Electronic Supplementary Material (ESI) for Chemical Communications.

A NIR-Emitting Cyanine with Large Stokes Shifts for Live Cell Imaging: Large Impact of Phenol Group on Emission

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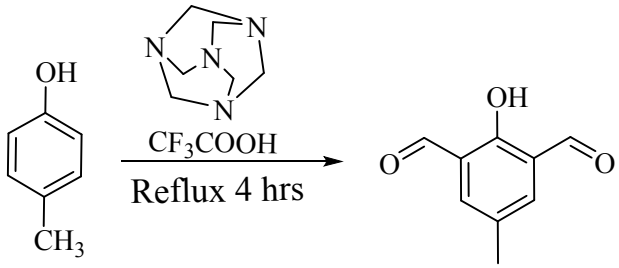
General Considerations: All starting materials and the essential solvents were purchased from Sigma-Aldrich, Ark Pharma, Fischer Scientific, Alfa-Asaer and Across Organics and directly used without further purifications. All deuterated solvents were purchased from Cambridge Isotopes and used as received. All NMR data were recorded on Varian 300 and 500 MHz instruments with all spectra referenced to deuterated solvents. Mass spectra were obtained from HP1100LC/MSD mass spectrometry. HRMS data were performed on an ESI-TOF MS system (Waters, Milford, MA).

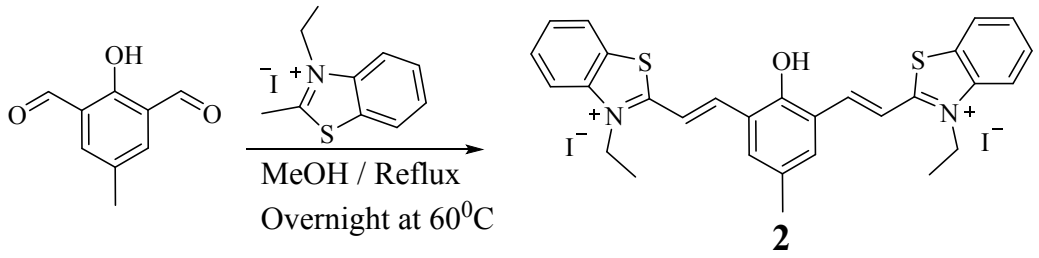
Synthesis of 2-hydroxy-5-methylisophthalaldehyde:



In a 250 mL round-bottomed (RB) flask,1 g (9.25 mmol) of p-cresol and 2.6 g (18.55 mmol) of hexamethylene tetraamine were mixed in a 250 mL RB flask and 30 mL trifluoroacetic acid was added. The mixture was then refluxed for 6 hrs. After completion of the reaction, the solution was cooled in an ice-water bath and was neutralized with KOH solution to the pH about 6 to get a yellow colored precipitate. The precipitate was then filtered, washed several times with water and dried. Pure yellow crystalline solid 2-hydroxy-5-methylisophthalaldehyde was obtained by recrystallization in methanol with >90 % yield.

^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.45 ppm (s, 1H), 10.21 ppm (s, 2H), 7.77ppm (s, 2H), 7.26 ppm (solvent), 2.38 ppm (s, 3H).

Synthesis of 2,2'-((2-hydroxy-5-methyl-1,3-phenylene)bis(ethene-2,1-diyl))bis(3-ethyl benzothiazole-3-ium) diiodide (compound 2):



2

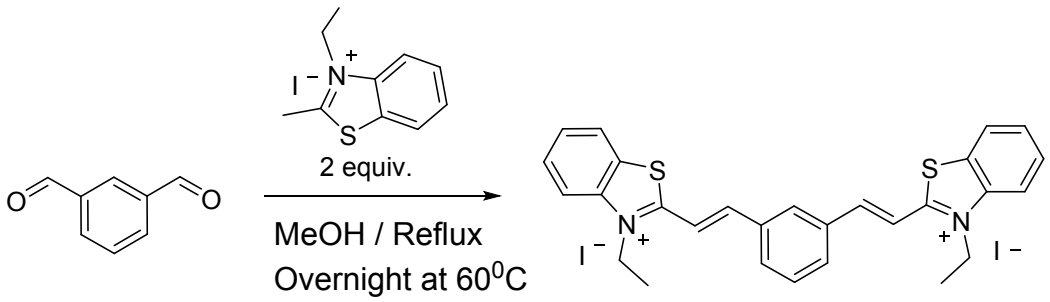
In a round-bottomed flask, 297 mg (0.98 mmol) of 1-ethyl-2methylbenzothiazolium salt was dissolved in 20 mL methanol, and 0.5 mL pyridine was added to it. The mixture was stirred at room temperature for 30 minutes, and 75 mg (0.46 mmol) of 2-hydroxy-5-methylisophthalaldehyde (dialdehyde) was added to the flask, and the mixture was heated to 60^{o}C and stirred overnight at 60^{o}C. After completion of the reaction, the solvent was evaporated using rotary evaporator and the crude solid obtained, was washed with 50 mL ethyl acetate which was filtered and dried to get dark-colored solid. The solid still contained excess unreacted benzothiazolium salt which was washed with water several times filtered and dried to get pure dark-colored solid compound 2 with 75% isolated yield. NMR and mass spectroscopy then characterized compound 2.

^{1}H NMR (500 MHz, DMSO-d$_6$): δ = 8.22 ppm (d, 2H), 8.08 ppm (m, 6H), 7.70ppm (t, 2H), 7.67 ppm (s, 2H), 7.60 ppm (t, 2H), 4.71 ppm (q, 4H), 2.45 ppm (solvent DMSO), 2.19 ppm (s, 3H) and 1.42 ppm (t, 6H).

^{13}C NMR (125 MHz, DMSO-d$_6$): δ = 171.78, 143.55, 141.13, 134.17, 130.03, 128.70, 124.86, 124.19, 117.07, 113.19, 44.79, 40.02 (solvent), 20.51, 14.68.

Synthesis of 2,2'-(1,3-phenylenebis(ethene-2,1-diyl))bis(3-ethylbenzo[d]thiazol-3-ium) iodide (compound 3):

In a 50 mL RB flask, 100 mg (0.33 mmol) of 1-ethyl-2methylbenzothiazolium iodide salt was dissolved in 30 mL methanol in a round-bottomed flask, and 0.5 mL pyridine was added. The mixture was stirred for about 30 minutes, and 22 mg (0.16 mmol) of isophthalaldehyde was added to it. After mixing, it was then stirred at 60^{0}C for overnight. When the reaction was completed, the solvent was evaporated in a rotary evaporator to get a red-colored solid, which was washed with ethyl acetate, filtered and dried. The solid obtained was again washed with water several times to remove unreacted excess benzothiazolium salt, filtered and dried to get pure compound **3** with the yield of 85%. NMR and mass spectroscopy characterized compound **3**.



^{1}H NMR (500 MHz, DMSO-d$_6$): δ = 8.68 ppm (s, 1H), 8.49 ppm (d,2H), 8.35 ppm (d, 2H), 8.30 ppm (m, 4H), 8.20 ppm (d, 2H), 7.92 ppm (t, 2H), 7.84 ppm (t, 2H), 7.78 ppm (t, 2H), 5.04 ppm (q, 4H), 2.48 ppm (solvent), 1.52 ppm (t, 6H).

^{13}C NMR (126MHz, DMSO-d$_6$): δ = 171.79, 147.97, 141.48, 135.33, 132.95, 131.79, 130.46, 130.22, 129.16, 129.08, 125.10, 117.39, 45.42, 40.02 (solvent), 14.87.

