(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 June 2009 (11.06.2009)

PCT

(10) International Publication Number
WO 2009/071444 A2

(51) International Patent Classification:
A61K 49/14 (2006.01) *A61K 51/08* (2006.01)

(21) International Application Number:
PCT/EP2008/065824

(22) International Filing Date:
19 November 2008 (19.11.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/988,840 19 November 2007 (19.11.2007) US
0722650.9 19 November 2007 (19.11.2007) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

(54) Title: NOVEL IMAGING METHOD

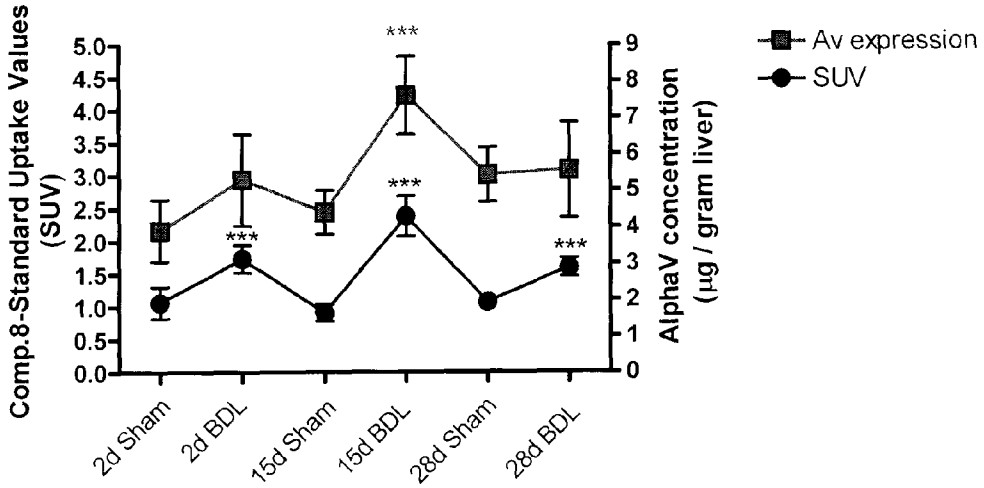

Figure 5

(57) Abstract: The present invention relates to a method useful in facilitating the identification of fibrogenesis in a subject. The method of the invention is particularly useful when applied as part of a method to diagnose fibrogenesis of the liver. The invention also provides a compound for use in a method for identification of fibrogenesis in a subject A further aspect of the invention is a compound for use in the preparation of a medicament for use in a method for identification of fibrogenesis in a subject.

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NOVEL IMAGING METHOD

Technical Field of the Invention

The present invention relates to an *in vivo* imaging method and in particular to a novel application of certain known *in vivo* imaging agents. Preferred methods of *in vivo* imaging of the invention are single-photon emission tomography (SPECT) and positron-emission tomography (PET).

Description of Related Art

There is a wide geographic distribution and high prevalence of insults with the potential to cause liver fibrosis; including chronic viral hepatitis, non-alcoholic steatohepatitis (NASH), parasitemia, inborn errors of metabolism, and toxic damage through alcohol consumption. All these factors mean that fibrosis, leading to cirrhosis and possible cancer of the liver, remains a major cause of morbidity and mortality worldwide. In the United Kingdom alone, liver disease is now the fifth most common cause of mortality, and its incidence is rising (Iredale 2003 BMJ Vol. 327 pp 143–147).

Two types of fatty liver disease exist; non-alcoholic fatty liver disease (NAFLD), and NASH. Around 24% of the US population is thought to have NAFLD, which progresses to NASH at low frequency. NAFLD is associated with the metabolic syndrome, which is linked with obesity, hyperlipidemia, hypertension and type II diabetes. It is believed that in the region of 47 million individuals in USA has the metabolic syndrome. An estimated 8.6 million of the US population are thought to have NASH, which may become associated with fibrosis and cirrhosis with 20-28% of patients with NASH developing cirrhosis over a decade. NAFLD is therefore very common and represents the less severe end of a spectrum of NAFLD that may progress to NASH, and ultimately to cirrhosis of the liver. Liver fibrosis is an indicator of a risk of progression from NASH to cirrhosis.

Currently-used approaches for the detection of liver fibrosis have some notable disadvantages. Liver biopsy analysed histologically for the pattern of collagen deposition is considered the gold standard for assessing liver disease stage and liver fibrosis. However, the procedure is associated with some morbidity, occasional mortality, high costs, sampling

errors and high inter-observer variability among hepatopathologists in categorising the degree of fibrosis. Sampling of liver in biopsy results in only 1/50,000th of the liver being assessed, which can lead to errors in stage diagnosis. Furthermore, as collagen is a marker of fibrotic tissue it is not an ideal target for active disease as it can be found both in the later stages of active fibrosis as well as where the disease process is resolving. There is currently no means by which liver fibrosis can effectively be characterized and monitored *via* a non-invasive procedure. This has a negative impact on early therapeutic intervention which may slow or halt liver fibrosis. Furthermore, in order to monitor disease progression in a timely manner, it is recommended to carry out repeat biopsies every 3-5 years. Available blood tests for detecting liver fibrosis are of limited value because they cannot be used for assessing the degree of fibrosis or for discriminating fibrosis from cirrhosis. Therefore there is no currently available method that can distinguish NAFLD from NASH, or satisfactorily quantify and characterise fibrosis in NASH.

Hepatic stellate cells (HSC) are widely regarded as the principal fibrocompetent cell in the liver. During progressive liver fibrosis, HSC activate and proliferate, but during resolution of fibrosis there is extensive HSC apoptosis that coincides with degradation of the liver scar. This progressive stage of the process of fibrosis is termed fibrogenesis. An upregulation of integrin expression on activated HSC has been reported (Zhou *et al* J. Biol. Chem. 2004; 279(23): 23996-24006). Activation of HSC is critical to the initiation and development of fibrogenesis and subsequently liver fibrosis. Markers of HSC activation therefore represent targeting opportunities for the imaging of fibrogenesis. In this regard, markers of the process that have recently become prominent are integrins (Zhou *et al* 2004 J. Biol. Chem.; 279: 23996-24006; Patsenker *et al* 2007 J. Hepatol.; 46(5): 878-887; Zhou *et al* 2006 J. Biol. Chem.; 281: 39757-39765; Carloni *et al* 1996 Gastroenterology: 110: 1127-36).

The use of suitably labelled integrin binders for *in vivo* imaging applications has previously been described.

WO 2004/020435 discloses integrin-binding piperidinyl compounds that are useful in the treatment of diseases associated with pathological upregulation or disregulation of cell proliferation resulting from expression of α_v integrin, or subtype thereof. WO 2004/020435 also discloses that the compounds of the invention may be conjugated to a

moiety suitable for *in vivo* imaging, and used as a non-invasive tumour imaging agent.

WO 2007/088041 discloses a class of small molecule integrin-binding compounds that are useful in the treatment and/or prevention of a disease, wherein the disease is preferably one mediated by $\alpha_v\beta_1$ integrin. Liver fibrosis is included as a particular disease where the compounds of WO 2007/088041 find use. In addition to treatment and prevention, WO 2007/0880041 teaches that the compounds disclosed therein may include an *in vivo* imaging moiety and be used for *in vivo* imaging.

WO 2003/006491, WO 2005/012335 and WO 2005/123767 relate to RGD peptide-based compounds that bind to receptors associated with angiogenesis, said receptors including integrins. The compounds disclosed comprise either an anti-neoplastic agent or an *in vivo* imaging moiety and are taught as being useful in the treatment and *in vivo* imaging of diseases associated with angiogenesis.

WO 2006/054904 discloses use of RGD peptides labelled with an *in vivo* imaging moiety as contrast agents that target the extracellular matrix (ECM). The contrast agents are said to be useful in the diagnosis and monitoring of diseases related to the excessive formation of collagen, including liver fibrosis. However, there is a disincentive to using RGD peptides for *in vivo* imaging of liver fibrosis as it is known that excretion of radiolabelled integrin-binding RGD peptides occurs primarily *via* the hepatobiliary system (Haubner 1999 J. Nuc. Med.; 40: 1061-71). Additionally, as mentioned above, collagen is not an ideal marker for active fibrosis as it can be found when the disease process is resolving as well as in the latter stages of active disease. It would be more advantageous to target an earlier, active stage, when application of a treatment regimen is probably more appropriate, and also likely to be more clinically effective, e.g. the early stages of fibrosis in NASH, before liver cirrhosis develops. There is therefore a need for a method to identify the early states of liver fibrosis (also termed "fibrogenesis") and thereby intervene in the disease process at a stage where it can be most effectively treated.

Summary of the Invention

The present invention relates to a method useful in facilitating the identification of

fibrogenesis in the liver of a subject. The invention also provides a compound for use in a method for identification of fibrogenesis in the liver of a subject. A further aspect of the invention is a compound for use in the preparation of a medicament for use in a method for identification of fibrogenesis in the liver of a subject. The present invention demonstrates that RGD peptide-based compounds can be effectively used to detect activated hepatic stellate cells (HSCs), thereby providing a method useful in the early diagnosis of liver fibrosis.

Brief Description of the Figures

Figure 1 and Figure 2 show Compound 6 binding specifically to both activated human hepatic stellate cells and EA-Hy926 membranes. Ki was determined as ~10nM in the EA-Hy926 membrane assay, and EC50 as ~1nM in the LX-2 cell assay. Specificity of binding in the LX-2 assay was shown by virtue of the specific inhibition of Compound 6 binding by cold Compound 6. A low affinity scrambled negative control does not bind to integrins or activated stellate cells.

Figure 3 and Figure 4 show Compound 3 binding specifically to both activated liver stellate cell membranes and EA-Hy926 membranes. Ki determined as ~1nM in the EA-Hy926 membrane assay, as observed and 59nM in the stellate cell membrane assay. Compound 8 seems to follow Compound 3 in affinity, Ki determined to 56nM in the activated stellate cell membrane assay and 6nM in the EA-Hy926 membrane assay. The negative control scrambled RGD peptide shows almost no binding to either cell type.

Figure 5 demonstrates specific uptake of Compound 8 by livers from bile duct ligation (BDL) rats compared to negative control scrambled RGD peptide, where no liver uptake was observed. The BDL liver uptake was also proportional to the degree of fibrogenesis, with maximum uptake being observed at 10 and 15 days post-surgery when fibrogenesis is at its highest level.

Figure 6 illustrates specific uptake of RGD-peptide into BDL rat liver vs. a negative control compound (RGD scrambled peptide). Data from this experiment showed that both Compound 3 and Compound 8 were significantly retained in the liver of BDL compared to sham animals at one hour post-injection compared to the negative control

where no significant difference in uptake between BDL and sham was observed.

Figure 7 shows significant alpha$_v$-integrin up-regulation in BDL on day 15 post-op. In conjunction with Figure 7, Figure 8 demonstrates correlation between alpha$_v$-integrin expression and compound uptake in liver of BDL animals at all time point post-surgery.

Detailed Description of the Invention

In one aspect, the present invention relates to a compound of Formula I for use in a method to determine of the presence, location and/or amount of fibrogenesis in the liver of a subject, said method comprising the following steps:

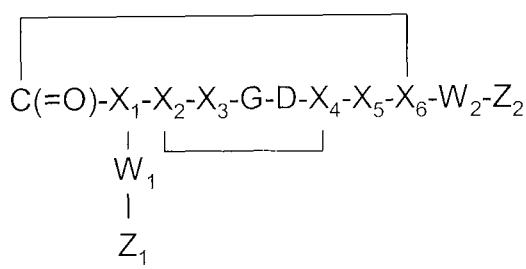
(i) providing a subject to whom a detectable quantity of a compound of Formula I has been administered;

(ii) allowing the compound of Formula I to bind to any fibrogenic tissue in said liver;

(iii) detection of signals emitted by said compound of Formula I by an *in vivo* imaging method; and,

(iv) generation of an image representative of the location and/or amount of said signals;

wherein said compound of Formula I is defined as follows:



(I)

wherein:

G represents glycine;

D represents aspartic acid;

X_1 represents an amino acid selected from aspartic acid, glutamic acid, lysine, homolysine or a C_{3-6} diaminoalkanoic acid, or derivatives thereof;

X_2 and X_4 independently represent amino acid residues whose side chains are linked together to form a cyclising bridge, such as cysteine or homocysteine forming disulphide or thioether bonds, or other amino acids capable of forming a cyclising bridge such as aspartic acid and lysine;

X_3 represents arginine, N-methylarginine or an arginine mimetic;

X_5 represents tyrosine, phenylalanine, 3-iodo-tyrosine C_{4-6} cycloalkylalanine or naphthylalanine, or derivatives thereof;

X_6, represents a thiol-containing amino acid that forms either a thioether bond or a thioacetal bond linking X_6 to the C(=O) group;

W_1 and W_2 are independently an optional linker moiety, wherein when present W_1 is linked to the amino acid side chain moiety of X_1 and W_2 when present is linked to the carboxy group of X_6; and,

Z_1 and Z_2 are independently an imaging moiety, a sugar moiety, and organic dye moiety or hydrogen, with the proviso that at least one of Z_1 and Z_2 is an imaging moiety.

For said compound of Formula I:

X_1 is preferably lysine.

X_2 and X_4 are preferably independently cysteine or homocysteine, and are most preferably both cysteine.

X_3 is preferably arginine.

X_5 is preferably cyclohexylalanine, phenylalanine or 3-iodo-tyrosine, and most preferably phenylalanine.

X_6 is preferably cysteine or homocysteine, and most preferably cysteine.

In a preferred embodiment of Formula I:

X_1 is lysine;

X_2 and X_4 are independently cysteine or homocysteine;

X_3 is arginine;

X_5 is phenylalanine or 3-iodo-tyrosine; and,

X_6 is cysteine or homocysteine.

In a most preferred embodiment of Formula I:

X_1 is lysine;

X_2 and X_4 are both cysteine;

X_3 is arginine;

X_5 is phenylalanine; and,

X_6 is cysteine.

In the present invention, the term "fibrogenesis" specifically relates to the active, progressive stage of fibrosis, when, amongst other things, hepatic stellate cells (HSC) are activated and express integrins. HSCs are widely regarded as the principal fibrocompetent cell in the liver. During fibrogenesis, HSC activate and proliferate, but during resolution of fibrosis there is extensive HSC apoptosis that coincides with degradation of the liver scar. Furthermore, during fibrogenesis, the deposition of extracellular matrix (ECM) components, such as collagen, has not yet taken place. The presence of ECM components is therefore characteristic of the later stages of fibrosis and of resolution of fibrosis. Targeting the disease process during fibrogenesis therefore provides a better indication of active disease where application of treatment is most appropriate.

In the present invention an "amino acid" consists of an amino group, a carboxyl group,

a hydrogen atom , and an amino acid side chain moiety, all bonded (in the case of an alpha amino acid) to a single carbon atom that is referred to as an alpha carbon. Amino acids include, but are not limited to, naturally-occurring amino acids. Naturally-occurring amino acids are those from which the amino acid units of naturally-occurring proteins are derived, and are well-known to those skilled in the art of the present invention. The term "derivatives thereof" when used herein in connection with an amino acid means an amino acid wherein the side chain is a derivative of a naturally-occurring amino acid side chain (see "Amino Acid Derivatives" 1999 Oxford University Press, Barrett, Ed.).

By the term "amino acid mimetic" is meant synthetic analogues of naturally occurring amino acids which are isosteres, i.e. have been designed to mimic the steric and electronic structure of the natural compound. Such isosteres are well known to those skilled in the art and include but are not limited to depsipeptides, retro-inverso peptides, thioamides, cycloalkanes or 1,5-disubstituted tetrazoles (see M. Goodman, Biopolymers, 24, 137, (1985)).

The term "cyclising bridge" refers to any combination of amino acids, or amino acids and -$(CH_2)_o$- or-$(CH_2)_o$-C_6H_4- groups, with functional groups allowing for the introduction of a bridge (where o represents a positive integer from 1 to 10). Preferred examples are disulphides, disulphide mimetics such as the -$(CH_2)_4$- carba bridge, thioacetal, thioether bridges (cystathione or lanthionine), bridges containing esters and ethers, and amide bridges. Preferably, one bridge forms a disulphide bond and a second bridge comprises a thioether (sulphide) bond. When the cyclising bridge is formed by two amino acids, as with X_2 and X_4, for instance the side chain of one of cysteine or homocysteine is linked to the side chain of one of cysteine, homocysteine, serine, threonine, or an aldehyde-containing amino acid to form the cyclising bridge.

An "arginine mimetic" is a synthetic analogue of naturally occurring arginine which is an isostere, in the same way as defined above for amino acid mimetic.

The peptide part of the compound of Formula I can be synthesised using all known methods of chemical synthesis but particularly useful is the solid-phase methodology of

Merrifield employing an automated peptide synthesiser (J. Am. Chem. Soc., 85: 2149 (1964)). Standard procedures for the synthesis strategy are described in E. Atherton & R.C. Sheppard, "Solid Phase Peptide Synthesis: a Practical Approach", 1989, IRL Press, Oxford.

A synthesis resin with an acid-labile linker group, to which the desired protected *C*-terminal amino acid residue is attached by amide bond formation, is used. For example, a so-called Rink amide AM resin with a (dimethoxyphenyl-aminomethyl)-phenoxy-derived linker may be applied (Rink, H. (1987), Tetrahedron Lett. 30, p.3787). Acidolytic cleavage of the peptide from this resin will yield a peptide amide. Alternatively, a O-Bis-(aminoetyl)ethylene glycol trityl resin (K. Barlos et al (1988), Liebigs Ann. Chem, p. 1079) can be used that upon acidolytic cleavage yields a peptide with a primary amine handle.

Labelling with an imaging moiety may be conveniently carried out by means of a "precursor compound", which is a derivative of said compound of Formula I, designed so that chemical reaction with a convenient chemical form of the desired imaging moiety/moieties occurs site-specifically; can be conducted in a minimal number of steps (ideally a single step); and without the need for significant purification (ideally no further purification), to give the desired compound of Formula I. Such precursor compounds are synthetic and can conveniently be obtained in good chemical purity. The precursor compound may optionally comprise one or more protecting groups for certain functional groups of Formula I.

By the term "protecting group" is meant a group which inhibits or suppresses undesirable chemical reactions, but which is designed to be sufficiently reactive that it may be cleaved from the functional group in question under mild enough conditions that do not modify the rest of the molecule. After deprotection the desired product is obtained. Protecting groups are well known to those skilled in the art and are suitably chosen from, for amine groups: Boc (where Boc is *tert*-butyloxycarbonyl), Fmoc (where Fmoc is fluorenylmethoxycarbonyl), trifluoroacetyl, allyloxycarbonyl, Dde [i.e. 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl] or Npys (i.e. 3-nitro-2-pyridine sulfenyl); and for carboxyl groups: methyl ester, *tert*-butyl ester or benzyl ester. For hydroxyl groups, suitable

protecting groups are: methyl, ethyl or *tert*-butyl; alkoxymethyl or alkoxyethyl; benzyl; acetyl; benzoyl; trityl (Trt) or trialkylsilyl such as tetrabutyldimethylsilyl. For thiol groups, suitable protecting groups are: trityl and 4-methoxybenzyl. The use of further protecting groups are described in 'Protective Groups in Organic Synthesis', Theorodora W. Greene and Peter G. M. Wuts, (Third Edition, John Wiley & Sons, 1999).

