Non-covalent Conjugates between Cationic Polyamino Acids and Gd^{III} Chelates: A Route for Seeking Accumulation of MRI-Contrast Agents at Tumor Targeting Sites

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Abstract: Three novel Gd chelates containing on their external surface pendant phosphonate and carboxylate groups, which promote the interaction with the positively charged groups of polyornithine and polyarginine, have been synthesized. Their solution structures have been assessed on the basis of ^{1}H- and ^{31}P-NMR spectra of the Eu and Yb analogues. A thorough investigation of the relaxometric (^{1}H and ^{17}O) properties of the Gd chelates has been carried out and the observed relaxivities have been accounted for the sum of three contributions arising from water molecules in the first, second, and outer coordination layers, respectively. It has been found that the occurrence of a tight second coordination coating renders the dissociation of the water molecule directly coordinated to the Gd ion more difficult. The binding interactions between the negatively charged Gd chelates and the positively charged groups of polyornithine (ca. 140 residues) and polyarginine (ca. 204 residues) have been evaluated by means of the proton relaxation enhancement (PRE) method. Although the binding interaction decreases markedly in the presence of competitive anions in the solution medium, the affinity is strong enough that in blood serum it is possible to meet the conditions where most of the chelate is bound to the polyamino acid substrate. On this basis one may envisage a novel route for a MRI location of tumors as it is known that positively charged polyamino acids selectively bind to tumors having a greater negative charge than non-tumor cells.

Keywords: lanthanides · MRI contrast agents · polycations

Introduction

In the last decade the growing impact of magnetic resonance imaging (MRI) as one of the most powerful tool in medical diagnosis has focused a renewed interest around Gd^{3+} complexes for their use as contrast agents (CA)[1–3] coupled to this methodology. In fact it has been demonstrated that the use of Gd -based CA adds relevant physiological information to the impressive anatomical resolution of the uncontrasted images. For this reason Gd complexes have entered into several protocols to assess abnormalities in blood/brain barrier and in renal clearance, as well as in angiographic applications.[4]

For a potential application as a potential CA for MRI a Gd complex must satisfy three basic requisites: i) high thermodynamic and kinetic stability to avoid the release of harmful Gd ions; ii) good solubility in water to allow administration of concentrated solutions; iii) marked ability to enhance the relaxation rate of solvent water protons.[5] It is customary to express the latter property in term of relaxivity (r_{1p}) which represents the increase of the water proton relaxation rate in the presence of 1 mM concentration of the CA.

Although the MRI gives a superb resolution it suffers from a limited sensitivity. For instance, in order to reach a 50% increase in the contrast with the commercially available CA, it is necessary to reach local concentration of the CA of the order of 0.5 mM.[6]

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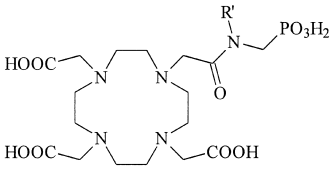
Whereas concentration of this order can be easily reached with the so-called T_1, general CA (administered at 0.1–0.3 mmol per kg), which homogeneously distribute in blood vessels and in the extracellular space, sensitivity problems arise when one wishes to pursue a target specificity. For instance targeting of receptor sites overexpressed by tumor cells, which are at best of the order of 10^{-9} moles for gram of tissue, appears a real challenge. It has been suggested that visualisation of tumors receptors may be possible by appending multiple gadolinium chelates on the surface of monoclonal antibodies.[6, 7] However, at least two problems seem to decrease the potential of this approach, namely i) the size of heavily substituted Ab may introduce additional difficulties in approaching the receptor sites, if they are not located on the vessels walls, and ii) the presence of several Gd chelates on the surface of the Ab may decrease its binding affinity for the receptor.

An alternative route may be pursued by addressing the accumulation of CA molecules in the proximity of the receptor sites on the basis of specific molecular recognition patterns.

In this work we have explored the potential of a system based on a positively charged polymer which display either a high binding affinity for the negatively charged surface of certain tumors[8] or a good ability to act as a collector of CA molecules. Therefore we proceeded with the synthesis of three novel Gd chelates containing phosphonates and carboxylate pendant groups on their external surface; these groups promote the interaction with the positively charged groups of polyamino acid chains of ornithine or arginine.

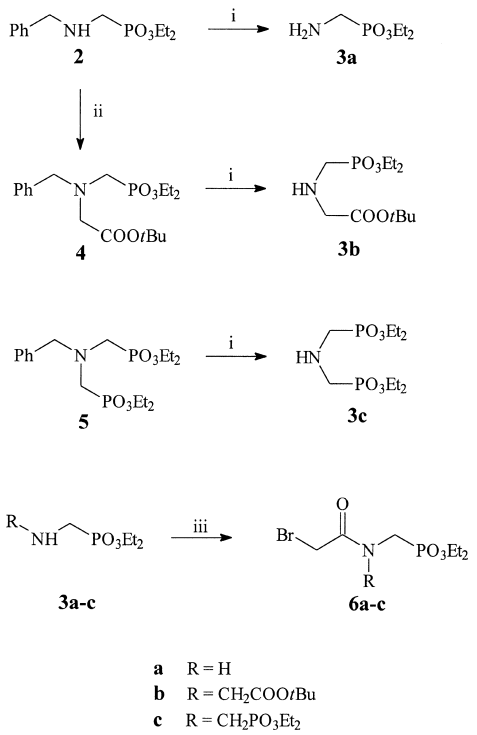
Results and Discussion

Synthesis of the ligands: The three ligands have been designed in order to display the same coordination cage for the Gd ion based on four nitrogens of tetraazacyclododecane ring, three oxygens of the acetate arms and one oxygen of a carbamoyl functionality. The latter group bears the recognition synthons in ligands **I–III** as shown below.



I: R' = -CH_2-PO_3H_2
II: R' = -CH_2-COOH
III: R' = -H

The synthesis of the ligands **I–III** is based on the alkylation of DO3A-tri-*tert*-butyl ester **7**, with the proper electrophile represented by a suitably N-substituted α-bromoacetamide (**6a–c**). The latter synthons have been synthesized according to the routes shown in Scheme 1. First, heating 1,3,5-tribenzylhexahydro-1,3,5-triazine with equimolar amount of diethyl phosphite at 100 °C for 3 h, without adding any solvent, afforded good yields of *N*-benzyl phosphoglycine diethyl ester (**2**). The latter compound was either debenzylated by treatment with ammonium formate/10% Pd/C in refluxing methanol to give phosphoglycine diethyl ester (**3a**), or alkylated with *tert*-butyl bromoacetate in acetonitrile in the presence of Hünig's base (diisopropylethylamine) to give *N*-benzyl-*N*-diethoxyphosphorylmethyl glycine *tert*-butyl ester (**4**). Compound **4** was then debenzylated to give *N*-diethoxyphosphorylmethyl glycine (**3b**). Heating benzylamine with 2 equiv of diethyl phosphite and formaldehyde yielded (**5**), which by successive catalytic hydrogen transfer gave the tetraethyl iminobis(methyl phosphonate) derivative (**3c**). Acylation of compounds **3a–c** with bromoacetyl bromide under the classical Schotten–Baumann conditions afforded the corresponding N-substituted α-bromoacetamides (**6a–c**), necessary for the next alkyaltion step.



a R = H
b R = $CH_2COOtBu$
c R = $CH_2PO_3Et_2$

Scheme 1. Synthesis of compounds **6a–c**. i) $HCOONH_4$, Pd/C, MeOH, reflux; ii) $BrCH_2COOtBu$, iPr_2NEt, CH_3CN, reflux; iii) $BrCH_2COBr$, CH_2Cl_2/aq. NaOH.

