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NMR relaxometric study of new Gd^{III} macrocyclic complexes and their interaction with human serum albumin

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Five novel Gd(III) complexes based on the structure of the heptadentate macrocyclic 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A) ligand have been synthesized and their ^{1}H and ^{17}O NMR relaxometric properties investigated in detail. The complexes have been functionalised on the secondary nitrogen atom of the macrocyclic ring with different pendant groups for promoting their ability to interact non-covalently with human serum albumin (HSA). The analysis of the proton relaxivity, measured as a function of pH and magnetic field strength, have revealed that the three complexes bearing a poly(ethylene glycol) (PEG) chain possess a single coordinated water molecule, whereas the complexes functionalised with 1-[3-(2-hydroxyphenyl)]-propyl and 1-[3-(2-carboxyphenyloxy)]-propyl pendant groups have two inner sphere water molecules. The water exchange rates, measured by variable temperature ^{17}O NMR, cover a broad range of values (from 18 to 770 ns) as a function of their charge, the chemical nature of the substituent and its ability to organize a second sphere of hydration near the water(s) binding site. All the complexes have shown some degree of interaction with HSA, with a stronger binding affinity measured for those bearing an aromatic moiety on the pendant group. However, upon binding the expected relaxation enhancement has not been observed and this has been explained with the displacement of the coordinated water molecules by the protein and formation of ternary adducts.

Introduction

The polyaminopolycarboxylate paramagnetic complexes have been intensively investigated as possible contrast agents (CA) for magnetic resonance imaging (MRI).[1–3] These substances are able to markedly increase the water proton relaxation rate in the tissues where they distribute thus enhancing the contrast between healthy and pathological districts. The Gd^{III} chelates are particularly suitable for this purpose because of the high magnetic moment and long electronic relaxation times of the metal ion, which results in a strong dipolar interaction with the water proton nuclei. The efficiency of this interaction depends on several structural and dynamic parameters of the metal complex, including the number q of inner sphere water molecules, their rate of exchange ($k = 1/\tau_M$), their distance r from the metal centre, the rotational mobility of the whole complex described in terms of the correlation time τ_R, the electronic relaxation times of the paramagnetic metal ion ($T_{1,2e}$), the relative diffusion coefficients of solute and solvent (D), and the distance of minimum approach between the water molecules in the outer coordination sphere of the complex and the paramagnetic centre (a).[4] The relaxation efficacy of a given paramagnetic complex is described by its relaxivity, the increment of the water protons' relaxation rate induced by one millimolar concentration of the metal chelate. A deep understanding of the relationship between the solution structure of the complexes and their relaxation parameters is crucial in order to enhance the contrast efficiency of these agents.[5] We have now synthesized and characterized, by relaxometric techniques, a series of Gd^{III} complexes with new tetraaza macrocyclic heptadentate ligands functionalised with different pendant groups at the secondary nitrogen atom. The macrocyclic ligand is based on the 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A) scaffold and it therefore contains seven potential donor atoms: the four nitrogen atoms of the macrocycle and three oxygen atoms of the carboxylate groups.[6] Since gadolinium ions are commonly nine coordinate, two water molecules are expected to complete the inner coordination sphere of the paramagnetic metal cation, as found for the corresponding lanthanide complexes of DO3A.[7,8] At the imaging fields (20–60 MHz proton Larmor frequency) the relaxivity of the low molecular weight Gd^{III} chelates are largely determined by the term $q\tau_R/r^6$ and by τ_M, which has an optimal value of about 30 ns at 298 K. In the case of GdDO3A a τ_M of about 90 ns was calculated by ^{17}O NMR data, short enough so as not to limit the observed relaxivity.[8] The present work is aimed at studying the effects of the introduction of pendant groups potentially able to bind human serum albumin (HSA) through non-covalent interactions on the relaxometric properties of the complexes. Pendant groups consisting of polyethyleneglycol (PEG) chains were chosen for the following reasons: i) to increase the solubility of the complexes in water; ii) to exploit the increase of the molecular weight for enhancing the inner sphere relaxivity, which strongly depends on the value of τ_R; iii) to check the possibility that the PEG chain favours the organization of a well defined second sphere of hydration, thus contributing to further increasing the relaxivity; iv) to assess, in the case the previous hypothesis is verified, if a modulation of the value of τ_M occurs; v) to investigate the extent of the binding with HSA and the concomitant gain in relaxivity.

For a better evaluation of the role of the PEG pendant groups, relaxometric properties of similar complexes characterized by the presence of propylene chains terminated with aromatic substituents have been also studied.

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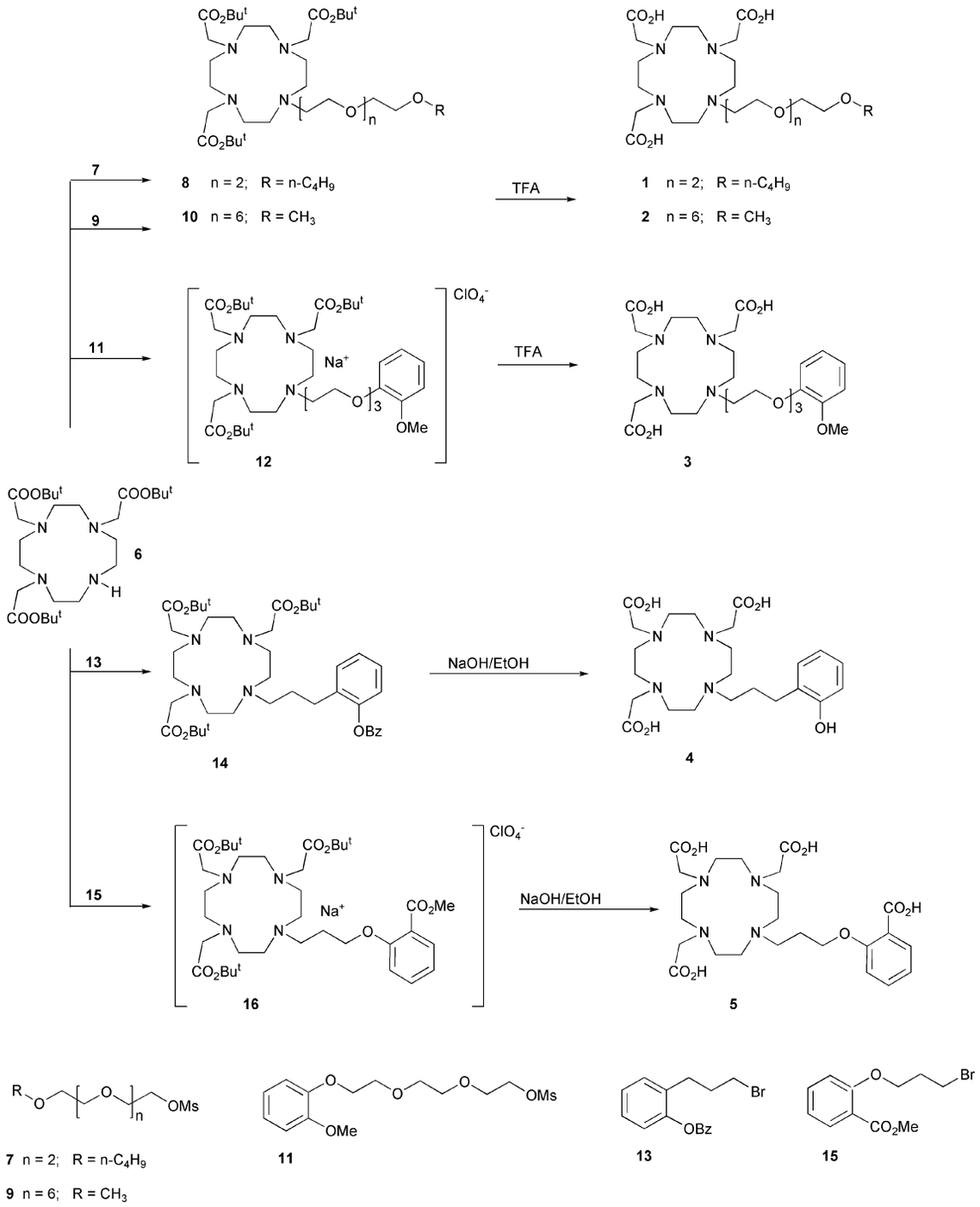


