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(54) Title: IMPROVEMENTS IN OR RELATING TO MODIFIED HAPTENS USEFUL AS IMAGING AND THERAPEU- TIC AGENTS (57) Abstract The present invention discloses a method for preserving a chemical compound associated with or attached to a strong beta emitter in an aqueous solution from radiolytic degradation by adding thereto a reducing oxidizing agent, preferably ascorbate ion, as a radioprotectant. For instance, degradation of haptens radiolabelled with a strong beta emitter, such as ⁹⁰ Yttrium, and stored in an aqueous solution can be substantially reduced by adding ascorbate ion to the solution. A method for preventing radiolytic degradation of compounds during the radiolabelling process is also provided.		

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IMPROVEMENTS IN OR RELATING TO MODIFIED HAPTENS
USEFUL AS IMAGING AND THERAPEUTIC AGENTS

BACKGROUND OF THE INVENTION

The present invention generally relates to in vivo imaging and treatment of disease using monoclonal antibodies. More particularly the invention relates to use of radioprotectant ions to decrease radiolytic degradation of haptens in aqueous solution.

Monoclonal antibodies are becoming increasingly important for in vivo use. Of substantial interest are their potential applications for the imaging and treatment of disease, particularly cancer. Monoclonal antibodies which are labeled, for example, with a chelate complex of a metal ion and a chelating agent are particularly useful for the imaging and treatment of certain disease states. Generally, such monoclonal antibodies are labeled by chemical conjugation with the chelating agent or, in the case of anti-hapten antibodies, by their ability to specifically bind with the metal chelate to form non-covalent hapten-antibody complexes.

Recent studies relating to the use of monoclonal antibodies for tumor imaging and therapy have shown that bifunctional antibodies have specificity for a hapten and, for example, a tumor target, to provide a mechanism for delivery of the hapten to the tumor target. Delivery of a hapten to a disease site with a bifunctional antibody system involves time dependent binding and release of the hapten from the antibody. Consequently, the usefulness of a particular hapten as an imaging or therapeutic agent is dependent upon the interaction of the hapten with the antibody utilized as well as with serum components, e.g. proteins, lipids, and other tissue compartments. For example, the inherent pharmacokinetic properties of a particular hapten and the affinity of an antibody for the

hapten may result in the hapten binding so tightly to the anti-hapten antibody as to be indistinguishable from covalently bound antibody conjugates, or in the hapten binding so loosely to the anti-hapten antibody as to be incapable of localization with the antibody at the disease site. Additionally, the inherent pharmacokinetic properties of a particular hapten may result in undesirable serum and tissue interactions such that the accumulation of the hapten in the liver, kidneys or intestines reaches intolerable levels. In such cases, the biodistribution and localization of the hapten substantially limit the potential clinical value of the bifunctional antibody delivery system.

Haptens which are useful for in vivo imaging and therapeutic agents can be derivatized to modify the inherent pharmacokinetic properties of the hapten, as is disclosed in copending parent U.S. Patent application 380,061 filed July 14, 1989. Some of the derivatized haptens have the characteristic shape of a dumbbell (DB). These DB-haptens can be radiolabeled and stored in an aqueous solution that serves as a quench buffer for the reaction of the hapten with the radioisotope. However, the radiolabeled DB-haptens tend to rapidly degrade in aqueous solution if the linker moiety joining the two halves of the dumbbell chelate contains a functional group readily destabilized by oxy-radicals formed during radiolysis of water molecules in the storage solution. The radiolytic effect is particularly marked and hard to control if the radioisotope is a strong beta emitter, such as ^{90}Y .

It is known to use radioprotectants in aqueous solutions to prevent degradation of radiolabeled compounds. For instance, dimethyl thiourea is used to protect compounds radiolabeled with low energy beta-emitters, such as tritium, and glycine is used to protect compounds radiolabeled with gamma emitters, such as ^{111}In . However, these radioprotectants do not satisfactorily protect certain haptens from radiolysis caused by strong beta-

emitters, such as ^{90}Y . For instance, the DB-chelate particles of this invention degrade in aqueous solution when labeled with ^{90}Y , especially those having a thiourea linker connecting the two halves of the dumbbell. Therefore, the need exists for a method of decreasing degradation of radioactive haptenic compounds stored in aqueous solution. Particularly, the need exists for a method of reducing the degradation rate in aqueous solution of DB-chelate haptens radiolabeled with a strong beta emitter, such as ^{90}Y .

SUMMARY OF THE INVENTION

The current invention is directed to a method for decreasing radiolytic degradation of compounds comprising radioactive substances in aqueous environments by adding an amount of a reducing antioxidant, such as ascorbate ion, as a radioprotectant in an amount sufficient to retard the radiolytic degradation of the compound in the aqueous environment. For example, sufficient radioprotectant can be added to substantially retard formation of oxy-radicals from water molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: represents the tumor uptake at 24 and 48 hours of the derivatized haptens shown in Table I in the pre-mix protocol.

Figure 2: represents the tumor to blood ratios at 24 and 48 hours of the derivatized haptens shown in Table I in a pre-mix protocol.

Figure 3: represents the biodistributions (percent dose/gram organ) at 4 hours for the derivatized haptens EOTUBE, TUBE NUBE and PHATUBE in a prelocalization protocol.

Figure 4: represents the biodistributions (percent dose/gram organ) at 24 hours for the derivatized haptens

EOTUBE, TUBE, NUBE, and PHATUBE in a prelocalization protocol.

Figure 5: represents the biodistributions (percent dose/gram organ) at 48 hours for the derivatized haptens EOTUBE, TUBE, NUBE and PHATUBE in a prelocalization protocol.

Figure 6: represents the tumor to organ ratios at 4 hours for the derivatized haptens EOTUBE, TUBE, NUBE and PHATUBE in a prelocalization protocol.

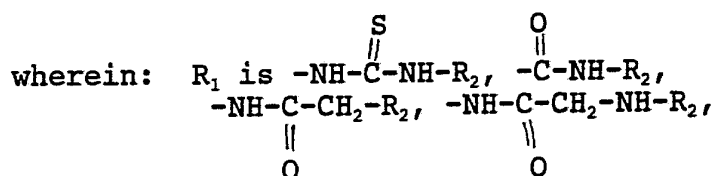
Figure 7: represents the tumor to organ ratios at 24 hours for the derivatized haptens EOTUBE, TUBE, NUBE and PHATUBE in a prelocalization protocol.

Figure 8: represents the tumor to organ ratios at 48 hours for the derivatized haptens EOTUBE, TUBE, NUBE and PHATUBE in a prelocalization protocol.

The invention has been summarized in order that the detailed description may be better understood and the contribution to the art may be better appreciated.

DETAILED DESCRIPTION OF THE INVENTION

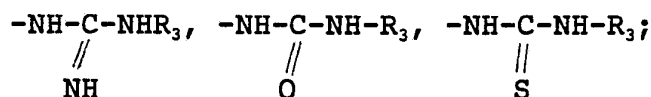
Haptens which can be derivatized, in accordance with the present invention, are generally selected from complexes of a chelating agent and a metal ion. Preferably, the hapten selected for use in the invention is a metal ion complex of ethylenediaminetetraacetic acid ("EDTA"), with the derivatized hapten being represented generally by Formula (I):



R_2 is hydrogen, an amino group, $-OC-(Y)$,

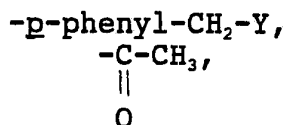
-p-phenyl-CH₂-Y, an unsubstituted C₁ to C₃₀ branched or straight chain alkyl group; a substituted C₁ to C₃₀ branched or straight chain alkyl, cycloalkyl, aryl or arylalkyl group, in which the substituents are one or more of any of:

hydroxy, carboxy, =O, =S, -(C=O)-, -(C=S)-, -S-, -SR₄, fluoro, chloro, bromo, iodo, amino, nitro, -SO₃H, -NHR₃, -NHR₄, -N(R₃)₂, -CONHR₃, -COOR₃, -SO₄, -PO₄, phenyl, benzyl, imidazolo, or a group of the formula:



wherein

R₃ is hydrogen, a C₁ to C₃₀ straight or branched chain alkyl group,



or a non-reactive functional group;

Y is EDTA-M₁⁺, DTPA-M₁⁺, DOTA-M₁⁺, HETA-M₁⁺, TRITA-M₁⁺, or TETA-M₁⁺;

R₄ is H or $\begin{array}{c} \text{-CH}_2\text{-C-NHR}_3 \\ \parallel \\ \text{O} \end{array}$

M⁺ and M₁⁺ are metal ions, provided that one such ion is radioactive and suitable for radiotherapy or radioimaging;

or a pharmaceutically acceptable salt thereof.

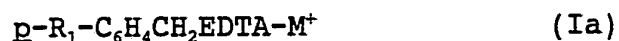
In the above Formula I, "DTPA" is diethylenetriaminepentaacetic acid, "DOTA" is 1,4,7,10-

tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid, "HETA" is 1,5,9,13-tetraazacyclohexadecane-N,N',N'',N'''-tetraacetic acid, "TRITA" is 1,4,7,10-tetraazacyclotridecane-N,N',N'',N'''-tetraacetic acid, and "TETA" is 1,4,8,11-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic acid.

Additionally, R₂ may further comprise non-reactive functional groups which may include substituents that exhibit specific tissue interactions, e.g., amino acids, polypeptides, nucleotides, polynucleotides, carbohydrates or lipids. As used for R₂ and R₃, the term "non-reactive functional group" refers to a functional group incapable of forming a covalent bond with tissue or serum components, and the anti-hapten antibody utilized, under physiological conditions.

In Formulae (I), the designation -OC—(—Y) is meant to relate to one skilled in the art that the moiety "Y", as defined in the formulae, is linked to the adjacent portion of the molecule through one of the carboxy groups of the Y moiety. On the other hand, the designation -Y, alone, is meant to indicate that the Y moiety is linked to the adjacent portion of the molecule through one of the methylene (-CH₂-) groups present on the Y moiety.

A preferred embodiment of the compounds of Formula I are the compounds of Formula (Ia):



wherein R₁ is $\begin{array}{c} \text{S} \\ \parallel \\ \text{-NH-C-NHR}_2 \end{array}$; $\begin{array}{c} \text{O} \\ \parallel \\ \text{-C-NH-R}_2 \end{array}$, or $\begin{array}{c} \text{O} \\ \parallel \\ \text{-NH-C-CH}_2\text{-R}_2 \end{array}$;

R₂ is hydrogen, an amino group, an unsubstituted C₁-C₃₀ branched or straight chain alkyl group, or a C₁-C₃₀ branched

or straight chain alkyl, cycloalkyl, aryl or arylalkyl group substituted by one or more substituents independently selected from the group consisting of :

-OH; -COOH; =O; =S; -S-; -Cl; -F; -Br; -I; -NH₂;

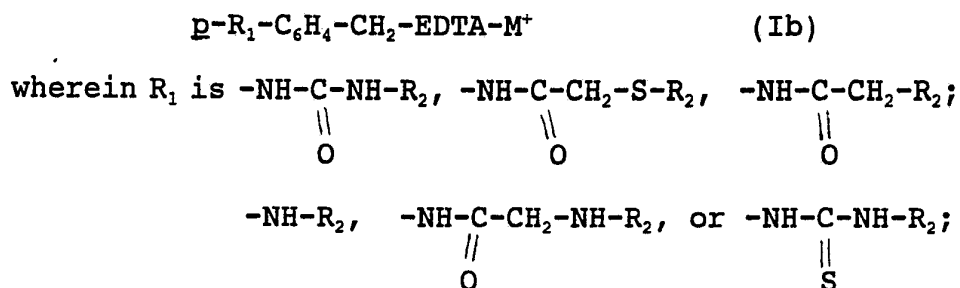
-NO₂; -SO₃H; -NH-R₃; -CONHR₃; -COOR₃; -SO₄; -PO₄;

phenyl; and benzyl;

R₃ is H, an alkyl group, or a non-reactive functional group; and M⁺ is a metal ion that is radioactive and suitable for radioimaging purposes; or a pharmaceutically-acceptable salt thereof. The compounds of Formula Ia are useful as radioimaging agents.

A further preferred group of compounds of Formula (Ia) occurs when R₁ is a group of the formula -NH-C(=S)-NHR₂.

Especially preferred compounds of the invention are those represented by Formula Ib:



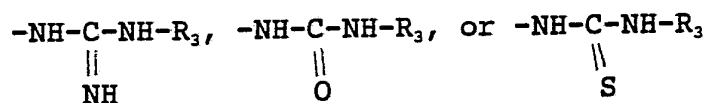
R₂ is -p-phenyl-CH₂-Y, -OC—(—Y),

a substituted C₁ to C₃₀ branched or straight chain alkyl, cycloalkyl, aryl or arylalkyl group, in which the substituents are one or more of any of:

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hydroxy, $-C=O$, $-C=S$, $-SR_4$, $-NHR_3$,
 $-NHR_4$, $-N(R_3)_2$, imidazolo, or

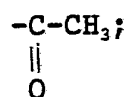
a group of the formula:



wherein

R_3 is $-p\text{-phenyl-CH}_2\text{-Y}$,

or



Y is $EDTA-M_1^+$, $DTPA-M_1^+$, $DOTA-M_1^+$, $HETA-M_1^+$, $TRITA-M_1^+$, or $TETA-M_1^+$;

R_4 is H , $CH_2-C-NHR_3$; M^+ and M_1^+ are metal ions, provided that one such ion is radioactive and suitable for radiotherapy or radioimaging; or a pharmaceutically-acceptable salt thereof. The compounds of Formula (Ib) are useful as radiotherapeutic and radioimaging agents.

One preferred group of compounds from the ones in Formula (Ib) are compounds wherein R_1 is a group of the formula $-NH-C(=S)-NH-R_2$, and especially so wherein R_2 is $p\text{-phenyl-CH}_2\text{-Y}$, a substituted C_1 to C_{30} branched or straight chain alkyl, cycloalkyl, aryl or arylalkyl group; wherein R_3 is $p\text{-phenyl-CH}_2\text{-Y}$ or acetyl; Y is $EDTA-M_1^+$, $DTPA-M_1^+$, $DOTA-M_1^+$, $HETA-M_1^+$, $TRITA-M_1^+$, or $TETA-M_1^+$; and wherein M^+ is a metal ion and M_1^+ is a radioactive metal ion suitable for use in radiotherapy or radioimaging.

Four other preferred groups of compounds within Formula (Ib) occurs when R_1 is a group of the formula $-NH-C(=O)-CH_2-S-R_2$, $-NH-C(=O)-CH_2-NH-R_2$, $-NH-C(=O)-NH-R_2$, or $p\text{-}$

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phenyl-CH₂-Y, respectively.

Additionally, pharmaceutical compositions are provided which comprise a compound of Formula (I), Formula (Ia), or Formula (Ib) associated with one or more pharmaceutically-acceptable vehicles therefor. The pharmaceutical compositions may further comprise, separate from or in combination with a hapten of the invention, an antibody capable of complexing with the hapten. The antibody used for the composition may be monoclonal, a synthetic blend of monoclonals, or polyclonal sera, or any fragments thereof. The antibodies may further be mono- or bispecific, as described below.

Further, the present invention provides methods for in vivo imaging and therapy which comprises administering to a warm blooded animal, especially a human being, sequentially, simultaneously, or as an antibody complex of the hapten, a predetermined effective dosage of a hapten of the invention, specifically those haptens of Formulas (I), (Ia) or (Ib), and a suitable anti-hapten antibody. The anti-hapten antibody may be monospecific for the hapten portion of the molecule or bispecific (or "bifunctional") with specificities for both the present hapten and the hapten of another substance or all.

As indicated above, the present invention, in one aspect, provides a method for modifying the pharmacokinetics of a hapten as an in vivo radioimaging or radiotherapeutic agent. The method comprises the selection of a hapten suitable for imaging or therapy and an antibody capable of complexing with the hapten, and the derivatization of the hapten to modify the affinity of the

haptent-antibody complex and the interaction of the haptent with serum and tissue components, thereby increasing or decreasing serum half-life, as desired. Another benefit of the present modified haptens of Formula I is that non-specific interactions, such as accumulation of radioactivity in non-target tissues, is reduced or eliminated. The derivatized haptent and the antibody are administered to a patient in a predetermined effective dosage for imaging or therapy. Preferably, the biodistribution and localization of the derivatized haptent, and complexes of the derivatized haptent and the antibody, are enhanced depending upon the particular diagnostic or therapeutic purpose.

In the context of the present invention, the term "haptent" refers to a molecule capable of specific reactivity with an antibody, but incapable of stimulating an immune response by antibody production, except in combination with or as part of a carrier protein molecule. As used herein, the term "pharmacokinetics" refers to the change in biodistribution of a molecule over a period of time. The terms "biodistribution" and "localization," as used herein, refer to the distribution of a molecule within individual organs of an animal at given time points, and the amount or concentration of a molecule present in an organ, respectively. Further, the term "localization" implies the presence, in excess of the concentration due to blood levels, of a molecule at a particular organ for a period of time as a result of the interaction between the molecule and tissue compartments.

For purposes of the present invention, the haptent

selected for use in the invention will generally depend upon the chemical, biological and physiological properties of the hapten and the desired application of the invention. For example, the selection of a particular hapten will depend upon whether the hapten will be used as an imaging or therapeutic agent, and generally involves consideration of chemical properties, such as water solubility, biological properties such as the rate of clearance from particular tissues, and physiological properties, such as the capability of binding to a receptor at a disease site.

The haptens generally selected for use in the invention are comprised of chelate complexes of a metal ion and a chelating agent. The chelating agent for the hapten portion of the claimed compounds is ethylenediaminetetraacetic acid ("EDTA"). EDTA, methods for preparing analogs and the starting materials for the compounds of the present invention are described in Meares et al., U.S. Patent No. 4,622,420 issued November 11, 1986, herein incorporated by reference.

Additionally the derivatized haptens may also include other chelating agents, such as diethylenetriaminepentaacetic acid ("DTPA"), DOTA, HETA, TRITA, TETA and analogs thereof. The methods for preparing DTPA and its analogs are discussed in the '420 patent. The methods for preparing DOTA, HETA, TRITA, TETA, and corresponding starting materials useful in the present invention are described in detail in Meares et al., U.S. Patent No. 4,678,667 issued July 7, 1987, herein incorporated by reference, and in Moi et al., J. Am. Chem. Soc., 110, 6266 (1988). The EDTA and DTPA analogs

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disclosed in U.S. Patent No. 4,622,420 are capable of forming physiologically stable chelates with a variety of metal ions. Likewise, the DOTA, HETA, TRITA, and TETA analogs described in U.S. Patent No. 4,678,667 and Moi, et al. are capable of forming physiologically stable chelates with various metal ions.

As used in Formulae (I), (Ia), and (Ib), "M⁺" and "M₁⁺", when referring to a metal ion means a metal atom in ionic form without particular reference to possible valences. The particular valence of the ion, as one skilled in the art will recognize, depends on the particular metal in question. Thus, the terms "M⁺" or "M₁⁺" are not meant to limit the metal ion to any particular state of valency. The preferred metal ions, as indicated below, however, are Indium (III) and ¹¹¹Indium(III) for M⁺, and ¹¹¹Indium(III) and ⁹⁰Yttrium (III) for M₁⁺.

Preferred for use as substituents on the derivatized haptens as shown in Formula I, (Ia), or (Ib) in the present invention are chelating agents that chelate with the radioactive metal ions of ^{195m}Pt, ⁵⁷Ni, ⁵⁷Co, ¹⁰⁵Ag, ⁶⁷Mn, ⁵²Fe, ¹¹¹In, ^{113m}In, ^{99m}Tc, ⁶⁸Ga, ⁶⁷Ga, ¹⁶⁹Yb, ⁵⁷Co, ¹⁶⁷Tm, ¹⁶⁶Tm, ¹⁴⁶Gd, ¹⁵⁷Dy, ^{95m}Nb, ¹⁰³Ru, ⁹⁷Ru, ⁹⁹Rh, ^{101m}Rh and ²⁰¹Tl, i.e., where M⁺ or M₁⁺ is any of the foregoing radioimaging or radiotherapeutic metallic ions. The foregoing radioactive metal ions are a preferred group of radioactive metal ions for M⁺ or M₁⁺, when such metal ions are required to be radioactive. It is preferred that the metal chelates complex with indium-111 ("¹¹¹In"), technetium-99m ("^{99m}Tc"), copper-67 ("⁶⁷Cu") gallium-67 ("⁶⁷Ga") and yttrium-90 ("⁹⁰Y"), and such metal ions are a more preferred group for M⁺ and M₁⁺ when such ions

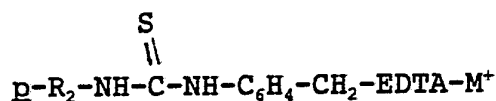
are required to be radioactive. The metal ion $^{111}\text{Indium}$ (III) is generally preferred for diagnostic application, i.e. imaging, while $^{90}\text{Yttrium}$ (III) is generally preferred for therapeutic application.

The compounds of the invention having designations DB-I to DB-X and DDB-I are particularly preferred compounds of those set forth in Formula (Ib). These particular compounds possess a unique "dumbbell" shape (thus, the designation "DB") due to the presence of the (chelating) hapten and a second, chelating portion on the derivatized hapten. For example, a DB compound could be complexed at the hapten (EDTA) end with cold (non-radioactive) indium (III) ion, in order to supply the appropriate hapten for the anti-benzyl EDTA-Indium antibodies used in the present invention, then subsequently the second chelating moiety (e.g., DTPA, DOTA) on the derivatized hapten could be loaded with a radioimaging (e.g., ^{111}In (III)) metallic nuclide or a radiotherapeutic (e.g., ^{90}Y (III)) metallic nuclide. Also, the same DB hapten could be used as a radioimaging agent to a target tumor(s) then as a radiotherapeutic dose to the imaged tumor(s). For instance, a DB hapten which at its metal chelate moiety was complexed with ^{111}In could in turn be complexed with an anti-(indium benzyl EDTA) antibody and administered to the patient. After imaging, the same antibody-DB hapten complex, wherein the DB hapten was chelated to ^{90}Y rather than ^{111}In , could be administered to the patient for radiotherapy of the imaged tumor. Alternatively, the ^{90}Y -chelated hapten could be administered without being complexed with antibody after a dose of $^{111}\text{Indium}$ -labelled

h¹⁴apten that is complexed with an antibody has been administered.

The preferred haptens of the invention comprise benzyl EDTA derivatives. Such haptens contain structural moieties that can conveniently complex a variety of metal ions, including ¹¹¹In (III), for the desired diagnostic or therapeutic application. Benzyl EDTA, analogs thereof, and methods for their preparation are described in U.S. 4,622,420.

In one embodiment, preferred compounds of the invention are p-thioureiodobenzyl EDTA derivatives or analogs thereof, which are derived from isothiocyanatobenzyl EDTA. Isothiocyanatobenzyl EDTA and isothiocyanatobenzyl DTPA, analogs thereof, and methods for their preparation are described in Meares et al., U.S. Patent No. 4,622,420, issued November 11, 1986, herein incorporated by reference. Such preferred haptens of the invention, have a structure represented by the following formula:



wherein R₂ is hydrogen; NH₂; an unsubstituted C₁-C₁₈ branched or straight chain alkyl group; or a C₁-C₁₈ branched or straight chain alkyl, cycloalkyl, aryl or arylalkyl group, substituted by one or more substituents independently selected from the group consisting of:

-OH; -NH₂; -COOH; -CONH₂; phenyl; benzyl;
-SO₃H; and -NO₂; and

M⁺ is a metal ion.

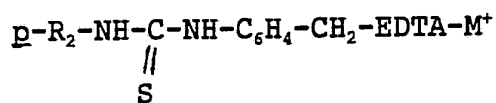
In addition, R₂ may further comprise non-reactive

functional groups including substituents that may exhibit specific tissue interactions, e.g., amino acids, polypeptides, nucleotides, polynucleotides, carbohydrates, or lipids.

The structures of a series of novel derivatized haptens which are preferred for use in the invention are shown in Table I. The abbreviations set forth in Table I will be used later to refer to these novel haptens.

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TABLE I

DERIVATIZED HAPTENS OF THE INVENTION



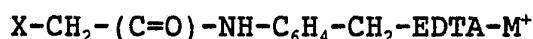
<u>R₂</u>	<u>HAPTEN ABBREVIATION</u>
-H	TUBE
-CH ₂ CH ₃	ETUBE
-(CH ₂) ₃ CH ₃	BUTUBE
-(CH ₂) ₇ CH ₃	OTUBE
-CH ₂ CH ₂ OH	EOTUBE
-CH ₂ CH ₂ NH ₂	NUBE
-(CH ₂) ₂ CO ₂ H	PATUBE
-(CH ₂) ₃ CO ₂ H	BATUBE
-(CH ₂) ₄ CH(NHC(=O)CH ₃)CONH ₂	BALTUBE
-(CH ₂) ₂ NHC(=O)(CH ₂) ₃ CO ₂ H	GNUBE
-C ₆ H ₅	PHTUBE
-CH ₂ C ₆ H ₅	BETUBE
-R-(C ₆ H ₄)CH ₂ CO ₂ H	PHATUBE
p-C ₆ H ₄ -CH ₂ -DTPAM ₁ ⁺	DB-III
-CH ₂ CH ₂ -NHC(=S)NH-C ₆ H ₄ -CH ₂ -DTPA-M ₁ ⁺	DB-IV
-(CH ₂) ₄ -NHC(=S)NH-C ₆ H ₄ -CH ₂ -DTPA-M ₁ ⁺	DB-V
-(CH ₂) ₂ -N-((CH ₂) ₂ -NH ₂)-(CH ₂) ₂ -NH-C(=S)	
-NH-p-(C ₆ H ₄ -CH ₂ DTPA)-M ₁ ⁺	DB-IX
-p-C ₆ H ₄ -CH ₂ -DOTA-M ₁ ⁺	DDB-I

(In the above compounds of Table I the benzyl EDTA moiety has a para-substituted phenyl ring. Furthermore, it is preferred that all compounds other than the DB and DDB compounds have M⁺ as ¹¹¹In (III). It is preferred that the

DB and DDB compounds have M^+ as indium (III) and M_1^+ as $^{111}\text{Indium}$ (III) or $^{90}\text{Yttrium}$ (III)).

