# Synthesis, Equilibrium and Kinetic Properties of $Gd^{3+}$ Complexes of Three DTPA-Bis(Amide) Derivatives Containing Stable Nitroxide Free Radical Substituents

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Two new DTPA-bis(amide) derivatives containing stable nitroxide free radicals, $H_3L^2$ and $H_3L^3$, were synthesized. The complexation properties of $L^2$ and $L^3$ with $Gd^{3+}$ were compared with those of $H_3L^1$, synthesized earlier. The sums of the protonation constants $\Sigma\log K_i^H$ of the DTPA-bis(amide) derivative ligands with stable nitroxide free radicals are somewhat higher than that for DTPA-bis(methylamide) ($H_3L^4$). The ligands $L^1$, $L^2$, and $L^3$ form complexes with a metal-to-ligand ratio of 1:1 with $Gd^{3+}$ (GdL), while the species formed with $Zn^{2+}$ are ZnL, ZnHL, $ZnH_2L$, and $Zn_2L$. The formation of the dinuclear complex $Zn_2L$ results in a lower selectivity of the ligands for $Gd^{3+}$ over $Zn^{2+}$ than was assumed for $L^4$. The relaxivities of the complexes $GdL^1$, $GdL^2$, and $GdL^3$ are close to the sums of the relaxivities of the complex $GdL^4$ and of the free ligands $L^1$, $L^2$, and $L^3$. The relaxivities of $GdL^1$, $GdL^2$, and $GdL^3$ are practically independent of temperature in the 7–30 °C range, which is probably a result of the relatively long lifetime of the water molecules in the inner spheres of the $[GdL(H_2O)]$ complexes. The kinetic stabilities of the $Gd^{3+}$ complexes were characterized by the rates of the exchange reactions taking place between the complexes GdL and $Lu^{3+}$ or $Zn^{2+}$. The reactions occur through the proton-assisted dissociation of the complexes, with the formation of a protonated intermediate. Another pathway is the direct reaction between the $Gd^{3+}$ complex and $Lu^{3+}$ or $Zn^{2+}$, which occurs with the formation of a dinuclear intermediate, GdLM. The exchange reactions proceed about five times more rapidly with $Zn^{2+}$ than with $Lu^{3+}$, indicating the importance of the formation of an intermediate containing an N–$Zn^{2+}$ bond, which is stronger than the N–$Lu^{3+}$ bond in an intermediate with similar structure. The exchange reactions of the complex $GdL^3$ formed with the disubstituted ligand $L^3$ are significantly slower than those of the complexes of the monosubstituted ligands $L^1$ and $L^2$.

## Introduction

The $Gd^{3+}$ complex of diethylenetriamine-*N,N,N′,N′′,N′′*-pentaacetic acid ($H_5DTPA$) is a clinically used contrast agent in magnetic resonance imaging (MRI). The negative charges on $Gd(DTPA)^{2-}$ are neutralized with methylglucosamine cations, so the osmotic pressure in the body fluids after intravenous administration of the contrast agent is relatively high.[1a−1c] Nonionic $Gd^{3+}$ complexes are formed with the ligands obtained by replacement of two terminal carboxylates of DTPA with uncharged functional groups.[2] Of the uncharged functionalities investigated, the amide group has proven to be most suitable because it forms the strongest bonds with lanthanide(III) ions ($Ln^{3+}$).[3,4] The nonionic complexes [Gd(DTPA-BMA)] and [Gd(DTPA-BMEA)] are clinically used MRI contrast agents ($H_3$DTPA-BMA = $H_3L^4$ and $H_3$DTPA-BMEA = $H_3L^5$, see Scheme 1).[2,5−7] The stability constants of the $Gd^{3+}$ complexes formed with the DTPA-bis(amide) derivative ligands are significantly lower than that of $[Gd(DTPA)]^{2-}$.[3,5−7] However, the selectivity of DTPA-BMA for $Gd^{3+}$ over the endogenous $Zn^{2+}$ was found to be significantly higher than that of DTPA, and as a consequence of the favorable selectivity, [Gd(DTPA-BMA)] is a safe contrast agent.[5]

The results of NMR spectroscopic and X-ray structural studies revealed that the octadentate DTPA-bis(amide) derivative ligands coordinate to $Ln^{3+}$ through three amine nitrogen atoms, and three carboxylate and two amide oxygen atoms.[6−11] The ninth coordination site is occupied by an $H_2O$ molecule, as found by fluorescence-lifetime measurements on the complexes of $Eu^{3+}$.[6,12] The rate of exchange of the $H_2O$ molecule between the nonionic complexes $[Gd(DTPA-BMA)(H_2O)]$ or $[Gd(DTPA-BMEA)(H_2O)]$

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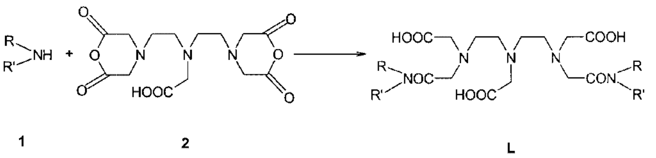
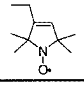


| R | H | H |  | H | $CH_3$ |
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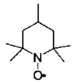
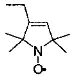
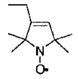
| R' |  |  |  | $CH_3$ | $CH_2CH_2OCH_3$ |
| 1 | a | b | c | | |
| Ligand | $L^1$ | $L^2$ | $L^3$ | $L^4$ | $L^5$ |

Scheme 1

and bulk water, as studied by $^{17}O$ NMR spectroscopy, was found to be about 10 times lower than that for $[Gd(DTPA)(H_2O)]^{2-}$.[10,13,14]

The successful use of [Gd(DTPA-BMA)] as a nonionic contrast agent has resulted in growing interest in the synthesis and study of new DTPA-bis(amide) derivative ligands and their $Gd^{3+}$ complexes. Through the use of different alkyl- and arylamines, a number of new ligands have been synthesized.[15−22] The structures and relaxation properties of the $Gd^{3+}$ complexes formed with these ligands are similar to those of [Gd(DTPA-BMA)] and [Gd(DTPA-BMEA)].

Besides the paramagnetic metal complexes, nitroxide-stable free radicals are also known to increase the relaxation rates of water protons. The relaxation properties of several five-membered pyrrolidine and six-membered piperidine derived free radicals have been studied as potential contrast agents.[23−27] In order to increase the relaxation effect of $Gd^{3+}$ complexes, Sosnovsky et al. synthesized a DTPA-bis-(amide) derivative ligand ($L^1$ in Scheme 1) labeled with two piperidine-*N*-oxyl free radicals.[28] The increase in the relaxation effect of the complex $GdL^1$ was not significant, but attachment of $GdL^1$ to bovine serum albumin resulted in a spin-labeled macromolecule with high relaxivity.[29] As far as we know, however, the complexation properties of the ligands with stable nitroxide free radicals, the stability constants, and the kinetic stability of the $Gd^{3+}$ complex have not yet been studied.