1,4,7,10-Tetraazacyclododecane-1,4,7-triacetic acid tri-*tert*-butyl ester hydrochloride (DO3A-*t*Bu·HCl, **7**) was therefore treated with electrophiles **6a–c** in *N*,*N*-dimethylformamide at 100 °C in the presence of potassium carbonate as base, to form compounds **8a–c**. (Scheme 2) Finally, removal of the ester groups, namely phosphonic ethyl and carboxylic *tert*-butyl esters, was carried out with trimethylsilyl bromide in methylene chloride and trifluoroacetic acid, followed by neutralisation with propylene oxide. The ligands **I–III** obtained were pure enough for the subsequent complexation reactions.

Characterisation of europium and ytterbium complexes: Sodium salts of the lanthanide complexes with **I–III** ligands were prepared in water at pH 6.5 (by addition of aqueous NaOH) by mixing equimolar amounts of the given ligand and the appropriate lanthanide chloride.

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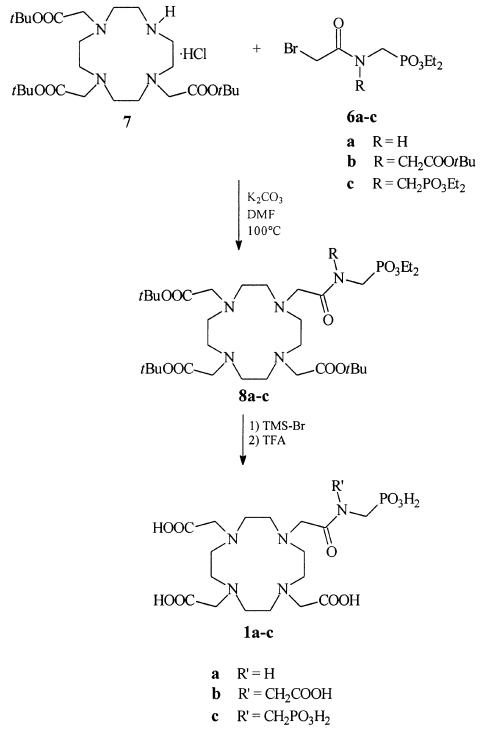

Scheme 2. Synthesis of the ligands **1a–c**.

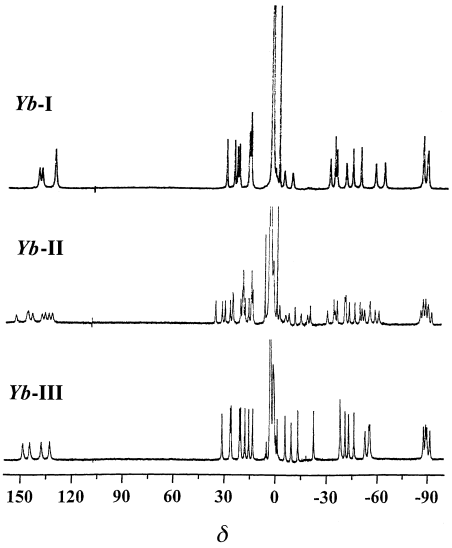

Figure 1. ^{1}H-NMR spectra of the Yb-**I–III** complexes recorded at 90 MHz, in D_2O, and at 25 °C. *tert*-Butyl alcohol was added as an internal reference ($\delta = 0$).

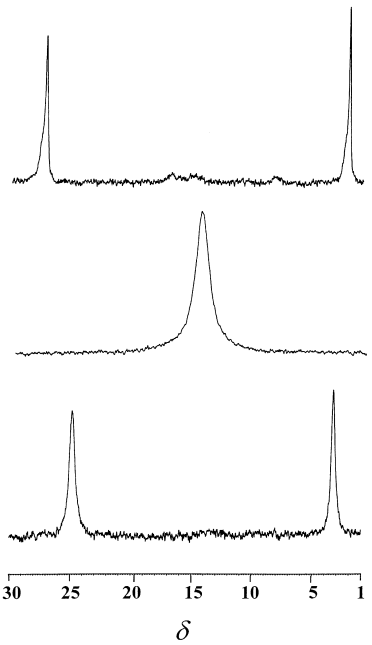

Figure 2. ^{31}P-NMR spectra of the Yb-**I–III** complexes recorded at 161.8 MHz, in D_2O, and at 25 °C. H_3PO_4 was used as an external reference ($\delta = 0$).

It is well established that the long electronic relaxation time of the Gd ion results in a severe induced line broadening of the resonances which prevents the detection of high resolution spectra of its complexes. Thus, insights into the solution structures of these complexes have been gained from the spectra of Eu and Yb complexes. The ^{1}H-NMR spectra of these complexes show close similarities with the parent DOTA and related complexes; it was previously reported that these complexes can adopt two diastereomeric structures, namely the square antiprismatic (M) and the twisted square antiprismatic (m) one.[9, 10] The two structures basically differ for the layout of the coordinating arms thus causing a marked difference in the relative orientation of the two square plans formed by the four nitrogens and four oxygens. The two plans make an angle of about 40° in M-type structures whereas the same angle is reduced to about 20° (and reversed) in m-type derivatives.[11] The overall structural differences between M and m structures are nicely reflected in their ^{1}H-NMR spectra. At ambient temperature all three Yb complexes display a M-type structure with ^{1}H-NMR resonances covering a region of about 210 ppm (Figure 1). In the asymmetrically substituted **II–III** ligands, the orientation of the substituent can generate a doubling of most of the resonances as a consequence of the formation of two structural isomers. This has been observed for Yb-**II** complex where two sets of ^{1}H-NMR resonances have been observed in the relative intensity ratio of 1:0.8. The observation of just one set of resonances for Yb-**III** has been accounted for in terms of the occurrence of a relatively fast rotation around the amide bond as confirmed by the detection of a single signal in the ^{31}P-NMR spectrum at RT at an intermediate position between the two ^{31}P-NMR resonances observed for Yb-**II** complex (Figure 2). Two ^{31}P-NMR resonances are of course also observed for Yb-**I**. Conversely to ytterbium complexes, the europium complexes show the occurrence of both M and m diastereoisomers in the relative intensity ratio of about 3:1 for all three derivatives. Analogously to the parent Eu-DOTA,[9] at 273 K, ^{1}H-NMR resonances of M-type structures cover a spectral window of about 60 ppm whereas those of m-type only about a half. The smaller spectral width covered by Eu complexes causes partial overlap especially in the case of Eu-**II** which shows a doubling of the resonances associated with the hindered rotation of the amide bond.

