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(54) **Title:** CONJUGATES WHICH BIND A BLOOD PROTEIN SUCH AS HUMAN SERUM ALBUMIN AND METHODS OF USING THE SAME IN DIAGNOSTIC AND THERAPEUTIC APPLICATIONS

(57) **Abstract:** Novel constructs and agents useful for diagnostic and therapeutic applications have been developed. The constructs comprise a chelator, a first linker and either a hydrophobic amino acid or a "hydrophobe" (hydrophobic non-amino acid), optionally attached to a second linker and a targeting vector. The structure of such compounds enables prolonged residence time in the blood, and therefore increases their usefulness in imaging and treating disease. The present invention is further directed to compounds which provide a high fraction bound to serumand/or to compounds which have high measured relaxivity in Seronorm™.

# CONJUGATES WHICH BIND A BLOOD PROTEIN SUCH AS HUMAN SERUM ALBUMIN AND METHODS OF USING THE SAME IN DIAGNOSTIC AND THERAPEUTIC APPLICATIONS

## CROSS REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims priority to and benefit of U.S. Provisional Application No. 60/931,090, filed May 21, 2007, the contents of which are hereby incorporated by reference in their entirety.

## FIELD OF THE INVENTION

**[0002]** The invention relates to conjugates that bind a blood protein such as human serum albumin (HSA), and are useful in diagnostic and/or therapeutic applications. The invention also includes methods for making and using such conjugates in therapeutic and diagnostic applications.

## BACKGROUND OF THE INVENTION

**[0003]** New and better compounds are needed for the diagnosis and treatment of disease. For example, the diagnosis and prognosis of coronary and peripheral vascular disease is important in medicine. X-ray angiography with contrast agents has been done in coronary artery imaging. Due to the dilution of these contrast agents in the blood and their rapid transit into the extravascular space, imaging must be performed rapidly during dosing. Intravenous digital subtraction angiography (IV-DSA) was a technique widely studied in the 1980s in an attempt to address the need for better imaging; however, this technique was largely unsuccessful. *See* Saccani S. et al., "Digital angiography and surgery of the carotid: our experience," *Acta Biomed Ateneo Parmense* 1987; 58:99-103; Fiedler V., "Image exactness in digital subtraction angiography," *ROFO Fortschr Geb Rontgenstr Nuklearmed* 1986; 145:708-713; Crocker EF Jr. et al., "The role of intravenous digital subtraction angiography in the evaluation of extra cranial carotid artery disease. Can the decision for carotid artery surgery be made solely on the basis of its findings?" *J Vasc Surg* 1986; 4:157-163; Ball JB Jr. et al., "Complications of intravenous digital subtraction angiography," *Arch Neurol* 1985; 42:969-972, all of which are hereby incorporated by reference in their entirety.

**[0004]** The applicability of magnetic resonance imaging (MRI) to the diagnosis of cardiovascular and cerebrovascular abnormalities has steadily increased in recent years, due in no small part to the development of sophisticated hardware and special pulse sequences that allow the vasculature to be imaged selectively in the presence of other tissues. Standard extracellular MRI contrast agents (in combination with fast, gated pulse sequences and

breath-holding on the part of the patient) have been employed in magnetic resonance angiography (MRA). Indeed, it appears that specialized agents for MRA are unnecessary for certain peripheral vascular examinations. *See*, *e.g.*, Botnar RM et al., "A fast 3D approach for coronary MRA," *J Magn Reson Imaging* 1999;10:821-825; van Geuns RJ et al., "Magnetic resonance imaging of the coronary arteries: clinical results from three dimensional evaluation of a respiratory gated technique," *Heart* 1999; 82:515-519; Earls JP et al., "MR angiography of the thoracic, abdominal, and extremity venous system," *Magn Reson Imaging Clin N Am* 1998; 6:417-435; Talagala SL et al., "Fast three-dimensional time-of-flight MR angiography of the intra-cranial vasculature," *J Magn Reson Imaging* 1995; 5:317-323; Siebert JE et al., "Physical principles and application of magnetic resonance angiography," *Semin Ultrasound CT MR* 1992;.13:227-245; Leiner T. et al., "Contrast-enhanced magnetic resonance angiography," *Ned Tijdschr Geneeskd* 1999; 143:1087-1093; Shetty AN et al., "3D Breath-hold contrast-enhanced MRA: a preliminary experience in aorta and iliac vascular disease," *J Comput Assist Tomogr* 1998; 22:179-185; all of which are hereby incorporated by reference in their entirety.

**[0005]** Blood pool agents for MRI diagnostics and other applications have been investigated in recent years. *See*, *e.g.*, WO 00/38738, directed to metal ion complexes of conjugates of bile acids with molecules having chelating activity as contrast agents in MRI, in particular as blood pool agents, which is hereby incorporated by reference in its entirety.

**[0006]** An additional attractive feature of MRA is the fact that employment of a lower dose of contrast agent is a positive attribute for any new technique. However, for cardiovascular magnetic resonance angiography (CVMRA), the situation is more complex for several reasons. For example, the heart is always in motion; the patient may not be able to endure breath-holding for more than 15-20 seconds; the hardware is not yet powerful and fast enough (non contrast techniques such as spiral K space sampling or techniques employing single pass CVMRA); and finally, spatial resolution must be at least 1.0 mm x 1.0 mm x 1.0 mm in order to achieve meaningful results. This forces a scan time of at least 7 minutes with hardware that is considered possible to construct. Thus, known contrast agents such as MS-325 (gadofosveset trisodium) a member of a class of blood pool agents for magnetic resonance angiography (MRA) have been used, for example, to diagnose vascular disease under the above constraints.

**[0007]** In view of these factors, an agent that provides T1 shortening (<50 ms) of principally the intravascular space for an extended period of time is highly desirable, and can greatly facilitate CVMRA. In order for this to be possible, the agent preferably should be restricted

from diffusing into the extravascular space, and preferably should undergo clearance from the blood with a half time less than that of extracellular space agents.

**[0008]** The major shortcoming of extracellular MRI agents, their short blood half time, is also faced by targeted imaging agents generally. For example in the case of a targeted diagnostic or therapeutic radiopharmaceutical, even if the radiometal is tightly chelated and the metal chelate is conjugated to a targeting vector, the concentration of the targeted radiopharmaceutical is decreased by distribution to the extravascular space, active and passive excretion via the kidney and possibly hepatobiliary excretion. These processes may be so rapid that residence time of the targeted radiopharmaceutical in the blood may be insufficient to permit its accumulation at an intravascular target or even an extravascular target site. It should be remembered that even if the radiopharmaceutical is distributed rapidly to the extravascular space, if clearance from the extravascular space via the kidney, for example, is rapid, targeting of an extravascular site will be ineffective. Therefore diagnostic and therapeutic compounds bearing a moiety capable of extending the blood half time and even the half time in the extravascular space may have an advantage in targeting sites of disease vs those that cannot. There is therefore a need for diagnostic and therapeutic compounds having superiority over existing compounds with respect to the fraction of the compound bound to serum and with respect to measured relaxivity in Seronorm™ (a solution of human serum albumin ("HSA") commercially available from Nycomed-Amersham).

## SUMMARY OF THE INVENTION

**[0009]** The present invention is directed, in one embodiment, to a compound for the imaging or treatment of a disease in a mammal, having the structure:

Ch– L1 – R – L2 – Q

wherein Ch is a chelating moiety, optionally complexed with M, a metal or a radionuclide;

L1 is a first linker;

R is a hydrophobic amino acid or a hydrophobic non-amino acid compound which renders the entire construct capable of binding a blood protein, particularly of binding human serum albumin (HSA); and

L2 is an optional second linker that is further linked to an optional targeting vector Q, which can direct the entire construct to a tissue bearing the target.

**[0010]** Note that the components CH, L1, R, L2 and Q need not be attached in the order shown but may also be attached according to, for example:

Ch-L1(L2-Q)-R ; and

Ch(L2-Q)-L1-R.

[0011] Ch is a chelating moiety, which is optionally complexed with a metal or a radionuclide. In one embodiment, Ch comprises DTPA, DOTA, EDTA, TETA, EHPG, HBED, NOTA, DOTMA, TETMA, PDTA, TTHA, LICAM, MECAM or derivatives thereof. In a preferred embodiment Ch comprises DOTA or a derivative thereof. In a more preferred embodiment Ch comprises DO3A or derivatives thereof. Most preferably, Ch comprises DO3A10CM, an abbreviation of 10-carbonylmethyl-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid. This designation indicates that the compound has 3 carboxylate moieties chelating the gadolinium or other metal, and a fourth chelating group one component of which is a carbonyl group which is not the carbonyl group of a carboxylic acid, but rather the carbonyl group of another functionality such as a carboxamide or a hydroxamate, or an acylhydrazine, or of a semicarbazide, or a semicarbazone for example. or related chelators. In another preferred embodiment Ch is a chelating moiety having high relaxivity. For example, Ch may be selected from the following table:

**Chart 4. Enhanced Relaxivity (ER) Chelate Systems**

CM-DOTMA CM-DOTA CEMP-DO3A 7MP-DO3A

7MP-CM-DO3A 4-CE-DTPA TE-DOTA 4-CEMP-DTTA

In a preferred embodiment Ch is selected from CE-DTPA; CM-DOTMA; CM-DOTA; 7MP-DO3A and TE-DOTA.

[0012] Optionally Ch is complexed with a metal M or a radionuclide. M may be a metal ion such as, for example, a paramagnetic or radioactive metal ion. In one embodiment M is a paramagnetic metal ion. Preferably in this embodiment M comprises $Mn^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Gd^{3+}$, $Eu^{3+}$, $Dy^{3+}$, $Pr^{3+}$, $Cr^{3+}$, $Co^{3+}$, $Fe^{3+}$, $Ti^{3+}$, $Tb^{3+}$, $Nd^{3+}$, $Sm^{3+}$, $Ho^{3+}$, $Er^{3+}$, $Pa^{4+}$ or $Eu^{2+}$. Most preferably in this embodiment M is $Gd^{3+}$.

[0013] In another embodiment M is a radioactive metal ion or a radionuclide. Preferably, in this embodiment M is selected from $^{18}F$, $^{124}I$, $^{125}I$, $^{131}I$, $^{123}I$, $^{77}Br$, $^{76}Br$, $^{99m}Tc$, $^{51}Cr$, $^{67}Ga$, $^{68}Ga$, $^{47}Sc$, $^{51}Cr$, $^{167}Tm$, $^{141}Ce$, $^{111}In$, $^{168}Yb$, $^{175}Yb$, $^{140}La$, $^{90}Y$, $^{88}Y$, $^{153}Sm$, $^{166}Ho$, $^{165}Dy$, $^{166}Dy$, $^{62}Cu$, $^{64}Cu$, $^{67}Cu$, $^{97}Ru$, $^{103}Ru$, $^{186}Re$, $^{188}Re$, $^{203}Pb$, $^{211}Bi$, $^{212}Bi$, $^{213}Bi$, $^{214}Bi$, $^{105}Rh$,

$^{109}$Pd, $^{117m}$Sn, $^{149}$Pm, $^{161}$Tb, $^{177}$Lu, $^{198}$Au or $^{199}$Au and the like. Preferably M is $^{99m}$Tc, $^{67}$Ga, $^{68}$Ga, $^{90}$Y, $^{88}$Y, $^{111}$In or $^{177}$Lu.

**[0014]** L1, the first linker, links Ch, the chelating moiety to one or more other components of the conjugate. Thus, L1 comprises functional groups sufficient to allow such linking. In a preferred embodiment L1 links the chelator to the hydrophobic molecule R. Preferably L1 comprises a substituted or unsubstituted alkyl chain, a polyethylene glycol derivative, one or more amino acids, a sugar, an aliphatic or aromatic spacer, a lipid molecule, or combinations of any of the foregoing. In a particularly preferred embodiment, L1 comprises an amino acid. For example, L1 may comprise Gly or Lys. Similarly, in a preferred embodiment L1 comprises trans-4-methylaminocyclohexanecarboxylic acid (referred to as a). In a more preferred embodiment L1 comprises two amino acids. Preferably L1 is Gly-Lys (referred to as GK); Gly-Gly (referred to as GG); Pro- trans-4-aminomethylcyclohexanecarbonyl (referred to as Pa); Asp- trans-4-aminomethylcyclohexanecarbonyl (referred to as Da); isonipecotic acid- trans-4-aminomethylcyclohexanecarbonyl (referred to as ia); Gly-Asp (referred to as GD); Gly-trans-4-aminomethylcyclohexanecarbonyl (referred to as Ga); Glu- trans-4-aminomethylcyclohexanecarbonyl (referred to as Ea); trans-4-aminomethylcyclohexanecarbonyl-Gly (referred to as aG); Asp-Asp (referred to as DD); Glu-Leu (referred to as EL); Asp-Leu (referred to as DL) or Leu-Leu (referred to as LL) . Of these, the most preferred are GK, a, Ga and aa.

**[0015]** R is a hydrophobic moiety which renders the entire conjugate able to bind to a blood protein such as HSA. Preferably R is a hydrophobic amino acid, a hydrophobic moiety derived from a hydrophobic amino acid or a hydrophobic organic group. In a preferred embodiment of the present invention, R comprises a hydrophobic amino acid. For example, R may be tryptophan or a derivative thereof; *l*-biphenylalanine (referred to as B) or *l*-3,3-diphenylalanine (referred to as e). In a particularly preferred embodiment both R and L1 comprise amino acids (or are derived from amino acids). In an even more preferred embodiment, L1 comprises two amino acids and R comprises a hydrophobic amino acid which binds HSA. For example, in preferred compounds of the invention L1-R comprises glycyl-trans-4-aminomethylcyclohexanecarbonyl-*l*-4-biphenyl (refereed to herein as GaB) or trans-4-aminomethylcyclohexanecarbonyl-*l*-4-biphenyl (referred to herein as aB) .

**[0016]** In another embodiment R is a hydrophobic organic group. Preferably in this embodiment R is a hydrophobic organic group of similar size to a hydrophobic amino acid.

More preferably, R is a hydrophobic aliphatic or aromatic acyl group In a preferred embodiment R is selected from:

R =

G =

* Indicates point of attachment of G in the general structure.

[0017] The compounds of the present invention optionally include L2, a second linker. In one embodiment L2 is a linker which includes sufficient functional groups to link the targeting vector Q to the remainder of the conjugate. Preferably L2 comprises a substituted or unsubstituted alkyl chain, a polyethylene glycol derivative, one or more amino acids, a sugar, an aliphatic or aromatic spacer, a lipid molecule, or combinations of any of the foregoing. L2 may be the same or different as L1. Preferably L2 comprises one or more amino acids.

**[0018]** Q is an optional targeting vector, which directs the entire conjugate to tissue bearing the target. For example, the target may be a site of disease or a pathological process, such as, for example, an angiogenic site in a tumor. Q may be a targeting protein, antibody, peptide or equivalent, derivative or analog thereof; Q may also be, *e.g.*, an enzyme or a molecule that binds to an enzyme or it may be an antibiotic. Q may be a protein such as an antibody, antibody fragment, diabody, triabody, a small molecule which functions as a targeting vector a carbohydrate or an aptamer. In a preferred embodiment Q and L1 both comprise amino acids.

**[0019]** Particularly preferred conjugates of the invention include DO3A10CM-a-B (where Ch is DO3A10CM, L1 is a and R is B); DO3A10CM- G-a-B (where Ch is DO3A10CM, L1 is G-a and R is B); DO3A10CM -D-a-B (where Ch is DO3A10CM, L1 is Da and R is B); DO3A10CM -G-L-B (where Ch is DO3A10CM, L1 is Gl and R is B); CE-DTPA-a-B (where Ch is CE-DTPA, L1 is a and R is B); CM-DOTMA-a-B (where Ch is CM-DOTMA, L1 is a and R is B); CM-DOTA-a-B (where Ch is CM-DOTA, L1 is a and R is B); and 7MP-DO3A-G-a-B (where Ch is 7MP-DO3A, L1 is G-A and R is B). Each of these conjugates may optionally further include a second linking group L2 and/or a targeting vector Q. Any of these conjugates may be optionally complexed with a metal M. In a preferred embodiment these conjugates are complexed with a paramagnetic metal, particularly Gd. In another preferred embodiment, these conjugates are complexed with a radioactive metal or radionuclide, particularly with a radioactive lanthanide.

**[0020]** Conjugates of the present invention are capable of binding a blood protein such as HSA, which increases the half time of the conjugate in the blood compared to a comparable agent unable to bind a blood protein. The increased blood half time results in conjugates better able to accumulate at target tissue. Additionally, where the conjugates of the invention are bound to a paramagnetic metal, the binding to a blood protein such as HSA results in an increase of the relaxivity of the chelator which is commonly referred to as enhanced relaxivity. Such conjugates, when bound to the blood proteins diffuse into the extravascular space much more slowly than those which cannot bind to proteins in the blood. Therefore their effective residence time in the blood is much longer and because they are bound to blood proteins such as HSA they exhibit enhanced relaxivity and enhanced signal vs chelators which are not protein bound, permitting improved imaging of the intravascular space and facilitating CVMRI.

**[0021]** In one embodiment, such conjugates also include a chelating moiety Ch whose relaxivity changes to a greater degree then that of a standard chelator when a molecule to

which it is conjugated to binds proteins in the blood. Such agents provide a T1 shortening (<50 ms) of only the intravascular space for an extended period of time, further facilitating CVMRI. Such chelating moieties, referred to herein as high or enhanced relaxivity chelators, include for example, : CM-DOTMA, CM-DOTA, CEMP-DO3A, 7MP-DO3A, 7MP-CM-DO3A, TE-DOTA and 4-CEMP-DTTA whose structures are shown in Chart 4 .

**[0022]** The present invention is also directed to a method of preparing a conjugate capable of binding a blood protein such as human serum albumin (HSA), wherein said method comprises the steps of: attaching a chelating moiety to the first linker, attaching said first linker to the hydrophobic group R via a protecting group having a carboxylic group thereto, attaching an optional second linker to said hydrophobic group, and attaching an optional targeting vector Q to said optional second linker. The conjugate may then be optionally complexed with M, a metal/radionuclide. The present invention is also directed to a method of preparing a conjugate capable of binding a blood protein such as human serum albumin wherein said method comprises attaching a hydrophobic group R which is capable of binding HSA to a first linker, then attaching the first linker to the chelating moiety Ch, then attaching another conjugatable group in the first linker to a second linker followed by attachment of the second linker to an optional molecule which can serve as a targeting vector. The conjugate may then be optionally complexed with M, a metal/radionuclide.

**[0023]** The present invention is also directed to a method of preparing a conjugate capable of binding a blood protein such as HSA, wherein said method comprises preparing the components of the entire construct as modules such as Ch-L1, R-P (where P is an optional protecting group for a reactive function other than that to be employed for assembling the construct) and L2-Q and then attaching the modules in any desired order to obtain constructs such as Ch – L1 – R – L2 – Q, Ch-L1(L2-Q)-R or Ch(L2-Q)-L1-R. The conjugate may then be optionally complexed with M, a metal/radionuclide.

**[0024]** The present invention is also directed to assembly on solid phase of the entire conjugate or a subset of the entire conjugate described above using orthogonal or quasiorthogonal series of protecting groups which allow creation of any of the conjugate Ch – L1 – R – L2 – Q, Ch-L1(L2-Q)-R, Ch(L2-Q)-L1-R or any part of the conjugate on solid phase in fully or partially protected form followed by simultaneous cleavage from the solid phase and deprotection of the desired construct. The conjugate may then be optionally complexed with M, a metal/radionuclide.

**[0025]** The present invention is further directed to compounds which provide a high fraction bound to serum. For example, compounds of the invention may have a fraction bound to

serum greater than about 0.87 (87%, expressed as a percentage). The present invention is also directed to compounds which have high measured relaxivity in Seronorm™. For example, compounds of the invention may have a measured relaxivity in Seronorm™ of greater than about 36.57. Still further, the present invention is also directed to compounds which have both high fraction bound to serum and high measured relaxivity. For example, compounds of the invention may have fraction bound to serum greater than about 87% and a measured relaxivity in Seronorm™ of greater than about 36.57.

**[0026]** The present invention is also directed to methods of using the conjugates of the invention. For example, conjugates of the invention may be used in methods of treating a disease in a mammal comprising the step of administering a composition comprising the conjugates of the present invention. Similarly, conjugates of the invention may be used in methods of imaging a site in a mammal, comprising the steps of administering a composition comprising the conjugates of the present invention and imaging the mammal.

## DEFINITIONS

**[0027]** As used herein, the term "therapeutic" includes at least partial alleviation of symptoms of a given condition. For example, treatment of an individual can result in a decrease in the size of a tumor or diseased area, or prevention of an increase in size of the tumor or diseased area or partial alleviation of other symptoms. Treatment can result in reduction in the number of blood vessels in an area of interest or can prevent an increase in the number of blood vessels in an area of interest. Treatment can also prevent or lessen the number or size of metastic outgrowths of the main tumor(s).

**[0028]** As used herein, the term "polypeptide" is used to refer to a compound of two or more amino acids joined through the main chain (as opposed to side chain) by a peptide amide bond (–C(=O)NH–). The term "peptide" is used interchangeably herein with "polypeptide" but is generally used to refer to polypeptides having fewer than 40, and preferably fewer than 25 amino acids.

**[0029]** As used herein, the term "relaxivity" means the ability of a compound to shorten the T1 (longitudinal relaxation time) of water protons or other atoms which can be detected by T1-weighted MRI imaging. The symbol for relaxivity is r and where the T1 relaxivity is quoted the symbol is r1. Usually this value is quoted as being measured at a given field strength, such as 20MHz, for example, and the value is given in units of $mM^{-1}sec^{-1}$. In the case of MRI agents

based on gadolinium chelates the value is assumed to be per gadolinium atom. In cases where there is more than one gadolinium atom in a MRI imaging agent the aggregate relaxivity may be quoted and often the relaxivity per gadolinium atom is also quoted in order to clarify the interpretation of the data.

**[0030]** As used herein, the term "high relaxivity" refers to values of r1 provided by MRI agents of a given class, particularly MRI agents based on gadolinium chelates, which are significantly greater than those provided by typical gadolinium chelate-based MRI agents under the same conditions. By significantly greater is meant at least 10% greater than the relaxivity of the comparison gadolinium chelate. By 'same conditions' is meant that the chelators in question are employed under the same conditions of measurement and if conjugated to another molecular species such as a targeting vector or a protein binding moiety, they are conjugated to the same molecular species.

**[0031]** As used herein the term 'enhanced relaxivity' refers to an increase in relaxivity, r1, which is obtained when a MRI agent, typically based on a gadolinium chelator, binds to a macromolecular species such as a blood protein. The comparison is made between the relaxivity of the MRI agent measured in water or optionally in aqueous solutions of a buffer such a pH 7 phosphate buffer or phosphate buffered saline solution and the relaxivity of the MRI agent in the presence of a defined concentration of a macromolecular target. In one preferred embodiment the macromolecular target is human serum albumin (HSA).

**[0032]** The term "binding" refers to the determination by standard assays, including those described herein, that a binding moiety such as a hydrophobic group, a small molecule, a peptide, or a polypeptide binds reversibly to a given target. Such standard assays include, but are not limited to, equilibrium dialysis, gel filtration, surface plasmon resonance and the monitoring of spectroscopic changes, such as increased or decreased absorption, a bathochromic or hypsochromic shift, an increase or decrease or an elimination of or an induction of fluorescence, for example, that result in the bound state.

**[0033]** "Homologues" of the binding species disclosed herein (including hydrophobic groups, small molecules, peptides or polypeptides) may be produced using any of the modification or optimization techniques described herein or known to those skilled in the art. Such homologues will be understood to fall within the scope of the present invention and the definition of "binding species" so long as the substitution, addition, or deletion of functionality, or in the case of peptidic species, amino acids or other such modification does not eliminate its ability to bind to the target. The term "homologous," as used herein, with regard to peptides and polypeptides refers to the degree of sequence similarity between two

polymers (*i.e.* polypeptide molecules or nucleic acid molecules). When the same nucleotide or amino acid residue or one with substantially similar properties (*i.e.* a conservative substitution) occupies a sequence position in the two polymers under comparison, then the polymers are homologous at that position. For example, if the amino acid residues at 60 of 100 amino acid positions in two polypeptide sequences match or are homologous, then the two sequences are 60% homologous. The homology percentage figures referred to herein reflect the maximal homology possible between the two polymers, *i.e.*, the percent homology when the two polymers are so aligned as to have the greatest number of matched (homologous) positions. Polypeptide homologues within the scope of the present invention will be at least 70% and preferably greater than 80% homologous to at least one of the binding sequences disclosed herein.

**[0034]** The term “specificity” refers to a binding polypeptide having a higher binding affinity for one target over another. Binding specificity may be characterized by a ratio of the dissociation equilibrium constants ($K_D$) or association equilibrium constants ($K_a$) for the two tested target materials.

**[0035]** The term “patient” as used herein refers to any mammal, especially humans.

**[0036]** The term “pharmaceutically acceptable” carrier or excipient refers to a non-toxic carrier or excipient that may be administered to a patient, together with a compound of this invention, and which does not destroy the pharmacological activity thereof.

**[0037]** The term “target” or “target molecule” refers to any substance that a binding moiety or binding polypeptide (including for example a targeting vector or hydrophobic compound) can bind to, such as a protein or polypeptide, a cell, a receptor, a carbohydrate, a lipid, an oligonucleotide, etc. As used herein, “target” also includes a family of receptors, such as, for example, protein-tyrosine kinase receptors.

**[0038]** The terms “therapeutic agent” or “therapeutic” refer to a compound or an agent having a beneficial, therapeutic or cytotoxic effect *in vivo*. Therapeutic agents include those compositions referred to as, for example, bioactive agents, cytotoxic agents, drugs, chemotherapy agents, radiotherapeutic agents, genetic material, etc.

**[0039]** The following common abbreviations are used throughout this specification: 9-fluorenylmethyloxycarbonyl (fmoc or Fmoc), 1-hydroxybenozotriazole (HOBt), N,N’-diisopropylcarbodiimide (DIC), acetic anhydride ($Ac_2O$), (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde), trifluoroacetic acid (TFA), Reagent B (TFA:$H_2O$:phenol:triisopropylsilane, 88:5:5:2), N,N-diisopropylethylamine (DIEA), O-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), O-(7-

azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorphosphate (HATU), N-hydroxysuccinimide (NHS), solid phase peptide synthesis (SPPS), dimethyl sulfoxide (DMSO), dichloromethane (DCM), dimethylformamide (DMF), and N-methylpyrrolidinone (NMP).

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

**[0040]** The present invention is directed, in one embodiment, to novel conjugates that exhibit superior ability to bind a blood protein such as Human Serum Albumin (HSA) and which may be used in diagnostic and/or therapeutic applications.

**[0041]** The present invention is further directed, in another embodiment, to novel conjugates that exhibit the ability to bind human serum at greater than about 87% and which may be used in diagnostic and/or therapeutic applications.

**[0042]** The present invention is further directed, in still another embodiment, to novel conjugates that exhibit a measured relaxivity in Seronorm™ of greater than about 36.57 and which may be used in diagnostic and/or therapeutic applications.

**[0043]** The present invention is further directed, in another embodiment, to novel conjugates that exhibit the ability to bind human serum at greater than about 87% and a measured relaxivity in Seronorm™ (a solution of human serum albumin commercially available from Nycomed-Amersham) of greater than about 36.57 which may be used in diagnostic and/or therapeutic applications.

**[0044]** The present invention is further directed, in yet another embodiment, to compounds that comprise one or more moieties that bind a blood protein such as HSA, to which are conjugated to a metal chelator. Most preferably, the compounds comprise one or more agents that bind the protein HSA, as well as a metal chelator linked to the HSA-binding portion by one or more linkers.

**[0045]** In one preferred embodiment the conjugates have the structure:

Ch– L1 – R – L2 – Q

wherein Ch is a chelating moiety, optionally complexed with M, a metal or a radionuclide;
L1 is a first linker;

R is a hydrophobic amino acid or a hydrophobic non-amino acid compound which renders the entire construct capable of binding a blood protein, particularly of binding human serum albumin (HSA); and

L2 is an optional second linker that is further linked to an optional targeting vector Q, which can direct the entire construct to a tissue bearing the target.

**[0046]** Note that the components CH, L1, R, L2 and Q need not be attached in the order shown but may also be attached according to, for example:

Ch-L1(L2-Q)-R ; and Ch(L2-Q)-L1-R.

**[0047]** In another preferred embodiment the conjugates comprise :

Ch-L1-R,

wherein Ch, L1 and R have the above meanings.

Chelating Moieties

**[0048]** Ch is a chelating moiety, which is optionally complexed with a metal or a radionuclide. In one embodiment, Ch is a paramagnetic metal chelate, which, when complexed with paramagnetic metal may serve as a contrast agent for use in MRI. Suitable chelators are known in the art and include, *e.g.*, acids with methylene phosphonic acid groups, methylene carbohydroxamine acid groups, carboxyethylidene groups, carboxymethylene groups or methylene carboxamide groups. Examples of useful chelators include, but are not limited to, diethylenetriamine pentaacetic acid (DTPA), 1,4,7,10-tetraazacyclotetradecane-1,4,7,10-tetraacetic acid (DOTA), 1-substituted 1,4,7,-tricarboxymethyl 1,4,7,10 teraazacyclododecane triacetic acid (DO3A), ethylenediaminetetraacetic acid (EDTA), and 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA).

**[0049]** Additional useful chelating ligands are ethylenebis-(2-hydroxy-phenylglycine) (EHPG), and derivatives thereof, including 5-Cl-EHPG, 5Br-EHPG, 5-Me-EHPG, 5-t-Bu-EHPG, and 5-sec-Bu-EHPG; benzodiethylenetriamine pentaacetic acid (benzo-DTPA) and derivatives thereof, including dibenzo-DTPA, phenyl-DTPA, diphenyl-DTPA, benzyl-DTPA, and dibenzyl DTPA; bis-2 (hydroxybenzyl)-ethylene-diaminediacetic acid (HBED) and derivatives thereof; the class of macrocyclic compounds which contain at least 3 carbon atoms, more preferably at least 6, and at least two heteroatoms (O and/or N), which macrocyclic compounds can consist of one ring, or two or three rings joined together at the hetero ring elements, e.g., benzo-DOTA, dibenzo-DOTA, and benzo-NOTA, where NOTA is 1,4,7-triazacyclononane N,N',N"-triacetic acid, benzo-TETA, benzo-DOTMA, where

DOTMA is 1,4,7,10-tetraazacyclotetradecane-1,4,7, 10-tetra(methyl tetraacetic acid), and benzo-TETMA, where TETMA is 1,4,8,11- tetraazacyclotetradecane-1,4,8,11-(methyl tetraacetic acid); derivatives of 1,3-propylenediaminetetraacetic acid (PDTA) and triethylenetetraaminehexaacetic acid (TTHA); derivatives of 1,5,10-N,N',N"-tris(2,3-dihydroxybenzoyl)-tricatecholate (LICAM) and 1,3,5-N,N',N"-tris(2,3-dihydroxybenzoyl) aminomethylbenzene (MECAM). A preferred chelator for use in the present invention is DTPA.

**[0050]** In one embodiment, Ch comprises DTPA, DOTA, EDTA, TETA, EHPG, HBED, NOTA, DOTMA, TETMA, PDTA, TTHA, LICAM, MECAM or derivatives thereof. In a preferred embodiment Ch comprises DOTA or a derivative thereof. In a more preferred embodiment Ch comprises DO3A or DO3A10CM.

**[0051]** In another embodiment Ch is a chelator for a radioactive metal or radionuclide. Such chelators include, for example, linear, macrocyclic, terpyridine, and $N_3S$, $N_2S_2$, or $N_4$ chelants (*see also* U.S. 5,367,080, U.S. 5,364,613, U.S. 5,021,556, U.S. 5,075,099, U.S. 5,886,142), and other chelators known in the art including, but not limited to, HYNIC, DTPA, EDTA, DOTA, TETA, and bisamino bisthiol (BAT) chelators (*see also* U.S. 5,720,934). For example, $N_4$ chelators are described in U.S. Patent Nos. 6,143,274; 6,093,382; 5,608,110; 5,665,329; 5,656,254; and 5,688,487. Certain $N_3S$ chelators are described in PCT/CA94/00395, PCT/CA94/00479, PCT/CA95/00249 and in U.S. Patent Nos. 5,662,885; 5,976,495; and 5,780,006. The chelator may also include derivatives of the chelating ligand mercapto-acetyl-acetyl-glycyl-glycine (MAG3), which contains an $N_3S$, and $N_2S_2$ systems such as MAMA (monoamidemonoaminedithiols), DADS ($N_2S$ diaminedithiols), CODADS and the like. These ligand systems and a variety of others are described in Liu and Edwards, *Chem Rev* 99, 2235-2268 (1999) and references therein.

