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(54) **Biotin-DOTA conjugates and their use in pretargeting methods**

Biotin-DOTA Konjugate und deren Verwendung in "Pretargeting" Verfahren

Biotine-DOTA conjugués et leur utilisation dans des procédés de préciblage

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EP-A- 0 251 494 **WO-A-88/08422**

- **CHEMICAL ABSTRACTS, vol. 106, no. 8, 23 February 1987, Columbus, Ohio, US; abstract no. 55742f, & HEPATOLOGY vol. 6, no. 4 , 1986 , BALTIMORE pages 723 - 728 P. VAN DER SLUIJS ET AL. 'DRUG TARGETING TO THE LIVER WITH LACTOSYLATED ALBUMINS: DOES THE GLYCOPROTEIN TARGET THE DRUG OR IS IT THE DRUG TARGETING THE GLYCOPROTEIN?'**
- **NUCL. MED. BIOL. vol. 18, no. 7 , 1991 pages 719 - 726 F. VIRZI ET AL. 'NEW INDIUM-111 LABELED BIOTIN DERIVATES FOR IMPROVED IMMUNOTARGETING.' cited in the application**
- **Goodwin D.A. et al.: J. NUCL. MED., vol. 33, 1992, pp. 880, anstract no. 232**

Remarks:
The file contains technical information submitted after the application was filed and not included in this specification

EP 0 646 019 B9

Description

Technical Field

[0001] The present invention relates to diagnostic or therapeutic agent conjugates for delivery to a target site by a target moiety.

Background of the Invention

[0002] Conventional cancer therapy is plagued by two problems. The generally attainable targeting ratio (ratio of administered dose localizing to tumor versus administration dose circulating in blood or ratio of administered dose localizing to tumor versus administered dose migrating to bone marrow) is low. Also, the absolute dose of radiation or therapeutic agent delivered to the tumor is insufficient in many cases to elicit a significant tumor response. Improvement in targeting ratio or absolute dose to tumor is sought.

Summary of the Invention

[0003] The present invention provides certain biotin-DOTA conjugates as targeting moiety-ligands useful in diagnostic and therapeutic pretargeting methods. Selection of moieties and methodologies used to enhance internalization (of chemotherapeutic drugs, for example) or to enhance retention at the target cell surface (of radionuclides, for example) is also discussed.

Detailed Description of the Invention

[0004] Prior to setting forth the invention, it may be helpful to set forth definitions of certain terms to be used within the disclosure.

[0005] Targeting moiety: a molecule that binds to a defined population of cells. The targeting moiety may bind a receptor, an oligonucleotide, an enzymatic substrate, an antigenic determinant, or other binding site present on or in the target cell population. Antibody is used throughout the specification as a prototypical example of a targeting moiety. Tumor is used as a prototypical example of a target in describing the present invention.

[0006] Ligand/anti-ligand pair: A complementary/anti-complementary set of molecules that demonstrate specific binding, generally of relatively high affinity. Exemplary ligand/anti-ligand pairs include zinc finger protein/dsDNA fragment, enzyme/inhibitor, hapten/antibody, lectin/carbohydrate, ligand/receptor, and biotin/avidin. Biotin/avidin is used throughout the specification as a prototypical example of a ligand/anti-ligand pair.

[0007] Anti-ligand: As defined herein, an "anti-ligand" demonstrates high affinity, and preferably, multivalent binding of the complementary ligand. Preferably, the anti-ligand is large enough to avoid rapid renal clearance, and contains sufficient multivalency to accomplish crosslinking and aggregation of targeting moiety-ligand conjugates. Univalent anti-ligands are also contemplated by the present invention. Anti-ligands of the present invention may exhibit or be derivitized to exhibit structural features that direct the uptake thereof, e.g., galactose residues that direct liver uptake. Avidin and streptavidin are used herein as prototypical anti-ligands.

[0008] Avidin: As defined herein, "avidin" includes avidin, streptavidin and derivatives and analogs thereof that are capable of high affinity, multivalent or univalent binding of biotin.

[0009] Ligand: As defined herein, a "ligand" is a relatively small, soluble molecule that exhibits rapid serum, blood and/or whole body clearance when administered intravenously in an animal or human. Biotin is used as the prototypical ligand.

[0010] Active Agent: A diagnostic or therapeutic agent ("the payload"), including radionuclides, drugs, anti-tumor agents, toxins and the like. Radionuclide therapeutic agents are used as prototypical active agents.

[0011] N_xS_y Chelates: As defined herein, the term "N_xS_y chelates" includes bifunctional chelators that are capable of (i) coordinately binding a metal or radiometal and (ii) covalently attaching to a targeting moiety, ligand or anti-ligand. Particularly preferred N_xS_y chelates have N_2S_2 and N_3S cores. Exemplary N_xS_y chelates are described in Fritzberg et al., Proc. Natl. Acad. Sci. USA 85:4024-29, 1988; in Weber et al., Bioconj. Chem. 1:431-37, 1990; and in the references cited therein, for instance.

[0012] Pretargeting: As defined herein, pretargeting involves target site localization of a targeting moiety that is conjugated with one member of a ligand/anti-ligand pair; after a time period sufficient for optimal target-to-non-target accumulation of this targeting moiety conjugate, active agent conjugated to the opposite member of the ligand/anti-ligand pair is administered and is bound (directly or indirectly) to the targeting moiety conjugate at the target site (two-step pretargeting). Three-step and other related methods described herein are also encompassed.

[0013] Clearing Agent: An agent capable of binding, complexing or otherwise associating with an administered moiety

(e.g., targeting moiety-ligand, targeting moiety-anti-ligand or anti-ligand alone) present in the recipient's circulation, thereby facilitating circulating moiety clearance from the recipient's body, removal from blood circulation, or inactivation thereof in circulation. The clearing agent is preferably characterized by physical properties, such as size, charge, configuration or a combination thereof, that limit clearing agent access to the population of target cells recognized by a targeting moiety used in the same treatment protocol as the clearing agent.

[0014] Target Cell Retention: The amount of time that a radionuclide or other therapeutic agent remains at the target cell surface or within the target cell. Catabolism of conjugates or molecules containing such therapeutic agents appears to be primarily responsible for the loss of target cell retention.

[0015] Conjugate: A conjugate encompasses chemical conjugates (covalently or non-covalently bound), fusion proteins and the like.

[0016] Permeability Enhancing Moiety: An agent capable of increasing the permeability at a target site characterized by a three dimensional cellular matrix. Exemplary permeability enhancing moieties function by one or more of the following mechanisms: inducing gaps in the endothelium of venules through action on the postcapillary bed; inducing such gaps through action on the entire capillary bed; disrupting cell-to-cell associations; mediating target cell inflammatory responses; or the like.

[0017] Intercellular Junction: An area of interacting adjacent plasma membranes. Intercellular junctions can be categorized functionally into: (1) adhering junctions that hold cells tightly together (for example, desmosomes); (2) impermeable junctions that hold cells tightly together and prevent leakage of molecules between cells (i.e., tight junctions); and (3) communicating junctions that mediate passage of small molecules between adjacent cells (for instance, gap junctions).

[0018] Immunogen: A substance which is capable, under appropriate conditions, of inducing a specific immune response and of reacting with the products of that response (e.g., a specific antibody, specifically sensitized T-lymphocytes or both).

[0019] Hapten Immunogen: A specific protein-free substance which has a chemical configuration such that it can interact with specific combining groups on an antibody or with the recognition site on a T-lymphocyte but which, unlike antigenic determinants, does not itself elicit an immune response (e.g., a detectable T-cell response or the formation of a detectable amount of antibody). When coupled with a carrier protein, it does elicit an immune response.

[0020] Lymphokine. Soluble protein mediators released by certain lymphocytes, which in turn can regulate other cell-mediated immune functions, such as lymphocyte transformation, macrophage activation or cytotoxicity on other cells.

[0021] Mitogen. A substance that induces mitosis and cell transformation, especially lymphocyte transformation.

[0022] A recognized disadvantage associated with in vivo administration of targeting moiety-radioisotopic conjugates for imaging or therapy is localization of the attached radioactive agent at both non-target and target sites. Until the administered radiolabeled conjugate clears from the circulation, normal organs and tissues are transitorily exposed to the attached radioactive agent. For instance, radiolabeled whole antibodies that are administered in vivo exhibit relatively slow blood clearance; maximum target site localization generally occurs 1-3 days post-administration. Generally, the longer the clearance time of the conjugate from the circulation, the greater the radioexposure of non-target organs.

[0023] These characteristics are particularly problematic with human radioimmunotherapy. In human clinical trials, the long circulating half-life of radioisotope bound to whole antibody causes relatively large doses of radiation to be delivered to the whole body. In particular, the bone marrow, which is very radiosensitive, is the dose-limiting organ of nonspecific toxicity.

[0024] In order to decrease radioisotope exposure of non-target tissue, potential targeting moieties generally have been screened to identify those that display minimal non-target reactivity, while retaining target specificity and reactivity. By reducing non-target exposure (and adverse non-target localization and/or toxicity), increased doses of a radiotherapeutic conjugate may be administered; moreover, decreased non-target accumulation of a radiodiagnostic conjugate leads to improved contrast between background and target.

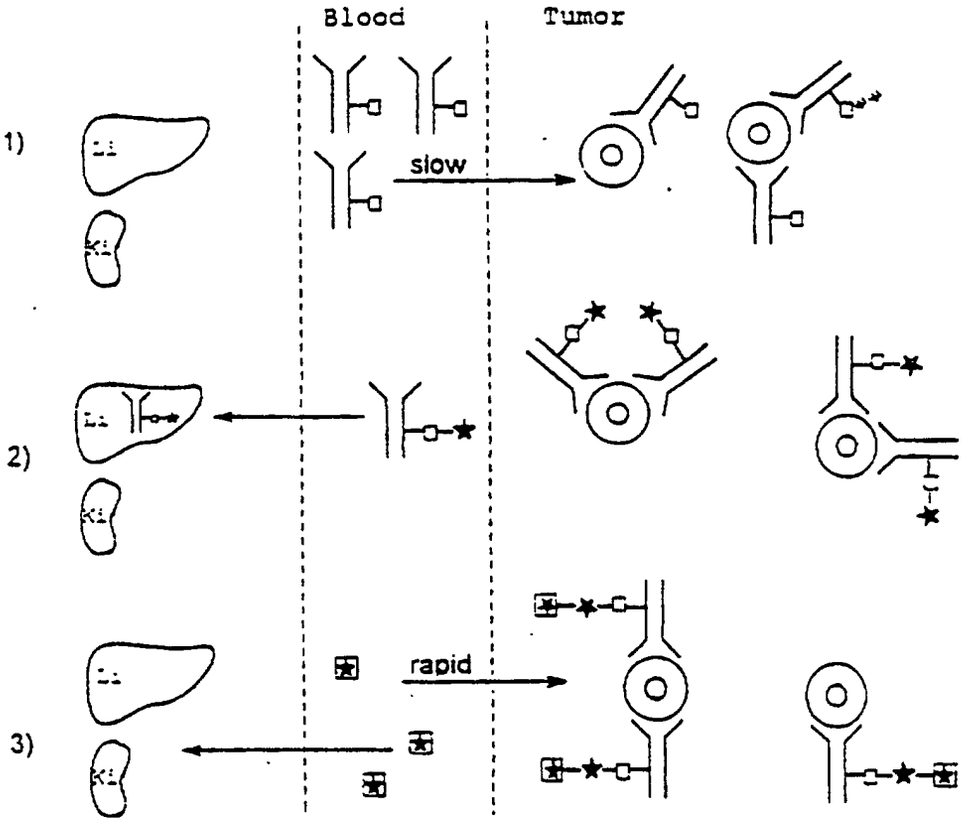
[0025] Therapeutic drugs, administered alone or as targeted conjugates, are accompanied by similar disadvantages. Again, the goal is administration of the highest possible concentration of drug (to maximize exposure of target tissue), while remaining below the threshold of unacceptable normal organ toxicity (due to non-target tissue exposure). Unlike radioisotopes, however, therapeutic drugs need to be taken into a target cell to exert a cytotoxic effect. In the case of targeting moiety-therapeutic drug conjugates, it would be advantageous to combine the relative target specificity of a targeting moiety with a means for enhanced target cell internalization of the targeting moiety-drug conjugate.

[0026] In contrast, enhanced target cell internalization is disadvantageous if one administers diagnostic agent-targeting moiety conjugates. Internalization of diagnostic conjugates results in cellular catabolism and degradation of the conjugate. Upon degradation, small adducts of the diagnostic agent or the diagnostic agent per se may be released from the cell, thus eliminating the ability to detect the conjugate in a target-specific manner.

[0027] One method for reducing non-target tissue exposure to a diagnostic or therapeutic agent involves "pretargeting" the targeting moiety at a target site, and then subsequently administering a rapidly clearing diagnostic or therapeutic agent conjugate that is capable of binding to the "pretargeted" targeting moiety at the target site. A description of some

embodiments of the pretargeting technique may be found in US Patent No. 4,863,713 (Goodwin et al.).

[0028] A typical pretargeting approach ("three-step") is schematically depicted below.



Targeting moiety
Anti-ligand
Ligand
Ligand-active agent
Binding site (i.e., receptor, antigenic determinant)
Liver
Kidney

Briefly, this three-step pretargeting protocol features administration of an antibody-ligand conjugate, which is allowed to localize at a target site and to dilute in the circulation. Subsequently administered anti-ligand binds to the antibody-ligand conjugate and clears unbound antibody-ligand conjugate from the blood. Preferred anti-ligands are large and contain sufficient multivalency to accomplish crosslinking and aggregation of circulating antibody-ligand conjugates. The clearing by anti-ligand is probably attributable to anti-ligand crosslinking and/or aggregation of antibody-ligand conjugates that are circulating in the blood, which leads to complex/aggregate clearance by the recipient's RES (reticuloendothelial system). Anti-ligand clearance of this type is preferably accomplished with a multivalent molecule; however, a univalent molecule of sufficient size to be cleared by the RES on its own could also be employed. Alternatively, receptor-based clearance mechanisms, e.g., Ashwell receptor hexose, e.g., galactose, mannose or the like, residue recognition mechanisms, may be responsible for anti-ligand clearance. Such clearance mechanisms are less dependent upon the valency of the anti-ligand with respect to the ligand than the RES complex/aggregate clearance mechanisms. It is preferred that the ligand-anti-ligand pair displays relatively high affinity binding.

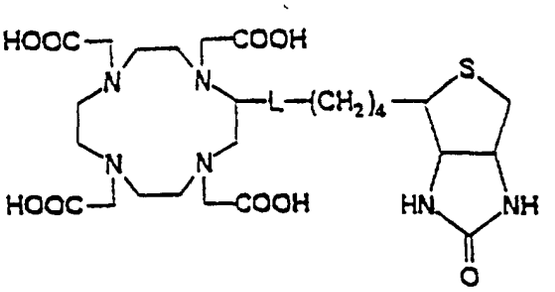
[0029] A diagnostic or therapeutic agent-ligand conjugate that exhibits rapid whole body clearance is then administered. When the circulation brings the active agent-ligand conjugate in proximity to the target cell-bound antibody-ligand-anti-ligand complex, anti-ligand binds the circulating active agent-ligand conjugate and produces an antibody-ligand : anti-ligand : ligand-active agent "sandwich" at the target site. Because the diagnostic or therapeutic agent is attached to a rapidly clearing ligand (rather than antibody, antibody fragment or other slowly clearing targeting moiety), this technique promises decreased non-target exposure to the active agent.

