$[GdPCP2A(H_2O)_2]^-$: A Paramagnetic Contrast Agent Designed for Improved Applications in Magnetic Resonance Imaging

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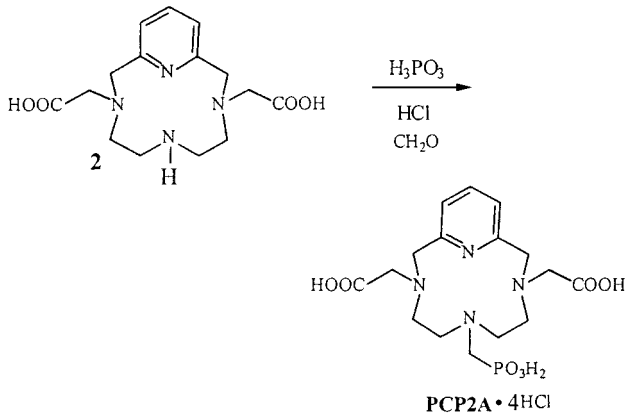
A novel ligand based on a pyridine-containing macrocycle bearing two acetic and one methylenephosphonic arms (PCP2A) has been synthesized. An efficient synthesis of PCP2A is based on the macrocyclization reaction between 2,6-bis(chloromethyl)pyridine and a 1,4,7-triazaheptane derivative bearing a methylenephosphonate group on N-4. The Gd(III) complex of PCP2A displays characteristic properties which make it a very promising contrast agent for improved applications in magnetic resonance imaging. In fact it shows (i) a very high stability constant (log $K_{GdPCP2A} = 23.4$) which should guarantee against the in vivo release of toxic free Gd(III) ions and free ligand molecules and (ii) a relaxivity that is about 2 times higher than the values reported for contrast agents currently used in the clinical practice. Its high relaxivity is the result of the presence of two water molecules in the inner coordination sphere and a significant contribution from water molecule(s) hydrogen bonded to the phosphonate group. Moreover, the inner sphere water molecules are involved in an exchange with the bulk water which is relatively fast. This property is important for the attainment of an even higher relaxivity once the molecular reorientation rate of the $[GdPCP2A(H_2O)_2]^-$ moiety is lengthened by means of conjugation to a macromolecular substrate.

Introduction

Recent years have seen an increased interest in the study of Gd(III) complexes aimed at providing more efficient contrast agents for magnetic resonance imaging (MRI) applications.[1−3] The basic requirements that a Gd(III) complex should have in order to be considered for these applications are high thermodynamic stability, good water solubility, low osmolality, and a marked ability to enhance the relaxation rate of solvent−water protons.[4,5] The latter property is usually evaluated "in vitro" and expressed in terms of relaxivity (r_{1p}), which corresponds to the relaxation enhancement of water protons promoted by the paramagnetic complex at a 1 mM concentration. Established theory provides a basis for understanding the relationships between the structural and dynamic properties of a given Gd(III) complex and its relaxivity. It has been shown that, at the imaging fields, high relaxivities can be obtained in the presence of long reorientational times (τ_r) of the complex allied to long electronic relaxation times (τ_s) and to exchange lifetimes of the coordinated water (τ_M) of the order of a few tenths of nanoseconds.[1,6] The molecular motion of the complex can be slowed by the formation of covalent or noncovalent conjugates between the Gd chelate and a slowly moving substrate such as a protein,[7] micelle,[8] polyamino acid,[9] polysaccharide,[10] or dendrimer.[11] Thus the first step usually deals with the identification of a Gd(III) chelate containing an appropriate synthon which will be successively conjugated to a macromolecular substrate to fully exploit its potential to relax water protons. It is then extremely important to find Gd(III) complexes displaying optimal values for hydration number q, τ_M, and τ_s because the amplification effect associated with the formation of the macromolecular adduct strictly depends on the relaxometric properties of the free complex.[12]

Scheme 1



PCP2A • 4HCl

Along this strategy, herein we report a novel Gd(III) chelate based on a pyridine containing a macrocyclic ligand with two acetic and one methylenephosphonic arms (PCP2A = pyridine containing a triaza macrocyclic

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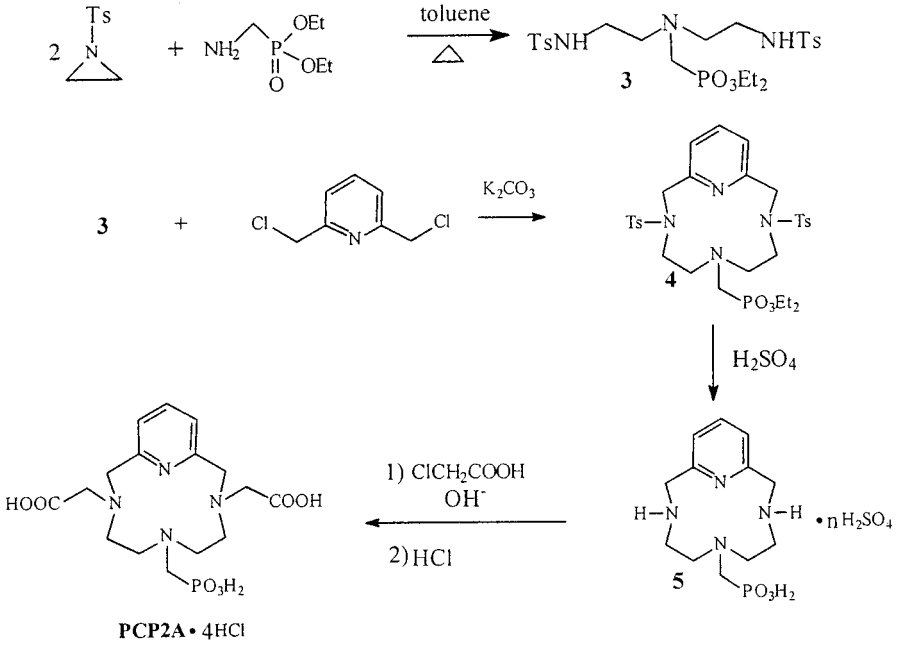
Scheme 2



phosphonic diacetic acid, Scheme 1). The heptadenticity of the ligand should allow the coordination of two water molecules ($q = 2$) in the inner sphere of the metal and consequently a higher relaxivity with respect to the Gd complexes with octadentate ligands ($q = 1$). Moreover, it has been previously shown that Gd complexes with ligands bearing phosphonate groups such as GdDOTP (H_8DOTP = 1,4,7,10-tetrakis(methylenephosphonic acid)-1,4,7,10-tetraazacyclododecane) or GdPCTP-[12] (H_3-PCTP-[12] = pyridine containing a triaza macrocyclic triphosphonic acid) display an additional contribution to the observed relaxivity arising from second-sphere water molecules.[13] This additional contribution is a consequence of the formation of hydrogen bonds between solvent molecules and the oxygens of the methylenephosphonic group. Thus the relaxivity of GdPCP2A should receive a significant contribution also from water molecule(s) in the second coordination sphere. Another interesting property of the Gd(III) complexes containing heptadentate ligands should deal with a relatively fast water-exchange rate, with respect to complexes with octacoordinating ligands. In fact, both heptacoordinated GdDO3A[14] (H_3DO3A = 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid) and GdPCTA[14] show τ_M values which are significantly shorter than those of octacoordinated GdDTPA and GdDOTA.[15,16] In the presence of short water-exchange lifetimes it should be possible to attain high relaxivity values because the molecular reorientational time (τ_r) of the complex will be drastically elongated upon the formation of a macromolecular adduct. One may envisage a synthetic route to obtain macromolecule targeting contrast agents through the introduction of suitable substituents[17−19] on either the acetate or the phosphonic methylenic carbons once it has been proven that the metal complex satisfies the expectations at the basis of its design.

