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Stereochemical Influence on the Stability of Radio-Metal Complexes In Vivo. Synthesis and Evaluation of the Four Stereoisomers of 2-(*p*-Nitrobenzyl)-*trans*-CyDTPA

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Abstract—Distinct differences in in vivo stability of the two diastereomeric C-Functionalized CyDTPA chelating agents, (CHX-A DTPA and CHX-B DTPA, both racemates), as recently reported prompted further investigation as to why differences in configuration produced striking effects on the in vivo stability of their yttrium complexes. To this end, the four individual component stereoisomers of CHX-A and CHX-B were synthesized and ability to bind yttrium was investigated both in vitro and in vivo. Published by Elsevier Science Ltd.

Introduction

Use of radiolabeled monoclonal antibodies (mAbs) for the diagnosis and therapy of malignancies continues to receive attention.[1–3] A variety of metallic radionuclides have been employed to expand the choices of emission characteristics and half-lives for diagnostic (γ and β^+ emitters) and therapeutic (β^- and α emitters) applications.[4,5]

The development of suitable bifunctional chelating agents for modification of proteins for radioimmunoimaging (RII) and radioimmunotherapy (RIT) then also continues to be a desirable goal. Considerable efforts have been concentrated on preparation of C- and N-functionalized polyaminocarboxylate chelating agents such as diethylenetriamine pentaacetic acid (DTPA).[5–7] These reagents form stable covalent bonds to proteins and can then bind radionuclides such as ^{111}In, ^{90}Y, ^{67}Cu, and ^{153}Sm, which are of potential interest for RII/RIT of cancer.[5,8–10] A critical requirement for success in this endeavor is the stable sequestration of the radionuclide during the delivery of the protein conjugate to the target site. Normal tissue uptake, metabolic processing and subsequent release of the radionuclide continues to be of concern.[11]

We had previously reported synthesis of C-functionalized CyDTPA's, CHX-A DTPA, and CHX-B DTPA (Fig. 1) and their evaluation with $^{205/206}$Bi for in vivo stability for use with the therapeutic α-emitter ^{212}Bi.[12–14] Both ligands were excellent at sequestering bismuth radionuclides, being statistically equivalent to the protein conjugate formed with 2-(*p*-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA) in stability, without the detraction of the slow formation kinetics inherent to the macrocycle. However, we later reported an unexpected result upon evaluation of these two ligands with ^{88}Y, used as a tracer to determine the usefulness of these ligands for sequestering the β-emitter ^{90}Y.[15] The ^{88}Y CHX-A DTPA complex, linked to mAb B3, was found to be equivalent in stability to previously reported ligands while the ^{88}Y CHX-B DTPA, under the same conditions, was remarkably unstable both in vitro and in vivo. Careful re-examination of the serum stability studies of CHX-A DTPA vs. CHX-B DTPA $^{205/206}$Bi complexes revealed a similar trend, but of a much lesser magnitude (insignificant due to ^{212}Bi $t_{1/2}$ = 60 min) that could only be detected after several days had elapsed.[16]

Clearly, since CHX-A DTPA and CHX-B DTPA are diastereomers, different chemical properties were to be expected and were even relied upon for the isolation of the two compounds. However, the very large differences in in vivo stability for complexing ^{88}Y coupled with the much smaller difference for complexing ^{212}Bi in an active biological milieu prompted further investigation of these ligands.

Having observed that stereochemistry could be a significant variable effecting in vivo complex stability, some concern was expressed that even using pairs of enantiomers, such as CHX-A DTPA itself, could be yet masking some additional, subtle level of stability, or instability, that could be resolved. To eliminate all doubt as to the results obtained from study of the stability of the yttrium complexes, stereospecific

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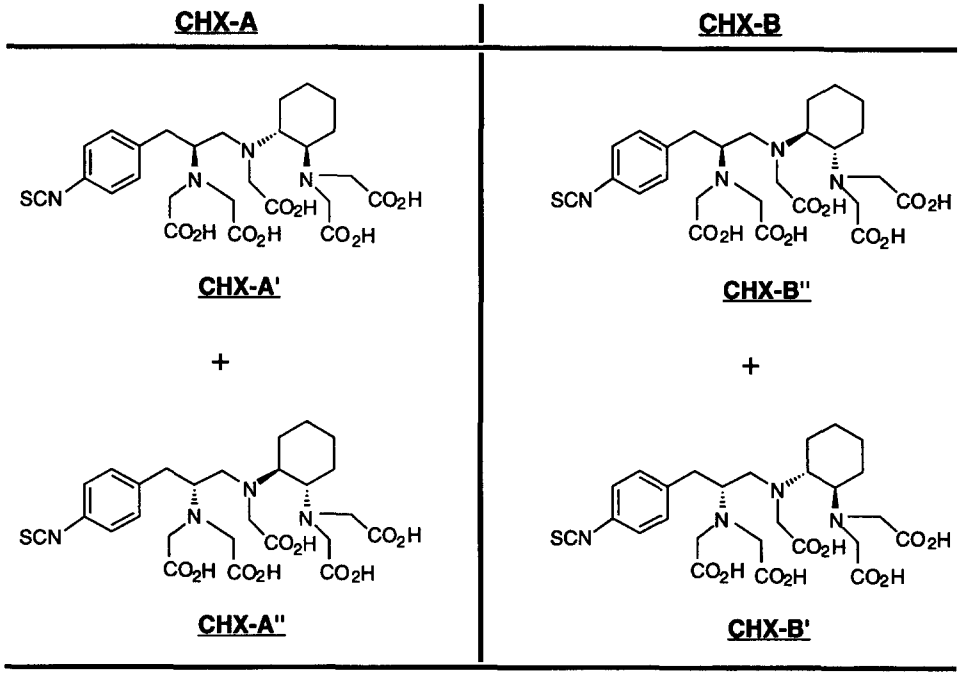

Figure 1. Structures of the CHX-A and CHX-B DTPA chelating agents and their individual component enantiomers.

synthesis and evaluation of each of the four stereoisomers that comprise CHX-A DTPA and CHX-B DTPA was proposed.

Herein, we report the correlated results for the evaluation of the ^{88}Y complexes of the four stereoisomers of the C-functionalized CHX-A DTPA and CHX-B DTPA, their individual serum stability, transchelation, and in vivo stability as determined by ^{88}Y deposition in the bone of mice.

Results and Discussion

Synthesis of the CHX-A″, -B″, and B′ stereoisomers (Figs 2 and 3) were performed using modifications of the chemistry reported for preparation of the previously prepared enantiomer, CHX-A′.[13] A consistent obstacle was encountered during the borane reduction of the amides, where a tendency for reduction of the aryl nitro group to the aniline was routinely experienced. This side reaction could be minimized only by careful titration with BH_3·THF and addition of excess hydride or excessive heating routinely resulted in complete reduction of the nitro group. This remedy did not completely eliminate the problem and occasionally contributed to incomplete reduction of the amide. Thus, the triamines were not isolated as pure compounds, but directly alkylated with *tert*-butyl bromoacetate, with purification then performed at the pentaacetic acid stage. This was accomplished first by preparative HPLC to remove gross contaminants originating from aniline formation or incomplete alkylation. In none of the syntheses was there any evidence of product due to epimerization of the amino acid chiral center during either carbamate deprotection or the borane reduction as determined by analytical HPLC (ΔR_t > 1 min).