[0030] Alternate pretargeting methods eliminate the step of parenterally administering an anti-ligand clearing agent. These "two-step" procedures feature targeting moiety-ligand or targeting moiety-anti-ligand administration, followed by administration of active agent conjugated to the opposite member of the ligand-anti-ligand pair. As an optional step "1.5" in the two-step pretargeting methods of the present invention, a clearing agent (preferably other than ligand or anti-ligand alone) is administered to facilitate the clearance of circulating targeting moiety-containing conjugate.

[0031] In the two-step pretargeting approach, the clearing agent preferably does not become bound to the target cell population, either directly or through the previously administered and target cell bound targeting moiety-anti-ligand or targeting moiety-ligand conjugate. An example of two-step pretargeting involves the use of biotinylated human transferrin as a clearing agent for avidin-targeting moiety conjugate, wherein the size of the clearing agent results in liver clearance of transferrin-biotin-circulating avidin-targeting moiety complexes and substantially precludes association with the avidin-targeting moiety conjugates bound at target cell sites. (See, Goodwin, D.A., Antibod. Immunoconj. Radiopharm., 4: 427-34, 1991).

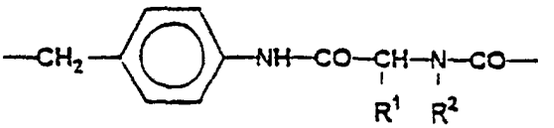
[0032] The two-step pretargeting approach overcomes certain disadvantages associated with the use of a clearing agent in a three-step pretargeted protocol. More specifically, data obtained in animal models demonstrate that in vivo anti-ligand binding to a pretargeted targeting moiety-ligand conjugate (i.e., the cell-bound conjugate) removes the targeting moiety-ligand conjugate from the target cell. One explanation for the observed phenomenon is that the multivalent anti-ligand crosslinks targeting moiety-ligand conjugates on the cell surface, thereby initiating or facilitating internalization of the resultant complex. The apparent loss of targeting moiety-ligand from the cell might result from internal degradation of the conjugate and/or release of active agent from the conjugate (either at the cell surface or intracellularly). An alternative explanation for the observed phenomenon is that permeability changes in the target cell's membrane allow increased passive diffusion of any molecule into the target cell. Also, some loss of targeting moiety-ligand may result from alteration in the affinity by subsequent binding of another moiety to the targeting moiety-ligand, e.g., anti-idiotype monoclonal antibody binding causes removal of tumor bound monoclonal antibody.

[0033] The first aspect of the present invention provides a biotin-DOTA conjugate a biotin-DOTA conjugate of the following formula:

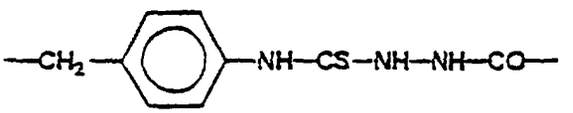


wherein a linker L is selected from the group comprising:

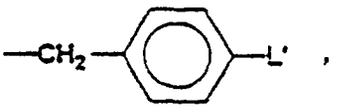
1) a D-amino acid containing linker of the formula



2) a linker of the formula

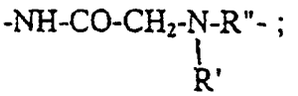


3) a linker of the formula



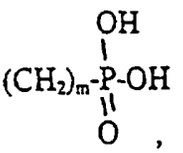
wherein L' is selected from the group comprising:

a) -NH-CO-$(CH_2)_n$-O-;
b) -NH-;
c)



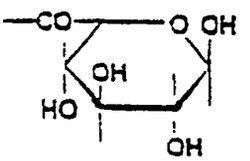
d) -NH-CS-NH-;
e) -NH-CO-$(CH_2)_n$-NH-; and
-NH-CO-Z-CH(COOH)-NH-CO where Z is -$(CH_2)_2$-, -CH_2-S-CH_2-, -CH_2-, or -$(CH_2)_n$-CO-O-CH_2- where n is from 1 to 4.

wherein R^1 is hydrogen, C_1-C_5 lower alkyl; C_1-C_5 lower alkyl substituted with one or more hydrophilic groups preferably $(CH_2)_m$-OH, $(CH_2)_m$-OSO_3, $(CH_2)_m$-SO_3, and



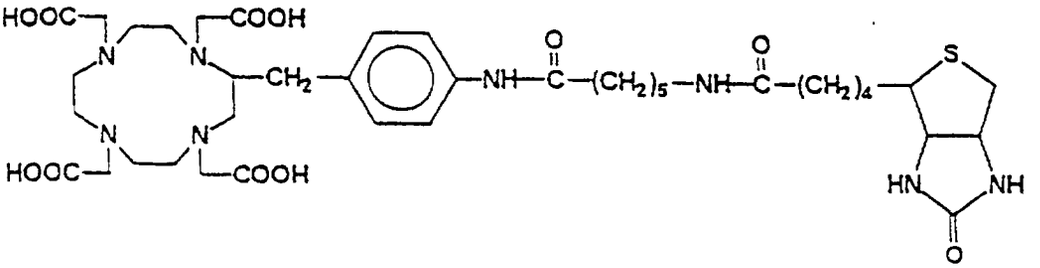
where m is 1 or 2;
glucuronide-substituted amino acids, or other glucuronide derivatives;

R^2 is hydrogen; C_1-C_5 lower alkyl; substituted C_1-C_5 lower alkyl having one or more substituents selected from the group comprising hydroxy, sulfate, and phosphonate; or a hydrophilic moiety;
R' is hydrogen; -$(CH_2)_2$-OH or a sulfate or



phosphonate derivative thereof; or
R" is a bond or -$(CH_2)_n$-CO-NH- ; and
n ranges from 0-5.

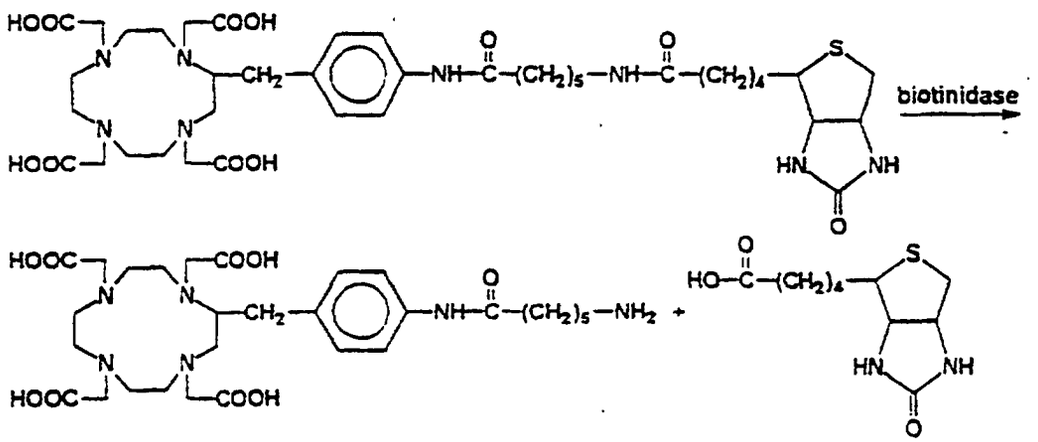
[0034] The 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetra acetic acid (DOTA)-biotin conjugate (DOTA-LC-biotin) depicted below has been reported to have desirable in vivo biodistribution and is cleared primarily by renal excretion.



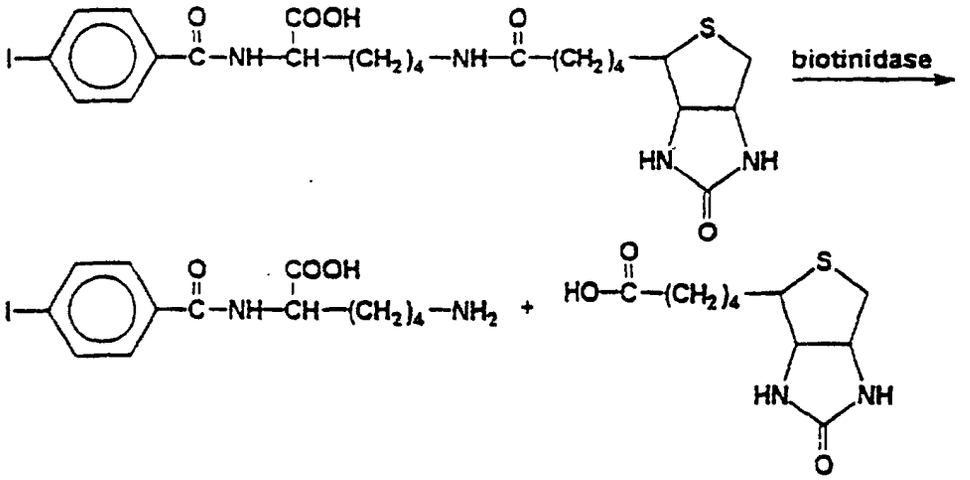
DOTA may also be conjugated to other ligands or to anti-ligands in the practice of the present invention.

[0035] Because DOTA strongly binds Y-90 and other radionuclides, it has been proposed for use in radioimmunotherapy. For therapy, it is very important that the radionuclide be stably bound within the DOTA chelate and that the DOTA chelate be stably attached to biotin. Only radiolabeled DOTA-biotin conjugates exhibiting those two characteristics are useful to deliver radionuclides to the targets. Release of the radionuclide from the DOTA chelate or cleavage of the biotin and DOTA conjugate components in serum or at non-target sites renders the conjugate unsuitable for use in therapy.

[0036] Serum stability of DOTA-LC-biotin (where LC refers to the "long chain" linker, including an aminocaproyl spacer between the biotin and the DOTA conjugate components) shown above, while reported in the literature to be good, has proven to be problematic. Experimentation has revealed that DOTA-LC-biotin is rapidly cleared from the blood and excreted into the urine as fragments, wherein the biotinamide bond rather than the DOTA-amide bond has been cleaved, as shown below.



[0037] Additional experimentation employing PIP-biocytin conjugates produced parallel results as shown below.

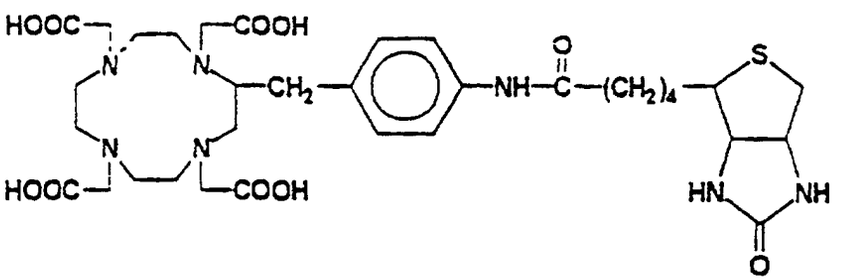


Cleavage of the benzamide was not observed as evidenced by the absence of detectable quantities of iodobenzoic acid in the serum.

[0038] It appears that the cleavage results from the action of serum biotinidase. Biotinidase is a hydrolytic enzyme that catalyzes the cleavage of biotin from biotinyl peptides. See, for example, Evangelatos, et al., "Biocinidase Radioassay Using an I-125-Biotin Derivative, Avidin, and Polyethylene Glycol Reagents," Analytical Biochemistry, 196: 385-89, 1991.

[0039] Drug-biotin conjugates which structurally resemble biotinyl peptides are potential substrates for cleavage by plasma biotinidase. Poor in vivo stability therefore limits the use of drug-biotin conjugates in therapeutic applications. The use of peptide surrogates to overcome poor stability of peptide therapeutic agents has been an area of intense research effort. See, for example, Spatola, Peptide Backbone Modification: A Structure-Activity Analysis of Peptide Containing Amide Bond Surrogates, "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," vol. 7, Weinstein, ed., Marcel Dekker, New York, 1983; and Kim et al., "A New Peptide Bond Surrogate: 2-Isoxazoline in Pseudodipeptide Chemistry," Tetrahedron Letters, 45 : 6811-14, 1991.

[0040] Elimination of the aminocaproyl spacer of DOTA-LC-biotin gives DOTA-SC-biotin (where the SC indicates the "short chain" linker between the DOTA and biotin conjugate components), which molecule is shown below:



DOTA-SC-biotin exhibits significantly improved serum stability in comparison to DOTA-LC-biotin. This result does not appear to be explainable on the basis of biotinidase activity alone. The experimentation leading to this conclusion is summarized in the Table set forth below.

Time Dependent Cleavage of DOTA-Biotin Conjugates

Time at 37°C	% Avidin Binding		
	PIP-Biocytin	Y-90-LC DOTA-Biotin	Y-90-SC DOTA-Biotin
5 Minutes	75%	50%	-
15 Minutes	57%	14%	-
30 Minutes	31%	12%	-

(continued)

Time Dependent Cleavage of DOTA-Biotin Conjugates			
Time at 37°C	% Avidin Binding		
	PIP-Biocytin	Y-90-LC DOTA-Biotin	Y-90-SC DOTA-Biotin
60 Minutes	-	0%	98%
20 Hours	-	0%	60%

where "-" indicates that the value was not measured.

[0041] The difference in serum stability between DOTA-LC-biotin and SOTA-SC-biotin might be explained by the fact that the SC derivative contains an aromatic amide linkage in contrast to the aliphatic amide linkage of the LC derivative, with the aliphatic amide linkage being more readily recognized by enzymes as a substrate therefor. This argument cannot apply to biotinidase, however, because biotinidase very efficiently cleaves aromatic amides. In fact, it is recognized that the simplest and most commonly employed biotinidase activity measuring method uses N-(d-biotinyl)-4-aminopenzoate (BPABA) as a substrate, with the hydrolysis of BPABA resulting in the liberation of biotin and 4-aminobenzoate (PABA). See, for example, B. Wolf, et al., "Methods in Enzymology," pp. 103-111, Academic Press Inc., 1990. Consequently, one would predict that DOTA-SC-biotin, like its LC counterpart, would be a biotinidase substrate. Since DOTA-SC-biotin exhibits serum stability, biotinidase activity alone does not adequately explain why some conjugates are serum stable while others are not. A series of DOTA-biotin conjugates was therefore synthesized by the present inventors to determine which structural features conferred serum stability to the conjugates.

[0042] Some general strategies for improving serum stability of peptides with respect to enzymatic action are the following: incorporation of D-amino acids, N-methyl amino acids and alpha-substituted amino acids.

[0043] In vivo stable biotin-DOTA conjugates are useful within the practice of the present invention. In vivo stability imparts the following advantages:

1) increased tumor uptake in that more of the radioisotope will be targeted to the previously localized targeting moiety-streptavidin; and
2) increased tumor retention, if biotin is more stably bound to the radioisotope.

In addition, the linkage between DOTA and biotin may also have a significant impact on biodistribution (including normal organ uptake, target uptake and the like) and pharmacokinetics.

