Supporting Information

Molecular magnetic resonance imaging of nitric oxide in biological systems

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Table S1. Relaxivity, oxidation states, and extinction coefficients of Mn complexes

Compound	r_1 ($mM^{-1}s^{-1}$)[a]	Oxidation state[b]	ε ($M^{-1}cm^{-1}$) / λ_{max} (nm)[c]
$MnCl_2$	4.6 ± 0.2	+2	n/a
Mn**Pc**	8.5 ± 0.4	+2	21544 / 300
Mn**TPPS$_4$**	7.9 ± 0.2	+3	81977 / 460
Mn**TMPyP**	4.7 ± 0.3	+3	97662 / 460
Mn**L1**	4.2 ± 0.1	+3	4002 / 300
Mn**L1F** (NORA)	2.7 ± 0.3	+3	33465 / 310
Mn**L1Me**	3.1 ± 0.3	+3	4000 / 300
Mn**L2**	2.5 ± 0.1	+3	11542 / 270
Mn**L3**	5.2 ± 0.2	+3	4520 / 300
Mn**L4**	3.8 ± 0.2	+3	3924 / 360
Mn**L5**	2.1 ± 0.2	+3	1098 / 400

[a] T_1 relaxivity at 7 T and 22 °C in 25 mM MOPS, pH 7.4, 100 mM KCl; error margins denote s.e.m. of five independent measurements. [b] Formal oxidation state of the metal center. [c] in 25 mM MOPS, pH 7.4, 100 mM KCl.

Table S2. Apparent relaxivity of NORA in labeled cells

Condition[a]	ΔR_1 (s^{-1})[b]	[Mn] (μM)[c]	r_1 ($mM^{-1}s^{-1}$)[d]
GFP	0.162 ± 0.006	26 ± 2	6.3 ± 0.5
GFP + 1400W	0.145 ± 0.002	27 ± 4	5.5 ± 0.8
GFP + thapsigargin	0.157 ± 0.005	27 ± 2	5.9 ± 0.4
iNOS	0.039 ± 0.007	23 ± 1	1.7 ± 0.3
iNOS + 1400W	0.070 ± 0.003	23 ± 2	3.0 ± 0.2
nNOS	0.180 ± 0.003	27 ± 3	6.6 ± 0.6
nNOS + thapsigargin	0.111 ± 0.004	28 ± 3	3.9 ± 0.5

[a] Cells expressing GFP, iNOS, or nNOS were incubated with 20 μM NORA for 30 minutes in the presence or absence of 100 μM 1400W or 1 μM thapsigargin, washed, and pelleted by centrifugation prior to MRI analysis. [b] R_1 measured from cell pellets in the experiments of Fig. 2, minus the R_1 of unlabeled cells, all measured at 7 T and 22 °C. [c] Following MRI, cells were digested in HNO_3 at 70 °C, diluted to total 2 v% HNO_3, and filtered to remove the remaining debris before analysis of apparent Mn content by ICP-MS. [d] Apparent r_1 values were computed by dividing ΔR_1 by [Mn] for each experiment. Error margins for ΔR_1 and [Mn] denote s.e.m. of three independent measurements; error margins for r_1 values were determined by propagation of uncertainty.

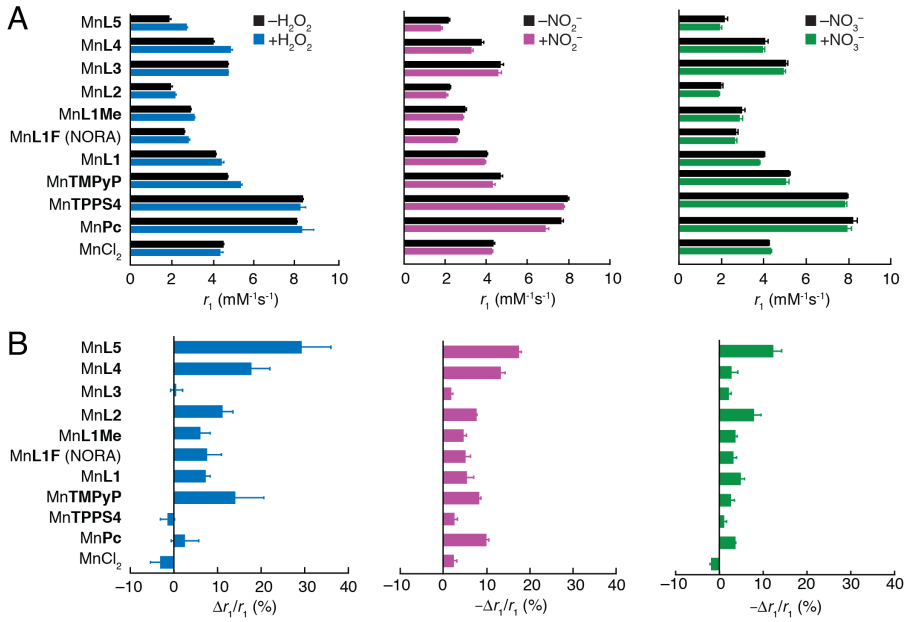


Fig. S1. Contrast agent interactions with biologically relevant reactive oxygen and nitrogen species. (A) Two-point relaxivity measurements were performed at 7 T and 22 °C in 25 mM MOPS, 100 mM KCl, following incubation for 60 minutes in the absence and presence of 100 μM H_2O_2 (left), 50 μM NO_2^- (middle), or 50 μM NO_3^- (right). (B) Percent change in r_1 corresponding to the measurements in (A). Error bars denote s.e.m. of three independent measurements.

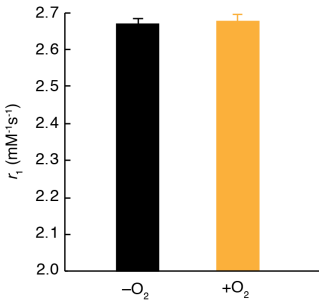


Fig. S2. NORA relaxivity in the presence and absence of O_2. Relaxivity of NORA was estimated from the R_1 of 40 μM contrast agent in deoxygenated *vs.* oxygenated 25 mM MOPS, pH 7.4. Error bars denote s.e.m. of three independent measurements.

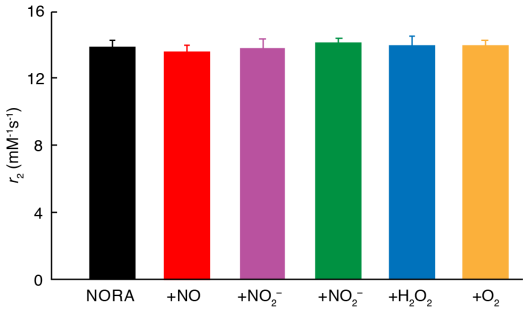


Fig. S3. Transverse relaxivity of NORA. 120 μM NORA in 25 mM MOPS (pH 7.4) was treated with DEA/NO (150 μM), $NaNO_2$ (150 μM), $NaNO_3$ (150 μM), H_2O_2 (150 μM) and O_2 and the corresponding r_2 values were computed from T_2 relaxation rate data at 22 °C and 7 T. Error bars denote s.e.m. of three independent measurements.

