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A Novel Series of Complexones with Bis- or Biazole Structure as Mixed Ligands of Paramagnetic Contrast Agents for MRI

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Abstract—We describe the syntheses, physicochemical properties and biological evaluation of a novel series of complexones containing bis- or biazoles moieties and two iminodiacetic acid units as novel ligands for paramagnetic lanthanides. The complexones were prepared by reaction of the corresponding 1,1′-bishaloethylbi- or bispyrazoles with methyl iminodiacetate and subsequent NaOH hydrolysis. 1,1′-Bisbromoethyl precursors were obtained by direct alkylation with an excess of 1,2-dibromoethane, or by heating the corresponding alcohol in HCl. Sigmoidal binding isotherms and MO calculations supported as most stable structures in solution, those containing two Gd(III) atoms bound per molecule of complexone with half saturation values $S_{0.5}$ (M^{-1}, 22 °C, pH 7.2) in the range $6.5\ 10^{-6} < S_{0.5} < 36.1\ 10^{-6}$. Relaxivity properties [r_1, r_2, $s^{-1}\ mM^{-1}$ Gd(III)] determined at 1.5 Tesla gave values ($12.0 < r_1 < 17.7$, $12.2 < r_2 < 20$), improving significantly the relaxivities of reference compounds such as Gd(III)EDTA (5.2, 5.6) or Gd(III)DTPA (4.30, 4.30). These improvements involve mainly increased hydration and slower rotational motions. In vitro toxicity experiments are reported.

Introduction

Even though Magnetic Resonance Imaging (MRI) methods inherently provide high intrinsic tissue contrast, the use of extrinsic contrast agents has become a routine in many diagnostic imaging procedures.[1] Very frequently, the paramagnetic lanthanide Gd(III) is used to increase locally the longitudinal relaxation rate of surrounding tissue water, highlighting the intensity of specific tissue areas in T_1 weighted images.[2] However, free Gd(III) is toxic in vivo and in vitro and Gd(III) chelates must be used in the clinic for safety reasons.[3,4] The first generation of Gd(III) ligands was derived from linear polyaminopolycarboxylates such as diethylenetriaminepentaacetic acid (DTPA) or from macrocycles such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA).[5] The corresponding Gd(III) complexes depicted very high thermodynamic and kinetic stability.[6] However, their capacity to induce water relaxation termed relaxivity,[7] remained ca. 4–5 $s^{-1}\ mM^{-1}$, well below the optimal values of approximately 100 $s^{-1}\ mM^{-1}$ predicted by theory.[8] Reduced relaxivity imposed the use of large doses of these agents, limiting the possibilities to visualize successfully low concentration molecular targets such as cell surface antigens, receptors, enzymes or even genes.[9]

To overcome these limitations it became necessary to increase the relaxivity of new generations of Gd(III) chelates, maintaining simultaneously their high thermodynamic stability. One approach towards this goal, is to maintain the basic chemical structures of Gd(III)DTPA or Gd(III)DOTA complexes but increase their relaxivity by restricting their rotational dynamics through conjugation to linear polymers, dextrans and proteins or through the production of dendrimeric derivatives.[3,10] An extensive series of macromolecular DTPA and DOTA derivatives were produced and characterized in this way, increasing relaxivities to ca. 15 $s^{-1}\ mM^{-1}$ per unit of bound Gd(III) chelate in linear polymers, ca. 19 $s^{-1}\ mM^{-1}$ in dendrimers and ca. 50 $s^{-1}\ mM^{-1}$ in those bound to serum albumin.[11] However, even these values remained below the optimal relaxivities predicted, suggesting that further improvements would

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require additional modifications of the chemical structure of the ligands.

With this aim, we proposed earlier the use of nitrogen containing heterocycles as ligands for paramagnetic contrast agents.[12] The approach relied on the high capability of nitrogen as electron donor,[13,14] a property which allowed us previously to design and prepare a series of pH sensitive probes for magnetic resonance spectroscopy.[15–17] However, few complexones had incorporated earlier heterocycles in their structures.[18–20] The first generation of azolic complexones **1**–**4**[12] contained only one iminodiacetic acid unit and one azole ring (Fig. 1). These ligands were able to form tetradentate complexes with Gd(III) involving two carboxylates, one amine group, and the heterocyclic nitrogen atom, showing improved relaxivity properties as compared to Gd(III) DTPA or Gd(III)DOTA. However, their thermodynamic stability was much lower than that of these octadentated reference ligands.

To improve the relaxivity and thermodynamic stability of this type of compounds we approach here the syntheses and physicochemical characterization of the second generation of heterocyclic complexones containing two pyrazole rings and two iminodiacetic acid units per molecule **5a**–**d** (Fig. 1). These dimeric structures, conceived initially to provide an octadentate geometry around the Gd(III) ion are shown here to fold over intramolecularly forming two tetradentadentate Gd(III) complexes per complexone molecule. The corresponding Gd(III) complexes of **5a**–**d** depicted remarkable improvements in relaxivity of ca. $25 < r_1 < 37$ and $25 < r_2 < 70$ s^{-1} mM^{-1} complexone as compared to the first series of heterocyclic complexones, and to Gd(III) DTPA or Gd(III) DOTA. Thermodynamic stability is also improved, being higher than the previous monomeric complexones but still lower than that of Gd(III) DTPA or Gd(III) EDTA reference compounds.

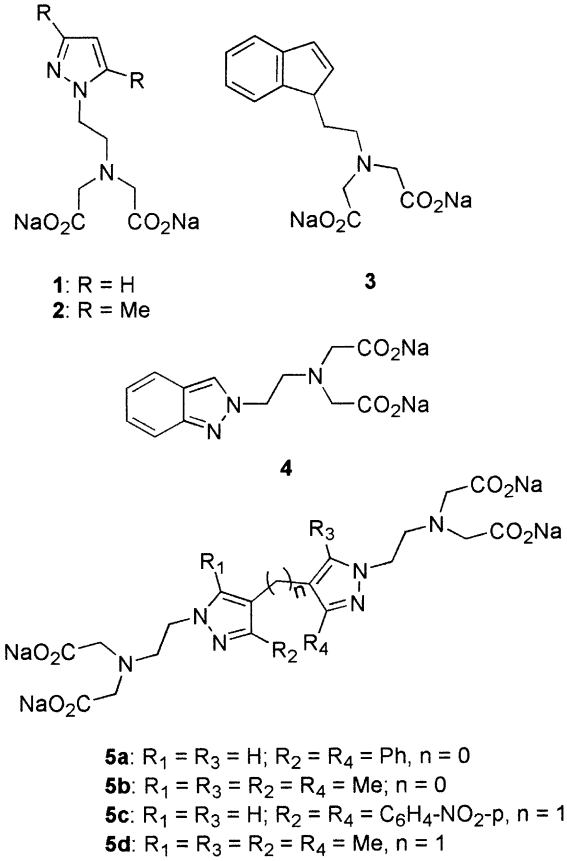


Figure 1. Structures of monoazolic complexones **1**–**4**[12] and bis- or biazolic complexones **5a**–**d**.

Results

Chemical syntheses

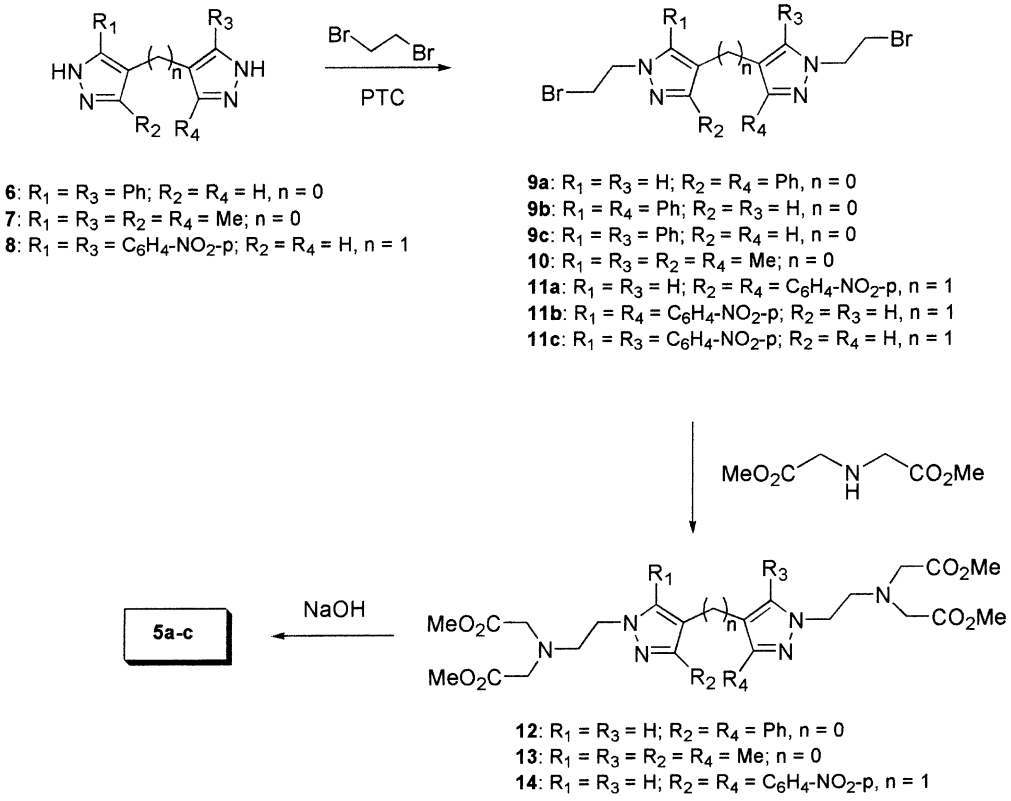
Compounds **5a**–**d** were prepared by reaction of the corresponding bi or bisazoles **6**–**8**,[21–23] with an excess of 1,2-dibromoethane using liquid-liquid phase transfer catalysis (Scheme 1). In all cases, the corresponding 3,3′-, 3,5′-, 5,5′-(2-bromoethylpyrazol-1yl) substituted isomers were isolated, being the 3,3′-regioisomers **9a**, **10** and **11a** the major products. The alkylation of **7** yielded the dibromoderivative **10**, the corresponding monosubstituted compound **15**, and a complex mixture of polymeric material.

