Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Synthesis and Relaxometric Characterization of a MRI Gd-Based Probe Responsive to Glutamic Acid Decarboxylase Enzymatic Activity

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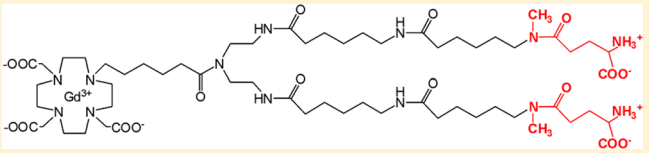
Supporting Information

ABSTRACT: Novel contrast agent based systems, which selectively visualize specific cells, e.g., neurons in the brain, would be of substantial importance for the fast developing field of molecular magnetic resonance imaging (MRI). We report here the synthesis and in vitro validation of a Gd(III)-based contrast agent designed to act as an MRI responsive probe for imaging the activity of the enzyme glutamic acid decarboxylase (GAD) present in neurons. Upon the action of the enzyme, the Gd(III) complex increases its hydration sphere and takes on a residual positive charge that promotes its binding to endogenous macromolecules. Both effects contribute in a synergic way to generate a marked relaxation enhancement, which directly reports enzyme activity and will allow activity detection of GAD positive cells in vitro and in vivo selectively.



INTRODUCTION

In the past 2 decades, paramagnetic Gd(III) complexes have been widely investigated in the context of their use as MRI contrast agents.[1−4] Their ability to enhance the contrast in MR images relies on the marked shortening effect on the water proton relaxation times in the surrounding region. Quantitatively this property is expressed by the relaxivity value (r_1), which represents the water proton relaxation rate enhancement in the presence of 1 mM concentration of the paramagnetic complex. The relaxivity of a given complex is determined by its chemical structure and molecular dynamics and is dependent on a number of environmental parameters such as field strength, temperature, interaction with other molecules, entrapment in defined compartments, etc. Although the relaxivity value is determined by the concomitant effects of several factors, it has been shown that the structure of the paramagnetic agent can be designed in a way to make it dependent only (or essentially) on a specific determinant. This approach may be exploited to endow the paramagnetic complex with the responsiveness toward a specific parameter of interest.[5−9] In a seminal paper, Meade and co-workers showed that the activity of β-galactosidase can be assessed by the relaxivity changes of a Gd complex properly functionalized with a galactose moiety that hinders the access of water molecules to the paramagnetic center.[10,11] The enzymatic cleavage removes the sugar moiety and restores the hydration of the Gd(III) ion, which in turn results in an increase of the observed relaxivity. Since then, this concept has been widely applied in the design of Gd-based responsive probes.[12−15]

Our motivation was to design a Gd-based probe responsive to the activity of glutamate decarboxylase (GAD). This enzyme catalyzes the α-decarboxylation reaction of L-glutamate (L-Glu) to yield γ-aminobutyric acid (GABA). GAD is particularly important in the central nervous system (CNS) where L-Glu and GABA act as the major excitatory and inhibitory neurotransmitters, respectively.

Variations in the GABA-mediated signaling has been associated with several pathological conditions such as Parkinson's disease, epilepsy, and psychiatric disorders.[16,17] Actually, the methods available for the identification and quantification of GAD expression and GAD activity, as well as for the evaluation of GAD positive neurons, are based on immunohistochemistry (IHC),[18−20] in situ hybridization (ISH), and fluorimetric/radiochemical measurements.[21−23] All of these methods rely on post-mortem extraction and preparation of brain samples. However, to the best of our knowledge, no probe for in vivo investigations has yet been reported.

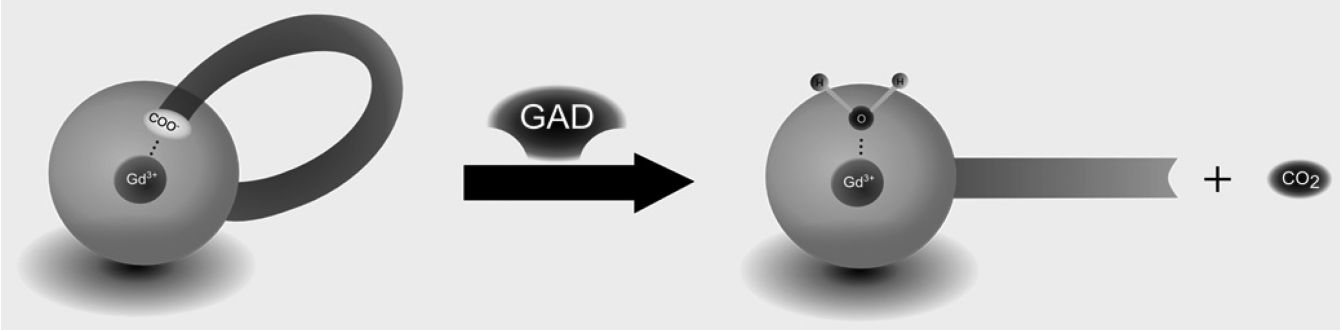
The MR probe here presented is then the first prototype of a GAD activity responsive system designed for an in vivo use. Our work has been driven by the idea of applying such a system to follow in vitro and in vivo stem cells differentiation into GABA-ergic neurons upon ex vivo labeling.[24]

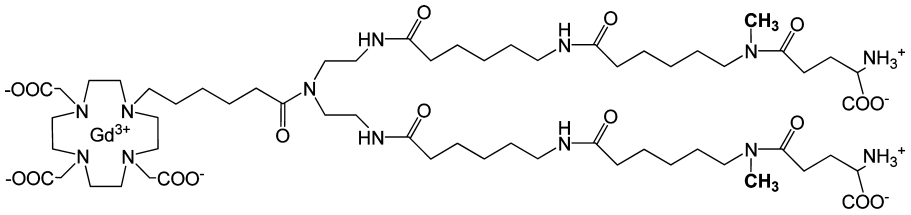
The design of the agent (Scheme 1) is in line with the mechanism of activation of the β-Gal contrast agent early developed by Meade and colleagues;[10,11] i.e., through the

Received: December 12, 2012
Published: March 8, 2013

dx.doi.org/10.1021/jm301831f | J. Med. Chem. 2013, 56, 2466−2477

Scheme 1. Schematic Representation of the GAD Responsive Probe Activation Mechanism





Gd-DO3A-GAD

Figure 1. Structure of the GAD responsive complex (**15**).

cleavage of the coordinated glutamate moieties by the GAD enzyme, an increased hydration at the Gd(III) center results in an enhanced relaxivity. Moreover, the cleavage of the glutamic moiety or moieties yields a complex with a residual positive charge that may further enhance the observed relaxivity through the electrostatic interactions with negatively charged macromolecules.

RESULTS

Synthesis of Gd-DO3A-GAD (15). The structure of Gd-DO3A-GAD (**15**) consists of a Gd-DO3A moiety bearing a long, highly flexible substituent that ends up with two glutamate residues. The DO3A cage acts as a heptadentate ligand leaving two coordination sites available on the Gd(III) ion for water molecules or other donor atoms containing units.[25] The two glutamate moieties are expected to compete with water for the two coordination sites, and thus, their presence results in generating a low relaxivity system. In fact, for Gd-DO3A derivatives, there is evidence, from relaxometric studies, that donor atoms on substituents at different distances from the N-anchoring site can enter the Gd(III) coordination cage. Several examples have been reported.[26−32] From our laboratory, a particularly instructive case deals with a derivative of Gd-DO3A bearing a flexible linker ending with a 2-pyridyldithio group that, upon covalent reaction with GSH, forms a species in which one of the glutathionyl carboxyl groups establishes an intramolecular coordinative bond with the Gd(III) metal ion.[33]

On this basis, it is expected that the terminal glutamate moiety enters the Gd(III) coordination sphere and the removal of the carboxylates by GAD yields an increase in the observed relaxivity. To avoid the cleavage of the peptidic bonds by endopeptidases, the amidic nitrogens formed upon conjugation of the glutamate residues have been selectively methylated. In order to avoid steric constrain in the access to GAD active site, the glutamate moieties have been separated from the Gd chelate by a long and flexible linker (Figure 1).

The ligand (**14**) has been synthesized according to the route shown in Scheme 2. The synthesis involved several critical steps, which were optimized to avoid the racemisation of the amino acid. First of all, it was necessary to orthogonally protect the L-glutamic acid functional groups in order to correctly derivatize the amino acid on the ω position, leaving intact the α carbon that has to be recognized by the enzyme. Thus, the amino group was protected by reaction of L-glutamic acid with benzyl chloroformiate in dimethoxyethane/water mixture at pH 9 (compound **1**). In order to esterify predominantly the α carboxylic group, **1** was stirred with DCC in tetrahydrofuran for 6 days at room temperature to get the cyclic anhydride, which was then opened by treatment with methanol in dichloromethane at room temperature. Besides the desired product, *N*-(benzyloxycarbonyl)-L-glutamic acid methyl α-emiester (**3**, 45%), the major impurities obtained were the corresponding methyl ω-emiester (28%) and the dimethyl ester (25%). No better results were obtained using acidic or basic conditions or decreasing the reaction temperature.

The crude mixture of **3** was used in the next step without any purification process because the dimethyl ester did not react at all and we found out that the methyl ω-emiester was much less reactive than the corresponding methyl α-emiester in the same coupling conditions (less than 5%). The free ω-carboxylic acid of **3** was activated in acetonitrile with DCC and NHS and then coupled in water with 6-*N*-methylaminoesanoic acid (**4**), obtained by refluxing *N*-methyl caprolactam in ethanol and 85% KOH for 4 days. During the coupling the pH was maintained at 8 in order to avoid the hydrolysis of the methyl ester and the racemization of the α carbon. The desired product (**5**) was purified by flash chromatography on silica gel and then coupled again with 6-aminohexanoic acid following exactly the same procedure as for **4**. In this way a protected glutamate residue with a flexible linker ready to enter the DO3A-like chelating cage was obtained.

