An NIR-Emitting Lysosome-Targeting Probe with Large Stokes' Shift via Coupling Cyanine and Excited-State Intramolecular Proton Transfer

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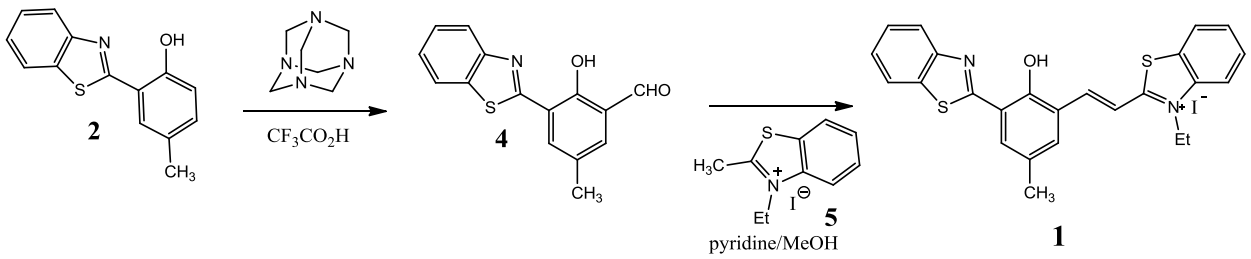
Materials. 2-Hydroxy-5-methylbenzaldehyde (98%) was purchased from Ark Farm. 2-Methylbenzo[d]thiazole (98%) and ethyl iodide were purchased from Alfa Aesar. 2-aminothiophenol (90%) was purchased from Aldrich chemical. Hexamethylenetetramine (99%) was purchased from Acros Organics. And methanol, trifluoroacetic acid and pyridine (all in analytical grade) were purchased from Fisher Scientific. Lyso-Tracker red DND-99 was purchased from ThermoFisher Scientific. All chemicals and reagents were used as received unless otherwise stated. Starting materials 2-(Benzo[d]thiazol-2-yl)-4-methylphenol (**2**) [1] and 4-(2-benzothiazolyl)phenol[2] was synthesized by using literature procedures.

Spectroscopic Measurements. NMR spectra were collected on a Varian 300 Gemini spectrometer. Mass spectrometric data were obtained on a HP1100LC/MSD mass spectrometry. HRMS data were performed on an ESI-TOF MS system (Waters, Milford, MA). UV-Vis spectra were acquired on a Hewlett-Packard 8453 diode-array spectrometer. Fluorescence spectra were obtained on a HORIBA Jobin Yvon NanoLog spectrometer.

1. Experimental Details for Chemical Synthesis

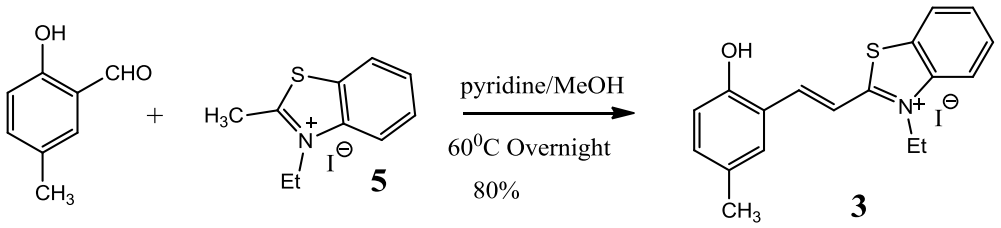


Scheme S1: Synthesis of compound **1**.

Synthesis of 3-(benzo[d]thiazol-2-yl)-2-hydroxy-5-methyl- benzaldehyde (4). To a round-bottomed flask was added compound **2** (0.455 g, 1.7 mmol), hexamethylenetetramine (0.546 g, 3.9 mmol), and trifluoroacetic acid (15 mL). The mixture was refluxed overnight. After the mixture was cooling down, the acid was neutralized with KOH solution. The precipitate was collected by filtration, and washed with water for several times. After drying under vacuum, **4** was obtained in ~100% yield, and had the following spectral properties. ^{1}H NMR (in $CDCl_3$): δ 10.46 (s, 1H), δ 8.01 (d, 1H), δ 7.90 (t, 2H), δ 7.68 (s, 1H), δ 7.53 (t, 1H), δ 7.44 (t, 1H), δ 7.26 (s, $CDCl_3$), δ 2.40 (s, 3H).

Synthesis of compound 2-[3-(benzo[d]thiazol-2-yl)-2-hydroxy-5-methylstyr-yl]-3-ehtylbenzo[d]thiazol-3-ium iodide (1). To the methanol solution (20 mL) of **5** (298 mg, 0.976 mmol) was added compound **4** (300 mg, 1.118 mmol) and pyridine (0.5mL). The resulting mixture was heated to reflux overnight. After removing the solvent on a rotary evaporator, the solid residue was collected by filtration, washed with ethyl acetate (50 mL), and dried under vacuum to give brown solid product **1** in 85% yield (melting point 239^{0}C -240^{0}C). The product had the following spectral properties. ^{1}H NMR (d_6-DMSO,): δ 13.35 (s, 1H), δ 8.42 (d, 1H), δ 8.34 (s, 1H), δ 8.30 (d, 1H), δ 8.21(d, 1H), δ 8.15 (s, 1H), δ 8.09 (d, 1H), δ 7.97 (s, 1H), δ 7.89 (t, 1H), δ 7.80 (t, 1H),

