Synthesis of C-Functionalized *trans*-Cyclohexyldiethylenetriaminepenta-acetic Acids for Labelling of Monoclonal Antibodies with the Bismuth-212 α-Particle Emitter

Martin W. Brechbiel* and Otto A. Gansow*
Inorganic and Radioimmune Chemistry Section, Radiation Oncology Branch, Building 10, Room B3B69, National Cancer Institute, NIH, Bethesda, MD, 20892, USA

Several C-functionalized cyclohexyldiethylenetriaminepenta-acetic acid derivatives have been prepared from (±)-4-nitrophenylalanine and (±)-*trans*-cyclohexane-1,2-diamine to produce two sets of diastereoisomeric enantiomers. A modification of this synthesis has been employed to prepare a single enantiomer in order to define the absolute configurations of the products.

Radioimmunotherapy against cancer by use of antibodies labelled with β-particle emitting radionuclides is under clinical investigation.[1–4] Monoclonal antibody (mAb) immunoconjugates employing α-particle emitters are of even greater potential therapeutic value in that α-decay produces cytocidal, densely ionizing radiation of very high energy over a range of a few cell diameters thus limiting toxicity to small tissue volumes.[5] We have discussed the exquisite and remarkably tumour-cell-specific cytotoxicity of ^{212}Bi-mAb conjugates *in vitro*.[6,7] Recent *in vivo* use of the short half-life ^{212}Bi ($t_{\frac{1}{2}}$ = 60.6 min, $E_{av}(\alpha)$ = 7.8 MeV) minimized systemic toxicity in a recent study of α-particle mediated radiotherapy of leukaemia in mice.[8]

A chelating agent is necessary for labelling of immunoprotein with ^{212}Bi.[9–11] Chemical constraints on the chelator to be employed are severe. Complexes must be thermodynamically stable and kinetically inert so that the radioisotope is irreversibly bound to protein for at least five half-lives to avoid renal toxicity caused by kidney deposition of released radiobismuth. Yet, preparation of the ^{212}Bi labelled immunoconjugate should be rapid and efficient to make effective use of carrier-free metal solutions and to minimize radiation damage. Thus, while bismuth chelates of the macrocyclic ligand DOTA† are inert both *in vitro*[12] and *in vivo*,[13] attempts to label DOTA linked to mAb with ^{212}Bi iodide according to established procedures were unsuccessful due to slow complex formation kinetics.[14] To obviate this difficulty, we have prepared a C-functionalized derivative of *trans*-cyclohexyldiethylenetriaminepenta-acetic acid (CyDTPA) which is both inert *in vivo* and useful for preparation of ^{212}Bi-mAb.

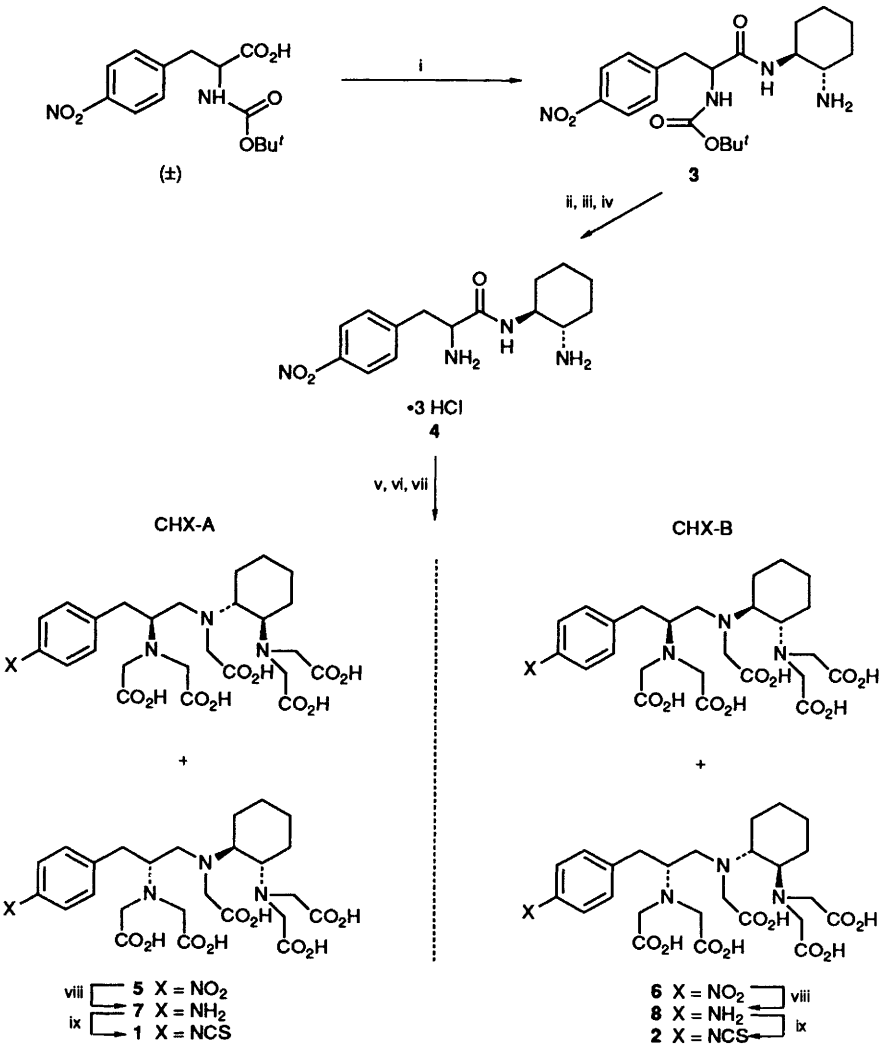
The rationale which guided our selection of CyDTPA for synthesis is simply stated. We observed that the seven-coordinate antibody-linked diethylenetriaminetetra-acetic acid produced by reaction of DTPA anhydrides with protein lysine residues formed bismuth complexes adequately stable in cell culture media but labile in animals.[7,15] An eight-coordinate ligand, 2-(*p*-isothiocyanatobenzyl)-DTPA, when similarly linked to mAb was seen to reduce significantly deposition of radiobismuth (^{206}Bi) in mouse kidneys, the kidney being the natural repository of bismuth.[16] Introduction of a C-4 methyl substituent onto the backbone of this DTPA to sterically hinder breakage of metal chelate rings further reduced kidney uptake.[13] We reasoned that fusion of the *trans*-cyclohexyl moiety into DTPA would additionally increase steric rigidity in the complex and improve the orientation of the chelating groups.[17] Moreover, the *trans*-cyclohexyl EDTA ligand was known to form trivalent metal complexes markedly more thermodynamically stable than those of EDTA itself.[18] Accordingly, we have synthesized by straightforward and efficient means a set of bifunctional CyDTPA ligands which incorporate the *p*-isothiocyanatobenzyl side chain for protein linkage.[19] We note that the parent CyDTPA ligand had been claimed in the patent literature and as well was suggested in a poster abstract while our study was in progress.[20,21] A preliminary account of the current study has appeared.[14]