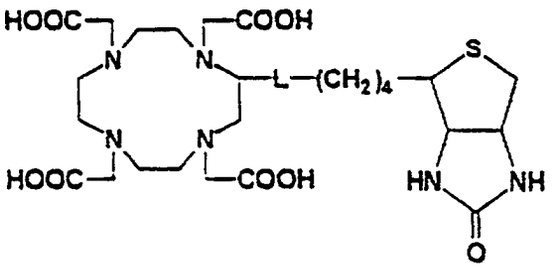
[0044] The strategy for design of the DOTA-containing molecules and conjugates of the present invention involved three primary considerations:

1) in vivo stability (including biotinidase and general peptidase activity resistance), with an initial cut of 100% stability for 1 hour;
2) renal excretion; and
3) ease of synthesis.

The DOTA-biotin conjugates of the present invention reflect the implementation of one or more of the following strategies:

1) substitution of the carbon adjacent to the cleavage susceptible amide nitrogen;
2) alkylation of the cleavage susceptible amide nitrogen;
3) substitution of the amide carbonyl with an alkyl amino group;
4) incorporation of D-amino acids as well as analogs or derivatives thereof; or
5) incorporation of thiourea linkages.

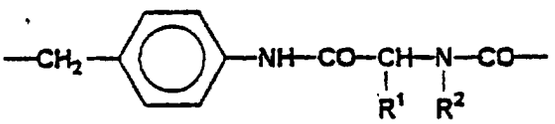
[0045] DOTA-biotin conjugates in accordance with the present invention may be generally characterized as follows: conjugates that retain the biotin carboxy group in the structure thereof and those that do not (i.e., the terminal carboxy group of biotin has been reduced or otherwise chemically modified. Structures of such conjugates represented by the following general formula have been devised:



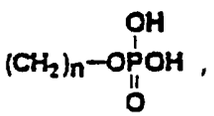
wherein L may alternatively be substituted in one of the following ways on one of the -CH_2-COOH branches of the DOTA structure: -CH(L)-COOH or -CH_2COOL or -CH_2COL). when these alternative structures are employed, the portion of the linker bearing the functional group for binding with the DOTA conjugate component is selected for the capabiiity to interact with either the carbon or the carboxy in the branch portions of the DOTA structure, with the serum stability conferring portion of the linker structure being selected as described below.

[0046] In the case where the linkage is formed on the core of the DOTA structure as shown above, L is selected according to the following principles, with the portion of the linker designed to bind to the DOTA conjugate component selected for the capability to bind to an amine.

[0047] A. One embodiment of the present invention includes linkers containing a D-amino acid spacer between a DOTA aniline amine and the biotin carboxy group shown above. Substituted amino acids are preferred for these embodiments of the present invention, because alpha-substitution also confers enzymatic cleavage resistance. Exemplary L moieties of this embodiment of the present invention may be represented as follows:



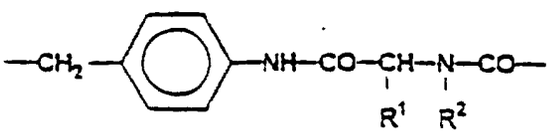
where R^1 is selected from lower alkyl, lower alkyl substituted with hydrophilic groups (preferably, $(CH_2)_n$-OH, $(CH_2)_n$-OSO_3, $(CH_2)_n$-SO_3,



where n is 1 or 2), glucuronide-substituted amino acids or other glucuronide derivatives; and

R^2 is selected from hydrogen, lower alkyl, substituted lower alkyl (e.g., hydroxy, sulfate, phosphonate or a hydrophilic moiety (preferably OH).

[0048] An embodiment of the present invention provides a biotin-DOTA conjugate as claimed in claim 1 where L is a D-amino acid-containing a biotin DOTA conjugate of claim 1 wherein L is a D-amino acid-containing a linker of the formula

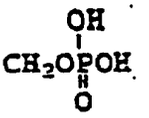


wherein R^1 and R^2 are as herein before defined.

[0049] For the purposes of the present disclosure, the term "lower alkyl" indicates an alkyl group with from one to five carbon atoms. Also, the term "substituted" includes one or several substituent groups, with a single substituent group preferred.

[0050] Preferred L groups of this embodiment of the present invention include the following:

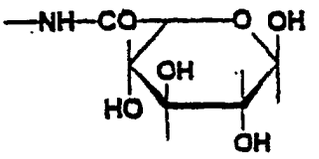
$R^1 = CH_3$ and R^2 = H (a D-alanine derivative);
$R^1 = CH_3$ and $R^2 = CH_3$ (an N-methyl-D-alanine derivative) ;
$R^1 = CH_2$-OH and R^2 = H (a D-serine derivative) ;
$R^1 = CH_2OSO_3$ and R^2 = H (a D-serine-O-sulfate-derivative) ; and
R^1 =



and R^2 = H (a D-serine-O-, phosphonate-derivative) ;

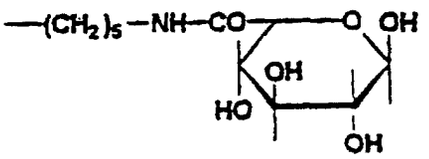
[0051] An embodiment of the present invention provides a biotin-DOTA conjugate of Claim 2 wherein R^1 is CH_3 and R^2 is H.

[0052] Other preferred moieties of this embodiment of the present invention include molecules wherein R^1 is hydrogen and $R^2 = -(CH_2)_nOH$ or a sulfate or phosphonate derivative thereof and n is 1 or 2 as well as molecules wherein R^1 is



;

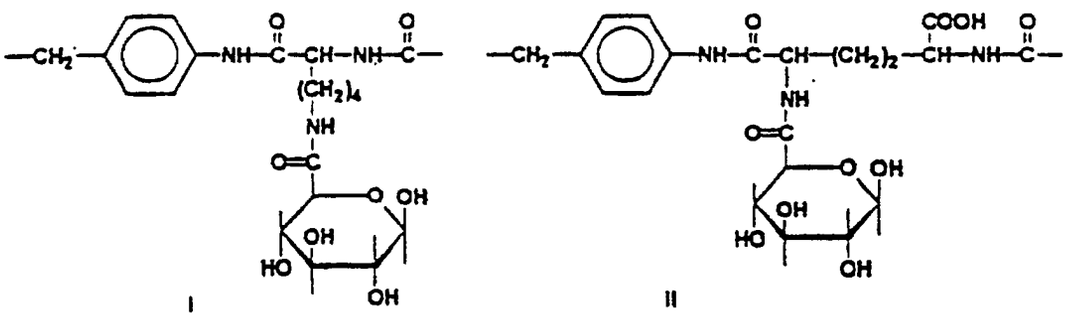
or $-(CH_2)_5-NH_2$; or



;

when R^2 = H.

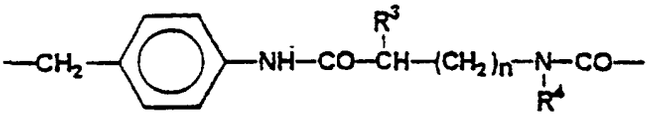
[0053] Preferred moieties incorporating the glucuronide of D-lysine and the glucuronide of amino pimelate are shown below as I and II, respectively.



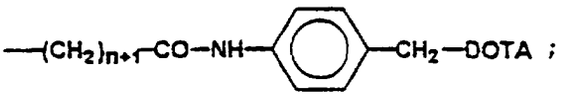
I II

[0054] A particularly preferred linker of this embodiment of the present invention is the D-alanine derivative set forth above.

[0055] B. Linkers incorporating alkyl substitution on one or more amide nitrogen atoms are also encompassed by the present invention, with some embodiments of such linkers preparable from L-amino acids. Amide bonds having a substituted amine moiety are less susceptible to enzymatic cleavage. Such linkers exhibit the following general formula:



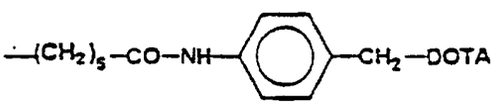
where R^4 is selected from hydrogen, lower alkyl, lower alkyl substituted with hydroxy, sulfate, phosphonate or the like and



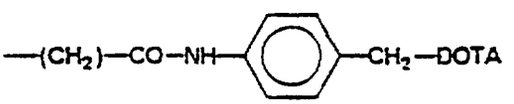
R_3 is selected from hydrogen; an amine; lower alkyl; an amino- or a hydroxy-, sulfate- or phosphonate-substituted lower alkyl; a glucuronide or a glucuronide-derivatized amino groups; and
n ranges from 0-4.

[0056] Preferred linkers of this embodiment of the present invention include:

R^3 = H and R^4 = CH_3 when n = 4, synthesizable as discussed in Example XXI;
R^3 = H and R^4 = CH_3 when n = 0, synchesizable from N-methyl-glycine (having a trivial name of sarcosine) as described in Example XXI;
R^3 = NH_2 and R^4 = CH_3, when n = 0;
R^3 = H and R^4 =

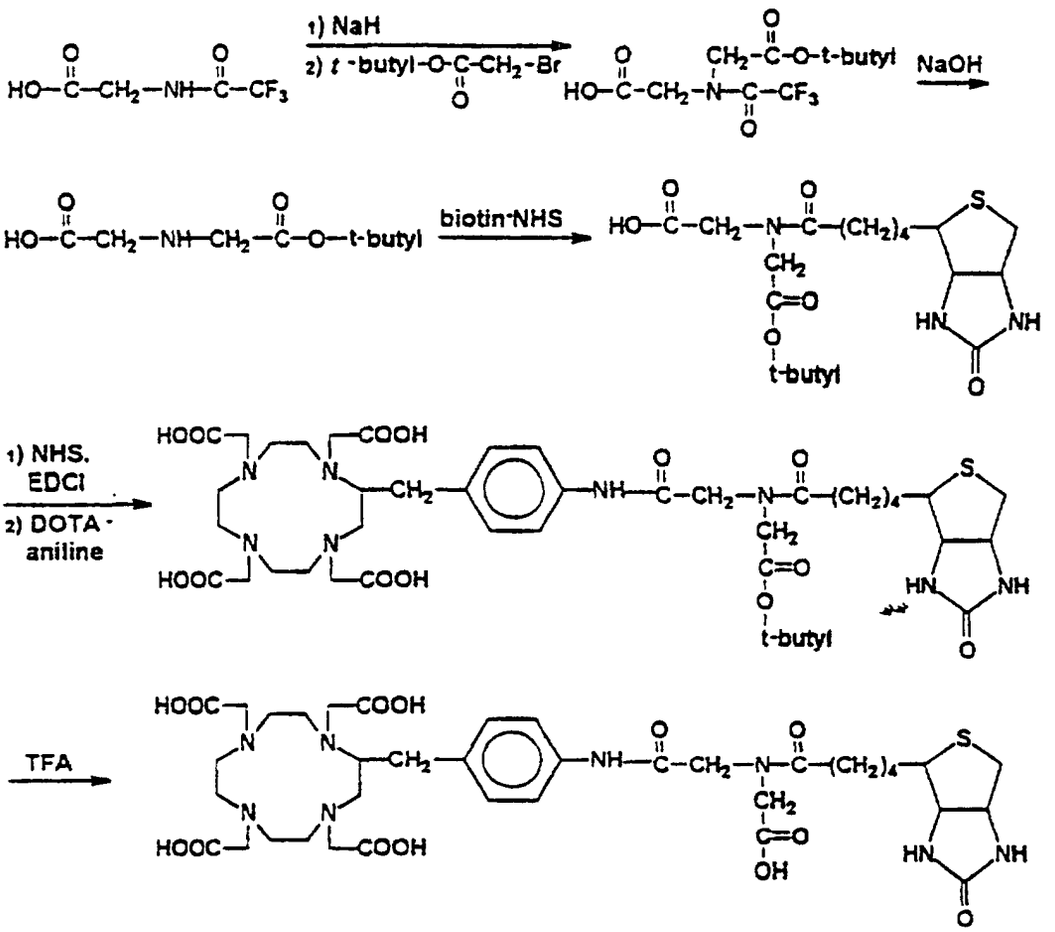


when n = 4 (Bis-DOTA-LC-biotin), synthesizable from bromohexanoic acid as discussed in Example XXI; and
R^3 = H and R^4 =



when n = 0 (bis-DOTA-SC-biotin), synthesizable from iminodiacetic acid.

[0057] The synthesis of a conjugate including a linker wherein R^3 is H and R^4 is $-CH_2CH_2OH$ and n is 0 is also described in Example XXI. Schematically, the synthesis of a conjugate of this embodiment of the present invention wherein n is 0, R^3 is H and R^4 is $-CH_2$-COOH is shown below.

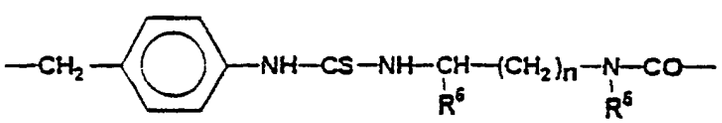


[0058] Bis-DOTA-LC-biotin, for example, offers the following advantages:

1) incorporation of two DOTA molecules en one biotin moiety increases the overall hydrophilicity of the biotin conjugate and thereby directs in vivo distribution to urinary excretion; and
2) substitution of the amide nitrogen adjacent to the biotin carboxyl group blocks peptide and/or biotinidase cleavage at that site.

[0059] Bis-DOTA-LC-biotin, the glycine-based linker and the N-methylated linker where R^3 = H, R^4 = CH_3, n = 4 are particularly preferred linkers of this embodiment of the present invention.

[0060] C. Another linker embodiment incorporates a thiourea moiety therein. Exemplary thiourea adducts of the present invention exhibit the following general formula:



where R^5 is selected from hydrogen or lower alkyl;

R^6 is selected from H and a hydrophilic moiety; and
n ranges from 0-4.

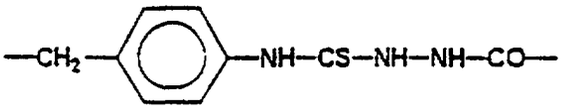
[0061] Preferred linkers of this embodiment of the present invention are as follows:

R^5 = H and R^6 = H when n = 5;

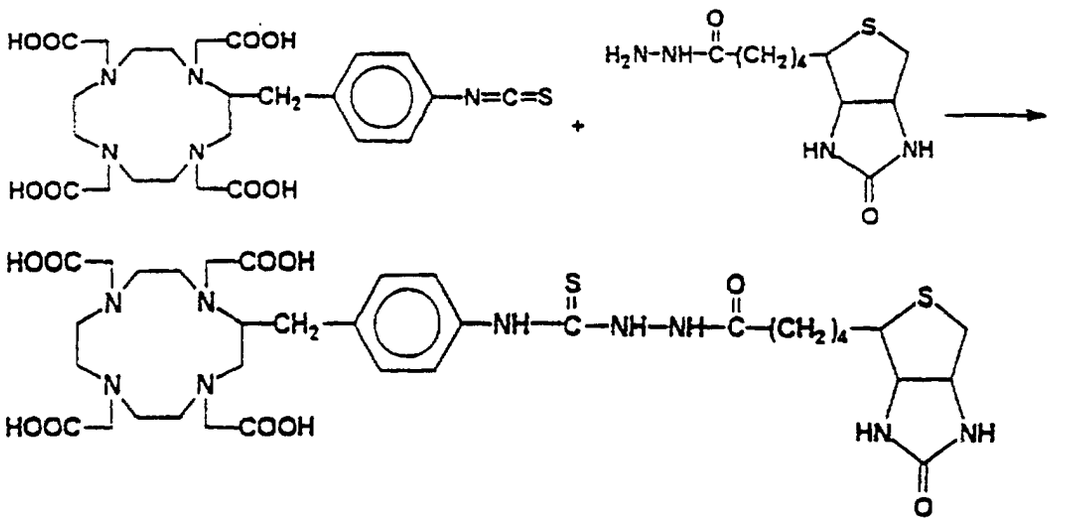
R^5 = H and R^6 = COOH when n = 5; and
R^5 = CH_3 and R^6 = COOH when n = 5.