Additionally preferred compounds of the invention are those provided in Table II:

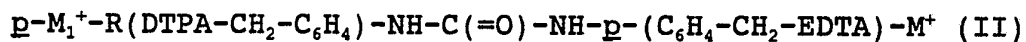
Table II



<u>X</u>	<u>ABBREVIATION</u>
$-\text{S}(\text{CH}_2)_4\text{SCH}_2\text{C}(=\text{O})\text{NHC}_6\text{H}_4\text{CH}_2-\text{DTPA}-M_1^+$	DB-I
$-\text{SCH}_2\text{CH}(\text{OH})\text{CH}(\text{OH})\text{CH}_2\text{SCH}_2\text{C}(=\text{O})\text{NHC}_6\text{H}_4\text{CH}_2-\text{DTPA}-M_1^+$	DB-II
$-\text{NH}-\text{C}_6\text{H}_4-\text{CH}_2-\text{DPTA}-M_1^+$	DB-VI
$-\text{NHCH}_2\text{CH}(\text{OH})\text{CH}_2\text{NHCH}_2\text{C}(=\text{O})\text{NHC}_6\text{H}_4-\text{CH}_2-\text{DTPA}-M_1^+$	77DB-VIII

(In the above Table II, the phenyl rings of the $-\text{NH}-\text{C}_6\text{H}_4-\text{DTPA}$ portions of the structures are substituted in the para position. Further, it is preferred that in the compounds of Table II, M^+ is indium (III) and M_1^+ is $^{90}\text{Yttrium}$ (III) or $^{111}\text{Indium}$ (III).)

Another preferred compound is DB X, of the Formula II:



It is further preferred for Formula II that M^+ is indium (III) and that M_1^+ is $^{90}\text{Yttrium}$ (III) or $^{111}\text{Indium}$ (III).

Another preferred group of compounds of the present

invention are the intermediate compounds. Specifically, these are compounds of Formulas I, (Ia), (Ib), and compounds designated as DB's I through X, DDB-I, TUBE, ETUBE, BUTUBE, OTUBE, EOTUBE, NUBE, PATUBE, BATUBE, BALTUBE, GNUBE, PHTUBE, BETUBE, and PHATUBE, wherein, as applicable, the metal ions M^+ or M_1^+ , or both ions are absent, that is, when one or (if applicable) both chelating agents on the haptens are unchelated.

Those skilled in the art will recognize that because the pharmacokinetic criteria for imaging and therapeutic agents may differ, the series of novel derivatized haptens provided by the invention permits the selection of haptens having desirable pharmacokinetics for a particular application of the invention. For example, a particular derivatized hapten may be desirable as a therapeutic agent because of its ability to accumulate at a disease site but may be undesirable as an imaging agent because of its inability to clear rapidly from circulation. Additionally, those skilled in the art will appreciate that the present invention is not limited to the specific derivatized haptens set forth in Tables I or II. The present invention contemplates, therefore, modifications to the structures of the novel derivatized haptens specifically disclosed, provided such structural modifications are intended to modify the inherent pharmacokinetic properties of these haptens for purposes of imaging or therapy.

In accordance with the invention, the derivatization of a hapten to modify the affinity of the hapten-antibody complex, and the interaction of the hapten with serum and tissue components, can be accomplished by standard

procedures well-known in the art. For example, hapten structure can be modified by derivatization of the aromatic amine in (S)-4-aminobenzyl EDTA by acylation, alkylation, Schiff's base formation (which is followed by reduction to the amine), conversion to the reactive isocyanate and subsequent conversion to a substituted urea conversion to a sulfonamide or diazotization and subsequent reaction with a nucleophile. These reactions can be accomplished with compounds containing simple aliphatic chains, or branched chains, or aromatic or heterocyclic compounds which may or may not contain functional groups. (See, e.g., March, J. Advanced Organic Chemistry, John Wiley & Sons, New York, (1985)). In carrying out the reaction, interfering functional groups may have to be blocked by standard methods which subsequently can be removed by well known methods. Additionally, in accordance with the invention, the structure of a hapten is preferably modified to enhance the biodistribution and localization of the hapten for a particular application of the invention. Further, it will be understood that haptens derivatized in accordance with the present invention retain the ability to bind the anti-hapten antibody selected for use.

The preferred haptens of the invention represented by Formulae (I), (Ia), and (Ib) are prepared using standard synthetic methods familiar to one skilled in the art. For example, the preferred thiourea-type compounds of the invention are prepared by reacting a precursor isothiocyanate compound with an amine. The most useful isothiocyanates for purposes of the present invention are isothiocyanatobenzyl EDTA ("ITCBE") and

isothiocyanatobenzyl DTPA ("ITCBD"), both of which are prepared according to the teachings of Meares et al., U.S. Patent No 4,622,420, issued November 11, 1986, herein incorporated by reference. Thus, one skilled in the art determines which compound of the invention to prepare and then reacts one, or possibly both, of ITCBE or ITCBD with the necessary amine compound(s) to obtain the desired structure. Likewise, to prepare the corresponding macrocyclic polyamine derivatives of the invention, the corresponding isothiocyanate derivatives of DOTA, HETA, TRITA, and TETA are described in U.S. Pat. No. 4,678,667, the teachings of which are herein incorporated by reference. The preparation of HETA, DOTA, TRITA and TETA also are disclosed in U.S. Pat. No. 4,678,667. An improved synthesis of DOTA is described in Moi et al., J. Am. Chem. Soc., 110, 6266 (1988). The corresponding isothiocyanate compounds useful as starting materials in the present invention are prepared in a manner completely analogous to the preparation of ITCBD and ITCBE.

To prepare the amide-type compounds of the invention, two starting materials are particularly useful: p-bromoacetamideobenzyl EDTA ("BABE"), and p-bromoacetamidobenzyl DTPA ("BABD"). These compounds are prepared according to the teachings of DeRiemer et al., J. Lab. Comp. and Radiopharm., 18, 1517 (1981) and U.S. Patent No. 4,622,420. The corresponding bromoacetamido-derivatives for HETA, DOTA, TRITA, and TETA are prepared in a manner completely analogous to the preparation of BABE and BABD. The preparation of these starting materials is described in U.S. Pat. No. 4,678,667, herein incorporated by reference.

The alpha-bromo moiety of these compounds renders the adjacent methylene moiety particularly susceptible to nucleophilic attack from groups such as free sulfhydryl or amino groups. Thus, to prepare the desired compound of the invention, one needs only to determine the appropriate intermediate R₂ moiety of Formulae (I), (Ia), or (Ib) and react it with the required starting material(s).

Typically, the desired compounds of the invention are prepared in aqueous buffer solutions at pH levels from about 6 to about 12. Preferred pH ranges are from about 8 to about 11. The progress of the reaction can be monitored easily using HPLC. The temperature is not crucial but preferably the reaction is run at temperatures from about 0° C. to about 60°C, preferably from about 20°C. to about 40°C.

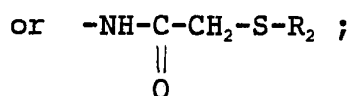
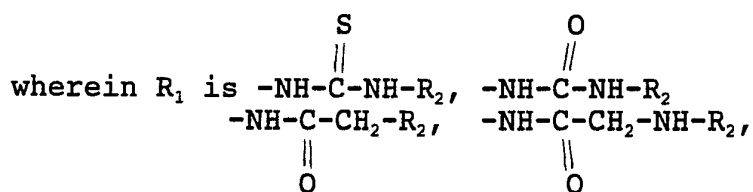
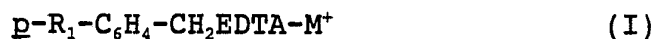
The product obtained from the reaction can be purified using standard chromatography techniques. The preferred separation method involves anion-exchange chromatography using, for example, a DEAE Sephadex A-25 resin (Pharmacia Fine Chemicals), an AG-1X8, formate column (BioRad Laboratories, Richmond California) or in the case of certain DB compounds, a C18 reversed phase column (LiChroprep® RP-18, EM Science Corp., Cherry Hill, N.J.). Of course, those skilled in the art will recognize other equally useful means for purification of the final products.

In addition, it will be recognized that many compounds of the present invention contain basic and acidic groups which are capable of forming pharmaceutically-acceptable acid or base addition salts. For example, those compounds

which contain a free carboxyl group are able to react with alkaline earth metal bases, for example sodium bicarbonate, sodium hydroxide, potassium carbonate, potassium bicarbonate, potassium hydroxide, calcium hydroxide, etc., to form pharmaceutically-acceptable salts which also are useful in the invention. Likewise, free amino groups present in the molecule are capable of reacting with acidic reagents to form the corresponding acid addition salts. For example, an amino-containing compound of the invention could be reacted with acids such as hydrochloric, sulfuric, tartaric, lactic, nitric, etc., to prepare the corresponding salt. "Pharmaceutically-acceptable" in this context refers to those salt forms which are useful in the treatment or diagnosis of a warm-blooded animal. Consequently, pharmaceutically-acceptable salt forms of appropriate compounds are also encompassed by the invention.

Although the methods described here and further elaborated in the accompanying non-limiting examples are the presently preferred method for preparing the compounds of the invention, one skilled in the art will recognize other means which may work equally as well. Thus, the methods provided for preparing compounds of Formulae (I), (Ia), or (Ib) are not to be construed as limiting on the means for preparing the desired compound. Other equally-acceptable means for preparing the compounds may be available and are meant to be encompassed by the present invention.

Thus, in another aspect of the invention there is provided a process for preparing a compound of Formula (I):



R_2 is hydrogen, an amino group,

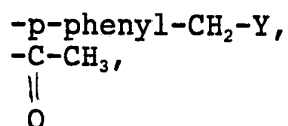
-p-phenyl-CH₂-Y, an unsubstituted C₁ to C₃₀ branched or straight chain alkyl group; a substituted C₁ to C₃₀ branched or straight chain alkyl, cycloalkyl, aryl or arylalkyl group, in which the substituents are one or more of any of:

hydroxy, carboxyl, =O, =S, -C=O,
 -C=S, -S-, -SR₄, fluoro, chloro,
 bromo, iodo, amino, nitro, -SO₃H,
 -NHR₃, -NHR₄, -N(R₃)₂, -CONHR₃,
 -COOR₃, -SO₄, -PO₄, phenyl, benzyl,
 imidazolo, or a group of the formula:

$$\begin{array}{ccc} -NH-C-NH-R_3 & -NH-C-NH-R_3 & -NH-C-NH-R_3; \\ || & || & || \\ NH & O & S \end{array}$$

wherein

R_3 is hydrogen, a C₁ to C₃₀ straight or branched chain alkyl group,



or a non-reactive functional group;

Y is EDTA-M₁⁺, DTPA-M₁⁺, DOTA-M₁⁺, HETA-M₁⁺, TRITA-

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M_1^+ , or TETA- M_1^+ ;

R_4 is H, $-\text{CH}_2-\text{C}-\text{NHR}_3$; M^+ is a metal ion, and

$$\begin{array}{c} \parallel \\ \text{O} \end{array}$$

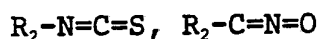
M_1^+ is a metallic radionuclide ion suitable for radiotherapy or radioimaging purposes, which comprises,

(A) in the case in which R_1 is $-\text{NH}(\text{C}=\text{S})\text{NH}-\text{R}_2$,

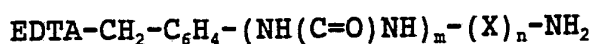
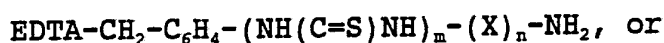
(1) reacting isothiocyanatobenzyl EDTA, or

a metal chelate thereof, with a compound of the formula $\text{H}_2\text{N}-\text{R}_2$, in which R_2 is as described above; or,

(2) reacting a compound of the formula



as appropriate, or a metal chelate thereof, with a compound of the formula:



or a metal chelate thereof, in which X is C_1 to C_{30} branched or straight chain alkyl, aryl or arylalkyl group optionally substituted with one or more of the substituents allowed in the definition of R_2 above, and n and m, independently, are 0 and 1;

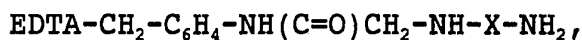
(B) if R_1 is $-\text{NH}(\text{C}=\text{O})\text{CH}_2-\text{S}-\text{R}_2$,

reacting p-bromoacetamidobenzyl-EDTA ("BABE"), or a metal chelate thereof, with a compound of the formula $\text{HS}-\text{R}_2$, in which R_2 , is as defined above;

(C) if R_1 is $-\text{NH}(\text{C}=\text{O})\text{CH}_2-\text{NH}-\text{R}_2$, reacting p-bromoacetamidobenzyl EDTA ("BABE") with a compound of the formula $\text{H}_2\text{N}-\text{R}_2$, in which R_2 is as defined above; or, if R_1 is $-\text{NH}(\text{C}=\text{O})\text{CH}_2-\text{NH}-\text{R}_2$ in which a substituent of R_2 is NHR_4 , reacting p-bromoacetamidobenzyl-Y, in

which Y is EDTA, DTPA, DOTA, HETA, TRITA or TETA;

or a metal chelate thereof, with a compound of the formula:



or metal chelate thereof, in which X is a C₁ to C₃₀ branched or straight chain alkyl, aryl, or arylalkyl group optionally substituted with one or more of the substituents allowed in the definition of R₂, above; and,

(D) if desired, salifying or preparing the metal chelate of the product obtained.

For purposes of the present invention, the antibodies selected for use are anti-hapten antibodies capable of specifically binding and complexing with the hapten selected for use. It will be appreciated by those skilled in the art that the same antibody can be utilized to complex with a hapten of the invention which may contain a broad range of functional groups.

Preferred for use in the invention are monoclonal antibodies, particularly monoclonal antibodies exhibiting the ability to bind preferentially to chelate complexes, said complexes comprising a specific metal ion and a chelating agent, e.g., complexes of ¹¹¹Indium and benzyl EDTA derivatives of the invention. Such monoclonal antibodies and methods for the preparation are described in Meares et al., U.S. Patent No. 4,722,892 issued February 2, 1988, the disclosure of which is herein incorporated by reference. However, those skilled in the art will appreciate that monoclonal or polyclonal antibodies may be utilized in the invention provided the antibodies selected exhibit the

requisite specificity for the hapten selected for use. Methods for the preparation of monoclonal and polyclonal antibodies are now quite well known in the art. See, e.g., Kohler, G. and Milstein, C., Nature 256, 495 (1975).

Particularly preferred for use in the invention are bifunctional monoclonal antibodies having a dual specificity, with one or more binding sites for a hapten of the invention and one or more binding sites for a disease site, e.g. a tumor target. Because such bifunctional antibodies have specificity for the hapten as well as for a disease site, they are capable of functioning both as carriers for the hapten and as disease site-associated receptors. Such bifunctional antibodies, therefore, provide an effective mechanism for the site-directed delivery and localization of the derivatized hapten to the disease site. The disease sites which such bifunctional antibodies recognize may be, for example, tumor-associated antigens such as alpha-fetoprotein ("AFP"), carcinoembryonic antigen ("CEA"), human choriongonadotropin ("HCG"), prostatic acid phosphatase ("PSA"), ferritin, bombesin, melanoma-associated antigens p97 and gp240, or milk fat globulins. Alternatively, the disease-sites for which such bifunctional antibodies are specific may be, for example, sites of infection or thrombosis. Bifunctional antibodies and methods for their preparation are described in European Patent Publication 105360, the disclosure of which is incorporated by reference. The preparation of bifunctional antibodies may be accomplished biologically, for example, by cell fusion techniques to produce polydomas, or chemically, by synthetic techniques, all of

which now are well-known in the art. See, for example, U.S. Pat. Nos. 4,470,925 and U.S. 4,444,878.

In certain applications of the invention, human monoclonal antibodies, particularly human bifunctional antibodies, having the requisite hapten-binding specificity are preferred. Human monoclonal antibodies can be prepared by techniques well known in the art and are produced by hybridomas which are, for example, the fusion product of a human B-lymphocyte with a human or mouse myeloma cell line. Human bifunctional antibodies can also be prepared by conventional techniques known in the art. See, Human Hybridomas and Monoclonal Antibodies, edited by E.G. Engleman, S.K. Fount, J. Larrick and A. Raubitschek, Plenum Press, New York (1985).

Also preferred for use in certain applications of the invention are chimeric monoclonal antibodies capable of complexing with the hapten selected for use. Chimeric antibodies are generally comprised of a variable region, i.e. binding region, derived from one mammalian species, e.g., mouse, and a constant region derived from a second and different mammalian species, e.g. human. Murine/human chimeric antibodies will be beneficial in certain applications of the invention because such antibodies are expected to retain the specificity and affinity of murine antibodies while being substantially less immunogenic. Such murine/human chimeric antibodies, which may be the product of fused immunoglobulin genes, can be prepared by recombinant DNA and gene transfection techniques well-known in the art. See, for example, European Patent Publication 173494, Patent Cooperation Treaty Publication WO 86/01533,

Cabilly et al., U.S. Patent No. 4,816,567, issued March 28, 1989, and Boss et al., U.S. Patent No. 4,816,397, issued March 28, 1989, the latter two of which are herein incorporated by reference. Chimeric monoclonal antibodies which are bifunctional are particularly preferred for use in certain applications of the invention. The chimeric bifunctional antibodies useful in the invention may be the product of fused immunoglobulin genes encoding for variable region specificity both for the hapten selected for use and a disease site, e.g., tumor target. Such antibodies may be able to be prepared by conventional recombinant DNA and gene transfection techniques. See, for example, European Patent Publication 012507. In addition, synthetic chimeric, bifunctional, or chimeric-bifunctional antibodies can be prepared from antibody half-molecules, or fragments thereof, cross-linked with a bifunctional cross-linking agent such as bis-(maleimido)-methyl ether ("BMME"), or other such cross-linking agents familiar to those skilled in the art.

Also, encompassed by the term "chimeric antibody", whether in reference to chimeric antibodies per se, cross-linked chimeric antibodies, or chimeric-bifunctional antibodies, is the concept of "humanized antibody", that is those antibodies in which the framework or complementarity determining regions ("CDR") have been modified to comprise the CDR of an immunoglobulin of different specificity as compared to that of the parent immunoglobulin. In a preferred embodiment, a murine CDR is grafted into the framework region of a human antibody to prepare the "humanized antibody". Particularly preferred CDR's

correspond to those representing sequences recognizing the antigens noted above for the chimeric and bifunctional antibodies. The reader is referred to the teaching of European Patent Publication EP A 0 239 400 (Sept. 30, 1987), incorporated herein by reference, for its teaching of CDR modified antibodies.

In addition, the invention encompasses within its scope immunoglobulins or immunoglobulin fragments to which are fused active proteins, for example, an enzyme of the type disclosed in Neuberger, et al., WO 86/01533, published March 13, 1986. The disclosure of such products is incorporated herein by reference.

Additionally, those skilled in the art will recognize that the present invention encompasses in any of the previously described antibody formats the use of antibody fragments including Fab, Fab' and F(ab)'₂ fragments, or any other antibody fragment retaining the essential binding function of the antibody selected for use. However, it will be recognized that in those utilities of the invention requiring bifunctional antibodies, Fab or Fab' fragments may only be suitably utilized if the haptens of the invention have some specificity of their own. Antibody fragments can be prepared by proteolytic enzyme digestion, for example, by pepsin or papain digestion, reductive alkylation, or recombinant techniques, all of which are well-known in the art. See, Nisonoff, A., Molecular Immunology, Sinauer Associates, Massachusetts (1982). Those skilled in the art will also appreciate that in certain applications of the invention it may be beneficial to use a mixture of two or more antibodies or fragments

thereof, or mixtures of ³⁰antibody fragments with whole antibody.

Further, it will be understood by those skilled in the art that for certain applications of the invention it may be beneficial to select a high affinity antibody, while in other applications of the invention the selection of a low affinity antibody will be advantageous. Accordingly, the present invention includes the selection of antibodies which may be characterized as having high or low affinity for the hapten selected for use, and in the case of bifunctional antibodies, high or low affinity for a disease site.

In accordance with the invention, the derivatized hapten and anti-hapten antibody can be administered to the patient sequentially, simultaneously, or in combination as a complex for purposes of in vivo imaging or therapy.

In one method of the invention, in which the anti-hapten antibody selected for use is a bifunctional antibody, the hapten of the invention and the antibody are administered separately. Preferably, the antibody is administered and permitted to distribute itself throughout the patient prior to administration of the derivatized hapten. After sufficient time has elapsed to permit localization of the bifunctional antibody at the disease site, the derivatized hapten is administered to the patient. The derivative hapten can be administered in multiple doses over a period of time or by slow infusion. The derivatized hapten antibody complex is thereafter formed in vivo and the diagnostic or therapeutic effect can be observed. Such methods are desirable for application of

the invention in which the bifunctional antibody selected for use requires a relatively long period of time for localization at disease sites, or the derivatized hapten has a relatively short serum half-life or a relatively short isotope half-life.

In an additional method of the invention, a first bifunctional anti-hapten antibody is selected for use and administered to the patient followed, after a period of time, by administration of a mixture of the derivatized hapten and a second carrier anti-hapten antibody. The derivatized hapten and second carrier antibody are administered to the patient after sufficient time has elapsed to permit localization of the first bifunctional antibody at the disease site. The second carrier antibody permits, in effect, transfer of the derivatized hapten to the prelocalized bifunctional antibody at the disease site. The administration of the derivatized hapten with a second carrier antibody may be required to certain applications of the invention in which the concentration of circulating bifunctional antibody is insufficient to bind the derivatized hapten for delivery to the disease site prior to clearance of the hapten from circulation.

Alternatively, the derivatized hapten and the antibody can be administered to the patient as a mixture. Accordingly, the derivatized hapten and the antibody can be combined in vitro to permit the formation of derivatized hapten-antibody complexes prior to administration to the patient. After administration of the complexes to the patient, the desired diagnostic or therapeutic effect can be observed.

Administration of the derivatized hapten and the antibody, whether administered separately or as a mixture, can be accomplished by intravenous, intraperitoneal, intralymphatic, subcutaneous or intra-muscular injection.

As indicated above, the novel derivatized haptens provided by the invention are useful in compositions for in vivo imaging and therapy. Thus, in an additional embodiment of the invention, compositions are provided for in vivo imaging and therapy comprising a hapten wherein the hapten is derivatized to modify the dissociation rate of a hapten-antibody complex and the interaction of the hapten with serum components and tissue compartment, thereby increasing or decreasing serum half-life and radiation doses to tissues. Accordingly, the pharmacokinetics of the compositions of the invention are modified for purposes of imaging and therapy. Preferably, the biodistribution and localization of the compositions of the invention are enhanced as compared to compositions comprising unmodified haptens. More specifically, the enhanced biodistribution and localization of the compositions of the invention result in an increased and more rapid accumulation of the derivatized hapten at the disease site, and improved clearance of the derivatized hapten from normal tissues.

Preferred for use in the compositions of the present invention are derivatized haptens having structures as previously described. Especially preferred are the series of novel hapten derivatives set forth in Tables I and II and Formula II above. As previously indicated, the series of novel hapten derivatives provided by the invention permits the selection of haptens most desirable for

particular applications of the invention.

The compositions of the present invention may further comprise an antibody capable of complexing with the hapten, preferably a monoclonal antibody. Particularly preferred for use in the compositions of the invention are bifunctional antibodies having hapten binding specificity as well as specificity for a disease site. Additionally, compositions comprising human antibodies or chimeric antibodies are particularly preferred in certain applications of the invention. Further, those skilled in the art will appreciate that the compositions of the invention may be comprised of antibody fragments or mixtures of antibodies or fragments thereof, as described earlier.

In further embodiments of the invention, methods are provided for in vivo imaging and therapy which comprise administering to a patient a predetermined effective dosage of a hapten, derivatized in accordance with the invention, and an antibody, or antibody fragment, capable of complexing with the hapten. The derivatized hapten and anti-hapten antibody, which may be administered sequentially, simultaneously, or as a complex, are prepared preferably for use in pharmaceutical compositions for injection.

The dosage for imaging for the present invention varies with each patient. The dosage must be as small as possible but yet still give significant signal above the background noise. For ^{111}In , a typical dosage for imaging is approximately 5 millicuries. The specific activity for the $^{111}\text{Indium}$ (III) labelled-compounds of the present

invention is approximately 5 millicuries/100 picomoles. Thus, the amount of the present compounds used depends on the complexing abilities of each compound. For instance, approximately 25 nanomoles of EOTUBE and approximately 4 nanomoles of DB III are required to give a dose of 5 millicuries. The dosage for therapeutic purposes is generally anywhere between approximately 5 to 40 millicuries of radionuclide. As the specific activity for ⁹⁰Yttrium (III) is also 5 millicuries/100 picomoles of the compounds of the present invention, typically 4 nanomoles of DB-III is required to achieve the desired dose.

Thus, the invention also provides pharmaceutical compositions, comprising a compound of Formula (I), (Ia), or (Ib), either with or without the appropriate quantity of antibodies discussed above, and one or more pharmaceutically-acceptable vehicles therefor. As those skilled in the art will recognize, "pharmaceutically-acceptable" refers to those vehicles such as carriers, diluents, or excipients recognized as useful in the diagnosis or therapy of a warm-blooded animal. Specifically, the term includes aqueous solution such as bicarbonate buffers, phosphate buffers, Ringer's solution and physiological saline, supplemented with 5% dextrose or human serum albumin, if desired. A preferred pharmaceutical vehicle is a glycinate buffer at physiological pH and isotonic with human serum.