Synthesis of C-Functionalized Cyclohexyl-DTPA.—The substituted (−)- or (±)-*p*-nitrophenylalanines, which have often been employed in bifunctional ligand preparations,[22–24] served as starting materials for our syntheses. Inclusion of the aryl nitro group as a backbone substituent preserves the octadenticity of DTPA and serves as a latent amine that may later be selectively derivatized to produce various protein linker groups.

Attempts to produce the amide **3** *via trans*-cyclohexyldiamine aminolysis of methyl *p*-nitrophenylalanine gave none of the desired product (EI-MS), although this method had been successful with ethylenediamine.[23] Reaction of carbamate protected (±)-*p*-nitrophenylalanine produced hydroxysuccinimidyl ester (HOSu, EDC, EtOAc, DMF; 25 °C) which immediately after isolation was added dropwise in dimethylformamide (DMF) to rapidly stirred neat (±)-*trans*-cyclohexane-1,2-diamine to produce the monoamide **3** (77%) (Scheme 1). This amide then was deprotected by treatment with $HCl_{(g)}$ in anhydrous dioxane and reduced to the diethylenetriamines (diens) **4** with borane–THF (86%).

Alkylation of C-functionalized diens **4** with excess chloro- or bromo-acetic acid to generate the corresponding DTPA produced a large number of products (HPLC) none of which were a DTPA (EI-MS). Whereas halogenoacetate methyl esters had been reported as satisfactory for alkylation of cyclic polyamines[25] to form macrocyclic ligands, employment here generated a mixture of compounds which were interpreted to arise from lactam formation [*m*/*z* (CI/NH_3) 549 (M^+ + 1)] with no measurable penta-ester present. Use of the *tert*-butyl ester was successful. Modification of a reported procedure[26] ($BrCH_2CO_2Bu^t$, Na_2CO_3, DMF) followed by treatment with trifluoroacetic acid, cleanly provided two DTPA derivatives in *ca.* 45:55 ratio (HPLC) which could be isolated by preparative HPLC. Additional purification *via* anion-exchange chromatography (AG1X8, 200–400 mesh, $ClCH_2CO_2H$ form) produced a pair of analytically pure bifunctional CyDTPA ligands, **5**, **6**, which were labelled CHX-A and CHX-B, respectively.

† Abbreviations used in this paper are as follows: DOTA = 1,4,7,10-tetraazacyclododecanetetraacetic acid, CyDTPA = *trans*-cyclohexyldiethylenetriaminepentaacetic acid, EDTA = ethylenediaminetetraacetic acid, HOSu = *N*-hydroxysuccinimide, EDC = 1,2-dichloroethane, DMF = dimethylformamide, THF = tetrahydrofuran, DTPA = diethylenetriaminetetraacetic acid, dien = diethylenetriamine.



Scheme 1 *Reagents and conditions:* i, HOSu, EDC, (±)-*trans*-cyclohexane-1,2-diamine; ii, $HCl_{(g)}$/dioxane; iii, BH_3·THF; iv, $HCl_{(g)}$/dioxane; v, $BrCH_2CO_2Bu^t$; vi, TFA; vii, HPLC; viii, H_2/Pd-C; ix, Cl_2CS

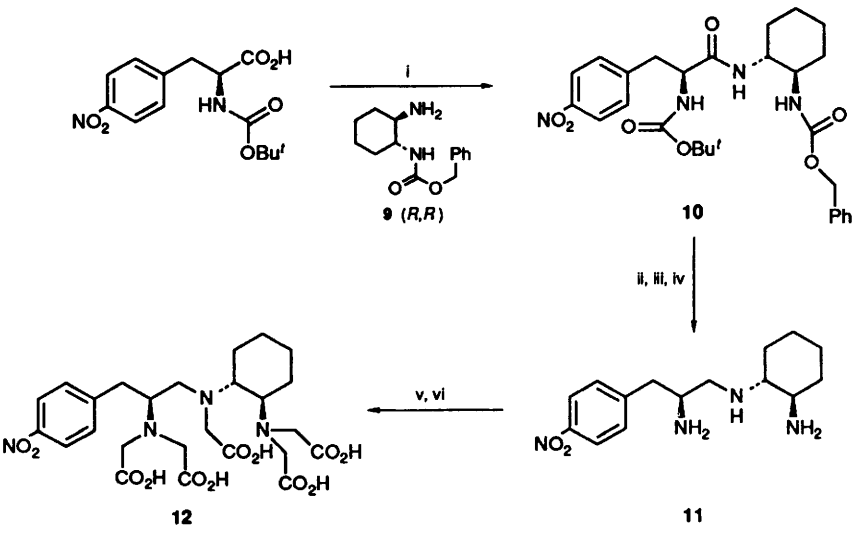
We expected that differences in stereochemistry were responsible for the two separable fractions in the HPLC chromatography. An examination of possible products of the synthesis revealed that two pairs of enantiomers should be produced and that the relationship between them would be diastereoisomeric. The two products from this sequence of reactions were consistently found (HPLC) to be in *ca.* 45:55 ratio rather than 50:50 ratio expected from the racemic amino acid starting material. In repeating the synthesis with (−)-*p*-nitrophenylalanine, we again produced a 45:55 ratio of products now indicative of racemization at the amino acid chiral centre.

