Supplementary Material (ESI) for Organic & Biomolecular Chemistry

SUPPORTING INFORMATION

Intracellular Imaging of Organelles With New Water-soluble Benzophenoxazine Dyes

*Jiney Jose,[1] Aurore Loudet,[1] Yuichiro Ueno,[1] Rola Barhoumi,[2] Robert C. Burghardt[2] and Kevin Burgess[1]***

Department of Chemistry, Texas A & M University, Box 30012, College Station, TX 77843, USA.
Tel: +1 979 845-4345; Fax: +1 979 845-8839; E-mail: burgess@tamu.edu

TABLE OF CONTENTS

General Experimental Methods

All reactions were carried out under an atmosphere of dry nitrogen. Glasswares were oven-dried prior to use. Unless otherwise indicated, common reagents or materials were obtained from commercial source and used without further purification. All solvents were dried prior to use with appropriate drying agents. Dry distilled DMF was obtained from Acros and used as such. Flash column chromatography was performed using silica gel 60 (230-400 mesh). Analytical thin layer chromatography (TLC) was carried out on Merck silica gel plates with QF-254 indicator and visualized by UV. Fluorescence spectra were obtained on a Varian Cary Eclipse fluorescence spectrophotometer at room temperature. Absorbance spectra were obtained on a Varian 100 Bio UV-Vis spectrophotometer at room temperature. IR spectra were recorded on a Bruker Tensor 27 spectrometer.

^{1}H and ^{13}C spectra were recorded on a Varian 300 (300 MHz ^{1}H; 75 MHz ^{13}C) or Varian 500 (500 MHz ^{1}H; 125 MHz ^{13}C) spectrometer at room temperature. Chemical shifts were reported in ppm relative to the residual $CDCl_3$ (δ 7.26 ppm ^{1}H; δ 77.16 ppm ^{13}C), CD_3OD (δ 3.31 ppm ^{1}H; δ 49.0 ppm ^{13}C), DMSO-d_6 (δ 2.54 ppm ^{1}H; δ 39.52 ppm ^{13}C) or acetone-d_6 (δ 2.05 ppm ^{1}H; δ 29.84 and 206.26 ppm ^{13}C) Coupling constants (J) were reported in Hertz.

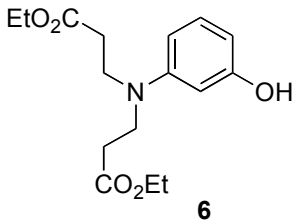
Photophysical Properties and Determination of Quantum Yields

Steady-state fluorescence spectroscopic studies were performed on a Cary Eclipse fluorometer. The slit width was 5 nm for both excitation and emission. The relative quantum yields of the samples were obtained by comparing the area under the corrected emission spectrum of the test sample with that of a standard dye in ethanol.[1] The quantum efficiencies of fluorescence were obtained from three measurements with the following equation:

$$\Phi_x = \Phi_{st} (I_x/I_{st}) (A_{st}/A_x) (\eta_x^2/\eta_{st}^2)$$

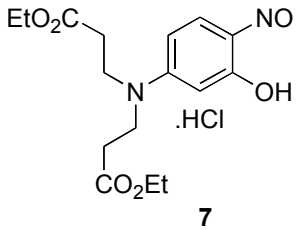
Where Φ_{st} is the reported quantum yield of the standard, **I** is the area under the emission spectra, **A** is the absorbance at the excitation wavelength and η is the refractive index of the solvent used, measured on a pocket refractometer from ATAGO. **X** subscript denotes unknown, and **st** denotes standard.[2] Molar extinction coefficients were obtained from the slope of a graph of absorbance *vs* concentration for each dye with five different concentrations (10^{-6} M). 0.25, 0.5, 1.0, 1.5, and 3.0 x 10^{-6} M were the concentration used.

Experimental Section



6

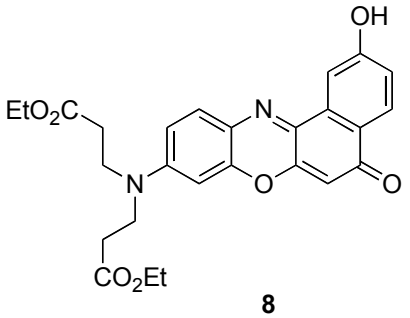
A solution of dicarboxylic acid (2.0 g, 7.9 mmol) in ethanol (100 mL) along with HCl (10 M, 0.2 mL) was refluxed for 12 h. The reaction mixture was cooled and ethanol evaporated under reduced pressure. The residue obtained was dissolved in dichloromethane (20 mL) and organic layer washed with water (5 mL x 5). The organic layer was dried under Na_2SO_4 and solvent evaporated to obtain a residue which was purified by passing through a short flash chromatography normal phase silica column eluting with 1/1 EtOAc/hexanes to yield **6** as an yellow oil (1.64 g, 67 %). R_f = 0.8 (1/1 EtOAc/hexanes). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.05 (t, 1H, *J* = 5.0 Hz), 6.99 (s, 1H), 6.24 (br, 2H), 4.13 (q, 4H, *J* = 7.1 Hz), 3.61 (t, 4H, *J* = 7.0 Hz), 2.58 (t, 4H, *J* = 7.0 Hz), 1.24 (t, 6H, *J* = 7.1 Hz); **^{13}C NMR** (125 MHz, $CDCl_3$) δ 172.9, 157.7, 148.4, 130.6, 105.0, 104.6, 99.9, 61.1, 47.1, 32.8, 14.4. **MS (ESI)** m/z calculated for $(M+H)^+$ 310.37 found 310.32 $(M+H)^+$; ν_{cm}^{-1} (neat) 3387, 2962, 1732 cm $^{-1}$



7

Sodium nitrite (0.24 g, 3.5 mmol) in water (3 mL) was added, over a period of 15 min, via a syringe pump at the rate of 0.2 mL/min, to a solution of **6** (1.0 g, 3.2 mmol) in HCl (3 mL, 10 M) and water (1.5 mL) at 0 ^{o}C. The mixture was stirred for 3 h at 0 ^{o}C and

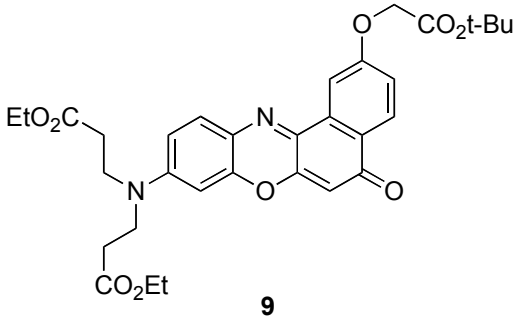
filtered to remove residual impurities. The filtrate was evaporated under reduced pressure to yield **7** as a yellow solid (0.92g, 77 %). The crude product was very moisture sensitive and therefore directly used without further purification for the next step.



8

1,6-Dihydroxynaphthol (0.34 g, 2.1 mmol) with HCl (0.1 mL, 10 M) was added to a solution of **7** (0.8 g, 2.1 mmol) in EtOH (30 mL) all in one portion. The reaction mixture was refluxed for 5 h. The solvent was evaporated and residue purified by flash chromatography eluting with 50 % EtOAc/hexane and 10 % MeOH/EtOAc to afford **8** as a red solid (372 mg, 37 %). R_f = 0.8 (10 % MeOH/EtOAc).

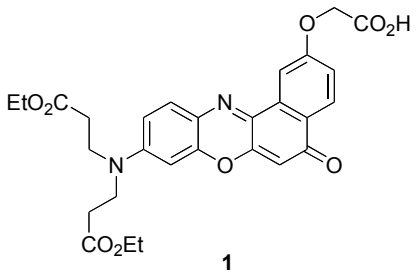
^{1}H NMR (300 MHz, acetone-d_6) δ 8.07 (d, 1H, J = 8.5 Hz), 8.0 (s, 1H), 7.62 (d, 1H, J = 8.5 Hz), 7.19 (dd, 1H, d, J = 9.1, 2.6 Hz), 6.89 (dd, 1H, d, J = 9.1, 2.6 Hz), 6.72 (s, 1H), 6.14 (s, 1H), 4.13 (q, 4H, J = 7.0 Hz), 3.89 (t, 4H, J = 7.0 Hz), 2.74 (t, 4H, J = 7.0 Hz), 1.22 (t, 6H, J = 7.0 Hz); **^{13}C NMR** (125 MHz, CD_3OD) δ 183.8, 172.1, 161.2, 153.5, 152.8, 146.8, 139.9, 134.3, 131.5, 127.6, 125.8, 125.5, 110.8, 106.9, 104.3, 97.1, 95.0, 60.8, 32.2, 29.6, 13.3. **MS (ESI)** m/z calculated for $(M)^+$ 478.17 found 478.33 $(M)^+$. ν_{cm}^{-1} (neat) 3427, 2944, 1736 cm^{-1}.



9

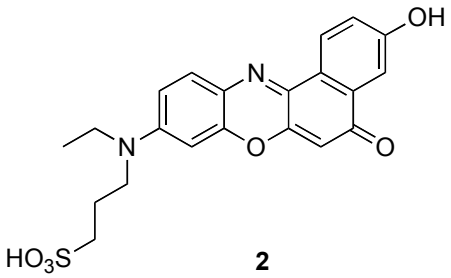
Compound **8** (0.42 g, 0.9 mmol) and Cs_2CO_3 (1.43 g, 4.4 mmol) was dissolved in CH_3CN (5 mL). Tertiary butyl bromoacetate (0.86 g, 4.4 mmol) was added after 5 min. and the reaction mixture heated to 60 ^oC for 6 h. The solvent was evaporated under reduced

pressure and the residue purified by flash chromatography eluting with 1/1 EtOAc/hexanes to afford **9** as a red solid (360 mg, 69 %). R_f = 0.6 (1/1 EtOAc/hexanes). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 8.12 (d, 1H, *J* = 9.3 Hz), 7.94 (s, 1H), 7.53 (d, 1H, *J* = 9.3 Hz), 7.17 (dd, 1H, *J* = 8.7, 4.2 Hz), 6.60 (dd, 1H, *J* = 8.7, 4.2 Hz), 6.43 (s, 1H), 6.22 (s, 1H), 4.86 (s, 2H), 4.11 (q, 4H, *J* = 4.8 Hz), 3.72 (br, 4H), 2.64 (br, 4H), 1.41 (s, 9H), 1.18 (t, 6H, *J* = 4.8 Hz); **^{13}C NMR** (125 MHz, $CDCl_3$) δ 183.0, 172.1, 168.9, 161.3, 152.2, 150.0, 146.8, 141.9, 133.8, 131.4, 128.8, 127.0, 125.7, 118.9, 110.3, 107.1, 106.4, 97.9, 83.3, 66.2, 61.4, 47.3, 33.1, 28.2, 14.3. **MS (ESI)** m/z calculated for $(M)^+$ 592.24 found 592.21 $(M)^+$. ν_{cm}^{-1} (neat) 3423, 2941, 1747 cm^{-1}.



