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The stereochemistry of amide side chains containing carboxyl groups influences water exchange rates in EuDOTA-tetraamide complexes

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Abstract

Many Eu(III) complexes formed with DOTA-tetraamide ligands (where DOTA is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) have sufficiently slow water exchange kinetics to meet the slow-to-intermediate condition required to serve as chemical exchange saturation transfer (CEST) contrast agents for MRI. This class of MRI contrast agents offers an attractive platform for creating biological sensors because water exchange is exquisitely sensitive to subtle ligand stereochemistry and electronic effects. Introduction of carboxyl groups or carboxyl ethyl ester

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groups on the amide substituents has been shown to slow water exchange in these complexes, but less is known about the orientation or position of these side-chain groups relative to the inner-sphere Eu(III)-bound water molecule. In this study, a series of Eu(III) complexes having one or more carboxyl groups or carboxyl esters at the δ -position of the pendant amide side chains were prepared. Initial attempts to prepare optically pure EuDOTA-[(S)-Asp]₄ resulted in a chemically pure ligand consisting of a mixture of stereochemical isomers. This was traced to racemization of (S)-aspartate diethyl ester during the synthetic procedure. Nevertheless, NMR studies of the Eu(III) complexes of this mixture revealed that each isomer had a different water exchange rate, differing by a factor of 2 or more. A second controlled synthesis and CEST study of EuDOTA-[(S)-Asp]₄ and *cis*-EuDOTA-[(S)-Asp]₂[(R)-Asp]₂ confirmed that the water exchange rates in these diastereomeric complexes are controlled by the axial versus equatorial orientation of the carboxyl groups on the amide side chains. These observations provide new insights toward the development of even more slowly water exchanging systems which will be necessary for practical in vivo applications.

Keywords

Lanthanide complexes; Macrocyclic conformations; MRI contrast agents; Water exchange

Introduction

Modulation of water exchange in lanthanide(III) complexes has been widely studied over the past few years largely driven by interest in optimizing their use as contrast agents for magnetic resonance imaging (MRI). In the case of gadolinium-based T_1 agents, the goal for some time has been to increase the rate of water exchange, whereas in the case of paramagnetic chemical exchange saturation transfer (PARACEST) agents [where the chemical exchange saturation transfer (CEST) signal is based on water molecule exchange], the goal has been the opposite, to decrease the rate of water exchange [1]. A fundamental requirement for CEST is that the rate of chemical exchange between two exchanging pools (k_{ex}) is less than or equal to the resonance frequency difference between the two pools ($\Delta\omega$), the so-called slow-to-intermediate exchange condition. This means that paramagnetic complexes with large $\Delta\omega$ values can meet the intermediate exchange requirement for CEST with faster-exchanging species. Larger $\Delta\omega$ values have the added benefit of reducing the chances of off-resonance saturation of bulk water signal. The inner-sphere water molecule in many EuDOTA-tetraamide complexes (where DOTA is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) typically displays both slow exchange kinetics and a large hyperfine shift (large $\Delta\omega$) and therefore easily meets the CEST requirement, $\Delta\omega > k_{ex}$ [2–4]. Exogenous paramagnetic agents of this type have been designed to be responsive to a variety of important biological parameters, including pH [5–9], temperature [7,10], redox chemistry [11, 12], enzyme activity [13–15], and the tissue distribution of glucose [16–18], and as gene reporters [19]. Although some PARACEST sensor designs have relied on exchanging –NH or –OH protons on the ligand, those based on water molecule exchange offer some advantages, including larger hyperfine shifts (larger $\Delta\omega$) and widely tunable water exchange rates [1]. Although several successes have been reported to date, it is important to identify complexes with even slower water exchange so the CEST signal can be activated using low-power RF pulses [3], clearly an advantage for clinical translation. The water exchange rate in Ln^{3+} complexes is known to be governed by some important factors, including coordination geometry [20–22], the size of the Ln^{3+} ion [23], and the electron density on ligating atoms [12, 24]. It was previously demonstrated that introduction of carboxyl groups or carboxyl ethyl esters on the amide substituent of EuDOTA-tetraamide complexes effectively slows water exchange in these systems [25]. It was hypothesized that the hydrogen-bonding network formed by carboxyl groups and

surrounding water molecules stabilizes second-sphere water molecules, whereas the added hydrophobicity of the ester group presumably makes it more difficult for outer-sphere water molecules to approach the inner-sphere coordination position. Both effects lead to slower water exchange rates. In the present study, we introduced a chiral center at the α -position of the amide arms to evaluate the effects of positioning the carboxyl or carboxyl ethyl ester into axial versus equatorial positions on the water exchange rates and CEST properties of these complexes (Fig. 1).

Materials and methods

General remarks

All solvents and reagents were purchased from commercial sources and used as received unless otherwise stated. High-resolution ^1H and ^{13}C NMR spectra were recorded with a Bruker Avance III spectrometer operating at 400.13 and 100.62 MHz, respectively. Infrared spectra were recorded with a Nicolet Avatar 360 Fourier transform infrared spectrophotometer. Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. The samples for CEST studies were prepared by dissolving the appropriate amount of the agents in water. The pH of the solutions used for high-resolution ^1H NMR and CEST experiments was adjusted to approximately 7 by adding a small amount of NaOH or gaseous HCl. All CEST spectra were recorded using an Agilent (Varian) INOVA 500 spectrometer.

