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(54) Title: FLUORESCENT IMAGING AGENTS

(57) **Abrégé/Abstract:**

Provided is a family of intramolecularly quenched imaging agents for use in both in vivo and in vitro imaging that contain at least one enzymatically cleavable oligopeptide and two fluorophores or a fluorophore and a quencher. When subjected to proteolytic cleavage, at least one fluorophore is unquenched and becomes capable of producing a fluorescent signal upon excitation with light of an appropriate wavelength. Also provided are in vivo and in vitro imaging methods using such imaging agents.



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(54) **Title:** FLUORESCENT IMAGING AGENTS

(57) **Abstract:** Provided is a family of intramolecularly quenched imaging agents for use in both in vivo and in vitro imaging that contain at least one enzymatically cleavable oligopeptide and two fluorophores or a fluorophore and a quencher. When subjected to proteolytic cleavage, at least one fluorophore is unquenched and becomes capable of producing a fluorescent signal upon excitation with light of an appropriate wavelength. Also provided are in vivo and in vitro imaging methods using such imaging agents.



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FLUORESCENT IMAGING AGENTS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of and priority to co-pending U.S. Provisional Patent Application No. 61/022,014, filed January 18, 2008, the entire contents of which are herein incorporated by reference.

5 BACKGROUND

[0002] Current approaches for assessing of molecular endpoints in disease usually require tissue and blood sampling, surgery, and in the case of experimental animals, sacrifice at different time points. Despite improvements in noninvasive imaging, more sensitive and specific imaging agents and methods are urgently needed. Imaging
10 techniques capable of visualizing specific molecular targets and/or entire pathways would significantly enhance the ability to diagnose and assess treatment efficacy of therapeutic interventions for many different disease states. Most current imaging techniques report primarily on anatomical or physiological information (e.g., magnetic resonance imaging (MRI), computed tomography (CT), and ultrasound). Newer modalities, such as, optical
15 imaging and new molecular imaging probes have the potential to revolutionize the way disease is detected, treated, and monitored.

[0003] Molecular imaging is a developing field in the imaging sciences that transcends the traditional boundaries of imaging structure or physiology, and has the potential to revolutionize current research and clinical practices towards real molecular medicine. The
20 one paradigm for molecular imaging involves the use of a “molecular” probe or agent that selectively targets a particular gene, protein, receptor or a cellular function, with the absence, presence, amount or concentration of the specific target being indicative of a particular disease state.

[0004] In particular, optical imaging offers several strong performance attributes that
25 make it a truly powerful molecular imaging approach, both in the research and clinical settings. Specifically, optical imaging is fast, safe, cost effective and highly sensitive. Scan times typically are on the order of seconds to minutes, there is no ionizing radiation, and the imaging systems are relatively simple to use. In addition, optical probes can be

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designed as dynamic molecular imaging agents that can alter their reporting profiles *in vivo* to provide molecular and functional information in real time. In order to achieve maximum penetration and sensitivity *in vivo*, the choice for most optical imaging in biological systems is within the red and near-infrared (NIR) spectral region, although
5 other wavelengths in the visible region can also be used. In the NIR wavelength range, absorption by physiologically abundant absorbers such as hemoglobin or water is minimized.

[0005] Although different types of optical imaging probes have been developed including (1) probes that become activated after target contact (e.g., binding or
10 interaction), (2) wavelength shifting beacons, (3) multicolor fluorescence probes, (4) probes that have high binding affinity to targets, i.e., that remain within a target region while non-specific probes are cleared from the body (Achilefu *et al.*, *Invest. Radiol.*, **35**:479-485, 2000; Becker *et al.*, *Nature Biotech.* 19:327-331, 2001; Bujai *et al.*, *J. Biomed. Opt.* 6:122-133, 2001; Ballou *et al. Biotechnol. Prog.* **13**:649-658, 1997; and Neri
15 *et al.*, *Nature Biotech.* **15**:1271-1275, 1997), and (5) fluorescent semiconductor based probes, there is still an ongoing need for imaging probes that, for example, are capable of providing high quality images and molecular information.

SUMMARY OF THE INVENTION

[0006] The present invention provides fluorescent imaging agents that have
20 significantly enhanced fluorescent properties upon activation and can be used for *in vivo* and *in vitro* imaging applications. The imaging agents have a fluorescent reporter system, which in certain embodiments, contains two fluorophores or a fluorophore and a quencher, where one fluorophore quenches the other fluorophore or the quencher quenches the fluorophore. Although the imaging agents may have no more than two fluorophores they
25 still have an extinction coefficient sufficient for *in vivo* imaging applications.

Furthermore, their size permits the agents to become quickly distributed through the tissues or body fluid of a subject, which allows the agent to travel to tissues or cells for activation by proteases, binding to cell surface receptors or internalization within cells.

[0007] The imaging agents have enhanced fluorescent properties upon activation and
30 under certain circumstances can have magnetic properties, for example, paramagnetic or superparamagnetic properties, so that the imaging agents can be used as MRI or multi-

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modality imaging agents (for example, optical imaging and magnetic resonance imaging). In addition, the imaging agents optionally can include diagnostic and or therapeutic moieties, for example, radioactive metals, so that the resulting imaging agents can be used like radiopharmaceuticals, nuclear imaging agents or multi-modality imaging agents (for example, in an optical imaging and nuclear imaging environment).

[0008] In one aspect, the invention provides an intramolecularly-quenched imaging agent. The agent comprises (a) an enzymatically cleavable oligopeptide comprising from about 2 to about 30 amino acid residues; (b) an optional biological modifier chemically linked to the enzymatically cleavable oligopeptide; and (c) either two fluorophores or one fluorophore and one quencher, each covalently linked, directly or indirectly, to the oligopeptide or to the optional biological modifier, wherein one fluorophore quenches the other fluorophore or the quencher quenches the fluorophore and upon enzymatic cleavage of the oligopeptide, at least one fluorophore becomes unquenched and is capable of producing a greater fluorescent signal when excited by electromagnetic radiation than before enzymatic cleavage of the oligopeptide.

[0009] The oligopeptide can comprise from about 2 to about 25 amino acid residues, from about 2 to about 14 amino acid residues, from about 4 to about 10 amino acid residues, or from about 5 to about 8 amino acid residues.

[0010] In certain embodiments, the imaging agent can be represented by Formula I:



wherein:

B is [M_m-ECO-M_m];
 ECO is an enzymatically cleavable oligopeptide;
 G is L-F;
 F is a fluorophore or quencher;
 M is a biological modifier;
 K is N-L;
 N is a non-fluorescent reporter;
 L, independently, for each occurrence, is a linker moiety or a bond;

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n is an integer from 0 to 3;

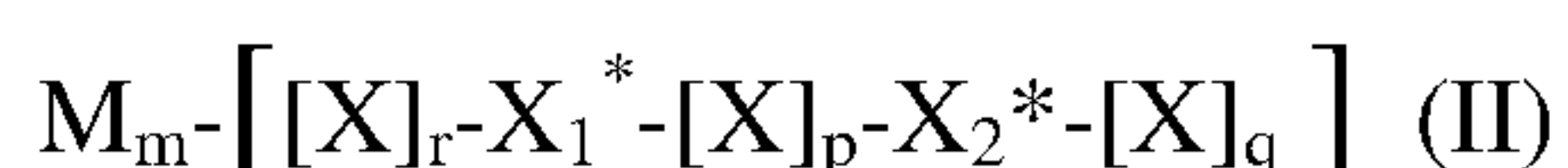
m, independently, for each occurrence, is 0 or 1, and optionally at least one m is 1; and

f, independently, for each occurrence, is an integer from 0 to 2, wherein the

5 total number of fluorophores F in the agent is no greater than 2.

[0011] Each F can be chemically linked, directly or through the linker moiety L, to a separate amino acid of the oligopeptide. Alternatively, at least one F is chemically linked, directly or through the linker moiety L, to M. In certain embodiments, ECO is a cyclic oligopeptide.

10 **[0012]** In addition, in certain embodiments, the imaging agent is represented by Formula II:



wherein:

X, independently, for each occurrence, is an amino acid residue;

15 X_1^* and X_2^* are each independently X-L-F;

L, independently, for each occurrence, is a linker moiety or a bond;

F is a fluorophore;

M is a biological modifier;

m is 0, 1 or 2;

20 r is an integer from 0 to 28;

p is an integer from 1 to 28;

q is an integer from 0 to 28; wherein the sum of r, p and q is no greater than

28.

[0013] Depending upon the circumstances, amino acid residue X_1^* is a lysine and/or
25 amino acid residue X_2^* is a lysine.

[0014] In another aspect, the invention provides an intramolecularly-quenched imaging agent represented by Formula III:



- 5 -

wherein:

ECO is an enzymatically cleavable oligopeptide;

G is L-F;

F, independently, is selected from a fluorophore or quencher, wherein at

5 least one F is a fluorophore;

L, independently for each occurrence, is selected from a linker moiety or a bond;

M is a biological modifier;

K is L-N;

10 N is a non-fluorescent reporter;

p is an integer from 1 to 4;

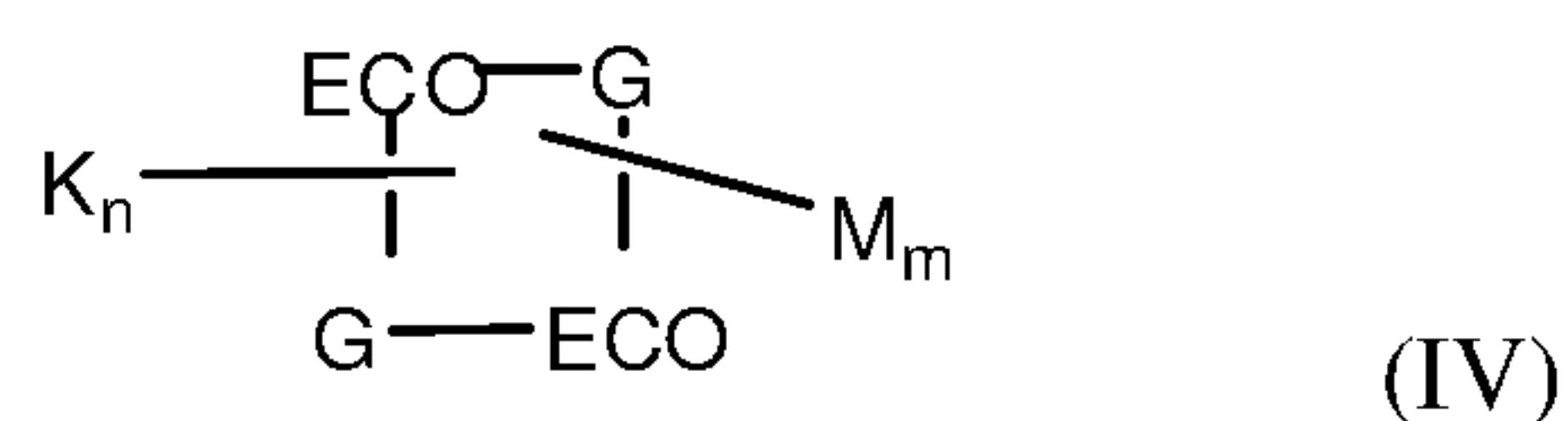
n is an integer from 0 to 3; and

g, independently, for each occurrence, is an integer from 1 to 2, wherein the sum of each occurrence of g is no greater than 2.

15 **[0015]** The oligopeptide can comprise from about 2 to about 25 amino acid residues, from about 2 to about 14 amino acid residues, from about 4 to about 10 amino acid residues, or from about 5 to about 8 amino acid residues. In certain embodiments, at least one F is chemically linked, directly or indirectly, to a lysine residue.

[0016] In another aspect the invention provides cyclic intramolecularly quenched
20 imaging agent comprising (a) a first fluorophore chemically linked, directly or indirectly, to the C-terminus of a first cleavable oligopeptide and chemically linked, directly or indirectly, to the N-terminus of a second, optionally cleavable, oligopeptide; (b) a second fluorophore chemically linked, directly or indirectly, to the N-terminus of the first cleavable oligopeptide and chemically linked directly or indirectly, to the C-terminus of
25 the second, optionally cleavable oligopeptide; and (c) optionally, at least one biological modifier chemically linked to the first or second oligopeptide or fluorophore.

[0017] In one embodiment, the imaging agent can be represented by Formula IV:



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wherein,

ECO, independently, for each occurrence, is an enzymatically cleavable oligopeptide;

G is L-F-L;

5 F, independently, for each occurrence, is a fluorophore;

L, independently, for each occurrence, is a linker moiety or a bond;

M is a biological modifier;

K is L-N;

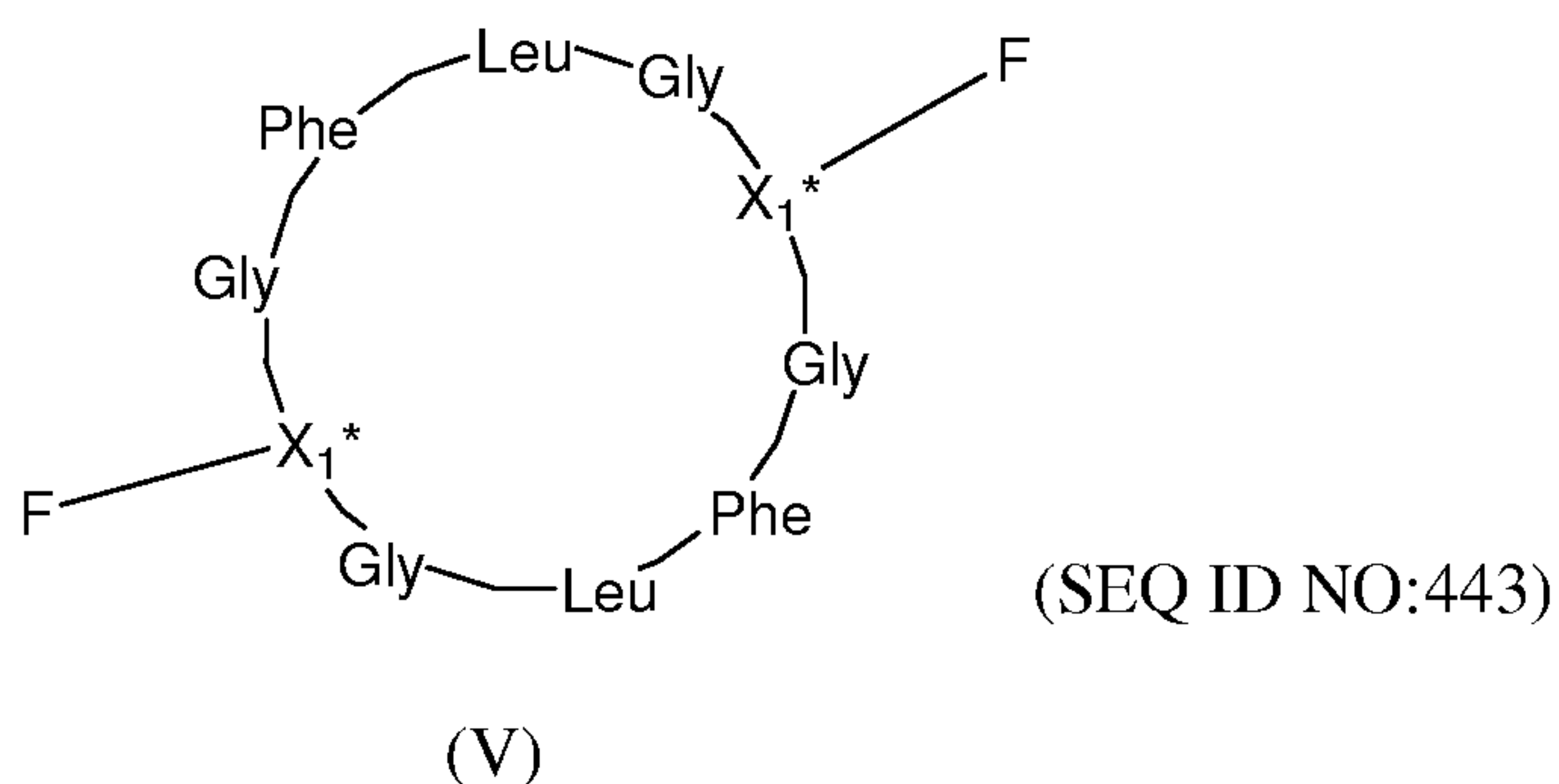
N is a non-fluorescent reporter;

10 n is an integer from 0 to 3; and

m is an integer from 0 to 3.

[0018] In another aspect, the invention provides an intramolecularly-quenched imaging agent comprising (a) an enzymatically cleavable oligopeptide comprising from 2 to 14 amino acid residues; (b) at least one biological modifier with a molecular weight of from about 5 kDa to about 35 kDa covalently linked to the enzymatically cleavable oligopeptide; and (c) two fluorophores, each covalently linked, directly or indirectly, to the oligopeptide at locations so that the fluorophores quench one another, and wherein, upon enzymatic cleavage of the oligopeptide, at least one fluorophore becomes unquenched and is capable of emitting a fluorescent signal when excited by
20 electromagnetic radiation.

[0019] In another aspect, the invention provides an intramolecularly-quenched imaging agent represented by Formula V:



25

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wherein

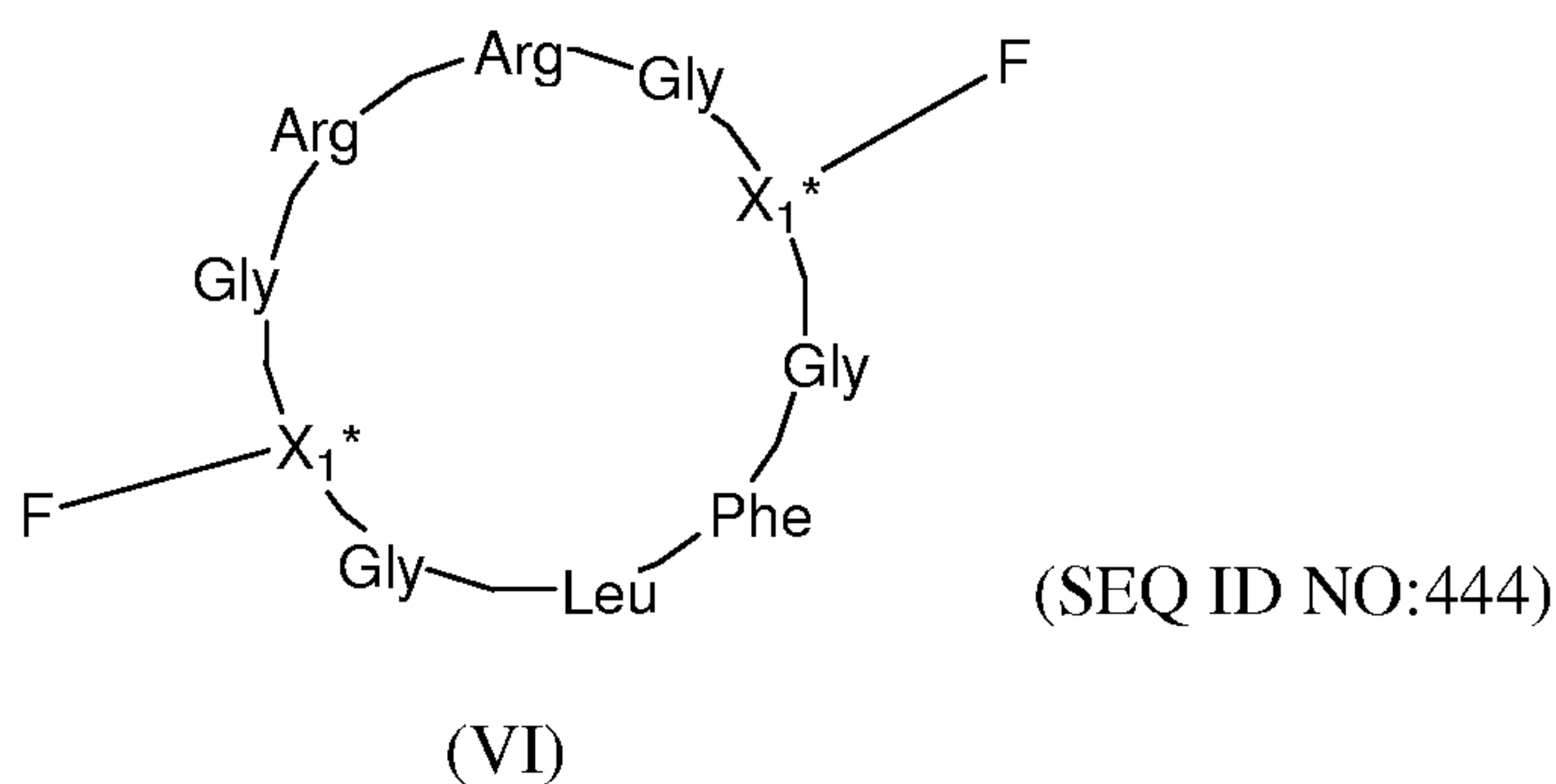
X_1^* independently, at each occurrence, is X-L-;

X is an amino acid residue;

L is a linker moiety or a bond; and

5 F, independently, at each occurrence, is a fluorophore.

[0020] In another aspect, the invention provides an intramolecularly-quenched imaging agent represented by Formula VI:



10 wherein

X_1^* , independently, at each occurrence, is X-L-;

X is an amino acid residue;

L is a linker moiety or a bond;

F, independently, at each occurrence, is a fluorophore.

15 [0021] In any of the foregoing imaging agents, the fluorophore preferably is a far-red or a near-infrared fluorophore. Exemplary fluorophores include carbocyanine fluorophore and indocyanine fluorophore. It is understood that when the agent comprises two fluorophores, they can be the same or different. Other useful fluorophores and quenchers, as well as useful linkers, biological modifiers, and non-fluorescent reporters are described
20 in more detail below.

[0022] In certain embodiments, the enzymatically cleavable oligopeptide is cleavable by at least one enzyme selected from the group consisting of a cathepsin, a matrix metalloprotease, a peptidase, a carboxypeptidase, a glycosidase, a lipase, a phospholipase, a phosphatase, a phosphodiesterase, a sulfatase, a reductase, and a bacterial enzyme.

25 [0023] In addition, the invention provides methods of imaging *in-vivo* or *in-vitro*. In

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one aspect, the invention provides a method for performing *in vivo* optical imaging in a subject, for example, a mammal, for example, an animal or a human. The method comprises the steps of (a) administering to a subject one of more of the foregoing imaging agents, (b) allowing the agent or agents to distribute within the subject; (c) exposing the
5 subject to light of a wavelength absorbable by at least one fluorophore in the agent; and (d) detecting a signal emitted by the fluorophore. The emitted signal can be used to construct an image, for example, a tomographic image. Furthermore, steps (c) - (d) can be repeated at predetermined intervals thereby to permit evaluation of the emitted signals of the agent in the subject over time. Similarly, steps (a) - (d) can be repeated at
10 predetermined time intervals thereby to permit evaluation of the emitted signals of the agent in the subject over time. The presence, absence, or level of emitted signal can be indicative of a disease state and or can be used to detect and/or monitor a disease.

[0024] In another aspect, the invention provides an *in vitro* imaging method. The method comprises (a) contacting a sample, for example, a biological sample, with any one
15 ore more of the foregoing imaging agents, (b) allowing the agent to bind to or associate with a biological target; optionally, removing unbound agents; and (d) detecting a signal emitted from the fluorophore thereby to determine whether the agent has been activated by or bound to the biological target.

[0025] The imaging agents can also be incorporated into a kit, for example, a kit with
20 optional instructions for using the agents during *in vivo* or *in vitro* imaging. The kit optionally comprises components that aid in the use of the imaging agents for the disclosed methods, such as buffers, and other formulating agents. Alternatively, the kit can comprise medical devices that aid in the administration and/or detection of the agents to subjects.

25 [0026] The imaging agents and methods disclosed herein provide various advantages and have broad applications in both research and clinical settings. For example, the imaging agents and methods permit the acquisition of molecular images and optionally, high resolution anatomical images. The imaging agents and methods may provide insights into specific molecular abnormalities that form the basis of many diseases, and can be
30 used to assess efficacy of established therapies at the molecular level. This, in turn, is

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expected to have an impact in drug development, drug testing, disease diagnosis, and choosing appropriate therapies and therapy changes in a given subject.

[0027] Other features and advantages of the invention will be apparent from the following figures, detailed description, and the claims.

5 BRIEF DESCRIPTION OF THE DRAWINGS

[0028] **Figure 1** depicts a planar image of bilateral tumors at 6 hours after administration of an exemplary imaging agent referred to as Q65.

[0029] **Figure 2** depicts fluorescence tomography bilateral images of tumors at 6 hours after administration of the exemplary imaging agent Q65.

10 [0030] **Figure 3** depicts planar fluorescence reflectance images of 4T1 Xenograft tumors using an exemplary agent referred to as Q66.

[0031] **Figure 4** depicts a planar fluorescence reflectance image of HT-29 Xenograft tumors using an exemplary agent referred to as Q91.

[0032] **Figure 5** depicts the enzymatic activation profile of the imaging agent Q65.

15 [0033] **Figure 6** depicts the enzymatic activation profiles of the exemplary imaging agents, referred to as Q92 (Figure 6A) and Q93 (Figure 6B).

[0034] **Figure 7A** depicts fluorescent images of control (**Figures 7A and 7C**) and ovariectomy induced osteoporosis (**Figures 7B and 7D**) in mice taken 4 hours (**Figures 7A and 7B**) and 24 hours (**Figures 7C and 7D**) after administering an exemplary imaging agent referred to as Q94. **Figure 7E** is a bar chart showing quantitation of tibial fluorescence of induced osteoporosis 4 hours and 24 hours after administration of imaging agent Q94.

[0035] **Figure 8** depicts an image produced by fluorescence reflectance imaging of cardiovascular disease using the exemplary imaging agent Q65.

25 [0036] **Figure 9** depicts an image of a mouse following carrageenan induced paw edema using the exemplary imaging agent Q91.

[0037] **Figure 10** is a schematic representation of an exemplary cyclic imaging agent that comprises two fluorophores separated by two peptides. Each peptide contains a

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proteolytic cleavage site where, when intact, the imaging agent is quenched and when the peptides are cleaved by exposure to one or more proteases, at least one fluorophore is no longer quenched by the other.

5 [0038] **Figure 11** is a bar chart showing the enzyme activation profile for exemplary imaging agents referred to as R20, R21, R23, R24, R26, and R27.

[0039] **Figure 12** shows images of mice on a control or low sodium diet using two exemplary imaging agents, wherein **Figure 12A** represents a mouse on a control diet having received the imaging agent R20, **Figure 12B** represents a mouse on a low sodium diet having received the imaging agent R20, **Figure 12C** represents a mouse on a control
10 diet having received the imaging agent R21, and **Figure 12D** represents a mouse on a low sodium diet having received the imaging agent R21.

[0040] **Figure 13** is a bar chart showing an enzyme activation panel for an exemplary imaging agent referred to as R22.

[0041] **Figure 14** show images created by fluorescent reflectance imaging after 6 hours
15 (**Figure 14A**) and after 24 hours (**Figure 14B**) and by tomographic imaging after 6 hours (**Figure 14C**) and after 24 hours (**Figure 14D**) using the exemplary imaging agent R22.

[0042] **Figure 15** is a bar chart showing enzyme activation profiles of exemplary imaging agents referred to as R51, R52, R53, R55, R56, R57, R58, and R59.

[0043] **Figure 16** depicts images and quantification of the images in ApoE -/- and
20 control mice using the exemplary imaging agent R51. **Figure 16A** shows images taken by fluorescent tomography, and **Figure 16B** is a bar chart showing quantification of the resulting images. **Figure 16C** is a reflectance image, and **Figure 16D** is a bar chart showing quantification of the resulting images.

[0044] **Figure 17** depicts images and quantification of the images in ApoE -/- and
25 control mice using the exemplary imaging agent R55. **Figure 17A** shows images taken by fluorescence tomography, and **Figure 17B** is a bar chart showing quantification of the resulting images.

DETAILED DESCRIPTION

[0045] This invention provides imaging agents that include one or more fluorescently

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labeled peptides. The fluorescence of the imaging agents is enhanced upon activation, e.g, an increase of fluorescence occurs, due to a cleavage of a peptide sequence by a protease after, before or during binding of the agent or following internalization of the imaging agent.

5 [0046] For example, the imaging agents of the present invention includes at least one oligopeptide comprising a proteolytic cleavage site, and two fluorophores or a single fluorophore and a quencher) covalently linked (directly or indirectly) to the oligopeptide or an optional biological modifier such that the fluorescence of at least one fluorophore is significantly quenched. Cleavage of the peptide by, for example, enzymatic cleavage, the
10 agent emits a fluorescent signal when excited by electromagnetic radiation of appropriate wavelength and frequency. In certain embodiments, the imaging agents contain no more than two fluorophores. For example, the imaging agents may contain a fluorescent reporter system consisting of, or consisting essentially of, (i) two fluorophores, (ii) two fluorophores and two quenchers, (iii) two fluorophores and one quencher, or (iv) one
15 fluorophore and one quencher.

[0047] As used herein, the term “quench” is understood to mean the process of partial or complete reduction of the fluorescent signal from a fluorophore. For example, a fluorescent signal can be reduced inter-molecularly or intra-molecularly through the placement of another fluorophore (either the same or a different fluorophore) in
20 fluorescent quenching proximity to the first fluorophore or the placement of a non-fluorogenic quenching chromophore molecule (quencher) in fluorescent quenching proximity to the first fluorophore. The agent is de-quenched (or activated), for example, through the enzymatic cleavage of a peptide sequence.

[0048] The peptide of the imaging agents optionally can be chemically linked to a
25 biological modifier. Furthermore the imaging agents optionally can be chemically linked to another non-fluorescent reporter. As used herein, the term “chemically linked” is understood to mean connected by an attractive force between atoms strong enough to allow the combined aggregate to function as a unit. This includes, but is not limited to, chemical bonds such as covalent bonds, non-covalent bonds such as ionic bonds, metallic
30 bonds, and bridge bonds, hydrophobic interactions, hydrogen bonds, and van der Waals interactions.

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[0049] In one aspect of the invention, the agents of the present invention are intramolecularly-quenched agents that include (a) an enzymatically cleavable oligopeptide comprising from about 2 to about 30 amino acid residues; (b) an optional biological modifier chemically linked to the enzymatically cleavable oligopeptide; and (c) either two
5 fluorophores or one fluorophore and one quencher, each covalently linked, directly or indirectly, to the oligopeptide or to the biological modifier, wherein one fluorophore quenches the other fluorophore or the quencher quenches the fluorophore and upon enzymatic cleavage of the oligopeptide, at least one fluorophore becomes unquenched and is capable of producing a greater fluorescent signal when excited by electromagnetic
10 radiation than before enzymatic cleavage of the oligopeptide.

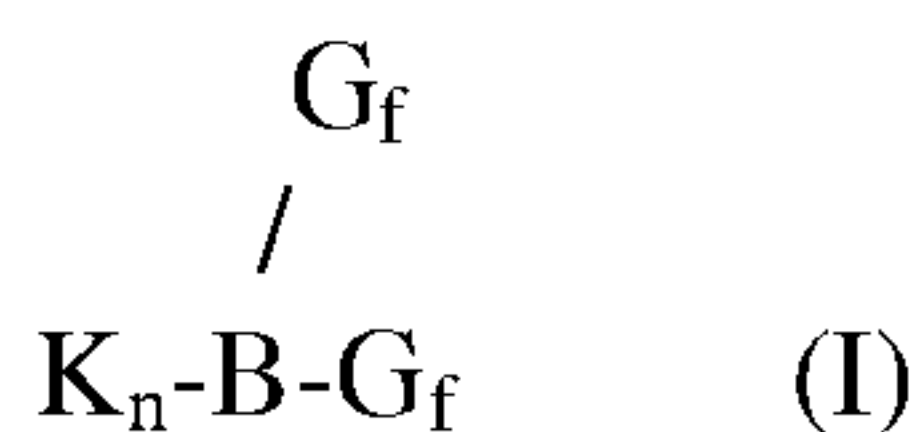
[0050] For example, the imaging agent can comprise one or more oligopeptides from about 2 to about 25 amino acid residues in length, from about 2 to about 14 amino acid residues in length, from about 5 to about 8 amino acid residues in length, or from about 4 to about 10 amino acid residues in length. In certain embodiments, the oligopeptide
15 comprises 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids.

[0051] The biological modifier, before being chemically linked to the enzymatically cleavable oligopeptide, can have a molecular weight less than about 35 kDa, for example, from about 10 kDa to about 35 kDa, from about 5 kDa to about 30 kDa, or from about 15
20 kDa to about 25 kDa. The biological modifier preferably is linked to the oligopeptide at a position that does not interfere with fluorescent quenching. For example, the biological modifier may be chemically linked to a moiety of the oligopeptide that is not positioned between two fluorophores that quench one another or between a fluorophore and a quencher.

25 [0052] In some embodiments, the imaging agents disclosed herein comprise two fluorophores, which may be the same or different. Imaging agents of the invention can further comprise a non-fluorescent reporter chemically linked to the enzymatically cleavable oligopeptide or the biological modifier.

[0053] In one embodiment, the imaging agent is represented by Formula I:

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wherein:

5

B is $[M_m-ECO-M_m]$;

ECO is the enzymatically cleavable oligopeptide;

G is L-F;

F is a fluorophore or quencher;

M is a biological modifier;

10

K is N-L;

N is a non-fluorescent reporter;

L, independently, for each occurrence, is a linker moiety or a bond;

n is an integer from 0 to 3 (for example, 0, 1, 2, or 3);

m, independently, for each occurrence, is 0 or 1, and optionally at least one

15

m is 1; and

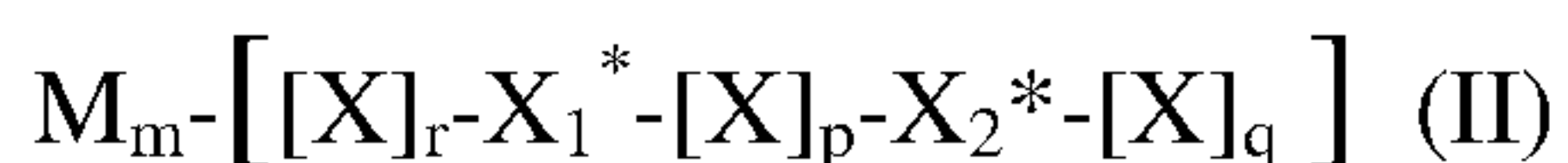
f, independently, for each occurrence, is an integer from 0 to 2, wherein the total number of fluorophores F in the agent is no greater than 2.

[0054] In one embodiment, f is 2 and at least one F is a fluorophore.

[0055] Each F may be covalently linked, directly or indirectly (for example, through a linker moiety L), to a different amino acid residue (for example, a lysine) of the oligopeptide. In another embodiment, at least one F is covalently linked, directly or indirectly (for example, through a linker moiety L), to the biological modifier M. It is understood that ECO can be a linear oligopeptide, or can be a cyclic oligopeptide.

[0056] In another aspect, the imaging agent is represented by formula II:

25



wherein:

X, independently, for each occurrence, is an amino acid residue;

 X_1^* and X_2^* are each independently X-L-F;

L, independently, for each occurrence, is a linker moiety or a bond;

30

F is a fluorophore;

M is a biological modifier;

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m is 0, 1 or 2;

r is an integer from 0 to 28;

p is an integer from 1 to 28;

q is an integer from 0 to 28; wherein the sum of r, p and q is no greater than

5 28.

[0057] In certain embodiments, the sum of r, p and q is 3, 4, 5, 6, 7, 8, 9 or 10. The amino acid residue X_2^* and/or X_1^* can be, for example, a lysine.

[0058] In another aspect, the imaging agent is represented by Formula III:



10

wherein:

ECO is an enzymatically cleavable oligopeptide;

G is L-F;

F, independently, is selected from a fluorophore or quencher, wherein at

15 least one F is a fluorophore;

L, independently, for each occurrence, is selected from a linker moiety or a bond;

M is a biological modifier;

K is L-N;

20 N is a non-fluorescent reporter;

p is an integer from 1 to 4;

n is an integer from 0 to 3; and

g, independently, for each occurrence, is an integer from 1 to 2, wherein the sum of each occurrence of g is no greater than 2.

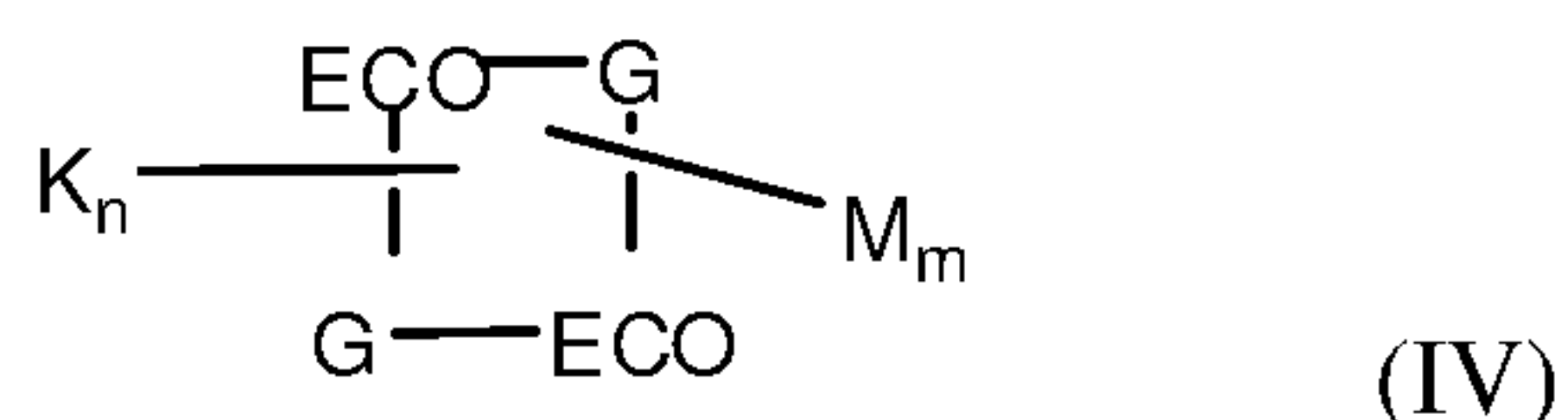
25 **[0059]** For example, ECO can be about 5 to about 8 amino acid residues in length. The integer n can be 0, 1, 2 or 3; g of formula III can be, independently for each occurrence, 1, 2, 3, 4 5 or 6. In certain embodiments, at least one F is chemically linked, directly or indirectly, to a lysine residue of the oligopeptide ECO. For example, F, for each occurrence, is covalently bound to the oligopeptide ECO.

30 **[0060]** In another aspect, the imaging agent is a cyclic intramolecularly quenched imaging agent comprising: a) a first fluorophore chemically linked, directly or indirectly,

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to the C-terminus of a first cleavable oligopeptide and chemically linked, directly or indirectly, to the N-terminus of a second, optionally cleavable oligopeptide; b) a second fluorophore chemically linked, directly or indirectly, to the N-terminus of the first cleavable oligopeptide and chemically linked directly or indirectly, to the C-terminus of the second, optionally cleavable, oligopeptide; and c) optionally, at least one biological modifier chemically linked to the first or second oligopeptide or fluorophore.

[0061] In one embodiment, the cyclic agents can be represented by Formula IV:



wherein

10 ECO, independently for each occurrence, is an enzymatically cleavable oligopeptide;

G is L-F-L;

F, independently, for each occurrence, is a fluorophore;

L, independently, for each occurrence, is a linker moiety or a bond;

15 M is a biological modifier;

K is L-N;

N is a non-fluorescent reporter;

n is an integer from 0 to 3 (for example 0, 1, 2 or 3); and

m is an integer from 0 to 3 (for example 0, 1, 2 or 3).

20 **[0062]** Also contemplated herein are stereoisomeric forms, mixtures of stereoisomeric forms, and pharmaceutically acceptable salt forms of the disclosed imaging agents.

I. Fluorophores

[0063] As used herein, the term “fluorophore” is understood to mean a fluorochrome, a dye molecule, a organic or inorganic fluorophore, or metal chelate. A fluorophore can include a far-red or a near-infrared fluorophore. In certain embodiments, imaging agents disclosed herein include a fluorophore selected from the group consisting of a carbocyanine fluorophore or an indocyanine fluorophore. Exemplary fluorophores include sulfonated fluorophores. It is understood that fluorophores can also be nanoparticles having fluorescent or luminescent properties.

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[0064] In certain embodiments, the fluorophores are near infrared fluorophores (NIRFs) with absorption and emission maximum between about 600 nm and about 900 nm. It is appreciated that the use of fluorophores with excitation and emission wavelengths in other spectrums can also be employed in the compositions and methods of the present invention.

5 [0065] For example, certain exemplary NIRFs have an extinction coefficient of at least 30,000 M⁻¹cm⁻¹ per fluorophore molecule in aqueous medium, or at least 50,000 M⁻¹cm⁻¹ per fluorophore molecule in aqueous medium. NIRFs preferably also have (1) high quantum yield (i.e., quantum yield greater than 5% in aqueous medium), (2) narrow excitation/emission spectrum, spectrally separated absorption and excitation spectra (i.e.,
10 excitation and emission maxima separated by at least 15 nm), (3) high chemical and photostability, (4) little or no nontoxicity, (5) good biocompatibility, biodegradability and excreatability, and (6) commercial viability and scalable production for large quantities (i.e., gram and kilogram quantities) required for *in vivo* and human use.

[0066] An extinction coefficient of the agents that include a fluorophore can be
15 calculated as the ratio of the absorbance of fluorophore at its absorption maxima (for example at ~750 nm for VivoTag-S-750, VisEn Medical) in a 1 cm path length cell to the concentration of particles, ($\epsilon = A/cl$, where A is absorbance, c is molar concentration and l is path length in cm).

[0067] In particular, certain carbocyanine or polymethine fluorescent fluorophores can
20 be used to produce the imaging agents of the invention, for example, those described in U.S. Patent No. 6,747,159; U.S. Patent No. 6,448,008; U.S. Patent No. 6,136,612; U.S. Patent No. 4,981,977; 5,268,486; U.S. Patent No. 5,569,587; U.S. Patent No. 5,569,766; U.S. Patent No. 5,486,616; U.S. Patent No. 5,627,027; U.S. Patent No. 5,808,044; U.S. Patent No. 5,877,310; U.S. Patent No. 6,002,003; U.S. Patent No. 6,004,536; U.S. Patent
25 No. 6,008,373; U.S. Patent No. 6,043,025; U.S. Patent No. 6,127,134; U.S. Patent No. 6,130,094; U.S. Patent No. 6,133,445; also WO 97/40104, WO 99/51702, WO 01/21624, and EP 1 065 250 A1; and Tetrahedron Letters 41, 9185-88 (2000).

[0068] Various useful, exemplary fluorophores are commercially available and include, for example: Cy5.5, Cy5 and Cy7 (GE Healthcare); AlexaFluor660, AlexaFluor680,
30 AlexaFluor750, and AlexaFluor790 (Invitrogen); VivoTag680, VivoTag-S680, and VivoTag-S750 (VisEn Medical); Dy677, Dy682, Dy752 and Dy780 (Dyomics);

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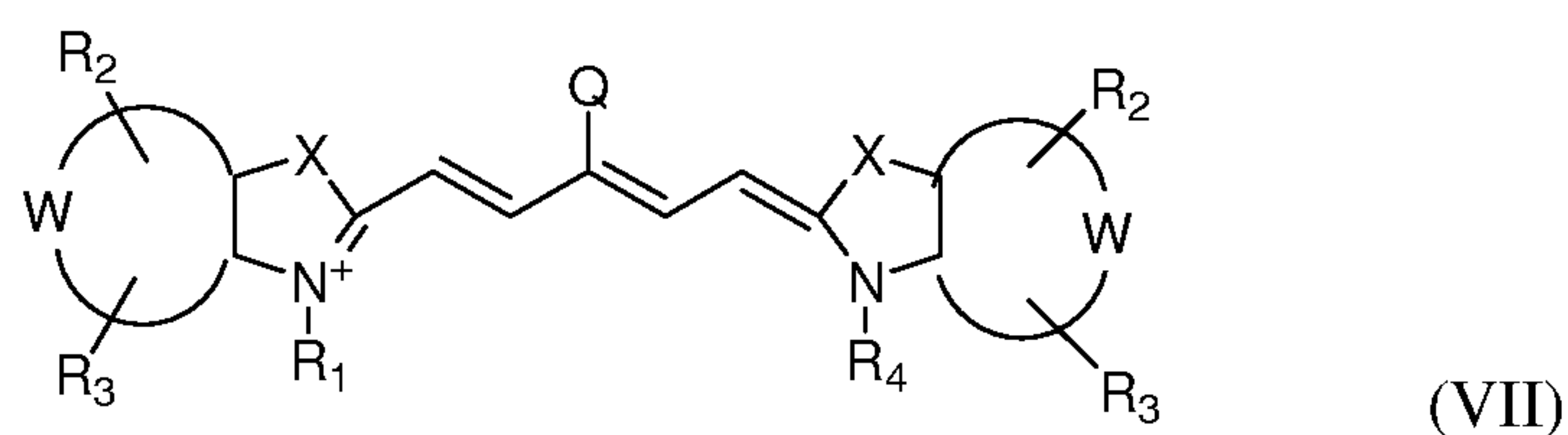
DyLight547, DyLight647 (Pierce); HiLyte Fluor 647, HiLyte Fluor 680, and HiLyte Fluor 750 (AnaSpec); IRDye800CW, IRDye 800RS, and IRDye 700DX (Li-Cor) ; and ADS780WS, ADS830WS, and ADS832WS (American Dye Source).

[0069] Table 1 lists a number of exemplary fluorophores useful in the practice of the invention together with their spectral properties.

TABLE 1

Fluorophore	$\epsilon_{\max} \text{ M}^{-1} \text{ cm}^{-1}$	Absorbance max (nm)
Cy5	250,000	649
Cy5.5	250,000	675
Cy7	250,000	743
AlexaFlour660	132,000	663
AlexaFlour680	184,000	679
AlexaFlour750	280,000	749
VivoTag-S680	220,000	674
VivoTag-S750	100,000	750
Dy677	180,000	673
Dy682	140,000	690
Dy752	270,000	748
Dy780	170,000	782
DyLight547	150,000	557
DyLight647	250,000	653

[0070] For example, certain useful fluorophores are represented by the following general Formula VII:



or a salt thereof, wherein:

X is independently selected from the group consisting of C(CH₂Y₁)(CH₂Y₂), O, S, and Se;

Y₁ and Y₂ are independently selected from the group consisting of H, C₁-C₂₀ aliphatic group and a C₁-C₂₀ aliphatic group substituted with -OR*, N(R*)₂ or -SR*;

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W represents a benzo-condensed, a naphtho-condensed or a pyrido-condensed ring;

R_1 is selected from the group consisting of $(CH_2)_xCH_3$, $(CH_2)_nSO_3^-$ and $(CH_2)_nSO_3H$, wherein x is an integer selected from 0 to 6 and n is an integer selected from 2 to 6;

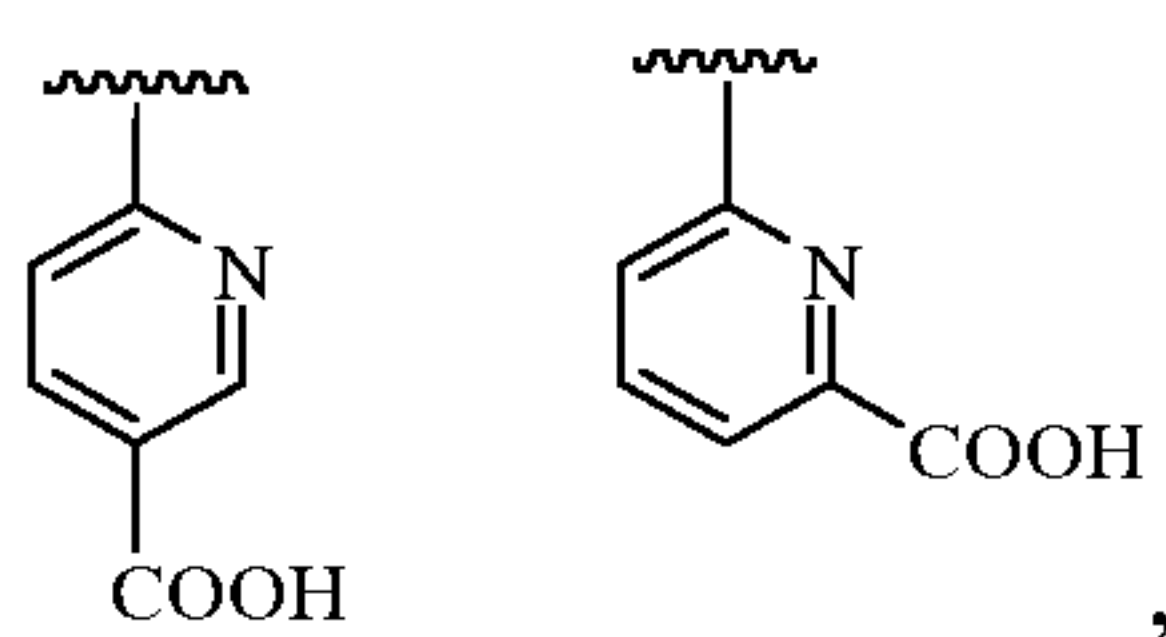
R_2 and R_3 independently are selected, for each occurrence, from the group consisting of H, carboxylate, carboxylic acid, carboxylic ester, amine, amide, sulfonamide, hydroxyl, alkoxyl, a sulphonic acid moiety and a sulphonate moiety;

R_4 is selected from the group consisting of $(CH_2)_xCH_3$, $(CH_2)_nSO_3^-$ and $(CH_2)_nSO_3H$, wherein x is an integer selected from 0 to 6 and n is an integer selected from 2 to 6; and

Q is selected from a group consisting of a heteroaryl ring substituted with a carboxyl group or 6-membered heteroaryl ring substituted with a carbonyl group.

[0071] In certain embodiments, Q can be selected from the group consisting of (i) a

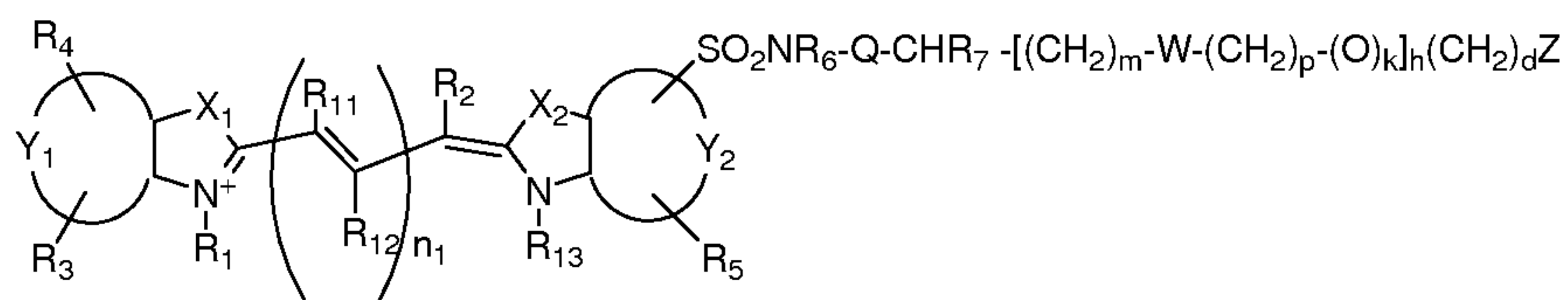
carboxyl functionalized heterocyclic ring, (ii) a carboxyl functionalized nitrogen containing heterocyclic ring, (iii) a carboxyl functionalized nitrogen containing 6-membered heterocyclic ring, such as pyridine, pyrimidone, pyrazine, and pyridazine, (iv) a carboxyl functionalized nitrogen containing 6-membered heterocyclic ring, such as pyridine, (v) a carbonyl functionalized nitrogen containing 6-membered heterocyclic ring, such as pyridine, (vi) an isonicotinic acid, nicotinic acid and picolinic acid, and a group selected from:



wherein the carboxyl group may also be in the form of an ester, an activated ester or carbonyl halide that is capable of reacting with nucleophiles, and can be, for example, a CO-O-benzotriazolyl, CO-ON-hydroxysuccinimidyl, CO-O-tetrafluorophenyl, CO-O-pentafluorophenyl, CO-O-imidazole, and CO-O-p-nitrophenyl.

