = 8.0$ Hz, 2 H); 8.34 (d, $J = 8.0$ Hz, 1 H). LCMS (A2): ES $m/z = 698$ $[M + H]^+$; $m/z = 696$ $[M - H]^+$; $m/z = 742$ $[M + HCO_2H - H]^+$; $t_R = 0.87$ min.

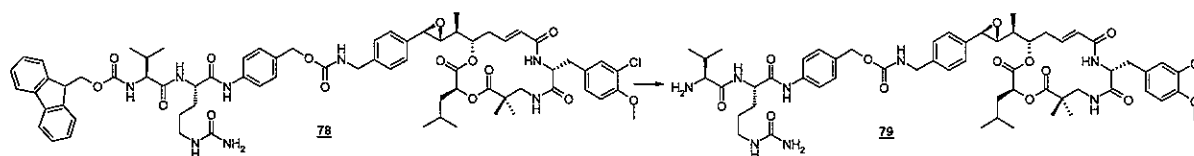
Compound 78: FmocVal-Cit-PABAC- α -aminocryptophycin 77



Compound **77** (10 mg, 14.32 μmol) is dissolved in anhydrous DMF (0.1 ml), followed by addition of a solution of the intermediate **73** (11 mg, 14.35 μmol ; prepared according to **WO**

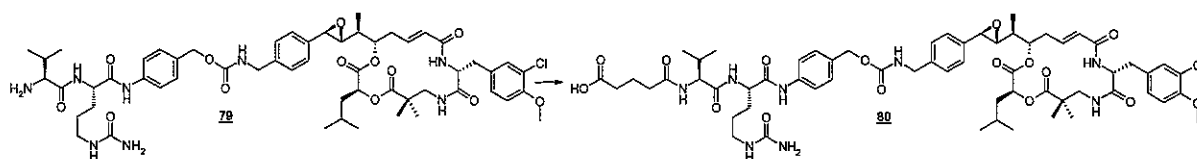
2006/110 476) in a mixture of acetonitrile (1 ml) and DMF (0.1 ml). The mixture is stirred for 3 hours at RT and then concentrated under reduced pressure. The residue is then purified by chromatography on silica gel, eluting with a 100/0 to 90/10 DCM/methanol mixture. Compound **78** is obtained in the form of a white solid (18 mg, 95%). LCMS (A5): ES $m/z = 1325 [M + H]^+$; $m/z = 1369 [M + HCO_2H - H]^-$; $t_R = 4.32$ min.

Compound 79: Val-Cit-PABAC- α -aminocryptophycin **77**

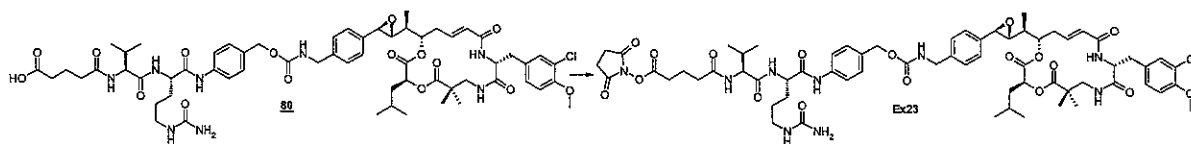


Compound **78** (42 mg, 31.68 μ mol) is dissolved in DMF (5 ml), followed by addition of piperidine (150 μ l, 1.51 mmol) and the mixture is stirred for 30 minutes at RT. The mixture is concentrated under reduced pressure and the residue is dissolved in a minimum amount of methanol and precipitated from ether. Compound **79** is thus obtained in the form of a white solid (21 mg, 60%). LCMS (A4): ES $m/z = 1103 [M + H]^+$; $m/z = 1101 [M - H]^-$; $m/z = 1147 [M + HCO_2H - H]^-$; $t_R = 3.67$ min.

Compound 80: Glutaric acid-Val-Cit-PABAC- α -aminocryptophycin **77**

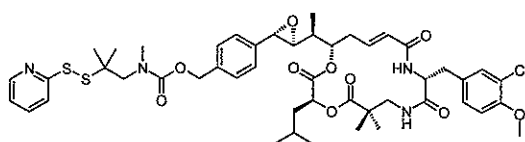


Compound **79** (20 mg, 18.12 μ mol) is dissolved in a mixture of DMF (100 μ l) and anhydrous DCM (500 μ l), followed by addition of glutaric anhydride (4 mg, 35.06 μ mol). The solution obtained is stirred overnight at RT and then concentrated under reduced pressure. The residue is taken up in a minimum amount of DCM and methanol, precipitated from a mixture of ether and pentane, and filtered on a sinter funnel. Compound **80** is thus obtained in the form of a white solid (23 mg, 104% crude), which is used in crude form in the following step. 1H NMR (500 MHz, DMSO- d_6): 0.78 (d, $J = 6.3$ Hz, 6 H); 0.84 (d, $J = 6.6$ Hz, 3 H); 0.86 (d, $J = 6.6$ Hz, 3 H); 1.00 (s, 3 H); 1.04 (d, $J = 6.9$ Hz, 3 H); 1.12 (s, 3 H); 1.22 to 1.47 (m, 3 H); 1.51 to 1.64 (m, 3 H); 1.72 (m, 3 H); 1.80 (m, 1 H); 1.99 (m, 1 H); 2.20 (m, 4 H); 2.28 (m, 1 H); 2.59 to 2.73 (m, 2 H); 2.90 to 3.07 (m, 5 H); 3.32 (partially masked m, 1 H); 3.81 (s, 3 H); 3.87 (broad s, 1 H); 4.19 (m, 3 H); 4.24 (m, 1 H); 4.38 (m, 1 H); 4.91 (dd, $J = 3.3$ and 9.6 Hz, 1 H); 4.97 (broad s, 2 H); 5.10 (dd, $J = 4.4$ and 10.7 Hz, 1 H); 5.40 (broad s, 2 H); 5.79 (broad d, $J = 15.1$ Hz, 1 H); 5.99 (broad m, 1 H); 6.47 (ddd, $J = 3.6$ and 11.3 and 15.1 Hz, 1 H); 7.05 (d, $J = 8.5$ Hz, 1 H); 7.17 (broad d, $J = 8.5$ Hz, 1 H); 7.20 to 7.34 (m, 8 H); 7.60 (broad d, $J = 8.2$ Hz, 2 H); 7.76 (broad t, $J = 5.4$ Hz, 1 H); 7.83 (d, $J = 8.5$ Hz, 1 H); 8.09 (broad d, $J = 7.1$ Hz, 1 H); 8.34 (d, $J = 8.0$ Hz, 1 H); 9.95 (broad s, 1 H); 11.98 (broad m, 1 H). LCMS (A2): ES $m/z = 1217 [M + H]^+$; $m/z = 1215 [M - H]^-$; $t_R = 1.03$ min.

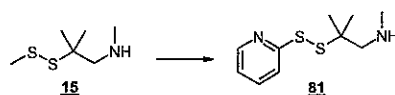
Example 23

Example 23 may be obtained by activating the acid 80 according to the method described for Example 22.

Example 24: 4-((2S,3S)-3-((S)-1-[(E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl)oxiranyl)benzyl [2-methyl-2-(pyridin-2-yl)disulfanyl]propyl]methylcarbamate

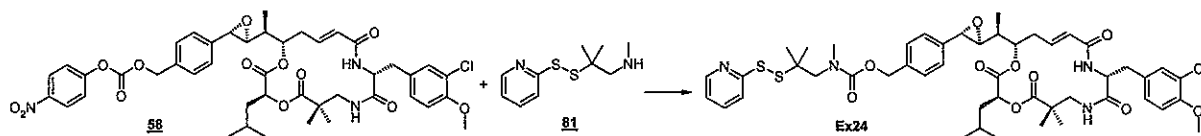


Compound 81: Methyl[2-methyl-2-(pyridin-2-yl)disulfanyl]propyl]amine



To a solution of the amine 15 (478 mg, 2.90 mmol) in MeOH (35 ml) is added TCEP (831 mg, 2.9 mmol). After reaction for 2 hours at RT, the solution obtained is added dropwise, under an argon atmosphere, to a solution of 2,2'-dipyridyl disulfide (960 mg, 4.36 mmol) in ethanol (70 ml). After reaction for a further 2 hours at RT, the mixture is concentrated under reduced pressure. The residue is taken up in DCM and washed with saturated aqueous NaHCO₃ solution. The organic phase is separated out and the aqueous phase is then extracted twice more with DCM. The combined organic phases are dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude product is purified by chromatography on silica gel, eluting with a 98/2 to 85/15 DCM/methanol mixture. Compound 81 is obtained in the form of a pale yellow oil (587 mg, 89%). ¹H NMR (500 MHz, DMSO-*d*₆): 1.25 (s, 6 H); 1.80 (broad m, 1 H); 2.19 (s, 3 H); 2.45 (s, 2 H); 7.22 (ddd, J = 1.5 and 5.0 and 7.0 Hz, 1 H); 7.74 to 7.85 (m, 2 H); 8.42 (ddd, J = 1.1 and 1.5 and 5.0 Hz, 1 H).

