Synthesis of macrocyclic bifunctional chelating agents: 1,4,7-tris(carboxymethyl)-10-(2-aminoethyl)-1,4,7,10-tetraazacyclododecane and 1,4,8-tris(carboxymethyl)-11-(2-aminoethyl)-1,4,8,11-tetraazacyclotetradecane†

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The convenient, synthetically useful bifunctional chelating agents, 1,4,7-tris(carboxymethyl)-10-(2-aminoethyl)-1,4,7,10-tetraazacyclododecane and 1,4,8-tris(carboxymethyl)-11-(2-aminoethyl)-1,4,8,11-tetraazacyclotetradecane, were obtained by reaction of ethyl bromoacetate with 1,4,7,10-tetraazacyclododecane and 1,4,8,11-tetraazacyclotetradecane, followed by reaction with *N*-(2-bromoethyl)phthalimide. This method is proven to be more efficient to prepare bifunctional chelating agents with aliphatic side arms in high yields, above 53%.

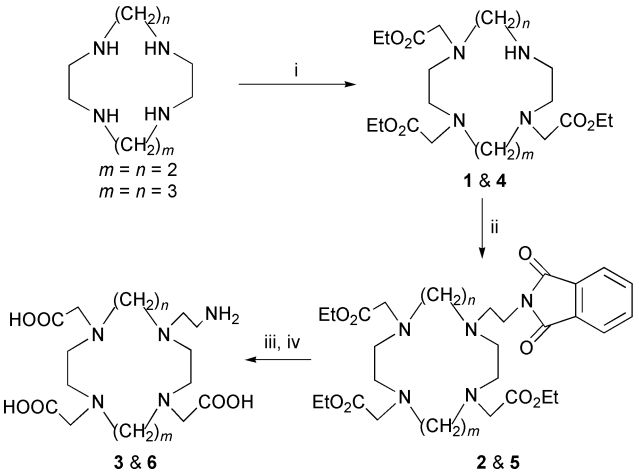
The continued interest in new macrocyclic bifunctional chelating agents stems mainly from their use in labelling monoclonal antibodies (mAbs) with radioactive metals for cancer diagnosis and therapy,[1–3] and with paramagnetic ions for magnetic resonance imaging.[4] Our ongoing studies are currently concerned with simple, convenient methods of ligand synthesis. The attachment of metal ions to proteins, such as monoclonal antibodies, can create new tools for use in biology and medicine.[5] The reagents used for such attachment are usually called "bifunctional chelating agents" because they incorporate a strong metal chelating group and a chemically reactive group. Bifunctional chelating agents are most often used to endow biological molecules with the nuclear,[6] physical[7] or chemical[8] properties of chelated metal ions. In the past decades, substantial progress has been made in the application of such reagents to problems such as cancer diagnosis,[9–11] therapy and DNA footprinting.[12] The bifunctional chelating agents play a major role in exploiting the properties of metal ions for clinical applications. This paper describes a novel approach for the synthesis of polyaza macrocyclic bifunctional chelating agents using inexpensive, readily available materials. The growing demand for large quantities of macrocyclic bifunctional chelating agents encouraged us to develop a convenient approach to obtain the aminoethyl derivatives of tetraazamacrocycles (Scheme 1).

Experimental

General procedures and starting materials

All the experiments were performed under nitrogen. TLC was run on plastic backed silica gel plates (0.2 mm thick silica gel 60 F254, E. Merck, Germany) using a 10 w/v aqueous ammonium acetate–CH_3OH (1 : 1 v/v) solution as eluent. HPLC was carried out on a Rainin Rabbit HPX System (Rainin Instruments, Woburn, MA) equipped with titanium piston washing pump heads. Solvents were mixed using a Dynamax dual-chamber dynamic mixer (Titanium). UV absorbance was measured using an absorbance/fluorescence monitor (ISCO model UA-5) at 254 or 354 nm. A Gilson model 201 fraction collector was used. Dynamax software on a Macintosh Plus computer controlled the HPLC system. Reversed-phase HPLC was performed at room temperature with a Dynamax 21.4 × 50 nm C_{18} column, generally using a gradient of CH_3OH or CH_3CN and 0.1 M ammonium acetate (pH 6) or 0.05% TFA with a flow rate of 1 mL min^{-1}. All the solvents for HPLC and reaction mixtures were filtered through a nylon 66 Millipore filter (0.45 μm) prior to use.