**[0052]** The chelator may also include complexes containing ligand atoms that are not donated to the metal in a tetradentate array. These include the boronic acid adducts of technetium and rhenium dioximes, such as are described in U.S. Patent Nos. 5,183,653; 5,387,409; and 5,118,797, the disclosures of which are incorporated by reference herein, in their entirety.

**[0053]** In another embodiment, disulfide bonds of a binding polypeptide of the invention are used as two ligands for chelation of a radionuclide such as $^{99m}Tc$. In this way the peptide loop is expanded by the introduction of Tc (peptide-S-S-peptide changed to peptide-S-Tc-S-peptide). This has also been used in other disulfide containing peptides in the literature (J. Q. Chen, A. Cheng, N. K. Owen, T. H. Hoffman, Y. Miao, S. S. Jurisson, T. P. Quinn. J. Nucl.

Med. 42, 1847-1855 (2001)) while maintaining biological activity. The other chelating groups for Tc can be supplied by amide nitrogens of the backbone, another cystine amino acid or other modifications of amino acids.

**[0054]** Particularly preferred metal chelators include those of Formula 20, 21, and 22 (for $^{111}$In and lanthanides such as paramagnetic gadolinium ($Gd^{3+}$) and radioactive lanthanides, such as, for example $^{177}$Lu, $^{90}$Y, $^{153}$Sm, and $^{166}$Ho) and those of Formula 23, 24, and 25 (for radioactive $^{99m}$Tc, $^{186}$Re, and $^{188}$Re) set forth below. These and other metal chelating groups are described in U.S. Patent Nos. 6,093,382 and 5,608,110, which are incorporated by reference herein in their entirety. Additionally, the chelating group of formula 22 is described in, for example, U.S. Patent No. 6,143,274; the chelating group of formula 24 is described in, for example, U.S. Patent Nos. 5,627,286 and 6,093,382, and the chelating group of formula 25 is described in, for example, U.S. Patent Nos. 5,662,885; 5,780,006; and 5,976,495.

HOOC COOH N N N N O COOH

(22)

NH HN N N COOH OH OH

(23a)

(23b)

(24a)

(24b)

(25)

**[0055]** In the above Formulas 20 and 21, R is alkyl, preferably methyl. In the above Formula 24, X is either $CH_2$ or O, Y is either $C_1$-$C_{10}$ branched or unbranched alkyl; Y is aryl, aryloxy, arylamino, arylaminoacyl; Y is arylkyl – where the alkyl group or groups attached to the aryl group are $C_1$-$C_{10}$ branched or unbranched alkyl groups, $C_1$-$C_{10}$ branched or unbranched hydroxy or polyhydroxyalkyl groups or polyalkoxyalkyl or polyhydroxy-polyalkoxyalkyl groups, J is C(=O)–, OC(=O)–, $SO_2$, NC(=O)–, NC(=S)–, N(Y), NC(=$NCH_3$)–, NC(=NH)–, N=N–, homopolyamides or heteropolyamines derived from synthetic or naturally occurring amino acids; all where n is 1-100. Other variants of these structures are described, for example, in U.S. Patent No. 6,093,382. The disclosures of each of the foregoing patents, applications and references are incorporated by reference herein, in their entirety.

**[0056]** The chelators may be covalently linked to the HSA-binding moiety via L1, or directly to the HSA binding moiety which is optionally further linked to L2 and/or Q, the targeting vector, as described previously, and then directly labeled with the radioactive metal of choice (*see*, *e.g.*, WO 98/52618, U.S. 5,879,658, and U.S. 5,849,261). Further examples of representative chelators and chelating groups contemplated by the present invention are described in, *e.g.*, WO 98/18496, WO 86/06605, WO 91/03200, WO 95/28179, WO 96/23526, WO 97/36619, WO 01/82974, WO 01/064708, WO 00/38738, PCT/US98/01473, PCT/US98/20182, and U.S. 4,899,755, U.S. 5,474,756, U.S. 5,846,519 and U.S. 6,143,274, each of which is hereby incorporated by reference in its entirety.

**[0057]** A preferred chelator used in conjugates of the present invention is 1-substituted 4,7,10-tricarboxymethyl 1,4,7,10 tetraazacyclododecane triacetic acid (DO3A). Another preferred chelating moiety is 1,4,7,10-tetraazacyclotetradecane-1,4,7,10-tetraacetic acid

(DOTA). The present invention also contemplates chelators comprising DOTA derivatives; *e.g.*, substituted derivatives of DOTA having 4 carboxyl groups chelating gadolinium or another metal and wherein the linkage of the macrocyclic system to the linker L1 is via either a group emanating from one of the alpha-carbon atoms of one of the chelating sidearms or from the macrocyclic carbon backbone.

**[0058]** Yet another chelator moiety is DO3A10CM, an abbreviation of 10-carbonylmethyl-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid. This designation indicates that the compound has 3 carboxylate moieties chelating the gadolinium or other metal, and a fourth chelating group one component of which is a carbonyl group which is not the carbonyl group of a carboxylic acid, but rather the carbonyl group of another functionality such as a carboxamide or a hydroxamate, or an acylhydrazine, or of a semicarbazide, or a semicarbazone for example.

**[0059]** It has been found that high relaxation rates provide increased contrast in the image. Increased contrast makes it possible to obtain better physiological information in a shorter period of time. This leads to significant advantages in terms of image quality and cost. Therefore, compounds with high relaxivity (as defined above) and a greater enhanced relaxivity upon binding to a macromolecular target provide a stronger signal enhancing effect per molecule than can be obtained with more typical relaxation agents that are used in contrast enhancement. One application that may take advantage of an enhanced relaxivity chelate is linking a compound or complex to a bioactive compound or targeting moiety that targets a particular tissue. Localization at the target via the targeting moiety can result in a higher signal enhancement than could be obtained if a comparable compound were linked that had normal relaxivity. Therefore, in a preferred embodiment Ch is a chelating moiety having high or enhanced relaxivity. Examples of high or enhanced relaxivity chelators that are useful for the present invention are described in, *e.g.*, WO 99/45967, WO 99/45968 (manganese chelates), WO 98/05625 and WO 98/05626, each of which is hereby incorporated by reference in its entirety. For example, Ch may be selected from the following table:

**Chart 4. Enhanced Relaxivity (ER) Chelate Systems**

CM-DOTMA CM-DOTA CEMP-DO3A 7MP-DO3A

7MP-CM-DO3A 4-CE-DTPA TE-DOTA 4-CEMP-DTTA

In a more preferred embodiment Ch is a chelator with enhanced relaxivity selected from CE-DTPA; CM-DOTMA; CM-DOTA; 7MP-DO3A and TE-DOTA.

**[0060]** A most preferred high relaxivity chelator for use in contrast agents for MRI is an aza ligand that is able to complex metal ions, in particular paramagnetic ions. Such an aza ligand should be able to form chelates with the transition metals or metals of the Lanthanide series, exhibiting a high level of affinity and selectivity for the relevant paramagnetic ions, as well as suitable pharmacokinetic properties such as excretion, binding to plasma proteins, metabolical inertia, and the like. Aza ligands that are particularly useful for the present invention are described in WO 03/008390, which is hereby incorporated by reference in its entirety.

**[0061]** Structures of some preferred chelating moieties of the present invention are shown in the charts below, and further discussed in the Examples. DO3A10CM was the standard chelating function that was employed for screening the candidate conjugates. Other chelators were employed in an attempt to optimize the conjugates for use in MRI. These chelates were chosen because they were found to display a greater increase in relaxivity than standard chelators in an assay that measures the relaxivity of the Gd-chelate when its motion is restricted artificially. The artificial restriction of the motion of the chelate is designed to mimic the binding of a chelator containing molecule to a blood protein, such as HSA (Human Serum Albumin). As described above the increase in relaxivity is often referred to as enhanced relaxivity – ER for short – by comparison with the unbound state. Thus, pairing up of one of the ER chelating molecules with a strong binder of HSA is expected to provide a greater increase in relaxivity upon binding the HSA than would be afforded by the compound comprising the same HSA binding molecule with the standard chelating function DO3A10CM.

**Structures of the standard chelates of lanthanides and other metals**

DO3A10CM

DTPA

**Structures of optimized chelates of lanthanides and other metals**

CM-DOTMA CM-DOTA CEMP-DO3A 7MP-DO3A

7MPCMDO3A 4-CE-DTPA TE-DOTA 4-CEMP-DTTA

**[0062]** Other chelators useful for the present invention will be apparent to those skilled in the art and/or on review of this application or references cited in this application.

Metals

**[0063]** Optionally the chelating moiety Ch is complexed with a metal M or a radionuclide. M may be a metal ion such as, for example, a paramagnetic or radioactive metal ion. In one embodiment M is a paramagnetic metal ion. Preferred paramagnetic metal ions have atomic numbers 21-29, 42, 44, or 57-83, including ions of the transition metal or lanthanide series which have one, and more preferably five or more, unpaired electrons and a magnetic moment of at least 1.7 Bohr magneton. Preferred paramagnetic metals include, but are not limited to, chromium (III), manganese (II), manganese (III), iron (II), iron (III), cobalt (II), nickel (II), copper (II), praseodymium (III), neodymium (III), samarium (III), gadolinium (III), terbium (III), dysprosium (III), holmium (III), erbium (III), europium (III) and ytterbium (III). Additionally, compounds of the present invention may also be conjugated with one or more superparamagnetic particles. Preferably in this embodiment M comprises $Mn^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Gd^{3+}$, $Eu^{3+}$, $Dy^{3+}$, $Pr^{3+}$, $Cr^{3+}$, $Co^{3+}$, $Fe^{3+}$, $Ti^{3+}$, $Tb^{3+}$, $Nd^{3+}$, $Sm^{3+}$, $Ho^{3+}$, $Er^{3+}$, $Pa^{4+}$ or $Eu^{2+}$.

**[0064]** One skilled in the art will be able to select a metal according to the dose required to detect target containing tissue, as well as considering other factors such as, *e.g.*, toxicity of the metal to the subject. *See*, Tweedle *et al.*, Magnetic Resonance Imaging (2nd ed.), vol. 1, Partain et al., eds. (W.B. Saunders Co. 1988), pp. 796-97. Generally, the desired dose for an individual metal will be proportional to its relaxivity, modified by factors such as the biodistribution, pharmacokinetics and metabolism of the metal.

**[0065]** The trivalent cation of gadolinium, referred to as Gd(III) or $Gd^{3+}$, is particularly preferred for MRI contrast agents, due to its high relaxivity and low toxicity, with the further advantage that it exists in only one biologically accessible oxidation state. This minimizes undesired metabolization of the metal by a patient. Gd(III) chelates have been used for clinical and radiologic MR applications since 1988, and approximately 30% of all MR examinations currently employ a gadolinium-based contrast agent. Another preferred metal is $Cr^{3+}$, which is advantageous and relatively inexpensive.

**[0066]** In another embodiment M is a radioactive metal ion or a radionuclide. Preferably, in this embodiment M is selected from $^{18}F$, $^{124}I$, $^{125}I$, $^{131}I$, $^{123}I$, $^{77}Br$, $^{76}Br$, $^{99m}Tc$, $^{51}Cr$, $^{67}Ga$, $^{68}Ga$, $^{47}Sc$, $^{51}Cr$, $^{167}Tm$, $^{141}Ce$, $^{111}In$, $^{168}Yb$, $^{175}Yb$, $^{140}La$, $^{90}Y$, $^{88}Y$, $^{153}Sm$, $^{166}Ho$, $^{165}Dy$, $^{166}Dy$, $^{62}Cu$, $^{64}Cu$, $^{67}Cu$, $^{97}Ru$, $^{103}Ru$, $^{186}Re$, $^{188}Re$, $^{203}Pb$, $^{211}Bi$, $^{212}Bi$, $^{213}Bi$, $^{214}Bi$, $^{105}Rh$, $^{109}Pd$, $^{117m}Sn$, $^{149}Pm$, $^{161}Tb$, $^{177}Lu$, $^{198}Au$ or $^{199}Au$ and the like. Preferably M is $^{99m}Tc$, $^{67}Ga$, $^{68}Ga$, $^{90}Y$, $^{88}Y$, $^{111}In$ or $^{177}Lu$.

## Linkers

**[0067]** As explained infra, one or more linkers (e.g. L1) may be present between the chelating agent and the hydrophobic compound, and optionally, one or more additional linkers (L2) may be present between the targeting vector and the remainder of the conjugate. Use of such linkers/spacers may improve the relevant properties of the conjugates (*e.g.*, by increasing serum stability, etc.). Linkers that are useful for the present invention may include, but are not restricted to, substituted or unsubstituted alkyl chains, polyethylene glycol derivatives, amino acid spacers, sugars, aliphatic or aromatic spacers, lipid molecules, or combinations of any of the foregoing.

**[0068]** In a particularly preferred embodiment, the conjugates of the invention include L1 and optionally L2. In a more preferred embodiment L1 (and L2 if present) comprise at least one amino acid. For example, L1 may comprise Gly or Lys. Similarly, in a preferred embodiment L1 comprises trans-4-methylaminocyclohexanecarboxylic acid (referred to as a). In a more preferred embodiment L1 comprises two amino acids. Preferably for compounds of Class 2 where (see Table 2) L1 can be Gly-Lys (referred to as GK), alternatively L1 can be

Lys(Gly) which indicates that the Gly is appended to the epsilon nitrogen of the Lys component of L1 instead of the alpha nitrogen of the Lys. In general when the linker comprises Lys attachment can be at the alpha and epsilon amines, at the alpha amine and carboxylic acid or at the carboxylic acid and epsilon amine. For compounds of Class 1 L1 in another more preferred embodiment is Gly-Gly (referred to as GG); Pro- trans-4 aminomethylcyclohexanecarbonyl (referred to as Pa); Asp- trans-4-aminomethylcyclohexanecarbonyl (referred to as Da); isonipecotic acid- trans-4-aminomethylcyclohexanecarbonyl (referred to as ia); Gly-Asp (referred to as GD); Gly-trans-4-aminomethylcyclohexanecarbonyl (referred to as Ga); Glu- trans-4-aminomethylcyclohexanecarbonyl (referred to as Ea); trans-4-aminomethylcyclohexanecarbonyl-Gly (referred to as aG); Asp-Asp (referred to as DD); Glu-Leu (referred to as EL); Asp-Leu (referred to as DL) or Leu-Leu (referred to as LL) . For compounds of Class 1 the most preferred are a, Ga and aa.

**[0069]** In a preferred embodiment L2 is present and includes sufficient functional groups to link the targeting vector Q to the remainder of the conjugate. Preferably L2 comprises one or more amino acids. In a preferred embodiment L2 comprises Gly or oligoglycines $(Gly)_n$ where n = 1-10, or Adoa (where Adoa refers to 8-amino, 3-6-dioxaoctanoyl) or $(Adoa)_n$ where n = 1-10, or combinations of Gly and Adoa or combinations of $(Gly)_n$ and Adoa, or combinations of $(Gly)_n$ and $(Adoa)_n$ or combinations of Gly and $(Adoa)_n$ in the order given or in the reverse order wherein the Adoa or $(Adoa)_n$ precedes the Gly or $(Gly)_n$ or in any other combination of the two linkers.

**[0070]** In general, methods disclosed herein as well as other known methods can be used to couple the metal chelate and the linker. *See, e.g.*, WO 95/28967, WO 98/18496, WO 98/18497 and discussions therein. The present invention contemplates linking of the chelate(s) on any position, provided the metal chelate retains the ability to bind the metal tightly in order to minimize toxicity. Similarly, a component of a compound of this invention may be modified or elongated in order to generate a locus for attachment to a metal chelate, provided such modification or elongation does not eliminate its ability to bind the target.

The Hydrophobic Group R

**[0071]** R is a hydrophobic moiety which renders the entire conjugate able to bind to a blood protein such as HSA. Preferably R is a hydrophobic amino acid, a hydrophobic moiety derived from a hydrophobic amino acid or a hydrophobic organic group. In a preferred embodiment of the present invention, R comprises a hydrophobic amino acid. In a preferred embodiment the hydrophobic amino acid is comprised of an entity containing an indole ring

substituted at the indole nitrogen with a hydrophobic substituent such as a substituted benzene sulfonamide or substituted benzamide moiety. In a particularly preferred embodiment R is tryptophan or a derivative thereof; *l*-biphenylalanine (referred to as B); or *l*-3,3-diphenylalanine (referred to as e). In a preferred embodiment both R and L1 comprise amino acids (or are derived from amino acids). In an even more preferred embodiment, L1 comprises two amino acids and R comprises a hydrophobic amino acid which binds HSA. For example, in preferred compounds of the invention L1-R comprises glycyl-trans-4-aminomethylcyclohexanecarbonyl-*l*-4-biphenylalanyl (referred to herein as GaB) or trans-4-aminomethylcyclohexanecarbonyl-*l*-4-biphenylalanyl (referred to herein as aB) .

**[0072]** In another embodiment R is a hydrophobic organic group. Preferably in this embodiment R is a hydrophobic organic group of similar size to a hydrophobic amino acid. More preferably, R is a hydrophobic molecule that contains one or more benzene rings or 6-membered carbon aryl groups. Preferably R is a hydrophobic aliphatic or aromatic carboxylic acid. In a preferred embodiment R is selected from:

R =

G =

$C_5H_{11}$

O

$CH_3O$

* Indicates point of attachment of G in the general structure.

[0073] In the compositions of the present invention, the purpose of the hydrophobic, blood protein binding portion is to prolong circulation of the conjugate in the bloodstream, in contrast with non-protein binding molecules of similar molecular weight. Typically, these non-protein binding molecules would be expected to be rapidly distributed from the blood stream to the extravascular space and then excreted from the body, *e.g.*, through the urine or the feces. In contrast, in the conjugates of the present invention, binding of the molecule to a protein in the blood, such as HSA, increases the blood residence time of the compound, because the molecular size of the bound blood protein is such that it is only very slowly excreted from the bloodstream, as compared to smaller molecules. When the compounds of the present invention are bound to the blood protein, they are unavailable to the pathways for

excretion of small molecules such as, for example, passive diffusion across the endothelial cell junctions into the extravascular space, or uptake by the kidneys or the liver via a first pass effect.

Targeting Moieties

**[0074]** The conjugates of the present invention may comprise an optional targeting vector Q, which directs the entire conjugate to tissue bearing the target. Thus, Q directs the conjugate to the appropriate site, or involves the compounds in a reaction, where the desired diagnostic or therapeutic activity will occur. Q may be a targeting protein, antibody, peptide or equivalent, derivative or analog thereof; Q may also be, *e.g.*, an enzyme or a molecule that binds to an enzyme or it may be an antibiotic. Q may be a protein such as an antibody, antibody fragment, diabody, or triabody, a small molecule which functions as a targeting vector a carbohydrate or an aptamer. In one embodiment, the targeting vector Q may be a peptide, equivalent, derivative or analog thereof which functions as a ligand that binds to a particular site, or which has binding affinity for a particular site or a specific metabolic function. The targeting vector may be a peptide that binds to a receptor or enzyme of interest. In another embodiment, the targeting vector may be an enzyme, or a molecule that binds to an enzyme. In another embodiment, the targeting moiety may be an antibiotic.

**[0075]** In a preferred embodiment, the targeting moiety is a peptide. For example, the targeting moiety may be a peptide hormone such as, for example, luetinising hormone releasing hormone (LHRH) such as that described in the literature [*e.g.*, Radiometal-Binding Analogues of Leutenizing Hormone Releasing Hormone PCT/US96/08695; PCT/US97/12084 (WO 98/02192)]; insulin; oxytosin; somatostatin; Neuro kinin-1 (NK-1); Vasoactive Intestinal Peptide (VIP) including both linear and cyclic versions as delineated in the literature, [*e.g.*, Comparison of Cyclic and Linear Analogs of Vasoactive Intestinal Peptide. D. R. Bolin, J. M. Cottrell, R. Garippa, N. Rinaldi, R. Senda, B. Simkio, M. O'Donnell. Peptides: Chemistry, Structure and Biology Pravin T. P. Kaumaya, and Roberts S. Hodges (Eds)., Mayflower Scientific LTD., 1996, pp. 174-175]; gastrin releasing peptide (GRP); bombesin and other known hormone peptides, as well as analogues and derivatives thereof.

**[0076]** Other useful targeting peptides include analogs of somatostatin which, for example, are Lanreotide (Nal-Cys-Thr-DTrp-Lys-Val-Cys-Thr-$NH_2$), Octreotide (Nal-Cys-Thr-DTrp-Lys-Val-Cys-Thr-ol), and Phe-Cys-Thr-DTrp-Lys-Val-Cys-Thr-ol. These analogues are described in the literature [*e.g.*, Potent Somatostatin Analogs Containing N-terminal Modifications, S. H. Kim, J. Z. Dong, T. D. Gordon, H. L. Kimball, S. C. Moreau, J.-P.

Moreau, B.A. Morgan, W. A. Murphy and J. E. Taylor; Peptides: Chemistry, Structure and Biology Pravin T. P. Kaumaya, and Roberts S. Hodges (Eds)., Mayflower Scientific LTD., 1996, pp. 241-243.]

**[0077]** Still other useful targeting peptides include Substance P agonists [*e.g.*, G. Bitan, G. Byk, Y. Mahriki, M. Hanani, D. Halle, Z. Selinger, C. Gilon, Peptides: Chemistry, Structure and Biology, Pravin T. P. Kaumaya, and Roberts S. Hodges (Eds), Mayflower Scientific LTD., 1996, pp. 697-698; G Protein Antagonists A novel hydrophobic peptide competes with receptor for G protein binding, Hidehito Mukai, Eisuke Munekata, Tsutomu Higashijima, J. Biol. Chem. 1992, 267, 16237-16243]; NPY(Y1) [*e.g.*, Novel Analogues of Neuropeptide Y with a Preference for the Y1-receptor, Richard M. Soll, Michaela, C. Dinger, Ingrid Lundell, Dan Larhammer, Annette G. Beck-Sickinger, Eur. J. Biochem. 2001, 268, 2828-2837; 99mTc-Labeled Neuropeptide Y Analogues as Potential Tumor Imaging Agents, Michael Langer, Roberto La Bella, Elisa Garcia-Garayoa, Annette G. Beck-Sickinger, Bioconjugate Chem. 2001, 12, 1028-1034; Novel Peptide Conjugates for Tumor-Specific Chemotherapy, Michael Langer, Felix Kratz, Barbara Rothen-Rutishauser, Heidi Wnderli-Allenspach, Annette G. Beck-Sickinger, J. Med. Chem. 2001, 44, 1341-1348]; oxytocin; endothelin A and endothelin B; bradykinin; Epidural Growth Factor (EGF); Interleukin-1 [Anti-IL-1 Activity of Peptide Fragments of IL-1 Family Proteins, I. Z. Siemion, A. Kluczyk, Zbigtniew Wieczorek, Peptides 1998, 19, 373-382]; and cholecystokinin (CCK-B) [Cholecystokinin Receptor Imaging Using an Octapeptide DTPA-CCK Analogue in Patients with Medullary Thyroid Carcinoma, Eur. J. Nucl Med. 200, 27, 1312-1317].

**[0078]** Literature which gives a general review of targeting peptides, can be found, for example, in the following: The Role of Peptides and Their Receptors as Tumor Markers, Jean-Claude Reubi, Gastrointestinal Hormones in Medicine, p. 899-939; Peptide Radiopharmaceutical in Nuclear Medicine, D. Blok, R. I. J. Feitsma, P. Vermeij, E. J. K. Pauwels, Eur. J. Nucl Med. 1999, 26, 1511-1519; and Radiolabeled Peptides and Other Ligands for Receptors Overexpressed in Tumor Cells for Imaging Neoplasms, John G. McAfee, Ronald D. Neumann, Nuclear Medicine and Biology, 1996, 23, 673-676 (somatostatin, VIP, CCK, GRP, Substance P, Galanin, MSH, LHRH, Arginine-vasopressin, endothelin). All of the literature and references cited are herein incorporated by reference in their entirety.

**[0079]** Other targeting peptides are disclosed in the following: Co-expressed peptide receptors in breast cancer as a molecular basis of in vivo multireceptor tumour targeting. Jean Claude Reubi, Mathias Gugger, Beatrice Waser. Eur. J. Nucl Med. 2002, 29, 855-862,

(includes NPY, GRP); Radiometal-Binding Analogues of Leutenizing Hormone Releasing Hormone PCT/US96/08695 (LHRH); PCT/US97/12084 (WO 98/02192) (LHRH); PCT/EP90/01169 (radiotherapy of peptides); WO 91/01144 (radiotherapy of peptides); and PCT/EP00/01553 (molecules for the treatment and diagnosis of tumours), all of which are herein incorporated by reference in their entirety.

**[0080]** Additionally, analogs of a targeting peptide can be used. These analogs include molecules that target a desired site receptor with avidity that is greater than or equal to the targeting peptide itself, as well as muteins, retropeptides and retro-inverso-peptides of the targeting peptide. One of ordinary skill will appreciate that these analogs may also contain modifications which include substitutions, and/or deletions and/or additions of one or several amino acids, insofar that these modifications do not negatively alter the biological activity of the peptides described therein. These substitutions may be carried out by replacing one or more amino acids by their synonymous amino acids. Synonymous amino acids within a group are defined as amino acids that have sufficiently similar physicochemical properties to allow substitution between members of a group in order to preserve the biological function of the molecule. Synonymous amino acids as used herein include synthetic derivatives of these amino acids (such as for example the D-forms of amino acids and other synthetic derivatives), and may include, for example, those listed in the following Table. In the chart and throughout this application amino acids are abbreviated interchangeably either by their three letter or single letter abbreviations, which are well known to the skilled artisan. Thus, for example, T or Thr stands for threonine, K or Lys stands for lysine, P or Pro stands for proline and R or Arg stands for arginine.

| Amino Acids | Synonymous Groups |
|---|---|
| Arg | His, Lys, Glu, Gln |
| Pro | Ala, Thr, Gly, N-methyl Ala, pipecolic acid, azetidine carboxylic acid |
| Thr | 3-hydroxy proline, 4-hydroxy proline, Ser, Ala, Gly, His, Gln |
| Lys | Lys, ornithine, Arg, diaminopropionic acid, HArg, His |

**[0081]** Deletions or insertions of amino acids may also be introduced into the defined sequences provided they do not alter the biological functions of said sequences. Preferentially such insertions or deletions should be limited to 1, 2, 3, 4 or 5 amino acids and should not remove or physically disturb or displace amino acids which are critical to the functional conformation. Muteins of the peptides or polypeptides described herein may have

a sequence homologous to the sequence disclosed in the present specification in which amino acid substitutions, deletions, or insertions are present at one or more amino acid positions. Muteins may have a biological activity that is at least 40%, preferably at least 50%, more preferably 60-70%, most preferably 80-90% of the peptides described herein. However, they may also have a biological activity greater than the peptides specifically exemplified, and thus do not necessarily have to be identical to the biological function of the exemplified peptides. Analogues of targeting peptides also include peptidomimetics or pseudopeptides incorporating changes to the amide bonds of the peptide backbone, including thioamides, methylene amines, and E-olefins. Also peptides based on the structure of a targeting peptide or its peptide analogues with amino acids replaced by N-substituted hydrazine carbonyl compounds (also known as aza amino acids) are included in the term analogues as used herein.

**[0082]** The targeting peptide may be attached to the linker via the N or C terminus or via attachment to the epsilon nitrogen of lysine, the gamma nitrogen or ornithine or the second carboxyl group of aspartic or glutamic acid.

**[0083]** Most preferably, the targeting peptide may be LHRH, insulin, oxytosin, somatostatin, NK-1, VIP, GRP, bombesin or any other hormone peptides known in the art, as well as analogs and derivatives thereof. For a discussion of other targeting peptides that are useful in the present invention, *see*, *e.g.*, WO 04/062574, incorporated herein by reference in its entirety.

Constructs of the Present Invention

**[0084]** Table 1, Table 2 and Table 2a list conjugates of the present invention complexed with Gd. The complexes are divided into two "classes" based on the structure of the HSA binding portion of the conjugates that were evaluated.

**[0085]** The first class includes conjugates of the formula: **chelator-linker L1- hydrophobic AA**, complexed with Gd and the tested compounds in this class are listed in Table 1. "Class 2" includes compounds having the formula: **chelator-linkerL1-hydrophobic non-AA**, complexed with Gd and the tested compounds in this class are listed in Tables 2 and 2a. The distinction between the linkerL1 and the hydrophobic AA or hydrophobic non-AA is somewhat formalistic as it was understood that all of these might contribute to binding of the constructs to HSA. The tables also include and evaluation of the enhanced relaxivity of the

constructs. The Examples provide details regarding the synthesis, screening and evaluation of representative compounds of the two classes.

**[0086]** The following explains the column headings in Table 1 and Table 2:

**[0087] Chelate class** refers to the type of chelating function that is present in the compound: S stands for standard, and ER stands for the chelating moieties expected to provide enhanced T1 relaxivity by comparison with the standard chelating function. Not all cases provided enhanced T1 relaxivity, by comparison with their standard chelator analogs..

**[0088] The parameter $g_i$** refers to the relaxation gain, which is the ratio of the T1 relaxivity of the substance in a solution of HSA called Seronorm™ (commercially available from Nycomed-Amersham) to the relaxivity of the same compound in water at a specified concentration. Values close to 1 indicate that no binding to protein is occurring and that the compound is unsuitable. On the other hand, higher values (generally, about 3 and above) are more desirable because they highlight, in an MRI image, the bloodstream (intravascular compartment) due to the much higher T1 relaxivity (which is the source of the MRI signal) when bound to the HSA in the blood.

**[0089] The parameter $r1_S$** is the T1 relaxivity of the substance in a solution of human serum albumin called Seronorm™ (commercially available from Nycomed-Amersham).

**[0090] The parameter $r1_w$** is the T1 relaxivity of the compound in water, where no protein is present.

**[0091] The parameter $F_b$** is the fraction of the compound bound to human serum albumin (HSA) under standard conditions of defined concentration and temperature. A value of 0.90 indicates that 90% of the molecules of the test compound are bound to HSA. A higher fraction bound ($F_b$) means that more of the compound should provide the higher signal characteristic of the bound molecule due to immobilization of the chelating function, or significant slowing of the chelating function upon binding of the chelate-HSA binding ligand to the HSA. Compounds in Class 1 and Class 2 were evaluated, and the results are as follows. The abbreviations used in these tables and throughout the application are known to those skilled in the art and/or set forth herein, particularly in the sections regarding chealators, linking groups and hydrophobic groups.