The ^{31}P-NMR spectra of the Eu-**I–III** complexes invariantly showed two sets of absorptions corresponding to M- and m-type structures. Analogously to what found previously for

the parent DOTA complex, the increase of temperature causes M ⇔ m isomeric exchange (Figure 3). For the purpose of this work we may assume that Gd^{III} chelates with **I–III** ligands should display an intermediate behavior between Eu and Yb complexes, that is they should be largely for the M-isomers, and that both isomers should display one coordinated water molecule as previously found for M- and m-type structures.[12]

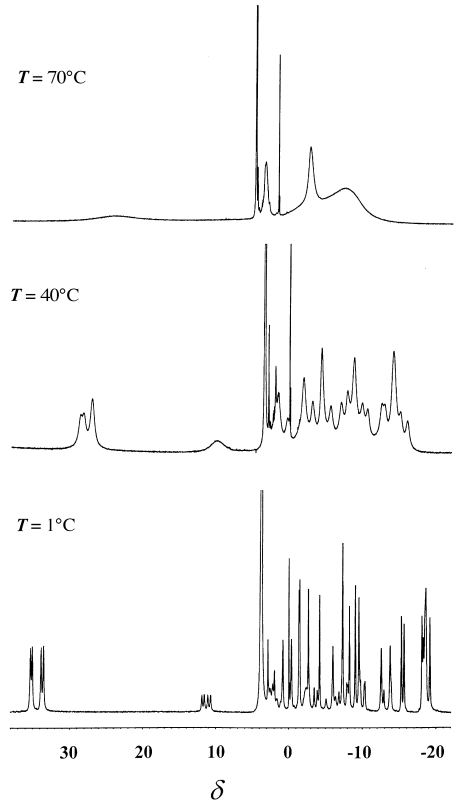

Figure 3. ^{1}H-NMR spectra of the Eu-**III** complexes recorded at 400 MHz, in D_2O. *tert*-Butyl alcohol was added as an internal reference ($\delta = 0$).

Relaxometric characterisation of Gd complexes: The relaxivities (at 20 MHz and 298 K) of Gd-**I**, -**II** and -**III** are 6.2, 6.0, and 5.6 $mM^{-1}s^{-1}$, respectively. These values appear significantly higher than those previously found for DOTA-monoamide complexes of similar size.[13] This behavior appears to be associated with the presence of the negatively charged phosphonate and carboxylate substituents which can bind water molecules on the surface of the paramagnetic complex.[14] The occurrence of such a second-coordination coating contribution to the overall relaxivity of these complexes is nicely demonstrated by the analysis of their $1/T_1$ NMRD profiles. (Figure 4). The observed relaxivity may be accounted for the sum of three contributions:[15]

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

Where r_{1p}^{1st} represents the contribution from the water molecule(s) directly coordinated to the Gd^{III} ion; r_{1p}^{2nd} and r_{1p}^{os} represent analogous contributions from water in the second coordination coating and from water molecules diffusing in the proximity of the paramagnetic complex, respectively. Whereas r_{1p}^{os} is rather constant for small-sized Gd complexes

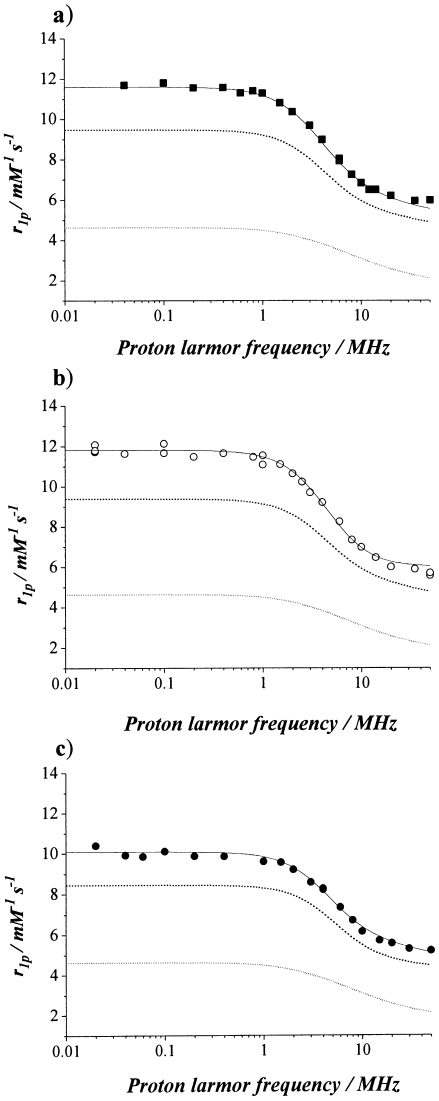

Figure 4. $1/T_1$ NMRD profiles of a 1 mM solution of a) Gd-**I**, b) Gd-**II**, and c) Gd-**III** at pH 7 and 25 °C. The solid curves through the data points were calculated with the parameters reported in Table 1. The fittings were obtaines with the assumption that one water molecule is in the first coordination sphere ($r_{Gd-H} = 3$ Å) and one in the second coordination layer ($r_{Gd-H} = 3.8$ Å). the lower dotted lines (••••) represent the outer sphere water contribution whereas the upper ones (- - - -) correspond to the sum of inner and outer sphere contriutions.

(ca. 2.0–2.5 $mM^{-1}s^{-1}$), r_{1p}^{1st} and r_{1p}^{2nd} are strongly dependent upon the molecular structure and dynamics.

Being the water molecule(s) in the second coordination coating associated with the paramagnetic complex by hydrogen bonding interactions, we may assume that their lifetime is of the order of nanoseconds. Its contribution to the overall relaxivity can then be assessed on the basis of the set of Solomon–Bloembergen–Morgan equations usually employed to evaluate the inner-sphere term:

$$r_{1p}^{1st\ or\ 2nd} = \frac{q^{1st\ or\ 2nd}}{55.5\,(T_{1M}^{1st\ or\ 2nd} + \tau_m^{1st\ or\ 2nd})} \quad (2)$$

$$\frac{1}{T_{1M}^{1st\ or\ 2nd}} \propto \frac{k}{r^6} \cdot f(\tau_c) \quad (3)$$

$$(\tau_c)^{-1} = (\tau_r)^{-1} + (\tau_s)^{-1} + (\tau_m^{1st\ or\ 2nd})^{-1} \quad (4)$$

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Where q is the number of water molecules in the first (q^{1st}) or in the second (q^{2nd}) coordination layers of the Gd ion, T_{1M} is the longitudinal relaxation time of their protons which is determined by the distances 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 (τ_m^{1st}) and the second (τ_m^{2nd}) coordination layer water.

The values of the parameters obtained from the best fitting procedure of the $1/T_1$ NMRD profiles are reported in Table 1. The determinants of the inner-sphere contribution display values which are very similar to those ones previously found for other Gd-DOTA-monoamide derivatives. Interestingly the second-layer term contributes for about 10–15% to the overall relaxivities of Gd-**I**–**III** complexes. The number of water molecules responsible for this contribution (and their relative distance from the paramagnetic center) cannot be determined from the available data. Among a number of possibilities, it may be informative to consider the case in which this contribution were brought about by only one water molecule entrapped between the inner-sphere water molecule and the phosphonate or carboxylate substituent[16] (Figure 5).

Table 1. Values of the parameters obtained from the best fitting procedure of the $1/T_1$ NMRD profiles.