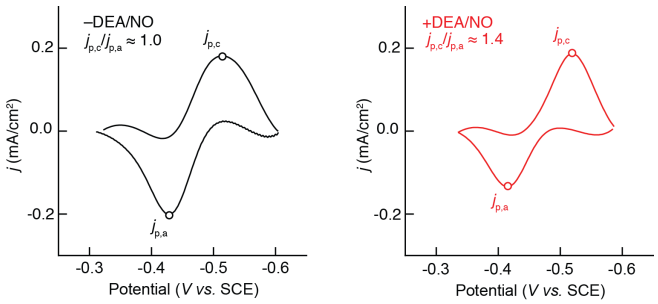


Fig. S4. Cyclic voltammetry of NORA in the absence and presence of DEA/NO. Cyclic voltammograms of 1 mM NORA in 25 mM MOPS, pH 7.4, 100 mM KCl before (left) and after (right) addition of 1 mM DEA/NO. Background subtraction was performed by fitting the baseline to a fourth order polynomial for both the cathodic and anodic traces. Potentials are reported with respect to a saturated calomel electrode (SCE). Peak cathodic and anodic current densities labeled on the graphs ($j_{p,c}$ and $j_{p,a}$, respectively) display different ratios in the absence and presence of DEA/NO, indicating a change in the free energy associated with manganese redox transitions in the presence of the NO donor.

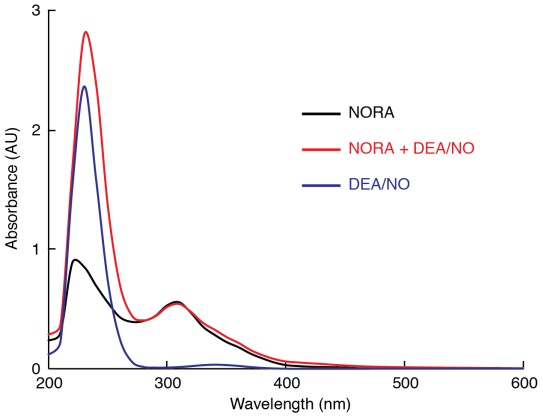


Fig. S5. Spectroscopic analysis of NORA stability in the presence of DEA/NO. Ultraviolet-visible spectra of 30 µM NORA in 25 mM MOPS, pH 7.4, 100 mM KCl in the absence (black) and presence (red) of 75 µM DEA/NO. The spectrum of 75 µM DEA/NO alone is shown in blue.

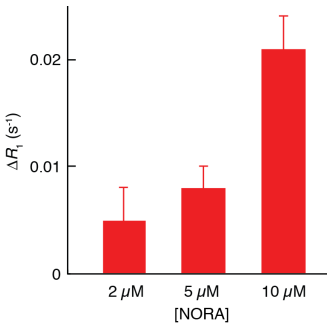


Fig. S6. Relaxation changes in iNOS-expressing cells labeled with varying concentrations of NORA. Cells expressing iNOS were incubated with varying concentrations of NORA, resulting in approximately proportional relaxation differences (ΔR_1) with respect to unlabeled cells. Error bars denote s.e.m. of three independent measurements.

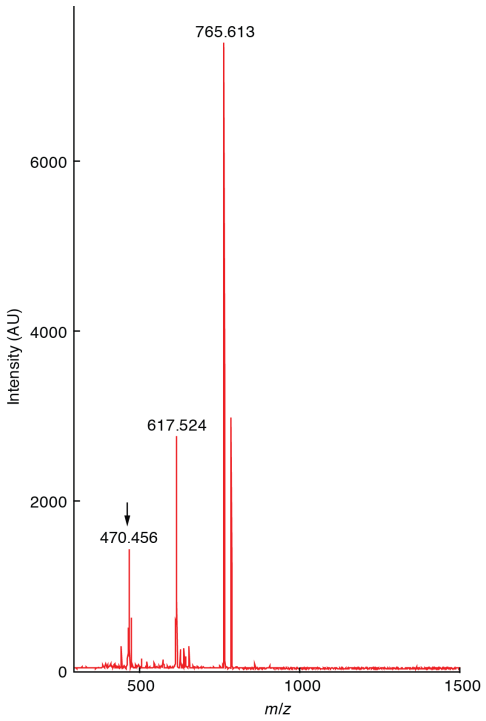


Fig. S7. Mass spectrometry of NORA-labeled iNOS-expressing cells. HEK cells expressing iNOS were incubated with 20 μM NORA for 30 min, pelleted, lyophilized, treated with acetonitrile, and centrifuged again. The supernatant was analyzed by matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry to ascertain integrity of NORA following this procedure, and is indeed visible as a peak at m/z = 470.456 (expected $[M+Cl]^-$: m/z = 470.976), with no indication of demetallation (expected 383.322) or fragmentation products.

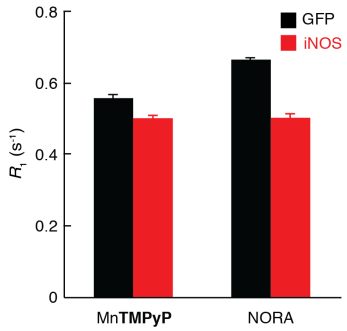


Fig. S8. Relaxation rates of MnTMPyP and NORA in cells. Relaxation rates measured from pelleted GFP- and iNOS-expressing cells, each preincubated with 20 μM of the contrast agents Mn**TMPyP** or NORA. Error bars denote s.e.m. of three independent measurements.

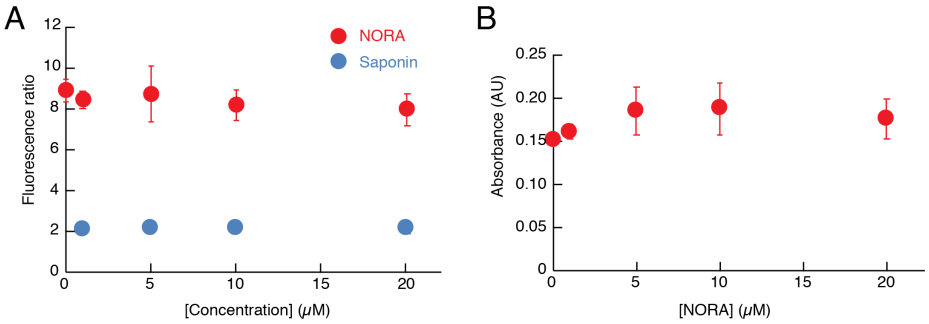


Fig. S9. Cytotoxicity and viability of cells exposed to NORA. (A) The ratio of fluorescence measurements at 530 nm, indicating calcein AM hydrolysis in viable cells, to 645 nm, indicating accumulation of Ethidium Homodimer III due to plasma membrane compromise; higher values indicate greater viability. Exposure to NORA for 60 min showed negligible effect at all tested concentrations compared to naïve cells. (B) To assess possible long-term effects due to exposure to NORA, cells were incubated with NORA at varying concentrations and then after 24 hr washed and assayed for mitochondrial enzymatic conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) into formazan, which reflects cell viability and proliferation. No significant reduction in MTT conversion was observed under any of the incubation conditions ($p \geq 0.81$, $n = 3$ replicates each).

