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Mechanistic Investigation of β-Galactosidase-Activated MR Contrast Agents

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We report a mechanistic investigation of an isomeric series of β-galactosidase-activated magnetic resonance contrast agents. Our strategy focuses on the synthesis of macrocyclic caged-complexes that coordinatively saturate a chelated lanthanide. Enzyme cleavage of the complex results in an open coordination site available for water that creates a detectable MR contrast agent. The complexes consist of a DO3A Gd(III) chelator modified with a galactopyranose at the N-10 position of the macrocycle. We observed significant differences in relaxometric properties and coordination geometry that can be correlated to subtle variations of the linker between the macrocycle and the galactopyranose. After synthesis and purification of the *R*, *S*, and racemic mixtures of complexes **1** and **3** and measurement of the hydration number, water residence lifetime, and longitudinal relaxation rates, we propose mechanisms for water exclusion from the lanthanide in the precleavage state. While the stereochemistry of the linker does not influence the agents' properties, the mechanism of water exclusion for each isomer is significantly influenced by the position of modification. Data for one series with a methyl group substituted on the sugar-macrocycle linker at the α-position suggests a steric mechanism where the galactopyranose sugar blocks water from the Gd(III) center. In contrast, our observations for a second series with methyl substitution at the β position of the sugar-macrocycle linker are consistent with a mechanism in which a bidentate anion occupies two available coordination sites of Gd(III) in the precleavage state.

Introduction

Magnetic resonance imaging (MRI) is receiving increased attention because it is noninvasive and provides information about biological structure and function of whole organisms over time.[1−5] A three-dimensional image of a specimen is acquired by using gradient coils that measure the ^{1}H NMR signal of bulk water protons, and it is a function of the local water concentration and the relaxation times of nuclear spins.[3−6] Spin−lattice, or T_1, contrast agents enhance images by decreasing the longitudinal relaxation time through interaction of the paramagnetic lanthanide with surrounding water protons.[6−9] The effect of a paramagnetic ion on relaxation times has been described using eq 1 where P_M equals the mole fraction of the agent, q equals the water hydration number, T_{1M} is the proton longitudinal relaxation time, and τ_m is the water residence lifetime. Importantly, this

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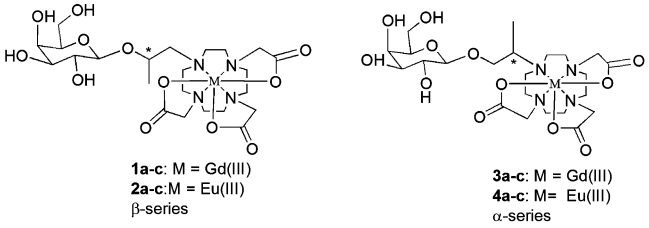
Chart 1. Complexes **1**−**4**[a]



[a] Complexes **1**−**4** are composed of a Ln(III) ion coordinated to a DO3A ligand where the N-10 position has a two-carbon linker to a galactopyranose sugar. Complexes **1** and **2** have a methyl group at the *β* position to the macrocycle (*β*-series). Complexes **3** and **4** have a methyl group at the α position (α-series).

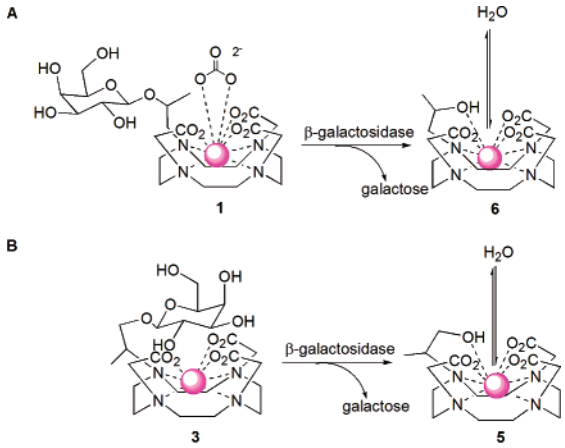
equation shows that the relaxation rate, and therefore image intensity, can be modulated by altering the hydration number *q*, since image intensity is proportional to relaxation rate.[3,10,11]

$$\frac{1}{T_{1S}} = \frac{P_M q}{T_{1M} + \tau_m} \quad (1)$$

We pioneered the development of bioactivated MR contrast agents to detect enzymatic activity and secondary messengers in vivo.[6,12,13] The first example of this class of agent was designed for in vivo detection of *β*-galactosidase (*β*-gal), an enzyme that is produced by the marker gene *LacZ*.[5,6,14−16] The complex, hereafter referred to as *β*-EGadMe, is a Gd(III) ion chelated by a tetraazamacrocycle (DO3A) functionalized at the N-10 position with a two-carbon linker to a galactopyranose sugar (Chart 1).[17] Upon cleavage of the galactopyranose by *β*-gal, the blocking group is removed, and the octadentate chelate leaves one coordination site open for water exchange. The subsequent increase in *q* from 0 to 1 results in an increase in relaxivity (dark to bright image), and the contrast agent is a marker for enzyme activity which can be correlated to gene expression.[5,6,14]

Here, we describe the synthesis and characterization of the *R* and *S* isomers of *β*-EGadMe, and the *R* and *S* isomers and racemic mixture of α-EGadMe (Chart 1).[18] The α- and *β*-series of complexes differ by the position of a single methyl group. We propose mechanisms for the enzymatic activation of these complexes to produce conditionally detectable MR contrast agents based on calculated τ_m, *q*, and relaxivity values in the presence and absence of carbonate. Our hypotheses are corroborated by NMRD and calculated binding constants for the two complexes with the bidentate

Scheme 1. (A) Proposed Mechanism of Activation for Complex **1** and (B) Proposed Mechanism of Activation for Complex **3**



anion, CO_3^{2-}. On the basis of the results of a systematic investigation of the coordination of the lanthanide centers, we propose two entirely different mechanisms that describe *q*-modulation initiated by the enzyme *β*-gal.

In order to investigate the proposed mechanisms, we synthesized the resolved and racemic mixtures of complexes **1** and **3** and the racemic *cleaved* agents **5** and **6** (the complex lacking the galactopyranose). The mechanism of water exclusion from the lanthanide of the α- and *β*-isomers depends on the position of a single methyl group (Chart 1). For the *β*-isomer, carbonate anions play a role in water exclusion prior to sugar cleavage. Carbonate ions, well-known to act as bidentate ligands, occupy two coordination sites on the Gd(III) ion in the *β*-isomer forming a coordinatively saturated complex (Scheme 1A).[19] Upon cleavage, the OH group coordinates to the Gd(III) ion, and carbonate is displaced resulting in a complex with $q = 1$.[20]

An entirely different mechanism is proposed for the α-isomer (Scheme 1B). The galactopyranose sterically hinders water access to the Gd(III) prior to cleavage. The cleaved complex has an octadentate chelate and is open to coordination by one water molecule resulting in an observed relaxivity increase upon cleavage.[5,6,14]

Experimental Section

Materials. All reagents were used as purchased. CH_2Cl_2, THF, and MeCN were dried using a solvent system from Glass Contour, San Diego, CA. All other solvents were used as purchased from Aldrich in anhydrous Sure-Seal bottles. Water was purified using a Millipore Milli-Q Synthesis water system. 1,4,7,10-tetraazocyclododecane (cyclen) was used as purchased from Strem. Prohance was obtained from Bracco Laboratories.

Synthesis. The synthetic procedure and characterization is described for preparation of the *R*-diastereomer for each compound **1** and compound **3**. The same procedure was performed for the preparation of the *S*-diastereomer and the racemate of each of the complexes. Those exact procedures and characterization are found in the Supporting Information.

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***R*-(+)-2-Bromopropan-1-ol (7a).** *R*-(+)-2-Bromopropionic acid (7.0 mL, 11.9 g, 0.078 mol) was combined with 50 mL of dry THF under N_2 and brought to 0 °C. 1.0 M BH_3·THF in THF (116 mL, 0.116 mol) was added dropwise at 0 °C over 10 min, and the mixture was allowed to stir until the ice bath melted and was then stirred at r.t. overnight. A 150 mL amount of 1:1 H_2O:THF was added slowly followed by 50 g of anhydrous K_2CO_3. After another 45 min of stirring, the mixture was transferred to a separatory funnel; the organic layer was washed with water (3 × 50 mL) and then brine (25 mL), and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). All organic layers were combined, dried over $MgSO_4$, and transferred to a 500 mL round-bottom flask for distillation. Pure product was obtained by distillation under static vacuum (vapor temp = 31 °C). Yield: 60%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.62 (d, 3H, C*H*$_3$CHBrCH$_2$OH, J = 8), 2.05 (s, 1H, CH$_3$CHBrCH$_2$O*H*), 3.59−3.75 (m, 2H, CH$_3$C*H*BrC*H*$_2$OH), 4.14−4.23 (m, 1H, CH$_3$CHBrC*H*$_2$OH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.1, 53.4, 68.9.

***R*-(+)-2-Bromopropan-1-β-D-galactose Tetraacetate (8a).** At 0 °C, 2.14 g (0.015 mol) **7a** was added to a solution of 5.00 g (0.013 mol) of β-D-galactose pentaacetate dissolved in 22 mL of dry CH_2Cl_2. BF_3·Et_2O (2.9 mL, 3.28 g, 0.023 mol) was added slowly over 40 min at 0 °C. The mixture was allowed to stir at 0 °C and then at r.t. overnight. After 23 h, 2.6 g of anhydrous K_2CO_3 was added and allowed to react for 1 h. The solid was filtered under vacuum and the solvent evaporated under reduced pressure. The product was isolated by flash column chromatography on silica gel in hexanes/ethyl acetate gradient (9:1−3:1). Yield: 49%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.51 (d, 3H, methyl, J = 6.5), 1.80−2.05 (m, 12H, OAc), 3.54−3.59 (m, 2H, CH$_3$CHBrC*H*$_2$O-sugar), 3.84−3.91 (m, 1H, CH$_3$C*H*BrCH$_2$O-sugar), 3.97−4.11 (m, 3H, H-5 and H-6) 4.45−4.49 (m, 1H, H-4), 4.93−4.95 (m, 1H, H-2), 5.09−5.13 (m, 1H, H-3), 5.23−5.29 (m, 1H, sugar anomeric H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.80, 20.88, 21.08, 22.45, 46.58, 61.41, 67.11, 68.70, 70.12, 70.68, 70.91, 75.08, 101.48, 170.21, 170.30. ESI-MS m/z = 492.8 (M + Na^+).

