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Electronic Supplementary Information

Rigid Mn(II) Chelate as Efficient MRI Contrast Agent for Vascular Imaging

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Experimental details:

2,6-Pyridinedicarboxylic acid, DL-2-Piperidinecarboxylic acid, Manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) was purchased from Sigma and used as received. Sodium borohydride ($NaBH_4$), thionyl chloride ($SOCl_2$) was purchased from Aladdin

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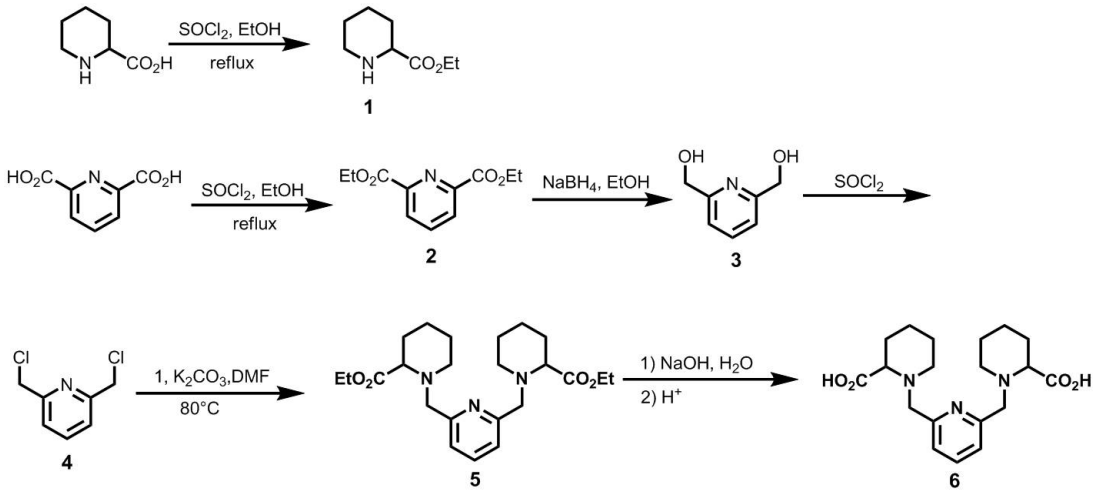
and used as received. Other reagents used in the synthesis, if not specified, were used as received. All reactions were carried out under an argon atmosphere. Reactions were monitored by TLC. ^{1}H and ^{13}C NMR spectra were recorded on a Brucker AM400. Mass spectrometry was obtained from ESI-MS (Finnigan TSQ Quantum Ultra). Elemental analysis was performed on a LEEMAN instrument Euro EA-3000 elemental analyzer. Single crystal data were collected using on a Xcalibur Eos CCD diffractometer (Mo-Kα, λ = 0.794 Å). Concentration of Mn^{2+} was measured using an atomic absorption spectroscopy (AAS) (AA800, Perkin-Elmer, USA). T_1 relaxivity was measured at 1.5 T on a clinical MR scanner (Siemens Sonata). *In vivo* MR imaging was carried out on a 3 T imaging system (Philips Medical System, Intera 3T). Inductively coupled plasma atomic emission spectroscopy (ICP-AES, IRIS Advantage, Thermo Electron, USA) method was used for the quantitative assessment of Mn^{2+} uptake by tissue *in vivo*. The synthetic process of the ligand is illustrated in Scheme S1.



Scheme S1: Synthesis route of the ligand

Ethyl piperidine-2-carboxylate (1)

To a solution of DL-2-Piperidinecarboxylic acid (5.0 g, 39.0 mmol) in 150 mL ethanol at 0 °C was added $SOCl_2$ (4.5 mL, 58 mmol) slowly. The result mixture was refluxed for 2 h and then concentrated under vacuum to give a crude oil. A 100 mL ethyl ether was added into the crude oil and stirred for several minutes, the suspension

was filtrated to afford **compound 1** as a white solid; 7.1 g, (93%). ^{1}H-NMR (400 MHz, DMSO-d_6, ppm) δ: 9.30 (d, 2H), 4.23 (q, J = 7.0 Hz, 2H), 4.06 (d, J = 10.2 Hz, 1H), 3.24 (d, J = 12.5 Hz, 1H), 2.89 (t, J = 10.7 Hz, 1H), 2.07 (d, J = 10.9 Hz, 1H), 1.83 – 1.44 (m, 5H), 1.24 (t, J = 7.1 Hz, 3H). ESI-MS (m/z): 158.15 $[M+H]^+$.

Diethyl pyridine-2,6-dicarboxylate (2)

To a solution of 2,6-Pyridinedicarboxylic acid (10 g, 60.0 mmol) in 120 mL ethanol at 0°C was added 10 mL $SOCl_2$ slowly. The result mixture was refluxed for 5 h and then concentrated under vacuum. The residue was taken up in ethyl ether. The organic phase was washed with a saturated aqueous solution of sodium bicarbonate then dried over sodium sulfate and concentrated. **Compound 2** was obtained as a white solid; 8.9 g, (66%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.26 (d, J = 7.8 Hz, 2H), 7.99 (t, J = 7.8 Hz, 1H), 4.46 (q, J = 7.1 Hz, 4H), 1.43 (t, J = 7.1 Hz, 6H). ESI-MS (m/z): 224.00 $[M+H]^+$.

Pyridine-2,6-diyldimethanol (3)

To a solution of **compound 2** (4.5 g, 20.0 mmol) in 80 mL ethanol at 0 °C was added $NaBH_4$ (3.3 g, 86.8 mmol) slowly. The reaction mixture was stirred at room temperature for 2 h and then refluxed for 5 h. The crude mixture was concentrated under vacuum and 100 mL saturated aqueous solution of potassium carbonate was added. The result mixture was stirred at 60 °C for 2 h and then extracted with chloroform (100 mL, three times). The organic phase dried over sodium sulfate and concentrated. **Compound 3** was obtained as a white solid; 2.4 g, (86%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.70 (t, J = 7.6 Hz, 1H), 7.21 (d, J = 7.6 Hz, 2H), 4.82 (s, 4H). ESI-MS (m/z): 139.92 $[M+H]^+$.

2,6-bis(chloromethyl)pyridine (4)

Compound 3 (2.40 g, 17.3 mmol) was added slowly to 20 mL of $SOCl_2$ at 0 °C. The reaction mixture was stirred for 1 h and then refluxed for 2 h. The crude mixture was

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concentrated under vacuum and 20 mL of H_2O was added. The solution was filtrated and saturated aqueous solution of sodium bicarbonate was added in drops into the filtrate. The precipitate was isolated by filtration to afford **compound 4** as a white solid; 2.57 g, (85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.93 (t, J = 7.8 Hz, 1H), 7.58 (d, J = 7.8 Hz, 2H), 4.81 (s, 4H). ESI-MS (m/z): 175.90 $[M+H]^+$.