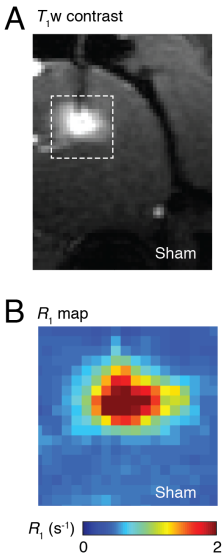
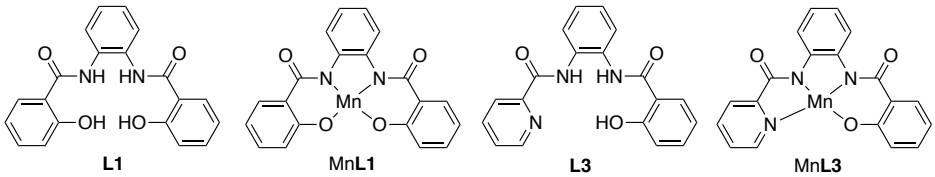
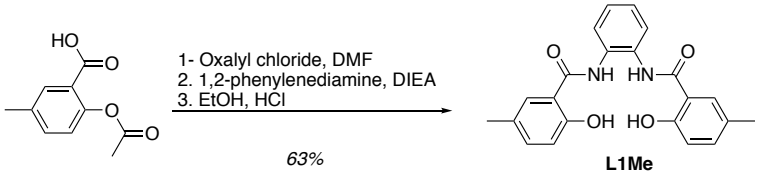


Fig. S9. MRI intensity and R_1 changes in sham-treated rat brains. (A) Representative T_1-weighted images of sham-treated rats infused with NORA. (B) R_1 map of sham-treated NORA-infused region corresponding to the box in (B), averaged over animals (n = 3).

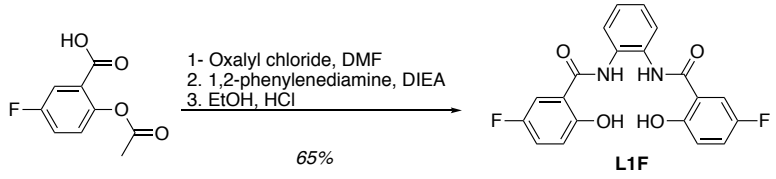
SUPPLEMENTAL METHODS



N,*N*'-(1,2-phenylene)bis(2-hydroxybenzamide) (**L1**) and *N*-(2-(2-hydroxybenzamido)phenyl)picolin amide (**L3**) and the manganese complexes Mn**L1** and Mn**L3** were synthesized as previously reported.[2]

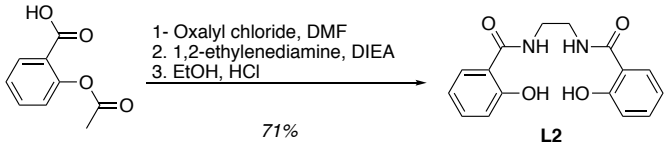


***N*,*N*'-(1,2-phenylene)bis(2-hydroxy-5-methylbenzamide), L1Me.** 2-acetoxy-5-methylbenzoic acid (2 g, 10 mmol) was dissolved in anhydrous dichloromethane (50 mL) and oxalyl chloride (1.44 g, 11 mmol) and dimethylforamide (10 µL) were added. After stirring the reaction solution at room temperature for 3 h, phenylenediamine (0.56 g, 5 mmol) and diisopropylethyl amine (2 mL, 11 mmol) were added at 0 °C and the reaction solution warmed to 40 °C and stirred for 18 hr. The reaction mixture was diluted with dichloromethane (100 mL) and washed with water (3 x 50 mL), the organic phase was dried over $MgSO_4$ and all volatiles removed in vacuum. The resulting white solid was dissolved in hot ethanol (10 mL) and treated with concentrated hydrochloric acid (5 mL) and stirred at 50 °C for 10 hr. The reaction mixture cooled to room temperature and the precipitated solid was filtered and washed with cold (0 °C) ethanol. The white solid was dried in vacuum to afford **L1Me**. Yield: 1.2 g (63%). MS (HRESI): *m/z* = 375.412 $[M-H]^-$. ^1H NMR (400 MHz, DMSO-d_6) δ 11.48 (s, 2H, OH), 10.39 (s, 2H, NH), 7.89-7.72 (m, 4H, aromatic), 7.26 (ddd, *J* = 15.6, 7.2, 2.9 Hz, 4H, aromatic), 6.88 (d, *J* = 8.3 Hz, 2H, CH_3), 2.26 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ 166.29, 156.10, 134.46, 131.12, 129.50, 127.83, 125.61, 125.34, 117.08, 116.53, 40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.90, 20.09.

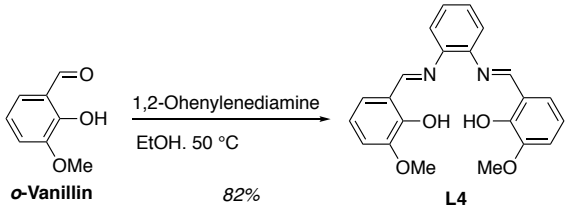


***N*,*N*'-(1,2-phenylene)bis(5-fluoro-2-hydroxybenzamide), L1F.** 2-acetoxy-5-fluorobenzoic acid (3 g, 0.015 mol) was dissolved in anhydrous dichloromethane (50 mL) and oxalyl chloride (2.1 g, 0.016 mol) was added. After adding catalytic amount of DMF (0.1 mL) the solution was stirred at room temperature for 2 hr. A solution of *o*-phenylenediamine (0.8 g, 7.5 mmol) and diisopropylethylamine (2 g, 15 mmol, 2.69 mL) was added dropwise and the resulting solution was stirred at room temperature for 18 hr. After removal of all volatiles the resulting residue was dissolved in EtOH (50 mL) and treated with hydrochloric acid (15 mL, 1 M). The resulting clear solution was stirred under reflux for 10 h, cooled to ambient temperature and the deposited white solid was filtered and washed with cold ethanol (3 x 5 mL, -10 °C) and dried in vacuum to afford **L1F** as white solid. Yield: 1.9 g (65%). MS (HRESI): *m/z* = 383.322 [M-

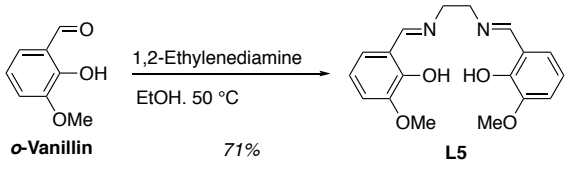
H]$^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.64 (s, 2H, OH), 10.47 (s, 2H, NH), 7.84-7.78 (m, 4H, aromatic), 7.35-7.29 (m, 4H, aromatic), 7.01 (dd, J = 9.0, 4.6 Hz, 2H, aromatic). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.34, 156.64, 154.78, 154.31, 131.34, 126.34, 125.76, 121.27 (d, J = 23.5 Hz), 119.16 (d, J = 7.6 Hz), 118.31, 115.63 (d, J = 24.5 Hz). ^{19}F NMR (376 MHz, DMSO-d_6) δ -124.29 (m).