CEST spectra

The standard method for characterizing the CEST properties of lanthanide complexes is to apply a long, frequency-selective presaturation pulse over a range of presaturation frequencies followed by an observe pulse to monitor the intensity of residual water. The resulting plot of the residual bulk water signal intensity, M_s/M_0 , versus saturation frequency is referred to as a CEST spectrum [26]. Such spectra are useful in comparing the CEST properties of such complexes (at equivalent concentrations) and in measuring the water and/or proton exchange rates that contribute to the CEST spectrum. In these studies, CEST spectra were acquired by applying a long, frequency-selective presaturation pulse over the range of ± 100 ppm to cover all potentially exchanging species, including the Eu^{3+} -bound water molecule that frequency appears at approximately 50 ppm with water set to 0 ppm. The applied B_1 field (in hertz) was calibrated prior to collection of the CEST spectra by measuring the 360° pulse width for bulk water protons as a function of the transmitter power level. CEST experiments were performed at four different B_1 values (9.4, 14.1, 18.8, and 23.5 μT).

Fitting CEST spectra to the Bloch equations modified for exchange

The water exchange rates were estimated by fitting the experimental CEST spectra to a three-pool model (Eu^{3+} -bound water molecule, ligand amide protons, and bulk water protons) using a numerical solution to the Bloch equations modified for exchange and written in MATLAB (The MathWorks, Natick, MA, USA) [3, 27]. T_1 of solvent water was measured using a standard inversion recovery sequence without a presaturation pulse, whereas T_2 was estimated from the full width at half height of the bulk water peak. The following parameters were included in the fitting procedure: applied field (B_1) in hertz, the presaturation time, concentrations of exchanging bound water protons and amide protons, T_1 (bulk water), T_2 (bulk water), and chemical shifts of the Eu^{3+} -bound water exchange peak and the complex $-\text{NH}$ protons. Since CEST was detected only from the square antiprism (SAP) isomer as evidenced by the water exchange peak near 50 ppm, the concentration of the complex was given as that fraction of the total concentration that exists in solution as the SAP isomer as measured by high-resolution NMR spectroscopy. It was assumed that the

twisted square antiprism (TSAP) isomer, when present in small amounts in the sample, had a negligible effect on the fitting results. The residence lifetime of the Eu^{3+} -bound water molecule was determined by averaging the fitting results measured at four B_1 values (9.4, 14.1, 18.8, and 23.5 μT). Initial estimates of τ_M (bound water lifetime) were provided to the program along with upper and lower boundaries, typically 10–500 μs . Water exchange rates are reported as bound water lifetimes, τ_M ($k_{\text{ex}} = 1/\tau_M$).

Omega plots

The water exchange rates were also estimated using the omega plot method [28]. Here, a plot of $[M_z/(M_0 - M_z)]$ versus $1/\omega_1^2$ (ω_1 in radians per second) was generated by measuring the bulk water signal intensity after a 5-s presaturation pulse on the Eu^{3+} -bound water exchange peak using a series of applied B_1 values (ω_1 is a frequency of B_1). The x -axis intercept ($-1/k_{\text{ex}}^2$) gives the Eu^{3+} -bound water exchange rate.

Synthesis

(S)-3-(2-Bromoacetyl)amino)propionic acid ethyl ester—(*S*)-3-Aminobutanoate ethyl ester (INDOFINE Chemical, Hillsborough, NJ, USA) (2.0 g, 0.015 mol) and potassium carbonate (8.4 g, 0.061 mol) were dissolved in dichloromethane (200 mL). The mixture was cooled to 273 K, and a solution of 2-bromoacetyl bromide (8.08 mL, 93.0 mmol) was added dropwise with stirring over 30 min. The reaction mixture was stirred at room temperature for 2 h and then quenched with water (200 mL). The reaction mixture was transferred to a separatory funnel, and the two phases were separated; the organic layer was washed with a 5 % citric acid solution (200 mL) and then with water (200 mL). The organic extract was dried (Na_2SO_4) and the solvents were removed in vacuo. The solid residue was dried under vacuum to afford (*S*)-3-(2-bromoacetyl)amino)propionic acid ethyl ester (arm-2) as a colorless solid. Yield 1.96 g (51 %); melting point 338–339 K. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.11 (1H, s, NH), 4.27 (1H, m, CH), 4.10 (2H, q, $^3J_{\text{H-H}} = \text{OCH}_2$), 3.79 (2H, s, BrCH_2), 2.48 (2H, dd, $^3J_{\text{H-H}} = 5$ Hz, $^4J_{\text{H-H}} = 3$ Hz, CHCH_2CO), 1.22 (3H, t, $^3J_{\text{H-H}} = 7$ Hz, OCH_2CH_3), 1.20 (3H, d, $^3J_{\text{H-H}} = 7$ Hz, CH-CH_3). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 171.4 (CO_2), 164.7 (C=O), 60.8 (OCH_2), 42.8 (CH_2CO_2), 39.6 (CH), 29.2 (BrCH_2), 19.7 (CH-CH_3), 14.2 (OCH_2CH_3). ν_{max} (cm^{-1}) (KBr disc): 3,289 (NH), 3,081, 1,718 (C=O), 1,646 (C=O), 1,557, 1,464, 1,375, 1,318, 1,198, 1,151, 1,103, 666. m/z [positive electrospray ionization mass spectrometry (ESI-MS $^+$): 252 (100 %, $[\text{M} + \text{H}^+]$); Anal. Found: C, 38.1; H, 5.67; N, 5.46. $\text{C}_8\text{H}_{14}\text{BrNO}_3$ requires C, 38.1; H, 5.6; N, 5.56.