1

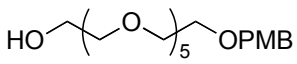
A solution of **9** (100 mg, 0.17 mmol) in trifluoroacetic acid (3 mL) and dichloromethane (3 mL) was stirred for 4 h. The excess trifluoroacetic acid was neutralized by adding sodium hydroxide solution (2 mL, 2 M). The solvent was evaporated and residue purified by flash chromatography eluting with 20 % MeOH/EtOAc to afford **1** as a purple solid (60 mg, 66 %). R_f = 0.6 (EtOAc). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.03 (d, 1H, *J* = 10.0 Hz), 7.88 (s, 1H), 7.61 (d, 1H, *J* = 10.0 Hz), 7.27 (dd, 1H, *J* = 10.0, 5.0 Hz), 6.82 (dd, 1H, *J* = 10.0, 5.0 Hz), 6.73 (s, 1H), 6.19 (s, 1H), 4.88 (s, 2H), 4.08 (q, 4H, *J* = 5.0 Hz), 3.73 (t, 4H, *J* = 5.0 Hz), 2.63 (t, 4H, *J* = 5.0 Hz), 1.19 (t, 6H, *J* = 5.0 Hz); **^{13}C NMR** (125 MHz, DMSO-d_6) δ 182.2, 172.3, 170.6, 161.3, 152.4, 151.3, 146.9, 140.1, 134.1, 131.7, 128.1, 126.0, 124.9, 118.7, 111.1, 107.3, 105.1, 97.8, 65.6, 60.9, 47.1, 32.4, 14.9. **MS (ESI)** m/z calculated for $(M+H)^+$ 537.19 found 537.18 $(M+H)^+$. ν_{cm}^{-1} (neat) 3389, 2954, 1721 cm^{-1}.



2

The nitroso compound **B** (1.0g, 3.1 mmol) and 1,7-dihydroxynaphthol (0.5 g, 3.1 mmol) was dissolved in dry DMF (20 ml) and heated to 140 ^{o}C for 4 h. The reaction mixture was cooled to 25 ^{o}C and DMF removed under reduced pressure. The residual material was purified by column chromatography eluting with 1/1 MeOH/EtOAc to afford **2** as a blue solid (0.94 g, 71 %). R_f = 0.3 (10% MeOH/EtOAc).

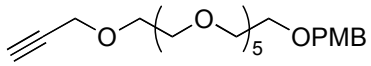
^{1}H NMR (500 MHz, CD_3OD) δ 8.55 (d, 1H, *J* = 8.8 Hz), 7.65 (d, 1H, *J* = 8.7 Hz), 7.56 (s, 1H), 7.23 (dd, 1H, *J* = 8.7, 2.7 Hz), 6.95 (dd, 1H, *J* = 8.7, 2.7 Hz), 6.74 (s, 1H), 6.35 (s, 1H), 3.66 (t, 2H, *J* = 8.3 Hz), 3.59 (q, 2H, *J* = 6.8 Hz), 2.92 (t, 2H, *J* = 8.3 Hz), 2.15 (br, 2H), 1.26 (t, 3H, *J* = 6.8 Hz); **^{13}C NMR** (125 MHz, CD_3OD) δ 184.3, 172.0, 159.9, 152.7, 151.3, 146.6, 138.9, 133.2, 130.7, 126.0, 125.9, 120.1, 110.9, 109.9, 104.3, 96.5, 49.4, 48.3, 45.2, 23.0, 11.5. **MS (ESI)** m/z calculated for $(M-H)^-$ 427.10 found 427.02 $(M-H)^-$. ν_{cm}^{-1} (neat) 3397, 2931, 1689 cm^{-1}.



C

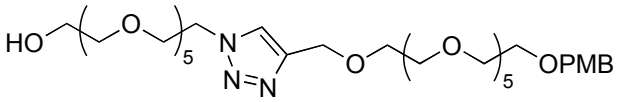
To a solution of hexaethylene glycol (4.3 g, 15.2 mmol), Ag_2O (5.29 g, 22.8 mmol) and potassium iodide (1.52 g, 9.14 mmol) in toluene (40 mL) was added *p*-methoxybenzyl chloride (2.62 g, 16.8 mmol) dropwise at 25 ºC. The reaction mixture was refluxed at 120 ºC for 22 h. After cooling to room temperature, the reaction was filtered through celite and washed with EtOAc. The solvent was evaporated and the residual material was purified by flash chromatography eluting with 50 % EtOAc/hexanes, 100 % EtOAc and 50 % acetone/EtOAc to afford **C** as a yellow oil (3.68 g, 60 %). R_f = 0.1 (100 % EtOAc with iodine stain). **^{1}H NMR** (500 MHz, $CDCl_3$) d 7.25 (d, 2H, *J* = 8.5 Hz), 6.85 (d, 2H, *J* = 8.5 Hz), 4.48 (s, 2H), 3.79 (s, 3H), 3.79-3.69 (m, 2H), 3.67-3.64 (m, 18H), 3.60-3.57

(m, 4H); **^{13}C NMR** (125 MHz, $CDCl_3$) d 159.1, 130.2, 129.3, 113.7, 72.8, 72.5, 70.6, 70.53, 70.51 (3C), 70.49 (2C), 70.46, 70.3, 69.0, 61.7, 55.2; **MS (ESI)** m/z calculated for $(M+Li)^+$ 409.24 found 409.24 $(M+Li)^+$.



10

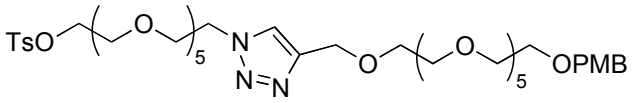
Sodium hydride (278 mg, 6.96 mmol, 60 % in mineral oil) was slowly added to a solution of **C** (2.0 g, 4.97 mmol) in THF (15 mL) at 0 ºC. The reaction was stirred at 0 ºC for 30 min, then propargyl bromide (1.1 g, 7.45 mmol, 80 % w/w in toluene) was added dropwise. After the stirring at 0 ºC for 30 min, the reaction mixture was warmed to room temperature and stirred at 25 ºC for additional 6 h. MeOH (1 mL) was added carefully at 0 ºC and water (5 mL) was added. The aqueous layer was extracted with CH_2Cl_2 (5 x 20 mL) and combined organic layer was dried over $MgSO_4$ and concentrated under the reduced pressure. The residue was purified by flash chromatography eluting with 50 % EtOAc/hexanes and 100 % EtOAc to afford **10** as an yellow oil (1.81 g, 83 %). R_f 0.5 (100 % EtOAc with iodine stain). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.26 (d, 2H, J = 8.5 Hz), 6.87 (d, 2H, J = 8.5 Hz), 4.49 (s, 2H), 4.20 (d, 2H, J = 2.5 Hz), 3.80 (s, 3H), 3.70-3.64 (m, 22H), 3.60-3.58 (m, 2H), 2.43 (t, 1H, J = 2.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.1, 130.3, 129.4, 113.7, 79.6, 74.5, 72.8, 70.6, 70.57 (2C), 70.54 (6C), 70.4, 69.1, 69.0, 58.4, 55.2; **MS (ESI)** m/z calculated for $(M+Li)^+$ 447.26 found 447.26 $(M+Li)^+$.



12

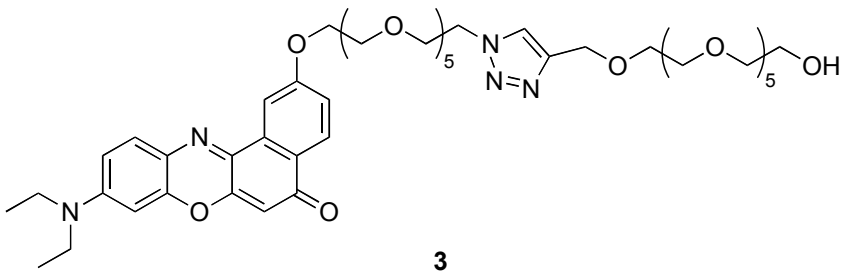
Copper powder (244 mg, 3.84 mmol), $CuSO_4$ (0.77 mL, 0.77 mmol, 1M in H_2O) and H_2O (12 mL) were added to a solution of **11** (1.18 g, 3.84 mmol) and **10** (2.2 g, 4.99 mmol) in THF (12 mL). The reaction mixture was stirred at 25 ºC for 24 h under the nitrogen. The solvents were evaporated under reduced pressure and the residual material was purified by flash chromatography eluting with 50 % acetone/EtOAc, 100 % acetone and 5 to 10 % MeOH/CH_2Cl_2 to afford **12** as an yellow oil (2.7 g, 94 %). R_f 0.2 (50 % acetone/EtOAc with iodine stain). **^{1}H NMR** (300 MHz, $CDCl_3$) δ 7.75 (s, 1H), 7.26 (d,

2H, *J* = 9.0 Hz), 6.86 (d, 2H, *J* = 9.0 Hz), 4.67 (s, 2H), 4.53 (t, 2H, *J* = 5.1 Hz), 4.48 (s, 2H), 3.86 (t, 2H, *J* = 5.1 Hz), 3.79 (s, 3H), 3.73-3.57 (m, 44H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.9, 144.6, 130.1, 129.2, 123.7, 113.5, 72.6, 72.4, 70.4, 70.32 (8C), 70.26 (8C), 70.0, 69.4, 69.2, 68.8, 64.3, 61.4, 55.1, 50.0; **MS (ESI)** m/z calculated for $(M+H)^+$ 748.42 found 748.44 $(M+H)^+$.



13

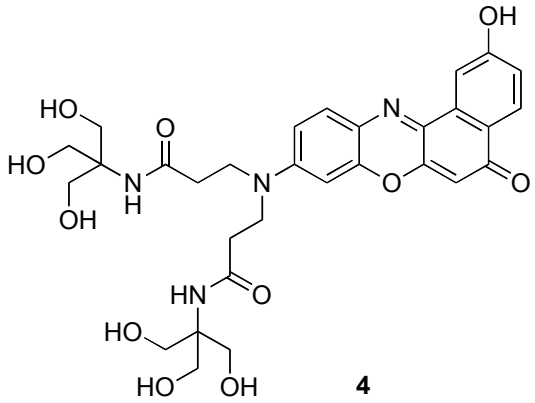
Compound **12** (1.5 g, 2.01 mmol), Et_3N (0.56 mL, 4.01 mmol) and Me_3N•HCl (19 mg, 0.20 mmol) were added in CH_3CN (20 mL) at 0 ºC. A solution of TsCl (574 mg, 3.01 mmol) in CH_3CN (12 mL) was then added by syringe and the reaction mixture was stirred at 0 ºC for 10 min and 12 h at 25 ºC. The reaction was quenched with water (12 mL) and the aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layer was dried over $MgSO_4$ and solvents were concentrated under the reduced pressure. The residue was purified by flash chromatography eluting with 50 % acetone/EtOAc and then 100 % acetone to afford product as a brown oil (1.6 g, 89 %). R_f = 0.3 (50 % acetone/EtOAc with iodine stain). **^{1}H NMR** (300 MHz, $CDCl_3$) δ 7.74 (d, 2H, *J* = 8.0 Hz), 7.72 (s, 1H), 7.30 (d, 2H, *J* = 8.0 Hz), 7.22 (d, 2H, *J* = 9.0 Hz), 6.82 (d, 2H, *J* = 9.0 Hz), 4.62 (s, 2H), 4.48 (t, 2H, *J* = 5.0 Hz), 4.44 (s, 2H), 4.10 (t, 2H, *J* = 5.0 Hz), 3.82 (t, 2H, *J* = 5.0 Hz), 3.75 (s, 3H), 3.65-3.53 (m, 42H), 2.40 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.9, 144.7, 132.7, 130.1, 129.7, 129.2, 127.8, 123.7, 113.5, 72.7, 70.5, 70.43, 70.39, 70.37, 70.34 (10C), 70.28 (3C), 70.25, 69.4, 69.3, 69.1, 68.9, 68.5, 64.4, 55.1, 50.0, 21.5 (1 carbon is missing for aromatic region); **MS (ESI)** m/z calculated for $(M+H)^+$ 902.43 found 902.45 $(M+H)^+$.