[0072] Other useful, exemplary fluorophores are represented by the general Formula VII:

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(VIII)

or a salt thereof, wherein:

X_1 and X_2 are independently selected from the group consisting of

5 $C(CH_2K_1)(CH_2K_2)$, O, S and Se;

K_1 and K_2 are independently selected from the group consisting of H, a C_1 - C_{20} aliphatic group and a C_1 - C_{20} aliphatic group substituted with $-OR^*$, $N(R^*)_2$ or $-SR^*$; or K_1 and K_2 together are part of a substituted or unsubstituted carbocyclic, or heterocyclic ring;

10 Y_1 and Y_2 are each independently a benzo-condensed ring, a naphtha-condensed ring or a pyrido-condensed ring;

n_1 is 1, 2, or 3;

15 R_2 , R_{11} and R_{12} are independently H, F, Br, Cl, C_1 - C_6 alkyl, C_1 - C_6 alkoxy, aryloxy, a nitrogen-containing heterocyclic ring, a nitrogen-containing heteroaromatic ring, a sulfonate, an iminium ion, or any two adjacent R_{12} and R_{11} substituents or R_2 and R_{11} substituents, when taken in combination, form a 4-, 5-, or 6-membered substituted or unsubstituted carbocyclic ring, substituted or unsubstituted non-aromatic carbocyclic ring or a substituted or unsubstituted carbocyclic aryl ring, wherein the carbocyclic rings are each independently
20 optionally substituted one or more times by C_1 - C_6 alkyl, halogen, or OR^* or SR^* ;

R_1 and R_{13} are $(CH_2)_xCH_3$, when x is an integer selected from 0 to 6; or R_1 and R_{13} are independently $(CH_2)_nSO_3^-$ or $(CH_2)_nSO_3H$ when n is an integer selected from 2 to 6;

25 R_3 , R_4 and R_5 are independently selected from the group consisting of H, carboxylate, carboxylic acid, carboxylic ester, amine, amide, sulfonamide, hydroxyl, alkoxyl, a sulphonic acid moiety and a sulphonate moiety;

Q is absent, or is selected from a carbonyl moiety or a substituted or unsubstituted C_1 - C_6 alkyl group, wherein 0-2 of the methylene groups of the alkyl group can optionally be replaced by NH, O or S, or a substituted or unsubstituted

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C₁-C₆ carbocyclic, non-aromatic carbocyclic, heterocyclic or non-aromatic heterocyclic ring wherein the heterocyclic rings contains 1-2 heteroatoms;

5 R₆ is selected from the group consisting of H, a substituted or unsubstituted C₁-C₂₀ aliphatic group, a substituted or unsubstituted aryl, a substituted or unsubstituted alkylaryl, wherein R₆ is optionally substituted with halogen, OR*, N(R*)₂ or SR*, when Q is absent, a carbonyl group, a substituted or unsubstituted C₁-C₆ alkyl group, wherein 0-2 of the methylene groups of the alkyl group are replaced by NH, O or S, or a substituted or unsubstituted C₁-C₆ carbocyclic, non-aromatic carbocyclic, heterocyclic or non-aromatic heterocyclic ring wherein the
10 heterocyclic rings contains 1-2 heteroatoms; or

R₆ is H, when Q is a carbonyl; and

R₇ is selected from the group consisting of H, a substituted or unsubstituted C₁-C₂₀ aliphatic group, a substituted or unsubstituted aryl, a substituted or unsubstituted alkylaryl, wherein R₇ is optionally substituted with halogen, OR*,
15 N(R*)₂ or SR*; or

R₆ and R₇, taken together form a 4-, 5-, 6- or 7-membered heterocyclic or non-aromatic heterocyclic ring optionally substituted with halogen, OR*, N(R*)₂ or SR*; or

NR₆, Q and CHR₇ together form a substituted or unsubstituted or
20 heterocyclic or non-aromatic heterocyclic ring system wherein the rings contain 1 or 2 heteroatoms, wherein rings are optionally substituted with -OR*, N(R*)₂ or -SR*; and

W is absent or is a group selected from the group consisting of -SO₂NR₆- Q-CHR₇-, -O-, -COO-, and -CONH-;

25 h = 0-70; k = 0 or 1; d = 0-12; m = 0-12; p = 0-12; and

Z is, or contains a N, O or S nucleophile functionality or is, or contains a functionality capable of reacting with N, O or S nucleophiles; and each R* is independently H or C₁₋₂₀ alkyl.

[0073] The imaging agents disclosed herein can include various fluorophore derivatives
30 and other forms of fluorophores, such as N-hydroxysuccinimide forms of fluorophores. The fluorophores may be chemically linked to enzymatically cleavable oligopeptides using chemistries known in the art.

[0074] Exemplary fluorophores that can be used in the synthesis of the imaging agents of the invention include, for example, those listed in Table 2.

5

TABLE 2

No.	Fluorophore
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No.	Fluorophore
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[0075] In another embodiment, the fluorophore is a nanoparticle having fluorescent or luminescent properties. For example, an enzymatically cleavable oligopeptide can be

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grafted onto nanoparticles comprising silicon in a form that has fluorescent or luminescent properties or fluorescent nanoparticles. Aggregates of crystalline silicon (as multiple or single crystals of silicon), porous silicon, or amorphous silicon, or a combination of these forms, can form the nanoparticle. Preferred fluorescent silicon nanoparticles have a diameter from about 0.5 nm to about 25 nm, more preferably from about 2 nm and about 10 nm. The size of nanoparticles can be determined by laser light scattering or by atomic force microscopy or other suitable techniques.

[0076] Fluorescent silicon nanoparticles can have excitation and emission spectra 200 nm to 2000 nm, however, preferred fluorescent silicon nanoparticles have excitation and emission maximum from about 400 nm to about 1200 nm (and preferably 500 nm-900 nm, for example, 500 nm-600 nm, 600 nm-700 nm, 700 nm-800 nm, or 800 nm-900 nm). Preferred fluorescent silicon nanoparticles also have extinction coefficients of at least 50,000 M⁻¹cm⁻¹ in aqueous medium. Although fluorescent silicon nanoparticles with excitation and emission maximum between 400 nm and 1200 nm are preferred, it will be appreciated that the use of fluorescent silicon nanoparticles with excitation and emission wavelengths in other spectrums can also be employed in the compositions and methods of the present invention. For example, in certain embodiments, the particles can have excitation approximately about 300-350 nm, and emission approximately about 400-450 nm.

[0077] Fluorescent silicon nanoparticles also have the following properties: (1) high quantum yield (i.e., quantum yield greater than 5% in aqueous medium), (2) narrow emission spectrum (i.e., less than 75 nm; more preferably less than 50 nm), (3) spectrally separated absorption and emission spectra (i.e., separated by more than 20 nm; more preferably by more than 50 nm), (3) have high chemical stability and photostability (i.e., retain luminescent properties after exposure to light), (4) are biocompatible (as discussed below) or can be made more biocompatible; (5) are non toxic or minimally toxic to cells or subjects at doses used for imaging protocols, (as measured for example, by LD₅₀ or irritation studies, or other similar methods known in the art) and/or (6) have commercial viability and scalable production for large quantities (i.e., gram and kilogram quantities) required for *in vivo* use, for example use in humans.

[0078] Fluorescent quantum dots are also contemplated, for example, enzymatically

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cleavable oligopeptide can be grafted onto a fluorescent quantum dot such as amine T2 MP EviTags (Evident Technologies) or Qdot Nanocrystals (Invitrogen). In general, fluorescent quantum dots are nanocrystals containing several atoms of a semiconductor material (including but not limited to those containing cadmium and selenium, sulfide, or tellurium; zinc sulfide, indium-antimony, lead selenide, gallium arsenide, and silica or ormosil, which have been coated with zinc sulfide to improve the properties of these fluorescent agents.

[0079] Fluorophores can include metal oxide nanoparticles that are fluorescent and can be used in a variety of *in vitro* and *vivo* applications. In an embodiment, an enzymatically cleavable oligopeptide is conjugated to at least one fluorescent metal oxide nanoparticle with one or more of the following features: (1) a polymer coating suitable for attaching a plurality of fluorophores thereby achieving large extinction coefficients (in excess of $1,000,000 \text{ M}^{-1} \text{ cm}^{-1}$), (2) a non-crosslinked polymer coating suitable for attaching from about 10 to about 300 fluorophores per particle, (3) a polymer coating suitable for attaching a plurality of fluorophores in a manner that does not significantly compromise the quantum yield of the fluorophores (e.g., the nanoparticles retain at least 50% of the fluorescent signal that is created by substantially the same number of free fluorophores when tested under the same conditions), and (4) a polymer coating that is amenable to efficient chemical linking of biomolecules with retention of their biological properties to yield molecular imaging agents. The fluorescent metal oxide nanoparticles can be highly stable molecular imaging agents *in vitro*, both before and after chemical linking of fluorophores and agents, but yet are labile and/or degradable *in vivo*.

[0080] In certain embodiments, one or more different fluorophore molecules can be covalently linked to the oligopeptide, or alternatively, two substantially similar fluorophores can be covalently linked to the oligopeptide, at fluorescence-quenching permissive locations to produce the imaging agents of the present invention.

[0081] In certain embodiments, a quencher is used to quench the fluorescent signal from the fluorophore covalently linked to the oligopeptide. For example, an agent can be designed such that the quencher quenches the fluorescence of the fluorophore of the imaging agent when the agent is in an unactivated state, so that the imaging agent exhibits little or no signal until it is activated. It is understood that the quencher can be a non-

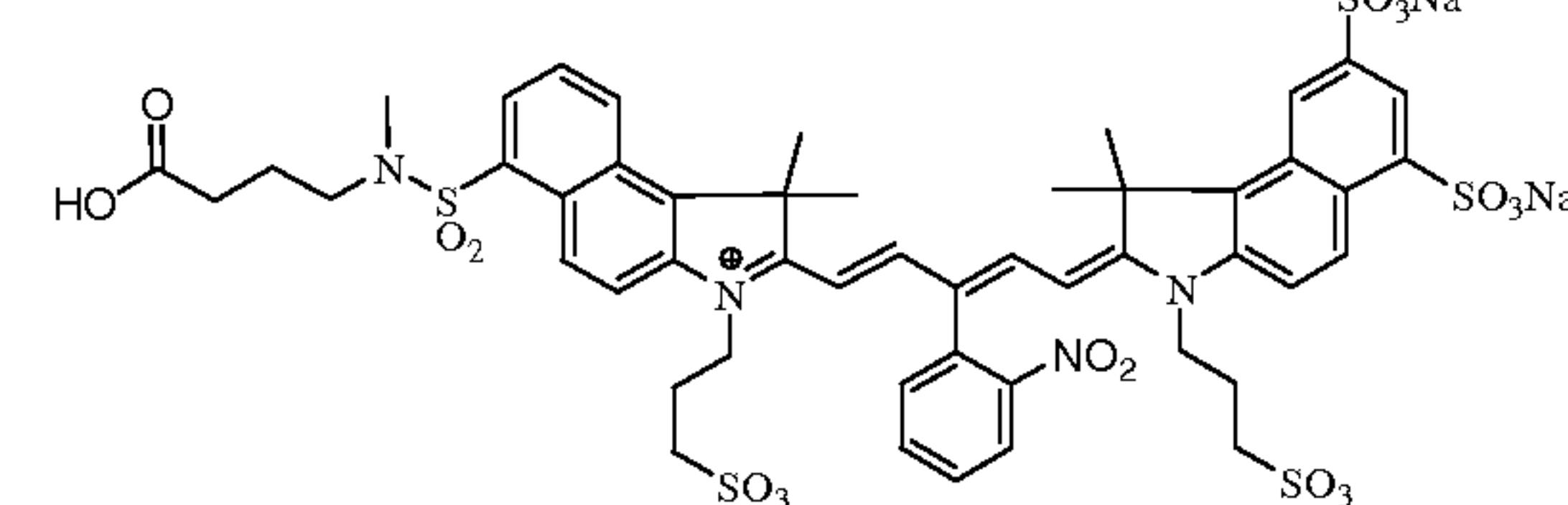
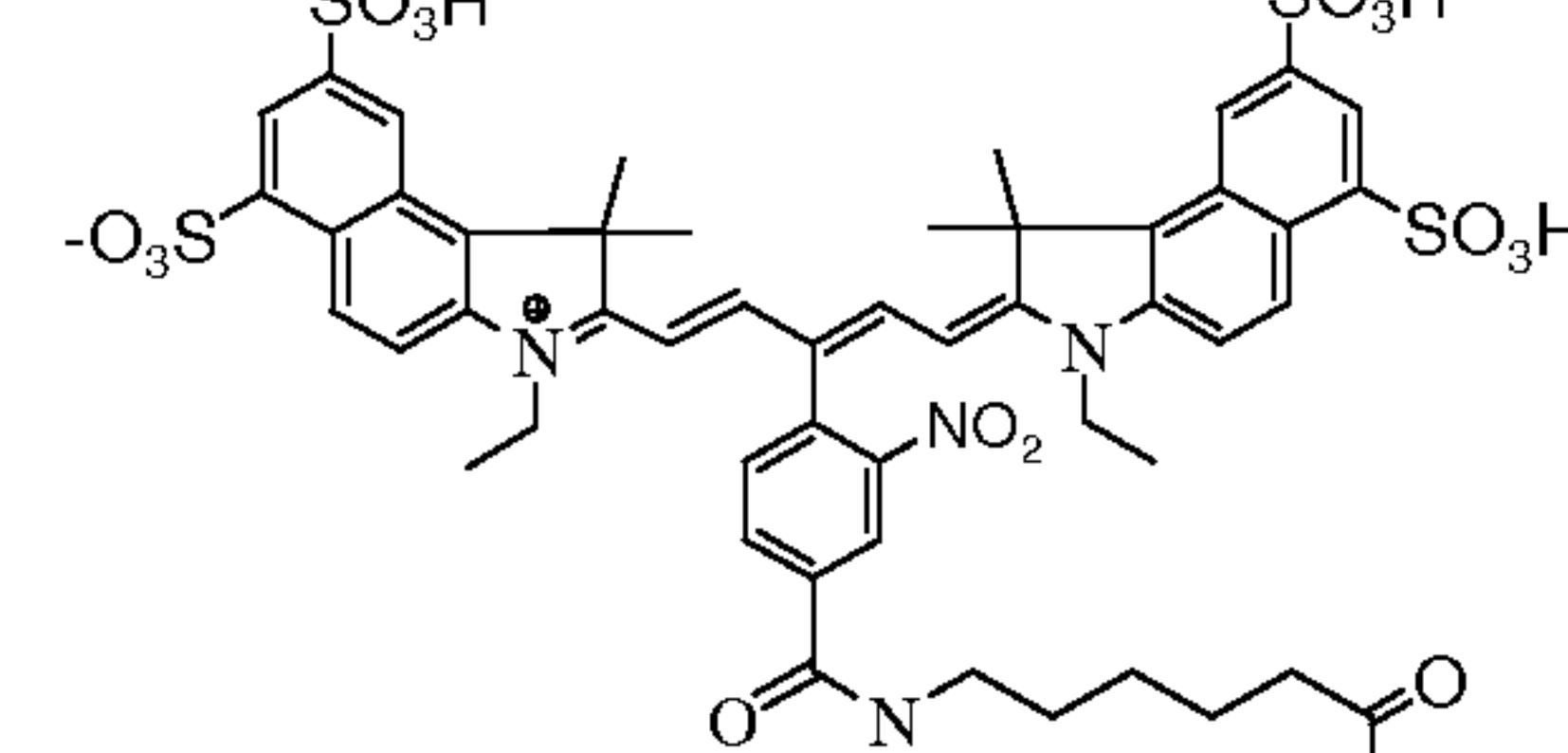
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fluorescent agent, which when suitably located relative to a fluorophore (i.e., at a fluorescence-quenching permissive location) is capable of quenching the emission signal from the fluorophore. As discussed above, it is understood that certain of the foregoing fluorophores can act to quench the fluorescent signal of another spaced apart fluorophore, when the two fluorophores are positioned at fluorescence-quenching interaction permissive locations.

[0082] A number of quenchers are available and known to those skilled in the art including, but not limited to 4-{[4-(dimethylamino)-phenyl]-azo}-benzoic acid (DABCYL), QSY[®]-7 (9-[2-[(4-carboxy-1-piperidiny]sulfonyl]phenyl]-3,6-bis(methylphenylamino)- xanthylum chloride) (Molecular Probes, Inc., OR), QSY[®]-33 (Molecular Probes, Inc., OR), ATTO612Q, ATTO580Q (ATTO-TEC, Germany); Black Hole Quenchers[®] (BioResearch Technologies, Novato, CA), QXL[™]680 Acid (AnaSpec, San Jose CA), and fluorescence fluorophores such as Cy5 and Cy5.5 (e.g., 2-[5-[3-[6-[(2,5-dioxo-1-pyrrolidiny]oxy]-6-oxohexyl]-1,3-dihydro-1,1-dimethyl-6,8-disulfo-2H-benz[e]indol-2-ylidene]-1,3-pentadienyl]-3-ethyl-1,1-dimethyl-6,8- disulfo-1H-benz[e]indolium, inner salt) (Schobel, *Bioconjugate* 10:1107, 1999). Other quenching strategies can be used, for example, using various solvents to quench fluorescence of the agents.

[0083] Exemplary fluorophores that can quench the emission of other fluorophores are represented in Table 3.

TABLE 3

No.	Quencher
1	
2	

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[0084] As with all the imaging agents discussed herein, the two fluorophores or the fluorophore and the quencher are located within the intact imaging agent at fluorescent-quenching interaction permissive positions. In other words, a first fluorophore is located close enough in the intact imaging agent to a second fluorophore (or quencher) to permit them to interact photochemically with one another so that the second fluorophore (or quencher) quenches the signal from the first fluorophore. In the case of the imaging agents with two fluorophores, one fluorophore preferably quenches the other fluorophore. For principles of quenching, see U.S. Patent No. 6,592,847.

II. Non-Fluorescent Reporters

[0085] The term “non-fluorescent reporter” as used herein, refers to a chemical moiety that is not fluorescent but which can be used to provide the contrast or signal in imaging and is detectable by a non-fluorescent imaging technique. In certain embodiments, other non-fluorescent reporters can be chemically linked with the imaging agents, or can be administered to a subject simultaneously or sequentially with the imaging agents of the invention. Such reporters can include photoluminescent nanoparticles, radioisotopes, superparamagnetic agents, X-ray contrast agents, and ultrasound agents. A reporter may also comprise therapeutic reporters such as porphyrins, Photofrin®, Lutrin®, Antrin®, aminolevulinic acid, hypericin, benzoporphyrin derivatives used in photodynamic therapy, and radionuclides used for radiotherapy.

(A) Radioactive Reporters

[0086] The imaging agents can include one or more radioactive labels. Radioisotopic forms of metals such as copper, gallium, indium, technetium, yttrium, and lutetium can be chemically linked to the metallic imaging agents and can be used for nuclear imaging or therapeutic applications. Exemplary radioactive labels include, without limitation, ^{99m}Tc , ^{111}In , ^{64}Cu , ^{67}Ga , ^{186}Re , ^{188}Re , ^{153}Sm , ^{177}Lu , and ^{67}Cu .

[0087] Other exemplary labels include, for example, ^{123}I , ^{124}I , ^{125}I , ^{11}C , ^{13}N , ^{15}O , and ^{18}F . Other exemplary labels can be therapeutic radiopharmaceuticals including for example, ^{186}Re , ^{188}Re , ^{153}Sm , ^{166}Ho , ^{177}Lu , ^{149}Pm , ^{90}Y , ^{212}Bi , ^{103}Pd , ^{109}Pd , ^{159}Gd , ^{140}La , ^{198}Au , ^{199}Au , ^{169}Yb , ^{175}Yb , ^{165}Dy , ^{166}Dy , ^{67}Cu , ^{105}Rh , ^{111}Ag , and ^{192}Ir .

[0088] Chelators or bonding moieties for diagnostic and therapeutic

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radiopharmaceuticals are also contemplated and can be chemically associated with the imaging agents. Exemplary chelators can be selected to form stable complexes with radioisotopes that have imageable gamma ray or positron emissions, such as ^{99m}Tc , ^{111}In , ^{64}Cu , and ^{67}Ga . Exemplary chelators include diaminedithiols, monoamine-

5 monoamidedithiols, triamide-monothiol, monoamine-diamide-monothiol, diaminedioximes, and hydrazines. Chelators generally are tetradentate with donor atoms selected from nitrogen, oxygen and sulfur, and may include for example, cyclic and acyclic polyaminocarboxylates such as diethylenetriaminepentaacetic acid (DTPA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), (DO3A), 2-benzyl-

10 DOTA, alpha-(2-phenethyl)1,4,7,10-tetraazacyclododecane-1-acetic-4,7,10-tris(methylacetic)acid, 2-benzyl-cyclohexyldiethylenetriaminepentaacetic acid, 2-benzyl-6-methyl-DTPA, and 6,6''-bis[N,N,N'',N''-tetra(carboxymethyl)aminomethyl]-4'-(3-amino-4-methoxyphenyl)-2,2':6',2''-terpyridine.

[0089] Chelators or bonding moieties for therapeutic radiopharmaceuticals can be

15 selected to form stable complexes with the radioisotopes that have alpha particle, beta particle, Auger or Coster-Kronig electron emissions, such as ^{186}Re , ^{188}Re , ^{153}Sm , ^{177}Lu , and ^{67}Cu . Chelators can be selected from diaminedithiols, monoamine-monoamidedithiols, triamide-monothiol, monoamine-diamide-monothiol, diaminedioximes, and hydrazines, cyclic and acyclic polyaminocarboxylates such as

20 DTPA, DOTA, DO3A, 2-benzyl-DOTA, alpha-(2-phenethyl)1,4,7,10-tetraazacyclododecane-1-acetic-4,7,10-tris(methylacetic)acid, 2-benzyl-cyclohexyldiethylenetriaminepentaacetic acid, 2-benzyl-6-methyl-DTPA, and 6,6''-bis[N,N,N'',N''-tetra(carboxymethyl)aminomethyl]-4'-(3-amino-4-methoxyphenyl)-2,2':6',2''-terpyridine.

25 (B) Magnetic Reporters

[0090] Other exemplary reporters can include a chelating agent for magnetic resonance agents. Such chelators can include for example, polyamine-polycarboxylate chelators or iminoacetic acid chelators that can be chemically linked to the agents.

[0091] Chelators for magnetic resonance imaging agents can be selected to form stable

30 complexes with paramagnetic metal ions, such as Gd(III), Dy(III), Fe(III), and Mn(II), are selected from cyclic and acyclic polyaminocarboxylates such as DTPA, DOTA, DO3A, 2-

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benzyl-DOTA, alpha-(2-phenethyl)1,4,7,10-tetraazacyclododecane-1-acetic-4,7,10-tris(methylacetic)acid, 2-benzyl-cyclohexyldiethylenetriaminepentaacetic acid, 2-benzyl-6-methyl-DTPA, and 6,6''-bis[N,N,N'',N''-tetra(carboxymethyl)aminomethyl]-4'-(3-amino-4-methoxyphenyl)-2,2':6',2''-terpyridine.

- 5 [0092] In one embodiment, the imaging agents are chemically linked to superparamagnetic metal oxide nanoparticles that are either (a) non-fluorescent or (b) are fluorescent and can be used in a variety of *in vitro* and *in vivo* applications. Fluorescent metal oxide nanoparticles that also have magnetic properties can be used for MRI, thus providing a multi-modality imaging agent.
- 10 [0093] In certain embodiments, the imaging agents can include a fluorescent and/or non-fluorescent superparamagnetic metal oxide nanoparticle with one or more of the following features: (1) a polymer coating suitable for attaching a plurality of agents (2) a non-crosslinked polymer coating suitable for attaching from about 10 to about 300 agents per particle, and (3) a polymer coating that is amenable to efficient chemical linking of the
- 15 agents with retention of their biological properties to yield molecular imaging agents. The agent modified metal oxide nanoparticle can be a highly stable molecular imaging agent *in vitro*, both before and after chemical linking of the agents, but yet are labile and/or degradable *in vivo*.

(C) Ultrasound Reporters

- 20 [0094] A non-fluorescent reporter can include gas-filled bubbles such as Levovist, Albunex, or Echovist, or particles or metal chelates where the metal ions have atomic numbers 21-29, 42, 44 or 57-83, for example to facilitate ultrasound imaging. Examples of such compounds are described in Tyler et al., Ultrasonic Imaging, 3, pp. 323-29 (1981) and D.P. Swanson, "Enhancement Agents for Ultrasound: Fundamentals,"
- 25 Pharmaceuticals in Medical Imaging, pp. 682-87 (1990).

(D) X-Ray Reporters

- [0095] Exemplary reporters can comprise of iodinated organic molecules or chelates of heavy metal ions of atomic numbers 57 to 83, for example, for X-ray imaging. Examples of such compounds are described in M. Sovak, ed., "Radiocontrast Agents," Springer-
- 30 Verlag, pp. 23-125 (1984) and U.S. Patent No. 4,647,447.

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III. Linkers

[0096] Linker or spacer moieties can be used to covalently link one or more fluorophores, quenchers, biological modifiers and non-fluorescent reporters to an enzymatically cleavable oligopeptide or to an optional biological modifier to produce
5 agents of the present invention. It is understood that there is no particular structural, size or content limitation of a linker, if present. Linkers can include, for example, a variety of functional groups such as maleimide, dithiopyridyl, thiol, azide, alkene, or alkyne that permit the assembly of molecules of diverse architecture.

[0097] Linkers can be homofunctional linkers or heterofunctional linkers. For example,
10 amine (NH₂)-functionalized moieties can be reacted with bifunctional cross-linkers designed to react with amino groups. Particularly useful conjugation reagents that can facilitate formation of a linker or facilitate covalent linkage between, for example, a fluorophore, and an enzymatically cleavable oligopeptide can include a N-hydroxysuccinimide (NHS) ester and/or a maleimide. The NHS ester can react with the
15 amine group of, for example, a peptide or fluorophore. The maleimide can react with the sulfhydryl group of another molecule. Other particularly useful linker moieties are bifunctional crosslinkers such as N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), long chain-SPDP, maleimidobenzoic acid-N-hydroxysuccinimide ester (MBS), succinimidyl trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate (SMCC),
20 succinimidyl iodoacetate (SIA).

[0098] In certain embodiments a linker, if present, may be a derivative of a diamine. A diamine moiety or derivative can provide a linker arm of varying lengths and chemistries for chemically linking molecules by derivatizing, optionally, with carboxylic acids. Non-limiting examples of diamines include ethylenediamine (EDA), propylenediamine,
25 spermidine, spermine, hexanediamine, and diamine-amino acids, such as homolysine, lysine, ornithine, diaminobutyric acid and diaminopropionic acid. In other embodiments, moieties of an imaging agent can be chemically linked to a dicarboxylic acid, for example, succinic acid, glutaric acid, suberic acid, or adipic acid. In one embodiment, the linker is aminoethylmaleimide.

30 [0099] In certain embodiments, a linker can be formed from an azide moiety that can react with substituted alkynes in an azide-acetylene Huisgen [3+2] cycloaddition. In

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certain embodiments the azide or alkyne linker can link a polyethyleneglycol (PEG) moiety to, for example, an enzymatically cleavable oligopeptide. Other contemplated linkers include propargylglycine, pentanoyl, pentynoic acid, propargylic acid, and/or propargylamine moieties.

5 [00100] In certain embodiments, fluorophores, quenchers, or other reporters are directly linked to the imaging agent using reactive NHS ester groups on the fluorophores, quenchers, or reporters which react with an amine group on the enzymatically cleavable oligopeptide. In certain other embodiments, carboxylic acid groups on the fluorophores, quenchers, or other reporters can be activated *in situ* by activating agents known in the art,
10 such as 2-(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU), 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N,N'-dicyclohexylcarbodiimide (DCC), N,N'-disuccinimidyl carbonate (DSC). In other embodiments, reporters including a sulfhydryl or thiol group, can be chemically linked to the agent via a bifunctional cross-linker that has a second moiety that can react with a
15 sulfhydryl (thiol) group. Such crosslinking agents include, for example and as described above, SPDP, long chain-SPDP, SIA, MBS, SMCC, and others that are well known in the art.

[00101] Useful linker moieties include both natural and non-natural amino acids, oligopeptides, for example, linear or cyclic oligopeptides, and nucleic acids.

20 [00102] The linker can be a peptide or peptide moiety which optionally includes a proteolytic or non-proteolytic cleavage site, such as an ester linkage, that can be cleaved due to pH changes at the site of interest.

IV Biological Modifiers

[00103] As used herein, the term "biological modifier" is understood to mean any moiety
25 that can be used to alter the biological properties of the imaging agent, such as, without limitations, making the imaging agent more water soluble or more dispersible in media for administration, increasing binding specificity, decreasing immunogenicity or toxicity, or modifying pharmacokinetic profile compared to the non-biological modifier modified agents.

30 [00104] In an embodiment, one or more biological modifiers can be chemically linked to

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the enzymatically cleavable oligopeptide or fluorophore. In one embodiment, the biological modifier is covalently linked to the enzymatically cleavable peptide at a position that is not between two amino acids covalently linked to a fluorophore or a quencher.

5 [00105] Exemplary biological modifiers include polyethylene glycol (PEG) and derivatives thereof (for example, alkoxy polyethylene glycol (for example, methoxypolyethylene glycol, ethoxypolyethylene glycol and the like), branched polypropylene glycol, polypropylene glycol, a graft copolymer of poly-lysine and methoxypolyethyleneglycol, amino acids, peptides, lipids, fatty acids, palmitate, 10 phospholipids, phospholipid-PEG conjugates, carbohydrates (such as dextran, amino-dextran, carboxymethyl-dextran), iron oxide nanoparticles, sulfonates, polysulfonates, cysteic acid, naphthylalanine, phenylalanine, and 3,3-diphenylpropylamine.

[00106] In general, the biological modifier may have a molecular weight of from about 2 kDa to less than 50 kDa, such as from about 5 kDa to about 40 kDa, such as from about 10 15 kDa to about 35 kDa, further such as from about 15 kDa to 30 kDa. In another embodiment, the biological modifier may have a molecular weight of from about 5 kDa to about 45 kDa, such as from about 5 kDa to about 40 kDa, such as from about 5 kDa to about 35 kDa, such as from about 5 kDa to about 30 kDa, such as from about 5 kDa to about 25 kDa, such as from about 5 kDa to about 20 kDa, such as from about 5 kDa to 20 about 15 kDa, further such as from about 5 kDa to 10 kDa. In another embodiment, the biological modifier may have a molecular weight of from about 2 kDa to less than 50 kDa, such as from about 2 kDa to about 45 kDa, such as from about 2 kDa to about 40 kDa, such as from about 2 kDa to about 35 kDa, such as from about 2 kDa to about 30 kDa, such as from about 2 kDa to about 25 kDa, such as from about 2 kDa to about 10 kDa, 25 further such as from about 2 kDa to 5 kDa.

[00107] In certain embodiments, as discussed above, the biological modifier may be a PEG moiety that has a molecular weight, for example, from about 0.5 kDa to about 50 kDa, about 5 kDa to about 35 kDa, or about 10 kDa to about 30 kDa. Alternatively, the PEG may be dPEG, functionalized at a discrete molecular weight, for example, of about 30 1100 daltons.

[00108] In certain embodiments, the PEG is methoxyPEG₍₅₀₀₀₎-succinimidylpropionate

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(mPEG-SPA), methoxyPEG₍₅₀₀₀₎-succinimidylsuccinate (mPEG-SS). Such PEGS are commercially available from Nektar Therapeutics or SunBiowest or LaysanBio or NOF.

[00109] In one embodiment, a PEG moiety may be conjugated to reactive amines on the enzymatically cleavable oligopeptide or fluorophore via a carboxyl functionality.

5 Alternatively, the PEG modifier can be conjugated to the enzymatically cleavable oligopeptide or fluorophore by using a thiol reactive cross linker and then reacting with a thiol group on the PEG.

[00110] In one embodiment, the PEG may be branched, or Y-shaped, as available from JenKem USA or NOF, or comb-shaped, or synthesized by coupling two or more PEGs to
10 a small molecule such as glutamic acid.

[00111] The omega position of PEG may include a hydroxyl group or a methoxy group and the PEG may also contain an amino group in the omega position. Such an amino group can in turn be coupled to a variety of agents. In another embodiment of the present invention, the biological modifier can be a pegylated poly-L-lysine or a pegylated poly-D-
15 lysine.

[00112] In other embodiments, the biological modifier can be polyvinylpyrrolidone (PVP)-type polymers. The biological modifier can be a functionalized polyvinylpyrrolidone, for example, carboxy or amine functionalized on one (or both) ends of the polymer (as available from Polymersource) or within the polymer chain.

20 **[00113]** Alternatively, the biological modifier can include Poly N-(2-hydroxypropyl)methacrylamide (HPMA), or functionalized HPMA (amine, carboxy, etc.), Poly(N-isopropyl acrylamide) or functionalized poly(N-isopropylacrylamide).

[00114] Biological modifiers can include straight or branched chain acyl groups, such as pentynoyl; acidic groups, such as succinyl; lower alkyl groups, such as methyl, ethyl,
25 propyl, etc.; carboxyalkyl groups, such as carboxyethyl; haloalkyl groups, such as trifluoromethyl; and the like.

[00115] In other embodiments, the biological modifier can include, but is not limited to, proteins, peptides, antibodies and antigen binding fragments thereof (for example, Fab, Fab', (Fab')₂ fragments), single chain antibodies or sFvs, oligonucleotides, aptamers,
30 glycoproteins, ligands for cell receptors, polysaccharides, cell receptors, enzyme

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substrates, enzyme cofactors, biotin, hormones, neurohormones, neurotransmitters, growth factors, cytokines, lymphokines, lectins, selectins, toxins, nucleic acids, oligonucleotides and derivatives thereof. Other biomolecules can also be used, such as folate-mediated targeting molecules(Leamon & Low, *Drug Discovery Today*, 6:44-51, 2001), transferrin, vitamins, carbohydrates and ligands that target internalizing receptors, including, but not limited to, asialoglycoprotein receptor, somatostatin, nerve growth factor, oxytocin, bombesin, calcitonin, arginine vasopressin, angiotensin II, atrial natriuretic peptide, insulin, glucagons, prolactin, gonadotropin, various opioids and urokinase-type plasminogen activator. Biomolecules such as integrin targeting agents are contemplated, such as $\alpha_v\beta_3$ and $GP\alpha_{IIb}\beta_3$, bombesin, CD4 and VCAM-1. Also contemplated are peptides for Hepsin, SPARC, PAR1, colon cancer, Factor 13.

[00116] Exemplary peptides for use as biological modifiers include: (Hepsin) Ile-Pro-Leu-Val-Leu-Pro-Leu (SEQ ID NO:1); (SPARC) Ser-Pro-Pro-Thr-Gly-Ile-Asn (SEQ ID NO:2); (VCAM1) Val-His-Pro-Lys-Gln-His-Arg (SEQ ID NO:3); (Cathepsin K) Val-His-Pro-Lys-Gln-His-Arg (SEQ ID NO:4); (E-selection binding peptide) Cys-Asp-Ser-Asp-Ser-Asp-Ile-Thr-Trp-Asp-Gln-Leu-Trp-Asp-Asp-Leu-Met-Lys (SEQ ID NO:5); and (Tat) Arg-Arg-Arg-Arg-Gly-Arg-Arg-Arg-Arg (SEQ ID NO:6).

[00117] Other contemplated biological modifiers include membrane, transmembrane, and nuclear translocation signal compounds and sequences, which can be derived from a number of sources including, without limitation, viruses and bacteria. Non-limiting examples include HIV-tat derived peptides, protamine, and polyArg and Arg-rich peptides.

[00118] Biological modifiers can also include synthetic compounds including, but not limited to, small molecule drugs, phototherapeutic molecules and derivatives thereof. Other contemplated biological modifiers include antibiotics such as vancomycin, clindamycin, chemotherapeutics such as doxorubicin, molecules such as glycine, derivatives of AMG706, Zactima™, MP-412, erlotinib, sorafenib, dasatinib, lestaurtinib, lapatinib, XL647, XL999, MLN518, PKC412, STI571, AMN107, AEE788, OSI-930, OSI-817, sunitinib, AG-013736; molecules that target/inhibit VEGF receptors, PDGF receptor, HER2, SSKI, EphB4, EGFR, FGFR, VEGFR-2, VEGFR-3, serine/threonine and receptor kinases, FLT-3, type III RTKs, c-KIT, Bcr-Abl, CSF-1R, CCR-2, RET, VEGF-2

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and photodynamic reagents including but not limited to Chlorin e6, Photofrin®, Lutrin®, Antrin®, aminolevulinic acid, hypericin, porphyrins, and porphyrin derivatives, for example, benzoporphyrin derivative.

[00119] The biological modifiers may, under certain circumstances, render the imaging agents more useful for biological imaging. For example, the biological modifier may render agents more water soluble, and/or more dispersible in media for administration and/or may have an increased binding specificity, and/or may be less immunogenic and/or less toxic, and/or have a reduced non-specific binding and/or altered biodistribution and/or pharmacokinetic profile as compared to a non-biologically modified agents. For example, incorporation of methoxypolyethylene glycol (mPEG) or polypeptides may function to modify the pharmacodynamics and blood clearance rates of the agents *in vivo*. Other biological modifiers may be chosen to accelerate the clearance of the agents from background tissue, such as muscle or liver, and/or from the blood, thereby reducing the background interference and improving image quality. Additionally, the biological modifiers may also be used to favor a particular route of excretion, e.g., via the kidneys rather than via the liver. The biological modifiers may also aid in formulating agents in pharmaceutical compositions or may be used to alter or preserve the signal reporting properties of the agents. In one embodiment of the present invention, the biological modifier can be a polyamino acid or a peptide, including cyclic peptides. In particular, chemical linking of polyethylene glycol (PEG) or a derivative thereof to agents can result in longer blood residence time (longer circulation) and decreasing immunogenicity.

V. Enzymatically Cleavable Oligopeptides

[00120] As used herein, the term “enzymatically cleavable oligopeptide” is understood to mean a peptide comprising two or more amino acids (as defined herein) that are linked by means of a enzymatically cleavable peptide bond. Also included are moieties that include a pseudopeptide or peptidomimetic. Examples of cleavable peptide substrates can be found in U.S. Patent No. 7,439,319.

[00121] The term “amino acid” as used herein is understood to mean an organic compound containing both a basic amino group and an acidic carboxyl group. Included within this term are natural amino acids (e.g., L-amino acids), modified and unusual amino acids (e.g., D-amino acids), as well as amino acids which are known to occur

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biologically in free or combined form but usually do not occur in proteins. Natural amino acids include, but are not limited to, alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, tryptophan, proline, and valine. Other amino acids include, but are not limited to, arginosuccinic acid, citrulline, cysteine sulfinic acid, 3,4-dihydroxyphenylalanine, homocysteine, homoserine, ornithine, carnitine, selenocysteine, selenomethionine, 3-monoiodotyrosine, 3,5-diiodotyrosine, 3,5,5'-triiodothyronine, and 3,3',5,5'-tetraiodothyronine.

- [00122]** Modified or unusual amino acids which can be used to practice the invention include, but are not limited to, D-amino acids, hydroxylysine, dehydroalanine, pyrrollysine, 2-aminoisobutyric acid, gamma aminobutyric acid, 5-hydroxytryptophan, S-adenosyl methionine, S-adenosyl homocysteine, 4-hydroxyproline, an N-Cbz-protected amino acid, 2,4-diaminobutyric acid, homoarginine, norleucine, N-methylaminobutyric acid, naphthylalanine, phenylglycine, .beta.-phenylproline, tert-leucine, 4-aminocyclohexylalanine, N-methyl-norleucine, 3,4-dehydroproline, N,N-dimethylaminoglycine, N-methylaminoglycine, 4-aminopiperidine-4-carboxylic acid, 6-aminocaproic acid, trans-4-(aminomethyl)-cyclohexanecarboxylic acid, 2-, 3-, and 4-(aminomethyl)-benzoic acid, 1-aminocyclopentanecarboxylic acid, 1-aminocyclopropanecarboxylic acid, and 2-benzyl-5-aminopentanoic acid.
- [00123]** As used herein, a "pseudopeptide" or "peptidomimetic" is a compound which mimics the structure of an amino acid residue or a peptide, for example, by using linking groups other than via amide linkages (pseudopeptide bonds) and/or by using non-amino acid substituents and/or a modified amino acid residue. A "pseudopeptide residue" means that portion of a pseudopeptide or peptidomimetic that is present in a peptide. The term "pseudopeptide bonds" includes peptide bond isosteres which may be used in place of or as substitutes for the normal amide linkage. These substitute or amide "equivalent" linkages are formed from combinations of atoms not normally found in peptides or proteins which mimic the spatial requirements of the amide bond and which should stabilize the molecule to enzymatic degradation. The following conventional three-letter amino acid abbreviations are used herein: Ala = alanine; Aca = aminocaproic acid, Ahx = 6-aminohexanoic acid, Arg = arginine; Asn = asparagines; Asp = aspartic acid; Cha =

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cyclohexylalanine; Cit = citrulline; Cys = cysteine; Dap = diaminopropionic acid; Gln = glutamine; Glu = glutamic acid; Gly = glycine; His = histidine; Ile = isoleucine; Leu = leucine; Lys = lysine; Met = methionine; Nal = naphthylalanine; Nle = norleucine; Orn = ornithine; Phe = phenylalanine; Phg = phenylglycine; Pro = proline; Sar = sarcosine; Ser = serine; Thi = Thienylalanine; Thr = threonine; Trp = tryptophan; Tyr = tyrosine; and Val = valine. Use of the prefix D- indicates the D-isomer of that amino acid; for example D-lysine is represented as D-Lys.

[00124] The peptides can be synthesized using either solution phase chemistry or solid phase chemistry or a combination of both (Albericio, Curr. Opin. Cell Biol., 8, 211-221 (2004), M. Bodansky, Peptide Chemistry: A Practical Textbook, Springer-Verlag; N.L. Benoiton, Chemistry of Peptide Synthesis, 2005, CRC Press).

[00125] Selective or orthogonal amine protecting groups may be required to prepare the agents of the invention. As used herein, the term "amine protecting group" means any group known in the art of organic synthesis for the protection of amine groups. Such amine protecting groups include those listed in Greene, "Protective Groups in Organic Synthesis" John Wiley & Sons, New York (1981) and "The Peptides: Analysis, Synthesis, Biology, Vol. 3, Academic Press, New York (1981). Any amine protecting group known in the art can be used. Examples of amine protecting groups include, but are not limited to, the following: 1) acyl types such as formyl, trifluoroacetyl, phthalyl, and p-toluenesulfonyl; 2) aromatic carbamate types such as benzyloxycarbonyl (Cbz or Z) and substituted benzyloxycarbonyls, 1-(p-biphenyl)-1-methylethoxycarbonyl, and 9-fluorenylmethyloxycarbonyl (Fmoc); 3) aliphatic carbamate types such as tert-butyloxycarbonyl (Boc), ethoxycarbonyl, diisopropylmethoxycarbonyl, and allyloxycarbonyl; 4) cyclic alkyl carbamate types such as cyclopentyloxycarbonyl and adamantyloxycarbonyl; 5) alkyl types such as triphenylmethyl and benzyl; 6) trialkylsilane such as trimethylsilane; and 7) thiol containing types such as phenylthiocarbonyl and dithiasuccinoyl. Also included in the term "amine protecting group" are acyl groups such as azidobenzoyl, p-benzoylbenzoyl, o-benzylbenzoyl, p-acetylbenzoyl, dansyl, glycyl-p-benzoylbenzoyl, phenylbenzoyl, m-benzoylbenzoyl, benzoylbenzoyl.

[00126] In certain embodiments the enzymatically cleavable oligopeptide can include

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oligo-L-arginine, oligo-L-lysine, oligo-L-aspartic acid or oligo-L-glutamic acid.

- [00127]** In certain embodiments the enzymatically cleavable oligopeptide includes lysine and arginine. In certain embodiments of the present invention, the enzymatically cleavable oligopeptide can include lysine, arginine and phenylalanine, or may include lysine, phenylalanine and glycine. In another embodiment, the enzymatically cleavable oligopeptide can include lysine, phenylalanine, leucine, and glycine, or can include ornithine, phenylalanine, leucine, and glycine. In one embodiment, the enzymatically cleavable oligopeptide may include diaminopropionic acid, ornithine, phenylalanine, leucine, and glycine.
- [00128]** Exemplary enzymatically cleavable oligopeptides are set forth in Table 4.

TABLE 4

Oligopeptide	SEQ ID NO.
Lys-Lys-Lys-Lys-Gly	7
Lys-Lys-Lys-Gly	8
Lys-Lys-Gly-Lys-Lys	9
Orn-Lys-Lys-Orn-Gly	10
Orn-Lys-Lys-Orn-Ahx	11
Orn-Lys-Lys-Orn-Ahx-Gly-Gly	12
Lys-Lys-Lys-Lys-Ahx	13
Lys-Lys-Lys-βAla	14
Lys-Lys-Lys-Lys-Gly-Gly-Gly-Gly-Gly	15
Gly-Gly-Lys-Lys-Lys-Lys-Lys-Gly	16
Lys-Lys-Lys-Lys-Lys-Lys-Lys-Gly	17
D-Lys-Lys-Lys-Lys-Lys-Lys-Lys-Gly	
Lys-Lys-Lys-Lys-Lys-Lys-Lys-Ahx	18
Lys-Lys-Lys-Lys-Lys-Lys-Lys-Gly-Gly-Gly-Gly-Gly	19
Lys-Lys-Lys-Lys-Lys-Lys-Lys-D-Lys-Gly-Gly-Gly-Gly-Gly	
Lys-Arg-Lys-Arg-Lys-Arg-Gly	20
Lys-Arg-Lys-Arg-Lys-Arg-Gly-Cys	21
Lys-Arg-Arg-Arg-Lys-Arg-Gly	22
Lys-Arg-Lys-Arg-Lys-Arg-Lys-Gly	23
Lys-Arg-Arg-Arg-Arg-Lys-Arg-Gly	24
Phe-Arg-Lys-Gly-Gly-Arg-Lys	25
Phe-Arg-Lys-Gly-Gly-Arg-D-Lys	
Phe-Arg-Lys-Gly-Gly-Arg-Arg-Lys	26
Phe-Arg-Lys-Gly-Gly-Arg-Lys-Ahx	27
Phe-Arg-Lys-Gly-Gly-Arg-Lys-Gly-Gly	28
Cha-Arg-Lys-Gly-Gly-Arg-Lys	29
Thi-Arg-Lys-Gly-Gly-Arg-Lys	30

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Oligopeptide	SEQ ID NO.
Thi-Arg-Lys-Gly-Gly-Arg-Lys-Gly	31
Phe-Gly-Lys-Arg-Arg-Lys	32
D-Phe-Gly-Lys-Arg-Arg-Lys	
Thi-Gly-Lys-Arg-Arg-Lys	33
Cha-Gly-Lys-Arg-Arg-Lys	34
Phe-Gly-Orn-Arg-Arg-Dap	35
Phe-Gly-Orn-Arg-Arg-Orn	36
Phe-Gly-Lys-Arg-Arg-Lys-Gly-Gly	37
Phe-Gly-Lys-Arg-Arg-Lys-Ahx	38
Phe-Gly-Lys-Arg-Arg-Lys-Arg-Arg-Arg-Ahx	39
Phe-Gly-Lys-Arg-Arg-Lys-Arg-Arg-Arg-D-Arg-Ahx	
Phe-Gly-Lys-Arg-Arg-Lys-Glu-Glu-Glu-Ahx	40
Phe-Gly-Lys-Arg-Arg-Lys-Arg-Arg-Arg-Ahx-Cys	41
Lys-Gly-Phe-Leu-Gly-βAla-Lys	42
D-Lys-Gly-Phe-Leu-Gly-βAla-Lys	
Orn-Gly-Phe-Leu-Gly-βAla-Orn	43
Lys-Gly-Phe-Leu-Gly-βAla-Lys-Gly	44
Lys-Gly-Phe-Leu-Gly-βAla-Lys-Ahx	45
Lys-Gly-Phe-Leu-Gly-βAla-Lys-Gly-Gly-Gly	46
Gly-Gly-Gly-Lys-Gly-Phe-Leu-Gly-βAla-Lys	47
Ahx-Lys-Gly-Phe-Leu-Gly-βAla-Lys-Ahx	48
Lys-Gly-Phe-Leu-Gly-βAla-Lys-Ahx-Cys	49
Gly-Phe-Leu-Gly-Lys	50
Lys-Phe-Leu-Gly-Lys-Ahx	51
Gly-Phe-Leu-Gly-Lys-Gly-Gly-Gly	52
Gly-Gly-Phe-Leu-Gly-Lys-Ahx	53
Gly-Phe-Leu-Gly-Lys-Cys	54
Gly-Phe-Leu-Gly-Lys-Ahx-Cys	55
Gly-Gly-Phe-Leu-Gly-Lys-Ahx-Cys	56
Gly-Phe-Leu-Gly-Orn	57
Gly-Phe-Leu-Gly-Lys-Arg-Arg-Arg-Arg-Cys	58
His-Gly-Pro-Asn-Lys-His-Gly-Pro-Asn-βAla	59
His-Gly-Pro-Asn-Orn-His-Gly-Pro-Asn-βAla	60
His-Gly-Pro-Asn-Lys-His-Gly-Pro-Asn-βAla	61
Lys-His-Gly-Pro-Asn-Lys-His-Gly-Pro-Asn-βAla	62
Orn-His-Gly-Pro-Asn-Lys-His-Gly-Pro-Asn-βAla	63
Orn-His-Gly-Pro-Asn-Orn-His-Gly-Pro-Asn-βAla	64
Phe-Gly-Gly-His-Gly-Pro-Asn-Lys-His-Gly-Pro-Asn-Ahx	65
His-Gly-Pro-Arg-Lys-His-Gly-Pro-Arg-βAla	66
His-Gly-Pro-Asn-Lys-His-Gly-Pro-Arg-βAla	67
His-Gly-Pro-Arg-Lys-His-Gly-Pro-Asn-βAla	68
His-Gly-Pro-Asn-Lys-His-Gly-Pro-Asn-Ahx	69
His-Gly-Pro-Arg-Lys-His-Gly-Pro-Arg-Gly-Gly-Gly-Phe-Gly	70
His-Gly-Pro-Arg-Orn-His-Gly-Pro-Arg-βAla	71
His-Gly-Pro-Cit-Lys-His-Gly-Pro-Asn-βAla	72

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Oligopeptide	SEQ ID NO.
His-Gly-Pro-Asn-Lys-His-Gly-Pro-Cit-βAla	73
His-Gly-Pro-Asn-Orn-His-Gly-Pro-Cit-βAla	74
His-Gly-Pro-Asn-Lys	75
His-Gly-Pro-Asn-Orn	76
Lys-His-Gly-Pro-Asn-Lys	77
Orn-His-Gly-Pro-Asn-Lys	78
His-Gly-Pro-Asn-Lys-Gly-Gly-Gly	79
His-Gly-Pro-Asn-Lys-Gln-Gly-Gly	80
His-Gly-Pro-Asn-Orn-Gly-Gly-Ahx	81
His-Gly-Pro-Asn-Lys-Arg-Arg-Arg-Ahx	82
His-Gly-Pro-Asn-Lys-Arg-Gly-Gly	83
Gly-Arg-Arg-Arg-Ahx-Orn-His-Gly-Pro-Asn-Lys-Gly	84
Gly-Arg-Arg-Arg-Ahx-D-Lys-His-Gly-Pro-Asn-Lys-Gly	
His-Gly-Pro-Arg-Lys	85
His-Gly-Pro-Arg-Orn	86
Lys-His-Gly-Pro-Arg-Lys	87
Orn-His-Gly-Pro-Arg-Lys	88
His-Gly-Pro-Arg-Lys-Ahx	89
His-Gly-Pro-Arg-Lys-Gly-Gly-Gly	90
His-Gly-Pro-Arg-Orn-Gly-Gly-Ahx	91
His-Gly-Pro-Arg-Lys-Arg-Arg-Arg-Ahx	92
His-Gly-Pro-Arg-Lys-Arg-Gly-Gly	93
Gly-Arg-Arg-Arg-Ahx-Lys-His-Gly-Pro-Arg-Lys-Gly	94
Gly-Lys-Arg-Arg-Ahx-Orn-His-Gly-Pro-Asn-Orn-Gly	95
Gly-Lys-Lys-Arg-Ahx-Orn-His-Gly-Pro-Asn-Orn-Gly	96
Gly-Arg-Arg-Arg-Lys-Ahx-His-Gly-Pro-Asn-Lys-Gly	97
Lys-Pro-Leu-Gly-Val-Arg-Lys	98
Lys-Gly-Pro-Leu-Gly-Val-Arg-Lys	99
Ahx-Lys-Pro-Leu-Gly-Val-Arg-Lys	100
Lys-Pro-Leu-Gly-Val-Arg-Lys-Ahx	101
Lys-Pro-Leu-Gly-Val-Arg-Lys-Gln-Ahx	102
Lys-Pro-Leu-Gly-Val-Arg-Orn	103
Lys-Pro-Leu-Gly-Val-Arg-Gly-D-Lys	
Orn-Pro-Leu-Gly-Val-Arg-Orn	104
Lys-Pro-Leu-Gly-Val-Arg-Lys-Cys	105
Lys-Gly-Pro-Leu-Gly-Val-Arg-Lys-Cys	106
Lys-Pro-Leu-Gly-Val-Arg-Lys-Gly-Gly-Gly	107
Lys-Gly-Pro-Leu-Gly-Val-Arg-Lys-Gly-Gly-Gly	108
Lys-Pro-Leu-Gly-Val-Arg-Lys-Arg-Arg-Arg	109
Lys-Gly-Pro-Leu-Gly-Val-Arg-Lys-Arg-Arg-Arg	110
Lys-Val-Arg-Leu-Gly-Pro-Lys	111
Lys-Gly-Val-Arg-Leu-Gly-Pro-Lys	112
Ahx-Lys-Val-Arg-Leu-Gly-Pro-Lys	113
Ahx-D-Lys-Val-Arg-Leu-Gly-Pro-D-Lys	
Lys-Val-Arg-Leu-Gly-Pro-Lys-Ahx	114