Synthesis of PCP2A

The synthesis of the ligand PCP2A has been pursued through two alternative approaches: (i) by direct introduction of the methylenephosphonate moiety on the previously described[20] 3,6,9,15-tetraazabicyclo[9.3.1]-pentadeca-1(15),11,13-triene-3,9-diacetic acid and (ii) by a macrocyclization reaction between 2,6-bis(chloromethyl)pyridine and a 1,4,7-triazaheptane derivative bearing a methylenephosphonate group on N-4 (Scheme 1).

According to route i, the functionalized macrocycle **2** was reacted with phosphorous acid and paraformaldehyde in the presence of hydrochloric acid.[21] Even changing the molar ratios of the reactants or varying the volume of the reaction mixture, the acidity of the medium, the rate of paraformaldehyde addition, or the overall reaction time does not allow the recovery of the PCP2A ligand in more than 5% yield, after a tedious and time-consuming purification workup. Route ii (Scheme 2) proved to work better, affording the ligand PCP2A in 45% overall yield.

The use of *N*-sulfonylaziridines in the synthesis of diethylenetriamine derivatives is well-documented in the literature.[22−24] Diethyl aminomethylphosphonate was envisaged as a good candidate to induce the ring opening of aziridines, leading to a 1,4,7-triazaheptane derivative bearing a methylenephosphonate group on N-4. Thus, *N*-tosylaziridine was reacted with diethyl aminomethylphosphonate[25] in refluxing toluene affording 1,7-ditosyl-4-diethoxyphosphorylmethyl-1,4,7-triazaheptane (**3**) in 80% yield after crystallization from toluene.

The presence of two electron-withdrawing groups on N-1 and N-7 facilitates the macrocyclization reaction between the derivative **3** and the commercially available bis-electrophilic reagent, 2,6-bis(chloromethyl)pyridine, using the experimental conditions previously reported:[26] i.e. potassium carbonate as a base in anhydrous acetonitrile in heterogeneous conditions. Under these conditions, the macrocycle **4** was obtained in 75% yield after TLC purification. Following the Richman and Atkins protocol,[27] compound **4** was obtained in less than 30% yield.

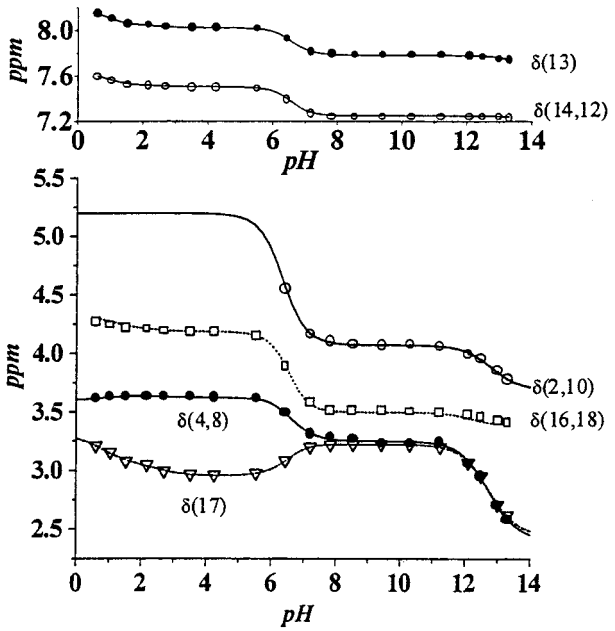
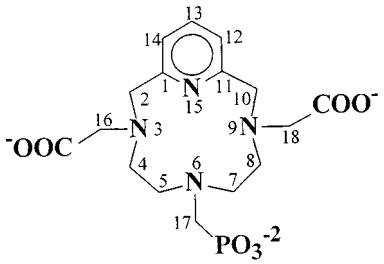


Figure 1. pH dependence of the ^{1}H NMR resonance of the ligand PCP2A recorded at 400 MHz and 25 °C.

Chart 1



Removal of the tosyl groups and hydrolysis of the diethoxyphosphoryl moiety were accomplished by heating **4** with concentrated sulfuric acid.[28] The crude compound **5**·nH$_2$SO$_4$ was alkylated by reaction with chloroacetic acid in the presence of sodium hydroxide at 100 °C and maintaining the pH of the solution at a value of about 10. After cooling to room temperature, the reaction mixture was treated with concentrated hydrochloric acid (pH = 0). The residue obtained after evaporation of the solvent (and further purification) corresponds to the pure ligand PCP2A as established by NMR and MS determinations.

Results and Discussion

Microscopic Protonation Sequence of PCP2A. It is well-established that, with polyamino carboxylate ligands, it is possible to assess the sequence of protonation by NMR spectroscopy.[29] This is due to the fact that protonation of a basic site results in a deshielding of the resonance of the adjacent nonlabile protons in the ^{1}H NMR spectrum. The plot of ^{1}H chemical shifts of the PCP2A ligand, as a function of the pH (Figure 1), shows that the first H$^+$ addition mainly involves the nitrogen opposite the pyridine (N-6, pK_5 = 12.8). In fact, the resonance corresponding to protons 17 (Chart 1) displays the largest downfield shift at pH > 10.5. The substitution of an acetic with a methylenephosphonic arm causes an increase of the basicity of this nitrogen (N-6) with respect to the PCTA-[12][26,30] ligand (pK_1 = 10.9) likely as a consequence of hydrogen bond formation between −HN-6−$^+$ and PO_3^{2-} groups. Since methylenic resonances 4/8, 2/10 display an analogous, although smaller, downfield shift in the same pH range, it is reasonable to suppose that some positive charge is located also on N-3 and N-9. In the pH range 5−8 there is a downfield shift of resonances 4/8, 16/18 coupled to an upfield shift of signal due to protons 17. This behavior is consistent with the occurrence of two protonation steps characterized by similar pK_a values (pK_4 = 6.7; pK_3 = 6.4). The first step affords a bis-protonated species on N-3 and N-9 following a shift of the former proton from N-6 to N-3 or N-9. The second step involves the protonation of one phosphonic oxygen. The overall protonation scheme is confirmed by recording the pH dependence of the ^{31}P NMR resonance (Figure 2). At the more basic pH, the protonation at N-6 causes a large upfield shift of the ^{31}P resonance whose position is (in part) recovered at pH 4−5 following the deprotonation of N-6 and the protonation of one of the oxygens of the phosphonate group. At pH < 4 the simultaneous shift of the ^{31}P and the protons 17 resonances is a consequence of the protonation of the second oxygen on the phosphonic group (pK_2 = 2.42), whereas the last observable protonation (pK_1 = 0.72) involves one acetic arm as shown from the downfield shift of the protons 16/18.