Since diastereoisomeric ligands may behave differently in biological systems, it seemed prudent to ascertain the precise configurations of CHX-A and CHX-B. Accordingly, a modified synthesis of the C-functionalized cyclohexyl-DTPA was performed by using (−)-*p*-nitrophenylalanine and (*R,R*)-*trans*-cyclohexane-1,2-diamine to generate a single enantiomer (Scheme 2). Carbamate-protected (−)-*p*-nitrophenylalanine was converted into the amide **10** by diimide coupling (HOBT, EDC, EtOAc, DMF) with *N*-benzyloxycarbonyl-(*R,R*)-*trans*-cyclohexane-1,2-diamine. The carbamate protecting groups were removed with 33% HBr in acetic acid after which the ammonium salt amide was reduced with borane–THF, and the triamine **11** alkylated as described above. After treatment with trifluoroacetic acid, analytical HPLC revealed that this enantiomer (*S,R,R*)-**12** corresponded with the first HPLC peak CHX-A **5** generated by the diamine route described above. With this information, the structural identity of the two products **5**, **6** was complete. Compound **12** and its corresponding enantiomer are the constituents of **5** and their diastereoisomeric pair of enantiomers must be **6**.

For linkage of ligands **5**, **6** to mAb, we chose to employ the isothiocyanato group to be consistent in biological evaluation of relative usefulness of these ligands with radio-bismuth *versus* other DTPA ligands.[27] Additionally, to allow accurate quantitation of amount of ligand linked to mAb, **5**, **6** were prepared with a ^{14}C label by alkylation with [^{14}C]-*tert*-butyl bromoacetate.

To form isothiocyanates, the nitro compounds **5**, **6** were hydrogenated (10% Pd/C) and the product anilines **7**, **8** were converted into the respective isothiocyanates **1**, **2** by treatment with thiophosgene.[28] Details of these synthetic procedures and of linkage to mAb have been extensively discussed.[15]

Conclusions.—The facile synthesis of the C-functionalized *p*-nitrobenzylcyclohexyl DTPA ligands reported here produces separable products **5**, **6**, whose configurations were characterized by preparation of a single enantiomer **12**. Reduction of the nitro group to an amine provides reagents which will allow considerable flexibility in choice of functionalities ultimately to be used for ligand to mAb linkage



Scheme 2 *Reagents:* i, HOBT, EDC; ii, HBr/HOAc; iii, BH_3·THF; iv, HCl/dioxane; v, $BrCH_2CO_2Bu^t$, Na_2CO_3; vi, TFA

Experimental

Anhydrous solvents (THF, dioxane and DMF) were employed throughout the synthesis. (±)-*trans*-Cyclohexane-1,2-diamine was distilled from 5 Å sieves prior to use. *tert*-Butyl bromo-[$^{14}C_1$]acetate (sp. act. 7.6 mCi mmol^{-1}) was provided by custom synthesis from Amersham. ^{1}H and ^{13}C NMR spectra were obtained by using a Varian 300XL or a Nicolet NTC500 spectrometer. Chemical shifts are reported in ppm on the δ scale relative to TMS, TSP, or the following ^{13}C signal of solvents as noted in the text: dioxane (δ 66.5), CD_3CN (δ 1.30). Circular dichroism data were recorded using a Jasco J-600 spectropolarimeter. Chemical ionization mass spectra (CI) were obtained by use of a Finnegan 3000 instrument. Electron impact mass spectra (EI) were recorded on an LKB9000 instrument. Fast atom bombardment (FAB) mass spectra were measured by the Mass Spectroscopy Laboratory, College of Chemistry, University of California, Berkeley, CA. Elemental analyses were performed by either Galbraith Laboratories (Knoxville, TN) or Atlantic Microlabs (Atlanta, Georgia). Analytical HPLC was performed by 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 μm particles, 4.6 × 250 mm) and a binary linear gradient of 0–100%B/25 min (solvent A=0.05 mol dm^{-3} Et_3N/HOAc, pH 5.5; solvent B=MeOH) at 1.0 cm^3 min^{-1} was used for all analyses. Preparative HPLC was performed by using a Waters DeltaPrep system with a Waters C-18 reverse phase column (15 μm particles, 100 Å pore size, 30 × 300 mm) with the gradient described above but at a flow rate of 40 cm^3 min^{-1}.

N-tert-*Butoxycarbonyl*-N′-(trans-2-*aminocyclohexyl*)-p-*nitrophenylalaninamide* **3**.—*N-tert*-Butoxycarbonyl-*p*-nitrophenylalanine (19.0 g, 61.3 mmol), *N*-hydroxysuccinimide (7.05 g, 61.33 mmol) and EDC (11.75 g, 61.3 mmol) were dissolved in a mixture of ethyl acetate (300 cm^3) and DMF (50 cm^3). After the mixture had been stirred under argon for 18 h, the precipitate formed was collected and vacuum dried. To obtain additional yield, the filtrate was extracted with water (100 cm^3), 5% $NaHCO_3$ (3 × 200 cm^3), and saturated brine (100 cm^3). The ethyl acetate solution was then dried ($MgSO_4$), filtered, and taken to dryness to leave an off-white solid which was combined with the vacuum dried precipitate. This active ester was dissolved in dry DMF (250 cm^3), filtered, and added dropwise over 24 h to freshly distilled (±)-*trans*-cyclohexane-1,2-diamine (500 cm^3) under argon with vigorous stirring. After addition, the cloudy solution was filtered and vacuum evaporated to provide a thick oil which was taken up in $CHCl_3$ (600 cm^3) and the solution extracted with 5% $NaHCO_3$ (3 × 200 cm^3) and washed with saturated brine (100 cm^3). After being dried ($MgSO_4$), the solution was filtered, evaporated to *ca.* 100 cm^3, diluted with light petroleum (200 cm^3) and vigorously stirred. The product solidified and the resulting suspension was cooled (4 °C) for 8 h and the solid product collected on a Buchner funnel whilst being copiously washed with light petroleum. The solid product was vacuum dried to give the monoamide **3** (19.15 g, 77%) (Found: C, 59.75; H, 7.35; N, 13.3. $C_{20}H_{30}N_4O_5$ requires C, 59.08; H, 7.45; N, 13.78%); δ_H([$^2H_6$]DMSO) 8.133 (d, 2 H, *J* 8.5), 7.453 (d, 2 H, *J* 8.5), 7.092 (d, 1 H, *J* 8.0, CON*H*CH), 5.872 (d, 1 H, *J* 8.5, O_2CN*H*), 4.426 (m, 1 H, CONHC*H*), 4.439 (br q, 1 H, CH_XCO), 3.250 (dd, 1 H, *J* 14.0, 6.0, PhCH_aH_b), 3.046 (dd, 1 H, *J* 13.5, 5.0, PhCH_aH_b), 2.361 (m, 1 H), 2.00–1.50 (br m, 4 H), 1.383 [s, 9 H, $(CH_3)_3$] and 1.350–1.00 (m, 4 H); δ_C([$^2H_6$]DMSO) 170.41 (CONH), 154.76 (O_2CNH), 146.15, 144.96, 129.81. 122.72, 79.09, 55.87, 54.90, 54.12, 37.96, 34.26, 31.45, 27.66 and 24.39; *m/z* (CI/NH_3) 407 (M^+ + 1).

