COMMONWEALTH of AUSTRALIA
Patents Act 1952

61781 6

APPLICATION FOR A STANDARD PATENT

I/We

Nihon Medi-Physics Co., Ltd.

of

2-1, Takatsukasa 4-chome, Takarazuka-shi, Hyogo-ken, Japan

hereby apply for the grant of a Standard Patent for an invention entitled:

Diethylenetriamine pentaacetic acid derivatives

which is described in the accompanying complete specification.

Details of basic application(s):-

Number	Convention Country	Date
139885/1988	Japan	7 June 1988
139886/1988	Japan	7 June 1988

The address for service is care of DAVIES & COLLISON, Patent Attorneys, of 1 Little Collins Street, Melbourne, in the State of Victoria, Commonwealth of Australia.

DATED this SIXTH day of JUNE 1989

To: THE COMMISSIONER OF PATENTS

....
a member of the firm of
DAVIES & COLLISON for
and on behalf of the
applicant(s)

Davies & Collison, Melbourne

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

DECLARATION IN SUPPORT OF CONVENTION OR NON—CONVENTION APPLICATION FOR A PATENT

In support of the Application made for a patent for an invention
entitled : "DIETHYLENETRIAMINE PENTAACETIC ACID DERIVATIVES"

Insert title of invention.

I, Katsuyuki Toki
~~We~~ of Nihon Medi-Physics Co., Ltd.
of 2-1, Takatsukasa 4-chome,
Takarazuka-shi, Hyogo-ken, Japan

Insert full name(s) and address(es) of declarant(s) being the applicant(s) or person(s) authorized to sign on behalf of an applicant company.

do solemnly and sincerely declare as follows :—

Cross out whichever of paragraphs 1(a) or 1(b) does not apply
1(a) relates to application made by individual(s)
1(b) relates to application made by company; insert name of applicant company.

1. (a) ~~I am~~ ~~We are~~ ~~the applicant xxx xxxxxx xxx xxxxx xxx~~

or (b) I am authorized by

Nihon Medi-Physics Co., Ltd.

the applicant.............. for the patent to make this declaration on its behalf. ~~their~~

Cross out whichever of paragraphs 2(a) or 2(b) does not apply
2(a) relates to application made by inventor(s)
2(b) relates to application made by company(s) or person(s) who are not inventor(s); insert full name(s) and address(es) of inventors.

2. (a) ~~I am~~ ~~We are~~ ~~the actual inventor xxx xxx xxx of the invention~~

or (b) Susumu KONDO   of
3109, Sumitomokagaku Kamiwaseda Shataku,
135, Iriyamazu, Ichihara-shi, Chiba-ken, Japan,

Miki KURAMI   of
314, Sumitomokagaku Anesaki Shataku,
1-9, Yushudainishi, Ichihara-shi,
Chiba-ken, Japan   and
Makoto AZUMA   of
750, Sainiro, Ichihara-shi, Chiba-ken, Japan

is are the actual inventor.S......... of the invention and the facts upon which the applicant............
is ~~xxx~~ entitled to make the application are as follows :—

State manner in which applicant(s) derive title from inventor(s)

The actual inventors have assigned the invention
to the said applicant.

Cross out paragraphs 3 and 4 for non-convention applications. For convention applications, insert basic country(s) followed by date(s) and basic applicant(s).

3.   The basic application...S...... as defined by Section 141 of the Act ~~was~~ were made

in .....Japan.. on the 7th June 1988...................
by Nihon Medi-Physics Co.,Ltd...
in .... Japan .... on the 7th June 1988...................
by Nihon Medi-Physics Co.,Ltd..
in .... on the ....
by...

4.   The basic application...S..... referred to in paragraph 3 of this Declaration ~~was~~ were
the first application.S....... made in a Convention country in respect of the invention the subject
of the application.

Insert place and date of signature.

Declared at Hyogo-ken   this   30th   day of May 1989

Signature of declarant(s) (no attestation required)

Note· Initial all alterations.

Nihon Medi-Physics Co., Ltd.

Katsuyuki Toki (signature)

Katsuyuki TOKI, President

DAVIES & COLLISON, MELBOURNE and CANBERRA.

(12) PATENT ABRIDGMENT (11) Document No. AU-B-36039/89
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 617816

(57) Claim

1.   A compound of the formula I:

$$HOOCH_2C \diagdown N-CH_2CH_2-N-CH_2CH_2-N \diagup CH_2COOH$$

with $HOOCH_2C$ and CH_2COOH on the left nitrogen, CH_2COOH on the central nitrogen, and CH_2COOH / $CH_2CONHC_nH_{2n}NH-A$ on the right nitrogen.

I

wherein n is an integer of 2 to 10, and

A is a monovalent group formed by reacting one of the two reactive groups of a cross linking reagent,

and physiologically acceptable salts thereof.

17.   A method of preparing a radioactive metal complex the method comprising treating a water soluble compound of a radioactive metal with a solution of a carrier comprising a compound as defined in claim 7.

7.   A compound of the formula II:

$$HOOCH_2C \diagdown \atop HOOCH_2C \diagup N-CH_2CH_2-\underset{\underset{CH_2COOH}{|}}{N}-CH_2CH_2-N \diagup^{CH_2COOH} \diagdown_{CH_2CONHC_nH_{2n}NH-A'-B}$$

II

wherein n is an integer of 2 to 10,

        A' is a bivalent linking group formed by reacting

        both the reactive groups of a cross linking

        reagent, and

        B is a residue of a polypeptide compound, and
physiologically acceptable salts thereof.

22.   A compound of the formula IIIa:

$$HOOCH_2C \diagdown \atop HOOCH_2C \diagup N-CH_2CH_2-\underset{\underset{CH_2COOH}{|}}{N}-CH_2CH_2-N \diagup^{CH_2COOH} \diagdown_{CH_2CONH(CH_2)_nNH_2}$$

IIIa

wherein n is an integer of 3 to 10.

13.   The compound according to claim 7, wherein the
polypeptide compound is a lipoprotein or glycoprotein.

617816

COMMONWEALTH OF AUSTRALIA
PATENTS ACT 1952
COMPLETE SPECIFICATION

NAME & ADDRESS
OF APPLICANT:

Nihon Medi-Physics Co., Ltd.
2-1, Takatsukasa 4-chome, Takarazuka-shi
Hyogo-ken
Japan

NAME(S) OF INVENTOR(S):

Susumu KONDO
Miki KURAMI
Makoto AZUMA

ADDRESS FOR SERVICE:

DAVIES & COLLISON
Patent Attorneys
1 Little Collins Street, Melbourne, 3000.

COMPLETE SPECIFICATION FOR THE INVENTION ENTITLED:

Diethylenetriamine pentaacetic acid derivatives

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

BACKGROUND OF THE INVENTION

Field of the invention

The present invention relates to novel diethylenetriamine pentaacetic acid (hereinafter, referred to as DTPA) derivatives and radioactive metal complex thereof as well as application of the latter in the diagnostic and therapeutic fields.

Background information

Substances labeled with ^{131}I have been widely used in the nuclear medicine for the detection of specific diseases, pharmacokinetic research and therapy of specific diseases using radioisotope. These substances, however, have many deficiencies such as (a) relatively long half-life of radioactivity, (b) emission of useless beta-ray besides gamma-ray, (c) radiation exposure of tissues other than a targetted tissue due to in vivo deiodination of ^{131}I and the like.

In view of said deficiencies, the use of other radioactive metal elements such as ^{111}In and ^{99m}Tc has been investigated as a substitute for ^{131}I. These metal elements are usually used in a conjugated form with a carrier material comprising a ligand compound bonded to a physiologically active substance. In order to label physiologically active high molecular compounds such as fibrinogen, TPA (Tissue plasminogen activator), monoclonal antibodies and fragments thereof with radioisotopes (RI) as the metal elements, bifunctional chelating agents (BFC) have been widely utilized. Commonly used BFCs include anhydride (1) of DTPA, activated ester (2) thereof and ethylenediamine tetraacetic acid benzyl isothiocyanate (3).

$$
\begin{array}{c}
\text{OCH}_2\text{C} \\
\diagup \qquad \diagdown \\
\text{O} \qquad\qquad \text{N-CH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{-N} \qquad \text{O} \qquad\qquad (1) \\
\diagdown \qquad \diagup \qquad\qquad | \qquad\qquad \diagdown \qquad \diagup \\
\text{OCH}_2\text{C} \qquad\qquad \text{CH}_2\text{COOH} \qquad \text{CH}_2\text{CO} \\
\text{CH}_2\text{CO}
\end{array}
$$

(1) structure: OCH$_2$C / O \ OCH$_2$C — N-CH$_2$CH$_2$-N-CH$_2$CH$_2$-N with CH$_2$COOH branch, /CH$_2$CO\ O \CH$_2$CO/

(2) HOOCH$_2$C \ N-CH$_2$CH$_2$-N-CH$_2$CH$_2$-N / HOOCH$_2$C, CH$_2$COOH, CH$_2$COOH, CH$_2$COO-N (succinimide ring with O, O)

(3) HOOCH$_2$C \ N-CH-CH$_2$-N / HOOCH$_2$C, CH$_2$, CH$_2$COOH, CH$_2$COOH, C$_6$H$_4$, SCN
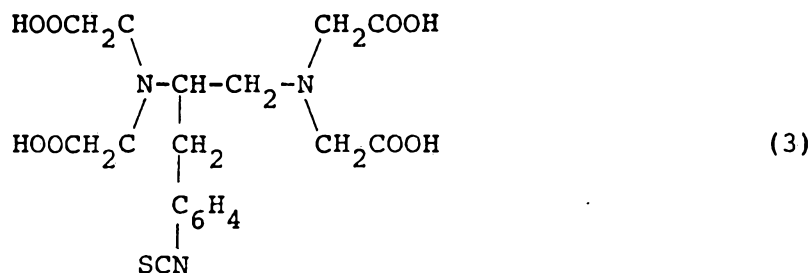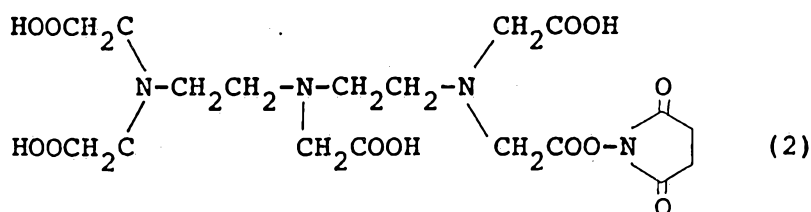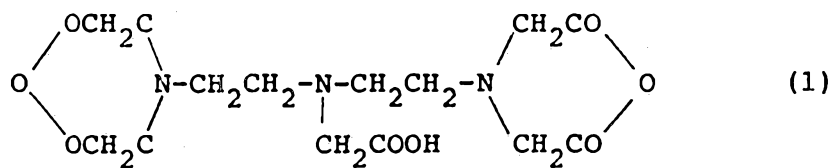
In all cases, these BFCs are bonded to the physiologically active substances by amide bond (-NHCO-). This means that the available and hence selectable bonding site is limited to amino groups. This also means that when the active site of the particular physiologically active substance contains lysine residue, a serious consequence such as loss of the physiological activity may occur. In addition, a grave problems, high uptake and long retention of RI in normal liver cells, may raise when amide bond is applied for labeling a monoclonal antibody with RI. The cause of this problem is entirely attributed to the fact

that the bondage between BFCs and physiologically active substances is limited to amide bond. More particularly, monoclonal antibodies are destined to be metabolized in normal liver cells. In this process RIs may dissociate from BFC-antibodies before their enzymatic cleavage of BFC-RI from antibodies because of greater strength of amide bond and retained as hydrolysates in liver cells. Since, however, BFC-RIs are water soluble complexes, even if they are cleaved from biololgically active substances, they are secreted into blood stream and then excreted through kidney, thus raising no problem. Therefore, it has been found necessary to develop BFCs which can provide various bonding modes not limited to amide bond.

