Supplementary Material (ESI) for Chemical Communications

trans-2-Aminocyclohexanol as a pH-Sensitive Conformational Switch in Lipid Amphiphiles

Barbora Brazdova,[†] Ningrong Zhang,[‡] Vyacheslav V. Samoshin,[†,*] and Xin Guo[‡,*]

Department of Chemistry[†], and Department of Pharmaceutics and Medicinal Chemistry[‡],

University of the Pacific, Stockton, California 95211

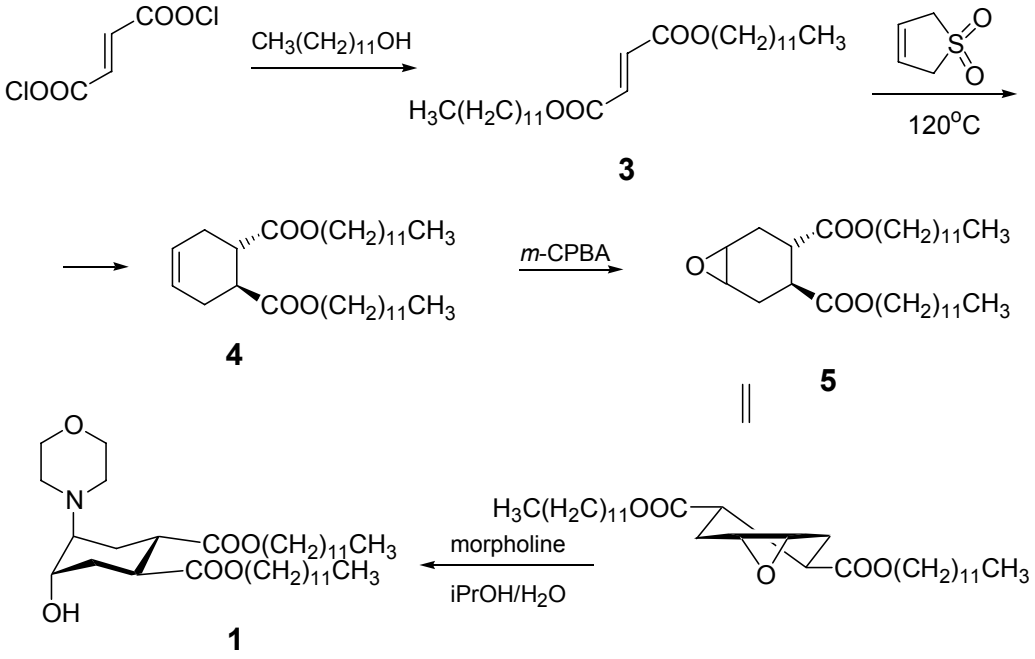
Supporting Information Available

1. Syntheses

General Procedures. Column chromatography was performed on silica gel (Sorbent Technologies, 40-75 μm). The reactions were monitored by TLC (silica gel, 8x2 cm plates with UV-indicator (254 nm), manufactured by Analtech Inc.). ^{1}H NMR and ^{13}C NMR spectra were acquired on Varian Mercury NMR-spectrometer (300 MHz). Exact mass measurements were performed on JEOL LC-Mate double-focusing mass spectrometer (Peabody, MA, USA) equipped with electrospray ionization source at a resolving power of 5000 with polyethyleneglycol as an internal reference. The MS spectra were obtained on Varian 1200LC triple quadrupole mass spectrometer (Walnut Creek, CA, USA) with electrospray ionization source in positive mode. Elemental analyses were carried out by Micro-Mass Facility, UC Berkeley. All solvents were purified by conventional techniques prior to use. Starting materials were purchased from Aldrich.

Lipids **1** and **2** were prepared (Scheme S1, p.2; Scheme S2, p.6) using the approach developed for the syntheses of reported *trans*-2-aminocyclohexanol-based conformational switches including crown ethers and podands.[1-6]

Scheme S1.



Didodecyl fumarate (3)

Fumaryl chloride (5g, 27 mmol) was refluxed for 2 h with 12 mL (10 g, 54 mmol) of 1-dodecanol in 50 mL of dry toluene under N_2. The reaction mixture was diluted with 200 mL of $CHCl_3$ and neutralized with solid $NaHCO_3$. The decanted organic layer was washed with 2 x 80 mL of water, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography ($CHCl_3$) to yield 11.0 g (90%) of oily solid. ^{1}H NMR (300 MHz, $CDCl_3$): 6.85 (s, 2H, HC=CH), 4.19 (t, 4H, OCH_2, dodecyl), 1.67 (quin, 4H, CH_2, dodecyl), 1.24-1.4 (m, 36H, CH_2, dodecyl), 0.88 (t, 6H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 165.14, 133.65, 65.54, 31.93, 29.65, 29.64, 29.58, 29.51, 29.36, 29.24, 28.56, 25.90, 22.70, 14.11.

Didodecyl 4-cyclohexene-*trans*-1,2-dicarboxylate (4)

Didodecyl fumarate (6g, 13.3 mmol), butadiene sulfone (2g, 16.9 mmol), and hydroquinone (110 mg, 1 mmol) were mixed and diluted with 10 mL of isopropanol. The mixture was heated in a sealed reactor at 120 ºC for 48 h. After cooling, 150 mL of H_2O and 70 mL of $CHCl_3$ were added and the mixture was neutralized with solid $NaHCO_3$ (~25 g). The organic layer was separated, washed with 100 mL of brine and with water, dried over $MgSO_4$, and

concentrated *in vacuo* to yield 6.3 g of crude product, which was purified by column chromatography ($CHCl_3$) to yield 4.19 g (62%) of yellowish oil. ^{1}H NMR (300 MHz, $CDCl_3$): 5.68 (m, 2H, H4+H5), 4.07 (m, 4H, OCH_2, dodecyl), 2.85 (m, 2H, H1+H2), 2.36-2.48 (m, 2H, H3e+H6e), 2.10-2.23 (m, 2H, H3a+H6a), 1.61 (br. quin, J = 6.6 Hz, 4H, CH_2, dodecyl), 1.2-1.4 (m, 36H, CH_2, dodecyl), 0.88 (t, J = 6.6 Hz, 6H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 174.85, 125.03, 64.82, 41.39, 31.95, 29.68, 29.66, 29.61, 29.56, 29.37, 29.29, 28.67, 25.91, 22.70, 14.09.