Specific pharmaceutical compositions, either with or without the appropriate quantity of antibodies discussed above, and especially so regarding the appropriate quantity of antibodies CHA255 or ECH037.2, include for instance:

a) a compound of Formula (Ia) wherein R_1 is a group of the formula

$-\text{NH}-\text{C}(=\text{S})-\text{NH}-\text{CH}_2-\text{CH}_2-\text{OH}$; and especially so when M^+ is $^{111}\text{Indium (III)}$;

b) a compound of formula (Ib) wherein R_1 is a group of the formula:

$M_1^+ -p-(\text{DTPA}-\text{CH}_2-\text{C}_6\text{H}_4)-\text{NH}-\text{C}(=\text{S})-\text{NH}-$; and especially so when M^+ is indium (III) and M_1^+ is $^{90}\text{Yttrium (III)}$ or $^{111}\text{Indium (III)}$; and

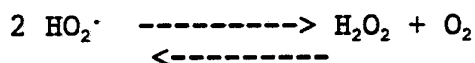
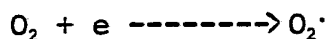
c) a compound of Formula (Ib) wherein R_1 is a group of the formula:

$-\text{NH}-\text{C}(=\text{S})-\text{NH}-p-(\text{C}_6\text{H}_4-\text{CH}_2-\text{DOTA})-M_1^+$; and especially so when M^+ is indium(III) and M_1^+ is $^{111}\text{Indium(III)}$ or $^{90}\text{Yttrium (III)}$.

Such pharmaceutical compositions are preferably in unit dosage form, each dosage containing, for example, from about 10 picomoles to about 10 millimoles of the desired hapten. Preferred formulations contain from about 100 picomoles to about 1 micromole of the derivatized hapten. The novel derivatized haptens and anti-hapten antibodies are effective over a wide dosage range depending upon factors such as the disease state involved, the antigen density at the disease site, the hapten-antibody affinity, the extent of non-specific binding interactions, the manner of administration and the condition of the patient.

To use dumbbell chelate compounds for purposes of radiotherapy, it is usually preferred to label the compounds with a strong beta emitter, such as $^{90}\text{Yttrium}$.

¹¹¹In, a gamma emitter giving off a particle having an energy level of about 172 to 247 keV is sufficient to generate oxy-radicals in aqueous solution, but ⁹⁰Y, a high energy beta-emitter that gives off a beta particle having a maximum energy of about 2.27 MeV, generates a greater number of oxy-radicals per particle emitted. The oxy-radicals interfere with efforts to attach the radionuclide to the hapten and degrade the haptens themselves both before and after the radionuclide has attached to them. While not wishing to be bound by theory, Applicants believe that the species active for promoting degradation of compounds generally formed in aqueous solution by the radiolytic effect of the radionuclide upon water are superoxide anion, hydroperoxy radical, and hydrogen peroxide, as is illustrated by the following reaction formulae:



Consequently compounds, such as the DB-chelates of this invention, that are labelled with ⁹⁰Y and stored in aqueous solutions are particularly susceptible to degradation over time.

It is believed that these oxygen-derived radicals, or oxy-radicals, help to degrade the DB-hapten compounds by attacking the linker groups that connect the two halves of the DB-chelate moiety, for example connecting

an indium loaded benzyl³⁷EDTA to a benzylDTPA or benzylDOTA. The radiolytic effect of the radionuclide upon the chelate compound is due, at least in part, to extensive formation of oxygen-derived radicals and species generated in the water by radiation energy from the radionuclide. Once radiolabeled and stored in aqueous solution, such as the quench buffer solution, the dumbbell chelates undergo degradation at the point of the moiety linking the two halves of the chelate compound. When the dumbbell chelates, such as DBIII, DB6, DBX and DDBIII, are radiolabeled with ⁹⁰Y and stored in aqueous solution the rate of degradation is especially high. As one skilled in the art will appreciate, virtually any radionuclide generates sufficient energy to cause radiolysis of water molecules.

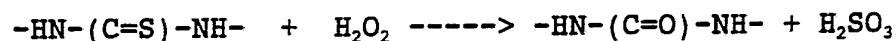
The higher the energy escaping from the decaying nucleus of the radionuclide, the greater is the risk of initiating degradation in a chemical compound associated with the radionuclide in the aqueous solution. For instance, the aqueous DB-chelates most subject to degradation by the radiolytic effect of the radionuclide in water are those in which the two halves of "dumbbell" are connected by a linking group having one of the following formulae:

Formula I	-NH-C(=S)-NH-
Formula II	-NH-C(=O)-CH ₂ -NH-
Formula III	-NH-C(=O)-NH-
Formula IV	-NH-(C=O)-NH-

Compounds containing the thiourea linker (Formula I), are

more susceptible to degradation than are any of the other compounds of this invention. However, the DBIII chelate appears to be more stable in aqueous solution than would be expected even though it contains the thiourea linker. Although the invention is not intended to be limited by any theory, it appears that the hydrogen peroxide formed by radiolysis of water reacts with a certain portion of the thiourea linkers in DBIII chelates to form a urea linker according to the following reaction:

(Formula V)



Postlabeling stability of aqueous radiolabeled compounds can be enhanced by addition to the quench buffer of one or more reducing anti-oxidant species to serve as a radioprotectant in aqueous solution. More particularly, addition of a reducing anti-oxidant species to an aqueous solution containing a compound labeled with ^{90}Y , such as one of the DB-chelate compounds of this invention, substantially retards radiolytic degradation of the compound, even a DB-chelate having a thiourea linker connecting the two halves of the dumbbell. Reducing anti-oxidants useful in the practice of this invention include, but are not limited to, the following: ascorbyl palmitate, hypophosphorous acid, monothioglycerol, sodium formaldehyde sulfoxylate, sodium metabisulfite, sodium thiosulfate, ascorbate, and sulfur dioxide. Ascorbate ion is the preferred radioprotectant species. Antioxidants can also be combined with known reducing agents to serve as radioprotectants in the

practice of this invention. Antioxidants that can be used in this fashion include, for example, butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, and tocopherol. One skilled in the art can readily supply a suitable reducing agent to combine with such antioxidants.

Certain of these radioprotectant molecules, for example ascorbate, ascorbyl palmitate and hypophosphorous acid, can chelate metals and thereby interfere with the radiolabeling process. Therefore, the radioprotectant is preferably added to the storage solution after the DB-chelate has already been radiolabeled. And preferably, radioprotectant is added to the quench buffer after the chelate has been incubated with the radioisotope molecule for a sufficient time and under suitable reaction conditions to allow the radiolabeling reaction to take place.

The proper reaction conditions, i.e., the incubation times and temperatures, necessary to attach the radioisotope to most chelating compounds can readily be determined by one skilled in the art from the examples herein and from the literature. However, the DDB-chelate DDBIII is particularly difficult to radiolabel in aqueous solution since the reaction forming the bond between the radionuclide and the hapten proceeds unusually slowly. For instance, to attach ^{90}Y to DDBIII, it has been found necessary to use a solution of radionuclide having a specific activity of at least 16.5 mCi/nmol. The reaction requires severe conditions, preferably a temperature of at least 65 °C for 20 minutes. Under

these conditions, radiolysis of water is sufficiently advanced that the oxy-radicals so formed rapidly degrade the DDBIII chelate during the radiolabeling procedure. Protection can be afforded to the hapten while the radionuclide is being attached by adding to the aqueous reaction mixture a reducing antioxidant that does not chelate metal ions. The preferred radioprotectant for this purpose is sodium thiosulfate.

To retard the rate of degradation of compounds subjected to strong beta radiation during storage, such as that generated by ^{90}Y , an effective amount of a reducing antioxidant is added into the storage liquid, for instance an amount effective to substantially reduce radiolysis of water molecules in the aqueous solution. Ascorbate has been found to reduce the degradation rate of DB-chelate compounds to no more than about 10 weight percent over a period of at least three days and sometimes as many as six days.

What constitutes an effective amount of the radioprotectant ion depends upon several factors. Factors to be considered in determining the amount of radioprotectant necessary to significantly retard degradation of a radiolabeled compound are the level of energy produced by emission of a single particle and the specific activity of the radionuclide, a higher energy level and a higher specific activity requiring addition of a greater concentration of the radioprotectant to the aqueous solution. Other factors to be considered in determining an effective amount are the length of storage time contemplated, the degradation rate desired, and the

nature of the functional group in the compound most subject to radiolytic attack, i.e., from oxy-radicals generated by radiolytic effect of the radionuclides upon water.

The DB-chelates of this invention containing thiourea linkers are more subject to degradation than are those containing urea linkers. One skilled in the art will appreciate that a higher concentration of the radioprotectant ion will be needed to achieve a given level of stability if the linker is one especially subject to the radiolytic effects of the decaying radionuclide in water, for example a thiourea group, than if the linker is a more stable moiety, for example a urea group. In general, however, the rate of degradation of any DB-chelate compound of this invention labeled with ^{90}Y and stored at room temperature can be reduced to no more than about 10 weight percent over a period of three days if the concentration of ascorbate in the aqueous storage solution is in the range from about 0.5 to 20.00, and preferably from about 0.68 to 12.05 mg per mCi of ^{90}Y . However, one skilled in the art will appreciate that in most cases the upper limit on the range of radioprotectant that can be effectively employed is determined by economic practicality.

In another embodiment of this invention, a therapeutic composition is provided comprising a radiolabelled substance stored in an aqueous environment and an amount effective to substantially reduce radiolytic decay of the substance of one or more reducing antioxidants. For therapeutic applications, such as

parenteral administration, it is preferred to adjust the pH as well as the osmolality of the aqueous storage buffer containing the radioprotectant species to values that are acceptable for parenteral administration to humans. It is well known that for therapeutic compositions used in parenteral administration in humans, the preferred pH is in the range between 7.2 and 7.6 and the preferred osmolality is in the range between about 275 and 300 mOsm per liter (Remington Pharmaceutical Sciences, Ed. Arthur Osol, Chapter 79 "Tonicity, Osmoticity, Osmolality and Osmolarity," Mack Publishing Co., 1980, pp. 1403-1419).

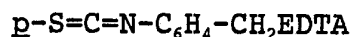
Many of the above-named radioprotectant species will affect the osmolality of an aqueous solution. Therefore, it is preferred in preparing a therapeutic composition containing a radiolabelled hapten for parenteral administration that the amounts and/or concentrations of the radioprotectant and the other species in the storage solution be adjusted to one another, using techniques well known to those skilled in the art, so that the pH and osmolality fall within these preferred ranges while maintaining radioprotection for the therapeutic compound.

The following non-limiting Examples are provided to further illustrate the invention.

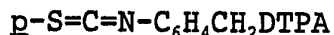
EXAMPLES

One of the starting materials used in some of the examples to prepare the novel hapten derivatives of the invention was isothiocyanatobenzyl EDTA ("ITCBE"), the

structure of which is shown below:



Also used as a starting material in the Examples is "ITCBD", isothiocyanatobenzyl DTPA, the structure of which is shown below:



A third starting material for the current claimed compounds is p-isothiocyanatobenzyl-1,4,7,10, - tetraazacyclodecone-N,N',N'',N'''-tetraacetic acid ("**DITC"). The precursor for this molecule, the p-aminobenzyl analogue, is described by M.K. Moi and C.F. Meares, J. Amer. Chem. Soc., 110, pp 6266-6267 (1988).

Unless stated otherwise, for in vitro and in vivo characterization, radioactive ¹¹¹indium complexes of the novel hapten derivatives of the invention were used. However, for DB and DDB complexes, the indium in the EDTA portion of the molecule is preferably the natural, non-radioactive isotope. For in vitro characterization, the monoclonal antibody, designated as CHA255 was used. This antibody was discussed above and has specificity for the indium complex of benzyl EDTA. However, as one skilled in the art would appreciate, alternative antibodies having specificity for indium benzyl EDTA complexes may be prepared and used interchangeably with CHA255.

For in vivo characterization, a bifunctional antibody was used in conjunction with the novel hapten derivatives of the invention. The bifunctional antibody used, designated as ECHO37.2, has a dual specificity for carcinoembryonic antigen (CEA) and the indium complex of

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benzyl EDTA. ECHO37.2 is made from the cell lines of the parent antibodies CHA255 and CEM 231.6.7 as described below in the Examples. The source of the CHA255 antibody was given above, CEM231.6.7 was developed at Hybritech. Those skilled in the art will appreciate, however, that alternative antibodies having dual specificity for CEA and indium benzyl EDTA complexes may be utilized and interchanged with ECH037.2. Such antibodies can additionally be made from quadromas and triomas (See for example, J. Martinis et al., PCT Publication No. WO 83/03679, EPO Application No. 83901672.2, and U.S. Patent Application No. 06/484,377 filed April 12, 1983, all of which are herein incorporated by reference) as ECH037.2 was, and by recombinant DNA means, (see for example M.J. Johnson et al., U.S. Patent Application No. 274,105, filed November 17, 1988, herein incorporated by reference or by combining F(ab') fragments by standard methods, for example, through a BMME linkage.

A. HAPTEN DERIVATIZATION

General Procedures for the Syntheses of Hapten Derivatives of the Invention.

All glassware and plasticware used in the syntheses was acid washed to remove metal ions. Glassware was washed with a mixture of acids (equal volumes of concentrated sulfuric and nitric acids) and plasticware was washed in 6M HCl. Both were rinsed exhaustively with deionized water before use. Metal-free plastic pipet tips from BioRad (Richmond, CA) were used to transfer solutions. Deionized water with a resistivity of at

least 15 Mohm-cm was used throughout. The water was obtained by passing water through a Milli Q Milli-RO 4 apparatus (Millipore Corporation, Bedford, MA). "Milli-Q water" refers to this deionized water. All reagents used were the purest commercially available. The different amines were all purchased from Aldrich Laboratories (Milwaukee, Wisconsin) and were used without further purification.

(S)-p-nitrobenzyl EDTA was prepared and converted to (S)-4-isothiocyanatobenzyl EDTA ("ITCBE") as described in Meares et al., U.S. Patent No. 4,622,420, and Meares, C.F., Anal. Biochem. 142, 68-75 (1984). The lyophilized ITCBE was resuspended in 0.3 M HCl (Ultrex, J.T., Baker Chemical, Phillipsburg, New Jersey) to a final concentration of roughly 50 mM ("Stock ITCBE solution"). This solution was stored at -70° C. Analogously, p-isothiocyanatobenzyl DTPA ("ITCBD") was prepared. Using the HPLC gradient system described below, ITCBD has a retention time of 5.0 minutes.

Similarly, for the preparation of the corresponding DOTA, HETA, TETA and TRITA haptens of the invention, one would use the corresponding isothiocyanato-derivative described in Meares et al., U.S. Patent No. 4,678,667, issued July 7, 1987, herein incorporated by reference.

Products were purified by anion exchange chromatography on either AG-1x8, formate form (BioRad Laboratories, Richmond, CA) or DEAE Sephadex A-25 ("A-25 column") (Pharmacia Fine Chemicals, Uppsala, Sweden). Columns were monitored at 280 nm on a LKB UVicord S detector. Fluorescamine (Floram™, Hoffman-LaRoche,

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Nutley, N.J.) was used to test for the presence of amines. The sample to be tested was spotted on Whatman 4 filter paper and then sequentially sprayed with 0.2 M sodium borate, pH 9.5 and 0.25 mg/mL Fluorescamine in acetonitrile. Fluorescence, indicating the presence of an amine, was detected by illuminating the sample with a short wave-length UV lamp. HPLC analyses were done on a Hewlett-Packard 1090 liquid chromatograph with a microbore C₁₈ column (Hewlett Packard #79916 OD opt 552) unless otherwise noted. Starting buffer was aqueous 50 mM triethylammonium acetate, 10 mM EDTA, pH 6.0. A 10 minute linear gradient from starting buffer to neat methanol was used throughout. The flow rate was kept constant at 0.4 mL/minute and column effluent was monitored at 280 nm. With this system, the ITCBE starting material had a retention time of 5.3 minutes unless otherwise noted.

Unless indicated otherwise, all reactions were performed in aqueous solutions at 40° C.

NMR spectra were obtained on a Varian model XL-300 300 MHz instrument with multinuclear capabilities. Samples were dissolved in D₂O and the pH adjusted with NaOD or DCl.

Chelate concentrations were determined by titration with an indium (III) solution spiked with radioactive ¹¹¹Indium(III), in a modification of the procedure described by Meares et al., Anal. Biochem. 142, 68-78 (1984). Standard indium solutions were prepared by dissolution of a precisely known mass of indium foil (Puratonic, Alfa Products, Danvers, Massachusetts) in

Ultrex HCl (J.T. Baker, Chemical Co., Phillipsburg, New Jersey). A 0.13 M ammonium citrate buffer, pH 6.0, was used for the titrations, and the TLC system used was that described by Meares et al., supra.

Unless indicated otherwise, labeling of the non-EDTA portions of the haptens with designations starting with "DB" with $^{111}\text{Indium(III)}$ and $^{90}\text{Yttrium(III)}$ were performed in the following manner: The hapten to be labeled is prepared as a 2 micromolar solution in 25 mg/mL glycine, pH 4.5-6.5. $^{111}\text{Indium(III)}$ labeling is done using a 2.5 mCi/mL solution of $^{111}\text{Indium(III)}$ in 0.088 M glycine. Equal volumes of the hapten solution and $^{111}\text{Indium(III)}$ solutions are combined, vortexed and allowed to stand at room temperature for at least 10 minutes. The labeling mixture then is neutralized by adding 0.22 M glycine, 0.2 M DTPA, 4% (w/v) NH_4Cl , pH 8.2, in volume equal to three times the volume of $^{111}\text{Indium(III)}$ solution added. $^{90}\text{Yttrium(III)}$ labeling is done by combining one volume of a solution of $^{90}\text{Yttrium(III)}$ (200 mCi/mL) in 70 mM HCl with 10 volumes of DB hapten chelating agent. The mixture is vortexed and allowed to stand at room temperature for at least 5 minutes. The mixture is quickly neutralized by adding it to a volume (equal to 14 times the volume of hapten chelating agent) of quench buffer, composed of 150 mM ascorbic acid, 50 mM Tris, and 0.1 mM DTPA (pH 7.4).

Radio-HPLC of the $^{111}\text{Indium(III)}$ and $^{90}\text{Yttrium(III)}$ labeled DB-chelates was done on an IBM model 9533 chromatograph using a C_{18} reversed phase column (IBM No. 8635308). Elution was with a 20 minute linear gradient

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from neat HPLC buffer A (50 mM aqueous triethylammonium acetate, 10mM EDTA, pH 6.0) to neat Buffer B (methanol) at a flow rate of 1 mL/min. Two different systems were used for radiodetection. $^{90}\text{Yttrium(III)}$ detection was done with a Canberra 2007p/802-3 sodium iodide crystal. $^{111}\text{Indium(III)}$ detection was done with a Ludlem model 177 Alarm ratemeter coupled with a model 44-6 side window G-M detector. Both systems were in-line and coupled to an integrator. Prior to injection, samples were diluted with HPLC buffer A, defined above. In this system, all $^{111}\text{Indium(III)}$ labeled hapten chelates appeared as a single, well-defined peak. $^{90}\text{Yttrium(III)}$ labeled DTPA species appeared as a doublet, with two peaks within 0.5 minutes of one another, with relative areas of 3:2 for the leading and trailing peak, respectively.

Absorbance measurements were made on a Hewlett Packard Model 8451A Diode Array spectrophotometer. (1 cm path length) cuvettes were used throughout, and samples were diluted in PBS, and measured against PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.2). Molar extinction coefficients were calculated for the metal-free chelating agents based on concentrations determined by indium titrations.

B. Synthesis of Compounds

EXAMPLE 1

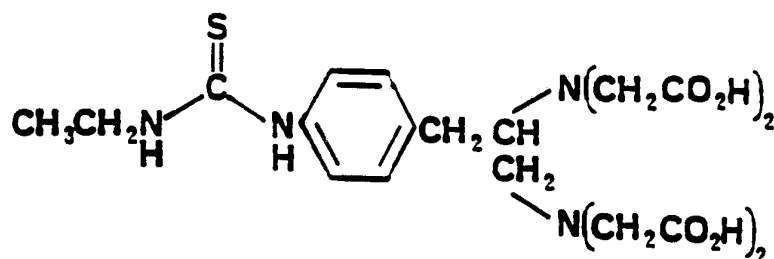
Synthesis of Hapten ETUBE

Stock ITCBE solution was diluted to a concentration of 20 mM using water. The diluted solution (2.5mL) was combined with 1.25 mL of an aqueous 200 mM ethylamine

solution and the pH adjusted to 11.8 with 10 N NaOH. The volume was adjusted to 5 mL with water and the mixture allowed to react for 15 minutes at room temperature, at which time it was checked by HPLC analysis. All of the ITCBE was converted to ETUBE, which had a retention time of 3.6 minutes.

The ETUBE crude product was purified by anion exchange chromatography on a 5 mL AG1x8 (formate form) column (5 mL, equilibrated with 0.5 M formic acid). The column was washed with 0.5 M formic acid until the effluent was fluorescamine negative, indicating that all of the excess amine was removed. ETUBE was eluted by washing the column with 8 M formic acid. Excess formic acid was removed from the collected product by lyophilization. The product was characterized by indium titration, HPLC and UV/visible absorbance. The material had an absorbance maximum at 244 nm, with $\epsilon = 2.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$

The structure of ETUBE is shown below:



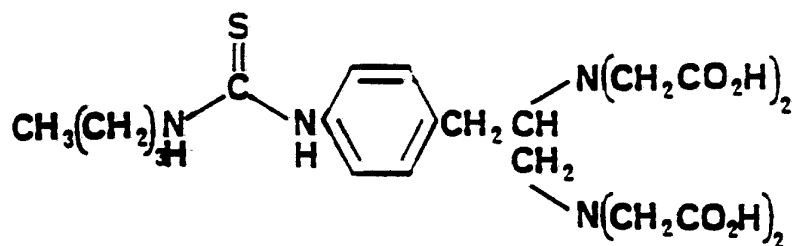
EXAMPLE 2

Synthesis of Hapten BUTUBE

BUTUBE was prepared exactly as described in Example 1, except that 4-aminobutane was used in place of ethylamine. The product had an HPLC retention time of 4.8 minutes and an absorbance maximum of 244 nm with $\epsilon =$

$1.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

The structure of BUTUBE is shown below:

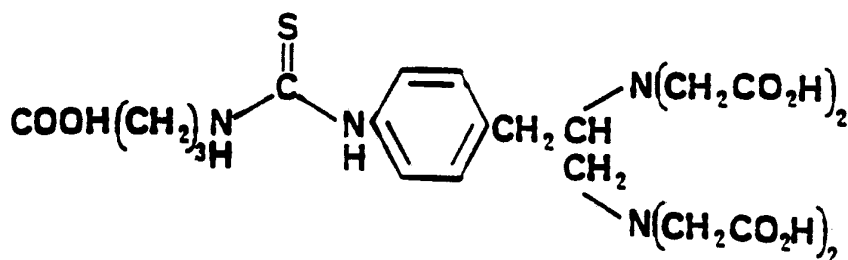


EXAMPLE 3

Synthesis of Hapten BATUBE

BATUBE was prepared exactly as described in Example 1, except that 4-aminobutyric acid was used in place of ethylamine. The product had an HPLC retention time of 3.1 minutes and an absorbance maximum of 244 nm with $\epsilon = 1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

The structure of BATUBE is shown below:

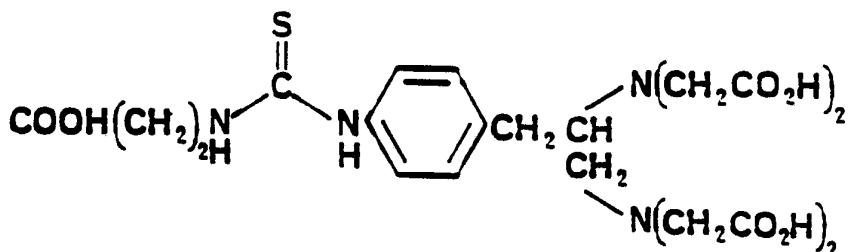


EXAMPLE 4

Synthesis of Hapten PATUBE

PATUBE was prepared exactly as described in Example 1, except that β -alanine was used in place of ethylamine. The product had an HPLC retention time of 3.0 minutes and an absorbance maximum at 244 nm with $\epsilon = 1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

The structure of PATUBE is shown below:

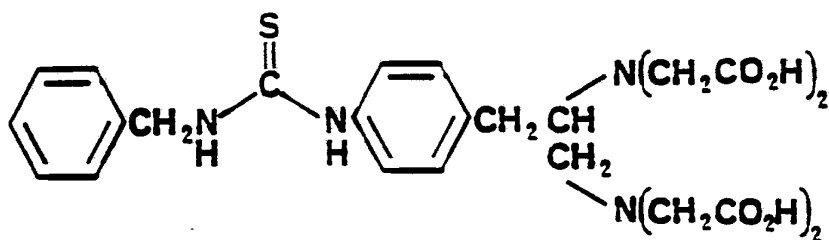


EXAMPLE 5

Synthesis of Hapten BETUBE

BETUBE was prepared exactly as described in Example 1, except that benzylamine was used in place of ethylamine. The product had an HPLC retention time of 5.1 minutes and an absorbance maximum at 244 nm with $\epsilon = 2.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

The structure of BETUBE is shown below:

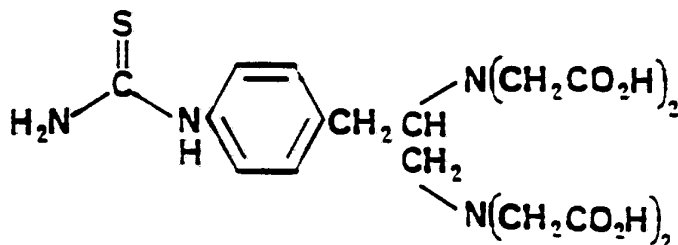


EXAMPLE 6

Synthesis of Hapten TUBE

A solution of ITCBE (2 mL, 42.7 mM) in 0.3 M HCl was lyophilized to dryness in an acid-washed flask. To this was added 1.2 mL of 5 M ammonium formate at a pH of 10-10.5. After 15 minutes at room temperature, the reaction was checked by HPLC; only about half of the starting chelating agent had converted to product (retention time 3.14 minutes). The pH of the mixture was then adjusted to 10.5 by the addition of 25% (w/v) NH_4OH . The reaction was stirred and checked by HPLC. All of the ITCBE had converted to the desired product, TUBE. The reaction mixture was taken to dryness by lyophilization.