The tetraazamacrocycles were synthesised in our laboratory by the standard procedure.[13] Bromoethyl acetate, *N*-(2-bromoethyl)phthalimide, sodium carbonate, caesium carbonate, HBr/HCOOH, P_4O_{10} and other solvents (Aldrich) were used as received.



Scheme 1 Synthesis of **3** and **6**. *Reagents:* (i) ethyl bromoacetate, CH_2Cl_2; (ii) *N*-(2-bromoethyl)phthalimide, MeCN, Na_2CO_3; (iii) HBr–HCOOH; (iv) P_4O_{10}.

† Electronic supplementary information (ESI) available: fragmentation pathways of **1** and **4** in EI-MS. See: http://www.rsc.org/suppdata/nj/b0/b006978g/

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Imaging plate and radiation counting

The imaging plate consisted of a flexible plastic coated with fine photostimulable phosphor crystals (BaFBr : Eu + 2) capable of storing a fraction of the absorbed incident energy from irradiation with electrons or photons. When later stimulated by visible or infrared radiation, these crystals emit photostimulated luminescence at an intensity proportional to the absorbed radiation energy. The imaging plate system has several advantages compared to other image sensors: ultra-high sensitivity, a wider dynamic range, superior linearity and better spatial resolution.

Gamma counting was carried out using a Beckman model 310 counter with appropriate energy windows set for ^{57}Co. TLC plates containing radiolabelled materials were visualised on a phosphor imager 445 SI imaging system (Molecular Dynamics, USA, model 410A).

NMR and mass spectrometry

NMR data were recorded on a Bruker AC 400 operating near 400 (^1H) or 100 (^{13}C) MHz or on a Bruker AC 200 operating near 200 (^1H) or 50 (^{13}C) MHz. Chemical shifts were relative to either HDO (4.70 ppm) or residual $CHCl_3$ (7.24 ppm).

Mass spectra were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical Instruments) at Veterinary Medical College (Nantes, France) using DCI or EI mode and FAB^+ mode. During mass spectrometry measurements, either 3-nitrobenzyl alcohol or dithiothreitol–dithioerythritol (3 : 1 w/w) was used as a matrix along with a small amount of *p*-toluenesulfonic acid. FAB spectra contained polyethylene glycol or polyethylene glycol methyl ether as references. Elemental analyses were performed at Benaras Hindu University (Varanasi, India).

Syntheses and spectroscopic data

1,4,7-Tris(carboethoxymethyl)-1,4,7,10-tetraazacyclododecane, 1. 1 was prepared by adding 2.24 equiv. of ethyl bromoacetate dropwise (in ~30 mL CH_2Cl_2) to a CH_2Cl_2 solution of 1 equiv. tetraazacyclododecane; the resulting solution was stirred at room temperature for 40 h and produced a mixture of two products. The tetrasubstituted product was removed by filtration; the resulting filtrate was washed with water and dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was purified on a silica gel open column by flash chromatography (10% MeOH–CH_2Cl_2), yielding 72% of tri-*N*-substituted tetraazacyclododecane **1**. FAB-MS: *m/z* calc. for $[M + H]^+$ 431, found 431. ^1H NMR (250 MHz, $CDCl_3$): δ 1.25 (2t, 9H), 2.91 (m, 12H), 3.12 (m, 4H), 3.45 (2s, 6H), 4.16 (q, 6H), 10.01 (br, 1H). ^{13}C NMR ($CDCl_3$): δ 14.6 (C-16), 47.7 (C-9, C-11), 49.7 (C-2 to C-6), 51.8 (C-8, C-12), 57.6 (C-13), 61.1 (C-15), 170.7 (C14b), 171.5 (C-14a, C-14c). EI-MS (% rel. int.): *m/z* 430 (27), 357 (88), 314 (30), 300 (30), 288 (76), 271 (21), 259 (50), 185 (97), 173 (68), 159 (34), 144 (62), 130 (100) (see ESI for identity of fragment peaks).