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Oligopeptide	SEQ ID NO.
Lys-Val-Arg-Leu-Gly-Pro-Orn	115
Orn-Val-Arg-Leu-Gly-Pro-Orn	116
Lys-Val-Arg-Leu-Gly-Pro-Lys-Cys	117
Lys-Gly-Val-Arg-Leu-Gly-Pro-Lys-Cys	118
Lys-Val-Arg-Leu-Gly-Pro-Lys-Gly-Gly-Gly	119
Lys-Gly-Val-Arg-Leu-Gly-Pro-Lys-Gly-Gly-Gly	120
Lys-Val-Arg-Leu-Gly-Pro-Lys-Arg-Arg-Arg	121
Lys-Val-Arg-Leu-Gly-Pro-Lys-Arg-Arg-Arg-D-Arg	
Lys-Gly-Val-Arg-Leu-Gly-Pro-Lys-Arg-Arg-Arg	122
Lys-Gly-His-Pro-Gly-Gly-Pro-Gln-Gly-Lys	123
His-Pro-Gly-Gly-Pro-Gln	124
Lys-Gly-His-Pro-Gly-Gly-Pro-Gln-Gly-Orn-Ahx	125
Gly-Lys-Gly-His-Pro-Gly-Gly-Pro-Gln-Lys	126
Lys-His-Pro-Gly-Gly-Pro-Gln-Lys	127
Lys-Gly-His-Pro-Gly-Gly-Pro-Gln-Gly-Lys-Cys	128
Lys-Gly-His-Pro-Gly-Gly-Pro-Gln-Lys-Gly-Gly	129
Lys-Gly-His-Pro-Gly-Gly-Pro-Gln-Lys-Gly-Gly-Arg-Arg	130
Lys-Gly-His-Pro-Gly-Gly-Pro-Gln-Lys-Gly-Arg-Arg-Arg	131
Lys-Gly-His-Pro-Gly-Gly-Pro-Gln-Lys-Ahx-Arg-Arg-Arg-Cys-Gly	132
Ile-His-Pro-Phe-His-Leu-Val-Ile-His	133
Lys-His-Pro-Phe-His-Leu-Val-Ile-His	134
Lys-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys	135
Lys-Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys	136
Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys	137
Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys	138
Thr-Pro-Phe-Ser-Gly-Gln	139
Glu-Pro-Phe-Trp-Glu-Asp-Gln	140
Leu-Val-Gly-Gly-Ala	141
Tyr-Pro-Gly-Gly-Pro-Gln	142
Val-Ala-Asp-Cys-Ala-Asp-Gln	143
Val-Ala-Asp-Cys-Ala-Asp-Arg-Gln	144
Val-Ala-Asp-Cys-Ala-Asp-Asp-Gln	145
Val-Ala-Asp-Cys-Arg-Asp-Gln	146
Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln	147
Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln	148
Ala-Pro-Glu-Glu-Ile-Met-Pro-Arg-Gln	149
Gly-Phe-Leu-Gly	150
Gly-Leu-Phe-Gly	151
Glu-Gly-Phe-Leu-Gly	152
Glu-Lys-Gly-Phe-Leu-Gly-Lys	153
Arg-Arg-Glu-Lys-Gly-Phe-Leu-Gly-Lys	154
Arg-Gly-Leu-Gly-Lys	155
Gly-Gly-Arg-Arg	156
Gly-Gly	
Gly-Phe-Cha-Gly	157

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Oligopeptide	SEQ ID NO.
Arg-Leu-Val-Gly-Phe-Asp	158
Arg-Gly- Phe-Phe-Leu	159
Arg-Gly-Phe-Phe-Pro	160
Ala-Phe-Leu-Gly	161
Phe-Pro-Ala-Met	162
Glu-Ala-Ala-Ala	163
Gly-Gly-Arg	
Gly-Arg	
Phe-Arg	
Glu-Lys-Arg-Arg-Lys	164
Succinyl-Glu-Lys-Arg-Arg-Lys	165
Val-Lys-Lys-Arg	166
Ala-Pro	
Ala-Ala-Lys His-Gly-Pro-Asn	167
His-Gly-Pro-Arg	168
Gly-Pro-Arg	
Gly-Pro-Arg-Lys	169
Gly-Pro-Asn	
Pro-Ala-Gly-Pro	170
Asn-Gly-Pro-Asn-Lys	171
His-Gly-Pro-Ile	172
His-Gly-Hyp-Asn	173
His-Gly-Pro-Cit	174
His-Gly-hPro-Asn Pro-Leu-Gly-Val-Arg	175
Gly-Pro-Leu-Gly-Val-Arg	176
Gly-Pro-Leu-Gly-Val-Arg-Glu	177
Gly-Pro-Leu-Gly-Val-Arg-Asp	178
Gly-Pro-Leu-Gly-Met-Arg	179
Pro-Leu-Gly-Glu-Arg-Gly	180
Pro-Leu-Gly-Leu-Ala-Gly	181
Gly-Val-Arg-Leu-Gly-Pro-Lys	182
Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln	183
Val-Pro-Met-Ser-Met-Arg-Gly-Gly	184
Ile-Pro-Val-Ser-Leu-Arg-Ser-Gly	185
Arg-Pro-Phe-Ser-Met-Ile-Met-Gly	186
Val-Pro-Leu-Ser-Leu-Thr-Met-Gly	187
Val-Pro-Leu-Ser-Leu-Tyr-Ser-Gly	188
Ile-Pro-Glu-Ser-Leu-Arg-Ala-Gly	189
Lys-His-Pro-Phe-His-Leu-Val-Ile-His-D-Lys	
Lys(COR)-His-Pro-Phe-His-Leu-Val-Ile-His -Lys; R = Me	190
Lys(COR)-His-Pro-Phe-His-Leu-Val-Ile-His -Lys; R = CF ₃	191
Lys(COR)-His-Pro-Phe-His-Leu-Val-Ile-His -Lys; R = Et	192
Lys(COR)-His-Pro-Phe-His-Leu-Val-Ile-His -Lys; R = mPEG20k	193
[R'CO] Lys-His-Pro-Phe-His-Leu-Val-Ile-His -Lys; R' = Me	194
[R'CO] Lys-His-Pro-Phe-His-Leu-Val-Ile-His -Lys; R' = carboxyethyl	195

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Oligopeptide	SEQ ID NO.
Lys-His-Pro-Phe-His-Leu-Leu-Tyr-His-Lys	196
Ile-His-Pro-Phe-His-Leu-Leu-Tyr-His-Lys	197
Lys-His-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Lys	198
Ile-His-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Lys	199
Lys-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Lys	200
Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Lys	201
Lys-His-Pro-Tyr(R)-His-Leu-Val-Ile-His-Lys; R = Me	202
Lys-His-Pro-Tyr(R)-His-Leu-Val-Ile-His-Lys; R = Et	203
Ile-His-Pro-Tyr(R)-His-Leu-Val-Ile-His-Lys; R = Me	204
Ile-His-Pro-Tyr(R)-His-Leu-Val-Ile-His-Lys; R = Et	205
Orn-His-Pro-Phe-His-Leu-Val-Ile-His-Lys	206
Dap-His-Pro-Phe-His-Leu-Val-Ile-His-Lys	207
Ahx-His-Pro-Phe-His-Leu-Val-Ile-His-Lys	208
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys	209
Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys	210
Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys	211
Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys	212
Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys	213
Pro-Leu-Ala-Gln-Ala-Val-Lys-Arg-Ser-Ser-Ser-Arg	214
Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Orn	215
Pro-Leu-Ala-Gln-Ala-Val-Orn-Arg-Ser-Ser-Ser-Arg	216
Ser-Pro-Leu-Ala-Asn-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys	217
Pro-Leu-Ala-Asn-Ala-Val-Lys-Arg-Ser-Ser-Ser-Arg	218
Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-Lys	219
Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-Lys	220
Ala-Pro-Glu-Glu-Ile-Met-Asp-Gln-Gln-Lys	221
Ile-Ser-Leu-Met-Lys-Arg-Pro-Pro-Gly-Phe	222
Gly-Lys-Asp-Glu-Val-Asp	223
Lys-His-Pro-Phe-His-Cha-Val-Ile-His-Lys	451
Lys(COR)-His-Pro-Phe-His-Cha-Val-Ile-His-Lys; R = Me, CF ₃	452

[00129] Other exemplary enzymatically cleavable oligopeptides include a Cys-S-S-Cys moiety.

[00130] In one embodiment of the present invention, the enzymatically cleavable oligopeptide can be a enzymatically cleavable cyclic peptide, which may for example, include about 4 to about 30 amino acids. In one embodiment of the present invention, the agent can include 2-5 cyclic peptides. In another embodiment of the present invention, the cyclic peptide may include an acyclic component.

[00131] Cyclic peptides can be prepared by methods known in the art, for example, by

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the linking the N-terminus of a linear peptide with the C-terminus of the linear peptide:
head-to-tail cyclization. Such linkages can be via a bond, for example, an amide bond or
through a linker. In one embodiment, cyclic peptides can be prepared by the linking the
N-terminus of a linear peptide with the side chain carboxylic acid of a linear peptide, by
5 the linking the side chain amine of a linear peptide with the C-terminus carboxylic acid of
a linear peptide, or by the linking the side chain amine of a linear peptide with the side
chain carboxylic acid of a linear peptide. For example, cyclic peptides can be prepared by
the linking the side chain thiol group of a linear peptide with the side chain thiol group of
a linear peptide; for example by forming a Cys-Cys disulfide bond. Cyclic peptides can
10 be made in solution phase or on solid phase and that cyclization of linear peptides can be
achieved in solution phase or on solid phase.

[00132] Cyclic peptides are described using conventional nomenclature. For example a
head to tail cyclic peptide containing Arg, Gly, Asp, Phe, Lys can be written as cyclo(Arg-
Gly-Asp-Phe-Lys)(SEQ ID NO:224); a cyclic peptide containing Cys, Arg, Gly, Asp, Cys
15 can be shown as follows: head to tail: cyclo(Cys-Arg-Gly-Asp-Cys)(SEQ ID NO:225);
disulphide: Cys-Arg-Gly-Asp-Cys (SEQ ID NO:225).

[00133] In certain embodiments, the enzymatically cleavable cyclic oligopeptide can
include oligo-L-arginine, oligo-L-lysine, oligo-L-aspartic acid or oligo-L-glutamic acid.
In certain embodiments the enzymatically cleavable cyclic oligopeptide includes lysine
20 and arginine. In another embodiment of the present invention, the enzymatically cleavable
cyclic oligopeptide includes lysine, arginine and phenylalanine. In one embodiment, an
enzymatically cleavable cyclic oligopeptide includes lysine, phenylalanine and glycine, or
includes lysine, phenylalanine, leucine, and glycine, or includes ornithine, phenylalanine,
leucine, and glycine. In another embodiment, the enzymatically cleavable cyclic
25 oligopeptide comprises diaminopropionic acid, ornithine, phenylalanine, leucine, and
glycine.

[00134] In one embodiment of the present invention, the enzymatically cleavable cyclic
oligopeptide may include one or more enzymatically cleavable oligopeptide units.

[00135] Exemplary enzymatically cleavable cyclic oligopeptides include those shown in
30 Table 5.

TABLE 5

Cyclic Oligopeptide	SEQ ID NO.
cyclo(Lys-Arg-Arg-Lys-Arg-Arg)	226
cyclo(D-Lys-Arg-Arg-Lys-Arg-Arg)	
cyclo(Lys-Arg-Arg-Arg-Lys-Arg-Arg)	227
cyclo(D-Phe-Lys-Arg-Arg-Lys-Arg-Arg-Gly)	
cyclo(Phe-Lys-Arg-Arg-Phe-Lys-Arg-Arg)	228
cyclo(D-Phe-Lys-Arg-Arg-D-PheLys-Arg-Arg)	
cyclo(Cys-Lys-Arg-Arg-Cys-Lys-Arg-Arg)	229
cyclo(Phe-Lys-Arg-Arg-Phe-Lys-Arg-Arg-D-Lys)	
cyclo(Phe-Lys-Arg-Arg-Phe-Lys-Arg-Arg-Gly-Gly-Gly)	230
cyclo(Lys-His-Gly-Pro-Asn-Lys-His-Gly-Pro-Asn-Gly)	231
cyclo(D-Lys-His-Gly-Pro-Asn-D-Lys-His-Gly-Pro-Asn-Gly)	
cyclo(Ahx-Lys-Arg-Arg-Lys-Arg-Arg)	232
cyclo(Lys-Arg-Arg-Lys-Lys-Arg-Arg-Lys)	233
cyclo(Gly-Lys-Arg-Arg-Lys-Lys-Arg-Arg-Lys-Gly)	234
cyclo(Gly-D-Lys-Arg-Arg-Lys-D-Lys-Arg-Arg-Lys-Gly)	

5 [00136] Such cyclopeptides may include peptide structures wherein one or two proteolytic events may be required for activation of the agent.

[00137] The enzymatically cleavable oligopeptide is cleavable by at least one enzyme chosen from hydrolases, elastases, cathepsins, matrix metalloproteases, peptidases, exopeptidases, endopeptidases, carboxypeptidases, glycosidases, lipases, nucleases, lyases, amylases, phospholipases, phosphatases, phosphodiesterases, sulfatases, serine
10 proteases, subtilisin, chymotrypsin, trypsin, threonine proteases, cysteine proteases, calpains, papains, caspases, aspartic acid proteases, pepsins, chymosins, glutamic acid proteases, renin, reductases, and parasitic, viral and bacterial enzymes.

VI. Exemplary Imaging Agents

15 [00138] Useful imaging agents can be created using one or more of the enzymatically cleavable oligopeptides, fluorophores, quenchers (if appropriate), biological modifiers and non-fluorescent reporters described hereinabove and coupled together using standard chemistries known in the art. The imaging agents can be water soluble or water dispersible (i.e., sufficiently soluble or suspendable in aqueous or physiological media

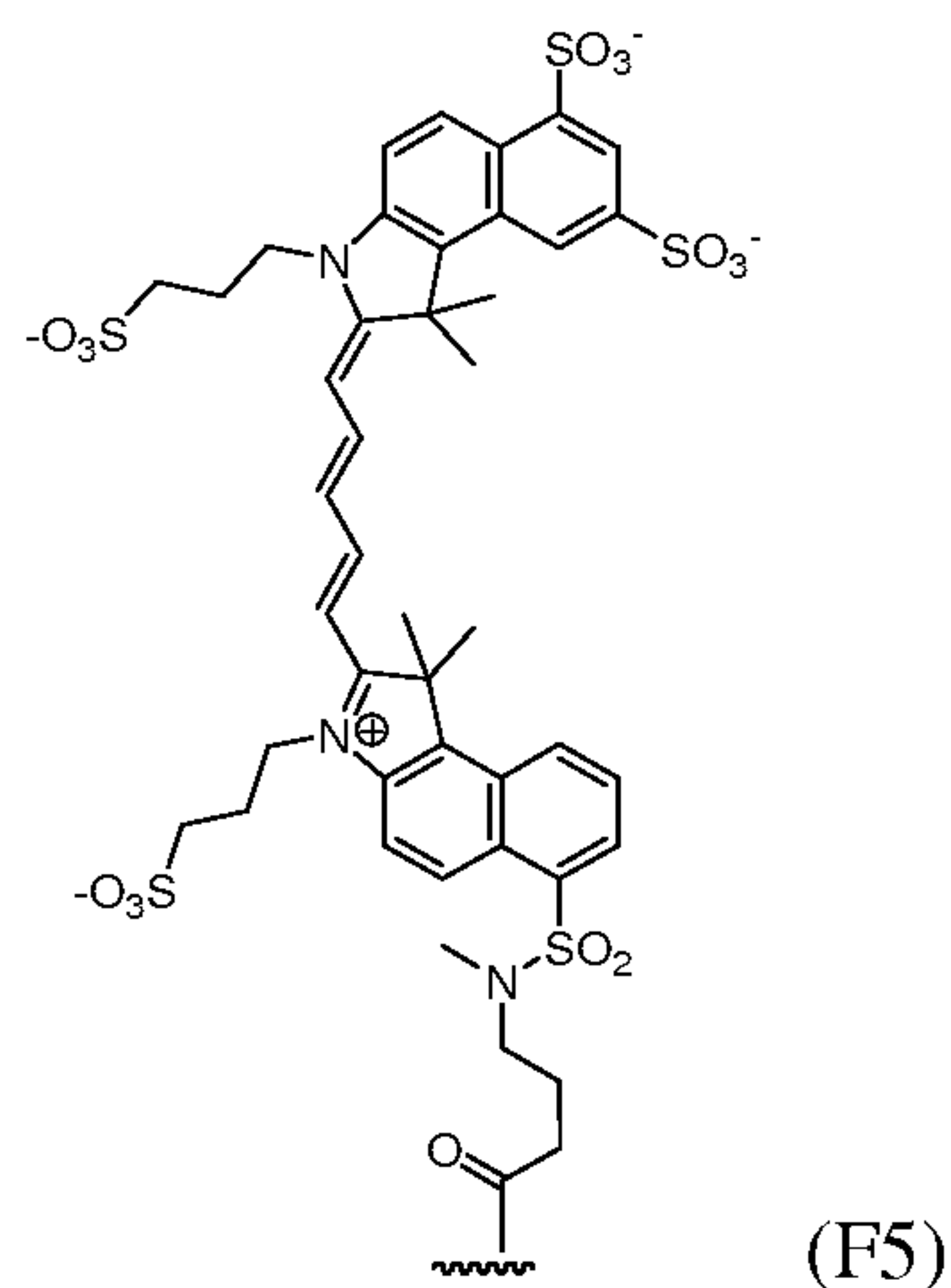
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solutions). The *in vivo* half-life of the agent can be designed to be at least about 10 minutes, but more preferably 30 minutes to several hours. The *in vivo* half-life of the agent preferably is a time (for example, at least about 30 minutes) sufficient to achieve good tissue exposure, target binding, and imaging signal. In a preferred embodiment, the agent imaging probe is water soluble or dispersible in aqueous media, and is biocompatible i.e., non-toxic having, for example, an LD₅₀ of greater than about 50 mg/kg body weight. The imaging agents also preferably do not have any undesired phototoxic properties and/or display low serum protein binding affinity.

[00139] In certain embodiments, the disclosed agents include peptide or linker moieties capable of releasing one, two or more fluorophores from the oligopeptide and/or biological modifier upon contact with an enzyme. Such imaging agents can include a biological modifier that can be released from a cleavable oligopeptide by an enzyme that is different from the one that cleaves the peptide.

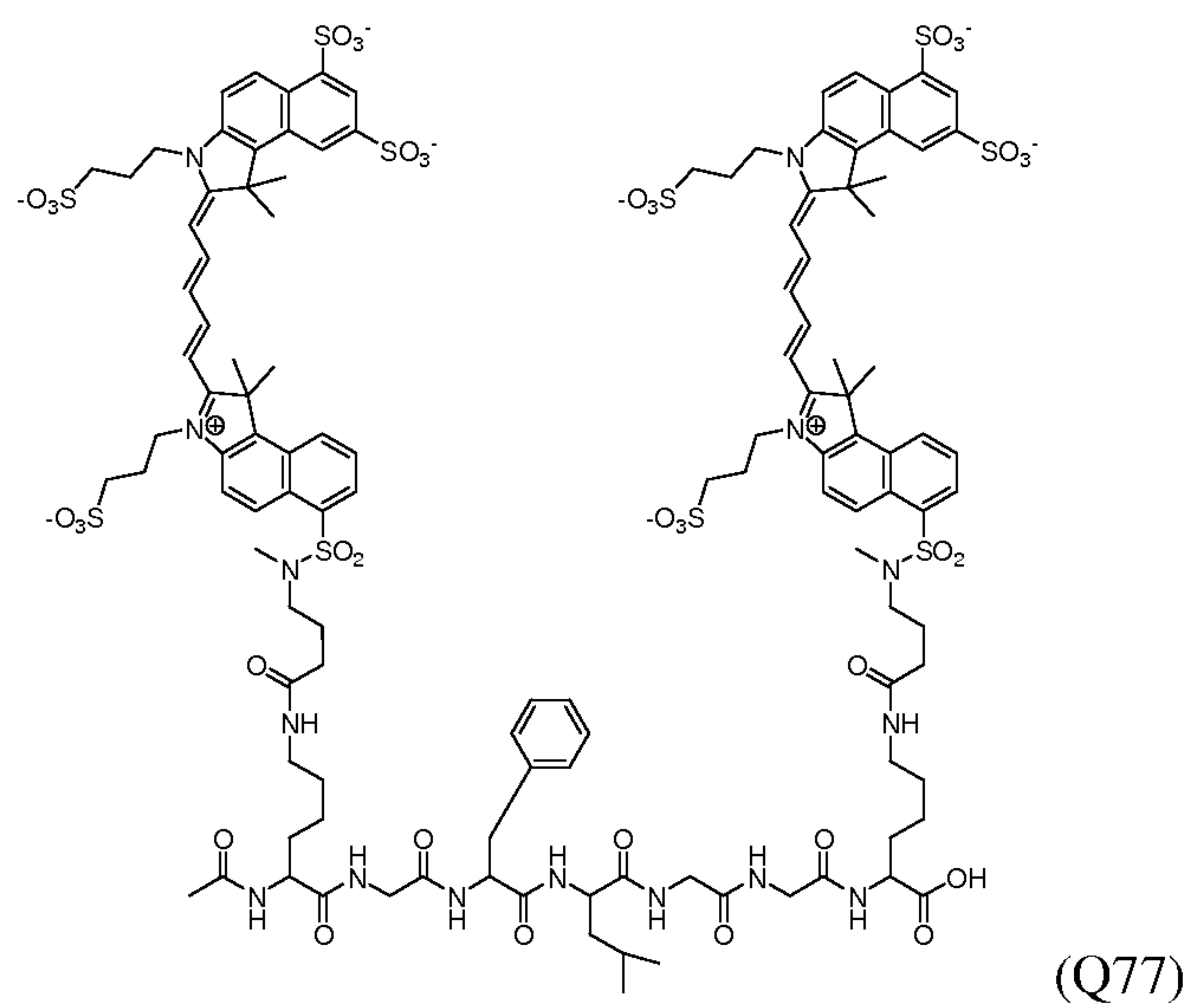
[00140] Certain preferred imaging agents are disclosed in the sections that follow.

[00141] One exemplary imaging agent represented by Formula Q77 includes [Ac]-Lys(F5)-Gly-Phe-Leu-Gly-Gly-Lys(F5)-[OH] (SEQ ID NO:235), wherein Ac is an acetyl group and F5 is represented by:

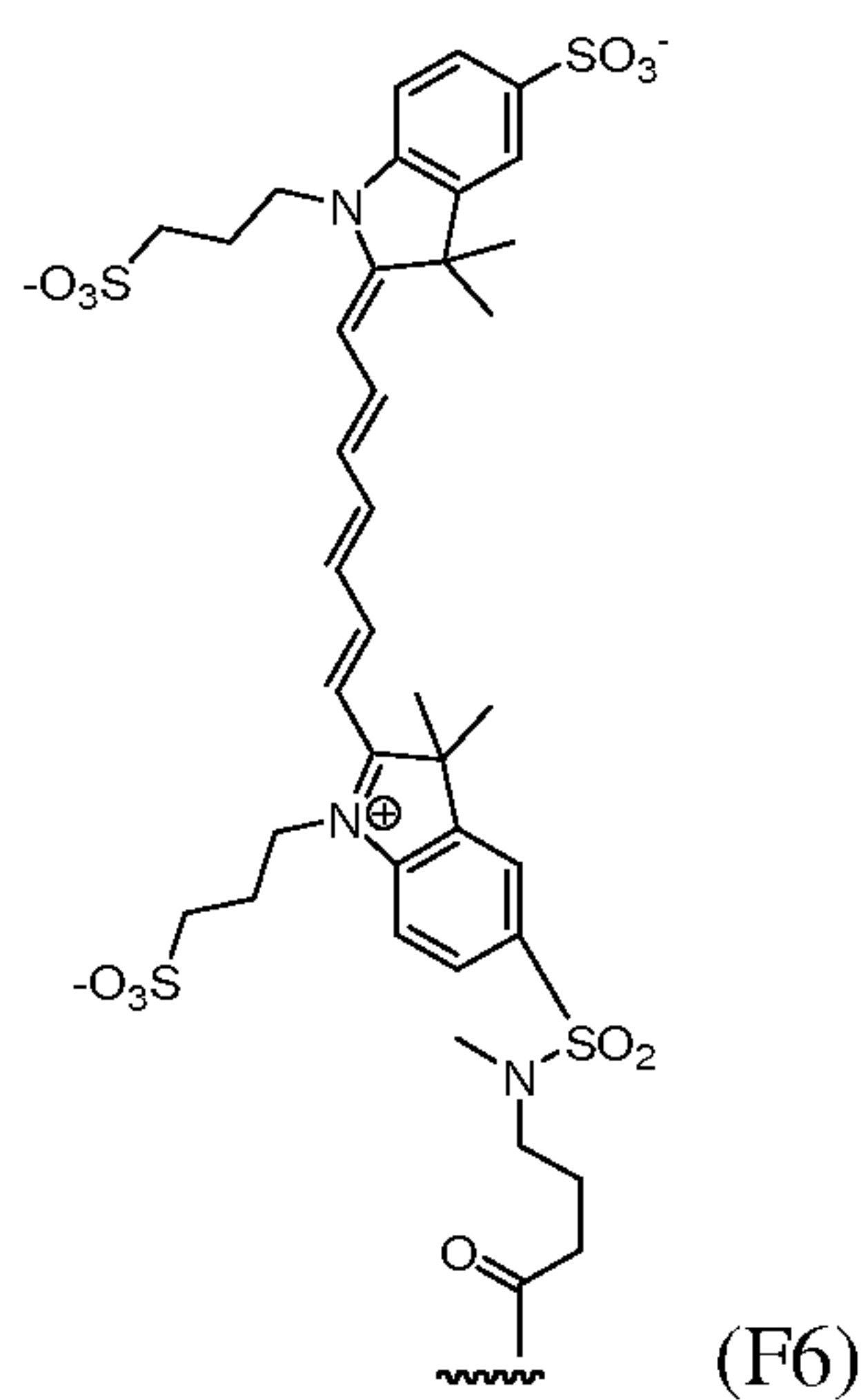


[00142] One exemplary imaging agent represented by Formula Q77 includes

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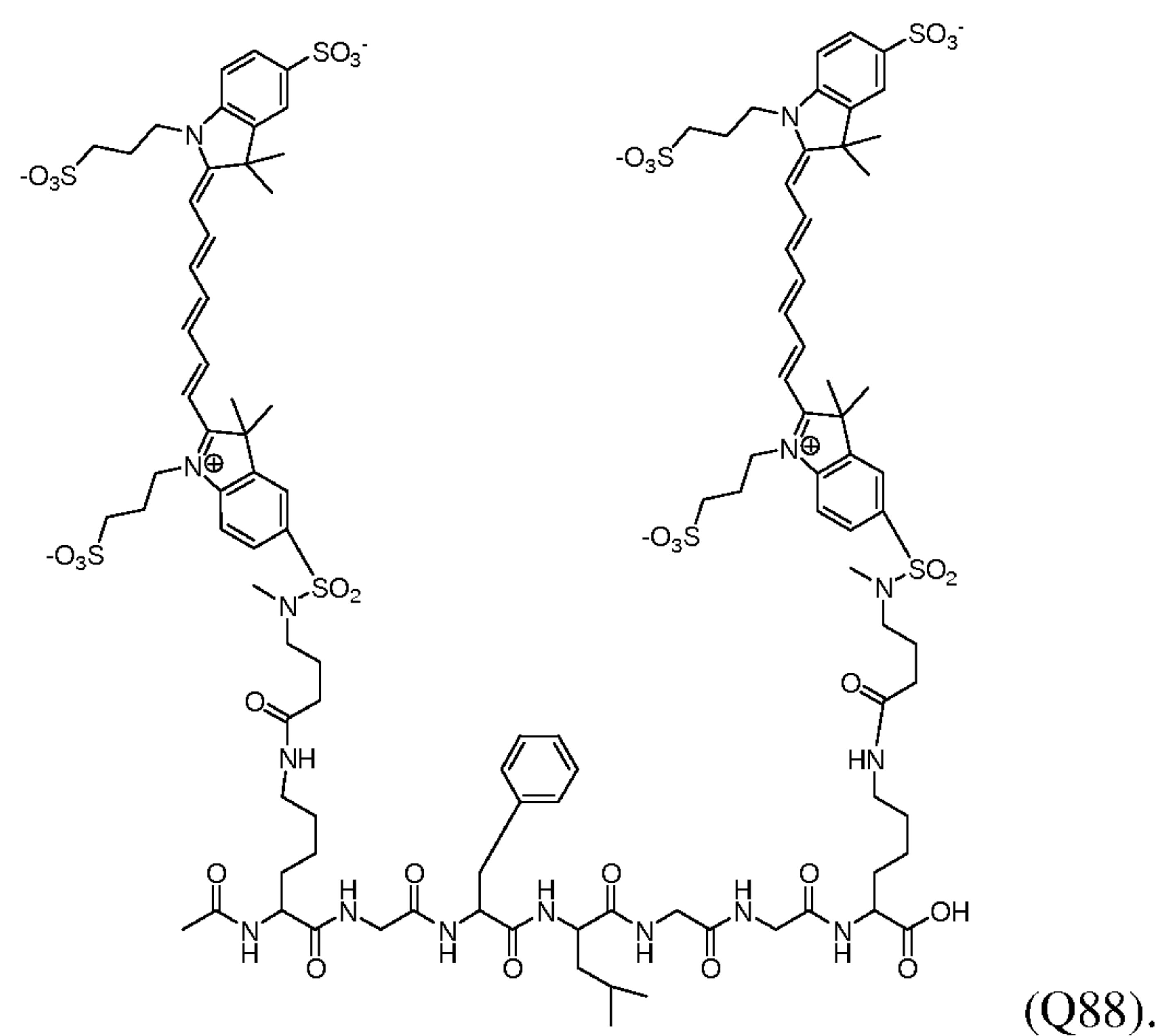
[00143] One exemplary imaging agent represented by Formula Q88 includes [Ac]-Lys(F6)-Gly-Phe-Leu-Gly-Gly-Lys(F6)-[OH] (SEQ ID NO:236), wherein Ac is an acetyl group and F6 is



5

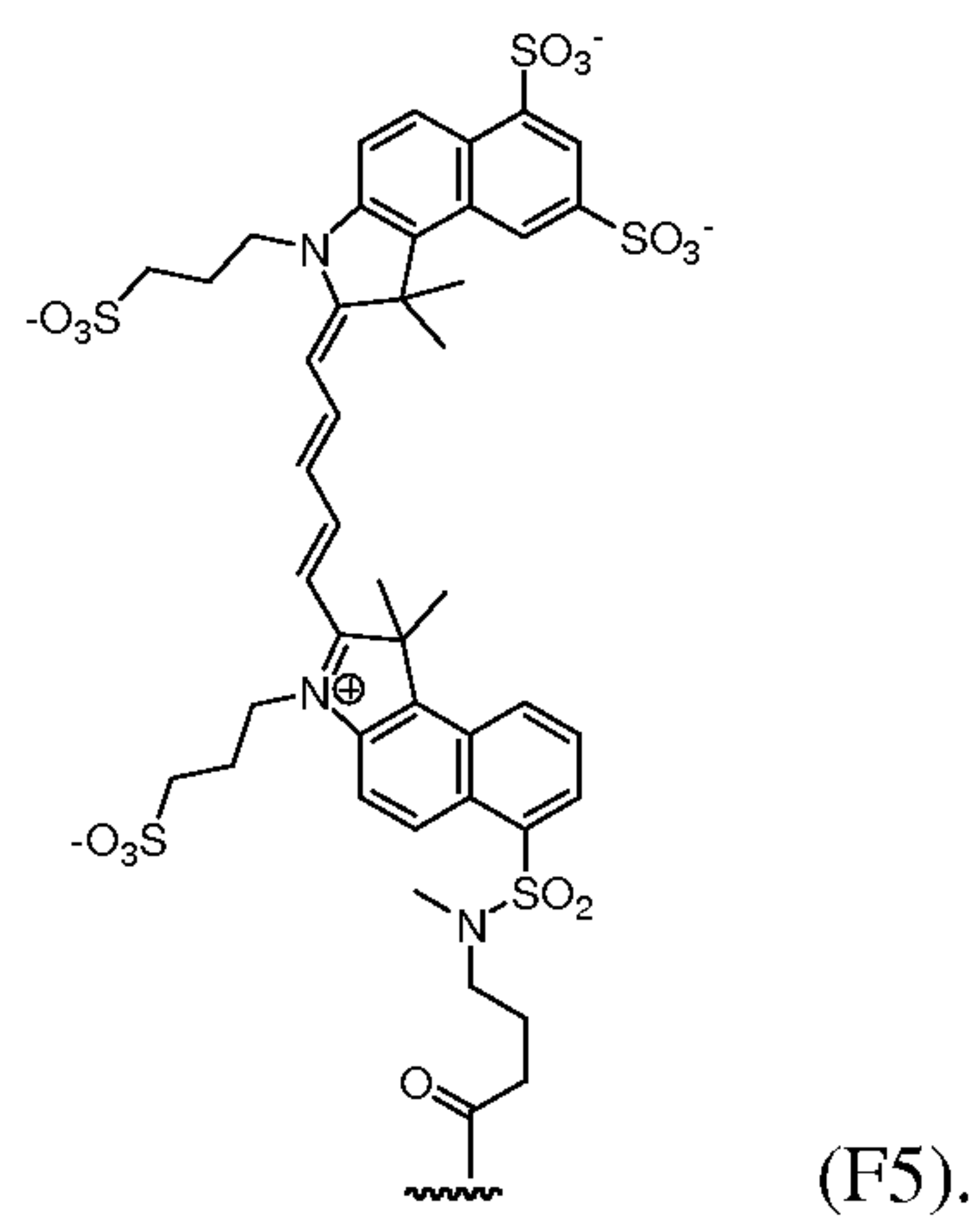
[00144] One exemplary imaging agent represented by Formula Q88 includes

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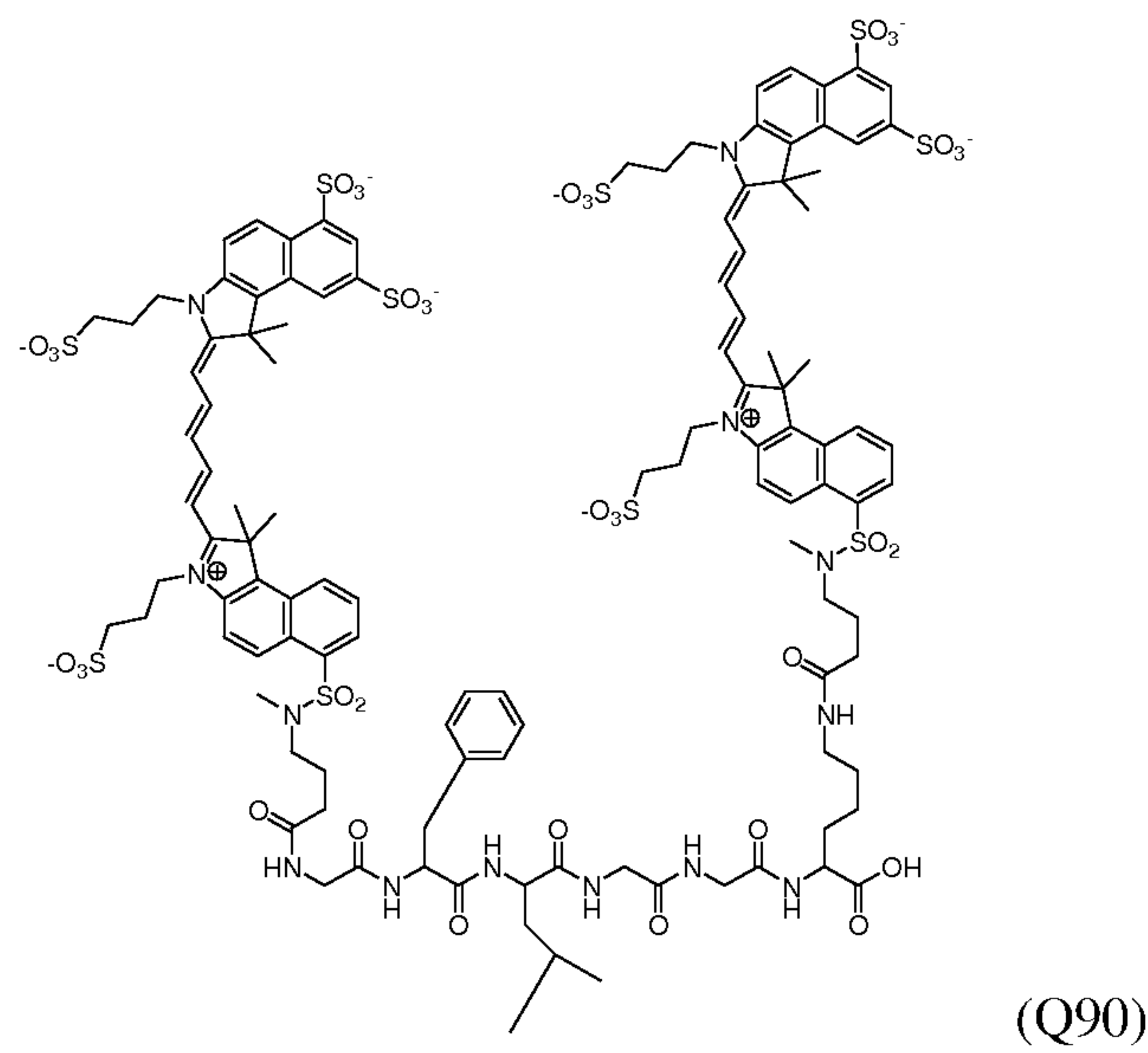
[00145] An exemplary imaging agent represented by Q90 includes

[F5]-Gly-Phe-Leu-Gly-Gly-Lys(F5)-[OH] (SEQ ID NO:237), wherein F5 is



5 [00146] An exemplary imaging agent represented by the Formula Q90 can be depicted as:

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[00147] Other exemplary imaging agents can include moieties set forth in Table 6.

TABLE 6

Imaging Agent	SEQ ID NO.
[Acetyl]-Lys(F5)-Lys-Lys-Lys(F5)-Lys-Lys-Gly-[OH]	238
[Acetyl]-Lys(F5)-Lys-Lys-Lys(F5)-Gly-[OH]	239
[F5]-His-Gly-Pro-Asn-Lys(F5)-[OH]	240
[F6]-Gly-Phe-Leu-Gly-Lys(F6)-[OH]	241
[F5]-His-Gly-Pro-Arg-Lys(F5)-[OH]	242
[F5]-His-Gly-Pro-Asn-Lys(F5)-His-Gly-Pro-Asn-βA-[OH]	243
[pentynoyl]-Lys(F5)-His-Pro-Gly-Gly-Pro-Gln-Lys(F5)-[OH]	244
[pentynoyl]-Lys(F5)-Gly-His-Pro-Gly-Gly-Pro-Gln-Gly-Lys(F5)-[OH]	245
[pentynoyl]-Lys(F5)-Val-Arg-Leu-Gly-Pro-Lys(F5)-[OH]	246
[pentynoyl]-Lys(F5)-Pro-Leu-Gly-Val-Arg-Lys(F5)-[OH]	247
[pentynoyl]-Phe-Gly-Lys(F5)-Arg-Arg-Lys(F5)-[OH]	248
[pentynoyl]-Phe-Arg-Lys(F5)-Gly-Gly-Arg-Lys(F5)-[OH]	249
[F5]-Gly-Phe-Leu-Gly-Lys(F5)-[OH]	250
Acetyl-Phe-Gly-Lys(F5)-Arg-Arg-Lys(F5)-Gly-[OH]	251
Acetyl-Phe-Arg-Lys(F5)-Gly-Gly-Arg-Lys(F5)-[OH]	252
cyclo(Lys(F5)-Arg-Arg-Arg-Lys(F5)-Arg-Arg)	253
cyclo(Phe-Lys(F6)-Arg-Arg-Phe-Lys(F6)-Arg-Arg-Gly-Gly-Gly)	254
cyclo(Ahx-Lys(F6)-Arg-Arg-Lys(F6)-Arg-Arg)	255
[Acetyl]-Lys(F5)-Lys-Lys-Lys(F5)-Lys-Lys-Gly-[mPEG20K]	256
[Acetyl]-Lys(F5)-Lys-Lys-Lys(F5)-Gly-[mPEG20K]	257
[F5]-His-Gly-Pro-Asn-Lys(F5)-[mPEG20K]	258
[F6]-Gly-Phe-Leu-Gly-Lys(F6)-[mPEG20K]	259
[F5]-His-Gly-Pro-Arg-Lys(F5)-[mPEG20K]	260
[F5]-His-Gly-Pro-Asn-Lys(F5)-His-Gly-Pro-Asn-βA-[mPEG20K]	261

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[F5]-His-Gly-Pro-Asn-Lys(F5)-[dPEG-1.1k]	262
[F5]-His-Gly-Pro-Asn-Lys(F5)-[mPEG-5k]	263
[F5]-His-Gly-Pro-Asn-Lys(F5)-[mPEG-10k]	264
[pentynoyl]-Lys(F5)-His-Pro-Gly-Gly-Pro-Gln-Lys(F5)-[mPEG20K]	265
[pentynoyl]-Lys(F5)-Gly-His-Pro-Gly-Gly-Pro-Gln-Gly-Lys(F5)-[mPEG20K]	266
[pentynoyl]-Lys(F5)-Val-Arg-Leu-Gly-Pro-Lys(F5)-[mPEG20K]	267
[pentynoyl]-Lys(F5)-Pro-Leu-Gly-Val-Arg-Lys(F5)-[mPEG20K]	268
[pentynoyl]-Phe-Gly-Lys(F5)-Arg-Arg-Lys(F5)-[mPEG20K]	269
[pentynoyl]-Phe-Arg-Lys(F5)-Gly-Gly-Arg-Lys(F5)-[mPEG20K]	270
[F5]-Gly-Phe-Leu-Gly-Lys(F5)-OH	271
[succinyl]-Lys(F5)-Gly-Phe-Leu-Gly-Lys(F5)-[NH ₂]	272
[mPEG-20k] (F6)-Gly-Phe-Leu-Gly-Lys(F6)-[NH ₂]	273
[mPEG-40k]-(F5)-Gly-Phe-Leu-Gly-Lys(F5)-[NH ₂]	274
[diphenylpropylamine]-(F6)-Gly-Phe-Leu-Gly-Lys(F6)-[NH ₂]	275
[dPEG-1.1k]-(F6)-Gly-Phe-Leu-Gly-Lys(F6)-[NH ₂]	276
[succinyl]-Lys(F6)-Arg-Arg-Lys(F6)-[NH ₂]	277
[mPEG-20k-succinyl]-Lys(F6)-Arg-Arg-Lys(F6)-[NH ₂]	278
[diphenylpropylamine-succinyl]-Lys(F6)-Arg-Arg-Lys(F6)-[NH ₂]	279
[dPEG-1.1k-succinyl]-Lys(F6)-Arg-Arg-Lys(F6)-[NH ₂]	280
[succinyl]-Lys(F6)-Ala-Arg-Arg-Lys(F6)-[NH ₂]	281
[mPEG-20k]-Lys(F6)-Ala-Arg-Arg-Lys(F6)-[NH ₂]	282
[succinyl]-Lys(F6)-Arg-Arg-Arg-Lys(F6)-[NH ₂]	283
[mPEG-20k-succinyl]-Lys(F6)-Ala-Arg-Arg-Lys(F6)-[NH ₂]	284
[succinyl]-Lys(F5)-Lys-Lys-Lys(F5)-[NH ₂]	285
[mPEG-20k]-Lys(F5)-Lys-Lys-Lys(F5)-[NH ₂]	286
[palmitoyl]-Lys(F5)-Phe-Arg-Lys(F5)-[NH ₂]	287
[Ac]-Lys(F5)-Lys-Lys-Lys(F5)-Gly-[Iron Oxide Nanoparticle]	288
[F6]-Gly-Pro-Leu-Gly-Val-Arg-Lys(F6)-[mPEG-20k]	289
[F6]-Gly-Pro-Leu-Gly-Val-Arg-Lys(F6)-[mPEG-40k]	290
[F6]-Gly-Pro-Leu-Gly-Val-Arg-Lys(F6)-[Y-PEG-40k]	291
[F6]-Gly-Pro-Leu-Gly-Val-Arg-Lys(F6)-[eda]	292
[F6]-Gly-Pro-Leu-Gly-Val-Arg-Lys(F6)-[PVP-6k]	293
[F6]-Gly-Pro-Leu-Gly-Val-Arg-Lys(F6)-[Dextran-10k]	294
[F6]-Gly-Pro-Leu-Gly-Val-Arg-Lys(F6)-Glu(mPEG-20k)-[mPEG-20k]	295
Ac-Phe-Gly-Lys(F5)-Arg-Arg-Lys(F5)-Gly-[mPEG20K]	296
Ac-Phe-Arg-Lys(F5)-Gly-Gly-Arg-Lys(F5)-[mPEG20K]	297
[F5]Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[OH]	298
[F5]Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = H	299
[F5]Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = Me	300
[F5]Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = Et	301
[F5]Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = mPEG20k	302
[F5]Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = mPEG10k	303
[F5]Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = mPEG5k	304

[F5]Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = dPEG24	305
[F5]Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[OH]	306
[F5]Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = H	307
[F5]Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = Me	308
[F5]Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = Et	309
[F5]Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = mPEG20k	310
[F5]Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = mPEG10k	311
[F5]Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = mPEG5k	312
[F5]Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R = dPEG24	313
[F5]Lys (COR)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[OH]; R= Me	314
[F5]Lys (COR)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[OH]; R= CF ₃	315
[F5]Lys (COMe)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R= mPEG20k	316
[F5]Lys (COCF ₃)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHR]; R= mPEG20k	317
[RCO]Lys (F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NH ₂]; R= carboxyethyl	318
[RCO]Lys (F5)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NH ₂]; R= carboxyethyl	319
[RCO]Lys (F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NH ₂]; R= Me	320
[RCO]Lys (F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NH ₂]; R= mPEG5k	321
[RCO]Lys (F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NH ₂]; R= mPEG20k	322
[RCO]Lys (F5)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NH ₂]; R= Me	323
[RCO]Lys (F5)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NH ₂]; R= mPEG5k	324
[RCO]Lys (F5)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NH ₂]; R= mPEG20k	325
[F5]Orn-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[OH]	326
Orn(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[OH]	327
[F5]Orn-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = H	328
[F5]Orn-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = Me	329
[F5]Orn-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG5k	330
[F5]Orn-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG20k	331
Orn(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR] R = H	332
Orn(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR] R = Me	333
Orn(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR] R = mPEG5k	334
Orn(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR] R = mPEG20k	335
[F5]Dap-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[OH]	336
Dap(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[OH]	337
[F5]Dap-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = H	338
[F5]Dap-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = Me	339
[F5]Dap-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG5k	340
[F5]Dap-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R =	341

mPEG20k	
Dap(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR] R = H	342
Dap(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR] R = Me	343
Dap(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR] R = mPEG5k	344
Dap(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR] R = mPEG20k	345
[F5]Ahx-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[OH]	346
Ahx(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[OH]	347
[F5]Ahx-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = H	348
[F5]Ahx-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = Me	349
[F5]Ahx-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG5k	350
[F5]Ahx-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG20k	351
Ahx(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = H	352
Ahx(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = Me	353
Ahx(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG5k	354
Ahx(F5)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG20k	355
[F5]Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[OH]	356
[F5]Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = H	357
[F5]Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = Me	358
[F5]Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG5k	359
[F5]Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG20k	360
[F5]Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[OH]	361
[F5]Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = H	362
[F5]Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = Me	363
[F5]Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG5k	364
[F5]Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG20k	365
[F5]Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[OH]	366
[F5]Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = H	367
[F5]Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = Me	368
[F5]Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG5k	369
[F5]Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG20k	370
[F5]Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[OH]	371
[F5]Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = H	372
[F5]Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = Me	373

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[F5]Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG5k	374
[F5]Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)[NHR]; R = mPEG20k	375
[F5]Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys(F5)	376
[F5]Pro-Leu-Ala-Gln-Ala-Val-Lys(F5)-Arg-Ser-Ser-Ser-Arg	377
[F5]Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Orn(F5)	378
[F5]Pro-Leu-Ala-Gln-Ala-Val-Orn(F5)-Arg-Ser-Ser-Ser-Arg	379
[F5]Ser-Pro-Leu-Ala-Asn-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys(F5)	380
[F5]Pro-Leu-Ala-Asn-Ala-Val-Lys(F5)-Arg-Ser-Ser-Ser-Arg	381
[F5]Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Gln-Lys(F5)	382
[F5]Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-Lys	383
[F5]Ala-Pro-Glu-Glu-Ile-Met-Asp-Gln-Gln-Lys	384
[F5]Lys-His-Pro-Phe-His-Cha-Val-Ile-His-Lys(F5)[OH]	453
[F5]Lys-His-Pro-Phe-His-Cha-Val-Ile-His-Lys(F5)[NHR]; R = mPEG5k	454
[F5]Lys-His-Pro-Phe-His-Cha-Val-Ile-His-Lys(F5)[NHR]; R = mPEG20k	455
[F5]Lys(COR)-His-Pro-Phe-His-Cha-Val-Ile-His-Lys(F5)[OH]; R = Me	456
[F5]Lys(COR)-His-Pro-Phe-His-Cha-Val-Ile-His-Lys(F5)[OH]; R = CF ₃	457

[00148] As used in Table 6, F5 is the fluorochrome as depicted above in agent Q77, F6 is the fluorochrome as depicted above in agent Q88, MPEG is methoxypolyethylene glycol of a specified molecular weight (for example, mPEG20K is a 20 kDa

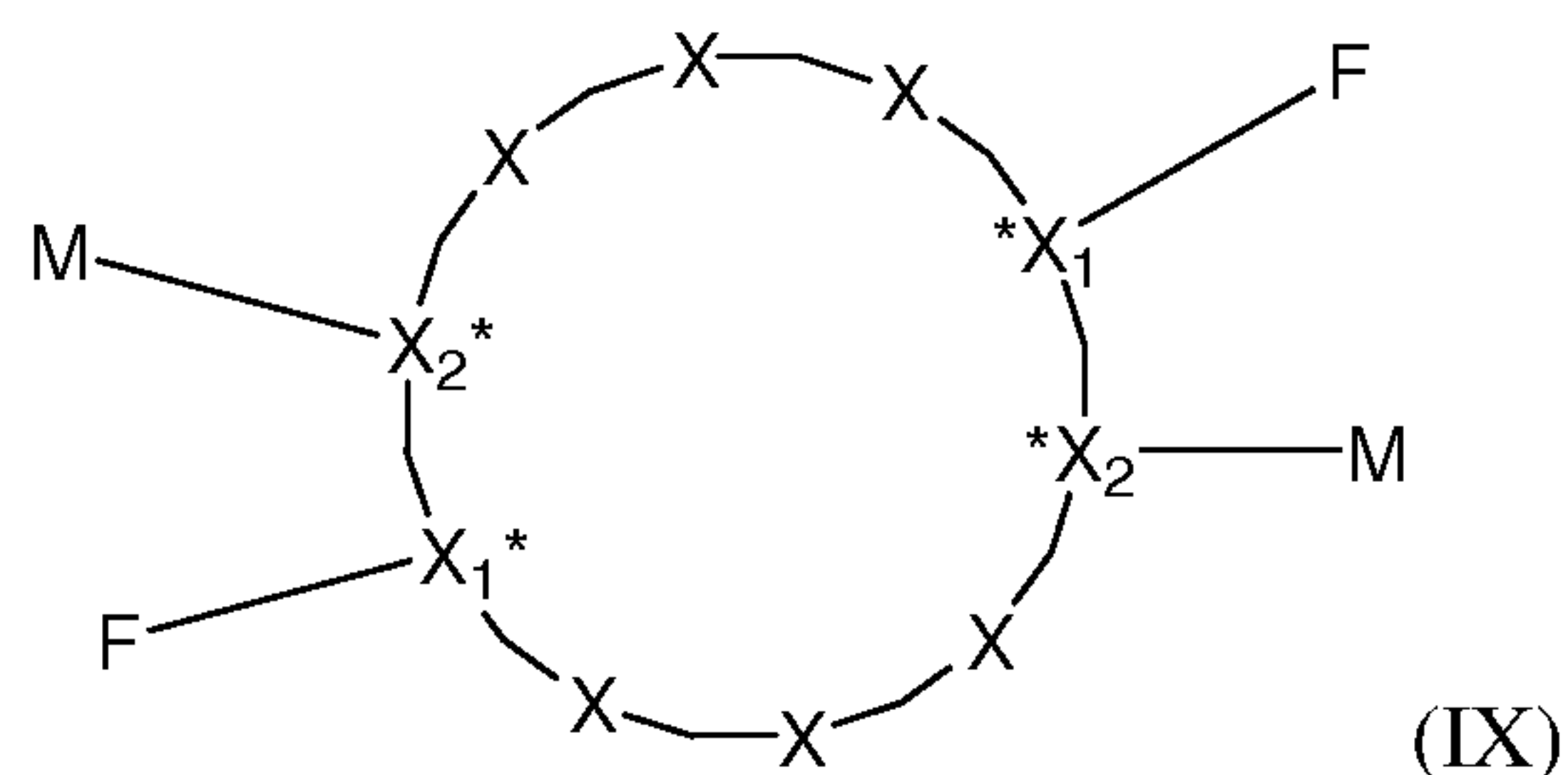
5 methoxypolyethylene glycol). Ahx is aminohexanoic acid, Orn is ornithine, and DAP is 2,3-diaminopropionic acid.