Subsequently, the reaction of the 2-bromoethyl derivatives **9a**, **10** and **11a** with four equivalents of methyl iminodiacetate gave compounds **12**–**14**. Basic hydrolysis of **12**–**14** yielded the corresponding complexones **5a**–**c**.

Bispyrazole **8** was synthesized from the aldehyde **16** via tosylhydrazone intermediate, as reported by Lepage and Lepage (Fig. 2).[24] The formation of only one isomer (*E*,*E*) of **16** was described then. However, we reproduced this reaction, in the same conditions and obtained a mixture of *E*,*E*/*E*,*Z* isomers, both compounds being separated by column chromatography on silica gel and characterized by spectroscopic methods.

^{1}H NMR of *E*,*E*-**16** depicted a characteristic singlet at 9.52 ppm corresponding to both isochronous CHO groups, while the spectrum of the *E*,*Z*-**16** isomer showed two singlets at 9.86 and 9.74 ppm derived from the two anisochronous CHO groups. According to the synthetic strategy employed to give pyrazole **6**, the reaction of **16** (*E*,*E*/*E*,*Z*) with tosylhydrazine in MeOH yielded the tosylhydrazone **17**, as the only *E*,*Z*-isomer. The assignment of **17** was based on NMR spectra. The ^{1}H NMR spectrum depicted two singlets at 2.26 and 2.09 ppm corresponding to the two different methyl groups. ^{13}C NMR confirmed this structure by showing the corresponding methyl resonances at 20.9 and 20.7 ppm, respectively. Compound **17** provides an interesting example where the rotational barrier for *cis*–*trans* interconversion of the isomers is low enough[25] to yield only the compound most stable thermodynamically. Indeed, theoretical conformational analysis (HF/6-31G*//HF/3-21G) of **17** showed a higher stabilization of the *E*,*Z* isomer through the formation of two intramolecular hydrogen bonds.

The synthesis of **5d** followed an alternative approach (Scheme 2). **18a**[26] reacted with paraformadehyde in



Scheme 1. Syntheses of complexones **5a**–**c**.

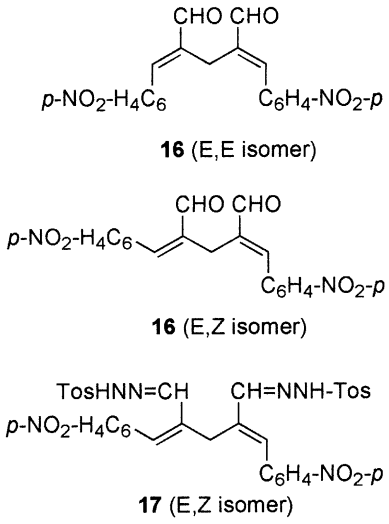


Figure 2. Structures of the isomers of **16** and **17**.

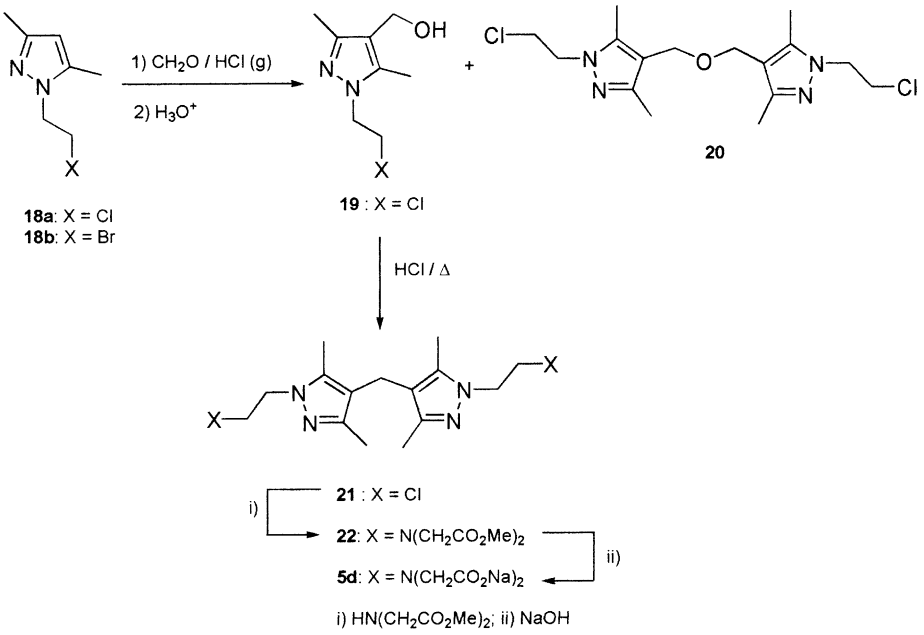
1,2-dichloroethane saturated with dry HCl to yield the corresponding 4-chloromethylpyrazole.[27] Subsequent acid hydrolysis gave the alcohol **19** and a small amount of the ether **20**. Chloromethylation of **18b** was carried out under the same conditions used for **18a**, to obtain a mixture of products. The mass spectrum of this mixture revealed the presence of the corresponding chloroethyl derivatives produced through Br–Cl exchange.

Chloroethyl derivative **21** was obtained by refluxing alcohol **19** in concd HCl. Compounds **22** and **5d** were obtained by reaction of **21** with methyl iminodiacetate following alkaline hydrolysis similar to that described above.

Physicochemical characterization

Gd(III) binding. Binding isotherms (22 °C) of complexones **5a–d** for Gd(III) were obtained spectrophotometrically by competition with Arsenazo III (ArsIII) as indicated in the Experimental.[12] Figure 3 shows a representative example of the results obtained with **5b**, but similar isotherms were obtained for complexones **5a**, **5c** and **5d**.

Gd(III) chloride titrations of the model solutions (0.15 M ionic strength, pH 6.5, 22 °C) containing ArsIII depicted typical hyperbolic saturation curves (solid lines), providing a value for the apparent dissociation constant of the Gd(III)–ArsIII complex of ca. 20 μM. However, when 1 mM complexones **5a–d** were present in the titration mixtures, competitive binding of Gd(III) to the complexone and to ArsIII caused a marked deviation from the typically hyperbolic Gd(III)–ArsIII titration curves. Under these conditions, Gd(III) did not bind appreciably to the complexones below 0.03–0.05 mM, but evident binding occurred at higher Gd(III) concentrations, as revealed by the sharp decrease in the saturation value of the Gd(III)–ArsIII complex (dashed line). This pattern suggested that the transition from free to fully saturated complexones occurred in a narrow range of Gd(III) concentrations, following a sigmoidal trend which indicated cooperative binding of more than one Gd(III) ion per mole of complexone (dotted lines). Non linear least squares fittings of the



Scheme 2. Synthesis of complexone **5d**.

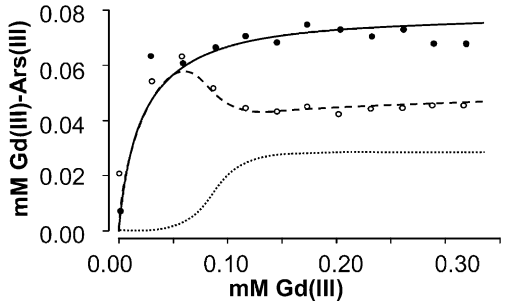


Figure 3. Binding of Gd(III) to ArsIII in the absence (solid lines) or presence (dashed line) of **5a**. Sigmoidal Gd(III) binding to **5a** (dotted line) is calculated as the difference between the solid and dashed lines. Binding parameters $S_{0.5}$ and n reflecting the half maximal saturation of the complexone and the degree of cooperativity can be estimated as indicated in the Experimental (c.f. Table 1).

experimental data to an heuristic model consisting of a single binding site in ArsIII and sigmoidal Gd(III) binding in the complexone, provided optimal fits of the results. This allowed to determine the corresponding parameters $S_{0.5}$ and cooperativity coefficients n for every complexone (Table 1).

MO calculations. We used ab initio molecular orbital calculations[28] to investigate the geometries of the Gd(III) complexes of **5a–d**. This approach has previously provided reliable results in the study of some lanthanide complexes with polyamino carboxylate and phosphinic ligands.[29–32] We calculated first the most stable structures of representative complexone **5b** in the absence of Gd(III) and in the presence of one or two Gd(III) atoms (Fig. 4). Similar calculations were performed with complexones **5a**, **5c** and **5d**.

In the absence of Gd(III), the most stable structure of **5b** was the fully open conformation (**A**, $E=-1764.731996$ a.u.) with both iminodiacetate moieties located at opposite sides of the molecule. In the presence of one

Table 1. Binding parameters obtained from titrations of solutions of ArsIII with Gd(III) chloride in the absence and presence of complexones **5a–d**

Compd	K_d[a] (μM)	$S_{0.5}$[a] (μM)	n[a]
ArsIII	23.3±8.3[b]		
5a		6.7±0.03[b]	6.5±1.0[b]
5b		9.0±0.02	3.2±0.9
5c		35.0±0.08	7.0±0.0
5d		36.1±0.04	4.9±1.9

[a]Binding parameters for complexones **5a–d** were determined by non linear least squares fittings of Gd(III) titrations (Fig. 1), as described in the Experimental.
[b]Results are given as the mean±SD of three independent determinations.

Gd(III) the complexone folded to create an internal cavity in which an hexacoordinated metal (**B**, $E=-1800.990569$ a.u.) was buried between the two iminodiacetate units. In this folded form, the pyrazole groups do not participate in chelation and the two phenyl (**5a**, **5c**) or methyl (**5b**, **5d**) groups fall inside the metal cavity causing steric hinderance and instability of the complex. When two Gd(III) atoms are available, the complexone folds into its most stable structure consisting of two tetradentate complexes of Gd(III), each one involving one azole ring, two carboxylates and one amino group from the closest iminodiacetic acid moiety (**C**, $E=-1836.086424$ a.u.). Under these conditions, both phenyl groups in **5a** and **5c** or methyl groups in **5b** and **5d**, remain outside the coordination centers avoiding steric hindrance and resulting in a more stable chelate.

