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(54) Title: UROKINASE PLASMINOGEN ACTIVATOR RECEPTOR TARGETED CONTRAST AGENT

(57) **Abrégé/Abstract:**

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(54) Title: UROKINASE PLASMINOGEN ACTIVATOR RECEPTOR TARGETED CONTRAST AGENT

(57) Abstract: The invention relates to contrast agents for detection of the Urokinase Plasminogen Activator Receptor (uPAR). More specifically the invention relates to contrast agents comprising a peptidic vector binding to the uPAR, labelled with an imageable moiety.



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uPAR-targeting contrast agents**Field of the invention**

The present invention relates to contrast agents for detection of the Urokinase Plasminogen Activator Receptor (uPAR). More specifically the invention relates to contrast agents comprising a peptidic vector binding to the uPAR, labelled with an imageable moiety. The contrast agents may be used to identify sites where uPAR is expressed to diagnose diseases associated with this receptor.

Background of invention

The interaction of the urokinase-type plasminogen activator (uPA) with cell surfaces is exclusively mediated by its glycolipid-anchored receptor (uPAR) to which uPA binds with high affinity.

uPAR is localised to the outer layer of the cell membranes via glycosyl phosphatidylinositol linkage (GPI anchor). It is a cysteine-rich heavily glycosylated protein composed of three homologous domains. uPA is composed of the catalytic C-terminal serine protease domain and a modular N-terminal part including a growth factor-like domain (GFD; aa 1-49) and a kringle domain (aa 50-135). The interaction between uPA and uPAR is primarily mediated by residues 19-31 of the GFD of uPA.

Since proteolytic degradation of the extracellular matrix has an established role in tumour invasion and metastasis, uPAR represents a potential target for diagnostic contrast agents. uPAR appears to be up-regulated in vivo in most human carcinomas examined to date, specifically, in the tumour cells themselves, in tumour-associated endothelial cells undergoing angiogenesis and in macrophages. uPAR overexpression in cancer patients is present in advanced disease and has been correlated with poor prognosis in numerous human carcinomas. The fact that uPAR expression is up-regulated only in pathological states involving extracellular matrix remodelling and cell motility such as cancer makes it an attractive marker for diagnosis.

WO 01/25410 describes diagnostically or therapeutically labelled uPAR-targeting proteins and peptides. The peptide or protein comprises at least 38 amino acid residues, including residues 13-30 of the uPAR binding site of uPA.

US 6,277,818 describes uPAR-targeting cyclic peptide compounds that may be conjugated with a diagnostic label. The peptides are based on the amino acid residues 20-30 of uPA.

- 5 US 6,514,710 is also directed to cyclic peptides having affinity for uPAR. The peptides may carry a detectable label. The peptide comprises 11 amino acids joined by a linking unit.

10 Ploug et al. in Biochemistry 2001, 40, 12457-12168 describes uPAR targeting peptides but not in the context of imaging, including amino acid sequences as described in the present document.

The efficient targeting and imaging of uPAR demands a selective high-affinity vector that is chemically robust and stable. These stringent conditions are met by the
15 contrast agents of the invention.

Summary of the invention

In view of the needs of the art the present invention provides contrast agents for detection of the Urokinase Plasminogen Activator Receptor (uPAR). More specifically
20 the invention relates to contrast agent comprising a peptide sequence binding to the uPAR with high affinity, labelled with an imageable moiety. Ploug discloses e.g. a uPAR targeting peptide with the sequence X-Phe-X-X-Tyr-Leu-Trp-Ser, wherein standard abbreviations for the amino acids are used and wherein X denotes an amino acid selected from a group of 25 amino acids.

25

Detailed description of the invention

Viewed from a first aspect the invention provides a uPAR targeting contrast agent of formula Ia,

30 $Z_1-W_1-X_0-X_1-Phe-X_2-X_3-X_4-Leu-Trp-X_5-X_6-W_2-Z_2$ (Ia)

wherein,

X_0 represents 1-5 amino acids,

X_1, X_2, X_3, X_4 and X_5 independently represent one amino acid,

Phe represents phenylalanine,

35 Leu represents leucine,

Trp represents tryptophan,

X_6 represents 0 to 5 amino acids or is identified by formula (Ib),

β -Ala-Lys(Z_1 - W_1 - X_0 - X_1 -Phe- X_2 - X_3 - X_4 -Leu-Trp- X_5) (Ib)

wherein the denotations are defined as for formula Ia,

and wherein

5 β -Ala represents β -Alanine,

Lys represents lysine, and wherein

W_1 and W_2 represent the same or different moiety, and are individually a spacer, a

biomodifier or is absent, and at least one Z_1 or Z_2 is present representing an

imageable moiety capable of detection either directly or indirectly in a diagnostic

10 imaging procedure.

The amino acids X_0 , X_1 , X_2 , X_3 , X_4 , X_5 and X_6 all represent natural or unnatural amino

acids, and are preferably natural, except X_1 which is preferably unnatural. All amino

acids are either in D or L form, with preferences as given below.

15

The X_0 - X_1 -Phe- X_2 - X_3 - X_4 -Leu-Trp- X_5 - X_6 component of the agent of formula Ia has

affinity for uPAR and is herein after denoted a peptidic vector. The peptidic vector of

the contrast agent has homology with the Growth Factor Domain of uPA but differs

from uPAR targeting vectors of the prior art in that it does not comprise an amino

20 acid sequence identical to an amino acid sequence found in human uPA.

X_0 represents 1 to 5, D or L amino acids. Preferably X_0 includes amino acids selected

from the group of alanine (Ala), threonine (Thr), glycine (Gly), aspartic acid (Asp) and

glutamic acid (Glu). The amino acids are preferably in the L form, except threonine

25 which is preferably in the D form. Most preferably X_0 represents L-Asp, D-Thr or Gly-

Gly-Asp.

X_1 preferably represents a β -cycloalkylalanine and is more preferably β -

cyclopentylalanine, β -cyclohexylalanine (Cha) or β -cycloheptylalanine, and is most

30 preferably β -cyclohexylalanine.

X_2 preferably represents serine (Ser) or alanine (Ala), more preferably serine and

most preferably D-serine.

35 X_3 preferably represents arginine (Arg), or an arginine mimetic such as N-

methylarginine(mArg), tyrosine (Tyr) or alanine, and more preferably D-arginine.

X_4 preferably represents tyrosine, alanine, leucine or cyclohexylalanine, more preferably tyrosine and most preferably L-tyrosine.

X_5 preferably represents serine, histidine (His), alanine, tyrosine or leucine, and more preferably L-serine or D-histidine.

X_6 preferably represents 0 to 5, D or L amino acids. Preferably X_6 includes amino acids selected from the group of glycine, aspartic acid, lysine, phenylalanine or β -alanine (β -Ala). Alternatively, X_6 comprises the group of formula (Ib)

10

β -Ala-Lys(Z_1 - W_1 - X_0 - X_1 -Phe- X_2 - X_3 - X_4 -Leu-Trp- X_5) (Ib),

15

wherein all symbols are as earlier defined and wherein the β -Ala of formula Ib is linked to X_5 of formula Ia. The peptide chain in brackets is linked via its X_5 to the ϵ -amino group of lysine. In this alternative the contrast agent will comprise a dimer synthesised on a modified lysine scaffold having its α -amino group prederivatized with β -alanine thus creating a pseudo symmetrical dimer with respect to the α -carbon atom of lysine. In this alternative, the dimer is preferably a homodimer, wherein the peptide sequences in the two monomers are the same. Most preferably X_6 is absent.

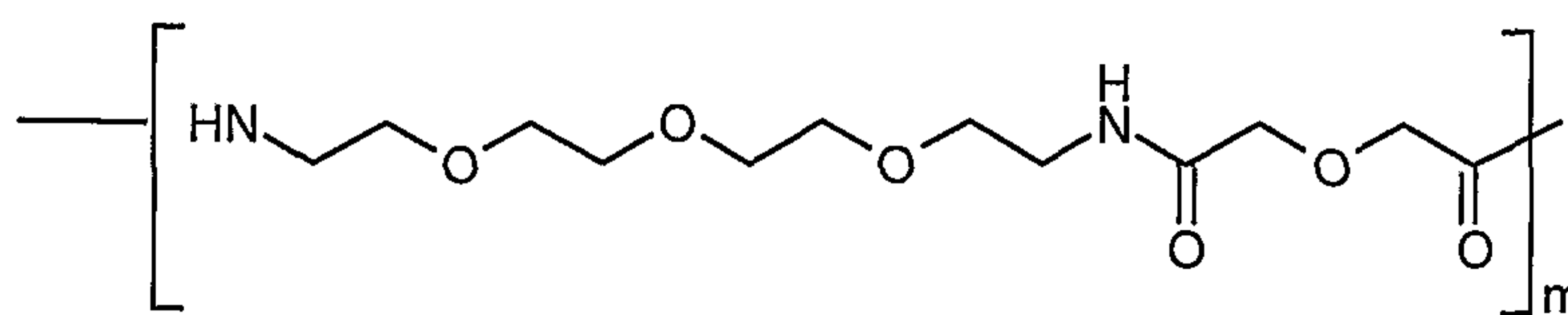
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Surprisingly, it has been found that the X_4 and X_5 positions of the peptidic vector, wherein Ploug uses tyrosine and serine respectively, may be replaced with other amino acids as outlined above.

25

W_1 and W_2 individually represent a moiety acting as spacer, a biomodifier moiety or both, or is absent, and is preferably based on a monodisperse polyethylene glycol (PEG) building block comprising 1 to 10 units of said building block. W_1 or W_2 may also represent 1 to 10 amino acid residues preferably comprising glycine, lysine, aspartic acid, serine or aminohexanoic acid. More preferably, W_1 or W_2 comprises both amino acid residues and a PEG-based structure, such as 1-10 amino acid residues in combination with a PEG-based structure. Preferably, either W_1 or W_2 represent a biomodifier and in a preferred embodiment at least one of W_1 or W_2 represents a unit comprised of the monodisperse PEG-based structure, 17-amino-5-oxo-6-aza-3,9,12,15-tetraoxaheptadecanoic acid of formula (II),

30



(II)

wherein m equals an integer from 1 to 10 and where the C-terminal end is an amide or acid moiety. As a biomodifier, W_1 or W_2 has the function of modifying the pharmacokinetics and blood clearance rates of the compounds. The biomodifier effects less uptake of the compounds in tissue i.e. muscle, liver etc. thus giving a better diagnostic image due to less background interference. The secretion is mainly through the kidneys which is a further advantage of the biomodifier.

