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(54) Title: A BIFUNCTIONAL DTPA-TYPE LIGAND

(57) Abstract

The subject matter of the present invention relates to bifunctional cyclohexyl DTPA ligands and methods for utilizing these compounds.

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A BIFUNCTIONAL DTPA-TYPE LIGAND

BACKGROUND OF THE INVENTION

Technical Field

The subject matter of the present invention relates to bifunctional cyclohexyl DTPA ligands and methods of using these compounds. Specifically, such ligands are useful for radiolabeling proteins with radioactive metals, and can consequently be utilized with respect to radioimmunoimaging and/or radioimmunotherapy.

Background Information

This invention relates to metal chelates and the use of metal-chelate protein conjugates.

Interest in the art of metal chelates and in methods for forming metal chelate-protein conjugates for diagnostic and therapeutic purposes continues. Representative type chelates and conjugates and methods for forming conjugates are disclosed, inter alia, in U.S. Pat. Nos. 4,454,106, 4,472,509, 4,339,426, in EPA 0 279 307 and in German Patent 1,155,122. Other proteins including antibodies, monoclonal antibodies and fragments thereof, monoclonal antibodies and fragments thereof which have been structurally altered by recombinant DNA techniques (i.e., chimeric antibodies), polyclonal antibodies, antigens, blood proteins, or proteins bound to blood lymphocytes or other cells can also be employed in the formation of conjugates.

A method for synthesis of bifunctional metal chelates for conjugation to proteins involves reduction of amino acid amides to ethylenediamines to form monosubstituted derivatives which are converted to bifunctional ethylenediaminetetraacetic acid (EDTA) chelates by alkylation with haloacetic acid. (Yeh et al., Anal. Biochem. 100: 152 (1979)). Similarly, a monosubstituted diethylenetriamine is synthesized by reaction of ethylenediamine with an amino acid ester and reduction of the resulting amide carbonyl. (Brechbiel et al. Inorg. Chem. 25: 2772-81 (1986)). Alkylation of the diethylenetriamine with haloacetic acid produces a monosubstituted bifunc-

with haloacetic acid produces a monosubstituted bifunctional diethylenetriaminepentaacetic acid (DTPA) chelate.

Another method of synthesis of a bifunctional DTPA involves reaction of a DTPA or EDTA carboxylate with a chloroformate ester to form a reactive anhydride. (Krejcarek et al., Biochem. Biophys Res. Commun. 77:581 (1977)). The dianhydride of DTPA used as a bifunctional chelate is prepared by dehydration of the parent DTPA. (Hnatowich et al., Int. J. Appl. Rad. Isot. 33:327 (1982)). The practice of using an EDTA chelate monosubstituted at the carbon-1 position to better retard the release of metal from chelate in vitro, than the unsubstituted EDTA chelate, has also been reported. (Meares et al., Anal. Biochem. 142:68 (1984)).

The prior art has formed metal-protein chelate conjugates by mixing monosubstituted bifunctional EDTA or DTPA chelates, or DTPA anhydrides with proteins followed by reaction with the metal to be chelated. (Krejcarek et al., Biochem. Biophys. Res. Commun. 77:581, (1987); Brechbiel et al., Inorg. Chem. 25:5783 (1986)). Imaging of tumor target sites in vivo with metal chelate conjugated monoclonal antibodies prepared according to these methods has been reported. (Khaw et al., Science 209:295, (1980); Sheinberg et al., Science 215:151, (1982)). Diagnosis of human cancer in vivo using metal chelate conjugated monoclonal antibody has also been reported. (Rainsbury et al., Lancet 2:694 (1983)). The use of chimeric antibodies and advantages thereof have been discussed by Morrison, S.L., Hospital Practice (Office Edition) 24:64-65, 72-74, 77-80 (1989). The potential efficacy of using a linking group within a chelate conjugated protein has also been discussed (Paik et al., J. Nucl. Med. 30:1693-1701 (1989)).

However, attempts to employ the tumor localizing properties of metal chelate conjugated monoclonal antibodies for therapeutic purposes have not found common usage. This is, in part, because metals may be (and often are) released from the metal chelate conjugate in vivo.

Particularly, radioactive metal salts may produce undesirable concentrations of toxic radionucleotides in bone marrow or kidney or the like, even if the conjugates are rigorously purged of adventitiously bonded metal. A process for purifying metal chelate protein conjugates of adventitiously bonded metals is disclosed in U.S. Pat. No. 4,472,509. The importance of using very strong metal chelates to firmly link radiometals to monoclonal antibodies and of rigorous purification of the conjugates to effect maximal tumor localization and minimize delivery to non-target tissues is discussed in Brechbiel et al., Inorg. Chem. 25:2772-81 (1986)). Undesirable localization of potentially therapeutic radionuclides released in mice in vivo from metal chelate conjugated polyclonal antibodies have precluded some therapy investigations in humans. (Vaughn et al., EIR-Bericht. Vol. 78, (1986)). Increased in vivo bone uptake of radiometal injected for therapy as a metal chelate conjugated monoclonal antibody has also been reported. (Hnatowich et al., J. Nucl. Med. 26:503 (1985)). The amount of potentially therapeutic doses in humans of radiometal chelated polyclonal antibody has been limited by bone marrow toxicity (Order et al., Int. J. Rad. Oncol. 12:277 (1986)). Kidney uptake of radiometal has recently been reported as preventing human use. (Macklis et al, Science, 240:1024-26 (1988)).