***R*-(+)-(2-(1,4,7,10-Tetraazacyclododecyl)propan)-1-β-D-galactose Tetraacetate (9a).** Cyclen (5.305 g, 0.0308 mol) was dissolved in 35 mL of dry $CHCl_3$ and brought to reflux. **8b** (5.623 g, 0.0120 mol) in 10 mL of dry $CHCl_3$ was added dropwise over 15 min, and the reaction mixture was refluxed for 15 h. TLC in 1:1 hexanes:ethyl acetate indicated no unreacted sugar. MS showed the presence of desired product as well as disubstituted side product. Solvent was removed under reduced pressure to yield a viscous yellow syrup. MeCN (10 mL) was added and the product isolated by flash column chromatography on silica gel in 1:9:90 sat. KNO_3:H_2O:MeCN. This product was carried on to the next step without separation from the salt. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.82−0.98 (d, 3H, methyl, J = 6.0), 1.85−2.18 (m, 12H, sugar acetate CH_3), 3.0−3.5 (br, 16H, cyclen), 3.95−4.04 (m, 3H, methylene and methine on linker), 4.18−4.28 (m, 3H, H-6 and H-5), 4.50 (d, 1H H-4), 4.92−4.99 (m, 1H, H-2), 5.02−5.15 (m, 1H, H-3), 5.42 (m, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$) δ 9.54, 20.72, 20.76, 20.90, 54.40, 60.33, 67.32, 68.72, 70.25, 71.43, 71.54, 110.2, 169.46, 169.93, 170.75, 171.48. ESI-MS m/z = 561.2 (M + H^+).

(*R*)-(+)-(2-(4,7,10-Trismethylcarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan)-1-β-D-galactose Tetraacetate (10a). A quantity of 4.663 g of mixture containing **9** and KNO_3 was combined with 4.6 g of anhydrous K_2CO_3 in 40 mL of dry MeCN. Methyl bromoacetate (2.3 mL, 3.72 g, 0.024 mol) was added, and the mixture was allowed to stir at room temperature for 20 h. TLC in 1:9:90 sat. KNO_3:H_2O:MeCN showed no unreacted **9**. The reaction mixture was filtered and the solvent removed under reduced pressure to yield a bright yellow viscous oil. The desired product was isolated by flash column chromatography on silica gel (1−8% MeOH in CH_2Cl_2). A yellow solid was obtained after removal of the solvents. Yield from **8b**: 33%. ^{1}H NMR (500 MHz $CDCl_3$) δ 1.26−1.30 (m, 3H, d, methyl, J = 6.0), 1.8−2.2 (m, 12H, OAc), 2.3−3.5 (br, 25H, cyclen, OC*H*$_3$), 3.85−3.98 (m, 2H, methylene on linker), 4.02−4.15 (m, 3H, methine on linker and H-6), 4.55−4.60 (m, 1H, H-4), 5.0 (m, 1H, H-5), 5.08 (br, 2H, H-3 and H-2).5.35 (m, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.72, 20.86, 20.95, 21.09, 51.78, 52.08, 52.51, 52.72, 55.79, 61.16, 67.09, 68.88, 70.77, 70.98, 71.15, 100.71, 169.96, 170.05, 170.37, 170.64, 170.98, 171.45. ESI-MS: m/z = 777.6 (M + H^+), 799.6 (M + Na^+).

Gd(III) (*R*)-(+)-(4,7,10-2-(Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan)-1-β-D-galactose (3a). A quantity of 112 mg (0.144 mmol) of **10a** was dissolved in 10 mL of water, and 11.5 mL (1.2 mmol) of 0.1 M NaOH was added. After 2 h, 0.06 g (0.16 mmol) of $GdCl_3$·$6H_2O$ was added and the pH was adjusted to 5.5. After stirring at r.t for 5 days at pH 5.5, the pH was adjusted to 9.5 to precipitate excess metal as the hydroxide. This solution was centrifuged and decanted, and the pH was adjusted to 7.2, and the product was lyophilized to a white powder and purified by preparative HPLC. The LC-MS of the isolated product showed a single peak at 13.23 min with m/z = 720.1 (M + H^+ ESI-MS) displaying the characteristic gadolinium isotope pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}Gd$: C 38.33, H 5.45, N 7.78. Found: C 38.24, H 5.28, N 7.53. Yield: 33%.

Gd(III) (*S*)-(−)-(4,7,10-2-(Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan)-1-β-D-galactose (3b). The *S*-diastereomer of **3** was synthesized according to the procedure described above beginning with 87.2 mg (0.112 mmol) of **10b.** The desired product was isolated by HPLC. The LC-MS of the isolated product showed a single peak at 16.20 min with a m/z = 720.1 (M + H^+ ESI-MS) displaying the characteristic gadolinium isotope pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}Gd$: C 38.33, H 5.45, N 7.78. Found: C 38.20, H 5.43, N 7.69. Yield: 35%.

Gd(III) (2-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan)-1-β-D-galactose (3c). The racemic mixture of **3** was synthesized according to the procedure described above beginning with 0.240 g (0.309 mmol) of **10c**. The LC-MS of the isolated product showed a single peak at 14.00 min with a m/z = 720.1 (M + H^+ ESI-MS) with the characteristic gadolinium isotope pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}Gd(H_2O)_{2.0}$: C 36.51, H 5.73, N 7.40. Found: C 36.37, H 5.39, N 7.09. Yield: 24%.

Eu(III) (*R*)-(+)-(2-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan)-1-β-D-galactose (4a). The europium complexes were synthesized according to the same procedure as for the Gd(III) complexes except that $EuCl_3$·$6H_2O$ was substituted for $GdCl_3$·$6H_2O$. The synthesis of **4a** began with 19.7 mg (0.025 mmol) of **10a**. The LC-MS of the isolated product showed a single peak at 14.77 min with m/z = 717.1 (M + H^+ ESI-MS) with the characteristic europium isotope pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}Eu(H_2O)_{1.0}$: C 37.66, H 5.63, H 7.64. Found: C 37.67, H 5.81, N 7.47. Yield: 40%.

Eu(III) (*S*)-(−)-(2-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan)-1-β-D-galactose (4b). The synthesis of **4b** was carried out according to the method described above beginning with 60.5 mg (0.078 mmol) of **10b**. The LC-MS of the isolated product showed a single peak at 14.35 min with a m/z = 717.1 (M + H^+ ESI-MS) with the characteristic europium isotope

pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}Eu(H_2O)_{2.0}(HBr)_{1.0}$: C 33.18, H 5.33, N 6.73. Found: C 33.56, H 5.01, N 6.68. Yield: 36%.

Eu(III) (2-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan)-1-β-D-galactose (4c). The synthesis of **4c** was carried out as described above beginning with 50.8 mg (0.065 mmol) of **10c**. The LC-MS of the isolated product showed a single peak at 14.38 min with a $m/z = 717.1$ ($M + H^+$ ESI-MS) with the characteristic europium isotope pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}$-Eu: C 38.18, H 5.77, N 7.45. Yield: 35%.

Kinetic Resolution of *R*-(+)-Propylene Oxide (11a). Racemic propylene oxide was kinetically resolved using Jacobsen's catalyst. A quantity of 0.354 g (0.008 equiv) of (*R*,*R*)-(+)-*N*,*N*′-bis(3,5-di-*tert*-butylsalicylidene)-1,2-cyclohexane diaminocobalt(II) was combined with 10 mL of toluene and 0.0654 mL (0.016 equiv) of concentrated acetic acid, and the mixture was stirred for 2 h. The toluene was evaporated under reduced pressure to yield a dark brown residue. Racemic propylene oxide (5.0 mL, 4.15 g, 0.71 mol) was added, and the mixture was cooled to 0 °C in an ice bath. 0.90 mL (0.7 equiv) H_2O was added dropwise with stirring, and then the ice bath was removed. The flask was sealed, stirred overnight, and equipped with a short path distillation apparatus. The collection flask was brought to −78 °C in a dry ice/acetone bath. The product was collected under slight static vacuum at 33 °C. The product was isolated in 24% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.27−1.29 (d, 3H, methyl, $J = 5$), 2.39−2.40 (q, 1H, methylene), 2.70 (m, 1H, methylene), 2.92−2.96 (m, 1H, methine). ^{13}C NMR (125 MHz, $CDCl_3$) δ 18.12, 48.16, 48.39.

***R*-(+)-1-Bromopropan-2-ol (12a).** A quantity of 17.5 mL (14.51 g, 0.25 mol) of **11a** was combined with 500 mL of dry THF. The solution was brought to 0 °C, and 800 mL of Li_2NiBr_4 (0.5 M in THF) was added slowly via syringe. The solution was stirred at 0 °C for 2 h and turned a dark blue-green color. The solution was poured into 1250 mL of phosphate buffer pH 7.0, and a bright green precipitate formed. The solid was filtered and washed with H_2O (500 mL). The filtrate was extracted with CH_2Cl_2 (9 × 500 mL) and dried with Na_2SO_4. Solvents were removed to yield an orange oil. This solution was distilled (vapor temperature = 49 °C), and a colorless distillate was collected, leaving behind an orange impurity. Yield: 37.7%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.25−1.32 (d, 3H, methyl, $J = 6.0$), 1.85 (br, 1H, O*H*), 3.32−3.38 (m, 1H, methylene), 3.45−3.51 (m, 1H, methylene), 3.94−4.02 (m, 1H, methine). ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.18, 40.77, 67.20.