In order to have 2 equiv of glutamate residues on the structure, DO3A-tris-*tert*-butyl ester was functionalized on the

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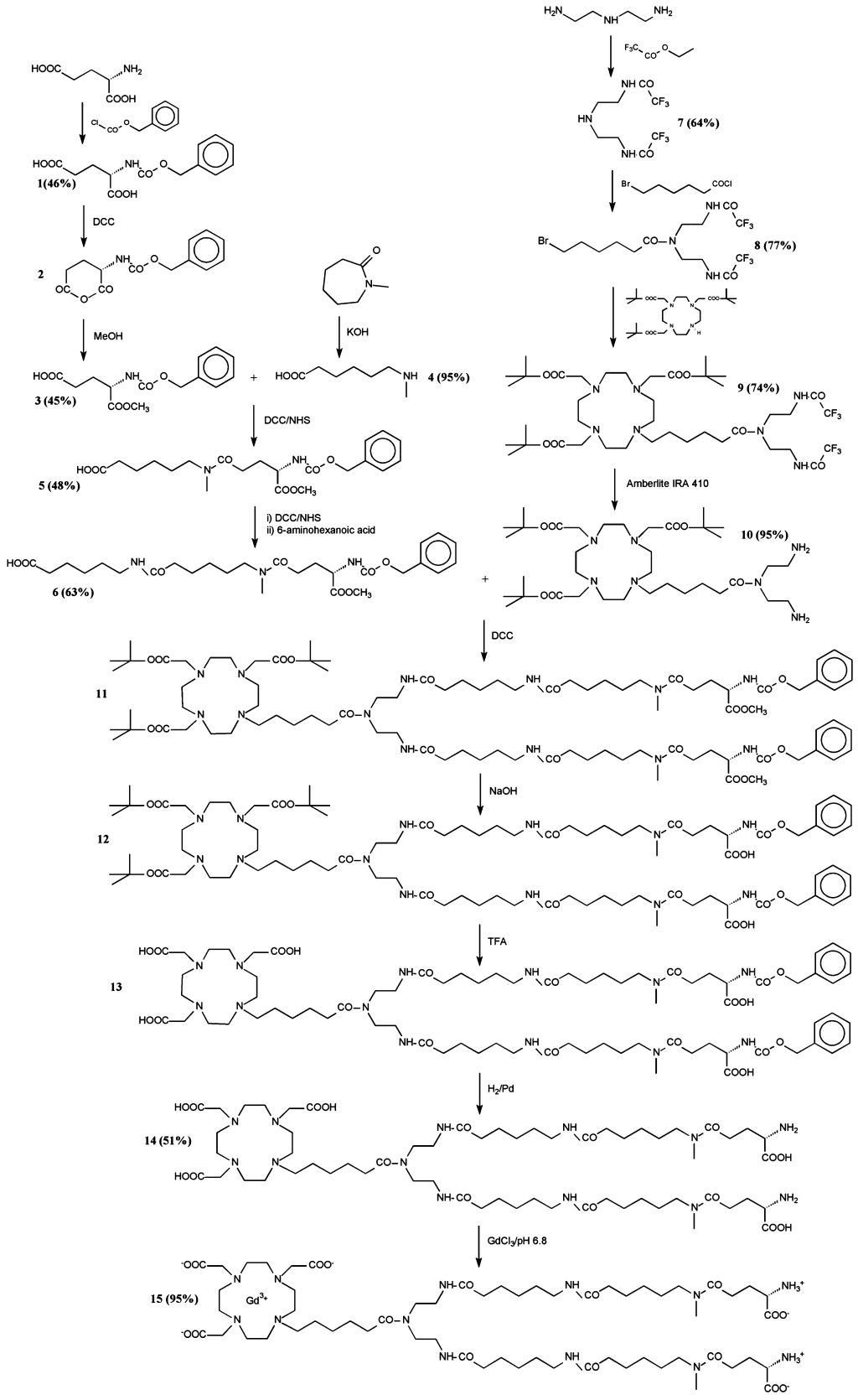
Scheme 2. Synthetic Route of Complex 15



fourth nitrogen with a diethylenetriamine unit. More specifically, both the primary amines of diethylenetriamine were protected with ethyl trifluoroacetate in tetrahydrofuran at 0 °C (**7**), while the secondary amine was acylated with 6-bromohexanoyl chloride in dichloromethane and K_2CO_3 at room temperature (**8**). **8** was then added dropwise to a solution of DO3A-tris-*tert*-butyl ester in acetonitrile to obtain compound **9**. After removal of the trifluoroacetyl groups by

dx.doi.org/10.1021/jm301831f | J. Med. Chem. 2013, 56, 2466−2477

treatment with Amberlite IRA 410 in a water/methanol mixture, both primary amino groups were finally reacted with **6** in the presence of DCC in dichloromethane. Compound **11** was directly used in the following deprotection steps as crude material. At first, the methyl ester groups were removed by hydrolysis in a water/methanol mixture at pH 12 for 5 h at room temperature. Then the *tert*-butyl groups were removed by dissolving **12** in neat trifluoroacetic acid and stirring the mixture for 20 h at room temperature. During this treatment a partial release of the benzyloxycarbonyl group was also observed. Therefore, the crude solid was dissolved in 5:1 water/methanol (10 mL) and loaded onto Amberchrom CG161 resin. Excess TFA and salts were removed, eluting with pure water, whereas the crude product and its impurities were collected, eluting with methanol. The cleavage of the remaining benzyloxycarbonyl groups was achieved by hydrogenation with Pd/C at atmospheric pressure and room temperature in methanol/water mixture. The previous removal of the TFA was necessary to complete the hydrogenation reaction. The deprotected ligand **14** was carefully purified to 98% by HPLC–MS on a semipreparative RP-C18 Atlantis column (⌀ 19 mm, height 100 mm, 5 μm particle size) with water/acetonitrile plus 0.1% TFA gradient.

The complexation with Gd(III) was done according to the method described in ref 34, allowing a slight excess of ligand (2%) to ensure that all Gd(III) is in the complexed form. The orange xylenol test confirmed complete complexation of Gd(III).[35]

Relaxometric and Potentiometric Characterization of the Gd Coordination Scheme. Figure 2 reports the relaxivity changes of **15** as a function of pH measured at 298 K and at the proton Larmor frequency of 20 MHz. At pH > 8, the observed relaxivity is very low (<2.5 mM^{-1} s^{-1}) and appears to be determined only by the outer/second sphere contributions. As the pH is moved toward the acid side, a relaxivity increase is observed likely due to a change in the hydration shell of the Gd complex. In order to better assess the hydration of the complex at different pH values, the variation of the ^{17}O transverse relaxation rate (R_{2p}) as a function of temperature was measured either at pH 5 and pH 9 (Figure 3a). Comparison of the experimental data with the profiles relative to two reference Gd complexes endowed with one (Gd-DOTA) and two (Gd-DO3A) inner sphere water molecules allowed us to realize that the hydration of **15** is very low at acid and basic pH values. On the basis of this analysis, the number of inner sphere water molecules was calculated to be slightly higher than zero (q = 0.3) at pH 5 and zero at pH 9. The finding that 0.3 inner-sphere water molecules contribute to the observed relaxivity at pH 5 may be accounted for in terms of the occurrence of different isomeric species containing one or more water molecules directly coordinated to the metal center. From the applied fitting procedure, such water molecule(s) appears to be in fast exchange (τ_M = 44 ns) with bulk water.

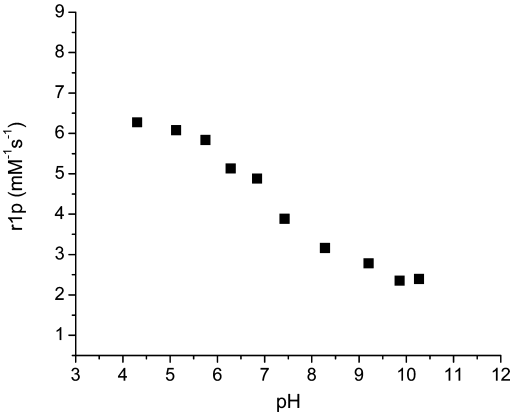


Figure 2. Proton relaxivity variation as a function of the solution pH measured at 20 MHz and 25 °C.

Further insight into the understanding of the determinants of relaxivity as a function of pH has been gained by acquiring the NMRD (nuclear magnetic resonance dispersion) profiles of **15** at pH 5 and pH 9 (Figure 3b).

A good fitting of the observed r_1 data to the values calculated on the basis of the Solomon–Bloembergen–Morgan theory for inner sphere contribution and of Freed model for outer sphere relaxation has been obtained, and the relative principal relaxometric parameters are reported in Table 1. The observed relaxivities have been assumed to receive contributions from water protons present in the second-coordination sphere of the chelate. This second-sphere contribution was analyzed on the basis of the Solomon–Bloembergen–Morgan model suitably modified by introducing a generic correlation time (τ^{ss}) that deals with the modulation of the dipolar interaction of the second-coordination sphere water molecules (exchange and/or rotation).

