Electronic Supplementary Material (ESI) for ChemComm.


**Supporting information**

# $^{19}$F is a sensitive reporter in assessing multiple conformations of lanthanide binding tags used for protein NMR analysis

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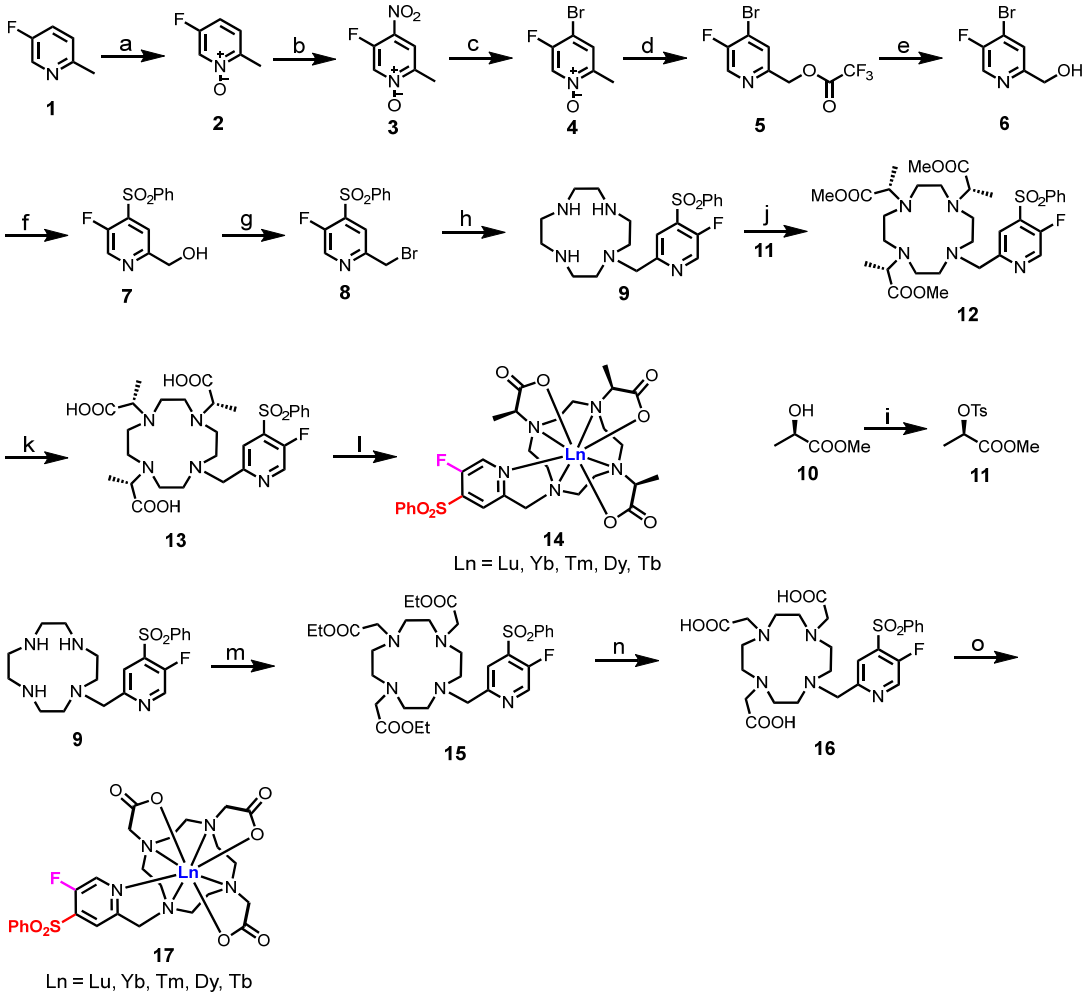
## General information

All $^{1}$H-NMR and $^{13}$C-NMR spectra of organic compounds were recorded on a Bruker Avance 400 MHz spectrometer. $^{19}$F-NMR spectra of lanthanide complexes were recorded on a Bruker Avance 400 MHz spectrometer equipped with a cryoprobe. $^{15}$N-HSQC spectra of protein-tag conjugates were recorded on a Bruker Avance 600 MHz spectrometer equipped with a QCI-cryoprobe. Chemical shifts of organic compounds were reported in ppm from an internal standard and coupling constants, J, in Hz (NMR description: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad).

## Organic synthesis




**Scheme S1.** Synthesis of T1 and T2. Reagents and conditions: a) $CH_3COOH$, 30 % $H_2O_2$, 80 °C; b) Nitrosonitric acid, conc. $H_2SO_4$, 120 °C; c) 33 % HBr in $CH_3COOH$, 80 °C; d) $(CF_3CO)_2O$, $CH_2Cl_2$, 45 °C; e) $K_2CO_3$, $H_2O$; f) $PhSO_2Na$; $CH_3CN$, $CH_3COOH$, Ar, 90 °C; g) $PBr_3$, $CH_2Cl_2$, 45 °C; h) cyclen, $CHCl_3$, rt;

i) TsCl, $CH_2Cl_2$, $Et_3N$; j) $K_2CO_3$, $CH_3CN$, Ar, 60 °C; k) KOH, THF, $H_2O$; HCl, $H_2O$; l) $Ln(NO_3)_3 \bullet 6H_2O$, $H_2O$, 120 °C, pH 7; m) $K_2CO_3$, $CH_3CN$, Ar, 60 °C; n) KOH, THF, $H_2O$; HCl, $H_2O$; o) $Ln(NO_3)_3 \bullet 6H_2O$, $H_2O$, 90 °C, pH 6.

**Synthesis of DO3MA(S)-4PS-5F-Py (T1)**

**2-methyl-5-fluoropyridine 1-oxiade (2).** 30% $H_2O_2$ (34 mL, 360.0 mmol) was added to a solution of 2-methyl-5-fluoropyridine (20 g, 180.2 mmol) in 100 mL $CH_3COOH$, and the mixture was stirred at 80 °C for 16 h. The mixture were concentrated under reduced pressure and used directly in next step.

**2-methyl-4-nitro-5-fluoropyridine 1-oxiade (3).** A mixture of **2** in 100 mL $H_2SO_4$ and 80 mL fuming $HNO_3$ was stirred at 110 °C for 3 h. Then the reaction solution was mixed with ice and water, and extracted with $CH_2Cl_2$. The extracts were washed with saturated $K_2CO_3$ solution, dried with $Na_2SO4$, filtered, and concentrated under reduced pressure to give 25.2 g (146.5 mmol) yellow powder (yield 81.3 %). $^1$H NMR (400 MHz, $CD_3Cl$), $\delta$ ppm: 8.33 (1H, d, *J* = 6.0 Hz), 8.07 (1H, d, *J* = 9.0 Hz), 2.52 (3H, s).

**2-methyl-4-bromo-5-fluoropyridine 1-oxiade (4).** A mixture of **3** (25 g, 145.4 mmol) in 60 mL $CH_3COOH$ solution containing 33% HBr was stirred at 80 °C for 12 h. Then the reaction solution was mixed with ice and water and pH was adjusted to around 8 by KOH. The solution was extracted by $CH_2Cl_2$, then the extracts was dried with $Na_2SO_4$, filtered, and concentrated to give 27 g (131.1 mmol) yellow compound (yield 90.0 %).

**2-methanol-4-bromo-5-fluoropyridine (6).** Compound **4** (25 g, 121.4 mmol) was dissolved in 200 mL $CH_2Cl_2$, then 51 mL trifluoroacetic anhydride (361.1 mmol, 3 eq) was added dropwise to the solution. The reaction mixture was heated to reflux for 12 h. The reaction solution was concentrated and mixed with water. The pH of solution was adjusted to about 9 with $K_2CO_3$ and stirred 5 h at room temperature. The pH of resulting solution was adjusted to 14 with KOH and extracted with $CH_2Cl_2$, washed with dilute KOH solution, dried with $Na_2SO_4$, filtered, and concentrated to give 15.5 g (75.2 mmol) yellow compound (yield 62.0 %). $^1$H NMR (400 MHz, $CD_3Cl$), $\delta$ ppm: 8.34 (1H, s), 7.63 (1H, d, *J* = 4.6Hz), 4.72 (2H, s), 4.45 (1H, s).

**2-methanol-4-phenylsulfonyl-5-fluoropyridine (7).** 250 mL $CH_3CN$ was mixed with **6** (15.0 g, 72.8 mmol), sodium benzenesulfinate (35.8 g, 218.5 mmol) and catalytic amount of $CH_3COOH$, then the

mixture was heated to reflux under argon atmosphere for 36 h. Then excess of $K_2CO_3$ was added into the reaction mixture to react with $CH_3COOH$. The resulting solution was filtered, washed with $CH_2Cl_2$, and concentrated to give 18.6 g (69.7 mmol) yellow compound (yield 95.7 %). $^1$H-NMR (400 MHz, $CDCl_3$) δ ppm： 8.47 (1H, s), 8.01 (2H, d, *J* = 7.4 Hz), 7.95 (1H, d, *J* = 5.2 Hz), 7.68 (1H, t, *J* = 7.0 Hz), 7.56 (2H, d, *J* = 7.5 Hz), 4.82 (2H, s), 2.97 (1H, s).

**2-bromomethyl-4-phenylsulfonyl-5-fluoropyridine (8).** 10.3 mL $PBr_3$ (108.6 mmol, 2.0 eq) was added dropwise to the solution of **7** (14.5 g, 54.3 mmol) in 300 mL $CH_2Cl_2$ and heated to reflux for 5 h. The reaction mixture was mixed with water and adjusted pH to 9 with $K_2CO_3$, extracted with $CH_2Cl_2$, dried with $Na_2SO_4$, filtered, and concentrated to give 16.4 g (49.7 mmol) yellow compound (yield 91.5 %). $^1$H NMR (400 MHz, $CD_3Cl$), δ ppm: 8.51 (1H, s), 8.10-8.06 (3H, q), 7.73 (1H, t, *J* = 7.4 Hz), 7.62 (2H, t, *J* = 7.7 Hz), 4.61 (2H, s).