A "linker moiety" of the present invention is a radical of Formula $-(L)_n-$ wherein:

each L is independently -C(=O)- , $-CR'_2-$, -CR'=CR'- , -C≡C- , $-CR'_2CO_2-$, $-CO_2CR'_2-$, -NR'-, -NR'CO- , -CONR'- , -NR' (C=O)NR'-, -NR'(C=S)NR'-, $-SO_2NR'-$, $-NR'SO_2-$, $-CR'_2OCR'_2-$, $-CR'_2SCR'_2-$, $-CR'_2NR'CR'_2-$, a C_{4-8} cycloheteroalkylene group, a C_{4-8} cycloalkylene group, a C_{5-12} arylene group, a C_{3-12} heteroarylene group, an amino acid, a polyalkyleneglycol, polylactic acid or polyglycolic acid moiety;

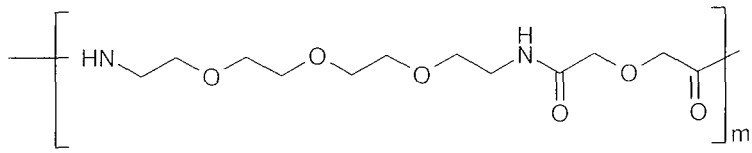
n is an integer of value 1 to 15;

each R' group is independently H or C_{1-10} alkyl, C_{3-10} alkylaryl, C_{2-10} alkoxyalkyl, C_{1-10} hydroxyalkyl, 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;

with the proviso that said linker moiety is a chain of no more than 100 atoms, preferably no more than 50 atoms. The linker moiety is most preferably a chain of between 10 and 50 atoms, and especially preferably a chain of between 10 and 30 atoms. Preferred L groups are -C(=O)-, $-CH_2-$, -NH-, -NHC(=O)-, -C(=O)NH-, $-CH_2-O-CH_2-$, and amino acids.

Preferably, the linker acts as a biomodifier moiety. A "biomodifier moiety" has the function of modifying the pharmacokinetics and blood clearance rates of the compound of Formula I. An example of a suitable biomodifier moiety is one based on a monodisperse PEG building block comprising 1 to 10 units of said building block. Additionally, said biomodifier moiety may also represent 1 to 10 amino acid residues. Preferred amino acid residues for said biomodifier moiety are charged amino acids such as lysine and glutamic

acid, or charged non-natural amino acids such as cysteic acid and phosphonoalanine. In addition, the amino acids glycine, aspartic acid and serine may be included. In a preferred embodiment, the biomodifier moiety comprises a monodisperse PEG-like structure, the 17-amino-5-oxo-6-aza-3,9,12,15-tetraoxaheptadecanoic acid of Formula II:



(II)

wherein m equals an integer from 1 to 10 and where the C-terminal unit is an amide moiety. The biomodifier moiety acts to modify the pharmacokinetics and blood clearance rates of the compounds. The function of the biomodifier moiety in the present invention is to decrease uptake in the tissues and increase excretion *via* the kidneys, thereby resulting in less background interference and giving a better *in vivo* image. The biomodifier moiety can further represent a moiety preferentially derived from glutaric and/or succinic acid and/or a polyethyleneglycol based unit and/or a unit of Formula II as illustrated above. The nature of the linker moiety should not interfere with the affinity of the compound of Formula I for its target receptors. In addition, the linker moiety should not act to increase the background liver uptake of the compound of Formula I, such as may occur if e.g. an overly-large polyethyleneglycol based unit were to be used.

Where either Z_1 or Z_2 is a sugar moiety it too may act as a biomodifier moiety. A "sugar moiety" is a carbohydrate group which is usually an aldehyde or a ketone derivative of a polyhydric alcohol. It may be a monomer (monosaccharide), such as fructose or glucose, or two sugars joined together to form a disaccharide. Disaccharides include sugars such as sucrose, which is made of glucose and fructose. The term sugar includes both substituted and non-substituted sugars, and derivatives of sugars. Preferably, the sugar is selected from glucose, glucosamine, galactose, galactosamine, mannose, lactose, fucose and derivatives thereof, such as sialic acid, a derivative of glucosamine. The sugar is preferably α or β. The sugar may especially be a manno- or galactose pyranoside. The hydroxyl groups on the sugar may be protected with, for example, one or more acetyl groups. The sugar moiety is preferably N-acetylated. Preferred examples of such sugars include N-acetyl

galactosamine, sialic acid, neuraminic acid, N-acetyl galactose, and N-acetyl glucosamine.

The "organic dye moiety" can be any organic dye that interacts with light in the electromagnetic spectrum with wavelengths from the ultraviolet light to the near-infrared. Preferred organic dye moieties include groups having an extensive delocalized electron system. A most preferred organic dye moiety is a cyanine dye (CyDye™). Cyanine dyes are compounds defined by a polyene chain containing an odd number of carbon atoms linked by alternating single and multiple, preferably double, carbon-carbon bonds, terminated at either end by an amino group, one of which is quaternised. The cyanine and analogues aryl-linker-aryl chromophores optionally carry pendant or fused ring substituents. General description of cyanine dyes and synthesis thereof are described in US 6048982, US 5268486 and EP 1037947. Cyanine dyes for the present invention are preferably selected from the group consisting of carbacyanines, oxacyanines, thiacyanines and azacyanines.

The "detection" step of the method described herein involves the of signals emitted by the "imaging moiety" of Formula I by means of a detector sensitive to said signals. This detection step can also be understood as the acquisition of signal data. Examples of signals emitted by the imaging moiety that are suitable for use in the present invention are (i) any that may be detected externally to the human body, such as gamma rays; or, (ii) *via* use of detectors designed for use *in vivo*, such as radiation detectors designed for intra-operative use. The "generation" step of the method described herein is carried out by a computer which applies a reconstruction algorithm to the acquired signal data to yield a dataset. This dataset is then manipulated to generate images showing areas of interest within the subject.

The imaging moiety of the compound of the invention is preferably chosen from:

(i) a radioactive metal ion;

(ii) a gamma-emitting radioactive halogen;

(iii) a positron-emitting radioactive non-metal; and,

(iv) a paramagnetic metal ion.

When the imaging moiety is a radioactive metal ion, i.e. a radiometal, suitable radiometals can be either positron emitters such as ^{64}Cu, ^{48}V, ^{52}Fe, ^{55}Co, ^{94m}Tc or ^{68}Ga; or γ-emitters such as ^{99m}Tc, ^{111}In, ^{113m}In, or ^{67}Ga. Preferred radiometals are ^{99m}Tc, ^{64}Cu, ^{68}Ga and ^{111}In. Most preferred radiometals are γ-emitters, especially ^{99m}Tc.

When the imaging moiety is a paramagnetic metal ion, 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). Preferred paramagnetic metal ions are Gd(III), Mn(II) and Fe(III), with Gd(III) being especially preferred.

When the imaging moiety of the compound of Formula I is a metal ion, it is preferably present as a metal complex of the metal ion with a synthetic ligand. By the term "metal complex" is meant a coordination complex of the metal ion with one or more ligands. It is strongly preferred that the metal complex is "resistant to transchelation", i.e. does not readily undergo ligand exchange with other potentially competing ligands for the metal coordination sites. Potentially competing ligands include other excipients in the preparation *in vitro* (e.g. radioprotectants or antimicrobial preservatives used in the preparation), or endogenous compounds *in vivo* (e.g. glutathione, transferrin or plasma proteins). The term "synthetic" has its conventional meaning, i.e. man-made as opposed to being isolated from natural sources e.g. from the mammalian body. Such compounds have the advantage that their manufacture and impurity profile can be fully controlled.

Suitable ligands for use in the present invention which form metal complexes resistant to transchelation include: chelating agents, where 2-6, preferably 2-4, metal donor atoms are arranged such that 5- or 6-membered chelate rings result (by having a non-coordinating backbone of either carbon atoms or non-coordinating heteroatoms linking the metal donor atoms); or monodentate ligands which comprise donor atoms which bind strongly to the metal ion, such as isonitriles, phosphines or diazenides. Examples of donor atom types which bind well to metals as part of chelating agents are: amines, thiols, amides, oximes, and phosphines. Phosphines form such strong metal complexes that even monodentate or bidentate phosphines form suitable metal complexes. The linear geometry of isonitriles and diazenides is such that they do not lend themselves readily to incorporation into

chelating agents, and are hence typically used as monodentate ligands. Examples of suitable isonitriles include simple alkyl isonitriles such as *tert*-butylisonitrile, and ether-substituted isonitriles such as MIBI (i.e. 1-isocyano-2-methoxy-2-methylpropane). Examples of suitable phosphines include Tetrofosmin, and monodentate phosphines such as *tris*(3-methoxypropyl)phosphine. Examples of suitable diazenides include the HYNIC series of ligands i.e. hydrazine-substituted pyridines or nicotinamides.

When the metal ion is technetium, suitable chelating agents which form metal complexes resistant to transchelation include, but are not limited to:

(i) diaminedioximes;

(ii) N_3S ligands having a thioltriamide donor set such as MAG_3 (mercaptoacetyltriglycine) and related ligands; or having a diamidepyridinethiol donor set such as Pica;

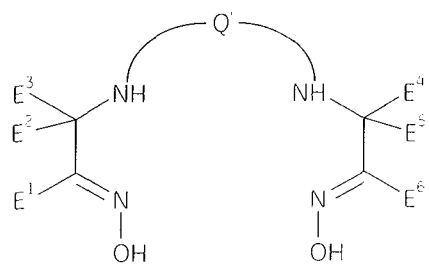
(iii) N_2S_2 ligands having a diaminedithiol donor set such as BAT or ECD (i.e. ethylcysteinate dimer), or an amideaminedithiol donor set such as MAMA;

(iv) N_4 ligands which are open chain or macrocyclic ligands having a tetramine, amidetriamine or diamidediamine donor set, such as cyclam, monoxocyclam dioxocyclam; and,

(v) N_2O_2 ligands having a diaminediphenol donor set.

Preferred chelating agents of the invention when the imaging moiety is technetium are diaminedioximes and tetraamines, preferred versions of which are now described in more detail.

Preferred diaminedioximes are of Formula VII:



(VII)

where E^1-E^6 are each independently an R* group;

each R* is H or C_{1-10} alkyl, C_{3-10} alkylaryl, C_{2-10} alkoxyalkyl, C_{1-10} hydroxyalkyl, C_{1-10} fluoroalkyl, C_{2-10} carboxyalkyl or C_{1-10} aminoalkyl, or two or more R* groups together with the atoms to which they are attached form a carbocyclic, heterocyclic, saturated or unsaturated ring, and wherein one or more of the R* groups is conjugated to the CBP;

and Q' is a bridging group of formula $-(J')_e-$;

where e is 3, 4 or 5 and each J' is independently -O-, -NR*- or $-C(R^*)_2-$ provided that $-(J')_e-$ contains a maximum of one J' group which is -O- or -NR*-.

Preferred Q' groups are as follows:

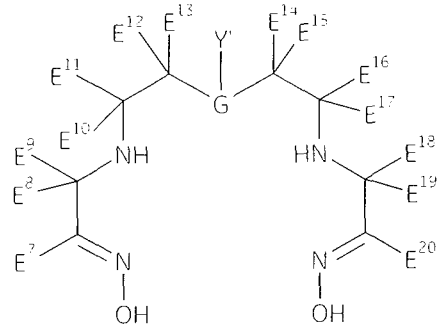
Q' = $-(CH_2)(CHR^*)(CH_2)-$ i.e. propyleneamine oxime or PnAO derivatives;

Q' = $-(CH_2)_2(CHR^*)(CH_2)_2-$ i.e. pentyleneamine oxime or PentAO derivatives;

Q' = $-(CH_2)_2NR^*(CH_2)_2-$.

E^1 to E^6 are preferably chosen from: C_{1-3} alkyl, alkylaryl alkoxyalkyl, hydroxyalkyl, fluoroalkyl, carboxyalkyl or aminoalkyl. Most preferably, each E^1 to E^6 group is CH_3.

The diaminedioxime is preferably conjugated at either the E^1 or E^6 R* group, or an R* group of the Q' moiety. Most preferably, it is conjugated at an R* group of the Q' moiety. When it is conjugated at an R* group of the Q' moiety, the R* group is preferably at the bridgehead position. In that case, Q' is preferably $-(CH_2)(CHR^*)(CH_2)-$, $-(CH_2)_2(CHR^*)(CH_2)_2-$ or $-(CH_2)_2NR^*(CH_2)_2-$, most preferably $-(CH_2)_2(CHR^*)(CH_2)_2-$. An especially preferred diaminedioxime has the Formula VIIa:



(VIIa)

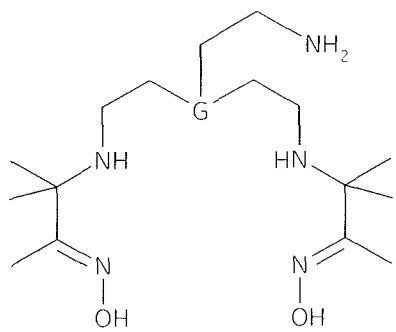
where:

E^7-E^{20} are each independently an R* group as defined above;

G is N or CR*; and

Y' is the point of attachment to the peptide portion of Formula I.

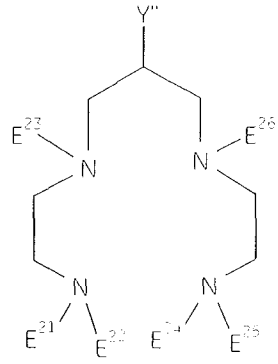
A preferred chelator of Formula VIIa is of Formula VIIb:



(VIIb)

where G is as defined above, and is preferably CH. A method for the preparation of Chelate I is disclosed in WO 03/006070.

Preferred tetraamine chelators are of Formula VIII:



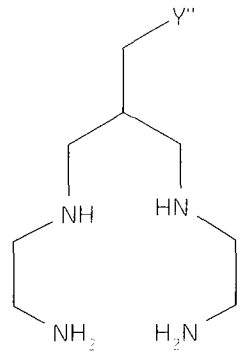
(VIII)

wherein:

Y" is the point of attachment to the rest of Formula I; and,

E^{21} to E^{26} are R* groups as previously defined.

A most preferred tetraamine chelate is of Formula VIIIa:



(VIIIa)

wherein Y" is as defined above. A method for the synthesis of a chelate of Formula VIIIa is disclosed in WO 06/008496.

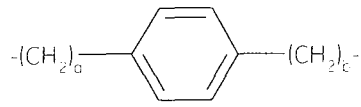
The above described ligands are particularly suitable for complexing technetium e.g. ^{94m}Tc or ^{99m}Tc, and are described more fully by Jurisson *et al* (Chem.Rev., 99, 2205-2218 (1999)). The ligands are also useful for other metals, such as copper (^{64}Cu or ^{67}Cu), vanadium (e.g. ^{48}V), iron (e.g. ^{52}Fe), or cobalt (e.g. ^{55}Co).

Other suitable ligands are described in Sandoz WO 91/01144, which includes ligands which are particularly suitable for indium, yttrium and gadolinium, especially macrocyclic aminocarboxylate and aminophosphonic acid ligands. Ligands which form non-ionic (i.e.

neutral) metal complexes of gadolinium are known and are described in US 4885363. Particularly preferred for gadolinium are chelates including DTPA, ethylene diamine tetraacetic acid (EDTA), triethylene tetraamine hexaacetic acid (TTHA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A) and derivatives of these.

When the imaging moiety is a metal ion present as part of a metal complex, an associated linker moiety is preferably present. The role of the linker moiety in this case is to distance the relatively bulky metal complex, which results upon metal coordination, from the active site of the peptide so that e.g. substrate binding is not impaired. This can be achieved by a combination of flexibility (e.g. simple alkyl chains), so that the bulky group has the freedom to position itself away from the active site and/or rigidity such as a cycloalkyl or aryl spacer which orientates the metal complex away from the active site. Preferred linker moieties in the context of these chelators have a backbone chain which contains 2 to 10 atoms, most preferably 2 to 5 atoms, with 2 or 3 atoms being especially preferred. A minimum linker group backbone chain of 2 atoms confers the advantage that the chelator is well-separated from the peptide so that any interaction is minimised. Furthermore, the peptide is unlikely to compete effectively with the coordination of the chelator to the metal ion. In this way, both the biological targeting characteristics of the peptide, and the metal complexing capability of the chelator are maintained. It is strongly preferred that the metal complex is bound to the peptide in such a way that the linkage does not undergo facile metabolism in blood. That is because such metabolism would result in the imaging metal complex being cleaved off before the labelled peptide reaches the desired *in vivo* target site. The peptide is therefore preferably covalently bound to the metal complex *via* linker moieties comprising linkages which are not readily metabolised. Suitable such linkages are carbon-carbon bonds, amide bonds, urea or thiourea linkages, or ether bonds.

Non-peptide linker groups such as alkylene groups or arylene groups have the advantage that there is no significant hydrogen bonding with the peptide part of Formula I so that the linker does not interact with the peptide. Preferred alkylene spacer groups are $-(CH_2)_q-$ where q is an integer of value 2 to 5. Preferably q is 2 or 3. Preferred arylene spacers are of Formula IX:



(IX)

where: a and b are each independently 0, 1 or 2.

A preferred linker moiety here is thus $-CH_2CH_2-(L)_p-$ where L is as defined above and p is an integer of value 0 to 3. Most preferably, $-(L)_p-$ is -CO- or -NR-. For Formula VIIb, when G is N and $-(L)_p-$ is -NH-, this grouping has the additional advantage that it stems from the symmetrical intermediate $N(CH_2CH_2NH_2)_3$, which is commercially available.

When the imaging metal is technetium, the usual technetium starting material is pertechnetate, i.e. TcO_4^- which is technetium in the Tc(VII) oxidation state. Pertechnetate itself does not readily form metal complexes, hence the preparation of technetium complexes usually requires the addition of a suitable reducing agent such as stannous ion to facilitate complexation by reducing the oxidation state of the technetium to the lower oxidation states, usually Tc(I) to Tc(V). The solvent may be organic or aqueous, or mixtures thereof. When the solvent comprises an organic solvent, the organic solvent is preferably a biocompatible solvent, such as ethanol or DMSO. Preferably the solvent is aqueous, and is most preferably isotonic saline.

When the imaging moiety is a gamma-emitting radioactive halogen, the radiohalogen is suitably chosen from ^{123}I, ^{131}I or ^{77}Br. ^{125}I is specifically excluded as it is not suitable for use as an imaging moiety for external *in vivo* imaging. A preferred gamma-emitting radioactive halogen for *in vivo* imaging is ^{123}I.

