Electronic Supplementary Material (ESI) for Dalton Transactions.
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Supplementary information

Mn(II) tags for DEER distance measurements in proteins via C-S attachment

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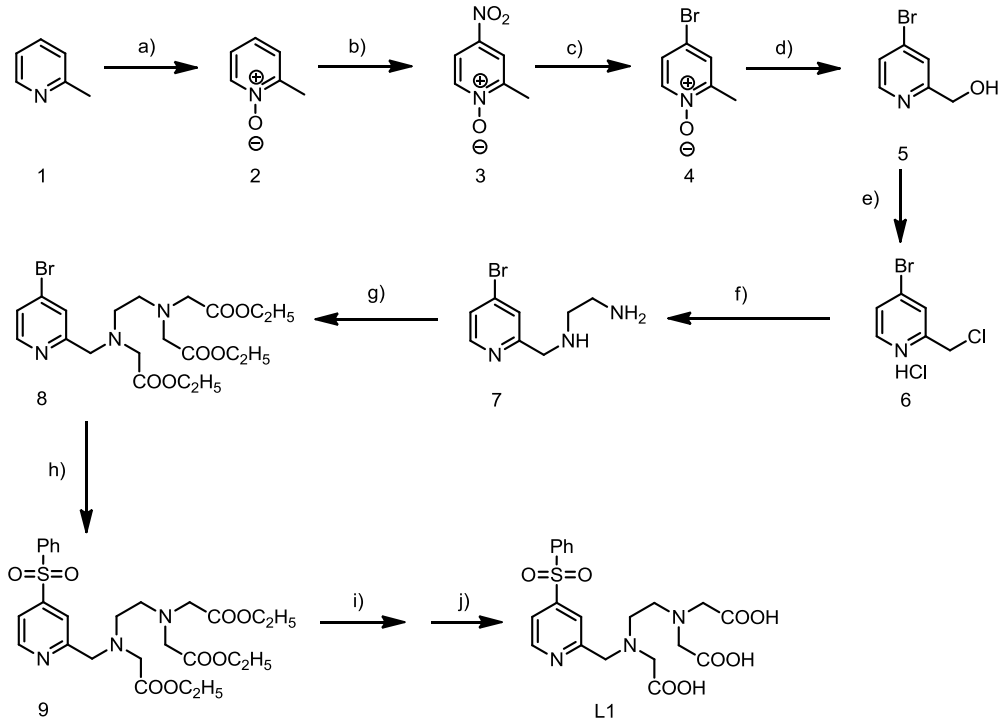
Content

1. Synthesis of the spin labels

Synthesis of L1 (Scheme 1)



Scheme 1. Synthesis of L1. a): AcOH, H_2O_2; b): H_2SO_4, HNO_3; c):CH_3COBr; d): $(CF_3CO)_2O$, CH_2Cl_2; e): $SOCl_2$; f): $NH_2CH_2CH_2NH_2$; g): $BrCH_3COOC_2H_5$, CH_3CN, K_2CO_3, Ar; h): CH_3CN, $PhSO_2Na$, TBAB, Ar; i): NaOH, C_2H_5OH, H_2O; j):H^+.

2-Methylpyridine 1-oxide (**2**). The title compound was synthesized using a modified protocol as published previously[1]. At 25ºC, 30 % H_2O_2 (20 mL) was added to a solution of 2-methylpyridine (**1**) (10 mL, 0.11 mol) in 50 mL CH_3COOH. The resulting mixture was stirred at 80 ºC for 9 h, and the solution was concentrated to one third of the original volume under reduced pressure. The solution left was poured into 100 mL ice water, and powder K_2CO_3 was added until the pH reached 12. The mixture was extracted with dichloromethane, and then washed with brine and the organic layer was dried over Na_2SO_4. The mixture was filtered, and the filtrate was concentrated and purified by column chromatography to obtain title compound **2** (8.3 g, 70.8%) as pale yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.15 (1H, d), 7.24 (1H, d), 6.93-6.67 (2H, m), 2.71 (3H, s).

2-Methyl-4-nitropyridine 1-oxide (**3**). This compound was synthesized according the published protocol[2]. A mixture of **2** (10 g, 0.09 mol), 30 mL concentrated H_2SO_4 and 35 mL fuming HNO_3 was stirred at 110 ºC for 7 h. The resulting mixture was then cooled down to room temperature and poured into to ice cold water. Powder K_2CO_3 was added to the above solution until pH reached 12. The mixture was extracted with ethyl acetate, and washed with brine. The organic layer was dried over Na_2SO_4. The mixture was filtered and the filtrate was concentrated to obtain **3** (11.2g, 79.3%) as pale yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.33 (1H, d), 8.16 (1H, s), 8.02 (1H, d Hz), 2.59 (3H, s).

4-Bromo-2-methylpyridine 1-oxide (**4**). Similar to the previous report[3], 35 mL acetyl bromide was added dropwise into the solution of **3** (3.5 g, 0.02 mol) in 35mL CH_3COOH at 60 ºC. Then the mixture was stirred at 80ºC for 6 h. The solution was added into to ice cold water, treated with K_2CO_3 until the pH reached 10. The mixture was extracted with ethyl acetate, and washed with brine. The organic layer was dried over Na_2SO_4. The resulting solution was filtered and the filtrate was concentrated to obtain **4** (3.1 g, 72.6 %) as reddish brown oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.12 (1H, d), 7.43 (1H, d), 7.29 (1H, dd), 2.50 (3H, s).

(4-Bromopyridin-2-yl)methanol (**5**). Similar to the previous report[4], trifluoroacetic anhydride (14 mL, 0.1 mol) in 20 mL of dichloromethane was added dropwise to the solution of **4** (3.1 g, 0.02 mol) in 40mL dichloromethane. The reaction mixture was refluxed for 16h. The resulting solution was cooled down to room temperature and the solvent was removed under reduced pressure. The left solid was dissolved in 20 mL H_2O, and powder K_2CO_3 was added until the pH reached 8.5. The resulting solution was stirred for 5 h at ambient temperature. Then the pH was adjusted to 12 with KOH, and the mixture was extracted with dichloromethane. The organic layer was washed with brine and dried over Na_2SO_4. The mixture was

filtered and the filtrate was concentrated to obtain **5** (1.9 g, 61.3 %) as yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.35 (1H, d), 7.52 (1H, s), 7.38 (1H, dd), 4.75 (2H, s).

4-Bromo-2-(chloromethyl)pyridine hydrochloride (**6**). Similar to the previous report[4], 1.8 mL thionyl chloride in 20mL dichloromethane was added dropwise to the solution of **5** (1.9 g, 0.01 mol) in 20mL dichloromethane and the temperature was kept below 5 ºC. The resulting solution was stirred at 40 °C for 4 h. Then the mixture was concentrated to obtain **6** (2.3 g, 91.6 %) as yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.37 (1H, d), 7.49 (1H, s), 7.36 (1H, d), 3.84 (2H, s).