Didodecyl 7-oxabicyclo[4.1.0]heptane-*trans*-3,4-dicarboxylate (5)

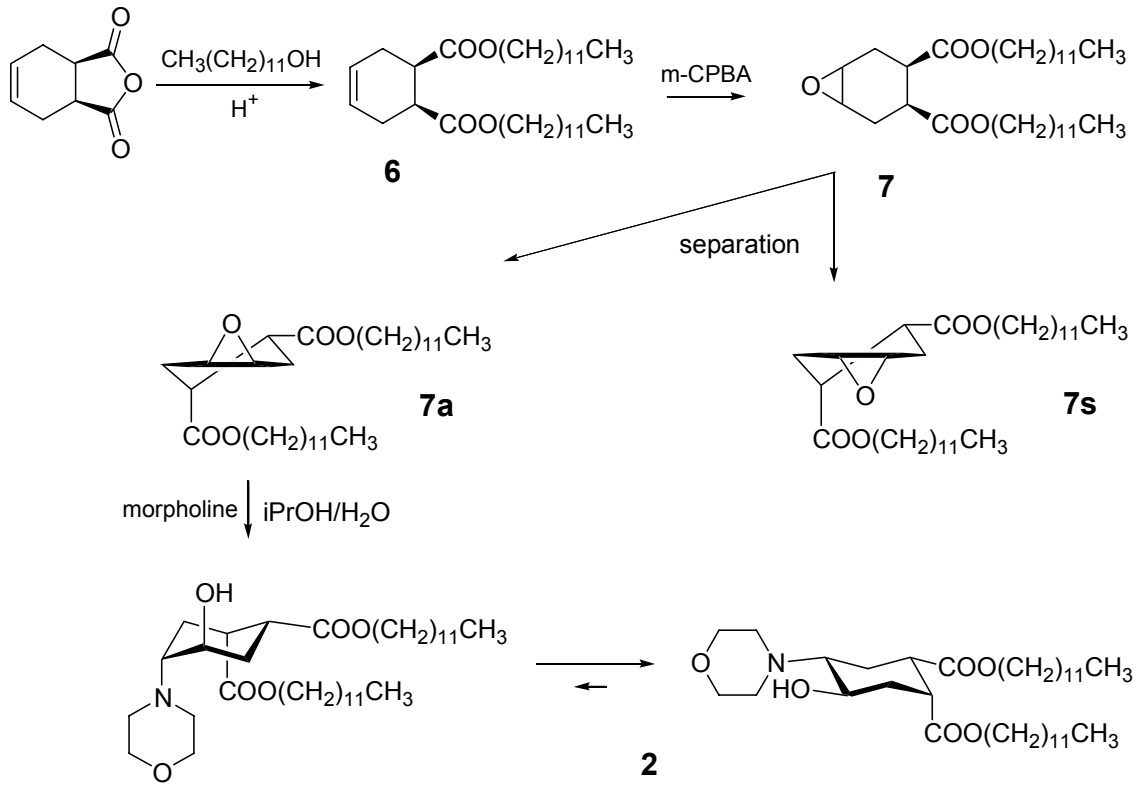
Diester **4** (11.1g, 22 mmol) was dissolved in 400 mL of dry CH_2Cl_2, and *m*-CPBA (16.4 g of 70 % tech. grade, 66 mmol) was added in small portions at 0^{o}C while stirring. The reaction mixture was kept at this temperature for 10 h. After the consumption of the starting material (TLC, $CHCl_3$), 100 mL of chloroform was added followed by 200 mL of saturated Na_2CO_3. The mixture was stirred for 30 min and then the organic phase was washed with 4 x 100 mL of Na_2CO_3. The organic layer was dried for 12 h over anhydrous Na_2SO_4 and evaporated to yield 9 g of yellowish oil. The epoxide **5** was purified by column chromatography ($CHCl_3$): yield 6.6 g (58 %). ^{1}H NMR (300 MHz, $CDCl_3$): 4.05 (m, 4H, OCH_2, dodecyl), 3.25 (m, 1H, H6), 3.19 (t, J = 4.3 Hz, 1H, H1), 2.84 (dt, J = 4.9, 10.5 Hz, 1H, H4), 2.61 (dt, J = 6.6, 10.6 Hz, 1H, H3), 2.47 (ddd, J = 14.9, 4.8, 1.8 Hz, 1H, H5e), 2.32 (ddd, J = 15.4, 6.6, 4.68 Hz, 1H, H2e), 2.06 (dd, J = 15.4, 10.7 Hz, 1H, H2a), 1.89 (ddd, J = 14.9, 10.8, 2.0 Hz, 1H, H5a), 1.59 (br. quin, 4H, CH_2, dodecyl), 1.24-1.34 (m, 36H, CH_2, dodecyl), 0.88 (t, J = 6.7 Hz, 6H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 174.70, 173.73 64.99, 51.86, 50.36, 40.14, 37.81, 31.94, 29.67, 29.66, 29.61, 29.55, 29.37, 29.28, 28.61, 27.29, 26.46, 25.90, 22.70, 14.10. MS/MS *m/z* (rel. intensity): 99.2 (12), 123.4 (22), 151.1 (37), 169.2 (43), 187.2 (100), 319.3 (18), 355.2 (68), 523.3 (33) $[M+H]^+$.

Didodecyl *cis*-4-hydroxy-*trans*-5-morpholylcyclohexane-*trans*-1,2-dicarboxylate (1)

Epoxide **5** (1.0 g, 1.9 mmol) and morpholine (0.9 mL, 10 mmol) were stirred 15 h at 40 ^{o}C in 5 mL of iPrOH:H_2O (1.5:1). The reaction mixture was concentrated on a rotary evaporator, and the product **1** was isolated as a clear oil by column chromatography (EtOAc:hexane, 1:2): yield 0.78 g (67 %). ^{1}H NMR (300 MHz, CD_3OD): 4.07 (br. t, J = 6.5 Hz, 4H, OCH_2, dodecyl), 4.00 (dt, J = 2.8, 5.2 Hz, 1H, H4), 3.69 (t, J = 4.7 Hz, 4H, OCH_2, morpholyl), 3.09 (dt, J = 4.3, 9.4 Hz, 1H, H2), 2.98 (dt, J = 3.7, 9.3 Hz, 1H, H1), 2.57 m (2H, CH_2N), 2.48 m (2H, CH_2N), 2.23 (dt, J = 3.1, 5.3 Hz, 1H, H5), 2.04 (ddd, J = 9.9, 6.6, 3.0 Hz, 1H, H3e), 1.99 (ddd, J = 10.2, 7.7, 3.2 Hz,

