(54) Title: CONJUGATES AND THEIR USES IN MOLECULAR IMAGING
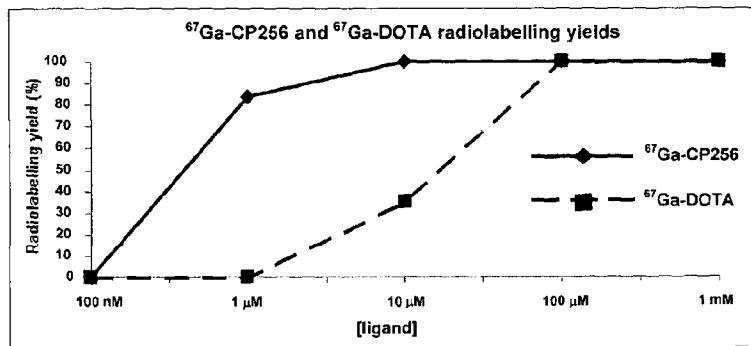
Figure 1

(57) Abstract: The present invention relates to bifunctional compounds, the bifunctional compounds for use in molecular imaging
and therapy, methods of molecular imaging using the bifunctional compounds and kits including the bifunctional compounds for
use molecular imaging. The bifunctional compounds have a tripodal hydroxypyridinone chelating portion and may be conjugated
to a targeting group so that the compounds target specific cells or tissues in a subject.

Conjugates and Their Uses in Molecular Imaging

Field of the Invention

The present invention relates to bifunctional compounds and in
5   particular compounds for use in molecular imaging and therapy.
The compounds may be conjugated to a targeting group so that the
compounds target specific cells or tissues in a subject.

Background of the Invention

10 Molecular imaging may be defined as the three-dimensional mapping
of molecular processes, such as gene expression, blood flow,
physiological changes (pH, [O_2] etc.), immune responses, and cell
trafficking, *in vivo*. It can be used to detect and diagnose
disease, select optimal treatments, and to monitor the effects of
15 treatments to obtain an early readout of efficacy. A number of
distinct technologies can in principle be used for molecular
imaging, including positron emission tomography (PET), single
photon emission tomography (SPET), optical (OI) and magnetic
resonance imaging (MRI). Combinations of these modalities are
20 emerging to provide improved clinical applications, e.g. PET/CT
and SPET/CT ("multi-modal imaging").

Radionuclide imaging with PET and SPET has the advantage of
extremely high sensitivity and small amounts of administered
25 contrast agents (e.g. picomolar *in vivo*), which do not perturb
the *in vivo* molecular processes. Moreover, the targeting
principles for radionuclide imaging can be applied also in
targeted delivery of radionuclide therapy. Typically the isotope
that is used as a radionuclide in molecular imaging is
30 incorporated into a molecule to produce a radiotracer that is
pharmaceutically acceptable to the subject. Many radiotracers
have been developed with a range of properties. For example,
fluorodeoxyglucose (^{18}F) is a labelled glucose derivative that is
frequently used in molecular imaging with PET.

35

WO 2009/021947 describes tripodal chelators for use as MRI
contrast agents. Hydroxypyridinone chelating groups with a

hydrophilic R group are described. The hydrophilic group is
required to help solubilise the chelator. In addition, the
chelator may be coupled to large molecules, such as a dendrimer,
in order to increase the relaxivity of the MRI contrast agent.

5

Summary of the Invention

It may be difficult to prepare radiotracers with sensitive
functional moieties. For example, incorporation of radioisotopes
into the radiotracer may involve elevated temperatures that would
10   disrupt protein structure. It may be desirable to include
sensitive functional moieties into radiotracers and so it is a
need to provide radiotracers that may be prepared using mild
conditions. As a result imaging conjugates with improved
functionality and improved molecular imaging properties may be
15   produced.

Currently, 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-
tetraacetic acid (DOTA) is a common chelator for gallium-68 (and
other metallic radioisotopes such as Ga-67, In-111, Cu-64, Lu-
20   177, Y-90) used in molecular imaging and targeted radionuclide
therapy. However, DOTA has a long radiolabelling time of around
30 minutes (relative to the half-life of ^{68}Ga ~ 68 minutes). In
addition, chelation of gallium by DOTA derivatives often requires
a high labelling temperature of around 95°C. The present
25   inventors have found bifunctional molecules that are able to
quickly chelate radionuclides at room temperature, whilst
retaining adequate or even enhanced stability towards
dissociation in the biological milieu. In addition to the metal
chelating portion, the bifunctional molecules have a reactive
30   portion to couple the bifunctional molecule to a functional
moiety, such as targeting group which can target, for example,
cells, tissues or biological molecules in the body.

At its most general, the present invention provides bifunctional
35   molecules for molecular imaging having a tripodal hexadentate
tris(hydroxypyridinone) chelating portion to couple to a
radionuclide or an imaging label and a reactive functionality to

couple to a targeting group for targeting specific cells or
tissues in a subject or to a delivery vehicle for delivering a
drug, toxin or other such molecule so that the *in vivo*
distribution and/or final location of the target group or
5   delivery vehicle may be monitored.

Accordingly, in a first aspect the present invention provides a
bifunctional compound for use in a method of molecular imaging,
wherein the bifunctional compound is represented by the formula:
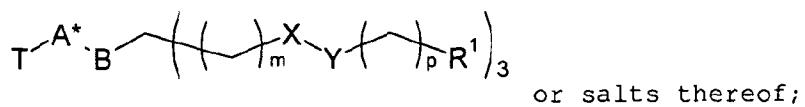
$$T-A^*-B \left(\left(\right)_m X-Y \left(\right)_p R^1 \right)_3$$

10   or salts thereof;

wherein one of X and Y is C=O and the other is NR;

wherein each m and p are independently selected from 0 to 6;

15

wherein R^1 is a chelating group capable of chelating a
radionuclide and is selected from:

20

wherein R, R^2, R^3 and R^4 are independently hydrogen or an
optionally substituted C_{1-7} alkyl group;

B is a linker group for linking the chelating group to the
25   reactive group, wherein is represented by the formula:
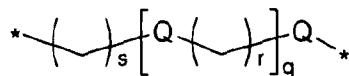
wherein each Q is independently selected from a group consisting
of $-NR^5-$, $-C(O)NR^5-$, $-C(O)O-$, $-NR^5C(O)NR^5-$, $-NR^5C(S)NR^5-$ and $-O-$,
each R^5 is independently hydrogen or an optionally substituted
C_{1-7} alkyl group, each q and s are independently selected from 0 to
5   6 and each r is independently selected from 1 to 6;

A* is a reacted reactive group that is coupled to T, T being a
targeting group capable of binding to a target of interest in the
subject;
10 wherein the method comprises the steps of:
        (a) administering the bifunctional compound to the subject
so that the bifunctional compound binds to the target of
interest; and
        (b) administering an imaging probe composition comprising a
15 radionuclide to the subject, so that the chelating group R^1 of the
bifunctional compound chelates the radionuclide at the target of
interest; and
        (c) detecting the radionuclide to image the target of
interest in the biological system.
20

The bifunctional compound of the first aspect may circulate the
biological system to a targeted location. Then, when the
radionuclide is introduced into the subject, the radionuclide may
quickly pass though the system and chelate with the bifunctional
25 compound. In this way, the radionuclide may be fixed in a
location of interest within a short time of being introduced into
the biological system so that the efficacy of the radionuclide is
maximised.

30 Preferably s is independently selected from 0 to 6, each r is
independently selected from 1 to 6, and q is selected from 1 to
6.

Preferably the method of molecular imagining is PET or SPET.
35

In another aspect, the present invention provides a bifunctional compound for chelating a radionuclide for use in molecular imaging, wherein the compound is represented by the formula:

$$A\text{-}B\text{-}\left(\left(\text{ }\right)_m X\text{-}Y\left(\text{ }\right)_p R^1\right)_3$$

or salts thereof;

wherein one of X and Y is C=O and the other is NR;

wherein each m and p are independently selected from 0 to 6;

wherein R^1 is a chelating group selected from:

,   or

wherein R, R^2, R^3 and R^4 are independently hydrogen or an optionally substituted C_{1-7} alkyl group;

B is a linker group for linking the chelating portion to the reactive group, wherein is represented by the formula:

wherein each Q is independently selected from a group consisting of $-NR^5-$, $-C(O)NR^5-$, $-C(O)O-$, $-NR^5C(O)NR^5-$, $-NR^5C(S)NR^5-$ and $-O-$, wherein each R^5 is independently hydrogen or an optionally substituted C_{1-7} alkyl group, each q and s are independently selected from 0 to 6 and each r is independently selected from 1 to 6;

A is a reactive group for coupling to a biological moiety, a
targeting group, a protein, a polypeptide or a delivery vehicle.

The bifunctional conjugate compounds provide a tripodal
5   hexadentate tris(hydroxypyridinone) chelating portion which is
able to chelate metallic radionuclides in a very short time (in
the order of 5 min or less) and is able to do so at room
temperature in water at around physiological pH. The reactive
portion is linked to the chelating portion and allows the
10 bifunctional molecule to be conjugated with a targeting group or
other vehicle, such as a polypeptide or other biomolecule, drug
or nanoparticle. The quick and low temperature labelling of the
chelating group allows the bifunctional molecule to be coupled
both to a radionuclide with a short half-life, such as ^{68}Ga
15 (T½ = 68 minutes) through the chelating portion and to a
temperature sensitive targeting group, such as a polypeptide
which may denature at radiolabelling temperatures above body
temperature.

20 The efficiency of the chelating reaction also allows labelling at
very low delivery vehicle concentration. In this way, a
significant proportion of the delivery vehicle is radiolabelled
with the radionuclide and, as a result, leading to a very high
specific activity. In this way, the bifunctional molecule
25 provides an excellent precursor for a radiolabelling conjugate.

Preferably the reactive group A is the protein-reactive
functional group. The protein reactive group may react with
proteins or modified proteins or peptides or other vehicles
30 derivatised for the purpose. Preferably the protein-reactive
group is a maleimide group, an aldehyde, an ester, or "click"
reagent such as an alkyne, azide, alkene, hydrazine, hydrazine
derivative, alkoxyamine, alkoxyamine derivative, aminoxy or thiol
group. Maleimide, aldehyde and ester groups efficiently react
35 with peptide thiol- or amine-containing residues (cysteine,
lysine) and so a conjugate can easily form. Other bioorthogonal
functional groups can be engineered into peptides and proteins

for the purpose of conjugating them with alkyne, azide, alkene, hydrazine, aminoxy or thiol groups.

Preferably R^1 is

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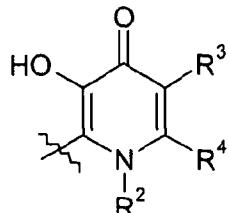
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Preferably R^2, R^3 and R^4 are independently H or CH_3. It is preferred to have relatively small substituents on the heterocyclic ring so that the substituents do not sterically

10 hinder the chelation site of the chelating group and so that the chelator may not become too lipophilic. So in preferred embodiments, R^2, R^3 and R^4 are H. However, one or more methyl substituents on the heterocyclic group may be used to tailor the solubility and/or the hydrophobicity of the molecule. Accordingly

15 one or more of R^2, R^3 and R^4 may be CH_3.

Preferably m is 2 or 3. Preferably p is 0 or 1. More preferably when m is 2, p is 1 or when m is 3, p is 0. This gives a tripodal arm length to the chelating group R_1 of 5 atoms (m + p = 3 plus X

20 and Y). When the tripodal arm length is 5 atoms, the tripodal group is suited to chelating radionuclides, such as gallium.

The linker group B connects the tripodal chelating portion and the reactive functionality (and the e.g. targeting group or

25 carrier vehicle when conjugated). The linking group may be tailored by varying the groups Q, q, r and/or s in order to vary, for example, the length of the linker.

The linker group may be arranged in either direction so that

30 either A or the quaternary carbon (attached to each arm having a chelating group) or both may be attached to Q. In preferred embodiments, B is arranged so that the bifunctional compound is represented by the formula:

The ordering of the atoms in the functional group represented by Q is not limited. In other words, when Q is an amide group, the

5   order of the group from the chelating portion towards the reactive functionality may be $-NR^5-$ then $-C(O)-$ or vice versa.

Preferably at least one Q is an amide ($-C(O)NR^5-$). Preferably q is 1, r is 2 and/or s is 1. More preferably q is 1, r is 2 and

10   s is 1. Preferably R^5 is hydrogen.

Preferably B is represented by one of the following formulae:

15
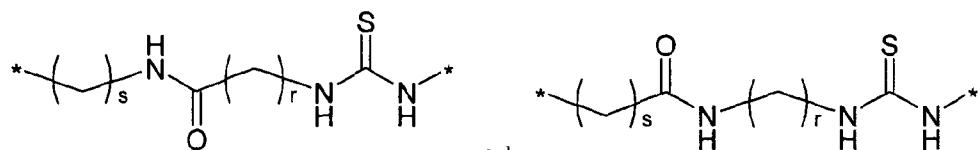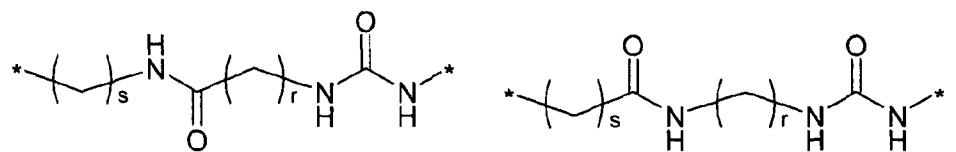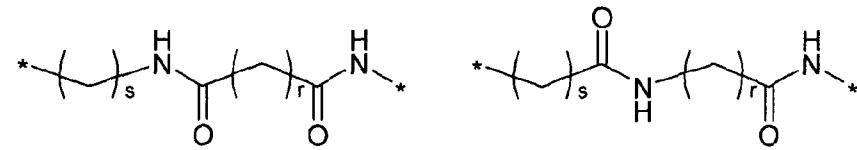
wherein r is selected from 1 to 6 and s selected from 0 to 6. Preferably r is 2 and/or s is 2.

20   In preferred embodiments, the bifunctional compound is represented by the following formula:
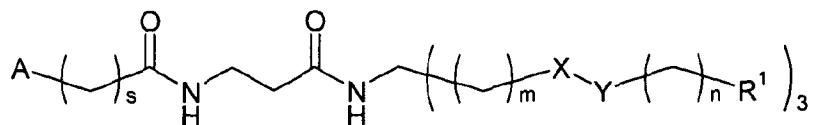
wherein A, s, m, X, Y, n and R^1 are as defined herein.

In preferred embodiments, the radionuclide is an isotope of
technetium, rhenium, copper, cobalt, gallium, yttrium, lutetium
5   or other lanthanide, indium, zirconium, scandium. More preferably
the radionuclide is Tc-99m, Re-186, Re-188, Co-57, In-111, Cu-60,
Cu-61, Cu-62, Cu-64, Cu-67, Tc-94m, Ga-68, Ga-67, Ga-66, Y-90,
Y-86, Sc-44, Sc-47, Fe-52, Sn-117m, Tb-149, Gd-153, Ho-166, Lu-
177, Zr-89, Bi-213 or Co-55 and more preferably the radionuclide
10 is gallium, and most preferably Ga-68 or Ga-67.

Preferably the targeting group is a member of a specific binding
pair that is capable of binding to a binding partner at the
target of interest. More preferably the targeting group and
15 target of interest are a receptor and ligand, or an antibody and
antigen, or metabolic probe e.g. glucose transporter/glucose,
hypoxia/hypoxia responsive moiety, or bone
mineral/bisphosphonate, or avidin (or streptavidin or related
protein) and biotin (or a biotin-like derivative), or RNA and
20 antisense RNA.

Preferably the targeting group is a peptide, protein, antibody,
aptamer or small molecule ligand capable of binding to a binding
partner at the target of interest. More preferably the targeting
25 group is a polypeptide capable of binding to phosphatidylserine
(PS) so that the bifunctional conjugate composition can be
employed in apoptosis or cell death imaging studies. More
preferably the targeting group includes Annexin V and the C2
domain of a synaptotagmin, TIMP-2, CEA, RGD peptide, somatostatin
30 receptor targeting peptide, bombesin, gastrin or VCAM targeting
peptide.

Preferably the target of interest is an *in vivo* molecular target.
More preferably the target of interest is a ligand or receptor
35 expressed on diseased cells or tissue (of which the abundance or
ligand occupancy is to be determined), a cell surface antigen
associated with a disease state, tumour markers, such as a cancer

specific marker or a tissue specific marker or a marker of a
normal process which is up- or down-regulated in a disease state.
Preferably the target of interest is a location, an organ, a tissue type
or physiological property in a subject undergoing molecular imaging.

5

Reactive group A may be for coupling to a delivery vehicle. A
delivery vehicle as defined herein is a molecular structure for
carrying a functional molecule, such as a small molecule drug or
toxin with the purpose of delivering the functional molecule for
10   example in a biological system. The functional molecule may be
contained within or bonded to a surface of the delivery vehicle.
The functional molecule is typically reversibly associated with
the delivery vehicle so that the functional molecule may be
released at a given location in the biological system. Many such
15   delivery vehicles are known in the art. Preferably the delivery
vehicle is a nanoparticle or liposome. Functional molecules, such
as pharmaceutical drugs may be bound to the surface of
nanoparticles or contained within liposomes.

20   Thus, when the bifunctional compounds of the present invention
are coupled to the delivery vehicle and a radionuclide is
chelated by the chelating group R^1, the passage of a drug (carried
by the delivery vehicle) through a subject may be monitored by
imaging of the radionuclide.

25

The reactive group may (in addition to a biological moiety, a
targeting group, a protein, a polypeptide or a delivery vehicle)
be for coupling to a secondary imaging label. Preferably the
secondary imaging label is an optical label, a paramagnetic
30   label, such as a SPIO, or a quantum dot. More preferably, the
imaging label is a paramagnetic label which is a MRI contrast
agent with a paramagnetic probe. Alternatively, the imaging label
is an optical label which is an organic molecule or metal complex
with fluorescent or luminescent properties. The label may, for
35   example in the case of an optical label or paramagnetic label,
allow for the bifunctional compound to be used in multi-modal
imaging.

The reactive group A may couple with further biological moieties, targeting groups, proteins, polypeptides or delivery vehicles. For example, the bifunctional compound may couple the

5   radionuclide to a targeting group for targeting specific cells in a subject and a liposome containing a pharmaceutical drug. In this way, targeted drug delivery may be monitored during its passage through the biological system.

10 The method of molecular imaging may include introducing the bifunctional compound (or conjugate thereof) into a biological system (e.g. a subject) either before the radionuclide is introduced into the biological system (i.e. pre-targeting) or after the radionuclide has been chelated to the chelating portion

15 of the bifunctional compound (i.e. pre-assembled).

When the bifunctional compound (or conjugate) is introduced into the biological system before the radionuclide, the bifunctional compound may circulate the biological system to a preferred or

20 targeted location. Then, when the radionuclide is introduced, the radionuclide may quickly pass though the system and chelate with the bifunctional molecule (or conjugate). In this way, the radionuclide may be fixed in a location of interest within a short time of being introduced into the biological system so that

25 the efficacy of the radionuclide is maximised.