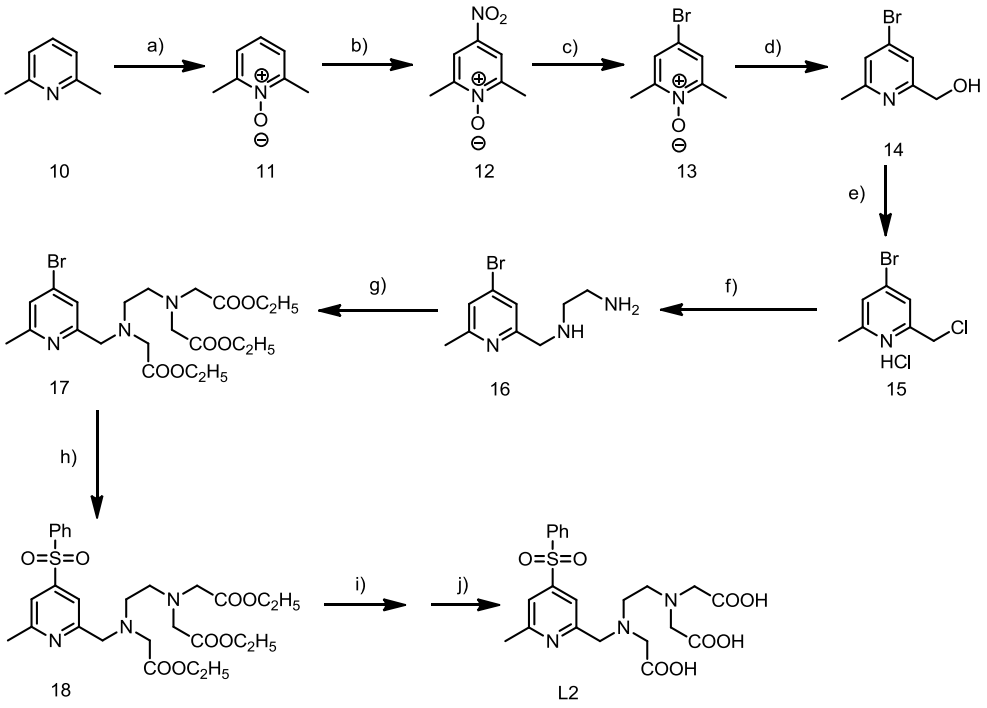
N-((4-bromopyridin-2-yl)methyl)ethane-1,2-diamine (**7**). **6 (**1.4 g, 5.76 mmol) in 25 mL acetonitrile was added dropwise to a solution of 6 mL ethanediamine in 20 mL acetonitrile. The reaction mixture was stirred at room temperature for 21 h. The mixture was concentrated and dissolved in 50 mL ethyl acetate. The organic layer was washed with brine and dried over Na_2SO_4. Then the mixture was filtered and the solved was removed under reduced pressure to obtain **7** (1.1 g, 83.1 %) as brown oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.42 (1H, dd), 7.48 (1H, d), 7.27 (1H, d), 3.92 (2H, s), 2.79 (4H).

Diethyl-2,2'-((2-(((4-bromopyridin-2-yl)methyl)(2-ethoxy-2-oxoethyl)amino)ethyl)azanediyl)diacetate (**8**). Under argon atmosphere, 4.0 mL ethyl 2-bromoacetate in 20 mL acetonitrile was added dropwise to a mixture containing **7** (0.9 g, 3.91 mmol), K_2CO_3 (4.5 g, 32.8 mmol), KI (0.2 g, 1.2 mmol) and 25 mL acetonitrile. Then the reaction mixture was stirred at room temperature for 24 h, and filtered. The filtrate was concentrated and purified by silicon column to obtain title compound **8** (1.2 g, 65.3 %) as yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.39 (1H), 7.68 (1H), 7.36-7.18 (1H, m), 4.19-4.14 (6H, m), 3.97 (2H, s), 3.58 (4H, s), 3.50 (2H, s), 2.92-2.88 (4H, m), 1.30-1.25 (9H, m).

Diethyl-2,2'-((2-((2-ethoxy-2-oxoethyl)((4-(phenylsulfonyl)pyridin-2-yl)methyl)amino)ethyl)azanediyl)diacetate (**9**). **8** (1.1 g, 2.25 mmol) sodium benzenesulfinate (1.2 g, 7.32 mmol) and tetrabutyl ammonium bromide (85 mg, 0.26mmol) was mixed with 35 mL acetonitrile. Under argon atmosphere, the reaction mixture was stirred at 80 °C for 15 h. The solvent was removed under reduced pressure and the left mixture was dissolved in ethyl acetate. The organic layer was washed with brine, and dried over Na_2SO_4. The solution was filtered, and the filtrate was concentrated and purified by column chromatography to obtain **9** (0.85 g, 71.5 %) as yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.71 (1H, d), 8.09 (1H, s), 8.01 (2H, d), 7.67-7.56 (4H, m), 4.20-4.13(6H, m), 4.09 (2H, s), 3.57 (4H, s), 3.51(2H, s), 2.90-2.87 (4H, m), 1.30-1.25 (9H, m).

2,2'-((2-((Carboxymethyl)((4-(phenylsulfonyl)pyridin-2-yl)methyl)amino)ethyl)azanediyl)diacetic acid (**L1**). A mixture of **9** (0.85 g, 1.55 mmol), NaOH (0.37 g, 9.25 mmol), 8 mL ethanol and 8 mL water was stirred at room temperature overnight. The resulting mixture was treated with Dowex H^+ ion exchange resin until pH reached 4. The mixture was filtered and the filtrate was concentrated to obtain **10** (0.68 g, 94.4 %) as white solid. ^{1}H-NMR (400 MHz, 90% H_2O + 10% D_2O) δ ppm：8.70 (1H, d), 7.97 (1H, s), 7.92 (2H, d), 7.83 (1H, d), 7.65 (1H, t), 7.54 (2H, t). ^{13}C-NMR (100 MHz, 90% H_2O + 10% D_2O) δ ppm: 172.41, 170.86, 155.89, 150.65, 150.32, 137.31, 135.28, 130.03, 128.13, 121.06, 120.65, 58.13, 56.55, 55.56, 51.42, 50.21. MS-ESI: 464.0 (M-1).

Synthesis of L2 (Scheme 2)



Scheme 2. Synthesis of L2. a) AcOH, H_2O_2; b) H_2SO_4, HNO_3; c) CH_3COBr; d) $(CF_3CO)_2O$, CH_2Cl_2; e): $SOCl_2$; f): $NH_2CH_2CH_2NH_2$; g): $BrCH_3COOC_2H_5$, CH_3CN, K_2CO_3, Ar; h): CH_3CN, $PhSO_2Na$, TBAB, Ar; i): NaOH, C_2H_5OH, H_2O; j): H^+

2,6-Dimethyl-4-nitropyridine 1-oxide (**12**). Similar to the previous report[1], compound **11** (7.3 g, 0.06 mol) was mixed with the solution of 20 mL H_2SO_4 and 25 mL fuming HNO_3, and the mixture was stirred at 110 °C for 6 h. The solution was cooled down to room temperature and then poured into to ice cold water. The above mixture was adjusted to pH 12 with K_2CO_3 and then extracted with ethyl acetate. Washed with brine, the organic layer was dried over Na_2SO_4. The mixture was filtered and the solvent was

removed to obtain **12** (7.4 g, 74.2 %) as pale yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.07 (2H, s), 2.58 (6H, s).

4-Bromo-2,6-dimethylpyridine 1-oxide (**13**). 35mL acetyl bromide was added dropwise into the solution of **12** (2.4 g, 0.01 mol) in 35 mL CH_3COOH at 60 ºC. Then the mixture was stirred at 80 ºC for 6 h. The solution was poured into ice cold water, and the pH was adjusted to 10 with K_2CO_3. The mixture was extracted with ethyl acetate, washed with brine and the organic layer was dried over Na_2SO_4. The solution was filtered and the solvent was removed under reduced pressure obtain **13** (2.0 g, 74.2 %) as pale yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 7.41 (2H, s), 2.53(6H, s).

(4-Bromo-6-methylpyridin-2-yl)methanol (**14**). Trifluoroacetic anhydride (8.0 mL, 0.06 mol) in 20 mL dichloromethane was added dropwise to the solution of **13** (2.0 g, 0.01 mol) in 25 mL dichloromethane at room temperature. Then the reaction mixture was refluxed for 15 h. The resulting mixture was cooled down to room temperature, and the solved was removed under reduced pressure. 15 mL of H_2O was added to the residue, and the pH was adjusted to 8.5 with K_2CO_3. The solution was then stirred for 5h at room temperature and the pH was increased to 12 with KOH. The resulting mixture was extracted with dichloromethane. The organic layer was washed with brine, dried over Na_2SO_4, and filtered. The solvent was removed under reduced pressure to **14** (1.2 g, 63.2 %) as yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 7.33 (1H, s), 7.29 (1H, s), 4.74 (2H, s), 2.58 (3H, s).