1H, H6e), 1.88 (ddd, *J* = 14.1, 5.5, 3.9 Hz, 1H, H6a), 1.79 (ddd, *J* = 13.4, 5.4, 4.7 Hz, 1H, H3a), 1.62 (br. quin, *J* = 6.4 Hz, 4H, CH_2, dodecyl), 1.26-1.39 (m, 36H, CH_2, dodecyl), 0.89 (t, *J* = 6.7 Hz, 3H, CH_3). ^{13}C NMR (300 MHz, CD_3OD): 176.44, 176.22, 68.33, 66.00, 65.95, 65.67, 65.35, 51.57, 41.08, 40.95, 33.12, 31.94, 30.83, 30.81, 30.74, 30.52, 30.40, 29.81, 29.80, 27.13, 27.11, 24.93, 23.77, 14.47. MS/MS *m/z* (rel. intensity): 221.1 (7), 267.1 (28), 281.1 (10), 355.1 (100), 474.4 (5), 541.8 (9), 542.8 (8), 563.9 (6), 591.7 (6), 610.1 (5) $[M+H]^+$. HRMS: $C_{36}H_{67}NO_6$ requires *m/z* $[M+H]^+$ 610.5041, found 610.5023. Calcd. for $C_{36}H_{67}NO_6$: C, 70.89; H, 11.07; N, 2.30. Found: C, 71.02; H, 11.86; N, 2.28.

Scheme S2



Didodecyl 4-cyclohexene-*cis*-1,2-dicarboxylate (6)

cis-Tetrahydrophthalic anhydride 10 g (66 mmol), 1-dodecanol (32 mL, 143 mmol), and *p*-toluensulfonic acid (0.5 g) were refluxed 12 h in 200 mL of toluene. Toluene was removed on rotary evaporator, and the residue was

purified by column chromatography (EtOAc:hexane, 1:4) to yield colorless oil: yield 30.5 g (91 %). ^{1}H NMR (300 MHz, $CDCl_3$): 5.65 (m, 2H, H4+H5), 4.05 (t, J = 6.7 Hz, 4H, OCH_2, dodecyl), 3.01 (br. t, J = 5.6 Hz, 2H, H1+H2), 2.54 (m, 2H, H3+H6), 2.34 (m, 2H, H3+H6), 1.58 (br. quin, J = 6.8 Hz, 4H, CH_2, dodecyl), 1.22-1.33 (m, 36H, CH_2, dodecyl), 0.86 (t, J = 6.8 Hz; 6H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 173.24, 125.18 , 64.74, 39.82, 31.89, 29.63, 29.61, 29.56, 29.52, 29.32, 29.24, 28.58, 25.91, 25.89, 22.65, 14.04.

Didodecyl 7-oxabicyclo[4.1.0]heptane-*cis*-3,4-dicarboxylates (7a) and (7s)

Diester **6** (10 g, 20 mmol) was epoxidized the same way as described above for the synthesis of epoxide **5**. Two products - *anti*-epoxide **7a** and *syn*-epoxide **7s** (3:1 by ^{1}H NMR) - were separated by column chromatography (EtOAc:hexane, 1:5).

Epoxide **7a**: yield 4.3 g (42 %). ^{1}H NMR (300 MHz, $CDCl_3$): 4.06 (t, J = 6.7 Hz, 4H, OCH_2, dodecyl), 3.23 (br. s, 2H, H1+H6), 2.90 (br. t, J = 5.1 Hz, 2H, H3+H4), 2.16-2.32 (m, 4H, H2+H5), 1.60 (br. quin, J = 6.7 Hz, 4H, CH_2, dodecyl), 1.19-1.38 (m, 36H, CH_2, dodecyl), 0.88 (t, J = 6.6 Hz, 6H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 172.99, 65.03, 51.61, 37.75, 31.90, 29.64, 29.62, 29.58, 29.53, 29.33, 29.25, 28.56, 25.92, 24.89, 22.66, 14.06. HRMS: $C_{32}H_{58}O_5$ requires *m/z* $[M+Na]^+$ 545.4177, found 545.4160.

Epoxide **7s**: yield 2.5 g (24 %). ^{1}H NMR (300 MHz, $CDCl_3$): 4.07 (t, J = 6.7 Hz, 4H, OCH_2, dodecyl), 3.16 (br. s, 2H, H1+H6), 2.64-2.76 (m, 4H, H2-H5), 2.07-2.17 (m, 2H, H2+H5), 1.60 (br. quin, J = 6.8 Hz, 4H, CH_2, dodecyl), 1.22-1.38 (m, 36H, CH_2, dodecyl), 0.88 (t, J = 6.7 Hz, 6H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 172.47, 64.84, 50.88, 37.62, 31.90, 29.63, 29.62, 29.57, 29.52, 29.32, 29.24, 28.53, 25.95, 24.94, 22.65, 14.05. HRMS: $C_{32}H_{58}O_5$ requires *m/z* $[M+Na]^+$ 545.4177, found 545.4164.

Didodecyl *trans*-4-hydroxy-*cis* -5-morpholylcyclohexane-*cis*-1,2-dicarboxylate (2)

anti-Epoxide **7a** (1.0 g, 1.9 mmol) and morpholine (0.9 mL, 10 mmol) were stirred 15 h at 40 ^{o}C in 5 mL of iPrOH:H_2O (1.5:1). The reaction mixture was concentrated on rotary evaporator, and the product **2** was isolated as a clear oil by column chromatography (EtOAc:hexane, 1:2): yield 0.77 g (66 %). ^{1}H NMR (300 MHz, CD_3OD): 4.06 (m, 4H, OCH_2, dodecyl), 3.68 (m, 4H, OCH_2, morpholyl), 3.53 (dt, J = 4.3, 10.4 Hz, 1H, H4), 3.25 (q, J = 4.1 Hz, 1H, H2), 2.70 (m, 2H, NCH_2, morpholyl), 2.65 (dt, J = 12.2, 4.3 Hz; 1H, H1), 2.53 (m, 2H, NCH_2, morpholyl), 2.40