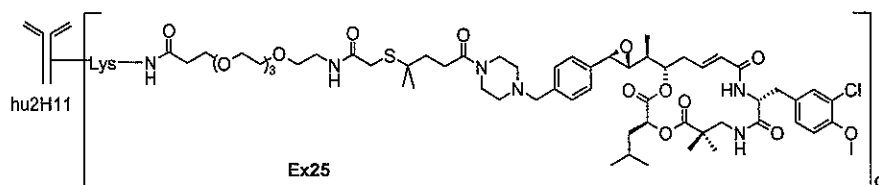
Example 24: 4-((2S,3S)-3-((S)-1-[(E)-(3S,10R,16S)-10-(3-Chloro-4-methoxybenzyl)-3-isobutyl-6,6-dimethyl-2,5,9,12-tetraoxo-1,4-dioxo-8,11-diazacyclohexadec-13-en-16-yl]ethyl)oxiranyl)benzyl [2-methyl-2-(pyridin-2-yl)disulfanyl]propyl]methylcarbamate



To a solution of the intermediate 58 (21 mg, 24.3 μmol) in anhydrous DCM are successively

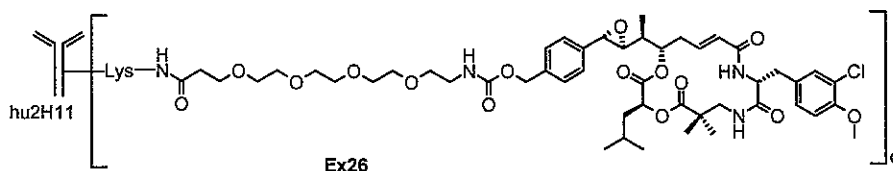
added the amine **81** (10 mg, 43.79 μ mol) and DIPEA (8 μ l, 45.43 μ mol). After 24 hours at RT, saturated aqueous NaHCO_3 solution is added and the mixture is extracted 3 times with DCM. The combined organic phases are dried over Na_2SO_4 , filtered and concentrated under reduced pressure. The crude product is purified by chromatography on silica gel, eluting with a 100/0 to 95/5 DCM/methanol mixture. Compound **Ex. 24** is thus obtained in the form of a colourless solid (7 mg, 30%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$): 0.82 (d, $J = 6.0$ Hz, 3 H); 0.84 (d, $J = 6.0$ Hz, 3 H); 0.97 (d, $J = 6.8$ Hz, 3 H); 1.03 (s, 3 H); 1.14 (s, 3 H); 1.25 (broad s, 6 H); 1.45 to 1.68 (m, 3 H); 1.88 (m, 1 H); 2.42 (partially masked m, 1 H); 2.58 to 2.75 (m, 2 H); 2.83 to 3.09 (m, 6 H); 3.34 (partially masked m, 1 H); 3.46 (broad s, 2 H); 3.81 (broad s, 4 H); 4.28 (m, 1 H); 4.91 to 5.17 (m, 4 H); 5.88 (broad d, $J = 15.2$ Hz, 1 H); 6.49 (m, 1 H); 7.05 (d, $J = 8.6$ Hz, 1 H); 7.15 to 7.38 (m, 8 H); 7.80 (broad m, 2 H); 8.39 (d, $J = 7.8$ Hz, 1 H); 8.43 (broad d, $J = 4.6$ Hz, 1 H). LCMS (A2): ES $m/z = 953$ $[\text{M} + \text{H}]^+$; $m/z = 477$ $[\text{M} + 2\text{H}]^{2+}$; $m/z = 997$ $[\text{M} + \text{HCO}_2\text{H} - \text{H}]^-$; $t_R = 1.23$ min.

Example 25: hu2H11-Ex17



According to the general method, 8.31 mg (0.057 μ mol, 1.468 ml) of naked antibody hu2H11 at a concentration of 5.66 mg/ml are treated with 5 eq. of compound **Ex. 17** (0.442 mg, 0.340 μ mol) dissolved in 37.3 μ l of DMA, such that the final antibody concentration is 3 mg/ml in the mixture. After purifying on Superdex 200 pg and concentrating on Amicon Ultra-15 (Ultracel 50k membrane, Millipore), 2.15 ml of conjugate are obtained at a concentration of 2.48 mg/ml with, on average, 2.2 cytotoxic units per antibody and a monomer purity of 99.7% in an aqueous pH 6.5 buffer containing 0.01 M of phosphate, 0.14 M of NaCl and 20% by volume of NMP. The final buffer change is performed in an aqueous pH 6.5 buffer containing 0.01 M of phosphate and 0.14 M of NaCl on 1 ml of conjugate. 1.5 ml of a solution of conjugate **Ex. 25** at a concentration of 1.06 mg/ml with, on average, 2.8 cryptophycin derivatives (determined by UV) per antibody and a monomer purity of 99.7% are thus obtained.

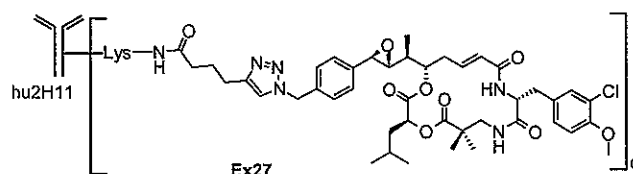
Example 26: hu2H11-Ex18



According to the general method, 7.8 mg (0.053 μ mol, 1.458 ml) of naked antibody hu2H11 at a concentration of 5.35 mg/ml are treated with 6 eq. of compound **Ex. 18** (0.578 mg, 0.531 μ mol, purity 60%) dissolved in 272 μ l of DMA, such that the final antibody concentration is 3 mg/ml in

the mixture. After purifying on Superdex 200 pg and concentrating on Amicon Ultra-15 (Ultracel 50k membrane, Millipore), 2.35 ml of conjugate are obtained at a concentration of 2.49 mg/ml with, on average, 2.5 cryptophycin derivatives per antibody and a monomer purity of 100% in an aqueous pH 6.5 buffer containing 0.01 M of phosphate, 0.14 M of NaCl and 20% by volume of NMP. The final buffer change is performed in an aqueous pH 6.5 buffer containing 0.01 M of phosphate and 0.14 M of sodium chloride on 1 ml of conjugate. 1.5 ml of a solution of conjugate **Ex. 26** at a concentration of 0.84 mg/ml with, on average, 2.22 cryptophycin derivatives (determined by UV) per antibody and a monomer purity of 99.9% are thus obtained.

Example 27: hu2H11-Ex19



According to the general method, 12 mg (81.7 μ mol, 1.172 ml) of naked antibody hu2H11 at a concentration of 10.24 mg/ml are treated with 2 $\times$ 5 eq. of compound **Ex. 19** (0.382 mg, 0.41 μ mol) dissolved in 44.3 μ l of DMA, such that the final antibody concentration is 3 mg/ml in the reaction medium. After purifying on Superdex 200 pg in the presence of 20% NMP and concentrating on Amicon Ultra-15 (Ultracel 50k membrane, Millipore), 1.04 ml of conjugates are obtained in an aqueous pH 6.5 buffer containing 0.01 M of phosphate, 0.14 M of NaCl and 20% by volume of NMP. The final buffer change is performed in an aqueous pH 6.5 buffer containing 0.01 M of histidine, 10% of sucrose (w/v) and 5% of NMP (v/v). 1.5 ml of a solution of conjugate **Ex. 27** at a concentration of 2.66 mg/ml with, on average, 1.4 cryptophycin derivatives (HRMS) per antibody and a monomer purity of 99.8% are thus obtained.

The examples given above were prepared with a particular cryptophycin derivative (often the derivative **D₁**), but may be applied to another derivative of the group **D₁-D₈** or to the cryptophycin derivative of general formula (II). Similarly, the examples of conjugates in Examples 10, 11, 12, 25, 26 and 27 may be applied to antibodies other than hu2H11.

Note: in said examples, -Lys- means that the attachment takes place on the ϵ -amino groups of the lysines of the antibody.

Example 28: Evaluation of the inhibition of proliferation of MDA-MB-231, MDA-A1 and HCT116 cell lines by the cytotoxic agents, study performed on the compounds of formula (II) of the type SZ_a with $Z_a = SMe$ or of the type $-C(=O)-Z_bR_b$ with $Z_bR_b = OMe$ or $OCH_2-CH=CH_2$

The MDA-MB-231, MDA-A1 or HCT116 cells in their exponential growth phase are trypsinized and resuspended in their respective culture medium (DMEM/F12 Gibco #21331, 10% FCS Gibco #10500-056, 2 mM glutamine Gibco #25030 for the MDA cells; DMEM Gibco #11960, 10% FCS Gibco #10500-056, 2 mM glutamine Gibco #25030 for the HCT116 cells). The cell suspension is

seeded in Cytostar 96-well culture plates (GE Healthcare Europe, #RPNQ0163) in the whole culture medium containing serum at a density of 5000 cells/well (MDA-MB-231, MDA-A1, HCT116). After incubation for 4 hours, successive dilutions of the cryptophycin derivatives are added to the wells at concentrations decreasing from 10^{-7} to 10^{-12} M (in triplicate for each concentration). The cells are cultured for 3 days at 37°C under an atmosphere containing 5% CO₂ in the presence of the cytotoxic agents. On the 4th day, 10 µl of a ¹⁴C-thymidine solution (0.1 µCi/well, Perkin Elmer #NEC56825000) are added to each well. The incorporation of ¹⁴C-thymidine is measured 96 hours after the start of the experiment using a microbeta radioactivity counter (Perkin Elmer). The data are expressed in the form of a percentage of survival by determining the ratio between the reduced count obtained with the cells treated with cytotoxic agent and the count obtained with the cells of the control wells (treated with the culture medium alone).