[00149] Figure 10 is a schematic representation of an exemplary cyclic imaging agent. The cyclic imaging agent comprises two peptides connected at each end to a fluorophore. A first peptide has a first fluorophore attached in the vicinity of its N-terminus and a second
10 fluorophore attached in the vicinity of its C-terminus, and a second oligopeptide has the first fluorophore attached in the vicinity of its C-terminus and the second fluorophore attached in the vicinity of its N-terminus. When intact, the fluorophore quenches the other fluorophore, and more preferably each fluorophore quenches the other fluorophore. It is understood, however, that one of the fluorophores could be replaced with a suitable non
15 fluorescent quencher. Although both peptides, as shown, contain a proteolytic cleavage site, it is understood that, under certain circumstances, only one of the two peptides may have a proteolytic cleavage site. Upon cleavage of the proteolytic cleavage site(s), the fluorophores are no longer constrained in fluorescent quenching proximity to one another and the fluorophores can now fluoresce upon excitation following exposure to light of

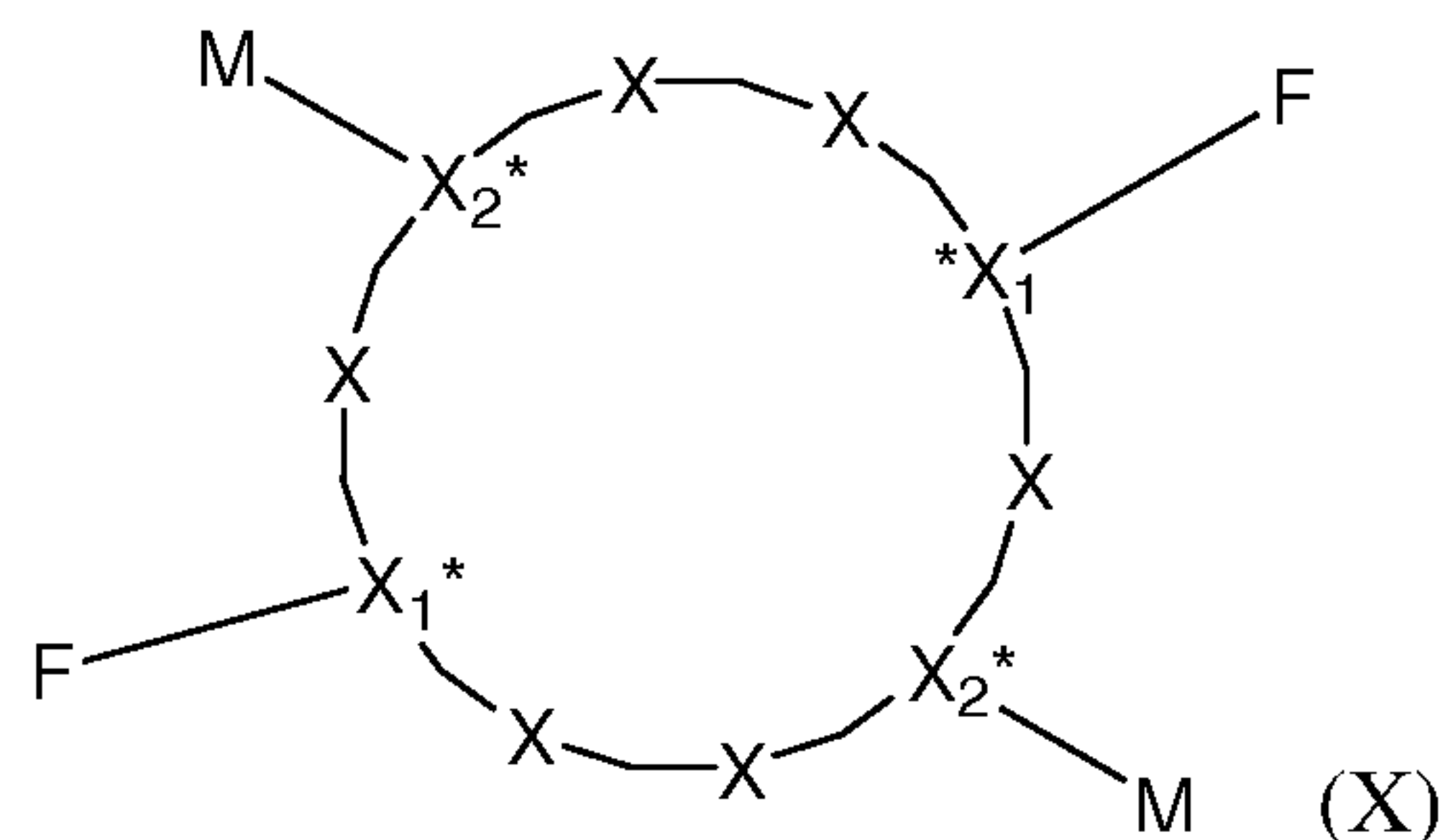
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an appropriate wavelength.

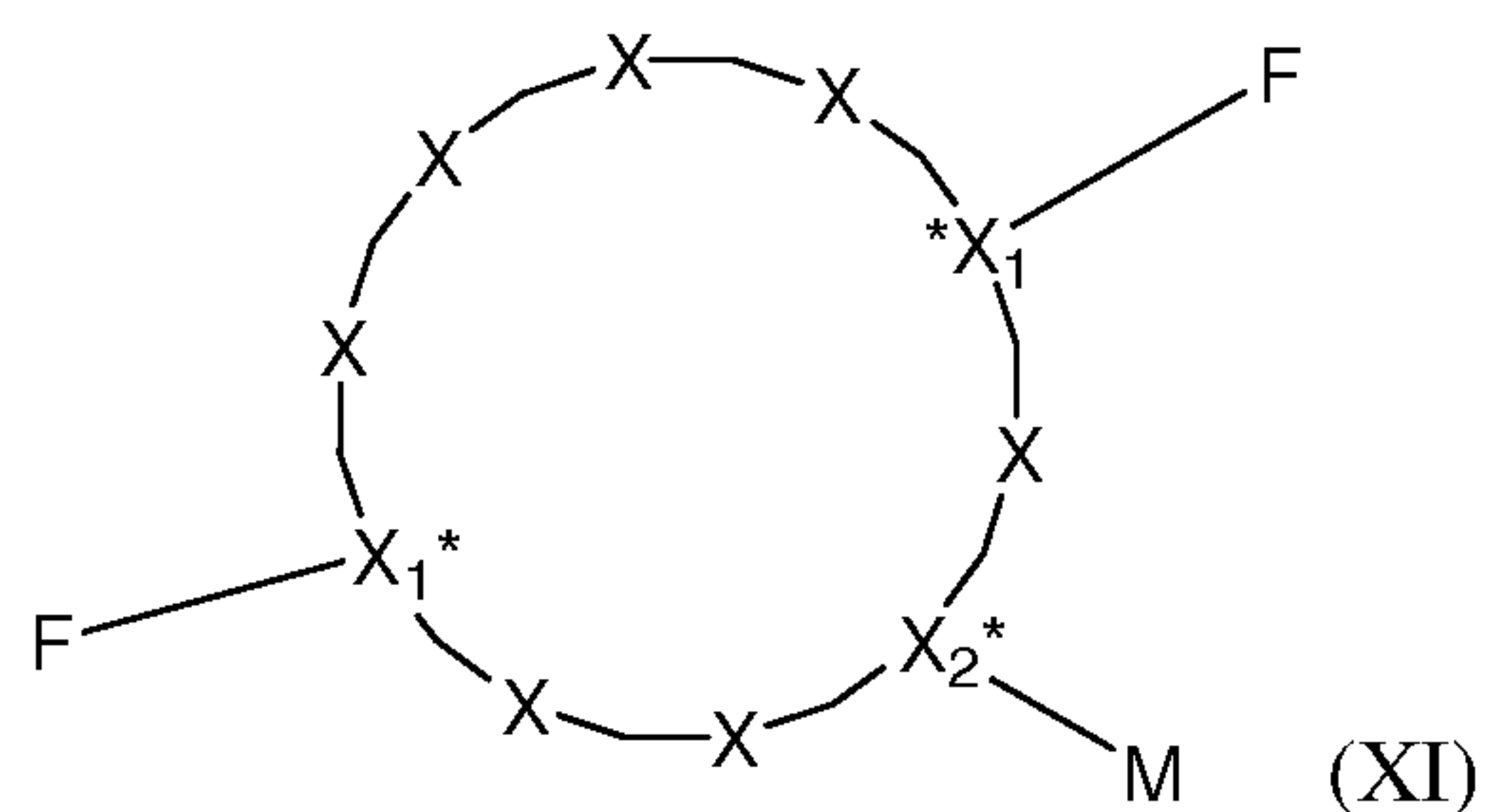
[00150] Exemplary cyclic imaging agents can include an enzymatically cleavable cyclic oligopeptide, for example, an agent represented by Formula IX, wherein, for each occurrence, X is an amino acid residue, M is a biological modifier, F is a fluorophore or
 5 quencher, X_1^* is X-L, X_2^* is X-L, and L is a linker moiety or a bond:



[00151] Another exemplary enzymatically cleavable cyclic oligopeptide is represented by Formula X, wherein each X, M, F, X_1^* and X_2^* is as defined for Formula IX:



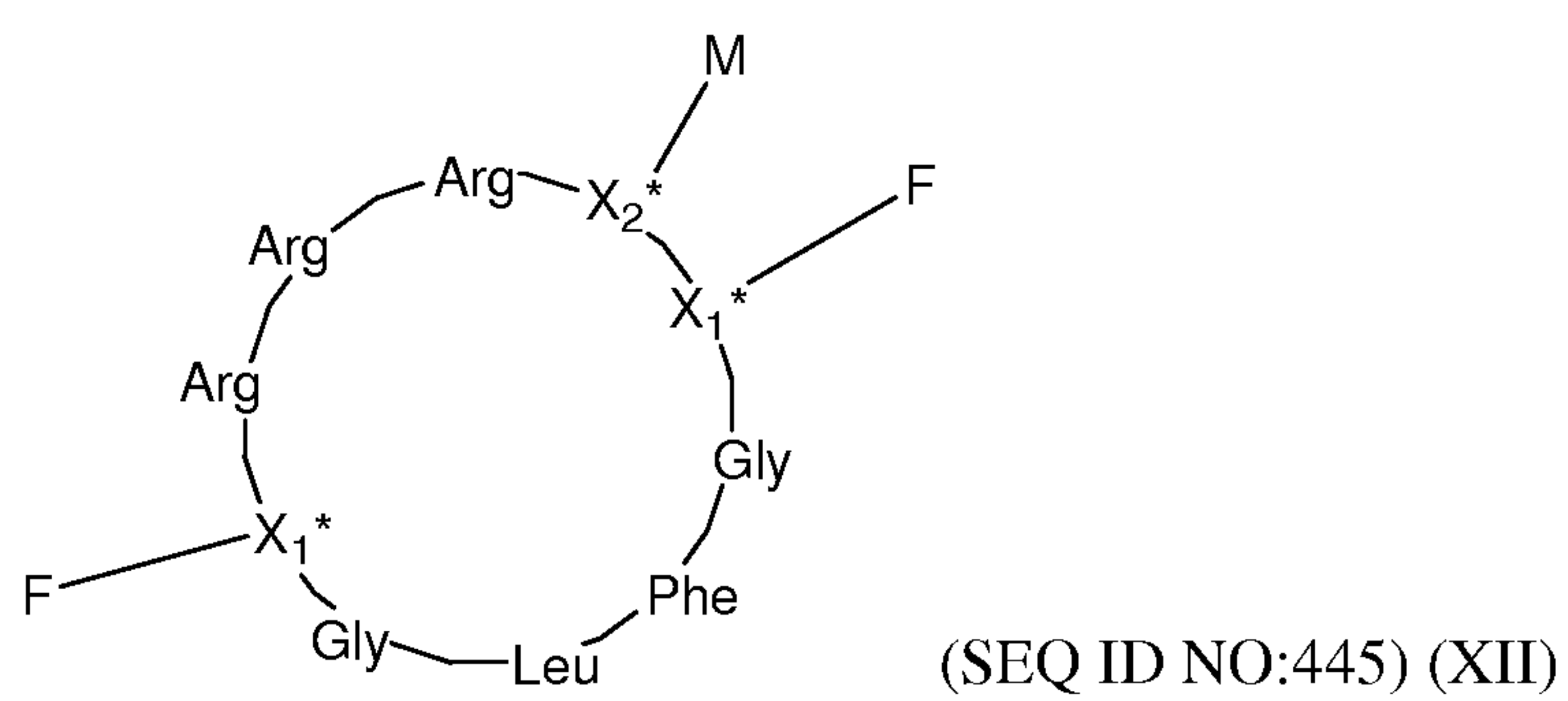
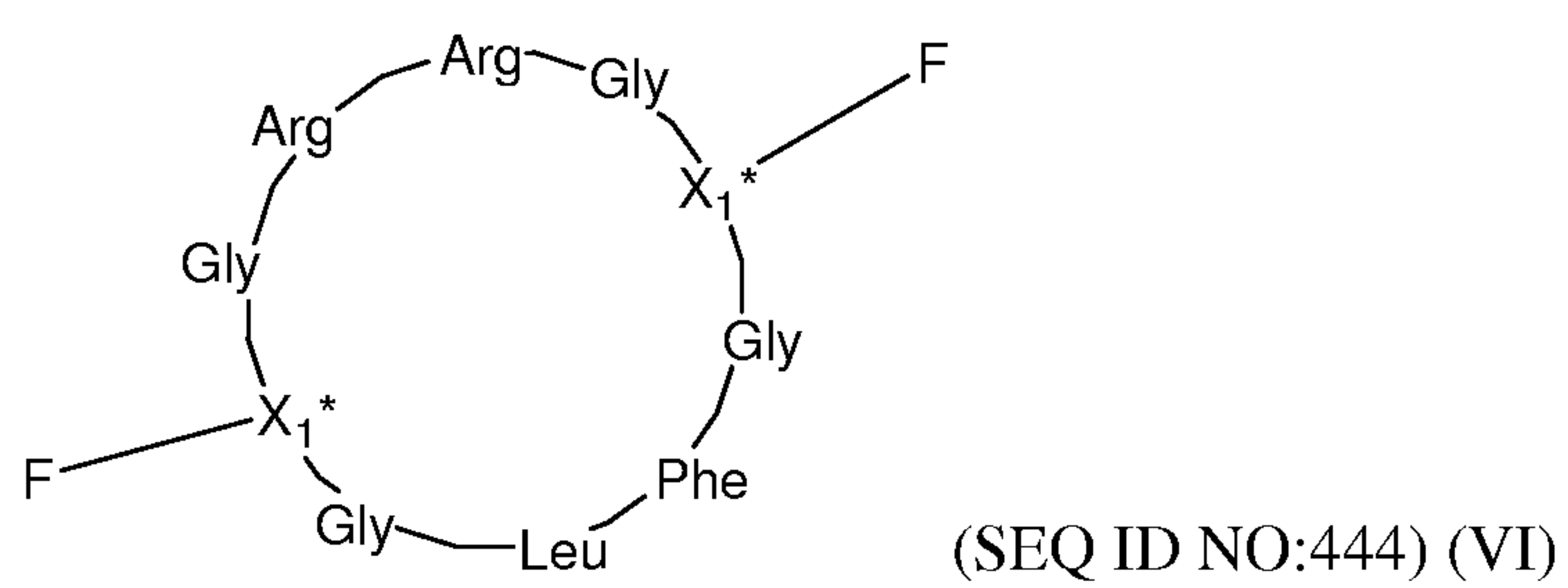
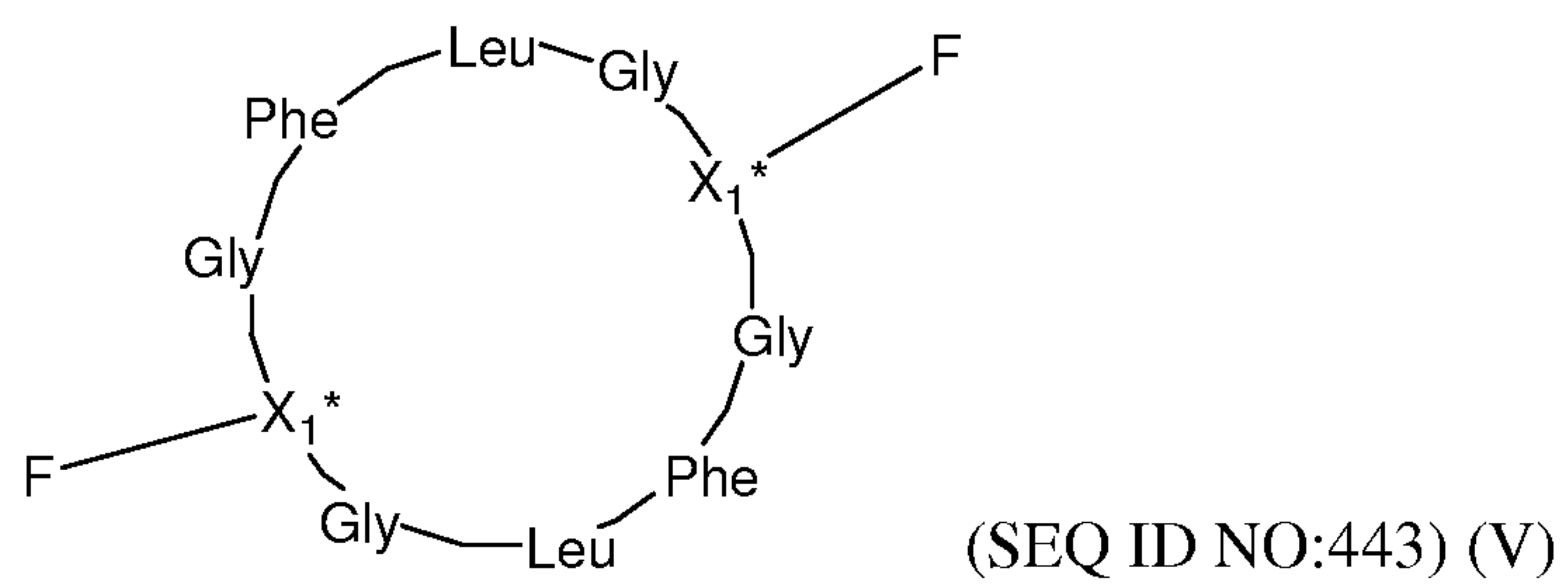
10 [00152] In another embodiment, an enzymatically cleavable cyclic oligopeptide is represented by Formula XI, wherein each X, M, F, X_1^* and X_2^* is as defined in Formula II, above:



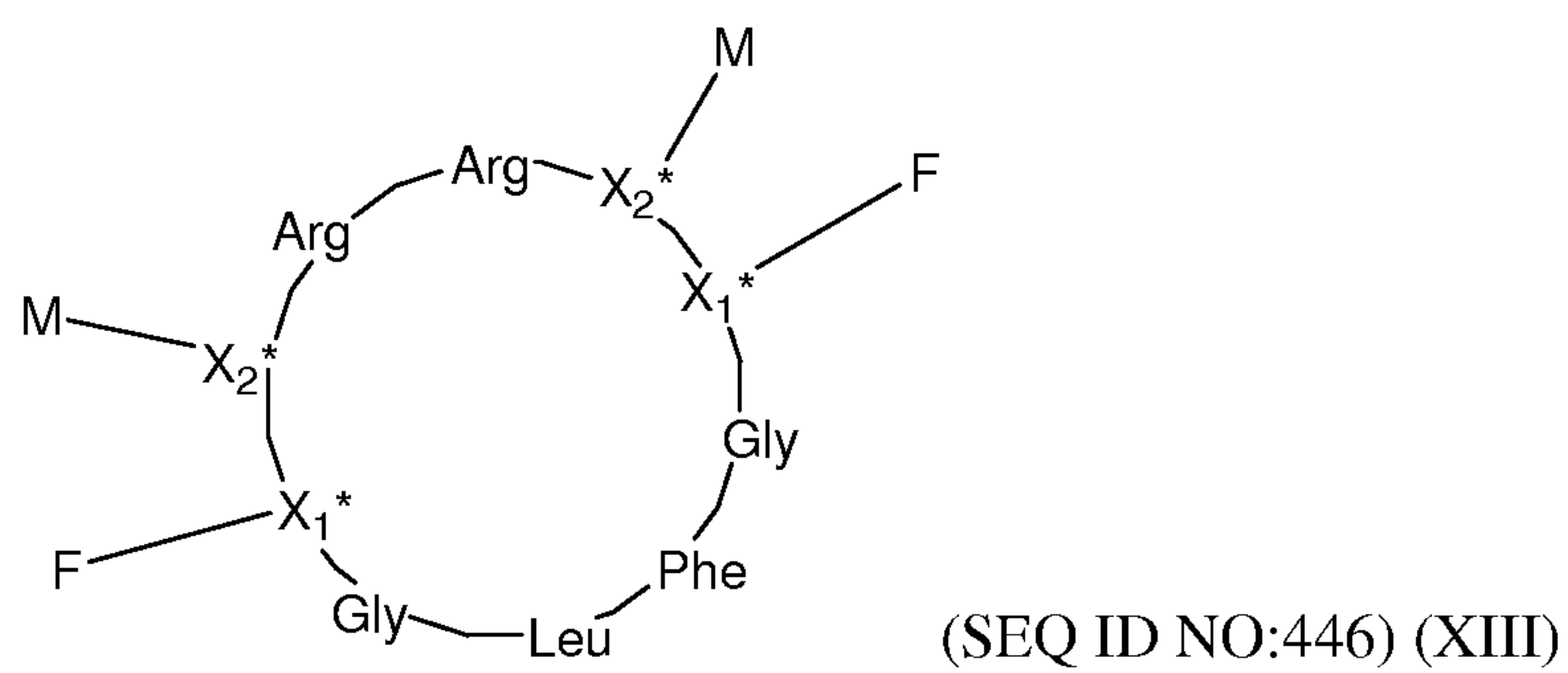
15 [00153] Other exemplary enzymatically cleavable cyclic oligopeptides are represented by Formulas V, VI, and XII-XVII wherein, each F, M, X_1^* , and X_2^* is as defined in Formula

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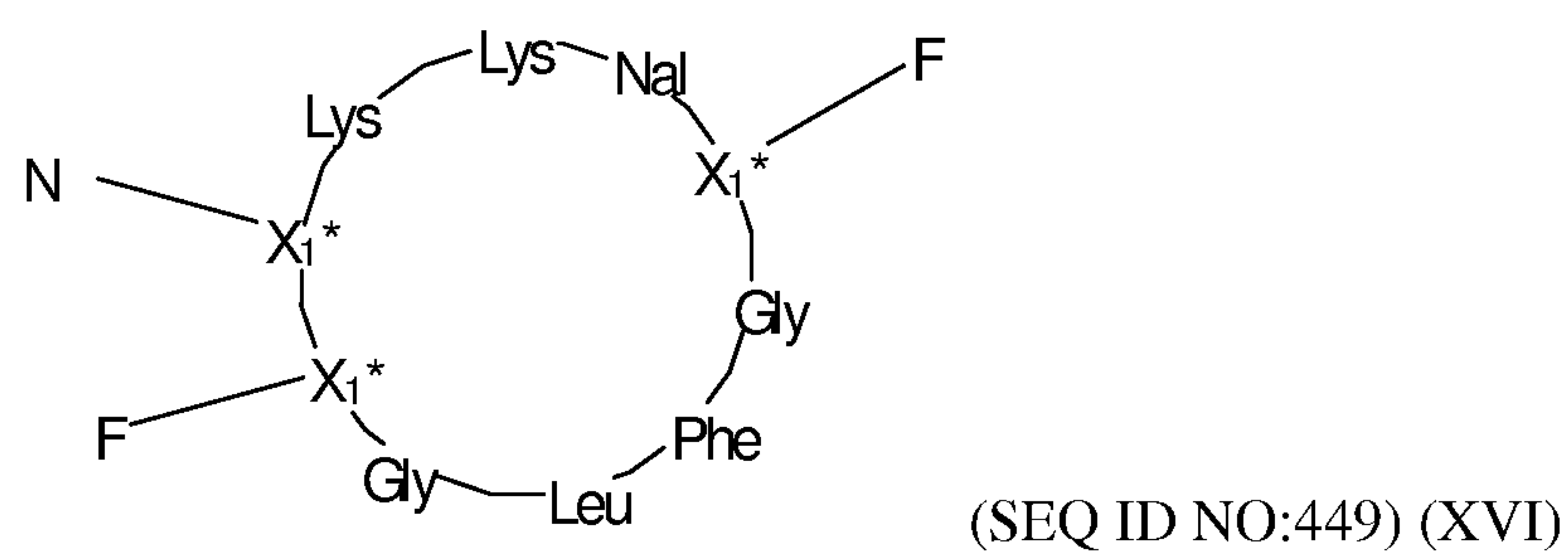
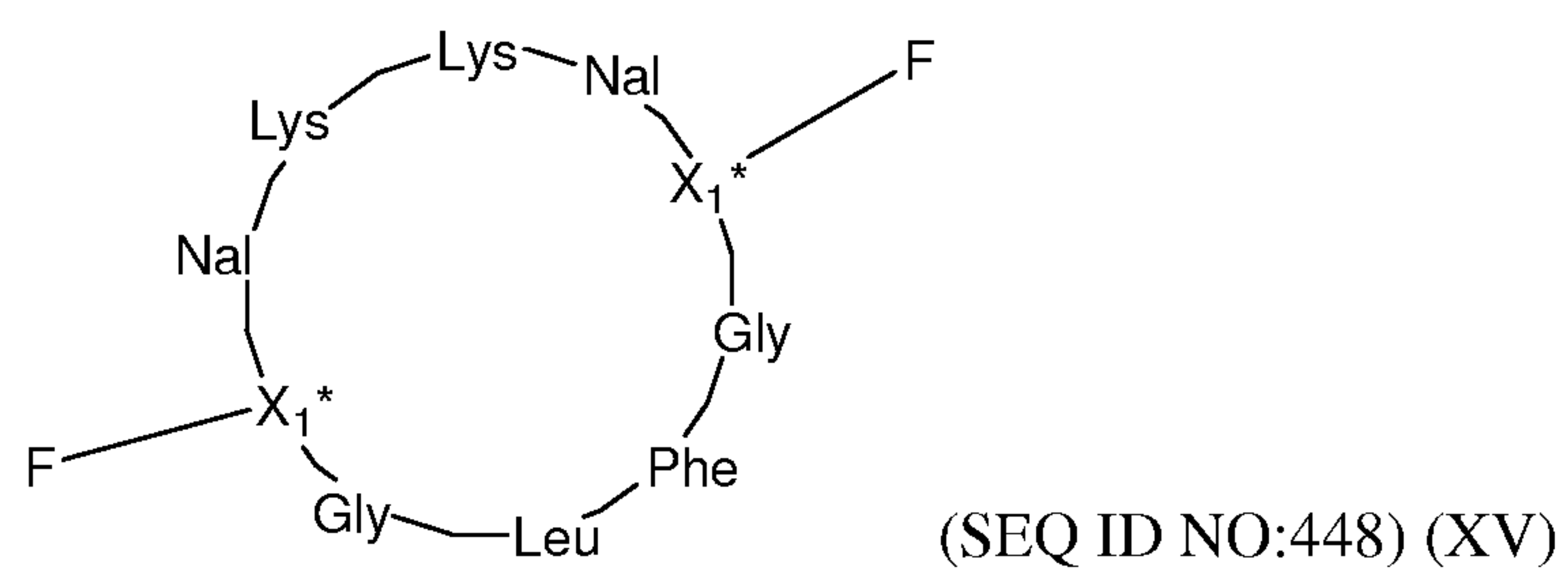
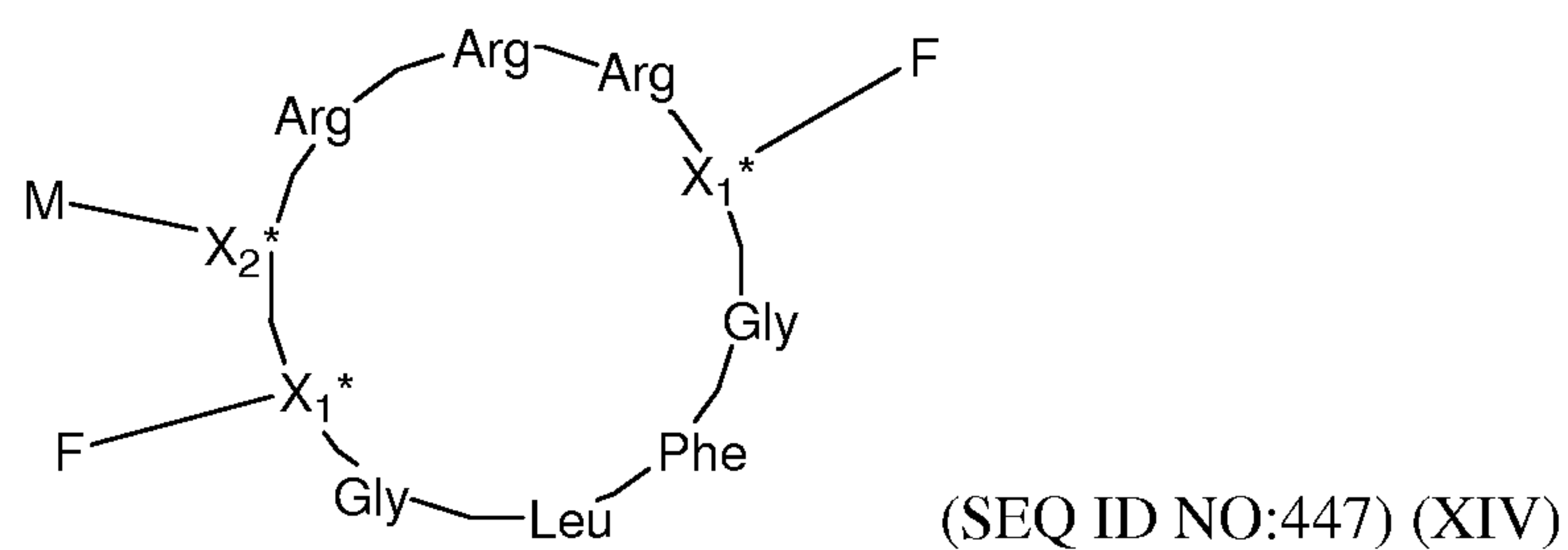
IX:



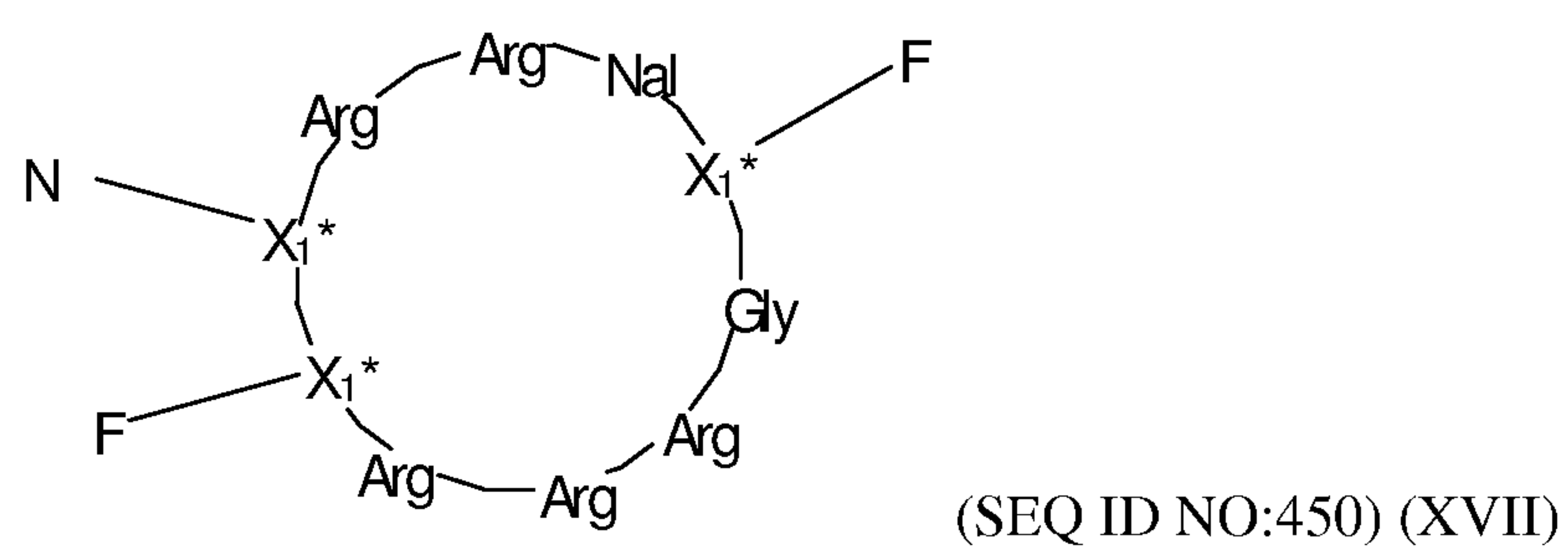
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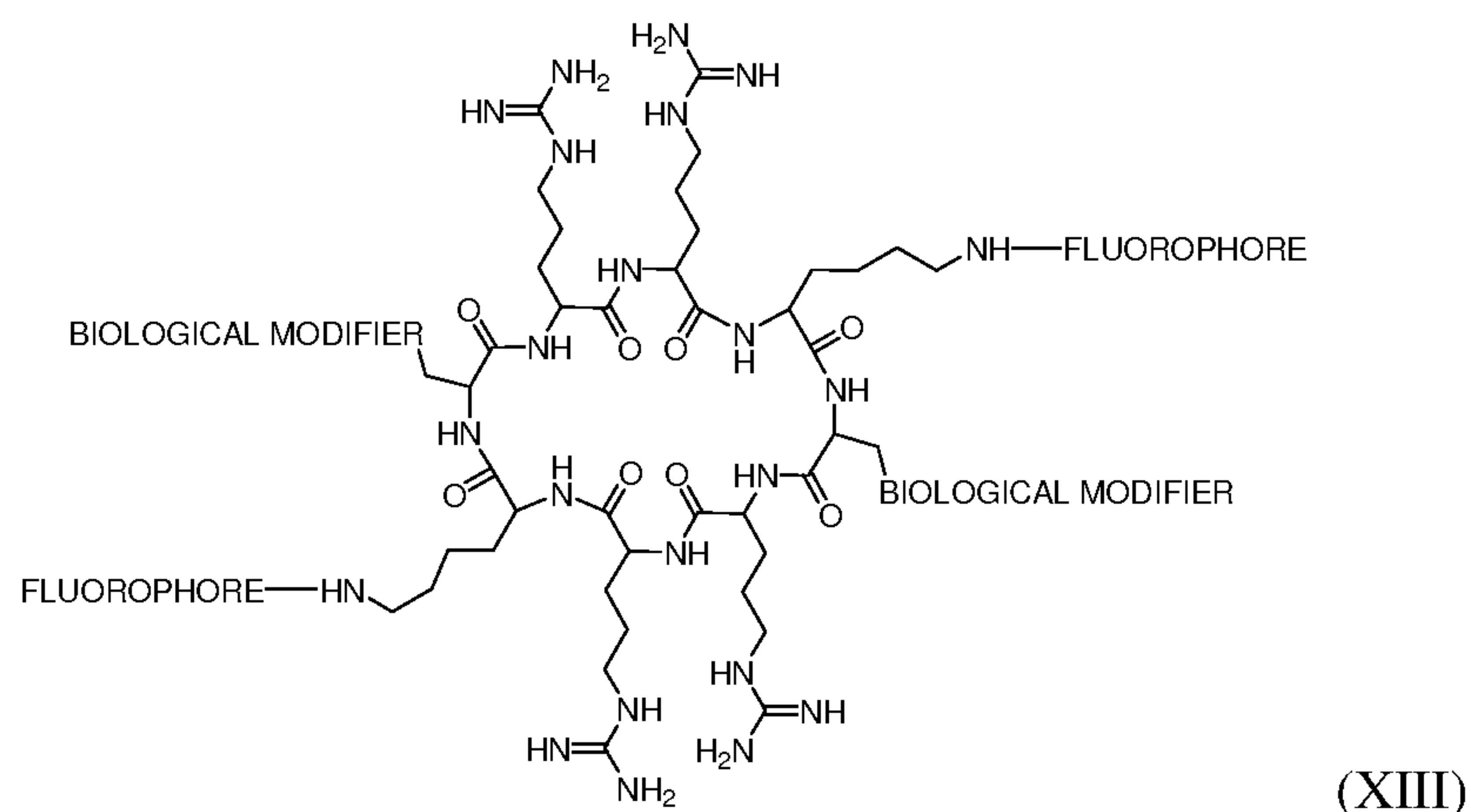


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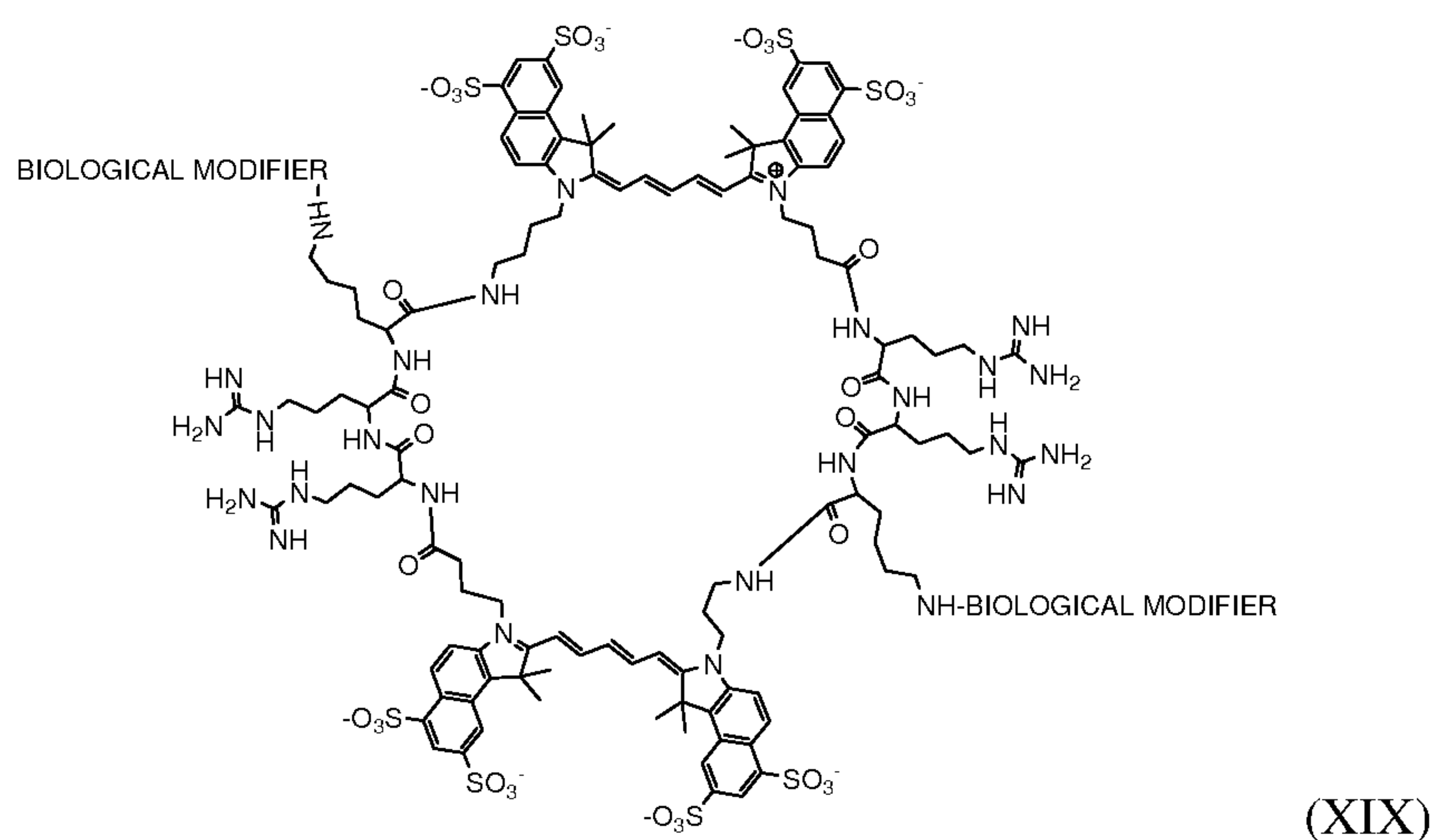


[00154] An enzymatically cleavable cyclic oligopeptide is represented by Formula XIII, wherein the fluorophore and modifiers are as discussed hereinabove:

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[00155] In an exemplary embodiment, an enzymatically cleavable cyclic oligopeptide imaging agent can also be represented as Formula XIX wherein the biological modifiers are as described hereinabove:



5

[00156] The resulting imaging agents preferably have a molecular weight from about 2 kDa to about 60 kDa, such as from about 5 kDa to about 50 kDa, such as from about 10 kDa to about 40 kDa, or from about 20 kDa to about 30 kDa. In certain embodiments, the imaging agent may have a molecular weight of from about 2 kDa to about 50 kDa, such as from about 2 kDa to about 45 kDa, such as from about 2 kDa to about 40 kDa, such as from about 2 kDa to about 35 kDa, such as from about 2 kDa to about 30 kDa, such as from about 2 kDa to about 25 kDa, such as from about 2 kDa to about 10 kDa, or such as from about 2 kDa to 5 kDa. In certain other embodiments, the intact imaging agent may have a molecular weight of from about 5 kDa to about 50 kDa, such as from about 5 kDa

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to about 45 kDa, such as from about 5 kDa to about 40 kDa, such as from about 5 kDa to about 35 kDa, such as from about 5 kDa to about 30 kDa, such as from about 5 kDa to about 25 kDa, such as from about 5 kDa to about 20 kDa, such as from about 5 kDa to about 15 kDa, or such as from about 5 kDa to about 10 kDa.

5 VII. Formulations

[00157] The imaging agents disclosed herein can be formulated into a pharmaceutical composition suitable for administration to a subject, for example, an animal and/or a human. The pharmaceutical composition can include one or more imaging agents and one or more excipients, for example, a stabilizer in a physiologically relevant carrier.

10 [00158] For *in vivo* use, the compositions of the present invention can be provided in a formulation suitable for administration to a subject, for example, an animal or a human. Accordingly, the formulations include the agents together with a physiologically relevant carrier suitable for the desired form and/or dose of administration. The term,
“physiologically relevant carrier” is understood to mean a carrier in which the agents are
15 dispersed, dissolved, suspended, admixed and physiologically tolerable, i.e., can be administered to, in, or on the subject’s body without undue discomfort, or irritation, or toxicity. The preferred carrier is a fluid, preferably a liquid, more preferably an aqueous solution; however, carriers for solid formulations, topical formulations, inhaled formulations, ophthalmic formulations, and transdermal formulations are also
20 contemplated as within the scope of the invention.

[00159] It is contemplated that the agents can be administered orally or parenterally. For parenteral administration, the agents can be administered intravenously, intramuscularly, cutaneously, percutaneously, subcutaneously, rectally, nasally, vaginally, and ocularly. Thus, the composition may be in the form of, *e.g.*, solid tablets, capsules, pills, powders
25 including lyophilized powders, colloidal suspensions, microspheres, liposomes granulates, suspensions, emulsions, solutions, gels, including hydrogels, pastes, ointments, creams, plasters, irrigation solutions, drenches, osmotic delivery devices, suppositories, enemas, injectables, implants, sprays, or aerosols. The pharmaceutical compositions can be formulated according to conventional pharmaceutical practice (see, *e.g.*, Remington: The
30 Science and Practice of Pharmacy, 20th edition, 2000, ed. A.R. Germaro, Lippincott

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Williams & Wilkins, Philadelphia, and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York).

5 [00160] It is understood that the formulation of the agents, the choice of mode of administration, the dosages of agents administered to the subject, and the timing between administration of the agents and imaging is within the level of skill in the art.

VIII. Imaging Methods

[00161] The present invention provides methods for *in vitro* and *in vivo* imaging using the imaging agents disclosed herein. For a review of optical imaging techniques, see, e.g., Alfano et al., *Ann. NY Acad. Sci.* 820:248-270 (1997); Weissleder, *Nature Biotechnology* 10 19, 316 - 317 (2001); Ntziachristos et al., *Eur. Radiol.* 13:195-208 (2003); Graves et al., *Curr. Mol. Med.* 4:419-430 (2004); Citrin et al., *Expert Rev. Anticancer Ther.* 4:857-864 (2004); Ntziachristos, *Ann. Rev. Biomed. Eng.* 8:1-33 (2006); Koo et al., *Cell Oncol.* 28:127-139 (2006); and Rao et al., *Curr. Opin. Biotechnol.* 18:17-25 (2007).

15 [00162] Optical imaging includes all methods from direct visualization without use of any device and use of devices such as various scopes, catheters and optical imaging equipment, for example computer based hardware for tomographic presentations. The imaging agents are useful with optical imaging modalities and measurement techniques including, but not limited to: endoscopy; fluorescence endoscopy; luminescence imaging; time resolved transmittance imaging; transmittance imaging; nonlinear microscopy; 20 confocal imaging; acousto-optical imaging; photoacoustic imaging; reflectance spectroscopy; spectroscopy; coherence interferometry; interferometry; optical coherence tomography; diffuse optical tomography and fluorescence mediated molecular tomography (continuous wave, time domain frequency domain systems and early photon), and measurement of light scattering, absorption, polarization, luminescence, fluorescence 25 lifetime, quantum yield, and quenching.

[00163] An imaging system useful in the practice of the invention typically includes three basic components: (1) an appropriate light source for inducing excitation of the imaging agent, (2) a system for separating or distinguishing emissions from light used for fluorophore excitation, and (3) a detection system. The detection system can be hand-held 30 or incorporated into other useful imaging devices, such as intraoperative microscopes. Exemplary detection systems include an endoscope, catheter, tomographic system, hand-

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held imaging system, or a intraoperative microscope.

[00164] Preferably, the light source provides monochromatic (or substantially monochromatic) light. The light source can be a suitably filtered white light, *i.e.*, bandpass light from a broadband source. For example, light from a 150-watt halogen
5 lamp can be passed through a suitable bandpass filter commercially available from Omega Optical (Brattleboro, VT). Depending upon the system, the light source can be a laser. See, *e.g.*, Boas *et al.*, *Proc. Natl. Acad. Sci. USA* **91**:4887-4891, 1994; Ntziachristos *et al.*, *Proc. Natl. Acad. Sci. USA* **97**:2767-2772, 2000; and Alexander, *J. Clin. Laser Med. Surg.* **9**:416-418, 1991. Information on lasers for imaging can be found, for example, at
10 Imaging Diagnostic Systems, Inc., Plantation, FL and various other sources. A high pass or bandpass filter can be used to separate optical emissions from excitation light. A suitable high pass or bandpass filter is commercially available from Omega Optical, Burlington, VT.

[00165] In general, the light detection system can be viewed as including a light
15 gathering/image forming component and a light/signal detection/image recording component. Although the light detection system can be a single integrated device that incorporates both components, the light gathering/image forming component and light detection/image recording component are discussed separately.

[00166] A particularly useful light gathering/image forming component is an endoscope.
20 Endoscopic devices and techniques which have been used for *in vivo* optical imaging of numerous tissues and organs, including peritoneum (Gahlen *et al.*, *J. Photochem. Photobiol. B* **52**:131-135, 1999), ovarian cancer (Major *et al.*, *Gynecol. Oncol.* **66**:122-132, 1997), colon and rectum (Mycek *et al.*, *Gastrointest. Endosc.* **48**:390-394, 1998; and Stepp *et al.*, *Endoscopy* **30**:379-386, 1998), bile ducts (Izuishi *et al.*,
25 *Hepatogastroenterology* **46**:804-807, 1999), stomach (Abe *et al.*, *Endoscopy* **32**:281-286, 2000), bladder (Kriegmair *et al.*, *Urol. Int.* **63**:27-31, 1999; and Riedl *et al.*, *J. Endourol.* **13**:755-759, 1999), lung (Hirsch *et al.*, *Clin Cancer Res* **7**:5-220, 2001), brain (Ward, *J. Laser Appl.* **10**:224-228, 1998), esophagus, and head and neck regions can be employed in the practice of the present invention.

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[00167] Other types of light gathering components are catheter-based devices, including fiber optics devices. Such devices are particularly suitable for intravascular imaging. See, e.g., Tearney *et al.*, Science 276:2037-2039, 1997; and Circulation 94:3013, 1996.

[00168] Still other imaging technologies, including phased array technology (Boas *et al.*, Proc. Natl. Acad. Sci. USA 91:4887-4891, 1994; Chance, Ann. NY Acad. Sci. 838:29-45, 1998), optical tomography (Cheng *et al.*, Optics Express 3:118-123, 1998; and Siegel *et al.*, Optics Express 4:287-298, 1999), intravital microscopy (Dellian *et al.*, Br. J. Cancer 82:1513-1518, 2000; Monsky *et al.*, Cancer Res. 59:4129-4135, 1999; and Fukumura *et al.*, Cell 94:715-725, 1998), confocal imaging (Korlach *et al.*, Proc. Natl. Acad. Sci. USA 96:8461-8466, 1999; Rajadhyaksha *et al.*, J. Invest. Dermatol. 104:946-952, 1995; and Gonzalez *et al.*, J. Med. 30:337-356, 1999) and fluorescence molecular tomography (FMT) (Nziachristos *et al.*, Nature Medicine 8:757-760, 2002; U.S. Patent No. 6,615,063, PCT WO 03/102558, and PCT WO 03/079015) can be used with the imaging agents of the invention. Similarly, the imaging agents can be used in a variety of imaging systems, for example, (1) the IVIS® Imaging Systems: 100 Series, 200 Series (Xenogen, Alameda, CA), (2) SPECTRUM and LUMINA (Xenogen, Alameda, CA), (3) the SoftScan® or the eXplore Optix™ (GE Healthcare, United Kingdom), (4) Maestro™ and Nuance™-2 Systems (CRI, Woburn, MA), (5) Image Station In-Vivo FX from Carestream Molecular Imaging, Rochester, NY (formerly Kodak Molecular Imaging Systems), (6) OV100, IV100 (Olympus Corporation, Japan), (7) Cellvizio Mauna Kea Technologies, France), (8) NanoSPECT/CT or HiSPECT (Bioscan, Washington, DC), (9) CTLM® or LILA™ (Imaging Diagnostic Systems, Plantation, FL), (10) DYNOT™ (NIRx Medical Technologies, Glen Head, NY), and (11) NightOWL Imaging Systems by Berthold Technologies, Germany.

[00169] A variety of light detection/image recording components, e.g., charge coupled device (CCD) systems or photographic film, can be used in such systems. The choice of light detection/image recording depends on factors including the type of light gathering/image forming component being used. It is understood, however, that the selection of suitable components, assembling them into an optical imaging system, and operating the system is within ordinary skill in the art.

[00170] For agents that have magnetic properties, MRI imaging well known in the art can

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also be applied in the practice of the invention. For a review of MRI techniques see Westbrook, Handbook of MRI Technique, 2nd Edition, 1999, Blackwell Science. It is possible that images obtained, for example, by optical imaging and by magnetic resonance imaging can be co-registered or fused with one another to provide additional information about the item being imaged. Furthermore, multi-modality imaging systems (i.e., combined optical and MR imaging systems) can be used to create combined optical MR images.

[00171] In addition, the compositions and methods of the present invention can be used for other imaging compositions and methods. For example, the agents of the present invention can be imaged by other imaging modalities, such as, X-ray, computed tomography (CT), MR imaging, ultrasound, positron emission tomography (PET), and single photon computerized tomography (SPECT).

[00172] In addition, the compositions and methods of the present invention can be used in combination with other imaging compositions and methods. For example, the agents of the present invention can be imaged by optical imaging protocols either alone or in combination with other traditional imaging modalities, such as, X-ray, computed tomography (CT), MR imaging, ultrasound, positron emission tomography (PET), and single photon computerized tomography (SPECT). For instance, the compositions and methods of the present invention can be used in combination with CT or MRI to obtain both anatomical and molecular information simultaneously, for example, by co-registration of with an image generated by another imaging modality. The compositions and methods of the present invention can also be used in combination with X-ray, CT, PET, ultrasound, SPECT and other optical and MR contrast agents or alternatively, the agents of the present invention may also include imaging agents, such as iodine, gadolinium atoms and radioactive isotopes, which can be detected using CT, PET, SPECT, and MR imaging modalities in combination with optical imaging. The imaging agents can be linked to or incorporated in the agents.

(A) In Vivo Imaging

[00173] With respect to optical *in vivo* imaging, such a method comprises (a) administering to a subject one or more imaging agents; (b) allowing the agent(s) to distribute within the subject; (c) exposing the subject to light of a wavelength absorbable

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by at least one fluorophore in the imaging agent; and (d) detecting an optical signal emitted by the fluorophore. The emitted optical signal can be used to construct an image. The image can be a tomographic image. Furthermore, it is understood that steps (a)-(d) or steps (c)-(d) can be repeated at predetermined intervals thereby to permit evaluation of the
5 subject over time.