Disubstituted bifunctional DTPA derivatives have proven useful for the labeling of proteins with radioactive metals (Kozak, et al., Cancer Research 49:2639-44 (1989)). The introduction of a second substituent on the carbon backbone of DTPA was seen to retard the loss of metal from the DTPA ligand when linked to an antibody and injected into the circulation of animals.

The usefulness of radionuclide materials in cancer therapy is disclosed in the article, Kozak et al., "Radionuclide-conjugated monoclonal antibodies: A Synthesis of Immunology, in Organic Chemistry and Nuclear Science" Trends in Biotechnology, 4(10):259-64 (1985). This article discusses the use of antibody conjugates to

deliver either alpha or beta radiation. The value of alpha radiation for bismuth-212 in radionuclide therapy is further discussed in the two articles, Kozak et al., "Bismuth-212-labeled anti-Tac monoclonal antibody: Alpha-particle-emitting Radionuclides as Modalities for Radioimmunotherapy," Proc. Natl. Acd. Sci. U.S.A. 83:474-478 (1986) and Gansow et al., "Generator-produced Bi-212 Chelated to Chemically Modified Monoclonal Antibody for Use in Radiotherapy," Am. Chem. So. Symposium Series 15:215-227 (1984). Ligands, for the secure linkage of bismuth to proteins, have not been available. (Macklis et al., Science 240:1024-2 (1988)).

Examples of other uses for chelated metal ions are disclosed in the following articles. Magerstadt et al., "Gd(DOTA): An alternative to Gd(DPTA) as a $T_{1/2}$ Relaxation Agent for NMR Imaging or Spectroscopy," Magnetic Resonance in Medicine 3:808-812 (1986), discloses the usefulness of gadolinium as a relaxation agent for NMR imaging. The article, Spirlet et al., "Structural Characterization of a Terbium (III) Complex with 1,4,8,11-Tetraazacyclotetradecane-1,4,8,11 tetraacetic acid. Lanthanide Ions and Conformation of the 14-Membered Ring," Inorgan. Chem. 23:4278-4783 (1984), disclosed the usefulness of lanthanide chelates.

All patents and publications referred to herein are hereby incorporated by reference.

It is evident from the above that there continues to be a need for more effective metal chelate protein conjugates that firmly link metals to proteins to minimize metal release and permit highly selective delivery of metals to targeted sites in vivo.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide novel polysubstituted cyclohexyl diethylenetriamines.

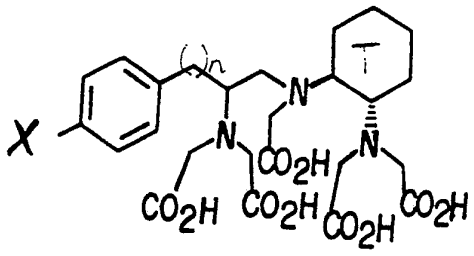
It is another object of the present invention to provide novel polysubstituted bifunctional cyclohexyl diethylenetriaminepentaacetic acid ligands or chelates.

It is yet another object of this invention to provide novel ligand and chelate-hapten conjugates.

It is still a further object of this invention to provide novel metal chelate protein conjugates.

It should be noted that the present invention overcomes an inherent synthetic barrier which limits the prior art to less than three ligand substituents without stereo-control.

The present invention includes a ligand comprising:



(I)

wherein n is an integer from 1 to 5;

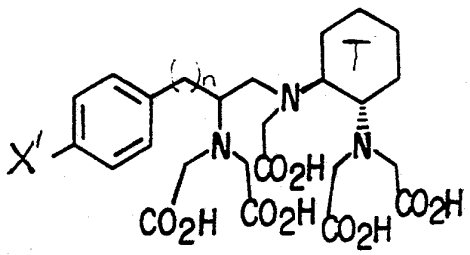
X equals $-NO_2$, $-NH_2$, -NCS, or $-NHCOCH_2-Z$;

T denotes the <u>trans</u> isomer of the cyclohexyldiamine substructure; and

Z is chloride, bromide or iodide.

The invention also includes a metal chelate of the ligand wherein n is an integer from 1 to 5, X equals $-NO_2$, $-NH_2$, -NCS, or $-NHCOCH_2-Z$, the metal is chosen from the elements consisting of Cu, Pb, In, Yt, Bi, the lanthanides, Au, Ag, and Ga, and Z is chloride, bromide or iodide.

In addition, the invention includes a ligand-hapten conjugate comprising:



(II)

wherein n is an integer from 1 to 5;

X' is NH-Q, NHCS-Q or $-NHCOCH_2$ where Q is a hapten chosen from the group consisting of steroids, enzymes, proteins, monoclonal antibodies, chimeric antibodies, or fragments thereof; and

T denotes the trans isomer of the cyclohexyldiamine substructure.

Another embodiment of the invention includes the ligand-hapten conjugate wherein n is an integer from 1 to 5, X' is -NH-L-Q, -NHCS-L-Q, or $-NHCOCH_2$-L-Q, where Q is a hapten selected from the group consisting of steroids, enzymes, proteins, monoclonal antibodies, chimeric antibodies, or fragments thereof, and L is a covalent linking group.