Where the imaging moiety is radioiodine, the compound of Formula I can be obtained by means of a precursor compound comprising a derivative which either undergoes electrophilic or nucleophilic iodination or undergoes condensation with a labelled aldehyde or ketone. Examples of the first category are:

(a) organometallic derivatives such as a trialkylstannane (e.g. trimethylstannyl or tributylstannyl), or a trialkylsilane (e.g. trimethylsilyl) or an organoboron compound (e.g. boronate esters or organotrifluoroborates);

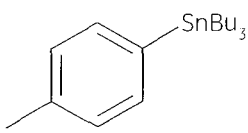
(b) a non-radioactive alkyl bromide for halogen exchange or alkyl tosylate, mesylate or triflate for nucleophilic iodination;

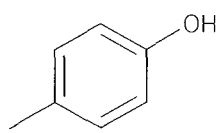
(c) aromatic rings activated towards nucleophilic iodination (e.g. aryl iodonium salt aryl diazonium, aryl trialkylammonium salts or nitroaryl derivatives).

Preferred such precursor compounds comprise: a non-radioactive halogen atom such as an aryl iodide or bromide (to permit radioiodine exchange); an organometallic precursor compound (e.g. trialkyltin, trialkylsilyl or organoboron compound); or an organic precursor such as triazenes or a good leaving group for nucleophilic substitution such as an iodonium salt. Preferably for radioiodination, the precursor compound comprises an organometallic precursor compound, most preferably trialkyltin.

Precursor compounds and methods of introducing radioiodine into organic molecules are described by Bolton (J.Lab.Comp.Radiopharm., 45, 485-528 (2002)). Suitable boronate ester organoboron compounds and their preparation are described by Kabalka *et al* (Nucl.Med.Biol., 29, 841-843 (2002) and 30, 369-373(2003)). Suitable organotrifluoroborates and their preparation are described by Kabalka *et al* (Nucl.Med.Biol., 31, 935-938 (2004)).

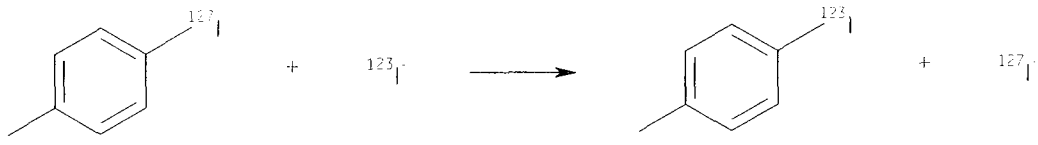
Examples of aryl groups to which radioactive iodine can be attached are given below:





Both contain substituents which permit facile radioiodine substitution onto the aromatic ring. A tyrosine residue permits radioiodination to be carried out using its inherent phenol group.

Alternative substituents containing radioactive iodine can be synthesised by direct iodination *via* radiohalogen exchange, e.g.



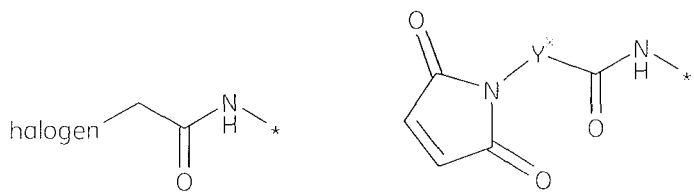
The radioiodine atom is preferably attached *via* a direct covalent bond to an aromatic ring such as a benzene ring, or a vinyl group since it is known that iodine atoms bound to saturated aliphatic systems are prone to *in vivo* metabolism and hence loss of the radioiodine.

When the imaging moiety is a positron-emitting radioactive non-metal, suitable such positron emitters include: ^{11}C, ^{13}N, ^{15}O, ^{17}F, ^{18}F, ^{75}Br, ^{76}Br or ^{124}I. Preferred positron-emitting radioactive non-metals are ^{11}C, ^{13}N, ^{18}F and ^{124}I, especially ^{11}C and ^{18}F, most especially ^{18}F.

Radiofluorination may be carried out *via* direct labelling using the reaction of ^{18}F-fluoride with a suitable chemical group in a precursor compound having a good leaving group, such as an alkyl bromide, alkyl mesylate or alkyl tosylate. ^{18}F can also be introduced by alkylation of N-haloacetyl groups with a $^{18}F(CH_2)_3OH$ reactant, to give -$NH(CO)CH_2O(CH_2)_3{}^{18}F$ derivatives. For aryl systems, ^{18}F-fluoride nucleophilic displacement from an aryl diazonium salt, aryl nitro compound or an aryl quaternary ammonium salt are suitable routes to aryl-^{18}F derivatives.

A ^{18}F-labelled compound of the invention may be obtained by formation of ^{18}F fluorodialkylamines and subsequent amide formation when the ^{18}F fluorodialkylamine is reacted with a precursor containing, e.g. chlorine, $P(O)Ph_3$ or an activated ester.

A further approach for radiofluorination, which is particularly suitable for radiofluorination of peptides, is described in WO 03/080544 and uses thiol coupling. A precursor compound comprising of one of the following substituents:



is reacted with a compound of Formula X:

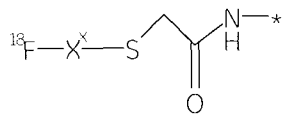
$$^{18}F\text{-}X^{X}\text{-}SH \qquad (X)$$

wherein Y^X is a linker moiety of formula $-(L^Y)_y-$ wherein L^Y is as previously defined for L, y is 1-10 and optionally includes 1-6 heteroatoms;

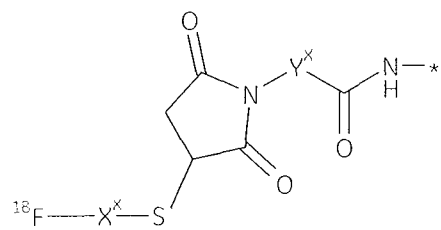
X^X is a linker of formula $-(L^X)_x-$ wherein L^X is as previously defined for L, x is 1-30 and optionally includes 1 to 10 heteroatoms; and,

* defines the point of attachment to the rest of the compound;

to give radiofluorinated imaging agents of formula (Xa) or (Xb) respectively:



(Xa)



(Xb)

wherein X^X, Y^X, and * are as defined above.

An additional approach particularly suitable for radiofluorination of peptides is described in WO 04/080492 and makes use of aminoxy coupling. Radiofluorination is carried out by reaction of a precursor compound of formula (XI) with a compound of formula (XIa):

R^1-* (XI)

$^{18}F\text{-}X^{XI}\text{–}R^2 \qquad (XIa)$

or,

by reaction of a precursor compound of formula (XII) with a compound of formula

(XIIa):

R^3-* (XII)

^{18}F-X^{XII}-R^4 (XIIa)

wherein;

X^{XI} and X^{XII} are linker groups -$(L^{XI})_z$- wherein L^{XI} is as previously defined for L, z is 1-10 and optionally includes 1-6 heteroatoms;

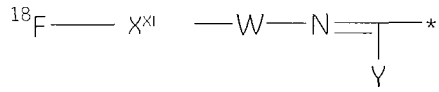
R^1 is an aldehyde moiety, a ketone moiety, a protected aldehyde such as an acetal, a protected ketone, such as a ketal, or a functionality, such as diol or N-terminal serine residue, which can be rapidly and efficiently oxidised to an aldehyde or ketone using an oxidising agent;

R^2 is a functional group which, under mild conditions such as aqueous buffer, reacts site-specifically with R^1 yielding a stable conjugate. R^2 can be ammonia derivatives such as primary amine, secondary amine, hydroxylamine, hydrazine, hydrazide, aminoxy, phenylhydrazine, semicarbazide, or thiosemicarbazide, and is preferably a hydrazine, hydrazide or aminoxy group;

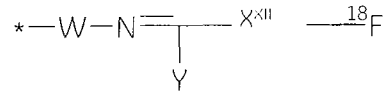
R^3 is a functional group which reacts site-specifically with R^4. R^3 can be ammonia derivatives such as primary amine, secondary amine, hydroxylamine, hydrazine, hydrazide, aminoxy, phenylhydrazine, semicarbazide, or thiosemicarbazide, and is preferably a hydrazine, hydrazide or aminoxy group;

R^4 is an aldehyde moiety, a ketone moiety, a protected aldehyde such as an acetal, a protected ketone, such as a ketal, or a functionality, such as diol or N-terminal serine residue, which can be rapidly and efficiently oxidised to an aldehyde or ketone using an oxidising agent;

to give a conjugate of formula (XIII) or (XIV), respectively:



(XIII)



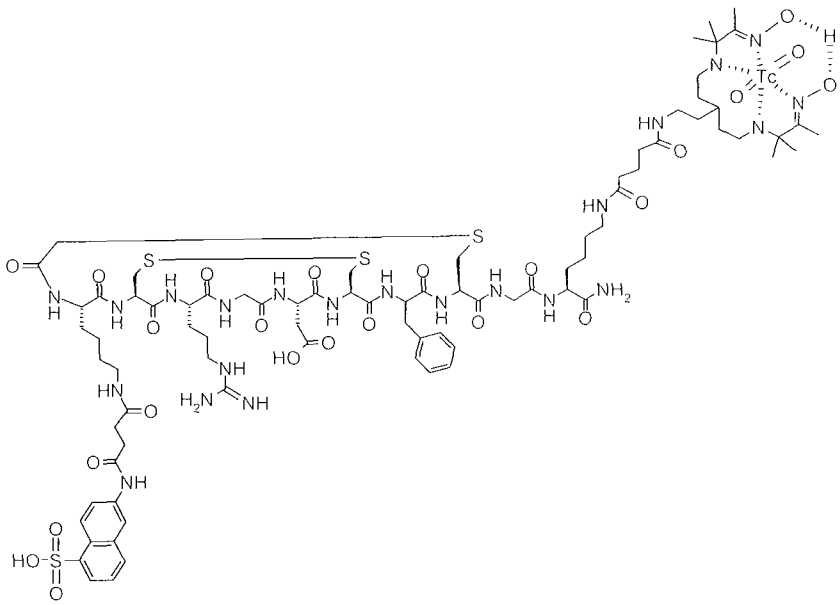
(XIV)

wherein W is -CO-NH- , -NH- , –O-, -NHCONH-, or –NHCSNH-, and is preferably -CO-NH- , -NH- or -O- ; Y is H, C_{1-6} alkyl or C_{5-6} aryl substituents, and wherein X^{XI}, X^{XII} and * are as previously defined.

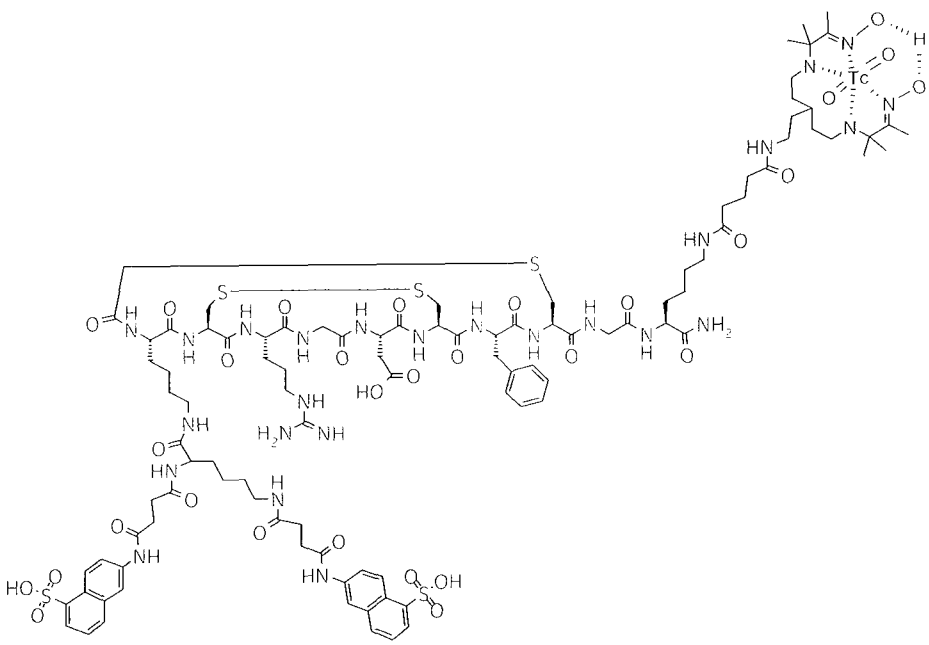
Further details of synthetic routes to ^{18}F-labelled derivatives are described by Bolton, J.Lab.Comp.Radiopharm., 45, 485-528 (2002).

Preferred imaging moieties are those which can be detected externally in a non-invasive manner following administration *in vivo*, such as by means of single photon emission tomography (SPECT), positron emission tomography (PET) and magnetic resonance imaging (MRI). Most preferred imaging moieties are radioactive, especially radioactive metal ions, gamma-emitting radioactive halogens and positron-emitting radioactive non-metals, particularly those suitable for imaging using SPECT or PET, e.g. ^{99m}Tc, ^{123}I, ^{11}C and ^{18}F.

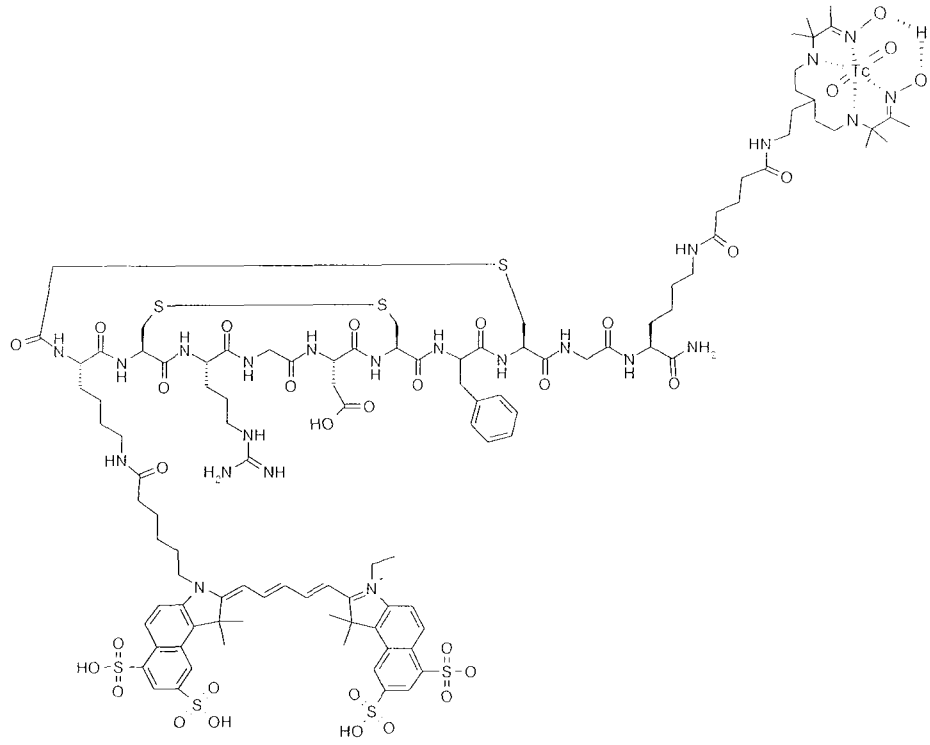
In one preferred embodiment, W_1 and W_2 both represent linker moieties, Z_1 represents an organic dye moiety, and Z_2 represents an imaging moiety. These compounds and methods for their preparation are presented in WO 2006/054904. Most preferably, Z_2 is a radioactive imaging moiety. Examples of these preferred compounds include the following:



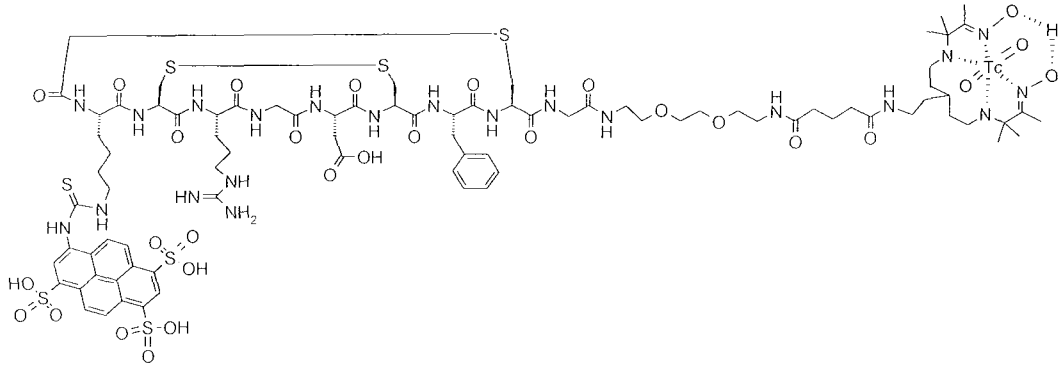
Compound 1



Compound 2



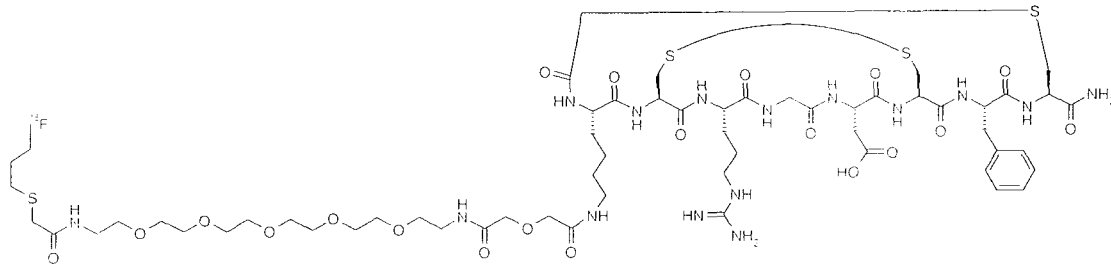
Compound 3



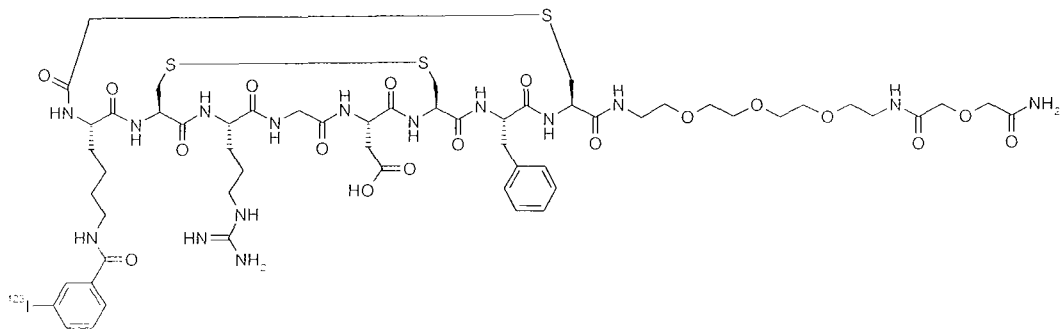
Compound 4

Methods for the preparation of Compounds 1 to 4 are detailed in WO 2006/054904.