On the other hand, the bonding modes between the biologically active substance and BFC-RI have also the importance in obtaining better quality of diagnosis or therapeutic effect. For example, TPA has a very short activity half-life of only a few minutes in blood stream. Therefore, when RI-labelled TPA is adopted for diagnosing blood clot, an RI having correspondingly shorter half-life such as ^{99m}Tc ($T_{1/2}$: 6 hours) should be selected, but ^{99m}Tc has rather long half-life. Since the target tissue for TPA-BFC-RI is the vascular blood clot to which the administered TPA-BFC-RI binds when contacted, if

inactivation of TPA in said TPA-BFC-RI precede decay of RI, the inactivated-TPA-BFC-RI remains in the blood stream. This will affect adversely the quality of diagnosis. Therefore, a bonding mode which allows rapid cleavage of BFC-RI from inactivated TPA and hence rapid excretion is desirable. While an unstable bond is preferred to a stable bond in the above example, there may be other examples in which the above relation is reversed. For instance, in the diagnostic agent for imaging tumors containing lesser vascularity, it is essential or at least desirable that a certain amount of RI necessary for diagnosis contacts and binds to the tumor lesion and accumulate.

In these cases, relatively stable bonding modes are preferred. Of course, when the tumor to be diagnosed or treated contains much vascularity, stable bonding modes are not preferred. Accordingly, there is a strong and continuous need for development of BFC which allows wide selection of bonding mode.
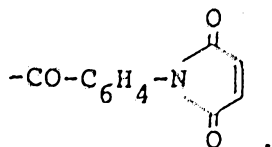
There has been proposed an alternative approach, different from the use of above-mentioned BFCs (1), (2) and (3), in which a carrier is prepared by forming Schiff base of DTPA-mono-(2-aminoethyl)amide with oxidized inulin (aldehydic inulin), followed by reduction (J. Med. Chem. 31, 898-901, 1988). In this approach, however, the bonding

modes of inulin is limited and therefore this approach is not satisfactory.

The DTPA-^{111}In complex has been used as a radioactive diagnostic agent. Aromatic amide derivative, i.e. 2-aminoethylanilide of DTPA was described in J. Labelled Comp. Radiopharm., $\underline{23}$, 1291 (1981).

SUMMARY OF THE INVENTION

In the first aspect, the present invention provides a compound of the formula I:

$$HOOCH_2C \diagdown N-CH_2CH_2-N-CH_2CH_2-N \diagup CH_2COOH$$

HOOCH$_2$C, CH$_2$COOH — N-CH$_2$CH$_2$-N-CH$_2$CH$_2$-N — CH$_2$COOH, CH$_2$COOH, CH$_2$CONHC$_n$H$_{2n}$NH-A

I

wherein n is an integer of 2 to 10, and

        A is a monovalent group formed by reacting one of

        the two reactive groups of a cross linking

        reagent,

and physiologically acceptable salts thereof. Such compound is useful as BFC for preparing a non-radioactive carrier for radioactive metal elements.

In the second aspect, the present invention provides a compound of the formula II:

$$\begin{array}{c} \text{HOOCH}_2\text{C} \\ \\ \\ \text{HOOCH}_2\text{C} \end{array} \diagdown\!\!\diagup \text{N-CH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{-N} \diagup\!\!\diagdown \begin{array}{c} \text{CH}_2\text{COOH} \\ \\ \\ \\ \text{CH}_2\text{COOH} \end{array}$$

CH_2COOH

CH_2CONHC_nH_{2n}NH-A'-B

II

wherein n is an integer of 2 to 10,

   A' is a bivalent linking group formed by reacting

      both the reactive groups of a cross linking

      reagent, and

   B is a residue of a polypeptide compound,

and physiologically acceptable salts thereof. Such compound

is useful as a non-radioactive carrier for radioactive metal

elements.

   In the third aspect, the present invention

provides a carrier for radioisotopes comprising at least one

compound of the formula II.

   In the fourth and fifth aspects, the present

invention provides a radioactive diagnostic agent comprising

the compound of the formula II labeled with a radioactive

metal element, and a radioactive therapeutic agent

comprising the compound of the formula II labeled with a

radioactive metal element, respectively.

DETAILED DESCRIPTION OF THE INVENTION

   In the above formulae, n may be any integer of 2

to 10 (both inclusive), preferredly 2 to 8, and more preferredly 2 to 6. The group C_nH_{2n} includes straight chain (i.e. polymethylene) groups and a branched chain (e.g. having one or more methyl side chains) groups.

In the above formula I, the group represented by A may be any monovalent group formed by reacting one of the two reactive groups of a cross linking regent. Preferred group A has a molecular weight of about 100 to about 1000 and/or has at least one physiologically cleavable linking group. In the more preferred group A, said physiologically cleavable linking group is selected from the group consisting of -O-, -COO-, -S-, -SS-, -SO- and -SO_2-.

Suitable examples of the group A includes:

$$-CO-CH_2CH_2-CO-O-CH_2CH_2-O-CO-CH_2CH_2-CO-O-N \overset{O}{\underset{O}{\diagdown}}$$

$$-CO-CH_2CH_2-S-S-CH_2CH_2-CO-O-N \overset{O}{\underset{O}{\diagdown}} -SO_3Na$$

$$-CO-O-CH_2CH_2-\overset{O}{\underset{O}{S}}-CH_2CH_2-O-CO-O-N \overset{O}{\underset{O}{\diagdown}}$$

$$-CO-CHOH-CHOH-CO-O-N \overset{O}{\underset{O}{\diagdown}} , \text{ and}$$

$$-CO-C_6H_4-N \underset{O}{\overset{O}{\diagdown}}$$
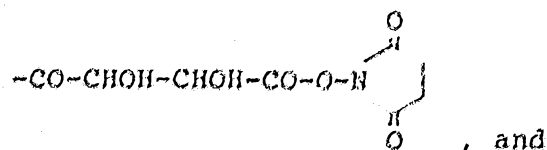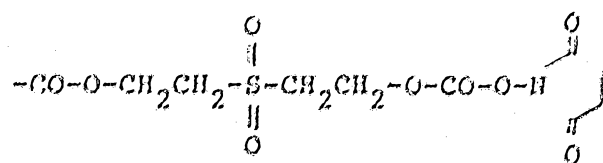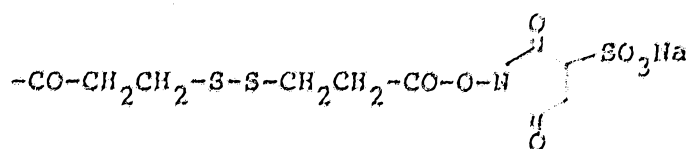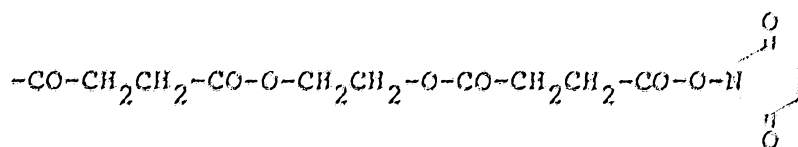
In the above formula II, the group represented by A' may be any bivalent group formed by reacting both the reactive groups of a cross linking reagent. Preferred group A' has a molecular weight of about 60 to about 900 and/or has at least one physiologically cleavable linking group. In the more preferred group A', said physiologically cleavable linking group is selected from the group consisting of $-O-$, $-COO-$, $-S-$, $-SS-$, $-SO-$ and $-SO_2-$. Suitable examples of the group A' includes:

$$-CO-CH_2CH_2-CO-O-CH_2CH_2-O-CO-CH_2CH_2-CO-$$

$$-CO-CH_2CH_2-S-S-CH_2CH_2-CO-$$

$$-CO-O-CH_2CH_2-\overset{O}{\underset{O}{\overset{\|}{S}}}-CH_2CH_2-O-CO-$$

$$-CO-CHOH-CHOH-CO- \quad , \text{ and}$$

$$-CO-C_6H_4-N \underset{O}{\overset{O}{\diagdown}}$$
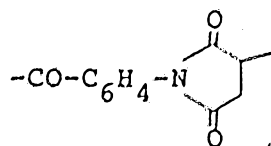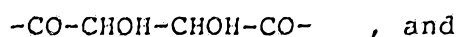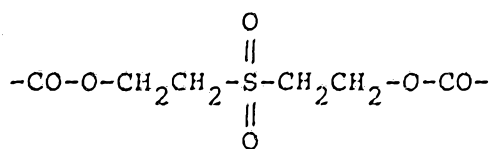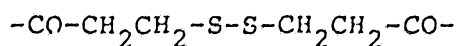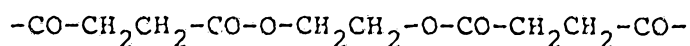
The cross linking reagent referred to in the
groups A and A' is intended to mean any compound having two
or more reactive groups which may react with an amino group,
a hydroxy group, a mercapt group, a dithio group, etc. to
form a bond to N, O or S atom thereof, at least one of said
reactive groups being capable of reacting with an amino
group. Such reactive groups include active esters, such as
succinimidyl ester, sulfosuccinimidyl ester, etc.
imidoesters, nitroarylhalides (these being capable of
reacting with an amino group), and disulfides, maleimides,
thiophthalimides, active halogens (these being capable of
reacting with a mercapt group), and so on. The preferred
cross linking reagent contains at least one bond easily
cleavable in vivo at the moiety other than the reactive
groups, or has at least one bond formed by the reactive
groups which is easily cleavable in vivo. Examples of such
bond are -O-, -COO-, -S-, -SS-, -SO-, $-SO_2-$, etc.