A further embodiment includes the situation where L of the ligand-hapten conjugate is selected from the group consisting of an organic radical, or a substituted aliphatic hydrocarbon chain. The chain may be interrupted by one or more hetero atoms selected from -O- or -S-, or one or more -NR'- groups, where R' is a hydrogen atom or a C-1 alkyl group, -CONR'- groups, -NR'CO- groups, cycloaliphatic, aromatic or heteroaromatic groups, or a mixture thereof.

A further embodiment includes the metal chelates of the ligand-hapten conjugate wherein n is an integer from 1 to 5, X' is equal to -NH-Q, -NHCS-Q or $-NHCOCH_2$-Q,

where Q is a hapten selected from the group consisting of steroids, enzymes, proteins, monoclonal antibodies, chimeric antibodies, or fragments thereof, and the metal is selected from the group consisting of Cu, Pb, In, Yt, Bi, the lanthanides, Au, Ag, and Ga.

An additional embodiment includes the metal chelates of the ligand-hapten conjugate wherein n is an integer from 1 to 5, X' is equal to -NH-L-Q, -NHCS-L-Q or $-NHCOCH_2$-L-Q, where Q is a hapten selected from the group consisting of steroids, enzymes, proteins, monoclonal antibodies, chimeric antibodies, or fragments thereof, and the metal is selected from the group consisting of Cu, Pb, In, Yt, Bi, the lanthanides, Au, Ag, and Ga, and L is a covalent linking group.

Another embodiment includes the situation where L of the metal chelates of the conjugate is selected from the group consisting of an organic radical or a substituted aliphatic hydrocarbon chain. The chain may be interrupted by one or more hetero atoms selected from -O- or -S- or by one or more -NR'- groups, where R' is a hydrogen atom or a C_{-1} alkyl group, -CONR'- groups, -NCR'O- groups, cycloaliphatic groups, aromatic or heteroaromatic groups, or a mixture thereof.

The present invention also includes the method of using the metal chelates of the ligand-hapten conjugate wherein said conjugate is administered to a patient as a therapeutic agent or diagnostic agent.

Furthermore, the present invention includes the method of using the metal chelates of the ligand-hapten conjugate possessing a linking group wherein the chelate as a therapeutic or diagnostic agent.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 represents a scheme for the preparation of a substituted cyclohexyl diethylenetriaminepentaacetic acid ligand (DTPA).

DETAILED DESCRIPTION OF INVENTION

Unless specifically defined otherwise, all technical or scientific terms used herein have the same

meaning as commonly understood by one or ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

The subject matter of the present invention relates to bifunctional cyclohexyl DTPA chelating agents or ligands and methods for using these compounds. Specifically, the ligands can be used in radioimmunoimaging and/or radioimmunotherapy, as the compounds may be utilized for labeling proteins with radioactive metals.

Monoclonal antibodies are immunoglobulins of well-defined chemical structure, in contrast to polyclonal antibodies which are heterogeneous mixtures of immunoglobulins. A characteristic feature of monoclonal antibodies is reproducibility of function and specificity, and such antibodies can be and have been developed for a wide variety of target antigens, including tumor cells. More recently, chimeric monoclonal antibodies and fragments have been prepared by recombinant techniques (Morrison, S.L., Hospital Practice (Office Edition) 24: 64-65, 72-74, 77-80 (1989)).

Methods for obtaining monoclonal antibodies or fragments have been extensively discussed and are well-known in the art. A useful text is Monoclonal Antibodies (R. H. Kennett, T. J. McKearn & K. B. Bechtol eds. 1980). See also Koprowski et al.(U.S. Pat. No. 4,196,265). The selection of a monoclonal antibody for the practice of this invention will depend upon the end use for which the metal chelate conjugated monoclonal antibody will be employed. Such selection is within the skill of the art.

A wide variety of organic chelating agents or ligands can be conjugated to monoclonal antibodies. Organic ligands to be conjugated to monoclonal antibodies may be chosen from among either the natural or synthetic amines, porphyrins, aminocarboxylic acids, iminocarboxylic acids, ethers, thiols, phenols, glycols and alcohols or

the polyamines, polyaminocarboxylic acids, polyiminocarboxylic acids, aminopolycarboxylic acids, iminopolycarboxylic acids, nitrilocarboxylic acids, dinitrilopolycarboxlic acid, polynitrilopolycarboxylic acids, ethylenediaminetetracetates, diethylenetriaminepenta or tetraacetates, polyethers, polythiols, cryptands, polyetherphenolates, polyetherthiols, ethers of thioglycols or alcohols, polyaminephenols, all either acyclic, macrocyclic, cyclic, macrobicyclic or polycyclic, or other similar ligands which produce highly stable metal chelates or cryptates.

The ligand of this invention possesses a nonmetal bonded organic functional group suitable for bonding to the monoclonal antibody. Functional groups may be chosen from among the carboxylic acid groups, diazotiazable amine groups, N-hydroxy-succinimidyl, esters, anhydrides, mixed anhydrides, maleimides, hydrazines, benzimidates, nitrenes, isothiocyanates, azides, sulfonamides, bromoacetamides, iodocetamides, carbodiimides, sulfonylchlorides, hydroxides, thioglyols, or any reactive functional group known in the art as a biomolecular conjugating or coupling agent.