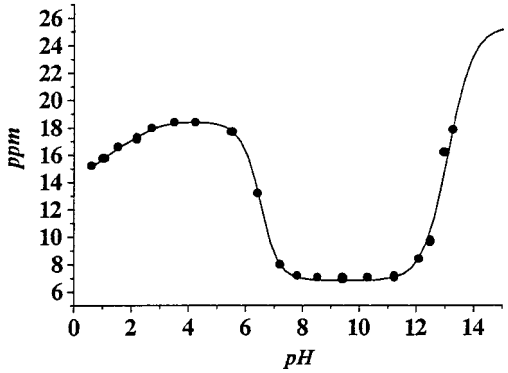


Figure 2. pH dependence of the ^{31}P NMR resonance of the ligand PCP2A recorded at 161.9 MHz and 25 °C.

Synthesis and Relaxometric Characterization of the GdPCP2A Complex. The Gd(III) complex was synthesized by adding stoichiometric amounts of Gd(III) chloride to the aqueous solution of the PCP2A ligand while maintaining the pH of the solution at 7.5 with 1 M NaOH. Formation of the complex has been followed by measuring the solvent proton relaxation rate ($1/T_1$). The presence of an excess of free Gd(III) ions, which yields a noticeable increase of the observed relaxation rate, can be easily removed by forming the insoluble hydroxide at basic pH followed by centrifugation of the resulting suspension.

The relaxivity (r_{1p}) of GdPCP2A, measured at 20 MHz and 298 K, is 8.3 mM^{-1} s^{-1}. This value is significantly higher than those reported for Gd complexes of similar size and based on heptadentated ligands such as GdDO3A and GdPCTA (r_{1p} = 6.0 and 6.9 mM^{-1} s^{-1}, respectively).[4] To characterize the different contributions to the observed relaxivity, the $1/T_1$ NMRD profile over an extended range of frequencies at 25 °C has been measured (Figure 3). Fitting of the NMRD profile was carried out by considering three contributions to the observed relaxivity:[31]

$$R_{1p}^{obs} = R_{1p}^{1st} + R_{1p}^{2nd} + R_{1p}^{os} \quad (1)$$

Where R_{1p}^{1st} represents the contribution from the water molecule(s) directly coordinated to the metal ion, R_{1p}^{2nd} is the contribution from water molecules in the second coordination sphere, and R_{1p}^{os} arises from the water molecules that diffuse in the proximity of the paramagnetic complex. The outer sphere R_{1p}^{os} term is rather constant for different small-sized Gd complexes and at 20 MHz and 25 °C corresponds to ca. 2−2.5 mM^{-1} s^{-1}.[6] As far as R_{1p}^{1st} and R_{1p}^{2nd} are concerned, both contributions can be evaluated on the basis of the Solomon−Bloembergen−Morgan theory:

$$R_{1p} = \frac{q}{55.5(T_{1M} + \tau_M)} \quad (2)$$

$$\frac{1}{T_{1M}} \propto \frac{k}{r^6} f(\tau_c) \quad (3)$$

$$\tau_c^{-1} = \tau_r^{-1} + \tau_s^{-1} + \tau_M^{-1} \quad (4)$$

where q is the number of water molecules in the first or second coordination layers of the Gd ion and T_{1M} is the longitudinal relaxation time of their protons which is determined by the distance from the paramagnetic center, r, and by the shorter of the three correlation times, τ_r being the molecular reorientational time, τ_s the electronic relaxation time, and τ_M the exchange lifetime for the inner and second coordination layer waters, respectively.

Table 1 shows the relaxometric parameters determined by fitting the NMRD profile to eqs 1−4 and to Freed's equation[32] for the outer sphere term. On the basis of the results obtained from the fitting procedure, this Gd complex possesses two water molecules in the inner coordination sphere whose protons are at a distance of 3 Å from the Gd ion, similar to what was previously found for the related compound with three acetate arms (GdPCTA). The hindrance of only one phosphonate group on the coordination cage does not cause a reduction of the number of the metal-bound water molecules as observed in the case of GdPCTP-[12] which contains three phosphonate moieties. The second sphere water molecules contribute ca. 10−15% (at 20 MHz) to the overall relaxivity, but from the available data we cannot determine their distance without fixing their number. In Table 1 we report a distance of 3.8 Å from the Gd(III) center obtained by imposing the presence of only one water molecule bound to the phosphonate group through a hydrogen bond. The other relaxometric parameters determined for GdPCP2A are very similar to those reported for related complexes containing heptadentate ligands[14] and $q = 2$.

Figure 4 shows the r_{1p} values measured at 20 MHz and 25 °C as function of the pH of the solution. The values of the observed relaxivities are constant in the pH range 3.0−9.5. The increase in r_{1p} observed at pH's lower than 3.0 caused by an increased hydration of the resulting species may reflect effects associated with the protonation of the phosphonate group. As previously found[14] for other Gd complexes with $q > 1$ (GdDO3A, GdPCTA) at basic pH (>10), the relaxivity of the GdPCP2A decreases by ca. 1.4 r_{1p} units. This behavior

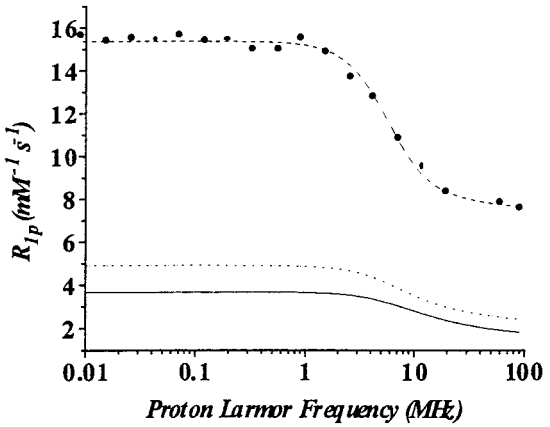


Figure 3. $1/T_1$ NMRD profiles of a 1 mM GdPCP2A solution at pH 7 and 25 °C. The dashed curve through the data points was calculated with the parameters reported in Table 1. The lower solid line represents the outer sphere water contributions, whereas the upper dotted line corresponds to the sum of second and outer sphere contributions.

Table 1

complex	q	Δ^2 $s^{-2}/10^{19}$	τ_v (ps)	τ_r (ps)	τ_M (ns)	ΔH_m (kJ mol^{-1})	ΔH_v (kJ mol^{-1})
GdPC2AP	2	3.5[a]	22[a]	68[a]	60[b]	26[b]	10[b]

[a] Best-fitting parameters obtained from the analysis of the NMRD profile by considering two inner sphere water molecules (q) whose protons are at an average metal distance of 3 Å and one second sphere water molecule whose protons are at a metal distance of 3.9 Å. [b] ^{17}O NMR best-fitting parameters obtained from the analysis of the temperature dependence of R_{2p} for a 2.7 mM solution of GdPCP2A by using a Gd−^{17}O scalar coupling constant of -3.8×10^6 rad s^{-1} and a Gd−^{17}O distance of 2.5 Å.

can be attributed to the formation of ternary complexes with dissolved carbonate ions. This hypothesis has been confirmed by measuring the pH dependence of the relaxivity in a closed cell under N_2 atmosphere and by using CO_2-free KOH (Figure 5). Under these conditions r_{1p} remains rather constant up to pH values of 11.5. The decrease of relaxivity observed at higher pH values is a consequence of the partial displacement of the coordinated water molecules by OH^-.