Although adequate for the experiments described herein, this reduction complication renders this route unacceptable for routine preparation of these chelating agents, particularly in light of the preparative HPLC requirement. The cleavage of the benzyl carbamate (CBZ) by treatment with 33% HBr in glacial acetic acid was suggested as the origin of the erratic borane reduction. Changing the CBZ group to a *tert*-butyl carbamate (BOC) was easily achieved and both carbamates could be cleanly removed with $HCl_{(g)}$ in dry dioxane (Fig. 4). Direct borane reduction of the deprotection product was now completely uneventful without any measurable aniline formation as determined by HPLC. After alkylation of this crude triamine, analytical HPLC of the pentaacetic acid, obtained after ester cleavage, indicated that the need for preparative HPLC had indeed been successfully eliminated. Final purification was accomplished by a sequence of cation- and anion-exchange chromatography.[13,17] The nitro group was then hydrogenated to the aniline which was directly converted to the isothiocyanate with thiophosgene.[6,13] A notable instability was observed for all four final products as determined by HPLC which has been presumed to due to a known disproportionation reaction leading to to bis-DTPA thiourea compound.[18] This condition, while precluding a detailed analysis of the final products, did not interfere with the studies discussed below.

Determination of serum stability of the ^{88}Y labeled stereoisomers CHX-A′,-A″,-B′,-B″ DTPAs revealed no

significant differences between the complexes formed from the enantiomeric pairs and the corresponding racemate (Fig. 5). These results essentially reproduced and confirmed that the ^{88}Y labeled CHX-B DTPA was significantly unstable in serum as compared to the ^{88}Y labeled CHX-A DTPA for both enantiomers and racemates.

Somewhat more interesting were the results from the transchelation experiments wherein each ^{88}Y complex was challenged with a large excess of DTPA (Fig. 6). At pH 7.2, this reaction was too slow (<0.5% per 24 h) to be relevant when applied to the topic of relatively short-lived radioisotopes. To obtain results during a meaningful time frame, the experiment was conducted at pH 5.0. Again, the general trend of CHX-B DTPAs being much less stable than the CHX-A DTPAs was confirmed and as before for serum stability, the results for the individual ^{88}Y labeled enantiomers were essentially identical to the results for the racemate. While small differences in rate could be suggested between enantiomers, particularly for CHX-B′ and CHX-B″, these were relegated to being due to experimental error and also occurring only after ca. 90% of the radionuclide had been lost. However, a curious result that continues to be studied was the biphasic rate of transchelation observed during the study for all of the compounds.

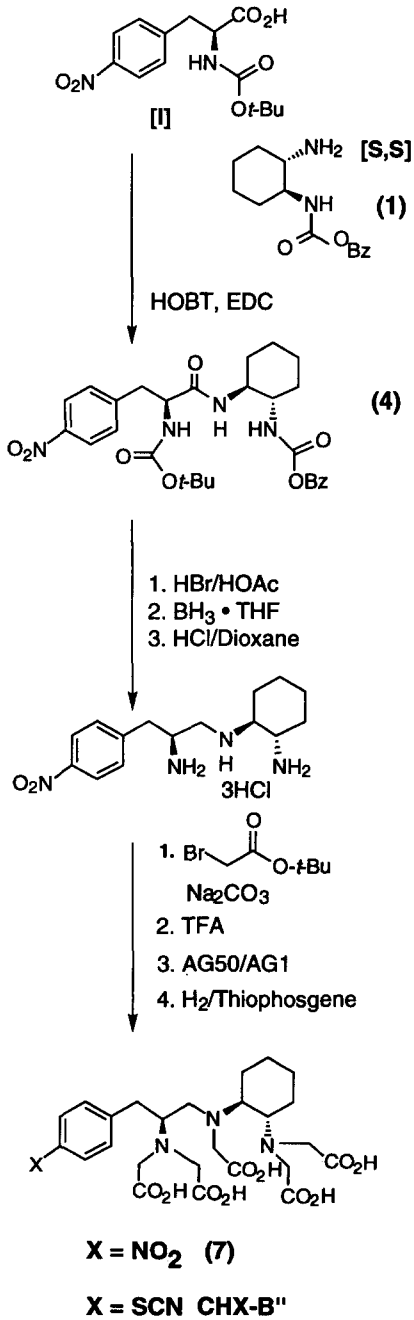

Figure 2. Stereospecific synthesis of CHX-B″ DTPA chelating agent.

A striking result was obtained upon in vivo evaluation of the ^{88}Y labeled B3 conjugates. Table 1 shows accumulation for processed femur at 24 and 168 h. Detailed biodistribution studies of these conjugates will be presented elsewhere.[19] The femurs were chosen as the most critical part of the body since yttrium is well known to be a bone-seeker when released in vivo.[20] While the general trend of the CHX-Bs being less stable than the CHX-As was observed, based on bone uptake, resolution of significant differences between the ^{88}Y labeled conjugates was clearly obtained. A large difference was readily observed between CHX-B′ and CHX-B″ at 24 h which continued to increase throughout the time course of the experiment with the CHX-B′ ^{88}Y labeled conjugate being least stable of all. Small, but significant differences were recorded between CHX-A″ and CHX-A′, wherein the former was found to be slightly more stable ($p < 0.05$) than the latter, consistantly with the lowest deposition of ^{88}Y to bone.

The influence of ligand or metal complex stereochemistry on the behavior of chelated radionuclides in vivo has been reported in the literature.[21–23] Two studies deal with differences that occur due to the diastereomeric nature of either the ligand itself or the complex formed being composed of two separable diastereomers. Not surprisingly, significant differences were observed for in vivo behavior. The metal complexes of the meso versus racemic *N,N′*-ethylenebis-[2-(*o*-hydroxyphenyl)glycine] (EHPG) were found to possess differences in HPLC retention, biodistribution localization and clearance, and stability constants as well.[21] Technetium and

Table 1. The percentage of injected dose per gram of processed bone of ^{88}Y CHX-A′, -A″, -B′, -B″ DTPA conjugated to B3 (mean ± 1 SD, $n = 10$)

Time	24 h	168 h
CHX-A′	1.68 ± 0.23	4.73 ± 0.71[a]
CHX-A″	1.54 ± 0.21	4.06 ± 0.61
CHX-B′	7.52 ± 0.84	21.91 ± 2.75[b]
CHX-B″	3.94 ± 0.72	12.13 ± 1.51[b]

[a] $p < 0.05$ compared with CHX-A″.
[b] $p <$ compared with CHX-A″ with student T-test.