In an alternative preferred embodiment, W_1 represents a linker moiety, Z_1 represents an imaging moiety, W_2 represents an optional linker moiety, and Z_2 is hydrogen. These compounds and methods for their preparation are presented in WO 2005/012335. Examples of these preferred compounds include the following:



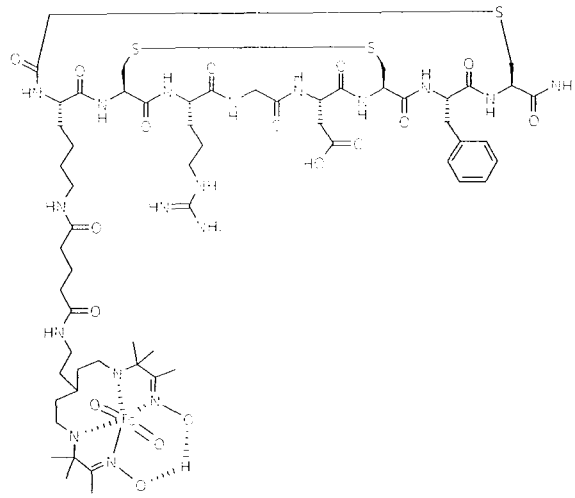
Compound 5



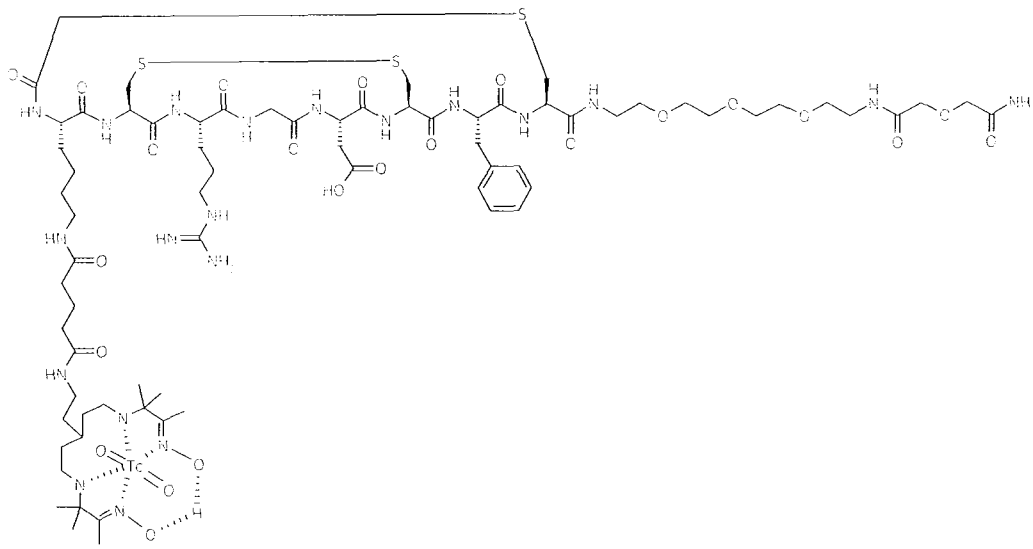
Compound 6

Methods for the preparation of Compounds 5 and 6 are detailed in WO 2005/012335

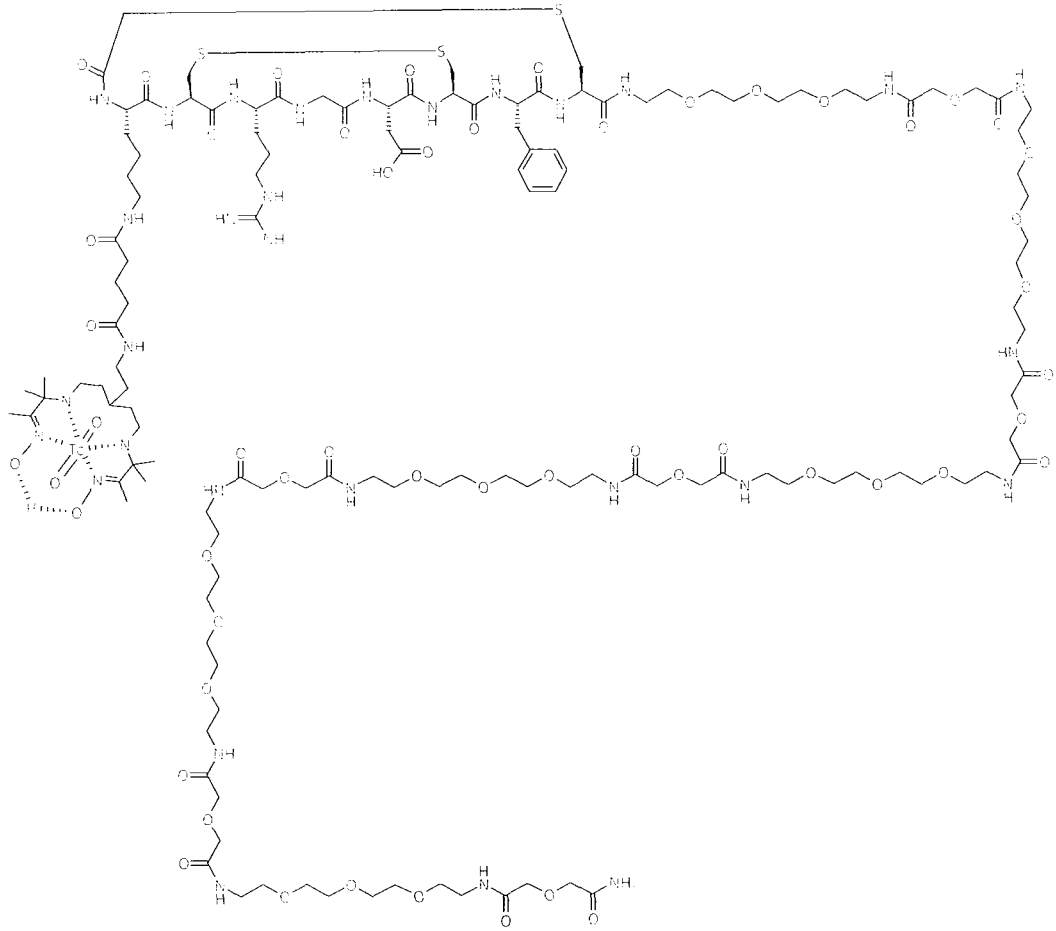
In a yet further alternative preferred embodiment, W_1 represents an optional linker moiety, Z_1 is hydrogen, W_2 represents a linker moiety, and Z_2 represents an imaging moiety. These compounds and methods for their preparation are presented in WO 2003/006491. Examples of these preferred compounds include the following:



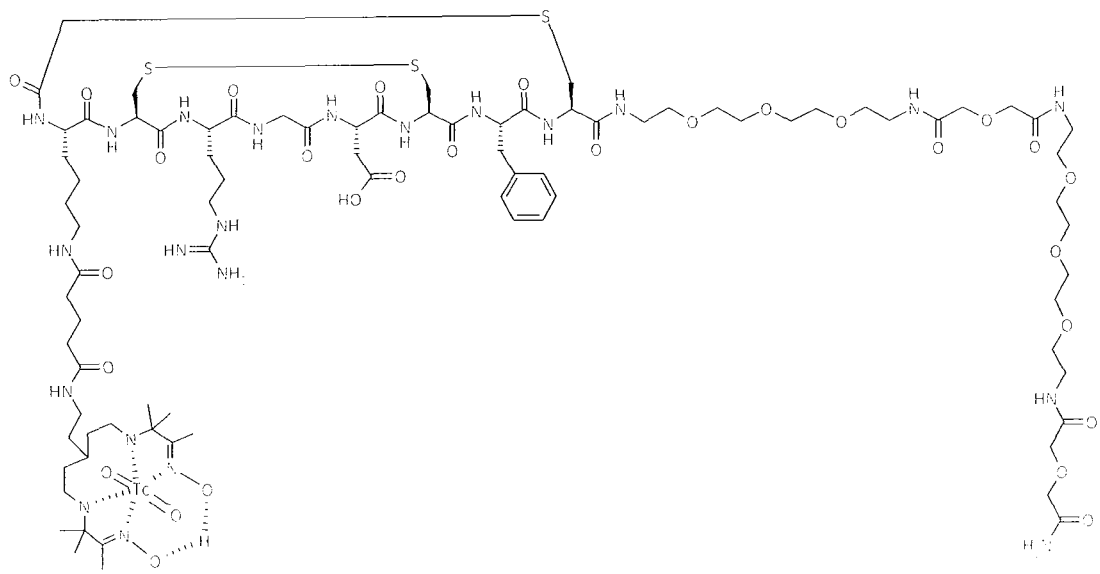
Compound 7



Compound 8



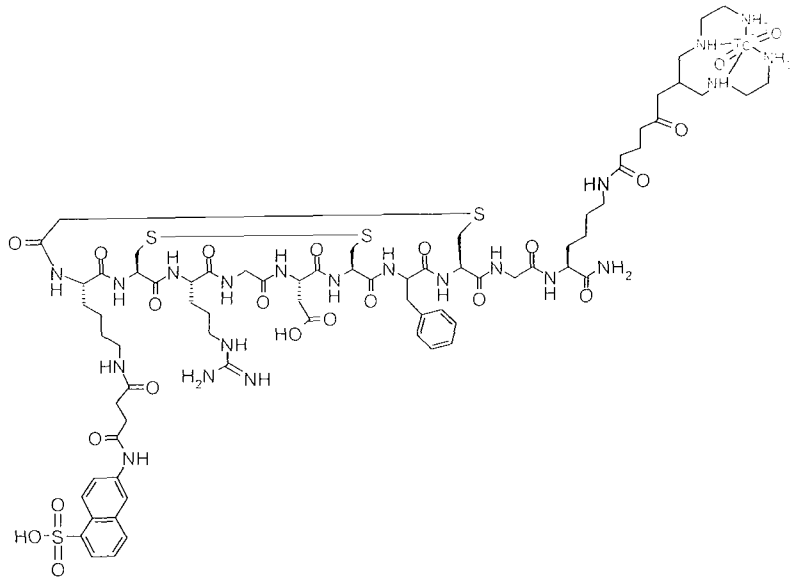
Compound 9



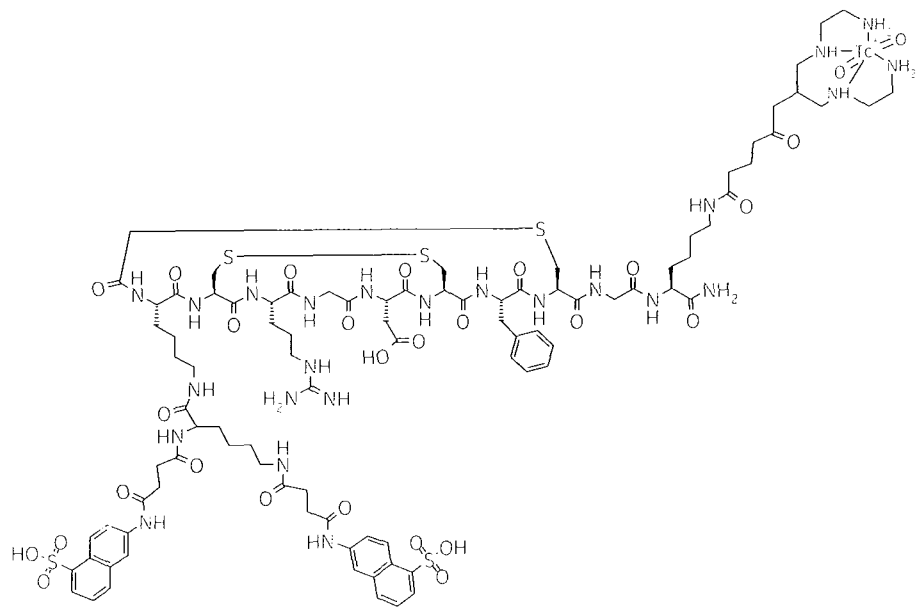
Compound 10

Methods for the preparation of Compounds 7 to 10 are detailed in WO 2003/006491.

For Compounds 1 to 4 and 7 to 10, it is furthermore alternatively preferred that the diamine dioxime chelate moiety is replaced with a tetraamine chelate moiety to form Compounds 1a to 4a and 7a to 10a:



Compound 1a



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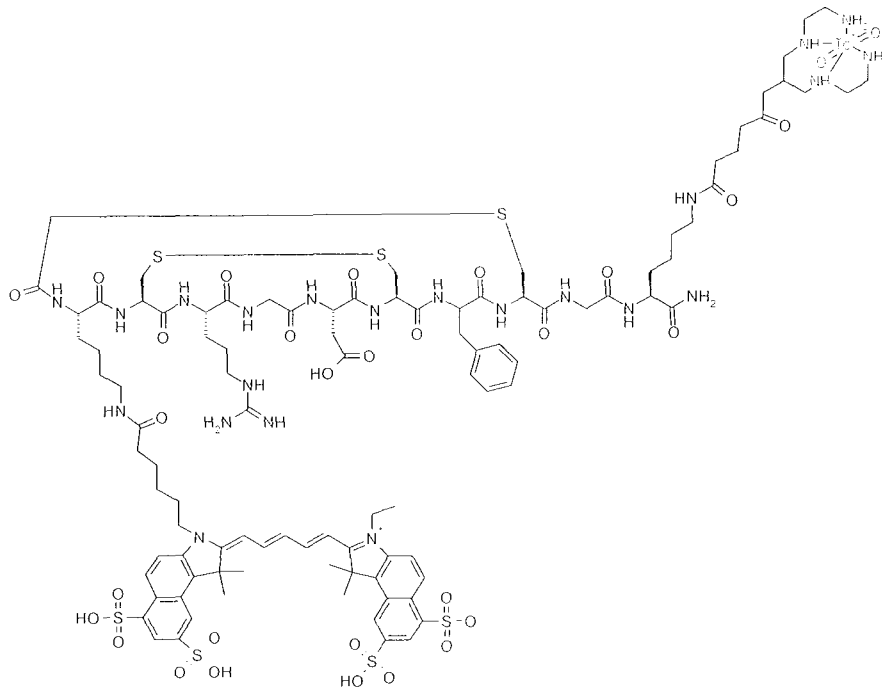

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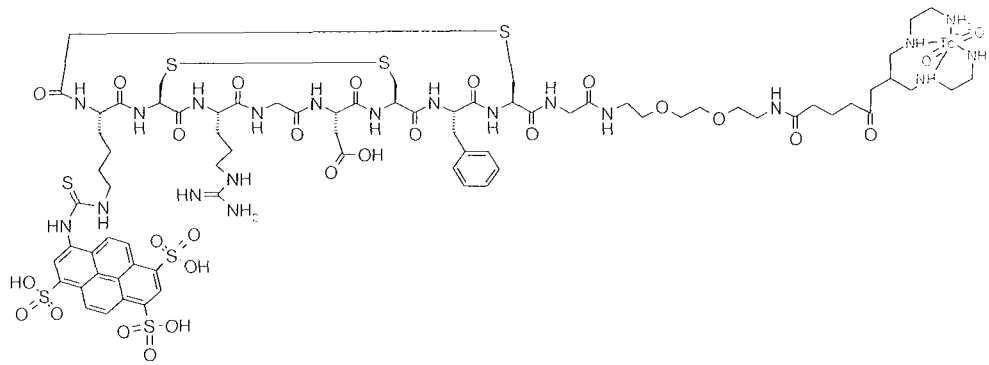
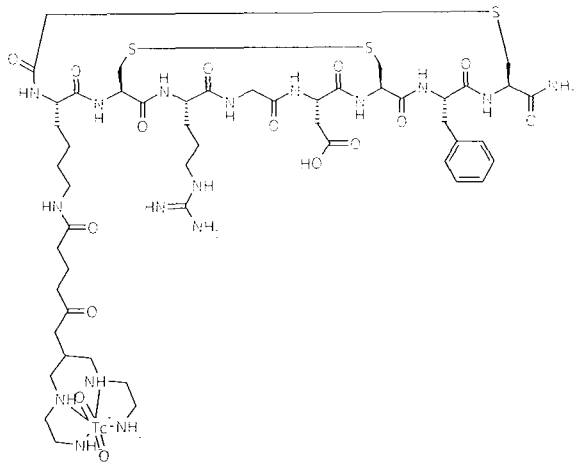
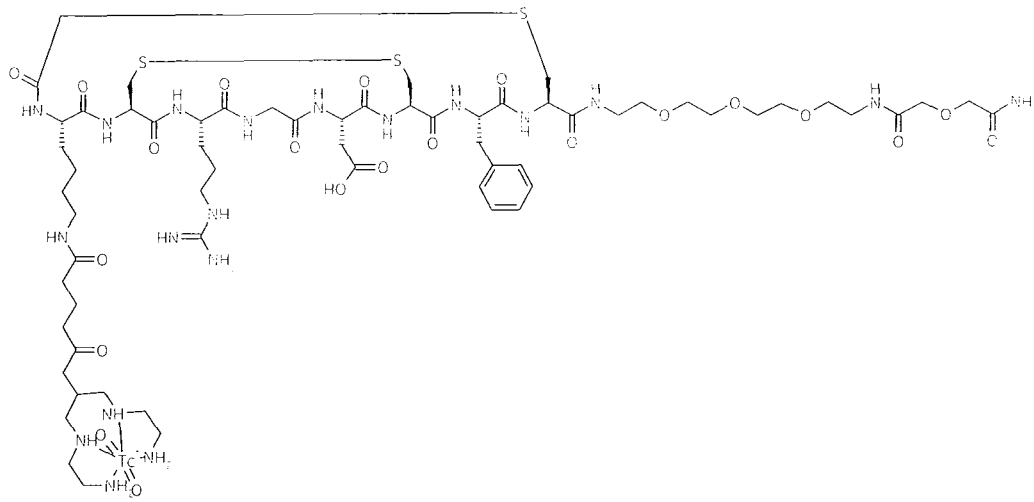