3

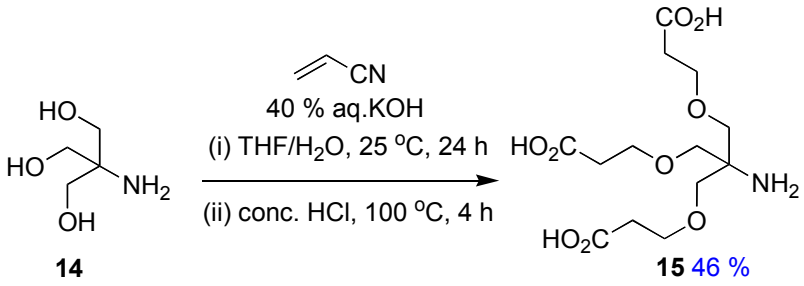
2-Hydroxy Diethyl Nile Red **D** (50.0 mg, 0.15 mmol) and K_2CO_3 (103.0 mg, 0.75 mmol) were dissolved in CH_3CN (5 mL). **13** (162.0 mg, 0.18 mmol) in CH_3CN (2 mL) was added drop wise in 5 min. to above solution at 25 ^oC. The reaction mixture was heated to 50 ^oC for 4 h. After completion of reaction the solvent was evaporated and residue was subjected to flash chromatography eluting with 30-40 % acetone/EtOAc and then with 10 % MeOH/CH_2Cl_2 to afford 150.0 mg of red colored material. Flash chromatography was performed to remove excess **13**.

Above red material (60.0 mg, 0.06 mmol) was dissolved in TFA/CH_2Cl_2 (1/1, 3 mL) and stirred at 25 ^oC for 1 h. Solvent was removed under reduced pressure and the residual material was dissolved in 5 mL of water. This solution was filtered to remove solid impurities and the filtrate was purified by reverse phase medium pressure liquid chromatography (MPLC) eluting with 30 % CH_3CN/H_2O to afford **3** as a dark red solid (35.0 mg, 58%). **^{1}H NMR** (500 MHz, CD_3OD) δ 8.06 (d, 1H, *J* = 8.9 Hz), 8.01 (s, 1H), 7.97 (d, 1H, *J* = 3.0 Hz), 7.54 (d, 1H, *J* = 8.9 Hz), 7.17 (dd, 1H, *J* = 9.2, 3.0 Hz), 6.79 (dd, 1H, *J* = 9.2, 3.0 Hz), 6.54 (d, 1H, *J* = 3.0 Hz), 6.17 (s, 1H), 4.61 (s, 2H), 4.55 (t, 2H, *J* = 5.0 Hz), 4.32-4.29 (br, 2H), 3.93 (br, 2H), 3.89 (t, 2H, *J* = 5.0 Hz), 3.77-3.74 (br, 2H), 3.70-3.68 (br, 2H), 3.66-3.52 (m, 40H), 1.27 (t, 6H, *J* = 7.0 Hz); **^{13}C NMR** (75 MHz, CD_3OD) δ 183.7, 161.9, 152.8, 151.8, 147.1, 144.6, 143.8, 138.1, 134.3, 131.3, 127.3, 125.1, 124.7, 118.1, 110.7, 106.4, 103.7, 96.0, 72.5, 70.7, 70.5, 70.4, 70.3, 70.2, 70.1, 69.6, 69.2, 67.9, 63.8, 61.0, 50.2, 44.9, 11.8; **MS (ESI)** m/z calculated for $(M+H)^+$ 944.49 found 944.49 $(M+H)^+$ **MS (ESI)** m/z calculated for $(M+Na)^+$ 966.47 found 966.45 $(M+Na)^+$; ν_{cm}^{-1} (neat) 3435, 2925, 1641 cm^{-1}.



4

Compound **E**[3] (25 mg, 0.06 mmol) and activating agent EDCI (28 mg, 0.24 mmol) was dissolved in pyridine (2 mL). Tris(hydroxymethyl)aminomethane (43 mg, 0.36 mmol) was added and reaction continued at 25 ^{o}C for 24 h. The solvent was evaporated and residue purified by reverse phase medium pressure liquid chromatography (MPLC) eluting with 3/2 CH_3CN/H_2O to afford **4** as a dark red solid (22 mg, 59 %). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 7.96 (d, 1H, J = 10.0 Hz), 7.87 (s, 1H), 7.61 (d, 1H, J = 10.0 Hz), 7.39 (s, 2H), 7.08 (dd, 1H, J = 10.0, 5.0 Hz), 6.84 (dd, 1H, J = 10.0, 5.0 Hz), 6.68 (s, 1H), 6.19 (s, 1H), 4.73 (br, 6H), 3.65 (br, 4H), 3.53 (s, 12H), 2.51 (br, 4H); **^{13}C NMR** (125 MHz, DMSO-d_6) δ 182.4, 172.1, 152.2, 152.2, 151.3, 146.9, 140.1, 134.4, 131.4, 128.2, 124.8, 119.2, 110.8, 110.2, 109.2, 105.2, 97.4, 63.2, 61.2, 48.1, 34.6. **MS (ESI)** m/z calculated for $(M+H)^+$ 629.25 found 629.23 $(M+H)^+$. ν_{cm}^{-1} (neat) 3412, 3316, 1693 cm^{-1}.

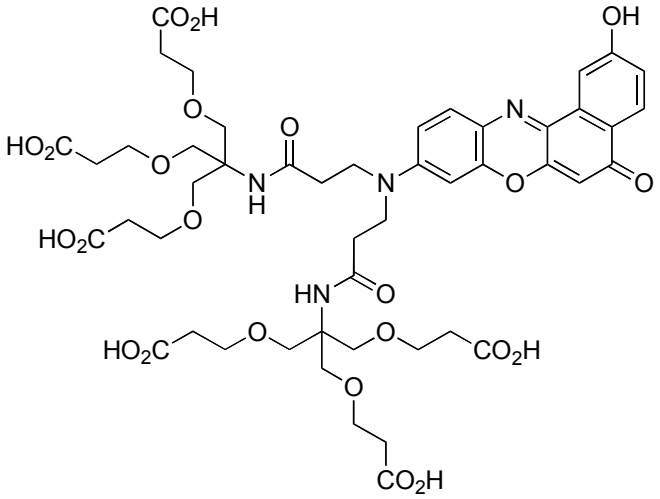


14 **15** 46 %

Tris(hydroxymethyl)aminomethane **14** (2g, 16.5 mmol) was dissolved in THF (10 mL) and aqueous 40 % KOH (1 mL). Acrylonitrile (5.4 ml, 82.5 mmol) was added to the above solution and stirred at 25 ^{o}C for 24 h. The solvent was evaporated and water (10 mL) added. The aqueous layer was extracted with dichloromethane (5 x 5 mL) and dried

over Na_2SO_4. The residue obtained was purified by flash chromatography eluting with 10% MeOH/EtOAc to obtain cyano derivative as a yellow oil (2.8 g, 61%).

^{1}H NMR (500 MHz, CD_3OD) δ 3.70 (t, 6H, *J* = 8.8 Hz), 3.48 (s, 6H), 2.74 (t, 6H, 8.8 Hz); **^{13}C NMR** (125 MHz, CD_3OD) δ 118.8, 71.8, 66.1, 56.1, 18.1. ν_{cm}^{-1} (neat) 3378, 2290, 1732, 1616 cm $^{-1}$. Above cyano derivative (1 g, 3.6 mmol) was dissolved in concentrated HCl (3 mL) and refluxed at 100 ^{o}C for 4 h. The reaction mixture was cooled to 25 ^{o}C and diluted with water (20 mL). The solvent was evaporated and residue purified by reverse phase medium pressure liquid chromatography (MPLC) eluting with 1/4 CH_3CN/H_2O to afford **15** as a colorless viscous solid (0.91 g, 75 %). **^{1}H NMR** (500 MHz, D_2O) δ 3.56 (t, 6H, *J* = 6.1 Hz), 3.43 (s, 6H), 2.41 (t, 6H, *J* = 6.1 Hz); **^{13}C NMR** (125 MHz, D_2O) δ 177.6, 68.1, 67.4, 59.7, 35.3. **MS (ESI)** m/z calculated for $(M)^+$ 337.14 found 337.18 $(M)^+$. ν_{cm}^{-1} (neat) 2927, 1732, 1615 cm^{-1}.



5

Compound **E**[3] (60 mg, 0.14 mmol), *N*-hydroxysuccinimide (81 mg, 0.7 mmol) and *N,N'*-diisopropylcarbodiimide (88 mg, 0.7 mmol) were dissolved in dry DMF (2 ml) and stirred at 25 ^{o}C for 24 h. Solvent was evaporated under reduced pressure and residue dissolved in EtOAc (5 mL) and washed with water (5 mL x 3). The organic layer was dried over $MgSO_4$ and solvent evaporated to obtain a red colored material.

The above red colored material was dissolved in DMF (2 ml) along with tricarboxylic acid **15** (479 mg, 1.4 mmol), DMAP (1 mg, 0.01 mmol) and triethylamine (0.2 mL, 1.4 mmol). The reaction mixture was stirred at 25 ^{o}C for 48 h. After removal of DMF under

reduced pressure the residue was dissolved in water (2 mL) and washed with EtOAc (2 mL x 3). The aqueous layer containing crude product was loaded on to a reverse phase MPLC column and purified using 3/2 CH_3CN/H_2O solvent mixture. The solvent was evaporated to afford **5** as a dark purple solid (43 mg, 28 %). **^{1}H NMR** (500 MHz, CD_3OD) δ 8.04 (br, 1H), 7.94 (br, 1H), 7.56 (br, 1H), 7.08 (br, 1H), 6.91 (s, 1H), 6.74 (s, 1H), 6.20 (s, 1H), 3.76 (br, 4H), 3.67 (br, 24H), 2.75 (br, 4H), 2.47 (s, 12H); **^{13}C NMR** (125 MHz, CD_3OD) δ 183.9, 175.6, 172.4, 161.0, 152.5, 151.3, 146.5, 139.1, 134.3, 130.8, 127.3, 125.1, 123.9, 118.0, 110.8, 108.4, 103.6, 96.9, 68.5 (2C), 67.2, 60.9, 35.4, 34.2; **MS (MALDI)** m/z calculated for $(M+3H)^+$ 1063.39 found 1063.35 $(M+3H)^+$.
ν_{cm}^{-1} (neat) 2942, 1722, 1621 cm^{-1}.

Cellular Uptake and Sub-cellular Localization Studies.

a. Cell culture

Clone 9 cells (American Type Culture Collection) were cultured as subconfluent monolayers on 75 cm^2 culture flask with vent caps in Ham's medium supplemented with 10 % fetal bovine serum (FBS) in a humidified incubator at 37 ºC with 5 % CO_2. Cells grown to subconfluence were enzymatically dissociated from the surface with trypsin and plated 2-3 days prior to the experiments in Lab-Tek two-well chambered coverglass slides (Nunc).

