Optimized pH-Responsive Cyanine Fluorochromes for Detection of Acidic Environments

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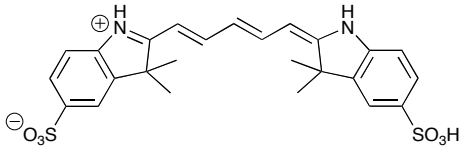
General Considerations. All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI), TCI America (Portland, OR) or Acros Organics USA (Morris Plains, NJ) and were used as received except 2,3,3-trimethylindolenine-5-sulfonic acid, which was prepared according to the literature.[1] All solvents were purchased from Sigma-Aldrich. High performance liquid chromatography (HPLC) was performed on a Waters Alliance 2695 instrument equipped with a 2996 photodiode array detector. All HPLC data were collected with a Grace-Vydac column (218TP5210) using a 15 min gradient from 0 to 100% buffer B and a flow rate of 0.3 mL/min, unless noted otherwise. Buffer A consisted of water and 0.1% TFA, whereas buffer B was composed of 90% acetonitrile, 9.9% water and 0.1% TFA. Nuclear magnetic resonance spectra were acquired on a Bruker DPX-400 spectrometer at ambient temperature and referenced to tetramethylsilane (TMS) as an internal standard. Absorption spectra were collected on a Varian Cary 50-Bio UV/visible spectrophotometer (Palo Alto, CA). All extinction coefficient measurements in 50 mM glycine buffer, pH 3.0, were performed in triplicate using dyes, which were purified by reverse phase C-18 and cation exchange chromatography. For determination of the extinction coefficients, fresh stock solution of the dyes were prepared for each trial by dissolution of 2-3 mg portions of the dyes, weighed on a Mettler AT201 analytical balance with an error of ±0.01 mg, in glycine buffer, pH 3.0, using a 10 mL volumetric flask. Typical standard deviations for the extinction coefficient measurements were less than 5%. Fluorescence data were collected on a Horiba Jobin Yvon Fluorolog-3 spectrofluorometer (Edison, NJ) and were corrected for the detector sensitivity. Quantum yield measurements of the protonated forms of the dyes in 50 mM glycine, pH 3.0, performed in triplicate following published procedures,[2] were collected using dilute samples (absorbance $\leq$ 0.10 OD) with Cy 5 ($\Phi = 0.27$)[1] in phosphate buffered saline as a standard. All samples for quantum yield determinations were excited at 635 nm. All pK_a titrations were collected on dilute dye solutions (absorbance using $\leq$ 0.10 OD) using integrated fluorescence emission data in a citric acid/phosphate buffer system in which the solution pH was systematically lowered by addition of aliquots of a 5 M citric acid stock solution to the dyes in 50 mM phosphate buffer with an initial pH of 9.0. High-resolution electrospray ionization (ESI) mass spectra were collected on a Bruker Daltonics APEXII 3 T fourier transform mass spectrometer in the Department of Chemistry Instrumentation Facility (DCIF) at the Massachusetts Institute of Technology.

References.

(1) Mujumdar, R. B.; Ernst, L. A.; Mujumdar, S. R.; Lewis, C. J.; Waggoner, A. S. *Bioconjugate Chem.* **1993**, *4*, 105-11.

(2) Demas, J. N.; Crosby, G. A. *J. Phys. Chem.* **1971**, *75*, 991-1024.

Synthesis of compound 1.



