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Preparation and *in vitro* evaluation of GdDOTA-(BOM)$_4$; a novel angiographic MRI contrast agent

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A novel Gd(III) complex, GdDOTA-(BOM)$_4$, has been prepared by a simple three-step procedure. The complex showed high T_1-relaxivity values in serum albumin solutions, blood and plasma, resulting from high affinity for serum albumin. The T_1-relaxivity in plasma, 67.4 s^{-1} mM^{-1} (20 MHz, 37 °C), makes it a promising candidate for angiographic applications of MRI.

Introduction

Paramagnetic materials have been investigated as MRI contrast agents (CAs) for more than two decades.[1] These materials enhance the contrast of the image indirectly by lowering the magnetic relaxation time of the water protons in the surrounding tissues.[2,3] The most frequently used CAs are stable gadolinium(III) (Gd) complexes with hydrophilic poly(aminocarboxylate) ligands resulting in rapid extracellular distribution and renal elimination. Gd(III) is preferred because of its favourable magnetic properties (seven unpaired electrons). Depending on the denticity of the ligand one or more water molecules might be directly coordinated to the paramagnetic centre.

One of the most commonly used CAs is GdDOTA (gadolinium 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate, DOTAREM™, Guerbet, France). At the magnetic fields usually employed in MRI (0.5–1.5 T), the efficacy (T_1-relaxivity) of this complex is mainly determined by the molecular reorientational time (τ_R). By increasing this value, and at the same time keeping all the other relaxation parameters constant, one would obtain a large increase in the T_1-relaxivity, provided that the water residence time (τ_M) is sufficiently low. This has been evaluated by linking Gd(III) complexes covalently to various macromolecules (albumin, polylysine and dextran)[4,5,6] and dendrimers,[7] or by preparing amphiphilic derivatives able to form micelles or liposomes in aqueous solutions.[8,9]

Another approach is to increase τ_R by the formation of non-covalent interactions between the Gd(III) complex and human serum albumin (HSA).[10] This interaction will, in addition to increased T_1-relaxivity, result in a prolonged residence time for the complex in the vascular system. This is advantageous for angiographic applications of MRI.

Earlier studies have shown that DOTA-like Gd(III) complexes with hydrophobic benzyloxymethyl (BOM) substituents (Fig. 1) bind non-covalently to HSA, with binding association constants (K_A) increasing with the number of substituents.[11] A high T_1-relaxivity, 53.2 s^{-1} mM^{-1}, has been reported for the GdDOTA-(BOM)$_3$-HSA adduct.

The synthesis of these Gd(III) complexes follows a laborious method developed by Aime *et al.*[12] Preparation and evaluation of the novel complex GdDOTA-(BOM)$_4$ constitute the subject of the present paper.

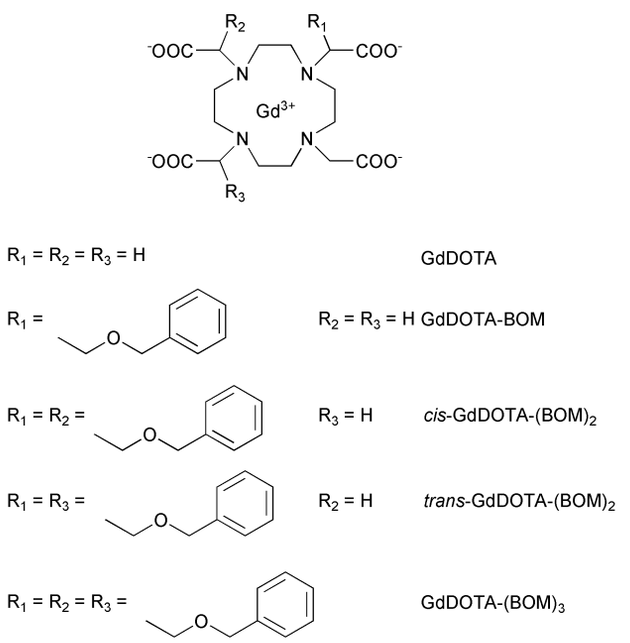

Fig. 1 Structure of GdDOTA and the previously reported GdDOTA-BOM derivatives. Nomenclature according to ref. 11.

Results and discussion

Synthesis

Syntheses of BOM substituted DOTA derivatives by reacting cyclen with five equivalents of potassium 3-benzyloxy-2-chloropropionate in DMF at 50 °C for 30 h has been reported previously.[12] Separation of mono-, di- and trisubstituted derivatives from the reaction mixture, followed by reaction with sodium bromoacetate, yielded DOTA-BOM, *cis*-DOTA-(BOM)$_2$, *trans*-DOTA-(BOM)$_2$ and DOTA-(BOM)$_3$ in moderate to low yields. The yield of the intermediate DO3A-(BOM)$_3$ (DO3A = 1,4,7-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane) was 8%. Neither the isolation nor the observation of DOTA-(BOM)$_4$ were reported.