The present invention is a derivative of diethylenetriaminepentaacetic acid (DTPA). It has been found that DTPA ligands tightly bind metal ions and that the DTPA derivative of this invention forms a chelate conjugated monoclonal antibody that is highly stable, both with respect to the metal chelate binding and with respect to chelate-antibody conjugate. These properties are of great importance, particularly for <u>in vivo</u> applications. For example, if the chelate releases the metal ion after introduction into the blood, these ions tend to be bound by transferrin, or the like, and be distributed generally in the circulatory system of the body. Moreover, the ions will ultimately tend to collect and remain in organs such as the liver and spleen, bone or kidney. These effects can have serious consequences depending on the toxicity of the metal and its radioactivity. Furthermore, if the

chelate does not form a highly stable conjugate with the antibody, there is a significant reduction in the amount of metal delivered to the target site and a corresponding decrease in efficacy. If the conjugate is used for diagnostic purposes, release of the metal can undesirably increase background radiation.

The metals which may be employed in the present invention may include radioactive or nonradioactive elements with a valance of two or higher. Monovalent metals generally do not form sufficiently stable chelates for the purposes of this invention. Representative radioactive elements may include d-block transition metals, the group IIIA, IVA, VA metals, the lanthanides or the actinides. Nonradioactive metals may be selected, for example, for their useful physical properties such as paramagnetism, fluorescence, or phosphorescence. Representative nonradioactive metals include most lanthanides and some first row d-block elements. While this invention is discussed in terms of metals or metal chelates, it will be understood that metal ions are, in fact, chelated in the conjugate.

If the metal chelate conjugated monoclonal antibody is to be used for imaging in vivo, a gamma or positron emitting radiometal, such as indium-111 (gamma) or gallium-68 (positron), can be used depending upon the chosen method of detection. For purposes of therapy, the radiometals may be alpha (e.g. bismuth-212), beta (e.g. Pb-212, Y-90 or Cu-67 scandium -47) or Auger electron emitter. An alpha emitter, such as bismuth-212 is desirably employed for therapy. Paramagnetism, fluorescence and phosphorescence can be used, for example, for in vitro tests. The choice of any particular metal and valence state is within the skill of the art.

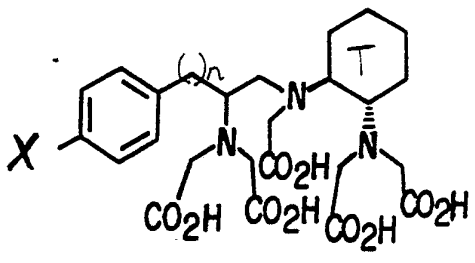
Metal chelation is carried out in a solution and, desirably avoids the use of strong acids or bases. Metal chelation for any chelate-antibody conjugate is carried out at a pH which does not significantly reduce the biological activity or specificity of the antibody.

Generally, the acceptable range is from about pH 3.2 to about pH 9, however, particular antibodies may have to be restricted to a narrower range. At a pH above 3.5, adventitious binding of metal ions to antibodies is substantially impaired for many metals. A preferred range, therefore, is often from about pH 3.2 to about pH 3.5. Factors peculiar to solutions of the metal employed, however, may permit a pH about 3.5. The selection of the appropriate pH within the range is within the skill of the art.

Linkage of Bismuth ions to antibodies by use of metal chelates is a particularly difficult process. Only the iodide complexes label ligand linked antibodies effectively (Kozak et al., Proc. Nat. Acad. Sci. 83:474-78 (1986)). Moreover, no chelating agents are currently available which bind bismuth securely in vivo (Macklis et al., Science, 240:1024-26 (1988)).

The functionalized DTPA ligand of the present invention is substituted at three carbon positions as is drawn in Formula I (shown below) in which X represents the nitro, amino, isothiocyanate or haloacetimide functional groups, n = 1 to 5, and the symbol T in the cyclohexane ring denotes the trans isomer of the diaminecyclohexyl substructure.

The structure of Formula I is as follows:



The invention also includes the metal chelates of the ligand of Formula I. Preferably, the metals are chosen from the elements including copper, lead, indium,

yttrium, bismuth, the lanthanides, gold, silver, and gallium.

Particularly preferred embodiments of the invention involve metal chelates of the ligand of Formula I with the radioactive isotopes In-111, Y-90, Bi-212, Pb-202, Pb-212, Ga-66, Ga-67, Ga-68, and Cu-67.

The presence of three substituents on the carbon backbone of DTPA induces a maximal control of the stereochemical predisposition of the ligand towards effective metal complexation. In particular, it is well known to introduce the trans structure of the cyclohexyldiamine substructure of Formula I to increase metal complex thermodynamic stability as opposed to the incorporation of similar cis cyclohexyldiamine isomer as is the practice for the well characterized cis and trans cyclohexyl-EDTA complexes. See data in A. E. Martell, Critical Stability Constants, Vol. 1, Plenum Press, New York, (1974). Additionally, the stereochemical constraint of the cyclohexane ring serves to direct and focus the lone pairs of the two amines to impart maximal overlap with available empty metal orbitals, as well as to maximize ligand-dipole to metal electrostatic bonding. This results in a DTPA ligand with optimized pre-control and pre-organization for metal ion binding thus reducing the entropy of metal complex formation as recorded in the reference above.

Other functionalized DTPA ligands which feature trisubstitution on the carbon backbone have been envisioned, but they lack the critical stereochemistry of the trans cyclohexyldiamine substructure of Formula I. See U.S. Patent No. 4,831,175.