As a biomodifier moiety W_1 or W_2 is preferably derived from glutaric and/or succinic acid and/or a PEG based unit, e.g. including the moiety of formula II. W_1 or W_2 can further act as a spacer, with biomodifier properties, linking the imageable moiety Z_1 or Z_2 to the peptidic vector. Other representative spacer elements include structural-type polysaccharides, storage-type polysaccharides, polyamino acids and methyl and ethyl esters thereof, and polypeptides, oligosaccharides and oligonucleotides, which may or may not contain enzyme cleavage sites. As a spacer moiety one role of W_1 or W_2 is to distance a relatively bulky imageable moiety from the receptor binding domain of the peptidic vector. Alternatively, in the simplest form W_1 or W_2 is a functional bond or comprises a functional group which permits facile conjugation of the imageable moiety to the peptidic vector, such groups include $-\text{NR}^a$ -, CO_2 -, $\text{N}(\text{C}=\text{S})$ -, $-\text{N}(\text{CO})$ -, $-\text{S}$ -, $-\text{O}$ -, $-\text{O}-\text{NH}_2$ and $-\text{CHO}$ wherein the R^a group is independently H, C_{1-10} alkyl, C_{3-10} alkylaryl, C_{2-10} alkoxyalkyl, C_{1-10} hydroxyalkyl, C_{1-10} haloalkyl and α -haloacetyl.

When Z_1 is absent and W_1 present, W_1 preferably has an N-terminal with a free amino group or an amino group comprising a metabolism inhibiting group. By the term metabolism inhibiting group is meant a biocompatible group which inhibits or suppresses in vivo metabolism of the peptide or amino acid at the amino terminus. Such groups are well known to those skilled in the art and are suitably selected from the group of acetyl, Boc, (tert-butyloxycarbonyl), Fmoc (fluorenylmethoxycarbonyl), benzyloxycarbonyl, trifluoroacetyl and allyloxycarbonyl. Preferred metabolism inhibiting groups are acetyl and benzyloxycarbonyl. When Z_2 and W_2 are absent, the

C-terminal of the peptidic vector is preferably an amide or carboxylic group, and preferably an amide group. Also when Z_2 is absent, and W_2 is present comprising amino acids, W_2 preferably ends with an amide or carboxylic group, and more preferably an amide group.

5

The contrast agent of the invention preferably comprises a linear peptide, meaning that there are no bridges between any of the amino acids, or between amino acids and other moieties of the agent that generate a cyclic structure. There are hence preferably no sulphide, thioether bridges or other bridges forming cyclic peptide

10 structures. The contrast agent preferably comprises a maximum of 20 amino acids.

In a preferred embodiment the peptidic vector represented by

X_0 - X_1 -Phe- X_2 - X_3 - X_4 -Leu-Trp- X_5 - X_6 of formula Ia comprises the sequence:

X_0 -Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser, such that the contrast agent has the formula

15 Z_1 - W_1 - X_0 -Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser- X_6 - W_2 - Z_2 , wherein the denotations are as earlier defined.

More preferably the peptidic vector comprises the sequence:

Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser, or

20 *Thr*-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser,

wherein italic font denotes D amino acids and wherein X_6 is absent.

One example of a contrast agent of the invention is:

25 Z_1 - W_1 -Gly-Gly-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-NH₂

wherein Z_1 and W_1 are as earlier defined, and wherein X_6 , W_2 and Z_2 are absent, and wherein the C-terminal is an amide group.

At least one of Z_1 and Z_2 is present and represents an imageable moiety. Z is hereinafter used to denote either Z_1 or Z_2 . Z can be any imageable moiety. The nature of Z will depend on the imaging modality utilised in the diagnosis. A wide range of moieties suitable for detection in *in vivo* imaging are known from e.g. WO 98/47541, the content of which is incorporated by reference.

35 Z is a moiety capable of detection either directly or indirectly in an *in vivo* diagnostic imaging procedure, such as radio or SPECT, PET, MR, x-ray, ultrasound or optical imaging. Z comprises e.g. a moiety which emits or may be caused to emit detectable

radiation (eg. by radioactive decay, fluorescence excitation, spin resonance excitation, etc.), a moiety which affects local electromagnetic fields (e.g. paramagnetic, superparamagnetic, ferrimagnetic or ferromagnetic species), a moiety which absorbs, emits or scatters radiation energy (e.g. chromophores, particles
 5 (including gas or liquid containing vesicles), a heavy element and compounds thereof, etc.), and a moiety which generates a detectable substance (eg. gas microbubble generators).

The imageable moiety Z may be represented by an entity M and a moiety Y₁, wherein
 10 M represents metal ions, paramagnetic metals, metal radio-nucleides, heavy metals and heavy metal oxides. The moiety Y₁ must be able to carry one or several moieties M. By carrying is meant any form of association between the moiety Y₁ and M such as a chemical bond, e.g. covalent bond or electrovalent or ionic bonds or by absorption or any other type of association, and preferably M is chelated by a
 15 chelating moiety Y₁.

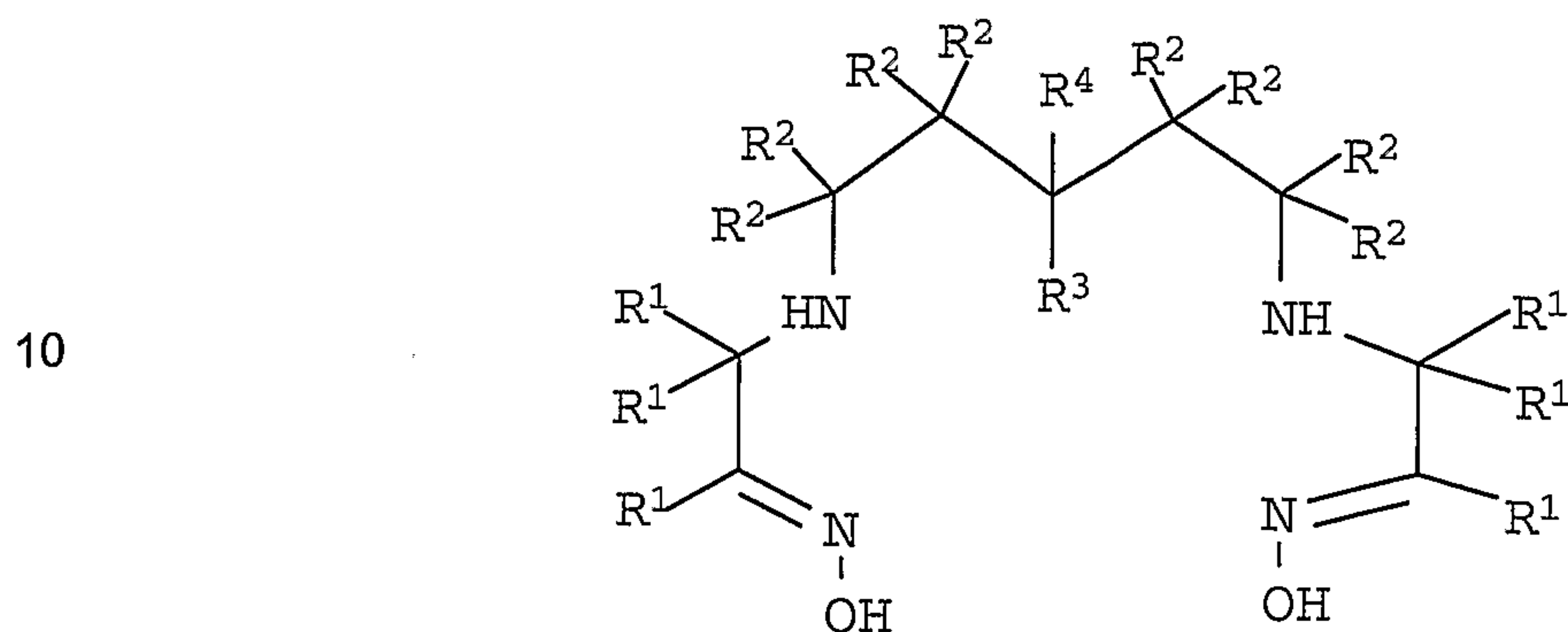
Imaging modalities and imageable moieties Z are described in more detail hereinafter:

20 In a first embodiment of this aspect, Z comprises one or more moieties M useful in the radio and SPECT imaging modality such as a radioactive metal ion or a gamma-emitting radioactive halogen, optionally carried by a moiety Y₁. Preferably M is a gamma emitter with low or no alpha- and beta-emission and with a half-life of more than one hour. Preferred groups M are the radionuclides ⁶⁷Ga, ¹¹¹In, ¹²³I, ¹²⁵I, ¹³¹I,
 25 ^{81m}Kr, ⁹⁹Mo, ^{99m}Tc, ²⁰¹Tl and ¹³³Xe. Most preferred is ^{99m}Tc.

M can further be represented by the following isotopes or isotope pairs: ⁴⁷Sc₂₁; ¹⁴¹Ce₅₈; ¹⁸⁸Re₇₅; ¹⁷⁷Lu₇₁; ¹⁹⁹Au₇₉; ⁴⁷Sc₂₁; ¹³¹I₅₃; ⁶⁷Cu₂₉; ¹³¹I₅₃ and ¹²³I₅₃; ¹⁸⁸Re₇₅ and ^{99m}Tc₄₃; ⁹⁰Y₃₉ and ⁸⁷Y₃₉; ⁴⁷Sc₂₁ and ⁴⁴Sc₂₁; ⁹⁰Y₃₉ and ¹²³I₅₃; ¹⁴⁶Sm₆₂ and ¹⁵³Sm₆₂; and
 30 ⁹⁰Y₃₉ and ¹¹¹In₄₉.

When M denotes a metallic radionuclide for radio or SPECT imaging then Y₁ preferably denotes a chelating agent suitable for forming a stable chelate with M. Such chelating agents are well known from the state of art and typical examples of
 35 such chelating agents are described in Table I of WO 01/77145.

Particularly preferred are chelating agents Y₁ of formula (III):



20 wherein:

