Supporting Information

Near-infrared Fluorescent pH-sensitive Probes via Unexpected Barbituric Acid Mediated Synthesis

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Experimental Section

General Considerations. All chemicals were purchased from commercial sources and were used without further purification. All reactions were routinely performed under argon. NMR spectra were recorded with either Varian Inova-600 (Varian Assoc., Palo Alto, CA) or a Varian Unity+300 spectrometer and the data were processed with VNMR software. Proton and carbon chemical shifts were measured in parts per million (ppm) downfield from an internal TMS standard. Proton spectra were obtained in DMSO-*d*6 with a 7200-Hz spectral width collected into 32K data points. Carbon spectra were obtained with a 33000-Hz spectral width collected into 64k data points. COSY experiment was obtained with a 9.2 μs π/2 proton pulse width. A data matrix with 2048 complex point in F2 and 512 real points in F1 dimension was collected with 16 transients per t1 increment. The proton-detected HMQC and HMBC spectra were recorded using a 0.3 s 1H-13C nulling period and 55 ms delay, respectively. The 90^{o} 1H pulse width was 9.2 μs and the 90^{o} 13C pulse width was 12.5 μs. Phase sensitive HMQC spectra were obtained by employing the Hypercomplex method while HMBC was processed in absolute mode. A 2 x 256 x 2048 data matrix with 32scans per t1 value was collected. Gaussian line broadening was used in weighting both the t2 and the t1 dimension. After two-dimensional Fourier transform, the spectra resulted in 512 x 2048 data points, which were phase and baseline corrected in both dimensions. The absorption spectra were recorded on Beckman Coulter DU640 spectrophotometer (Fullerton, CA, USA) and fluorescence spectra were recorded on Fluorolog III fluoremeter (Horiba Jobin Yvon, Edison, NJ, USA). The molar extinction coefficient was obtained using Beer's law at 0.1-0.6 μM concentration of the dye. The relative fluorescence quantum yield was determined using the equation:

$$\Phi_{F(x)} = (A_s/A_x)\,(F_x/F_s)\,(n_x/n_s)^2\Phi_{F(s)}$$

where $\Phi_{F(X)}$ is the fluorescence quantum yield, *A* is the absorbance, *F* is the area under the emission curve, *n* is the refractive index of the solvents using in measurement, and the subscripts *s* and *x* represent the standard and unknown, respectively. Indocyanine green (ICG) was used as a reference standard, which has the value of 0.078 in MeOH.[1]

The compounds were dissolved in methanol (0.2 mL) and added to water (100 mL) under Ar atmosphere to exclude CO2 interference with the measurements. The solution was basified with dilute aq. NaOH and the desired pH was attained by titrating the solution with aqueous HCl, or acidification of the solution followed by titration with NaOH at relatively low ionic strengths (I = 0.02-0.05 M). The pH of the solution was continuously measured using Accumet pH meter AB15 (Fisher Sci.) At each pH, point absorption and fluorescent measurements were determined. The pKa values were calculated from the sigmoidal dose-response curve fit implemented in software Prism 5.0 (GraphPad Software Inc., La Jolla, CA).

General Procedures for the synthesis of cyanine dyes 2a-2c. Precursor chloro dye **1a** was prepared according to the published procedure.[2]

Method A. To a solution of sodium hydride (33 mg, 1.4 mmol) in DMF (3 mL) under argon was added a solution of barbituric acid (155 mg, 1.2 mmol) in DMF (3 mL) and stirred at rt for 1h. The resultant mixture was then added a solution of **1a** or **1b** (0.37 mmol) in DMF (10mL) and stirred at rt for 24h. Workup and reverse phase column chromatography (water/AcCN) afforded **2a** or **2b** as blue solid.

Method B. A mixture of **1a** (0.10 g, 0.12 mmol) and barbituric acid derivative (0.86 mmol) was stirred at room temperature in the presence of TEA (0.7 mL) in AcCN (10 mL) for 24 hrs. The reaction progress was monitored by visible/near-infrared spectroscopy for aliquots diluted with

methanol until absorption of the starting chloro cyanine disappeared. The product was precipitated from the reaction mixture upon addition of diethyl ether followed by filtration. The precipitate was then dissolved in MeOH and further purified on the Biotage SP4 purification system eluting water/AcCN. Fractions with an absorption maxima at 690 nm were collected and solvent was removed by lyophilization to give blue solid.

Dye 2a: Yields, 71% (**Method A**), 83% (**Method B**); ^{1}H NMR δ 1.94 (s, 6H), 2.04 (m, 2H), 2.38 (m, 1H), 2.59 (m, 3H), 2.69 (m, 1H), 2.79 (m, 1H), 2.91 (m, 1H), 4.29 (m, 2H), 6.08 (br d, J = 14 Hz, 1H), 7.41 (t, J = 7 Hz, 1H), 7.54 (t, J = 7 Hz, 1H), 7.72 (d, J = 9 Hz, 1H), 7.76 (s, 1H), 7.98 (m, 2H), 8.25 (br d, J = 14 Hz, 1H), 8.29 (d, J = 8 Hz, 1H), 10.62 (s, 1H); ^{13}C NMR δ 23.2, 27.5, 30.7, 39.5, 42.0, 48.1, 96.1, 111.2, 122.0, 123.9, 127.2, 129.9, 136.3, 141.1, 162.5; HRMS *m/z* calcd for $C_{31}H_{30}N_3O_8S$ 604.1748, found 604.1749; UV-Vis (MeOH); λ_{max} = 690 nm, pH > 4 (ε = 1.3 x 10^5 cm^{-1} M^{-1}); UV-Vis (MeOH) λ_{max} = 605 nm, pH < 4; λ_{em} = 700 nm; ϕ_F (MeOH): 0.079.