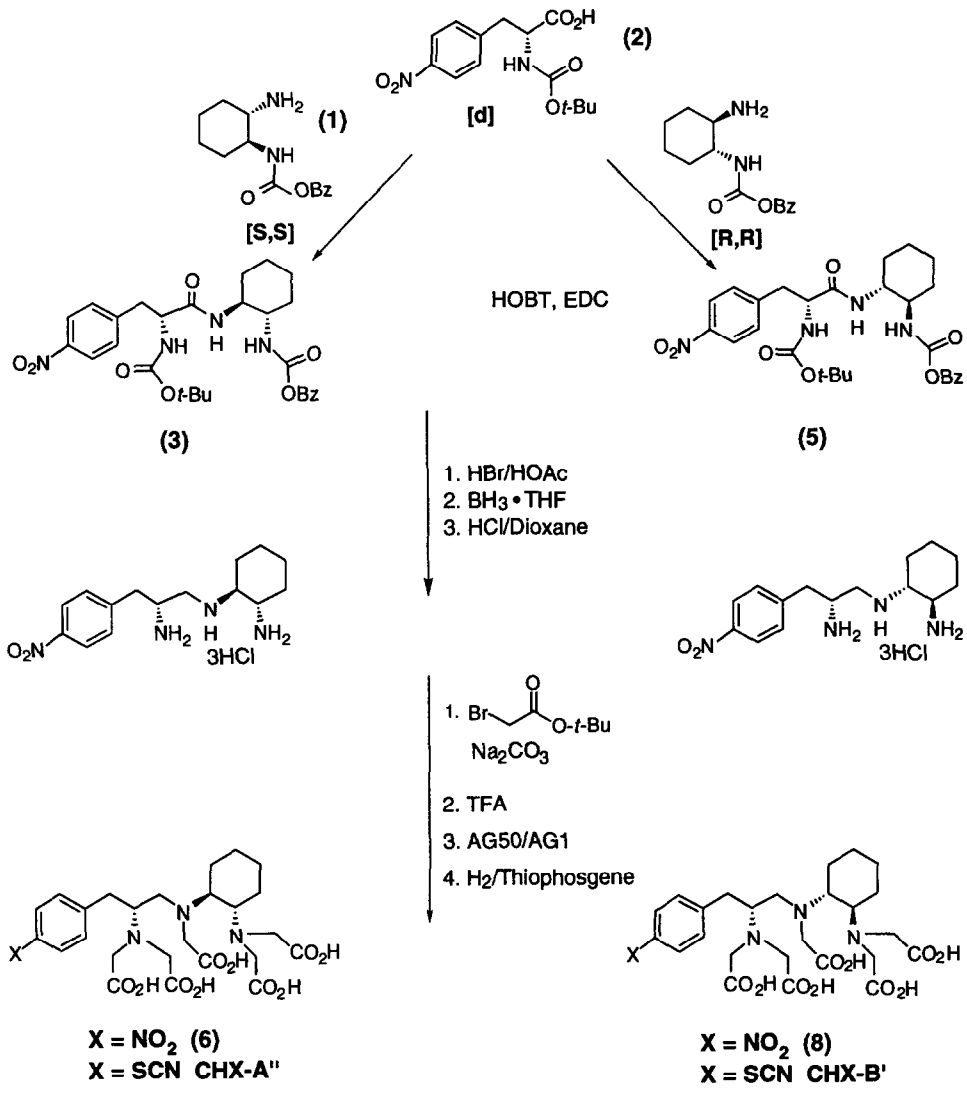

Figure 3. Stereospecific syntheses of the CHX-A″ and the CHX-B′ DTPA chelating agents.

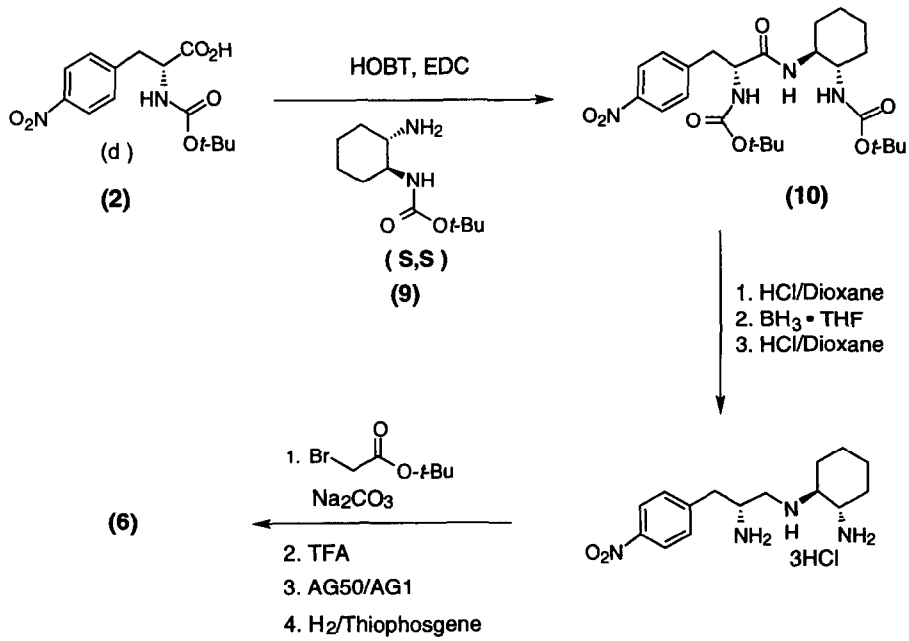

Figure 4. Modified stereospecific synthesis of the *p*-nitro precursor of CHX-A″ DTPA chelating agent.

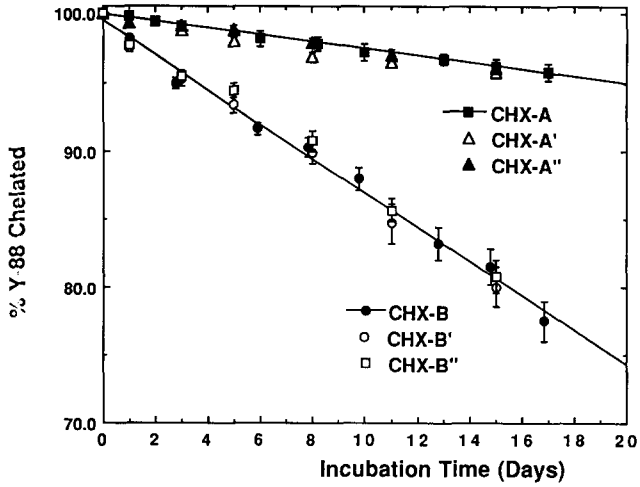

Figure 5. Serum stability of the ^{88}Y complexes of the four stereoisomers CHX-A′, -A″, -B′, B″ DTPA and their respective racemates over 17 days.