The fitting procedure yields quite a substantial agreement with the hydration parameters obtained from ^{17}O R_{2p} vs T analysis. The most relevant feature coming from the fitting procedure of NMRD profiles is the presence of second sphere water molecules. Actually, at pH 5, their number appears to be 3 times higher than at pH 9, indicating that at acidic pH the second coordination sphere favors hydrogen-bonded water molecules whose protons are at a distance (4 Å is the value used in the fitted NMRD profiles) that is not too different from the values usually found for inner-sphere water molecules. The relaxivity decrease observed at basic pH may then be ascribed to a marked reduction in both inner and second hydration sphere. In summary, both ^{17}O R_{2p} vs T and ^{1}H NMRD analysis highlighted a very low inner sphere hydration of the Gd(III) ion in the paramagnetic complex, which is consistent with the occurrence of intramolecular coordination of the carboxylic groups of the glutamate terminal moieties leading to a q = 0 system.

Further support to the occurrence of the proposed coordination scheme has been gained from potentiometric investigations. The ligand has four high pK values (11.4, 9.8, 9.5, and 8.6 assigned as protonation constants of ring N, glutamic NH_2 and ring N and glutamic NH_2 donor atoms, respectively). In complex **15** two protonation constants have been found for the NH_2 groups (log K_1^H = 9.19 and log K_2^H = 6.90; see Figure S1 and Table S1 in Supporting Information). The log K_2^H value of **15** is almost 2 orders of magnitude lower than the protonation constants of glutamic NH_2 groups in the free ligand and may be assigned to the NH_2 group of the glutamic acid residue in which the carboxylic group is involved in the coordination to the Gd(III) metal ion. The log K_1^H of **15** may be ascribed to the second NH_2 moiety on the pendent glutamic acid residue that is not coordinated to Gd(III). This pK value is comparable to the protonation constant of the NH_2 group of the glutamic acid (log K_1^H = 9.41, Table S1).

Responsiveness to GAD. To test the responsiveness to GAD, water proton relaxation rates have been measured at 20

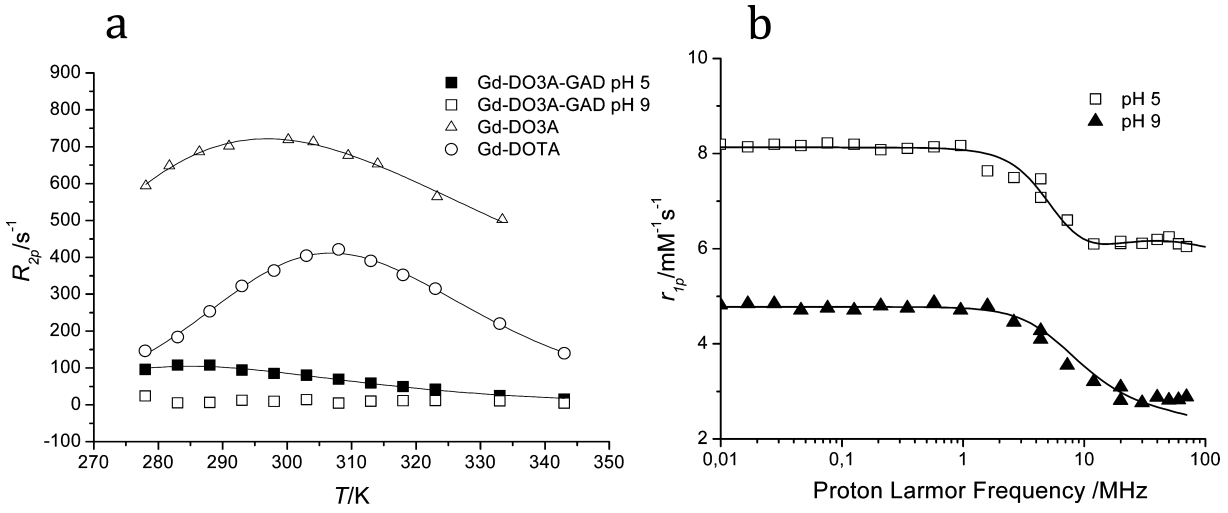


Figure 3. (a) Temperature dependence of the paramagnetic contribution to transversal ^{17}O relaxation rate measured at 14.1 T on 10 mM solutions of **15** at pH 5 (■) and pH 9 (□), of Gd-DOTA (○), and of Gd-DO3A (△). (b) $1/T_1$ ^{1}H NMRD profiles of **15** registered at 25 °C and pH 5 (□) or pH 9 (▲). The data are normalized to 1 mM concentration of the paramagnetic complexes. The solid curves through the data points were calculated with the parameters reported in Table 1.

Table 1. Principal Relaxometric Parameters Governing the Relaxometric Behavior of 15 Obtained through the Analysis of NMRD Profiles Reported in Figure 3b and ^{17}O R_{2p} vs T Data Reported in Figure 7a[a]

	Δ^2 (s^{-2})	τ_V (ps)	τ_R (ps)	τ_M (ns)	q	q^{ss}	τ^{ss} (ps)	r_{Gd-H} (Å)	r_{Gd-H}^{ss} (Å)
pH 5	4.17×10^{19}	25.0	309	44[b]	0.3[b]	3.3	60[c]	3.1[c]	4.0[c]
pH 9	4.20×10^{19}	21.0	309		0[b]	1.1	60[c]	3.1[c]	4.0[c]

[a]Experimental data have been fitted to the Solomon−Bloembergen−Morgan (for NMRD) and Swift and Connick (for ^{17}O R_{2p} vs T)[51] equations. Δ^2 is the the square of the zero-field splitting. τ_V is the correlation time describing the modulation of the zero-field splitting. τ_R is the reorientational correlation time. τ_M is the exchange lifetime of the coordinated water molecule. q is the number of inner sphere water molecules. q^{ss} is the number of second sphere water molecules. r_{Gd-H} is the distance between Gd ion and protons of the inner sphere water molecule(s), and r_{Gd-H}^{ss} is the distance between Gd ion and protons of the second sphere water molecule(s). [b]Fixed to the value found with ^{17}O R_{2p} vs T analysis. [c]Fixed during the fitting procedure.

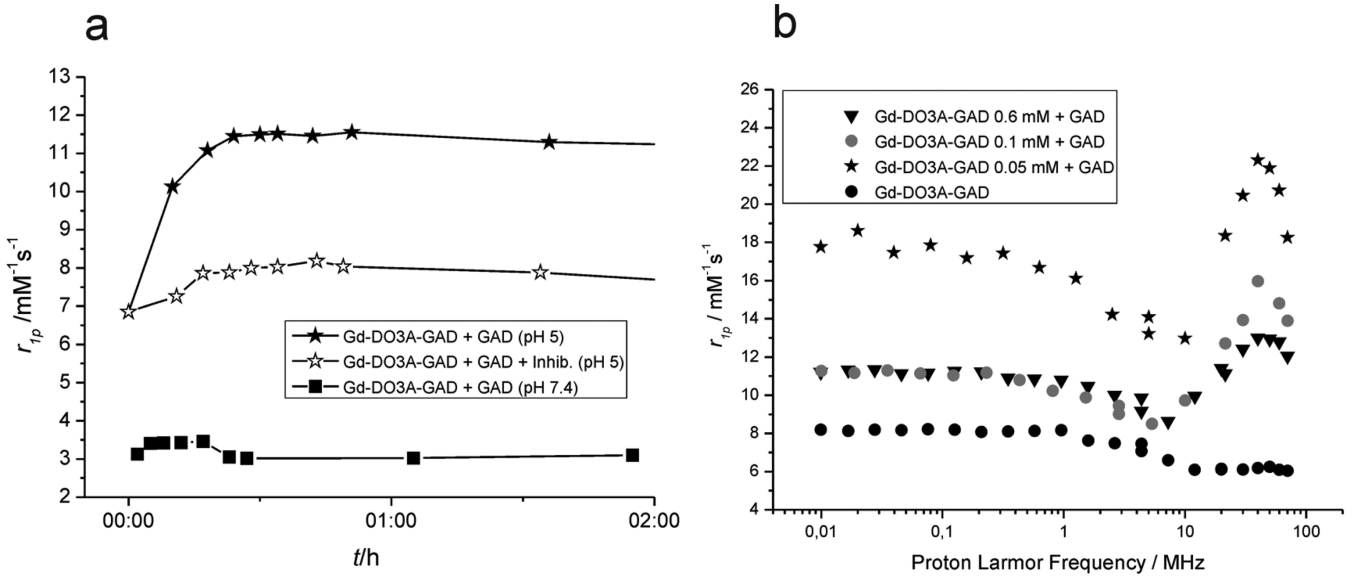


Figure 4. (a) Variation of the relaxivity of **15** (0.5 mM) over time in the presence of GAD (6 mg/mL) at pH 5 (★) and pH 7 (■) and in the presence of GAD (6 mg/mL) and the inhibitor chelidamic acid (0.5 mM) at pH 5 (☆). The measurements were carried out at 20 MHz and 25 °C. (b) $1/T_1$ ^{1}H NMRD profiles of **15** (●) and **15** at 0.05 mM (★), 0.1 mM (gray circle), 0.6 mM (▼) after 1 h of incubation with GAD enzyme (6 mg of crude extract/ml) registered at 25 °C and pH 5. The data are normalized to 1 mM concentration of the paramagnetic complex. The molar fractions of the bound complex in the different conditions are 0.79 (★), 0.43 (gray circle,) and 0.33 (▼).