Scheme 1

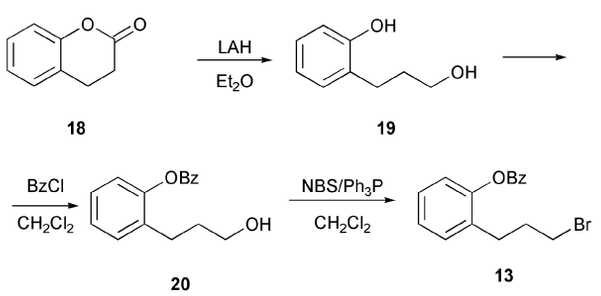
Results and discussion

Synthesis of the ligands

N-alkylation of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris-(1,1-dimethylethyl) ester **6**[9] with suitable reagents, followed by deprotection of the *tert*-butyl ester functions, allows a convenient access to the new ligands **1**–**5** (Scheme 1). However, alkylation conditions and purification of the intermediate triester had to be optimized for any single ligand. Thus, ligands **1** and **3** were prepared by alkylation of **6** with methanesulfonates **7** and **11**, respectively, carried out in CH_3CN and solid Na_2CO_3 as a base. Subsequent treatment of the crude triesters **8** and **12**, dissolved in CH_2Cl_2, with 30% aqueous $NaClO_4$ afforded the corresponding sodium complexes which were isolated by column chromatography. The preparation of the complexes is necessary in order to simplify the chromatographic purification and allow higher yields of isolated products. Decomplexation of the sodium complex of **8** was achieved by continuous extraction of the complex, after Cl^- for ClO_4^- counterion exchange in H_2O/CH_3OH = 9 : 1 (v/v) with *n*-pentane[10] to give the free ligand **8** in 58% yield. This last compound was hydrolyzed with CF_3COOH at room temperature to afford **1** quantitatively as a white solid. Compound **12** was analogously isolated and characterized as $NaClO_4$ complex in 85% yield after purification by column chromatography. This product was first decomplexed and then hydrolyzed with CF_3COOH, as described in the case of **8**, to afford ligand **3** in quantitative yield. On the other hand, alkylation of **6** with PEG-350 monomethylether methanesulfonate **9** was carried out in DMF at 110 °C for 24 h to give triester **10** in 70% yield as an oil. Hydrolysis of **10** by CF_3COOH in CH_2Cl_2 at room temperature and crystallization of the residue with THF/*n*-heptane yielded **2** as a light white solid. Alkylation of **6** with 2-(3-bromopropyl)phenyl benzoate **13** was carried out in CH_3CN at

reflux and solid Na_2CO_3 as a base to give triester **14** in 58% yield, whereas triester **16**, obtained by alkylation of **6** with methyl 2-(3-bromopropoxy)-benzoate **15**, was conveniently isolated and characterized after treatement with $NaClO_4$. Hydrolysis of **14** and **16** with NaOH in 96% EtOH at reflux directly afforded ligands **4** and **5** in quantitative yields. It is also worth noting that the alkylating agents employed here were prepared in high yields from commercially available or easily obtained precursors, which is particularly helpful in view of possible large-scale preparation of the new ligands. Indeed, methanesulfonates **7**, **9** and **11** were prepared from triethyleneglycol monobutylether, PEG-350 monomethylether (commercially available reagents) and triethyleneglycol mono-2-methoxyphenyl ether **17**,[11] respectively, following standard procedures as described in the Experimental section.

2-(3-Bromopropyl)phenyl benzoate **13** was prepared from 3-(2-hydroxyphenyl)-1-propanol **19**[12] through benzoylation of the phenolic hydroxyl group followed by bromination of the protected phenol **20** with *N*-bromosuccinimide (NBS) and triphenylphosphine (Scheme 2). Methyl 2-(3-bromopropoxy)-benzoate **15** was prepared by alkylation of commercially available methyl salicylate with an excess of 1,3-dibromopropane in refluxing acetonitrile and solid Na_2CO_3 as a base.



Scheme 2

^{1}H NMR relaxometric studies

The parent complex GdDO3A has been shown to be present in aqueous solution as a mixture of two coordination isomers differing in the hydration number, *i.e.* $q = 2$ (predominant species) and $q = 1$ (largely minor species).[8] The relaxivity at 25 °C and 20 MHz is 6.0 mM^{-1} s^{-1}, about 28% higher than for the monoaquo GdDOTA complex (4.7 mM^{-1} s^{-1}).[1–3] Under identical experimental conditions the relaxivities of the Gd^{III} complexes with **1**–**3** are 3.9, 4.6 and 4.1 mM^{-1} s^{-1}, respectively. This suggests a lower hydration for Gd·**1**–**3** complexes ($q < 2$) that may arise from partial displacement of the coordinated water molecules by oxygen atom(s) of the PEG chain. A similar effect has recently been observed in the case of PEG-functionalised complexes based on the hydroxypyridonate (HOPO) binding unit.[13] Although we cannot exclude the occurrence of an effective fractional q value arising from a mixture of coordination isomers with $q = 0$ (low concentration) and $q = 1$ (predominant), we assume for simplicity that the complexes are monoaquo species. Support for this hypothesis has been gained from the pH-dependent behaviour of the relaxivity (*vide infra*). On the other hand, the r_{1p} values of Gd·**4**–**5** (25 °C, 20 MHz) are 7.4 and 7.5 mM^{-1} s^{-1}, respectively and thus fully consistent with the relaxivity expected for diaquo complexes. This is clearly demonstrated by the linear plot of the relaxivity *versus* the molecular weight for a series of Gd^{III} complexes with $q = 2$ (Fig. 1).[14]