	Δ^2 [$s^{-2}/10^{19}$]	τ_v [ps]	τ_r [ps]	τ_m [μs]	R_{1st} [Å]	q_{1st}	R_{2nd} [Å]	q_{2nd}
Gd-**I**	1.8 ± 0.3	21 ± 0.25	97 ± 0.3	1.6 ± 0.8	3	1	3.8	1
Gd-**II**	1.6 ± 0.2	23 ± 0.4	91 ± 0.3	1.6 ± 0.5	3	1	3.7	1
Gd-**III**	3.3 ± 1.0	17 ± 0.5	78 ± 0.5	1.5 ± 0.2	3	1	3.8	1

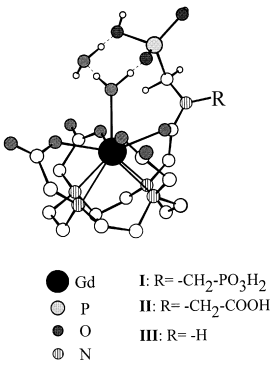

Figure 5. Schematic representation of Gd **I**–**III** chelates showing the entrapment of a water molecule between the inner-sphere water molecule and the phosphonate or carboxylate substituent.

Based on this model and in order to account for the observed contribution, the water protons should be at 3.7/3.8 Å from the paramagnetic center. For two molecules with the same distances this would correspond to a change in distance from 3.8 to 4.3 Å. There is also an effect of this tight second coordination layout on the exchange rate of the first sphere water as it has been assessed from the measurement of transverse ^{17}O-NMR relaxation times as function of temperature. It is well established that such data when fitted to the values calculated on the basis of Swift–Connick equations yields good estimates of the exchange lifetime of the coordinated water molecule.[17] In fact for systems endowed with τ_m in the μ-second range, R_{2p} values of solvent ^{17}O-nuclei describe a bell-shaped curve whose low temperature limb is dominated by the exchange lifetime and the high temperature one by the electronic relaxation time, respectively. As shown in Figure 6, Gd-**I**–**III** complexes display at high temperatures values which are smaller than the theoretical calculated values. From the fitting of the data in the 273–320 K range, τ_m^{298K} values between 1.5 and 2.0 μs are estimated for all the three complexes. These values are about 2–3 times longer than those found for related Gd-DOTA monoamide complexes previously reported.[14] We believe that the overall behavior (i.e., longer τ_m^{298K} and lack of fitting of the high temperature data) can be explained in terms of effects associated with tightly bound second coordination water that makes the dissociation of the water molecule directly bound to the Gd ion more difficult. At high temperature this effect is removed and the observed R_{2p} values are then lower than the fitting values expected as a consequence of the occurrence of a faster exchange rate.

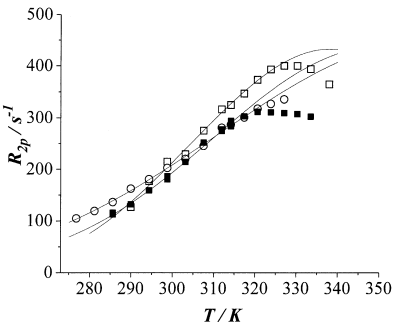

Figure 6. Temperature dependence of the paramagnetic contribution to the water ^{17}O NMR transverse relaxation rate (R_{2p}) for Gd-**I** (□), Gd-**II** (○), and Gd-**III** (■) (22.7 mM) at 2.1 T and pH 7.

Binding of Gd-I–III to polyamino acids: As stated in the introduction, Gd-**I**–**III** complexes were designed to form strong ion pairs with positively charged groups on macromolecular substrates like polylysine, polyornithine and polyarginine.

The setup of the interaction would allow the accumulation of Gd-based contrast agents at the targeting sites of the polyamino acid substrate while maintaining the advantages of the small-sized complex to get free access to the site of interest and in the elimination pathway as well.

Although several systems of different length have been tested, most of the work has been concentrated on a polyornithine containing (on the average) 140 amino acid residues and on a polyarginine derivative containing about 204 arginine residues. As the interaction is driven essentially by electrostatic forces, the binding is strongly pH dependent and, fortunately, at neutral pH is close to the highest attainable value. At acidic pH the interaction is weaker because of the protonation of phosphonate and carboxylate moieties as well as the affinity decreases at higher pH following the deprotonation of the aminic groups. The strength of the binding interaction has been assessed by the

proton relaxation enhancement (PRE) method.[18] Upon interacting with a macromolecule the relaxation induced by a Gd complex usually increases as a consequence of the elongation of the molecular reorientational time τ_r [Eqs. (2–3)]. Quantitatively, in the presence of a reversible interaction between the paramagnetic species and the macromolecule, the observed enhancement depends on both the molar fraction of the macromolecular adduct and the solvent-water relaxation in the all-bound limit, r_{1p}^b. Thus, the determination of the binding parameter k_A (thermodynamic association constant) and n (number of independent and equivalent sites characterised by a given k_A value) is pursued by measuring the solvent proton relaxation rates in the presence of variable chelate/macromolecule ratios. In Table 2, the results obtained from two sets of independent titrations for each Gd chelate/polyamino acid system are presented. In the first titration, the amount of chelate is maintained constant (0.2 mM) and R_1 values are measured in the presence of increasing concentrations of the polyamino acid, whereas in the second titration the amount of macromolecule is kept constant and the paramagnetic chelate is varied. The former measurement yields a more precise information on k_A and r_{1p}^b, whereas the latter one gives an indication of the number of binding sites. From the data reported in Table 2, one can draw the conclusion that the binding strength is dependent upon the residual negative charge on the complex being the highest for Gd-**I** and the lowest for Gd-**III** either for polyornithine or for polyarginine. Polyarginine binds less strongly than polyornithine. The binding is very strong in pure water and decreases in saline for the competition of the chloride ions for the positively charged amino or guanidino groups on the polyamino acid chain and of the sodium ions for the phosphonate or carboxylate groups on the surface of the Gd chelate. A further decrease in the binding affinity occurs when the measurements are carried out in blood serum because of the presence of different species as other macromolecules (serum albumin) or anions (lactate, phosphate, etc.), which are able to interact either with the negatively charged Gd complexes or the polyamino acid chain. Fortunately, the affinity of these Gd complexes for the human serum albumin (HSA), is relatively low (Gd-**I**/HSA: $k_A = 380\ M^{-1}$, $n = 1$)so that the titrations carried out in the two media (120 mM NaCl and human serum) appear very similar (Figure 7). Therefore, assuming the same k_A for the adduct Gd-**I**/polyornithine in 120 mM NaCl and in human serum and, considering the weak affinity of the complex for the albumin, we can estimate the distribution of the Gd-**I** in physiological conditions. For instance, when the contrast agent (Gd-**I**) was administered at the standard dose (0.1 mmol kg^{-1}) and the polyornithine at a dose ten times lower, about 85 % of the Gd complex results bound to the polyamino acid chain. This high binding selectivity makes the adduct Gd-**I**/polyornithine a good candidate for the purposes which are at the base of this project.

Table 2. Results obtained from two sets of independent titrations for each Gd chelate/polyamino acid system.