It was anticipated that a combination of higher temperature and greater excess of alkylating agent was needed to obtain tetrasubstitution of cyclen. *tert*-Butyl protected acid **2**[13] rather than potassium 3-benzyloxy-2-chloropropionate was used to ease the purification.

The reaction with cyclen (**1**) was performed with seven equivalents of *tert*-butyl 3-benzyloxy-2-bromopropionate (**2**) in DMF at 100 °C and in the presence of potassium carbonate (Scheme 1). The resulting *tert*-butyl protected DOTA-(BOM)$_4$ (**3**) was easily purified by flash chromatography; yield: 30%. TLC analysis confirmed the purity of the compound, and it was characterised by electrospray MS and NMR (^{1}H and ^{13}C). The large number of signals in the ^{13}C NMR spectrum can be

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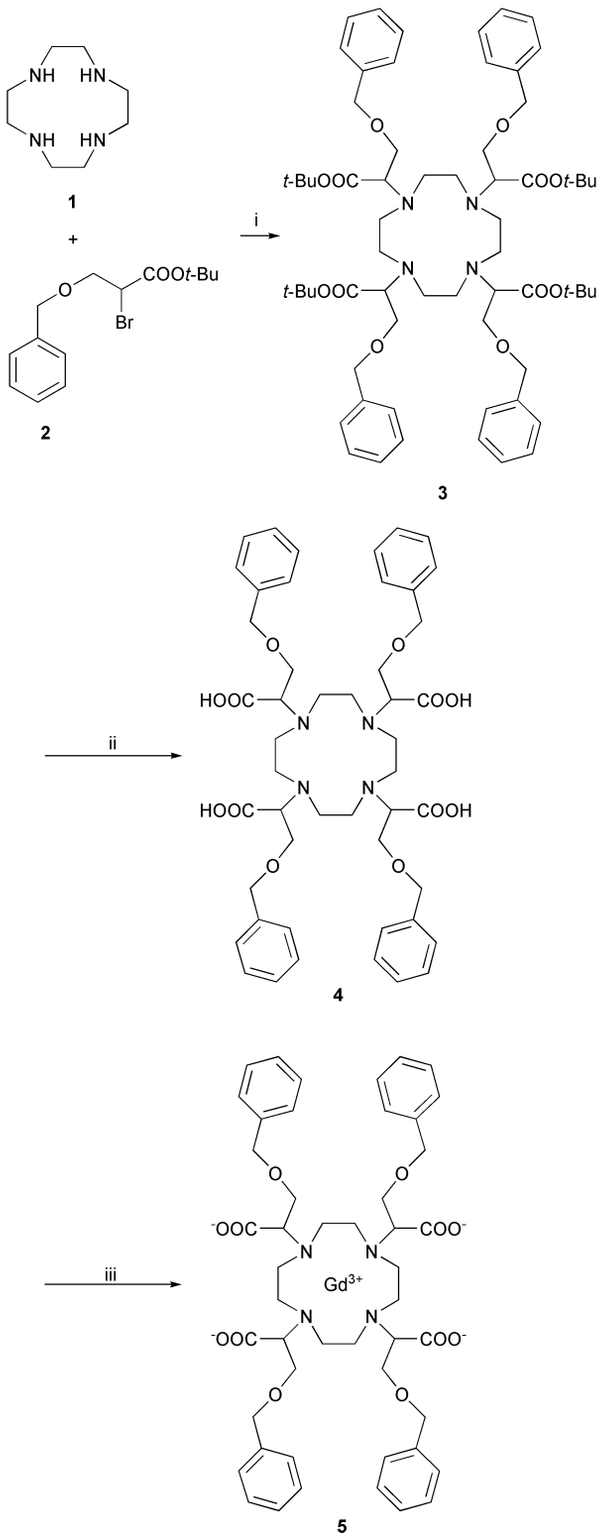

Scheme 1 Reagents and conditions: i, K_2CO_3, DMF, 100 °C; ii, TFA, rt; iii, $GdCl_3 \cdot 6H_2O$, H_2O, pH 7.8, 80 °C.

explained by the fact that the product is a mixture of diastereomers. No attempts were made to isolate the products with a lesser degree of substitution.