4-Bromo-2-(chloromethyl)-6-methylpyridine hydrochloride (**15**). Below 5 ºC, 0.8 mL thionyl chloride in 15 mL dichloromethane was added dropwise to the solution of **14** (1.2 g, 5.9 mmol) in 20 mL dichloromethane. The reaction mixture was stirred at 40 ºC for 4 h, and then the solution was concentrated to obtain **6** (1.4 g, 93.3 %) as yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 7.37 (1H, s), 7.20 (1H, s), 3.81 (2H, s), 2.55 (3H, s).

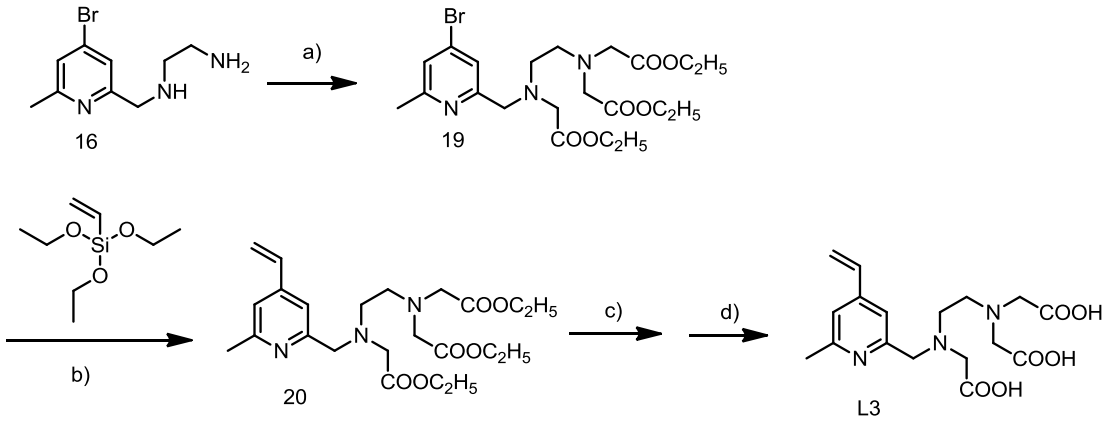
N-((4-bromo-6-methylpyridin-2-yl)methyl)ethane-1,2-diamine (**16**). **15** (1.4 g, 5.45 mmol) in 25 mL acetonitrile was added dropwise to the solution of 6 mL ethanediamine in 20 mL acetonitrile. The reaction mixture was stirred at room temperature for 20h. The solvent was then removed and the solid left was dissolved in 50mL ethyl acetate. The organic layer was washed with brine and dried over Na_2SO_4. Then the mixture was filtered and the filtrate was concentrated to obtain **16** (0.8 g, 60.2 %) as pale brown oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 7.57 (2H, s), 3.91 (2H, s), 2.85 (2H, t), 2.73 (2H, t), 2.56 (3H, s).

Diethyl-2,2'-((2-(((4-bromo-6-methylpyridin-2-yl)methyl)(2-ethoxy-2-oxoethyl)amino)ethyl)azanediyl)diacetate (**17**). 4.0 mL ethylbromo-acetate in 20 mL acetonitrile was added dropwise to the mixture of **16** (0.8 g, 3.28 mmol), K_2CO_3 (4.5 g, 32.8 mmol), KI (0.2 g, 1.2 mmol) and 25 mL acetonitrile. Under argon atmosphere, the reaction mixture was stirred at room temperature for 22 h. The solution was filtered, and the filtrate was concentrated and purified by chromatography to obtain **17** (1.4 g, 83.8 %) as reddish brown oil. ^{1}H-NMR (400 MHzm, $CDCl_3$) δ ppm: 7.48 (1H, d), 7.13 (1H, d), 4.18-4.15 (6H, m), 3.90 (2H, s), 3.58 (4H, s), 3.48 (2H, s), 2.91-2.86 (4H, m), 2.51 (3H, s), 1.30-1.25 (9H, m).

Diethyl-2,2'-((2-((2-ethoxy-2-oxoethyl)((6-methyl-4-(phenylsulfonyl)pyridin-2-yl)methyl)amino)ethyl)azanediyl)diacetate (**18**). **17** (1.2 g, 2.39 mmol), sodium benzenesulfinate (1.2 g, 7.32 mmol) and tetrabutyl ammonium bromide (85 mg, 0.26 mmol) was mixed with 35 mL acetonitrile. Under argon atmosphere, the reaction mixture was stirred at 80 ºC for 17 h. The solvent was then removed under reduced pressure and the left was diluted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4. The mixture was filtered, and the filtrate was concentrated and purified by column chromatography to obtain **18** (0.68 g, 50.8 %) as yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm:7.99 (2H, d, J=8.64 Hz), 7.86 (1H, s), 7.66-7.64 (1H, m), 7.58-7.55 (2H, m), 7.48 (1H, s), 4.19-4.13 (6H, m), 4.01 (2H, s), 3.56 (4H, s), 3.48 (2H, s), 2.88-2.85 (4H, m), 2.60 (3H, s), 1.29-1.26 (9H, m).

2,2'-((2-((Carboxymethyl)((6-methyl-4-(phenylsulfonyl)pyridin-2-yl)methyl)amino)ethyl)azanediyl)diacetic acid (**L2**). The mixture of **18** (1.1 g, 1.95 mmol), NaOH (0.44 g, 11 mmol), 8 mL ethanol and 8 mL water was stirred at room temperature overnight. The reaction mixture was then treated with Dowex H^+ ion exchange resin until the pH reached 4. The mixture was filtered and the filtrate was concentrated to obtain **L2** (0.85 g, 90.7 %) as white solid. ^{1}H-NMR (400 MHz, 90% H_2O + 10% D_2O) δ ppm: 7.89 (3H, t), 7.82 (H, s), 7.64 (H, t), 7.53 (2H, t), 4.21 (2H, s), 3.65 (4H, s), 3.45 (2H, s), 3.32-3.22 (4H, m), 2.55 (3H, s). ^{13}C-NMR (100 MHz, 90% H_2O + 10% D_2O) δ ppm: 173.00, 170.68, 160.53, 154.97, 151.83, 137.04, 135.33, 130.00, 128.14, 121.48, 118.95, 57.22, 56.29, 55.08, 51.55, 50.01, 36.69, 22.12. MS-ESI: 478.0 (M-1).

Synthesis of L3 (Scheme 3)



Scheme 3. Synthesis of L3. a): $BrCH_3COOC_2H_5$, DIPEA; b): DMF, $Pd(OAc)_2$, PPh_3, TBAF; c): NaOH, H_2O; d): H^+.

Diethyl-2,2'-((2-(((4-Bromo-6-methylpyridin-2-yl)methyl)(2-ethoxy-2-oxoethyl)amino)ethyl)azaediyl)diacetate (**19**). Compound **16** (2.5 g) and 9.0 mL N, N-diisopropylethylamine (DIPEA) was added into a solution of 40 mL CH_3CN. Ethyl 2-bromoacetate (5.5 mL in 20 mL CH_3CN) was added dropwise into the above mixture under stirring. The resulting solution was heated to 60 ºC for 10 h and then filtered. The filtrate was evaporated, and the resulting oil residue was purified by chromatography on silica gel eluting **19** as oil liquid (2.66 g, 51.8 %).^{1}H-NMR (400MHz, $CDCl_3$) δ ppm: 7.56 (1H, s), 7.21(1H, s), 4.19 (6H, m), 3.95(2H, s), 3.57 (4H, s), 3.52 (2H, s), 2.89 (4H, m), 2.50 (3H, s), 1.27 (9H, m).

