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Supporting Information

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Multilocus binding increases relaxivity of protein bound MRI contrast agents

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Experimental

General. 2,4,6-Trimethylphenylboronic acid was purchased from Frontier Scientific, Inc. (Logan, UT). All other reagents were purchased from Sigma-Aldrich (Milwaukee, WI) and used without further purification unless otherwise stated.

The synthesis of compound 1e (see Scheme 1)

N,N-Bis[2-[N',N'-bis[(tert-butoxycarbonyl)methyl]-amino]ethyl]-L-aspartic acid (1a). Compound **1a** was prepared according to a published method.[1]

Tert-Butyl (2R)-2-{bis[2-(bis{[(tert-butyl)oxycarbonyl]methyl}amino)ethyl]amino}-3-(N-{[benzyloxycarbonyl]methyl}carbamoyl)propanoate (1b). To a solution of compound **1a** (20 g, 0.027 mol) in a mixture of CH_3CN and CH_2Cl_2 (7:1, 120 ml), under nitrogen, was added Glycine benzyl ester hydrochloride (6.0 g, 0.030 mol), and HOBt (4.6 g, 0.030 mol). The basicity of the mixture was adjusted by the addition of diisopropylethylamine in a dropwise fashion (monitored with wet pH sticks). The "pH"

was adjusted to ~8.0 accordingly. DIC (4.72 ml, 0.030 mol) was added to the mixture and the "pH" was again measured and adjusted to ~8.5. The reaction mixture was stirred at room temperature for 24 h. The reaction mixture was filtered (to remove the existing white precipitate) and the filtrate was concentrated under vacuo to give an oil. The oil was loaded onto slurry-packed silica gel column (200 g in 400 ml 10% EtOAc/90% Hexane) and flash purified using an EtOAc/Hexane gradient consisting of 250 ml 10% EtOAc-90% Hexane, followed by 250 ml 20% EtOAc-80% Hexane. Normal Phase TLC (30% EtOAc in Hexane, PMA stain) showed product at an R_f of 0.5. Fractions which showed one product spot on TLC were combined and concentrated to an oil to give 15 g of compound **1b** (63% yield, HPLC purity >95%).

2-((3R)-3-{bis[2-(bis{[(tert-butyl)oxycarbonyl]methyl}amino)ethyl]amino}3-3[(tert-butyl)oxycarbonyl]propanoylamino)acetic acid (1c). 15.0 g of compound **1b** was dissolved in 5%TEA in EtOAc (100 ml) and added to 10% Pd/C (3.0 g), which was in a nitrogen purged Parr bottle. The solution was placed on a Parr apparatus and evacuated and purged with hydrogen three times. The solution was pressurized to 45 psi and shaken overnight. The reaction mixture was filtered through celite 545 and concentrated to provide 13.9 g of crude carboxylic acid **1c** as a light yellow oil. After column chromatography (normal phase silica gel, hexane/ethyl acetate solvent system) one obtains 9.0 g of purified carboxylic acid **1c**: on a HPLC-MS with UV (220 nm) and +ESI detection with a gradient of eluant A (98% 50 mM ammonium formate-2% CH_3CN) with 0% eluant B (10% 50 mM ammonium formate-90% CH_3CN) rising to 75% eluant B over 5 min (0.8 mL/min, Vydac C4 column, 4.6 x 50 mm, 5 μm), **1c** elutes at 4.71 min (95% total peak area at 220 nm, positive ion, m/z = 789.3 $[M+H]^+$).