***R*-(+)-1-Bromopropan-2-β-D-galactose Tetraacetate (13a).** A quantity of 15.61 g (0.040 mol) of β-D-galactose pentaacetate was combined with 4.4 mL (6.67 g, 1.2 equiv) of **12a** in 40 mL of dry CH_2Cl_2 at 0 °C. To this solution was added 7.6 mL (8.51 g, 1.5 equiv) of $BF_3{\cdot}Et_2O$ dropwise over 15 min. This solution was allowed to stir for 1 h at 0 °C, and the ice bath was removed. The reaction mixture was allowed to warm to room temperature and stirred overnight. After 18 h, 6.0 g of anhydrous K_2CO_3 was added and stirred for an additional 1 h. The solids were filtered and washed with CH_2Cl_2 (1 × 50 mL), and the solvents were removed under reduced pressure. The resulting viscous brown oil was purified by flash column chromatography on silica gel in hexanes:ethyl acetate (gradient: 9:1−3:1). Single crystals of compound **13a** were obtained from a solution of ether/petroleum ether, methanol, and ethyl acetate. Yield: 31%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.26−1.32 (d, 3H, methyl, $J = 6.5$), 1.85−2.32 (m, 12H, OAc), 3.24−3.32 (m, 1H, C*H*$_2$Br), 3.50−3.12 (m, 1H, C*H*$_2$Br), 3.89−4.02 (m, 2H, methine on linker, H-5), 4.05−4.22 (m, 2H, H-6), 4.52−4.64 (m, 1H, H-4), 4.98−5.08 (m, 1H, H-3), 5.24−5.34 (m, 1H, H-2), 5.40 (m, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.11, 20.96, 21.05, 21.10, 36.25, 61.62, 67.23, 69.06, 71.05, 76.62, 76.91, 100.95, 169.41, 170.24, 170.35, 170.52. ESI-MS: $m/z = 492.9$ ($M + H^+$).

***R*-(+)-1-(1,4,7,10-Tetraazacyclododecyl)propan-2-β-D-galactose Tetraacetate (14a).** A quantity of 2.2247 g (0.0047 mol) of **13a** was dissolved in 100 mL of dry $CHCl_3$ under N_2. In a separate flask, 2.4858 g (0.014 mol, 2.5 equiv) of cyclen was dissolved in 100 mL of dry $CHCl_3$ under N_2. The two solutions were combined and refluxed overnight after which time the MS of crude reaction mixture indicated the presence of the desired product. The solvent was removed under reduced pressure to yield a viscous yellow oil. This product was purified by flash column chromatography on silica gel in 9:0.9:0.1 MeCN:H_2O:sat. KNO_3. The product was carried on to the next step without separation from the salt. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.1−1.3 (d, 3H, methyl, $J = 6.5$), 1.98−2.38 (m, 12H, OAc), 2.7 (s, methylene on linker), 2.8−3.8 (br, 16H, cyclen), 3.98−4.28 (m, 2H, H-6), 4.30−4.38 (m, 1H, H-4), 4.66 (m, 1H, H-5), 4.92−5.0 (m, 1H, H-3), 5.05−5.12 (m 2H, H-2 and H-1), 5.42 (s, anomeric). ESI-MS: $m/z = 561.3$ ($M + H^+$).

***R*-(+)-1-(4,7,10-Trismethylcarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan-2-β-D-galactose Tetraacetate (15a).** A quantity of 1.1172 g (0.0020 mol) of **14a** was combined with 1.1 g (4 equiv) of K_2CO_3 in 70 mL of dry MeCN. A quantity of 0.6 mL (0.99 g, 0.0065 mol, 3 equiv) of methyl bromoacetate was added, and the reaction mixture was stirred overnight. TLC in 9:0.9:0.1 MeCN:H_2O:sat. KNO_3 showed almost no **14a**. The solvents were removed in vacuo, and the pale yellow oil that remained was purified by flash column chromatography on silica gel. The mobile phase was a gradient of 1% to 8% MeOH in CH_2Cl_2. Yield over last two reactions beginning with **13a**: 50%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.22−1.30 (d, 3H, methyl, $J = 6.5$), 1.97−2.14 (m, 12H, OAc), 2.65−3.08 (m, 16H, cyclen), 3.61−3.82 (m, 9H, OC*H*$_3$), 3.87−4.19 (br, methylene and methine on linker and H-6), 4.58−4.65 (m, 1H, H-4), 5.03−5.08 (m, 2H, H-3 and H-2), 5.40 (s, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.79, 20.91, 21.00, 48.26, 50.83, 51.85, 52.10, 52.76, 56.05, 62.35, 67.34, 68.78, 70.64, 71.56, 98.71, 170.36, 170.84, 171.44, 176.61. ESI-MS: $m/z = 777.2$ ($M + H^+$), 799.3 ($M + Na^+$).

Gd(III) *R*-(+)-1-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan-2-β-D-galactose (1a). A quantity of 116.7 mg (0.150 mmol) of **15a** was dissolved in 10 mL of H_2O. A quantity of 2.4 mL of 0.5 M aqueous NaOH (8 equiv) was added, and the solution was stirred for 2.5 h. $GdCl_3{\cdot}6H_2O$ (0.062 g, 1.1 equiv) was added, and the pH was brought down to 5.5 with 5 w/v % HCl. This solution was stirred overnight, and the pH was brought up to 12 with 0.1 M NaOH in order to precipitate excess Gd(III) as the hydroxide. The reaction mixture was centrifuged, and the supernatant was lyophilized to a white powder which was purified by preparative HPLC. The LC-MS of the isolated product showed a single peak at 13.35 min with $m/z = 720.1$ ($M + H^+$ ESI-MS) with the appropriate isotope pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}$-$Gd(H_2O)_{2.5}$: C 36.08, H 5.79, N 7.32. Found: C 36.01, H 5.60, N 7.04. Yield: 42%.

Gd(III) *R*-(−)-1-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan-2-β-D-galactose (1b). The *R*-isomer of β-EGadMe was synthesized using the procedure described above beginning with 410.9 mg (0.530 mmol) of **15b**. The LC-MS of the isolated product showed a single peak at 13.23 min with a $m/z = 720.1$ ($M + H^+$ ESI-MS) with the characteristic gadolinium isotope pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}Gd(H_2O)_{0.5}$: C 37.86, H 5.53, 7.68. Found: C 37.87, H 5.52, 7.53.

Gd(III) 1-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan-2-β-D-galactose (1c). The racemic mixture of β-EGadMe was synthesized using the procedure described above

beginning with 468.2 mg (0.603 mmol) of **15c**. The LC-MS of the isolated product showed a single peak at 12.07 min with a m/z = 720.1 ($M + H^+$ ESI-MS) with the characteristic gadolinium isotope pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}Gd$: C 38.33, H 5.45, N 7.78. Found: C 37.97, H 5.76, N 7.55. Yield: 40%.

Eu(III) *R*-(+)-1-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan-2-β-D-galactose (2a). The europium complexes were synthesized according to the same procedure as for the Gd(III) complexes except that $EuCl_3 \cdot 6H_2O$ was substituted for $GdCl_3 \cdot H_2O$. The synthesis of **2a** began with 105.9 mg (0.135 mmol) of **15a**. The LC-MS of the isolated product showed a single peak at 12.51 min with m/z = 717.1 ($M + H^+$ ESI-MS) with the characteristic europium isotope pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}$-Eu: C 38.61, H 5.49, N 7.83. Found: C 38.66, H 5.57, N 7.47. Yield: 28%.

Eu(III) *R*-(−)-1-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan-2-β-D-galactose (2b). The *R*-enantiomer of the Eu(III) analogue of β-EGadMe was synthesized using the procedure described above beginning with 218.4 mg (0.281 mmol) of **15b**. The LC-MS of the isolated product showed a single peak at 12.63 min with an m/z = 717.1 ($M + H^+$ ESI-MS) with the characteristic europium isotope pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}$-$Eu(H_2O)_2$: C 36.75, H 5.77, N 7.45. Found: C 36.70, H 5.61, N 7.09. Yield: 29%.

Eu(III) 1-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))propan-2-β-D-galactose (2c). The racemic mixture of the Eu(III) analogue of β-EGadMe was synthesized using the method described above beginning with 244.9 mg 0.316 mmol) of **15c**. The LC-MS of the isolated product showed a single peak at 11.78 min with a m/z = 717.1 ($M + H^+$ ESI-MS) with the characteristic europium isotope pattern. Anal. Calcd for $C_{23}H_{39}N_4O_{12}$-Eu: C 38.61, H 5.49, N 7.83. Found: C 38.92, H 5.74, N 7.71. Yield: 38%.

(2-Bromo-1-methyl-ethoxy)-*tert*-butyl-dimethyl-silane (16). 7c (2.116 g, 0.015 mol) was combined with 2.55 g (0.037 mol, 2.5 equiv) of imidazole in 25 mL of DMF. TBDMS-Cl (2.8 g, 0.019 mol, 1.2 equiv) was added and stirred for an additional 3 h at which time TLC in 1:1 hexanes:ethyl acetate showed no more starting material. The DMF was removed under reduced pressure, and the product was obtained after purification by column chromatography on silica gel in neat hexanes. Yield: 63%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.015−0.076 (m, 6H, Si(CH_2)$_2$*t*-Bu), 0.06−1.00 (m, 9H, Si(CH_2)$_2$*t*-Bu), 1.55−1.70 (d, 3H, methyl, J = 6.8), 3.52−3.65 (m, 1H, methylene), 3.68−3.82 (m, 1H, methine), 3.90−4.08 (m, 1H, methylene). ^{13}C NMR (125 MHz, $CDCl_3$) δ 18.52, 26.10, 26.43, 49.54, 69.10.