**1-((5-fluoro-4-(phenylsulfonyl)pyridin-2-yl)methyl)-1,4,7,10-tetraazacyclododecane (9).** 15.8 g **8** (48.0 mmol) in 140 mL $CHCl_3$ was added dropwise to the solution of 1,4,7,10-tetraazacyclododecane (12.4 g, 72.0 mmol) in 140 mL $CHCl_3$ and stirred at room temperature overnight. The mixture was purified by chromatography column to give the title product as white solid (12.5 g, 29.7 mmol, yield 61.9 %). $^1$H-NMR (400MHz, $CDCl_3$), δ ppm: 8.43 (1H, s), 8.17 (1H, d, *J* = 5.5 Hz), 8.05 (2H, d, *J* = 8.2 Hz), 7.67 (1H, t, *J* = 7.4 Hz), 7.57 (2H, t, *J* = 7.7 Hz), 3.85 (2H, s), 2.87-2.59 (19 H, brs). MS-ESI (+): 422.3.

**(R)-ethyl-2-tosyloxypropanoate (11).** A solution of (R)-ethyl 2-hydroxypropanoate (25.0 g, 240 mmol) and p-toluenesulfonyl chloride (55.0 g, 288 mmol) in 500 mL $CH_2Cl_2$ was stirred at 0-10 °C for 10 min. 43.8 mL triethylamine (312 mmol) was then added dropwise with vigorous stirring and cooling. The reaction mixture was then stirred for 5 h at room temperature, and filtered. The filtrate was washed with water, dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The residue was purified by chromatography column (petroleum ether: ethyl acetate=50:1, and then 10:1). The title product (55.6 g, 215.5 mmol, 89.8 %) was a pale yellow oil. $^1$H-NMR spectrum (400 MHz, CDCl3), δ ppm: 7.82 (2H, d, *J* = 8.2 Hz), 7.35 (2H, d, *J* = 8.0 Hz), 4.94 (1H, t, *J* = 6.9 Hz), 4.13 (2H, t, *J* = 7.1 Hz), 2.45 (3H, s), 1.52 (3H, d, *J* = 6.9 Hz), 1.20 (3H, t, *J* = 8.3 Hz).

**(2S,2'S,2''S)-triethyl-2,2',2''-(10-((5-fluoro-4-(phenylsulfonyl)pyridin-2-yl)-methyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)tripropanoate (12).** The mixture of **9** (5.1 g, 12.0 mmol), $K_2CO_3$ (9.9 g, 72.0 mmol, 6.0 eq), **11** (14.0 g, 54.0 mmol, 4.5 eq) and 80 mL $CH_3CN$ was stirred at 55-60 °C

under argon atmosphere for 36 h. The mixture was then filtered and concentrated. The residue was purified by chromatography column ($CH_2Cl_2$ : methanol : ammonium hydroxide = 5 : 1 : 0.1) to give a yellow solid (7.5 g, 11.0 mmol) and the yield was about 92 %. $^1$H-NMR (400 MHz, $CDCl_3$), δ ppm: 8.42 (1H, s), 8.05 (2H, d, *J* = 7.6 Hz), 7.82 (1H, d, *J* = 8.2 Hz), 7.68 (1H, t, *J* = 7.2 Hz), 7.58 (2H, t, *J* = 7.4 Hz), 5.30 (2H, s), 3.65 (9H, s), 3.51 (3H, q, *J* = 7.4 Hz), 2.84-2.97 (6H, br), 2.62-2.68 (10H, br), 1.28 (9H, d, *J* = 7.4 Hz). MS-ESI (+): 680.3, 681.3, 702.4, 703.3.

**(2S,2'S,2''S)-2,2',2''-(10-((5-fluoro-4-(phenylsulfonyl)pyridin-2-yl)-methyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)tripropanoic acid (13, DO3MA(S)-4PS-5F-Py, T1). 12** (7.4 g, 10.9 mmol) and KOH (3.7 g, 65.3 mmol, 6.0 eq) was mixed with 50 mL $H_2O$ and 50 mL THF. The solution was stirred at room temperature for 12 h. The reaction mixture was adjusted to pH 2 with HCl and then concentrated, extracted with 80 mL $CH_3OH$ and filtered. The filtrate was concentrated to give 6.9 g yellow solid (10.8 mmol, yield 98.0 %). MS-ESI (+): 638.3, 639.3. High resolution ESI (+): 638.2656, 639.2679, 640.2683.

**Formation of lanthanide complexes (14, T1-Ln).** The free tag **13** (400 mg, 0.6 mmol) and 0.7 mmol $Ln(NO_3)_3$•$6H_2O$ was dissolved in 18 mL $H_2O$, and the pH was adjusted to 7 with KOH solution. The mixture was heated to 120 °C for 5 h in a high-pressure reactor. After cooling to room temperature, the reaction solution was adjusted to pH 10 with $K_2CO_3$, and then concentrated, extracted with 18 mL $CH_3OH$ and filtered to remove the inorganic salt. The filtrate was concentrated, extracted with $CH_3OH$ again, to give a yellow powder (0.5 mmol, yield ~ 90 %).

**Triethyl 2,2',2''-(10-((5-fluoro-4-(phenylsulfonyl)pyridin-2-yl)-methyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (15).** 3.0 mL ethyl 2-bromoacetate (27.0 mmol, 4.5 eq) was added to the mixture of **9** (2.5 g, 6.0 mmol), $K_2CO_3$ (5.0 g, 36.0 mmol, 6.0 eq) and 60 mL $CH_3CN$. The mixture solution was stirred and heated to reflux at 60 °C for 16 h under argon atmosphere. The reaction mixture was filtered, concentrated under reduced pressure and then purified by column separation to give a yellow solid (3.8 g, 5.5 mmol, yield 92 %). MS-ESI (+): 702.4, 703.4.

**2,2',2''-(10-((5-fluoro-4-(phenylsulfonyl)pyridin-2-yl)-methyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (16, DO3A-4PS-5F-Py, T2).** 3.7 g **15** (5.4 mmol) was mixed with KOH (1.8 g, 32.6 mmol, 6.0 eq), 25 mL $H_2O$ and 25 mL THF and stirred at room temperature for 12 h. The pH of reaction mixture was adjusted to 2 with HCl solution, concentrated, extracted with 30 mL $CH_3OH$

and filtered. The filtrate was concentrated under reduced pressure to give a yellow solid (3.1 g, 5.3 mmol, yield 96 %). MS-ESI (+): 596.3, 597.3, 618.3, 619.3.

**Symthesis of lanthanide complexes (17, T2-Ln).** The synthesis procedure was similar to that of T1-Ln complexes.

**MS-ESI (+):**

**T1-Lu:** $[M+K]^+$, m/z (calc.) = 848.1, m/z (meas.) = 848.2
**T1-Yb:** $[M+K]^+$, m/z (calc.) = 847.1, m/z (meas.) = 847.2
**T1-Tm:** $[M+K]^+$, m/z (calc.) = 842.1, m/z (meas.) = 842.2
**T1-Tb:** $[M+K]^+$, m/z (calc.) = 832.1, m/z (meas.) = 832.1
**T1-Dy:** $[M+K]^+$, m/z (calc.) = 837.1, m/z (meas.) = 837.1
**T2-Lu:** $[M+K]^+$, m/z (calc.) = 806.1, m/z (meas.) = 806.1
**T2-Yb:** $[M+H]^+$, m/z (calc.) = 767.1, m/z (meas.) = 767.2
**T2-Tm:** $[M+H]^+$, m/z (calc.) = 762.1, m/z (meas.) = 762.1
**T2-Tb:** $[M+H]^+$, m/z (calc.) = 752.1, m/z (meas.) = 752.2
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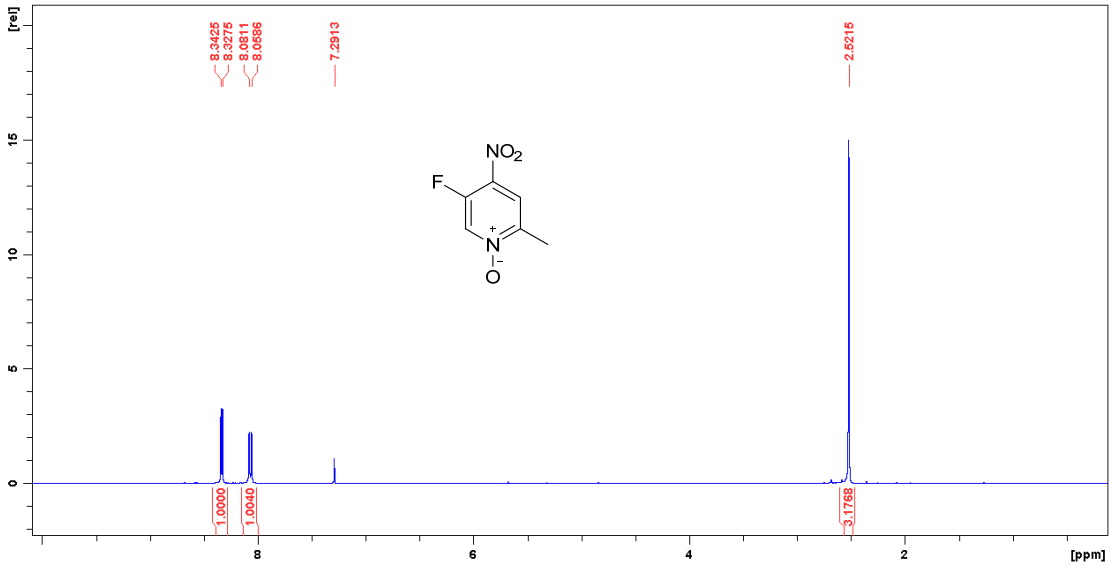