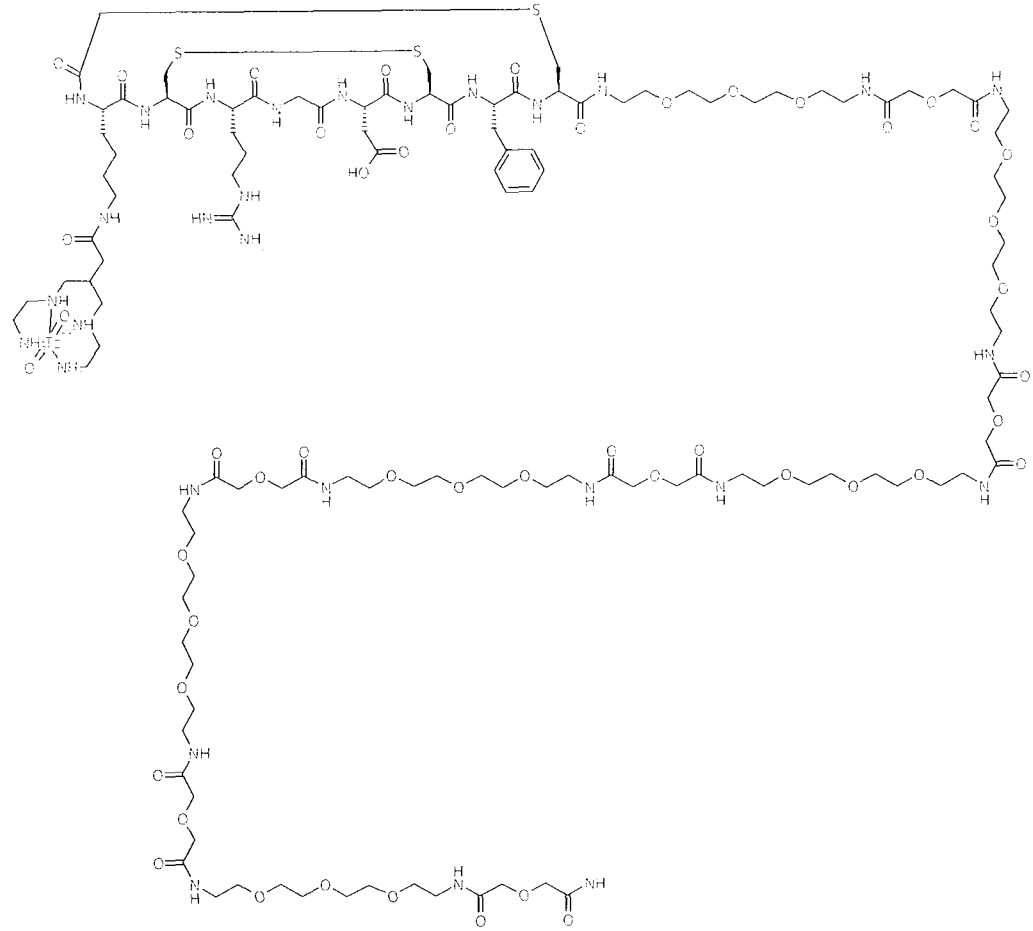
Compound 4a



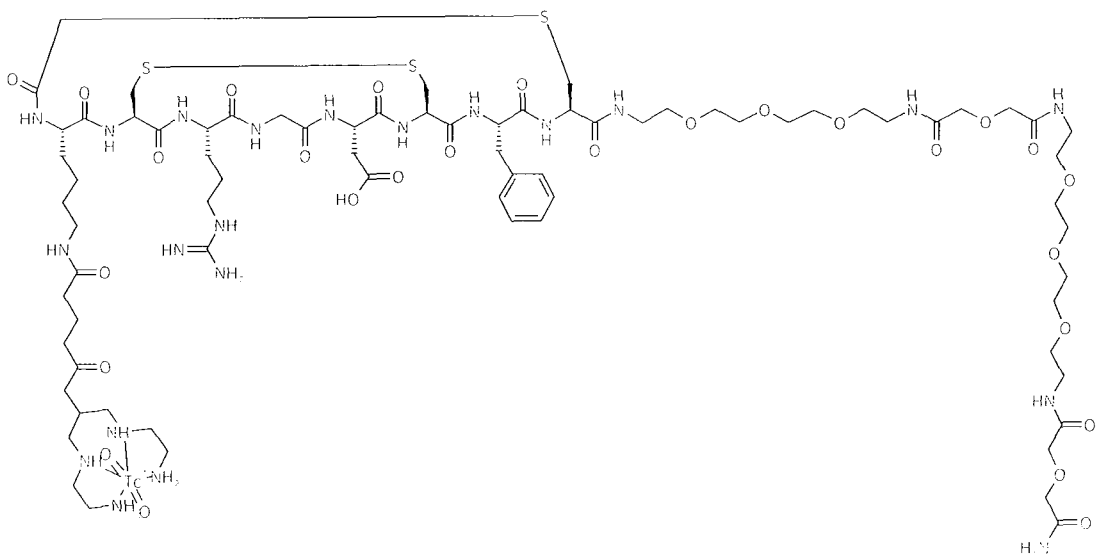
Compound 7a



Compound 8a



Compound 9a



Compound 10a

Methods for the preparation of Compounds 1a-10a are analogous to those for Compounds 1-10, except that the tetraamine chelate of Compounds 1a-10a is used in place of the diamine dioxime chelate of Compounds 1-10. Conjugation of the tetraamine chelate is achieved using normal peptide coupling with a Boc-protected species.

A number of further modified compounds were synthesised with the aim of improving the biodistribution, i.e. primarily to reduce background liver uptake. Details of the synthesis of Compounds 11-15 are provided in Examples 8-12, below. Compound 11 was designed to assess the effect of changing from a diamine dioxime chelate to a tetraamine chelate. In Compound 12 cysteic acid groups were added. Compound 13 has an additional PEG moiety at the N-terminal side of the peptide. Compound 14 has an additional PEG moiety at the C-terminal side of the peptide. In Compound 15 the tetraamine chelate was used in addition to a number of glutamic acid residues.

The method described herein begins by "providing" a subject to whom a detectable quantity of a compound of Formula I has been administered. The purpose of the method of the invention is the provision of a diagnostically-useful image. Therefore, administration to the subject of the compound of Formula I can be understood to be a preliminary step necessary for facilitating generation of said image. Preferably said subject is a mammal, and most preferably a human. Most preferably, said subject is the intact mammalian body *in vivo*. Therefore, in a preferred embodiment, the compound of Formula I has been administered as a pharmaceutical composition which comprises said compound together with a biocompatible carrier, in a form suitable for mammalian administration. A preferred route of administration is intravascular administration. In an alternative embodiment, administration of a detectable quantity of a compound of Formula I is carried out as part of the method.

Following the providing step and preceding the detection step, the compound of Formula I is allowed to bind to any fibrogenic tissue in said subject. For example, when the subject is an intact mammal, the compound of Formula I will dynamically move through the mammal's body, coming into contact with various tissues therein. Once the compound comes into contact with any fibrogenic tissue, a specific interaction takes place such that clearance of the compound from fibrogenic tissue takes longer than from non- fibrogenic

tissue. A certain point in time will be reached when detection of compound specifically bound to fibrogenic tissue is enabled as a result of the ratio between compound bound to fibrogenic tissue versus that bound in non- fibrogenic tissue. An ideal such ratio is at least 2:1.

The "biocompatible carrier" is a fluid, especially a liquid, in which the compound of Formula I is suspended or dissolved, such that the composition is physiologically tolerable, i.e. can be administered to the mammalian body without toxicity or undue discomfort. The biocompatible carrier medium is suitably an injectable carrier liquid such as sterile, pyrogen-free water for injection; an aqueous solution such as saline (which may advantageously be balanced so that the final product for injection is either isotonic or not hypotonic); an aqueous solution of one or more tonicity-adjusting substances (e.g. salts of plasma cations with biocompatible counterions), sugars (e.g. glucose or sucrose), sugar alcohols (e.g. sorbitol or mannitol), glycols (e.g. glycerol), or other non-ionic polyol materials (e.g. polyethyleneglycols, propylene glycols and the like). The biocompatible carrier medium may also comprise biocompatible organic solvents such as ethanol. Such organic solvents are useful to solubilise more lipophilic compounds or formulations. Preferably the biocompatible carrier medium is pyrogen-free water for injection, isotonic saline or an aqueous ethanol solution. The pH of the biocompatible carrier medium for intravenous injection is suitably in the range 4.0 to 10.5.

Such pharmaceutical compositions are suitably supplied in either a container which is provided with a seal which is suitable for single or multiple puncturing with a hypodermic needle (e.g. a crimped-on septum seal closure) whilst maintaining sterile integrity. Such containers may contain single or multiple patient doses. Preferred multiple dose containers comprise a single bulk vial (e.g. of 10 to 30 cm^3 volume) which contains multiple patient doses, whereby single patient doses can thus be withdrawn into clinical grade syringes at various time intervals during the viable lifetime of the preparation to suit the clinical situation. Pre-filled syringes are designed to contain a single human dose, or "unit dose", and are therefore preferably a disposable or other syringe suitable for clinical use. Where the pharmaceutical composition is a radiopharmaceutical composition, the pre-filled syringe may optionally be provided with a syringe shield to protect the operator from radioactive dose. Suitable such radiopharmaceutical syringe shields are known in the art

and preferably comprise either lead or tungsten.

The pharmaceutical composition may be prepared from a kit. Alternatively, it may be prepared under aseptic manufacture conditions to give the desired sterile product. The pharmaceutical composition may also be prepared under non-sterile conditions, followed by terminal sterilisation using e.g. gamma-irradiation, autoclaving, dry heat or chemical treatment (e.g. with ethylene oxide).

In a further aspect of the invention, the compound of Formula I may be employed for use in the preparation of a medicament for the determination of the presence, location and/or amount of fibrogenesis in an organ or body area of a subject. Preferred and most preferred embodiments of Formula I, organ or body area, subject and method of administration are as defined above.

The invention is useful for assessment of the presence, location and/or amount of activated HSC, providing an indicator of fibrogenesis. This is particularly advantageous because fibrogenic tissue is a better marker of early active disease than fibrotic tissue, the latter also being present where the disease process is resolving. Identification of the disease process can therefore be done at a stage when implementation of treatment can be most efficacious.

These advantages are demonstrated in the following non-limiting examples.

Brief Description of the Examples

Example 1 demonstrates that Compound 2 binds specifically to activated human HSC *in vitro*.

Example 2 describes the methods used to set up the rat bile duct ligation model and associated sham-operated model, as well as histopathological validation.

Example 3 demonstrates a correlation between uptake of Compound 4 and fibrogenesis. Markers of fibrogenesis are high in the rat bile duct ligation (BDL) model (described in Example 2) at 15 days postoperatively, and uptake of Compound 4 into fibrogenic livers in rat BDL model is highest at 15 days postoperatively.

Example 4 demonstrates that Compound 4 binding to BDL fibrogenic liver is

specifically inhibited by cold Compound 4.

Example 5 demonstrates a correlation between liver uptake of Compound 4 and the liver expression of α_v integrin.

Example 6 shows that Compound 8 binding to BDL fibrogenic liver is specifically inhibited by cold Compound 8.

Example 7 demonstrates a correlation of α_v expression with uptake of Compound 8.

Examples 8-12 describe the synthesis of Compounds 11-15.

Abbreviations used in the Examples

BCA	Bicinchoninic acid assay
BDL	Bile duct ligation
Boc	tert-Butyloxycarbonyl
BSA	Bovine serum albumin
DIEA	Diisopropylethylamine
DMF	Dimethylformamide
EA-Hy966	Human umbilical vein endothelial cells
EGTA	Ethylene glycol tetraacetic acid
ESI-MS	Electrospray ionization mass spectrometry
FBS	Fetal bovine serum
Fmoc	9H-Fluoren-9-yl methoxycarbonyl
MBHA	p-Methylbenzhydrylamine
DCC	1,3-Dicyclohexylcarbodiimide
DMSO	Dimethyl sulfoxide
HATU	(*N*-[(Dimethylamino)-1*H*-1,2,3triazolo-[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosohate *N*-oxide
HPLC	High performance liquid chromatography
HOAt	1-Hydroxy-7-azabenzotriazole
HSC	Hepatic stellate cells
i.d.	Injected dose
i.v.	Intravenously

ITLC	Instant thin layer chromatography
K_d	Dissociation constant
LC-MS	Liquid chromatography-mass spectroscopy
NMM	*N*-Methylmorpholine
NMP	1-methyl-2-pyrrolidinone
NPP	p-Nitrophenyl phosphate
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PEI	Polyethylenimine
PMSF	Phenylmethylsulphonyl fluoride
PyAOP	7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
RIPA	RadioImmuno Precipitation Assay
s.c.	Subcutaneously
SUV	Standard uptake value
TFA	Trifluoroacetic acid
UV	Ultraviolet

Examples

Example 1: Binding of Compound 6 to Activated Human HSC

1(i) Binding to membranes prepared from EA-Hy926 cells

The inhibition constant of Compound 6 was measured using a previously-described membrane binding assay (Indrevoll et al, Bioorg & Med Chem Lett, 2006, 16, 6190-6193).

In brief, membranes from the human endothelial adenocarcinoma cell line EA-Hy926 were prepared and the K_d calculated for the purified membrane fraction. A competitive binding assay was then established to measure inhibition constants for cold Compound 6. ^{125}I-echistatin was used as the labelled ligand and cold echistatin as a reference standard.

A total of sixteen dilutions of cold test compound (either cold echistatin or cold Compound 6) were prepared and mixed with a combination of ^{125}I-echistatin and membrane prior to incubation for 1 hour at 37°C. Following several washes, the bound

material was harvested on a filter using a Skatron micro harvester. The filterspots were finally excised and counted in a Packard γ-counter.

Figure 1 illustrates binding of ^{125}I-echistatin versus cold Compound 6 and echistatin on EA-Hy926 membranes.

1(ii) Binding to Activated Human Hepatic Stellate Cells

Activated human hepatic stellate cells, LX-2 (provided by Prof. Scott L. Friedman, Mount Sinai School of Medicine, New York), were cultured in 12-well plates (Nunc) to confluence in Dulbeccos Modified Eagels Medium containing 10% FBS, penicillin, streptomycin and glutamine. The cells were washed twice in a cold buffer containing 50mM Tris, pH 7,4, 150 mM NaCl, 5 mM $MnCl_2$, 1 mM $CaCl_2$ and 0,01% BSA. The cells were further incubated in the buffer containing a trace amount of labelled compound (either 0.1nM ^{125}I Compound 6 or 0.1nM ^{125}I-echistatin) together with various concentrations of cold compound for 60 min at 4°C with shaking. Following incubation, the unbound material was removed by washing the cells 3 times with cold buffer. The cells were detached from the wells by adding 0.1M NaOH and transferred to a tube to be counted in a gamma-counter (Packard). Figure 2 illustrates the observed activity values.

Example 2: Binding of Compound 3 and Compound 8 to Activated Rat HSC

2(i) Binding to membranes prepared from EA-Hy926 cells

The inhibition constants for Compound 3 and Compound 8 were determined using the method described in Example 1. Figure 3 illustrates binding of ^{125}I-echistatin versus cold Compound 3, Compound 8 and echistatin on EA-Hy926 membranes.

2(ii) Cells and cultivation

An activated rat liver stellate cell line (immortalized) was a kind gift from Professor Trond Berg at University of Oslo. The cells were grown in 150mm culture flasks to confluence in Dulbeccos modified Eagels medium containing 10% FBS, glutamine and Pennicillin/Streptomycin.

2(iii) Preparation of membranes

The cells were washed twice in ice cold PBS, pH 7.4, added 10ml PBS and scraped off with a rubber policeman and transferred to a 50 ml vial on ice. Another 10ml PBS were added to the flasks, scraped and combined with the first cells. The cells were centrifuged at 2000rpm at 4°C for 10 min. The supernatant was poured off and the pellet resuspended in 3 ml PBS. All pellets were combined into one tube, centrifuged as above and the final pellet frozen at -70°C immediately.

The cell pellets were resuspended in ice-cold homogenization buffer (10 times the amount of cells, for example 2 g pellet to 20 ml homogenization buffer) containing 50 mM Tris-HCl, 5mM $MgCl_2$, 1mM EGTA, pH7,4 and in addition protease inhibitors 10ug/ml leupeptin, 10ug/ml pepstatin, 200 ug/ml Bacitracin, 0,5 ug/ml aprotinin and 100uM PMSF. The cells were homogenized on ice with 3 times 10 strokes in a Dounce homogenizer, pestle B. The homogenate was centrifuged for 5 min at 2100 rpm at 4°C and the supernatant was poured off. The pellet was resuspended in 10 ml homogenization buffer and rehomogenized 2 times 10 strokes before centrifugation at 2100 rpm in 10 min. This supernatant was combined with the first one and centrifuged at 16500 rpm in a Beckman Coulter Centrifuge with JA-17 rotor (29000xg). The pellet was resuspended in 20 ml homogenization-buffer and the centrifugation step was repeated.

The supernatant was poured off and the pellet resuspended in 3 ml binding buffer containing 10mM Hepes, 135mM NaCl, 4,8 mM KCl, 1,7 mM $MgSO_4$, 2,5mM $CaCl_2$, 1.0 mM NaH_2PO_4, pH 7,4. Following removal of samples for protein measurements, 3 ml more of binding buffer was added. Aliquots of 300ul were frozen immediately at -80°C.

2(iv) Protein measurement

100 ul of the well mixed membrane prep was diluted 1:10 and a serial dilution and the protein content measured according to the instructions in BCA Protein Assay Reagent kit (Pierce No.23225).

2(v) Experimental design testing binding to integrins on stellate cell membranes

An experiment was set up in a 96-well plate. To each well 60 ul buffer (50mM Tris, pH 7,4, 150 mM NaCl, 5 mM $MnCl_2$, 1 mM $CaCl_2$, 0,01% BSA), 20ul cold Compound 3, Compound 8 or cold echistatin, 20 ul ^{125}I-echistatin and 50 ul membrane solution (diluted 1:30 in buffer corresponding to about 1ug membrane per well) were added and incubated at 37°C for 60 min with shaking. Following incubation, unbound material was washed away with PBS in a Skatron cell harvester and the bound material concentrated into a filter spot. This filterspot was cut off and added a tube that finally was counted in a gamma-counter. The filters were pre-soaked in 0.3% PEI in water for at least 4 hours. Figure 4 shows the results obtained.

Example 3: Bile Duct Ligation (BDL) and Sham Animals

3(i) Animal Model Set-up

Outbred male Sprague Dawley rats (180 – 200g; Charles River) were used in all bile duct ligation (BDL) and sham studies. After 6 days acclimatization rats were divided into 2 groups (BDL group and sham group).

For the BDL animals, the abdomen was shaved and swabbed with betadine solution followed by 5mg/kg carprofen subcutaneously (s.c.) and 5mg/kg bupronorphine s.c. and under Isoflurane anaesthesia a mid-line laparotomy was performed and the common bile duct located. Bile duct was double ligated, the first ligation made between the junction of the hepatic ducts and the second above the entrance of the pancreatic ducts.

The second group (sham animals) abdomen was shaved and swabbed with betadine solution followed by 5mg/kg carprofen s.c. and 5mg/kg bupronorphine s.c. Animals underwent sham surgery where bile duct was manipulated and a suture passed under the bile duct.

Before closing 2-3 ml saline was administered into the peritoneum of each animal. Fascia and skin were closed and animals administered with 2mg/kg metaclopromide s.c, 5mg/kg Baytril s.c., and ~ 2ml saline s.c. Carprofen was given (5mg/kg) as required over the next couple of days. Animals were closely monitored for the duration of the experiment.

