Supporting Information

Di-nuclear Nonionic Magnetic Resonance Contrast Agents using Pyrazinyl Linking Centers

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Experimental Section

Instruments and materials

NMR spectra were recorded on a 300 or 600 MHz Bruker NMR spectrometer. Luminescence spectra of Eu^{3+} and Tb^{3+} derivatives were collected using a Perkin Elmer LS55 spectrophotometer. The luminescence delay curves were obtained at a Lecroy Wave Runner 6100 digital oscilloscope (1 GHz) using a tunable laser (pulse width = 4 ns, gate = 50 ns) as the excitation source [Continuum Sunlite optical parametric oscillator (OPO)]. Mass spectra (MS) were obtained at an autoflex III TOF/TOF MALDI-MS and a Quattro Premier XE Electron Spray Ionization source (ESI-MS).Elemental analysis were performed using a VarioEL Element Analyzer. The concentration of Gd^{3+} was determined by ICP-MS. The longitudinal relaxation times (T_1) of the two complexes were determined on MQ-20 Minispec (Bruker) at 20 MHz and 32.0 ± 0.1°C in 50 mM HEPES at pH 6.5 using an inversion-recovery pulse sequence or NMI20-Analyst NMR Analyzing & Imaging system (Shanghai Niumag Corporation).

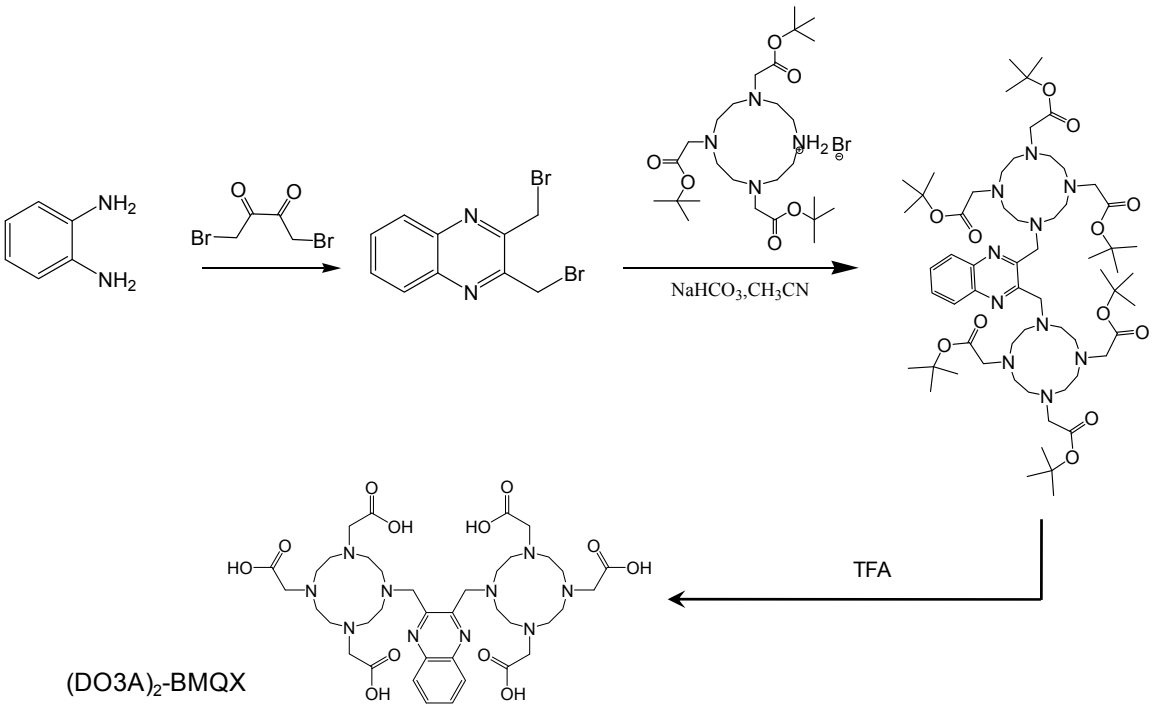
1,4,7,10-Tetrazacyclododecane, europium chloride hexahydrate, and terbium trifluoromethanesulfonate were purchased from Strem chemicals. Gadolinium chloride hexahydrate were purchased from Alfa Aesar. Acetonitrile was

distilled by calcium hydride and stored over 4 Å molecular sieves. THF were distilled by Lithium aluminium hydride and stored over 4 Å molecular sieves. Other reagents and solvents were commercially available and used without further purification. All reactions were carried out under N_2 atmosphere unless otherwise noted and were monitored by thin-layer chromatography (TLC) on plates precoated with silica gel 60 F-254 (0.25 mm). The synthesis of 1,4,7-tri(tert-butoxymethane)-1,4,7,10-tetraazacyclododecane (^{t}Bu-DO3A.HBr) followed the precedures in literatures[1].

Synthesis of compounds

The synthesis of $(DO3A)_2$-BMQX and $(DO3A)_2$-BMP followed the procedures in Supporting Schemes 1 and 2.

Scheme S1. The synthesis of $(DO3A)_2$-BMQX.



2,3-bis(bromomethyl)quinoxaline (1)

1,4-dibromo-2,3-butanedione (1.46 g, 6.00 mmol) was dissolved in dry THF (8.0

mL) and added to a solution of 1,2-phenylenediamine (0.681 g, 6.30 mmol) in dry THF (4.0 mL) at 0 °C with stirring. The reaction was warmed to room temperature and stirred for another 4h. After concentration in vacuo, the crude material was purified by column chromatography on silica gel (ethyl acetate:petroleum ether=1:12) to yield a white crystalline solid (1.67 g, 5.29 mmol, 88%). ^{1}H NMR(600 MHz, $CDCl_3$) δ 8.08 (m, 2H), 7.80 (m, 2H), 4.93 (s, 4H).