N-[2-*Amino*-3-(p-*nitrophenyl*)*propyl*]-trans-*cyclohexane*-1,2-*diamine Trihydrochloride* **4**.—The amide **3** (10.21 g, 25.1 mmol) was deprotected by additon to dry dioxane (300 cm^3) previously saturated with $HCl_{(g)}$. This suspension was stirred under argon for 8 h and then left at 4 °C for 12 h. A precipitate formed and was collected under an argon atmosphere and vacuum dried. This solid was washed into a three-necked flask with dry THF (100 cm^3) which was cooled in an ice-bath and 1 mol dm^3 BH_3/THF (150 cm^3) was added *via* a syringe; the reaction mixture was then heated to 50 °C for 48 h. The reaction was again cooled in an ice-bath and MeOH (40 cm^3) was slowly added. The solution was vacuum rotary evaporated to a gum and the residue was dissolved in 100% EtOH (100 cm^3) and again vacuum rotary evaporated to a gum and finally taken to a solid under high vacuum. Dry dioxane (150 cm^3), previously saturated with $HCl_{(g)}$, was added and the resulting suspension was vigorously refluxed for 5 h. The suspension was then stirred at room temperature under argon for 12 h. A precipitate formed and was collected, washed with ether, and dried *in vacuo* to give the dien **4** (8.69 g, 86%); HPLC t_R 16.3 min [Found: C, 44.9; H, 6.6; N, 13.85. $C_{15}H_{24}N_4O_2(HCl)_3$ requires C, 44.83; H, 6.79; N, 13.95%]; δ_H(D_2O, pH 1.0) 8.282 (d, 2 H, *J* 8.0), 7.597 (d, 2 H, *J* 8.0), 4.070 (m, 1 H, HNCH), 3.70–3.10 (m, 6 H), 2.20 (m, 2 H), 1.821 (m, 2 H) and 1.70–1.30 (m, 4 H); δ_C(D_2O, pH 1.0, Ref =

dioxane) 147.87, 142.98, 131.11, 124.88, 60.43, 59.89, 53.25, 52.94, 51.55, 51.25, 47.43, 46.67, 36.77, 29.93, 27.94, 23.53, 23.45, 23.30 and 23.19; *m/z* (CI/NH_3) 293 ($M^+ + 1$).