Calculation of quantum yield:

Zinc Phthalocyanine ($\Phi_{ref.} \approx 0.20$) was used as the standard of reference for calculation of quantum yield of compound **2**, and quinine sulfate (($\Phi_{ref.} \approx 0.53$) was used as a standard of reference for compound **3**. Quantum yield of probes was calculated using an equation shown below, where 'A' is absorbance at the excitation wavelength, 'I' is an integrated area of emission, 'Φ_{ref}' is a quantum yield of reference compound and 'η' is the refractive index of solvent used.

$$\Phi_{sample} = \Phi_{ref} * {I_{sample}}/{I_{ref}} * {A_{ref}}/{A_{sample}} * {\eta_{sample}^2}/{\eta_{ref}^2}$$

NMR Study:

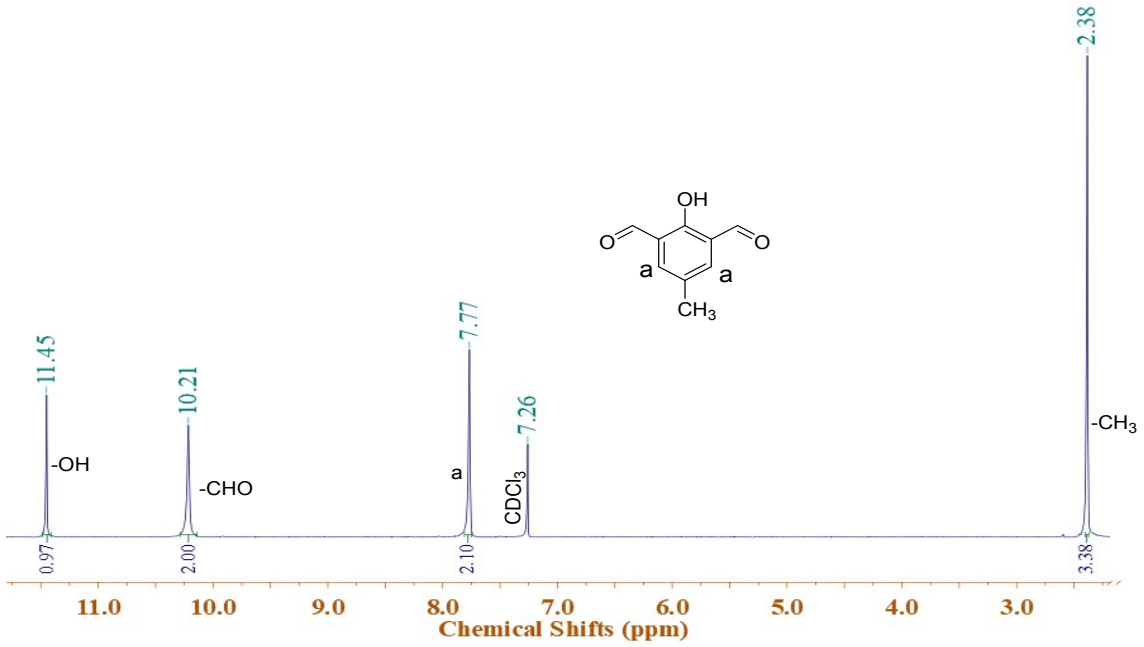


Figure S1. ^{1}H NMR spectra of 2-hydroxy-5-methylisophthalaldehyde in $CDCl_3$.

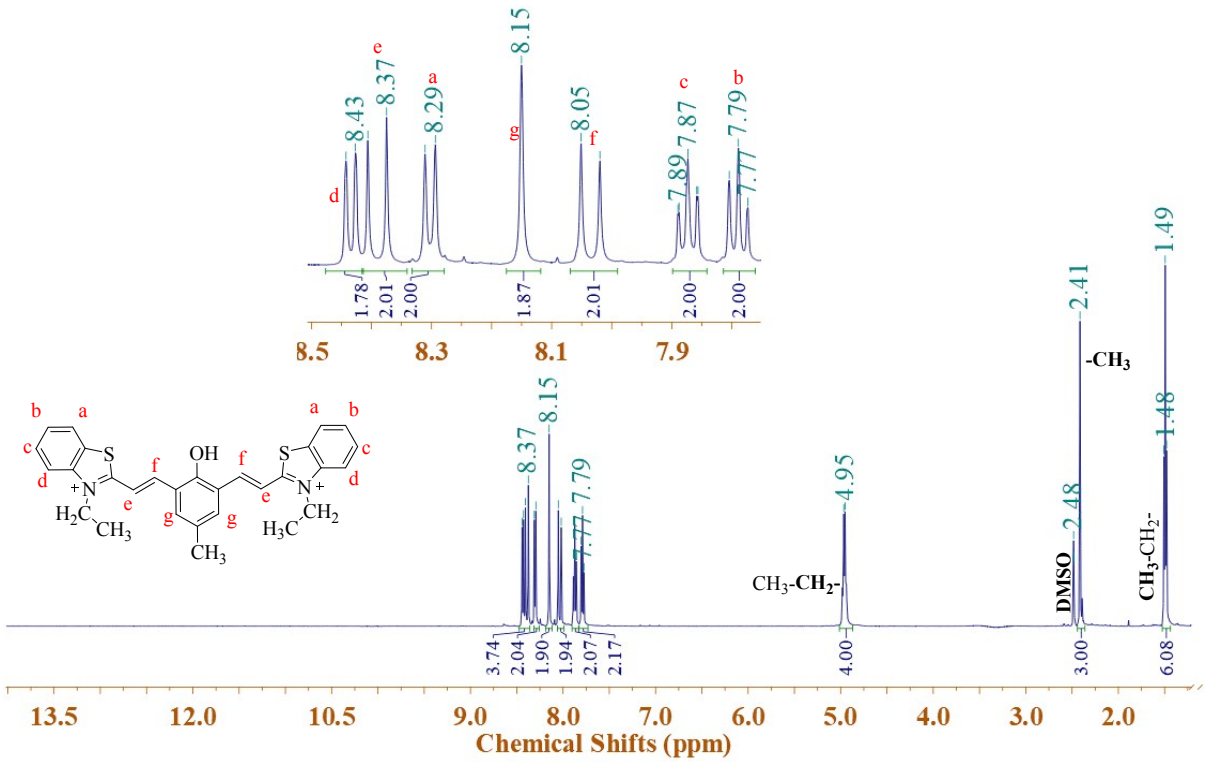


Figure S2. ^{1}H NMR spectra of compound **2**. The top inset is an expanded region from 7.6 ppm to 8.5 ppm for clarity.

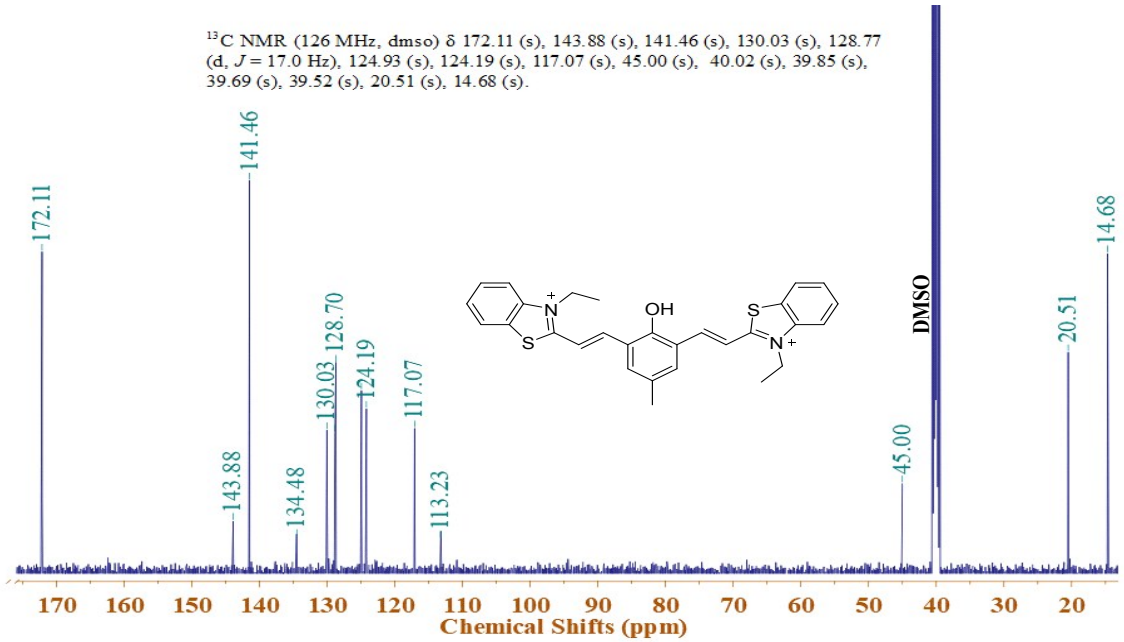


Figure S3. ^{13}C NMR spectra of compound **2** in DMSO-d6.