(^{t}Bu-DO3A)$_2$–BMQX (2)

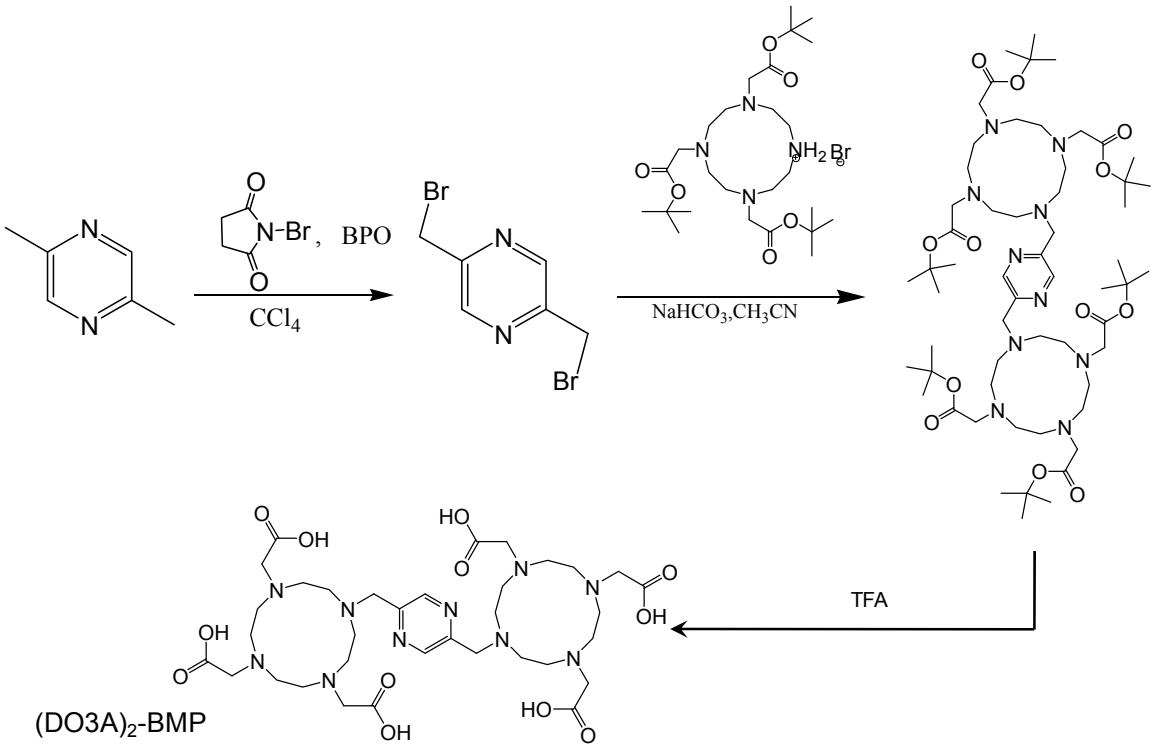
^{t}Bu-DO3A·HBr (1.22g, 2.05 mmol) was suspended in dry acetonitrile (7 ml). $NaHCO_3$ (0.682g, 8 mmol) was then added in. The mixture was then slowly heated to reflux temperature and maintained at reflux for 40 min. 2,3-Bis(bromomethyl) quinoxaline (0.348 g, 1.1 mmol) was added to the mixture and stirred at 85 °C overnight. The inorganic salt was filtered and the filtrate was concentrated after the reaction was terminated and the mixture was cooled to room temperature. The residue was purified using silica gel column chromatography eluting with DCM / MeOH = 15:1 to give a pale yellow solid (0.67g, 57% yield). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.90, 172.19, 153.36, 140.15, 128.91, 128.15, 82.11, 57.26, 56.58, 55.61, 52.82, 51.95, 50.52, 49.81, 27.79. MS(MALDI-DHB): m/z 1205.8 $[M + Na]^+$, 1183.8 $[M + H]^+$

(DO3A)$_2$–BMQX (3)

The *tert*-butyl protected compound **2** (1.42 g, 1.2 mmol) was treated with trifluoroacetic acid (40 ml, 540 mmol) at room temperature for 24 h followed by evaporation to remove the solvent. The residue was washed repeatedly with methanol (3 x 30 ml) and dichloromethane (3 x 30 ml). A pale green solid was precipitated from a minimum volume of methanol solution with diethyl ether at 0 °C. The precipitate was isolated by filtration and dried under reduced pressure to give the target product (DO3A)$_2$–BMQX (1.0 g). ^{13}C NMR (151 MHz, D_2O) δ 173.95, 169.07, 162.51, 162.28, 139.50, 131.55, 131.55, 128.03, 118.82, 116.89, 114.95, 113.02, 55.34, 54.85, 52.73, 51.34, 48.65, 48.09, 47.34, 41.88. ES$^-$ MS (CH_3OH): m/z 845.8 $[M - H]^-$, 422.4 $[M - 2H]^{2-}$. Anal. Calc for $C_{38}H_{58}N_{10}O_{12}·4CF_3COOH·3H_2O$, C, 40.71;

H, 5.05; N, 10.32. Found C, 39.59; H, 5.287; N, 10.45. UV-vis (H_2O) λ_{max} = 321 nm (ε 4305 M^{-1} cm^{-1})

Scheme S2. The synthesis of $(DO3A)_2$-BMP.



2,5-bis(bromomethyl)pyrazine (4)

N-bromosuccinimide (3.55 g, 20 mmol) was added to a solution of 2,5-dimethylpyrazine (1.08 g, 10 mmol) in CCl_4 (100 ml). The mixture was refluxed under a nitrogen atmosphere for 45 min. Benzoyl peroxide (50 mg) was then added in one portion. Dibenzoyl peroxide (44 and 30 mg) was further supplied after a stirring of 4 h and 8h, respectively, at 87 °C. The reaction mixture was kept at reflux overnight and then cooled to room temperature. The succinimide as a precipitate was filtered off and washed with CCl_4. The filtrate was concentrated in vacuo, and passed through a column of silica gel with CH_2Cl_2 /hexane =5:1 to afford the dibromo-substituted compounds (1.1 g), which were used in the following reactions without further purification. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.667 (s, 2H), 4.564 (s, 4H). 8.852 (s, 1H), 8.410 (s, 1H), 6.652 (s, 1H), 2.631 (s, 3H).

(^{t}Bu-DO3A)$_2$-BMP (5)

A mixture of ^{t}Bu-DO3A·HBr (2.1 g, 3.53 mmol) and $NaHCO_3$ (1.43 g, 17 mmol) in dry acetonitrile (20 ml) was slowly heated. After a reflux of 40 min at 84 °C, dibrom-substitutional compound **4** (0.915 g) was added and kept reflux overnight. Then the mixture was cooled to room temperature. The inorganic solid was filtered off and the filtrate was evaporated to dryness to afford crude product (2.5 g), which was then purified by column chromatography eluting with CH_2Cl_2 /methanol = 70:5 to give the pure compound (^{t}Bu-DO3A)$_2$-BMP (1.14 g). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.69, 170.46, 153.11, 143.32, 82.15, 58.04, 56.24, 55.67, 52.75, 51.47, 50.53, 49.42, 47.64, 27.98. MS(MALDI-DIT): m/z 1155.7 [M + Na]$^+$.