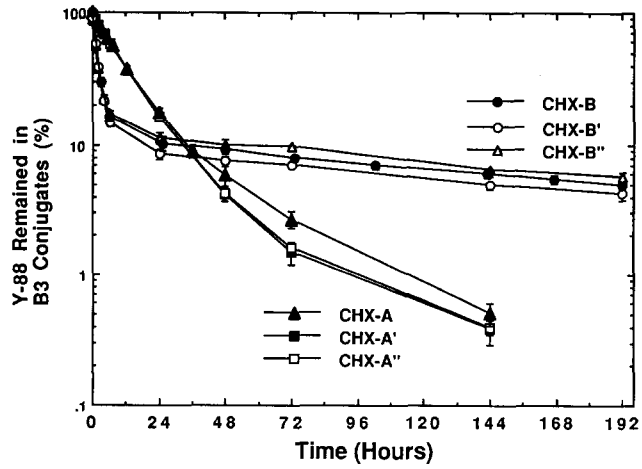

Figure 6. Transchelation of ^{88}Y from the ^{88}Y complexes of the four stereoisomers CHX-A′, -A″, -B′, B″ DTPA and their respective racemates conjugated to B3 versus excess EDTA.

rhenium complexes with 2,3-bis(mercaptoacetamido)-propanoic acid (map) formed diastereomers that also had chromatographic and biological differences.[22] The only literature report that has examined differences in properties between enantiomers was directed towards the determination of the potential immunogenicity of C-functionalized DOTA ligands conjugated to protein. Relevant to this report are the $t_{1/2}$ blood clearance curves reported for the (R)- versus (S)-DOTA ^{111}In labeled conjugates of rabbit IgG which were reported as being signicantly different ($p < 0.05$).[23] However, no attempts were made to examine differences in stability between the two complexes in that study.

The significance of the results obtained herein are of paramount importance and implies that if one chooses the proper stereochemistry, the geometry of the coordination sphere can be optimized to obtain ligands that could form radionuclide complexes conjugated to protein of yet greater stability. The results obtained through the use of in vitro stability experiments (Figs 5 and 6) serve to demonstrate that small, yet significant differences could not be detected through use of these techniques and that only in vivo experimentation was effective in obtaining these results. Use of chelating agents for RIT and RII must meet the minimum requirements of kinetic and thermodynamic stability in vivo. Failure to meet this fundamental prerequisite leads to dissociation of the isotope in vivo and its subsequent deposition into radiation sensitive organs which contributes to toxicity. The small, but reproducible increase in in vivo stability that occurs with use of the CHX-A″ then might allow for RIT with decreased toxicity to patients in treatment of cancer. An overall practical result of synthesis and use of CHX-A″ is the elimination of the tedious and wasteful synthesis of the racemate wherein both CHX-A and CHX-B were prepared and separated by preparative HPLC.

These results indicate that stereocontrol of the configuration of the chelating agent can be employed to refine existing chelating agents in general and may be a factor in the design of new bifunctional chelating agents.

Conclusions

Ligand stereochemistry, largely discounted as a factor in the stability of polyaminocarboxylate ligands used for RII and RIT, was found to be relevant to the stability of the yttrium complexes formed by the four stereoisomers CHX-A′,-A″,-B′,-B″. To the best of our knowledge, differences in stability between metal complexes formed from enantiomeric ligands conjugated to protein has not been examined in the literature and implies that either the metal ion itself, or the protein, an inherent chiral reagent, can exert a stereochemical influence to produce complexes that are inequivalent or diastereomeric in a biological milieu. Limitations of in vitro modeling via serum stability and transchelation experiments were demonstrated by the inability of these methods to record the results comparable to those obtained via biodistribution studies. Further detailed studies of the physical solution chemistry of the four stereoisomers are planned to ascertain whether there is any relationship between stability constants or acid catalyzed dissociation of the lanthanide complexes formed with these ligands and their in vivo behavior.

Experimental

Materials and methods

All chemicals and solvents were purchased from either Fluka, Sigma, or Aldrich. The L-*p*-nitrophenylalanine was obtained from Research Organics while the D-*p*-nitrophenylalanine was purchased from Bachem (CA). The *trans*-(*R*,*R*)- and (*S*,*S*)-1,2-diaminocyclohexanes were purchased from Fluka. Production and isolation of mAb B3 has been described in detail elsewhere.[24] ^{88}Y was obtained from Los Alamos National Labora-

tory. Ion-exchange resins were purchased from Bio-Rad.

^1H and ^{13}C NMR data were obtained using a Varian Gemini 300 instrument and chemical shifts are reported in ppm on the d scale relative to TMS, TSP, or solvent. Proton chemical shifts are annotated as follows: ppm (multiplicity, integral, coupling constant (Hz)). Chemical ionization mass spectra (CI-MS) were obtained on a Finnnegan 3000 instrument. Fast atom bombardment mass spectra (FABMS) were taken on a Extrel 400. Optical rotations were measured using a Rudolf Autopol III polarimeter. Infrared spectra were obtained using a Beckman 4240 spectrophotometer with the samples prepared as Nujol mulls. Melting points were obtained on a Thomas Hoover Unimelt capilaary melting point apparatus. Elemental analysis was performed by Atlantic Microlabs (Norcross, Georgia).

Analytical HPLC was performed using a Beckman gradient system equipped with Model 114M pumps controlled by System Gold software and a Model 165 dual wavelength detector set at 254 and 280 nm. An Altex C-18 reverse-phase column (5 (m particles, 4.6 (250 mm) and a binary gradient of 0–100% B/25 min (Solvent A = 0.05 M Et_3N/HOAc, pH 5.5, Solvent B = MeOH) at 1.0 mL/min was used for all analyses. Preparative HPLC was performed using a Waters DeltaPrep system. A Waters C-18 reverse-phase column (15 μm particles, 100 Å pore size, 30 × 300 mm) using the gradient described above at a flow rate of 40 mL/min. Size-Exclusion HPLC were performed using a system comprised of a Dionix GPM-2 pump, a Gilson 112 UV/VIS detector monitoring either 254 or 280 nm, and an on-line γ-detector from IN/US Systems. The system was controlled and data collection performed by IN/US WinFlow software.

Ligand synthesis. The racemic CHX-A and CHX-B DTPAs and the CHX-A′ DTPA were prepared as previously reported.[13] Syntheses of the remaining three stereoisomers (Figs 2 and 3) via modification of those methods, the details of which and relevent characterization data for all compounds is provided below.