When the radionuclide is chelated to the bifunctional compound (or conjugate) before introduction into the biological system, the bifunctional compound may be used to monitor the passage of

30 the bifunctional compound or the conjugate having a biological moiety, a targeting group, a protein, a polypeptide a carrier vehicle or a secondary imaging label attached to the compound through the biological system.

35 Preferably the biological moiety, targeting group, protein, polypeptide or delivery vehicle are bound to the bifunctional compound to form a bifunctional compound conjugate before

introduction of the bifunctional compound composition into the biological system or subject.

Preferably the biological system is a subject undergoing
5   diagnosis or therapy for a disease or condition. The therapy may be radionuclide therapy. In particular the therapy may be cancer treatment using the radionuclide or a chemotherapeutic agent associated with the bifunctional molecule, for example via the delivery vehicle to which it is attached. Preferably the
10   bifunctional molecule is associated with or bound to a therapeutic agent. Alternatively, the subject may be a subject in whom the biodistribution of the targeting molecule or delivery vehicle is being evaluated.

15   In a second aspect, the present invention provides a bifunctional compound conjugate for chelating a radionuclide for use in molecular imaging, wherein the conjugate is represented by the formula:

$$T\overset{A^*}{\underset{B}{\frown}}\left(\left(\right)_m X\text{-}Y\left(\right)_p R^1\right)_3$$

or salts thereof;
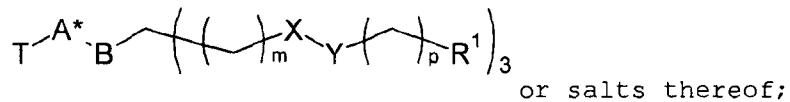
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wherein one of X and Y is C=O and the other is NR;

wherein m and p are independently 0 to 6;

25   wherein R^1 is a chelating group selected from:
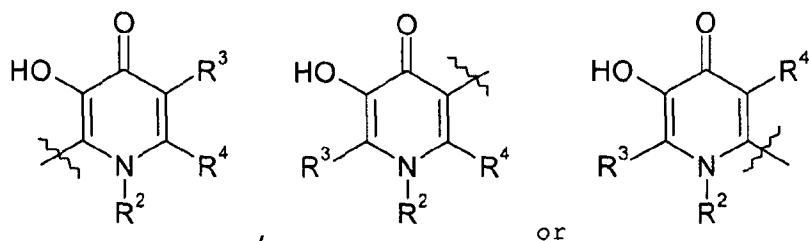
wherein R, R^2, R^3 and R^4 are independently hydrogen or an
30   optionally substituted C_{1-7} alkyl group;

B is a linker group for linking the chelating portion to the reactive group, wherein is represented by the formula:
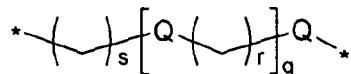
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wherein each Q is independently selected from a group consisting of $-NR^5-$, $-C(O)NR^5-$, $-C(O)O-$, $-NR^5C(O)NR^5-$, $-NR^5C(S)NR^5-$, $-O-$, wherein each R^5 is independently hydrogen or an optionally substituted C_{1-7} alkyl group, each q and s are independently

10   selected from 0 to 6 and each r is independently selected from 1 to 6; and

A* is a reacted reactive group for coupling to T, wherein T is a biological moiety, a targeting group, a protein, a polypeptide or

15   a delivery vehicle.

In a further aspect, the present invention provides a kit for use in a method of molecular imaging or therapy comprising: a bifunctional molecule composition having a bifunctional compound

20   as described herein, wherein the compound is linked to a biological moiety, a targeting group, a protein, a polypeptide or a delivery vehicle through reactive group A; and an imaging composition comprising a radionuclide as described herein.

25   In another aspect, the present invention provides a kit for use in a method of molecular imaging or therapy comprising: a bifunctional molecule composition having a bifunctional compound as described herein; and a biological moiety, a targeting group, a protein, a polypeptide or a delivery vehicle for coupling to

30   the bifunctional molecule. The kit may also include an imaging composition comprising a radionuclide as described herein.

In another aspect, the present invention provides a method of molecular imaging a target of interest in a biological system,

35   the method comprising:
            (a) administering to a subject a composition comprising

13

bifunctional compound as described herein, wherein the compound
is linked to a biological moiety, a targeting group, a protein, a
polypeptide or a delivery vehicle through reactive group A;

(b) administering to the subject an imaging composition
5   comprising a radionuclide, wherein the chelating group R^1 of the
bifunctional compound is capable of chelating the radionuclide;
and

(c) detecting the radionuclide to image the target of
interest in the biological system.
10

In another aspect, the invention provides a method of molecular
imaging a target of interest or passage of interest in a
biological system, the method comprising:

(a) chelating a radionuclide to the bifunctional molecule
15   through the chelating group as described herein, wherein the
compound is linked to a biological moiety, a targeting group, a
protein, a polypeptide or a delivery vehicle through reactive
group A, to form a radiolabelled bifunctional molecule compound;

b) administering to a subject a composition comprising the
20   radiolabelled bifunctional compound; and

(c) detecting the radionuclide to image the target or
passage of interest in the biological system.

Preferably the radionuclide is a radionuclide capable of
25   delivering radiotherapy to the target of interest. Preferably the
radionuclide is Cu-67, Re-186, Re-188, Y-90, Lu-177, Sc-47 or
Bi-213.

The preferred and optional features of any one aspect may be
30   applied to any other aspect. In particular, the preferred
features of the bifunctional compounds apply to the method of
molecular imaging and vice versa.

Embodiments of the present invention will now be described by way
35   of example and not limitation with reference to the accompanying
figures.

Brief Description of the Figures

Figure 1. shows the radiolabelling yield of chelating portion CP256 with ^{67}Ga after 5 minutes at room temperature compared to the radiolabelling yield of DOTA with ^{67}Ga after 40 minutes at

5   80°C;

Figure 2. shows the elution profiles of ^{67}Ga-CP256 from a PD10 column for radiolabelled solutions at 2.27 µM (A) and 0.227 µM (B) of CP256 after incubation with apotransferrin, compared to ^{67}Ga-citrate (C).

10   **Figure 3.** shows retention of ^{67}Ga-citrate, ^{67}Ga-CP256, ^{67}Ga-DOTA and ^{67}Ga-DTPA to 30 kDa MWCO filters, after incubation with apotransferrin.

Figure 4. shows the radioactive elution profile, using a PD10 size exclusion cartridge, of C2Ac and C2Ac-(7) after 15 minutes

15   incubation with Gallium-68 in acetate buffer; and

Figure 5. The ^{68}Ga-labelled conjugate showed specific calcium dependent binding to phosphatidylserine (PS) in a red blood cell binding assay

Figure 6. shows a PET scan of a mouse 90 minutes after

20   administration of (a) C2Ac-(7)-^{68}Ga and (b) unchelated "free" ^{68}Ga.

Figure 7. Size exclusion radiochromatography (PD10 column) showing chelation of ^{68}Ga by CP256 in serum: a) control, ^{68}Ga chloride incubated in serum; b) control, preformed ^{68}Ga-CP256 complex incubated with serum; c) control, ^{68}Ga incubated in

25   buffer; and d) experimental sample, ^{68}Ga chloride added to serum to which CP256 (23 µM or above) had been added.

Figure 8. PD10 size exclusion chromatography profile of ^{111}In after incubation of ^{111}In-acetate and ^{111}In-CP256 in serum, compared to ^{111}In-CP256 in buffer.

30   **Figure 9.** Biodistribution of ^{67}Ga-CP256-SER4 (Group 1), 67Ga-citrate 5 min after injection of SER4 (Group 2), and 67Ga-citrate 5 min after injection of CP256-SER4 (Group 3), in wild type mice (top) or sialoadhesin knockout mice (bottom).

Figure 10. Biodistribution of ^{68}Ga-CP256-SER4 (Group 1), ^{68}Ga

35   acetate injected 1 h after injection of SER4 (Group 2), and ^{68}Ga acetate injected 1 h after injection of CP256-SER4 (Group 3), in wild type mice.

Figure 11. Fig. 11A (left): PET/CT scan (maximum intensity
projection) of a mouse from group 3 showing pre-targeting using
CP256-SER4: 8 week-old C57B1/6 injected with CP256-SER4 followed
by Ga-68 acetate, showing liver/spleen uptake. Image taken at
5   1.5h post injection. Fig. 11B (right): for comparison, mouse
injected with Ga-68 acetate only, showing retention in blood pool
and accumulation in joints.

Detailed Description

10   *A: Targeting the site of interest*
Where the bifunctional compound is for coupling to a targeting
molecule, a wide range of primary targeting groups may be linked
to the bifunctional compounds to direct the bifunctional
compounds with the chelating group to different targets of
15   interest in the biological system in question.

In one aspect, the target of interest will comprise a member of a
specific binding pair that is capable of specifically binding to
the primary targeting group in the system, such that they will be
20   members of a pair of molecules which have particular specificity
for each other and which in normal conditions bind to each other
in preference to binding to other molecules. Examples of
specific binding pairs are well known in the art and include for
example receptors and ligands, enzymes and substrates, antibodies
25   and antigens. Thus, primary targeting groups may be peptides,
proteins or other biological molecules, such as aptamers, or
small molecule ligands, that bind to specific *in vivo* molecular
targets. Classes of targets of interest include ligands or
receptors or transporters expressed on diseased cells or tissue,
30   cell surface antigen associated with disease states, or tumour
markers, e.g. cancer specific markers or tissue specific markers.

One group of primary targeting groups are polypeptides capable of
binding to phosphatidylserine (PS) so that the bifunctional
35   molecule conjugates of the present invention can be employed in
apoptosis or cell death imaging studies. Examples of such
polypeptides include Annexin V and the C2 domain of a

synaptotagmin. Polypeptides that comprise one or more C2 domains
are well known in the art. While some polypeptides have only one
C2 domain, others have two or more C2 domains, and the domains
are generally described by attaching a letter (in alphabetical
5   order) to the end of the name (e.g., C2A, C2B, and so on). For a
protein that contains only one C2 domain, the domain is simply
referred to as C2 domain. While the examples below use the C2A
domain of rat synaptotagmin I, other C2 domains that are capable
of binding to PS could be employed instead, for example a C2A
10   domain of a synaptotagmin of another species. Further examples
of proteins that contain a C2 domain include but are not limited
to synaptotagmin 1-13, protein kinase C family members of
serine/threonine kinases, phospholipase A2, phospholipase δ1,
cofactors in the coagulation cascade including factors V and
15   VIII, and members of the copine family. Human synaptotagmins
include synaptotagmin 1-7, 12 and 13.

Other proteins that may be employed in the present invention
include polypeptides capable of specifically binding to markers
20   that are expressed by cancer cells, thereby enabling the cancer
cells to be imaged using the bioconjugates. By way of example,
the present invention can employ an anti-CD33 antibody, or
fragment thereof, for imaging cancer cells expressing CD33 such
as cells of myelomonocytic lineage and leukaemic cells, see
25   Emberson et al., J. Immunol. Methods. 305(2):135-51, 2005. A
further example is the use of a tissue inhibitor of
metalloproteinases (TIMPs), such as TIMP-2, for imaging matrix
metalloproteinase expression, as expression of metalloproteinases
has been implicated in metastatic processes, see Giersing et al.,
30   Bioconjug Chem. 12(6): 964-71, 2001. A further example of a
polypeptide that can be used to make conjugates according to the
present invention is complement receptor 2 (CR2). Antibodies
capable of binding to the glycoprotein carcinoembryonic antigen
(CEA) may also be used as primary targeting groups as members of
35   this family of glycoproteins are expressed on colorectal cancer
cells, gastric cancer cells, pancreatic cancer cells, lung cancer
cells, medullary thyroid cancer cells and breast cancer cells.

A further example may exploit the affinity or the peptide
sequence arginine-glycine-aspartic acid (RGD) for the $\alpha_v\beta_3$
integrin expressed highly in the endothelium of tissues
5   undergoing angiogenesis as is commonly seen in tumours,
atherosclerotic plaque and repairing diseased tissue such as
infarcted myocardium, by linking an RGD peptide derivative to the
bifunctional compound by means of a suitable reactive group A.

10   A further example may exploit the affinity of the peptide
octreotide or other related analogue of somatostatin, which may
bind to the somatostatin receptor expressed highly at the surface
of cancer cells e.g. in carcinoid, medullary thyroid carcinoma
and other neuroendocrine tumours, by linking a somatostatin
15   analogue peptide to the bifunctional compound by means of a
suitable reactive group A.

Another example may exploit the affinity of antibodies to cell
adhesion molecules. One example is the affinity of the
20   monoclonal antibody, SER 4, to the macrophage adhesion molecule,
sialoadhesin. Sialoadhesin is found on the surface of
macrophages, and, for example, in high amounts on macrophages of
the spleen, liver, lymph node, bone marrow colon and lungs.

25   The skilled person will be able to use other targeting groups
such as bombesin, gastrin, or VCAM targeting peptide.

Preferably, the primary targeting group may be linked to the
bifunctional compound as a conjugate having a functional group
30   for binding to the bifunctional compound and the reactive group
for linking to the primary targeting group. Various
possibilities for achieving this will be apparent to the skilled
person. By way of example, in one embodiment, a reactive group
for site-specific linkage to the primary targeting group may be
35   employed, for example a maleimide group for site-specific linkage
to thiol groups in a biomolecule such as a peptide, polypeptide
or antibody. The maleimide group can then be site-specifically

linked to a thiol group, for example of a cysteine residue
incorporated site-specifically into the peptide or protein for
the purpose. In another example the reactive group in the
bifunctional chelator would be an aldehyde or ketone group able
5   to react site-specifically with a protein or peptide to which a
hydrazine or similarly reactive group has been site-specifically
incorporated (e.g. using a hydrazinonicotinic acid derivative) to
form a hydrazone or similar link.

10   In some cases, the primary targeting group may comprise a
suitable polypeptide or protein, or a fragment or domain thereof.
Accordingly, while for convenience the methods herein are
generally described by reference to "polypeptides", this should
be taken to include shorter sequences of amino acids (e.g., from
15   3 to 10 amino acids in length to 30, 40 or 50 amino acids in
length), sometimes referred to in the art as peptides. The term
should also be taken to include polypeptides having secondary,
tertiary or quaternary structure, generally referred to as
proteins, as well as multidomain proteins. In some embodiments,
20   and in the examples provided below, the polypeptides used as
primary targeting groups are protein domains. "Protein domains"
are fragments of a full length protein that have the ability to
retain structure independent of the full length protein,
typically forming a stable and folded three-dimensional
25   structure. Many proteins consist of several structural protein
domains and it is common for a particular domain to be found in a
range of related proteins. Protein domains vary in length from
between about 25 amino acids up to 500 amino acids in length, or
from 50 amino acids to 250 amino acids, or from 75 amino acids to
30   150 amino acids.

In the present invention, where the polypeptide is an antibody,
this term describes an immunoglobulin whether natural or partly
or wholly synthetically produced. The term also covers any
35   polypeptide or protein comprising the antigen binding domain of
an antibody; antibody fragments which comprise an antigen binding
domain such as Fab, scFv, Fv, dAb, Fd; and diabodies. It is

possible to take monoclonal and other antibodies and use
techniques of recombinant DNA technology to produce other
antibodies or chimeric molecules which retain the specificity of
the original antibody. Such techniques may involve introducing
5   DNA encoding the immunoglobulin variable region, or the
complementarity determining regions (CDRs), of an antibody to the
constant regions, or constant regions plus framework regions, of
a different immunoglobulin. See, for instance, EP 0 184 187 A,
GB 2,188,638 A or EP 0 239 400 A.
10

Antibodies can be modified in a number of ways and the term
"antibody molecule" should be construed as covering any specific
binding member or substance having an antibody antigen-binding
domain with the required specificity. Thus, this term covers
15   antibody fragments and derivatives, including any polypeptide
comprising an immunoglobulin binding domain, whether natural or
wholly or partially synthetic. Chimeric molecules comprising an
immunoglobulin binding domain, or equivalent, fused to another
polypeptide are therefore included. Cloning and expression of
20   chimeric antibodies are described in EP 0 120 694 A and EP 0 125
023 A. It has been shown that fragments of a whole antibody can
perform the function of binding antigens. Examples of binding
fragments are (i) the Fab fragment consisting of VL, VH, CL and
CH1 domains; (ii) the Fd fragment consisting of the VH and CH1
25   domains; (iii) the Fv fragment consisting of the VL and VH
domains of a single antibody; (iv) the dAb fragment (Ward, E.S.
et al., Nature 341, 544-546 (1989)) which consists of a VH
domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a
bivalent fragment comprising two linked Fab fragments (vii)
30   single chain Fv molecules (scFv), wherein a VH domain and a VL
domain are linked by a peptide linker which allows the two
domains to associate to form an antigen binding site (Bird et al,
Science, 242; 423-426, 1988; Huston et al, PNAS USA, 85: 5879-
5883, 1988); (viii) bispecific single chain Fv dimers
35   (PCT/US92/09965) and (ix) "diabodies", multivalent or
multispecific fragments constructed by gene fusion (WO 94/13804;
Holliger et al, P.N.A.S. USA, 90: 6444-6448, 1993). Fv, scFv or

diabody molecules may be stabilised by the incorporation of
disulphide bridges linking the VH and VL domains (Reiter et al,
Nature Biotech, 14: 1239-1245, 1996). Minibodies comprising a
scFv joined to a CH3 domain may also be made (Hu et al, Cancer
5   Res., 56: 3055-3061, 1996).

Affibodies may also be used as a primary targeting group.
Affibodies are small high affinity proteins that may bind
specifically to a large number of target proteins or peptides.
10   Affibodies imitate monoclonal antibodies in many respects and are
classed as antibody mimetics. The selection of affibody should
not be limited and many affibodies are known in the art, such as
unconjugated anti-TNF-α affibody. Anti-TNF-α affibody may be
produced in E. coli and targets human cytokine tumor necrosis
15   factor α, TNF-α. TNF- α is a pleiotropic inflammatory cytokine.
The cytokine is produced by several types of cells, but
especially by macrophage. It acts as a key mediary in the local
inflammatory immune response and has further immune regulatory
functions. TNF-α is an acute phase protein which initiates a
20   cascade of cytokines and increases vascular permeability, thereby
recruiting macrophage and neutrophils to a site of infection.
Therefore, TNF-α is an important target for inflammation in
atherosclerotic lesions and inflammations.

25   Each bifunctional compound may be linked to a plurality of
primary targeting groups, thereby increasing (a) the affinity of
the primary targeting groups for the target of interest as the
overall affinity increases as the product of the affinity of each
targeting group for the target and/or (b) the rate of binding to
30   the target, i.e. the likelihood of binding ensuing from each
encounter between target and primary targeting group in vivo.

B: Imaging probesThe imaging probes that are capable of binding
to the bifunctional compounds comprise a radionuclide (bound
35   through the chelating group).

The imaging probes (metallic radioisotopes) used in accordance
with the present invention have the property of binding to the
chelating portion of the bifunctional compounds either *in vitro*
or *in vivo*, in either the pre-targeted or pre-assembled
5   approaches. One possibility for achieving this is to use
radionuclides that have an intrinsic binding affinity for
chelating portion of the bifunctional compounds. By way of
example, $^{68}Ga^{3+}$ is capable of binding to the hydroxypyridinone
groups of the chelating portion and can be used in PET imaging
10   techniques.