**Table 1: Gd Complexes of Conjugates of the present invention in Class 1: Chelator-Linker-Hydrophobic Amino Acid**

| Compound | Chelate Class | $g_i$ | r1 Seronorm | $r1_W$ | $F_b$ |
|---|---|---|---|---|---|

| Compound | Chelate Class | $g_i$ | r1 Seronorm | $r1_W$ | $F_b$ |
|---|---|---|---|---|---|
| Gd(CM-DOTMA)-GaB 2Na | ER | 5.1 | 30.7 | 6.01 | 0.87 |
| Gd(CM-DOTMA)-aB Meg | ER | 6.04 | 35.57 | 5.89 | 0.83 |
| Gd(CM-DOTMA)-aB Na | ER | 6.18 | 36.02 | 5.82 | 0.85 |
| Gd(DO3A10CM)Ga(d,l)B Meg[b] | S | 4.25 | 21.14 | 4.97 | 0.86 |
| Gd(DO3A10CM)Ga(d)B Meg[b] | S | 4.29 | 21.51 | 5.01 | 0.82 |
| Gd(DO3A10CM)Ga[N-(4-phenyl)benzyl]Gly Meg[b] | S | 4.23 | 21.78 | 5.15 | 0.85 |
| Gd-DO3A10CM-D-a-B | S | 3.36 | 24.83 | 7.38 | 0.76 |
| Gd-DO3A10CM-G-L-B | S | 3.99 | 20.4 | 5.11 | 0.65 |
| Gd(DO3A10CM)Ga(l)B Meg[a] | S | 4.12 | 21.71 | 5.27 | 0.81 |
| Gd-DO3A10CM-G-G-$N^{in}$-2,4,6-trimethylbenzoyl-Trp | S | 4.7 | 21.83 | 4.65 | 0.82 |
| Gd-DO3A10CM-G-G-$N^{in}$-2,4,6-trimethylbenz-enesulfonyl-Trp | S | 4.46 | 20.14 | 4.52 | 0.89 |
| Gd(DO3A10CM)aB Meg | S | 3.35 | 17.45 | 5.22 | 0.76 |
| Gd-4-CEMP-DTTA-GaB Meg | ER | 4.77 | 33.71 | 7.06 | 0.95 |
| Gd-7MP-DO3A-GaB Meg | ER | 5.55 | 33.95 | 6.12 | 0.89 |
| Gd(CEMP-DO3A)-GaB Meg | ER | 3.43 | 16.1 | 4.7 | 0.87 |
| Gd(CEMP-DO3A)-GaB | ER | 3.42 | 14.39 | 4.21 | 0.92 |
| Gd(CE-DTPA)-aB 3Meg | S | 5.67 | 33.3 | 5.87 | 0.91 |
| Gd-TE-DOTA-aB Meg | ER | 6.08 | 42.09 | 6.92 | 0.96 |
| Gd-TE-DOTA Meg | ER | 1.63 | 7.01 | 4.29 | 0.01 |
| Gd(CM-DOTA)-aB Meg | ER | 6.4 | 30.36 | 4.75 | 0.95 |
| Gd(CE-DTPA)-GaB 3Meg | S | 3.26 | 21.41 | 6.56 | 0.88 |
| Gd-DO3A10CM-Ga-(p-Cl)-Bip Meg[c] | S | 4.46 | 24.12 | 5.41 | 0.89 |
| Gd-7MPCMDO3A-aB Meg | ER | 3.93 | 20.1 | 5.12 | 1 |

**Table 2: Gd Complexes of Conjugates of the present invention in Class 2: Structure Chelator-Linker-Hydrophobic Acyl Group**

| Compound | Chelate Class | $g_i$ | r1 Seronorm | $r1_W$ | $F_b$ |
|---|---|---|---|---|---|
| [(Fl-1)-K[G-(Gd-DO3A10CM)]-OH](Meg) | S | 4.68 | 22.4 | 4.79 | 0.91 |

| Compound | Chelate Class | $g_i$ | r1 Seronorm | $r1_W$ | $F_b$ |
|---|---|---|---|---|---|
| [(Fl-1)-K[G-(Gd-DO3A10CM)]-O-](Na$^+$) | S | 4.08 | 24.15 | 5.91 | 0.91 |
| Gd-CE-DTPA-(Fl-9)-OH Meg | S | 4.57 | 23.9 | 5.23 | 0.61 |
| Gd(CEDTPA)-K(Fl-9)OH Meg | S | 4.28 | 24.59 | 5.74 | 0.73 |
| Gd(DO3A10CM)-GK-(Fl-9) –OH Meg[a] | S | 4.54 | 25.08 | 5.52 | 0.84 |
| Gd(DO3A10CM)-GK(Fl-9)-OH Meg[a] | S | 5.31 | 26.32 | 4.95 | 0.79 |
| Gd-DO3A10CM-G-K-(3,5-di-t-butylbenzoyl) | S | 3.87 | 17.06 | 4.4 | 0.86 |
| Gd-DO3A10CM-G-K(4-n-pentylcyclohexanoyl) | S | 3.67 | 16.45 | 4.49 | 0.86 |
| Gd-DO3A10CM-K-(Fl-1)-OH Meg | S | 5.44 | 27.49 | 5.06 | 0.91 |
| Gd-DO3A10CM-K-(Fl-1)O$^-$ Na$^+$ | S | 5.75 | 27.68 | 4.81 | 0.9 |

**[0092]** The following shows the hydrophobic R groups of carboxylic acids that were selected for preparation and testing as the R (hydrophobic non-amino acid) portion of conjugates of the invention :

**Structures of Selected Hydrophobic R Groups Constituting the Class 2 Compound Library**

GENERAL STRUCTURE

R=

1 2 3 4 5 6

7 8 9 10 11 12

13 14 15 16 17 18

19 20 21 22 23

24 25 26 27 28

*** Indicates point of attachment of R in the general structure.**

**[0093]** Table 2b shows the primary screening results for the Gd complexes of conjugates of the invention in Class 2 with the structure chelator-linker-hydrophobic carboxylic acid. In

particular, the listed conjugates of the general structure DO3A10CM-G-K-hydrophobic carboxylic acid were prepared and evaluated. The "Group Number" refers to the groups as labeled in the chart above.

**Table 2a. Gd Complexes of Conjugates of the present invention in Class 2: Structure Chelator-Linker-Hydrophobic Carboxylic Acid**

| Compound Name | R Group Number | $g_i$ | $r1_S$ | $r1_W$ | $F_b$ |
|---|---|---|---|---|---|
| Gd(DO3A10CM)GK-27 | 27 | 4.94 | 24.62 | 4.98 | 0.68 |
| Gd(DO3A10CM)GK-21 | 21 | 4.42 | 20.89 | 4.73 | 0.79 |
| Gd(DO3A10CM)GK-22 | 22 | 3.87 | 17.06 | 4.40 | 0.86 |
| Gd(DO3A10CM)GK-13 | 13 | 3.67 | 16.45 | 4.49 | 0.86 |
| Gd(DO3A10CM)GK-24 | 24 | 3.51 | 17.49 | 4.98 | 0.73 |
| Gd(DO3A10CM)GK-7 | 7 | 3.46 | 16.04 | 4.64 | 0.66 |
| Gd(DO3A10CM)GK-14 | 14 | 3.39 | 18.55 | 5.46 | 0.65 |
| Gd(DO3A10CM)GK-25 | 25 | 3.39 | 21.48 | 6.33 | 0.53 |
| Gd(DO3A10CM)GK-11 | 11 | 3.28 | 15.46 | 4.72 | 0.55 |
| Gd(DO3A10CM)GK-23 | 23 | 3.24 | 15.28 | 4.71 | 0.60 |
| Gd(DO3A10CM)GK-5 | 5 | 3.24 | 16.17 | 4.99 | 0.58 |
| Gd(DO3A10CM)GK-10 | 10 | 3.01 | 14.47 | 4.81 | 0.38 |
| Gd(DO3A10CM)GK-26 | 26 | 2.98 | 14.99 | 5.03 | 0.46 |
| (i) d(DO3A10CM)GK-4 | 4 | 2.93 | 16.03 | 5.48 | 0.50 |
| Gd(DO3A10CM)GK-18 | 18 | 2.58 | 11.96 | 4.64 | ND† |
| Gd(DO3A10CM)GK-12 | 12 | 2.50 | 11.26 | 4.51 | ND |
| Gd(DO3A10CM)GK-9 | 9 | 2.39 | 13.85 | 5.80 | 0.30 |
| Gd(DO3A10CM)GK-19 | 19 | 2.37 | 13.57 | 5.74 | ND |
| Gd(DO3A10CM)GK-6 | 6 | 2.32 | 10.03 | 4.32 | ND |
| Gd(DO3A10CM)GK-17 | 17 | 2.30 | 10.73 | 4.66 | ND |
| Gd(DO3A10CM)GK-15 | 15 | 2.09 | 9.78 | 4.68 | ND |
| Gd(DO3A10CM)GK- | 16 | 2.08 | 9.60 | 4.63 | ND |

| | | | | | |
|---|---|---|---|---|---|
| 16 | | | | | |
| Gd(DO3A10CM)GK-28 | 28 | 1.92 | 9.98 | 5.20 | ND |
| Gd(DO3A10CM)GK-8 | 8 | 1.90 | 10.85 | 5.72 | ND |
| Gd(DO3A10CM)GK-3 | 3 | 1.80 | 8.62 | 4.78 | ND |
| Gd(DO3A10CM)GK-2 | 2 | 1.67 | 7.85 | 4.71 | ND |
| Gd(DO3A10CM)GK-1 | 1 | 1.52 | 7.28 | 4.78 | ND |

† ND = not determined

**[0094]** Conjugates DO3A10CM-D-a-B; DO3A10CM-G-a-B(Meg); DO3A10CM-G-L-B; CM-DOTMA-a-B; CE-DTPA-a-B; 7MP-DO3A-G-a-B and CM-DOTA-a-B, optionally conjugated to a second linker L2 and/or a targeting vector Q are preferred. CM-DOTMA-a-B; CE-DTPA-a-B; 7MP-DO3A-G-a-B and CM-DOTA-a-B, optionally conjugated to a second linker L2 and/or a targeting vector Q are particularly preferred. As described herein such conjugates may be complexed with a metal, or a paramagnetic metal such as for example, Gd, or others described above or with a radionuclide from amongst those described above.

**[0095]** Compounds of the invention with fractions bound to serum of at least 0.87 are particularly preferred. A higher fraction bound to serum will result in a longer lifetime in blood, thus providing a more persistant signal. Similarly, compound so f the invention with higher relaxivity values are also particularly preferred. Higher relaxivity value will result in more signal for a given compound after administration. Table 6 in Example 8 shows fraction bound to serum and relaxivity data collected for representative compounds of the present invention compared to a reference compound, MS-325, whose structure is presented below. As shown in Table 6, Gd-(TE-DOTA)-aB Meg provides a relaxivity ($r_1$) in Seronorm™ (HSA) of 42.09, well above the comparator MS-325 ($r_1$ Seronorm = 36.57). Such a high relaxivity will provide more signal for a given compound after administration. Furthermore, Gd-(TE-DOTA)-aB Meg provides an $F_b$ of 0.996, well above the $F_b$ of the comparator MS-325 ($F_b$ = 0.87)

MS-325 (Gadofosveset Trisodium)

**MS-325**

**[0096]** As further shown in Table 6, the following compounds each provide higher bound fractions than the MS325 comparator: Gd-(10CM-DO3A)-G-G-W-(Nin-2,4,6-trimethylbenzenesulfonyl)-$O^-$ Meg; Gd-(10CM-DO3A)-aaB a; Gd-(10CM-DO3A)-aak a,b; Gd-(10CM-DO3A)-DDk a; Gd-(10CM-DO3A)-EEB a,b; Gd-(10CM-DO3A)-Pak a,c; Gd-(10CM-DO3A)-Pak a,b,c; Gd-(10CM-DO3A)-Dak a,b meg; Gd-(10CM-DO3A)-DDj a,c; Gd-(10CM-DO3A)-DDX c; Gd-(10CM-DO3A)-Paj a,c; Gd-(10CM-DO3A)-aaX; Gd-(10CM-DO3A)-GGm Meg; Gd-(10CM-DO3A)-Gao Meg; Gd-(10CM-DO3A)-GGo Meg a; Gd-(10CM-DO3A)-Pao Meg; Gd-(10CM-DO3A)-Pam Meg; Gd-(10CM-DO3A)-Gam Meg a; Gd-(10CM-DO3A)-aao Meg a; Gd-(10CM-DO3A)-aam Meg a;Gd-(10CM-DO3A)-Gan Meg; Gd-(10CM-DO3A)-Ga-(p-Cl)-Bip; Gd-(CEMP-DO3A)-GaB; Gd-(CEMP-DO3A)-aB Na; Gd-4-CEMP-DTTA-GaB Meg; Gd-(CE-DTPA)-GaB 3Meg; Gd-(CE-DTPA)-aB 3Na; Gd-(CE-DTPA)-aB 3Meg; Gd-(CM-DOTA)-aB Meg; Gd-(CM-DOTA)-aB Na; Gd-(7MP-DO3A)-GaB; Gd-(7MP-DO3A)-GaB Meg; Gd-(7MP-CM-DO3A)-aB Meg; Gd-(TE-DOTA)-aB Meg; Gd-(10CM-DO3A)-K-(1-fluorenecarboxamido)O- Na+; Gd-(10CM-DO3A)-K-(1-fluorenecarboxamido)- OH Meg; [(Fl-1)-K{G-[Gd-(10CM-DO3A)]}-OH](Meg); and [(Fl-1)-K[G-{Gd-(10CM-DO3A)]}-O-](Na+).

Uses of Conjugates of the Invention

**[0097]** The conjugates of the present invention are useful for to detect and treat various medical conditions. For example, the conjugates of the present invention can be employed for diagnostic imaging. In particular, because the conjugates bind a blood protein such as

HSA they are useful for prolonged imaging of the intravascular space, or simply the vasculature, because of their ability to be confined to that region for longer periods of time. Thus compounds of the present invention are useful for imaging the vascular space by, for example, MRI or nuclear medicine imaging, depending on the type of metal complexed with the chelator. Methods of the invention include the steps of administering to a patient a pharmaceutical preparation that includes a conjugate of the invention complexed with a metal or a radionuclide and imaging the compound after administration to the patient. In a preferred embodiment, the imaging step includes magnetic resonance imaging, or nuclear imaging. In these methods, the administering step may include, *e.g.*, inhalation, transdermal absorption, intramuscular injection, subcutaneous injection, intravenous injection, intraperitoneal injection, intraarterial injection or parenteral administration.

**[0098]** Even in the case where a fraction of the compound is in the extravascular space, it is still possible to image the vasculature, because the extravascular space contains little HSA. Therefore, for conjugates useful for MRI (e.g. those complexed with a paramagnetic metal ion ), the T1 relaxivity, r1, of the compound in the extravascular space will be similar to that found in water, whereas the r1 of the compound in the vascular space, which contains HSA, will be high because the compound is bound to HSA in that region.

**[0099]** In one embodiment, conjugates of the present invention which are useful for MRI (e.g. those that are complexed with a paramagnetic metal ion) are useful as CVMRA agents in therapeutic and diagnostic applications. The invention further includes novel methods for preparation of such agents.

**[00100]** In one embodiment, the conjugates of the present invention useful for MRI exhibit superior relaxivity. The term $r1_S$ is defined as the relaxivity of the compound measured in Seronorm™ (a commercially available preparation of human serum albumin) at 20 MHz in $mM^{-1}s^{-1}$. The term $r1_w$ is defined as the relaxivity of the compound measured in water at 20 MHz in $mM^{-1}s^{-1}$. Agents of the invention were shown to have superior properties using the methods of parallel synthesis and mass primary screening of candidate compounds, as described in the Examples.

**[00101]** The total signal in blood symbolized as $\mathbf{S_{blood}}$ is a function of the relaxivity-concentration product for the bound and unbound test compound in the blood. Expressing this as a function of $\mathbf{r1_w}$ gives:

$$\mathbf{S_{blood} = (1-F_b)*r1_w*C_{blood} + g_i*r1_w*F_b*C_{blood}} \qquad \text{(I)}$$

where:

- $\mathbf{F_b}$ is the fraction of the construct bound to protein in blood

- $\mathbf{r1_w}$ is the T1 relaxivity of the test compound measured in water at a standard concentration of the test compound

- $\mathbf{r1_S}$ is the T1 relaxivity of the test compound measured in Seronorm solution at a standard concentration of the test compound

- $\mathbf{C_{blood}}$ is the concentration of the test compound in blood

- $\mathbf{g_i}$ is the standard relaxation gain or the relaxation enhancement defined as the ratio r1s/r1w under a defined standard of concentration and temperature.

**[00102]** The first term of the addition equation, $(1-\mathbf{F_b})*\mathbf{r1_w}*\mathbf{C_{blood}}$, gives the contribution of the *unbound* compound to the total signal in blood. The second term, $\mathbf{g_i}*\mathbf{r1_w}*\mathbf{F_b}*\mathbf{C_{blood}}$, which contains the multiplier $\mathbf{g_i}$, gives the contribution of the HSA-bound compound to the total signal in blood. It can be seen that when $\mathbf{F_b}$ is high, most of the signal in the blood is due to the bound fraction of the compound. This signal is magnified by the factor of $\mathbf{g_i}$ (the relaxation gain). This parameter is also referred to as the enhanced relaxivity or relaxation enhancement which is realized upon binding of the contrast agent to the macromolecule. In the regime of MRI imaging field strengths generally employed (0.3 T to 1.5 T) this increase of relaxivity is realized by a significant increase in the rotational correlation time $\tau_r$, which has a effect dominant effect on the T1 relaxation rate of protons by the gadolinium atom in the MRI contrast agent.

**[00103]** For example, if the compound is 80% bound ($\mathbf{F_b}$ = 0.8) to HSA in blood, most of the gadolinium atoms in the solution and their associated bound water molecules are rotating at a significantly reduced rate (longer $\tau_r$). This leads to an increase in the relaxation rate of the associated water and the water in the inner and out sphere of the gadolinium atoms of the bound chelate. If the relaxation gain $\mathbf{g_i}$ is assumed to be 3.0, which is a modest value

(see **Table 1**, infra) and $\mathbf{r1_w} = 6$ (an arbitrarily chosen value), substitution in the equation above gives:

$$\mathbf{S_{blood}} = (1-0.8)*6*\mathbf{C_{blood}} + 3.0*6*0.8*\mathbf{C_{blood}}$$

Further combining terms gives:

$$\mathbf{S_{blood}} = 1.2*\mathbf{C_{blood}} + 14.4*\mathbf{C_{blood}} = 15.6\mathbf{C_{blood}}$$

**[00104]** Now the ratio of the signal due to the bound fraction of the test molecule in blood to the unbound fraction is 14.4/1.2 = 12 (the concentration terms cancel). By comparison, the available signal with a compound of the same concentration in blood that does <u>not</u> bind HSA and possesses the same $\mathbf{r1_W}$ is:

$$\mathbf{S_{blood}} = (1-0)*\mathbf{r1_W}*\mathbf{C_{blood}}$$

because $\mathbf{F_b}$ is 0 (the compound is 0% bound) and the second term is therefore zero.

Hence,

$$\mathbf{S_{blood}} = 6*\mathbf{C_{blood}}$$

**[00105]** The ratio of the signal for the two compounds is, therefore:

$$\mathbf{S_{protein\ binding\ compound}}/\mathbf{S_{protein\ non\text{-}binding\ compound}} = 15.5\mathbf{C_{blood}}/6\mathbf{C_{blood}} = 2.583 \text{ or } 258\%$$

This ratio is time dependent and, except in the period immediately after administration, is a likely underestimate (the concentration of the non-protein binding compounds in blood is reduced versus their protein binding congeners with time).

**[00106]** Thus, it can be seen that there are many advantages of the blood protein-bound conjugates of the invention, including confinement to the vascular compartment for a longer period, and the higher signal strength available due to protein binding of the chelate functionalized protein binding molecule.

**[00107]** In a preferred embodiment of the present invention, the compound is first administered, and clearance of unbound compound from blood is then allowed to occur

before the vascular space is imaged. However, this process is not always strictly necessary. In the case where imaging is conducted after a waiting period, the administrator must account for the fraction of compound that may migrate to the extravascular space, which also contains HSA.

**[00108]** If the concentration of the compound in the extravascular space were to be equal to that in the intravascular space (an unlikely circumstance), both areas would provide signal in the MRI, and the ability to distinguish the extravascular space from the intravascular space might thus be diminished. However, even in such a case, signal intensity in the intravascular space would still be greater in most instances. This is because the concentration of HSA in the extravascular spaces (which has been measured) is generally between 10-16 g/L, except in the gut, where it is 25-30 g/L. These values should be compared with the value of ~25g/L in the vascular space (which includes both the volume of plasma and the other blood components, *e.g.*, erythrocytes and white cells). Therefore, independent of the fractional distribution of the compound in the vascular space versus the extravascular space, the present invention provides an advantage for signal strength in the blood due to the higher concentration of HSA in blood, except in the case of the gut.

**[00109]** Imaging according to the present invention should preferably be conducted at time point early enough to allow binding of the constructs of the present invention to intravascular HSA (on the order of one circulation time, *i.e.* 1-2 min) and clearance of unbound compound to the extravascular space ($T_{1/2}$ ~ 16 min). Both of these rates of clearance are much faster than the rate of clearance of intravascular HSA (with or without the molecule bound) to the extravascular space (4%/h).

**[00110]** Thus, utilizing the conjugates of the invention diagnostic imaging methods, and in particular, MRI imaging, can be employed to provide morphological imaging of the vasculature for detection of a variety of conditions, including, for example, vascular abnormalities, stenosis or even sites of angiogenesis that can be characterized. The morphology and permeability of tumor vasculature can be similarly assessed in accordance with the present invention. The constructs of the present invention are applicable to MRI; they are also applicable to other forms of imaging such, as *e.g.*, ultrasound imaging, wherein metal chelators facilitate the process, and/or light imaging; and additionally to other diagnostic and therapeutic applications that may be contemplated by those skilled in the art.

**[00111]** The conjugates of the present invention are applicable to other modes of diagnostic imaging as well as to therapeutic applications. For example, where the conjugates of the invention are complexed with metals having different properties than, for example,

paramagnetic metals, other applications can be contemplated by those of ordinary skill in the art. For example, the HSA- binding portion of the molecule may be used to prolong the residence time in blood of the constructs such as, *e.g.*, targeted pharmaceuticals, including therapeutics and diagnostics. Also, radiometals may be chelated by the chelating function and a construct that utilizes the HSA binding sequence and the chelator bound to a targeting function which directs the construct to tumor sites or to other diseased tissues. The radiometal may also be useful for diagnosis or radiotherapy. Because the conjugates of the invention bind to a blood protein such as HAS, the blood residence time is prolonged, allowing more opportunity for binding of the radiopharmaceutical to the target site.

**[00112]** The present invention also contemplates the delivery of other compounds such as, *e.g.*, chemotherapeutics. For example, a conjugate of the invention which includes a targeting vector that targets a specific type of tumor can be linked to a chemotherapeutic. Thus, the conjugate, by virtue of the fact that it binds to the intravascular HSA, can prolong the blood residence time of the construct. Such an increase of blood residence time is advantageous for many reasons; for example, it allows more opportunity for binding of the conjugate to the target site because the HSA-bound construct is essentially in a depot form, where it is slowly released to the target, as opposed to being cleared in a first pass clearance.

**[00113]** The constructs of the present invention may also be used to target genetic material to specific cells. The conjugates of the invention may further include genetic material and may be used to localize genetic material to cells or tissue containing the desired target. Thus such constructs may be useful in gene therapy. The genetic material may include nucleic acids, such as RNA or DNA, of either natural or synthetic origin, including recombinant RNA and DNA and antisense RNA and DNA. Types of genetic material that may be used include, for example, genes carried on expression vectors such as plasmids, phagemids, cosmids, yeast artificial chromosomes (YACs) and defective or “helper” viruses, antigene nucleic acids, both single and double stranded RNA and DNA and analogs thereof, such as phosphorothioate and phosphorodithioate oligodeoxynucleotides. Additionally, the genetic material may be combined, for example, with lipids, proteins or other polymers. Delivery vehicles for genetic material may include, for example, a virus particle, a retroviral or other gene therapy vector, a liposome, a complex of lipids (especially cationic lipids) and genetic material, a complex of dextran derivatives and genetic material, etc.

**[00114]** The present invention is directed, in another embodiment, to novel methods for preparing and using such conjugates in diagnostic and therapeutic applications.

Magnetic Resonance Imaging (MRI)

**[00115]** MRI contrast agents including conjugates of the invention may be used in the same manner as conventional MRI contrast reagents. When imaging target-containing tissue such as, for example, a site of angiogenesis, certain MR techniques and pulse sequences may be preferred to enhance the contrast of the site to the background blood and tissues. These techniques include (but are not limited to), for example, black blood angiography sequences that seek to make blood dark, such as fast spin echo sequences (*see, e.g.*, Alexander *et al.*, *Magnetic Resonance in Medicine*, 40(2): 298-310 (1998)) and flow-spoiled gradient echo sequences (*see*, *e.g.*, Edelman et al., *Radiology*, 177(1): 45-50 (1990)). These methods also include flow independent techniques that enhance the difference in contrast, such as inversion-recovery prepared or saturation-recovery prepared sequences that will increase the contrast between target containing tissue, such as an angiogenic tumor, and background tissues. Finally, magnetization transfer preparations may also improve contrast with these agents (*see, e.g.*, Goodrich *et al.*, *Investigative Radiology*, 31(6): 323-32 (1996)).

**[00116]** The labeled reagent may be administered to the patient in the form of an injectable composition. The method of administering the MRI contrast agent is preferably parenterally, meaning intravenously, intraarterially, intrathecally, interstitially, or intracavitarilly. For imaging active angiogenesis, intravenous or intraarterial administration is preferred.

**[00117]** For MRI, it is contemplated that the subject will receive a dosage of contrast agent sufficient to enhance the MR signal at the target (*e.g.*, a site of angiogenesis) at least 10%. After injection of the composition including the MRI reagent, the patient is scanned with a MRI machine to determine the location of any sites containing the target. In therapeutic settings, upon target localization, a cytotoxic or therapeutic agent can be immediately administered, if necessary, and the patient can be subsequently scanned to determine the location of any targeted sites.

Nuclear Imaging (Radionuclide Imaging) and Radiotherapy

**[00118]** The present invention may also be useful in the field of nuclear medicine. Conjugates of the present invention may be complexed with a radioactive metal or other radionuclide appropriate for scintigraphy, SPECT or PET imaging or with a radionuclide appropriate for radiotherapy.

**[00119]** For use as a PET agent, a conjugate of the present invention may be complexed with one of the various positron emitting metal ions, such as $^{51}Mn$, $^{52}Fe$, $^{60}Cu$, $^{68}Ga$, $^{72}As$, $^{94m}Tc$, or $^{110}In$. The constructs of the present invention can also be labeled by

halogenation using radionuclides, such as $^{18}F$, $^{124}I$, $^{125}I$, $^{131}I$, $^{123}I$, $^{77}Br$, and $^{76}Br$. Preferred metal radionuclides for scintigraphy or radiotherapy include $^{99m}Tc$, $^{51}Cr$, $^{67}Ga$, $^{68}Ga$, $^{47}Sc$, $^{51}Cr$, $^{167}Tm$, $^{141}Ce$, $^{111}In$, $^{168}Yb$, $^{175}Yb$, $^{140}La$, $^{90}Y$, $^{88}Y$, $^{153}Sm$, $^{166}Ho$, $^{165}Dy$, $^{166}Dy$, $^{62}Cu$, $^{64}Cu$, $^{67}Cu$, $^{97}Ru$, $^{103}Ru$, $^{186}Re$, $^{188}Re$, $^{203}Pb$, $^{211}Bi$, $^{212}Bi$, $^{213}Bi$, $^{214}Bi$, $^{105}Rh$, $^{109}Pd$, $^{117m}Sn$, $^{149}Pm$, $^{161}Tb$, $^{177}Lu$, $^{198}Au$ and $^{199}Au$. The choice of metal or halogen will be determined based on the desired therapeutic or diagnostic application. For example, for diagnostic purposes the preferred radionuclides include $^{64}Cu$, $^{67}Ga$, $^{68}Ga$, $^{99m}Tc$, and $^{111}In$. For therapeutic purposes, the preferred radionuclides include $^{64}Cu$, $^{90}Y$, $^{105}Rh$, $^{111}In$, $^{117m}Sn$, $^{149}Pm$, $^{153}Sm$, $^{161}Tb$, $^{166}Dy$, $^{166}Ho$, $^{175}Yb$, $^{177}Lu$, $^{186/188}Re$, and $^{199}Au$.

**[00120]** Preferably, a radioactive lanthanide, such as, for example, $^{177}Lu$, $^{90}Y$, $^{153}Sm$, $^{111}In$, or $^{166}Ho$ is used with DOTA or DO3A in radiopharmaceutical conjugates of the invention.

**[00121]** $^{99m}Tc$ is preferred for diagnostic applications because of its low cost, availability, imaging properties, and high specific activity. The nuclear and radioactive properties of $^{99m}Tc$ make this isotope an ideal scintigraphic imaging agent. This isotope has a single photon energy of 140 keV and a radioactive half-life of about 6 hours, and is readily available from a $^{99}Mo$-$^{99m}Tc$ generator. Other appropriate radionuclides for preferred embodiments of the present invention will be easily determined by one of ordinary skill in the art.

**[00122]** The metal radionuclides may be chelated by any of the chelating moieties discussed herein, *supra*. One skilled in the art can select an appropriate chelator for a desired radionuclide.

**[00123]** Complexes of radioactive technetium are particularly useful for diagnostic imaging and complexes of radioactive rhenium are particularly useful for radiotherapy. In forming a complex of radioactive technetium with the reagents of this invention, the technetium complex, preferably a salt of $^{99m}Tc$ pertechnetate, is reacted with the reagent in the presence of a reducing agent. Preferred reducing agents are dithionite, stannous and ferrous ions; the most preferred reducing agent is stannous chloride. Means for preparing such complexes are conveniently provided in a kit form comprising a sealed vial containing a predetermined quantity of a reagent of the invention to be labeled and a sufficient amount of reducing agent to label the reagent with $^{99m}Tc$. Alternatively, the complex may be formed by reacting a conjugate of the invention with a pre-formed labile complex of technetium and another compound known as a transfer ligand. This process is known as ligand exchange and is well known to those skilled in the art. The labile complex may be formed using such

transfer ligands as tartrate, citrate, gluconate or mannitol, for example. Among the $^{99m}$Tc pertechnetate salts useful with the present invention are included the alkali metal salts such as the sodium salt, or ammonium salts or lower alkyl ammonium salts.

**[00124]** Preparation of the complexes of the present invention where the metal is radioactive rhenium may be accomplished using as starting materials rhenium compounds wherein the metal is in the +5 or +7 oxidation state. Examples of compounds in which rhenium is in the Re(VII) state are $NH_4ReO_4$ or $KReO_4$. Re(V) is available as, for example, $[ReOCl_4](NBu_4)$, $[ReOCl_4](AsPh_4)$, $ReOCl_3(PPh_3)_2$ and as $ReO_2(pyridine)_4^+$ (Ph is phenyl; Bu is n-butyl). Other rhenium reagents capable of forming a rhenium complex may also be used.

**[00125]** Radioactively-labeled scintigraphic imaging agents provided by the present invention must contain a suitable amount of radioactivity. In forming $^{99m}$Tc complexes, it is generally preferred to form radioactive complexes in solutions containing radioactivity at concentrations of from about 0.01 millicurie (mCi) to 100 mCi per mL.

**[00126]** Generally, the unit dose to be administered has a radioactivity of about 0.01 mCi to about 100 mCi, preferably 1 mCi to 20 mCi. The solution to be injected at unit dosage is from about 0.01 mL to about 10 mL.

**[00127]** After injection of the radionuclide imaging agent into the patient, a PET camera or a gamma camera calibrated for the gamma ray energy of the nuclide incorporated in the imaging agent is used to image areas of uptake of the agent and quantify the amount of radioactivity present in the site. Imaging of the site *in vivo* can take place in a matter of a few minutes. However, imaging can take place, if desired, in hours or even longer, after the radiolabeled peptide is injected into a patient. In most instances, a sufficient amount of the administered dose will accumulate in the area to be imaged within about 0.1 of an hour to permit the taking of scintiphotos.

**[00128]** The radiotherapeutic and radiodiagnostic compositions of the invention can include physiologically acceptable buffers, and can require radiation stabilizers to prevent radiolytic damage to the compound prior to injection. Radiation stabilizers are known to those skilled in the art, and may include, for example, para-aminobenzoic acid, ascorbic acid, gentistic acid and the like.

