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Toward Optimized High-Relaxivity MRI Agents: The Effect of Ligand Basicity on the Thermodynamic Stability of Hexadentate Hydroxypyridonate/Catecholate Gadolinium(III) Complexes

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The thermodynamic stabilities of the Gd^{III} complexes of five hexadentate ligands, which incorporate the 2,3-dihydroxyterephthalamide and 2,3-hydroxypyridonate chelating moieties, have been determined by potentiometric and spectrophotometric titration. The ligands were chosen to span a range of basicities while maintaining a similar tripodal structural motif, facilitating a study of the effect of ligand basicity on the thermodynamic stability of the Gd^{III} complexes. The relative stability of the five complexes is found to be highly pH dependent, with the most acidic ligands forming the most stable complexes at low pH and more basic ligands forming more stable complexes at high pH. The most stable Gd^{III} complex at a physiological pH of 7.4 is formed with a ligand of intermediate basicity and is of stability comparable to that of Gd^{III} complexes that feature eight-coordinate amino–carboxylate ligands and are currently used as magnetic resonance imaging contrast agents in diagnostic medicine. A single-crystal X-ray structure of the intermediate compound 3-hydroxy-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylic acid ethyl ester is described: This compound crystallizes in the triclinic space group $P\bar{1}$ with $a = 7.4801(3)$ Å, $b = 8.0671(3)$ Å, $c = 8.3457(4)$ Å, $\alpha = 72.242(2)°$, $\beta = 80.693(2)°$, $\gamma = 69.943(3)°$, $V = 449.60(3)$ Å^3, $Z = 2$, and $R = 0.042$.

Introduction

The Gd^{III} cation is particularly well suited as a contrast agent in diagnostic medical MRI due to its high magnetic moment and favorable electronic relaxation rate.[1,2] However, Gd^{III} is highly toxic in the quantities required for MRI and must be encapsulated within a ligand that prevents release of the free cation in vivo.[3] At the same time, it is desirable to maximize relaxivity. This requires a water exchange rate which closely matches the Larmor frequency and as many water molecules as possible coordinated to the Gd^{III} center. Thus, a compromise has to be reached between relaxivity and stability. This situation is further complicated by the fact that the relaxivity of some $q > 1$ complexes is reduced by the presence of proteins and endogenous anions, such as phosphate and carbonate, due to replacement of the coordinated water molecules by exogenous ligands.[1,4,5] Currently, all commercial contrast agents, such as $[Gd(DTPA)(H_2O)]^{2-}$ and $Gd(DTPA\text{-}BMA)(H_2O)$,[6] are amino−carboxylate complexes that feature a nine-coordinate Gd^{III} center bound by an octadentate ligand and one inner sphere water molecule. Target-specific contrast agents that are currently in clinical trials, such as the blood pool imaging agent MS-325[7,8] and the hepatobiliary agent $[Gd(BOPTA)(H_2O)]^-$,[9] also fall in

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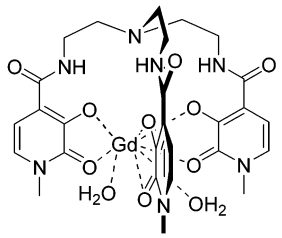

Figure 1. Structure of Gd-**1**, Gd(TREN-1-Me-3,2-HOPO)(H_2O)$_2$, illustrating the six oxygen donors from the ligand and the two water molecules, which bind to the Gd^{III} center.

this group. Since target-specific complexes can be expected to exhibit increased residence times in vivo, relative to nonspecific contrast agents, inhibition of slow release of gadolinium by high complex stability may then be particularly important. Therefore, in the pursuit of new target-specific contrast agents of increased relaxivity, it is important to understand how complex stability can be maximized while also achieving fast water exchange *and* increasing the number of bound water molecules.

It is important to distinguish between thermodynamic stability and kinetic lability. It has been shown that $[Gd(DOTA)(H_2O)]^-$ and related complexes have high thermodynamic stability as well as very high kinetic inertness in vivo, slowing the release of Gd^{III}, whereas $[Gd(DTPA)(H_2O)]^{2-}$ and related amide derivatives are far more kinetically labile.[3,10,11] Nevertheless, the thermodynamic stability of the latter complexes is sufficient to prevent the release of toxic quantities of Gd^{III} in vivo. For such complexes it has also been shown that a series of competing equilibria with physiological metals and ligands must be taken into account in order to satisfactorily account for their toxicity in mice.[11] For example, the complex Gd(DTPA-BMA)(H_2O) is appreciably less toxic than $[Gd(DTPA)(H_2O)]^{2-}$ despite the fact that the latter complex is considerably more stable in water at neutral pH. This apparent anomaly is explained by the competing equilibria with physiological metal ions. Metathesis is much more significant than just dissociation of the Gd^{III} complex; consequently DTPA-BMA, for example, is far more selective than DTPA for Gd^{III} relative to H^+, Ca^{II}, Zn^{II}, and Cu^{II}.[11]

We have previously reported Gd^{III} complexes of a series of hydropyridinonate (HOPO) ligands derived from TREN-1-Me-3,2-HOPO (H_3**1**) (Figure 1). X-ray crystallography of Gd-**1** showed that the Gd^{III} center is bound by six oxygen donors from the ligand and two solvent water molecules to give an overall eight-coordinate Gd^{III} center, as shown in Figure 1.[12] Complexes of this family/series exhibit unusually short water residence lifetimes, τ_m, of a few nanoseconds, which can lead to exceptionally high values of relaxivity.[12−17] Furthermore, unlike other $q = 2$ complexes, their relaxivity appears to be unaffected by the presence of the physiological bidentate chelators acetate, lactate, and malonate.[16] These complexes are also of very high thermodynamic stability: Gd-**1** has a higher pGd value than Gd(DTPA-BMA)(H_2O) or $[Gd(DTPA)(H_2O)]^{2-}$, and is significantly more selective for Gd^{III} over Ca^{II} and Zn^{II} relative to amino−carboxylate ligands.[12] This high stability is remarkable for hexadentate ligands, compared particularly with the hexadentate amino−carboxylate ligand EDTA, whose Gd^{III} complex is of much lower stability and is highly toxic in mice.[11]

The favorable thermodynamic properties of Gd-**1** can be attributed to several effects. First, the Gd^{III} cation is highly oxophilic and will bind more strongly to the six oxygen donors provided by the HOPO ligand rather than the mixture of nitrogen and oxygen donors offered by a hexadentate amino−carboxylate ligand such as EDTA. Second, the two donor atoms on each 1-Me-3,2-HOPO arm are predisposed to bind to Gd^{III} in a five-membered chelate ring. Such an arrangement of donor atoms is expected to favor larger cations such as Gd^{III} and Ca^{II} over smaller cations such as Zn^{II} and Cu^{II}.[18−20] A third and very important effect is the basicity (or log K_a) of the donor atoms, which has been demonstrated to correlate well with the formation constant of the ligand with various Lewis acids.[18,19] This study is concerned with evaluating the effect of varying ligand basicity on the stability of the Gd^{III} complexes for a series of hexadentate ligands (Chart 1) composed of HOPO or related bidentate donors.