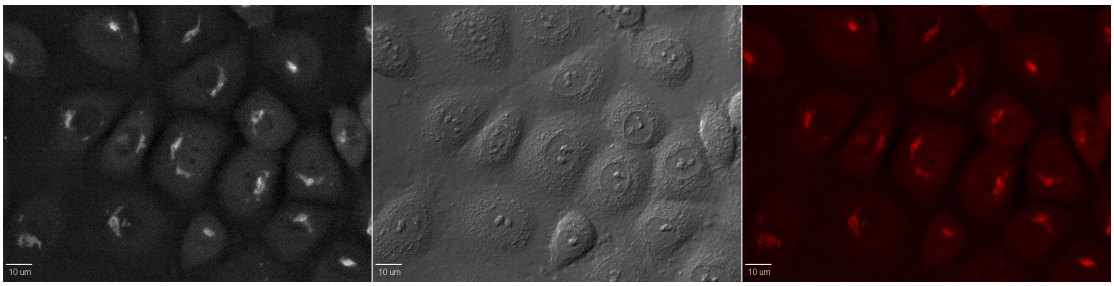
b. Fluorescence microscopy

Subcellular localization of Nile Red derivatives and BODIPY TR ceramide complexed to BSA was measured on living Clone 9 cells using a Stallion Dual Detector Imaging System consisting of an Axiovert 200M inverted fluorescence microscope, CoolSnap HQ digital cameras and Intelligent Imaging Innovations (3I) software. Digital images of Nile Red dyes, MITO tracker green labeled mitochondria, BODIPY TR ceramide complexed to BSA labeled Golgi, and LysoTracker Blue DND-22 were captured with a C-APO 63X/1.2 W CORR D=0.28M27 objective with the following filter sets: Exciter BP560/40, Dichroic FT 585, Emission BP 630/75 for Nile Red derivatives and BODIPY TR ceramide complexed to BSA; Exciter BP470/20, Dichroic FT 493, Emission BP 505-530 for MITO tracker green; and Exciter G 365, Dichroic FT 395, Emission BP 445/50 for LysoTracker Blue DND-22.

Clone 9 cells were incubated for 30 min to one hour at 37 ºC in ACAS with various concentration of probes (solution in PBS or DMSO). After the incubation period, the cells were washed several times with phosphate-buffered saline (PBS, pH 7.4) before imaging (in ACAS). To identify the subcellular localization of the probe, the cells were costained with MITO tracker green (0.1 μL/mL), a mitochondria marker, or LYSO tracker blue (100 nM), a lysozome marker. A parallel experiment with BODIPY TR ceramide complexed to BSA was also carried out to confirm localization of Nile Red derivatives **4** and **D** in Golgi.

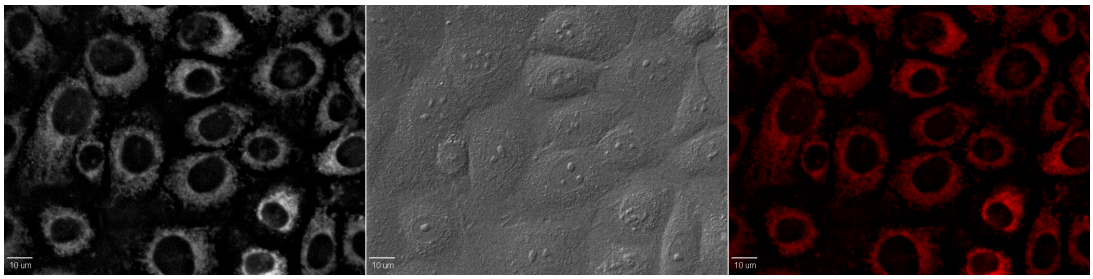
Nile Red D

Clone 9 cells were incubated with 2 μM of dye in ACAS medium for 30 min at 37 ºC. After the incubation period, the cells were washed several times with PBS and kept in ACAS (1 mL) for imaging.

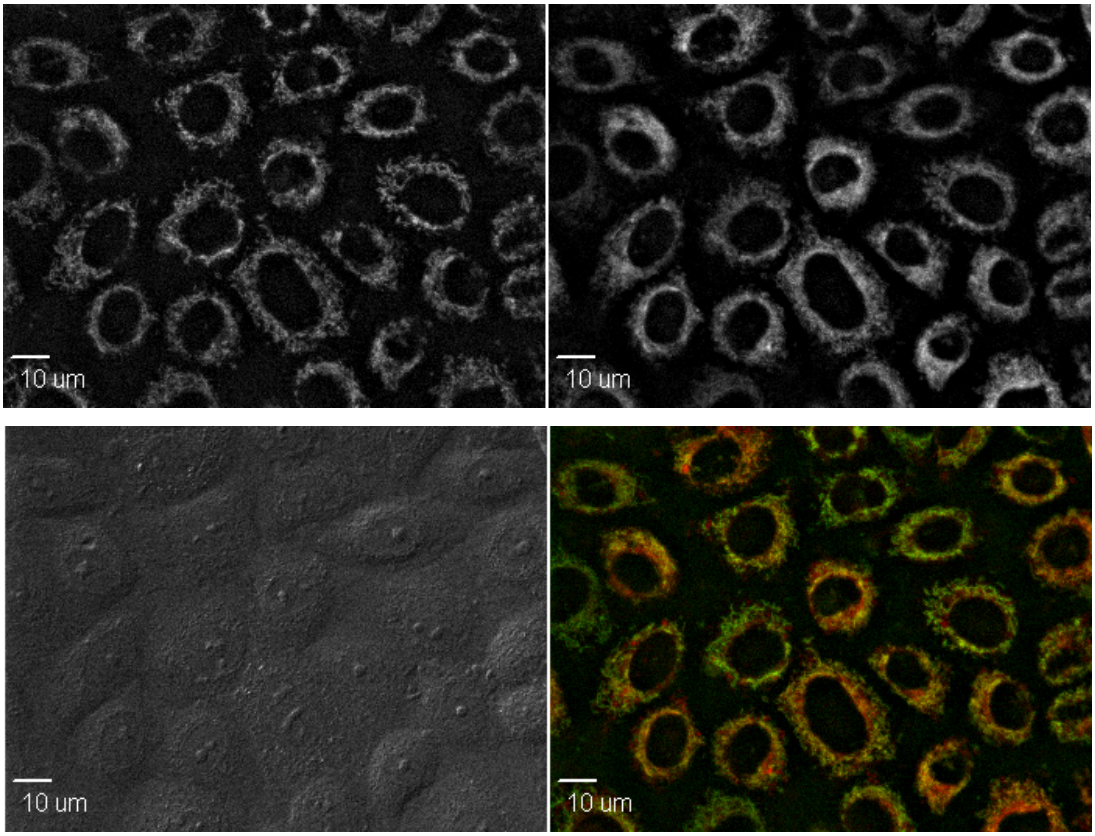


Nile Red derivative 3

Clone 9 cells were incubated with 10 μM of dye (stock soln in PBS) in ACAS medium for 30 min at 37 ºC. The cells were washed several times with PBS and kept in ACAS (1 mL) for imaging.

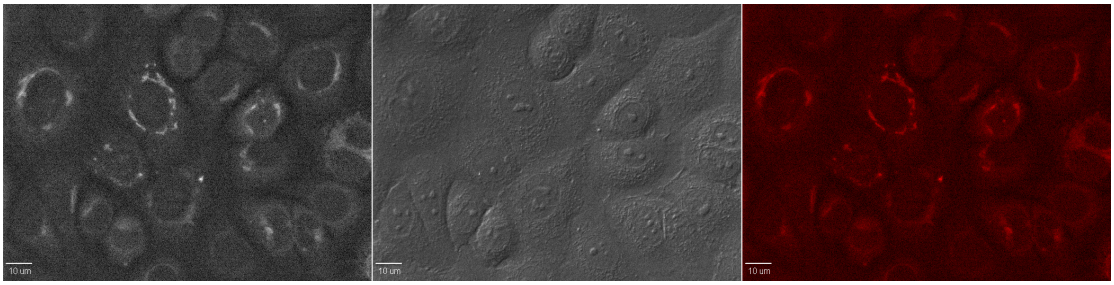


The cells were costained with MITO tracker green (10 nM, 5 min, 37 ºC).



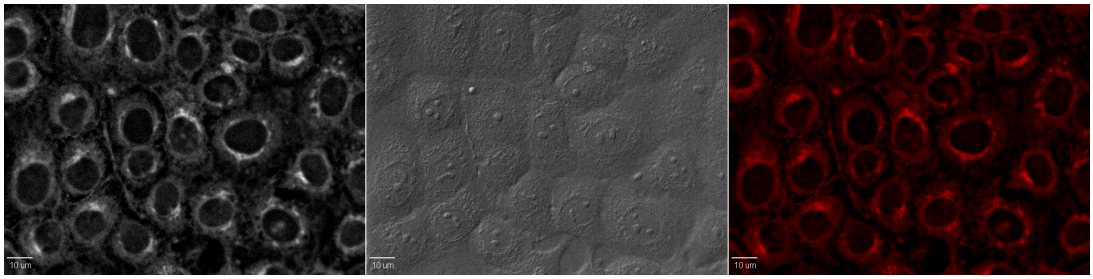
Nile Red derivative 4

Clone 9 cells were incubated with 10 μM of dye (stock soln in PBS) in ACAS medium for 30 min at 37 ºC. The cells were washed several times with PBS and kept in ACAS (1 mL) for imaging.



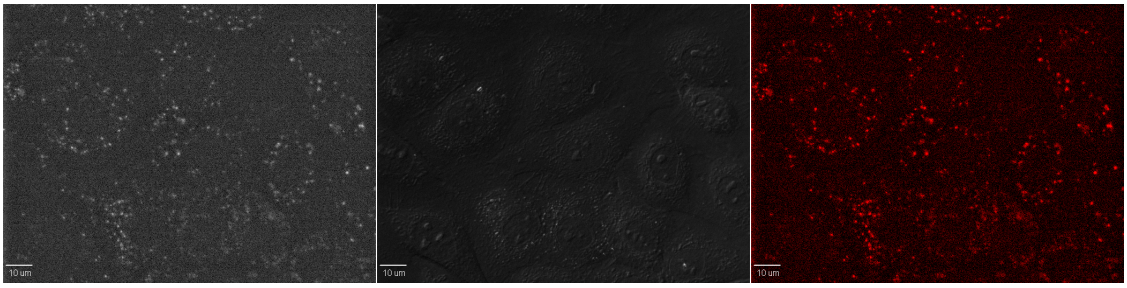
Golgi Marker

Clone 9 cells were incubated with 5 µM of ceramide-BSA for 30 minutes at 4 ºC in ACAS. The cells were then rinsed several time with ice cold ACAS medium and incubated in fresh ACAS medium for another 30 minutes at 37 ºC.

