

## Headgroup engineering in mechanosensitive membrane probes

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### Supplementary Information

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## 1. Materials and methods

As in ref. S1, Supporting Information. Briefly, reagents for synthesis were mostly purchased from Fluka, Sigma-Aldrich, and Apollo Scientific; buffers and salts of the best grade available from Fluka or Sigma-Aldrich were used as received. Reactions were performed under N<sub>2</sub> or Ar atmosphere when specified. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), N-(dodecanoyl)-sphing-4-enine-1-phosphocholine (Sphingomyelin) and cholesterol were purchased from Avanti Polar Lipids.

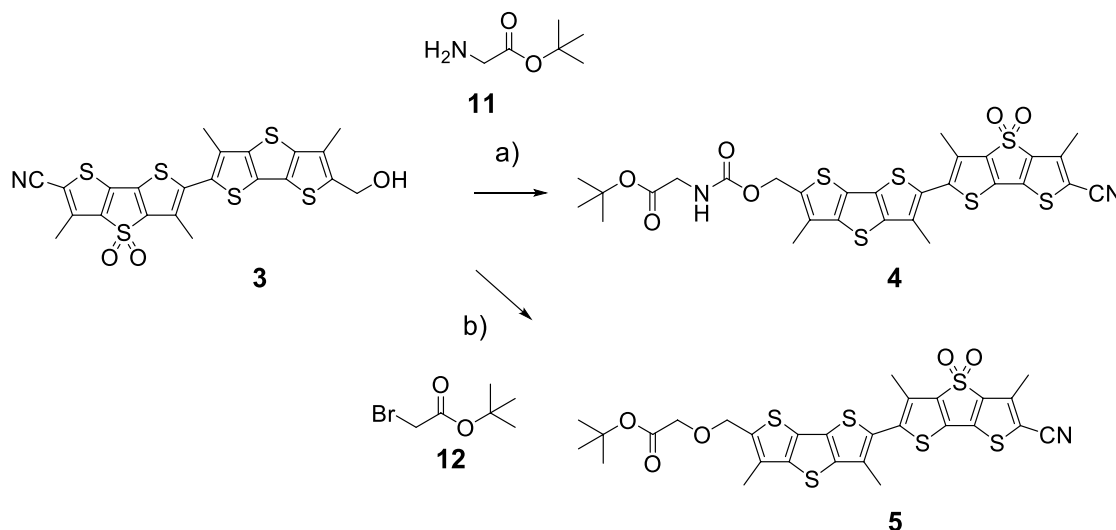
Unless stated otherwise, column chromatography was carried out on silica gel (SiliaFlash® P60, SILICYCLE, 230–400 mesh). Analytical (TLC) and preparative thin layer chromatography (PTLC) were performed on TLC Silica gel 60 F254 (Merck) and SilicaPlate TLC (SILICYCLE, 1000  $\mu$ m), respectively. UV-Vis spectra were recorded on a JASCO V-650 spectrophotometer equipped with a stirrer and a temperature controller (25 °C) and are reported as maximal absorption wavelength  $\lambda$  in nm. Fluorescence measurements were performed with a FluoroMax-4 spectrofluorometer (Horiba Scientific) equipped with a stirrer and a temperature controller. Fluorescence spectra were corrected using instrument-supplied correction factors, unless stated otherwise. Melting points (Mp) were recorded on a Büchi Melting Point M-565. IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer (ATR, Golden Gate) and are reported as wavenumbers  $\nu$  in cm<sup>-1</sup> with band intensities indicated as s (strong), m (medium), w (weak), br (broad). <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded (as indicated) either on a Bruker 300 MHz, 400 MHz or 500 MHz spectrometer and are reported as chemical shifts ( $\delta$ ) in ppm relative to TMS ( $\delta$  = 0). Spin multiplicities are reported as a singlet (s), doublet (d), triplet (t), quartet (q) and quintet (quint) with coupling constants ( $J$ ) given in Hz, or multiplet (m). <sup>1</sup>H and <sup>13</sup>C resonances were assigned with the aid of additional information from 1D & 2D NMR spectra (H, H-COSY, DEPT 135, HSQC and HMBC). ESI-MS for the characterization of new compounds was performed on an ESI API 150EX (AB/MDS Sciex) and is reported as mass-per-charge ratio  $m/z$  (intensity in

%, [assignment]). LUVs were prepared using a Mini-Extruder from Avanti Polar Lipids (pore size 100 nm). The images of the GUVs were taken with a Confocal Leica SP5 STED using a excitation white light laser, using two excitation wavelengths (480 nm or 560 nm) and detection at 505-630 nm or 585-630 nm. Alternatively, imaging was performed with a spinning-disk confocal microscope based on Nikon Ti with two excitation lasers, 488 nm and 561 nm and a 600/50 emission filter. Confocal images were performed using the Nikon Eclipse Ti A1R microscope, with a laser 488 nm for excitation and 585/50 nm filter for the emission. FLIM images were generated with FLIM upgrade kit (PicoQuant) installed on the same microscope described before, with a laser 485 (PicoQuant, LDH-D-C-485) with 20 MHz. Fluorescence was collected passing through 600/50nm filter. FLIM images were analyzed using SymPhoTime 64 software (PicoQuant). LC-MS (ESI) were recorded using a Thermo Scientific Accela HPLC with a LCQ Fleet three-dimensional ion trap mass spectrometer (both ESI, thermo scientific) and a diode array detector. The chromatographic column used was a Thermo C18 Hypersil gold column 1.9  $\mu$ m, 5 cm x 2.1 mm, using a gradient elution from 30% to 90% CH<sub>3</sub>CN / H<sub>2</sub>O (both with 0.01% TFA) in 4 minutes at a flow rate 0.75 mL/min.

**Abbreviations.** AcOH: Acetic acid; aq: Aqueous solution; CDI: Carbonyldiimidazole; CF: 5(6)-Carboxyfluorescein; CL: Cholesterol; DMSO: Dimethylsulfoxide; DOPC: 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine; DPPC: 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine; Et<sub>2</sub>O: Diethyl ether; EtOAc: Ethyl acetate; FLIM: Fluorescence-lifetime imaging microscopy; GUVs: Giant unilamellar vesicles; ITO: Indium tin oxide; L<sub>d</sub>: Liquid disordered; LUVs: Large unilamellar vesicles; MeOH: Methanol; PE: Petroleum ether; PTLC: Preparative of thin layer chromatography; rt: Room temperature; sat: Saturated; SM: Sphingomyelin; S<sub>o</sub>: Solid ordered; TBTA: Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine; TFA: Trifluoroacetic acid; THF: Tetrahydrofuran; Tris: Tris(hydroxymethyl)aminomethane.

## 2. Supporting text

### 2.1. Synthesis



**Scheme S1.** a) 1. CDI, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C to rt, 2 h, 2. **11**, CH<sub>2</sub>Cl<sub>2</sub>, rt, 1 h, 63%; b) 1. NaH, THF, -20 °C, 1 h, 2. **12**, THF, rt, 2 h, 75%.

**Compound 1, 14.** This compound was prepared following the reported procedure.<sup>S1</sup>

**Compound 3.** Preparation of this compound will be published in due course.<sup>S2</sup>

**Compound 8.** This compound was prepared following the reported procedure.<sup>S3</sup>

**Compound 9.** This compound was prepared following the reported procedure.<sup>S4</sup>

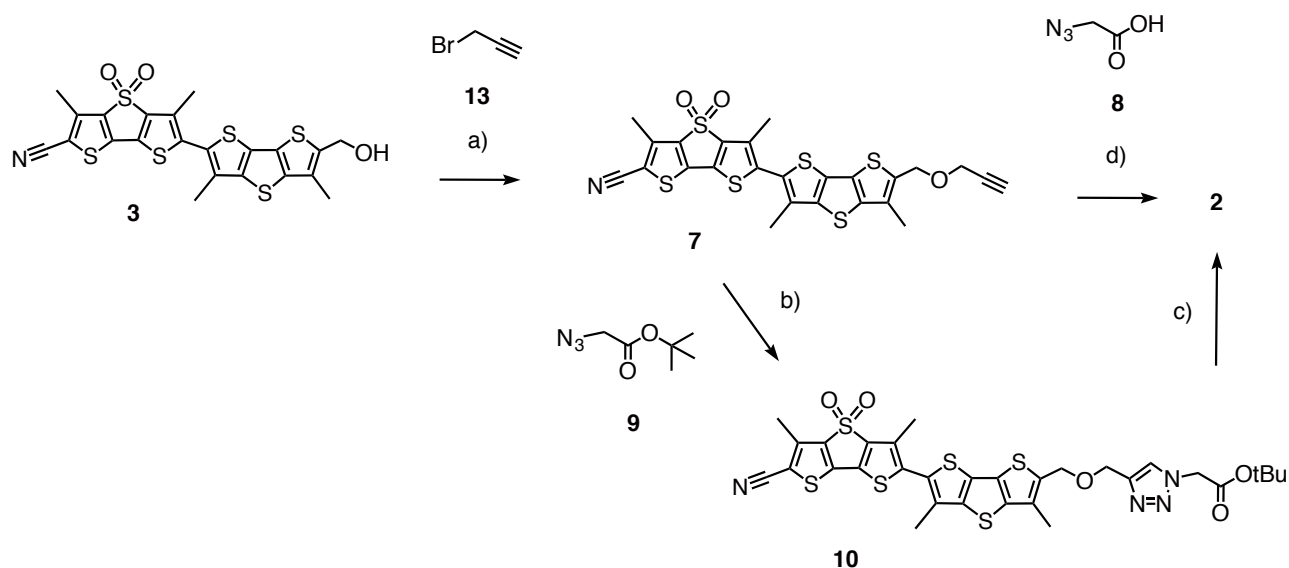
**TBTA** was prepared following the reported procedure.<sup>S5</sup>