(DO3A)$_2$-BMP (6)

The *tert*-butyl protected compound **5** (1.14 g, 1.01 mmol) was dissolved in trifluoroacetic acid (15 ml, 202 mmol) and stirred at 0 °C for 1h. The stirring was kept for another 24 h at room temperature before all volatiles being removed under reduced pressure. The residue was dissolved in methanol (30 ml x 2) and CH_2Cl_2 (30 ml x 2) and evaporated to dryness repeatedly. The resulted residue was dissolved in minimum methanol, followed by the addition of diethyl ether at 0 °C. The solid product was filtered and washed with diethyl ether to afford the target product (DO3A)$_2$-BBMP (0.9 g). ^{13}C NMR (151 MHz, D_2O) δ 174.48, 173.91, 168.72, 162.52, 162.28, 146.28, 145.12, 142.43, 141.83, 118.83, 116.90, 114.96, 113.03, 61.57, 54.91, 54.63, 53.26, 52.70, 51.12, 50.95, 49.99, 47.68, 41.87. ES-MS (CH_3OH): m/z 795.9 [M - H]$^-$, 817.9 [M +Na - 2H] $^-$. Anal. Calc for $C_{34}H_{56}N_{10}O_{12}$·4CF_3COOH·3H_2O, C, 38.60; H, 5.09; N, 10.72. Found C, 38.97; H, 5.791; N, 10.86. UV-vis (H_2O) λ_{max} = 275 nm (ε 4546 M^{-1} cm^{-1}).

Preparation of Ln^{3+} complexes

The Gd^{3+}, Eu^{3+}, and Tb^{3+} complexes were prepared by mixing solutions of hydrated Ln^{3+} and ligands in a 2 : 1 ratio at pH 6.5. Stirring was continued for 24 h at 77℃.The complexation reaction was completed .The free Gd^{3+} was controlled with the xylenol orange test.

(Gd-DO3A)$_2$-BMQX: ES$^-$ MS (H_2O): m/z 1155.2 [M - H]$^-$,.UV-vis (H_2O) λ_{max} = 334 nm (ε 4128 M^{-1} cm^{-1}), λ_{max}= 247 nm (ε 122,704 M^{-1} cm^{-1}). IR (FT) γ_{max} 3426,1615,1398,1207,1132,1085 cm^{-1}.

(Gd-DO3A)2-BMP : ES$^-$ MS (H_2O): m/z 1105.2 [M - H]$^-$,.UV-vis (H_2O) λ_{max} = 296 nm (ε 2840 M^{-1} cm^{-1}),. IR (FT) γ_{max} 3409,1610,1397,1206,1130,1086 cm^{-1}.

Proton T_1 measurements

The T_1 relaxation times of (Gd-DO3A)$_2$–BMQX and (Gd-DO3A)$_2$-BMP at different concentrations were measured using an inversion–recovery pulse sequence on a NMI20-Analyst NMR Analyzing & Imaging system (Shanghai Niumag Corporation). The relaxivity was derived from the plots of $1/T_1$ versus the concentration of Gd(III) complex based on Equation 1[2].

$$(1/T_1)_{obsd} = (1/T_1)_d + [M]*r_1 \qquad (1)$$

where $(1/T_1)_{obsd}$ and $(1/T_1)_d$ are the values in the presence and absence of the agent, respectively; [M] is the concentration of the agents at the observed T_1 values; and r_1 is the relaxivity of the agent in a unit of $mM^{-1}s^{-1}$.

The temperature dependence of the longitudinal proton relaxivity of the two agents was obtained on a MQ-20 Minispec (Bruker) at 20 MHz.

Luminescence

The steady state emission spectra of the (Eu-DO3A)$_2$–BMQX and (Tb-DO3A)$_2$-BMP were collected on a Perkin-Elmer LS 55 luminescence spectrometer. The luminescence lifetimes of the agens (0.3 mM) were measured at a Lecroy Wave Runner 6100 digital oscilloscope (1 GHz) using a tunable laser (pulse width = 4 ns, gate = 50 ns) as the excitation source [Continuum Sunlite optical parametric oscillator (OPO)] in 50 mM HEPES-NaOH or HEPES-NaOD buffer at pH 6.5. The hydration number q of the agents were deduced from the luminescence lifetimes using Equation 2:

$$q = ([1/\tau_{H2O} - 1/\tau_{D2O}] - B) * A \qquad (2)$$

where A and B are 1.2 ms^{-1} and 0.25 ms for Eu^{3+} and 5 ms^{-1} and 0.06 ms for Tb^{3+},

respectively; τ_{H2O} and τ_{D2O} are the luminescence lifetimes of Ln^{3+} in H_2O and D_2O, respectively[3,4].

MTT assay

The cytotoxicity of $(Gd\text{-}DO3A)_2$–BMQX and $(Gd\text{-}DO3A)_2$-BMP was investigated by MTT assay using HeLa cells. In brief, HeLa cells with a density of 3000 cells/well were seeded in 96-well plates and cultured for 24 h. Then different concentrations of the agents were added in and co-incubated for another 24 h. After three washes with PBS 0.5% MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (5 mg/mL) was added to each well. An additional incubation of 4 h was allowed. Then the media was discarded and 150 μL of DMSO was added to each well to dissolve the formazan crystals for 10 minutes. The absorbance was measured at 490 nm on a Labsystem Multiskan microplate reader. The experiments were conducted in triplicate and repeated twice, the results are shown as the mean ± standard deviation (SD) of three independent experiments.

In Vivo MRI imaging

The T_1-weighted images were acquired on a 1.5 T GE Signa human clinical scanner using a FSE sequence. For in vivo MRI test , the rat was first anesthetized by intraperitoneal injection of chloral hydrate solution (10 wt%), and then 0.5mL of (Gd-DO3A)2–BMQX solution was administrated intravenously into a rat at a dose of 0.1mmol/kg. The imaging parameters are as follows: repetition time (TR) =500 ms;echo time (TE) = 16.51 ms; field of view (FOV)=20*20cm ; 320 *192 matrix size; 4mm slice thickness; number of acquisitions (NEX) = 2.

Reference

(1) Mizukami, S.; Tonai, K.; Kaneko, M.; Kikuchi, K. *J Am Chem Soc* **2008**, *130*, 14376-14377.

(2) Lauffer, R. B. *Chem Rev* **1987**, *87*, 901-927.

(3) Horrocks, W. D.; Sudnick, D. R. *J Am Chem Soc* **1979**, *101*, 334-340.

(4) Beeby, A.; M. Clarkson, I.; S. Dickins, R.; Faulkner, S.; Parker, D.; Royle, L.; S. de Sousa, A.; A. Gareth Williams, J.; Woods, M. *J Chem Soc, Perkin Trans 2* **1999**, 493-504.