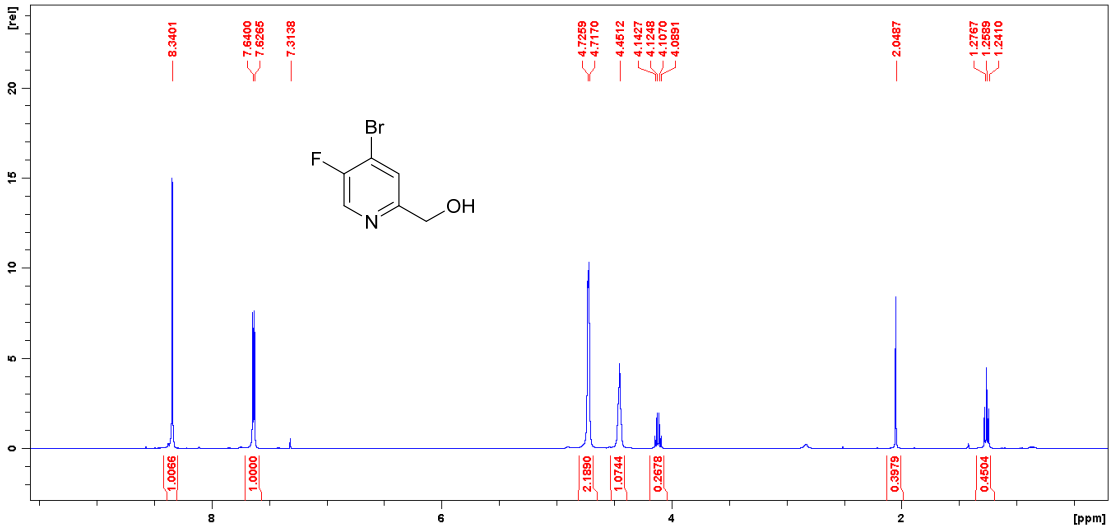
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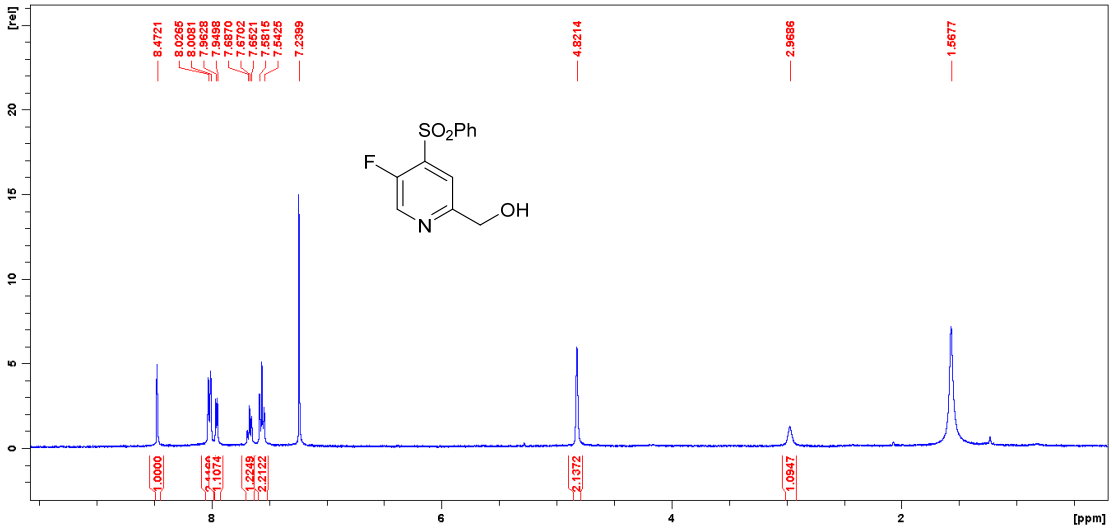
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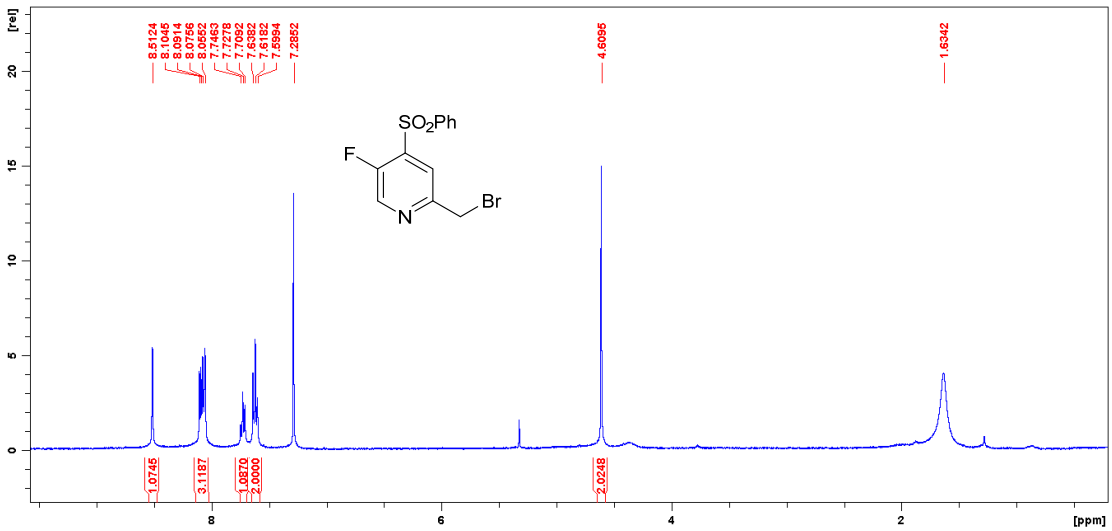
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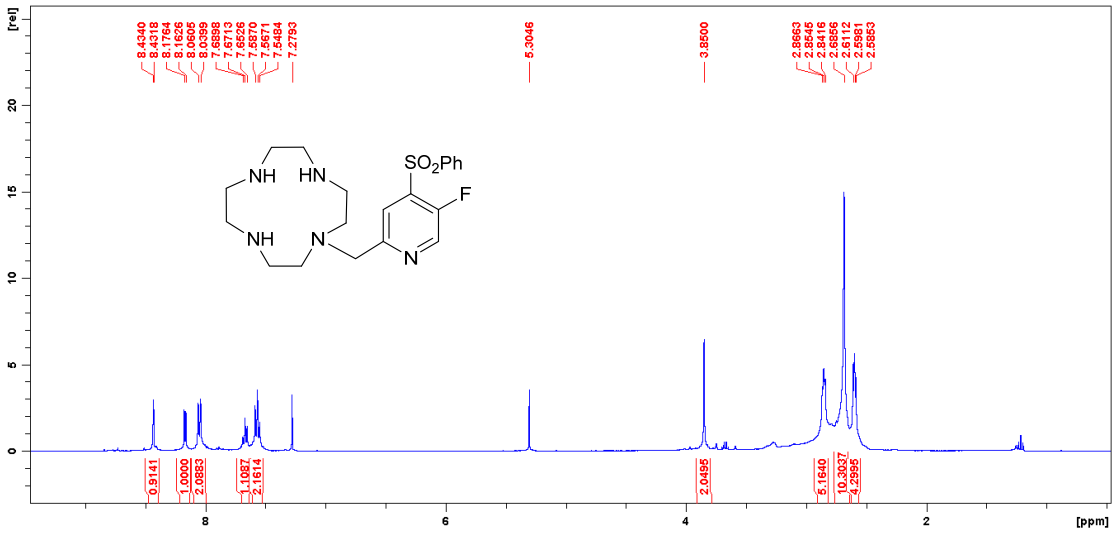
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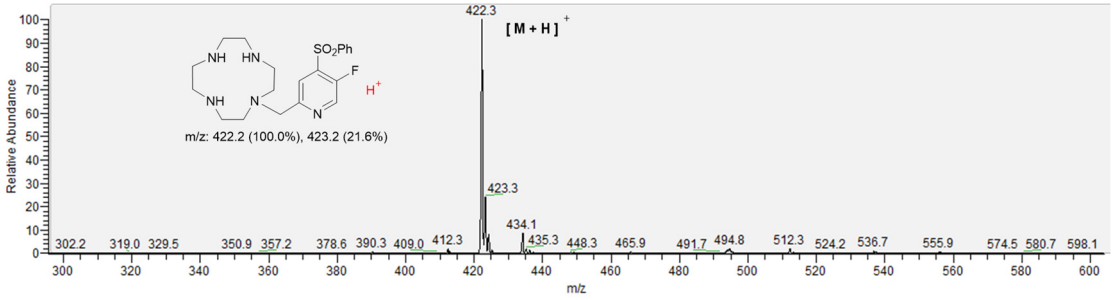
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NH
N
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H+
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Relative Abundance
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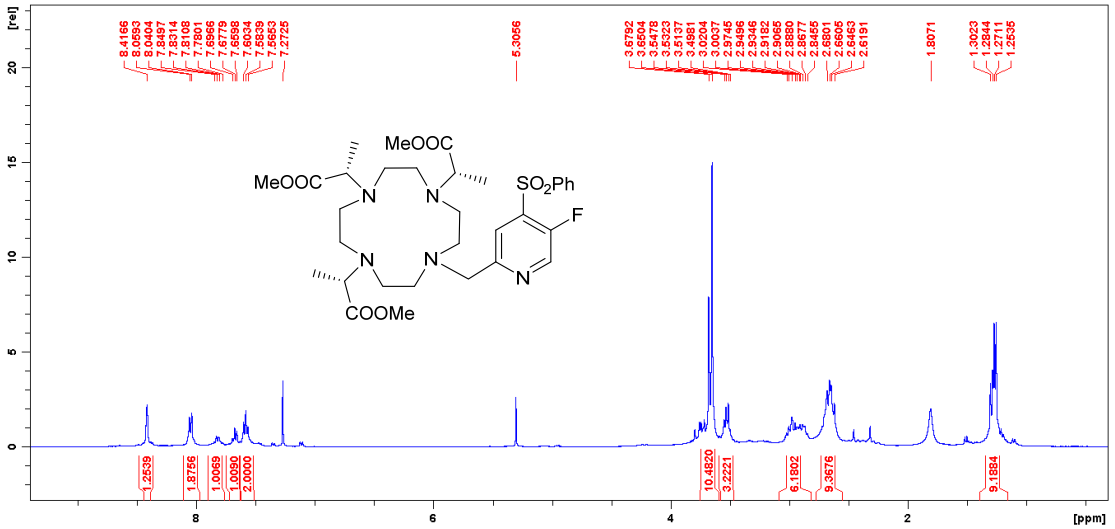
434.1
m/z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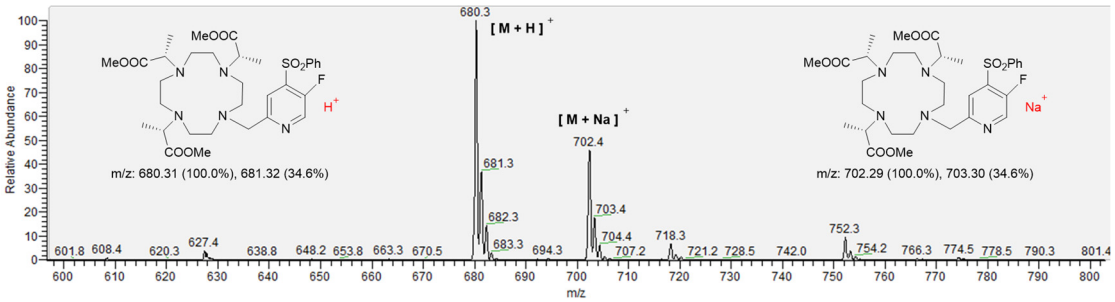
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SO2Ph
F
N
N
N
COOMe
H+
m/z: 680.31 (100.0%), 681.32 (34.6%)
[M + Na]+
702.4
Relative Abundance
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682.3
703.4
704.4
752.3
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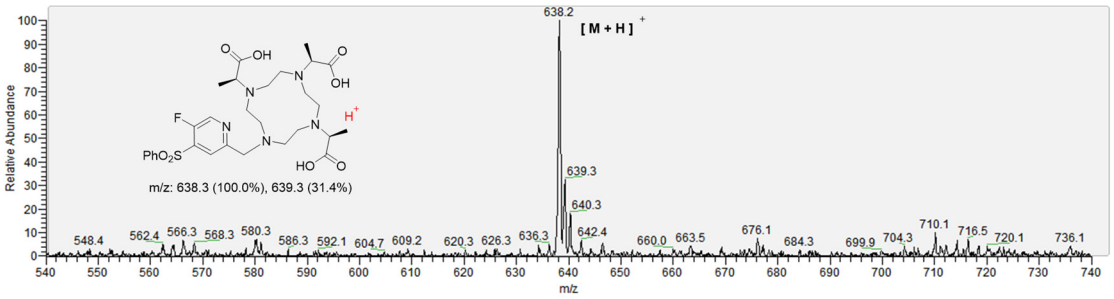
Na+
m/z: 702.29 (100.0%), 703.30 (34.6%)
m/z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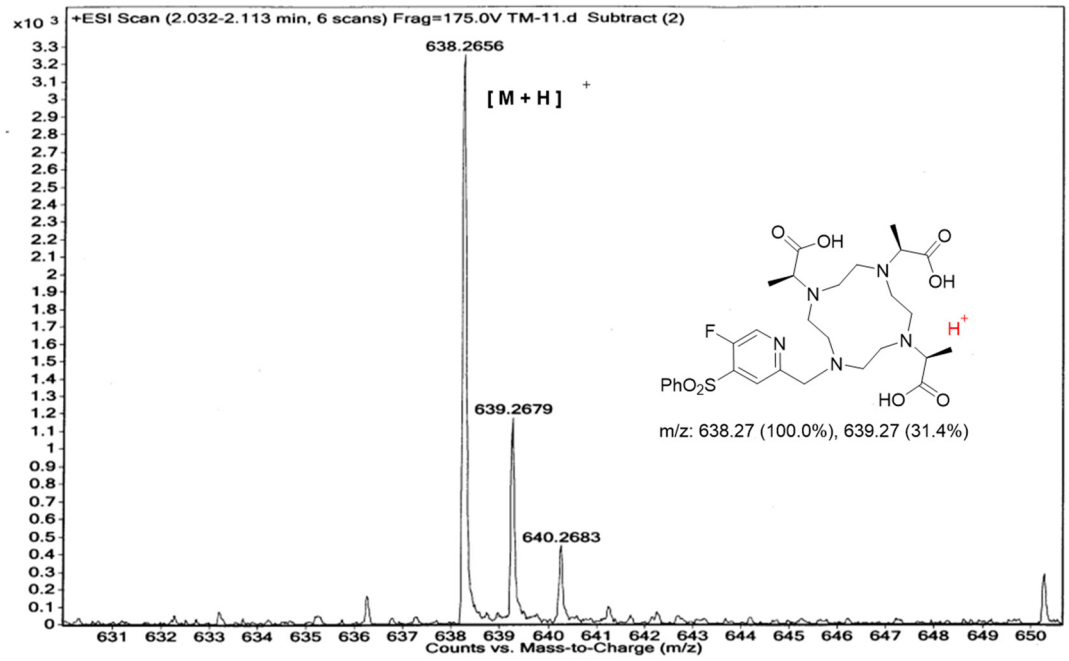
640.3
642.4
m/z: 638.3 (100.0%), 639.3 (31.4%)
Relative Abundance
m/z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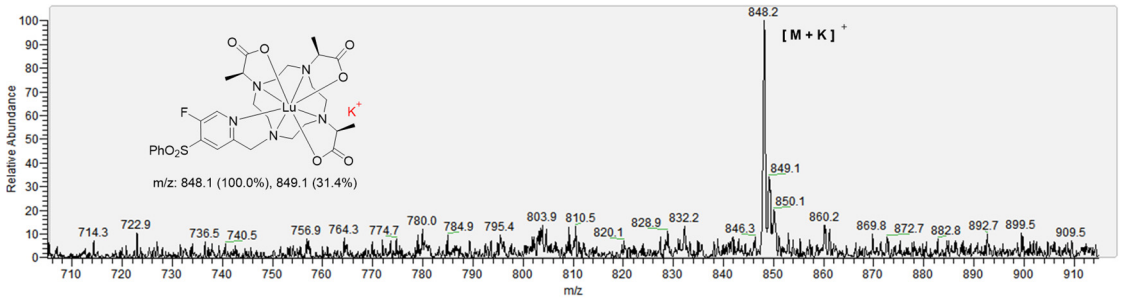
640.2683
m/z: 638.27 (100.0%), 639.27 (31.4%)
Counts vs. Mass-to-Charge (m/z)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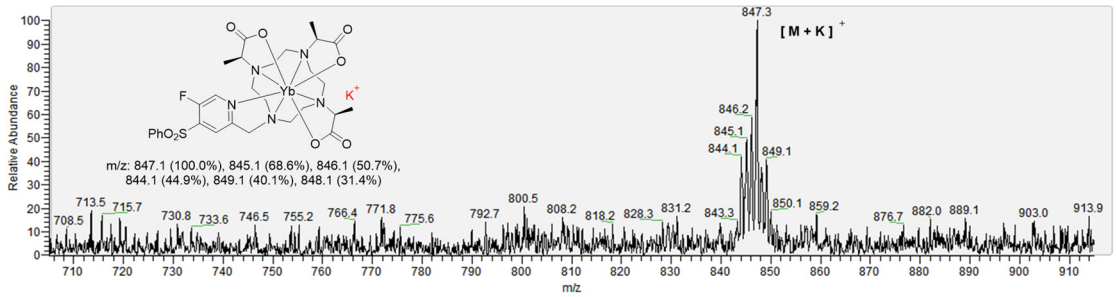
Relative Abundance
m/z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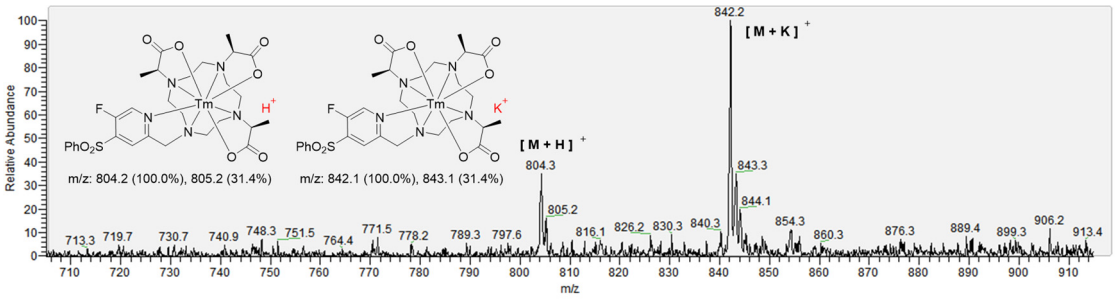
Relative Abundance
m/z