(ddd, J = 13.2, 4.2, 3.3; 1H, H3e), 2.33 (ddd, J = 11.9, 9.7, 3.7 Hz; 1H, H5), 2.14 (dt, J = 13.0, 3.7 Hz; 1H, H6e), 1.83 (q, J = 12.5 Hz; 1H, H6a), 1.63 (m, 1H, H3a), 1.61 (m, 4H, CH_2, dodecyl), 1.27-1.35 (m, 36H, CH_2, dodecyl), 0.89 (t, J = 6.7 Hz; 6H, CH_3). ^{13}C NMR (300 MHz, CD_3OD): 174.79, 174.74, 69.62, 68.48, 66.54, 65.99, 50.33, 43.69, 42.40, 35.49, 33.13, 30.86, 30.82, 30.78, 30.75, 30.53, 30.44, 29.84, 29.70, 27.20, 27.18, 23.78, 23.03, 14.49. MS/MS *m/z* (rel. intensity): 201.5 (5), 238.1 (13), 256.12 (100), 378.3 (11), 424.1 (85), 442.3 (27), 592.5 (90), 610.5 (8) $[M+H]^+$. HRMS: $C_{36}H_{67}NO_6$ requires $[M+H]^+$ 610.5041, found 610.5032. Calcd. for $C_{36}H_{67}NO_6$: C, 70.89; H, 11.07; N, 2.30. Found: C, 71.10; H, 11.84; N, 2.27.

2. NMR Studies on Acid-triggered Conformation Switch

Acid titration

The conformation change of the *trans*-2-aminocyclohexanol moiety in the lipids was monitored by their ^{1}H NMR spectra. Triflouroacetic acid (TFA) in CD_3OD (1:3 v/v) was added in 10 μL portions to dilute CD_3OD solutions (< 0.1 M) of lipid **1** or **2** in NMR tube. The solution was carefully mixed, pH was measured using the capillary microelectrode MI-412 (Microelectrodes, Inc.; Bedford, NH), and the ^{1}H NMR spectrum was acquired.

Figure S1. ^{1}H NMR of 1 at the apparent pH 8.1 in CD_3OD.

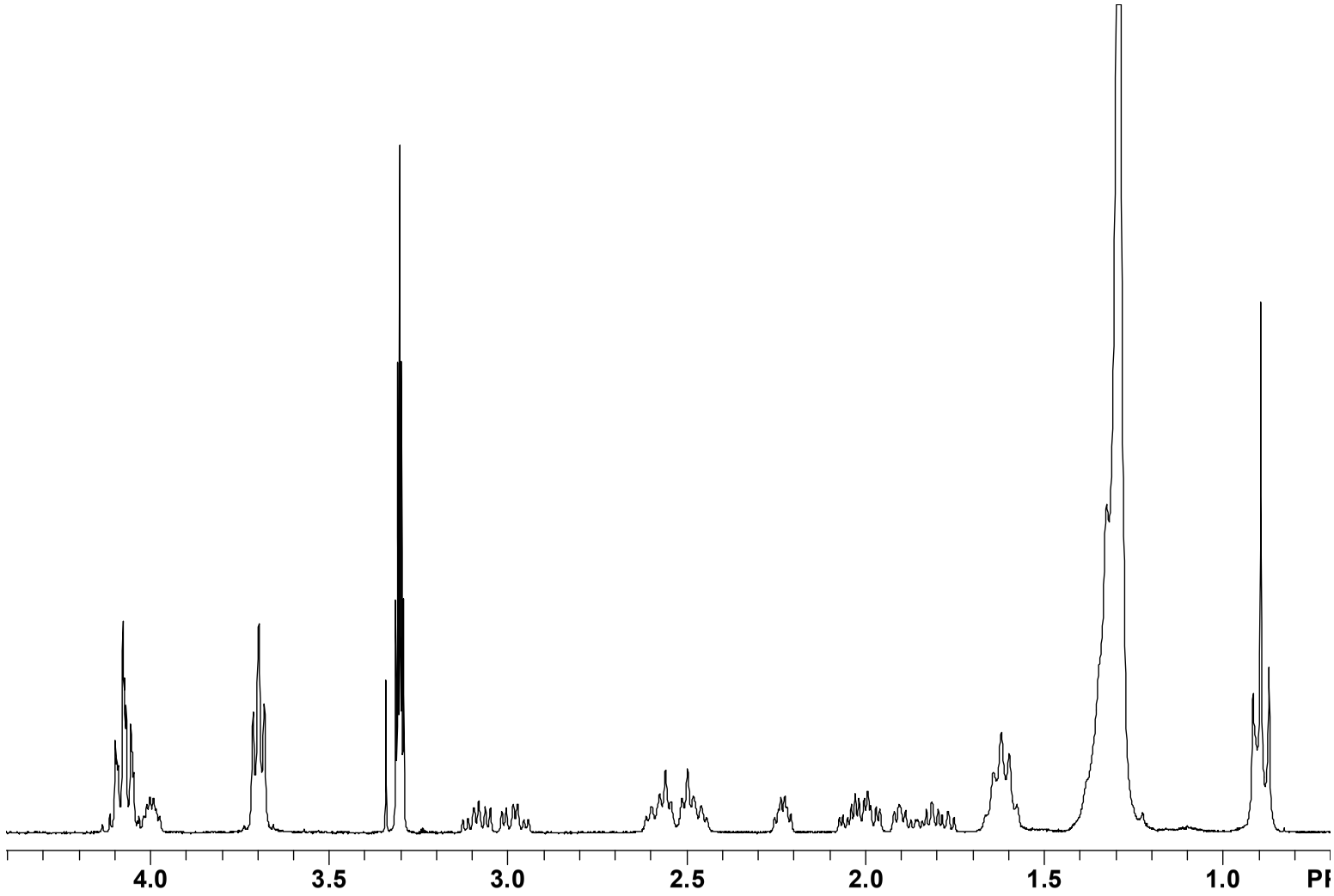


Figure S2. ^{1}H NMR of 1 at the apparent pH 5.5 in CD_3OD.