**Compound 4.** Glycine *t*-butyl ester hydrochloride (35 mg, 0.20 mmol) was treated with aqueous sodium hydroxide (10 M, 50 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The CH<sub>2</sub>Cl<sub>2</sub> solution was back washed with brine, dried over MgSO<sub>4</sub>, filtered and concentrated in vacuum to obtain **11**. To a solution of CDI (18 mg, 0.11 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (0.5 mL) at 0 °C was slowly added a solution of **3**

(50 mg, 93  $\mu$ mol) in  $\text{CH}_2\text{Cl}_2$  (2 mL). The mixture was stirred under Ar atmosphere at rt for 2 h. Then a solution of **11** (25 mg, 0.19 mmol) in  $\text{CH}_2\text{Cl}_2$  (1 mL) was added and the mixture was stirred at rt for 1 h. An aqueous solution of HCl (1 M, 30 mL) was added and the organic layer was separated. The aqueous layer was extracted with  $\text{CH}_2\text{Cl}_2$  (3 x 30 mL), and the combined organic phase was washed with water (3 x 10 mL) and brine (3 x 10 mL). The organic phase was dried over  $\text{MgSO}_4$ . The crude mixture was purified by flash chromatography on silica gel ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  20:1). The product **4** (40 mg, 63%) was obtained as a red powder.  $R_f(\text{CH}_2\text{Cl}_2/\text{MeOH}$  20:1): 0.39; Mp: decomposition > 200  $^\circ\text{C}$ ; IR (neat): 3025 (br), 2013 (m), 1843 (s), 1370 (s), 1304 (s), 1220 (m);  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ): 5.33 (s, 2H), 5.23 (t,  $^3J(\text{H,H}) = 5.4$  Hz, 1H), 3.91 (d,  $^3J(\text{H,H}) = 5.4$  Hz, 2H), 2.64 (s, 3H), 2.44-2.43 (m, 6H), 2.37 (s, 3H), 1.49 (s, 9H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ): 164.3 (2 x C), 146.0 (C), 143.7 (C), 143.1 (C), 142.6 (C), 140.9 (C), 138.0 (C), 133.5 (C), 133.3 (C), 132.5 (2 x C), 132.0 (C), 130.5 (C), 126.4 (C), 116.4 (C), 111.0 (C), 110.3 (C), 102.1 (C), 79.4 (C), 72.4 ( $\text{CH}_2$ ), 60.0 ( $\text{CH}_2$ ), 28.0 (3 x  $\text{CH}_3$ ), 14.1 ( $\text{CH}_3$ ), 13.1 ( $\text{CH}_3$ ), 12.9 ( $\text{CH}_3$ ), 12.4 ( $\text{CH}_3$ ); MS (ESI+,  $\text{CHCl}_3$ ): 690 (50,  $[\text{M}+\text{H}]^+$ ), 516 (100,  $[\text{M}-\text{C}_7\text{H}_{12}\text{NO}_4]^+$ ).

**Compound 5.** A solution of **3** (100 mg, 0.19 mmol) in dry THF (5 mL) was added dropwise to a suspension of NaH (37 mg, 0.93 mmol, 60% in mineral oil) in THF (3 mL) under Ar atmosphere at -20  $^\circ\text{C}$ . After stirring for 1 h at -20  $^\circ\text{C}$ , **12** (183 mg, 0.930 mmol) was added and the mixture was slowly brought to rt and stirred for 2 h. Water (50 mL) was added and the product was extracted with  $\text{CH}_2\text{Cl}_2$  (3 x 60 mL). The combined organic extracts were dried over  $\text{MgSO}_4$  and the solvent was evaporated. The crude mixture was purified by flash chromatography on silica gel ( $\text{CH}_2\text{Cl}_2$ ). The product **6** (88 mg, 75%) was obtained as an orange-red powder.  $R_f(\text{CH}_2\text{Cl}_2)$ : 0.26; Mp: 100-101  $^\circ\text{C}$ ; IR (neat): 3751 (s), 2924 (s), 1750 (w), 1148 (s), 844 (s);  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ): 4.86 (s, 2H), 4.06 (s, 2H), 2.64 (s, 3H), 2.44 (s, 3H), 2.42 (s, 3H), 2.37 (s, 3H), 1.52 (s, 9H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ): 169.5 (C), 145.2 (C), 143.7 (C), 141.2 (C), 140.2 (C), 138(C), 135.0 (C), 132.4 (C), 132.3 (C), 132.0 (C), 131.2 (C), 130.0 (C), 129.1 (C), 128.0 (C), 127.7 (C),

125.6 (C), 112.5 (C), 108.9 (C), 81.9 (CH<sub>2</sub>), 67.2 (CH<sub>2</sub>), 65.9 (C), 28.3 (3 x CH<sub>3</sub>), 14.1 (CH<sub>3</sub>), 13.1 (CH<sub>3</sub>), 12.8 (CH<sub>3</sub>), 12.4 (CH<sub>3</sub>); MS (ESI<sup>+</sup>, CHCl<sub>3</sub>): 647 (50, [M+H]<sup>+</sup>), 516 (100, [M-C<sub>6</sub>H<sub>11</sub>O<sub>3</sub>]<sup>+</sup>).



**Scheme S2.** a) 1. NaH, THF, -20 °C, 1 h, 2. **13**, THF, rt, 2 h, 80%; b) **9**, sodium ascorbate, CuSO<sub>4</sub> • 5 H<sub>2</sub>O, TBTA, CH<sub>2</sub>Cl<sub>2</sub>, rt, 30 min, 90%; c) TFA, CH<sub>2</sub>Cl<sub>2</sub>, rt, 1 h, 50%; d) **8**, sodium ascorbate, CuSO<sub>4</sub> • 5 H<sub>2</sub>O, TBTA, CH<sub>2</sub>Cl<sub>2</sub>, rt, 10 min, 90%.

**Compound 7.** A solution of **3** (100 mg, 0.19 mmol) in dry THF (5 mL) was added dropwise to a suspension of NaH (37 mg, 0.93 mmol; 60% in mineral oil) in THF (3 mL) under Ar atmosphere at -20 °C. After 1 h **13** (139 mg, 0.930 mmol, 80% in toluene) was added and the mixture was stirred for 2 h at rt. Water (50 mL) was added and the product was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 60 mL). The combined organic extracts were dried over MgSO<sub>4</sub> and the solvent was evaporated. The crude mixture was purified by flash chromatography on silica gel (CH<sub>2</sub>Cl<sub>2</sub>). The product **7** (85 mg, 80%) was obtained as a red powder. *R<sub>f</sub>*(CH<sub>2</sub>Cl<sub>2</sub>): 0.33; Mp: 90-91 °C; IR (neat): 3282 (s), 2922 (w), 2209 (w), 1727 (s), 1441 (w), 1412 (m), 1311 (m), 1369 (m), 1146 (w), 1064 (m), 667 (m); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.85 (s, 2H), 4.25 (d, <sup>4</sup>*J*(H,H) = 2.2 Hz, 2H), 2.64 (s, 3H), 2.54 (t, <sup>4</sup>*J*(H,H) = 2.2 Hz, 1H), 2.44 (s, 3H), 2.42 (s, 3H), 2.38 (s, 3H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): 145.3 (C), 143.7 (C), 143.1 (C), 142.4 (C), 141.3 (C), 140.2 (C), 138.1 (C), 135.0 (C),

132.5 (C), 132.1 (C), 131.2 (C), 130.0 (C), 129.1 (C), 125.5 (C), 112.0 (C), 109.0 (C), 108.9 (C), 79.3 (C), 75.2 (CH), 64.2 (CH<sub>2</sub>), 56.7 (CH<sub>2</sub>), 14.1 (CH<sub>3</sub>), 13.2 (CH<sub>3</sub>), 12.9 (CH<sub>3</sub>), 12.5 (CH<sub>3</sub>); MS (ESI<sup>+</sup>, CHCl<sub>3</sub>): 571 (30, [M+H]<sup>+</sup>), 516 (100, [M-C<sub>3</sub>H<sub>3</sub>O]<sup>+</sup>).