Relative Abundance
m/z
[ M + H ] +
[ M + K ] +
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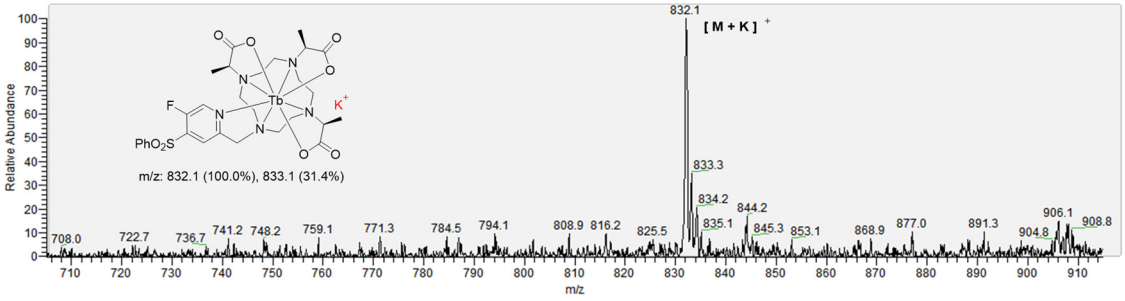
m/z: 842.1 (100.0%), 843.1 (31.4%)