In order to determine the exchange lifetime of the coordinated water molecules, transverse ^{17}O NMR relaxation times of an aqueous solution of GdPCP2A as a function of the temperature have been measured. In fact, it is well-established that water $^{17}O-R_{2p}$ data fitted to the values calculated on the basis of Swift−Connick equations yield good estimates of this exchange lifetime (τ_M).[16] Figure 6 shows the $^{17}O-R_{2p}$ profile of a 2.7 mM GdPCP2A solution recorded at 9.4 T and pH 7. It shows a maximum of R_{2p} at low temperature (ca. 280 K) typical of a Gd complex with short τ_M values. At ambient temperature the exchange lifetime obtained from the analysis of the variable temperature profile (Table 1) is ca. 60 ns, considering two water molecules in the inner coordination sphere. It is interesting to note that the substitution of an acetate arm with a phosphonate one on the pyridine-containing macrocycle causes only a slight effect on the exchange lifetime τ_M. In fact, the GdPCTA-[12] complex shows a τ_M value of 70 ns.[14] On the other hand in the three-phosphonate derivative (GdPCTP-[12]) only one, fast exchanging water molecule has been found. Likely the short τ_M value (6 ns)[14] found in GdPCTP-[12] may be accounted for in terms of the

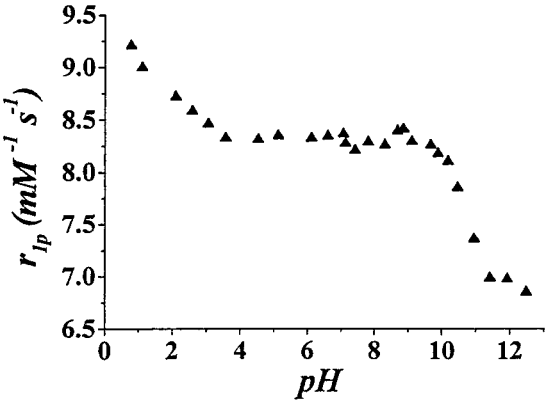


Figure 4. pH dependence of the longitudinal water proton relaxivity (r_{1p}) for a 1 mM solution (20 MHz, 25 °C) of GdPCP2A.

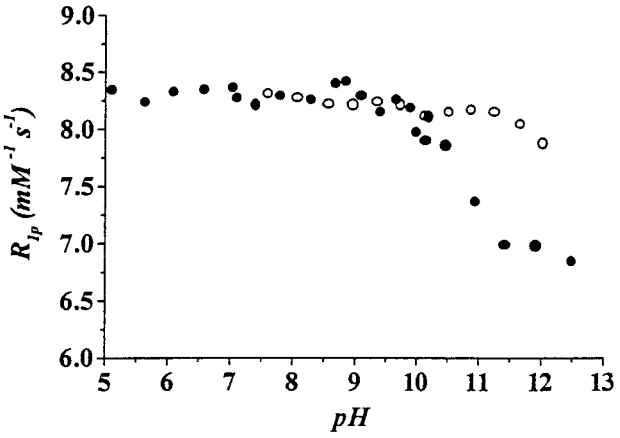


Figure 5. pH dependence of the longitudinal water proton relaxivity (r_{1p}) for a 1 mM solution of GdPCP2A with (●) or without (○) carbonate.

occurrence of an associative mechanism for the exchange of the coordinated water in this octacoordinated complex. Thus, upon replacing all of the acetate arms with the isosteric methylenephosphonic substituents, there is a decrease in the hydration state of the lanthanide ion analogous to what was observed on going from the DOTA to DOTP complexes.

The exchange lifetime τ_M of the GdPCP2A appears short enough to avoid any quenching of the relaxivity enhancement expected once this moiety would be endowed with a long reorientation time (τ_r). To support the latter statement, a NMRD profile has been calculated using the parameters reported in Table 1 but whose τ_r value has been set equal to 3×10^{-8} s (the reorientation time commonly assumed for human serum albumin). As shown in Figure 7, the calculated profile shows a relaxivity peak of ca. 120 mM^{-1} s^{-1} at the imaging fields. For comparison, in the same figure, also reported is an analogous NMRD profile calculated for an analogously immobilized GdDOTA system which shows a maximum relaxivity of 45 mM^{-1} s^{-1}. Values of such magnitude have already been experimentally obtained in several systems based on macromolecular conjugates of DOTA-like systems.[17−19] The "quenching" of the expected relaxation enhancement often shown upon binding to a DOTA-like Gd complex (slowly moving system) has been associated with the occurrence of a long residence lifetime of the coordinated water (i.e. $T_{1M} < \tau_M$ in eq 2).

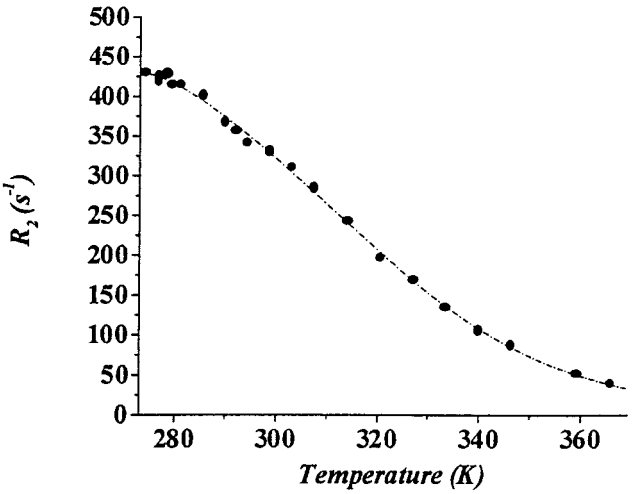


Figure 6. Temperature dependence of the transverse water ^{17}O relaxation rate at 9.4 T and pH 7 for a 2.7 mM solution of GdPCP2A. The solid curve through the data points was calculated with the parameters reported in Table 1.

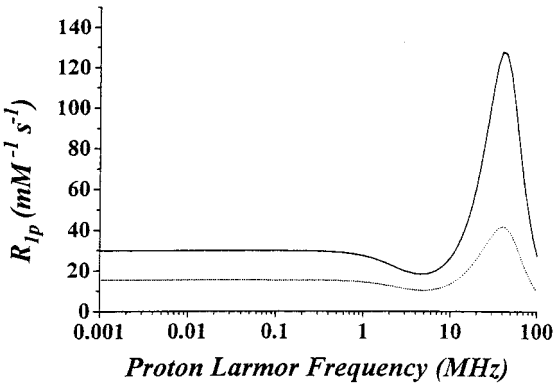


Figure 7. Curves represent two NMRD profiles calculated for immobilized GdDOTA (dotted line) and GdPC2AP systems. τ_r has been set equal to 3×10^{-8} s. The other parameters used for GdDOTA were taken from ref 4, whereas parameters for GdPCP2A were taken from Table 1.