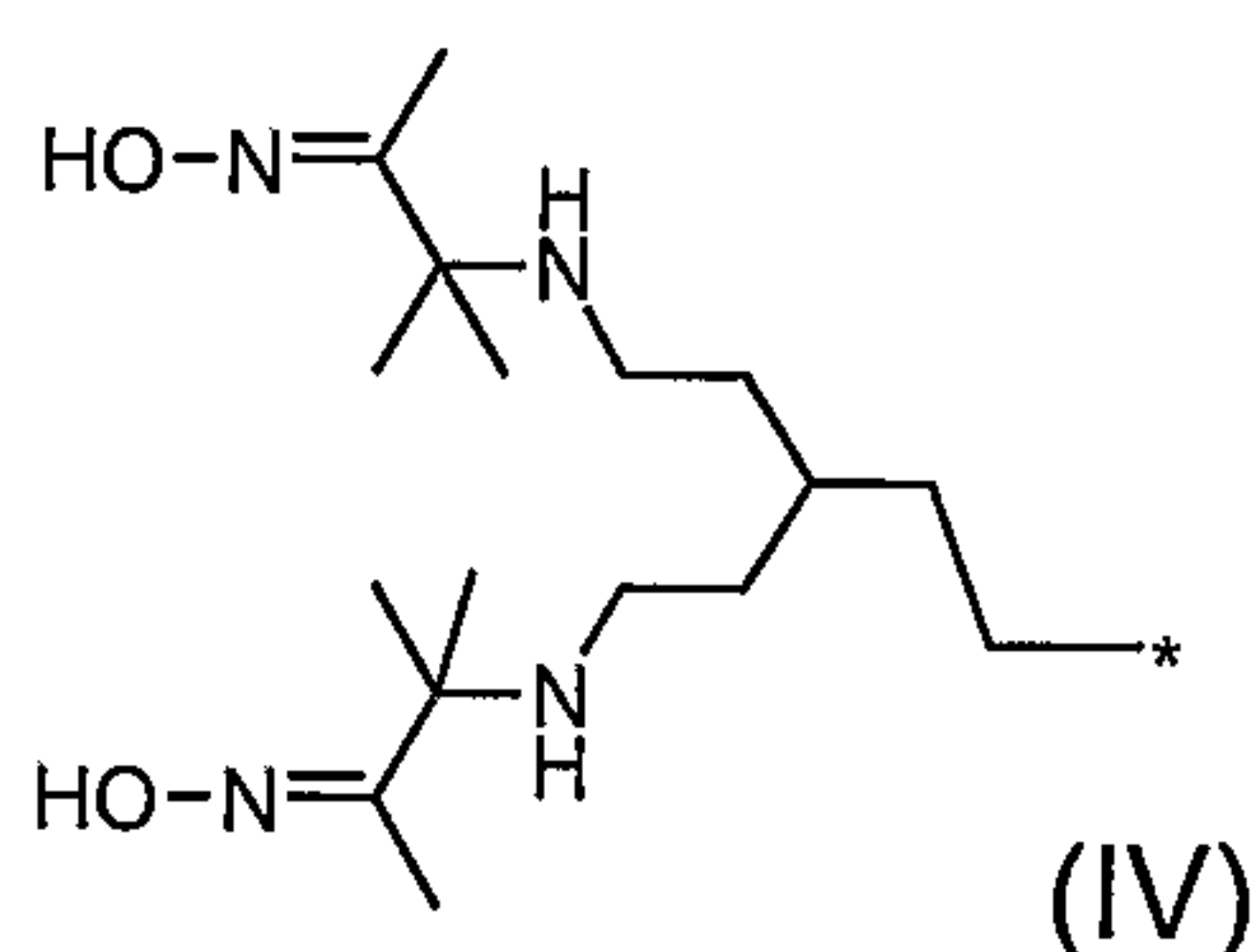
(III)

each R^1 , R^2 , R^3 and R^4 is independently H or C_{1-10} alkyl, C_{3-10} alkylaryl, C_{2-10} alkoxyalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkylamine, C_{1-10} fluoroalkyl, or 2 or more R groups, together with the atoms to which they are attached form a carbocyclic, heterocyclic, saturated or unsaturated ring.

25

More particularly preferred are chelating agents Y_1 of formula (III) where R^1 , R^2 and R^3 are hydrogen or methyl groups and R^4 is an alkyl or an alkylamine group. More preferably Y_1 is the chelate of formula (IV), herein denoted cPN216, and most preferably the imaging moiety M is ^{99m}Tc . The star denotes a possible linking site.

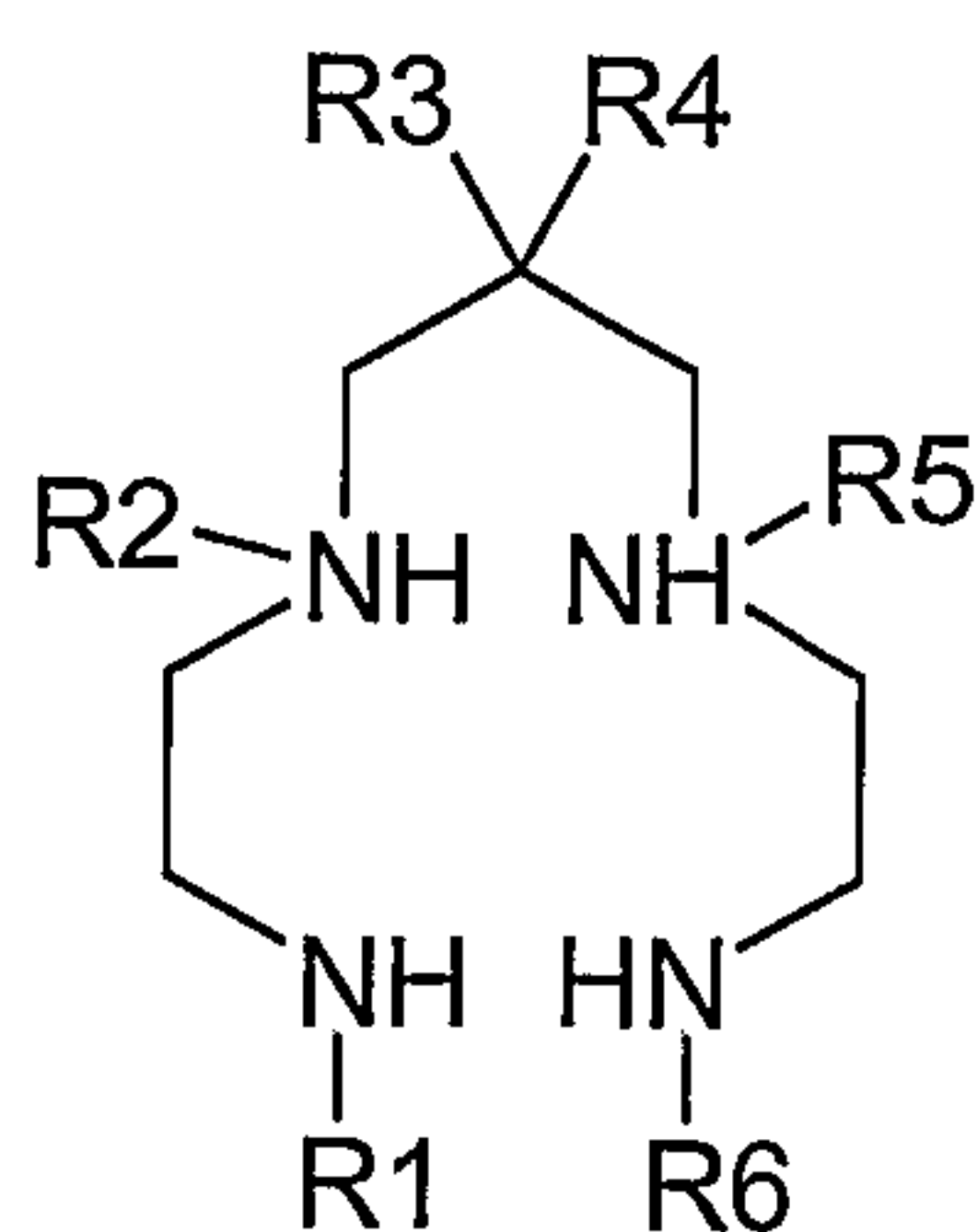
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Other preferred chelating agents are of formula (V)

45



50

(V)

wherein R1-R6 independently represent H, alkyl, aryl or a combination thereof, where R1-R6 groups contain one or more functional moieties such that the chelate can be conjugated to W₁ or W₂ of formula (Ia). Suitable functional moieties are e.g. alkylamine, alkylsulphide, alkoxy alkyl carboxylate, arylamine, aryl sulphide or alpha-haloacetyl.

If Z represent a gamma-emitting radioactive halogen such as ¹²³I, ¹²⁵I, ¹³¹I and ⁷⁷Br, wherein ¹²⁵I is preferred, it may be covalently linked to W₁ or W₂ by a substitution or addition reaction well known from the state of art, or alternatively to X₀ or X₆ if W₁ or W₂ is absent, or directly to X₅ if X₆ is absent.

In a second embodiment, the compound of formula (Ia) comprises a moiety Z useful in the PET imaging modality. Z then comprises a radioemitter with positron-emitting properties, preferably a positron-emitting radioactive non-metal. Preferred groups Z comprise either of the positronemitters ¹¹C, ¹⁸F, ¹³N, ¹⁵O, ⁷⁷F, ⁷⁵Br, ⁷⁶Br and ¹²⁴I. ¹⁸F is specifically preferred. Suitable metallic positron-emitters are ⁶⁴Cu, ⁴⁸V, ⁵²Fe, ⁵⁵Co, ^{94m}Tc, ⁶⁸Ga or ⁸²Rb, ⁶⁸Ga being preferred, which can be chelated with a chelating agent Y₁.

Non-metal radionuclides such as those comprising ¹⁸F may be covalently linked to the moiety W₁ or W₂ when present or alternatively to X₀, X₅ or X₆ by a substitution or addition reaction well known from the state of art.

When Z is an ¹⁸F-labelled aldehyd, thiol or aminooxy-group then W₁ or W₂ preferably has a functional group comprising an alpha-halo acetyl moiety, an aldehyd or an aminooxy group. Thiol coupling chemistry and aldehyd and aminooxy coupling chemistry, ¹⁸F-synthons and labelled peptides prepared using this chemistry are described in WO 03/080544 and WO 04/080492, the content of which is incorporated herein by reference. Alternatively, ¹⁸F may be introduced by other moieties comprising ¹⁸F such as e.g. ¹⁸F-fluorobenzaldehyde. The linking site, W₁, W₂, X₀, X₅ or X₆ would then preferably comprise an aminooxy group.

When M denotes a metallic positron-emitter for PET imaging then Y₁ denotes a chelating agent suitable for forming a stable chelate with M. Such chelating agents are well known from the state of art and typical examples of such chelating agents are described in Table I of WO 01/77145 and in the previous embodiment directed to radio and SPECT imaging.

In a preferred embodiment Y_1 is the DOTA chelating agent and M is ^{68}Ga which can be readily introduced in to the chelate using microwave chemistry.

- 5 In a third embodiment, Z comprises a moiety Y_1 carrying one or more moieties M useful in the MR imaging modality. M here denotes a paramagnetic metal, suitable such metal ions include: Gd(III), Mn(II), Cu(II), Cr(III), Fe(III), Co(II), Er(II), Ni(II), Eu(III) or Dy(III). Gd(III), Dy(III), Fe(III) and Mn(II) are particularly preferred. Y_1 denotes a chelating agent, in particular a chelating agent such as acyclic or cyclic
- 10 polyaminocarboxylates (e.g. DTPA, DTPA-BMA, DOTA and DO3A) as described e.g. in US 4,647,447 and WO 86/02841. M may also denote metal oxides such as superparamagnetic, ferrimagnetic or ferromagnetic species which are adsorbed by or bound to Y_1 , e.g. such that Y_1 function as a coating to the metal oxide. Metal oxides for use as MR contrast agents are described e.g. in US patent 6,230,777 which is
- 15 hereby incorporated by reference.

In a fourth embodiment Z represents an imageable moiety useful in the X-ray imaging modality. Z here comprises a heavy metal such as tungsten, gold and bismuth preferably in the form of oxides. Z can also be represented by iodinated aryl

20 derivatives particularly well known as X-ray contrast agents, e.g. IopamironTM and OmnipaqueTM. These agents can be linked via their acid or amine functions to the peptidic vector of formula (Ia), optionally via W_1 or W_2 .

In a further embodiment the compound of formula (Ia) comprises Z in the form of

25 gas-filled microvesicles. Such ultrasound imaging agents can be utilised in the imaging of receptors e.g. when they are functionalised for binding to a peptide as described in the state of art e.g. in WO98/18500.