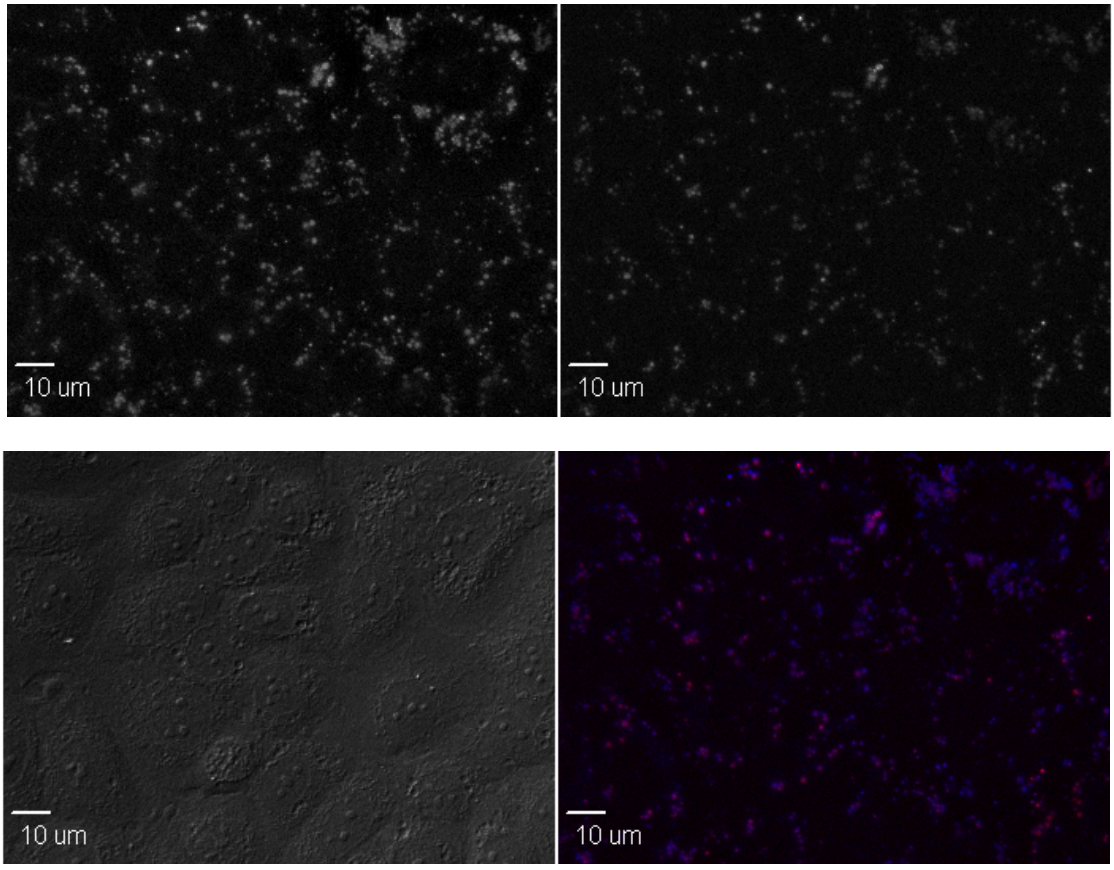


Nile Red derivative 5

When Clone 9 cells were incubated with derivative **5** (10 µM, 30 min, 37 ºC), no uptake could be observed. Therefore, the cells were incubated with a 1:3 dye:azoR$_8$ complex in ACAS medium for 60 min at 37 ºC. The complex dye **5** : azoR$_8$ was preformed by mixing both components at room temperature for 30 min. The cells were washed several times with PBS and kept in ACAS (1 mL) for imaging.



The cells were costained with LYSO tracker blue (100 nM, 5 min, 37 ºC).


10 um
10 um
10 um
10 um

Mechanism of Uptake of 3.

a. Fluorescence Imaging

Studies on the mechanism of uptake of Nile Red derivative **3** was done on a Zeiss 510 META NLO Multiphoton Microscope System consisting of an Axiovert 200 MOT microscope. Digital images of **3** and Fluo 4 were captured with a 40x / 1.3 oil objective with the following filter sets: Excitation 543 nm; Emission LP 560 and; Excitation 488 nm; Emission BP 500-550, respectively.

b. Measurement of the Mitochondrial Potential After Treatment with 3

Nile Red derivative 3 and JC-1

Clone 9 cells were plated on a 96 well plated and allowed to grow for 2 days before treatment. Thereafter, the cells were incubated for 30 min with 1 μM of Nile red **3** at 37 °C, washed, then co-incubated with JC1 (5 μg/mL) for another 30 min.

As a control of fully depolarized mitochondria, cells were first treated with Nile Red **3**, washed, then co-incubated with JC1 (5 μg/mL) and 100 μM CCCP for an additional 30 min at 37 °C.

Mitochondrial membrane polarization was also measured on untreated cells. Thus, Clone 9 cells were treated with JC1 (5 μg/mL) for 30 min at 37 °C.

Depolarization of the mitochondrial membrane was also induced on a set of untreated cells. Thus, Clone 9 cells were treated with 100 μM CCCP for 30 min at 37 °C.

The cells were then analyzed on a BioTek Synergy 4 Microplate Reader using the Gen5 software. Fluorescence emission from JC-1 was measured looking at Exc. 485 nm, Emis. 530 nm; and Exc. 535 nm, Emis. 590 nm. The membrane potential was then obtained from the ratio red to green fluorescence from JC-1.

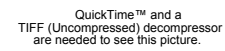
c. Intracellular calcium concentration

Nile Red derivative 3 and Fluo4

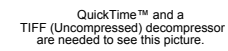
Clone 9 cells were incubated with 10 μM of **3** (stock soln in PBS) and 3 μM of Fluo4 in ACAS medium for 60 min at 37 ºC. The cells were washed several times with PBS and kept in ACAS (1 mL) for imaging.

In parallel, to estimate the intracellular concentration of calcium in Clone 9 cells (before treatment with **3**), Clone 9 cells were incubated with 3 μM of Fluo4 in ACAS medium for 60 min at 37 ºC.

*a. before treatment with **3**.*



*b. After treatment with **3***



Calcium level and ROS

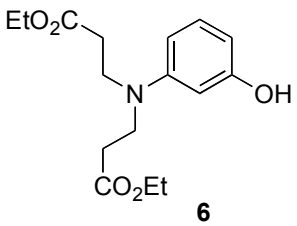
Clone 9 cells were plated on a 96 well plates 2 days before treatment in Ham's medium and incubated at 37 °C. Twelve hours before treatment, the medium was removed and replaced with ACAS.

Thereafter, the cells were treated respectively as follows, with:

- Nile Red **3** (10 µM; n = 16),
- Fluo 4 (3 µM; n = 16), to measure the intracellular concentration of Ca before treatment
- CM-H_2DCFDA (2 µg/mL, n = 16), to measure the intracellular concentration of ROS before treatment
- Nile Red **3** + Fluo 4 (10 µM 3 + 3 µM Fluo 4; n =16), to measure the intracellular concentration of Ca after treatment
- Nile Red **3** + CM-H_2DCFDA (10 µM 3 + 2 µg/mL CM-H_2DCFDA; n =16),), to measure the intracellular concentration of Ca after treatment
- Untreated cells (n = 8), to determine cell autofluorescence

and incubated at 37 °C for 1h. After the incubation period, the cells were washed and analyzed on a Biotek multiplate reader, Exc. 488 nm, Emis. 524 nm.

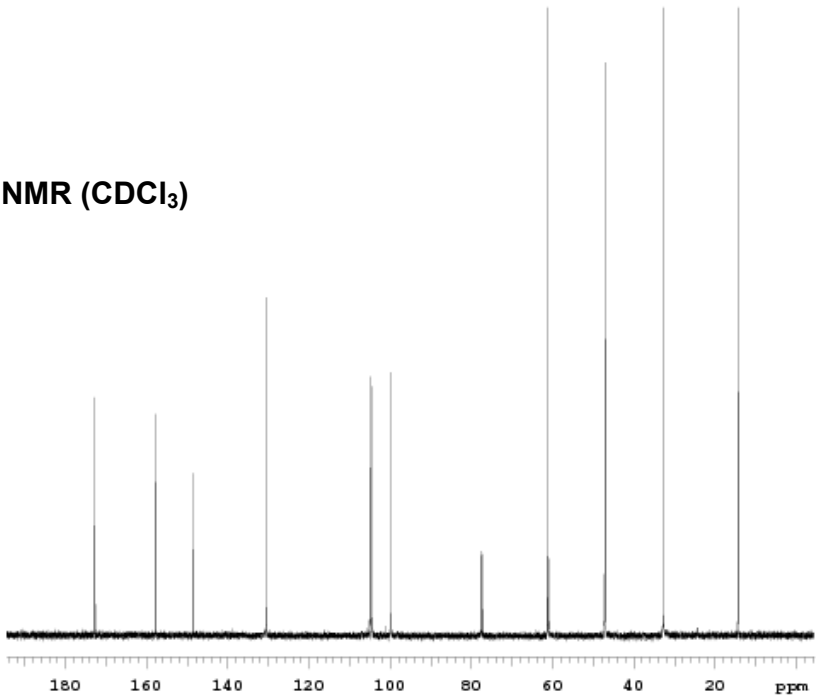
Copies of ^{1}H and ^{13}C Spectra

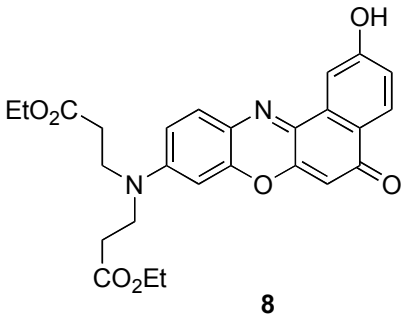


6

^{1}H NMR ($CDCl_3$)

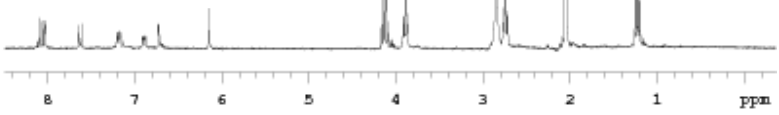
^{13}C NMR ($CDCl_3$)



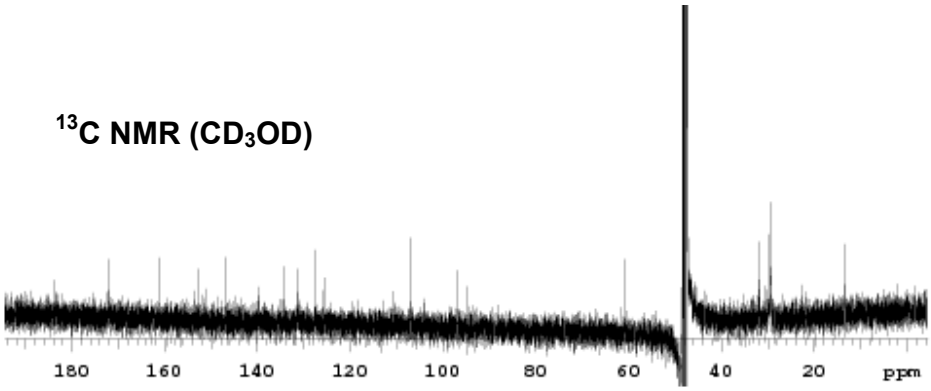


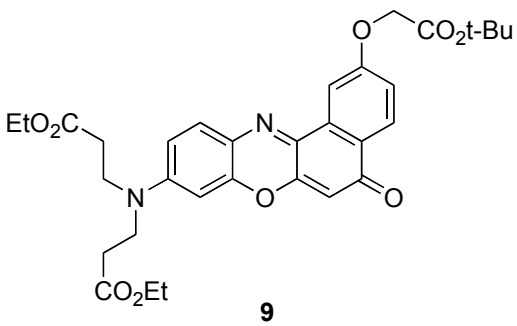
8

^{1}H NMR (acetone-d_6)



^{13}C NMR (CD_3OD)