Diethyl-2,2'-((2-(2-ethoxy-2-oxoethyl)((6-methyl-4-vinylpyridin-2-yl)methyl)amino)ethyl)azanediyl)diacetate (**20**) 0.8 g **19** was dissolved in 25 mL DMF, and then 0.75 mL triethoxyvinylsilane, 6.5 mL tetrabutylammonium fluoride (TBAF) in THF stock, 22 mg $Pd(OAc)_2$ and 85 mg PPh_3 (5 mmol) were added stepwise into the above mixture under argon atmosphere. The resulting solution was heated to 90 ºC for 3 h and then cooled down to room temperature. The solution was mixed with 120 mL water and the resulting solution was extracted with ethyl acetate. The combined organic phases were washed with brine, dried with anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The resulted yellowish oil was purified by chromatography on silica using petroleum ether (b.p. 60~90 ºC): ethyl acetate 4:1. 0.41 g yellowish oil was obtained with the yield about 57.7 %. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 7.40 (1H, s), 7.02 (1H, s), 6.65 (1H, dd, J=10.96Hz, J=17.76Hz), 5.99 (1H, J=17.76Hz), 5.46 (1H, J=10.96Hz), 4.14 (6H, m), 3.94 (2H, s), 3.58 (4H, s), 3.47 (2H, s), 2.89 (4H, m), 2.53 (3H, s), 1.24 (9H, m).

2,2'-((2-((carboxymethyl)((6-methyl-4-vinylpyridin-2-yl)methyl)amino)ethyl)azanediyl)diacetic acid (**L3**) 0.6 g **20** was first mixed with 5 mL ethanol and 5 mL H_2O, and then 0.23 g NaOH in 2 mL H_2O was added into the above mixture. The resulting solution was stirred at room temperature overnight. Dowex H^+ ion exchange resin was added to the above mixture and the solution was filtered till the pH decreased to 3. The solution was evaporated under reduced pressure and the solid was suspended in 10 mL acetone and filtered. 0.36 g white solid was obtained and the yield was 75 %. ^{1}H-NMR (400 MHz, D_2O, pD > 12) δ ppm: 7.20 (1H, s), 7.11 (1H, s), 6.61 (1H, dd, J=19.0 Hz, J=11.6 Hz), 5.96 (1H, J=19.0 Hz), 5.42 (1H, J=11.6 Hz), 3.56 (2H, s), 3.02 (2H, s), 2.81 (4H, s), 2.47 (4H, m), 2.32 (3H, s). ^{13}C-NMR (100MHz, D_2O, pD > 12) δ ppm: 178.3, 158.6, 157.5, 138.2, 134.3, 119.7, 118.8, 59.8, 58.7, 58.4, 51.3, 50.4, 23.1. MS (ESI): 364.2(M-1).

2. General procedure for site-specific labeling of proteins with spin labels

1mL 1.0 mM solution of ubiqituin T22C/G47C or G35C/E64C mutant and 0.1 mM TCEP in 20 mM Tris buffer at pH 8.0 was mixed with 10 equivalents of spin label (L1, L2 or L3) that was in 50 mM stock in aqueous solution, and the pH value of the above mixture was adjusted to 8.2 using 2.0 M NaOH. The resulting solution was incubated at room temperature for about 10 hours. Subsequently, the ligation product was purified through anion exchange column. The overall yield of the doubly spin labled protein was about 70%.

The reactivity of cysteine in a protein depends on its local structural environment. L3 has the lowest reactivity towards protein thiols, while T22C is less reactive towards the tags than G47C, E64C and G35C. The thiol chemoreactivity of the cysteine mutants in ubiquitin is E64C, G47C > G35C >> T22C. For the modified protein the chemoreactivity of these tags is L2 > L1 > L3.

3. Sample preparation for DEER measurements

The protein samples containing paramagnetic metal ions were prepared by monitoring the ^{15}N-HSQC spectroscopy of ^{15}N-labelled proteins with the addition of paramagnetic ions. In particular, ^{15}N-HSQC spectrum of 0.10 mM ^{15}N-labeled protein sample (T22C/G47C and its bioconjugates with L1 and L2, respectively) was first recorded in 20 mM MES buffer at pH 6.5 and $MnCl_2$ in 10 mM stock solution was added up to [metal ion]:[protein] = 1.8:1. ^{1}H-protein NMR spectra were recorded for the samples of T22C-L3/G47C-L3, and G35C/E64C conjugates with L1 and L2, respectively. The protein signals were monitored by addition of 10 mM $MnCl_2$ till the [metal ion]:[protein] reached 1.8:1. All NMR spectra were recorded on a 600 MHz Bruker Avance spectrometer equipped with a QCI-cryoprobe at 298K.

The prepared protein samples were lyophilized and re-dissolved in D_2O/glycerole-d_8 (7/3 volume/volume) for DEER measurement.