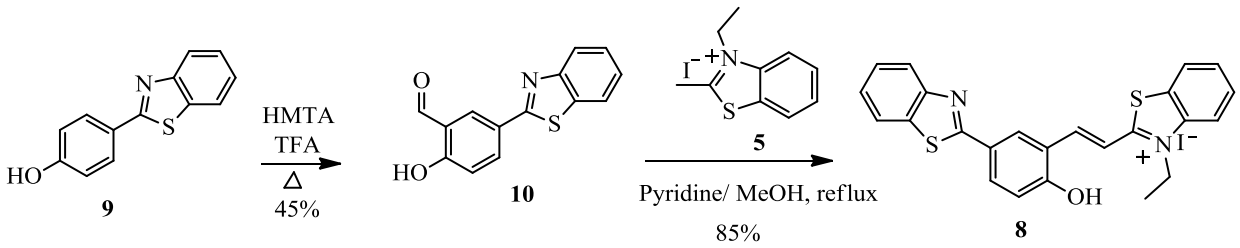
δ 7.62 (t, 1H), δ 7.53 (t, 1H), δ 4.98 (q, 2H), δ 2.49 (s, 6H), δ 2.44 (s, 3H), δ 1.52 (t, 3H). HRMS (m/z): $[M-I]^+$ calcd for $C_{25}H_{21}N_2OS_2$, 429.1095; found, 429.0998.



Scheme S2: Synthesis of compound **3**.

Synthesis of 3-ethyl-2-(hydroxy-5-methylstyryl)benzo[d]thiazol-3-ium iodide (3).

To a 50 mL round-bottomed flask was added compound **5** (92 mg, 0.301 mmol), methanol (20 mL), 2-hydroxy-5-methylbenzaldehyde (50 mg, 0.367 mmol) and 0.5 mL of pyridine. The mixture was refluxed overnight at 60^0C. After completing reaction, solvent was evaporated on a rotary evaporator and the solid residue was washed with ethyl acetate (50 mL), filtered and dried under vacuum to give 0.127 g **3** as yellow solid (isolation yield ~80%). ^{1}H NMR (d_6-DMSO): δ 10.62 (s, 1H), δ 8.36 (d, 1H), δ 8.29 (s, 1H), δ 8.24 (d, 1H), 7.852 (m, 4H), 7.21 (d, 1H), δ 6.91 (d, 1H), δ 4.91 (q, 2H), δ 2.28 (s, 3H), δ 1.46 (t, 3H). HRMS (m/z): $[M-I]^+$ calcd for $C_{18}H_{18}NOS$, 296.1109; found, 296.1154.



Scheme S3: Synthesis of compound **8**.

Synthesis of compound 5-(benzo[d]thiazol-2-yl)-2-hydroxybenzaldehyde (10). To a 100 mL round-bottomed flask was added 4-(2-benzothiazolyl)phenol[2] (**9**) (200 mg, 0.88 mmol), HMTA (250 mg, 1.8 mmol) and 40 ml of TFA. The mixture was reflux for 18 hours. The reaction mixture was cooled down to room temperature and pH was adjusted to 6.5 by using NaOH with occasional cooling. Crude product (as yellowish solid) was collected by vacuum filtration. The product was further purified on a silica gel column with MeOH/dichloromethane (2:98) eluent to afford **10** as a light yellow powder (100 mg, 45% yield). ^{1}H NMR ($CDCl_3$): δ 11.29 (broad, 1H), δ 10.04 (s, 1H), δ 8.36 (s, 1H), δ 8.22 (d, 1H), δ 8.08 (d, 1H), δ 7.92 (d, 1H), δ 7.51 (m, 1H), δ 7.41 (m, 1H), δ 7.26 (s, 1H), δ 7.15 (d, 1H).

Synthesis of compound 2-[5-(benzo[d]thiazol-2-yl)-2-hydroxystyryl]-3-ehtylbenzo[d]thiazol-3-ium iodide (8). To a 50 mL round-bottomed flask was charged with 3-ethyl-2-methylbenzo[d]thiazolium iodide **5** (50 mg, 0.164 mmol), 20 ml methanol and 0.5 ml of pyridine, and 5-(benzo[d]thiazol-2-yl)-2-hydroxybenzaldehyde **10** (48 mg, 0.188 mmol). The mixture was refluxed overnight at 60^{0}C. After removing the solvent on a rotary evaporator, the residue was washed with ethyl acetate (~50 mL) to give **8** as a yellow-greenish solid. ^{1}H NMR (d_6-DMSO): δ 11.78 (s, 1H), δ 8.41 (t, 2H), δ 8.33 (d, 2H), δ 8.23 (s, 1H), δ 8.12 (m, 2H), δ 8.01 (d, 1H), δ 7.88 (t, 1H), δ 7.79 (t, 3H), δ 7.53 (t, 1H), δ 7.44 (t, 1H) δ 7.25 (d, 1H), δ 4.95(q, 2H), δ 2.48 (s, 6H), δ 1.50 (t, 3H). HRMS (m/z): $[M-I]^+$ calcd for $C_{24}H_{19}N_2OS_2$, 415.0939; found, 415.0969.