[00174] The illuminating and/or detecting steps (steps (c) and (d), respectively) can be performed using an endoscope, catheter, tomographic system, planar system, hand-held imaging system, goggles, or an intraoperative imaging system or microscope.

[00175] Before or during these steps, a detection system can be positioned around or in
10 the vicinity of a subject (for example, an animal or a human) to detect signals emitted from the subject. The emitted signals can be processed to construct an image, for example, a tomographic image. In addition, the processed signals can be displayed as images either alone or as fused (combined) images.

[00176] In addition, it is possible to practice an *in vivo* imaging method that selectively
15 detects and images one or more molecular imaging probes, including the imaging agents simultaneously. In such an approach, for example, in step (a) noted above, two or more imaging probes whose signal properties are distinguishable from one another are administered to the subject, either at the same time or sequentially, wherein at least one of the molecular imaging probes is a agent. The use of multiple probes permits the recording
20 of multiple biological processes, functions or targets.

[00177] The subject may be a vertebrate, for example, a mammal, for example, a human. The subject may also be a non-vertebrate (for example, *C. elegans*, *drosophila*, or another model research organism, etc.) used in laboratory research.

[00178] Information provided by such *in vivo* imaging approaches, for example, the
25 presence, absence, or level of emitted signal can be used to detect and/or monitor a disease in the subject. Exemplary diseases include, without limitation, autoimmune disease, bone disease, cancer, cardiovascular disease, environmental disease, dermatological disease, immunologic disease, inherited disease, infectious disease, metabolic disease, neurodegenerative disease, ophthalmic disease, and respiratory disease. In addition, *in*
30 *vivo* imaging can be used to assess the effect of a compound or therapy by using the imaging agents, wherein the subject is imaged prior to and after treatment with the

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compound or therapy, and the corresponding signal/images are compared.

[00179] The invention also features an *in vivo* imaging method where labeled cells are administered to the recipient. The cells can be labeled with the imaging agents *ex vivo*. The cells can be derived directly from a subject or from another source (e.g., from another
5 subject, cell culture, etc.). The imaging agents can be mixed with the cells to effectively label the cells and the resulting labeled cells administered to the subject into a subject in step (a). Steps (b)-(d) then are followed as described above. This method can be used for monitoring trafficking and localization of certain cell types, including T-cells, tumor cells, immune cells and stem cells, and other cell types. In particular, this method may be used
10 to monitor cell-based therapies.

[00180] The methods of the invention can be used to determine a number of indicia, including tracking the localization of the agent in the subject over time or assessing changes or alterations in the metabolism and/or excretion of the agent in the subject over time. The methods can also be used to follow therapy for such diseases by imaging
15 molecular events and biological pathways modulated by such therapy, including but not limited to determining efficacy, optimal timing, optimal dosing levels (including for individual patients or test subjects), and synergistic effects of combinations of therapy.

[00181] The methods and compositions described herein can be used to help a physician or surgeon to identify and characterize areas of disease, such as arthritis, cancers and
20 specifically colon polyps, or vulnerable or unstable plaque, atherosclerosis, to distinguish diseased and normal tissue, such as detecting tumor margins that are difficult to detect using an ordinary operating microscope, e.g., in brain surgery, to help dictate a therapeutic or surgical intervention, e.g., by determining whether a lesion is cancerous and should be removed or non-cancerous and left alone, or in surgically staging a disease, e.g.,
25 intraoperative lymph node staging, sentinel lymph node mapping, or assessing intraoperative bleeding.

[00182] The methods and compositions described herein can also be used in the detection, characterization and/or determination of the localization of a disease, especially early disease, the severity of a disease or a disease-associated condition, the staging of a
30 disease, and/or monitoring a disease. The presence, absence, or level of an emitted signal can be indicative of a disease state.

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[00183] The methods and compositions disclosed herein can also be used to monitor and/or guide various therapeutic interventions, such as surgical procedures, and monitoring drug therapy, including cell based therapies. The methods can also be used in prognosis of a disease or disease condition.

- 5 **[00184]** With respect to each of the foregoing, examples of such disease or disease conditions that can be detected or monitored (before, during or after therapy) include inflammation (for example, inflammation caused by arthritis, for example, rheumatoid arthritis), cancer (for example, colorectal, ovarian, lung, breast, prostate, cervical, testicular, skin, brain, gastrointestinal, pancreatic, liver, kidney, bladder, stomach,
- 10 leukemia, mouth, esophageal, bone), cardiovascular disease (for example, atherosclerosis and inflammatory conditions of blood vessels, ischemia, hypertension, stroke, thrombosis, disseminated intravascular coagulation), dermatologic disease (for example, Kaposi's Sarcoma, psoriasis, allergic dermatitis), ophthalmic disease (for example, macular degeneration, diabetic retinopathy), infectious disease (for example, bacterial, viral, fungal
- 15 and parasitic infections, including Acquired Immunodeficiency Syndrome, Malaria, Chagas Disease, Schistosomiasis), immunologic disease (for example, an autoimmune disorder, lymphoma, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, lupus erythematosus, myasthenia gravis, Graves disease), central nervous system disease (for example, a neurodegenerative disease, such as Parkinson's disease or Alzheimer's disease,
- 20 Huntington's Disease, amyotrophic lateral sclerosis, prion disease), inherited diseases, metabolic diseases, environmental diseases (for example, lead, mercury and radioactive poisoning, skin cancer), bone-related disease (for example, osteoporosis, primary and metastatic bone tumors, osteoarthritis), neurodegenerative disease, and surgery-related complications (such as graft rejection, organ rejection, alterations in wound healing,
- 25 fibrosis or other complications related to surgical implants).

- [00185]** The methods and compositions described herein, therefore, can be used, for example, to determine the presence and/or localization of tumor cells, the presence and/or localization of inflammation, including the presence of activated macrophages, for instance in atherosclerosis or arthritis, the presence and in localization of vascular disease
- 30 including areas at risk for acute occlusion (i.e., vulnerable plaques) in coronary and peripheral arteries, regions of expanding aneurysms, unstable plaque in carotid arteries,

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and ischemic areas. The methods and compositions of the invention can also be used in identification and evaluation of cell death, injury, apoptosis, necrosis, hypoxia and angiogenesis. The methods and compositions can also be used for drug delivery and to monitor drug delivery, especially when drugs or drug-like molecules are chemically
5 attached to the imaging agents.

(B) *In Vitro* Methods

[00186] With respect to *in vitro* imaging, the imaging agents can be used in a variety of *in vitro* assays. For example, an exemplary *in vitro* imaging method comprises: (a) contacting a sample, for example, a biological sample, with one or more imaging agents of
10 the invention; (b) allowing the agent(s) to interact with a biological target in the sample; (c) optionally, removing unbound agents; (d) in the case of fluorescent agents, illuminating the sample with light of a wavelength absorbable by a fluorophore of the agents; and (e) detecting a signal emitted from fluorophore thereby to determine whether the agent has been activated by or bound to the biological target.

15 [00187] After an agent has been designed, synthesized, and optionally formulated, it can be tested *in vitro* by one skilled in the art to assess its biological and performance characteristics. For instance, different types of cells grown in culture can be used to assess the biological and performance characteristics of the agent. Cellular uptake, binding or cellular localization of the agent can be assessed using techniques known in the art,
20 including, for example, fluorescent microscopy, FACS analysis, immunohistochemistry, immunoprecipitation, in situ hybridization and Forster resonance energy transfer (FRET) or fluorescence resonance energy transfer. By way of example, the agents can be contacted with a sample for a period of time and then washed to remove any free agents. The sample can then be viewed using an appropriate detection device such as a fluorescent
25 microscope equipped with appropriate filters matched to the optical properties of a fluorescent agent. Fluorescence microscopy of cells in culture or scintillation counting is also a convenient means for determining whether uptake and binding has occurred. Tissues, tissue sections and other types of samples such as cytospin samples can also be used in a similar manner to assess the biological and performance characteristics of the
30 agents. Other detection methods including, but not limited to flow cytometry, immunoassays, hybridization assays, and microarray analysis can also be used.

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[00188] The invention will now be illustrated by the following examples, which are given for the purpose of illustration only and without any intention of limiting the scope of the present invention.

EXAMPLES

- 5 [00189] Representative materials and methods that may be used in preparing the materials of the invention are described below. All chemicals and solvents (reagent grade) were used as commercially obtained without further purification. HPLC Analysis: Analytical RP HPLC was performed on a Waters 2695 system using either a Phenomenex Phenyl-hexyl column (3 μ , 100 x 4.6 mm) or C18 column (5 μ , 50 x 4.6 mm) with a flow
10 rate of 1 to 2 mL/min. Chromatograms were monitored at 254 nm and 675 nm. Preparative HPLC was performed on a Varian system using either a Phenomenex Phenyl-hexyl column or a Phenomenex C18 column (250 x 10 mm) at 4.7 mL/min using similar gradient as the analytical run.

15 [00190] **Example 1: Synthesis of the Succinimidyl Ester Form of Fluorophore Conjugated Peptides**

[00191] The imaging agents set forth in Table 7 were made in accordance with the principles discussed in this Example.

TABLE 7

Imaging Agent	SEQ ID NO.
Acetyl-Phe-Arg-Lys(F5)-Gly-Gly-Arg-Lys(F5)-OH	385
Acetyl-Phe-Gly-Lys(F5)-Arg-Arg-Lys(F5)-Gly-OH	386
F5-Gly-Phe-Leu-Gly-Lys(F5)-OH	387
Acetyl-Phe-Arg-Lys(F5)-Gly-G-Arg-Lys(F5)-[OH]	388
pentynoyl-Phe-Arg-Lys-(F5)-Gly-Gly-Arg-Lys(F5)-[OH]	389
pentynoyl-Phe-Gly-Lys(F5)-Arg-Arg-Lys(F5)-[OH]	390
pentynoyl-Lys(F5)-Pro-Leu-Gly-Val-Arg-Lys(F5)-[OH]	391
pentynoyl-Lys(F5)-Gly-Phe-Leu-Gly- β A-Lys(F5)-[OH]	392
pentynoyl-Lys(F5)-Val-Arg-Leu-Gly-Pro-Lys(F5)-[OH]	393
pentynoyl-Lys(F5)-Gly-His-Pro-Gly-Gly-Pro-Gln-Gly-Lys(F5)-[OH]	394
pentynoyl-Lys(F5)-His-Pro-Gly-Gly-Pro-Gln-Lys(F5)-[OH]	395
[F5]-His-Gly-Pro-Arg-Lys(F5)-[OH]	242
[F5]-Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NH ₂]	396
[F5]-His-Gly-Pro-Asn-Lys(F5)-[OH]	240
[F5]-His-Gly-Pro-Asn-Lys(F5)-His-Gly-Pro-Asn- β A-[OH]	243

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Imaging Agent	SEQ ID NO.
[F5]-Ile-His-Pro-Phe-His-Leu-Leu-Tyr-His-Lys(F5)-[OH]	397
[F5]-Ile-His-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Lys(F5)-[OH]	398
Acetyl-Lys(F5)-Lys-Lys-Lys(F5)-Gly-[OH]	399
Acetyl- Lys(F5)-Lys-Lys-Lys(F5)-Lys-Lys-Gly-[OH]	400
[F5]-Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[OH]	401
succinyl-Lys(F5)-Gly-Phe-Leu-Gly-Lys(F5)-[NH ₂]	402
succinyl-Lys(F6)-Gly-Phe-Leu-Gly-Lys(F6)-[NH ₂]	403
succinyl-Lys(F5)-Arg-Arg -Lys(F5)-[NH ₂]	404
succinyl-Lys(F6)-Arg-Arg -Lys(F6)-[NH ₂]	405
succinyl- Lys(F6)-Ala-Arg-Arg -Lys(F6)-[NH ₂]	406
succinyl- Lys(F5)-Lys-Lys -Lys(F5)-[NH ₂]	407
succinyl- Lys(F6)-Arg-Arg-Arg -Lys(F6)-[NH ₂]	408
[F5]-His-Gly-Pro-Ile-Lys-[OH]	409
[F5]-Asn-Gly-Pro-Ile-Lys-[OH]	410
[F6]-Gly-Pro-Leu-Gly-Val-Arg-Lys(F6)-[OH]	411
[F5]-Gly-Val-Arg-Leu-Gly-Pro-Lys(F5)=[OH]	412
[F5]-Gly-Pro-Leu-Gly-Val-Arg-Lys(F5)-[OH]	413
[F6]-His-Gly-Pro-Asn-Lys(F6)-[OH]	414

wherein F5 was the fluorophore described in the imaging agent of Formula Q77, and F6 was the fluorophore described in the imaging agent of Formula Q88.

Part A. Preparation of fluorophore conjugated peptide

- 5 **[00192]** Individual peptide (as set forth in Table 7; 0.45 μ mol) and fluorophore No. 3 from Table 2 (1.0 μ mol) were combined in 100 μ L of N,N-dimethylformamide (DMF) with 1 μ L of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of
- 10 methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μ L of 0.1 M sodium bicarbonate and purified by HPLC using a 10% to 35% gradient of acetonitrile in 25 mM triethylammonium acetate, pH 7, on a 10 mm X 250 mm 300 Å C18 column. The purified peptide was characterized by LC-MS.

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Part B. Preparation of the Succinimidyl Ester of the Labeled Peptide at the C-terminus

[00193] The fluorophore labeled peptide (0.25 μ mol) was dissolved in 50 μ L DMF containing 0.5 mg of disuccinimidyl carbonate (DSC) and 0.5 μ L NMM. The solution was placed on rotator shielded from light and rotated at room temperature for 4 h. The peptide succinimidyl ester was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant and dried briefly under vacuum.

Example 2: Synthesis of mPEG-5kDa Conjugated Peptide

[00194] Methoxypolyethylene glycol amine (mPEG-NH₂, Laysan Bio, molecular weight 5 kDa, 2.5 mg, 0.50 μ mol) was dissolved in 100 μ L DMF and 1 μ L NMM. The solution was added to 0.25 μ mol of the labeled peptide succinimidyl ester (from Example 1) and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC on a 10 mm X 250 mm 300 Å C18 column. The pegylated peptide was characterized by RP-18 HPLC and SEC HPLC.

Example 3: Synthesis of mPEG-20kDa Conjugated Peptide

[00195] Methoxypolyethylene glycol amine (mPEG-NH₂, Laysan Bio, molecular weight 20 kDa, 10 mg, 0.50 μ mol) was dissolved in 100 μ L DMF and 1 μ L NMM. The solution was added to 0.25 μ mol of the labeled peptide succinimidyl ester (from Example 1) and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC on a 10 mm X 250 mm 300 Å C18 column. The pegylated peptide was characterized by RP-18 HPLC and SEC HPLC.

Example 4: Synthesis of mPEG-30kDa Conjugated Peptide

[00196] Methoxypolyethylene glycol amine (mPEG-NH₂, Laysan Bio, molecular weight 30 kDa, 15 mg, 0.50 μ mol) was dissolved in 100 μ L DMF and 1 μ L NMM. The solution was added to 0.25 μ mol of the labeled peptide succinimidyl ester (from Example 1) and

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the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC on a 10 mm X 250 mm 300 Å C18 column. The pegylated peptide was characterized by RP-18 HPLC and SEC HPLC.

Example 5: Synthesis of Pegylated poly-L-lysine Conjugated Peptide

[00197] The peptide succinimidyl ester (0.25 μ mol) from Example 1, was dissolved in 50 μ L DMSO and added to a solution containing approximately 400,000-500,000 Da pegylated poly-L-lysine (0.17 μ mol) in 1.5 mL HEPES buffer, pH 7. The solution was rotated at room temperature for 4 hours, then 20 μ L of acetic anhydride and 1.0 mL of carbonate/bicarbonate buffer, pH 9 was added and rotation continued for 2 hours. The polymer conjugated peptide was purified from unreacted peptide by gel filtration using P-100 gel (BioRad) and elution with 1xPBS. Purity of the product was assessed by RP-18 HPLC and SEC HPLC.

Example 6: Synthesis of Pegylated Poly-D-lysine Conjugated Peptide

[00198] The peptide succinimidyl ester (0.25 μ mol) from Example 1, is dissolved in 50 μ L DMSO and added to a solution containing approximately 300,000-500,000 Da pegylated poly-L-lysine (0.17 μ mol) in 1.5 mL HEPES buffer, pH 7. The solution is rotated at room temperature for 4 hours, then 20 μ L of acetic anhydride and 1.0 mL of carbonate/bicarbonate buffer, pH 9 added and rotation continued for 2 hours. The polymer conjugated peptide is purified from unreacted peptide by gel filtration using P-100 gel (BioRad) and eluted with 1xPBS. Purity of the product is assessed by RP-18 HPLC and SEC HPLC.

Example 7: Synthesis of a Branched PEG Peptide Conjugate

[00199] Methoxypolyethylene glycol amine (mPEG-NH₂, Laysan Bio, molecular weight 20 kDa, 100 mg, 5.0 μ mol) was dissolved in 1000 μ L DMF. The solution was added to 175 μ L of DMF containing N-fmoc glutamic acid (0.9 mg, 2.5 μ mol) and 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU, 1.9 mg, 5 μ mol) followed by addition of 2.1 μ L (12 μ mol) of diisopropylethylamine. The solution

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was stirred at room temperature for 2 hours, and the resulting product was precipitated by addition of 10 mL of methyl tertiary-butyl ether. The solvent was decanted and the branched PEG was purified by HPLC on a 21 mm X 250 mm 300 Å C18 column.

[00200] The fmoc group of the purified product was deprotected with 20% v/v diethylamine in DMF for 30 minutes, followed by precipitation with methyl tertiary-butyl ether to yield amine-functionalized branched PEG. The amine-functionalized branched PEG (15 mg, 0.50 µmol) was dissolved in 100 µL DMF and 1 µL NMM. The solution was added to 0.25 µmol of the labeled peptide (from Example 1) along with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 µmol) and hydroxybenzotriazole (HOBt, 0.2 mg, 1.5 µmol) and the solution was placed on rotator shielded from light and rotated at room temperature for 16 h. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 µL water and purified by HPLC using a 10% to 85% gradient of acetonitrile in 25 mM triethylammonium bicarbonate, pH 8.5, on a 21 mm X 250 mm 300 Å C18 column. The branched PEG conjugated peptide was further purified by strong anion exchange chromatography. The branched PEG conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

Example 8: Synthesis of a Polyvinylpyrrolidone Peptide Conjugate

[00201] Carboxy functionalized polyvinylpyrrolidone (6 kDa, Polymersource, 20 mg, 3.3 µmol) was dissolved in 200 µL DMF with disuccinimidyl carbonate (5 mg, 20 µmol) and DMAP (0.5 mg, 5 µmol) and rotated at room temperature for 4 hours. The succinimidyl ester functionalized polyvinylpyrrolidone was precipitated with 1.5 mL of ethyl acetate.

[00202] The labeled peptide from Example 1A above (0.75 µmol) along with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 2.5 mg, 13 µmol) and hydroxybenzotriazole (HOBt, 2.5 mg, 19 µmol) were dissolved in 750 µL of 0.1 M MES buffer, pH 6. To the solution was added 200 µL of 1 M ethylene diamine dihydrochloride in 1 M HEPES buffer, pH 7 and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The amine functionalized peptide was purified by HPLC.

[00203] The amine functionalized peptide (0.5 µmol) described in the paragraph above,

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was dissolved in 200 μ L DMF containing 20 mg of the succinimidyl ester functionalized polyvinylpyrrolidone described above along with DMAP (0.5 mg, 5 μ mol). The solution was rotated at RT for 24 hours. The polyvinylpyrrolidone conjugated peptide was precipitated by addition of 1.5 mL of MTBE and isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC. The polyvinylpyrrolidone conjugated peptide was further purified by strong anion exchange chromatography. The polyvinylpyrrolidone conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

Example 9: Synthesis of an Aminodextran Peptide Conjugate

10 [00204] Aminodextran (Invitrogen, molecular weight 10 kDa, 19 mg, 1.9 μ mol) was dissolved in 150 μ L 0.1 mM MES pH 6. The solution was added to 0.75 μ mol of the labeled peptide (from Example 1) along with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 2 mg, 10 μ mol) and hydroxybenzotriazole (HOBt, 2 mg, 15 μ mol) and the solution was placed on rotator shielded from light and rotated at
15 room temperature for 16 hours. The aminodextran conjugated peptide was purified by HPLC. The aminodextran conjugated peptide was further purified by gel filtration chromatography using Biogel P10 (Biorad) and eluting with 1XPBS. The aminodextran conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

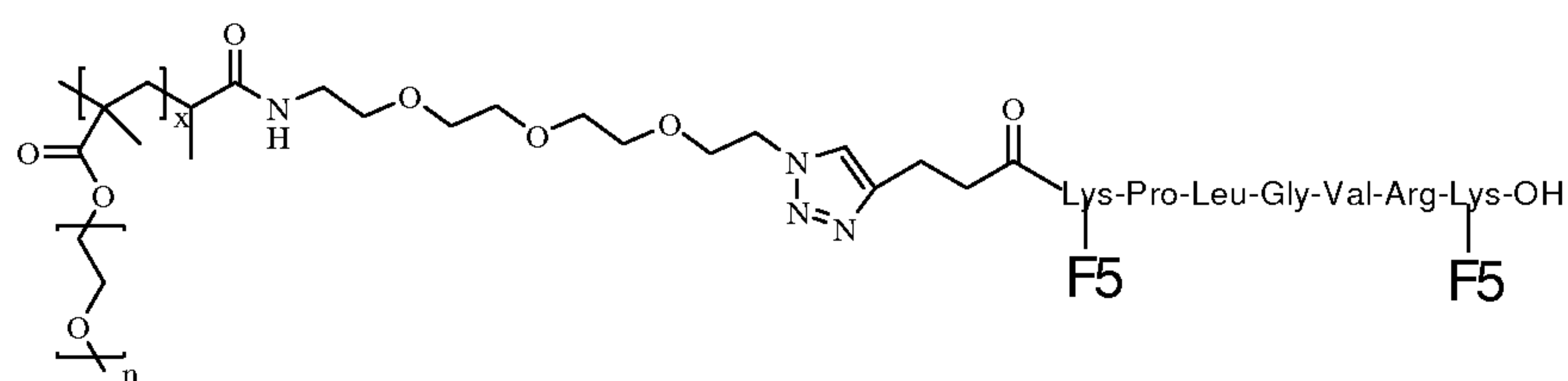
Example 10: Synthesis of an iron Oxide Nanoparticle Peptide Conjugate

20 [00205] A solution of amine-functionalized iron oxide nanoparticles (16 mg total iron) in 8 mL 0.1 mM MES pH 6 was combined with 0.5 μ mol of the labeled peptide (from Example 1A above) along with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 5 mg, 25 μ mol) and hydroxybenzotriazole (HOBt, 5 mg, 38 μ mol) and the solution was placed on rotator shielded from light and rotated at room temperature
25 for 16 hours. The iron oxide nanoparticle conjugated peptide was purified by gel filtration chromatography using Biogel P100 (Biorad) and eluting with 1XPBS. The aminodextran conjugated peptide was characterized by SEC HPLC.

Example 11: Synthesis of Exemplary Imaging Agent

[00206] This example describes the synthesis of an exemplary imaging agent denoted by
30 Formula XXII.

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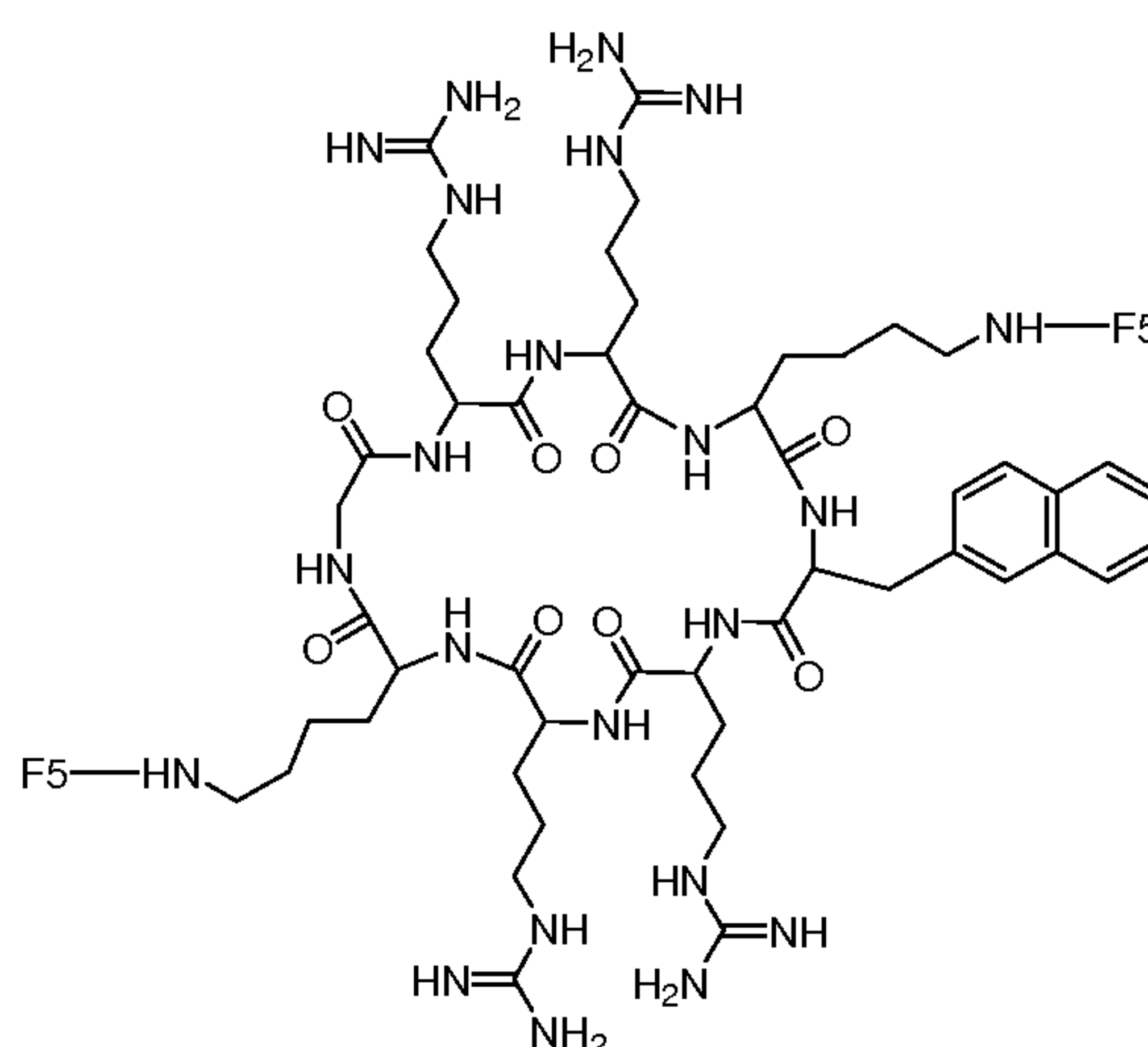
(XXII)

[00207] The fluorophore labeled, alkyne-modified peptide Lys-Pro-Leu-Gly-Val-Arg-Lys (SEQ ID NO:415) (400 μmol) was combined with the azide 11-azido-3,6,9-trioxaundecan-1-amine (azido-PEG-amine, $n=3$) in 200 μL of 65 mM triethylammonium chloride, 25 mM sodium phosphate, pH 5 to which 0.2 mg of nano-sized copper powder was added. The mixture was rotated at room temperature for 1 hour, filtered, and purified by HPLC on a 10 mm X 250 mm 300 Å C18 column. The purified peptide was characterized by MALDI MS (calculated 3135.05, found 3135.13).

[00208] The resulting amine functionalized peptide (0.16 μmol) was dissolved in 200 μL DMSO with 0.35 μmol PolyPEG NHS ester (Warwick Effect Polymers) and 0.2 μL diisopropylethylamine. The solution was rotated at RT for 24 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μL water and purified by HPLC on a 10 mm X 250 mm 300 Å C18 column. The pegylated peptide was characterized by RP-18 HPLC and SEC HPLC.

Example 12: Cyclic Peptide Imaging Agent

[00209] This example describes the synthesis of the cyclic imaging agent represented by Formula XXIII.



(XXIII)

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[00210] The peptide Arg(Pbf)-Arg(Pbf)-Lys(boc)-Nal-Arg(Pbf)-Arg(Pbf)-Lys(boc)-Gly-OH (SEQ ID NO:416) is prepared using solid phase synthesis as known in the art of solid phase peptide synthesis using the Fmoc protection/deprotection strategy. The resin used is Fmoc-Gly-HMPB-MBHA resin that is commercially available. The peptide is cleaved off the resin using 1%TFA in methylene chloride to retain acid labile side chain protecting groups. Head-tail cyclization is effected by heating a solution of the peptide in DMF in the presence of Hunig's base and HBTU. After cyclization is achieved, the side chain protecting groups are removed under acidic conditions such as 95% TFA-5% water or other similar deprotection cocktails known in the art. Fluorophore 3 from Table 2 is coupled to the two lysine side chain amines and the product is isolated and purified by reversed phase HPLC.

Example 13: Synthesis of Exemplary Imaging Agent

[00211] This Example describes the synthesis of the exemplary imaging agent [F5]Lys(COCF₃)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[OH] (SEQ ID NO:417).

[00212] Starting material peptide Lys(COCF₃)- His-Pro-Phe-His-Leu-Val-Ile-His-Lys-(OH) (SEQ ID NO:418) (6 μmol) and fluorophore No. 3 from Table 2 (20 μmol) were combined in 2 mL of N,N-dimethylformamide (DMF) with 6 μL of N-methylmorpholine (NMM). The solution was shielded from light and magnetically stirred at room temperature for 16 hOURS. The labeled peptide was precipitated by addition of 2 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 1.2 mL of 0.1 M sodium bicarbonate and stirred at room temperature for 4 hrs. The mixture then was purified by HPLC on a 10 mm X 250 mm phenyl hexyl column. The purified peptide was characterized by LC-MS (ESI+, (M+3)/3: 1,131.02).

Example 14: Synthesis of Exemplary Imaging Agent

[00213] This Example describes the synthesis of the exemplary imaging agent [F5]Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[OH] (SEQ ID NO:298) (agent R20).

[00214] Product agent from Example 13 (2 μmol) was dissolved in 0.9 mL of water and 0.3 mL of concentrated ammonium hydroxide. The solution was shielded from light and magnetically stirred at room temperature for 16 hours. The mixture then was purified by

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HPLC on a 10 mm X 250 mm phenyl hexyl column. The purified peptide was characterized by LC-MS (ESI+, (M+3)/3: 1,099.03)

Example 15: Synthesis of Exemplary Imaging Agent

[00215] This Example describes the synthesis of the imaging agent [F5]Lys-His-Pro-Phe-
5 His-Leu-Val-Ile-His-Lys(F5)-[NHmPEG20k (SEQ ID NO:419) (agent R21).

[00216] A mixture of product agent from Example 13 (9 μ mol), methoxypolyethylene glycol amine (mPEG-NH₂, Laysan Bio, molecular weight 20 kDa, 220 mg, 11 μ mol) was dissolved, EDC (52 μ mol), HOBt (74 μ mol), NMM (40 μ L) in 6 mL of DMF and was shielded from light and magnetically stirred at room temperature for 3 days. The
10 pegylated peptide was precipitated by addition of 20 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dissolved in 3 mL of 0.1 M aqueous sodium bicarbonate and stirred at room temperature for 4 hrs. Concentrated ammonium hydroxide (1.2 mL) was added and the resulting mixture was stirred at room temperature for 16 hours. The mixture was purified by HPLC. The
15 pegylated peptide was further purified by anion exchange chromatography and characterized by RP-HPLC.

Example 16: Synthesis of Exemplary Imaging Agent

[00217] This Example describes the synthesis of the imaging agent [F5]Ile-His-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Lys(F5)-[OH] (SEQ ID NO:420).

[00218] Starting material peptide, Ile- His-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Lys-(OH) (SEQ ID NO:421) (1.9 μ mol) and fluorophore No. 3 from Table 2 (9 μ mol) were combined in 900 μ L of N,N-dimethylformamide (DMF) with 5 μ L of N-methylmorpholine (NMM). The solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by
25 addition of 5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 0.9 mL of 0.1 M hydroxylamine and stirred at room temperature for 2 hrs. To this solution was added 0.3 mL of 0.1 M aqueous sodium bicarbonate and the mixture was stirred at room temperature for 3 days. The mixture was purified by HPLC. The purified
30 peptide was characterized by LC-MS (ESI+, (M+3)/3: 1,124.03).

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Example 17: Synthesis of Exemplary Imaging Agent

[00219] This Example describes the synthesis of the imaging agent [F5]Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHmPEG5k] (SEQ ID NO:304) (Agent R27)

[00220] A mixture of product from Example 13 (2.5 μ mol), methoxypolyethylene glycol amine (mPEG-NH₂, Laysan Bio, molecular weight 5 kDa, 30 mg, 6 μ mol), EDC (78 μ mol), HOBt (111 μ mol), NMM (15 μ L) in 2 mL of DMF was shielded from light and magnetically stirred at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 10 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dissolved in 0.8 mL of 0.1 M aqueous sodium bicarbonate and stirred at room temperature for 5 hours. Concentrated ammonium hydroxide (0.6 mL) was added and the resulting mixture was stirred at room temperature for 16 hours. The mixture was purified by HPLC using a 10% to 85% gradient of acetonitrile in 25 mM triethylammonium bicarbonate, pH 8.5, on a 10 mm X 250 mm 300 Å C18 column. The pegylated peptide was characterized by RP-HPLC.

15 **Example 18: Synthesis of Exemplary Imaging Agent**

[00221] This Example describes the synthesis of the imaging agent [F5]Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHdPEG₂₄] (SEQ ID NO:305) (Agent R26)

[00222] A mixture of product agent from Example 13 (1.0 μ mol), methyl-PEG₂₄-Amine (MA(PEG)₂₄ Thermo Scientific, 5.5 μ mol), EDC (15 μ mol), HOBt (17 μ mol), NMM (20 μ L) in 1 mL of DMF was shielded from light and magnetically stirred at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 10 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dissolved in 0.7 mL of 0.1 M aqueous sodium bicarbonate and stirred at room temperature for 2 hours. The main component of the mixture was isolated by preparative HPLC using a 10% to 85% gradient of acetonitrile in 25 mM triethylammonium bicarbonate, pH 8.5, on a C18 column. The combined fractions were treated with concentrated ammonium hydroxide (1.0 mL) and the resulting mixture was stirred at room temperature for 5 days. The mixture was purified by HPLC using a 10% to 35% gradient of acetonitrile in 25 mM triethylammonium bicarbonate, pH 8.5 on C18 column. The pegylated peptide was characterized by RP-HPLC.

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Example 19: Synthesis of Exemplary Imaging Agent

[00223] This Example describes the synthesis of the imaging agent [F5]Lys(Ac)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[OH] (SEQ ID NO:314) (Agent R23)

5 [00224] To a solution of product agent from Example 14 (1.0 μmol) in DMF (1 mL) was added N-methylmorpholine (30 μL) and a solution of succinimidyl acetate (2.8 μmol) in acetic acid (4 μL). The solution was placed on rotator shielded from light and rotated at room temperature for 17 hours. The peptide was precipitated by addition of ether followed by centrifugation and decanting of the supernatant. The crude product was dissolved in 1 mL of 0.1 M aqueous sodium bicarbonate and stirred at room temperature
10 overnight. The mixture then was purified by HPLC using a 20% to 35% gradient of acetonitrile in 25 mM triethylammonium bicarbonate, pH 8.5 on phenyl hexyl column. The purified peptide was characterized by LC-MS (ESI+, (M+3)/3: 1,113.03).

Example 20: Synthesis of Exemplary Imaging Agent

[00225] This Example describes the synthesis of the exemplary imaging agent
15 [F5]Lys(COCF₃)-His-Pro-Phe-His-Cha-Val-Ile-His-Lys(F5)-[OH] (SEQ ID NO:458).

[00226] Starting material peptide Lys(COCF₃)-His-Pro-Phe-His-Cha-Val-Ile-His-Lys-(OH) (7.8 μmol) and a fluorophore No. 3 of Table 2 (20 μmol) were combined in 2 mL of N,N-dimethylformamide (DMF) with 100 μL of N-methylmorpholine (NMM). The solution was shielded from light and magnetically stirred at room temperature for 16
20 hours. The labeled peptide was precipitated by addition of 20 mL of ether followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 1.2 mL of 0.1 M sodium bicarbonate and stirred at room temperature for 6 hours. The mixture then was purified by HPLC using a 10% to 40% gradient of acetonitrile in 25 mM triethylammonium bicarbonate, pH 8.5 on a phenyl hexyl column.
25 The purified peptide was characterized by LC-MS (ESI+, (M+3)/3: 1,144.37).

Example 21: Synthesis of Exemplary Imaging Agent

[00227] This Example describes the synthesis of the exemplary imaging agent [F5]Lys-His-Pro-Phe-His-Cha-Val-Ile-His-Lys(F5)-[OH] (SEQ ID NO:430) (Agent R24).

[00228] Combined pure fractions of product from Example 20 (1 μmol , in 4 mL of HPLC
30 solvent) were combined with 0.4 mL of concentrated ammonium hydroxide. The solution

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was shielded from light and stirred at room temperature for 16 hours. The mixture was concentrated *in vacuo* and then was purified by HPLC using a 10% to 40% gradient of acetonitrile in 25 mM triethylammonium bicarbonate, pH 8.5 on a phenyl hexyl column. The purified peptide was characterized by LC-MS (ESI+, (M+3)/3: 1,112.37).

5 **Example 22: Synthesis of Exemplary Imaging Agents**

[00229] This Example describes the synthesis of the exemplary imaging agents denoted as Q65, Q66, Q91, Q92, Q93, Q94, R22, R51, R52, R53, R55, R56, R57, R58 and R59.

10 **(Agent Q65) Pentynoyl-Lys(F5)-Gly-Phe-Leu-Gly-βAla-Lys(F5)-PEG20kDa (SEQ ID NO:422)**

[00230] The peptide [pentynoyl]-Lys-Gly-Phe-Leu-Gly-βAla-Lys-[OH] (SEQ ID NO:459) (Tufts University Core Facility, 0.45 μmol) and fluorophore No. 3 from Table 2 (1.0 μmol) were combined in 100 μL of N,N-dimethylformamide (DMF) with 1 μL of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μL of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

20 [00231] The labeled peptide (25 μmol) then was combined with methoxy polyethylene glycol amine (mPEG-NH₂, Laysan Bio, 10 mg, 0.50 μmol) and dissolved in 100 μL DMF with 1 μL NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μmol) and hydroxybenzotriazole (HOBt, 0.2 mg, 1.5 μmol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 25 16 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μL water and purified by HPLC. The mPEG conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

30 **(Agent Q66) Pentynoyl-Lys(F6)-Gly-Phe-Leu-Gly-βAla-Lys(F6)-PEG20kDa (SEQ ID NO:423)**

[00232] The peptide [pentynoyl]-Lys-Gly-Phe-Leu-Gly-βAla-Lys-[OH] (SEQ ID NO:460) (Tufts University Core Facility, 0.45 μmol) and fluorophore No. 2 (Table 2, 1.0

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μmol) were combined in 100 μL of N,N-dimethylformamide (DMF) with 1 μL of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μL of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

[00233] The labeled peptide (25 μmol) was then combined with methoxy polyethylene glycol amine (mPEG-NH₂, Laysan Bio, 10 mg, 0.50 μmol) and dissolved in 100 μL DMF with 1 μL NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μmol) and hydroxybenzotriazole (HOBt, 0.2 mg, 1.5 μmol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μL water and purified by HPLC. The mPEG conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

(Agent 91) Pentynoyl-Lys(F5)-Pro-Leu-Gly-Val-Arg-Lys(F5)-PEG20kDa (SEQ ID NO:424)

[00234] The peptide [pentynoyl]-Lys-Pro-Leu-Gly-Val-Arg-Lys (SEQ ID NO:461) (Tufts University Core Facility, 0.45 μmol) and fluorophore No. 3 from Table 2) (1.0 μmol) were combined in 100 μL of N,N-dimethylformamide (DMF) with 1 μL of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μL of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

[00235] The labeled peptide (25 μmol) was then combined with methoxy polyethylene glycol amine (mPEG-NH₂, Laysan Bio, 10 mg, 0.50 μmol) and dissolved in 100 μL DMF with 1 μL NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC,

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0.25 mg, 1.3 μ mol) and hydroxybenzotriazole (HOBt, 0.2 mg, 1.5 μ mol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was
 5 dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC. The mPEG conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

(Agent Q92) [F5]-His-Gly-Pro-Arg-Lys(F5)-[PEG20kDa] (SEQ ID NO:425)

10 **[00236]** The peptide [H]-His-Gly-Pro-Arg-Lys-[OH] (SEQ ID NO:85) (Tufts University Core Facility, 0.45 μ mol) and fluorophore 3 (Table 2, 1.0 μ mol) were combined in 100 μ L of N,N-dimethylformamide (DMF) with 1 μ L of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was
 15 precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μ L of 0.1 M sodium bicarbonate and purified by HPLC on a 10 mm X 250 mm 300 Å C18 column. The purified peptide was characterized by LC-MS.

[00237] The labeled peptide (25 μ mol) was then combined with methoxy polyethylene glycol amine (mPEG-NH₂, Laysan Bio, 10 mg, 0.50 μ mol) and dissolved in 100 μ L DMF with 1 μ L NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μ mol) and hydroxybenzotriazole (HOBt, 0.2 mg, 1.5 μ mol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 h. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by
 25 centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC on a 21 mm X 250 mm 300 Å C18 column. The mPEG conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

30 **(Agent Q93) [F5]-His-Gly-Pro-Asn-Lys(F5)-His-Gly-Pro-Asn- β A-[PEG20kDa] (SEQ ID NO:426)**

[00238] The peptide [H]-His-Gly-Pro-Asn-Lys-His-Gly-Pro-Asn-[OH] (SEQ ID

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NO:462) (Tufts University Core Facility, 0.45 μ mol) and fluorophore No. 3 from Table 2 (1.0 μ mol) were combined in 100 μ L of N,N-dimethylformamide (DMF) with 1 μ L of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μ L of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

[00239] The labeled peptide (25 μ mol) was then combined with methoxy polyethylene glycol amine (mPEG-NH₂, Laysan Bio, 10 mg, 0.50 μ mol) and dissolved in 100 μ L DMF with 1 μ L NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μ mol) and hydroxybenzotriazole (HOBt, 0.2 mg, 1.5 μ mol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 h. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC. The mPEG conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

20 **(Agent Q94) [F5]-His-Gly-Pro-Asn-Lys(F5)-[PEG20kDa] (SEQ ID NO:427)**

[00240] The peptide [H]-His-Gly-Pro-Asn-Lys-[OH] (SEQ ID NO:75) (Tufts University Core Facility, 0.45 μ mol) and fluorophore No. 3 from Table 2 (1.0 μ mol) were combined in 100 μ L of N,N-dimethylformamide (DMF) with 1 μ L of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μ L of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

30 [00241] The labeled peptide (25 μ mol) was then combined with methoxy polyethylene glycol amine (mPEG-NH₂, Laysan Bio, 10 mg, 0.50 μ mol) and dissolved in 100 μ L DMF with 1 μ L NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC,

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0.25 mg, 1.3 μ mol) and hydroxybenzotriazole (HOBt, 0.2 mg, 1.5 μ mol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was
5 dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC. The mPEG conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

(Agent R22) [F6]-Gly-Pro-Leu-Gly-Val-Arg-Lys(F6)-[YPEG 2x20kDa] (SEQ ID NO:434)

10 **[00242]** The peptide [H]-Gly-Pro-Leu-Gly-Val-Arg-Lys-[OH] (SEQ ID NO:463) (Tufts University Core Facility, 0.45 μ mol) and fluorophore No. 2 of Table 2 (1.0 μ mol) were combined in 100 μ L of N,N-dimethylformamide (DMF) with 1 μ L of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16
15 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μ L of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

[00243] The labeled peptide (25 μ mol) was then combined Y-PEG-amine 2X20k (Y-shape PEG Amine, MW 40000, JenKem USA, 40 mg, 0.50 μ mol) and dissolved in 100
20 μ L DMF with 1 μ L NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μ mol) and hydroxybenzotriazole (HOBt, 0.2 mg, 1.5 μ mol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 1.5
25 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC. The Y-PEG conjugated peptide was further purified by strong anion exchange chromatography. The Y-PEG conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

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(Agent R51) [mPEG-20kDa-Suc]-Lys(F6)-Gly-Phe-Leu-Gly-Lys(F6)-[NH₂] (SEQ ID NO:435)

[00244] The peptide [succinyl]-Lys-Gly-Phe-Leu-Gly-Lys-[NH₂] (SEQ ID NO:464) (Tufts University Core Facility, 0.45 μmol) and fluorophore No. 2 of Table 2 (1.0 μmol) were combined in 100 μL of N,N-dimethylformamide (DMF) with 1 μL of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μL of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

[00245] The labeled peptide (25 μmol) was then combined with methoxy polyethylene glycol amine (mPEG-NH₂, Laysan Bio, 10 mg, 0.50 μmol) and dissolved in 100 μL DMF with 1 μL NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μmol) and hydroxybenzotriazole (HOBt, 0.2 mg, 1.5 μmol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μL water and purified by HPLC. The mPEG conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

(Agent R52) [dPEG-1kDa-Suc]-Lys(F6)-Gly-Phe-Leu-Gly-Lys(F6)-[NH₂] (SEQ ID NO:436)

[00246] The peptide [succinyl]-Lys-Gly-Phe-Leu-Gly-Lys-[NH₂] (SEQ ID NO:465) (Tufts University Core Facility, 0.45 μmol) and fluorophore No. 2 of Table 2 (1.0 μmol) were combined in 100 μL of N,N-dimethylformamide (DMF) with 1 μL of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μL of 0.1 M sodium bicarbonate and

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purified by HPLC. The purified peptide was characterized by LC-MS.

[00247] The labeled peptide (25 μmol) was then combined with Methyl-PEG₂₄-Amine (MA(PEG)24 Thermo Scientific, 0.54 mg, 0.50 μmol) and dissolved in 100 μL DMF with 1 μL NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μmol) and hydroxybenzotriazole (HOBT, 0.2 mg, 1.5 μmol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The discrete-pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The discrete-pegylated peptide was dried briefly under vacuum, dissolved in 350 μL water and purified by HPLC. The dPEG conjugated peptide was characterized by RP-18 HPLC and LC-MS

(Agent R53) [dppa-Suc]-Lys(F6)-Gly-Phe-Leu-Gly-Lys(F6)-[NH₂] (SEQ ID NO:437)

[00248] The peptide [succinyl]-Lys-Gly-Phe-Leu-Gly-Lys-[NH₂] (SEQ ID NO:466) (Tufts University Core Facility, 0.45 μmol) and fluorophore 2 (Table 2, 1.0 μmol) were combined in 100 μL of N,N-dimethylformamide (DMF) with 1 μL of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μL of 0.1 M sodium bicarbonate and purified by HPLC on a 10 mm X 250 mm 300 Å C18 column. The purified peptide was characterized by LC-MS.

[00249] The labeled peptide (25 μmol) was then combined with 3,3-diphenylpropylamine (dppa, 0.11 mg, 0.50 μmol) and dissolved in 100 μL DMF with 1 μL NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μmol) and hydroxybenzotriazole (HOBT, 0.2 mg, 1.5 μmol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 h. The dppa conjugated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The dppa conjugated peptide was dried briefly under vacuum, dissolved in 350 μL water and purified by HPLC. The dppa conjugated peptide was characterized by RP-18 HPLC and LC-MS.

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(Agent R55) [mPEG-20kDa-Suc]-Lys(F6)-Arg-Arg-Lys(F6)-[NH₂] (SEQ ID NO:438)

5 **[00250]** The peptide [succinyl]-Lys-Arg-Arg-Lys-[NH₂] (SEQ ID NO:467) (Tufts University Core Facility, 0.45 μmol) and fluorophore No. 2 of Table 2 (1.0 μmol) were combined in 100 μL of N,N-dimethylformamide (DMF) with 1 μL of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16
10 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μL of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

15 **[00251]** The labeled peptide (25 μmol) was then combined with methoxy polyethylene glycol amine (mPEG-NH₂, Laysan Bio, 10 mg, 0.50 μmol) and dissolved in 100 μL DMF with 1 μL NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μmol) and hydroxybenzotriazole (HOBt, 0.2 mg, 1.5 μmol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE,
20 isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μL water and purified by HPLC. The mPEG conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

(Agent R56) [dPEG-1kDa-Suc]-Lys(F6)-Arg-Arg-Lys(F6)-[NH₂] (SEQ ID NO:439)

25 **[00252]** The peptide [succinyl]-Lys-Arg-Arg-Lys-[NH₂] (SEQ ID NO:468) (Tufts University Core Facility, 0.45 μmol) and fluorophore No. 2 of Table 2 (1.0 μmol) were combined in 100 μL of N,N-dimethylformamide (DMF) with 1 μL of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The
30 solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μL of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

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[00253] The labeled peptide (25 μ mol) was then combined with Methyl-PEG₂₄-Amine (MA(PEG)₂₄ Thermo Scientific, 0.54 mg, 0.50 μ mol) and dissolved in 100 μ L DMF with 1 μ L NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μ mol) and hydroxybenzotriazole (HOBT, 0.2 mg, 1.5 μ mol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The discrete-pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The discrete-pegylated peptide was dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC. The dPEG conjugated peptide was characterized by RP-18 HPLC and LC-MS

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(Agent R57) [dppa-Suc]-Lys(F6)-Arg-Arg-Lys(F6)-[NH₂] (SEQ ID NO:440)

[00254] The peptide [succinyl]-Lys-Arg-Arg-Lys-[NH₂] (SEQ ID NO:469) (Tufts University Core Facility, 0.45 μ mol) and fluorophore No. 2 of Table 2 (1.0 μ mol) were combined in 100 μ L of N,N-dimethylformamide (DMF) with 1 μ L of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide was dried briefly under vacuum, dissolved in 200 μ L of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

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[00255] The labeled peptide (25 μ mol) was then combined with 3,3-diphenylpropylamine (dppa, 0.11 mg, 0.50 μ mol) and dissolved in 100 μ L DMF with 1 μ L NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μ mol) and hydroxybenzotriazole (HOBT, 0.2 mg, 1.5 μ mol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The dppa conjugated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The dppa conjugated peptide was dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC. The dppa conjugated peptide was characterized by RP-18 HPLC and LC-MS.

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(Agent R58) [mPEG-20kDa-Suc]-Lys(F6)-Ala-Arg-Arg-Lys(F6)-[NH₂] (SEQ ID NO:441)

[00256] The peptide [succinyl]-Lys-Ala-Arg-Arg-Lys-[NH₂] (SEQ ID NO:470) (Tufts University Core Facility, 0.45 μmol) and fluorophore No. 2 of Table 2 (1.0 μmol) were
5 combined in 100 μL of N,N-dimethylformamide (DMF) with 1 μL of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide
10 was dried briefly under vacuum, dissolved in 200 μL of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

[00257] The labeled peptide (25 μmol) was then combined with methoxy polyethylene glycol amine (mPEG-NH₂, Laysan Bio, 10 mg, 0.50 μmol) and dissolved in 100 μL DMF with 1 μL NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μmol) and hydroxybenzotriazole (HOBt, 0.2 mg, 1.5 μmol) were added and
15 the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μL water and purified by HPLC. The mPEG
20 conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

(Agent R59) [mPEG-20kDa-Suc]-Lys(F6)-Arg-Arg-Arg-Lys(F6)-[NH₂] (SEQ ID NO:442)

[00258] The peptide [succinyl]-Lys-Ala-Arg-Arg-Lys-[NH₂] (SEQ ID NO:471) (Tufts University Core Facility, 0.45 μmol) and fluorophore No. 2 of Table 2 (1.0 μmol) were
25 combined in 100 μL of N,N-dimethylformamide (DMF) with 1 μL of N-methylmorpholine (NMM) and 0.25 mg of N,N-dimethylaminopyridine (DMAP). The solution was placed on a rotator shielded from light and rotated at room temperature for 16 hours. The labeled peptide was precipitated by addition of 1.5 mL of methyl-t-butyl ether (MTBE) followed by centrifugation and decanting of the supernatant. The solid peptide
30 was dried briefly under vacuum, dissolved in 200 μL of 0.1 M sodium bicarbonate and purified by HPLC. The purified peptide was characterized by LC-MS.

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[00259] The labeled peptide (25 μ mol) was then combined with methoxy polyethylene glycol amine (mPEG-NH₂, Laysan Bio, 10 mg, 0.50 μ mol) and dissolved in 100 μ L DMF with 1 μ L NMM. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.25 mg, 1.3 μ mol) and hydroxybenzotriazole (HOBT, 0.2 mg, 1.5 μ mol) were added and the solution was placed on rotator shielded from light and rotated at room temperature for 16 hours. The pegylated peptide was precipitated by addition of 1.5 mL of MTBE, isolated by centrifugation and decanting of the supernatant. The pegylated peptide was dried briefly under vacuum, dissolved in 350 μ L water and purified by HPLC. The mPEG conjugated peptide was characterized by RP-18 HPLC and SEC HPLC.

