

Electronic Supplementary Information for
Ratiometric ^{19}F MR-based Method for Quantification of Ca^{2+} Using
Responsive Paramagnetic Probes

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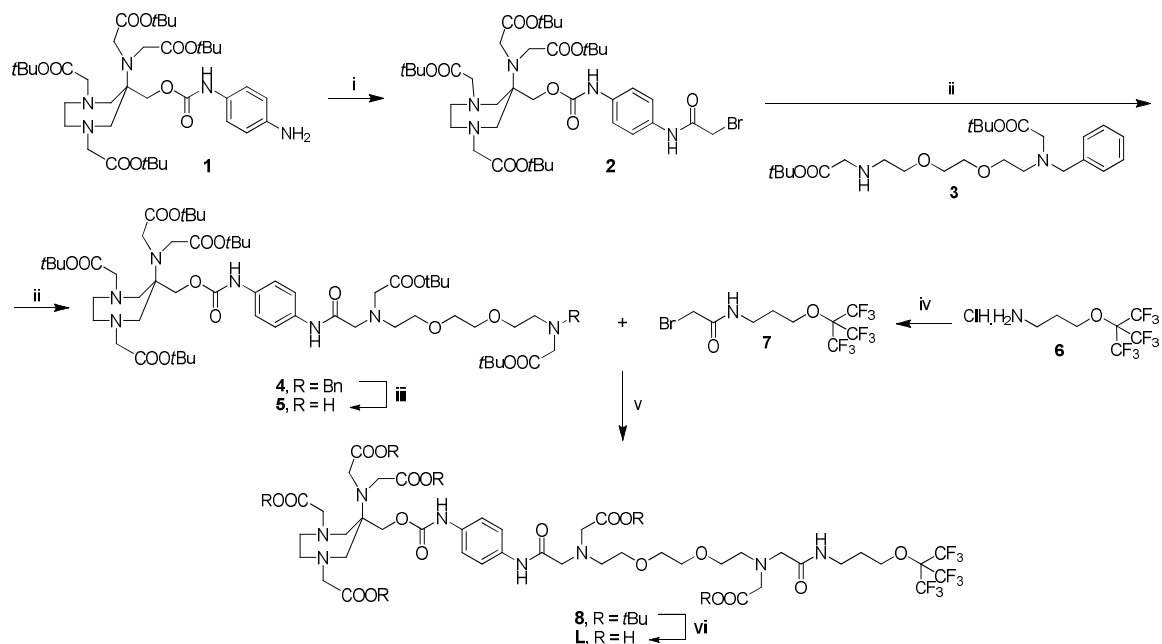
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General remarks

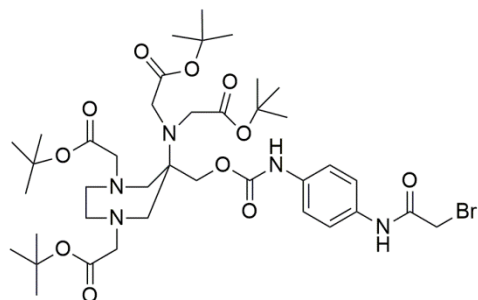
Commercially available reagents and solvents were used without further purification. Purification by column chromatography was performed with silica gel 60 (0.03–0.2 mm) from Carl Roth (Germany). ^1H , ^{13}C and ^{19}F NMR spectra were recorded on a Bruker Avance III 300 MHz spectrometer at 25 °C. Processing was performed using TopSpin 2.1 (Bruker GmbH) and ACD/SpecManager 9.0 (Advanced Chemistry Development, Inc.). The NMR spectra were obtained either in CDCl_3 or D_2O , using the deuterium lock frequency. High resolution mass spectra were recorded on a Bruker Daltonics APEX II (FT-ICR-MS) with an electrospray ionization source. Reversed-phase HPLC purification was performed on a Varian PrepStar Instrument (Australia) with PrepStar SD-1 pump heads. Analytical reversed-phase HPLC was performed with an Atlantis C18 column (4.6 x 150 mm, 5 μm particle size). Semipreparative reversed-phase HPLC was conducted with a Polaris 5 C18-A column (250 x 21.2 mm). Elution conditions are described in Table S1. Compounds **1**, **3** and **6** were prepared as previously reported.¹⁻³ The concentration of Dy^{3+} in analysed solutions was determined using the bulk magnetic susceptibility shift method,⁴ while the concentration of Y^{3+} was determined by ^{19}F NMR titration with Ca^{2+} , following the variation of the signal until saturation occurred.



Scheme S1. Preparation of L. i) BrAcOH, DCC, CH_2Cl_2 , rt, 81%; ii) NaHCO_3 in MeCN, 55°C, 73%; iii) H_2 , $\text{Pd}(\text{OH})_2/\text{C}$ 10% in EtOH, 98%; iv) 2-bromoacetyl bromide, NaHCO_3 sat., CH_2Cl_2 , 62%; v) NaHCO_3 , MeCN, 55°C, 63%; vi) formic acid, 55°C, 50%.

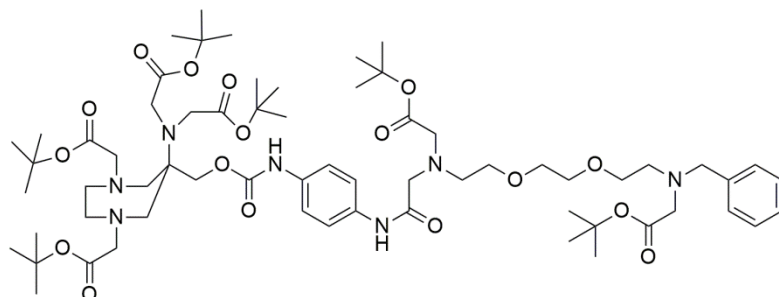
Synthetic procedures for preparation of ligands and complexes

Synthesis of di-tert-butyl 2,2'-(6-(bis(2-(tert-butoxy)-2-oxoethyl)amino)-6-((((4-(2-bromoacetamido)phenyl)carbamoyl)oxy)methyl)-1,4-diazepane-1,4-diyl)diacetate (2)



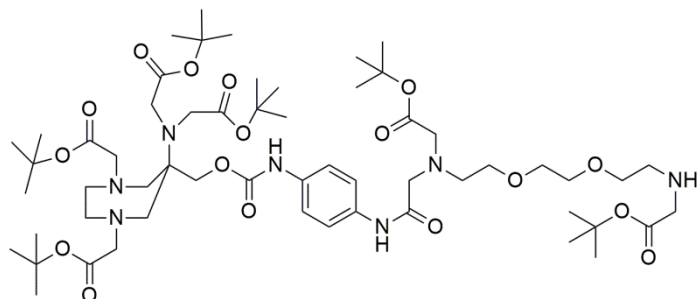
Compound **1** (3.80 g, 5.17 mmol) was dissolved in CH_2Cl_2 (50 mL) in a 100 mL round bottom flask. Afterwards, DCC (3.59 g, 25.8 mmol) and bromoacetic acid (5.33 g, 25.8 mmol) were added and the reaction mixture was stirred overnight at room temperature. Then, the formed insoluble salts were removed by filtration on paper and the solvent was removed *in vacuo*. The crude was purified by column chromatography (SiO_2 , gradient 1-4 % MeOH in CH_2Cl_2) obtaining the pure product as a yellow thick oil (3.59 g, 81 % yield). ^1H NMR (300 MHz, CDCl_3): δ 1.44 (s, 36H, CH_3), 2.69-2.81 (b, 6H, CH_2N), 3.08-3.13 (d, 2H, CH_2N), 3.28 (s, 4H, CH_2CO), 3.73 (s, 4H, CH_2CO), 4.03 (s, 2H, CH_2OH), 4.22 (s, 2H, CH_2Br), 7.37-7.40 (d, 2H, CH_{Ar}), 7.50-7.53 (d, 2H, CH_{Ar}); ^{13}C NMR (75 MHz, CDCl_3): δ 28.1 (CH_3), 33.9 (CH_2Br), 51.4, 62.2 (CH_2CO), 53.5, 58.1, 58.9 (CH_2N) 63.2 (C_q), 67.5 (CH_2OH), 80.7-80.9 (C_{qtBu}), 119.0, 120.9 (CH_{Ar}), 132.7, 135.1 (C_{Ar}), 153.5 (OCONH), 157.3 (NHCO), 170.8, 172.8 (COO); HRMS: m/z calculated for $\text{C}_{39}\text{H}_{63}\text{BrN}_5\text{O}_{11}^+$ ($[\text{M}+\text{H}]^+$): 856.3702, found: 856.3685.

Synthesis of di-tert-butyl 3-benzyl-12-(2-((4-(((6-(bis(2-(tert-butoxy)-2-oxoethyl)amino)-1,4-bis(2-(tert-butoxy)-2-oxoethyl)-1,4-diazepan-6-yl)methoxy)carbonyl)amino)phenyl)amino)-2-oxoethyl)-6,9-dioxa-3,12-diazatetradecane-1,14-dioate (4)



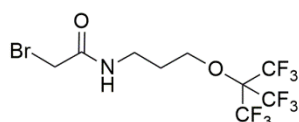
Amine **3** (1.24 g, 2.65 mmol) was dissolved in acetonitrile (50 mL) in a 100 mL round bottom flask and sodium bicarbonate (0.67 g, 7.96 mmol) was then suspended. Thereafter, compound **2** (2.5 g, 2.92 mmol) was dissolved in acetonitrile (20 mL) and added slowly to the suspension. The reaction mixture was then stirred overnight at 55 °C. The inorganic salts were removed by filtration on paper and the solvent was removed *in vacuo*. The crude product was then purified by column chromatography (SiO₂, gradient 1-5 % MeOH in CH₂Cl₂) providing **4** a light brown thick oil (2.42 g, 73 % yield). ¹H NMR (300 MHz, CDCl₃): δ 1.36-1.37 (s, 54, CH₃), 2.58-2.61 (b, 2H, CH₂N), 2.69-2.85(b, 6H, CH₂N), 3.02-3.06 (d, 2H, CH₂N), 3.20 (s, 6H, CH₂CO), 3.32 (s, 2H, CH₂O), 3.39 (s, 2H, CH₂O), 3.45-3.48 (s, 8H, CH₂CO), 3.66 (s, 4H, CH₂O), 3.74-3.75 (d, 2H, CH₂Ph), 4.16 (s, 2H, CH₂OH) 7.13-7.31 (d, 7H, CH_{Ar}), 7.45-7.52 (d, 2H, CH_{Ar}); ¹³C NMR (75 MHz, CDCl₃): δ 26.9-27.0 (CH₃), 50.3 (CH₂CO), 51.8, 61.0 (CH₂CO), 52.3, 53.6, 54.5 55.5, 56.1, 57.4, 57.7, 58.8 (CH₂N) 66.2 (C_q), 67.8 (CH₂OH), 69.01-69.17 (CH₂O), 79.4-79.6, 80.4 (C_{qtBu}), 117.8, 119.0, 119.3, 125.8, 127.0, 127.7 (CH_{Ar}), 132.4, 133.0, 138.0 (C_{Ar}), 152.2 (OCONH), 168.4 (NHCO), 169.5, 169.7, 171.5 (COO); HRMS: m/z calculated for C₆₄H₁₀₅N₇O₁₇²⁺ ([M+2H]²⁺): 621.8778, found: 621.8776.