Results and Discussion

The ligands used in this study are shown in Chart 1. The same tripodal backbone motif is maintained throughout, so that steric effects within the ligand remain constant. The ligands were chosen to represent a wide range of basicities. HOPO donors have previously been shown to exhibit a protonation constant (log K_a) of between 6 and 8 with a second of about zero,[12,21] and the 2,3-dihydroxyterephthalamide (TAM) substituents have a log K_a value typically greater than 10.5 and a second one which is 6−8. [22]

Ligand Syntheses and Characterization. The syntheses for H_3**1** and H_4**3** have been reported previously,[12,13] whereas H_3**2**, H_5**4**, and H_6**5** were synthesized for this study using the routes detailed in Scheme 1.

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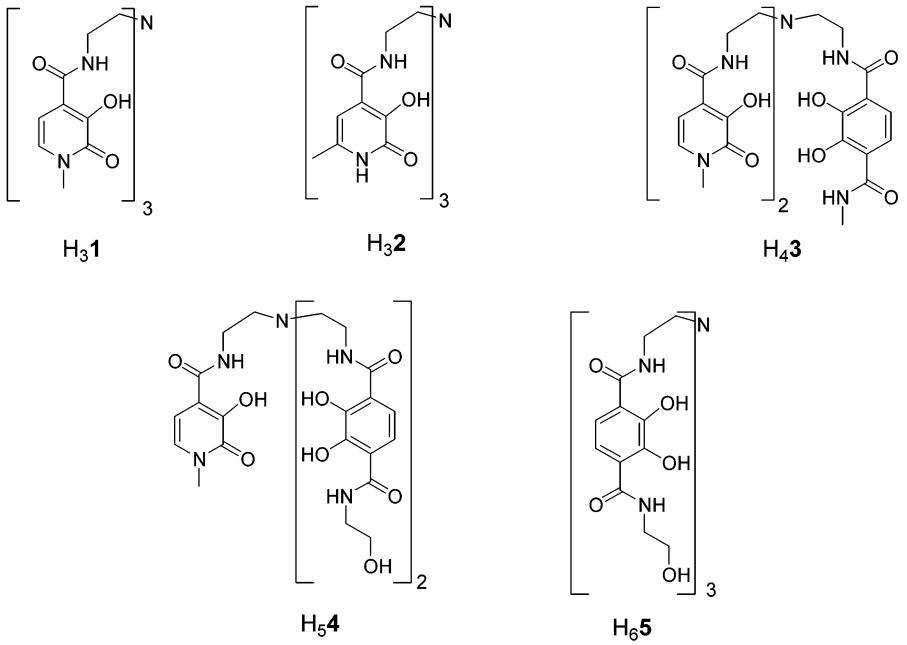
Chart 1. Ligands Used in This Study



The precursor of 6-Me-3,2-HOPO, **6**, was synthesized via an adaptation of a previously reported procedure.[23] A single-crystal X-ray structure determination was undertaken on this compound in order to verify the position of the methyl group on the HOPO ring. Crystals suitable for X-ray diffraction were grown by evaporation of the MeOH solution. The structure (Figure 2) confirmed the presence of the HOPO ring with a methyl substituent in the 6-position. Additionally, the ^{1}H NMR spectrum of a bulk sample of **6** showed just one 3H singlet resonance at 2.07 ppm, indicating that the crystal structure was typical of the bulk material.

Subsequent benzyl protection and ester hydrolysis of **6** gave the carboxylic acid **7** in a combined yield of 46% for the two steps. Benzylation of **6** proved to be particularly sensitive to the conditions employed with a bis-benzylated byproduct, in which the second benzyl group is substituted on the HOPO nitrogen, invariably being a major product of the reaction when carried out in a purely organic solvent. However, the desired mono-benzylated product was formed in reasonable reaction time when biphasic conditions were employed using cetylpyridinium chloride as the phase transfer catalyst.

Reaction of **7** with 2-mercaptothiazoline and dicyclohexylcarbodiimide (DCC) resulted in the formation of the thiazolide activated compound **8** as yellow crystals. Subsequent reaction of **8** with tris(2-aminoethylamine) (TREN) gave **9** in 76% yield. Final deprotection was achieved by hydrogenation using a Pd/C catalyst to give the protonated ligand H_3**2**·HCl in 78% yield.

The benzyl-protected precursor **10** was used in the synthesis of H_5**4** and H_6**5** rather than the methyl-protected variant which was previously used in the synthesis of H_4**3**.[13] This is because the methyl-protected ligands were found to be particularly difficult to deprotect in later steps. **10** was synthesized from the carboxylic acid by treatment with oxalyl chloride followed by reaction with 2-mercaptothiazoline. It has two sites liable to reaction with amines; however, by adding ethanolamine dropwise to a large excess of **10** the *mono*-substituted product **11** was formed in 79% yield.

