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The Applicant/Nominated Person has entitlement from the actual inventor as follows:-

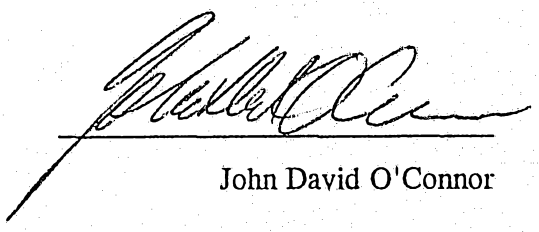
The Applicant/Nominated Person is the assignee of the actual inventor.

The Applicant/Nominated Person is entitled to rely on the application listed in the Declaration under Article 8 of the PCT as follows:

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The basic application listed on the Declaration under Article 8 of the PCT is the first application made in a Convention country in respect of the invention.

Dated 7 September 1995



John David O'Connor

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TECHNETIUM-99M LABELED PEPTIDES FOR DIAGNOSTIC IMAGING

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(56) Prior Art Documents
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(57) Claim

1. A reagent for preparing a radiolabeled peptide for imaging sites within a mammalian body, said reagent comprising:

a) a platelet factor 4 peptide or a platelet factor 4 peptide fragment;

b) a radiolabel-binding moiety covalently linked to the peptide or fragment; and

c) from one to five basic amino acid residues.

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(57) Abstract

This invention relates to reagents, radiolabeled reagents and methods for producing such reagents and radiolabeled reagents. Specifically, the invention relates to technetium-99m (Tc-99m) labeled peptides that specifically bind to sites of infection, inflammation, thrombosis, atherosclerosis and neoplatic growth *in vivo*, methods and kits for making such peptides, and methods for using such peptides to image sites in a mammalian body.

TECHNETIUM-99m LABELED PEPTIDES FOR DIAGNOSTIC IMAGING

Background of the Invention

1. Field of the Invention

This invention relates to radiodiagnostic reagents and peptides, methods of using these radiodiagnostic reagents and methods for producing such labeled radiodiagnostic agents. Specifically, the invention relates to technetium-99m (Tc-99m) labeled reagents, methods and kits for making such reagents, and methods for using such reagents to image sites in a mammalian body.

2. Description of the Prior Art

There is a clinical need to be able to determine the location and/or extent of sites of focal or localized infection. In a substantial number of cases conventional methods of diagnosis (such as physical examination, X-ray, CT and ultrasonography) fail to identify such sites (e.g., an abscess). Although biopsy may be resorted to, it is preferable to avoid such invasive procedures, at least until they are diagnostically appropriate to identify the pathogen responsible for an abscess at a known location. Identifying the site of such "occult" infection is important because rapid localization and identification of the problem is critical to effective therapeutic intervention.

In the field of nuclear medicine, certain pathological conditions can be localized or the extent of such conditions determined by imaging the internal distribution of administered radioactively-labeled tracer compounds (i.e. radiotracers or radiopharmaceuticals) that accumulate specifically at the pathological site. A variety of radionuclides are known to be useful for radioimaging, including ^{67}Ga, ^{99m}Tc (Tc-99m), ^{111}In, ^{123}I, ^{125}I, ^{169}Yb and ^{186}Re.

However, an abscess may be caused by any one of many possible pathogens, so that a radiotracer specific for a particular pathogen would have

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limited scope. On the other hand, infection is almost invariably accompanied by inflammation, which is a general response of the body to tissue injury. Therefore, a radiotracer specific for sites of inflammation would be expected to be useful in localizing sites of infection caused by any pathogen, as well as being useful for localizing other inflammatory sites.

One of the main phenomena associated with inflammation is the localization of leukocytes (white blood cells), usually monocytes and neutrophils, at the site of inflammation. A radiotracer specific for leukocytes would be useful in detecting leukocytes at the site of a localized infection. Currently approved nuclear medicine procedures for imaging sites of infection use either indium-111 labeled leukocytes (^{111}In-WBC) (*see, e.g.* Peters, 1992, J. Nucl. Med. 33: 65-67) or gallium-67 (^{67}Ga) citrate (*see, e.g.* Ebright *et al.*, 1982, Arch. Int. Med. 142: 246-254). A major disadvantage of using ^{111}In-labeled WBCs is that the preparation of the radiotracer requires a number of technical steps: sterile removal of autologous blood, sterile isolation of the leukocytes from the blood, sterile labeling of the leukocytes using conditions that do not damage the cells (since damaged WBC are taken up by the reticuloendothelial system when re-injected) and sterile return (re-injection) of the (now labeled) leukocytes to the patient. Furthermore, a delay of 12 to 48 hours between injection and imaging may be required to obtain optimum imaging. While Tc-99m labeled leukocytes have been used to shorten this delay period (*see, e.g.* Vorne *et al.*, 1989, J. Nucl. Med. 30: 1332-1336), ex-corporeal labeling is still required. A preferred radiotracer would be one that does not require removal and manipulation of autologous blood components.

Alternatively, ^{67}Ga-citrate can be administered by intravenous injection. However, this compound is not specific for sites of infection or inflammation. Moreover, a delay of up to 72 hours is often required between injection of the radiotracer and imaging. In addition, the γ-(gamma) emissions energies of ^{67}Ga are not well suited to conventional gamma cameras.

Radiolabeled monoclonal and polyclonal antibodies raised against human leukocytes (including monocytes, neutrophils, granulocytes and other cell types)

have been developed. Tc-99m labeled antigranulocyte monoclonal antibodies (*see*, *e.g.* Lind *et al.*, 1990, J. Nucl. Med. 31: 417-473) and ^{111}In-labeled non-specific human immunoglobulin (*see*, *e.g.* LaMuraglia *et al.*, 1989, J. Vasc. Surg. 10: 20-28) have been tested for the detection of inflammation secondary to infection. ^{111}In-labeled IgG shares the disadvantages of ^{111}In-labeled WBC, in that 24-48 hours are required between injection and optimal imaging. In addition, all radiolabeled antibodies are difficult to produce and face protracted approval procedures, as they are routinely classified as biologics by regulatory agencies.

Small readily synthesized molecules are preferred as routinely-used radiopharmaceuticals. There is clearly a need for small synthetic molecules that can be directly injected into a patient and will image sites of infection and inflammation by localizing at sites where leukocytes have accumulated. One kind of small, readily synthesized molecule useful in such applications are peptides.

The sensitivity of imaging methods using radioactively-labeled peptides is much higher than other techniques known in the art, since the specific binding of the radioactive peptide concentrates the radioactive signal over the area of interest, for example, an inflammatory site. In addition, methods for achieving high-yield chemical synthesis of small peptides is well known in the art.

One class of peptides known to bind to leukocytes are chemotactic peptides that cause leukocytes to move up a peptide concentration gradient (*see* Wilkinson, 1988, Meth. Enzymol. 162: 127-132). These compounds bind to receptors on the surface of leukocytes with very high affinity. These peptides are derived from a number of sources, including complement factors, bacteria, tuftsin, elastin, fibrinopeptide B, fibrinogen Bβ, platelet factor 4 and others. Small synthetic peptides derived from these chemotactic compounds and radiolabeled would be very useful as radiotracers for imaging sites of inflammation in vivo.

Radiolabeled peptides have been reported in the prior art.

Zoghbi *et al.*, 1981, J. Nucl. Med. 22: 32 (Abst) disclose formyl peptide chemotactic factors (fMLF) derived from bacteria coupled to ^{111}In-labeled transferrin.

Jiang *et al.*, 1982, Nuklearmedizin 21: 110-113 disclose a chemotactic

formylated peptide (fMLF) radiolabeled with ^{125}I.

Fischman *et al.*, 1991, J. Nucl. Med. 32: 482-491 relates to chemotactic formyl peptide (fMLF) - ^{111}In-labeled DTPA conjugates.

EPC 90108734.6 relates to chemotactic formyl peptide (fMLF) - ^{111}In-labeled DTPA conjugates.