**[00129]** The present invention also contemplates a single, or multi-vial kit that contains all of the components needed to prepare the radiopharmaceuticals of this invention, other than the radionuclide. A single-vial kit preferably contains a conjugate of the invention, a source of a stannous salt (if reduction is required, *e.g.,* when using technetium), or other

pharmaceutically acceptable reducing agent, and is appropriately buffered with pharmaceutically acceptable acid or base to adjust the pH to a value of about 3 to about 9. The quantity and type of reducing agent used would depend highly on the nature of the exchange complex to be formed. The proper conditions are well known to those who are skilled in the art. It is preferred that the kit contents be in lyophilized form. Such a single vial kit may optionally contain labile or exchange ligands such as glucoheptonate, gluconate, mannitol, malate, citric or tartaric acid and can also contain reaction modifiers such as diethylenetriamine-pentaacetic acid (DPTA), ethylenediamine tetraacetic acid (EDTA), or α-, β-, or γ-cyclodextrin that serve to improve the radiochemical purity and stability of the final product. The kit may also contain stabilizers, bulking agents such as mannitol, that are designed to aid in the freeze-drying process, and other additives known to those skilled in the art.

**[00130]** A multi-vial kit preferably contains the same general components but employs more than one vial in reconstituting the radiopharmaceutical. For example, one vial may contain all of the ingredients that are required to form a labile Tc(V) complex on addition of pertechnetate (*e.g.*, the stannous source or other reducing agent). Pertechnetate is added to this vial, and after waiting an appropriate period of time, the contents of this vial are added to a second vial that contains the conjugate of the invention, as well as buffers appropriate to adjust the pH to its optimal value. After a reaction time of about 5 to 60 minutes, the complexes of the present invention are formed. It is advantageous that the contents of both vials of this multi-vial kit be lyophilized. As above, reaction modifiers, exchange ligands, stabilizers, bulking agents, etc. may be present in either or both vials.

Pharmaceutical Compositions and Administration

**[00131]** Conjugates of the invention may be administered by any convenient route customary for diagnostic or therapeutic agents; *e.g.*, parenterally, enterally or intranasaly, and preferably by infusion or bolus injection, or by depot or slow release formulation. In a preferred embodiment, the composition may be formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Other pharmaceutically acceptable carriers include, but are not limited to, sterile water, saline solution, buffered saline (including buffers like phosphate or acetate), alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, paraffin, etc. Where necessary, the composition may also include a

solubilizing agent and a local anesthetic such as, *e.g.*, lidocaine to ease pain at the site of the injection; and further may include preservatives, stabilizers, wetting agents, emulsifiers, salts, lubricants, *etc*. as long as they do not react deleteriously with the active compounds. Similarly, the composition may comprise conventional excipients, *i.e.*, pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral or intranasal application which do not deleteriously react with the active compounds. Generally, the ingredients will be supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as, *e.g.*, an ampoule or sachette indicating the quantity of active agent in activity units. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade "water for injection" or saline. Where the composition is to be administered by injection, an ampoule of sterile water for injection or saline may be provided so that the ingredients may be mixed prior to administration.

## EXAMPLES

**[00132]** In the following examples, the synthesis and evaluation of several representative conjugates of the general formula **chelator-linkerL1-hydrophobic compound** is included. The conjugates and their Gd complexes were prepared and screened for enhanced relaxivity (ER). Points of interest for the experiments were the hydrophobic compound/HSA binder, the linker, and the gadolinium chelator.

### EXAMPLE 1 Synthesis and Screening of Gd-(DO3A10CM)-Ga(*l*)B Meg (Class 1)

**[00133]** The compound (final compound referred to as compound **(12)**) was prepared as follows:

Boc-NH COOH H — $Cs_2CO_3$, Benzyl bromide, DMF, RT, 15 h → BocNH $COOCH_2C_6H_5$ H

1 2

**[00134]** To a solution of *N*-*t*-butoxycarbonyl-L-biphenylalanine (**1**) (10.00 g, 30.55 mmol) in methanol-water (100 mL, 1:1) was added cesium carbonate (9.95 g, 30.54 mmol),

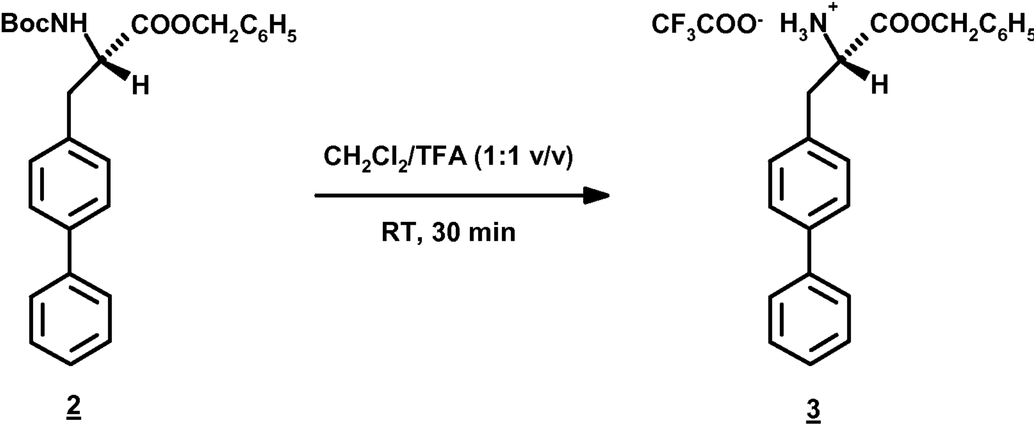
and the mixture was stirred for 20 min at room temperature. The reaction mixture was evaporated to dryness and the evaporation was continued with dry DMF (20 mL, twice) to dryness. The solid material was dissolved in dry DMF (100 mL), and benzyl bromide (5.75 g, 33.62 mmol) was added dropwise from an additional funnel. After the addition, the reaction mixture was stirred at room temperature for 15 h. The salts were removed by filtration, and the filtrate was evaporated to a paste which was taken-up in ethyl acetate (200 mL). The organic layer was washed with water (2 x 200 mL), dried ($Na_2SO_4$) and evaporated to a semi-solid which was then triturated with hexane to provide a colorless solid. The material was filtered, washed with hexane and dried to provide12.3 g (96.4% yield) of N-*t*-Butyloxycarbonyl-L-biphenylalanine benzyl ester **(2).**

**[00135]**

**HPLC:** $t_R$ 22.15 min Column: YMC C18, 4.6 mm x 250 mm, S-10P µm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 45% B, then linear gradient to 95% B over 25 min; Flow rate: 1.0 mL/min, Detection: UV @230 nm).

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.42 (s, 9 H, t-$C_4H_9$), 3.15 (m, 2 H, $CH_2$CH), 4.67 (m, 1 H, C*H*), 5.04 (d, 2 H, N*H*), 5.18 (dd, 2 H, $CH_2C_6H_5$) and 7.08-7.55 (m, 14 H, Ar*H*).

**MS:** 432.2 $(M+H)^+$.




**[00136]** To a solution of N-*t*-Butyloxycarbonyl-L-biphenylalanine benzyl ester **(2)** (10 g, 23.17 mmol) in $CH_2Cl_2$ (50 mL) was added phenol (1.5 g) followed by trifluoroacetic acid (50 mL), and the mixture was stirred at room temperature for 30 min. Volatiles were

removed by evaporation and the paste thus obtained was dissolved in ether (50 mL), and allowed to stand at room temperature. A white crystalline solid separated out and was filtered, washed with ether and dried. This provided 9.89 g (96% yield)of L-biphenylalanine benzyl ester trifluoroacetate salt (L-Bip-OBzl•TFA) **(3).**

**HPLC:** $t_R$ 8.12 min Column: YMC C18, 4.6 mm x 250 mm, S-10P μm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA);Elution: Initial condition: 45% B, linear gradient to 95% B over 25 min; Flow rate: 1.0 mL/min, Detection: UV @230 nm.

**$^1$H-NMR (500 MHz, DMSO-d$_6$):** δ 3.05-3.21 (2dd, 2 H, C$H_2$CH), 4.41 (t, 1 H, $CH_2CH$), 5.12 (dd, 2 H, C$H_2C_6H_5$), 7.21-7.65 (m, 14 H, Ar$H$) and 8.58 (bs, 3H, $NH_3^+$).

**MS:** 332.2 $(M+H)^+$.

CF3COO- H3N+ COOCH2C6H5 H BocNH H COOH H HATU, DIEA, CH2Cl2 RT, 2 h BocNH CONH COOCH2C6H5 H

3 4

**[00137]** A mixture of L-Bip-OBzl•TFA **(3)** (9.00 g, 20.21 mmol), HATU [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, PerSeptive Biosystems] (11.52 g, 30.32 mmol) and *trans*-4-N-*t*-butoxycarbonylaminomethyl-cyclohexanecarboxylic acid (Boc-a) (5.72 g, 21.23 mmol) in dichloromethane (30 mL) was added diisopropylethylamine (8.00 g, 61.90 mmol), and the solution was stirred at room temperature for 2 h. The reaction mixture was then evaporated on a rotary evaporator to remove dichloromethane and the residue was taken-up in ethyl acetate (200 mL). The organic layer was washed sequentially with dilute HCl (0.05 N, 2 x 200 mL), $NaHCO_3$ (5%, 2 x 200 mL) and water (2 x 200 mL). The organic layer was then dried ($Na_2SO_4$), filtered and evaporated to provide *trans*-4-N-*t*-butoxycarbonylaminomethylcyclohexane-carboxyl-L-biphenylalanine benzyl ester (Boc-a-Bip-OBzl) **(4)** (11.0 g ~ quantitative yield) as a light brown foamy solid.

**HPLC:** $t_R$ 20.14 min (Column: YMC-C18, 4.6 mm x 250 mm, S-10P µm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA);; Elution:I Initial condition: 45% B, then linear gradient to 95% B over 25 min; Flow rate: 1.0 mL/min, Detection: UV @ 230 nm).

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 0.92 (m, 2 H, cyclohex-C*H*$_2$), 1.45 (s & m, 11 H, *t*-$C_4H_9$ & cyclohex-C*H*$_2$), 1.60-2.09 (m, 6 H, cyclohex-C*H*$_2$), 2.95 (m, 2 H, bip-C*H*$_2$CH), 3.15 (m, 2 H, bocNHC*H*$_2$), 4.55 (m, 1 H, bip-α*H*), 4.95 (m, 1 H, bocN*H*), 5.18 (m, 2 H, C*H*$_2C_6H_5$), 5.92 (d, 1 H, CHN*H*CO) and 7.01-7.60 (m, 14 H, Ar*H*).

**MS:** 571.4 $(M+H)^+$

BocNH, H, CONH, COOCH$_2$C$_6$H$_5$, H, H — **4** —[$CH_2Cl_2$/TFA (1:1 v/v), RT, 30 min]→ $H_3N^+$, H, CONH, COOCH$_2$C$_6$H$_5$, H, H, $CF_3COO^-$ — **5**

**[00138]** A solution of Boc-a-Bip-OBzl **(4)** (11.00 g, 20.20 mmol) in dichloromethane (50 mL) was treated with phenol (1.5 g) followed by trifluoroacetic acid (50 mL), and the mixture was stirred at room temperature for 30 min. The volatiles were removed by rotary evaporation and the residue was triturated with ether. The solid was filtered and washed with ether to provide *trans*-4-aminomethylcyclohexanecarboxyl-L-biphenylalanine benzyl ester trifluoroacetate salt (a-Bip-OBzl•TFA) **(5)** in nearly quantitative yield (11.80 g) as a colorless solid..

**HPLC:** $t_R$ 9.50 min. Column: YMC C18 4.6 mm x 250 mm, S-10P µm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 45% B, then linear gradient to 95% B over 25 min; Flow rate: 1.0 mL/min; Detection: UV @ 230 nm).

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 0.94 (m, 2 H, cyclohex-C$H_2$), 1.45 (m, 2 H, cyclohex-C$H_2$), 1.60-2.15 (m, 6 H, cyclohex-C$H_2$), 2.75 (m, 2 H, bip-C$H_2$CH), 3.15 (m, 2 H, bocNHC$H_2$), 4.85 (m, 1 H, bip-α*H*), 5.18 (m, 2 H, C$H_2C_6H_5$), 5.92 (d, 1 H, CHN*H*CO) and 6.91-7.71 (m, 14 H, Ar*H*).

**MS:** 471.3 $(M+H)^+$

HH

Boc-Gly-OH

HATU/DIEA/$CH_2Cl_2$
RT, 2 h

5 6

**[00139]** To a mixture of a-Bip-OBzl•TFA **(5)** (11.80 g, 20.20 mmol), Boc-Gly (5.31 g, 30.31 mmol), and HATU (11.52 g, 30.32 mmol) in dichloromethane (50 mL) was added N,N-diisopropylethylamine (7.88, 60.98 mmol). The mixture was stirred at room temperature for 2 h. The volatiles were removed on a rotary evaporator, and the residue obtained was taken up in ethyl acetate (200 mL). The organic layer was washed sequentially with dilute HCl (0.5 N, 2 x 150 mL), aqueous sodium carbonate (5%, 2 x 150 mL) and water (150 mL). The organic layer was then separated, dried ($Na_2SO_4$), filtered and evaporated on a rotary evaporator to provide N-*t*-Butoxycarboxylglycyl-*trans*-4-aminomethylcyclohexanecarbonyl-L-biphenylalanine benzyl ester (Boc-Gly-a-Bip-OBzl) **(6)** (12.60 g, quantitative yield) as an off-white foam .

**HPLC:** $t_R$ 16.33 min; Column: YMC C18 4.6 mm x 250 mm, S-10P µm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 45% B, then linear gradient to 95% B over 25 min; Flow rate: 1.0 mL/min, Detection: UV @ 230 nm.

**$^1$H-NMR ($CDCl_3$):** δ 0.94 (m, 2 H, cyclohex-C$H_2$), 1.45 (s & m, 12 H, $C_4H_9$ & cyclohex-C$H_2$), 1.70-2.15 (m, 5 H, cyclohex-C$H_2$), 3.15 (m, 4 H, bip-C$H_2$CH & bocNHC$H_2$), 3.75 (d, 2 H, gly-C$H_2$), 4.95 (m, 1 H, bip-α$H$), 5.18 (m, 3 H, bocN$H$ & C$H_2C_6H_5$), 5.92 (d, 1 H, CHN$H$CO), 6.32 (t, 1 H, $CH_2$CON$H$$CH_2$) and 6.91-7.59 (m, 14 H, Ar$H$).

**MS:** 628.6 $(M+H)^+$

BocHN–CH2–CONH–(cyclohexyl)–CONH–CH(CH2-biphenyl)–COOCH2C6H5 (6) → TFA/CH2Cl2 (1:1 v/v), RT, 30 min → H3N+–CH2–CONH–(cyclohexyl)–CONH–CH(CH2-biphenyl)–COOCH2C6H5 CF3COO- (7)

**[00140]** A solution of Boc-Gly-a-Bip-OBzl **(6)** (12.5 g, 19.91 mmol) and phenol (0.5 g) in dichloromethane (50 mL) was treated with trifluoroacetic acid (50 mL) and stirred at room temperature for 30 min. The volatiles were removed and the residual trifluoroacetic acid was co-evaporated with chloroform to provide Gly-a-Bip-OBzl (TFA salt) (7) (13.00 g, quantitative yield) as a light brown foam. This was triturated and washed with ether, filtered and dried.

**[00141]**

**HPLC:** $t_R$ 9.41 min; Column: YMC-C18, 4.6 mm x 250 mm, S-10P μ, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 45% B, then linear gradient to 95% B over 25 min; Flow rate: 1.0 mL/min, Detection: UV @ 230 nm).

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 0.88 (m, 2 H, cyclohex-C$H_2$), 1.25 to 2.11(m, 8 H, $C_4H_9$ & cyclohex-C$H_2$ & C$H$), 3.12 (m, 4 H, bip-C$H_2$CH & CONHC$H_2$), 3.79 (d, 2 H, gly-C$H_2$), 4.85 (m, 1 H, bip-α$H$), 5.18 (m, 2 H, C$H_2C_6H_5$), 6.55 (d, 1 H, CHN$H$CO), 6.32 (t, 1 H, $CH_2$CON$H$$CH_2$), 6.91-7.89 (m, 15 H, $CH_2$CON$H$$CH_2$ & Ar$H$) and N$H_3^+$.

**MS:** 528.3 $(M+H)^+$.

**[00142]** To a mixture ofGly-a-Bip-OBzl•TFA **(7)** (13.00 g, 20.32 mmol), DO3A10CM-1,4,7-tris-*t*-butyl ester **(8)** (19.26 g, 30.48 mmol) and HATU (11.58 g, 30.48 mmol) in dichloromethane (50 mL) was added N,N-diisopropylethylamine (7.98 g, 60.98 mmol). The mixture was stirred at room temperature for 2 h. The volatiles were removed on a rotary evaporator and the residue was taken up in ethyl acetate (200 mL). The organic layer was washed sequentially with dilute HCl (0.05 N, 2 x 150 mL), aqueous sodium carbonate (5%, 2 x 150 mL) and water (150 mL). The organic layer was then separated, dried ($Na_2SO_4$), filtered and evaporated on a rotary evaporator to provide DO3A10CM-(1,4,7-tris-*t*-butyl ester)-glycyl-*trans*-4-aminomethylcyclohexanecarbonyl-L-biphenyl-alanine benzyl ester **(9)** (19.80 g, 90.40 % yield) as an off-white foam.

**TLC:** $R_f$ 0.42 (silica gel, 5% methanol in dichloromethane)

**HPLC:** $t_R$ 13.44 min; Column: YMC-C18, 4.6 mm x 250 mm, S-10P μm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 45% B, then linear gradient to 95% B over 25 min; Flow rate: 1.0 mL/min, Detection: UV @ 230 nm.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 0.95 (m, 2 H, cyclohex-C$H_2$), 1.48 ( m & s, 29 H, $C_4H_9$ & cyclohex-C$H_2$), 1.75 to 2.11 (m, 6 H, cyclohex-C$H_2$ & C*H*), 2.15 to 3.85 (m, 30 H, macrocyclic-H, bip-C$H_2$CH, CONHC$H_2$ and gly-C$H_2$), 4.95 (m, 1 H, bip-α*H*), 5.18 (2d, 2 H, C$H_2C_6H_5$), 6.05 (d, 1 H, CHN*H*CO), 6.55 (t, 1 H, $CH_2$CON*H*$CH_2$), 6.85 (t, 1 H, $NCH_2$CON*H*), 7.09-7.59 (m, 14 H, Ar*H*).

**MS:** 1082.7 $(M+H)^+$

Pd-C (Degussa, 10%)/[$H_2$]
Ethanol

RT, 4 h

DO3A10CM (tri-*t*-butyl ester)-Gly-a-Bip-OBzl **(9)** (19.5 g, 18.03 mmol) in ethanol (100 mL) was mixed with Pd-C catalyst (5 g, Degussa type, 10%) and hydrogenated on a Parr

hydrogenator at 40 psi for 5 h. Filtration through Celite removed the catalyst, and the filtrate was evaporated to provide DO3A10CM (1,4,7-tris-*t*-butyl ester)-Gly-a-Bip-OH **(10)** (17.0 g, 95% yield) as an off-white solid.

**HPLC:** $t_R$ 8.10 min; Column: YMC C18, 4.6 mm x 250 mm, S-10P µm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA);Elution: Initial condition: 45% B, linear gradient to 95% B in 25 min; Flow rate: 1.0 mL/min, Detection: UV @ 230 nm.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 0.91 (m, 2 H, cyclohex-C$H_2$), 1.48 (m & s, 29 H, $C_4H_9$ & cyclohex-C$H_2$), 1.65 to 4.22 (m, 36 H, cyclohex-C$H_2$, macrocyclic-*H*, bip-C$H_2$CH, CONHC$H_2$ and gly-C$H_2$), 4.75 (m, 1 H, bip-α*H*), 6.51 (bd, 1 H, CHN*H*CO), 6.69 (bt, 1 H, $CH_2$CON*H*$CH_2$), 7.12 (bt, 1 H, N$CH_2$CON*H*), 7.23-7.57 (m, 9 H, Ar*H*).

**MS:** 992.7 $(M+H)^+$

**[00143]**

10

1.) TFA/Phenol/Water/(i-$C_3H_7)_3SiH$ (88:5:5:2)
RT, 4 h

2.) Anion-exchange column purification
(AG 1-X2 resin, quaternary ammonium,
formate form)

11

[00144] DOTA(tri-*t*-butyl ester)-Gly-a-Bip-OH **(10)** (17.0 g, 17.15 mmol) was treated with trifluoroacetic acid:water:phenol:triisopropylsilane (88:5:5:2) (Reagent B) (300 mL) with stirring for 4 h under a nitrogen atmosphere. After removal of the solvent the paste thus obtained was triturated with ether (50 mL), and the mixture was centrifuged and the ether layer was decanted. This process was repeated twice to completely remove phenol. The resulting solid was dried to provide 13.5 g (95.5% yield) of DO3A10CM-Gly-a-Bip-OH as an off-white solid. The material, was purified by anion exchange (Bio-Rad, AG1-X2 resin, 100-200 mesh, formate form) by elution of the column with 4 L each of 0.1M, 0.2M and 0.3M aqueous formic acid followed by elution with 0.4M formic acid (~ 12 L) whereupon the desired compound was eluted (eluant monitored by UV detector at 230 nm). Product-containing fractions were combined and the volatiles were removed by rotary evaporation to provide a foam The foam was dissolved in water and freeze-dried to provide DO3A10CM-Glycyl-*trans*-4-aminomethylcyclohexanecarbonyl-L-Biphenylalanine (DO3A10CM-Gly-a-Bip-OH) **(11)** (12.2 g, 86.3% yield) DO3A10CM- as a colorless crystalline solid.

**HPLC:** $R_t$ 39.35 min (Column: YMC-C18, 4.6 mm x 250 mm, S-10P μm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 0% B, then linear gradient to 50% B in 50 min; Flow rate: 1.0 mL/min, Detection: UV @ 230 nm).

**$^1$H-NMR (500 MHz, DMSO-d$_6$):** δ 0.82-2.08 (m, 10 H, cyclohex-C*H*$_2$), 2.60-4.25 (m & s, 30 H, macrocyclic-*H*, bip-C*H*$_2$CH, CONHC*H*$_2$ and gly-C*H*$_2$), 4.45 (m, 1 H, bip-α*H*), 7.23-7.67 (m, 9 H, Ar*H*), 8.15 (m, 2 H, CHN*H*CO & $CH_2$CON*H*$CH_2$) and 8.51 (bt, 1 H, $CH_2$CON*H*$CH_2$).

**MS:** 824.5 $(M+H)^+$

11

1.) Gd $(OAc)_3$, pH 4.5, RT, 16 h
2.) Preparative HPLC purification
3.) Elution through AG 50W-X8 resin column (sulfonic acid, meglumine form)

12

**[00145]** DO3A10CM-Gly-a-Bip-OH **(11)** (6 g, 7.28 mmol) was dissolved in water (150 mL), gadolinium acetate tetrahydrate (4.43 g, 10.91 mmol) was added and the mixture was stirred for 30 min. The pH of the solution was adjusted to 4.50 with 1N NaOH, and the mixture was stirred at ambient temperature for 16 h. The excess gadolinium was precipitated as gadolinium oxide by raising the pH of the reaction mixture to 8.50 (addition of 0.5N NaOH) and stirring overnight. The gadolinium oxide was filtered and the product-containing

filtrate was treated with 0.1N HCl to lower the pH to 4.50. The product was purified in three portions by reverse phase HPLC as follows: A reversed phase C18 preparative column (Rainin, 41.4 x 250 mm, 8μ, 60Å), was equilibrated with water (no TFA). The sample was applied to the column, and the column was eluted with water-acetonitrile (8:2 v/v), and then a linear gradient elution from 20% acetonitrile to 50% acetonitrile in 60 min was performed. The fractions were analyzed on an analytical reversed phase C18 column, and the fractions containing the product in >99.5% purity were pooled and freeze-dried to provide the Gd-DO3A10CM-G-a-B-OH as a colorless fluffy solid. The gadolinium chelate was converted into its meglumine salt by passing it through a cation exchange column (Bio-Rad, AG 50W-X8 resin, sulfonic acid, meglumine form). The fractions containing the product (detected by UV at 230 nm) were collected and lyophilized to provide Gd-DO3A10CM-Glycyl-*trans*-4-aminomethylcyclohexanecarboxyl-L-Biphenylalanine (Gd-DO3A10CM-Gly-a-Bip-OH•Meg) **(12)** as a colorless fluffy solid. The entire sample purified in this manner provided 5.49 g (82% yield) of compound **12**.

**[00146]**

**HPLC:** $t_R$ 39.82 min; Column: YMC C18 4.6 mm x 250 mm, S-10P μm, 120Å; **Eluents:** A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA)**;** Elution: Initial condition: 0% B, linear gradient to 50% B in 50 min; Flow rate: 1.0 mL/min, Detection: UV @ 230 nm.

**MS:** 979.6 with an isotopic pattern for Gd $(M+H)^+$ ; 196.0 $(M+1)^+$ for meglumine

**Elemental Analysis:** Calcd for $C_{41}H_{54}N_7O_{11}Gd{\bullet}C_7H_{17}NO5{\bullet}4.0\ H_2O$: C, 46.29; H, 6.39; N, 9.00; Gd, 12.63.

Found: C, 46.13; H, 6.52; N, 8.78; Gd, 12.48; %Na: <0.02; % $H_2O$: 6.12.

**[00147]** The product **(12)** was then screened using an IBM PC20-minispec relaxometer for relaxivity in water and Seronorm™, and for HSA binding by the centrifree micro partition method.

Materials and Methods

**[00148]** *Determination of* $^{20}r1_S$, $^{20}r1_W$ *and calculation of* $g_i^{32}$

Seronorm™ (Batch # 508471, Nycomed, distributed in the U.S. by Accurate Chemical and Scientific Corp., Westbury, NY) was reconstituted with 80% of the recommended volume of deionized, distilled water. 0.4 mL of 2.5 mM Gd-conjugate and 1.6 mL of Seronorm™ or water were added to an NMR Tube, 7.5 mm x 180mm, (Bruker, Spectrospin, Milton, Ontario, Canada, Cat# 022-0910) and mixed (Seronorm™ now diluted to 100% of the recommended volume). The Seronorm™/Gd-chelate-peptide and water-peptide mixtures were warmed for not longer than 20 min at 38°C prior to the relaxivity measurement, so that the sample was at the same temperature as the PC20 relaxometer (also referred to as the NMR). Prior to measurement of sample T1's (in triplicate), the NMR signal was optimized (SOP 830-210 IBM PC/20 Multispec Relaxometer 12/18/97). T1 is also measured for a pure water sample and a fresh aqueous Seronorm™ sample. The average T1 for each Seronorm™/Gd-conjugate sample was used in the equation below to calculate relaxivity/mM in Seronorm™ $r1_S$.

**$r1_S$ = (1/T1 sample in Seronorm™ – 1/T1 Seronorm™)/[Gd-conjugate]**

The average T1 for each water/peptide sample was used in the equation below to calculate water r1 relaxivity/mM.

**$r1_W$ = (1/T1 sample in water – 1/T1 water)/[Gd-conjugate-peptide]**

Where [Gd-conjugate] is measured in the Seronorm™ sample by ICP.

The standard relaxation gain of a peptide in Seronorm™ is defined by the equation below:

**$g_i = r1_S / r1_W$**

*Determination of Fraction Bound to Seronorm™ by the Centrifree Method*

After the PC20 relaxivity measurements, the samples were cooled to room temperature, the Seronorm™/Gd-conjugate mixture was removed from the NMR tubes and 1 mL was placed in a Centrifree Micropartition Device (Millipore, Beverly, MA). The device was centrifuged at 500xg for 45 min in a fixed angle rotor (Beckman Model J2-21M, JA-20 rotor) and 0.5 mL was removed from below the filter for Gd measurement by ICP. The [Gd] in 0.5 mL of the uncentrifuged Seronorm™/peptide mixture was measured as a control. The fraction of Gd-chelate-peptide bound was calculated using the equation below:

**Fraction Bound ($F_b$) = [Gd]control – [Gd]Seronorm™/Gd-conjugate) / [Gd]control**

<u>Results</u>

**[00149]** It was found that the peptidic moiety glycyl–*trans*–4–aminomethylcyclohexanecarbonyl – *l* – 4 – biphenylalanyl (GaB) is a useful linker-hydrophobic compound moiety, and this compound or its des-glycyl congener aB was appended to several gadolinium chelating systems in an attempt to provide a compound with high relaxivity.

**[00150]** The following were evaluated for Gd-(DO3A10CM)-Ga(I)B Meg: mouse tolerance, MRA in rabbits, blood T1 and imaging in rabbits and plasma T1 lowering *ex vivo* in monkeys. The compound tested displayed an acceptable tolerance profile in mice (MLD = 3 mmol/kg). It is surmised that the buildup of too much hydrophobic functionality in a local region of the molecule will inevitably lead to a suboptimal tolerance profile. Results are shown below, in Table 3:

**Table 3: Evaluation of Gd-(DO3A10CM)-Ga(I)B Meg**

| Compound | $r1_S$ | $F_{b,s}$[g] | $F_{b,r}$[g] | $F_{b,m}$[g] | Δr1 in rabbit (in vivo-2T) | | Plasma r1 monkey (ex vivo-0.5T) | | MLD (mice-mmol/Kg) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1' | 15' | 1' | 15' | |
| Gd-(DO3A10CM)-Ga(I)B Meg | 21.7 | 0.81 | 0.71[e] | 0.76[g] | 16 | 6 | 18[b] | 11[b] | 3.0 |

b = 0.05 mmol/Kg dose, , e – 0.15 mM,
g – 0.12 mM. $F_{b,s}$-$F_{b,r}$-$F_{b,m}$ refer to the fraction bound in human, rabbit and monkey serum albumin respectively.

**[00151]** The compound was optimized using ligand diversity, *i.e.*, the *d* and *l* isomers of the compound were prepared as their meglumine salts. The *d,l* mixture was constituted from the individual compounds. HPLC purification gave the pure ligand which was converted to the chelate and purified by prep HPLC. The results of primary screening for the new compounds prepared using the strategy of ligand diversity are shown in Table 4.

**Table 4. Primary Screening Results for Analogs of Gd-(DO3A10CM)-Ga(I)B Meg**

| Compound | $g_i$ | $r1_S$ | $r1_W$ | $F_b$ |
|---|---|---|---|---|
| Gd(DOTA)Ga(I)B Meg[a] | 4.12 | 21.71 | 5.27 | 0.81 |
| Gd(DOTA)Ga(d)B Meg[b] | 4.29 | 21.51 | 5.01 | 0.82 |
| Gd(DOTA)Ga(d,I)B Meg[b] | 4.25 | 21.14 | 4.97 | 0.86 |

[a]MLD = 3.0 mmol/Kg, [b]MLD evaluation not conducted.

[00152] In each case the optimization strategy met its goal. The racemic compound Gd-DO3A10CM-G-a-(*d,l*)-Bip displayed $g_i$, $r1_S$ and $r1_W$ values essentially identical to those found for either the d- or l-isomer alone. This indicates that the binding of this compound does not appear to be chiroselective. Also indicated is that methods for synthesis of racemic amino acids can be employed to prepare the *d,l*-Bip, thus reducing the cost of a Bip-based compound.

[00153] Optimization of the compound was further conducted via chelate diversity. A number of enhanced relaxivity gadolinium chelates were identified, which, when conjugated to HSA binding compounds, were hypothesized to provide higher relaxivity than Gd-DO3A10CM-monoamides. The structures of these are given below:

**Chart 4. Enhanced Relaxivity (ER) Chelate Systems**

CM-DOTMA CM-DOTA CEMP-DO3A 7MP-DO3A

7MP-CM-DO3A 4-CE-DTPA TE-DOTA 4-CEMP-DTTA

[00154] Results of the optimization are shown above, in Table 1 and below, in Table 5.