The structure of TUBE is shown below:



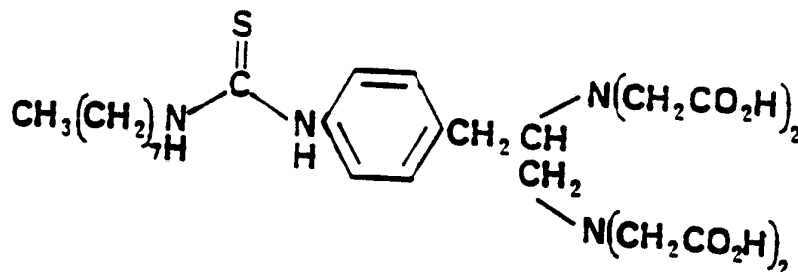
EXAMPLE 7

Synthesis of Hapten OTUBE

A 20 mM solution of ITCBE in 0.3 M HCl was added to a 20 mM aqueous octylamine solution (1.26 mL) in 40%

(v/v) aqueous ethanol. The pH was adjusted to 11.8 with 10 N NaOH, and the volume increased to 5 mL by the addition of 40% (v/v) aqueous ethanol. The reaction mixture was stirred for 15 minutes at 40°C. The HPLC retention time of product was 7.4 minutes, and the absorbance maximum was 244 nm. Low aqueous solubility precluded an accurate determination of the molar extinction coefficient.

The structure of OTUBE is shown below:

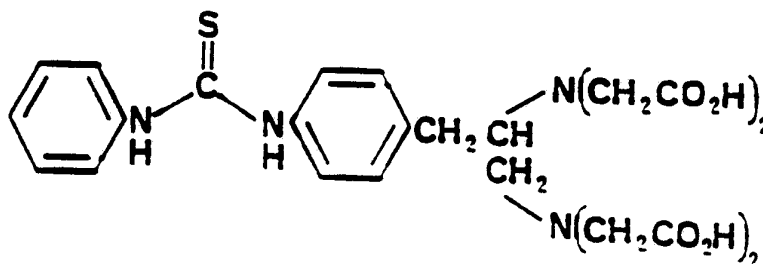


EXAMPLE 8

Synthesis of Hapten PHTUBE

Diluted Stock ITCBE solution was added to 200 mM aqueous solution of aniline (1.25 mL) and the pH was adjusted to 7.6 with 10 N NaOH. Addition of water brought the volume up to 5 mL, and the reaction was allowed to proceed for 75 minutes. The product was purified as described in Example 1, and had an HPLC retention time of 4.4 minutes. The product has an absorbance maximum at 258 nm with $\epsilon = 2.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

The structure of PHTUBE is shown below:

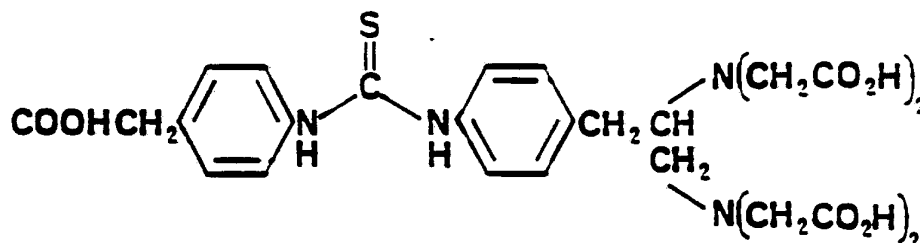


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EXAMPLE 9Synthesis of Hapten PHATUBE

PHATUBE was prepared exactly as described in Example 7, except that p-aminophenylacetic acid was used as the amine reagent. The product had an HPLC retention time of 3.5 minutes, with an absorbance maximum at 256 nm with $\epsilon = 2.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

The structure of PHATUBE is shown below:

EXAMPLE 10Synthesis of Hapten EOTUBE

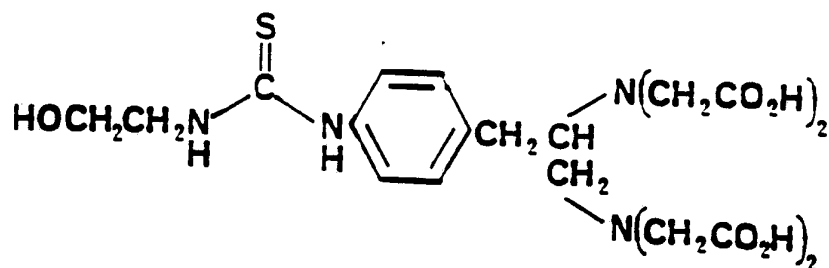
EOTUBE was prepared exactly as described in Example 1, except that ethanolamine was used in place of ethylamine, and the final pH of the reaction was 11.0. The product had an HPLC retention time of 3.1 minutes.

The product was purified by anion exchange chromatography on an 11 mL column of DEAE Sephadex A-25, eluted with a 110 mL linear gradient of 0.1 to 1 M ammonium formate, pH 6. Fractions containing the product were pooled and lyophilized. The product had an absorbance maximum at 246 nm with $\epsilon = 1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

The structure was verified by ^{13}C NMR spectroscopy. The peak corresponding to the carbon in the isothiocyanate moiety in ITCBE was at 139 ppm, and was replaced by a peak at 182 ppm in EOTUBE. This latter

peak corresponds to the carbon in the thiourea linkage. The aromatic region (128-138 ppm) of the spectrum of ITCBE shows four peaks, while that of EOTUBE shows three. In the aliphatic region, there are five peaks in common for ITCBE and EOTUBE, and an additional two peaks at 64 and 49 ppm in the EOTUBE spectrum. The latter peaks correspond to the carbons adjacent to the hydroxyl and thiourea moieties, respectively.

The structure of EOTUBE is shown below:

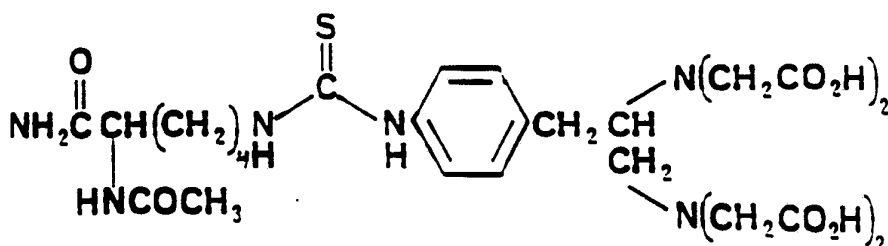


EXAMPLE 11

Synthesis of Hapten BALTUBE

BALTUBE was prepared as described in Example 1, except that N- α -acetyl-L-lysine amide was used as the amine reagent, and the reaction was done at pH 11.5.

Purification of BALTUBE was done as described in Example 1. The product had an absorbance maximum of 224 nm with $\epsilon = 2.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The structure was verified by ^{13}C and NMR spectroscopy and is shown below:



The NMR spectra for BALTUBE are as follows:

$^{13}\text{C}(\text{D}_2\text{O})$: δ 171.4, 179.6, 176.8, 176.6, 171.9, 168.2, 138.0, 132.9, 128.8, 63.1, 58.4, 57.4, 56.3, 55.4, 55.0,

47.1, 46.0, 35.2, 33.4, 30.5, 25.2, 24.5; proton NMR (D_2O , 300 MHz) δ 7.18, 7.11, 4.2-2.4 (multiple peaks).

The amide of N- α -acetyl-L-lysine was prepared via the methyl ester using a procedure described by Yeh, S.M. et al. Anal. Biochem. 100, 152-159 (1979). N- α -Acetyl-L-lysine methyl ester (1 g) was dissolved in 45 mL of anhydrous methanol and the solution saturated with gaseous NH_3 . The reaction was kept under a nitrogen atmosphere and saturated with ammonia twice a day for 2-3 days. The reaction was followed by TLC on silica plates (PolyGram Sil G/UV, Brinkmann Instruments, Inc., Westbury, New York) with a solvent comprised of ethanol: 25% ammonium hydroxide (4:1, v/v). Spots were visualized by a ninhydrin spray reagent. The methyl ester had an R_f of 0.3. NMR indicated the presence of an additional material which could not be visualized by ninhydrin. Analysis of the NMR indicated that the side product was a seven-membered cyclic amide formed by an intramolecular reaction. Attempts to eliminate the side product by presaturating the methanol prior to the addition of the methyl ester were unsuccessful.

When the reaction was complete it was taken to dryness by rotary evaporation and the solid redissolved in sufficient water to make a 10 mM solution. The amide was purified by chromatography on a 90 mL column of Sephadex CM-25 (Pharmacia Fine Chemicals, Uppsala, Sweden), previously equilibrated with 10 mM trimethylammonium formate, pH 5. Before application, the pH of the sample was adjusted to less than 8 with 10 mM trimethylammonium formate. The cyclic amide side product

flowed through the column as the sample was applied and during a wash with 2 column volumes of 10 mM trimethylammonium formate. The desired product then was eluted with a 900 mL linear gradient of trimethylammonium formate, pH 5, from 10 to 500 mM. Fluorescamine was used to test the individual fractions for the presence of amine. Those fractions which were fluorescamine positive were pooled and lyophilized. The NMR spectra were consistent with the expected structure for N-acetyl-L-lysine amide. The concentration of amine was determined by assay with trinitrobenzenesulfonic acid (TNBS) (Glazer, A.N. *et al.*, Chemical Modification of Proteins, pp 76-77, American Elsevier, New York, (1975)).

EXAMPLE 12

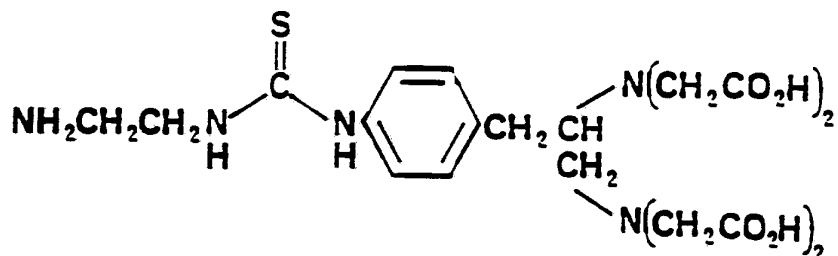
Synthesis of Hapten NUBE

Ethylenediamine dihydrochloride (115 mg, 875 μ mole) was added to 108 μ l (1.624 μ mole) of ethylenediamine. To the resulting solution was added 2.5 mL of 42.2 mM ITCBE (105 μ mole) in 0.3 M HCl with stirring. The pH was adjusted to 9-10 with triethylamine or hydrochloric acid. HPLC analysis showed that the reaction occurred instantaneously. The HPLC retention time of NUBE was 2.67 minutes under the HPLC conditions in Example 1.

The reaction mixture was diluted to 500 mL with water and applied to a 15 mL DEAE Sephadex chromatography column equilibrated in 20 mM triethylammonium formate, pH 6-7. The column then was eluted with a 500 mL linear gradient from 20 to 250 mM triethylammonium formate. Peak fractions (as determined by absorbance at 250 nm)

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were pooled and lyophilized. When compared with the ^{13}C NMR spectrum of ITCBE, new resonances appeared at 183.0, 44.7, and 41.8 ppm, corresponding to the thiourea and ethylenediamine methylene carbons, respectively. In addition, one signal near the 140 ppm region of the ITCBE spectrum (assigned to the isothiocyanate carbon) did not appear in the NUBE spectrum.

The structure of NUBE is shown below:



EXAMPLE 13

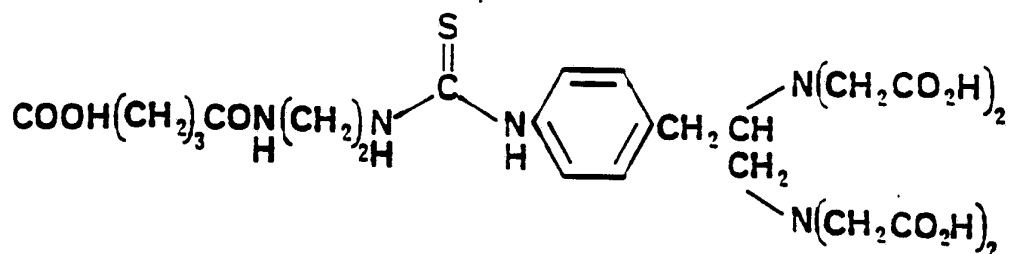
Synthesis of Hapten GNUBE

A solution of 16 mg (140 μ mole) glutaric anhydride in 100 μ l dimethylacetamide was added to a stirred 35 mM solution (800 μ l, 28 μ mole, pH 8) of NUBE, prepared as described in Example 12. The reaction was allowed to proceed 20 hours at room temperature, then stopped by dilution into 250 mL 20 mM triethylammonium formate, pH 6.5. The HPLC retention time, under the condition described in Example 1, was 3.19 minutes.

The solution was then applied to a 15 mL DEAE Sephadex A-25 column equilibrated in 20 mM triethylammonium formate, pH 6.5 and eluted with a 500 mL gradient from 200 to 800 mM of the same buffer. The peak

fractions, as determined⁵⁹ by absorbance at 250 nm, were pooled and lyophilized. When compared with the ¹³C spectrum of NUBE, new resonances appeared at 180.6, 178.7, 37.7, 35.9, and 23.6 ppm, corresponding to the acid, amide, and three methylene carbons from the glutaramate substituent, respectively. In addition, the NUBE resonance appearing at 44.7 ppm shifted to 46.8 ppm in GNUBE, presumably due to acylation of the adjacent amine.

The structure of GNUBE is shown below:



EXAMPLE 14

Synthesis of Hapten DB-I

p-Bromoacetamidobenzyl-EDTA ("BABE") is prepared according to the procedure outlined in U.S. Patent No. 4,622,420, which is incorporated herein by reference. The BABE then labeled with 10% molar excess of cold indium (III) trichloride in 1 M NaOAc, pH 8.0, such that the final pH is about 4. A trace amount of ¹¹¹Indium was added to follow the labeling procedure. The conditions for the labeling were as follows:

2000 μ l 1 M NaOAc, pH 8.0

1089 μ l 100 mM InCl₃ in 50 mM HCl

120 μ l ¹¹¹In

900 μ l BABE (110 mM)

The above reagents were added in the order listed to an acid-washed container containing an acid-washed stirring

bar. The pH is adjusted to 4.0 with either 10 N NaOH or 1 N NaOH and the reaction is stirred for 30 minutes. An ED3A (ethylenediamine triacetate Pierce Chemical Co., Rockford, Illinois) column was equilibrated in 50 mM NaOAc, pH 4.0 buffer. A 6 mL column was packed (about 3-fold capacity) and loaded to about 34.4% of the resin capacity with the reaction mixture and the product was eluted with the sodium acetate buffer. In-BABE with a purity of about 65% was obtained.

p-Bromoacetamidobenzyl-DTPA ("BABD") is prepared analogously to the procedures taught for BABE, described earlier, in U.S. Pat. No. 4,622,420. BABD (47.1 μ moles, 145 mM) was reacted in 23.55 mL of 5 mM 1,4-butanedithiol (plus a 40-fold excess of 1,4-butanedithiol) in 0.1 M NaHCO₃, pH 8.2. The reaction was followed by HPLC. After the reaction was complete, the reaction mixture was centrifuged at 3200 RPM for ten minutes and the supernatant was acidified with 2 M formic acid to a final concentration of 0.5 M formic acid. The resulting precipitate was stored at -70° C. The reaction was then extracted with 40 mL portions of diethyl ether until the extracts were negative for free sulfhydryl groups, as determined by testing with DTNB. (DTNB is 5,5'-Dithiobis(2-nitrobenzoic acid). See for example, G.E. Meares and R.E. Feeney, Chemical Modification of Proteins, Holden-Day Inc., San Francisco, California, p. 220 (1971).) The aqueous layer was lyophilized overnight and stored at -70° C. The yield of the "MBD" obtained was 73.5%

MBD (31.5 μ moles; 19.1 mM in 0.1 M NaHCO₃, pH 8.2)

was reacted with 63.0 μ moles In-BABE (100 mM in 0.3 M HCl), prepared as indicated above. The final volume was 6.3 mL. The reagents were added as indicated below:

4.0 mL 0.1 M NaHCO_3 , pH 8.2

1.6 mL MBD with the pH being adjusted as necessary to 8.2 with saturated Na_2CO_3 .

0.63 mL In-BABE, again adjusting the pH to 8.2 with saturated Na_2CO_3 , as necessary.

The reaction time was about 1 to 2 hours as determined by HPLC. The reaction mixture is applied to a 2 mL chromatography column (BioRad Affi-gel-501 column equilibrated with 0.1 M NaHCO_3 , pH 8.2). About 30 mL of effluent were collected and the pH of the eluent was adjusted to 6.0 with 23 M formic acid. The effluent then immediately was applied to a 5 mL A-25 column equilibrated in 10 mM ammonium formate, pH 6.0 buffer. The column was washed with 10 mM buffer to make certain that the sample did not elute. A 50 mL gradient from 10 mM to 1.0 M ammonium formate, pH 6.0 was run. The HPLC retention time of the product was 5.08 minutes.

EXAMPLE 15

Synthesis of Hapten DB-II

DB-II was prepared by reacting DTT (dithiothreitol) with BABD, followed by reaction with BABE-In.

BABD (833 μ l, 100 mM in 0.2 M HCl) was added to 832 μ l of DTT (0.5 M in NaHCO_3 , pH 8). The pH was adjusted to 8.2 and the reaction was complete within 2 hours as determined by HPLC. The reaction was acidified with 23 M formic acid and then was extracted with diethyl ether

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until DTNB negative. The product was lyophilized overnight then dissolved in 1.0 mL water. The reaction product, designated as MBD-2, then was reacted with In-BABE, prepared in Example 14. The reaction conditions were as follows:

4440 μ l 0.1 M NaHCO_3 , pH 8.2

900 μ l MBD-2 (33mM)

520 μ l In-BABE (113 mM)

The reaction was complete within 2 hours as determined by HPLC. The reaction mixture was purified on a 5.5 mL organomecurial agarose column (BioRad Affi-gel 501) eluted with 0.1 M NaHCO_3 , pH 8.2. The pH of the eluted material was adjusted to pH 6 and further purified on a 5 mL A-25 column with a 100 mL gradient from 10 mM to 1M ammonium formate, pH 6 buffer. The product was obtained in about 78% purity as determined on HPLC and had a retention time of 4.1 minutes.

EXAMPLE 16

Synthesis of Hapten DB-III

Process 1

DB-III is prepared by reacting p-aminobenzyl EDTA ("ABE") with p-isothiocyanatobenzyl-DTPA ("ITCBD"), both starting materials prepared according to the teaching of U.S. Patent No. 4,622,420.

ABE was labeled with a 20% molar excess of indium (III) chloride dissolved in 0.1 M NaOAc, (pH 6). The reagents were added as follows:

2310 μ l 0.1 M NaOAc, pH 6

1410 μ l 100 mM InCl_3

900 μ l 129.6 mM ABE

The pH was adjusted to 6 with 10 N NaOH and the reaction continued for 1 hour at room temperature. The indium-labeled ABE then was passed through a 28 mL ED3A (ethylenediaminetriacetate) column and eluted with 10 mM NaOAc, pH 6 buffer. The mixture was lyophilized overnight and re-dissolved in 1 mL of milli-Q water. Throughout the procedure, the pH of the solution was maintained at 6 or greater to prevent the indium (III) from equilibrating between the EDTA and DTPA moieties.

ITCBD (1.2 mL, 51 mM) was lyophilized prior to the addition of indium-labeled ABE. The addition was made as follows: Sodium bicarbonate (1.5 mL, 0.1 M, pH 8.2) was added to 1 mL of 70.3 mM ABE-In prepared above. The pH was adjusted to 8 with saturated Na_2CO_3 and then the solution was added to the lyophilized ITCBD. The mixture was reacted at 40° C. and the reaction was completed after 2 hours as determined by HPLC. The product was obtained at 78.4% purity and had an HPLC retention time of 3.04 minutes. The pH of the sample was adjusted to 6 with formic acid, diluted to 20 mLs with milli-Q water and purified through a 20 mL A-25 column equilibrated with 10 mM ammonium formate, pH 6, buffer. A 200 mL gradient of 10mM to 1 M ammonium formate, pH 6, buffer was used to elute the product. The extinction coefficient at 260 nm in water, pH 7 was 20,380 $\text{M}^{-1} \text{cm}^{-1}$.

Radiolabeling the product, using $^{111}\text{Indium}$ (III) and $^{90}\text{Yttrium}$ (III), followed by radio-HPLC, showed 100% and 98% of the activity associated with the DB-III for the $^{111}\text{Indium}$ (III) and $^{90}\text{Yttrium}$ (III) labeled compounds,

respectively. The $^{90}\text{Yttrium}$ -labeled compound showed the characteristic doublet associated with $^{90}\text{Yttrium}$ -labeled DTPA chelates in this HPLC system. The labeled DB-III was highly bound to antibody CHA255. Specifically, the $^{111}\text{Indium}$ (III)-labelled species was 96% bound and the $^{90}\text{Yttrium}$ (III)-labelled species was 97% bound.

Process 2

p-Aminobenzyl EDTA and p-isothiocyanatobenzyl DTPA were prepared as described in the literature (Meares et al., U.S. Patent 4,622,420, filed Nov. 11, 1986, herein incorporated by reference and Anal. Biochemistry, **142**, 68-78 (1984)). By HPLC analysis, with the 10 cm column and a linear gradient from neat buffer A to neat B, these compounds had retention times of 2.7 and 5.0 min. for the amine and isothiocyanate, respectively.

p-Aminobenzyl EDTA was complexed with Indium(III) by combining 0.38 mmol of the chelating agent with 13.9 mL of a 30 mM solution (0.42 mmol) of InCl_3 in 1.2 M HCl such that the final volume was 26 mL. The pH of the resulting solution was adjusted by the sequential addition of 1.3 mL of 10 N NaOH and 2.7 mL of 1M sodium carbonate. After filtration through a 0.2 μm acrodisc filter (to removed Indium hydroxide, an isocratic HPLC analysis on a 20 cm C_{18} column was performed. Elution with 8% Buffer B/92% Buffer A clearly showed that the indium was complexed with p-Aminobenzyl EDTA (retention time 2.7 min.) and that no uncomplexed p-Aminobenzyl EDTA (retention time 2.5 min) remained.

To form DB, the indium complexed p-Aminobenzyl EDTA

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(0.35 mmol) was reacted in excess with p-isothiocyanatobenzyl DTPA (0.31 mmol) overnight in aqueous solution at pH 5.8 in a volume of 38 mL. HPLC analysis, with a 10 cm column and a gradient from neat Buffer A to neat Buffer B, was used to monitor the reaction. The reaction was determined to be complete when all of the p-isothiocyanatobenzyl DTPA species was reacted. With this system, HPLC retention times were 2.7 min, 3.2 min, and 5.0 min, respectively, for indium-complexed p-Aminobenzyl EDTA, DB III and p-isothiocyanatobenzyl DTPA. The reaction mixture was frozen and lyophilized to give crude product.

Pure product was obtained by serial purification on C₁₈ (LiChroprep®RP18 EM Science) and DEAE-A25 resins (Pharmacia Fine Chemicals). For the C₁₈ column purification, an isocratic mixture of 88% Buffer A (50 mM aqueous triethylammonium acetate, pH 6.0) and 12% Buffer B (methanol) was used. The crude product was split in half and each half purified on a 2.5 x 33 cm column with elution at a flow rate of 15 mL/min. Eluent was monitored at 280 nm and individual fractions then analyzed by HPLC on a 10 cm column with a linear gradient from neat Buffer A to neat Buffer B. Fractions containing > 90% DB III were pooled and taken to dryness by rotary evaporation. Products from the two reversed phase columns were then pooled and subsequently purified by anion exchange chromatography.

Anion exchange chromatography was done on a 1.5 x 12.5 cm column of DEAE sephadex A-25, in the formate form. The sample was taken up in water and applied to

the column equilibrated with 0.1 M ammonium formate, pH 8.2.

After washing the column with 2 column volumes of 0.1M ammonium formate, pH 8.2, DB III was eluted with a linear gradient (total volume = 500 mL) from 0.1 to 1.0 M ammonium formate, pH 8.2. Eluate was monitored at 280 nm, and those fractions containing product further analyzed by HPLC using a 20 cm column and a 20 minute gradient from neat buffer A to neat B. Those fractions containing a single peak with >95% purity were further analyzed by isocratic elution on a 20 cm column with a mixture of 88% Buffer A/12% Buffer B. Those fractions containing DB III were combined and lyophilized.

Yield (based on $\epsilon = 20,400 \text{ cm}^{-1} \text{ M}^{-1}$ at 260 nm in neutral aqueous solution) was 129 μmol (42%). The product was 95.5% pure, as determined by HPLC with a 20cm column and a linear gradient from neat Buffer A to Buffer B. Radiolabeling the product, followed by radioHPLC, showed 100 and 98 percent of the activity associated with the DB III for the $^{111}\text{Indium}$ (III) and $^{90}\text{Yttrium}$ (III) labeled compounds respectively. Retention times were 14.3 min. and 14.5/14.8 min. The $^{90}\text{Yttrium}$ (III) labeled compound showed the characteristic doublet associated with $^{90}\text{Yttrium}$ (III) labeled DTPA chelates in this HPLC system. A high percentage (96% and 97%, respectively) of the labeled DB III bound to antibody CHA255 for the $^{111}\text{Indium}$ (III) and $^{90}\text{Yttrium}$ (III) labeled species. The ^{13}C NMR spectrum had a signal at δ 182 ppm, consistent with a thiourea bond (isothiocyanate = 137 ppm) as well as signals corresponding to indium benzyl EDTA and benzyl

DTPA. UV analysis of the product showed an absorbance maximum at 262 nm, associated with the diphenyl thiourea moiety of DB III. The ^1H and ^{13}C NMR of DB-III were determined as follows: Samples were freshly prepared using distilled D_2O as the solvent in acid-washed NMR tubes. The pH was adjusted as necessary with NaOD or DCl. The pH reported is the actual pH meter reading without correction for the activity of D_2O . p-Dioxane was used as an internal standard for the ^{13}C NMR spectrum (downfield shift of 66.5 ppm) and the HDO peak was used as a reference for the proton spectrum (4.70 ppm). All spectra were recorded on a Varian Model XL-300 NMR spectrometer.