References

1. Wang, J.; Chen, W.; Liu, X.; Wesdemiotis, C.; Pang, Y. *J. Mater. Chem. B.* **2014,** *2*, 3349-3354.
2. Puranik, N. V.; Puntambekar, H. M.; Srivastava, P. *Medicinal Chemistry Research* **2016**, 25, 805-816.

2. NMR and Mass Spectra:

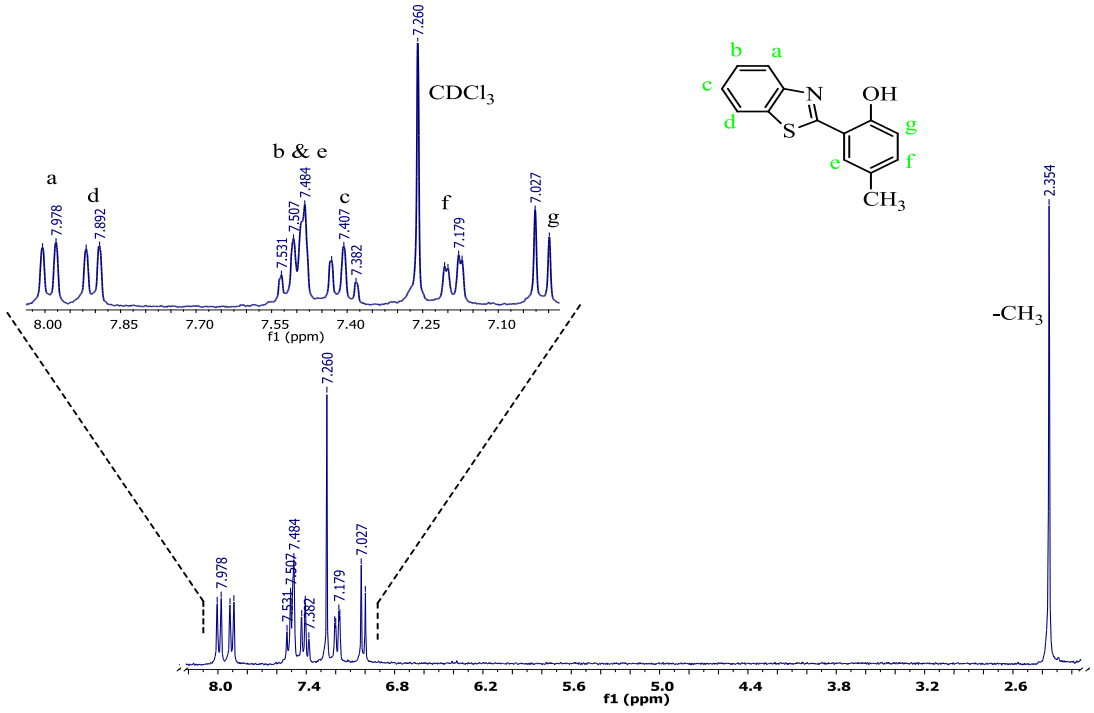


Figure S1: ^{1}H NMR of **2** in $CDCl_3$. The top inset is expanded region from 7.00 ppm to 8.10 ppm for clarity.

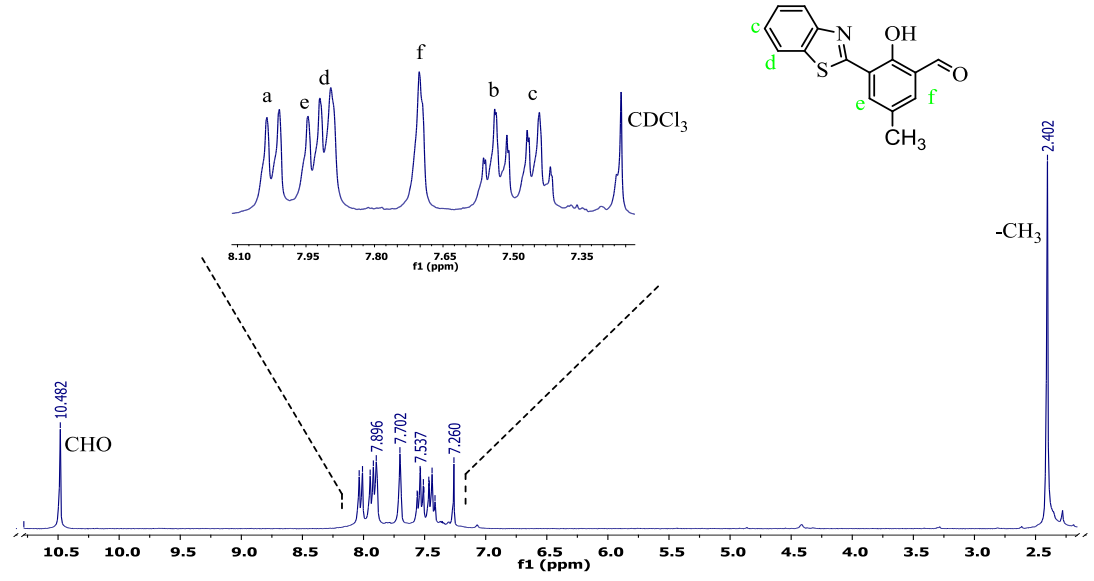


Figure S2: ^{1}H NMR of **4** in $CDCl_3$. The top inset is expanded region from 7.25 ppm to 8.21 ppm.