U.S. Patent No. 4,986,979 relates to the use of radiolabeled chemotactic formyl peptides (fMLF) to radiolabel leukocytes ex-corporeally *via* a photoaffinity label.

PCT W090/10463 relates to the use of radiolabeled chemotactic formyl peptides (fMLF) to radiolabel leukocytes ex-corporeally *via* a photoaffinity label.

The use of labeled formyl-methionyl-leucyl-phenylalanyl (fMLF) peptides known in the aforementioned art suffers from the serious drawback that this peptide causes superoxide release from neutrophils (Niedel and Cuatrecasas, 1980, Formyl Peptide Chemotactic Receptors of Leukocytes and Macrophages, *in* Curr. Top. Cell. Reg. 17: 137-170) and at sufficient doses can cause leukocytopenia (Jiang *et al.*, 1982, Nuklearmed. 21: 110-113).

Platelet factor 4 is a naturally-occurring chemotactic peptide, consisting of 70 amino acids and known in the prior art to bind to neutrophils and monocytes, cell types known to be associated with sites of inflammation and infection *in vivo*.

Thorbecke & Zucker, 1989, European Patent Application No. 88111962.2 disclose compositions and methods for modulating immune responses comprising administering an immunomodulating amount of platelet factor 4 or peptides derived therefrom.

Deuel *et al.*, 1977, Proc. Natl. Acad. Sci. USA 74: 2256-2258 disclose the amino acid sequence of human platelet factor 4.

Deuel *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78: 4584-4587 disclose that platelet factor 4 is chemotactic for neutrophils and monocytes *in vitro*.

Osterman *et al.*, 1982, Biochem. Biophys. Res. Comm. 107: 130-135 disclose that the carboxyl-terminal tridecapeptide of platelet factor 4 has chemotactic properties.

Holt & Niewiarowski, 1985, Sem. Hematol. 22: 151-163 provide a review

of the biochemistry of platelet α-granule proteins, including platelet factor 4.

Goldman *et al.*, 1985, Immunol. 54: 163-171 reveal that fMLF receptor-mediated uptake is inhibited in human neutrophils by platelet factor 4 and a carboxyl-terminal dodecapeptide thereof.

Bebawy *et al.*, 1986, J. Leukocyte Biol. 39: 423-434 describe the platelet factor 4-mediated chemotactic response of neutrophils *in vitro*.

Loscalzo *et al.*, 1985, Arch. Biochem. Biophys. 240: 446-455 describe the biochemical interaction between platelet factor 4 and glycosaminoglycans such as heparin.

Maione *et al.*, 1989, Science 247: 77-79 disclose that angiogenesis is inhibited by recombinant human platelet factor 4 and peptide fragments thereof.

The use of chelating agents for radiolabeling polypeptides, and methods for labeling peptides and polypeptides with Tc-99m are known in the prior art and are disclosed in co-pending U.S. Patent Applications Serial Nos. 07/653,012, 07/807,062, 07/851,074, 07/871,282, 07/886,752, 07/893,981, 07/902,935, 07/955,466, and 08/044,825, which are hereby incorporated by reference.

Summary of the Invention

The present invention provides scintigraphic imaging reagents that are radioactively-labeled peptides. The reagents of the invention are comprised of peptides that specifically bind to sites *in vivo*, in particular, sites of infection and inflammation, as well as thrombotic, atherosclerotic and tumor sites, that are covalently linked to a radioisotope complexing group wherein the complexing group binds a radioisotope.

The invention provides reagents that bind to such a multiplicity of sites *in vivo*, radiolabeled complexes of such reagents with technetium-99m, methods for preparing such complexes, methods for using such radiolabeled complexes for imaging sites of infection, inflammation, thrombosis, atherosclerosis and neoplastic growth within a mammalian body, and methods for preparing the reagents of the invention.

In a first aspect of the present invention, there is provided a reagent for preparing a radiolabeled peptide for imaging sites within a mammalian body comprising

a) a platelet factor 4 peptide or a platelet factor 4 peptide fragment;

b) a radiolabel-binding moiety covalently linked to the peptide or fragment; and

c) from one to five basic amino acid residues.

In one embodiment of the first aspect of the present invention, the radiolabel-binding moiety has a formula

I

Cp(aa)Cp

wherein Cp is a protected cysteine residue and (aa) stands for an amino acid, and wherein the radiolabel-binding moiety is covalently linked to the specific binding peptides. In a preferred embodiment, the amino acid is glycine. In another preferred embodiment, the radiolabel-binding moiety is linked to the specific peptide *via* one or more amino acids.

In a second aspect, the present invention provides a reagent comprising a platelet factor 4 peptide or a platelet factor 4 peptide fragment, said peptide or fragment being covalently linked to a radiolabel-binding moiety having a formula selected from the group consisting of:

II

A-CZ(B)-[C(R1R2)]n-X

wherein A is H, HOOC, H2NOC, (peptide)-NHOC, (peptide)-OOC or R4;

B is H, SH, -NHR3, -N(R3)-(peptide), or R4;

X is H, SH, -NHR3, -N(R3)-(peptide) or R4;

Z is H or R4;

R1, R2, R3 and R4 are independently H or lower straight or branched chain or cyclic alkyl;

n is 0, 1 or 2;

and where B is -NHR3 or -N(R3)-(peptide), X is SH, and n is 1 or 2;

where X is -NHR3 or -N(R3)-(peptide), B is SH, and n is 1 or 2;

where B is H or R^4, A is HOOC, H_2NOC, (peptide)-NHOC, (peptide)-OOC,

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X is SH, and n is 0 or 1;

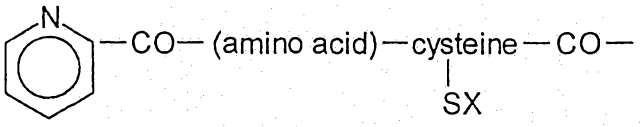
where A is H or R^4, then where B is SH, X is -NHR^3 or -$N(R^3)$-(peptide) and where X is SH, B is -NHR^3 or -$N(R^3)$-(peptide);

where X is H or R^4, A is HOOC, H_2NOC, (peptide)-NHOC, (peptide)-OOC and B is SH;

where Z is methyl, X is methyl, A is HOOC, H_2NOC, (peptide)-NHOC, (peptide)-OOC, B is SH and n is 0;

and wherein the thiol moiety is in the reduced form;

III

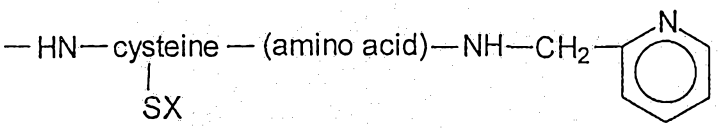


wherein X = H or a protecting group;

(amino acid) = any amino acid;

[For purposes of this invention, radiolabel-binding moieties having this structure will be referred to as picolinic acid (Pic)-based moieties];

IV

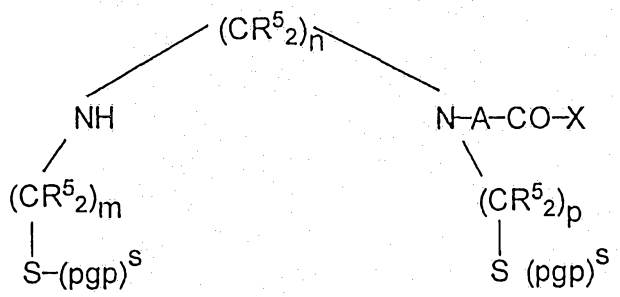


wherein X = H or a protecting group;

(amino acid) = any amino acid;

[For purposes of this invention, radiolabel-binding moieties having this structure will be referred to as picolylamine (Pica)-based moieties];

V



wherein each R^5 is independently H, CH_3 or C_2H_5;

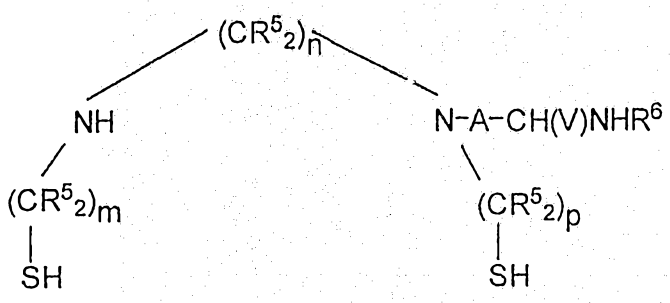
each $(pgp)^s$ is independently a thiol protecting group or H;

m, n and p are independently 2 or 3;

A = linear or cyclic lower alkyl, aryl, heterocyclyl, a combination

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VI



wherein each R^5 is independently H, lower alkyl having 1 to 6 carbon atoms, phenyl, or phenyl substituted with lower alkyl or lower alkoxy;

m, n and p are independently 1 or 2;

A = linear or cyclic lower alkyl, aryl, heterocyclyl, a combination thereof or a substituted derivative thereof;

V = H or -CO-peptide;

R^6 = H or peptide;

and wherein when V = H, R^6 = peptide and when R^6 = H, V = -CO-peptide.