A preferred group of the cross linking reagents is
represented by the formula

X-Q-Y
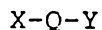
wherein X and Y are the same or different reactive groups,
and Q is a carbon skeleton, with the proviso that Q contains
at least one of O and S atoms in the basic skeleton, or
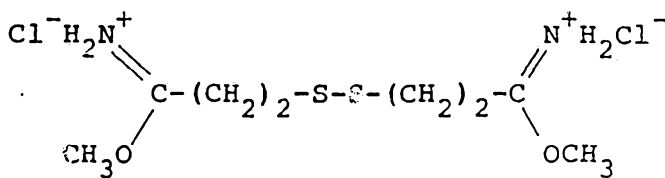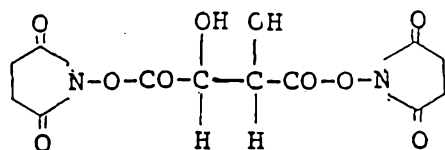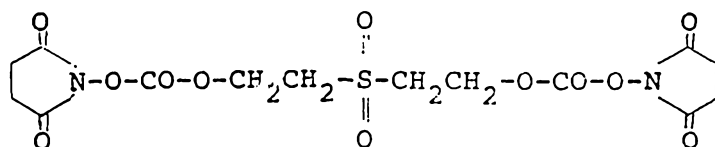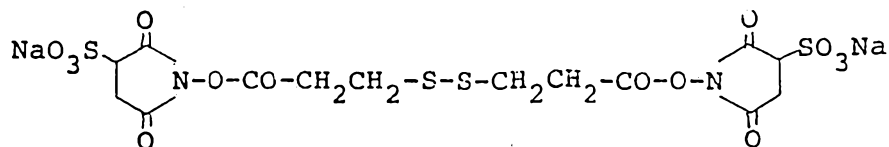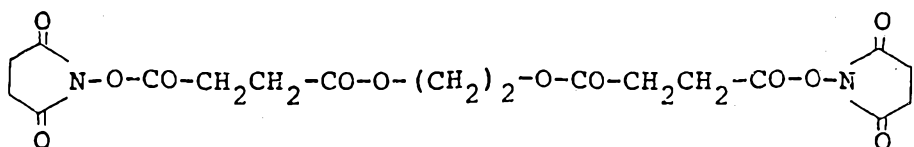where it does not contain the said atom in the basic

skeleton, at least one of X and Y is a reactive group which forms a bond easily cleavable in vivo. The said carbonic skeleton contains 2 to 20, preferredly 3 to 16, more preferredly 4 to 12 carbon atoms, and may contain heteroatom(s) as a member of an heteroaromatic nucleus or in the form of a functional group such as a carbonyl.
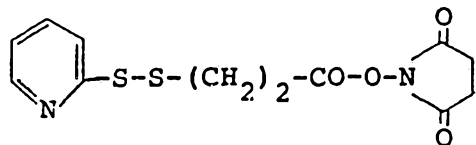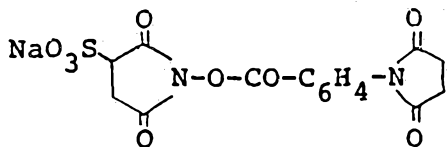
Suitable examples of the bifunctional bridging reagent are as follows:

- bis[2-(succinimidoxycarbonyloxy)ethyl]sulfone (BSOCOES),
- disuccinimidyltartarate (DST),
- 3,3'-dithiobis(succinimidylpropionate) (DSP),
- 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP),
- ethyleneglycolbis(succinimidylsuccinate) (EGS),
- ethyleneglycolbis(sulfosuccinimidylsuccinate) (sulfoEGS),
- bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl]sulfone (sulfoBSOCOES),
- dimethyl-3,3'-dithiobispropionimidate.2HCl (DTBP),
- dimethyladipimidate.2HCl (DMA),
- dimethylpimelimidate.2HCl (DMP),
- dimethylsuberimidate.2HCl (DMS),
- m-maleimidobenzoylsulfosuccinimide ester (sulfoMBS),
- N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP),
- 2-iminothiolane.HCl,
- bis(maleimido)methylether (BMME),
- sulfosuccinimidyl-2-(m-azido-o-nitrobenzamido)ethyl-1,3'-dithiopropionate (SAND),

- sulfosuccinimidyl-2-(p-azidosalicylamido)ethyl-1,3'-
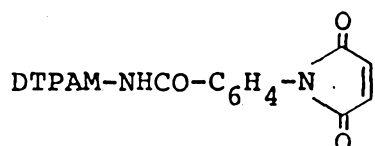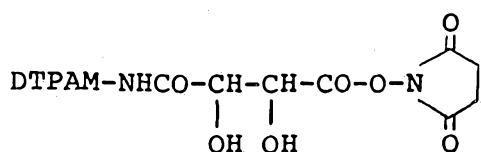dithiobispropionate (SASD).

Particularly suitable examples of the cross
linking reagent include the following compounds.

$$N-O-CO-CH_2CH_2-CO-O-(CH_2)_2-O-CO-CH_2CH_2-CO-O-N$$

$$NaO_3S \quad N-O-CO-CH_2CH_2-S-S-CH_2CH_2-CO-O-N \quad SO_3Na$$

$$N-O-CO-O-CH_2CH_2-S-CH_2CH_2-O-CO-O-N$$

$$N-O-CO-\underset{H}{\overset{OH}{C}}-\underset{H}{\overset{OH}{C}}-CO-O-N$$

$$Cl^-H_2N^+ \quad C-(CH_2)_2-S-S-(CH_2)_2-C \quad N^+H_2Cl^- \\ CH_3O \qquad\qquad\qquad OCH_3$$

$$Cl^-H_2N^+ \quad C-(CH_2)_6-C \quad N^+H_2Cl^- \\ CH_3O \qquad\qquad OCH_3$$

$$\begin{array}{c} H_2C-CH_2 \\ H_2C \quad\quad C=N^+H_2Cl^- \\ S \end{array}$$

$$NaO_3S-\text{(succinimide ring)}-N-O-CO-C_6H_4-N\text{(maleimide ring)}$$

$$\text{(pyridine)}-S-S-(CH_2)_2-CO-O-N\text{(succinimide ring)}$$
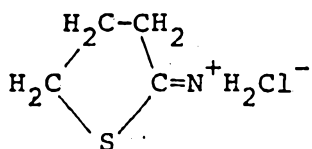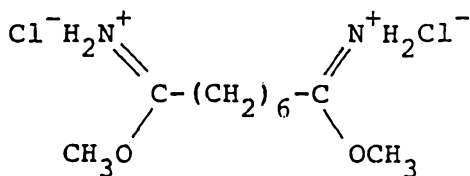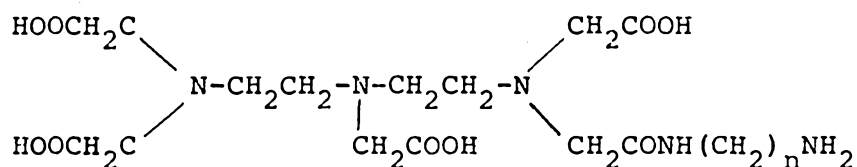
The term "polypeptide compound" represented by B
refers to compounds having multiplicity of peptide bond
formed by natural or unnatural amino acids and optionally
one or more non-peptide moiety such as sugar, lipid or
phosphoric ester and include both compounds known and
unknown to have any particular physiolosical activity (e.g.
therapeutic activity). Such compounds are polypeptides,
simple proteins and conjugated proteins (lipoproteins and
glycoproteins, etc.). The preferred polypeptide compounds
include serum proteins (e.g. albumin, fibrinogen, etc.),
enzymes (e.g. urokinase, strptokinase, TPA, etc.), peptide
hormones (e.g. adrenal cortical hormone, thyroid stimulating
hormone, insulin, etc.), antibodies (e.g. IgG, IgE, IgM,
IgA, IgD and the fragments Fab, Fab', $F(ab')_2$, etc.),
peptide antibiotics (e.g. bleomycin, mitomycin, etc.).
Those exhibiting specific distribution, accumulation or
behavior in vivo are preferred.

The radioisotope may be either metal or
non-metal, but metal is preferred. Specific examples of the

radioactive metal atom include diagnostic gamma-ray-emitting nuclide (e.g. ^{111}In, ^{99m}Tc, ^{67}Ga, etc.), a diagnostic positron-emitting nuclide (e.g. ^{68}Ga, ^{62}Cu, ^{62}Zn, etc.), a therapeutic beta-ray-emitting nuclide (e.g. ^{90}Y, ^{186}Re, ^{188}Re, ^{153}Sm, ^{212}Pb, etc.) and a therapeutic alpha-ray-emitting nuclide (e.g. ^{211}Bi, etc.).

Examples of physiologically acceptable salts of the compounds (I) and (II) include alkali metal salts such as sodium salts, potassium salts, etc., and ammonium salts.