Complex	Polyamino acid	k_a [M^{-1}]	r_{1p}^b	n	Medium
Gd-**I**	Orn_{140}	$>4\times10^5$	13.0 ± 0.2	17	water, pH 7.4
Gd-**II**	Orn_{140}	$(2.8\pm2)\ 10^5$	12.7 ± 0.6	26	water, pH 7.4
Gd-**III**	Orn_{140}	$(7.9\pm2)\ 10^4$	11.00.1	22	water, pH 7.4
Gd-**I**	Orn_{140}	$(7.0\pm1)\ 10^3$	12.9 ± 0.2	17	120 mM, $NaCl_{aq}$
Gd-**II**	Orn_{140}	$(7.7\pm0.7)\ 10^2$	12.4 ± 0.2	26	120 mM, $NaCl_{aq}$
Gd-**III**	Orn_{140}	$(4.0\pm0.5)\ 10^2$	9.9 ± 0.3	22	120 mM, $NaCl_{aq}$
Gd-**II**	Arg_{204}	$(7.8\pm2)\ 10^4$	15.5 ± 0.2	23	water, pH 7.4
Gd-**III**	Arg_{204}	$(3.3\pm0.3)\ 10^3$	15.1 ± 0.2	60	water, pH 7.4
Gd-**I**	Arg_{204}	$(1.2\pm0.2)\ 10^3$	20.1 ± 0.4	25	120 mM, $NaCl_{aq}$
Gd-**II**	Arg_{204}	$(8.5\pm0.8)\ 10^2$	17.0 ± 0.2	23	120 mM, $NaCl_{aq}$
Gd-**III**	Arg_{204}	$(1.7\pm0.2)\ 10^2$	13.7 ± 0.3	60	120 mM, $NaCl_{aq}$

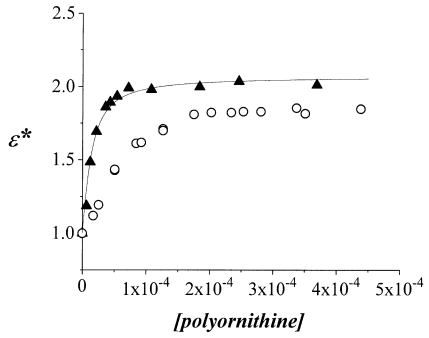

Figure 7. Enhancement factor ($\varepsilon^* = \frac{R^*_{1obs} - R^*_{1d}}{R_{1obs} - R_{1d}}$) as a function of the polyornithine concentration measured in a 0.2 mM solution of Gd-**I** in $NaCl_{aq}$ 120 mM (▲) and in human serum (○). R^*_{1obs} is the observed relaxation rate of the solution recorded at 20 MHz and 25 °C and R_{1d} is the diamagnetic contribution to the relaxivity. The asterisk indicates the presence of the polyamino acid in the solution.

The number of binding sites is significantly smaller than the value expected on the basis of a simple pairing of opposite electric charges. Structural effects are likely to become important and then for Gd-**I** containing two phosphonate moieties (four negative residual charges at pH 7.0) only 17 and 11 sites become available on polyornithine and polyarginine, respectively. On the other hand the less demanding two negatively charged Gd-**III** complex displays a number of binding sites very close to that shown by the adducts with Gd-**II**. For the latter complex the additional presence of the carboxylate group endows the system with a higher binding affinity either toward polyornithine or polyarginine in respect to Gd-**III**.

In general one may note that the obtained r_{1p}^b values are not very high if compared with the relaxivities of some macromolecular adducts formed by Gd complexes and proteins such as HSA.[19–22] The reason can be found in the long exchange lifetime of the coordinated water in such systems which acts as a "quenching" factor for the attainable relaxivity [Eq. (2)]. Moreover, at neutral pH, the polyamino acid chains is largely present as random coils characterized by a reorientational time τ_r significantly shorter than that of the less flexible proteins of similar molecular weights. This shorter τ_r value corresponds in a lower attainable r_{1p}^b value. Although these drawbacks may be (in part) overcome by an improved design of the ligand and of the cationic substrate, the obtained relaxivities for Gd-**I**–**III** adducts should not represent a limitation to proceed with an in vivo testing of the proposed procedure.

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Conclusion

The results reported in this paper show that it is possible to pursue the formation of supramolecular adducts formed by negatively charged Gd chelates endowed with a good affinity for positively charged polyamino acid substrates. These findings may provide insights for novel methods for the location of tumors. In fact it is known that positively charged polyamino acids bind selectively to tumors having a greater net negative charge than non-tumor cells, that is polyanionic charged tumors such as brain tumors, small cell carcinoma of the lung, and melanoma.[8] Although one may envisage an analogous method for targeting polyanionic charged tumors based on the use of polylysine containing some of its ε-NH_2 groups covalently conjugated with Gd–DTPA chelates, the non-covalent approach tackled in this paper appears to have several advantages. First of all, it may allow an easy route for the delivery and accumulation of the contrast agent through the separate administration of the polyamino acid and the Gd chelate, thus overcoming the problems associated to the limited accessibility to the targeting sites expected for the large conjugates. One may think of a method by which the administration of the polyamino acid is made through the injection in the arterial system in the area of the suspected tumors (so that the concentration of the toxic polyamino acid can be kept sufficiently low). Following the accumulation of the cationic polyamino acid as a consequence of the selective binding to the tumor cell surface and excretion of the unbound polyamino acid chains, one may proceed with the administration of the paramagnetic chelate whose high affinity for the polyamino acid will allow its specific recognition.

Experimental Section

High-resolution ^{1}H-NMR spectra of the Eu complex and the ^{31}P-NMR spectra of the Yb complexes were measured in D_2O with a JEOL EX-400 (400 MHz and 161.8 MHz), the ^{1}H-NMR spectra of the Yb-complexes were measured with a JEOL EX-90 (90 MHz). High-resolution ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra of the other compounds were measured in $CDCl_3$ or D_2O with a Bruker AC 200 (200 MHz, 50.3 MHz, and 80.9 MHz, respectively). Mass spectra were obtained with a VG 7070 EQ spectrometer (CI-MS, isobutane). 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, two 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 proton $1/T_1$ NMRD profiles were measured over a continuum of magnetic field strength from 0.00024 to 1.2 T (corresponding to 0.01–50 MHz proton Larmor frequency) on the Koenig–Brown field-cycling relaxometer installed at the NMR relaxometry laboratory of the University of Torino (Italy). The relaxometer works under complete computer control with an absolute uncertainty in $1/T_1$ of ±1%. Details of the instrument and of the data acquisition procedure are given elsewhere.[23]

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 D_2O as external lock. Experimental settings were: spectral width 10000 Hz, pulse width 7 μs, acquisition time 10 ms, 1000 scans, and 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.

The polyornithine (MW = 27650 Da) and the polyarginine (MW = 39400) were purchased from Sigma (St. Louis, MO, USA) and were used without any further purification

***N*-Benzylphosphoglycine diethyl ester (2)**: Prepared in 89% yield according to the procedure reported by Lewis et al.[24] Colorless oil; slowly decomposed on standing at room temperature. ^{1}H NMR (200 MHz, $CDCl_3$, TMS): δ = 1.21 (t, ^3J(H,H) = 7.1 Hz, 6H), 1.71 (br s, 1H), 2.93 (d, ^3J(H,H) = 12.4 Hz), 3.84 (s, 2H), 4.13 (m, 4H), 7.29 (m, 5H); ^{13}C NMR (50.3 MHz, $CDCl_3$, TMS): δ = 16.3, 44.0 (d, ^1J(C,P) = 155 Hz), 54.6 (d, ^2J(C,P) = 17.2 Hz), 61.9 (d, ^2J(C,P) = 6.2 Hz), 127.1, 128.1, 128.3, 139.1.