***N*,*N*'-(ethane-1,2-diyl)bis(2-hydroxybenzamide), L2.** 2-acetoxybenzoic acid (2 g, 11 mmol) was dissolved in anhydrous dichloromethane (30 mL) and oxalyl chloride (1.58 g, 0.016 mol) was added. After adding catalytic amount of DMF (0.1 mL) the solution was stirred at room temperature for 2 hr. A solution of 1,2-ethylenediamine (0.3 g, 3 mmol) and diisopropylethylamine (2 g, 15 mmol, 2.69 mL) was added dropwise and the resulting solution was stirred at room temperature for 18 hr. After removal of all volatiles the resulting residue was dissolved in EtOH (50 mL) and treated with hydrochloric acid (15 mL, 1 M). The resulting clear solution was stirred under reflux for 10 h, cooled to ambient temperature and the deposited white solid was filtered and washed with cold ethanol (3 x 5 mL, -10 °C) and dried in vacuum to afford **L2** as white solid. Yield: 1.48 g (71%). MS (HRESI): *m/z* = 299.311 [M-H]$^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.55 (s, 2H, OH), 8.96 (s, 2H, NH), 7.82 (dd, J = 7.9, 1.7 Hz, 2H, aromatic), 7.39 (ddd, J = 8.6, 7.2, 1.7 Hz, 2H, aromatic), 6.97-6.64 (m, 4H, aromatic), 3.50 (d, J = 5.6 Hz, 4H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ 169.37, 160.10, 133.71, 127.71, 118.53, 117.35, 115.16, 40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.90, 38.53.

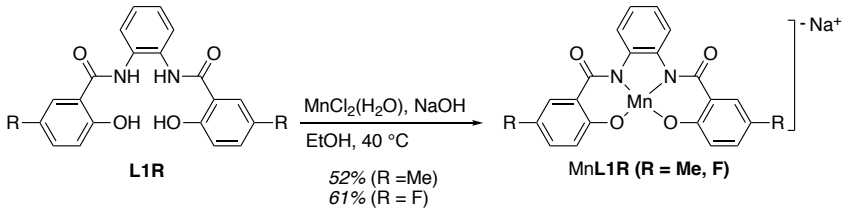


6,6'-(1,2-phenylenebis(azaneylylidene))bis(methaneylylidene))bis(2-methoxyphenol), L4. o-Vanillin (1.5 g, 10 mmol) was dissolved in ethanol (15 mL) and warmed to 50 °C while stirring. Phenylenediamine (0.53 g, 5 mmol) was added and the reaction solution was stirred at 50 °C for additional 4 hr. The deposited orange solid was filtered and washed with ethanol and dried in vacuum to afford **L4**. Yield: 1.52 g (82%). MS (HRESI): *m/z* = 375.412 [M-H]$^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.99 (s, 2H, OH), 8.92 (s, 2H, HC=N), 7.43 (m, 2H, aromatic), 7.25 (dd, J = 7.9, 1.5 Hz, 1H, aromatic), 7.16-7.11 (m, 1H, aromatic), 6.91 (t, J = 7.9 Hz, 1H, aromatic), 3.81 (s, 6H, OCH_3). ^{13}C NMR (101 MHz, DMSO) δ 164.34, 150.59, 147.86, 142.09, 127.81, 123.77, 119.81, 119.32, 118.55, 115.42, 55.66, 40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89.



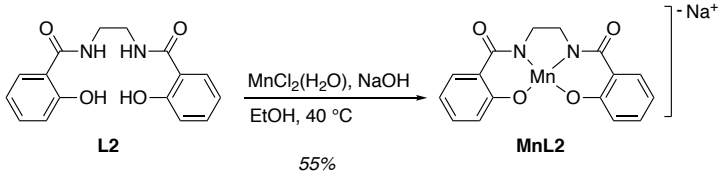
6,6'-(ethane-1,2-diylbis(azaneylylidene))bis(methaneylylidene))bis(2-methoxyphenol), L5. o-Vanillin (1.5 g, 10 mmol) was dissolved in ethanol (15 mL) and warmed to 50 °C while stirring. Ethylenediamine (0.3 g, 5 mmol) was added and the reaction solution was stirred at 50 °C for additional 4 hr. The deposited yellow solid was filtered and washed with ethanol and dried in vacuum to afford **L5**. Yield: 1.13 g (71%). MS (HRESI): *m/z* = 325.332 $[M-H]^-$. ^{1}HNMR (400 MHz, $CDCl_3$): δ 13.57 (s, 2H, OH), 8.33 (s, 2H, HC=N), 6.90 (dd, *J* = 7.8, 1.6 Hz, 2H, aromatic), 6.84 (dd, *J* = 7.8, 1.7 Hz, 2H, aromatic), 6.78 (t, *J* = 7.8 Hz, 2H, aromatic), 3.96 (s, 4H, CH_2), 3.88 (s, 6H, OCH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 129.16, 113.90, 110.79, 85.66, 80.94, 80.56, 76.57, 39.84, 39.52, 39.20, 22.01, 18.57.

General procedure for synthesizing manganese complexes MnL1Me, MnL1F and MnL2: Ligand was dissolved in hot ethanol (2 mL) and $MnCl_2$ (1 equiv.) was added. The reaction solution was stirred at 40 °C for 10 min and solution of NaOH (3 equiv.) in ethanol was added and the reaction mixture was stirred at 40 °C for additional 3 hr. The resulting mixture was stored at -20 °C for 10 hr and the deposited solid was filtered and washed with cold ethanol and dried in vacuum to afford manganese complexes as brown solid.



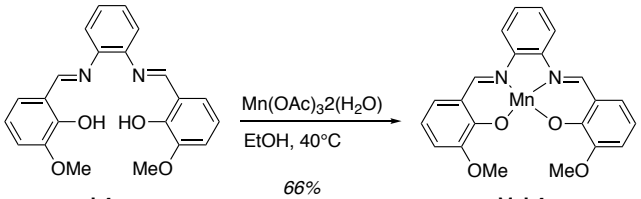
Mn**L1Me.** HRMS: Theory *m/z* = 427.3123 ($[M]^-$), Found *m/z* = 427.3101. Elemental analysis for $C_{22}H_{16}MnN_2NaO_4$ (450.31 g/mol): Calcd. C, 58.68; H, 3.58; N, 6.22; found C, 58.53; H, 3.55; N, 618.

Mn**L1F.** HRMS: Theory *m/z* = 435.2400 ($[M]^-$), Found *m/z* = 435.2508. Elemental analysis for $C_{20}H_{10}F_2MnN_2NaO_4$ (458.23 g/mol): Calcd. C, 52.42; H, 2.20; N, 6.11; found C, 52.28; H, 2.26; N, 6.07.

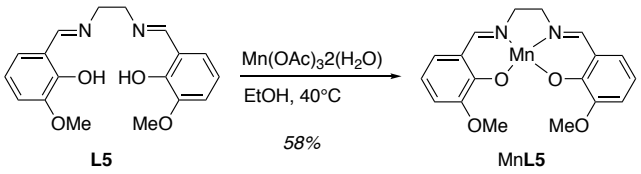


Mn**L2**. HRMS: Theory *m/z* = 351.2161 ($[M]^-$), Found *m/z* = 351.2381. Elemental analysis for $C_{16}H_{12}MnN_2NaO_4$ (374.21 g/mol): Calcd. C, 51.36; H, 3.23; N, 7.49; found C, 51.42; H, 3.27; N, 7.44.

General procedure for synthesizing manganese complexes MnL4 and MnL5: Ligand was dissolved in hot ethanol (2 mL) and $Mn(OAc)_3$•$2(H_2O)$ (1 equiv) was added and the reaction mixture cooled to room temperature and stirred for 30 min. Triethyl amine (3 equiv) was added and the reaction solution was stirred for additional 1 hr and stored at -20 °C overnight. The precipitated solid was filtered and washed with cold ethanol to afford manganese complexes as dark brown solids.