***N*-Benzyloxycarbonyl-(*S*,*S*)-cyclohexane-1,2-diamine (1)**. Benzylchloroformate (7.48 g, 43.9 mmol) in diethyl ether (100 mL) was added dropwise to (*S*,*S*)-1,2-diaminocyclohexane (5.0 g, 43.9 mmol) in diethyl ether (1 L) over 6 h. The resulting suspesion was rotary-evaporated to solid, which was taken up in ethyl acetate (300 mL) and vigorously stirred with 1 M HCl (300 mL). After achieving complete solution, the aqueous portion was basified with solid Na_2CO_3 to pH 9.0 and extracted with ethyl acetate (3 × 150 mL). The combined extracts were dried over Na_2SO_4, filtered, and rotary evaporated to obtain a white solid (2.5 g, 23%). ^1H NMR ($CDCl_3$) δ 7.361 (m, 5H), 5.111 (s, 2H), 4.691 (m, 1H), 3.212 (m, 1H), 2.352 (dt, 1H, *J* = 10.2, 3.6), 1.969 (br.t, 2H, *J* = 21.3), 1.710 (m, 2H), 1.488 (s, 2H), 1.340–1.090 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 156.887, 136.791, 128.776, 128.412, 66.848, 58.166, 55.616, 35.276, 32.848, 25.137(2C); MS (CI/NH_3) 249 (M^++1); $[\alpha]^{23}_D$ +21.62° (*c* 1.11, MeOH); mp 106–108 °C; Anal. calcd for $C_{13}H_{20}N_2O_2$: C, 67.70; H, 8.13; N, 11.28. Found: C, 67.98; H, 8.10; N, 11.10.

***tert*-Butyloxycarbonyl-D-*p*-nitrophenylalanine (2)**. D-*p*-Nitrophenylalanine (15.8 g, 75.2 mmol) was treated with triethylamine (15.7 mL, 113 mmol) and 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON) (18.54 g, 75.2 mmol) in 50% aqueous dioxane for 18 h. The solution was diluted with H_2O (200 mL) and extracted with ethyl acetate (3 × 150 mL). The aqueous solution was cooled in an ice bath and acidified to pH 2.5 with 1 M HCl. The solution was then extracted with ethyl acetate (3 × 150 mL). The combine extracts were dried over Na_2SO_4, filtered, and rotary evaporated to obtain the carbamate as a thick yellow oil that solidified upon standing to yield a yellow-brown solid (20 g, 86%). ^1H NMR (DMSO-d_6) δ 8.166 (d, 2H, *J* = 8.7), 7.541 (d, 2H, *J* = 8.7), 7.228 (d, 1H, *J* = 9.0), 4.183 (m, 1H), 3.179 (dd, 1H, *J* = 13.8, 5.1), 2.958 (dd, 1H, *J* = 13.5, 10.8), 1.296 (s, 9H); ^{13}C NMR (DMSO-d_6) δ 173.374, 155.646, 146.721, 146.478, 130.692, 123.346, 78.236, 54.557, 36.221, 28.025; MS (CI/NH_3) 328 (M^++18); $[\alpha]^{23}_D$ 4.76° (*c* 1.05, MeOH); mp 74–76 °C; Anal. calcd for $C_{14}H_{18}N_2O_6$: C, 54.18; H, 5.86; N, 9.03. Found: C, 54.29; H, 6.00; N, 8.93.

***N*-(*S*,*S*)-*trans*-2-Benzyloxycarbonylaminocyclohexyl-*N*-*tert*-butyloxycarbonyl-D-*p*-nitrophenylalanine amide (3)**. Carbamate **2** (2.375 g, 7.66 mmol), amine **1** (1.90 g, 7.66 mmol), 1-hydroxybenzotriazole (HOBT) (1.037 g, 7.66 mmol) were dissolved in ethyl acetate (200 mL) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (1.48 g, 7.7 mmol) in *N*,*N*-dimethylformamide (DMF) (150 mL) was added. After 18 h, ethyl acetate (150 mL) was added and the solution extracted sequentially with salt solution (100 mL), 5% HCO_3^- (2 × 100 mL), salt solution (100 mL), 1 N HCl (2 × 100 mL), and salt solution (100 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated to ca. 75 mL. The volume was doubled with hexanes and the suspension stoppered at 4 °C for 18 h. The product was collected and dried in vacuo to leave a white solid (3.6 g, 87%). ^1H NMR ($CDCl_3$) δ 8.144 (d, 2H, *J* = 8.7), 7.40–7.25 (m, 7H), 6.551 (d, 1H, *J* = 7.5), 5.069 (dd, 2H, *J* = 21.6, 12.0), 4.944 (d, 2H, *J* = 7.8), 4.349 (m, 1H), 3.568 (m, 1H), 3.344 (m, 1H), 3.234 (dd, 1H, *J* = 13.8, 6.3), 2.885 (m, 1H), 1.986 (br.t, 2H, *J* = 14.7), 1.751 (m, 2H), 1.396 (s, 9H), 1.32–1.00 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 170.528, 157.052, 154.980, 146.866, 144.836, 136.023, 130.183, 128.183, 128.156, 127.93, 123.536, 80.417, 66.799, 55.305, 54.813, 54.646, 38.575, 32.400, 32.053, 28.207, 24.708, 24.433; MS (CI/NH_3) 541 (M^++1); $[\alpha]^{23}_D$ +8.36° (*c* 0.96, DMSO); mp 201–202 °C decomp.; Anal. calcd for $C_{28}H_{36}N_4O_7$: C, 62.20; H, 6.66; N, 10.40. Found: C, 61.97; H, 6.69; N, 10.44.

***N*-(*S,S*)-*trans*-2-Benzyloxycarbonylaminocyclohexyl-*N*-*tert*-butyloxycarbonyl-L-*p*-nitrophenylalanine amide (4).** *tert*-Butyloxycarbonyl-L-*p*-nitrophenylalanine (3.213 g, 10.4 mmol), amine **1** (2.57 g, 10.4 mmol) HOBT (1.4 g, 10.3 mmol), and EDC (1.986 g, 10.4 mmol) were reacted as described for **3** to obtain the product as a white solid (5.16 g, 92%). ^{1}H NMR ($CDCl_3$) δ 8.100 (d, 2H, J = 8.4), 7.30–7.015 (m, 7H), 6.545 (d, 1H, J = 7.2), 5.031 (dd, 2H, J = 26.1, 12.3), 4.978 (m, 2H), 4.320 (m, 1H), 3.591 (m, 1H), 3.394 (m, 1H), 3.122 (dd, 1H, J = 13.7, 5.4), 2.883 (dd, 1H, J = 13.8, 7.2), 2.057 (m, 2H), 1.752 (m, 2H), 1.370 (s, 9H), 1.30–1.10 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 170.709, 156.931, 155.010, 146.837, 144.608, 144.526, 130.265, 128.444, 128.151, 128.006, 123.438, 80.032, 66.851, 54.916, 54.719(2C), 38.457, 32.543, 32.248, 28.220, 24.740, 24.442; MS (CI/NH_3) 541 (M^++1); $[\alpha]^{23}_D$ +32.35° (*c* 1.02, DMSO); mp 206–207 °C decomp; Anal. calcd for $C_{28}H_{36}N_4O_7$: C, 62.20; H, 6.66; N, 10.40. Found: C, 62.24; H, 6.64; N, 10.24.