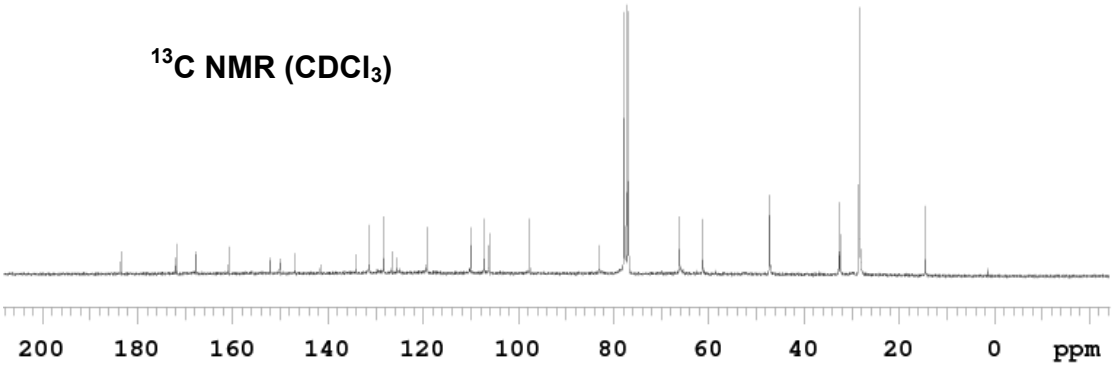


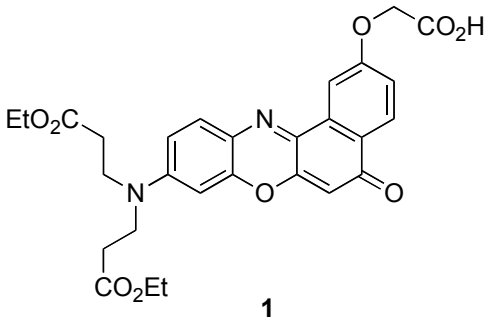


9

^{1}H NMR ($CDCl_3$)

^{13}C NMR ($CDCl_3$)

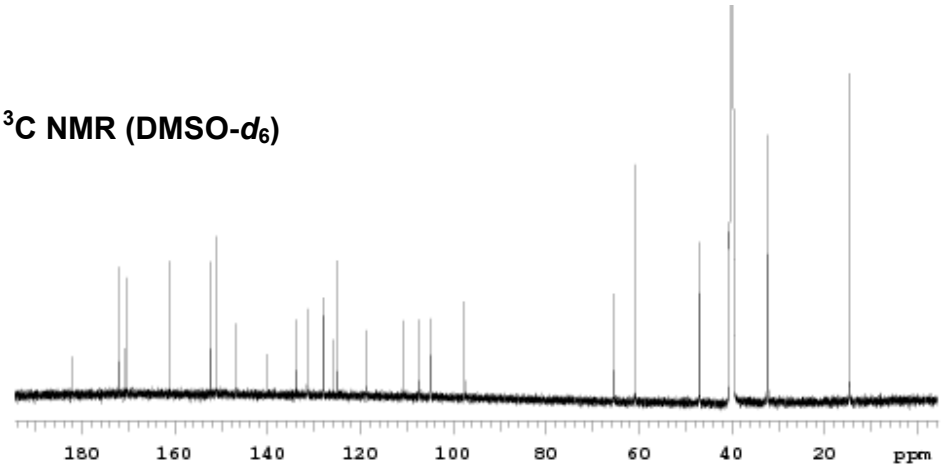


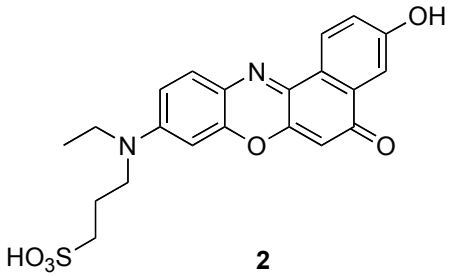


1

^{1}H NMR (DMSO-d_6)

^{13}C NMR (DMSO-d_6)

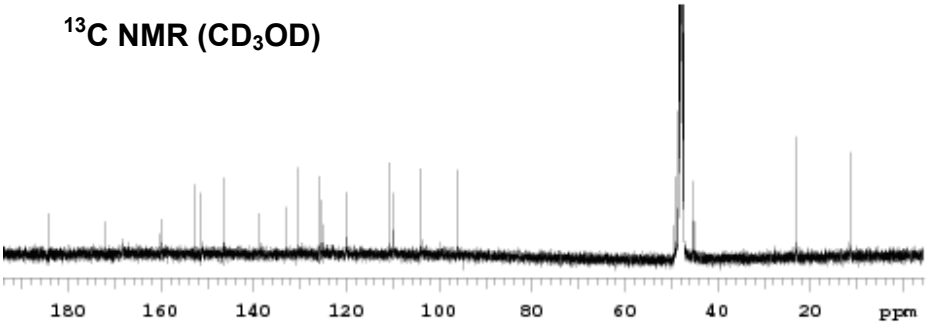


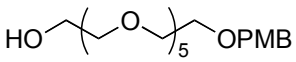


2

^{1}H NMR (CD_3OD)

^{13}C NMR (CD_3OD)





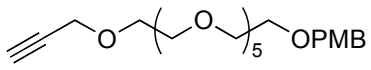
C

^{1}H NMR ($CDCl_3$)

QuickTime™ and a
decompressor
are needed to see this picture.

^{13}C NMR ($CDCl_3$)

QuickTime™ and a
decompressor
are needed to see this picture.



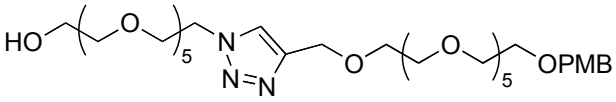
10

^{1}H NMR ($CDCl_3$)

QuickTime™ and a
decompressor
are needed to see this picture.

^{13}C NMR ($CDCl_3$)

QuickTime™ and a
decompressor
are needed to see this picture.



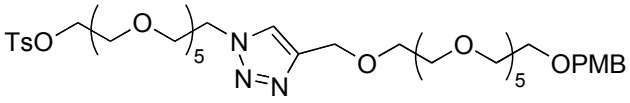
12

^{1}H NMR ($CDCl_3$)

QuickTime™ and a
decompressor
are needed to see this picture.

^{13}C NMR ($CDCl_3$)

QuickTime™ and a
decompressor
are needed to see this picture.



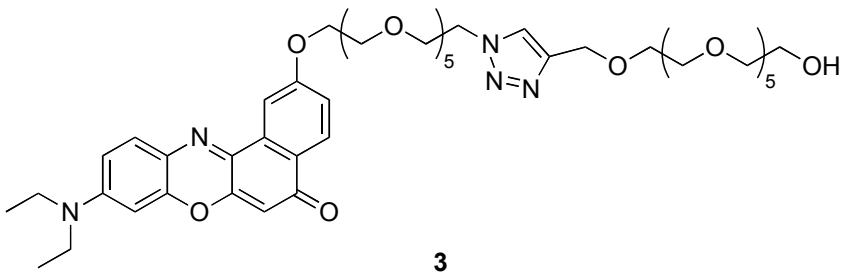
13

^{1}H NMR ($CDCl_3$)

QuickTime™ and a
decompressor
are needed to see this picture.

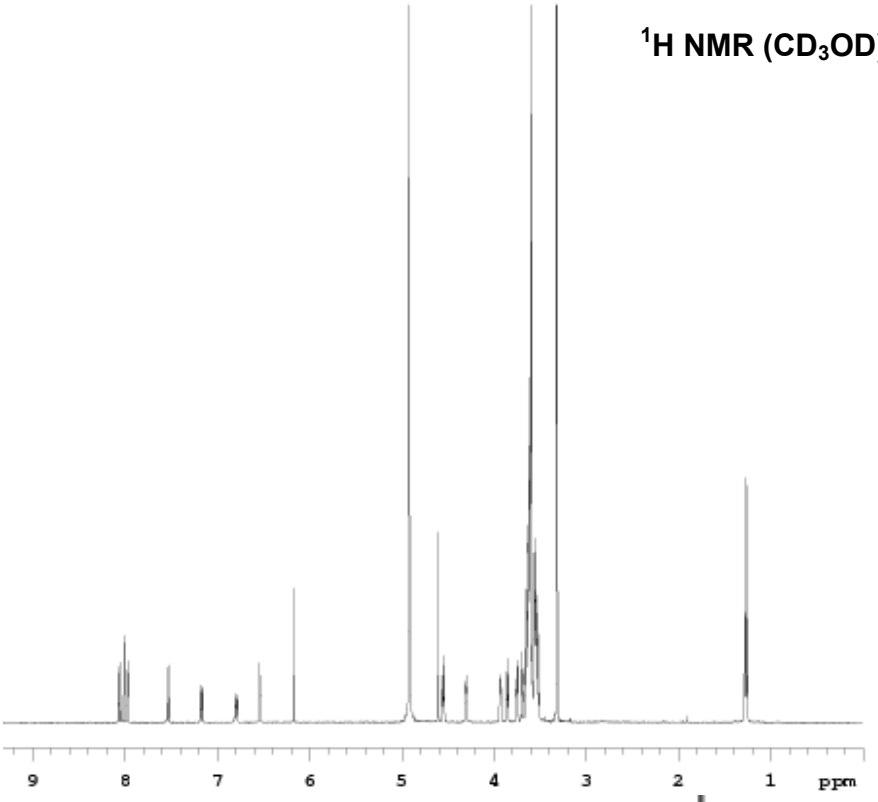
^{13}C NMR ($CDCl_3$)

QuickTime™ and a
decompressor
are needed to see this picture.

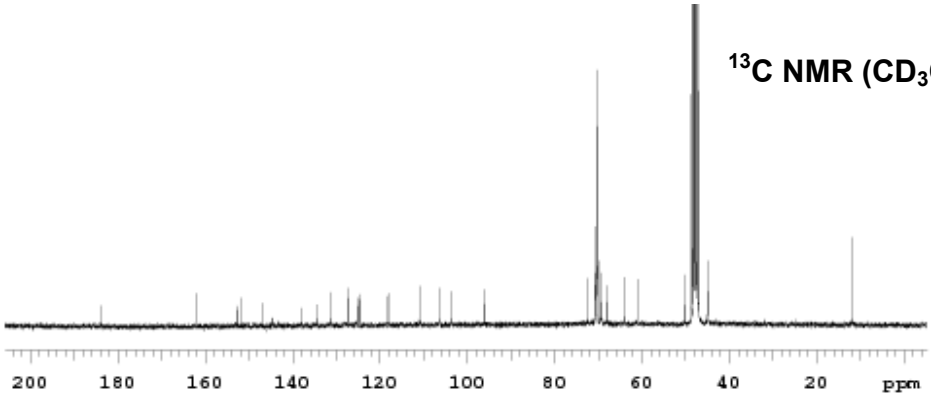


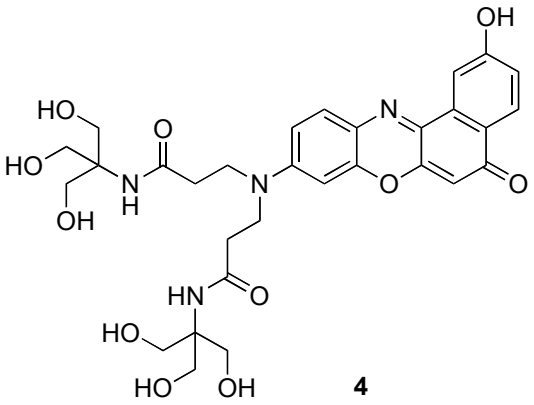
3

^{1}H NMR (CD_3OD)