Table III	IC ₅₀ (nM)		
	HCT116	MDA-MB-231	MDA-A1
Compound 1	0.032	0.041	1.07
Compound 7	0.315	0.303	7.946
Compound 8	0.052	0.104	0.828
Compound 13	0.336	0.529	9.763
Compound 19	0.152	0.149	2.705
Compound 30	0.031	0.03	11.95
Compound 40	0.12	0.293	1.579
Compound 44	0.135	0.156	15.552
Compound 57	1.131	1.185	29.01
Compound 62	0.035	0.095	10.88
Compound 77	0.105	0.170	15.06

Example 29: Evaluation of the inhibition of proliferation of the MDA-MB-231, MDA-A1 and HCT116 cell lines by the antibody-cytotoxic agent conjugates

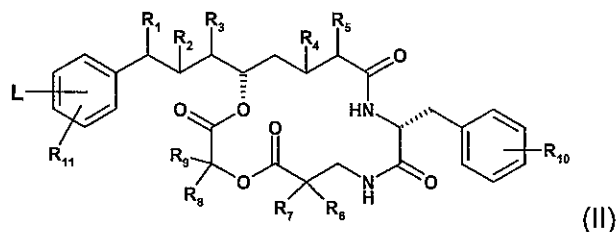
MDA-MB-231, MDA-A1 or HCT116 cells in their exponential growth phase are trypsinized and resuspended in their respective culture medium (DMEM/F12 Gibco #21331, 10% FCS Gibco #10500-056, 2 nM glutamine Gibco #25030 for the MDA cells; DMEM Gibco #11960, 10% FCS Gibco #10500-056, 2 mM glutamine Gibco #25030 for the HCT116 cells). The cell suspension is seeded in Cytostar 96-well culture plates (GE Healthcare Europe, #RPNQ0163) in the whole

culture medium containing serum at a density of 5000 cells/well (MDA-MB-231, MDA-A1, HCT116). After incubation for 4 hours, successive dilutions of the cryptophycin derivatives are added to the wells at concentrations decreasing from 10^{-7} to 10^{-12} M (in triplicate for each concentration). The cells are cultured at 37°C in an atmosphere containing 5% CO₂ in the presence of the antibody-cytotoxic agent immunoconjugates for 3 days. On the 4th day, 10 µl of a ¹⁴C-thymidine solution (0.1 µCi/well, Perkin Elmer #NEC56825000) are added to each well. The incorporation of ¹⁴C-thymidine is measured 96 hours after the start of the experiment with a microbeta radioactivity counter (Perkin Elmer). The data are expressed in the form of a percentage of survival by determining the ratio between the reduced count obtained with the cells treated with the immunoconjugate and the count obtained with the cells of the control wells (treated with the culture medium alone). In certain experiments, the naked antibody hu2H11 was added to the wells at a concentration of 1 µM at the start of the experiment and the inhibition of proliferation was measured as described previously.

Table IV	IC ₅₀ (nM), MDA-MB-231	
	Conjugate alone	in the presence of naked antibody
Ex.9	0.150	0.856
Ex.25	0.710	4.918

CLAIMS

1. Cryptophycin derivative of formula (II):



in which:

- R_1 represents a halogen atom and R_2 represents a group $-OH$, an acyl group derived from an amino acid **AA** or a group (C_1-C_4) alkanoyloxy; or alternatively R_1 and R_2 form an epoxide unit;

- **AA** denotes a natural or unnatural amino acid;

- R_3 represents a group (C_1-C_6) alkyl;

- R_4 and R_5 both represent H or together form a double bond $CH=CH$ between C13 and C14;

- R_6 and R_7 represent, independently of each other, H or a group (C_1-C_6) alkyl;

- R_8 and R_9 represent, independently of each other, H or a group (C_1-C_6) alkyl;

- R_{10} represents at least one substituent of the phenyl nucleus chosen from: H, a group OH, (C_1-C_4) alkoxy, a halogen atom or a group $-NH_2$, $-NH(C_1-C_6)$ alkyl or $-N(C_1-C_6)$ alkyl₂;

- R_{11} represents at least one substituent of the phenyl nucleus chosen from H and a group (C_1-C_4) alkyl;

- **L** represents a linker in the ortho (o), meta (m) or para (p) position, preferably in the para position, of the phenyl nucleus bearing the unit RCG_1 chosen from:

$-G'X(CR_{13}R_{14})_t(OCH_2CH_2)_y(CR_{15}R_{16})_uQRCG_1$;

$-G'X(CR_{13}R_{14})_t(OCH_2CH_2)_yY'(CR_{15}R_{16})_uQRCG_1$;

$-G'X(CR_{13}R_{14})_t(CR_{17}=CR_{18})(CR_{15}R_{16})_u(OCH_2CH_2)_yQRCG_1$;

$-G'X(CR_{13}R_{14})_t(OCH_2CH_2)_y(CR_{17}=CR_{18})(CR_{15}R_{16})_uQRCG_1$;

$-G'X(CR_{13}R_{14})_t$ -phenyl- $(CR_{15}R_{16})_uY'QRCG_1$; $-G'X(CR_{13}R_{14})_t$ -furyl- $(CR_{15}R_{16})_uY'QRCG_1$;

$-G'X(CR_{13}R_{14})_t$ -oxazolyl- $(CR_{15}R_{16})_uY'QRCG_1$; $-G'X(CR_{13}R_{14})_t$ -thiazolyl- $(CR_{15}R_{16})_uY'QRCG_1$;

$-G'X(CR_{13}R_{14})_t$ -thienyl- $(CR_{15}R_{16})_uY'QRCG_1$; $-G'X(CR_{13}R_{14})_t$ -imidazolyl- $(CR_{15}R_{16})_uY'QRCG_1$;

$-G'X(CR_{13}R_{14})_t$ -piperazinyl-CO $(CR_{15}R_{16})_uY'QRCG_1$; $-G'X(CR_{13}R_{14})_t$ -piperidyl-methyl-NR₁₂-CO $(CR_{15}R_{16})_uY'QRCG_1$;

$-G'X(CR_{13}R_{14})_t$ -piperidyl- $(CR_{15}R_{16})_uY'QRCG_1$; $-G'X(CR_{13}R_{14})_t$ -piperidyl-NR₁₂- $(CR_{15}R_{16})_uY'QRCG_1$;

$-G'X(CR_{13}R_{14})_t$ -triazolyl- $(CR_{15}R_{16})_uY'QRCG_1$; $-G'X(CR_{13}R_{14})_t$ -triazolyl- $(CR_{15}R_{16})_uY'QRCG_1$;

$-G'X(CR_{13}R_{14})_t$ -triazolyl- $(CR_{15}R_{16})_uY'QRCG_1$;

-G' X (CR₁₃R₁₄)_t-phenyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-furyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-oxazolyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-thiazolyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-thienyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-imidazolyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-piperazinyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-piperidyl-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-piperidyl-methyl-NR₁₂-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-piperidyl-NR₁₂-(CR₁₅R₁₆)_u Q RCG₁; -G' X (CR₁₃R₁₄)_t-triazolyl-(CR₁₅R₁₆)_u Q RCG₁;

or

-G'' Y (CR₁₃R₁₄)_t(OCH₂CH₂)_y(CR₁₅R₁₆)_u Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t(OCH₂CH₂)_y-Y'-(CR₁₅R₁₆)_u Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t(CR₁₇=CR₁₈)(CR₁₅R₁₆)_u(OCH₂CH₂)_y Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t(OCH₂CH₂)_y(CR₁₇=CR₁₈)(CR₁₅R₁₆)_u Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t-phenyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-furyl-(CR₁₅R₁₆)_u Y' Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t-oxazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-

thiazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-thienyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-imidazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t-piperazinyl-CO(CR₁₅R₁₆)_u Y' Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-piperidyl-methyl-NR₁₂-CO(CR₁₅R₁₆)_u Y' Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t-piperidyl-(CR₁₅R₁₆)_u Y' Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-piperidyl-NR₁₂-(CR₁₅R₁₆)_u Y' Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t-triazolyl-(CR₁₅R₁₆)_u Y' Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t-phenyl-(CR₁₅R₁₆)_u Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-furyl-(CR₁₅R₁₆)_u Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t-oxazolyl-(CR₁₅R₁₆)_u Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-thiazolyl-(CR₁₅R₁₆)_u Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t-thienyl-(CR₁₅R₁₆)_u Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-imidazolyl-(CR₁₅R₁₆)_u Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t-piperazinyl-(CR₁₅R₁₆)_u Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-piperazinyl-(CR₁₅R₁₆)_u Q CCR₁;

G'' Y (CR₁₃R₁₄)_t-piperidyl-(CR₁₅R₁₆)_u Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-piperidyl-methyl-NR₁₂-(CR₁₅R₁₆)_u Q RCG₁;

-G'' Y (CR₁₃R₁₄)_t-piperidyl-NR₁₂-(CR₁₅R₁₆)_u Q RCG₁; -G'' Y (CR₁₃R₁₄)_t-triazolyl-(CR₁₅R₁₆)_u Q RCG₁;

in which formulae:

G' represents a group -CH=CH- or -(CH₂)_n;

G'' represents a group -(CH₂)_n;

n represents an integer ranging from 1 to 6;

X represents a single bond or a group -CO-, -COO- or -CONR₁₂-, the group CO being attached to G';

Y represents a group -O-, -OCO-, -OCOO-, -OCONR₁₂-, -NR₁₂-, -NR₁₂CO-, -NR₁₂CONR'₁₂-, -NR₁₂COO- or -S(O)_q-, the atom O and the group NR₁₂ being attached to G'';

q represents an integer that may be 0, 1 or 2;

Y' represents a group -O-, -OCO-, -OCOO-, -OCONR₁₂-, -NR₁₂-, -NR₁₂CO-, -NR₁₂CONR'₁₂-, -NR₁₂COO-, -S(O)_q-, -CO-, -COO-, or -CONR₁₂;

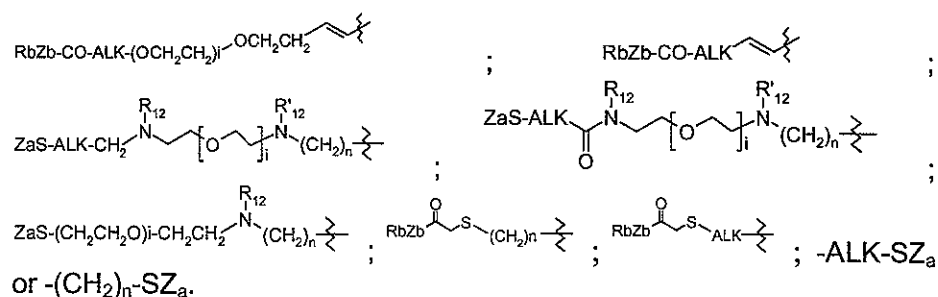
Y' represents a group -O-, -OCO-, -OCOO-, -OCONR₁₂-, -NR₁₂-, -NR₁₂CO-, -NR₁₂CONR'₁₂-, -NR₁₂COO-, -S(O)_q-, -CO-, -COO-, or -CONR₁₂;