**Compound 10.** To a solution of **7** (89 mg, 0.14 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (400 μL), a solution of **9** (15 mg, 0.14 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (200 μL) was added dropwise. Then solutions of sodium ascorbate (50 μL of 10 mM in water), CuSO<sub>4</sub>·5H<sub>2</sub>O (50 μL of 1 mM in water) and TBTA (10 μL of 10 mM in CH<sub>2</sub>Cl<sub>2</sub>) were added to the reaction mixture. The reaction mixture was stirred for 30 min at rt. A saturated aqueous solution of NaHCO<sub>3</sub> (30 mL) was added and organic layer was separated. The aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 30 mL), and the combined organic phase was washed with a saturated aqueous solution of NaHCO<sub>3</sub> (3 x 10 mL), water (3 x 10 mL) and brine (3 x 10 mL), the organic phase was dried over MgSO<sub>4</sub>. The crude was purified by flash chromatography (SiO<sub>2</sub>, CH<sub>2</sub>Cl<sub>2</sub>/MeOH 20:1) to afford compound **10** as a dark red powder (89 mg, 90%). *R*<sub>f</sub> (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 20:1): 0.2; Mp: 123-124 °C; IR (neat): 2925 (m), 2213 (s), 2174 (s), 1370 (m), 1314 (s), 1236 (m), 1144 (s), 1091 (w); <sup>1</sup>H NMR (400 MHz, CD<sub>2</sub>Cl<sub>2</sub>): 7.68 (s, 1H), 5.03 (s, 2H), 4.77 (s, 2H), 4.71 (s, 2H), 2.56 (s, 3H), 2.38 (s, 3H), 2.34 (s, 3H), 1.46 (s, 9H); <sup>13</sup>C NMR (125 MHz, CD<sub>2</sub>Cl<sub>2</sub>): 167.4 (C), 147.0 (C), 146.9 (C), 145.4 (C), 145.0 (C), 144.2 (C), 143.1 (C), 142.2 (C), 140.0 (C), 138.0 (C), 134.6 (C), 134.4 (C), 134.2 (C), 133.0 (C), 131.3 (C), 130.5 (C), 127.6 (C), 126.1 (CH), 114.4 (C), 111.0 (C), 85.6 (C), 67.2 (CH<sub>2</sub>), 65.1 (CH<sub>2</sub>), 53.5 (CH<sub>2</sub>), 29.9 (3 x CH<sub>3</sub>), 15.8 (CH<sub>3</sub>), 14.8 (CH<sub>3</sub>), 14.6 (CH<sub>3</sub>), 14.1 (CH<sub>3</sub>); MS (ESI<sup>+</sup>, CHCl<sub>3</sub>): 728 (50, [M+H]<sup>+</sup>), 516 (100, [M-C<sub>3</sub>H<sub>3</sub>O]<sup>+</sup>).

**Compound 2, Procedure a.** A solution of TFA (0.5 mL) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was added dropwise to a solution of **10** (20 mg, 27 μmol) in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) at rt under Ar. The reaction was followed via TLC, upon the disappearance of starting material after 1 h, the crude was washed H<sub>2</sub>O, dried over Na<sub>2</sub>SO<sub>4</sub> and solvent evaporated. PTLC purification (CH<sub>2</sub>Cl<sub>2</sub>/MeOH/AcOH 20:1:0.1, *R*<sub>f</sub> =

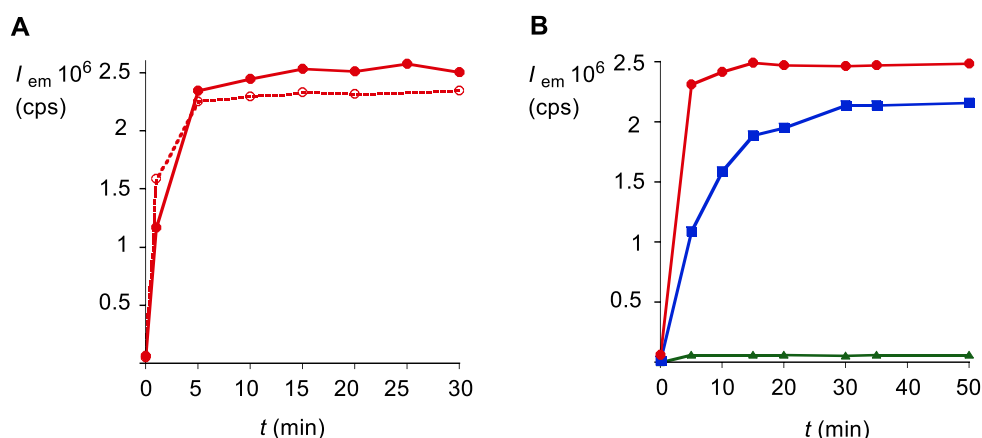
0.25 with CH<sub>2</sub>Cl<sub>2</sub>/MeOH/AcOH 20:1:0.1) followed by triturations firstly with CH<sub>2</sub>Cl<sub>2</sub> and then with hexane gave **2** (9 mg, 50%) as a dark red powder.

**Procedure b.** To a solution of **7** (57 mg, 98 μmol) in CH<sub>2</sub>Cl<sub>2</sub> (260 μL), a solution of **8** (10 mg, 98 μmol) in H<sub>2</sub>O (100 μL) was added dropwise. Then solutions of sodium ascorbate (32 μL of 10 mM in water), CuSO<sub>4</sub>·5H<sub>2</sub>O (32 μL of 1 mM in water) and TBTA (7 μL of 10 mM in CH<sub>2</sub>Cl<sub>2</sub>) were added to the reaction mixture. The reaction mixture was stirred for 10 min at rt, then CH<sub>2</sub>Cl<sub>2</sub> was evaporated. Formed precipitates were washed with aqueous NaHCO<sub>3</sub> solution, water, MeOH and CH<sub>2</sub>Cl<sub>2</sub>. The precipitate afforded **2**, while the combined MeOH and CH<sub>2</sub>Cl<sub>2</sub> filtrates were purified by PTLC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH/AcOH 20:1:0.1) to give **2** (59 mg, 90%) as a dark red powder. *R<sub>f</sub>* (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 20:1): 0.25; Mp: decomposition > 200 °C; IR (neat): 3624 (m), 2743 (m), 2241 (w), 1775 (s), 1656 (s), 1236 (m), 1267 (m), 1004 (m); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>/25% ND<sub>4</sub>OD in D<sub>2</sub>O 1:0.05): 7.96 (s, 1H), 4.77 (s, 2H), 4.70 (s, 2H), 4.60 (s, 2H), 2.48 (s, 3H), 2.34 (s, 3H), 2.32 (s, 3H), 2.31 (s, 3H); <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>/25% ND<sub>4</sub>OD in D<sub>2</sub>O 1:0.05): 168.1 (C), 143.7 (C), 143.1 (C), 142.4 (C), 142.4 (C), 141.6 (C), 140.8 (C), 139.8 (C), 138.3 (C), 137.0 (C), 133.2 (C), 131.7 (C), 130.3 (C), 128.7 (C), 127.9 (C), 125.9 (CH), 125.2 (2 x C), 112.5 (C), 109.5 (C), 64.4 (CH<sub>2</sub>), 62.7 (CH<sub>2</sub>), 53.6 (CH<sub>2</sub>), 14.3 (CH<sub>3</sub>), 12.6 (CH<sub>3</sub>), 12.1 (CH<sub>3</sub>), 12.0 (CH<sub>3</sub>); MS (ESI+, MeOH): 672 (20, [M+H]<sup>+</sup>), 516 (100, [M-C<sub>5</sub>H<sub>6</sub>N<sub>3</sub>O<sub>3</sub>]<sup>+</sup>).



## 2.2. Fluorescence properties in LUVs

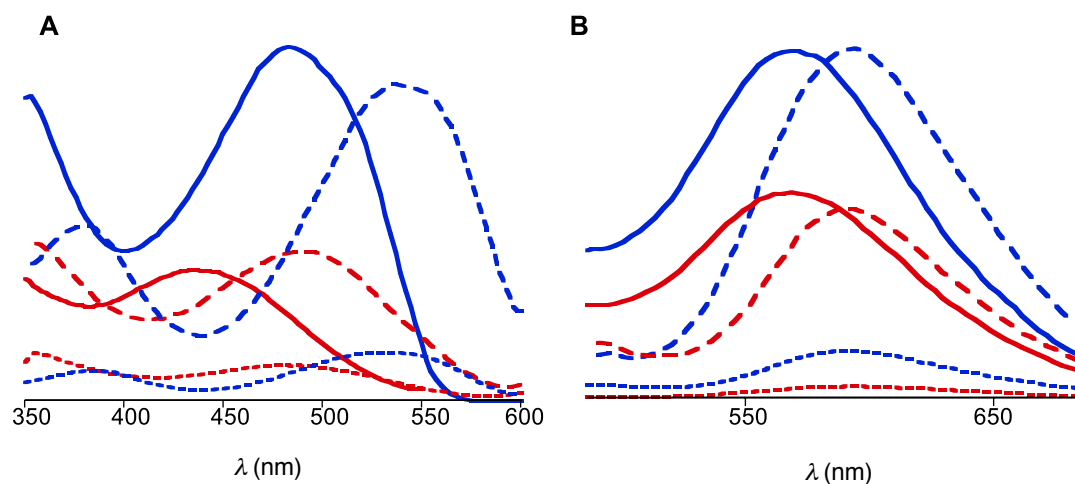
The large unilamellar vesicles (LUVs) used in this studies were prepared according to the procedure reported in ref. S1. To a gently stirred buffer solution (2 mL, 10 mM Tris, 100 mM NaCl, pH 7.4) in a glass cuvette, DOPC or DPPC LUVs (5  $\mu$ L, 75  $\mu$ M lipid) and the fluorophore (2  $\mu$ L of 0.5 mM in DMSO) were added. The final concentration was 0.5  $\mu$ M for amphiphiles **1**, **2** and **4**. The solution was stirred at 55  $^{\circ}$ C and the emission intensities were measured at different time ( $\lambda_{\text{ex}}$ : 412 nm;  $\lambda_{\text{em}}$ : 576 nm for amphiphiles **2**;  $\lambda_{\text{ex}}$ : 420 nm;  $\lambda_{\text{em}}$ : 600 nm for amphiphiles **1** and **4**), until no changes were detected. Fluorescence of probe **4** did not increase much upon addition of DPPC LUVs (55  $^{\circ}$ C), thus indicating very poor partitioning in DPPC membranes. The emission intensity maximum was reached in 10 min for **2** and in 30 min for **1**. Spectral corrections were not applied.