The second preferred linker recited above can be prepared using either L-lysine or D-lysine. Similarly, the third preferred linker can be prepared using either N-methyl-D-lysine or N-methyl-L-lysine.

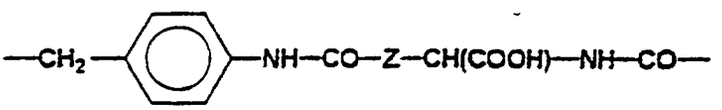
[0062] Another thiourea adduct of minimized lipophilicity is



which may be formed via the addition of biotinhydrazide (commercially available from Sigma Chemical Co., St. Louis, Missouri) and DOTA-benzylisothiocyanate a known compound synthesized in one step from DOTA-aniline), with the thiourea-containing compound formed as shown below.



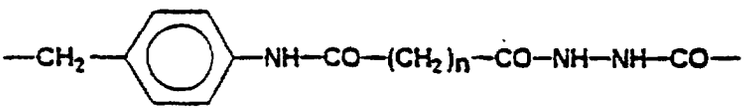
[0063] D. Amino acid-derived linkers of the present invention with substitution of the carbon adjacent to the cleavage susceptible amide have the general formula set forth below:



wherein Z is - $(CH_2)_2$-, conveniently synthesized form glutamic acid; or

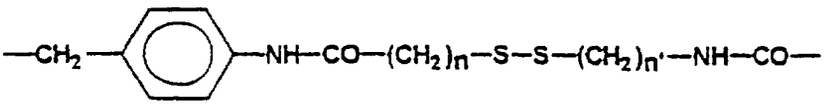
Z = -CH_2-S-CH_2-, synthesizable from cysteine and iodo-acetic acid; or
Z = -CH_2-, conveniently synthesized form aspartic acid; or
Z = -$(CH_2)_n$-CO-O-CH_2-, where n ranges from 1-4 and which is synthesizable from serine.

[0064] E. Another exemplary linker embodiment of the present invention has the general formula set forth below:



and n ranges from 1-5.

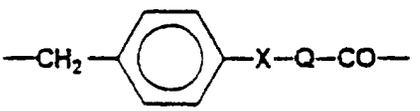
[0065] F. Another embodiment involves disulfide-containing linkers, which provide a metabolically cleavable moiety (-S-S-) to reduce non-target retention of the biotin-DOTA conjugate. Exemplary linkers of this type exhibit the following formula:



where n and n' preferably range between o and E.

[0066] The advantage of using conditionally cleavable linkers is an improvement in target/non-target localization of the active agent. Conditionally cleavable linkers include enzymatically cleavable linkers, linkers that are cleaved under acidic conditions, linkers that are cleaved under basic conditions and the like. More specifically, use of linkers that are cleaved by enzymes, which are present in non-target tissues but reduced in amount or absent in target tissue, can increase target cell retention of active agent relative to non-target cell retention. Such conditionally cleavable linkers are useful, for example, in delivering therapeutic radionuclides to target cells, because such active agents do not require internalization for efficacy, provided that the linker is stable at the target cell surface or protected from target cell degradation.

[0067] G. Ether, thioether, ester and thioester linkers are also useful in the practice of the present invention, because such linkages are acid cleavable and therefore facilitate improved non-target retention. Exemplary linkers of this type have the following general formula:



where X is O or S; and

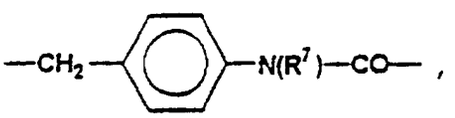
Q is a bond, a methylene group, a -CO- group or -CO-$(CH_2)_n$-NH-; and
n ranges from 1-5.

Other such linkers have the general formula:

$$-CH_2\text{-}X\text{-}Q,$$

where Q and X are defined as set forth above.

[0068] H. Another amino-containing linker of the present invention is structured as follows:



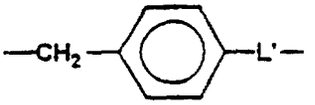
where R^7 is lower alkyl, preferably methyl.

In this case, resistance to enzymatic cleavage is conferred by the alkyl substitution on the amine.

[0069] I. Polymeric linkers are also contemplated by the present invention. Dextran and cyclodextran are preferred polymers useful in this embodiment of the present invention as a result of the hydrophilicity of the polymer, which leads to favorable excretion of conjugates containing the same. Other advantages of using dextran polymers are that such polymers are substantially non-toxic and non-immunogenic, that they are commercially available in a variety of sizes and that they are easy to conjugate to other relevant molecules. Also, dextran-linked conjugates exhibit advantages when non-target sites are accessible to dextranase, an enzyme capable of cleaving dextran polymers into smaller units while non-target sites are not so accessible.

[0070] Other linkers of the present invention are produced prior to conjugation to DOTA and following the reduction

of the biotin carboxy moiety. These linkers of the present invention have the following general formula:

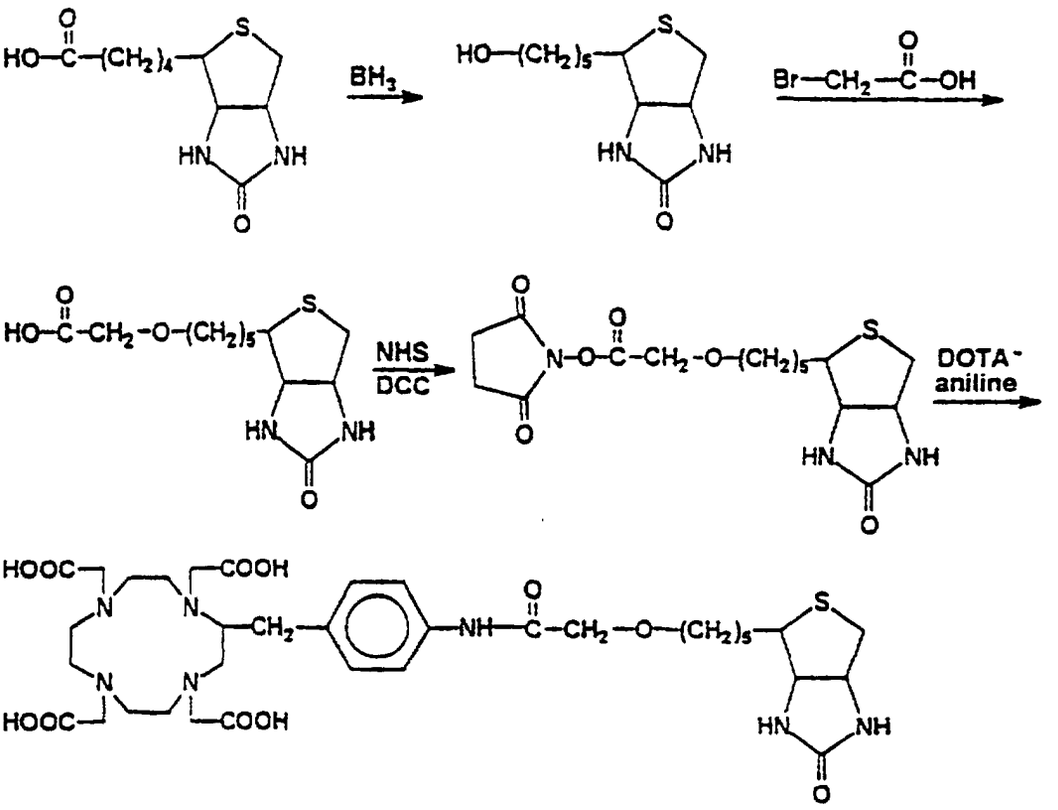


[0071] Embodiments of linkers of this aspect of the present invention include the following:

[0072] J. An ether linkage as shown below may be formed in a DOTA-biotin conjugate in accordance with the procedure indicated below.

$$L' = -NH\text{-}CO\text{-}(CH_2)_n\text{-}O-$$

where n ranges from 1 co 5, with 1 preferred.



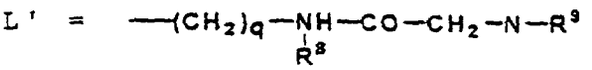
This linker has only one amide moiety which is bound directly to the DOTA aniline (as in the structure of DOTA-SC-biotin). In addition, the ether linkage imparts hydrophilicity, an important factor in facilitating renal excretion.

[0073] K. An amine linker formed from reduced biotin (hydroxybiotin or aminobiotin) is shown below, with conjugates containing such a linker formed, for example, in accordance with the procedure described in Example XXI.

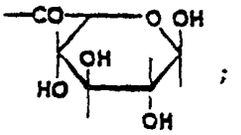
$$L' = -NH-$$

This linker contains no amide moieties and the unalkylated amine may impart favorable bicdistribution properties since unalkylated DOTA-aniline displays excellent renal clearance.

[0074] L. Substituted amine linkers, which can form conjugates via amino-biotin intermediates, are shown below.



where R^8 is H; $-(CH_2)_2-OH$ or a sulfate or phosphonate derivative thereof; or



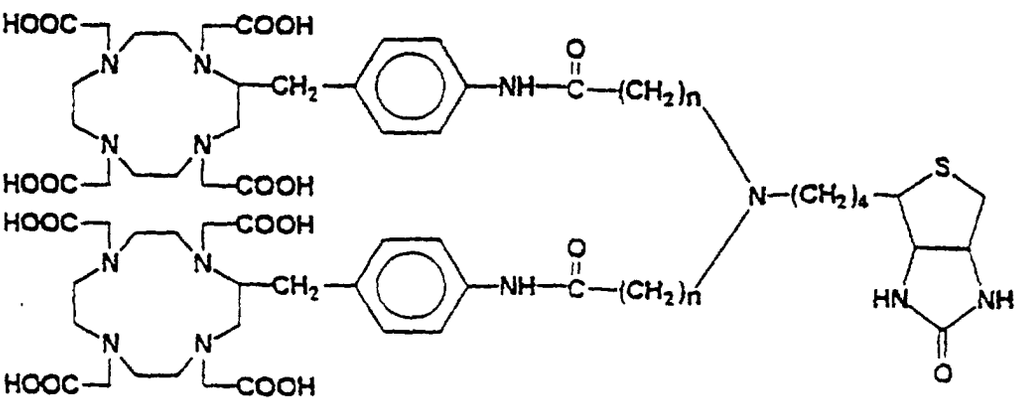
or the like; and R^9 is a bond or $-(CH_2)_n-CO-NH-$, where n ranges from 0-5 and is preferably 1 and where q is 0 or 1. These moieties exhibit the advantages of an amide only directly attached to DOTA-aniline and either a non-amide amine imparting a positive charge to the linker in vivo or a N-alkylated glucuronide hydrophilic group, each alternative favoring renal excretion.

[0075] M. Amino biotin may also be used as an intermediate in the production of conjugates linked by linkers having favorable properties, such as a thiourea-containing linker of the formula:

L' = -NH-CS-NH-

Conjugates containing this thiourea linker have the following advantages: no cleavable amide and a short, fairly polar linker which favors renal excretion.

[0076] A bis-DOTA derivative of the following formula can also be formed from amino-biotin.



where n ranges from 1 to 5, with 1 and 5 preferred. This molecule offers the advantages of the previously discussed bis-DOTA derivatives with the added advantage of no cleavable amides.

[0077] Additional linkers of the present invention which are employed in the production of conjugates characterized by a reduced biotin carboxy moiety are the following:

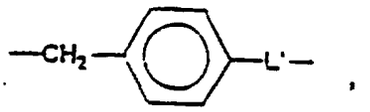
L = $-(CH_2)_4-NH-$,

wherein the amine group is attached to the methylene group corresponding to the reduced biotin carboxy moiety and the methylene chain is attached to a core carbon in the DOTA ring. Such a linker is conveniently synthesizable from lysine.

L = $-(CH_2)_q-CO-NH-$,

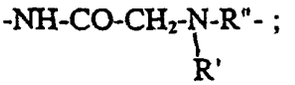
wherein q is 1 or 2, and wherein the amine group is attached to the methylene group corresponding to the reduced biotin carboxy moiety and the methylene group(s) are attached to a core carbon in the DOTA ring. This moiety is synthesizable from amino-biotin.

[0078] An embodiment of the present invention provides a biotin-DOTA conjugate as claimed in claim 1 wherein L is a linker of the formula:



wherein L' is selected from the group comprising:

a) -NH-CO-$(CH_2)_n$-O-;
b) -NH-;
c)



d) -NH-CS-NH-;
-NH-CO-$(CH_2)_n$-NH-; and -NH-CO-Z-CH(COOH)-NH-CO where Z is -$(CH_2)_2$,
-CH_2-S-CH_2-, -CH_2-, or -$(CH_2)_n$-CO-O-CH_2- where n is from 1 to 4 or the bis-DOTA derivative thereof, wherein n, R' and R" are as herein before defined.

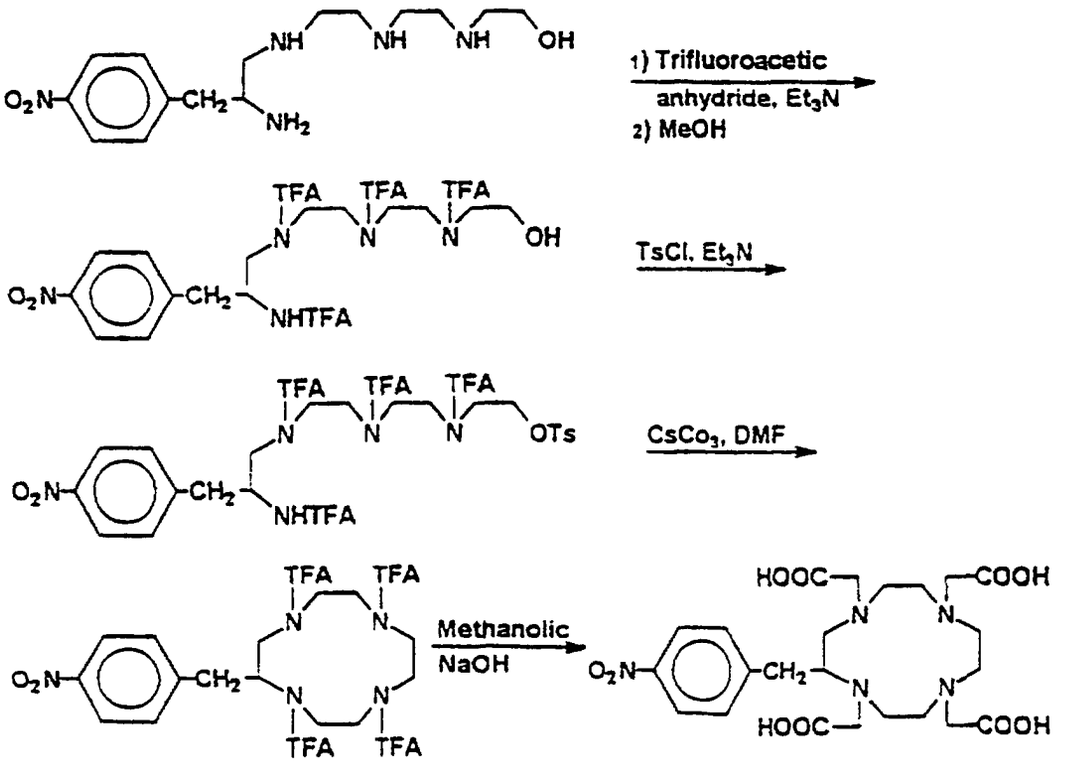
[0079] The linkers set forth above are useful to produce conjugates having one or more of the following advantages:

- bind avidin or streptavidin with the same or substantially similar affinity as free biotin;
- bind metal M^{+3} ions efficiently and with high kinetic stability;
- are excreted primarily through the kidneys into urine;
- are stable to bodily fluid amidases;
- penetrate tissue rapidly and bind to pretargeted avidin or streptavidin; and
- are excreted rapidly with a whole body residence half-life of less than about 5 hours.