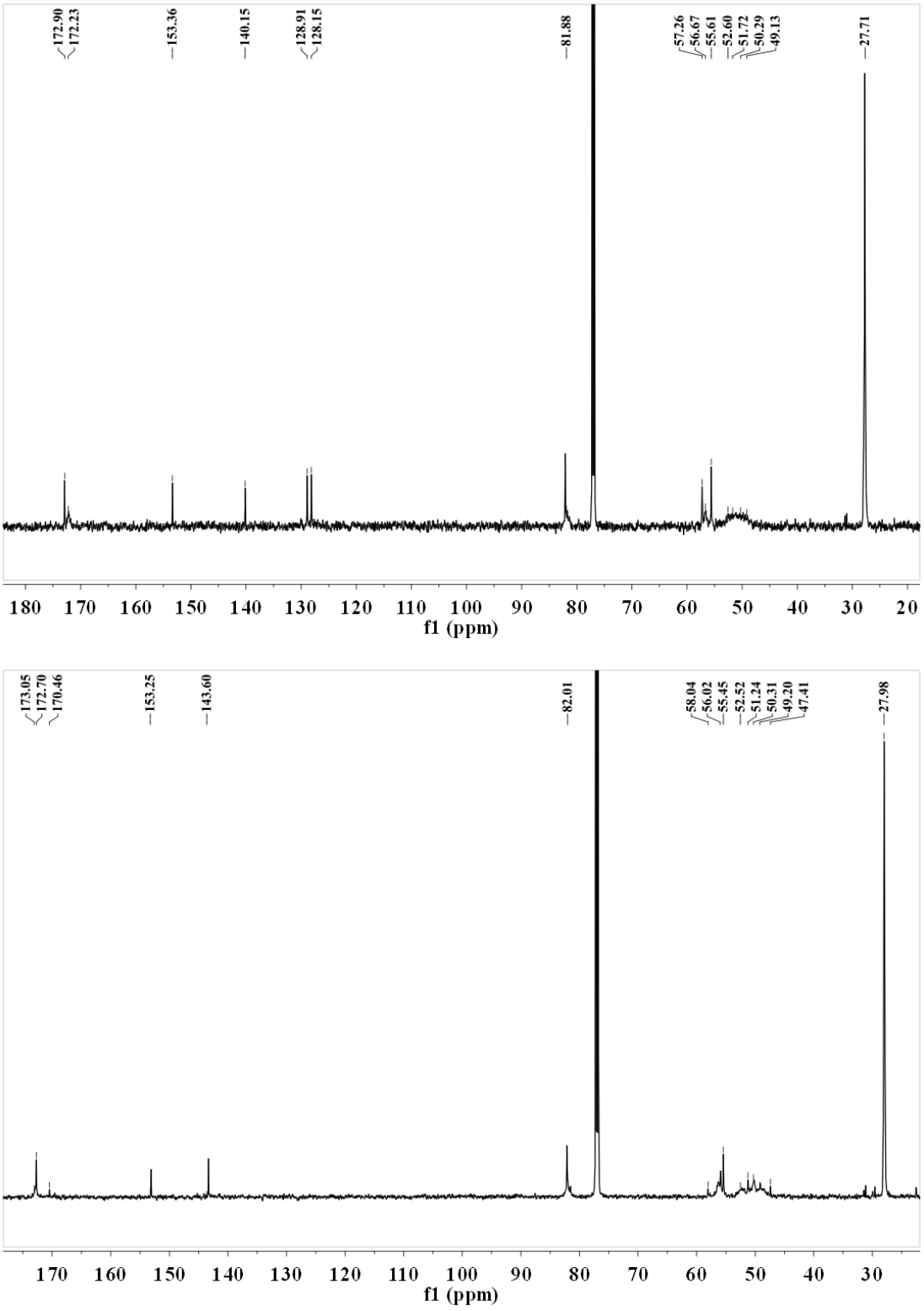


Figure S1. The ^{13}C NMR spectrum of (^{t}Bu-DO3A)$_2$–BMQX (top) and (^{t}Bu-DO3A)$_2$-BMP (bottom).

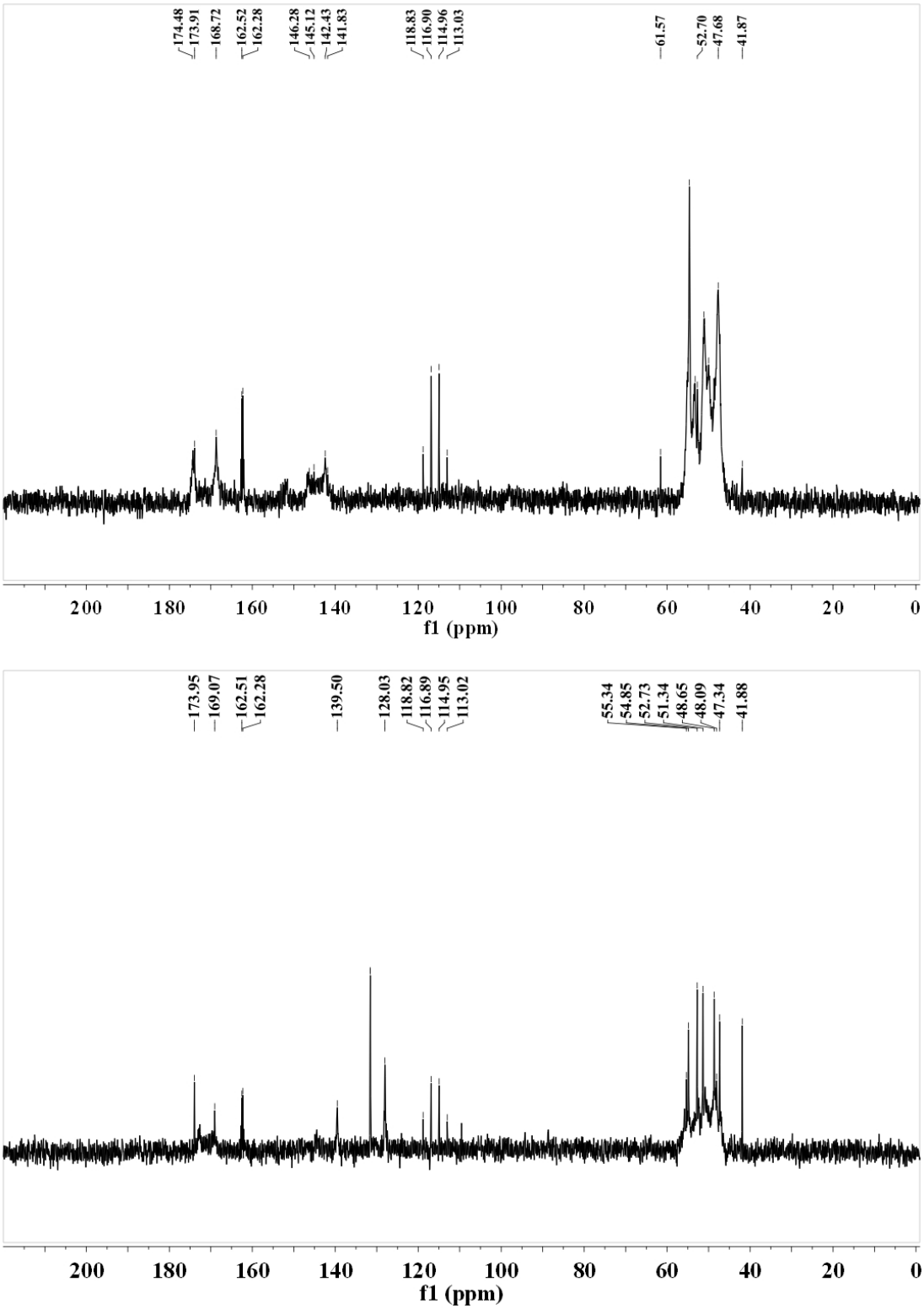


Figure S2. The ^{13}C NMR spectrum of $(DO3A)_2$-BMP (top) and $(DO3A)_2$-BMQX (bottom).