[For purposes of this invention, radiolabel-binding moieties having these structures will be referred to as "BAT" moieties]. In a preferred embodiment, the peptide is covalently linked to the radiolabel-binding moiety *via* an amino acid,

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most preferably glycine, and the radiolabel is technetium-99m.

The invention also comprises complexes of the peptides of the invention with Tc-99m, kits for preparing the peptides of the invention radiolabeled with Tc-99m, methods for radiolabeling the peptides of the invention with Tc-99m and methods for using the radiolabeled peptides of the invention for imaging sites of infection, inflammation, thrombosis, atherosclerosis and neoplastic growth within a mammalian body by gamma scintigraphy.

The invention provides scintigraphic imaging agents that are complexes of the peptide reagents of the invention with Tc-99m and methods for radiolabeling the peptide reagents of the invention with Tc-99m. Radiolabeled complexes provided by the invention are formed by reacting the peptide reagents of the invention with Tc-99m in the presence of a reducing agent. Preferred reducing agents include but are not limited to dithionite ion, stannous ion and ferrous ion. Complexes of the invention are also formed by labeling the peptide reagents of the invention with Tc-99m by ligand exchange of a prereduced Tc-99m complex as provided herein.

The invention also provides kits for preparing scintigraphic imaging agents that are the peptide reagents of the invention radiolabeled with Tc-99m. Kits for labeling the peptide reagents of the invention with Tc-99m are comprised of a sealed vial containing a predetermined quantity of a peptide reagent of the invention and a sufficient amount of reducing agent to label the reagent with Tc-99m.

This invention provides methods for preparing peptide reagents of the invention by chemical synthesis *in vitro*. In a preferred embodiment, specific binding peptides are synthesized by solid phase peptide synthesis.

This invention provides methods for using scintigraphic imaging agents that are Tc-99m labeled peptide reagents for imaging sites of inflammation and infection within a mammalian body by obtaining *in vivo* gamma scintigraphic images. These methods comprise administering an effective diagnostic amount of Tc-99m labeled peptide reagents of the invention and detecting the gamma radiation emitted by the Tc-99m label localized at the inflammation site within the

mammalian body.

In addition to localizing at sites of infection and inflammation, leukocytes are also known to localize at sites to thrombosis (*see, for example,* Kwaan *et al.*, 1989, "Pathogenesis of Thrombosis", in Clinical Thrombosis, Kwaan & Samama, eds, CRC Press: Boca Raton, La., p.35). Therefore, in addition to providing radiotracers useful for imaging sites of infection and inflammation, the PF4-derived radiotracers of the invention also provide radiotracers useful for imaging sites of deep vein thrombosis and pulmonary embolism.

Furthermore, it is known that PF4 binds to heparin and other glycosaminoglycans (*see* Loscalzo *et al.*, 1985, *ibid.*). In atherosclerosis, excessive cell proliferation, in particular smooth muscle cell proliferation, leads to an overabundance of extracellular matrix-derived material, a major component of which are glycosaminoglycans. Therefore, the radiolabeled PF4-derived peptides of the invention also provide radiotracers useful for imaging sites of atherosclerosis.

Also, it has been shown that PF4 and PF4 fragments can inhibit tumor-related angiogenesis (*see* Sharpe *et al.*, 1990. J. Natl. Cancer Inst. 82: 848-853 *and* Maione *et al.*, 1990, *ibid.*). Thus, the radiolabeled PF4-derived peptides of the invention are also useful for imaging tumor sites.

The specific binding peptides of the invention may also be covalently linked to a polyvalent linking moiety. Polyvalent linking moieties of the invention are comprised of at least 2 linker functional groups capable of covalently bonding to specific binding peptides or Tc-99m binding moieties. Preferred linker functional groups are primary or secondary amines, hydroxyl groups, carboxylic acid groups or thiol-reactive groups such as 2-haloacetyl groups and maleimido groups. In preferred embodiments, the polyvalent linking moieties are comprised of *bis*-succinimdylmethylether (BSME), 4-(2,2-dimethylacetyl)benzoic acid (DMAB), *tris*(succinimidylethyl)amine (TSEA), *tris*-acetamidoethylamine and 1,10-*bis*acetamido-4, 7-dioxa-decane.

A polyvalent linker covalently linked to at least two platelet factor 4 peptides or platelet factor 4 peptide fragments forms a multimer, wherein the molecular weight of the multimer is less than about 20,000 daltons.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred

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embodiments and the claims.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides Tc-99m labeled peptide reagents for imaging target sites within a mammalian body that specifically bind to sites of infection, inflammation, thrombosis, atherosclerosis and neoplastic growth within a mammalian body, the specific binding peptides being covalently linked to a radiolabel binding moiety wherein the radiolabel binding moiety binds a radioisotope.

The peptide reagents of this invention specifically bind to sites of infection, inflammation, thrombosis, atherosclerosis and neoplastic growth within a mammalian body. These reagents may also bind to leukocytes, preferably monocytes and neutrophils and most preferably to neutrophils. For purposes of this invention, the term "bind to leukocytes" is intended to mean that the specific binding peptides and peptide reagents of the present invention are capable of accumulating at sites of infection or inflammation in mammalian body sufficient to allow detection of the accumulation of radiolabeled complexes prepared from the reagents of the invention as disclosed herein at sites of infection or inflammation by gamma scintigraphy.

Also included in the meaning of the term "binds to leukocytes" herein is that the specific binding peptides and peptide reagents of the present invention are capable of accumulating at sites of thrombosis, due to the fact that leukocytes may be localized at such thrombotic sites.

The reagents of the invention may also bind to heparin and other glycosaminoglycans that are present at sites of atherosclerosis. For purposes of this invention, the term "bind to heparin and other glycosaminoglycans" is intended to mean that the specific binding peptides of the present invention are capable of accumulating at sites of atherosclerosis, due to the fact that extracellular matrix material, including glycosaminoglycans may be localized at such atherosclerotic sites.

The PF4-derived peptide reagents of the invention being capable of

inhibiting tumor-related angiogenesis, the specific binding peptides of the present invention are capable of accumulating at tumor sites *in vivo*, due to the fact that such peptides may localize to such tumor sites.

Preferred peptides of the invention include platelet factor 4 and peptides derived therefrom. For purposes of this invention, the term "peptides derived therefrom" is intended to encompass peptides having an amino acid sequence homologous to all or a portion of the platelet factor 4 amino acid sequence. Also intended to be circumscribed by this term are peptide fragments of platelet factor 4, whether generated by proteolytic degradation of native platelet factor 4 protein or by chemical synthesis of a portion of the platelet factor 4 amino acid sequence. Peptide fragments useful in the practice of this invention include those fragments capable of specifically binding to sites of infection and inflammation in a mammalian body. Examples of such peptides are presented hereinafter in the Examples, and PLY.Orn.Orn.II.Orn.Orn.LLES.