Diethyl 1,1'-(pyridine-2,6-diylbis(methylene))dipiperidine-2-carboxylate (5)

Compound 1 (1.0 g，5.71 mmol), **4** (3.6 g，13.7 mmol) and anhydrous potassium carbonate (K_2CO_3, 10 g, 73 mmol) was dissolved in 80 mL dry acetonitrile. The reaction mixture was refluxed until full conversion was observed by TLC. The crude product was purified by chromatography on silica gel (ethyl acetate/hexane = 1/2) and **compound 5** was obtained as a colorless oil; 1.9 g, (80%). ^{1}H-NMR (400MHz, $CDCl_3$, ppm) δ：8.04（t，J = 7.8 Hz， 1H), 7.56 (d, J = 7.8 Hz, 2H)，4.20（m，6H）, 3.90(d, J =14.0, 2H), 3.60(m, 2H), 3.20(m, 2H), 3.00(m, 2H), 2.26(m, 2H), 1.86(m, 4H), 1.65-1.40(m, 6H), 1.26(t, J = 7.2 Hz, 6H). ^{13}C-NMR (100MHz, $CDCl_3$, ppm) δ: 173.86, 158.26, 136.78, 121.05, 77.37, 77.05, 76.74, 64.58, 62.14, 60.31, 50.64, 29.62, 25.30, 22.47, 14.31. ESI-MS (m/z): 418.13 $[M+H]^+$, 440.06 $[M+Na]^+$.

1,1'-(pyridine-2,6-diylbis(methylene))dipiperidine-2-carboxylic acid (6)

Compound 5 (1.2 g，2.87 mmol) and NaOH (275 mg, 6.88 mmol) was stirred in ethanol (25 mL, 80% aq.) at room temperature until full conversion was observed by TLC. pH value of the result mixture was adjusted to 5.0 by adding HCl (1.0 M aq.), and the solvent was evaporated to afford a stringy solid. The residue was taken up in 30 mL chloroform and then dried over sodium sulfate and concentrated. **Compound 6** was obtained as a white solid; 1.0 g, (97%). ^{1}H NMR (400 MHz, D_2O) δ: 8.04 (t, J = 7.8 Hz ,1H), 7.56 (d, J = 7.8 Hz, 2H), 4.81 – 4.65 (m, 2H), 4.54 – 4.34 (m, 2H), 3.83 (d, J = 9.8 Hz, 2H), 3.53 (t, J = 15.1 Hz, 2H), 3.14 (dd, J = 27.5, 17.1 Hz, 2H), 2.28 (d, J = 13.9 Hz, 2H), 2.14 – 1.50 (m, 10H). ^{13}C-NMR (100 MHz, D_2O, ppm) δ: 173.24,

149.86, 139.79, 125.28, 66.43, 59.00, 52.60, 26.74, 21.68, 20.79. ESI-MS (m/z): 362.31 $[M+H]^+$.

Complexation Procedure

To a degassed aqueous solution of **compound 6** (361 mg, 1.0 mmol) and NaOH (80 mg，2.0 mmol) was added a solution of $MnCl_2 \cdot 4H_2O$ (213 mg，1.0 mmol) in water. The resultant solution was stirred at room temperature for 5 h, and then the pH was adjusted to 7.0. After water was evaporated under vacuum, the residue was then taken up in 30 mL chloroform (1% methanol), the insoluble salts was removed by filtration and the filtrate was concentrated to afford the Mn^{2+} complex as a white solid; 0.3 g, (72%). Further purification of the complex was carried out by sephadex column chromatography (Sephadex LH-20, $MeOH/H_2O$ = 1/2, v/v) to ensure there was no excess Mn^{2+} before using for measurement in solution, *in vitro* and *in vivo*, except the preparation of single crystals for X-ray analysis. ESI-MS (m/z): 415.21 $[MnL+H]^+$, 441.75 $[Mn_3L_2]^{2+}$, 457.78 $[MnL+2H_2O+Li]^+$. Elemental analysis for $Mn \cdot C_{19}H_{25}N_3O_4 \cdot 6H_2O$ found (calculated): C, 44.26 (43.68); N 7.97 (8.04); H 6.18 (7.14); Mn 11.40 (ICP-AES, calculated: 10.52).

X-ray crystallography

Single crystals for the X-ray analysis were prepared by a slow vapor diffusion of acetone into the methanol solution (drops of H_2O) of the Mn(II) complexes (without purification through Sephadex LH-20 column) at room temperature. Selected crystals were mounted on glass capillaries at 145.0 K, The diffraction data were collected on a Xcalibur Eos CCD diffractometer (Enraf- Nonius) using Mo KR (λ = 0.794 Å) and analyzed with the Olex2, the structure was solved with the ShelXS structure solution program using Direct Methods and refined with the ShelXL refinement package using Least Squares minimization.

Stability Constant Determination

Titration pH measurements of the ligand in the absence and presence of Mn^{2+} were

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performed with a METTLER TOLEDO pH meter equipped with a LE438 semi-micro electrode. The electrode was calibrated before each titration. A plot of pH (measured) versus V_{KOH} gave a working slope. The temperature of each solution, maintained in a covered, water-jacketed vessel, was kept constant at 25.0 ± 0.1 °C by a NESLAB RTE7 circulating bath, and the ionic strength was kept constant at 0.10 M KCl. MilliQ water used in this experiment was freshly boiled and carbon dioxide was excluded by bubbling nitrogen through it.[1]

The ligand solutions (1-2 mM) were titrated with KOH (0.1 M) over a pH range from 3 to 12 collecting about 40 data points per titration. The titration data was fit to a model of a ligand with four ionizable groups using the program HYPERQUAD, the value of p*K*w was fixed at 13.77. Equimolar metal/ligand solutions were titrated (40 data points per titration) over the pH range 3-12 with KOH for Mn^{2+}, and the stability constants were determined by analysis of the titration curve with HYPERQUAD. The Mn(II) data was fit to a model containing three metal-ligand species: MnL, MnHL, and MnH_2L.

T_1 relaxivity measurement

Solution of the complex was prepared by dissolving 9.6 mg of the Mn^{2+} complex in 5 mL H_2O, concentration of Mn^{2+} in the result solution was measured to be 258 mg/L by elemental analyses using atomic absorption spectroscopy. T_1 relaxivities of a series of the solution ([Mn^{2+}]: 1.5, 1.2, 0.9, 0.6, 0.3, 0.25, 0.2, 0.08 mM) were measured at 1.5 T on a clinical MR scanner (Siemens Sonata) at room temperature as described before.[2] The T_1-weighted images were acquired with a conventional spin echo acquisition (TE = 5.3 ms) with TR values ranging from 20 to 1000 ms. Relaxivity values of r_1 were calculated through the curve fitting of 1/relaxation time (s^{-1}) versus the manganese concentration (mM).

In vivo MRI studies

All studies involving animals were approved by the Animal Care and Use Committee of the Institute. MR imaging was carried out on a 3 T imaging system (Philips

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Medical System, Intera 3T) by using a rat coil (Philips) for transmission and reception of the signal. Sprague-Dawley (SD) Rats (180 - 220 g) were anaesthetized by pentobarbital sodium at the dose of 40 mg/kg body weight. Dynamic T_1-weighted images of chest and cervical region were obtained before and after intravenous injection (via tail vein) of a total 0.5 mL solution of the complex with the dosages of 0.5 mmol/kg body weight. The MRI signal intensity enhancements were monitored before and after injection of MRI agents up to 90 min, with images taken every 2~7 min. Signal intensity of liver and kidney was measured at each time point and plotted against time. T_1-weighted images were obtained with a T1W_TFE_IP sequence, TR = 5 ms, TE = 2 ms, field of view (FOV) = 160 mm × 160 mm, slice thickness = 0.9 mm, Flip angle = 30°. The intensity enhancement (IE) of region of interest (ROI) at point *t* was calculated as: IE (%) =100(ROI_t –ROI_o)/ ROI_o, where ROI_t and ROI_o correspond to the normalized signal intensity measured at time *t* and pre-injection.