Removal of the *tert*-butyl groups was accomplished in neat TFA at room temperature, followed by precipitation with diethyl ether. Complexation was achieved by reacting the ligand **4** with equimolar amounts of Gd(III) chloride hexahydrate in aqueous solution at neutral pH. Limited solubility of both ligand **4** and GdDOTA-$(BOM)_4$ (**5**) required high dilution and some heating. Excess Gd(III) was precipitated as the hydroxide (pH ~ 9).

Table 1 T_1-relaxivities (r_1) of GdDOTA-$(BOM)_4$ in the presence of BSA (20 MHz, pH 7.4)

[BSA]/mM	r_1(25 °C)/s^{-1} mM^{-1}	r_1(37 °C)/s^{-1} mM^{-1}
0.6	33.7	38.0
3.5	49.7	57.1

Relaxometric characterisation of GdDOTA-$(BOM)_4$

The T_1-relaxivity of GdDOTA-$(BOM)_4$ in aqueous solution was determined to 8.7 s^{-1} mM^{-1} (20 MHz, 25 °C, pH 7.4). This value was as predicted when plotting the T_1-relaxivity as a function of the molecular weight of the series of BOM substituted GdDOTA derivatives (Fig. 2). The T_1-relaxivity was 6.8 s^{-1} mM^{-1} at 37 °C. This somewhat lower value is a consequence of the decrease in τ_R with increasing temperatures.

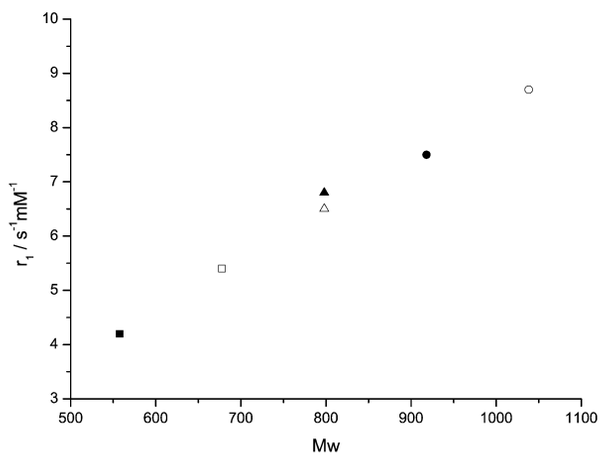

Fig. 2 T_1-relaxivity (r_1) as a function of *M*w of the series of GdDOTA-BOM derivatives (20 MHz, 25 °C). ■ GdDOTA,[3] □ GdDOTA-BOM,[11] △ *trans*-GdDOTA-$(BOM)_2$,[11] ▲ *cis*-GdDOTA-$(BOM)_2$,[11] ● GdDOTA-$(BOM)_3$,[11] ○ GdDOTA-$(BOM)_4$.

Initial studies to evaluate the effect of GdDOTA-$(BOM)_4$ were performed in Bovine Serum Albumin (BSA) solutions. Two different concentrations of BSA were employed. Concentrations of 0.6 mM and 3.5 mM were used to mimic physiological conditions and to study conditions with maximal protein binding, respectively. Higher BSA concentrations would probably give rise to formation of protein aggregates.[14] In both cases the Gd(III) concentration was kept at 0.10 mM. The T_1-relaxivity data from these experiments are presented in Table 1.

The high values obtained with the BSA concentration mimicking physiological conditions suggested a strong binding of the complex to BSA. It has earlier been reported that the T_1-relaxivity of GdDOTA-$(BOM)_3$ at HSA concentrations of >2.9 mM is largely dominated by the contribution from the complex–protein adduct. The present results indicate that this is also true for GdDOTA-$(BOM)_4$ at 3.5 mM BSA. The T_1-relaxivities are comparable to that calculated for the GdDOTA-$(BOM)_3$-HSA adduct, 53.2 s^{-1} mM^{-1} (20 MHz, 25 °C).[11]

As can be seen from Table 1, the T_1-relaxivities of GdDOTA-$(BOM)_4$ in the presence of BSA were higher at 37 °C than at 25 °C. To investigate this further the T_1-relaxivity of GdDOTA-$(BOM)_4$ in 3.5 mM BSA was measured as a function of temperature (Fig. 3).