Synthesis of di-tert-butyl 3-(2-((4-(((6-(bis(2-(tert-butoxy)-2-oxoethyl)amino)-1,4-bis(2-(tert-butoxy)-2-oxoethyl)-1,4-diazepan-6-yl)methoxy)carbonyl)amino)phenyl)amino)-2-oxoethyl)-6,9-dioxa-3,12-diazatetradecane-1,14-dioate (5)



Compound **4** (2.42 g, 1.95 mmol) was dissolved in ethanol (30 mL) in a reinforced glass vial round bottom flask. To this solution Pd(OH)₂ on carbon (10 % w/w) was added and the hydrogenation was carried out on a Parr apparatus (H₂ pressure = 3 bar c.ca) for 6 hours at room temperature. The catalyst was removed by filtration on celite and the solvent was evaporated *in vacuo*. The product **5** was obtained as a light brown thick oil and was used in next step without further purification (2.20 g, 98 % yield). ¹H NMR (300 MHz, CDCl₃): δ 1.29-1.30 (s, 54, CH₃), 2.51-2.54 (b, 2H, CH₂N), 2.66-2.80(b, 6H, CH₂N), 2.94-2.99 (d, 2H, CH₂N), 3.13 (s, 6H, CH₂CO), 3.28-3.30 (s, 4H, CH₂O), 3.43 (s, 10H, CH₂CO), 3.58 (s, 4H, CH₂O), 4.07 (s, 2H, CH₂OH) 7.23-7.26 (d, 2H, CH_{Ar}), 7.46-7.43 (d, 2H, CH_{Ar}); ¹³C NMR (75 MHz, CDCl₃): δ 28.0 (CH₃), 48.4 (CH₂N), 51.2, 54.7, 57.2, 58.8, 59.8, 63.1 (CH₂), 62.0 (CH₂COO) 67.2 (C_q), 69.9 (CH₂OH), 70.2 (CH₂O), 80.4, 80.6, 80.9, 81.4 (C_{qtBu}), 116.8, 120.7 (CH_{Ar}), 133.4, 134.3 (C_{Ar}), 153.4 (OCONH), 169.4 (NHCO), 170.5, 170.7, 171.0, 172.6 (COO); HRMS: m/z calculated for C₅₇H₉₉N₇O₁₇²⁺ ([M+2H]²⁺): 576.8543, found: 576.8542.

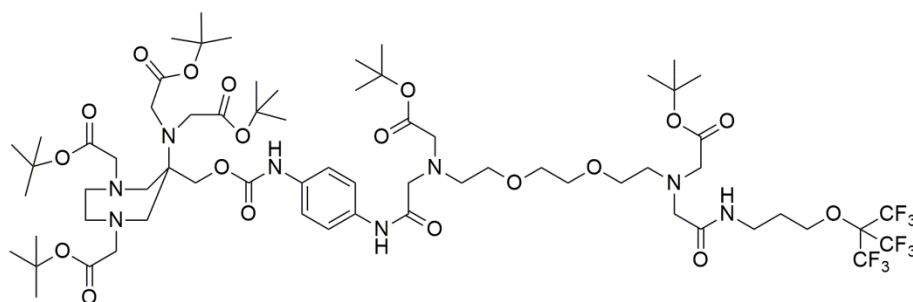
Synthesis of 2-bromo-N-(3-((1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)propyl)acetamide (7)



Amino salt **6** (2.50 g, 7.59 mmol) was dissolved in CH₂Cl₂ (20 mL) in a 100 mL round bottom flask. An aqueous solution of sat. NaHCO₃ (30 mL) was poured in the flask and the

biphasic mixture was vigorously stirred. While the flask was kept in an ice bath at c.ca 0 °C, 2-bromoactyl bromide (how much?) was dissolved in CH₂Cl₂ (15 mL) and slowly added dropwise to the mixture. The reaction was allowed to reach room temperature and left stirring overnight. The organic phase was then recovered from the biphasic solution with a separatory funnel. The water phase was extracted with CH₂Cl₂ (3x20 mL) and the combined organic phases were washed with sat. NaHCO₃ (2x20 mL) and brine (2x20 mL). The organic phase was finally treated with anh. Na₂SO₄, the salts were removed by filtration on paper and the solvent evaporated *in vacuo*. The pure product was obtained as transparent oil that turned in a white wax-like solid once reached room temperature (1.94 g, 62 % yield). ¹H NMR (300 MHz, CDCl₃): δ 1.95-2.04 (q, J = 0.02 ppm, 2H, CH₂), 3.40-3.46 (q, J = 0.02 ppm, 2H, CH₂N), 3.89 (s, 2H, CH₂Br), 4.11-4.15 (t, J = 0.02 ppm, 2H, CH₂O); ¹³C NMR (75 MHz, CDCl₃): δ 28.3 (CH₂), 29.1 (CH₂Br), 36.6 (CH₂N), 67.4 (CH₂O), 78.6-80.6 (C_q), 114.3, 118.3, 122.1, 126.0 (CF₃), 166.9 (COO); ¹⁹F NMR (282 MHz, CDCl₃): δ -71.1 (CF₃); HRMS: m/z calculated for C₉H₉BrF₉NO₂Na⁺ ([M+Na]⁺): 435.9565, found: 435.9557.

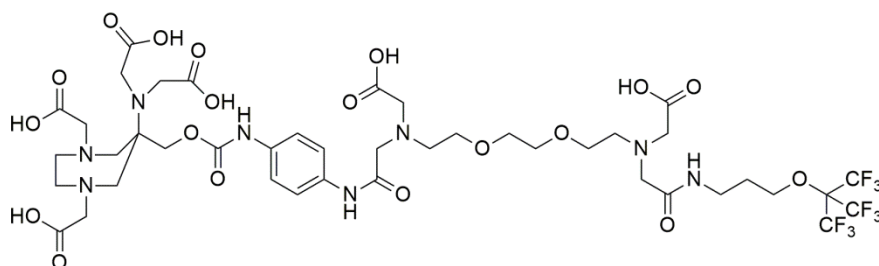
Synthesis of di-tert-butyl 3-(2-((4-(((6-(bis(2-(tert-butoxy)-2-oxoethyl)amino)-1,4-bis(2-(tert-butoxy)-2-oxoethyl)-1,4-diazepan-6-yl)methoxy)carbonyl)amino)phenyl)amino)-2-oxoethyl)-12-(2-((3-((1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)propyl)amino)-2-oxoethyl)-6,9-dioxa-3,12-diazatetradecane-1,14-dioate (8)



Compound **5** (2.20 g, 1.91 mmol) was dissolved in acetonitrile (50 mL) in a 100 mL round bottom flask. Sodium bicarbonate (0.48 g, 5.73 mmol) and **7** (1.19 g, 2.87 mmol) were added to the solution and the reaction mixture was stirred overnight at 55 °C. The inorganic salts were removed by filtration on paper and the solvent was evaporated *in vacuo*. The crude product was then purified by column chromatography (SiO₂, gradient 1-5 % MeOH in CH₂Cl₂) obtaining the pure product as a light brown thick oil (1.8 g, 63 % yield). ¹H NMR (300 MHz, CDCl₃): δ 1.25-1.29 (s, 54, CH₃), 1.75 (b, 2H, CH₂), 2.51 (b, 2H, CH₂N), 2.64-

2.76 (b, 6H, CH₂N), 2.95 (d, 2H, CH₂N), 3.10-3.13 (s, 10H, CH₂N), 3.27 (s, 6H, CH₂O/CH₂CO), 3.39 (s, 8H, CH₂O/CH₂CO), 3.55 (s, 4H, CH₂O), 3.91 (b, 2H, CH₂OC(CF₃)₃), 4.05 (s, 2H, CH₂OH) 7.23-7.41 (d, 4H, CH_{Ar}); ¹³C NMR (75 MHz, CDCl₃): δ 27.9-27.9 (CH₃), 29.7 (CH₂), 35.1 (CH₂N), 51.4 (CH₂CO), 54.3, 54.8, (CH₂N), 57.0, 57.4, 58.7, 59.1, 59.9 (CH₂N), 62.0 (CH₂CO), 63.1, 67.6 (CH₂O), 69.0, 70.0 (CH₂O), 67.0 (C_q), 80.3, 80.5, 81.1, 81.3 (C_{qtBu}), 114.4, 118.3, 122.1, 126.0 (CF₃), 116.8, 120.7 (CH_{Ar}), 133.4, 134.3 (C_{Ar}), 153.4 (OCONH), 169.3 (NHCO), 170.4, 170.6, 171.6, 172.5 (COO); ¹⁹F NMR (282 MHz, CDCl₃): δ -70.6 (CF₃); HRMS: m/z calculated for C₆₆H₁₀₇F₉N₈O₁₉²⁺ ([M+2H]²⁺): 743.3749, found: 743.3742.

Synthesis of 3-(2-((4-(((6-(bis(carboxymethyl)amino)-1,4-bis(carboxymethyl)-1,4-diazepan-6-yl)methoxy)carbonyl)amino)phenyl)amino)-2-oxoethyl)-12-(2-((3-((1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-yl)oxy)propyl)amino)-2-oxoethyl)-6,9-dioxo-3,12-diazatetradecane-1,14-dioic acid (L)



Compound **8** (1.50 g, 1.01 mmol) was dissolved in formic acid (20 mL) in a 100 mL round bottom flask and stirred overnight at 55 °C. The hydrolyzed acid was then evaporated *in vacuo*, the residue was re-dissolved in water and pH was adjusted 7.0 with aqueous NaOH (1 M). The crude product was purified by HPLC (Method A). The fractions containing the product were concentrated and freeze-dried, obtaining **L** as a white solid (0.58 g, 50 % yield). ¹H NMR (300 MHz, D₂O): δ 1.83-1.87 (t, 2H, CH₂), 2.66-2.70 (t, 2H, CH₂N), 2.88 (b, 2H, CH₂N), 3.16-3.32 (s, 14H, CH₂N), 3.39-3.62 (s, 20H, CH₂O/CH₂CO), 4.06 (t, 2H, CH₂OC(CF₃)₃), 4.13 (s, 2H, CH₂OH) 7.35-7.38, 7.44-7.47 (d, 4H, CH_{Ar}); ¹³C NMR (75 MHz, D₂O): δ 28.6 (CH₂), 35.3 (CH₂N), 52.1 (CH₂CO), 53.4, 54.1, 54.6 (CH₂N), 58.0, 58.4, 59.0, 59.5, 60.7 (CH₂N), 62.1 (CH₂CO), 67.5 (C_q), 68.5, 68.2 (CH₂O), 69.4 (CH₂O), 118.2, 120.7, 122.1 (CF₃), 120.7 (CH_{Ar}), 132.7, 134.7 (C_{Ar}), 155.2 (OCONH), 173.4 (NHCO),

174.4, 175.4, 179.0, 179.3, 180.0 (COO); ^{19}F NMR (282 MHz, D_2O): δ -70.5 (CF_3); HRMS: m/z calculated for $\text{C}_{42}\text{H}_{57}\text{F}_9\text{N}_8\text{O}_{19}\text{Ca}^{2+}$ ($[\text{M}+\text{Ca}]^{2+}$): 594.1606, found: 594.1596.