Accumulation of Mn(II) in tissues

To assess the accumulation of contrast agents in the tissues, the amount of manganese in liver, kidney, spleen and brain of rats at pre-injection, 24 h and 48 h post-injection of the complex through tail vein was analyzed by ICP-AES. In brief, each rat was anaesthetized by pentobarbital sodium at the dose of 40 mg/kg body weight, after heart perfusion by normal saline, rats were humanely sacrificed. liver, kidney, spleen and brain (including cerebellum) tissues were removed, weighed, and completely digested in 2.0 ml solution of 75% hydrochloric acid and 25% nitric acid (3 : 1) for 48 h. After centrifugation, the solution was diluted with MilliQ water, Mn(II) concentration in the sample was determined by calculating with a standard curve obtained using Mn(II) calibration standard. The percent-injected dose per gram (%ID/gram) were calculated according to references.[3, 4] The tissue Mn from the control group (without complex administration) was deducted as a background from the calculation.

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4. T. J. Wadas, C. D. Sherman, J. H. Miner, J. R. Duncan and C. J. Anderson, *Magnet Reson Med*, 2010, **64**, 1274-1280.

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Figures:

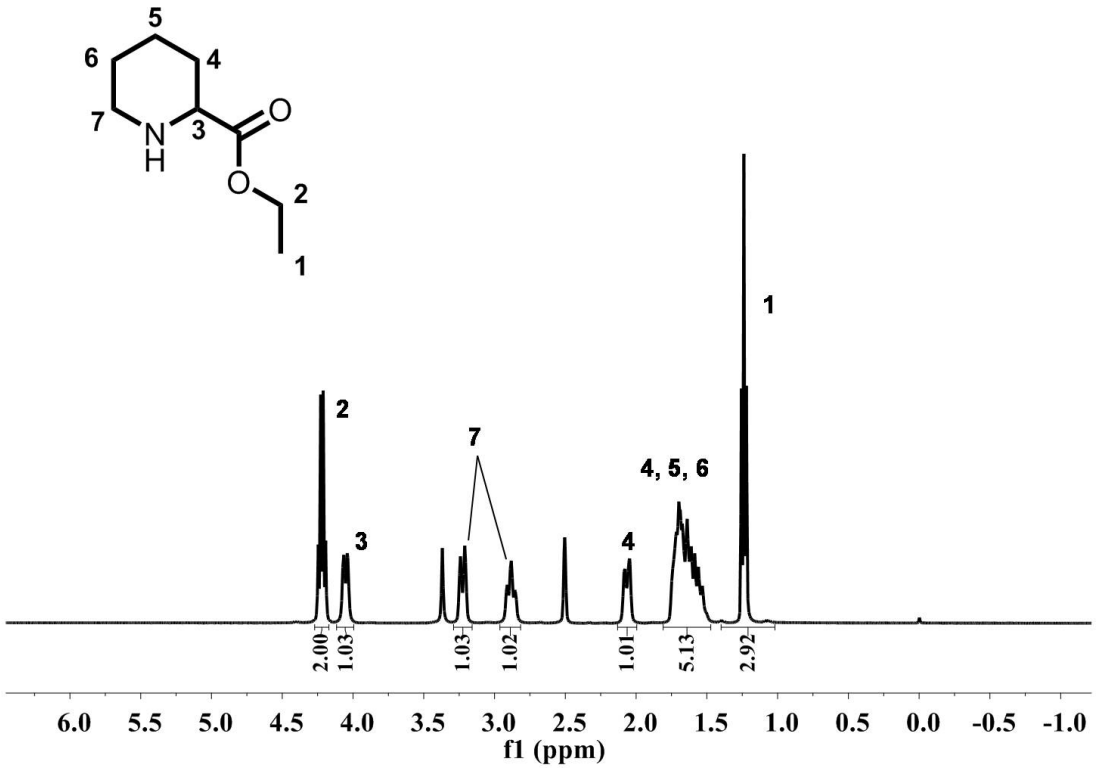

Figure S1. ^{1}H NMR spectrum of compound 1

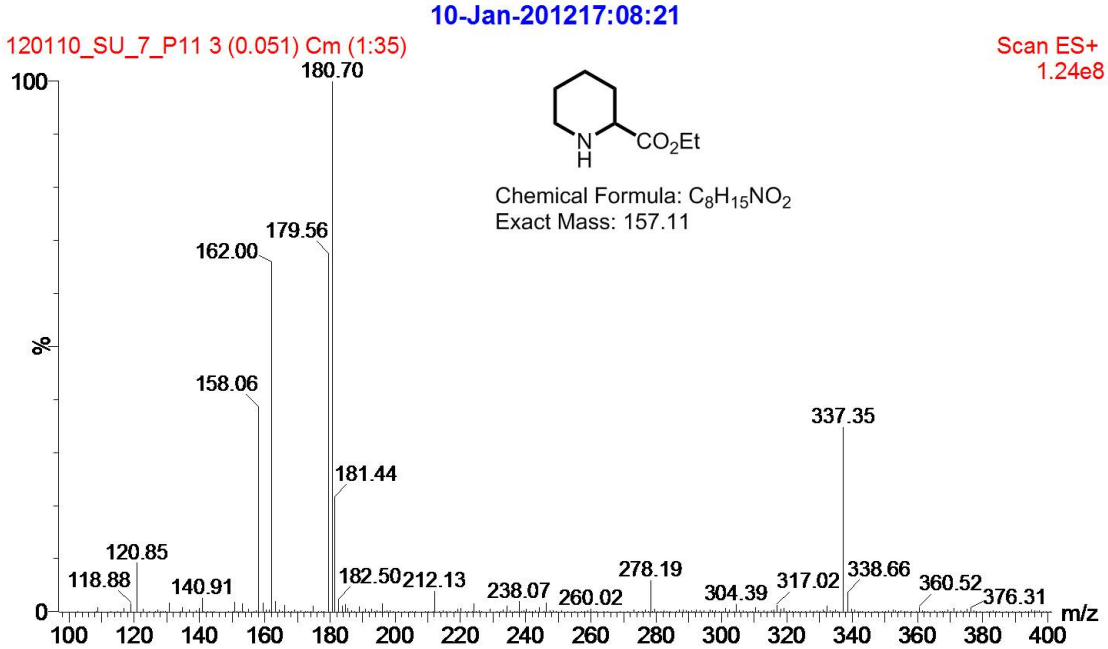

Figure S2. ESI-MS spectrum of compound 1

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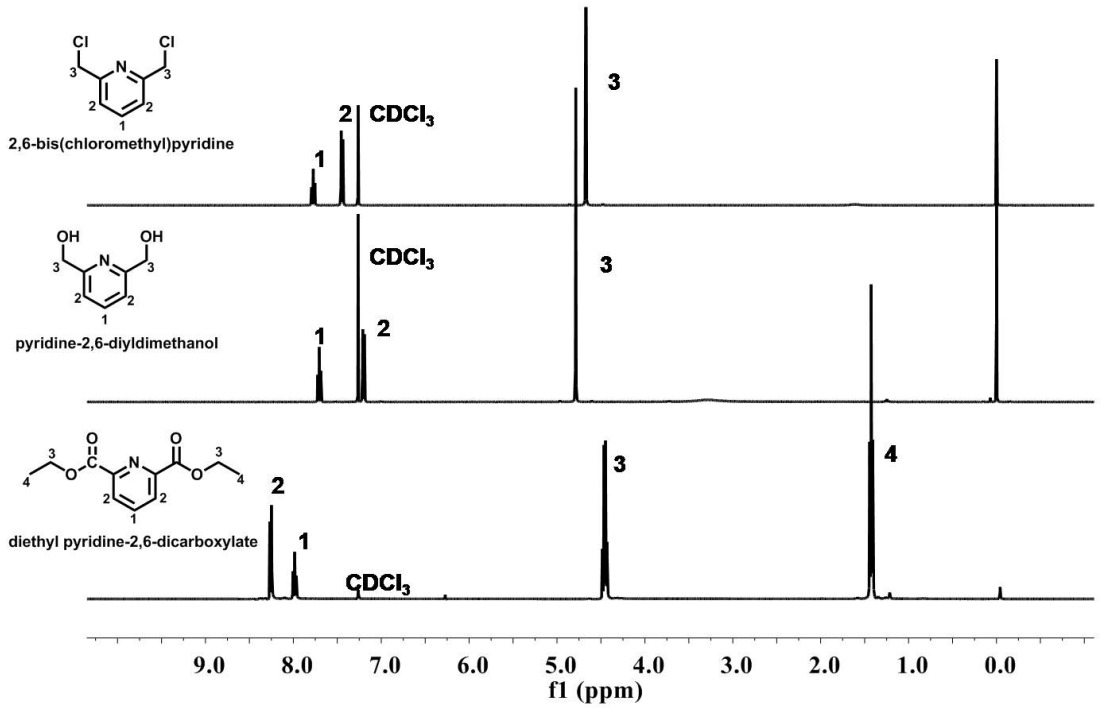

Figure S3. ^{1}H NMR spectra of compound 2, 3 and 4

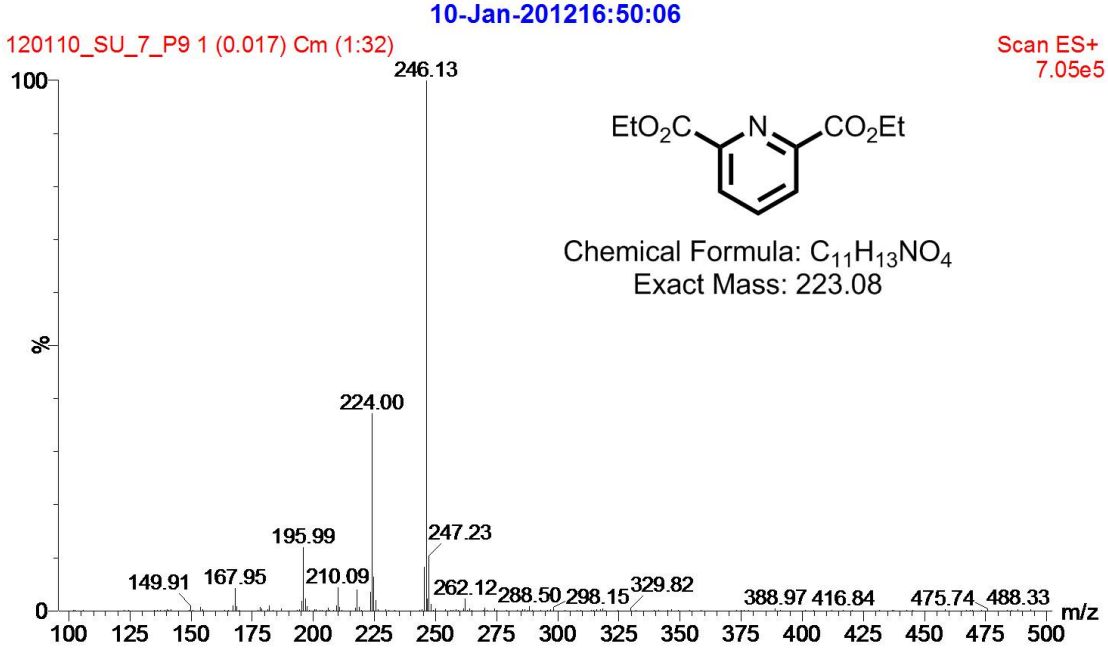

Figure S4. ESI-MS spectrum of compound 2

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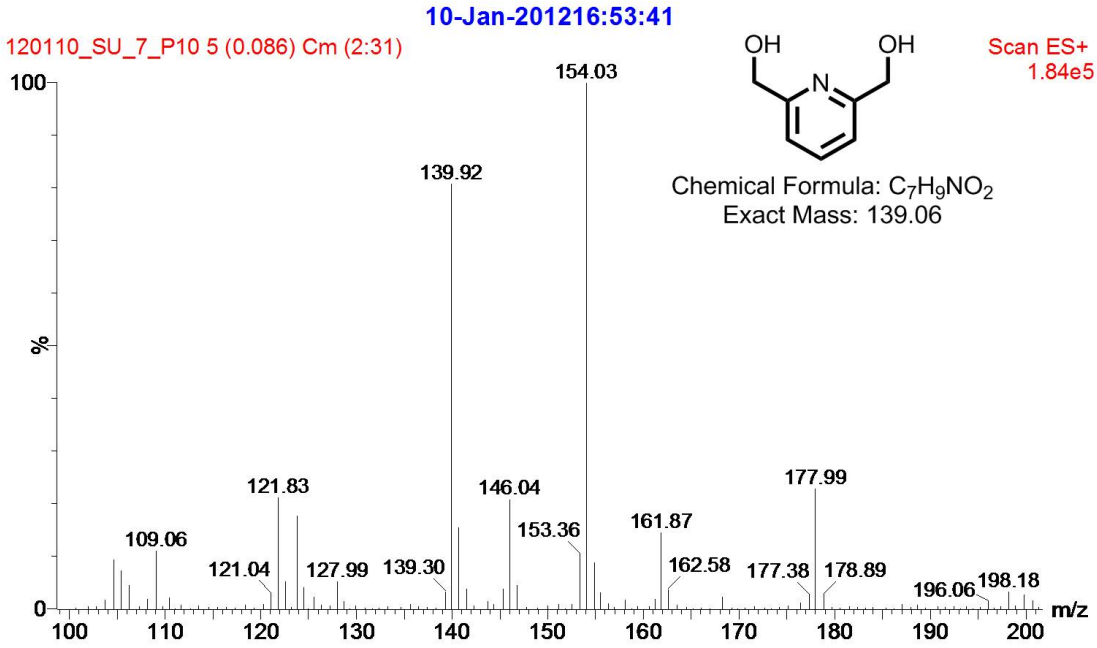