Relative Abundance
m/z
[ M + K ] +
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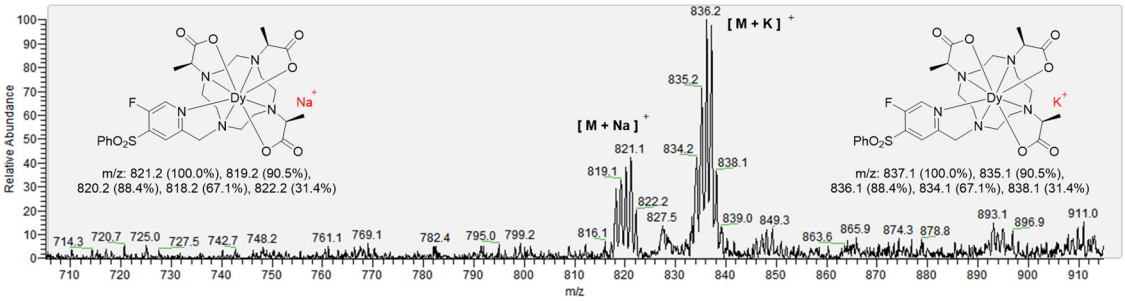
Relative Abundance
m/z
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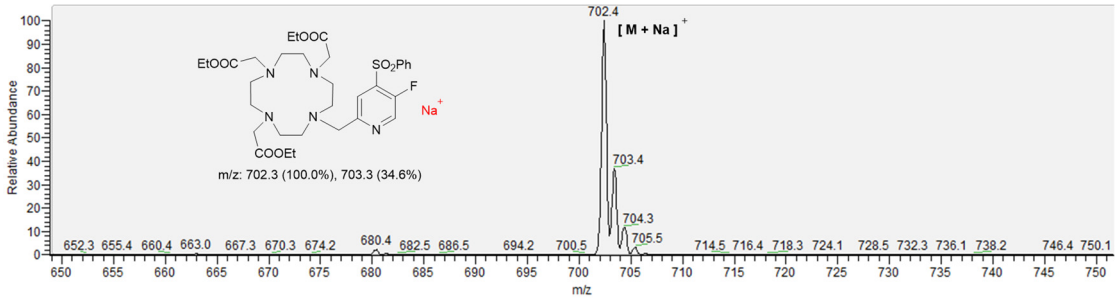
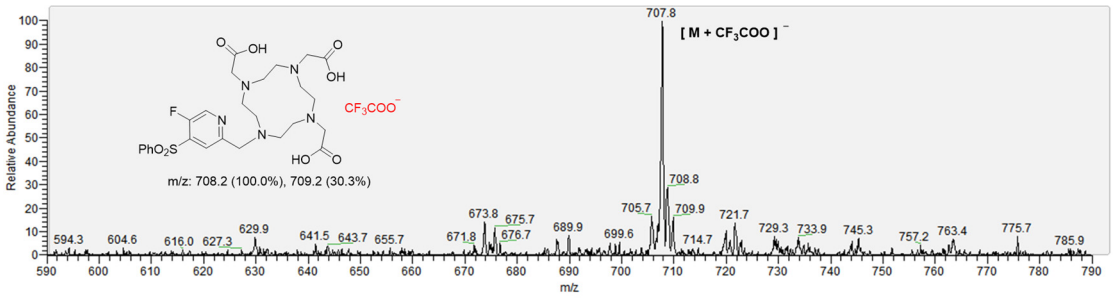
[ M + K ] +
m/z: 821.2 (100.0%), 819.2 (90.5%),
820.2 (88.4%), 818.2 (67.1%), 822.2 (31.4%)
m/z: 837.1 (100.0%), 835.1 (90.5%),
836.1 (88.4%), 834.1 (67.1%), 838.1 (31.4%)


Relative Abundance
m/z
[ M + Na ] +
m/z: 702.3 (100.0%), 703.3 (34.6%)


Relative Abundance
m/z
[ M + CF3COO ] −
m/z: 708.2 (100.0%), 709.2 (30.3%)

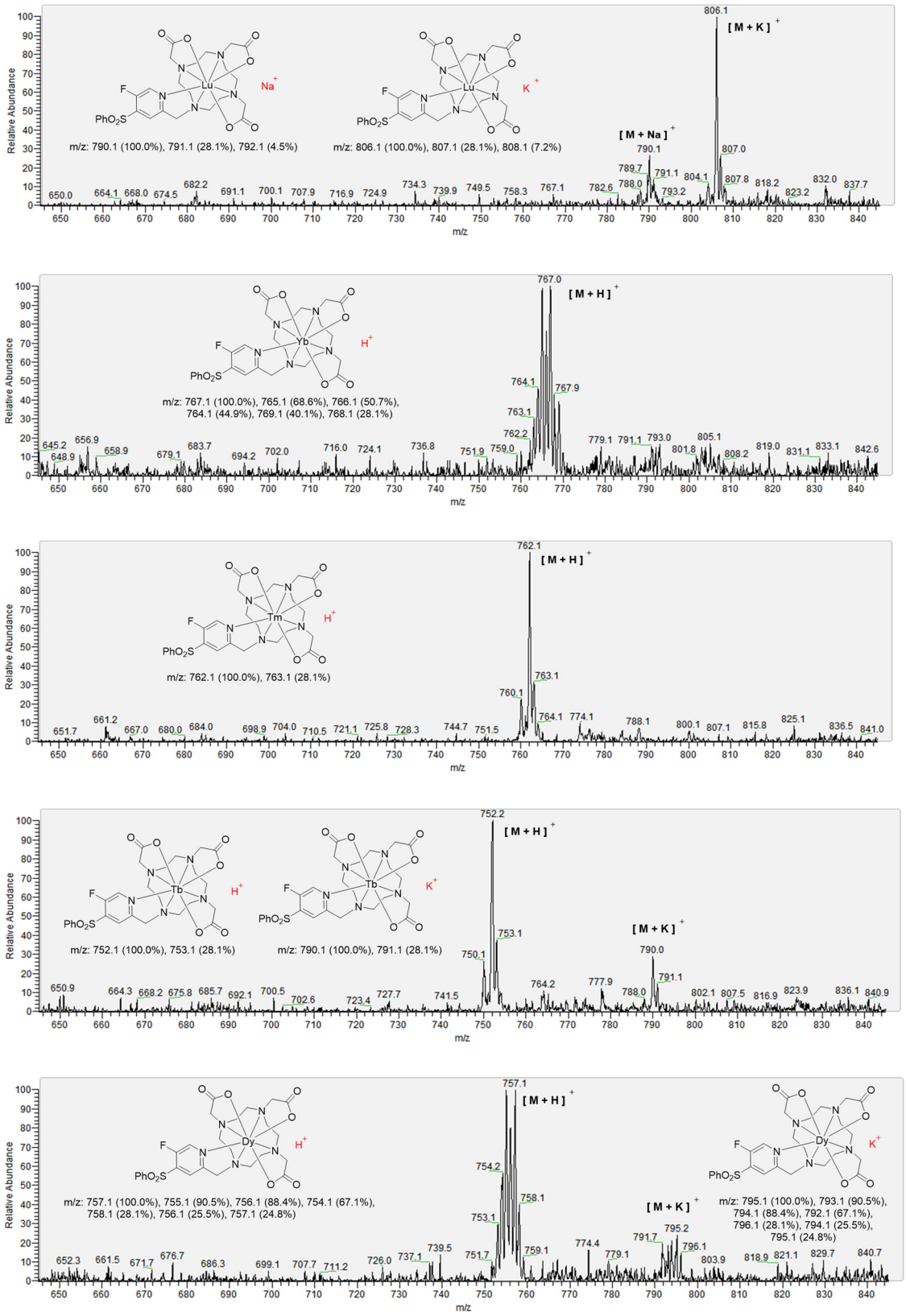



**Fig S1.** 1D proton NMR and Mass spectra of intermediate and target compounds in the synthesis.

**Protein expression and purification**

The plasmid for expression of ubiquitin G47C was constructed using a PET3a vector for expression under control of the T7 promoter. *E coli* BL21 (Rosetta) strain was used for protein expression. Recombinant human ubiquitin G47C mutant was cloned into the PET3a vector and the protein was expressed in *E. coli* BL21 (Rosetta) strain with induction by isopropyl-D-1-thiogalactopyranoside (IPTG). Uniformly $^{15}$N-labelled protein was prepared by growing cells in M9 medium following an established high cell-density protocol.[1] The protein was purified through a DEAE column and then a Superdex75 column. About 15 mg protein was obtained per 250 mL M9 medium.

**Site-specific labelling of ubiquitin G47C**

0.3 mM 1 mL $^{15}$N-ubiquitin G47C was mixed with 3 equivalents of T1-Ln or T2-Ln tag (in 20 mM stock of aqueous solution) and 1 eq tris(2-carboxyethyl)phosphine (TCEP) in 20 mM Tris buffer. The pH of reaction mixture solution was adjusted to about 8.5. The ligation reaction was monitored by recording $^{15}$N-HSQC spectra and mass spectra. After incubation for about 10 h at room temperature, excess of free paramagnetic tag was removed by a small PD-10 desalting column. The overall yield of ligated protein was about 85%.

**NMR measurements**

Samples for 1D $^{19}$F NMR measurements generally contained 1.0 mM T1-Ln or T2-Ln complexes (Ln = Lu, Yb, Tm, 20 mM for Tb and Dy complexes), 10 % $D_2O$ (v/v) at pH about 8.0. Samples of L-Cys modified T1-Ln or T2-Ln (Ln = Lu, Yb, Tm) complexes were prepared by incubation the mixture of 1.0 mM complexes and 5 eq L-Cys at pH 8.5 and room temperature for 10 h. All $^{19}$F NMR spectra were recorded at 293 K with Bruker Avance 400 MHz ($^1$H frequency) NMR spectrometer equipped with a cryoprobe. 1D $^{19}$F NMR spectra was recorded with 2048 scans for 1.0 mM tag-Ln and 128 scans for 20 mM tag-Ln complexes, respectively. 2D $^{19}$F-EXSY spectra were recorded with standard *noesyph* pulse program with mixing time from 1 ms to 30 ms for 20 mM lanthanide complexes. $^{19}$F-EXSY spectra were acquired with 2048 data points in F2 dimension, 200 increments in F1 dimension and 64 scans per increment. The spectra width of EXSY spectra was 15 ppm for tag-Lu complex, 60 ppm for tag-Ln complex (Ln=Yb, Tb, Dy), and 100 ppm for tag-Tm complex. The population of each isomer of T1-Ln or T2-Ln

complexes was determined by the peak volume ratio of each isomer to the sum of two isomers. $^{15}$N-HSQC spectra of protein-tag conjugates were recorded in 20 mM MES buffer at pH 6.4 and 298 K with Bruker Avance 600 MHz spectrometer equipped with a QCI-cryoprobe. PCSs of backbone amide protons were measured as the chemical shift differences between paramagnetic species and free protein.