Figure 5(A) illustrates in more detail the optimal geometry calculated for one of the azole moieties of the dimeric Gd(III) complex of **5a–d**.

The Gd(III) atom is located above the O1–O2–N plane by 0.703 Å (**5a**), 0.641 Å (**5b**), 0.689 Å (**5c**) and 0.627 Å (**5d**). The phenyl and *p*-nitrophenyl rings of complexones **5a** and **5c** deviate from coplanarity with the azole

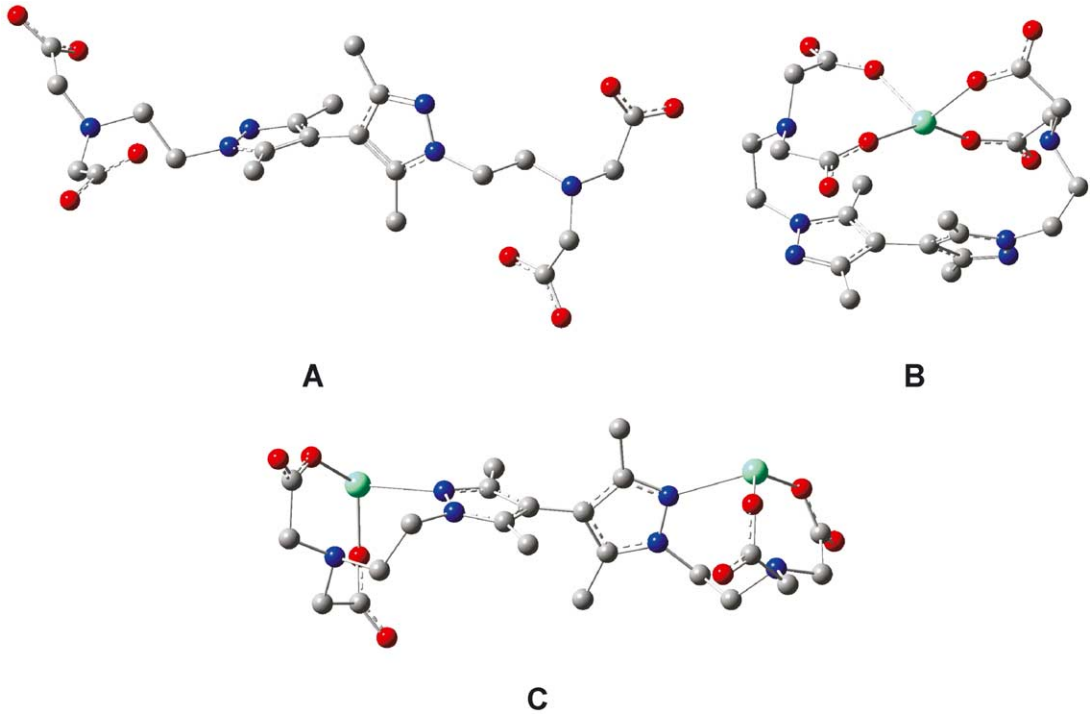


Figure 4. Optimized structures of biazolic complexone **5b** calculated in the absence (A) and presence of one (B) or two (C) Gd(III) atoms. Calculations were performed using the HF/3-21G basis set for the ligand, and the 46 + 4f[7] ECP with the [5s4p3d]-GTO valence basis set for the metal, as indicated in the Experimental. The Gd(III) atoms are shown in green.

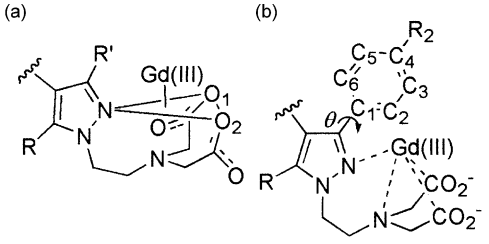


Figure 5. Coordination geometry around the metal in **5a–d** complexes: (a) distance from the metal to the N–O1–O2 plane of the ligand; (b) effect of the phenyl ring.

ring by $\theta = 60.63°$ and $\theta = 58.42°$, making the distance from Gd(III) to the O1–O2–N plane to increase in those complexones containing aromatic rings (Fig. 5B). This effect is due to the participation of the π electrons of the aromatic substituent in the complexation. Distances from the phenyl carbons to Gd(III) were (**5a**; **5c**, Å); (C1 3.091; 3.213, C2 3.023; 3.080, C3 3.604; 3.736, C4 4.155; 4.371, C5 4.218; 4.501, C6 3.734; 3.971). These distances are longer in **5c** than in **5a**, because of the electron withdrawal effect of the *p*-nitro group. Interestingly, we have described previously metal-phenyl interactions in complexes of Rh(I) with bis(pyrazol-1-yl)phenylmethane.[33]

Because of the importance of hydration determining the relaxivity properties, we calculated the structures of the hydrated complexes of **5a–d** Gd(III)–4(5)H_2O, EDTA Gd(III)–2(3)H_2O and DTPA Gd(III)–H_2O. Figure 6 shows optimized hydrated structures for **5b** (B) and its

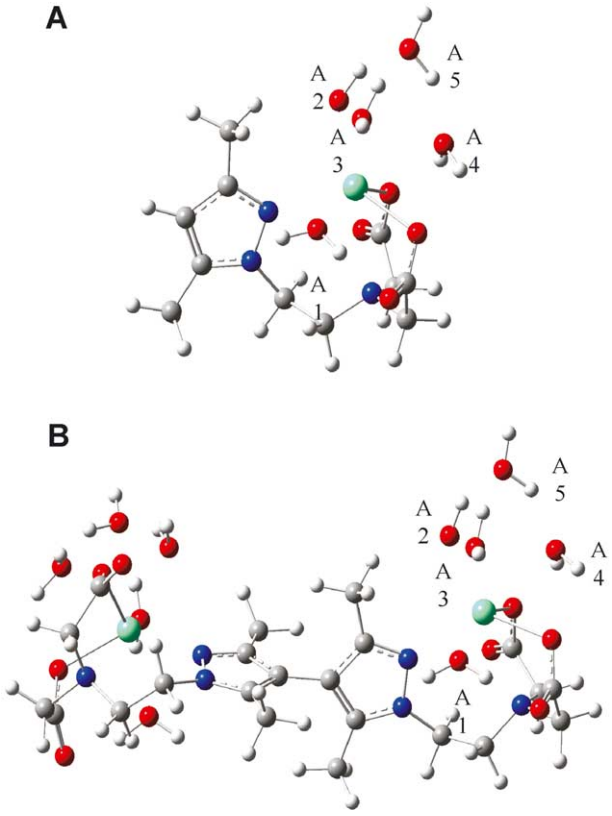


Figure 6. Calculated structures of **2** (A) and **5b** (B) in the presence of five water molecules (A1–A5) in the inner coordination sphere of Gd(III). Note that A5 is excluded from the coordination center. Calculations were performed as in Figure 4.

monomeric analogue **2** (A) in the presence of five water molecules.

In **5b**, optimized Gd(III)–O water distances were: Gd(III)–$(OH_2)_{A1}$ 2.452 Å; Gd(III)–$(OH_2)_{A2}$ 2.434 Å; Gd(III)–$(OH_2)_{A3}$ 2.391 Å; Gd(III)–$(OH_2)_{A4}$ 2.603 Å; Gd(III)–$(OH_2)_{A5}$ 3.741 Å. In the case of **2**, Gd(III)–O water distances were: Gd(III)–$(OH_2)_{A1}$ 2.454 Å; Gd(III)–$(OH_2)_{A2}$ 2.427 Å; Gd(III)–$(OH_2)_{A3}$ 2.393 Å; Gd(III)–$(OH_2)_{A4}$ 2.628 Å; Gd(III)–$(OH_2)_{A5}$ 3.730 Å. Even though five water molecules were included in the calculations, only four molecules $(OH_2)_{A1}$–$(OH_2)_{A4}$ became directly coordinated to Gd(III) in the optimized structures, the fifth one $(OH_2)_{A5}$ being excluded from the first coordination sphere in all cases. When only four water molecules were included in the calculation, all four water molecules showed very similar distances to the metal ion ($2.43 < Gd(III)–O < 2.53$ Å). Table 2 summarizes some relevant geometrical parameters of these molecules.

The distances (Å) calculated by ab initio methods match well the experimental measurements for Gd(III)DTPA(H_2O).[34] Some examples include (Å experimental, Å calculated) the Gd–O_{water} distance (2.490, 2.595) and the distances from Gd to the oxygen atoms and nitrogen atoms of the iminodiacetate and aminoacetate carboxylates Gd(III)-O (2.363–2.437, 2.311–2.449) and Gd(III)–N (2.629–2.710, 2.719–2.770). These results provide a measure of confidence on the geometrical parameters calculated for the Gd(III) complexes of **5a–d** for which no experimental X-ray diffraction measurements are available yet.