***N*-(*R,R*)-*trans*-2-Benzyloxycarbonylaminocyclohexyl-*N*-*tert*-butyloxycarbonyl-D-*p*-nitrophenylalanine amide (5).** Carbamate **2** (2.93 g, 9.45 mmol), *N*-benzyloxycarbonyl-(*R,R*)-cyclohexane-1,2-diamine (2.35 g, 9.47 mmol), HOBT (1.28 g, 9.46 mmol), and EDC (1.89 g, 9.84 mmol) were reacted as described for **3** to obtain the product as a white solid (4.80 g, 94%). ^{1}H NMR ($CDCl_3$) δ 7.996 (d, 2H, J = 8.4), 7.20–7.05 (m, 7H), 6.423 (d, 1H, J = 7.8), 5.042 (dd, 2H, J = 24.9, 12.3), 4.868 (d, 2H, J = 7.8), 4.211 (m, 1H), 3.457 (m, 1H), 3.287 (m, 1H), 3.018 (dd, 1H, J = 13.8, 5.7), 2.778 (dd, 1H, J = 13.8, 7.5), 1.946 (m, 2H), 1.646 (m, 2H), 1.265 (s, 9H), 1.20–1.00 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 170.761, 156.980, 154.978, 146.883, 144.654, 136.096, 130.292, 128.470, 128.173, 128.031, 123.462, 80.172, 66.846, 54.842, 54.705(2C), 38.438, 32.516, 32.220, 28.191, 24.704, 27.409; MS (CI/NH_3) 541 (M^++1); $[\alpha]^{23}_D$ 32.43° (*c* 1.05, DMSO); mp 208 °C decomp; Anal. calcd for $C_{28}H_{36}N_4O_7$: C, 62.20; H, 6.66; N, 10.40. Found: C, 62.10; H, 6.68; N, 10.43.

***N*-[(*R*)-2-Amino-3-(*p*-nitrophenyl)propyl]-*trans*-(*S,S*)-cyclohexane-1,2-diamine-*N,N,N′,N″,N″*-pentaacetic acid (6).** Amide **3** (3.40 g, 6.3 mmol) was deprotected with 33% HBr in glacial acetic acid (Fluka) (30 mL) and the resulting intermediate isolated as described.[13] This material was then washed into a three-necked flask with tetrahydrofuran (THF) (25 mL), the solution cooled in an ice bath, after which 1 M BH_3·THF (21 mL) was added and the reaction was then heated to 50 °C. After 24 h, the progress of the reaction was checked by HPLC and 1 M BH_3·THF (5 mL) was added. After another 24 h, 1 M BH_3·THF (5 mL) was added. After a final 24 h, the reaction was halted by addition of methanol, and the solvent removed to leave a gummy residue. This material was taken up in dioxane (100 mL), the solution saturated with $HCl_{(g)}$, and refluxed for 12 h. After cooling at 4 °C for 6 h, the precipitated crude triamine was collected, washed with Et_2O, and vacuum dried.

The crude triamine (1.00 g) was suspended in DMF (30 mL) and Na_2CO_3 (2.37 g, 22.4 mmol) was added. While heating to ca. 80 °C, *tert*-butyl bromoacetate (2.92 g, 14.97 mmol) was added after which the reaction was heated for 18 h. The crude penta-ester was isolated and treated with trifluoroacetic acid (TFA) (30 mL) as previously reported.[13] The TFA was removed by rotary evaporation and the tarry residue held under vacuum for 24 h to minimize remaining TFA. The residue was taken up in HPLC buffer and the penta-acid was separated from major impurities by preparative HPLC. The HPLC fraction, in buffer and MeOH, was concentrated and then further purified by ion-exchange chromatography[17] after which the product was isolated as a white powder (320 mg). ^{1}H NMR (D_2O) δ 8.202 (d, 2H, J = 8.7), 7.538 (d, 2H, J = 8.7), 4.00–2.85 (m, 19H), 2.165 (m, 2H), 1.799 (br.d, 2H, J = 8.7), 1.46–1.15 (m, 4H); ^{13}C NMR (D_2O) δ 180.121, 150.911, 149.386, 133.565, 126.873, 65.687, 64.712, 64.335, 62.109, 59.666, 56.785, 54.554, 53.337, 33.703, 25.111, 24.509, 24.112, 22.972; Anal. HPLC R_T = 10.35 min; MS (FAB/glycerol) 583 (M^++1); $[\alpha]^{23}_D$ 50.68° (*c* 0.59, H_2O); mp 165–168 °C decomp; Anal. calcd for $C_{25}H_{34}N_4O_{12}\cdot 6H_2O$: C, 43.47; H, 6.66; N, 8.10. Found: C, 43.17; H, 6.86; N, 8.45.

***N*-[(S)-2-Amino-3-(*p*-nitrophenyl)propyl]-*trans*-(*S,S*)-cyclohexane-1,2-diamine-*N,N,N′,N″,N″*-pentaacetic acid (7).** Amide **4** (4.70 g, 8.7 mmol) was deprotected with 33% HBr in glacial acetic acid as described for **6**. This material was then treated with 1 M BH_3·THF (31 mL) and after each of the next two 24 h periods had elapsed, after an HPLC assay, 1 M BH_3·THF (3 mL) was added. The crude triamine was isolated as described for **6**.

The crude triamine (1.00 g) was suspended in DMF (30 mL) and Na_2CO_3 (2.37 g, 22.4 mmol) was added. While heating to ca. 80 °C, *tert*-butyl bromoacetate (2.92 g, 14.97 mmol) was added after which the reaction was heated for 18 h. The product was isolated as a white solid as described above (295 mg). ^{1}H NMR (D_2O) δ 8.211 (d, 2H, J = 9.0), 7.512 (d, 2H, J = 9.0), 3.60–2.72 (m, 19H), 2.525 (m, 1H), 2.045 (m, 1H), 1.76–1.55 (m, 2H), 1.34–1.19 (m, 1H), 1.18–0.092 (m, 3H); ^{13}C NMR (D_2O) δ 181.839, 152.625, 149.175, 133.450, 126.832, 68.761, 64.672, 63.686, 63.441, 63.301, 62.294, 55.459, 54.225, 53.457, 33.854, 25.021, 24.759, 24.094, 23.971; Anal. HPLC R_T = 11.36 min; MS (FAB/glycerol) 583 (M^++1); $[\alpha]^{23}_D$ +30.10° (*c* 0.79, H_2O); mp 165–168 °C decomp; Anal. calcd for $C_{25}H_{34}N_4O_{12}\cdot 5H_2O$: C, 44.60; H, 6.55; N, 8.33. Found: C, 44.96; H, 6.57; N, 8.12.

***N*-[(*R*)-2-Amino-3-(*p*-nitrophenyl)propyl]-*trans*-(*R,R*)-cyclohexane-1,2-diamine-*N,N,N′,N″,N″*-pentaacetic acid (8).** Amide **5** (4.42 g, 8.19 mmol) was deprotected with 33% HBr in glacial acetic acid as described for **6**. This material was then treated with 1 M BH_3·THF (27.5 mL) for 24 h and after the next three 24 h periods, 2 × 6 mL, and 10 mL of hydride were added. The crude triamine was isolated as described for **6**.