The compounds of formula (I) can be obtained by reacting a DTPA amide derivative having the following formula

$$\begin{array}{c} HOOCH_2C \\ \\ HOOCH_2C \end{array} \!\!\! N\text{-}CH_2CH_2\text{-}N\text{-}CH_2CH_2\text{-}N \!\!\! \begin{array}{c} CH_2COOH \\ \\ CH_2CONH(CH_2)_nNH_2 \end{array}$$

with the middle N bearing CH_2COOH

III

wherein n is an integer of 2 to 10 or a salt thereof with a cross linking reagent which will provide a group A thereto. Any of the above mentioned cross linking reagent can be used in this reaction. This reaction is carried out by combining a compound (III) and the cross linking reagent in an appropriate solvent such as a phosphate buffer and then stirring at a moderate temperature such as between cooling and slight

heating, for example at room temperature for several tens minutes to a few hours, for example for 30 - 60 minutes. Usually the resultant compounds (I) are used without isolation for the preparation step of the compounds (II), but if particularly required, they may be isolated. Isolation is carried out by applying any combination of purification methods such as chromatography and a separation methods such as precipitation by addition of a non aqueous solvent. Preferred examples of the compounds (I) are as follows:

(DTPAM- represents a DTPA monoalkylamide group of the formula:

$$
\begin{array}{ccc}
\text{HOOCH}_2\text{C} & & \text{CH}_2\text{COOH} \\
& N\text{-CH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{-N} & \\
\text{HOOCH}_2\text{C} & \text{CH}_2\text{COOH} & \text{CH}_2\text{CONHC}_n\text{H}_{2n}
\end{array}
$$

$$\text{DTPAM-NHCO-CH}_2\text{CH}_2\text{-CO-O-CH}_2\text{CH}_2\text{-O-CO-CH}_2\text{CH}_2\text{-CO-O-N}$$

$$\text{DTPAM-NHCO-CH}_2\text{CH}_2\text{-S-S-CH}_2\text{CH}_2\text{-CO-O-N} \quad \text{SO}_3\text{Na}$$

$$\text{DTPAM-NHCO-O-CH}_2\text{CH}_2\text{-}\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}\text{-CH}_2\text{CH}_2\text{-O-CO-O-N}$$

$$\text{DTPAM-NHCO-CH-CH-CO-O-N} \begin{array}{c} O \\ \parallel \\ \\ \parallel \\ O \end{array}$$
$$\quad\quad\quad\quad | \;\; |$$
$$\quad\quad\quad\quad OH \; OH$$

$$\text{DTPAM-NHCO-C}_6\text{H}_4\text{-N} \begin{array}{c} O \\ \parallel \\ \\ \parallel \\ O \end{array}$$

Among compounds of the formula III, those represented by the formula IIIa:

$$\begin{array}{c} \text{HOOCH}_2\text{C} \\ \diagdown \\ \\ \text{N-CH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{-N} \\ \diagup \quad\quad\quad | \quad\quad\quad \diagdown \\ \text{HOOCH}_2\text{C} \quad\quad \text{CH}_2\text{COOH} \quad \text{CH}_2\text{CONH(CH}_2)_n\text{NH}_2 \end{array}$$

with CH_2COOH group.

IIIa

wherein n is an integer of 3 to 10, are novel.

The compounds (III) may be prepared by reacting DTPA or a reactive derivative at one or more carboxyl groups thereof (the remaining carboxyl groups may be protected by any carboxyl protecting groups conventionally used in the peptide synthesis) with diamine $\text{H}_2\text{NC}_n\text{H}_{2n}\text{NH}_2$ or a reactive derivative at one or more amino group thereof (the remaining amino group amy be protected by any amino protecting groups are present), subjecting the resultant product to removal reaction of the protecting group. DTPA is commercially

available. Examples of the reactive derivatives at the

carboxyl group of DTPA include acid halides, acid

anhydries(including mixed acid anhydrides), active esters,

active amides, etc., conventionally adopted for peptide

synthesis. Among the acid halides, acid chloride is the

most frequently used. Examples of the acid anhydrides

include cyclic anhydrides and mixed anhydrides, such as

dialkylphosphoric acid mixed anhydride, dialkylphosphorous

acid mixed hydride, alkylcarbonic acid mixed anhydride

aliphatic carboxylic acid (e.g. pivalic acid,

trichloroacetic acid) mixed anhydride etc. with cyclic

anhydrides being preferred. Examples of the activated

esters include methyl ester, ethyl ester, cyanomethyl ester,

p-nitrophenyl ester, an ester with N-hydroxysuccinimide etc.

Examples of the activated amides include an amide with

imidazole, dimethylimidazole or triazole. Examples of the

reactive derivatives at the amino group of diamine are

Schiff's bases with an aldehyde (e.g. acetaldehyde,

isopentanal, benzaldehyde), a reaction product with a silyl

compound (e.g. trimethylsilyl chloride,

trimethylsilylacetamide), a reaction product with a

phosphorus compound (e.g. phosphorus trichloride, phosphorus

oxychloride)etc., conventionally adopted for peptide

synthesis.

Examples of the protecting groups for residual carboxyl group of DTPA include those conventionally adopted in the peptide synthesis, such as phthalimido ester, succinimidomethyl ester pivaloyloxymethyl ester, benzyl ester or trimethylsilyl ester. Examples of the protecting groups for residual amino group of the compound (II) include those conventionally adopted in the peptide synthesis, such as benzyloxycarbonyl, t-butoxycarbonyl, benzyl, trityl, phthaloyl, trifluoroacetyl, trimethyl silyl etc.

Also, the reaction may be effected using DTPA as such, i.e. in the form of carboxylic acid, in the presence of a condensing agent conventionally used for peptide synthesis, such as $SOCl_2$, SO_2Cl_2, PCl_3, PCl_5, $POCl_3$, PBr_3, or N,N'-dicyclohexyl carbodiimide (DCC), N-cyclohexyl-N'-morpholinoethyl carbodiimide, N,N'-diisopropyl carbodiimide, $ClCO_2CH_3$, $ClCO_2C_2H_5$, $BrCO_2CH_3$, $(CH_3CO)_2O$, N-ethyl-benzisoxazolium salts, 2-chloro-1-methylpyridinium salt, N,N'-carbonyl diimidazole (CDI), etc.

The reaction may be usually carried out in an inert solvent. Examples of the solvent include dioxane, methylene chloride, chloroform, ether, tetrahydrofuran (THF), acetone, dimethylformamide (DMF), dimethylsulfoxide (DMSO), pyridine, acetonitrile, benzene, toluene, xylene etc. In order to avoid the formation of by-product, it is desirable to limit the molarity of one of the compounds used

in the reaction or to restrict the reaction conditions. The protecting groups are removed by any conventional removal method, for example hydrolysis, reduction, reaction with hydrazine or the like. The reaction product may be separated and recovered by any conventional methods such as concentration- crystallization, chromatography, etc. The compound (III) wherein C_nH_{2n} is CH_2CH_2 is known, and may be prepared according to the known method [J. Med. Chem., 31, pp. 898-901 (1988)].

The compounds of formula (II) may be obtained by reacting a compound of formula (I) or its salt with a polypeptide compound which will provide a group B thereto. As the polypeptide compound, those illustrated above are used. This reaction is carried out by combining a compound (I) and a polypeptide compound in an appropriate solvent such as a phosphate buffer and then stirring at a moderate temperature such as between cooling and slight heating, for example at room temperature for several tens minutes to a few hours, for example for 30 - 60 minutes. Usually, the compounds (I) are used in situ, i.e. in the form contained in the reaction solution used for preparation thereof. After the reaction is completed, any unreacted reactive group in the cross linking agent is decomposed by an appropriate stopping (or quenching) agent such as ammonium acetate and then the desired compound (II) is separated and

recovered by an appropriate separation methods such as chromatography.

The compounds (II) labeled with radioactive metal element are obtained by adding an aqueous solution of a water-soluble compound of radioactive metal to a solution of a compound (II). As the water-soluble compound of the radioactive metal, halide, for example, is used. Labeling is effected in the conventional manner.

The compounds (II) are useful as a carrier for a radioactive element. The compounds (II), which has four carboxyl groups in the part derived from DTPA, allowing thereby to capture metallic atoms, is suitable for labeling by different radioisotopes. The compounds (II) also provide an extremely high labeling efficiency. Therefore, the compounds (II) are useful as a carrier for a radioactive element.

On using as a carrier, the compounds (II) may be stored in the form of an aqueous solution, but are advantageously stored in the solid form as a lyophilisate. In the latter case, the compounds (II) are dissolved in a sterilized water, a physiological saline, a buffer, etc. prior to use. In addition, if required, an auxiliary solubilizing agent (e.g. an organic solvent), a pH controlling agent (e.g. an acid, a base, a buffer), a stabilizer, a preserving agent, an isotonizing agent, a

reducing agent, an oxidizing agent (for maintaining the atomic valence), etc. may be added.

The compound (II) labeled with radioactive metal element may be used as a radioactive diagnostic agent by external measurement and quantitation of the radioactivity emitted therefrom. Further, in the radiotherapy for treating malignant neoplasm such as cancer, they may be used as a therapeutic agent. For these use, it is advantageous to choose, as the polypeptide moiety, such a compound as specifically accumulates at a particular organ or tissue of a diagnostic or therapeutic target. Such compounds include, for example, peptide hormones, antibodies and fragments thereof. For instance, anti-myocardial myosin antibody is used for diagnosing myocardial infarction. The amount of the radioactive metal is one that can afford information sufficient to diagnose, or one that can provide the desired therapeutic effect, but desirably one that can keep a radiation exposure of any other organs or tissues as low as possible. Administration is usually effected through an intravenous route, but depending upon the purpose, any other administration route may be employed.

Since the compounds (II) of the present invention have allowed the group A' provided by the cross linking reagent to intervene between the DTPA moiety and the polypeptide moiety, expansion of a range of application is

realized by selecting an appropriate group to impart the compounds (II) any property suitable for the purpose. For instance, by including a group easily cleavable in vivo in the bridging reagent or by binding the bridging reagent using such a group, a blood clearance of the radioactive metal is promoted, which leads to decrease in undesired side effects. Also, by using a substance react in an aqueous solvent as the bridging reagent, the reaction under a mild condition is enabled and binding to a less stable polypeptide compound is facilitated.

Moreover, since the DTPA moiety and the terminal amine in the compounds (III) are joined via a fatty chain (C_nH_{2n}) an improved molecular flexibility and a better stability upon the radioactive metal binding thereto are provided, in comparison with those joined via an aromatic nucleus. In addition, they have various advantages such as rapider excretion, lesser antigenicity, easier purification, etc.

Additionally, by choosing suitably the compound in the polypeptide moiety, a specific distribution and an accumulation at a particular organ may be enabled. This advantage can not be seen in a known compound having a sugar such as inulin in place of peptide compound.

The compounds of the present invention are metabolized in vivo (e.g. in liver) into DTPA and rapidly

(e.g. 90% within one hour) excreted into urine. Therefore, a retention time in vivo is extremely short and so that a period for exerting toxicity is short. For instanace, when they were administered to an animal (a rat) at a dosage of 300 microgram/kg, no animal died and no abnormality was observed in behaviors during this experiment and in an autopsy. Since the above administered amount is about 20 times the estimated amount in clinical dosage, the above results indicate that the compounds of the present invention have extremely high safety.

The present invention is now illustrated in more detail by way of the following Examples.

In the Examples, % is given by weight %, unless otherwise defined. The following abbreviation are employed.

DTPA = diethylenetriaminepentaacetic acid;

hxnDTPA = N-[2-bis(carboxymethyl)aminoethyl]-N-[2-carboxymethyl-2-(6-aminohexyl)carbamoylmethyl]glycine [or diethylenetriaminepentaacetic acid mono(6-aminohexyl)-amide];

etnDTPA = N-[2-bis(carobxymethyl)aminoethyl]-N-[2-carboxymethyl-2-(2-aminoethyl)carbamoylmethyl]glycine [or diethylenetriaminepentaacetic acid mono(2-aminoethyl)-amide];

prnDTPA = N-[2-bis(carobxymethyl)aminoethyl]-N-[2-carboxymethyl-2-(3-aminopropyl)carbamoylmethyl]glycine [or

diethylenetriaminepentaacetic acid mono(3-aminopropyl)-amide];

DTSSP = 3,3'-dithiobis(sulfosuccinimidylpropranate);

EGS = ethyleneglycolbis(succinimidylsuccinate);

AMFab = anti-myocardial myosin monoclonal antibody fragment.