The linear dependence of r_{1p} from the molecular weight implies that all the complexes present the same hydration number and that their relaxivity is predominantly determined by the value of τ_R, which is strongly correlated with the molecular dimension of the complexes. Thus, relatively low values of r_{1p} are only observed for those complexes featuring oxygen atoms associated with the PEG chain and this is better

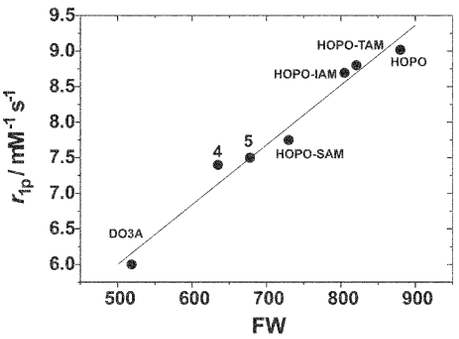


Fig. 1 Plot of ^{1}H relaxivity (r_{1p}; mM^{-1} s^{-1}) at 20 MHz and 25 °C *versus* molecular weight for several Gd(III) complexes with $q = 2$.[14] The linear relationship represents an indication that the compounds possess the same number of inner sphere water molecules.

explained by the fact that the substituent is involved in the coordination rather than by the predominance in solution of octacoordinated monoaquo species. The pH dependence of the relaxivity, measured at 25 °C and 20 MHz, confirms the different behaviour of the complexes Gd·**1**–**3** and Gd·**4**–**5**. In the former, r_{1p} is nearly constant in the range 4–12, whereas for the latter a significant decrease is observed from 8–10. In $q = 1$ complexes the coordinated water molecule cannot be displaced by the carbonate anions originating from dissolved CO_2 in the aerated solutions and the relaxivity does not change with pH.[7,15] In $q = 2$ complexes the chelating carbonate anions can remove the two inner sphere water molecules and the relaxivity decreases to a value close to that typical of pure outer sphere complexes (*ca.* 3.0 mM^{-1} s^{-1}) (Fig. 2).[7,15] The small inflection point at pH close to 9 observed in the pH profile in the case of the complexes Gd·**1**–**3** could result either from the presence in solution of a low concentration of the $q = 2$ species or from competition of carbonate with PEG for the coordination site(s) on the metal ion.

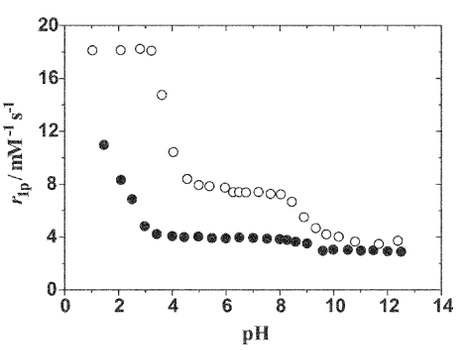


Fig. 2 Plot of ^{1}H relaxivity (r_{1p}; mM^{-1} s^{-1}) at 20 MHz and 25 °C *versus* pH for Gd·**1** (filled circles) and Gd·**4**(open circles).

^{17}O NMR relaxometric studies

The analysis of the temperature dependence of the transverse relaxation rate of the ^{17}O water nuclei is a well established procedure for obtaining an accurate evaluation of the inner sphere water molecules' exchange dynamics.[16] Moreover, in favorable cases (intermediate or fast exchange conditions) the electronic relaxation parameters can also be derived with good accuracy. We have measured the ^{17}O variable-temperature (VT) profiles at 2.1 (or 9.4) T and pH = 7 for the complexes Gd·**1**–**5** and analyzed the data in terms of the established theory (Swift–Connick equations). The derived best fit parameters are listed in Table 1 and representative profiles are shown in Fig. 3. The mean residence lifetimes show a marked dependence on the nature of the pendant group: their values (at 298 K) are within the wide interval 18–770 ns. The parent GdDO3A complex presents a τ_M value of *ca.* 90 ns, shorter by a factor of three with respect to GdDOTA and GdDTPA and the exchange mech-

Table 1 Parameters obtained from least-squares fits of ^{17}O NMR Data at 2.1 T and pH = 7

	τ_M/ns	ΔH_M/kJ mol^{-1}	τ_V/ns	ΔH_V/kJ mol^{-1}	$\Delta^2/10^{19}$ s^{-2}
Gd·**1**	180	44.0	19	8.5	13
Gd·**2**	216	49.0	20	5.0	6
Gd·**3**	500	50.1	18	8.5	9
Gd·**4**	770	56.0	10	8.0	12
Gd·**5**	18	40.8	12	7.0	13

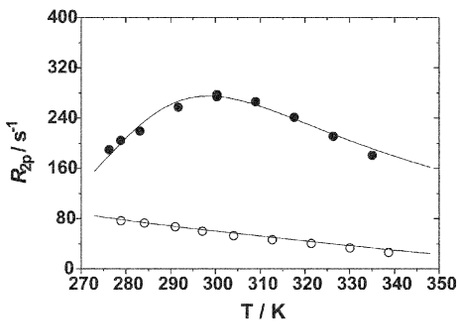


Fig. 3 Temperature dependence of the paramagnetic contribution (R_{2p}) to the transverse ^{17}O water relaxation rate of Gd·**3** (33 mM; open circles) and Gd·**5** (10 mM; filled circles) at 2.1 T and pH = 7.

anism has been found to be of a dissociative nature.[7] The exchange lifetimes for Gd·**1**–**5** cover a broad range of values which are a function of the *N*-substituent. In related macrocyclic octadentate complexes, the τ_M value has been found to be modulated by the relative proportion of the isomeric species present in their aqueous solutions,[17] the overall electric charge,[18] the nature of the counterion[19] and the accessibility of the solvent in the proximity of the gadolinium cation.[20] In the present case we have to consider: i) the partial water displacement by the PEG group. The $q = 1$ complexes often show longer τ_M values; ii) the ability of the PEG chain to promote the formation of a second hydration shell next to the metal center.[13] This network of H-bonded solvent molecules may involve the bound water molecule(s) and slow down either the departure of the leaving and/or the approach of the incoming water molecules; iii) the different basicity of the nitrogen atom of the macrocycle bearing the pendant group. This effect has been recently shown to modulate the water exchange rate in related GdDO3A derivatives.[21] Particularly striking is the difference in τ_M between Gd·**4** and Gd·**5**, which is difficult to rationalize based on the available data. We may only make the hypothesis of a possible involvement of the hydroxy group of the phenolic moiety in an H-bonding interaction with the coordinated water molecules that increases their exchange lifetimes by a factor of about 3–4. On the other hand, the much shorter τ_M of Gd·**5** could result from the predominance in solution of a conformational isomer endowed with fast water exchange rate and from the presence, around neutral pH, of an overall negative charge arising from the deprotonation of the carboxylic group of the aromatic moiety.