Dye 2b: Yields, 92% (**Method B**); ^{1}H NMR δ 1.75 (m, 2H), 1.80 (m, 4H), 1.95 (s, 6H), 2.49 (m, 2H), 2.65 (m, 4H), 4.16 (m, 2H), 6.04 (br d, J = 13 Hz, 1H), 7.42 (t, J = 8 Hz, 1H), 7.56 (t, J = 8 Hz, 1H), 7.66 (d, J = 9 Hz, 1H), 7.75 (s, 1H), 7.91 (d, J = 8 Hz, 1H), 7.92 (d, J = 9 Hz, 1H), 8.28 (br d, J = 14 Hz, 1H), 8.30 (d, J = 9 Hz, 1H), 10.67 (s, 1H); ^{13}C NMR δ 20.3, 22.4, 23.5, 25.7, 26.8, 27.2, 42.8, 50.6, 96.3, 111.1, 121.9, 124.0, 127.3, 129.3, 136.4, 140.2, 162.2; HRMS *m/z* calcd for $C_{31}H_{32}N_3O_6S$ 574.2006, found 574.2005; UV-Vis (MeOH): λ_{max} = 690 nm, pH > 4 (ε = 1.6 x 10^5 cm^{-1} M^{-1}); UV-Vis (MeOH) λ_{max} = 605 nm, pH < 4; λ_{em} = 700 nm; ϕ_F (MeOH): 0.064.

Dye 2c: Yield, 46% (**Method B**); ^{1}H NMR δ 1.78 (m, H), 2.00 (s, 6H), 2.44 (m, 1H), 2.72 (m, 2H), 3.27 (s, 3H), 3.70 (s, 3H), 4.58 (m, 2H), 6.75 (br d, J = 14 Hz, 1H), 7.31 (s, 1H), 7,64 (t, J = 8 Hz, 1H), 7.74 (t, J = 8 Hz, 1H), 8.02 (d, J = 9 Hz, 2H), 8.18 (m, 2H), 8.44 (d, J = 8 Hz, 1H),

8.53 (br d, J = 14 Hz, 1H); HRMS *m/z* calcd for $C_{33}H_{36}N_3O_6S$ 602.2319, found 602.2317; UV-Vis (MeOH): λ_{max} = 605 nm.

Figure S1. ^{1}H-NMR spectrum of **2a** at 600 MHz in DMSO-d_6 at 25 ^{o}C.

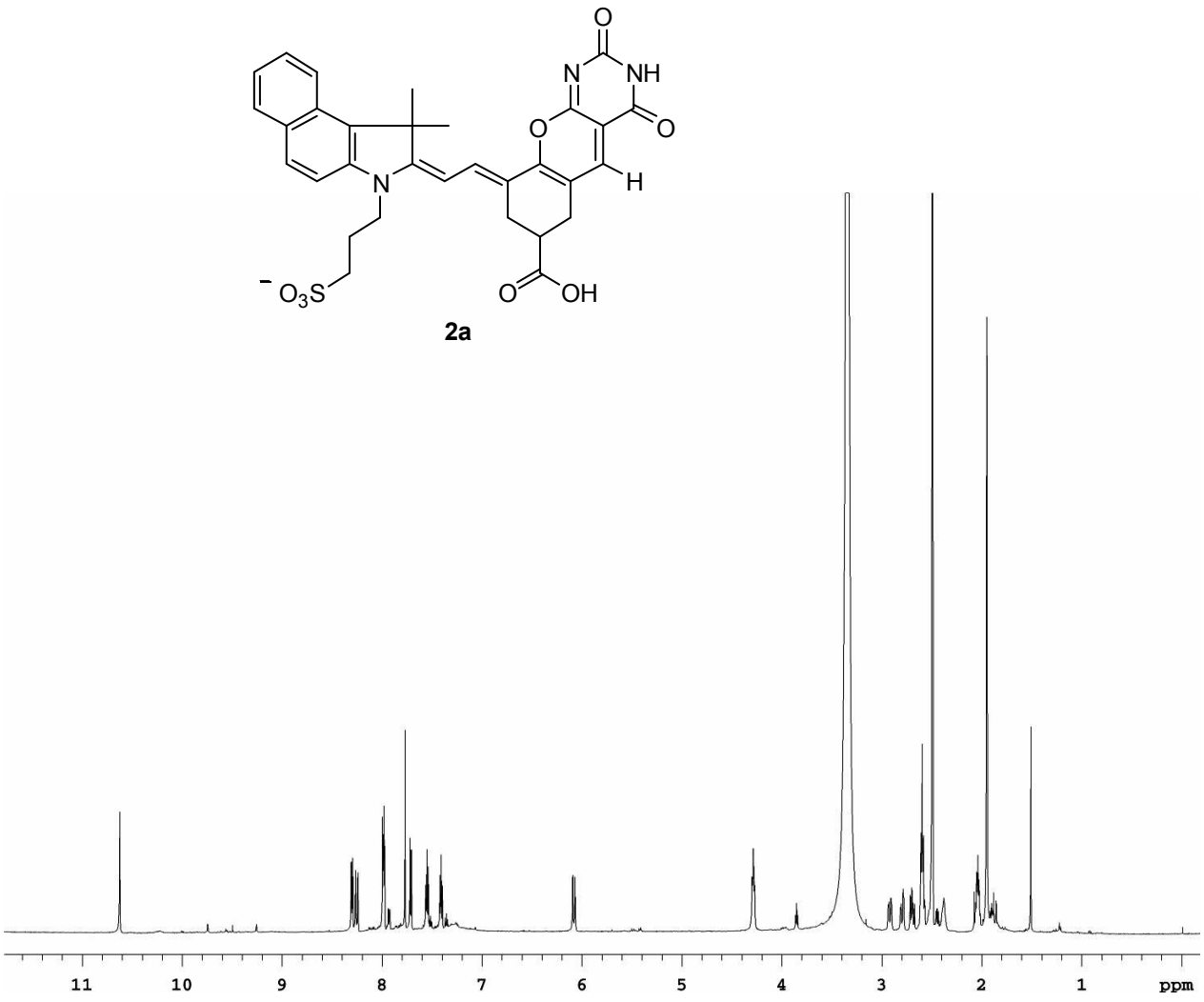


Figure S2. ^{13}C-NMR spectrum of **2a** at 600 MHz in DMSO-d_6 at 25 ^{o}C.

