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Supporting Information

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Multimodal MR/Optical Imaging Contrast Agent Sensitive to NADH

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Supporting Information

Experimental Section

General: Reagents were obtained from commercial suppliers and used directly, unless otherwise noted. Cyclen (1,4,7,10-Tetraazacyclododecane) was obtained from Macrocyclics. NADH was purchased from CalBioChem and NAD^+/NADH Quantitation Kit (K337-100) was purchased from Biovision. Dry solvents, where indicated, were obtained from either Aldrich or Fisher Scientific as anhydrous Sure-Seal bottles. Water was purified using a Millipore Milli-Q Synthesis purifier (18.0 MΩ cm, Barnstead). pH value was measured with a Beckman Φ240 pH/Temp meter. HRMS was performed with a LTQ-ORBIRAP (Thermo scientific) using 50% acetonitrile and 50% water with 0.1% Formic acid as solvent under ESI conditions: injection amount (loop injection): 10 μL; flow rate: 50 μL/min; electrospray voltage: 5 kV; capillary temperature: 275 °C. The absorption spectra were taken with a Cary 100-Bio UV-vis spectrophotometer and are expressed in wavenumbers (cm^{-1}). Emission spectra were recorded with a FluoroMax-P (JOBIN YVON Inc.). NMR spectra were recorded using a Bruker FT-500 spectrometer (499.7 MHz for ^1H, 125.7 MHz for ^{13}C, and 67.8 MHz for ^{17}O) and were routinely run using broadband decoupling at 298.0 K.

Synthesis of 5'-{1-[4,7,10-tris(*tert*-butoxycarboxymethyl)-1,4,7,10-tetraazacyclododecyl]methyl}-1,3,3-trimethylspiro[indoline-2,3'-[3*H*]naphth[2,1-*b*][1,4]oxazine: A solution of 1,4,7-Tris(*tert*-butoxycarboxymethyl)-1,4,7,10-tetraazacyclododecane, hydrobromide salt (0.631 g, 1.059 mmol) and sodium carbonate (0.281 g, 2.649 mmol) in 23 mL of dry THF was stirred in a flask equipped with a calcium chloride drying tube. 5'-Bromomethyl-1,3,3-trimethylspiro[indoline-2,3'-[3*H*]naphtha[2,1-*b*][1,4]oxazine (0.372 g, 0.883 mmol) in 7.0 mL of dry THF was added

to the flask dropwise. The mixture was refluxed for 4 hours. The solid was filtered out and the filtrate was evaporated in *vacuo* to dryness. The crude product was purified by column chromatography on silica gel with dichloromethane/methanol (97/3) as eluent to give 0.639 grams (85% yield) of tris-*tert*-butyl ester of spironaphthoxazine-DO3A as a yellow solid. HRMS *m*/*z* calculated for $C_{49}H_{70}O_7N_6$ $(M+H)^+$ 855.5379 was found to be 855.5399. ^{1}H NMR ($CDCl_3$) δ_H 0.99 (3H, s, $C(CH_3)_2$), 1.45-1.47 (27H, m, $C(CH_3)_3$), 1.62 (3H, s, $C(CH_3)_2$), 2.12-2.50 (10H, m, NCH_2), 2.86-3.05 (15H, m, NCH_2 & NCH_3), 4.07 (2H, s, NCH_2), 4.58 (1H, s, N=CH), 6.64 (1H, s, ArH), 6.83 (1H, s, ArH), 7.06 (1H, s, ArH), 7.20 (1H, s, ArH), 7.51 (1H, s, ArH), 7.64 (1H, s, ArH), 7.90 (1H, s, ArH), 8.01 (1H, s, ArH), 8.52 (1H, s, ArH). ^{13}C NMR ($CDCl_3$) δ_C 24.84, 28.03, 28.15, 28.22, 35.22, 46.22, 50.21, 51.09, 52.41, 56.17, 56.82, 56.99, 76.31, 82.67, 83.06, 108.19, 119.41, 119.96, 121.99, 122.42, 126.09, 126.17, 127.45, 127.72, 128.42, 128.74, 131.56, 136.13, 137.39, 148.58, 150.79, 163.91, 173.10, 173.85, 173.92.