Relaxivity. Table 3 depicts the results of longitudinal (T_1) and transversal (T_2) relaxation time measurements and the corresponding r_1 and r_2 relaxivities of Gd(III) chloride solutions and the Gd(III) complexes of **5a–d**, EDTA, DTPA and IDA performed at 1.5 Tesla as indicated in the Experimental. A 10-fold excess of the indicated ligand was used in these measurements to minimize the effects of free Gd(III). As expected, the free ligand did not contribute appreciably to the observed relaxation times. r_1 and r_2 values of the corresponding Gd(III) complexes followed the order **5c** > / = **5d** > **5b** > **5a** > aqua > EDTA > DTPA > IDA. When expressed per mol of Gd(III) ion, r_1 and r_2 relaxivity values of complexes **5a–d**, were significantly higher than Gd(III) chloride solutions of the same concentration, two to three fold higher than the Gd(III)EDTA and Gd(III)DTPA reference complexes and 4- to 6-fold higher than Gd(III)IDA. It should be noted here that relaxivity of Gd(III)DTPA is strongly dependent of ionic strength. While in the presence of 155 mM NaCl solution the r_1 and r_2 values are 4.3, in absence of saline solution, as it happens in distilled water solution, the values decrease to 3.5 (see Table 3). Moreover, since the Gd(III) complexes of **5a–d** appear to contain two Gd(III) atoms per mol of complexone, r_1 and r_2 relaxavities may be doubled when expressed per mole of complexone. These values remain to our knowledge, among the largest reported for compounds of this molecular weight[3,10] and similar to those of the Gd(III) chelates of DOTA and DTPA conjugated to synthetic polymers of ca. 20 kDa[35–37] or to those of dendrimeric materials.[38,39]

Using the same methodology, we investigated the relaxivities of some of these complexes in rat plasma containing or not 5 mM complexones and 0.5 mM Gd(III). The following values (T_1, T_2, s) were obtained; rat plasma (2.01 ± 0.03, 0.77 ± 0.05), **5a** (0.16 ± 0.04, 0.10 ± 0.04), **5b** (0.14 ± 0.06, 0.09 ± 0.02) and **5d** (0.13 ± 0.01, 0.08 ± 0.01). These values correspond to relaxivities (ion r_1, ion r_2 s^{-1} mM^{-1}) of **5a** (11.83 ± 0.33, 18.12 ± 0.01), **5b** (13.63 ± 0.08, 20.06 ± 0.05) and **5d** (13.94 ± 0.12, 20.84 ± 0.013). The relaxivity values obtained in plasma were similar than those found in aqueous solution suggesting no significant enhancement derived from binding of the complexes to plasma components.

Biological evaluation

In vitro toxicity of complexones **5a** and **5b** was assayed in cultures of C6 cells by monitoring the release of lactic dehydrogenase (LDH) to the incubation medium (Fig. 7). Cells were incubated for 60 min with ligands **5a**, **5b**

Table 2. Optimized structural parameters (Å) for the Gd(III) complexes of **5a–5d**, EDTA and DTPA as refined by ab initio calculations

Ligand	d(Gd–O_1)[a]	d(Gd–O_2)[a]	d(Gd–O_3)[a]	d(Gd–O_4)[a]	d(Gd–O_5)[b]	d(Gd–N_1)[c]	d(Gd–N_2)[c]	d(Gd–N_3)[d]	d(Gd–N_{Azol})[e]	d(Gd–O_{water})[f]	(L_o/2)[g]
5a	2.249	2.284	2.249	2.284		2.794	2.794		2.596	2.427–2.530	7.77
5b	2.261	2.293	2.261	2.293		2.734	2.734		2.587	2.427–2.497	7.84
5c	2.245	2.278	2.245	2.278		2.770	2.770		2.614	2.431–2.520	8.14
5d	2.261	2.294	2.261	2.294		2.736	2.736		2.589	2.429–2.492	8.34
EDTA[h]	2.304	2.312	2.304	2.312		2.632	2.632			2.499	4.35[i]
	2.316	2.302	2.382	2.318		2.722	2.731			2.482–2.858	
DTPA	2.383	2.449	2.341	2.311	2.350	2.770	2.719	2.807		2.595	4.43

[a] d(Gd–O_{1-4}), distance (Å) from Gd to the oxygen atoms of the iminodiacetate carboxylate groups.
[b] d(Gd–O_5), distance (Å) from Gd to the oxygen atoms of the central aminoacetate carboxylate group.
[c] d(Gd–N_{1-2}), distance (Å) from Gd to the nitrogen atoms (N_1,N_2) of the iminodiacetate groups.
[d] d(Gd–N_3), distance (Å) from Gd to the nitrogen atom (N_3) of the central aminoacetate group.
[e] d(Gd–N_{azol}), distance from Gd to the azolic nitrogen atom.
[f] d(Gd–O_{water}), distance from Gd(III) to the oxygen of the water molecule in complexes with $q = 1$. In complexes where $q > 1$, numbers indicate the range of distances from Gd(III) to the different oxygens of water molecules.
[g] L_o, molecular diameter.
[h] Upper numbers refer to Gd(III)EDTA–$2H_2O$ and lower numbers refer to Gd(III) EDTA–$3H_2O$.
[i] 4.89 Å for $HEDTA^{3-}$ ligand.

Table 3. Longitudinal and transversal relaxation times (T_1 and T_2) and relaxivities (r_1 and r_2) of aqueous solutions of **5a–d**, EDTA, DTPA and IDA and of the corresponding Gd(III) complexes determined at 1.5 Tesla[a]

Additions to model solution[b]	T_1 (s)[c]	T_2 (s)[c]	Ion r_1 (s^{-1} mM^{-1})	mol r_1 (s^{-1} mM^{-1})	Ion r_2 (s^{-1} mM^{-1})	Mol r_2 (s^{-1} mM^{-1})
None	3.81±0.06	2.10±0.01				
Gd(III)	0.22±0.001	0.17±0.002	8.3±0.02	8.3±0.02	10.8±0.1	10.8±0.1
5a	3.84±0.006	2.30±0.005				
5a + Gd(III)	0.16±0.005	0.15±0.001	12.0±0.4	24.0±0.8	12.2±0.1	24.4±0.2
5b	3.6±0.1	2.07±0.01				
5b + Gd(III)	0.14±0.001	0.11±0.001	14.6±0.08	29.2±0.1	16.9±0.03	33.80±0.06
5c	3.26±0.08	2.39±0.01				
5c + Gd(III)	0.10±0.005	0.06±0.001	18.5±0.1	37.0±0.2	35.0±0.01	70.0±0.01
5d	3.80±0.02	2.36±0.01				
5d + Gd(III)	0.11±0.001	0.10±0.001	17.7±0.1	35.4±0.2	20.0±0.02	40.0±0.04
EDTA	3.81±0.01	2.04±0.001				
EDTA + Gd(III)	0.35±0.001	0.30±0.001	5.2±0.02	5.2±0.02	5.6±0.01	5.6±0.01
DTPA	3.61±0.03	1.39±0.001				
DTPA + Gd(III)	0.41±0.001	0.35±0.001	4.3±0.01	4.3±0.01	4.3±0.01	4.3±0.01
	0.06±0.01	0.05±0.001	3.5±0.03[d]	3.5±0.03[d]	3.6±0.02[d]	3.6±0.02[d]
IDA	3.60±0.2	1.29 ±0.001				
IDA + Gd(III)	0.45±0.002	0.36±0.002	3.9±0.04	3.9±0.04	4.0±0.01	4.0±0.01

[a]Determined in a Bruker Minispec 1.5 T using the inversion recovery (T_1) and Carr Purcell Meiboom Gill (T_2) sequences as described in the Experimental.
[b]Model solutions contained 100 mM Tris/HCl (pH 6.5, 37 °C), 155 mM NaCl and where indicated 5 mM complexone or 5 mM complexone and 0.5 mM Gd(III).
[c]Results are expressed as mean±SD of at least three independent T_1 or T_2 measurements in each sample.
[d]Solution contained 5 mM pure complex Gd(III)DTPA in distilled water.

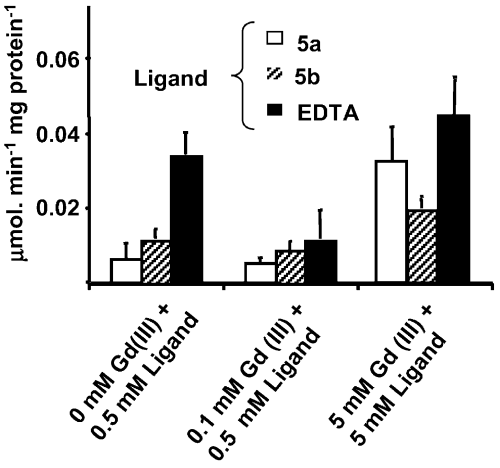


Figure 7. Release of LDH to the incubation medium in cultures of C6 cells incubated with increasing concentrations of Gd(III) and complexones **5a**, **5c** or EDTA.

and EDTA in the absence of Gd(III) or in the presence of increasing concentrations of Gd(III) and complexones. The release of LDH induced by the Gd(III) complexes of **5a** or **5b** was smaller than that induced by Gd(III)EDTA, indicating a lower toxicity.

Discussion

Causes of improved relaxivity

The heterocyclic complexones prepared in this study present important improvements in relaxivity as compared to the earlier series.[12] It is possible to understand this on the basis of the dynamics and structure of the corresponding Gd(III) complexes as described by the Solomon–Bloembergen–Morgan theory of paramagnetic relaxation[3,8,40] and MO calculations, respectively.[32]

Considering inner sphere effects only, the theory of paramagnetic relaxation indicates that longitudinal (r_1) and transversal (r_2) relaxivities of a Gd(III) complex are described by the expressions;

$$r_1 = 1/T_1^{IS} = q \cdot P_m/(T_{1M} + \tau_m) \quad (1)$$

$$r_2 = \left(1/T_2^{IS}\right) = P_m/\tau_m\left(\left(T_{2M^{-2}} + \tau_{m^{-1}} + \Delta\omega_m^2\right)/\left(\tau_{m^{-1}} + T_{2M^{-1}}\right)^2 + \Delta\omega_m^2\right) \quad (2)$$

where q is the number of coordinated water molecules to Gd(III), P_m represents the mole fraction of bound water nuclei, T_{1M} or T_{2M} are the T_1 or T_2 of bound water molecules, τ_m is the residence time of the water molecules in the inner sphere of the Gd(III) complex and $\Delta\omega_m^2$ refers to the squared difference of chemical shifts between metal bound and bulk water molecules.