Compound 1d. To a solution of triethylenetetramine (61.9 mg, 0.42 mmol) and diisopropylethylamine (0.40 ml) in DMF (10 mL) were added a solution of **1c** (2.0 g, 2.54 mmol) in CH_2Cl_2 (30 mL), HOBt (389 mg, 2.54 mmol) and DIC (320 mg, 2.54 mmol). The mixture was stirred at room temperature overnight. The solvent was removed at reduced pressure to give the reaction mixture as a yellow oil. The reaction mixture was submitted to Prep-HPLC on C4 column (eluant: 0.1% TFA-H_2O/0.1% TFA-

CH_3CN) to give a crude product as a white solid (1.12 g, 83% yield): on a HPLC-MS with UV (220 nm) and +ESI detection with a gradient of eluant A (0.1% TFA-98% H_2O-2% CH_3CN) with 0% eluant B (0.1% TFA-49% CH_3CN-49% IPA) rising to 75% eluant B over 5 min (0.8 mL/min, Vydac C4 column, 4.6 x 50 mm, 5 μm), **1e** elutes at 4.93 min (96.7% total peak area at 220 nm, positive ion, *m/z* = 1615.6 $[M+2H]^{2+}$, 1077.3 $[M+3H]^{3+}$).

Compound 1e. To compound **1d** (1.12 g, 0.35 mmol) was added a solution of TFA (72 ml), H_2O (1.6 mL), TIS (1.6 mL), CH_2Cl_2 (1.6 mL), dodecanethiol (1.6 ml) and anisole (1.6 mL). The mixture was stirred at room temperature for 3 h. The solvents were concentrated to about 5 mL. To the remaining solution was added ether to precipitate out the product. The crude product was submitted to Prep-HPLC on C18 column (eluant: 0.1% TFA-H_2O/0.1% TFA-CH_3CN) to give **1e** as a white solid (0.39 g, 53 % yield): on a HPLC-MS with UV (220 nm) and +ESI detection with a gradient of eluant A (0.1% TFA-98% H_2O-2% CH_3CN) with 0% eluant B (0.1% TFA-49% CH_3CN-49% IPA) rising to 50% eluant B over 5 min (0.8 mL/min, Vydac C4 column, 4.6 x 50 mm, 5 μm), **1e** elutes at 0.79 min (85.7% total peak area at 220 nm, positive ion, *m/z* = 1054.3 $[M+2H]^{2+}$, 703.5 $[M+3H]^{3+}$).

The synthesis of compound 2e (see Scheme 2)

4-Mesitylbenzoic acid (2a). To a solution of 2,4,6-Trimethylphenylboronic acid (10.0 g, 61 mmol) and 4-bromobenzoic acid (12.9 g, 64 mmol) in 1-propanol (150 mL) and DME (200 mL) were added triphenylphosphine (0.128 g, 0.49 mmol), 2M sodium carbonate solution (37 mL, 74 mmol) and water (30 mL). To the mixture was added palladium acetate (82 mg, 0.37 mmol) under nitrogen atmosphere. The mixture was heated to reflux for overnight. After heat source was removed, 100 mL of water was added and stirred for 2.5 h while cooling to room temperature. The darkened mixture was diluted with 150 mL of ethyl acetate and the two phases were separated. The organic layer was washed several times with saturated sodium bicarbonate solution until TLC indicated that

4-bromobenzoic acid (R_f = 0.55, eluant: CH_2Cl_2/CH_3OH = 5) was completely removed. The solution was extracted three times with 200 mL of 1N NaOH solution. To the combined aqueous layers was added about 50 mL of 12 N HCl to pH 3. The resultant precipitate were filtered, washed with water, and dried to give **2a** as a white solid (8.81g, 60.2 % yield): R_f = 0.75 (eluant: CH_2Cl_2/CH_3OH = 5); ^{1}H NMR ($CDCl_3$) δ 1.997 (s, 6 H), 2.340 (s, 3 H), 6.961 (s, 2 H), 7.274 (d, J = 8.1Hz, 2 H), 8.177 (d, J = 8.1Hz, 2 H).