:

| Table 5. Primary Screening Results for Compounds Prepared Employing Chelate Diversity for Optimization of Gd-(DO3A10CM)-Ga(I)B Meg | | | | |
|---|---|---|---|---|
| | | | | |
| **Compound** | $g_i$ | $r1_S$ | $r1_W$ | $F_b$ |
| Gd(DO3A10CM)-GaB 2Meg | 4.12 | 21.71 | 5.27 | 0.81 |
| Gd(DO3A10CM)aB 2Meg | 3.35 | 17.45 | 5.22 | 0.76 |
| **CEMP – Derivatives** | | | | |
| Gd(CEMP-DO3A)-GaB* | 3.42 | 14.39 | 4.21 | 0.92 |
| Gd(CEMP-DO3A)-aB Meg | 3.43 | 16.10 | 4.70 | 0.87 |
| Gd(CEMP-DO3A)-aB Na | 0.96 | 14.96 | 15.54 | 0.96 |
| Gd-4-CEMP-DTTA-GaB Meg | 4.77 | 33.71 | 7.06 | 0.95 |
| **CE-DTPA Derivatives** | | | | |
| Gd(CE-DTPA)-GaB 3Meg | 3.26 | 21.41 | 6.56 | 0.88 |

| Table 5. Primary Screening Results for Compounds Prepared Employing Chelate Diversity for Optimization of Gd-(DO3A10CM)-Ga(I)B Meg | | | | |
|---|---|---|---|---|
| | | | | |
| **Compound** | $g_i$ | $r1_S$ | $r1_W$ | $F_b$ |
| Gd(CE-DTPA)-aB 3Na | 5.56 | 29.44 | 5.29 | 0.94 |
| Gd(CE-DTPA)-aB 3Meg | 5.67 | 33.30 | 5.87 | 0.91 |
| **CM-DOTMA Derivatives** | | | | |
| Gd(CM-DOTMA)-GaB 2Na | 5.10 | 30.70 | 6.01 | 0.87 |
| Gd(CM-DOTMA)-aB Na | 5.74 | 34.41 | 6.00 | 0.86 |
| Gd(CM-DOTMA)-aB Na | 6.18 | 36.02 | 5.82 | 0.85 |
| Gd(CM-DOTMA)-aB Meg | 6.04 | 35.57 | 5.89 | 0.83 |
| **CM-DOTA Derivatives** | | | | |
| Gd(CM-DOTA)-aB Meg | 6.40 | 30.36 | 4.75 | 0.95 |
| Gd(CM-DOTA)-aB Na | 6.64 | 30.78 | 4.64 | 0.94 |
| **7MP-DO3A Derivatives** | | | | |
| Gd-7MP-DO3A-GaB* | 5.84 | 33.99 | 5.82 | 0.88 |
| Gd-7MP-DO3A-GaB Meg | 5.55 | 33.95 | 6.12 | 0.89 |
| **7MP-CM-DO3A Derivative** | | | | |
| Gd-7MP-CM-DO3A-aB Meg | 3.93 | 20.10 | 5.12 | 1.00 |
| **TE-DOTA Derivatives** | | | | |
| Gd-TE-DOTA-aB Meg | 6.08 | 42.09 | 6.92 | 0.96 |
| Gd-TE-DOTA Meg | 1.63 | 7.01 | 4.29 | 0.01 |

* neutralization of the Na salt to pH 4.5 was followed by prep HPLC.

## EXAMPLE 2 Synthesis and Screening of Gd-(DO3A10CM)-GK(Fl-9)-OH Meg (Class 2)

[00155] The strategy for preparing the lysine-based compounds required the use of the Dde protecting group for the quasi-orthogonal deprotection of the ω-amino group of lysine (to which the hydrophobes would be appended). The compound was prepared as follows:

**Fmoc-Lys-(Boc)-OBn–$N^{\alpha}$-(9-Fluorenylmethoxycarbonyl)-$N^{\omega}$-(Boc)-lysine benzyl ester**

A 250 mL round bottomed flask equipped with a magnetic stir bar and septum capped inlet was charged with water (30 mL), sodium bicarbonate (4.20 g, 50 mmol, 2.38 equiv) and tetrabutylammonium hydrogen sulfate (3.60 g, 10.62 mmol, 0.504 equiv). The mixture was stirred (gas evolution). Then dichloromethane (45 mL) was added to the stirred mixture followed by Fmoc-Lys-(Boc)-OH (9.87 g, 21.06 mmol, Advanced ChemTech) which was added in small portions over a 5 minute period. Then benzyl bromide (3.602 g, 21.06 mmol, Aldrich) was added via syringe dropwise over a 1 minute period. The mixture was stirred rapidly for 18h at ambient temperature using a vent needle in the septum cap to relieve pressure from evolved carbon dioxide. TLC analysis was performed using both

dichloromethane/ethylacetate 9/1 v/v and dichloromethane/methanol 9/1 v/v; this showed that the reaction was essentially complete. (The former system allows us to detect the consumption of benzyl bromide (solvent front) and formation of the product ($R_f$ = 0.5); the latter system allows us to detect the tetrabutylammonium salt of Fmoc-Lys(Boc)-OH ($R_f$=0.3).)

The organic layer was separated and the aqueous layer was extracted with 50 mL of dichloromethane. The dichloromethane layers were combined, volatiles were removed and the residue was redissolved in 200 mL of ethyl acetate. The organic phase was washed with water (4 x 200 mL) to remove tetrabutylammonium salts (visible by development in the iodine chamber) and then dried (sat. brine, then $MgSO_4$). The volatiles were removed to give a residue which was applied (as a solution in 60 mL of dichloromethane) to a 35 cm high x 54 mm wide column of Merck 9385 silica gel packed in dichloromethane. The column was eluted with dichloromethane (1300 mL), dichloromethane-ethyl acetate 95/5 v/v (1000 mL), dichloromethane-ethyl acetate 90/10 v/v (1000 mL) and then dichloromethane-ethyl acetate 85/15 v/v for the remainder of the elution. The fraction size was 50 mL and the desired compound was obtained in 48-84. Fractions 56-74 were taken as the heart cut. The volatiles were removed to provide a foam which was triturated with ether-hexanes to initiate precipitation. Then the volatiles were removed on the rotary evaporator employing an ambient temperature water bath and the resulting solid was pumped on at 7 mm Hg for two days. This provided the desired material (10.66 g) in 90.6% yield as an amorphous white solid.

This compound is known in the literature (Matsoukas, John M.; Hondrelis, John; Agelis, George; Barlos, Kleomenis; Gatos, Dimitrios; Ganter, Renee; Moore, Diana; Moore, Graham J. Novel Synthesis of Cyclic Amide-Linked Analogs of Angiotensins II and III. J. Med. Chem. (1994), 37(18), 2958-69.) but the $^1$H-NMR obtained for this sample is given for reference. Registry Number: 159541-28-3.

**$^1$H-NMR (500 MHz, DMSO-$d_6$)** δ 7.86 (d, 2H), 7.82 (d, 1H), 7.71 (d, 1.77H), 7.58 (app t, 0.25H), 7.4 (app t, 2H), 7.33 (m, 7H), 6.77 (app t, 0.66H), 5.13 (s with superimposed mult, 2H), 4.30 (m, 2H), 4.21 (t, 1H), 4.07 (m, 1H), 2.89 (m, 2H), 1.68 (m, 2H), 1.36 s, with superimposed multiplet).

On larger scale after it is ascertained that all of the starting material is consumed the organic layer was separated and the volatiles were removed by rotary evaporation and pumping at high vacuum. Then the residue was taken up in ethyl acetate and extracted (6X) with water (to remove all tetrabutylammonium salts) and the organic layer was dried. The volatiles were removed and the residue triturated with ether-hexanes and vigorously agitated by stirring overnight. Filtration gave the desired material, $N^{\alpha}$Boc-$N^{\omega}$-Fmoc-Lys-OBn which was isolated in high yield and purity.

**$N^{\alpha}$-Fmoc-Lys-OBn•TFA**

$N^{\alpha}$-(9-Fluorenylmethoxycarbonyl)-$N^{\omega}$-(Boc)-lysine benzyl ester (10.5 g, 18.79 mmol) was dissolved in $CH_2Cl_2$/anisole 45/5 v/v (50 mL) with stirring. To this was added trifluoroacetic acid (50 mL) and the mixture was stirred 1.5 h at ambient temperature. The volatiles were removed on the rotary evaporator and then by pumping at 0.1 mm Hg for 0.5 h. Then anhydrous diethyl ether (250 mL) was added, the mixture was agitated and the side of the flask was scratched with the oil using a glass rod. This led to formation of a solid which was broken up and well agitated. The flask was cooled in the freezer for 1 h and the solid was collected. Pumping overnight removed residual ether. This gave 10.53 g (97.87% yield) of the desired product as a white solid. The HCl salt (which we have prepared on a small scale previously) is a known compound: (Matsoukas, John M.; Hondrelis, John; Agelis, George; Barlos, Kleomenis; Gatos, Dimitrios; Ganter, Renee; Moore, Diana; Moore, Graham J. Novel Synthesis of Cyclic Amide-Linked Analogs of Angiotensins II and III. J. Med. Chem. (1994), 37(18), 2958-69.)

**$^1$H-NMR (500 MHz, DMSO-$d_6$,):** δ 7.9 (overlapping d, 5.4H), 7.7 (br d, 1.85H), 7.58 (app t, 0.25 H), 7.40 (app t, 2.12 H), 7.33 (m, 6.95H), 6.91 (m, 0.09H), 5.12 (s with shallow overlapping m, 2H), 4.31 (m, 2H), 4.21 (app t, 1.0H), 4.07 (m, 1.0H), 3.95 (m, 0.13H), 1.68 (m, 1.93H), 1.53(m, 2.14H), 1.36 (m, 2.04H), 1.07 (t, 0.07H, residual ether $CH_3$, totals to ~1% diethyl ether). **Note:** Non-integer integrals and fractional integrals result from conformational isomerism.

COOH
2
Fmoc—N(H)—CH(COOBn)—(CH$_2$)$_4$—$NH_3^+$ $CF_3COO^-$
1
HATU/DIEA/$CH_2Cl_2$
RT, 2 h
3

**N$^{\alpha}$-Fmoc-N$^{\omega}$-(Fluorene-9-Carbonyl)-Lysine Benzyl Ester (3).**

A 100 mL round-bottomed flask equipped with magnetic stir bar and septum capped inlet was charged with Fmoc-Lys-O-Bz•TFA (1) (3.00 g, 5.24 mmol), HATU (2.097 g, 5.502 mmol, 1.05 equiv., Perseptive Biosystems), fluorene-9-carboxylic acid (2) (1.156g, 5.502 mmol, 1.05 equiv, Aldrich Chemical) and dichloromethane (25 mL) with stirring in that order. The mixture was stirred at ambient temperature and diisopropylethylamine (2.099 g, 16.244 mmol, 3.10 equiv. Aldrich) was added dropwise over a period of 30 seconds. The mixture was stirred 5 min and then DMF (2 mL) was added. The mixture was stirred an additional 3 h; during this time a copious precipitate formed and the mixture could not be stirred. Then dichloromethane (70 mL) was added, the solid was broken up and the mixture was vigorously stirred for 3h. The solid was collected and dried by pumping at high vacuum to give 2.26 g of the product as a white solid.

The deep yellow mother liquor was examined by TLC employing E. Merck silica gel plates and dichloromethane-ethyl acetate 9/1 v/v as the developing solvent. This showed the presence of the major product (same $R_f$ as the collected solid) and a higher $R_f$ impurity later identified as 9-fluorenone; the starting acid was consumed. The mother liquor was processed by washing with 0.5M $NaHSO_4$ (200 mL), saturated $NaHCO_3$ (2 x 200 mL), 5% $Na_2CO_3$ (2 x 200 mL), 0.5M $NaHSO_4$ (200 mL), saturated brine (200 mL) and dried ($MgSO_4$). Removal of the drying agent and evaporation gave 1.11 g of a yellow solid. This was dissolved in 25 mL of dichloromethane and applied to a 29 mm diameter x 25 cm height column of Merck 9385 silica gel packed in dichloromethane. The column was eluted with 400 mL of dichloromethane, 95/5 dichloromethane-ethyl acetate 600 mL and then with dichloromethane-ethyl acetate 9/1 v/v (600 mL) in 20 mL fractions. Fractions 7-9 contained 9-fluorenone (0.070 g). This is a contaminant in the starting 9-fluorene carboxylic acid. Fractions 47-90 contained the desired product 0.84 g. The combined yield of product was 3.10 g (90.9% yield).

**TLC:** (E Merck Silica gel 60 GF, $CH_2Cl_2$-EtOAc 9/1 v/v) $R_f$ = 0.56, $R_f$ of 9-fluorenone = 0.98, $R_f$ fluorene-9-carboxylic acid = 0.26 (trails from 0.12 to 0.44).

**$^1$H-NMR (500 MHz, DMSO-$d_6$) δ** 8.38 (t, 1H), 7.87 and 7.86 (overlapping app doublets, 5H), 7.72 (d, 1.88 H), 7.59 (app t, 0.27H), 7.51 (d, 2H), 7.43-7.26 (13H), 5.14 (s with low intensity m overlapping, 2H), 4.79 (s, 1H), 4.33 (m, 2H), 4.23 (t, 1H), 4.12 (m, 1H), 3.40 (br s, 1H may be due to residual water), 3.12 (m, 2H), 1.76 and 1.70 (pair of m, 2H), 1.46 and 1.39 (pair of m, 2H) ppm.

**$^{13}$C-NMR (125 MHz, DMSO-$d_6$) δ** 172.4, 169.4, 156.3, 143.8, 143.7, 143.0, 141.3, 140.7, 136.0, 128.4, 128.0, 127.8, 127.60, 127.64, 127.2, 127.1, 125.24, 125.21, 124.8, 120.12, 120.07, 65.89, 65.71, 54.74, 54.03, 46.66, 38.57, 30.34, 28.71, 22.98 ppm.

**Mass Spectrum ESI+** 783.2 $(M+Cs)^+$ (Cs from residual CsI used by the vendor for machine calibration), 689.3 $(M+K)^+$, 674.2 $(M+1+Na)^+$, 673.3 $(M+Na)^+$, 652.3 $(M+1+H)^+$, 651.3 $(M+H)^+$, 567.2 (unassigned), 489.2 (unassigned), 456.2, 455.2 (M - 9-fluorene-methyleneoxy radical)$^+$, 441 (estimated, not delineated by the operator, unassigned), 429.3 $(M\text{-}Fmoc+2H)^+$.

**Elemental analysis:** Found: C, 76.14%; H, 5.69%; N, 4.06%.
Calc. for $C_{42}H_{38}N_2O_5$: C, 77.52%; H, 5.89%; N, 4.30%. $^1$H-NMR indicates 0.51 mol water.
Calc'd for $C_{42}H_{38}N_2O_5 \bullet 0.51\ H_2O$ C, 76.44%; H, 5.96%; N, 4.24 %.

Fmoc—NH … COOBn (3) → 20% Piperidine/$CH_3CN$, RT, 60 min → $H_2N$ … COOBn (4)

**[00156]** To a suspension of the Fmoc derivative **(3)** (10 g, 15 mmol) in acetonitrile (50 mL) was added 25 % piperidine in acetonitrile (150 mL, final concentration of piperidine 20%), and the mixture was stirred at room temperature for 60 min. Acetonitrile was removed

in vacuo; the residue was stirred with hexane (150 mL) for 10 min and the hexane layer was decanted. This process was repeated four times. The resulting solid was filtered, washed once more with hexane (50 mL) and dried to afford $N^{\varepsilon}$-(9-fluorenecarbonyl)-lysine benzyl ester **(4)** (6.25 g, 93 % yield).

**TLC:** $R_f$ 0.45 (silica gel, $CH_2Cl_2$:MeOH, 95:5 v/v)

**HPLC:** $t_R$: 5.24 min Column: YMC ODS-A 4.6 mm x 250 mm, S-10P μm, 120Å particle; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 50 % B, then linear gradient to 100% B in 20 min; Flow rate: 1.0 mL/min, Detection: UV @ 254 nm.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.2-1.65 (8H, $NH_2$ and $3CH_2$), 3.05 (bs, 2H, $CH_2$), 3.33 (m, 1H, CH), 4.76 (s, 1H, CH), 5.09 (s, 2H, benzylic), 5.28 (bs, 1H, NH), 7.31(s, 7H, ArH), 7.42 (m, 2H, ArH), 7.65 (m, 2H, ArH), 7.75 (m, 2H, ArH).

**MS:** 429.2 $(M+H)^+$



**[00157]** To a mixture of DO3A10CM-(tri-t-butyl ester)-glycine **(5)** (8.8 g, 14 mmol) and $N^{\varepsilon}$-(9-fluorenecarbonyl)- lysine benzyl ester **(3)** (5.13 g, 12 mmol) in anhydrous methylene chloride (100 mL) was added HATU (5.32 g, 14 mmol), and the mixture was stirred for 5

min. Diisopropylethyl amine (5.46 g, 42 mmol) was added to the reaction mixture and stirring was continued for 6 h. Methylene chloride was removed in vacuo, the residue was dissolved in ethyl acetate (150 mL), washed with saturated sodium bicarbonate solution (3 x 100 mL), sodium bisulfate solution (0.5 molar, 2 x 100 mL) and finally with brine (100 mL). The ethyl acetate layer was dried and solvent removal afforded the coupled product as a light yellow glassy solid (13g). This was purified by column chromatography over silica gel (Merck 9385, 350 g) using methylene chloride and methanol to afford the required product (8.6 g, 69 % yield) DO3A10CM-(tri-t-butyl ester)-glycinyl-$N^{\varepsilon}$-(9-fluorenecarbonyl)-lysine benzyl ester **(6)**.

**TLC:** $R_f$, 0.6 (silica gel, 6 % MeOH/ $CH_2Cl_2$)

**HPLC:** $t_R$ 9.37 min. (Column: YMC ODS-A, 4.6 mm x 250 mm, S-10P µm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA);; Elution:: Initial condition: 50 % B, then linear gradient to 100% B in 20 min; Flow rate: 1.0 ml/min, Detection: UV @ 254 nm).

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.385-1.436 (m, 27H, t-$C_4H_9$), 1.4-1.8 (m, 6H, $CH_2$), 4.35 (bs, 1H, $CHCOOCH_2C_6H_5$), 4.88s (s, 1H, CH), 5.12 (s, $CH_2$, benzylic), 6.6, 6.8 and 7.1 (broad singlets, 3H, NH), 7.3-7.8(m, 13H, ArH).

**MS:** $(M+H)^+$ 1040.6, $(M+Na)^+$ 1062.5.

6

1.) $H_2$/Pd - 50 psi 16h
2.) TFA:$H_2O$:TIPS (88:5.5:2 v/v/v) RT, 4h
3.) Prep HPLC

7

[00158] To a solution of **(6)** (8.30 g, 8 mmol) in methanol (80 mL) was added palladium catalyst (3.0 g, 10% DeGussa type), and the mixture was hydrogenated at 50 psi for 16 h. The catalyst was filtered off, the volatiles were removed in vacuo and the residue was dried. This was treated with TFA:$H_2O$:triisopropylsilane (88:5.5:2 v/v/v) (120 mL) for 4h under a nitrogen atmosphere. The volatiles were removed in vacuo, the residue was triturated with ether (2 x 100 mL) to provide a solid which was collected, washed with ether (50 mL) to afford DO3A10CM-glycinyl-$N^{\varepsilon}$ -(9-fluorenecarbonyl) -lysine **(7)** as the TFA salt (6.9 g, yield 86 %). This material was further purified on a reversed phase C18 preparative LC column (Rainin, 41.4 x 250 mm, 8μ particle, 60Å pore size) which was equilibrated with 0.1% aqueous TFA. The column was then eluted with water-acetonitrile solvent mixture (both containing 0.1 % TFA), starting a linear gradient from 10% acetonitrile to 80% acetonitrile in 80 min. The fractions were analyzed on an analytical reversed phase C18 column, and those containing the pure product were pooled and freeze-dried to provide DO3A10CM-glycinyl-$N^{\varepsilon}$-(9-fluorenecarbonyl)-lysine **(7),** (4.7 g, yield, 58 %) as a colorless fluffy solid.

**HPLC:** $t_R$ 13.14 min; Column: YMC ODS-A 4.6 mm x 250 mm, S-10P μm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 10 % B, linear gradient to 100% B over 20 min; Flow rate: 1.0 mL/min, Detection: UV @ 254 nm.

**$^1$H-NMR (500 MHz, DMSO):** δ 1.32-1.74 (m, 6H, $CH_2$), 2.80-3.95 (m, 28H,$CH_2$), 4.20 (bs, 1H, $C\underline{H}COOH$), 5.12 (s, $CH_2$, benzylic), 4.76 (s, 1H, CH), 7.25-7.55 (m, 6H, ArH), 7.85 (m,2H, ArH), 8.30, 8.40 and 8.62 (broad singlets, 3H, NH).

**MS:** 782.3 $(M+H)^+$, $(M+Na)^+$ 804.3, 590.3, 391.7.

• 2 $CF_3COOH$

7

1.) $Gd(OAc)_3$, pH 4.5
2.) Prep.HPLC
3.) AG50-X2 ion exchange resin Meglumine form

8

**[00159]** DO3A10CM-glycinyl-$N^{\varepsilon}$-(9-fluorenecarbonyl)-lysine **(7)** (4.3 g, bis-trifluoroacetate salt, 5.5 mmol) was dissolved in water (100 mL) and gadolinium acetate tetrahydrate (2.43 g, 6 mmol) was added. The mixture was stirred for 30 min. The pH of the solution was adjusted to 4.50 with 1N NaOH, and the mixture was stirred at ambient temperature for 24 h. HPLC analysis indicated the completion of the reaction. The solution containing the product was loaded onto a reversed phase C18 preparative column (Rainin, 41.4 x 250 mm, 8μ, 60Å) which was equilibrated with water. The column was then eluted with 10% aqueous acetonitrile and then a linear gradient from 10% acetonitrile to 80% acetonitrile in 80 min was run. The fractions were analyzed on an analytical reversed phase C18 column and the fractions containing the product in > 99.5% purity were pooled and freeze-dried to provide (Gd-DOTA)-Glycinyl-$N^{\varepsilon}$-(9-fluorenecarbonyl)-lysine, meglumine salt **(8)** as a colorless fluffy solid. To convert this Gd-chelate into its meglumine salt form, it was applied to a sulfonic acid cation exchange column (Bio-Rad, AG 50W-X8 resin)which had been converted into the meglumine form and the column was washed with water. The

fractions containing the product (the eluent was monitored with an UV detector at 254 nm) were collected and lyophilized to provide Gd-DO3A10CM-Glycinyl-$N^{\varepsilon}$-(9-fluorenecarbonyl)-lysine meglumine salt **(8)** (4.2g, 63 % yield) as a colorless fluffy solid..

**HPLC:** $t_R$ 10.40 min (Column: YMC ODS-A, 4.6 mm x 250 mm, S-10P μm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: initial condition: 10 % B, linear gradient to 80 % B over 20 min; Flow rate: 1.5 ml/min, Detection: UV @ 220 nm).

**MS:** 937.2 isotopic pattern for Gd $(M+H)^+$ and 195.9 meglumine $(M+1)^+$ .

**Elemental Analysis:** Calc. for $C_{38}H_{48}N_7O_{11}Gd \bullet C_7H_{17}NO_5 \bullet 5.5\ H_2O$: C, 44.32; H, 6.28; N, 9.11; Gd, 12.78 Found: C, 44.18, H,5.96; N, 9.01; Gd, 12.01; %Na: <0.24;

**[00160]** The compound was screened as described above in Example 1. Results showed that MDL > 3.0 mmol/Kg in mice. Other results are shown in Table 2.

**EXAMPLE 3 – Synthesis of Gd(CM-DOTMA)-aB $Na^+$ (Class 1)**

**[00161]** The compound was prepared as follows: a suspension of L-malic acid **(1)** (Aldrich Chemical Company, 120.0 g, 0.895 mol) in 2,2-dimethoxyethane (1200 mL) was heated under reflux with stirring until all of the material went into solution (2 h). The solution was concentrated under reduced pressure using a rotary evaporator and the resulting oil was dissolved in about 1000 mL of 7:3 hot hexane/isopropyl ether and filtered. The solid that separated from the cooled filtrate was collected and washed with 7:3 hexane/isopropyl ether (2 x 50 mL). The combined filtrates were again concentrated to about 250 mL, yielding

more of the product L-malic acid acetonide **(2)** as colorless crystalline solid. Yield: 131.0 g (84%).

**m. p.** 114-116° C.

**$^{1}$H-NMR (500 MHz, $CDCl_3$):** δ 1.5 and 1.55 (2s, 6H, -C-C$\underline{H}_3$), 2.8 (AB qd, 2H, -C$\underline{H}_2$-COOH), 4.6( dd, 1H, -C$\underline{H}$-$CH_2$COOH) and 11.1 (bs, 1H -COO$\underline{H}$). $^{13}$C-NMR (CDC13) δ 25.46, 26.37, 35.63, 70.08, 111.1, 171.7 and 174.9.

**MS:** 197 $(M+Na)^+$.

**Elemental Analysis:** Calc. for $C_7H_{10}O_5$: C, 48.28; H, 5.79; O, 45.94%.
Found: C, 48.18; H, 5.69.

$C_6H_5CH_2Br$

$K_2CO_3$/18-Crown-6

$CH_3CN$

2 → 3

**[00162]** A solution of the acetonide **2** (30.0 g, 0.172 mol) in dry acetonitrile was treated with 18-crown-6 (0.462 g, 1.75 mmol) and powdered potassium carbonate (23.8 g, 0.172 mol) and stirred under nitrogen with an overhead stirrer. Benzyl bromide (29.0 g, 0.17 mol) was added neat, and stirring was continued under nitrogen at RT for an additional 20 h. Acetonitrile was evaporated and the paste obtained was dissolved in water (300 mL). The aqueous solution was extracted with ethyl acetate (3 x 200 mL) and the organic layer was washed with water (3 x 200 mL), saturated sodium chloride (1 x 300 mL) and dried. Removal of the solvent furnished a colorless solid. The solid was recrystallized from hexane: isopropyl ether (7:3) to yield the benzyl ester of acetonide of L-malic acid **3** as colorless needles. Yield: 41.7 g (91.8%).

**m. p.** 62-63° C.

**$^1$H-NMR (500 MHz, $CDCl_3$)**: δ 1.5 and 1.55 (2s, 3H, -C-C<u>H</u>$_3$), 2.9 (ABqd, 2H, -C<u>H</u>$_2$-COOBn), 4.7 (dd, 1H, -CH-CH$_2$_COOBn), 5.15 (q, 2H, -C<u>H</u>$_2$-Ph) and 7.3 (m, 5H, Ar-H).

**$^{13}$C-NMR (125 MHz, $CDCl_3$)**: δ 26.58, 27.4, 67.74, 71.36, 111.9, 129.1, 129.2, 136.0, 169.8 and 172.7.

**MS:** 287.2 $(M+Na)^+$

**Elemental Analysis:** Calc. for $C_{14}H_{16}O_5$: C, 63.63; H, 6.1; O, 30.27%.
Found: C, 63.62; H, 6.03.

2N $H_2SO_4$, Dioxane
RT, 30 min

COOCH$_2$Ph
3

COOH
OH
H
PhCH$_2$OOC
4

**[00163]** A solution of the acetonide **3** (100.0g, 0.378 mol) in dioxane (250 mL) was treated with 2N $H_2SO_4$ and stirred vigorously at RT. Initially, the reaction mixture was cloudy and after about 30 min. the solution became clear. TLC indicated that the reaction was complete. The crude reaction mixture was saturated with sodium sulfate and extracted with ethyl acetate (5 x 300 mL). The combined organic layers were washed with saturated sodium chloride and dried ($Na_2SO_4$). Filtration followed by evaporation of the solvent yielded a colorless oil which solidified slowly on standing. The solid was again recrystallized from isopropyl ether/hexane (2:1) to yield the 3-benzyloxycarbonyl-2-hydroxy propionic acid [the monobenzyl ester of (L)-malic acid] **4** as a colorless solid. Yield: 77.0 g (90.9%).

**m. p.** 68-69° C.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 2.9 (m, 2H, -C<u>H</u>$_2$-COOBn), 4.5 (d, 1H, -C<u>H</u>OH), 5.15 (q, 2H, -C<u>H</u>$_2$Ph) and 7.3 (m, 5H, Ar-H).

**$^{13}$C-NMR (500 MHz, $CDCl_3$):** δ 38.03, 66.69, 66.90, 128.1, 128.2, 128.4, 134.9, 170.7 and 176.8.

**MS:** 247.1 $(M+Na)^+$, 225.2 $(M+H)^+$

**Elemental Analysis:** Calc. for $C_{11}H_{12}O_5$: C, 58.93; H, 5.39; O, 35.68%. Found: C, 59.4; H, 5.41%.

HOOC, OH, H, HOOC (**4**) —[$Ac_2O/Et_3N$, $CH_2Cl_2$, RT, 20h]→ HOOC, OAc, H, $PhCH_2OOC$ (**5**)

**[00164]** A solution of hydroxy acid **4** (77.0 g, 0.34 mol) in dry $CH_2Cl_2$ was treated with anhydrous triethylamine (70.7 g, 0.7 mol) and was cooled to 0° C. Acetic anhydride was added with stirring while maintaining the reaction temperature below 10° C. After the addition (about 10 min), the reaction mixture was allowed to come to RT and stirred for 20 h. Water (200 mL) was added and the mixture stirred for 1 h. The reaction mixture was cooled in ice and treated with 4N HCl (200 mL) and stirred for 30 min more. The organic layer was separated and the aqueous layer was extracted with $CH_2Cl_2$ (3 x 200 mL). The combined organic layer was washed with water (2 x 300 mL), saturated NaCl (1 x 500 mL) and dried ($Na_2SO_4$). Evaporation of the solvent under reduced pressure yielded 2-acetoxy-3-benzyloxycarbonyl propionic Acid **(5)** as a pale brown oil. This was dried overnight under vacuum (<0.1 mm) and employed immediately thereafter for the next step in the sequence. Yield: 90.5g (100%).

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 2.1(s, 3H, $-O-CO-C\underline{H}_3$), 2.9 (d, 2H, $-C\underline{H}_2-COOBn$), 5.15(q, $-C\underline{H}_2-Ph$), 5.5 (t, 1H-$C\underline{H}$-OAc), 7.3 (m, Ar-H) and 10.6 (bs, 1H, $-COO\underline{H}$).

**MS:** 267.2 $(M+H)^+$

**[00165]** To a solution of the acid **(5)** (94.0 g, 0.35 mol) in dry $CH_2Cl_2$ (1400 mL), anhydrous $MgSO_4$ (168.5 g, 1.5 mol) was added and stirred. To the above solution, *t*-BuOH (130.0g, 1.77 mol) was added followed by concentrated. $H_2SO_4$ in portions to maintain the temperature at about 25° C. After the addition, the flask was tightly stoppered and stirred at RT for 24 h. The reaction mixture was poured into saturated $NaHCO_3$ (68.0g, 0.8 mol in 1500 mL of water) and stirred vigorously to neutralize the acid. The organic layer was separated, washed with water (1 x 500 mL), and then with saturated NaCl (1 x 500 mL). The dried ($Na_2SO_4$) organic layer was filtered and evaporated to give the crude *t*-butyl-2-acetoxy-3-benzyloxycarbonyl propionate product **(6)** as a pale brown oil. The oil was kept under high vacuum for 20 h (< 0.1 mm) and was then taken on to the next step without purification. Yield: 100.0 g (89%).

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 2.1(s, 3H, -O-CO-C$\underline{H}_3$), 2.9 (d, 2H, -C$\underline{H}_2$-COOBn), 5.15(q, -C$\underline{H}_2$-Ph), 5.5 (t, 1H-C$\underline{H}$-OAc), 7.3 (m, Ar-H) and 10.6 (bs, 1H, -COO$\underline{H}$)

**MS:** 345.4 $(M+Na)^+$, 323.4 $(M+H)^+$, 267.2 $(M+H -C_4H_8)^+$

**[00166]** A solution of the acetate **6** (100.0 g, 0.31 mol) in a mixture of *i*-PrOH/*t*-BuOH (400 mL/100 mL) was cooled in an ice-bath and was treated with neat anhydrous hydrazine (12.7 g, 0.37 mol). After 30 min an additional amount of hydrazine (3.5 g, 0.1 mol) was added and the mixture was stirred for 30 min. Then another portion of hydrazine (0.34 g,

0.01 mol) was added and the mixture was stirred for 30 min. Silica gel TLC analysis indicated the completion of the reaction. The total reaction time was 2 h. The volatiles were removed under reduced pressure and the residue was taken up in water (500 mL). The oil that separated was extracted with ethyl acetate (3 x 250 mL) and the combined organic layers were washed with water (3 x 200 mL), saturated NaCl (1 x 250 mL) and dried ($Na_2SO_4$). Evaporation of the solvent yielded a pale yellow oil. The oil was dissolved in hot hexane (500 mL) and was filtered. The filtrate was cooled in an ice-bath. The crystals that separated were filtered and washed with ice-cold hexane (2 x 100 mL). The resultant compound *t*-butyl-3-benzyloxycarbonyl-2-hydroxy propionate,**(7)** obtained as a colorless solid, was dried at RT under vacuum for 20 h. Yield: 53.8 g (62%).