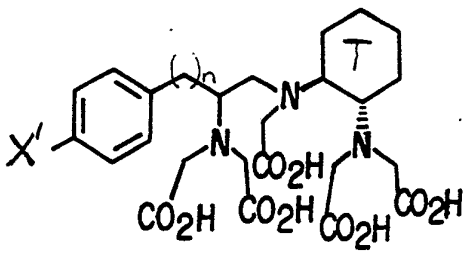
A principal advantage of the ligand of Formula I is that it forms complexes with bismuth ions that are stable in vivo. Such complexes of other substituted DTPA ligands are not stable in vivo, thus precluding their use in cancer therapy when linked to antibodies.

An additional feature of the ligands of this invention is that they form stable complexes in vivo with a wide variety of other radiometals which are used in

cancer detection and therapy. Such metal ions include trivalent indium, yttrium, or scandium and divalent lead and copper. Indium-111 is often used for tumor imaging. Thus, a patient could be imaged with the In-111 antibody conjugate of the ligand of this invention and thereafter treated with the bismuth-212 complex of the same antibody chelate conjugate, thus facilitating calculation of the dose of radioactivity transported to the patients tumor and so increasing likelihood of the effective application of the therapy. With dosimetry information, multiple dosing therapies can be designed.

Another embodiment of the invention is a ligand-hapten conjugate as is drawn in Formula II (shown below) in which the T in the cyclohexane ring denotes the trans isomer of the cyclohexyldiamine substructure, n is 1 to 5, and X' is -NH-Q, $-NHCOCH_2-Q$ or NHCS-Q, where Q is a hapten chosen from the group consisting of steroids, enzymes, or proteins. Of particular interest within the subset of proteins are monoclonal antibodies, chimeric monoclonal antibodies, and the fragments thereof.

The structure of Formula II is as follows:



A further embodiment of the invention is a ligand-hapten conjugate as is drawn in Formula II (shown above) in which the T in the cyclohexane ring denotes the trans isomer of the diamino cyclohexane substructure, n is 1 to 5, X' is -NH-L-Q, or -NHCS-L-Q, where Q is a hapten chosen from the group consisting of steroids, enzymes, or proteins, and L is a covalent linking group. Of particular interest within the subset of proteins are monoclonal antibodies, chimeric monoclonal antibodies, and the fragments thereof. Such conjugates may be utilized as therapeutic and diagnostic agents.

The nature of the L group as a linker group is such that it may be varied widely without affecting the usefulness of the compounds of Formula I and Formula II, and the metal complexes thereof. Thus, L may be any suitable organic radical and may be, for instance, an optionally substituted aliphatic hydrocarbon chain, optionally interrupted by one or more hetero atoms selected from -O- or -S-or by one or more -NR'- groups (where R' is a hydrogen atom or a C-1 alkyl group), -CONR'- groups, -NR'CO- groups, cycloaliphatic groups, aromatic groups, heteroaromatic groups, or a mixture thereof.

Yet another embodiment of the present invention includes the metal chelates of the conjugate of Formula II. Preferably, the metals are chosen from the elements including copper, lead, indium, yttrium, bismuth, the lanthanides, gold, silver, and gallium. Such metal chelates may also be utilized as therapeutic and diagnostic agents.

Particularly preferred embodiments of the present invention involve metal chelates of the ligand of Formula II with the radioactive isotopes In-111, Y-90, Bi-212, Pb-202, Pb-212, Ga-66, Ga-67, Ga-68, and Cu-67.

The metal chelate conjugated antibodies of this invention can be administered in vivo in any suitable pharmaceutical carrier. As noted earlier, a physiologic normal saline solution can appropriately be employed.

Often the carrier will include a minor amount of carrier protein such as human serum albumin to stabilize the antibody. The concentration of metal chelate conjugated antibodies within the solution will be a matter of choice. Levels of 0.5 mg per ml are readily attainable but the concentrations may vary considerably depending upon the specifics of any given application. Appropriate concentrations of biologically active materials in a carrier are routinely determined in the art.

The effective dose of radiation or metal content to be utilized for any application will also depend upon the particulars of that application. In treating tumors, for example, the dose will depend, inter alia, upon tumor burden, accessibility and the like. Somewhat similarly, the use of metal chelate conjugated antibodies for diagnostic purposes will depend, inter alia, upon the sensing apparatus employed, the location of the site to be examined and the like. In the event that the patient has circulating antigen in addition to those located at the site, the circulating antigens can be removed prior to treatment. Such removal of antigens can be accomplished, for example, by the use of unlabeled antibodies, or by plasmapheresis in which the patient's serum is treated to removed antigens.

The invention can be better illustrated by the use of the following non-limiting examples, all of which relate to the synthesis represented in Figure 1.

Example 1

Preparation of BOC-p-nitro phenylalanine trans-cyclohexyldiamine monoamide

The typical procedure used to prepare the active ester was to dissolve the BOC acid, N-hydroxysuccinamide, and EDC (48 mmol) in ethyl acetate ((400mL). The mixture was stirred for 12 hours. The reaction solution was then filtered, and the filtrate was washed sequentially with saturated salt solution, 1M HCl, 5% $NaHCO_3$, and saturated salt solution (200 mL each). The organic layer was separated and dried over $MgSO_4$. After

filtering, the solution was rotary evaporated to a solid. The solid was taken up in DMF (200 mL) and added dropwise to trans-1,2-diaminocyclohexane over a period of 18 hours. The precipitated diamide was filtered off, and the solution was rotary evaporated to a thick oil. The residue was taken up in chloroform and washed, as above, to remove any of the starting materials. The chloroform solution was dried as before, filtered, and concentrated to a gel-like consistency. This material was poured onto a Buchner funnel and triturated with petroleum ether to leave the product as a light tan solid.