The following show the results of the ^{13}C spectrum in ppm (δ) (wherein the EDTA end was complexed with non-radioactive Indium (III), and the DTPA end was not complexed with either Indium (III) or $^{90}\text{Yttrium}$ (III) (not all peaks are given) ^{13}C NMR: (D_2O , Dioxane standard, pH 6.5) δ : 179.5, 177.6, 176.0, 175.8, 175.5, 170.9, 170.3, 170.2, 136.5, 135.3, 134.4, 130.2, 130.1, 126.8, 126.7, (Dioxane) 66.5, 62.5, 61.0, 59.7, 57.7, 57.2, 56.7, 55.4, 53.9, 53.5, 53.1, 51.5, 48.5, 32.3, 31.9; ^1H NMR (D_2O , pH 6.5, HOD reference, 300 Mhz) δ : 8.36 (formate), 7.27, 7.23, and 2.5 - 4.0.

EXAMPLE 17

Synthesis of Hapten DB-IV

DB-IV is prepared by reacting NUBE with ITCBD in a manner analogous to that previously described for the preparation of the other DB-haptens.

The volumes of NUBE and ITCBD used were lyophilized prior to mixing. Aqueous Na_2CO_3 solution (50 μl), pH 11-12 was added to NUBE followed by addition to ITCBD. The reaction was performed at 40° C. The quantities used were as follows:

25 μl ITCBD (51 mM)

125 μl NUBE (20 mM), with 100% excess NUBE added

50 μl final volume of Na_2CO_3 , pH 11-12

The reaction was monitored by HPLC (C_{18} reversed phase column, as described above) and was complete within about one-half hour. The product had a retention time of 3.42 minutes.

EXAMPLE 18

Synthesis of Hapten DB-V

ITCBE was reacted with 1,4-diaminobutane to prepare BUBE. The BUBE then was loaded with cold indium (III) chloride (as above) and subsequently reacted with ITCBD to prepare DB-V.

In particular, 80 μl 1,4-diaminobutane (800 μmoles) was mixed with 300 μl of 3 N HCl so that the pH of the mixture was about 10.5. ITCBE (1 mL, 39 mM) was added dropwise with stirring to the mixture. An additional 10 μl of 1,4-diaminobutane was added to keep the pH at 10.5. When the reaction was complete as indicated by HPLC, the reaction mixture was diluted with milli-Q water and the pH was adjusted to 3 with 0.3 N HCl. The product was purified on column loaded with AG-1X8 resin. 1,4-diaminobutane was eluted with 50 mM formic acid until the eluent tested negative with fluorescamine. The product

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then was eluted with 1 N formic acid. Those fractions containing the product were combined and lyophilized.

The BUBE prepared above then was labeled with cold indium (III). Excess indium was removed using an ethylenediaminetriacetate column. ITCBD (0.5 mL, 39 mM), prepared as indicated earlier, was lyophilized and then re-dissolved in 1 mL of 0.1 M NaCHO₃. The pH was adjusted to 7 with 1 N NaOH. The In-BUBE (28 μ moles) prepared above was dissolved in 0.5 mL of 0.1 M NaHCO₃, pH 8. The two solutions were mixed and the pH was adjusted to 10.5 with 1 N NaOH. The resultant reaction mixture was stirred at room temperature for about 3 hours, when by HPLC indicated the reaction was complete.

The reaction mixture was purified on an A-25 column using a 200 mL gradient running from 10 mM to 1 M ammonium formate, pH 6 buffer. Fractions containing the desired product were combined, lyophilized and then dissolved in 1 mL 100 mM ammonium formate, pH 6, and characterized. The product obtained had an HPLC retention time of 3.83 minutes with a purity of about 88.6%. This product showed an absorbance maximum at 246 nm with an extinction coefficient of 26,700 M⁻¹ cm⁻¹.

EXAMPLE 19

Synthesis of Hapten DB-VI

p-aminobenzyl-DTPA ("ABD") was prepared in a manner analogous to the preparation of ABE described above, using the teaching of Meares et al., U.S. Patent No. 4,622,420. BABE, prepared as indicated earlier, was indium-labeled as follows: Indium chloride (30.6 mM in

1.2 N HCl, 2.0 mL) was added to a solution of BABE (144 mM, 400 μ l) and the pH was adjusted to about 6.0 with 10 N and 1 N NaOH. The mixture was incubated for 30 minutes and then purified over an ethylenediaminetriacetate column. The product was eluted with 10 mM sodium acetate. Column fractions containing the desired product were combined and lyophilized.

The indium-loaded BABE (20 μ moles in 1 mL H₂O) was added to a solution of ABD (20 μ moles) in H₂O (0.5 mL). The pH was adjusted to 5.6 and the reaction was incubated at 37°C. overnight. When HPLC analysis indicated that the reaction was complete, the reaction mixture was purified on an A-25 column using a gradient running from 0.1 to 1.0 M triethylammonium formate, pH 8.7, to elute the material. Fractions containing the desired product were combined and lyophilized. The DB-VI obtained had an HPLC retention time of 3.3 minutes. The ¹³C NMR spectrum showed the following chemical shifts (dioxane as the internal standard): δ 178.8, 178.6, 178.3, 177.7, 175.6, 173.1, 173.0, 172.1, 149.4, 138.1, 133.1 (CH), 132.7 (CH), 127.0, 125.6 (CH), 116.8 (CH), 65.5 (CH), 64.0 (CH), 62.5 (CH₂), 60.6 (CH₂), 60.2 (CH₂), 59.6 (CH₂), 56.8 (CH₂), 56.5 (CH₂), 56.0 (CH₂), 55.7 (CH₂), 54.4 (CH₂), 51.3 (CH₂), 50.7 (CH₂), 35.0 (CH₂), 34.2 (CH₂).

The proton NMR (HOD as an internal standard) showed resonances at 7.31 (d, 1H, J= 8.4 Hz), 7.19 (d, 1H, J=8.4 Hz), 7.08 (d, 1H, J=8.4 Hz), 6.7 (d, 1H, J=8.4 Hz), 3.94 (s, 2H), 2.70 (m, 2H), 2.55 (m, 1H).

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EXAMPLE 20

Synthesis of Hapten DB-VIII

DB-VIII is prepared by reacting p-bromoacetamidobenzyl DTPA ("BABD") with indium-loaded DHPBE which is prepared from BABE and 1,3-diamino-2-hydroxypropane. The BABD is prepared in a manner analogous to that previously taught for the preparation of BABE and generally follows the teaching of DeRiemer, L.H., et al., J. Lab. Comp. and Radiopharm, 18, 1517 (1981) and U.S. Patent No. 4,622,420.

In particular, 1,3-diamino-2-hydroxypropane (28 mg) was dissolved in 50 mM HEPES buffer, pH 10 (1 mL) then BABE (24 mM, 1 mL), prepared as noted above, was added dropwise. The reaction was stirred overnight at room temperature until HPLC indicated that the reaction was complete. The reaction mixture was diluted to 60 mL and applied to a Sephadex DEAE A-25 column (10 mL) which was equilibrated with 100 mM triethylammonium formate, pH 8.7. The column was eluted with a 400 mL gradient running from 100 mM to 1.0 M triethylammonium formate, pH 8.7. The fractions were analyzed by HPLC and those fractions containing the product were combined and lyophilized. The product was dissolved in water (1 mL, 24 μ moles) and indium chloride (30.6 mM, 400 μ l) was added. The pH was raised to about 5.8 and the solution was incubated for 30 minutes. Because the mixture showed that the product and starting material had the same retention times, excess indium chloride (600 μ l) was added. The pH was adjusted to about 6.0 and the material was applied to an ethylenediaminetriacetate column. The

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column was eluted with 10 mM sodium acetate and the fractions containing the product were combined and lyophilized.

The indium loaded DHPBE (20 μ moles) obtained was dissolved in water (1 mL) and added dropwise to BABD (40 μ moles). The pH was adjusted to about 9.0 and the reaction was stirred at 37°C for about six days. HPLC indicated that several products were formed. After repeated purifications over A-25 and BioRad Affi-gel-102 columns, a single product, designated DB-VIII was obtained. This product had an HPLC retention time of 3.2 minutes.

EXAMPLE 21

SYNTHESIS OF DB-IX

a) Reaction of tris(2-aminoethyl)amine and ITCBE:

Tris(2-aminoethyl)amine (280 μ L) was dissolved in water (17 mL) and ITCBE (1.6 mL, 117mM) was added dropwise. The reaction was stirred at room temperature for 30 minutes and HPLC analysis indicted the reaction was complete. The reaction mixture was diluted with water (100 mL final volume) and loaded onto an Sephadex DEAE A-25 column(=35mL). The column was washed with water until the eluant was fluorescamine negative (the flow through contained a large amount of product). The column was eluted with a gradient (500 mL) from 100mM to 1M triethylammonium formate, pH 8.6. Column fractions were combined based on HPLC analysis. The product was fluorescamine positive and the NMR data indicated that it was the desired material. This desired material was

called Compound A.

b) Indium loading of Compound A: Compound A was dissolved in water (4 mL) and InCl_3 (30 mM, 5.9 mL) was added. The solution was stirred for 45 minutes and then the pH adjusted to 9. The solution was filtered through a 0.2 μm acrodisc and analyzed by HPLC. Using the 20 cm HP reversed phase column and isocratic elution with 92% Buffer A and 8% Buffer B, the indium-loaded material eluted at 4.5 minutes and the metal free material at 4.2 minutes.

c) Reaction of Compound A and ITCBD: ITCBD (84 mM, 400 μL) was neutralized with 1M Na_2CO_3 and this solution was added to Indium-loaded Compound A (4 mL, concentration unknown, but excess amine). The reaction was stirred at room temperature, pH 8, overnight. HPLC analysis indicated that one new product (3.9 minute) had formed, with a small amount of ITCBD and a large amount of the amine still present. The reaction mixture was diluted to 100 mL with water and loaded onto an Sephadex DEAE A-25 column (35 mL). The column was washed with water (50 mL) and eluted with a gradient (500 mL) of from 100mM to 1M ammonium formate, pH 8. The column fractions were analyzed by HPLC and combined. HPLC analysis indicates that this compound does bind both indium (4.1 min) and yttrium (4.3, 4.4 min). This compound (16 μmol) was labeled with $^{111}\text{Indium}$ and analyzed for CHA-255 binding as described elsewhere in these Examples. The CHA binding was 91% and the Ambis scan of the TLC plate showed the

presence of Indium labelling.

EXAMPLE 22

SYNTHESIS OF DB-X

a) Indium p-aminobenzyl-EDTA (In-ABE) (50mM, 250 μ l) was mixed with p-aminobenzyl-DTPA (137mM, 100 μ l) and the solution diluted with 0.5M NaHCO₃, pH 9 (350 μ l). Excess N,N-disuccinimidyl carbonate was added and the reaction vortexed periodically. Three products were evident in the HPLC analysis (3.2, 3.7, and 4.2 min) in a 1:2:1 ratio, consistent with urea formation. The reaction mixture was diluted to 50mL with water and applied to an A-25 column (12 mL). The column was eluted with a gradient (200 mL) of 100mM to 1M ammonium formate, pH 8. Fractions were combined based on HPLC analysis and the fraction containing the 3.7 min peak analyzed. HPLC analysis showed that this compound bound both indium (4.2 min) and yttrium (4.3 and 4.4 min). UV analysis showed the compound had an absorbance maximum at 260 nm. Incorporation of ¹¹¹Indium (as explained above) was >95% and CHA-255 binding of the ¹¹¹Indium (III) was 94%. The ¹³C NMR spectrum contained a signal at δ 156.5, consistent with a urea carbon, as well as signals corresponding to Indium benzyl EDTA and benzyl DTPA. The proton NMR spectrum was consistent with the assigned structure.

NMR (¹³C, D₂O, pH=4.5) δ : 177.2, 176.7, 176.6, 176.3, 171.2, 171.1, 155.8, 138.3, 138.0, 132.2, 131.1, 130.7(CH), 121.7(CH), 63.4(CH), 62.0(CH), 60.6(CH₂), 58.7(CH₂), 58.2(CH₂), 57.7(CH₂), 55.8(CH₂), 55.0(CH₂), 54.3(CH₂), 54.0(CH₂), 52.4(CH₂), 49.5(CH₂), 33.0(CH₂),

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32.5(CH₂); (proton NMR, D₂O, pH=4.5) δ : 7.25(d, 2H, J = 7.8 Hz), 7.23(d, 2H, J = 7.8 Hz), 7.15(d, 2H, J = 7.8 Hz), 7.09(d, 2H, J = 7.8 Hz), 2.20 - 3.80(32H).

EXAMPLE 23

Preparation of DDB-I

A. Preparation of t-Boc-(L)-p-nitrophenylalanine

BOC-ON (12.9 g., 47.6 x 1.1), dissolved in 28.6 mL of dioxane, was added to a slurry of (L)-p-nitrophenylalanine (10.0 g, 47.6 mmol) and triethylamine (9.95 mL, 47.6 x 1.5 mmol) in water (28.6 mL) at room temperatures under nitrogen atmosphere. The reaction mixture became dark orange after 30 minutes. After stirring for 1.5 h, water (100 mL) and ether (100 mL) were added. The aqueous layer was washed with ether (4x100mL) and acidified to pH 1 using 3 M HCl. The aqueous layer was then extracted with ethyl acetate (5x100 mL). The organic layer was dried over anhydrous sodium sulfate and rotoevaporated to give 11.8 g (79.2%) of yellowish waxy solid:

TLC [developed in 5:1 chloroform:methanol, silica gel (Sybron-Brickman)] R_f =0.66 (R_f of (L)-nitrophenylalanine=0.06).

B. Preparation of t-Boc-(L)-p-nitrophenylalnine-N-hydroxysuccinimide ester .

Solid N-hydroxysuccinimide (4.30 g, 37.7 mmol) was added to a solution of t-Boc-(L)-p-nitrophenylalanine (11.70 g, 37.7 mmol) in dioxane (187 mL) under a nitrogen

atmosphere. Solid DCC (8.54 g, 1.1 x 37.7 mmol) was added at room temperature. After stirring for 5.5 hours, the precipitate (urea) was filtered off and the filtrate was air-dried. The residue was taken up in hot 2-propanol and crystallized. The light brown powdery crystals were collected to give 10.66 g (69.4%) of product.

TLC[silica gel developed in chloroform:methanol (20:1)] R_f =0.64 (R_f of t-Boc-(L)-p-nitrophenylalanine=0.50).

C. Preparation of t-Boc-(L)-p-nitrophenyl-glycyl-glycyl-glycine.

A solution of t-Boc-(L)-p-nitrophenylalanine-N-hydroxysuccinimide ester (10.0 g, 24.6 mmol in 49.2 mL of DMF) was added to a solution of glycyl-glycyl-glycine (7.2 g, 24.6 x 1.5 mmol) and sodium bicarbonate (3.10 g, 24.6 x 1.5 mmol) in 98 mL of water at room temperature. After stirring for 5 h, water (500 mL) was added. Upon acidification to pH 1 using concentrated HCl and cooling overnight, precipitate was observed. The precipitate was collected and dried under vacuum to give 10.78 g (91.1%) of product.

D. Preparation of (L)-p-nitrophenyl-glycyl-glycyl-glycine TFA salt.

t-Boc-(L)-p-nitrophenyl-glycyl-glycyl-glycine (10.0 g, 20.8 mmol) was dissolved in neat trifluoroacetic acid (100 mL) at 0° C. After stirring for 1 h, the acid was removed under reduced pressure to give clear yellow solid

product in quantitative yield (10.3 g).

E. Preparation of 11-p-Nitrobenzyl-1,4,10,12-tetraazadodecanol.

Over a period of 45 min, $\text{BH}_3\text{-THF}$ (1M) (171.7 mL, 17×10.1 mmol) was added to stirring solution of (L)-p-nitrophenyl-glycyl glycyl glycine-TFA salt (5.0 g, 10.1 mmol) in 50 mL of dry THF at 0°C under a nitrogen atmosphere. After complete addition, the reaction mixture was refluxed for 26.5 hours. The reaction mixture was then cooled in an ice-bath and methanol (50 mL) was added slowly followed by saturation with HCl(g) . After additional reflux for 1 hour, the reaction mixture was rotoevaporated to give a semi-solid residue. An aqueous solution of 2 M sodium hydroxide (25 mL) and water (50 mL) was added to the residue to adjust to pH 10 and the resultant solution was extracted with (800 mL) chloroform using a continuous extraction apparatus for 25.5 hours. The organic extract was dried over anhydrous sodium sulfate and rotoevaporated under high vacuum to give 2.02 g (61.5%) of a thick brown oil.

The NMR spectrum shows no side reduction of the nitro group; IR shows complete reduction of the amide and carboxyl carbonyl functionalities. NMR (CDCl_3 , 300 MHz) δ 2.2 (br.s, 6H), 2.5-3.0 (m, 14H), 3.1 (m, 1H), 3.6 (t, 2H), 7.36 (d, 2H), 8.15, (d, 2H).

F. Preparation of 11-p-Nitrobenzyl-N,N',N'',N''',O-(pentatoluenesulfonyl)-4,7,10,12-tetraazadodecanol.

Tosyl chloride (4.83 g, $1.1 \times 5 \times 4.62$ mmol) was added

in small portions to a solution of 11-p-nitrobenzyl-1,4,10,12,-tetraaza-dodecanol in 15 mL of triethylamine and 15 mL of acetonitrile with vigorous stirring under a nitrogen atmosphere at room temperature. After 5 h, the cloudy reaction mixture was rotoevaporated and the residue was taken up in chloroform (100 mL). The organic layer was washed with 1 N HCl (4x50 mL) and dried over anhydrous sodium sulfate. The organic phase was rotoevaporated then prepurified by vacuum flash chromatography (silica gel in dichloromethane) step eluted with 350 mL dichloromethane and 605 mL dichloromethane:ethyl acetate (10:1). The latter effluent from the column was rotoevaporated and purified by low pressure silica gel column chromatography to give 1.76 g (34.8%) of the title product:

^1H NMR ("300"-MHz CDCl_3) δ 2.30 (s, 3H), 2.50 (m, 12 H), 2.60-3.50 (m, 14H), 3.75 (m, 1H), 4.2 (t, 2H), 5.30 (d, 1H), 7.00-7.90 (m, 24H).

G. Preparation of 2-p-Nitrobenzyl-N,N',N'',N'''-(tetratoluenesulfonyl)-1,4,7,10-tetraazacyclododecane.

Cesium carbonate (0.52 g, 1.60 mmol) was added to a stirring solution of 11-p-nitrobenzyl-N,N',N'',N''',O-(pentatoluenesulfonyl)-4,7,10,12-tetraazadodecanol (1.75 g, 1.60 mmol) in dry DMF (160 mL) (commercial grade) under a nitrogen atmosphere. The resulting mixture was heated to 60° C. After 5 h, the reaction mixture was rotoevaporated under high vacuum. The residue was taken up in chloroform washed with water (3x50 mL) and dried

over sodium sulfate. The organic layer was prepurified by vacuum flash chromatography using silica gel eluted with 300 mL dichloromethane:ethyl acetate (10:1). The effluent was combined and purified by low pressure liquid chromatography (silica gel, Lobar) step eluted sequentially with dichloromethane:ethyl acetate(100:1), dichloromethane:ethyl acetate (25:1), dichloromethane:ethyl acetate(1:1). The appropriate fractions were combined and rotoevaporated to give 0.48 g (32.5%) of product:

^1H NMR (in CDCl_3) δ 2.40-2.60 (m, 12H), 3.00-4.20 (m, 16H), 4.50 (m, 1H), 7.00-7.50 (m, 12H), 7.60-7.90 (m, 6H), 8.15 (d, 2H).

H. Preparation of 2-p-Nitrobenzyl-1,4,7,10-tetraazacyclododecane.

Concentrated sulfuric acid (3 mL) was added to a mixture of 2-p-nitrobenzyl-N,N',N'',N'''(tetratoluenesulfonyl)-1,4,7,10-tetraazacyclododecane (0.10g, 0.108 mmol) and phenol (0.16g, 16 x 0.108 mmol) at room temperature under nitrogen atmosphere. The mixture was immediately heated to 100°C. The reaction slowly turn to reddish brown. After 48 h, the reaction mixture was poured over 20 mL of ice and saturated aqueous sodium hydroxide was added to raise the pH to 3-4. Final volume of the mixture was approximately 50 mL. Analysis of the total reaction mixture was carried out on reversed phase column chromatography (Microsorb C-18, IBM system). Preparative scale purification was carried out on the Lobar column

(C-18 support eluted with 20% acetonitrile in water containing 0.1% trifluoroacetic acid) the yield ranges from 64.8 to 82.5%. NMR (D₂O, pH 7, 300 Mhz): δ 2.80-3.50 (m, 17H), 7.50 (d, 2H), 8.20 (d, 2H).

I. Preparation of 2-p-Nitrobenzyl-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (p-Nitrobenzyl-DOTA).

Bromoacetic acid (0.24 g, 4 x 0.43 mmol) was added to a solution of 2-p-nitrobenzyl-1,4,7,10-tetraazacyclododecane (0.13 g, 0.43 mmol) in water (3 mL), pH 10, at room temperature. The mixture was heated to 60 °C and the pH was maintained at 10 by the addition of 2 M KOH. After 5 h, the reaction mixture was neutralized with 3 M HCl and analyzed by reversed phase HPLC (Microsorb C-18, IBM system). Preparative scale purification was carried out on the Millipore liquid chromatography system using C-18 reversed phase column (LiChroprep® RP 18, EM Science) eluted with 20% MeOH in 0.1M ammonium acetate at pH6, to give 0.103 g of the title product. The yield for this step ranges from 23.8 to 34.3%.

HPLC analysis shows identical retention time as the authentic sample of p-nitrobenzyl-DOTA. NMR (300 Mhz, D₂O, pH 5): δ 2.75-4.00 (M, 25H), 7.5 (d, 2H), 8.20 (d, 2H).

J. Preparation of 2-p-Aminobenzyl-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid.

2-p-Nitrobenzyl-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (50 mg, 0.093 mmol) was

dissolved in water (20 mL) and the pH was adjusted to 11 using aqueous KOH. The mixture was cooled in an ice bath and 120 mg of 5% Pd/C was added. The system was first flushed with nitrogen then with hydrogen gas. The system was maintained under a hydrogen atmosphere for 4 h.

(During the course of the reaction, the reaction mixture gave a transient greenish color.) The reaction mixture was neutralized with 3M HCl and filtered through a Gelman acrodisc (0.2 micron). The filtrate was fluorecamine positive, indicating the presence of amino groups. After lyophilization, the solid residue was analyzed by C-18 HPLC eluted with 20% MeOH in 0.1 M ammonium acetate at pH 6. (retention time = 4.2 min, R_f for the starting material = 6.71 min).

K. Preparation of p-Isothiocyanatobenzyl-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid, (i.e. DITC).

Redistilled thiophosgene (0.0345 mL, 0.450 mmol) was added to a solution of 2-p-aminobenzyl-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (45 μ moles) in 0.450 mL of 3 M HCl at room temperature. Completion of reaction was determined with a fluorecamine test. After 5 h, the excess thiophosgene was extracted with ether (3x5 mL). The aqueous layer was rotoevaporated to give yellow solid product.

The IR spectrum shows characteristic isothiocyanate N=C=S stretch at 2100 cm^{-1} .

HPLC analysis shows retention time at 5.5 min. Upon reaction with ammonia, the product formed has a retention

time of 2.756 min.

L. Preparation of DDB-I.

Indium aminobenzyl-EDTA, (In-ABE) wherein the In(III) used is non-radioactive ($0.9 \times 15 \mu\text{mol}$, 0.8 mL of 16.6 mM soln) was added to previously prepared p-isothiocyanatobenzyl-DOTA (DITC) ($15 \mu\text{mol}$, 300 μL of 50 mM soln, pH 6 adjusted by adding 1 M K_2CO_3). After stirring for 19 h at room temperature the reaction was stopped by proceeding to the purification step. The reaction mixture was purified by C-18 reversed phase column (LiChroprep® RP18, EM Science) eluted with a step gradient of 8% and 16% MeOH in 0.1 M ammonium acetate, pH 6. The product eluted at 16% MeOH to give $2.5 \mu\text{mol}$ (16.6%) isolated yield. FABMS: 1061, $[\text{M}+\text{H}]^+$; HPLC retention time = 3.40 min (Retention time for In-ABE = 2.74, DITC = 5.60 min.)

M. CHA-255 Assay for DDB-I

A small amount of (20 μL) of a solution of 20 μM DDB-I (400 pmol) in ammonium glycinate (pH 9.5, 220 mM) was mixed with 2 μL of a $^{90}\text{YCl}_3$ solution (8.2 pmol). The pH of the resultant solution was 8. After incubation for 10 minutes, 2.9 mL of 150 mM ascorbate quench buffer was added. A 10 μL aliquot of this mixture was mixed with a solution containing 1 mL PBS, 10 μL of 5% Triton-X, and 10 μL of CHA-255 (0.2 mg/mL) .01 mM, pH 7.4. After incubation for 15 minutes, the solution was analyzed (Centrifree® method as described herein).