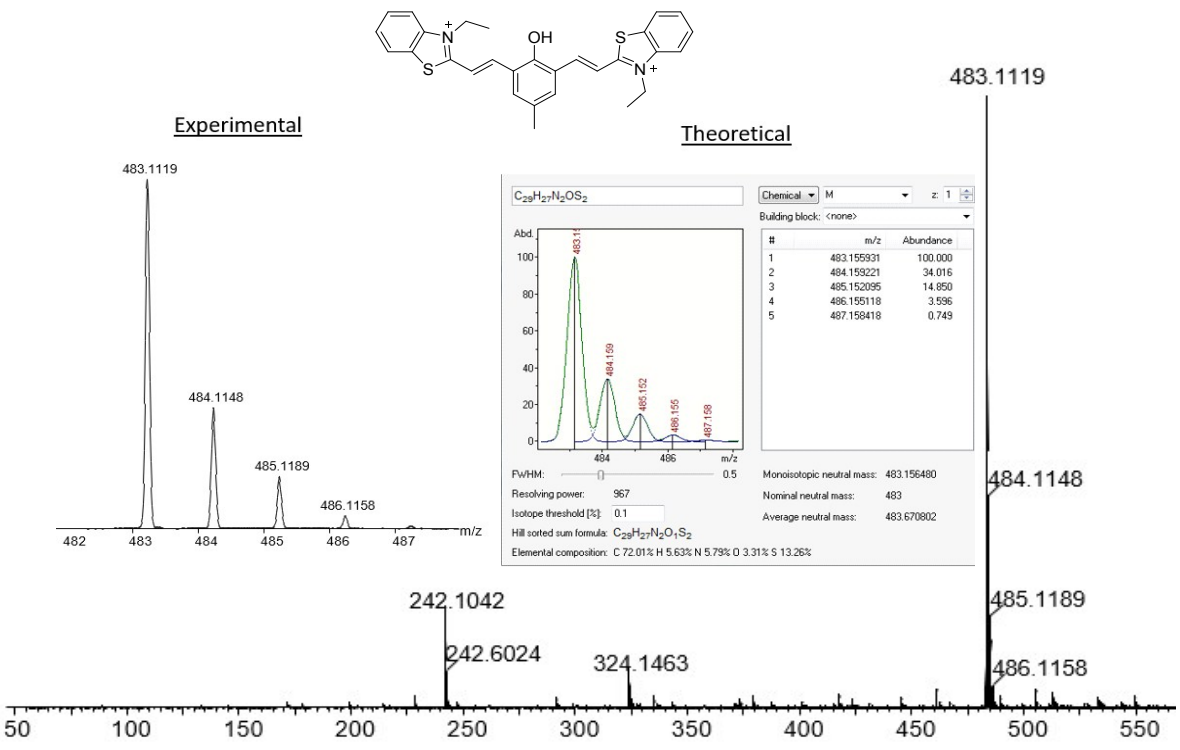


Figure S4. ESI-TOF mass spectra of compound **2**.

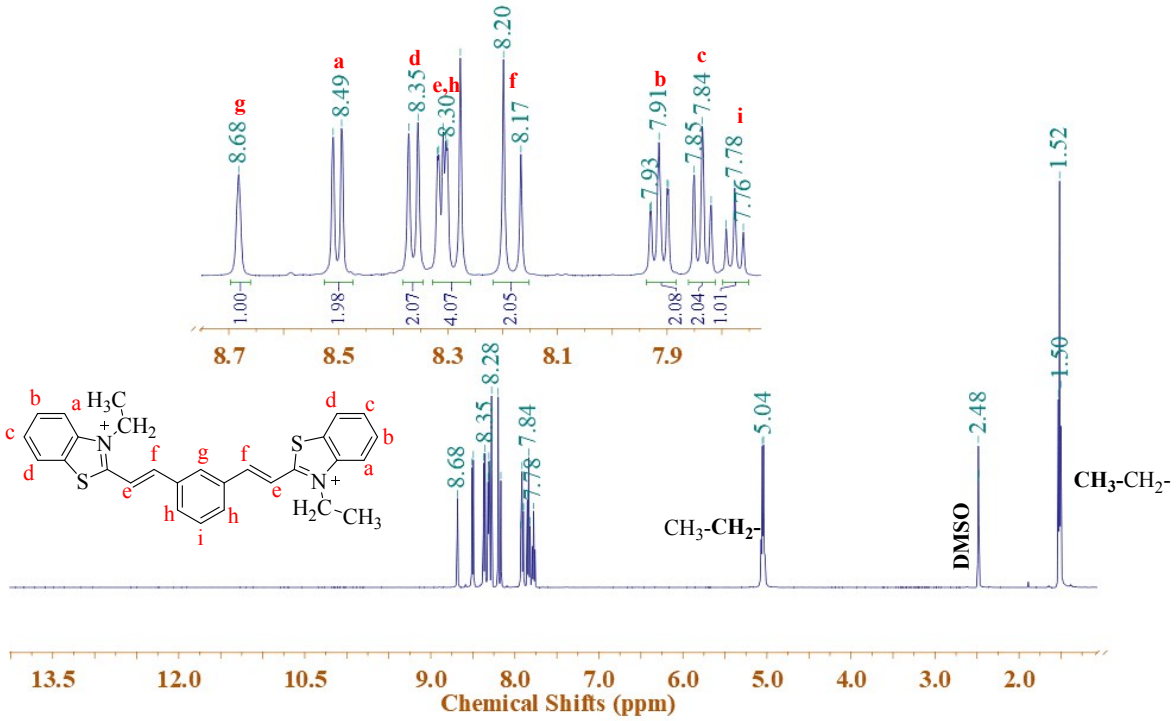


Figure S5. ^{1}H NMR spectra of compound **3**.

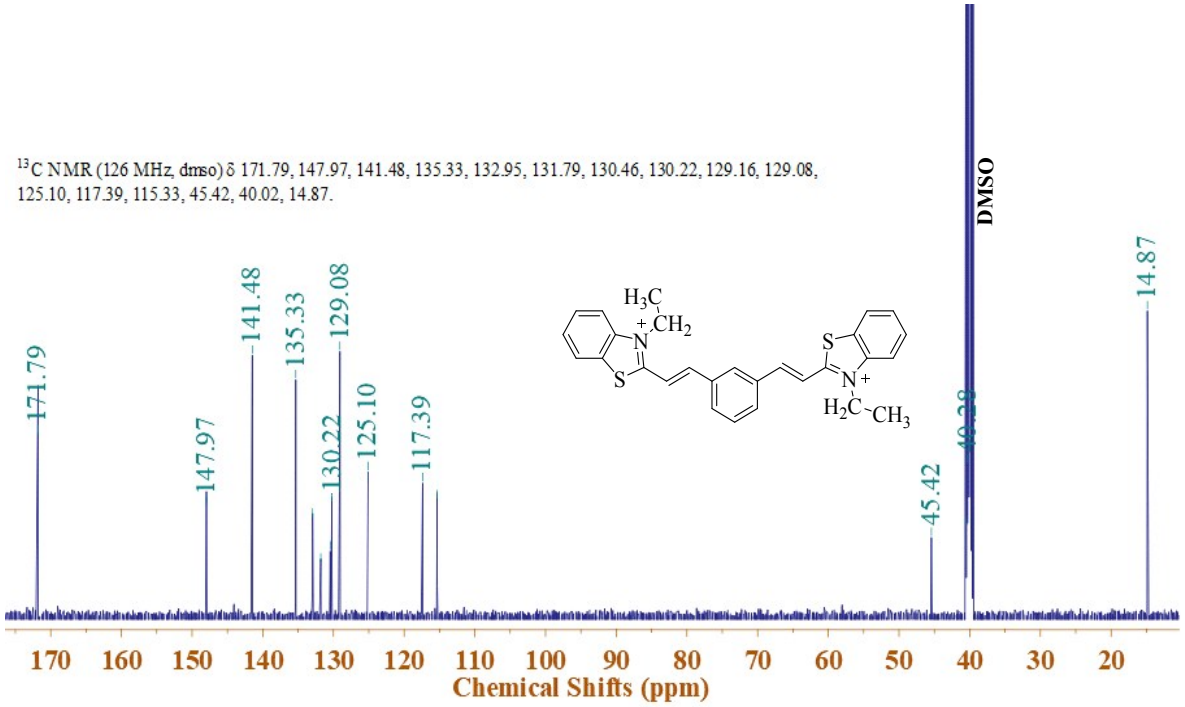


Figure S6. ^{13}C NMR spectra of compound **3** (125MHz).