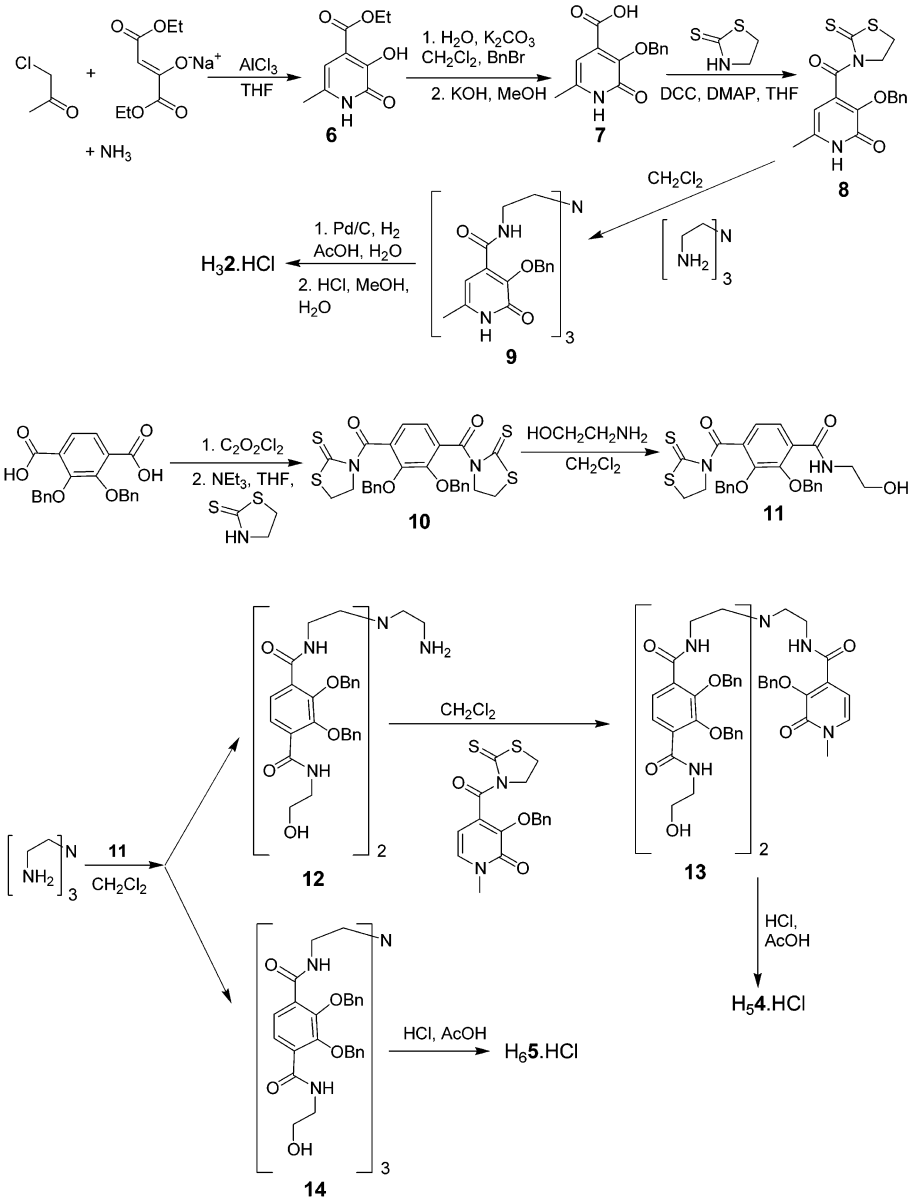
Reaction of TREN with **11** produced a crude mixture of mono-, bis-, and tris-substituted derivatives, which were separated by elution down a silica column to give **12** and **14**. The former was then combined with 3-benzyloxy-1-methyl-2-oxo-1,2-dihydropyridine-4-carbonyl(2-mercaptothiazolide) to give **13**. The yields of **13** and **14** relative to the starting material TREN were 18% and 34%, respectively, with the yield of the mono-substituted intermediate unknown, since it could not be removed from the silica column. **13** and **14** were both deprotected by stirring in AcOH and HCl to give the desired ligands H_5**4**·HCl and H_6**5**·HCl in 77% and 67% yield, respectively.

Thermodynamic Evaluation. The ligand log K_a's were determined by potentiometric titration, except for those of H_6**5**, which were determined by spectrophotometric titration. All Gd^{III} affinities were determined by spectrophotometric titration. Binding to Gd^{III} for ligands H_3**1**, H_3**2**, H_4**3**, and H_5**4** was found to be a kinetically labile process. However, the spectrophotometric titration of Gd-**5** exhibited poor reversibility, suggesting that the system is slow to reach equilibrium. Thus, only approximate values for equilibrium constants of Gd-**5** could be determined.

The results of the study are shown in Table 1. The overall basicity (sum of the log K_a values) of the ligands follows the order H_3**1** < H_3**2** < H_4**3** < H_5**4** < H_6**5**, with the highest log K_a values for H_5**4** and H_6**5** lying outside the range that can be accurately measured by potentiometric titration. As expected, the basicity of the ligands increases with increasing number of TAM substituents. Additionally, the ligand **2** is significantly more basic than **1**, which apparently reflects

(23) Feist, D. *Chem. Ber.* **1902**, *35*, 1540.

Scheme 1. Synthesis of Ligands H_3**2**, H_5**4**, and H_6**5**[a]



[a] The syntheses of H_3**1** and H_4**3** are reported elsewhere.[12,13]

the effect of the methyl substituent para to the 3-hydroxy group of the HOPO ring.

The pGd (where pGd = −log [Gd_{free}]) for complexes Gd-**1** to Gd-**4** has been calculated as a function of pH for a standard set of conditions (Figure 3, initial concentrations: [Gd] = 10^{-6} M, [L] = 10^{-5} M). Under these conditions, the theoretical minimum pGd will be 6, which occurs under highly acidic conditions when the complex is completely dissociated. At pH = 3 the most acidic ligand, H_3**1**, releases the least free Gd^{III}. As the pH is increased and competition with protons becomes less significant, the pGd of each ligand increases to approach its maximum limit when the pH exceeds the highest log K_a. Thus, the more acidic the ligand, the lower the pH at which it will approach its maximum pGd value. At a pH of 11, Gd-**1** exhibits the lowest pGd, with the highest pGd's observed for Gd-**3** and Gd-**4**, and it is apparent from Figure 3 that the latter two ligands have not reached their maximum pGd values by this point.

The pGd values of each complex at the physiological pH of 7.4 are given in Table 1. It is clear that the most stable complex under these conditions is that formed with **3**. This ligand apparently has near optimal basicity within the current series. Ligands H_3**1** and H_3**2** are more acidic and therefore bind to Gd^{III} less strongly, while the extra basicity of **4** and **5** causes considerable competition with H^+ at pH 7.4. The pGd values of the commercial contrast agents [Gd(DTPA)-$(H_2O)]^{2-}$, [Gd(DOTA)$(H_2O)]^-$, and Gd(DTPA-BMA)(H_2O) were calculated for the same conditions, and found to be 19.1, 20.4, and 15.5, respectively, using literature values for the protonation and Gd^{III} complex formation constants.[11,24] Thus, the stability of Gd-**3** (pGd = 20.1) compares very favorably with these commercial complexes, despite its having only six donor atoms.