In summary, the presence of two water molecules in the inner coordination sphere, which exchange faster than in systems with $q = 1$, coupled to a sufficiently long electronic relaxation time make GdPCP2A a very promising candidate for the attainment of very high relaxivities upon conjugation to macromolecular substrates.

Determination of the Thermodynamic Stability Constant of the Gd Complex. As anticipated in the Introduction, one of the important requisites that a Gd complex must have in order to be considered for in vivo MRI applications is represented by a high stability constant. In fact this is the requisite which is expected to limit toxicity problems related to the release of free Gd(III) ions and free ligand molecules as well. Determination of the GdPCP2A thermodynamic stability constant has been carried out using an NMR method.[33−35] This method is based on the measurement of the relaxation rate (R_1^{obs}) of GdL aqueous solutions in the presence of variable amounts of a ligand L′ which is able to form a complex of similar stability but endowed with a different relaxivity. The observed relaxation rates of these solutions are then functions of the speciation of Gd(III) complexes. The concentration of the various

species present in these solutions depends on the following equilibria:

$$Gd + L \rightleftarrows GdL$$

$$Gd + L' \rightleftarrows GdL'$$

$$GdL + H \rightleftarrows GdHL$$

$$GdL' + H \rightleftarrows GdHL'$$

$$H_{n-1}L + H \rightleftarrows H_nL$$

$$H_{n-1}L' + H \rightleftarrows H_nL'$$

each equilibrium being characterized by the relative constants K_{GdL}, K_{GdHL}, $K_{GdL'}$, $K_{GdHL'}$, K_{H_nL}, K_{HL}, $K_{H_nL'}$, $K_{HL'}$, etc. Working at pH $\approx$ 8 it has been possible to neglect the formation of the species GdHL and GdHL′. In fact, protonation of Gd complexes with heptadentate or octadentate polyamino carboxylate ligands occurs only at pH < 4. The pH dependence of the relaxivity of the GdPCP2A complex gives support to this approximation. Figure 4 shows that the formation of the protonated complex which is characterized by higher relaxivity occurs only at pH < 3.

On the basis of these assumptions, the system is simply described by the following set of equations:

$$C_{TL} = [GdL] + [L]\alpha_L \quad (5)$$

$$C_{TL'} = [GdL']^{\cdot} + [L']\alpha_{L'} \quad (6)$$

$$C_{TGd} = [GdL] + [GdL'] \quad (7)$$

$$R_1^{obs} = r_{1pGdL}[GdL] \times 10^3 + R_{1w} \quad (8)$$

$$K_{GdL} = \frac{[GdL]}{[Gd][L]};\ K_{GdL'} = \frac{[GdL']}{[Gd][L']} \quad (9, 10)$$

where C_{TL}, $C_{TL'}$ and C_{TGd} represent the total concentrations of the ligand L, ligand L′, and metal ion, respectively; α_L and $\alpha_{L'}$ are given by:

$$\alpha_L = 1 + K_{HL}[H] + K_{HL}K_{H^2L}[H]^2 + K_{HL}K_{H^2L}K_{H^3L}[H]^3... \quad (11)$$

$$\alpha_{L'} = 1 + K_{HL'}[H] + K_{HL'}K_{H^2L'}[H]^2 + K_{HL'}K_{H^2L'}K_{H^3L'}[H]^3... \quad (12)$$

In the calculation of α_L and $\alpha_{L'}$ the PCP2A protonation constants determined by NMR were used. r_{1pGdL} and $r_{1pGdL'}$ are the millimolar relaxivities of the two Gd complexes (mmol^{-1} s^{-1}) and R_{1w} is the diamagnetic contribution to the observed relaxivity (0.38 s^{-1} at 20 MHz and 25 °C). By working out this set of equations the final expression of the unknown stability constant is:

$$K_L = [K_L\alpha_L(C_{TL'}r_{1pGdL} \times 10^3 - C_{TL'}r_{1pGdL'} \times 10^3 + R_1^{obs} - r_{1pGdL}C_{TM} \times 10^3 - 0.38)(C_{TM}r_{1pML'} \times 10^3 + R_1^{obs} - 0.38)]/[\alpha_{L'}(R_1^{obs} - r_{1pGdL} \times 10^3C_{TM} - 0.38)(-C_{TL}r_{1pGdL} \times 10^3 + C_{TL}r_{1pML'} \times 10^3 - r_{1pML'} \times 10^3C_{TM} + R_1^{obs} - 0.38)] \quad (13)$$

DTPA (log K_{GdDTPA} = 22.46, r_{1p} = 4.7 mmol^{-1} s^{-1}at 25 °C and 20 MHz) was particularly suitable as a competitive ligand for the determination of the GdPCP2A stability constant. The solutions containing variable GdPCP2A/DTPA ratios were allowed to equilibrate for 4−5 days at 50 °C before the relaxivity measurements were carried out. Insertion of the observed R_1^{obs} data into eq 13 yielded a log $K_{GdPCP2A}$ = 23.4. The high stability constant value found for this complex with respect to other heptadentate ligands such as GdDO3A[4] or GdPCTA[26] (both showing a log K_{GdL} = 21) could be ascribed to the presence of the methylenephosphonic arm on the pyridine-containing tetraza macrocycle. This observation is confirmed by an analogous difference found between the stability constant of GdDOTP and GdDOTA.

Another "in vitro" experiment which may anticipate possible toxicological problems associated with the release of Gd(III) ions is provided by the measurement of the relaxivity of GdPCP2A in a 3 mM phosphate buffer solution (pH = 7; 25 °C). In fact, the decrease in the relaxivity, observed when a Gd complex is dissolved in a phosphate buffer with respect the pure water solution, may be accounted for the precipitation of $GdPO_4$. The relaxivity for the GdPCP2A does not change in the presence of phosphate ions, after 3 days; this result anticipates its good stability in serum. On the contrary, as shown by Rongved et al.,[36] the relaxivity of the less stable GdEDTA measured under the same conditions is significantly lower with respect to that observed in the absence of the competitive phosphate ions.

Conclusions

GdPCP2A possesses a number of favorable properties which make it a very promising complex in the development of the field of contrast agents for MRI. First of all, it displays one of the highest stability constants until now reported for a Gd complex. This high stability is strongly suggestive that no in vivo release of toxic free Gd(III) ions and free ligand will occur. Next, its relaxivity is 2 times higher than those shown by the contrast agents currently used in clinical practice (Figure 8). This remarkable capability to relax water protons is the result of the collective effects underlying the design of GdPCP2A, namely (i) the presence of two water molecules in the inner coordination sphere; (ii) a nonnegligible contribution from water molecule(s) in the second coordination sphere thanks to the ability of the phosphonate group to form hydrogen bonds; and (iii) the outer sphere contribution. Moreover GdPCP2A displays a fast exchange of the coordinated water molecules, and this property strongly suggests its use as a paramagnetic synthon for conjugation to a macromolecular substrate in order to obtain high relaxivities. There is a clear need of systems endowed with powerful relaxo-