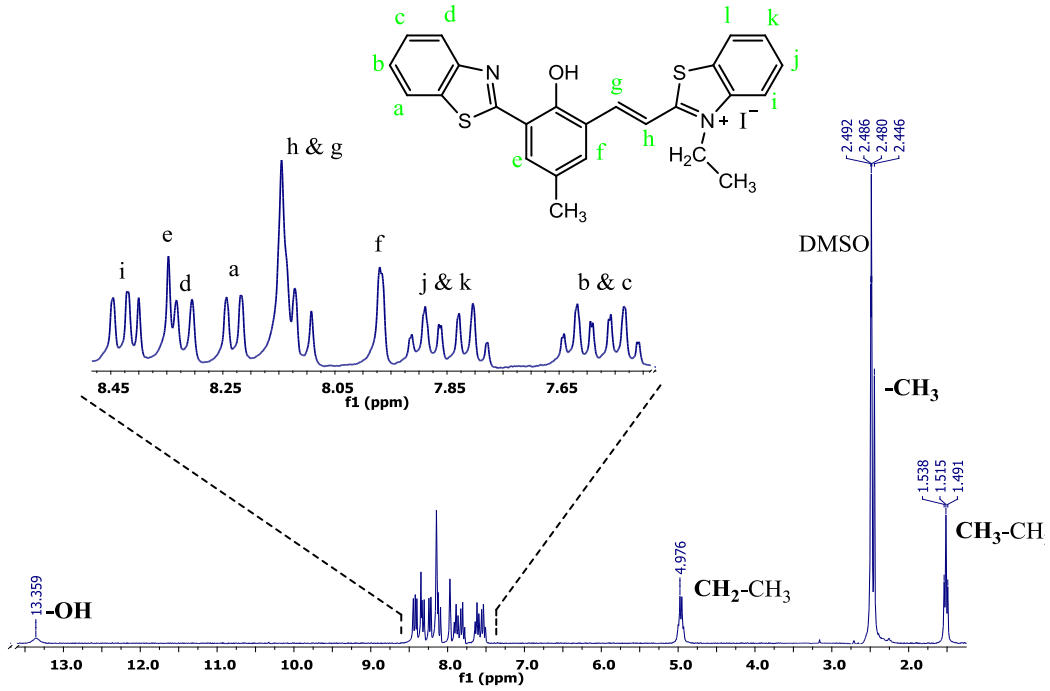


Figure S3: ^{1}H NMR spectra of **1** in d_6-DMSO. The top inset shows the expanded region from7.4 ppm to 8.7 ppm.

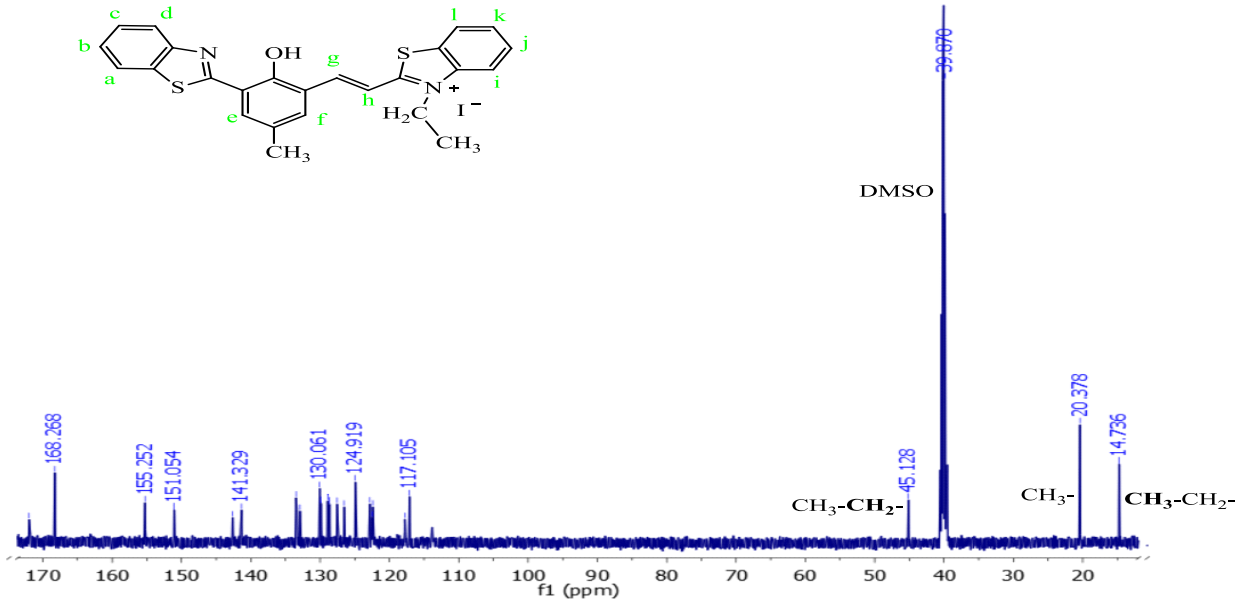


Figure S4: ^{13}C NMR spectra of **1** taken in 500 MHz NMR Spectrometer in d_6-DMSO.