10 **Example 23: Tumor Imaging**

[00260] NU/NU mice 6-8 weeks old (Charles River Laboratory, Wilmington, MA) were injected SC with 2 x 10⁶ 4T-1 or HT-29 cells bilaterally in each mammary fat pad. When tumors reached an approximate size of 3 x 3 mm (around 7 days after cell injection), mice were injected intravenously with 2-4 nmoles of each imaging agent (5 mice/probe and 2 mice/ no probe as control) in 100 μ L volume via tail vein. Imaging was conducted at 24 hours using a Fluorescence Molecular Tomography system (VisEn Medical, Woburn MA).

[00261] Pentynoyl-Lys(F5)-Gly-Phe-Leu-Gly- β Ala-Lys(F5)-PEG20kDa (SEQ ID NO:422) (Agent Q65) was used to image tumors *in vivo*. Figure 1 shows the planar image of bilateral 4T1 tumors 6 hours after administration of the agent. Figure 2 shows a fluorescence molecular tomography bilateral image of 4T1 tumors 6 hours after administration of agent Q65.

[00262] Pentynoyl-Lys(F6)-Gly-Phe-Leu-Gly- β Ala-Lys(F6)-PEG20kDa (SEQ ID NO:423) (Agent Q66) was also used to image tumors *in vivo*. Figure 3 shows the image of the 4T1 tumors at 5, 24 and 52 hours after administration of agent Q66.

[00263] Pentynoyl-Lys(F5)-Pro-Leu-Gly-Val-Arg-Lys(F5)-PEG20kDa (SEQ ID NO:424) (Agent Q91) was also used to image tumors *in vivo*. Figure 4 shows planar fluorescence reflectance images of HT-29 xenograft tumors at 2, 6 and 24 hour after administration of Agent Q91.

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Example 24: Enzyme Activation Profile of Imaging Agent Q65

[00264] In this Example, the enzyme activation profile of imaging agent pentynoyl-Lys(F5)-Gly-Phe-Leu-Gly- β Ala-Lys(F5)-PEG20kDa (SEQ ID NO:422) (Agent Q65) was characterized.

- 5 [00265] The reactions were performed with appropriate buffer at room temperature in a 96-well plate with a final assay volume of 250 μ L and probe and enzyme concentrations of 0.5 μ M and 0.05 μ M, respectively. The buffer for Cathepsin B was 25 mM MES (pH 5.0), 1 mM DTT. Recombinant human Cathepsin B (R&D Systems) was activated in buffer of 25 mM MES (pH 5.0) and 5 mM DTT for 15 minutes at room temperature.
- 10 Recombinant human Cathepsin L (R&D Systems) was activated in buffer of 50 mM MES (pH 6.0), 5 mM DTT, for 15 minutes on ice. Recombinant mouse Cathepsin Z (R&D Systems) was activated in buffer of 25 mM NaOAc (pH 3.0), 5 mM DTT for 5 minutes at room temperature. The buffer for plasmin (CalBiochem) and trypsin (Sigma) was PBS at pH 7.4.
- 15 [00266] The plate was incubated at room temperature and the activity monitored at 0, 1, 3, 5, and 24 hours with a Gemini (Molecular Devices) plate reader at excitation wavelength 663 nm, emission 690 nm, and cut off 665 nm. The activity was calculated as the fold of activation by comparing the fluorescence of the enzyme with probe to the fluorescence of probe alone.
- 20 [00267] The results are shown in Figure 5, which show that the imaging agent Q65 is activated by Cathepsin B but not by plasmin, trypsin and Cathepsin Z.

Example 25: Enzyme Activation Profile of Imaging Agents Q92 and Q93

- [00268] In this Example, the enzyme activation profile of imaging agents [F5]-His-Gly-Pro-Arg-Lys(F5)-[PEG20kDa] (SEQ ID NO:425) (Agent Q92) and [F5]-His-Gly-Pro-
- 25 Asn-Lys(F5)-His-Gly-Pro-Asn- β A-[PEG20kDa] (SEQ ID NO:426) (Agent Q93) were characterized.

- [00269] The reactions were performed with appropriate buffer at room temperature in a 96-well plate with a final assay volume of 250 μ L and probe and enzyme concentrations of 0.5 μ M and 0.05 μ M respectively. The buffer for activated humanized rabbit Cathepsin
- 30 K (Merck) was 50 mM MES (pH 5.0), 2.5 mM EDTA, 2.5 mM DTT. The buffer for

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Cathepsin B was 25 mM MES (pH 5.0), 1 mM DTT. The recombinant human Cathepsin B (R&D Systems) was activated in buffer of 25 mM MES (pH 5.0) and 5 mM DTT for 15 minutes at room temperature. The recombinant human Cathepsin L (R&D Systems) was activated in buffer of 50 mM MES (pH 6.0), 5 mM DTT, for 15 minutes on ice. The
5 buffer for MMP-2 (BIOMOL) and MMP-9 (BIOMOL) was 50 mM Tris (pH 7.5), 10 mM CaCl₂, 150 mM NaCl, and 0.05% Brij-35 detergent. The plate was incubated at room temperature and the activity was monitored at 0, 1, 3, 5, and 24 hours with a Gemini (Molecular Devices) plate reader at excitation wavelength 663 nm, emission 690 nm, and cut off 665 nm. The released fluorescent unit was determined by subtracting the
10 fluorescence of the probe alone from the total fluorescence.

[00270] The results for Q92 are presented in Figure 6A, and the results for Q93 are shown in Figure 6B, which show that both Q92 and Q93 are activated primarily by Cathepsin K.

Example 26: Bone Imaging

15 [00271] BALB/c female mice 7 weeks old (Charles River Laboratory, Wilmington, MA) were subjected to ovariectomy or sham surgery to induce osteoporosis. After one to two weeks after surgery, the mice were injected intravenously with imaging agent [F5]-His-Gly-Pro-Asn-Lys(F5)-[PEG20kDa] (SEQ ID NO:427) (Agent Q94). Imaging was conducted at various time points using a Fluorescence Molecular Tomography system
20 (VisEn Medical, Bedford, MA). The results are shown in Figure 7.

[00272] Figure 7A shows images of an ovariectomized mouse (OVX) compared to a control (sham) mouse at 4 and 24 hours post-injection. The quantitation of absolute fluorescence of osteoporosis for the sham and ovariectomized mice is shown in Figure 7B. The increased probe signal detected in the tibia region appears to be due to increased
25 Cathepsin K levels in bone loss induced by the ovariectomy procedure.

Example 27: Cardiovascular Disease Imaging

[00273] This Example show the imaging of cardiovascular disease using the imaging agent Pentynoyl-Lys(F5)-Gly-Phe-Leu-Gly-βAla-Lys(F5)-PEG20kDa (SEQ ID NO:422) (Agent Q65).

30 [00274] High cholesterol fed apoE^{-/-} mice were injected with 2 nmol of imaging agent

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and *in vivo* imaging was performed with *ex vivo* fluorescence reflectance imaging. Figure 8 shows fluorescence reflectance imaging of cardiovascular disease in inflamed atherosclerotic plaques of mice 24 hours post-injection of agent Q65. Fluorescence was detected in plaque-laden vascular sections in the aortic root, the arch and carotid arteries.

5 **Example 28: Inflammation Imaging (Carrageenan Induced Paw Edema)**

[00275] This Example shows the imaging of inflammation in a mouse model. Briefly, BALB/c female mice 6-8 weeks old (Charles River Laboratory, Wilmington, MA) were injected subcutaneously with a 1% solution of carrageenan in PBS. After 1 minute post-injection, mice were injected the imaging agent Q91. Imaging was conducted at various
10 time points using a Fluorescence Molecular Tomography system (VisEn Medical, Woburn MA). An image is shown in Figure 9, which shows that significantly more fluorescence is observed in the mouse paw injected with carrageenan compared to the control paw (no injection).

Example 29: Enzyme Activation Profiles of Agents R20, R21, R23, R24, and R27

15 [00276] In this Example, the enzyme activity profiles of the agents ([F5]-Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[OH]) (SEQ ID NO:401) (R20), ([F5]-Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHmPEG20k]) (SEQ ID NO:428) (R21), ([F5]-Lys(Ac)-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[OH]) (SEQ ID NO:429) (R23), ([F5]-Lys-His-Pro-Phe-His-Cha-Val-Ile-His-Lys(F5)-[OH]) (SEQ ID NO:430) (R24), and
20 ([F5]-Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHmPEG5k]) (SEQ ID NO:433) (R27) were characterized.

[00277] The reactions were performed with appropriate buffer in a 96-well plate with a final assay volume of 250 μ L and probe and enzyme concentrations of 1 μ M and 100 nM, respectively. The reactions were incubated at 37 C for Renin and room temperature for
25 the other proteases. The buffer for human neutrophil Cathepsin G (BIOMOL) was 100 mM Tris (pH 7.5), 1.6 M NaCl. The buffer for Cathepsin D (BIOMOL) was 100 mM Formic Acid (pH 3.3). The buffer for recombinant human neutrophil elastase (Innovative Research) was 100 mM Tris (pH 7.5). The buffer for plasmin (CalBiochem) and trypsin (Sigma) was 1X phosphate buffered saline (PBS) (pH 7.4). The buffer for recombinant
30 human Renin (rhRenin) (Proteos) and recombinant rat Renin (rrRenin) (Proteos) was 50

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mM MOPS (pH 7.4), 100 mM NaCl, with freshly prepared 0.002% tween-20. The plate was incubated at the appropriate temperature and the activity was monitored at 24 hours with a Gemini (Molecular Devices) plate reader at excitation wavelength 663 nm, emission 690 nm, and cut off 665 nm. The released fluorescent unit was determined by subtracting the fluorescence of the probe alone from the total fluorescence.

[00278] The results are shown in Figure 11, which show that agents R20, R23, and R24 were activated primarily by recombinant human rennin. Agents R21 and R27 were activated by recombinant human rennin, human neutrophil elastase, human neutrophil cathepsin, and trypsin.

10 **Example 30: Renal Imaging**

[00279] This example shows the *in vivo* imaging of kidneys in mice on a control diet versus a low sodium diet.

[00280] C57BL6 mice (Jackson Laboratories) were fed a sodium deficient diet (0.02% Na) and water was replaced with a solution of amiloride (0.1 mg/5mL or 5 mg/kg/day) for 2 days. Control C57BL6 mice were fed regular chow diet and water. Imaging agents [F5]-Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[OH] (SEQ ID NO:401) (R20) and ([F5]-Lys-His-Pro-Phe-His-Leu-Val-Ile-His-Lys(F5)-[NHmPEG20k] (SEQ ID NO:428) (R21) were injected intravenously at different concentrations (0.2, 0.7, 2 and 4 nmol/mouse) following 2 days on a low salt diet. Imaging was conducted at 24 hrs using a Fluorescence Molecular Tomography system (VisEn Medical, Woburn MA).

[00281] The results are shown in Figure 12, which demonstrate that for both imaging agents, the kidneys in the mice on the low sodium diet (Figures 12B and 12D) gave an increased signal relative to the kidneys in the mice on the control diet (Figures 12A and 12C).

25 **Example 31: Enzyme Activation of Imaging Agent R22**

[00282] In this Example, the enzyme activation profile of the imaging agent [F6]-Gly-Pro-Leu-Gly-Val-Arg-Lys(F6)-[YPEG 2x20kDa] (SEQ ID NO:434) (R22) was characterized.

[00283] Activation by recombinant enzymes was carried out in 250 μ L volumes set up in 96 well plates. MMPs were activated prior to the reaction according to manufacturers'

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protocols. Reactions contained 0.5 μ M of R22 and 0.05 μ M of activated enzyme in the appropriate activation buffer for each enzyme and incubated at room temperature for 24 hours. The plates were read at 24 hours with excitation/emission wavelength 740/770 nm. Fluorescence readings were performed using a Gemini (Molecular Devices) Fluorescence Plate Reader. Wells in which no enzyme was added were used as controls. Fold activation was obtained by dividing the enzyme-activated read-out (in relative fluorescence units) by its corresponding control well.

[00284] An example of the activation of the R22 by MMPs and other proteases is shown in Figure 13, which shows that R22 is activated primarily by recombinant human MMP-13 and trypsin.

Example 32: Tumor Imaging With Agent R22

[00285] This Example describes the imaging of tumors *in vivo* using imaging agent R22.

[00286] Male and female NU/NU Mice (Charles River Laboratories, Wilmington, MA), 6-8 weeks of age, were injected with tumor cells bilaterally in the first or second mammary fat pads (human colorectal cancer cell line HT-29: 3×10^6 cells/site). Once the tumors reached the desired volume, as measured with calipers (100 mm^3) mice were injected intravenously with 2 nmoles of compound R22. Imaging was performed at 6 and 24 hours later using the Fluorescence Tomography System (FMT2500) (VisEn Medical, Bedford, MA). For *in vivo* imaging, mice were anesthetized by gas anesthesia (isoflurane/oxygen mixture), placed in the FMT2500 system imaging chamber one at a time and imaged using the reflectance and tomographic modes. Examples of imaging with the agent in the HT-29 tumor model are shown in Figure 14.

[00287] The images taken by reflectance imaging after 6 hours are shown in Figure 14A and after 24 hours in Figure 14B. Tomographic images taken after 6 hours are shown in Figure 14C and after 24 hours in Figure 14D.

Example 33: Enzyme Activation Profiles of Agents R51, R52, R55, R56, R57, R58, and R59

[00288] This Example shows the enzymatic activation profiles for compounds ([mPEG-20kDa-Suc]-Lys(F6)-Gly-Phe-Leu-Gly-Lys(F6)-[NH₂]) (SEQ ID NO:435) (R51), ([dPEG-1kDa-Suc]-Lys(F6)-Gly-Phe-Leu-Gly-Lys(F6)-[NH₂]) (SEQ ID NO:436) (R52),

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([dppa-Suc]-Lys(F6)-Gly-Phe-Leu-Gly-Lys(F6)-[NH₂]) (SEQ ID NO:437) (R53),
 ([mPEG-20kDa-Suc]-Lys(F6)-Arg-Arg-Lys(F6)-[NH₂]) (SEQ ID NO:438) (R55),
 ([dPEG-1kDa-Suc]-Lys(F6)-Arg-Arg-Lys(F6)-[NH₂]) (SEQ ID NO:439) (R56), ([dppa-
 Suc]-Lys(F6)-Arg-Arg-Lys(F6)-[NH₂]) (SEQ ID NO:440) (R57), ([mPEG-20kDa-Suc]-
 5 Lys(F6)-Ala-Arg-Arg-Lys(F6)-[NH₂]) (SEQ ID NO:441) (R58), and ([mPEG-20kDa-
 Suc]-Lys(F6)-Arg-Arg-Arg-Lys(F6)-[NH₂]) (SEQ ID NO:442) (R59).

[00289] Compounds at a concentration of 0.5 uM were incubated with Cathepsin B, Cathepsin K, Cathepsin L, or Cathepsin S at a concentration of 0.05 uM. The reaction buffers used were as follows: Cathepsin K – 50 mM MES, pH 5.0, 2.5 mM EDTA, 2.5
 10 mM DTT; Cathepsin B – 25 mM MES, pH 5.0, 1 mM DTT; Cathepsin S – 50 mM NaOAc, pH 4.5, 5 mM DTT, 0.25M NaCl; Cathepsin L – 50 mM MES, pH 6, 5 mM DTT. The activity was monitored for 24 hours with a Gemini (Molecular Devices) fluorescence plate reader at excitation 663 nm and emission 690 nm with a cut off of 690 nm. Figure
 15 indicates that activation of imaging agent R51 by various Cathepsin proteases is greater than that of the other imaging agents.

Example 34: Imaging of Macrophage Activity in Atherosclerotic Plaque

[00290] Age-matched C57BL6 wild type female mice (Charles river Laboratories) were fed a normal diet and ApoE -/- knockout female mice (Jackson Laboratories) were fed a high cholesterol diet for 10-20 weeks. The mice were injected intravenously with
 20 compounds of formula R51 ([mPEG-20kDa-Suc]-Lys(F6)-Gly-Phe-Leu-Gly-Lys(F6)-[NH₂]) (SEQ ID NO:435) and R55 ([mPEG-20kDa-Suc]-Lys(F6)-Arg-Arg-Lys(F6)-[NH₂]) (SEQ ID NO:438). The mice were then imaged at 6 and 24 hours by fluorescence molecular tomography or fluorescence reflectance using an FMT2500 (VisEn Medical, Bedford, MA).

25 **[00291]** Figure 16 shows an example of fluorescence imaging using compound R51. Figure 16A shows fluorescence imaging of the aortic arch in ApoE -/- and wild type mice at 6 hours post-injection. Figure 16B compares the quantified fluorescence of wild type versus ApoE -/- knockout mice at 6 hours post-injection with compound R51. Figure 16C displays the reflectance imaging of the same mice with compound R51 at 6 hours. Figure
 30 16D compares the quantified fluorescence of wild type versus ApoE -/- knockout mice at 6 hours post-injection with compound R51. Imaging agent R51 produced a greater

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fluorescent signal in the ApoE knockout mice over controls demonstrating the use of R51 in imaging cardiovascular disease models.

[00292] Figure 17A shows an example of fluorescence molecular tomographic imaging of mice at 6 hours post-injection with R55. Figure 17B compares the quantified
5 fluorescence of wild type versus ApoE knockout mice at 6 hours post-injection with compound R55. Imaging agent R51 produced a greater fluorescent signal in the ApoE knockout mice over controls, demonstrating the use of R51 in imaging cardiovascular disease models.

INCORPORATION BY REFERENCE

10 [00293] All publications, patents, and patent applications cited herein are hereby expressly incorporated by reference in their entirety and for all purposes to the same extent as if each was so individually denoted.

EQUIVALENTS

15 [00294] The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are intended to be embraced therein.

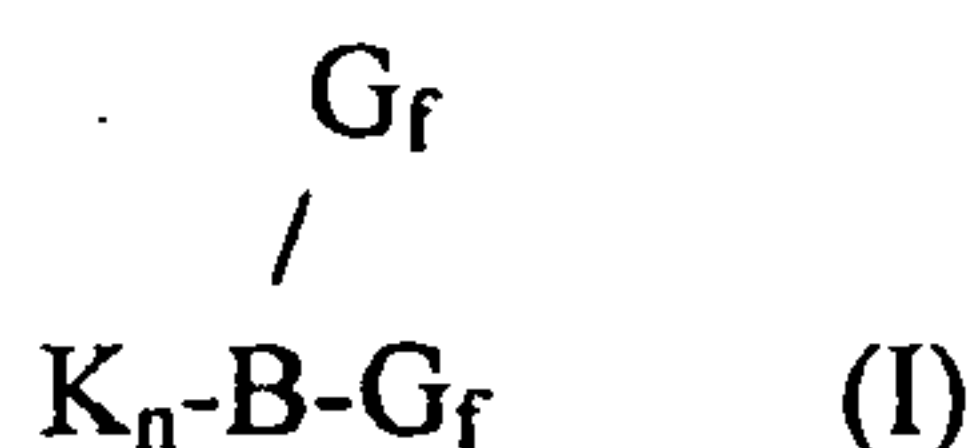
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1 CLAIMS

1 1. An intramolecularly-quenched imaging agent comprising:

2 (a) an enzymatically cleavable oligopeptide comprising from about 2 to about 30 amino
3 acid residues;4 (b) an optional biological modifier chemically linked to the enzymatically cleavable
5 oligopeptide; and6 (c) either two fluorophores or one fluorophore and one quencher, each covalently
7 linked, directly or indirectly, to the oligopeptide or to the optional biological modifier,
8 wherein one fluorophore quenches the other fluorophore or the quencher quenches the
9 fluorophore and upon enzymatic cleavage of the oligopeptide, at least one fluorophore
10 becomes unquenched and is capable of producing a greater fluorescent signal when
11 excited by electromagnetic radiation than before enzymatic cleavage of the
12 oligopeptide.1 2. The agent of claim 1, wherein the oligopeptide comprises from about 2 to about 25 amino
2 acid residues, from about 2 to about 14 amino acid residues, from about 4 to about 10
3 amino acid residues, or from about 5 to about 8 amino acid residues.1 3. The agent of claim 1 or 2, further comprising a non-fluorescent reporter covalently
2 associated with the enzymatically cleavable oligopeptide or the optional biological
3 modifier.

1 4. The agent of any one of claims 1-3, represented by Formula I:



6 wherein:

7 B is [M_m-ECO-M_m];

8 ECO is an enzymatically cleavable oligopeptide;

9 G is L-F;

10 F is a fluorophore or quencher;

11 M is a biological modifier;

12 K is N-L;

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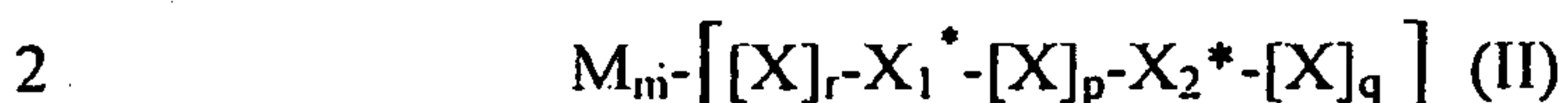
13 N is a non-fluorescent reporter;
 14 L, independently, for each occurrence, is a linker moiety or a bond;
 15 n is an integer from 0 to 3;
 16 m, independently, for each occurrence, is 0 or 1, and optionally at least one m is
 17 1; and
 18 f, independently, for each occurrence, is an integer from 0 to 2, wherein the total
 19 number of Fs in the agent is no greater than 2.

1 5. The agent of claim 4, wherein each F is chemically linked, directly or through the linker
 2 moiety L, to a separate amino acid of the oligopeptide.

1 6. The agent of claim 4, wherein at least one F is chemically linked, directly or through the
 2 linker moiety L, to M.

1 7. The agent of any one of claims 4-6, wherein ECO is a cyclic oligopeptide.

1 8. The agent of any one of claims 1-6, wherein the agent is represented by Formula II:



3 wherein:

4 X, independently, for each occurrence, is an amino acid residue;

5 X_1^* and X_2^* are each independently X-L-F;

6 L, independently, for each occurrence, is a linker moiety or a bond;

7 F is a fluorophore;

8 M is a biological modifier;

9 m is 0, 1 or 2;

10 r is an integer from 0 to 28;

11 p is an integer from 1 to 28;

12 q is an integer from 0 to 28; wherein the sum of r, p and q is no greater than 28.

1 9. The agent of claim 8, wherein the amino acid residue X_2^* is lysine.

1 10. The agent claim 8 or 9, wherein the amino acid residue X_1^* is lysine.

1 11. An intramolecularly-quenched imaging agent represented by Formula III:



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wherein:

ECO is an enzymatically cleavable oligopeptide;

G is L-F;

F, independently, is selected from a fluorophore or quencher, wherein at least one F is a fluorophore;

L, independently for each occurrence, is selected from a linker moiety or a bond;

M is a biological modifier;

K is L-N;

N is a non-fluorescent reporter;

p is an integer from 1 to 4;

n is an integer from 0 to 3; and

g, independently, for each occurrence, is an integer from 1 to 2, wherein the sum of each occurrence of g is no greater than 2.

12. The agent of claim 11, wherein the oligopeptide comprises from 5 to 8 amino acid residues.

13. The agent of claim 11 or 12, wherein at least one F is chemically linked, directly or indirectly, to a lysine residue.

14. A cyclic intramolecularly quenched imaging agent comprising:

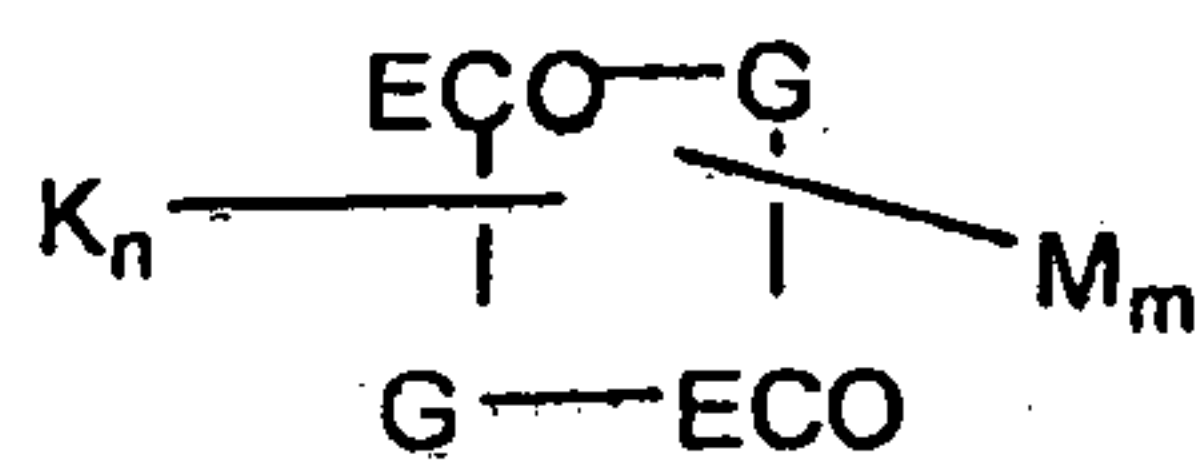
a) a first fluorophore chemically linked, directly or indirectly, to the C-terminus of a first cleavable oligopeptide and chemically linked, directly or indirectly, to the N-terminus of a second, optionally cleavable, oligopeptide;

b) a second fluorophore chemically linked, directly or indirectly, to the N-terminus of the first cleavable oligopeptide and chemically linked directly or indirectly, to the C-terminus of the second, optionally cleavable oligopeptide; and

c) optionally, at least one biological modifier chemically linked to the first or second oligopeptide or fluorophore.

15. The agent of claim 14, represented by Formula IV:

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IV

wherein

ECO, independently, for each occurrence, is an enzymatically cleavable oligopeptide;

G is L-F-L;

F, independently, for each occurrence, is a fluorophore;

L, independently, for each occurrence, is a linker moiety or a bond;

M is a biological modifier;

K is L-N;

N is a non-fluorescent reporter;

n is an integer from 0 to 3; and

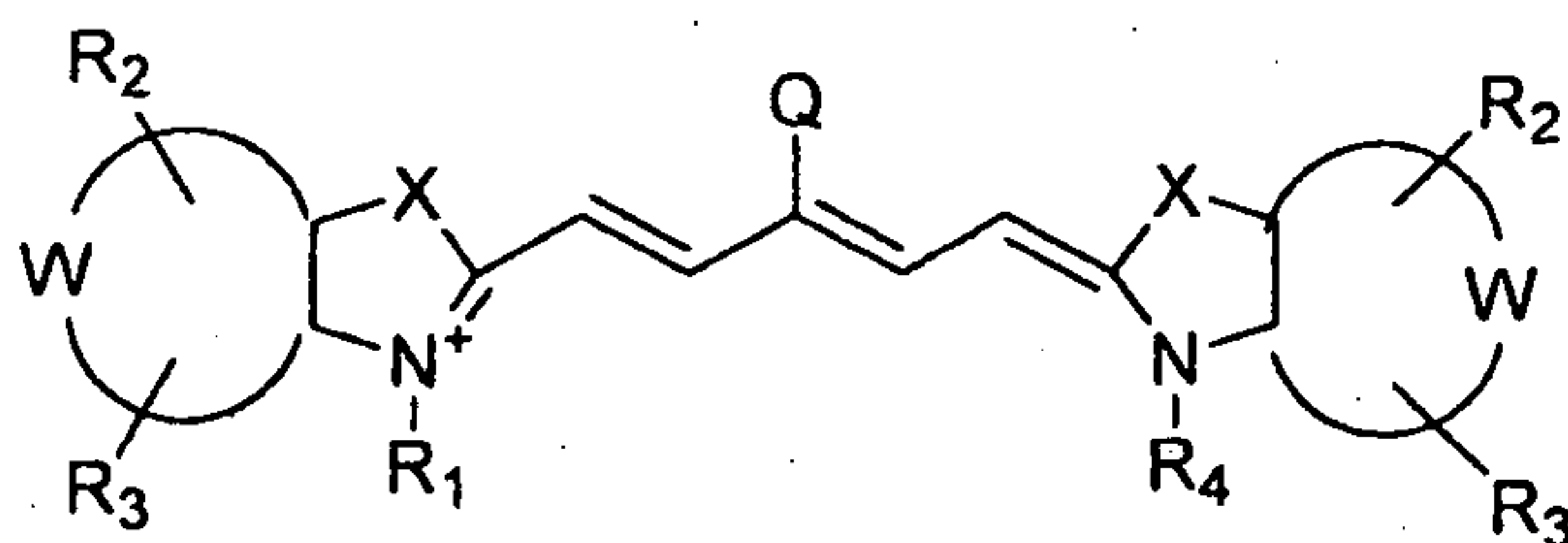
m is an integer from 0 to 3.

16. The agent of any one of claims 1-15, wherein F is a far-red or a near-infrared fluorophore.

17. The agent of claim 16, wherein the fluorophore is a carbocyanine fluorophore.

18. The agent of claim 16, wherein the fluorophore is an indocyanine fluorophore.

19. The agent of any one of claims 1-16, wherein the fluorophore is represented by the following general Formula VII:



(VII)

or a salt thereof, wherein:

X is independently, for each occurrence, selected from the group consisting of C(CH₂Y₁)(CH₂Y₂), O, S, and Se;

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Y₁ and Y₂ are independently selected from the group consisting of H and a C₁-C₂₀ aliphatic group optionally substituted with -OR*, N(R*)₂ or -SR*, wherein R* is H or alkyl;

W represents a benzo-condensed, a naphtho-condensed or a pyrido-condensed ring;

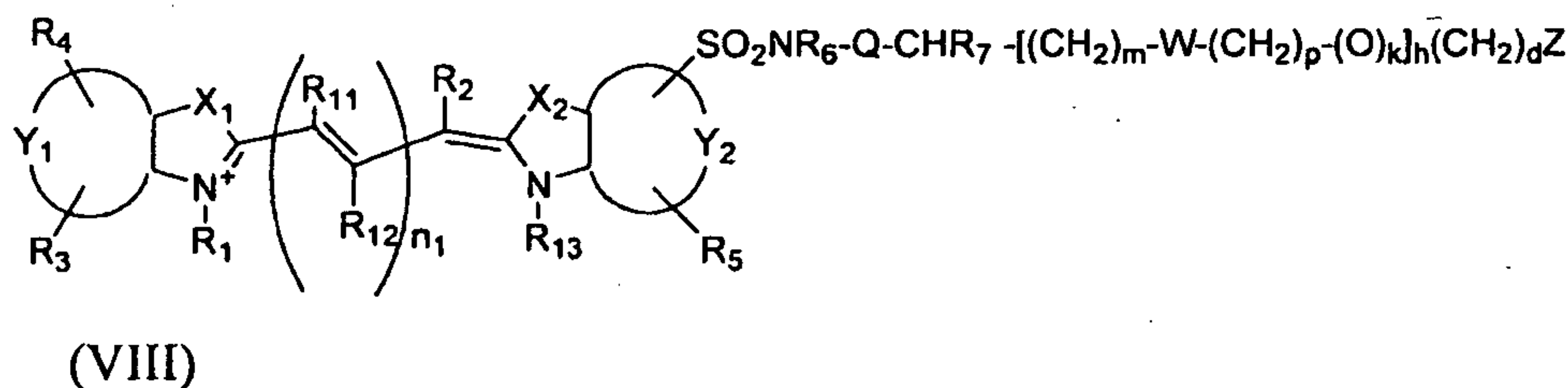
R₁ is selected from the group consisting of (CH₂)_xCH₃, (CH₂)_ySO₃⁻ and (CH₂)_ySO₃H, wherein x is an integer selected from 0 to 6 and y is an integer selected from 2 to 6;

R₂ and R₃ independently are selected, for each occurrence, from the group consisting of H, carboxylate, carboxylic acid, carboxylic ester, amine, amide, sulfonamide, hydroxyl, alkoxyl, a sulphonic acid moiety and a sulphonate moiety;

R₄ is selected from the group consisting of (CH₂)_xCH₃, (CH₂)_ySO₃⁻ and (CH₂)_nSO₃H, wherein x is an integer selected from 0 to 6 and y is an integer selected from 2 to 6; and

Q is selected from a group consisting of a heteroaryl ring substituted with a carboxyl group or 6-membered heteroaryl ring substituted with a carbonyl group.

20. The agent of any one of claims 1-16, wherein the fluorophore is represented by the general Formula VIII:



or a salt thereof, wherein:

X₁ and X₂ are independently selected, for each occurrence from the group consisting of C(CH₂K₁)(CH₂K₂), O, S and Se;

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K₁ and K₂ are independently selected from the group consisting of H, a C₁-C₂₀ aliphatic group optionally substituted with -OR*, N(R*)₂ or -SR*; or K₁ and K₂ together form part of a substituted or unsubstituted carbocyclic, or heterocyclic ring;

Y₁ and Y₂ are each independently a benzo-condensed ring, a naphtha-condensed ring or a pyrido-condensed ring;

n₁ is 1, 2, or 3;

R₂, R₁₁ and R₁₂ are independently selected from the group consisting of H, F, Br, Cl, C₁-C₆ alkyl, C₁-C₆ alkoxy, aryloxy, a nitrogen-containing heterocyclic ring, a nitrogen-containing heteroaromatic ring, a sulfonate, an iminium ion, or any two adjacent R₁₂ and R₁₁ substituents or R₂ and R₁₁ substituents, when taken in combination, form a 4-, 5-, or 6-membered substituted or unsubstituted carbocyclic ring, substituted or unsubstituted non-aromatic carbocyclic ring or a substituted or unsubstituted carbocyclic aryl ring, wherein the carbocyclic rings are each independently optionally substituted one or more times by C₁-C₆ alkyl, halogen, or OR* or SR*;

R₁ and R₁₃ are (CH₂)_xCH₃, when x is an integer selected from 0 to 6; or R₁ and R₁₃ are independently (CH₂)_nSO₃⁻ or (CH₂)_nSO₃H when n is an integer selected from 2 to 6;

R₃, R₄ and R₅ are independently selected from the group consisting of H, carboxylate, carboxylic acid, carboxylic ester, amine, amide, sulfonamide, hydroxyl, alkoxy, a sulphonic acid moiety and a sulphonate moiety;

Q is absent, or is selected from a carbonyl moiety or a substituted or unsubstituted C₁-C₆ alkyl group, wherein 0-2 of the methylene groups of the alkyl group can optionally be replaced by NH, O or S, or a substituted or unsubstituted C₁-C₆ carbocyclic, non-aromatic carbocyclic, heterocyclic or non-aromatic heterocyclic ring wherein the heterocyclic rings contains 1-2 heteroatoms;

R₆ is selected from the group consisting of H, a substituted or unsubstituted C₁-C₂₀ aliphatic group, a substituted or unsubstituted aryl, a substituted or unsubstituted alkylaryl, wherein R₆ is optionally substituted with halogen, OR*, N(R*)₂ or SR*, when Q is absent, a carbonyl group, a substituted or unsubstituted C₁-C₆ alkyl group, wherein 0-2 of the methylene groups of the alkyl group are replaced by NH, O or S, or a substituted or unsubstituted C₁-C₆ carbocyclic, non-aromatic carbocyclic, heterocyclic

- 101 -

or non-aromatic heterocyclic ring wherein the heterocyclic rings contains 1-2 heteroatoms; or

R_6 is H, when Q is a carbonyl; and

R_7 is selected from the group consisting of H, a substituted or unsubstituted C_1 - C_{20} aliphatic group, a substituted or unsubstituted aryl, a substituted or unsubstituted alkylaryl, wherein R_7 is optionally substituted with halogen, OR^* , $N(R^*)_2$ or SR^* ; or

R_6 and R_7 , taken together form a 4-, 5-, 6- or 7-membered heterocyclic or non-aromatic heterocyclic ring optionally substituted with halogen, OR^* , $N(R^*)_2$ or SR^* ; or

NR_6 , Q and CHR_7 together form a substituted or unsubstituted or heterocyclic or non-aromatic heterocyclic ring system wherein the rings contain 1 or 2 heteroatoms, wherein rings are optionally substituted with $-OR^*$, $N(R^*)_2$ or $-SR^*$; and

W is absent or is a group selected from the group consisting of $-SO_2NR_6-Q-CHR_7-$, $-O-$, $-COO-$, and $-CONH-$;

$h = 0-70$; $k = 0$ or 1 ; $d = 0-12$; $m = 0-12$; $p = 0-12$;

Z is a N, O or S nucleophile functionality moiety or is, or contains a functionality capable of reacting with N, O or S nucleophiles; and each R^* is independently H or C_{1-20} alkyl.

21. The agent of any one of claims 4-20, wherein L comprises a moiety selected from the group consisting of an amido bond, amino-polyethylene glycol-carboxylic acid, amino-polyethylene glycol azide, diaminoPEG, cysteic acid, glutamic acid, aminocaproic acid, ethylenediamine, propylenediamine, spermidine, spermine, hexanediamine, and a diamine-amino acid.

22. The agent of any one of claims 1-21, wherein the biological modifier has a molecular weight from about 10 kDa to about 35 kDa.

23. The agent of any one of claims 1-22, wherein the biological modifier is selected from the group consisting of polyethylene glycol, methoxypolyethylene glycol, branched polypropylene glycol, polypropylene glycol, a graft copolymer of poly-lysine and methoxypolyethyleneglycol, a fatty acid, a lipid, and a phospholipid.

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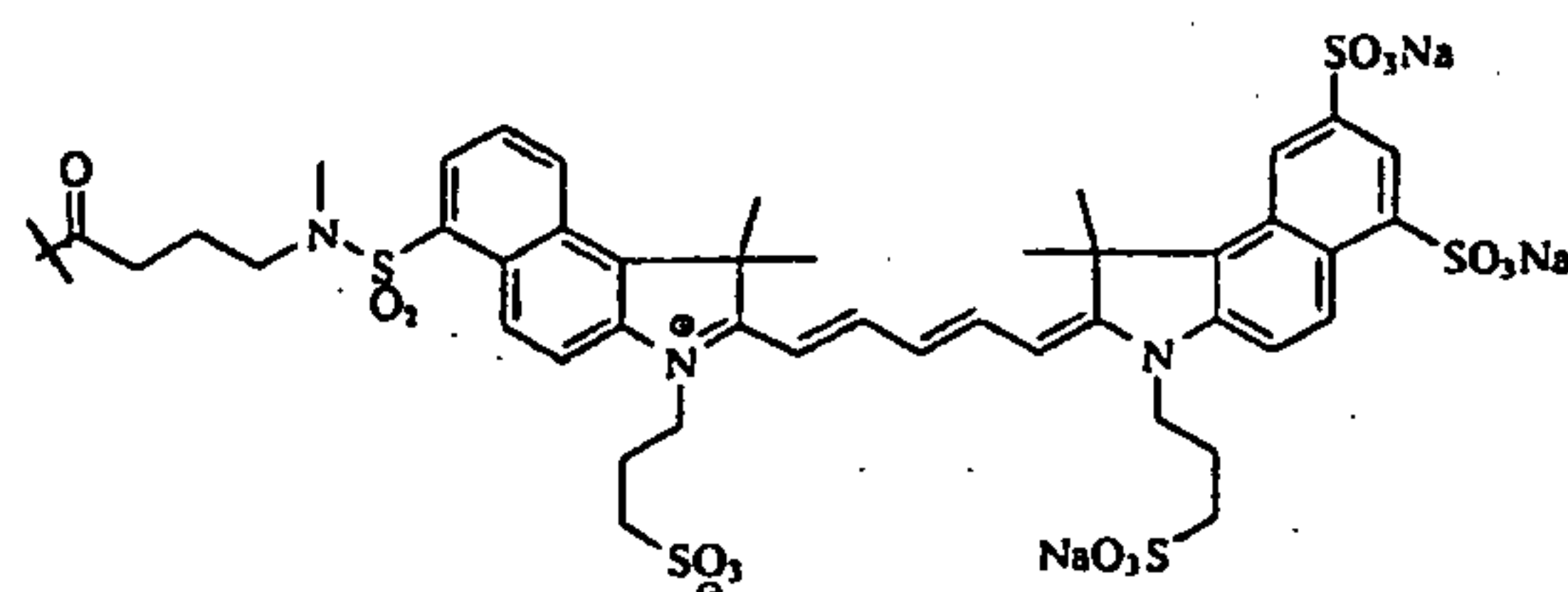
- 1 24. The agent of any one of claims 1-22, wherein the biological modifier is selected from the
2 group consisting of an amino acid, a peptide, a carbohydrate, a dextran, a sulfonate and a
3 polysulfonate.
- 1 25. The agent of any one of claims 1-22, wherein the biological modifier is selected from the
2 group consisting of glutamic acid, cysteic acid, naphthylalanine, phenylalanine,
3 diphenylpropylamine, 4,4-diphenylcyclohexanol, glucosamine, mannosamine,
4 galactosamine, arginine, lysine, homolysine and leucine.
- 1 26. The agent of any one of claims 1-25, wherein the biological modifier is covalently linked
2 to the enzymatically cleavable peptide at a position that is not between two amino acids
3 covalently linked to a fluorophore or a quencher.
- 1 27. The agent of any one of claims 1-26, wherein the fluorophores are the same.
- 1 28. The agent of any one of claims 1-26, wherein the fluorophores are different.
- 1 29. The agent of any one of claims 1-28 wherein the biological modifier is covalently linked
2 to the enzymatically cleavable oligopeptide through an acyl moiety.
- 1 30. The agent of any one of claims 1-28, wherein the biological modifier is covalently linked
2 to the enzymatically cleavable oligopeptide by a 3+2 cycloaddition reaction between an
3 azide moiety chemically linked to the biological modifier and an alkyne moiety
4 chemically linked to an N-terminus of the enzymatically cleavable oligopeptide or
5 wherein the biological modifier is chemically linked to the enzymatically cleavable
6 oligopeptide by a 3+2 cycloaddition reaction between an alkyne moiety chemically linked
7 to the biological modifier and an azide moiety covalently linked to the N-terminus of the
8 enzymatically cleavable oligopeptide.
- 1 31. The agent of any one of claims 1-28, wherein the biological modifier is covalently linked
2 to the enzymatically cleavable oligopeptide by an amide coupling reaction between the
3 biological modifier and the C-terminus of the enzymatically cleavable oligopeptide.
- 1 32. The agent of any one of claims 1-28, wherein the biological modifier is covalently linked
2 to the enzymatically cleavable oligopeptide by a thiol coupling reaction between the
3 biological modifier and the C-terminus of the enzymatically cleavable oligopeptide.

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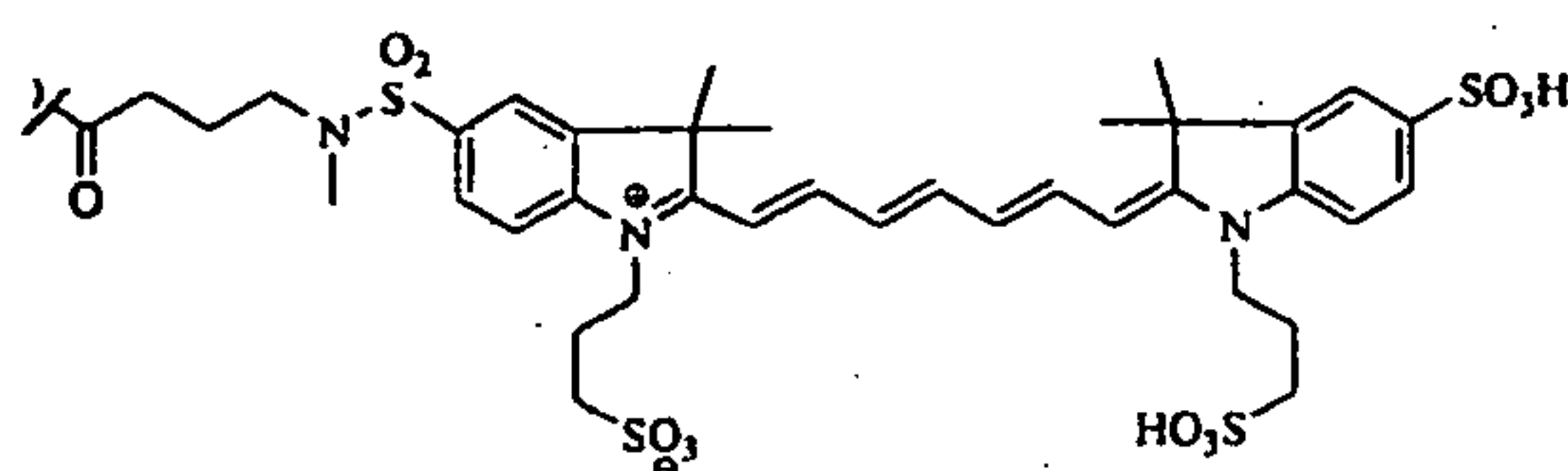
- 1 33. The agent of any one of claims 1-32, wherein the enzymatically cleavable oligopeptide is
 2 cleavable by at least one enzyme selected from the group consisting of a cathepsin, a
 3 matrix metalloprotease, a peptidase, a carboxypeptidase, a glycosidase, a lipase, a
 4 phospholipase, a phosphatase, a phosphodiesterase, a sulfatase, a reductase, and a
 5 bacterial enzyme.
- 1 34. The agent of any one of claims 4-33, wherein N is a radioisotopic metal selected from the
 2 group consisting of copper, gallium, indium, technetium, yttrium, and lutetium.
- 1 35. The agent of claim 34, wherein the radioisotopic metal is selected from the group
 2 comprising ^{99m}Tc , ^{111}In , ^{64}Cu , ^{67}Ga , ^{186}Re , ^{188}Re , ^{153}Sm , ^{177}Lu , and ^{67}Cu .
- 1 36. The agent of any one of claims 4-35, wherein N is a therapeutic radiopharmaceutical.
- 1 37. The agent of claim 36, wherein the therapeutic radiopharmaceutical comprises a
 2 radioisotopic metal selected from the group comprising ^{186}Re , ^{188}Re , ^{153}Sm , ^{166}Ho , ^{177}Lu ,
 3 ^{149}Pm , ^{90}Y , ^{212}Bi , ^{103}Pd , ^{109}Pd , ^{159}Gd , ^{140}La , ^{198}Au , ^{199}Au , ^{169}Yb , ^{175}Yb , ^{165}Dy , ^{166}Dy , ^{67}Cu ,
 4 ^{105}Rh , ^{111}Ag , and ^{192}Ir .
- 1 38. The agent of any one of claims 4-36, wherein N is a magnetic reporter selected from the
 2 group comprising Gd(III), Dy(III), Fe(III), Mn(II) and metal oxide nanoparticles.
- 1 39. The agent of any one of claims 4-36, wherein N is an X-ray reporter.
- 1 40. An intramolecularly-quenched imaging agent comprising:
 2 a) an enzymatically cleavable oligopeptide comprising from 2 to 14 amino acid
 3 residues;
 4 b) at least one biological modifier with a molecular weight of from about 5 kDa to
 5 about 35 kDa covalently linked to the enzymatically cleavable oligopeptide; and
 6 c) two fluorophores, each covalently linked, directly or indirectly, to the oligopeptide
 7 at locations so that the fluorochromes quench one another, and wherein, upon
 8 enzymatic cleavage of the oligopeptide, at least one fluorophore becomes
 9 unquenched and is capable of emitting a fluorescent signal when excited by
 10 electromagnetic radiation.
- 1 41. The agent of claim 40, wherein the fluorophores are the same.

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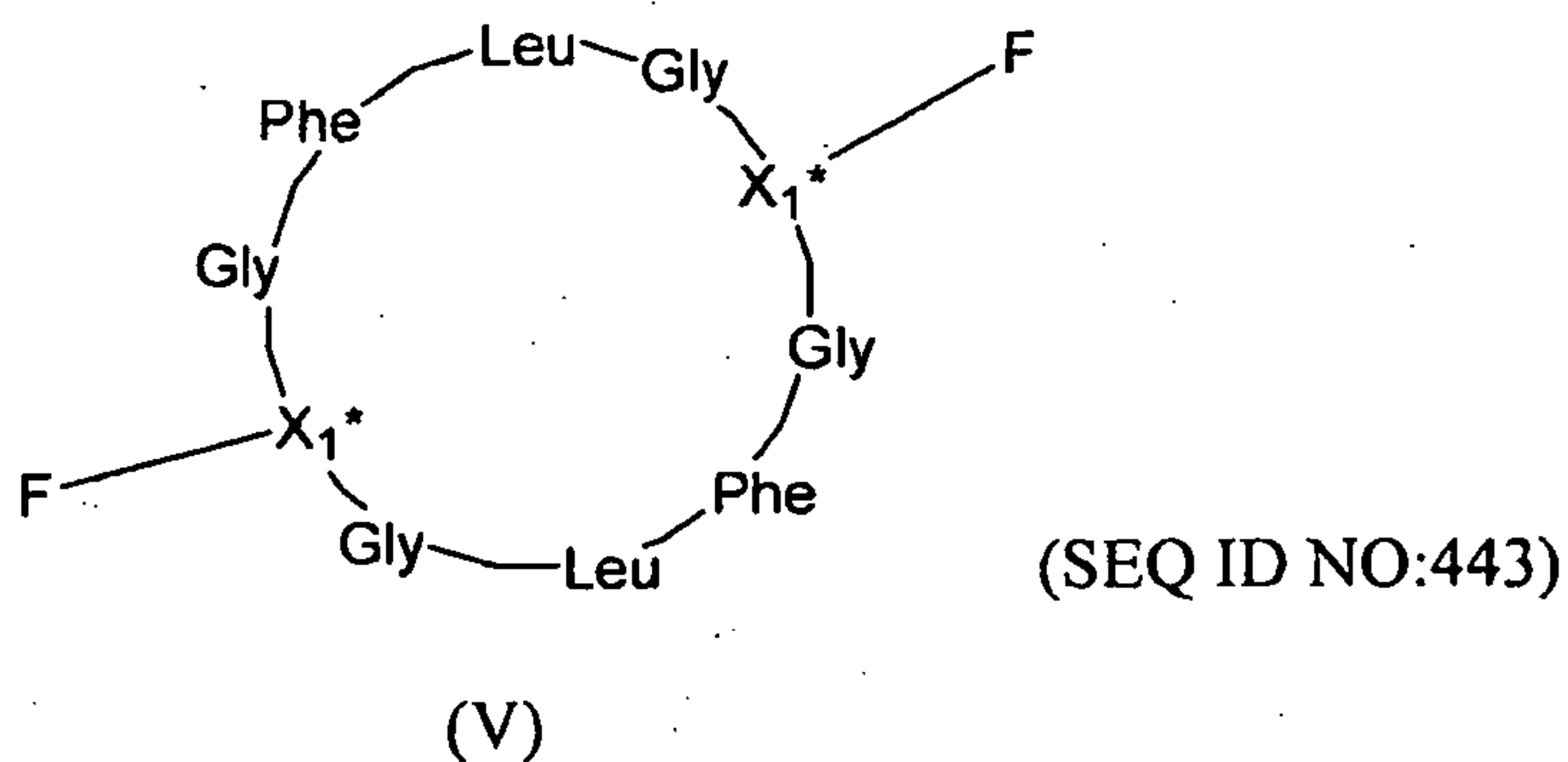
42. The agent of any one of claims 1-41, wherein at least one fluorophore when covalently linked to the oligopeptide an optional biological modifier is:



43. The agent of any one of claims 1-41, wherein at least one fluorophore when covalently linked to the oligopeptide an optional biological modifier is:



44. An intramolecularly-quenched imaging agent represented by Formula V:



wherein

X_1^* independently, at each occurrence, is X-L-;

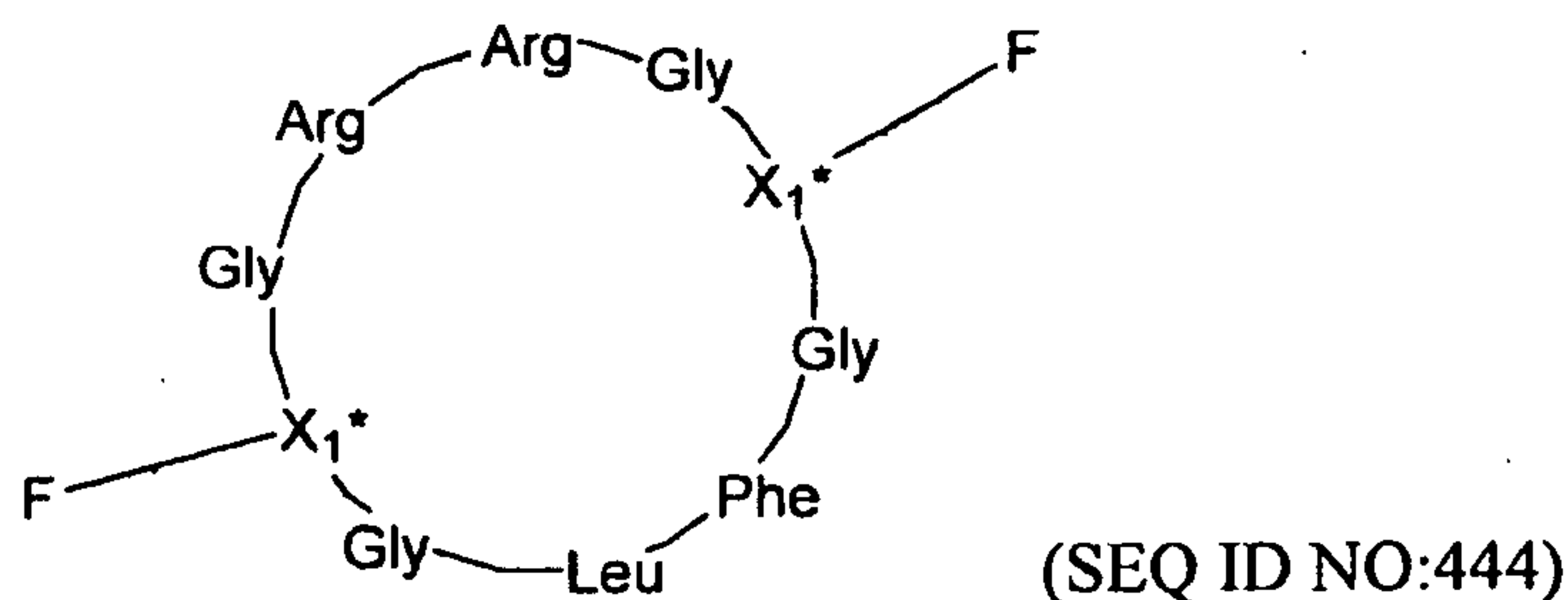
X is an amino acid residue;

L is a linker moiety or a bond; and

F, independently, at each occurrence, is a fluorophore.