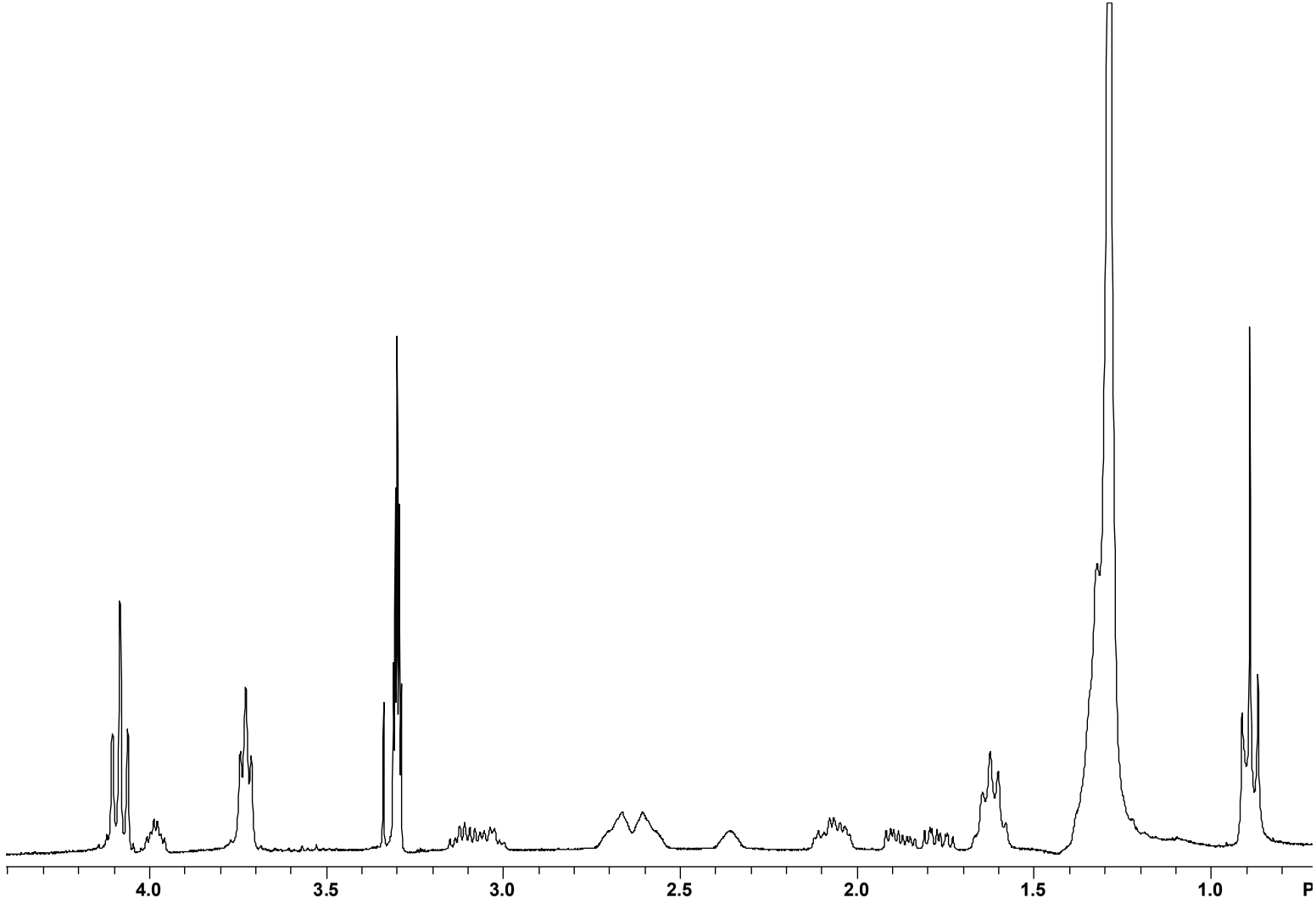


Figure S3. ^{1}H NMR of 1 at the apparent pH 4.6 in CD_3OD.