The crude triamine (1.5 g) was suspended in DMF (30 mL) and Na_2CO_3 (3.55 g, 33.5 mmol) was added. While heating to ca. 80 °C, *tert*-butyl bromoacetate (4.38 g, 22.5 mmol) was added after which the reaction was heated for 18 h. The product was isolated as a white solid as described above (388 mg). ^{1}H NMR (D_2O) δ 8.208 (d, 2H, J = 8.7), 7.512 (d, 2H, J = 8.7), 3.65–2.80 (m, 19H), 2.570 (m, 1H), 2.05 (m, 1H), 1.80–1.55 (m, 2H), 1.40–1.20 (m, 1H), 1.15–0.95 (m, 3H); ^{13}C NMR (D_2O) δ 181.900, 152.150, 149.185, 133.469, 126.852, 68.741, 64.574, 63.722, 63.402, 63.342, 62.396, 55.549, 54.443, 53.413, 33.475, 25.102, 24.638, 24.022, 23.891; Anal. HPLC R_T = 11.31 min; MS (FAB/Glycerol) 583 (M^++1); $[\alpha]^{23}_D$ −29.61° (*c* 0.61, H_2O); mp 165–168 °C decomp; Anal. calcd for $C_{25}H_{34}N_4O_{12}\cdot 6H_2O$: C, 43.47; H, 6.66; N, 8.10. Found: C, 43.49; H, 6.52; N, 8.24.

***N*-[(*R*)-2-Amino-3-(*p*-isothiocyanatophenyl)propyl]-*trans*-(*S*,*S*)-cyclohexane-1,2-diamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (CHX-A″)**. Pentaacid **6** (54 mg, 93 (mol) was dissolved in H_2O (3 mL) and the pH was adjusted to ca. 9 with Na_2CO_3. The yellow solution was injected into a 50 mL Schlenk flask, previously charged with 10% Pd/C (50 mg) suspended in H_2O (5 mL) and flushed with H_2, and attached to an atmospheric hydrogenator. After H_2 uptake ceased, the reaction was filtered through a fine frit with a pad of Celite 577, and the filtrate was then lyophilized. The residue was taken up in H_2O (3 mL) and thiophosgene (11 μL, 143 μmol) in $CHCl_3$ (10 mL) was added in one portion with vigorous stirring which was continued for 2 h. The volatiles were then removed by rotary evaporation and the aqueous solution lyophilized to leave an off-white solid (63 mg). ^{1}H NMR (D_2O) δ 7.436 (s, 4H), 4.15–2.45 (br.m, 15H), 2.23 (m, 2H), 1.91 (m, 2H), 1.57–1.35 (m, 4H); IR spectra 2100 cm^{-1} (SCN); Anal. HPLC R_T = 22.37 min; MS (FAB/glycerol) 595 (M^++1).

***N*-[(*S*)-2-Amino-3-(*p*-isothiocyanatophenyl)propyl]-*trans*-(*S*,*S*)-cyclohexane-1,2-diamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (CHX-B″)**. Pentaacid **7** (77 mg, 132 μmol) was hydrogenated and the reduction product was treated with thiophosgene (20 μL, 143 μmol) as described for CHX-A″ to leave an off-white solid (61.3 mg). ^{1}H NMR (D_2O) δ 7.302 (s, 4H), 4.16–1.05 (br.m, 23H); IR 2100 cm^{-1} (SCN); Anal. HPLC R_T = 17.33 min; MS (FAB/glycerol) 595 (M^++1).

***N*-[(*R*)-2-Amino-3-(*p*-isothiocyanatophenyl)propyl]-*trans*-(*R*,*R*)-cyclohexane-1,2-diamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (CHX-B′)**. Pentaacid **8** (52 mg, 89 μmol) was hydrogenated and the reduction product was treated with thiophosgene (11 μL, 143 μmol) as described for CHX-A″ to leave an off-white solid (58.3 mg). ^{1}H NMR (D_2O) (7.322 (s, 4H), 4.16–0.98 (br.m, 23H); IR 2100 cm^{-1} (SCN); Anal. HPLC R_T = 17.21 min; MS (FAB/glycerol) 595 (M^++1).

***N*-*tert*-Butyloxycarbonyl-(*S*,*S*)-cyclohexane-1,2-diamine (9)**. (*S*,*S*)-1,2-Diaminocyclohexane (15.00 g, 132 mmol) was dissolved in dry dioxane (900 mL) and BOC-ON (32.4 g, 132 mmol) in dioxane (100 mL) was added and the reaction was stirred at room temperature for 18 h. The solvent was removed by rotary evaporation to leave a syrup that was taken up in ethyl acetate (500 mL) and H_2O (300 mL). The aqueous layer was acidified to ca. pH 2.5 with 3 N HCl and the product was extracted into the acidic phase. The layers were immediately separated and the aqueous layer basified with solid Na_2CO_3 (pH 9.5). The product was extracted back into ethyl acetate (3 × 100 mL). The combined extracts were dried over Na_2SO_4, filtered, and rotary evaporated to isolate the carbamate as a yellow-tan solid (13.85 g, 49%). ^{1}H NMR ($CDCl_3$) δ 4.385 (br.s, 1H), 3.070 (br.d, 1H, J = 7.8), 2.256 (dt, 1H, J = 9.9, 3.9), 1.907 (m, 2H), 1.636 (m, 2H), 1.523 (br.s, 2H), 1.383 (s, 9H), 1.325–0.950 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 156.462, 79.416, 57.680, 55.737, 35.216, 32.969, 28.416, 25.198, 25.076; MS (CI/NH_3) 215 (M^++1); $[\alpha]^{23}_D$ +42.99° (*c* 0.98, MeOH); mp 110–111 °C; Anal. calcd for $C_{11}H_{22}N_2O_2$: C, 61.63; H, 10.37; N, 13.07. Found: C, 61.58; H, 10.29; N, 12.94.

***N*-(*S*,*S*)-*trans*-2-*tert*-Butyloxycarbonylaminocyclohexyl-*N*-*tert*-butyloxycarbonyl-D-*p*-nitrophenylalanine amide (10)**. Acid **2** (13.40 g, 43.2 mmol), amine **9** (9.25 g, 43.2 mmol), HOBT (5.85 g, 42.9 mmol), and EDC (8.60 g, 44.9 mmol) were reacted as described above for **3** to obtain the product as a tan solid (18.8 g, 86%). ^{1}H NMR (DMSO-d_6) δ 8.159 (d, 2H, J = 9.0), 7.851 (d, 1H, J = 7.8), 7.521 (d, 2H, J = 9.0), 6.753 (d, 1H, J = 8.7), 6.536 (d, 1H, J = 7.8), 4.190 (m, 1H), 3.444 (m, 1H), 3.200 (m, 1H), 3.019 (dd, 1H, J = 10.8, 3.9), 2.855 (dd, 1H, J = 12.4, 9.6), 1.80 (m, 1H), 1.71 (m, 1H), 1.61 (m, 2H), 1.344 (s, 9H), 1.302 (s, 2H), 1.270 (s, 6H), 1.225–1.100 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 170.885, 155.646, 155.039, 146.721, 146.357, 130.814, 130.692, 123.407, 123.225, 78.478, 77.568, 55.225, 54.496, 53.161, 52.129, 38.104, 36.161, 31.850, 28.207, 28.025, 24.261; MS (CI/NH_3) 507 (M^++1); $[\alpha]^{23}_D$ +24.67° (*c* 0.96, MeOH); mp 188–190 °C decomp; Anal. calcd for $C_{25}H_{38}N_4O_7$: C, 59.26; H, 7.58; N, 11.06. Found: C, 59.86; H, 7.41; N, 10.95.