(2-(1,4,7,10-Tetraazacyclododecyl)-1-methyl-ethoxy)-*tert*-butyl-dimethyl-silane (18). 16 (1.8274 g, 0.0073 mol) was combined with 3.2 g (0.019 mol, 2.5 equiv) of cyclen in 50 mL of dry $CHCl_3$. The solution was refluxed overnight. Product was purified by flash column chromatography on silica gel with 9:0.9:0.1 MeCN:H_2O: sat. KNO_3. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.015−0.13 (m, 6H, Si(CH_2)$_2$*t*-Bu), 0.91−1.01 (m, 12H, Si(CH_2)$_2$*t*-Bu, methyl), 2.8−3.6 (br, 17H, cyclen and methylene on linker), 3.62 (m, 1H, methine), 3.80 (m, 1H, methylene in linker). ESI-MS: m/z = 345.2 ($M + H^+$).

(2-(4,7,10-Trismethylcarboxymethyl-(1,4,7,10-tetraazacyclododecyl))-1-methyl-ethoxy)-*tert*-butyl-dimethyl-silane (17). A quantity of 0.4747 g (0.0014 mol) of **17** was dissolved in 50 mL of dry MeCN. To this were added 0.4 mL (0.658 g, 0.0043 mol, 3 equiv) of methyl bromoacetate and 0.8 g (4 equiv) of K_2CO_3. After being stirred for 16 h, the salts that had formed were filtered and washed with MeCN (1 × 15 mL). The solvents were removed in vacuo to yield a yellow oil. The product was obtained after purification by flash column chromatography on silica gel using CH_2Cl_2 with a gradient of 0% to 8% MeOH. Yield over two reactions beginning with **16**: 60%. ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.13−0.01 (m, 6H, Si(CH_2)$_2$*t*-Bu), 0.60−0.78 (m, 12H, Si-(CH_2)$_2$*t*-Bu, methyl), 1.98−3.40 (br, 19H, cyclen and linker), 3.42−3.62 (m, 9H, OCH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 17.81, 18.71, 25.61, 26.21, 45.08, 51.21, 51.34, 51.51, 52.06, 52.12, 52.17, 53.30, 54.64, 54.99, 55.09, 55.72, 165.47, 170.77, 171.05, 171.15, 173.01, 173.74. ESI-MS: m/z = 561.3 ($M + H^+$), 583.3 ($M + Na^+$).

Gd(III) (2-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))-1-methyl-ethoxy (5). Deprotection and metalation of **17** were performed in one pot. A quantity of 148 mg (0.264 mmol) of **23** was dissolved in 10 mL of H_2O, and the pH was brought up to 12 with 0.5 M NaOH. After 3 h of stirring, analysis of the crude mixture by LC-MS showed no protected ligand. A quantity of 0.11 g (0.296 mmol, 1.1 equiv) of $GdCl_3 \cdot 6H_2O$ was added and the pH adjusted to 5.5 with 5 wt % HCl and stirred for several days. The pH was adjusted to 11.0 to precipitate excess metal as $Gd(OH)_3$. This was centrifuged and the supernatant decanted and lyophilized to a white powder. The desired product was isolated by HPLC. The LC-MS of the isolated product showed a single peak at 16.50 min with m/z = 562.1 ($M + H^+$) with the expected isotope pattern. Calculated for $C_{17}H_{28}N_4Gd$: C 36.56, H 5.23, N 10.03. Found: C 36.73, H 5.16, N 9.70. Yield: 38%.

Eu(III) (2-(4,7,10-Triscarboxymethyl-(1,4,7,10-tetraazacyclododecyl))-1-methyl-ethoxy (19). Synthesized according to the procedure described above beginning with 0.1225 g (0.219 mmol) of **23** and substituting $EuCl_3 \cdot 6H_2O$ for $GdCl_3 \cdot 6H_2O$. The final complex was purified by HPLC. The LC-MS of the isolated product showed a single peak at 16.57 min with m/z = 557.2 ($M + H^+$), 577.2 ($M + Na^+$). Anal. Calcd for $C_{17}H_{28}N_4Gd(H_2O)_{1.5}$: C 35.23, H 5.39, N 9.67. Found: C 35.13, H 5.43, N 9.69. Yield: 45%.

Cleaved β-EGadMe (6). This complex known commercially as Prohance was obtained from Bracco Laboratories and was purified by HPLC. The LC-MS of the isolated product showed a single peak at 15.98 min with m/z = 562.1 ($M + H^+$). Anal. Calcd for $C_{17}H_{28}N_4Gd$: C 36.56, H 5.23, N 10.03. Found: C 36.51, H 5.26, N 9.84.

HPLC. All complexes were purified by preparative LC on a Varian Prostar system equipped with a two-channel 325 UV−visible diode array detector and an HP 1046A fluorescence detector. A Waters 19 × 250 mm Atlantis dC_{18} 10 μm column was used as the solid phase, and the mobile phase consisted of ultrapure H_2O and HPLC grade acetonitrile purchased from Aldrich. The gradient profile was as follows: 0−10% MeCN in H_2O over 20 min, 10−98% MeCN in H_2O over 5 min, hold 5 min, 98−0% MeCN over 5 min, hold 5 min. All experiments were run with a flow rate of 15 mL/min. Injected samples were 3−4 mL at 30−50 mg/mL of crude complex.

LC-MS. Analytical LC-MS was used to characterize the purified lanthanide complexes. A Varian Prostar system consisting of a 410 autosampler with a 100 μL sample loop, two 210 pumps with 5 mL/min heads, a 363 fluorescence detector, a 330 photodiode array detector, and a 1200L single quadrupole ESI-MS. All runs were performed with a 1.0 mL/min flow rate using a Waters 4.6 × 250 mm Atlantis dC_{18} 5 μm column, with a 3:1 split directing one part to the MS and three parts to the series-connected light detectors. The mobile phase and gradient profile were the same as for the preparative HPLC.

Luminescence Lifetime Measurements. The measurement of q was performed in water and buffered solutions separately. Solutions were prepared at 1.0 mM in H_2O and D_2O for each Eu-

Table 1. *q* Values for Eu(III) Complexes at 1.0 mM[d]

complex	$q(H_2O)$	q(MOPS)[b]	$q(MOPS/NaHCO_3)$[c]
2a	1.5	-	-
2b	1.2	-	-
2c	1.6	1.7	0.98
4a	0.5	-	-
4b	0.5	-	-
4c	0.6	0.3	0.5
6	1.3[a]	-	-
19	1.3[e]	-	-

[a] Hydration number was determined using the Tb(III) complex.[40] [b] 100 mM MOPS pH 7.3. [c] 100 mM MOPS and 24 mM $NaHCO_3$ pH 7.3. [d] All of the reported *q* values are nonintegers which is common for these calculations.[21] [e] The hydration number of compound **19** was calculated with correction for the O−H oscillator on the coordinating hydroxyl arm.

(III) complex. For the *q* experiments performed in 100 mM MOPS buffer, each solution was 1.0 mM complex in both MOPS/H_2O (pH 7.3) and MOPS/D_2O (pD 7.3). The solutions for *q* experiments performed in the presence of HCO_3^- were prepared in a similar way except that the buffer contained 24 mM $NaHCO_3$ and $NaDCO_3$, respectively. The luminescence lifetime decay was measured on a Hitachi F4500 fluorimeter. The sample was excited with 15 iterations at 394 nm, and the luminescence decay was monitored at 614 nm in phosphorescence lifetime (short) mode. The average of the 15 iterations was calculated and fit to a monoexponential decay function to give luminescence lifetime, τ. $\tau(H_2O)$ and $\tau(D_2O)$ were used to determine *q* from eq 2 given by Horrocks et al.[21] and are listed in Table 1.

Luminescence Titration. To 3.0 mL of a 1.0 mM solution of Eu(III) complex in 100 mM MOPS buffer at pH 7.3 were added aliquots of a 500 mM solution of $NaHCO_3$ in the same buffer. The concentration of HCO_3^- varied from 0 mM to 40 mM such that the overall volume change was <8%. At each concentration, the corrected luminescence emission spectra were obtained on a Hitachi F4500 fluorimeter with 5−10 iterations. The average peak intensity for the emission at $\lambda = 614$ nm was recorded and plotted versus $[HCO_3^-]$ (Figure 1). Data analysis was performed using Origin 7.0 iterative least-squares fitting as described by Bruce et al.[22]

^{17}O Transverse Relaxation Rate Measurements. Samples of each complex were 18 mM in water enriched with 1% $^{17}OH_2$, and the ^{17}O spectra were obtained at 54 MHz at temperatures ranging from 1 °C to 75 °C in 5 °C increments. The ^{17}O transverse relaxation rate was determined by obtaining the line width at half of the peak height, $\Delta\nu_{1/2}$, of the ^{17}O water signal and fit according to Swift and Connick theory.[23,24] The calculated τ_m values for each complex are listed in Table 2.

Relaxivity. A 2 mM stock solution of each of **1**, **3**, **5**, and **6** were prepared in 100 mM MOPS buffer at pH 6.99 and 100 mM MOPS/24 mM $NaHCO_3$ buffer at pH 7.3. Carbonate ions are physiologically present in ~24 mM, and we used this concentration in our experiments.[25] The solutions were diluted to concentrations of 0, 0.25, 0.5, 1.0, and 2.0 mM solutions. T_1 was determined on a Bruker mq60 Minispec relaxometer with an inversion recovery pulse sequence with the appropriate recycle delays. All experiments were performed in duplicate at 60 MHz and 37 °C. The relaxivity was determined by plotting $1/T_1$ v [Gd(III)] and fitting the data with a linear function.