Figure S5. ESI-MS spectrum of compound 3

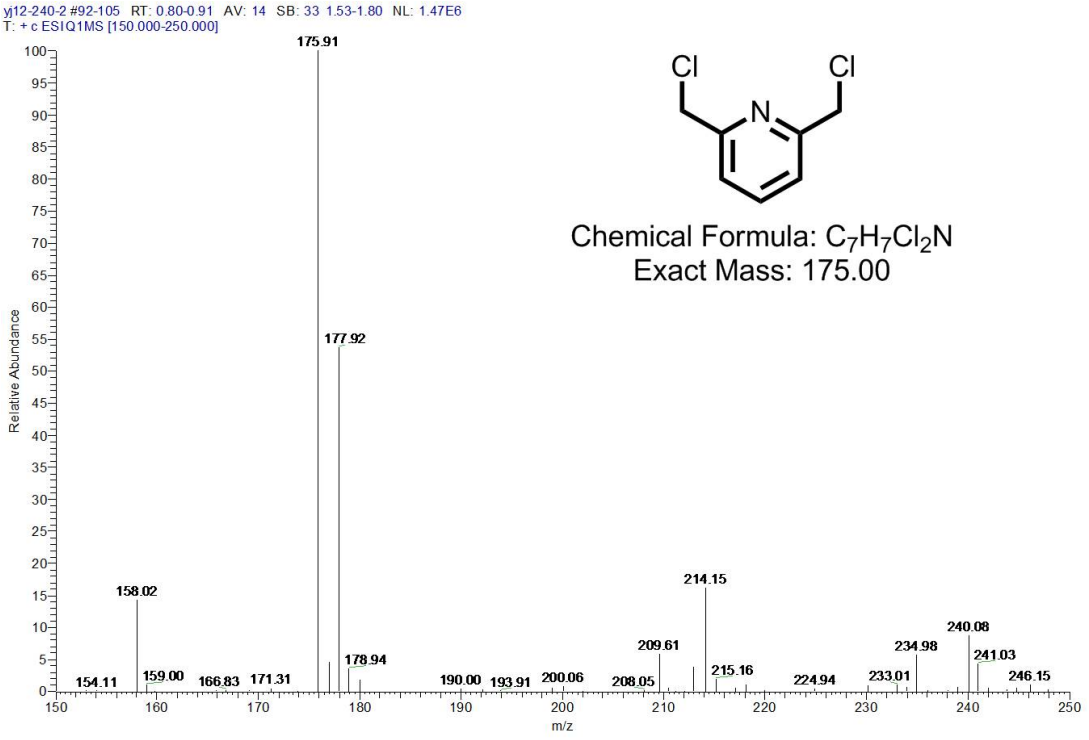

Figure S6. ESI-MS spectrum of compound 4

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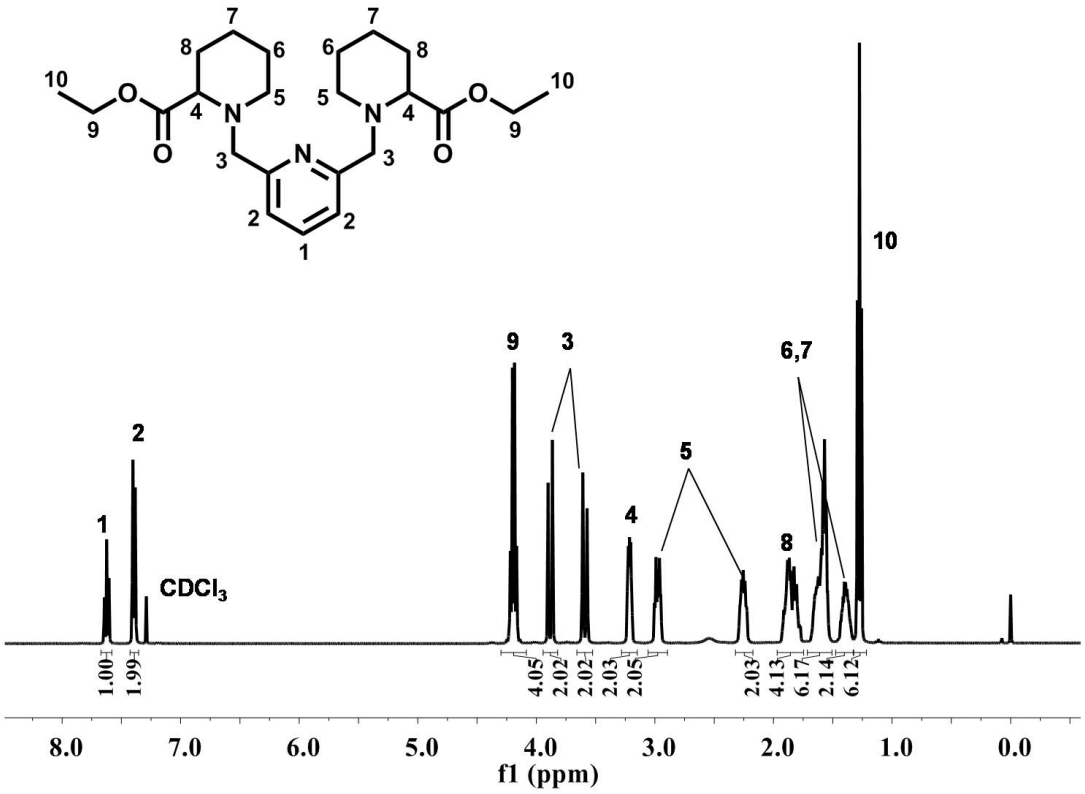