1,4,7-Tris(carboethoxymethyl)-10-[*N*-2-(ethyl)phthalimide]-1,4,7,10-tetraazacyclododecane, 2. 2 was obtained by adding an equivalent of *N*-(bromoethyl)phthalimide in acetonitrile to a solution of **1** using 8 equiv. of Na_2CO_3 in MeCN at 60–70 °C for 12 h under N_2 atmosphere. The reaction mixture was brought to room temperature, filtered, and evaporated under reduced pressure. The product was dissolved in chloroform and washed several times with 100 mL of water and the organic phase was dried over magnesium sulfate, filtered and evaporated to dryness, yielding 93% of **2**. FAB-MS: *m/z* calc. for $[M + H]^+$ 604, found 604. ^1H NMR (400 MHz, $CDCl_3$): δ 1.27 and 1.30 (2t, 9H, $J_{H\text{-}H} = 7.1$), 2.86–3.09 (m, 18H), 3.38–3.46 (2s, 6H), 3.58 (t, 2H, $J_{H\text{-}H} = 6.7$), 4.11 and 4.15 (2q, 6H, $J_{H\text{-}H} = 7.1$) 7.72 (dd, 2H, $J_{H\text{-}H} = 3.1$) 7.83 (dd, 2H, $J_{H\text{-}H} = 6.0$ and $J_{H\text{-}H} = 3.4$ Hz). ^{13}C NMR ($CDCl_3$): δ 14.2 (C-16), 28.2 (C-17), 39.2 (C-18), 47.3 (C9, C-11), 49.2 (C-2 to C-6), 51.3 (C-8, C-12), 57.0 (C-13), 60.7 (C-15), 123.4 (C-21), 131.7 (C-20), 134.2 (C-22), 167.7 (C-19), 170.3–171.1 (C-14). Anal. calc. for $C_{30}H_{45}N_5O_8$: C, 59.62; H, 7.51; N, 11.60; O, 21.20; found: C, 59.66; H ,7.55; N, 11.54; O, 21.35%.

1,4,7-Tris(carboxymethyl)-10-(2-aminoethyl)-1,4,7,10-tetraazacyclododecane, 3. 3, an amino derivative, was prepared from **2** (0.70 mmol) by treating with a mixture of 48% HBr (100 mL) and glacial acetic acid (100 mL, pH ~2.5–3.0); the resulting mixture was refluxed for 12 h. After evaporation to dryness the solid residue was treated with 1 M HBr (100 mL) and the undissolved residue of phthalic acid was removed by filtration. The filtrate was evaporated to dryness and redissolved in hot 48% HBr (75 mL). On gradual addition of glacial acetic acid (200 mL) to the stirred and ice-cooled solution the crude amine hydrobromide precipitated. It was collected, washed thoroughly with ethanol and ether, and finally dried over P_4O_{10} at 1 mm Hg for several hours. The amino derivative was dissolved in alkaline aqueous solution (pH 11) with stirring at 60 °C for 4 h and the reaction was monitored on HPLC. After the reaction was completed the pH was adjusted to 6 using 6 M HCl and water was removed to dryness. The obtained glassy solid was redissolved in 1 L of water and the product was lyophilised. Yield 80%. Reversed-phase C_{18} HPLC: solvent A: 0.1 M ammonium acetate, pH 6; solvent B: MeOH; 15–65% B, 0–25 min; 65–100% B, 30–35 min; 100–15% B, 35–40 min; product peak at 6 min. FAB-MS: *m/z* calc. for $[M + H]^+$ 390, found 390. ^1H NMR (D_2O): 2.92–3.43 (m, 20H, H-2, H-12, H-15 and H-16), 3.71–3.73 (3s, 6H, H-13).

1,4,8-Tris(carboethoxymethyl)-1,4,8,11-tetraazacyclotetradecane, 4. The tri-N-substituted cyclam **4** was prepared by adding 2.7 equiv. of ethyl bromoacetate to a solution of tetraazacyclotetradecane in dichloromethane with stirring at room temperature for 48 h, yielding 74% tri-N-substituted product. The remaining tetrasubstituted product was removed by filtration at the end of the reaction. The resulting filtrate was washed with water and purified on a silica gel column by flash chromatography (10% MeOH–CH_2Cl_2). FAB-MS: *m/z* calc. for $[M + H]^+$ 459, found 459. ^1H NMR (400 MHz, $CDCl_3$): δ 1.26 (2t, 9H), 1.69 (q, 2H), 2.06 (q, 2H), 2.63–3.54 (m, 22H), 4.15 (2 q, 6H), 10.01 (br, 1H). ^{13}C NMR ($CDCl_3$): δ 14.6 (C-18), 23.1 (C-13), 23.8 (C-6), 46.7, 47.6 (C-10, C-12), 48.9–54.3 (C-2 to C-5, C-7, C-9 and C-14), 54.4–55.4 (C-15), 60.7–61.2 (C-17), 171.5 (C-16b), 172.1 (C-16a, C-16c). EI-MS (% rel. int.): *m/z* 458 (27), 385 (56), 371 (35), 273 (18), 199 (48), 173 (24), 130 (100) (see ESI for identity of fragment peaks).