Synthesis of 5′-{1-[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecyl]methyl}-1,3,3-trimethylspiro[indoline-2,3′-[3*H*]naphth[2,1-*b*][1,4]oxazine (ligand): A solution of the tris-*tert*-butyl ester of spironaphthoxazine-DO3A (0.233 g, 0.273 mmol) in 5 mL of dichloromethane and 5 mL of trifluoroacetic acid was stirred for 24 hours. The solvent was evaporated in *vacuo* to dryness. The residue was dissolved in methanol, then the solvent was evaporated to dryness (the process was repeated for three times) to yield 0.18 grams (96% yield) of acid of spironaphthoxazine-DO3A as a black-brown solid. HRMS *m*/*z* calculated for $C_{37}H_{46}O_7N_6$ $(M+H)^+$ 687.3500 was found to be 687.3524. ^{1}H NMR (DMSO-d_6) δ_H 0.82-0.94 (3H, m, $C(CH_3)_2$), 1.54 (3H, s, $C(CH_3)_2$), 2.83 (1H, s, NCH_2), 2.93 (2H, s, NCH_2), 3.21-3.30 (16H, m, NCH_2 & NCH_3), 3.75-4.02 (6H, m, NCH_2), 4.65-

4.91 (3H, m, CH=N & NCH_2), 6.64 (1H, s, ArH), 6.76 (1H, s, ArH), 7.08-7.29 (2H, m, ArH), 7.67 (1H, s, ArH), 7.79 (1H, s, ArH), 8.12 (1H, s, ArH), 8.29 (1H, s, ArH), 8.43 (1H, s, ArH). ^{13}C NMR (DMSO-d_6) δ_C 21.31, 25.25, 27.15, 30.27, 35.20, 46.20, 46.32, 49.56, 50.01, 50.19, 51.06, 51.62, 53.51, 54.00, 54.48, 55.51, 75.81, 107.70, 109.90, 113.76, 116.11, 118.47, 122.24, 126.75, 126.90, 128.70, 129.88, 131.34, 136.23, 136.75, 148.05, 149.26, 150.48, 164.80, 170.00, 170.33, 172.10.

Synthesis of Gd-5′-{1-[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecyl]methyl}-1,3,3-trimethylspiro[indoline-2,3′-[3*H*]naphth[2,1-*b*][1,4]oxazine: A solution of the acid of spironaphthoxazine-DO3A (ligand) (0.356 g, 0.519 mmol) in 10 mL of water was cooled to 0 °C. The pH of the solution was brought to 6.5 with NaOH (1 N). A small amount of methanol (~1.0 mL) was added to increase the solubility of the acid in water. A solution of $GdCl_3$•$6H_2O$ (0.231 g, 0.621 mmol) in 5 mL of water was prepared and the pH of the solution was brought to 6.5. The gadolinium chloride solution was added to the flask dropwise. The pH was kept above 5.5 during metal addition. The solution was allowed to warm to room temperature while stirring, and the pH was adjusted periodically to keep it around 6.0. After 3 days at room temperature, the pH showed no change and the reaction was considered complete.[S1] The solvent was evaporated in *vacuo*. The crude product was purified by column chromatography on silica gel with methanol/water (4/1) as eluent to give 0.362 grams (83% yield) of complex **1** as a brown solid. HRMS *m*/*z* calculated for $C_{37}H_{43}O_7N_6Gd$ $(M+H)^+$ 839.2698 was found to be 839.2368 with appropriate isotope pattern.