Example 1:

Preparation of a derivative of diethylenetriaminepentaacetic acid (n=6 : hxnDTPA)

Anhydride of DTPA (5.3g, 14.8 mmol) was suspended in dimethylformamide (150ml) (hereinafter referred to as solution A). To a solution of 6-[N-(t-butoxycarbonyl)-amino]hexylamine hydrochloride 1/4 hydrate (1.5g, 5.83mmol) dissolved in dimethylformamide (50ml) was added 5N aqueous NaOH (1.17ml) (hereinafter referred to as solution B). The solution B was added dropwise to the solution A at room temperature under stirring over a period of 30 minutes and then allowed to react for another 30 minutes. The reaction solution became clear, and dimethylformamide was distilled off to give a syrupy residue. The syrupy residue was treated with 8ml of water and then 6ml of concentrated hydrochloric acid in order to hydrolyzed unreacted anhydride and remove the protecting group. Thereafter, hxnDTPA was fractionated by column chromatography using a cation exchange resin (S-Sepharose FF, H$^+$ form). The resulting fraction was concentrated and acetone was concentrated and

acetone was added thereto to give strongly hygroscopic white
precipitates (yield: 1.3g). This product was identified as
hxnDTPA by the following analysis. TLC: stationary phase:
silica gel, developing solvent: 10 % aqueous solution of
ammonium acetate/methanol = 1/3, a single spot appeared at
Rf = 0.33 by I_2 coloring and nynhydrin coloring.

Analysis: $C_{20}H_{37}O_9N_5 \cdot 3HCl \cdot 2H_2O \cdot 2[CH_3COCH_3]$

	C	H	N	Cl
Found :	42.1 %	7.3 %	9.1 %	14.4 %
Calcd.:	41.47 %	7.49 %	9.30 %	14.12 %

FD-Mass spectrum: The parent peak (hxnDTPA.H^+) was
recognized at m/e = 492.

^{13}C-NMR: measured in D_2O using an external standard method
where a signal of dioxane was 67.4 ppm. The measurement
modes employed were complete decoupling and off-resonance.

Chemical shifts of detected peaks /ppm	Assignment (underlined C-atom)
173.80(s)	$\begin{array}{c}\diagdown\!\!\diagup N-CH_2CH_2\diagdown\\[2pt]\qquad\qquad N-CH_2-\underline{C}OOH\\[2pt]\diagdown\!\!\diagup N-CH_2CH_2\diagup\end{array}$
169.58(s)	$-N\!-\!(-CH_2\underline{C}OOH)_2$
169.31(s)	$-NHCOCH_2-\overset{\mid}{N}-CH_2\underline{C}OOH$
165.96(s)	$-NH\underline{C}OCH_2-\overset{\mid}{N}-$

56.95(t) $-NHCO\underline{C}H_2-N-$

55.92(t) $-NHCOCH_2-N-\underline{C}H_2COOH$

$$\begin{array}{c} \diagdown N-CH_2CH_2 \diagdown \\ \diagup \hspace{3cm} N-\underline{C}H_2-COOH \\ 54.49(t) \hspace{1cm} \diagdown N-CH_2CH_2 \diagup \end{array}$$

53.75(t) $-N-(\underline{C}H_2\underline{C}OOH)_2$

50.19(t) $H_2N-\underline{C}H_2CH_2CH_2CH_2CH_2\underline{C}H_2-NHCOCH_2-$

40.14(t) $\diagdown N-\underline{C}H_2CH_2-N-CH_2\underline{C}H_2-N\diagup$

40.07(t) $\diagdown N-CH_2\underline{C}H_2-N-\underline{C}H_2CH_2-N\diagup$

28.47(t)

27.17(t) $H_2N-CH_2\underline{C}H_2\underline{C}H_2\underline{C}H_2\underline{C}H_2CH_2-NHCOCH_2-$

26.04(t)

25.75(t)

The splitting mode of peak at measurement in the
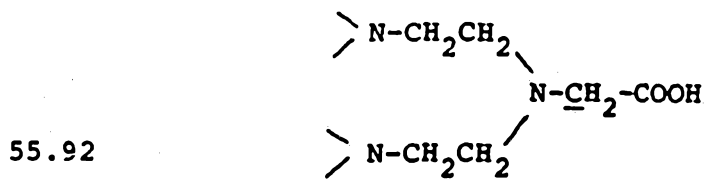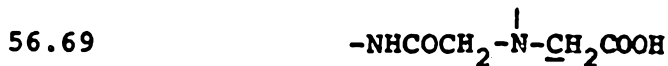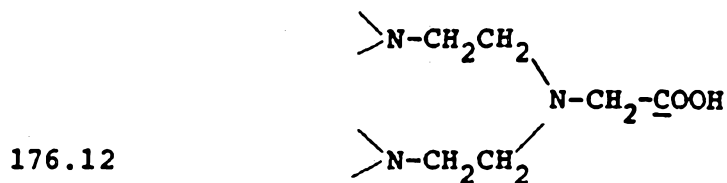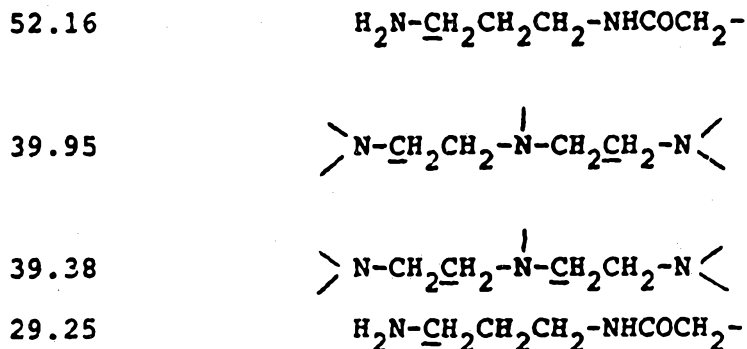off-resonance mode is shown in parentheses. s: singlet, t:
triplet

Example 2:

Preparation of a derivative of diethylenetriaminepentaacetic acid (n=3: prnDTPA)

Anhydride of DTPA (2.5 g, 7.0 mmol) was suspended in dimethylformaide (70 ml) (hereinafter referred to as solution A). To a solution of 2-[N-(t-butoxycarobonyl)-amino]propylamine hydrochloride 1/4 hydrate (0.59 g, 2.74 mmol) dissolved in dimethylformamide (24 ml) was added 5N aqueous NaOH (0.55 ml) (hereinafter referred to as solution B). The solution B was added dropwise to the solution A at room temperature under stirring over a period of 30 minutes and then allowed to react for another 30 minutes. The reaction solution became clear, and dimethylformamide was distilled off to give a syrupy residue. The syrupy residue was treated with 3.8 ml of water and then 2.8 ml of concentrated hydrochloric acid in order to hydrolyze unreacted anhydride and remove the protecting group. Thereafter, prnDTPA was fractionated by column chromatography using a cation exchange resin (S-Sepharose FF, H$^+$ form). The obtained fraction was concentrated and acetone was added thereto to give strongly hygroscopic white precipitates (yield;: 0.85 g). This product was identified as prnDTPA by the following analysis. FD-Mass spectrum: The parent peak (prnDTPA·H$^+$) was recognized at m/e=450.

^{13}C-NMR : measured in D_2O using tetramethylsilylpropionic acid as an external standard.

Chemical shifts of detected peaks	Assignment (underlined C-atom)
176.12	
171.87	$-N-(-CH_2\underline{C}OOH)_2$
171.76	$-NHCOCH_2-N-CH_2\underline{C}OOH$
169.03	$-NH\underline{C}OCH_2-N-$
59.08	$-NHCO\underline{C}H_2-N-$
58.35 ⎫ 58.16 ⎭	$-N-(-\underline{C}H_2COOH)_2$
56.69	$-NHCOCH_2-N-\underline{C}H_2COOH$
55.92	
52.43	$H_2N-CH_2CH_2\underline{C}H_2-NHCOCH_2-$

52.16	$H_2N-\underline{C}H_2CH_2CH_2-NHCOCH_2-$
39.95	$\diagdown N-\underline{C}H_2CH_2-\overset{\mid}{N}-CH_2\underline{C}H_2-N\diagup$
39.38	$\diagup N-CH_2\underline{C}H_2-\overset{\mid}{N}-\underline{C}H_2CH_2-N\diagdown$
29.25	$H_2N-\underline{C}H_2CH_2CH_2-NHCOCH_2-$
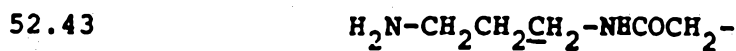
Example 3:

Preparation of a derivative of diethylenetriamine-pentaacetic acid (n=2 : etnDTPA).

Anhydride of DTPA (2.5 g, 7.0 mmol) was suspended in dimethylformamide (70 ml) (hereinafter referred to as solution A). To a solution of 2-[N-(t-butoxycarbonyl)-amino]ethylamine hydrochloirde 1/4 hydrate (0.55 g, 2.74 mmol) dissolved in dimethylformamide (24 ml) was added 5N aqueous NaOH (0.55 ml) (hereinafter referred to as solution B). The solution B was added dropwise to the solution A at room temperature under stirring over a period of 30 minutes and then allowed to react for another 30 minutes. The reaction solution became clear, and dimethylformamide was distilled off to give a syrupy residue. The syrupy residue was treated with 3.8 ml of water and then 2.8 ml of concentrated hydrochloric acid in order to hydrolyze

unreacted anhydride and remove the protecting group.
Thereafter, etnDTPA was fractionated by column
chromatography using a cation exchange resin (S-Sepharose
FF, H$^+$ form). The resultant fraction was concentrated and
acetone was added thereto to give strongly hygroscopic white
precipitates (yield: 0.85 g). This product was identified
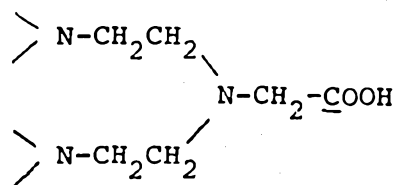as etnDTPA by the following analysis.

TLC: stationary phase: silica gel, developing solvent: 10 %
aqueous solution of ammonium acetate/methanol = 1/1, a
single spot was observed at Rf=0.48 by I_2 coloring and
nynhydrin coloring.