Y' represents a group -O-, -OCO-, -OCOO-, -OCONR₁₂-, -NR₁₂-, -NR₁₂CO-, -NR₁₂CONR'₁₂-, -NR₁₂COO-, -S(O)_q-, -CO-, -COO-, or -CONR₁₂;

q represents an integer that may be 0, 1 or 2;

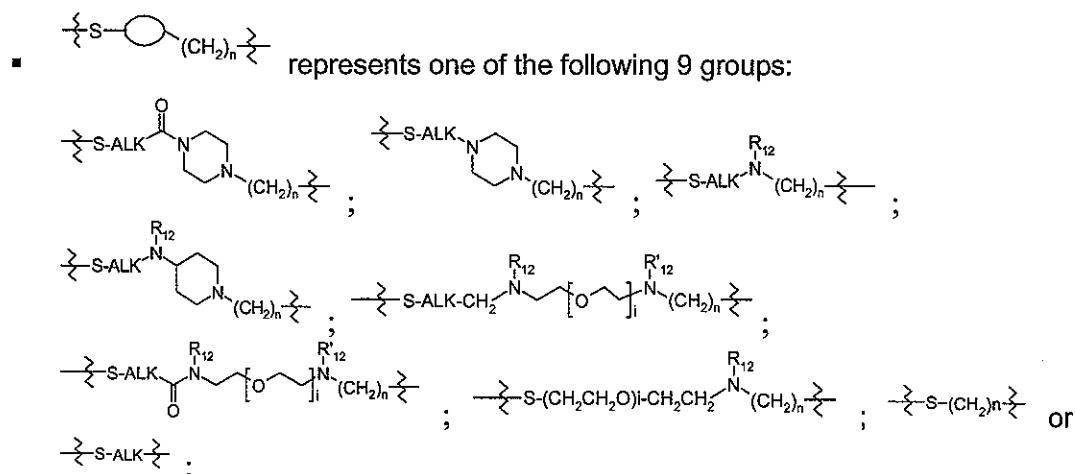
Y' represents a group -O-, -OCO-, -OCOO-, -OCONR₁₂-, -NR₁₂-, -NR₁₂CO-, -NR₁₂CONR'₁₂-, -NR₁₂COO-, -S(O)_q-, -CO-, -COO-, or -CONR₁₂;

-NR₁₂COO-, -S(O)_q-, -CO-, -COO-, or -CONR₁₂;



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in which formulae:



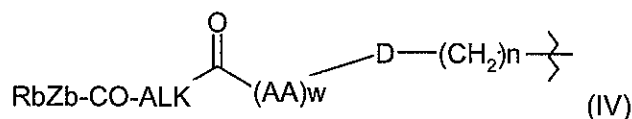
- n represents an integer ranging from 1 to 6;

- **ALK** represents a group (C₁-C₁₂)alkylene;

▪ **R₁₂** and **R'₁₂** represent, independently of each other, H or a group (C₁-C₆)alkyl, more particularly a methyl group;

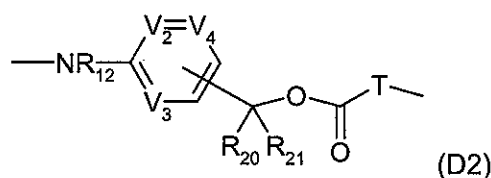
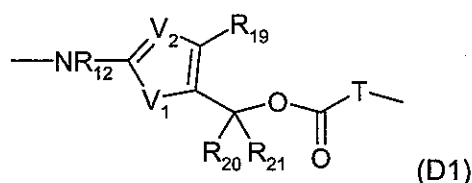
- *i* represents an integer ranging from 1 to 20, more particularly from 1 to 10, even more particularly from 1 to 8, or from 1 to 6, and even more particularly from 2 to 5;

or alternatively L is a linker of formula (IV):

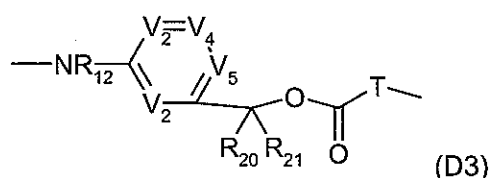


in which:

- **(AA)_w** represents a sequence of **w** amino acids **AA** connected together via peptide bonds;
- **w** represents an integer ranging from 1 to 12 and preferably from 1 to 6;
- **n** represents an integer ranging from 1 to 6;
- **D** represents one of the following units:



(D2)



for which:

R_{12} represents H or a group (C_1-C_6) alkyl;

R_{19} , R_{20} , R_{21} and R_{22} represent, independently of each other, H, a halogen atom, -OH, -CN or a group (C_1-C_4) alkyl;

T attached to $(CH_2)_n$ represents NR_{12} or O;

V_1 represents O, S or NR_{12} ;

V_2 represents CR_{22} or N;

V_3 , V_4 and V_5 are chosen, independently of each other, from CR_{22} and N;

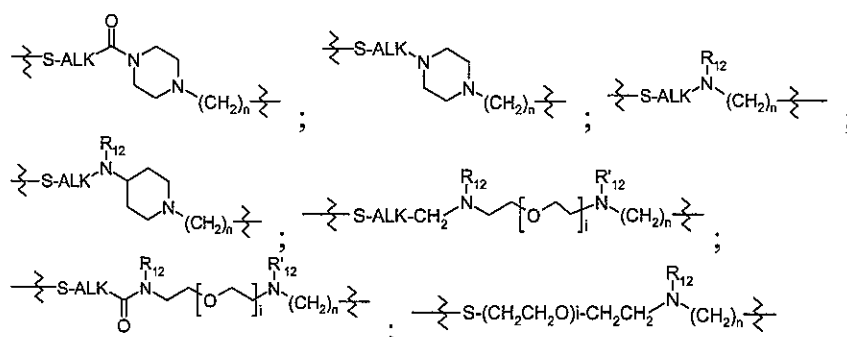
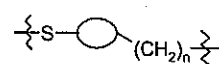
▪ Z_a represents H or the group $-SR_a$, R_a represents a group (C_1-C_6) alkyl, (C_3-C_7) cycloalkyl, aryl, heteroaryl or (C_3-C_7) heterocycloalkyl;

▪ Z_b represents a single bond, -O- or -NH-, R_b representing H or a group (C_1-C_6) alkyl, (C_3-C_7) cycloalkyl, aryl, heteroaryl or (C_3-C_7) heterocycloalkyl.

2. Cryptophycin derivative according to Claim 1, in which R_{10} represents at least one substituent on the phenyl nucleus chosen from: H, a group OH, (C_1-C_4) alkoxy, a halogen atom.

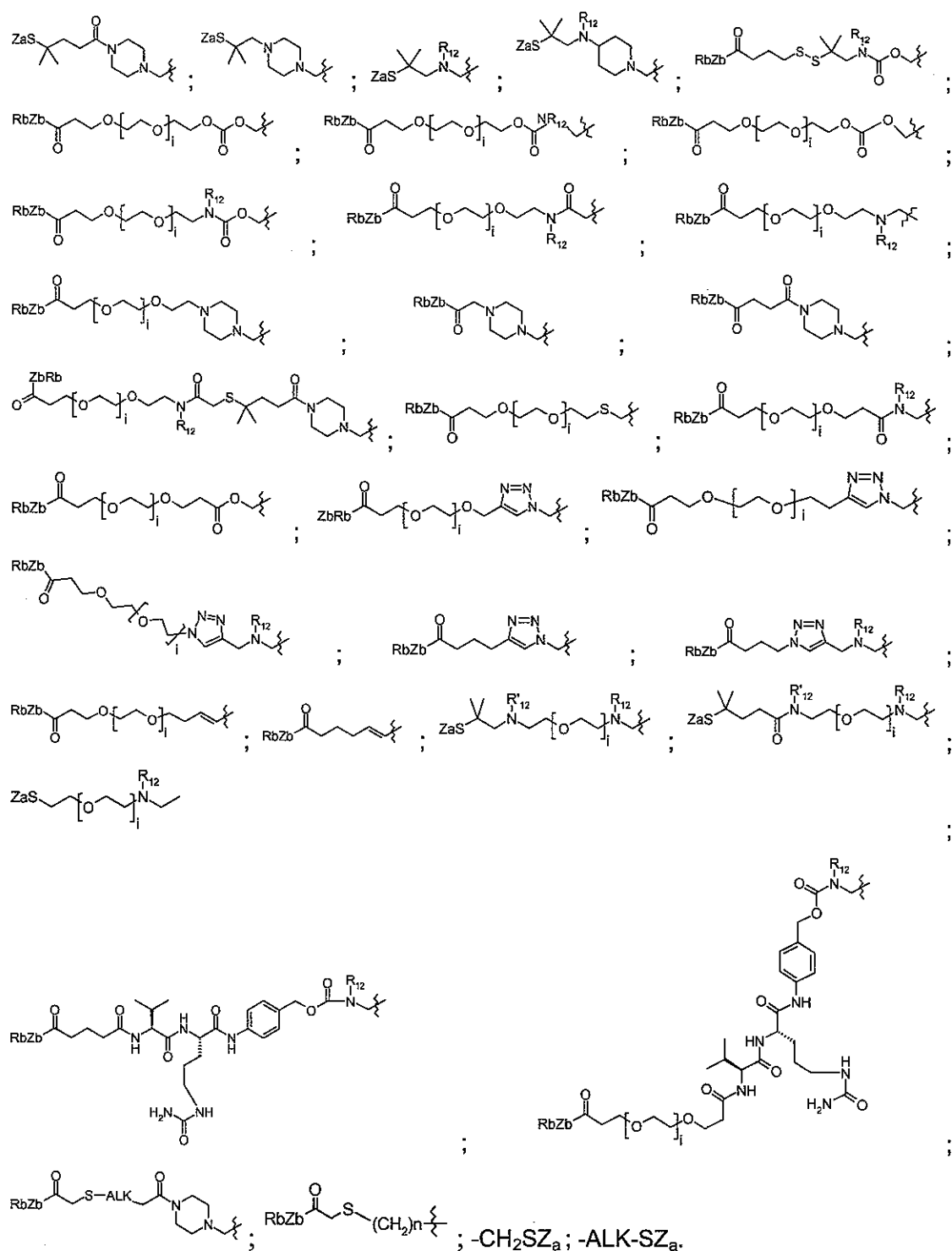
3. Cryptophycin derivative according to Claim 1 or 2, in which RCG1 represents $-SZ_a$ or $-C(=O)-Z_bR_b$.