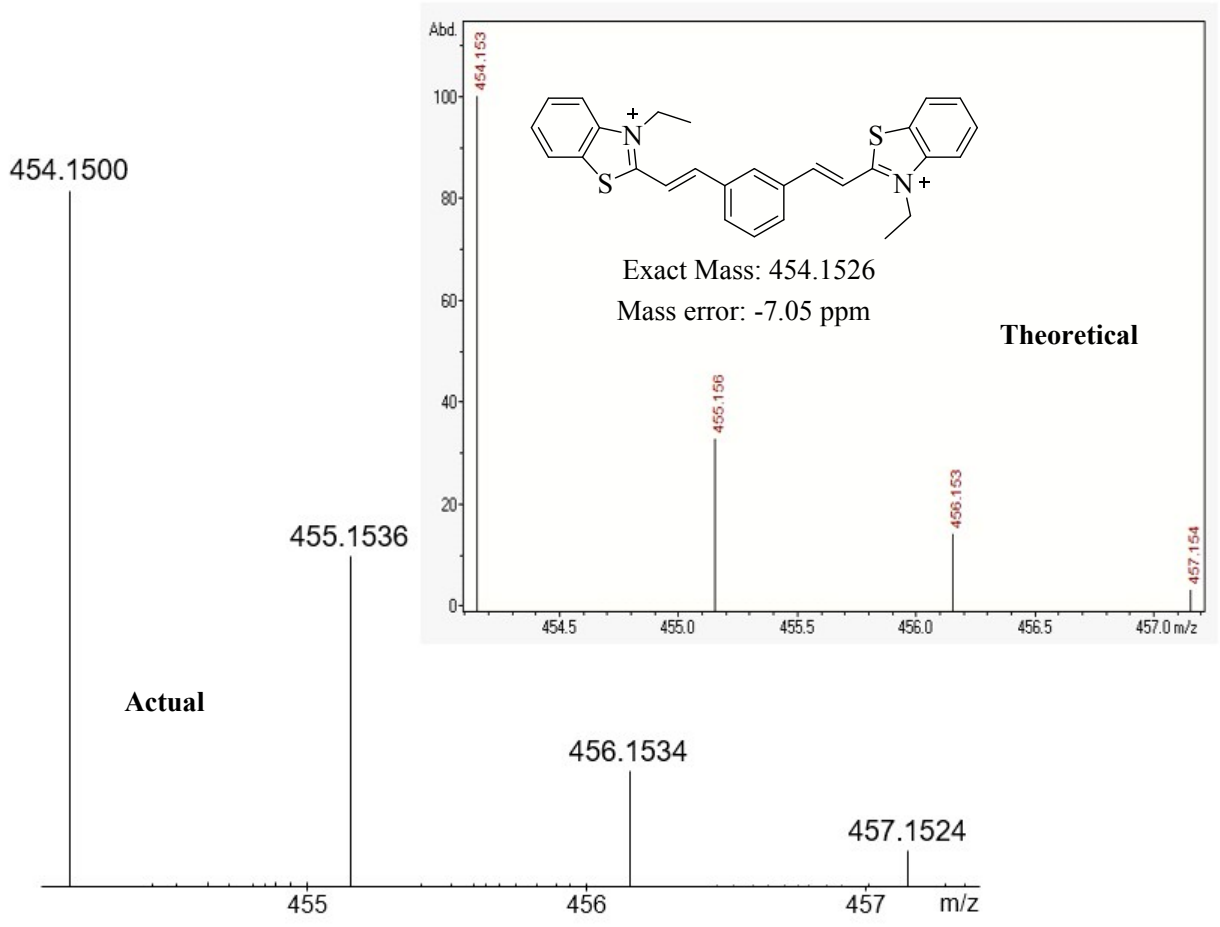


Figure S7. ESI-TOF mass spectra of compound **3**.

Study of Photophysical properties:

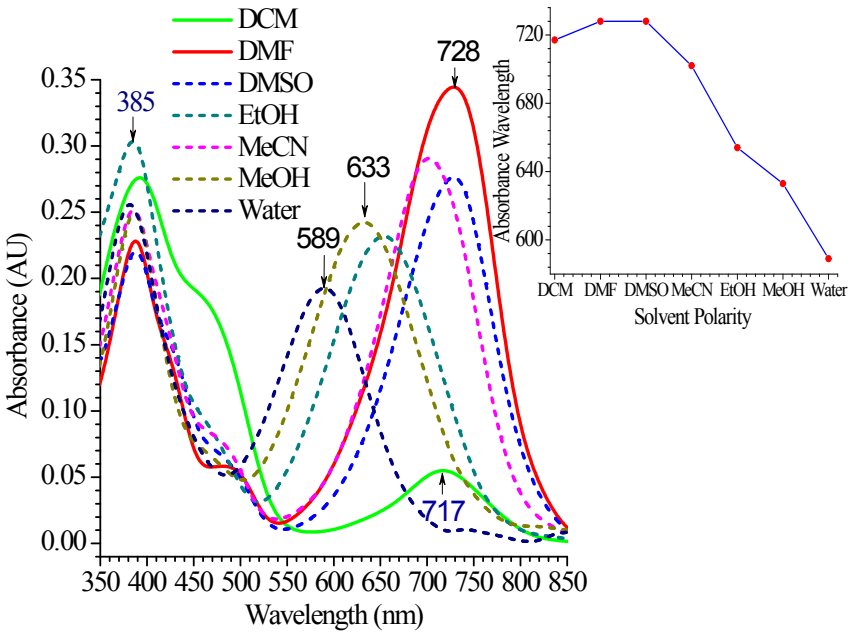


Figure S8. UV-absorbance spectra of compound **2** (10 μM) in different solvents.

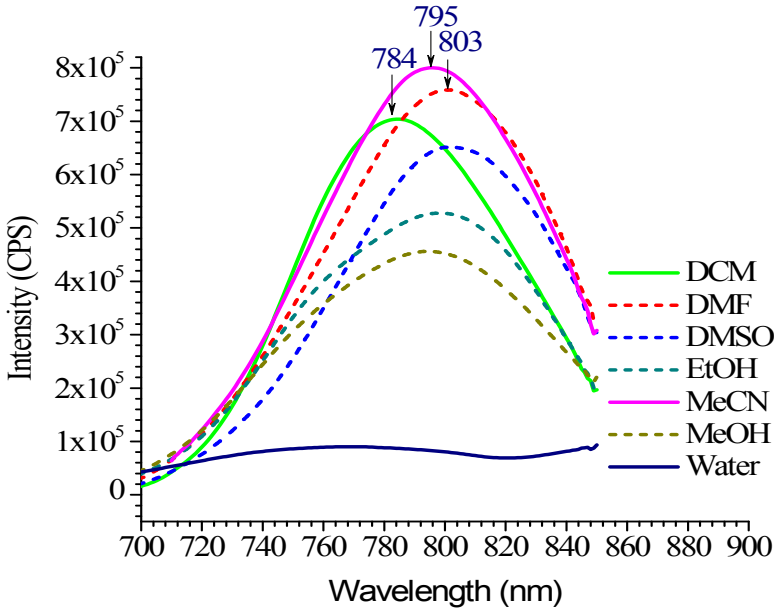


Figure S9. Fluorescence emission spectra of compound **2** (10 μM) in various solvents.

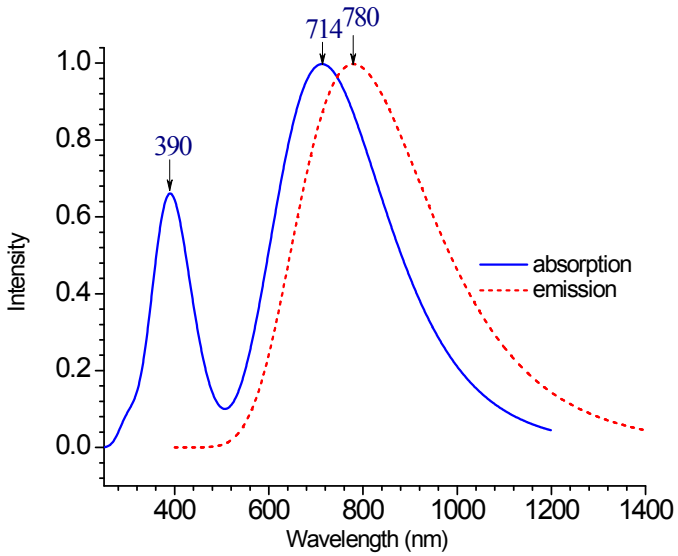


Figure S10. The calculated absorption/emission spectra of *keto* tautomer of **2** at the B3LYP/6-31+G(d) level in (CH_2Cl_2). The spectra were generated by using TD-SCF method, after the molecular geometry was optimized at the B3LYP/6-31+G(d) level.