To a solution of 2,3,3-trimethylindolenine-5-sulfonic acid (1.20 g, 5.0 mmol) and malondialdehyde tetrabutylammonium salt (780 mg, 2.5 mmol) in 10 mL of MeOH was added *p*-toluenesulfonic acid monohydrate (475 mg, 2.5 mmol). This solution was sealed in a thick-walled glass pressure reactor and heated to 60-70 °C for 2 h. After cooling, the dark blue reaction mixture was dried by rotary evaporation and the crude product was taken up in water and passed through Dowex 50WX8 hydrogen form cation exchange resin to separate the product from the tetrabutylammonium counterions. Following solvent removal by rotary evaporation, the blue solid was subjected to reverse-phase C-18 column chromatography (Isolute 70 g RPC18 cartridge) eluting with increasing concentrations of CH_3CN (5, 10, and 15%) in H_2O to give **1** (74 mg, 5.7%) as a dark blue solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.96 (broad s, 2H), 7.82 (s, 2H), 7.59 (d, 2H, J = 8.1 Hz), 7.14 (d, 2H, J = 8.0 Hz), 6.46 (t, 1H, J = 12.3 Hz), 6.11 (broad d, 2H, J = 13.7 Hz), 1.50 (s, 12H). HRMS-ESI $[M+H]^+$: calcd for $C_{25}H_{27}N_2O_6S_2^+$ 515.130, found 515.1298.

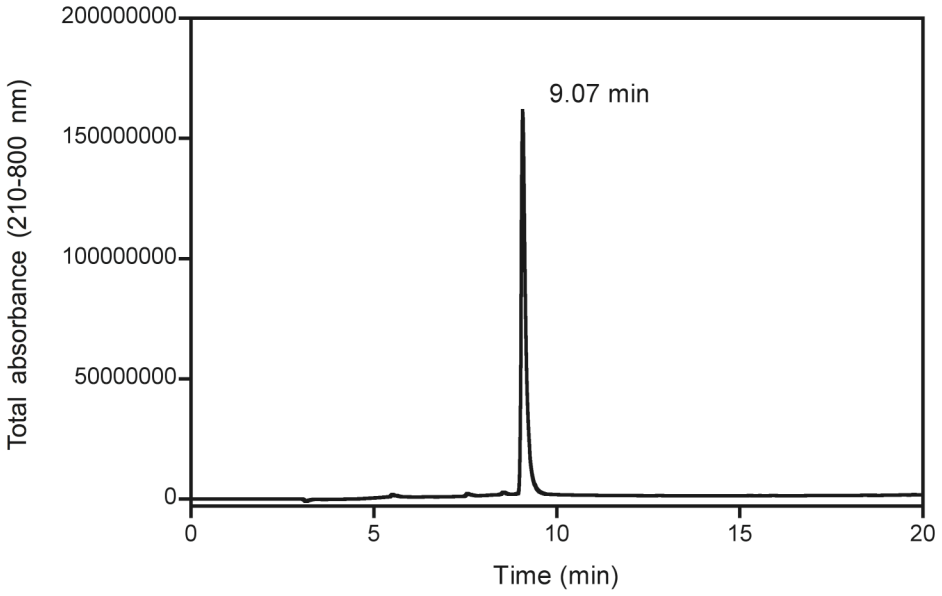


Figure S1. HPLC trace of compound **1** using a Grace-Vydac column (218TP54), and a gradient from 0 to 100% buffer B over 20 min with a flow rate of 1 mL/min.