In a sixth, and preferred, embodiment of the present invention the moiety Z of

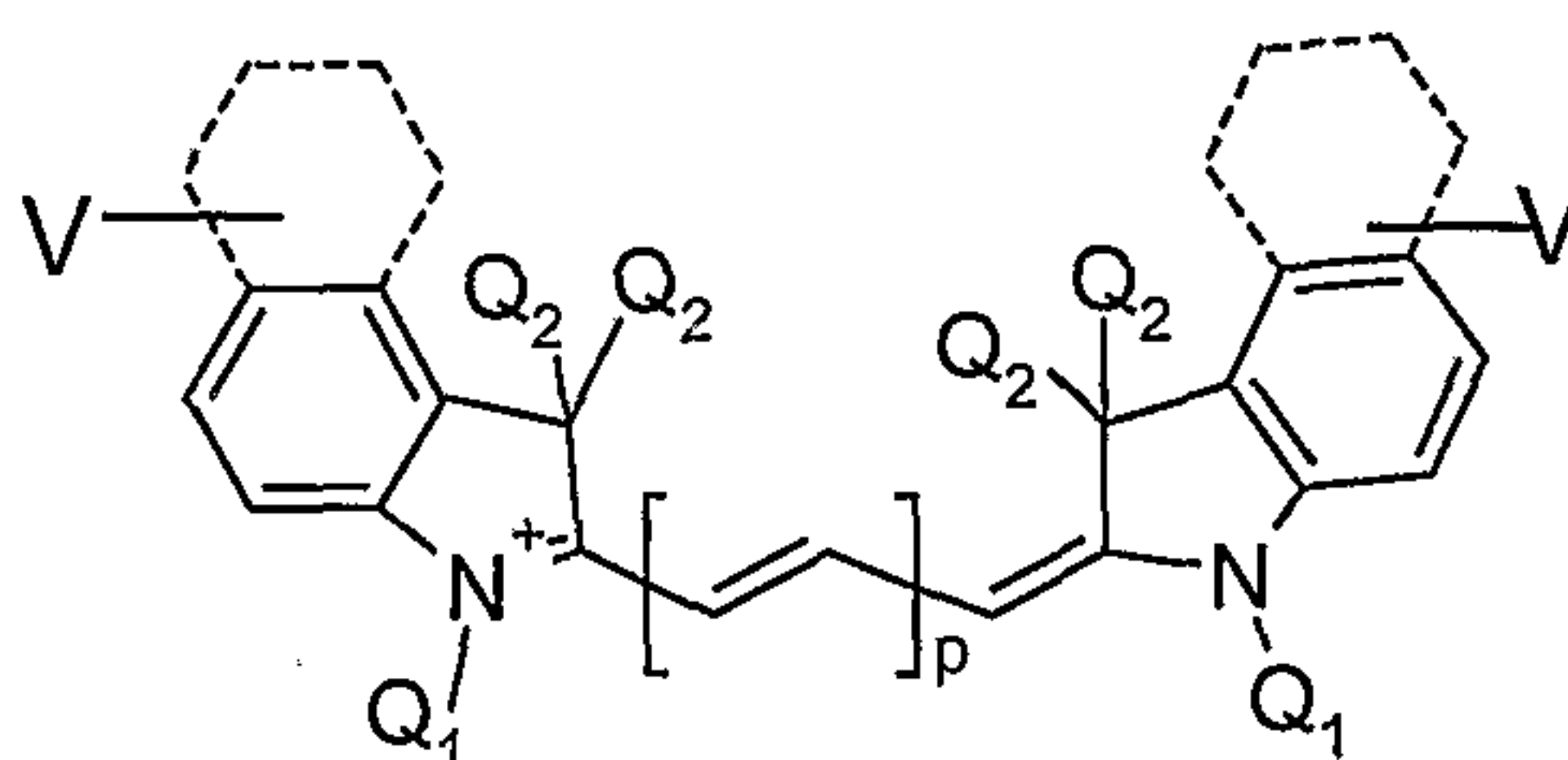
30 formula (Ia) may be any moiety capable of detection either directly or indirectly in an optical imaging procedure. The detectable moiety can be a light scatterer (e.g. a coloured or uncoloured particle), a light absorber or a light emitter. More preferably Z is represented by a dye such as a chromophore or a fluorescent compound. The moiety Z can be any dye that interacts with light in the electromagnetic spectrum with

35 wavelengths from the ultraviolet light to the near-infrared. In a preferred version Z has fluorescent properties.

Preferred organic dye moieties include groups having an extensive delocalized electron system, e.g. cyanines, merocyanines, indocyanines, phthalocyanines, naphthalocyanines, triphenylmethines, porphyrins, pyrilium dyes, thiapyrilium dyes, squarylium dyes, croconium dyes, azulenium dyes, indoanilines,

- 5 benzophenoxazinium dyes, benzothiaphenothiazinium dyes, anthraquinones, naphthoquinones, indathrenes, phthaloylacridones, trisphenoquinones, azo dyes, intramolecular and intermolecular charge-transfer dyes and dye complexes, tropones, tetrazines, bis(dithiolene) complexes, bis(benzene-dithiolate) complexes, iodoaniline dyes and bis(S,O-dithiolene) complexes. From the fluorescent dyes the
- 10 group of cyanine dyes is preferred. Even more preferred are the groups of carbacyanines, oxacyanines, thiacyanines and azacyanines. The groups of Cy5- and Cy7-dyes are the most preferred fluorescent dyes, and these can be linked to the peptidic vector by a *N*-hydroxysuccinimide ester (NHS-ester). Fluorescent proteins, such as green fluorescent protein (GFP) and modifications of GFP that have different
- 15 absorption/emission properties are also useful. Complexes of certain rare earth metals (e.g., europium, samarium, terbium or dysprosium) are used in certain contexts, as are fluorescent nanocrystals (quantum dots).

- Preferred dyes are selected from the group of carbacyanines, and even more
- 20 preferred are the carbacyanine dyes of the indole type. Preferred dyes of this type are illustrated by formula VI:



(VI)

- 25 wherein the Q₁ groups are the same or different and are substituted or unsubstituted lower alkyl groups, e.g. C₁ to C₆ alkyl groups which are optionally substituted. The alkyl groups are substituted e.g. with carboxy, sulphonic acid, amine, ammonium or ester groups, such as heterocyclic ester groups (e.g. NHS-ester). The Q₂ groups are the same or different and are lower alkyl groups, such as C₁ to C₆ alkyls, preferably
- 30 methyl groups, optionally substituted with e.g. carboxy or sulphonic acid groups. Optional aromatic groups are indicated by dotted lines, to include both structures comprising condensed benzo rings and condensed naphtho rings. Either rings are

substituted or unsubstituted. The rings may be substituted with a group V selected from sulphonic acid groups, carboxylic groups, hydroxyl groups, alkyl(sulphoalkyl)amino groups, bis(sulphoalkyl)amino groups, sulphoalkoxy groups, sulphoalkylsulphonyl group, alkyl or substituted alkyl or sulphoalkylamino groups.

5 p is a positive integer 1, 2, 3 or 4. Preferably, the cyanine dye is a pentamethine or a heptamethine dye with carbon-bridges of 5 and 7 carbon atoms, respectively.

Q₁, Q₂ and V are potential linking sites for the linking of the dye to the peptidic vector, optionally via W₁ and/or W₂, the Q₁ and V group being preferred linking sites. In a
10 preferred aspect one Q₁ group is linked to the peptidic vector while the other Q₁ group is an optionally substituted lower alkyl group.

Viewed from a second aspect the invention provides uPAR targeting compounds of formula (VII)

15

W₁-X₀-X₁-Phe-X₂-X₃-X₄-Leu-Trp-X₅-X₆-W₂ (VII)

wherein the denotations are defined as for formula Ia, and wherein at least one of W₁ and W₂ is present and represents a biomodifier as described. The compounds of
20 formula VII may be linked to an imageable moiety, as described in the first aspect, or may have other uses, such as in therapy.

The compounds of the present invention can be synthesised using the known methods of chemical synthesis but particularly useful is the solid-phase
25 methodology of Merrifield employing an automated peptide synthesiser (J. Am. Chem. Soc., 85: 2149 (1964)). Typically, the desired sequences are assembled by solid-phase peptide synthesis. Standard procedures for the synthesis strategy employed for the examples of this invention are described in E. Atherton & R.C. Sheppard, "Solid phase peptide synthesis: a practical approach", 1989, IRL Press,
30 Oxford.

For example, resins with a variety of acid-labile linker groups are used that will yield peptides with a C-terminal acid, amine or amide. The amino protecting group is then removed and the second amino acid in the sequence is coupled using a suitable
35 condensation reagent. Amino acids with semi-permanent amino protecting groups and permanent protecting groups for the functional side chains are employed.

Amino-deprotection and coupling cycles are then repeated in alternating steps until the sequence of interest is assembled.

Alternatively, the peptides can be synthesised through solution peptide synthesis methods known in the art, either in a step-wise manner from the carboxyl terminus and/or through the application of segment condensation or ligation methods, employing comprehensive or minimal protection strategies. Combined solution-solid phase segment condensation approaches can also be applied.

- Generally, the reactive side-chain groups present (for example amino, hydroxyl, guanidino and carboxyl groups) will be protected during overall synthesis as indicated above. A wide choice of protecting groups for amino acids is known (see, e.g., Greene, T.W. & Wuts, P.G.M. (1991) Protective groups in organic synthesis, John Wiley & Sons, New York). Amino protecting groups which may be employed include 9-fluorenylmethoxycarbonyl (Fmoc) and *t*-butyloxycarbonyl (Boc). Side-chain protecting groups which may be employed include *t*-butyl (*t*Bu), trityl (Trt), Boc, and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc). It will be appreciated that a wide range of other such groups are known in the art.
- Finally the permanent side-chain protecting groups are removed and the peptide is cleaved from the resin, usually simultaneously through treatment with a suitable acidic reagent, e.g. trifluoroacetic acid (TFA).

W_1 and/or W_2 can be conjugated to the peptidic vector using known methods of chemical synthesis. Particularly useful is the direct conjugation of W_1 and/or W_2 to the peptidic vector by amide bond formation, as for preparation of the peptidic vector. Alternatively, a nucleophile substitution reaction where a leaving group on the peptide N-terminus is replaced by a nucleophilic group on W_1 and/or W_2 may be used. Such a leaving group may be a bromide attached in alpha position to a carbonyl group, and such a nucleophile may be nitrogen.

Z can be conjugated directly to the peptide using the same methods as for the conjugation of W_1 and/or W_2 to the peptidic vector. In the case where Z is attached to the peptide via W_1 or W_2 any methods of chemical synthesis may be used in the conjugation of Z and W_1 or W_2 . Particularly useful is the automatic coupling of Z yielding e.g. an amide bond between the peptide and the imager moiety. Methods

for linking a reporter moiety to a peptide is well known in the art and will depend on the reporter chosen and on which spacers are used.

5 The peptidic vector and the contrast agent may be purified using high performance liquid chromatography (HPLC) and characterised by mass spectrometry and analytical HPLC.