***N*-Phosphoglycine diethyl ester (3a)**:[25] In a 50 mL flask diethyl ester **2** (1.98 g, 5.35 mmol) was dissolved in ethanol (15 mL). Ammonium formate (1.68 g, 26.8 mmol), dissolved in distilled water (3 mL), was added, followed by 10% Pd/C (0.51 g, moistened with 0.5 mL water). The mixture was stirred and heated to reflux for 1 h. Another portion of ammonium formate (1.68 g, 26.8 mmol) in water (3 mL) was added and reflux maintained for further 1 h. The catalyst was then removed by filtration, and the filtrate evaporated in vacuo. The residue was partitioned between CH_2Cl_2/10% aq. Na_2CO_3. The organic layers were separated, dried over Na_2SO_4, filtered, and evaporated, obtaining **3a** (0.72 g, 81%). Colorless oil; ^{1}H NMR (200 MHz, $CDCl_3$, TMS): δ = 1.19 (t, ^3J(H,H) = 7.1 Hz, 6H), 1.31 (s, 2H), 2.85 (d, ^3J(H,H) = 10.2 Hz, 2H), 3.99 (m, 4H); ^{13}C NMR (50.3 MHz, $CDCl_3$, TMS): δ = 16.0 (d, ^2J(C,P) = 4.7 Hz), 37.3 (d, ^1J(C,P) = 149 Hz), 61.5 (d, ^2J(C,P) = 5.6 Hz).

***N*-Benzyl-*N*-diethoxyphosphorylmethylglycine *tert*-butyl ester (4)**: Compound **2** (2.010 g, 7.79 mmol) and diisopropylethylamine (2.01 g, 15.57 mmol) were dissolved in anhydrous acetonitrile (10 mL). *tert*-Butyl bromoacetate (1.68 g, 8.56 mmol) was added dropwise during 5 min. The reaction mixture was stirred 30 min at room temperature and refluxed 4 h, after which TLC analysis (SiO_2, $CHCl_3$/AcOEt 1:1) showed complete conversion. The solvent was removed in vacuo and the residue partitioned between CH_2Cl_2/10% aq. Na_2CO_3. The organic layers were separated, dried over Na_2SO_4, filtered, and evaporated. The oily residue was submitted to flash chromatography (SiO_2, $CHCl_3$/AcOEt 1:1), obtaining pure **4** (1.98 g, 68.7%). Colorless oil; ^{1}H NMR (200 MHz, $CDCl_3$, TMS): δ = 1.08 (t, ^3J(H,H) = 7 Hz, 6H), 1.12 (s, 9H), 2.95 (d, ^3J(H,H) = 10.2 Hz, 2H), 3.26 (s, 2H), 3.72 (s, 2H), 3.89 (m, 4H), 7.09 (m, 5H); ^{13}C NMR (50.3 MHz, $CDCl_3$, TMS): δ = 16.2 (d, ^2J(C,P) = 5.2 Hz), 27.9, 48.3 (d, ^1J(C,P) = 161 Hz), 54.8 (d, ^2J(C,P) = 4.2 Hz), 59.2 (d, ^2J(C,P) = 5.6 Hz), 61.5 (d, ^2J(C,P) = 5.4 Hz), 80.5, 127.0, 128.0, 128.8, 138.1, 169.9; CI-MS: found: 372 $[M+H]^+$; calcd for $C_{18}H_{30}NO_5P$: m/z 371.

***N*-Diethoxyphosphorylmethylglycine *tert*-butyl ester (3b)**: Following the procedure adopted for the synthesis of **3a**, from **4** (1.98 g, 5.35 mmol), ammonium formate (2 × 1.68 g), and 10% Pd/C (0.51 g), except for the reaction time requiring only 1.5 h to reach completion (1.15 g, 77.2%). Colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$, TMS): δ = 1.34 (t, ^3J(H,H) = 7.1 Hz, 6H), 1.46 (s, 9H), 2.16 (br s, 1H), 3.0 (d, ^3J(H,H) = 12.6 Hz, 2H), 3.37 (s, 2H), 4.16 (m, 4H); ^{13}C NMR (50.3 MHz, $CDCl_3$, TMS): δ = 16.4 (d, ^2J(C,P) = 5.6 Hz), 28.0, 44.4 (d, ^1J(C,P) = 157 Hz), 52.3, 62.0 (d, ^2J(C,P) = 6.6 Hz), 80.9, 170.9; CI-MS: found: 282 $[M+H]^+$; calcd for $C_{11}H_{24}NO_5P$: m/z 281.

Tetraethyl *N*-benzyliminobis(methanephosphonate) (5): In a 50 mL round-bottomed flask benzylamine (0.98 g, 9.33 mmol) and diethyl phosphite (2.57 g, 18.7 mmol) were mixed and cooled to 0–5 °C (ice bath). Aqueous 37% formaldehyde (2.10 mL, 28.0 mmol) was added at a rate to maintain the reaction temperature under 10 °C (about 15 min). The resulting emulsion was stirred at room temperature for 30 min, then heated to 100 °C for 1 h. Removal in vacuo of water and excess formaldehyde gave analytically pure **5** (3.66 g, 96.3%). Colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$, TMS): δ = 1.31 (t, ^3J(H,H) = 7.1 Hz, 6H), 3.16 (d, ^3J(H,H) = 9.3 Hz, 4H), 3.96 (s, 2H), 4.11 (m, 8H), 7.31 (m, 5H); ^{13}C NMR (50.3 MHz, $CDCl_3$, TMS): δ = 16.3 (d, ^2J(C,P) = 5.1 Hz), 49.3 (dd, ^1J(C,P) = 157.2 Hz, ^2J(C,P) = 7.4 Hz), 60.8 (t, ^2J(C,P) = 6.0 Hz), 127.3, 128.1, 129.1, 137.8; CI-MS: found: 408 $[M+H]^+$; calcd for $C_{17}H_{31}NO_6P_2$: m/z 407.

Tetraethyl iminobis(methanephosphonate) (3c): Following the procedure adopted for the synthesis of **3a**, from **5** (3.66 g, 8.99 mmol), ammonium formate (2 × 2.88 g), and 10% Pd/C (0.50 g), except for the reaction time requiring a total of 12 h to reach completion (1.16 g, 88.0%). Colorless oil.

^{1}H NMR (200 MHz, $CDCl_3$, TMS): $\delta = 1.32$ (t, ^3J(H,H) = 7.1 Hz, 6H), 2.53 (brs, 1H), 3.09 (d, ^3J(H,H) = 10.8 Hz, 4H), 4.13 (m, 8H); ^{13}C NMR (50.3 MHz, $CDCl_3$, TMS): $\delta = 16.3$ (d, ^2J(C,P) = 4.9 Hz), 45.7 (dd, ^1J(C,P) = 153 Hz, ^2J(C,P) = 12.9 Hz), 62.0; CI-MS: found: 318 $[M+H]^+$; calcd for $C_{10}H_{25}NO_6P_2$: *m/z* 317.