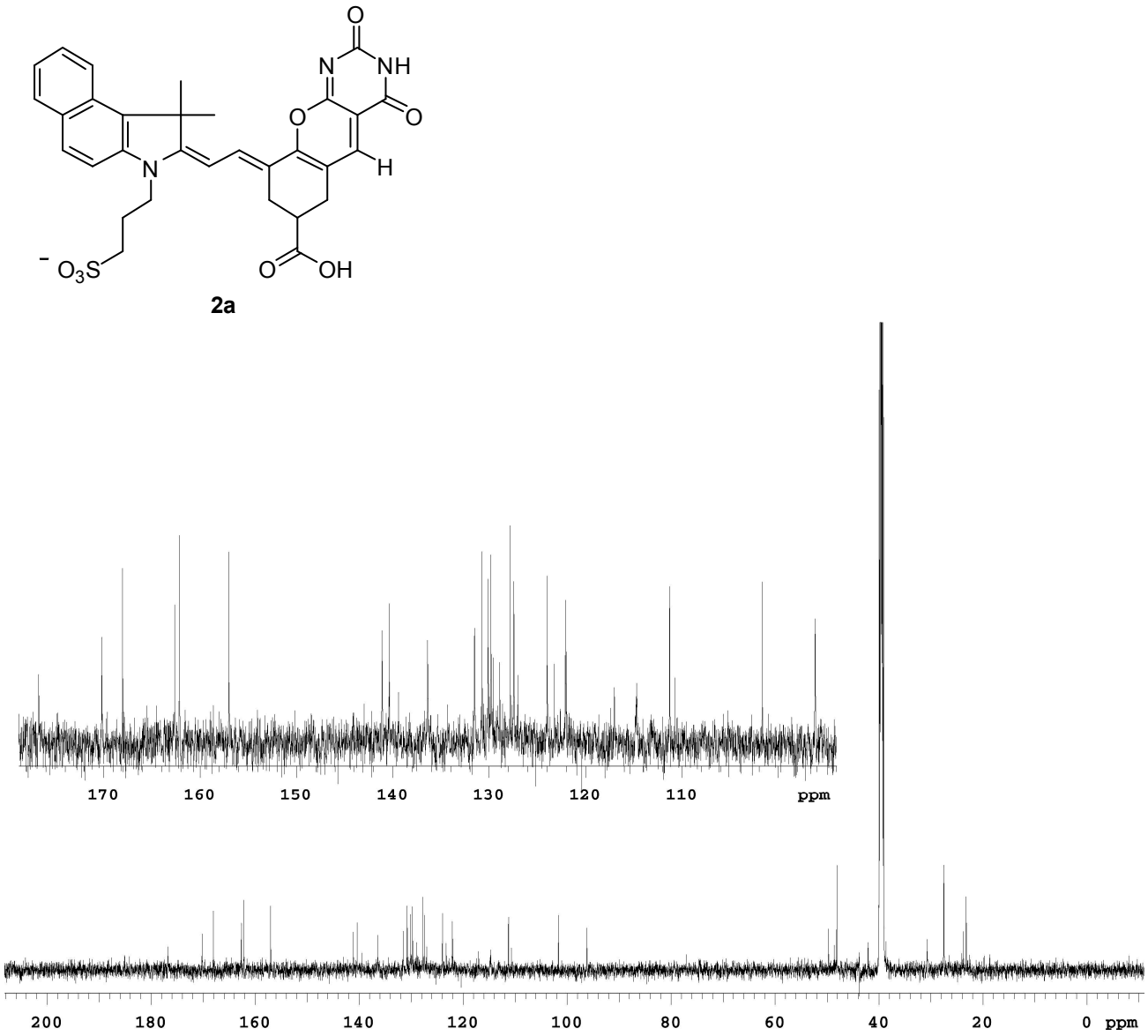
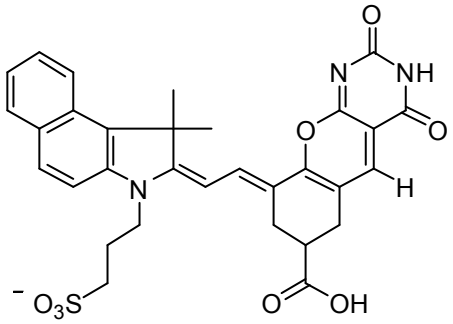


Figure S3. Expansion of (a) (b) COSY, (c) (d) HMQC, and (e) (f) (g) (h) HMBC of 600 MHz spectra of **2a** in DMSO-d_6 at 25° C. Connections for one-bond and long range proton-carbon correlations are indicated.