Figure S7. ^{1}H NMR spectrum of compound 5

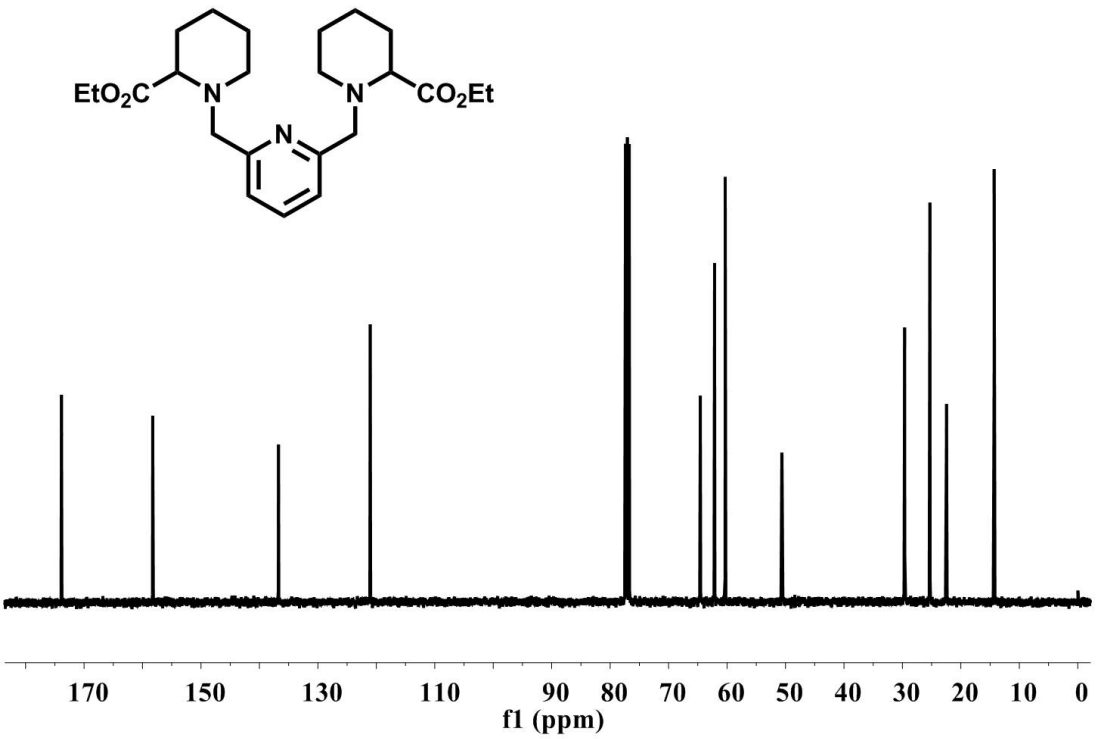

Figure S8. ^{13}C NMR spectrum of compound 5

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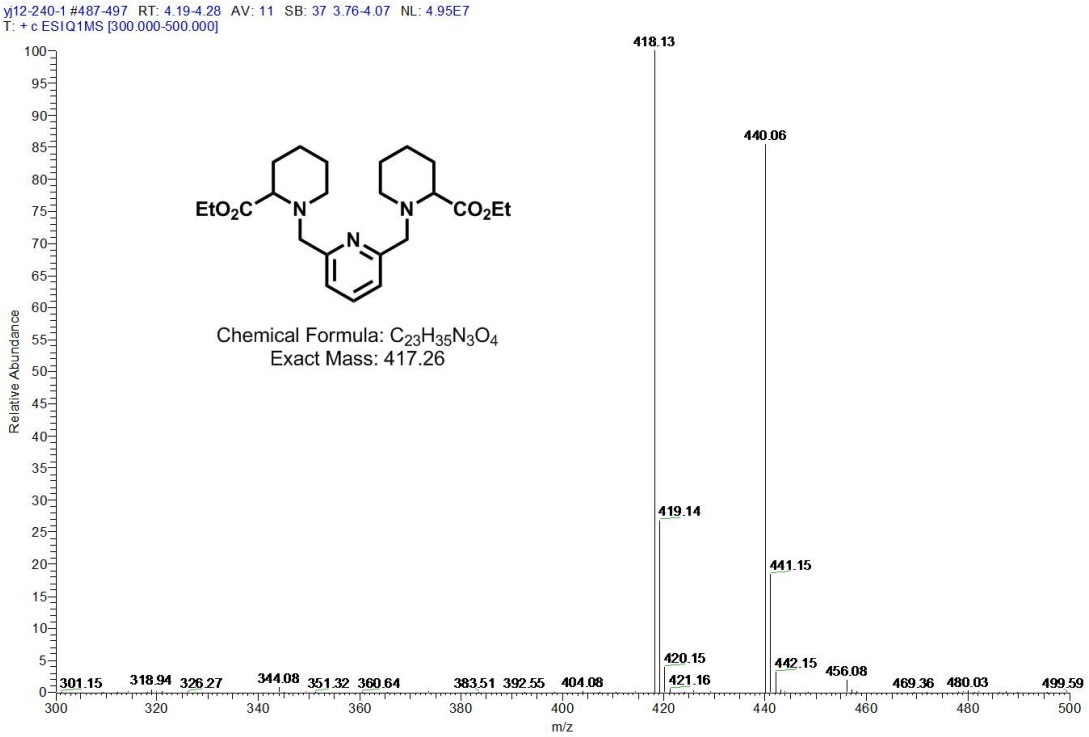