**Figure S1.** (A and B) Partition kinetics in DOPC (empty circles) and DPPC LUVs (filled symbols) of probes **2** (red circles), **1** (blue square) and **4** (green triangle).

To a gently stirred, thermostated buffer (2 mL, 55  $^{\circ}$ C, 10 mM Tris, 100 mM NaCl, pH 7.4) in a glass cuvette, DPPC LUVs (5  $\mu$ L, 75  $\mu$ M lipid) and the probe **2**, **1** or **4** (0.5  $\mu$ M) were added. The solution was stirred at 55  $^{\circ}$ C for 30 minutes before the emission and the excitation spectra were acquired ( $\lambda_{\text{ex}}$ : 420 nm,  $\lambda_{\text{em}}$ : 600 nm for **1** and **4**;  $\lambda_{\text{ex}}$ : 412 nm,  $\lambda_{\text{em}}$ : 576 nm for **2**). The temperature was then lowered to 25  $^{\circ}$ C and the solution was kept at this temperature for 10 min before the

emission and excitation spectra were acquired under the same conditions. Spectral corrections were not applied for emission spectra.



**Figure S2.** Excitation (A) and emission (B) spectra in DPPC LUVs at 55 °C (red) and 25 °C (blue) of probes **2** (solid), **1** (dashed) and **4** (dotted) line.

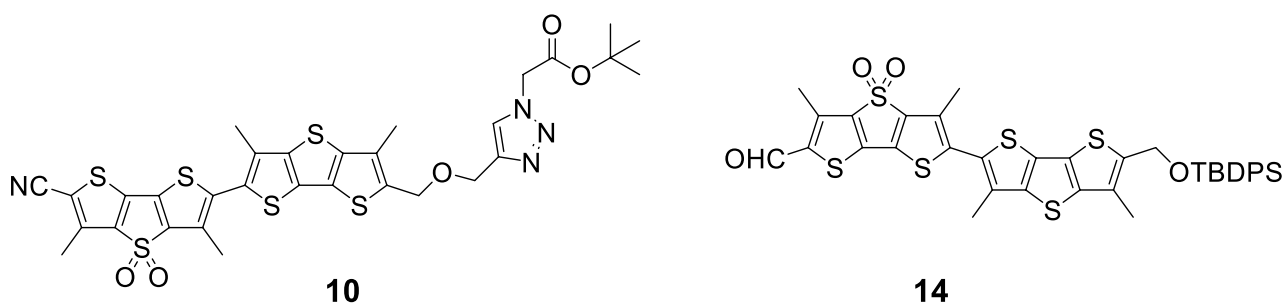
**Table S1.** Spectroscopic properties of probes **1** and **2** in  $L_d$  and  $S_o$  DPPC and DOPC membranes.

Compound	$\lambda_{ex}^{L_d}$ (nm) <sup>a</sup>	$\lambda_{ex}^{S_o}$ (nm) <sup>b</sup>	$\Delta\lambda_{ex}$ (nm) <sup>c</sup>	$I_{S_o}/I_{L_d}$ <sup>d</sup>
<b>1</b>	490	540	50	2.1
<b>2</b>	435	485	50	2.7

<sup>a</sup>Excitation maximum in DOPC LUVs at 25 °C. <sup>b</sup>Excitation maximum in DPPC LUVs at 25 °C.

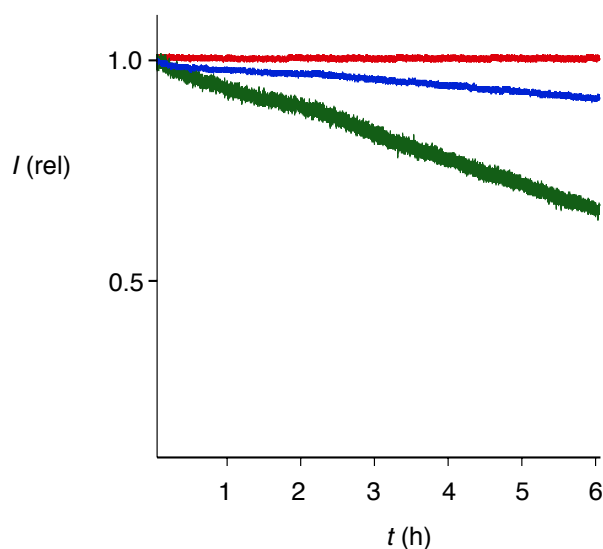
<sup>c</sup> $\Delta\lambda_{ex}$ : Difference in excitation maximum in DPPC LUVs and DOPC LUVs, at 25 °C. <sup>d</sup> $I_{S_o}$  and  $I_{L_d}$  are fluorescent intensities in DPPC LUVs at 25°C and DPPC LUVs at 55 °C.

## 2.3. Photostability



**Chart S1.** Compound **10** and **14** used to assess photostability of probes **1** and **2**.

Due to the poor solubility of compound **1** and **2** in EtOAc, the photostability experiments were carried out on compounds **10** and **14**. Solutions of **14** (25  $\mu$ M in EtOAc), **10** (20  $\mu$ M in EtOAc) and CF (100 nM in 10 mM Tris, 100 mM NaCl, pH 7.4) were prepared, which have the same absorbance at 435 nm. They were excited at  $435 \pm 5$  nm by the Xenon lamp of a Fluoromax. During the time of illumination, the emission intensity at 576 nm were recorded as a function of time.



**Figure S3.** Normalized emission intensity of **14** (blue), **10** (red) and CF (green) as a function of time.

## 2.4. Quantum yields

Due to low solubility of amphiphilic compounds in EtOAc, quantum yields were evaluated of the intermediates **10**, **5** and **4**. Solutions of **10** (12.5  $\mu\text{M}$ ), **5** (13.0  $\mu\text{M}$ ) and **4** (12.8  $\mu\text{M}$ ) were prepared in air saturated EtOAc. The sample concentration was adjusted to have the absorbance < 0.1 at the absorption maximum. Fluorescence quantum yields were evaluated based on external standards using equations S1 and S2:

$$\Phi_f^i = \frac{F^i f_s n_i^2}{F^s f_i n_s^2} \Phi_f^s \quad \text{S1}$$

$$f_x = 1 - 10^{-A_x} \quad \text{S2}$$

in which  $\Phi_f^i$  and  $\Phi_f^s$  are the photoluminescence quantum yield of the sample and the standard, respectively (Rhodamine 6G = 0.94; ref. S6).  $F^i$  and  $F^s$  are the integrated emission intensity (areas) of the sample and the standard.  $f_x$  are the absorption factors in equation S2, where  $A_x$  is absorbance,  $n_i$  is the refractive index of the sample,  $n_s$  is the refractive index of the standard.

**Table S2.** Quantum yields of **10**, **5** and **4**.

Compound	$\phi$ (%) <sup>a</sup>
<b>10</b>	25
<b>5</b>	27
<b>4</b>	27

<sup>a</sup>Quantum yields measured in EtOAc. Measured using Rhodamine 6G (EtOH) as a standard.

## 2.5. GUVs lifetime measurements

Giant unilamellar vesicles (GUVs) were prepared using a modified protocol of the electroformation technique.<sup>S7</sup> A mixture of N-(dodecanoyl)-sphing-4-enine-1-phosphocholine (Sphingomyelin) 58 mol%, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) 25 mol%, cholesterol 17 mol% and **1** or **2** (from DMSO solution) 0.1 mol% were mixed in CHCl<sub>3</sub> (500 µL, total lipid concentration 2 mg/mL). For the co-labelling experiments the commercially available probe ATTO647N was added (0.01 mol%) in addition to probe **1** or **2** (0.1 mol%). The obtained solution was heated at 50 °C together with two ITO-coated glass slides, previously cleaned with bidistilled H<sub>2</sub>O, EtOH and CHCl<sub>3</sub>. The lipid solution containing **1** or **2** (µL) was spread using a syringe on the conductive sides of the both ITO slides and dried by keeping the slides for 2 h at 55 °C. The chamber was assembled using the ITO slides separated by O-ring, filled with a 129 mOsm sucrose solution and blocked with silicone elastomer. An electric field (10 Hz, 1.1 V) was then applied for 2 h at 55 °C. Lifetimes were determined by FLIM. Average amplitude lifetimes are reported, i.e., the average lifetime obtained by the exponential fits, weighted by the amplitudes.