10 The requirements of the compounds of the invention to function as efficient contrast agents of the uPA/uPAR system is that on the one hand the peptidic vector should have a high affinity for the receptor, and on the other hand that the vector should "stay" on the receptor as long as necessary. Thus the contrast agent/uPAR system should preferably exhibit slow dissociation kinetics (the so-called "off-rate"), which may conveniently be expressed as the dissociation rate constant K_{diss} . The contrast agents of the invention have been found to have greatly improved receptor-binding kinetics. Based thereon, the contrast agents of the invention which are especially preferred have a dissociation rate constant (K_{diss}) relative to the Growth Factor Domain of uPA (GFD) of preferably < 50 , more preferably < 20 , even more preferably < 10 , and most preferably < 1 .

20 The contrast agents of the invention are preferably able to inhibit the binding of uPA to cell-surface uPAR. The contrast agent of the invention is preferably equipotent with diisopropyl fluorophosphates inactivated uPA. The parameter IC_{50} gives the concentration where 50 % of the uPA are competed off. Preferred contrast agents of the invention have an $\text{IC}_{50} < 50$ nM, preferably < 25 nM, more preferably < 10 nM, and 25 even more preferably < 3 nM.

Contrast agents according to the invention are preferably used to identify sites where uPAR is expressed to diagnose diseases associated with up-regulation of the receptor. The receptor is preferably more than 50 % more abundant in the diseased 30 tissue than in surrounding tissue. More preferably the receptor is more than two times more abundant in diseased tissue than in surrounding tissue. Even more preferably, the receptor is more than 5 times more abundant in diseased tissue than in surrounding tissue.

35 Viewed from a further aspect the invention provides a method for detecting the presence of uPAR on the surface of a cell, in a tissue, in an organ or in a biological

sample suspected of overexpressing uPAR due to a pathological state. The method comprises the steps of

a) contacting the cell, tissue, organ or biological sample with the contrast agent of the invention and

5 b) detecting the presence of the imageable moiety associated with the cell, tissue, organ or sample. In this method both the contacting and the detection may be conducted in vitro, alternatively the contacting is in vivo and the detection in vitro, preferably the contacting and the detection is in vivo.

10 A preferred method includes generating an image of a human or animal body by diagnostic imaging involving administering a contrast agent as described to said body, e.g. into the vascular system, and generating an image of at least a part of said body, to which said contrast agent has distributed.

15 Viewed from a still further aspect the invention provides a method of generating enhanced images of a human or animal body by imaging, previously administered with a contrast agent composition comprising a contrast agent as defined, which method comprises generating an image of at least part of said body.

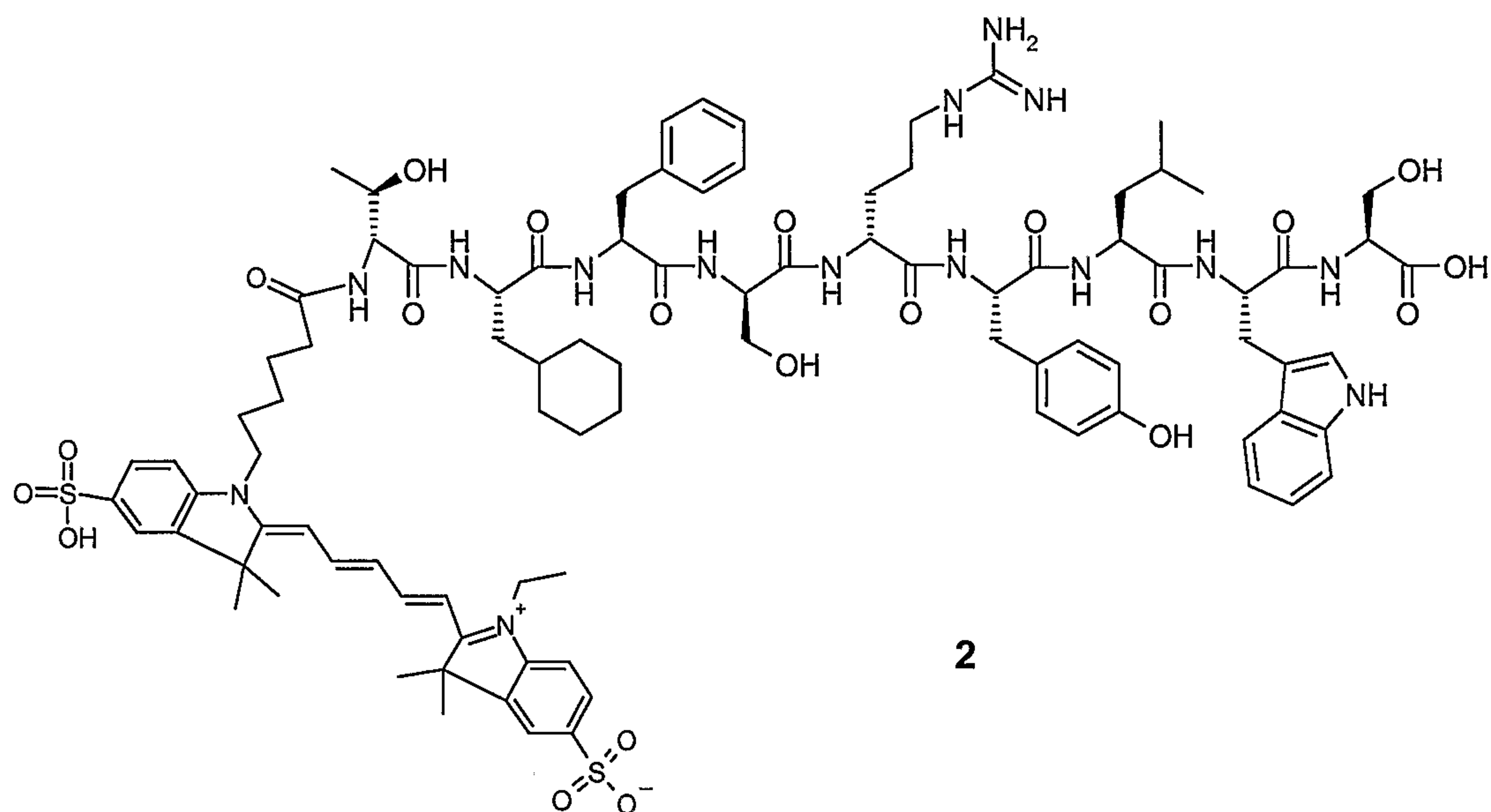
20 The new contrast agents of the invention may be used as contrast agents in any imaging modality, depending on the imageable moiety chosen. Use of the contrast agents in diagnostic imaging is hence an aspect of the invention. A preferred aspect is contrast agents as described for use in imaging of and diagnosing of different forms of cancer and metastasis, e.g. breast, skin, colorectal, pancreatic, prostate,
25 lung or ovarian cancer. Alternatively, the contrast agent may be used for detection of diseases where activated macrophages are present such as vulnerable plaque in atherosclerosis.

The present invention also provides a pharmaceutical composition comprising an
30 effective amount, e.g. an amount effective for enhancing image contrast in in vivo imaging of a contrast agent of the invention, or a salt thereof, together with one or more pharmaceutically acceptable adjuvants, excipients or diluents.

Viewed from a further aspect the invention provides the use of a contrast agent of the
35 invention for the manufacture of a contrast enhancing agent for use in a method of diagnosis involving administration of said contrast enhancing agent to a human or animal body and generation of an image of at least part of said body.

The present invention will now be further illustrated by way of non-limiting examples in which the following abbreviations are used:

- 5 BAEEG: Bis-(aminoethyl)ethylene glycol
- Boc: *t*-butoxycarbonyl
- Cy: Cyanine
- DEG: Diethyleneglycol
- DMF: N,N-dimethylformamide
- 10 Fmoc: 9-fluorenylmethoxycarbonyl
- HATU: 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
- HPLC: High performance liquid chromatography
- Krytox 222: 4, 7, 13, 16, 21, 24-hexaoxa-1, 10-diazabicyclo-(8, 8, 8)hexacosane
- 15 LC: Liquid chromatography
- MS: Mass spectroscopy
- NHS: *N*-hydroxysuccinimide
- NMM: N-methylmorpholine
- PEG: Polyethylene glycol
- 20 PBS: Phosphate buffer
- Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonyl
- PyAOP: (7-Azabenzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
- Rink Amide MBHA resin: 4-methylbenzhydrylamin linked to a polystyrene matrix
- RP-HPLC: Reversed phase HPLC
- 25 RT: Room temperature
- Sep-Pak: Separation column with a C18-resin
- TFA: Trifluoroacetic acid
- TIS: Triisopropylsilane

Example 1:**Cy5(bis-SO₃)-*Thr-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-OH* (2)**

5

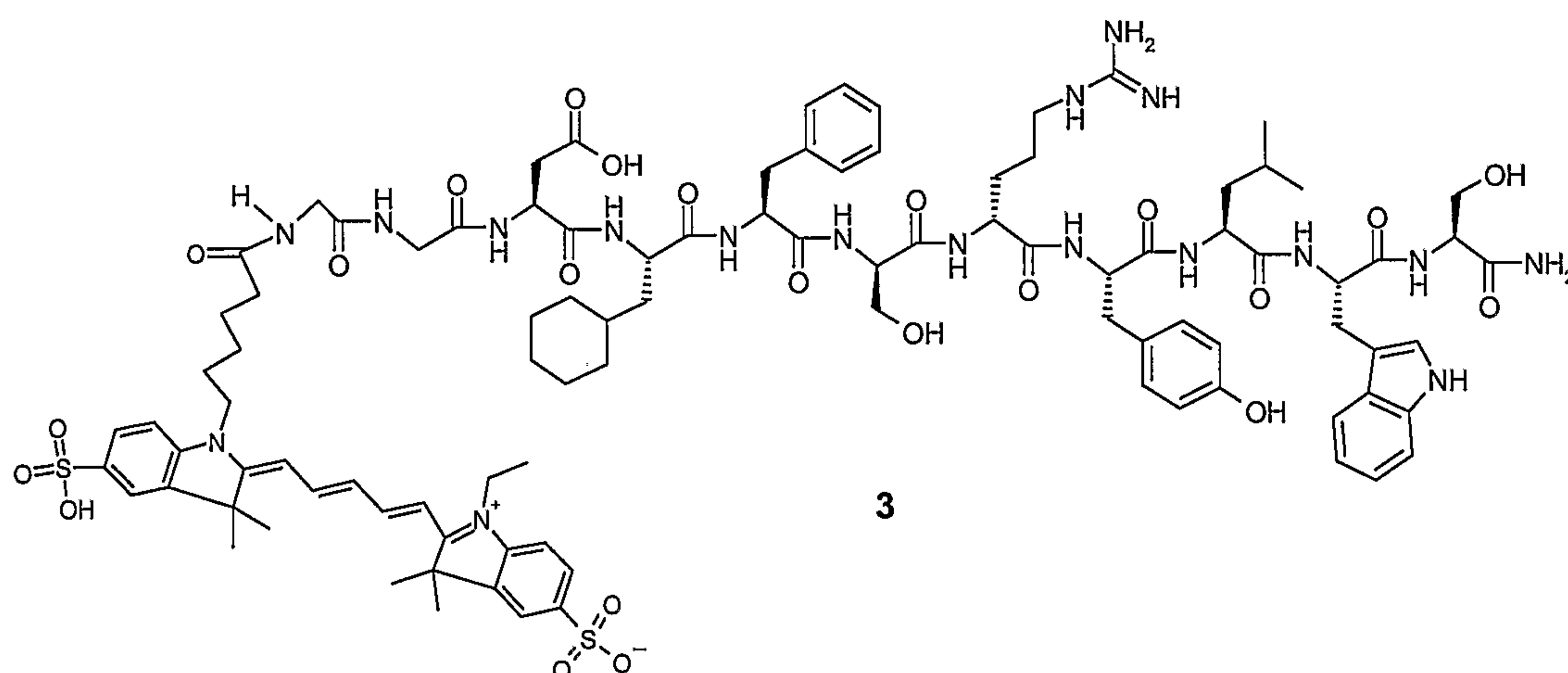
Synthesis of H-*Thr-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-OH* (1)

The peptide corresponding to the above sequence was synthesized by standard solid-phase peptide chemistry.