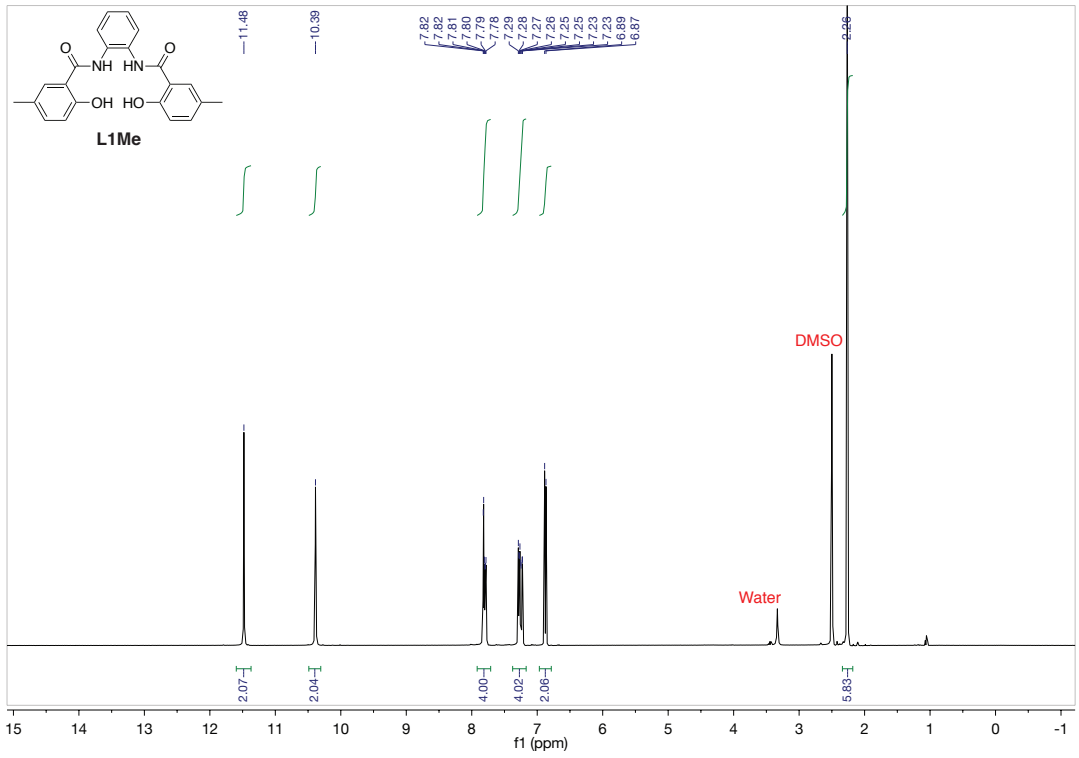


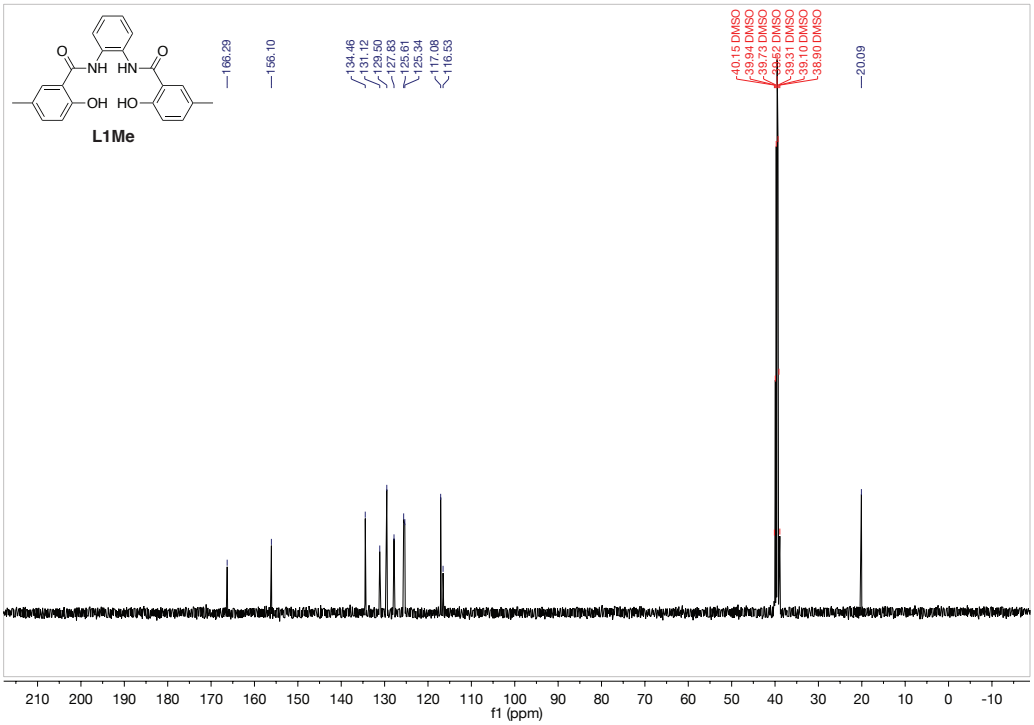
Mn**L4.** HRMS: Theory *m/z* = 429.0647 ([M]$^+$), Found *m/z* = 429.0563. Elemental analysis for $C_{24}H_{21}MnN_2O_6$ (488.08 g/mol for [M+OAc]): Calcd. C, 59.02; H, 4.33; N, 5.74; found C, 59.11; H, 4.35; N, 5.69. IR (KBr, cm^{-1}): 1601 ($\nu_{C=N}$, vs).