**Table S3.** Fluorescence lifetimes for FLIM imaging of GUVs.

Compound	GUVs <sup>a</sup>	$\langle \tau \rangle$ (ns) <sup>b</sup>
<b>1</b>	DOPC	2.79
	SM + 30% CL	5.73
<b>2</b>	DOPC	2.70
	SM + 30% CL	5.79

<sup>a</sup>Vesicle composition. <sup>b</sup>Average value  $\langle \tau \rangle = \sum \alpha_i \tau_i$ .

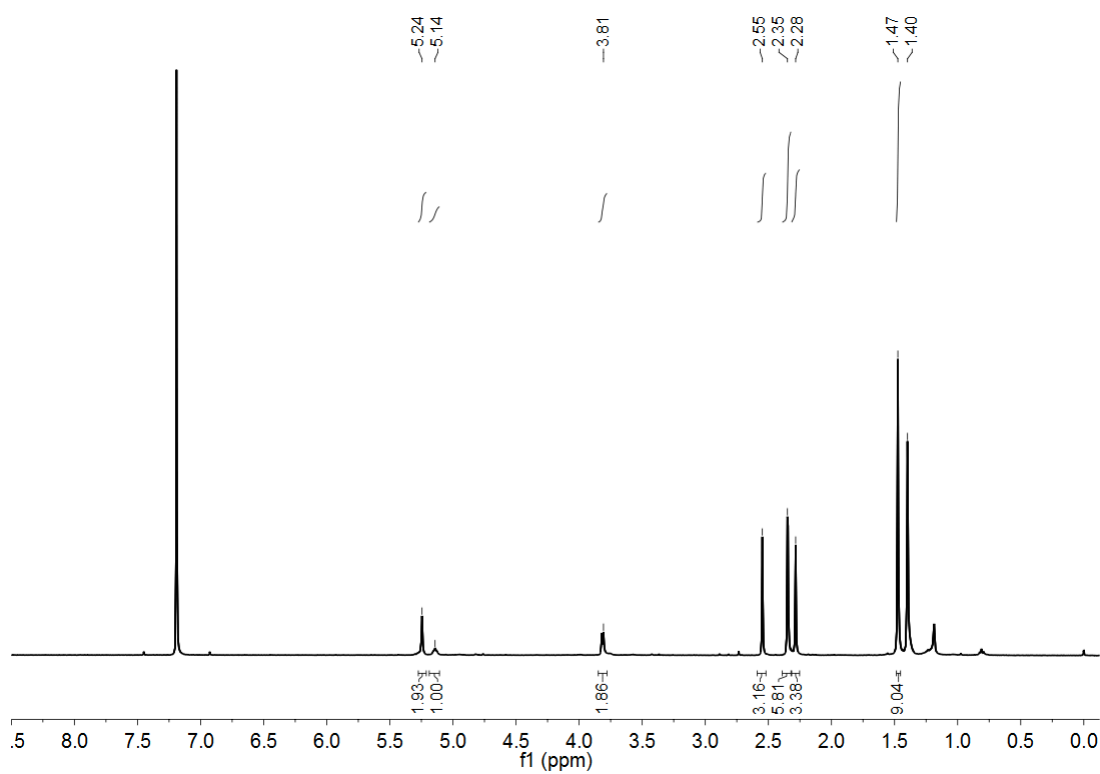
## **2.6. Confocal and FLIM imaging of cells**

MDCK cells were spread on 35 mm glass bottom dishes (Mattek Corporation, P35G-0.170 14-C) and grew on DMEM medium (GlutaMAX supplement, 10566016) for 72 h until they reached about 70% confluency. Before imaging, the medium was replaced by medium without phenol red (Leibovitz's Medium, 21083027). Confocal and FLIM images of cultured cells were taken after 2 minutes of addition of probe **1** and **2** (final concentration of 1  $\mu$ M). Confocal images were performed using the Nikon Eclipse Ti A1R microscope, with a laser 488 nm for excitation and 585/50 nm filter for the emission.

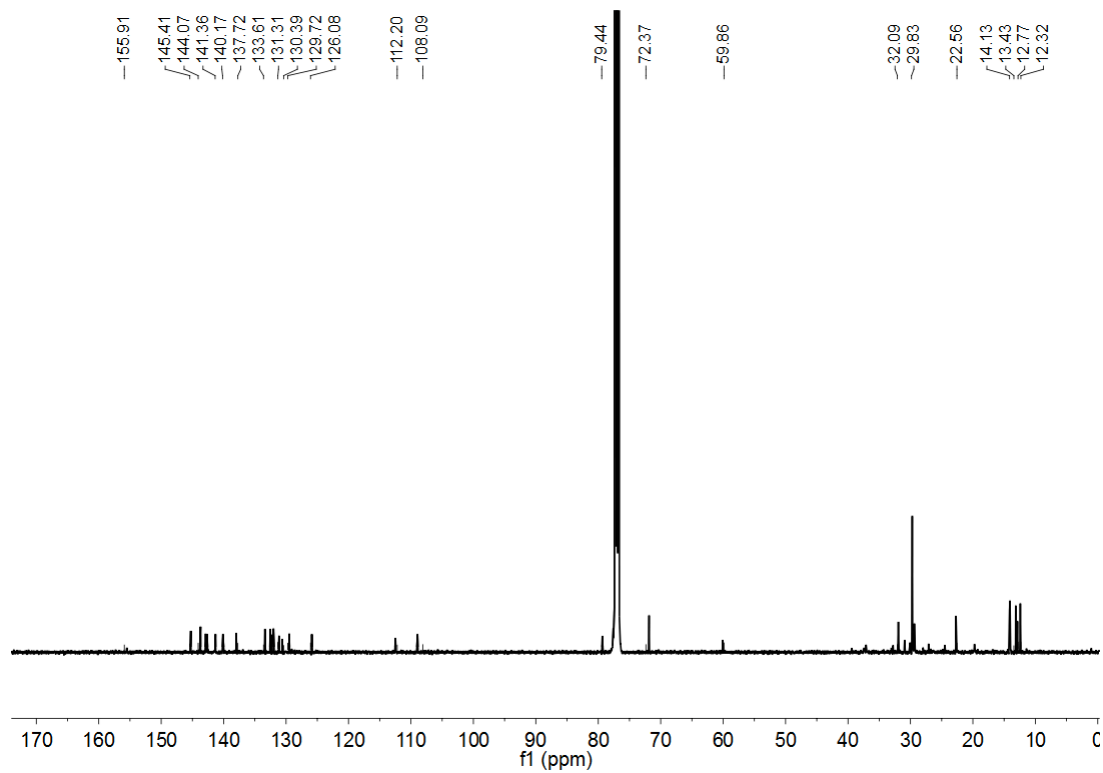
### 3. Supporting references

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- S2 Q. Verolet, M. Dal Molin, A. Colom, A. Roux, L. Guénée, N. Sakai and S. Matile, unpublished.
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#### 4. NMR spectra