NMRD

The analysis of the magnetic field dependence of the water proton longitudinal relaxation times represents a powerful tool for investigating the relaxometric properties of paramagnetic compounds.[22] The NMRD profiles of Gd·**1**–**3** are consistent with the results of the previous experiments as they reproduce quite closely the behaviour of complexes analogous with $q = 1$.[2] The analysis of the data was performed in terms of the Solomon–Bloembergen–Morgan (SBM) theory of paramagnetic relaxation, by using for τ_M the values previously obtained (see Table 1), and yielded the parameters listed in Table 2. Representative profiles for Gd·**3** and Gd·**4** are shown in Fig. 4. The rotational correlation times are typical of complexes

Table 2 Best fit relaxation parameters determined by analysis of NMRD profiles at 25 °C and pH = 7[a]

	Gd·**1**	Gd·**2**	Gd·**3**	Gd·**4**	Gd·**5**
$\Delta^2/10^{19}$ s^{-2}	12	6.0	9.5	13.0	12
τ_V/ps	18	18	18	13	11
τ_R/ps	90	86	85	120	116
r/Å	3.10	3.10	3.16	3.00	3.00

[a] The parameters a and D have been fixed in the fitting procedure to the standard values of 3.8 Å and 2.24×10^{-5} cm^2 s^{-1}, respectively.

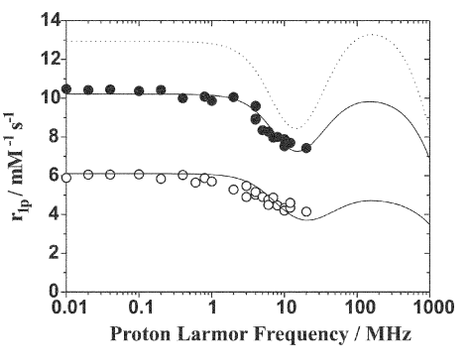


Fig. 4 Nuclear magnetic relaxation dispersion (NMRD) profiles for Gd·**3** (open circles) and Gd·**4** (filled circles) at 25 °C and pH = 7. The dotted line represents the calculated NMRD profile of Gd·**4** with a τ_M value lowered by one order of magnitude.

of this molecular weight and the parameters describing the electronic relaxation are quite similar to those evaluated from ^{17}O VT experiments. On the other hand, the experimental profiles of Gd·**4**–**5** were nicely fitted with similar parameters, but by assuming a hydration number $q = 2$. A limiting effect of the exchange lifetime on the relaxivity is observed only in the case of Gd·**4** where τ_M is of the order of μs (Fig. 4).

Interaction with HSA

The non covalent binding of Gd(III) complexes with HSA can be conveniently studied by relaxometric methods on a low resolution NMR spectrometer operating at a fixed frequency.[23] Titration of a dilute aqueous solution of the complex with the protein causes an increase of the relaxation rate when a binding interaction occurs, as a result of the increase of the reorientational correlation time of the macromolecular adduct. An analysis of the data according to the *proton relaxation enhancement* (PRE) method provides good estimates of the association constant, K_A, and of the relaxivity of the bound complex, r_{1p}^b.[23] All the complexes investigated show some degree of interaction with HSA, as shown in Fig. 5. The hydrophilic complexes Gd·**1**–**2** present only a weak interaction with the protein and this might be related to the lack of a specific, strong binding site (estimated K_A of *ca.* 1500 M^{-1} by assuming the presence of a single class of equivalent binding sites). More likely, the binding for these complexes simply involves charged domains on the surface of the protein. On the other hand, Gd·**3**–**5** present aromatic groups able to interact more strongly with the well defined hydrophobic sites (I and II) of HSA. In fact, in this latter case, the association constants are of the same

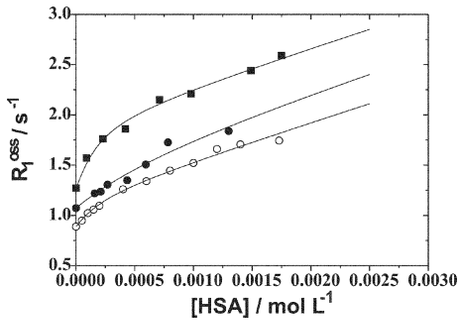


Fig. 5 Longitudinal ^{1}H water proton relaxation rates as a function of [HSA] for Gd·**1** (0.12 mM, filled circles), Gd·**3** (0.18 mM, open circles) and Gd·**5** (0.12 mM, squares) at 20 MHz and 25 °C.

order of magnitude of those previously found for GdDOTA derivatives bearing benzyloxypropionic groups ($K_A \approx 6750$, 5870 and 9290 M^{-1} for Gd·**3**–**5**, respectively).[24] It must be noted that the relaxivity values of the adducts are rather similar and much lower than those expected for macromolecular paramagnetic complexes ($r_{1p}^b = 6.6$, 6.8, 6.6, 10.6 and 12.5 mM^{-1} s^{-1} for Gd·**1**–**5**, respectively). This suggests that the binding might involve water displacement by carboxylic groups of the HSA with formation of ternary complexes lacking any coordinated water molecules. This has been previously observed in related complexes with $q > 1$ and represents a strong limitation for the attainment of high relaxivity through the lengthening of τ_R.[25–27]

Conclusions

The relaxometric properties of GdDO3A derivatives bearing an appendage group on the secondary nitrogen atom of the macrocycle show a marked dependence on the nature of the substituent. Lower r_{1p} values are found for the complexes functionalised with a PEG chain and this is the result of a partial water displacement by an oxygen atom of the PEG that lowers the hydration number of the metal ion. As a consequence, the $q = 1$ and $q = 2$ complexes exhibit different pH-dependent relaxivity behaviour. In the diaquo complexes the water molecules are displaced by the chelating carbonate anions present in the aerated solutions at basic pH and r_{1p} significantly decreases. This effect is substantially reduced in the case of the monoaquo complexes.

The mean residence lifetime of the bound water molecule(s) is modulated as a function of the nature of the pendant substituent. This, in turn, reflects the differences in the structure and dynamics of the H-bonded array of water molecules in the outer hydration sphere of the complexes.

All the complexes show some degree of binding affinity to HSA, with large differences in the K_A values. Clearly, the design of functionalised DO3A derivatives has to be optimised in view of the attainment of high relaxivity for the corresponding Gd-complexes. The introduction of a PEG chain in the substituent has to be carefully designed so as to avoid coordination to the metal centre and water displacement. A possibility is the introduction of a rigid spacer. The nature of the pendant aromatic group also needs to be optimised in order to enhance the targeting ability towards HSA. Finally, it is plausible that the presence of a negatively charged group on the substituent may help to decrease the ease of formation of ternary complexes with bioactive anions in physiological conditions.