N-[2-*Amino*-3-(p-*nitrophenyl*)*propyl*]-trans-*cyclohexane*-1,2-*diamine*-N,N′,N″-*penta-acetic Acids* (CHX-A, CHX-B) **5**, **6**.—The triamine **4** (5.54 g, 13.8 mmol) and Na_2CO_3 (13.2 g, 0.124 mol) were suspended in DMF (40 cm^3) and heated under argon to *ca.* 65 °C after which *tert*-butyl bromoacetate (16.15 g, 82.8 mmol) was added. The reaction mixture was then heated to *ca.* 90 °C for 12 h. After being cooled, the solution was poured into CH_2Cl_2 (60 cm^3) and washed with water (3 × 200 cm^3), dried ($MgSO_4$), filtered and vacuum rotary evaporated to provide a thick dark oil. Trifluoroacetic acid (80 cm^3) was added and the solution that formed was stirred for 12 h. The acid solution was then vacuum rotary evaporated to leave a dark gum which was dissolved in 0.5 mol dm^{-3} Et_3N–$HOAc_{(aq)}$ (500 cm^3) for preparative HPLC separation (*ca.* 8 cm^3 per run) and purification of the two sets of enantiomers (CHX-A, CHX-B). Analytical HPLC: t_R 11.59 and 12.46 min, respectively. Preparative HPLC: t_R 11.45 and 12.20 min, respectively for the two compounds. The two separated fractions were next treated identically as follows. The HPLC fraction was concentrated to *ca.* 50 cm^3 and was loaded onto an AG50W-X8 cation exchange column (200–400 mesh, H^+ form, 2.6 × 25 cm). The column was washed with water until the eluent was neutral and the product eluted with 2 mol dm^{-3} NH_4OH (1 dm^3). The basic eluent fraction was vacuum rotary evaporated to leave a solid yellow residue which was further purified *via* an AG1X8 cation exchange column (200–400 mesh, $ClCH_2CO_2H$ form, 1.6 × 35 cm). The solid (250–400 mg per run) was taken up in water (3–5 cm^3) and loaded onto the column which was then washed with water (11 18 × 150 mm test tube fractions). A 2 dm^3 gradient of 0.0–1.0 mol dm^{-3} chloroacetic acid was then run to elute the column (88 fractions). The purified pentaacetic acid was routinely found within fractions 45–88. The column was then washed with water until the eluent was neutral and the above process repeated as necessary. A fresh column was poured for the second preparative HPLC fraction. The relevant fractions from each run for each compound were combined and rotary evaporated to leave, after cooling, a solid mass. Diethyl ether (500 cm^3) and water (30 cm^3) were added and the biphasic mixture was poured into a separatory funnel. The layers were separated and the aqueous layer was extracted with ether (2 × 600 cm^3). The aqueous fraction was then loaded into a continuous extractor and extracted with ether for 72 h. The aqueous fraction was finally lyophilized to leave a fluffy white material for each fraction (CHX-A, 470 mg; CHX-B, 535 mg; 12.5% total). For CHX-A **5** (Found: C, 51.55; H, 5.85; N, 9.6. $C_{25}H_{34}N_4O_{12}$ requires C, 51.53; H, 5.89; N, 9.62%); δ_H(D_2O, pH 1.0) 8.248 (d, 2 H, *J* 8.5), 7.571 (d, 2 H, *J* 8.5), 4.40–3.40 (m, 13 H), 3.322 (dd, 1 H, *J* 14.0, 5.5), 3.15–2.95 (m, 3 H), 2.132 (br s, 2 H), 1.82 (br s, 2 H) and 1.55–1.15 (m, 4 H); δ_H(D_2O, pH 7.0) 8.223 (d, 2 H, *J* 8.5), 7.488 (d, 2 H, *J* 8.5), 4.749 (d, 1 H, *J* 12.0), 3.57–3.40 (m, 3 H), 3.313 (d, 2 H, *J* 2.5), 3.17–2.90 (m, 4 H), 2.82–2.50 (m, 4 H), 2.381 (d, 1 H, *J* 12.5), 2.256 (d, 1 H, *J* 17.5), 1.945 (br d, 2 H), 1.85–1.55 (br m, 3 H) and 1.45–0.90 (br m, 4 H); δ_H(500 MHz, D_2O, pH 11.0) 8.252 (d, 2 H, *J* 8.0), 7.485 (d, 2 H, *J* 8.0), 3.855 (d, 1 H, *J* 15.5), 3.510 (d, 1 H, *J* 17.5), 3.446 (d, 2 H, *J* 17.0), 3.307 (d, 2 H, *J* 8.0), 3.105 (m, 1 H), 3.026 (t, 4 H, *J* 16.5), 2.80–2.50 (m, 3 H), 2.381 (d, 1 H, *J* 13.5), 2.254 (d, 1 H, *J* 17.0), 1.934 (br d, 2 H), 1.83–1.55 (br m, 3 H), 1.372 (m, 1 H) and 1.10–0.90 (m, 3 H); δ_C(D_2O, pH 11.0, Ref = dioxane) 180.210 (CO_2), 180.095 (CO_2), 180.015 (CO_2), 179.828 (CO_2), 179.768 (CO_2), 148.15, 146.19, 130.05, 123.89, 65.63 (CH_2CO_2), 64.73 (CH_2CO_2), 64.53 (CH_2CO_2), 62.49 ($2CH_2CO_2$), 59.66, 56.78, 54.53, 53.54, 33.037, 25.065, 24.59, 24.21 and 23.049; *m/z* [EI/sample was prepared by treating **5** with an excess of bis(trimethylsilyl)trifluoroacetamide in dry acetonitrile] 942 (M^+) and 927 ($M^+ - 15$).

CHX-B **6** (Found: C, 51.3; H, 5.6; N, 9.55. $C_{25}H_{34}N_4O_{12}$ requires C, 51.53; H, 5.89; N, 9.62%); δ_H(D_2O, pH 1.0) 8.276 (d, 2 H, *J* 8.5), 7.606 (d, 2 H, *J* 8.5), 4.25–3.55 (m, 10 H), within which was discernable at 4.122 (dd, *J* 22.0, 17.5), 3.869 (d, *J* 18.0), and 3.625 (d, *J* 18.0), 3.52–3.25 (m, 3 H), 3.092 (dd, 1 H, *J* 14.0, 9.0), 3.003 (dd, 1 H, *J* 19.5, 3.0), 2.866 (br t, 1 H, *J* 10.0), 2.285 (br d, 1 H), 1.85–1.60 (m, 4 H) and 1.40–0.85 (m, 4 H); δ_H(D_2O, pH 7.0) 8.229 (d, 2 H, *J* 8.5), 7.498 (m, 2 H), 4.00–2.45 (complex series of multiplets, 17 H), 2.143 (d, 1 H, *J* 13.0), 1.90 (br m, 1 H), 1.633 (br s, 2 H) and 1.50–0.95 (m, 4 H); δ_H(500 MHz, D_2O, pH 11.0) 8.228 (d, 2 H, *J* 8.0 Hz), 7.492 (br t, 2 H, *J* 8.5), 3.863 (d, 1 H, *J* 15.5), 3.646 (d, 1 H, *J* 17.0), 3.550 (d, 2 H, *J* 16.5), 3.350 (m, 1 H), 3.319 (s, 2 H), 3.250 (d, 2 H, *J* 17.0), 3.22–3.00 (m, 2 H), 2.932 (d, 2 H, *J* 17.5), 2.768 (q, 2 H, *J* 6.0), 2.712 (dd, 1 H, *J* 29.0, 17.5), 2.511 (dd, 1 H, *J* 34.0, 13.0), 2.136 (d, 1 H, *J* 13.0), 1.895 (m, 1 H), 1.626 (m, 2 H) and 1.40–0.85 (m, 4 H); δ_C(D_2O, pH 11.0, Ref = dioxane) 180.25 (CO_2), 180.14 (CO_2), 179.95 (CO_2), 179.88 ($2CO_2$), 148.15, 146.21, 130.14, 123.93, 68.61 (CH_2CO_2), 64.62 (CH_2CO_2), 63.69 (CH_2CO_2), 63.37 (CH_2CO_2), 63.31 (CH_2–CO_2), 62.49 (CH_2CO_2), 55.59, 54.22, 53.44, 33.75, 24.82, 24.59, 23.94 and 23.71; *m/z* [EI/sample was prepared by treating **6** with excess of bis(trimethylsilyl)trifluoroacetamide] 927 ($M^+ - 15$).