The T_1-relaxivity increased when raising the temperature from 5 °C to 37 °C. A plateau was reached at temperatures between 37 °C and 50 °C. Above 50 °C there is an increasing risk of denaturation of the protein. The results can be explained by the influence of τ_M on the T_1-relaxivity, as described earlier for other GdDOTA-BOM derivatives.[11] Gd(III) complexes containing one coordinated water molecule ($q = 1$) have been shown to

Table 2 T_1-relaxivities (r_1) of GdDOTA-(BOM)$_4$ in blood and plasma (20 MHz).

	r_1(25 °C)/s^{-1} mM^{-1}	r_1(37 °C)/s^{-1} mM^{-1}
Blood	54.3	62.5
Plasma	57.4	67.4

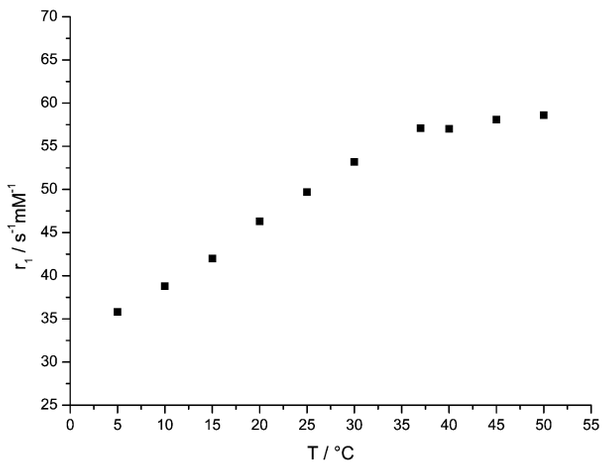

Fig. 3 T_1-relaxivity (r_1) of GdDOTA-(BOM)$_4$ in the presence of 3.5 mM BSA as a function of temperature (20 MHz, pH 7.4).

exhibit relatively long τ_M values, due to the dissociative mechanism of water exchange.[10] This means that the T_1-relaxivity enhancement expected as a result of the increase in τ_R by the formation of the GdDOTA-(BOM)$_4$-BSA adduct is to some extent quenched by the suboptimal τ_M value. When increasing the temperature this value decreases, resulting in higher T_1-relaxivities. At one point optimal water exchange conditions appear, and the T_1-relaxivity becomes dependent on τ_R. This effect has not been observed for low molecular weight Gd(III) complexes, except for some neutral GdDTPA-bisamide derivatives (DTPA = diethylenetriamine pentaacetic acid).

These results indicated that GdDOTA-(BOM)$_4$ might have a potential as a "blood-pool" imaging agent. To further study this potential, measurements of the T_1-relaxivity in human whole blood and plasma were carried out. The results are summarised in Table 2.

The T_1-relaxivities in both blood and plasma were considerably higher than the values obtained in 0.6 mM BSA, and even somewhat higher than those obtained in 3.5 mM BSA. The explanation might be that blood and plasma contain additional proteins and lipoproteins other than albumin, increasing the amount of bound Gd(III) complex. Another explanation can be that GdDOTA-(BOM)$_4$ binds more strongly to HSA than to BSA.

In conclusion, a novel Gd(III) complex, GdDOTA-(BOM)$_4$, has been prepared. The complex exhibits strong interactions with albumin and other blood components resulting in high T_1-relaxivity values. The T_1-relaxivity is dependent on the temperature, with increasing values from 5 °C to 37 °C. The T_1-relaxivity obtained in blood, 62.5 s^{-1} mM^{-1} (20 MHz, 37 °C), makes it an interesting candidate as an MRI contrast agent for angiographic applications.

Experimental

Synthesis

Reagents were obtained from Aldrich Chemical Co. Inc., USA or Fluka Chemie AG, Switzerland, and used as received. Cyclen (1,4,7,10-tetraazacyclododecane) was a gift from Bracco S.p.A (Milan, Italy).

NMR spectra were obtained on a Bruker Spectrospin Avance DPX300 (Bruker GmbH, Germany). Electrospray (ES) FT-ICR-MS mass spectra were recorded on a Bruker BioApex 4.7 T (Bruker GmbH, Germany).

tert-Butyl DOTA-(BOM)$_4$ (3)