We recently synthesized two new DTPA-bis(amide) derivatives containing stable nitroxide free radical moieties ($L^2$ and $L^3$ in Scheme 1) and compared their complexation properties and their selectivities for $Gd^{3+}$ over $Zn^{2+}$ with those of $L^1$. The kinetic stabilities of the $Gd^{3+}$ complexes were also studied by investigation of the rates of the exchange reactions between the $Gd^{3+}$ complexes and $Lu^{3+}$ or $Zn^{2+}$. We set out to obtain information on how the presence of bulky free radicals affects the equilibria and kinetic properties of the complexes.

## Results and Discussion

### Protonation and Stability Constants

The titration curves of $L^1$, $L^2$, and $L^3$ reveal two different buffer regions, in the pH ranges about 4−7 and 8−10. Typical titration curves of $L^1$ in the absence and in the presence of equivalent amounts of $Gd^{3+}$ or $Zn^{2+}$ are shown in Figure 1. The number of titration data points used to calculate the protonation constants of $L^1$, $L^2$, and $L^3$ were 213, 180, and 279, respectively. The protonation constants ($K_i^H$) are defined as described in Equation (1), where $i$ = 1, 2, ... 5.

$$K_i^H = [H_iL]/\{[H_{i-1}L][H^+]\} \qquad (1)$$

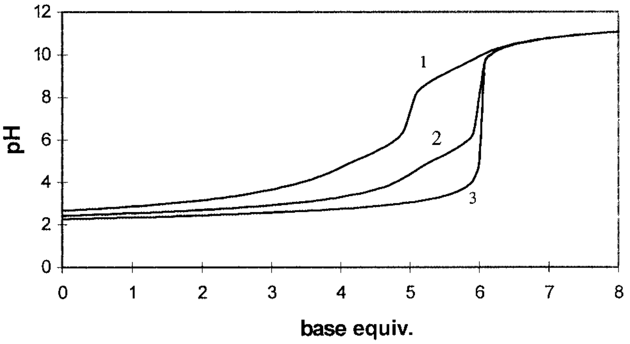


Figure 1. Titration curves of the ligand $L^1$ (1) alone or in the presence of $Zn^{2+}$ (2) or $Gd^{3+}$ (3), $[L^1] = [Zn^{2+}] = [Gd^{3+}] = 1 \times 10^{-3}$ M

The calculated protonation constants are presented in Table 1, which also gives the known protonation constants for $L^4$ and $L^5$ for comparison.

Table 1. Protonation constants of the ligands $L^1$, $L^2$, $L^3$ (25 °C, 0.1 M KCl), $L^4$ and $L^5$ (25 °C, 0.1 M $NaClO_4$)

| | $L^1$ | $L^2$ | $L^3$ | $L^4$ [a] | $L^5$[b] |
|---|---|---|---|---|---|
| $\log K_1^H$ | 9.13 (0.07) | 8.99 (0.03) | 9.46 (0.05) | 9.37 | 9.40 |
| $\log K_2^H$ | 5.40 (0.09) | 5.07 (0.04) | 5.82 (0.07) | 4.38 | 6.22 |
| $\log K_3^H$ | 3.87 (0.09) | 3.81 (0.04) | 4.21 (0.07) | 3.31 | 3.99 |
| $\log K_4^H$ | 2.90 (0.08) | 2.57 (0.05) | 3.01 (0.07) | 1.43 | 2.26 |
| $\log K_i^H$ | 21.30 | 20.44 | 22.50 | 18.49 | 21.87 |

[a] Ref.[6] [b] Ref.[7]

The first protonation constants of the different DTPA-bis(amide) derivative ligands are quite similar. The second protonation constants are somewhat lower for $L^1$ and $L^2$, containing two monosubstituted -C(O)NHR amide groups, than for $L^3$, containing two disubstituted $-C(O)NR_2$ amide groups. Similar results were found for $L^4$ and $L^5$, so the amide group does not decrease the $\log K_2^H$ value of the DTPA derivative so significantly if both amide hydrogens are replaced with alkyl (or aryl) groups.[7] This result was explained by the assumption of the formation of more hydrogen bonds in the ligands containing amide [-C(O)NHR] hydrogen atoms.[7]

The titration curves of the ligands obtained in the presence of equivalent amounts of $Zn^{2+}$ (Figure 1) could be fitted with lower values of the standard deviations and fitting parameters (the difference between the experimentally ascertained and the calculated volumes of base needed to attain a given pH value) if formation of the species ZnL, ZnHL and $ZnH_2L$ was assumed. The stability constants are defined as described in Equation (2), where $i = 0$, 1 or 2.

$$K_{\mathrm{MH_iL}} = [\mathrm{MH}_i\mathrm{L}]/\{[\mathrm{M}][\mathrm{H}_i\mathrm{L}]\} \tag{2}$$

The stability constants, calculated by use of 214, 202, and 220 data points for $L^1$, $L^2$, and $L^3$, respectively, are presented in Table 2. The formation of mono- and diprotonated complexes is probably a consequence of the presence of more basic donor atoms in the ligands than the number of $Zn^{2+}$ coordination sites. The complexes with $Zn^{2+}$ are mainly formed in the pH range from 3−6, whereas the formation of $Gd^{3+}$ complexes starts at pH values lower than 1.7 (the extent of complex formation at pH = 1.7 is about 50−60%) and it is completed at pH values around 3. Through the use of the titration data obtained in the pH range from 1.7−3 it was possible to calculate reliable stability constants, as indicated by the relatively low values of the standard deviations and fitting parameters. Similarly to the other DTPA−bis(amides) $L^4$ and $L^5$, the ligands $L^1$, $L^2$, and $L^3$ form only the species GdL, since the coordination number of $Gd^{3+}$ practically agrees with the number of ligand donor atoms. This is probably the reason why the complexes GdL do not form dinuclear complexes in the presence of excess $Gd^{3+}$. In the $ZnL^-$-$Zn^{2+}$ systems, however, the formation of dinuclear complexes $Zn_2L$ is possible because of the existence of free functional groups of the coordinated ligands. The differences between the titration curves obtained in the absence and the presence of excess $Zn^{2+}$ clearly indicate the competition between $H^+$ and $Zn^{2+}$ for the ZnL complexes. The stability constants of the dinuclear complexes $Zn_2L$ [Equation (3)] were calculated from the titration data (42, 61 and 100 data points for the systems $ZnL^1-Zn^{2+}$, $ZnL^2-Zn^{2+}$, and $ZnL^3-Zn^{2+}$, respectively), and the $\log K_{\mathrm{M_2L}}$ values are presented in Table 2.

$$K_{\mathrm{M_2L}} = [\mathrm{M_2L}]/\{[\mathrm{ML}][\mathrm{M}]\} \tag{3}$$

The formation of the dinuclear complexes $Zn_2L$ results in more successful competition of $Zn^{2+}$ with $Gd^{3+}$ for the ligand, as found experimentally by Puttagunta et al.[30] These findings also mean that the selectivity of the DTPA-bis(amide) derivative ligands for $Gd^{3+}$ over $Zn^{2+}$ is lower than calculated by Cacheris et al.,[5] who assumed the formation only of the complexes ZnL and ZnHL.