Determination of hydration number (q): ^{17}O NMR measurements were performed at 67.8 MHz in 5 mm NMR sample tubes on a Bruker FT-500 spectrometer (Bruker,

Billerica, MA) at 80 °C, following the method described in the literature except for using Gd(III) complexes instead of Dy(III) complex[S2] Although gadolinium complex has a very large line broadening effect on the ^{17}O signal at room temperature (Fig. S1), the peak shape significantly narrows (Fig. S2) at high temperature, when the exchange between Gd(III) bound water and bulk water is fast on the ^{17}O NMR time scale, and at high Gd(III) concentration, which is necessary for sufficient accuracy.[S2] Thus for Gd(III) complexes in high concentration, the observed ^{17}O chemical shift at high temperature (~ 80 ^{o}C) can be used directly to evaluate *q*. In this paper all measurements were conducted with natural abundance of ^{17}O in the sample. A sample of pure D_2O (0.5 mL) was placed in the spectrometer at 80 °C, and left for 10 min in order to reach the temperature. A ^{17}O NMR spectrum was recorded with the deuterium frequency lock and then the sample was substituted by a sample of **1** in D_2O (20 mM). The pH of the sample was checked and corrected to 7.0 (for $GdCl_3$, pH 3.5) by addition of a freshly prepared solution of NaOH. The ^{17}O NMR shift was measured with the same spectrometer settings. After cooling to room temperature, a small amount of NADH aqueous solution was added to the NMR tube containing **1** at a ratio of Gd(III) : NADH of 1 to 1 (the concentrations of Gd(III) and NADH were adjusted according to the volume change). The tube was placed in the dark for 20 min and then incubated in the spectrometer at 80 °C for 10 min, and the ^{17}O NMR shift was measured with the same spectrometer settings. For each complex or compound, two samples with similar concentration were prepared for measurement of ^{17}O NMR shift. The average of the two numbers is the hydration number of that complex or compound (Table S1 and S2).

Calculation of hydration number using sample **1** as an example: All equations, terms and definition are from the literature.[S2] From the total amounts of Gd (1.0×10^{-5} mol) and water (0.508 mL (0.0281 mol), including the complex **1** and water introduced during the pH adjustments), P_m was determined to be 3.56×10^{-4}. The difference in ^{17}O chemical shifts of the water resonances was -0.985 ppm (δ_{obs}). Thus, $\Delta = -0.985/P_m = -2766.85$ ppm [see eqn (2) in the literature[S2]]. At 80 °C, F ≈ -68.19 ppm [see eqn (5) in the literature[S2]] and therefore $q = \Delta/(<S> \cdot F) = -2766.85/(31.5 \times (-68.19) = 1.29$ [see eqn (3) in the literature[S2]].

R_1 relaxivity: T_1 values were determined using an inversion recovery pulse sequence with 10-15 data points at 60 MHz and 37 °C on a Bruker mq60 Minispec (Bruker, Billerica, MA). Each solution was incubated for 10 min at 37 °C in the dark before T_1 measurement. The resulting curves were fit to a monoexponential function to obtain T_1. The longitudinal (r_1) relaxivity was determined as the slope of the line for plots of $1/T_1$, against increasing gadolinium concentration with a correlation coefficient greater than 0.99. A small amount of concentrated NADH aqueous solution was added to Gd(III) solutions thus the ratio of Gd(III)/NADH in each solution is 1:1. The mixtures were put in dark for 20 min and then incubated for 10 min at 37 °C. T_1 values of these solutions were measured with the same procedure.

MRI: MRI was performed on a Bruker Biospec 7 T system (300 MHz, 21 °C, Bruker, Billerica, MA). The magnet was equipped with the standard gradient set (95 mT m^{-1} maximum gradient) and 72 mm internal diameter (ID) volume coil. Parameters for the images were TR = 1000 ms and TE = 10.3 ms for T 1 weighted (T1W) images. For all images a spin echo sequence was used with a field view (FOV) of 4.2 cm^2, slice thickness

1.5 mm, and a 128 × 128 matrix. After images of these solutions were taken, a small amount of concentrated NADH aqueous solution was added to Gd(III) solutions to reach a ratio of Gd(III)/NADH in each solution of 1:1. The mixtures were imaged immediately with the same experimental settings and then put in the dark. Then images were taken 10 and 30 minutes later with the same experimental settings.