In Cp(aa)Cp-containing peptides, the Cp is a protected cysteine where the S-protecting groups are the same or different and may be but not limited to:

-CH_2-aryl (aryl is phenyl or alkyl or alkyloxy substituted phenyl);

-CH-(aryl)$_2$, (aryl is phenyl or alkyl or alkyloxy substituted phenyl);

-C-(aryl)$_3$, (aryl is phenyl or alkyl or alkyloxy substituted phenyl);

-CH_2-(4-methoxyphenyl);

-CH-(4-pyridyl)(phenyl)$_2$;

-$C(CH_3)_3$;

-9-phenylfluorenyl;

-CH_2NHCOR (R is unsubstituted or substituted alkyl or aryl);

-CH_2-NHCOOR (R is unsubstituted or substituted alkyl or aryl);

-CONHR (R is unsubstituted or substituted alkyl or aryl);

-CH_2-*S*-CH_2-phenyl.

Radiolabel binding moieties comprising cysteine-sulfur protecting groups designated "(pgp)^S", such as the bisamino, bisthiol moieties of the invention, are also described by the above-mentioned listing of protecting groups.

The preferred protecting group has the formula -CH_2-NHCOR wherein R

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is a lower alkyl having 1 and 8 carbon atoms, phenyl or phenyl-substituted with lower alkyl, hydroxyl, lower alkoxy, carboxy, or lower alkoxycarbonyl.

Labeling with Tc-99m is an advantage of the present invention because the nuclear and radioactive properties of this isotope make it an ideal scintigraphic imaging agent. This isotope has a single photon energy of 140 keV and a radioactive half-life of about 6 hours, and is readily available from a ^{99}Mo-^{99m}Tc generator. Other radionuclides known in the prior art have effective half-lives which are much longer (*for example*, ^{111}In, which has a half-life of 67.4 h) or are toxic (*for example*, ^{125}I).

Peptides of the present invention may be chemically synthesized *in vitro*. Peptides of the present invention can generally advantageously be prepared on a peptide synthesizer. The peptides of this invention can be synthesized wherein the radiolabel binding moiety is covalently linked to the peptide during chemical *in vitro* synthesis, using techniques well known to those with skill in the art. Such peptides covalently-linked to the radiolabel binding moiety upon synthesis are advantageous because specific sites of covalent linkage can be determined therein.

Radiolabel binding moieties of the invention may be introduced into the target specific peptide during peptide synthesis. For embodiments [*e.g.*, Pic-Gly-Cys(protecting group)-] comprising picolinic acid (Pic-), the radiolabel-binding moiety can be synthesized as the last (i.e., amino-terminal) residue in the synthesis. In addition, the picolinic acid-containing radiolabel-binding moiety may be covalently linked to the ϵ-amino group of lysine to give, for example, αN(Fmoc)-Lys-ϵN[Pic-Gly-Cys(protecting group)], which may be incorporated at any position in the peptide chain. This sequence is particularly advantageous as it affords an easy mode of incorporation into the target binding peptide.

Similarly, the picolylamine (Pica)-containing radiolabel-binding moiety [-Cys(protecting group)-Gly-Pica] can be prepared during peptide synthesis by including the sequence [-Cys(protecting group)-Gly-] at the carboxyl terminus of the peptide chain. Following cleavage of the peptide from the resin the carboxyl terminus of the peptide is activated and coupled to picolylamine. This synthetic route requires that reactive side-chain functionalities remain masked (protected)

and do not react during the conjugation of the picolylamine.

Examples of small synthetic peptides containing the Pic-Gly-Cys- chelator are provided in the Examples hereinbelow. This invention provides for the incorporation of these chelators into virtually any platelet factor 4-derived peptide, resulting in a Tc-99m radiolabeled peptide.

This invention also provides specific-binding small synthetic peptides which incorporate bisamine bisthiol (BAT) chelators which may be labeled with Tc-99m.

In forming a complex of radioactive technetium with the peptide reagents of this invention, the technetium complex, preferably a salt of Tc-99m pertechnetate, is reacted with the peptide reagents of this invention in the presence of a reducing agent; in a preferred embodiment, the reducing agent is stannous chloride. Complexes and means for preparing such complexes are conveniently provided in a kit form comprising a sealed vial containing a predetermined quantity of the peptide reagent of the invention that are to be labeled and a sufficient amount of reducing agent to label the reagent with Tc-99m. Alternatively, the complex may be formed by reacting the peptide reagents of this invention with a pre-formed labile complex of technetium and another compound known as a transfer ligand. This process is known as ligand exchange and is well known to those skilled in the art. The labile complex may be formed using such transfer ligands as tartrate, citrate, gluconate or mannitol, for example. Among the Tc-99m pertechnetate salts useful with the present invention are included the alkali metal salts such as the sodium salt, or ammonium salts or lower alkyl ammonium salts. The reaction of the peptide reagents of this invention with Tc-pertechnetate or preformed Tc-99m labile complex can be carried out in an aqueous medium at room temperature. When an anionic complex is formed in an aqueous medium, the radiolabeled complex is in the form of a salt with a suitable cation such as sodium cation, ammonium cation, mono, di- or tri-lower alkyl amine cation. or any pharmaceutically acceptable cation.

In a preferred embodiment of the invention, a kit for preparing technetium-labeled peptides is provided. The peptide reagents of the invention can be

chemically synthesized using methods and means well-known to those with skill in the art and described hereinbelow. Peptides are covalently linked to a radiolabel binding moiety wherein the radiolabel binding moiety binds a radioisotope. An appropriate amount of the peptide reagent is introduced into a vial containing a reducing agent, such as stannous chloride or a solid-phase reducing agent, in an amount sufficient to label the reagent with Tc-99m. An appropriate amount of a transfer ligand as described (such as tartrate, citrate, gluconate or mannitol, for example) can also be included. Technetium-labeled peptides according to the present invention can be prepared by the addition of an appropriate amount of Tc-99m or Tc-99m complex into the vials and reaction under conditions described in Example 2 hereinbelow.

Radioactively labeled pedptide reagents provided by the present invention are provided having a suitable amount of radioactivity. In forming the Tc-99m radioactive complexes, it is generally preferred to form radioactive complexes in solutions containing radioactivity at concentrations of from about 0.01 millicurie (mCi) to 100 mCi per ml.

Technetium-labeled peptide reagents provided by the present invention can be used for visualizing sites of inflammation, including abscesses and sites of "occult" infection. The Tc-99m labeled peptides provided by the present invention can also be used for visualizing sites of inflammation caused by tissue ischemia, including such disorders as inflammatory bowel disease and arthritis. The peptides of the invention can also be used to visualize atherosclerotic and thrombotic sites, as well as being useful for localizing tumor sites *in vivo*, particularly sites of primary or metastatic tumors too small to be otherwise detected.

In accordance with this invention, the technetium-labeled peptides or complexes either as a complex or as a salt with a pharmaceutically acceptable counterion are administered in a single unit injectable dose. Any of the common carriers known to those with skill in the art, such as sterile saline solution or plasma, can be utilized after radiolabeling for preparing the injectable solution to diagnostically image various organs, tumors and the like in accordance with this invention. Generally, the unit does to be administered has a radioactivity of about 0.01 mCi to about 100 mCi, preferably 1 mCi to 20 mCi. The solution to be injected at unit dosage is from about 0.01 ml to about 10 ml. After intravenous administration, imaging of the organ or tumor *in vivo* can take place in a matter of a few minutes. However, imaging can take place, if desired, in hours or even longer, after injecting into patients. In most instances, a sufficient amount of the administered dose will accumulate in the area to be imaged within about 0.1 of an hour to permit the taking of scintiphotos. Any conventional method of scintigraphic imaging for diagnostic purposes can be utilized in accordance with this invention.

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The technetium-labeled peptides and complexes provided by the invention may be administered intravenously into any conventional medium for intravenous injection such as an aqueous saline medium, or in blood plasma medium. Such medium may also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, buffers, preservatives and the like. Among the preferred media are normal saline and plasma.

The methods for making and labeling these compounds are more fully illustrated in the following Examples. These Examples illustrate certain aspects of the above-described method and advantageous results. These Examples are shown by way of illustration and not by way of limitation.