General procedure for the preparation of LnL complex

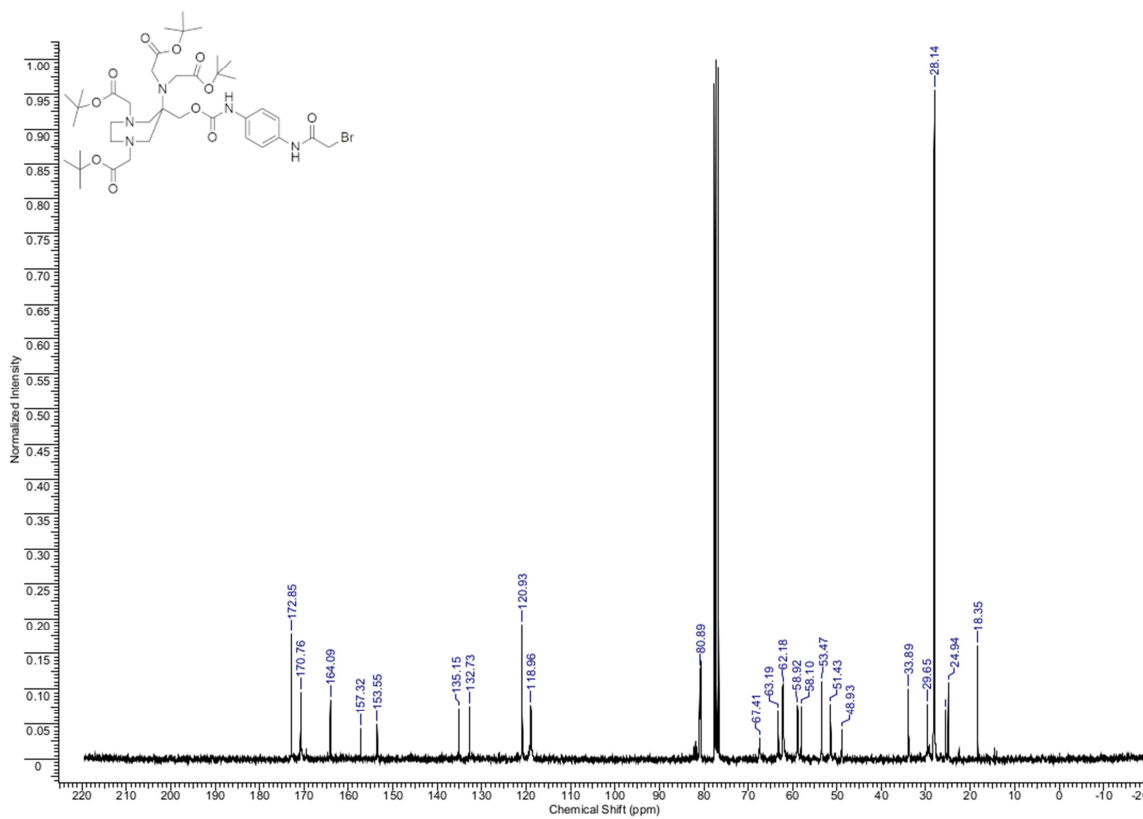
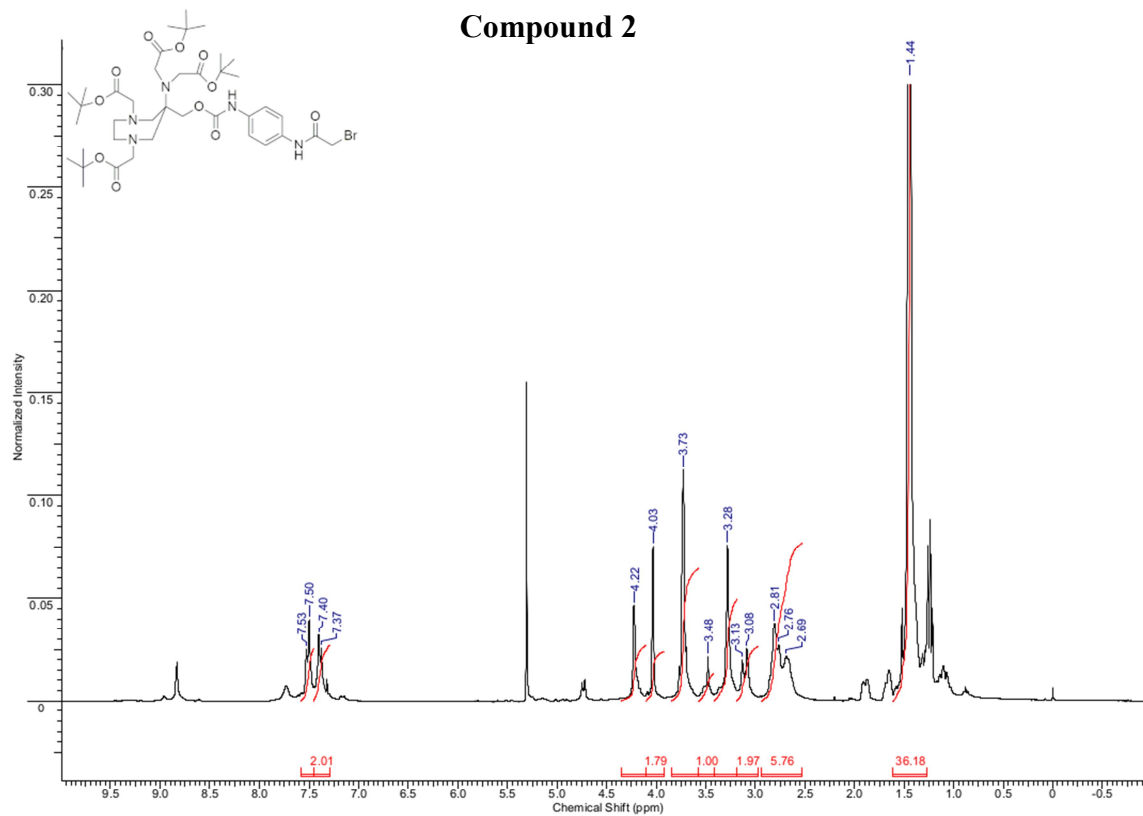
A stoichiometric amount of the LnCl_3 (Dy^{3+} , Ln^{3+}) dissolved in water was added to an aqueous solution containing the ligand **L**. The pH was adjusted to 7.4 and the solution stirred overnight at room temperature. Then, the solution was treated two times with Chelex® to remove the excess of lanthanide. Finally, the pH was adjusted again to 7.4 and the solution was freeze-dried to obtain the complex as a white solid in quantitative yield.

DyL: ^{19}F NMR (282 MHz, D_2O 10 % in H_2O): δ -72.75 (CF_3); LRMS (ESI): m/z calculated for $\text{C}_{42}\text{H}_{51}\text{DyF}_9\text{N}_8\text{Na}_2\text{O}_{19}^-$ ($[\text{M}-\text{H}]^-$): 1352.2, found 1352.3.