The radionuclide probes used in accordance with the present
invention may use a range of different radionuclides depending on
the application for which the probes are intended. Examples of
15   radionuclides that may form part of the probes of the present
invention include radionuclides of technetium, rhenium, copper,
cobalt, gallium, yttrium, lutetium, indium, zirconium, scandium,
namely Tc-99m, Ga-67, In-111 (SPET), Cu-64, Cu-60, Cu-61, Cu-62,
Tc-94m, Ga-68, Co-55, Zr-89, Sc-44 (PET), Cu-67, Re-186, Re-188,
20   Y-90, Lu-177, Sc-47 or Bi-213 (radionuclide therapy). The
present invention may employ the radionuclides alone or in
combinations.

The radionuclide may be in any form, such as a solvate, hydrate,
25   salt or weak chelate. Solvates, hydrates, salts and weak
chelates of radionuclides are known in the art. When the
radionuclide is to be administered to a subject in a radionuclide
composition (for example, when the bifunctional compound chelates
the radionuclide in the subject after administration), the
30   radionuclide should be in a pharmaceutically acceptable form.
For example, the radionuclide may be a citrate or acetate salt of
the metal ion.

The present invention may also involve the use of secondary
35   imaging labels, such as an optical label or a paramagnetic label
(through the reactive group A) that may be linked to or
associated with the bifunctional compounds, for example to enable

multi-modal imaging to be carried out. Examples of optical probes include fluorophores such as fluorescein and luminescent molecules and complexes such as lanthanide complexes and tricarbonylrhenium-quinolinate conjugates. The possibility to

5   incorporate optical probes as well as radionuclides and MR contrast agents provides the opportunity to combine modalities to enhance diagnosis and detection, for example the location of disease at the whole body level can be identified by whole body scanning with PET or SPECT while the same tracer may be detected

10   visually during surgery to assist identification of diseased tissue. Similarly, combined PET and MR imaging can provide the advantage of high sensitivity (PET, SPET), quantification of signal (PET) and anatomical resolution (MR) and measurement of the microenvironment (MR contrast enhancement).

15

C: Conjugation chemistry
The bifunctional compounds of the present invention may use a range of different chemistries and techniques for linking the biological moiety, targeting group, label, protein or polypeptide

20   to the bifunctional compounds and/or for introducing further groups and properties into the bifunctional compounds of the present invention.

Thus, in the present invention, the reactive group provides a way

25   of coupling the targeting group to the bifunctional compound, for example when the reactive group is a maleimide group (to bind to the thiol side chain of cysteine in the protein/peptide sequence)

Uses of the present invention

30   The bifunctional compounds of the present invention may be used for the molecular imaging of diseases, such as cancer, cardiovascular disease, immunological disease and associated conditions, such as transplant, inflammation, leukocyte tracking, stem cell tracking, allergy and infection. The bifunctional

35   compounds may also be employed for radionuclide therapy for treating cancer and arthritis.

The applications of the bifunctional compounds and radionuclides
of the present invention include a wide range of imaging and
spectroscopic applications that can employ the radionuclide
and/or a further label, for example, in multi-modal imaging

5   studies. As described herein, the bifunctional compounds are
particularly useful for *in vivo* imaging applications such as cell
death imaging, for example using bifunctional compounds for the
detection of apoptosis. This might be useful in a number of
different medical or research applications, for example in the

10   fields of oncology (e.g. in monitoring response to chemotherapy),
cardiovascular medicine (e.g. in imaging damaged myocardium post
myocardial infarction) or graft rejection (e.g. in imaging
cardiac allograft rejection).

15   The present invention is particularly relevant to nuclear
medicine imaging techniques, such as Single Photon Emission
Tomography (SPET), an imaging technique that detects gamma rays
emitted from a radionuclide to produce a three dimensional image
of the distribution of the radionuclide in a sample or subject,

20   and Positron Emission Tomography (PET), an imaging technique that
provides three-dimensional images by detecting pairs of gamma
rays emitted indirectly by a positron-emitting radionuclide
introduced into a sample or subject. By way of example SPET
studies can be carried out using In-111 and PET studies using Ga-

25   68. The skilled person, however, will be aware of other suitable
SPET and PET radionuclides that can be employed in the present
invention. Generally, the present invention may be employed for
positron emission tomography (PET), single photon emission
tomography (SPET), optical (OI) and/or magnetic resonance imaging

30   (MRI) by appropriate selection of imaging probe, or for
radionuclide therapy by selection of the appropriate radionuclide
(e.g. Y-90, Lu-177).

The bifunctional compounds of the present invention may be used
35   in methods of multi-modal imaging, that is where information or
images are derived from two different techniques, either by the
detection of the imaging probe capable of detection using two

different techniques or by providing a second label at the site
in the biological system where the bifunctional compounds become
localised, most conveniently by linking or associating the second
label with the bifunctional compounds as explained in detail

5   above. Multi-modal studies will be co-registered and may entail
simultaneous imaging with two modalities or may need to take
place in two steps, but generally employ the same sample so that
spatial information obtained using the two techniques can be
compared. Examples of multi-modal imaging include PET/CT,

10   SPET/CT, PET/MR and SPET/MR

Examples

Example 1 - Synthesis of chelator CP256 (Compound 52)

5   The tris tert-butyl esters **61** and **62** were synthesised by
following established literature methods (G. R. Newkome, R. K.
Behera, C. N. Moorefield and G. R. Baker, _J Org Chem_, 1991, **56**, 7162-
7167).
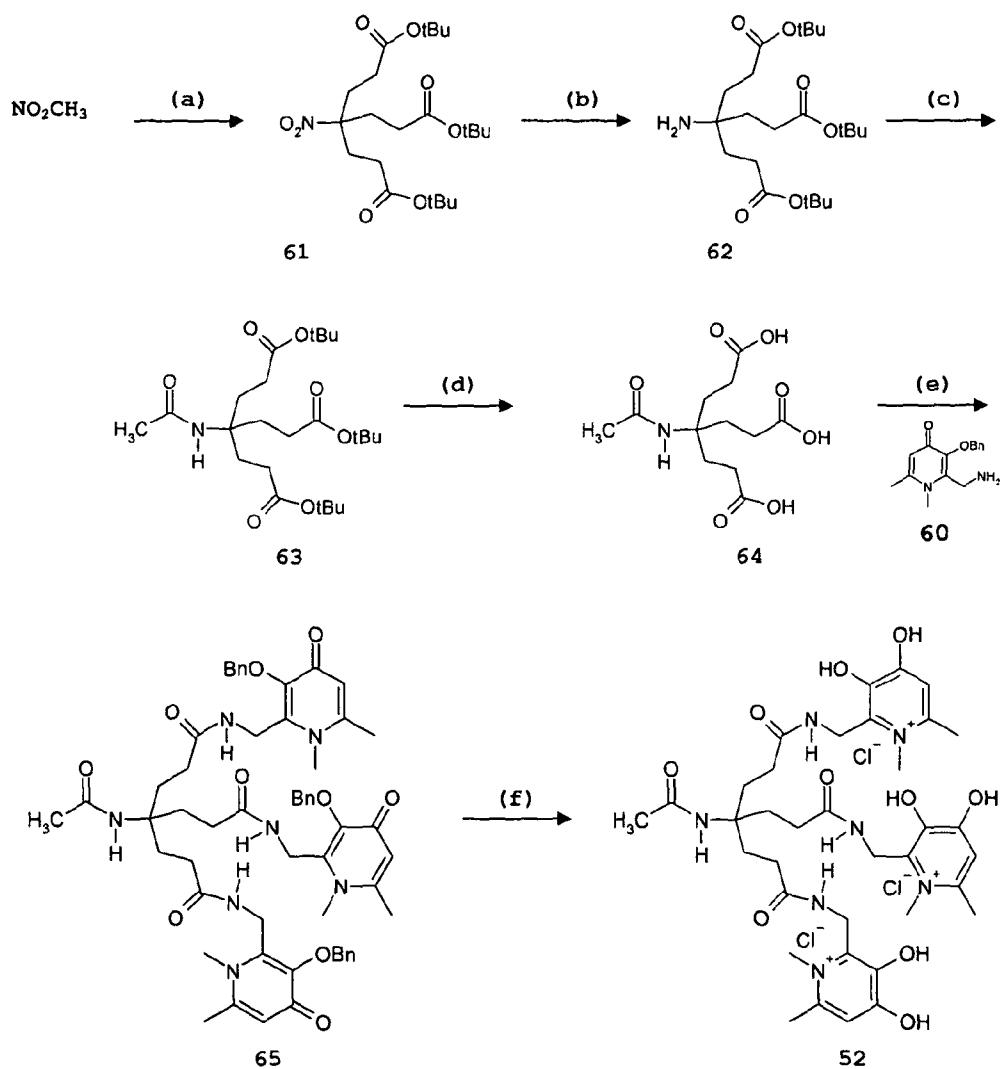
Synthesis of **52**. Materials and methods: (a) Triton B, Tert-butyl acrylate, DME,
70°C; (b) T-1 Raney Ni, H₂, 25°C, EtOH; (c) CH₃COCl, Et₃N, CH₂Cl₂, 0-25°C, (d) HCOOH;
(e) **60**, DCCI, HoBt, DMF 25°C; (f) Pd/C, H₂, HCl, CH₃OH, 25°C.

Nitro tripod **61** was produced by the reaction of nitromethane and tert-butyl acrylate in the presence of benzyltrimethylammonium hydroxide and recrystallised from ethanol in 57% yield. The

5　nitro group of **61** was then reduced to an amine to form **62** by hydrogenation with a T-1 Raney Nickel catalyst,[5] which was specially prepared from a nickel/aluminium alloy. Acetylation of **62** with acetyl chloride and purification by column chromatography gave **63** in 72% yield. The tert-butyl groups were then removed

10　with formic acid and the product, compound **64** was recrystallised from ethanol/petroleum spirit with a yield of 93%.

The conjugation of the bidentate moieties to the tripodal framework and subsequent deprotection to create **52** is shown

15　schematically above. 1 equivalent of tri-acid **64** was reacted with 3.6 equivalents of bidentate compound **60** in a coupling reaction with DCCI and HOBt in DMF. After removal of the solvent, the protected hexadentate ligand **65** was purified by column chromatography in 50% yield. The benzyl protecting groups

20　were removed by catalytic hydrogenation (Pd/C) in an acidic solution (HCl) of methanol. After removal of the catalyst, the tris HCl salt of **52** was precipitated from methanol/diethylether and dried under vacuum, resulting in 0.4 g (74%) of **52** as a white solid.

25

Experimental Details

All chemicals were purchased from Sigma Aldrich and Acros Organics and were reagent grade or better. Solvents were purchased from Fisher Scientific, Sigma Aldrich and VWR. NMR

30　solvents were purchased from GOSS Scientific and NMR tubes were manufactured by Wilmad. TLC plates (silica gel 60 UV$_{254}$ on alumina backing) and silica gel for column chromatography were purchased from Merck.

35　Samples were dried in a vacuum oven (Gallenkamp, rated to $\leq 250°C$) connected to a vacuum pump (BOC-Edwards.) Samples were hydrogenated on a Parr hydrogenator (room temperature) at 40 psi

with automatic shaking. Infrared Spectra were acquired on a
Perkin Elmer Spectrum one FTIR using KBr disks. The software
used was called "Spectrum."

5   ^{1}H-NMR spectra were acquired on a 400 MHz Ultra-shield magnet
(Bruker) and with XWin-NMR software. Spectra were analysed on
MestReNova sotware version 5.3 (Mestrelab Research.) Mass
spectrometry samples were submitted to the EPSRC Mass
Spectrometry Centre, Swansea or the Mass Spectrometry facility,
10 King's College, London. Samples at EPSRC were run on a Waters
ZQ4000 quadrupole mass spectrometer (ESI), a Finnigan MAT 95 XP
high resolution double focusing mass spectrometer (EI, CI) or a
Micromass Quattro II quadrupole mass spectrometer (EI.) Samples
at King's College were run on a Thermofisher LCQ DECA XP ion trap
15 mass spectrometer.

Isotopic distributions were calculated using Molecular Weight
Calculator version 6.46. The resulting graph data were extracted
and processed in Microsoft Excel 2003.
20

2-Chloromethyl-5-hydroxypyran-4(1H)-one (Chlorokojic acid) (54)
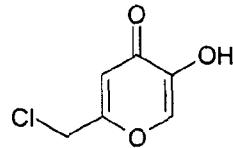
25 50 g kojic acid (350 mmol, 1 equiv) was added to 220 ml thionyl
chloride with stirring. The reaction vessel was cooled with ice
and the mixture was stirred for 1 hour until a bright yellow
suspension had formed. The mixture was vacuum filtered to give a
crude white solid after washing with petroleum spirit. This
30 solid was recrystallised from hot water to give 40 g of off-white
crystals in 71% yield.
^{1}**H NMR** (DMSO-d$_6$) σ 9.2 (s, 1H **3-OH**) 8.1 (s, 1H **2-H**) 6.5 (s, 1H **5-
H**) 4.6 (s, 2H **6-CH$_2$**-Cl)

2-Methyl-5-hydroxypyran-4(1H)-one (Allomaltol) (55)
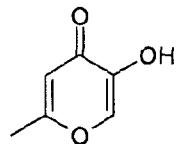
5 20 g chlorokojic acid (**54**) (125 mmol, 1 equiv) was added to 100
   ml distilled water and stirred at 50°C. Zinc dust (17 g) was
   slowly added to the solution and the temperature was increased to
   70°C. 40 ml of concentrated HCl was added dropwise into the
   stirring mixture slowly over a period of 90 minutes. During this
10 time, the chlorokojic acid dissolved. After stirring for a
   further 3 hours at 70-80°C, the now dark brown solution was hot-
   filtered to remove any solids. After cooling, the product was
   extracted with 5 X 70 ml portions of CH_2Cl_2. The solution was
   dried (Na_2SO_4) and the solvent was removed in vacuo. The crude
15 solid was recrystallised from hot isopropanol, resulting in 8.63
   g of beige crystals in 55% yield.
   ^{1}H NMR (DMSO-d_6) σ 9.0 (s, 1H **2-H**) 8.0 (s, 1H **3-OH**) 6.2 (s, 1H **5-
   H**) 2.2 (s, 3H **6-CH₃**)

20 **2-Hydroxymethyl-3-hydroxy-6-methyl-pyran-4(1H)-one (56)**
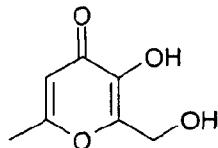
   3 g allomaltol (**55**) (23.8 mmol, 1 equiv) was added to a stirred
25 solution of 1.05 g NaOH in 25 ml distilled water. 2.2 ml of a
   37% solution of formaldehyde was added dropwise over 10 minutes.
   The solution was stirred for a further 18 hours at room
   temperature. The pH was adjusted to 1 with concentrated HCl at
   which point a white precipitate formed. To allow complete
30 precipitation, the solution was cooled at 4°C for 24 hours. The
   solid was filtered and washed with cold diethyl ether and dried
   in vacuo resulting in 2.92 g of a white solid in 78% yield.

^{1}H NMR (DMSO-d$_6$) σ 8.8 (s, 1H **3-OH**) 6.2 (s, 1H **5-H**) 4.3 (s, 2H **2-CH$_2$-OH**) 2.2 (s, 3H **6-CH$_3$**)

2-Hydroxymethyl-3-benzylroxy-6-methyl-pyran-4(1H)-one (57)
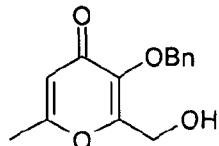
To 60 ml of CH$_3$OH was added 10 g of **56** (64 mmol, 1 equiv) and the solution was heated to reflux. A solution of 2.82 g NaOH (70 mmol, 1.1 equiv) in 6 ml H$_2$O was added slowly to the refluxing solution. 12.05 g benzyl bromide (70 mmol, 1.1 equiv) was dropped slowly into the refluxing solution over 30 minutes. The solution was refluxed for a further 16 hours. The solvent was removed in vacuo and the residue taken up into 180 ml CH$_2$Cl$_2$. The insoluble NaBr was filtered off. The organic layer was washed with two 100 ml portions of 5% aqueous NaOH and then with 2 X 100 ml portions of water, dried (Na$_2$SO$_4$) and evaporated to leave a clear yellow oil. The oil was recrystallised from CH$_2$Cl$_2$ and petroleum spirit to give 9.8 g of a white solid in 62% yield.
m/z 247 (M+H)$^+$ [ES$^+$]

^{1}H NMR (CDCl$_3$) σ 7.25 (m, 5H 3-O-CH$_2$-**Bnz**) 6.1 (s, 1H **5-H**) 5.1 (s, 2H 3-O-**CH$_2$**-Bnz) 4.2 (m, 2H **2-CH$_2$**-OH) 2.15 (s, 3H **6-CH$_3$**)

1,6-Dimethyl-2-hydroxymethyl-3-benzyloxypyridin-4(1H)-one (58)
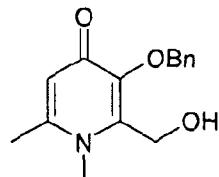
4.92 g **57** (20mmol, 1 equiv) was added to 60 ml CH$_2$Cl$_2$ with stirring. 3.4 g 3,4-dihydro-2-H-pyran (42.5 mmol, 2.2 equiv) was added followed by 60 mg p-toluenesulphonic acid as a catalyst.

After stirring for 3 hours the mixture was added to a separating
funnel and washed with 2 X 40 ml Na$_2$CO$_3$ (5%) followed by 2 X 40 ml
distilled water. The organic layer was dried (Na$_2$SO$_4$) and
evaporated to give a yellow oil, which was redissolved in 20 ml
5   EtOH. 200 ml NH$_2$CH$_3$ (40%, aqueous) was added and the mixture
stirred at 70°C for 18 hours. The solvent was removed in vacuo
and the resulting oil was redissolved in 40 ml EtOH. 2 ml
concentrated HCl (37%) was added and the solution was refluxed
for 4 hours. The solvent was removed in vacuo and 100 ml
10   distilled water was added to the residue. The solution was
washed with 2 X 50 ml diethylether. The pH of the aqueous phase
was then adjusted to 9 with 10 N aqueous NaOH. The solution was
added to a separating funnel and extracted with 4 X 100 ml CH$_2$Cl$_2$.
The organic layer was dried with anhydrous Na$_2$SO$_4$ and the solvent
15   was removed to give a crude brown solid, which was recrystallised
from CH$_3$OH and diethyl ether to give 2.8 g of white crystals in
54% yield.

m/z 260 (M+H)$^+$ [ES$^+$]

^{1}**H NMR** (DMSO) σ 7.3-7.5 (m, 5H 3-O-CH$_2$-**Bnz**) 6.15 (s, 1H **5-H**) 5.4
20   (m, 1H 2-**CH$_2$-OH**) 5.0 (s, 2H 3-O-**CH$_2$**-Bnz) 4.5 (m, 2H 2-**CH$_2$-OH**) 3.58
(s, 3H **6-CH$_3$**) 2.28 (s, 3H 1-N-**CH$_3$**)

1,6-Dimethyl-2-phthalimidomethyl-3-benzyloxypyridin-4(1H)-one
(59)
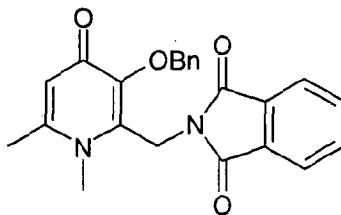
25

8.6 g triphenylphosphine (32 mmol, 1.2 equiv) and 4.8 g
phthalimide (32 mmol, 1.2 equiv) were added to a stirred
suspension of **58** (7 g, 27 mmol, 1 equiv) in 150 ml THF at 0°C.
6.47 g Diisopropyl azodicarboxylate [DIAD] (32 mmol, 1.2 equiv)
30   was added dropwise over 30 minutes. The solution was allowed to
warm to room temperature and was stirred for a further 17 hours,
after which time the solution was filtered. The solid was washed

with cold THF to give 9.92 g of a white amorphous solid in 95%
yield.