The high affinity of **3** for Gd^{III} was verified via competition against DTPA. Throughout this titration, the pH as well as the total concentrations of **3** and Gd^{III} were kept constant

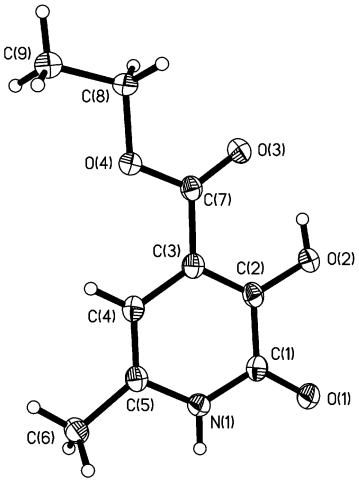


Figure 2. X-ray crystal structure of 3-hydroxy-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylic acid ethyl ester **6**, with 50% thermal displacement ellipsoids. In this precursor to the 6-Me-3,2-HOPO, the methyl group is positioned on the 6-position of the ring and not on the N.

whereas the concentration of the competing DTPA was progressively increased. The concentrations of free and complexed **3** were determined spectroscopically. This method enables direct and more precise measurement of the pM of a ligand at pH 7.4 without a large pH extrapolation. As can be seen in Figure 4, the concentration of DTPA necessary to generate equal partition of Gd between the two ligands (log([DTPA]/[**3**]) when log([GdDTPA]/[Gd-**3**]) = 0) directly gives the pM of **3** since that of DTPA is known. While it was necessary to perform this experiment at pH 6 to ensure constant electrolyte concentration (much higher concentrations of DTPA are required to compete at pH 7.4), this experiment confirms the high affinity of **3** for Gd^{III}. A pGd of 16.3 was determined at pH 6.0. This pGd was extrapolated to 20.0 at pH 7.4 using the protonation constants of the ligand and its metal complex.

It has previously been shown that it is necessary to consider selectivity for Gd^{III} over physiological metals in order to satisfactorily relate thermodynamic parameters to toxicity.[11] Our previous studies have shown that H_3**1** is highly selective for Gd^{III} over the physiological competitor metal centers Ca^{II} and Zn^{II}, which is thought to be largely due to the higher Lewis acidity of Gd^{III} compared to the two physiological metal ions.[12] This selectivity is greater than that of $[Gd(DTPA)(H_2O)]^{2-}$, $[Gd(DOTA)(H_2O)]^{-}$, and Gd-(DTPA-BMA)(H_2O), which are already in medical use, implying that Gd-**1** would not release toxic quantities of Gd^{III} in vivo. It is expected that **2**, **3**, **4**, and **5** will exhibit increasing selectivity for Gd^{III} over Ca^{II} and Zn^{II} since the difference in formation constants between strong and weak Lewis acids typically increases as ligand basicity increases.[18,19]

Conclusion

In summary, we have demonstrated that ligand donor atom basicity has a very significant effect on the stability of Gd^{III} complexes. Thus, the basicity of the donor atoms is clearly a critical factor that must be considered in the design of safe MRI contrast agents of increased relaxivity. In particular, optimization of donor atom basicity enables the formation of highly stable Gd^{III} complexes with ligands containing relatively few donor atoms. Such complexes are expected to exhibit highly desirable relaxivity parameters due to the increased number of inner sphere water molecules. These complexes also have very fast rates of water exchange due to a small difference in energy between the ground state and the nine-coordinate intermediate.[13] Optimization of the basicity of the donor atoms of the hexadentate ligand system described herein has facilitated the attainment of stabilities equal to or greater than those of commercially available contrast agents which feature eight-coordinate ligands.

Experimental Section

General Considerations. Starting materials were obtained from commercial suppliers and used without further purification unless otherwise noted. Flash silica gel chromatography was performed using ICN silica gel (40−63 μm, 60 Å). Microanalyses were performed by the Microanalytical Services Laboratory, College of Chemistry, Univeristy of California at Berkeley, Berkeley, CA 94720. Mass spectra were recorded at the Mass Spectrometry Laboratory, College of Chemistry, University of California at Berkeley, Berkeley, CA 94720. Unless otherwise stated, NMR spectra were recorded at room temperature on either AMX-300, AM-400, AMX-400, or DRX-500 Brüker FT spectrometers. Melting points were measured on a Büchi melting point apparatus and are uncorrected. Syntheses of H_3**1** and H_4**3** are described elsewhere.[12,13]

Ligand Syntheses. 3-Hydroxy-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylic Acid Ethyl Ester (6). This compound was synthesized by adapting a previously reported procedure.[23] Sodium diethyloxylacetate (42.1 g, 200 mmol) was dissolved in THF (500 mL) and then placed into a 1-L three-neck round bottom flask. Chloroacetone (16 mL, 200 mmol) was added to the mixture. After 10 min, NH_3 gas was bubbled through the reaction mixture. Finally, $AlCl_3$ (2.67 g, 20 mmol) was slowly and carefully added. The reaction mixture was stirred under ambient conditions for 5 days. The resulting orange solid was filtered off and suspended in 1 M HCl (500 mL) so that the pH < 3. The resulting suspension was stirred for 30 min and the precipitate filtered off, washed with distilled water, and recrystallized from hot EtOH (approximately 1 L) to yield colorless crystals (yield: 15.7 g, 40%). Mp: 227−229 °C. ^{1}H NMR (d_6-DMSO, 300 MHz): δ = 1.24 (t, 3H, CH_3), 2.07 (s, 3H, CH_3), 4.22 (q, 2H, CH_2), 6.07 (s, 1H, CH) ppm. Anal. Calcd (found) for $C_9H_{11}O_4N$: C, 54.82 (55.06); H, 5.62 (5.53); N, 7.11 (7.07). EI-MS (+): *m*/*z* 198 $[M + H]^+$.