The stability constants of the complexes of $L^1$, $L^2$, and $L^3$ with $Gd^{3+}$ and $Zn^{2+}$ are higher than those of the complexes with $L^4$ and $L^5$. This result can at least in part be interpreted in terms of the different media. In 0.1 M $NaClO_4$, $Na^+$ forms complexes with $L^4$ and $L^5$ more stable than those of $K^+$ with $L^1$, $L^2$, and $L^3$. The stronger competition of $Na^+$ with $Gd^{3+}$ or $Zn^{2+}$ for the ligand may result in some decrease in the stability constants in relation to the equilibrium constants observed in KCl solution.[31] Further, the basicity of $L^1$, $L^2$, and $L^3$ is higher than that of $L^4$ and $L^5$, as expressed in the values of the protonation constants.

Table 2. Stability constants (log*K*) of the complexes $Gd^{3+}$ and $Zn^{2+}$ formed with the ligands $L^1$, $L^2$, $L^3$ (25 °C, 0.1 M KCl), $L^4$ and $L^5$ (25 °C, 0.1 M $NaClO_4$)

| | $L^1$ | $L^2$ | $L^3$ | $L^{4[a]}$ | $L^{5[b]}$ |
|---|---|---|---|---|---|
| **GdL** | 18.51 (0.05) | 17.80 (0.07) | 19.87 (0.05) | 16.38 | 17.68 |
| **ZnL** | 14.06 (0.03) | 13.63 (0.03) | 15.18 (0.02) | 12.04 | − |
| **ZnHL** | 10.20 (0.02) | 9.05 (0.01) | 10.64 (0.01) | 6.66 | − |
| **$ZnH_2L$** | 8.00 (0.02) | 6.36 (0.06) | 7.67 (0.01) | − | − |
| **$Zn_2L$** | 3.97 (0.09) | 3.57 (0.06) | 3.30 (0.02) | − | − |

[a] Ref.[6] [b] Ref.[7]

## Relaxivities of the Ligands and Complexes

The relaxivities of the ligands with stable nitroxide free radicals, measured at 9 MHz, are significantly lower than those of their $Gd^{3+}$ complexes. For $L^1$, $L^2$, and $L^3$, the relaxivities were found to be 0.68, 0.57, and 0.88 $\mathrm{mM^{-1}\cdot s^{-1}}$, respectively. The relaxivities of the five- and six-membered nitroxide stable free radicals are around 0.26 $\mathrm{mM^{-1}\cdot s^{-1}}$ (25 °C, 9 MHz). Comparison of these values indicates that the relaxivities of $L^1$ and $L^2$ are a little higher than the sums of the relaxivities of the free radicals attached to the DTPA-bis(amide), while that of $L^3$ is somewhat lower (the error in the measurement of the relaxivities is ≤ 5%). On the basis of EPR studies, Sosnowsky et al. assumed a weak exchange interaction between the free radicals attached to EDTA or DTPA.[28]

The relaxivities of the complexes $GdL^1$, $GdL^2$, and $GdL^3$ are 7.46, 6.11, and 6.60 $\mathrm{mM^{-1}\cdot s^{-1}}$, respectively. Since the relaxivity of [Gd-DTPA-BMA] is about 5.5 $\mathrm{mM^{-1}\cdot s^{-1}}$ (25 °C, 9 MHz), the attachment of the nitroxide free radicals to the Gd-DTPA-bis(amide) complexes resulted in a weak increase in relaxivity. The interaction between the unpaired electron of the free radicals and $Gd^{3+}$ must be negligible because the free radicals are not coordinated in the inner sphere of $Gd^{3+}$ and some magnetic interaction is possible only if the free radical is in the inner sphere.[32]

The relaxivities of $L^1$, $L^2$, and $L^3$ decrease with increasing temperature. The temperature dependence of the relaxivity of the complex $[Gd(DTPA)]^{2-}$ is similar: the relaxivity decreases with increasing temperature (Figure 2). The relaxation effect of free radicals (and partly the $Gd^{3+}$ complexes) arises from the translational diffusion of the water molecules near the paramagnetic centers, which results in the so-called outer-sphere contribution. An increase in temperature increases the rates of diffusion, which results in a decrease in the relaxivity values.[1a,1b] The exchange between the water molecule(s) coordinated in the inner sphere of $Gd^{3+}$ complexes and the bulk water also produces an increase in the relaxivities. The contribution of the inner sphere effect is determined predominantly by the rotation correlation time of the complex at suitable rates of water exchange {e.g. for $[Gd(DTPA)]^{2-}$}. Increasing temperatures bring about decreases in the rotation correlation times and in relaxivities, as observed for $[Gd(DTPA)]^{2-}$.[1a,1b,10] However, the relaxivities of the complexes $GdL^1$, $GdL^2$, and $GdL^3$ in the 7−30 °C range exhibit practically no change with increasing temperature (Figure 2). Similarly, the relaxivities of $GdL^4$ were found to be practically independent of temperature.[10] An increasing temperature also results in decreases in the rotation correlation times for the $Gd^{3+}$ complexes of the DTPA-bis(amide) derivatives. However, the rates of water exchange on the complexes of the DTPA-bis(amide) derivatives are about 10 times lower at 25 °C than those for $[Gd(DTPA)]^{2-}$, and the slower water exchange results in lower relaxivity values.[10,13,19,33] Increasing temperature causes an increase in the rate of water exchange, which would result in an increase of the relaxivity but this increase is compensated by the decrease in the relaxivity due to the shortening of the rotation correlation time. These results indicate that the $Gd^{3+}$ complexes formed with the DTPA-bis(amide) derivatives containing stable nitroxide free radicals behave similarly to the complexes of the other DTPA-bis(amide)derivatives, which regularly display inner-sphere lifetimes of the water molecules longer than those seen for the ionic $Gd^{3+}$ complexes such as $[Gd(DTPA)]^{2-}$ and $[Gd(DOTA)]^{-}$.[10,13,19,33]