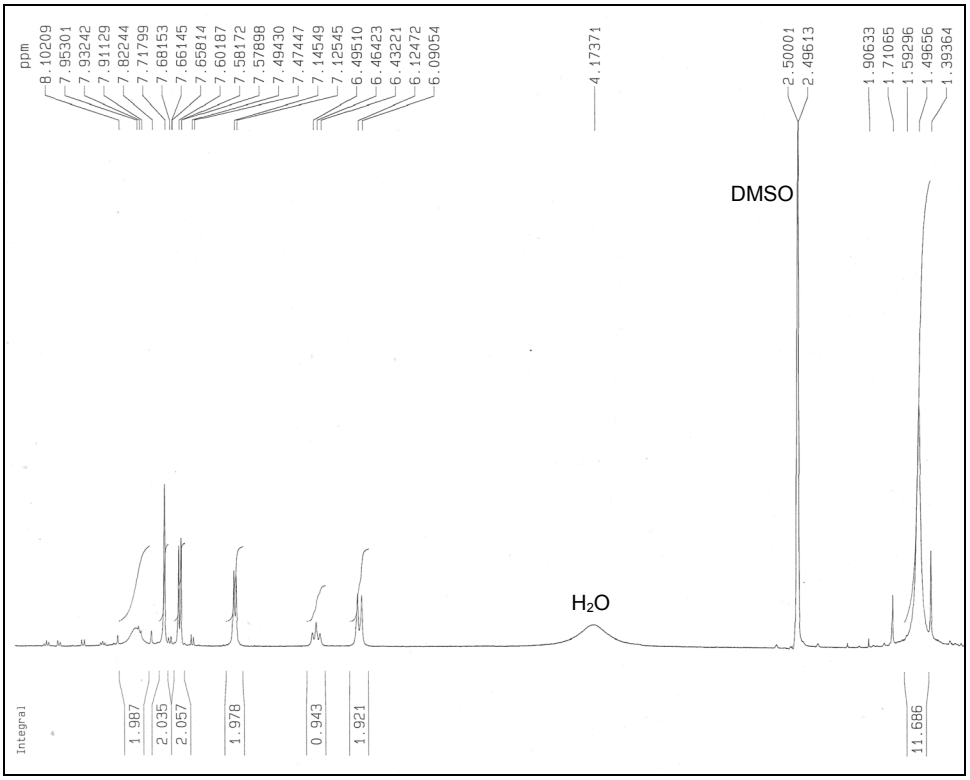
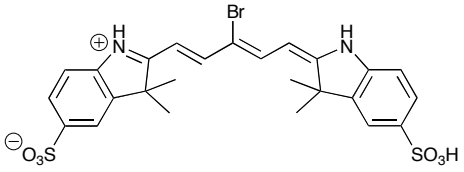


Figure S2. ^{1}H NMR Spectrum of compound **1** in DMSO-d_6**.**

Synthesis of compound 2.



To a solution of 2,3,3-trimethylindolenine-5-sulfonic acid (1.20 g, 5.0 mmol) was added bromomalonaldehyde (378 mg, 2.5 mmol) in 10 mL of MeOH. This solution was sealed in a thick-walled glass pressure reactor and heated to 60-70 °C for 6 h. After cooling, the dark blue solution was dried by rotary evaporation and purified by reverse-phase C-18 column chromatography (Isolute 70 g RPC18 cartridge) eluting with increasing concentrations of CH_3CN (5,10, and 15%) in H_2O. The purified product was then subjected to cation exchange chromatrography (Dowex 50WX8 hydrogen form resin) yielding pure **2** (195 mg, 13.1%) after lyophylization. ^{1}H NMR (400 MHz, DMSO-d_6): δ

8.22 (broad d, 2H, J = 12.3 Hz), 7.80 (s, 2H), 7.63 (d, 2H, J = 8.1 Hz), 7.19 (d, 2H, J = 8.1 Hz), 6.24 (broad d, 2H, J = 11.6 Hz), 1.61 (s, 12H). HRMS-ESI $[M+H]^+$: calcd for $C_{25}H_{26}BrN_2O_6S_2^+$ 593.0411, found 593.0409.