2a

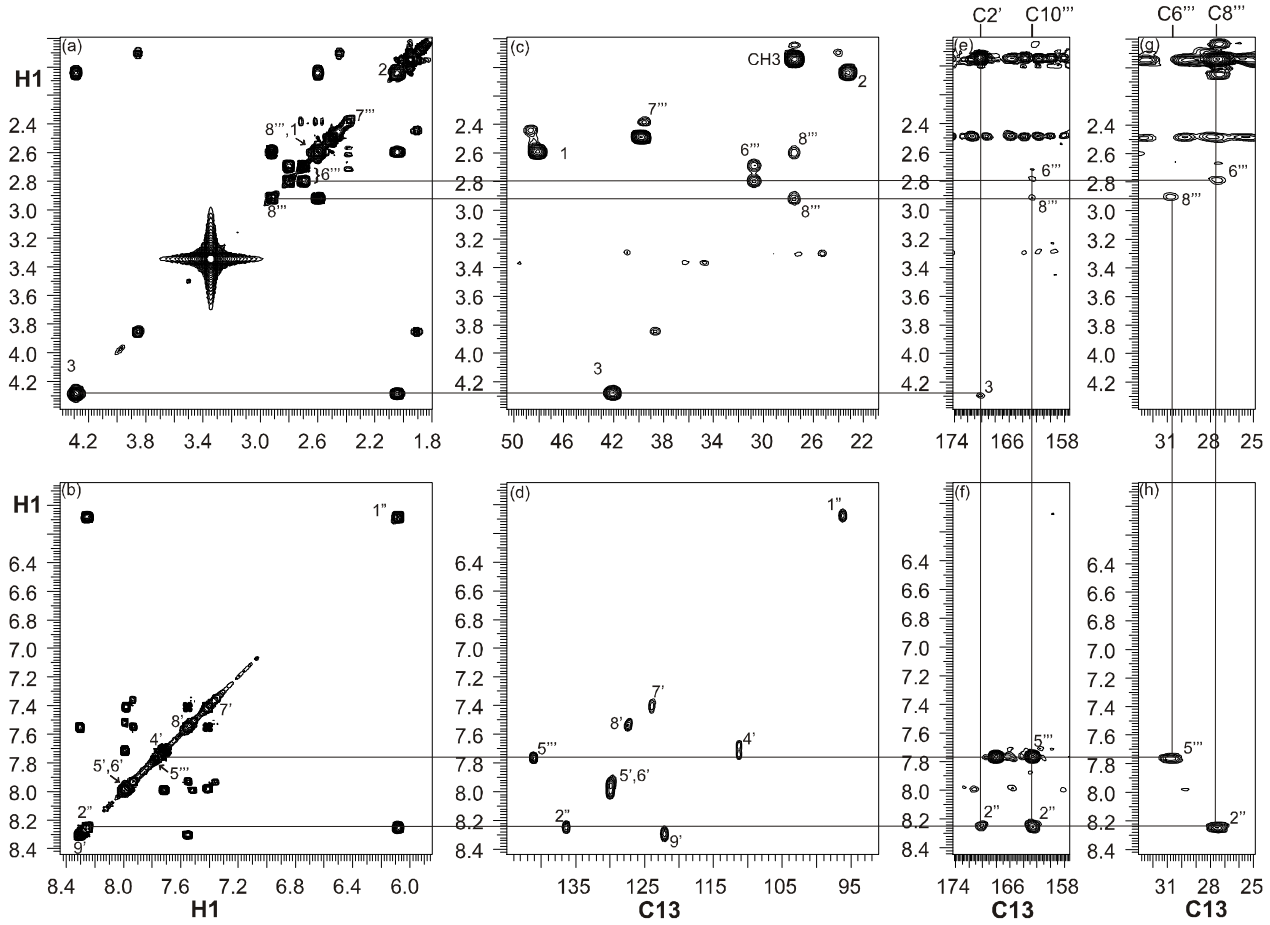
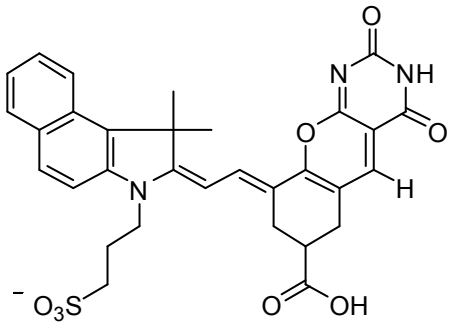


Figure S4. Demonstrates the reversibility of the protonation-deprotonation of dye **2a**. Fluorescence of the solutions was measured using 1.5 mL plastic acrylic cuvettes at 550 nm excitation with scan from 565 nm to 850 nm. The dye was predissolved in ethanol and redissolved in water. A base 0.1N NaOH was added to the dye solution till pH reached 7.9. Then 0.2N HCl was added to lower the pH till it reached 3.03. Finally, the pH was raised up with 0.1 N NaOH to 10.13.



2a

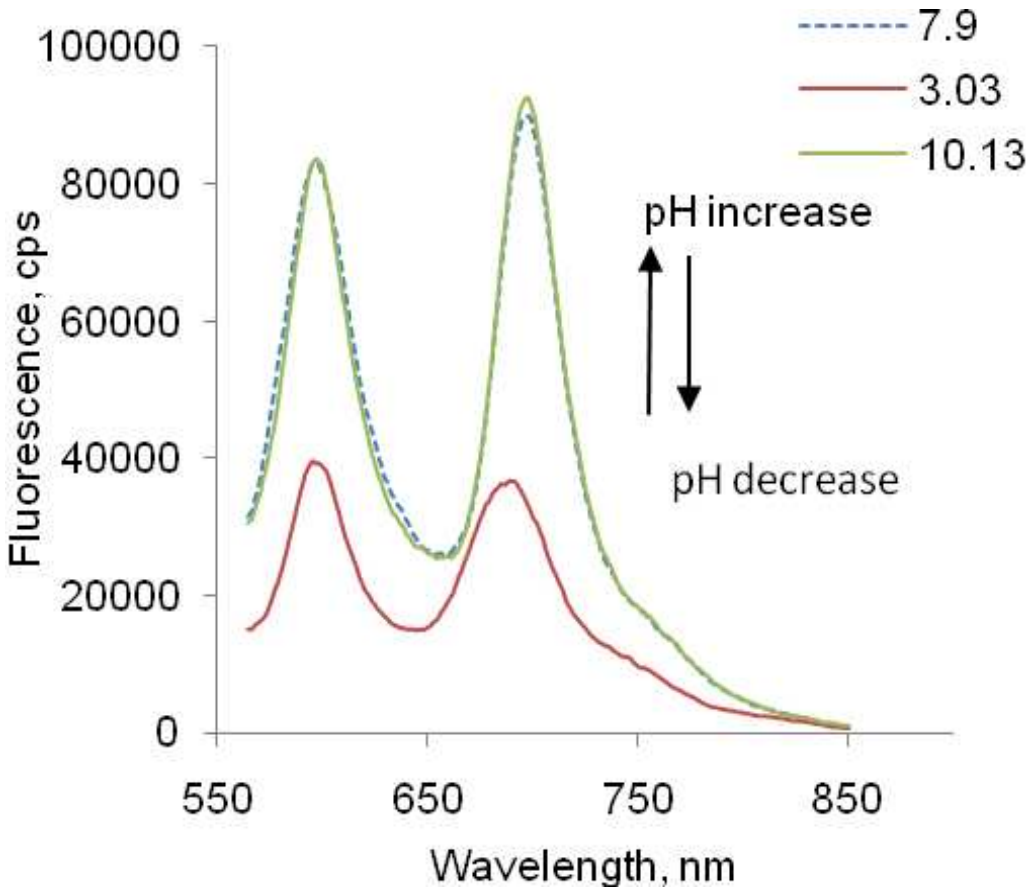
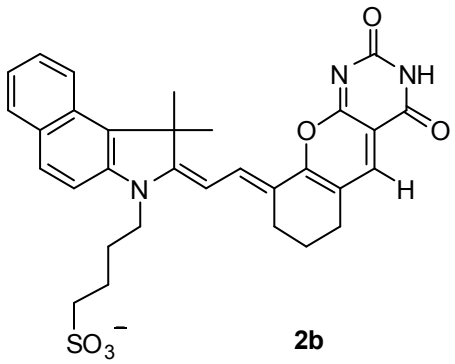