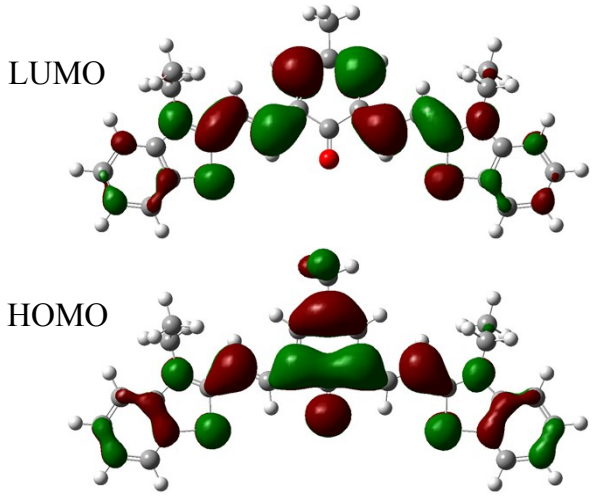


Figure S11. Calculated molecular HOMO/LUMO orbitals for compound **2** in its keto- tautomer after deprotonation at the B3LYP/6-31+G(d) level.

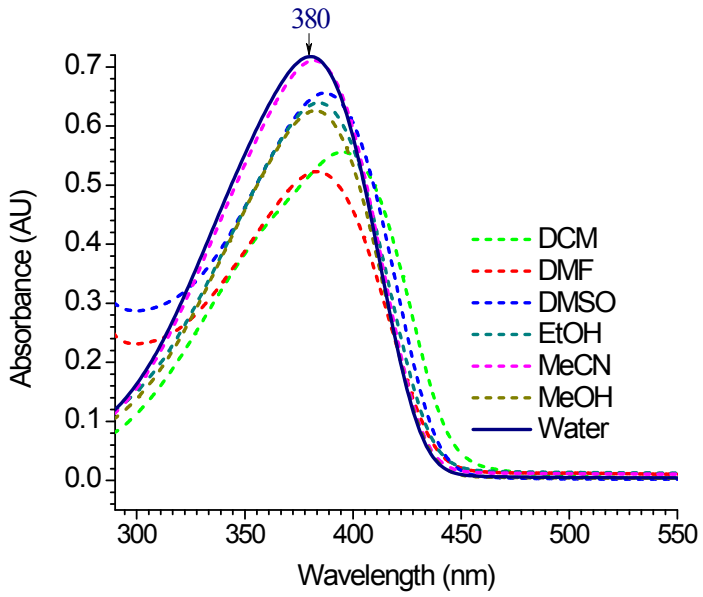


Figure S12. UV absorbance spectra of compound **3** in different solvents.

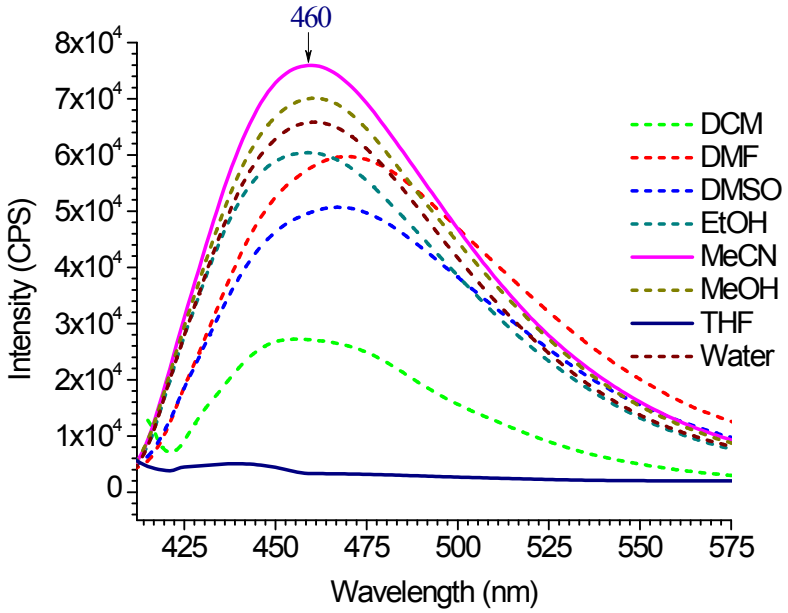


Figure S13. Fluorescence emission spectra of compound **3** in various solvents.

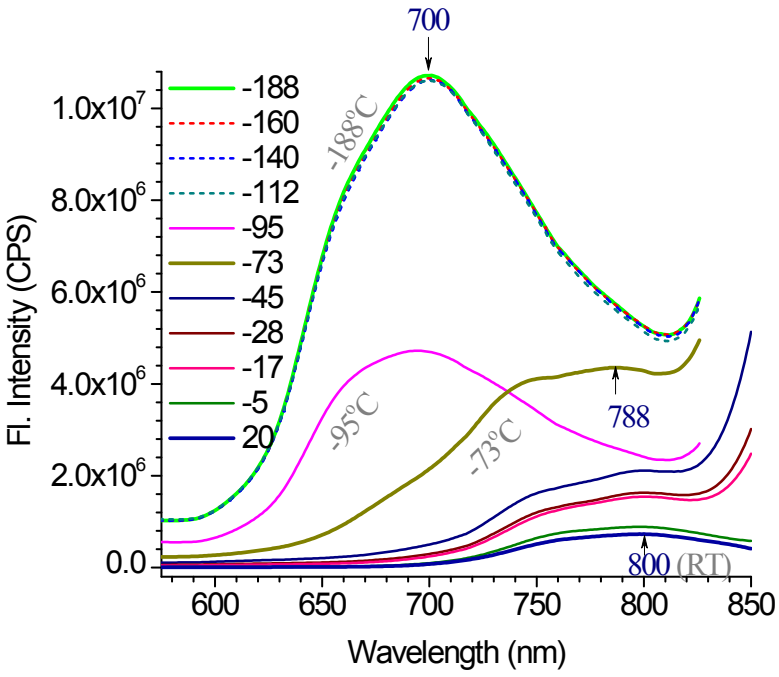


Figure S14. Low-temperature fluorescence spectra of compound **2** (λ_{ex} = 450).

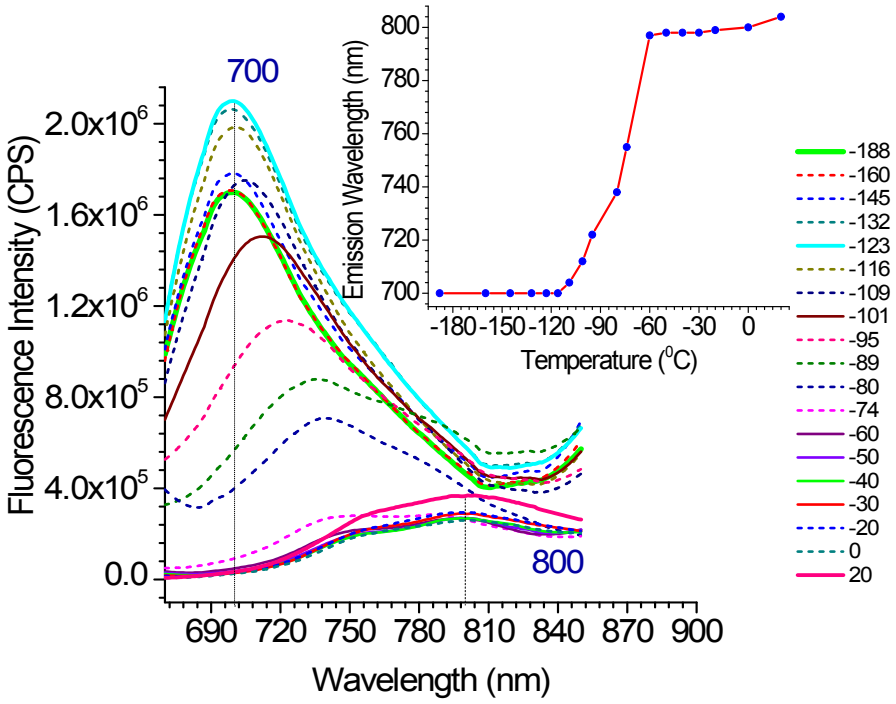


Figure S15. Low-temperature fluorescence spectra of compound **2** (λ_{ex} = 650).