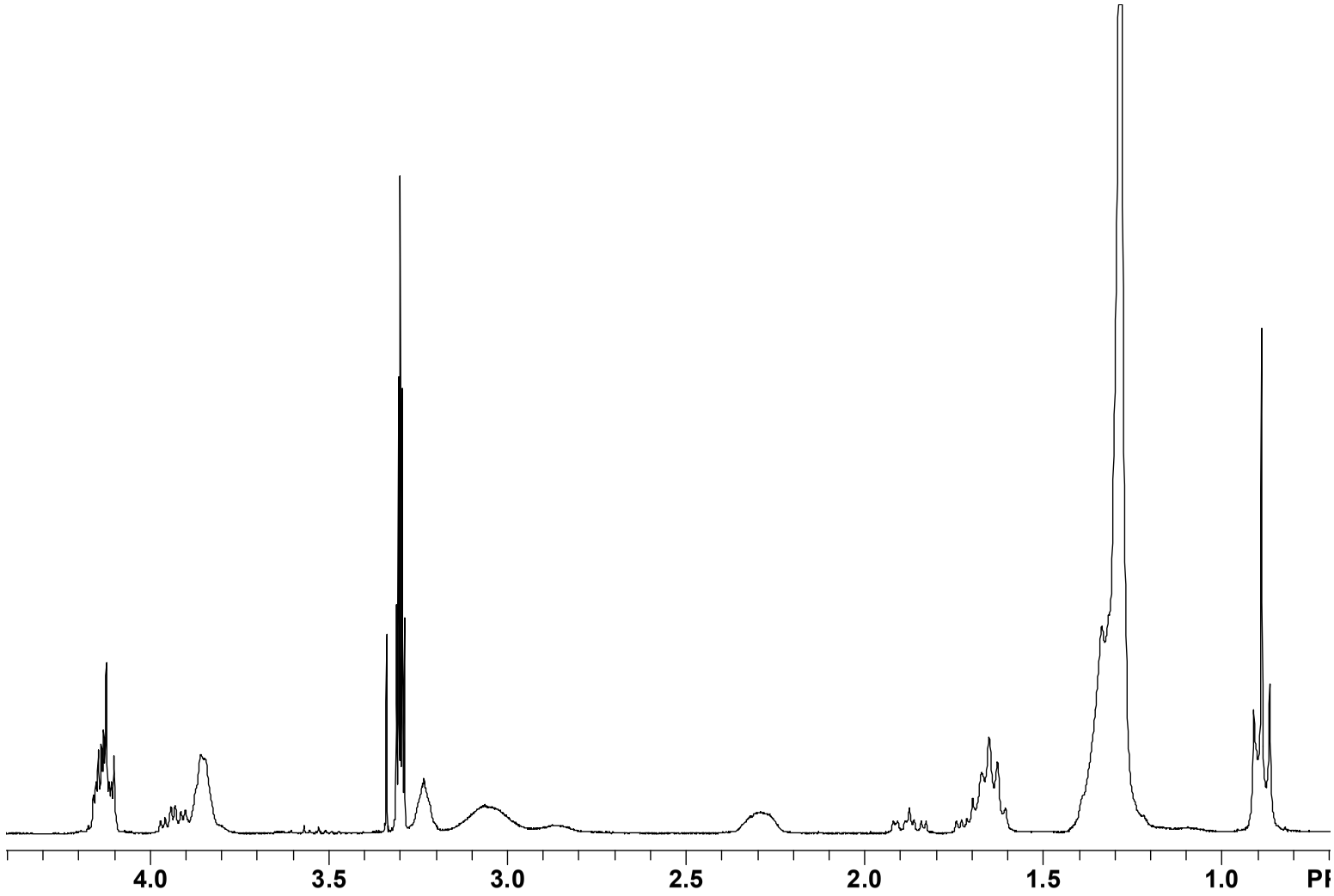


Figure S4. ^{1}H NMR of 1 at the apparent pH 1.0 in CD_3OD.