^{1}H NMR (CDCl$_3$) σ 7.65 (m, 4H 2-CH$_2$-**Phthalimide**) 7.15 (m, 5H
3-O-CH$_2$-**Bnz**) 6.25 (s, 1H 5-**H**) 5.35 (s, 2H 3-O-CH$_2$-**Bnz**) 4.75 (s, 2H
2-**CH$_2$**-Phthalimide) 3.5 (s, 3H 1-N-**CH$_3$**) 2.1 (s, 3H **6-CH$_3$**)

1,6-Dimethyl-2-aminomethyl-3-benzyloxypyridin-4(1H)-one (60)
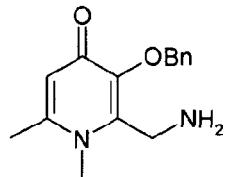
10 g of **59** (26 mmol, 1 equiv) was dissolved in 100 ml EtOH. 17
ml N$_2$H$_4$ (5.5% aqueous) was added and the solution was refluxed for
3 hours. **59** did not fully dissolve until refluxing had begun.
The solution was allowed to cool, at which point it solidified.
Water was added and the pH adjusted to 1 with conc. HCl. After
overnight cooling at 4°C, the solution was filtered. The pH of
the filtrate was adjusted to 14 with 10 N NaOH and extracted with
5 X 100 ml CH$_2$Cl$_2$. The extracts were combined and dried over
Na$_2$SO$_4$. The solvent was removed in vacuo to give 6.2 g of a white
solid in 92% yield.

m/z 259 (M+H)$^+$ [ES$^+$]

^{1}H NMR (CDCl$_3$) σ 7.25 (m, 5H 3-O-CH$_2$-**Bnz**) 6.35 (s, 1H 5-**H**) 5.25
(s, 2H 3-O-CH$_2$-**Bnz**) 3.7 (s, 2H 2-**CH$_2$**-NH$_2$) 3.6 (s, 3H 1-N-**CH$_3$**)
2.3 (s, 3H **6-CH$_3$**)

Di-*tert*-butyl 4-Nitro-4-[2-(*tert*-butoxycarbonyl)ethyl]-heptanedioate (61)
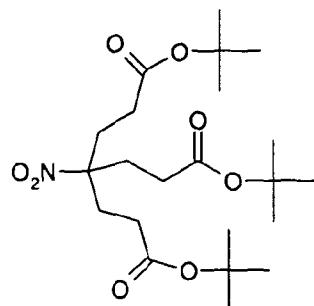
1.53 g nitromethane (25 mmol, 1 equiv) was added to 10 ml 1,2-dimethoxyethane (DME.) 0.25 ml of benzyltrimethylammonium hydroxide (Triton B) was added and the solution heated to 70°C. 9.93 g Tert-butyl acrylate (77.5 mmol, 3.1 equiv) was added in 1 ml portions along with another 2 X 0.25 ml portions of Triton B to keep the temperature between 70-80°C. Following complete addition of the reagents, the solution was heated at 70°C for a further hour. The solvent was removed in vacuo and the residue redissolved in 60 ml CHCl$_3$ and placed in a separating funnel. The organic layer was washed with 20 ml HCl (10% aqueous) followed by 3 X 20 ml conc. NaCl solution. The organic layer was dried with Na$_2$SO$_4$, filtered and the solvent was removed in vacuo. The crude product was recrystallised from hot EtOH, filtered and washed with ice cold EtOH to give 6.4 g of white crystals in 57% yield.

m/z 463 (M+NH$_4$)$^+$ [CI]

^{1}**H NMR** (CDCl$_3$) σ 2.2 (s, 12H [**CH$_2$-CH$_2$**-COO-tbu]$_3$) 1.45 (s, 27H [CH$_2$-CH$_2$-COO-**tbu**]$_3$)

IR (KBr) 2975s (CH$_2$), 1723s (C=O), 1536s (NO$_2$) cm^{-1}

T-1 Raney Nickel catalyst

To a 1 L three necked round bottomed flask was added 600 ml of a 10% solution of NaOH followed by 40 g of Nickel-Aluminium alloy. The solution was stirred with a magnetic stirrer bar and heated at a temperature of 90 - 95°C. After 40 minutes of heating the nickel began to adhere to the magnetic stirrer, impeding the stirring process. At this point stirring was continued manually

with a glass rod. Heating was discontinued after one hour and
the nickel was left to settle. Once the Nickel catalyst had
settled and the solution had cooled, the supernatant was decanted
and discarded. The catalyst was washed with 3 X 200 ml distilled
5   water and 5 X 50 ml ethanol. Care was taken to ensure that the
catalyst was covered with liquid and not exposed to the air
during the washing steps. The catalyst was then stored in a
refrigerator at 4°C under 50 ml ethanol.

10   **Di-*tert*-butyl 4-Amino-4-[2-(*tert*-butoxycarbonyl)ethyl]-
heptanedioate (62)**
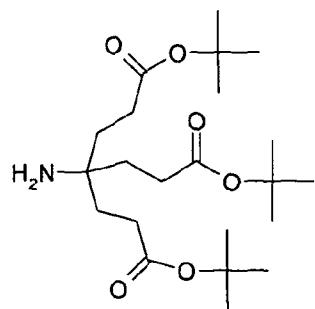
15   5 g of **61** (11.24 mmol, 1 equiv) was dissolved in 100 ml EtOH. 4
g of T-1 Raney Nickel catalyst was added. The solution was
hydrogenated at 40 psi (room temperature) for 7 days. During
this period, the H_2 pressure was maintained at 40 psi, and the
reaction stopped once the pressure ceased to reduce any further.
20   **61** did not fully dissolve at first but did during the period of
hydrogenation. The solution was filtered through celite and the
solvent was removed in vacuo to give 4.5 g of an oil in
quantitative yield, which solidified upon standing over a few
days.
25   **m/z** 416 $(M+H)^+$ $[ES^+]$

^{1}H NMR ($CDCl_3$) σ 2.2 (m, 6H [CH_2-**CH_2**-COO-tbu]$_3$) 1.6 (m, 6H [**CH_2**-CH_2-
COO-tbu]$_3$) 1.45 (s, 27H [CH_2-CH_2-COO-**tbu**]$_3$)

IR (KBr) 3376s (NH_2) 2978s (CH_2), 1730s (C=O) cm^{-1}

4-Acetylamino-4-(2-*tert*-butoxycarbonyl-ethyl)-heptanedioic acid
di-*tert*-butyl ester (63)
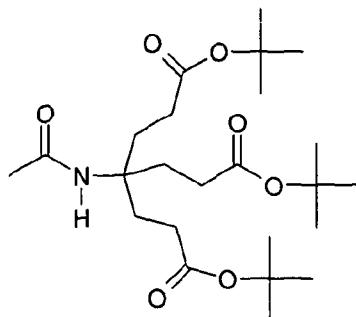
5

To 5 ml CH_2Cl_2 was added 1 g of triester **62** (2.4 mmol, 1 equiv)
and 0.3 g triethylamine (2.9 mmol, 1.2 equiv.) The solution was
cooled to 0°C with an ice bath and 0.23 g acetyl chloride (2.9
mmol, 1.2 equiv) was added dropwise. The solution was stirred
10 for a further 4 hours and allowed to warm slowly to room
temperature. The solution was poured into a separating funnel
and washed with 2 X 15 ml HCl (5% aqueous) and 1 X 15 ml
concentrated NaCl. The solution was dried over Na_2SO_4 and the
solvent was removed in vacuo. The residue was dissolved in
15 minimal EtOAc and loaded onto a silica column, where it was
purified by column chromatography with EtOAc and hexane (1:1) to
give 0.79 g of a white solid in 72% yield.

m/z 458 $(M+H)^+$ 475 $(M+NH_4)^+$ $[ES^+]$

^{1}**H NMR** (DMSO-d_6) σ 7.2 (s, 1H [CO**NH**]) 2.0 (m, 6H [CH$_2$-**CH$_2$**-COO-
20 tbu]$_3$) 1.8 (s, 3H [**CH$_3$**-CO]) 1.7 (m, 6H [**CH$_2$**-CH$_2$-COO-tbu]$_3$) 1.35 (s,
27H [CH$_2$-CH$_2$-COO-**tbu**]$_3$)

4-Acetylamino-4-(2-carboxy-ethyl)-heptanedioic acid (64)
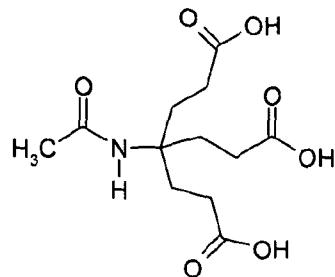
5   1.5 g of triester **63** (3.28 mmol) was added to 20 ml formic acid
(96%) and stirred for 4 hours. After this time the formic acid
was removed by azeotropic distillation with 5 X 20 ml portions of
toluene. The crude residue was recrystallised from EtOH and
petroleum spirit to give 0.9 g of off white crystals in 93%

10   yield.

m/z 290 (M+H)$^+$ [ES$^+$]

^{1}**H NMR** (DMSO-d$_6$) σ 12 (s, 3H [CH$_2$-COOH]) 7.2 (s, 1H [N-**H**]) 2.1 (m,
6H [CH$_2$-**CH$_2$**-COOH]$_3$) 1.8 (m, 6H [**CH$_2$**-CH$_2$-COO-tbu]$_3$) 1.7 (s, 3H [**CH$_3$-
CO**])

15

**4-Acetylamino-4-{2-[(3-benzyloxy-1,6-dimethyl-4-oxo-1,4-dihydro-
pyridin-2-ylmethyl)-carbamoyl]-ethyl}-heptanedioic acid bis-[(3-
benzyloxy-1,6-dimethyl-4-oxo-1,4-dihydro-pyridin-2-ylmethyl)-
amide] (65)**

20
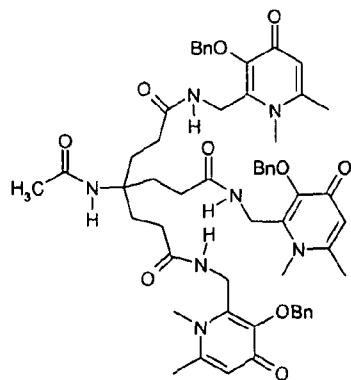
To 10 ml anhydrous DMF in a 100 ml round bottomed flask were
added 0.4 g **64** (1.38 mmol, 1 equiv) 1.29 g **60** (4.98 mmol, 3.6

equiv) 0.67 g HOBt (4.98 mmol, 3.6 equiv) and 1.03 g DCCI (4.98
mmol, 3.6 equiv.) The mixture was stirred for 48 hours at room
temperature under an atmosphere of N_2. The solution was filtered
to remove the precipitated DCU. DMF was removed in vacuo. The
5   residue was dissolved in methanol and purified by column
chromatography (silica gel) with a mobile phase of 4:1
$CHCl_3/CH_3OH$. The fractions were combined, dried (Na_2SO_4) and the
solvent was removed in vacuo to give 0.7 g of a white solid in
50% yield.

10   **m/z** 1010 (M+H)$^+$ 1032 (M+Na)$^+$ [ES$^+$]

^{1}**H NMR** (DMSO-d$_6$) σ 8 (t, 3H [CONH]) 7.3 (m, 15H [CH$_2$-Bnz]) 7.15
(s, 1H [CONH]) 6.2 (s, 3H [5-H pyridinone]) 5.05 (s, 6H [CH$_2$-Bnz])
4.3 (d, 6H [CONH-CH$_2$-pyridinone]) 3.35 (s, 9H [6-CH$_3$ pyridinone])
2.2 (s, 9H [N-CH$_3$ pyridinone]) 2.0 (m, 6H [CH$_2$-CH$_2$-CONH-
15   pyridinone]) 1.75 (m, 6H [CH$_2$-CH$_2$-CONH-pyridinone]) 1.7 (s, 3H
[CH$_3$-CONH-tripod])

4-Acetylamino-4-{2-[(3-hydroxy-1,6-dimethyl-4-oxo-1,4-dihydro-
pyridin-2-ylmethyl)-carbamoyl]-ethyl}-heptanedioic acid bis-[(3-
20   **hydroxy-1,6-dimethyl-4-oxo-1,4-dihydro-pyridin-2-ylmethyl)-amide]**
tris hydrochloride salt (52) (CP256)
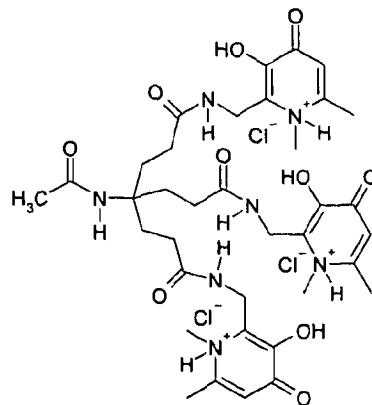
25   0.64 g of **65** (0.63 mmol, 1 equiv) was dissolved in 40 ml CH_3OH in
a thick walled hydrogenation flask. 2.3 ml of HCl (1.25 M in
CH_3OH) was added to the solution. 0.2 g of Pd/C catalyst was
added and the solution was hydrogenated for 18 hours (room
temperature, 40 psi.) The flask was removed with care and the

37

solution filtered to remove Pd/C. The solvent was removed in
vacuo. The residue was redissolved in minimal methanol. The
product (52) was precipitated with diethylether. Once the
precipitate had settled, the supernatant was removed and the

5   precipitate was washed with cold diethyl ether. This process was
repeated 3 times and solvent removed in vacuo to give 0.4 g of
the tris hydrochloride salt of 52 as a white solid in 74% yield.
m/z 740.4 (M+H)$^+$ 370.7 (M+2H)$^{2+}$

^{1}H NMR (DMSO-d$_6$) σ 8.8 (t, 3H [CONH]) 7.25 (s, 1H [CONH]) 7.1 (s,

10   3H [5-H pyridinone]) 4.5 (d, 6H [CONH-CH$_2$-pyridinone]) 3.8 (s, 9H
[6-CH$_3$ pyridinone]) 2.55 (s, 9H [N-CH$_3$ pyridinone]) 2.1 (m, 6H
[CH$_2$-CH$_2$-CONH-pyridinone]) 1.8 (m, 6H [CH$_2$-CH$_2$-CONH-pyridinone])
1.7 (s, 3H [CH$_3$-CONH-tripod])

15   *Example 2 - Characterisation of gallium and iron complexes of*
CP256 and Ga-67 labelling procedure

Formation of the Ga-CP256 and Fe-CP256 complexes is evident from
the mass spectral data shown in Table 1 and Table 2. The

20   protonated form of each complex was detected and the isotopic
distribution from the mass spectrum matches the calculated values
recorded in table 1. The Ga-CP256 complex has two molecular
species for (M + H)$^+$, at m/z 806 and m/z 808, due to gallium
having two stable isotopes with atomic masses of 69 and 71. No

25   evidence of Ga-CP256 complexes with Ga:ligand ratios other than
1:1 was found.

The mass spectral data for Fe-CP256 complex match the predicted
isotopic distributions.

30

Molecular formulae and monoisotopic masses

CP256:	C$_{36}$ H$_{49}$ N$_7$ O$_{10}$	(M = 739.3541)
Ga-CP256:	C$_{36}$ H$_{46}$ N$_7$ O$_{10}$ Ga	(M = 805.2562)
Fe-CP256:	C$_{36}$ H$_{46}$ N$_7$ O$_{10}$ [^{56}Fe]	(M = 792.2656)

Sample	LC retention time (mins)	Species	m/z (calculated)	m/z (found)
CP256	5.1	$(M + H)^+$	740.3614	740.3631
		$(M + 2H)^{2+}$	370.6843	370.6865
Ga-CP256	5.3	$(M + H)^+$	806.2635	806.2636
		$(M + Na)^+$	828.2454	828.2448
		$(M + 2H)^{2+}$	403.6354	403.6367
		$(M + H + Na)^{2+}$	414.6263	414.6266
		$(M + 2Na)^{2+}$	425.6173	425.6180
Fe-CP256	5.4	$(M + H)^+$	793.2728	793.2733
		$(M + Na)^+$	815.2548	815.2545
		$(M + 2H)^{2+}$	397.1401	397.1401
		$(M + H + Na)^{2+}$	408.1310	408.1316
		$(M + 2Na)^{2+}$	419.1220	419.1230

Table 1. Summary of LC-MS results

Radiolabelling CP256 with Ga-67 gave a product whose HPLC showed a single radioactive peak with an elution time identical with that of the "cold" Ga-CP256 complex.

Isotopic distribution for $(C_{36}H_{46}N_7O_{10}Ga + H)^+$	
m/z: Calculated/(found)	% Relative abundance: Calculated/(found)
806.2635/ (806.2635)	100.00/(100.00)
807.2635/ (807.2655)	42.59/(41.73)
808.2635/ (808.2634)	77.28/(76.99)
809.2635/ (809.2653)	30.34/(29.20)
810.2635/ (810.2670)	7.56/(7.36)

Isotopic distribution for $(C_{36}H_{46}N_7O_{10}Fe + H)^+$	
m/z: Calculated/(found)	% Relative abundance: Calculated/(found)
791.2775/ (791.2779)	6.33/(5.47)
792.2775/ (792.2768)	2.69/(2.92)
793.2775/ (793.2741)	100.00/(100.00)
794.2775/ (794.2765)	44.72/(42.20)
795.2775/ (795.2782)	12.14/(10.88)

Isotopic distribution for $(C_{36}H_{49}N_7O_{10} + 2H)^{2+}$	
m/z: Calculated/(found)	% Relative abundance: Calculated/(found)
370.6843/(370.6858)	100.00/(100.00)
371.1843/(371.1869)	42.64/(51.38)
371.6843/(371.6887)	10.93/(14.26)

Table 2. Calculated and experimental isotopic distributions of Ga-CP256 and Fe-CP256 and the CP256 ligand.

Experimental Details

Preparation of complexes

Ga-CP256

1 mg CP256 was dissolved in 1 ml methanol to give a ligand concentration of 1.18 mM (based on tris HCl adduct of CP256 with RMM of 848). This solution was added to 0.3 mg anhydrous $Ga(NO_3)_3$ (RMM 255) to give a 1:1 metal to ligand stoichiometry at a concentration of 1.18 mM.

Fe-CP256

The Fe^{3+} complex was produced in an analogous procedure to the Ga^{3+} complex, but with $Fe(NO_3)_3.9H_2O$ (RMM = 386) as the metal source.