4. Mass spectroscopy analysis of protein-spin labels

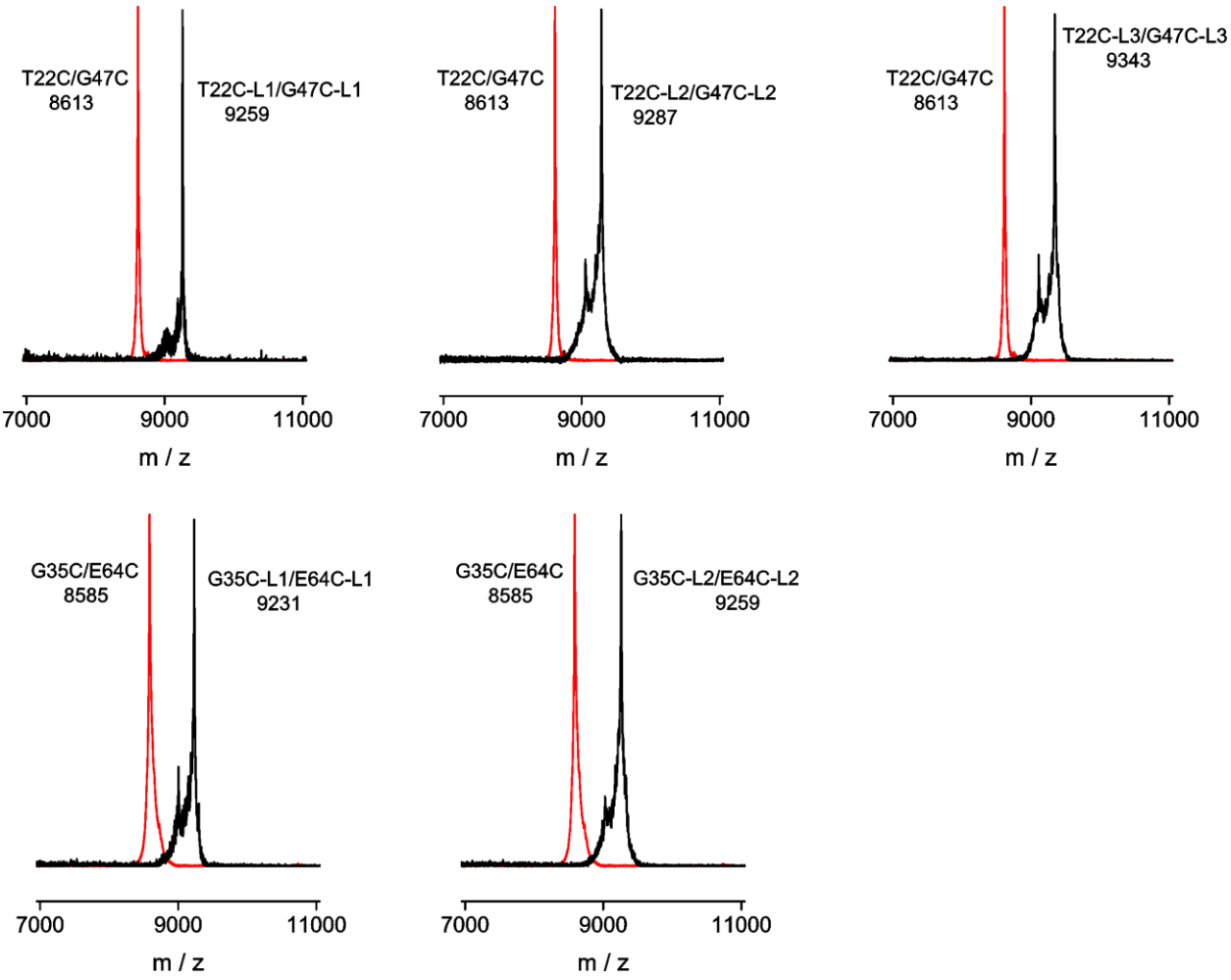


Fig. S1. MALDI-TOF spectra of ubiquitin T22C/G47C and G35C/E64C doubly labeled with L1, L2 and L3, respectively, where the molecular mass signal of the free protein and the labeled protein are in red and black, respectively. The difference of molecular mass between free protein and protein-spin label indicates the protein was labeled at two sites.

5. NMR spectra of proteins labeled with L1 and L2

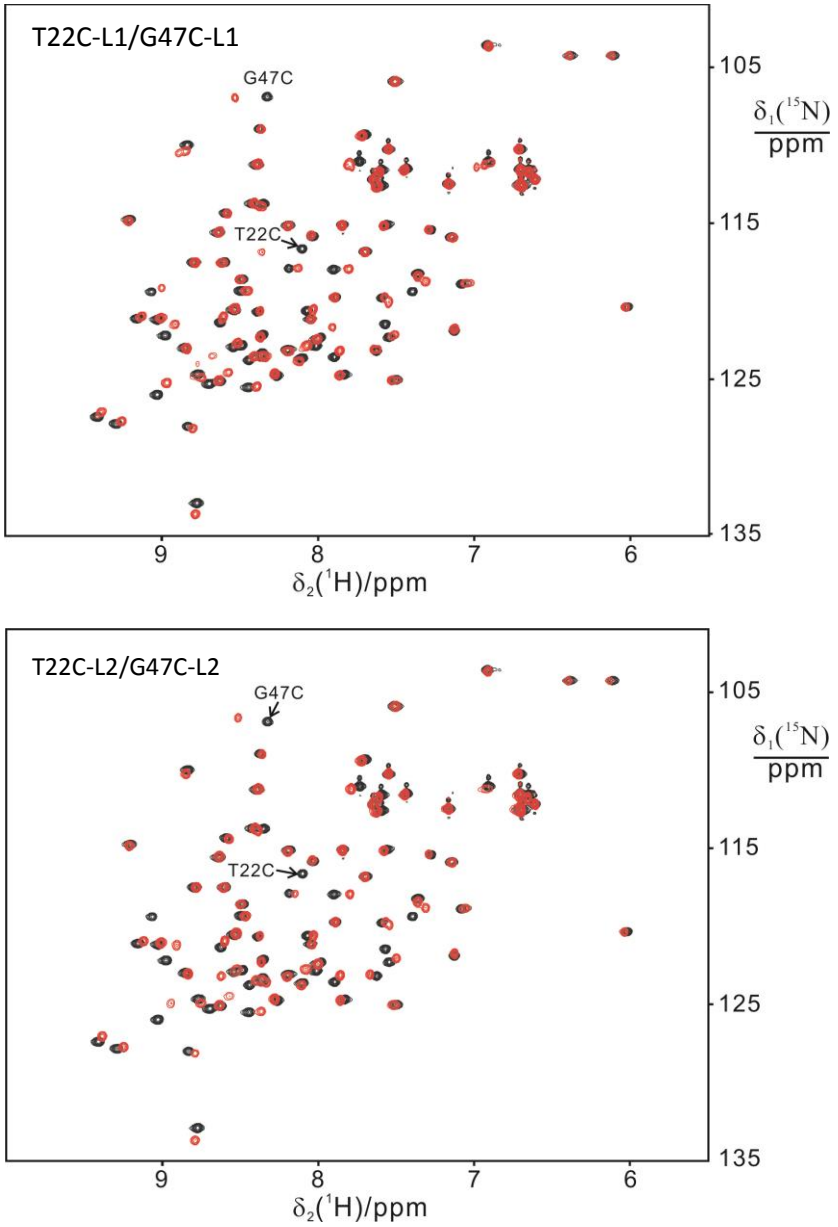


Fig S2. Superposition of ^{15}N-HSQC spectra of ubiquitin T22C/G47C (black) before (black) and after labeling with L1 or L2 (red), respectively. All NMR spectra were recorded for 0.10 mM protein samples in 20 mM MES, pH 6.4 on a 600 MHz NMR spectrometer at 298K.

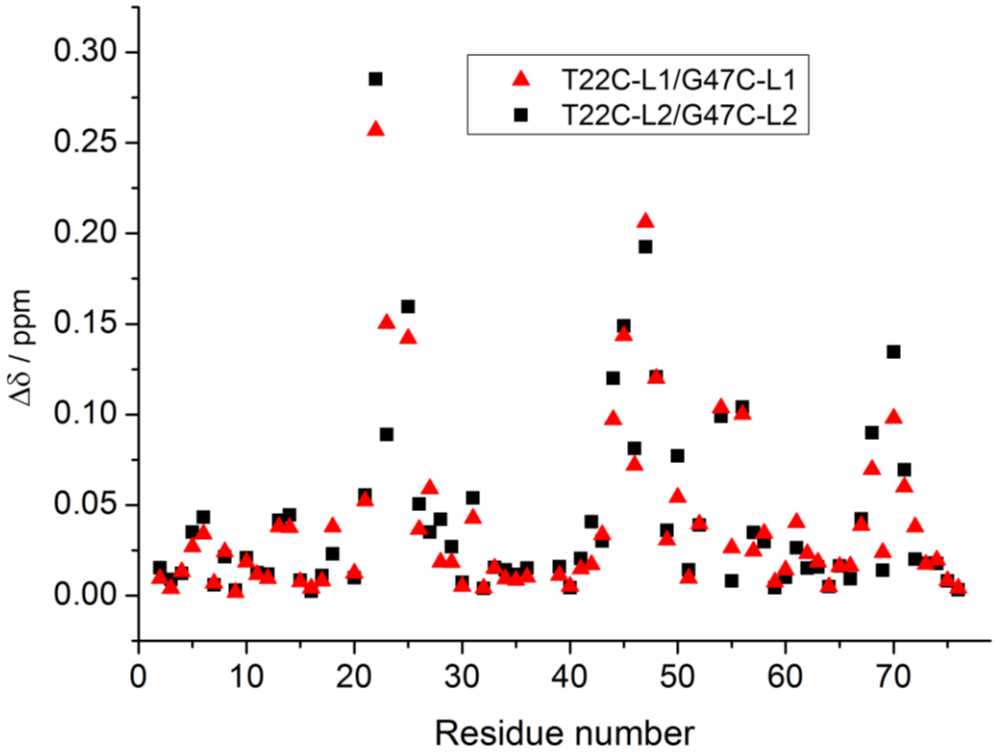


Fig. S3. The chemical shifts changes of protein backbone amides for human ubiquitin T22C/G47C labeled with L1 (red) and L2 (black), respectively. The chemical shift differences were calculated as $\Delta\delta = \sqrt[2]{(\Delta\delta_{HN})^2 + (\Delta\delta_N/10)^2}$. The chemical shift mapping suggests that the two labels in T22C/G47C produce negligible structural changes in protein.