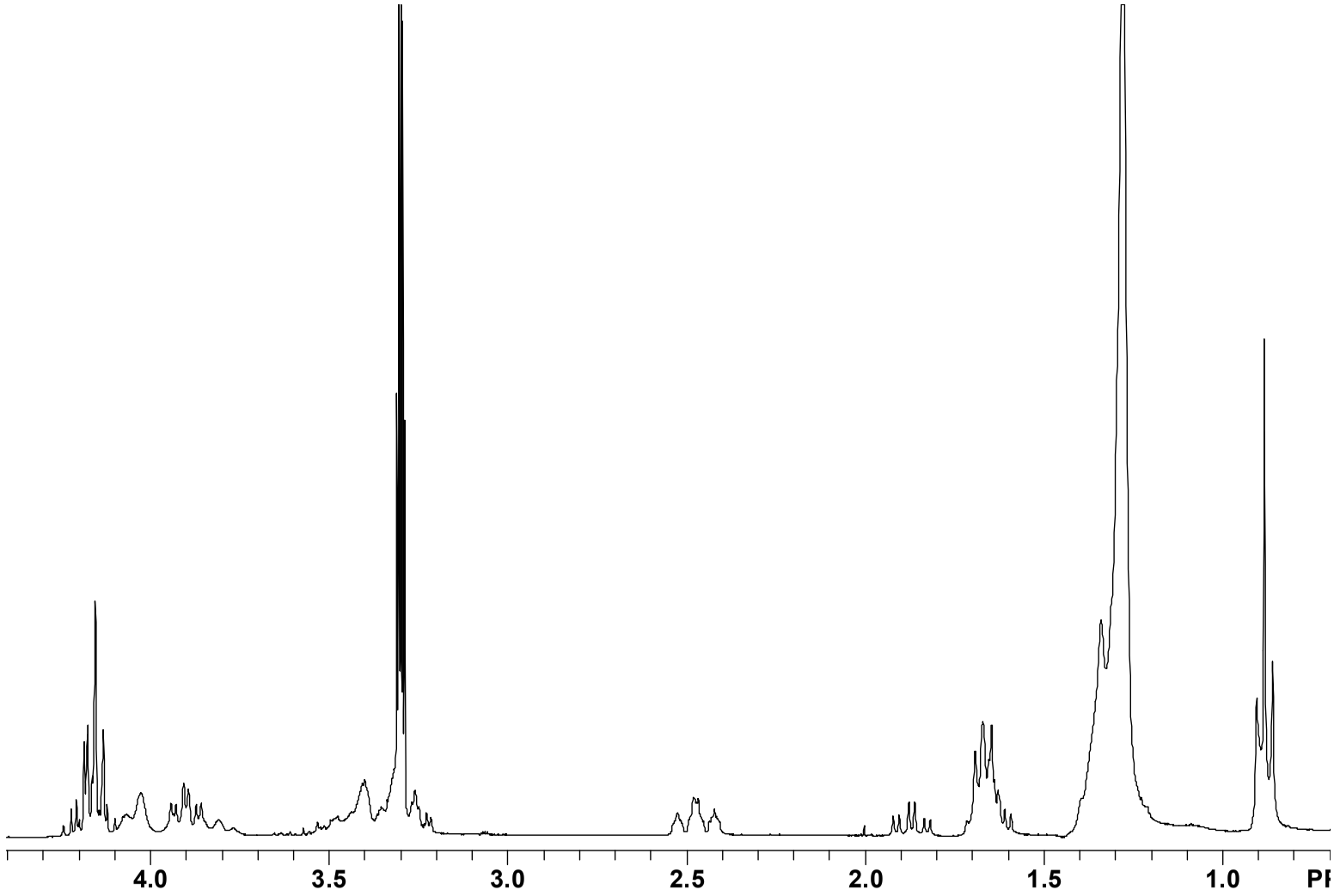


Figure S5. ^{1}H NMR of 2 at the apparent pH 8.3 in CD_3OD

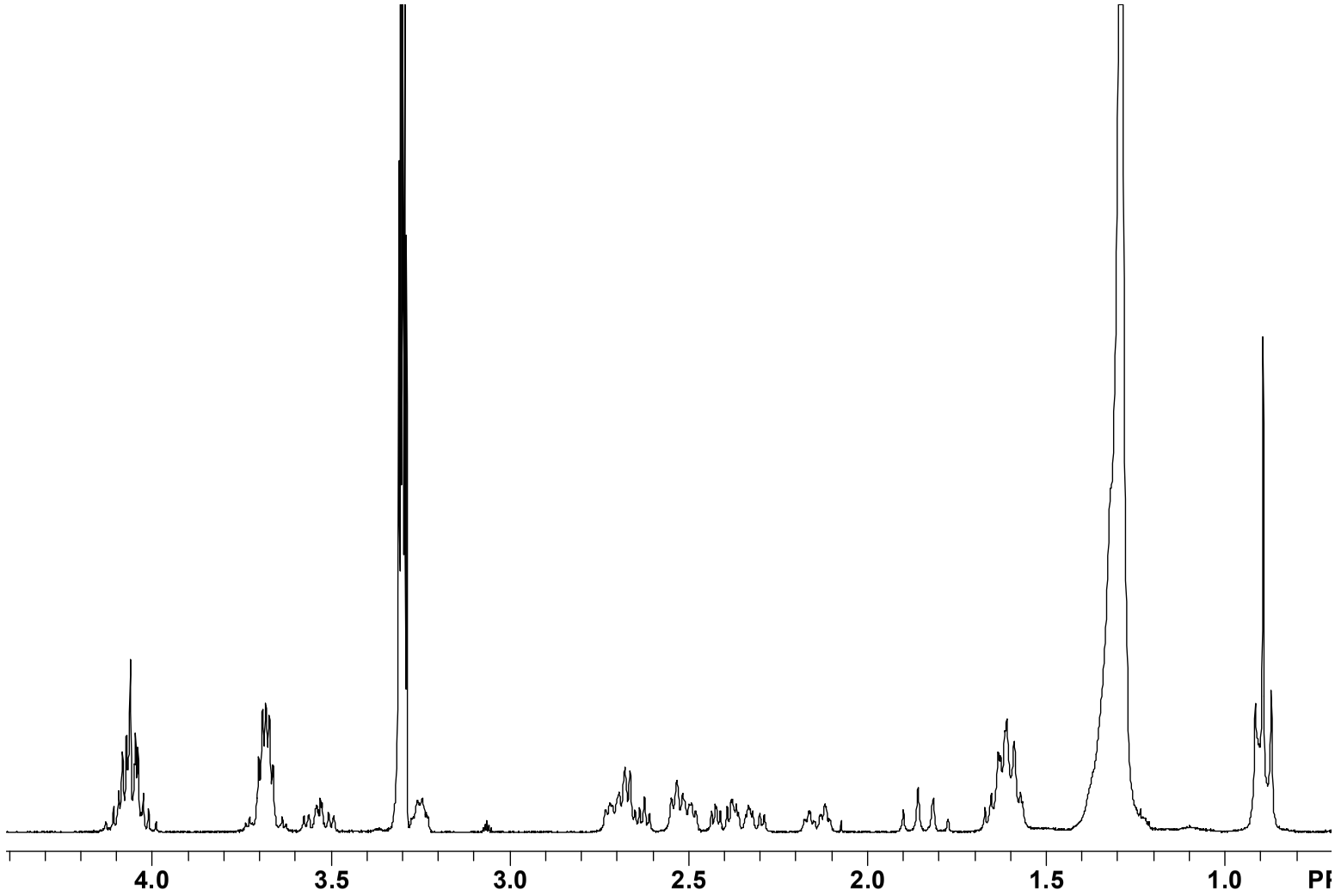
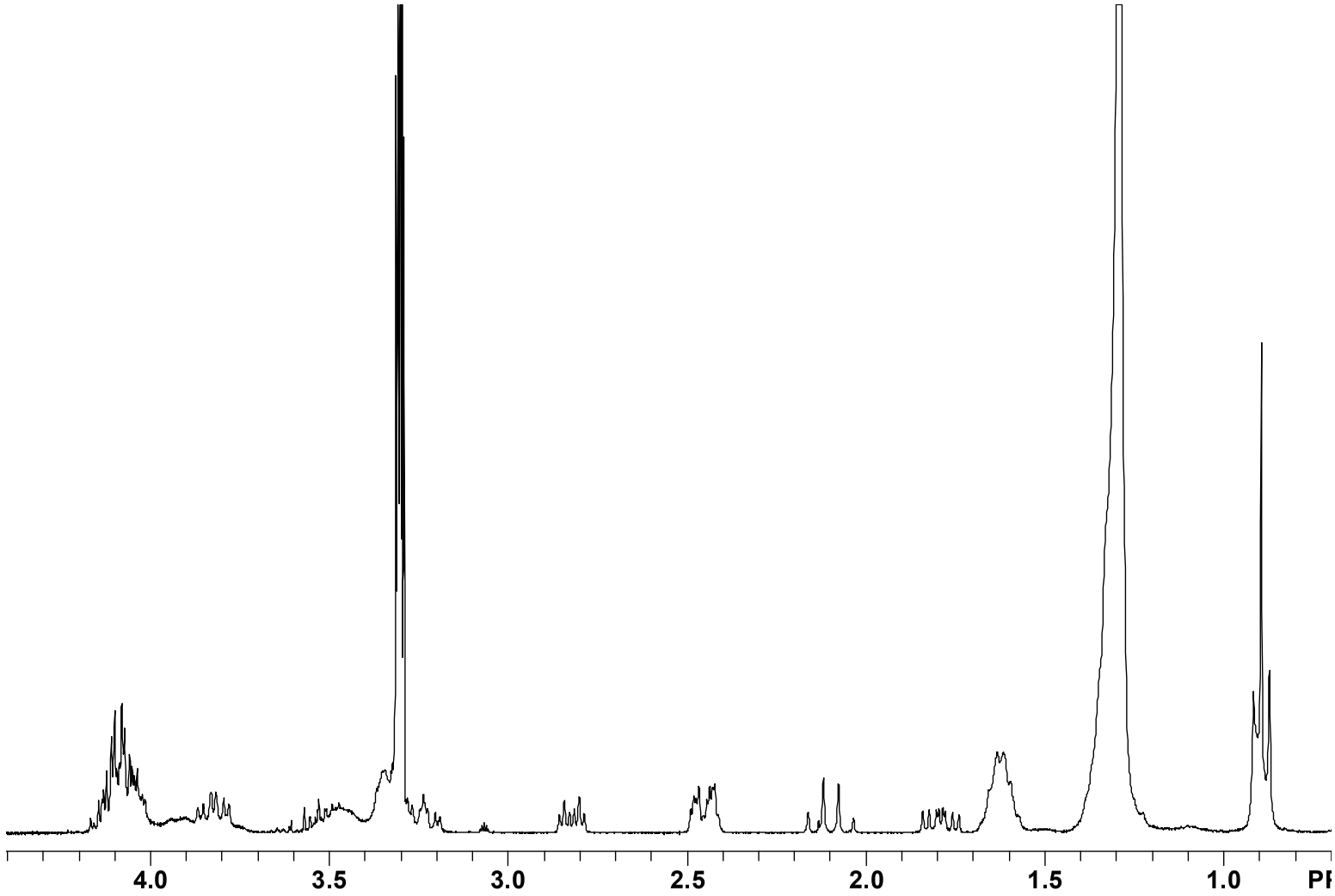


Figure S6. ^{1}H NMR of 2 at the apparent pH 2.0 in CD_3OD



3. Studies on Liposomes

Preparation of liposomes [7]