3(ii) Administration of Test Compound and Biodistribution

On the appropriate day post surgery BDL and sham animals were removed and put under isoflurane anaesthesia, then each animal was injected with 0.3ml intravenously (i.v.) *via* tail vein (~3MBq). At the appropriate time point post-injection of the test item, each animal was re-anaesthetised with isoflurane, sacrificed by cervical dislocation weighed and the weight recorded *via* a barcode scanning system. Each animal was dissected and the following organs and tissues were removed and counted using BASIL counter protocol 40 or manual counting:

Bone*	Muscle*
Blood*	Kidneys
Bladder & Urine (B/U)	Lung
Liver*	Spleen
Stomach & contents	Small and large intestine (SI & LI)
Heart	Thyroid
Skin*	Carcass
Injection site	

*weighed samples

The recorded activity in a whole organ (e.g., liver) was corrected for background radioactivity and for radioactive decay and the biodistribution of radioactivity calculated by reference to Formula 1:

$$\%\ i.d.\ Organ = \frac{A}{B}\ x\ 100$$

where:

A = counts per second measured in organ

B = total counts per second measured in all samples (excluding the injection site)

The percentage of injected radioactivity in the weighed tissue samples (e.g., blood) was calculated to give % i.d. in the entire tissue by reference to Formula 2:

$$\%\ i.d.\ tissue = \frac{(Z_s\ x\ W_b\ x\ F)/B}{W_s}\ x\ 100$$

where:

Z_s = counts per second in sample

W_s = weight of sample in grams

W_b = weight of animal in grams immediately after sacrifice

B = total counts per second measured in all the samples (excluding the injection site)

F = tissue specific factor representing the mass of the tissue as a proportion of the total body weight of the animal

Tissue	**F**
Bone	0.05
Muscle	0.43
Blood	0.058
Skin	0.18
Fat	0.07

3(iii) Histopathological Evaluation

Sections from the liver of each animal were fixed in 10% buffered neutral formalin solution at room temperature, embedded in paraffin, and 5-μm thick sections were prepared. Sections were stained with Mayer's Hematoxylin and Eosin (H&E) for evaluation of biliary hyperplasia, necrosis and inflammation, and Gomori's trichrome stain for fibrosis (collagen stain) using standard staining protocols previously described (Gomori 1950 Am. J. Clin. Pathol. 20:661-663).

Histological grading of liver damage was assessed by semi-quantitatively by evaluating the extent and severity of each lesions, then scoring the lesion from 0 – 5 as follows: no lesion = 0, minimal = 1, mild = 2, moderate = 3, marked = 4, severe = 5. Histomorphological evaluation of the tissues was performed on a blind basis with respect to both days post surgery, and procedure (i.e. sham vs. bile duct ligation). All

data were peer reviewed by an additional pathologist. Histological analysis was performed by light microscopy. Sections were viewed under bright-field illumination using a 40X, 100X or 400X objective lens.

Table 4 summarises the histopathological evaluation:

Table 4

Treatment	Pathology	Day 3	Day 5	Day 10	Day 15	Day 28
Sham	All	0	0	0	0	0
BDL	Biliary Hyperplasia	1	1	2	3	3
	Inflammation	0/1	0/1	0/1	0/1	0/1
	Necrosis	1	0/1	0/1	0/1	0/1
	Fibrosis	1	2	4	4	2

The data presented supports the use of this model, particularly at day 15, as a suitable model of hepatic fibrosis in which to identify molecules which show specific uptake associated with fibrogenesis.

Example 4: Compound 8 taken up into fibrogenic livers in rat BDL model (@15 days when markers of fibrogenesis are high) vs. sham

The BDL and sham animal models were set up as described in Example 3. Uptake of Compound 8 was calculated as Standard Uptake Value (SUV) as follows:

$$SUV = \frac{(Z_s / W_s)}{(Z_t / W_t)}$$

Z_s = counts per second in sample

W_s = weight of sample in grams

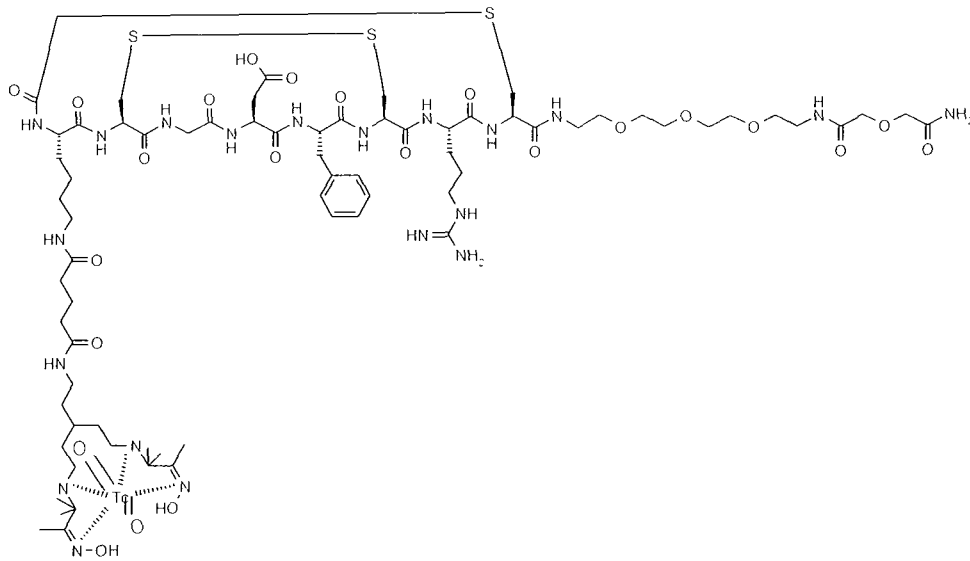
Z_t = total counts per second measured in all the samples (excluding the injection site)

W_t = weight of animal in grams

The percentage of radioactivity in the carcass was calculated by reference to the

counts/second in the residual carcass after dissection and correcting for the sampled tissues remaining in the carcass. Biological variability in body composition may have resulted in slight inaccuracies in the estimation of the tissue values and hence possible over- or under-correction of the carcass value. Where the carcass value has been over-corrected this occasionally resulted in negative values.

Figure 5 summarises the retention of radioactivity expressed as SUV in the livers of BDL- and sham-operated animals 1 hour of tail vein injection of Compound 8 at one of 2, 5, 10, 15, 28 days post-operatively, and a scrambled negative control (structure below) at 2 days post-operatively.



The results demonstrate significant retention of radioactivity in BDL as compared with sham-operated animals at all time points tested with the maximum retention of radioactivity observed in the livers from BDL animals at 10 and 15 days post-operatively. There was no significant difference in liver uptake in the sham-operated animals at any time point post-operatively.

Example 5: At 15 days post-op in BDL model, Compound 3 and Compound 8 are taken up into fibrogenic livers vs. sham animals and vs. negative control

The BDL and sham animal models were set up as described in Example 3. At 15 days post-operatively, one of Compound 3, Compound 8 or a negative control was

administered to each animal and liver uptake assessed as described in Example 3.

The structure of the negative control was as follows:

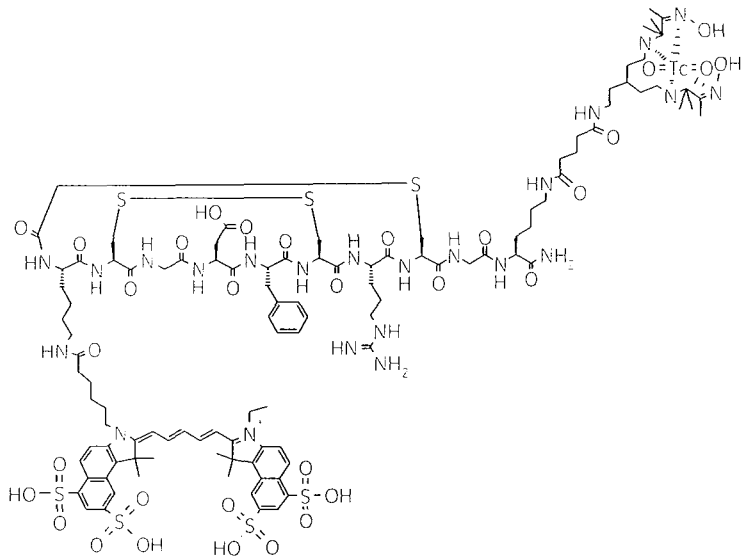


Figure 6 illustrates significantly higher liver uptake of Compounds 3 and 8 in the BDL model as compared with the sham-operated animals.

Example 6: Compound 8 binding to BDL fibrogenic liver is specifically inhibited by cold Compound 8

The biodistribution of radioactivity was investigated in 15 days post-operatively in BDL and sham-operated male Sprague Dawley rats (procedures described in Example 3) 1 hour following of intravenous injection *via* tail vein of ~3MBq of Compound 8, both before and after addition of 1:100 and 1:10,000 fold excess of cold Compound 8. At the appropriate time point post-injection of the test Item, each animal was re-anaesthetised with isoflurane, sacrificed by cervical dislocation and weighed. Each animal was dissected and organs and tissues (see Tables 5 and 6 below) were removed and counted using BASIL counter protocol 40 or manual counting.

The principal sites of accumulation of activity (in % i.d.) at one hour post-injection the BDL animals are summarized in Table 5 below:

Table 5

BDL 60 minutes	Compound 8		100-fold excess		10K-fold excess	
	Mean	SD±	Mean	SD±	Mean	SD±
Bone	3.02	0.53	2.97	0.16	3.18	0.40
Muscle	10.76	2.37	8.76	0.19	9.61	1.40
Blood	1.47	0.15	1.61	0.09	1.60	0.13
Kidneys	3.78	0.08	4.20	0.26	3.61	0.29
Bile & Urine	40.98	0.37	38.27	0.70	41.58	6.57
Lung	0.43	0.02	0.50	0.01	0.50	0.08
Liver	12.73	7.10	16.10	3.33	9.81	2.08
Spleen	0.51	0.00	0.57	0.04	0.52	0.10
Stomach	0.89	0.07	0.96	0.12	1.69	0.72
Small & Large Intestine	8.32	1.50	7.41	0.48	8.58	1.73
Heart	0.13	0.00	0.13	0.01	0.14	0.01
Thyroid	0.04	0.02	0.04	0.02	0.06	0.01
Skin	12.58	2.02	12.02	0.41	12.99	0.63
Carcass	4.36	0.88	6.47	1.83	6.14	0.60
Injection Site	1.32	0.26	1.75	0.35	1.68	0.07

The principal sites of accumulation of activity (in % i.d.) at one hour post-injection the sham animals are summarized in Table 6 below:

Table 6

BDL 60 minutes	Compound 8		+ 100-fold excess		+ 10K-fold excess	
	Mean	SD±	Mean	SD±	Mean	SD±
Bone	3.10	0.19	3.06	0.63	2.42	0.10
Muscle	10.43	0.82	10.92	0.40	8.41	1.02
Blood	1.55	0.14	1.37	0.09	1.40	0.16
Kidneys	4.07	0.13	4.13	0.11	3.65	0.52
Bile & Urine	44.65	2.79	43.25	2.87	46.52	4.68
Lung	0.46	0.07	0.38	0.04	0.39	0.05
Liver	3.71	0.66	3.52	0.39	3.03	0.31
Spleen	0.29	0.02	0.29	0.06	0.27	0.02
Stomach	0.90	0.11	1.21	0.15	1.92	1.07

Small & Large Intestine	7.86	0.12	8.42	0.60	7.54	1.01
Heart	0.12	0.02	0.10	0.01	0.10	0.01
Thyroid	0.05	0.02	0.05	0.00	0.04	0.01
Skin	14.37	0.71	13.97	0.93	12.69	1.37
Carcass	8.45	0.67	9.32	1.01	11.63	1.90
Injection Site	1.66	0.12	1.44	0.06	1.71	0.15

Example 7: Compound 8 Uptake and Alpha-v Upregulation in BDL day 15

The BDL and sham animal models were set up as described in Example 3. At each of 2, 15 and 28 days post-operatively, ~3MBq Compound 8 was administered to animals and the liver uptake assessed as described in Example 3.

The expression of α_v integrin in the animals' livers was measured in homogenised livers of BDL- and sham-operated animals. At each of 2, 15 and 28 days post-operatively, animals were sacrificed and their livers removed and stored at -70°C.

Pre-weighed livers were removed from -70°C freezer and placed into pre-cooled mortar and pestle in the presence of low amount of liquid nitrogen. Liver was crushed to almost a homogenous powder (during the crushing of the liver a small amount of liquid nitrogen was added to keep the tissue frozen at all times). The crushed livers were placed into a pre-cooled medicine measure and put into -70°C freezer until further use. 1g of frozen crushed tissue was added directly into 10ml of ice cold RIPA buffer (Sigma; 150 mM NaCl, 1.0% Igepal CA-630 (detergent), 0.5% sodium deoxycholate (anionic detergent), 0.1% SDS, 50 mM Tris, pH 8.0) containing 1 mM EDTA, 0.25M sucrose and freshly added protease inhibitor cocktail (Sigma) (10µl cocktail/ml of lysis buffer). The mixture was homogenized with a sharp blade homogenizer at 4° C and kept on wet ice for 30 minutes.

The homogenate was transferred into a pre-chilled glass tissue Dounce and three up and down strokes of the pestle were applied at 4° C. The tissue homogenate was then transferred into pre-cooled centrifuge tubes and centrifuged for 30 minutes at 30,000 rpm and 4°C. Supernatants were removed (total cell lysate) and placed on wet ice.

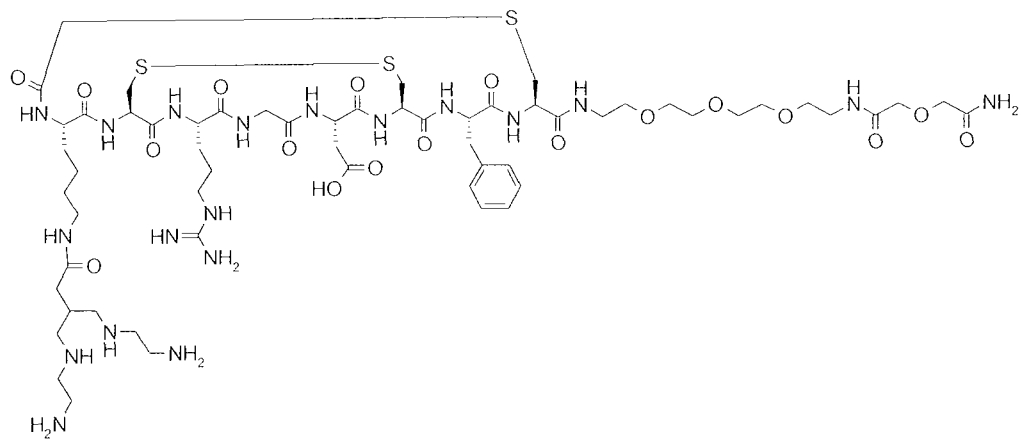
Relative protein concentration was determined in each sample using a commercial protein assay kit with BSA standards according to the manufacturer's instruction (Pierce, USA) (BSA aliquots should be made in modified RIPA buffer that was used for the homogenisation). Supernatant from each sample was further diluted between 1:50 and 1:200 in H_2O and protein concentration was determined using a 99 multi-well Plate reader (iEMS). Each sample was divided into 1ml aliquots and stored at -70°C until further use.

Anti-alpha-v integrin capture antibody (BD; catalogue number: 611013) was diluted to appropriate concentration in binding solution (0.05 M tris, 0.138 M NaCl and 0.0027 M KCl) and either 50 or 100 µl was added to each well of an enhanced protein-binding ELISA plate (Nunc-Immuno™ plates 96-well plate, MaxiSorp). The amount of antibody used was 200 ng/well. Plates were sealed and incubated either for 1 hour at 37°C or overnight at 4°C. Plates were emptied and the remaining sites for protein binding on the microtiter plate were saturated by incubating with blocking buffer (3% BSA/TBS with 0.02% sodium azide approx. 300µl per well). Plates were sealed and incubated for 1 hour at 37°C then washed 3 times with TBS/0.01% Tween-20. Neat liver homogenate supernatant and/or different concentrations of purified alpha-v integrin standards were prepared in blocking buffer and added in triplicate (100µl per well) into the coated plates then sealed and incubated for 2 hrs at 37°C. Plates were washed 4 times with TBS/0.05%Tween-20 and a second anti-integrin detection antibody (Chemicon AB1930) diluted in blocking buffer was added to wells and incubated for 1 hour at 37°C. Plates were washed 4 times with TBS/0.05%Tween-20 and secondary antibody alkaline phosphatase conjugated (Sigma; catalogue number: A7539) specific to the detection antibody was added to the wells and incubated for 1 hr at 37°C. Plates were washed 4 times with TBS/0.05%Tween-20 and 100 µl of NPP substrate solution to each well and incubate either for 2 hr at room temperature or overnight at 4°C. Hydrolysis was monitored qualitatively by visual inspection or quantitatively with a microtiter plate reader. Hydrolysis of NPP appears yellow after suggested incubation time has elapsed, optical densities at target wavelengths (405 nm) was measured on an ELISA plate reader (Spectra-Max Plus). Hydrolysis was stopped by adding 50 µl of 5 M sodium hydroxide NaOH.

For quantitative results, the signal of unknown samples was compared against those of the standard curve. Statistical analyses were performed using GraphPad PRISM, Version 4.0. Differences between groups were analysed by the non-parametric One-Way ANOVA. In all statistical analyses, a probability value of less than 0.05 ($P<0.05$) was considered significant.

Figure 7 and Figure 8 illustrate α_v expression in BDL vs. sham animals and demonstrate a correlation of α_v expression with uptake of Compound 8.