N-[2-*Amino*-3-(p-*aminobenzyl*)*propyl*]-trans-*cyclohexane*-1,2-*diamine*-N,N′,N′,N″,N″-*penta-acetic Acid* **7**.—The nitro compound **5** (53 mg, 0.091 mmol) was dissolved in water (3 cm^3) and the solution injected into a flask attached to an atmospheric hydrogenation apparatus. The contents of the flask prior to injection were 10% Pd/C (100 mg) suspended in water (5 cm^3) previously saturated with H_2 and stirred rapidly *via* a magnetic stir bar. The reaction was allowed to proceed until H_2 uptake halted and the suspension was filtered through a pad of Celite 577. The filtrate was lyophilized to leave an off-white solid (42 mg, 84%); HPLC t_R 14.34 min; δ_H(D_2O, pH 1.0) 7.447 (dd, 4 H, *J* 17.0, 8.5), 3.90–3.53 (m, 13 H), 3.388 (m, 2 H), 3.224 (dd, 1 H, *J* 14.5, 7.0), 3.15–3.00 (m, 3 H), 2.14 (br m, 2 H), 1.823 (br s, 2 H) and 1.53–1.15 (m, 4 H); δ_H(D_2O, pH 11.0) 7.084 (d, 2 H, *J* 8.5), 6.824 (d, 2 H, *J* 8.5), 3.854 (d, 1 H, *J* 15.5), 3.502 (d, 1 H, *J* 17.0), 3.455 (d, 1 H, *J* 17.5), 3.434 (d, 1 H, *J* 17.5), 3.284 (dd, 2 H, *J* 22.0, 15.5), 3.041 (d, 1 H, *J* 17.0), 3.009 (d, 1 H, *J* 15.5), 2.921 (d, 1 H, *J* 17.0), 2.809 (dd, 1 H, *J* 13.0, 3.0), 2.76–2.40 (m, 5 H), 2.341 (d, 1 H, *J* 17.0), 1.940 (br d, 1 H, *J* 12.0), 1.800 (br d, 1 H, *J* 10.0), 1.74–1.55 (br m, 2 H), 1.357 (m, 1 H), 1.034 (m, 3 H), one proton is obscured by the series of doublets in the range δ 3.1–2.9 and is recorded by integration; *m/z* (FAB/thioglycerol/glycerol) 553 ($M^+ + 1$) and 575 ($M^+ + Na$).

N-[2-*Amino*-3-(p-*aminophenyl*)*propyl*-trans-*cyclohexane*-N,N′,N′,N″,N″-*penta-acetic Acid* **8**.—The nitro compound **6** (100 mg, 0.172 mmol) was converted into the aniline as described above for **7** (91 mg, 96%); HPLC t_R 13.56 min; δ_H(D_2O, pH 1.0) 7.480 (dd, 2 H, *J* 19.0, 8.5), 4.15–3.68 (m, 10 H), 3.533 (d, 1 H, *J* 18.5), 3.462 (br, t, 1 H, *J* 8.0), 3.320 (dd, 1 H, *J* 15.0, 6.5), 3.230 (dd, 1 H, *J* 14.0, 6.5), 3.084 (dd, 1 H, *J* 14.0, 8.5), 3.007 (dd, 1 H, *J* 15.0, 4.5), 2.836 (dd, 1 H, *J* 11.0, 10.0), 2.29 (br d, 1 H), 1.90–1.67 (m, 3 H), 1.45–1.14 (m, 3 H) and 0.985 (m, 1 H); δ_H(D_2O, pH 11.0) 7.075 (m, 2 H), 6.820 (m, 2 H), 3.90–2.40 (m, 17 H), 2.057 (d, 1 H, *J* 13.0), 1.90 (br d, 1 H), 1.632 (br m, 2 H) and 1.40–0.95 (m, 4 H); *m/z* (FAB/thioglycerol/glycerol) 553 ($M^+ + 1$) and 575 ($M^+ + Na$).

N-[2-*Amino*-3-(p-*isothiocyanatophenyl*)*propyl*]-trans-*cyclohexane*-1,2-*diamine*-N,N′,N′,N″,N″-*penta-acetic Acid* **1**.—The aniline **7** (36.5 mg, 66 μmol) was dissolved in water (5 cm^3) in a 100 cm^3 round-bottom flask and stirred vigorously while

thiophosgene (8 mm^3, 0.1 mmol) in $CHCl_3$ (10 cm^3) was added in one portion. The biphasic mixture was stirred for 2 h after which the organic layer was removed by vacuum rotary evaporation at room temperature. The aqueous residue was lyophilized to leave an off-white powder (36 mg, 92%); ν_{max}/cm^{-1} 2100 (SCN); HPLC t_R 22.11 min; $\delta_H(D_2O$, pH 5.0) 7.376 (s, 4 H), 4.10–2.50 (br m, 15 H), 2.20 (m, 2 H), 1.85 (m, 2 H) and 1.45 (m, 4 H); *m/z* (FAB/thioglycerol/glycerol) 595 ($M^+ + 1$) and 617 ($M^+ + Na$).

N-[2-*Amino*-3-(p-*isothiocyanatophenyl*)*propyl*]-trans-*cyclohexane*-1,2-*diamine*-*N*,*N*′,*N*′,*N*″,*N*″-*penta*-*acetic Acid* **2**.—The aniline **8** (96 mg, 174 μmol) was converted into the corresponding isothiocyanate by treatment with thiophosgene (20 mm^3, 0.26 mmol) as described for **1** (97 mg, 94%); ν_{max}/cm^{-1} 2100 cm^{-1} (SCN); HPLC t_R 17.13 min; $\delta_H(D_2O$, pH 6.0) 7.302 (s, 4 H), 4.10–1.00 (br complex series of multiplets, 23 H); *m/z* (FAB/thioglycerol/glycerol) 595 ($M^+ + 1$) and 617 ($M^+ + Na$).