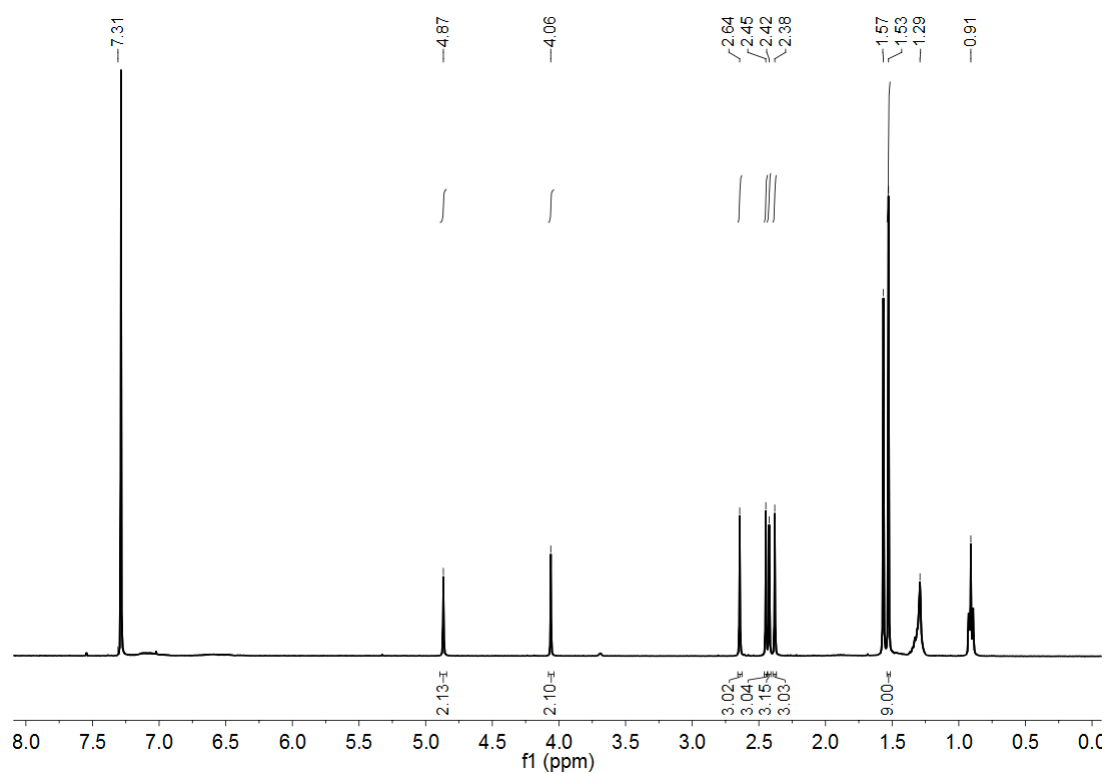


**Figure S4.** <sup>1</sup>H NMR spectrum of **4** in CDCl<sub>3</sub>.

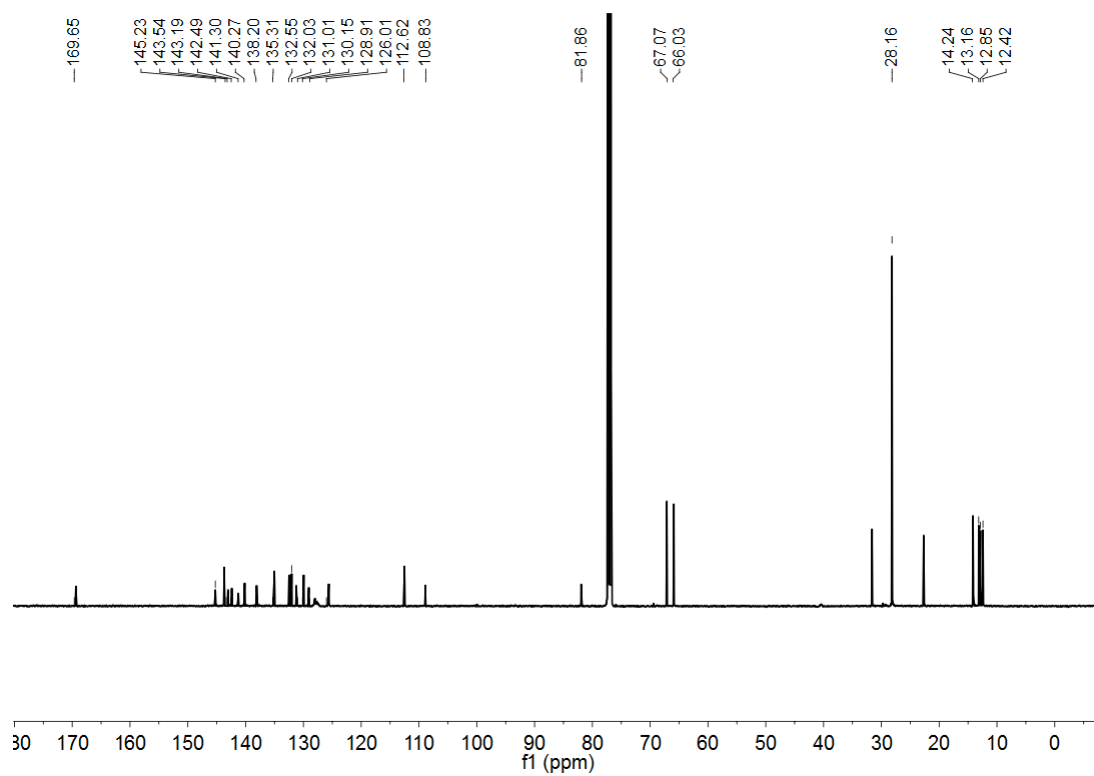


**Figure S5.** <sup>13</sup>C NMR spectrum of **4** in CDCl<sub>3</sub>.

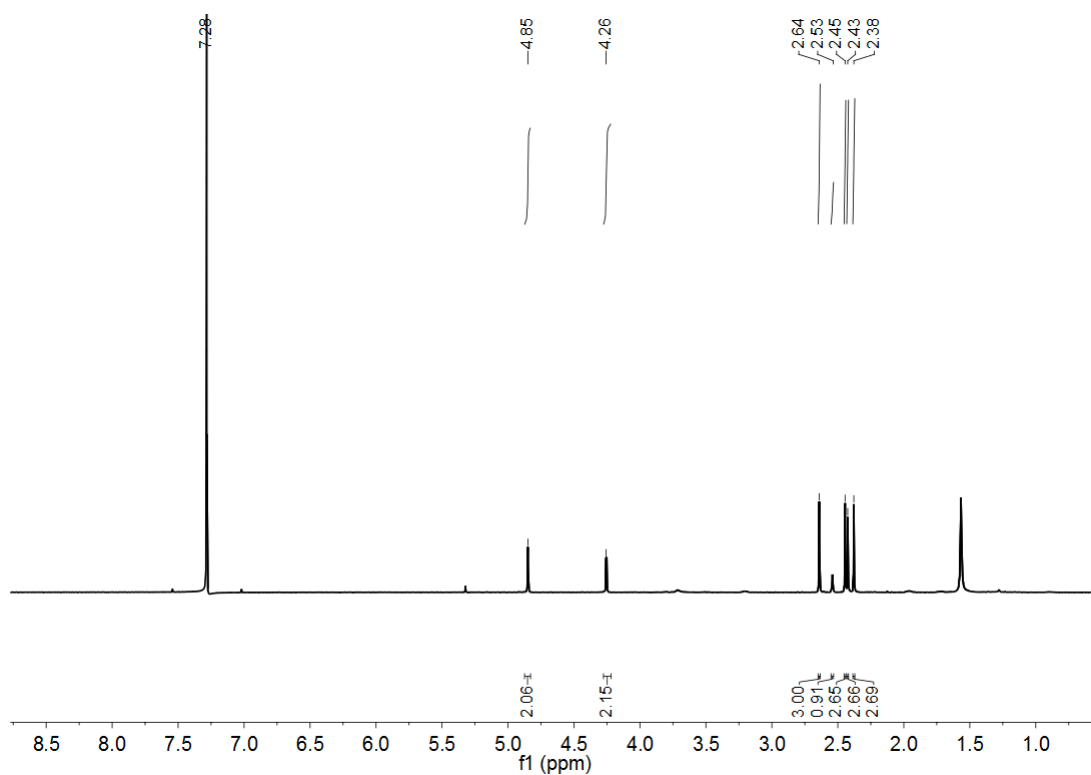




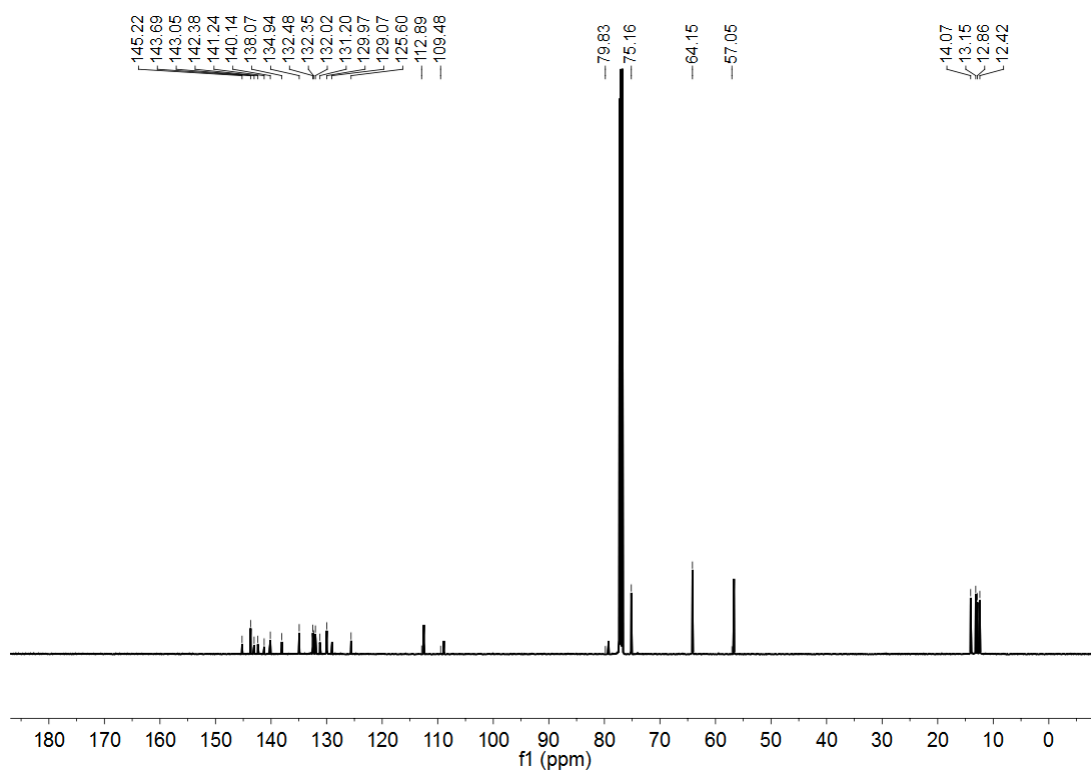
**Figure S6.** <sup>1</sup>H NMR spectrum of **5** in CDCl<sub>3</sub>.