(S)-2-(2-Bromoacetyl)amino)succinic acid diethyl ester—(*S*)-2-(2-Bromoacetyl)amino)succinic acid diethyl ester (arm-4) was synthesized in a manner analogous to that described for arm-2 using (*S*)-aspartic acid diethyl ester hydrochloride (INDOFINE Chemical, Hillsborough, NJ, USA) (10.0 g, 0.044 mol). The compound was obtained as a yellow oil. Yield 11.1 g (81 %). ^1H NMR (400 MHz CDCl_3): δ (ppm) 7.44 (1H, m, NH), 4.81 (1H, m, NHCH), 4.26 (2H, m, OCH_2), 4.18 (2H, m, OCH_2), 3.90 (2H, d, $^5J_{\text{H-H}} = 2$ Hz, BrCH_2), 2.83–3.09 (2H, m, CH_2CO_2), 1.29 (3H, t, $^3J_{\text{H-H}} = 7$ Hz, CH_2CH_3), 1.28 (3H, t, $^3J_{\text{H-H}} = 7$ Hz, CH_2CH_3). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 170.7 (CHCO_2), 170.0 (CH_2CO_2), 165.4 (CONH), 62.1 (OCH_2), 61.2 (OCH_2), 49.2 (CH), 36.0 (CHCH_2), 28.6 (BrCH_2), 14.2 (OCH_2CH_3), 14.0 (OCH_2CH_3). This product was not characterized further because the NMR spectra were clean and the data agreed with previously published results on this same compound [29].

(S,S,S,S)-1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrakis(3-acetoaminobutyric acid ethyl ester)—Under a nitrogen atmosphere at ambient temperature, potassium carbonate (2.8 g, 0.021 mol) was added to a solution of 1,4,7,10-tetraazacyclododecane (Strem Chemicals, Newburyport, MA, USA) (0.3 g, 1.7 mmol) and

arm-2 (1.8 g, 7.0 mmol) in anhydrous acetonitrile (200 mL). The reaction mixture was stirred at 338 K for 48 h. After it had been cooled to room temperature, the reaction mixture was filtered. The solvents were removed in vacuo. The solid residue was dissolved in chloroform (100 mL) and water (100 mL). The mixture was transferred to a separatory funnel, and the two phases were separated; the organic phase was dried (Na_2SO_4), and the solvents were removed in vacuo. The solid residue was dried under a vacuum and (S,S,S,S)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(3-acetoaminobutyric acid ethyl ester) (**2**) was obtained as pale yellow solid (1.30 g, 87 %). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.48 (4H, s, NH), 4.34 (4H, m, CH), 4.10 (8H, m, OCH_2), 3.11 (8H, s, NCH_2CO), 2.76 (16H, s, ring CH_2), 2.50 (8H, m, OCH_2CH_3), 1.23 (12H, t, $^3J_{\text{H-H}} = 7$ Hz, OCH_2CH_3), 1.21 (12H, d, $^3J_{\text{H-H}} = 7$ Hz, CHCH_3). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 171.6 (CO_2), 169.8 (C=O), 60.6 (OCH_2), 59.3 (ring CH_2), 52.7 (NCH_2CO), 42.0 (CH_2CO_2), 40.4 (CH), 20.2 (CH-CH_3), 14.2 (OCH_2CH_3). ν_{max} (cm^{-1}) (KBr disc): 3,291 (NH), 2,979, 1,735 (C=O), 1,663 (C=O), 1,553, 1,456, 1,375, 1,301, 1,187, 1,092, 1,029. m/z (ESI-MS⁺): 858 (100 %, $[\text{M} + \text{H}^+]$), 880 (12 %, $[\text{M} + \text{Na}^+]$). Anal. Found C, 55.1; H, 8.1; N, 12.5. $\text{C}_{40}\text{H}_{72}\text{N}_8\text{O}_{12}$ requires C, 56.1; H, 8.5; N, 13.1.

(S,S,S,S)-1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrakis(3-acetoaminobutyric acid)—Ligand **2** was dissolved in 5 mL of 3 M HCl. The reaction mixture was stirred at ambient temperature for 48 h, after which lyophilization of the resulting solution afforded (S,S,S,S)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(3-acetoaminobutyric acid) (**1**) as a yellow oil (0.40 g, 66 %). ^1H NMR (400 MHz, D_2O): δ (ppm) 4.13 (4H, m, CHCH_3), 3.61 (8H, s, NCH_2CO), 3.10 (16H, s, ring CH_2), 2.45 (8H, d, $^3J_{\text{H-H}} = 7$ Hz, CH_2COO), 1.08 (12H, d, $^3J_{\text{H-H}} = 7$ Hz, CHCH_3). ^{13}C NMR (100 MHz, D_2O): δ (ppm) 175.3 (CO_2), 168.2 (C=O), 54.8 (NCH_2CO), 50.7 (ring CH_2), 42.8 (CH_2CO_2), 40.3 (CH), 19.3 (CH-CH_3). m/z (ESI-MS⁺): 745 (100 %, $[\text{M} + \text{H}^+]$), 767 (10 %, $[\text{M} + \text{Na}^+]$), 783 (12 %, $[\text{M} + \text{K}^+]$). Anal. Found C, 44.2; H, 7.1; N, 12.7. $\text{C}_{32}\text{H}_{52}\text{N}_8\text{O}_{12} \cdot 4\text{HCl}$ requires C, 43.4; H, 6.4; N, 12.6.