EXAMPLE 1

Solid Phase Peptide Synthesis

Solid phase peptide synthesis (SPPS) was carried out on a 0.25 millimole (mmole) scale using an Applied Biosystems Model 431A Peptide Synthesizer and using 9-fluorenylmethyloxycarbonyl (Fmoc) amino-terminus protection, coupling with dicyclohexylcarbodiimide/hydroxybenzotriazole or 2-(1H-benzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorphosphate/hydroxybenzotriazole (HBTU/HOBT), and using *p*-hydroxymethylphenoxymethyl-polystyrene (HMP) resin for carboxyl-terminus acids or Rink amide resin for carboxyl-terminus

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amides. Where appropriate N-α-acetyl groups were introduced by treating the resin-bound peptide with acetic anhydride in N-methylpyrrolidinone (NMP). Resin-bound products were routinely cleaved using a solution comprised of trifluoroacetic acid, water, thioanisole, ethanedithiol, and triethylsilane, prepared in ratios of 100 : 5 : 5 : 2.5 : 2 for 1.5 - 3 h at room temperature. Crude peptides were purified by preparative high pressure liquid chromatography (HPLC) using a Waters Delta Pak C18 column and gradient elution using 0.1% trifluoroacetic acid (TFA) in water modified with acetonitrile. Acetonitrile was evaporated from the eluted fractions which were then lyophilized. The identity of each product was confirmed by fast atom bombardment mass spectroscopy (FABMS).

EXAMPLE 2

A General Method for Radiolabeling with Tc-99m

Peptides (0.1 mg) prepared as in Example 1 was dissolved in 0.1 mL of solvent as described in the footnotes to Table I. Tc-99m gluceptate was prepared by reconstituting a Glucoscan vial (E.I. DuPont de Nemours, Inc.) with 1.0 mL of Tc-99m sodium pertechnetate containing up to 200 mCi and allowed to stand for 15 minutes at room temperature. 25 μL of Tc-99m gluceptate was then added to the peptide reagent and the reaction allowed to proceed at 100°C or at room temperature for 30 min and then filtered through a 0.2 μm filter.

The purity of the Tc-99m labeled peptide reagent was determined by HPLC using a Vydak 218TP54 analytical column (RP-18, 5 micron, 220 x 4.6 mm) or a Waters DDelltaPak analytical column (C18, 5 micron, 39mm x 50mm) and eluted as described in the footnotes in Table I. Radioactive components were detected by an in-line radiometric detector linked to an integrating recorder. Tc-99m gluceptate and Tc-99m sodium pertechnetate elute between 1 and 4 minutes under these conditions, whereas the Tc-99m labeled peptide eluted after a much greater amount of time.

The following Table illustrates successful Tc-99m labeling of peptides prepared according to Example 1 using the method described herein.

TABLE I

Peptides	SEQ ID#	FABMS MH^+	Radiochemical Yield	HPLC R_t(min)
$C_{Mob}GC_{Acm}$PLYKKIIKKLLES	1	2028	97%[1]	Bound
[DTPA]$C_{Acm}GC_{Acm}$PLYKKIIKKLLES	2	2468	91%[1]	11.6
PicGC_{Acm}PLYKKIIKKLLES	3	1910	81%[1]	12.9,13.3
$C_{Acm}GC_{Acm}$GGPLYKKIIKKLLES	4	2093	96%[1]	12.6
(Ac$CC_{Acm}GC_{Acm}$PLYKKIIKKLLES)$_2$BSME		4483	97%[3]	11.6
AcKKKKK$C_{Acm}GC_{Acm}$GGPLYKKIIKKLLES	5	2276	98%[1]	11.3
[BAT].GGPLYKKIIKKLLES	6	2006	91%[4]	9.5
(Ac$CGC_{Acm}GC_{Acm}$GGPLYKKIIKKLLES)$_2$BSME		4483	98%[1]	11.6
[BAT].KKLLKKLYKKIIKKLLES	7	2533	97%[4]	17.4
AcKKKKKCGCGGPLYKKIIKKLLES	8	2633	99%[1]	14.8
Ac$KKC_{Acm}GC_{Acm}$GGPLYKKIIKKLLES	9	2391[¶]	100%[1]	15.5
AcKKKKK$C_{Acm}GC_{Acm}$QAPLYKKIIKKLLES	10	2861	100%[1]	15.1
Ac$KC_{Acm}GC_{Acm}$GGPLYKKIIKKLLES	11	2263	100%[1]	16.0
Ac$KC_{Acm}GC_{Acm}$QAPLYKKIIKKLLES	12	2348	100%[1]	16.0
AcKKK$C_{Acm}GC_{Acm}$GGPLYKKIIKKLLES	13	2519	100%[1]	15.0, 15.2
Ac$KKC_{Acm}GC_{Acm}$QAPLYKKIIKKLLES	14	2476	100%[1]	15.6

TABLE I (cont'd.)

Peptides	SEQ ID#	FABMS MH^+	Radiochemical Yield	HPLC R_t(min)
$(AcCKKC_{Acm}GC_{Acm}PLYKKIIKKLLES)_2BSME$		4996¶	99%[1]	15.1-15.7
$AcKKKKC_{Acm}GC_{Acm}GGPLYKKIIKKLLES$	15	2647	98%[1]	14.9, 15.1
$AcKKKKC_{Acm}GC_{Acm}QAPLYKKIIKKLLES$	16	2732	100%[1]	15.2
$AcKKKC_{Acm}GC_{Acm}QAPLYKKIIKKLLES$	17	2604	99%[1]	15.4
$AcC_{Acm}GC_{Acm}KKIIKKLLES$	18	1647	99%[1]	16.3, 16.8
$AcC_{Acm}GC_{Acm}PLYQQIIQQLLES$	19	1965	99%[1]	17.1
AcKKKKK.[BAT].GGPLYKKIIKKLLES	20	2778	97%[2]	15.6
RGCQAPLYKKIIKKLLES	21	2089	96%[2]	15.6
$AcRC_{Acm}GC_{Acm}QAPLYKKIIKKLLES$	22	2377	93%[1]	16.0
AcKK.(ϵ-K).GCGGPLYKKIIKKLLES		2275	98%[2]	15.1
$AcC_{Acm}GC_{Acm}QAPLYKKIIKKLLES$	23	2820	100%[1]	16.6
(2-ketogulonyl).(ϵ-K).GCGGPLYKKIIKKLLES		2153	N.D.	N.D.

¶ = M.W. detected by electrospray mass spectrometry

Single-letter abbreviations for amino acids can be found in G. Zubay, Biochemistry (2d. ed.), 1988 (MacMillen Publishing: Y; Orn = ornithine; Ac = acetyl; Pic = picolinoyl (pyridine-2-carbonyl); Acm = acetamidomethyl; Mob = 4-Methoxybe [DTPA] = diethylenetriaminepentaacetic acid; BSME = *bis*-succinimidylmethyl ether; ϵ-K = a lysine residue in a peptide in v the peptide bond involves the ϵ-amino group on the lysine sidechain rather than the α-amino group;

*The following labeling conditions were used with the appropriate peptides:

1. The peptide is dissolved in water and labeled at 100°C.
2. The peptide is dissolved in water and labeled at room temperature.
3. The peptide is dissolved in 1:1 mixture of ethanol:water and labeled at 100°C.
4. The peptide is dissolved in 1:1 mixture of ethanol:water and labeled at room temperature.