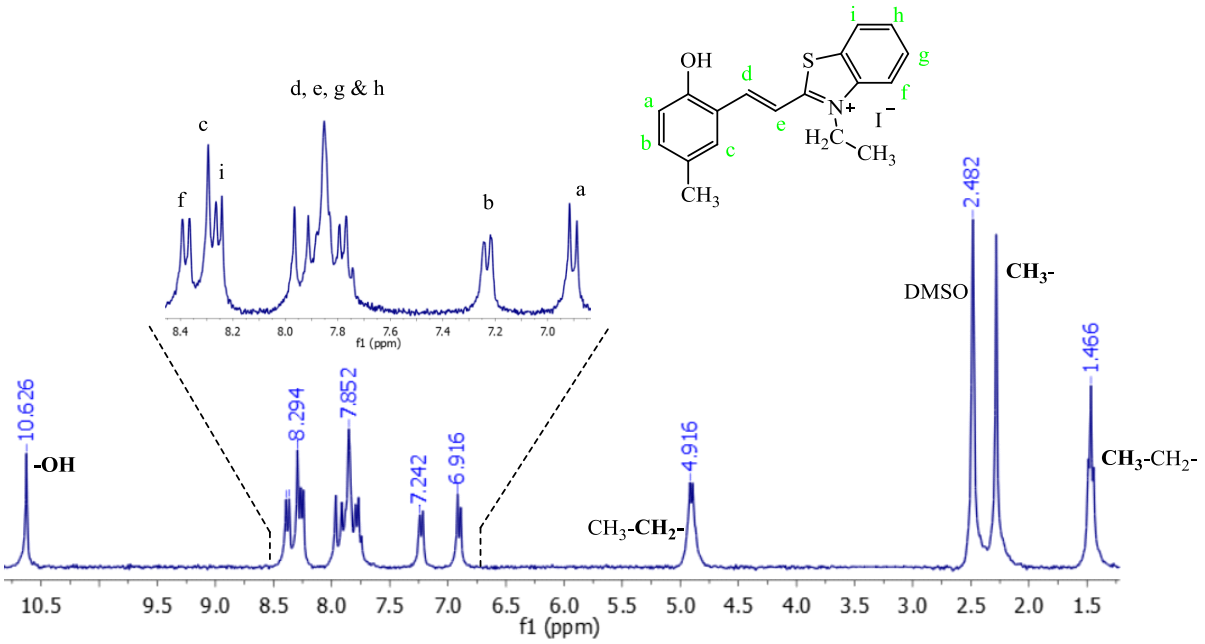


Figure S5: ^{1}H NMR spectra of **3** in d_6-DMSO. Top inset is expanded region from 6.8 ppm to 8.5 ppm for clarity

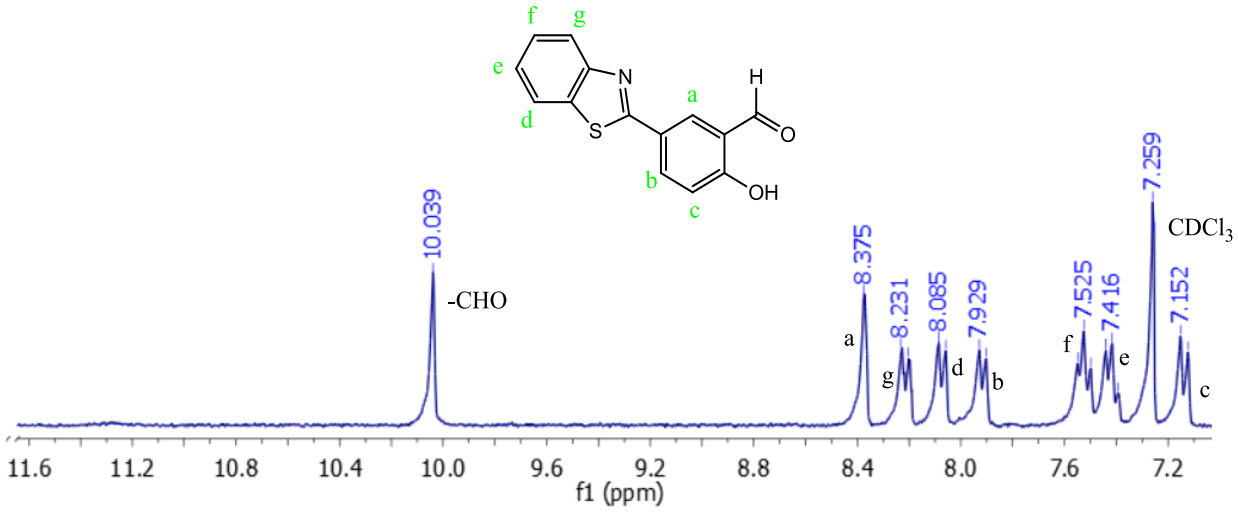


Figure S6: ^{1}H NMR spectra of **10** in $CDCl_3$.