10 **Conjugation of Cy5(bis SO₃) Mono NHS ester to peptide (1)**

Cy5(bis SO₃) mono NHS ester (1,18 mg, 0,0017 mmol) was dissolved in DMF and peptide (1) (2 mg, 0,0015 mmol) was added as a solid to the solution, followed by the addition of NMM (0,55 µl, 0,005 mmol). The reaction vessel was wrapped in foil and placed on a shaker apparatus for 16 hours. The desired product was confirmed by

15 electrospray MS: [M+H]⁺ of product expected at 1850.85 m/z, found at 1850.9 m/z.

Example 2:**Cy5(bis-SO₃)-Gly-Gly-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-NH₂ (3).**

5

Synthesis of Cy5(bis-SO₃)-Gly-Gly-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-NH₂ (3).

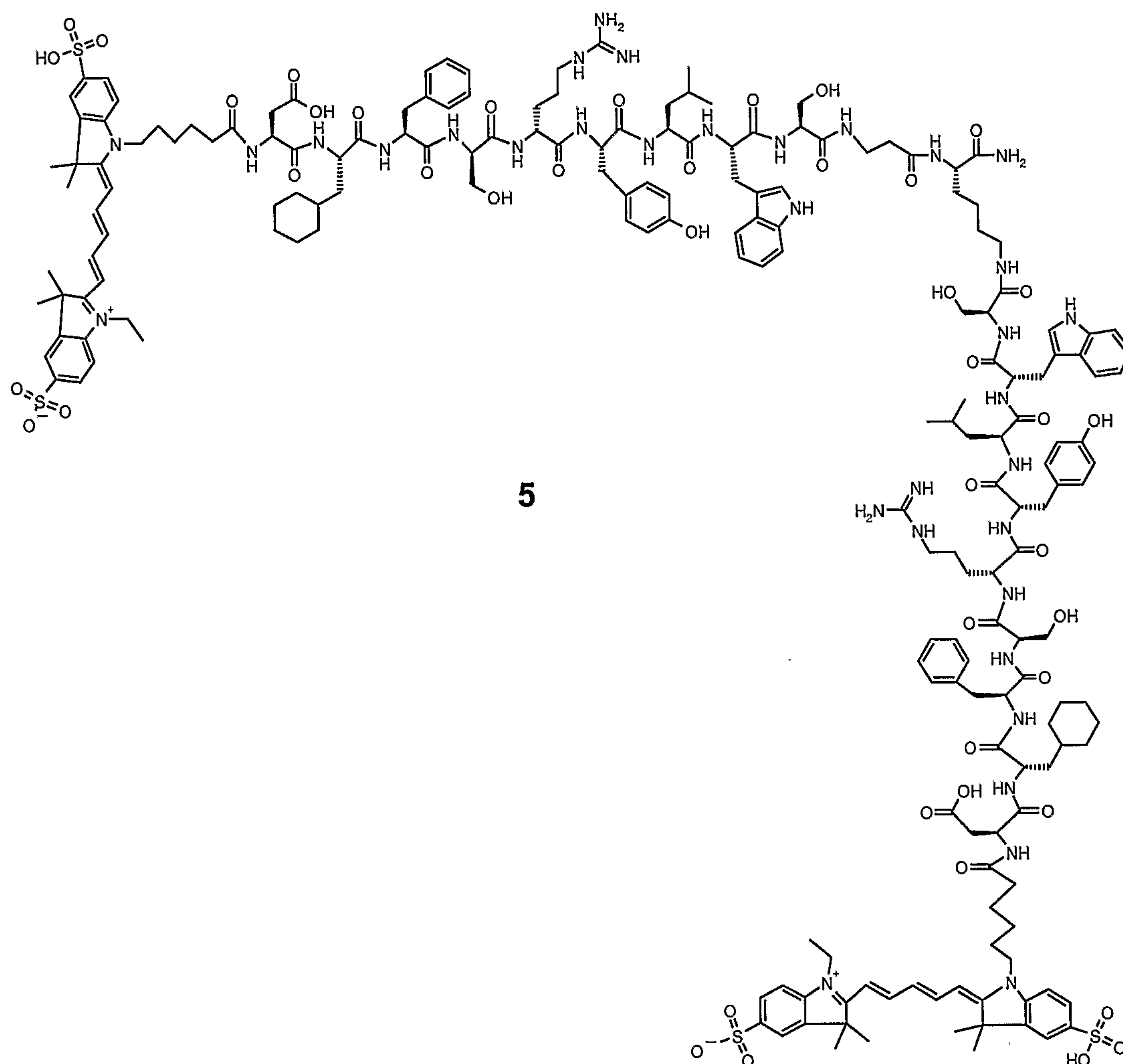
The amino acids in the above sequence were assembled by standard solid-phase peptide chemistry. Cy5(bis SO₃) mono NHS ester (0.5 eq) was then dissolved in

10 DMF and added to the H-Gly-Gly-Asp(OtBu)-Cha-Phe-Ser(tBu)-Arg(Pmc)-Tyr(tBu)-Leu-Trp(Boc)-Ser(tBu)-Rink Amide MBHA resin (1 eq) followed by addition of NMM (3 eq). The reaction vessel was wrapped in foil and placed on a shaker apparatus for 16 hours. The Cy5 dye conjugated peptide was de-protected and cleaved from the resin by treatment with TFA containing 2.5 % water and 2.5 % TIS for 1 hour. The

15 desired product was confirmed by electrospray MS: [M+H]⁺ of product expected at 1977.88 m/z, found at 1977.8 m/z.

Example 3:

Cy5(bis-SO₃)-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-βAla-Lys(Cy5(bis-SO₃)-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser)-NH₂ (5).

**5**

5

Synthesis of H-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-βAla-Lys(H-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser)-NH₂ (4).

The peptide corresponding to the above sequence was assembled by standard solid-phase peptide chemistry.

10

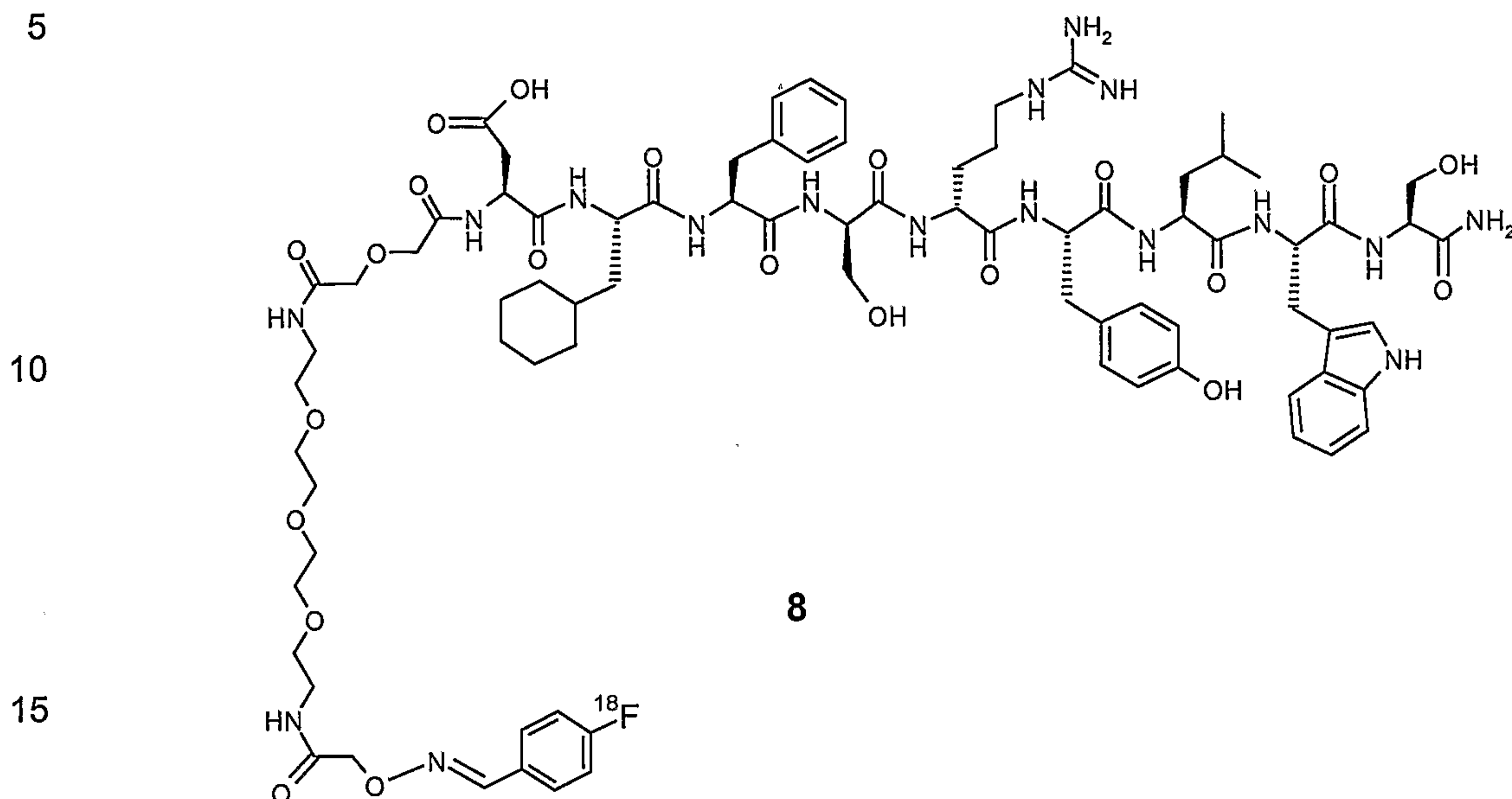
Conjugation of Cy5(bis SO₃) mono NHS ester to peptide (4).

The Cy5(bis SO₃) mono NHS ester (2.2 eq) is dissolved in DMF and peptide (4) (1 eq) is added as a solid to the solution followed by the addition of NMM (3 eq). The reaction vessel is wrapped in foil and placed on a shaker apparatus for 24 hours to afford the desired bis-conjugated product. The product is analyzed by RP-HPLC and electrospray-MS.

15

Example 4

N-(4-¹⁸F-fluorobenzylidene)aminooxyacetyl-PEG(4)-diglycoloyl-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-NH₂ (8).