Experimental

Solvents were purified by using standard methods and dried if necessary. All commercially available reagents were used as received. TLC was carried out on silica gel Si 60-F_{254}. Column chromatography was carried out on silica gel Si 60, mesh size 0.040–0.063 mm (Merck, Darmstadt, Germany). NMR spectra of organic compounds were recorded using Bruker AC 300 or Bruker AC 200 spectrometers. Melting points (uncorrected) were determined with a Büchi SMP-20 capillary melting point apparatus.

Note: Perchlorate salts are potentially dangerous substances. Although we never experienced any problems during manipulation of the perchlorate complexes of the ligands described here, they should be handled with care.

Triethyleneglycol-monobutylether methanesulfonate 7

A solution of methanesulfonyl chloride (6.3 g, 55 mmol) in pyridine (10 cm^3) was added dropwise to a stirred solution of triethyleneglycol-monobutyl ether (10.3 g, 55 mmol) in pyridine (60 cm^3), the temperature being kept between 0 and 5 °C. The reaction mixture was stirred for 4 h at 0 °C. After addition of ice (150 g) and acidification with 37% aqueous HCl, the mixture was extracted with CH_2Cl_2 (3 × 50 cm^3). The organic phase was washed with water, brine and then dried over Na_2SO_4. Evaporation of the solvent afforded **7** (13.9 g, 96%) as a yellowish thick oil. δ_H (300 MHz, $CDCl_3$, Me_4Si) 0.89 (3 H, t, *J* 7.3), 1.35 (2 H, m), 1.54 (2 H, m), 3.05 (3 H, s), 3.43 (2 H, m), 3.50–3.80 (10 H, m), 4.36 (2 H, m).

10-(3,6,9-Trioxatridecyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester 8

A suspension of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester **6** (0.51 g, 1.0 mmol), methanesulfonate **7** (0.35 g, 1.2 mmol) and, Na_2CO_3 (0.26 g, 2.5 mmol) in anhydrous CH_3CN (20 cm^3) was refluxed for 72 h under magnetic stirring. The mixture was cooled to room temperature, filtered and the filtrate was evaporated. The residue was dissolved in CH_2Cl_2 (50 cm^3), washed with water (2 × 30 cm^3) and then with 30% aqueous $NaClO_4$ (3 × 30 cm^3). The solvent was removed under reduced pressure and the product purified by column chromatography (silica gel, CH_2Cl_2/MeOH 98 : 2) to afford the sodium perchlorate complex of **8** as yellowish thick oil. This product was dissolved in methanol (30 cm^3) and stirred overnight at room temperature in the presence of solid KCl (0.23 g, 3.0 mmol). The suspension was filtered through Celite® and the solvent was removed under reduced pressure. The residue was dissolved in methanol (3 cm^3) and transferred in a liquid–liquid extractor. Water (30 cm^3) was added and the mixture was continuously extracted overnight with *n*-pentane (200 cm^3). The solvent was removed under reduced pressure to afford **8** (0.41 g, 58%) as a yellowish oil. δ_H (300 MHz, $CDCl_3$, Me_4Si) 0.9 (3 H, t, *J* 8.7), 1.34 (2 H, m), 1.44 (27 H, s), 1.60 (2 H, m), 2.66 (4 H, m), 2.79 (12 H, br s), 3.27 (m, 4H), 3.42–3.48 (m, 4H), 3.54–3.63 (12 H, m); δ_C (75 MHz, $CDCl_3$, Me_4Si) 14.2, 19.6, 28.6, 32.1, 52.2, 52.3, 52.6, 53.2, 55.1, 55.2, 56.6, 57.1, 62.1, 69.4, 69.6, 70.4, 70.8, 70.9, 71.0, 71.5, 72.9, 81.1, 171.4; *m*/*z* (FAB+) 703 (M + H^+. $C_{36}H_{70}N_4O_9$ requires 702).

10-(3,6,9-Trioxatridecyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid 1

A solution of triester **8** (154 mg, 0.22 mmol) in trifluoroacetic acid (5 cm^3) was stirred for 4 h at room temperature. The solvent was removed under reduced pressure and the residue was triturated with diethyl ether (50 cm^3) to yield **1** (117 mg, 97%) as a white solid. δ_H (300 MHz, CD_3OD, Me_4Si) 0.93 (3H, t, *J* 7), 1.39 (2 H, m), 1.55 (2 H, m), 3.06 (8 H, m), 3.38–3.58 (24 H, m), 3.85 (2 H, t, *J* 4.2), 3.98 (2 H, s); *m*/*z* (FAB+) 535 (M + H^+. $C_{24}H_{46}N_4O_9$ requires 534).

Polyethyleneglycol (PEG) 350-monomethylether methanesulfonate 9

A solution of anhydrous PEG 350-monomethylether (5.0 g, 14.3 mmol) and triethylamine (1.82 g, 18 mmol) in dry CH_2Cl_2 (30 cm^3) was cooled to 0 °C. A solution of methanesulfonyl

chloride (2.31 g, 17.7 mmol) in CH_2Cl_2 (20 cm^3) was slowly added under stirring and the reaction was then maintained at 4 °C for 24 h. The solvent was evaporated and the crude material was purified by column chromatography (neutral aluminium oxide, CH_3CN) to give **9** (6.15 g, 97%) as a viscous oil. δ_H (300 MHz, $CDCl_3$, Me_4Si) 3.06 (3 H, s), 3.35 (3 H, s), 3.50–3.76 (26 H, m), 4.33–4.38 (2 H, m).

10-[PEG 350 monomethyl ether]-1,4,7,10-tetrazacyclododecane-1,4,7-triacetic acid tri *tert*-butyl ester 10

A solution of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester **6** (0.8 g, 1.55 mmol) and PEG 350 monomethyl ether methanesulfonate **9** (0.69 g, 1.55 mmol) in dry DMF (20 cm^3) was heated for 24 h under nitrogen at 110 °C. The solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (gradient elution of concentrated NH_3 and CH_2Cl_2–MeOH 9 : 1) to give **10** (0.91 g, 70%) as a pale yellow oil. δ_H (200 MHz, $CDCl_3$, Me_4Si) 1.40–1.48 (27 H, m), 2.26–2.90 (18 H, m), 3.02 (4 H, s), 3.12 (2 H, s), 3.37 (3 H,s), 3.48–3.73 (26 H, m); δ_C (50.3 MHz, $CDCl_3$, Me_4Si) 28.1, 49.6, 50.5, 52.8, 55.6, 56.3, 58.9, 67.2, 69.7, 70.4, 71.8, 82.1, 172.5, 172.8; *m/z* (EI) 836 (M^+. $C_{41}H_{80}N_4O_{13}$ requires 836), 792, 748, 704, 660, 616, 514.