4. Cryptophycin derivative according to Claims 1 to 3, in which represents one of the following 7 groups:



5. Cryptophycin derivative according to Claims 1 to 4, in which n is 1.

6. Cryptophycin derivative according to Claim 1, in which L is chosen from:



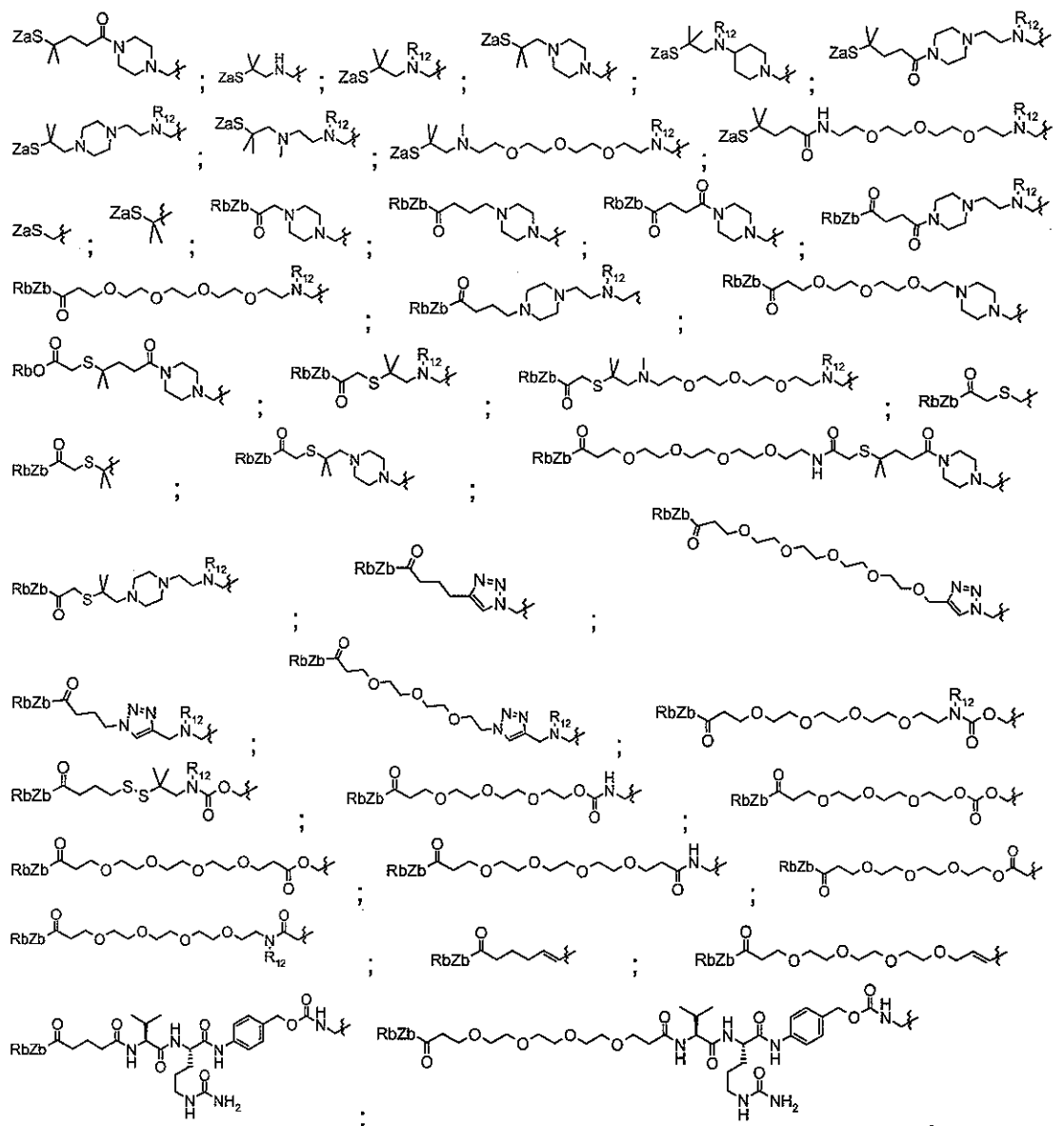
- **R₁₂** and **R'₁₂** represent, independently of each other, H or a group (C₁-C₆)alkyl, more particularly a methyl group;
- **i** represents an integer ranging from 1 to 20, more particularly from 1 to 10, even more particularly from 1 to 8, or from 1 to 6, and even more particularly from 2 to 5;
- **Z_a** represents H or a group -SR_a, **R_a** representing a group (C₁-C₆)alkyl, (C₃-C₇)cycloalkyl, aryl, heteroaryl or (C₃-C₇)heterocycloalkyl;
- **Z_b** represents a single bond, -O- or -NH-, **R_b** representing H or a group (C₁-C₆)alkyl, (C₃-C₇)cycloalkyl, aryl, heteroaryl or (C₃-C₇)heterocycloalkyl.

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7. Cryptophycin derivative according to Claim 1, in which L is chosen from:

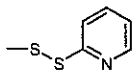


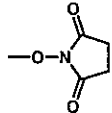
R_{12} representing H or a group (C_1 - C_6)alkyl, more particularly a methyl group.

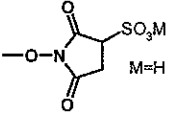
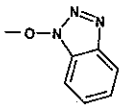
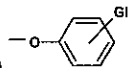
8. Cryptophycin derivative according to Claim 7, in which R_{12} represents H.

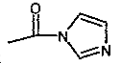
9. Cryptophycin derivative according to Claim 7, in which R_{12} represents (C_1 - C_6)alkyl, more particularly a methyl group.

10. Cryptophycin derivative according to Claims 1 to 9, in which Z_a represents H or

—S(C₁–C₆)alkyl, especially —SMe, ou —S-heteroaryl, especially  or Z_bR_b

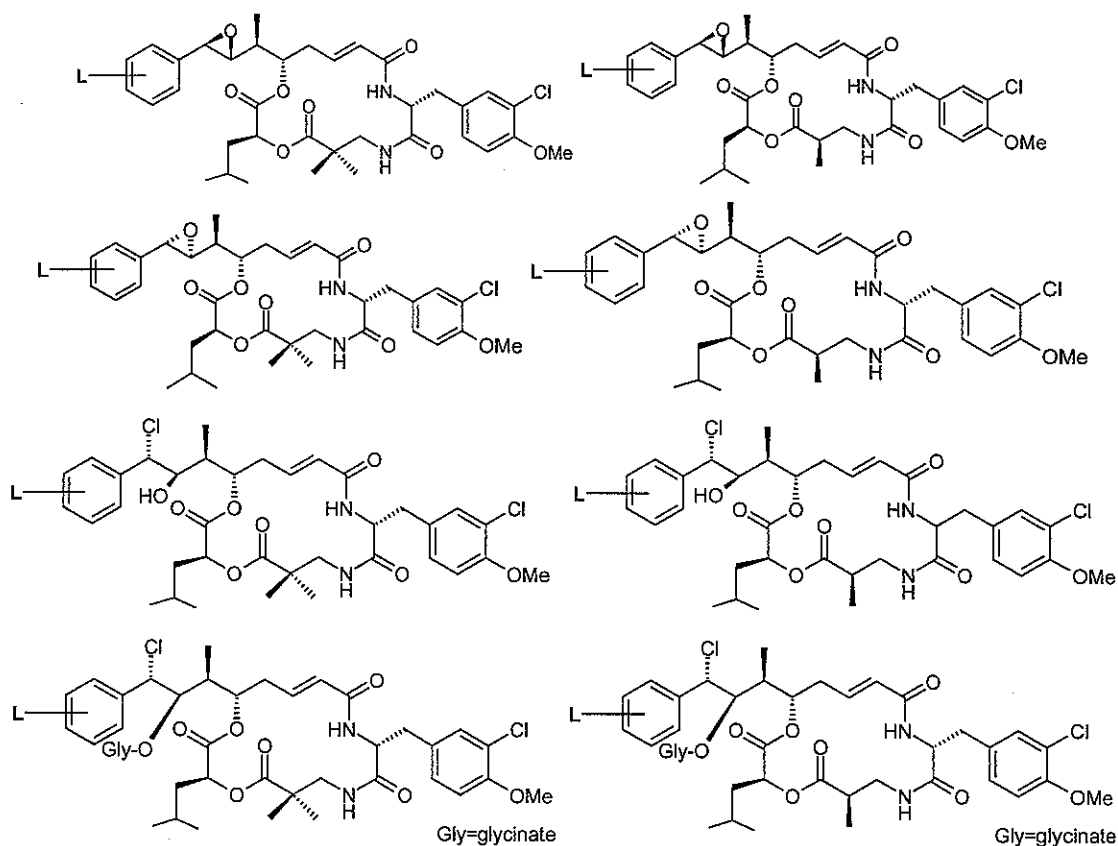
represents —O(C₁–C₆)alkyl, —OH, —OCH₃, —OCH₂CH=CH₂,  or

 M=H or cation,  or the group  in which **Gl** represents at least one

electroinductive group, or alternatively in which —C(=O)Z_bR_b represents .