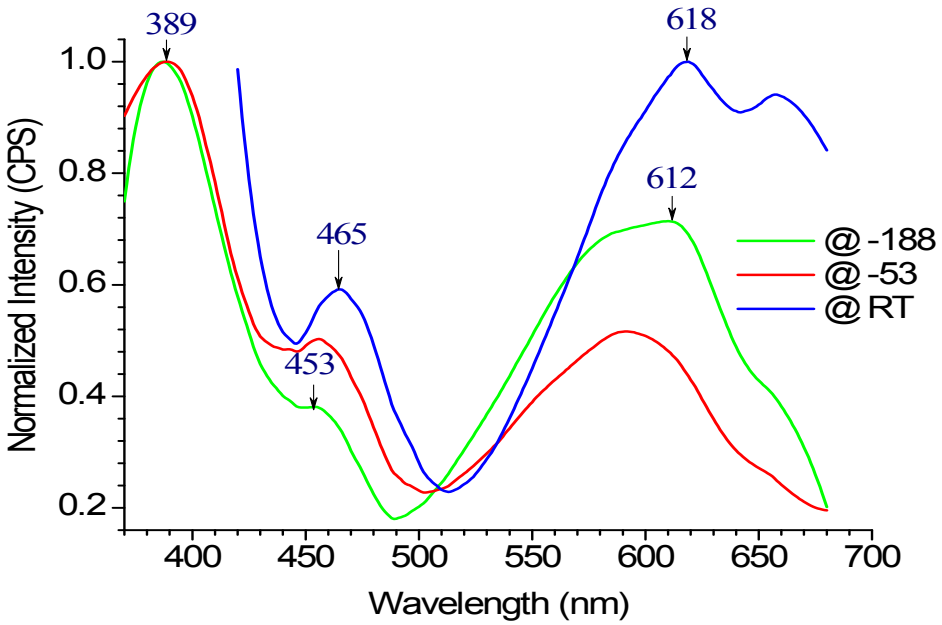


Figure S16. Excitation spectra of comound **2** at different temperature.

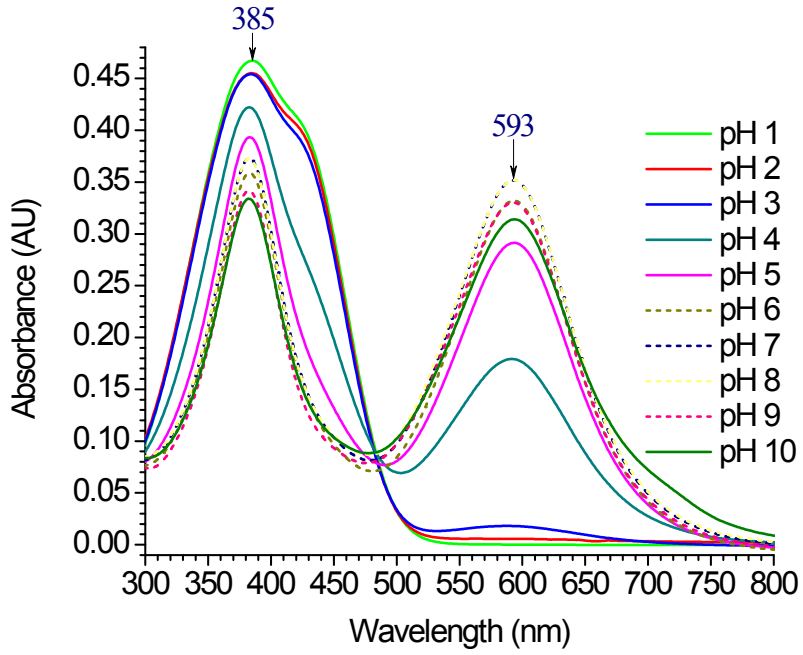


Figure S17. UV-absorbance spectra of compound **2** (10 μM) in different pH buffer in water.

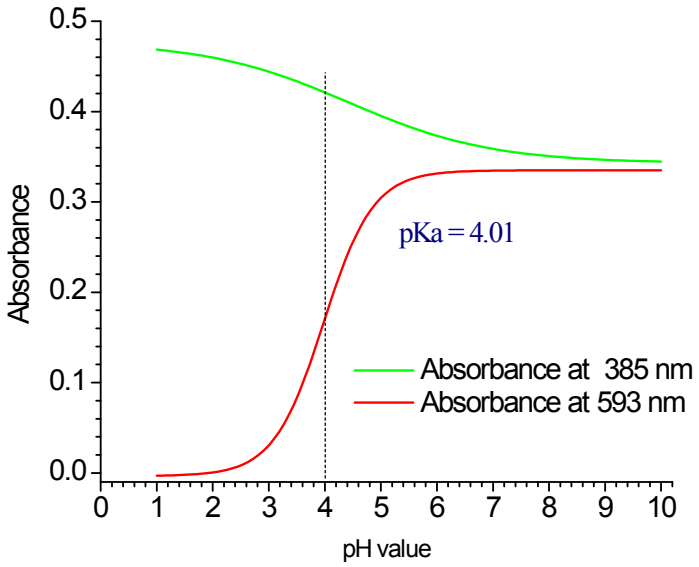
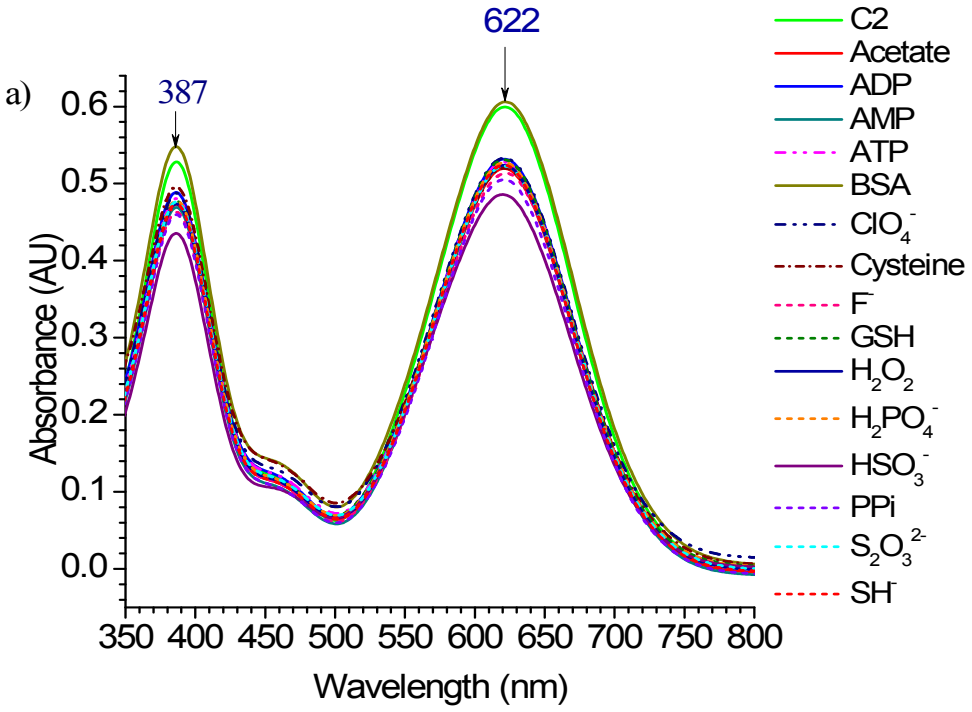


Figure S18. Boltzmann's curve fitting of absorbance of compound **2** to determine the pK_a value.