2',4',6'-Trimethyl-biphenyl-4-carboxylic acid {2-[2-(2-amino-ethylamino)-ethylamino]-ethyl}-amide (2b). To a solution of 4-Mesitylbenzoic acid (**2a**, 0.7 g, 2.91 mmol) and triethylenetetramine (4.26 g, 29.1 mmol) in CH_2Cl_2 (200 mL) were added HOBt (0.89 g, 5.83 mmol) and DIC (0.74 g, 5.83 mmol). The mixture was stirred at room temperature for overnight. The resultant precipitate was filtered and the solvent was removed under reduced pressure to give the reaction mixture as a yellow oil which contains **2b** as well as triethylenetetramine. The reaction mixture was submitted to Prep-HPLC on C4 column (eluant: 0.1% TFA-H_2O/0.1% TFA-CH_3CN) to give the TFA salts of **2b** as a white material (0.63 g, 26.3% yield): on a HPLC-MS with UV (220 nm) and +ESI detection with a gradient of eluant A (0.1% TFA-H_2O) with 10% eluant B (0.1% TFA-CH_3CN) rising to 80% eluant B over 7 min (0.3 mL/min, Intertsil C4 column, 30 x 2.0 mm, 5 μm), **2b** elutes at 5.46 min (85% total peak area at 220 nm, positive ion, *m/z* = 369.1 $[M+H]^+$).

***N*-[2-(2-Amino-ethylamino)-ethyl]-*N*'-(2',4',6'-trimethyl-biphenyl-4-ylmethyl)-ethane-1,2-diamine (2c)**. To solution of **2b** (0.63 g, 0.76 mmol) in ether (100 mL) and THF (100 mL) was added LAH (1.30 g, 34.2 mmol) slowly at room temperature. The mixture was refluxed for 2 h and then stirred at room temperature for overnight. To the mixture was added water dropwise to quench LAH. The resultant precipitate was removed by filtration and the solvent was removed at reduced pressure to give reaction mixture as colorless oil. The reaction mixture was submitted to Prep-HPLC on C4 column (eluant: 0.1% TFA-H_2O/0.1% TFA-CH_3CN) to give the TFA salts of **2c** as a white foam (180 mg, 29.2% yield): on a HPLC with UV (220 nm) with a gradient of

eluant A (0.1% TFA-H_2O) with 5% eluant B (0.1% TFA-CH_3CN) rising to 80% eluant B over 22 min (1.0 mL/min, Vydac protein & peptide C18 column, 4.6 x 150 mm, 5 μm), **2c** elutes at 14.83 min (95% total peak area at 220 nm); LC-MS (*m/z)* = 354.4 $[M+H]^+$.

Compound 2d. To a solution of the TFA salts of **2c** (180 mg, 0.22 mmol) and diisopropylethylamine (287 mg, 2.2 mmol) in DMF (50 mL) were added a solution of **1c** (1.88 g, 2.38 mmol) in CH_2Cl_2 (50 mL), HOBt (370 mg, 2.38 mmol) and DIC (301 mg, 370 mmol). The mixture was stirred at room temperature for overnight. The solvent was removed at reduced pressure to give the reaction mixture as a yellow oil. The reaction mixture was submitted to Prep-HPLC on C4 column (eluant: 0.1% TFA-H_2O/0.1% TFA-CH_3CN) to give **2d** as a white solid (0.36 g, 47% yield): on a HPLC with UV (220 nm) with a gradient of eluant A (0.1% TFA-H_2O) with 5% eluant B (0.1% TFA-CH_3CN) rising to 80% eluant B over 22 min and then holding at 80% B for 8 min (1.0 mL/min, Vydac protein & peptide C4 column, 4.6 x 150 mm, 5 μm), **2d** elutes at 25.68 min (95% total peak area at 220 nm); LC-MS (*m/z)* = 1719.4 $[M+2H]^{2+}$, 1147.2 $[M+3H]^{3+}$.