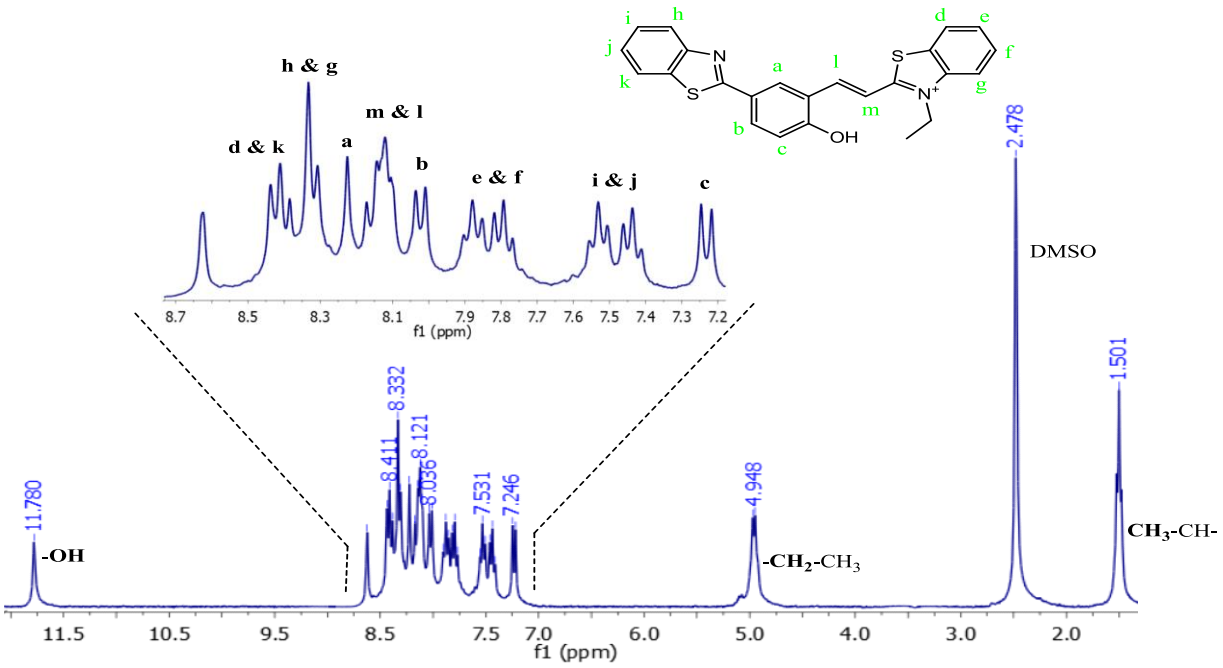


Figure S7: ^{1}H NMR spectra of **8** in d_6-DMSO. Top inset shows the expanded region from 7.18 to 8.66 ppm.

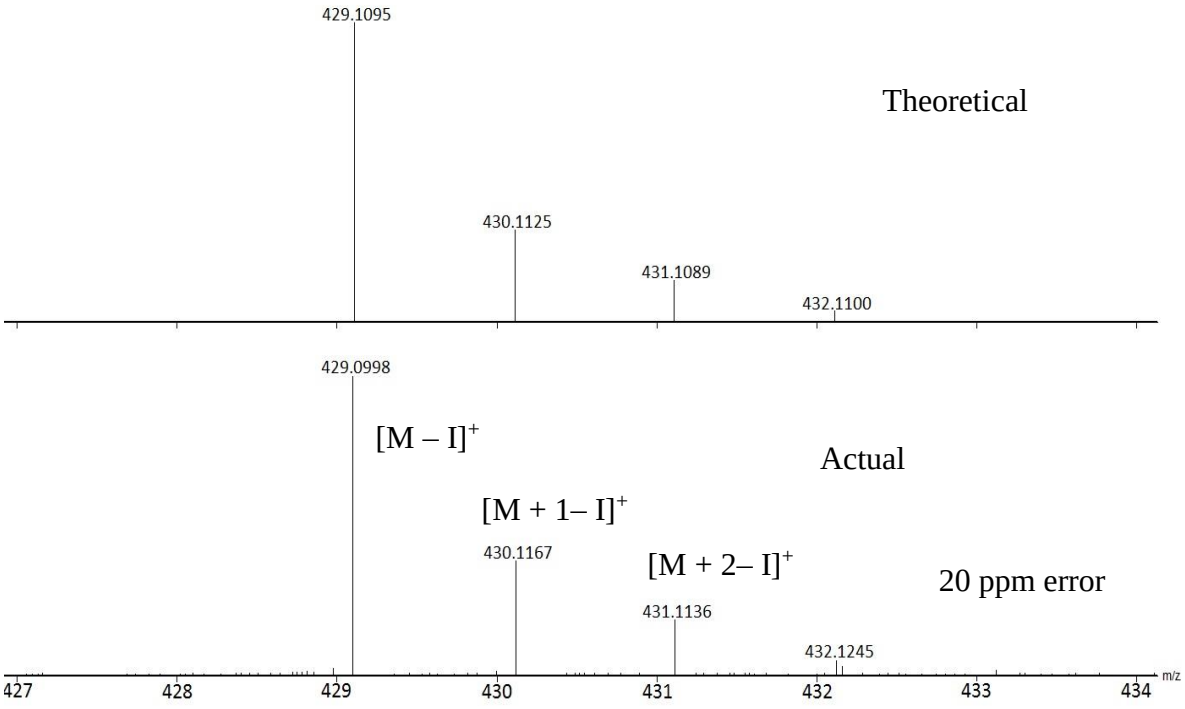


Figure S8: TOF MS ES+ Mass Spectra of **1**.