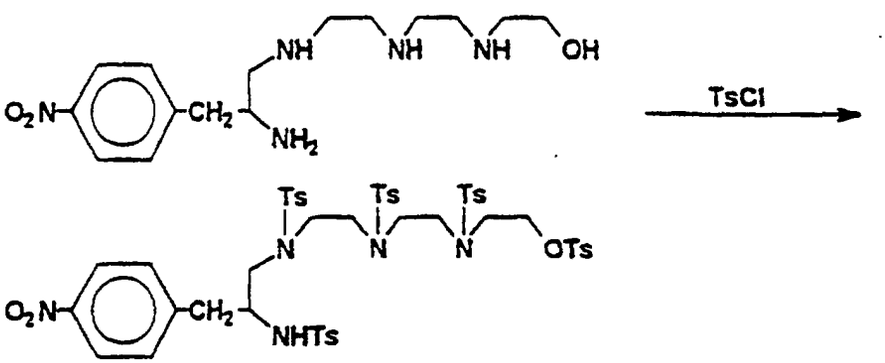
[0080] The second aspect of the present invention provides the use of a conjugate as described in any one of claims 1 to 4 in the preparation of an agent for use in increasing active agent localization to a target cell.

[0081] Synthetic routes to an intermediate of the DOTA-biotin conjugates depicted above, nitrobenzyl-DOTA, have been proposed. These proposed synthetic routes a produce the intermediate compound in suboptimal yield, however. For example, Renn and Meares, "Large Scale Synthesis of Bifunctional Chelating Agent Q-(p-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-N,N',N",N"'-tetra acetic acid, and the Determination of its Enantiomeric Purity by Chiral Chromatography," Bioconj. Chem., 3: 563-9, 1992, describe a nine-step synthesis of nitrobenzyl-DOTA, including reaction steps that either proceed in low yield or involve cumbersome transformations or purifications. More specifically, the sixth step proceeds in only 26% yield, and the product must be purified by preparative HPLC. Additionally, step eight proceeds in good yield, but the process involves copious volumes of the coreactants.

[0082] These difficulties in steps 6-8 of the prior art synthesis are overcome in the practice of the present invention through the use of the following synthetic alternative therefor.



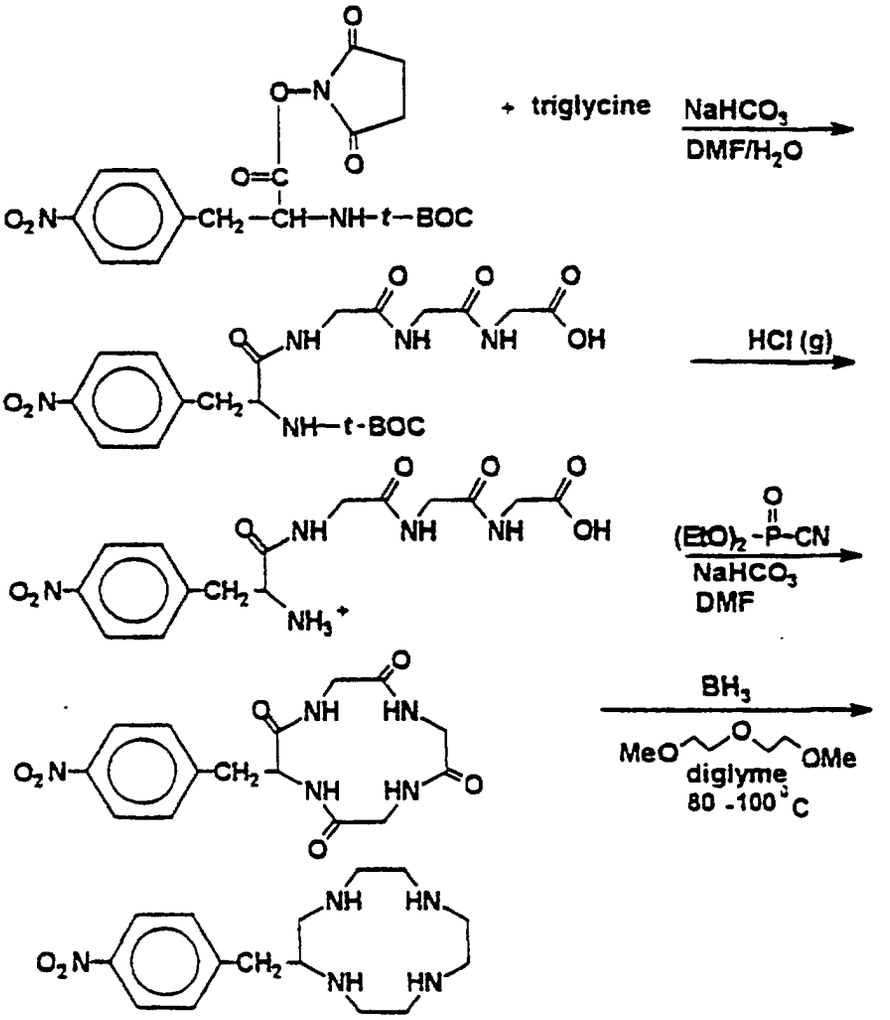
[0083] The poor yield in step six of the prior art synthesis procedure, in which a tetra amine alcohol is converted to a tetra-toluenesuifonamide toluenesulfonate as shown below, is the likely result of premature formation of the toluenesulfonate functionality (before all of the amine groups have been converted to their corresponding sulfonamides.



Such a sequence of events would potentially result in unwanted intra- or inter-molecular displacement of the reactive toluene sulfonate by unprotected amine groups, thereby generating numerous undesirable side-products.

[0084] This problem is overcome in the aforementioned alternative synthesis scheme of the present invention by reacting the tetra-amine alcohol with trifluoroacetic anhydride. Trifluoroacetates, being much poorer leaving groups than toluenesulfonates, are not vulnerable to analogous side reactions. In fact, the easy hydrolysis of trifluoroacetate groups, as reported in Greene and Wuts, "Protecting Groups in Organic Synthesis," John Wiley and Sons, Inc., New York, p. 94, 1991., suggests that addition of methanol to the reaction mixture following consumption of all amines should afford the tetra-fluoroacetamide alcohol as a substantially exclusive product. Conversion of the tetra-fluoroacetamide alcohol to the corresponding toluenesulfonate provides a material which is expected to cyclize analogously to the tetra-toluenesulfonamide toluenesulfonate of the prior art. The cyclic tetra-amide product of the cyclization of the toluenesulfonate of tetra-fluoroacetamide alcohol, in methanolic sodium hydroxide at 15-25°C for 1 hour, should afford nitrobenzyl-DOTA as a substantially exclusive product. As a result, the use of trifluoracetamide protecting groups circumvents the difficulties associated with cleavage of the very stable toluenesulfonamide protecting group, which involves heating with a large excess of sulfuric acid followed by neutralization with copious volumes of barium hydroxide.

[0085] Another alternative route to nitro-benzyl-DOTA is shown below.



This alternative procedure involves the cyclizaton of p-nitrophenylalanyltriglycine using a coupling agent, such as diethylcyanophosphate, to give the cyclic tetraamide. Subsequent borane reduction provides 2-(p-nitrobenzyl)-1,4,7,10-tetraazacyclododecane, a common precursor used in published routes to DOTA including the Renn and Meares article referenced above. This alternative procedure of the present invention offers a synthetic pathway that is considerably shorter than the prior art Renn and Meares route, requiring two rather than four steps

Example I

Two-Step Pretargeting In Vivo

[0086] A ^{186}Re-chelate-biotin conjugate (Re-BT) (MW ≈ 1000; specific activity = 1-2 mCi/mg) and a biotin-iodine-131 small molecule, PIP-Biocytin (PIP-BT, MW approximately equal to 602; specific activity = 0.5-1.0 mCi/mg), were examined in a three-step pretargeting protocol in an animal model. Like Re-BT, PIP-BT has the ability to bind well to avidin and is rapidly cleared from the blood, with a serum half-life of about 5 minutes. Equivalent results were observed for both molecules in the two-step pretargeting experiments described herein.

[0087] NR-LU-10 antibody (MW ≈ 150 kD) was conjugated to streptavidin (MW ≈ 66 kD) and radiolabeled with ^{125}I/PIP-NHS. The experimental protocol was as follows:

Time 0	inject (i.v.) 200 μg NR-LU-10 StrAv Conjugate;
Time 24-48 h	inject (i.v.) 60-70 fold molar excess of

radiolabeled biotinyl
molecule;

and perform biodistribution at 2, 6, 24, 72, 120 hours after injection of radiolabeled biotinyl molecule.

[0088] NR-LU-10-streptavidin has shown very consistent patterns of blood clearance and tumor uptake in the LS-180 animal model. When either PIP-BT or Re-BT is administered after allowing the LU-10-StrAv conjugate to localize to target cell sites for at least 24 hours, the tumor uptake of therapeutic radionuclide is high in both absolute amount and rapidity. For PIP-BT administered at 37 hours following LU-10-StrAv (I-125) administration, tumor uptake was above 500 pMOL/G at the 40 hour time point and peaked at about 700 pMOL/G at 45 hours post-LU-10-StrAv administration.

[0089] This almost instantaneous uptake of a small molecule therapeutic into tumor in stoichiometric amounts comparable to the antibody targeting moiety facilitates utilization of the therapeutic radionuclide at its highest specific activity. Also, the rapid clearance of radionuclide that is not bound to LU-10-StrAv conjugate permits an increased targeting ratio (tumor:blood) by eliminating the slow tumor accretion phase observed with directly labeled antibody conjugates. The pattern of radionuclide tumor retention is that of whole antibody, which is very persistent.

[0090] Experimentation using the two-step pretargeting approach and progressively lower molar doses of radiolabeled biotinyl molecule was also conducted. Uptake values of about 20% ID/G were achieved at no-carrier added (high specific activity) doses of radiolabeled biotinyl molecules. At less than saturating doses, circulating LU-10-StrAv was observed to bind significant amounts of administered radiolabeled biotinyl molecule in the blood compartment.

EXAMPLE II

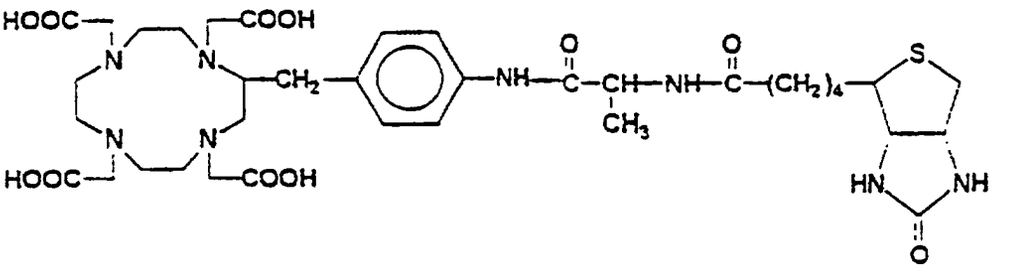
Synthesis of DOTA-Biotin Conjugates

A. Synthesis of Nitro-Benzyl-DOTA.

[0091] The synthesis of aminobenzyl-DOTA was conducted substantially in accordance with the procedure of McMurry et al., Bioconjugate Chem., 3: 108-117, 1992. The critical step in the prior art synthesis is the intermolecular cyclization between disuccinimidyl N-(tert-butoxycarbonyl)iminodiacetate and N-(2-aminoethyl)-4-nitro phenyl alanamide to prepare 1-(tert-butoxycarbonyl)-5-(4-nitrobenzyl)-3,6,11-trioxo-1,4,7,10-tetraazacyclododecane. In other words, the critical step is the intermolecular cyclization between the bis-NHS ester and the diamine to give the cyclized dodecane. McMurry et al. conducted the cyclization step on a 30 mmol scale, dissolving each of the reagents in 100 ml DMF and adding via a syringe pump over 48 hours to a reaction pot containing 4 liters dioxane.

[0092] A 5x scale-up of the McMurry et al. procedure was not practical in terms of reaction volume, addition rate and reaction time. Process chemistry studies revealed that the reaction addition rate could be substantially increased and that the solvent volume could be greatly reduced, while still obtaining a similar yield of the desired cyclization product. Consequently on a 30 mmol scale, each of the reagents was dissolved in 500 ml DMF and added via addition funnel over 27 hours to a reaction pot containing 3 liters dioxane. The addition rate of the method employed involved a 5.18 mmol/hour addition rate and a 0.047 M reaction concentration.

[0093] B. Synthesis of a D-alanine-linked conjugate with a preserved biotin carboxy moiety. A reaction scheme to form a compound of the following formula is discussed below.



[0094] The D-alanine-linked conjugate was prepared by first coupling D-aianine (Sigma Chemical Co.) to biotin-NHS ester. The resultant bictinyl-D-alanine was then activated with 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDCI) and N-hydroxysuccinimide (NHS). This NHS ester was reacted in situ with DOTA-aniline to give the desired product which was purified by preparative HPLC.