5   Sample Analysis

Samples were analysed by LC-MS. The HPLC system was an Agilent 1200 system with degasser, quaternary pump, UV-visible variable wavelength detector, and an autosampler. The mass spectrometer was an Agilent 6520 Accurate-Mass Q-TOF LC/MS. Samples were

10 acquired using Agilent Masshunter workstation acquisition software and data were analysed using Agilent Masshunter Qualitative analysis software. The HPLC column used was an Agilent Zorbax SB C18 cartridge column (2.1mm X 30mm, 3.5 μm.) The eluent flow rate was 0.5 ml/min and the mobile phase was a

15 gradient using mass spectrometry grade reagents: A = H_2O + 0.1% formic acid (Fluka, 34673) and B = methanol + 0.1 % formic acid (Fluka, 34671). The gradient is given in Table 3. Spectra were acquired using electrospray ionisation in positive mode using reference mass correction with

20 hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine
($C_{18}H_{18}N_3O_6P_3F_{24}$, m/z = 922.0098 [M + H]$^+$). Isotopic distributions were calculated using Molecular Weight Calculator version 6.46.

Time (min)	A (%)	B (%)
0	95	5
1	95	5
10	5	95
20	5	95
23	95	5
30	95	5

25 **Table 3.** Gradient for LC-MS analysis of CP256 and the Fe^{3+} and Ga^{3+} complexes.

HPLC of Radiolabelled Samples

HPLC Analysis

HPLC analyses were carried out with FC3600 detector and γ detector probe connected in series. The HPLC system, was an Agilent 1200

5   series with quadruple pump, degasser, UV detector and manual syringe injector. The HPLC column used for all analyses was an Agilent Eclipse XDB-C18 (5 μm, 4.6 mm X 150 mm) with a guard column. The analytical software used for radio-TLC and radio-HPLC was Laura (Lablogic). All general reagents and consumables

10   were purchased from Sigma-Aldrich or from Fisher Scientific. ^{67}Ga-Citrate solution for injection was obtained from Mallinkrodt Medical, 74 MBq/ml (at ref. date; contains 6.5 mM sodium citrate, 135 mM NaCl, 0.9% Benzyl alcohol).

15   *Radiolabelling procedure for HPLC analysis*

A 1 mg/ml stock solution of CP256 was prepared in PBS buffer in a 1 ml plastic microcentrifuge tube. Radiolabelling solutions were prepared by adding a 10 μl aliquot of the stock solution to 90 μl of labelling buffer in a plastic microcentrifuge tube to give a

20   total ligand concentration of 0.1 mg/ml in 100 μl. This solution was radiolabelled by adding 10 μl ^{67}Ga-citrate. Radiolabelling conditions are shown in table 4.

LIGAND	LABELLING BUFFER	LABELLING TEMPERATURE	LABELLING TIME	LIGAND CONC. (M)	MEASURED pH
CP256	PBS	25°C	1 min	1.18 X 10^{-4}	6.6

25   **Table 4.** Radiolabelling conditions for CP256 with ^{67}Ga

HPLC mobile phase

Linear gradient of 10% B at 0 minutes to 90% B at 15 minutes. (A = 0.1% TFA in H$_2$O, B = 0.1% TFA in Methanol.) The flow-rate

30   was 1 ml/min.

HPLC sample injection

20 μl of the sample was injected manually into the HPLC using a
25 μl HPLC syringe.

5   ***Example 3 - CP256 labelling efficiency with Ga-67 data***

The radiolabelling results in table 5, measured by Thin Layer
Chromatography (TLC) show that CP256 radiolabels with ^{67}Ga at room
temperature at lower concentrations than DOTA does at elevated
10   temperature, with 100% radiolabelling at a concentration as low
as 10 μM whereas the radiolabelling yield for DOTA at 10 μM is
only 35%. These results are shown graphically in figure 1.

LIGAND CONCENTRATION	RADIOTRACER LABELLING YIELD	
	CP256	DOTA
1 mM	100	100
100 μM	100	100
10 μM	100	35
1 μM	84	0
100 nM	0	0
10 nM	0	0

15   **Table 5.** Radiolabelling yields of ^{67}Ga-CP256 and ^{67}Ga-DOTA. Calculated
from TLC analysis.

Experimental Details

All general reagents and consumables were purchased from Sigma-
20   Aldrich or from Fisher Scientific. Specialist chemicals and
consumables were purchased as follows: Acetate buffer solution pH
4.6 (Sigma Aldrich 31048-1L); ^{67}Ga-Citrate solution for injection
(Mallinkrodt Medical) 74 MBq/ml (at ref. date; contains 6.5 mM
sodium citrate, 135 mM NaCl, 0.9% Benzyl alcohol); glass TLC
25   plates (silica gel 60, F_{254}, 250 mm X 750 mm) (Merck); ITLC-SG
strips (Pall Sciences).

Procedure for spotting and running TLC plates

Silica TLC plates and ITLC-SG strips were spotted with 1 µl of
radiolabelled complex at the origin, which was marked at 5 mm in
pencil. Spots were allowed to dry on silica gel TLC plates but
5   not for ITLC-SG strips. The plates were placed in a developing
chamber (100 ml wide necked sample bottle) filled with a 3 mm
depth of the mobile phase. Stationary phases and mobile phases
are listed in table 8. The TLC plate was developed until the
solvent front had reached a distance of up to 65 mm. The plates
10 were removed from the developing chamber and dried in an oven set
at 80°C. Radiochemical yields were determined using either Laura
software or the instant imager software.

TLC and ITLC analyses were carried out on a Mini-Scan TLC scanner
15 with FC3600 detector and γ detector probe. The analytical
software used was Laura (Lablogic). Radioactive samples were
counted for 10 seconds (window 101-110) on a 1282 Compugamma
Gamma Counter (LKB Wallac) using Ultroterm software. The dose
calibrator used for measuring radioactivity in samples was a CRC-
20 25R (Capintec.) Electronic autoradiography was performed on a
Packard Instant Imager with Imager version 2.05 software. The
gas mixture was 1% isobutane, 2.5% carbon dioxide and 96.5% argon
(Air products.)

25 Radiolabelling procedure for radiochemical yield determination by
TLC analysis

1.25 mM stock solutions of each ligand were prepared in the
appropriate labelling buffer, as listed in table 6. The stock
solution was diluted 10 fold by successive addition of the
30 radiolabelling buffer to create extra solutions with ligand
concentrations of 125 µM, 12.5 µM, 1.25 µM, 125 nM and 12.5 nM.
Eighty microlitres of each solution was added to a plastic
microcentrifuge tube and radiolabelled with 20 µl of ^{67}Ga-Citrate
(1.5 MBq) to produce radiolabelled solutions with final ligand
35 concentrations of 1 mM, 100 µM, 10 µM, 1 µM and 100 nM. A metal
heater block (containing oil in the wells) was used to heat the

^{67}Ga-DOTA solution. Radiolabelling conditions are shown in table
6.

LIGAND	LABELLING BUFFER	LABELLING TEMPERATURE	LABELLING TIME	MEASURED pH
DOTA	Acetate	80°C	45 min	4.6
CP256	PBS	25°C	1 min	6.6

5   **Table 6.** Radiolabelling conditions for each ligand. Final
concentrations of each ligand in the labelling mixture were 1 mM, 100
µM, 10 µM, 1µM, 100 nM and 10 nM.

Example 4 - CP256 labelled with Ga-67 - kinetic stability

10

Stability of ^{67}Ga-CP256 in apo-transferrin by PD10 size exclusion
Figure 2 shows the elution profiles of ^{67}Ga-CP256 from a PD10
column for radiolabelled solutions at 100 µM (A) and 10 µM (B) of
CP256. After dilution and incubation in apo-transferrin, the

15   concentrations of CP256 in these solutions were 227 µM and 2.27
nM respectively. After 4 hours incubation in apo-transferrin,
the profile is unchanged from that of the incubation in the
reference buffer, indicating no binding of ^{67}Ga to transferrin or
loss of ^{67}Ga from the ligand, for either of the 2.27 µM or 227 nM

20   solutions.

Radiopharmaceutical stability using 30 kDa MWCO filters
As shown in table 7 and figure 3, residual binding of all of the
radiolabelled tracers to the filter membrane was low ($\leq$ 5%.)

25   Seventy three percent of the radioactivity from the ^{67}Ga-citrate
incubation in apo-transferrin was associated with the MWCO filter
after 4 hours. The MWCO method also showed negligible
transchelation of ^{67}Ga to apo-transferrin ($\leq$ 1%) in the ^{67}Ga-CP256
and ^{67}Ga-DOTA solutions over the 4 hour incubation period. In the

30   case of the 100 µM CP256 solution (2.27 µM in apo-transferrin)
the background binding from the incubation in the reference
buffer was higher by 1%. ^{67}Ga-DTPA showed some dissociation over
the 4 hour incubation period, with 10% extra binding of the

radioactivity in this solution to the filter when compared with
the solution in the reference buffer.

TRACER	% RADIOACTIVITY BOUND TO MWCO FILTER	
	IN REFERENCE BUFFER	APO-TRANSFERRIN INCUBATION, AFTER 4 HOURS
^{67}Ga-CITRATE	1.7 ± 0.5	73.0 ± 4.1
^{67}Ga-CP256 (10 μM)	3.5 ± 1.3	3.9 ± 0.8
^{67}Ga-CP256 (100 μM)	4.9 ± 0.5	3.8 ± 0.5
^{67}Ga-DOTA	1.5 ± 1.7	2.6 ± 0.8
^{67}Ga-DTPA	1.5 ± 0.6	11.4 ± 1.4

5   **Table 7**. Binding of ^{67}Ga-citrate, ^{67}Ga-CP256, ^{67}Ga-DOTA and ^{67}Ga-DTPA to
30 kDa MWCO filters.

Experimental Details

Specialist chemicals and consumables were purchased as follows:
10 human apo-transferrin (Sigma Aldrich T5391-10MG); Sartorius
Vivaspin 500 30 kDa MWCO filters (Fisher Scientific FDP-875-
025B); PD10 columns (GE Healthcare 17-0851-01); UV
spectrophotometry of apo-transferrin was performed on a Cary UV
spectrophometer with Cary WinUV software. The wavelength was set
15 at 280nm.

RADIOTRACER	MOBILE PHASE	STATIONARY PHASE
^{67}Ga-CP256	Methanol/citric acid (0.5 M)/ammonium formate (10% w, v) [40/20/40]	Silica gel
^{67}Ga-DOTA	Sodium citrate (aq) [0.3 M]	Silica gel
^{67}Ga-DTPA	Methanol/citric acid (0.5 M) ammonium formate (10% w, v) [40/20/40]	Silica gel
	Methanol/acetic acid (0.5 M) ammonium formate (10% w, v) [40/20/40]	Silica gel
	Acetic acid [0.5 M]	Silica gel
	9/1/0.1 methanol/glacial acetic acid/sodium citrate [0.3 M]	ITLC-SG
	Sodium citrate [0.1 M]	ITLC-SG

Table 8. Details of mobile phases and stationary phases used for TLC and ITLC quality control of ^{67}Ga-CP256, ^{67}Ga-DOTA and ^{67}Ga-DTPA.

Preparation of ^{67}Ga-citrate reference solution

A reference solution of ^{67}Ga-citrate was produced by incubating 20 μl ^{67}Ga-citrate in 200 μl PBS. This solution was incubated at 37°C in a metal heating block and samples were removed for analysis in the same manner as for the serum incubation.

Preparation of apo-transferrin solution

50 mM TRIS-HCl buffer (pH 7.5) was prepared by dilution of a 1 M stock solution. Sodium bicarbonate was added to a concentration of 25 mM. A 2.5 mg/ml (32 μM) solution of apo-transferrin was prepared in this buffer.

Preparation of ^{67}Ga-tracers

Solutions of all tracers were radiolabelled at a concentration of
100 µM. A 10 µM CP256 solution was also prepared. See table 9
for radiolabelling conditions.

5

A 10 µl aliquot of each radiolabelled tracer was added to 30 µl
of an appropriate buffer to neutralise the pH to between 7 and
7.5. This buffer was 2 M TRIS-HCl (pH 7.5) in the case of those
tracers labelled in the acetate buffer. For preparations of [67]Ga-

10   CP256 and ^{67}Ga-Citrate the buffer was PBS. Details can be seen in
Table 9.

LIGAND	LIGAND CONC. AFTER RADIOLBELLING	RADIOLABELLING BUFFER	DILUTION BUFFER	FINAL LIGAND CONC.
DOTA	100 µM	Acetate	TRIS	25 µM
DTPA	100 µM	Acetate	TRIS	25 µM
CP256	100 µM	PBS	PBS	25 µM
	10 µM	PBS	PBS	2.5 µM

Table 9. Tracer radiolabelling conditions.

15

Tracer incubation in Apo-Transferrin

Apo-transferrin solution (200 µl) in a plastic microcentrifuge
tube was incubated at 37°C in a metal heating block. To this was
added 20 µl of each pH-neutralised tracer. 20 µl of each tracer

20   was also incubated in a reference buffer (containing no apo-
transferrin) of 50 mM TRIS-HCl containing 25 mM sodium
bicarbonate.

Tracer Incubation in Reference Buffer

25   The 50 mM TRIS-HCl solution (200 µl, not containing apo-
transferrin) was incubated in a heating block at 37°C. The
appropriate pH adjusted tracer (20 µl) was added to the
incubation. Aliquots were taken at appropriate intervals for
analysis.

30

Gel filtration using a PD10 column.

Apo-transferrin incubations

Equilibration and elution of PD10 columns was with 25 mM sodium
bicarbonate added to PBS elution buffer. The standard elution

5   profiles of ^{67}Ga-citrate, ^{67}Ga-CP256, ^{67}Ga-DOTA and ^{67}Ga-DTPA were
determined by adding 20 µl of each tracer that had been incubated
in the reference buffer (50 mM TRIS-HCl containing 25 mM sodium
bicarbonate) and eluting the columns as described above.

10   *To calculate the percentage of total eluted activity for each*
     0.5 ml fraction
Total eluted radioactivity for each column was calculated by
adding together the counts for each of the 30 fractions. Eluted
activity for each fraction was then calculated by the following

15   equation:

$$\frac{\text{Counts per fraction}}{\text{Total counts (for all 30 fractions)}} \quad X \ 100$$

     UV elution profile of Apo-transferrin from a PD10 gel filtration
20   *column*
The elution profile of Transferrin from a PD10 column was
determined by UV spectrophometry. Apo-transferrin (non
radioactive, 500 µl of the 2.5 mg/ml solution) was loaded onto a
PD10 column, eluted with PBS buffer and 0.5 ml fractions were

25   collected. The samples were loaded into a 1 ml quartz cuvette
and diluted to 0.9 ml with an extra 0.4 ml of PBS. Absorbance
was determined at 280 nm with PBS as a reference standard.

     Determination of tracer stability using 30 kDa MWCO
30   *microcentrifuge filters*
PBS (100 µl, containing 25 mM bicarbonate) was applied to the top
section (filter section) of the tube. The incubation solution
(5 µl) for each tracer (after 4 hours) was added and the tube was
spun in a microcentrifuge at 10,000 g for 8 minutes. Another 100

35   µl PBS was applied to wash and the tube was spun again at 10,000
g for a further 8 minutes. The filter section of the tube was

removed and counted separately from the tube in a gamma counter.
Percentage binding of the radioactivity to the MWCO filter was
calculated using the following equation:

$$\frac{\text{Counts from filter section}}{\text{Counts from filter section} + \text{counts from tube section}} \times 100$$

5

The analysis was done in triplicate after 4 hours for the
apo-transferrin incubations. The analysis was done 6 times (6
tubes) for the incubations in the 50 mM TRIS-HCl reference
10   buffer. Average values were taken as described above.

Example 5 - Synthesis of CP256-maleimide conjugates
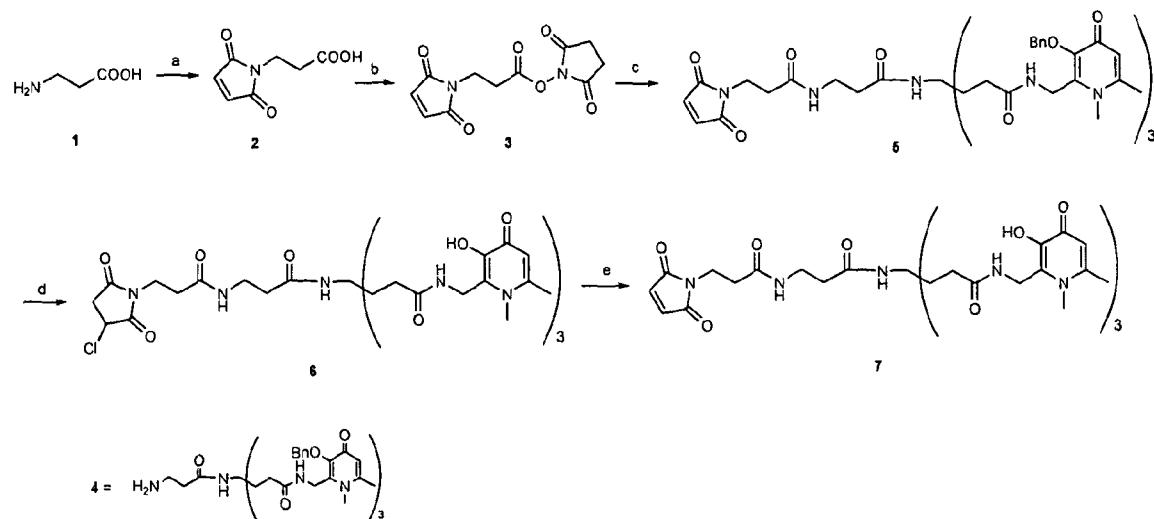
15

Synthesis of maleimide linked hexadentate chelator 7. a) maleic
anhydride/DMF; b) DCC/NHS/DMF; c) 4/DMF; d) BCl₃/DCM; e) Et₃N/DCM.

Experimental Details
20   The synthesis of compound 4 has been reported previously (Zhou T.
et al., J Med Chem. 2006, 49, 4171-4182).

Synthesis of succinimido 3-maleimidopropanoate 3
A solution of β-alanine (1.8 g, 20 mmol) and maleic anhydride
25   (2 g, 20 mmol) in DMF (30 mL) was stirred at room temperature for

3h. After the solid was completely dissolved,
N-hydroxysuccinimide (NHS; 2.3 g, 20 mmol) and
dicyclohexylcarbodiimide (DCC; 4.6 g, 24 mmol) was added into the
solution and the mixture was stirred at room temperature

5   overnight. The solution was filtered and the precipitate was
washed with water (100 ml) and dichloromethane (100 ml). The
filtrate was collected and the organic layer was washed with 3x50
ml 5% NaHCO$_3$ and one time with brine. The organic layer was dried
with Na$_2$SO$_4$ and dichloromethane removed under reduced pressure to

10   obtain a white solid (55%). This solid does not need further
purification and can be directly used for next coupling step. ^{1}H
NMR (CDCl$_3$, 400MHz): δ 2.82 (s, 4H, two succinimido-CH$_2$),
3.02 (t, J=7.0 Hz, 2H, COCH$_2$), 3.94 (t, J=7.0 Hz, 2H, NCH$_2$),
6.74 (s, 2H, two CH).