3-Benzyloxy-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylic Acid (7). K_2CO_3 (9.06 g, 65 mmol) and **6** (11.8 g, 60 mmol) were suspended in H_2O (650 mL), and the flask was immersed in an ultrasonic bath for 30 min in order to aid dissolution. This solution was added to a solution of benzyl bromide (7.8 mL, 65 mmol) in CH_2Cl_2 (500 mL) in a 2-liter three-neck round bottom flask. Cetylpyridinium chloride (9.09 g, 30 mmol) was added as a phase transfer catalyst for this reaction. The solution was stirred with an overhead stirrer, at 40 °C for 1 day until the reaction was complete. The two layers were separated, and the aqueous layer was extracted twice with CH_2Cl_2 (100 mL). The organic layers were combined, and the solvents were removed. Purification of this crude

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Table 1. Thermodynamic Parameters for the Ligands **1**−**5**[a]

quotient	constant	**1**	**2**	**3**[f]	**4**	**5**
[LH]/[L][H]	log K_{LH} (tertiary N)[c]	4.97(1)	5.26(4)	4.98(6)	4.94(3)	<5
[LH_2]/[LH][H]	log K_{LH2}	5.83(1)	6.12(3)	5.75(1)	5.90(4)	5.34(2)
[LH_3]/[LH_2][H]	log K_{LH3}	6.97(1)	7.50(2)	7.05(1)	7.02(4)	6.83(3)
[LH_4]/[LH_3][H]	log K_{LH4}	8.19(2)	8.73(6)	8.14(3)	8.18(6)	7.96(5)
[LH_5]/[LH_4][H]	log K_{LH5}			11.42(7)	11.1(1)	11.6(6)
[LH_6]/[LH_5][H]	log K_{LH6}				>12	>12
[LH_7]/[LH_6][H]	log K_{LH7}					>12
[GdL]/[Gd][L]	log K_{GdL}	19.22(5)	20.28(1)	24.1(1)	>23	>17
[GdHL]/[GdL][H]	log K_{GdLH}	3.53(9)	4.09(4)	5.6(2)	7.7(1)	
[GdH_2L]/[GdHL][H]	log K_{GdLH2}	3.48(9)	3.19(4)	4.6(2)	5.91(8)	
[GdH_3L]/[GdH_2L][H]	log K_{GdLH3}				5.21(8)	
[GdH_4L]/[GdH_3L][H]	log K_{GdLH4}				3.59(8)	
	pGd (pH = 3.0)[d]	7.4	7.2	7.0	6.0	6.0
	pGd (pH = 7.4)[d]	19.2[b]	19.5	20.1[e]	15.2	∼13
	pGd (pH = 11.0)[d]	20.2	21.2	24.5	22.6	∼18

[a] Numbers in parentheses give the uncertainty for each value in units of the least significant digit. All constants are determined at 25 °C and 0.1 M KCl. [b] The original pGd for **1** in ref 12 (20.3) is slightly corrected by this result. [c] log K_{LH} is assigned as protonation of the tertiary nitrogen on the basis of spectrophotometric and potentiometric data; the spectral changes for this protonation step were smaller than and at different wavelengths different from those observed for the other log K_a values. [d] Total concentrations for pGd calculations: [Gd] = 10^{-6} M, [L] = 10^{-5} M. [e] pGd confirmed from a competition titration against DTPA. [f] See ref 13.

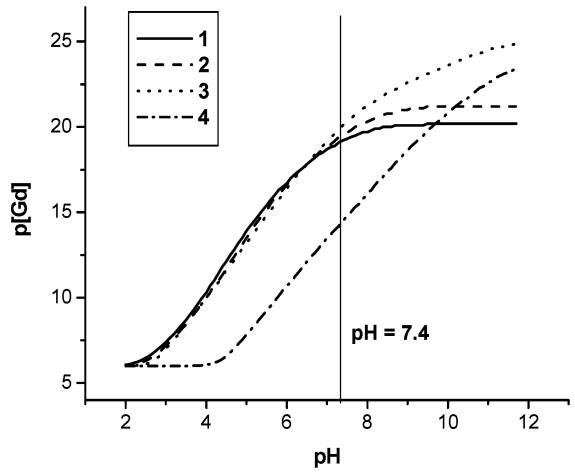

Figure 3. Plots of the change of pGd with pH for ligands **1** through **4**, between pH 2 and 11 (see Chart 1 for ligand structures). Calculated for total concentrations; [Gd] = 10^{-6} M, [L] = 10^{-5} M.

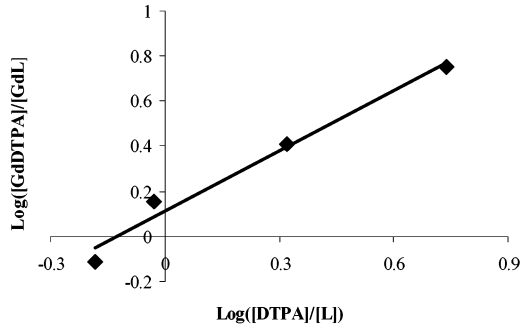

Figure 4. Competition titration of **3** against DTPA (see Chart 1 for ligand structures). The *x*-intercept indicates the difference in pGd between the two ligands. Experimental conditions: 25 °C, pH = 6, [KCl] = 0.1 M.

product (3-benzyloxy-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylic acid ethyl ester) was possible by column chromatography, although it was found to be more convenient to purify after the subsequent ester hydrolysis reaction. ^{1}H NMR ($CDCl_3$, 300 MHz) of 3-benzyloxy-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylic acid ethyl ester: δ = 1.29 (t, 3H, CH_3), 2.35 (s, 3H, CH_3), 4.30 (q, 2H, CH_2), 5.26 (d, 2H, CH_2), 6.22 (s, 1H, CH), 7.35 (m, 5H, Ph) ppm. The product was dissolved in a solution of KOH (16.68 g, 297 mmol) in MeOH (300 mL) and the solution heated under reflux for 24 h or until the reaction was judged to be complete by TLC. The solution was filtered and acidified to pH = 1 with 6 M HCl. The white solid was filtered off, washed with 100 mL of H_2O, and recrystallized from EtOH (400 mL) to give a white crystalline solid (yield of combined benzylation and hydrolysis reactions: 7.06 g, 46% from **6**). ^{1}H NMR (d_6-DMSO, 300 MHz): δ = 2.11 (s, 3H, CH_3), 5.07 (d, 2H, CH_2), 5.97 (s, 1H, CH), 7.30 (m, 5H, Ph) ppm. Anal. Calcd (found) for $C_{14}H_{13}O_4N$: C, 64.88 (64.73); H, 5.02 (5.15); N, 5.41 (5.37). EI-MS (+): *m*/*z* 259 $[M]^+$.