A dichloromethane solution of suitable lipids was transferred into a Pyrex brand glass tube (25 mm x 125 mm). Dichloromethane was evaporated under reduced pressure (70 mm Hg) at room temperature to form a lipid film at the bottom of the tube. The lipid film was placed under high vacuum for 24 h to remove residual dichloromethane. The lipid film was then hydrated with a buffer containing the ANTS fluorophore (50 mM ANTS, 50 mM DPX, 5 mM HEPES, pH 7.4) by 5 min of intermittent agitation with a vortex at room temperature. The tube containing the lipid suspension was filled with argon and sealed. The lipid suspension was rapidly frozen by submergence into acetone/dry ice, followed by melting in water bath at room temperature for 15 min. The freeze-thawing cycle was repeated 10 times and the resultant liposomes were extruded eleven times through a 0.2-μm polycarbonate membrane (Nucleopore, Pleasanton, CA) with a hand-held extrusion device (Avanti Polar Lipids, Alabaster, AL). The extruded liposomes were separated from the unencapsulated material using a Sephadex G-75 column with an elution buffer composed of 5 mM HEPES and 145 mM NaCl, pH 7.4. The hydrodynamic diameter and the ζ-potential of the liposomes were measured with a Malvern Zeta 3000 Dynamic Light Scattering Instrument (Malvern Instruments Ltd., Worcestershire, UK), using the automatic algorithm mode.

Colloidal stability of mPEG2000-Ceramide/1/POPC liposomes [8]

After 8 months of storage at 4 ºC and 48 h of incubation at 37 ºC, the mPEG2000-Ceramide/**1**/POPC liposomes showed no significant change in hydrodynamic diameter or polydispersity index, and kept a near-zero ζ-potential (Table S1).

Table S1. Colloidal Properties of mPEG2000-Ceramide/**1**/POPC liposomes[†].

Sample Treatment	Date of Measurements	Diameter (nm)	Polydispersity index	ζ-Potential (mV)
shortly after preparation	11/08/2006	144±1.8	0.12	4.3±1.3
8 month storage at 4 °C	08/23/2007	151±1.6	0.10	-3.6±0.6
further incubation at 37 °C for 48 h	08/25/2007	143±0.7	0.11	4.5±1.3

[†]The lipid bilayer comprises 5 mol% of a PEG-lipid conjugate, N-palmitoyl-sphingosine-1-[succinyl-(methoxypolyethyleneglycol) 2000] (mPEG2000-Ceramide), 24 mol% **1**, and 71 mol% POPC. Samples were measured in 10 mM HEPES buffer, pH 7.4. Hydrodynamic diameter and □-potential are reported as mean ± standard deviation of three measurements.

Liposome leakage assay [9]

The ANTS/DPX fluorescent assay was used to measure the contents release of the liposomes. Fluorescence measurements were taken on a Quatamaster Fluorometer (Photon Technology International, Lawrenceville, NJ). A small aliquot of liposomes (100 μL, 5 mM total lipid concentration) was injected into a magnetically stirred quartz cuvette containing 3 mL of an aqueous buffer (pH 7.4 or 5.5) at room temperature. The pH 7.4 buffer contains 5 mM HEPES and 145 mM NaCl. The pH 5.5 buffer contains 50 mM citric acid and 100 mM NaCl. Fluorescent intensity (λ_{ex} = 350 nm, λ_{em} = 550 nm) was recorded every one second for the first five minutes; in the following one hour, measurements were taken at longer intervals (5-15 min). At the end of each assay, liposomes were lysed with 2 % dodecyloctaethylene glycol monoether ($C_{12}E_8$). The raw fluorescent intensity data were converted to ASCII format and processed to percentage of contents leakage with Windows Excel software as previously reported. [9]

Liposome Serum Stability Assay [10]

An aliquot of a liposome preparation was mixed with three volume equivalent of fetal bovine serum and incubated at 37 ºC. After a certain incubation time period, a 200 µL aliquot of the mixture was diluted in 3 ml of a HEPES buffer (5 mM HEPES, 145 mM NaCl, pH 7.4, room temperature) in a polystyrene fluorimeter cuvette. The fluorescence of the diluted mixture minuts the fluorescence of the blank buffer was taken as F_t. F_t of a liposome sample measured immediately after mixing with fetal bovine serum was taken as F_0. After each F_t measurement, the detergent dodecyloctaethylene glycol monoether ($C_{12}E_8$) was added to the cuvette to lyse the liposomes. The fluorescence thus measured minus the fluorescence of the blank buffer was taken as F_{100}. The fluorescence of liposome samples was monitored on a Quatamaster fluorometer (Photon Technology International, NJ). The raw fluorescent intensity data were converted to ASCII format and processed to percentage of contents leakage with Windows Excel software as previously reported.[10] Each data point represents the average and standard deviation of three independent measurements.

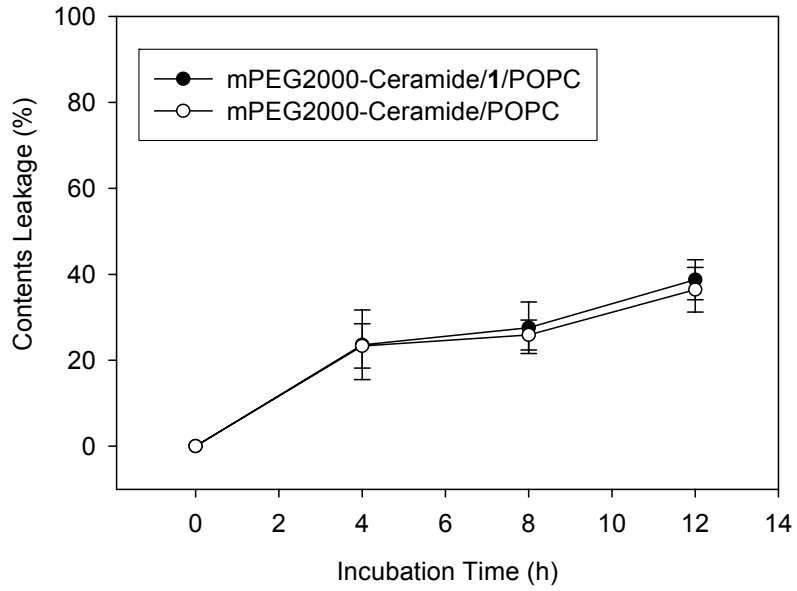


Figure S7. Contents leakage from liposomes in 75% fetal bovine serum.

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