FD-Mass spectrum: the parent peak (etnDTPA·H$^+$) was
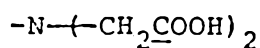   confirmed at m/e = 436.

^{13}C-NMR: measured using the same method and mode as those of
        Example 1.

Chemical shifts of detected peaks	Assignment (underlined C-atom)
173.81(s)	$>$N-CH$_2$CH$_2\backslash$ $\quad$ N-CH$_2$-$\underline{C}$OOH $>$N-CH$_2$CH$_2/$
169.86(s)	-N-(-CH$_2$$\underline{C}$OOH)$_2$

169.75 (s) $-NHCOCH_2-\overset{|}{N}-CH_2\underline{C}OOH$

167.97 (s) $-NH\underline{C}OCH_2-\overset{|}{N}-$

56.78 (t) $-NHCO\underline{C}H_2-\overset{|}{N}-$

55.93 (t) $-NHCOCH_2-\overset{|}{N}-\underline{C}H_2COOH$

54.48 (t)

$$\diagup N-CH_2CH_2 \diagdown$$
$$N-\underline{C}H_2-COOH$$
$$\diagup N-CH_2CH_2 \diagup$$

53.57 (t) $-N-(-\underline{C}H_2\underline{C}OOH)_2$

53.49 (t)

50.48 (t) $H_2N-CH_2\underline{C}H_2-NHCOCH_2-\overset{|}{N}-$

50.21 (t) $H_2N-\underline{C}H_2CH_2-NHCOCH_2-\overset{|}{N}-$

39.44 (t) $\diagup N-\underline{C}H_2CH_2-\overset{|}{N}-CH_2\underline{C}H_2-N\diagdown$

37.57 (t) $\diagdown N-CH_2\underline{C}H_2-\overset{|}{N}-\underline{C}H_2CH_2-N\diagup$

The splitting mode of a peak at measurement in the
off-resonance mode is shown in parentheses.   s: singlet, t:
triplet

Example 4:

Preparation of EGS-hxnDTPA.

To a solution of hxnDTPA hydrochloride in DMF (1 x 10^{-4} mol/ml, 2ml) was added 40 µl of 5N NaOH, then a solution of EGS in DMF (1 x 10^{-4} mol/ml, 2 ml), and the resultant mixture was stirred at room temperature for 30 minutes. Thereafter, EGS-hxnDTPA was fractionated by column chromatography using TSK G-1000H (eluent: DMF). The solvent was distilled off from the obtained fraction, and acetone was added thereto to give strongly hygroscopic white precipitates.

Example 5:

Preparation of bovine IgG-(DTSSP)-hxnDTPA.

To a neutral aqueous solution of hxnDTPA (2 x 10^{-4} mol/ml, 50 µl) was added 0.2 M phosphate buffer (pH 9.2, 200 µl), then a solution of DTSSP in 0.1 M phosphate buffer (pH 7.5, 5.75 x 10^{-5} mol/ml, 175 µl, 1.01 x 10^{-5} mol), and the resultant mixture was stirred at room temperature for 30 seconds. This solution (110 µl) was added to a solution of bovine IgG in 0.05M phosphate buffered saline (pH 7.5, 15 mg/ml, 1.6 ml), and the resultant mixture was stirred at room temperature for 30 minutes. Then, 30 µl of 1M aqueous ammonium acetate was added thereto, and the mixture was stirred at room temperature for 10 minutes to decompose the

unreacted DTSSP. A monomeric fraction was separated by gel chromatography using Sephacryl S-300SF [2.2cm (column inside diameter) x 75 cm (length), elution buffer: 0.2 M citric acid (pH 5.8), elution rate: about 15.5 ml/h] to give the desired bovine IgG-(DTSSP)-hxnDTPA.

Example 6:

Preparation of bovine IgG-(EGS)-hxnDTPA.

To a neutral aqueous solution of hxnDTPA (2×10^{-4} mol/ml, 50 µl) was added 0.2 M phosphate buffer (pH 9.2, 200 µl), then a solution of EGS in dimethylsulfoxide (1.01×10^{-4} mol/ml, 120 µl, 1.3×10^{-5} mol), and the mixture was stirred at room temperature for about one minute. This solution (50 µl) was added to a solution of bovine IgG in 0.05 M phosphate buffered saline (pH 7.5, 15 mg/ml, 1.6 ml), and the resultant mixture was stirred at room temperature for one hour. Then, 30 µl of 1 M aqueous ammonium acetate was added thereto, and the mixture was stirred at room temperature for 10 minutes to decompose the unreacted EGS. A monomeric fraction was separated by gel chromatography using Sephacryl S-300SF [2.2 cm (column inside diameter) x 75 cm (length), elution buffer: 0.2 M citric acid (pH 5.8), elution rate: about 16 ml/h] to give the desired bovine IgG-(EGS)-hxnDTPA.

Example 7:

Preparation of AMFab-(DTSSP)-hxnDTPA.

To a neutral aqueous solution of hxnDTPA (2×10^{-4} mol/ml, 60 µl) was added 0.2 M phosphate buffer (pH 9.2, 0.42 ml), then a solution of DTSSP in 0.2 M phosphate buffer (pH 9.2, 30.43 mg/ml, 0.12 ml), and the obtained mixture was stirred at 4 °C for 30 minutes. This solution (0.45 ml) was added to a mixture of a solution of AMFab in 0.05 M phosphate buffer (pH7.8, 15 mg/1.5 ml) and 0.2 M phosphate buffer (pH 9.2, 0.45 ml), and the mixture was stirred at 4 °C for three hours. Then, 0.1 ml of 1M aqueous ammonium acetate was added thereto and the mixture stirred at 4 °C for one hour to decompose the unreacted DTSSP. A monomeric fraction was separated by gel chromatography using Sephacryl S-200SF [2.2 cm (column inside diameter) x 50 cm (length), elution buffer: 0.1 M citric acid (pH 6), elution rate: about 15.5 ml/h] to give the desired AMFab-(DTSSP)-hxnDTPA.

Example 8:

Preparation of AMFab-(DTSSP)-etnDTPA.

To a neutral aqueous solution of etnDTPA (1×10^{-4} mol/ml, 87.5 µl) was added 0.2 M phosphate buffer (pH 9.2, 263 µl), then a solution of DTSSP in 0.2 M phosphate buffer (pH 9.2, 5×10^{-5} mol/ml, 100 µl), and the mixture was stirred at 4 °C for 30 minutes. This resultant solution (180 µl) was added to a mixture of a solution of AMFab in 0.05 M phosphate buffered saline (pH 7.8, 10.8 mg/ml, 0.58 ml) and 0.2 M phosphate buffer (pH 9.2, 180 µl), and the

resulting mixture was stirred at 4 °C for three hours.
Then, 100 µl of 1 M aqueous ammonium acetate was added
thereto and the mixture was stirred at 4 °C for one hour to
decompose the unreacted DTSSP. A monomeric fraction was
separated by gel chromatography with TSKG-2000SM [0.75 cm
(column inside diameter) x 30 cm (length) + 0.75 cm (guard
column inside diameter) x 7.5 cm (length), elution buffer:
0.1 M citric acid (pH 6), elution rate: about 0.75 ml/min.]
to give the desired AMFab-(DTSSP)-etnDTPA.

Example 9:

Preparation of bovine IgG-(DTSSP)-hxnDTPA^{111}In and
biodistribution in rats.

To a solution of bovine IgG-(DTSSP)-hxnDTPA (2.5
ml, corresponding to 1.56 mg of IgG) obtained according to
the procedure of Example 5 was added a solution fo ^{111}InCl$_3$
(0.2 ml, 2 mCi/ml) to give the bovine IgG-(DTSSP)-hxn-
DTPA-^{111}In. To examine the labeling efficiency, it was
subjected to TLC using silica gel as a stationary phase and
10 % aqueous $CH_3COONH_4/CH_3OH = 1/3$ as a developing solvent,
and scanning was carried out using a radiochromatoscanner.
99.5 % of the radioactivity was detected at the origin,
while the remaining 0.5 % was observed near Rf=0.35
corresponding to ^{111}In-hxnDTPA etc.

From the above results, it may be said that the
bovine IgG-(DTSSP)-hxnDTPA-^{111}In prepared according to the

above method has a labeling efficiency of 99.5 %.

The bovine IgG-(DTSSP)-hxnDTPA-^{111}In prepared according to the above method was injected intravenously to several SD female rats (dose: 0.1 ml per animal, corresponding to about 58 μg of IgG, and to about 15 μCi as ^{111}In), and the time course of the biodistribution with the lapse of time was investigated.

As the control, the same examination as above was carried out using ^{111}In-labeled, bovine IgG-DTPA obtained by conventional DTPA anhydride method (a labeling efficiency of an administration sample was 97.3 %).

The results are shown in the following Table. From the values shown in the Table, it is understood that, those employing DTSSP as spacer have a high renal accumulation, but exhibit a rapid blood clearance resulting rapid urinary excretion, and a low non-specific distribution in the remaining whole body (bone, muscle, skin, etc.). Further, their low hepatic accumulation clearly demonstrates an effect resulted by incorporation of -S-S- between the protein and chelate.

Table 1: Bovine IgG-(DTSSP)-hxnDTPA-^{111}In

	Injected Dose / Organ(%) Time after administration (hours)			
Organs	1	6	24	48
Liver	6.781	4.013	2.901	2.721
Spleen	0.398	0.167	0.111	0.143
Kidneys	4.758	17.66	15.47	13.61
Heart	0.701	0.291	0.051	0.029
Lungs	1.588	0.552	0.133	0.069
Stomach	0.300	0.170	0.057	0.045
Small intestine	4.086	5.724	0.536	0.366
Large intestine	0.833	6.172	4.451	0.717
Urine	7.901	36.73	58.05	59.59
Feces	0.000	0.107	10.78	17.88
Carcass	28.62	17.77	6.278	4.431
Whole blood	71.23	18.75	2.026	0.712

The total amount of blood is calculated as 6.4 % based on a body weight of rat.

Table 2: Bovine IgG-DTPA-^{111}In

	Injected Dose / Organ (%) Time after administration (hours)			
Organs	1	6	24	48
Liver	7.904	8.031	9.152	10.85
Spleen	0.498	0.455	0.458	0.574
Kidneys	1..73	1.943	3.217	4.531
Heart	0.882	0.835	0.544	0.418
Lungs	2.446	1.783	1.132	0.860
Stomach	0.332	0.414	0.457	0.369
Small intestine	2.786	4.117	3.003	2.287
Large intestine	0.620	2.800	4.032	2.222
Urine	2.653	3.255	4.603	7.744
Feces	0.000	0.001	2.889	7.037
Carcass	26.19	36.61	50.56	50.24
Whole blood	97.90	73.25	35.75	21.73

The total amount of blood is calculated as 6.4 % based on a body weight of rat.