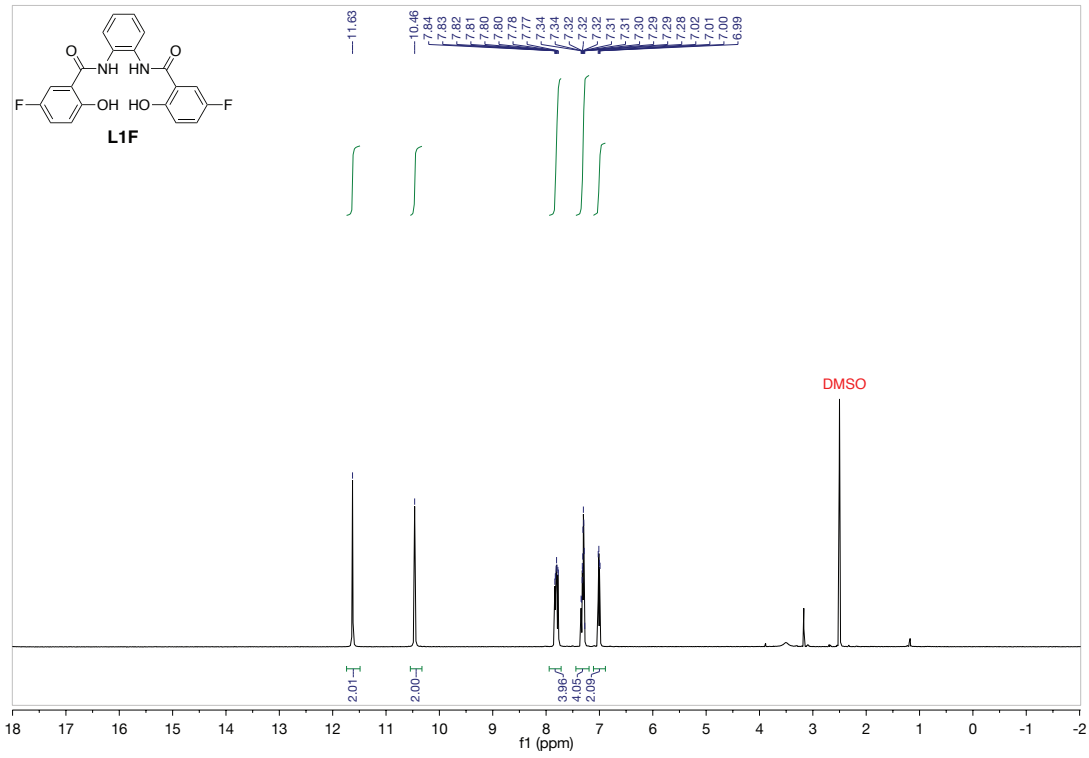


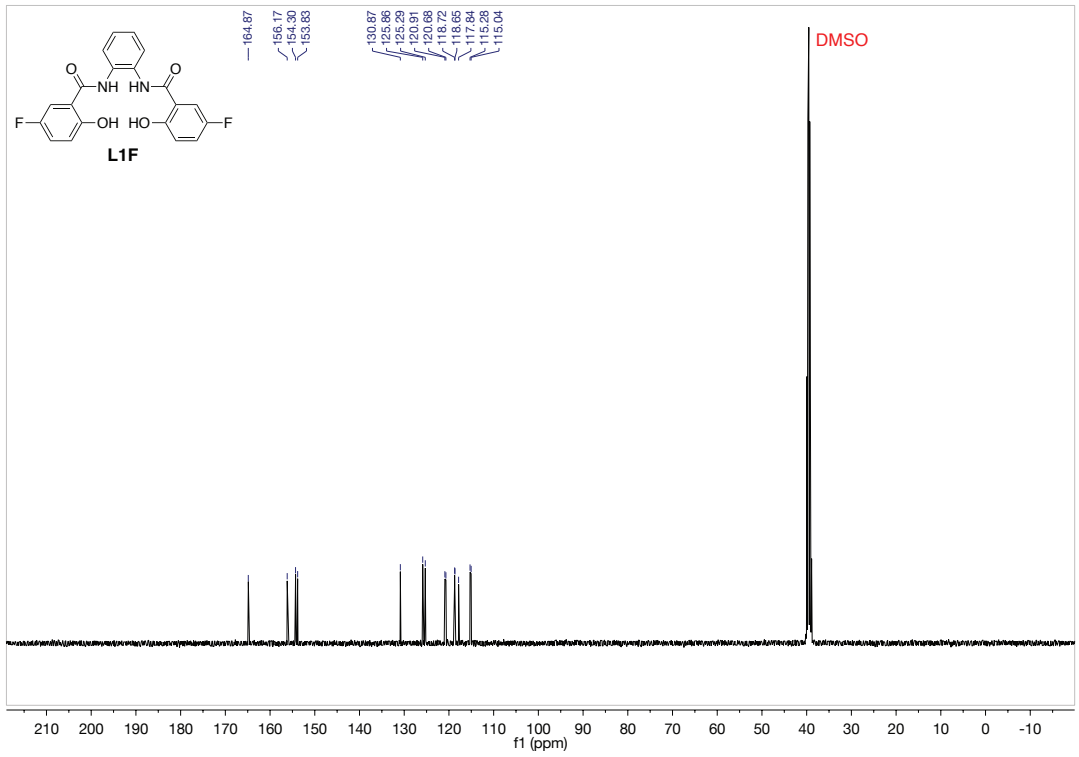
Mn**L5.** HRMS: Theory *m/z* = 381.2947 ([M]$^+$), Found *m/z* = 381.2871. Elemental analysis for $C_{20}H_{21}MnN_2O_6$ (440.08 g/mol for [M+OAc]): Calcd. C, 54.55; H, 4.81; N, 6.36; found C, 54.59; H, 4.79; N, 6.30. IR (KBr, cm^{-1}): 1586 ($\nu_{C=N}$, vs).