6. Echo decay kinetics and raw DEER data

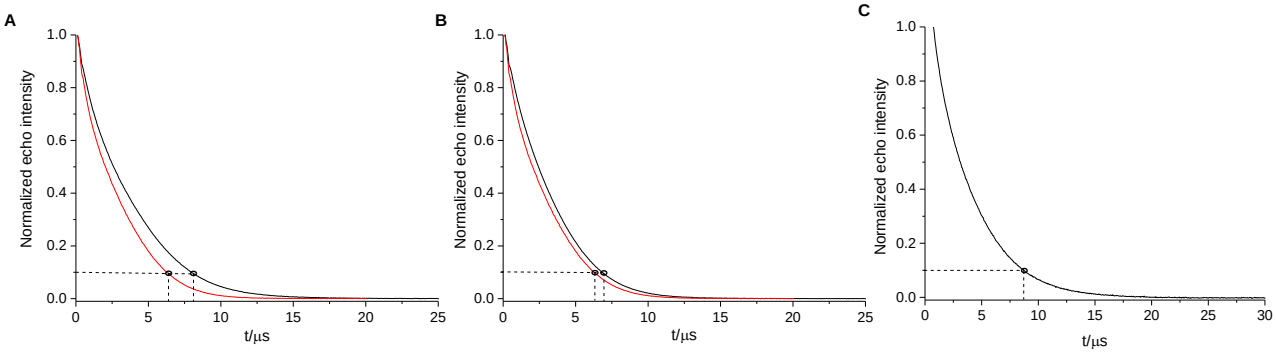


Fig. S4. Two pulse -echo decay of : A. T22C/G47C doubly labeled with L1-Mn^{2+} (black) and L2-Mn^{2+} (red); B. G35C/E64C doubly labeled with L1-Mn^{2+} (black) and L2-Mn^{2+} (red); C. T22C/G47C doubly labeled with L3-Mn^{2+} recorded at the maximum of the ED-EPR spectra. The open circles represent the echo at 10% of its maximum intensity.

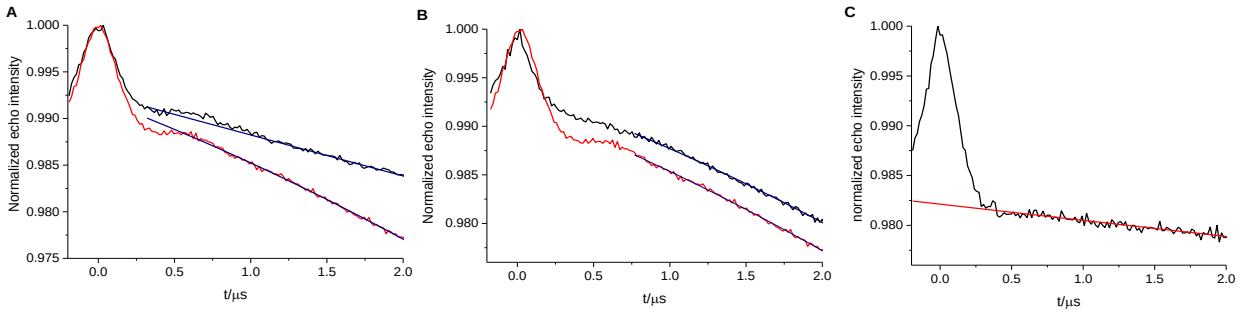


Fig.S5. Experimental DEER traces and background fit. A. T22C/G47C doubly labeled with L1-Mn^{2+} (black) and L2-Mn^{2+} (red); B. G35C/E64C doubly labeled with L1-Mn^{2+} (black) and L2-Mn^{2+} (red); C. T22C/G47C doubly labeled with L3-Mn^{2+}.

7. Simulations of ED-EPR spectra and experimental ELDOR detected NMR spectra

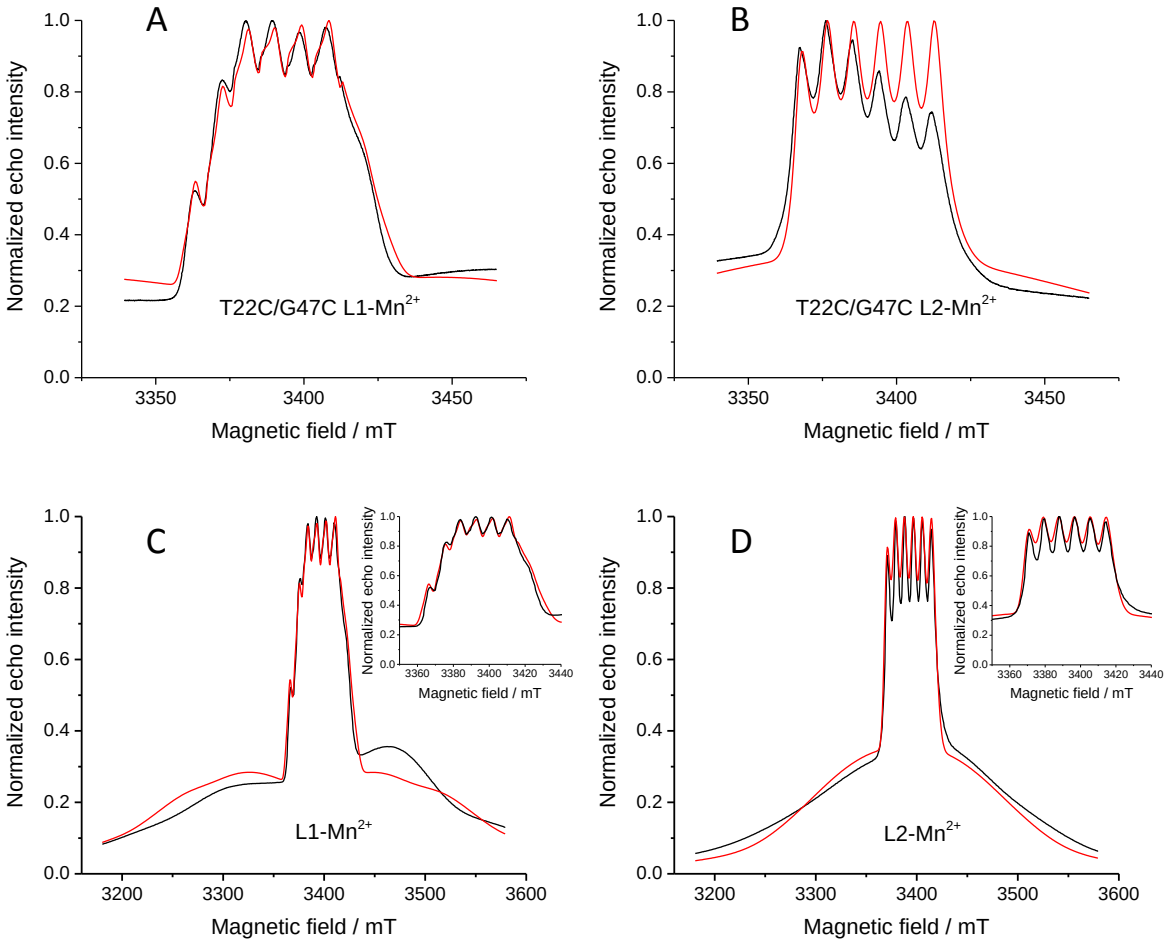


Fig. S6. W-band experimental and simulated ED-EPR spectra of T22C/G47C L1-Mn^{2+} (A) , T22C/G47C L2-Mn^{2+} (B), L1-Mn^{2+} (C) and L2-Mn^{2+} (D) . In (C) and (D) the inset shows a blow up of the central transition region. For the T22C/G47C samples the lengths of the π/2 and π pulses were 30 and 60 ns, optimized on the central transition and the τ value used was 750 ns. For L1-Mn^{2+} and L2-Mn^{2+} the π/2 and π pulse lengths were 15 ns and 30 ns and τ = 550 ns. The simulation parameters are given in Table S1. The simulations are of the absorption continuous wave EPR spectrum and not the ED-EPR spectrum and as such they not take into account the different nutation frequencies of the various transitions and their potentially different phase memory time. Therefore discrepancies are expected, particularly in the relative intensities of the central

transition and the other transitions. The simulations were obtained with Easyspin.[5] In the simulations we focused on the total width of the spectrum and the lineshape of the central transition. The experimental continuous wave EPR spectrum tends to undermine the contribution of broad lines because it appears as the first derivative of the absorption spectrum. Although the absorption spectrum can be recovered by integration, it is highly susceptible to baseline distortions and therefore ED-EPR is preferred.