(S,S,S,S)-1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrakis(aspartate diethyl ester)—Potassium carbonate (3.00 g, 21.7 mmol), 1,4,7,10-tetraazacyclododecane (Strem Chemicals, Newburyport, MA, USA) (0.30 g, 1.7 mmol), and arm-4 (2.17 g, 7.0 mmol) were suspended in anhydrous acetonitrile (60 mL) under a nitrogen atmosphere. The reaction mixture was stirred at 338 K for 48 h and then allowed to cool to room temperature. The mixture was filtered, and the solvents were removed under reduced pressure. The yellow oily residue was taken up in chloroform (50 mL) and washed with water (2×20 mL). The organic extracts were collected, dried over Na_2SO_4 , and the solvent chloroform was removed under reduced pressure. The residue was purified by column chromatography over silica gel and elution with 10 % methanol in chloroform afforded (S,S,S,S)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(aspartate diethyl ester) (**4**) as a pale yellow oil (0.93 g, 50 %). ^1H NMR (400 MHz CDCl_3): δ (ppm) 7.76 (4H, m, NH), 4.47 (4H, m, NHCH), 4.11 (8H, m, OCH_2), 4.06 (8H, m, OCH_2), 3.10 (8H, m, CH_2CO_2), 2.85 (8H, s, NCH_2CO), 2.74 (16H, s, ring CH_2), 1.18 (24H, t, $^3J_{\text{H-H}} = 7$ Hz, OCH_2CH_3). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 171.1 (CHCO_2), 170.8 (CH_2CO_2), 170.7 (CONH), 61.7 (OCH_2), 60.3 (OCH_2), 59.1 (ring CH_2), 53.2 (CH), 48.5 (NCH_2CO), 36.2 (CHCH_2), 14.1 (OCH_2CH_3), 14.0 (OCH_2CH_3). Anal. Found C, 40.63; H, 7.5; N, 10.14. $\text{C}_{48}\text{H}_{80}\text{N}_8\text{O}_{20} \cdot 10 \text{H}_2\text{O} \cdot \text{KBr}$ requires C, 41.53; H, 7.26; N, 8.07.

(S,S,S,S)-1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrakisaspartate—(S,S,S,S)-1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrakisaspartate (**3**) was prepared by hydrolysis of **4** as described for **1** (0.80 g, 0.73 mmol). The compound was obtained as a light yellow oil (0.38 g, 60 %). ^1H NMR (400 MHz, D_2O): δ (ppm) 4.66 (4H, m, NHCH),

3.74 (8H, m, CH_2CO), 3.24 (16H, s, ring CH_2), 2.81 (8H, s, NCH_2CO). ^{13}C NMR (100 MHz, D_2O): δ (ppm) 173.9 (CHCO_2), 173.5 (CH_2CO_2), 172.9 (CONH), 54.9 (CH), 50.5 (ring CH_2), 48.7 (NCH_2CO), 35.4 (CHCH_2). Anal. Found C, 37.02; H, 6.28; N, 9.59. $\text{C}_{32}\text{H}_{40}\text{N}_8\text{O}_{20}\cdot\text{HCl}\cdot 9\text{H}_2\text{O}$ requires C, 36.14; H, 6.35; N, 10.54.

General procedure for the preparation of Eu^{3+} complexes

The ligand was dissolved in 5 mL of methanol (for the esters) or water (for the acids), followed by addition of an equimolar quantity of the EuCl_3 dissolved in water. The pH of the reaction mixture was adjusted to approximately 6–7 (measured with a pH electrode for aqueous samples or pH paper for samples in methanol) and the mixture was stirred at 323 K for 48 h. Complexation was monitored by detection of any free Eu^{3+} ions using the colorimetric indicator xylenol orange [30]. No free Eu^{3+} was detected in the final solutions. The solvent was evaporated under a vacuum, and the resulting solid residue was dissolved in water (5 mL), filtered through a 2- μm membrane filter, and lyophilized to afford the europium complexes.