3-Benzyloxy-6-methyl-2-oxo-1,2-dihydropyridine-4-carbonyl-(2-mercaptothiazolide) (8). 7 (5.39 g, 21.1 mmol) was dissolved in dry THF (200 mL). Then 2-mercaptothiazoline (2.71 g, 23.2 mmol) and DMAP (0.25 g, 2.0 mmol) were added and the solution was stirred under an atmosphere of N_2 for 1 h. Then dicyclohexylcarbodiimide (DCC, 5.12 g, 25.3 mmol) was added in small portions over 3 h. The reaction mixture was stirred for 16 h and then left to stand at 0 °C for a further 24 h. The dicyclohexylurea (DCU) was removed by filtration, and the solvents were removed. The remaining residue was dissolved in CH_2Cl_2, the mixture filtered, and the product purified by elution down a silica column (eluent: CH_2Cl_2 with increasing gradient of MeOH). The solvents were removed from the resulting solution by rotary evaporation, and the residue was recrystallized from acetone, yielding yellow crystals (3.78 g, 56%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.32−7.47 (m, 5H, aromatic Bn), 5.98 (s, 1H, CH), 5.34 (s, 2H, Bn CH_2), 4.34 (t, *J* = 7.3 Hz, 2H, CH_2), 2.92 (t, *J* = 7.3 Hz, 2H, CH_2), 2.34 (s, 3H, CH_3) ppm. Anal. Calcd (found) for $C_{17}H_{16}N_2O_3S_2$: C, 56.65 (56.50); H, 4.47 (4.51); N, 7.77 (7.70). FAB-MS (+): *m*/*z* 361 $[M + H]^+$.

Benzyl-Protected TREN-6-Me-3,2-HOPO (9). 8 (1.5 g, 4.3 mmol) was dissolved in CH_2Cl_2 (30 mL). To this were added successive portions of tris(2-aminoethylamine) with a 4 h delay between subsequent additions until the solution turned from yellow to colorless. The solution was purified by two separate elutions down a silca column (solvent: CH_2Cl_2 with increasing gradient of MeOH from 0% to 8%). Removal of the solvent afforded a white glassy solid (yield: 0.91 g, 76%). ^{1}H NMR (d_6-DMSO, 400 MHz): δ = 8.12 (t, *J* = 7.2 Hz, 3H, NH), 7.20−7.37 (m, 15H, Bn), 5.95 (s, 3H, HOPO-H), 5.12 (s, 6H, Bn CH_2), 3.14 (m, 6H, TREN CH_2), 2.42 (m, 6H, TREN CH_2), 2.08 (s, 9H, CH_3) ppm. Anal. Calcd (found) for $C_{48}H_{51}N_7O_9$: C, 66.27 (65.97); H, 5.91 (5.91); N, 11.27 (11.17). FAB-MS (+): *m*/*z* 870 $[M + H]^+$.

TREN-6-Me-3,2-HOPO (2). 5% Pd on carbon (0.235 g) was added to a solution of **9** (0.360 g, 0.414 mmol) in AcOH (10 mL) and H_2O (5 mL) and the mixture stirred under an atmosphere of H_2 under ambient conditions for 2 h. The reaction mixture was then filtered and the solvent removed. The remaining residue was converted to the Cl^- salt by dissolving in MeOH (10 mL) and one drop of concentrated HCl, and the solvent was then removed (×3). Excess HCl was then removed by dissolving the residue in MeOH (10 mL) and removing the solvent under reduced pressure (×3). The remaining residue was taken up in MeOH (2 mL) and added to a rapidly stirring solution of Et_2O (200 mL) to afford a white precipitate, which was filtered off and dried under vacuum (yield: 0.225 g, 78%). ^{1}H NMR (D_2O, 500 MHz): $\delta = 5.87$ (s, 3H, HOPO-H), 3.79 (m, 6H, TREN CH_2), 3.61 (m, 6H, TREN CH_2), 1.96 (s, 9H, CH_3) ppm. Anal. Calcd (found) for TREN-6-Me-3,2-HOPO·HCl·$3H_2O$, $C_{27}H_{34}N_7O_8Cl\cdot 3H_2O$: C, 46.39 (46.09); H, 5.91 (5.82); N, 14.02 (13.80). FAB-MS (+): m/z 600 $[M]^+$.

2,3-Dibenzyloxy-1,4-dicarbonyl(2-mercaptothiazolide) (10). Oxalyl chloride (14 mL, 160 mmol) and DMF (1 drop) were added to a suspension of 2,3-dibenzyloxy-1,4-dicarboxylic acid (20 g, 52 mmol)[25] in dry toluene (250 mL). After 24 h of stirring under an atmosphere of N_2, a light brown solution was obtained. The solvent was removed by rotary evaporation and the residue dissolved in dry THF (200 mL). The solution was cooled to −35 °C, and a solution of 2-mercaptothiazoline (12.6 g, 104 mmol) and NEt_3 (10.1 g, 100 mmol) in THF (100 mL) was added dropwise over a period of 2 h. The solution was filtered at 0 °C to remove NEt_3·HCl. The solvents were removed, and the residue was dissolved in CH_2Cl_2 (50 mL) and filtered through a silica plug to yield a bright yellow solution. The solvent was removed, and the product was recrystallized from acetone (500 mL) to afford bright yellow prisms (yield: 22.9 g, 76%). ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 7.20-7.36$ (m, 12H, aromatic H), 5.08 (s, 4H, Bn CH_2), 4.32 (t, $J = 7.3$ Hz, 4H, CH_2), 2.96 (t, $J = 7.3$ Hz, 4H, CH_2) ppm. Anal. Calcd (found) for $C_{28}H_{24}N_2O_4S_4$: C, 57.91 (57.92); H, 4.17 (4.13); N, 4.82 (4.74). FAB-MS (+): m/z 581 $[M + H]^+$.

2,3-Dibenzyloxy-1-carbonyl(2-ethanolamide)-4-carbonyl(2-mercaptothiazolide) (11). A solution of ethanolamine (0.32 g, 5.26 mmol) in CH_2Cl_2 (100 mL) was added dropwise to a rapidly stirring solution of **10** (15.0 g, 26.3 mmol) in CH_2Cl_2 (150 mL) under ambient conditions. The reaction mixture was stirred for 8 h, and then the product was isolated by column chromatography (two columns required, eluent: CH_2Cl_2 with increasing gradient of MeOH, the product was the second yellow band). The solvents were removed to yield a yellow oil which solidified upon standing over several hours (yield: 2.17 g, 79%). ^{1}H NMR (D_2O, 500 MHz): $\delta = 8.17$ (t, $J = 5.4$ Hz, NH), 7.93 (d, $J = 8.2$ Hz, TAM CH), 7.39 (m, 10H, Bn H), 7.23 (d, $J = 8.2$ Hz, 2H, TAM CH), 5.14 (s, 2H Bn CH_2), 5.13 (s, 2H Bn CH_2), 4.40 (t, $J = 7.3$ Hz, 2H, thiazolide CH_2), 3.62 (t, $J = 4.7$ Hz, 2H, OCH_2), 3.39 (q, $J = 5.4$ Hz, 2H, NCH_2), 2.97 (t, $J = 7.3$ Hz, thiazolide CH_2) ppm. Anal. Calcd (found) for $C_{25}H_{26}N_2O_5S_2$: C, 62.05 (61.81); H, 5.01 (5.06); N, 5.36 (5.14). FAB-MS (+): m/z 523 $[M + H]^+$.