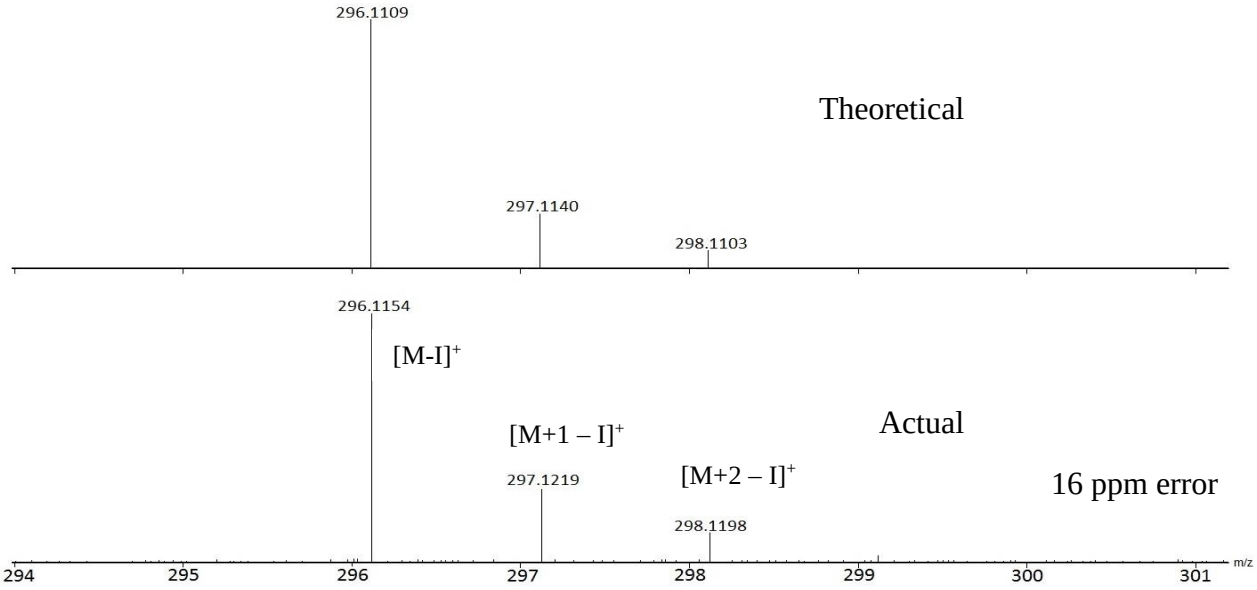


Figure S9: TOF MS ES+ Mass Spectra of **3**.

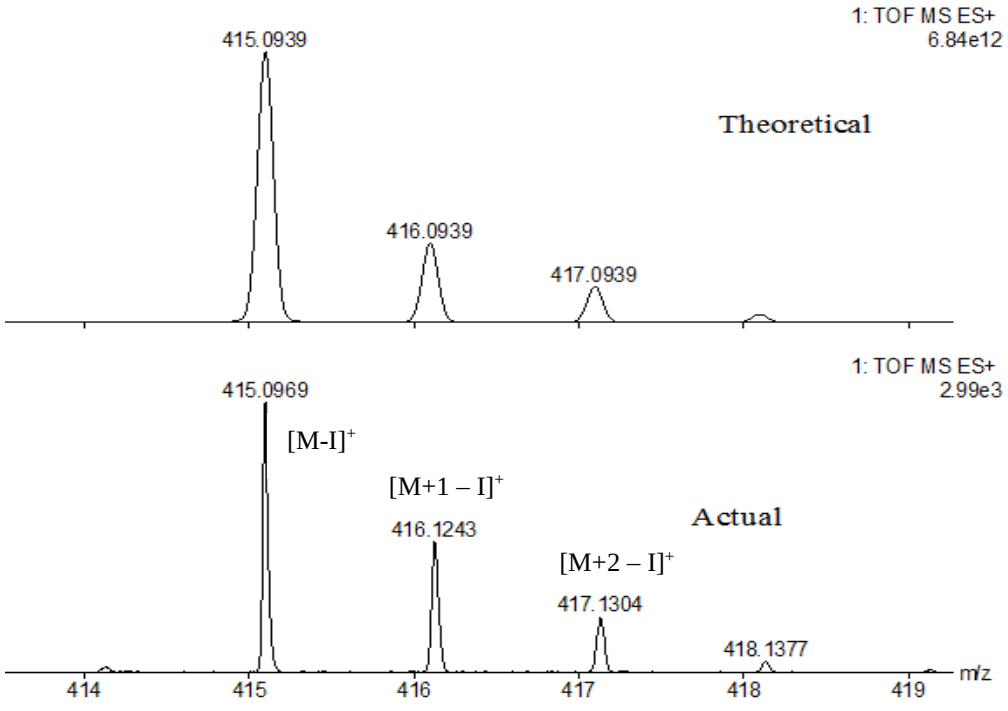


Figure S10: TOF MS ES+ Mass Spectra of **8**.