Longitudinal relaxivities r_1 of different Gd(III) complexes are dominated normally, for the same P_m, by the hydration number q and the value of T_{1M} (ms or s range), since τ_m values are much shorter (ns range). At clinical fields, T_{1M} is approximated by the expression;

$$1/T_{1M} = K/r^6\left[3\tau_c/\left(1 + \omega_i^2\tau_c^2\right)\right] \quad (3)$$

where K is a constant, r is the distance between the unpaired electrons of Gd(III) and the hydrogens of the coordinated water molecule, ω_i is the observation fre-

quency and τ_c is the reorientational correlation time of dipolar interaction between the unpaired electron and the water molecule described by

$$1/\tau_c = 1/T_{1e} + 1/\tau_m + 1/\tau_r \quad (4)$$

In this expression, T_{1e} is the relaxation time of the unpaired electron ($1 < T_{1e} < 10$ ns), τ_m is the residence time of water in the complex ($100 < \tau_m < 2500$ ns) and τ_r is the rotational correlation time of the complex ($50 < \tau_r < 500$ ps).[3,8]

Concerning the hydration number q, the Gd(III) chelates of **5a–d** have $q = 4$ (c.f. Fig. 6), as compared to $q = 9$ in the aqua Gd(III), $q = 5$ in Gd(III)IDA, $q = 4$ in Gd(III)NOVAN[41] or $q = 1$ in Gd(III)DTPA or Gd(III)-DOTA complexes.[3,8] However, it is possible to show here that the increased relaxivity observed in the Gd(III) complexes of **5a–d** as compared to Gd(III)DTPA or Gd(III)DOTA is not exclusively due to their increased hydration. Indeed, r_1 values of bis- and bi-azolic Gd(III) complexes vary importantly within the series ($12.0 < r_1 < 18.5$ s^{-1} mM^{-1}, Table 3), while the hydration number of the different complexes remains the same. Moreover, the Gd(III) complexes **5a–d** depict higher r_1 than those of molecules with even higher hidration numbers such as the nonahydrated Gd(III) aqua complex and the pentahydrated Gd(III)IDA complex (Table 3). Thus, the increased r_1 values observed must include additional contributions of T_{1M}, τ_m or both (c.f. 1).

The possible changes in T_{1M} may include modifications in r or τ_c (c.f. 3). The value of r influences greatly r_1 since it enters eq 3 as the sixth power. The calculated distances r between Gd(III) and the oxygen atom of the water molecules in the Gd(III) complexes **5a–d** are similar to those found in Gd(III)DTPA complex (Table 2), suggesting that distances from Gd(III) to the hydrogens of water molecules are in the same range also. Therefore, a significant decrease in r can be discarded as a relevant contribution to increased relaxivity in these Gd(III) complexes. Consequently, the dominant change in T_{1M} must occur in τ_c (c.f. 3 and 4), which at 1.5 Tesla is normally controlled by the value of the rotational correlation time τ_r expressed as:

$$\tau_r = 4\pi a^3 \eta / 3kT \quad (5)$$

where a is the molecular radius of gyration, η the microviscosity, k the Boltzman constant and T the absolute temperature. It is possible to compare the rotational correlation times of two molecules τ_{r1} and τ_{r2} with molecular radii a_1 and a_2, using expression 6 under the assumption that both molecules behave as rigid rotors in a medium of identical microviscosity and temperature;

$$\tau_{r1}/\tau_{r2} = a_1^3/a_2^3 \quad (6)$$

Therefore, from the known values of $\tau_r = 51$ ps of Gd(III)DTPA[3] and molecular radii of the Gd(III) complexes of DTPA[34] and **5a–d** ($L_o/2$ in Table 2) it is possible to calculate using 6 the values of τ_r (ps) for complexes of **5a** (303), **5b** (310), **5c** (345), **5d** (370). These calculated τ_r values of **5a–d** Gd(III) chelates are rather similar to those measured for much larger molecular weight linear polymers of Gd(III)DTPA (232 ps),[8,10,42] sugar derivatives as Gd(III)DTPA-BENGALAA (265 ps)[43] and slightly smaller than those measured for some Gd(III)DO3A dendrimers (580–870 ps).[44] It is interesting to note that the calculated τ_r values of the series increase in the order **5a** < **5b** < **5c** < **5d** in parallel with the r_1 relaxivities shown in Table 3. For the same hydration value through the series, this trend suggests an important contribution of τ_r to the observed r_1. Indeed, measured r_1 values of the heterocyclic complexones reported here, are similar to those of ca. 16–18 s^{-1} mM^{-1} of texaphyrins, a series of porphyrin analogues with similarly high hydration number ($q = 3.5$) and τ_r (ca. 295 ps).[20,45] However, although the effects of q and τ_r appear to dominate the observed increases in relaxivity, additional contributions from more favorable τ_m or T_{1e} values or the formation of intermolecular Gd(III) complexes involving more than one complexone molecule may also be considered.[11]

The particularly large values of transversal relaxivity r_2 determined for the Gd(III) complexes **5a–d** merit special consideration. In addition to the circumstances affecting r_1, the squared $\Delta\omega^2$ term containing the added differences in chemical shifts between the four bound water molecules and the bulk solvent (c.f. eq 2), must contribute importantly as described for the former series **1–4**.[12] In this respect, the particularly large r_2 relaxivity found in Gd(III)**5c** (ca. 70 s^{-1} mM^{-1}) is remarkable. This effect may involve; (i) a higher polarization of the four water molecules bound to Gd(III) due to the electron withdrawal effects of the *p*-nitro group and (ii) a more favorable water exchange in a less crowded coordination cavity as revealed by the longer distances calculated from Gd(III) to the benzene ring.

Considerations on Gd(III) binding

Present results reveal also some interesting aspects on the relationship between chemical structure and thermodynamic stability of heterocyclic Gd(III) complexes. In particular, cooperative Gd(III) binding to the same complexone molecule represents to our knowledge, a novel property of these ligands. The $S_{0.5}$ values of **5a** and **5b** are lower than those of **5c** and **5d**, suggesting that the presence of the methylene bridge decreases the stability of the Gd(III) complexes. Complexones **5a** and **5c** depicted higher cooperativity values than **5b** and **5d**, indicating that participation of the phenyl rings in both chelation centers favors an easier folding of the complex. In general, compounds **5a–d** show significantly improved Gd(III) binding capacity as compared to the earlier series of azolic complexones.[12] However, their $S_{0.5}$ values remain well below the stability constants for Gd(III) of classical complexones such as DTPA or DOTA.[3,4]

Concluding remarks

In summary, we described the syntheses, physicochemical properties and toxicological evaluation of a novel series of heterocyclic complexones. These ligands appear to form two intramolecular Gd(III) complexes depicting large r_1 and r_2 relaxivity values but their unfavorable binding properties for Gd(III) do not advise their use as Gd(III) chelators in the clinic. However, they may become useful clinical chelators of less toxic metals similar to dipyridoxaldiphosphate DPDP,[46,47] a ligand currently employed in the clinic to provide delayed release and organ selectivity of Mn^{2+}.

Experimental

General

Melting points were obtained on a microscope hot stage and are uncorrected. Elemental analyses were performed with a Perkin-Elmer 240 apparatus. Mass spectra were carried out on a GC/mass spectrometer Schimadzu QP-5000 at 70 eV. IR spectra were determined on a Philips PU-9700 spectrophotometer. NMR spectra were obtained with a Bruker DRX-400 (400.13 MHz for ^{1}H, and 100.03 MHz for ^{13}C) and Bruker AC-200 (200.13 MHz for ^{1}H, and 50.33 MHz for ^{13}C). ^{1}H and ^{13}C chemical shifts (δ) in $CDCl_3$ are given from internal tetramethylsylane and ^{13}C chemical shifts (δ) in D_2O are given from external DMSO-d_6 with an accuracy of ±0.01 ppm for ^{1}H and ±0.1 ppm for ^{13}C. The residual water signal in ^{1}H NMR spectra obtained in D_2O solution was suppressed when necessary using a 1 s (low power, 0.5 watts) presaturating pulse applied with decoupler. ^{1}H–^{1}H coupling constants (J) are accurate to ±0.2 Hz for ^{1}H NMR spectra. TLC chromatography was performed on DC-Aulofolien/Kieselgel 60 F_{245} (Merck) and column chromatography through silica gel Merck 60 (230–400 mesh). Methyl iminodiacetate hydrochloride was basified with solid Na_2CO_3 in the minimum amount of water prior to use. D_2O (99.9 D) was purchased from Appollo Scientific (Stockport, UK). The rest of the products were obtained from Aldrich.

4,4′-Methylenebis[5-(4-nitrophenyl)-1*H*-pyrazole] (8). A mixture of *p*-nitrobenzaldehyde (4.53 g; 30 mmol), 2-methoxy-2,3-dihydro-4*H*-pyrane (1.82 g; 16 mmol), AcOH (2 mL), H_2O (3 mL) and piperidine (two drops) was refluxed for 24 h. After cooling, the aqueous layer was decanted and the solvent was evaporated in vacuo. The residue was purified by column chromatography on silica gel. Elution with hexane/AcOEt (85:15) gave **16** (1.02 g; 19 %) as a white solid (*E*,*E*;[24] *E*,*Z* isomers). *E*,*Z* isomer (mp 143–145 °C AcOEt/hexane): IR (KBr): ν 1685, 1605, 1510 cm^{-1}. MS *m*/*z* (%): 366 (M^+, 12), 338 (32), 321 (24), 215 (53), 202 (91), 189 (21), 161 (31), 128 (27), 115 (100), 89 (34), 77 (31), 63 (30). ^{1}H NMR (200 MHz, $CDCl_3$, δ): 9.86 (s, 1H, CHO), 9.74 (s, 1H, CHO), 8.32 (d, 2H, J = 8.8 Hz, AA′XX′ system, aromatics), 8.24 (d, 2H, J = 8.8 Hz, AA′XX′ system, aromatics), 7.68–7.60 (2 d, 4H, AA′XX′ system, aromatics), 7.44 (s, 1H, CH=C), 7.40 (s, 1H, CH=C), 3.66 (s, 2H, CH_2).