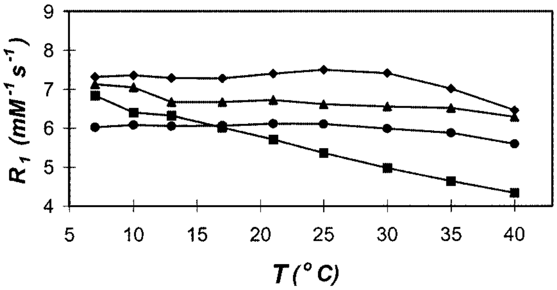


Figure 2. Temperature dependence of the relaxivities of the $Gd^{3+}$ complexes (pH = 5, [GdL] = 1 × $10^{-3}$ M, 9 MHz) (◆ $GdL^1$, ● $GdL^2$, ▲ $GdL^3$, ■ $GdDTPA^{2-}$)

### Kinetic Stabilities of $Gd^{3+}$ Complexes

As far as the clinical use of $Gd^{3+}$ complexes as MRI contrast agents is concerned, high kinetic stability is an important requirement. Since both $Gd^{3+}$ and the aminopolycarboxylate ligand are toxic, the extent of dissociation of the complexes in the body has to be practically negligible. It has been proposed that the kinetic stabilities, measured in 0.1 M HCl (or $HClO_4$) are characterized by the rates of dissociation of the complexes.[34,35] In 0.1 M acid, the dissociation occurs predominantly through the proton-assisted dissociation of the complexes, and the role of direct reactions taking place through direct attack of the endogenous ions (e.g., $Cu^{2+}$ or $Zn^{2+}$) on the complexes is neglected. In the dissociation of the DTPA and DTPA derivative complexes of $Gd^{3+}$, however, the direct reactions can be important, particularly at physiological pH values.[36,37]

The significance of the proton- and metal ion-assisted dissociation of complexes can be directly compared by study of the rates of the exchange reactions taking place between the $Gd^{3+}$ complexes and some other $Ln^{3+}$, $Cu^{2+}$ and $Zn^{2+}$ species.[36,38] As the exchanging ion in this study we used $Lu^{3+}$, which forms more stable complexes than $Gd^{3+}$ with $L^1$, $L^2$, and $L^3$, as indicated by the results of our preliminary experiments. $Cu^{2+}$ could not be used as the exchanging ion because of its redox reactions with the free radicals. Of the endogenous ions, $Zn^{2+}$ was used; this is present in the body fluids as an exchangeable species at relatively high concentrations.[39]

The rates of the exchange reactions were studied [in Equation (4), L = $L^1$, $L^2$ or $L^3$, and M = $Lu^{3+}$ or $Zn^{2+}$].

$$GdL + M^{z+} \rightarrow ML^{(z-3)+} + Gd^{3+} \tag{4}$$

In the kinetic studies, the concentrations of the exchanging ions $Lu^{3+}$ and $Zn^{2+}$ were 20−60 times higher than the concentrations of the $Gd^{3+}$ complexes. Under such conditions, the reactions can be regarded as pseudo-first order and the rates of the reactions can be expressed as in Equation (5), where $k_p$ is a pseudo-first order rate constant, and $[GdL]_t$ is the total concentration of the $Gd^{3+}$ complex.

$$-d[GdL]_t/dt = k_p\,[GdL]_t \tag{5}$$

The rates of the exchange reactions were studied at different concentrations of the exchanging $Lu^{3+}$ and $Zn^{2+}$ and at different pH values in the 4−5.2 range.

As can be seen in Table 2, the stability constants of the complexes ZnL formed with $L^1$, $L^2$, and $L^3$ are significantly lower than those of the respective $Gd^{3+}$ complexes. Were only the complexes with a metal to ligand ratio of 1:1 formed, the extent of formation of $Zn^{2+}$ complexes would be low. However, the relaxivities of the samples indicate that at equilibrium the $Gd^{3+}$ is mostly present in the form of $Gd^{3+}$·aq species, which is possible only if some other $Zn^{2+}$-containing species is also formed besides the complexes ZnL, ZnHL, and $ZnH_2L$. This species is the dinuclear complex $Zn_2L$, as found in the equilibrium studies. In order to obtain reliable pseudo-first order rate constants, the rate data were used until about 60% conversion in the exchange reactions, and the $k_p$ values were calculated by use of Equation (12). The relaxivities of the complexes GdL and the $Gd^{3+}$·aq ion were measured in separate experiments. The relaxivities of the complexes are presented above, while that of $Gd^{3+}$·aq is $R_{Gd}$ = 16.9 mM$^{-1}$·s$^{-1}$. The pseudo-first order rate constants obtained are presented in Figure 3 and 4. The $k_p$ values determined at different $Lu^{3+}$ and $Zn^{2+}$ concentrations are plotted against the $H^+$ concentration. As shown in Figure 3, the rates of the exchange reactions between the $Gd^{3+}$ complexes and $Lu^{3+}$ increase with rising $H^+$ concentration. The dependence can be expressed by a second degree equation for the $H^+$ concentration. However, the $k_p$ values increase very slightly with increasing $Lu^{3+}$ concentration. For the reactions with $Zn^{2+}$ (Figure 4) the $k_p$ values are directly proportional to the $H^+$ concentration and increase with increasing $Zn^{2+}$ concentration.

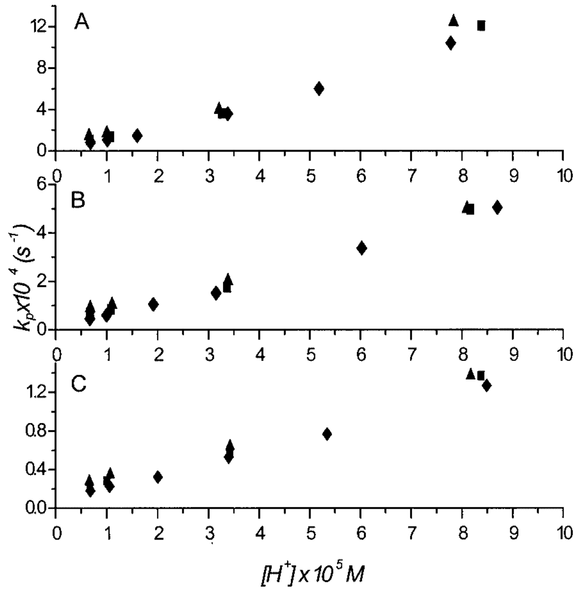


Figure 3. Rates of exchange reactions between $Gd^{3+}$ complexes ($5 \times 10^{-4}$ M) and $Lu^{3+}$; $[Lu^{3+}]$ = 0.01 M (◆), 0.02 M (■), and 0.03 M (▲) A: $GdL^1$; B: $GdL^2$, C: $GdL^3$ (25 °C, 1.0 M KCl)