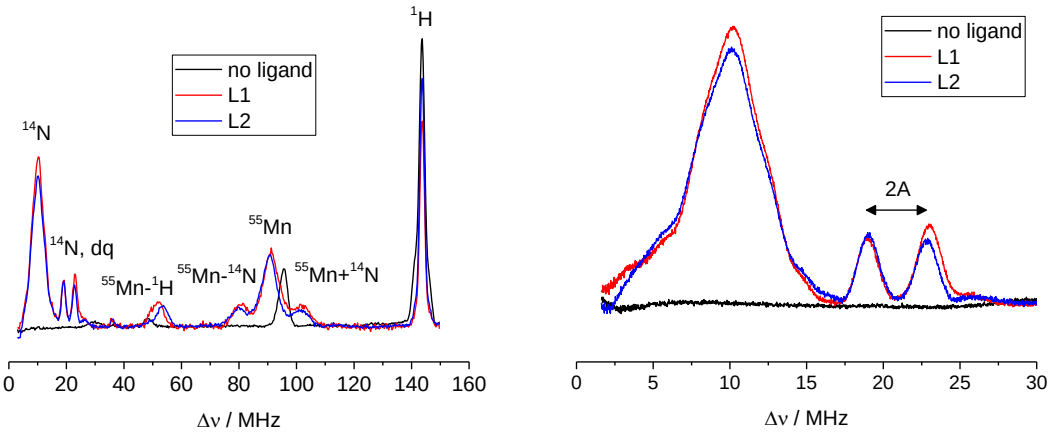


Fig. S7. W-band, 10 K, ELDOR detected NMR of L1- Mn^{2+}-and L2-Mn^{2+} (150 mM:250mM respectively). Left panel: full scale, right panel: focus on the ^{14}N region with the splitting of the two double quantum transitions giving the hyperfine coupling, A. The signals are assigned in the figure. The spectra are shown after background subtraction and multiplication of the intensities by -1. The sequence used was $t_{sat} - t - \pi/2 - \tau - \pi - \tau - (\tau - t_{comp} - \pi - \tau - echo - \tau - t_{comp} - \pi - \tau - echo)_N$. The length of the saturation pulse was 100 μs and the echo detection pulses were π/2 = 100 ns, π = 200 ns, τ = 1 μs, t = 1 μs, t_{comp} = 100 ns and B_0 = 3378 mT. CPMG sequence was used to increase the signal/noise ratio.[6]

Table S1. The parameters used to simulate the spectra shown in Fig. S6. The values in parenthesis denote the D and E strain.

sample	D, MHz	E, MHz	A_{iso}, MHz[a]	g[a]
T22C/G47CL1-Mn^{2+}	3060(600)	459 (200)	247	2.0026
L1-Mn^{2+}	3060(600)	459(200)	254	2.0019
T22C/G47CL2-Mn^{2+}	1920 (600)	576 (200)	252	2.0019
L2-Mn^{2+}	1860(900)	434 (600)	247	2.0007

[a] The difference in A_{iso} , the isotropic hyperfine coupling constant , and the g values are most probably due to the different sweep rates of the field for the small and wide range sweeps and should not considered significant.

8. Binding affinity assay of L1 and L2 with Mn^{2+} by high resolution NMR spectroscopy

The solution of 100 mM $MnCl_2$ was made at by dissolving the $MnCl_2$ $4H_2O$ in MiliQ water without adjusting the pH.

Interaction of L1 with Mn^{2+}

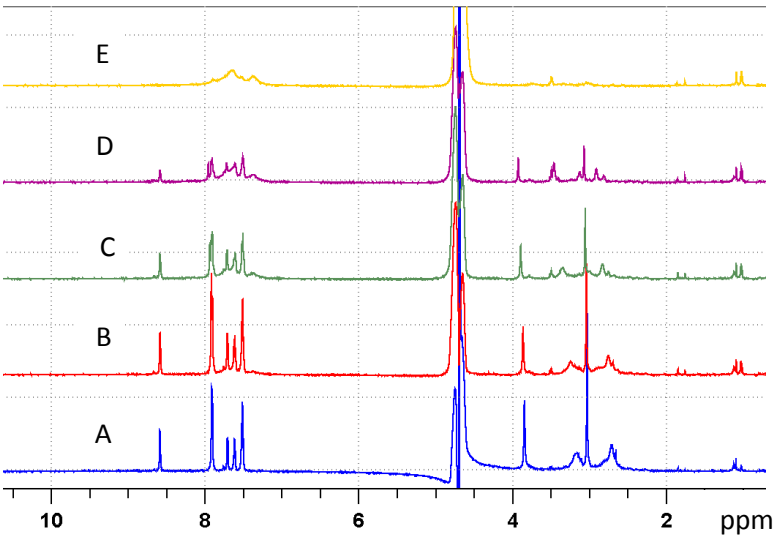


Fig. S8. Binding Mn^{2+} to L1 in D_2O analyzed by 1D ^{1}H-NMR spectra. A) 1.6 mM L1 in D_2O at pD 10.0; B) A + 25% $MnCl_2$; C) A + 50% $MnCl_2$; D) A + 75% $MnCl_2$; E) A + 100% $MnCl_2$. This experiment shows that the complex formed between L1 and Mn^{2+} is in slow exchange at the NMR time scale, indicating a tight binding with a K_d below the micromolar range. All the spectra were recorded at 298K on a 600 MHz NMR spectrometer. The pH during the titration was not adjusted.

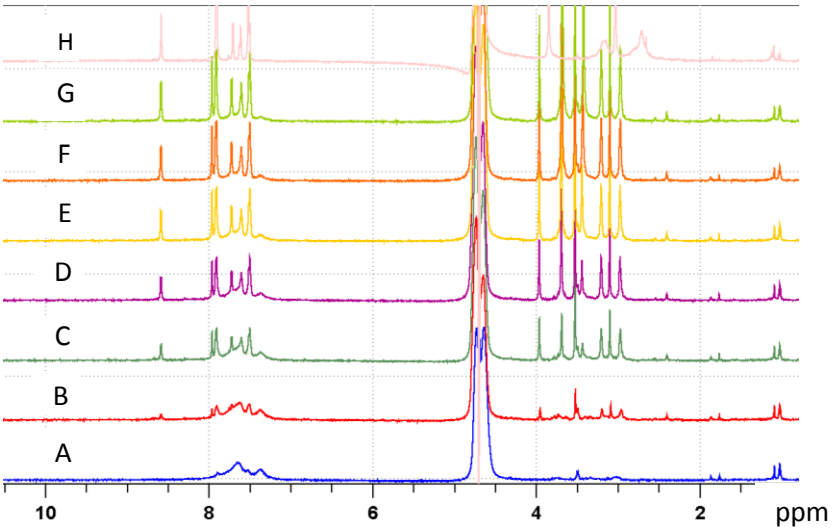


Fig. S9. Binding competition experiment of EDTA and L1 with Mn^{2+} determined by 1D ^{1}H-NMR spectra. A) the same as Fig. S8E; B) A + 25% EDTA (pH 6.0); C) A + 50% EDTA (pH 6.0); D) A + 75 % EDTA (pH 6.0); E) A + 100 % EDTA (pH 6.0); F) A + 125 % EDTA (pH 6.0); G) A + 150 % EDTA (pH 6.0); H) Fig. S8A. The experiments indicate that L1 has similar binding affinity to EDTA for Mn^{2+} since the certain amount of manganese(II) bound L1 is still present with

excess of EDTA as shown F) to G). All the spectra were recorded at 298K with 600 MHz NMR spectrometer. The pH during the titration was not adjusted.