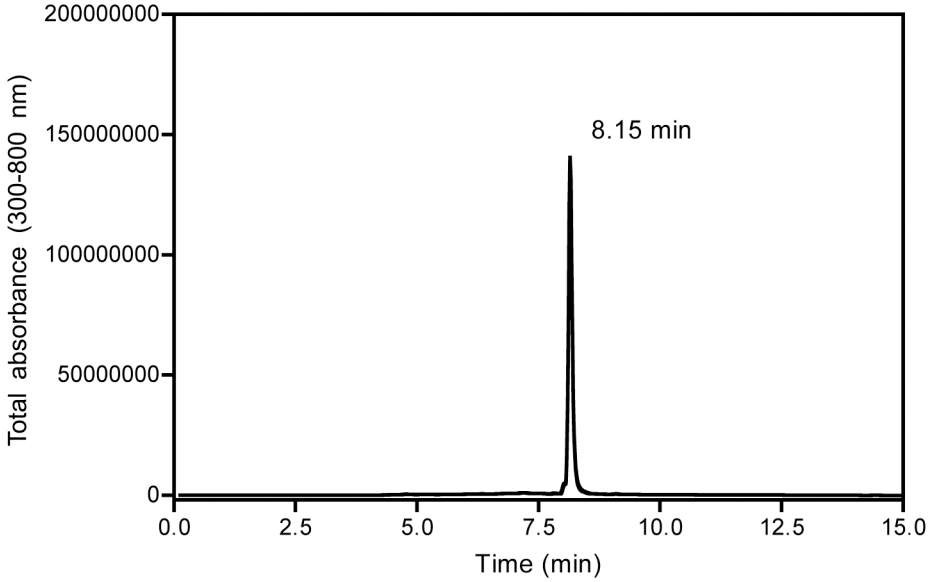


Figure S3. HPLC trace of compound **2**.

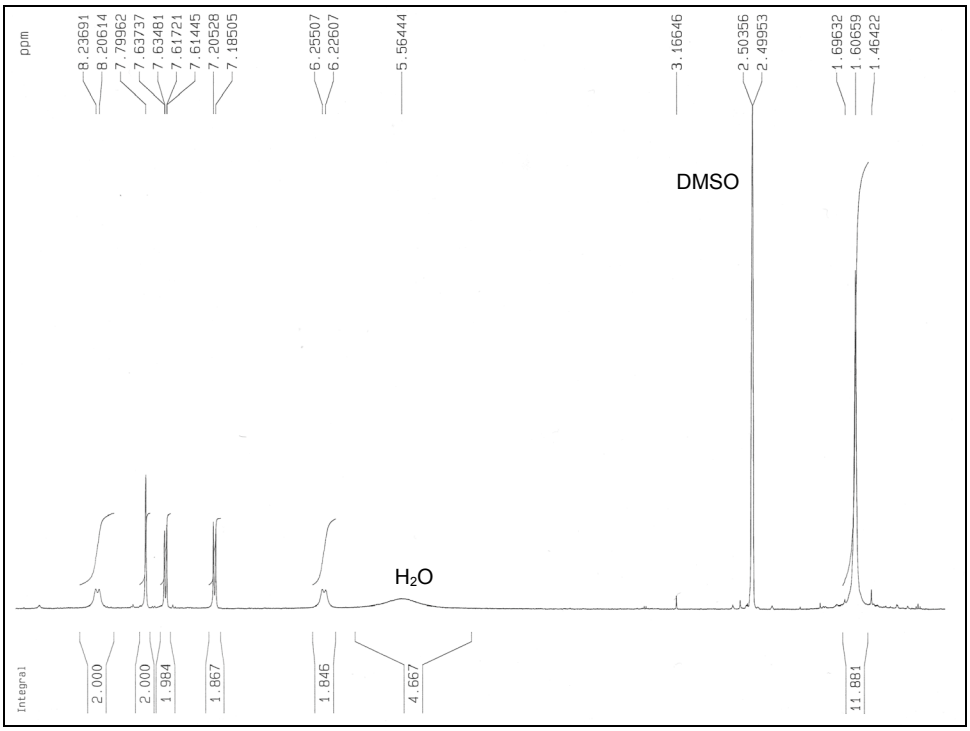
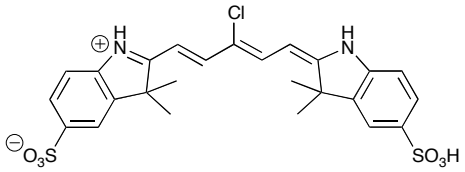


Figure S4. ^{1}H NMR Spectrum of compound **2** in DMSO-d_6.