10-[PEG 350 monomethylether]-1,4,7,10-tetrazacyclododecane-1,4,7-triacetic acid 2

A solution of **10** (0.9 g, 1.07 mmol) in trifluoroacetic acid (2 cm^3) and CH_2Cl_2 (8 cm^3) was stirred for 12 h at room temperature. Solvents were removed under reduced pressure and the residue was taken up in THF (10 cm^3). Addition of *n*-heptane to this solution allowed the formation of a light precipitate which was collected by centrifugation to afford **2** (0.6 g, 90%) as a white solid. δ_H (200 MHz, D_2O, Me_4Si) 2.62–3.00 (16 H, m), 3.15 (3 H, s), 3.20–3.73 (34 H, m); δ_C (50.3 MHz, D_2O, Me_4Si) 50.1, 50.7, 53.2, 54.1, 55.1, 56.1, 56.9, 60.4, 67.0, 71. 9, 72.3, 73.3, 170.8, 176.3; *m/z* (FAB+) 669 (M + H^+. $C_{29}H_{56}N_4O_{13}$ requires 668), 691 (M + Na^+).

Triethyleneglycol-mono-2-methoxyphenylether methanesulfonate 11

A solution of methanesulfonyl chloride (1.34 g, 11.7 mmol) in anhydrous CH_2Cl_2 (10 cm^3) was added dropwise to a stirred solution of triethyleneglycol-mono-2-methoxyphenylether **17**[11] (3 g, 11.7 mmol) and [2,2,2]-diazabicyclooctane (DABCO) (4 g, 35 mmol) in anhydrous CH_2Cl_2 (160 cm^3), the temperature being kept between 0 and 5 °C. The reaction mixture was stirred for 4 h at 0 °C. After addition of ice (100 g) and acidification with 37% aqueous HCl, the mixture was extracted with CH_2Cl_2 (3 × 50 cm^3), and the organic phase was washed with water, brine and then dried over Na_2SO_4. Evaporation of the solvent afforded **11** (3.6 g, 92%) as a colourless viscous oil. δ_H (300 MHz, $CDCl_3$, Me_4Si) 3.05 (3 H, s), 3.59–4.21 (12 H, m), 3.85 (3 H, s), 6.90 (4 H, m).

10-{1-[8-(2-Methoxyphenoxy)-3,6-dioxa]-octyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester sodium perchlorate complex 12

A suspension of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester **6** (257 mg, 0,50 mmol), methanesulfonate **11** (167 mg, 0,55 mmol) and Na_2CO_3 (212 mg, 2.0 mmol) in anhydrous CH_3CN (20 cm^3) was refluxed 72 h under magnetic stirring. The mixture was cooled to room temperature, filtered and the filtrate was evaporated. The residue was dissolved in CH_2Cl_2 (50 cm^3), washed with water (2 × 30 cm^3) and then with 30% aqueous $NaClO_4$ (3 × 30 cm^3). The solvent was removed under reduced pressure and the product purified by column chromatography (silica gel, CH_2Cl_2/MeOH 95/5) to afford the sodium perchlorate complex **12** (371 mg, 85%) as a white solid. δ_H (300 MHz, $CDCl_3$, Me_4Si) 1.42 (9 H, s). 1.44 (18 H, s), 2.10–2.80 (18 H, m), 2.98 (4 H, s), 3.20–3.30 (2 H, m), 3.57 (2 H, m), 3.67 (4 H, m), 3.82 (5 H, m), 4.13 (2 H, t, *J* 4), 6.87–6.90 (4 H, m); δ_C (75 MHz, $CDCl_3$, Me_4Si) 28.3, 28.4, 49.8, 52.5, 55.9, 56.2, 56.7, 67.1, 68.5, 70.0, 70.1, 82.5, 112.4, 114.0, 121.2, 121.9, 172.3, 172.8; *m/z* (FAB+) 776 ($[M - ClO_4]^+$. $C_{39}H_{68}N_4O_{10}NaClO_4$ requires 875).

10-{1-[8-(2-Methoxyphenoxy)-3,6-dioxa]-octyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid 3

A solution of the perchlorate complex **12** (0.31 g, 0.35 mmol) in methanol (30 cm^3) was stirred overnight at room temperature in the presence of solid KCl (0.38 g, 5 mmol). The suspension was filtered through Celite® and the solvent was removed under reduced pressure. The residue was dissolved in methanol (3 cm^3) and transferred in a liquid–liquid extractor. Water (30 cm^3) was added and the mixture was continuously extracted overnight with *n*-pentane (200 cm^3). The solvent was removed under reduced pressure affording a residue (0.25 g) which was dissolved in trifluoroacetic acid (5 cm^3) and stirred at room temperature for 4 hours. The solvent was removed under reduced pressure and the residue was triturated with diethyl ether (50 cm^3) to yield **3** (0.19 g, 93%) as a white solid. δ_H (300 MHz, CD_3OD, Me_4Si) 2.90–3.00 (8 H, m), 3.40–3.49 (8 H, m), 3.60–3.74 (10 H, m), 3.83 (3 H, m), 3.86 (2 H, m), 3.92 (2 H, m), 4.07 (2 H, m), 4.14 (2 H, m), 6.90 (4 H, m); δ_H (75 MHz, CD_3OD, Me_4Si) 52.2, 52.6, 53.7, 55.0, 55.9, 56.5, 67.0, 69.9, 70.8, 71.2, 71.4, 113.8, 115.4, 122.3, 122.8, 149.7, 150.9, 169.2, 174.2;

m/z (FAB+) 585 (M + H^+. $C_{27}H_{44}N_4O_{10}$ requires 584), 607 (M + Na^+).

3-(2-Hydroxyphenyl)-1-propanol 19

A solution of commercially available 3,4-dihydrocoumarin **18** (7.41 g, 50 mmol) in dry Et_2O (40 cm^3) was added dropwise at room temperature to a stirred suspension of $LiAlH_4$ (2.28 g, 60 mmol) in dry Et_2O (40 cm^3). The reaction mixture was refluxed for 4 h and then stirring was continued at room temperature overnight. The excess of $LiAlH_4$ was destroyed by careful addition of THF/H_2O (1 : 1 v/v, 5 cm^3); the mixture was stirred for 30 min, then 5% aqueous H_2SO_4 (30 cm^3) was added dropwise until the gas evolution ceased. The organic layer was separated and the aqueous phase was extracted with Et_2O (2 × 30 cm^3). The combined organic layers were washed with H_2O (30 cm^3), 5% aqueous $NaHCO_3$ (30 cm^3) and brine (30 cm^3). The organic phase was dried over Na_2SO_4 and the solvent evaporated *in vacuo* to afford a yellow residue which was purified by column chromatography (silica-gel, CH_2Cl_2/AcOEt 7 : 1) to give **19** (6.62 g, 87%) as a pale yellow oil. This compound had been previously synthesized following a different procedure.[12]

δ_H (300 MHz, $CDCl_3$, Me_4Si) 1.83–1.91 (2 H, m), 2.45 (1 H, s), 2.77 (2 H, t, *J* 6.6), 3.63 (2 H, t, *J* 5.9 Hz), 6.82–6.88(2 H, m), 7.00 (1 H, s), 7.08–7.15 (2 H, m); δ_C (75 MHz 300 MHz, $CDCl_3$, Me_4Si) 25.8, 32.8, 61.4, 116.3, 121.2, 127.9, 128.0, 131.0, 154.8.