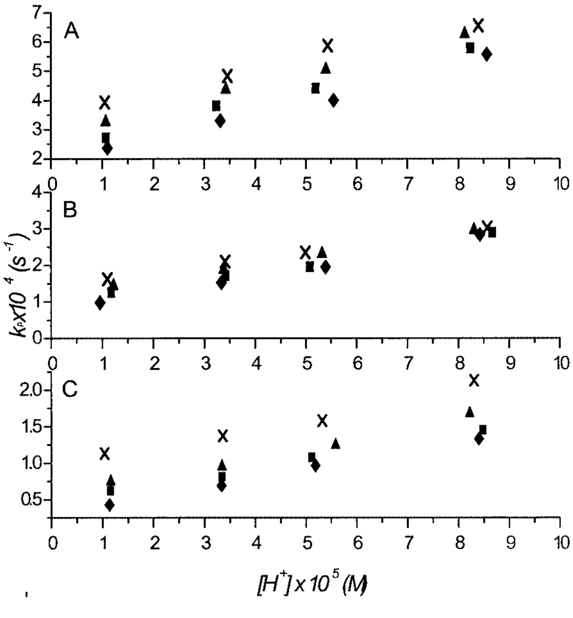


Figure 4. Rates of exchange reactions between $Gd^{3+}$ complexes ($5 \times 10^{-4}$ M) and $Zn^{2+}$; $[Zn^{2+}]$= 0.01 M (◆), 0.015 M (■), 0.02 M (▲), and 0.03 M (×) A: $GdL^1$; B: $GdL^2$, C: $GdL^3$ (25 °C, 1.0 M KCl)

These findings are similar to those obtained for the exchange reactions of the DTPA and DTPA-mono(amide) complexes of $Gd^{3+}$.[36–38] The increases in the rates of the exchange reactions with increasing $H^+$ concentration can be interpreted in terms of the relatively slow, proton-assisted dissociation of $Gd^{3+}$ complexes, which is followed by the fast reaction between the free ligand and the exchanging $Lu^{3+}$ or $Zn^{2+}$. It can be assumed that $H^+$ protonates a carboxylate group of the ligand, which may be followed by the transfer of proton to a N atom when a glycinate functionality of the ligand becomes free, and the dissociation of the ligand can take place step by step.

The formation and dissociation of protonated complexes can be expressed as follows:

$$\mathrm{GdL} + \mathrm{H^+} \overset{K_{GdHL}}{\rightleftharpoons} \mathrm{GdHL^+} \xrightarrow{k_{GdHL}} \mathrm{Gd^{3+}} + \mathrm{HL^{2-}} \qquad (6)$$

The dissociation may occur more rapidly if a second proton is attached to the monoprotonated complex:

$$\mathrm{GdHL} + \mathrm{H^+} \xrightarrow{k^H_{GdHL}} \mathrm{Gd^{3+}} + \mathrm{H_2L^-} \qquad (7)$$

The increase in the exchange rate with increasing $Lu^{3+}$ or $Zn^{2+}$ concentration can be explained in terms of the direct attack of the exchanging metal ion on the complex, in which a dinuclear complex, GdLM, is formed, the ligand being transferred slowly, step by step, to the attacking ion:[36]

$$\mathrm{GdL} + \mathrm{M^{z+}} \overset{K_{GdLM}}{\rightleftharpoons} \mathrm{GdLM} \xrightarrow{k_{GdLM}} \mathrm{ML} + \mathrm{Gd^{3+}} \qquad (8)$$

From consideration of the reaction pathways [Equations (6)–(8)], the rates of the exchange reactions can be expressed as in Equation (9):

$$-d[\mathrm{GdL}]_t/dt = k_{\mathrm{GdHL}}[\mathrm{GdHL}] + k^{\mathrm{H}}_{\mathrm{GdHL}}[\mathrm{GdHL}][\mathrm{H^+}] + k_{\mathrm{GdLM}}[\mathrm{GdLM}] \qquad (9)$$

The pseudo-first order rate constant $k_p$ can be expressed from Equations (5) and (9). By use of the expression for the total concentration of the complex, $[\mathrm{GdL}]_t = [\mathrm{GdL}] + [\mathrm{GdHL}] + [\mathrm{GdLM}]$, the equations defining the protonation constant $\{K_{\mathrm{GdHL}} = [\mathrm{GdHL}]/[\mathrm{GdL}][\mathrm{H^+}]\}$ and the stability constant of dinuclear complex $\{K_{\mathrm{GdLM}} = [\mathrm{GdLM}]/[\mathrm{GdL}][\mathrm{M}]\}$, $k_p$ can be expressed as in Equation (10), where the rate constants $k_1 = k_{\mathrm{GdHL}}K_{\mathrm{GdHL}}$ and $k_2 = k^{\mathrm{H}}_{\mathrm{GdHL}}K_{\mathrm{GdHL}}$ characterize the rates of the proton-assisted dissociation of the complexes, and $k_3 = k_{\mathrm{GdLM}}K_{\mathrm{GdLM}}$ is characteristic for the rates of exchange occurring through the direct attack of $Lu^{3+}$ or $Zn^{2+}$ on the complex:

$$k_p = \{k_1[\mathrm{H^+}] + k_2[\mathrm{H^+}]^2 + k_3[\mathrm{M}]\}/\{1 + K_{\mathrm{GdLM}}[\mathrm{M}] + K_{\mathrm{GdHL}}[\mathrm{H^+}] \qquad (10)$$

In the denominator of Equation (10), the term $K_{\mathrm{GdHL}}[\mathrm{H^+}]$ can be neglected since the protonation constants of the $Gd^{3+}$ complexes are very low (the formation of protonated complexes cannot be detected by pH potentiometry.)

The rate constants $k_1$, $k_2$, and $k_3$ and the stability constants $K_{GdLM}$ were calculated by fitting the $k_p$ values, obtained at different $H^+$ and exchanging metal concentrations, to Equation (10), and are given in Table 3. The $K_{GdLM}$ values are relatively low and so could not be calculated for all the exchange reactions. Because of the low $K_{GdLM}$ value in the denominator of Equation (10), $K_{GdLM}[M] << 1$ when the error in the calculated $K_{GdLM}$ is high (in some cases the error was higher than the constant itself).