To a solution of tosylhydrazine (1.55 g; 8.36 mmol) in MeOH (10 mL) was added **16** (1.53 g; 4.18 mmol, mixture of *E*,*E*/*E*,*Z* isomers) and the reaction mixture was refluxed for 1 h and 30 min. After cooling, the solid was filtered to give **17** (1.71 g; 58%, only *E*,*Z* isomer) as a yellow solid (mp 208–210 °C, MeOH). IR (KBr): ν 3480, 3200, 1590, 1505, 1340, 1160, 1065 cm^{-1}. ^{1}H NMR (200 MHz, DMSO-d_6, δ): 8.15 (d, 2H, J = 7.6 Hz, AA′XX′ system, aromatics), 8.10 (d, 2H, J = 7.6 Hz, AA′XX′ system, aromatics), 7.91 (s, 1H, CH=C), 7.73 (s, 1H, CH=C), 7.69 (d, 2H, J = 8.2 Hz, AA′XX′ system, aromatics), 7.48–7.40 (2 d, 4H, AA′XX′ system, aromatics), 7.32 (d, 2H, J = 8.3 Hz, AA′XX′ system, aromatics), 7.17 (s, 1H, CH=N), 7.13–7.06 (2 d, 4H, AA′XX′ system, aromatics), 6.31 (s, 1H, CH=N), 3.48 (s, 2H, CH_2), 2.26 (s, 3H, CH_3), 2.09 (s, 3H, CH_3).

A mixture of **17** (3.17 g; 4.51 mmol), sodium methoxide (1.1 g; 20.3 mmol) and triethylen glycol (7 mL) was heated at 100 °C for 2 h. After cooling, the reaction crude was poured into ice-water and AcOH (3 mL). The solid obtained was filtered, and washed with saturated solution of $NaHCO_3$, H_2O and MeOH. Finally, the solvent of the filtrate was removed in vacuo to give **8** (0.56 g; 33%) as a brown solid (mp 129–140 °C, decomp) and 1.2 g of polymeric material. IR (KBr): ν 3390, 3210, 1595, 1505, 1335, 1855 cm^{-1}. MS *m*/*z* (%): 390 (M^+, 100), 168 (95). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 8.21 (d, 4H, J = 8.8 Hz, AA′XX′ system, aromatics), 7.87 (d, 4H, J = 8.8 Hz, AA′XX′ system, aromatics), 7.46 (s, 2H, H_3), 4.09 (s, 2H, CH_2).

Alkylation of bi and bispyrazoles with 1,2-dibromoethane

General procedure. A mixture of bi- or bispyrazole (1 equivalent), (40%) NaOH (3 equivalents), BTBA (0.025 equivalents) and dibromoethane (10 equivalents) was refluxed until the consumption of the starting material was detected by TLC. The organic layer was then separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic extracts were washed with water, dried over $MgSO_4$ and evaporated in vacuo. The residue was purified by column chromatography on silica gel.

3,3′-Diphenyl-1,1′-bis(2-bromoethyl)-4,4′-bipyrazole (9a). According to the general procedure we used bipyrazole **6** (2.2 g; 7.69 mmol), NaOH (0.92 g; 23.07 mmol), BTBA (62 mg; 0.19 mmol), 1,2-dibromoethane (23.13 g; 123.04 mmol) and H_2O (1.5 mL) and the mixture was refluxed for 1 h and 30 min. Elution with CH_2Cl_2/MeOH (98:2) gave **9a** (1.17 g; 30%) as a white solid (mp 137–139 °C, EtOH), **9b** (0.69 g; 18%) as a yellow oil and **9c** (0.25 g; 6%) as a white solid (mp 170–175 °C, CH_2Cl_2/hexane). **9a**: IR (KBr): ν 1600, 1515, 1510, 1440, 1415, 1350, 1320, 1270, 1180, 950, 775, 720, 695 cm^{-1}. MS *m*/*z* (%): 502 (M^+ + 2, 31), 500 (M^+, 68), 498 (M^+ − 2, 32), 233 (100), 77 (10), 63 (51). ^{1}H NMR (200 MHz, $CDCl_3$, δ): 7.61–7.48 (m, 4H, aromatics), 7.28 (m, 2H, H_5), 7.25–7.16 (m, 6H, aromatics), 4.46 (t, 4H, J = 6.2 Hz, CH_2–N(Azole)), 3.74 (t, 4H, J = 6.2 Hz,

CH_2-Br); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 150.6, 133.2, 131.5, 128.2, 127.5, 110.6, 53.6, 30.4. Anal. calcd for $C_{22}H_{20}Br_2N_4$: C, 52.81; H, 4.04; N, 11.20. Found: C, 53.03; H, 4.23; N, 11.08. **9b**: IR (KBr): ν 1495, 1445, 1305, 1215, 940, 760, 700 cm^{-1}. MS: *m/z* (%): 502 ($M^+ + 2$, 47), 500 (M^+, 100), 498 ($M^+ - 2$, 48), 392 (20), 286 (39), 107 (26), 77 (41). ^1H NMR (200 MHz, $CDCl_3$, δ): 7.45 (s, 1H, H_3), 7.44–7.38 (m, 2H, aromatics), 7.31 (m, 6H, aromatics), 7.13–7.08 (m, 3H, aromatics and H_5), 4.36 (t, 2H, $J = 6.4$ Hz, CH_2–N(Azol-H_5)), 4.35 (t, 2H, $J = 6.8$ Hz, CH_2–N(Azol-H_3)), 3.67 (t, 2H, $J = 6.8$ Hz, $BrCH_2CH_2$–N(Azol-H_3)), 3.65 (t, 2H, $J = 6.4$ Hz, $BrCH_2CH_2$–N(Azol-H_5)); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 150.6, 143.6, 141.5, 139.7, 133.3, 130.6, 129.9, 129.3, 128.6, 128.1, 127.7, 127.4, 112.2, 110.6, 53.4, 50.3, 30.3, 29.7. **9c**: IR (KBr): ν 1485, 1440, 1395, 1325, 1285, 1230, 970, 935, 860, 790, 750, 700, 630 cm^{-1}. MS *m/z* (%): 502 ($M^+ + 2$, 46), 500 (M^+, 100), 498 ($M^+ - 2$, 46), 392 (17), 286 (68), 107 (49), 77 (37). ^1H NMR (200 MHz, $CDCl_3$, δ): 7.33–7.21 (m, 8H, aromatics and H_3), 7.03–6.99 (m, 4H, aromatics), 4.23 (t, 4H, $J = 6.9$ Hz, CH_2–N(Azole)), 3.56 (t, 4H, $J = 6.9$ Hz, CH_2-Br); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 140.8, 138.7, 129.9, 129.6, 128.7, 128.6, 112.2, 50.1, 29.3.

1,1′-Bis(2-bromoethyl)-3,3′,5,5′-tetramethyl-4,4′-bipyrazole (10). According to the general procedure, we employed bipyrazole **7** (0.96 g; 5.05 mmol), NaOH (0.6 g; 15.2 mmol), BTBA (40 mg; 0.26 mmol), 1,2-dibromoethane (15.03 g; 80.4 mmol) and H_2O (1.5 mL) and the mixture was refluxed for 1 h. Elution with CH_2Cl_2/MeOH (98:2) gave **10** (0.22 g; 11%) as a yellow solid (mp 112–114 °C, hexane) and **15** (0.15 g; 8%) as a yellow oil. **10**: IR (KBr): ν 1735, 1620, 1540, 1500, 1460, 1435, 1400, 1310, 1270, 1130, 955, 900, 825, 720, 640 cm^{-1}. MS *m/z* (%): 404 (M^+, 36), 296 (15), 229 (14), 190 (100), 107 (29). ^1H NMR (400 MHz, $CDCl_3$, δ): 4.27 (t, 4H, $J = 6.7$ Hz, CH_2–N(Azole)), 3.62 (t, 4H, $J = 6.7$ Hz, CH_2–Br), 1.98 (s, 6H, CH_3), 1.93 (s, 6H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 147.6, 137.6, 109.7, 49.6, 30.2, 11.9, 9.7. Anal. calcd for $C_{14}H_{20}Br_2N_4$: C, 41.60; H, 5.00; N, 13.86. Found: C, 42.08; H, 5.11; N, 13.77. **15:** ^1H NMR (200 MHz, $CDCl_3$, δ): 4.42 (t, 2H, $J = 6.4$ Hz, CH_2–N(Azole)), 3.76 (t, 2H, $J = 6.5$ Hz, CH_2–Br), 2.15 (s, 6H, CH_3), 1.98 (s, 3H, CH_3), 1.93 (s, 3H, CH_3).

4,4′-Methylenebis[1-(2-bromoethyl)-3-(4-nitrophenyl)pyrazole] (11a). According to the general procedure we employed bispyrazole **8** (575 mg; 1.47 mmol), NaOH (176 mg; 4.41 mmol), BTBA (12 mg; 0.037 mmol), 1,2-dibromoethane (2 mL; 23.52 mmol) and the mixture was refluxed for 2 h. Elution with hexane/AcOEt (98:2) gave **11a** (115 mg; 13%) as a white solid (mp 270–272 °C, CH_2Cl_2/hexane). IR (KBr): ν 1595, 1500, 1330, 1110, 855, 705 cm^{-1}. MS *m/z* (%): 606 ($M^+ + 2$, 25), 604 (M^+, 49), 602 ($M^+ - 2$, 24), 107 (58), 80 (100). ^1H NMR (400 MHz, $CDCl_3$, δ): 8.23 (d, 4H, $J = 8.9$ Hz, AA′XX′ system, aromatics), 7.80 (d, 4H, $J = 8.9$ Hz, AA′XX′ system, aromatics), 7.28 (s, 2H, H_5), 4.49 (t, 4H, $J = 6.0$ Hz, CH_2–N(Azole)), 4.04 (s, 2H, CH_2), 3.76 (t, 4H, $J = 6.0$ Hz, CH_2–Br). One further elution with the same eluent gave 64 mg of a product which could be identified as compound **11b** and 13 mg of product **11c**. Further purification of these products was not pursued.