2-(3-Hydroxypropyl)phenyl benzoate 20

Neat benzoyl chloride (0.58 cm^3, 5 mmol) was added *via* syringe to a stirred solution of **19** (1.52 g, 10 mmol) and triethylamine (2 cm^3) in anhydrous CH_2Cl_2 (20 cm^3). The solution was stirred at room temperature for 15 min then a second portion of benzoyl chloride (0.58 cm^3, 5 mmol) was added and stirring was continued overnight. The reaction mixture was transferred into a separatory funnel, diluted with CH_2Cl_2 (30 cm^3) and washed with H_2O (2 × 30 cm^3) and brine (30 cm^3). The organic phase was dried over Na_2SO_4 and the solvent evaporated to afford a yellow residue which was purified by column chromatography (silica-gel, CH_2Cl_2/AcOEt 9 : 1) to give **20** (1.83 g, 71.4%) as a

colourless thick oil. δ_H (300 MHz, $CDCl_3$, Me_4Si) 1.83–1.92 (2 H, m), 2.69 (2 H, t, *J* 6.95), 3.62 (2 H, t, *J* 5.20), 7.13–7.33 (4 H, m), 7.50–7.68 (3 H, m), 8.20–8.22 (2 H, m); δ_C (75 MHz 300 MHz, $CDCl_3$, Me_4Si) 26.2, 32.7, 61.9, 122.4, 126.1, 127.1, 128.6, 129.3, 130.0, 130.3, 133.6, 133.7, 149.1, 165.2.

2-(3-Bromopropyl)phenyl benzoate 13

Solid *N*-bromosuccinimide (NBS) (0.21 g, 1.2 mmol) was added portionwise to a stirred solution of 2-(3-hydroxypropyl)phenyl benzoate **20** (0.25 g, 1 mmol) and triphenylphosphine (0.31 g, 1.2 mmol) in dry CH_2Cl_2 (15 cm^3). During the addition the temperature was kept at 0 °C and then the reaction mixture was maintained at this temperature and under stirring for 1 hour. The solvent was removed under reduced pressure and the brown waxy solid residue was taken up in diethyl ether (50 cm^3). The red brown solid thus formed was collected by filtration, the filtrate was concentrated and purified by column chromatography (silica gel, light petroleum/diethyl ether = 9 : 1) to afford **13** (0.22 g, 68%) as a colourless oil. δ_H (300 MHz, $CDCl_3$, Me_4Si) 2.15 (2 H, m). 3.36 (2 H, t, *J* 5.1), 3.76 (2 H, t, *J* 8.6), 7.15–7.30 (4 H, m), 7.45–7.60 (3 H, m), 8.20–8.22 (2 H, m),

10-{1-[3-(2-Carboxyphenyl)]-propyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester 14

A suspension of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester **6** (0.32 g, 0,63 mmol), alkyl bromide **13** (0.20 g, 0,63 mmol) and Na_2CO_3 (0.21 g, 2 mmol) in anhydrous CH_3CN (20 cm^3) was refluxed for 24 h under magnetic stirring. The mixture was cooled to room temperature, filtered and the filtrate was evaporated. The residue was dissolved in CH_2Cl_2 (50 cm^3) and washed with water (2 × 30 cm^3). The solvent was removed under reduced pressure and the residue purified by column chromatography (silica gel, CH_2Cl_2/MeOH 95 : 5) to afford compound **14** (0.276 g, 58%) as a white solid. δ_H (300 MHz, $CDCl_3$, Me_4Si) 1.37 (9 H, s), 1.41 (18 H, s), 1.60–2.38 (6 H, m), 2.40–2.80 (8 H, m), 2.90–3.20 (14 H, m), 7.10–7.20 (4 H, m), 7.53 (2 H, m), 7.67 (1 H, m), 8.20 (2 H, m); δ_C (75 MHz 300 MHz, $CDCl_3$, Me_4Si), 28.1, 28.4, 28.7, 50.0, 51.3, 53.2, 53.6, 54.3, 56.1, 56.7, 82.7, 83.0, 122.8, 126.6, 127.6, 129.1, 130.0, 130.3, 133.8, 134.2, 149.3, 165.3, 172.9, 173.8; *m*/*z* (FAB+) 754 (M + H^+. $C_{42}H_{64}N_4O_8$ requires 753), 776 (M + Na^+).

10-{1-[3-(2-Hydroxyphenyl)]-propyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid 4

A solution of triester **14** (0.25 g, 0.33 mmol) and NaOH (0.16 g, 4 mmol) in 96% EtOH (20 cm^3) was stirred at reflux for 4 h. Evaporation of the solvent afforded a residue which was dissolved in water (5 cm^3) and acidified to pH = 1 with 10% aqueous HCl. The precipitated benzoic acid was washed out with $CHCl_3$ (2 × 30 cm^3) and the aqueous phase was evaporated *in vacuo*. The residue was taken up with absolute ethanol (10 cm^3), the undissolved solid was removed by filtration and the solvent was evaporated under reduced pressure to yield **4** (0.12 g, 76%) as a white solid. δ_H (300 MHz, CD_3OD, Me_4Si) 2.12 (2 H, m), 2.70 (2 H, t, *J* 6), 2.88 (2 H, m), 3.10 (8 H, m), 3.40 (8 H, m), 3.63 (4 H, m), 4.23 (2 H, s), 6.75 (2 H, m), 7.06 (2 H, m); *m*/*z* (FAB+) 481 (M + H^+. $C_{28}H_{36}N_4O_7$ requires 480), 503 (M + Na^+).

2-(3-Bromopropoxy)benzoic acid methyl ester 15

A suspension of methyl salicylate (1.52 g, 10 mmol), 1,3 dibromopropane (10 g, 50 mmol) and K_2CO_3 (3.12 g, 20 mmol) in dimethylformamide (30 cm^3) was stirred at 70 °C for 16 h. The reaction mixture, diluted with water (100 cm^3), was transferred into a separatory funnel and extracted with diethyl ether (3 × 50 cm^3). The combined organic layers were washed with 10% aqueous NaOH (40 cm^3) and water (2 × 50 cm^3), dried with Na_2SO_4 and the solvent was evaporated *in vacuo*. The residue was purified by column chromatography (silica gel, CH_2Cl_2) to afford **15** (1,72 g, 63%) as a colourless oil. δ_H (300 MHz, $CDCl_3$, Me_4Si) 2.35 (2 H, m), 3.70 (2 H, t, *J* 6.2), 3.88 (3 H, s), 4.18 (2 H, t, *J* 5.6), 6.97(2 H, m), 7.46 (1 H, m), 7.81 (1 H, dd, *J* 8.0, 2.0).