1,4,8-Tris(carboethoxymethyl)-11-[*N*-2-(ethyl)phthalimide]-1,4,8,11-tetraazacyclotetradecane, 5. 5 was prepared from tri-substituted **4** in the same way as compound **2** (yield 92%). FAB-MS: *m/z* calc. for $[M + H]^+$ 632, found 632. ^1H NMR (250 MHz, $CDCl_3$): 1.26 (t, 9H, $J_{H\text{-}H} = 7.1$), 1.61 (m, 4H), 2.70–3.21 (m, 18H), 3.35–3.41 (3s, 6H), 3.62 (m, 2H), 4.13–4.16 (2q, 6H, $J_{H\text{-}H} = 7.1$), 7.75 (dd, 2H, $J_{H\text{-}H} = 6.0$ and $J_{H\text{-}H} = 3.1$), 7.89 (dd, 2H, $J_{H\text{-}H} = 6.0$ and $J_{H\text{-}H} = 3.1$ Hz). ^{13}C NMR ($CDCl_3$): 14.1 (C-18), 22.8, 23.5 (C-6, C-13), 28.2 (C-19), 39.1–55.6 (C-2 to C-5, C-7 to C-12, C-14, C-15, C-20), 60.8 (C-17), 123.3 (C-23), 131.2 (C-22), 134.2 (C-24), 167.5 (C-21), 170.8–172.0 (C-16). Anal. calc. for $C_{32}H_{49}N_5O_8$: C, 60.84; H, 7.82; N, 11.09; O, 20.26; found: C, 60.90; H, 7.85; N, 11.14; O, 20.21%.

1,4,8-Tris(carboxymethyl)-11-(2-aminoethyl)-1,4,8,11-tetraazacyclotetradecane, 6. The aminoethyl derivative **6** was obtained from an aqueous solution of **5** and subjected to cleavage and hydrolysis in the same way as compound **3**. Reversed-phase C_{18} HPLC: solvent A: 0.1 M ammonium

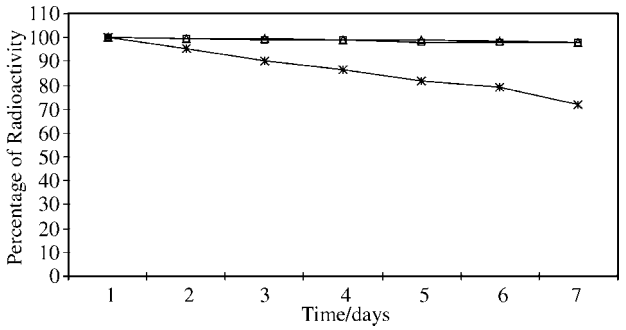

Fig. 1 Serum stability studies of ^{111}In and ^{153}Sm complexes of chelates **3** and **6**: (△) 1,4,7-tris(carboxymethyl)-10-(2-amino ethyl)-1,4,7, 10-tetraazacyclododecane, (□) 1,4,8,11-tris(carboxymethyl)-11-(2-aminoethyl)-1,4,8,11-tetraazaclyclotetradecane, (*) diethylenetriamine pentaacetic acid.

acetate, pH 6; solvent B: MeOH; 15–65% B, 0–25 min; 65–100% B, 30–35 min; 100–15% B, 35–40 min; product peak at 6.54 min (yield 80%). FAB-MS: *m/z* calc. for $[M + H]^+$ 418, found 418. ^{1}H NMR (D_2O): δ 1.51 (m, 2H, H-18), 1.65 (m, 2H, H-17), 2.25 (t, 2H, H-13, $J_{H\text{-}H} = 5.2$), 2.35 (t, 2H, H-6, $J_{H\text{-}H} = 5.2$ Hz), 2.47–2.76 (m, 12H), 3.01–3.06 (m, 4H), 3.14 (s, 4H), 3.21 (s, 2H).

Complexation with the radionuclides ^{111}In and ^{153}Sm

Radiolabelling of **3** and **6** with ^{111}In (1 mCi, in 100 μL of 0.05 M HCl) was done using the following procedure. A stock solution of carrier-free $^{111}InCl_3$ (20 μL, in sodium acetate buffer, pH 5.5 adjusted with 1 M sodium acetate) was added to a stock solution of **3** or **6** (20 μL of 50 mM, 0.1 μmol). Then 16 μL of 0.1 M sodium acetate buffer, pH 7, was added. The pH was checked to be 7. After 3 h, the mixture was analysed by using a TLC system (1 : 1 MeOH–10% NH_4OAc). Radichemical purity was above 97%.