Compound 2e. To a solution of **2d** (0.19 g, 0.055 mmol) in CH_2Cl_2 (4.5 mL) and anisole (4.5 mL) was added dropwise 4.5 mL of 12 N HCl. The mixture was stirred at room temperature for 3 h. To the mixture was added 40 mL of water and washed three times with ether. The aqueous solution was lyophilized to give crude product. The crude product was submitted to Prep-HPLC on C18 column (eluant: 100 mM $AcONH_4$/CH_3CN) to give **2e** as a white solid (50 mg, 39 % yield): on a HPLC with UV (220 nm) with a gradient of eluant A (0.1% TFA-H_2O) with 5% eluant B (0.1% TFA-CH_3CN) rising to 80% eluant B over 22 min and (1.0 mL/min, Vydac protein & peptide C4 column, 4.6 x 150 mm, 5 μm), **2e** elutes at 10.33 min (95% total peak area at 220 nm); LC-MS (*m/z)* = 1158.6 $(M+2H)^{2+}$, 772.8 $(M+3H)^{3+}$.

The synthesis of compound 3e (see Scheme 3)

Compound 3a. To a solution of **2a** (1.5 g, 6.24 mmol) and triethylenetetramine (0.43 g, 2.97 mmol) in CH_2Cl_2 (60 mL) were added HOBt (0.96 g, 6.24 mmol) and DIC (0.79 g,

6.24 mmol). The mixture was stirred at room temperature for overnight. The resultant precipitate was filtered and dried to give **3a** as a white solid (1.45 g, 82.5% yield): on a HPLC-MS with UV (220 nm) and +ESI detection with a gradient of eluant A (0.1% TFA-H_2O) with 10% eluant B (0.1% TFA-CH_3CN) rising to 80% eluant B over 7 min and then holding at 80% for 5 min (0.3 mL/min, Intertsil C4 column, 30 x 2.0 mm, 5 μm), **3a** elutes at 7.25 min (55% total peak area at 220 nm, positive ion, *m/z* = 591.3 $[M+H]^+$).

***N*-(2',4',6'-Trimethyl-biphenyl-4-ylmethyl)-*N*'-(2-{2-[(2',4',6'-trimethyl-biphenyl-4-ylmethyl)-amino]-ethylamino}-ethyl)-ethane-1,2-diamine (3b)**. To solution of **3a** (0.45 g, 0.76 mmol) in ether (20 mL) and THF (80 mL) was added LAH (0.33 g, 8.68 mmol) slowly at room temperature. The mixture was refluxed for 2 h and then stirred at room temperature for overnight. To the mixture was added water dropwise to quench LAH. The resultant precipitate was removed by filtration and the solvent was removed at reduced pressure to give reaction mixture as a pale yellow oil. The reaction mixture was submitted to Prep-HPLC on C4 column (eluant: 0.1% TFA-H_2O/0.1% TFA-CH_3CN) to give the TFA salts of **3b** as a white solid (140 mg, 18.4% yield): on a HPLC-MS with UV (220 nm) and +ESI detection with a gradient of eluant A (0.1% TFA-H_2O) with 10% eluant B (0.1% TFA-CH_3CN) rising to 80% eluant B over 7 min and then holding at 80% for 4 min (0.3 mL/min, Intertsil C4 column, 30 x 2.0 mm, 5 μm), **3b** elutes at 6.52 min (90% total peak area at 220 nm, positive ion, *m/z* = 564.6 $[M+H]^+$).

Compound 3c. To a solution of the TFA salts of **3b** (50 mg, 0.049 mmol) and diisopropylethylamine (38 mg, 0.294 mmol) in DMF (30 mL) were added a solution of **1c** (193 mg, 0.245 mmol) in CH_2Cl_2 (30 mL), HOBT (37.5 mg, 0.245 mmol) and DIC (31 mg, 0.245 mmol). The mixture was stirred at room temperature for overnight. The solvent was removed at reduced pressure to give the reaction mixture as a brown oil. The reaction mixture was submitted to Prep-HPLC on C4 column (eluant: 0.1% TFA-H_2O/0.1% TFA-CH_3CN) to give a crude product as a pale yellow solid: on a HPLC-MS with UV (220 nm) and +ESI detection with a gradient of eluant A (0.1% TFA-H_2O) with 10% eluant B (0.1% TFA-CH_3CN) rising to 80% eluant B over 7 min and then holding at

80% for 5 min (0.3 mL/min, Intertsil C4 column, 30 x 2.0 mm, 5 μm), **3c** elutes at 10.21 min (90% total peak area at 220 nm, positive ion, *m/z* = 1824.2 $[M+2H]^{2+}$, 1216.3 $[M+3H]^{3+}$, 912.5 $[M+4H]^{4+}$).