HPLC methods:

general: solvent A = 0.1% CF_3COOH/H_20
solvent B_{90} = 0.1% CF_3COOH/90% CH_3CN/H_20
solvent flow rate = 1ml/min

Vydak column = Vydak 218TP54 RP-18, 5μ, 220mm x 4.6mm analytical column with guard column

Waters column = Waters DeltaPak C18, 5μ, 39mm x 150mm

Method: Vydak or Waters column 100% A to 100% B_{90} in 10-20 min

EXAMPLE 3

Scintigraphic Imaging and Biodistribution of Tc-99m Labeled Peptides

In order to demonstrate the effectiveness of Tc-99m labeled peptide reagents as provided above, New Zealand white rabbits were innoculated intramuscularly in the left calf with a potent strain of *E. coli*. After 24 h, the animals were sedated by i.m. injection of ketamine and xylazine, and then injected i.v. with Tc-99m labeled peptide (≤ 150μg, 2-10 mCi). The animals were positioned supine in the field of view of a gamma camera (LEAP collimator/photopeaked for Tc-99m) and imaged over the first hour post-injection, and then at approximately 1h intervals over the next three hours post injection. Animals were allowed to recover between image acquisitions and re-anesthetized as needed.

Upon completion of the final imaging, each animal was sacrificed by overdose of phenobarbital i.v. and dissected to obtain samples of blood and of infected and control muscle tissue. The tissue samples were weighed, and along with a standard amount of the injected dose, were counted using a gamma

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counter, and the percent injected dose (per gram of tissue) remaining in the tissues was determined. Ratios of percent of injected dose per gram of infected *versus* non-infected muscle tissue, and of infected muscle tissue *versus* blood, were calculated for each peptide. These results are presented in the following Table. Scintiphotos of whole body and leg images of a rabbit injected with a Tc-99m labeled reagent of the invention, having the formula

*acetyl*KKKKKC$_{Acm}$GC$_{Acm}$GGPLYKKIIKKLLES

are presented in Figure 1.

The ratios of infected/control muscle seen in the Table clearly demonstrate the specific and preferential localization of radiolabeled peptides of the invention at sites of infection, compared with normal tissues. The images shown in Figure 1 also clearly show the much greater extent of localization of radioactivity in the leg bearing the experimentallly-infected site compared to the non-infected contralateral leg.

TABLE II

Peptide Reagents	Infected Muscle (%ID/g)	Control Muscle (%ID/g)	Ratio of Infected/ Control	Blood (%ID/g)	Ratio of Infected/ Blood
Ac.CGGGPLYKKIIKKLLES	0.0235	0.0050	4.70	0.032	0.74
(*Ac*.$CC_{Acm}GC_{Acm}$PLYKKIIKKLLES)$_2$BSME	0.0082	0.0011	7.45	0.010	0.84
Ac.$KKKKKC_{Acm}GC_{Acm}$GGPLYKKIIKKLLES	0.0061	0.0019	5.60	0.006	0.93
[BAT].GGPLYKKIIKKLLES	0.0021	0.0003	7.00	0.004	0.50

(%ID/g) = percent injected dose per gram tissue; other abbreviations are as in the previous Tables.

EXAMPLE 4

In Vivo Imaging of Deep Vein Thrombosis in a Canine Model using Tc-99m Labeled Compound Thrombus Scintigraphic Imaging Agents

Mongrel dogs (25-35lb., fasted overnight) are sedated with a combination of ketamine and aceprozamine intramuscularly and then anesthetized with sodium pentabarbital intravenously. An 18-gauge angiocath is inserted in the distal half of the right femoral vein and an 8mm Dacron®-entwined stainless steel embolization coil (Cook Co., Bloomington IN) is placed in the femoral vein at approximately mid-femur in each animal. The catheter is removed, the wound sutured and the placement of the coil documented by X-ray. The animals are then allowed to recover overnight.

One day following coil placement, each animal is re-anesthetized, intravenous saline drips placed in each foreleg and a urinary bladder catheter inserted to collect urine. The animal is placed supine under a gamma camera which is equipped with a low-energy, all purpose collimator and photopeaked for Tc-99m. Images are acquired on a nuclear medicine computer system.

Tc-99m labeled reagent [185-370 mBq (5-10 mCi) Tc-99m and 0.2-0.4mg reagent] is injected into one foreleg intravenous line at its point of insertion. The second line is maintained for blood collection. Anterior images over the legs are acquired for 500,000 counts or 20 min (whichever was shorter), at approximately 10-20 min. and at approximately 1, 2, 3 and 4h post-injection. Following the collection of the final image, each animal is deeply anesthetized with pentobarbital. Two blood samples are collected on a cardiac puncture using a heparinized syringe followed by a euthanasing dose of saturated potassium chloride solution administered by intercardiac or bolus intravenous injection. The femoral vein containing the thrombus and samples of thigh muscle are then carefully dissected out. The thrombus is then dissected free of the vessel and placed in a pre-weighed test tube. The thrombus samples are then weighed and counted in a gamma well counter in the Tc-99m channel. Known fractions of the injected doses are counted as well.

Fresh thrombus weight, percent injected dose (%ID)/g in the thrombus and blood obtained just prior to euthanasia and thrombus/blood and thrombus/muscle

ratios are determined. Thrombus/background ratios were determined by analysis of the counts/pixel measured in regions-of-interest (ROI) drawn over the thrombus and adjacent muscle from computer-stored images. These results are used to demonstrate thrombus-specific localization of radioactivity and the efficacy of scintigraphic imaging to localize sites of thrombus formation *in vivo*.

EXAMPLE 5

Localization and *In Vivo* Imaging of Atherosclerotic Plaque using the Hypercholesterol Rabbit Model

New Zealand White (NZW) rabbits of both sexes and weighing 2-3kg are divided into two groups. The control group is housed and fed commercial rabbit chow (Purina). The HC group is fed a standardized, cholesterol-rich diet (rabbit chow mixed to a 1% w/w concentration of cholesterol) from seven weeks until 28 weeks of age. All animals are given water *ad libitum*.

Approximately 250-400μg of a peptide as described above is labeled with 140-160mCi of Tc-99m and prepared in unit doses of 7-8mCi (12.5-20.0 μg/rabbit; 6-7μg/kg) in 0.2mL volume doses. Adult rabbits are dosed with Tc-99m labeled peptide intravenously in a lateral ear vein by slow bolus infusion (approximately 0.1mL/min). A gamma camera fitted with a pin-hole collimator (5mm aperture) and energy window set for Tc-99m and programmed to accumulate 500,000 counts or scan for a desired time is used. Shortly before imaging, animals are anesthetized with a mixture of ketamine and xylazine (5:1, 1mL/kg intramuscularly).

Gamma camera images are collected at 40°-45° just above the heart (left anterior oblique [LAO] view) to delineate the aortic arch and view the descending aorta. Images are acquired at 1 and 2h and occasionally at 3 and 5h after injection. Supplementary anesthesia is injected as needed prior to each image collection.

At 2.5 h (after a 2h scan), animals are sacrificed with an intravenous dose of sodium pentobarbital. Upon necropsy, the aorta is removed and branching vessels dissected free from the aortic valve to the mid-abdominal region. Using a parallel hole collimator, the aorta is imaged *ex corpora*. Next, the aortae are

opened longitudinally and stained with Sudan IV, thereby turning atherosclerotic plaque a deep red brick color. Lipid-free and uninjured aortic endothelium retains its normal, glistening white-pink appearance under these conditions.

Uptake in the plaqued region of the aorta *versus* the non-plaqued aorta seen in the *in vivo* or *ex corpora* images congruent with the localization of plaque observed by staining to aortae are used to demonstrate the utility of the radiolabeled peptides of the invention to provide images of atherosclerosis.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

The claims defining the invention are as follows:

1. A reagent for preparing a radiolabeled peptide for imaging sites within a mammalian body, said reagent comprising:

a) a platelet factor 4 peptide or a platelet factor 4 peptide fragment;

b) a radiolabel-binding moiety covalently linked to the peptide or fragment; and

c) from one to five basic amino acid residues.