As a control, a similar assay was done for $^{90}\text{Yttrium}$ -

DOTA as described below:

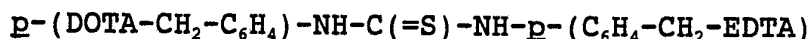
Two hundred microliters of a 20 μ M DOTA (400 pmols) solution in ammonium glycinate (pH 9.5, 220 mM) was mixed with 20 μ L of $^{90}\text{YCl}_3$ (82 pmol). The pH of the resultant solution was 8. After incubation for 10 minutes, 380 μ L of the quench buffer described above was added. A 10 μ L aliquot of this mixture was mixed with a solution containing 1mL PBS, 10 μ L 5% Triton-X, and 10 μ L of CHA-255 (0.2 mg/mL). After incubation for 15 minutes, the solution was analyzed (Centrifree® method).

The results from these experiment are as follows:

<u>Chelate</u>	<u>Ab/Chelate</u>	<u>[CHA-255]</u>	<u>[Chelate]</u>	<u>% bound to CHA-255</u>
^{90}Y -DOTA	9	12.5nM	1.37nM	9%
^{90}Y -DDB-I	9	12.5nM	1.37nM	95%

These results confirm the presence of the In-benzyl EDTA moiety in the DDB-I structure.

The structure of DDB-I (minus the metal ions) is shown below:



C. MONOCLONAL ANTIBODY PREPARATION

PREPARATION 1

Preparation of Monoclonal Antibody CHA255

Antibody producing hybridoma cell lines were prepared as follows. Spleen cells from BALB/c mice multiply immunized with the antigen were fused with a variant of the P3.653 myeloma cell line. See Gerhard, Monoclonal Antibodies, edited by Kenneth et al., Plenum

Press, New York (1980). The resulting hybridomas were screened by a solid phase second antibody radioimmunoassay for their ability to bind indium aminobenzyl-EDTA (Wang et al., Journal of Immunological Methods, 18, 157, (1977)). Based on their high titers and relatively high affinity as determined by inhibition of binding by unlabeled antigen, a monoclonal antibody designated as CHA255 was chosen for further study and injected intra-peritoneally into BABL/c mice for ascites production. The monoclonal antibodies were purified from mouse ascites by ion-exchange chromatography on DEAE-cellulose as described by Parham et al., J. Immunol. Meth., 53, 133 (1982). Monoclonal antibody CHA255 is further described by Reardon, D.T. et al., Nature 316, pp 265-268 (1985) and Meares et al., U.S. Patent No. 4,722,892, issued February 2, 1988, herein incorporated by reference.

Preparation 2

Preparation of Bifunctional Antibody ECHO37.2

Bifunctional antibody ECHO37.2 was prepared by the formation of a tetradoma by the biological fusion of the hybridoma cell line expressing monoclonal antibody CHA225, prepared as described in Example 2A, and a hybridoma cell line, designated CEM231.6.7, expressing antibody having specificity for carcinoembryonic antigen (CEA). Hybridoma cell line 231.6.7 was deposited with the American Type Culture Collection under the Budapest Treaty on January 7, 1988 (ATCC Accession No. HB 9620). Thus, the bifunctional antibody produced has specificity

for both indium benzyl-EDTA and CEA. The tetradoma which expresses the bifunctional antibody designated as ECH037.2 was prepared by biological fusion of the hybridoma cell lines CHA255 and CEM231.6.7 as described by Burnett, K.G. et al. in Biotechnology: Applications and Research, pp. 401-409, edited by Cheremisinoff and Ouellette, Technomic Publishing Company, Pennsylvania (1985). ECH037.2 was purified from culture fluid in a two step process. IgG molecules were first isolated from other components of the culture medium by chromatography on DEAE-cellulose, as described by Parham et al., J. Immunol. Meth., 53, 133 (1982), followed by further purification by HPLC on hydroxylapatite (BioRad Laboratories, Richmond, CA). The ECH037.2 bifunctional antibody was eluted using a gradient from 10 mM to 160 mM sodium phosphate, pH 6.8. Both buffers also contained 10 mM Ca^{2+} to prevent column degradation. The bifunctional antibody ECH037.2 was identified by the pattern of protein bands generated with electrophoresis in 7.5% acrylamide gels (SDS), and the ability of the antibody to bind both indium-benzyl EDTA chelates and CEA.

Preparation 3

Hapten capacity assay:

To determine the ability of the antibody to bind indium benzyl-EDTA, the antibody and the In(III) chelate of (S)-4-p-nitrobenzyl EDTA were combined in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.5) containing 1.5 mg/mL normal serum albumin. Antibody concentration was 0.15 mg/mL and the Indium (S)-4-p-nitrobenzyl EDTA

concentration was 3 μ M. The chelate was radiolabeled at a specific activity of 0.5-3.0 μ Ci/nmol with ^{111} Indium. After a 5 minute incubation at room temperature, duplicate 400 μ l aliquots of the mixture were transferred to separate Centrifree[®] ultrafiltration devices (NMW cutoff = 30,000; available from Amicon, Danvers, Massachusetts). These were centrifuged at 1500 g for 10 minutes. Duplicate 50 μ l pre-Centrifree[®] samples were counted and averaged. This value was called "TC". Duplicate 50 μ l aliquots of the Centrifree[®] filtrates were counted and averaged. This value was called "F". The percent of theoretical capacity was calculated from the following equation:

$$(1 - F/TC) \times M$$

M was assigned a value of 150, 100, 200 or 300 for intact antibody (CHA255), Fab fragment, F(ab)'₂, bifunctional antibody and intact bifunctional antibody (ECHO37.2), respectively.

To test the ability of the antibody to bind CEA, an immunoreactivity assay was designed to determine the percentage of the radiolabeled anti-CEA antibody preparation which bound to CEA. This value was determined by a two site immunoassay employing solid phase CEA bound to a polystyrene bead by a second antibody reactive with CEA, essentially as described in U.S. 4,376,110. Antigen positive and negative beads were prepared by incubating CEA beads, obtained from the commercially available TANDEM[®]-R CEA test kit (Hybritech Incorporated, San Diego, California) in high CEA and zero calibrators. After unbound CEA antigen was washed off

the beads, approximately 1 ng of the antibody radiolabeled with ^{125}I to a specific activity of $10\ \mu\text{Ci}/\mu\text{g}$ was added. The percent immunoreactivity was determined by calculating the percent of counts bound to the bead in the presence of antigen. A non-specific binding control was determined by the percent of counts bound to beads that have no CEA antigen present.

Preparation 4

Antibody CHA255 Binding Assay

As indicated, the monoclonal antibody CHA255 is specific for the In(III)-benzyl EDTA complex. The affinity and characterization have been described previously in Reardon et al. in Nature, 316, 265 (1985). Binding assays were performed to verify the identity of the haptens designated as DB-I to DB-VIII and DDB-I. Under the conditions of the assay, neither $^{90}\text{Yttrium}$ (III) or $^{111}\text{Indium}$ (III) DTPA, alone, bound to CHA255. For the $^{90}\text{Yttrium}$ (III) hapten chelate, in particular, CHA255 binding required the presence of the Indiumbenzyl-EDTA moiety as provided in the compounds of the invention. The labeled DB and DDB hapten chelates were combined with antibody CHA255 in PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) containing $44\ \mu\text{g}/\text{mL}$ of human serum albumin and 0.05% Triton-X-100 detergent. Concentrations of the radiolabeled hapten chelating agent and antibody are 5 nM and 133 nM, respectively. The mixture is vortexed and allowed to stand at room temperature for 15 minutes, after which it is transferred to an Amicon Centrifree® device, with a NMW limit of

30,000, and centrifuged to separate antibody-bound chelate from free hapten chelate. The species bound to the antibody is retained by the membrane and the free chelate flows through the membrane into the filtrate. The extent of antibody-hapten chelate binding is then determined.

C. IN VITRO CHARACTERIZATION OF DERIVATION HAPTENS

Procedure 5

Determination of Hapten-Antibody Dissociation Rates

Hapten-antibody dissociation rates were determined using monoclonal antibody CHA255, prepared as described in Preparation 1, by measuring the quantity of $^{111}\text{Indium}$ (III) chelate released from the antibody hapten complex as a function of time after addition of a 100 fold excess (over antibody binding sites) of $^{115}\text{indium}$ loaded hapten. Thus, for each hapten, a 700 μl solution was prepared containing: antibody at a concentration of 160 nM; indium-loaded hapten, trace labeled with $^{111}\text{Indium}$ (total concentration = 160 nM); normal human serum albumin at a total concentration of 1.5 mg/mL; and isotonic phosphate buffered saline. (10 mM PO_4^{2-} , 150 mM NaCl, pH 7.2). $^{115}\text{Indium}$ loaded hapten was added to a final concentration of 32 μM . Reactions were stirred by placing the sample container in a rack mounted on a vortexer head.

At various times after addition of the excess hapten, 400 μl portions of the reaction solutions were transferred to Centrifree® tubes (Amicon Corporation, Danvers, Massachusetts) and centrifuged at 1500xg for eight to ten minutes. The reaction time was taken to be

the time between addition of the excess hapten and the time the centrifuge reached the desired velocity. Six time points, each in duplicate, were obtained over a range of about two reaction half-lives for each rate constant determination. A 200 μ l portion from each centrifuged and uncentrifuged solution was then counted for ^{111} Indium. The relative concentration of free hapten was taken to be the ratio of counts in the centrifuged solution to the uncentrifuged solution at each timepoint. Times were recorded to the nearest tenth of a minute.

Room temperature rate constants were measured without the aid of a constant temperature bath. The ambient temperature remained at $22^{\circ}\pm 1^{\circ}$ C.

Rate constants and standard deviation were obtained from a plot of the logarithm of relative complex concentration vs. time. Data were analyzed using the linear regression module of the RS/1 Program (BBN Software Products Corporation, Cambridge, Massachusetts).

Control samples were included in each experiment to determine: A) that the initial condition of 100% of labeled hapten bound to antibody was met, and B) that in the absence of antibody, 100% of counts were able to penetrate the Centrifree® membrane. If control A showed that a significant portion of counts did not bind antibody initially, the average number of counts in the control duplicates was subtracted from all centrifuged and uncentrifuged sample solutions. If control B showed that not all counts penetrated the membrane, each count ratio (i.e. the relative concentration of free hapten) was divided by the average of the fraction penetrating

the membrane in the control duplicates. The antibody-hapten dissociation rates, expressed as reaction half-lives ($t_{1/2}$ minutes) shown in Table III.

TABLE III

22°C Dissociation Half-Lives of Selected Haptens

<u>Hapten Abbreviation</u>	<u>$t_{1/2}$ (min)</u>	<u>S.D.*</u>	<u>-r**</u>
GNUBE	98.8	1.2	0.999
BETUBE	94.2	3.0	0.995
BALTUBE	90.9	1.2	0.999
BUTUBE	90.6	2.0	0.998
PATUBE	87.8	2.6	0.996
TUBE	84.2	2.5	0.996
PHTUBE	75.0	0.8	0.999
BATUBE	74.3	1.8	0.997
ETUBE	72.6	1.3	0.998
PHATUBE	71.5	0.8	0.999
EOTUBE	59.7	1.5	0.997
NUBE	45.6	0.8	0.998

* standard deviation

** correlation coefficient

As shown in Table III, haptens of the invention dissociate from the antibody at rates which vary over a wide range. Thus, a hapten may be selected or designed to dissociate slowly from the antibody for applications such as therapy in which total dose to the target is the most important consideration. Conversely, haptens with shorter half-lives may be more advantageous for applications such as imaging, in which signal (at target) to noise (for example, blood pool) is the most important factor, or in protocols which require transfer of haptens between antibodies.

D. IN VIVO CHARACTERIZATION OF DERIVATIZED HAPTENS

Procedure 6

Determination of Hapten Biodistributions

Two protocols were used for examining the biodistribution of the derivatized haptens in the presence of the bifunctional antibody ECH037.2. In a premix protocol, ECH037.2 and the derivatized hapten were combined prior to injection in the animals. In the prelocalization protocol, ECH037.2 was injected into the animals, and the antibody was allowed to prelocalize for 24 hours prior to injection of the radiolabeled hapten.

Biodistribution studies were performed in nude mice carrying subcutaneous implants of the human colon carcinoma cell line T-380 which expresses the carcinoembryonic antigen (CEA) recognized by ECH037.2. Briefly, Balb/C Nu/Nu mice were injected subcutaneously with a minced preparation of T-380 tumor implants and

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maintained for approximately two weeks, or until the tumor implant had grown to a size of approximately 0.5 grams as described by Hagan, P.L., et al. J. Nuc. Med., 26 1418-1423 (1985). Mice were kept in sterile environment to prevent complications due to bacterial or viral infections. Biodistributions were performed as described by Hapern, S., et al. , Cancer Res. 43, 5347-5355 (1983). Briefly, the radiolabeled haptens were injected intravenously in normal mice, and the pharmacokinetics were evaluated at several time points post-injection. At these times, mice were sacrificed and weighed. Organs were removed, their weight recorded, and each organ was then washed in sterile PBS, pH 7.0, and fixed in formalin solution. Samples of blood, urine and feces also were collected and their weight similarly recorded. The organs, or a portion thereof, were transferred to clean tubes and their radioactive content was determined in a well-type gamma-counter.

Relative radioactive content of each organ was expressed as a percentage of injected dose per organ.

Procedure 7

Preparation of Samples for Biodistribution--Premix Protocol

To indium-label the haptens of the invention 10 μ l of 0.1 mM ligand was combined with X μ l of $^{111}\text{InCl}_3$ (in 50 mM HCl) and (x+10) μ l of 0.26 M ammonium citrate, pH 6.0. X is calculated by the following equation:

$$X = (200 \mu\text{Ci}) / (A \mu\text{Ci}/\mu\text{l})$$

where A is the specific activity of the $^{111}\text{Indium}$ (III).

The labeling was done in an acid-washed microfuge tube. After combining all of the components above, the solution was vortexed and allowed to stand at room temperature for 15 minutes.

Incorporation of the $^{111}\text{Indium}$ (III) into the chelating agent was determined by TLC on silica in a methanol/aqueous ammonium acetate solvent system as described by Meares, et al. (Anal. Biochem., 142, 68-75 (1984)). Greater than 95% incorporation of the radioactivity into the chelate was required for the sample to be submitted for a biodistribution assay.

Prior to injection the $^{111}\text{Indium}$ (III)-labeled hapten was diluted into freshly prepared isotonic sodium bicarbonate (1.39% w/v) containing 1 mg/mL normal human serum albumin (Buminate, 25% solution, Travenol Laboratories, Glendale, California) to which the bifunctional antibody had been added. The final composition of the solution was 0.2 mg/mL ECH037.2, 0.5 μM chelate, 0.1 $\mu\text{Ci}/\mu\text{l}$ $^{111}\text{Indium}$, all in a volume of 2.0 mL. For the biodistribution studies, 100 μl of the solution was injected into the tail vein of each animal.

Table IV and V below summarize the results of the biodistribution (expressed as percentage of injected dose/gram of organ) of the different haptens as determined using the premix protocol described above.

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Table IV

COMPARISON OF VARIATIONS OF HAPTENS USED IN A PREMIX
PROTOCOL WITH 20 μ g ECHO37.2 IN NUDE MICE BEARING T-380 TUMORS

(% DOSE PER GRAM AT 48 HOURS)

<u>HAPTEN</u>	<u>BLOOD</u>	<u>KIDNEY</u>	<u>LIVER</u>	<u>TUMOR</u>	<u>URINE*</u>	<u>FECES*</u>	<u>TOTAL TUMOR/ RECOV</u>	<u>BLOOD</u>
ETUBE	5.9	1.2	2.4	10.4	36.0	15.2	66.6	1.76
BETUBE	5.5	1.5	1.9	7.5	12.0	29.0	54.2	1.36
PATUBE	7.6	1.7	2.4	9.8	36.0	8.9	61.7	1.29
BATUBE	7.9	1.5	2.0	12.1	30.4	21.1	68.3	1.53
BUTUBE	5.8	1.2	3.8	5.7	27.0	26.5	69.3	0.98
OTUBE	0.5	0.2	0.6	0.7	10.9	68.4	81.4	1.40
TUBE	3.8	1.1	1.6	6.5	48.8	9.8	69.5	1.71
EOTUBE	4.7	1.4	3.1	14.5	49.3	7.9	72.3	3.09
BALTUBE	5.2	1.9	2.2	12.9	26.5	13.8	57.8	2.48
PHTUBE	5.4	1.7	2.3	10.6	24.5	21.7	62.4	1.96
PHATUBE	3.7	0.8	1.1	6.9	22.4	34.9	66.4	1.86
NUBE	2.0	2.5	1.9	8.1	52.6	12.9	74.1	4.0
GNUBE	8.0	1.5	2.4	12.0	26.9	17.8	63.1	1.49

* Urine and Feces are percent of total injected dose

TABLE V

COMPARISON OF VARIATIONS OF HAPTENS USED IN A PREMIX
 PROTOCOL WITH 20 μ g ECHO37.2 IN NUDE MICE BEARING T-380 TUMORS
 (% DOSE PER GRAM AT 24 HOURS)

<u>HAPTEN</u>	<u>BLOOD</u>	<u>KIDNEY</u>	<u>LIVER</u>	<u>TUMOR</u>	<u>URINE*</u>	<u>FECES*</u>	<u>TOTAL RECOV</u>	<u>TUMOR/ BLOOD</u>
ETUBE	10.4	2.4	3.5	8.0	25.8	5.9	52.2	0.77
BETUBE	9.0	2.5	2.7	8.0	8.4	17.4	44.6	0.89
PATUBE	12.2	2.2	3.3	10.6	23.6	5.0	53.7	0.87
BATUBE	11.8	2.5	3.3	11.5	20.0	6.9	50.8	0.97
BUTUBE	9.0	1.7	3.4	5.3	22.0	16.8	63.8	0.59
OTUBE	1.1	0.5	0.8	1.1	8.9	56.5	68.9	1.00
TUBE	8.0	2.4	4.0	8.5	23.7	4.1	48.2	1.06
EOTUBE	9.3	2.8	3.8	12.5	25.9	6.2	55.6	1.34
BALTUBE	11.0	3.6	4.0	13.0	16.3	6.8	52.0	1.18
PHTUBE	8.9	2.9	3.6	12.4	20.3	13.9	57.2	1.39
PHATUBE	6.2	1.2	1.7	8.4	21.7	22.0	59.7	1.35
NUBE	5.9	4.0	3.1	8.5	42.5	5.9	66.3	1.44
GNUBE	13.0	2.7	3.4	9.0	14.2	10.6	52.0	0.70

* Urine and Feces are percent of total injected dose

Figure 1 shows the absolute uptake in the tumor and Figure 2 shows the variation in the tumor/blood ratio for the different haptens. In the figures, the designations "e", "be", "pa", "ba", "bu", "o", "eo", "bal", "ph", and "pha" refer to ETUBE, BETUBE, PATUBE, BATUBE, BUTUBE, OTUBE, EOTUBE, BALTUBE, PHTUBE, and PHATUBE, respectively. As shown in Tables IV and V and Figures 1 and 2, the biodistribution obtained is dependent upon the identity of the hapten. For example, if the simplest hapten TUBE is compared to those derivatives with

increasing aliphatic chain length, (ETUBE, BUTUBE and OTUBE), some trends in physiologic behavior may be observed. The biodistribution of ETUBE is very similar to that of TUBE, but as chain length increases (and hydrophobicity increases as shown by the HPLC data determined in Examples 1 through 22 and shown in Table VI below), the amount of hapten in the tumor diminishes (eg., 8.0, 5.3, and 1.1 percent injected dose/gram at 24 hrs. and 10.4, 5.7 and 0.7 percent injected dose/gram at 48 hrs. for ETUBE, BUTUBE and OTUBE, respectively). For the same series of compounds a dramatic increase is seen in the amount of material excreted through the gut and into the feces. For example, at 48 hrs. post injection, 15.2, 26.5 and 68.4 percent of the injected material was excreted in the feces for ETUBE, BUTUBE and OTUBE, respectively. In general, as the lipophilicity (as indicated by HPLC retention time) of the compounds increased, so did the amount of material excreted through the intestines and out as feces in the premix protocol. This is shown in Table VI, below.

TABLE VI

<u>Compound</u>	<u>HPLC Retention Time</u>	<u>Percent Injected</u> <u>Dose in Feces</u>	
		<u>24 hrs.</u>	<u>48 hrs.</u>
NUBE	2.6 min	5.9	12.9
TUBE	2.8	4.1	9.9
PATUBE	3.0	5.0	8.9
BATUBE	3.1	6.9	21.1
EOTUBE	3.1	6.2	7.9
GNUBE	3.2	10.6	17.8
BALTUBE	3.5	8.5	13.8
PHATUBE	3.5	22.0	34.9
ETUBE	3.6	6.7	15.2
PHTUBE	4.5	13.9	27.1
BUTUBE	4.8	16.8	26.5
BETUBE	5.1	17.4	29.0
OTUBE	7.4	56.5	68.4

As can be seen from Table VI, those compounds with HPLC retention times greater than 4 minutes were judged to be undesirable as potential imaging agents because of the difficulty which may be encountered in trying to image haptens in the abdominal cavity due to the high levels observed in the intestines. It is also interesting to compare the compounds ETUBE, PATUBE, EOTUBE and NUBE in which the terminal carbon has a carboxyl, a hydroxyl or an amino group within this series. The compound EOTUBE stands out with its outstanding tumor uptake (Figure 1) and good tumor/blood ratios (Figure 2).

Procedure 8Preparation of Samples for BiodistributionPrelocalization Protocol

To indium-label the chelating agents for the prelocalization protocol, 5 μ l of 0.1 mM chelating agent was combined with X μ l of $^{111}\text{InCl}_3$ (in 50 mM HCl) and (x+5) μ l of 0.26 M ammonium citrate, pH 6.0. X was calculated by the following equation:

$$X = 200 \mu\text{Ci}/A \mu\text{Ci}/\mu\text{l}$$

where A is the specific activity of the $^{111}\text{InCl}_3$ solution. The solution was vortexed and allowed to stand at room temperature for 15 minutes, after which incorporation of $^{111}\text{Indium}$ label into the chelating agent was determined as described for the premix protocol. If at least 95 percent of the $^{111}\text{Indium}$ (III) was bound by the chelating agent, the material was then diluted to a final volume of 2 mL with isotonic (1.39 percent, w/v) sodium bicarbonate containing 1 mg/mL normal human serum albumin. This material (100 μ l) then was injected into each tail vein of mice which had each been injected 24 hours earlier with 5 μ g of ECH037.2 in 100 μ l isotonic sodium bicarbonate containing 1 mg/mL normal human serum albumin.

Four of the haptens, TUBE, EOTUBE, NUBE and PHATUBE were further subjected to the prelocalization biodistribution protocol as described above. These results are summarized in Figures 3, 4 and 5, and the tumor/organ ratios are shown in Figures 6-8.

As can be seen in Figures 3-5, PHATUBE is

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characterized by the high degree of fecal excretion, when compared to the other compounds, which agrees with the results seen with the premix protocol. Thus, although the tumor/organ values found for this hapten are exceptional (Figures 6-8), the high degree of fecal excretion for all time points suggests that this hapten would not be ideal as an imaging agent.

The biodistribution of EOTUBE is characterized by high tumor uptake ratios at all time points (Figures 3-5) and high tumor/organ ratios at 24 hours (Figure 7). For this reason, this hapten was selected as the optimal imaging agent from the series of four compounds examined using this protocol.

Table VII shows the average tissue localization properties of ¹¹¹Indium-labeled DB-I, DB-II, and DB-III when combined with 20 µg of ECH037.2 and injected into nude mice carrying T-380 tumor implants. Each entry (% Dose per gram) for 24 hours represents the average of 5 replicates for DB-I, DB-II and DB-III and the data for DB-I, DB-II, and DB-III at 48 hours is the average of 6 replicates. The standard deviations are shown for each value.

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Table VII

Tissue Localization of DB-I, DB-II, and DB-III

% Dose per Gram

24 Hours Post-Injection

<u>Hapten</u>	<u>Blood</u>	<u>Kidney</u>	<u>Liver</u>	<u>Tumor</u>	<u>Urine*</u>	<u>Feces*</u>
DB-I	10.1 $\pm$ 1.6	2.4 $\pm$.57	3.6 $\pm$ 1.3	7.7 $\pm$.8	25.4	4.1
DB-II	8.03 $\pm$ 2.7	2.6 $\pm$.70	3.6 $\pm$ 1.3	5.6 $\pm$ 1.3	40.9	2.9
DB-III	12.2 $\pm$ 3.0	3.4 $\pm$.94	3.3 $\pm$.74	15.6 $\pm$ 2.4	19.0	4.0

48 Hours Post-Injection

DB-I	5.9 $\pm$ 1.6	2.8 $\pm$ 1.4	3.9 $\pm$ 1.5	8.2 $\pm$ 1.85	30.0	7.9
DB-II	4.4 $\pm$ 1.9	1.6 $\pm$.61	2.58 $\pm$ 1.7	6.9 $\pm$ 2.9	50.1	4.89
DB-III	8.2 $\pm$ 1.1	2.7 $\pm$.36	3.2 $\pm$.92	17.5 $\pm$ 3.1	32.6	5.2

*Urine/Feces Data is % dose/organ

Table VIII shows summaries of tissue localization properties of ¹¹¹Indium (III) Labeled DB-III, NUBE and TUBE. The data is obtained by injecting nude mice bearing T-380 tumor implants with 20 μ l of ECH037.2 and the noted hapten, using a premix protocol.