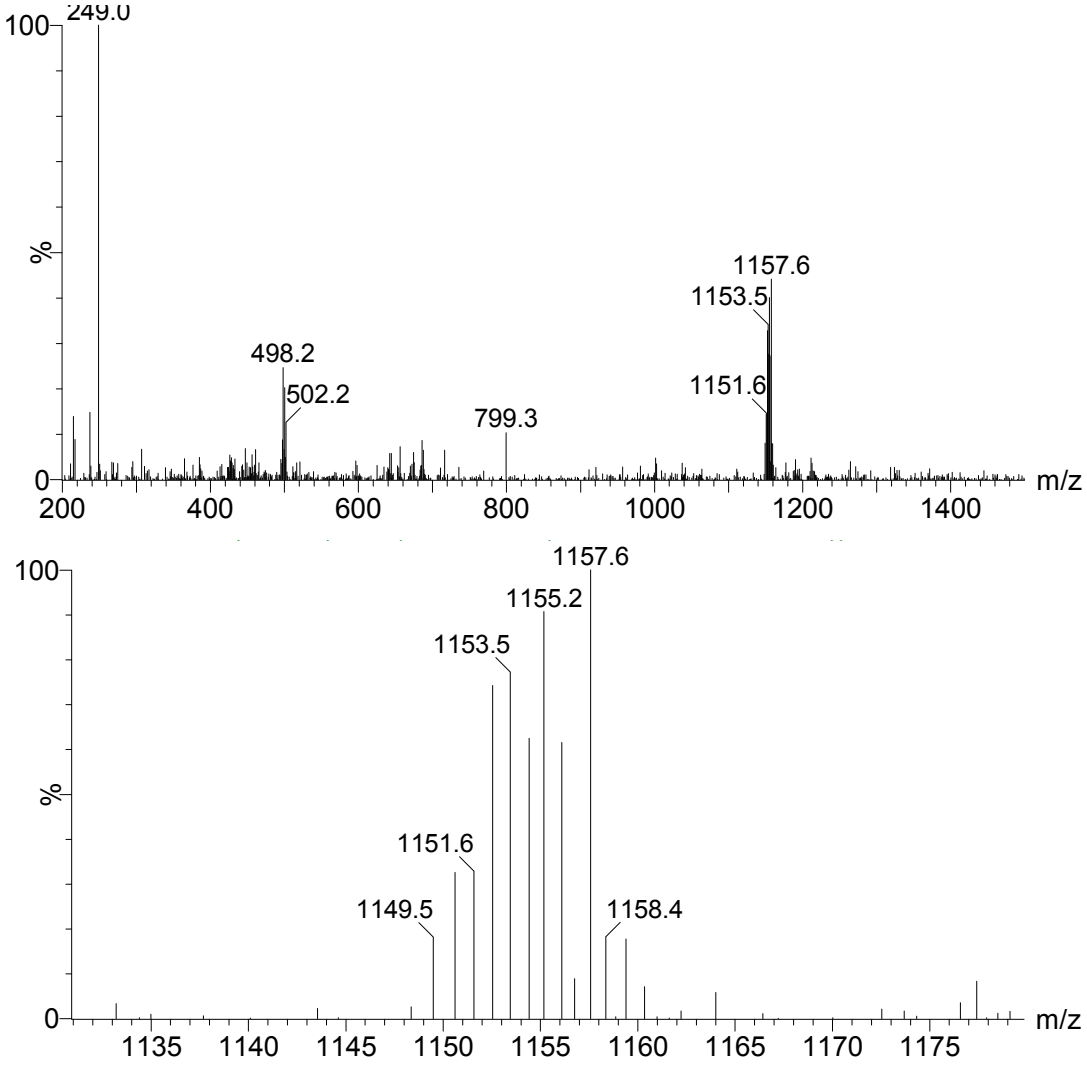


Figure S3. The mass spectrum of (Gd-DO3A)$_2$-BMQX (top) and expanded view of the target agent in mass spectrum of complex (bottom). The calculated molecular weights are 1153 Dalton for the (Gd-DO3A)$_2$-BMQX agent. The multiple molecular weights in the spectra represent the distribution of Gd isotopes.