Finally, 5.7 μL of this solution was mixed with 2 mL of healthy human serum to study the transchelation of indium metal ion. The rate of transchelation is determined by polyacrylamide gel electrophoresis under physiological conditions.

Chelates **3** and **6** were radiolabelled with ^{153}Sm under the same conditions as ^{111}In except that the incubation time for samarium was 6 h at room temperature.

Results and discussion

Alkylation reactions performed at room temperature in dichloromethane did not require drastic conditions to provide 70–80% yields of the trisubstituted products. Macrocycles **2** and **5** were obtained from the reaction of trisubstituted tetraazamacrocycles and *N*-(bromoethyl)phthalimide in MeCN using anhydrous Na_2CO_3 as base. The hydrolysis of the phthaloyl protective group and the esters of macrocycles **2** and **5** by acid hydrolysis,[14] followed by treatment in alkaline aqueous solution (pH > 11) yielded 2-aminoethyl substituted triacids **3** and **6**.

Synthesis of a polyaza macrocyclic ring is crucial for successful preparation of a macrocyclic bifunctional chelating agent. Previously reported protection methodologies[15–20] for preparing 12- and 14-membered polyaza macrocycles included reactions of benzyl chloroformate and *p*-toluenesulfonyl chlorides with the polyaza macrocycles, 1,4,7,10-teraazacyclododecane and 1,4,8,11-teraazacyclotetradecane, to allow for mono-N-alkylation with the required side chain. The use of these methodologies for synthesis of bifunctional chelating agents or the preparation of ligands with sterically hindered side arms requires protection and deprotection steps, which make the total procedure longer. Two-step alkylation reactions between a polyaza macrocycle (cyclen and cyclam), ethyl bromoacetate and *N*-(bromoethyl)phthalimide require no protection or activation steps in the synthetic approach described here. This alkylation scheme is simpler, much more convenient and is applicable to the preparation of other pendant functions or coordinating groups to hold metal ions of desired electronic configurations with appropriate polyaza macrocycles. The reactive group (the isothiocyanato group, which is prepared by reacting with $CSCl_2$ to form a thiourea linkage with amino groups of proteins or antibodies) attaches the metal chelate covalently to biological molecules or any polymeric substance for clinical applications.

The radioisotopes ^{111}In and ^{153}Sm form more stable complexes with polyaminopolycarboxylate, as is well established in the literature.[21] In terms of complexation the ionic size plays a very important role. Ligands having a macrocyclic cavity that can accommodate the large metal ions are potentially strong binders. These effective sequestering agents are usually multidentate in nature. These factors, coupled with the polydentate nature of the ligand, favour the formation of complexes with only one ligand per metal atom, therefore minimising the formation of multiple species in solution, which is evidenced by metal binding assays. The complexation studies were carried out at pH 5.5 at room temperature in sodium acetate buffer (0.1 M, pH 6). The complexation with macrocyclic chelate **3** and **6** was determined by the TLC method. This method allows easy and rapid analyses to be carried out in parallel. The complexation kinetics with ^{111}In and ^{153}Sm were observed to be rather slow with the chelates **3** and **6**. The amount of chelation reaches 97% in 4–6 h for indium and samarium, with the latter exhibiting slower kinetics. The complexation was monitored periodically on a phosphor imager by imaging the TLC plate spotted with free metal ion and metal chelates. Reacting either ^{111}In or ^{153}Sm with chelates **3** and **6** in a 1 : 1 molar ratio at room temperature (23 °C) forms only 70 and 62% of metal chelate with R_f 0.6 and 0.5 after 2 h; at 3 h it increases to 90 and 80%, respectively, while free metal ion does not migrate on the TLC plate using 10% NH_4OAc–MeOH (1 : 1) as eluent, which suggests the complexation is slow at ambient temperature.

The rate of decomplexation of the metal chelate (complexes of ^{111}In and ^{153}Sm) were studied in serum under physiological conditions over a 7-day period. It is clear that In and Sm chelates are more stable than the DTPA chelate in serum. Over the 7-day period no measurable loss (Fig. 1) of metal ion from the macrocyclic bifunctional chelator was observed. The macrocyclic bifunctional chelating agents are superior to the anionic chelates with DTPA and nitrobenzyl-DTPA for use *in vivo*. Similarly high stability with respect to In loss was also observed with ^{111}In complexes of 9-N_3-triacetates, for which fast rates of association were also defined.[21]

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