Synthesis of compound 3.



To a solution of 2,3,3-trimethylindolenine-5-sulfonic acid (1.20 g, 5.0 mmol) was added chloromalonaldehyde (266 mg, 2.5 mmol) in 10 mL of MeOH. This solution was sealed in a thick-walled glass pressure reactor and heated to 60-70 °C for 6 h. After cooling, the dark blue solution was dried by rotary evaporation and purified by reverse-phase C-18 column chromatography (Isolute 70 g RPC18 cartridge) eluting with increasing concentrations of CH_3CN (10, 20, and 30%) in H_2O. The purified product was then subjected to cation exchange chromatrography (Dowex 50WX8 hydrogen form resin) yielding pure **3** (188 mg, 13.7%) after lyophylization. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.15 (broad d, 2H, J = 11.5 Hz), 7.80 (s, 2H), 7.63 (d, 2H, J = 8.1 Hz), 7.20 (d, 2H, J = 8.1 Hz), 6.23 (broad d, 2H, J = 12.9 Hz), 1.60 (s, 12H). HRMS-ESI $[M+H]^+$: calcd for $C_{25}H_{26}ClN_2O_6S_2^+$ 549.0915, found 549.0908.

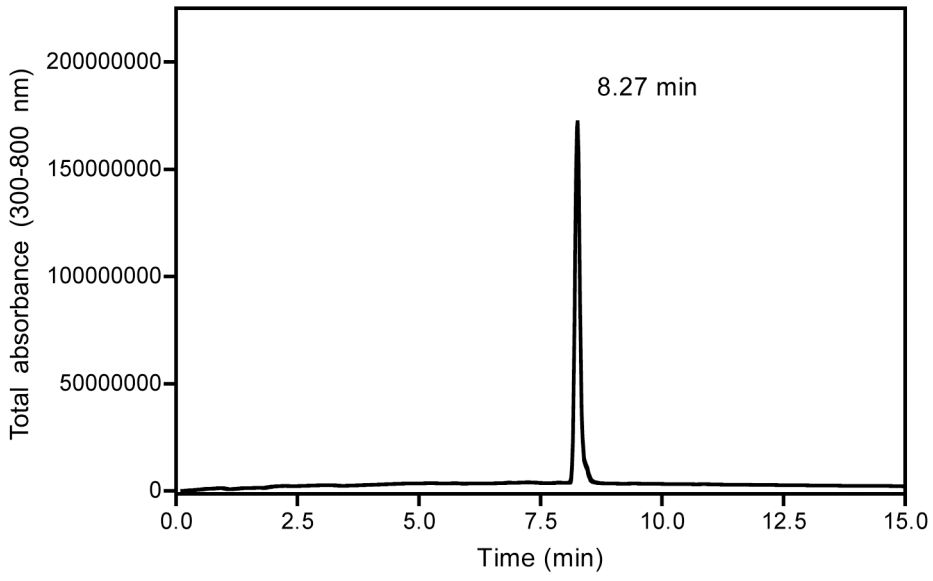


Figure S5. HPLC trace of compound **3**.