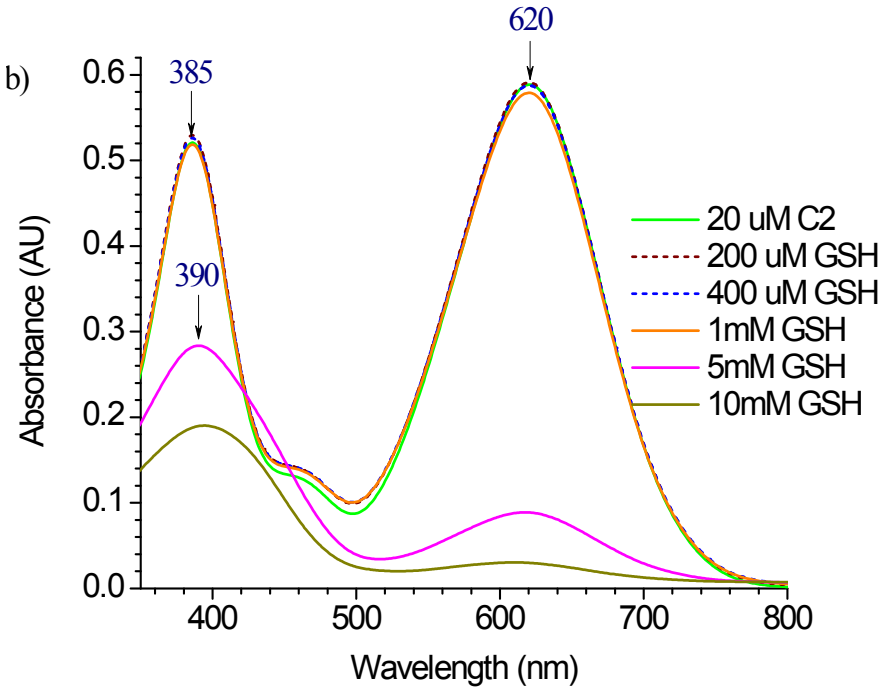
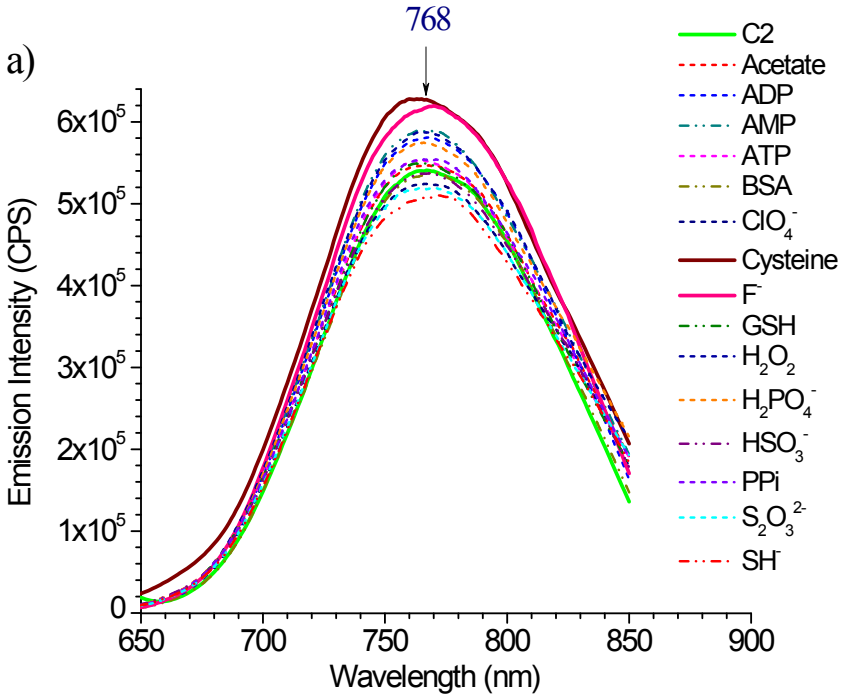


Figure S19. UV absorbance spectra of compound **2** (C2): (a) in the presence of one equivalent (10 μM) of different anions, and (b) in presence of different concentration of glutathione (GSH) in in EtOH/PBS (1:1 by v/v).



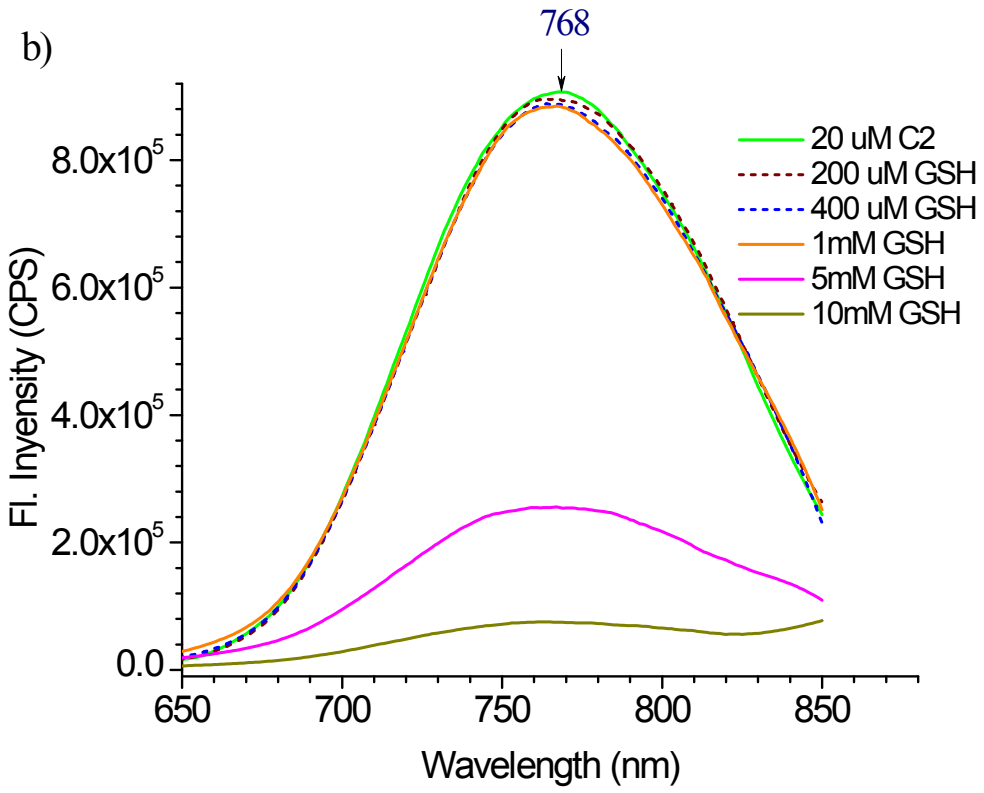


Figure S20. Fluorescence emission spectra of compound **2** (C2): (a) in the presence of one equivalent (10 μM)of different anions in EtOH/PBS (1:1 by v/v) and b) in the presence of different concentration of glutathione (GSH) in EtOH/PBS (1:1 by v/v).

Cell viability test for compound 2:

Biocompatible photophysical properties (NIR absorption and emission) of compound **2** encouraged us to find the application of compound **2** in live cells imaging. Before using in live cells imaging, Cell cytotoxicity of compound **2** was acquired using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay. Normal Human Lung Fibroblasts (NHLF) cells 5×10^3 per well were seeded in a 96-well plate in DMEM low glucose media with 10% FBS and incubated 24 hrs. at 37^0 C with 5% CO_2. After 24 hrs., the cells were starved overnight with 0.4% FBS DMEM low glucose media. The cells were then treated with a series of dilution of compound **2** (0-1 mM; n=8). A final concentration of 0.5 mg/mL of MTT per well was used followed by incubation at 37^0C for 2 hours. The absorbance was detected using Epoch Bio-Tek microplate reader at 570nm, and the IC_{50} value was calculated using GraphPad Prism. The IC50 value for **2** was found to be 24.72 μM.

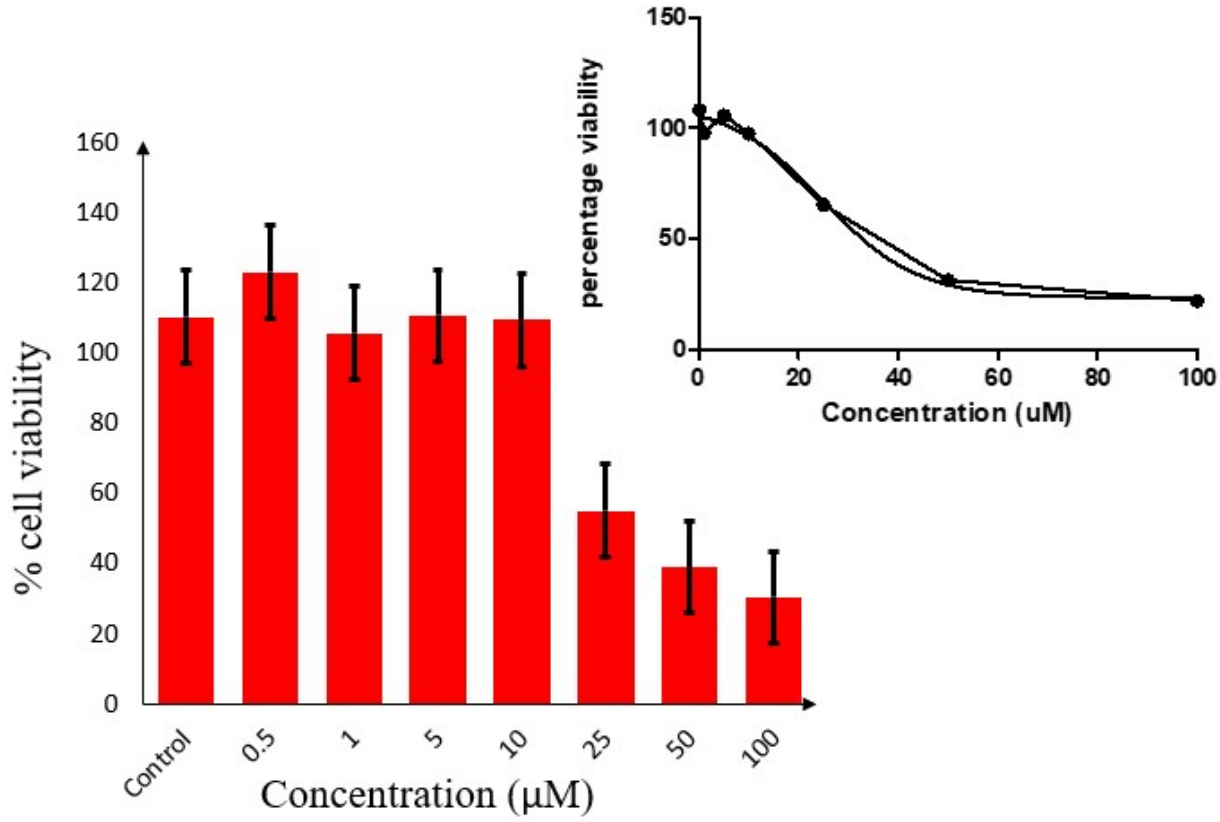


Figure S21. Cell viability test for compound **2** in NHLF cells (IC_{50}) was found to be 24.72 μM.