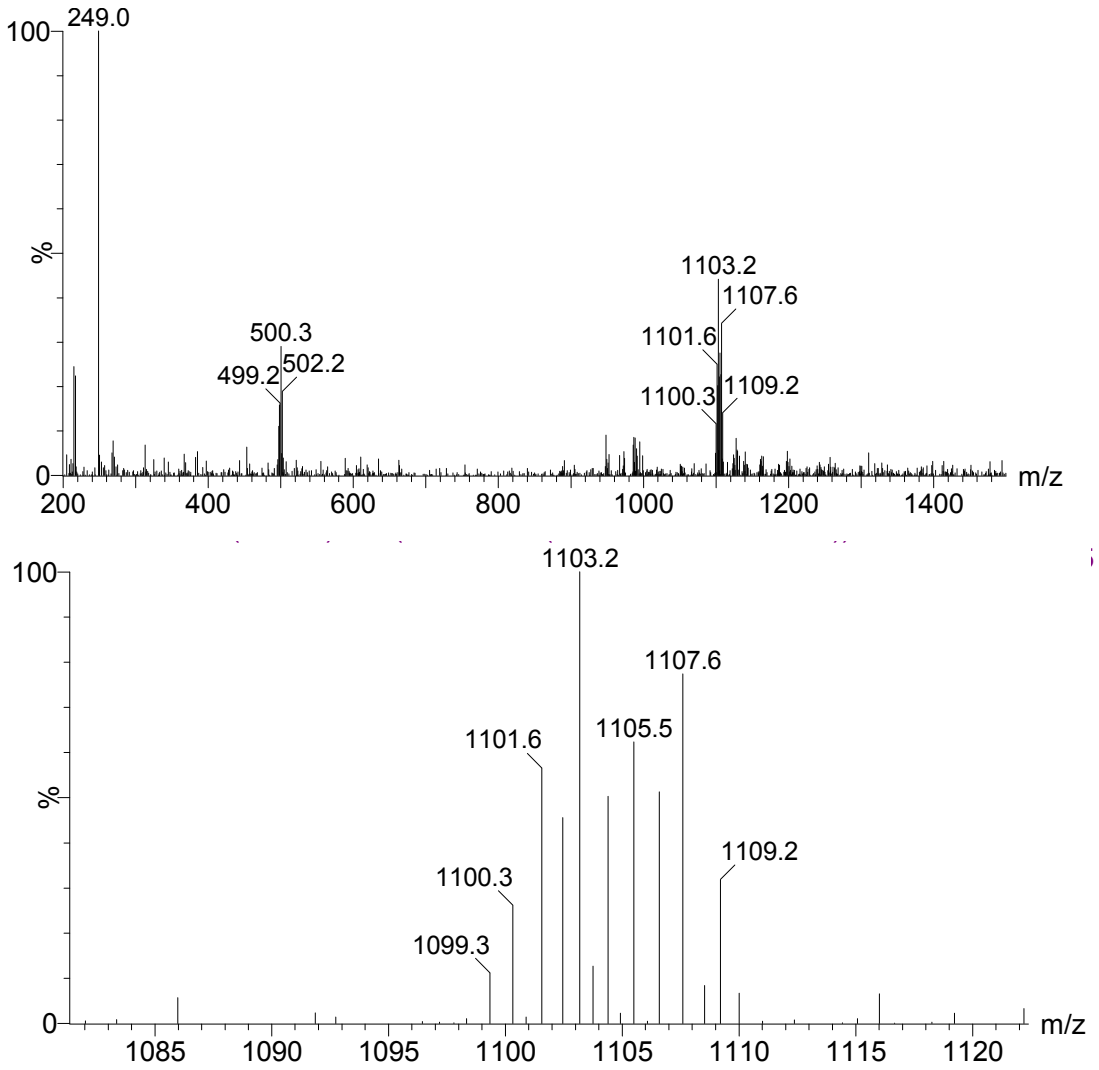


Figure S4. The mass spectrum of $(Gd\text{-}DO3A)_2$-BMP (top) and expanded view of the target agent in mass spectrum of complex (bottom).The calculated molecular weights are 1103 Dalton for the $(Gd\text{-}DO3A)_2$-BMP agent, The multiple molecular weights in the spectra represent the distribution of Gd isotopes.

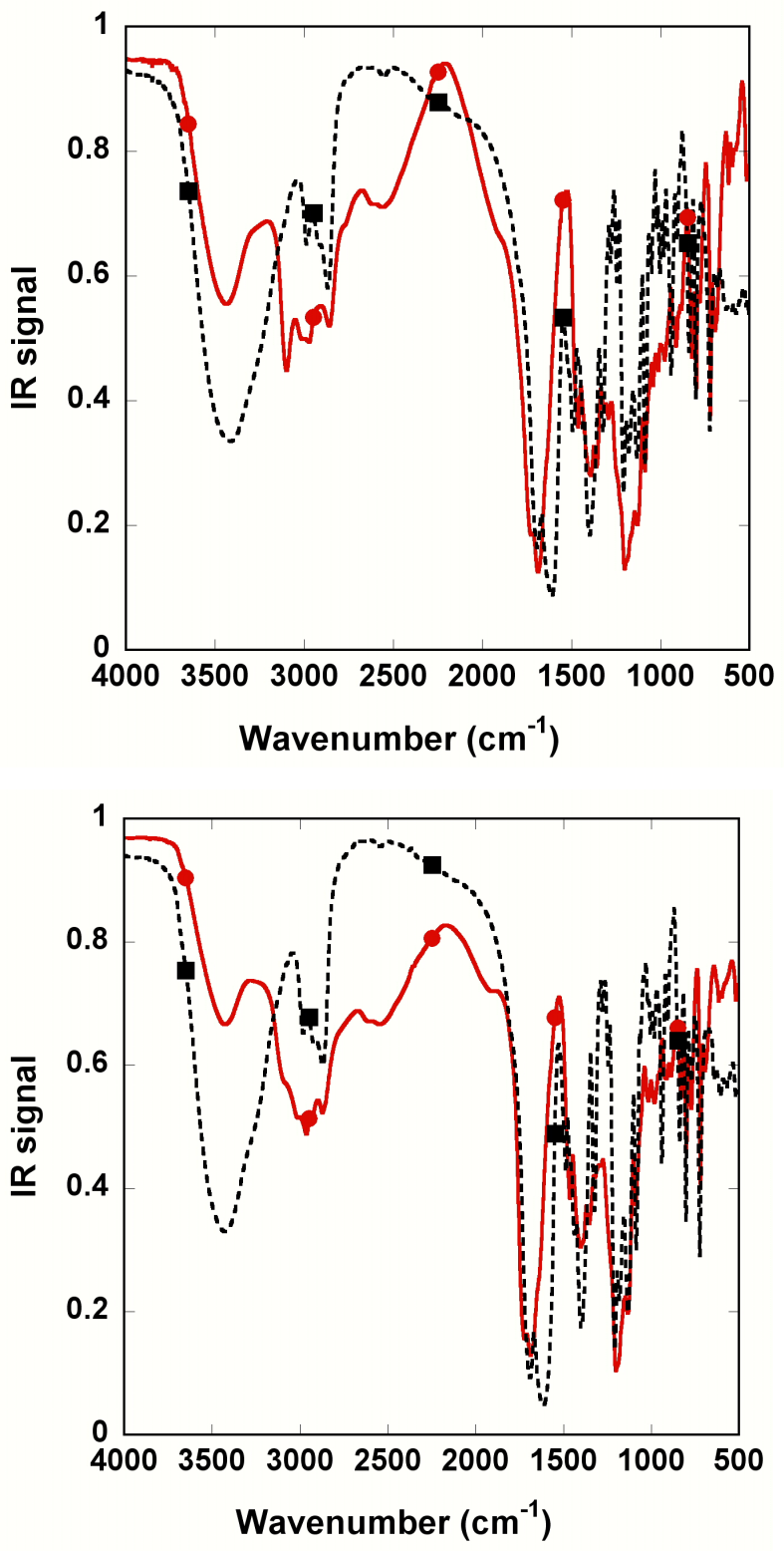


Figure S5. The infrared spectra of $(DO3A)_2$-BMP (top) and $(DO3A)_2$-BMQX (bottom) with (black) and without (red) the chelated Gd^{3+}.