MHz and 25 °C upon time on solutions of **15** incubated at 37 °C and in the presence of GAD obtained from crude acetone powder extract from *Clostridium perfringens* bacteria (0.04 U/mg solid). Even if the sequence homology of mammalian GAD

dx.doi.org/10.1021/jm301831f | J. Med. Chem. 2013, 56, 2466−2477

with nonmammalian species is rather low (for example, mammalian GAD vs *E. coli* GAD shows only ~24% identity), the region of the active site is highly conserved.[36]

The extract has been sonicated to induce complete dissolution and purified from suspended impurities and cell debris by centrifugation at 12 000 rpm for 20 min at 4 °C. Further purification steps with protamine sulfate and ammonium sulfate[37,38] were avoided. In a typical preparation from 20 mg/mL crude extract, an amount of ~1.3 mg/mL of total proteins was recovered.

In Figure 4a, the activation process is followed by reporting of the millimolar relaxivity values (r_1) of solutions of **15** (0.6 mM) after the addition of GAD extract (6 mg/mL) in MES (pH 5) or PBS (pH 7.2) buffers with or without the addition of the GAD inhibitor chelidamic acid (0.5 mM).[39] It is known that the optimal pH for bacterial GAD activity is 4.5−5.5.[40,41] In acidic conditions, the relaxivity of the solution increases to a "plateau" value that is ~60% higher than the starting relaxivity in a few tens of minutes. Conversely, no significant increase of relaxivity has been observed at pH values where the bacterial enzyme is not active (PBS buffer). Moreover, the addition of one of the most potent competitive inhibitors of GAD (chelidamic acid) consistently delays the observed increase in relaxivity, providing evidence of the specificity of the activation of the Gd probe operated by the GAD enzyme. As chelidamic acid could in principle form a ternary complex with **15**, leading to a reduction of its relaxivity, ^{1}H relaxivity measurements on solutions containing the latter complex in the presence of increasing concentrations of the GAD inhibitor have been carried out at acidic and neutral pH. The control experiment was also carried out on the parent Gd-DO3A complex that, being endowed with two inner sphere water molecules, better represents the Gd probe after GAD promoted cleavage of the glutamate carboxylates. As depicted in Figure 5, the relaxivity of both Gd complexes remains constant at acidic and neutral pH even in the presence of a 20-fold excess of chelidamic acid. These results indicate that no significant interaction takes place between the GAD inhibitor chelidamic acid and Gd-DO3A containing systems.

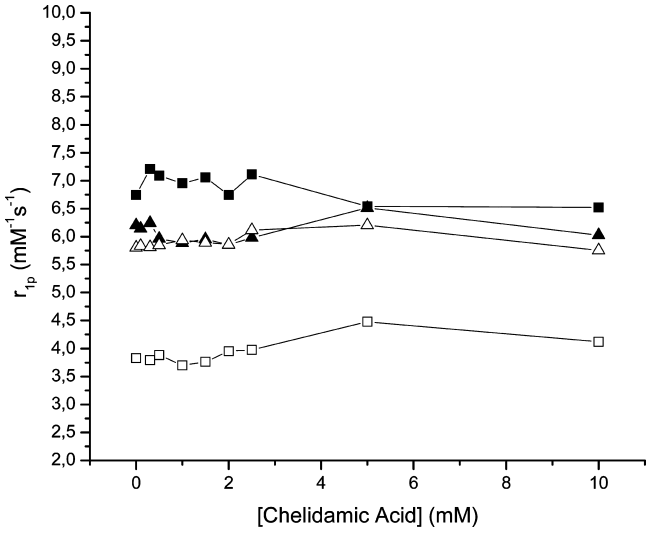


Figure 5. Variation of the relaxivity of **15** (squares) and Gd-DO3A (triangles) as a function of the addition of increasing concentrations of chelidamic acid measured at pH 5 (closed symbols) and pH 7 (open symbols) at 20 MHz and 25 °C. The data are normalized to 1 mM concentration of the paramagnetic complexes.

Further evidence of the coordination of the glutamate carboxylate in **15** and of its removal upon GAD attack has been achieved through high resolution ^{1}H NMR experiments. The relevant results are the following:

(i) In the ^{1}H NMR spectrum of the DO3A-GAD ligand the methyl groups yield two resonances (singlets) at 2.91 and 3.04 ppm, respectively (Figure 6a). The presence of two signals results from the hindered rotation around the amide bond (in fact, upon increasing the temperature to 353 K, the two signals markedly broaden as a consequence of the incipient "free" rotation around the C−N bond (Figure S2)).

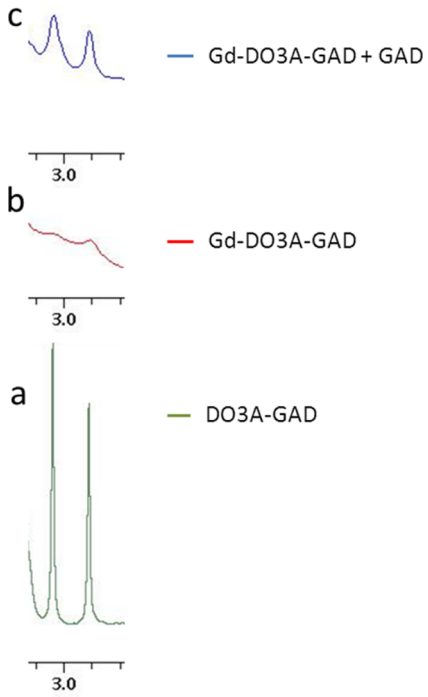


Figure 6. Sections of the ^{1}H NMR spectra registered at 14.2 T, 25 °C, and pH 5. The peaks of the methyl groups in the ligand (a), in complex **15** (b), and in **15** that underwent 24 h of enzymatic cleavage (c) are shown. Solvent was D_2O, and TMSPA was used as the internal chemical shift reference.

(ii) In the ^{1}H NMR spectrum of **15** only resonances from protons at relatively long distances from the paramagnetic metal center can be detected, and they are very broad indeed. The methyl resonances can be assigned to the two broad humps centered at about the same chemical shift values found in the parent ligand (Figure 6b).

(iii) The ^{1}H NMR spectrum of the solution of **15** that underwent GAD enzymatic activation for 24 h at 37 °C and pH 5 and was deprived of GAD enzyme by centrifugation in Vivaspin cartridge (MWCO = 3000 Da), has been acquired. Two relatively sharp resonances have emerged at the chemical shifts of the methyl groups of the ligand as detected in (i) (Figure 6c).

The complete NMR spectra are reported in Supporting Information (Figure S3). The sharpening of the methyl resonances in Figure 6c is an indication of the partial removal

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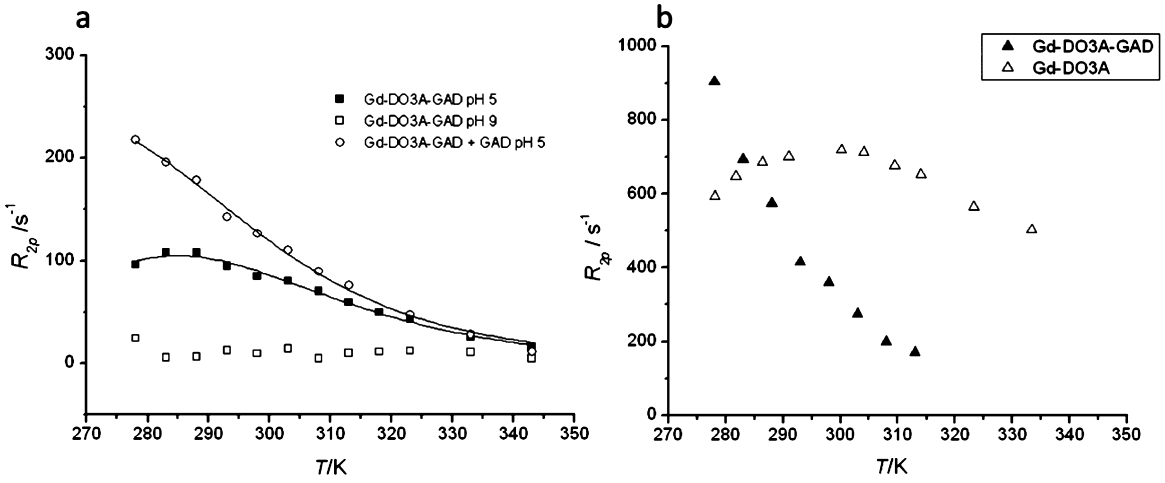

Figure 7. (a) Temperature dependence of the paramagnetic contribution to transversal ^{17}O relaxation rate measured at 14.1 T on 10 mM solutions of **15** at pH 5 (■) and pH 9 (□) and on **15** at 10 mM after 5 h of incubation with GAD extract (20 mg/mL) at pH 5 and 37 °C (○). (b) Contribution to the ^{17}O R_{2p} relaxation rate of the completely bound complex **15** calculated from the molar fraction of bound species at 10 mM Gd complex and 20 mg/mL GAD extract (▲) compared to the profile of 10 mM Gd-DO3A (△).