**Exchange rates between isomers determined by 2D $^{19}$F-EXSY spectra**

In the 2D $^{19}$F-EXSY spectra, the peak intensity of cross peaks ($I_{AB}$, $I_{BA}$) and diagonal peaks ($I_{AA}$, $I_{BB}$) were closely related to the isomer population ($P_A$, $P_B$), exchange rates ($k_{ex}$), and mixing time (t), which could be described as following equation,[2-3]

$$I_{AA} = P_A(P_A + P_B\exp(-k_{ex}t))\exp(-R_1t) \quad (1)$$

$$I_{BB} = P_B(P_B + P_A\exp(-k_{ex}t))\exp(-R_1t) \quad (2)$$

$$I_{AB} = I_{BA} = P_AP_B(1 - \exp(-k_{ex}t))\exp(-R_1t) \quad (3)$$

where $R_1$ is the longitudinal relaxation rates of lanthanide complexes. To eliminate the influence of $R_1$ to fitting, the peak intensity ratio of cross peak to diagonal peak ($I_{BA}/I_{AA}$, $I_{AB}/I_{BB}$) was derived based on the foregoing equations.

$$\frac{I_{BA}}{I_{AA}} = \frac{1 - \exp(-k_{ex}t)}{P_A/P_B + \exp(-k_{ex}t)} \quad (4)$$

$$\frac{I_{AB}}{I_{BB}} = \frac{1 - \exp(-k_{ex}t)}{P_B/P_A + \exp(-k_{ex}t)} \quad (5)$$

Based on the two equations, the exchange rates $k_{ex}$ and isomer population, $P_A$, $P_B$, could be determined readily with various mixing time.

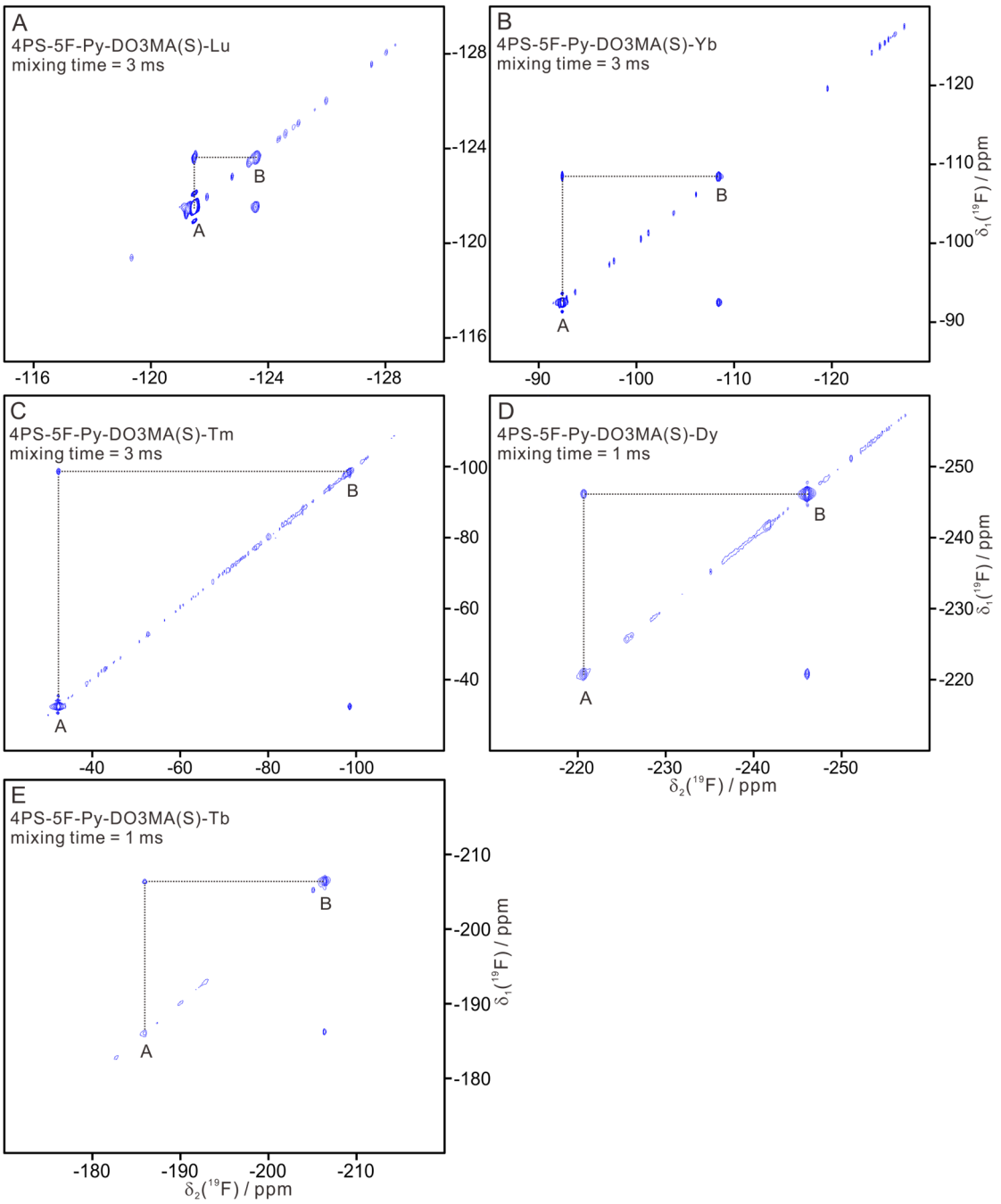



**Fig S2.** 2D $^{19}$F-EXSY spectra recorded for the 20 mM 4PS-5F-Py-DO3MA(S)-Ln (T1-Ln) complexes (A, Lu; B, Yb; C, Tm; D, Dy; E, Tb) in aqueous solution at pH 8.0 and 293 K. The mixing time was 3 ms for T1-Lu, T1-Yb, and T1-Tm complexes, and 1 ms for T1-Tb and T1-Dy complex.

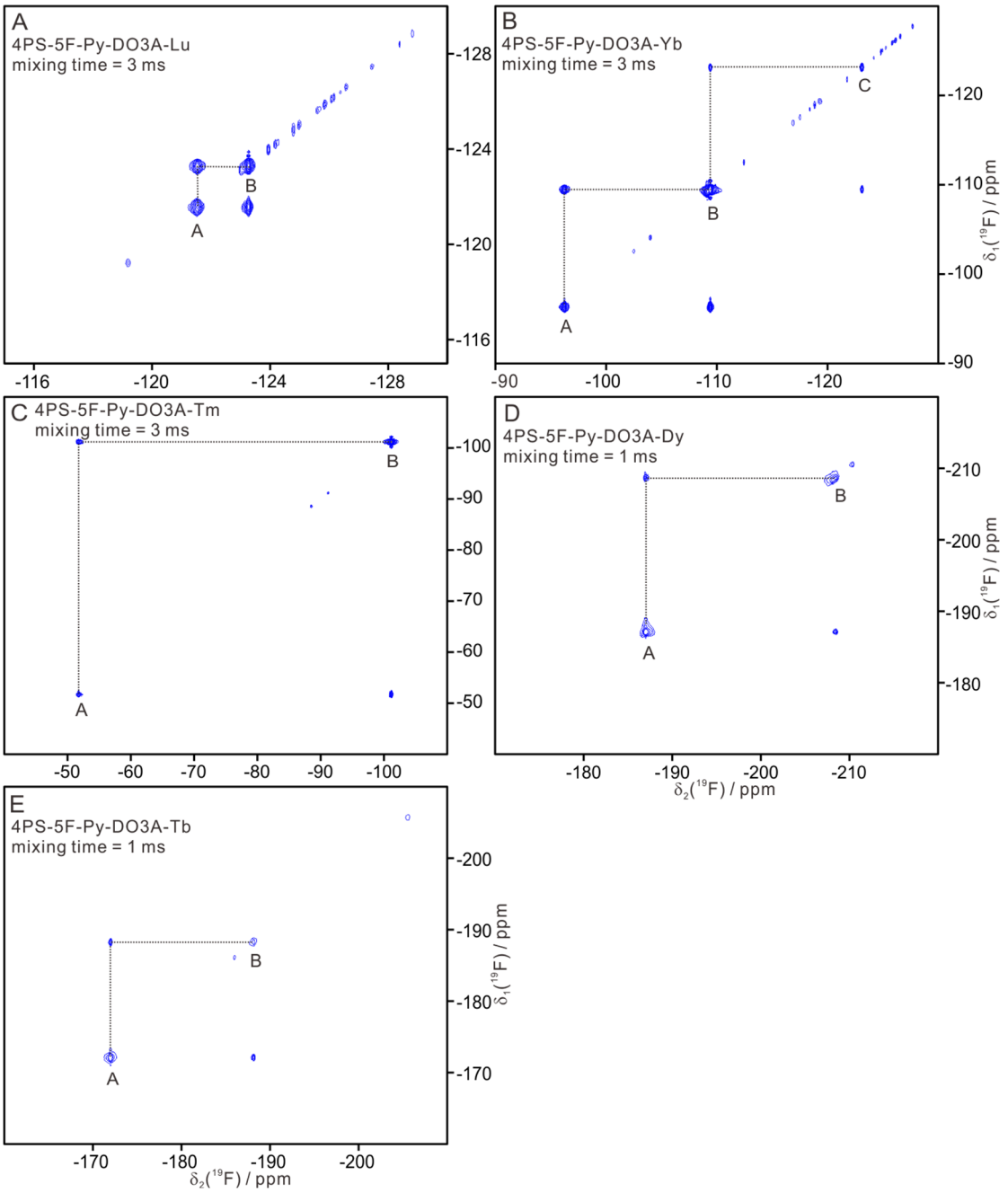



**Fig S3.** 2D $^{19}$F-EXSY spectra recorded for the 20 mM 4PS-5F-Py-DO3A-Ln (T2-Ln) complexes (A, Lu; B, Yb; C, Tm; D, Dy; E, Tb) in aqueous solution at pH 8.0 and 293 K. The mixing time was 3 ms for T1-Lu, T1-Yb, and T1-Tm complexes, and 1 ms for T1-Tb and T1-Dy complex.