N-*Benzyloxycarbonyl*-(R,R)-*cyclohexane*-1,2-*diamine* **9**.—(*R*,*R*)-*trans*-cyclohexane-1,2-diamine (5.0 g, 43.8 mmol) was dissolved in diethyl ether (800 cm^3) in a 1 dm^3 flask under an argon atmosphere. The solution was cooled in an ice-bath and benzylchloroformate (6.82 g, 40 mmol) in diethyl ether (60 cm^3) was added dropwise over 3 h during which time a large quantity of precipitate formed. The flask was allowed to warm to room temperature after which the suspension was stirred for 18 h. The white solid was collected by vacuum filtration and washed with a small quantity of ether after which it was transferred to a beaker. The material was partitioned between 0.1 mol dm^{-3} HCl (300 cm^3) and ethyl acetate (100 cm^3). After clearing, the mixture was poured into a separatory funnel and the aqueous portion was retained and washed with additional ethyl acetate (100 cm^3). The acidic solution was then treated with solid Na_2CO_3 to raise the pH to *ca.* 9 after which it was extracted with $CHCl_3$ (3 × 100 cm^3). The combined $CHCl_3$ extracts were dried ($MgSO_4$), filtered, and vacuum rotary evaporated to leave a white solid (1.65 g, 16.6%) (Found: C, 67.45; H, 8.05; N, 11.05. $C_{14}H_{20}N_2O_2$ requires C, 67.70; H, 8.13; N, 11.89%); $\Delta\varepsilon_{290}$ −0.2278 and $\Delta\varepsilon_{245}$ 0.1330; $\delta_H(CDCl_3)$ 7.279 (s, 5 H, aryl), 5.032 (s, 2 H, CH_2O), 4.741 (br m, 1 H, NH), 3.128 (m, NHC*H*), 2.275 (ddd, 1 H, *J* 10.10, 4, H_2NCH), 1.900 [m, 2 H, HC*H*CH(NH)CH(NH_2)*H*CH], 1.630 [m, 2 H, *H*CHCH(NH)-CH(NH_2)HC*H*], 1.345 (s, 2 H, NH_2) and 1.28–0.70 (m, 4 H, CH_2CH_2); $\delta_C(CDCl_3)$ 155.58 (C=O), 135.62 (Ph), 127.51 (Ph), 127.43 (Ph), 127.09 (Ph), 65.71 (CH_2–O), 57.19 (CH–NH), 54.56 (CH–NH_2), 34.34 (*C*H_2–CH), 31.81 (CH–*C*H_2), 24.114 (*C*H_2CH_2CH) and 24.03 ($CHCH_2CH_2$); *m/z* (CI/NH_3) 249 ($M^+ + 1$).

N-(R,R)-trans-2-*Benzyloxycarbonylaminocyclohexyl*-N-tert-*butyloxycarbonyl*-p-*nitrophenylalaninamide* **10**.—*N*-Hydroxybenzotriazole (0.85 g, 6.25 mmol) and *N*-*tert*-butyloxycarbonyl-*p*-nitrophenylalanine (1.94 g, 6.25 mmol) were mixed in ethyl acetate (300 cm^3) in a round-bottom flask under an argon atmosphere. The carbamate amine **9** (1.55 g, 6.25 mmol) was added and after the solution had cleared, EDC (1.198 g, 6.25 mmol) was added along with DMF (50 cm^3). After 18 h, the solution was poured into a separatory funnel with ethyl acetate (100 cm^3) and sequentially extracted with water (100 cm^3), 5% aqueous $NaHCO_3$ (2 × 100 cm^3), saturated brine (100 cm^3), 1 mol dm^{-3} HCl (2 × 100 cm^3), and saturated brine (100 cm^3). The organic phase was dried ($MgSO_4$), filtered, and after concentration to *ca.* 100 cm^3 was diluted with hexanes (100 cm^3). The precipitating suspension was left at 4 °C for 18 h after which the product was collected and dried *in vacuo* (3.01 g, 89%) (Found: C, 62.0; H, 6.8; N, 10.3. $C_{28}H_{36}N_4O_7$ requires C, 62.19; H, 6.72; N, 10.36%); $\Delta\varepsilon_{280}$ −0.3612 and $\Delta\varepsilon_{245}$ 0.1378; $\delta_H([^2H_6]$-DMSO) 8.147 (d, 2 H, *J* 8.5, aryl), 7.842 (d, 2 H, *J* 8.0, NH), 7.518 (d, 2 H, *J* 8.5, aryl), 7.313 (s, 5 H, aryl), 7.043 (br d, 1 H, *J* 8.0, NH), 6.828 (d, 1 H, *J* 8.5, NH), 5.034 (d, 1 H, *J* 12.5, C*H*H–O), 4.941 (d, 1 H, *J* 12.5, CH*H*–O), 4.203 (m, 1 H, CH–C=O), 3.525 (m, 1 H, C*H*–NH), 3.320 (m, 1 H, C*H*–NH), 3.032 (dd, 1 H, *J* 13.0, 4.5, C*H*H–CHC=O), 2.851 (dd, 1 H, *J* 12.5, 10.5, CH*H*–CH–C=O), 1.581 (m, 1 H, CH_2CHH–CH), 1.739 (m, 1 H, CH_2–CH*H*–CH), 1.624 (m, 2 H, CH_2–CH_2–CH) and 1.261 [br s, 13 H, $(CH_3)_3$, $CH_2(CH_2)_2CH_2$]; $\delta_C([^2H_6]$DMSO) 170.65 (CHC=O), 155.87 (NHC=O), 154.85 (NHC=O), 146.45 (aryl), 146.13 (aryl), 137.09 (aryl), 130.53 (aryl), 128.18 (aryl), 127.53 (aryl), 122.94 (aryl), 77.98 (C–O), 65.15 (CH_2–O), 55.20 (CH), 53.66 (CH), 52.08 (CH), 37.82 (Ph–*C*H_2–CH), 31.86 (CH_2–*C*H_2–CH), 31.48 (CH–*C*H_2–CH_2), 27.93 [$(CH_3)_3$] and 24.16 (CH_2–*C*H_2–*C*H_2–CH_2); *m/z* (CI/NH_3) 541 ($M^+ + 1$).