**m. p.** 67-68° C.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.42 (9s, 9H, -C-$CH_3$), 2.8 (m, 2H, -$CH_2$-COOBn), 4.35 (t, 1H, -CH-OH), 5.15 (q, 2H, -$CH_2$Ph) and 7.3 9m, 5H, Ar-H).

**$^{13}$C-NMR (125 MHz, $CDCl_3$):** δ 27.82, 38.93, 66.6, 67.34, 82.95, 128.3, 128.5, 135.5, 170.2 and 172.5.

**MS:** 303.0, $(M+Na)^+$

**Elemental Analysis:** Calc. for $C_{15}H_{20}O_5$: C, 64.27; H, 7.19; O, 28.54%.
Found: C, 64.27; H, 7.35%

$Tf_2O$/2,6-lutidine
$CH_2Cl_2$
7 → 8

**[00167]** To a solution of the alcohol **7** (21.0 g, 0.075 mol) in dry dichloromethane (100 mL), 2,6-lutidine (9.1 g, 0.085 mol) was added and the mixture was cooled to -10° C. Trifluoromethanesulfonic anhydride (23.3 g, 0.0825 mol) was added dropwise with stirring under nitrogen over a period of about 15-20 min. After the addition, the reaction mixture was

stirred at 0° C for 1 h. The reaction mixture was poured into crushed ice (250 g) and the organic layer was washed with ice-cold 2N HCl (2 x 100 mL), ice-cold water (2 x 100 mL) and dried with anhydrous calcium sulfate in a freezer before its use. The *t*-butyl-3-benzyloxycarbonyl-2-O-[trifluoromethanesulfonyl) propionate **(8)** was kept as a solution in dichloromethane. The triflate **8** was found to be very unstable at room temperature and therefore was used immediately as a solution in dichloromethane as soon as it was prepared.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.5 (s, 9H, *t*-butyl $CH_3$), 3.2(d, 2H, -C<u>H</u>$_2$-COOBn), 5.2 (q, 2H, -COOC<u>H</u>$_2$Ph), 5.4 (triplet, 1H, -C<u>H</u>-), and 7.4 (m, 5H, Ar-H).

**[00168]** A solution of 1,4,7,10-tetraazacyclododecane (TACD) **(9)** (31.54 g, 0.18 mol) in dry dichloromethane (100 mL) was cooled in an ice-bath and a solution of the triflate **8** (nominally 0.075 mol ) in dry dichloromethane (filtered from the drying agent directly into a cold-jacketed addition funnel that was kept at 0° C) was added rapidly with vigorous stirring under nitrogen. The reaction mixture was stirred at 0° C for 30 min and then allowed to come to RT and stirred overnight. The reaction mixture was diluted with 200 mL of ethyl acetate and filtered through a bed of celite and washed with 1:1 dichloromethane/ethyl acetate (2 x 50 mL). The combined organic layers were washed with water (3 x 200 mL) and dried ($Na_2SO_4$). Removal of the solvent yielded the crude monoalkylated TCAD **(10)** as a pale brown paste. This was used directly in the next step in order to avoid decomposition by adventitious moisture. Yield: 28.57 g (87.8%, crude).

**TLC:** $R_f$ 0.18 (1:1:0.1-$CHCl_3$/I-PrOH/$NH_4OH$).

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.48 (s, 9H, -C-C<u>H</u>$_3$), 2.5-3.0 (m, 21H, -N-C<u>H</u>$_2$ and –C<u>H</u>$_2$-COOBn), 3.8 (t, 1H, -C<u>H</u>-COO-*t*-Bu), 5.15 (q, 2H, -C<u>H</u>$_2$-Ph) and 7.3 (m, 5H, Ar-H).

**MS:** 435.3 $(M+H)^+$.

**[00169]** To a suspension of 4Å molecular sieves (50.0 g) in dry dichloromethane (200 mL), *t*-butyl-(D)-lactate **(11)** (Fluka, 51.4 g, 0.352 mol) and 2,6-lutidine (46.0 g, 0.43 mol) were added with stirring until the solids dissolved. The resulting solution was cooled to -20° C and trifluoromethanesulfonic anhydride (119.1 g, 0.42 mol) in dry dichloromethane (50 mL) was added dropwise under nitrogen with stirring over a period of ~30 min. The reaction mixture was stirred for 1 h more at -20° C,decanted into a separatory funnel, washed with 2N ice-cold HCl (2 x 200 mL), water (2 x 200 mL) and dried. The solvent was evaporated under reduced pressure at RT and the resulting brown liquid was filtered through a 300 g bed of flash silica gel packed in a sintered glass funnel which was washed with 1L of 20% ethyl acetate in hexane. The filtrate was concentrated under reduced pressure at RT to constant weight. The product, *t*-butyl-O-(trifluoromethanesulfonyl)-(D)-lactate **(12)** was obtained as pale yellow oil. The purity of the product was established by silica gel TLC and $^1$H-NMR analysis. Yield: 97.8 g (100%).

**[00170]**

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.5 (s, 9H, -C-C<u>H</u>$_3$), 1.65 (d, 3H, -C<u>H</u>-CH$_3$) and 5.2 (q, 1H, -C<u>H</u>-Me).

**[00171]** A solution of the monoalkylated TACD **10** (38.2 g, 0.063 mol) in dry acetonitrile (300 mL) was treated with 150.0 g of 4Å molecular sieves and stirred while being cooled in an ice-bath. The triflate **12** (97.0 g, 0.35 mol) was added dropwise from an addition funnel under nitrogen with stirring. After the addition, the reaction mixture was allowed to come to RT and stirred for an additional 20 h under nitrogen. The solvent was evaporated under reduced pressure at RT and the residue was dissolved in 500 mL of ethyl acetate and treated with saturated sodium carbonate solution (500 mL). The organic layer was separated and washed with water (2 x 250 mL) and dried ($Na_2SO_4$). Evaporation of the solvent yielded a brown oil which was chromatographed over flash silica gel (2 Kg) using chloroform-methanol as the eluent. The product was eluted with 2% methanol in chloroform to provide fully protected CM-DOTMA **13** as a golden-yellow foam. Yield: 72.2 g (82.8%).

**[00172]**

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.3 (bs, 9H, -CH-C<u>H</u>$_3$), 1.48 (s, 36H, -C-C<u>H</u>$_3$), 2.4-2.8 (m, 18H, -C<u>H</u>$_2$), 3.5 (m, 3H, -C<u>H</u>-Me), 4.2 (d, 1H, -C<u>H</u>-$CH_2$COOBn), 5.2 (AB q, 2H, -C<u>H</u>$_2$-Ar) and 7.4 (m, 5H, Ar-H).

**MS:** 819.9 $(M+H)^+$.

**Elemental Analysis:** Calc. $C_{45}H_{74}N_4F_3O_{13}SNa$: C, 54.53; H, 7.53; N, 5.65; F 5.75; S, 3.24, Na, 2.32%. Found: C, 54.75; H, 7.57; N, 5.54; F, 5.36; S, 3.11; Na, 2.48%.

$H_2$-Pd/C, 50 psi
$CH_3OH:H_2O$ 9:1 v/v
RT, 4 h

<u>13</u> → <u>14</u>

**[00173]** A solution of the fully protected CM-DOTMA **13** (25.0 g, 0.025 mol) in 90% methanol-water (50 mL) was hydrogenated in the presence of Pd/C (5.0 g, 10%; 50% by weight water) at 50 psi for 4 h. The catalyst was filtered off through a pad of Celite™ and washed with 90% methanol-water (2 x 25 mL). The combined filtrates were concentrated

under reduced pressure to obtain CM-DOTMA tetra-t-butyl ester, monoacid **(14)** as a colorless foam. The product was thoroughly dried under vacuum overnight (< 0.1 mm) and used in the next step without further purification. Yield: 19.45 g (85%).

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.2 (m, 9H, CH-C$\underline{H}_3$), 1.5 (s, 9H, C-C$\underline{H}_3$), 2.2-3.2 (m, 18H, -C$\underline{H}_2$), 3.7 (m, 3H, -C$\underline{H}$-Me) and 4.2 (m, 1H, -CH2-C$\underline{H}$-COO-*t*-Bu).

**MS:** 729.5 $(M+H)^+$.

HOOC / N-Boc / H — 15

1.) $Cs_2CO_3$ - $CH_3OH$ / $H_2O$
2.) $PhCH_2Br$ / DMF
RT, 20h

$PhCH_2OOC$ / N-Boc / H — 16

**[00174]** A solution of N-Boc-(L)-biphenylalanine **(15)** (Advanced Chem Tech, 46.0 g, 0.135 mol) in methanol (250 mL) stirred and cesium carbonate (43.88 g, 0.135 mol) in water (250 mL) was added. The solid slowly dissolved to give a pale yellow homogeneous solution. The water and methanol were removed under reduced pressure to give a colorless solid that was dried under vacuum for 2 h. The solid was suspended in dry DMF (500 mL) and cooled in ice. Benzyl bromide (23.0 g, 0.135 mol) was added with stirring under nitrogen and the mixture was stirred for 20 h at RT. DMF was removed under reduced pressure and the residue was taken up in 500 mL of water and extracted with ethyl acetate (5 x 200 mL). The organic layer was washed with saturated aqueous sodium carbonate (1 x 200 mL), water (2 x 200 mL), and dried ($Na_2SO_4$). Removal of the solvent yielded N-Boc-(L)-biphenyl alanine benyl ester **(16)** (56.0 g; 96% yield) as a colorless solid, which did not require further purification.

**[00175]**

**TLC:** $R_f$ 0.75 (silica gel, 2% methanol in $CH_2Cl_2$).

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.42 (s, 9 H, t-$C_4H_9$), 3.15 (m, 2 H, $CH_2CH$), 4.67 (m, 1 H, C*H*), 5.04 (d, 2 H, N*H*), 5.18 (dd, 2 H, $CH_2C_6H_5$) and 7.08-7.55 (m, 14 H, Ar*H*).

**MS:** 432.2 $(M+H)^+$.

[00176] trans-4-Aminomethylcyclohexanecarboxylic acid **(17)** (31.0 g, 0.197 mol) was added to a solution of sodium carbonate (23.32 g, 0.22 mol) in water (350 mL) with stirring. Upon dissolution of the solid the solution was cooled in an ice bath and a solution of di-t-butyldicarbonate (48.0 g, 0.22 mol in 350 mL of THF). was added dropwise with rapid stirring. After the addition, the ice bath was removed and the solution was allowed to stir at RT for 4 h. The volatiles were removed under reduced pressure, the residue was taken up in 500 mL of water, the aqueous solution was extracted with ethyl acetate (4 x 100 mL) and the organic layer was discarded. The pH of the solution was adjusted to ~3.0 by addition of saturated aqueous $KHSO_4$ solution; this operation resulted in the formation of a precipitate. The precipitated solid was filtered off and washed with water (4 x 100 mL) and air-dried. The solid was dissolved in ethyl acetate (500 mL) and washed sequentially with water (3 x 200 mL) and saturated sodium chloride solution (300 mL) and dried ($Na_2SO_4$). Evaporation of the volatiles provided 4-Boc-aminomethylcyclohexane-carboxylic acid **(18)** as a colorless fluffy solid. Yield: 50.67 g (100%).

[00177] Boc-biphenylalanine **(16a)** (13.93 g, 0.03 mol) was added to a stirred solution of phenol (2.5 g) in TFA (50 mL). The mixture was stirred for 30 min at RT and then the TFA was removed under reduced pressure. The residue was triturated with 100 mL of anhydrous ether and the solid that separated was filtered and washed with anhydrous ether (2

x 25 mL). The solid, L-biphenylalanine benzyl ester•TFA (**16b**) was dried under vacuum (< 0.1 mm Hg) for 2 h before use.

**[00178]**

**[00179]** **HPLC:** $t_R$ 8.72 min; Column: YMC C18-ODS 4.6 mm x 250 mm; S-10P µ particle; 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 45% B, then linear gradient 45-95% B over 25 min; Flow rate: 1 mL/min; Detection: UV @ 230 nm.

**$^1$H-NMR (500 MHz, DMSO-d$_6$):** δ 3.05-3.21 (2dd, 2 H, C$H_2$CH), 4.41 (t, 1 H, $CH_2$C$H$), 5.12 (dd, 2 H, C$H_2C_6H_5$), 7.21-7.65 (m, 14 H, Ar$H$) and 8.58 (bs, 3H, $NH_3^+$).

**MS:** 332.2 $(M+H)^+$.

**[00180]**

*trans*-4-N-*t*-Butyloxycarbonylaminomethylcyclohexanecarboxyl-L-biphenylalanine benzyl ester (Boc-a-Bip-OBn) (**19**).

A mixture of Bip-OBn•TFA (**16b**) (9.00 g, 20.21 mmol), HATU [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (11.52 g, 30.32 mmol) and *trans*-4-N-*t*-butyloxy-carbonylaminomethylcyclohexane-carboxylic acid (Boc-a) (17) (5.72 g, 21.23 mmol) in dichloromethane (30 mL) was added diisopropylethylamine (8.00 g, 61.90 mmol) and the solution was stirred at room temperature for 2 h. The reaction mixture was then evaporated on a rotary evaporator to remove dichloromethane and the residue was taken-up in ethyl acetate (200 mL). The organic layer was washed with dil.HCl (0.05 N, 2 x 200 mL) followed by washing with $NaHCO_3$ (5%, 2 x 200 mL) and water (2 x 200 mL). The organic layer was then dried ($Na_2SO_4$), filtered and evaporated to provide a light brown foamy solid in nearly quantitative yield (11.00 g). The material was sufficiently pure for use in the following step.

**TLC:** $R_f$ 0.55 (silica gel, 5% methanol in dichloromethane).

**MS:** 571.4 $(M+H)^+$.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 0.92 (m, 2 H, cyclohex-C$H_2$), 1.45 (s & m, 11 H, *t*-$C_4H_9$ & cyclohex-C$H_2$), 1.60-2.09 (m, 6 H, cyclohex-C$H_2$), 2.95 (m, 2 H, bip-C$H_2$CH), 3.15 (m, 2 H, bocNHC$H_2$), 4.55 (m, 1 H, bip-α*H*), 4.95 (m, 1 H, bocN*H*), 5.18 (m, 2 H, C$H_2C_6H_5$), 5.92 (d, 1 H, CHN*H*CO) and 7.01-7.60 (m, 14 H, Ar*H*).

PhCH$_2$OOC ... NHBoc **19** → 1.) TFA/Phenol 2.) Ether trituration → PhCH$_2$OOC ... $CF_3COO^-$ $NH_3^+$ **20**

**[00181]** N-(*trans*-4-aminomethylcyclohexanecarbonyl)-L-biphenylalanine benzyl ester trifluoroacetic acid salt (**20**).

**[00182]** To a solution of phenol (4.1g, 0.0432 mol) in anhydrous TFA (150 mL), N-(*trans*-4-N-Boc-aminomethylcyclohexanecarbonyl)-L-biphenylalanine benzyl ester **(19)** (12.32 g, 0.0216 mol) was added and the mixture was stirred at RT for 30 min. TFA was removed under reduced pressure and the residue was triturated with anhydrous ether (250 mL). Compound **(20)** that separated as a solid was filtered, washed with anhydrous ether (4 x 50 mL) and dried at RT under high vacuum (4 h, 0.05 mm). It was used in the next step without purification.

**HPLC:** $t_R$ 9.50 min (Column: YMC C18, 4.6 mm x 250 mm, S-10P μm particle, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 45% B, then linear gradient to 95% B over 25 min; Flow rate: 1.0 mL/min, Detection: UV @ 230 nm.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 0.94 (m, 2 H, cyclohex-C$H_2$), 1.45 (m, 2 H, cyclohex-C$H_2$), 1.60-2.15 (m, 6 H, cyclohex-C$H_2$), 2.75 (m, 2 H, bip-C$H_2$CH), 3.15 (m, 2 H, bocNHC$H_2$),

4.85 (m, 1 H, bip-α*H*), 5.18 (m, 2 H, $CH_2C_6H_5$), 5.92 (d, 1 H, CHN*H*CO) and 6.91-7.71 (m, 14 H, Ar*H*).

**MS:** 471.3 $(M+H)^+$.

BnOOC H NH₂ O N H H H 20

t-BuOOC COOH H t-BuOOC N COO-t-Bu N N 14 $H_3C$ H N H $CH_3$ $H_3C$ COO-t-Bu H

HATU / DIEA / $CH_2Cl_2$ -10° C, 1 h

BnOOC O H N O COO-t-Bu H H H N H t-Bu-OOC N COO-t-Bu N N H $H_3C$ H N $CH_3$ 21 t-Bu-OOC $CH_3$ H

**[00183]** CM-DOTMA acid **(14)** (19.45 g, 0.0216 mol) was dissolved in anhydrous dichloromethane (100 mL) and cooled to –10° C under a nitrogen atmosphere. HATU (9.12 g, 0.024 mol) was added followed by diisopropylethylamine (3.1 g, 0.024 mol) and the mixture was stirred for 10 min at –10° C. A suspension of N-(*trans*-4-aminomethylcyclohexanecarbonyl)-L-biphenylalanine benzyl ester trifluoroacetic acid salt **(20)** and diisopropylethylamine (3.1 g, 0.024 mol) in anhydrous dichloromethane (50 mL) was added in one lot to the activated acid above and the reaction was continued for 1 h at –10° C. The solution was cloudy after the addition of the amine but gradually clarified in about 5 min. At the end of the reaction the solvent was removed under reduced pressure and saturated aqueous sodium carbonate (500 mL) was added to the residue.. The aqueous mixture was extracted with ethyl acetate (5 x 200 mL) and then combined. The organic layer was washed with water (3 x 200 mL), saturated sodium carbonate (2 x 200 mL), and dried ($Na_2SO_4$). Removal of the solvent yielded a brown paste (25.0 g) which was chromatographed over flash silica gel (2 Kg). Elution with 2% methanol in chloroform yielded the product as a golden yellow paste (22 g, 86% yield). Though the resultant product CE-DOTMA-a-B **(21)** was homogeneous by silica gel TLC analysis, HPLC analysis indicated a purity of only 80%. Repeated attempts to further purify the material were unsuccessful. It was decided to purify at the next stage in the sequence.

**TLC:** $R_f$ 0.41 (9:1, $CHCl_3$/MeOH).

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 0.92, (m, 2H), 1.23 (d, 9H, CH-C<u>H</u>$_3$), 1.48 (2s and a m, 38H, -*t*-butyl methyls and –C<u>H</u>$_2$), 1.6-3.2 (m, 28H, -N-C<u>H</u>$_2$ and other-C<u>H</u>$_2$s), 3.6 (m, 3H, -C<u>H</u> - (Me)-COO-*t*-Bu), 4.2 (dd, 1H, -C<u>H</u>-$CH_2$-CO-NH), 4.9 (m, 1H, -C<u>H</u>-COOBn), 5.2 (Abq, 2H, -$CH_2$-Ph), 5.9 (d, 1H, -N<u>H</u>-CH-COOBn), 6.5 (t, 1H, -N<u>H</u>-CO-$CH_2$), 7.1 (d, 2H, Ar-H), 7.3 (m, 6H, Ar-H), 7.4 (m, 4H, Ar-H) and 7.55 (m, 2H, Ar-H).

**MS:** 1181.8 $(M+H)^+$.

HH

21 → 22

1.) H2-Pd/C, 50 psi
CH3OH:H2O 9:1, v/v
RT, 4h
2.) TFA:Anisole 8:1, v/v
RT, 20h

**[00184]** A hydrogenation bottle was charged with a solution of fully protected CM-DOTMA-a-B **(21)** (19.0 g, 0.016 mol) in $CH_3OH$:Water 9:1v/v , (100 mL) and Pd/C (10% Pd content, 50% water by weight, 3.8 g). The vessel was evacuated and refilled with hydrogen several times. Then the hydrogenation was conducted for 4 h at 50 psi at ambient temperature. . The catalyst was filtered off through a bed of Celite™ and washed with $CH_3OH$:$H_2O$ 9:1 v/v (2 x 20 mL). The combined filtrate was concentrated under reduced pressure to a foam which was kept under high vacuum (< 0.05 mm) for 4 h. The foam was then dissolved in anhydrous TFA (200 mL) containing anisole (25 mL) and stirred for 20 h at RT. The volatiles were removed under reduced pressure and the residue was triturated with 500 mL of anhydrous ether. The solid that precipitated was filtered off and washed with anhydrous ether (2 x 100 mL). The analytical HPLC chromatogram of the product CE-DOTMA-a-B ligand **(22)** (14.1 g; > 100%) displayed two peaks when a linear gradient system or an isocratic elution method was employed. Attempted purification by preparative

HPLC (several runs) provided only low yields of the predominant product.. Hence it was decided to form the Gd chelate and then purify the chelate.

**[00185]**

**HPLC:** $t_R$ 17.15 min (major isomer), 15.27 (minor isomer); Column: YMC C18-ODS; 4.6 mm x 250 mm, S-10P μ; 120Å; . Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA);Elution: Isocratic 30% B.

**$^{13}$C-NMR (DMSO-d$_6$):** δ 28.65, 29.53, 29.65, 36.36, 37.03, 43.68, 44.86, 44.98, 45.39, 45.62, 45.71, 45.77, 45.81, 45.9, 45.93, 45.98, 47.09, 47.2, 47.31, 47.37, 47.47, 47.64, 53.18, 57.09, 58.41, 126.4, 126.5, 127.3, 129.8, 137.2, 138.2, 140.0, 158.1, 158.3, 169.8, 171.8, 173.3 and 175.3.

**MS:** 867.6 $(M+H)^+$.

**Elemental Analysis:** Calc. $C_{44}H_{62}N_6O_{12}$•1.8 TFA•4 $H_20$ ; C, 49.96; H, 6.32; F, 8.97; N, 7.34; O, 27.40%. Found: C, 50.15; H, 6.02; F, 8.49; N, 7.06%.

**[00186]**

1.) Gd(OAc)3, N-methyl-(D)-glucamine pH 4-6 28 h
2.) N-methyl-(D)-glucamine pH 9, filter
3.) Preparative HPLC

22 → 23 •2 N-methyl-(D)-glucamine

A solution of the ligand **(22)** (14.1 g, 0.012 mol; calculated based on CMDOTA-a-B•2 TFA•$2H_2O$) in water (200 mL) was treated with solid Gd $(OAc)_3$ tetrahydrate (6.09 g, 0.015 mol) and a saturated solution of N-methylglucamine was added with stirring until the pH of the solution was adjusted to 6.0. The reaction mixture was then allowed to stir at RT and the pH of the reaction mixture was monitored hourly. Initially, the pH dropped to about 4 and then was brought back to 6 by the addition of meglumine; after 8 h the pH of the reaction mixture was stable (pH 6.0). The reaction was continued for 20 h more at RT and then the

pH of the reaction was adjusted to 9.0 addition of aqueous meglumine. The precipitated gadolinium oxide was filtered off using a 0.22 μ filter and the filtrate was purified by preparative HPLC to obtain Gd-CEDOTMA-a-B **(23)** (4.2 g, 34% yield of the major isomer).

**HPLC:** Analytical: $t_R$ 19.89 min (purity >99%). Column: YMC C18-ODS 250 mm x 4.6 mm; S-10P μ; 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Isocratic 30% B for 30 min; Flow Rate: 1mL/min; Detection: UV @ 230 nm; Fluorescence: 280 Ex, Em 320 nm.

Preparative: Column: 50 mm X 250 mm YMC C18- ODS; S-10P μ; 120Å Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 0% B, then linear gradient 0-60% B over 60 min.Flow rate: 75 mL/min, Detection: UV @ 230 nm;

**MS:** 1022.4 $(M+H)^+$.

**Elemental Analysis:** Calc. for $C_{44}H_{59}N_6O_{12}Gd{\bullet}2(C_7H_{17}NO_5){\bullet}3.2H_2O$: C, 47.41; H, 6.82; N, 7.63; O, 27.44; Gd, 10.70%. Found: C, 47.89; H, 6.68; N, 7.60; Gd, 11.02; $H_2O$, 4.09%.

**[00187]** The final product **(23)** was then screened using an IBM PC20-minispec relaxometer for relaxivity in water and Seronorm™, and for HSA binding by the centrifree micro partition method. As in Example 1, the following were tested: mouse tolerance, MRA in rabbits, blood T1 and imaging in rabbits and plasma T1 lowering *ex vivo* in monkeys. The compound tested displayed an acceptable tolerance profile in mice (MLD = 3 mmol/kg) Results appear in Table 1 and Table 5.

## EXAMPLE 4 –Synthesis and Screening of Gd-TE-DOTA-a-B Meg (Class 1)

[00188] The compound was prepared as follows: To a solution of **1** (100 mg, 0.192 mmol) in $H_2O$ (1.5 mL) was added $Gd(OAc)_3 \cdot 4H_2O$ (85.7 mg, 0.21 mmol, Aldrich) in $H_2O$ (1.5 mL). The pH of the solution was maintained at 4.5 by adding 1N NaOH and it was stirred at RT for 2 days. It was directly used for the next step without further purification. [For related compounds see: Jacques, Vincent; Gilsoul, Dominique; Comblin, Vinciane; Desreux, Jean F. Rigidified macrocyclic lanthanide chelates for magnetic resonance imaging. Journal of Alloys and Compounds (1997), 249(1-2), 173-177. Desreux, Jean F.; Tweedle, Michael F.; Ratsep, Peter C.; Wagler, Thomas R.; Marinelli, Edmund R. Preparation of hepatobiliary tetraazamacrocyclic magnetic resonance contrast agents. U.S. (1994), 12 pp. US 5358704 A 19941025]

**HPLC:** $t_R$ 6.5 min. Column: YMC C18 4.6 mm x 250 mm, S-10P µm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Isocratic 4% B; Flow rate: 1.0 ml/min; Detection: UV @ 230 nm.

**MS:** 677.1 $(M+H)^+$.

[00189] To a crude mixture of **(2)** in $H_2O$ (3 mL) was added thiophosgene (44 mg, 0.384 mmol) in $CHCl_3$ (1.5 mL) in one portion. The resulting mixture was stirred at RT for 2h. The process was monitored by HPLC. The layers were separated and the pH of the aqueous layer was adjusted from 1.8 to 7 by adding 1N NaOH. This solution was directly used for the next step without further purification.

**HPLC:** $t_R$: 6.3 min; Column: YMC C18 4.6 mm x 250 mm, S-10P µm, 120Å;

Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA), Elution: Isocratic 20% B; Flow rate 1.0 ml/min.; Detection: UV @ 230 nm.

**MS:** 719.1 $(M + H)^+$.

**[00190]** To a solution of **4** (40 mg, 0.081 mmol) in MeOH (5 mL) was added $Et_3N$ (8.2 mg, 0.081 mmol). The solvent was evaporated and THF (2 mL) was added. This suspension was added to the above crude **3** in $H_2O$ and the suspension was stirred at RT for 16 h. $CH_3CN/H_2O$ (1:1, v/v; 2 mL) was added and the solution became homogeneous. The pH of the solution was adjusted from 6 to 9 and it was stirred at RT for 16 h (final pH of the solution was 7). It was purified by prep. HPLC using a YMC C-18 (250 mm x 20mm) reverse phase HPLC column. The column was eluted with 5% $CH_3CN/H_2O$ (both containing 0.05% TFA) for 5 min., 5-20% in 10 min., and finally 20-50% in 60 min. The desired fractions were collected and lyophilized. This gave 25 mg of the product with purity of 89% (HPLC). The material was then purified by DEAE-sephadex resin. The compound in 5 mM triethylammonium bicarbonate solution was loaded onto the reconditioned resin and it was eluted with a gradient of aqueous triethylammonium bicarbonate solution (5 mM-800 mM, ) total volume of 360 mL). The desired fractions were combined and lyophilized to give 25 mg of **(5)** (97% pure as determined by analytical HPLC), as a white fluffy solid.

**HPLC:** $t_R$: 6.7 min; Column: YMC C18 4.6 mm x 250 mm, S-10P μm, 120Å; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA);Elution: Isocratic 40% B, Flow rate: 1.0 mL/min; Detection: UV at 230 nm.

**MS:** 1099.4 $(M + H)^+$.

**[00191]** BioRad AG 50-X8 ion exchange resin (100 mL) in a glass column fitted with a conductivity meter at the column outlet, was reconditioned as follows: The column was washed with 400 mL of 3 N HCl, $H_2O$ (to pH 7, 400 mL of 2 N NaOH, $H_2O$ to pH 7, then 400 mL of 3 N HCl and finally rinsed with $H_2O$ to pH 7. N-methyl-D-glucamine (35 g in 100 mL of $H_2O$) was passed through the column; then the column was washed with a large excess of $H_2O$ until the conductivity was <0.9μS/cm The Gd-chelate **(5)** (25 mg) in 20 mL of $H_2O$ was applied to the column and the eluate was monitored by UV at 230 nm, employing a

Knauer Variable Wavelength UV Detection using a 0.4 mm flow cell. The fractions containing the compound were combined and lyophilized. A total of 25 mg of the compound **6** was obtained as a white fluffy solid.

**HPLC:** $t_R$ 6.8 min; Column: YMC C18 4.6 x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: 40% B; Flow rate: 1.0 mL/min; UV @ 230 nm.

**MS:** $(M+H)^+$ 1099.4.

**[00192]** After synthesis, the compound was evaluated as described in Examples 1-3 above. Results are shown in Table 1.

## EXAMPLE 5 – Synthesis and Screening of Gd(CE-DTPA)-a-B 3Meg (Class 1)

The compound was prepared as follows: To a solution of **1** (7.0 g, 11.75 mmol) in $CH_2Cl_2$ (120 mL) was added **2** (11.6 g, 15.3 mmol,) (L-Glutamic acid and L-lysine as useful building blocks for the preparation of bifunctional DTPA-like ligands. Anelli, Pier Lucio; Fedeli, Franco; Gazzotti, Ornella; Lattuada, Luciano; Lux, Giovanna; Rebasti, Fabrizio.

Bioconjugate Chemistry (1999), 10(1), 137-140), HATU [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (5.95 g, 15.3 mmol), and $Et_3N$ (3.1 g, 30.9 mmol, Aldrich). The clear solution was stirred at room temperature for 3 h. The volatiles were evaporated and the residue was dissolved in EtOAc (1000 mL). It was washed with 5 % $NaHCO_3$ (2 x 400 mL), 0.05 N HCl (2 x 400 mL), $H_2O$ (1 x 200 mL), and dried ($MgSO_4$). Evaporation of solvent afforded 16.7 g (excess of theory) of **3** (containing some ethyl acetate).

**MS**: 1199 $(M+H)^+$.

**HPLC:** $t_R$ 27.2 min; Column: YMC C18 4.6 x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 45% B, then linear gradient to 95% B over 25 min.; Flow rate: 1.0 ml/min.; Detection: UV @ 230 nm.

To a solution of **3** (16.7 g, 13.9 mmol) in EtOH (80 mL) was added palladium, on carbon (10 wt. % (dry basis), wet, Degussa type E101 NE/W, 1.4 g, Aldrich). The mixture was hydrogenated at 45 psi for 20 h. The catalyst was filtered through a Celite™ cake and the volatiles were evaporated to provide 15.3 g (99% yield) of compound **4**.

**HPLC:**$t_R$: 21.3 min. Column: YMC C18, 4.6 x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition:45% B, then linear gradient to 95% B over 25 min.;; Flow rate: 1.0 ml/min.; Detection: UV @ 230 nm.