Interaction of L2 with Mn^{2+}

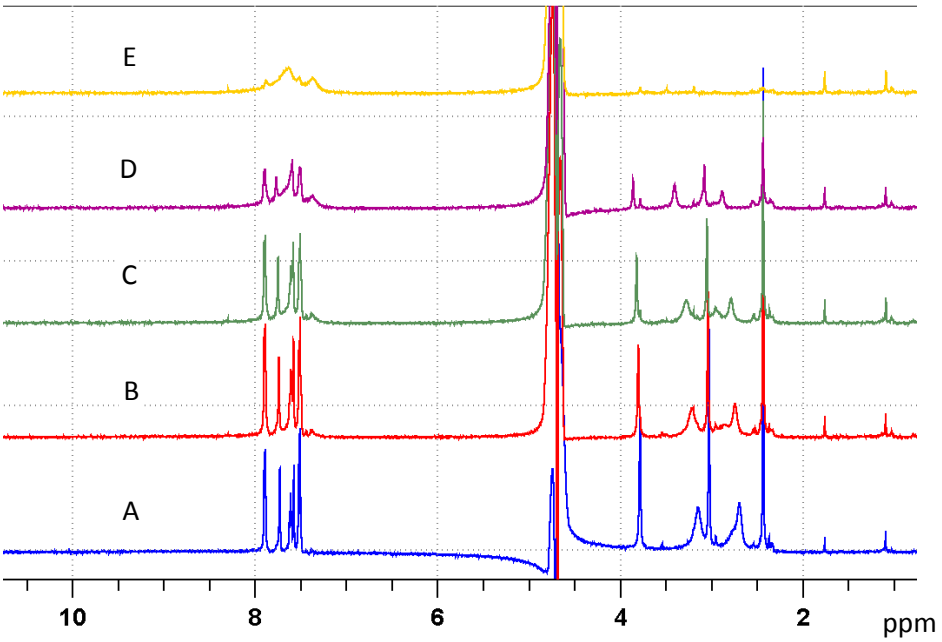


Fig. S10. Binding of Mn^{2+} to L2 wit in D_2O analyzed by 1D ^{1}H-NMR spectra. A) 1.6 mM L2 in D_2O at pD 10.0; B) A + 25% $MnCl_2$; C) A + 50% $MnCl_2$; D) A + 75% $MnCl_2$; E) A + 100% $MnCl_2$. The experiment shows that the complex formed between L2 and Mn^{2+} is in slow exchange at NMR time scale, indicating a tight binding with a K_d below micromolar range. All the spectra were recorded at 298K on a 600 MHz. NMR spectrometer. The pH during the titration was not adjusted.

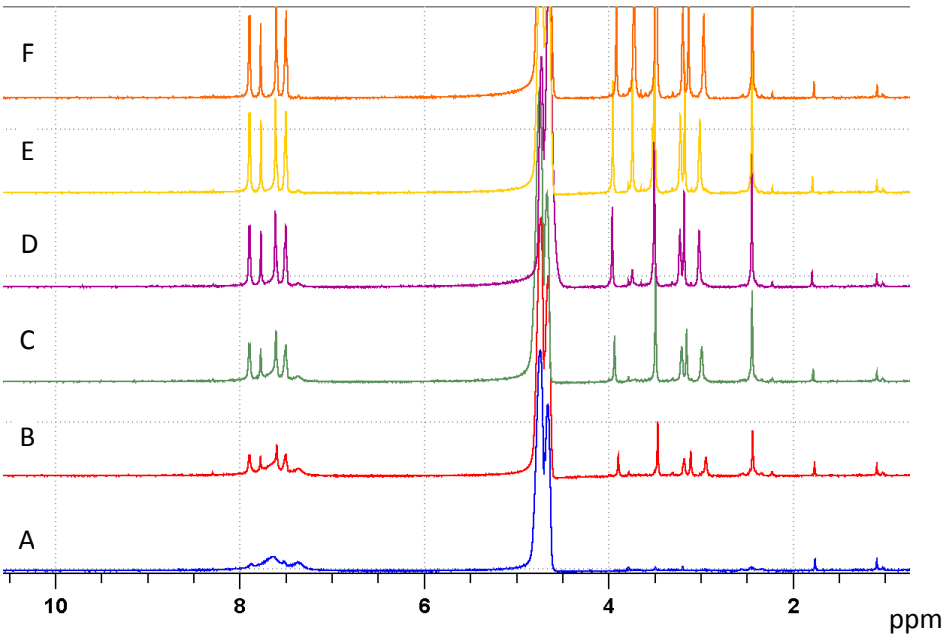


Fig. S11. Binding competition experiment of EDTA and L2 with Mn^{2+} determined by 1D ^{1}H-NMR spectra. A) the same as Fig. S10E; B) A + 25% EDTA (pH 6.0); C) A + 50% EDTA (pH 6.0); D) A + 75 % EDTA (pH 6.0); E) A + 100 % EDTA (pH 6.0); F) A + 150 % EDTA (pH 6.0). The experiments indicate that L2 has a lower binding affinity than EDTA

for Mn^{2+} but in a similar order since the free NMR signals of L2 still increase with increase of EDTA as shown D) to F). All the spectra were recorded at 298K on a600 MHz spectrometer. The pH during the titration was not adjusted.

Binding measurements by EPR

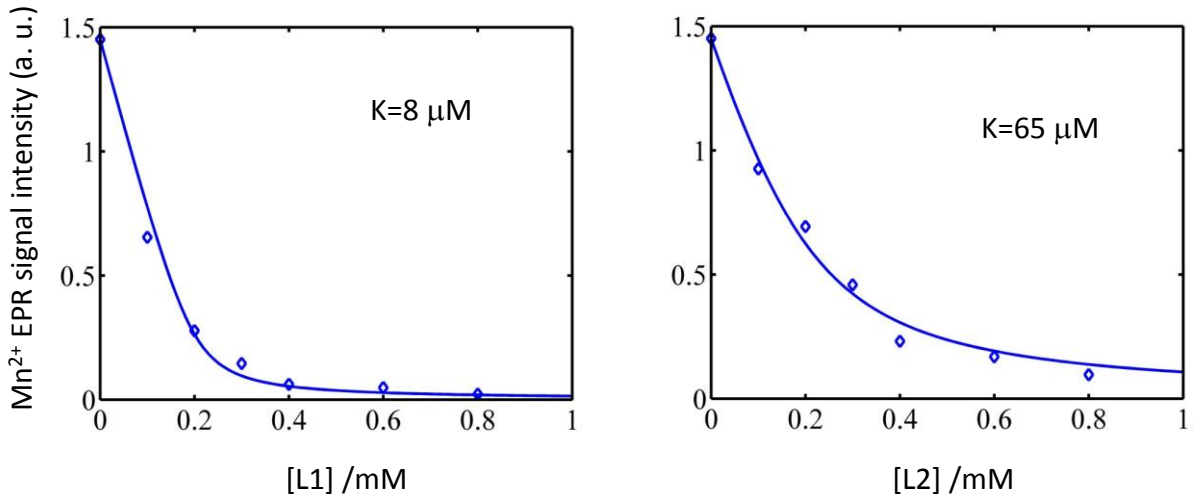


Fig. S12. Measurements of the binding constant of Mn^{2+} to L1 and L2 using X-band EPR spectra at room temperature. Here the intensity of the Mn^{2+} EPR spectrum is measured as a function of [L1] (left) and [L2] (right). The solid line represents the calculated curve with the K values noted on the Figure. All samples were in H_2O and the pH was adjusted to 7.

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