[0095] More specifically, a mixture of D-alanine (78 mg, 0.88 mmol, 1.2 equivalents), biotin-NHS ester (250 mg, 0.73

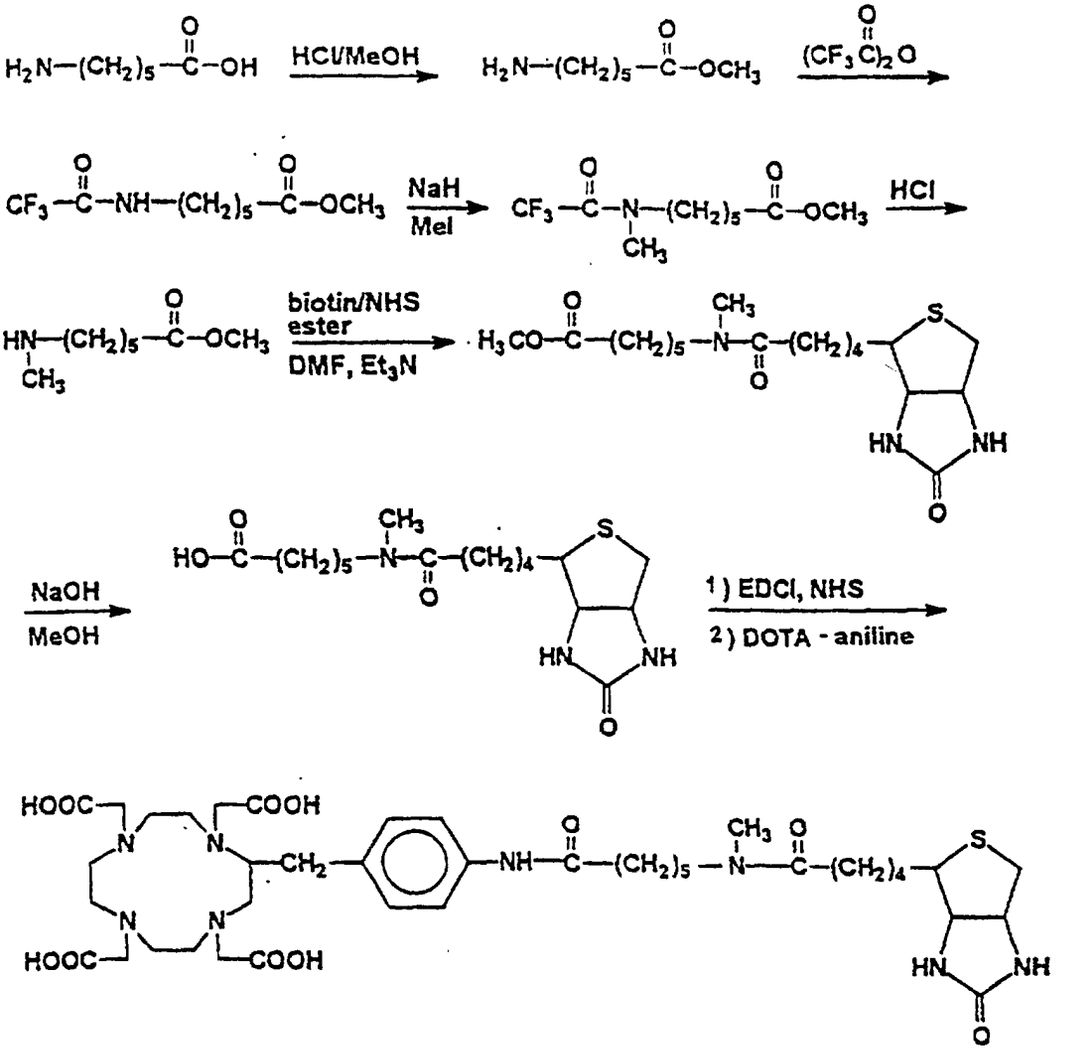
mmol, 1.0 equivalent), triethylamine (0.30 ml, 2.19 mmol; 3.0 equivalents) in DMF (4 ml) was heated at 110°C for 30 minutes. The solution was cooled to 23°C and evaporated. The product solid was acidified with glacial acetic acid and evaporated again. The product biotinyl-D-alanine, a white solid, was suspended in 40 ml of water to remove excess unreacted D-alanine, and collected by filtration. Biotinyl-D-alanine was obtained as a white solid (130 mg, 0.41 mmol) in 47% yield.

[0096] NHS (10 mg, 0.08 mmol) and EDCI (15 mg, 0.07 mmol) were added to a solution of biotinyl-D-alanine (27 mg, 0.08 mmol) in DMF (1 ml). The solution was stirred at 23°C for 60 hours, at which time TLC analysis indicated conversion of the carboxyl group to the N-hydroxy succinimidyl ester. Pyridine (0.8 ml) was added followed by DOTA-aniline (20 mg, 0.04 mmol). The mixture was heated momentarily at approximately 100°C, then cooled to 23°C and evaporated. The product, DOTA-aniline-D-alanyl-biotinamide was purified by preparative HPLC.

C. Synthesis of N-hydroxyethyl-linked conjugate.

[0097] Iminodiacetic acid dimethyl ester is condensed with biotin-NHS-ester to give biotinyl dimethyl iminodiacetate. Hydrolysis with one equivalent of sodium hydroxide provides the monomethyl ester after purification from under and over-hydrolysis products. Reduction of the carboxyl group with borane provides the hydroxyethyl amide. The hydroxyl group is protected with t-butyl-dimethyl-silylchloride. The methyl ester is hydrolysed, activated with EDCI and condensed with DOTA-aniline to form the final product conjugate.

[0098] D. Synthesis of N-Me-LC-DOTA-biotin. A reaction scheme is shown below.



[0099] Esterification of 6-Aminocaproic acid (Sigma Chemical Co.) was carried out with methanolic HCl. Trifluoroacetylation of the amino group using trifluoroacetic anhydride gave N-6-(methylcaproyl)-trifluoroacetamide. The amide

nitrogen was methylated using sodium hydride and iodomethane in tetrahydrofuran. The trifluoroacetyl protecting group was cleaved in acidic methanol to give methyl 6-methylamino-caproate hydrochloride. The amine was condensed with biotin-NHS ester to give methyl N-methyl-caproylamido-biotin. Saponification afforded the corresponding acid which was activated with EDCI and NHS and, in situ, condensed with DOTA-aniline to give DOTA-benzylamido-N-methyl-caproylamido-biotin.

1. Preparation of methyl 6-aminocaproate hydrochloride. Hydrogen chloride (gas) was added to a solution of 20.0 g (152 mmol) of 6-aminocaproic acid in 250 ml of methanol via rapid bubbling for 2-3 minutes. The mixture was stirred at 15-25°C for 3 hours and then concentrated to afford 27.5 g of the product as a white solid (99%):

H-NMR (DMSO) 9.35 (1 H, broad t), 3.57 (3H, s), 3.14 (2H, quartet), 2.28 (2H, t), 1.48 (4H, multiplet), and 1.23 ppm (2H, multiplet).

2. Preparation of N-6-(methylcaproyl)-trifluoroacetamide. To a solution of 20.0 g (110 mmol) of methyl 6-aminocaproate hydrochloride in 250 ml of dichloromethane was added 31.0 ml (22.2 mmol) of triethylamine. The mixture was cooled in an ice bath and trifluoroacetic anhydride (18.0 ml, 127 mmol) was added over a period of 15-20 minutes. The mixture was stirred at 0-10°C for 1 hour and concentrated. The residue was diluted with 300 ml of ethyl acetate and saturated aqueous sodium bicarbonate (3 x 100 ml). The organic phase was dried over anhydrous magnesium sulfate, filtered and concentrated to afford 26.5 g of the product as a pale yellow oil (100%):

H-NMR (DMSO) 3.57 (3H, s), 3.37 (2H, t), 3.08 (1.9H, quartet, N-CH_3), 2.93 (1.1H, s, N-CH_3), 2.30 (2H, t), 1.52 (4H, multiplet), and 1.23 ppm (2H, multiplet).

3. Preparation of methyl 6-N-methylamino-caproate hydrochloride. To a solution of 7.01 g (29.2 mmol) of N-6-(methylcaproyl)-trifluoroacetamide in 125 ml of anhydrous tetrahydrofuran was slowly added 1.75 g of 60% sodium hydride (43.8 mmol) in mineral oil. The mixture was stirred at 15-25°C for 30 minutes and then 6.2 g (43.7 mmol) of iodomethane was added. The mixture was stirred at 15-25°C for 17 hours and then filtered through celite. The solids were rinsed with 50 ml of tetrahydrofuran. The filtrates were combined and concentrated. The residue was diluted with 150 ml of ethyl acetate and washed first with 5% aqueous sodium sulfite (2 x 100 ml) and then with 100 ml of 1 N aqueous hydrochloric acid. The organic phase was dried over anhydrous magnesium sulfate, filtered and concentrated to afford a yellow oily residue. The residue was diluted with 250 ml of methanol and then hydrogen chloride (gas) was rapidly bubbled into the mixture for 2-3 minutes. The resultant mixture was refluxed for 18 hours, cooled and concentrated. The residue was diluted with 150 ml of methanol and washed with hexane (3 x 150 ml) to remove mineral oil previously introduced with NaH. The methanol phase was concentrated to afford 4.91 g of the product as a yellow oil (86%):

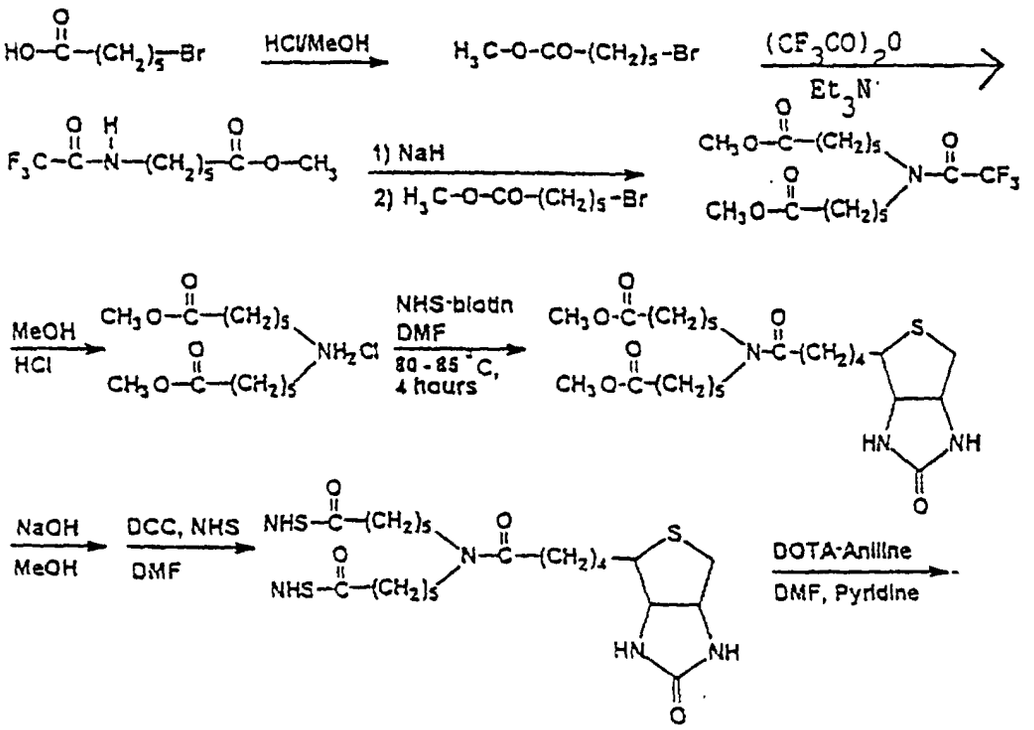
H-NMR (DMSO) 8.80 (2H, broad s), 3.58 (3H, s), 2.81 (2H, multiplet), 2.48 (3H, s), 2.30 (2H, t), 1.52 (4H, multiplet), and 1.29 ppm (2H, multiplet).

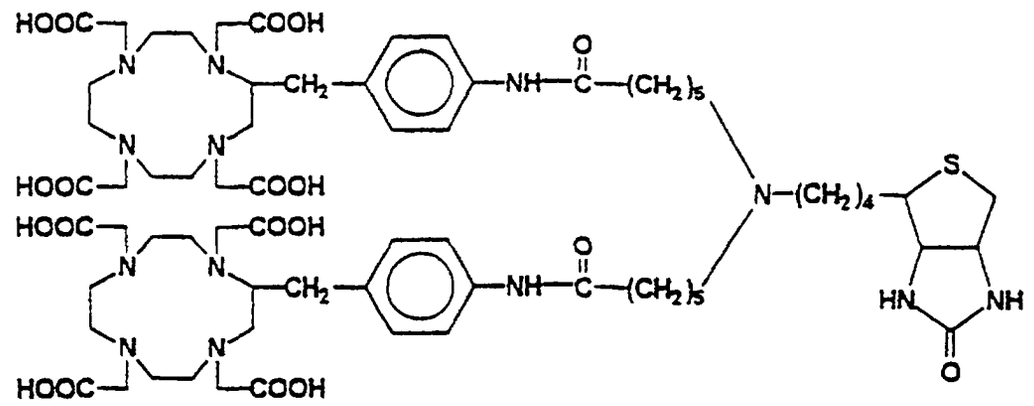
4. Preparation of methyl 6-(N-methylcaproylamidobiotin. N-hydroxysuccinimidyl biotin (398 mg, 1.16 mmol) was added to a solution of methyl 6-(N-methyl) aminocaproate hydrochloride (250 mg, 1.28 mmol) in DMF (4.0 ml) and triethylamine (0.18 ml, 1.28 mmol). The mixture was heated in an oil bath at 100°C for 10 minutes. The solution was evaporated, acidified with glacial acetic acid and evaporated again. The residue was chromatographed on a 25 mm flash chromatography column manufactured by Ace Glass packed with 50 g silica (EM Science, Gibbstown, New Jersey, particle size 0.40-0.63 mm) eluting with 15% MeOH/EtOAc. The product was obtained as a yellow oil (390 mg) in 79% yield.

5. Preparation of 6-(N-methyl-N-biotinyl) amino caproic acid. To a solution of methyl 6-(N-methylcaproylamidobiotin (391 mg, 1.10 mmol) in methanol (2.5 ml) was added a 0.95 N NaOH solution (1.5 ml). This solution was stirred at 23°C for 3 hours. The solution was neutralized by the addition of 1.0 M HCl (1,6 ml) and evaporated. The residue was dissolved in water, further acidified with 1.0 M HCl (0.4 ml) and evaporated. The gummy solid residue was suspended in water and agitated with a spatula until it changed into a white powder. The powder was collected by filtration with a yield of 340 mg.

6. Preparation of DOTA-benzylamido-N-methylcaproylamido-biotin. A suspension of 6-(N-methyl-N-biotinyl)amino caproic acid (29 mg, 0.08 mmol) and N-hydroxysuccinimide (10 mg, 0.09 mmol) in DMF (0.8 ml) was heated over a heat gun for the short time necessary for the solids to dissolve. To this heated solution was added EDCI (15 mg, 0.08 mmol). The resultant solution was stirred at 23°C for 20 hours. To this stirred solution were added aminobenzyl-DOTA (20 mg, 0.04 mmol) and pyridine (0.8 ml). The mixture was heated over a heat gun for 1 minute. The product was isolated by preparative HPLC, yielding 3 mg.

[0100] E. Synthesis of a bis-DOTA conjugate with a preserved biotin carboxy group. A reaction scheme is shown below.





1. Preparation of methyl 6-bromocaproate (methyl 5-bromohexanoate). Hydrogen chloride (gas) was added to a solution of 5.01 g (25.7 mmol) of 6-bromocaproic acid in 250 ml of methanol via vigorous bubbling for 2-3 minutes. The mixture was stirred at 15-25°C for 3 hours and then concentrated to afford 4.84 g of the product as a yellow oil (90%):

H-NMR (DMSO) 3.58 (3H, s), 3.51 (2H, t), 2.29 (2H, t), 1.78 (2H, pentet), and 1.62-1.27 ppm (4H, m).