Results

The four ligands (**1–4**) were prepared from the corresponding chiral (*S*)-amines by reaction with bromoacetyl bromide followed by alkylation of 1,4,7,10-tetraazacyclododecane (see Scheme 1). The respective Eu^{3+} complexes were prepared in aqueous solution using standard complexation conditions. High-resolution ^1H NMR spectra of the complexes showed that the SAP isomer dominates in all four complexes, as evidenced by the appearance of an axial ethylene proton resonance (commonly referred to as the H_4 protons) near 25 ppm in these spectra (Fig. 2). The spectra of **Eu(1)** and **Eu(2)** showed a single H_4 resonance near 25–26 ppm, whereas the spectra of **Eu(3)** and **Eu(4)** showed multiple H_4 resonances between 23 and 26 ppm, reflecting some unknown mixture of SAP isomers (Fig. 2). Notably, the proton resonances of the two ester complexes [**Eu(2)** and **Eu(4)**] were broader than those of the corresponding acid complexes [**Eu(1)** and **Eu(3)**], likely reflecting small differences in molecular weights of the respective ester versus acid complexes or differences in the ability to form hydrogen bonds with the solvent. The multiple H_4 resonances in the spectra of **Eu(3)** and **Eu(4)** suggest that these complexes consist of multiple stereochemical isomers [31], perhaps due to racemization of the starting (*S*)-aspartic acid diethyl ester or, more likely, racemization of arm-4 prior to or during alkylation of 1,4,7,10-tetraazacyclododecane. Transesterification of ligands **2** and **4** could have occurred during complex formation in methanol, leading to mixtures of ethyl and methyl esters and hence complex NMR spectra. However, since only a single H_4 resonance appeared in the NMR spectrum of **Eu(2)** (indicating no transesterification had occurred) and the H_4 resonance pattern was quite similar in the NMR spectra of **Eu(3)** (no ester groups present) and **Eu(4)** (ester groups present), we conclude that transesterification was not the origin of these signals. A previous report describing the synthesis of optically pure (*R*)-*t*- Bu_4 -DOTAGA, an analogous 1,4,7,10-tetraazacyclododecane-based ligand with one asymmetric carbon at the α -position of a single side chain, showed that racemization can occur during alkylation of 1,4,7,10-tetraazacyclododecane under similar experimental conditions [32]. So if one assumes that the origin of these multiple H_4 resonances was due to racemization of the (*S*)-aspartic acid diethyl ester side arms and complete racemization of arm-4 occurred prior to alkylation of 1,4,7,10-tetraazacyclododecane, then six possible europium complexes could contribute to the spectrum: $\text{Eu}(\text{S},\text{S},\text{S},\text{S})$ (one), $\text{Eu}(\text{S},\text{S},\text{S},\text{R})$ (up to four), $\text{Eu}(\text{S},\text{S},\text{R},\text{R})$ (two to four), $\text{Eu}(\text{S},\text{R},\text{S},\text{R})$ (two), $\text{Eu}(\text{R},\text{R},\text{R},\text{S})$ (up to four), and $\text{Eu}(\text{R},\text{R},\text{R},\text{R})$ (one), where the values in parentheses refer to the number of magnetically nonequivalent H_4 resonances expected for each complex based on symmetry arguments. Given that $\text{Eu}(\text{S},\text{S},\text{S},\text{S})$ and $\text{Eu}(\text{R},\text{R},\text{R},\text{R})$ are indistinguishable by NMR spectroscopy, the

maximum number of possible unique H₄ resonances should be no more than 13. Close inspection of the NMR spectra of Eu(**3**) and Eu(**4**) indicates that eight distinct H₄ resonances are visible, so either some isomers have overlapping chemical shifts (most likely) or the distribution of all possible isomers was not random.

To verify that racemization was the origin of these NMR observations, the same complexes were prepared again starting with either (*S*)-aspartic acid diethyl ester hydrochloride or (*R*)-aspartic acid diethyl ester hydrochloride, except in the second synthesis, arm-4 was freshly prepared and used immediately to alkylate 1,4,7,10-tetraazacyclododecane under milder reaction conditions (ambient temperature and 24 h). Two specific complexes were targeted for synthesis: Eu(**3**-*S,S,S,S*) [all four amides were derived from (*S*)-aspartic acid] and Eu(**3**-*R,S,R,S*) [where the 1,7-*trans*-amides were derived from (*S*)-aspartic acid and the 4,10-*trans*-amides were derived from (*R*)-aspartic acid]. In the latter case, a 1,7-diprotected 1,4,7,10-tetraazacyclododecane was first prepared using well-established selective protection chemistry [33]. The 4,10-amines of this derivative were alkylated first using the bromomethyl derivative of (*S*)-aspartic acid ethyl ester, the 1,7-protection groups were removed, and subsequently the 1,7-amines were alkylated using the bromomethyl derivative of (*R*)-aspartic acid ethyl ester. The high-resolution ¹H NMR spectra of Eu(**3**) (first synthesis) and the newly prepared Eu(**3**-*S,S,S,S*), and Eu(**3**-*R,S,R,S*) are compared in Fig. 3. It is clear from the NMR spectra of Eu(**3**-*S,S,S,S*) and Eu(**3**-*R,S,R,S*) that these complexes exist largely as single isomers of high symmetry and each isomer has a unique chemical shift (25.5 and 24.7 ppm, respectively). This supports our hypothesis that racemization of arm-4 during alkylation of 1,4,7,10-tetraazacyclododecane was indeed the origin of the multiple H₄ resonances in the spectrum of Eu(**3**). The smaller H₄ resonances detected in the baseline of the spectra of Eu(**3**-*S,S,S,S*) and Eu(**3**-*R,S,R,S*) illustrate that a small amount of racemization did occur even during the second, more carefully controlled synthesis.