**[00193]**

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 0.95-1.85 (m, 8H, $CH_2$'s on cyclohexyl); 1.40 (s, 45H, t-Bu's; 2.05 (m, 2H, $\underline{CH_2}CH_2$CONH-); 2.15-2.25 (m, 1H, $CONHCH_2\underline{CH}$ on cyclohexyl); 2.40-2.50 (m, 1H, $\underline{CH}$CONH on cyclohexyl); 2.55-2.65 (m, 2H, $CH_2\underline{CH_2}$CONH-); 2.65-2.80 (m, 8H, $NCH_2$'s); 3.00-3.15 (m, 2H, $CH_2$ on biphenyl); 3.15-3.38 (m, 2H, $CH_2CH_2CONH\underline{CH_2}$); 3.48-3.32 (m, 9H, $NCH_2CO_2$tBu & $NHCO_2$tBu); 4.79-4.85 (m, 1H, $NH\underline{CH}CO_2$Bn); 6.19 (d, 1H, $CO\underline{NH}$CH); 6.87 (m, 1H, $CO\underline{NH}CH_2$); 7.00-7.53 (m, 14H, CH's on biphenyl and benzyl).

**MS:** 1109 $(M+H)^+$.

**[00194]** Compound **4** (15.3 g, 13.8 mmol) was dissolved in 400 mL of TFA/$H_2O$/triisopropylsilane (95:2.5:2.5, v/v/v). The mixture was stirred at room temperature for 4 h. The volatiles were evaporated to provide a pasty residue. Ether (400 mL) was added, the mixture was stirred for 10 min and the precipitate was filtered. This was repeated 3X to remove all the organic impurities. The grayish solid was dried *in vacuo* to give 12.5 g of a crude material. This was dissolved in $CH_3CN$:$H_2O$:1N HCl 1:4:1 v/v/v and purified by preparative HPLC employing a Rainin Dynamax-60 A C-18 column 250 mm x 41.4 mm equipped with a guard column.. The column was eluted at 50 mL/min and a gradient elution ($CH_3CN$/$H_2O$ (both containing 0.1 % TFA) 15 % to 75 % over 120 min) was employed. The product-containing fractions were lyophilized to give pure **5** as a white fluffy solid (7.3 g, 53% yield).

**HPLC:** $t_R$ 12.3 min; Column: YMC C18 4.6 x 250 mm, S-10P μ, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 25% B , then linear gradient to 65% B over 20 min.; Flow rate: 1.0 mL/min.; Detection: UV at 230 nm.

**$^1$H-NMR (500 MHz, $D_2O$):** δ 0.85-1.70 (m, 9H, $CH_2$'s on cyclohexyl & CONHCH$_2$<u>CH</u> on cyclohexyl); 1.85-1.92 (m, 1H, <u>CH</u>CONH on cyclohexyl); 1.85-1.95 (m, 2H, <u>CH$_2$</u>CH$_2$CONH-); 2.30-2.40 (m, 2H, CH$_2$<u>CH$_2$</u>CONH-); 2.90-3.10 (m, 8H, NCH$_2$'s); 3.35-3.40 (m, 4H, $CH_2$ on biphenyl & CH$_2$CH$_2$CONH<u>CH$_2$</u>); 3.48-3.52 (m, 1H, NH<u>CH</u>CO$_2$H); 3.92-3.40 (s, 8H, NCH$_2$COOH); 4.60-4.65 (m, 1H, CONHCHCOOH); 7.30-7.65 (m, 9H, CH's on biphenyl).

**MS:** 828.4 $(M+H)^+$.

**[00195]** To a suspension of **5** (6 g, 6.0 mmol) in $H_2O$ (300 mL) was added 1N NaOH solution to adjust the pH to 5. Then a solution of $Gd(OAc)_3$ (3.3 g, 8.0 mmol) in $H_2O$ (10 mL) was added and the pH of the mixture was maintained at 5 by adding 1N NaOH. The cloudy solution was stirred at room temperature for 16 h. This solution was purified by preparative HPLC employing a Rainin Dynamax-60 A C-18 column (250 mm x 41.4 mm) equipped with guard column.. The column was eluted at 50 mL/min and a gradient elution (0% $CH_3CN$:$H_2O$ v/v over 10 min., then ramp from 0-20 % over 10 min., then ramp to 75 %

over 30 min) was employed. Pure product-containing fractions were lyophilized to give compound **6** as a white fluffy solid (4.8 g, 81.3% yield).

**HPLC:** $t_R$ 12.2 min; Column: YMC C18 4.6 x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 25% B, then linear gradient to 65% B over 20 min., Flow rate: 1.0 ml/min.; Detection: UV at 230 nm.

**MS:** 983.3 $(M+H)^+$.

**Elemental Analysis:** Calc. for $C_{40}H_{47}N_5O_{14}GdNa_3{\cdot}9H_2O$: C, 39.70; H, 5.41; N, 5.79; Na, 5.70; Gd 12.99. Found: C, 39.75; H, 5.29; N, 5.72; Na, 5.45; Gd, 13.15.

**[00196]** A glass column was packed with 100 mL of BioRad AG50W-X8 ion exchange resin and the outlet was connected to a conductivity meter. The column was washed with 400 mL of 3N HCl, rinsed with $H_2O$ to pH 7, washed with 400 mL of 2N NaOH, rinsed with $H_2O$ to pH 7, then washed with 400 mL of 3N HCl and then rinsed with $H_2O$ until pH 7. Next, the column was washed with a solution of 70 g of N-methyl-D-glucamine in 300 mL of $H_2O$ followed by distilled deionized water until the conductivity was <0.9 μS/cm. The Gd-chelate **6** (3.6 g, 3.7 mmol) in 400 mL of $H_2O/CH_3CN$ 80/20 (v/v) was applied to the column and the eluate was monitored by UV at 230 nm. The product-containing fractions were combined and lyophilized. A total of 5.3 g (88% yield) of the desired meglumine salt **7** was obtained as a white fluffy solid.

**HPLC:** $t_R$ 12.3 min.Column: YMC C18, 4.6 x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 25% B, then linear gradient to 65% B in 20 min., Flow rate: 1.0 mL/min; Detection: UV @ 230 nm.

**MS:** 983.4 $(M+H)^+$.

**Elemental Analysis:** Calc. for $C_{61}H_{101}N_8O_{29}Gd{\cdot}3H_2O$: C, 45.18; H, 6.65; N, 6.91; Gd, 9.70. Found: C, 44.88; H, 6.59; N, 6.72; Gd, 9.95.

[00197] The compound was analyzed as described above in Examples 1-4. Results are shown in Tables 1 and 5.

## EXAMPLE 6 – Synthesis of Gd-(7-MP-DO3A)-GaB (Meglumine salt) (Class 1)

[00198] To a solution of 1,4,7,10-tetraazacyclododecane (TACD) (21.5 g, 0.125 mol) in $CHCl_3$ (200 mL) was added benzyl-2-bromoacetate (11.45 g, 7.92 mL, 0.05 mol) in $CHCl_3$ (50 mL) dropwise over a period of 2 h and the reaction mixture was stirred at 4°C for 24 h. Chloroform was evaporated and the residue was treated with a saturated solution of sodium bicarbonate (200 mL). The mixture was extracted with methylene chloride and the organic phase was washed with water. The organic layer was dried with sodium sulfate. Methylene chloride was removed to give a viscous oil, which was vacuum-dried for 24 h to provide crude 1-benzyloxycarbonylmethy-1,4,7,10-tetraazacyclododecane **(1)** (12.0 g, 75% yield) which was used in the next step without further purification.

**$^1$H-NMR (500 MHz, $CDCl_3$):** $\delta$ 2.55 and 2.75 (m, 16 H, $NC\mathit{H}_2C\mathit{H}_2N$), 3.48 (s, 2 H, $NC\mathit{H}_2CO_2CH_2Ph$),5.12(s, 2 H, $C\mathit{H}_2Ph$), 7.32 (m, 5 H, Ar*H*).

**MS:** 321 $(M+H)^+$.

**[00199]** A round-bottomed flask was charged with the benzyl ester **1** (11.0 g, 0.034 mol) in toluene (300 mL) and dimethylformamide diethyl acetal (7.3 g, 8.5 mL, 0.049 mol). The mixture was heated at 85°C for 12 h with stirring. The volatiles were evaporated on a rotary evaporator to provide a yellow oil which was treated with dry ether (200 mL). A gummy solid formed during the addition of ether and the yellow ether solution was filtered through Celite™. The filter cake was washed with ether (2 x 100 mL). The combined ether washings were concentrated to give crude 1-benzyl (1,4,7,10-tetraazatricyclo[5.5.1.0<4,1,3>-tridec-10-yl)acetate **(2)** (10.2 g, 89% yield) as a thick yellow oil which was used in the next step without further purification.

**$^1$H-NMR (500 MHz, $CDCl_3$):** $\delta$ 2.60 and 3.09 (m, 16 H, $NC\mathit{H}_2C\mathit{H}_2N$), 3.52 (s, 2 H, $NC\mathit{H}_2CO_2CH_2Ph$), 4.81 (s, 1 H, CH), 5.12 (s, 2 H, $C\mathit{H}_2Ph$), 7.32 (m, 5 H, Ar*H*).

**MS:** 331 $(M+H)^+$.

**[00200]** A solution of benzyl (1,4,7,10-tetraazatricyclo[5.5.1.0<4,1,3>-tridec-10-yl)acetate **(2)** (10.0 g, 0.06 mol) in THF (100 mL) and water (25 mL) was stirred at room temperature for 18 h. The volatiles were removed under vacuum and the light yellow oil obtained was dried under vacuum to provide compound **3** (9.0 g, 85.7%) which was used in the next step without further purification.

**$^1$H-NMR (500 MHz, $CDCl_3$):** $\delta$ 2.60-2.9 and 3.4-3.9 (m and s, 18 H, $NC\mathit{H}_2C\mathit{H}_2N$ and $NC\mathit{H}_2CO_2CH_2Ph$ ), 5.12 (s, 2 H, $C\mathit{H}_2Ph$), 7.32 (m, 5 H, Ar*H*), 8.12 (s, 1H, CHO).

**MS:** 349.2 $(M+H)^+$.

**[00201]** A stirred solution of 1-benzyloxycarbonylmethy-7-formyl -1,4,7,10-tetraazacyclododecane **(3)** (9.0 g , 0.0258 mol) in acetonitrile (80 mL) was treated with diisopropylethylamine (13.0 g , 17.6 mL, 0.1 mol) and t-butyl bromoacetate (11.6 g, 8.8 mL, 0.06 mol) RT for 24 h. The volatiles were removed on a rotary evaporator and the residue was treated with water (150 mL) and the entirety of the contents of the flask was extracted with ethyl acetate (250 mL). The ethyl acetate layer was separated and washed sequentially with aqueous $NaHCO_3$ solution (200 mL), water (200 mL) and dried ($Na_2SO_4$). The volatiles were removed and the oil obtained was chromatographed over silica gel. The column was packed with hexane-ethyl acetate (7:3, v/v) and eluted sequentially with hexane-ethyl acetate (7:3, v/v; 600 mL), hexane-ethylacetate (1:1 v/v; 600 mL), and finally with pure ethyl acetate. The product-containing fractions were collected, the volatiles removed and the residue was dried under vacuum to give **4** (8.7 g, 59% yield) as a thick yellow oil.

**[00202]**

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.45 (s, 18 H, $C_4H_9$), 2.65-2.95 (m, 12 H, $NCH_2CH_2N$), 3.42 and 3.49 (m, 2H, $NCH_2CH_2N$, $NCH_2CO_2CH_2Ph$ and $NCH_2CO_2C_4H_9$), 5.12 (s, 2 H, $CH_2Ph$), 7.32 (m, 5 H, Ar*H*), 8.02 (s, 1 H, CHO).

**MS:** 577.5 $(M+H)^+$.

**HRMS (FAB):** m/z, Calc. for $C_{30}H_{48}N_4O_7$ 599.3421 $(M+Na^+)$; Found: 599.3422.

**[00203]** A mixture of 1-benzyloxycarbonylmethy-7-formyl- 4,10-di-t-butoxycarbonylmethyl-1,4,7,10-tetraazacyclododecane **(4)** (5.0 g, 8.6 mmol), hydroxylamine hydrochloride (0.8 g, 11.5 mmol) and water (0.3 mL) in isopropanol (35 mL) was refluxed for 12 h. The progress of the reaction was monitored by TLC using $CH_2Cl_2$:$CH_3OH$ (95:5, v/v). At completion of the reaction the isopropanol and water were removed on a rotary evaporator and the resulting viscous oil was dried under vacuum. This was treated with chloroform (200 mL) and the light precipitate that formed was filtered and the chloroform solution was concentrated to a viscous oil. The oil was dried under vacuum to give a foamy solid. This was triturated with ether (75 mL) and the precipitated white solid was filtered and dried to provide 3.52 g (74% yield) of benzyl ester **5**.

**$^1$H-NMR (500 MHz, CDCl$_3$):** δ 1.49 (s, 18 H, $C_4H_9$), 2.60-2.9 (m and 16 H, $NCH_2CH_2N$), 3.45 (s, 4 H, $NCH_2CO_2C_4H_9$), 3.52 (s, 2 H, $NCH_2CO_2CH_2Ph$), 5.12 (s, 2 H, $CH_2Ph$), 7.32 (m, 5 H, Ar*H*).

**MS:** 549 $(M+H)^+$.

**[00204]** To a mixture of the benzyl ester **5** (3.0 g,5.47 mmol) and diisopropylethylamine (3.25 g, 4.38 mL, 25 mmol) in acetonitrile (10 mL) was added the triflate **A** (Phillion, D. P., US 4740608 (1988)), (3.0 g, 8.42 mmol) in acetonitrile (5 mL). The mixture was stirred at 40°C for 6 h. After the reaction, acetonitrile was evaporated and the residue was treated with a saturated solution of sodium carbonate (25 mL). The mixture was extracted with ethyl acetate (2 x 100 mL), washed with water (100 mL) and dried ($Na_2SO_4$). The ethyl acetate was evaporated and the oil obtained was dried under vacuum to provide a foam which was triturated with hexane (3 x 75 mL) at 50°C to give 1-benzyloxycarbonymethyl-4,10-di-t-butoxycarbonymethyl-7-di-t-butoxyphosphinylmethyl-1,4,7,10-tetraazacyclododecane **(6)** (3.0 g, 73% yield) as a white solid. An analytical sample was obtained by crystallization from hexane-ethyl acetate.

**$^1$H-NMR (500 MHz, CDCl$_3$):** δ 1.42 (s, 18 H, $C_4H_9$), 1.51(s, 18 H, $O{=}PC_4H_9$), 2.10-2.95 (m, 24 H, $NCH_2CH_2N$, $NCH_2CO_2$ and $NCH_2PO$), 5.10 (s, 2 H, $CH_2C_6H_5$), 7.35 (m, 5 H, $CH_2C_6H_5$).

**MS:** 755.5 $(M+H)^+$.

**HRMS (FAB):** m/z, Calcd. for $C_{38}H_{67}N_4O_9PNa$ 777.4543 (M+Na); Found: 777.4599.

**[00205]** To a solution of 1-benzyloxycarbonymethyl-4,10-di-t-butoxycarbonymethyl-7-di-t-butoxyphosphinylmethyl-1,4,7,10-tetraazacyclododecane **(6)** (1.0 g, 0.0078 mmol) in THF (20 mL) was added Pd-C 10% (0.5 g, Degussa type ~ 50% water), and the mixture was hydrogenated at 45 psi for 12 h. The catalyst was filtered through Celite™ and the filter cake

was washed with THF (2 x 30 mL). The combined THF washings were concentrated on a rotary evaporator to give the acid as a thick, viscous oil which was dried under vacuum for 24 h to give 1-carboxymethyl-4,10-di-t-butoxycarbonymethyl-7-di-t-butoxyphosphinylmethyl-1,4,7,10-tetraazacyclododecane **(7)** (0.84 g, 95% yield) as a foamy solid which was used without further purification.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.42 (s, 18 H, $C_4H_9$), 1.51(s, 18 H, O=$PC_4H_9$), 2.50-2.95 (m, 24 H, $NCH_2CH_2N$ and $NCH_2CO_2$ and $NCH_2PO$).

**MS (FAB):** 687 $(M+Na)^+$.

**HRMS (FAB):** m/z, Calc. for $C_{31}H_{61}N_4O_9P$ 665.4254 $(M+H^+)$; Found: 665.4256.

**[00206]** To a cooled (0°C) mixture of 1-carboxymethyl-4,10-di-t-butoxycarbonymethyl-7-di-t-butoxyphosphinylmethyl-1,4,7,10-tetraazacyclododecane **(7)**, (0.832 g; 1.0 mmol) and HATU (0.38 g, 1.0 mmol) in $CH_2Cl_2$ (10 mL) was added DIEA (0.39 g, 0.55 mL, 3.0 mmol) and the mixture was stirred at 0°C for 5 min. The tripeptide **8** (0.51 g, 0.967 mmol) was added and the mixture was stirred at 0°C for 1 h. The volatiles were evaporated and the residue was treated with $NaHCO_3$ solution (10 mL) and the resulting mixture was extracted with $CH_2Cl_2$ (2 x 15 mL). The organic phase was washed with water and dried ($Na_2SO_4$). Evaporation of the volatiles gave an oil, which was vacuum-dried to give a foamy solid which was triturated with water to provide a granular solid which was filtered. The solid was again triturated with water, filtered and air-dried to provide compound **9** (1.12 g, 97% yield) which was used directly in the next step.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 0.942 (m, 2 H, cyclohex ), 1.42 (s, 18 H, $C_4H_9$), 1.51 (s, 18 H, O=$PC_4H_9$), 1.62-3.4 (m, 32 H, $NCH_2CH_2N$, cyclohex, $NCH_2CO_2$ and $NHCOCH_2NH$), 4.92 (m, 1 H, $CHCH_2Ar$), 5.15 (q, 2 H, $CH_2C_6H_5$), 6.0 (d, 1 H, CONHCH), 6.75 and 6.9 (2 x bt, 2 H, $CONHCH_2$), 7.0-7.55 (m, 14 H, ArH).

**MS:** 1197 $(M+Na)^+$.

**[00207]** To a solution of compound **9** (1.12 g, 0.95 mmol) in THF (15 mL) was added Pd-C 10% (0.5 g, Degussa type ~ 50% water), and the mixture was hydrogenated at 45 psi for 12 h. The catalyst was filtered through Celite™ and the filter cake was washed with THF (2 x 30 mL). The combined THF washings were concentrated on a rotary evaporator to give the product **10** as a thick, viscous oil which was vacuum-dried for 24 h to give **10** (0.92 g, 85% yield) as a foamy solid..

**$^{1}$H-NMR (500 MHz, $CDCl_3$):** δ 0.94 (m, 4 H, cyclohex ), 1.42 (s, 18 H, $C_4H_9$), 1.51(s, 18 H, $O{=}PC_4H_9$), 1.62-3.4 (m, 32 H, $NCH_2CH_2N$, cyclohex, $NCH_2CO_2$, $NCH_2PO$ and $NHCOCH_2NH$), 4.80 (m, 1 H, $CHCH_2Ar$), 6.45 (m, 1 H, CHNHCO) 6.75 and 7.10 (2 x bt, 2 H, $CONHCH_2$), 7.28-7.62 (m, 9 H, ArH).

**MS:** 1107 $(M+Na)^+$.

**[00208]** TFA (1.5 mL) was added to compound **10** (0.35 g, 0.32 mmol) and the mixture was stirred at RT for 6 h. Ether (50 mL) was then added and the precipitated solid was filtered and dried under vacuum to give compound **11** (0.25 g, 72% yield) as a white solid.

**HPLC:** $t_R$ 25.8 min Column: YMC C18, 4.6 x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 0% B, then linear gradient to 80% B over 40 min; Flow rate: 1.0 mL/min, Detection: UV @ 230 nm.

**$^{1}$H-NMR (500 MHz, DMSO$d_6$):** δ 0.81-2.1 (m, 14 H, cyclohex, $NHCH_2CO$ and $CONHCH_2CO$) 2.75-3.7 (m, 16 H, $NCH_2CH_2N$), 3.48 (s, 2 H, $NCH_2CO_2CH_2Ph$),5.12 (s, 2 H, $CH_2Ph$), 7.32 (m, 5 H, Ar*H*).

**MS:** 860 $(M+H)^+$.

**[00209]** To a solution of compound **11** (0.25 g, 0.23 mmol) in water (5 mL) $Gd(OAc)_3$ (0.125 g, 0.3 mmol) in water (2 mL) was added. The initial pH was 2.57 and was adjusted to 5.0 by addition of 1N NaOH solution. The solution became turbid and the mixture was

allowed to stir for 24 h. The progress of the reaction was followed by HPLC. Upon completion of the reaction the solution was centrifuged to allow decantation of the liquid layer as a clear solution. This was purified by preparative HPLC. Fractions containing the pure product were collected and lyophilized to give the product **12** (0.125 g, 52% yield) as a white solid.

**HPLC:** $t_R$ 25.80 min Column: YMC C18 4.6 x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 0% B, then linear gradient to 60% B over 30 min; Flow rate: 1.0 mL/min, Detection: UV @ 230 nm.

**MS:** 1015 $(M+H)^+$.

**Elemental Analysis:** Calc. for $C_{40}H_{55}N_7O_{12}PGd{\bullet}2\ H_20$: C, 45.75; H, 5.66; N, 9.34; Gd 14.97; O, 21.33; P, 2.95%.

To a solution of compound **11** (108 mg, 1.0 mmol) in water (5 mL), $Gd(OAc)_3$ (48 mg, 1.2 mmol) in water (2 mL) was added. The initial pH was 2.50 and the pH of the solution was adjusted to 5.0 by the addition of aqueous meglumine solution. The solution became turbid and was stirred for 24 h. The progress of the reaction was followed by HPLC. The solution was centrifuged in order to allow decantation of the liquid phase as a clear solution. The decanted solution was purified by preparative HPLC. Fractions containing the pure product were collected and freeze dried to give compound **12a** (50 mg) as a white solid.

**HPLC:** $t_R$ 25.25 min; Column: YMC C18, 4.6 x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution:: Initial condition: 0% B, then linear gradient to 80% B over 40 min; Flow rate: 1.0 mL/min, Detection: UV @ 230 nm.

**Elemental Analysis:** Calc. for $C_{40}H_{55}N_7O_{12}PGd{\bullet}2\ C_7H_{17}NO_5{\bullet}6H_2O$: C, 42.88; H,6.73; N, 8.33; Gd, 10.40; O,29.62, P, 2.05%.
Found: C, 43.21; H, 6.56; N, 8.10; Gd, 8.80%.

**[00210]**

**[00211]** The product was analyzed as described above in Examples 1-5. Results are shown in Tables 1 and 5.

# EXAMPLE 7: Preparation of [(Fl-1)-K(Gd-DOTA)-OH]Meg (Class 2)

A 500 mL round-bottomed flask equipped with a stir bar was charged with tetrabutylammonium hydrogen sulfate (25 mmol, 8.5 g, 0.5 equiv), $NaHCO_3$ (139.3 mmol, 11.7 g, 2.8 equiv) and water (80 mL)(caution, gas evolution). The mixture was stirred for 30 min and Boc-Lys(Fmoc)-OH (50 mmol, 23.46 g, 1 equiv) in $CH_2Cl_2$ (80 mL) was added. The mixture was stirred for 20 min, and benzyl bromide (8.9 g, 52 mmol, 6.2 mL, 1.04 equiv) was added in one portion.. After 18.5 h, HPLC analysis showed that the reaction was complete. The organic layer was separated and the aqueous layer was extracted with EtOAc (150 mL). The organic layers were combined, the volatiles were removed and the residue was dissolved in EtOAc (250 mL). The organic phase was washed with water (3 x 250 mL) to remove the tetrabutylammonium salts, and then dried with saturated brine followed by $Na_2SO_4$). The drying agent was filtered and the volatiles were removed to provide the crude product **1** as an oil in 100% yield.

[00212]

[00213] Ether (300 mL) was added to the crude oil **1** with agitation. The solution turned cloudy. Hexane (50 mL) was added to the solution. This resulted in formation of a white precipitate. The whole solution was kept in a refrigerator for 15 h. This resulted in the formation of a well-defined solid which was collected and dried overnight. The yield of pure solid product **1** was 26.7g (95.6%).

[00214]

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.5 (s, 9H, -C(C<u>H</u>$_3$)$_3$ on Boc group), 1.2-1.9 ( m, <u>CH$_2$</u> on Lysine), 3.2 (s, 2H, C<u>H</u>$_2$-N(Fmoc)) 4.17-4.82 (m, 4H, <u>CH</u>$_2$ –O-CO) <u>CH</u>-CH$_2$, and <u>CH</u>-CO), 5.2 (dd, 2H, C<u>H</u>$_2$-Ph), 7.3-7.8 (m, 13H all aromatic H).

**MS**: 597.2 $(M+K)^+$, 581.3 $(M+Na)^+$, 559.3 $(M+H)^+$, 459.3 $(M\text{-}Boc+H)^+$.

[00215] Boc-Lys(Fmoc)-benzyl ester (**1**) (14.5 g, 26.0 mmol) was dissolved in $CH_2Cl_2$ (80 mL) and anisole (6 mL). The mixture was stirred for 5 min. TFA (80 mL) was added and the mixture was stirred for 1.5 h at ambient temperature. HPLC analysis indicated complete reaction. The volatiles were removed, and ether (200 mL) was added. A solid formed after 15 min of stirring and was collected after 3h. This was dried overnight to give pure Lys(Fmoc)-OBn•TFA (14.4 g, 97% yield).

**HPLC:** $t_R$ 8.44 min; Column: YMC C18 4.6 x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Isocratic 50% B; Flow rate: 1.0 mL/min; Detection: UV @ 254 nm.

**$^1$H-NMR** (500 MHz, $CDCl_3$): δ 1.5 (s, 9H), 1.2-1.9 (m, CH$_2$ on Lysine), 3.1 (s, 2H, C<u>H</u>$_2$-N(Fmoc)), 3.95-4.40 (m, 4H, <u>CH</u>$_2$ –O-CO, <u>CH</u>-CH$_2$, and <u>CH</u>-CO ), 5.1 (dd, 2H, C<u>H</u>$_2$-Ph), 7.3-7.8 (m, 13H all aromatic H).

**MS:** 481.2 $(M+Na)^+$, 459.3 $(M+1)^+$.

[00216] Compound **2** (13.0 g, 22.7 mmol, 1.0 equiv), 1-fluorenecarboxylic acid (5.0 g, 23.8 mmol,1.05 equiv) and HATU (9.0 g, 23.8 mmol, 1.05 equiv) were charged to a 500 mL round-bottomed flask. Then $CH_2Cl_2$ (135 mL) was added. The resulting suspension was

stirred for 5 min after which DIEA (6.14 g, 8.28 mL, 23.8 mmol, 2.10 equiv) was added, followed by DMF (5 mL). After 30 min, stirring became difficult and an additional 50 mL portion of $CH_2Cl_2$ was added. The mixture was stirred for 3 h upon which HPLC analysis indicated completion of the reaction. The flask was cooled to ~10°C, the solid was collected. and was washed with cold $CH_2Cl_2$ and 50/50 hexane/$CH_2Cl_2$. The solid was dried overnight to give ~ 18 g of crude product. HPLC and NMR analysis indicated the presence of impurities. Therefore the product was washed with EtOAc/hexane 50/50 (3 x 200 mL), EtOAc (2 x 200 mL) andether (1 x 200 mL). This provided the pure product 3 (14.5 g, 98% yield).

**HPLC**: $t_R$ 36.4 min. Column: YMC C18, 4.6 mm x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 30% B, then linear gradient to 100% B over 35 min, hold 10 min at 100% B, then ramp to 30% B over 5 min; Flow rate: 1.0 mL/min; Detection: UV @ 254 nm.

**$^1$H-NMR (500 MHz, DMSO-d$_6$):** δ 1.2-1.9 (m, $\underline{CH_2}$ on Lysine), 3.0 (s, 2H, C$\underline{H_2}$-N(Fmoc)), 4.0-4.5 (m, 6H, $\underline{CH_2}$ –O-CO, $\underline{CH}$-$CH_2$, -C-$\underline{CH_2}$-C-and $\underline{CH}$-CO), 5.2 (q, 2H, C$\underline{H_2}$-Ph), 7.3-8.1 (m, all aromatic H).

**MS**: 696.4 $(M+K)^+$, 573.4 $(M+Na)^+$, 651.4 $(M+1)^+$.

**[00217]** Compound **3** (11.62 g) was suspended in $CH_3CN$ (100 mL)and the stirred suspension was cooled to 0°C in an ice bath. A solution of 40% piperidine in $CH_3CN$ (100 mL) was added to the mixture and the mixture was stirred for 40 min. The flask was allowed to rise to room temperature and the reaction mixture was stirred for 3 h. The volatiles were removed; hexane was added and the suspension was stirred for 10 min. The solid was collected by filtration and washed with hexane (2 x 200 mL). Then the solid was dissolved in $CHCl_3$ (45 mL), and applied to a 300 g silica gel column which was eluted with $CHCl_3$, 1% MeOH in $CHCl_3$, 2% MeOH in $CHCl_3$, 5% MeOH in $CHCl_3$ and 10% MeOH in $CHCl_3$. The product-containing fractions were combined and the volatiles were removed to provide compound **4** (5.8 g ,74% yield).

**HPLC**: $t_R$ 16.69 min. Column: YMC C18, 4.6 mm x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 30% B, then linear gradient to 100% B over 35 min, hold 10 min at 100% B, then ramp to 30% B over 5 min; Flow rate: 1.0 mL/min; Detection: UV @ 254 nm.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.3-1.9(m, $\underline{CH_2}$ on Lysine), 2.7(m, 2H, $C\underline{H_2}$-$NH_2$), 4.2 (s, 2H, C-$C\underline{H_2}$-C), 4.9 (m, 1H, $\underline{CH}$), 5.2 (q, 2H, $C\underline{H_2}$-Ph), 6.8-8.0 (m, all the aromatic H).

**MS**: 429.4 $(M+H)^+$.

**[00218]** Compound **4** (4.60 g, 10.75 mmol, 1.0 equiv.), HATU (6.13 g, 16.13 mmol, 1.5 equiv.), $CH_2Cl_2$ (80 mL) and DO3A10CM-1,4,7-tris t-butyl-ester•NaCl (10.5 g, 16.13 mmol, 1.5 equiv.) were added to a 500 mL round-bottomed flask. The flask was cooled to 0°C and DIEA (4.17 g, 32.25 mmol, 3.0 equiv.) was added. The reaction mixture was stirred at 0°C for 15 min and then at room temperature for 19 h. HPLC analysis indicated complete reaction. The volatiles were removed, the residue was dissolved in EtOAc and washed with saturated $NaHCO_3$ solution (3 x 250 mL), 3% $NaHCO_3$ solution (2 x 250 mL), and saturated brine (2 x 250 mL). The organic phase was dried over $Na_2SO_4$. After removal of the volatiles, a 14 g portion of a pale yellow glass was isolated. The weight of material was greater than that calculated based on quantitative yield due to the presence of the excess DO3A10CM-1,4,7-tris-t-butyl-ester•NaCl employed in the synthesis. This crude product **5** was directly used without further purification.

**HPLC**: $t_R$ 13.7 min; Assay 72% purity (largest impurity peak 9%); YMC C18, 4.6 mm x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 50% B, then linear gradient to 100% B over 25 min, hold for 10 min at 100% B then ramp to 50% B over 5 min; Flow rate: 1.0mL/min; Detection: UV @ 254 nm;

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.3-1.6 (m, $\underline{CH_2}$ on Lysine), 1.5 (m, 27 H, $C(\underline{CH_3})_3$), 2.0-4.0 (m, macrocycle, $C\underline{H_2}$-NH, $\underline{CH_2}$-COO, C-$C\underline{H_2}$-C), 4.5 (1H, $\underline{CH}$), 5.2 (q, 2H, $C\underline{H_2}$-Ph), 7.0-8.0 (m, all aromatic H).

**MS:** 983.6 $(M+H)^+$.

**[00219]** A hydrogenation bottle was charged with crude compound **5** (14 g), $CH_3OH$ (100 mL) and ~3 g of 10% Pd/C catalyst. The bottle was evacuated and refilled several times with hydrogen. Then compound **5**was subjected to hydrogenolysis at 50 psi $H_2$ for 3.5 h at ambient temperature. HPLC analysis indicated complete hydrogenolysis. After removal of the catalyst and solvent, 12 g (94% yield) of the crude product **6** was obtained. This was used without further purification.