15

Synthesis of compound 5
The activated acid **3** (6 mmol) and amine **4** (5 mmol) in anhydrous
DMF (50 ml) was stirred at room temperature for one day. The
solvent was removed under reduced pressure and the residue was

20   purified on a silica gel column using chloroform:methanol (8:2)
as an eluent to afford a white foam (81%). ^{1}H NMR (CDCl$_3$, 400MHz):
δ 2.05 (brs, 6H, CH$_2$), 2.15 (s, 9H, CH$_3$), 2.23 (brs, 2H, CH$_2$),
2.29 (brs, 6H, CH$_2$), 2.41 (t, J=6.6 Hz, 2H, CH$_2$), 3.32 (brs, 2H,
CH$_2$), 3.36 (s, 9H, CH$_3$), 3.73 (t, J=6.6 Hz, 2H, CH$_2$), 4.35 (s, 6H,

25   CH$_2$), 4.87 (s, 6H, CH$_2$), 6.10 (s, 3H, CH), 6.65 (s, 2H, CH), 6.76
(brs, 1H, NH), 7.23-7.30 (m, 15H, ArH), 7.72 (brs, 3H, NH). ^{13}C
NMR (CDCl$_3$, 100MHz): 20.81 (CH$_3$), 30.70 (CH$_2$), 31.71 (CH$_2$), 34.43
(CH$_2$), 34.62 (CH$_2$), 34.82 (CH$_2$), 36.31 (CH$_3$), 36.68 (CH$_2$), 57.99,
73.42 (CH$_2$), 118.25 (CH), 128.30 (CH), 128.52 (CH), 128.58 (CH),

30   134.23 (CH), 136.97, 141.05, 146.35, 148.04, 170.43, 170.66,
171.70, 173.10, 173.23. ESI-MS: 1190.40 (M+1)$^+$

Synthesis of compound 6
To the benzyl protecting compound **5** in anhydrous DCM under N$_2$ was

35   added boron trichloride (12 equiv) in ice-bath and the mixture
was stirred at room temperature for 2 days. The excess BCl$_3$ was
quenched by adding MeOH followed by 1 mL concentrated

hydrochloric acid. The mixture was evaporated to obtain brown
solid, which dissolved in methanol and precipitated with the
addition of acetone. This procedure was repeated three times to
afford white solid (72%). ^{1}H NMR (DMSO-d_6, 400MHz): δ 1.75-1.84
5   (m, 6H, CH$_2$), 2.08-2.13 (m, 6H, CH$_2$), 2.21 (t, J=7.1 Hz, 2H, CH$_2$),
2.32 (t, J=7.5 Hz, 2H, CH$_2$), 2.57 (s, 9H, CH$_3$), 2.85 (dd, J=4.1,
8.3 Hz, 1H, one H of maleic CH$_2$), 3.16 (q, J=6.5 Hz, 2H, CH$_2$),
3.34 (dd, J=8.5, 18.3Hz, 1H, one H of maleic CH$_2$), 3.58 (t,
J=7.5Hz, 2H, CH$_2$), 3.89 (s, 9H, CH$_3$), 4.57 (d, J=5.0 Hz, 6H, CH$_2$),
10   4.99 (dd, J=4.1, 8.5 Hz, 1H maleic CH), 7.28 (s, 3H, CH), 7.30
(brs, 1H, NH), 8.09 (t, J=5.6 Hz, 1H, NH), 8.92 (t, J=5.1 Hz, 3H,
NH). ^{13}C NMR (DMSO-d_6, 100MHz): δ 20.56 (CH$_3$), 29.26, (CH$_2$), 29.98
(CH$_2$), 32.67 (CH$_2$), 34.71 (CH$_2$), 35.27 (CH$_2$), 35.58 (CH$_2$), 35.83
(CH$_2$), 39.00 (CH$_2$), 39.05 (CH$_3$), 49.82 (CH), 56.79, 112.73 (CH),
15   139.90, 142.69, 148.55, 159.59, 169.12, 170.08, 173.18, 173.36,
173.44. ESI-MS: 956.27 (M+1)$^+$. HRMS: Calcd for C$_{44}$H$_{59}$ClN$_9$O$_{13}$ (M+1)$^+$,
956.3921; Found, 956.3953.

Synthesis of compound 7

20   To compound **6** (20 mg) in DCM (20 mL) was added excess Et$_3$N and the
mixture was stirred at room temperature overnight. After
evaporation to remove the solvent, the residue was loaded on a
solid phase extraction column by dissolving in 1ml 0.1% formic
acid solution. The column was firstly washed with 0.1% formic
25   acid (10 mL) to remove triethylamine hydrochloride salt, followed
by elution of methanol (10 mL). The fraction of the methanol
solution was concentrated and dried by vacuum oven to yield a
white powder (80%). ^{1}H NMR (CD$_3$OD, 400MHz): δ 1.94-2.03 (m, 6H,
CH$_2$), 2.20-2.29 (m, 6H, CH$_2$), 2.32 (t, J=6.3 Hz, 2H, CH$_2$), 2.38
30   (s, 9H, CH$_3$), 2.42 (t, J=7.0 Hz, 2H, CH$_2$), 3.31 (q, J=6.7 Hz, 2H,
CH$_2$), 3.66 (s, 9H, CH$_3$), 3.69 (t, J=7.1Hz, 2H, CH$_2$), 4.58 (s, 6H,
CH$_2$), 5.25 (s, 3H, NH), 6.29 (s, 3H, CH), 6.81 (s, 2H, CH). ^{13}C
NMR (CD$_3$OD, 100MHz): δ 21.03 (CH$_3$), 30.11, (CH$_2$), 31.48 (CH$_2$),
35.43 (CH$_2$), 35.69 (CH$_2$), 36.02 (CH$_2$), 37.21 (CH$_3$), 37.49 (CH$_2$),
35   59.17, 63.70 (CH$_2$), 114.58 (CH), 129.59, 132.25, 135.57 (CH),
147.38, 148.87, 171.12, 172.26, 172.98, 173.33, 175.89. ESI-MS:

920.33 (M+1)⁺. HRMS: Calcd for $C_{44}H_{58}N_9O_{13}$ (M+1)⁺, 920.4154; Found, 920.4146.

Example 6 - Conjugation of CP256-chlorosuccinimide (6) or CP256-
5   **maleimide (7) with C2Ac protein**

C2A is a small protein that recognises phosphatidylserine displayed on cells undergoing apoptotis. It has been engineered with a cysteine residue to facilitate site-specific labelling
10 with thiol-reactive agents (see Tavare R, Torres Martin de Rosales R, Blower PJ, Mullen GED. Efficient site-specific radiolabeling of a modified C2A domain of synaptotagmin I with [⁹⁹ᵐTc(CO)₃]⁺ : a new radiopharmaceutical for imaging cell death. Bioconjugate Chem 2009; 20:2071-2081). The resulting engineered
15 protein (C2Ac) was conjugated with CP256 using either 6 or 7. The same product was obtained for both conjugation reactions.

The average molecular weight of C2Ac is 14997 and so the C2Ac-(7) conjugate was expected to have an average molecular weight of
20 15918.44. The found average molecular weight was 15970.85, which corresponds to the C2Ac-(7) conjugate with an additional iron atom and without 3 hydrogen atoms.

Experimental Details
25 C2Ac (2 mg in a solution of 2mg/mL) was treated with a 3:1 molar excess of chlorosuccinimide-hexadentate chelator (6) or maleimide-hexadentate chelator (7) for 4 hours at room temperature. The mixture was purified using a PD-10 column pre-equilibrated with PBS that had been stored over Chelex and
30 analysed by LC-MS.

Example 7 - Labelling of C2Ac protein conjugate with ⁶⁸Ga and
functional data on labelled protein

35 The C2Ac-(7) conjugate was radiolabelled with ⁶⁸Ga according to the method described herein. Figure 4 shows a high degree of chelation after only 15 minutes of incubation with ⁶⁸Ga in acetate

buffer. The ^{68}Ga-labelled conjugate showed specific calcium dependent binding to phosphatidylserine (PS) in a red blood cell binding assay (see Fig. 5)

5   *Experimental Details*

Unchelated ^{68}Ga in buffer, was prepared as follows. The generator was eluted with in 2ml 0.1M HCl to give 25 MBq ^{68}Ga eluate. 1.5ml of conc. HCl (36%) was added to the elution to give a final concentration of 4M HCl to form gallium tetrachloride anion, and

10   the solution passed through an anion exchange column (SAX SPEC, Chromafix 30-PS-HCO3, 45mg, Macherey-Nagel, Germany), trapping all activity. The column was then washed with 250 µl 5M NaCl and the activity then eluted with 300 µl distilled water and the eluate (pH < 1, ~70 MBq) buffered by adding 5 µl of 1M NaOH and

15   30 µl of 1M ammonium acetate buffer, pH 6, raising pH to 7. 100 µl of PBS, pH 6 was then added.

200 µL of the preconcentrated and buffered ^{68}Ga solution was added to 100 µg (in 100 µl) of the C2Ac-(7) chelator conjugate. At 5

20   minute intervals, 2 µl of the mixture was spotted on ITLC-SG and the chromatogram developed using 0.1 M citrate buffer as the mobile phase.

The binding of radiolabeled C2Ac-(7) to PS on red blood cells

25   (RBC) was performed according to a literature method (see Tait, J. F., Gibson, D. F., and Smith, C. (2004) Measurement of the affinity and cooperativity of annexin V-membrane binding under conditions of low membrane occupancy. Anal. Biochem.329, 112-119). A commercial preparation of preserved human RBC was

30   obtained from Beckman-Coulter (High Wycombe, UK). Calcium titrations of RBC were performed in a buffer of 50 mM HEPES-sodium, pH 7.4, 100 mM NaCl, 3 mM NaN$_3$, with 1 mg/mL BSA as carrier protein. Reactions were prepared with 1 nM ^{68}Ga labelled C2AcH and calcium; RBC were then added, and the reaction (1 ml)

35   was incubated for 8 min at RT.

The cells were then centrifuged (3 min at 7500 RCF), the
supernatant was removed, and the cells were resuspended in 1 ml
assay buffer containing the same concentration of calcium used
during the incubation step. The cells were centrifuged again, the
5   supernatant was removed, and the pellet was resuspended in 0.7 ml
assay buffer plus 10 mM ethylenediaminetetraacetic acid (EDTA) to
release ^{68}Ga labelled C2Ac-(7) bound in a calcium-dependent
manner. After centrifugation to remove the RBC, the released
labelled C2Ac-(7) in the supernatant was measured using a gamma
10   counter. The EC50 was calculated as described in the literature
using the equation $Y = [Ca]^N/([Ca]^N + EC50^N)$ where $Y = B/Bmax$, B
is the observed amount of radiolabelled protein bound at a given
calcium concentration, and $Bmax$ is the concentration of
radiolabelled protein bound at saturating calcium concentrations.
15

Curve fitting was performed using a nonlinear curve fit by a
routine based on the Levenberg-Marquardt algorithm using
Kaleidagraph (Synergy Software, Reading, US).

20   ***Example 8 - PET scanning and biodistribution in mice with***
^{68}Ga-C2Ac conjugate

PET images taken at 1.5 h after injection of the ^{68}Ga-C2Ac-(7)
conjugate and unchelated ("free") ^{68}Ga into mice are shown in
25   Figs. 6a and 6b respectively. The presence of ^{68}Ga is shown by
bright white areas in the image. Injection of the ^{68}Ga-C2Ac-(7)
conjugate showed rapid clearance of radioactivity from blood and
tissues with uptake almost exclusively in kidney with a small
amount of activity excreted into the bladder (Fig. 6a). This
30   contrasts with the images obtained with injection of "free" ^{68}Ga
in which activity was seen slowly clearing from blood pool
predominantly to remodelling bone (Fig. 6b).

	Average (n = 4)	Std dev
Spleen	0.46	0.11
Stomach	0.77	0.31
Intestines	0.42	0.06
Cecum	0.34	0.05
Liver	0.94	0.15
Blood	0.33	0.18
Kidneys	155.29	27.21
Thymus	0.27	0.09
Heart	0.47	0.15
Lungs	0.63	0.15
Muscle	0.39	0.23
Femur (L)	0.40	0.10
Tail	0.85	0.29

Table 10. % of injected radioactivity per gram in mouse tissues
90 min post injection of ^{68}Ga-C2Ac-(7) conjugate.

5   *Experimental Details*

The ^{68}Ga labelled C2Ac-(7) conjugate was synthesised as described
above. Unchelated ^{68}Ga in buffer, was prepared using the
pre-concentration (and buffering to pH 7) method described in
example 7. TLC analysis was performed. No gallium hydroxide
10   precipitate was detected by the time of injection. The solutions
for injection were filtered through a 0.22 μm filter and injected
into the tail veins of female C57B/6 mice (n = 4, 10 MBq in 100
μl for each animal). PET/CT scans were acquired 90 min post
injection using a NanoPET/CT scanner (Bioscan, Paris, France)
15   with PET acquisition time 1800 s, coincidence relation: 1-3.
Image reconstruction: OSEM with SSRB 2D LOR, energy window: 400-
600 keV, filter: Ramlak cutoff 1, number of iterations/subsets:
8/6. Animals were killed at 90 min and explanted organs counted
in a gamma counter to determine biodistribution.
20

Example 9 - Labelling of CP256 with ^{68}Ga in serum

Figure 7 shows size exclusion radiochromatography (PD10 column)
of chelation of ^{68}Ga by CP256 in serum and control experiments to

show ^{68}Ga-chloride incubated in serum, preformed ^{68}Ga-CP256-(7)
complex incubated with serum and ^{68}Ga incubated in buffer.

As a measure of the efficiency of chelation of ^{68}Ga with CP256,
5   and to demonstrate the feasibility of the pre-targeting approach
described above, the labelling was conducted in serum thus
presenting the chelation process with challenge from serum
transferrin. Addition of ^{68}Ga chloride to a mixture of CP256 and
serum gave a size exclusion chromatogram similar to that of ^{68}Ga-
10   CP256 when the CP256 sample concentration was 23 μM or above,
whereas addition of ^{68}Ga to serum alone gave a chromatogram
consistent with a mixture of ^{68}Ga-transferrin complex and "free"
gallium. This shows that CP256 competes effectively with serum
transferrin. Moreover, if the ^{68}Ga was equilibrated first with
15   serum, and then CP256 was added, again a chromatogram similar to
that of ^{68}Ga-CP256 was observed, showing that CP256 is able to
extract ^{68}Ga from ^{68}Ga-transferrin complex. The ethanol
precipitation and ITLC experiments confirmed that in serum
containing CP256, there was no measurable protein binding and no
20   detectable free ^{68}Ga. All ^{68}Ga remained at the origin on ITLC of
the supernatants. These observations suggest that ^{68}Ga and CP256
conjugates could be used in a novel "pretargeting" mode in vivo,
whereby a targeted CP256 conjugate is administered and allowed to
bind to target, followed by injection of ^{68}Ga to locate the
25   pretargeted CP256.

Experimental Details
Two different radiolabelling techniques were used. In method 1,
100 μl of chelator (CP-256, 2.35mM, 1.17mM, 589μM, 117μM, 23μM
30   and 11.7μM; pH 7 - 7.5 in PBS/bicarbonate buffer) was mixed with
300 μl of human serum. After 10-15 minutes 20 μl of 68Ga eluate
was added and the solution incubated at 37°C in a plastic tube. In
the second method, 20 μl of ^{68}Ga solution was added to the 300 μl
of human blood serum. After 15-20 minutes 100 μl of chelator (Cp-
35   256) was added. At each time point 50μl of the incubation
solution was applied to the top of the PD-10 column and eluted
the column with PBS collecting 0.5 ml fractions. Fractions were

counted in a gamma counter (Perkin Elmer wizard 31480 automatic
gamma counter, 10 seconds per sample). The incubated solution
were also analysed by ethanol precipitation, counting the pellet
and supernatant after centrifugation to determine protein bound
5   ^{68}Ga, and the centrifuged supernatants were analysed by ITLC-SG
with 0.1 M sodium citrate as mobile phase. In these conditions
"free" ^{68}Ga migrates with the solvent front (Rf = 1) and ^{68}Ga-CP256
remains at the origin (Rf = 0).

10   *Example 10 - Labelling of CP256 with ^{111}In.*

^{111}In-chloride formed a complex with CP256 (589 μM) in about 90%
yield after 5 min, as determined by ITLC-SG(HSA). The complex
showed <2% release of In-111 or binding to serum proteins when
incubated in human serum for 2 h, as determined by PD10 size
15   exclusion chromatography (Fig. 8).

Experimental Details

A 50 μl aliquot of acetate buffer (pH=6) was added to vial
containing ^{111}InCl$_3$ (7MBq, Mallinckrodt Medical) in HCl. A 589 μM
20   solution of CP256 was prepared by dissolving 0.5 mg of CP256 in 1
ml of PBS. A 100 μl aliquot of this CP256 solution was mixed with
50 μl of the ^{111}In solution. After incubation for 5 minutes the
speciation of ^{111}In was analysed using ITLC-SG strips that had
been previously saturated with human serum albumin (HSA) and
25   dried. The chromatograms were developed with water, ethanol and
ammonia in the ratio of 5:2:1. Under these conditions ^{111}In stays
at the origin whereas ^{111}In-CP256 moves to the solvent front.

Example 11 - in vivo imaging of antibody-CP256 conjugate via the
30   *pretargeting mode with ^{67}Ga.*

A monoclonal IgG antibody, SER4, against a sialoadhesin antigen
expressed by pro-inflammatory macrophages, was conjugated with
CP256-malemide (7) by first reducing antibody disulfide bonds
with 2-mercaptoethanol, then treating the thiol groups so formed
35   with CP256-maleimide (7) to give CP256-SER4.

The typical biodistribution in mice of SER4 labelled with ^{99m}Tc

(unpublished data) observed previously reflects rapid clearance
from circulation with uptake predominantly in spleen and to some
extent also liver.

5   CP256-SER4 was labelled with ^{67}Ga to give ^{67}Ga-CP256-SER4. Groups
of wild type mice were injected with either directly labelled
^{67}Ga-CP256-SER4 (group 1), or SER4 (unconjugated) followed 5 min
later by ^{67}Ga citrate (group 2) or CP256-SER4 followed by ^{67}Ga
citrate.

10

The same experiments were also performed using sialoadhesin
knockout mice. All animals were killed after 3 h and
biodistribution of radioactivity determined.

15   The results are shown in Fig. 9. For each organ, biodistribution
data for Group 1 are shown in left hand column, biodistribution
data for Group 2 are shown in the central column, and
biodistribution data for Group 3 are shown in the right hand
column.

20

Group 1 showed strong targeting of ^{67}Ga-CP256-SER4 to spleen (36%
ID/g), whereas group 2 showed very low spleen uptake of ^{67}Ga
citrate (4% ID/g). On the other hand, group 3 showed that pre-
injection of CP256-SER4 was able to increase spleen uptake of ^{67}Ga
25   citrate from 4% ID/g to 10% ID/g, suggesting that the ^{67}Ga
followed the location of antibody conjugate to a significant
extent.