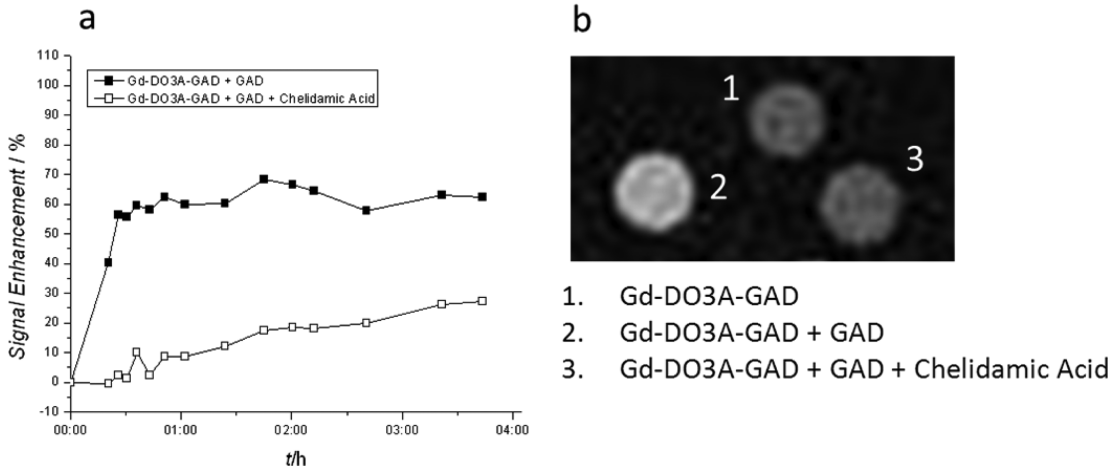

Figure 8. (a) Magnetic resonance signal enhancement observed over time after the addition of GAD enzyme (6 mg/mL crude extract) to 0.2 mM **15** with (□) or without (■) the addition of the GAD inhibitor chelidamic acid (0.5 mM). (b) T_1-weighted spin−echo ^{1}H magnetic resonance image acquired at 1 T (TR/TE/NEX (80/7.2/20), FOV 2 cm, 1 slice 2 mm) after the solutions were gently mixed for 1 h.

of the paramagnetic effect due to the closeness to the Gd(III) ion. In the parent **15**, the involvement of the terminal glutamate carboxylate(s) in the coordination scheme causes the methyl resonances to markedly broaden. The decarboxylation of the terminal glutamate moieties by GAD hampers the formation of the coordination ring, thus causing an increase of the distance between the paramagnetic center and the methyl groups. In turn, the increased distance results in a significant sharpening of the methyl resonances in the ^{1}H NMR spectrum.

Some further insight into the characteristics of the species obtained by GAD activation was attained by analyzing the NMRD profiles of **15** (measured at different concentrations of the Gd probe) after 1 h of activation at pH 5 (Figure 4b). The shape of the corresponding profiles (high relaxivity peak centered at 40 MHz) indicates that the derivative formed upon the GAD action is able to reversibly interact with the macromolecular components present in the preparation. This hypothesis is also supported by the observation that lowering the ratio between **15** and the added enzyme resulted in an increased relaxivity of the activated species. From the relaxation data acquired at different ratios between **15** and the added enzyme, it was possible to estimate the relaxivity of the completely bound species (21.4 mM^{-1} s^{-1} at 20 MHz and 25 °C) and the molar fractions of the bound complex in the different samples (see caption of Figure 4b).

Moreover, ^{17}O R_{2p} vs T data analysis of a 10 mM solution of **15** after 5 h of incubation with GAD (20 mg/mL) at pH 5 and 37 °C (Figure 7a) unambiguously indicate an increase of the Gd complex hydration. The expected formation of a complex with two inner sphere water molecules, upon enzyme activated glutamate decarboxylation, is not yet completely achieved. In fact the observed R_{2p} vs T data, which indicate the occurrence of fast exchange between the coordinated and "bulk" water, do not reach the R_{2p} values of the parent Gd-DO3A. Nevertheless, from the calculation of the molar fraction of the bound complex (0.15) at the ratio **15**/GAD extract used for this experiment, it is possible to rescale the transversal relaxivity values in order to obtain those of the completely bound Gd compex. This calculation (Figure 7b) restores, for the activated **15** complex, R_{2p} values similar to those of a q = 2 Gd complex (i.e., Gd-DO3A).

dx.doi.org/10.1021/jm301831f | J. Med. Chem. 2013, 56, 2466−2477

The GAD responsiveness of solutions of **15** can be appreciated through the acquisition of T_1-weighted magnetic resonance images. In Figure 8 magnetic resonance images obtained at 1 T (40 MHz proton larmor frequency) at different times of reaction are reported. This magnetic field is particularly suitable to assess MRI response to GAD activity of the herein developed probe because the relaxivity peak observed upon GAD activation is centered exactly at 40 MHz (Figure 4b). A phantom consisting of three capillaries containing the following solutions was used: (1) **15** in MES buffer (pH 5); (2) **15** and GAD enzyme in MES buffer (pH 5); (3) **15**, GAD enzyme and chelidamic acid in MES buffer (pH 5) (Figure 8). The MRI experiment is consistent with the relaxometric measurements. The solution contained in capillary 2 shows ∼60% signal enhancement with respect to capillary 1. As expected, the presence of the inhibitor "quenches" the relaxation enhancement, and the signal intensity of capillary 3 is very close to that observed for capillary 1.

DISCUSSION AND CONCLUSIONS

^{1}H and ^{17}O NMR relaxometric and high resolution ^{1}H NMR results show that the substituent containing two glutamate moieties is responsible for a marked reduction of the Gd(III) ion hydration in **15** with respect to the parent Gd-DO3A. The relaxivity (r_1) differences observed on going from pH 5 to pH 9 appear to indicate the occurrence, in acidic conditions, of an equilibrium between one (or more) species with higher hydration and one (or more) species in which all the coordination sites on the Gd(III) ion are taken by donor atoms from the ligand. At basic pH only, the equilibrium is fully shifted toward structure(s) that contain no inner sphere water molecules (q = 0). However, q = 0 species are still largely dominant at pH 5 because a much higher relaxivity would have been expected for a q = 2 Gd complex. One may calculate that the molecular weight increase from Gd-DO3A to **15** would have led to a r_1 of approximately 14−15 mM^{-1} s^{-1}.[42,43] The analysis of NMRD profiles of **15** at pH 5 and pH 9 indicates an important contribution from water molecules in the second coordination sphere that is particularly relevant at acidic pH. It appears that the occurrence of protonated moieties generates a system stabilized by hydrogen-bonded water molecules.[44,45]

As shown in Figures 4 and 8, the action of GAD yields a Gd complex endowed with an enhanced relaxivity. The evidence that the observed relaxation enhancement is the result of the specific GAD enzymatic activity relies on the observation of a "quenching" effect associated with the presence of chelidamic acid, a well-known inhibitor of GAD activity. Furthermore, the probe is activated only at acidic pH conditions, which are those known as necessary for bacterial GAD to work. Bacterial GAD has been used for our in vitro enzymatic activity tests because of its commercial availability. Of course, we expect that the herein reported results on the mechanism of action of the responsive Gd probe can be translated to mammalian GAD working at neutral pH. Indeed, **15** has been shown to be a good reporter of the GAD activity in GABAergic neurons in vitro and in vivo.[24]

The product of GAD activity on **15** displays a relaxometric NMRD profile that is typical of a paramagnetic macromolecular system. We do not expect the formation of a polymer structure because of the unlikely chemical transformation necessary for such a polymerization. One may consider that the release of CO_2 from **15** upon GAD enzyme action leads to a product characterized by a residual positive charge, which is likely the determinant responsible for binding of the Gd(III) complex to macromolecular structures present in the GAD protein preparation. Further investigations would be necessary to elucidate the macromolecules involved. We used a GAD preparation from *Clostridium perfringens*, which contains negatively charged macromolecules. Binding of cationic Gd(III) complexes to negatively charged macromolecules, with a consequent increase in the observed relaxivity, is well documented in the literature. As an example, Geninatti et al. reported that the binding of a spermidin functionalized positively charged Gd(III) complex to DNA plasmids in vivo results in a dramatic relaxivity enhancement.[46]

In summary, **15** represents a new entry in the family of MRI Gd-based enzyme responsive agents that have the potential for in vivo applications.[24] It exploits the change in the hydration of the paramagnetic metal ion that scales up with the relaxivity of the complex. This approach was shown to be highly versatile, as it has already been applied in the design of several enzyme-responsive agents. The formation of a net positive charge upon GAD action on **15** is an additional advantage of the system, as it promotes the interaction with slowly moving negatively charged macromolecules. This behavior, on one hand, may favor the accumulation of the Gd(III) containing product at the cellular site of its formation, and on the other hand, it is useful because the paramagnetic adduct is characterized by high relaxivity response at 0.5−1.5 T magnetic field strength.

EXPERIMENTAL SECTION

Materials. Analytical grade reagents were purchased from Sigma-Aldrich and Merck and used without further purification. Chemical reactions were monitored either by analytical HPLC (Merck Hitachi) using a Lichrosorb RP-select B 250 mm × 4 mm Merck column or by thin-layer chromatography (TLC) on Merck plates precoated with silica gel 60 F254 (0.25 mm). Spots were visualized either by UV light or by permanganate solution. Flash chromatography separations were performed on silica gel 60 (230−400 mesh, Merck). Mass spectra were acquired using a VCT Plus electrospray ionization ion trap (ESI-IT) mass spectrometer. Semipreparative HPLC−MS was performed on a Waters autopurification FractionLynx system with a multiwavelength detector and ESI mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker spectrometer at 600/150 MHz. Chemical shifts reported are relative to trimethylsilane (TMS).

Synthesis of Probe 15. Scheme 2 reports the synthetic route that has been followed for the preparation of **15**. Ligand **14** and the gadolinium complex **15** possess a purity higher than 95% determined through analytical HPLC−MS method on a Waters FractionLynx system equipped with Waters 2996 diode array (at λ = 220 nm) and Waters 3100 mass detector using XTerraC8 column 3.5 μm RPdC18 4.6/150: solvent A, 7 mM CH_3COONH_4, pH 7; solvent B, CH_3CN; 1 mL/min; 2.49 min, 5% B → 10% B; 20.00 min, 10% B → 25%B (method A).