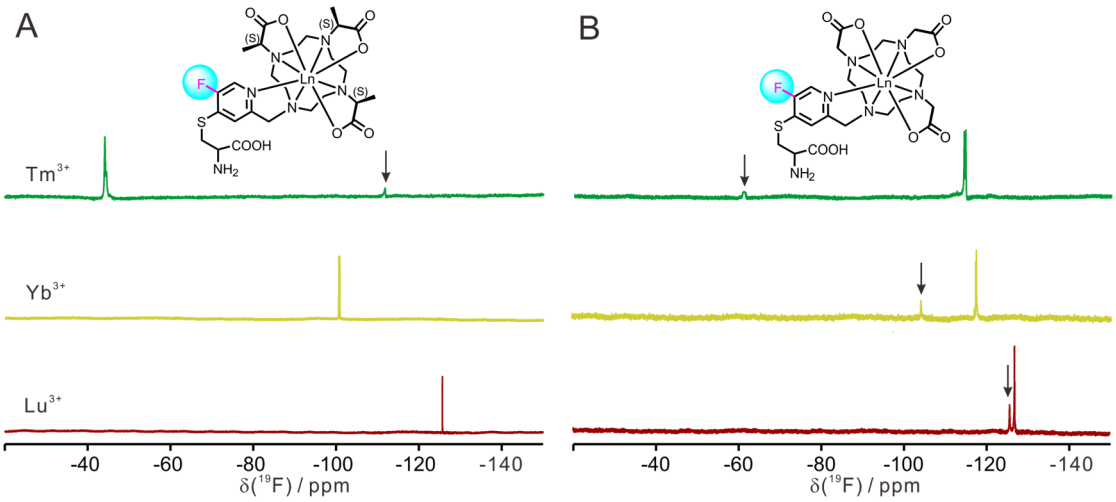



**Fig S4.** 1D $^{19}$F NMR spectra of the 1.0 mM L-Cys-5F-Py-DO3MA(S)-Ln (A) and L-Cys-5F-Py-DO3A-Ln (B) (Ln = Lu, Yb, Tm) complexes recorded at 293 K and pH 8.0. Arrows denote the minor species.

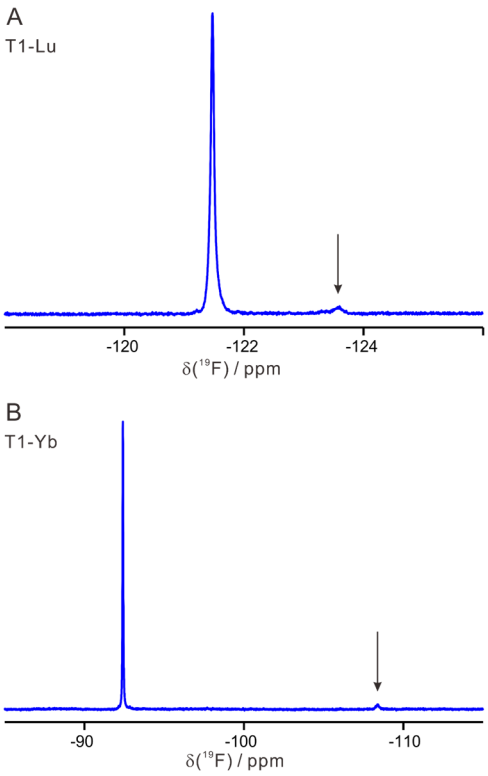
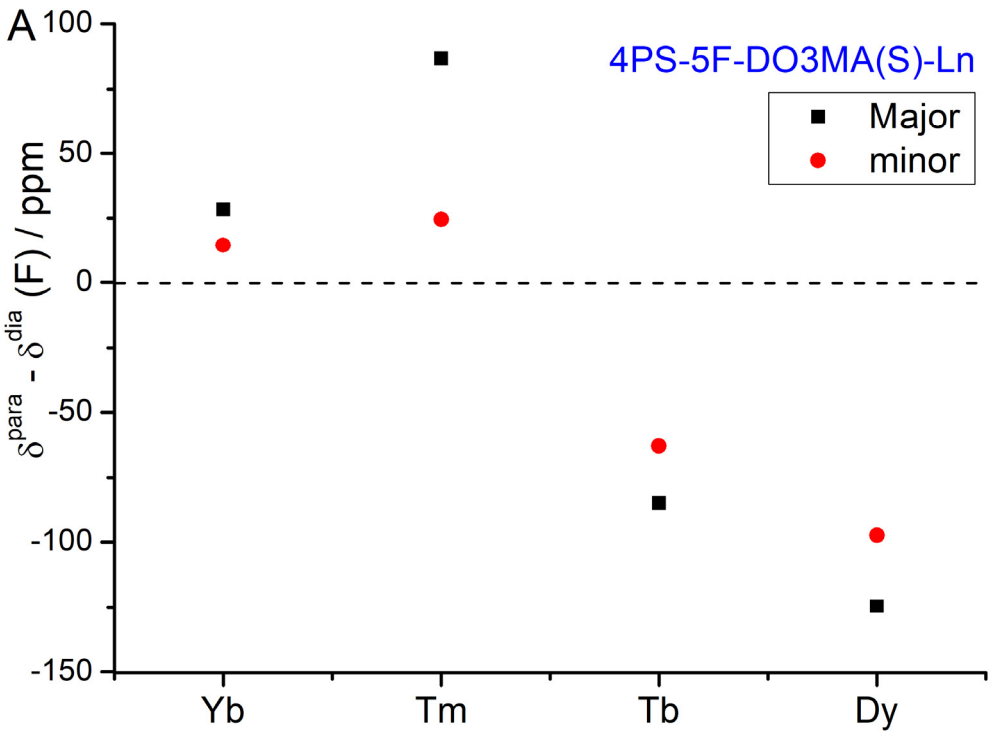



**Fig S5.** 1D $^{19}$F NMR spectra of 20 mM T1-Lu (A) and T1-Yb (B) recorded at pH 8.0 and 293 K. Arrows denote the minor species. The abundance of minor species is about 4 % for T1-Lu and 5 % for T1-Yb complex based on the integral ratio of minor species to the sum of two species. Notably, the minor species shows larger linewidth than the major species in these two complexes, which makes the minor species more difficult to observe in the 1D $^{19}$F-NMR spectra.

**Table S1.** $^{19}F$ chemical shifts and populations of two isomers of reaction products of L-cysteine with T1-Ln and T2-Ln complexes determined by 1D $^{19}F$ NMR spectra at 293 K and pH 8.0 in aqueous solution. For reaction product of L-cysteine with T1-Lu complex, no significant signal of minor species was observed due to the too low abundance. $\Delta\delta^{AB}$ was the absolute value of the chemical shift difference between the two species in the lanthanide complexes.

| | | isomer A (SAP) | | isomer B (TSAP) | | |
|---|---|---|---|---|---|---|
| Complex | $Ln^{3+}$ | δ (ppm) | Population (%) | δ (ppm) | Population (%) | $\Delta\delta^{AB}$ |
| T1-Ln | Lu | -125.68 | 100 | - | - | |
| | Yb | -100.78 | 95 | -115.87 | 5 | 15.09 |
| | Tm | -44.15 | 88 | -111.87 | 12 | 67.72 |
| T2-Ln | Lu | -125.64 | 28 | -126.81 | 72 | 1.17 |
| | Yb | -104.50 | 21 | -117.58 | 79 | 13.08 |
| | Tm | -60.74 | 14 | -114.86 | 86 | 54.12 |



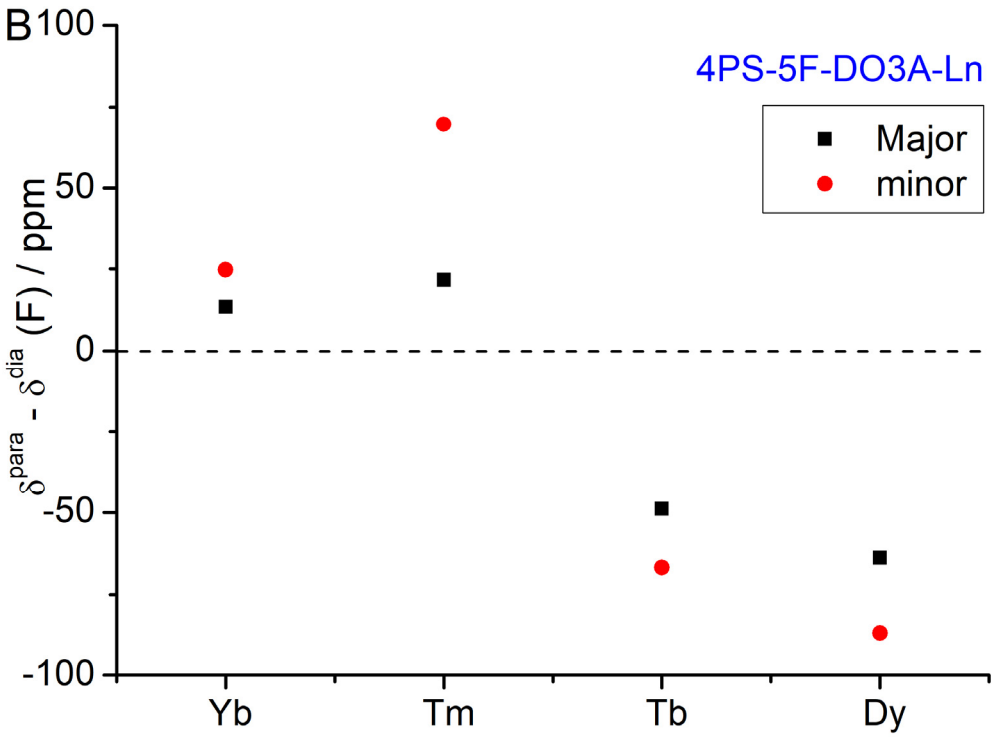



**Fig S6.** Chemical shift difference of $^{19}F$ signals between paramagnetic species and diamagnetic species for the major isomers (black square) and minor species (red circle) of 4PS-5F-Py-DO3MA(S)-Ln (A) and 4PS-5F-Py-DO3A-Ln (B), respectively.