Cell culture and staining: Normal human lungs fibroblast (NHLF) cells and oligodendrocytes cells were maintained in Dulbecco's modified eagle's medium (DMEM) with 10% FBS at 37^0C in 5% CO_2 humidified incubator. The solution of probe **2** and MitoTracker Green FM was made in DMSO. The final concentration of 1 μM solution of probe **2**, 200 nM concentrations of MitoTracker Green were applied in cells for *in vivo* imaging. For cell studies, the NHLF cells were plated in a separate imaging petri dish with DMEM media and they were treated with probe **2** and Red MitoTracker green for 30 minutes at 37^oC. Cells were then washed three times with 1x PBS.

The excitation wavelength of compound **2** was 640 nm laser with 700 – 735 nm filter for emission. MitoTracker green was excited at 488 nm with a 525/50 nm bandpass emission filter. An Okolab Bold Cage Incubator at 37^oC was used for the incubation of imaging cells, and images were processed using NIS Elements. Fiji ImageJ software was used to calculate the colocalization coefficient of compound **2** with commercial trackers.

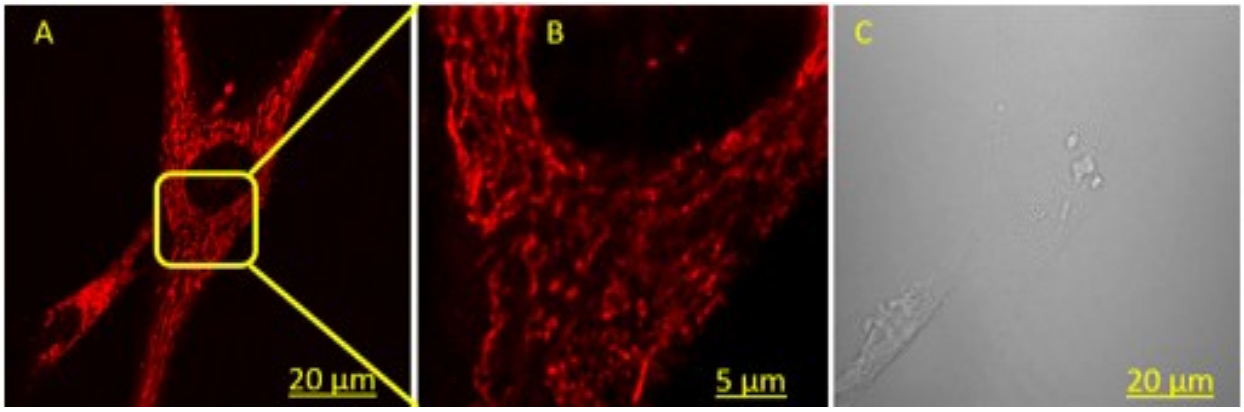


Figure S22. Confocal fluorescence images for normal human lungs fibroblast (NHLF) cells stained with compounds **1b** and **2.** A) **2** under 100x magnification, B) 4 times digitally enhanced image of yellow rectangular box in A, C) bright-field to show cells for compound **2**, D) **1b** under 100x magnification, E) 4 times digitally enhanced image of yellow rectangular box in D and F) bright-field to show cells for compound **1b**. Compound 2 was excited with 640 nm laser with the emission filter of 680-735 nm, and **1b** was excited at 405 nm lesser with emission filters of 680 - 720 nm.

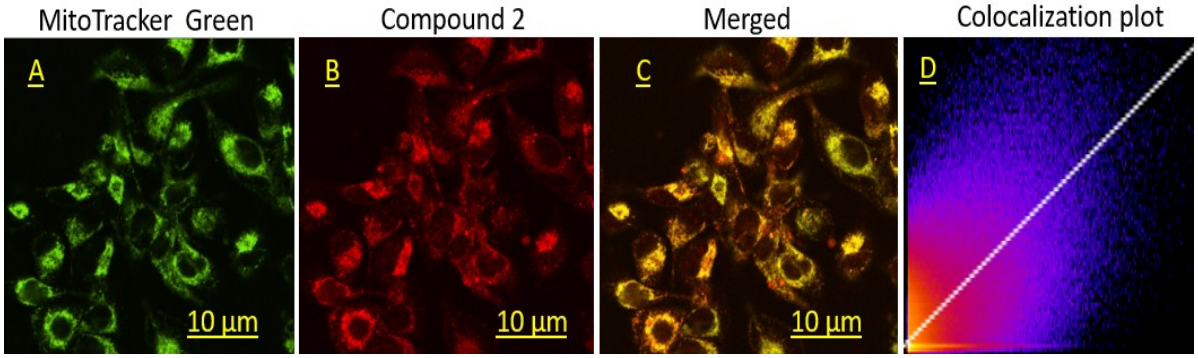


Figure S23. Images of oligodendrocyte cells stained with A) MitoTracker Green (200 *n*M), B) compound **2** (1 µM), C) merged A and B under magnification of 100x and D) correlation plot between Compound **2** and MitoTracker Green. Excitation for MitoTracker Green was 488 nm with the emission of 525 nm, for compound **2** excitation was 640 nm with 680-735 nm filters for emission,

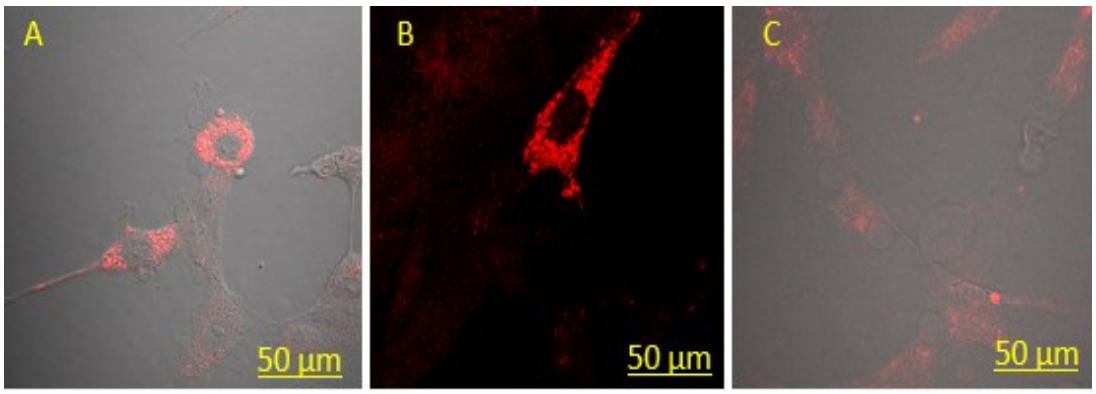


Figure S24. Images of fixed NHLF cells stained with compound **2** (1 µM). A) cells were stained with compound **2** first for 30 minutes and fixed, B) cells were fixed first, permeabilized and stained for 30 minutes and C) cells were fixed and treated with dye for 30 minutes. The compound **2** was excited at 640 nm excitation laser with the emission filter of 680-735 nm.

It was assumed that the probe **2** could be a functional dye to stain mitochondria as it had double positive charges, which would have strong interaction with the negatively charged mitochondrial matrix. In order to shed some light on its staining interaction with mitochondria, we further tested the probe **2** in fixed cells. The probe **2** remained inside the cells in a non-uniform pattern when cells were treated with the dye before fixing them (Figure S24A). After cells being fixed, the cells could be stained with **2** along with permeabilizer (to drive the probe molecules into cells)(Figure S24B). However, compound **2** alone did not show the ability to stain the fixed cells (Figure S24C), as the cellular functions (e.g. *mitochondrial potential gradient* or *endocytosis*) would have been lost upon fixation of cells. Therefore, probe **2** could be considered as a "functional mitochondrial probe" which is accumulated into mitochondria due to the potential gradient ($\Delta\psi_m$) of the mitochondrial matrix.