20 **Synthesis of N-Boc-aminooxyacetyl-PEG(4)-diglycoloyl-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-NH₂ (7)**

Boc-aminooxyacetyl-PEG(4)-diglycolic acid can be prepared by a skilled artisan using an active ester of Boc-aminooxyacetic acid and reacting it with the corresponding amino-PEG(4)-diglycolic acid. The obtained product Boc-aminooxyacetyl-PEG(4)-diglycolic acid (1,4 eq) and PyAOP (1,2 eq) is dissolved in DMF. NMM (2 eq, 200 μ L) is added and the mixture is stirred for 5 min. A solution of peptide (6) (1 eq) and NMM (4 eq) in DMF is added and the reaction mixture is stirred for 30 min. DMF is evaporated in vacuo, and the product is purified using preparative RP-HPLC. The fractions containing the desired product is adjusted to pH 5 using 0.3 % ammonia (NH₃)/water prior to lyophilisation in order to prevent removal of the Boc protecting group. The product is analyzed by PR-HPLC and electrospray-MS.

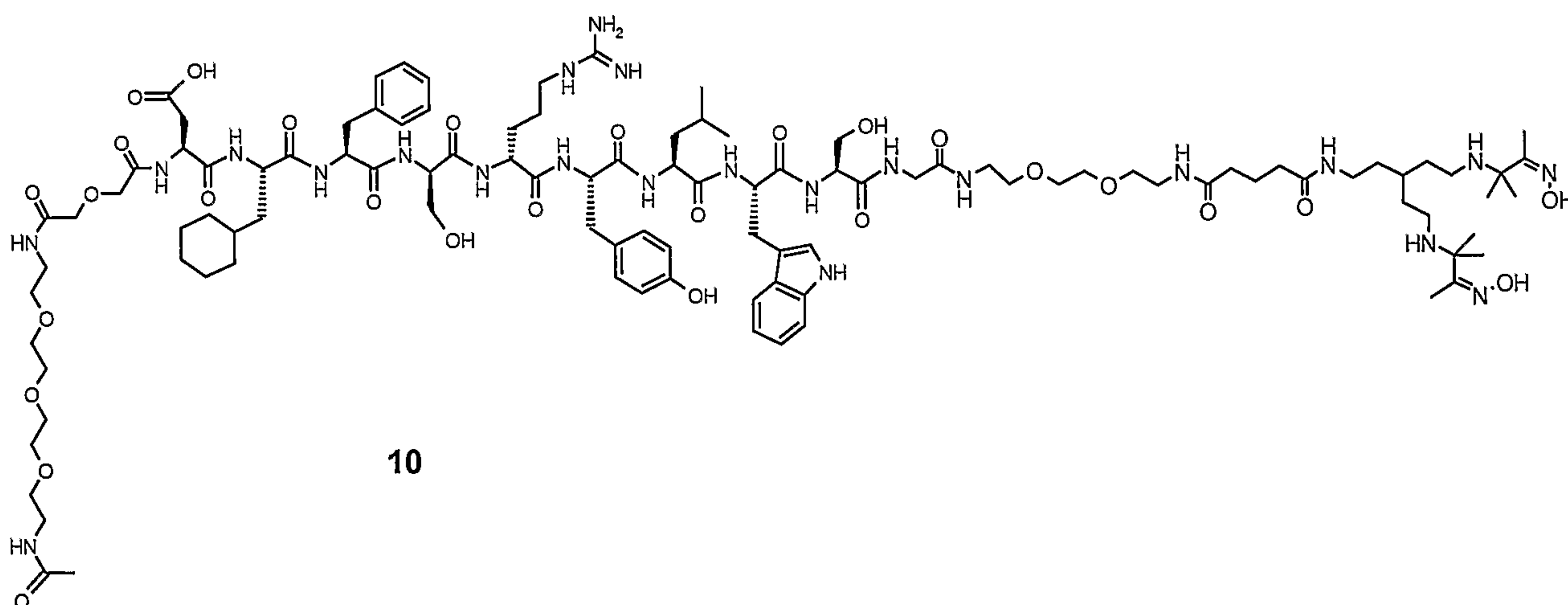
¹⁸F labelling of peptide (7)

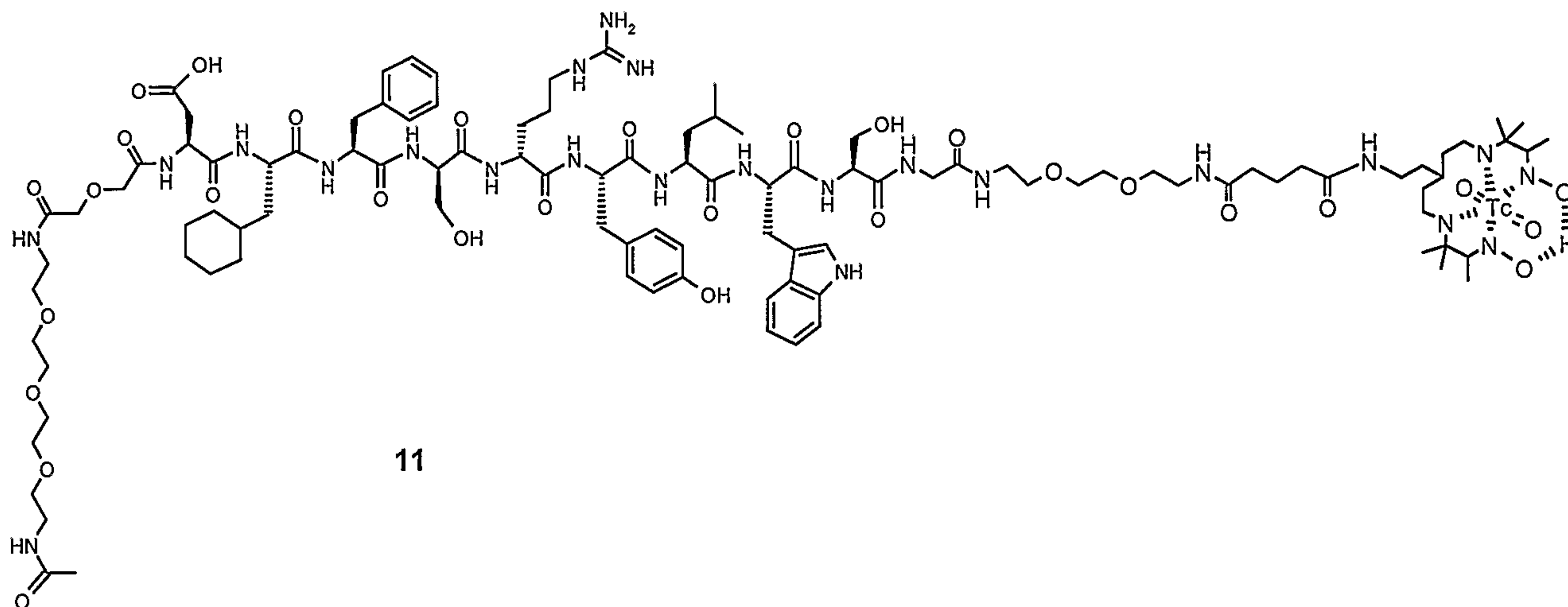
35 ¹⁸F-Fluoride is azeotropically dried in the presence of Kryptofix 222 (5 mg in 0.5 ml ACN) and potassium carbonate (50 μ l of 0.1 M solution) by heating to 110°C under a flow of nitrogen for 20 min. During this time, 3 x 0.5 ml ACN is added and removed.

The mixture is cooled to <40°C and trimethylammonium benzaldehyde triflate, synthesised according to the procedure described by Haka et al in J. Labelled Cpd.& Radiopharms 1989 27(7) 823, (1 mg in 0.4 ml dimethylsulfoxide) is added. The reaction vessel is sealed and heated to 90°C for 15 min to effect labelling. The crude 4-18F-fluorobenzaldehyde solution is then cooled to room temperature. Meanwhile, peptide (7) (6 mg) is treated with 5% water in TFA (200 μ l) for 5 min at room temperature to remove the Boc-protecting group. The solvents are removed in vacuo. The Boc-deprotected peptide is redissolved in 0.1M ammoniumacetate solution (pH4, 0.4 ml) and combined with the crude 4-18F-fluorobenzaldehyde solution in the reaction vessel. The vessel is sealed and heated to 70°C for 15 min to effect conjugation. After cooling to room temperature, the crude conjugate is purified by prep HPLC. The fraction containing the desired conjugate is diluted with 10 ml water and loaded onto a C18 Sep-Pak (pre-prepared by washing sequentially with 10 ml ethanol and 20 ml water). The Sep-Pak is rinsed with 10 ml water, then eluted with 2 ml ethanol. The ethanol is removed in vacuo and the product (8) is formulated in PBS.

Example 5

20 **Acetyl-PEG(4)-diglycoloyl-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-Gly-BAEEG-Glut-cPN216 (10).**





Synthesis of Acetyl-PEG(4)-diglycoloyl-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-Gly-BAEEG-NH₂ (9)

The peptidyl resin corresponding to the above sequence is synthesized by standard solid-phase peptide chemistry. Acetyl-PEG(4)-diglycolic acid (5 eq) and PyAOP (4.5 eq) is dissolved in DMF. NMM (10 eq, 200 μ L) is added and the mixture is stirred for 5 min. The mixture is then added to the peptide resin pre-swollen in DMF and the reaction is continued over night. The peptide is then de-protected and cleaved from the resin using TFA containing 2.5 % water and 2.5 % TIS for 1 hour. The product is analyzed by RP-HPLC and electrospray-MS.

Conjugation of cPN216 to peptide (9).

Peptide (9) (1 eq) is dissolved in DMF and cPN216-glutaryl-tetrafluorothiophenyl ester (2 eq) is added followed by NMM (3 eq). After stirring over night the reaction mixture is worked up by removing the solvent under reduced pressure and the product (10) is purified by preparative RP-HPLC. The product is analyzed by RP-HPLC and electrospray-MS.