General procedure for acylation of compounds 3a–c: In a 50 mL round-bottomed flask compound **3** was dissolved in methylene chloride (5 mL per g) and NaOH (2 equiv, in 5 mL distilled water) was added. Bromoacetyl bromide (1.3–1.5 equiv, in 3 mL CH_2Cl_2) was slowly dropped into the stirred heterogeneous mixture, while maintaining the temperature $< 10\,^\circ$C (ice bath). After 1 h vigorous stirring, TLC analysis (SiO_2, AcOEt/MeOH 9:1) showed complete disappearance of compound **3**. The reaction mixture was diluted with water (10 mL) and the crude product is thoroughly extracted with methylene chloride. The organic extracts were washed subsequently with 10% aq. Na_2CO_3, 2M HCl, and water, dried and evaporated in vacuo to give **6a–c**.

***N*-Bromoacetylphosphoglycine diethyl ester (6a)**: Obtained from **3a** in 97% yield. White solid. ^{1}H NMR (200 MHz, $CDCl_3$, TMS): $\delta = 1.35$ (t, ^3J(H,H) = 6.9 Hz, 6H), 3.74 (dd, ^2J(H,H) = 12.4 Hz, ^2J(H,H) = 6.0 Hz, 1H), 3.91 (s, 2H), 4.16 (m, 4H), 7.26 (brs, 1H); ^{13}C NMR (50.3 MHz, $CDCl_3$, TMS): $\delta = 16.2$ (d, ^2J(C,P) = 5.1 Hz), 28.1, 35.1 (d, ^1J(C,P) = 157 Hz), 62.8 (d, ^2J(C,P) = 6.4 Hz), 166.4; CI-MS: found: 288–290 $[M+H]^+$; calcd for $C_7H_{15}BrNO_4P$: *m/z* 287–289.

***N*-Bromoacetyl-*N*-diethoxyphosphorylmethylglycine *tert*-butyl ester (6b)**: Obtained from **3b** in 91% yield. Colorless oil. The signals in NMR spectra indicated the presence of two rotational isomers (amide bond) with a ratio of 58:42 at 300 K. ^{1}H NMR (200 MHz, $CDCl_3$, TMS): $\delta = 1.28$ (t, ^3J(H,H) = 7.0 Hz, 6H), 1.35 (t, ^3J(H,H) = 7.1 Hz, 6H), 1.42 (s, 9H), 1.44 (s, 9H), 3.75 (s, 2H), 3.78 (d, ^3J(H,H) = 9.6 Hz, 2H), 3.86 (d, ^3J(H,H) = 10.8 Hz, 2H), 3.99 (s, 2H), 4.09 (m, 4H), 4.10 (m, 2H), 4.24 (s, 2H), 4.25 (s, 2H); ^{13}C NMR (50.3 MHz, $CDCl_3$, TMS): $\delta = 16.2$ (d, ^2J(C,P) = 5.0 Hz), 16.3 (d, ^2J(C,P) = 5.1 Hz), 25.2, 25.7, 27.8, 27.9, 41.5 (d, ^1J(C,P) = 157 Hz), 45.3 (d, ^1J(C,P) = 157 Hz), 49.1, 50.8, 62.5 (d, ^2J(C,P) = 6.9 Hz), 62.8 (d, ^2J(C,P) = 7.2 Hz), 82.2, 83.0, 166.7, 167.0, 167.2, 167.3; CI-MS: found: 402–404 $[M+H]^+$; calcd for $C_{13}H_{25}BrNO_6P$: *m/z* 401–403.

Tetraethyl *N*-bromoacetyliminobis(methanephosphonate) (6c): Obtained from **3c** in 92% yield. Colorless oil. The signals in NMR spectra indicated a slow rotation (on NMR time scale) around the amide bond. ^{1}H NMR (200 MHz, $CDCl_3$, TMS): $\delta = 1.30$ (m, 12H), 3.99–4.06 (m, 6H), 4.14 (m, 8H); ^{13}C NMR (50.3 MHz, $CDCl_3$, TMS): $\delta = 16.2$ (d, ^2J(C,P) = 4.5 Hz), 16.3 (d, ^2J(C,P) = 4.8 Hz), 41.1 (d, ^1J(C,P) = 156 Hz), 44.3 (d, ^1J(C,P) = 160 Hz), 62.5 (d, ^2J(C,P) = 5.4 Hz), 62.8 (d, ^2J(C,P) = 5.7 Hz), 166.6; CI-MS: found: 438–440 $[M+H]^+$; calcd for $C_{12}H_{26}BrNO_7P_2$: *m/z* 437–439.

General procedure for the synthesis of compounds 8a–c: In a 50 mL round-bottomed flask 1,4,7,10-tetrazacyclododecane-1,4,7-triacetic acid tri-*tert*-butyl ester hydrochloride (DO3A-*t*Bu·HCl, **7**, 1 mmol) and finely powdered K_2CO_3 (2 mmol) were suspended in anhydrous DMF (2 mL) and stirred at room temperature for 30 min. Compound **6** (1.2 mmol) was added portionwise and the resulting mixture was heated to 100 °C for 2–16 h. The light brown suspension was filtered, and the inorganic salts washed with ethanol (10 mL). The filtrate and washings are combined and evaporated in vacuo. The oily residue was submitted to silica gel chromatography (CH_2Cl_2/MeOH/28% aq. NH_3 9:1.5:0.15) obtaining analytically pure **8**.

10-[*N*-(Diethoxyphosphorylmethyl)aminocarbonylmethyl]-1,4,7,10-tetrazacyclo-dodecane-1,4,7-triacetic acid tri *tert*-butyl ester (8a): Obtained from **6a** and **7** in 42% yield. Light yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$, TMS): $\delta = 1.30$ (t, ^3J(H,H) = 7.1 Hz, 6H), 1.44 (s, 27H), 2.37 (m, 16H), 2.86 (s, 4H), 2.94 (s, 4H), 3.70 (m, 2H), 4.15 (m, 4H), 8.57 (bt, ^3J(H,H) = 6.2 Hz, 1H); ^{13}C NMR (50.3 MHz, $CDCl_3$, TMS): $\delta = 16.4$ (d, ^2J(C,P) = 4.9 Hz), 27.7, 27.9, 34.1 (d, ^1J(C,P) = 157 Hz), 49.1–50.9 (brs, 4s), 55.5, 55.6, 56.2, 62.4 (d, ^2J(C,P) = 5.2 Hz), 81.7, 81.8, 162.2, 171.9, 172.3; CI-MS: found: 722 $[M+H]^+$; calcd for $C_{33}H_{64}N_5O_{10}P$: *m/z* 721.