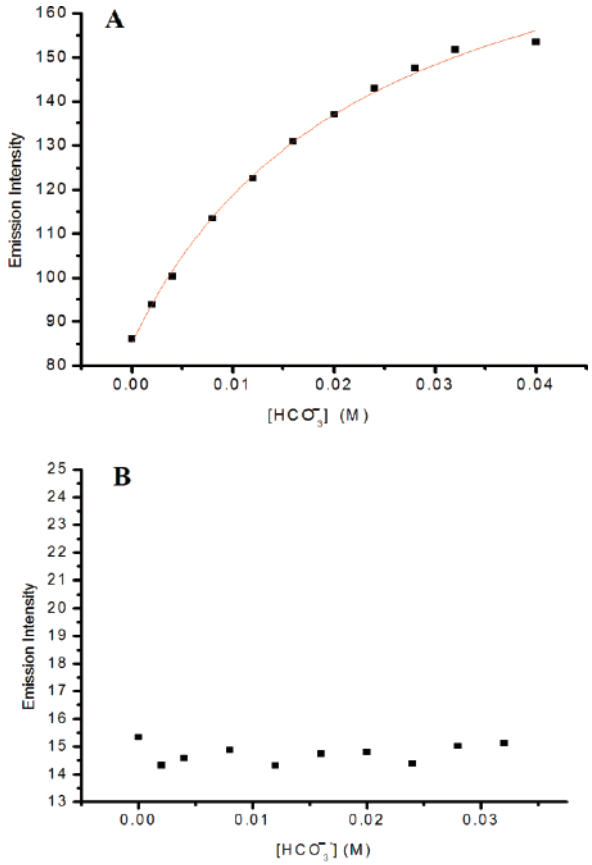

Figure 1. (A) Emission peak intensity dependence on $[HCO_3^-]$ added for complex **2c**. (B) Emission peak intensity dependence on $[HCO_3^-]$ added for complex **4c**.

Table 2. τ_m Values for *β*-gal MR Agents with and without the Sugar Moiety Present Determined from a Fit of R_{2p} vs Temperature Using Swift and Connick Theory

complex	τ_m at 298 K
1	43 ± 4 ns[a,c]
3	-
5	85 ± 20 ns[b,c]
6	345 ns[41]

[a] $\Delta H = 39 \pm 2$ kJ mol^{-1}, $T_{1e}^{298} = 43 \pm 4$ ns, $\Delta E_{T_{1e}} = -0.01 \pm 3.30$ kJ mol^{-1}. [b] $\Delta H = 39 \pm 3$ kJ mol^{-1}, $T_{1e}^{298} = 12.7 \pm 0.3$ ns, $\Delta E_{T_{1e}} = 27 \pm 3$ kJ mol^{-1}. [c] Experimental $R_{2p}[H_2O]/([Gd])$ values were fitted to $(T_{2M} + \tau_m)^{-1}$ calculated according to eq 4; $A/\hbar$ was fixed to -3.8×10^6 rad/s.[32] T_{1e}^{298} is smaller than the value extrapolated from the NMRD profiles due to the use of an approximated low field theory and the possible presence of further relaxation mechanisms at high fields.

ICP−MS. The concentration of each sample for relaxivity measurements was determined by ICP−MS for ^{157}Gd(III). A quantity of 10 μL of each sample was diluted to 1 mL with concentrated nitric acid for digestion. This sample was diluted with water to an appropriate concentration for ICP−MS analysis. Concentrations were obtained on a VG Elemental PQExcell spectrometer that was standardized with eight concentrations spanning 0−50 ppb Gd(III). In(III) (1 ppb) was used as an internal standard in each sample.

NMRD. Longitudinal water proton relaxation rates (R_1) were measured with a Stelar Spinmaster FFC-2000-1T fast field cycling relaxometer in the 0.01−45 MHz proton Larmor frequency range

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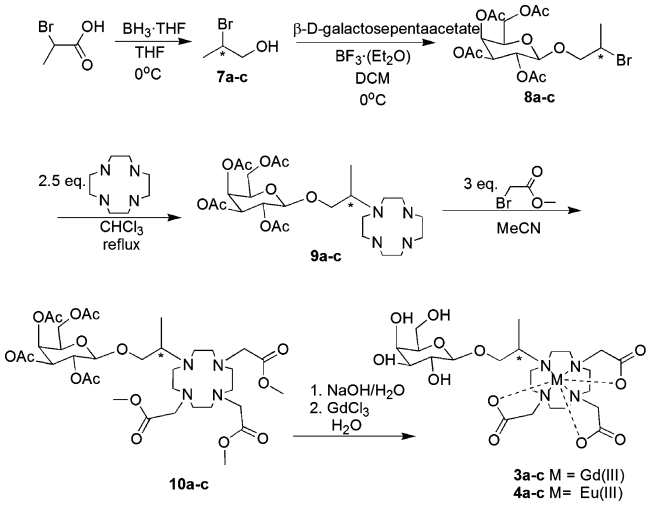
Scheme 2. Synthesis of Complexes **3a**−**c** and **4a**−**c**[a]



[a] **a**: *R*-(+)-diastereomer; **b**: *S*-(−)-diastereomer; **c**: racemic mixture.

at 293, 298, and 310 K. Standard field cycling protocol was used. R_1 values were obtained with an error smaller than 1%. Proton nuclear magnetic relaxation dispersion (NMRD) profiles were thus obtained by plotting proton relaxation rates as a function of applied magnetic field. NMRD data, subtracted from the diamagnetic contribution of buffer alone and normalized to 1 mM Gd(III), were analyzed in terms of inner-sphere and outer-sphere effects according to existing theories.[26]

Results

The coordination chemistry of a series of substituted Gd(III)DO3A complexes has been investigated to determine the optimal ligand geometry for the enzymatic activation of a MR contrast agent. We have discovered that the placement of a single methyl group on a two-carbon linker between the macrocycle and sugar has a significant impact on the observed relaxation properties. Further, the stereochemistry of linker substitution may influence the relaxivity of the complexes, and we investigated whether the observed differences were due to one of the diastereomers of a series or if they were characteristic of both isomeric components. To this end, we synthesized the *R*, *S*, and racemic mixtures of **1** and **3** and characterized each separately. A chiral center was introduced at the beginning of each of the syntheses and confirmed by ^{13}C NMR after substitution to the DO3A macrocycle and ^{1}H NMR of the final Eu(III) complexes. Complexes **5, 6**, and **19** were independently synthesized from complexes **1a**−**c**, **2a**−**c**, **3a**−**c**, and **4a**−**c**.

Synthesis. Complex **3** was synthesized according to Scheme 2 beginning with the reduction of 2-bromopropionic acid to the alcohol with BH_3·THF. Compound **7** was coupled with β-D-galactose pentaacetate using BF_3·Et_2O at 0 °C to prepare 2-bromopropan-1-β-D-galactose tetraacetate. The thermodynamic product (2-bromopropan-1-α-D-galactose tetraacetate) was present in small amounts and removed during purification by column chromatography as confirmed by ^{1}H NMR spectroscopy.

Compound **9** was prepared by refluxing **8** with cyclen in $CHCl_3$ overnight. In some cases there was evidence of di- or trialkylation side products, and these were removed during purification as confirmed by TLC (9:0.9:0.1 MeCN:H_2O: sat. KNO_3(aq)), ESI-MS, and ^{1}H NMR. Since this reaction proceeds via a S_N2 mechanism, the chirality at the methyl substitution point should be maintained but reversed.

Evidence of racemization has been observed in the literature for similar reactions where the reaction center is also a chiral center.[27,28] These systems, unlike the reaction of compound **8** with cyclen, consist of multisite substitution (tri or tetra) or substitution onto a nitrogen with compromised nucleophilicity due to backbone substitution. However, it was necessary to confirm that the stereocenter was maintained without racemization for reaction of compound **8** with cyclen to produce compound **9**. Since compounds **9a** and **9b** are diastereomers, ^{13}C NMR was used. As expected for a pure diastereomer, the ^{13}C spectra for compounds **9a** and **9b** consist of one set of peaks for the sugar linker. However, the spectrum obtained for compound **9c** shows two sets of peaks consistent with a mixture of diastereomers. Importantly, this shows that stereopurity can be maintained after monoalkylation of cyclen when a bromide leaving group is used.

Complete alkylation of **9** to yield **10** was achieved in 24 h by addition of 3 eq. of methyl bromoacetate to **9** in dry MeCN with K_2CO_3 as base. Deprotection of the ligand and insertion of the lanthanide were performed in one pot with addition of aqueous NaOH and MCl_3·$6H_2O$ as the lanthanide source (M = Gd(III), Eu(III)). All complexes (**3a**−**c** and **4a**−**c)** were purified by HPLC and determined to be analytically pure by LC-MS (Figure 2) and elemental analysis. Each of the HPLC traces for the pure diastereomers exhibits one peak; however, the racemate under the same conditions exhibits two as does a mixture of pure *R*- and pure *S*-α-EGadMe.

In order to further confirm the chiral purity at the methyl-substituted center, ^{1}H NMR spectra for the Eu(III) complexes, **4a**−**c**, were obtained. The axial protons of the macrocycle are characteristically shifted upfield by the paramagnetic Eu(III), creating a spectral footprint region for each compound. As can be seen in the boxed spectral region in Figure 3, the pure *R*-diastereomer (bottom) and the pure *S*-diastereomer (top) produce distinctly different peaks in this spectral region, and combining the two produces the spectrum for the racemate (middle).