Compound 3e. To a solution of **3c** (0.58 g, 0.159 mmol) in CH_2Cl_2 (5 mL) and anisole (5 mL) was added dropwise 10 mL of 12 N HCl. The mixture was stirred at room temperature for 3 h. To the mixture was added 40 mL of water and washed three times with ether. The aqueous solution was lyophilized to give crude product. The crude product was submitted to Prep-HPLC on C18 column (eluant: 100 mM $AcONH_4/CH_3CN$) to give **3e** as a white solid (11 mg, 2.7 % yield): on a HPLC-MS with UV (220 nm) and +ESI detection with a gradient of eluant A (buffer =3 mM TEA/AcOH, pH = 4, 85% buffer-5% CH_3CN-10% MeOH-0.1 mM EDTA) with 0% eluant B (5% Buffer-85% CH_3CN-10% MeOH)) rising to 25% B over 20 min and then to 100% over 12 min (1.0 mL/min, AQS-C8 C8 column, 4.6 x 250 mm, 5 μm) elutes at 23.43 min (100% total peak area at 220 nm, positive ion, *m/z*= 1263.2 $(M+2H)^{2+}$, 842.4 $(M+3H)^{3+}$, 632.2 $(M+4H)^{4+}$).

Metal complex formation. The gadolinium complexes **1, 2,** and **3**, were prepared in an analogous manor. All were formed in situ and not purified further to remove inorganic salts. The general procedure is described for Compound **1** as an example. Compound **1e** (50 mg) was dissolved in water (4.7 mL) such that the concentration would be in the 1 – 10 mM range. The concentration of chelatable material in solution was determined by photometric titration of an aliquot of **1e** in a solution containing the indicator xylenol orange in sodium acetate buffer at pH 4.8. The dye solution is orange but turns purple in the presence of uncomplexed Gd(III). This solution was titrated with standardized $Gd(NO_3)_3$ solution and the absorbance monitored at 572 nm until a positive endpoint was reached. In this manner, the concentration of **1e** was determined to be 3.73 mM (or 70 μmol of chelatable equivalents). Solid $GdCl_3 \bullet 6H_2O$ (22.7 mg, 61 μmol) was added to **1e** and the pH adjusted to 6.8 using 1 N NaOH. An additional 37 μL of a 244.7 mM $GdCl_3$ solution (9.05 μmol) was added to fully chelate the ligand. The solution was stirred for 10 minutes at pH 6.8. Analysis by LC/MS showed full complexation, and colorimetric analysis with xylenol orange not detect any uncomplexed gadolinium.