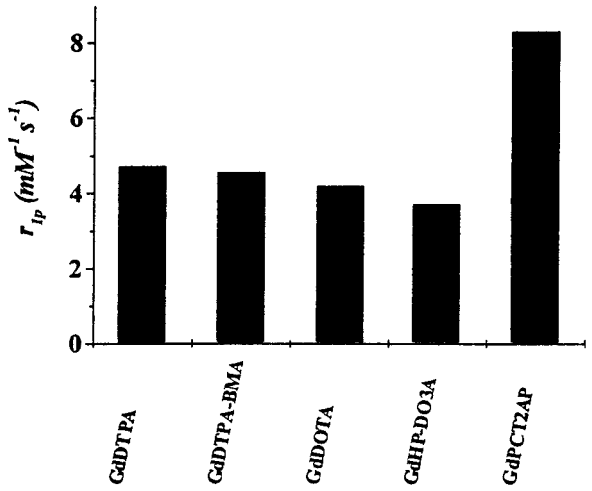


Figure 8. Comparison among the relaxivity (25 °C, 20 MHz) shown by commercial Gd-based contrast agents (GdDTPA: Magnevist, Shering; GdDOTA: Dotarem, Guerbet; GdDTPA-BMA: Omniscan, Nycomed-Amersham; GdHP-DO3A: ProHance, Bracco) and GdPCP2A.

metric characteristics to be used as tracers to visualize small tumor lesions[37] and as receptors in neo-angiogenesis[38] processes and, finally, to report successful transfection in gene therapy.[39]

Experimental Section

^{1}H and ^{13}C NMR spectra were obtained on Brucker AC 200 (200 and 50.2 MHz, respectively) and JEOL EX-400 (400 and 100.4 MHz) spectrometers. ^{31}P NMR spectra were run on Varian XL200 (81.0 MHz) and JEOL EX-400 (161.9 MHz) spectrometers. The NMR pH titration was made up in D_2O (99.8%), and the pD was adjusted with DCl or CO_2-free NaOD (Sigma). Mass spectra were recorded with a VG 7070 EQ spectrometer (at 70 eV; *m*-nitrobenzyl alcohol as matrix in the FAB^+ ionization). Elemental analyses were performed with a Perkin-Elmer 240 apparatus. Melting points were determined with a Büchi 520 apparatus and are uncorrected.

The longitudinal water proton relaxation rate was measured on the Stelar Spinmaster spectrometer (Stelar, Mede (PV), Italy) operating at 20 MHz, by means of the standard inversion−recovery technique (16 experiments, 2 scans). A typical 90° pulse width was 3.5 μs and the reproducibility of the T_1 data was ±0.5%. The temperature was controlled with a Stelar VTC-91 air-flow heater equipped with a copper−constantan thermocouple (uncertainty of ±0.1 °C). The NMRD profile was measured on a continuum of magnetic field strength from 0.00024 to 0.24 T (corresponding to 0.01−10 MHz proton Larmor frequency) on a Spinmaster FFC, fast field cycling NMR relaxometer (Stelar, Mede (PV), Italy) installed at the LIMA Laboratory (Bioindustry Park, Ivrea (TO), Italy). Variable-temperature ^{17}O NMR measurements were recorded on a JEOL EX-400 (9.4 T) spectrometer, equipped with a 5-mm probe, using D_2O as external lock. Experimental settings were: spectral width 10880 Hz, pulse width 7 μs, acquisition time 10 ms, 256 scans, no sample spinning. Solutions containing 2.6% of ^{17}O isotope (Yeda, Israel) were used. The observed transverse relaxation rates (R^{O}_{2obs}) were calculated from the signal width at half-height.

All chemical were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO).

4-Diethoxyphosphorylmethyl-1,7-ditosyl-1,4,7-triazaheptane (3). A solution of diethyl aminomethylphosphonate (6.80 g, 40.6 mmol) and *N*-tosylaziridine (20.32 g, 103.1 mmol) in dry toluene (200 mL) was refluxed for 6 h while monitoring by TLC on silica gel (ethyl acetate/methanol 9:1). The solvent was evaporated under vacuum and the residue was crystallized from toluene to afford pure **3** (mp 125 °C, 18.25 g, 32.5 mmol 80% yield). ^{1}H NMR ($CDCl_3$): δ 7.79 (AA'BB' system, 4H), 7.31 (AA'BB' system, 4H), 6.03 (m, 2H, exchangeable with D_2O), 4.16 (dq, 4H, ^3J = 7.3 Hz, $^3J_{H-P}$ = 8.1 Hz), 2.93 (m, 4H), 2.81 (d, 2H, $^2J_{H-P}$ = 9.9 Hz), 2.68 (m, 4H), 2.42 (s, 6H), 1.34 (t, 6H, ^3J = 7.3 Hz). ^{13}C NMR ($CDCl_3$): δ 142.9 (C), 137.0 (C), 129.5 (CH), 127.0 (CH), 62.3 (d, CH_2, $^2J_{C-P}$ = 6.9 Hz), 55.3 (d, CH_2, $^3J_{C-P}$ = 6.3 Hz), 49.6 (d, CH_2, $^1J_{C-P}$ = 165.1 Hz), 40.9 (CH_2), 21.3 (CH_3), 16.3 (d, CH_3, $^3J_{C-P}$ = 4.8 Hz). MS (EI): *m*/*z* 562 (M). Anal. ($C_{23}H_{36}N_3O_7PS_2$) C: calcd, 49.18; found, 49.05. H: calcd, 6.46; found, 6.56. N: calcd, 7.48; found, 7.52.

6-Diethoxyphosphorylmethyl-3,9-ditosyl-3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene (4). To a solution of 2,6-bis(chloromethyl)pyridine (5.14 g, 29.2 mmol), in anhydrous acetonitrile (150 mL), compound **3** (14.89 g, 26.5 mmol) and potassium carbonate (14.00 g, 101.3 mmol) were added and the mixture was refluxed under vigorous stirring and nitrogen atmosphere. After 16 h (TLC, ethyl acetate as eluant) the solvent was evaporated and the residue dissolved in methylene dichloride (100 mL). The mixture was filtered through Celite; the organic layer was washed with aqueous 2 N sodium hydroxide (40 mL), water (40 mL) and dried over anhydrous sodium sulfate. After evaporation of the solvent, the residue (17.6 g) was purified by flash silica gel chromatography [ethyl acetate → ethyl acetate/methanol (9:1) as eluant] to give pure compound **4** (14.52 g, 21.8 mmol, 75%) as a white foam after high vacuum removal of the volatiles. ^{1}H NMR ($CDCl_3$): δ 7.72 (AA'BB' system and t, 5H, ^3J = 8.2 Hz), 7.33 (AA'BB' system and d, 6H, ^3J = 8.2 Hz), 4.44 (s, 4H), 4.11 (dq, 4H, ^3J = 7.3 Hz, $^3J_{H-P}$ = 8.0 Hz), 3.20 (m, 4H), 2.84 (d, 2H, $^2J_{H-P}$ = 9.8 Hz), 2.54 (m, 4H), 2.45 (s, 6H), 1.33 (t, 6H, ^3J = 7.3 Hz). ^{13}C NMR ($CDCl_3$): δ 154.8 (C), 143.3 (C), 138.4 (C), 135.6 (CH), 129.9 (CH), 127.3 (CH), 123,7 (CH), 61.8 (d, CH_2, $^2J_{C-P}$ = 6.9 Hz), 54.3 (CH_2), 52.8 (d, CH_2, $^3J_{C-P}$ = 5.3 Hz), 51.0 (d, CH_2, $^1J_{C-P}$ = 162.0 Hz), 44.5 (CH_2), 21.3 (CH_3), 16.3 (d, CH_3, $^3J_{C-P}$ = 5.4 Hz). MS (EI): *m*/*z* 665 (M). Anal. ($C_{30}H_{41}N_4O_7PS_2$) C: calcd, 54.20; found, 54.15. H: calcd, 6.22; found, 6.30. N: calcd, 8.43; found, 8.49.