Synthesis of **1a**−**c** and **2a**−**c** began with the kinetic resolution of propylene oxide with Jacobsen's catalyst (Scheme 3). This step was followed by ring opening of the epoxide with Li_2NiBr_4 to produce **12**. Compound **13** was synthesized by coupling β-D-galactose pentaacetate to the alcohol using BF_3·Et_2O. Upon the addition of the sugar to the chirally resolved bromide, compounds **13a** and **13b** become diastereomers. ^{13}C NMR was used to confirm the

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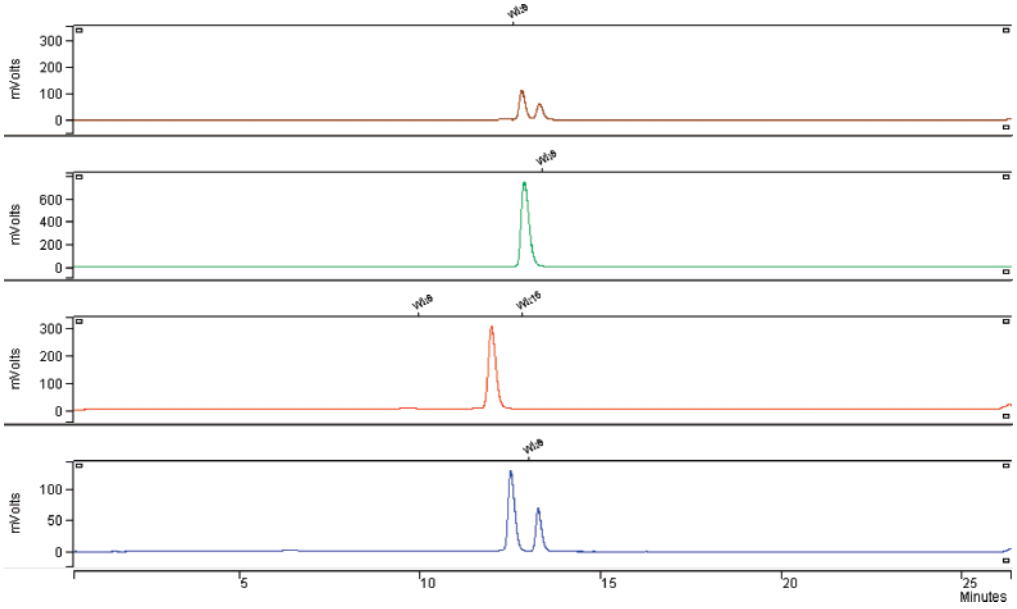

Figure 2. Fluorescence chromatograms for $\lambda_{ex} = 275$ nm and $\lambda_{em} = 314$ nm (listed from top to bottom) of racemic-α-EGadMe (compound **3a**), (*R*)-α-EGadMe (compound **3a**), (*S*)-α-EGadMe (compound **3b**), and a mixture of *R*-α-EGadMe and *S*-α-EGadMe (compounds **3a** and **3b**). One peak is observed in two places for each of the pure diastereomers, and two peaks are seen for the mixture and the complex synthesized from racemic starting material.

diastereomeric purity of compounds **13a** and **13b**. For the spectra obtained for **13a** and **13b,** only one set of peaks were observed. However for compound **13c**, two sets of peaks were observed, one set for each of the diastereomers. The crystal structures for the two diastereomers were determined, further confirming the isomeric purity of the isolated compounds (see Supporting Information). This stereocenter is not involved in any reactions after this point, so the confirmed stereochemistry should not be compromised during the subsequent steps.

The alkylation of cyclen and deprotection and metalation of the ligand were performed according to the procedures described above for the α-series. Notably, the stereochemistry of the methyl substitution site is maintained upon addition to cyclen and is *not* reversed as in the α-series since the chiral center is not the reaction center.

Complexes **5** and **19** were synthesized according to Scheme 4. Compound **7** was TBDMS-protected with addition of TBDMS-Cl and imidazole to the alcohol in dry DMF. Alkylation of the cyclen, ligand deprotection, and metalation were carried out according to a similar procedure as described above for the synthesis of α-EGadMe. Complex **6** is the commercially available contrast agent known as Prohance (Bracco Laboratories) and was purified from a buffered solution using HPLC. After purification, the complex was characterized by LC-MS and elemental analysis.

Luminescence. Since the Eu(III) and Gd(III) ions are similar in charge and ionic radius, the Eu(III) derivative of a Gd(III) complex is often used in luminescence studies to determine the number of bound water molecules, *q*.[21,29] The luminescence lifetime decay spectrum was measured for each complex in both H_2O and D_2O, and *q* was calculated. The vibronic coupling of the Eu(III) to O−H oscillators is much greater than to O−D oscillators, so the luminescence lifetime in H_2O is much shorter than in D_2O.[30] Equation 2, given by Supkowski et al., relates the values of *q* to the difference in these luminescence lifetimes.[21]

$$q = 1.11[\tau^{-1}(H_2O) - \tau^{-1}(D_2O) - 0.31] \qquad (2)$$

The results shown in Table 1 reveal a significant difference in the number of water molecules coordinated to complexes **2** and **4**. Compound **2** has a *q* greater than 1; however, compound **4** has a *q* less than 1. We propose the number of water molecules bound to **4a**−**c** to be zero even though *q* was calculated to be ≈0.5 by this technique. This conclusion is supported by relaxivity data, NMRD fits, and VT ^{17}O NMR. The slightly inflated calculated hydration number may be due to interactions of the metal center with the O−H oscillators on the galactopyranose which in this complex is likely positioned directly over the open coordination site of the Gd(III) ion.

There is no difference between the *q* of the *R*/*S*-diastereomers of the same (α/*β*)-isomer. Therefore, hydration studies in the presence and absence of a coordinating anion (CO_3^{2-}) were performed using the racemic mixtures. Significantly, the *q* of complexes **2c** and **4c** in the presence and absence of 24 mM $NaHCO_3$ show a difference in the interaction of each of these complexes with a coordinating anion. While *q* of complex **4c** remains the same (within the error of the calculation) upon introduction of CO_3^{2-}, complex **2c** decreases by 58% (1.7 to 0.98). These observations are consistent with the relaxivity values obtained for the complexes in the same buffers.

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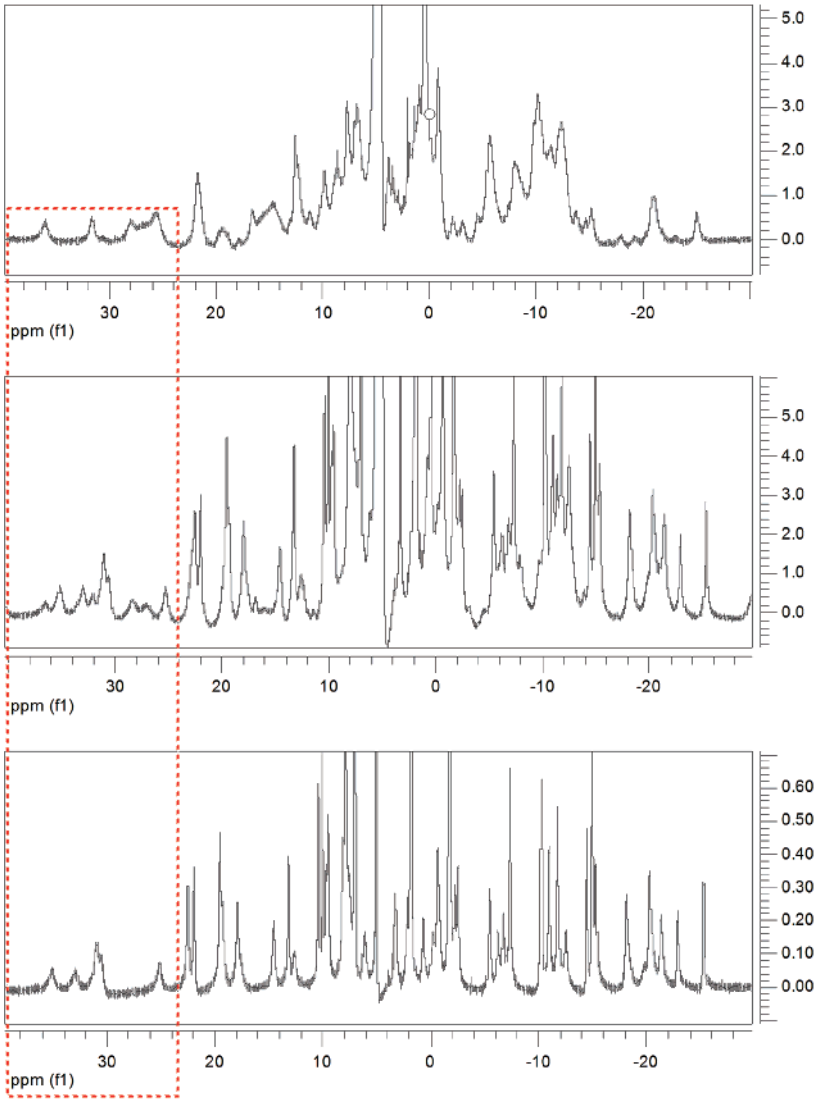


Figure 3. ^{1}H NMR spectra of Eu(III) analogues at 500 MHz and 25 °C of *S*-α-EGadMe (top), racemic α-EGadMe (middle), and *R*-α-EGadMe (bottom). The boxed area shows axial protons of the major isomer that are shifted because of the paramagnetic lanthanide. This shows that the two diastereomers produce distinctly different NMR signals in this spectral region, and combining the spectra for the pure *R*- and *S*-diastereomers produces the middle spectrum for the racemate.