Cell uptake: P388D1 cells were plated at 500,000 cells/mL into 35mm culture dishes and were maintained in RPMI-1640 media supplemented with L-Glutamine and 10% FBS at 37 °C in a 5% CO_2 atmosphere overnight. Solutions of complex **1** (0.103 mM or 0.28 mM) in the same media were prepared and incubated in 37 °C waterbath for 20 min before use. After removal of culture media, the Gd(III) containing solution was transferred into the dish. The cells were incubated with complex **1** for 1 h at 37 °C in 5% CO_2 atmosphere. After removal of the media containing **1**, cells were washed three times with 1X PBS and were placed in PBS for confocal imaging or MRI.

Confocal Microscopy: Cells containing **1** in the dark were imaged with a Zeiss LSM 5 Pascal confocal microscope equipped with a LD-neofluar 40×/0.6 corr objective. An excitation wavelength of 458 nm (25% power) was used with a 458/514 nm HFT beam splitter, and a 505 nm low pass filter. A 1024 × 1024 matrix (230.3 × 230.3 μm^2 in-plane resolution), pinhole of 1 Airy unit, and a scan speed of 2 (56.2 μs pixel time) were used. After imaging, a small amount of concentrated NADH aqueous solution was added to the cells to reach a ratio of Gd(III)/NADH in the media of 1:1. The samples were imaged immediately with the same experimental settings and then put in the dark. Then images were taken 5, 10, and 20 minutes later with the same experimental settings.

[S1]. J. A. Duimstra, F. J. Femia, T. J. Meade, *J. Am. Chem. Soc.* **2005**, *127*, 12847–12855.

[S2]. K. Djanashvili, J. A. Peters, *Contrast Media Mol. Imaging* **2007**, *2*, 67–71.

Table S1 Hydration number of complex **1** before and after addition of NADH

	Spirooxazine-Gd-DO3A		Spirooxazine-Gd-DO3A + NADH**	
[Gd(III)] (mol)	1.0×10^{-5}	0.99×10^{-5}	1.0×10^{-5}	0.99×10^{-5}
D_2O (mL(mol))	0.508 (0.0281)	0.505 (0.0279)	0.528 (0.0292)	0.525 (0.029)
P_m	3.56×10^{-4}	3.55×10^{-4}	3.42×10^{-4}	3.41×10^{-4}
^{17}O Shift (ppm)	-0.972	-0.925	-1.504	-1.423
δ_{obs} (ppm)*	-0.985	-0.938	-1.517	-1.436
Δ (ppm)	-2766.85	-2642.25	-4435.67	-4211.14
q	1.29	1.23	2.06	1.96
q (average)	1.26 ± 0.03		2.01 ± 0.05	

* δ_{obs} (D_2O) = 0.013 ppm
** 20 μL of NADH (0.52 M) was added to the system.

Table S2 Hydration number of Gd-DOTA and $GdCl_3 \cdot 6H_2O$

	Gd-DOTA		$GdCl_3 \cdot 6H_2O$	
[Gd(III)] (mol)	1.11×10^{-5}	1.19×10^{-5}	1.05×10^{-5}	1.01×10^{-5}
D_2O (mL(mol))	0.508 (0.0281)	0.505 (0.0279)	0.505(0.0279)	0.510 (0.0282)
P_m	3.95×10^{-4}	4.27×10^{-4}	3.76×10^{-4}	3.61×10^{-4}
^{17}O Shift (ppm)	-0.894	-1.002	-7.787	-7.847
δ_{obs} (ppm)*	-0.907	-1.015	-7.8	-7.86
Δ (ppm)	-2298.38	-2376.01	-20744.68	21772.85
q	1.07	1.11	9.65	10.13
q (average)	1.09 ± 0.02		9.89 ± 0.24	