2. Preparation of N,N-bis-(methyl 6-hexanoyl)-amine hydrochloride. To a solution of 4.01 g (16.7 mmol) of N-(methyl 6-hexanoyl)-trifluoroacetamide (prepared in accordance with section D.2. herein) in 125 ml of anhydrous tetrahydrofuran was added 1.0 g (25 mmol) of 60% sodium hydride in mineral oil. The mixture was stirred at 15-25°C for 1 hour and then 3.50 g (16.7 mmol) of methyl 6-bromocaproate was added and the mixture heated to reflux. The mixture was stirred at reflux for 22 hours. NMR assay of an aliquot indicated the reaction to be incomplete. Consequently, an additional 1.00 g (4.8 mmol) of methyl 6-bromocaproate was added and the mixture stirred at reflux for 26 hours. MNR assay of an aliquot indicated the reaction to be incomplete. An additional 1.0 g of methyl 6-bromocaproate was added and the mixture stirred at reflux for 24 hours. NMR assay of an aliquot indicated the reaction to be near complete. The mixture was cooled and then directly filtered through celite. The solids were rinsed with 100 ml of tetrahydrofuran. The filtrates were combined and concentrated. The residue was diluted with 100 ml of methanol and washed with hexane (3x 100 ml) to remove the mineral oil introduced with the sodium hydride. The methanol phase was treated with 6 ml of 10 N aqueous sodium hydroxide and stirred at 15-25°C for 3 hours. The

mixture was concentrated. The residue was diluted with 100 ml of deionized water and acidified to pH 2 with concentrated HCl. The mixture was washed with ether (3 x 100 ml). The aqueous phase was concentrated, diluted with 200 ml of dry methanol and then hydrogen chloride gas was bubbled through the mixture for 2-3 minutes. The mixture was stirred at 15-25°C for 3 hours and then concentrated. The residue was diluted with 50 ml of dry methanol and filtered to remove inorganic salts. The filtrate was concentrated to afford 1.98 g of the product as a white solid (38%):

H-NMR (DMSO) 8.62 (2H, m) 3.58 (6H, s), 2.82 (4H, m) 2.30 (4H, t), 1.67-1.45 (8H, m) and 1.38-1.22 ppm (4H, m).

3. Preparation of N,N=bis-(methyl 6-hexanoyl)-biotinamide. To a solution of 500 mg (1.46 mmol) of N-hydroxysuccinimidyl biotin in 15 ml of dry dimethylformamide was added 600 mg (1.94 mmol) of N,N-bis-(methyl 6-hexanoyl) amine hydrochloride followed by 1.0 ml of triethylamine. The mixture was stirred at 80-85'CC for 3 hours and then cooled and concentrated. The residue was chromatographed on silica gel, eluting with 20% methanol/ethyl acetate, to afford 620 mg of the product as a near colorless oil (85%):

H-NMR ($CDCl_3$) 5.71 (1H, s), 5.22 (1H, s), 4.52 (1H, m), 4.33 (1H, m), 3.60 (3H, s), 3.58 (3H, s), 3.34-3.13 (5H, m), 2.92 (1H, dd), 2.75 (1H, d), 2.33 (6H, m) and 1.82-1.22 ppm (18H, m); TLC-R_f 0.39 (20:80 methanol/ ethyl acetate).

4. Preparation of N,N-bis-(6-hexanoyl)-biotinamide. To a solution of 610 mg (0.819 mmol) of N,N-bis-(methyl 6-hexanoyl)-biotinamide in 35 ml of methanol was added 5.0 ml of 1N aqueous sodium hydroxide. The mixture was stirred at 15-25°C for 4.5 hours and then concentrated. The residue was diluted with 50 ml of deionized water acidified to pH 2 with 1N aqueous hydrochloric acid at 4°C. The product, which precipitated out as a white solid, was isolated by vacuum filtration and dried under vacuum to afford 482 mg (84%):

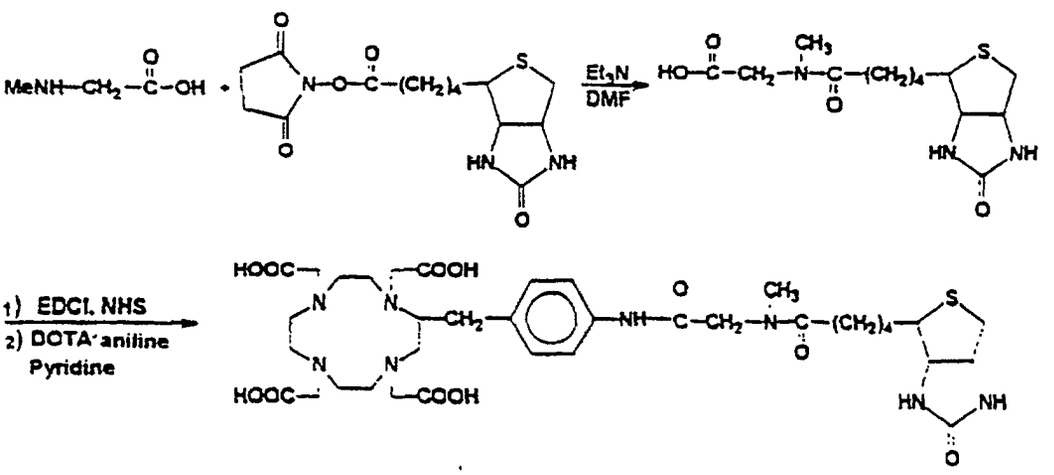
H-NMR (DMSO) 6.42 (1H, s), 6.33 (1H, s), 4.29 (1H, m), 4.12 (1H, m), 3.29-3.04 (5H, m), 2.82 (1H, dd), 2.57 (1H, d), 2.21 (6H, m) and 1.70-1.10 ppm (18H, m).

5. Preparation of N',N'-bis-(N-hydroxy-succinimidyl 6-hexanoyl)-biotinamide. To a solution of 220 mg (0.467 mmol) of N,N-bis-(6-hexanoyl)-biotinamide in 3 ml of dry dimethylformamide was added 160 mg (1.39 mmol) of N-hydroxysuccinimide followed by 210 mg (1.02 mmol) of dicyclohexyl-carbodiimide. The mixture was stirred at 15-25°C for 17 hours and then concentrated. The residue was chromatographed on silica gel, eluting with 0.1:20:80 acetic acid/methanol/ethyl acetate, to afford 148 mg of the product as a foamy off-white solid (48%):

H-NMR (DMSO) 6.39 (1H, s), 6.32 (1H, s), 4,29 (1H, m), 4,12 (1H, m), 3.30-3.03 (5H, m), 2.81 (9H, dd and s), 2.67 (4H, m), 2.57 (1H, d),. 2.25 (2H, t), 1.75-1.20 (18H, m); TLC-R_f 0.37 (0.1:20:80 acetic acid/methanol/ethyl acetate).

6. Preparation of N,N-bis-(6-hexanoylamidobenzyl-DOTA)-biotinamide. To a mixture of 15 mg of DOTA-benzylamine and 6.0 mg of N',N'-bis-(N-hydroxy-succinimidyl 6-hexanoyl)-biotinamide in 1.0 ml of dry dimethylformamide was added 0.5 ml of dry pyridine. The mixture was stirred at 45-50°C for 4.5 hours and at 15-25°C for 12 hours. The mixture was concentrated and the residue chromatographed on a 2.1 x 2.5 cm octadecylsilyl (ODS) reverse-phase preparative HPLC column eluting with a --20 minute gradient profile of 0.1:95:5 to 0.1:40:60 trifluoroacetic acid:water:acetonitrile at 13 ml/minute to afford the desired product. The retention time was 15.97 minutes using the aforementioned gradient at a flow rate of 1.0 ml/minute on a 4.6 mm x 25 cm ODS analytical HPLC column.

[0101] F. Synthesis of an N-methyl-glycine linked conjugate. A reaction scheme for this synthesis is shown below.



[0102] The N-methyl glycine-linked DOTA-biotin conjugate was prepared by an analogous method to that used tc prepare D-alanine-linked DOTA-biotin conjugates. N-methyl-glycine (trivial name sarcosine, available from Sigma Chemical Co.) was condensed wich biotin-NHS ester in DMF and triethylamine to obtain N-methyl glycyl-biotin. N-methyl-glycyl biotin was then activated with EDCI and NHS. The resultant NHS ester was not isolated and was condensed in situ with DOTA-aniline and excess pyridine. The reaction solution was heated at 60°C for 10 minutes and then evaporated. The residue was purified by preparative HPLC to give [(N-methyl-N-biotinyl)-N-glycyl]-aminobenzyl-DOTA.

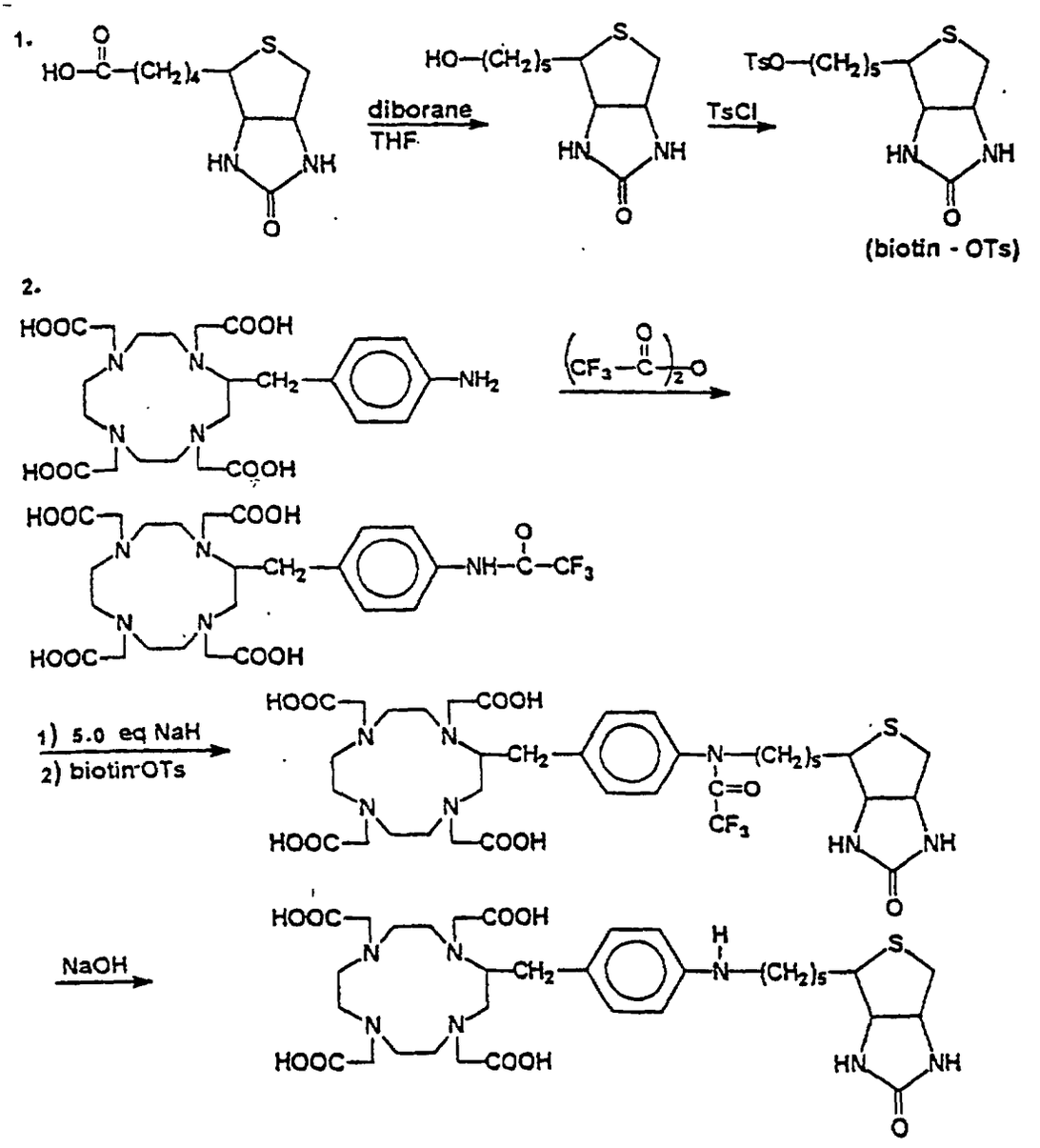
1. Preparation of (N-methyl)glycyl biotin. DMF (8.0 ml) and triethylamine (0.61 ml, 4.35 mmol) were added to solids N-methyl glycine (182 mg, 2.05 mmol) and N-hydroxy-succinimidyl biotin (500 mg, 1.46 mmol). The mixture was heated for 1 hour in an oil bath at 85°C during which time the solids dissolved producing a clear and colorless solution. The solvents were then evaporated. The yellow oil residue was acidified with glacial acetic acid, evaporated and chromatographed on a 27 mm column packed with 50 g silica, eluting with 30% MeOH/EtOAc 1% HOAc to give the product as a white solid (383 mg) in 66% yield.

H-NMR (DMSO): 1.18-1.25 (m, 6H, $(CH_2)_3$), 2.15, 2.35 (2 t's, 2H, CH_2CO), 2.75 (m, 2H, SCH_2), 2.80, 3.00 (2 s's, 3H, NCH_3), 3.05-3.15 (m, 1H, SCH), 3.95, 4.05 (2 s's, 2H, CH_2N), 4.15, 4.32 (2 m's, 2H, 2CHN's), 6.35 (s, NH), 6.45 (s, NH).

2. Preparation of [(N-methyl-N-biotinyl)glycyl] aminobenzyl-DOTA. N-hydroxysuccinimide (10 mg, 0.08 mmol) and EDCI (15 mg, 6.08 mmol) were added to a solution of (N-methylglycyl biotin (24 mg, 0.08 mmol) in DMF (1.0 ml). The solution was stirred at 23 °C for 64 hours. Pyridine (0.8 ml) and aminobenzyl-DOTA (20mg, 0.04 mmol) were added. The mixture was heated in an oil bath at 63°C for 10 minutes, then stirred at 23 °C for 4 hours. The solution was evaporated. The residue was purified by preparative HPLC to give the product as an off white solid (8 mg, 0.01 mmol) in 27% yield.

H-NMR (D_2O): 1.30-1.80 (m, 6H), 2.40, 2.55 (2 t's, 2H, CH_2CO), 2.70-4.2 (complex multiplet), 4.35 (m, CHN), 4.55 (m, CHN), 7.30 (m, 2H, benzene hydrogens), 7.40 (m, 2H, benzene hydrogens).'

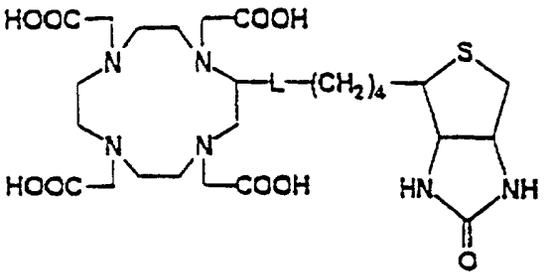
[0103] G. Synthesis of a short chain amine-linked conjugate with a reduced biotin carboxy group. A two-part reaction scheme is shown below.