N-[(*S*)-2-*Amino*-3-(p-*nitrophenyl*)*propyl*]-(R,R)-trans-*cyclohexane*-1,2-*diamine Trihydrochloride* **11**.—The amide **10** (2.63 g, 4.87 mmol) was transferred to a 50 cm^3 round-bottom flask and 33% HBr in acetic acid (Fluka) was added. The reaction was stirred under argon until there was no evidence for the starting material by TLC and until a precipitate formed. The suspension was diluted with diethyl ether and the ammonium salt filtered off. The latter was then transferred to a flask, dried *in vacuo*, and then washed into a 250 cm^3 three-necked flask with THF (70 cm^3). The flask was cooled in an ice-bath while 1 mol dm^{-3} BH_3/THF was injected in *via* a syringe. The ice-bath was removed and the reaction mixture was warmed to *ca.* 50–55 °C for 48 h. The flask was cooled in an ice-bath and methanol (20 cm^3) was added to decompose the remaining hydride. The solution was vacuum rotary evaporated to a gummy residue which was then refluxed with dioxane (100 cm^3) which had been previously saturated with $HCl_{(g)}$. After the mixture had been cooled at 4 °C for 2 h, the precipitate was collected, washed with diethyl ether, and dried *in vacuo* to leave an off-white powdery product (1.55 g, 79%); HPLC t_R 16.3 min [Found: C, 44.8; H, 6.85; N, 13.7. $C_{15}H_{24}N_4O_2(HCl)_3$ requires C, 44.83; H, 6.79; N, 13.95%]; $\delta_H(D_2O$, pH 12.0, Ref = TSP) 8.198 (d, 2 H, *J* 8.0, aryl), 7.489 (d, 2 H, *J* 8.0, aryl), 3.159 (m, 1 H, CH), 3.006 (m, 1 H, CH), 2.719 (m, 2 H, CH_2–N), 2.440 (m, 2 H, aryl-CH_2), 2.186 (m, 1 H, CH), 1.904 (br d, 1 H, CH_2–C*H*H–CH), 1.802 (br d, 1 H, CH–C*H*H–CH_2), 1.652 (br s, 2 H, CH_2–CH*H*–CH, CH–CH*H*–CH_2) and 1.30–0.95 [m, 4 H, $CH_2(CH_2)_2CH_2$]; $\delta_C(D_2O$, pH 12.0, Ref = TSP) 150.83 (aryl), 149.29 (aryl), 133.27 (aryl), 119.60 (aryl), 65.97, 56.84, 54.70, 44.55, 43.55, 37.15, 32.89, 27.57 and 27.49; *m/z* (CI/NH_3) 293 ($M^+ + 1$).

N-[(S)-2-*Amino*-3-(p-*nitrophenyl*)*propyl*]-trans-(R,R)-*cyclohexane*-1,2-*diamine*-N,N′,N′,N″,N″-*penta*-*acetic Acid* **12**.—The triamine **11** (0.5 g, 1.25 mmol) was alkylated as described above for **5**, **6** with Na_2CO_3 (1.59 g, 15 mmol) and *tert*-butyl bromoacetate (1.46 g, 7.5 mmol) in DMF (30 cm^3). The crude product was isolated as above and purified directly by means of the described ion-exchange methodology to yield the single enantiomer product (288 mg, 39%); HPLC t_R 11.57 min (Found: C, 51.25; H, 6.0; N, 9.5. $C_{25}H_{34}N_4O_{12}$ requires C, 51.53; H, 5.89; N, 9.62%); $\Delta\varepsilon_{303}$ −0.6804 and $\Delta\varepsilon_{257}$ 0.1291; $\delta_H(D_2O$, pH 12.0) 8.183 (d, 2 H, *J* 9.0), 7.477 (d, 2 H, *J* 9.0), 3.40–2.92 (m, 11 H), 2.831 (m, 2 H), 2.626 (m, 2 H), 2.450 (m, 1 H), 2.250 (m, 1 H), 1.996 (m, 2 H), 1.688 (m, 2 H) and 1.104 (m, 4 H); $\delta_C(D_2O$, pH 12.0, Ref = TSP) 183.28 (CO_2), 182.69 (CO_2), 182.48 (CO_2), 152.27 (aryl), 148.75 (aryl), 133.06 (aryl), 126.53 (aryl), 64.51, 63.86, 58.14, 57.39, 56.46, 38.34, 28.06, 287.91, 27.32 and 26.63; *m/z* (FAB/glycerol) 583 ($M^+ + 1$).

[^{14}C]-N-[2-*Amino*-3-(p-*nitrophenyl*)*propyl*]-trans-*cyclohexane*-1,2-*diamine*-N,N′,N′,N″,N″-*penta*-*acetic Acid* [^{14}C]-CHX-

A, **5a**; [^{14}C]-CHX-B **6a**.—A modified procedure for the preparation of **5**, **6** was followed. The triamine **4** (1.0 g, 2.5 mmol) and Na_2CO_3 (2.253 g, 21.3 mmol) were combined in DMF (5 cm^3) and heated to *ca.* 90 °C under argon. *tert*-Butyl bromo[1-^{14}C]acetate (2.5 mCi per ampoule, sp. act.: 10.5 mCi $mmol^{-1}$) was added with two 1-cm^3 rinses of DMF for each ampoule. After 2 h, *tert*-butyl bromoacetate (2.68 g, 13.7 mmol) was added and the reaction was continued for 18 h. After cooling to room temperature, the solution was extracted with CH_2Cl_2 (30 cm^3) and the extract washed with water (3 × 100 cm^3), dried ($MgSO_4$) and filtered directly into a 100 cm^3 Schlenk flask. The contents were then frozen in liquid nitrogen. The flask was then attached to two liquid nitrogen traps in line and the system was evacuated. After disconnection of the vacuum pump system, the frozen material was allowed to reach room temperature after which it was gently heated to transfer all volatile materials to the traps. With the transfer completed, trifluoroacetic acid (20 cm^3) was added to the contents of the flask and the solution stirred for 18 h. The solution was vacuum rotary evaporated to afford a thick gum which was dissolved in the aqueous HPLC buffer. The separation and purification procedures from this point were identical with those employed for the non-radiolabelled ligands. The specific activities for [^{14}C]-CHX-A **5a** and -CHX-B **6a** were determined by measuring the UV absorbance of a solution of known concentration (ε 7730 l mol^{-1} cm^{-1} at 280 nm) followed by counting of the ^{14}C activity in that same solution against a set of ^{14}C quenched standards. The specific activity of two fractions were 0.48 and 0.50 mCi $mmol^{-1}$, respectively.

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Paper 1/06007D
Received 27*th November* 1991
Accepted 28*th January* 1992