Table 3. Rate constants $k_1$ ($M^{-1}\cdot s^{-1}$), $k_2$ ($M^{-2}\cdot s^{-1}$) and $k_3$ ($M^{-1}\cdot s^{-1}$) characterizing the exchange reactions between the complexes GdL and $Lu^{3+}$ or $Zn^{2+}$ (25 °C, 1.0 M KCl)

| Complex | $Lu^{3+}$ | $Zn^{2+}$ |
|---|---|---|
| $GdL^1$ | $k_1 = 6.1 \pm 0.4$,<br>$k_2 = (9.6 \pm 0.7) \times 10^4$,<br>$k_3 = (3.3 \pm 0.2) \times 10^{-3}$ | $k_1 = 5.6 \pm 0.2$,<br>$k_3 = (2.1 \pm 0.1) \times 10^{-2}$,<br>$K_{GdLZn} = 22 \pm 3$ |
| $GdL^2$ | $k_1 = 3.4 \pm 0.2$,<br>$k_2 = (2.9 \pm 0.4) \times 10^4$,<br>$k_3 = (2.4 \pm 0.2) \times 10^{-3}$,<br>$K_{GdLLu} = 3.0 \pm 1.8$ | $k_1 = 3.1 \pm 0.1$,<br>$k_3 = (9.2 \pm 0.4) \times 10^{-3}$,<br>$K_{GdLZn} = 27 \pm 3$ |
| $GdL^3$ | $k_1 = 1.4 \pm 0.1$,<br>$k_3 = (6.6 \pm 0.4) \times 10^{-4}$ | $k_1 = 1.2 \pm 0.1$,<br>$k_3 = (3.1 \pm 0.1) \times 10^{-3}$ |
| $Gd(DTPA)^{2-}$ [a] | $k_1 = 0.58$,<br>$k_2 = 9.7 \times 10^4$,<br>$k_3 = 4.9 \times 10^{-4}$,<br>$K_{GdLEu} = 20$ | $k_3 = 5.6 \times 10^{-2}$ |
| $GdL^4$ [a] | | $k_1 = 12.7$,<br>$k_3 = 7.8 \times 10^{-3}$ |

[a] Ref.[42]

The rate constants $k_1$ obtained for a given complex GdL in the reactions with $Lu^{3+}$ and $Zn^{2+}$ are practically equal, because the rates of the proton-assisted dissociation of the complexes are independent of the nature of the exchanging ion.

The exchange reactions occur more rapidly with $Zn^{2+}$ than with $Lu^{3+}$. This is a result of the higher value of $k_3$, which is about five times greater for the reactions with $Zn^{2+}$ than for $Lu^{3+}$. $Zn^{2+}$ is more active than $Lu^{3+}$ in the direct reaction because a $N-Zn^{2+}$ bond, which is stronger than the $N-Lu^{3+}$ bond, is formed in the dinuclear intermediate before the rate-determining step, and transfer of the other donor atoms from the $Gd^{3+}$ to the $Zn^{2+}$ is more probable.

Comparison of the values of $k_1$ and $k_3$ for the complexes $GdL^1$, $GdL^2$, $GdL^3$, and $GdL^4$ shows that the attachment of the free radicals to the DTPA-bis(amide) generally has only a slight effect on $k_3$. However, the $k_1$ values characteristic for the proton-assisted dissociation decrease by a factor of about 10 in the sequence $GdL^4$, $GdL^1$, $GdL^2$, and $GdL^3$. This sequence means that the kinetic stabilities are higher for the DTPA-bis(amide) derivative complexes containing free radicals.

The equilibrium and kinetic properties of the $Gd^{3+}$ complexes indicate that the number of substituted amide hydrogens influences both the thermodynamic and kinetic stabilities. The stability constants of the $Gd^{3+}$ complexes formed with the ligand $L^3$, containing two disubstituted amide groups, are higher than those of $GdL^2$, in which the ligand contains two monosubstituted amide groups. The trend in the kinetic stabilities is similar. The rate of proton-assisted dissociation is higher for $GdL^2$ than for $GdL^3$, so the complex formed with the disubstituted amide has the higher kinetic stability.

## Experimental Section

### Synthesis of the Ligands

DTPA dianhydride **2** (Scheme 1) was treated with paramagnetic amines RNHR′ **1** for $L^1$, $L^2$ and $L^3$ according to published procedures.[28,29,40,41] Since five-membered nitroxides are more stable than six-membered ones,[42] the syntheses were carried out with the five-membered ring nitroxide radical **1b** to give the biradical $L^2$. Since the preliminary results were promising, we tried to increase the number of the paramagnetic centers by treatment of DTPA dianhydride **2** with the biradical **1c** to yield the tetraradical $L^3$ (Scheme 1). For comparison, the ligand $L^1$ was synthesized by means of the reaction between **1a** and **2**, as carried out by Sosnowsky et al.[28]

A solution of the corresponding amine **1a**, **1b**, or **1c** (4.0 mmol) in dry DMF (30 mL) was added dropwise at 40 °C to a stirred solution of DTPA dianhydride **2** (758 mg, 2.2 mmol) in dry dimethylformamide (DMF, 60 mL), and the resulting mixture was stirred further at this temperature for 2 h. The mixture was left stand overnight and then filtered, and the filtrate was evaporated to dryness in vacuo. The residue was suspended in acetone (100 mL) and left stand at room temperature for 2 h. The turbid solution was then decanted from the residue, was again suspended in acetone (100 mL), and the mixture was left stand at 4 °C for 2 h. The resulting gummy material was filtered off and left stand over $P_2O_5$ in a vacuum desiccator overnight. The glassy semisolid was purified by flash column chromatography with $CHCl_3$/MeOH, 3:1, followed by $CHCl_3$/MeOH, 1:1 and $CHCl_3$/MeOH, 1:3. The effluents gathered with $CHCl_3$/MeOH, 1:1 and $CHCl_3$/MeOH, 1:3 were pooled and the solvents were evaporated to dryness. The crystalline residue was taken up in $Et_2O$ (20 mL) and filtered, and the filter cake was washed with acetone (30 mL), then with a 1:1 mixture of acetone/$Et_2O$ (30 mL), and air-dried.

**DTPA-Bis(amide) $H_3L^2$:** A yellow solid, 532 mg (38%); m.p. 130−133 °C; $R_f$ = 0.28 (MeOH). IR (Nujol): $\tilde{\nu}$ = 3270, 1710, 1640, 1530 $cm^{-1}$. $C_{32}H_{53}N_7O_{10}$ (695.81): calcd. C 55.24, H 7.68, N 14.09; found C 55.05, H 7.86, N 14.00.

**DTPA-Bis(amide) $H_3L^3$:** $Et_2O$ was used instead of acetone during the workup. A yellow solid, 800 mg (40%); m.p. 140−142 °C; $R_f$ = 0.37 (MeOH). IR (Nujol): $\tilde{\nu}$ = 3400, 1710, 1650 $cm^{-1}$. $C_{50}H_{81}N_9O_{12}$ (1000.25): calcd. C 60.04, H 8.16, N 12.60; found C 60.19, H 8.14, N 12.74.

Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed with a Fisons EA 1110 CHNS elemental analyzer. The IR (Specord 75) spectra were in each case consistent with the assigned structure. Flash column chromatography was performed on Merck Kieselgel 60 (0.040−0.063 mm). Qualitative TLC was carried out on commercially prepared plates (20 × 20 × 0.02 cm) coated with Merck Kieselgel $GF_{254}$. DTPA dianhydride **2** was purchased from Aldrich. Compounds **1b** and **1c** were prepared as published earlier.[43,44]

**Equilibrium Measurements**

For the preparation of $GdCl_3$ and $LuCl_3$ stock solutions, $Gd_2O_3$ and $Lu_2O_3$ (99.9%, Fluka) were dissolved in 1:1 HCl and the excess of acid was evaporated off. $ZnCl_2$ solution was prepared by dissolving $ZnCl_2$ in doubly distilled water. The concentrations of the metal chloride solutions were determined by complexometry with standardized $K_2H_2$edta, with xylenol orange as indicator ($H_4$edta = ethylenediaminetetraacetic acid).

The concentrations of the ligands $L^1$, $L^2$, and $L^3$ were determined by pH-potentiometry, on the basis of the titration curves obtained in the presence and absence of an excess of $CaCl_2$ ($c_{Ca}/c$L ≈ 40, when all the dissociable protons of the ligands dissociate).

The protonation constants of the ligands were determined by pH-potentiometric titrations with a Radiometer PHM 93 Reference pH-meter, an ABU 80 autoburette, and PHG 211 glass and K 401 calomel electrodes. The titration vessel was thermostatted at 25 °C. The titrated solutions (15 mL, 1 mM) were stirred and $N_2$ was bubbled through them. The titrations were carried out in the pH range 1.7−11.5. For the calibration of the pH-meter, KH phthalate (pH = 4.008) and borax (pH = 9.180) buffers were used. The titrations were performed in 0.1 M KCl solutions with standardized KOH solution. To obtain $H^+$ concentrations from the measured pH values, the method proposed by Irving et al. was used.[45] A 0.01 M HCl solution (0.1 M for KCl) was titrated with standardized KOH solution. The differences between the measured and calculated pH values were used to calculate the $H^+$ concentrations from the pH values obtained in the titration experiments.

The stability constants of the complexes GdL and ZnL were determined by pH-potentiometry. The titrations were performed at metal to ligand ratios of 1:1 in 1.0 mM solutions (0.1 M KCl) in the pH range 1.7−11.5.

The formation equilibria of the dinuclear complexes $Zn_2L$ were studied by pH-potentiometry. The solutions of ligands ($1.5 \times 10^{-3}$ M) were titrated with 0.2 M KOH in the presence of an excess of $Zn^{2+}$ ($3.1 \times 10^{-3}$ M). In the knowledge of the stability constants of the complexes ZnL, ZnHL, and $ZnH_2L$ and on the basis of the competition of $H^+$ and $Zn^{2+}$ for ZnL, the stability constants of the complexes $Zn_2L$ were determined.

The protonation and stability constants of the complexes were calculated with the program PSEQUAD.[46]

**Measurement of Relaxation Times**

The relaxation times of the water protons were measured at 9 MHz with an MS-4 NMR spectrometer. The temperature of the sample holder was controlled with a thermostatted air stream. The longitudinal relaxation times ($T_1$) were measured by the inversion recovery method (180°-τ-90°), with five to nine measurements at six to eight different τ values at 25 °C. In the study of the temperature dependence (7−40 °C) of the relaxation times, the concentration of the complexes $GdL^1$, $GdL^2$, and $GdL^3$ was 1.0 mM, while the concentration of the free radical ligands $L^1$, $L^2$, and $L^3$ was 10.0 mM. In the preparation of the solutions of complexes, a slight ligand excess was used. The pH of the solutions was around 5. To characterize the relaxation behavior of the free ligands and the complexes GdL, the water proton relaxation rates ($1/T_1$) were used as in Equation (11), where $T_{1m}$ and $T_{1w}$ are the relaxation times, measured in the presence and absence of the paramagnetic compound:

$$1/T_1 = 1/T_{1m} - 1/T_{1w} \tag{11}$$

The relaxivity ($R_1$) of the solutions is the increase in the relaxation rates observed on an increase in the concentration of the paramagnetic species ($c_p$) by 1.0 mM (i.e., $R_1 = (1/T_1) \times (1/c_p)$ $\text{mM}^{-1}\cdot\text{s}^{-1}$).

**Kinetic Studies**

The kinetic stabilities of the complexes (GdL) were characterized by the rates of the exchange reactions occurring between the paramagnetic complex and the diamagnetic $Lu^{3+}$ or $Zn^{2+}$. The progress of the exchange reactions was followed by measurement of the relaxation rates, since the relaxivities of the complexes GdL and the ion $Gd^{3+}$.aq formed in the exchange reactions differ considerably. The exchanging metal ions were used in large excess in order to guarantee pseudo-first order conditions. The ionic strength of the solutions was kept constant (1.0 M for KCl). The exchange rates were studied in the pH range about 4−5.2. In the reactions with $Zn^{2+}$ or $Lu^{3+}$, boric acid (0.01 M) − mannitol (0.04 M) or *N*-methylpiperazine (0.01 M) buffers were used, respectively. The pseudo-first order rate constants ($k_p$) were calculated by fitting the relaxation rate data to the Equation (12), where $R_t$ is the relaxivity at time *t*, and $R_0$ and $R_p$ are the relaxivities at the start of the reaction and at equilibrium, respectively.

$$R_t = (R_0 - R_p)\exp(-k_p t) + R_p \tag{12}$$

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[1a] B. Lauffer, *Chem. Rev.* **1987**, *87*, 901. [1b] P. Caravan, J. J. Ellison, T. J. McMurry, R. B. Lauffer, *Chem. Rev.* **1999**, *99*, 2293. [1c] *The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging* (Eds.: A. E. Merbach, E. Toth); John Wiley & Sons, LTD, New York **2001**.

[2] S. C. Quay, *Diamine-DTPA Paramagnetic Constant Agents for MR Imaging*, U.S. Patent No. 4, **1987**, 687.

[3] A. D. Sherry, W. P. Cacheris, K.-T. Kuan, *Magn. Reson. Med.* **1988**, *8*, 191.

[4] C. Paul-Roth, K. N. Raymond, *Inorg. Chem.* **1995**, *34*, 1408.

[5] W. P. Cacheris, S. C. Quay, S. M. Rocklage, *Magn. Reson. Imag.* **1990**, *8*, 467.

[6] E. N. Rizkalla, G. R. Choppin, W. P. Cacheris, *Inorg. Chem.* **1993**, *32*, 582.

[7] H. Imura, G. R. Choppin, W. P. Cacheris, L. A. LeLearie, T. J. Dumn, D. H. White, *Inorg. Chim. Acta* **1997**, *258*, 227.