45. An intramolecularly-quenched imaging agent represented by Formula VI:

- 105 -



(VI)

wherein

X_1^* , independently, at each occurrence, is X-L-;

X is an amino acid residue;

L is a linker moiety or a bond;

F, independently, at each occurrence, is a fluorophore.

46. The agent of any one of claims 1-45, wherein the agent has a molecular weight from about 2 kDa to 60 kDa.

47. The agent of any one of claims 1-46, wherein the agent has no greater than two fluorophores.

48. The agent of any one of claims 1-47, wherein the agent is represented by a structure included in Table 6

49. A method of *in vivo* optical imaging, the method comprising:

- (a) administering to a subject an agent of any one of claims 1-48;
- (b) allowing the agent to distribute in the subject;
- (c) exposing the subject to light of a wavelength absorbable by at least one fluorophore in the agent; and
- (d) detecting a signal emitted by the fluorophore.

50. The method of claim 49, wherein the emitted signal is used to construct an image.

51. The method of claim 50, wherein the image is a tomographic image.

52. The method of claim 51, wherein steps (c) - (d) are repeated at predetermined intervals thereby to permit evaluation of the emitted signals from the agent in the subject over time.

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- 1 53. The method of claim 49, wherein steps (a) - (d) are repeated at predetermined intervals
2 thereby to permit evaluation of the emitted signals from the agent in the subject over time.
- 1 54. The method of claim 49, wherein the subject is an animal or a human.
- 1 55. The method of claim 49, wherein, in step (a), two or more imaging agents whose signal
2 properties are distinguishable from one another are administered to a subject.
- 1 56. The method of claim 49, wherein the exposing and detecting steps are performed using an
2 endoscope, catheter, tomographic system, hand-held optical imaging system, or an
3 intraoperative microscope.
- 1 57. The method of claim 49, wherein the presence, absence, or level of emitted signal is
2 indicative of a disease state.
- 1 58. The method of claim 49, wherein the method is used to detect and/or monitor a disease.
- 1 59. The method of claim 58, wherein the disease is selected from the group consisting of
2 bone disease, cancer, cardiovascular disease, atherosclerosis, restinosis, cardiac ischemia,
3 myocardial reperfusion injury, environmental disease, dermatological disease,
4 immunologic disease, inherited disease, infectious disease, inflammatory disease,
5 metabolic disease, neurodegenerative disease, ophthalmic disease, and respiratory
6 disease.
- 1 60. The method of claim 49, wherein, in step (a), cells labeled with the agent are administered
2 to the subject.
- 1 61. The method of claim 60, wherein the signal emitted by the agent is used to monitor
2 trafficking and localization of the cells.
- 1 62. An *in vitro* imaging method, the method comprising:
2 (a) contacting a sample with an agent of any one of claims 1-48;
3 (b) allowing the agent to bind to or associate with a biological target;
4 (c) optionally, removing unbound agent; and
5 (d) detecting a signal emitted from the fluorophore thereby to determine whether the
6 agent has been activated by or bound to the biological target.
- 1 63. The method of claim 62, wherein the sample is a biological sample.