* δ_{obs} (D_2O) = 0.013 ppm

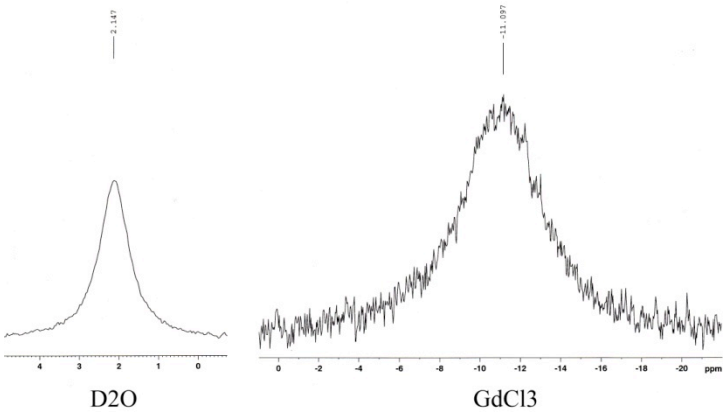


Fig 1 ^{17}O chemical shift of $GdCl_3$ (20mM) in D_2O at 298K.

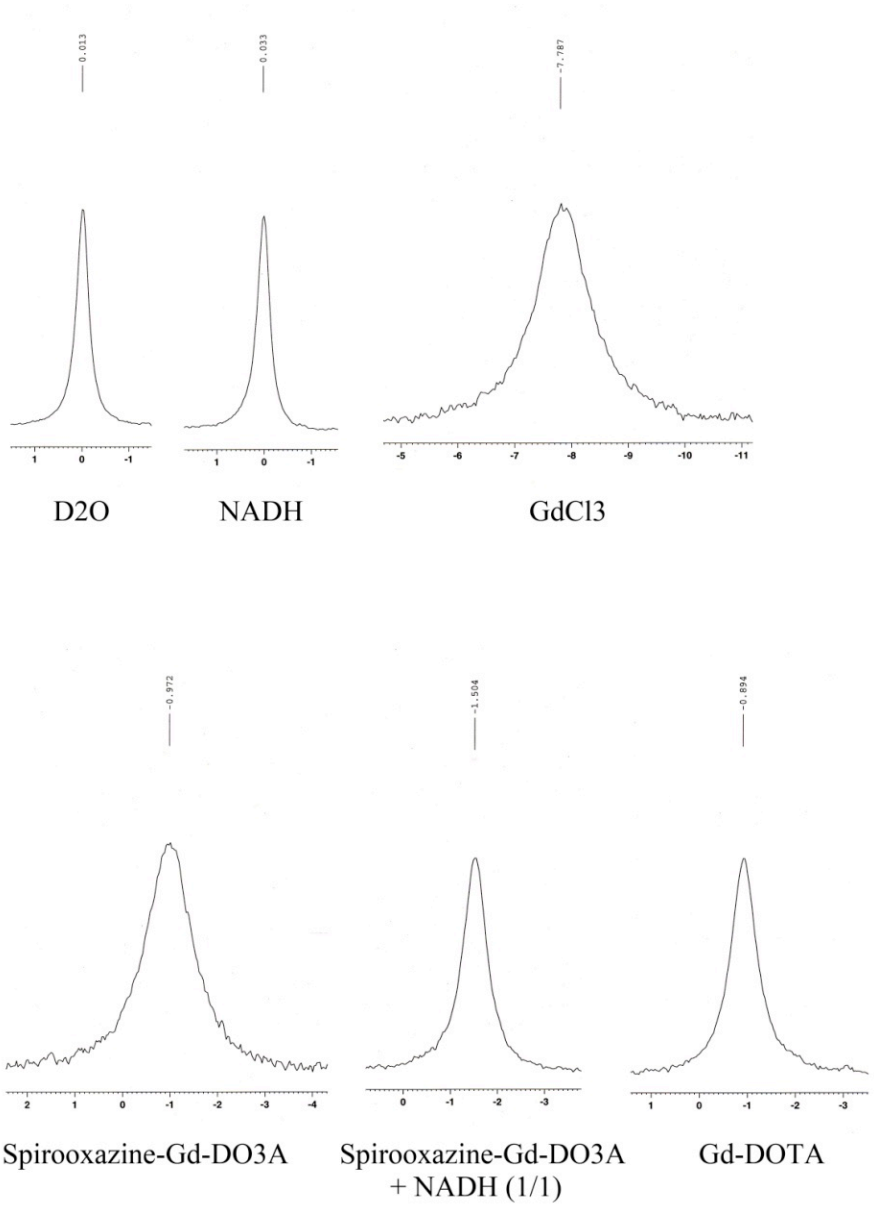
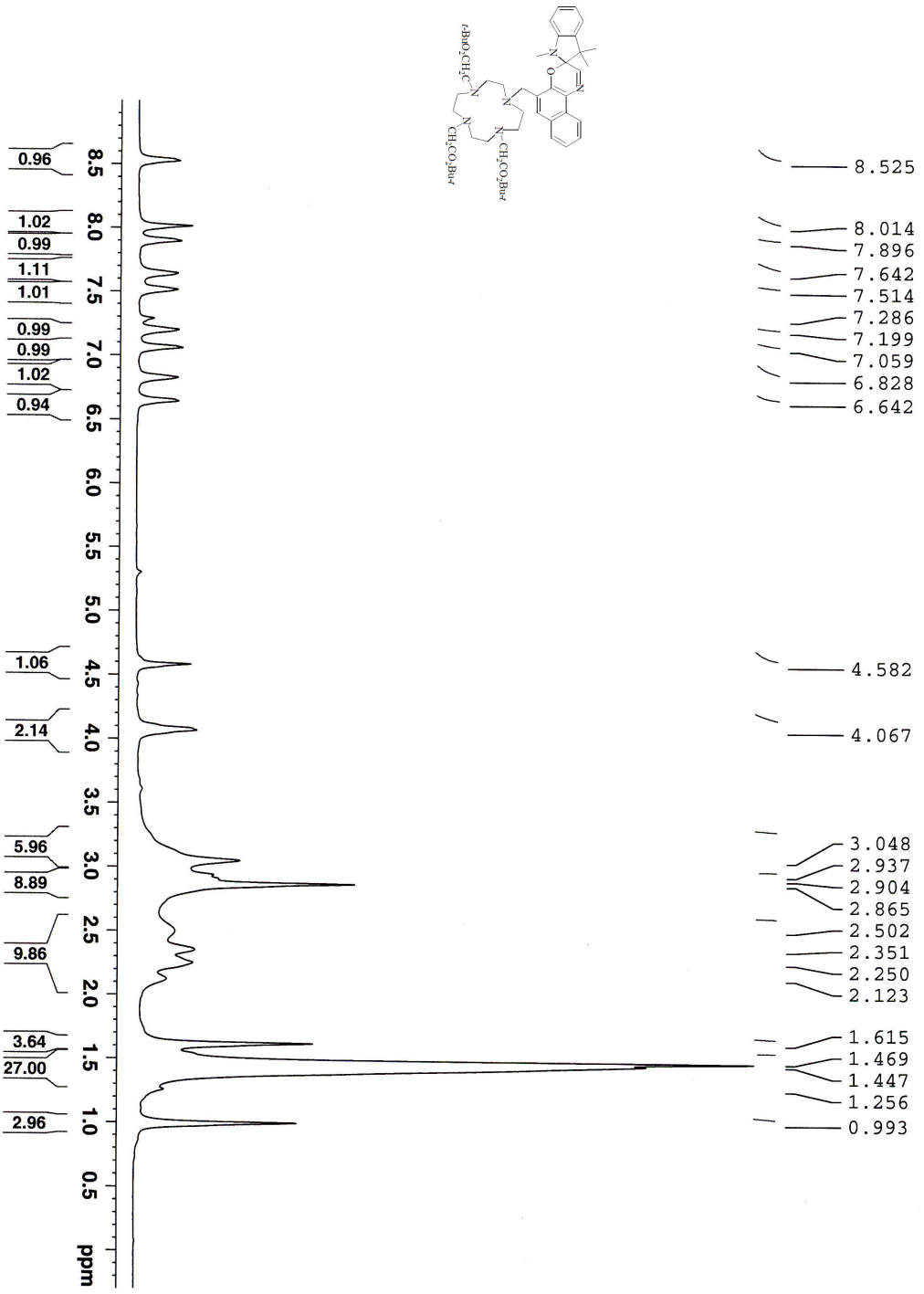