***N*-(Benzyloxycarbonyl)-L-glutamic Acid (1).** A solution of benzyl chloroformiate (100 g, 0.58 mol) in dimethoxyethane (165 mL) was slowly dripped into a solution of L-glutamic acid (86.5 g, 0.58 mol) in water (330 mL) and ethanol (330 mL), maintaining the pH at 9 with 2 N NaOH. When the pH remained constant without requiring addition of NaOH, the solution was brought to pH 7 with 37% HCl and concentrated in vacuum to remove the organic solvent. The aqueous solution was washed with dichloromethane (5 × 50 mL) and then brought to pH 1 with 37% HCl obtaining an amorphous precipitate. After the mixture was stirred fro one night in water the white precipitate was filtered off, washed with water, and dried with P_2O_5, yielding the desired product (76.1 g, 46.0%). ^{1}H NMR (600 MHz, DMSO-d_6, 25 °C, TMS): δ (ppm) = 7.59 (d, 1H, NH), 7.31 (m, 5H, Ar-H), 5.01 (s, 2H, CH_2-Ar), 3.99 (m, 1H, CH), 2.29 (m, 2H, HOOC-CH_2), 1.95 (m, 1H, CH_2-CH), 1.74 (m, 1H, CH_2-CH) ppm. ^{13}C NMR (150 MHz, DMSO-d_6, 25 °C, TMS): δ (ppm) = 176.4

dx.doi.org/10.1021/jm301831f | J. Med. Chem. 2013, 56, 2466−2477

(COOH), 176.42 (COOH), 158.9 (HNCOO), 139.7 (Ar), 131.1 (Ar), 130.6 (Ar), 130.2 (Ar), 68.2 (CH), 55.8 (CH_2-Ar), 32.8 (CH_2), 28.80 (CH_2) ppm. MS [M + H^+] calcd, 282.1; found, 282.0.

***N*-(Benzyloxycarbonyl)-L-glutamic Acid Anhydride (2).** A solution of *N*,*N*′-dicyclohexylcarbodiimide DCC (66.4 g, 0.32 mol) in tetrahydrofuran (200 mL) was quickly dropped into a solution of **1** (82.05 g, 0.29 mol) in tetrahydrofuran (200 mL) under vigorous stirring. The formation of a white precipitate was immediately observed (*N*,*N*′-dicyclohexylurea DCU). After 6 days of stirring at room temperature (room temperature), the suspension was filtered and the solid was washed several times with tetrahydrofuran. The solution was concentrated to afford a yellowish oil still containing some DCU (85.5 g). The product was used in the following step without any further purification. ^{1}H NMR (600 MHz, $CDCl_3$, 25 °C, TMS): δ (ppm) = 7.36 (m, 5H, Ar-H), 5.14 (s, 2H, CH_2-Ar), 4.45 (m, 1H, CH), 2.48 (m, 2H, HOOC-CH_2), 1.93 (m, 1H, CH_2-CH), 1.84 (m, 1H, CH_2-CH) ppm.

***N*-(Benzyloxycarbonyl)-L-glutamic Acid Methyl α-Emiester (3).** Methanol (120 mL) was added to a solution of **2** (57.9 g, 0.22 mol) in dichloromethane (220 mL), and the solution was stirred at room temperature overnight. The solution was concentrated to afford a yellow oil (64.0 g) that was used in the following step without any purification. The main impurities were the methyl ω-emiester (28%, based on the HPLC area ratio), easier to remove in the next step because it is much less reactive and the dimethyl ester (25%) is not reactive at all. ^{1}H NMR (600 MHz, $CDCl_3$, 25 °C, TMS): δ (ppm) = 7.39 (m, 5H, Ar-H), 5.09 (s, 2H, CH_2-Ar), 4.42 (m, 1H, CH), 3.73 (s, 3H, CH_3), 2.44 (m, 2H, HOOC-CH_2), 2.21 (m, 1H, CH_2-CH), 1.99 (m, 1H, CH_2-CH). ^{13}C NMR (150 MHz, $CDCl_3$, 25 °C, TMS): δ (ppm) = 177.5 (COOH), 172.8 ($COOCH_3$), 156.3 (HNCOO), 136.2 (Ar), 129.2 (Ar), 128.3 (Ar), 67.2 (CH), 53.1 (CH_3), 51.9 (CH_2-Ar), 30.0 (CH_2), 28.4 (CH_2). MS [M + H^+] calcd, 296.3; found, 296.1.

6-*N*-Methylaminoesanoic Acid (4). A solution of *N*-methyl caprolactam (8.12 g, 0.064 mol) in ethanol (80 mL) was dropped into a solution of 85% KOH (80 mL). The solution was refluxed for 4 days and then concentrated to eliminate ethanol and washed with dichloromethane (2 × 60 mL) to eliminate the unreacted *N*-methyl caprolactam. The aqueous solution was directly used in the following step (8.8 g, 95%). ^{1}H NMR (600 MHz, D_2O, 25 °C, TMS): δ (ppm) = 2.50 (t, 2H, CH_2-NH), 2.27 (s, 3H, CH_3), 2.17 (t, 2H, CH_2-COOH), 1.56 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 1.30 (m, 2H, CH_2). ^{13}C NMR (150 MHz, D_2O, 25 °C, TMS): δ (ppm) = 186.3 (COOH), 59.7 (CH_2-NH), 52.6 (CH_2-COOH), 36.8 (CH_2), 30.4 (CH_2), 27.9 (CH_2), 19.1 (CH_3). MS [M + H^+] calcd, 146.1; found, 146.1.

***N*^{7}-Methyl-7-aza-8-oxo-11-methyloxycarbonyl-11-benzyloxycarbonylamino-11-(*S*)-unecanoic Acid (5).** DCC (2.83 g, 0.014 mol) and *N*-hydroxysuccinimide NHS (1.47 g, 0.013 mol) were added to a stirred solution of **3** (4.7 g, 0.012 mol) in acetonitrile (60 mL) at room temperature. The resulting mixture was stirred for 16 h. Then DCU was filtered off and washed several times with acetonitrile. The organic solution was then dropped into a solution of **4** (2.0 g, 0.016 mol) in water (30 mL), maintaining the pH at 8 with 2 N NaOH. After 3 h at room temperature, the acetonitrile was removed under reduced pressure, the aqueous solution was brought to pH 2 with 37% HCl, and the desired product was extracted with dichloromethane (3 × 50 mL). The organic phase was dried over Na_2SO_4 and concentrated to give a yellowish oil (5.4 g). The crude material was purified by flash chromatography on silica gel, eluting with 95:5 dichloromethane−methanol to afford the title product as a clear oil (1.84 g, 48%). ^{1}H NMR (600 MHz, $CDCl_3$, 25 °C, TMS): δ (ppm) = 7.33 (m, 5H, Ar-H), 5.07 (m, 2H, CH_2-Ar), 4.32 (m, 1H, CH), 3.71 (s, 3H, $COOCH_3$), 3.31 (m, 1H, CH_2-NCH_3), 3.19 (m, 1H, CH_2-NCH_3), 2.87 (s, 3H, N-CH_3), 2.40 (t, 2H, CH_2-COOH), 2.32 (m, 2H, CH_2-CON), 2.19 (m, 1H, CH_2-CH), 2.02 (m, 1H, CH_2-CH), 1.61 (m, 2H, CH_2), 1.50 (m, 2H, CH_2), 1.28 (m, 2H, CH_2). ^{13}C NMR (150 MHz, $CDCl_3$, 25 °C, TMS): δ (ppm) = 180.0 (COOH), 175.9 ($CONCH_3$), 174.5 ($COOCH_3$), 158.5 (HNCOO), 138.6 (Ar), 130.7 (Ar), 130. 5 (Ar), 130.3 (Ar), 69.2 (CH), 56.1 ($COOCH_3$), 54.7 (CH_2-Ar), 51.9 (CH_2-N), 50.0 (CH_2-COOH), 36.2 (CH_2), 31.8 (CH_2), 29.5 (CH_2), 29.0 (CH_2), 28.4 (CH_2), 26.7 (N-CH_3). MS [M + H^+] calcd, 423.4; found, 423.3; [M + Na^+] found, 445.3.