Figure S5. ^{1}H-NMR spectrum of **2b** at 600 MHz in DMSO-d_6 at 25 ^{o}C.



2b

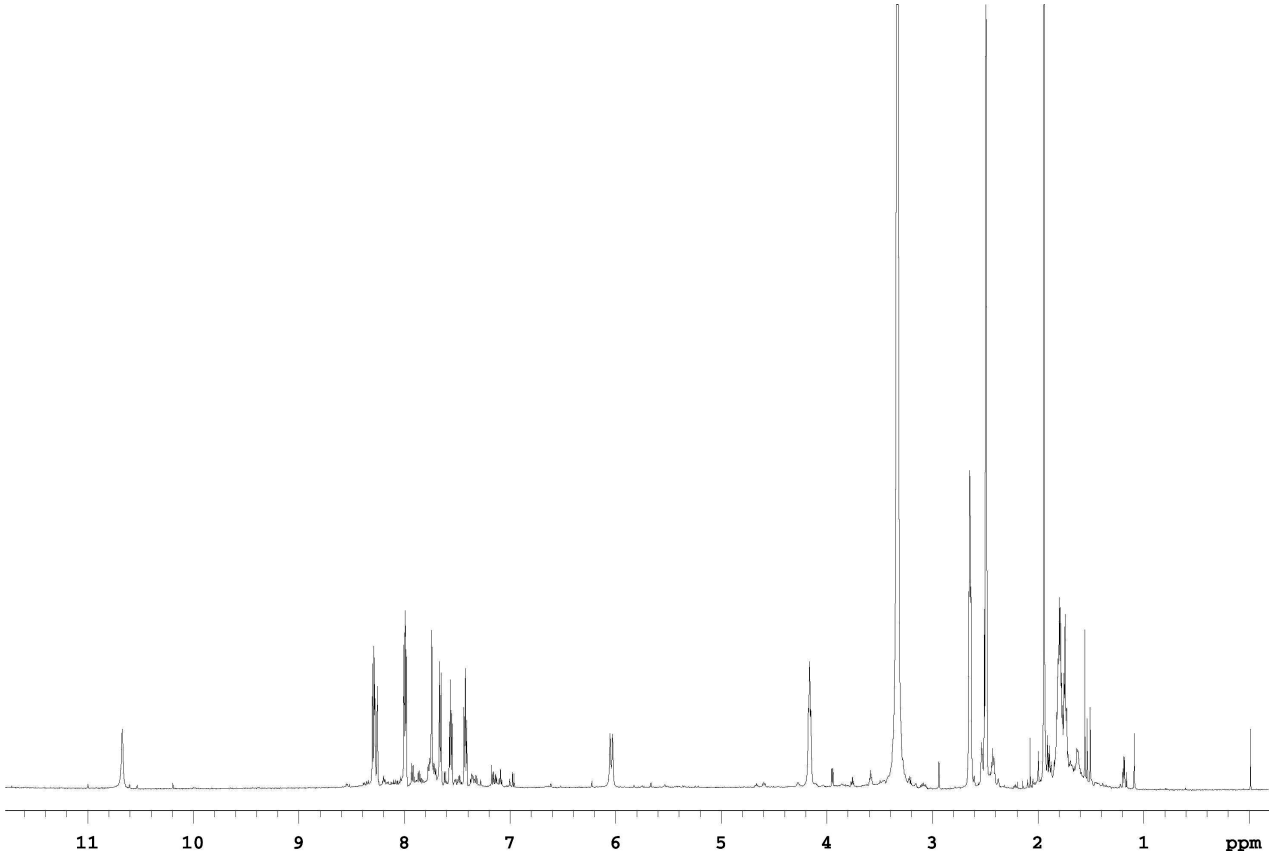


Figure S6. ^{13}C-NMR spectrum of **2b** at 600 MHz in DMSO-d_6 at 25 ^{o}C.