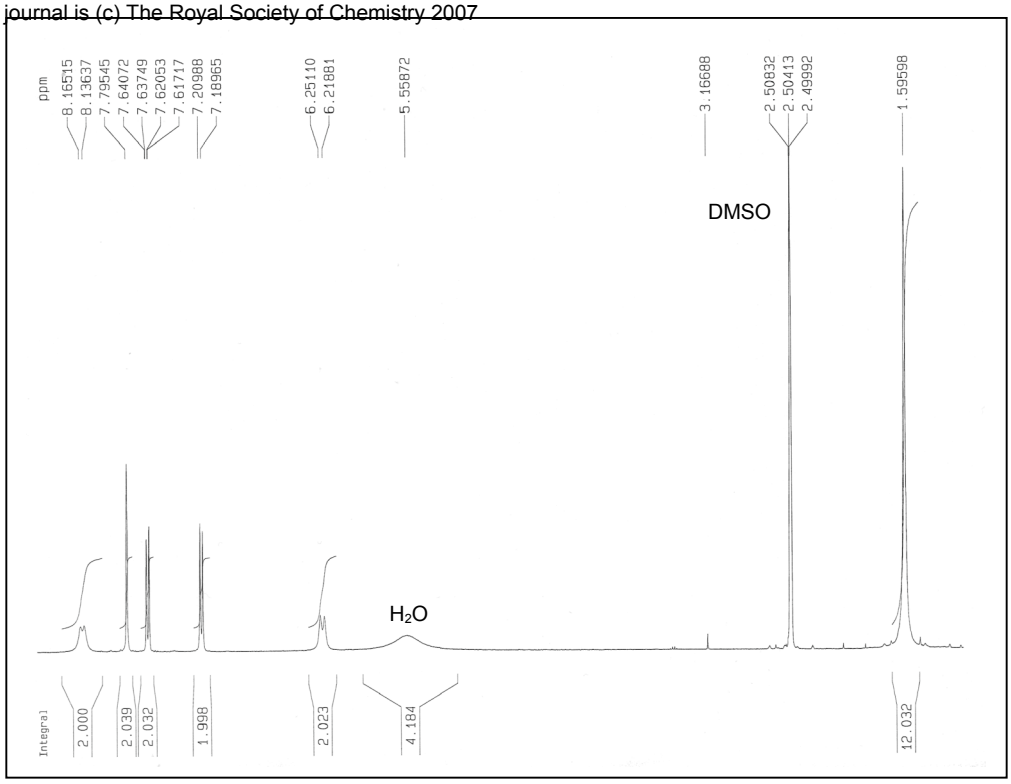


Figure S6. ^{1}H NMR Spectrum of compound **3** in DMSO-d_6.

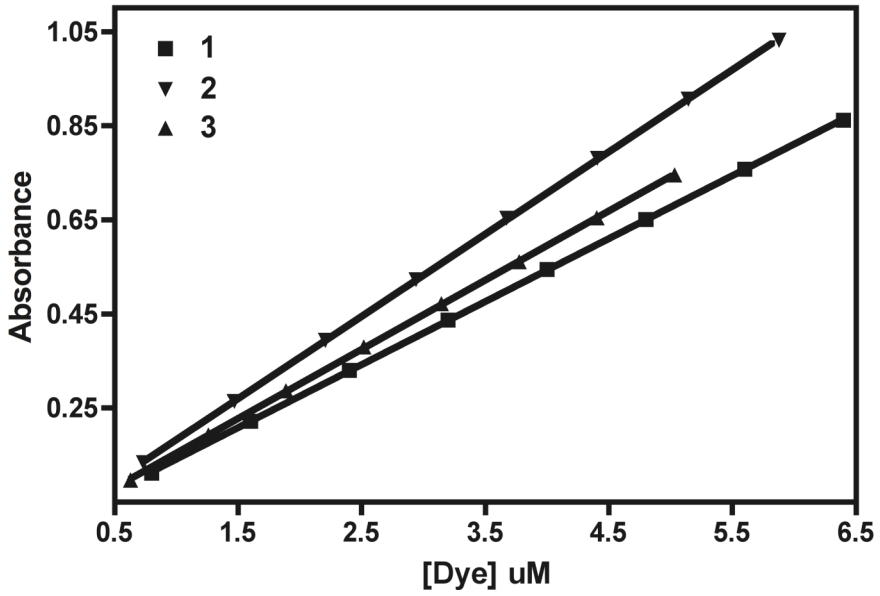


Figure S7. Absorbance titrations of dyes **1-3** at their respective absorption maxima in 50 mM glycine buffer at pH 3.0. The linear Beer-Lambert traces indicate no dye aggregation occurs in the concentration range investigated.