(Alternate route from di-BOC precursor 10) *N*-[(R)-2-Amino-3-(*p*-nitrophenyl)propyl]-*trans*-(*S*,*S*)-cyclohexane-1,2-diamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (6). Bis-carbamate **10** (7.5 g, 14.8 mmol) was added to dry dioxane (250 mL) previously saturated with $HCl_{(g)}$ and cooled in an ice bath. The suspension was left stirring at room temperature for 18 h. The volume was then doubled with diethyl ether, and the flask cooled (4 °C) for 4 h. The precipitate was then collected, washed with diethyl ether, and vacuum dried for 24 h. The solid was washed into a three-necked flask with THF (150 mL), cooled in an ice bath, and 1 M BH_3·THF (75 mL) was injected. After stirring at room temperature for 2 h, the reation was heated at 50–52 °C for 48 h. The excess hydride was decomposed with methanol, and the solution rotary evaporated to a gum which was held under vacuum for 18 h. This material was taken up in dioxane (50

mL), added to dioxane (200 mL), the combined solution saturated with $HCl_{(g)}$, refluxed for 6 h, and the resulting suspension left stirring for 12 h. The precipitate was collected, washed with diethyl ether, and dried under vacuum. The crude triamine (3.0 g) was suspended in DMF (40 mL) and Na_2CO_3 (7.92 g, 74.7 mmol) was added. While heating to ca. 80 °C, *tert*-butyl bromoacetate (8.745 g, 44.85 mmol) was added after which the reaction was heated for 18 h. The final product was isolated as described above (1.98 g, 23%). All characterization data for this compound were identical to that obtained previously for **6**.

Conjugation of ligands to protein. The mAb B3 was concentrated to ca. 5 mg/mL and dialyzed into 0.1 M HEPES, 0.15 M NaCl, pH 8.5. Solutions of the mAb were then conjugated to each of the four enantiomers and the racemates, respectively, and purified as previously described.[15,24] The average number of chelates per antibody for each enantiomer was determined to range between 1.5–2.0 for the four preparations.[25]

Radiolabeling of B3-CHX-DTPA conjugates. Carrier free ^{88}Y was first purified by ion-exchange chromatography (Re.Spec resin, EiChrom). In brief, stock solution was mixed with an equal volume of concentrated HNO_3 and heated to dryness. The activity was dissolved in 2 M HNO_3 and passed through the resin. The fractions containing the ^{88}Y were collected, heated to dryness, taken up in 0.1 M HNO_3 and used for radiolabeling. The pH of the ^{88}Y solution was adjusted to ca. 6.0 with 3 M NH_4OAc after which the B3 conjugate was added. After incubating for 30 min, 5 μL 0.1 M EDTA was added and the radiolabeled protein preparation was purified by HPLC using a TSK-3000 column eluted with Phosphate Buffered Saline (PBS) at 1.0 mL/min. Radiochemical yields were 85–90% for all preparations and specific activities ranged from 1.5–1.6 mCi/mg. Immunoreactivity of each preparation prior to and after radiolabeling was determined by described methods.[26]

Serum stability

The *p*-nitro form of the CHX-A, -A′, -A″, -B, -B′, -B″ DTPAs were labeled with ^{88}Y and their serum stability was evaluated over 17 days. Briefly, for each isomer, 25 μL of each stock ligand solution (4×10^{-4} M) were mixed with 55–60 (Ci of ^{88}Y in 0.1 M HNO_3, and the pH was adjusted to 5.5–6.5 with 3 M NH_4OAc. The reaction mixture was incubated at 37 °C for 30 min. The complexes were purified by Chelex-100 resin (Bio-Rad, 3 × 15 mm), eluted from the resin with H_2O (0.5 mL), and diluted to 1.0 mL. In every case, labeling efficiencies were ≥90%.

Aliquots (100 μL) of each ^{88}Y-labeled ligand stock solution (1.0×10^{-5} M) were then mixed with 9.9 mL of normal human serum (final concentration 1.0×10^{-7} M), passed through a sterile 0.22 μm filter (Millipore, Bedford, MA), aliquoted (0.8 mL) into 12 sterile culture tubes and incubated at 37 °C in a CO_2-enriched atmosphere (5% CO_2).

The percentage of dissociated ^{88}Y was assessed by paper chromatography (Whatman No.1) using PBS as the mobile phase. All ^{88}Y-labeled ligands showed R_f values ca. 1.0, whereas in the control experiments, less than 1% of free activity migrated above the top half of the paper ($R_f > 0.5$).

Transchelation

Reaction mixtures were prepared by mixing appropriate amounts of each ^{88}Y labeled B3 conjugate prepared above such that the concentration of components was 1×10^{-7} M for the chelate, 0.01 M in DTPA, and 1 mg/mL in human IgG (carrier protein to prevent nonspecific adsorption of B3), at pH 5.0 (0.1 M NH_4OAC) or at 7.2 (0.1 M HEPES). The mixtures were sterilized by filtering them (0.22 μm filters) into sterile culture tubes which were then tightly capped, and incubated in a 37 °C water bath.

Percent ^{88}Y transchelated to DTPA from each ^{88}Y labeled B3 CHX conjugate was measured by size-exclusion HPLC (7.8 mm × 30 cm, TosoHaas, Japan) eluting with PBS at 1.0 mL/min.

Biodistribution (femur deposition)

A detailed report of the complete biodistribution of the four stereoisomers conjugated to B3 and labeled with ^{88}Y will be presented elsewhere.[19] Nontumor bearing athymic mice (10 mice per time point) were given iv 1.5, 1.7, 1.7, and 1.5 (Ci of the ^{88}Y CHX-A′, -A″, -B′, and -B″ radiolabeled B3 conjugate, respectively, 2 μg of protein total. The mice were sacrificed and a modification of the reported method was applied for determining uptake of ^{88}Y in cortical bones.[15,26] In brief, a femur was removed from each, broken longitudinally, incubated with PBS for 30 min and then washed again with PBS. The bone was then washed with PBS/10% SDS for 1 h at 56 °C, centrifuged, and then washed again with PBS. The activity in the bone was counted after being blotted dry and weighed to determine percent injected dose per gram of processed bone.

Statistical analysis of the results was performed using One-way analysis of variance (ANOVA, with pairwise comparison using the Bonferroni method (Sigmastat, Jandel Scientific, San Rafael, CA).

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