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Table VIII

Tissue Localization of DB-III, NUBE and TUBE

24, 48 and 72 Hours Post-Injection

% Dose per Gram

	<u>DB-III</u>		<u>NUBE</u>		<u>TUBE</u>	
	<u>Blood</u>	<u>Tumor</u>	<u>Blood</u>	<u>Tumor</u>	<u>Blood</u>	<u>Tumor</u>
24 hrs.	12.2	15.6	5.8	11.7	8.0	8.5
48 hrs.	8.2	17.5	3.3	12.2	3.8	6.5
72 hrs.	6.6	18.1	0.7	10.5	1.7	5.3

Example 24

Radiolabeling of haptens DBIII, DB6, and DBX with ^{90}Y was accomplished by incubating 2 mCi of $^{90}\text{YCl}_3$ (10ul of 200 mCi/ml) with 0.2 nmol of hapten (2 nmol/ml solution in 25 mg/ml glycine formulation). The formulation containing the YCl_3 was in 70 mM hydrochloric acid having an activity concentration of 200mCi/ml for DBIII, DB6 and DBX, and 1500 mCi/ml for DDBIII. The incubation times were as follows: (DBIII, 5 min; DB6, 5 min; and DBX, 15 min). The radiolabeling reaction was quenched with 1.39 ml of a quench buffer containing either ascorbate, glycine, or citrate as a radioprotectant. The ascorbate buffer was formulated to contain 150 mM of ascorbate; 50 mM of tris-buffer (Aldrich Chemicals, tris(hydroxymethyl) amino methane), and 0.1 mM of DPTA and was at a pH of 7.42. The glycine buffer was formulated to contain 220 mM of glycine; 0.4 volume percent of ammonium chloride, and 0.2 mM of DPTA at a pH of 8.2. And the citrate buffer contained 130 mM of sodium citrate, 0.1 mM of

DPTA, and 0.10 M of dimethyl thiourea at a pH of 8.5.

For ^{90}Y radiolabeling of DDBIII, 8 μl of DDBIII (200 μM or 1.6 nmol) solution was incubated with 16 mCi of $^{90}\text{YCl}_3$ (approximately 1500 mCi/ml) at pH of 6.0 in 17.5 μl of acetate buffer to quench the reaction. The acetate quench buffer contained 440 mM of acetate at a pH of 8.42 and 110 mM of thiosulfate as non-chelating radioprotectant. The incubation was conducted at 65 $^{\circ}\text{C}$ for 20 minutes. 6 μl samples of the solution containing radiolabeled ^{90}Y DDBIII was mixed with either 6 μl of the ascorbate buffer or 1.5 ml of the glycine buffer prepared as described above to make the radioprotectant storage solution.

The post-labeled stability of the ^{90}Y haptens at room temperature in the radioprotectant storage solution was monitored for one to six days by means of two analytical procedures. In one procedure, using methods well known in the art, a C18-RP radio HPLC was used to determine the percentage of the compound that remained intact and the percentage that had degraded at the linker position. In the second procedure a competitive antibody binding assay was used to determine what percentage of the compound (including what had degraded) was able to bind to the antibody CHA 255 for which the haptens tested were known to be specific.

In the binding assay a known aliquot of the ^{90}Y DB-hapten was incubated with an excess of CHA255 for about 15 minutes to form the CHA255-DB-hapten complex. Then the CHA255-bound DB-hapten was separated from the

unassociated radiolabeled chelates by a Centrifree® filtration unit (Amicon, Danvers, Mass.). Counting the various fractions provided the percentage of DB hapten having both the haptenic moiety and the chelating moiety intact.

The post-labeled stability of ^{90}Y DBIII in each of the three radioprotectants tested are summarized in Table IX below:

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Table IX

**Comparison of Postlabeling Stability of ⁹⁰Y-DB
Chelate in Citrate and Ascorbate Buffer**

<u>Day</u>	<u>Quench Buffer</u>	<u>% of ⁹⁰YBDIII Intact</u>	<u>% of Hapten Binding CHA255</u>
0	citrate *	74.8	86.70
1	citrate	4.2	50.50
0	ascorbate **	81.7	95.74
1	ascorbate	69.5	92.52
2	ascorbate	58.8	89.30
3	ascorbate	50.7	85.20

* the specific activity is 10 mCi/nmol

** the specific activity is 16.5 mCi/nmol (more severe condition)

These results show that even though the specific activity of the radionuclide in the ascorbate-containing buffer solution was more than one-third greater than that in the citrate-containing solution, in the former at least 50 volume percent of the DB-chelate remained intact for three days. By contrast, the citrate-containing quench buffer was unable to prevent almost total degradation of the ⁹⁰Y labeled DB-III chelate in one days time. The binding studies on the other hand showed that in the citrate solution about fifty percent of the compound remained intact, while in the ascorbate solution about 93 percent of the compound tested retained intact offer one day. This apparent discrepancy resulted from a portion of DB-III compound having been changed by radiolytic effect to another more stable DB species capable of binding the radionuclide by the reaction of Formula 5 above.

Example 25

Tests were conducted to compare the protection afforded by three different radioprotectants to DB-chelates known to suffer degradation in aqueous solution at room temperature when labelled with ^{90}Y . Solutions containing glycine, citrate, or a combination of dimethyl thiourea (DMTU) and citrate as radioprotectant were prepared as described in Example 24 above.

The results of these tests are summarized in Table X below:

Table X

**The Postlabeled Stability of $^{90}\text{YDBIII}$
in Various Buffers**

<u>Day</u>	<u>Spec. Act. (mCi/nmol)</u>	<u>Buffer/ Radioprotectant</u>	<u>% of $^{90}\text{YDBIII}$ Intact</u>	<u>% of Hapten Binding</u>
				<u>CHA255</u>
0	10.0	glycine	83.2	95.2
1	10.0	glycine	42.4	85.7
2	10.0	glycine	39.1	82.2
3	10.0	glycine	36.8	79.5
0	10.0	citrate	74.8	86.70
1	10.0	citrate	4.2	50.50
0	10.0	citrate/DMTU*	46.3	95.4
1	10.0	citrate/DMTU	2.7	79.0
0	16.5	ascorbate	81.7	95.74
1	16.5	ascorbate	69.5	92.52
2	16.5	ascorbate	58.8	89.30
3	16.5	ascorbate	50.7	85.20
* 0.1 M DMTU				

None of these radioprotectants proved as effective as ascorbate against the radiolytic effects of ^{90}Y .

Comparison of the results of Table X with those of Table IX above shows that $^{90}\text{Y DBIII}$ degrades at a rate of about 15.5% per day when glycine is added as a radioprotectant

to the aqueous quench buffer, but at a rate of only about 10% per day when ascorbate is added to the quench buffer. DMTU, a radioprotectant known to provide radiolytic stability for compounds labeled with tritium (K.S. Kirshenbaum, M. Blanchette and M. Rigdon, pp. 8-9, "Dupont Biotech Update"), failed to provide radiolytic stability for DB-chelate labeled with ^{90}Y .

Example 26

Yet another study was conducted to compare the amount of protection against the radiolytic effect of ^{90}Y in aqueous storage solution at room temperature provided by various radioprotectant ions on different linker groups in DB-chelate compounds. Samples of DB-chelates containing three different linking groups were labeled with ^{90}Y as described in Example I above and stored in either ascorbate- or glycine-containing aqueous buffer for three days. The three DB-haptens used were DB6 (linker group Equation 2), DBX (linker group Equation 3), and DDBIII (linker group Equation 4). The buffers were prepared and the postlabeled stability of the ^{90}Y haptens was monitored using the techniques described in Example 24 above.

The results of the comparative studies are summarized in Table XI below:

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Table XI

Comparison of Ascorbate and Glycine Radioprotectants for
Y-90 Labeled DB-Chelates

<u>Hapten</u>	<u>Day</u>	<u>Spec. Act.</u> <u>(mCi/nmol)</u>	<u>Buffer</u> <u>Radioprotectant</u>	<u>% of ⁹⁰Y-</u> <u>Hapten Intact</u>	<u>%</u> <u>Binding</u>
DB6	0	10.0			
DB6	1	10.0	ascorbate	73.3	73.0
DB6	2	10.0	ascorbate	66.5	70.6
DB6	3	10.0	ascorbate	69.1	70.6
			ascorbate	69.3	68.7
DB6	0	10.0			
DB6	1	10.0	glycine	68.2	67.3
DB6	2	10.0	glycine	34.3	33.5
DB6	3	10.0	glycine	18.3	17.5
DB6	3	10.0	glycine	11.7	13.3
DBX	0	10.0	ascorbate	89.5	89.1
DBX	1	10.0	ascorbate	88.4	87.8
DBX	2	10.0	ascorbate	86.4	86.7
DBX	3	10.0	ascorbate	86.9	85.9
DBX	0	10.0			
DBX	1	10.0	glycine	89.7	88.7
DBX	2	10.0	glycine	89.2	86.0
DBX	3	10.0	glycine	85.4	84.1
			glycine	84.0	82.8
DDBIII	0	10.0			
DDBIII	1	10.0	ascorbate	97.5	93.6
DDBIII	2	10.0	ascorbate	93.4	92.7
DDBIII	3	10.0	ascorbate	92.4	92.1
DDBIII	6	10.0	ascorbate	91.4	91.6
			ascorbate	90.9	89.5
DDBIII	0	10.0			
DDBIII	1	10.0	glycine	93.3	92.3
DDBIII	2	10.0	glycine	90.6	89.7
DDBIII	3	10.0	glycine	88.2	87.2
			glycine	85.8	83.6

As can be seen by the results in Table XI above, the postlabeled stability of ⁹⁰Y-labeled haptens in aqueous storage solution depends on both the linker group in the hapten and the radioprotectant selected for use in the aqueous storage buffer. ⁹⁰YDBX and ⁹⁰YDDBIII, which both contain a carbonyl functional group in the linker (i.e., -NH-C(=O)-NH-) are less susceptible to radiolytic degradation in aqueous solution than is ⁹⁰YDBIII, which

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contains a sulfonyl functional group in the linker (i.e.,
-NH-C(=S)-NH-).

Therefore, compared with stability in glycine, compounds containing the carbonyl functional group (90YDBX and 90YDDBIII) showed less increase in stability in ascorbate as radioprotectant than did the compound containing the sulfonyl functional group (⁹⁰YDB6). However, a storage solution containing sufficient ascorbate consistently provided better stability over three days time for all the ⁹⁰Y-labeled haptens than did other storage solutions. And DBIII remained over 90 percent intact for six days when stored in an ascorbate buffer solution.

This conclusion is supported by calculation of the rate constants for degradation of the three DB-haptens stored with various radioprotectants from data presented in Tables IX-XI above, as shown in Table XII below:

Table XII

Rate Constant for the Postlabeled Degradation of
Various ⁹⁰Y-Haptens in Different Quench Buffers

<u>Hapten</u>	<u>Buffer *</u> <u>(10 mCi/nmol SA)</u>	<u>Rate Constant</u> <u>(% Degraded Per Day)</u>
DBIII	citrate	-90.0
DBIII	citrate/DMTU	-90.0
DBIII	glycine	15.5
DBIII *	ascorbate	10.3
DB6	glycine	18.8
DB6	ascorbate	1.3
DBX	glycine	1.9
DBX	ascorbate	0.9
DDBIII	glycine	2.5
DDBIII	ascorbate	2.0

Citrate and the combination of citrate and DMTU afforded very little protection for 90YDBIII, which contains the highly susceptible sulfonyl functional group in its linker; whereas ascorbate was effective in reducing the rate of dissociation to about 10 percent per day. In all cases, use of ascorbate as radioprotectant yielded a smaller rate of degradation at room temperature than did glycine.

Example 27

A series of tests were conducted to determine the level of protection afforded by ascorbate to the same DB-chelates as in Example 26 when they were labeled with ^{111}In , a gamma particle emitter having an energy associated with the gamma particle of about 172 to 247 keV. ^{111}In -labeled DBIII, DB6 and DBX were stored for three days in aqueous buffer solution containing either glycine or ascorbate as radioprotectant. The ascorbate and glycine storage buffers were prepared as described in Example 24 above. The DB-haptens were radiolabeled by incubating 0.75 mCi of ^{111}In formulated in glycine solution with 0.2 nmol of hapten. The formulation of the glycine solution was 88 mM glycine and 30mM hydrochloric acid (activity concentration of 300 ul of 2.5 mCi/ml). The DB-hapten was formulated in 25 mg/ml glycine solution to make a solution of 2 nmol of hapten/ml solution. Each of the haptens was incubated for 30 minutes at room temperature and then 300 ul of the reaction mixture was

quenched with 300 ul of either the glycine- or ascorbate-containing buffer solution. Stability of the radiolabelled haptens in aqueous solution was monitored for three days using the methods described in Example 24 above.

Results of the postlabeled stability studies of ^{111}In -DB haptens stored in ascorbate and glycine are summarized for comparison in Table XIII below:

Table XIII

**Postlabeled Stability Studies of ^{111}In -Haptens
in Ascorbate or Glycine Quench Buffers**

<u>Hapten</u>	<u>Day</u>	<u>% of ^{111}In Haptens Intact</u>	
		<u>Ascorbate Buffer</u>	<u>Glycine Buffer</u>
DBIII	0	84.10	82.90
DBIII	1	65.60	71.90
DBIII	2	42.50	62.80
DBIII	3	30.50	54.40
DB6	0	76.00	75.70
DB6	1	75.10	73.70
DB6	2	76.10	75.20
DB6	3	76.00	73.40
DBX	0	91.80	92.30
DBX	1	91.90	91.10
DBX	2	93.20	93.00
DBX	3	94.30	94.40

Surprisingly, these results show that ascorbate buffer did not protect the DBIII hapten from radiolytic degradation caused by the gamma emitter as well as glycine did. In the cases of DB6 and DBX, glycine was nearly as effective as a radioprotectant in the storage

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solution as ascorbate. By comparison with these results, the superior level of protection against degradation of DB-chelate compounds labeled with a high energy beta emitter provided by reducing antioxidants, such as ascorbate, is unexpected.

Example 28

A series of storage buffers was prepared to discover which formulations would yield a quench and storage solution containing sufficient ascorbate to act as a radioprotectant, yet having a final pH and osmolality within the range acceptable for therapeutic compositions used for parenteral administration to humans. Each solution was made by mixing together glycine, ascorbic acid (Aldrich Chemicals), tris buffer, DTPA (Baker Chemicals) in the proportions shown in Table XIV below:

Table XIV

Optimization of Storage Solution Containing
Radioprotectant For Use in Pharmaceutical Compositions

Glycine/ or H ₂ O	Ascorbic acid (mM)	Tris Buffer (mM)	DTPA (mM)	pH	Osmolality (mOsm)
2 ml H ₂ O	250	100	0.1	7.2	437
2 ml H ₂ O	180	50	0.1	7.18	370
2 ml H ₂ O	100	50	0.1	7.27	189
2 ml H ₂ O	140	50	0.1	7.19	241
2 ml glycine*	150	50	0.1	7.34	288
2 ml glycine	160	50	0.1	7.35	302

* 2 ml of 25 mg/ml glycine

It will be apparent to those skilled in the art that changes and modifications will be possible without departing from the spirit and scope of the invention. It is intended that the following claims be interpreted to embrace all such modifications.

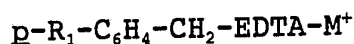
113

We claim:

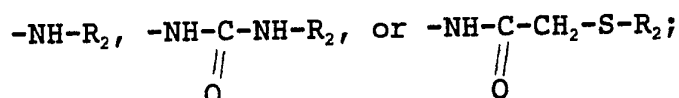
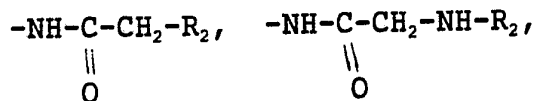
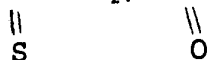
1. A method for preserving a chemical compound in an aqueous environment containing a radioactive source comprising adding to said aqueous environment sufficient reducing anti-oxidant to retard the radiolytic degradation of the compound.
2. The method of claim 1 wherein sufficient reducing anti-oxidant is added to retard formation of oxy-radicals in said aqueous environment.
3. The method of claim 1 wherein the antioxidant is selected from the group consisting of ascorbyl palmitate, hypophosphorous acid, monothioglycerol, sodium formaldehyde sulfoxylate, sodium metabisulfite, sodium thiosulfate, ascorbate ion and sulfur dioxide.
4. The method of claim 1 wherein the antioxidant comprises a reducing agent and a compound selected from the group consisting of butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, and tocopherol.
5. The method of claim 1 wherein the radioactive source is a beta particle emitter and the reducing anti-oxidant comprises ascorbate ion.
6. The method of claim 3 wherein the source is a radioactive metal ion attached to said compound.

7. The method of claim 2 or 3 wherein the compound comprises a metal chelate and the source is a radioactive metal ion attached thereto.
8. The method of claim 5 wherein the rate of degradation is retarded to no more than about 10 weight per cent per day for up to six days.
9. The method of claim 5 wherein the amount of ascorbate is an amount sufficient to substantially retard radiolysis of the aqueous solution.
10. The method of claim 5 wherein the radioactive source is ⁹⁰Yttrium.
11. The method of claim 10 wherein the rate of degradation is retarded to no more than 10 weight per cent per day for up to three days.
12. The method of claim 2 wherein the compound comprises a urea or thiourea functional group.

13. The method of claim 2 wherein the compound is of the formula:



wherein R_1 is $-\text{NH}-\text{C}-\text{NH}-\text{R}_2$, $-\text{C}-\text{NH}-\text{R}_2$,



R_2 is hydrogen, an amino group, $-\text{OC}-(-\text{Y})$, p -phenyl- $\text{CH}_2\text{-Y}$, an unsubstituted C_1 to C_{30}

branched or straight chain alkyl group; a

substituted C_1 to C_{30} branched or

straight chain alkyl, cycloalkyl,

aryl or arylalkyl group, in which the

substituents are one or more of any of:

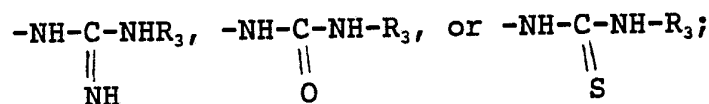
hydroxy, carboxy, $=\text{O}$, $=\text{S}$, $-(\text{C}=\text{O})-$, $-(\text{C}=\text{S})-$, $\text{S}-$

$-\text{SR}_4$, fluoro, chloro, bromo, iodo, amino,

nitro, $-\text{SO}_3\text{H}$, $-\text{NHR}_3$, $-\text{NHR}_4$, $-\text{N}(\text{R}_3)_2$, $-\text{CONHR}_3$, $-$

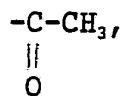
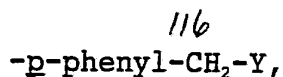
COOR_3 , $-\text{SO}_4$, $-\text{PO}_4$, phenyl, benzyl, imidazolo,

or a group of the formula:



R_3 is hydrogen, a C_1 to C_{30} straight or

branched chain alkyl group,



or a non-reactive functional
group;

Y is EDTA-M₁⁺, DTPA-M₁⁺, DOTA-M₁⁺, HETA-M₁⁺,
TRITA-M₁⁺, or TETA-M₁⁺;

R₄ is H or $-\text{CH}_2-\text{C}-\text{NHR}_3$; and

$$\begin{array}{c} || \\ \text{O} \end{array}$$

M⁺ and M₁⁺ are metal ions provided that one
such ion is radioactive and suitable for radiotherapy or
radioimaging;

or a pharmaceutically acceptable salt
thereof.

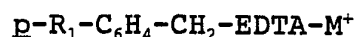
14. The method of claim 13 wherein R₁ is a group of the
formula $-\text{NH-C(=S)-NHR}_2$.

15. The method of claim 13 or 14 wherein the radioactive
substance is ⁹⁰Yttrium and the reducing antioxidant comprises
ascorbate in the range from about 0.5 to 15 mg per mCi of
specific activity of the said ⁹⁰Yttrium.

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16. The method of claim 15 wherein the pH of the aqueous environment is in the range between about 7.2 and 7.6 and the osmolality is in the range between about 275 and 300 mOsm per liter.

17. The method of claim 2 wherein the compound is of the formula:



wherein R_1 is $-NH-\overset{\overset{S}{\parallel}}{C}-NHR_2$; $-C-\overset{\overset{O}{\parallel}}{NH}-R_2$, or $-NH-\overset{\overset{O}{\parallel}}{C}-CH_2-R_2$;

in which R_2 is hydrogen, an amino group, an unsubstituted C_1-C_{30} branched or straight chain alkyl group, or a C_1-C_{30} branched or straight chain alkyl, cycloalkyl, aryl or arylalkyl group substituted by one or more substituents independently selected from the group consisting of:

$-OH$, $-COOH$, $=O$, $=S$, $-S-$, $-Cl$, $-F$, $-Br$, $-I$, $-NH_2$,
 $-NO_2$, $-SO_3H$, $-NH-R_3$, $-CONHR_3$, $-COOR_3$, $-SO_4$, $-PO_4$,
 phenyl, and benzyl;

R_3 is H, an alkyl group, or a non-reactive functional group;
 and M^+ is a metal ion that is radioactive and suitable for radioimaging, or a pharmaceutically-acceptable salt thereof.

18. The method of claim 17 wherein R_1 is a group of the formula

$$-NH-C(=S)-NHR_2.$$

19. The method of claim 17 or 18 wherein the radioactive source is $^{90}\text{Yttrium}$ chelated to said compound, and the amount of ascorbate is in the range from about 0.5 to 15 mg per mCi of specific activity of the said $^{90}\text{Yttrium}$.

20. The method of claim 19 wherein the pH of the aqueous environment is in the range between about 7.2 and 7.6 and the osmolality is in the range between about 275 and 300 mOsm per liter.
21. A therapeutic composition made by the method of claim 1.
22. A therapeutic composition made by the method of claim 11.
23. A method of labelling a chemical compound with a radioisotope in an aqueous environment comprising contacting said compound with said radioisotope in an aqueous solution maintained at reaction conditions, said solution containing sufficient reducing antioxidant to substantially retard radiolysis of water molecules.
24. The method of claim 23 wherein the compound comprises a metal chelate, the radioisotope comprises a radioactive metal ion, and the reducing antioxidant is non-chelating.
25. The method of claim 24 wherein the reducing antioxidant is selected from the group consisting of hypophosphorous acid, monothioglycerol, sodium formaldehyde sulfoxylate, sodium metabisulfite, sodium thiosulfate, ascorbate ion and sulfur dioxide.
26. The method of claim 25 wherein the compound contains a urea or thiourea linker and the radioisotope is a strong beta particle emitter.

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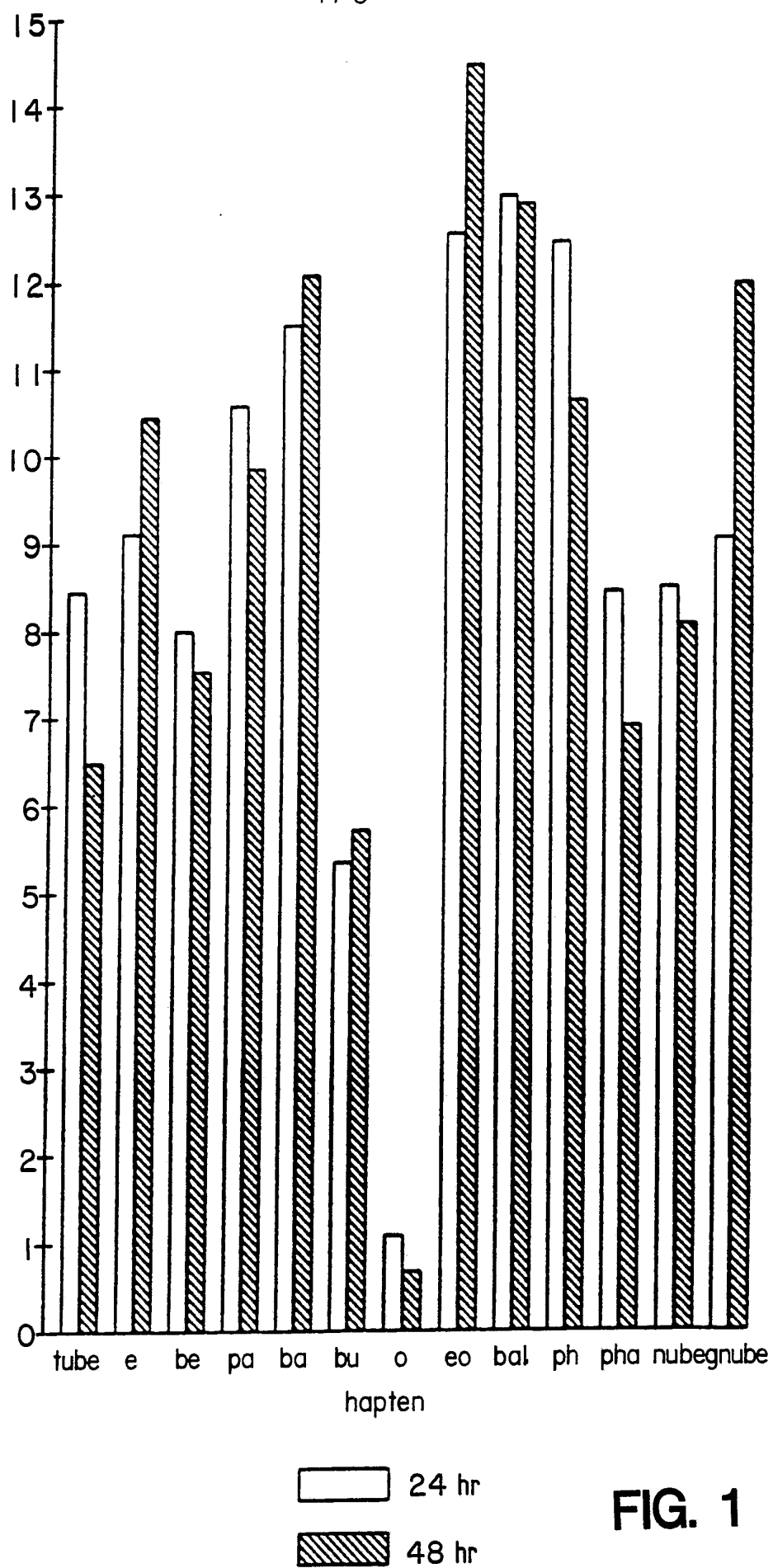