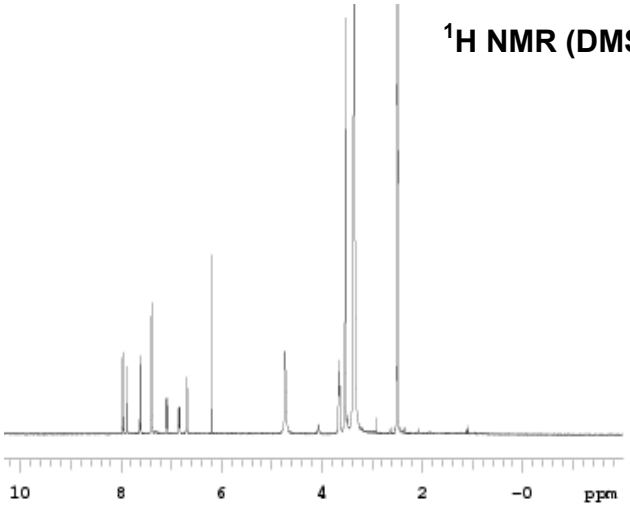
^{13}C NMR (CD_3OD)



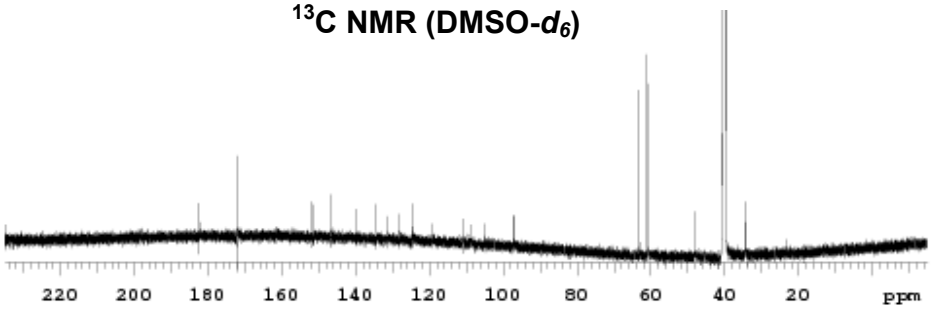


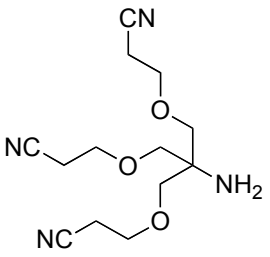
4

^{1}H NMR (DMSO-d_6)

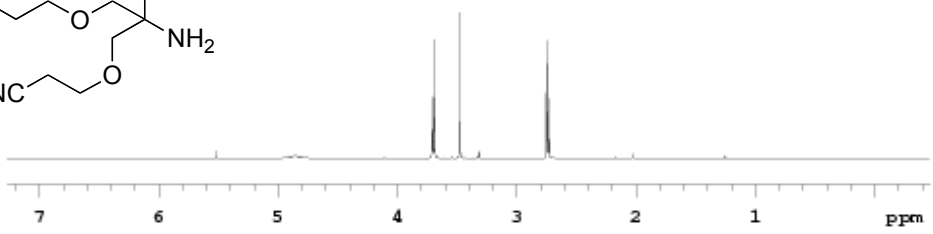


^{13}C NMR (DMSO-d_6)

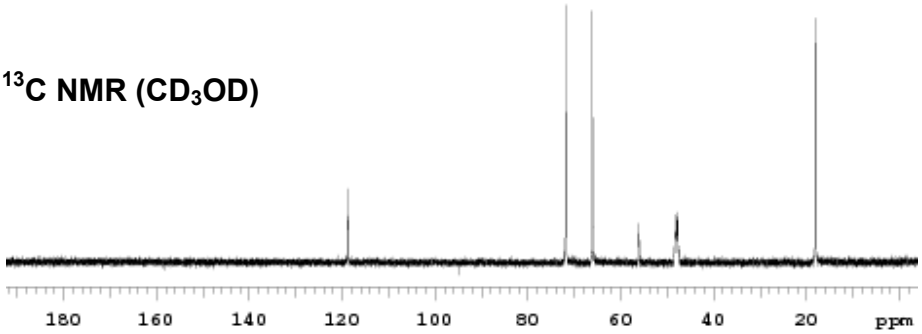


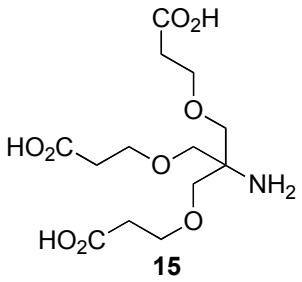


^{1}H NMR (CD_3OD)



^{13}C NMR (CD_3OD)

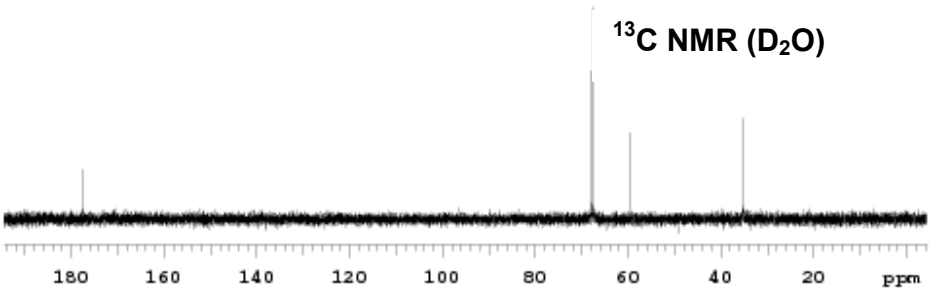


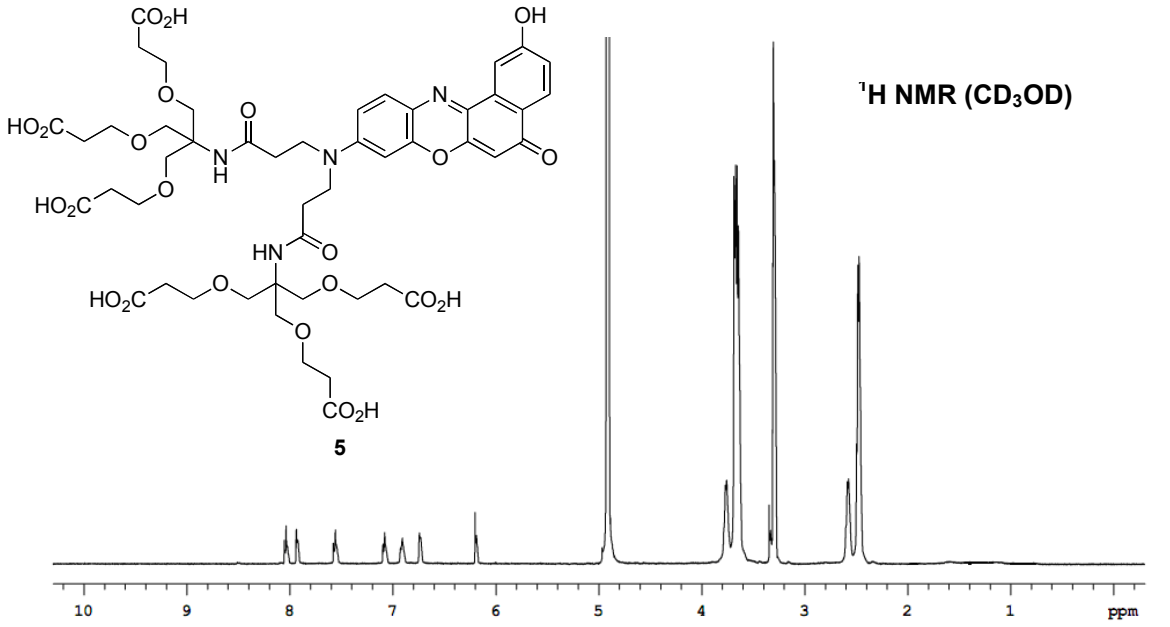


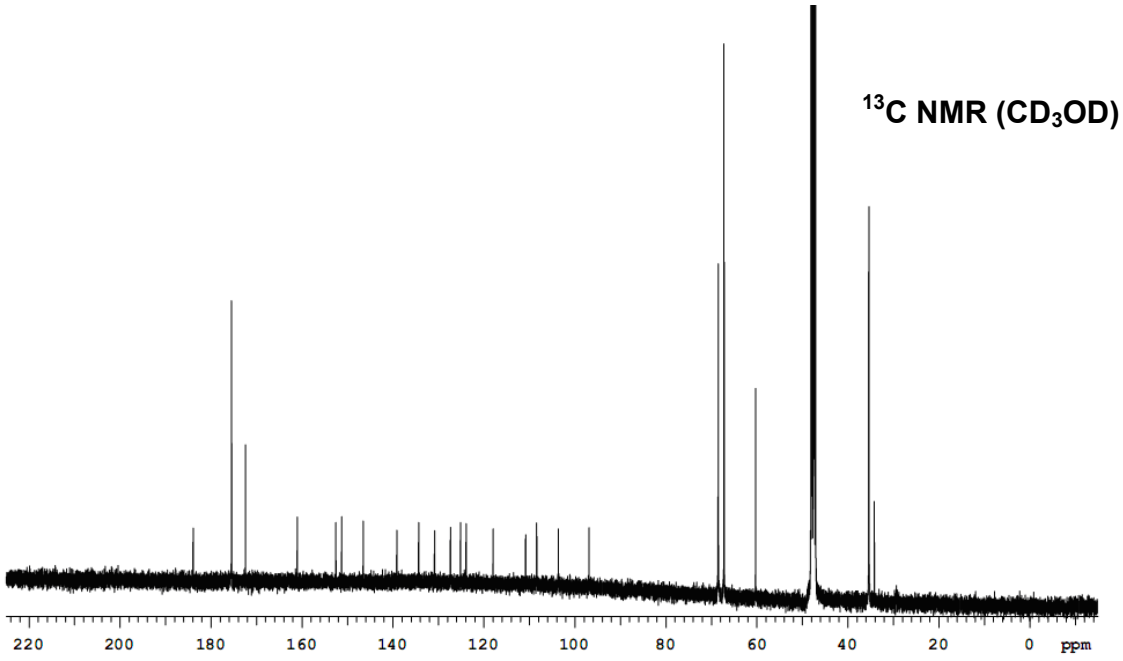
15

^{1}H NMR (D_2O)

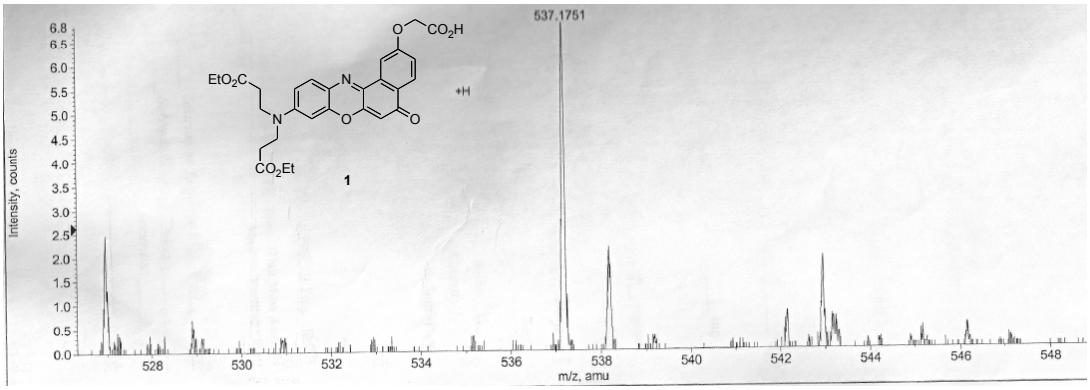
^{13}C NMR (D_2O)