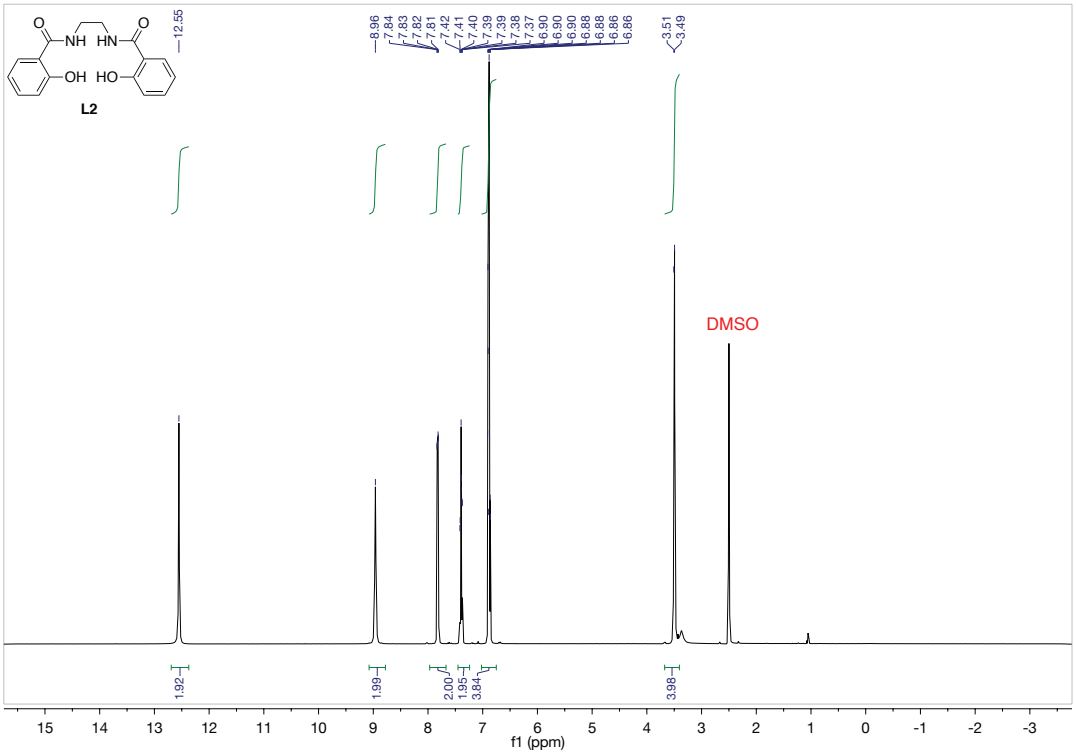
NMR SPECTRA OF SYNTHETIC COMPOUNDS

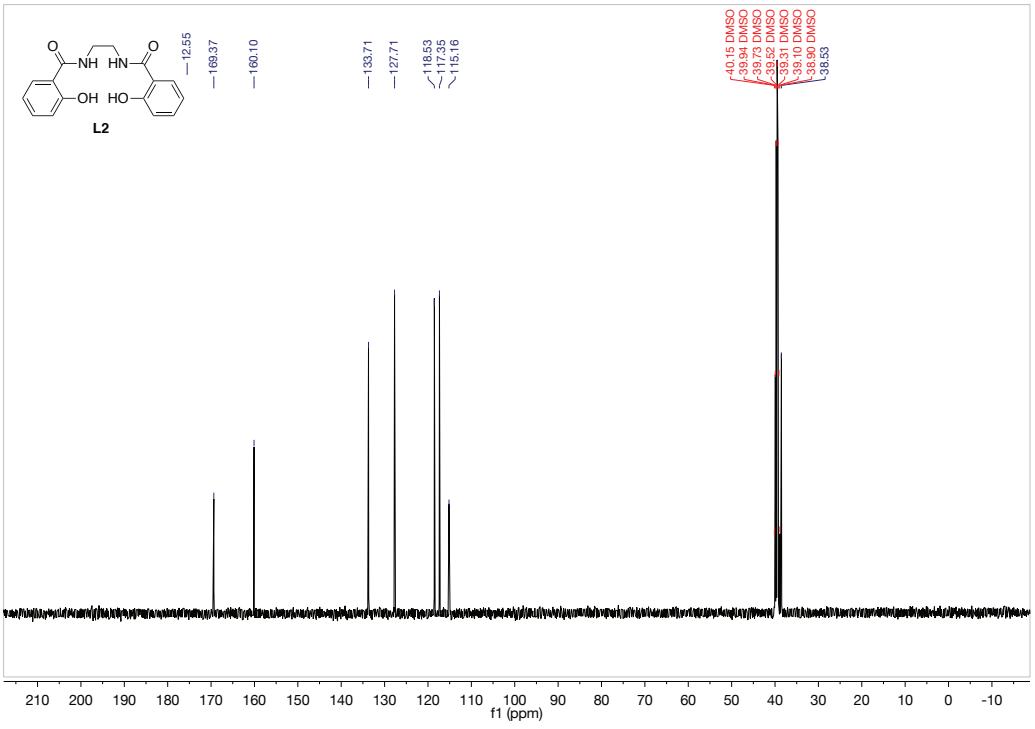


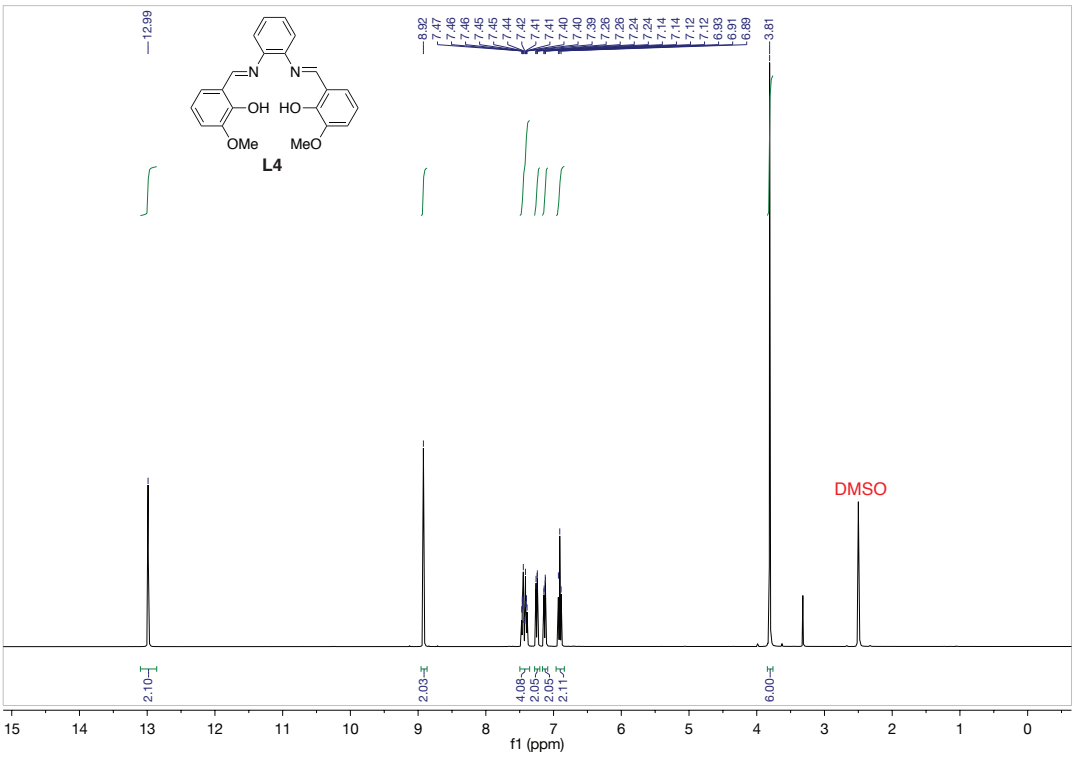


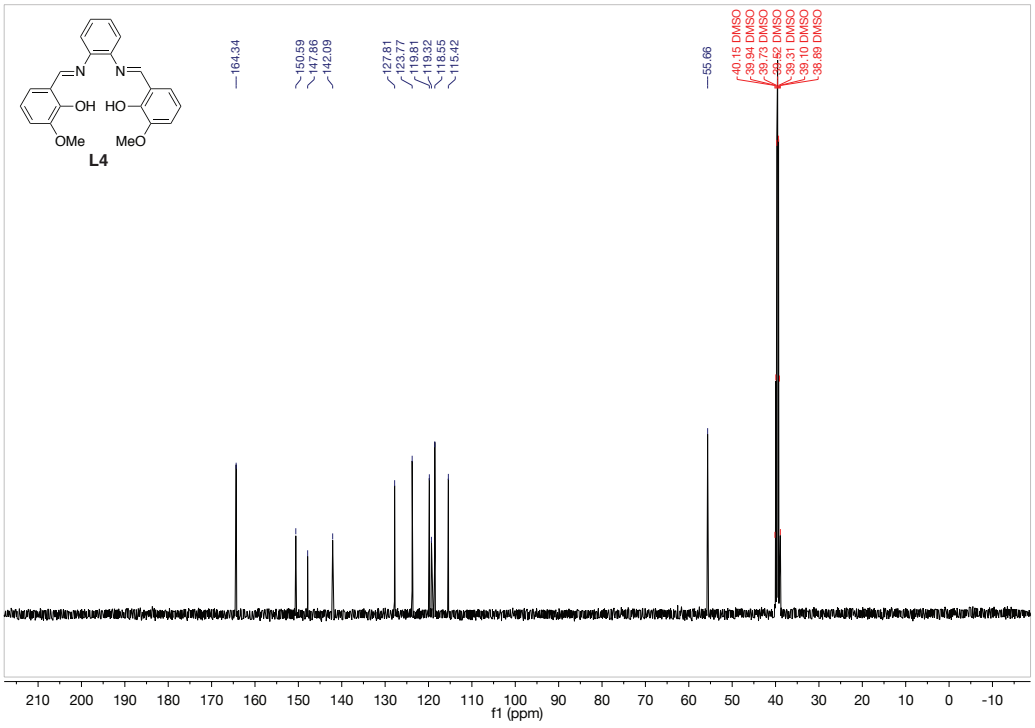

L1F
DMSO
f1 (ppm)