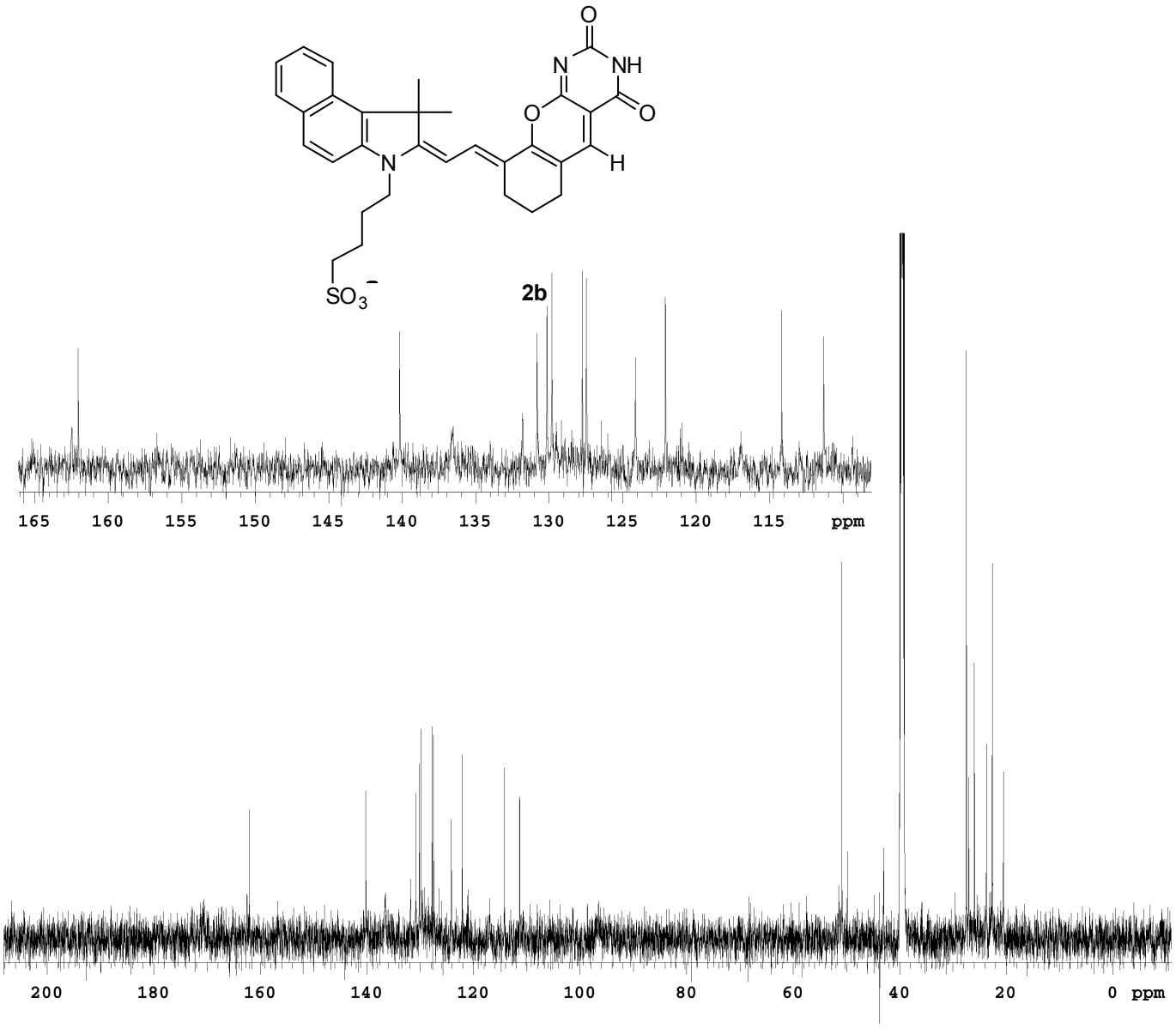
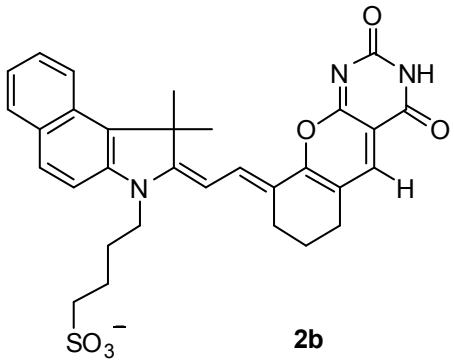


Figure S7. Expansion of (a) (b) COSY, (c) (d) HMQC, and (e) (f) (g) (h) HMBC of 600 MHz spectra of **2b** in DMSO-d_6 at 25° C.