Figure S9. ESI-MS spectrum of compound 5

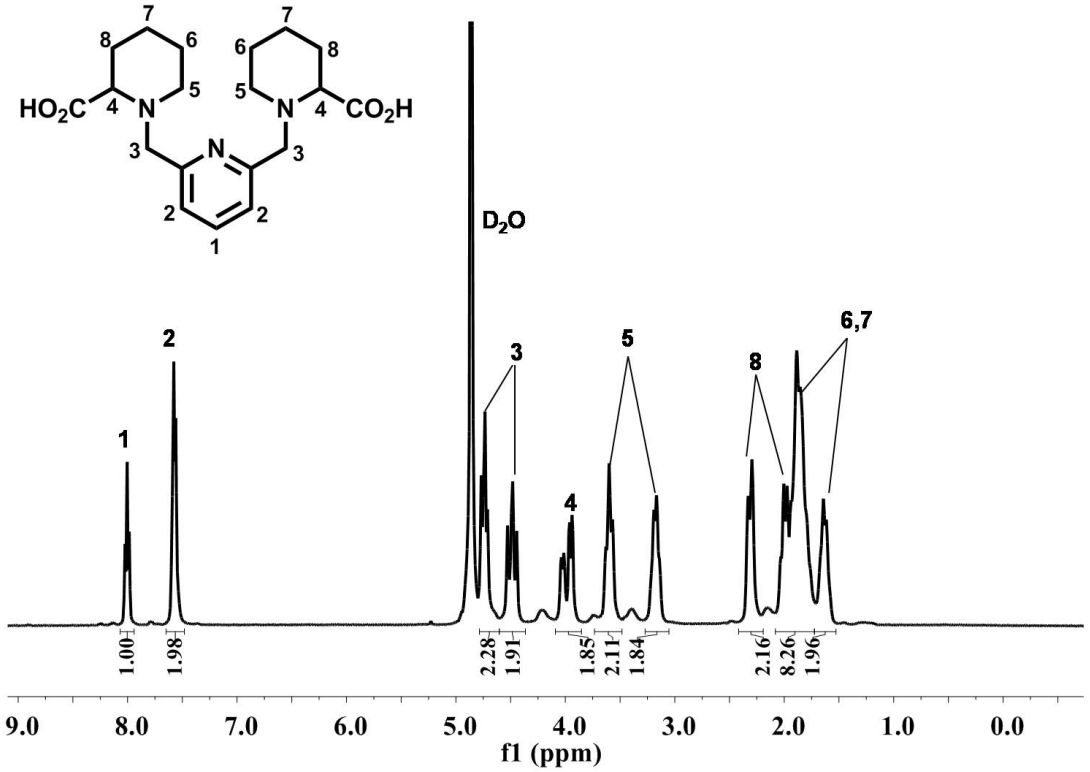

Figure S10. ^{1}H NMR spectrum of compound 6

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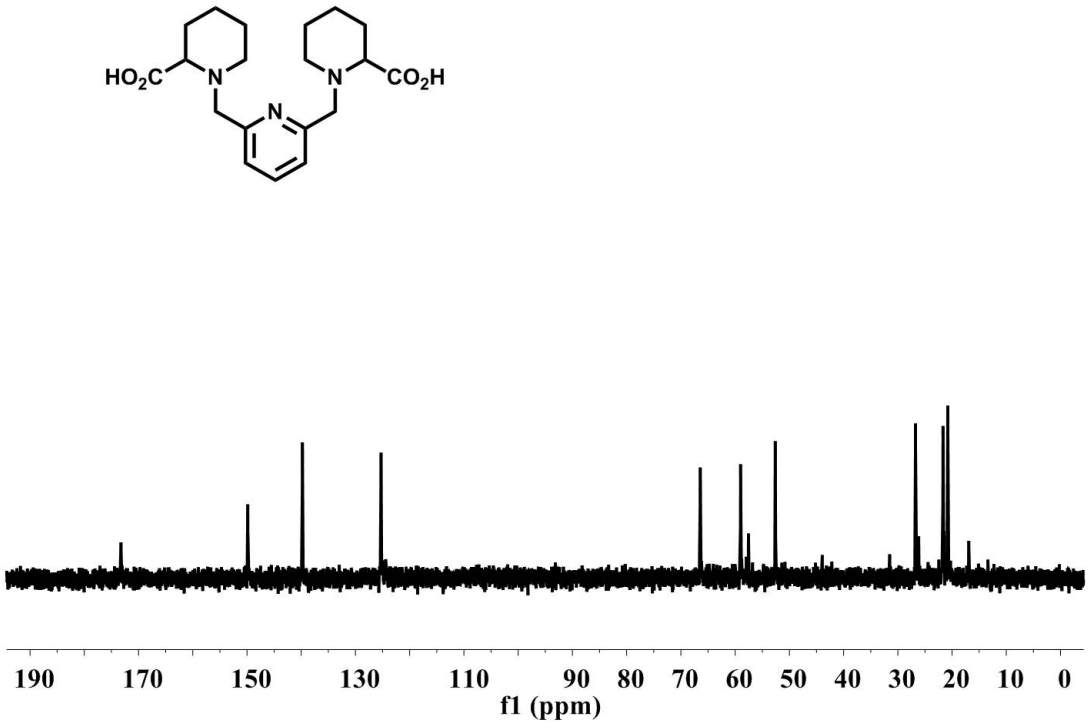

Figure S11. ^{13}C NMR spectrum of compound 6

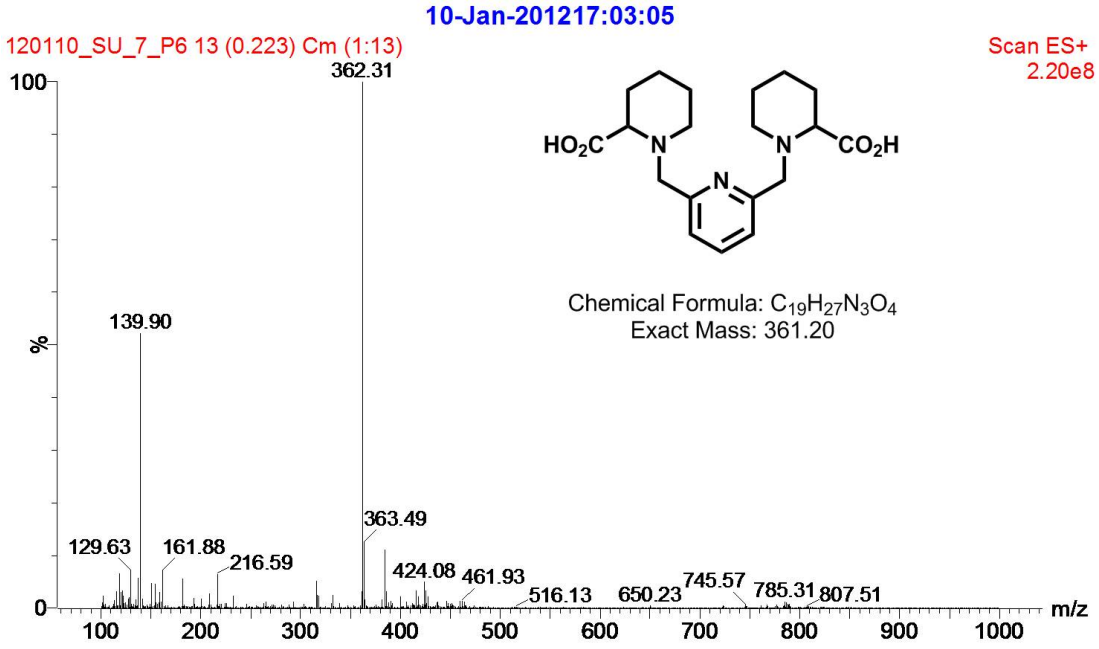

Figure S12. ESI-MS spectrum of compound 6

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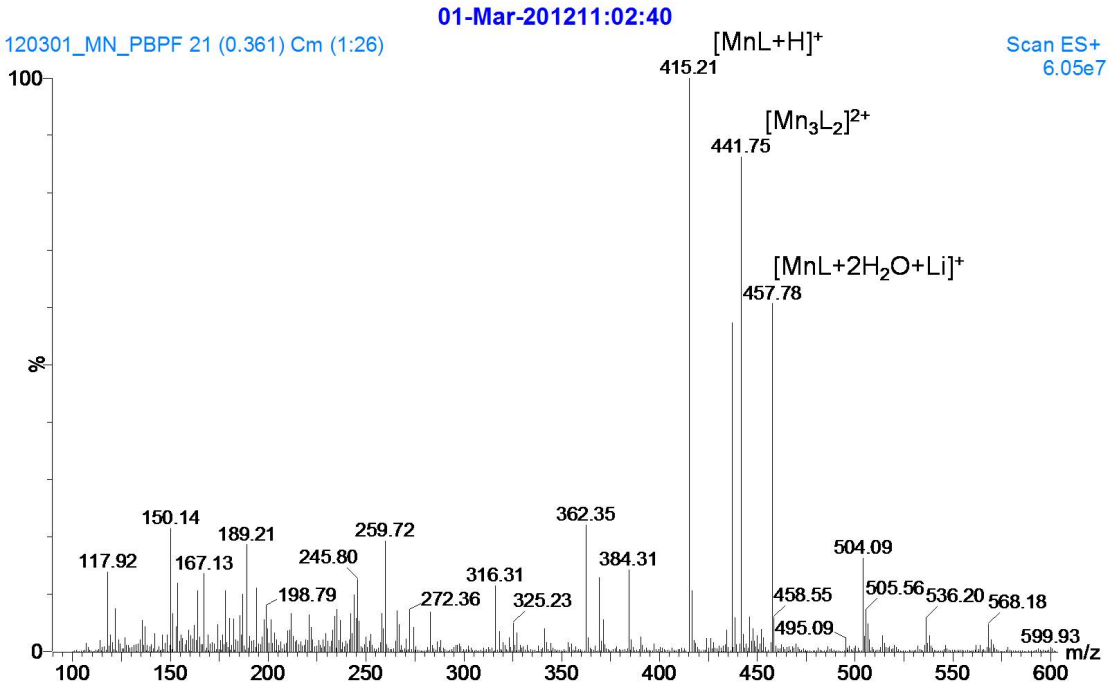

Figure S13. ESI-MS spectrum of Mn-ligand complex without purification through Sephadex LH-20 column

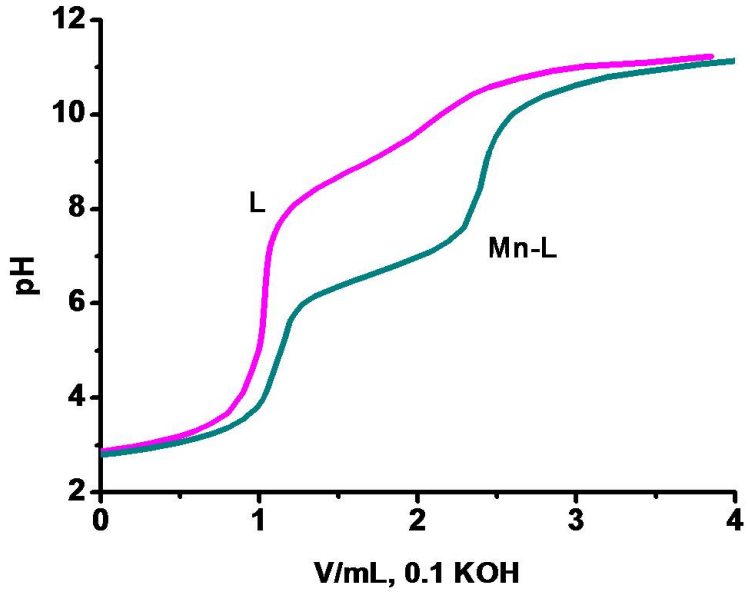

Figure S14. Titration plot of measured pH versus V_{KOH} (I = 0.1 M KCl, 25ºC)