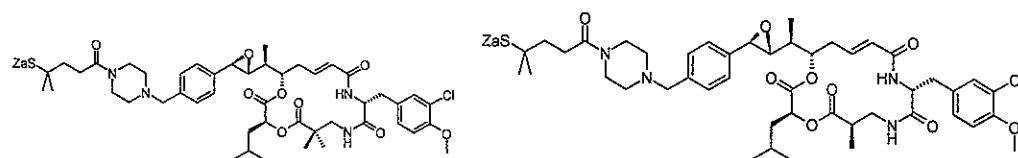
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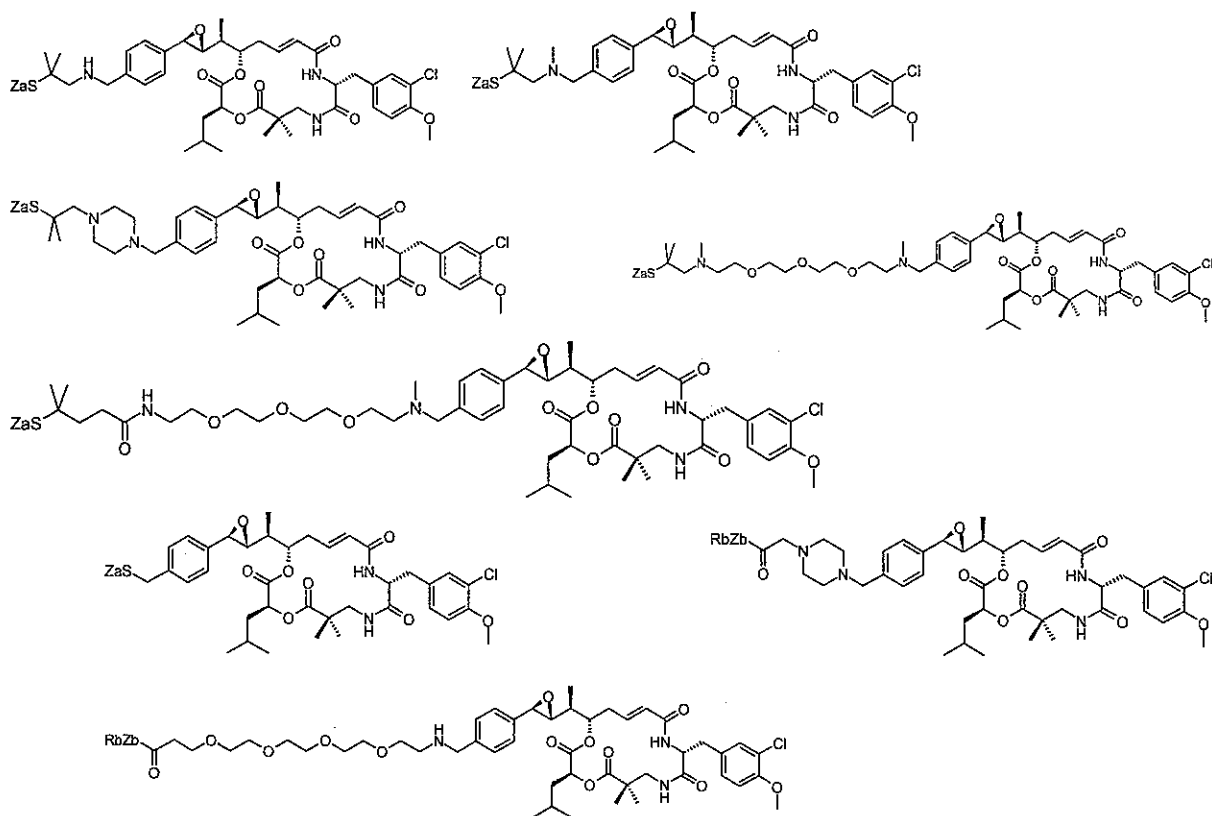
11. Cryptophycin derivative according to one of Claims 1 to 10 defined according to one of the following formulae, **L** preferably being in the para position:



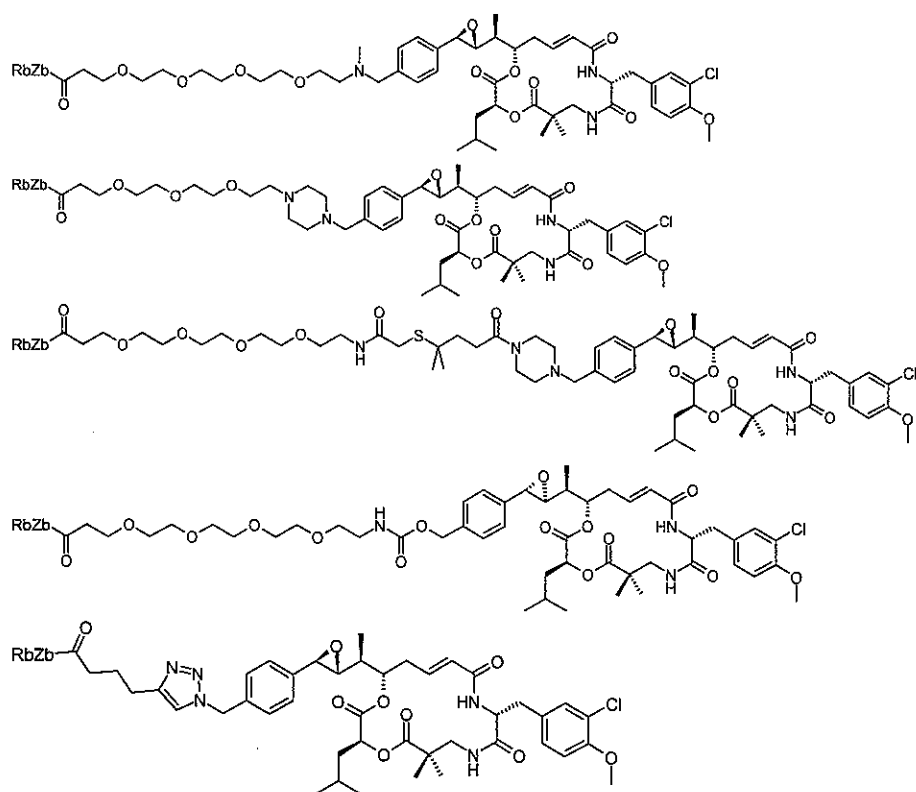
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12. Cryptophycin derivative according to Claim 1, chosen from:

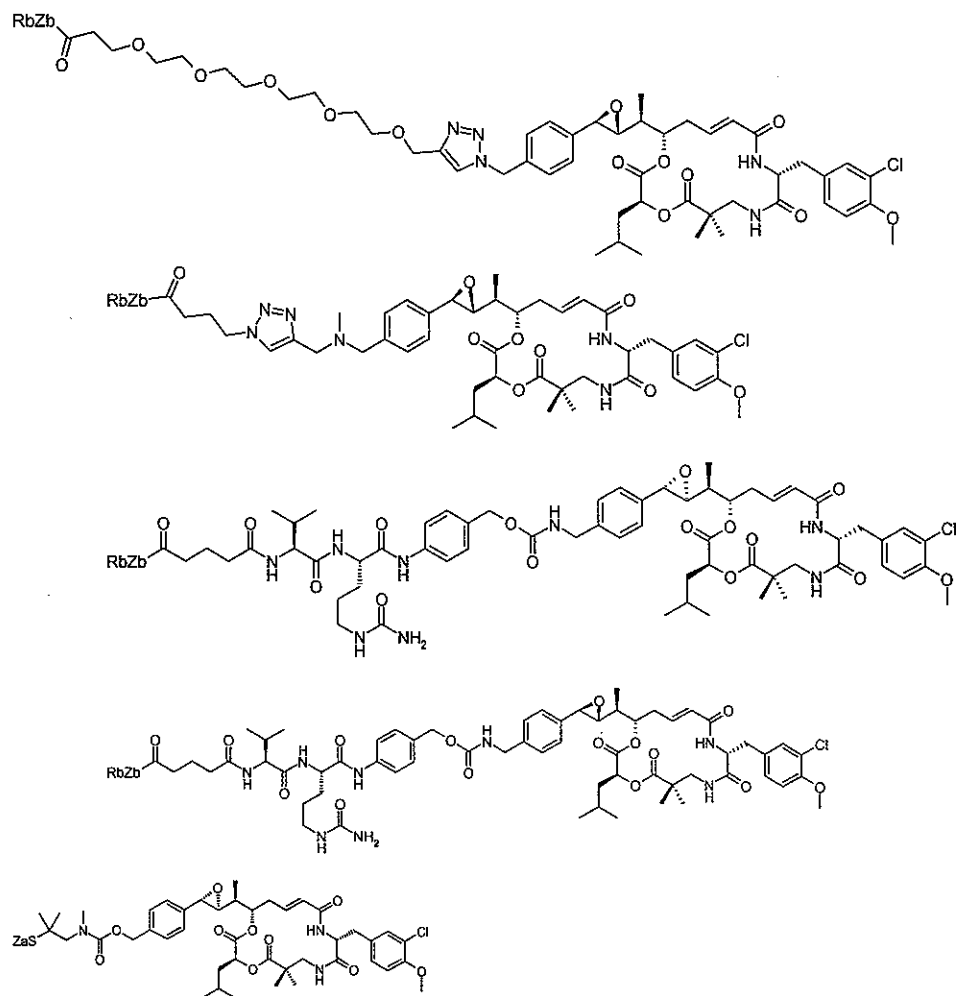




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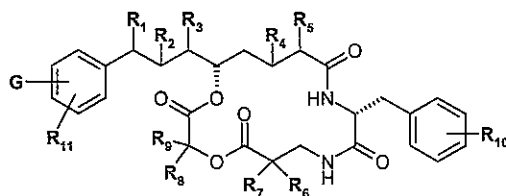
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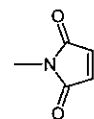
Z_a, Z_b and R_b being as defined in Claim 1 or 10.