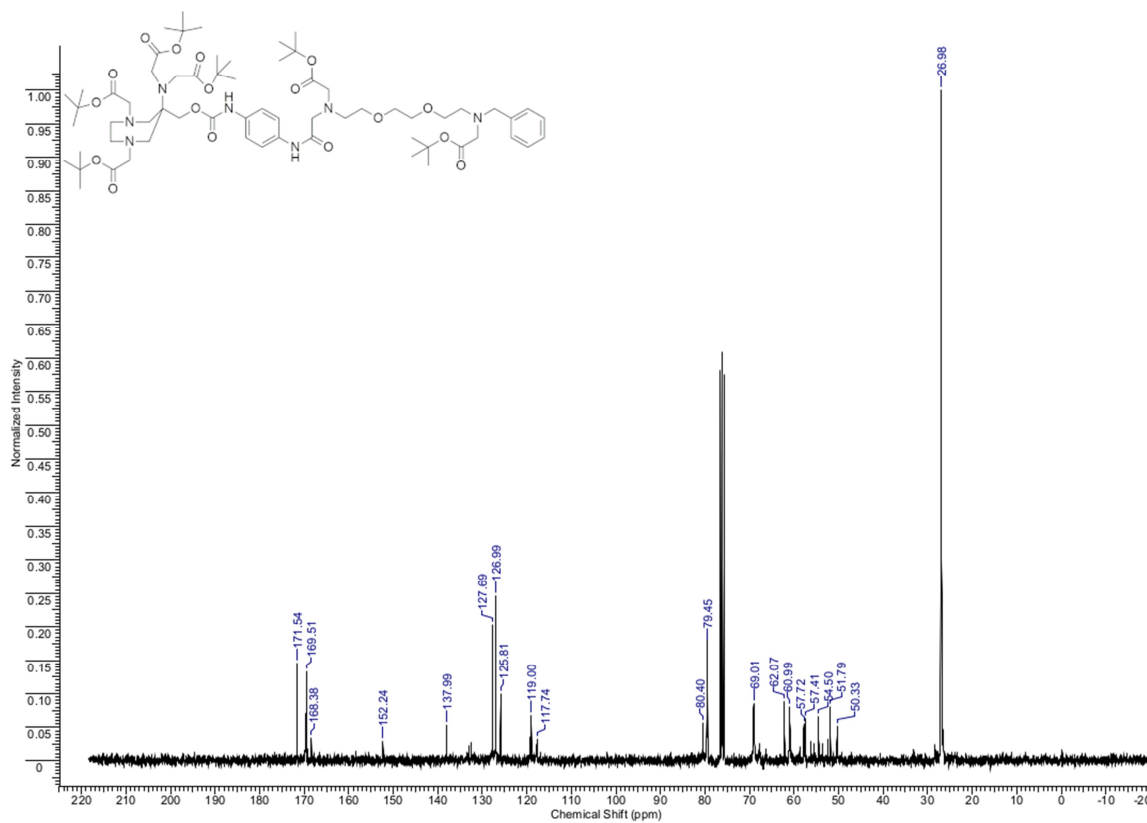
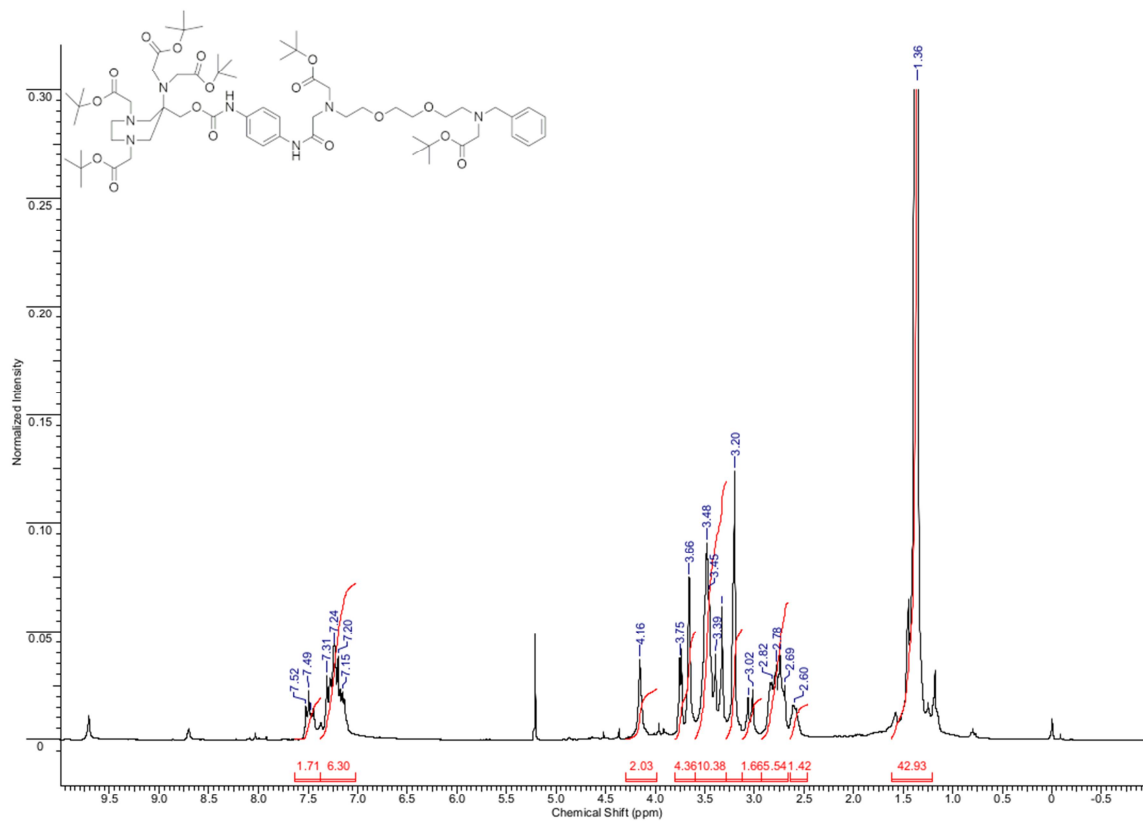
YL: ^{19}F NMR (282 MHz, D_2O 10 % in H_2O): δ -70.40 (CF_3); LRMS (ESI): m/z calculated for $\text{C}_{42}\text{H}_{51}\text{F}_9\text{N}_8\text{Na}_2\text{O}_{19}\text{Y}^-$ ($[\text{M}-\text{H}]^-$): 1277.2, found 1277.3.

^1H , ^{13}C and ^{19}F NMR spectra

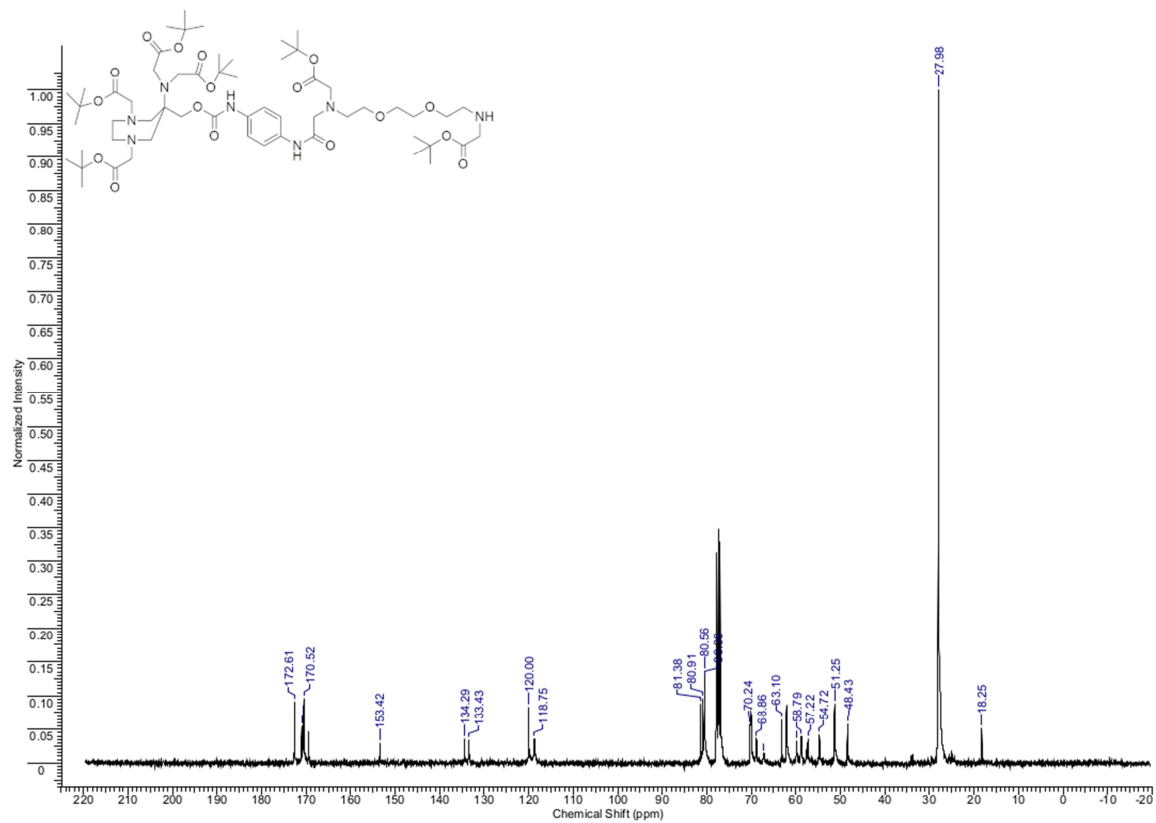
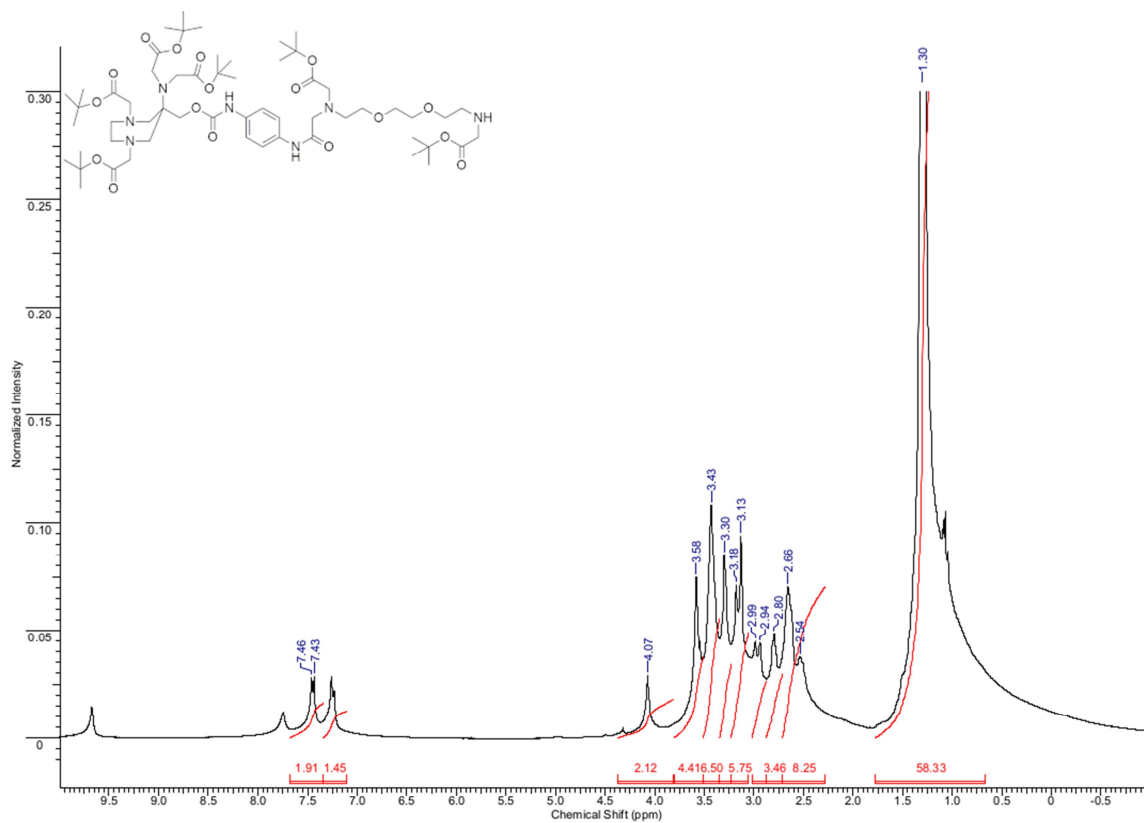
Compound 2