2. A reagent comprising a platelet factor 4 peptide or a platelet factor 4 peptide fragment, said peptide or fragment being covalently linked to a radiolabel-binding moiety having a formula selected from the group consisting of:

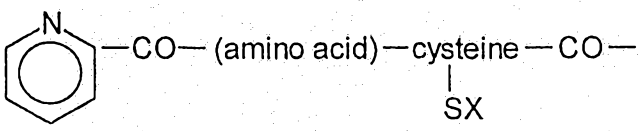
II

$$A\text{-}CZ(B)\text{-}[C(R^1R^2)]_n\text{-}X$$

wherein A is H, HOOC, H_2NOC, (peptide)-NHOC, (peptide)-OOC or R^4;
B is H, SH, $-NHR^3$, $-N(R^3)$-(peptide), or R^4;
X is H, SH, $-NHR^3$, $-N(R^3)$-(peptide) or R^4;
Z is H or R^4;
R^1, R^2, R^3 and R^4 are independently H or lower straight or branched chain or cyclic alkyl;
n is 0, 1 or 2;

and where B is $-NHR^3$ or $-N(R^3)$-(peptide), X is SH, and n is 1 or 2;
where X is $-NHR^3$ or $-N(R^3)$-(peptide), B is SH, and n is 1 or 2;
where B is H or R^4, A is HOOC, H_2NOC, (peptide)-NHOC, (peptide)-OOC, X is SH, and n is 0 or 1;
where A is H or R^4, then where B is SH, X is $-NHR^3$ or $-N(R^3)$-(peptide) and where X is SH, B is $-NHR^3$ or $-N(R^3)$-(peptide);
where X is H or R^4, A is HOOC, H_2NOC, (peptide)-NHOC, (peptide)-OOC and B is SH;
where Z is methyl, X is methyl, A is HOOC, H_2NOC, (peptide)-NHOC, (peptide)-OOC, B is SH and n is 0;
and wherein the thiol moiety is in the reduced form;

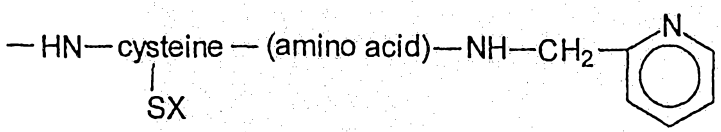
III



wherein X = H or a protecting group;
(amino acid) = any amino acid;

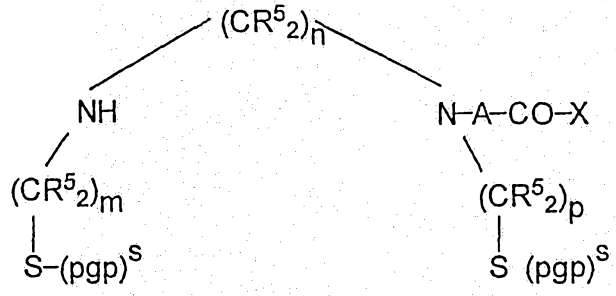
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IV



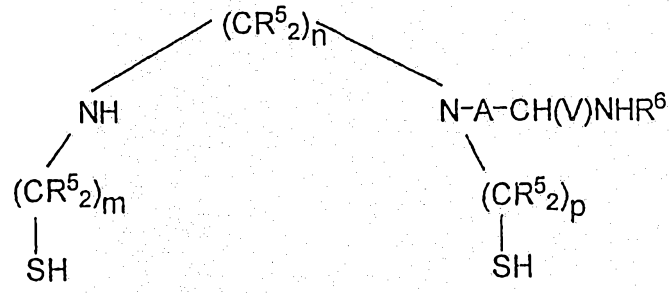
wherein X = H or a protecting group;
(amino acid) = any amino acid;

V



wherein each R^5 is independently H, CH_3 or C_2H_5;
each $(pgp)^s$ is independently a thiol protecting group or H;
m, n and p are independently 2 or 3;
A = linear or cyclic lower alkyl, aryl, heterocyclyl, a combination thereof or a substituted derivative thereof;

VI



wherein each R^5 is independently H, lower alkyl having 1 to 6 carbon atoms, phenyl, or phenyl substituted with lower alkyl or lower alkoxy;
m, n and p are independently 1 or 2;
A = linear or cyclic lower alkyl, aryl, heterocyclyl, a combination thereof or a substituted derivative thereof;
V = H or -CO-peptide;
R^6 = H or peptide;
and
wherein when V = H, R^6 = peptide and when R^6 = H, V = -CO-peptide.

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3. The reagent of claim 2, wherein the peptide or fragment and the radiolabel binding moiety are covalently linked through from one to about 20 amino acids.

4. The reagent of claim 1, wherein the radiolabel-binding moiety has a formula

Cp(aa)Cp

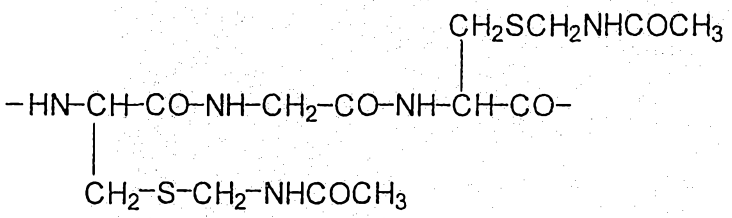
wherein Cp is a protected cysteine having a protecting group and (aa) is an α- or β-amino acid.

5. The reagent of claim 4, wherein the protecting group has a formula

$-CH_2-NH-CO-R$

wherein R is a lower alkyl having 1 to 6 carbon atoms, 2-pyridyl, 3-pyridyl, 4-pyridyl, phenyl, or phenyl substituted with lower alkyl, hydroxy, lower alkoxy, carboxy, or lower alkoxycarbonyl.

6. The reagent of claim 1, wherein the radiolabel-binding moiety has a formula:



7. The reagent of any of claims 1 through 6, wherein the peptide is selected from the group consisting of PLYKKIIKKLLES, PLY.Orn.Orn.II.Orn.Orn.LLES, QAPLYKKIIKKLLES and KKIIKKLLES.

8. The reagent of any of claims 1 through 7, further comprising a radiolabel.

9. The reagent of claim 8, wherein the radiolabel is technetium-99m.

10. A multimer having a molecular weight of less than about 20,000 daltons, which comprises a polyvalent linker covalently linked to at least two platelet factor 4 peptides or platelet factor 4 peptide fragments.

11. The multimer of claim 10, wherein the linker is selected from the group consisting of *bis*-succinimidylmethylether, 4-(2,2-dimethylacetylbenzoic acid, diethylenetriaminepentaacetic acid, *tris*(succinimidylethyl)amine, *tris*-acetamidoethylamine and 1,10-*bis*acetamido-4,7-dioxa-decane, a derivative of *bis*-succinimidylmethylether, a derivative of 4-(2,2-dimethylacetyl)benzoic acid, a derivative of diethylenetriaminepentaacetic acid, a derivative of *tris*(succinimidylethyl)amine, a derivative of *tris*-acetamidoethylamine and a derivative of 1,10-*bis*acetamido-4,7-dioxadecane.

12. A complex formed by reacting the reagent of any of claims 1 through 7 with technetium-99m in the presence of a reducing agent.

13. The complex of claim 12, wherein the reducing agent is selected from the group consisting of dithionite ion, a stannous ion, and a ferrous ion.

14. A complex formed by labeling the reagent of any of claims 1 through 7 with technetium-99m by ligand exchange of a prereduced technetium-99m complex.

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15. A kit for preparing a radiopharmaceutical preparation, said kit comprising a sealed vial containing a predermined quantity of the reagent of any of claims 1 through 7 and a sufficient amount of a reducing agent to label the reagent with technetium-99m.

16. Use of the reagent of any of claims 1 through 7 for preparing a medicament for imaging a site within a mammalian body.

17. A process for preparing the reagent of any of claims 1 through 7 by chemical *in vitro* synthesis.

18. The process of claim 17, wherein the radiolabel binding moiety is covalently linked to the peptide or fragment during *in vitro* chemical synthesis.

19. The process of any of claims 17 or 18, wherein the synthesis is solid phase peptide synthesis.

20. A method of labeling the reagent of any of claims 1 through 7, comprising the step of reacting the reagent with Tc-99m in the presence of a reducing agent.

21. The method of claim 20, wherein the reducing agent is selected from the group consisting of a dithionite ion, a stannous ion, and a ferrous ion.