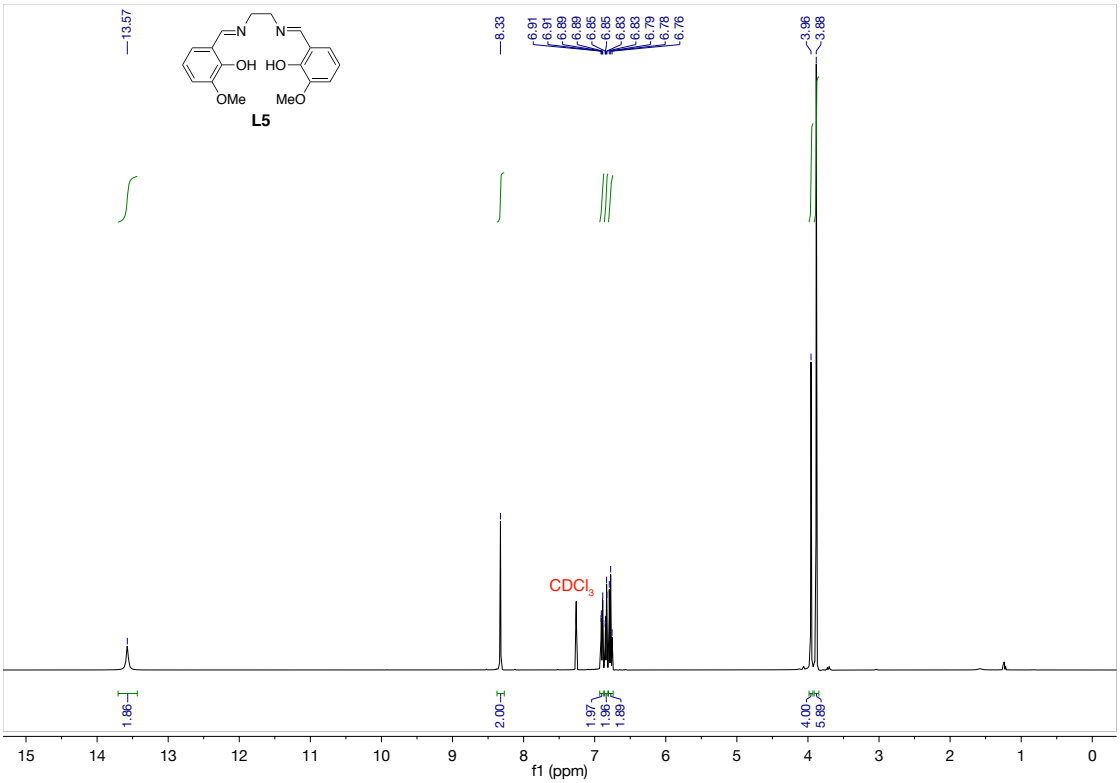

L1F
DMSO
f1 (ppm)

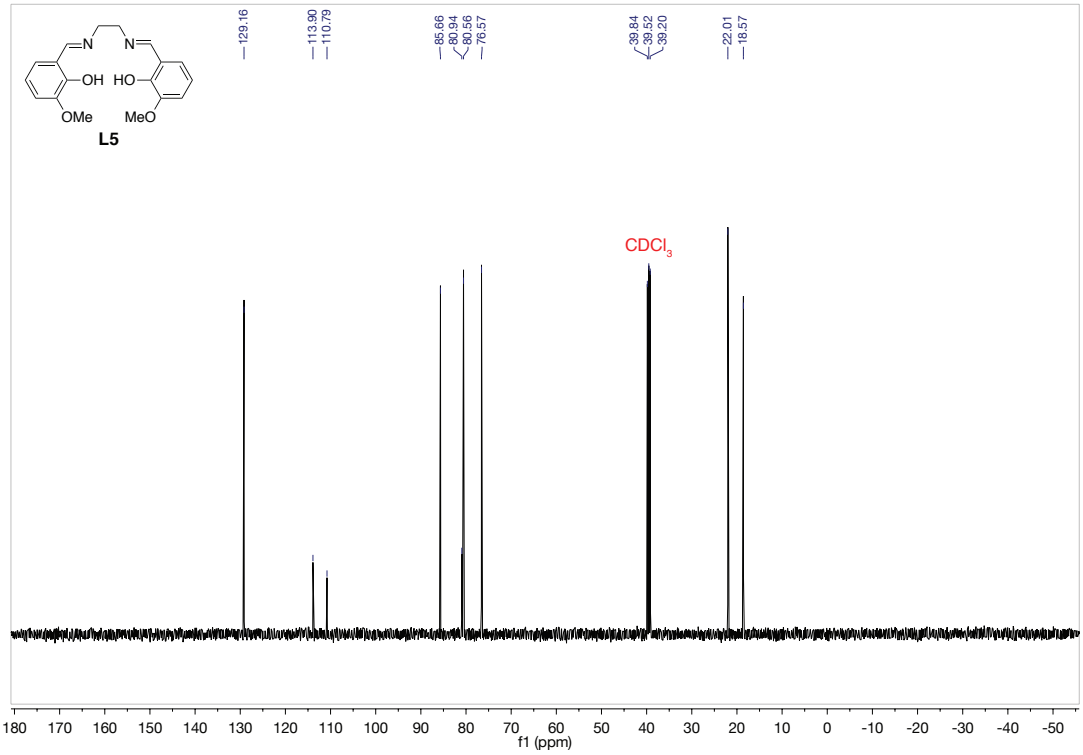

L2
12.55
8.96
7.84
7.83
7.82
7.81
7.42
7.41
7.40
7.39
7.39
7.38
7.37
6.90
6.90
6.90
6.88
6.88
6.86
6.86
3.51
3.49
DMSO
1.92
1.99
2.00
1.95
3.84
3.98
f1 (ppm)


L2
12.55
169.37
160.10
133.71
127.71
118.53
117.35
115.16
40.15 DMSO
39.94 DMSO
39.73 DMSO
39.52 DMSO
39.31 DMSO
39.10 DMSO
38.90 DMSO
38.53
f1 (ppm)


12.99
8.92
7.47
7.46
7.46
7.45
7.45
7.44
7.42
7.41
7.41
7.40
7.40
7.39
7.26
7.26
7.24
7.24
7.14
7.14
7.12
7.12
6.93
6.91
6.89
3.81
OH HO
OMe MeO
L4
DMSO
2.10
2.03
4.08
2.05
2.05
2.11
6.00
f1 (ppm)


OH HO
OMe MeO
L4
164.34
150.59
147.86
142.09
127.81
123.77
119.81
119.32
118.55
115.42
55.66
40.15 DMSO
39.94 DMSO
39.73 DMSO
39.52 DMSO
39.31 DMSO
39.10 DMSO
38.89 DMSO
f1 (ppm)


13.57
8.33
6.91
6.91
6.89
6.89
6.85
6.85
6.83
6.83
6.79
6.78
6.76
3.96
3.88
OH HO
OMe MeO
L5
CDCl3
1.86
2.00
1.97
1.96
1.89
4.00
5.89
f1 (ppm)


129.16
113.90
110.79
85.66
80.94
80.56
76.57
39.84
39.52
39.20
22.01
18.57
OH HO
OMe MeO
L5
CDCl3
f1 (ppm)