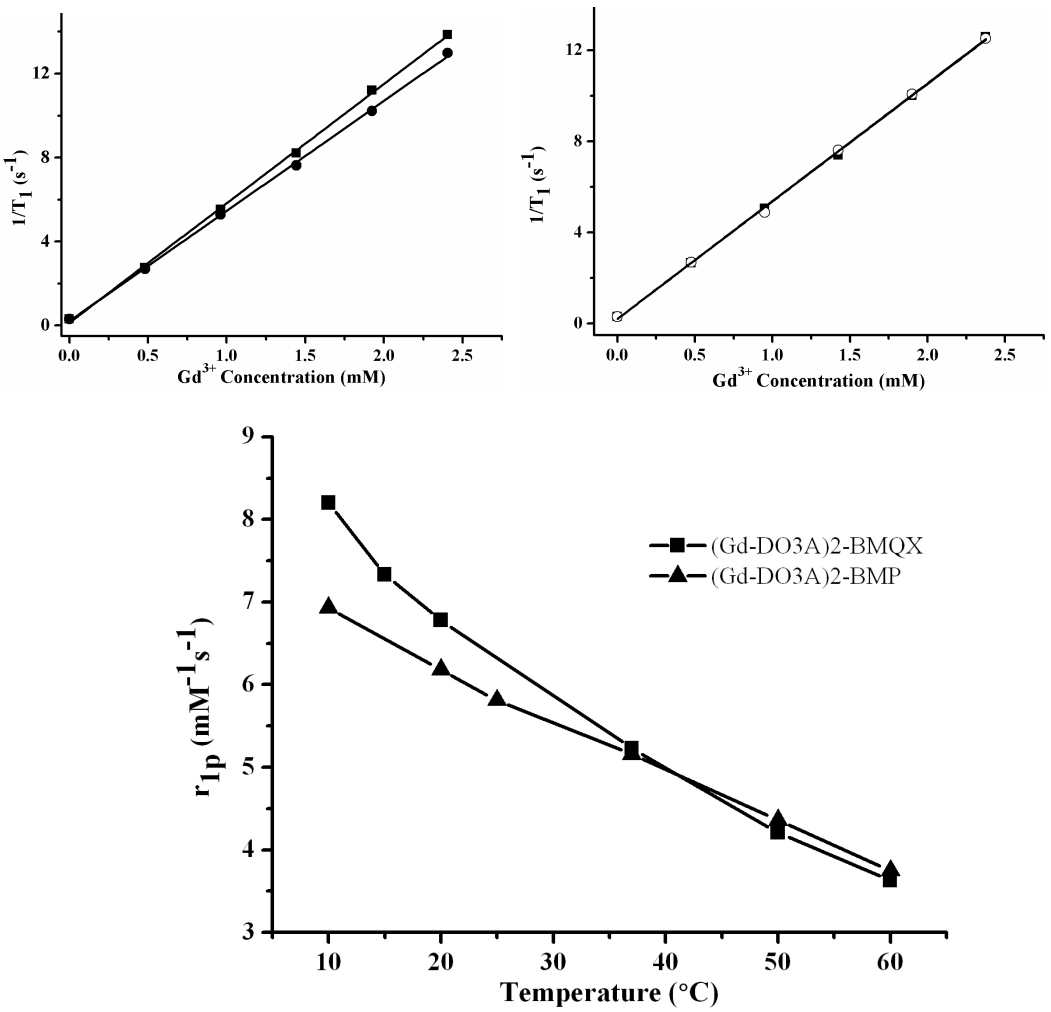


Figure S6. (top, left) The R_1 relaxation time of the H_2O as a function of $(Gd\text{-}DO3A)_2$-BMQX in the absence (●) and presence (■) of 10 equiv. of La^{3+} at 37 °C and 20 MHz. (top, right) The R_1 relaxation time of the H_2O as a function of $(Gd\text{-}DO3A)_2$-BMP in the absence (○) and presence (■) of 10 equiv. of La^{3+} at 37 °C and 20 MHz. The slopes of the lines represent the relaxivity of the contrast agents in a unit of $mMGd^{-1}s^{-1}$. The results show that the mutual influence of electronic relaxation exists in $(Gd\text{-}DO3A)_2$-BMQX but not in $(Gd\text{-}DO3A)_2$-BMP. This difference is due to the closer Gd-Gd distance in $(Gd\text{-}DO3A)_2$-BMQX than that in $(Gd\text{-}DO3A)_2$-BMP. (bottom) Temperature dependence of the relaxivity of $(Gd\text{-}DO3A)_2$-BMQX (■) and $(Gd\text{-}DO3A)_2$-BMP (▲) at pH=7.0 and 20 MHz.