Fig 2 ^{17}O chemical shift of Gd complexes in D_2O at 353K.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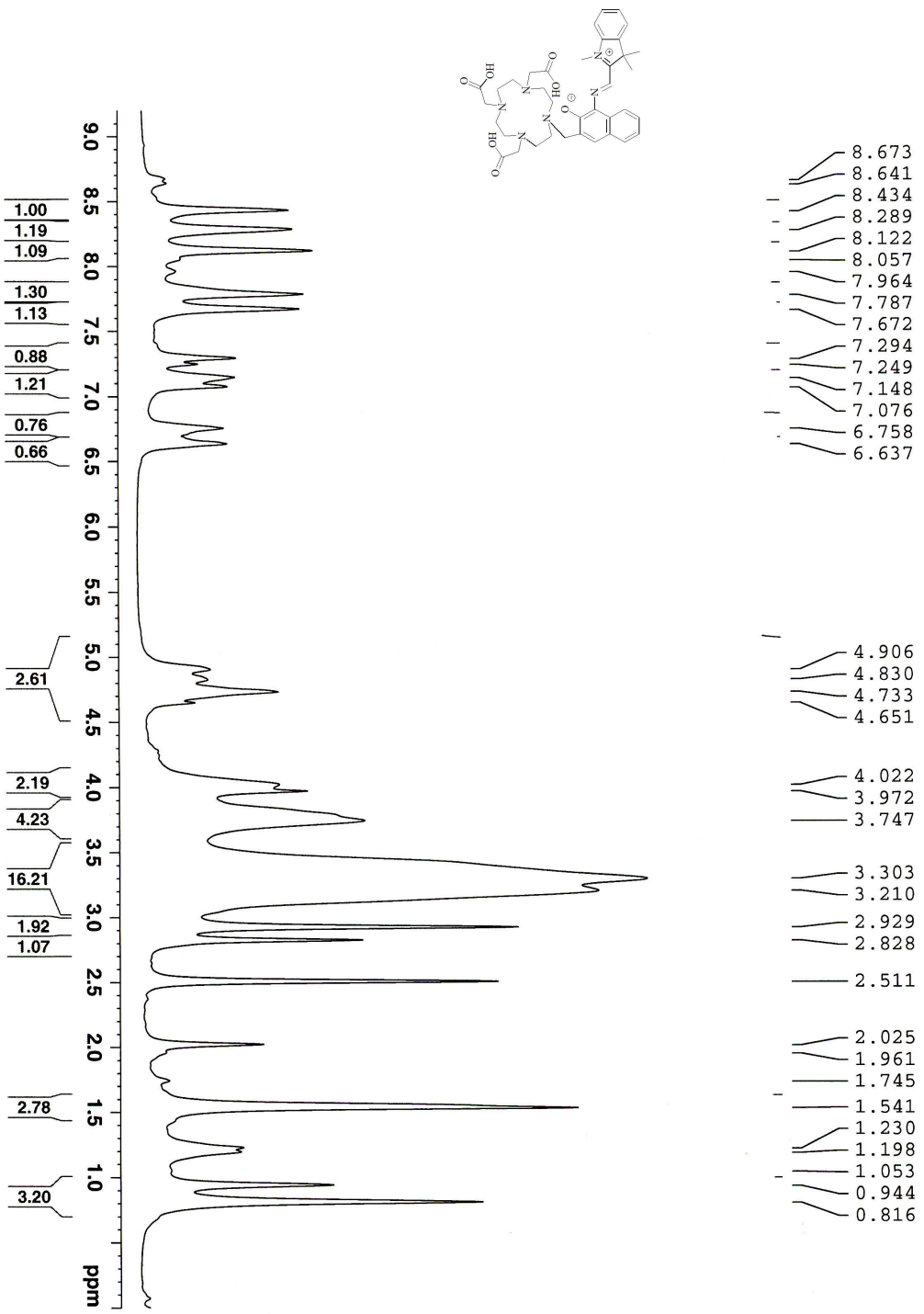
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