[8] C. F. G. C. Geraldes, A. M. Urbano, M. C. Alpoim, M. A. Hoefnagel, J. A. Peters, *J. Chem. Soc., Chem. Commun.* **1991**, 656.

[9] C. F. G. C. Geraldes, A. M. Urbano, M. A. Hoefnagel, J. A. Peters, *Inorg. Chem.* **1993**, *32*, 2426.

[10] S. Aime, M. Botta, M. Fasano, S. Paoletti, P. L. Anelli, F. Uggeri, M. Virtuani, *Inorg. Chem.* **1994**, *33* 4707.

[11] L. Ehnebom, B. F. Pedersen, *Acta Chem. Scand.* **1992**, *46*, 126.

[12] C. F. G. C. Geraldes, A. D. Sherry, W. P. Cacheris, K.-T. Kuan, R. D. Brown, III, S. Koenig, M. Spiller, *Magn. Reson. Med.* **1988**, *8*, 191.

[13] G. Gonzalez, D. H. Powell, U. Tissierez, A. E. Merbach, *J. Phys. Chem.* **1994**, *98*, 53.

[14] E. Toth, L. Helm, A. E. Merbach, R. Heidinger, K. Hegetschweiler, A. Janossy, *Inorg. Chem.* **1998**, *37*, 4104.

[15] S. W. A. Bligh, A. H. M. S. Chowdhury, M. McPartlin, I. J. Scowen, R. A. Bulman, *Polyhedron* **1995**, *14*, 567.

[16] S. W. A. Bligh, A. H. M. S. Chowdhury, D. Kennedy, C. Luchinat, G. Parigi, *Magn. Reson. Med.* **1999**, *41*, 767.

[17] C. Geze, C. Mouro, F. Hindre, M. Le Plouzennec, C. Moinet, R. Rolland, L. Aldereghi, A. Vacca, G. Simonneaux, *Bull. Soc. Chim. Fr.* **1996**, *133*, 267.

[18] H. Lammers, F. Maton, D. Pubanz, M. W. van Laren, H. van Bekkum, A. E. Merbach, R. N. Muller, J. A. Peters, *Inorg. Chem.* **1997**, *36*, 2527.

[19] S. Aime, F. Benetollo, G. Bombieri, S. Colla, M. Fasano, S. Paoletti, *Inorg. Chim. Acta* **1997**, *254*, 63.

[20] H. Lammers, A. M. van der Heijden, H. van Bekkum, C. F. G. C. Geraldes, J. A. Peters, *Inorg. Chim. Acta* **1998**, *268*, 249.

[21] X. Zhao, R. Zhuo, Z. Lu, W. Liu, *Polyhedron* **1997**, *16*, 2755.

[22] Y.-M. Wang, Y.-J. Wang, R.-S. Sheu, G.-C. Liu, W.-C. Liu, J.-H. Liao, *Polyhedron* **1999**, *18*, 1147.

[23] H. F. Bennett, R. D. Brown, III, S. H. Koenig, H. M. Swartz, *Magn. Reson. Med.* **1987**, *4*, 93.

[24] H. F. Bennett, R. D. Brown, III, J. F. W. Keana, S. H. Koenig, H. M. Swartz, *Magn. Reson. Med.* **1990**, *14*, 40.

[25] E. G. Janzen, R. A. Towner, *Aminoxyl Radicals as MRI Contrast Agents, in Bioactive Spin Labels* (Ed.: R. I. Zhadorov), Springer Verlag, Berlin, **1999**, p. 573.

[26] P. Vallet, Y. Van Haverbeke, P. A. Bonnet, G. Subra, J.-P. Chapat, R. N. Muller, *Magn. Reson. Med.* **1994**, *32*, 11.

[27] R. A. Carvalho, J. A. Peters, C. F. G. C. Geraldes, *Inorg. Chim. Acta* **1997**, *262*, 167.

[28] G. Sosnovsky, S. W. Li, U. M. Rao, R. C. Brasch, *Z. Naturforsch., Teil B* **1985**, *40*, 1558.

[29] G. Sosnovsky, N. U. M. Rao, J. Lukszo, R. C. Brasch, *Z. Naturforsch., Teil B* **1986**, *41*, 1170.

[30] N. R. Puttagunta, W. A. Gibby, V. L Puttagunta, *Invest. Radiol.* **1996**, *31*, 619.

[31] H. Maumela, R. D. Hancock, L. Carlton, J. H. Reibenspies, K. P. Wainwright, *J. Am. Chem. Soc.* **1995**, *117*, 6698.

[32] C. Lescop, E. Belorizky, D. Luneau, P. Ray, *Inorg. Chem.* **2002**, *41*, 3375.

[33] F. Botteman, G. M. Nicole, L. V. Elst, S. Laurent, A. E. Merbach, R. N. Muller, *Eur. J. Inorg. Chem.* **2002**, 2686.

[34] P. Wedeking, K. Kumar, M. F. Tweedle, *Magn. Reson. Imaging* **1992**, *10*, 641.

[35] K. P. Pulukkody, T. J. Norman, D. Parker, L. Royle, C. J. Brown, *J. Chem. Soc., Perkin Trans.* **1993**, *2*, 605.

[36] L. Sarka, L. Burai, E. Brücher, *Chem. Eur. J.* **2000**, *6*, 719.

[37] E. Brücher, *Top. Curr. Chem.* **2002**, *221*, 103.

[38] G. L. Rothermel, Jr., E. N Riskalla, G. R. Choppin, *Inorg. Chim. Acta* **1997**, *262*, 133.

[39] P. M. May, P. W. Linder, D. R. Williams, *J. Chem. Soc., Dalton Trans.* **1977**, 588.

[40] G. C. F. G. C. Geraldes, R. Delgado, A. M. Urbano, J. Costa, F. Jasanada, F. Nepveu, *J. Chem. Soc., Dalton Trans.* **1995**, 327.

[41] F. Jasanada, F. Nepveu, *Tetrahedron Lett.* **1992**, *33*, 5745.

[42] R. C. Brasch, M. T. McNamara, R. L. Ehman, W. R. Couet, T. N. Tozer, G. Sosnovsky, N. U. Maheswara Rao, I. Prakash, *Eur. J. Med. Chem.* **1989**, *24*, 335.

[43] H. O. Hankovszky, K. Hideg, L. Lex, *Synthesis* **1981**, 147.

[44] K. Hideg, H. O. Hankovszky, L. Lex, Gy. Kulcsár, *Synthesis* **1980**, 911.

[45] H. M. Irving, M. G. Miles, L. Pettit, *Anal. Chim. Acta* **1967**, *28*, 475.

[46] L. Zekany, I. Nagypal, in *Computational Methods for Determination of Formation Constants* (Ed.: D. J. Legett), Plenum, New York, **1985**, p. 291.

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