Reaction of haloethyl derivatives of bi- or bispyrazole with methyl iminodiacetate

General procedure. A mixture of the corresponding haloethyl derivative (1 equivalent) and methyl iminodiacetate (2 equivalents) was heated at 110 °C until the consumption of the starting material was detected by TLC. After cooling, the mixture was extracted with CH_2Cl_2 and the organic layer was dried over $MgSO_4$. Organic solvent was evaporated in vacuo and the residue was purified by column chromatography on silica gel.

[(2-{1′-[2-(Bismethoxycarbonylmethylamino)ethyl]-3,3′-diphenyl-1′*H*-[4,4′]bipyrazolyl-1-yl}ethyl)methoxycarbonylmethylamino] acetic acid methyl ester (12). According to the general procedure we used **9a** (1 g; 2 mmol) and methyl iminodiacetate (1.29 g; 8 mmol) the mixture being heated at 110 °C for 8 h and 15 min. Elution with CH_2Cl_2/MeOH (98:2) gave **12** (0.844 g; 65%) as a colorless oil. IR (KBr): ν 1750, 1450, 1210, 1190, 1160 cm^{-1}. MS *m/z* (%): 660 (M^+, 15), 601 (12), 587 (23), 286 (25), 174 (100), 128 (43), 116 (35). ^1H NMR (200 MHz, $CDCl_3$, δ): 7.52–7.47 (m, 4H, aromatics), 7.34 (s, 2H, H_5), 7.20–7.13 (m, 6H, aromatics), 4.20 (t, 4H, $J = 6.2$ Hz, CH_2–N(Azole)), 3.61 (s, 12H, CH_3), 3.45 (s, 8H, CH_2–CO_2Me), 3.22 (t, 4H, $J = 6.2$ Hz, CH_2–N); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 171.6, 149.7, 133.6, 131.7, 128.1, 127.3, 127.2, 110.8, 55.6, 54.9, 51.7, 51.5. Anal. calcd for $C_{34}H_{40}N_6O_8 \cdot C_{12}H_6N_6O_{14}$ (1·2 picric acid): C, 49.47; H, 4.16; N, 15.05. Found: C, 50.08; H, 4.54; N, 14.58.

[(2-{1′-[2-(Bismethoxycarbonylmethylamino)ethyl]-3,5,3′,5′-tetramethyl-1′*H*-[4,4′]bipyrazolyl-1-yl}ethyl)methoxycarbonylmethylamino] acetic acid methyl ester (13). According to the general procedure we used **10** (0.31 g; 0.76 mmol) and methyl iminodiacetate (0.39 g; 3.03 mmol) and the mixture was heated at 110 °C for 2 h. Elution with AcOEt/hexane (1:1) gave **13** (0.22 g; 51%) as a colorless oil. IR (film): ν 1735, 1420, 1200, 1020, 745 cm^{-1}. MS *m/z* (%): 564 (M^+, 20), 187 (68), 174 (100), 146 (54), 128 (91). ^1H NMR (400 MHz, $CDCl_3$, δ): 4.08 (t, 4H, $J = 6.7$ Hz, CH_2–N(Azole)), 3.61 (s, 12H, OCH_3), 3.41 (s, 8H, CH_2CO_2Me), 3.09 (t, 4H, $J = 6.7$ Hz, CH_2–N), 2.00 (s, 6H, CH_3), 1.95 (s, 6H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 171.5, 147.1, 138.0, 110.0, 55.5, 54.5, 51.5, 48.4, 12.2, 9.8.

({2-[1′-[2-(Bismethoxycarbonylmethylamino)ethyl]-3,3′-bis(4-nitrophenyl)-1′*H*-[4,4′]bipyrazolyl-1-yl]ethyl}methoxycarbonylmethylamino) acetic acid methyl ester (14). According to the general procedure we employed **11a** (33 mg; 0.055 mmol) and methyl iminodiacetate (39 mg; 0.24 mmol) and the mixture was heated at 110 °C for 5 h. Elution with CH_2Cl_2/EtOH (99:1) gave **14** (28 mg; 67%) as a yellow oil. ^1H NMR (200 MHz, $CDCl_3$, δ): 8.21 (d, 4H, $J = 8.9$ Hz, AA′XX′ system, aromatics), 7.79 (d, 4H, $J = 8.7$ Hz, AA′XX′ system, aromatics), 7.38 (s, 2H, H_5), 4.22 (t, 4H, CH_2–N(Azole)), 4.02 (s,

2H, CH_2), 3.66 (s, 12H, OCH_3), 3.46 (s, 8H, $CH_2CO_2CH_3$), 3.22 (t, 4H, CH_2–Br).

{[2-(4-{1-[2-(Bismethoxycarbonylmethylamino)ethyl]-3,5-dimethyl-1*H*-pyrazol-4-ylmethyl}3,5-dimethylpyrazol-1-yl)ethyl]methoxycarbonylmethylamino} acetic acid methyl ester (22). According to the general procedure we used **20** (340 mg; 1.033 mmol) and methyl iminodiacetate (733 mg; 4.55 mmol) and the mixture was heated at 110 °C for 24 h. Elution with CH_2Cl_2/EtOH (98:2) gave **22** (118 mg; 20%) as a yellow oil. IR (film): ν 1725, 1420, 1210, 915, 735 cm^{-1}. MS *m*/*z* (%): 578 (M^+, 2), 505 (3), 187 (24), 174 (60), 146 (40), 128 (62), 114 (43), 86 (38). ^1H NMR (400 MHz, $CDCl_3$, δ): 3.95 (t, 4H, *J* = 6.5 Hz, CH_2–N(Azole)), 3.57 (s, 12H, OCH_3), 3.41 (s, 8H, CH_2CO_2Me), 3.27 (s, 2H, CH_2), 2.90 (t, 4H, *J* = 6.6 Hz, CH_2–N), 2.05 (s, 6H, CH_3), 1.86 (s, 6H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 171.5, 146.0, 136.1, 114.0, 55.4, 54.6, 51.4, 47.8, 18.1, 11.9, 9.3.

Basic hydrolysis

General procedure. A mixture of corresponding ester (1 equivalent) and (0.6%; H_2O MQ) NaOH (4 equivalents) was stirred at room temperature or heated at 50–60 °C until consumption of the starting material was detected by TLC. After cooling, the reaction mixture was washed with CH_2Cl_2 and the water was removed in vacuo.

[(2-{1′-[2-(Biscarboxymethylamino)ethyl]-3,3′-diphenyl-1′*H*-[4,4′]bipyrazolyl-1-yl}-ethyl)carboxymethylamino] acetic acid tetrasodium salt (5a). According to the general procedure we used **12** (800 mg; 1.21 mmol) and NaOH (194 mg; 4.85 mmol). The mixture was stirred at room temperature for 48 h to give **5a** (772 mg; 92%) as a white solid. IR (KBr): ν 2150, 1575, 1405 cm^{-1}. ^1H NMR (200 MHz, D_2O, δ): 7.62 (s, 2H, H_5), 7.12 (s br, 10H, aromatics), 4.20 (t, 4H, CH_2–N(Azole)), 3.12 (s, 8H, CH_2–CO_2Na), 3.01 (t, 4H, CH_2–N); ^{13}C NMR (100 MHz, D_2O, δ): 177.4, 149.4, 131.8, 131.3, 127.4, 126.9, 126.6, 109.6, 57.5, 53.4, 48.8, 48.6.

[(2-{1′-[2-(Biscarboxymethylamino)ethyl]-3,5,3′,5′-tetramethyl-1′*H*-[4,4′]bipyrazolyl-1-yl}ethyl)carboxymethylamino] acetic acid tetrasodium salt (5b). According to the general procedure we used **13** (800 mg; 1.4 mmol) and NaOH (224 mg; 5.6 mmol). The mixture was stirred at room temperature for 72 h to give **5b** (768 mg; 90%) as a white solid. IR (KBr): ν 1590, 1310 cm^{-1}. ^1H NMR (400 MHz, D_2O, δ): 3.93 (t, 4H, *J* = 7.5 Hz, CH_2–N(Azole)), 2.91 (s, 8H, CH_2CO_2Na), 2.68 (t, 4H, *J* = 7.5 Hz, CH_2–N), 1.77 (s, 6H, CH_3), 1.69 (s, 6H, CH_3); ^{13}C NMR (100 MHz, D_2O, δ): 180.4, 149.2, 141.2, 111.2, 60.4, 55.3, 48.2, 12.7, 10.8.

({2-[1′-[2-(Biscarboxymethylamino)ethyl]-3,3′-bis(4-nitrophenyl)-1′*H*-[4,4′]bipyrazolyl-1-yl]ethyl}carboxymethylamino) acetic acid tetrasodium salt (5c). According to the general procedure we used **14** (28 mg; 0.037 mmol) and NaOH (6 mg; 0.148 mmol). The reaction mixture was heated at 60 °C for 31 h to give **5c** (23 mg; 78%) as a yellow solid. IR (KBr): ν 3440, 1600, 1390, 1340 cm^{-1}. ^1H NMR (200 MHz, D_2O, δ): 8.06 (d, 4H, *J* = 8.7 Hz, AA′XX′ system, aromatics), 7.52 (s, 2H, H_5), 7.39 (d, 4H, *J* = 8.7 Hz, AA′XX′ system, aromatics), 4.11 (t, 4H, CH_2–N(Azole)), 3.98 (s, 2H, CH_2), 3.08 (s, 8H, CH_2CO_2Na), 2.84 (t, 4H, CH_2N).