[0104] The biotin carboxyl group is reduced with diborane in THF to give a primary alcohol. Tosylation of the alcohol with tosyl chloride in pyridine affords the primary tosylate. Aminobenzyl DOTA is acylated with trifluoroacetic anhydride in pyridine to give (N-trifluoroacetyl)aminobenzyl-DOTA. Deprotonation with 5.0 equivalents of sodium hydride followed by displacement of the biotin tosylate provides the (N-trifluoracetamido-N-descarboxylbiotinyl)aminobenzyl-DOTA. Acidic cleavage of the N-trifluoroacetamide group with HCl(g) in methanol provides the amine-linked DOTA-biotin conjugate.

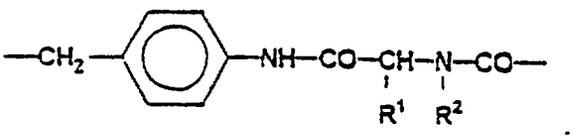
Claims

1. A biotin-DOTA conjugate of the following formula:

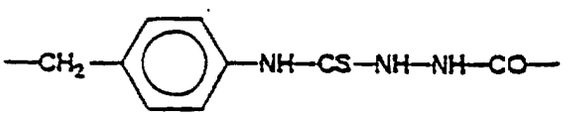


wherein a linker L is selected from the group comprising:

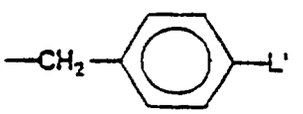
1) a D-amino acid containing linker of the formula



2) a linker of the formula

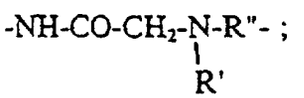


3) a linker of the formula



wherein L' is selected from the group comprising:

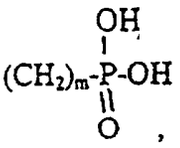
a) -NH-CO-$(CH_2)_n$-O-;
b) -NH- ;
c)



d) -NH-CS-NH-;
e) -NH-CO-$(CH_2)_n$-NH-; and
f) -NH-CO-Z-CH(COOH)-NH-CO where Z is -$(CH_2)_2$-, -CH_2-S-CH_2-, -CH_2-, or -$(CH_2)_n$-CO-O-CH_2- where n is from 1 to 4

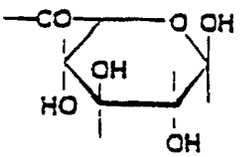
wherein R^1 is hydrogen, C_1-C_5 lower alkyl; C_1-C_5 lower alkyl substituted with one or more hydrophilic groups

preferably $(CH_2)_m$-OH, $(CH_2)_m$-OSO_3, $(CH_2)_m$-SO_3, and



where m is 1 or 2; glucuronide-substituted amino acids, or other glucuronide derivatives;

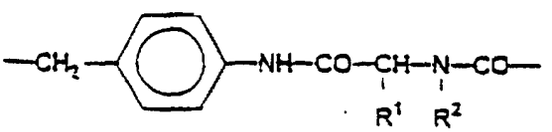
R^2 is hydrogen; C_1-C_5 lower alkyl; substituted C_1-C_5 lower alkyl having one or more substituents selected from the group comprising hydroxy, sulfate, and phosphonate; or a hydrophilic moiety;
R' is hydrogen; -$(CH_2)_2$-OH or a sulfate or



;

phosphonate derivative thereof; or
R" is a bond or -$(CH_2)_n$-CO-NH- ; and
n ranges from 0-5.

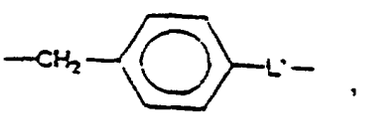
2. A biotin-DOTA conjugate of Claim 1 wherein L is a D-amino acid-containing a linker of the formula



wherein R^1 and R^2 are as herein before defined.

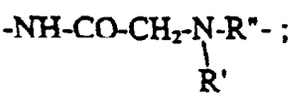
3. A biotin-DOTA conjugate of Claim 2 wherein R^1 is CH_3 and R^2 is H.

4. A biotin-DOTA conjugate of Claim 1 wherein L is a linker of the formula



, wherein L' is selected from the group comprising:

a) -NH-CO-$(CH_2)_n$-O-;
b) -NH-;
c)



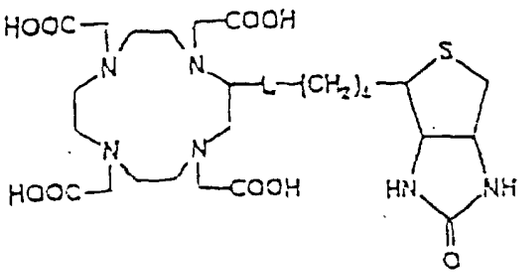
d) -NH-CS-NH-; -NH-CO-$(CH_2)_n$-NH-; and -NH-CO-Z-CH(COOH)-NH-CO where Z is -$(CH_2)_2$, -CH_2-S-CH_2-, -CH_2-, or -$(CH_2)_n$-CO-O-CH_2- where n is from 1 to 4

or the bis-DOTA derivative thereof, wherein n, R' and R" are as herein before defined.

5. The use of a conjugate as claimed in any one of Claims 1 to 4 in the preparation of an agent for use in increasing active agent localization to a target cell.

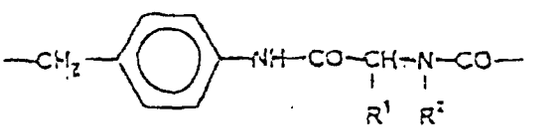
Patentansprüche

1. Biotin-DOTA-Konjugat der folgende Formel:

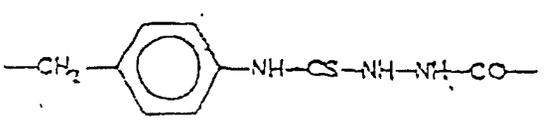


wobei ein Linker L ausgewählt ist aus der Gruppe, die umfaßt:

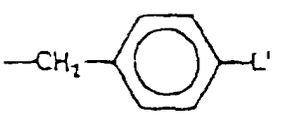
1) einen eine D-Aminosäure enthaltenden Linker der Formel



2) einen Linker der Formel



3) einen Linker der Formel

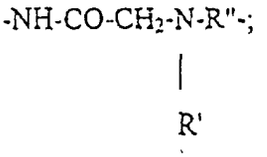


wobei L' ausgewählt ist aus der Gruppe, welche umfaßt:

a) -NH-CO-$(CH_2)_n$-O-;

b) -NH-;

c)

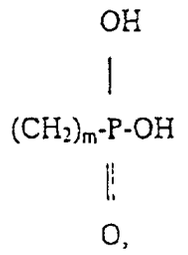


d) -NH-CS-NH-;

e) -NH-CO-$(CH_2)_n$-NH-; und

f) -NH-CO-Z-CH(COOH)-NH-CO, wobei Z -$(CH_2)_2$-, -CH_2-S-CH_2-, -CH_2- oder -$(CH_2)_n$-CO-O-CH_2- ist, wobei n von 1 bis 4 ist,

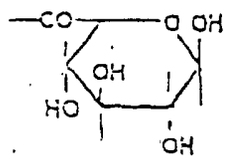
wobei R^1 Wasserstoff; C_1-C_5-Niederalkyl; C_1-C_5-Niederalkyl, substituiert mit einer oder mehreren hydrophilen Gruppen, vorzugsweise $(CH_2)_m$-OH, $(CH_2)_m$-OSO_3, $(CH_2)_m$-SO_3 und



wobei m 1 oder 2 ist; Glucuronid-substituierte Aminosäuren oder andere Glucuronid-Derivate ist;

R^2 Wasserstoff; C_1-C_5-Niederalkyl; substituiertes C_1-C_5-Niederalkyl mit einem oder mehreren Substituenten, die ausgewählt sind aus der Gruppe, die Hydroxy, Sulfat und Phosphonat umfaßt; oder eine hydrophile Einheit ist;

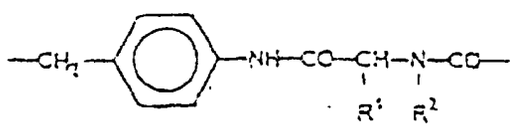
R' Wasserstoff; -$(CH_2)_2$-OH oder ein Sulfat- oder Phosphonat-Derivat davon ist; oder



R" eine Bindung oder -$(CH_2)_n$-CO-NH- ist; und

n im Bereich von 0-5 liegt.

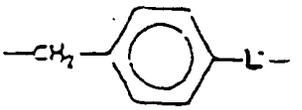
2. Biotin-DOTA-Konjugat nach Anspruch 1, **dadurch gekennzeichnet, daß** L ein eine D-Aminosäure enthaltender Linker der Formel



ist,
wobei R^1 und R^2 sind, wie hierin zuvor definiert.

3. Biotin-DOTA-Konjugat nach Anspruch 2, **dadurch gekennzeichnet, daß** R^1 CH_3 ist und R^2 H ist.

4. Biotin-DOTA-Konjugat nach Anspruch 1, **dadurch gekennzeichnet, daß** L ein Linker der Formel

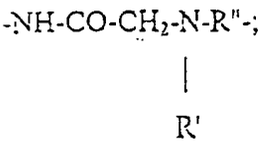


ist, wobei L' ausgewählt ist aus der Gruppe, welche umfaßt:

a) -NH-CO-$(CH_2)_n$-O-;

b) -NH-;

c)



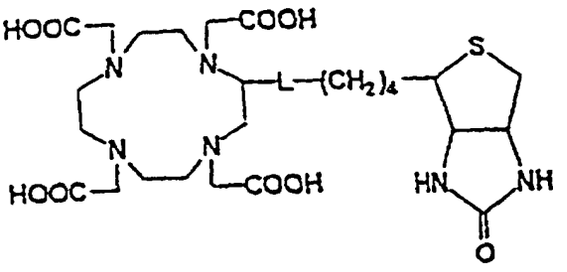
d) -NH-CS-NH-; -NH-CO-$(CH_2)_n$-NH-; und -NH-CO-Z-CH(COOH)-NH-CO, wobei Z-$(CH_2)_2$, -CH_2-S-CH_2-, -CH_2- oder -$(CH_2)_n$-CO-O-CH_2- ist, wobei n von 1 bis 4 ist,

oder das Bis-DOTA-Derivat davon, wobei n, R' und R" sind. wie hierin zuvor definiert.

5. Verwendung eines Konjugats nach einem der Ansprüche 1 bis 4 zur Herstellung eines Mittels zur Verwendung bei der Erhöhung der Lokalisierung von aktivem Agens zu einer Zielzelle.

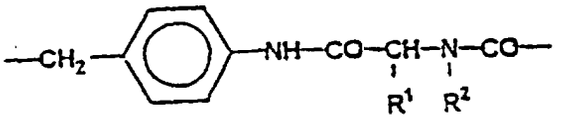
Revendications

1. Conjugué biotine-DOTA répondant à la formule suivante :

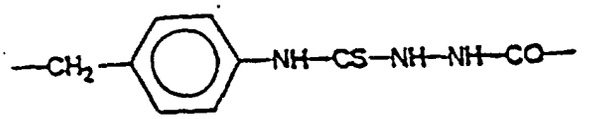


dans laquelle un groupe de liaison L est choisi dans le groupe consistant en :

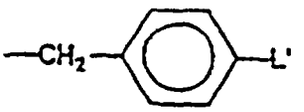
1) un groupe de liaison, contenant un D-amino-acide, de formule



2) un groupe de liaison de formule

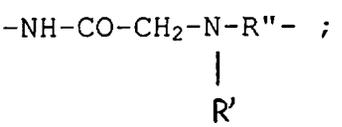


3) un groupe de liaison de formule



dans laquelle L' est choisi dans le groupe comprenant :

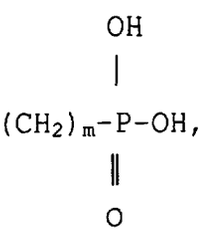
a) -NH-CO-$(CH_2)_n$-O- ;
b) -NH- ;
c)



d) -NH-CS-NH- ;
e) -NH-CO-$(CH_2)_n$-NH ; et
f) -NH-CO-Z-CH(COOH)-NH-CO

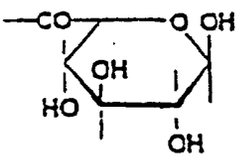
dans laquelle Z représente un groupe -$(CH_2)_2$-, -CH_2-S-CH_2-, -CH_2- ou -$(CH_2)_n$-CO-O-CH_2- dans lequel n a une valeur de 1 à 4,

R^1 représente l'hydrogène ; un groupe alkyle inférieur en C_1 à C_5 ; un groupe alkyle inférieur en C_1 à C_5 substitué avec un ou plusieurs groupes hydrophiles, de préférence $(CH_2)_m$-OH, $(CH_2)_m$-OSO_3, $(CH_2)_m$-SO_3, et



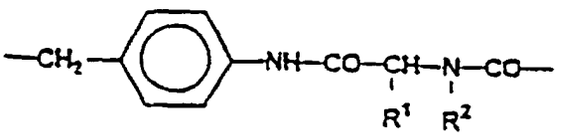
dans laquelle m est égal à 1 ou 2 ;
des amino-acides substitués avec un glucuronide, ou d'autres dérivés de glucuronide ;
R^2 représente l'hydrogène ; un groupe alkyle inférieur en C_1 à C_5 ; un groupe alkyle inférieur en C_1 à C_5

substitué, ayant un ou plusieurs substituants choisis dans le groupe comprenant des substituants hydroxy,. sulfate et phosphonate ; ou un groupement hydrophile ;
R' représente l'hydrogène ; un groupe $-(CH_2)_2-OH$ ou un sulfate ou un groupe



un de ses dérivés consistant en un phosphonate ; ou
R" représente une liaison ou un groupe $-(CH_2)_n-CO-NH-$; et
n a une valeur de 0 à 5.

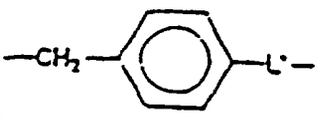
2. Conjugué biotine-DOTA suivant la revendication 1, dans lequel L représente un groupe de liaison, contenant un D-amino-acide, de formule



dans laquelle R^1 et R^2 répondent aux définitions précitées.

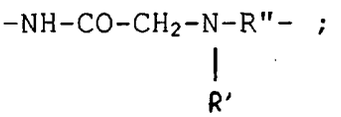
3. Conjugué biotine-DOTA suivant la revendication 2, dans lequel R^1 représente un groupe CH_3 et R^2 représente H.

4. Conjugué biotine-DOTA suivant la revendication 1, dans lequel L représente un groupe de liaison de formule



dans laquelle L' est choisi dans le groupe comprenant :

a) $-NH-CO-(CH_2)_n-O-$;
b) $-NH-$;
c)



d) $-NH-CS-NH-$;
e) $-NH-CO-(CH_2)_n-NH$; et
f) $-NH-CO-Z-CH(COOH)-NH-CO$

dans laquelle Z représente un groupe $-(CH_2)_2$, $-CH_2-S-CH_2-$, $-CH_2-$ ou $-(CH_2)_n-CO-O-CH_2-$ dans lequel n a une valeur de 1 à 4,
ou son dérivé de bis-DOTA, dans lequel n, R' et R" répondent aux définitions précitées.

5. Utilisation d'un conjugué suivant l'une quelconque des revendications 1 à 4 dans la préparation d'un agent destiné à être utilisé pour accroître la localisation d'un agent actif à une cellule cible.