Benzyl-Protected TREN-1-Me-HOPO-TAM$_2$ (13) and Benzyl-Protected TREN-TAM$_3$ (14). A solution of **11** (1.50 g, 2.81 mmol) in CH_2Cl_2 (100 mL) was added dropwise to a rapidly stirring solution of TREN (0.229 g, 1.57 mmol) in CH_2Cl_2 (100 mL) over 2 h. The resulting solution was eluted down a silica column (eluent: 99:1 CH_2Cl_2:NEt_3 with increasing gradient of MeOH to 10%). The first compound that eluted was **14**; this was further purified by column chromatography (eluent: CH_2Cl_2 with increasing gradient of MeOH to 10%) to give a white foam (yield: 0.38 g, 18% relative to TREN). ^{1}H NMR (d_6-DMSO, 400 MHz): $\delta = 8.33$ (t, 3H, NH), 8.22 (t, 3H, NH), 7.28−7.39 (m, 36H, aromatic H), 5.05 (s, 12H, Bn CH_2), 4.77 (t, 3H, OH), 3.23−3.34 (m, 24H, CH_2) ppm. FAB-MS (+): m/z 1356 $[M + 1]^+$. Crude **12** was also collected from the first column; this was dissolved in CH_2Cl_2 (10 mL) and added to a solution of excess 3-benzyloxy-1-methyl-2-oxo-1,2-dihydropyridine-4-carbonyl(2-mercaptothiazolide) (0.270 g, 0.75 mmol) in CH_2Cl_2 (100 mL). After checking that the reaction had gone to completion by TLC, the solution was passed down a silica column (eluent: CH_2Cl_2 with 1% NEt_3 and an increasing gradient of MeOH to 5%). The solvents were then removed to yield a colorless oil (yield: 0.63 g, 34% relative to TREN). ^{1}H NMR (d_6-DMSO, 400 MHz): $\delta = 8.31$ (br t, 2H, TAM NH), 8.22 (br t, 2H, TAM NH), 8.14 (t, 1H, HOPO NH), 7.45 (d, $J = 7.0$ Hz, 1H, HOPO CH) 7.29−7.40 (m, 29H, aromatic H), 6.21 (d, $J = 7.0$ Hz, 1H, HOPO CH), 5.19 (s, 2H, HOPO Bn CH_2), 5.03 (s, 8H, TAM Bn CH_2), 4.75 (t, 2H, OH), 3.45 (s, 3H, HOPO CH_3), 3.24−3.33 (m, 20H, CH_2) ppm. Anal. Calcd (found) for $C_{68}H_{71}N_7O_{13}\cdot 2H_2O$: C, 66.38 (66.60); H, 6.14 (6.20); N, 7.97 (7.83). FAB-MS (+): m/z 1195 $[M + 1]^+$.

TREN-1-Me-HOPO-TAM$_2$ (4). 13 (0.470 g, 0.382 mmol) was dissolved in AcOH (5 mL) and concentrated HCl solution (5 mL), and the mixture was stirred for 3 days under ambient conditions. The solvents were removed, and the residue was dissolved in 6.0 M HCl and stirred for another 24 h (this was done in order to hydrolyze acetyl ester that had been found to have formed during the deprotection reaction). The solvents were removed, and the residue was taken up in MeOH (5 mL), which was subsequently removed by rotary evaporation (×3). The residue was then taken up in MeOH (3 mL) and added to a rapidly stirring solution of Et_2O (200 mL), to yield a white precipitate, which was filtered off and dried under vacuum (yield: 0.248 g, 77%). ^{1}H NMR (d_6-DMSO, 300 MHz): $\delta = 9.14$ (br s, 2H, TAM NH), 8.93 (br s, 2H, TAM NH), 8.70 (br s, 1H, HOPO NH), 7.41 (d, $J = 8.8$ Hz, 2H, TAM CH), 7.34 (d, $J = 8.8$ Hz, 2H, TAM CH), 7.13 (d, $J = 7.3$ Hz, 1H, HOPO CH), 6.43 (d, $J = 7.3$ Hz, 1H, HOPO CH), 3.36−3.75 (m, 23H, aliphatic H) ppm (also evidence of approximately 0.15 equiv of NEt_3, presumably present from the column in previous reaction). Anal. Calcd (found) for $C_{33}H_{42}N_7O_{13}Cl\cdot 0.15NEt_3HCl\cdot 2.5H_2O$: C, 48.14 (48.36); H, 5.89 (5.91); N, 11.84 (11.84). FAB-MS (+): m/z: 744 $[M + H]^+$.

TREN-TAM$_3$ (5). 14 (0.210 g, 0.154 mmol) was dissolved in AcOH (5 mL) and concentrated HCl solution (5 mL), and the mixture was stirred for 3 days under ambient conditions. The solvents were removed, and the residue was dissolved in 6.0 M HCl and stirred for another 24 h (this was done in order to hydrolyze acetyl ester that had been found to have formed during the deprotection reaction). The solvents were removed, and the residue was taken up in MeOH (5 mL), which was subsequently removed by rotary evaporation (×3). The residue was then taken up in MeOH (3 mL) and added to a rapidly stirring solution of Et_2O (200 mL), to yield a white precipitate, which was filtered off and dried under vacuum (yield: 0.093 g, 67%). ^{1}H NMR (d_6-DMSO, 500 MHz): $\delta = 9.05$ (br s, 3H, NH), 8.89 (br s, 3H, NH), 7.38 (d, $J = 8.5$ Hz, 3H, TAM CH), 7.30 (d, $J = 8.5$ Hz, 3H, TAM CH), 3.74 (br s, 6H, CH_2), 3.29−3.54 (m, 18H, CH_2) ppm. Anal. Calcd (found) for $C_{36}H_{46}N_7O_{15}Cl\cdot 3H_2O$: C, 47.71 (47.79); H, 5.78 (5.67); N, 10.82 (10.65). FAB-MS (+): m/z 816 $[M + 1]^+$.