2b

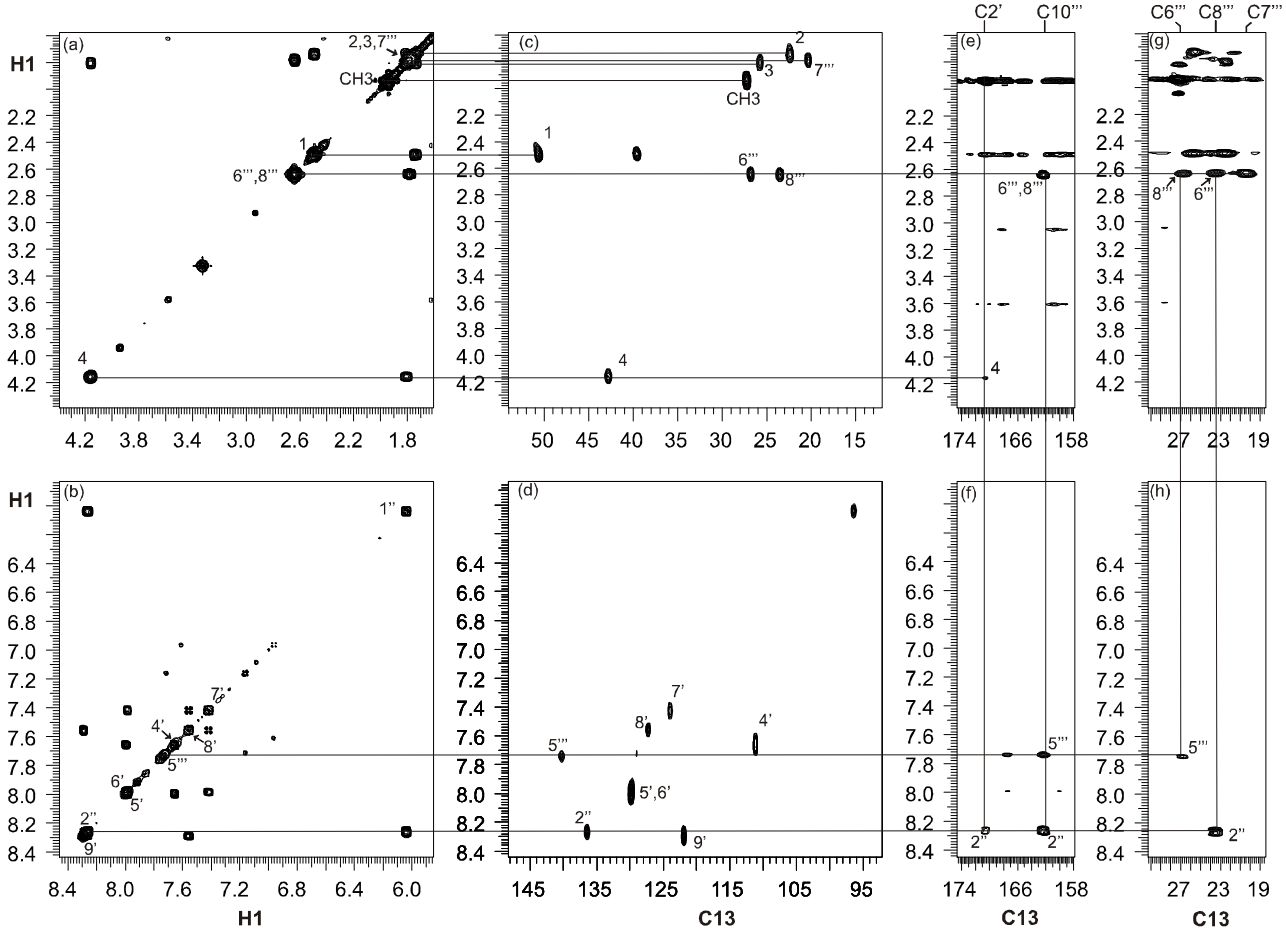
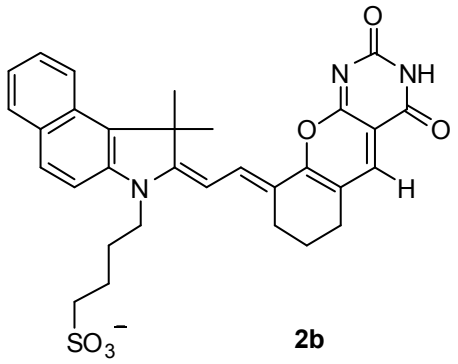


Table S1. Proton and carbon chemical shift assignments of **2a** and **2b** in DMSO-d_6 at 25° C.

	2a		**2b**	
	δ_H	δ_C	δ_H	δ_C
1	2.59	48.1	2.49	50.6
2	2.04	23.2	1.75	22.4
3	4.29	42.0	1.81	25.7
4			4.16	42.8
1'-CH_3	1.94	27.5	1.95	27.2
4'	7.72	111.2	7.66	111.1
5'	7.97	129.9	7.91	129.3
6'	7.96	129.9	7.92	129.3
7'	7.41	123.9	7.42	124.0
8'	7.54	127.2	7.56	127.3
9'	8.29	122.0	8.30	121.9
1"	6.08	96.1	6.04	96.3
2"	8.25	136.3	8.28	136.4
5'''	7.76	141.1	7.75	140.2
6'''	2.69, 2.79	30.7	2.65	26.8
7'''	2.38	39.5	1.80	20.3
8'''	2.59, 2.91	27.5	2.66	23.5
10'''		162.5		162.2

Figure S8. Absorption (top left) and emission spectra (top right) and pKa curve (bottom) of **2b** as a function of pH.



2b

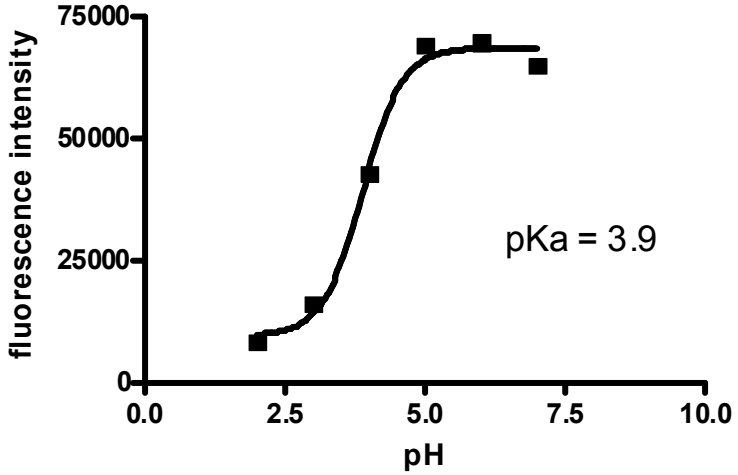


Figure S9. ^{1}H-NMR spectrum of **2c** at 300 MHz in DMSO-d_6 at 25 ^{o}C.