13. Cryptophycin derivative of formula (III):

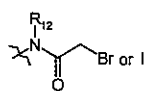


10 in which the groups R₁ to R₁₁ have the same meanings as in Claim 1, 11 or 12 and G represents a group -(CH₂)_nY, which is in an ortho (o), meta (m) or para (p) position on the phenyl nucleus bearing the unit CR₁, preferably in the para position, n being an integer ranging from 1 to 6 and Y denoting -N₃; -NR₁₂-CH₂-C≡CH in which R₁₂

represents H or a group (C₁-C₆)alkyl; -OMs; -OC(=O)-O-(4-nitrophenyl),



or

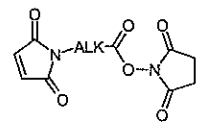


15 , R₁₂ representing H or (C₁-C₆)alkyl, more particularly a methyl group.

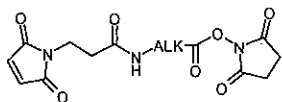
14. Cryptophycin derivative according to Claim 13, in which Y represents $-N_3$; $-NR_{12}-CH_2-C\equiv CH$ in which R_{12} represents H or a group $(C_1-C_6)alkyl$; $-OMs$ or $-OC(=O)-O-(4-nitrophenyl)$.
- 5 15. Cryptophycin derivative according to one of Claims 1 to 12 or according to formula (III) of Claim 13 with $G = -(CH_2)_nY$ with $Y = -Cl, -N_3, -OH, -NH_2, maleimido, haloacetamido$ intended to be conjugated to a binding agent.
- 10 16. Cryptophycin derivative according to Claim 15, in which the binding agent is a ligand, a protein, an antibody, more particularly a monoclonal antibody, a protein or antibody fragment, a peptide, an oligonucleotide or an oligosaccharide.
- 15 17. Process for preparing a binding agent to which is attached at least one cryptophycin derivative, referred to as a conjugate, which consists in:
- (i) placing in contact and leaving to react an aqueous solution of an optionally buffered binding agent and a solution of the cryptophycin derivative as defined in one of Claims 1 to 16;
- (ii) and then in optionally separating the conjugate formed in step (i) from the cryptophycin derivative and/or the unreacted binding agent and/or any aggregates formed.
- 20 18. Process according to Claim 17, in which:
- in the presence of a cryptophycin derivative comprising a reactive chemical group RCG1 of the type $-SZ_a$, the binding agent comprises:
 - disulfide chemical groups in the case where RCG1 represents $-SH$;
 - 25 ▪ thiol chemical groups in the case where RCG1 represents $-SZ_a$ with $Z_a \neq H$;
 - maleimido or iodoacetamido chemical groups in the case where RCG1 represents $-SH$;
 - in the presence of a cryptophycin derivative comprising a reactive chemical group RCG1 of the type $-C(=O)-Z_bR_b$, the cryptophycin derivative is reacted with the amino functions of the binding agent, especially the ϵ -amino groups borne by the side chains of the lysine residues (Lys) of an antibody;
 - 30 ➤ in the presence of a cryptophycin derivative of formula (III) with $G = -(CH_2)_nY$, the binding agent comprises groups $-SH$ when $Y = -Cl$ or $-maleimido$, groups $-C\equiv CH$ when $Y = -N_3$ or carboxylic acid groups when $Y = -OH$ or $-NH_2$;
 - 35 ➤ in the presence of a cryptophycin derivative comprising a reactive chemical group RCG1 of maleimido or haloacetamido type, the binding agent comprises thiol chemical groups.

- in the presence of a cryptophycin derivative comprising a reactive chemical group RCG1 of the type -SH, the binding agent is modified with a modifying agent chosen from

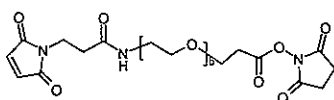
succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate;



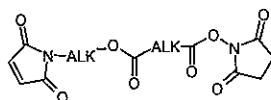
sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate;



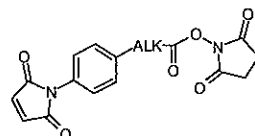
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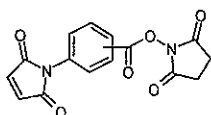
;



;

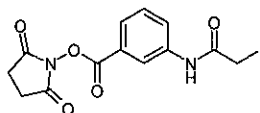


;

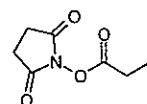


ALK denoting a group (C₁-C₁₂)alkylene and b being an integer

between 0 and 2000;



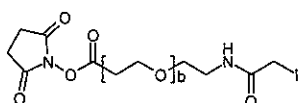
;



;

succinimidyl-N-

succinimidyl-3-(N-bromoacetamido)propionate;



10

, b being an integer between 0 and 2000.

20. Conjugate that may be obtained via the process according to one of Claims 17 to 19.
21. Conjugate according to Claim 20, characterized by a DAR determined from the deconvolution of the HRMS spectrum of greater than 1, preferably between 2 and 10 and more particularly between 2 and 7.
22. Conjugate according to Claim 20, characterized by a DAR determined using a UV spectrophotometer of greater than 0.5, preferably greater than 1, more particularly between 1 and 10 and even more particularly between 2 and 7, the DAR being calculated by the equation:

$$\text{DAR} = \text{CD} / \text{CA}$$

in which:

$$\text{CD} = [(\epsilon_{A\lambda 1} \times A_{\lambda 2}) - (\epsilon_{A\lambda 2} \times A_{\lambda 1})] / [(\epsilon_{D\lambda 2} \times \epsilon_{A\lambda 1}) - (\epsilon_{A\lambda 2} \times \epsilon_{D\lambda 1})]$$

$$\text{CA} = [A_{\lambda 1} - (\text{CD} \times \epsilon_{D\lambda 1})] / \epsilon_{A\lambda 1}$$

and

$A_{\lambda 1}$ and $A_{\lambda 2}$ denoting, respectively, the absorbances of the conjugate solution, respectively, at the wavelengths $\lambda 1$ and $\lambda 2$;

$\varepsilon_{D \lambda 1}$ and $\varepsilon_{D \lambda 2}$ denoting, respectively, the molar absorption coefficients of the cryptophycin derivative before conjugation at the wavelengths $\lambda 1$ and $\lambda 2$, these coefficients being measured on the compounds of formula (II) of the type $-SZ_a$ with $Z_a = -SMe$ or of the type $-C(=O)-Z_bR_b$ with $Z_bR_b = -OMe$ or $-OCH_2-CH=CH_2$;

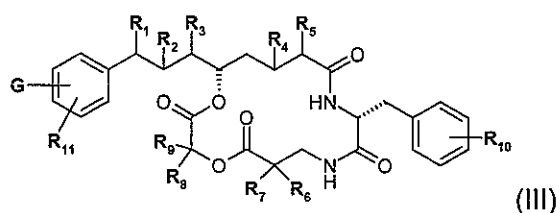
$\varepsilon_{A \lambda 1}$ and $\varepsilon_{A \lambda 2}$ denoting, respectively, the molar absorption coefficients of the naked antibody before conjugation at the two wavelengths $\lambda 1$ and $\lambda 2$.

23. Conjugate according to Claim 22, in which $\lambda 1 = 280$ nm and $\lambda 2$ is chosen within the specific wavelength range 246 nm - 252 nm.

24. Conjugate solution that may be obtained via the process according to one of Claims 17 to 19 or comprising the conjugate as defined in one of Claims 20 to 23.

25. Use of a cryptophycin derivative according to one of Claims 1 to 13, for the preparation of a binding agent to which is attached at least one of the said cryptophycin derivatives.