3,9-Bis(carboxymethyl)-6-dihydroxyphosphorylmethyl-3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene·4HCl (PCP2A). Compound **4** (2.98 g, 4.5 mmol) was dissolved in concentrated sulfuric acid (15 mL) at 80 °C. The mixture was heated to 200 °C in 9 min and left at this temperature for 1 more min. The resulting brown solution was cooled to 0 °C and diethyl ether (60 mL) was added. The precipitate was filtered and washed with diethyl ether (4 × 20 mL). After high vacuum desiccation, compound **5**·$n$$H_2SO_4$ (1.45 g) was submitted to the next step without further purification. ^{1}H NMR spectrum, registered in D_2O, revealed the complete absence of the tosyl group resonances. To a solution of compound **5**·$n$$H_2SO_4$ (1.45 g) and chloroacetic acid (1.42 g, 15 mmol) in water (25 mL) aqueous 15% sodium hydroxide (14 mL) and 2 drops of phenolphthalein were added. The reaction mixture was heated at 100 °C for 24 h, maintaining a pH = 10 (persistent pink color of phenolphthalein) with sodium hydroxide. The solution was then cooled to room temperature and acidified with 4 N hydrochloric acid until pH = 0. The solvent was evaporated and the residue purified by column chromatography on XAD 1600 [water → water/methanol (7:3)]. PCP2A·4HCl was obtained as a white solid after evaporation of the solvent [mp = 220 °C dec, 1.52 g, 2.7 mmol, 60% overall yields from **4**]. ^{1}H NMR (D_2O): δ 7.78 (t, 1H, ^3J = 8.0 Hz), 7.26 (d, 2H, ^3J = 8.0 Hz), 4.64 (s, 4H), 3.90 (s, 4H), 3.36 (m, 4H), 2.82 (m, 4H), 2.73 (m, 4H, $^2J_{H-P}$ = 10.0 Hz). ^{13}C NMR (D_2O): δ 172.9 (C), 153.1 (C), 144.3 (CH), 125.4 (CH), 61.4 (CH_2), 59.1 (CH_2), 55.7 (CH_2), 54.5 (CH_2), 52.5 (d, CH_2, $^1J_{C-P}$ = 147.6 Hz). ^{31}P NMR (D_2O): δ 22.6. MS (FAB^+): *m*/*z* 439 (M + Na)$^+$, 417 (M + H)$^+$. Anal. ($C_{16}H_{29}N_4O_7Cl_4P$) C: calcd, 34.18; found, 34.31. H: calcd, 5.20; found, 5.11. N: calcd, 9.96; found, 9.89.

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References

(1) Aime, S.; Botta, M.; Fasano, M.; Terreno, E. Lanthanide(III) chelates for NMR applications. *Chem. Soc. Rev.* **1998**, *27*, 19−29.

(2) *Magnetic Resonance in Medicine*; Rinck, P. A., Ed.; Blackwell Scientific Publications: Oxford, 1993.

(3) Kumar, K.; Tweedle, M. F. Macrocyclic polyaminocarboxylate complexes of lanthanides as magnetic resonance imaging contrast agents. *Pure Appl. Chem.* **1993**, *65*, 515−520.

(4) Caravan, P.; Ellison, J. J.; McMurry, T. J.; Lauffer, R. B. Gadolinium(III) chelates as MRI contrast agents: Structure, dynamic and applications. *Chem. Rev.* **1999**, *99*, 2293−2352.

(5) Peters, J. A.; Huskens, J.; Raber, D. J. Lanthanide induced shifts and relaxation rate enhancements. *Prog. NMR Spectrosc.* **1996**, *28*, 283−350.

(6) Lauffer, R. B. Paramagnetic Metal Complexes as Water Proton Relaxation Agents for NMR Imaging: Theory and Design. *Chem. Rev.* **1987**, *87*, 901−927.

(7) Brasch, R. C. Rationale and applications for macromolecular Gd-based contrast agents. *Magn. Reson. Med.* **1991**, *22*, 282−287.

(8) Aime, S.; Barbero, L.; Botta, M. Trends in NMR studies of paramagnetic Gd(III) complexes as potential contrast agents in MRI. *Magn. Reson. Imaging* **1991**, *9*, 843−847.

(9) Vexler, V. S.; Clément, O.; Schmitt-Willich, H.; Brasch, R. C. J. Effect of varying the molecular weight of the MR contrast agent Gd-DTPA-polylysine on blood pharmacokinetics and enhancement patterns. *Magn. Reson. Imaging* **1994**, *4*, 381−388.

(10) Rebizak, R.; Schaefer, M.; Dellacherie. Polymeric conjugates of Gd3+-dietylenetriaminepentaacetic acid and dextran, Synthesis, characterization and paramagnetic properties. *Bioconjugate Chem.* **1997**, *8*, 605−610.

(11) Wiener, E. C.; Brechbiel, M. W.; Brothers, H.; Magin, R. L.; Gansow, O. A.; Tomalia, D. A.; Lauterbur, P. C. Dendrimer-based metal chelates: anew class of magnetic resonance imaging contrast agents. *Magn Reson. Med.* **1994**, *31*, 1−8.

(12) Aime, S.; Botta, M.; Fasano, M.; Geninatti, Crich S.; Terreno, E. ^{1}H and ^{17}O NMR relaxometric investigations of paramagnetic contrast agents for MRI. Clues for higher relaxivities. *Coord. Chem. Rev.* **1999**, *185*−*186*, 321−333.

(13) Aime, S.; Botta, M.; Geninatti, Crich S.; Giovenzana, G. B.; Pagliarin, R.; Piccinini, M.; Sisti, M.; Terreno, E. Towards MRI contrast agents of improved efficacy. NMR relaxometric investigations of the binding interactions to HSA of a novel hepadentate macrocyclic triphosphonate Gd(III)-complex. *J. Biol. Inorg. Chem.* **1997**, *2*, 470−479.

(14) Aime, S.; Botta, M.; Geninatti, Crich S.; Giovenzana, G. B.; Pagliarin, R.; Sisti, M.; Terreno, E. NMR relaxometric studies of Gd(III) complexes with heptadentate macrocyclic ligands. *Magn. Reson. Chem.* **1998**, *36*, S200−S208.

(15) Mickei, K.; Helm, L.; Brucher, E.; Merbach, A. ^{17}O NMR study of water exchange on $[Gd(DTPA)(H2O)]^{2-}$ and $[Gd(DOTA)(H2O)]^{-}$ related to NMR imaging. *Inorg. Chem.* **1993**, *32*, 3844−3850.