Example 10:

Preparation of bovine IgG-(EGS)-hxnDTPA-^{111}In and

biodistribution in rats.

To a solution of bovine IgG-(EGS)-hxnDTPA (2.5 ml,

corresponding to 2.1 mg of IgG) obtained according to the

procedure of Example 6 was added a solution of ^{111}InCl$_3$ (0.2

ml, 2mCi/ml) to give the bovine IgG-(EGS)-hxnDTPA-^{111}In.

In order to examine the labeling efficiency, it

was subjected to TLC according to the method of Example 9.

As a result, 99.7 % of the radioactivity was present at the

origin, while the remaining 0.3 % was observed near Rf=0.35

corresponding to ^{111}In-hxnDPTA etc. Therefore, it may be

said that the labeling efficiency was 99.7 %.

The bovine IgG-(EGS)-hxnDTPA-^{111}In prepared by the

above method was used to investigate the time course of the

biodistribution in rats with the elapse of time according to

the method of Example 9.

The results are shown in the following Table.

From the values shown in the Table, it is understood that

adoption of EGS as a spacer (i.e. incorporating an ester

bond between the protein and chelate) results in

acceleration of blood clearance and thus promotion of

urinary excretion. It may be said that, at the same time,

an effect of reducing accumulation in liver and the

remaining whole body was obtained.

Table 3: Bovine IgG-(EGS)-hxnDTPA-^{111}In

| | Injected Dose / Organ (%) | | | |
| | Time after administration (hours) | | | |
Organs	1	6	24	48
Liver	6.942	5.491	4.699	4.587
Spleen	0.445	0.304	0.229	0.245
Kidneys	1.125	1.448	1.582	1.919
Heart	0.693	0.753	0.313	0.147
Lungs	1.909	1.401	0.636	0.352
Stomach	0.266	0.322	0.259	0.142
Small intestine	3.032	4.480	2.015	1.228
Large intestine	0.514	5.136	5.546	2.408
Urine	5.303	21.03	39.80	49.81
Feces	0.000	0.001	8.065	16.68
Carcass	25.46	29.14	25.33	17.75
Whole blood	69.48	56.08	19.98	8.057

The total amount of blood is calculated as 6.4 % based on a body weight of rat.

Table 4: Bovine IgG-DTPA-^{111}In

Organs	Injected Dose / Organ (%) Time after administration (hours)			
	1	6	24	48
Liver	7.904	8.031	9.152	10.85
Spleen	0.498	0.455	0.458	0.574
Kidneys	1.373	1.943	3.217	4.531
Heart	0.882	0.835	0.544	0.418
Lungs	2.446	1.783	1.132	0.860
Stomach	0.332	0.414	0.457	0.369
Small intestine	2.789	4.117	3.003	2.287
Large intestine	0.620	2.800	4.032	2.222
Urine	2.653	3.255	4.603	7.744
Feces	0.000	0.001	2.889	7.037
Carcass	26.19	36.61	50.56	50.24
Whole blood	97.90	73.25	35.75	21.73

The total amount of blood is calculated as 6.4 % based on a body weight of rat.

Example 11:

Preparation of AMFab-(DTSSP)-hxnDTPA-^{111}In.

To a solution of AMFab-(DTSSP)-hxnDTPA (1 ml, corresponding to 0.5 mg of Fab) obtained by the method of Example 7 was added a solution of ^{111}InCl$_3$ (1 ml, 2 mCi/ml) to prepare AMFab-(DTSSP)-hxnDTPA-^{111}In.

In order to examine the labeling efficiency, it was subjected to TLC using silica gel as a stationary phase and 10% CH$_3$COONH$_4$/MeOH = 1/1 as a developing solvent, and scanning was carried out using radiochromato-scanner. 98.7 % of the radioactivity was present at the origin, while the remaining 1.3 % was detected near Rf=0.5 corresponding to ^{111}In-hxnDTPA etc. Therefore, it may be said that AMFab-(DTSSP)-hxnDTPA-^{111}In prepared by the above method has a labeling efficiency of 98.7%.

AMFab-(DTSSP)-hxnDTPA-^{111}In prepared by the above method was measured for its affinity constant by a radioimmunometric assay using major myocardial myosin as an antigen giving superior result of Ka = 1.5 x 10^{8}M^{-1} as compared with Ka = 1.0 x 10^{8}M^{-1} for an antibody prepared by a conventionally adopted anhydrous DTPA method.

Example 12:

Preparation of AMFab-(DTSSP)-etnDTPA-^{111}In.

To a solution of AMFab-(DTSSP)-etnDTPA (1 ml, corresponding to 0.5 mg of Fab) obtained by the method of

Example 8 was added a solution of $^{111}InCl_3$ (1 ml, 2 mCi/ml) to give AMFab-(DTSSP)-etnDTPA-^{111}In.

In order to examine the labeling efficiency, TLC was effected according to the method of Example 9. As the result, 98.2 % of the radioactivity was present at the original point, while the remaining 1.8 % was detected near Rf 0.5 corresponding to ^{111}In-etnDTPA etc. Therefore, it may be said that AMFab-(DTSSP)-etnDTPA-^{111}In prepared by the above method has a labeling efficiency of 98.2 %.

In a manner similar to Example 11, an affinity constant of AMFab-(DTSSP)-etnDTPA-^{111}In was measured and also a satisfactory result of Ka = 1.8 x $10^8 M^{-1}$ was obtained.

Example 13:

The removal efficiency of unreacted bifunctional ligand(s).

AMFab-(DTSSP)-hxnDTPA and AMFab-(DTSSP)-etnDTPA prepared in Examples 7 and 8 respectively were estimated for the removal efficiency of unreacted free bifunctional ligand(s) after the reaction. As a control, an anhydrous DTPA conventionally adopted as a bifunctional ligand was used. A purification procedure used was a high performance liquid chromatography presently regarded as the most efficient one (column: TSK G-2000SW and TSK G-3000SW, elution rate 0.75 ml/min.). The results are shown in the following Table.

Table 5:

Column: TSK G-2000SW

Mode of binding	Eluting buffer	
	citric acid	0.1 M citric acid in saline
CA-DTPA	17.7 %	17.5 %
AMFab-(DTSSP) hxnDTPA	2.3 %	-
AMFab-(DTSSP) etnDTPA	1.8 %	-

Column: TSK G-2000SW

Mode of binding	Eluting buffer	
	citric acid	0.1 M citric acid in saline
CA-DTPA	15.2 %	15.4 %
AMFab-(DTSSP) hxnDTPA	-	-
AMFab-(DTSSP) etnDTPA	-	-

1) The above % indicates a rate of peak shown by free ligand-^{111}In when the isorated monomeric fraction was labelled with ^{111}In.

2) All the above eluting buffers have a pH of 6.

From the results shown in the above Table, it is understood that the purification efficiency obtained by the method of this invention is much higher than that by any conventional method, and therefore any other purification method is not required to be used jointly. It may be said that this provides an excellent economical advantage.

Example 14:

Preparation of AMFab-(DTSSP)-prnDTPA.

To a neutral aqueous solution of prnDTPA (1×10^4 mol/ml, 36.6 µl) was added 0.2 M phosphate buffer (pH 9.2, 163.4 µl) and then a solution of DTSSP in 0.2 M phosphate buffer (pH 9.2; 2.09×10^{-5} mol/ml, 100 µl), and the resultant mixture was stirred at 4 °C for 30 minutes. To this solution was added a solution of AMFab in 0.05 M phosphate buffer saline (pH 7.8; 10.8 mg/ml, 500 µl) and the mixture was stirred at 4 °C for three hours. Then, 50 µl of 1 M aqueous ammonium acetate was added thereto and the mixture was stirred at 4 °C for one hour to decompose unreacted DTSSP. Then, gel column chromatography using TSKG-2000SW [0.75 cm (column inside diameter) x 30 cm (length) + 0.75 cm (guard column inside diameter) + 7.5 cm (length), elution buffer: 0.1 M citrate buffer (pH 6), elution rate: 0.75 ml/min.] was effected to separate a monomeric fraction, which gave the AMFab-(DTSSP)-prnDTPA.

Example 15:

Preparation of AMFab-(DTSSP)-prnDTPA-^{111}In and

biodistribution and excretion in rats.

     To a solution of AMFab-(DTSSP)-prnDTPA (1 ml,

corresponding to 0.5 mg as Fab) obtained according to the

procedure of Example 14·was added a solution of ^{111}InCl$_3$ (1

ml, 2 mCi/ml) to give AMFab-(DTSSP)-prnDTPA-^{111}In. In order

to examine the labeling efficiency, it was subjected to TLC

using silica gel as a stationary phase and 10 % aqueous

CH$_3$COONH$_4$/CH$_3$OH = 1/1 as a developing solvent, and scanning

was carried out using a radiochromato-scanner. 97.6 % of

the radioactivity emitted was detected at the origin , while

the remaining 2.4 % was observed near Rf 0.45 corresponding

to prnDTPA-^{111}In etc. Therefore, it may be said that

AMFab-(DTSSP)-prnDTPA-^{111}In prepared by the above method has

a labeling efficiency of 97.6 %.

     The AMFab-(DTSSP)-prnDTPA-^{111}In prepared according

to the above method was injected intravenously to several SD

female rats (dose: 0.1 ml per animal, corresponding to about

25 µg as Fab, and to about 100 µCi as ^{111}In), and the

time course of the biodistribution was investigated. Also,

at 3 hours after injection, a portion of urine was analyzed

by HPLC [column: TSK G-2000SW, 0.75 cm (column inside

diameter) x 30 cm (length) + 0.75 cm (guard column

inside diameter) x 7.5 cm (length), elution buffer:

0.1 M phosphate buffer (pH 7) containing 0.1 %
polyethyleneglycol, elution rate: 0.75 ml/min, detector: a
sigle channel analyzer (gamma-ray of 171 keV, 245 KeV
detectable)] to examine metabolites in urine.

For the control, the same examination as above was
carried out using ^{111}In-labeled, AMFab-DTPA obtained by
conventional DTPA anhydride method (a labeling efficiency of
an administration sample was 85.8 %).