Example 8: Synthesis of Compound 11

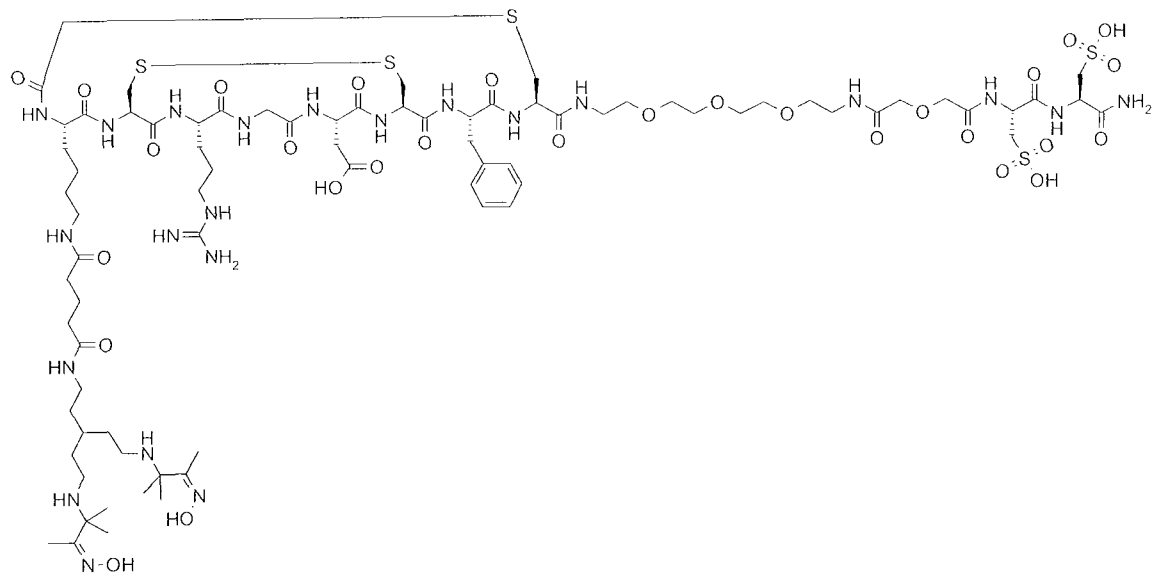


Boc-tetraamine-N-hydroxysuccinimide ester (WO 2006/008496) (36 mg, 0.050 mmol) was preactivated with HOAt (1.4 mg, 0.010 mmol) and NMM (17 μL, 0.15 mmol) in DMF (0.5 mL) for 10 min and then added to a solution of Cys2-6; c[CH_2CO-Lys-Cys-Arg-Gly-Asp-Cys-Phe-Cys]-PEG(4)-Diglycoloyl-NH_2 (WO 03/006491) (12.6 mg, 0.010 mmol) in DMF (0.5 mL). The reaction mixture was stirred at room temperature for 3 days then concentrated *in vacuo*. The Boc protection groups were removed by addition of a TFA/water/triisopropylsilane (95:2.5:2.5, 10 mL) solution for 90 min. The mixture was concentrated and the crude product was precipitated from ether and purified by preparative HPLC (column Phenomenex Luna C18(2) 250 × 21.2 mm, 5μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 10-20% B over 40 min; flow rate 10 mL/min, UV detection at 214 nm and 254 nm) affording 10.4 mg after lyophilisation. Analysis by LC-MS (column Phenomenex Luna C18(2) 20 × 2 mm, 3 μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 10-20% B

over 5 min; flow rate 0.6 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 2.02 min, *m/z* 1458.7 $(MH)^+$ confirmed the structure.

Labelling with ^{99m}Tc was carried out by adding the following to a nitrogen-purged P46 vial: 100 µg of IGF precursor 1 or 2 in MeOH: 0.5 ml $Na_2CO_3/NaHCO_3$ buffer (pH 9.2); 0.5 ml TcO_4^- from a Drytec™ ^{99m}Tc generator; 0.1 ml $SnCl_2$/MDP solution (containing 10.2 mg $SnCl_2$ and 101 mg methylenediphosphonic acid in 100ml N_2 purged saline).

Example 9: Synthesis of Compound 12



The synthesis was run on a Rink amide AM resin on 0.3 mmol scale (0.432 g, loading 0.71 mmol/ g). The first three coupling steps were carried out in a manual nitrogen bubbler apparatus. The Fmoc group on the resin was cleaved by standard protocol (20% piperidine in DMF). Fmoc-Cys (trityl)-OH (702 mg, 1.20 mmol) was coupled to the resin in DMF by standard coupling reagents HATU and DIEA. Completion of coupling was checked by standard Kaiser test. After Fmoc cleavage the coupling was repeated introducing the second cysteine. The resin was suspended in a dichloromethane/TFA/triisoprolylsilane (94:5:1, 10 mL) solution. After 3 min the yellow solution was drained off. The step was repeated 6 times assuring complete deprotection of the side chain thiol functions. The resin was washed with

dichloromethane and dried (nitrogen flow for 30 min). A mixture of 20% formic acid (18 mL) and 35% hydrogen peroxide (2 mL) was allowed to stand for 1 h at room temperature and then cooled to 0 °C. The resin was washed with an aliquot of the performic acid solution (2 x 4 mL) and then kept in the performic acid solution (5 mL) for 12 h at 5 °C with gentle shaking. An aliquot of the resin was cleaved (TFA/water/triisopropylsilane, 95:2.5:2.5) and analysed by LC-MS (column Phenomenex Luna C18(2) 50 × 2 mm, 3μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 5-50% B over 5 min; flow rate 0.3 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 2.48 min, *m/z* 564.3 $(MNa)^+$ confirming the structure.

After Fmoc removal (standard protocol) of the resin above, Fmoc-amino-PEG(4)-Diglycolic acid (796 mg, 1.50 mmol) was coupled to the resin in DMF using standard coupling reagents (HATU and DIEA). After 6 h an aliquot was cleaved (described in previous paragraph) and analysed by LC-MS (column Phenomenex Luna C18(2) 50 × 2 mm, 3μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 5-50% B over 5 min; flow rate 0.3 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 3.28 min, *m/z* 832.3 $(MH)^+$ showing complete conversion.

All subsequent amino acids were coupled using an ABI433A automated peptide synthesiser using SlowMoc coupling protocol on ~0.25 mmol resin from above to give H-Lys(Boc)-Cys(tBu)-Arg(Pmc)-Gly-Asp(OtBu)-Cys(tBu)-Phe-Cys(Trt)-PEG4-Diglycoloyl-CyA-CyA-Rink amide resin.

A solution of chloroacetic acid (142 mg, 1.50 mmol) and DCC (155 mg, 0.75 mmol) in dichloromethane (10 mL) was stirred at room temperature for 15 min, then filtered and concentrated. The residue was taken up in DMF and added to the peptide resin above. The peptide was cleaved off the resin using a TFA/water/triisopropylsilane solution (95:2.5:2.5, 10 mL) for 2 h. The solution was concentrated and the crude peptide precipitated from ether and dried in vacuo to give 340 mg. Analysis by LC-MS (column Phenomenex Luna C18(2) 50 × 2 mm, 3μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 0-50% B over 5 min; flow rate 0.3 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 3.09 min, *m/z* 1710.6 $(MH)^+$ confirmed the

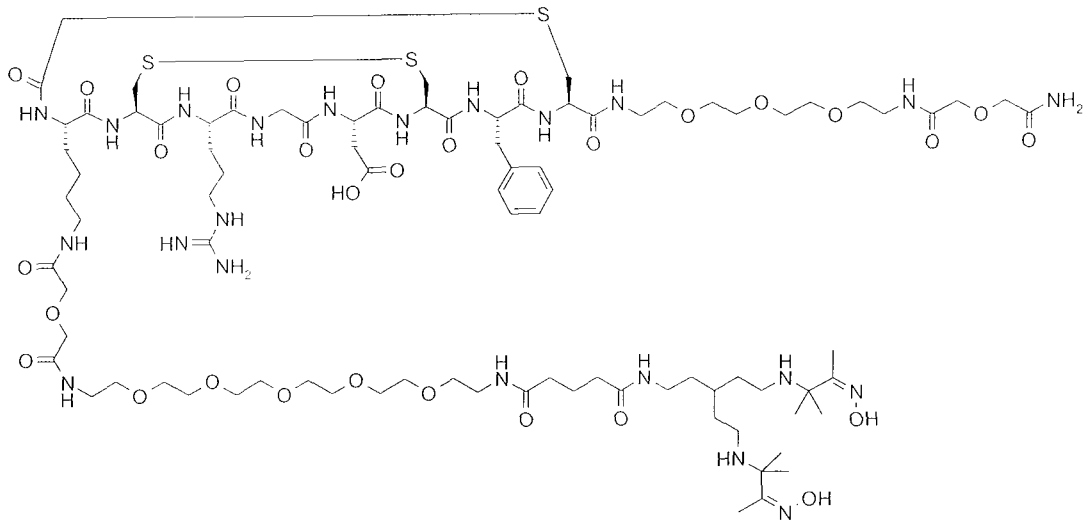
chloroacetylated peptide.

To a solution of the peptide (68 mg) in 50% acetonitrile (64 mL) was added 2.5% ammonia until pH was 8.5. The mixture was stirred at room temperature for 16 h. LC-MS analysis confirmed complete thioether cyclisation. Acetonitrile was removed under reduced pressure and the aqueous solution was lyophilised. Isolated material was taken up in a 5% solution of DMSO in TFA (100 mL) and stirred at room temperature for 60 min. The mixture was concentrated and the crude product isolated by precipitation from ether followed by purification by preparative HPLC (column Phenomenex Luna C18(2) 250 × 21.2 mm, 5μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 5-20% B over 60 min; flow rate 10 mL/min, UV detection at 214 and 254 nm) to give 48 mg of solid material. Analysis by LC-MS (column Phenomenex Luna C18(2) 50 × 2 mm, 3μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 0-30% B over 5 min; flow rate 0.3 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 3.18 min, *m/z* 1560.5 $(MH)^+$ confirmed the disulfide bridge formation.

A partially dissolved mixture of the peptide (5 mg), Chelate I-Glutaryl tetrafluorothiophenol ester (WO 03/006491) (5 mg) and DIEA (2.7 µL) in NMP (1.5 mL) was heated at 40 °C overnight. The mixture was purified by preparative HPLC (column Phenomenex Luna C18(2) 250 × 21.2 mm, 5μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 0-30% B over 40 min; flow rate 10 mL/min, UV detection at 214 and 254 nm) affording 2.9 mg pure product. Analysis by LC-MS (column Phenomenex Luna C18(2) 20 × 2 mm, 3 μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 0-30% B over 5 min; flow rate 0.6 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 3.83 min, *m/z* 1000.7 $(MH_2)^{2+}$ confirmed the structure.

Labelling with ^{99m}Tc was carried out as described in WO 2003/006491.

Example 10: Synthesis of Compound 13



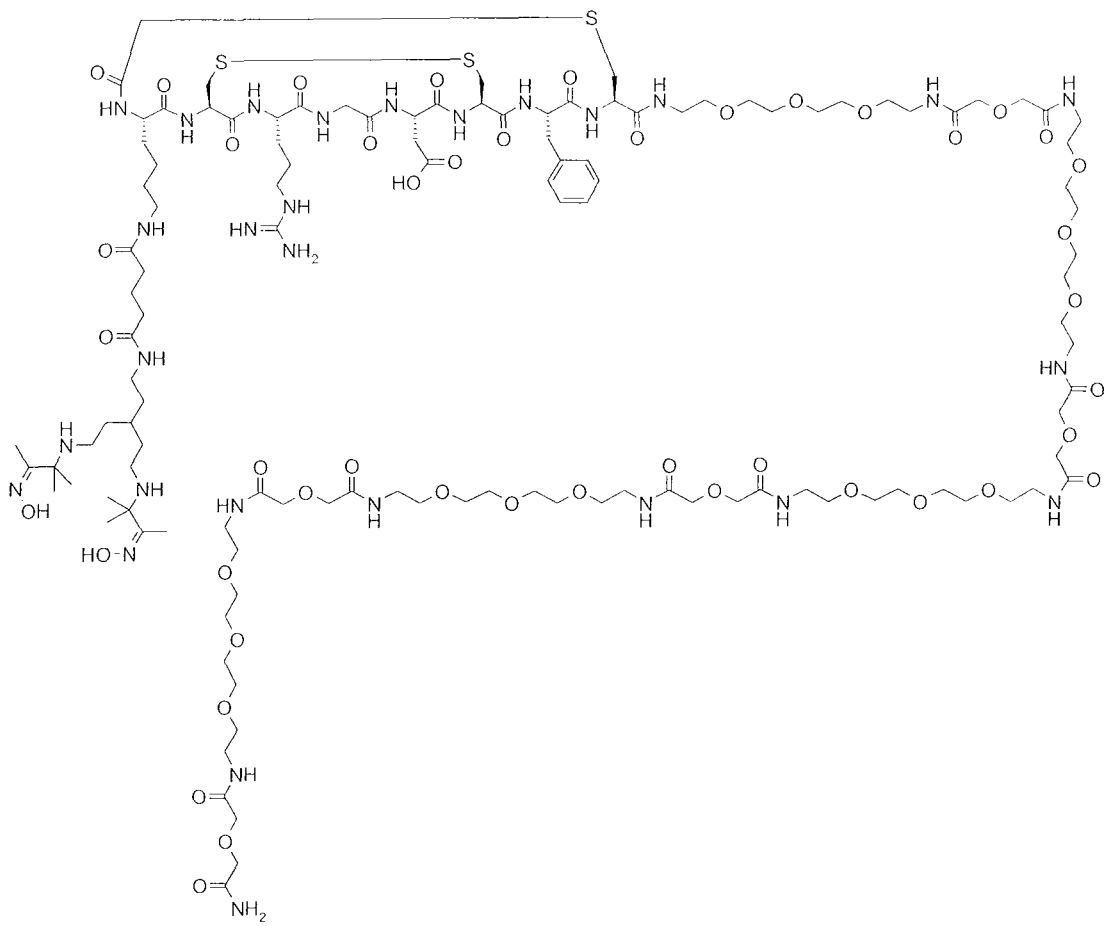
Boc-PEG(6)-Diglycolic acid (50 mg, 0.10 mmol) was coupled to Cys2-6; c[CH_2CO-Lys-Cys-Arg-Gly-Asp-Cys-Phe-Cys]-PEG(4)-Diglycoloyl-NH_2 (WO 03/006491) (25.2 mg, 0.020 mmol) in DMF using standard coupling reagents (HATU and NMM). The reaction mixture was stirred overnight and concentrated *in vacuo*. The Boc protection groups were removed by addition of a TFA/water/triisopropylsilane (95:2.5:2.5, 10 mL) solution for 45 min at room temperature. The mixture was concentrated followed by precipitation of crude material from ether. The product was purified by preparative HPLC (column Phenomenex Luna C18(2) 250 × 21.2 mm, 5μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 10-30% B over 40 min; flow rate 10 mL/min, UV detection at 214 and 254 nm) affording 5.5 mg. Analysis by LC-MS (column Phenomenex Luna C18(2) 20 × 2 mm, 3 μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 10-25% B over 5 min; flow rate 0.6 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 3.01 min, *m/z* 1636.0 $(MH)^+$ confirmed the product.

Chelate I-Glutaric acid (WO 03/006491) (15.6 mg, 0.034 mmol) was coupled to the peptide (5.5 mg, 0.0034 mmol) in DMF using standard coupling reagents (PyAOP (7-Azabenzotriazol-1-yloxy-tris-(pyrrolidino)phosphonium hexafluorophosphate) and NMM). After 4 h the mixture was purified by preparative HPLC (column Phenomenex Luna C18(2) 250 × 21.2 mm, 5μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 15-30% B over 40 min; flow rate 10 mL/min, UV detection at 214 and 254 nm) affording 3.8 mg. Analysis by LC-MS (column

Phenomenex Luna C18(2) 20 × 2 mm, 3 μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 15-30% B over 5 min; flow rate 0.6 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 2.13 min, *m/z* 1039.0 $(MH_2)^{2+}$ confirmed the structure.

Labelling with ^{99m}Tc was carried out as described in WO 2003/006491.

Example 11: Synthesis of Compound 14



The synthesis was carried out in a manual nitrogen bubbler apparatus on a Rink Amide MBHA resin (loading 0.72 mmol/g) on a 0.80 mmol scale. The Fmoc group on the resin was cleaved by standard protocol. Fmoc-PEG(4)-Diglycolic acid (850 mg, 1.6 mmol) was coupled to the resin using standard coupling reagents (PyAOP and DIEA) in DMF. The conjugation reaction followed by Fmoc deprotection was repeated until 5 PEG(4)-Diglycoloyl groups were introduced on the resin.

Fmoc-Cys(Trt)-OH (1.0 mmol) was coupled to the resin above (0.2 mmol) using standard coupling reagents (PyAOP and NMM) in DMF/ dichloromethane (1:1, 6 mL). After 2.5 h an aliquot of the resin was cleaved off (described above) and analysed by LC-MS (column Phenomenex Luna C18(2) 20 × 2 mm, 3 μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 10-80% B over 5 min; flow rate 0.6 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 2.3 min, *m/z* 1794.1 $(MH)^+$ showing complete conversion.

All subsequent amino acids were coupled using an ABI433A automated peptide synthesiser using SlowMoc coupling protocol on ca 0.1 mmol resin from above to give H-Lys(Boc)-Cys(tBu)-Arg(Pbf)-Gly-Asp(OtBu)-Cys(tBu)-Phe-Cys(Trt)-(PEG(4)-Diglycoloyl)$_5$-Rink amide resin.

Chloroacetic anhydride (2.00 mmol) was synthesised by stirring a solution of chloroacetic acid (378 mg, 4.00 mmol) and DCC (412 mg. 2.00 mmol) in dichloromethane (10 mL) for 1 h followed by filtration. The solution was concentrated and the residue taken up in DMF (3 mL) and added to the peptide resin above. NMM (220 µL, 2.00 mmol) was added and the mixture was left for 3 h. An aliquot of the resin was cleaved (described above) and analysed by LC-MS that confirmed complete conversion. The peptide was cleaved off the resin by addition of a TFA/water/triisopropylsilane solution (95:2.5:2.5, 10 mL) for 3 h. The solution was concentrated.

The residue from above was taken up in water (100 mL) and extracted with ether. To the aqueous solution was added acetonitrile (100 mL) and pH was adjusted to 8 by addition of dilute ammonia. The reaction was left over night. The acetonitrile was evaporated off and the crude product isolated by lyophilisation giving 154 mg. Analysis by LC-MS confirmed complete thioether cyclisation.

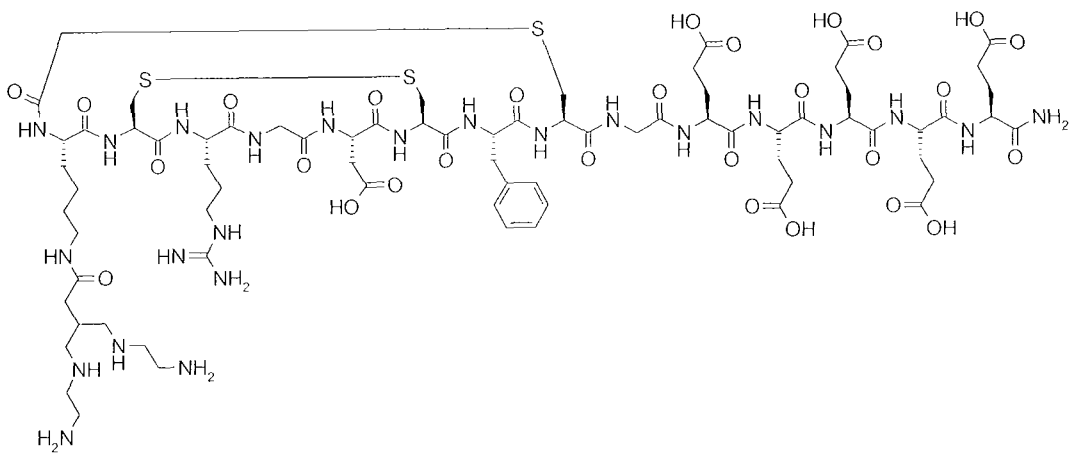
Isolated material from above was taken up in a 5% solution of DMSO in TFA (80 mL) and stirred at room temperature for 60 min. The mixture was concentrated and the crude product isolated by precipitation from ether followed by purification by preparative HPLC (column Phenomenex Luna C18(2) 250 × 21.2 mm, 5μm, solvents: A=

water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 15-25% B over 60 min; flow rate 10 mL/min, UV detection at 214 and 254 nm) affording 5 mg of pure material. Analysis by LC-MS confirmed the disulfide bridge formation.