Example 2

Preparation of p-Nitrobenzyl-"CHX" diethylenetriamine

The BOC group was cleaved by stirring the amide (4.6 g) overnight in dioxane (300 mL) saturated with HCl. Addition of diethyl ether (200 mL), followed by cooling to 4°C, added significant precipitate. The dihydrochloride was collected on a Buchner funnel under argon and vacuum dried.

The amide dihydrochloride was suspended in THF (50 mL) in a three neck round bottom flask held in an ice bath. The flask was fitted with a condenser, thermometer, and a septum. Diborane/THF (6 eqv's) were injected into the flask, and the temperature was raised to 50°C and maintained there until the reduction was complete. The progress of the reaction was monitored by HPLC using a ten minute gradient of 100% 0.1M HOAc in water to 100% 0.1M HOAc in methanol. The column was a Waters DeltaPak C_{18}.

After the reaction was finished, the solution was cooled to room temperature, and methanol (50mL) was added to decompose any excess hydride. The solution was taken to dryness on a rotary evaporator, and the residue was taken up in 100% ethanol (100 mL). This solution was taken to dryness using a high vacuum rotary evaporator. Dioxane (150 mL), previously saturated with HCl, was added to the solid and the suspension was refluxed for four hours. The final suspension was left at 4°C for 18 hours.

The product was collected on a Buchner funnel under argon and then vacuum dried.

Example 3

Preparation of p-Nitrobenzyl CHX DTPA

The triamine (1.0 g, 2.49 mmol) was dissolved in DMF (25 mL) with sodium carbonate (1.992 g), and tert-butyl bromoacetate (2.915 g, 14.95 mmol) was added. The solution was heated to about 80°C overnight under argon, after which the reaction mixture was poured into H_2O (100 mL) and extracted with CH_2Cl_2 (100 mL). The organic layer was washed with water (3 x 100 mL), separated, dried over $MgSO_4$, filtered, and rotary evaporated to an oil. The oil was further concentrated to a thick oil by high vacuum rotary evaporation. A CI-MS of this oil indicated that the penta ester was by far the principal product (greater than 70%).

The oil was treated with TFA (25 mL) overnight. The excess reagent was removed by rotary evaporation. HPLC revealed essentially two major peaks, 10.95 minutes and 11.9 minutes. The HPLC method was a gradient of 0.05M Et_3N/HOAc to 100% MeOH over 25 minutes. Preparative HPLC was performed to separate and collect the two peaks. After completion of the pre-HPLC, the HPLC buffer was removed by ion-exchange chromatography (AG50 Wx8 200/400 mesh H+ form). Analytical HPLC of the two separated fractions indicated two separated pure products. After lyophilization, the two fractions were each treated with bis(trimethylsilyl) trifluoracetamide and EI-MS's were obtained. Each EI-MS gave the same M+ which in turn corresponded to the desired pentaacetic acid, each peak therefore being a separated pair of diastereomers now labeled a CHX-A or CHX-B.

Example 4

Preparation of p-Aminobenzyl CHX DTPA-A, -B

Atmospheric hydrogenation of each fraction was performed using 100 mg of each nitro compound with 10% Pd/C (100 mg) at pH 8.5. The reaction was allowed to proceed until the H_2 uptake had halted. The reaction

mixture was filtered on a fine frit with Celite 577. The filtrate was lyophilized to leave an off-white residue.

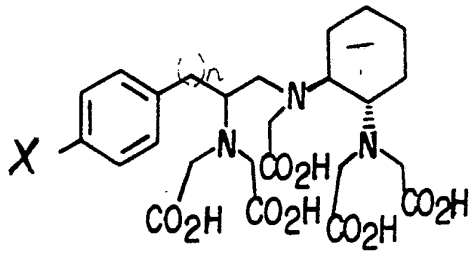
Example 5

Preparation of p-Isothiocyanatobenzyl CHX DTPA-A, -B

Each fraction was dissolved in H_2O (5mL) and treated with thiophosgene (20uL) in $CHCl_3$ (10mL) with maximum stirring under argon for two hours. The organic layer was removed by room temperature rotary evaporation, and the aqueous layer was lyophilized to leave an off-white solid. I.R. spectrum of each fraction showed a good, strong band at 2100 cm^{-1} for the aryl SCN group.

WHAT IS CLAIMED IS:

1. A ligand comprising:

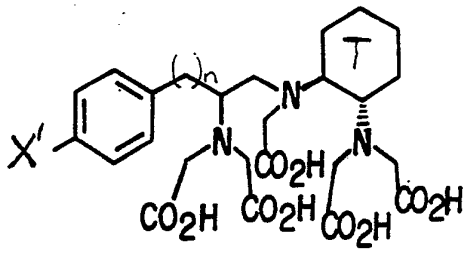


(I)

wherein n is an integer from 1 to 5;
X is $-NO_2$, $-NH_2$, -NCS or $-NHCOCH_2-Z$;
T denotes the trans isomer of the cyclohexyldiamine substructure; and
Z is chloride, bromide or iodide.