The CEST spectra of Eu(**1**) and Eu(**2**) and the products resulting from the first synthesis of Eu(**3**) and Eu(**4**) are shown in Fig. 4. Several differences are apparent in these spectra. First, the CEST water exchange peaks of the two ester complexes lie further upfield than those of the corresponding acid complexes [53 ppm versus 49 ppm for Eu(**1**) and Eu(**2**), respectively, and 54 ppm versus 46 ppm for Eu(**3**) and Eu(**4**), respectively]. It is well known that the magnitude of paramagnetic shifts such as those observed here depend not only on the geometry of the complex but also on the magnetic susceptibility of the Eu³⁺ ion (Eq. 1), which in turn is influenced by weak ligand fields [34–36]:

$$\delta_i^{pc} = \frac{1}{3N_A r_i^3} \left[(\chi_{zz} - \chi_{xx}) (3 \cos^2 \theta_i - 1) \right]. \quad (1)$$

The small differences in the paramagnetic shifts observed here for Eu(**1**) versus Eu(**2**) and Eu(**3**) versus Eu(**4**) are consistent with our previous observations that ester derivatives provide weaker ligands for Eu³⁺ (smaller Δ*ω*) than the corresponding acid derivatives [37]. On the basis of a previous finding of an inverse correlation between Δ*ω* and water exchange lifetimes [37], one would predict from these observations that the complexes prepared from the ester ligands would have the slowest water exchange in each pair. Second, the width of each water exchange CEST peak also differs among the four complexes and, somewhat surprisingly, the widths of the water exchange peaks in the CEST spectra of Eu(**3**) and Eu(**4**), samples known to contain mixtures of isomers, were not the broadest among these four CEST spectra. These data were used to estimate the bound water lifetimes in the four complexes by (1) fitting these CEST spectra to the Bloch equations modified for exchange [27] and (2) by the omega plot method [28]. A simple three-pool exchange model (Eu³⁺-bound water, ligand –NH protons, bulk water) was used in the Bloch fitting of all CEST

spectra, even though multiple isomers were known to be present in Eu(3) and Eu(4) and it was likely that each of these isomers may have different water exchange kinetics. Despite these limitations, all four CEST spectra fit quite well to this over-simplistic three-pool model. The results are summarized in Table 1. The bound water lifetimes obtained by use of the omega plot method (this requires no assumption of the number of exchanging pools) gave results similar to those obtained from the fits to the Bloch equations. This suggests that these two fitting methods cannot differentiate differences in bound water lifetimes when the chemical shifts of the exchanging peaks are all clustered in the same region. The τ_M^{298} values obtained for Eu(1) and Eu(2) were both in the range of 80–100 μ s. In comparison, other acid/ester complexes, including EuDOTA-(Gly)₄ versus EuDOTA-(GlyOEt)₄ [28] and EuDOTA-(Me₂Gly)₄ versus EuDOTA-(Me₂GlyOEt)₄ [25], consistently show differences in τ_M^{298} values of about twofold, with the ester analog always showing slower water exchange than the corresponding acid complexes. The τ_M^{298} values estimated for Eu(3) and Eu(4) were considerably larger and, as expected, the ester analog displayed a water lifetime (approximately 440 μ s) nearly twofold longer than that of the acid analog (approximately 260 μ s).

Given these favorably long “averaged” water exchange lifetimes, further studies were conducted to determine whether some of the individual isomers known to be present in these samples might have more favorable exchange lifetimes than others. The low-field region of the ¹H NMR spectrum of Eu(3) collected at 277 K is shown in Fig. 5. At this temperature, four new resonances were also evident between 57 and 63 ppm characteristic of Eu³⁺-OH₂ coordinated water molecules in slow exchange with bulk water. Also shown in Fig. 5 are the corresponding CEST peaks of this same sample collected at 277 K. Four well-resolved CEST exchange peaks were clearly visible in these spectra at chemical shifts identical to those seen for the Eu³⁺-OH₂ resonances in the high-resolution ¹H spectrum. This offered the opportunity to examine whether the water exchange lifetimes in these four Eu(3) isomers differ at 277 K. To achieve this, five CEST spectra were collected using different *B*₁ values and an omega plot was generated for each of the four resolved CEST peaks. That analysis gave τ_M^{277} values for each of the four water exchange peaks of 554, 714, 556, and 309 μ s, for the low to high field peaks, respectively. These data illustrate that the Eu³⁺-OH₂ exchange lifetime can vary substantially depending on which isomer is present. These differences are likely influenced by the juxtaposition of the aspartate carboxyl groups relative to the Eu³⁺-OH₂ protons in each isomer. The position and orientation of these noncoordinated carboxyl groups would also likely influence the position and number of second-sphere water molecules around each complex.

Finally, CEST spectra of the newly prepared Eu(3-*S,S,S,S*) and Eu(3-*R,S,R,S*) complexes were also collected to determine the bound water lifetimes in these isomer-enriched samples at 298 K (Fig. 6). Again, the chemical shifts of the exchanging water peaks differed in these two species, entirely consistent with the chemical shift differences measured for the H₄ resonances in these two species. In the high-resolution ¹H NMR and CEST spectra, the H₄ and CEST water exchange peaks were shifted slightly further downfield in the more symmetrical Eu(3-*S,S,S,S*) species. This indicates that the Eu³⁺ ion experiences a somewhat stronger ligand field in Eu(3-*S,S,S,S*) than in Eu(3-*R,S,R,S*). A fit of these individual CEST spectra to the Bloch equations gave bound water lifetimes of 195 and 327 μ s for Eu(3-*S,S,S,S*) and Eu(3-*R,S,R,S*), respectively, at 298 K. As before, the isomer that produced the weaker ligand field resulted in a Eu³⁺ species with the slower water exchange [37].