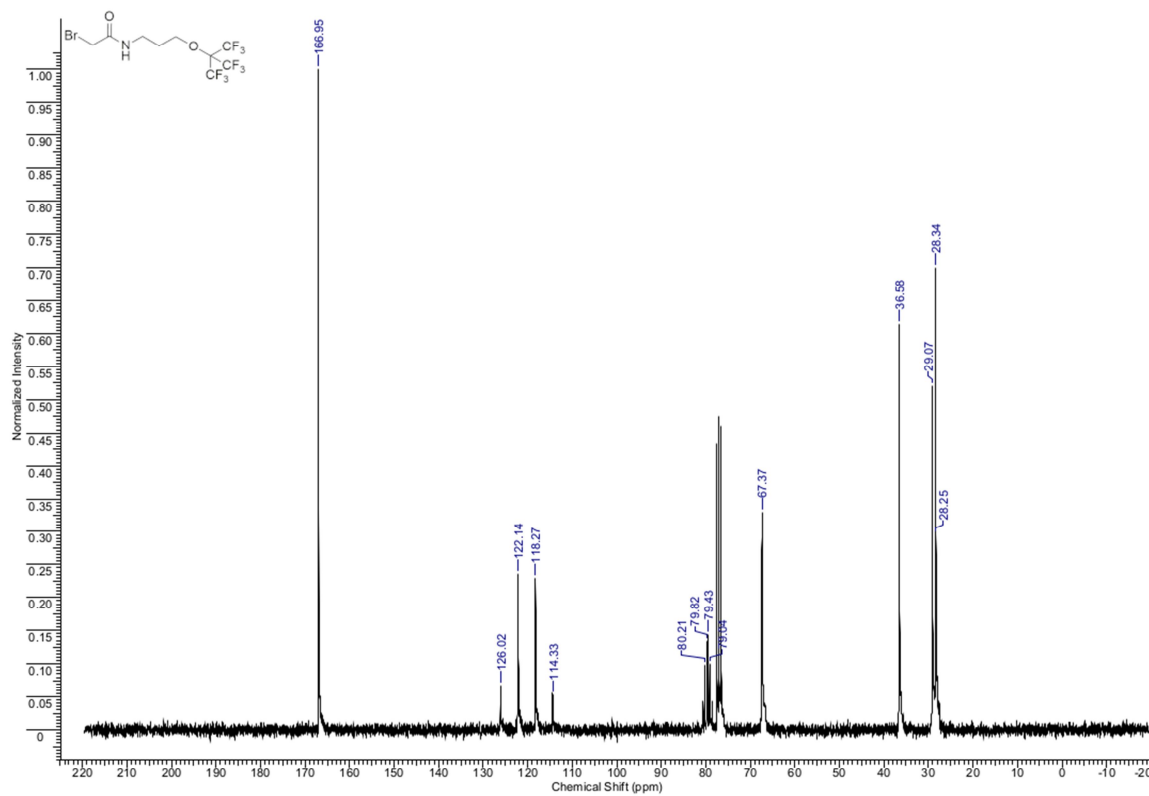
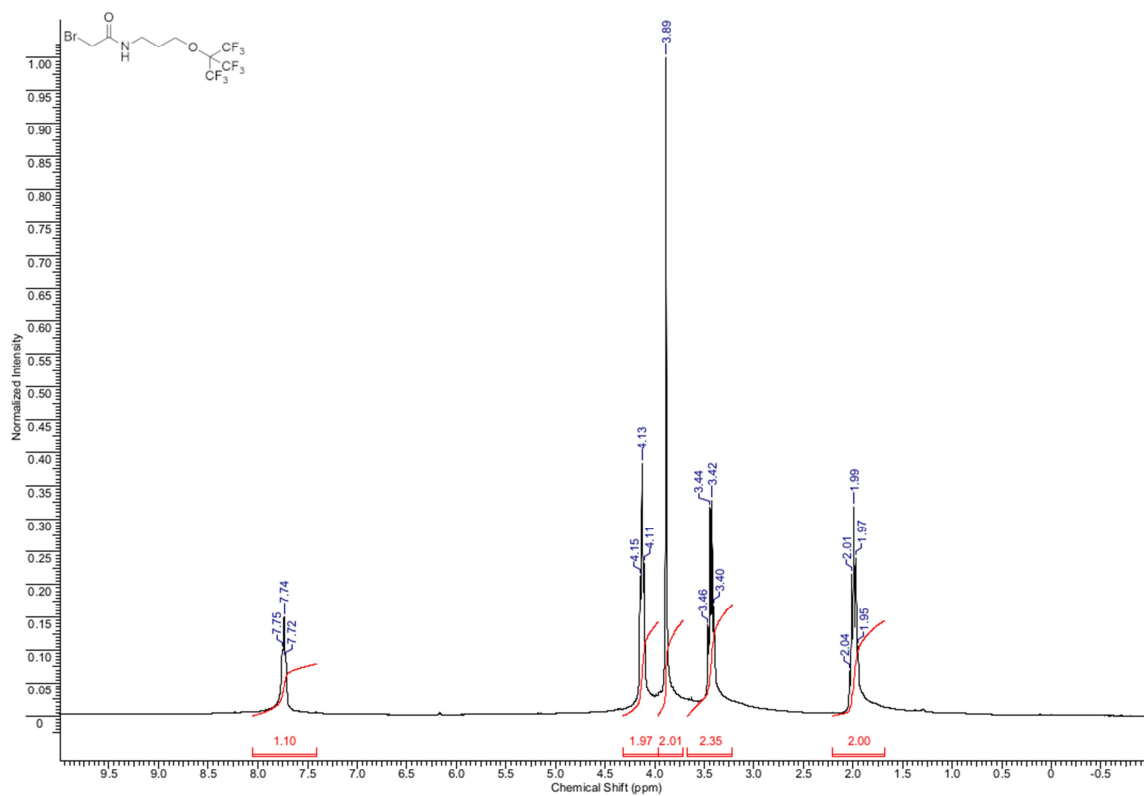
Compound 4