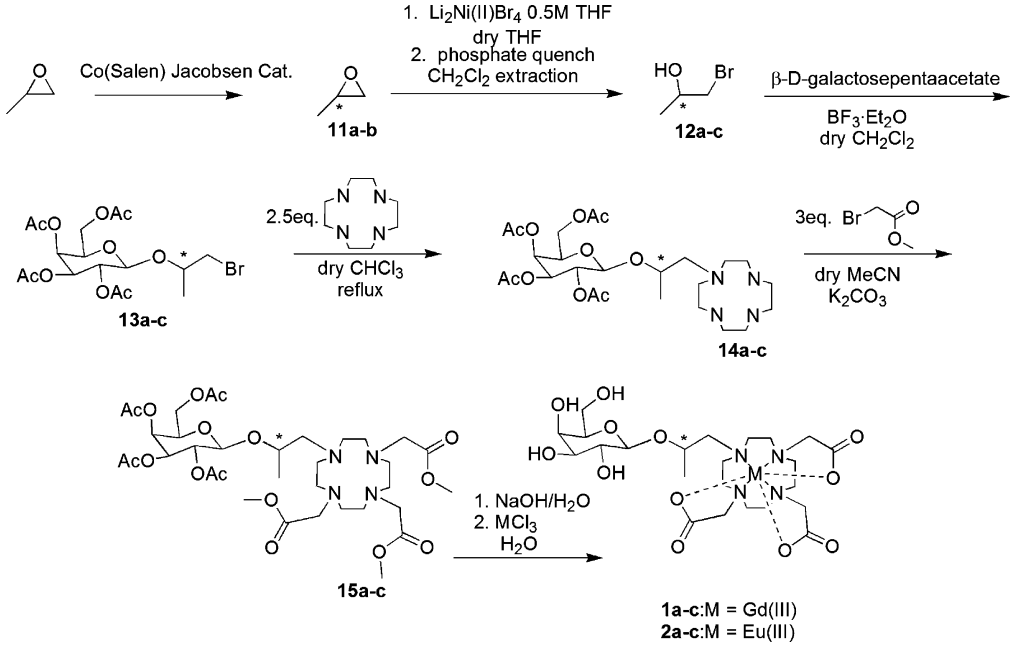
The hydration number for complex **5** was calculated using eq 3 from Supkowski et al. where n_{O-H} is the number of O−H oscillators present in the first coordination sphere of the Eu(III). This term corrects for the contribution of O−H oscillators on the luminescence lifetime decay of the Eu(III). The hydration number, 1.3 ± 0.1, calculated for complex **5** from this equation is in agreement with the literature value for cleaved *β*-EGadMe which is Prohance.

$$q = 1.11[\tau^{-1}(H_2O) - \tau^{-1}(D_2O) - 0.31 - n_{O-H}(0.44)] \quad (3)$$

In order to verify that CO_3^{2-} coordination causes a change in q for complex **2**, the luminescence emission at $\lambda = 614$ nm was examined with gradual titration of HCO_3^- for complexes **2c** and **4c**. An estimated anion binding constant (K_a) for the complex with carbonate (pH 7.3, 100 mM MOPS) for 1:1 ternary metal−ligand−anion complex formation can be calculated. For complex **2c** which showed a characteristic increase in the $\lambda = 614$ nm emission with increased HCO_3^- concentration (Figure 1A), the binding constant was determined to be $K_a = 44.5\ M^{-1}$ for the Eu(III) complex of *β*-EGadMe to HCO_3^- at pH 7.3. Since carbonate (CO_3^{2-}) is the anionic species that is actually thought to bind to the lanthanide, the binding constant calculated and reported here for HCO_3^- is probably much lower than the actual value for CO_3^{2-}. In contrast, complex **4c** was unchanged with titration of HCO_3^- (Figure 1B), and it was concluded that there is no binding affinity of complex **4** for carbonate.

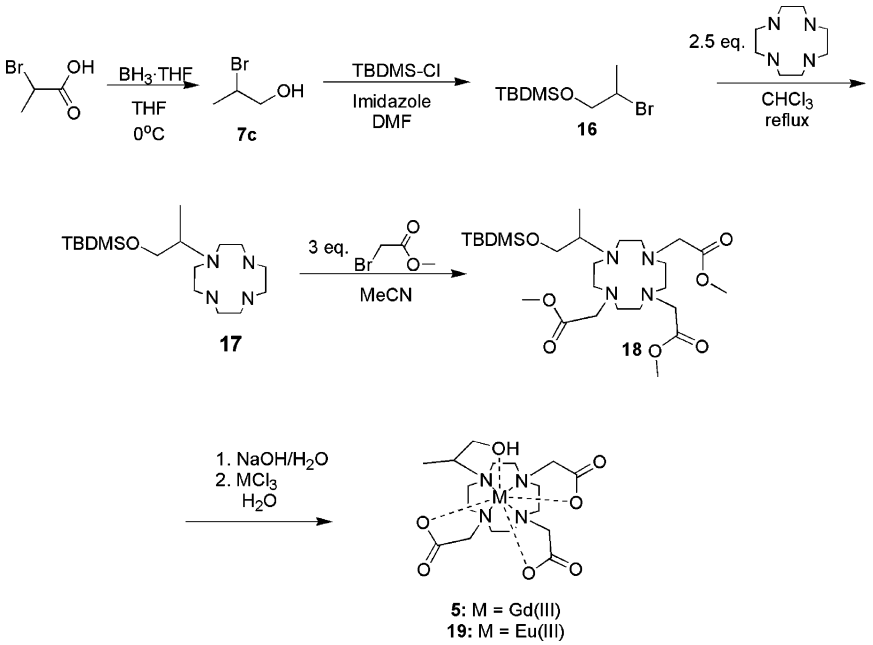
VT ^{17}O NMR. A water molecule that is coordinated to the metal center is in constant exchange with bulk water, and the rate of exchange is equal to the inverse of its residence lifetime, $k_{ex}^{-1} = \tau_m$.[31] The exchange rate can be determined directly from variable temperature ^{17}O NMR.[23] The water exchange rates (Table 2) of the racemic complexes were determined by fitting plots of R_{2p} versus temperature

Scheme 3. Synthesis of Complexes **1a**−**c** and **2a**−**c**[a]



[a] **a**: *R*-(+)-diastereomer; **b**: *S*-(−)-diastereomer; **c**: racemic mixture.

Scheme 4. Synthesis of Complexes **5** and **19**



(Figure 4) according to Swift and Connick theory,[23,24] using eq 4,[32]

$$\frac{1}{T_{2M}} \cong \frac{S(S+1)}{3}\left(\frac{A}{\hbar}\right)^2 (T_{1e}^{-1} + \tau_m^{-1})^{-1} \qquad (4)$$

where S is the electron spin quantum number (7/2 for Gd(III)), $A/\hbar$ is the hyperfine coupling constant between the Gd(III) ion and the oxygen nucleus, and T_{1e} is the electron relaxation time, the temperature dependence of which is described with the following equation, analogous to the temperature dependence of the exchange time:

$$\frac{1}{T_{1e}} \cong \frac{1}{T_{1e}^{298}} \exp\left(\frac{\Delta E_{T_{1e}}}{R}\left(\frac{1}{T} - \frac{1}{298\ \mathrm{K}}\right)\right) \qquad (5)$$

$$\frac{1}{\tau_m} \cong \frac{1}{\tau_m^{298}} \frac{T}{298\ \mathrm{K}} \exp\left(\frac{\Delta H}{R}\left(\frac{1}{298\ \mathrm{K}} - \frac{1}{T}\right)\right) \qquad (6)$$

The line width of the ^{17}O NMR signal was measured for variable temperature (VT), proton-decoupled ^{17}O NMR. R_{2p} at each temperature is calculated using the equation: $R_{2p} =$

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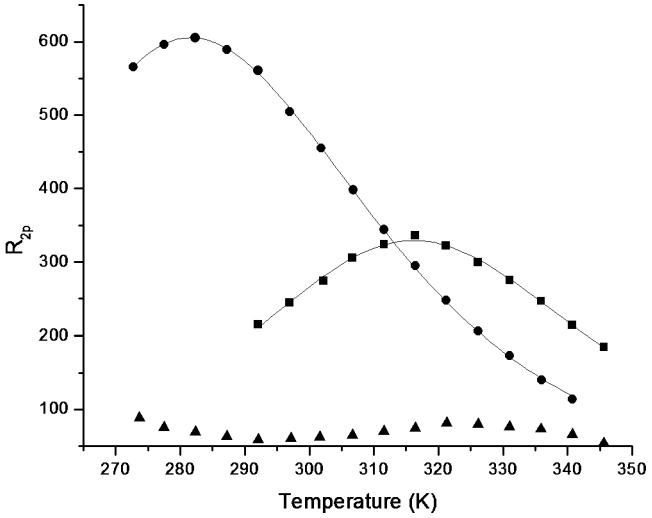

Figure 4. R_{2p} (s^{-1} mM^{-1}) dependence on temperature for complexes **1** (●) and **5** (■) demonstrates that these complexes do have inner-sphere water molecules that are in exchange with the bulk. The lack of R_{2p} dependence temperature observed for compound **3** (▲) confirms that α-EGadMe does not have any inner-sphere water molecules.

Table 3. Relaxivity Values for Gd(III) Complexes at 37 °C and 60 MHz[a]

complex	r_1 H_2O	r_1 MOPS[b]	r_1 MOPS/$NaHCO_3$[c]
1a	4.1 ± 0.5	3.7	2.62 ± 0.06
1b	4.0 ± 0.2	4.39 ± 0.01	2.9 ± 0.1
1c	4.95 ± 0.04	4.46 ± 0.07	2.8 ± 0.4
3a	1.35 ± 0.04	1.85 ± 0.08	2.05 ± 0.04
3b	1.71 ± 0.03	2.4 ± 0.1	2.6 ± 0.2
3c	1.76 ± 0.03	2.08 ± 0.08	2.07 ± 0.08
5	2.955 ± 0.007	3.32 ± 0.08	3.245 ± 0.007
6	2.44 ± 0.03	2.50 ± 0.05	3.237 ± 0.005

[a] Error is standard deviation of duplicate measurements. [b] 100 mM MOPS pH 6.99. [c] 100 mM MOPS and 24 mM $NaHCO_3$ pH 7.3.

$\Delta_{\nu 1/2}\pi$. R_{2p} was plotted versus temperature and corrected for background by subtracting the plot obtained for a water control.

The plots for complexes **1** and **5** exhibit characteristic R_{2p} dependencies on temperature that are expected for metal complexes of their size and coordination state.[9,33] The water residence lifetime (τ_m) for **1** and **5** were calculated from these curves and are shown in Table 2. Notably, the plot for complex **3** shows no dependence of R_{2p} on temperature. Since the temperature-dependent effect of the Gd(III) on the ^{17}O transverse relaxation time is a through-bond interaction, it is clear that there are no water molecules coordinated to the metal center of **3**.