Chelate I-Glutaric acid (WO 03/006491) (9 mg, 0.02 mmol) was coupled to the peptide above using standard coupling reagents (PyAOP and DIEA) in DMF over night. The product was purified by preparative HPLC (column Phenomenex Luna C18(2) 250 × 21.2 mm, 5 μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 10-30% B over 60 min; flow rate 10 mL/min, UV detection at 214 and 254 nm) affording 1.3 mg. Analysis by LC-MS (column Phenomenex Luna C18(2) 20 × 2 mm, 3 μm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 10-60% B over 5 min; flow rate 0.6 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 1.8 min, *m/z* 1430.1 $(MH_2)^{2+}$ confirmed the structure.

Labelling with ^{99m}Tc was carried out as described in WO 2003/006491.

Example 12: Synthesis of Compound 15



The peptide sequence H-Lys(Boc)-Cys(tBu)-Arg(Pbf)-Gly-Asp(OtBu)-Cys(tBu)-Phe-Cys(Trt)-Gly-[Glu(OtBu)]$_5$-NH_2 was assembled on an ABI 433A automated peptide synthesiser on a Rink amide MBHA resin (0.1 mmol) using SlowMoc coupling method.

A solution of chloroacetic acid (378 mg, 4.00 mmol) and DCC (412 mg, 2.00 mmol) in dichloromethane (10 mL) was stirred at room temperature for 1 h, then filtered and concentrated. The residue was taken up in DMF and added to the peptide resin

described above. NMM (220 µl, 2.00 mmol) was added and the reaction left for 2 h. An aliquot of the resin was cleaved (described above) and analysed by LC-MS (column Phenomenex Luna C18(2) 20 × 2 mm, 3 µm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 20-30% B over 5 min; flow rate 0.6 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 2.2 min, *m/z* 1820.7 $(MH)^+$ confirming complete conversion. The peptide was cleaved off the resin by addition of a TFA/water/triisopropylsilane solution (95:2.5:2.5, 10 mL) for 2 h. The solution was concentrated and the crude product isolated by precipitation from ether followed by purification by preparative HPLC (column Phenomenex Luna C18(2) 250 × 21.2 mm, 5 µm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 20-40% B over 60 min; flow rate 10 mL/min, UV detection at 214 and 254 nm). The semi pure product was isolated by lyophilisation and taken up in 50% acetonitrile/ water (6 mL). pH was adjusted to 8 by addition of dilute ammonia and the reaction was stirred overnight. The acetonitrile was evaporated off under reduced pressure and the product isolated by lyophilisation giving 14.8 mg. Analysis by LC-MS [column Phenomenex Luna C18(2) 20 × 2 mm, 3 µm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 10-60% B over 5 min; flow rate 0.6 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 2.0 min, *m/z* 1785.0 $(MH)^+$ confirmed the thioether cyclisation.

The isolated peptide above was taken up in a 5% solution of DMSO in TFA (8 mL) and stirred at room temperature for 15 min. The mixture was concentrated and the crude product precipitated from ether followed by purification by preparative HPLC (column Phenomenex Luna C18(2) 250 × 10 mm, 10 µm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 10-20% B over 60 min; flow rate 5 mL/min, UV detection at 214 and 254 nm) affording 2 mg pure material. Analysis by LC-MS (column Phenomenex Luna C18(2) 20 × 2 mm, 3 µm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 10-60% B over 5 min; flow rate 0.6 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 1.0 min, *m/z* 1670.8 $(MH)^+$ confirmed the peptide.

To a solution of the peptide from above (2 mg, 1 µmol) and Boc-tetraamine-N-hydroxysuccinimide ester (WO 2006/008496) (8.6 mg, 0.012 mmol) in DMF was added

DIEA (3 µL, 0.02 mmol). The reaction mixture was stirred overnight and subjected to purification by preparative HPLC (column Phenomenex Luna C18(2) 250 × 21.2 mm, 5 µm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 20-80% B over 60 min; flow rate 10 mL/min, UV detection at 214 and 254 nm. The pure product was lyophilised and dissolved in a mixture of TFA and dichloromethane (1:1, 2 mL). After 2 h the solvent was evaporated and the residue taken up in acetonitrile/water and lyophilised to afford 1.0 mg of the pure product. Analysis by LC-MS (column Phenomenex Luna C18(2) 20 × 2 mm, 3 µm, solvents: A= water/0.1% TFA and B= acetonitrile/0.1% TFA; gradient 0-20% B over 5 min; flow rate 0.6 mL/min, UV detection at 214 and 254 nm, ESI-MS) t_R= 4.2 min, *m/z* 935.5 $(MH_2)^{2+}$ confirmed the structure.

Labelling with ^{99m}Tc was carried out as described for Compound 11 in Example 8 above.

Claims

1) A compound of Formula I for use in a method to determine of the presence, location and/or amount of fibrogenesis in the liver of a subject, said method comprising the following steps:

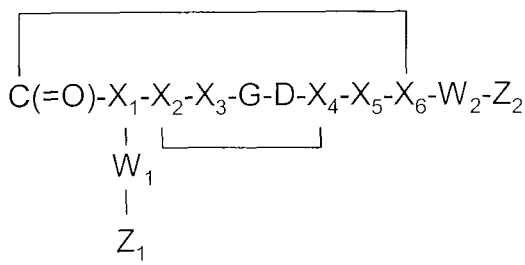
(i) providing a subject to whom a detectable quantity of a compound of Formula I has been administered;

(ii) allowing the compound of Formula I to bind to any fibrogenic tissue in said liver;

(iii) detection of signals emitted by said compound of Formula I by an *in vivo* imaging method; and,

(iv) generation of an image representative of the location and/or amount of said signals;

wherein said compound of Formula I is defined as follows:



(I)

wherein:

G represents glycine;

D represents aspartic acid;

X_1 represents an amino acid selected from aspartic acid, glutamic acid, lysine, homolysine or a C_{3-6} diaminoalkanoic acid, or derivatives thereof;

X_2 and X_4 independently represent amino acid residues whose side chains are linked together to form a cyclising bridge, such as cysteine or homocysteine forming

disulphide or thioether bonds, or other amino acids capable of forming a cyclising bridge such as aspartic acid and lysine;

X_3 represents arginine, N-methylarginine or an arginine mimetic;

X_5 represents tyrosine, phenylalanine, 3-iodo-tyrosine C_{4-6} cycloalkylalanine or naphthylalanine, or derivatives thereof;

X_6, represents a thiol-containing amino acid that forms either a thioether bond or a thioacetal bond linking X_6 to the C(=O) group;

W_1 and W_2 are independently an optional linker moiety, wherein when present W_1 is linked to the amino acid side chain moiety of X_1 and W_2 when present is linked to the carboxy group of X_6; and,

Z_1 and Z_2 are independently an imaging moiety, a sugar moiety, and organic dye moiety or hydrogen, with the proviso that at least one of Z_1 and Z_2 is an imaging moiety.

2) The compound of claim 1 wherein X_1 is lysine.

3) The compound of either one of claims 1 or 2 wherein X_2 and X_4 are independently cysteine or homocysteine.

4) The compound of any one of claims 1 to 3 wherein X_3 is arginine.

5) The compound of any one of claims 1 to 4 wherein X_5 is cyclohexylalanine, phenylalanine or 3-iodo-tyrosine.

6) The compound of any one of claims 1 to 5 wherein X_6 is cysteine or homocysteine.

7) The compound of any one of claims 1 to 6 wherein:

X_1 is lysine;

X_2 and X_4 are both cysteine;

X_3 is arginine;

X_5 is phenylalanine; and,

X_6 is cysteine.

8) The compound of any one of claims 1 to 7 wherein at least one of W^1 and W^2 is present and said linker moiety is a radical of Formula $-(L)_n-$ wherein:

each L is independently –C(=O)- , -CR'$_2$- , -CR'=CR'- , -C≡C- , -CR'$_2$CO$_2$- , -CO$_2$CR'$_2$- , -NR'-, -NR'CO- , -CONR'- , -NR' (C=O)NR'-, -NR'(C=S)NR'-, -SO$_2$NR'- , -NR'SO$_2$- , -CR'$_2$OCR'$_2$- , -CR'$_2$SCR'$_2$- , -CR'$_2$NR'CR'$_2$- , a C_{4-8} cycloheteroalkylene group, a C_{4-8} cycloalkylene group, a C_{5-12} arylene group, a C_{3-12} heteroarylene group, an amino acid, a polyalkyleneglycol, polylactic acid or polyglycolic acid moiety;

n is an integer of value 1 to 15;

each R' group is independently H or C_{1-10} alkyl, C_{3-10} alkylaryl, C_{2-10} alkoxyalkyl, C_{1-10} hydroxyalkyl, 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.

9) The compound of any one of claims 1 to 8 wherein at least one of W^1 and W^2 is present and said linker moiety represents a monodisperse PEG building block comprising 1 to 10 units of said building block.

10) The compound of any one of claims 1 to 8 wherein at least one of W^1 and W^2 is present and said linker moiety represents 1 to 10 amino acid residues.

11) The compound of any one of claims 1 to 8 wherein one of Z_1 and Z_2 is a sugar moiety.

12) The compound of any one of claims 1 to 8 wherein one of Z_1 and Z_2 is an organic dye moiety.

13) The compound of any one of claims 1 to 12 wherein said imaging moiety is chosen from:

(i) a radioactive metal ion;

(ii) a gamma-emitting radioactive halogen;

(iii) a positron-emitting radioactive non-metal; and,

(iv) a paramagnetic metal ion.

14) The compound of claim 13 wherein said imaging moiety is selected from (i) to (iii).

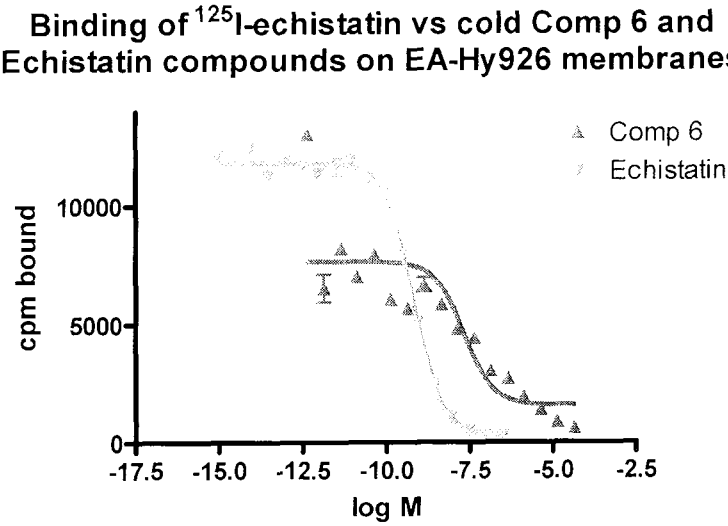
15) The compound of claim 14 wherein said imaging moiety is selected from ^{99m}Tc, ^{123}I, ^{11}C and ^{18}F.

16) The compound of any one of claims 1 to 15 which is provided as a pharmaceutical composition together with a biocompatible carrier, in a form suitable for mammalian administration.

17) The compound of claim 16 for use in the method as defined in claim 1, wherein said subject is an intact mammalian body *in vivo*.

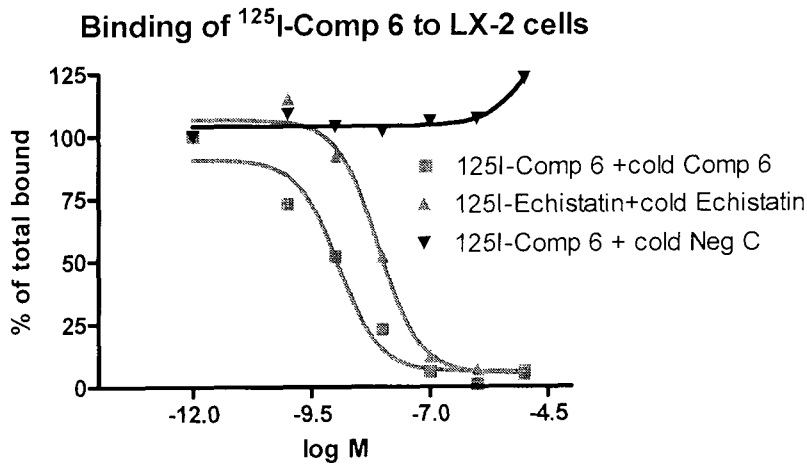
18) The compound of claim 16 for use in the method as defined in claim 1, wherein said subject is a human.

19) A compound of Formula I, as defined in any one of claims 1 to 16 for use in the preparation of a medicament for the determination of the presence, location and/or amount of fibrogenesis in an organ or body area of a subject.



	Comp 6	Echistatin
KI	1.1057e-008	3.2455e-010

Figure 1



	125I-Comp 6 +cold Comp 6	125I-echistatin+cold ech
EC50	1.1669e-009	8.1405e-009

Figure 2

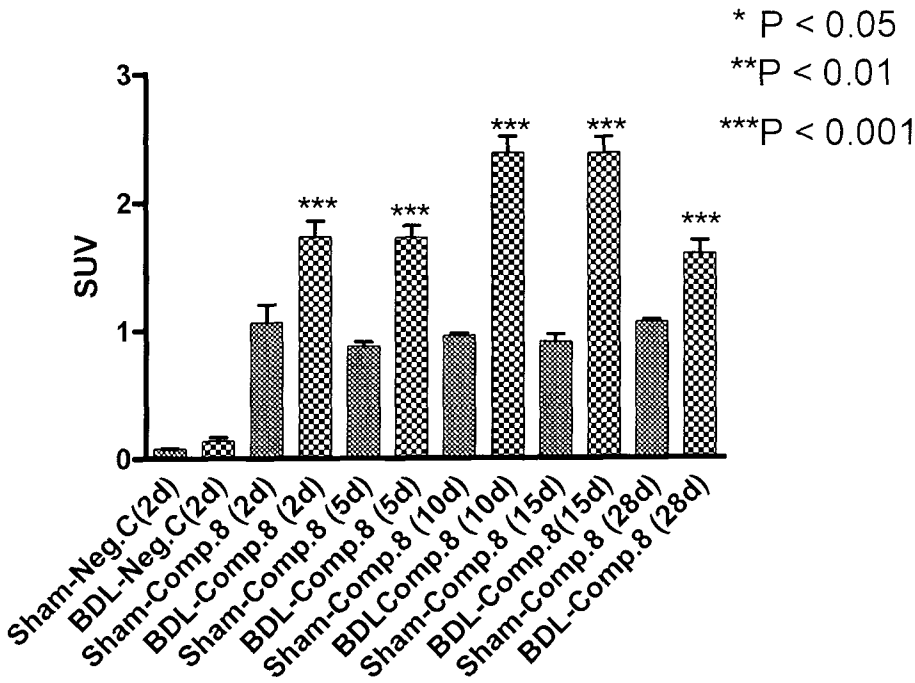

* P < 0.05
**P < 0.01
***P < 0.001
SUV
3
2
1
0
***
***
***
***
Sham-Neg.C(2d)
BDL-Neg.C(2d)
Sham-Comp.8 (2d)
BDL-Comp.8 (2d)
Sham-Comp.8 (5d)
BDL-Comp.8 (5d)
Sham-Comp.8 (10d)
BDLComp.8 (10d)
Sham-Comp.8 (15d)
BDL-Comp.8(15d)
Sham-Comp.8 (28d)
BDL-Comp.8 (28d)

Figure 3

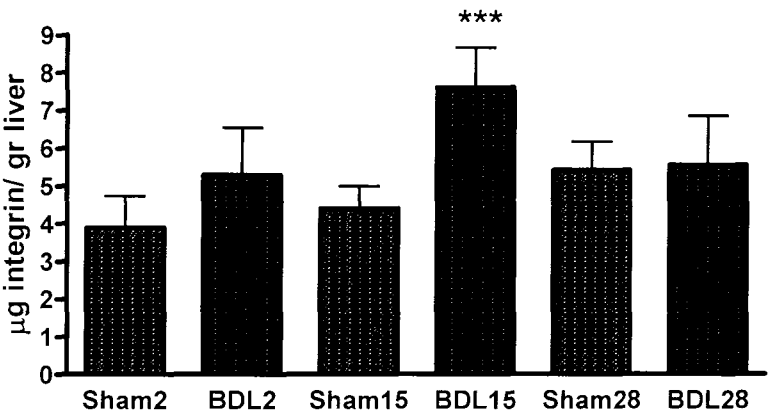


Figure 4

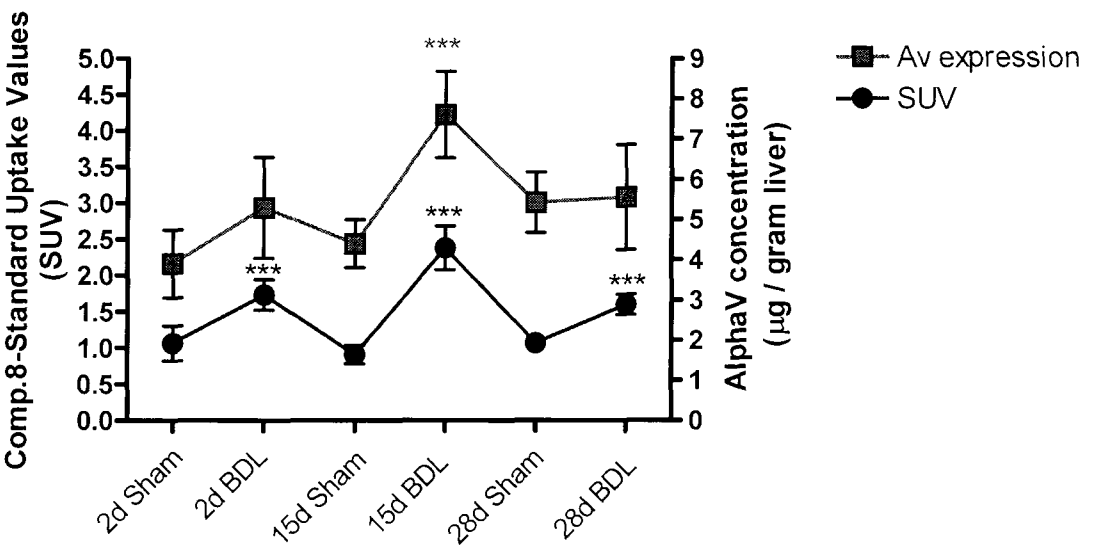


Figure 5