{[2-(4-{1-[2-(Biscarboxymethylamino)ethyl]-3,5-dimethyl-1*H*-pyrazol-4-ylmethyl}-3,5-dimethylpyrazol-1-yl)ethyl]-carboxymethylamino} acetic acid tetrasodium salt (5d). According to the general procedure we used **14** (81 mg; 0.14 mmol) and NaOH (22.5 mg; 0.56 mmol). The reaction mixture was stirred at room temperature for 18 h to give **5d** (80 mg; 94%) as a white solid. IR (KBr): ν 3440, 1600, 1410 cm^{-1}. ^1H NMR (200 MHz, D_2O, δ): 4.02 (t, 4H, *J* = 7.4 Hz, CH_2–N(Azole)), 3.36 (s, 2H, CH_2), 3.14 (s, 8H, CH_2CO_2Na), 2.79 (t, 4H, *J* = 7.3 Hz, CH_2–N), 2.05 (s, 6H, CH_3), 1.84 (s, 6H, CH_3); ^{13}C NMR (100 MHz, D_2O, δ): 178.3, 146.2, 137.6, 113.5, 57.7, 52.7, 45.4, 16.1, 9.8, 7.7.

[1-(2-Chloroethyl)-3,5-dimethyl-1*H*-pyrazol-4-yl]methanol (19). A stream of dry hydrogen chloride was passed through a solution of **18**[12] (1.0 g; 6.3 mmol), paraformaldehyde (0.23 g) in 1,2-dichloroethane (5 mL) for 2 h. Subsequently, the reaction mixture was refluxed for 2 h. Concd HCl (5 mL) was added and the water layer was made alkaline with Na_2CO_3 and extracted with CH_2Cl_2. The combined organic extracts were washed with water, dried over $MgSO_4$ and evaporated in vacuo. The residue was purified by recrystallization to give **19** (763 mg; 67%) as a white solid (mp 95–97 °C, CH_2Cl_2/Hexane). IR (KBr): ν 3260, 1565, 1475, 1450, 1325, 1300, 995, 765 cm^{-1}. MS *m*/*z* (%): 190 ($M^+ + 2$, 18), 188 (M^+, 54), 171 (52), 139 (100), 126 (49), 109 (60), 97 (19), 56 (23). ^1H NMR (400 MHz, $CDCl_3$, δ): 4.47 (s br, 2H, CH_2O), 4.25 (t, 2H, *J* = 6.4 Hz, CH_2–N(Azole)), 3.84 (t, 2H, *J* = 6.3 Hz, CH_2Cl), 2.28 (s, 3H, CH_3), 2.24 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 147.6, 138.4, 116.0, 54.7, 49.7, 42.7, 11.5, 9.4. Anal. calcd for $C_8H_{13}ClN_2O$: C, 50.92; H, 6.95; N, 14.85. Found: C, 51.12; H, 6.85; N, 14.91. Traces of ether **20** were isolated in this reaction. MS *m*/*z* (%): 358 (M^+, 2), 200 (25), 171 (100), 109 (29).

4,4′-Methylenebis[1-(2-chloroethyl)-3,5-dimethylpyrazole] (20). A suspension of **19** (990 mg; 5.25 mmol) in concd HCl (0.5 mL) was refluxed for 1 h. After cooling was added of H_2O (10 mL), neutralized with Na_2CO_3, extracted with CH_2Cl_2 and organic layer was dried over $MgSO_4$. The organic solvent was evaporated in vacuo to give **10** (680 mg; 79%) as a white solid (mp 99–101 °C, CH_2Cl_2/hexane). IR (KBr): ν 1555, 1465, 1425, 1380, 1310, 1280, 745, 655 cm^{-1}. MS *m*/*z* (%): 328 (M^+, 19), 313 (88), 279 (4), 171 (100), 135 (21), 108 (15). ^1H NMR (200 MHz, $CDCl_3$, δ): 4.23 (t, 4H, *J* = 6.2 Hz, CH_2–N), 3.80 (t, 4H, *J* = 6.2 Hz, CH_2Cl), 3.39 (s, 2H, CH_2), 2.10 (s, 6H, CH_3), 2.03 (s, 6H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, δ): 147.0, 136.5, 114.0, 49.6, 43.0, 18.1, 12.0, 9.5. Anal. calcd for $C_{15}H_{22}Cl_2N_4$: C, 54.70; H, 6.75; N, 17.02. Found: C, 54.64; H, 6.66; N, 16.94.

Gd(III) binding studies. Binding isotherms (22 °C, pH 6.5) of Gd(III) to ArsIII and to the different complexones were determined spectrophotometrically at 680 nm

using a microplate spectrophotometer (Molecular Devices Spectramax, Sunnyvale, CA, USA). Gd(III) chloride (up to 0.35 mM in 5-μM steps) was added into model solutions containing 20 mM HEPES (pH 6.7), 100 mM NH_4Cl and 0.1 mM ArsIII in the absence and presence of 1 mM complexones **5a–d**, monitoring at 680 nm the increase in concentration of the ArsIII–Gd(III) complex ($\varepsilon = 1.1 \cdot 10^4$ M^{-1} cm^{-1}).[12,48,49] Binding simulations and not linear least squares regressions were programmed using the Mathematica 4.1 program (Wolfram Research Inc., Campaign, IL, USA) implemented on a Pentium IV platform. Binding parameters were obtained by non linear least squares fittings of the titration curves to a mathematical model which considered the competition between hyperbolic binding to ArsIII (y_1) and sigmoidal binding to the complexone (y_2). Hyperbolic binding of Gd(III) to ArsIII is given by the equation $y_1 = A^*[\mathrm{Gd(III)}]/([\mathrm{Gd(III)}] + K_d)$ where K_d and A represent the apparent dissociation constant and saturation values of ArsIII. Sigmoidal binding of Gd(III) to the complexone is described by the equation $y_2 = \mathrm{B}\ [\mathrm{Gd(III)}]^n/([\mathrm{Gd(III)}]^n + S_{0.5'}{}^n)]$ where $S_{0.5'}$ is the apparent Gd(III) concentration for half maximal saturation of the complexone in the presence of competing ArsIII and n is a parameter reflecting the degree of cooperativity in the binding. The amount of Gd(III) bound to ArsIII in the presence of complexone is given by $y = y_1 - y_2$. It is possible to calculate the apparent Gd(III) concentration for half maximal saturation of the complexone in the abscence of the competing ArsIII, $S_{0.5}$ by the expression $S_{0.5} = S_{0.5}'/(1 + [\mathrm{ArsIII}]/K_d)$.

Ab initio calculations. Ab initio molecular orbital calculations were performed with the Gaussian 98 package.[28] The $46 + 4f^7$ core electrons of the gadolinium atoms were described by the quasi-relativistic pseudopotential of Dolg et al.[50,51] and the valence electrons by a (7s6p5d)/[5s4p3d] Gaussian basis set. For the ligands 3-21G and 6-31G(d,p) basis sets were applied. The geometries of the systems were obtained as follows. Initially, we performed a conformational analysis of each free ligand with the molecular mechanics MMFF94 model implemented in the Spartan suite of programs (PC Spartan Pro v1.03, Wavefunction Inc., Irvine, CA, USA) in order to select the lowest energy conformer. This conformer was then fully optimized at the RHF/3-21G level. As none of the empirical (MMFF94, Sybyl) or semiempirical (AM1, PM3) available models is conveniently parametrized for lanthanide atoms, we studied the coordination modes and conformations of the ligand groups starting from the optimized structure of the Gd(III)-iminodiacetate complex at the HF level. So, we performed full optimizations of molecular systems without symmetry constraints for different conformations of the $–CH_2–CH_2$-azole arm at the RHF level, with the $46 + 4f^7$ core electrons ECP and the [5s4p3d]-GTO valence basis set for the metal and 3-21G basis set for the ligands. It has been tested that geometry optimizations using the 3-21G basis set for the ligand, followed by single point energy calculation with higher basis set provide a satisfactory representation of energetic properties.[29–31,51,52]

Determination of relaxivities. ^{1}H NMR relaxation times T_1 and T_2 (37 °C, pH = 6.5) of the water protons in aqueous solutions of complexones **5a–d** containing or not Gd(III), were measured at 1.5 Tesla in a Bruker Minispec NMR spectrometer. T_1 values were determined by the inversion-recovery method (d1-π-τ-π/2-aq) and T_2 values were determined by the Carr–Purcell–Maiboom–Gill sequence (d1-π/2-[τ-π-τ]$_n$-aq) using in both cases not less than 13 different τ values. Three different measurements of T_1 or T_2 were performed in every sample. Typically, 5 mM complexones were dissolved in 100 mM Tris/HCl, 150 mM NaCl containing or not 0.5 mM Gd(III). In addition, some determinations were performed in rat plasma. In these cases, blood was drained from the inferior caval vein of well fed, anesthetized (Nembutal 50 mg/kg), female Wistar rats (250–300 g) using an heparinized syringe. Plasma was recovered as the supernatant of blood centrifugation at 5000g (4 °C, 5 min) and added 5 mM complexone or 5 mM complexone and 0.5 mM Gd(III) chloride. Relaxivities $r_{1(2)}$ were calculated according to the expression:

$$r_{1(2)} = \Delta\left(1/T_{1(2)}\right)/[\mathrm{Gd(III)}]$$

where, Δ is the difference in longitudinal or transversal relaxation rates ($1/T_{1(2)}$) of the water protons in the presence and absence of Gd(III), and [Gd(III)] the concentration of Gd(III) expressed in mM.

In vitro toxicity studies. Toxicity was investigated by measuring the amount of intracellular LDH released to the medium in cultures of glioma C6 cells.[12] Cells were grown to confluence in DMEM medium containing 5% fetal calf serum and incubated for one h in the absence and presence of increasing concentrations of Gd(III) and **5a**, **5c** and EDTA (Fig. 7). The amount of LDH released to the medium was measured spectrophotometrically in a microplate reader (Molecular Devices Spectramax, Sunnyvale, CA, USA) at 340 nm, using an incubation mixture containing 50 mM HEPES pH 7.2, 5 mM sodium pyruvate and 0.35 mM NADH. Results are the mean of three independent experiments.

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