Discussion

In a previous study [38], we observed two different coordination isomers (SAP and TSAP) for EuDOTA-(*sec*-butylamide)₄ and hypothesized that the bulky ethyl group would be positioned in a pseudo-equatorial position in the major species (SAP) and in a pseudo-axial position (presumably higher energy) in the less-favored TSAP isomer. Following these same arguments for Eu(1) and Eu(2), the carboxyl groups (CH₂CO₂⁻ or ester) are bulkier than the methyl groups (CH₃) so one would predict that these groups would have a preference for a pseudo-equatorial position (Fig. 7). An *S* configuration in the α-carbon confers a Δ orientation and an *R* configuration confers a Λ orientation on the pendant arms. The molecules reported here were prepared from starting materials with an *S* configuration, which, if they remain in this configuration, makes Δ(λλλλ) the favored SAP isomer. We observed only one SAP isomer in the high-resolution ¹H NMR spectra of Eu(1) and Eu(2), which indicates that the other SAP isomers [Λ(δδδδ)] appear to be strongly disfavored, as predicted. Carboxyl groups in a pseudo-equatorial position appear to be effective in organizing second coordination sphere solvent water molecules through hydrogen bonding, which helps increase the inner-sphere water molecule residence lifetime. The evidence for this is the longer bound water lifetimes found for Eu(1) and Eu(2) compared with the lifetime for EuDOTA-(*sec*-butylamide)₄, which has a methyl group instead of a carboxyl group at the same position (unpublished results).

In the case of Eu(3) and Eu(4), one would predict that the smaller α-carboxyl (–CO₂⁻ or ester) group would favor a pseudo-axial position since the other substituent (–CH₂–CO₂⁻), being somewhat bulkier, should favor a pseudo-equatorial position (Fig. 7, bottom). Thus, using the same arguments as described above for Eu(1) and Eu(2), one would predict that the favored SAP isomer Eu(3-*S,S,S,S*) would favor a Λ(δδδδ) configuration. However, given the observation that several SAP isomers coexist in the racemized sample [likely Eu(3-*S,S,S,S*), Eu(3-*S,S,S,R*), Eu(3-*S,S,R,R*), Eu(3-*S,R,S,R*), and Eu(3-*R,R,R,S*)], this suggests that any energy differences between the Δ(λλλλ) and Λ(δδδδ) configurations must be small.

A second factor that may play a role in influencing the axial versus equatorial position of –CO₂⁻ or –CH₂–CO₂⁻ groups could be any difference in the ability of these side-chain carboxyl groups to form hydrogen bonds with the Eu³⁺–OH₂ molecule. In an attempt to address this question, two molecular models of Eu(3-*S,S,S,S*) were built in HyperChem™. Model 1 had all four (*S*)-aspartate –CH₂–CO₂⁻ groups positioned equatorially (the Λ(δδδδ) configuration) and two of the four axial –CO₂⁻ groups were hydrogen bonded to the Eu³⁺–OH₂ water protons. Model 2 had two of the *S*-aspartate –CH₂–CO₂⁻ groups positioned equatorially and two positioned axially, with the carboxyls of the two axial –CH₂–CO₂⁻ groups hydrogen bonded to the Eu³⁺–OH₂ protons. Energy minimization of these two structures using the Fletcher–Reeves molecular mechanics optimization algorithm in HyperChem™ showed that model 2 was approximately 24 kcal/mol more stable than model 1. This likely reflects greater flexibility of the –CH₂–CO₂⁻ groups to align properly with the Eu–OH₂ protons to form the stablest hydrogen bonds. If this molecular mechanics prediction is correct, then this configuration would also result in the stablest Eu³⁺–OH₂ water molecule and the slowest water exchange system. This may also explain why water exchange was also observed to be about twofold slower in the Eu(3-*R,S,R,S*) complex versus the Eu(3-*S,S,S,S*) complex. In the mixed Eu(3-*R,S,R,S*) complex, molecular modeling further illustrates that two –CH₂–CO₂⁻ groups (either *R,R* or *S,S* at the 1,7-*trans* positions) favor an axial position simply on the basis of steric crowding, even in the absence of hydrogen bonding with the Eu³⁺–OH₂ protons. Thus, both hydrogen-bonding and steric considerations appear to play a role in determining the lowest-energy configuration and water exchange rates in these paramagnetic complexes. The possibility that the orientation of the side-chain carboxyl groups may be influenced by intramolecular hydrogen bonding

with the –NH amide proton on each side chain was also considered. The energy-minimized structures that included such intramolecular hydrogen bonds indicated that the structure without carboxyl-to-amide proton hydrogen bonding had the lowest energy (46 kcal/mol) compared with four hydrogen bonds between the β -carboxyl groups and the amides (96 kcal/mol) or four hydrogen bonds between the α -carboxyl groups and the amides (201 kcal/mol). These results suggest that such intramolecular hydrogen bonds actually destabilize the complexes, likely by making them more rigid. Given the observation that the ethyl ester complexes consistently display slower water exchange kinetics than the corresponding acid derivatives, it may prove useful to prepare mixed-ester derivatives of Eu(**3-*R,S,R,S***) where only two –CH₂–CO₂[–] groups (either *R,R* or *S,S* in the 1,7-*trans* positions) remain as free carboxyl groups and are positioned axially for hydrogen bonding with the Eu-OH₂ protons, whereas the remaining six side chains are in the form of esters. Collectively, these observations move us closer toward the design and construction of optimized PARACEST agents that have metal-ion-bound water lifetimes (τ_M) in the 1–2-ms range at physiologically relevant temperatures.