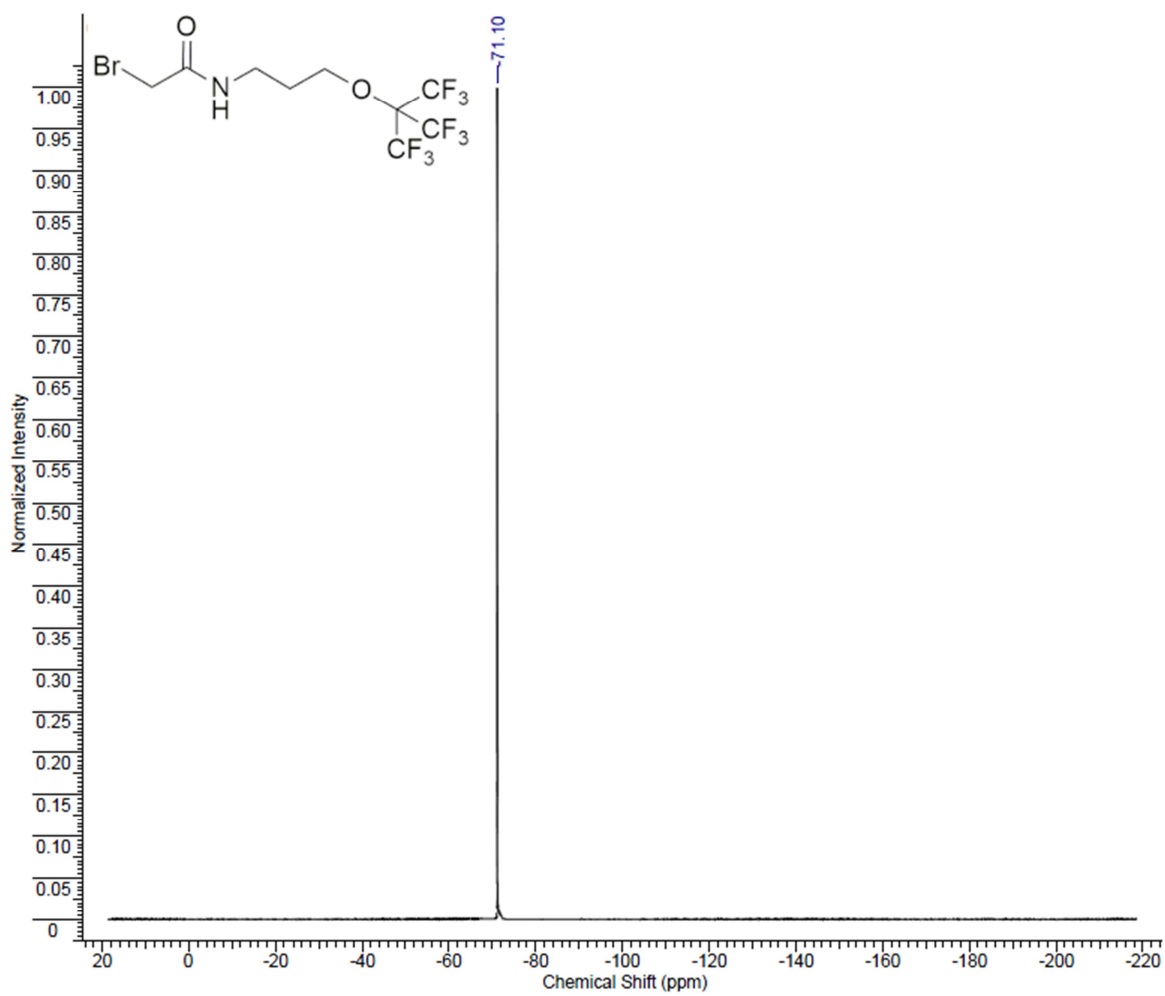


Compound 5

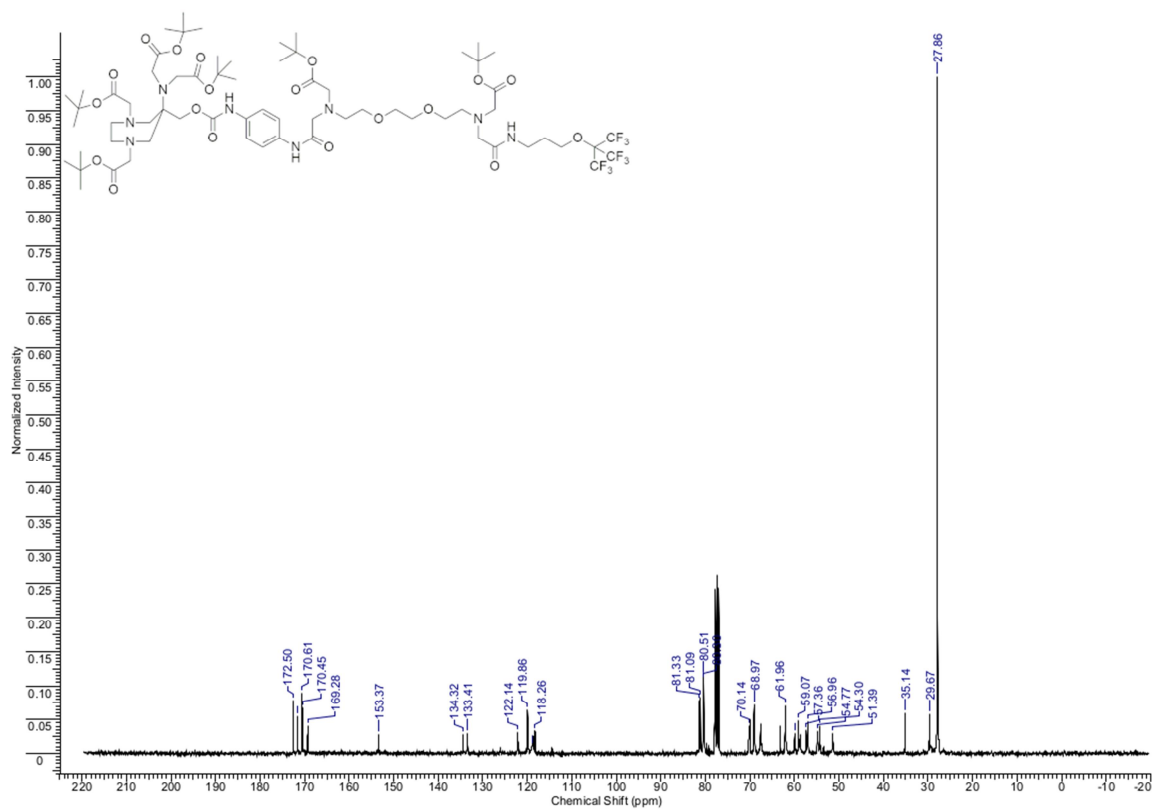
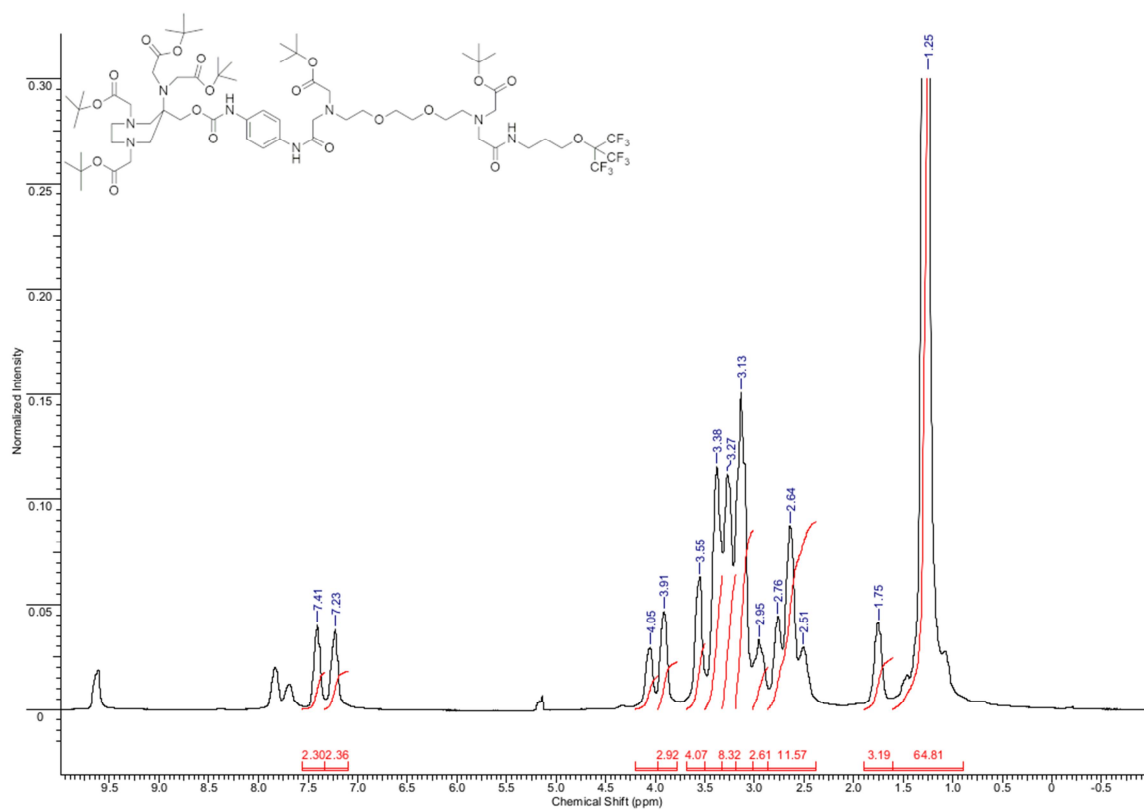


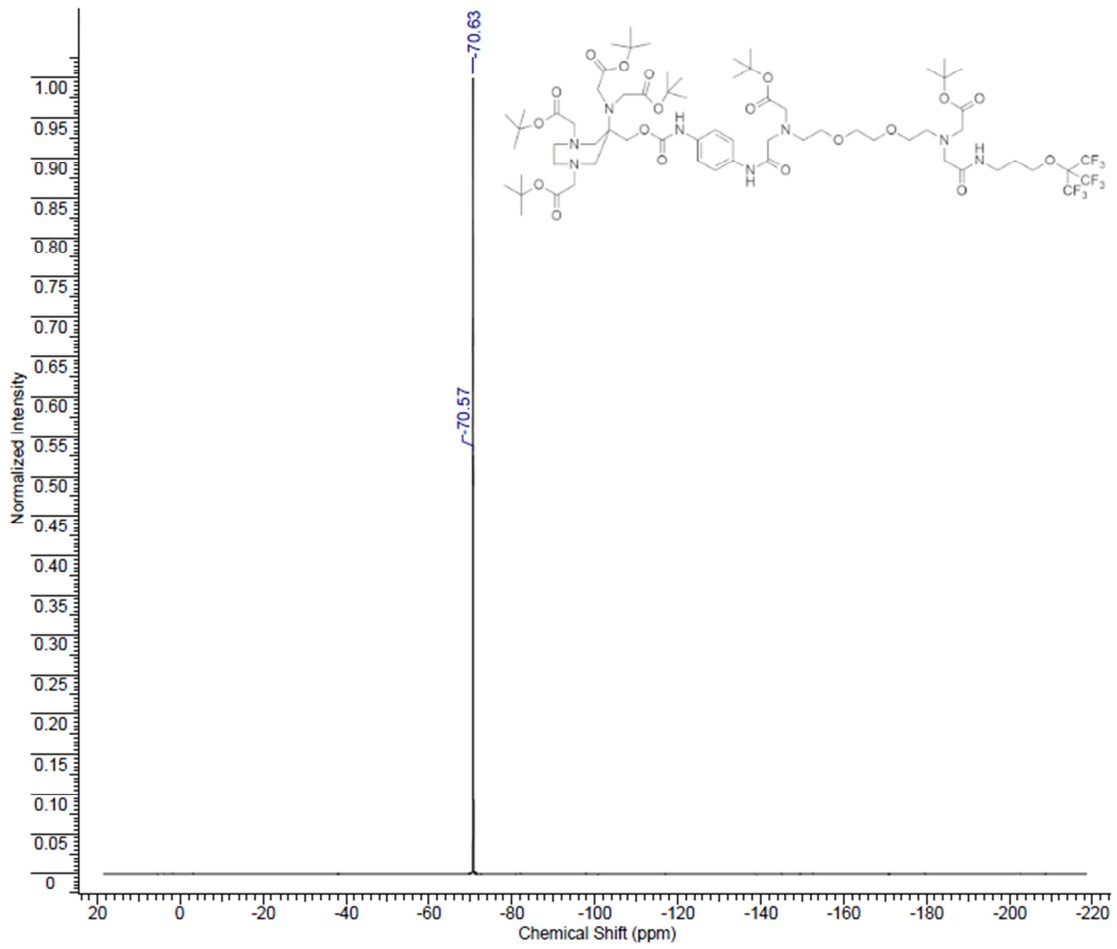
Compound 7



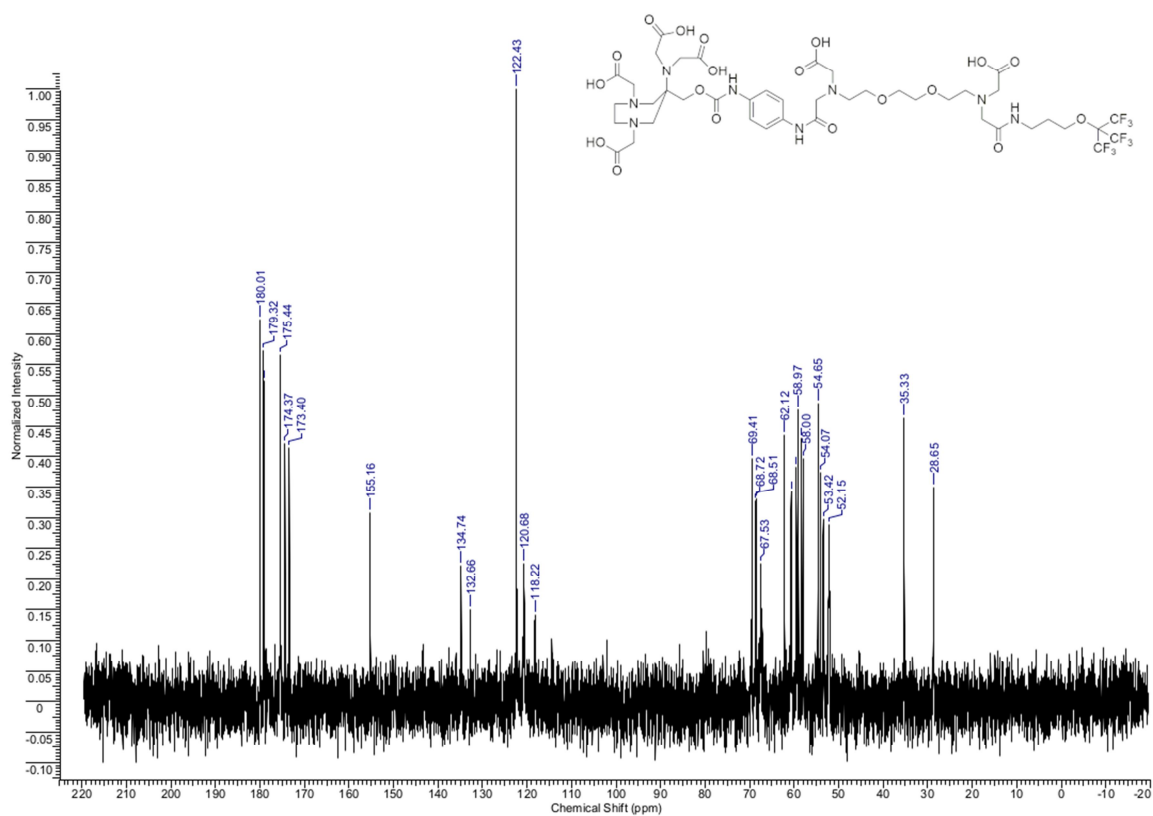
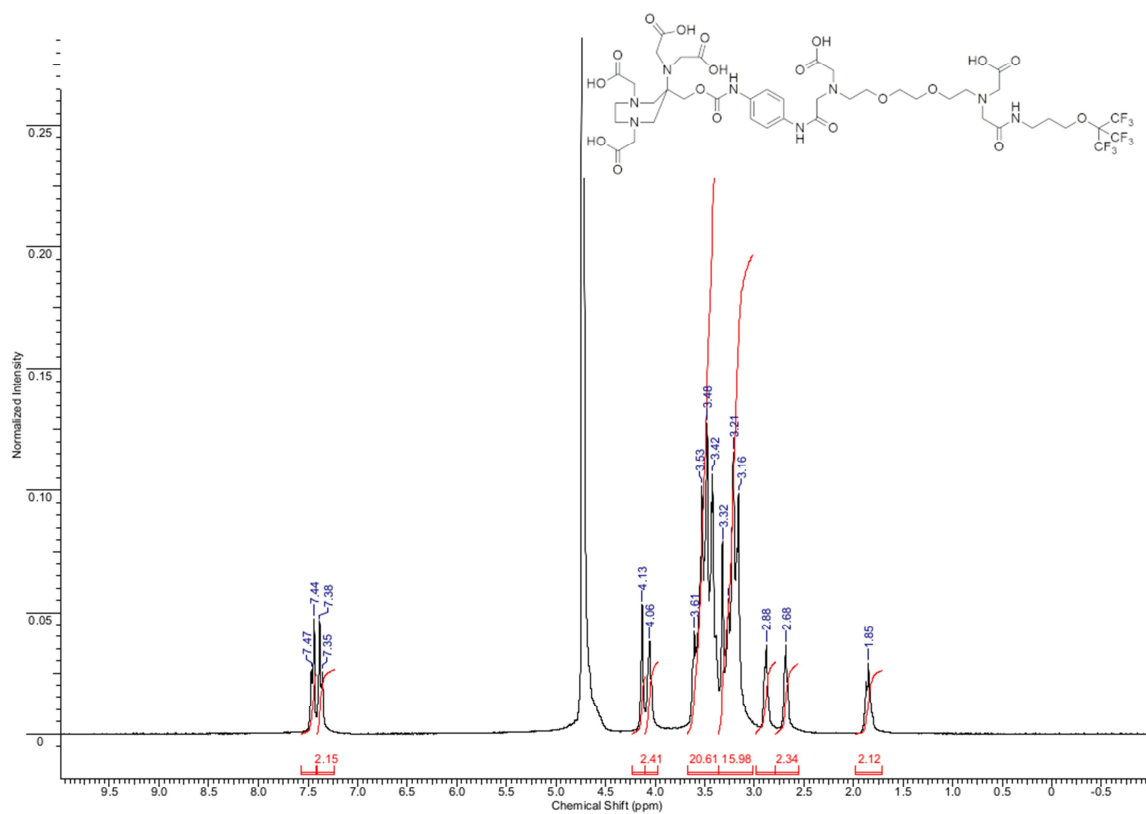