FIG. 1

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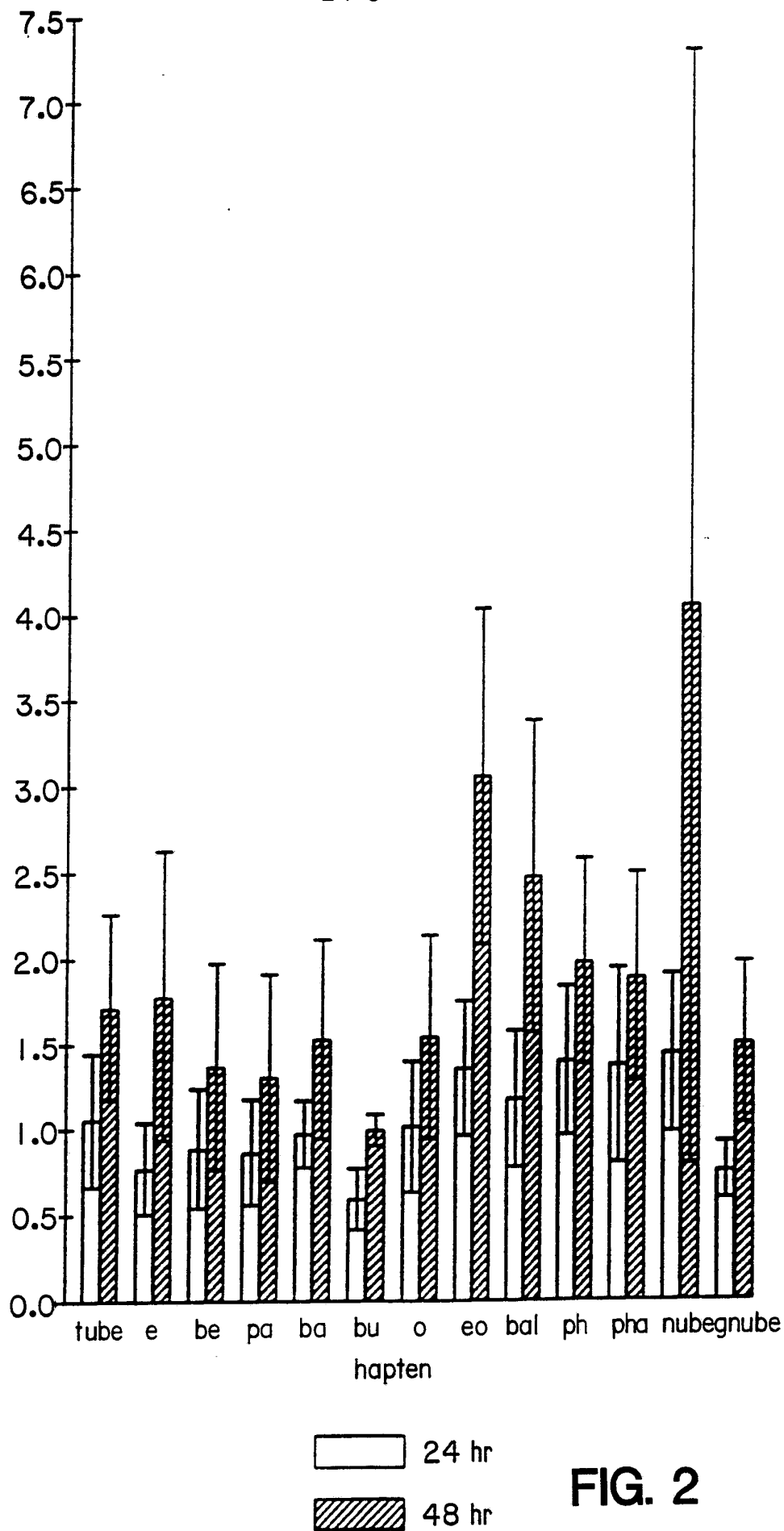


FIG. 2

SUBSTITUTE SHEET

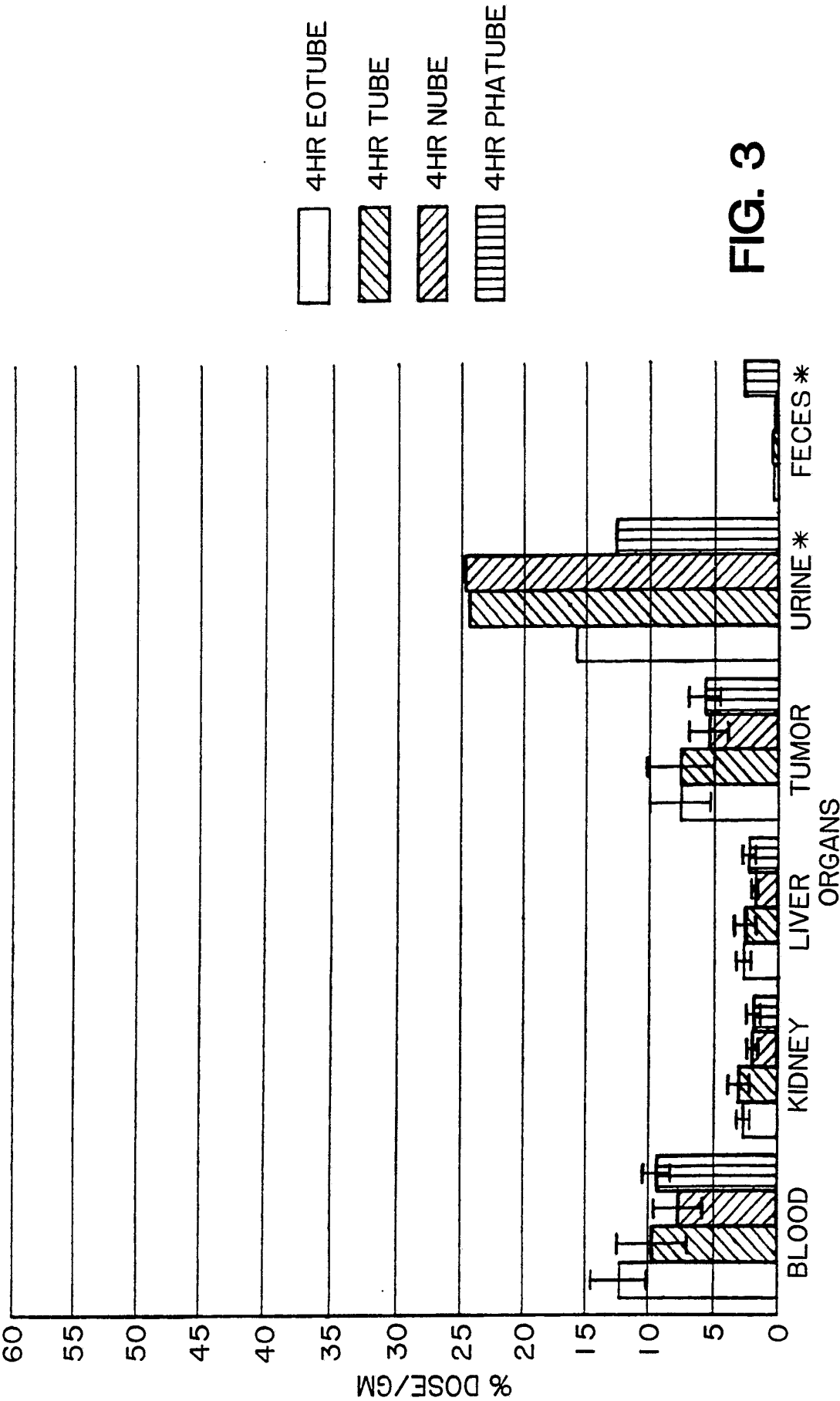


FIG. 3

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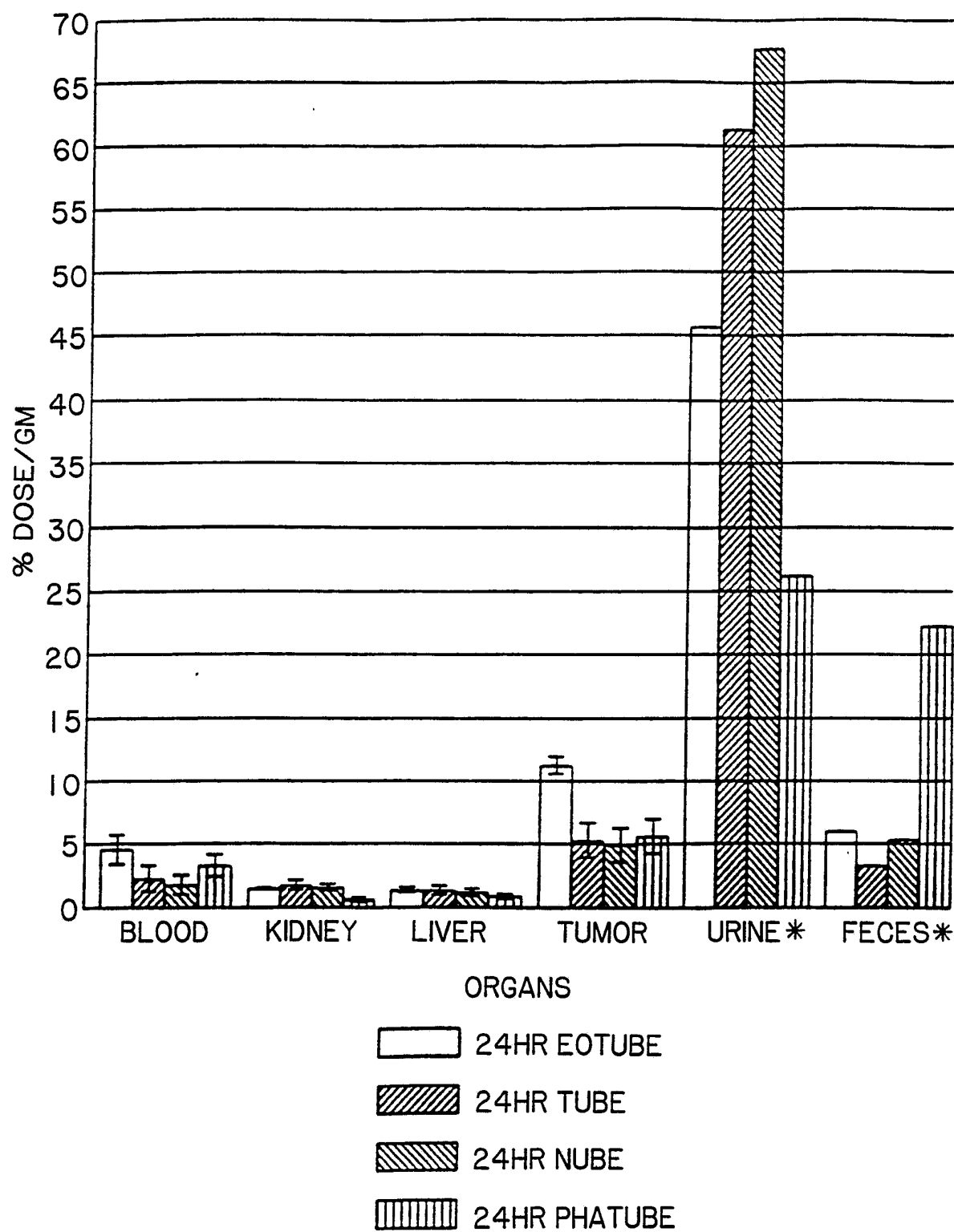


FIG. 4

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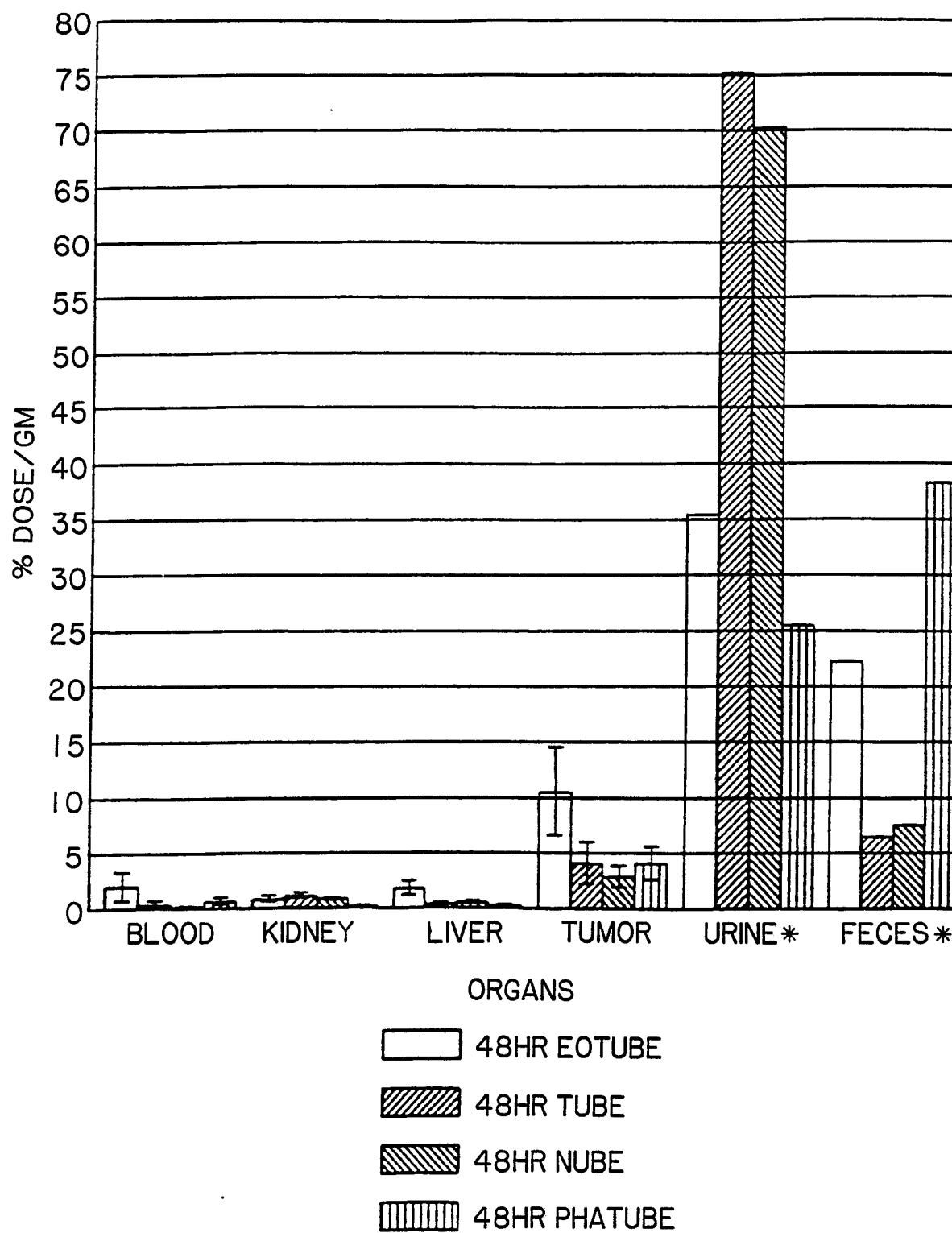
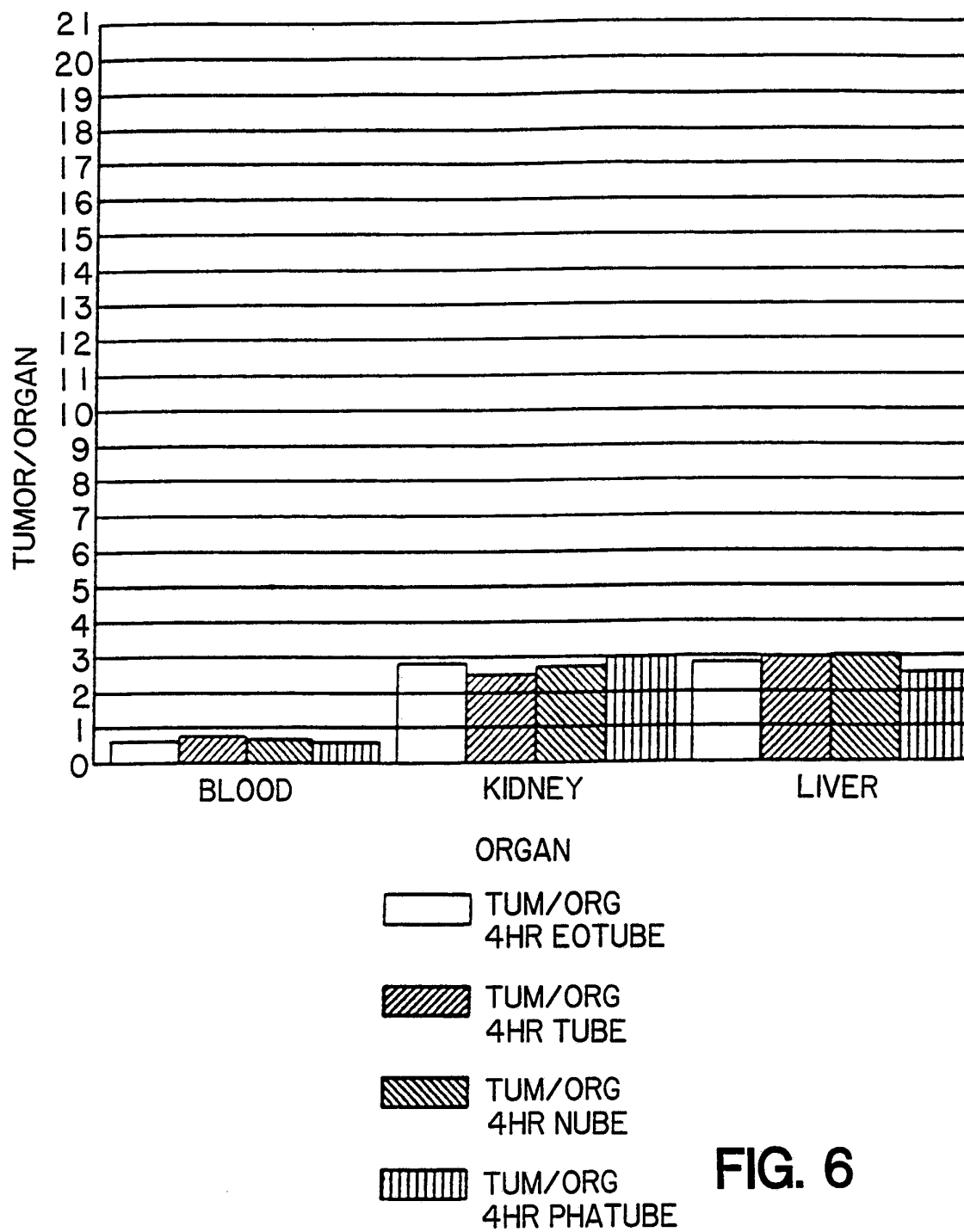
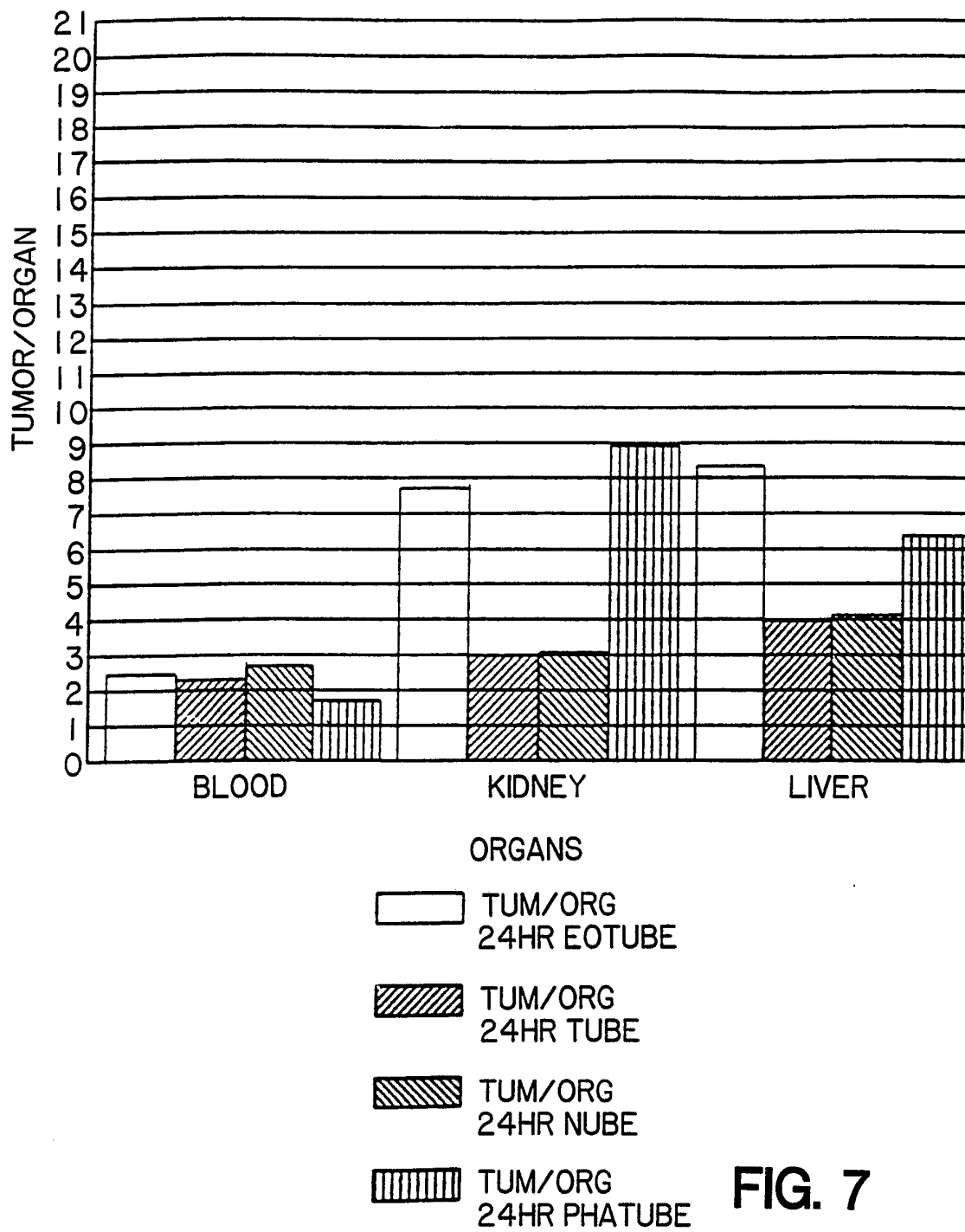


FIG. 5

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**FIG. 6**

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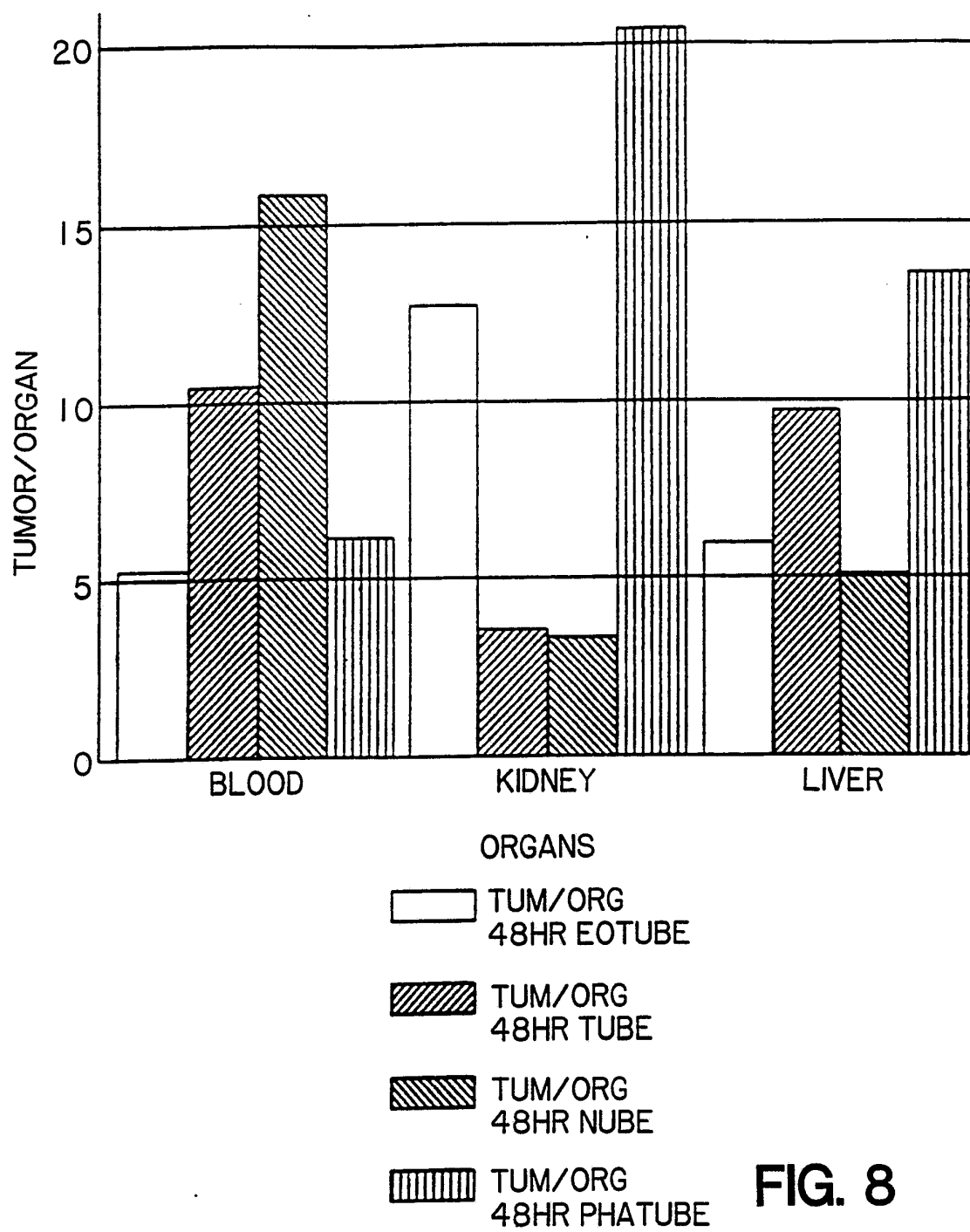


FIG. 8