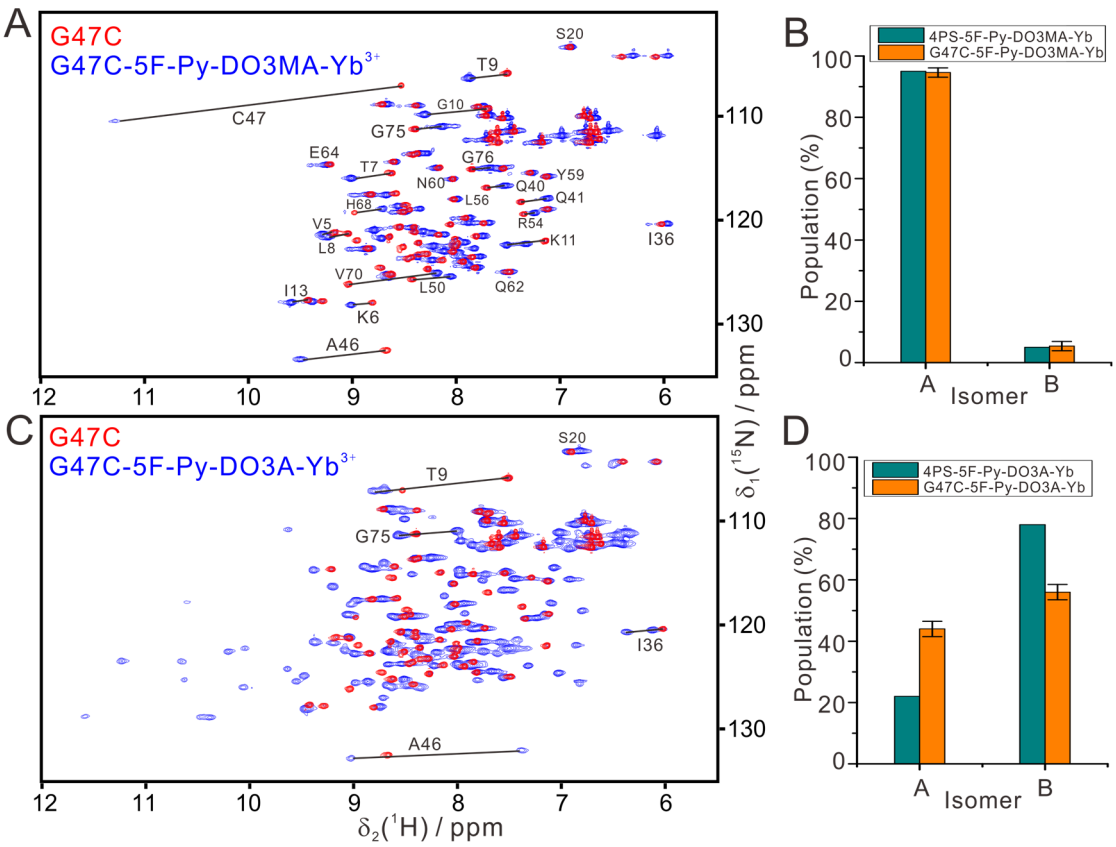



**Fig S7.** Isomeric species of two tag-Yb complexes evaluated in ubiquitin G47C conjugates. Overlay of $^{15}$N-HSQC spectra recorded for 0.2 mM Ub G47C-tag-Yb (blue) and 0.2 mM Ub G47C (red) in 20 mM MES buffer at pH 6.4 and 298 K for (A) 4PS-5F-PyDO3MA-Yb and (C) 4PS-5F-PyDO3A-Yb. Comparison of populations of two isomers in free tag-Yb (cyan) and Ub G47C-tag-Yb (orange) for (B) 4PS-5F-PyDO3MA-Yb and (D) 4PS-5F-PyDO3A-Yb, respectively.

**Table S2.** $\Delta\chi$-tensor parameters of ubiquitin G47C-5F-Py-DO3MA(S)-Ln (Ln = $Yb^{3+}$, $Tm^{3+}$) conjugates.[a]

| $Ln^{3+}$ | $\Delta\chi_{ax}$ | $\Delta\chi_{rh}$ | α | β | γ | x | y | z | Q |
|---|---|---|---|---|---|---|---|---|---|
| Yb | -14.6 | -8.8 | 165.9 | 130.2 | 18.2 | 14.707 | 38.042 | 22.957 | 0.130 |
| Tm | 50.9 (22.2) | 23.6 (13.0) | 44.1 | 127.1 | 144.8 | 14.707 | 38.042 | 22.957 | 0.066 |

[a] The tensor parameters were obtained by fitting the PCSs of backbone amide protons in regions of a regular secondary structure to the crystal structure of ubiquitin (PDB code: 1UBI)[4]. The tensor parameters of ubiquitin G47C-Py-DO3MA(S)-Tm[5] are shown in brackets for

comparison. The $\Delta\chi$-tensors are in units of $10^{-32}$ $m^3$, Euler angles in units of degrees relative to the crystal structure of ubiquitin (PDN code: 1UBI). x, y, z are the coordinates of fitted $Ln^{3+}$ ion in crystal structure of ubiquitin. Q-factor was calculated as $Q = \sqrt{\frac{\sum(PCS^{calc} - PCS^{obs})^2}{\sum(PCS^{obs})^2}}$, where $PCS^{obs}$ and $PCS^{calc}$ are observed and back-calculated PCSs.

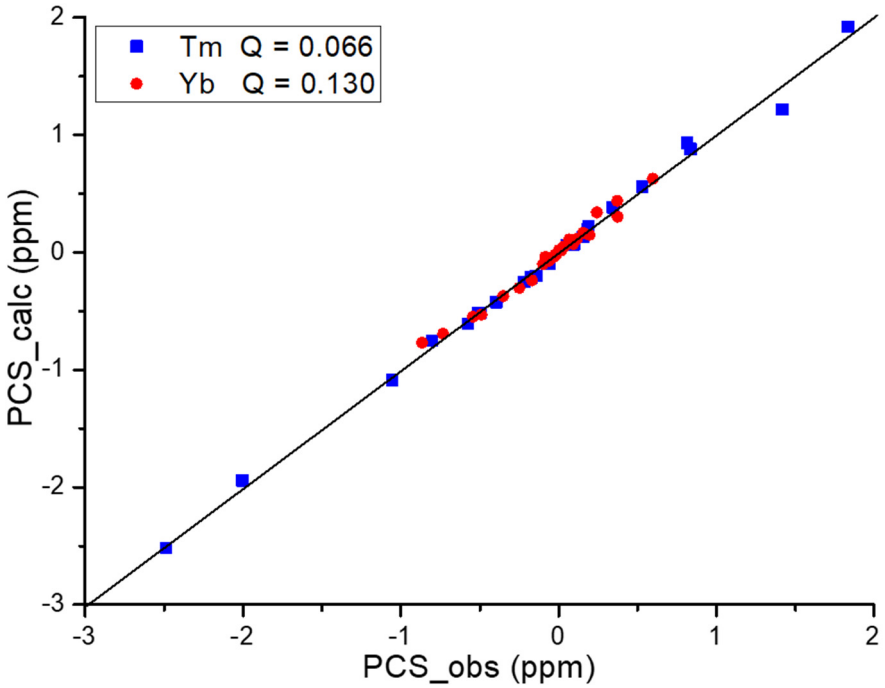



**Fig S8.** Correlations of the observed PCSs (PCS_obs) and back-calculated PCSs (PCS_calc) using Numbat program. The Quality factors were shown as insert in figure. PCSs were measured for the protein samples of 0.2 mM ubiquitin G47C-5F-PyDO3MA(S)-Ln (Yb as red, Tm as blue) in 20 mM MES buffer at pH 6.4 and 298K.

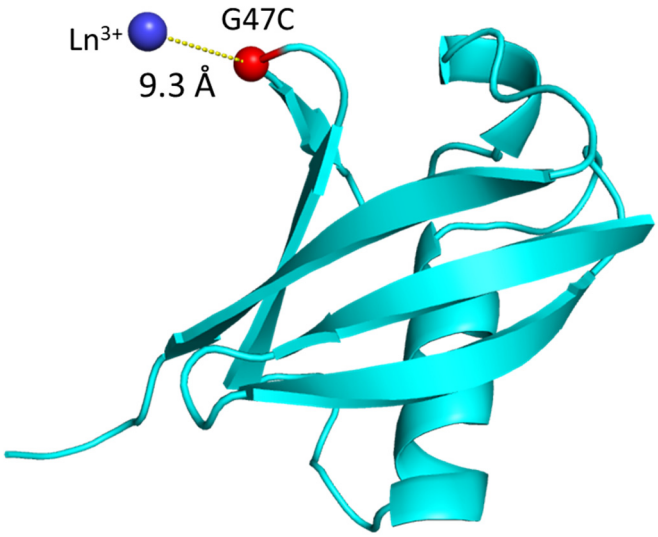



**Fig S9.** Structural representation of the fitted $Ln^{3+}$ position (blue) in ubiquitin G47C-5F-Py-DO3MA(S)-Ln complexes, of which the distance between fitted $Ln^{3+}$ position and Cα atom of G47C (red) is shown (PDB code: 1UBI).

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