Group 1 also showed high activity of ^{67}Ga-CP256-SER4 in
30   circulating blood (31% ID/g), whereas ^{67}Ga-citrate (group 2) did
not (7% ID/g); Pre-administration of CP256-SER4 (group 3)
increased blood pool retention from 7% to 16% ID/g. The knockout
mice did not show the spleen targeting behaviour, consistent with
this pattern being associated with sialoadhesin expression. These
35   experiments demonstrate the feasibility of imaging pretargeted
unlabelled bioconjugates of CP256 by later injections of ^{67}Ga
citrate.

Experimental details

For the conjugation of CP256 to SER4: To 2mg (5mg/mL) of SER4
antibody in phosphate buffered saline was added 25 µL of 50 mM
5   EDTA. The resulting solution was left at room temperature for 1 h
in order to chelate metal ions present in the solution.

The antibody was then washed by centrifugation at 3000 rpm for 3
x 15 min in a 50,000 mwco Vivaspin2 ultracentrifugation tube. The
10   antibody was resuspended between centrifugation steps and the
volume adjusted to 2 ml by addition of 0.1M metal-free phosphate
buffer, pH 7. The concentrated and washed antibody was collected
in approximately 300 µL 0.1 M phosphate buffer.

15   For the reduction of the antibody, 2-mercaptoethanol (5 µL) was
diluted to 20 µL in 0.1 M phosphate buffer, pH 7. 3.8 µL (1000
fold excess) of the diluted 2-mercaptoethanol was added to the
antibody and the reduction allowed to proceed for 30 min at room
temperature.
20

The volume was adjusted to 1 ml by addition of 0.1 M phosphate
buffer and the removed by applying the reduced antibody to a
disposable size exclusion column (PD10). Fractions containing the
reduced antibody were combined and CP256-maleimide (0.77 mg
25   dissolved in 8 µL DMSO, a 40 fold excess) was added immediately.
The conjugation reaction was heated at 37°C for 30 min. The
conjugated antibody was then washed using a 50,000 mwco Vivaspin2
ultracentrifugation tube as before but using 0.1 M ammonium
acetate buffer, pH6 to remove the excess ligand. The concentrated
30   CP256-antibody was collected in 0.1 M ammonium acetate buffer,
pH6.

^{67}Gallium radiolabelling was performed by addition of 45 µL ^{67}Ga
citrate (0.6-0.75 MBq) to 90 µL (90 µg) CP256-SER4.
35   Radiolabelling was allowed to proceed for 10 min before dilution
to 350 µL with phosphate buffered saline. Injections containing
25 µg of labelled antibody were drawn up. Analysis of the

radiolabelled antibody was performed by size exclusion HPLC using 0.1 M phosphate buffer containing 2 mM EDTA as the mobile phase to ensure good radiolabelling had been achieved prior to injection into animals.

5

Groups of wild type mice (C57B1/6, male 8-10 weeks, n = 2 to 3 per group) were injected with either directly labelled ^{67}Ga-CP256-SER4 (group 1), or SER4 (unconjugated) followed 5 min later by ^{67}Ga citrate (group 2) or CP256-SER4 followed by ^{67}Ga citrate. The activity of ^{67}Ga and the amount of antibody used in each case was equivalent. The same experiments were also performed using knockout mice (Sialoadhesin KO, C57B1/6, male 8-10 weeks, n= 2 to 3 per group). All animals were killed after 3 h and biodistribution of radioactivity determined by explanting tissues and weighing and counting.

Example 12 - in vivo imaging of antibody-CP256 conjugate via the pretargeting mode with ^{68}Ga.

SER4, conjugated with CP256-malemide (7) as described in Example 11, was labelled with ^{68}Ga as described above in example 7, to give ^{68}Ga-CP256-SER4. On three separate occasions groups of wild type mice (C57B1/6, male 8-10 weeks, n= 2 to 3 per group) were injected with either ^{68}Ga-CP256-SER4 (group 1), or SER4 (unconjugated) followed 1 h later by ^{68}Ga acetate(group 2) or CP256-SER4 followed 1 h later by ^{68}Ga acetate (group 3).

All animals were killed 1.5 h after injection of ^{68}Ga and biodistribution of radioactivity determined. The results are shown in Fig. 10. For each organ, biodistribution data for Group 2 are shown in left hand column, biodistribution data for Group 3 are shown in the central column, and biodistribution data for Group 1 are shown in the right hand column.

Group 1 showed strong targeting of ^{68}Ga-CP256-SER4 to spleen (20-50% ID/g over the three experiments), whereas group 2 showed very low spleen uptake of ^{68}Ga acetate (2-5% ID/g). On the other hand,

group 3 showed that pre-injection of CP256-SER4 was able to increase spleen uptake of ^{68}Ga acetate from 2-5% ID/g to 17-40% ID/g, suggesting that the ^{68}Ga followed the location of antibody conjugate to a significant extent. These experiments demonstrate

5   the feasibility of imaging pretargeted unlabelled bioconjugates of CP256 by later injections of ^{68}Ga acetate.

A NanoPET/CT image of a mouse from group 3 is shown in Fig. 11A. The mouse was image using the pre-targeting method of the

10   invention using CP256-SER4: 8 week-old C57B1/6 injected with CP256-SER4 followed by Ga-68 acetate. The figure shows targeted liver/spleen uptake. For comparison, a mouse injected with Ga-68 acetate only is shown in Fig. 11 B, showing retention in blood pool and accumulation in joints.

15

Experimental details
The antibody was conjugated to CP256 as described in Example 11. For radiolabelling, ^{68}Ga was eluted from a ^{68}Ge/^{68}Ga generator and concentrated as described in Example 7 to give ^{68}Ga acetate. To

20   150 µL (105 µg) CP256-SER4 was added 150 µL ^{68}Ga acetate (16 MBq). The radiolabelling reaction was allowed to proceed for 5 min at room temperature. Injections containing 35 µg of the radiolabelled antibody were drawn up. Analysis of the radiolabelled antibody was carried out as described in Example

25   11.

Groups of wild type mice (C57B1/6, male 8-10 weeks, n= 2 to 3 per group) were injected with either directly labelled ^{68}Ga-CP256-SER4 (group 1), or SER4 (unconjugated) followed 1 h later by ^{68}Ga

30   acetate (group 2) or CP256-SER4 followed by ^{68}Ga acetate. The activity of ^{68}Ga and the amount of antibody used in each case was equivalent. All animals were killed after 2-3 h and biodistribution of radioactivity determined by explanting tissues and weighing and counting. One animal from each group was scanned

35   using a NanoPET/CT scanner (Bioscan). Scans were initiated immediately after injection of ^{68}Ga acetate or ^{68}Ga-CP256-SER4 and continued for 1.5 h.

References:

The following references are expressly incorporated by reference for all purposes in their entirety.

5   1. Emberson et al., J. Immunol. Methods. 305(2):135-51, 2005

2. Giersing et al., Bioconjug Chem. 12(6): 964-71, 2001

3. Bird et al, Science, 242; 423-426, 1988

4. Huston et al, PNAS USA, 85: 5879-5883, 1988

5. Holliger et al, P.N.A.S. USA, 90: 6444-6448, 1993

10   6. Reiter et al, Nature Biotech, 14: 1239-1245, 1996

7. Hu et al, Cancer Res., 56: 3055-3061, 1996

8. Newkome et al., *J Org Chem*, 1991, **56**, 7162-7167

9. Zhou T. et al., J Med Chem. 2006, 49, 4171-4182

10. Tavare et al., Bioconjugate Chem 2009; 20:2071-2081

15   11. Tait et al., Anal. Biochem. 329, 112-119

It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common

20   general knowledge in the art, in Australia or any other country.

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word

25   "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1.   A method of diagnostic molecular imaging or therapy, the method comprises the steps of:

5   (a) administering a bifunctional compound or a bifunctional molecule to a subject so that the bifunctional compound or bifunctional molecule binds to a target of interest in the subject; wherein

   (i) the bifunctional compound is represented by the formula:

$$T\text{-}A^*\text{-}B\text{-}\left(\text{-}CH_2\text{-}\right)_m X\text{-}Y\left(\text{-}\right)_p R^1\Big)_3$$

10   or salts thereof;

wherein one of X and Y is C=O and the other is NR;

wherein each m and p are independently selected from 0 to 6;

wherein R^1 is a chelating group capable of chelating a radionuclide and is selected from:

15

   ,   or
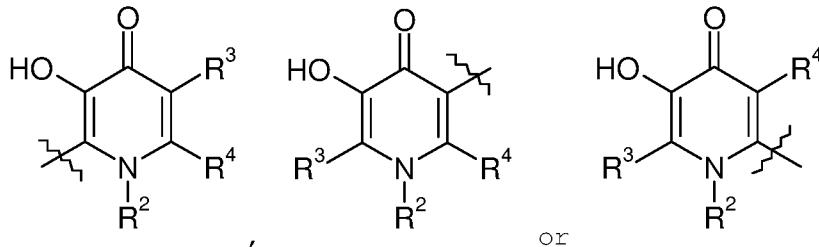
wherein R, R^2, R^3 and R^4 are independently hydrogen or a C_{1-7} alkyl group;

B is a linker group for linking the chelating group to the

20   reactive group, and is represented by the formula:

wherein each Q is independently selected from a group consisting of $-NR^5-$, $-C(O)NR^5-$, $-C(O)O-$, $-NR^5C(O)NR^5-$, $-NR^5C(S)NR^5-$ and $-O-$, each R^5 is independently hydrogen or a C_{1-7} alkyl group, each q and

25   s are independently selected from 0 to 6 and each r is independently selected from 1 to 6;

A* is a reacted reactive group that is coupled to T, T being a targeting group capable of binding to the target of interest in the subject;

64

and (ii) wherein the bifunctional molecule comprises a bifunctional compound as defined above and a radionuclide bound through a chelating group of the bifunctional compound; the method further comprises the steps of:

5   and

(b) where a bifunctional compound has been administered, further administering an imaging probe composition comprising a radionuclide to the subject, so that the chelating group R^1 of the bifunctional compound chelates the radionuclide at the target of

10   interest; and

(c) optionally detecting the radionuclide to image the target of interest in the biological system.

2. A bifunctional compound precursor, a bifunctional compound or

15   a bifunctional molecule, wherein:

(a) the bifunctional compound precursor is represented by the formula:

$$A\text{-}B\text{-}CH_2\left(\left(CH_2\right)_m X\text{-}Y\left(CH_2\right)_p R^1\right)_3$$

or salts thereof;
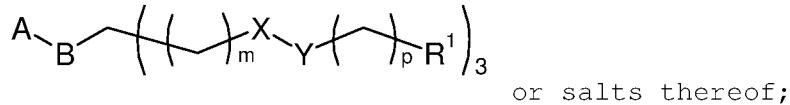
Wherein:

20   (i)   X is C=O and Y is NR, m is selected from 0 to 6, and p is
           selected from 1 to 6; or

(ii)   Y is NR and X is C=O, and m and p are independently
           selected from 0 to 6;

wherein R^1 and B are as defined in claim 1;

25   A is a reactive group for coupling to a biological moiety, a
targeting group, a protein, a polypeptide or a delivery vehicle;

(b) the bifunctional compound is represented by the formula:

$$T\text{-}A^*\text{-}B\text{-}CH_2\left(\left(CH_2\right)_m X\text{-}Y\left(CH_2\right)_p R^1\right)_3$$

or salts thereof;
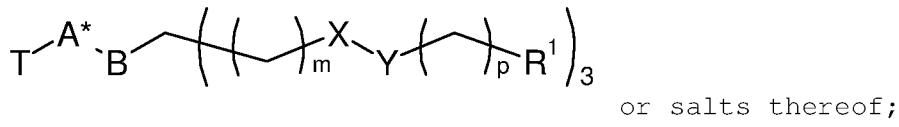
Wherein:

30   (i)   X is C=O and Y is NR, m is selected from 0 to 6, and p is
           selected from 1 to 6; or

(ii)   Y is NR and X is C=O, and m and p are independently
           selected from 0 to 6;

wherein R^1, B and A* are as defined in claim 1; and

(c) the bifunctional molecule comprises a bifunctional compound and a radionuclide bound through a chelating group of the bifunctional compound, wherein the bifunctional compound of the bifunctional molecule is represented by the formula:

$$T-A^*-B-\left(\left(\ \right)_m X-Y\left(\ \right)_p R^1\right)_3$$

or salts thereof;

wherein one of X and Y is C=O and the other is NR;

wherein each m and p are independently selected from 0 to 6;

wherein R^1, B, A* and T are as defined in claim 1.

3.   The method of diagnostic molecular imaging or therapy according to claim 1, or the bifunctional compound precursor, bifunctional compound or bifunctional molecule according to claim 2, wherein s is independently selected from 0 to 6, each r is independently selected from 1 to 6, and q is selected from 1 to 6.

4.   The method of diagnostic molecular imaging according to claim 1 or the bifunctional compound precursor, bifunctional compound or bifunctional molecule according to claim 2 for use in a method of molecular imaging, wherein the method of molecular imaging is PET or SPET.

5.   The method of diagnostic molecular imaging or therapy according to any one of claim 1 or 3 to 4 or the bifunctional compound precursor, bifunctional compound or bifunctional molecule according to any one of claims 2 to 4, wherein the reactive group A is a protein-reactive functional group, and optionally the reactive group is selected from the group consisting of a maleimide, aldehyde, ester, hydrazine, hydrazine derivative, alkoxyamine, alkoxyamine derivative, alkyne, alkene and azide group.

6. The method of diagnostic molecular imaging or therapy according to any one of claims 1 or 3 to 5 or the bifunctional compound precursor, bifunctional compound or bifunctional molecule according to any one of claims 2 to 5, wherein R^1 is

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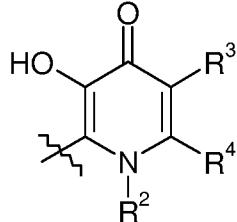
7. The method of diagnostic molecular imaging or therapy according to any one of claims 1 or 3 to 6 or the bifunctional compound precursor, bifunctional compound or bifunctional molecule according to any one of claims 2 to 6, wherein B is represented by one of the following formula:

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and

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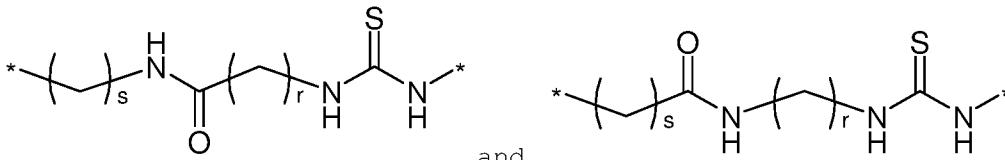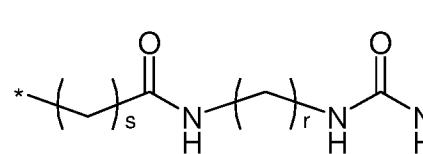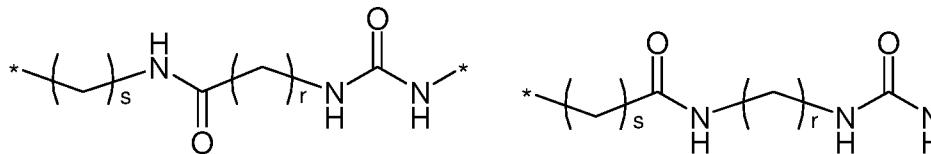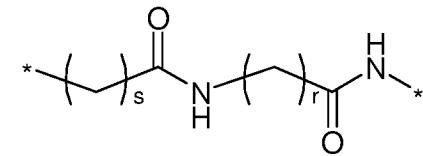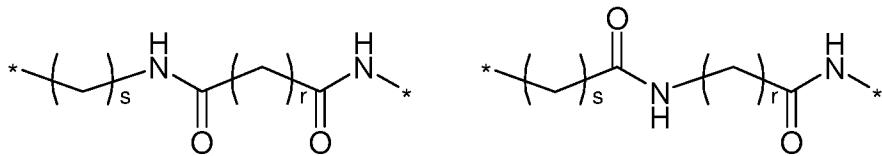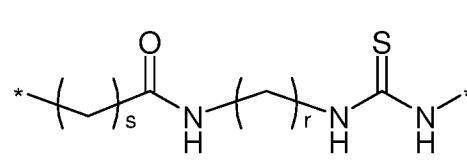
8. The method of diagnostic molecular imaging or therapy according to any one of claims 1 or 3 to 7 or the bifunctional compound precursor, bifunctional compound or bifunctional molecule according to any one of claims 2 to 7, wherein the group
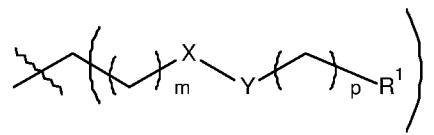
is represented by the group

67

or   .
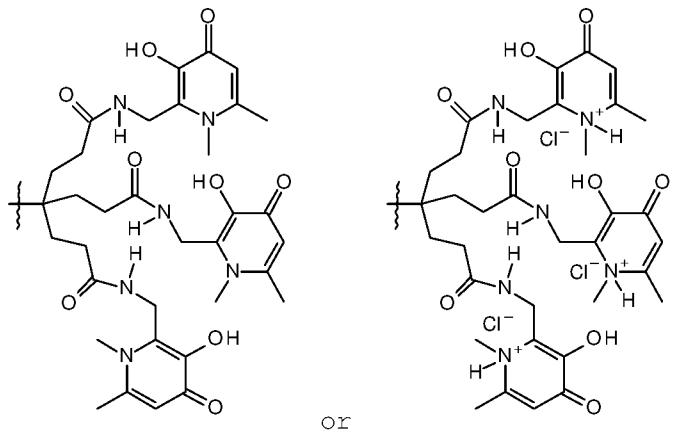
9. The method of diagnostic molecular imaging or therapy
according to any one of claims 1 or 3 to 8 or the bifunctional
compound precursor, bifunctional compound or bifunctional
molecule according to any one of claims 2 to 8, which comprises a
bifunctional compound or bifunctional molecule, wherein the
bifunctional compound is represented by the formula:

or salts thereof.
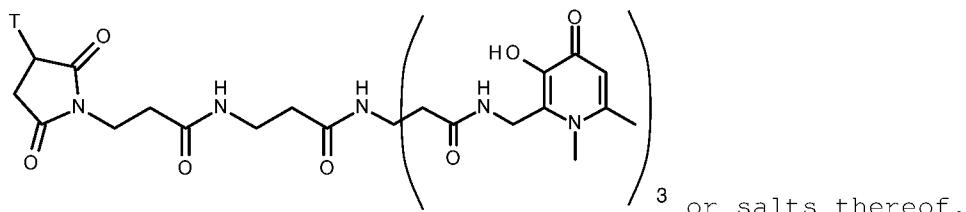
10. The method of diagnostic molecular imaging or therapy
according to any one of claims 1 or 3 to 9 or the bifunctional
compound precursor, bifunctional compound or bifunctional
molecule according to any one of claims 2 to 9, which comprises a
bifunctional compound or bifunctional molecule , wherein the
targeting group and target of interest are a receptor and ligand,
or an antibody and antigen, or metabolic probe e.g. glucose
transporter/glucose, hypoxia/hypoxia responsive moiety, or bone
mineral/bisphosphonate, or avidin (or streptavidin or related
protein)/biotin or a biotin-like derivative, or RNA/antisense RNA

11. The method of diagnostic molecular imaging or therapy
according to any one of claims 1 or 3 to 10 or the bifunctional

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compound precursor, bifunctional compound or bifunctional
molecule according to any one of claims 2 to 10, wherein the
targeting group is a peptide, protein, antibody, aptamer or small
molecule ligand capable of binding to binding partner at the
target of interest.