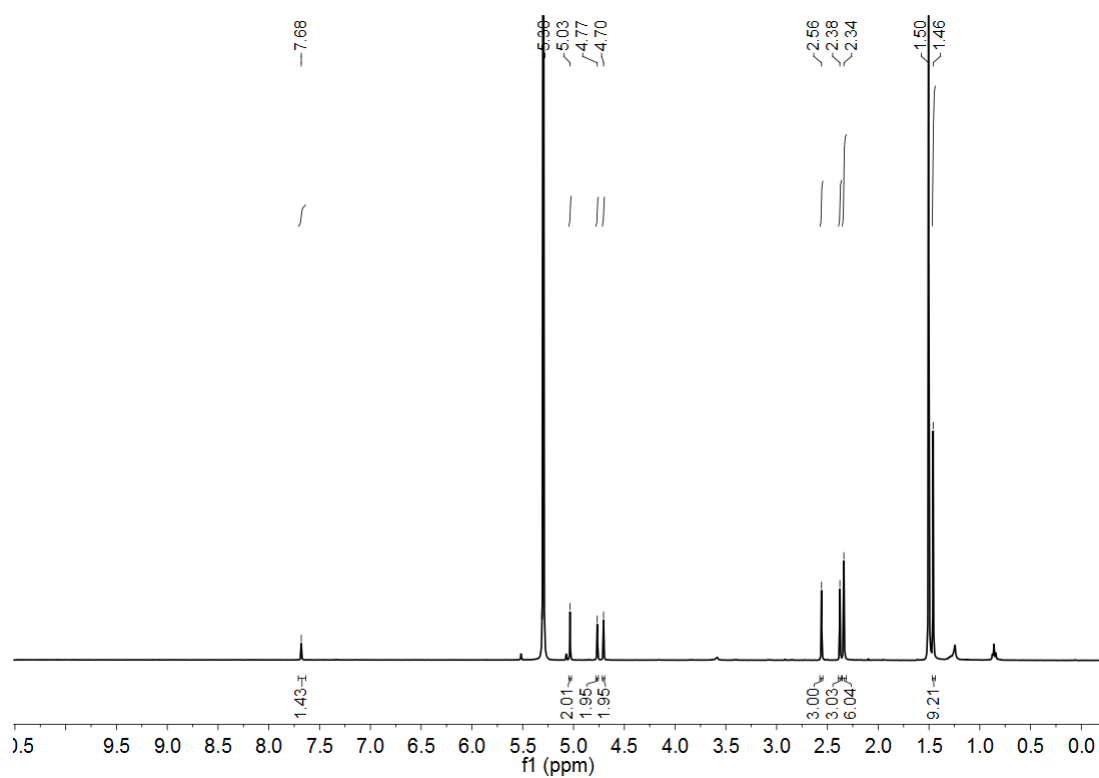
**Figure S7.** <sup>13</sup>C NMR spectrum of **5** in CDCl<sub>3</sub>.



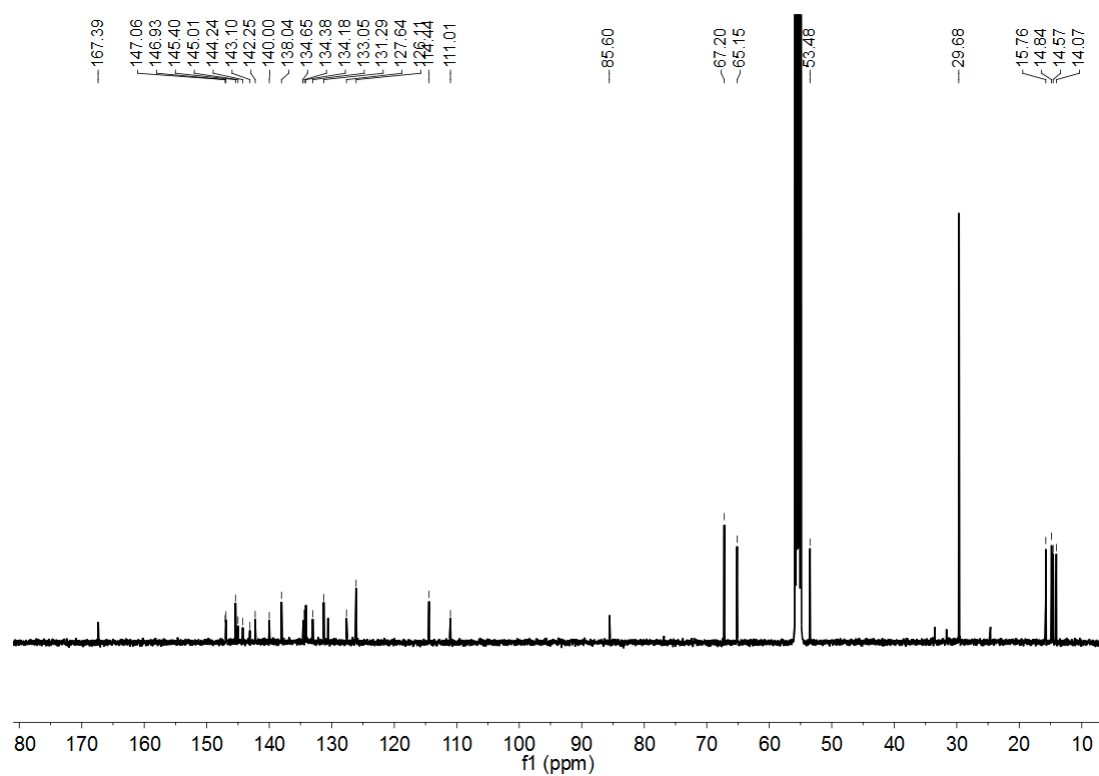
**Figure S8.** <sup>1</sup>H NMR spectrum of **7** in CDCl<sub>3</sub>.



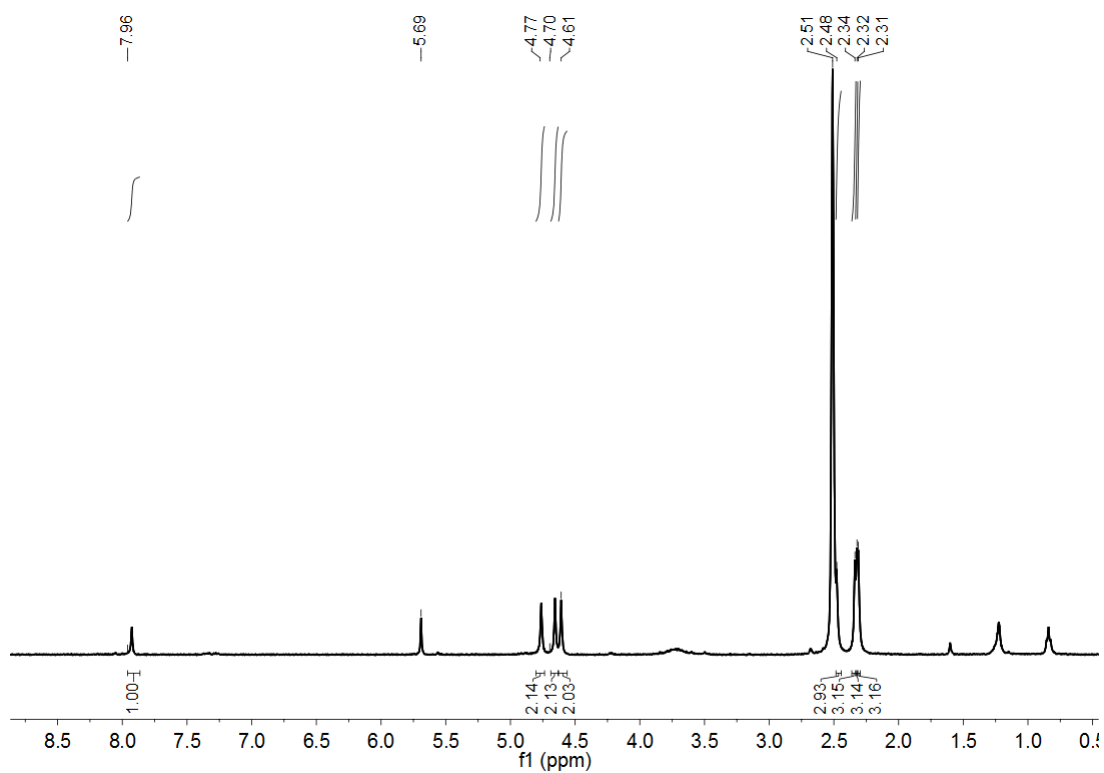
**Figure S9.** <sup>13</sup>C NMR spectrum of **7** in CDCl<sub>3</sub>.