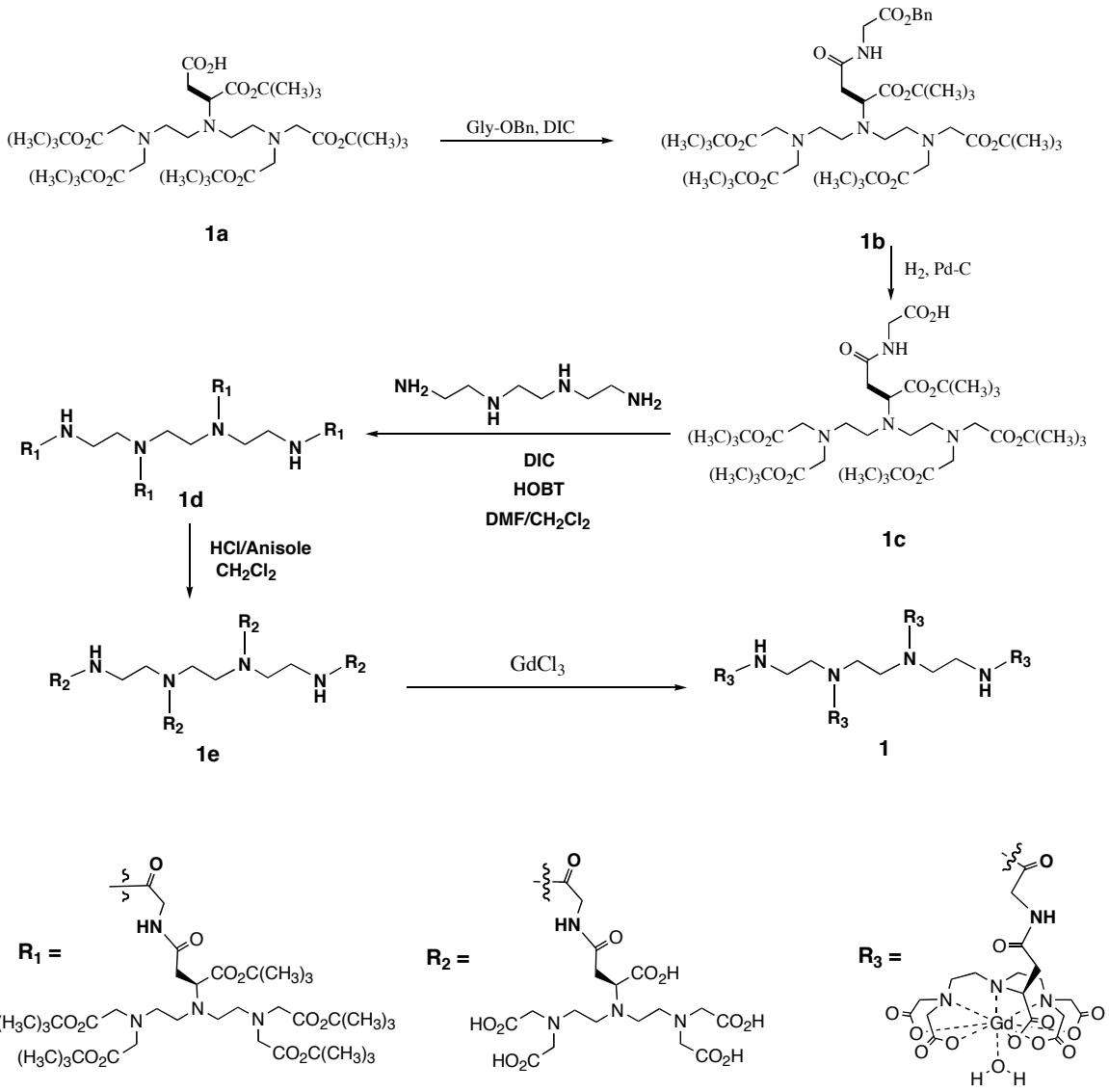
Compound 1 on a HPLC-MS with UV (220 nm) and +ESI detection with a gradient of eluant A (50 mM ammonium formate) with 0% eluant B (10% 50 mM ammonium formate-90% CH_3CN:) at 5 min rising to 50% B over 15 min (15 µL/min, Keystone HyPurity C18 column, 0.5 x 150 mm, 5 µm) elutes at 11.26 min (90.0% total peak area at 220 nm, positive ion, *m/z*= 1363.9 $(M+2H)^{2+}$, 909.4 $(M+3H)^{3+}$).

Compound 2. was prepared similarly to compound **1**. On a HPLC-MS with UV (254 nm) and +ESI detection with a gradient of eluant A (50 mM ammonium formate) with 1% eluant B (9:1 CH_3CN:50 mM ammonium formate) rising to 20% B over 11 min (2.0 mL/min, Kromasil C4 column, 4.6 x 150 mm, 3.5 µm), **3** elutes at 6.58 min (91.0% total peak area at 254 nm, positive ion, *m/z* = 1467.7 $[M+2H]^{2+}$, 978.9 $[M+3H]^{3+}$.