A suspension of cyclen (**1**) (121 mg, 0.7 mmol), *tert*-butyl 3-benzyloxy-2-bromopropionate (**2**) (1.55 g, 4.9 mmol) and potassium carbonate (677 mg, 4.9 mmol) in DMF (10 ml) was heated to 100 °C under argon. After 24 h the mixture was cooled and filtered. The filtrate was evaporated *in vacuo*, and the residue was submitted to flash chromatography (SiO_2, CH_2Cl_2/MeOH 9 : 1). Yield: 230 mg (30%). Mass spectrum (ES): *m*/*z* 555 [[M + 2H]$^{2+}$, $C_{64}H_{94}N_4O_{12}$], 527, 499, 471, 443. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.25–1.50 (m, 36H), 2.20–2.75 (m, 9H), 2.90–3.20 (m, 7H), 3.40–4.00 (m, 12H), 4.25–4.75 (m, 8H), 7.10–7.35 (m, 20H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 27.70, 27.85, 27.98, 28.01, 45.75, 46.67, 47.00, 47.84, 60.67, 61.05, 61.25, 61.51, 61.99, 64.85, 65.29, 65.66, 73.27, 73.48, 73.60, 81.93, 82.11, 82.22, 83.34, 127.20, 127.30, 127.45, 127.52, 127.56, 127.67, 127.97, 128.24, 128.27, 128.32, 128.51, 136.60, 137.44, 137.60, 168.80, 172.63, 172.62.

DOTA-(BOM)$_4$ (4)

tert-Butyl DOTA-(BOM)$_4$ (**3**) (183 mg, 0.16 mmol) was dissolved in trifluoroacetic acid (5 ml). After stirring at room temperature for 16 h the solution was evaporated *in vacuo*. The residue was dissolved in methanol (5 ml) and diethyl ether (50 ml) was slowly added. The resulting precipitate was washed with diethyl ether and dried. Yield: 73 mg (50%). Mass spectrum (ES): *m*/*z* 885 [[M + H]$^+$, $C_{48}H_{61}N_4O_{12}$], 795, 705, 617, 443 [M + 2H]$^{2+}$. ^{1}H NMR (300 MHz, D_2O): δ 2.25–3.50 (m, 16H), 3.51–4.50 (m, 12H), 4.70–5.25 (m, 8H), 7.25–8.25 (m, 20H). ^{13}C NMR (75 MHz, D_2O): δ 48.02, 48.26, 64.40, 69.54, 73.78, 128.98, 129.13, 129.22, 129.54, 139.19, 178.95.

GdDOTA-(BOM)$_4$ (5)

DOTA-(BOM)$_4$ (**4**) (70 mg, 0.08 mmol) and $GdCl_3 \cdot 6H_2O$ (30 mg, 0.08 mmol) was dissolved in water (20 ml) and the pH was adjusted to 7.8. After stirring at room temperature for 4 h the temperature was raised to 80 °C for 1 h. The temperature was then allowed to fall to room temperature overnight. The pH of the solution was adjusted to 9.2, and the resulting precipitate removed by filtration. The pH of the filtrate was adjusted to 7.4. The filtrate was evaporated *in vacuo* yielding 60 mg of a slightly yellow solid.

Mass spectrum (ES): *m*/*z* 1040 [[M + 2H]$^+$, $C_{48}H_{58}N_4O_{12}Gd$].

Relaxometric characterisation of GdDOTA-(BOM)$_4$

The relaxation measurements were performed at 20 MHz on a Bruker Minispec mq 20 NMR Analyzer (Bruker Analytik GmbH, Rheinstetten, Germany). The temperature was controlled with a HAAKE DC10 circulator (Gebrüder HAAKE GmbH, Karlsruhe, Germany). The T_1-relaxation times were obtained by the inversion recovery method, and the T_1-relaxivity (r_1) was calculated using eqns. (1) and (2),

$$R_1^{obs} = \frac{1}{T_1} \quad (1)$$

$$r_1 = \frac{R_1^{obs} - R_1^{m}}{C} \quad (2)$$

where R_1^{obs} and R_1^{m} are the relaxation rates in s^{-1} of the sample and the matrix, respectively, and C is the Gd(III) concentration in mM.

Bovine Serum Albumin (BSA) (Fraction V) was obtained from Aldrich Chemical Co. Inc., USA, and used without further purification. The molecular weight was about 66 kDa.

The solutions of BSA were prepared in aqueous phosphate buffer (50 mM, pH 7.4). Aqueous solutions of GdDOTA-$(BOM)_4$ were added to a Gd(III) concentration of 0.10 mM. Blanks were made by adding the same amount of distilled water to the BSA solutions.

Human whole blood (EDTA treated) was used for the measurements in blood. Plasma was obtained by ultracentrifugation of the blood. Small amounts (0.11 ml) of an aqueous solution of GdDOTA-$(BOM)_4$ were added to the blood and plasma to obtain final Gd(III) concentrations of 0.093 mM. Blood and plasma were used as blanks.

The Gd(III) concentrations of the aqueous solutions of GdDOTA-$(BOM)_4$ were determined by mineralisation with hydrochloric acid (37%), followed by T_1-measurements. The T_1-relaxivity of Gd(III) under these conditions is 13.5 s^{-1} mM^{-1}.

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