2. A metal chelate of the ligand of claim 1 wherein n is an integer from 1 to 5, X is $-NO_2$, $-NH_2$, -NCS, or $-NHCOCH_2-Z$, the metal is chosen from the group of elements consisting of Cu, Pb, In, Yt, Bi, the lanthanides, Au, Ag, and Ga, and Z is chloride, bromide and iodide.

3. A ligand-hapten conjugate comprising:



(II)

wherein n is an integer from 1 to 5;
X' is NH-Q, NHCS-Q or $-NHCOCH_2-Q$ where Q is a hapten chosen from the group consisting of steroids, enzymes, proteins, monoclonal antibodies, chimeric antibodies, and fragments thereof; and

T denotes the trans isomer of the cyclohexyldiamine substructure.

4. The ligand-hapten conjugate of claim 3 wherein n is an integer from 1 to 5, X' is -NH-LQ, -NHCS-L-Q or $-NHCOCH_2-L-Q$, where Q is a hapten selected from the group consisting of steroids, enzymes, proteins, monoclonal antibodies, chimeric antibodies, and fragments thereof, and L is a covalent linking group.

5. The ligand-hapten conjugate of claim 4 where L is selected from the group consisting of an organic radical, and a substituted aliphatic hydrocarbon chain.

6. The ligand-hapten conjugate of claim 5 wherein said substituted aliphatic hydrocarbon chain is interrupted by at least one hetero atom selected from -O- and -S- or by at least one -NR'- group, where R' is a hydrogen atom or a C-1 alkyl group, -CONR'- group, -NR'CO- group, cycloaliphatic, aromatic or heteroaromatic group, or a mixture thereof.

7. The metal chelate of the conjugate of claim 3 wherein n is an integer from 1 to 5, X' is -NH-Q, -NHCS-Q, or $-NHCOCH_2-Q$, where Q is a hapten selected from the group consisting of steroids, enzymes, proteins, monoclonal antibodies, chimeric antibodies, and fragments thereof, and the metal is selected from the group consisting of Cu, Pb, In, Yt, Bi, the lanthanides, Au, Ag, and Ga.

8. The metal chelate of the conjugate of claim 3 wherein n is an integer from 1 to 5, X' is -NH-L-Q, or -NHCS-L-Q, or $-NHCOCH_2-L-Q$, where Q is a hapten selected from the group consisting of steroids, enzymes, proteins, monoclonal antibodies, chimeric antibodies, and fragments thereof, the metal is selected from the group consisting of Cu, Pb, In, Yt, Bi, the lanthanides, Au, Ag, and Ga, and L is a covalent linking group.

9. The metal chelate of claim 8 wherein L is selected from the group consisting of an organic radical and a substituted aliphatic hydrocarbon chain.

10. The metal chelate of claim 9 where said substituted aliphatic hydrocarbon chain is interrupted by

at least one hetero atom selected from -O- and -S-, or by at least one -NR'- group, where R' is a hydrogen atom or a C-1 alkyl group, -CONR'- group, -NR'CO- group, cycloaliphatic, aromatic or heteroaromatic group, or a mixture thereof.

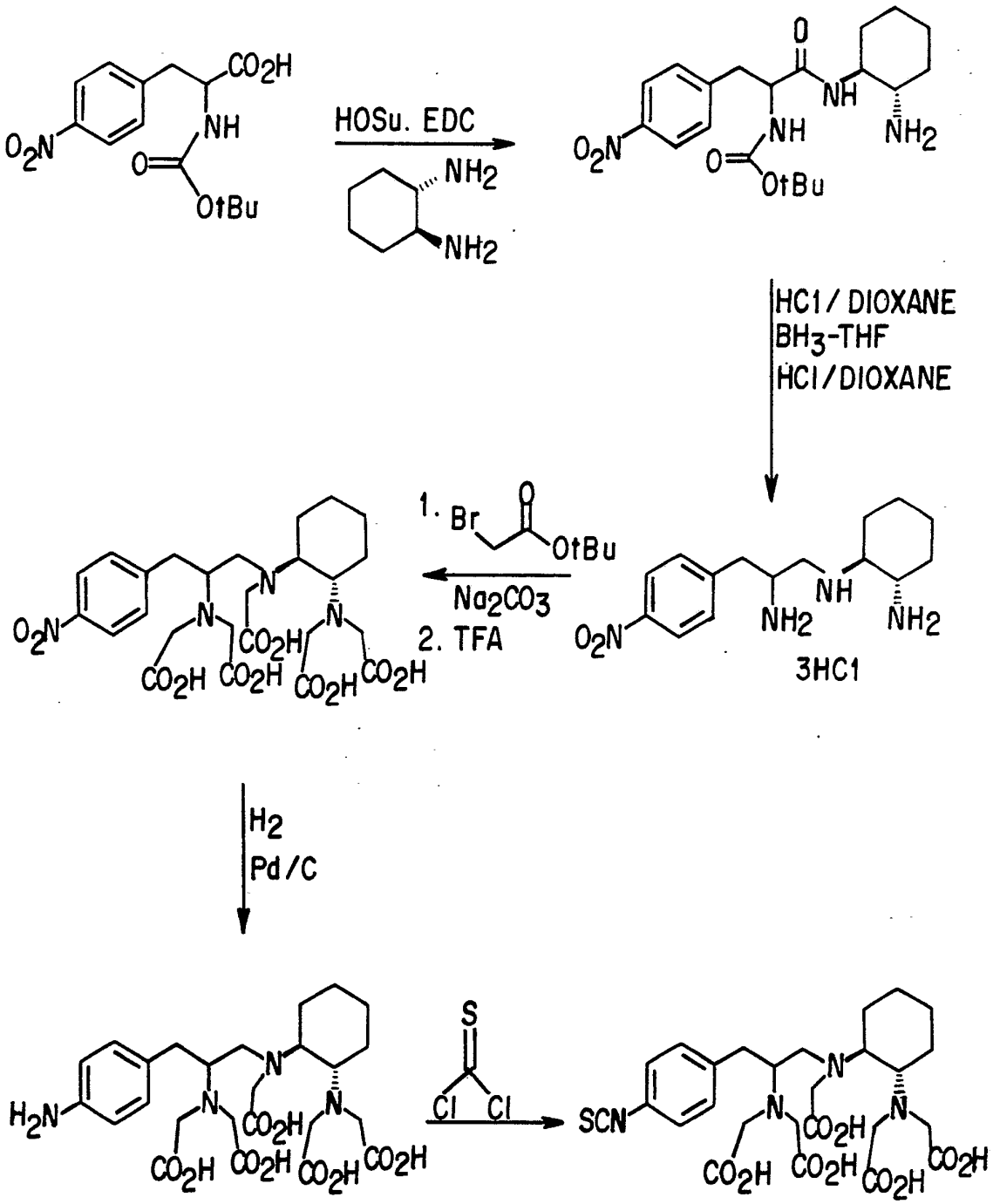
11. The method of using the conjugate of claim 7 wherein said conjugate is administered to a patient as a therapeutic or diagnostic agent.