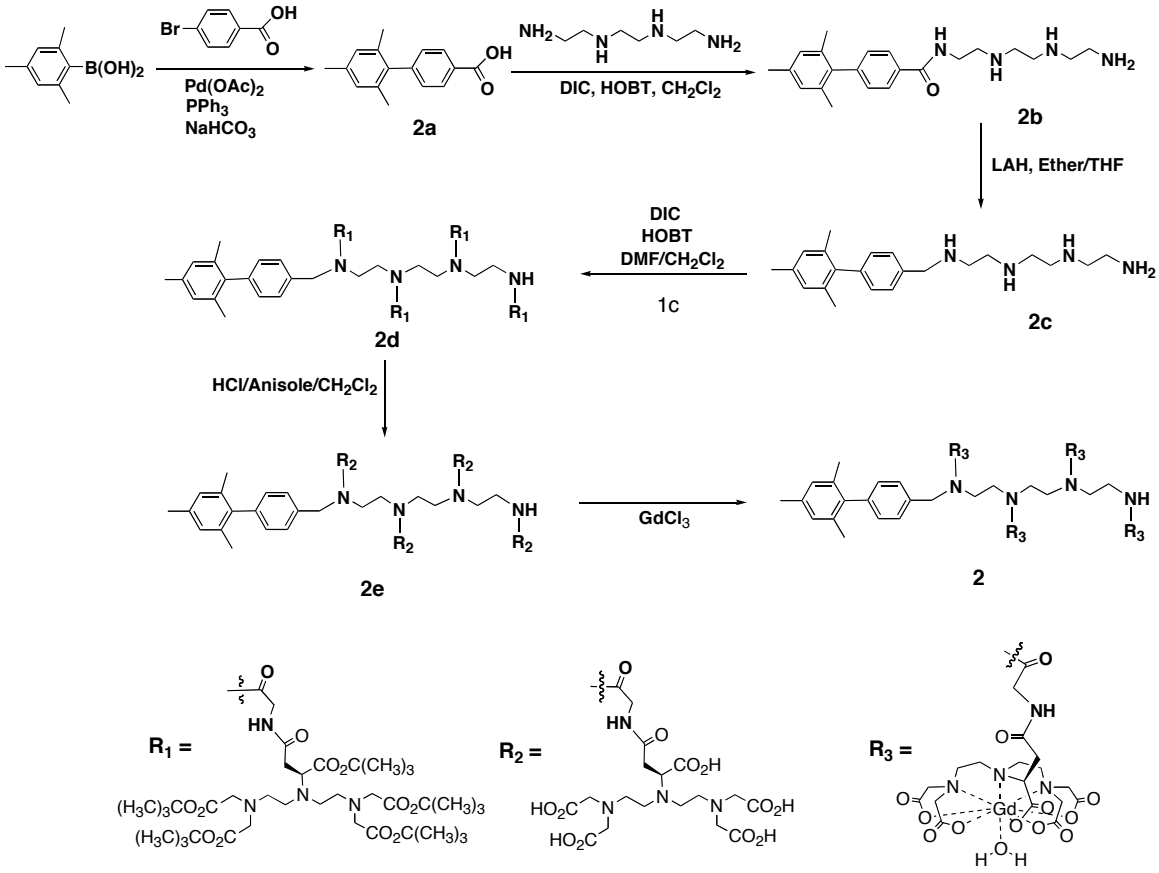
Compound 3 was prepared similarly to compound **1**. On a HPLC-MS with UV (254 nm) and +ESI detection with a gradient of eluant A (50 mM ammonium formate) with 5% eluant B (9:1 CH_3CN:50 mM ammonium formate) rising to 50% B over 30 min (0.8 mL/min, Kromasil C4 column, 4.6 x 150 mm, 3.5 µm), **3** elutes at 13.51 min (90.7% total peak area at 254 nm, positive ion, *m/z* = 1572.0 $[M+2H]^{2+}$, 1048.1 $[M+3H]^{3+}$,786.7 $[M+2H]^{2+}$).