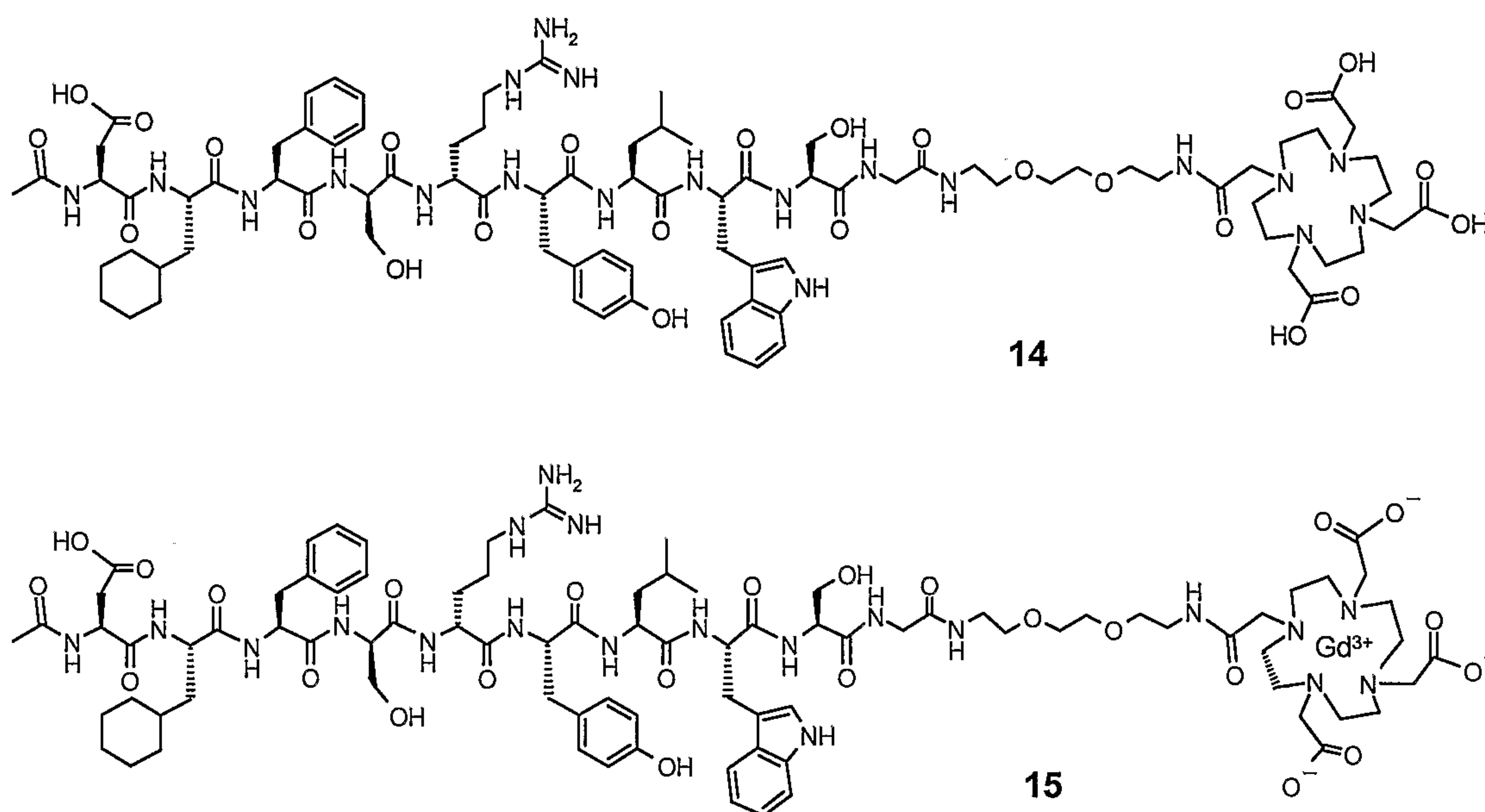
^{99m}Tc-labelling of peptide (10)

Peptide (10) (0.1 mg) is reconstituted in saline or methanol (0.1 ml) and transferred into a freeze dried Toolbox kit of excipients. The Toolbox kit, designed to provide generic radio labelling conditions for amine based chelates, contained stannous chloride dehydrate (16 μ g), methylene diphosphonic acid (25 μ g), sodium hydrogen carbonate (4500 μ g), sodium carbonate (600 μ g), sodium para-aminobenzoate (200 μ g), Kit pH= 9.2. Sodium Pertechnetate (^{99m}Tc) injection (2.1 GBq) in saline (3 ml) is then added, the kit is inverted a few times to dissolve the contents and is then left to

incubate at room temperature for 15-20 min. A sample is analyzed immediately by HPLC and ITLC and the ^{99m}Tc -labelled peptide (**11**) is to be administered to the trial subject between 1-3 hours after reconstitution of the kit.

5 **Example 6**

Gadolinium(III) complex of acetyl-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-Gly-BAEG-Glut-DOTA (15).



10

Synthesis of Acetyl-Asp-Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser-Gly-DEG-NH₂ (**12**)

The peptide corresponding to the above sequence is synthesized by standard solid-phase peptide chemistry.

15 Synthesis of Tri-tBu-DOTA (**13**)

Tri-tBu-DO3A (1,4,7,10-tetraazacyclododecane-N,N',N''-triacetic acid, tri-tert-butyl ester, 10 mmol) and bromoacetic acid (10 mmol) was dissolved in MeOH (50 ml). K₂CO₃ (30 mmol) dissolved in water (50 ml) was added (pH 11 in the MeOH/water mixture) and the reaction was stirred for 24 hours and then heated at 40 degrees for another 24 hours. The crude product was purified by RP-HPLC (Phenomenex Luna 5 micron, C18, 250 x 21.2 mm, gradient 5-50% B over 40 min at 10 ml/min). The desired product was confirmed by electrospray-MS: [M+Na]⁺ of product expected at 595.4 m/z, found at [M+Na]⁺ 595.3 m/z and by ¹H-NMR at 80 degrees.

20

Synthesis of DOTA-conjugated peptide (14)

Tri-*t*Bu-DOTA (**13**) (1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid, tri-*t*ert-butyl ester 1eq) is activated with HATU (1 eq) in DMF in the presence of and NMM (3 eq) for 5 min. The mixture is added to peptide (**12**) and the reaction is
5 continued over night. The solvent is removed in vacuo and the product is *t*Bu-deprotected in TFA containing 5% water. The product is purified by RP-HPLC and analyzed by RP-HPLC and electrospray-MS.

Complexation with Gd(III)

10 The DOTA-conjugated peptide (**14**) (1 eq) is reacted with GdCl₃ (1 eq) in an aqueous solution at pH 6.5 at RT. Uncomplexed Gd(III) is removed by centrifugation of the solution at basic pH. The product (**15**) is isolated by lyophilization.

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CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 24

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THIS IS VOLUME 1 OF 2
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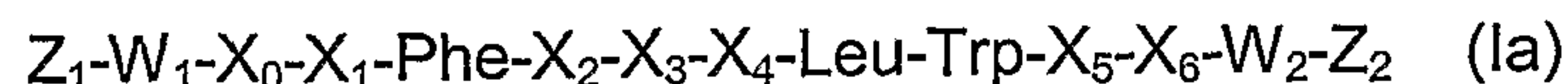
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Claims:

1. A uPAR targeting contrast agent of formula Ia,



5 wherein,

X_0 represents 1-5 amino acids,

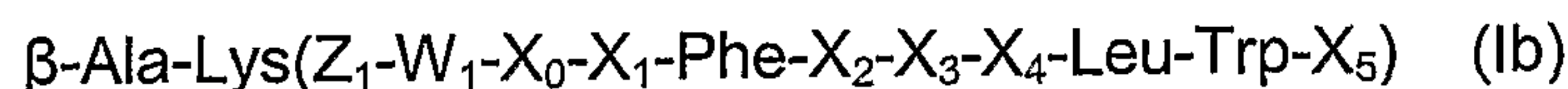
X_1, X_2, X_3, X_4 and X_5 independently represent one amino acid,

Phe represents phenylalanine,

Leu represents leucine,

10 Trp represents tryptophan,

X_6 represents 0 to 5 amino acids or is identified by formula (Ib),



wherein the denotations are defined as for formula Ia,

15 and wherein

β -Ala represents β -Alanine,

Lys represents lysine,

β -Ala of formula Ib is linked to X_5 of formula Ia, and wherein

20 W_1 and W_2 represent the same or different moiety, and are individually a spacer, a biomodifier or is absent, and at least one Z_1 or Z_2 is present representing an imageable moiety capable of detection either directly or indirectly in a diagnostic imaging procedure.

2. A contrast agent as claimed in claim 1 wherein X_0 represents 1 to 5 amino acids selected from the group of alanine, threonine, glycine, aspartic acid and glutamic acid.

3. A contrast agent as claimed in claim 1 or 2 wherein X_1 represents a β -cycloalkylalanine.

30

4. A contrast agent as claimed in any of the claims 1 to 3 wherein X_2 represents serine or alanine.

5. A contrast agent as claimed in any of claims 1 to 4 wherein X_3 represents arginine, an arginine mimetic, tyrosine or alanine.

35

6. A contrast agent as claimed in any of claims 1 to 5 wherein X_4 represents tyrosine, alanine, leucine or cyclohexylalanine

7. A contrast agent as claimed in any of claims 1 to 6 wherein X_5 represents
5 serine, histidine, alanine, tyrosine or leucine.

8. A contrast agent as claimed in any of claims 1 to 7 wherein X_6 comprises amino acids selected from the group of glycine, aspartic acid, lysine, phenylalanine or β -alanine or is absent.

10

9. A contrast agent as claimed in any of claims 1 to 8 comprising the peptide sequence $Z_1-W_1-X_0$ -Cha-Phe-Ser-Arg-Tyr-Leu-Trp-Ser- $X_6-W_2-Z_2$, wherein Cha represents cyclohexylalanine, Ser represents serine, Arg represents arginine, Tyr represents tyrosine and all other denotations are as earlier defined.

15

10. A contrast agent as claimed in any of the previous claims wherein at least one of W_1 and W_2 comprises 1-10 units of a monodispers PEG building block or 1 to 10 amino acid residues.

20 11. A contrast agent of any of the previous claims wherein one or both of Z_1 and Z_2 represent an imageable moiety for use in *in vivo* diagnosis comprising a moiety detectable in radio, SPECT, PET, x-ray, MR, ultrasound or optical imaging.

12. A contrast agent as claimed in claim 11 wherein one or both of Z_1 and Z_2
25 comprise a moiety M representing a gamma emitting moiety for radio or SPECT imaging selected from ^{67}Ga , ^{111}In , ^{123}I , ^{125}I , ^{131}I , $^{81\text{m}}\text{Kr}$, ^{99}Mo , $^{99\text{m}}\text{Tc}$, ^{201}Tl and ^{133}Xe .

13. A contrast agent as claimed in claim 11 wherein one or both of Z_1 and Z_2
comprise a moiety M representing a radio emitter with positron emitting properties for
30 PET imaging selected from ^{11}C , ^{18}F , ^{13}N , ^{15}O , ^{77}F , ^{75}Br , ^{76}Br , ^{124}I , ^{64}Cu , ^{48}V , ^{52}Fe , ^{55}Co , $^{94\text{m}}\text{Tc}$, ^{68}Ga or ^{82}Rb .

14. A contrast agent as claimed in claim 11 wherein one or both of Z_1 and Z_2
comprise a moiety M representing a paramagnetic metal selected from the group of
35 Gd(III), Mn(II), Cu(II), Cr(III), Fe(III), Co(II), Er(II), Ni(II), Eu(III) or Dy(III) or a metal oxide such as a superparamagnetic, ferromagnetic or ferromagnetic species for MR imaging.

15. A contrast agent as claimed in claim 11 wherein at least either of Z_1 and Z_2 represent a light scatterer, a light absorber or a light emitter for optical imaging.

- 5 16. A pharmaceutical composition comprising a contrast agent of any of the previous claims, together with one or more pharmaceutically acceptable adjuvants, excipients or diluents.

17. Contrast agents of any of the claims 1-15 for use in diagnostic imaging.

10

16. A method of generating images of a human or animal body by diagnostic imaging involving administering a contrast agent as claimed in any of claims 1-15 to said body, and generating an image of at least a part of said body to which said contrast agent is administered.