(16) Powell, D. H.; Ni Dhubhghaill, O. M.; Pubanz, D.; Helm, L.; Lebedev, Y. S.; Schlaepfer, W.; Merbach, A. E. Structural and dynamic parameters obtained from ^{17}O NMR, EPR, and NMRD studies of monomeric and dimeric Gd^{3+} complexes of interest in magnetic resonance imaging: an integrated and theoretically self-consistent approach. *J. Am. Chem. Soc.* **1996**, *118*, 9333−9346.

(17) Lauffer, R. B.; Parmelee, D. J.; Dunham, S. U.; Ouellet, H. S.; Dolan, R. P.; Witte, S.; McMurry, T. J.; Walovitch, R. C. MS-325: albumin-targeted contrast agent for MR angiography. *Radiology* **1998**, *207*, 529−538.

(18) Toth, E.; Connac, F.; Helm, L.; Adzamli, K.; Merbach, A. E. Direct assessment of water exchange on a Gd(III) chelate bound to a protein. *J. Biol. Inorg. Chem.* **1998**, *3*, 606−613.

(19) Aime, S.; Botta, M.; Fasano, M.; Geninatti, Crich S.; Terreno, E. Gd(III) complexes as contrast agents for magnetic resonance imaging,: a proton relaxation enhancement study of the interaction with human serum albumin. *J. Biol. Inorg. Chem.* **1996**, *1*, 312−319.

(20) Kim, W. D.; Hrncir, D. C.; Kiefer, G. E.; Sherry, A. D. Synthesis, crystal structure and potentiometry of pyridine-containing tetraazamacrocyclic ligands with acetate pendant arms. *Inorg. Chem.* **1995**, *34*, 2225−2232.

(21) Lazar, I.; Hrncir, D. C.; Kim, W. D.; Kiefer, G. E.; Sherry, A. D. Optimized synthesis, structure, and solution dynamics of 1,4,7,-10-tetraazacyclododecane-1,4,7,10-tetrakis(methylenephosphonic acid) (H_8DOTP). *Inorg. Chem.* **1992**, *31*, 4422−4424.

(22) Cernerud, M.; Skrinning, A.; Bergere, I.; Moberg, C. Bis-(ethylsulfonamide)amines via nucleophilic ring-opening of chiral aziridines. Application to Ti-mediated addition of diethylzinc to benzaldehyde. *Tetrahedron: Asymmetry* **1997**, *20*, 3437−3441.

(23) Espana, E. G.; Micheloni, M.; Paoletti, P. Synthesis of the new bis-azamacrocycle 1,2-bis(1,4,8,12-tetraazacyclotetradecanyl)-ethane and of its dicopper(II)complex. *Gazz. Chim. Ital.* **1985**, *115*, 399−340.

(24) Hata, K.; Doh, M. K.; Kashiwabara, K.; Fujita, J. Preparation and absorption and circular dichroism spectra of cobalt(III) complexes with n,n,n',n'-tetrakis(2-aminoethyl)-1,2-ethanediamine, -1,3-propanediamine, -1,4-butanediamine, and -(R,R)- and -(R,S)-pentanediamine. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 190−195. Gauss, W.; Moser, P.; Schwarzenbach, G.; N, N,N',N'-Tetrakis-(*β*-aminoäthyl)-äthylendiamin. *Helv. Chim. Acta* **1952**, *35*, 2359−2363.

(25) Ferrari, M.; Jommi, G.; Miglierini, G.; Pagliarin, R.; Sisti, M. Asymmetric synthesis of diethyl α-amino-α-alkyl-phosphonates by alkylation of the chiral Schiff base derived from (+)-ketopinic acid and diethylaminomethyl phosphonate. *Synth. Commun.* **1992**, *22*, 107−123.

(26) Aime, S.; Botta, M.; Geninatti Crich, S.; Giovenzana, G. B.; Jommi, G.; Pagliarin, R.; Sisti, M. Synthesis and NMR studies of three pyridine-containing triaza macrocyclic triacetate ligands and their complexes with lanthanide ions. *Inorg. Chem.* **1997**, *36*, 2992−3000.

(27) Richman, J. E.; Atkins, T. J. Nitrogen analogues of crown ethers. *J. Am. Chem. Soc.* **1974**, *96*, 2268.

(28) Lazar, I. Rapid and high yield detosylation of linear and macrocyclic *p*-toluenesulfonamides. *Synth. Commun.* **1995**, *25*, 3181−3185

(29) Sudmeier, J. L.; Reilley. Nuclear magnetic resonance studies of protonation of some polyaminocarboxylate compounds containig asymmetric carbon atoms. *Anal. Chem.* **1964**, *36*, 1707−1712.

(30) Geraldes, C. F. G. C.; Sherry, A. D.; Cacheris, W. P. Synthesis, protonation sequence and NMR studies of polyazamacrocyclic methylenephosphonates. *Inorg. Chem.* **1989**, *28*, 3336−3341.

(31) Banci, L.; Bertini, I.; Luchinat, C. *Nuclear and electron relaxation*; VCH: Weinheim, 1991.

(32) Freed, J. H. Dynamic effects of pair correlation functions on spin relaxation by translational diffusion in liquids. II. Finite jumps and independent T_1 processes. *J. Chem. Phys.* **1978**, *68*, 4034−4037.

(33) Cortes, S.; Brucher, E.; Geraldes, C. F. G. C.; Sherry, A. D. Potentiometry and NMR Studies of 1,5,9,-triazacyclododecane-N,N',N''-triacetic acid and its metal ion complexes. *Inorg. Chem.* **1990**, *29*, 5−9.

(34) Brucher, E.; Cortes, S.; Chavez, F.; Sherry, A. D. Synthesis, Equilibrium and kinetic properties of the Gadolinium(III) complexes of three triazacyclodecanetriacetate ligands. *Inorg. Chem.* **1991**, *30*, 2092−2097.

(35) Sherry, A. D.; Ren, J.; Huskens, Brucher E.; Toth, E.; Geraldes, C. F. C. G.; Castro, M. M. C. A.; Chacheris, W. P. Characterization of lanthanide(III) DOTP complexes: Thermodynamics, protonation and coordination to alkali metal ions. *Inorg. Chem.* **1996**, *35*, 4604−4612.

(36) Rongved, P.; Fritzell, T. H.; Strande, P.; Klaveness, J. Polysaccharides as carriers for magnetic resonance imaging contrast agents: synthesis and stability of a new amino acid linker derivative. *Carbohydr. Res.* **1996**, *287*, 77−89.

(37) Wiener, E. C.; Konda, S.; Shadron, A.; Brechbiel, M.; Gansow, O. Targeting Dendrimer-chelates to tumors and tumor cells expressing the high affinity folate receptor. *Invest. Radiol.* **1997**, *32*, 748−754.

(38) Lemieux, G. A.; Yarema, K. J.; Jacobs, C. L.; Bertozzi, C. R. Exploiting differences in sialoside expression for selective targeting of MRI contrast agents. *J. Am. Chem. Soc.* **1999**, *121*, 4278−4279.

(39) Louie, A. Y.; Huber, M. M.; Ahrens, E. T.; Rothbacher, U.; Moats, R.; Jacobs, R. E.; Fraser, S. E.; Meade, T. J. In vivo visualisation of gene expression using magnetic resonance imaging. *Nat. Biotechnol.* **2000**, *18*, 321−325.

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