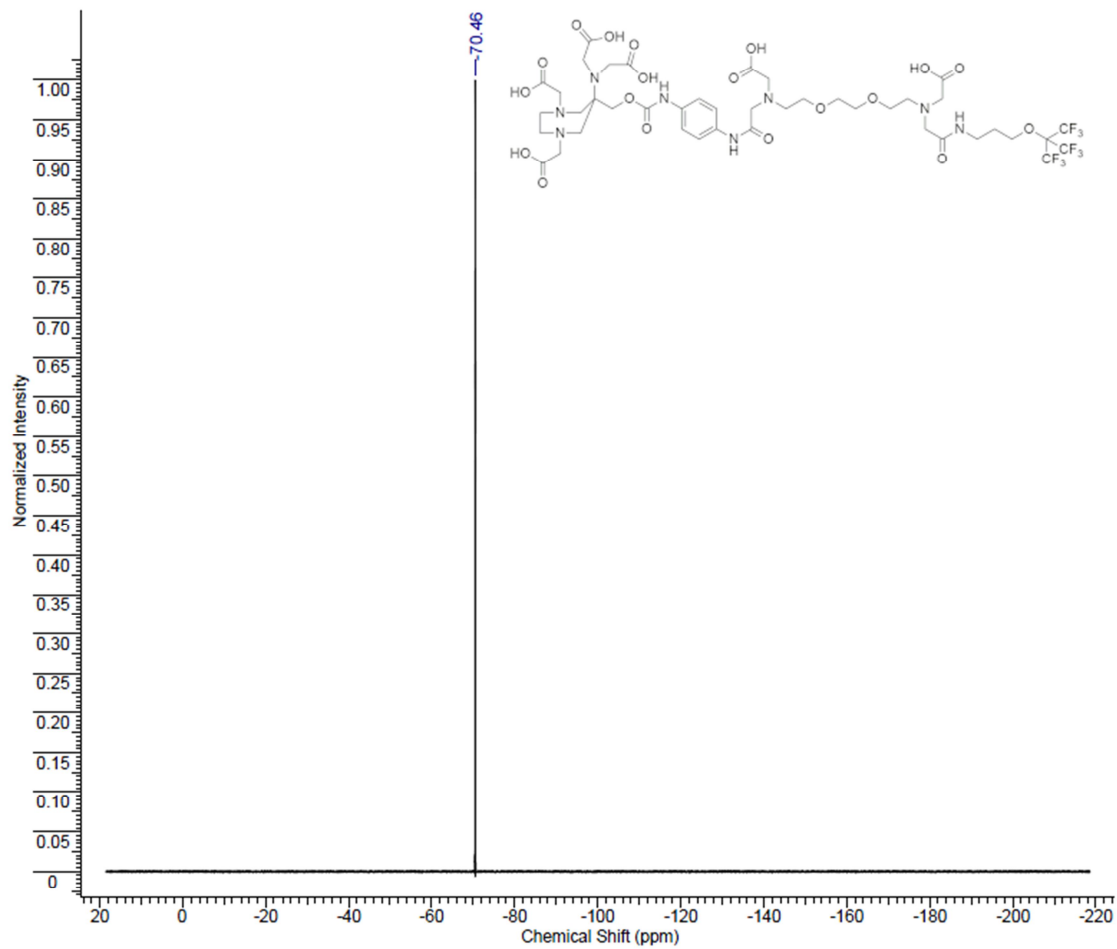
Compound 8





Compound L





HPLC purification and characterization

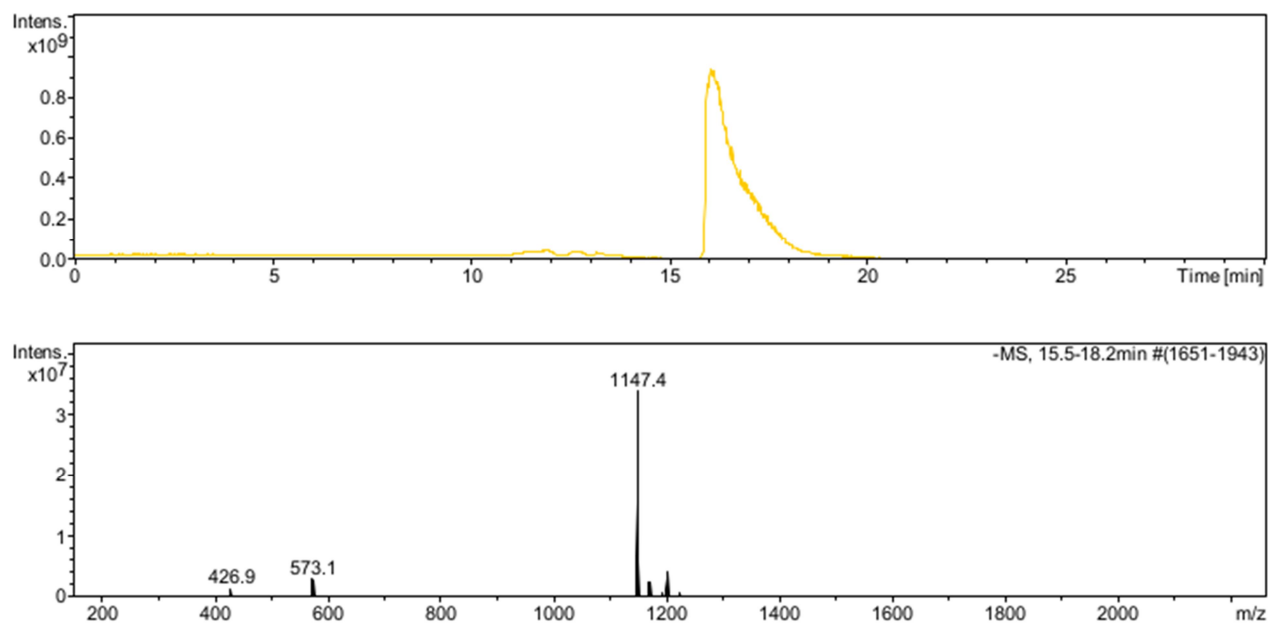


Figure S1: Analytical LC-MS ESI total ion chromatogram (TIC) for ligand **L**. The method is described in Table S1.

Table S1: Elution condition for analytical LC MS of ligand **L**. Solvent A = CH₃COOH 1 % in water, Solvent B = CH₃COOH 1% in acetonitrile. Flow rate = 1 mL/min.

Time (min)	% Solv. A	% Solv. B
0	95	5
5	95	5
20	10	90
25	0	100
29	0	100
30	95	5

Table S2: Elution condition for HPLC semi-preparative method for the purification of ligand L. Solvent A = water, Solvent B = acetonitrile. Flow rate = 10 mL/min.

Time (min)	% Solv. A	% Solv. B
0	95	5
5	95	5
20	10	90
28	0	100
33	0	100
35	95	5

^{19}F NMR Ca^{2+} titrations

All ^{19}F NMR spectra were recorded on a 7T (282 MHz) Bruker Avance III NMR spectrometer at 25 °C, with the same sequence and parameters (number of scans = 256, receiver gain = 2050).

^{19}F NMR titrations of [DyL] and [YL] with Ca^{2+} , Mg^{2+} and Zn^{2+} . Two solutions of **DyL** or **YL** with starting $[\text{Ln}^{3+}] = 3.0$ mM in HEPES buffer (pH 7.4) were titrated using a 50 mM CaCl_2 solution. A coaxial capillary, containing 100 mM NaF was inserted in the samples' tubes to be used as reference. ^{19}F NMR spectra were acquired after every CaCl_2 addition. The signal intensity for the peak of the complex was calculated by integrating the region between -72.5 and -73.0 ppm for **DyL** and between -70.0 and -70.5 ppm for **YL**. The obtained values were normalized for each measurement using the integral of the peak of the NaF (region between -120.0 and -121.0 ppm).

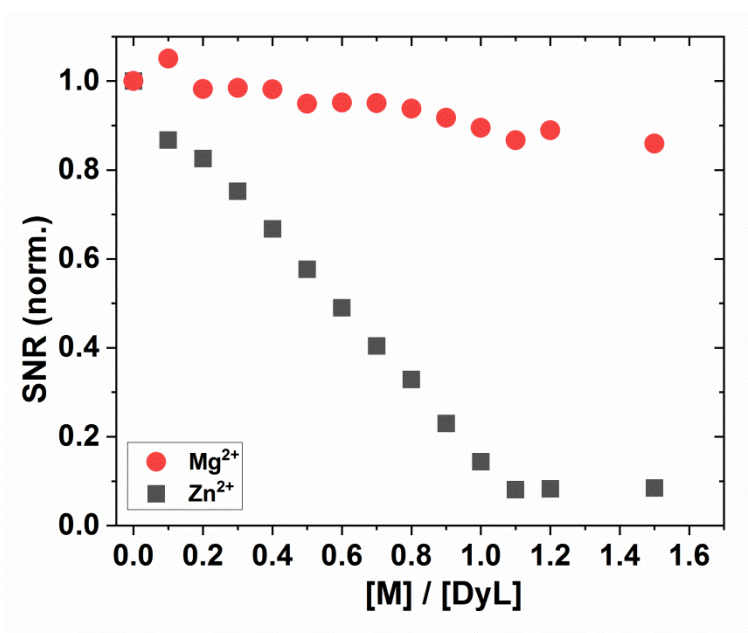


Figure S2. ^{19}F NMR titration of **DyL** (3 mM) with Mg^{2+} and Zn^{2+} in isotonic buffer at pH 7.4 (50 mM HEPES and 150 mM NaCl).