***N*^{14}-Methyl-7,14-diaza-8,15-dioxo-18-methyloxycarbonyl-18-benzyloxycarbonylamino-18-(*S*)-octadecanoic Acid (6).** DCC (0.97 g, 0.00468 mol) and NHS (0.36 g, 0.00312 mol) were added to a stirred solution of **5** (1.10 g, 0.0026 mol) in acetonitrile (20 mL) at room temperature. The resulting mixture was stirred for 16 h. Then DCU was filtered off and washed several times with acetonitrile. The organic solution was then dropped into a solution of 6-aminohexanoic acid (0.51 g, 0.0039 mol) in water (5 mL), maintaining the pH at 8 with 2 N NaOH. After the mixture was stirred for 4 h at room temperature, the acetonitrile was removed under reduced pressure and the aqueous solution was brought to pH 2 with 37% HCl. The crude product was extracted with dichloromethane (3 × 50 mL). The organic phase was dried over Na_2SO_4 and concentrated to give a yellowish oil (1.6 g) that was purified by flash chromatography on silica gel, eluting with 10:1 dichloromethane−methanol to yield the desired product as a clear oil (0.88 g, 63%). ^{1}H NMR (600 MHz,$CDCl_3$, 25 °C, TMS): δ (ppm) = 7.34 (m, 5H, Ar-H), 5.09 (m, 2H, CH_2-Ar), 4.34 (m, 1H, CH), 3.73 (s, 3H, $COOCH_3$), 3.34 (m, 1H, CH_2-NCH_3), 3.27 (t, 2H, CH_2NH), 3.20 (m, 1H, CH_2-NCH_3), 2.91 (s, 3H, N-CH_3), 2.41 (t, 2H, CH_2-COOH), 2.32 (m, 2H, CH_2-$CONCH_3$), 2.21 (m, 1H, CH_2-CH), 2.14 (t, 2H, CH_2-CONH), 2.05 (m, 1H, CH_2-CH), 1.61 (m, 4H, CH_2), 1.52 (m, 4H, CH_2), 1.35 (m, 2H, CH_2), 1.25 (m, 2H, CH_2). ^{13}C NMR (150 MHz, $CDCl_3$, 25 °C, TMS): δ (ppm) = 178.9 (COOH), 175.6 ($CONCH_3$), 174.9 (CONH), 174.3 ($COOCH_3$), 158.5 (HNCOO), 138.5 (Ar), 131.2 (Ar), 103.9 (Ar), 130.7 (Ar), 69.2 (CH), 56.3 ($COOCH_3$), 54.7 (CH_2-Ar), 51.6 (CH_2-NCH_3), 50.0 (CH_2-COOH), 41.3 (CH_2NH), 39.0 (CH_2), 37.2 (CH_2), 36.1 (CH_2), 31.9 (CH_2), 31.2 (CH_2), 29.8 (CH_2), 28.9 (CH_2), 27.9 (CH_2), 27.4 (CH_2), 26.3 (N-CH_3). MS [M + H^+] calcd, 536.6; found, 536.5; [M + Na^+] found, 558.4; [M + K^+] found, 574.4.

1,5-Bis(trifluoroacetylamino)-3-azapentane (7). A solution of ethyl trifluoroacetate (44.7 g, 0.315 mol) in tetrahydrofuran (50 mL) was slowly dropped into a cooled solution (0−10 °C) of diethylenetriamine (18.0 g, 0.175 mol) in tetrahydrofuran (150 mL). The reaction mixture was allowed to reach room temperature and stirred overnight. The solvent was removed under reduced pressure and the crude oil was stirred in diisopropyl ether (50 mL) to obtain a white solid, which was then filtered off, washed with diethyl ether (2 × 50 mL), and dried over P_2O_5, (33.2 g, 64%). ^{1}H NMR (600 MHz, DMSO-d_6, 25 °C, TMS): δ (ppm) = 3.21 (t, 4H, CH_2-NHCO), 2.82 (t, 2H, N*H*CO), 2.67 (t, 1H, CH_2-N*H*-CH_2), 2.62 (t, 4H, CH_2-NH-CH_2). ^{13}C NMR (150 MHz, DMSO-d_6, 25 °C, TMS): δ (ppm) = 118.8 (q, CF_3), 160.1 (q, CO), 49.8 (CH_2-NHCO), 48.1 (CH_2-NH-CH_2). MS [M + H^+] calcd, 296.1; found, 296.1.

1,5-Bis(trifluoroacetylamino)-3-*N*-[6-bromohexanoyl]-3-azapentane (8). **7** (11.8 g, 0.04 mol) was dissolved in dichloromethane (100 mL), and K_2CO_3 (6.9 g, 0.05 mol) was added. The resulting mixture was cooled at 4 °C, and 6-bromohexanoyl chloride (6.0 mL, 0.04 mol) was slowly added. After the mixture was stirred for 2 h at room temperature, salts were filtered off and washed with dichloromethane (2 × 20 mL). The organic solution was washed with 5% $NaHCO_3$ (30 mL), water (30 mL), 0.1 N HCl (30 mL), water (30 mL), and brine (30 mL). The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure (14.5 g, 76.8%). ^{1}H NMR (600 MHz, $CDCl_3$, 25 °C, TMS): δ (ppm) = 3.60 (m, 4H, CH_2-NHCO), 3.53 (m, 4H, CH_2NCO), 3.39 (t, 2H, CH_2-Br), 2.33 (t, 2H, CH_2-CO), 1.85 (q, 2H, -CH_2-), 1.60 (q, 2H, -CH_2-), 1.44 (q, 2H, -CH_2-). ^{13}C NMR (150 MHz, $CDCl_3$, 25 °C, TMS): δ (ppm) = 171.8 (CON), 160.6 (q, $COCF_3$), 160.4 (q, $COCF_3$), 118.0 (q, CF_3), 118.2 (q, CF_3), 49.7 (CH_2-NHCO), 47.6 (CH_2-NHCO), 42.0 (CH_2-NH-CH_2), 41.0 (CH_2-NH-CH_2), 35.8 (CH_2), 34.9 (CH_2), 34.6 (CH_2), 29.9 (CH_2), 26.6 (CH_2). MS [M + H^+] calcd, 474.2; found, 474.1.

1-[9-(Trifluoroacetylamino)-7-*N*-[2-(trifluoroacetylamino)-ethyl]-6-oxo-7-azanonyl]-1,4,7,10-tetraazacyclododecane-4,7,10-triacetic(1,1-dimethylethyl ester) (9). DO3A-tris-*tert*-butyl ester[47](10.3 g, 0.020 mol) was dissolved in acetonitrile (200 mL), and K_2CO_3 (3.45 g, 0.025 mol) was added. A solution of **8** (9.5 g, 0.020

dx.doi.org/10.1021/jm301831f | *J. Med. Chem.* 2013, 56, 2466−2477

mol) in acetonitrile (50 mL) was then added. The resulting mixture was stirred at room temperature for 1 week. Then the salts were removed by filtration and washed with acetonitrile (2 × 40 mL). The organic solution was concentrated in vacuo, yielding the desired product (13.5 g, 74%).

1-[7-*N*-[2-Aminoethyl]-9-amino-6-oxo-7-azanonyl]-1,4,7,10-tetraazacyclododecane-4,7,10-triacetic(1,1-dimethylethyl ester) (10). **9** (13.5 g, 0.015 mol) was dissolved in methanol (200 mL). Amberlite IRA 410 (50 mL) and water (100 mL) were then added, and the resulting mixture was stirred at room temperature for 16 h. The resin was filtered off, washed with methanol, and the solution was concentrated under reduced pressure, yielding the desired product (10.2 g, 95.4%). ^{1}H NMR (600 MHz, $CDCl_3$, 25 °C, TMS): δ (ppm) = 3.90−1.80 (b m, 34H, CH_2 DO3A + spacer), 1.60 (b m, 6H, CH_2 spacer), 1.42 (s, 27H, CH_3). MS [M + H$^+$] calcd, 715.0; found, 714.9.

1-[5-[[*N*,*N*′-Bis[3,10,17-triaza-4,11,18-trioxo-21-methyloxycarbonyl-21-benzyloxycarbonyl-*N*-methylaminoeinecosane]]-aminocarbonyl]pentyl]-1,4,7,10-tetraazacyclododecane-4,7,10-triacetic(1,1-dimethylethyl ester) (11). DCC (0.232 g, 1.125 mmol) was added to a solution of **10** (0.32 g, 0.45 mmol) and **6** (0.53 g, 0.99 mmol) in dichloromethane (8 mL). After the mixture was stirred for 30 min at room temperature, precipitation of DCU was observed. The mixture was stirred at room temperature for 4 days. The precipitate was filtered off and washed with cold dichloromethane. The organic solution was washed with 5% $NaHCO_3$ (2 × 10 mL), water (2 × 10 mL) and dried over Na_2SO_4. The organic solvents were removed under reduced pressure, and the crude product (1.06 g) was used in the following step without any further purification. MS [M + H$^+$] calcd, 1750.2; found, 1750.3.

1-[5-[[*N*,*N*′-Bis[3,10,17-triaza-4,11,18-trioxo-21-carboxyl-21-benzyloxycarbonyl-*N*-methylaminoeinecosane]]-aminocarbonyl]pentyl]-1,4,7,10-tetraazacyclododecane-4,7,10-triacetic(1,1-dimethylethyl ester) (12). A solution of **11** (1.06 g, ∼60%) in methanol (20 mL) and water (20 mL) was stirred at room temperature for 5 h, maintaining the pH constant at 12 with 1 N NaOH. The organic solvent was concentrated. The aqueous solution was brought to pH 5 with 1 N HCl and evaporated, giving a yellow residue (0.54 g), which was used in the following step without purification. MS [M + H$^+$] calcd, 1722.2; found, 1722.8.

1-[5-[[*N*,*N*′-Bis[3,10,17-triaza-4,11,18-trioxo-21-carboxyl-21-benzyloxycarbonyl-*N*-methylaminoeinecosane]]-aminocarbonyl]pentyl]-1,4,7,10-tetraazacyclododecane-4,7,10-triacetic Acid (13). **12** (0.47 g, 0.27 mmol) was dissolved in neat TFA (3 mL) at 5−10 °C, and triisopropylsilane (10 μL) was added. The mixture was stirred at room temperature for 20 h. Diethyl ether (30 mL) was slowly added in order to precipitate the desired product, which was filtered and washed with diethyl ether (2 × 5 mL) and dried in vacuo (0.54 g). During this treatment a partial removal of the benzyloxycarbonyl group was also observed. Therefore, the crude solid was dissolved in 5:1 water−methanol (10 mL) and loaded onto Amberchrom CG161 resin. Excess of TFA and salts were removed, eluting with water, whereas the crude product and its impurities were collected, eluting with methanol as a yellowish oil that was used in the following step without any further purification (0.37 g). MS [M + H$^+$] calcd, 1553.9; found, 1553.8; [M + H$^+$] − CBz found, 1418.8; [M + H$^+$] − 2CBz found, 1284.8.