10-[*N*-(*tert*-Butoxycarbonylmethyl)-*N*-(diethoxyphosphorylmethyl)aminocarbonyl-methyl]-1,4,7,10-tetrazacyclododecane-1,4,7-triacetic acid tri *tert*-butyl ester (8b): Obtained from **6a** and **7** in 48% yield. Yellow oil. The signals in NMR spectra indicate the presence of two rotational isomers (amide bond) in a 60:40 ratio at 300 K. ^{1}H NMR (200 MHz, $CDCl_3$, TMS): $\delta = 1.28$ (t, ^3J(H,H) = 7.1 Hz, 6H), 1.30 (t, ^3J(H,H) = 7.1 Hz, 6H), 1.42 (s, 9H), 1.44 (s, 9H), 2.10–2.50 (m, 16H+16H), 2.85–3.00 (m, 8H+8H), 3.75 (s, 2H), 3.78 (d, ^3J(H,H) = 9.6 Hz, 2H), 3.86 (d, ^3J(H,H) = 10.8 Hz, 2H), 3.99 (s, 2H), 4.10 (m, 4H), 4.24 (m, 4H); CI-MS: found: 836 $[M+H]^+$; calcd for $C_{39}H_{74}N_5O_{12}P$: *m/z* 835.

10-[*N*,*N*-Bis(diethoxyphosphorylmethyl)aminocarbonyl-methyl]-1,4,7,10-tetraza-cyclododecane-1,4,7-triacetic acid tri *tert*-butyl ester (8c): Obtained from **6a** and **7** in 44% yield. Light yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$, TMS): $\delta = 1.30$ (t, ^3J(H,H) = 7.4 Hz, 6H), 1.34 (t, ^3J(H,H) = 7.4 Hz, 6H), 1.45 (s, 9H), 1.46 (s, 18H), 2.22–2.47 (m, 16H), 2.80 (s, 2H), 2.86 (s, 2H), 2.93 (s, 4H), 3.91–4.20 (m, 12H); ^{13}C NMR (50.3 MHz, $CDCl_3$, TMS): $\delta = 16.4$ (d, ^2J(C,P) = 5.1 Hz), 27.8, 27.9, 40.1 (d, ^1J(C,P) = 109 Hz), 43.2 (d, ^1J(C,P) = 110 Hz), 48.9–52.1 (brs, 4s), 54.8, 55.6, 55.9, 62.2 (d, ^2J(C,P) = 5.3 Hz), 62.7 (d, ^2J(C,P) = 5.3 Hz), 81.7, 81.8, 162.2, 171.5, 172.5; CI-MS found: 872 $[M+H]^+$; calcd for $C_{38}H_{75}N_5O_{13}P_2$: *m/z* 871.

General procedure for hydrolysis of compounds 8a–c: Compounds **8a–c** (1 mmol) were dissolved in anhydrous methylene chloride (5 mL); after cooling to 0–5 °C (ice-bath), trimethylsilyl bromide (10 mmol) was slowly dropped into. A mildly exothermic reaction occurred, and the homogeneous solution was stirred overnight at room temperature. The volatile materials were removed in vacuo and the semisolid residue was redissolved in neat trifluoroacetic acid (5 mL). The mixture was stirred overnight at room temperature, then excess CF_3COOH was evaporated under reduced pressure; the residue was taken up in methanol (3 mL) and diethyl ether (15 mL) added dropwise. The crude ligand **1a–c** (trifluoroacetate salt) precipitated as fine white powders, isolated by careful centrifugation. The product, re-suspended in hot methanol was additioned of propylene oxide (0.5 mL), which caused free **1a–c** to precipitate as white microcrystalline solids. The latter were isolated by filtration and repeatedly washed with cold methanol and finally with diethyl ether, followed by drying in vacuo.

10-[*N*-(Dihydroxyphosphorylmethyl)aminocarbonylmethyl]-1,4,7,10-tetra-zacyclo-dodecane-1,4,7-triacetic acid (1a): Obtained in 85% yield from **8a**. White crystals, m.p.: 165 °C (dec.); ^{1}H NMR (400 MHz, D_2O, pH 8): $\delta = 3.16$ (m, 4H), 3.21 (m, 4H), 3.28 (d, ^3J(H,H) = 12.4 Hz, 2H), 3.42 (s, 2H), 3.46 (m, 8H), 3.63 (s, 2H), 3.86 (s, 4H); ^{13}C NMR (50.3 MHz, D_2O, pH 8): $\delta = 40.0$ (d, ^1J(C,P) = 138 Hz), 49.8, 50.0, 52.9, 53.2, 56.4, 57.8, 58.7, 172.3, 173.1, 179.5; ^{31}P NMR (D_2O, pH 8): $\delta = 13.6$; FAB-MS: calcd for $C_{17}H_{32}N_5O_{10}P$: 497, found: 520 $[M+Na]^+$, 498 $[M+H]^+$.

10-[*N*-(Dihydroxyphosphorylmethyl)-*N*-(hydroxycarbonylmethyl)-amino-carbonylmethyl]-1,4,7,10-tetrazacyclododecane-1,4,7-triacetic acid (1b): Obtained in 98% yield from **8b**. White crystals, m.p.: 233 °C (dec.). The NMR spectra showed (as in **6b** and **8b**) the presence of two rotational isomers in approx. 55:45 ratio. ^{13}C NMR (50.3 MHz, D_2O, pH 8): $\delta = 47.0$ (d, ^1J(C,P) = 141 Hz), 49.0 (d, ^1J(C,P) = 139 Hz), 50.1–50.8 (brs, 2s), 52.0–53.4 (brs, 2s), 55.2, 53.5, 58.1, 58.2, 58.3, 58.4, 172.3, 172.9, 177.7, 178.4, 179.2, 179.3; ^{31}P NMR (D_2O, pH 8): $\delta = 14.9$, 13.5; FAB-MS: calcd for $C_{19}H_{34}N_5O_{12}P$: 555, found: 578 $[M+Na]^+$, 556 $[M+H]^+$.

10-[*N*,*N*-Bis(dihydroxyphosphorylmethyl)aminocarbonylmethyl]-1,4,7,10-tetraza-cyclododecane-1,4,7-triacetic acid (1c): Obtained in 95% yield from **8c**. White crystals, m.p.: 242 °C (dec.); ^{1}H NMR (400 MHz, D_2O, pH 8): $\delta = 3.22$ (m, 8H), 3.41 (m, 10H), 3.54 (s, 2H), 3.56 (d, ^3J(H,H) = 10.6 Hz, 2H), 3.77 (s, 4H), 3.79 (s, 2H); ^{13}C NMR (50.3 MHz, D_2O, pH 8): $\delta = 46.6$ (d, ^1J(C,P) = 131 Hz), 48.0 (d, ^1J(C,P) = 129 Hz), 50.6, 52.3, 55.9, 58.1, 58.3, 59.1, 170.5, 174.6, 178.6; ^{31}P NMR (D_2O, pH 8): $\delta = 14.9$, 12.9; FAB-MS: calcd for $C_{18}H_{35}N_5O_{13}P_2$: 591; found: 614 $[M+Na]^+$, 592 $[M+H]^+$.

Preparation of the Ln complexes: The complexation has been carried out by adding stoichiometric amounts of the corresponding $LnCl_3$ to the aqueous solutions of the ligands at neutral pH and at room temperature. The formation of Yb- or Eu-**I–III** complexes was verified by recording ^{1}H-NMR spectra. The synthesis of the Gd-**I–III** complexes was followed by measuring the solvent proton relaxation rate ($1/T_1$). The presence of an excess of free Ln ions may be quickly removed by centrifugation of the solution brought to basic pH.

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