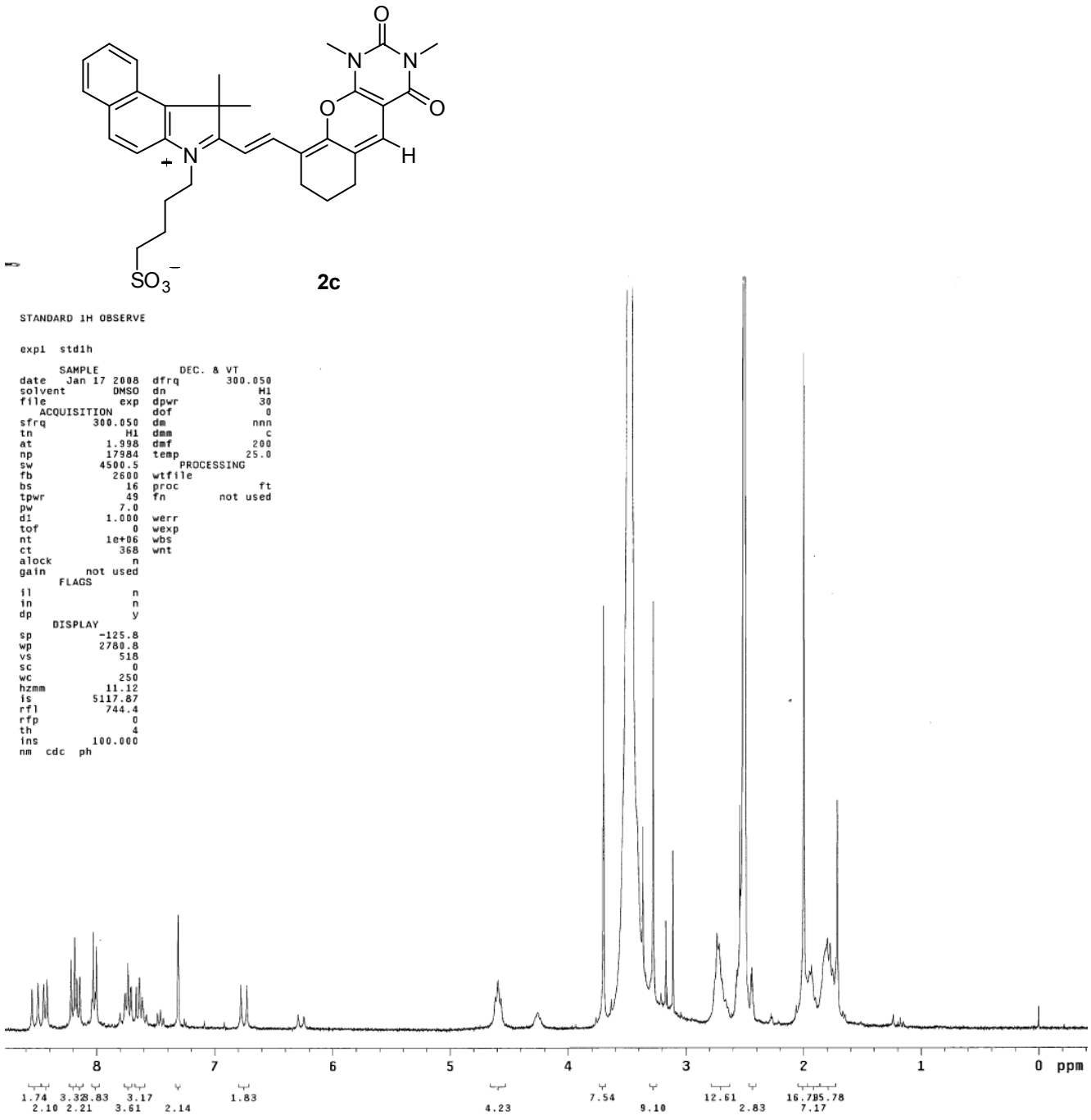
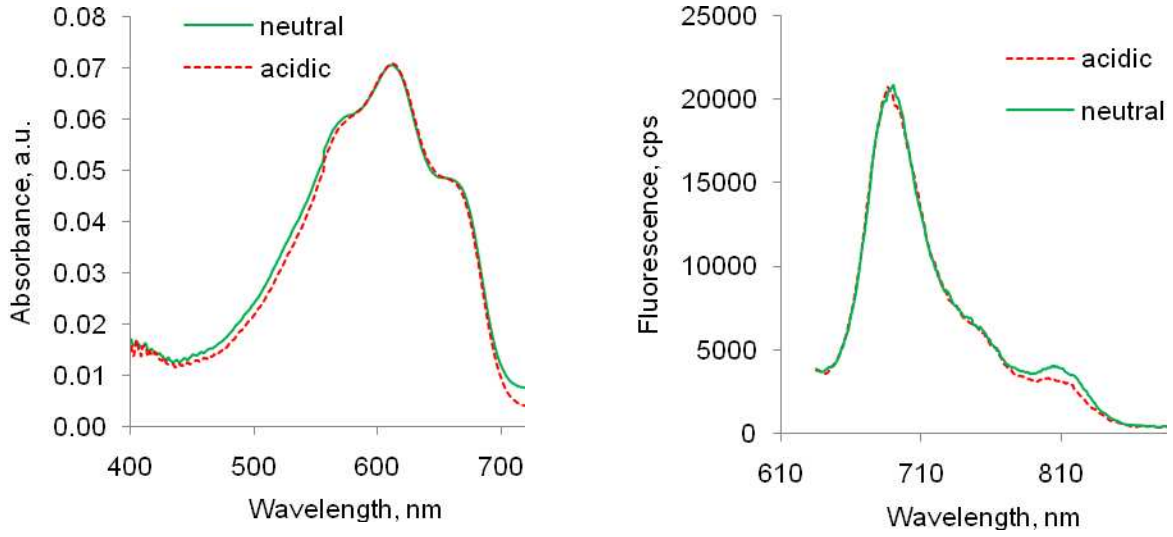


Figure S10. Absorption (left) and emission spectra (right, excitation at 616 nm) of **2c** in water at neutral (pH 6.9) and acidic (pH=2.5, HCl) conditions show complete overlap indicating the absence of protonation/deprotonation.



References

1. Benson, R. C.; Kues, H. A., Fluorescence properties of indocyanine green as related to angiography. *Phys Med Biol* **1978,** 23, (1), 159-63.
2. Mason, J. C. The Synthesis of Novel Near-Infrared Heptamethine Cyanine Dyes. Georgia State University, Atlanta, 2001.