The results are shown in the following Table.
From the values shown in the Table, it may be said that,
there are few apparent differences in the biodistribution
for two reasons, i.e. for one reason that the labeling
efficiency of AMFab-DTPA-^{111}In employed as a control is low
(in the preparation of AMFab-DTPA, unreacted free DTPA is
difficult to be removed, so that free DTPA-^{111}In would
remain contained as much as 14.2 %. Once free DTPA-^{111}In is
administered, it rapidly disappears from blood to be
excreted into urine.) and the other reason that AMFab per
se, which has only a small molecular weight (about 50,000),
tends to be subjected to excretion by glomerular filtration
(difficult to be resorbed in tubulus) in rats. However, the
results of HPLC urinalysis for three hours after
administration indicate that, in case DTSSP was adopted as a
spacer, although only a few % of free prnDTPA-^{111}In is
contained in the administration samples, the radioactive

components derived from molecules with low molecular weight was c :tected no less than 48.3 % in urine (the remaining 51.7 % was those excreted in the form of Fab-(DTSSP)-prnDTPA-^{111}In, for which the molecular weight of Fab was small as mentioned above). This occurred because the spacer part was subjected to any metabolisis to liberate the prnDTPA-^{111}In moiety followed by excretion into urine. In case Fab-DTPA-^{111}In as a control substance was injected to rats, only the low molecular radioactive components whose amount corresponded to that of free DTPA-^{111}In contained in the administration sample were detected in urine, and it was ,confirmed that, for Fab=DTPA prerpared by the conventional DTPA anhydride method, the bondage between protein and chelate was too strong to be metabolized in vivo. Thus, the analysis of urinary excretion clearly demonstrated an effect of incorporation of -S-S- between protein and chelate.

Table 6: AMFab-DTSSP-prnDTPA-^{111}In

	Injected Dose / Organ (%)		
	Time after administration (hours)		
Organs	1	3	6
Liver	4.74	3.81	3.26
Spleen	0.32	0.20	0.17
Kidneys	6.95	8.51	19.38
Heart	0.53	0.26	0.14
Lungs	1.79	1.36	0.51
Stomach	0.32	0.29	0.16
Small intestine	2.08	2.13	0.86
Large intestine	0.75	0.69	2.82
Urine	33.80	54.04	58.11
Feces	-	-	0.05
Carcass	25.67	17.41	9.12
Whole blood*	39.27	15.89	6.29

*   The total amount of blood was calculated as 6.4 % based
on the body weight of rat.

Table 7: AMFab-DTPA-^{111}In

	Injected Dose / Organ(%)		
	Time after administration (hours)		
Organs	1	3	6
Liver	3.96	3.54	3.53
Spleen	0.27	0.21	0.17
Kidneys	10.80	11.69	12.63
Heart	0.57	0.31	0.13
Lungs	1.56	0.99	0.51
Stomach	0.24	0.22	0.20
Small intestine	2.08	1.95	1.57
Large intestine	0.49	1.73	4.31
Urine	37.86	49.75	58.89
Feces	-	-	-
Carcrass	23.55	23.70	15.55
Whole blood*	31.38	12.35	4.31

* The total amount of blood was calculated as 6.4 % based on the body weight of rat.

Table 8: Urinalysis in rat receiving AMFab-(DTSSP)-prnDTPA-^{111}In and AMFab-DTPA-^{111}In (3 hours after administration).

Sample	Retention time(min.)/Relative ratio % (Ratio against injected dose (% ID) *)	
AMFab-(DTSSP)-prnDTPA-^{111}In	13.48/51.7 % (27.9 %ID)	18.08/48.3 % (26.1 %ID)
AMFab-DTPA-^{111}In	13.47/72.8 % (36.2 %ID)	16.80/27.2 % (13.5 %ID)

* Calculated values based on the urinary excretion at 3 hr postinjection shown in Tables 5 and 6, i.e., AMFab-(DTSSP)-prnDTPA-^{111}In : 54.04 % and AMFab-DTPA-^{111}In : 49.75 %.

Example 16:

Preparation of EGS-prnDTPA.

An aqueous solution of prnDTPA.hydrochloride (1 x 10^{-4} mol/ml) was neutralized by adding 40 μl of 5 M NaOH and then lyophilized. The produced white solid was solublized by adding 2 ml of DMF, and a solution of EGS in DMF (1 x 10^{-4} mol/ml, 2 ml) was added. The mixture was stirred at room temperature for 30 minutes. Thereafter, gel column chromatography using TSKG-2000HxL [0.75 cm (column inside diameter) x 30 cm (length) + 0.6 cm (guard column inside

diameter) x 4 cm (length), eluent: DMF elution rate: 0.5 ml/min., sample loading amount: each time 500 µl] was effected to fractionate EGS-prnDTPA. The resultant fraction was concentrated by distilling off the solvent and acetone was added to give strongly hygroscopic white precipitates.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A compound of the formula I:

$$HOOCH_2C,\quad CH_2COOH$$
$$N-CH_2CH_2-N-CH_2CH_2-N$$
$$HOOCH_2C \qquad CH_2COOH \qquad CH_2CONHC_nH_{2n}NH-A$$

I

wherein n is an integer of 2 to 10, and

A is a monovalent group formed by reacting one of the two reactive groups of a cross linking reagent,

and physiologically acceptable salts thereof.

2. The compound according to claim 1, wherein n is an integer of 2 to 6.

3. The compound according to claim 1, wherein A has a molecular weight of 100 to 1000.

4. The compound according to claim 1, wherein A contains at least one bond easily cleavable in vivo at the moiety other than at the reactive group or has at least one bond formed by the reactive group which is easily cleavable in vivo.

5. The compound according to claim 4, wherein said cleavable bond is within a group selected from the group consisting of -O-, -COO-, -S-, -SS-, -SO- and -SO$_2$-.

6. The compound according to claim 4, wherein A is selected from the group consisting of

$$-CO-CH_2CH_2-CO-O-CH_2CH_2-O-CO-CH_2CH_2-CO-O-N \overset{O}{\underset{O}{\bigcirc}}$$

$$-CO-CH_2CH_2-S-S-CH_2CH_2-CO-O-N \overset{O}{\underset{O}{\bigcirc}} SO_3Na$$

$$-CO-O-CH_2CH_2-\overset{O}{\underset{O}{S}}-CH_2CH_2-O-CO-O-N \overset{O}{\underset{O}{\bigcirc}} .$$

$$-CO-CHOH-CHOH-CO-O-N \overset{O}{\underset{O}{\bigcirc}} \text{ , and}$$

$$-CO-C_6H_4-N \overset{O}{\underset{O}{\bigcirc}} .$$

7. A compound of the formula II:

$$\begin{array}{c} HOOCH_2C \\ \\ HOOCH_2C \end{array} N-CH_2CH_2-N-CH_2CH_2-N \begin{array}{c} CH_2COOH \\ \\ CH_2CONHC_nH_{2n}NH-A'-B \end{array}$$

$$\underset{CH_2COOH}{}$$

II

wherein n is an integer of 2 to 10,

A' is a bivalent linking group formed by reacting both the reactive groups of a cross linking reagent, and

B is a residue of a polypeptide compound, and physiologically acceptable salts thereof.

8. The compound according to claim 7, wherein n is an integer of 2 to 6.

9. The compound according to claim 7, wherein A' has a molecular weight of 100 to 1000.

10. The compound according to claim 7, wherein A' contains at least one bond easily cleavable in vivo at the moiety other than at the reactive group or has at least one bond formed by the reactive group which is easily cleavable in vivo.

11. The compound according to claim 10, wherein said cleavable bond is within a group selected from the group consisting of -O-, -COO-, -S-, -SS-, -SO- and -SO$_2$-.

12. The compound according to claim 10, wherein A' is selected from the group consisting of
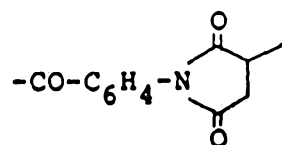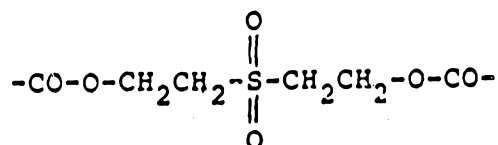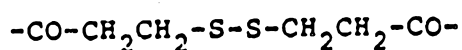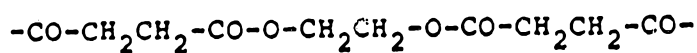
$$-CO-CH_2CH_2-CO-O-CH_2CH_2-O-CO-CH_2CH_2-CO-$$

$$-CO-CH_2CH_2-S-S-CH_2CH_2-CO-$$

$$-CO-O-CH_2CH_2-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{S}}-CH_2CH_2-O-CO-$$

$$-CO-CHOH-CHOH-CO- \quad , \text{ and}$$

$$-CO-C_6H_4-N\underset{}{\overset{}{\diagdown}}$$

13. The compound according to claim 7, wherein the polypeptide compound is a lipoprotein or glycoprotein.

14. The compound according to claim 7, wherein B is a protein having biological activity.

15. The compound according to claim 7, wherein B is selected from the group consisting of serum proteins, enzymes, peptide hormones, peptide antibiotics, antibodies and fragments thereof.

16. The compound according to claim 15, wherein said serum proteins are immunoglobulins.

17. A method of preparing a radioactive metal complex the method comprising treating a water soluble compound of a radioactive metal with a solution of a carrier comprising a compound as defined in claim 7.

18. A radioactive diagnostic agent comprising the compound according to claim 7 labelled with a radioactive metal element.

19. The agent according to claim 18, wherein said radioactive metal element is selected from the group consisting of ^{111}In, ^{99m}Tc, ^{67}Ga, ^{68}Ga, ^{62}Cu and ^{62}Zn.

20. A radioactive therapeutic agent comprising the compound according to claim 7 labelled with a radioactive metal element.

21. The agent according to claim 20, wherein said radioactive metal element is selected from the group consisting of ^{90}Y, ^{186}Re, ^{188}Re, ^{153}Sm, ^{212}Pb and ^{211}Bi.

22. A compound of the formula IIIa:

$$\begin{array}{c}
\text{HOOCH}_2\text{C} \\
\hspace{2em} \diagdown \\
\hspace{4em} \text{N--CH}_2\text{CH}_2\text{--N--CH}_2\text{CH}_2\text{--N} \\
\hspace{2em} \diagup \hspace{3em} | \hspace{4em} \diagdown \\
\text{HOOCH}_2\text{C} \hspace{3em} \text{CH}_2\text{COOH} \\
\end{array}$$

with the substituents CH_2COOH at the upper right nitrogen and $CH_2CONH(CH_2)_nNH_2$ at the lower right nitrogen.

IIIa

wherein n is an integer of 3 to 10.

23.   A compound according to claim 1, or claim 7, or a
radioactive diagnostic or therapeutic agent comprising a
said compound, substantially as hereinbefore described
with reference to the Examples.

~~24.   The steps, features, compositions and compounds~~
disclosed herein or referred to ~~or~~ indicated in the
specification and/or ~~claims~~ of this application,
individually or ~~collectively~~, and any and all combinations
~~of any two or more of said steps or features.~~

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