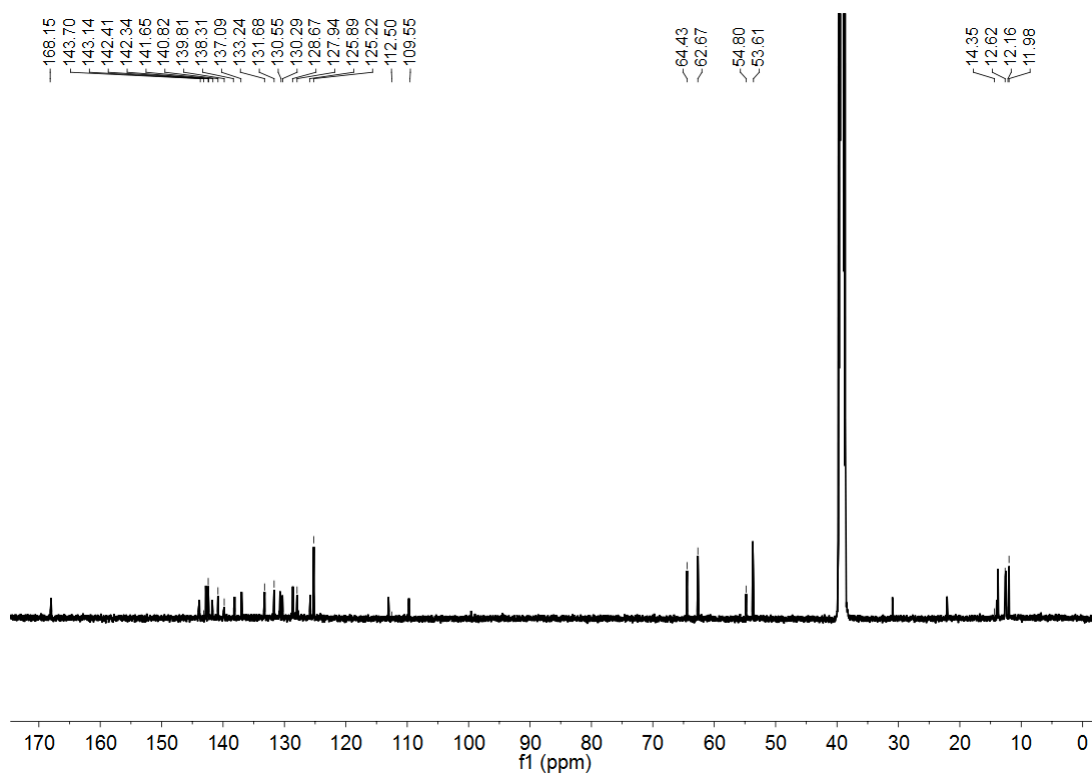
**Figure S10.** <sup>1</sup>H NMR spectrum of **10** in CD<sub>2</sub>Cl<sub>2</sub>.



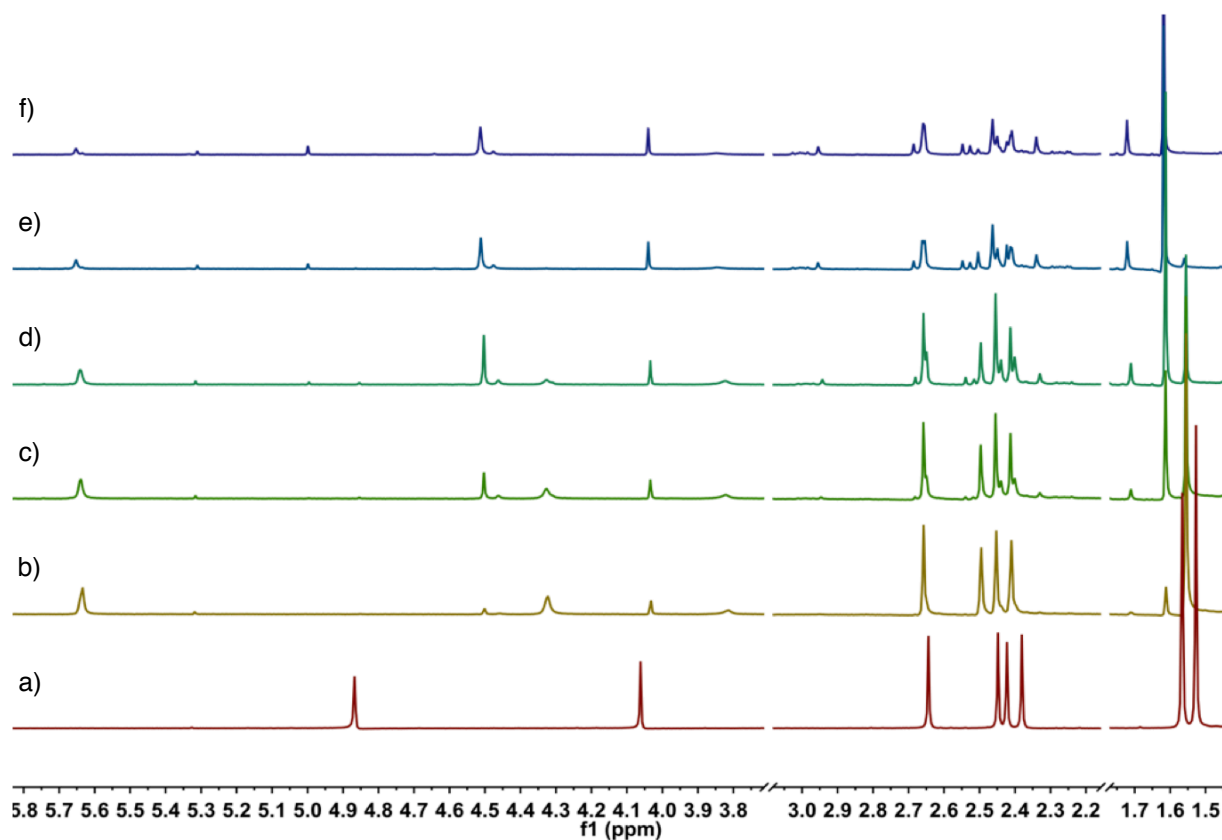
**Figure S11.** <sup>13</sup>C NMR spectrum of **10** in CD<sub>2</sub>Cl<sub>2</sub>.



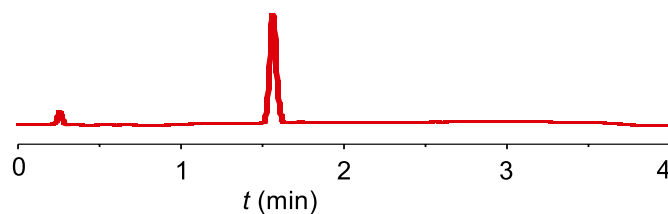
**Figure S12.** <sup>1</sup>H NMR spectrum of **2** in DMSO-*d*<sub>6</sub>/25% ND<sub>4</sub>OD in D<sub>2</sub>O 1:0.05.



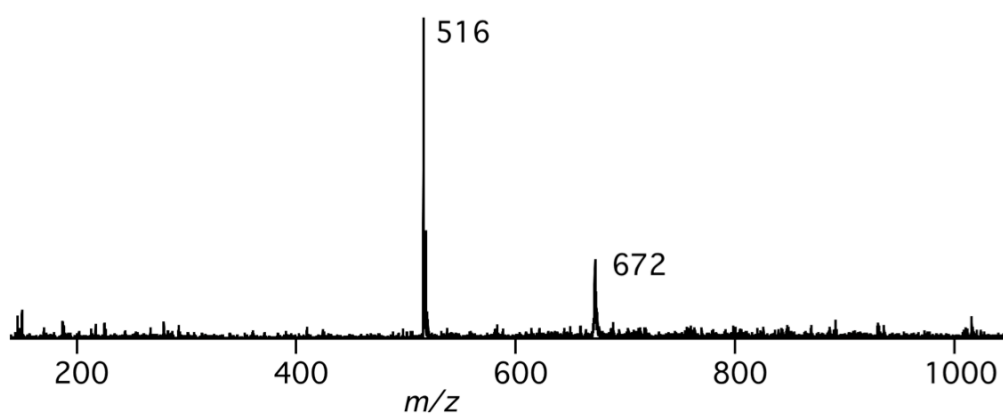
**Figure S13.** <sup>13</sup>C NMR spectrum of **2** in DMSO-*d*<sub>6</sub>/25% ND<sub>4</sub>OD in D<sub>2</sub>O 1:0.05.



**Figure S14.**  $^1\text{H}$  NMR spectra changes of **6** in  $\text{CDCl}_3$  (a), with 10% TFA after 0.25 h (b), 0.5 h (c), 1.5 h (d), 3 h (e) and 5 h (f).



**Figure S15.** Reversed-phase HPLC of compound **2**,  $R_t = 1.6$  min, column Hypersil gold, S-1.9  $\mu\text{m}$  particles, Thermo C-18 (50 mm x 2.1 mm), elution gradient: from 30% of A ( $\text{CH}_3\text{CN} + 0.01\%$  TFA) and 90% of B ( $\text{H}_2\text{O} + 0.01\%$  TFA) to 90% of A and 10% of B in 4 min, flow rate: 0.75 mL/min.



**Figure S16.** ESI MS of compound **2**: 672,  $[M+H]^+$ ; 516,  $[M-C_5H_6N_3O_3]^+$  (= intermediate **6**, Scheme 1).