References

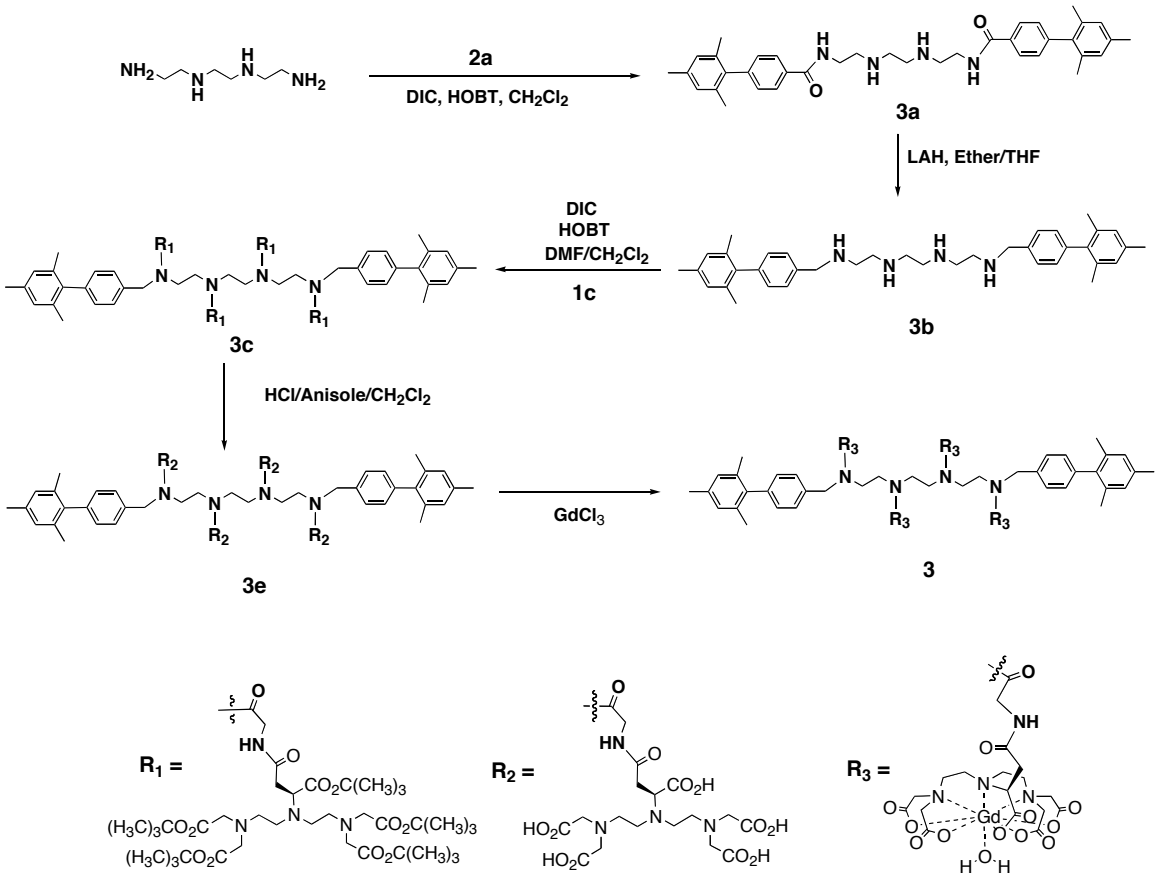
1. Amedio, J. C.; Van Wagenen, G,, Jr.; Zavlin, G.; Gyorkos, A.; Peterson, S. A. *Synthetic Communications*, **2000**, *30*, 3755.



Scheme 1. Synthesis of compound **1**.



Scheme 2. Synthesis of compound **2**.



Scheme 3. Synthesis of compound **3**.