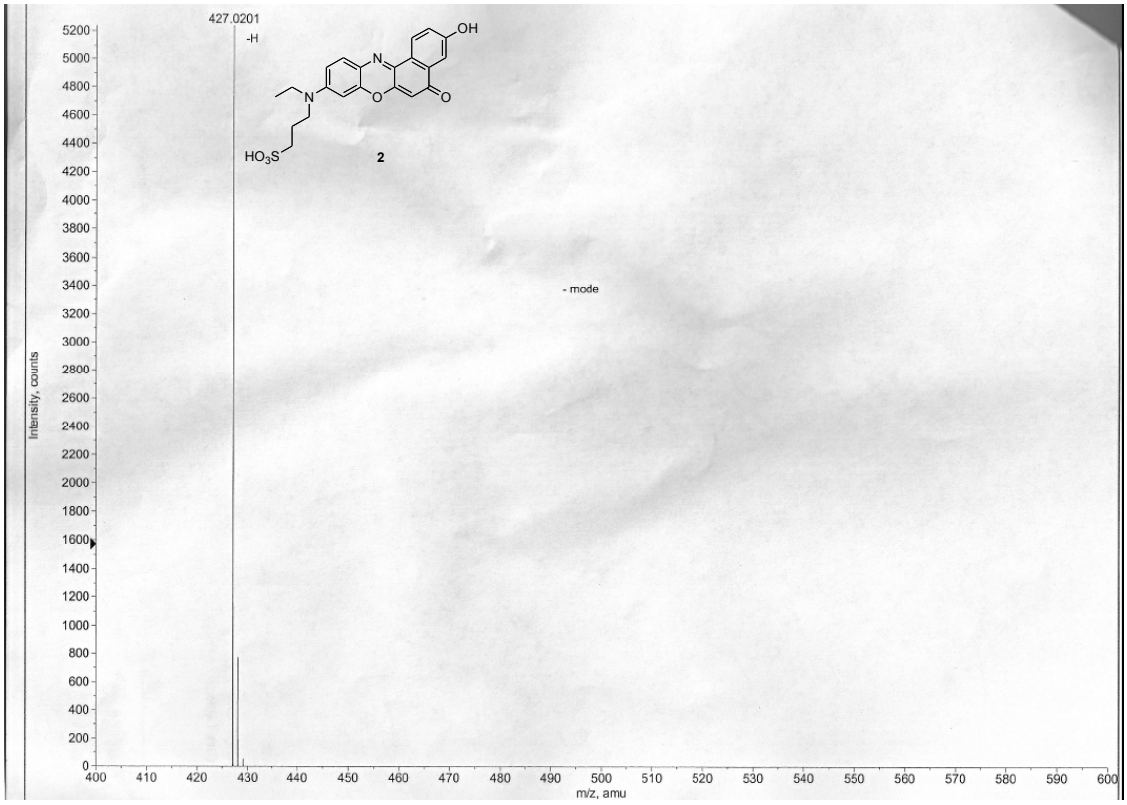


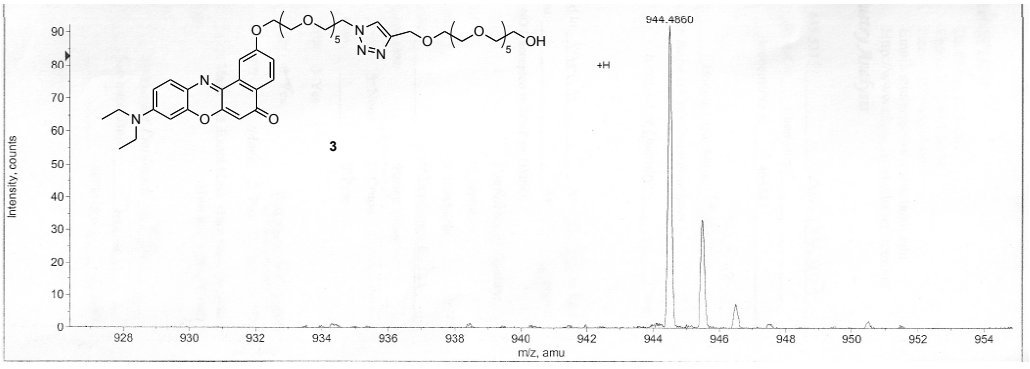

CO2H
OH
HO2C
O
N
H
HN
CO2H
5
1H NMR (CD3OD)
10
9
8
7
6
5
4
3
2
1
ppm

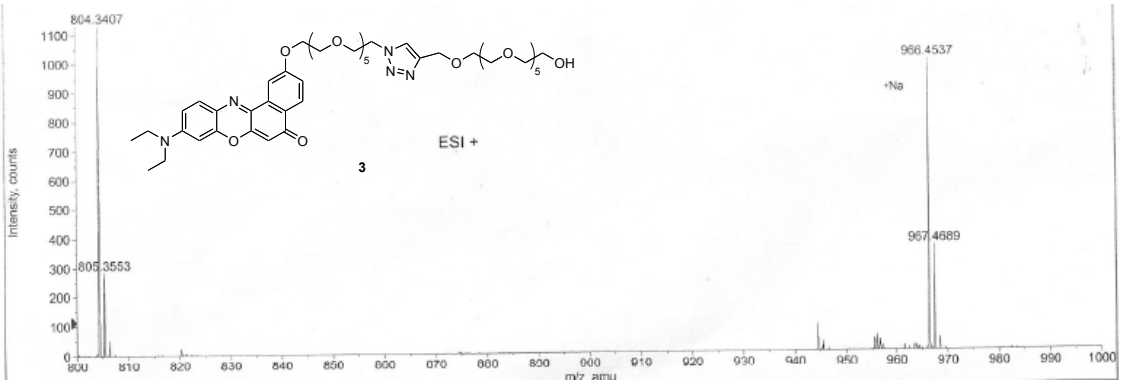

13C NMR (CD3OD)
220
200
180
160
140
120
100
80
60
40
20
0
ppm

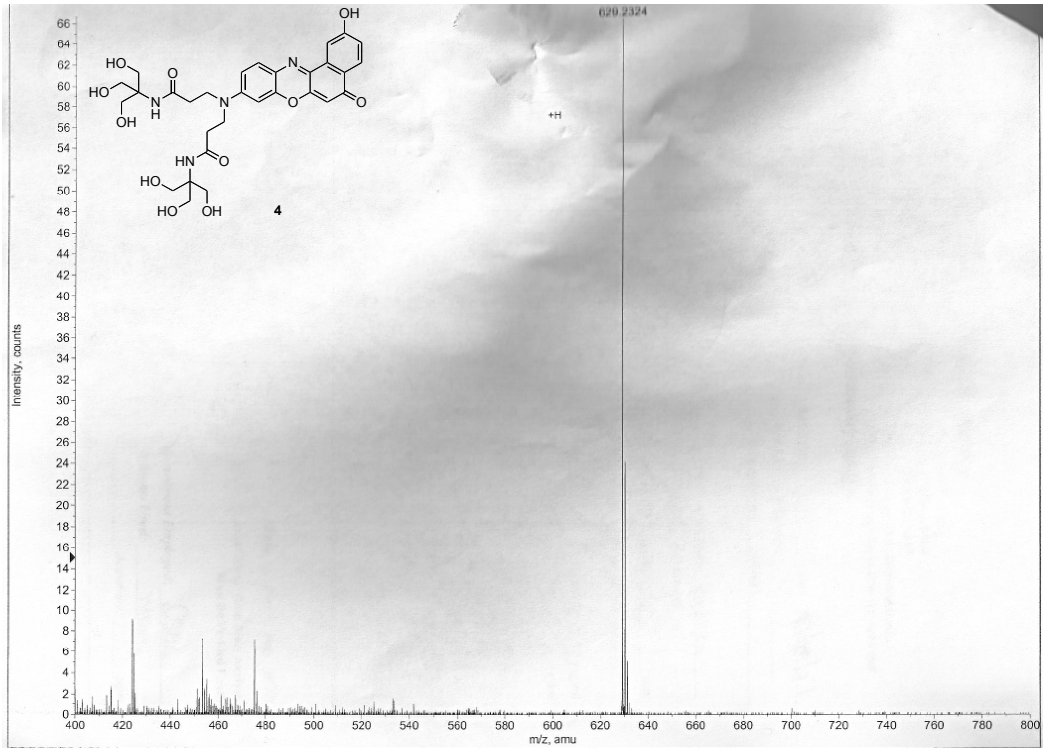
Copies of Mass Spectra for Dyes 1-5

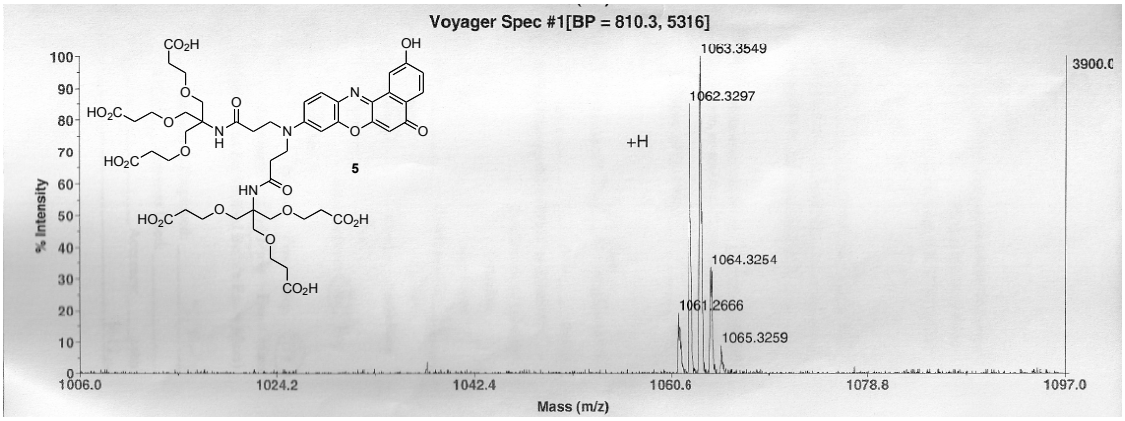







804.3407
966.4537
+Na
967.4689
805.3553
ESI +
3
Intensity, counts
m/z, amu


620.2324
+H
4
Intensity, counts
m/z, amu


Voyager Spec #1[BP = 810.3, 5316]
1063.3549
1062.3297
+H
1064.3254
1061.2666
1065.3259
5
% Intensity
Mass (m/z)

References

(1) Rurack, K. *Springer Series on Fluorescence* **2008**, *5*, 101-145.
(2) Williams, A. T. R.; Winfield, S. A.; Miller, J. N. *Analyst* **1983**, *108*, 1067-71.
(3) Jose, J.; Burgess, K. *J. Org. Chem.* **2006**, *71*, 7835-7839.