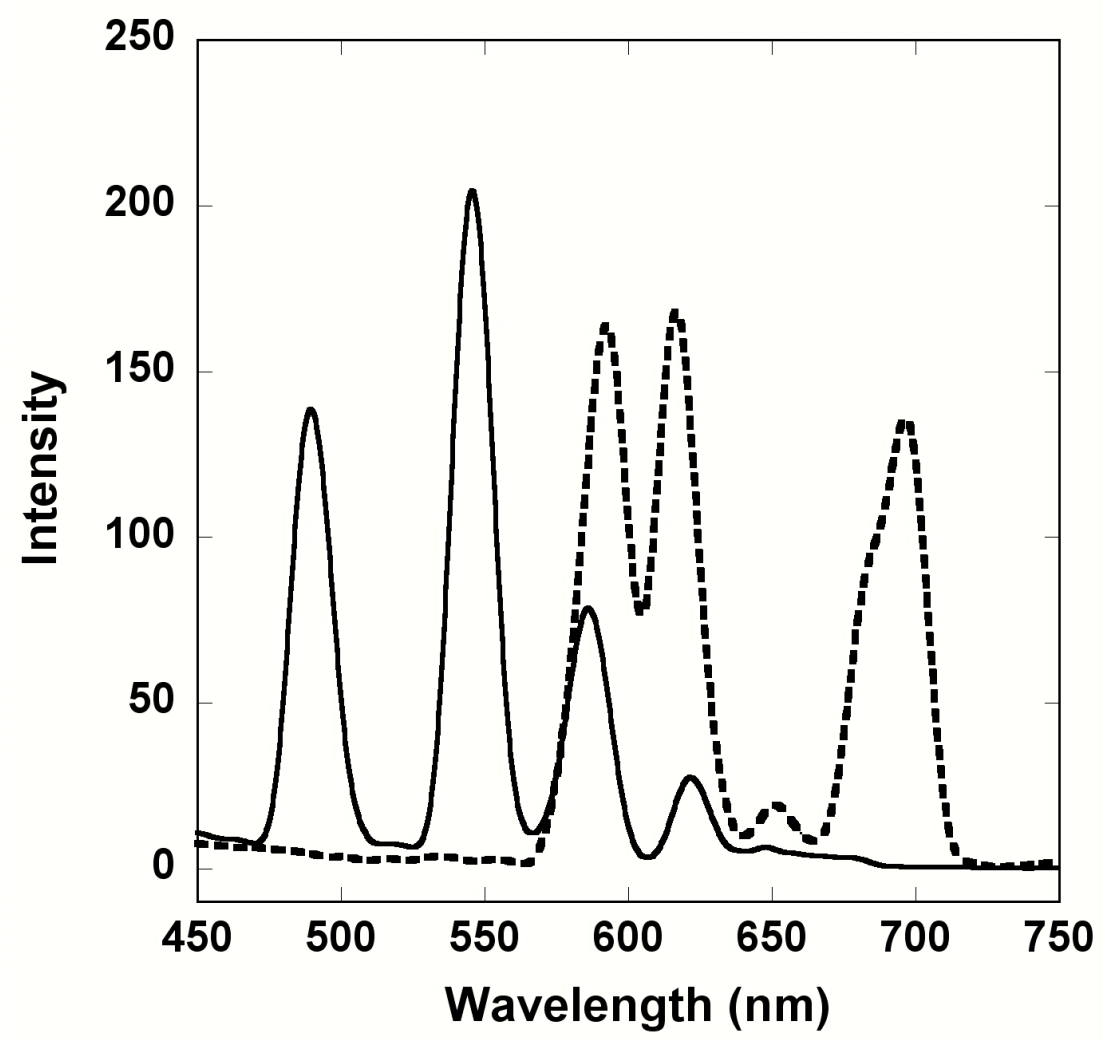


Figure S7. The emission luminescence spectra of $(Tb\text{-}DO3A)_2$-BMP (solid) and $(Eu\text{-}DO3A)_2$-BMQX (dashed). The excitation wavelengths were 290 nm for $(Tb\text{-}DO3A)_2$-BMP and 340 nm for $(Eu\text{-}DO3A)_2$-BMQX. $(Tb\text{-}DO3A)_2$-BMP possessed typical Tb^{3+} luminescence spectra, which showed two intense peaks at 489.5 and 545.5 nm originating from the $^5D_4 \rightarrow {}^7F_6$ and $^5D_4 \rightarrow {}^7F_5$ transitions and two weaker bands at 586 and 622 nm rooting in the $^5D_4 \rightarrow {}^7F_4$ and $^5D_4 \rightarrow {}^7F_3$ transitions, respectively. $(Eu\text{-}DO3A)_2$-BMQX possessed typical Eu^{3+} emission spectra, which showed intense peaks at 592, 616.5, and 696.5 nm corresponding to the $^5D_0 \rightarrow {}^7F_1$, $^5D_0 \rightarrow {}^7F_2$ and $^5D_0 \rightarrow {}^7F_4$ transitions and one weaker band at 651 nm corresponding to the $^5D_0 \rightarrow {}^7F_3$ transitions, respectively.

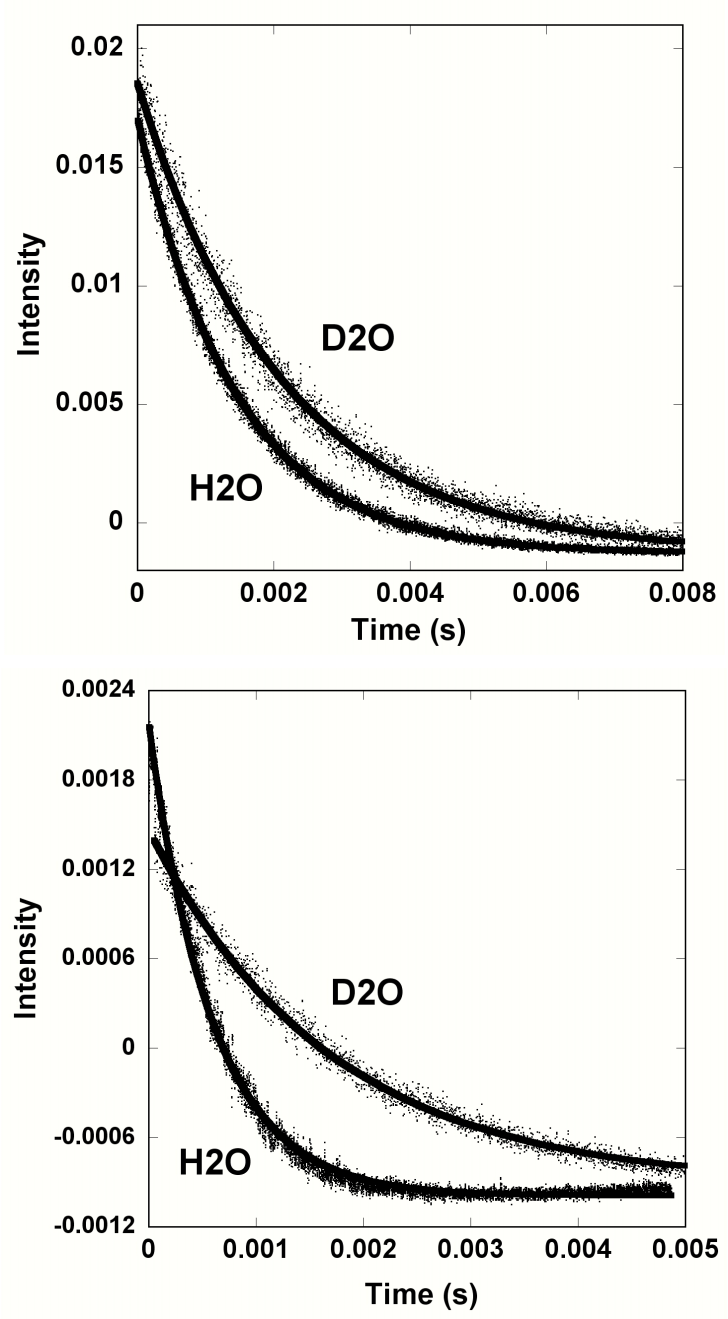


Figure S8. Luminescence decay of (Tb-DO3A)$_2$-BMP (top) and (Eu-DO3A)$_2$-BMQX (bottom) in H_2O and D_2O are shown. The solid lines were generated by fitting the data with an exponential equation. The resulted luminescence lifetimes of (Tb-DO3A)$_2$-BMP were 1.44 and 2.12 ms in H_2O and D_2O solutions, respectively, which correlated to a hydration number of 0.81 for Tb^{3+}. The resulted luminescence lifetimes of (Eu-DO3A)$_2$-BMQX were 0.598 and 1.68 ms in H_2O and D_2O solutions, respectively, which correlated to a hydration number of 0.99 for Eu^{3+}.