1-[5-[[*N*,*N*′-Bis[3,10,17-triaza-4,11,18-trioxo-21-carboxyl-21-aminoeinecosane]]-*N*-methylaminocarbonyl]pentyl]-1,4,7,10-tetraazacyclododecane-4,7,10-triacetic Acid (14). Compound **13** (0.37 g, ∼60%) was dissolved in 1:1 methanol−water (20 mL) and 5% Pd/C (50 mg) was added. The reaction was carried out under hydrogen atmospheric pressure at room temperature for 6 h. The mixture was then filtered, and the solvents were evaporated, giving a yellowish solid as crude product (300 mg). The product was purified through HPLC−MS on a semipreparative RP-C18 Atlantis column (⌀ 19 mm, height 100 mm, 5 μm particle size), eluting with water−acetonitrile plus 0.1% TFA gradient. Fractions containing the pure product (>95%) were combined, evaporated, and then lyophilized to give a white solid (0.156 g, 51%). ^{1}H NMR (600 MHz, D_2O, 25 °C, TMS): δ (ppm) = 4.02 (m, 4H, 2 CH + CH_2-COOH DO3A), 3.7−3.15 (b m, 36H, CH_2 DO3A+spacer), 3.04 (s, 3H, CH_3), 2.91 (s, 3H, CH_3), 2.65 (b m, 4H, CH_2 spacer), 2.38 (m, 2H, CH_2 spacer), 2.21 (m, 12H, CH_2 spacer), 1.78 (m, 2H, CH_2 spacer), 1.65−1.42 (b m, 20H, CH_2 spacer), 1.40−1.20 (b m, 10H, CH_2 spacer). ^{13}C NMR(150 MHz, D_2O, 25 °C, TMS): δ (ppm) = 179.7−178.4, 176.5, 175.5, 173.8, 170.8, 165.4−164.7, 57.1, 56.3, 54.4, 54.0, 52.1, 52.1, 50.6, 50.1, 50.0, 49.1, 46.6, 41.3, 39.3, 38.9, 37.8, 37.5, 35.8, 34.8, 31.1, 30.6−30.1, 29.1, 28.2−26.8, 26.0, 24.8. HPLC method A: retention time = 4.99 min. MS [M + H$^+$] calcd, 1285.8; found, 1285.9; [M + H^{2+}] found, 643.1; [M + H^{3+}] found, 429.1; [M + H^{4+}] found, 322.1. purity 96%.

1-[5-[[*N*,*N*′-Bis[3,10,17-triaza-4,11,18-trioxo-21-carboxyl-21-aminoeinecosane]]-*N*-methylaminocarbonyl]pentyl]-1,4,7,10-tetraazacyclododecane-4,7,10-triacetate (3−) Gadolinate (15). The complexation was performed with $GdCl_3$ in aqueous solution at pH 7 by the method of the addition of the ligand.[34] An equimolar amount of $GdCl_3$ (20 mg, 0.0518 mmol) solution in water (0.5 mL) was added to a solution of ligand **14** (120 mg, 0.0518 mmol, 60%) in water (10 mL), maintaining the pH at 6.8 with 0.1 N NaOH. The mixture was allowed to stir at room temperature until the pH remained constant at 6.8. Then the solution was filtered on a 22 μm Millipore and lyophilized to give a white solid (150 mg). The amount of residual free Gd^{3+} ion was assessed by the xylenol orange UV method,[35] and the corresponding amount of ligand was added in order to prevent the presence of free Gd^{3+} ions. HPLC method A: retention time = 9.33 min. MS [M + H$^+$] calcd, 1439.7; found, 1439.8; [M + H^{2+}], 720.3; found, 720.2. Purity, 98%.

Water Proton Relaxation Measurements. The longitudinal water proton relaxation rate was measured at 25 °C by using a Stelar Spinmaster (Stelar, Mede, Pavia, Italy) spectrometer operating at 20 MHz, by means of the standard inversion−recovery technique. The temperature was controlled with a Stelar VTC-91 air-flow heater equipped with a copper constantan thermocouple (uncertainty of 0.1 °C). The relaxometric characterization of the field-dependent relaxometry of the paramagnetic Gd(III) probe solutions was carried out through the acquisition of the NMRD profiles. The proton $1/T_1$ NMRD profiles were measured at 25 °C on a fast field-cycling Stelar relaxometer over a continuum of magnetic field strengths from 0.000 24 to 0.47 T (corresponding to 0.01−20 MHz proton Larmor frequencies). The relaxometer operates under computer control with an absolute uncertainty in $1/T_1$ of ±1%. Additional data points in the range 20−70 MHz were obtained on the Stelar Spinmaster spectrometer. The concentration of the solutions used for the relaxometric characterization was determined according to a previously reported relaxometric method.[48]

^{17}O NMR Measurements. Variable temperature ^{17}O NMR measurements were recorded at 14.1 T on a Bruker 600 spectrometer, equipped with a 5 mm probe, by using a D_2O insert as internal lock. Experimental settings were the following: spectral width 8136 Hz, 90° pulse (12 μs), acquisition time 10 ms, 1024 scans, and no sample spinning. Aqueous solutions containing 2.6% of ^{17}O isotope (Yeda, Israel) were used. The observed transverse relaxation rates (R^O_{2obs}) were calculated from the signal width at half-height ($\Delta\nu_{1/2}$): $R^O_{2obs} = \pi\Delta\nu_{1/2}$. Paramagnetic contributions to the observed transversal relaxation rate (R_{2p}) were calculated by subtracting from R^O_{2obs} the diamagnetic contribution measured at each temperature on pure water enriched with 2.6% ^{17}O isotope.

Equilibrium Measurements. $GdCl_3$ stock solution was prepared by dissolving Gd_2O_3 (Fluka, 99.9%) in 6 M HCl and evaporating the excess of acid. The concentration of the $GdCl_3$ solution was determined by complexometric titration with standardized Na_2H_2EDTA and xylenol orange as indicator. The concentration of the H_3DO3A-GAD solution was determined by pH-potentiometric titrations in the presence and absence of a 40-fold excess of Ca^{2+}. The stock solution of **15** complex was prepared by stepwise mixing of the stoichiometric amounts of $GdCl_3$ and H_3DO3A-GAD solutions at pH 7.0 and 85 °C (the pH of the solution was maintained by additions of the standardized 0.2 M KOH). pH-potentiometric titrations were made with standardized 0.2 M KOH.

The protonation constants of H_3DO3A-GAD (K_i^H = [H_iL]/[$H_{i-1}L$][H$^+$]) and of the corresponding Gd complex (**15**) (K_{MHiL}=

dx.doi.org/10.1021/jm301831f | J. Med. Chem. 2013, 56, 2466−2477

$[MH_iL]/[MH_{i-1}L][H^+]$) were determined by pH-potentiometric titration in 0.0025 M ligand and of the Gd complex and in the pH range 1.7−12.0. All the equilibrium measurements were performed at 25 °C and at constant ionic strength maintained by 0.1 M KCl. The calculations of log K_i^H and log K_{MHiL} were performed by using the milliliter base−pH data obtained in the pH ranges 1.7−12.0 and 3.5−12.0, respectively.

The pH-potentiometric titrations were carried out with a Metrohm 785DMP Titrino titration workstation with the use of a Metrohm-6.0233.100 combined electrode. The titrated solutions (10 mL) were thermostated at 25 °C. The samples were stirred with a magnetic stirrer. To avoid the effect of CO_2, N_2 gas was bubbled through the solutions. For the calibration of the pH meter, KH phthalate (pH 4.005) and borax (pH 9.177) buffers were used. The equilibrium calculation was performed by using the $[H^+]$ values evaluated from measured pH in the pH-potentiometric titrations. For the calculation of the H^+ concentration from the measured pH values, the method proposed by Irving et al. was used.[49] A 0.01 M HCl (0.1 M KCl) solution was titrated with 0.2 M KOH, and the $[H^+]$ was calculated point by point. The difference between the measured and calculated pH (−log $[H^+]$) values was used to calculate the $[H^+]$ from the pH values determined in the pH-potentiometric experiments. For the calculation of the equilibrium constants the PSEQUAD program was used.[50]

Magnetic Resonance Images. Magnetic resonance images were acquired on glass capillaries filled with aqueous solutions of the different samples embedded in an agar phantom by using an Aspect M2 system (Aspect Imaging, Shoam, Israel) operating at 1 T equipped with a horizontal bore MRI magnet using a standard T_1 weighted multislice spin echo sequence (TR = 80 ms, TE = 7.2 ms, NEX = 20, FOV = 2 cm, 1 slice, slice thickness = 2 mm).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Potentiometric equilibrium studies and ^{1}H NMR high resolution spectra of the DO3A-GAD ligand and its Gd complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written with contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by grants from ENCITE EU-FP7 (Grant HEALTH-F5-2008-201842), Regione Piemonte (Project PIIMDMT), MIUR (Project PRIN 2009235JB7), the Italian Consortium CIRCMSB, and the Volkswagen Foundation (Grant I/83 443).

■ ABBREVIATIONS USED

GAD, glutamic acid decarboxylase; L-Glu, L-glutamate; CNS, central nervous system; ISH, in situ hybridization; β-Gal, β-galactosidase; GSH, glutathione; NHS, *N*-hydroxysuccinimide; NMRD, nuclear magnetic resonance dispersion; DCU, dicyclohexylurea; DO3A, 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid

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