12. The method of diagnostic molecular imaging or therapy
according to claim 11 or the bifunctional compound precursor,
bifunctional compound or bifunctional molecule according to claim
11, wherein the targeting group includes Annexin V and the C2
domain of a synaptotagmin, TIMP-2, CEA, RGD peptide, somatostatin
receptor targeting peptide, bombesin, gastrin or VCAM targeting
peptide.

13.   The method of diagnostic molecular imaging or therapy
according to any one of claims 1 or 3 to 12 or the bifunctional
compound precursor, bifunctional compound or bifunctional
molecule according to any one of claims 2 to 12, which comprises
a bifunctional compound or bifunctional molecule, wherein the
target of interest is a ligand or receptor expressed on diseased
cells or tissue, a cell surface antigen associated with a disease
state, tumour markers, such as a cancer specific marker, or a
tissue specific marker, or the target of interest is a location,
an organ, a tissue type, infectious agent or physiological
property in a subject undergoing molecular imaging.

14.   The method of diagnostic molecular imaging or therapy
according to any one of claims 1 or 3 to 13 or the bifunctional
compound precursor, bifunctional compound or bifunctional
molecule according to any one of claims 2 to 13, which comprises
a bifunctional molecule, wherein the radionuclide is an isotope
of scandium, iron, cobalt, copper, gallium, yttrium, zirconium,
technetium, indium, tin, rhenium, gadolinium, terbium, holmium,
bismuth or lutetium, optionally wherein the radionuclide is an
isotope of gallium, Ga-68, Ga-67 or Bi-213.

15. The method of diagnostic molecular imaging according to any

one of claims 1 or 3 to 14 or the bifunctional compound
precursor, bifunctional compound or bifunctional molecule
according to any one of claims 2 to 14, which comprises a
bifunctional compound precursor of formulae:

5

 or salts thereof.
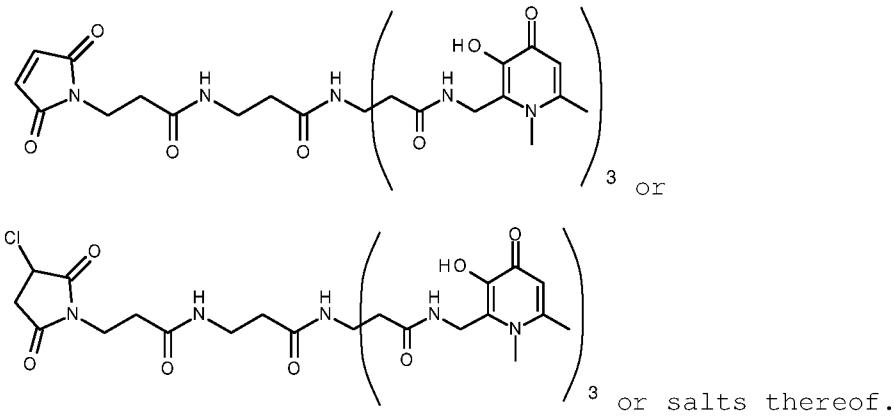
16. A kit for use in a method of diagnostic molecular imaging or
therapy comprising: a bifunctional compound composition having a
10   bifunctional compound according to any one of claims 2 to 15; and
a composition comprising a radionuclide.

17. A gallium-ligand complex or use of a gallium-ligand complex
in a method of diagnostic molecular imaging or therapy, wherein
15   the gallium-ligand complex comprises a ligand represented by one
of the following formulae:

or ; and
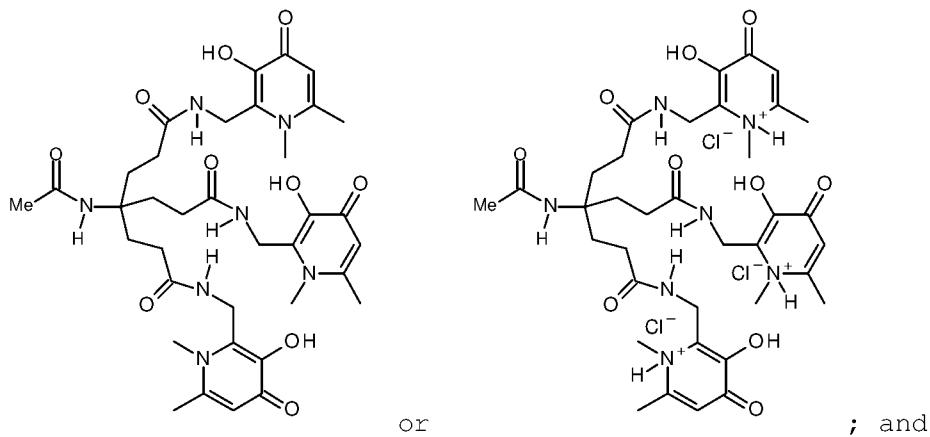
a gallium isotope complexed to the ligand.

20

70

Figure 1

Figure 2

Stability of ^{67}Ga-Tracers using 30 kDa MWCO filters

Figure 3

PD-10 fractionation of C2Ac and C2Ac-(7) after 15 min incubation with Ga-68 in acetate buffer

Figure 4

Figure 5

a)   b)
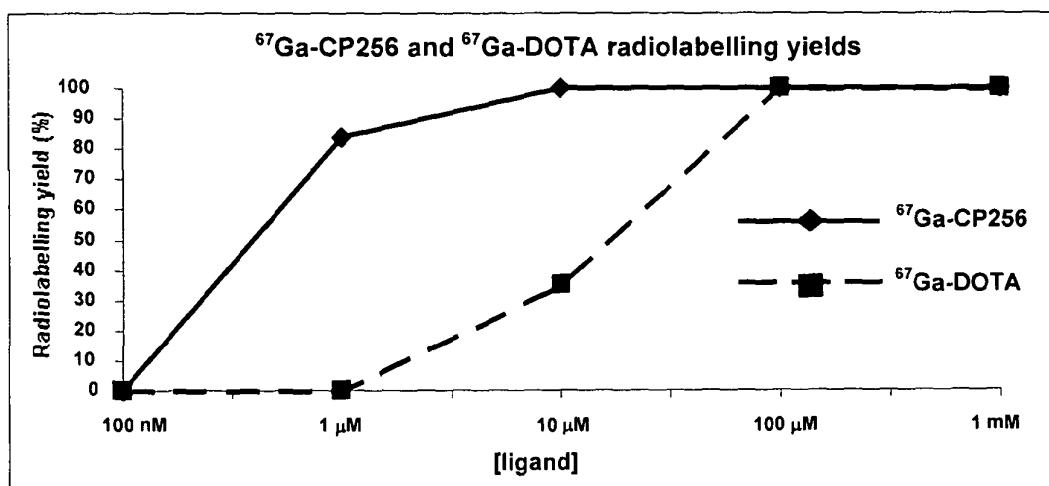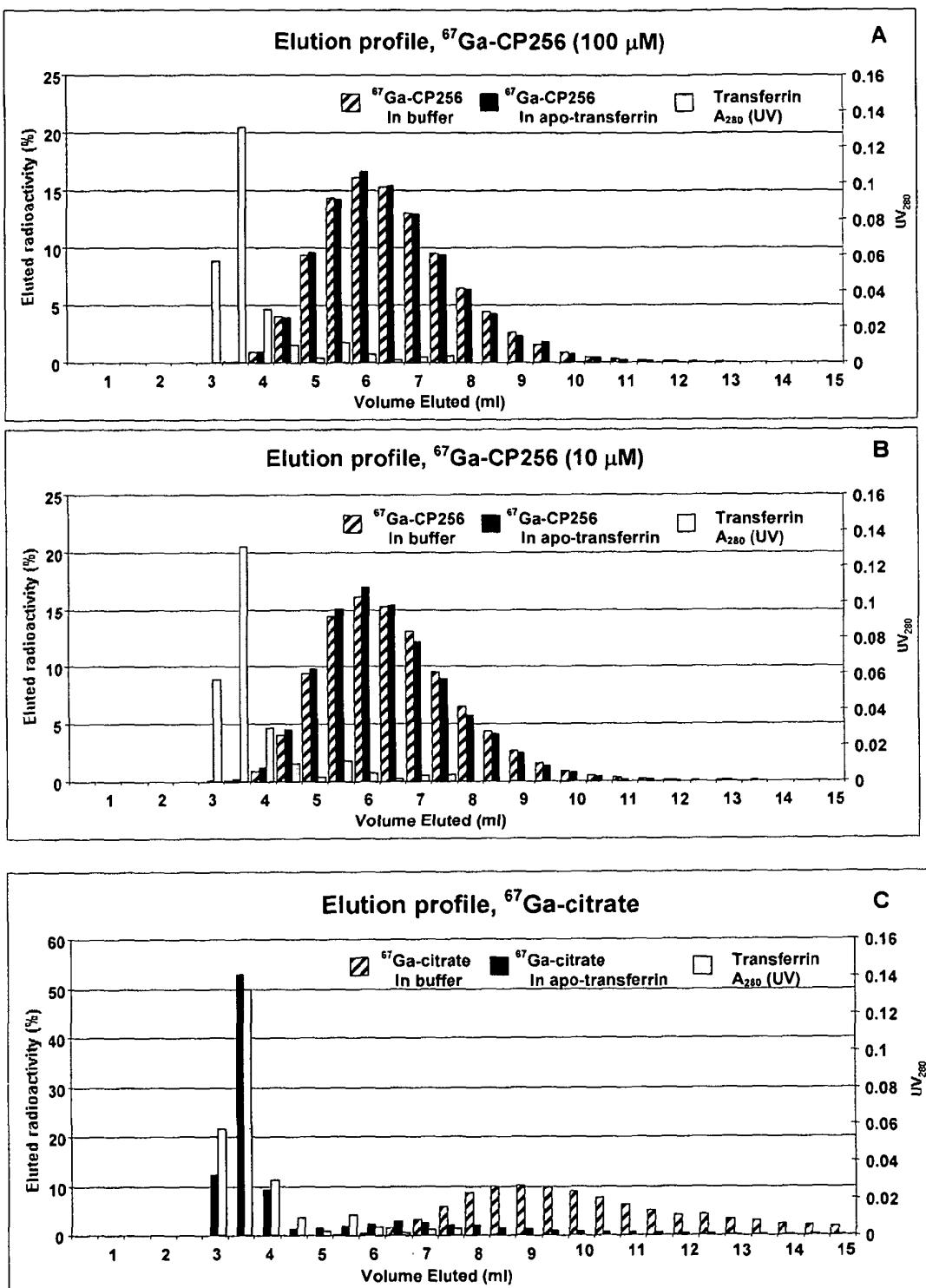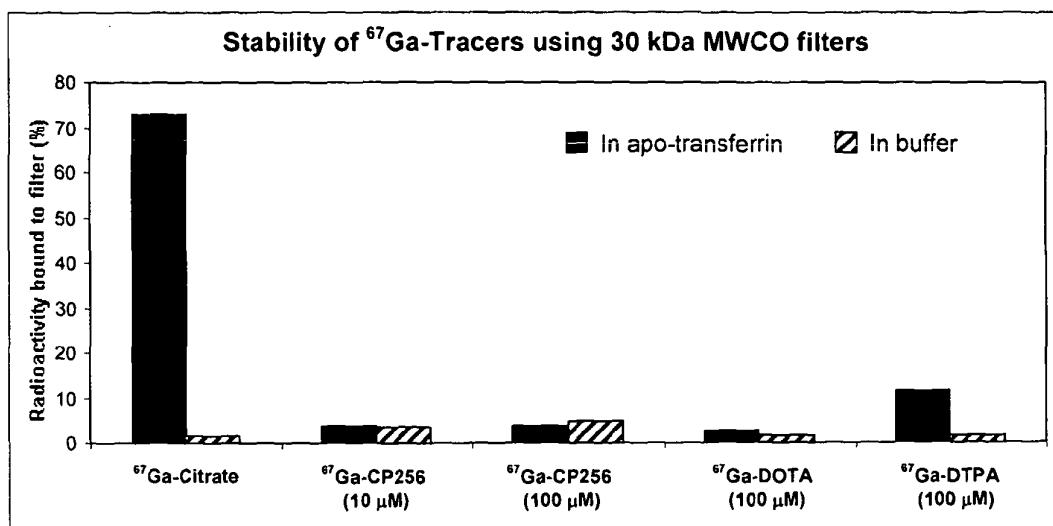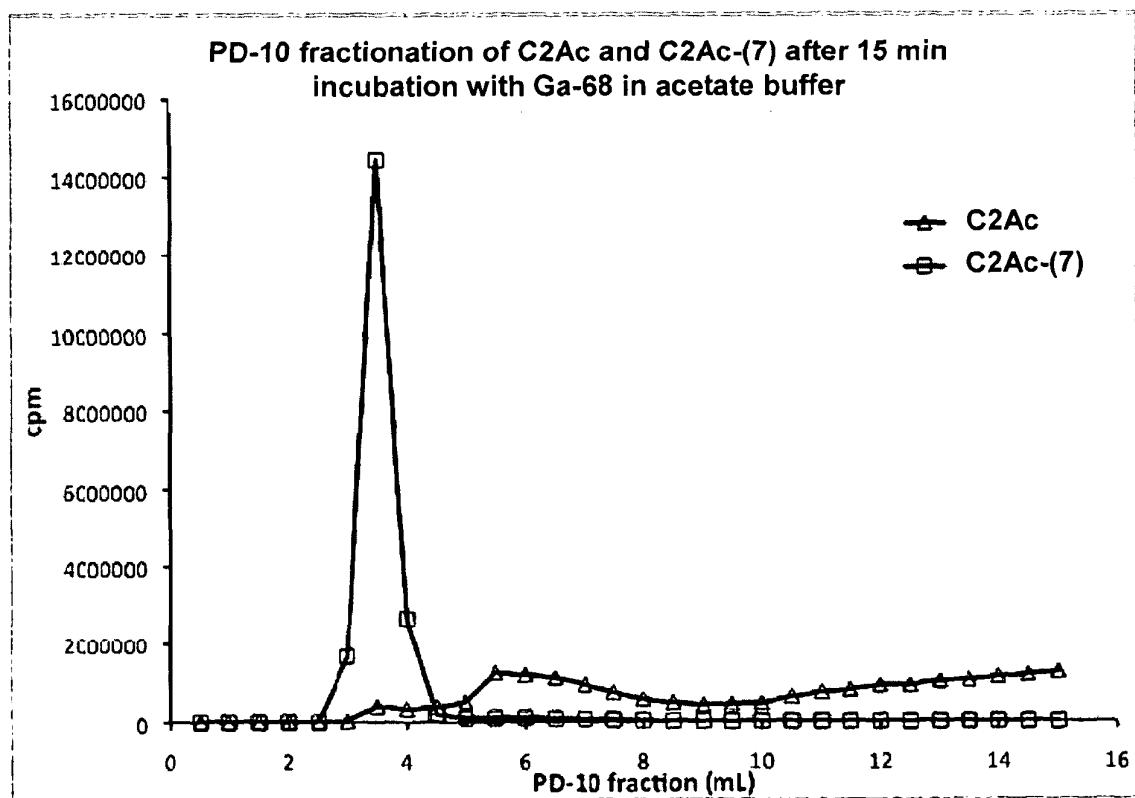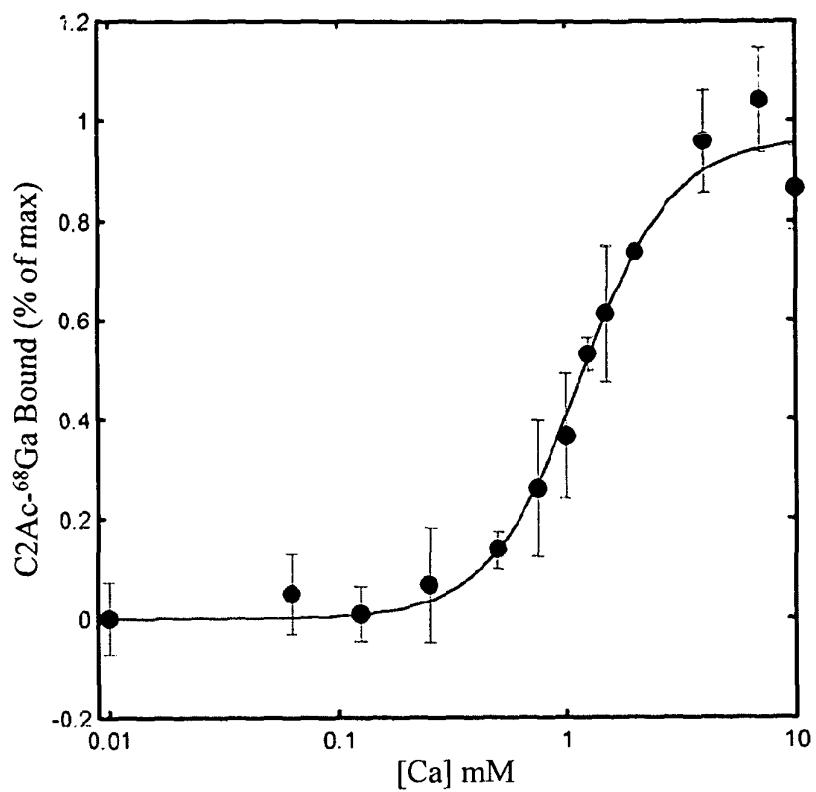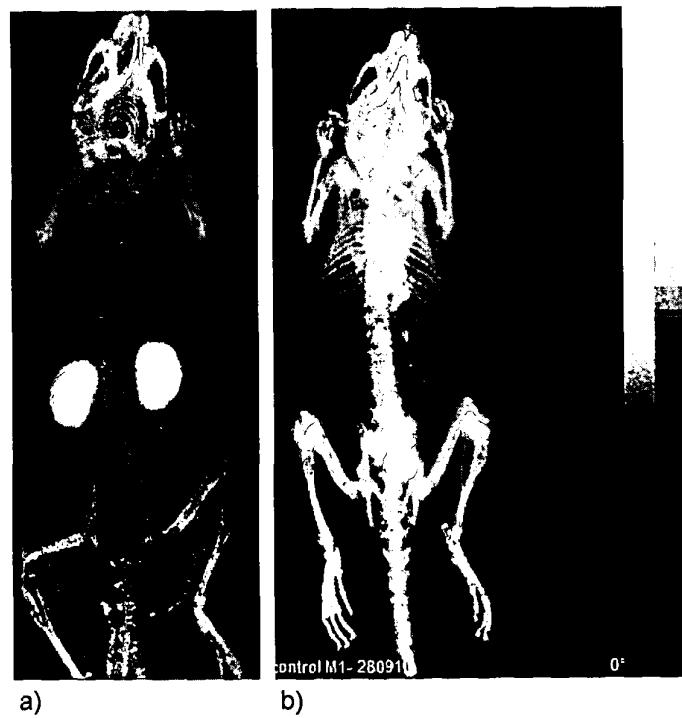
Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11