10-{1-[3-(2-Carbomethoxyphenyl)]-propyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester sodium perchlorate complex 16

A suspension of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester **6** (0.257 g, 0,5 mmol), alkyl bromide **15** (0.138 g, 0.5 mmol) and Na_2CO_3 (0.212 g, 2 mmol) in anhydrous CH_3CN (20 cm^3) was refluxed for 40 h under magnetic stirring. The mixture was cooled to room temperature, filtered and the filtrate was evaporated. The residue was dissolved in CH_2Cl_2 (50 cm^3), washed with water (2 × 30 cm^3) and then with 30% aqueous $NaClO_4$ (3 × 30 cm^3). The solvent was removed under reduced pressure and the residue purified by column chromatography (silica gel, CH_2Cl_2/MeOH 95 : 5) to afford the complex **16** (0.31 g, 76%) as a white solid. δ_H (300 MHz, $CDCl_3$, Me_4Si) 1.39 (9 H, s), 1.40 (18 H, s), 1.95 (2 H, m), 2.10–3.50 (26 H, m), 3.85 (3 H, s), 4.03 (2 H, t, *J* 9), 6.92 (2 H, m), 7.41 (1 H, m), 7.78 (1 H, dd, *J* 8.0, 1.8); δ_C (75 MHz, $CDCl_3$, Me_4Si) 28.2, 28.3, 28.5, 50.5, 51.5, 52.2, 56.2, 57.0, 67.7, 82.7, 83.1, 94.2, 94.4, 113.7, 120.6, 120.8, 132.0, 133.8, 158.4, 173.0, 174.0; *m*/*z* (FAB+) 729 ([M − ClO_4]$^+$. $C_{37}H_{62}N_4O_9NaClO_4$ requires 828).

10-{1-[3-(2-Carbomethoxyphenyl)]-propyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid 5

A solution of **16** (0.29 g, 0.35 mmol) in methanol (30 cm^3) was stirred overnight at room temperature in the presence of solid KCl (0.38 g, 5 mmol). The suspension was filtered through Celite® and the solvent was removed under reduced pressure. The residue was dissolved in methanol (3 cm^3) and transferred into a liquid–liquid extractor. After addition of water (30 cm^3) the mixture was continuously extracted overnight with *n*-pentane (200 cm^3). The solvent was removed under reduced pressure to afford a residue, which was stirred at reflux for 4 h with NaOH (0.086 g, 2.3 mmol) in 96% EtOH (20 cm^3). Evaporation of the solvent afforded a residue which was dissolved in water (5 cm^3), acidified to pH = 1 with 10% aqueous HCl and evaporated *in vacuo*. The residue was taken up with absolute ethanol (10 cm^3), the solid was removed by filtration and the filtrate was evaporated under reduced pressure to yield **5** (0.17 g, 89%) as a white solid. δ_H (300 MHz, CD_3OD, Me_4Si) 2.27 (2 H, m), 3.13 (4 H, m), 3.50 (18 H, m), 4.18 (2 H, t, *J* 5), 4.25 (2 H, s), 7.05 (2 H, m), 7.50 (1 H, m), 7.82 (1 H, dd, *J* 8, 2); ^{13}C NMR (CD_3OD, 75 MHz) 175.1, 170.1, 136.1, 134.3, 122.8, 115.4, 67.0, 56.2, 54.3, 53.9, 51.6, 51.1, 50.8, 50.0, 25.1; *m*/*z* (FAB+) 525 (M + H^+. $C_{24}H_{36}N_4O_9$ requires 524).

Synthesis of the Gd(III) complexes

The complexes were prepared by mixing stoichiometric amounts of the ligand and of gadolinium chloride and by adjusting the pH to 7 with NaOH. The solutions were kept at room temperature under vigorous stirring for about one hour until the pH stabilized. The compounds were then purified by precipitation by addition of acetone. The absence of free metal ion was assessed by the constant value of the water proton relaxation rate, measured at 25 °C and 20 MHz, after addition of a small excess of the free ligand.

Relaxometric measurements

Water proton relaxivity measurements. The water proton $1/T_1$ longitudinal relaxation rates (20 MHz, 25 °C) were measured with a Stelar Spinmaster Spectrometer (Mede, Pv, Italy) on 0.1–1.5 mM aqueous solutions of the complexes. ^1H spin-lattice

relaxation times T_1 were acquired by the standard inversion-recovery method with typical 90° pulse width of 3.5 μs, 16 experiments of 4 scans. The reproducibility of the T_1 data was ±1%. The temperature was controlled with a Stelar VTC-91 air-flow heater equipped with a copper–constantan thermocouple (uncertainty of 0.1 ± °C). Titration experiments with HSA (Sigma, St. Louis, Mo, USA): different amounts of the protein were added to a dilute (0.1–0.2 mM) aqueous solution of the complexes at pH = 7.2 and the water proton relaxation rate was measured after each addition at 25 °C. The starting pH was adjusted by either HCl or KOH. Moreover, the pH of the solutions was controlled before and after the measurement. The $1/T_1$ nuclear magnetic relaxation dispersion profiles of water protons were measured over a continuum of magnetic field strength from 0.00024 to 0.28 T (corresponding to 0.01–12 MHz proton Larmor frequency) on the fast field-cycling Stelar Spinmaster FFC relaxometer installed at the "Laboratorio Integrato di Metodologie Avanzate", Bioindustry Park del Canavese (Colleretto Giacosa, To, Italy). The relaxometer operates under complete computer control with an absolute uncertainty in the $1/T_1$ values of ±1%. Additional data points at 20 and 90 MHz were recorded on the Stelar Spinmaster and on a JEOL EX-90 spectrometer, respectively. The concentration of the aqueous solutions of the complexes utilized for the measurements was in the range 0.5–1.0 mM.

VT ^{17}O relaxation measurements. Variable-temperature ^{17}O NMR measurements were recorded on a JEOL EX-90 (2.1 T) spectrometer, equipped with a 5 mm probe, by using a D_2O external lock. Experimental settings were: spectral width 10000 Hz, pulse width 7 μs (90°), acquisition time 10 ms, 1000 scans and no sample spinning. Aqueous solutions of the paramagnetic complexes (pH = 6.3) containing 2.6% of ^{17}O isotope (Yeda, Israel) were used. The observed transverse relaxation rates (R_2) were calculated from the signal width at half height.

Theory. The theory behind the analysis of the water ^{1}H and ^{17}O relaxation properties can be found in several recent publications.[1,18,28]

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