**HPLC**: $t_R$ 6.77 min; Assay 94% purity; Column: YMC C18, 4.6 mm x 250 mm, S-10P µm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Initial condition: 50% B, then linear gradient to 100% B over 25 min, hold for 10 min at 100% B then ramp to 50% B over 5 min; Flow rate: 1.0mL/min; Detection: UV @ 254 nm.

**$^1$H-NMR (500 MHz, $CDCl_3$):** (Crude compound): δ 1.3-1.6 (m, $\underline{CH_2}$ on Lysine), 1.5 (m, 27 H, C($\underline{CH_3}$)$_3$), 2.0-4.0 (m, macrocycle, C$\underline{H_2}$-NH, $\underline{CH_2}$-COO, C-C$\underline{H_2}$-C), 4.5 (1H, $\underline{CH}$), 7.0-8.0 ( m, all aromatic H).

**MS:** 915.5 $(M+Na)^+$, 893.5 $(M+H)^+$.

**[00220]** Compound **6** (12 g) was treated with Reagent B (TFA : $H_2O$ : Phenol : i-$(C_3H_7)_3SiH$, 88:5:5:2, v/v/w/v) (200 mL) for 18h. HPLC analysis indicated complete reaction. The volatiles were removed and ether was added to precipitate the product. The precipitated product was collected and washed with ether and the resulting material was purified by preparative reversed phase HPLC using a C18 preparative column. This provided the pure product ligand **7** (4.1 g, 53% yield). An additional 1.1 g of 95% pure product was also obtained.

**HPLC**: $t_R$ 9.64 min; Assay: 99.6% purity; Column: YMC C18, 4.6 mm x 250 mm, S-10P µm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Isocratic 28% B; Flow rate: 1.0 mL/min; Detection: UV @ 254 nm.

**$^1$H-NMR (500 MHz, $CDCl_3$):** δ 1.3-1.8 (m, 6H, $\underline{CH_2}$ on Lysine), 3.0-4.2 (m, macrocycle, C$\underline{H_2}$-NH, $\underline{CH_2}$-COO, C-C$\underline{H_2}$-C), 4.5 (1H, $\underline{CH}$), 7.3-8.5 (m, all aromatic H).

**MS:** 747.4 $(M+Na)^+$, 725.4 $(M+1)^+$.

**Elemental Analysis**: Calc. for $C_{38}H_{51}N_7O_{11}$•2.1 $H_2O$•1.7 TFA: C, 49.48; H, 5.68; N, 8.79;O, 25.93; F, 10.13. Found: C, 49.66; H, 5.73; N, 8.79; F, 9.73.

**[00221]** (Fl-1)-K(DO3A10CM)-OH **7** (3.9 g, 5.37 mmol) was dissolved in water (135 mL), and $Gd(OAc)_3$•$4H_2O$ (2.38 g, 5.86 mmol, 1.09 equiv.) was added. The mixture was stirred for 20 min. The pH of the solution was adjusted to ~4.5 with 1N NaOH. The mixture was stirred at ambient temperature for 18h. Analytical reverse phase HPLC showed the reaction was completed. The product was purified by reversed phase preparative HPLC using a preparative scale C18 column. A total of 3.9 g pure product was obtained. A cation exchange column (Bio-Rad AG50W-X8 resin in the meglumine form) was prepared. A dilute aqueous solution of the free acid **8a** was applied to the column and the column was washed with water and fractions were collected. The fractions containing the desired product, compound **8b**, were collected, combined and lyophilized to give the pure product (3.95 g) as a colorless fluffy solid. The yield from compound **7** was 91%. HPLC analysis indicated no free gadolinium ion in the final product.

**HPLC**: $t_R$ 8.18 min; Assay: >99% purity; Column: YMC C18, 4.6 mm x 250 mm, S-10P μm, 120Å pore; Eluents: A: $H_2O$ (0.1% TFA), B: $CH_3CN$ (0.1% TFA); Elution: Isocratic 28% B; Flow rate: 1.0 mL/min; Detection: UV @ 254 nm.
**MS:** $(M+Na)^+$ 902.3, $(M+1)^+$ for Gd-chelate 880.3, $(M+Meg)^+$ 1075.4, $(M+1)^+$ 880.3, $(Meg+1)^+$ 195.9.

**Elemental Analysis**: Calc.for $GdC_{36}H_{45}N_6O_{10}$•$C_7H_{17}NO_5$•3.0$H_2O$: C, 45.77; H, 6.08; N, 8.69; O, 25.52; Gd, 13.94 %. Found: C, 46.32; H, 6.14; N, 8.63; Gd, 13.80; Na, 0.08; % $H_2O$ 5.05.

**[00222]** The compound was analyzed as described above in Examples 1-5. Results are shown in Table 2.

**EXAMPLE 8: Compounds Having High Fraction Bound to Serum and/or Higher Measured Relaxivity in Seronorm™ Versus Comparator MS325 (Gadofosveset Trisodium),**

[00223] Compounds of the present invention were analyzed to compare binding to human serum and measured relaxivity in Seronorm™ versus the reference compound MS-325.

[00224] A higher fraction bound to serum will result in a longer lifetime in blood, thus providing a more persistant signal. Higher relaxivity value will result in more signal for a given compound after administration.

[00225] Table 6 below shows fraction bound to serum and relaxivity data collected for representative compounds of the present invention:

Table 6: Fraction Bound to Serum and Relaxivity

| No. | Compound | Cpd Class | Chelator Type | Relaxation gain determined | r1 Sero-norm | $r1_W$ | $F_b$ |
|---|---|---|---|---|---|---|---|
| 1 | **MS325 - comparison compound** | 0 | DTPA | 6.96 | 36.57[b] | 5.26 | 0.87[b] |
| 2 | Gd-(10CM-DO3A)-G-G-W-(Nin-2,4,6-trimethylbenzoyl)-O⁻ Meg | 1 | 10CM-DO3A | 4.75 | 23.7[c] | 4.99 | 0.87[b] |
| 3 | Gd-(10CM-DO3A)-G-G-W-(Nin-2,4,6-trimethylbenzene sulfonyl)-O⁻ Meg | 1 | 10CM-DO3A | 4.85 | 25.32[c] | 5.22 | 0.92[a] |
| 4 | Gd-(10CM-DO3A)-aaB Na | 1 | 10CM-DO3A | 3.401 | 30.62[c] | 9 | 0.93[a] |
| 5 | Gd-(10CM-DO3A)-aak Meg | 1 | 10CM-DO3A | 2.634 | 34.99[b] | 13.28 | 0.96[a] |
| 6 | Gd-(10CM-DO3A)-DDk Meg | 1 | 10CM-DO3A | 2.424 | 31.99[c] | 13.2 | 0.91[a] |
| 7 | Gd-(10CM-DO3A)-EEB Meg | 1 | 10CM-DO3A | 2.07 | 24.17[c] | 11.68 | 0.88[a] |
| 8 | Gd-(10CM-DO3A)-Pak Na | 1 | 10CM-DO3A | 1.575 | 25.23[c] | 16.02 | 0.96[a] |
| 9 | Gd-(10CM- | 1 | 10CM- | 1.344 | 19.38[c] | 14.42 | 0.95[a] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | DO3A)-Pak Meg | | DO3A | | | | |
| 10 | Gd-(10CM-DO3A)-Dak Meg | 1 | 10CM-DO3A | 3.57 | 19.99[c] | 5.6 | 0.94[a] |
| 11 | Gd-(10CM-DO3A)-DDj Na | 1 | 10CM-DO3A | 1.023 | 18.94[c] | 18.51 | 0.91[a] |
| 12 | Gd-(10CM-DO3A)-DDX Na | 1 | 10CM-DO3A | 1.01 | 16.82[c] | 16.65 | 0.95[a] |
| 13 | Gd-(10CM-DO3A)-Paj Na | 1 | 10CM-DO3A | 1.809 | 27.38[c] | 15.14 | 0.89[a] |
| 14 | Gd-(10CM-DO3A)-aaX Na | 1 | 10CM-DO3A | 1.61 | 14.11[c] | 8.78 | 0.96[a] |
| 15 | Gd-(10CM-DO3A)-GGm Meg | 1 | 10CM-DO3A | 3.81 | 25.7[c] | 6.75 | 0.92[a] |
| 16 | Gd-(10CM-DO3A)-Gao Meg | 1 | 10CM-DO3A | 2.8 | 21.3[c] | 7.62 | 0.88[a] |
| 17 | Gd-(10CM-DO3A)-GGo Meg | 1 | 10CM-DO3A | 2.44 | 21.35[c] | 8.73 | 0.9[a] |
| 18 | Gd-(10CM-DO3A)-Pao Meg | 1 | 10CM-DO3A | 2.33 | 20.83[c] | 8.93 | 0.88[a] |
| 19 | Gd-(10CM-DO3A)-Pam Meg | 1 | 10CM-DO3A | 2.05 | 21.5[c] | 10.5 | 0.93[a] |
| 20 | Gd-(10CM-DO3A)-Gam Meg | 1 | 10CM-DO3A | 1.99 | 24.52[c] | 12.32 | 0.94[a] |
| 21 | Gd-(10CM-DO3A)-aao Meg | 1 | 10CM-DO3A | 1.82 | 19.11[c] | 10.51 | 0.93[a] |
| 22 | Gd-(10CM-DO3A)-aam Meg | 1 | 10CM-DO3A | 1.37 | 27.29[c] | 19.87 | 0.97[a] |
| 23 | Gd-(10CM-DO3A)-Gan Meg | 1 | 10CM-DO3A | 2.6 | 23.16[c] | 8.92 | 0.99[a] |
| 24 | Gd-(10CM-DO3A)-Ga-(p-Cl)-Bip Na | 1 | 10CM-DO3A | 4.46 | 24.12[c] | 5.41 | 0.89[a] |
| 25 | **CEMP – Derivatives** | | CEMP-DO3A | | | | |
| 26 | Gd-(CEMP-DO3A)-GaB | 1 | CEMP-DO3A | 3.42 | 14.39[c] | 4.21 | 0.92[a] |
| 27 | Gd-(CEMP-DO3A)-aB Na | 1 | CEMP-DO3A | 0.96 | 14.96[c] | 15.54 | 0.96[a] |
| 28 | Gd-4-CEMP-DTTA-GaB Meg | 1 | CEMP-DO3A | 4.77 | 33.71[b] | 7.06 | 0.95[a] |
| 29 | **CE-DTPA Derivatives** | | CE-DTPA | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 30 | Gd-(CE-DTPA)-GaB 3Meg | 1 | CE-DTPA | 3.26 | 21.41[c] | 6.56 | 0.88[a] |
| 31 | Gd-(CE-DTPA)-aB 3Na | 1 | CE-DTPA | 5.56 | 29.44[c] | 5.29 | 0.94[a] |
| 32 | Gd-(CE-DTPA)-aB 3Meg | 1 | CE-DTPA | 5.67 | 33.3[b] | 5.87 | 0.91[a] |
| 33 | **CM-DOTMA Derivatives** | | CM-DOTMA | | | | |
| 34 | Gd-(CM-DOTMA)-GaB 2Na | 1 | CM-DOTMA | 5.1 | 30.7[c] | 6.01 | 0.87[b] |
| 35 | Gd-(CM-DOTMA)-aB Na | 1 | CM-DOTMA | 5.74 | 34.41[b] | 6 | 0.86[c] |
| 36 | Gd-(CM-DOTMA)-aB Na | 1 | CM-DOTMA | 6.18 | 36.02[b] | 5.82 | 0.85[c] |
| 37 | Gd-(CM-DOTMA)-aB Meg | 1 | CM-DOTMA | 6.04 | 35.57[b] | 5.89 | 0.83[c] |
| 38 | **CM-DOTA Derivatives** | | CM-DOTA | | | | |
| 39 | Gd-(CM-DOTA)-aB Meg | 1 | CM-DOTA | 6.4 | 30.36[c] | 4.75 | 0.95[a] |
| 40 | Gd-(CM-DOTA)-aB Na | 1 | CM-DOTA | 6.64 | 30.78[c] | 4.64 | 0.94[a] |
| 41 | **7MP-DO3A Derivatives** | | 7MP-DO3A | | | | |
| 42 | Gd-(7MP-DO3A)-GaB | 1 | 7MP-DO3A | 5.84 | 33.99[b] | 5.82 | 0.88[a] |
| 43 | Gd-(7MP-DO3A)-GaB Meg | 1 | 7MP-DO3A | 5.55 | 33.95[b] | 6.12 | 0.89[a] |
| 44 | **7MP-CM-DO3A Derivative** | | 7MP-CM-DO3A | | | | |
| 45 | Gd-(7MP-CM-DO3A)-aB Meg | 1 | 7MP-CM-DO3A | 3.93 | 20.1[c] | 5.12 | 1[a] |
| 46 | **TE-DOTA Derivatives** | | TEDOTA | | | | |
| 47 | Gd-(TE-DOTA)-aB Meg | 1 | TE-DOTA | 6.08 | 42.09[a] | 6.92 | 0.96[a] |
| 48 | Gd-(TE-DOTA) Meg | 1 | TE-DOTA | 1.63 | 7.01[c] | 4.29 | 0.01[c] |
| 49 | Gd-(10CM-DO3A)-K-(1-fluorenecarboxamido)$O^-$ $Na^+$ | 2 | 10CM-DO3A | 5.75 | 27.68[c] | 4.81 | 0.9[a] |
| 50 | Gd-(10CM- | 2 | 10CM- | 5.44 | 27.49[c] | 5.06 | 0.91[a] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | DO3A)-K-(1-fluorenecarboxamido)-$O^-$ Meg | | DO3A | | | | |
| 51 | [(Fl-1)-K{G-[Gd-(10CM-DO3A)]}-$O^-$](Meg) | 2 | 10CM-DO3A | 4.68 | 22.4[c] | 4.79 | 0.91[a] |
| 52 | [(Fl-1)-K[G-{Gd-(10CM-DO3A)]}-$O^-$]($Na^+$) | 2 | 10CM-DO3A | 4.08 | 24.15[c] | 5.91 | 0.91[a] |
| 53 | Gd-[(10CM-DO3A)-G-K-22] Meg | 2 | 10CM-DO3A | 3.87 | 17.06[c] | 4.4 | 0.86[c] |
| 54 | Gd-[(10CM-DO3A)-G-K-13] Meg | 2 | 10CM-DO3A | 3.67 | 16.45[c] | 4.49 | 0.86[c] |

**Key for bound fraction ($F_b$) values:** a = bound fraction > MS325, b = bound fraction = MS325, c = bound fraction < MS325

**Key for R1 Seronorm™ values: a** = relaxivity in Seronorm™ > MS325, b = relaxivity in Seronorm™ below but within 10% of MS325 value, c = relaxivity in Seronorm™ <90% of MS325 value

[00226] The following explains the column headings in Table 6:

[00227] **Chelator type** refers to the chelator used in the compound.

[00228] **Relaxation gain determined** refers to the relaxation gain, which is the ratio of the T1 relaxivity of the substance in a solution of HSA called Seronorm™ (commercially available from Nycomed-Amersham) to the relaxivity of the same compound in water at a specified concentration. Values close to 1 indicate that no binding to protein is occurring and that the compound is unsuitable. On the other hand, higher values are more desirable because they highlight, in an MRI image, the bloodstream (intravascular compartment) due to the much higher T1 relaxivity (which is the source of the MRI signal) when bound to the HSA in the blood.

[00229] **The parameter $r1_S$** is the T1 relaxivity of the substance in a solution of human serum albumin called Seronorm™ (commercially available from Nycomed-Amersham)

[00230] **The parameter $r1_W$** is the T1 relaxivity of the compound in water, where no protein is present.

[00231] **The parameter $F_b$** is the fraction of the compound bound to human serum albumin (HSA) under standard conditions of defined concentration and temperature. A value

of 0.90 indicates that 90% of the molecules of the test compound are bound to HSA. A higher fraction bound ($F_b$) means that more of the compound should provide the higher signal characteristic of the bound molecule due to immobilization of the chelating function, or significant slowing of the chelating function upon binding of the chelate-HSA binding ligand to the HSA.

**[00232]** As shown in Table 6, Gd-(TE-DOTA)-aB Meg provides a relaxivity ($r_1$) in Seronorm$^{TM}$ (HSA) of 42.09, well above the comparator MS-325 ($r_1$ Seronorm = 36.57). Such a high relaxivity will provide more signal for a given compound after administration. Furthermore, Gd-(TE-DOTA)-aB Meg provides an $F_b$ of 0.996, well above the $F_b$ of the comparator MS-325 ($F_b$ = 0.87).

**[00233]** As further shown in Table 6, the following compounds each provide higher bound fractions than the MS325 comparator: Gd-(10CM-DO3A)-G-G-W-(Nin-2,4,6-trimethylbenzenesulfonyl)-$O^-$ Meg; Gd-(10CM-DO3A)-aaB Na;Gd-(10CM-DO3A)-aak Meg; Gd-(10CM-DO3A)-DDk Meg; Gd-(10CM-DO3A)-EEB Meg; Gd-(10CM-DO3A)-Pak Na; Gd-(10CM-DO3A)-Pak Meg; Gd-(10CM-DO3A)-Dak Meg; Gd-(10CM-DO3A)-DDj Na; Gd-(10CM-DO3A)-DDX Na; Gd-(10CM-DO3A)-Paj Na; Gd-(10CM-DO3A)-aaX Na; Gd-(10CM-DO3A)-GGm Meg; Gd-(10CM-DO3A)-Gao Meg; Gd-(10CM-DO3A)-GGo Meg; Gd-(10CM-DO3A)-Pao Meg; Gd-(10CM-DO3A)-Pam Meg; Gd-(10CM-DO3A)-Gam Meg; Gd-(10CM-DO3A)-aao Meg; Gd-(10CM-DO3A)-aam Meg; Gd-(10CM-DO3A)-Gan Meg; Gd-(10CM-DO3A)-Ga-(p-Cl)-Bip Na; Gd-(CEMP-DO3A)-GaB; Gd-(CEMP-DO3A)-aB Na; Gd-4-CEMP-DTTA-GaB Meg; Gd-(CE-DTPA)-GaB 3Meg; Gd-(CE-DTPA)-aB 3Na; Gd-(CE-DTPA)-aB 3Meg; Gd-(CM-DOTA)-aB Meg; Gd-(CM-DOTA)-aB Na; Gd-(7MP-DO3A)-GaB; Gd-(7MP-DO3A)-GaB Meg; Gd-(7MP-CM-DO3A)-aB Meg; Gd-(TE-DOTA)-aB Meg; Gd-(10CM-DO3A)-K-(1-fluorenecarboxamido)O- Na+; Gd-(10CM-DO3A)-K-(1-fluorenecarboxamido)-$O^-$ Meg; [(Fl-1)-K{G-[Gd-(10CM-DO3A)]}-$O^-$](Meg); and [(Fl-1)-K[G-{Gd-(10CM-DO3A)]}-$O^-$] (Na+).

**[00234]** Definitions of abbreviations for unnatural amino acids as used herein are presented below:

c = 8-aminocaprylic acid j=L-O-benzyl-tyrosine, k=L-2,6-dichlorobenzyl-tyrosine. m = L-($N^{in}$-2,4,6-trimethybenzensulphonyl)-tryptophan, n = L-($N^{in}$-2,4,6-triisopropybenzensulphonyl)-tryptophan, o = L-($N^{in}$-2,4,6-trimethylbenzoyl)-tryptophan, p = O-benzyl-L-threonine, X= L-2-naphthylalanine.

| Table 7: Abbreviations and structures for unnatural amino acids enumerated in Table 6 | |
|---|---|
| | |
| c | j |
| | |
| k | m |
| | |
| n | O |
| | |
| p | X |

We claim:

1. A composition for the imaging and/or treatment of a disease in a mammal, comprising a compound having the formula:

Ch – L1 – R – L2

wherein Ch is a chelating moiety optionally complexed with M, a metal or a radionuclide;

L1 is a first linker;

R is a hydrophobic compound; and

L2 is an optional second linker

wherein said compound is selected from the group consisting of: Gd-(10CM-DO3A)-G-G-W-(Nin-2,4,6-trimethylbenzenesulfonyl)-O$^-$ Meg; Gd-(10CM-DO3A)-aaB Na;Gd-(10CM-DO3A)-aak Meg; Gd-(10CM-DO3A)-DDk Meg; Gd-(10CM-DO3A)-EEB Meg; Gd-(10CM-DO3A)-Pak Na; Gd-(10CM-DO3A)-Pak Meg; Gd-(10CM-DO3A)-Dak Meg; Gd-(10CM-DO3A)-DDj Na; Gd-(10CM-DO3A)-DDX Na; Gd-(10CM-DO3A)-Paj Na; Gd-(10CM-DO3A)-aaX Na; Gd-(10CM-DO3A)-GGm Meg; Gd-(10CM-DO3A)-Gao Meg; Gd-(10CM-DO3A)-GGo Meg; Gd-(10CM-DO3A)-Pao Meg; Gd-(10CM-DO3A)-Pam Meg; Gd-(10CM-DO3A)-Gam Meg; Gd-(10CM-DO3A)-aao Meg; Gd-(10CM-DO3A)-aam Meg; Gd-(10CM-DO3A)-Gan Meg; Gd-(10CM-DO3A)-Ga-(p-Cl)-Bip Na; Gd-(CEMP-DO3A)-GaB; Gd-(CEMP-DO3A)-aB Na; Gd-4-CEMP-DTTA-GaB Meg; Gd-(CE-DTPA)-GaB 3Meg; Gd-(CE-DTPA)-aB 3Na; Gd-(CE-DTPA)-aB 3Meg; Gd-(CM-DOTA)-aB Meg; Gd-(CM-DOTA)-aB Na; Gd-(7MP-DO3A)-GaB; Gd-(7MP-DO3A)-GaB Meg; Gd-(7MP-CM-DO3A)-aB Meg; Gd-(TE-DOTA)-aB Meg; Gd-(10CM-DO3A)-K-(1-fluorenecarboxamido)O- Na+; Gd-(10CM-DO3A)-K-(1-fluorenecarboxamido)-O$^-$ Meg; [(Fl-1)-K{G-[Gd-(10CM-DO3A)]}-O$^-$](Meg); and [(Fl-1)-K[G-{Gd-(10CM-DO3A)]}-O$^-$] (Na+) and salt or alternative salt forms thereof; and

optionally further comprises a targeting vector Q.

2. The composition of claim 1 wherein the compound having the formula: Ch – L1 – R – L2 is Gd-(TE-DOTA)-aB Meg or alternative salt forms thereof, optionally further comprising a targeting vector Q.

3. The composition of claim 1 wherein the relaxivity in human serum albumin is 10% greater than the relaxivity of gadofosveset trisodium.

4. The composition of claim 1 wherein the fraction bound to serum is greater than the fraction of gadofosveset trisodium bound to serum.

5. The composition of claim 1 complexed with a metal.

6. The composition of claim 5 wherein the metal is a paramagnetic metal ion.

7. The composition of claim 6 wherein the paramagnetic metal ion is selected from the group consisting of $Mn^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Gd^{3+}$, $Eu^{3+}$, $Dy^{3+}$, $Pr^{3+}$, $Cr^{3+}$, $Co^{3+}$, $Fe^{3+}$, $Ti^{3+}$, $Tb^{3+}$, $Nd^{3+}$, $Sm^{3+}$, $Ho^{3+}$, $Er^{3+}$, $Pa^{4+}$ or $Eu^{2+}$.

8. The composition of any one of claims 1 or 2 complexed with gadolinium.

9. The composition of claim 1 complexed with a radionuclide.

10. A method of imaging a tissue or organ of a mammal comprising the steps of:
administering a composition of claim 1 complexed with a metal useful for magnetic resonance imaging and
imaging a tissue or organ by magnetic resonance imaging.

11. A method of imaging at least a portion of the cardiovascular system of a mammal comprising the steps of:
administering a composition of claim 1 complexed with a metal useful for magnetic resonance imaging and
imaging a tissue or organ by cardiovascular magnetic resonance angiography.

12. A method of imaging a tissue or organ of a mammal comprising the steps of:
administering a composition of claim 1 complexed with a radionuclide suitable for nuclear imaging and
imaging a tissue or organ by nuclear imaging.

13. The method of claim 12 where in the nuclear imaging is selected from the group consisting of: scintigraphy, SPECT and PET imaging.

14. The composition of claim 9 wherein the radionuclide is selected from the group consisting of $^{51}Mn$, $^{52}Fe$, $^{60}Cu$, $^{68}Ga$, $^{72}As$, $^{94m}Tc$, or $^{110}In$, $^{99m}Tc$, $^{51}Cr$, $^{67}Ga$, $^{68}Ga$, $^{47}Sc$, $^{51}Cr$, $^{167}Tm$, $^{141}Ce$, $^{111}In$, $^{168}Yb$, $^{175}Yb$, $^{140}La$, $^{90}Y$, $^{88}Y$, $^{153}Sm$, $^{166}Ho$, $^{165}Dy$, $^{166}Dy$, $^{62}Cu$, $^{64}Cu$, $^{67}Cu$, $^{97}Ru$, $^{103}Ru$, $^{186}Re$, $^{188}Re$, $^{203}Pb$, $^{211}Bi$, $^{212}Bi$, $^{213}Bi$, $^{214}Bi$, $^{105}Rh$, $^{109}Pd$, $^{117m}Sn$, $^{149}Pm$, $^{161}Tb$, $^{177}Lu$, $^{198}Au$ and $^{199}Au$.

15. The method of claim 12, wherein the metal is selected form the group consisting of $^{64}Cu$, $^{67}Ga$, $^{68}Ga$, $^{99m}Tc$, and $^{111}In$.

16. A method of treating a patient in need thereof with radiotherapy comprising administering a compound of claim 1 complexed with a radionuclide suitable for radiotherapy.

17. The method of claim 16, wherein the radionuclide is selected from the group consisting of $^{64}Cu$, $^{90}Y$, $^{105}Rh$, $^{111}In$, $^{117m}Sn$, $^{149}Pm$, $^{153}Sm$, $^{161}Tb$, $^{166}Dy$, $^{166}Ho$, $^{175}Yb$, $^{177}Lu$, $^{186/188}Re$, and $^{199}Au$.

18. The composition of claim 1, wherein Q and L1 both comprise amino acids.

19. The composition of claim 1 further comprising a chemotherapeutic.

20. The composition of claim 1, where in the targeting vector is a peptide.

21. The composition of claim 20, where in the targeting peptide is selected from the group consisting of: peptide hormones, analogs of somatostatin, Substance P agonists, oxytocin; endothelin A and endothelin B; bradykinin; Epidural Growth Factor (EGF); Interleukin-1; and cholecystokinin (CCK-B).

22. A method of preparing a composition of claim 1, wherein said method comprises the steps of: attaching a chelating moiety to the first linker, attaching said first linker to the hydrophobic group R via a protecting group having a carboxylic group thereto, attaching an optional second linker to said hydrophobic group, and attaching an optional targeting vector Q to said optional second linker.

23. A method of preparing a composition of claim 1, wherein said method comprises attaching a hydrophobic group R which is capable of binding HSA to a first linker, then attaching the first linker to the chelating moiety Ch, then attaching another conjugatable group in the first linker to a second linker followed by attachment of the second linker to an optional molecule which can serve as a targeting vector.

24. A method of preparing a composition of claim 1, wherein said method comprises preparing the components of the entire construct as modules such as Ch-L1, R-P (where P is an optional protecting group for a reactive function other than that to be employed for assembling the construct) and L2-Q and then attaching the modules in any desired order to obtain constructs such as Ch – L1 – R – L2 – Q, Ch-L1(L2-Q)-R or Ch(L2-Q)-L1-R.

25. The method of any one of claims 22-24 further comprising complexing the composition with a metal or a radionuclide.

26. A composition for the imaging and/or treatment of a disease in a mammal, comprising a compound having the formula:

$$Ch - L1 - R - L2 - Q$$

wherein Ch is a chelating moiety optionally complexed with M, a metal or a radionuclide;

L1 is a first linker;

R is a hydrophobic compound; and

L2 is an optional second linker that is further linked to an optional targeting moiety Q wherein said compound has a fraction bound to serum of greater than about 0.87 and/or a relaxivity in human serum albumin of greater than about 36.57.

27. The composition of claim 26 wherein Ch-L1-R-L2 is selected from the group consisting of: Gd-(10CM-DO3A)-G-G-W-(Nin-2,4,6-trimethylbenzenesulfonyl)-$O^-$ Meg; Gd-(10CM-DO3A)-aaB Na;Gd-(10CM-DO3A)-aak Meg; Gd-(10CM-DO3A)-DDk Meg; Gd-(10CM-DO3A)-EEB Meg; Gd-(10CM-DO3A)-Pak Na; Gd-(10CM-DO3A)-Pak Meg; Gd-(10CM-DO3A)-Dak Meg; Gd-(10CM-DO3A)-DDj Na; Gd-(10CM-DO3A)-DDX Na; Gd-(10CM-DO3A)-Paj Na; Gd-(10CM-DO3A)-aaX Na; Gd-(10CM-DO3A)-GGm Meg; Gd-(10CM-DO3A)-Gao Meg; Gd-(10CM-DO3A)-GGo Meg; Gd-(10CM-DO3A)-Pao Meg; Gd-(10CM-DO3A)-Pam Meg; Gd-(10CM-DO3A)-Gam Meg; Gd-(10CM-DO3A)-aao Meg; Gd-(10CM-DO3A)-aam Meg; Gd-(10CM-DO3A)-Gan Meg; Gd-(10CM-DO3A)-Ga-(p-Cl)-Bip Na; Gd-(CEMP-DO3A)-GaB; Gd-(CEMP-DO3A)-aB Na; Gd-4-CEMP-DTTA-GaB Meg; Gd-(CE-DTPA)-GaB 3Meg; Gd-(CE-DTPA)-aB 3Na; Gd-(CE-DTPA)-aB 3Meg; Gd-(CM-DOTA)-aB Meg; Gd-(CM-DOTA)-aB Na; Gd-(7MP-DO3A)-GaB; Gd-(7MP-DO3A)-GaB Meg; Gd-(7MP-CM-DO3A)-aB Meg; Gd-(TE-DOTA)-aB Meg; Gd-(10CM-DO3A)-K-(1-fluorenecarboxamido)O- Na+; Gd-(10CM-DO3A)-K-(1-fluorenecarboxamido)-$O^-$ Meg; [(Fl-1)-K{G-[Gd-(10CM-DO3A)]}-$O^-$](Meg); and [(Fl-1)-K[G-{Gd-(10CM-DO3A)]}-$O^-$] (Na+) and salt or alternative salt forms thereof; and
optionally further comprises a targeting vector Q.

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/64359

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC(8) - A61B 6/00 (2008.04)
USPC - 600/431
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61B 6/00 (2008.04)
USPC - 600/431

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/450 (Text Search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB, USPT, USOC, EPAB, JPAB); DialogPRO (Engineering) and Google Scholar: contrast agent, conjugate, HSA, human serum albumin, meglumine, l-biphenylalanine, hydrophobic, bind, tryptophan, trans-4-methylaminocyclohexanecarboxylic acid, 10CM-DO3A, protein, aminocyclohexanecarboxylic acid, linker, DOTA

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 2007/0003479 A1 (McMurry et al.) 04 Jan 2007 (04.01.2007); para [0012], [0018]-[0021], [0029]-[0031], [0034], [0036], [0041], [0045]-[0048], [0065], [0148]-[0150]; claim 103, 107, 115 | 1-27 |
| Y | US 2006/0241018 A1 (DE HAEN et al.) 26 Oct 2006 (26.10.2006); para [0005], [0011]-[0013], [0017], [0018], [0023], [0056], [0070], [0074]-[0079], [0105], [0106], [0148], [0196] | 1-27 |
| Y | US 2006/0269479 A1 (COLTON et al.) 30 Nov 2006 (30.11.2006); para [0302] | 1-25 and 27 |
| Y | COMBLIN, et al. Designing New MRI Contrast Agents: a Coordination Chemistry Challenge. Coordination Chemistry Reviews. Oct 1999, Vol. 185-186, pages 451-470; abstract; Fig 1 | 1-25 and 27 |

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