12. The method of using the metal chelate of claim 8 wherein said chelate is administered to a patient as a therapeutic or diagnostic agent.

13. Use of the ligand haptan conjugate of claim 7 for the preparation of a medicament to be administered to a patient as a therapeutic or diagnostic agent.

14. Use of the metal chelate of claim 8 for the preparation of a medicament to be administered to a patient as a therapeutic or diagnostic agent.

FIG. 1


HOSu. EDC
HC1 / DIOXANE
BH3-THF
HCI/DIOXANE
1. Br OtBu
Na2CO3
2. TFA
3HC1
H2
Pd/C
S
Cl Cl
SCN

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/01919

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

(See Attachment)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/1.1,9,85.91,94.3; 435/188; 514/12,21; 530/388,390,391,408 409; 534/16; 552/502; 556/1,40,110; 558/17; 562/435,443,450,

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8].

Structure search in File Registry; File CA

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	WO, A, 86/06605 **(LAUFFER ET AL.)** 20 November 1986. See page 11, line 11.	1-14
Y	EP, A, 0,279,307 **(JOHNSON ET AL.)** 24 August 1988. See pages 4-10, 12 and 23.	1-14
Y	US, A, 4,454,106 **(GANSOW ET AL.)** 12 June 1984. See column 3.	1-14
A	US, A, 4,849,505 **(STAVRIANOPOULOS)** 18 July 1989. See column 36.	1-14
Y	Inorganic Chemistry, Vol. 25, No. 16, issued 1986, **BRECHBIEL ET AL.**, "Synthesis of 1-(p-Isothiocyanatobenzyl) Derivatives of DTPA and EDTA. Antibody Labeling and Tumor-Imaging Studies", pages 2772-2781. See 2nd column on page 2772.	1-14

* Special categories of cited documents: [10]

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

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

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

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

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

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

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

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

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21 June 1991	22 JUL 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Kay Kim, Ph.D. (vsh)

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

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

1. ☐ Claim numbers . ... . , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers .. , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

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

(See attachment)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. 1-14

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ATTACHMENT TO PCT/ISA/210
SUPPLEMENTAL SHEET
VI. UNITY OF INVENTION IS LACKING

UNITY OF INVENTION

Group I. Claims 1 and 2, drawn to a chelator ligand, classified in Class 534, 556, 558 or 562, subclasses 10;1,40;110;, 170, or 435, 443 and 450, respectively.

Group II. Claims 3-10, drawn to ligand-hapten conjugates classified in Classes 435, 530 or 552, subclasses 188, 388+ or 502 respectively.

Group III. Claims 11-14, drawn to method of use of the second invention above, classified in Classes 424 and 514, subclasses 1.1, 9, 85.91, 94.3 and 12, 21 respectively.

Inventions of Group I and Group II encompasses distinct chelating agents and hapten Q, respectively, represented by the following:

Group I. A) species of ligand wherein the X is NO2, NH2 and NHCOCH2-Z, and

B) species of ligand wherein the X is NCS.

Group II. A) species of conjugates wherein the hapten Q is steroids,

B) species of conjugates wherein the hapten Q is enzymes, and

C) species of ligand wherein the hapten Q is proteins of antibodies.

ATTACHMENT TO PCT/ISA/210/ (Second Sheet)
I. CLASSIFICATION OF SUBJECT MATTER

IPC(5): A61K 49/02; C12N 9/96; A61K 37/02, 39/44; C07K 17/02; C07F 19/00; C07J 43/00; C07F 1/00, 5/00, 7/24, 9/94, 11/00; C07C 331/28; C07C 205/06, 229/40, 229/76

U.S. CL.: 424/1.1, 9, 85.91, 94.3; 435/188; 514/12, 21; 530/388, 390, 391, 408, 409; 534/16; 552/502; 556/1, 40, 110; 558/17; 562/435, 443, 450