X-ray Crystallography. Crystals of **6** were obtained as colorless blocks from slow evaporation of its MeOH solution. Selected crystals were mounted in Paratone N oil on the ends of quartz

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Table 2. Crystal Data for the 6-Me-3,2-HOPO Ethyl Ester

empirical formula	$C_9H_{11}NO_4$	vol (Å^3)	449.60(3)
fw	197.194	Z	2
cryst syst	triclinic	ρ_{calcd} (g cm^{-1})	1.545
space group	$P\bar{1}$	T (K)	135(2)
a (Å)	7.4801(3)	λ(Mo Kα) (Å)	0.71073
b (Å)	8.0671(3)	μ(Mo Kα) (cm^{-1})	1.21
c (Å)	8.3457(4)	R1 [$I > 2\sigma(I)$][a]	0.0420
α (deg)	72.242(2)	wR2[a]	0.1147
β (deg)	80.693(2)		
γ (deg)	69.943(2)		

[a] $R1 = \sum||F_o| - |F_c||/\sum|F_o|$. $wR2 = (\sum[w(F_o^2 - F_c^2)^2]/\sum[wF_o^4])^{1/2}$. $w = 1/[\sigma^2(F_o^2) + (0.0830P)^2 + 3.7868P]$ where $P = (F_o^2 + 2F_c^2)/3$.

capillaries and frozen into place under a low-temperature nitrogen stream (N_2-cooling retained during data collection). All measurements were made on a Siemens SMART/CCD X-ray diffractometer equipped with a CCD area detector with graphite-monochromated Mo Kα radiation ($\lambda = 0.71072$ Å). Procedures for crystal indexing, data collection, and data reduction for this instrument have been described elsewhere.[26] Structure solutions were obtained using direct methods with SHELXS-86, and structure refinement was performed with SHELXTL-97. All non-hydrogen atoms were refined with anisotropic thermal displacement parameters, and hydrogen atoms were placed at calculated positions. Crystallographic parameters for the structure are given in Table 2, and selected bond lengths and angles are listed in Table 3.

Solution Thermodynamics. Experimental protocols and equipment followed closely those of a previous study of related ligands.[14] Ligand acidity and Gd^{III} coordination properties were examined by potentiometric (pH vs total proton concentration) and spectrophotometric (absorbance vs pH) titrations, with data analysis using the Hyperquad[27] and pHAB[28] suite of computer programs. All experiments were performed at 25 °C and 0.1 M KCl. Each determination resulted from at least three independent experiments (an experiment consists of two titrations, first with acid and then in reverse with base). Equilibration times were 90 s for ligand-only titrations and 300 s for ligand−metal titrations.

Protonation constants for ligands H_3**1**, H_3**2**, and H_5**4** were determined by potentiometric titration using ligand concentrations between 0.3 and 1.0 mM in the pH range 3−10.5. In the case of H_6**5**, spectrophotometry was used (280−400 nm) with a ligand concentration of 40 μM. The log K_a corresponding to protonation of the tertiary amine nitrogen atom could not be detected by spectrophotometric titration, and the solubility of the ligand did not allow for potentiometric titration.

Gd^{III} complexation to the various ligands was investigated spectrophotometrically at molar ratios between 1:1 and 1:3.75 for Gd:L (ligand concentrations 25−100 μM), analyzing selected spectral data over the wavelength range 240−450 nm (typically 70 wavelengths included in each analysis). Ligands H_3**1** and H_3**2** form protonated Gd^{III} complexes ($GdLH_2$ and GdLH) over the pH range 1.5−3, necessitating a correction for liquid−liquid junction potentials observed in the course of pH electrode calibration, performed as in the former work.[14] The resulting stability constants for these protonated species, i.e., $GdLH_2$ and GdLH, were included as fixed parameters in subsequent determinations of the constant for the neutral species, GdL. In the case of **4**, all species (GdL, GdLH, $GdLH_2$, $GdLH_3$, and $GdLH_4$) could be monitored over the pH range 3−10. The presence of multiple absorbing species was indicated by factor analysis and by low correlation between the stability constants of the different species. The spectrophotometric titration of the Gd^{III} complex of **5** showed poor reversibility. However, averaging the values from forward and reverse titrations of three separate experiments in the pH range 3−7.5 allowed calculation of an approximate value for pGd at pH 7.4.

Table 3. Selected Bond Lengths and Angles for **6**

Bond Lengths			
O4−C7	1.3240(18)	C3−C4	1.423(2)
O4−C8	1.4700(18)	C3−C7	1.485(2)
O3−C7	1.2248(18)	O2−C2	1.3429(19)
N1−C1	1.3613(19)	C4−C5	1.350(2)
N1−C5	1.3791(19)	C2−C1	1.454(2)
O1−C1	1.2494(17)	C9−C8	1.500(2)
C3−C2	1.377(2)	C5−C6	1.496(2)
Bond Angles			
C7−O4−C8	115.49(11)	O1−C1−C2	123.14(13)
C1−N1−C5	126.05(13)	N1−C1−C2	115.25(12)
C2−C3−C4	120.37(14)	O3−C7−O4	123.67(13)
C2−C3−C7	118.01(14)	O3−C7−C3	123.20(13)
C4−C3−C7	121.62(13)	O4−C7−C3	113.12(12)
C5−C4−C3	120.27(13)	O4−C8−C9	107.13(12)
O2−C2−C3	125.27(14)	C4−C5−N1	118.21(13)
O2−C2−C1	114.91(13)	C4−C5−C6	125.83(13)
C3−C2−C1	119.82(14)	N1−C5−C6	115.95(13)
O1−C1−N1	121.61(13)		

The affinity of **3** for Gd^{III} was verified by competition against DTPA. Varying volumes of DTPA stock solution were added to solutions of identical **3**, metal, and electrolyte concentrations. All solutions were brought to pH 6.00 ± 0.01 by the addition of standardized 0.1 M KOH and diluted to identical volumes. A molar ratio of 1:1 for Gd:**3** (ligand concentration 60 μM) and molar ratios of 1:0.5 up to 1:500 for **3**:DTPA were used. An equilibration time of 24 h ensured that thermodynamic equilibrium was attained. The concentrations of free and complexed **3** in each solution were determined spectroscopically by factor analysis and least squares refinement of 100 wavelengths between 325 and 375 nm using as references solutions of free and fully complexed ligand at identical concentrations and pH. In certain cases, when the ratio of DTPA to ligand was too high, the concentration of complexed ligand could not be accurately determined. Those points were removed from further calculations (Figure 4).

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