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Abbreviations

Arm-2	(<i>S</i>)-3-(2-Bromoacetyl-amino)propionic acid ethyl ester
Arm-4	(<i>S</i>)-2-(2-Bromoacetyl-amino)succinic acid diethyl ester
CEST	Chemical exchange saturation transfer
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
ESI-MS+	Positive electrospray ionization mass spectrometry
MRI	Magnetic resonance imaging
PARACEST	Paramagnetic chemical exchange saturation transfer
SAP	Square antiprism
TSAP	Twisted square antiprism

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Fig. 1. Chemical structures of the four DOTA-tetraamide ligands used in this study. The stereochemistry at each asymmetric α -carbon in each of the four arms should be *S* unless some racemization occurred during the synthesis (see the text)

Fig. 2.

High-resolution ^1H NMR spectra of the four EuDOTA-tetraamide complexes (20 mM in D_2O) recorded at 400 MHz and 298 K. The most highly downfield shifted resonances between 22 and 27 ppm reflect an axial proton from each of the four ethylene bridges in 1,4,7,10-tetraazacyclododecane (commonly referred to as the H_4 resonances). **1** is (*S,S,S,S*)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(3-acetoaminobutyric acid), **2** is (*S,S,S,S*)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(3-acetoaminobutyric acid ethyl ester), **3** is (*S,S,S,S*)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakisaspartate, and **4** is (*S,S,S,S*)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(aspartate diethyl ester)

Fig. 3.

A comparison of high-resolution ¹H NMR spectra of Eu(**3**) (first preparation containing multiple isomers) with Eu(**3-S,S,S,S**) and Eu(**3-R,S,R,S**) (second preparation). All samples were 20 mM complex dissolved in D₂O and were recorded at 400 MHz and 298 K. The spectra of Eu(**3-S,S,S,S**) and Eu(**3-R,S,R,S**) show one dominant H₄ resonance in the most downfield region, consistent with the presence of one predominant square antiprism coordination isomer

Fig. 4.

Top: the full chemical exchange saturation transfer (CEST) spectra of Eu(1), Eu(2), Eu(3), and Eu(4). The Eu(3) and Eu(4) samples consisted of a mixture of stereochemical isomers, whereas the Eu(1) and Eu(2) samples consisted largely of a single stereochemical isomer. The *symbols* represent the recorded data points (298 K, pH ~ 7, $B_1 = 23.5 \mu\text{T}$) and the *solid lines* represent the fit of these data to the Bloch equations for a three-pool exchange model (Eu-bound water, ligand -NH protons, and bulk water). The chemical shift of solvent water was set to 0 ppm

Fig. 5. High-resolution ¹H NMR spectrum (*left*) and CEST spectra (*right*) of Eu(**3**) recorded at 277 K. The CEST spectra were collected using five different B_1 values (4.7, 7, 9.4, 11.7, and 24 μ T, from *top* to *bottom*) and a presaturation time of 8 s

Fig. 6. High-resolution ^1H NMR spectra of Eu(3-S,S,S,S) and Eu(3-R,S,R,S) (*left*; only the lowfield H_4 resonances are shown) and CEST spectra of these same two complexes (*right*; only the water exchange peak is shown). The spectra were collected on 20 mM samples of the two isomers in water (298 K, pH 7.7, $B_1 = 14.1 \mu\text{T}$) at 400 MHz

amides of 3-aminobutanoate or the ester

amides of aspartate or the diester

Fig. 7.

Top: helicity preference for Eu(1) and its ester Eu(2). One would predict that a bulkier $-\text{CH}_2-\text{CO}_2^{-}$ group (compared with a methyl group) would prefer to be in a pseudo-equatorial position in the more favored structure [this would make the complex with Δ helicity favored for (*S*)-3-aminobutanoate as the side arms]. *Bottom:* Helicity preference for Eu(3) or its aspartate diethyl ester Eu(4). Similarly, the bulkier $-\text{CH}_2-\text{CO}_2^{-}$ groups (compared with $-\text{CO}_2^{-}$) would again prefer to be in a pseudo-equatorial position in the favored isomer (in this case, the complex with Δ-helicity favored for (*S*)-aspartate as the side-arms). All molecules are drawn as (*S*)-isomers

Scheme 1.

The synthetic scheme used to prepare the four DOTA-tetraamide ligands. Both starting materials for preparing the side arms were in the *S* configuration. *cyclen* 1,4,7,10-tetraazacyclododecane

Table 1

The chemical shifts ($\Delta\omega$) and water residence lifetimes (τ_M^{298}) for the inner-sphere water molecule in four EuDOTA–tetraamide complexes (where DOTA is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid)

R	$\Delta\omega$ (ppm)	τ_M^{298} (SAP; μ s)	
		3-pool fitting	Omega plots
1 3-BuAc amide	53	81 ± 8	77
2 3-BuAcOEt amide	49	103 ± 1	80
3 Asp	54	266 ± 23^a	223^a
4 AspOEt ₂	46	446 ± 33^a	440^a

SAP square antiprism

^a Average lifetime of multiple species