26. Use of a cryptophycin derivative of formula (III):

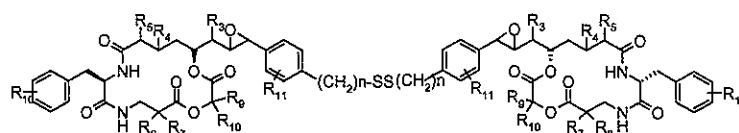


in which:

G represents a group $-CH=CH_2$ or $-(CH_2)_nY$, which is in an ortho (o), meta (m) or para (p) position on the phenyl nucleus bearing the unit CR_1 , preferably in the para position;

Y represents $-OH$; $-Cl$; $-N_3$; $-NH_2$; $-SH$; $-COOH$; $-NR_{12}-CH_2-C\equiv CH$ in which R_{12} represents H or a group $(C_1-C_6)alkyl$, more particularly a methyl group; $-OLG$ in which LG represents a leaving group; $-OC(=O)-O-(4-nitrophenyl)$; maleimido,

or of formula:



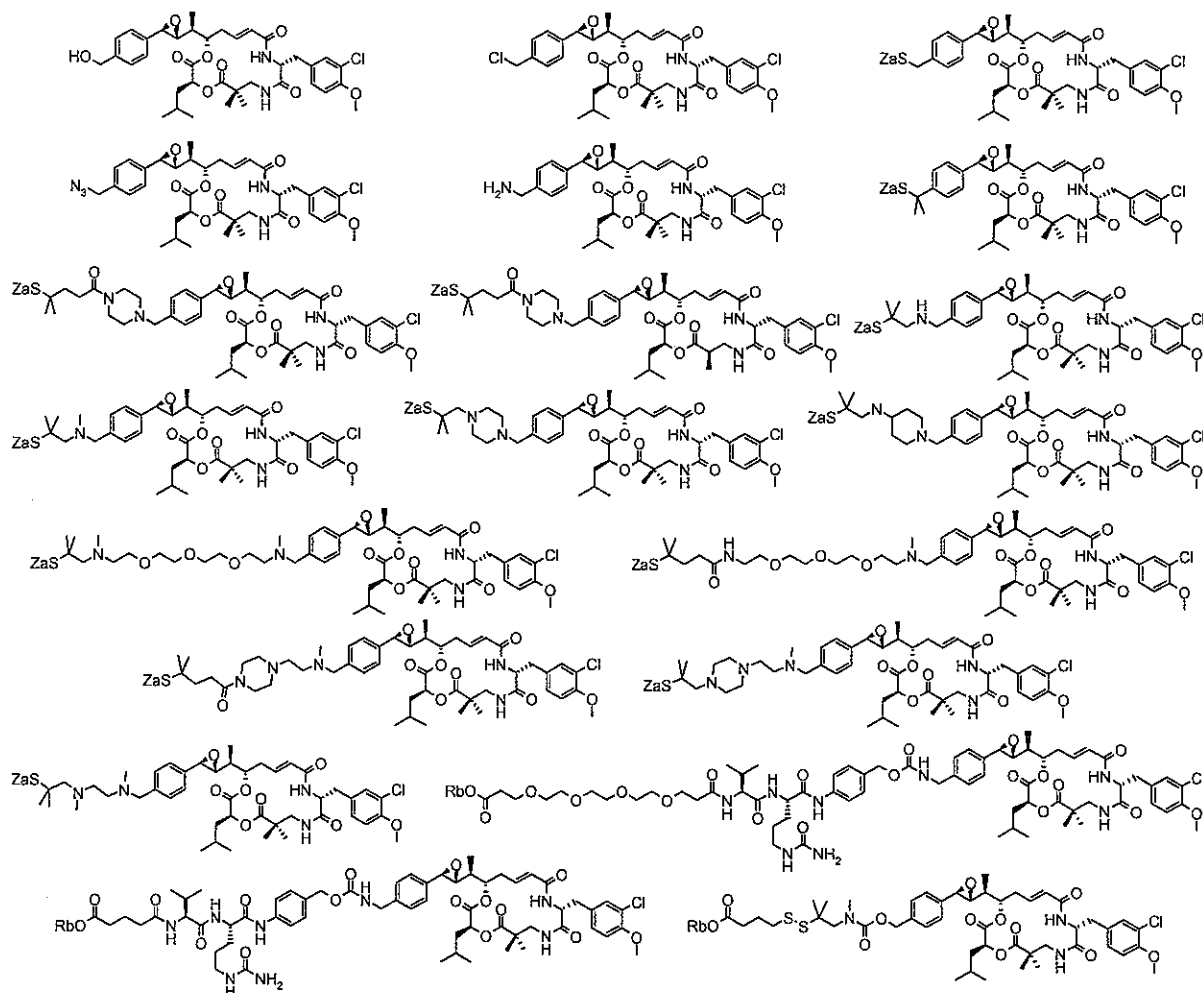
the groups R_1 to R_{11} having the same meaning as in Claim 1, 11 or 12 and n being an

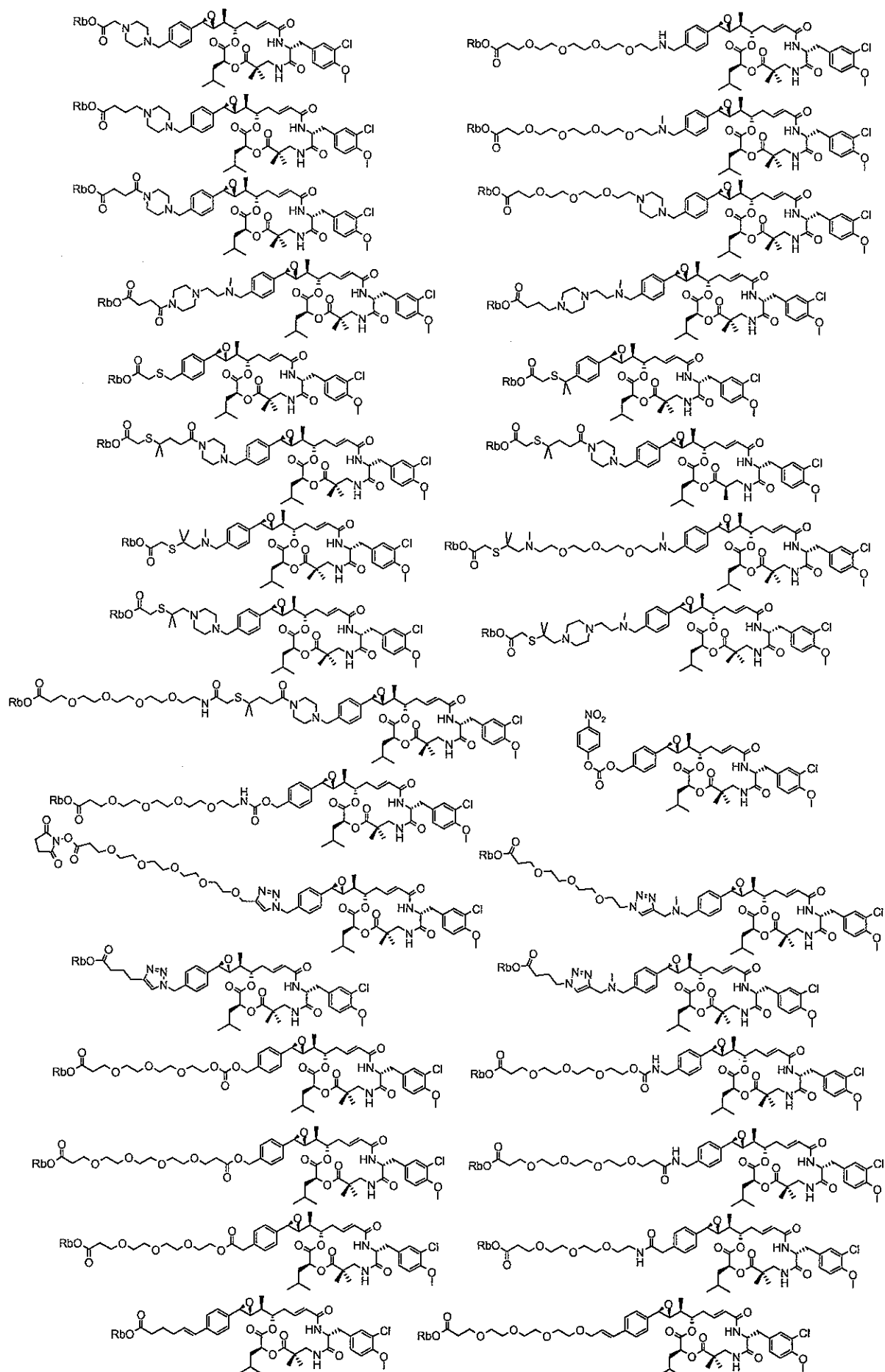
integer ranging from 1 to 6,

for the preparation of a binding agent to which is attached at least one of the said cryptophycin derivatives.

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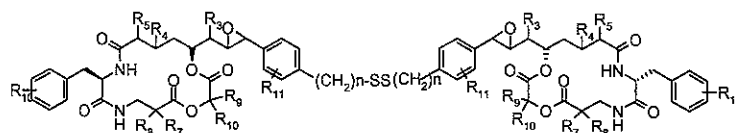
27. Use of a cryptophycin derivative chosen from one of the following:





Z_a , Z_b and R_b being as defined in Claim 1 or 10, for the preparation of a binding agent to which is attached at least one cryptophycin derivative.

28. Cryptophycin derivative of formula:



the groups R_1 to R_{11} having the same meanings as in Claim 1, 11 or 12 and n being an integer ranging from 1 to 6.

29. Cryptophycin derivative according to one of Claims 1 to 15, for use as an anticancer agent.

30. Conjugate according to Claims 20 to 23, for use as an anticancer agent.

31. Conjugate solution according to Claim 24, for use as an anticancer agent.

32. Use of a cryptophycin derivative according to one of Claims 1 to 15, for the preparation of an anticancer medicament.

33. Use of a conjugate according to Claims 20 to 23, for the preparation of an anticancer medicament.

34. Use of a conjugate solution according to Claim 24, for the preparation of an anticancer medicament.

Figure 1

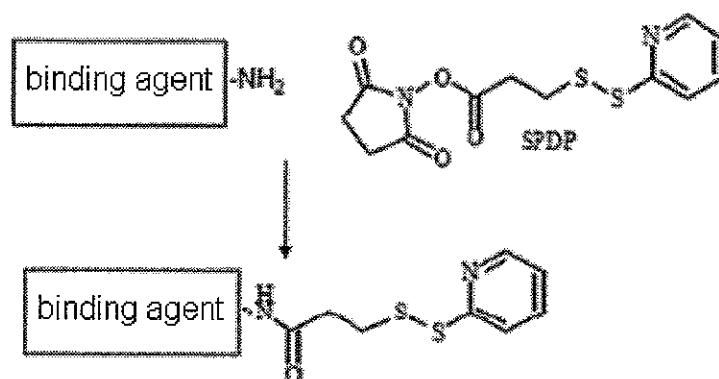


Figure 2