Relaxometry. Bidentate anions can block water access to the Gd(III) ion, and therefore the relaxivity of each new complex was determined in pH = 6.99, 100 mM MOPS and pH = 7.3, 100 mM MOPS/24 mM $NaHCO_3$. The data shown in Table 3 illustrate a difference in the role of the CO_3^{2-} in the water-blocking mechanism of **3** as compared to **1**.

The presence of the carbonate anion results in a significant decrease in relaxivity for **1** while having no effect on complex **3**. This anion influences the relaxivity of complexes that are substituted at the β-position from the macrocycle (β-isomer) and not the α-isomer. On the basis of this evidence, it can be reasoned that the relaxivity difference observed upon conversion of complex **1** to **6** is brought about via an anion coordination mechanism (Scheme 1A).

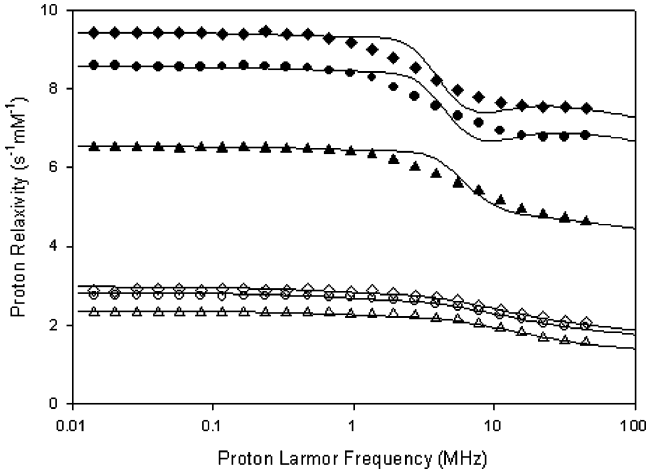

Figure 5. Water proton relaxivity data for complexes **1** (solid symbols) at 293 (◆), 298(●), and 310 (▲) K and **3** (open symbols) at 293 (◇), 298 (○), and 310 (△) K. The solid lines are best-fit curves to the SBM theory.

The relaxivity of **3** is independent of carbonate concentration, eliminating the possibility of the anion coordination mechanism, and we conclude that the galactopyranose sugar is preventing water access via a blocking mechanism (Scheme 1B). The relaxivity of **3** is significantly lower than that of **1** even in the presence of carbonate. The impact of achieving such a minimal relaxivity for **3** is significant because it affords a greater increase in relaxivity upon activation. The stereochemistry of the substitution center does not significantly influence the relaxivity in either of these cases.

NMRD. Metal hydration was investigated using NMRD which consists of measuring the field-dependent longitudinal relaxation rates down to fields as low as 0.01 MHz. The NMRD data for **1** and **3** were acquired at 293, 298, and 310 K in water and were normalized to 1 mM Gd(III) after subtraction of the diamagnetic contribution (Figure 5). As expected, the relaxivity values of **1** are significantly larger than those of complex **3**. Moreover, the values for complex **1** are in agreement with what is expected for the case of a fast exchanging water molecule in the first coordination sphere whereas the values for complex **3** are in agreement with what is expected for the absence of an exchanging water molecules in the first coordination sphere.

The observed values must be analyzed considering the presence of both inner- and outer-sphere contributions.[10,11,34] The former is due to the dipolar interaction between unpaired electrons and protons anchored at a distance *r* from the metal ion. The latter is due to the dipolar interaction with water protons freely diffusing up to a distance of closest approach, *d*, and both contributions depend on temperature.[35] Outer-sphere relaxation decreases with increasing temperature

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because it is dependent on the diffusion coefficient. Inner-sphere relaxation may increase or decrease depending on whether the proton residence lifetime τ_M is larger (slow exchange) or smaller (fast exchange) than T_{1M} (see eq 1). If the system is in the slow exchange regime, τ_M decreases significantly with increasing temperature and a large increase in the inner-sphere relaxation is observed with increasing temperature. However, the temperature dependence of **1** and **3** show that the relaxation rates decrease with increasing temperature. This indicates that τ_M does not dominate T_{1M} and that these complexes are in the fast exchange regime. This is in agreement with the ^{17}O measurements that have τ_M values of the order of 10^{-8} s. As a result, τ_M can be neglected in eq 1 and the NMRD data are sensitive to solvent accessibility to the metal center through inner-sphere contributions, outer-sphere contributions, or both.

The NMRD profiles were fitted according to the Solomon−Bloembergen−Morgan theory for the inner-sphere contribution and to the Freed model for the outer-sphere contribution.[26] All profiles of **1** exhibit an inflection point that corresponds to the ω_s dispersion. NMRD profiles depend on structural parameters such as the presence and distance of water molecules coordinated to a paramagnetic metal, and dynamic parameters like the rotational correlation time of the complex, τ_R, and the electron relaxation time.[26] The latter is well-known to be field dependent in Gd(III) complexes, and such field dependence is usually described using transient ZFS (Δ_t) and the correlation time τ_v.[26] A minimization was performed where the fit parameters were τ_R, Δ_t, and τ_v, and the number of coordinated water molecules, q, with an assumed metal-proton distance, r, of 3.05 Å. The distance of closest approach d was fixed to 3.9 Å as expected for these systems. Differences up to 10% in d were observed to have little influence on the best fit values of the other parameters.

The profiles cannot be fitted with an assumption of no coordinated water molecule ($q = 0$). A reasonably good fit, shown in Figure 5, is obtained assuming that one water molecule ($q = 1$) is coordinated to the lanthanide. The corresponding best fit values: for τ_R are 155, 145, 89 ps; for Δ_t are 0.031, 0.034, 0.042 cm^{-1}; for τ_v are 33, 30, 24 ps at 293, 298, and 310, respectively. Fits performed on the assumption of two coordinated water molecules, besides being of much worse quality, provide τ_R values too small for the investigated complex with respect to what is expected from its molecular weight, and Δ_t values too large from what is usually obtained for Gd(III) complexes.[26] Therefore, the NMRD profiles strongly suggest that one water molecule in fast exchange is bound to the lanthanide.

From the fit it is apparent that the ω_s dispersion is less sharp in the experimental data than expected for the Lorentzian dispersion in the Solomon−Bloembergen−Morgan theory. This is a consequence of the presence of static ZFS effects that are neglected in the above theory. Data were also fitted using the equations derived in the limit of slow rotation where ZFS is considered.[36,37] Indication of the presence of one coordinated fast-exchanging water molecule is obtained, and the values of the other parameters are only slightly affected. Therefore, even if approximated theories are used (due to the lack of a general minimization program where static and transient ZFS are considered in fast rotating systems), the obtained best fit values can be considered quite reliable. It has been shown that when the fast rotational motion is included as source of modulation of static ZFS, significant changes are only expected for the electronic parameters Δ_t and τ_v.[38,39]

In comparison, the NMRD profiles of complex **3** show an inflection point at larger frequencies to those of complex **1**. This is in agreement with a larger contribution from outer-sphere relaxation. The profiles were successfully fit including diffusing water molecules with a distance of closest approach for protons at 4.5 Å and two anchored protons at distances as large as 4.7 Å. Because of the slight dependence on the electron relaxation parameters, Δ_t and τ_v were fit to the values obtained for complex **1**. Most importantly, no first-sphere exchangeable protons are visible by NMRD for complex **3**. The two protons at 4.7 Å correspond to $q = 0.07$ coordinated water molecules.

Discussion

Our strategy for the development of enzymatically activated MR agents is based on preparing chelates of lanthanides that are coordinatively saturated prior to enzyme cleavage. As a result, the agents have low relaxivity[4] (low background) in an acquired MR image. In order to optimize agents that are coordinatively saturated and subsequently processed in vivo, it is necessary to understand the spatial orientation of the chelator and the mechanism by which that chelator blocks water from the lanthanide. Further, the role of physiologically relevant bidentate anions is important in this design strategy.

A new series of Gd(III) contrast agents designed to detect β-galactosidase have been synthesized and characterized. The position of a single methyl group substituted on the linker between the sugar and the Gd(III) chelate has significant impact on the mechanism by which the agent excludes water access prior to enzymatic cleavage. For these complexes, the stereochemistry at the substitution center does not appear to affect either of these properties.

The relaxivity of the β-series of complexes, **1a**−**c**, demonstrates a dependence on carbonate concentration. This dependence suggests a structure whereby the galactopyranose moiety is positioned away from the open coordination sites of the lanthanide, allowing the anion coordination mechanism to dominate (Scheme 1A). Conversely, agents **3a**−**c** are

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proposed to have a structure in which the galactose moiety resides over the macrocycle, blocking water access to the metal center in the uncleaved state (Scheme 2B). Our results indicate that it is the mechanism that produces the greatest image enhancement upon sugar cleavage because the uncleaved complex shows only outer-sphere relaxation contributions. The blocking mechanism appears to be more effective (lower relaxivity, lower background) than the anion coordination mechanism, and the resulting difference is dramatic. The lower background for the α-isomer provides an improved relaxivity increase between complexes **3** and **5** (α-isomer) than between complexes **1** and **6** (*β*-isomer). These observations are important for the development of new probes that can detect biologically important events in whole animals by MR imaging.

Acknowledgment. We thank Keith MacRenaris for assistance with ICP−MS measurements and Dr. Matthew Hartings for figures in the Table of Contents. This work was supported by NIH Grant 5 U54 CA90810, NIH Grant RO1 A147003, and DOD Grant DAMD 17-02-1-0693.

Supporting Information Available: Characterization for compounds **7b**,**c**, **8b**,**c**, **9b**,**c**, **10b**,**c**, **11b**, **12b**,**c**, **13b**,**c**, **14b**,**c**, **15b**,**c** and CIF file (CCDC 657215, 657216) for the single-crystal X-ray diffraction data of compounds **13a** and **13b**. This information is available free of charge via the Internet at http://pubs.acs.org.

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