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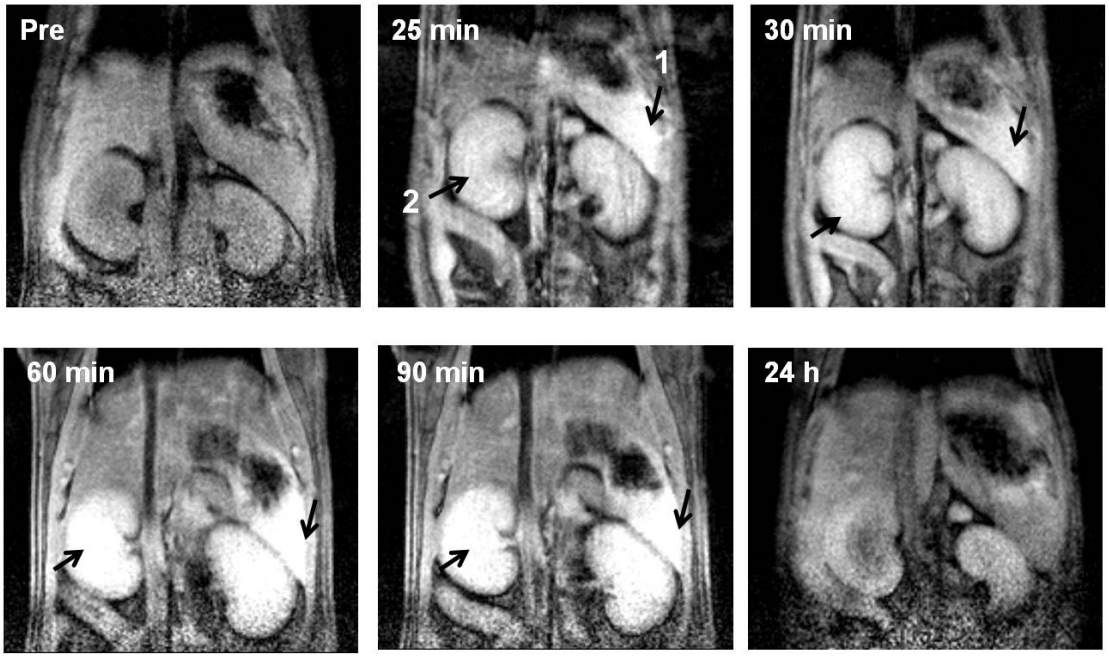

Figure S15. T_1-weighted MR image (Philips Medical System, Intera 3 T, TR = 5 ms, TE = 2 ms) of SD rat (1: spleen, 2: kidney) at pre-injection (A), (B) 25 min, (C) 30 min, (D) 60 min, (E) 90 min and (F) 24 h after administration of the complex (0.5 mmol/kg).

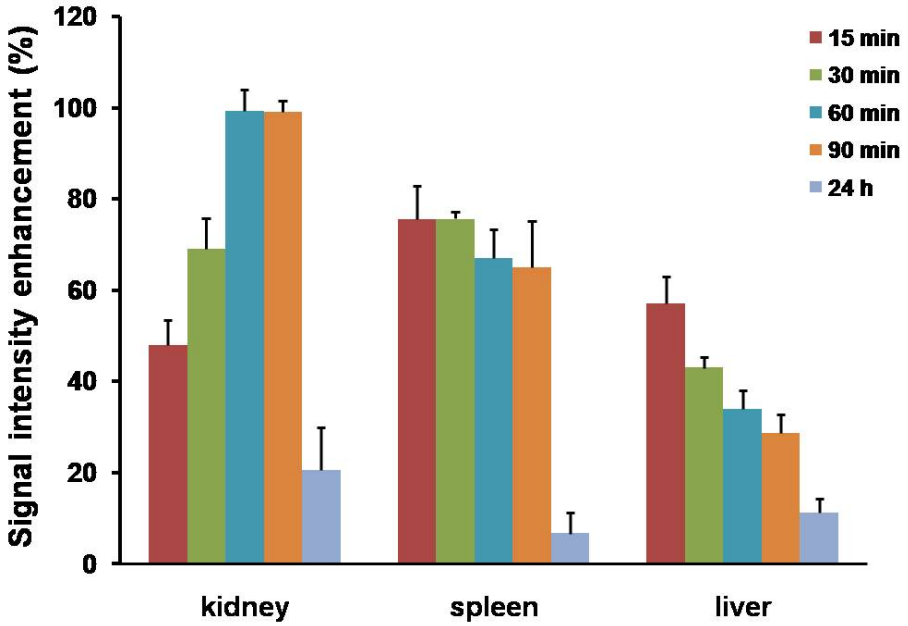

Figure S16. Signal intensity enhancement of kidney, spleen and liver at different time points post-injection of the complex

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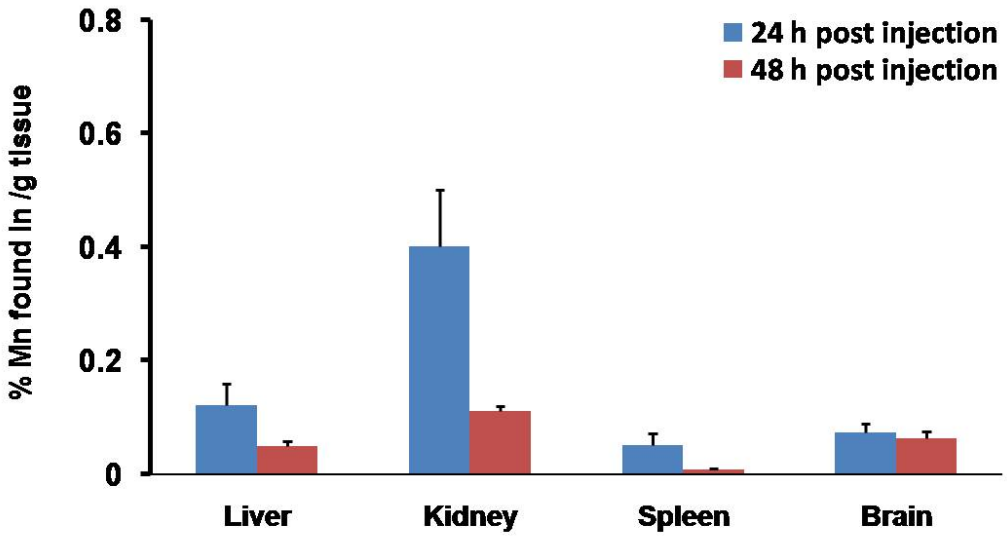

Figure S17. Measurements of Mn in liver, kidney, spleen and brain at 24 and 48 h post-injection of the complex as percent injected dose per gram of tissue.