22. A composition of matter having a formula:
$C_{Acm}GC_{Acm}$GGPLYKKIIKKLLES.

23. A composition of matter having a formula:
$(AcCC_{Acm}GC_{Acm}PLYKKIIKKLLES)_2$-BSME.

24. A composition of matter having a formula:
$AcKKKKKC_{Acm}GC_{Acm}$GGPLYKKIIKKLLES.

25. A composition of matter having a formula:
$C_{Mob}GC_{Acm}$PLYKKIIKKLLES.

26. A composition of matter having a formula:
[DTPA]$C_{Acm}GC_{Acm}$PLYKKIIKKLLES.

27. A composition of matter having a formula:
PicGC_{Acm}PLYKKIIKKLLES.

28. A composition of matter having a formula:
[BAT]GGPLYKKIIKKLLES.

29. A composition of matter having the formula:
$(AcCKKC_{Acm}GC_{Acm}PLYKKIIKKLLES)_2$-BSME.

30. A composition of matter having a formula:
[BAT]KKLLKKLYKKIIKKLLES.

31. A composition of matter having a formula:
$AcKKC_{Acm}GC_{Acm}$GGPLYKKIIKKLLES.

32. A composition of matter having a formula:
$AcKKC_{Acm}GC_{Acm}$QAPLYKKIIKKLLES.

33. A composition of matter having a formula:
$AcC_{Acm}GC_{Acm}$QAPLYKKIIKKLLES.

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34. A Tc-99m labeled reagent substantially as hereinbefore described with reference to any one of Examples 2 to 4.

35. A peptide reagent substantially as hereinbefore described with reference to Table 1 or Table 2.

36. A complex formed by reacting the reagent of claim 35 with technetium-99m in the presence of a reducing agent.

37. A complex formed by labeling the reagent of claim 35 with technetium-99m by ligand exchange of a prereduced technetium-99m complex.

38. A method of imaging a site within a mammalian body using the reagent of claim 35 substantially as hereinbefore described with reference to Examples 4 and 5.

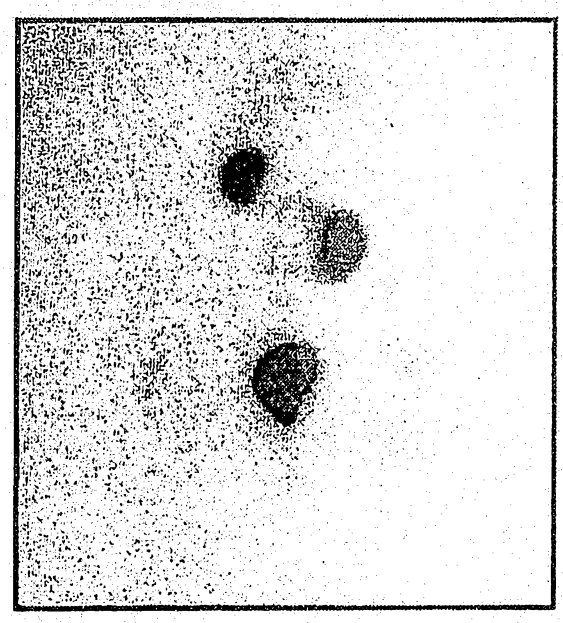
Dated 27 July, 1998
Diatech, Inc.

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

AUSTRALIAN PATENT OFFICE

Figure 1

Upper Torso



Lower Extremities



SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/01894

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K49/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,93 17719 (DIATECH) 16 September 1993 cited in the application see abstract see page 11 - page 12; claims 1-7,12,17-29; examples 1-3 see page 20 - page 22	1-30,33
P,X	WO,A,93 25244 (DIATECH) 23 December 1993 cited in the application see abstract see page 13, line 33 - line 34 see page 40, line 32 - line 45 see page 42, line 17 - line 30; claims	1-21,28
P,X	WO,A,93 21962 (DIATECH) 11 November 1993 cited in the application see the whole document	1-3, 6-21,26, 27,30

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 1994	07. 12. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hoff, P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 13572 (DIATECH) 20 August 1992 cited in the application	1-8, 11-21,24
Y	see the whole document, in particular page 5 line 30 and claims	2,9,10
Y	EP,A,0 453 082 (HYBRITECH) 23 October 1991 see abstract see page 4, line 11 - page 5, line 57; claims; example 1	9,10
Y	WO,A,90 10463 (NEORX CORPORATION) 20 September 1990 see abstract see page 6, line 29 - page 18, line 32 see page 29, line 33 - page 30, line 10; claims 1-17,29-33,72-75; example 1	2
Y	WO,A,92 05154 (MALLINCKRODT MEDICAL) 2 April 1992	2
A	see abstract; claims; examples	26
Y	INORGANIC CHEMISTRY, vol.29, no.13, 1990 pages 2948 - 2951 N. BRYSON ET AL. 'PROTECTING GROUPS IN THE PREPARATION OF THIOLATE COMPLEXES OF TECHNETIUM'	2
A	see the whole document	26
Y	WO,A,89 10759 (MALLINCKRODT) 16 November 1989	2
A	see page 2, line 29 - page 9, line 5 see page 13, line 4 - line 26; claim 1	27,30
Y	BIOCONJUGATE CHEMISTRY, vol.1, no.2, 1990 pages 132 - 137 K.E.BAIDOO ET AL. 'SYNTHESIS OF A DIAMINEDITHIOL BIFUNCTIONAL CHELATING AGENT FOR INCORPORATION OF TECHNETIUM-99M INTO BIOMOLECULES' see the whole document	2
A	CHINESE CHEMICAL LETTERS, vol.2, no.4, 1991 pages 285 - 288 B.Z. LI ET AL. 'A NEW BIFUNCTIONAL CHELATING AGENT N,N'-BIS(L-CYSTEINYL)-L-LYSINE FOR RADIOLABELLING OF MONOCLONAL ANTIBODIES WITH TECHNETIUM-99M' see the whole document	1-25,28, 29,31-33

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol.107, no.1, 1982 pages 130 - 135 D.G. OSTERMAN ET AL. 'THE CARBOXYL-TERMINAL TRIDECAPEPTIDE OF PLATELET FACTOR 4 IS A POTENT CHEMOTACTIC AGENT FOR MONOCYTES' cited in the application see the whole document ---	1-25,28, 29,31-33
A	WO,A,92 02240 (REPLIGEN CORPORATION) 20 February 1992 see the whole document -----	1-25,28, 29,31-33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/ 01894

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 15 is directed to a diagnostic method practised on the human/animal body the search has been carreid out and based on the alleged effects of the compound/composition

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.Claims: 1-3,6-21(partially); and 4,5,22-25,28,29,31-33(completely)
2.Claims: 1-3,6-21(partially)
3.Claims: 1-3,6-21(partially); and 26(completely).
4.Claims: 1-3,6-21 (partially)
5.Claims: 1-3,6-21 (partially),
6.Claims: 1-3,6-21 (partially), 27,30 (completely)

1. [X] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[X] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 94/01894

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9317719	16-09-93	NONE		
WO-A-9325244	23-12-93	AU-B-	4528793	04-01-94
WO-A-9321962	11-11-93	AU-B-	4107693	29-11-93
		CA-A-	2111863	11-11-93
WO-A-9213572	20-08-92	AU-A-	1411792	07-09-92
		EP-A-	0570493	24-11-93
		JP-T-	6503352	14-04-94
EP-A-0453082	23-10-91	AU-B-	637202	20-05-93
		AU-A-	7272291	12-09-91
		JP-A-	6092873	05-04-94
		US-A-	5273743	28-12-93
WO-A-9010463	20-09-90	US-A-	4986979	22-01-91
		EP-A-	0463116	02-01-92
		JP-T-	4504129	23-07-92
WO-A-9205154	02-04-92	AU-A-	8721591	15-04-92
WO-A-8910759	16-11-89	AU-A-	3778989	29-11-89
		EP-A-	0381713	16-08-90
WO-A-9202240	20-02-92	EP-A-	0541716	19-05-93
		JP-T-	6504262	19-05-94