FIGURE 1

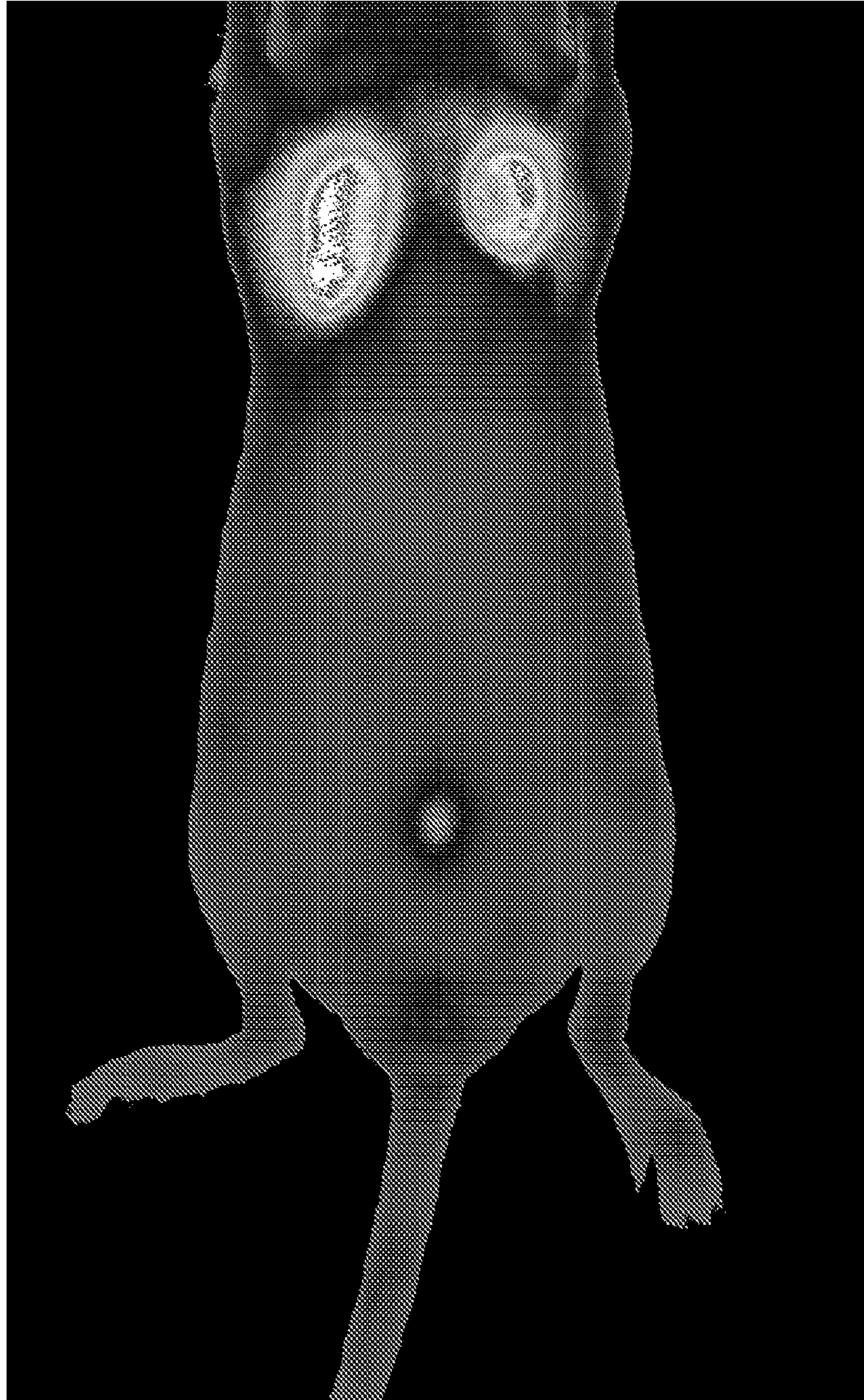


FIGURE 2

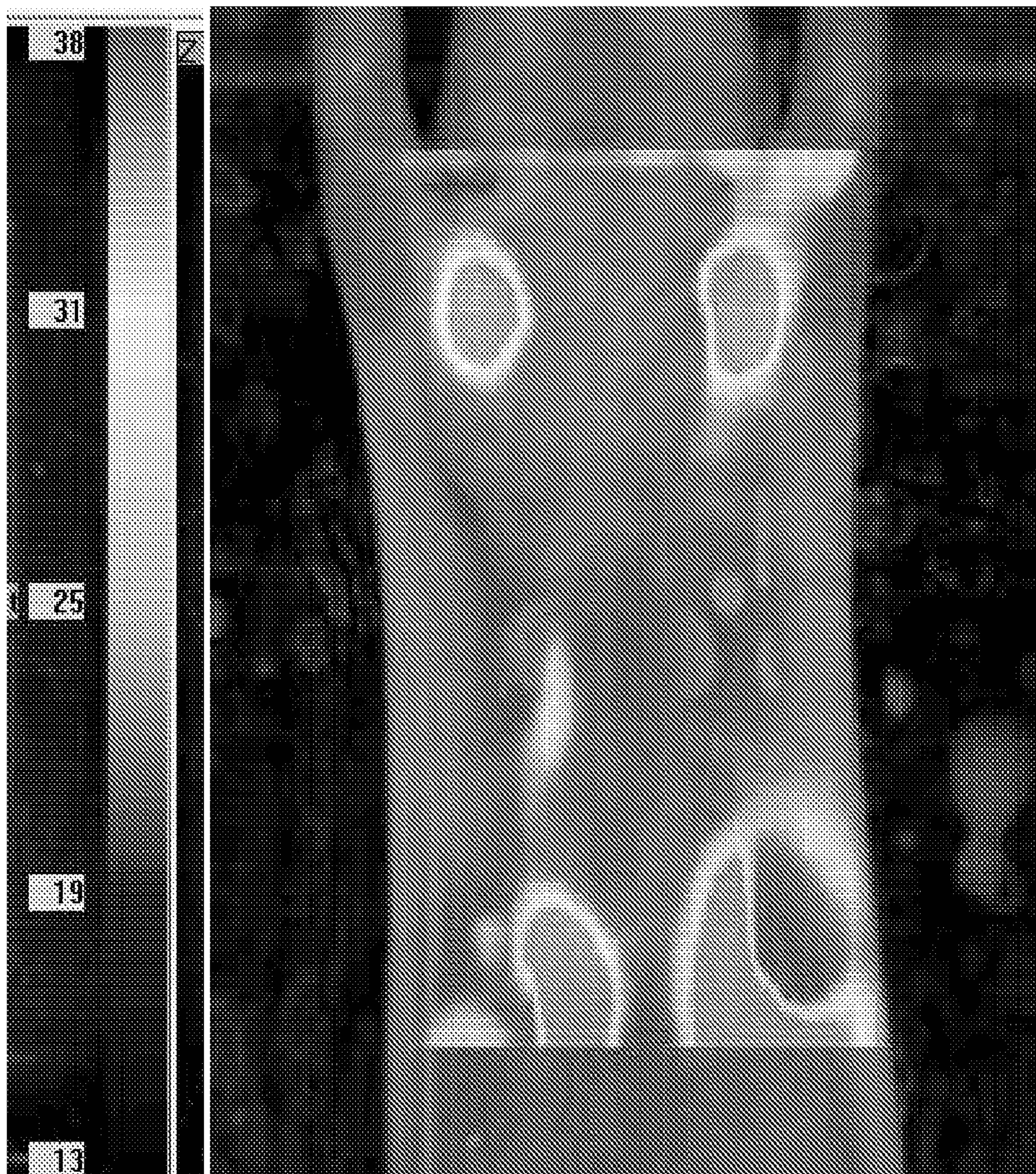


FIGURE 3

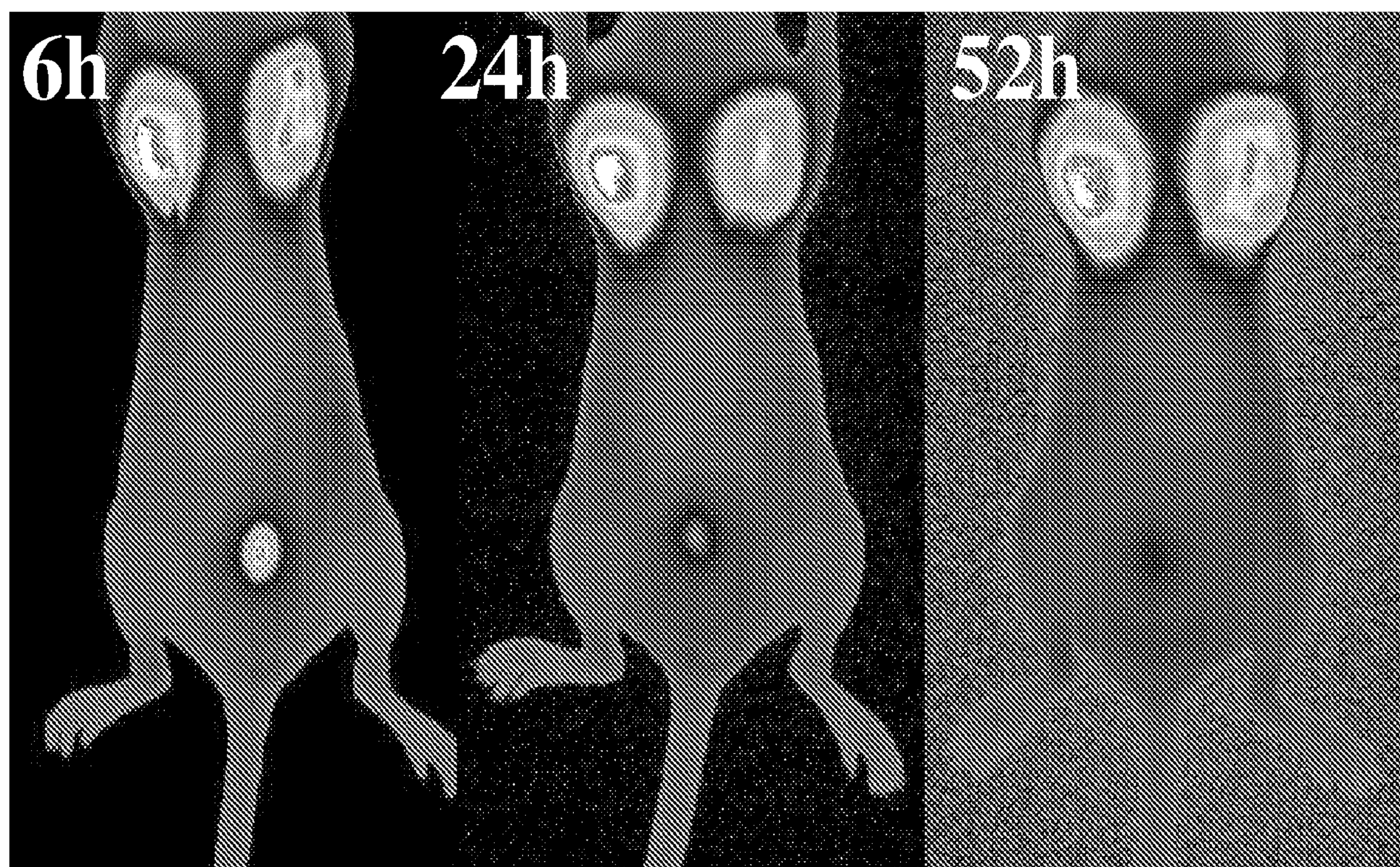


FIGURE 4

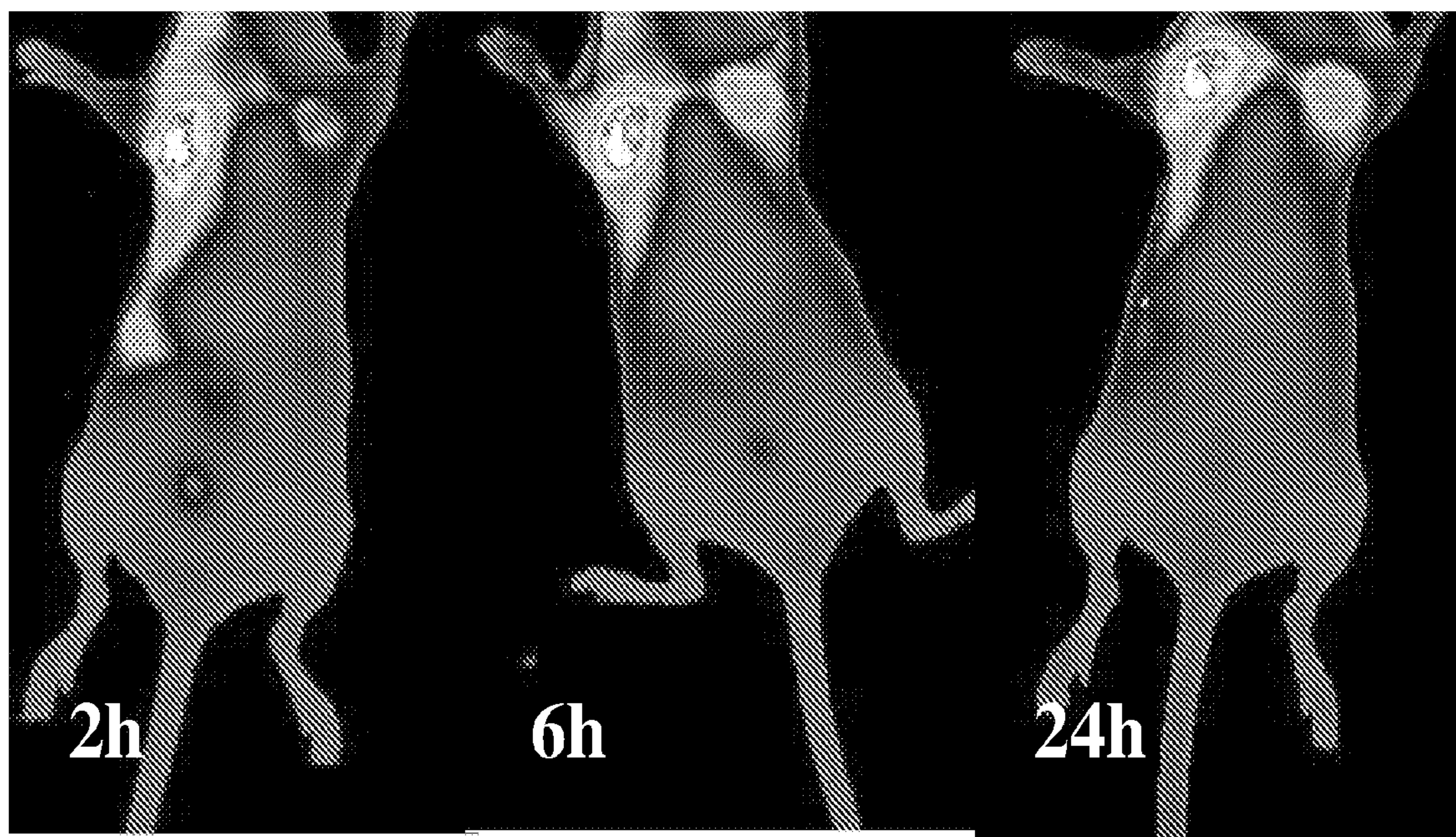


FIGURE 5

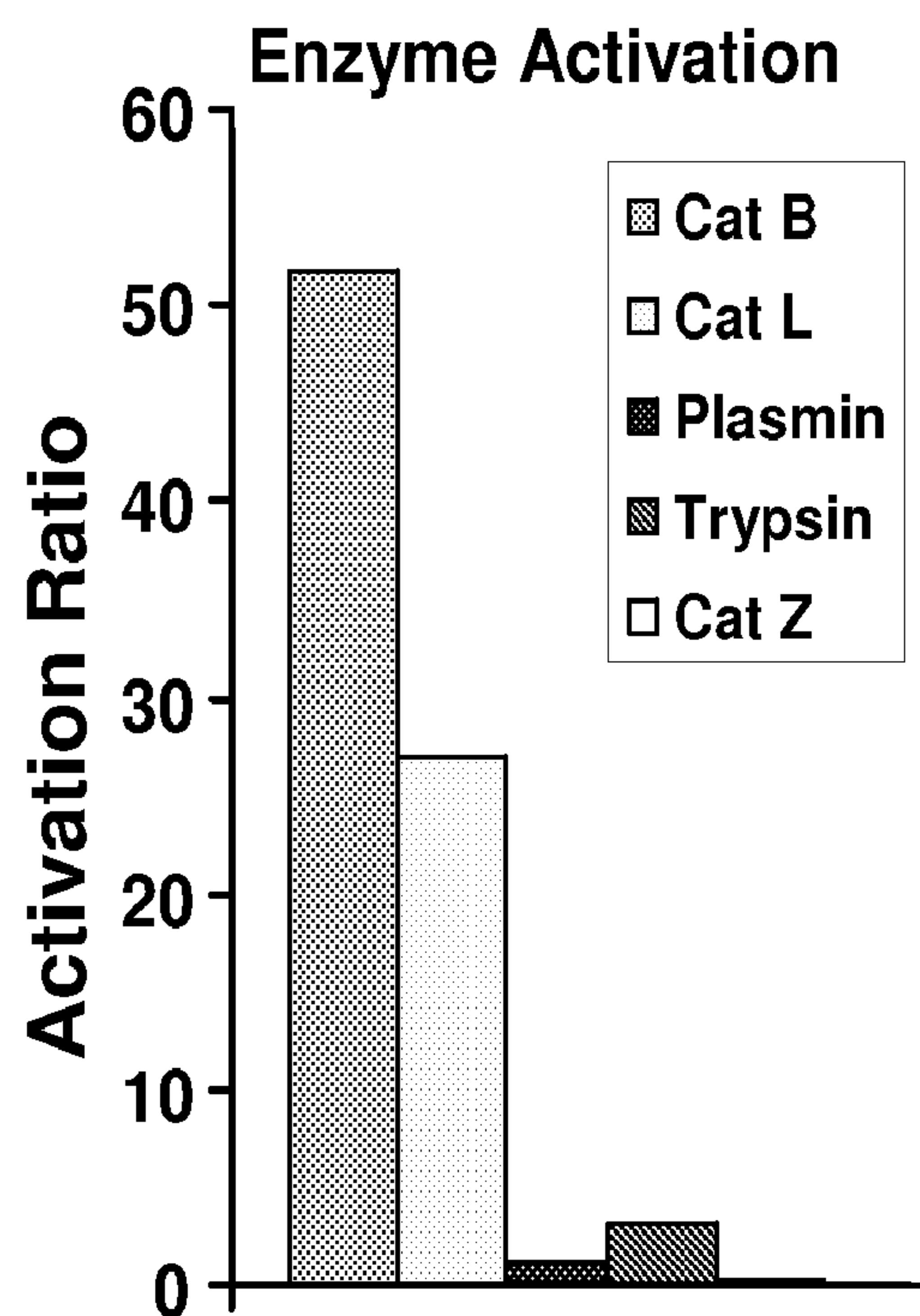


FIGURE 6

Figure 6A

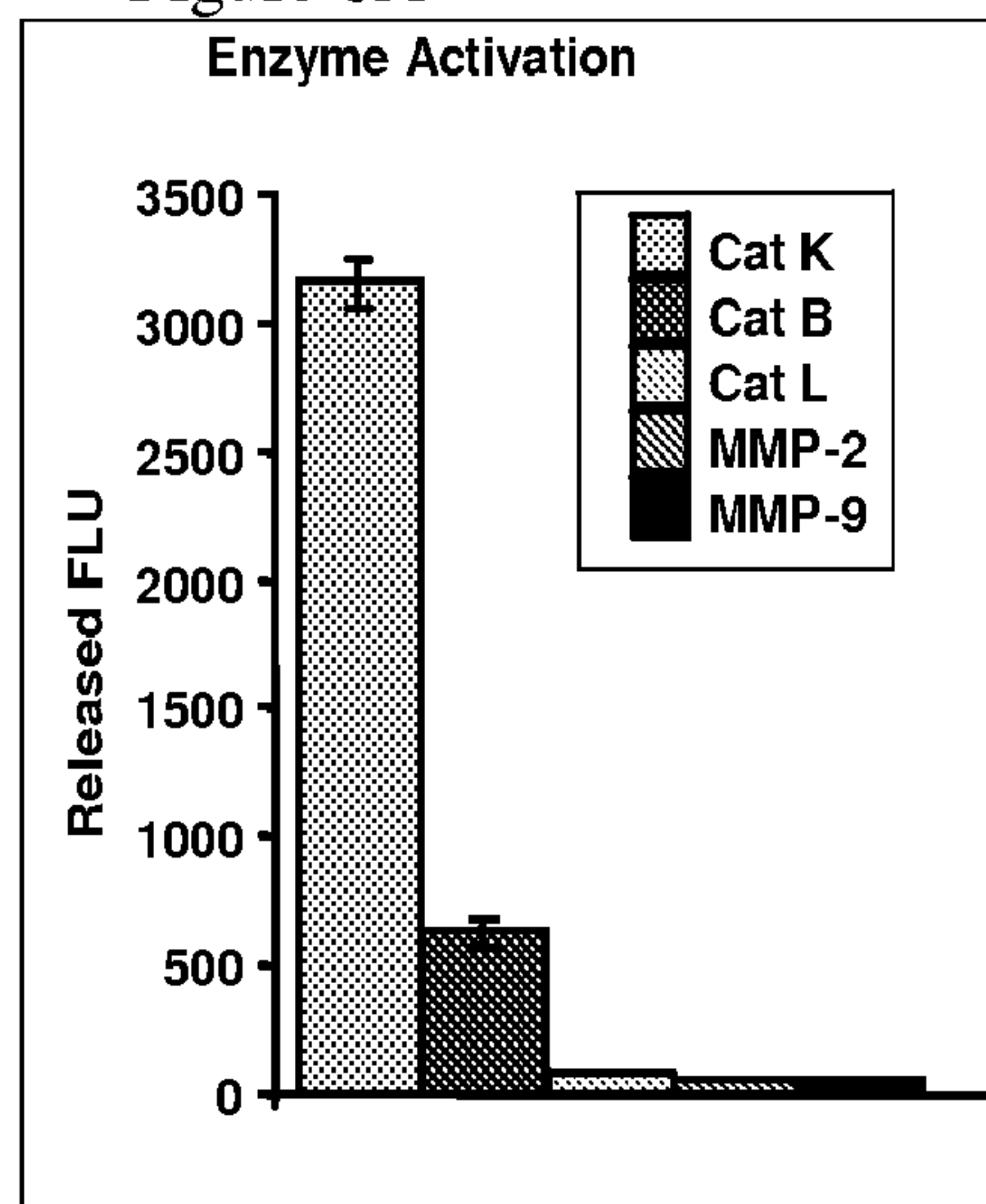


Figure 6B

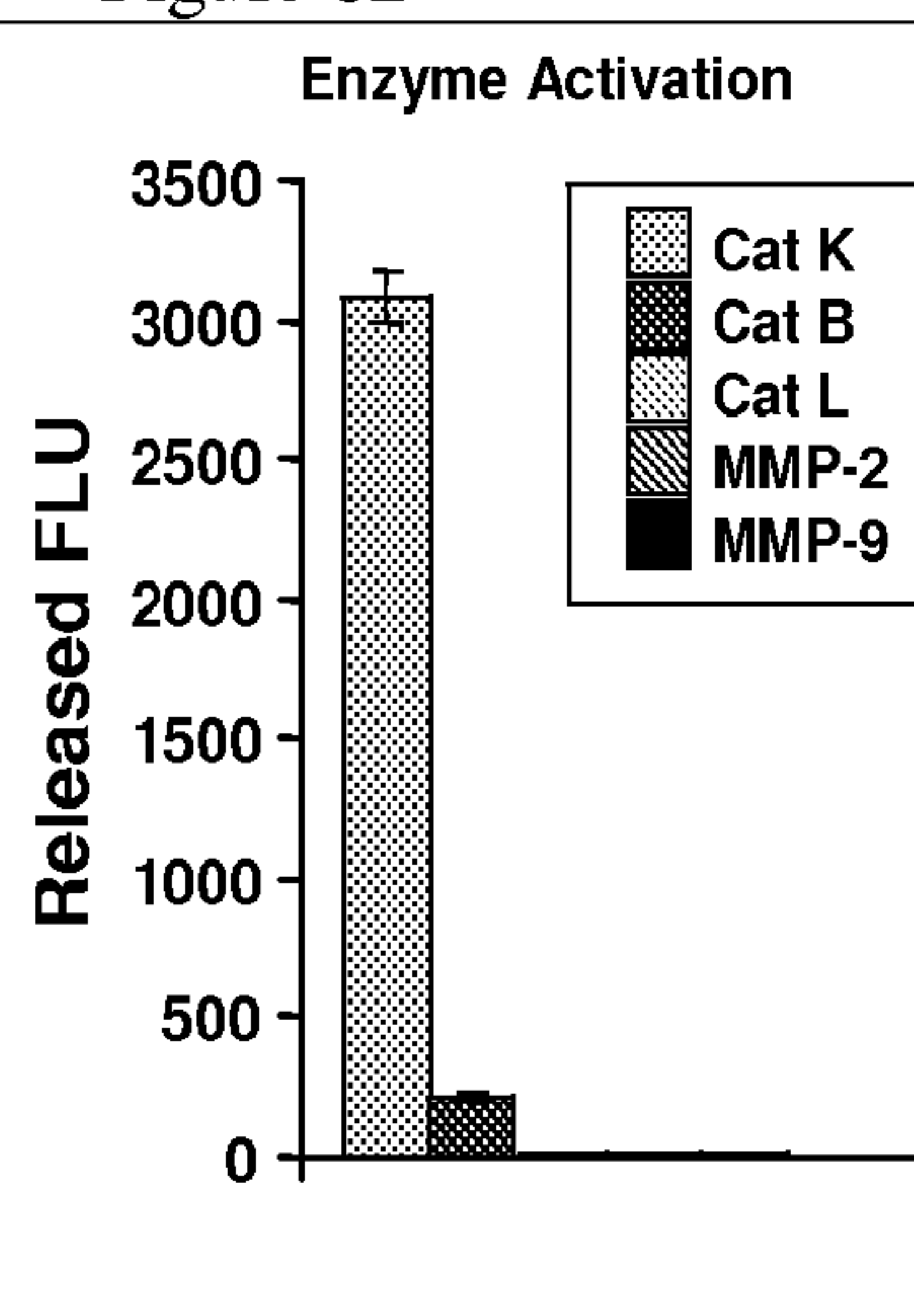


FIGURE 7

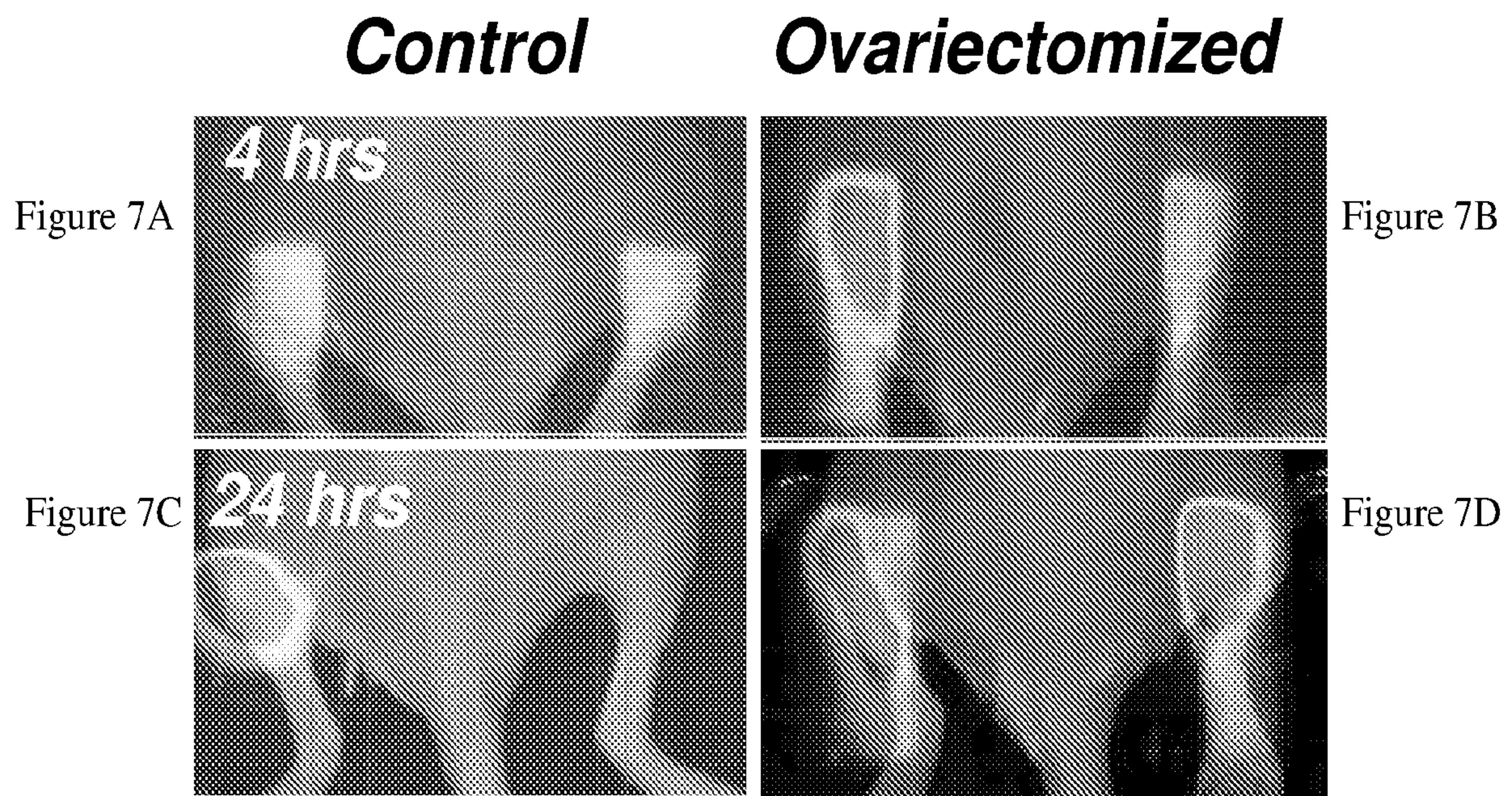


Figure 7E

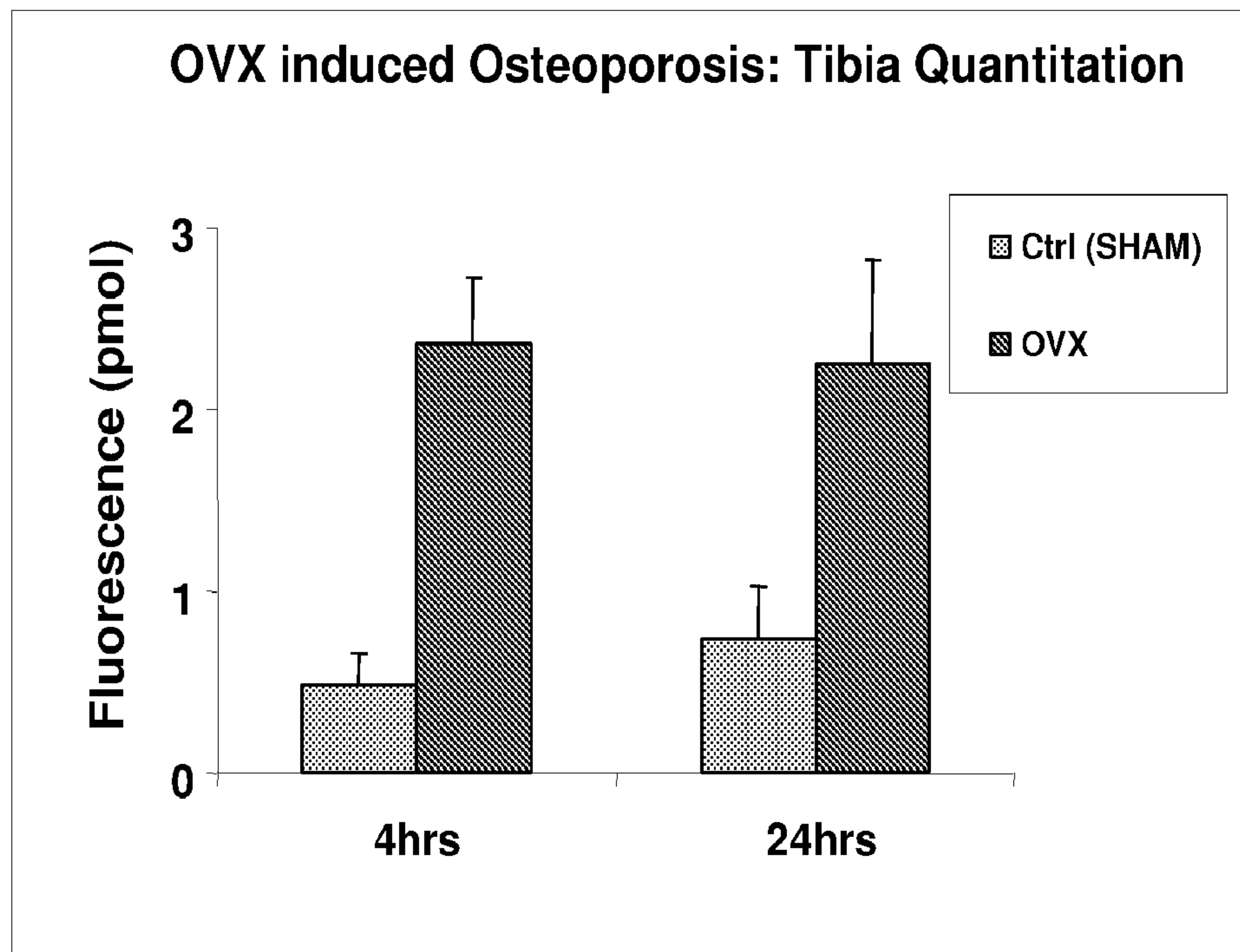


FIGURE 8

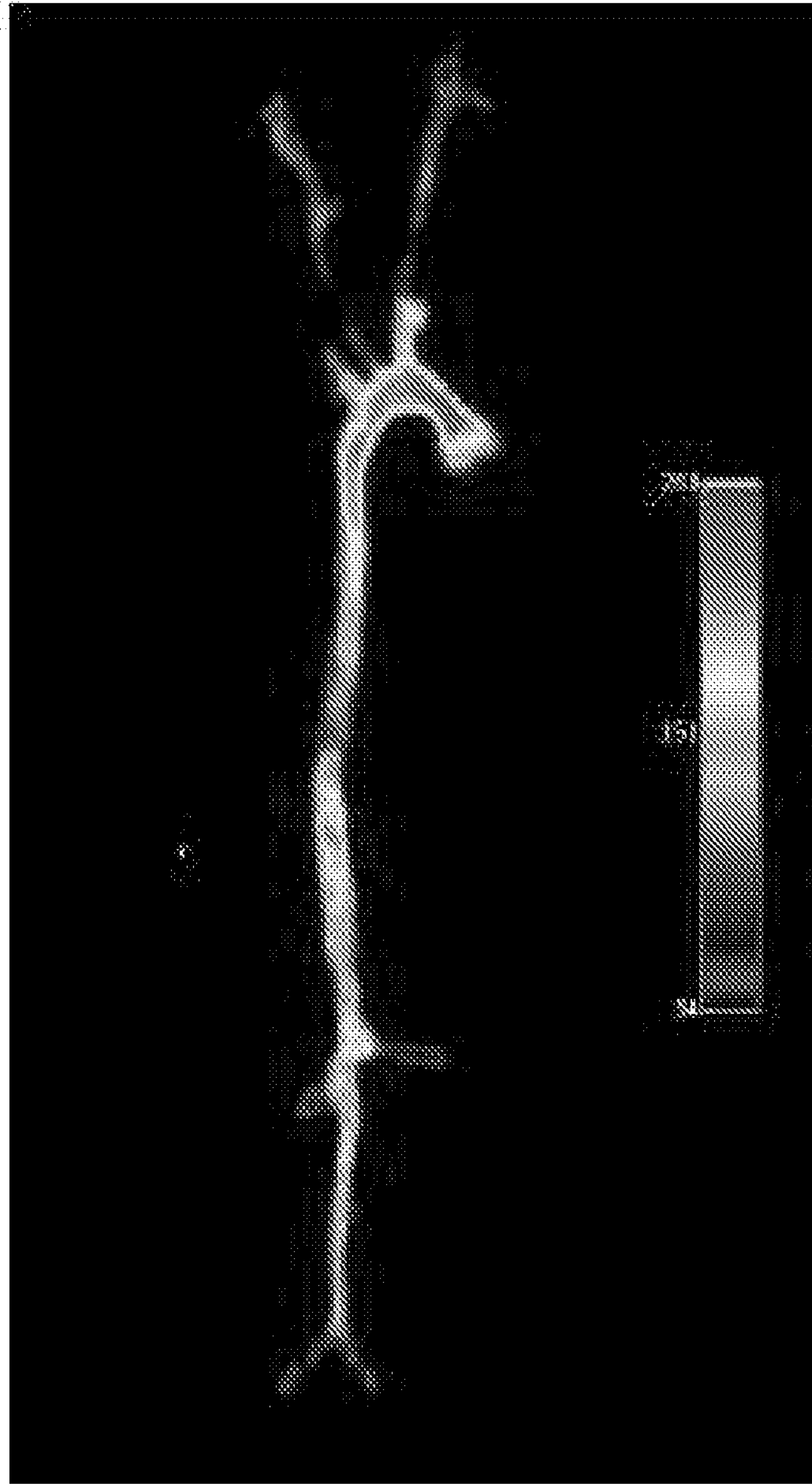


FIGURE 9

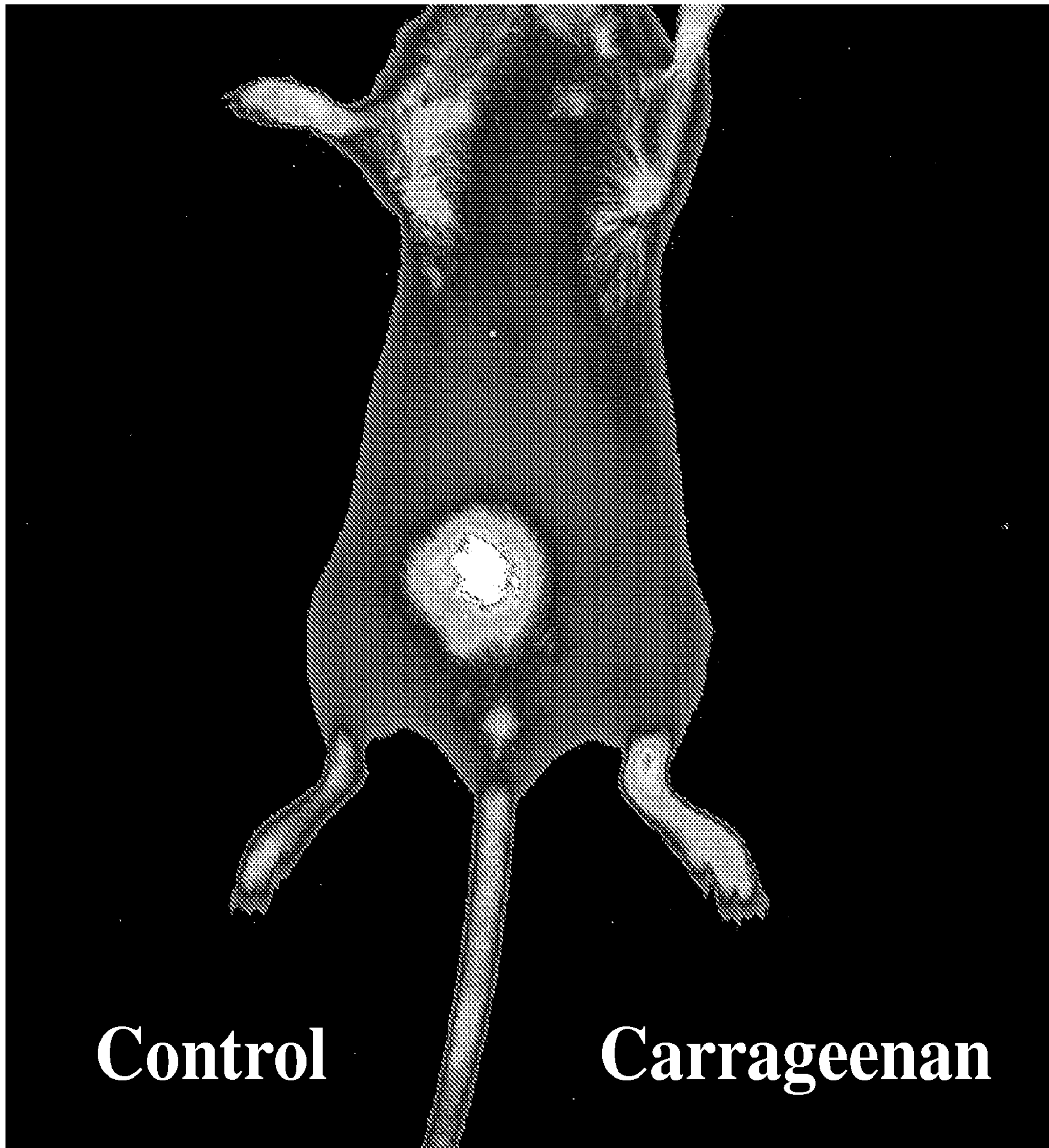


FIGURE 10

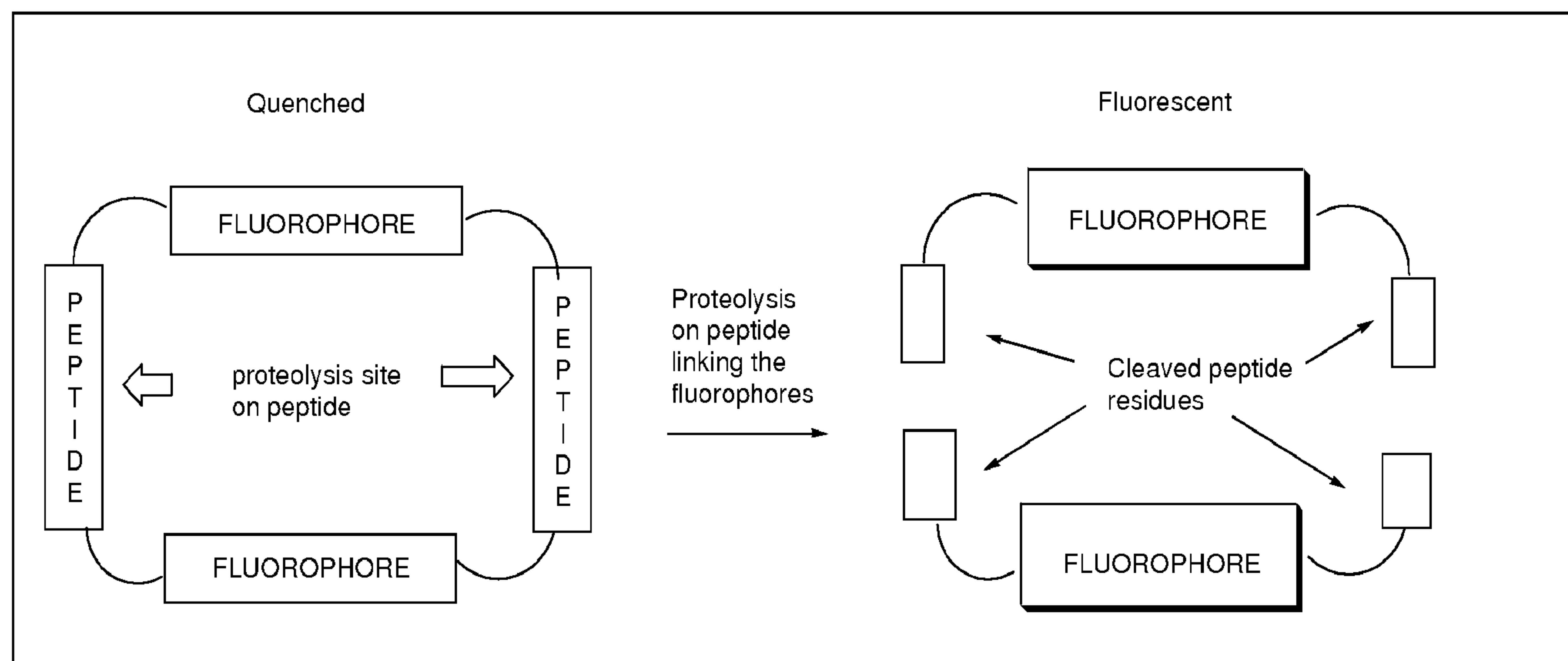


FIGURE 11

Figure 11A

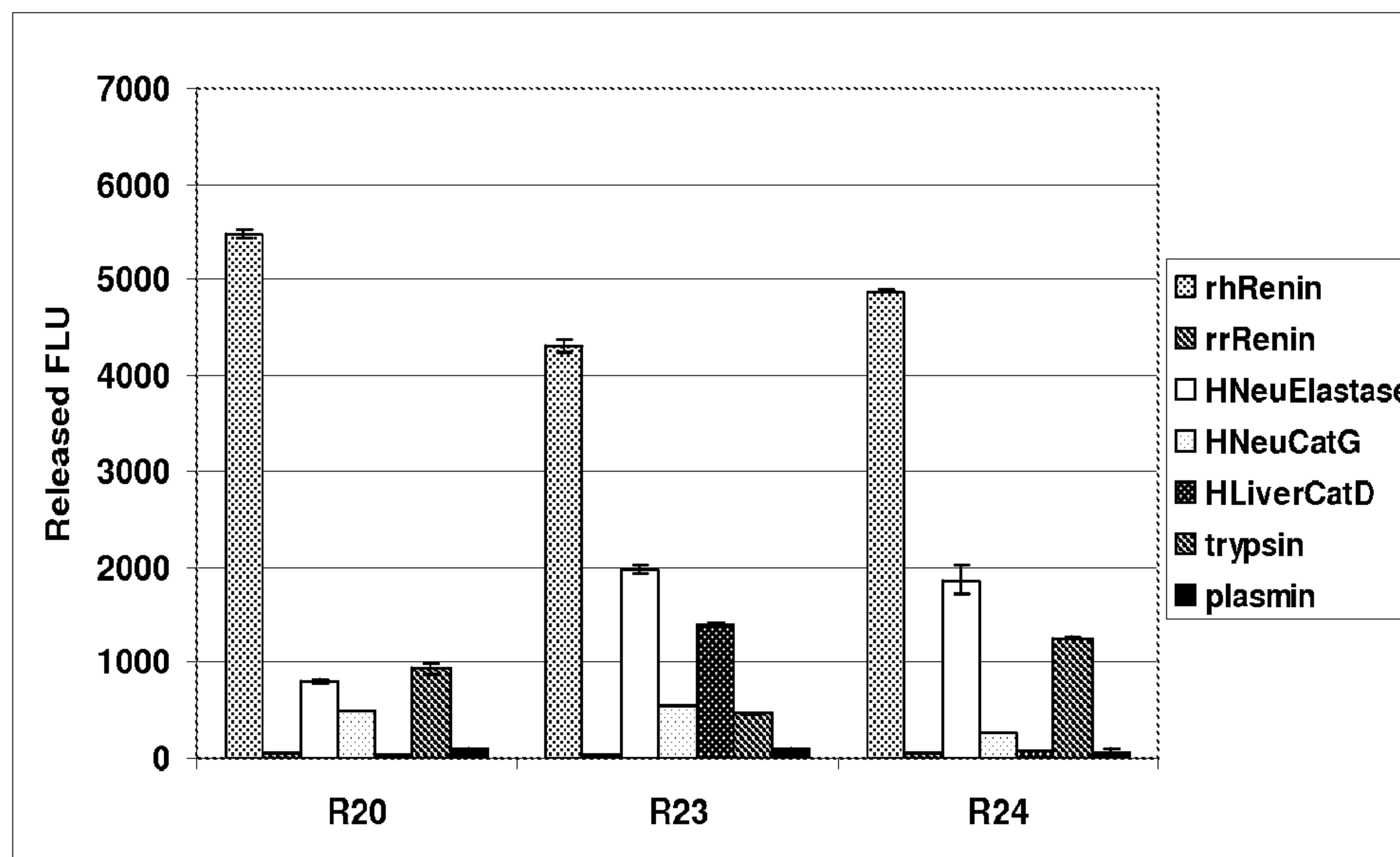


Figure 11B

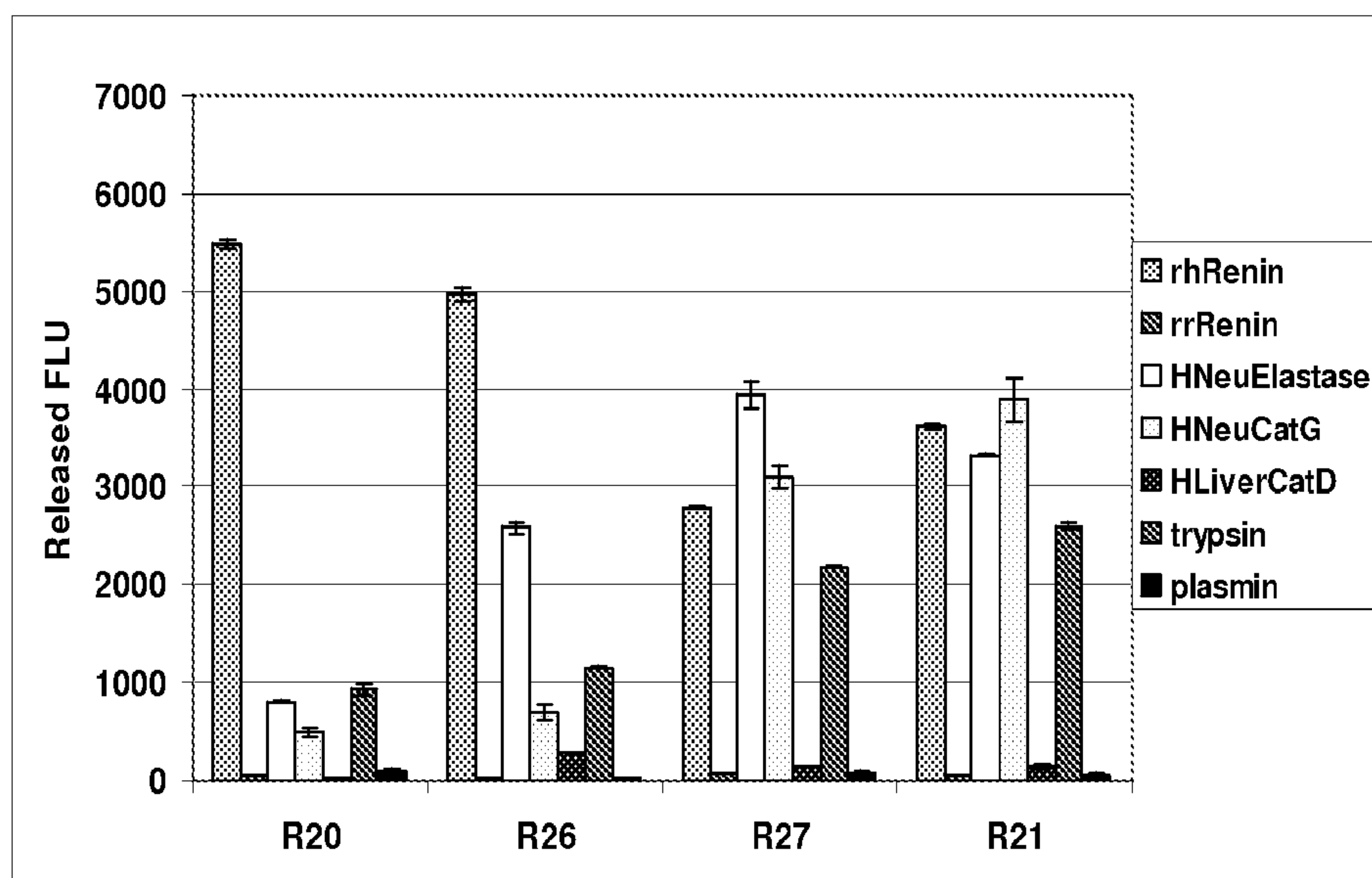


FIGURE 12

Control Diet

Low Sodium Diet

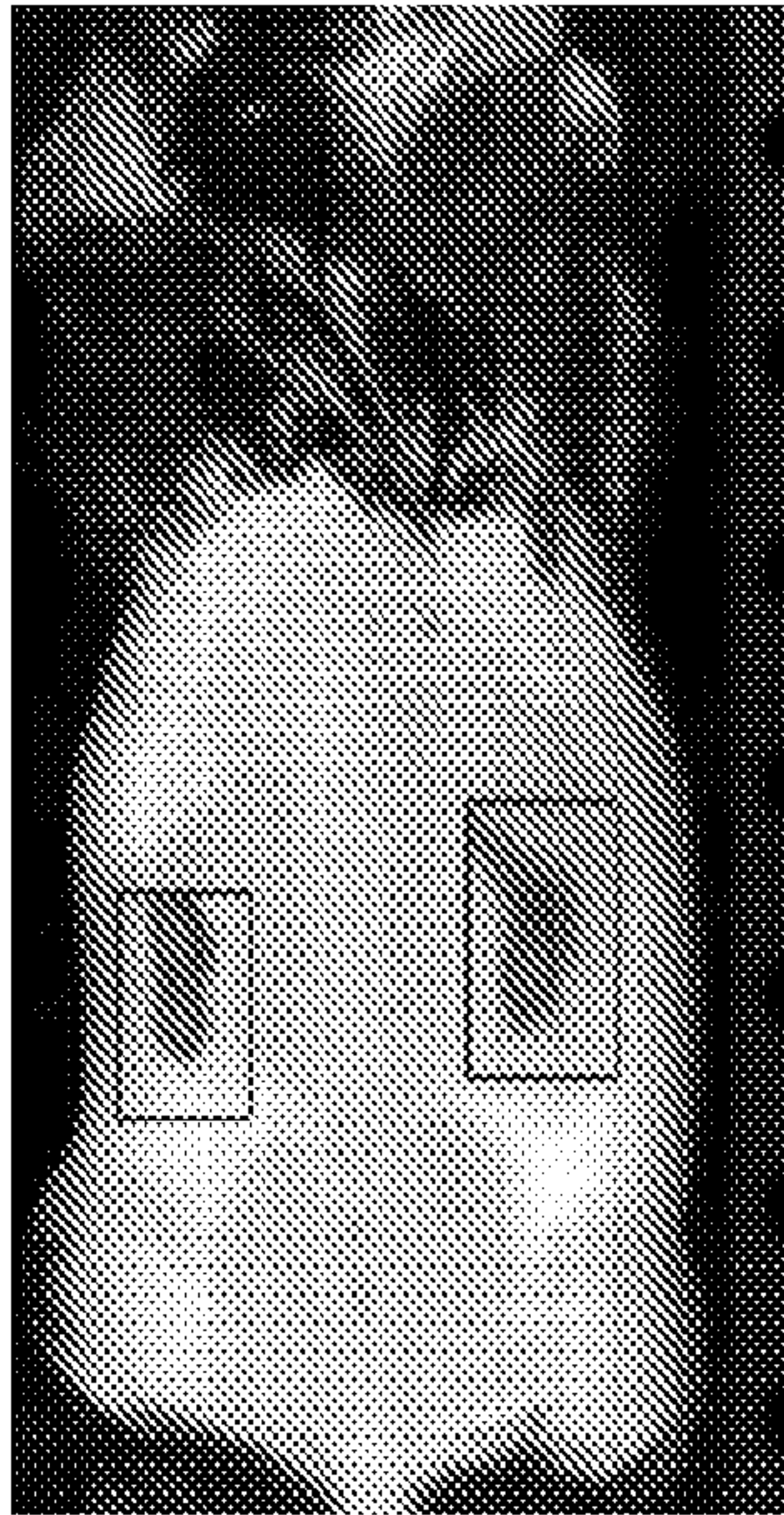


Figure 12A

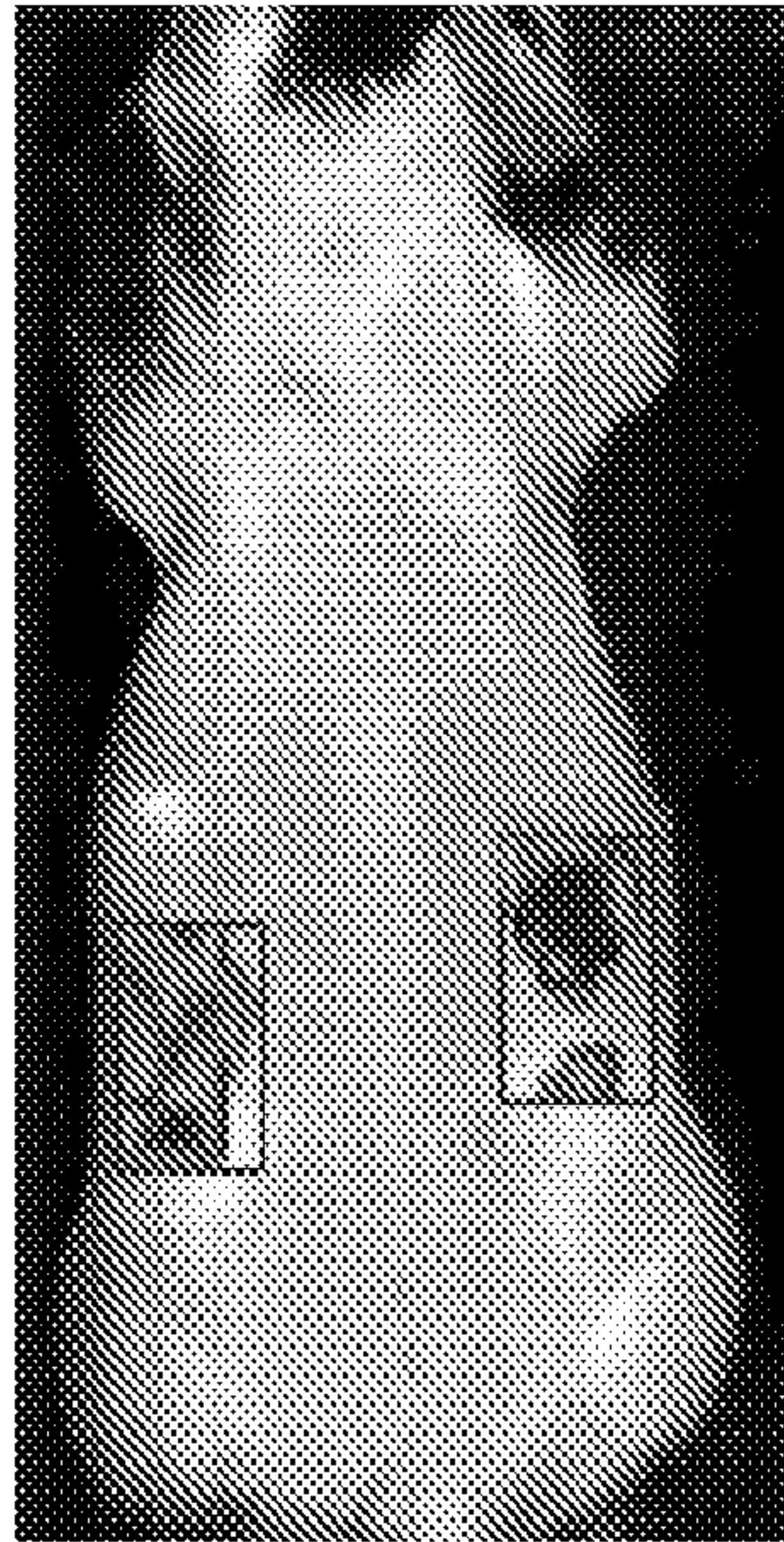


Figure 12B

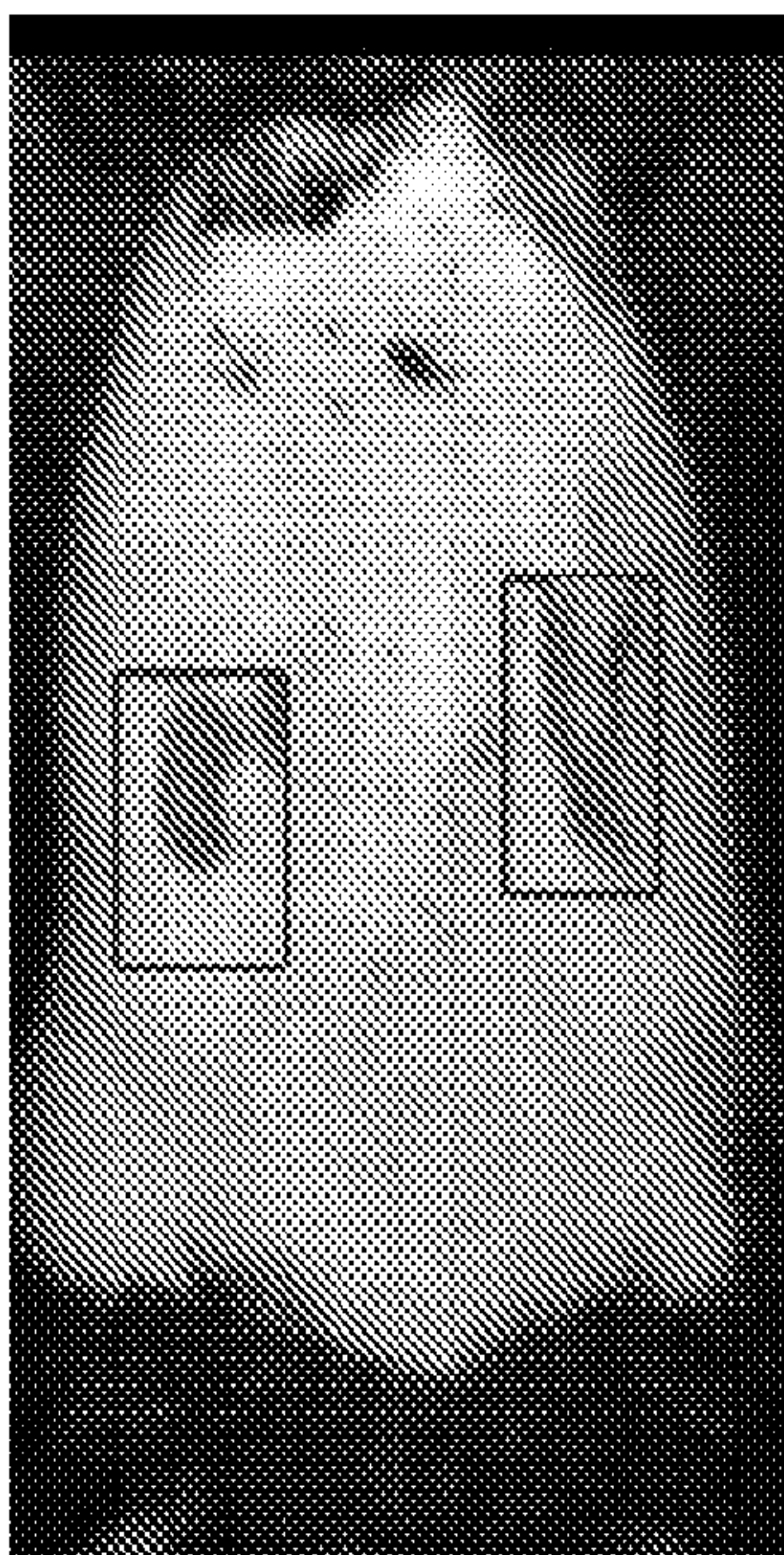


Figure 12C

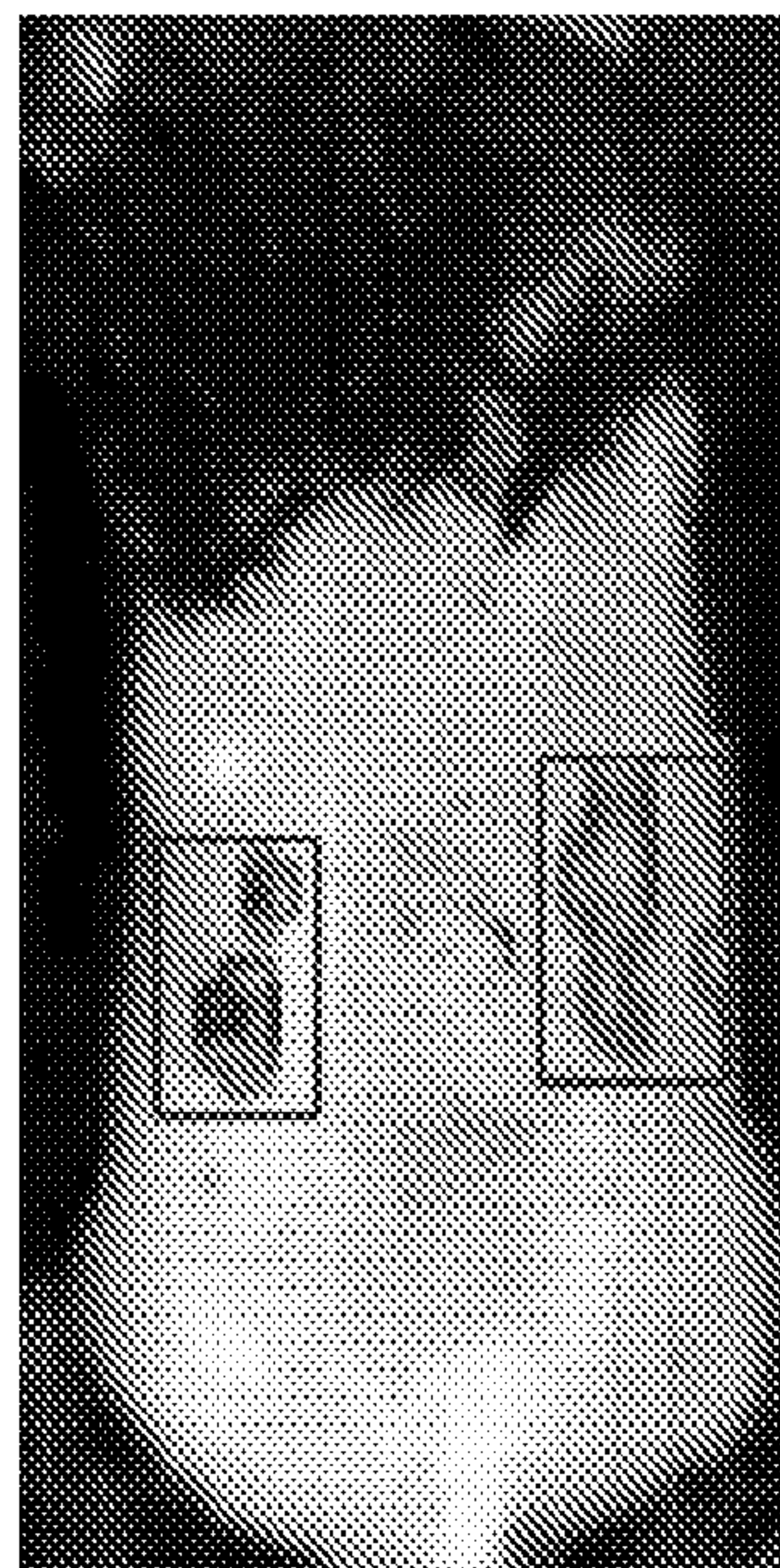


Figure 12D

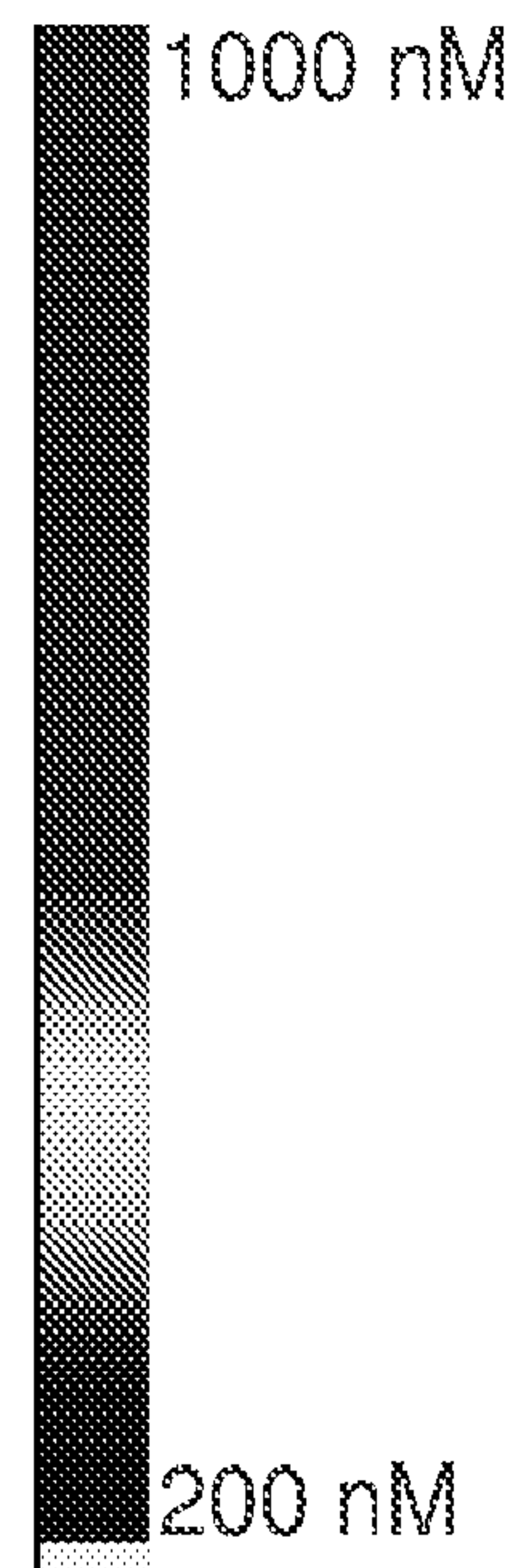


FIGURE 13

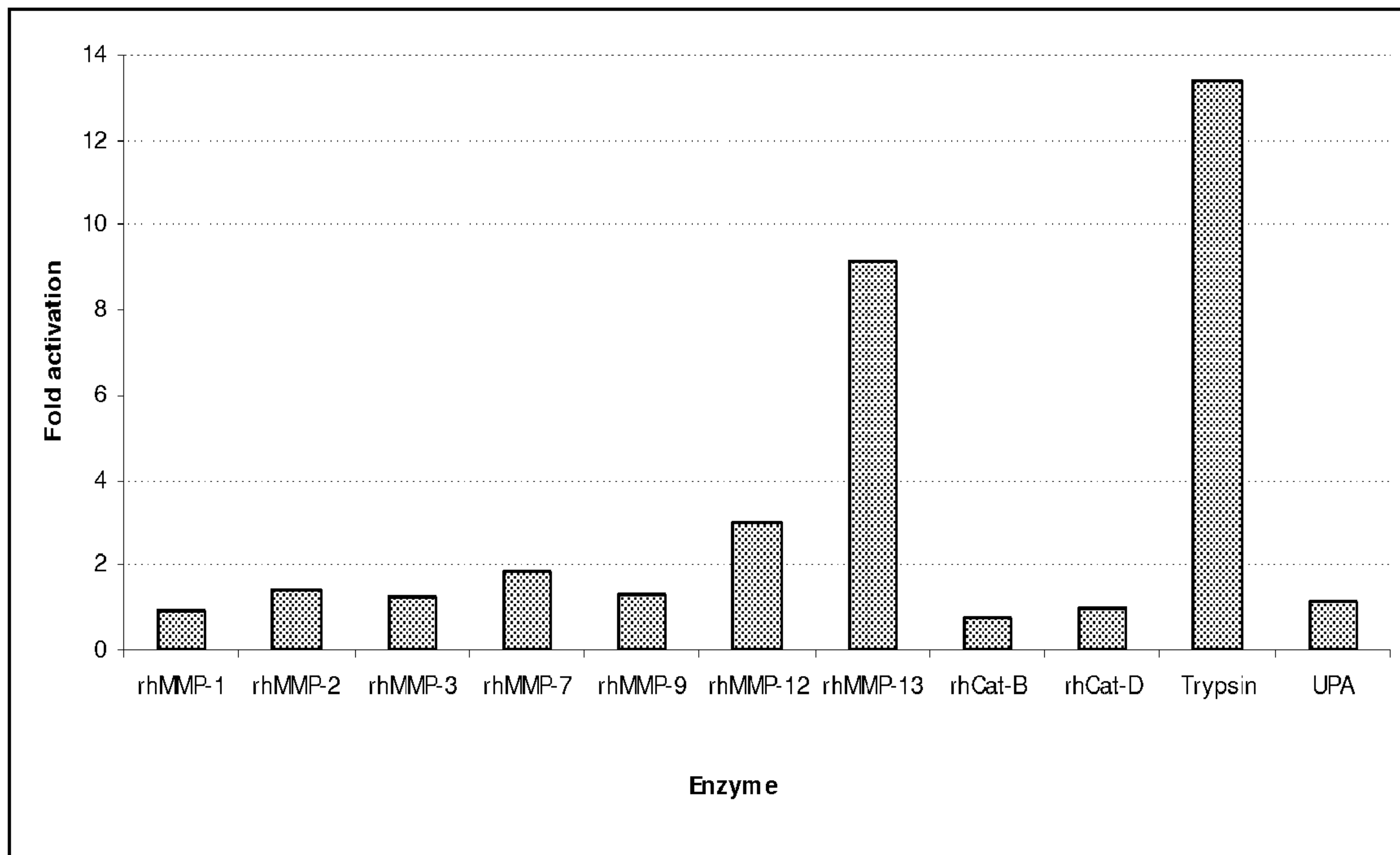


FIGURE 14

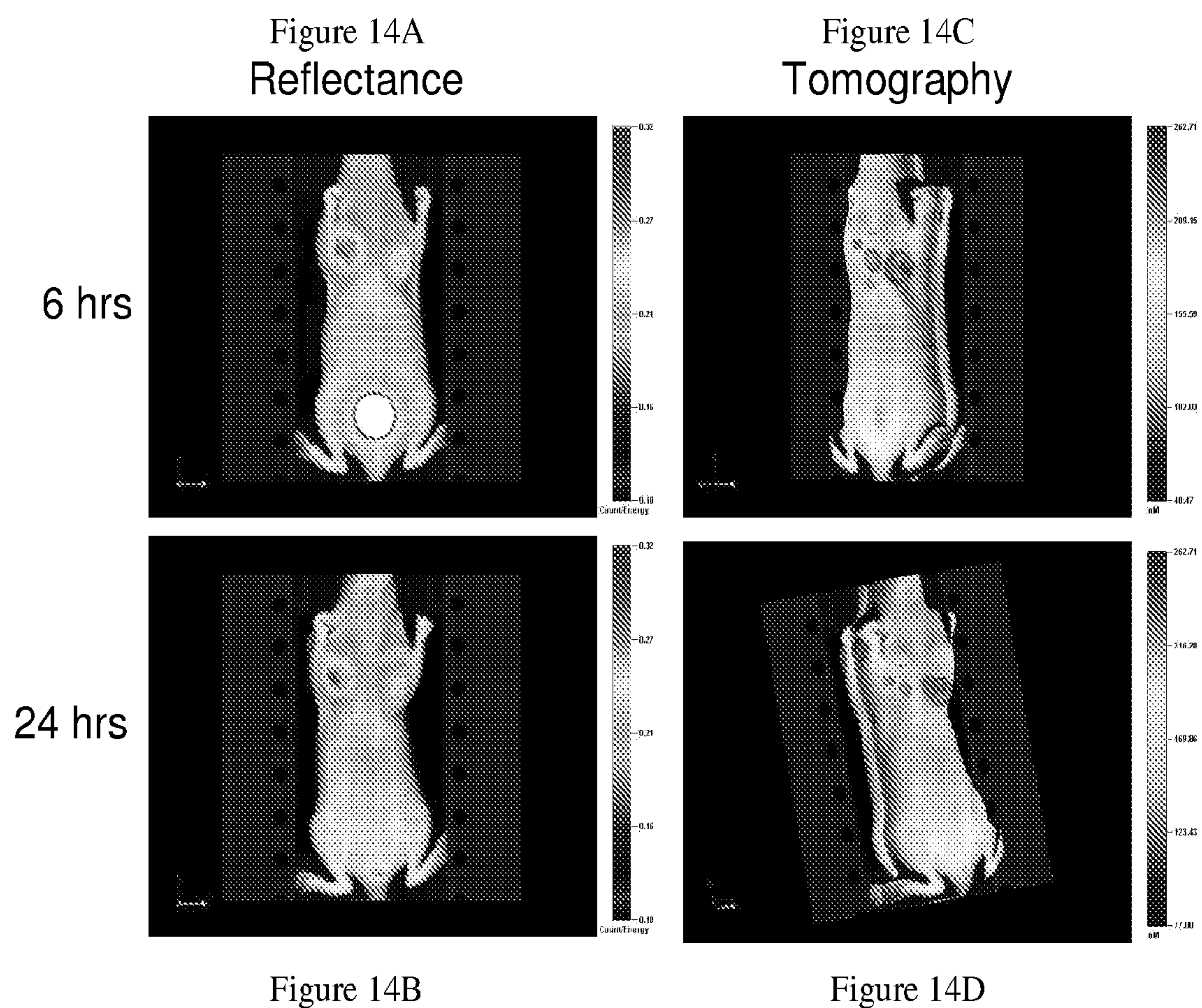


FIGURE 15

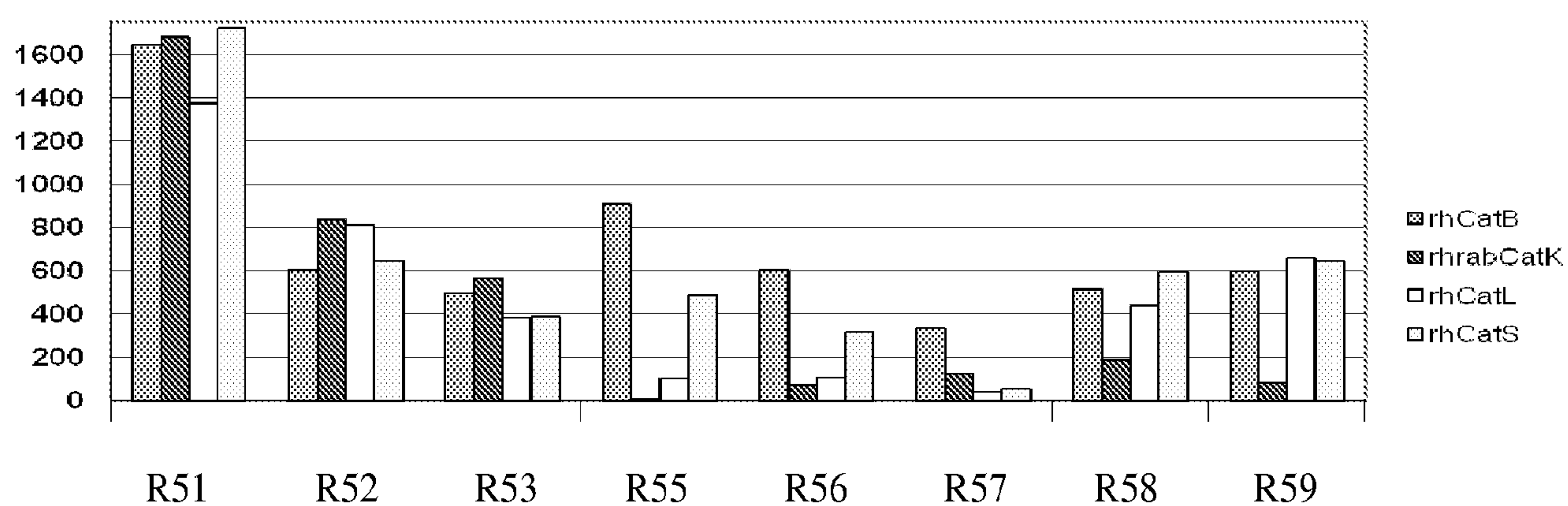


FIGURE 16

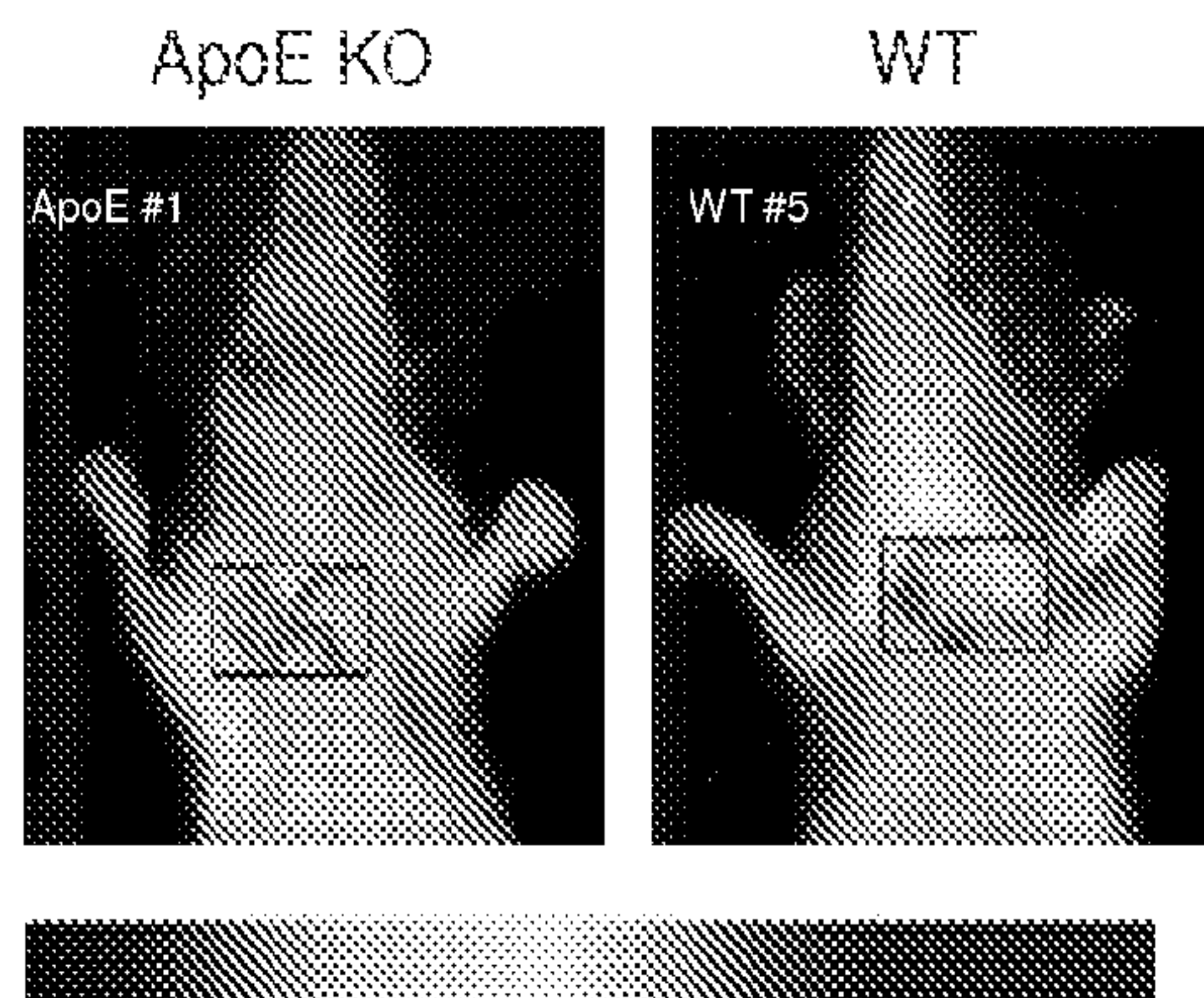


Figure 16A

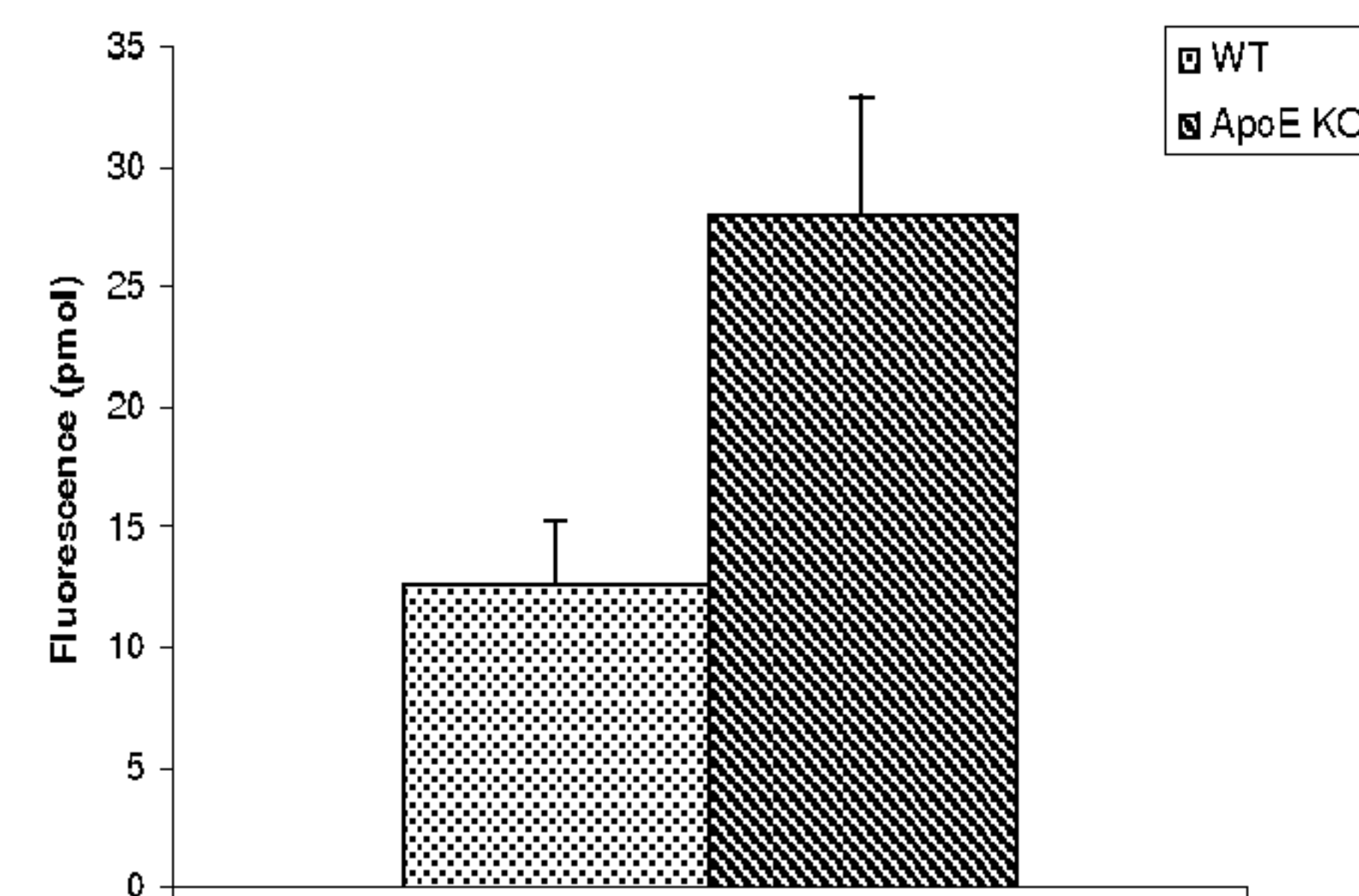


Figure 16B

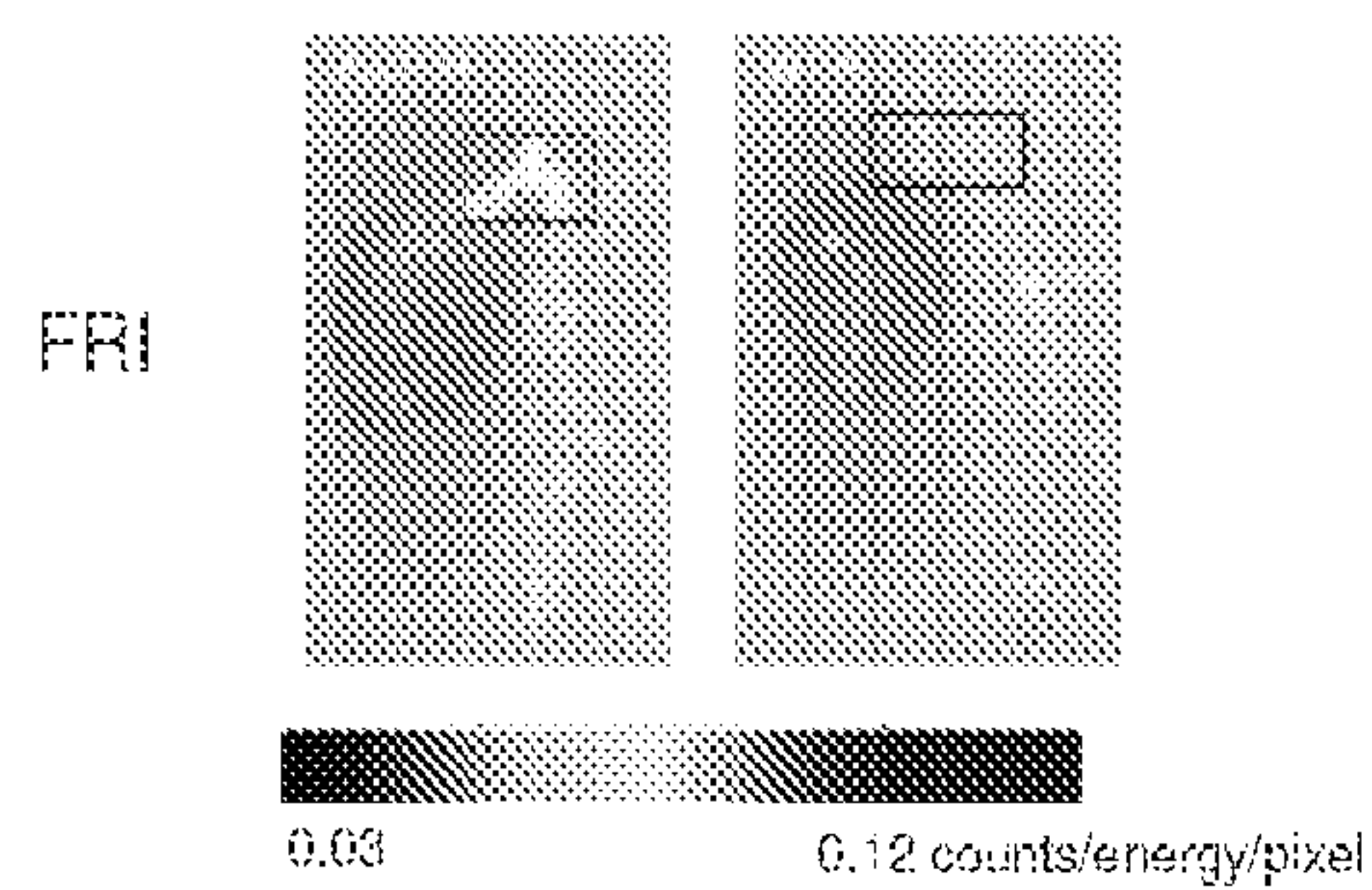


Figure 16C

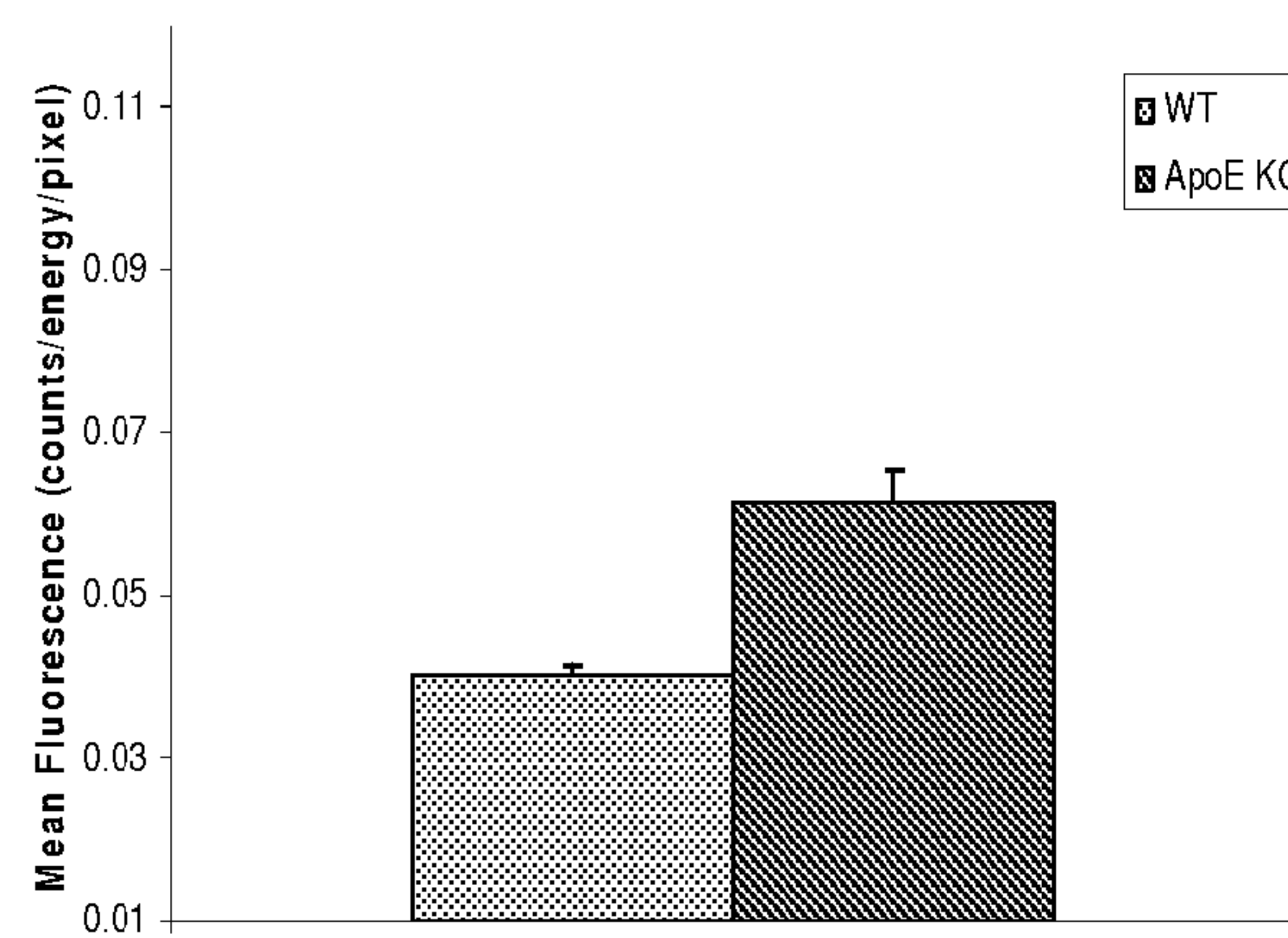


Figure 16D

FIGURE 17

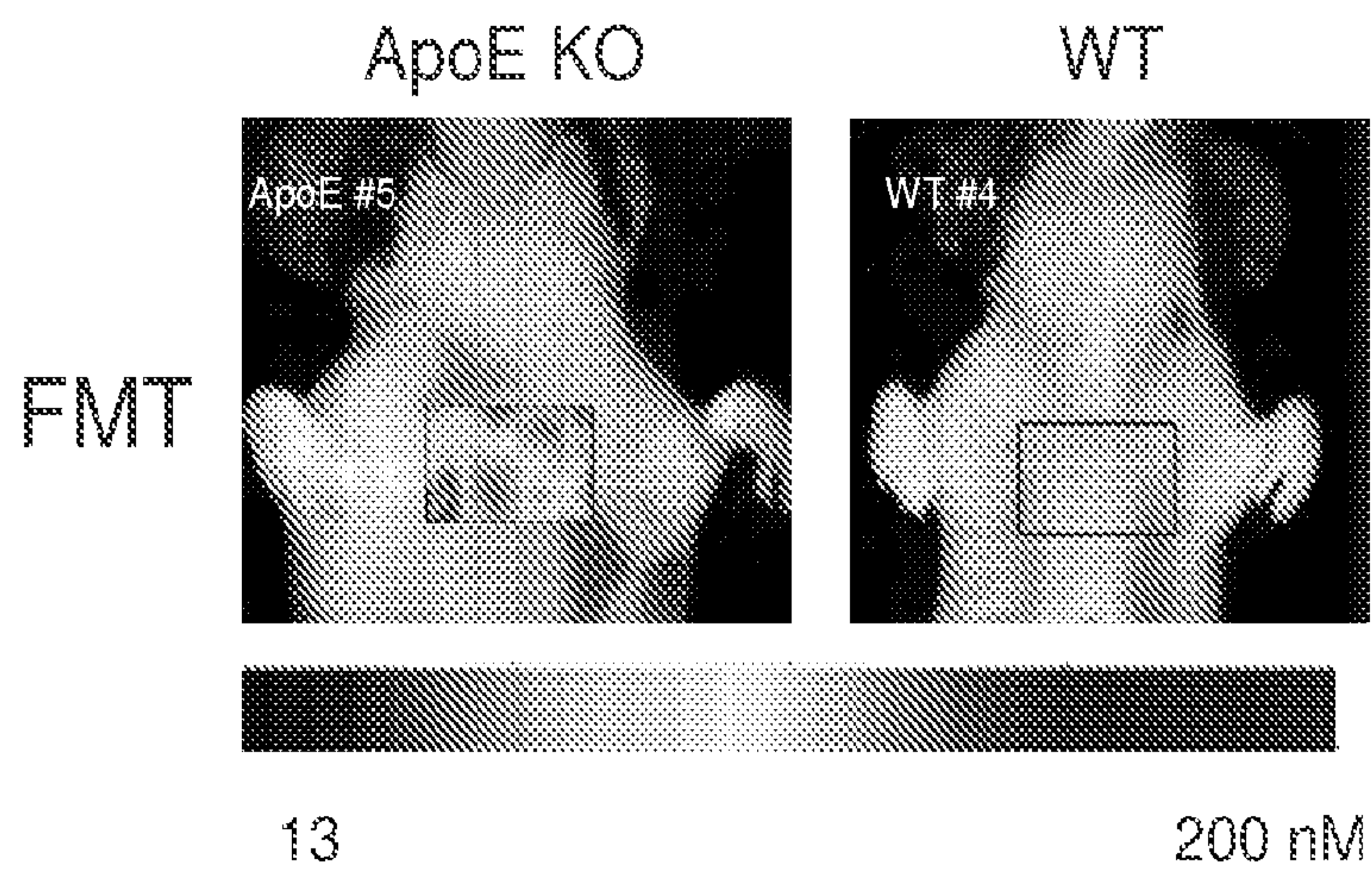


Figure 17 A

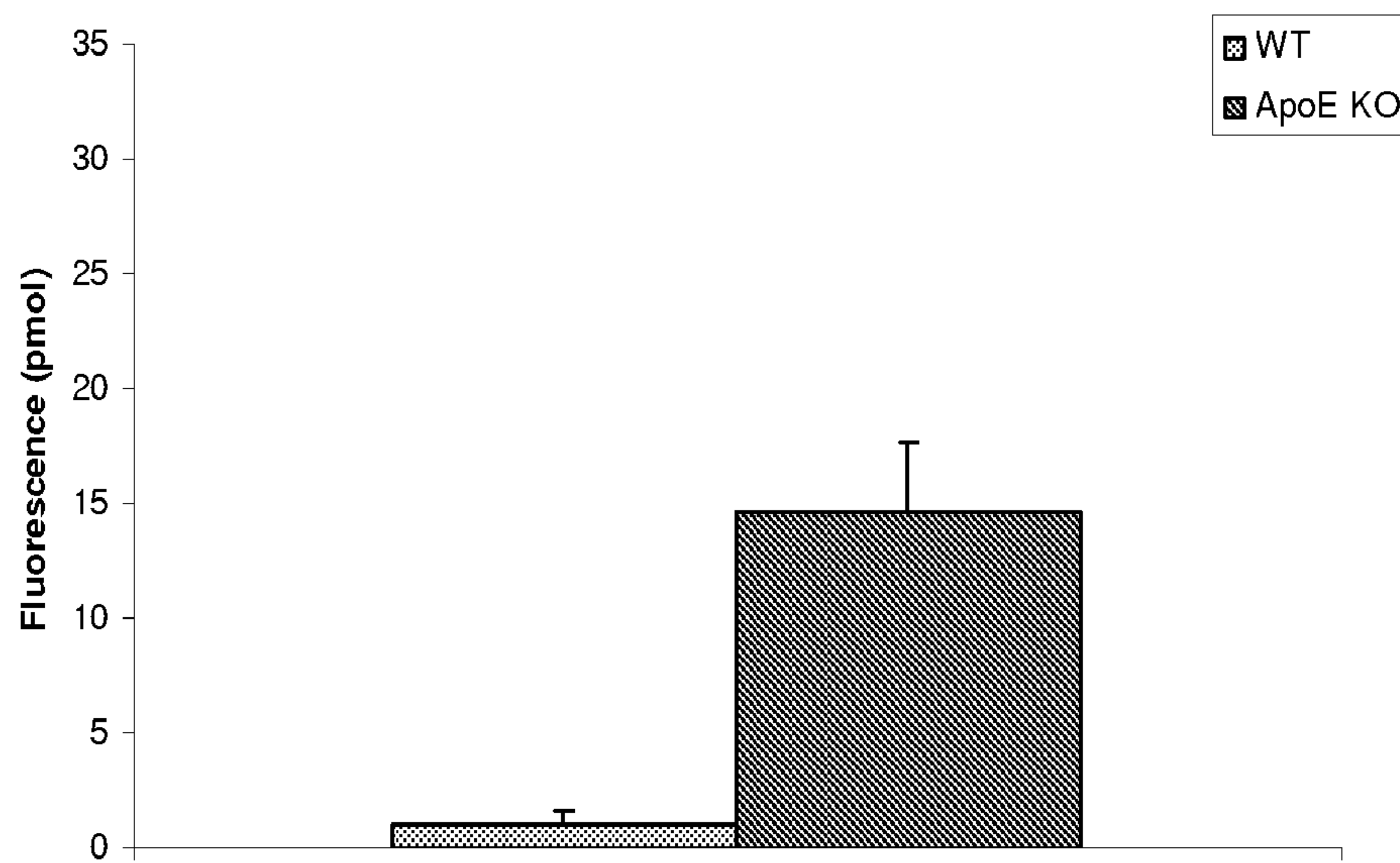


Figure 17B