^{19}F NMR Ca^{2+} titrations of mixed [DyL]/[YL] (50:50, 70:30, 90:10). Three solutions containing DyL mixed with YL in different ratios (50:50, 70:30, 90:10, maintaining [DyL] = 2.0 mM) in HEPES buffer (pH 7.4) were titrated using a 20 mM CaCl_2 solution. ^{19}F NMR spectra were acquired after every CaCl_2 addition. The $A_{\text{Dy}}/A_{\text{Y}}$ ratio was calculated by integrating the region between -72.5 and -73.0 ppm for DyL and between -70.0 and -70.5 ppm for YL. All titration points were normalized for the highest value.

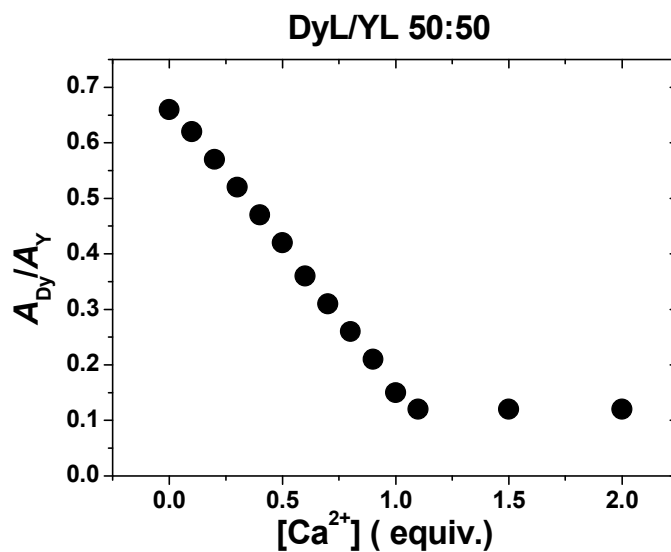


Figure S3. ^{19}F NMR titration for the mixed DyL/YL 50:50 sample.

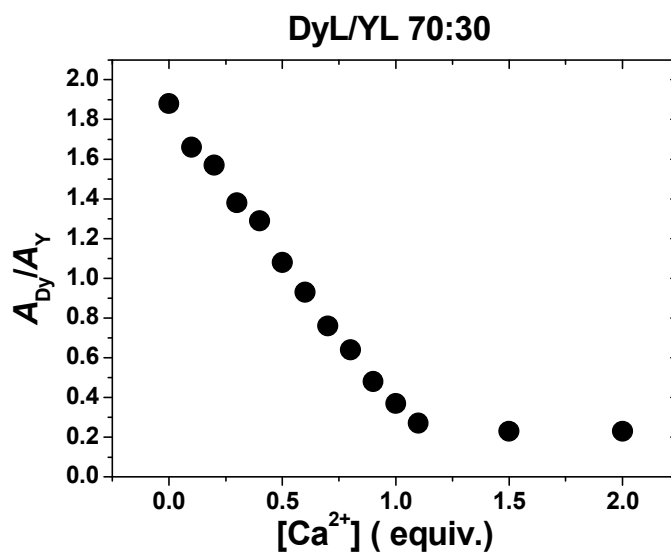


Figure S4. ^{19}F NMR titration for the mixed DyL/YL 70:30 sample.

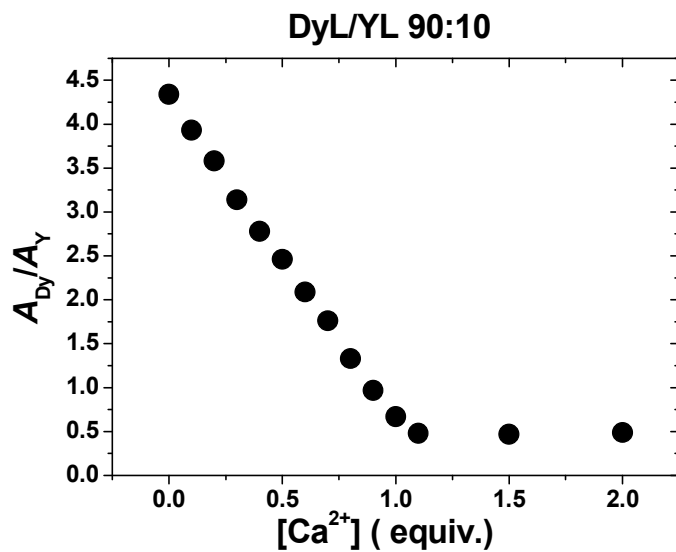


Figure S5. ¹⁹F NMR titration for the mixed **DyL/YL 90:10** sample.

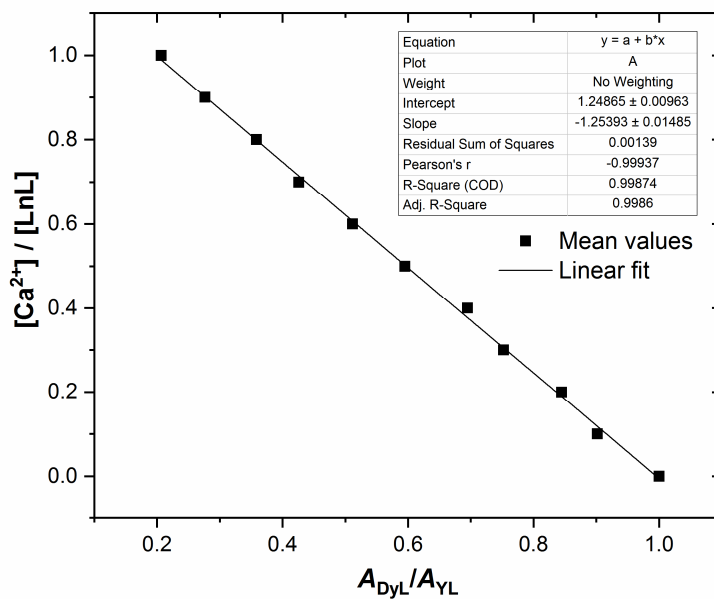


Figure S6. Linear fit of the mean normalized values for the three titrations with **DyL/YL** mixed samples.

Values obtained from the fit according to the Equation 1 from the main manuscript: $a = 1.25$, $b = -1.25$, with $R = 0.99$.

^{19}F CSI experiments

^{19}F CSI acquisition

MRI measurements were performed on a Bruker BioSpec 70/30 USR magnet (software version Paravision 5.1) using a Bruker volume coil (RF RES 300 1H 075/040 QSN TR). The phantom consisted of 6 x 400 μL vials. Each vial contained 5 mM **DyL** + 0.5 mM **YL** in HEPES (50 mM) at pH 7.4, mixed with 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 equivalents of CaCl_2 for samples 1-6 (1.0 equivalent = 5.5 mM), respectively.

^{19}F MR images were acquired using 2D CSI pulse sequence with the following parameters: field of view (FOV) = 80 x 80 mm, spatial resolution 2.5 x 2.5 mm, k-space weighted acquisition, slice thickness 200 mm, repetition time (TR) = 182.2 ms, 512 spectral points acquired with a bandwidth of 10 ppm and total acquisition time (TA) = 60 min 44 sec.

Data analysis

For quantification of the different signals in the CSI measurements, Lorentzian lineshapes were fitted to the acquired signals, using the AMARES algorithm,⁵ as implemented in the jMRUI 5.2 software package.⁶

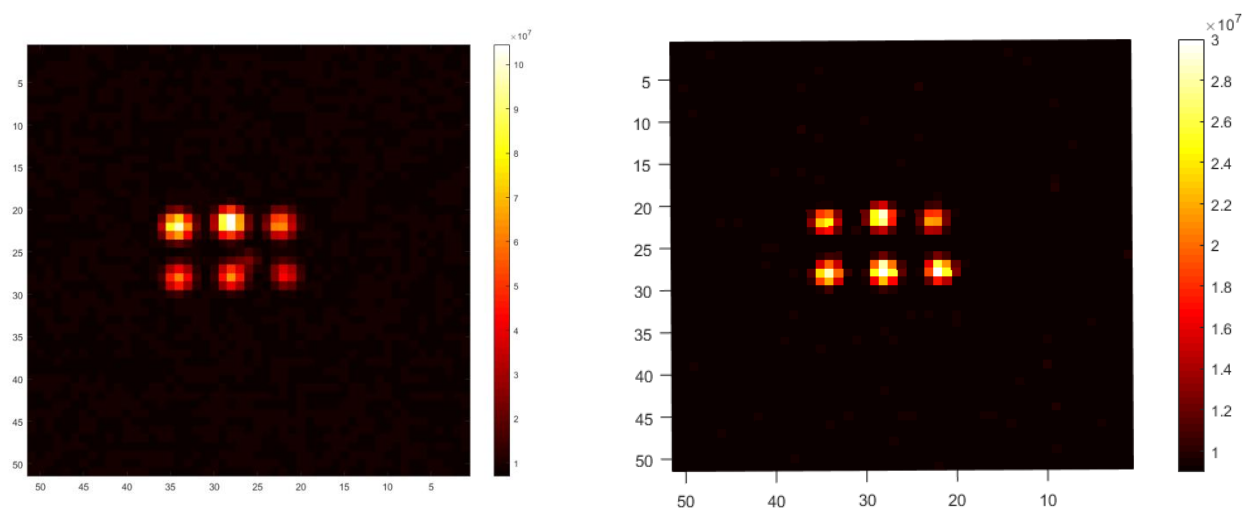


Figure S7. The CSI images of the phantom samples **1-6** obtained for the A_{Dy} (left) and A_{Y} (right) frequencies.

Table S3: Averaged values obtained for the A_{Dy}/A_Y ratio and calculated $[Ca^{2+}]$ from CSI experiments.

Sample tube	Actual $[Ca^{2+}]$ (mM)	A_{Dy}/A_Y ratio (norm.)	Calc. $[Ca^{2+}]$ (mM)
1	0	0.97 ± 0.07	0.04 ± 0.65
2	1.1	0.84 ± 0.04	1.35 ± 0.79
3	2.2	0.59 ± 0.06	2.89 ± 0.37
4	3.3	0.49 ± 0.04	3.54 ± 0.24
5	4.4	0.38 ± 0.03	4.32 ± 0.20
6	5.5	0.24 ± 0.02	5.29 ± 0.13

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