3. Optical Spectra:

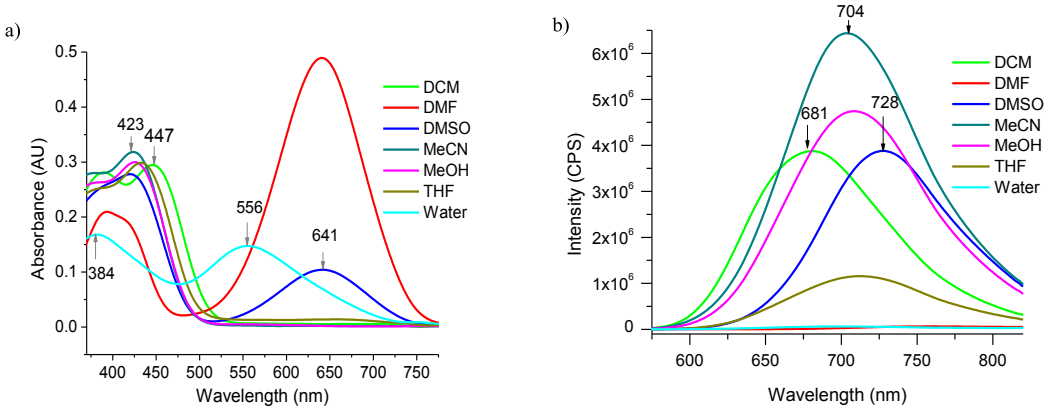


Figure S11: (a) UV- absorption and (b) fluorescence emission spectra of compound **1** in different solvents (concentration 10 μM). The emission spectra were acquired by exciting the compound's solution at 423 nm in different solvents.

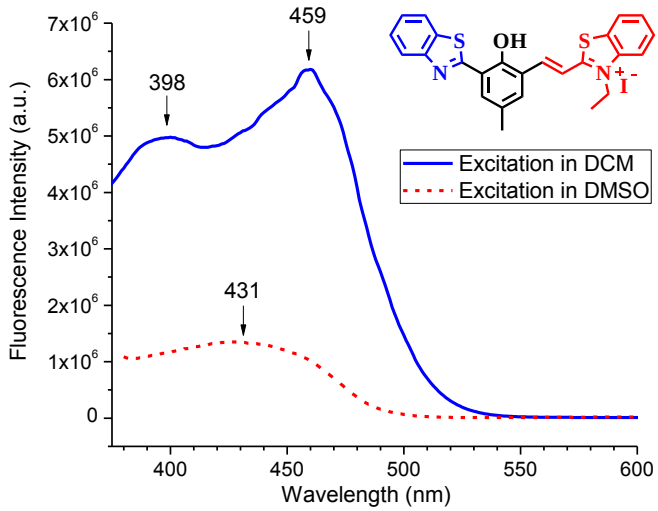


Figure S12: Excitation spectrum of **1** in dichloromethane (DCM) and DMSO.

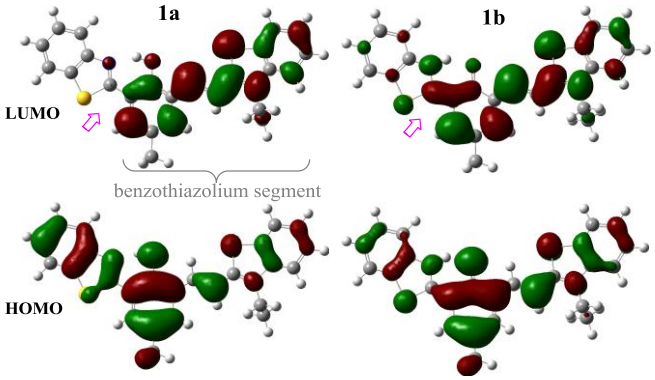


Figure S13(a): Calculated molecular HOMO-LUMO orbitals for **1a** and **1b** at the B3LYP/6-31+G(d) level. Double arrows indicate the orbital overlap between the benzothiazole and phenol unit. The impact of ESIPT was clearly visible. In the LUMO orbital of **1a**, the electrons were localized on the benzothiazolium cyanine segment, showing that the benzothiazole segment had little contribution to the excited state. The result was in agreement with the experimental finding that the absorption of **1a** was determined mainly by the benzothiazolium cyanine segment, and the effective chromophore was approximated by **3** (Scheme 1 & Figure 1). In the LUMO orbital of *keto* tautomer **1b**, however, the electron was delocalized beyond benzothiazolium cyanine segment, whose emission would occur at a longer wavelength (than the excited **1a**).

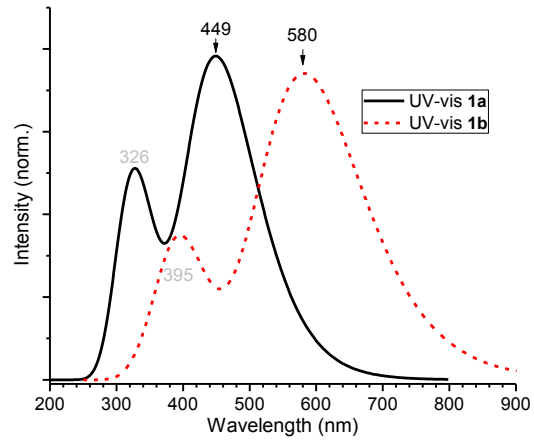


Figure S13 (b): The calculated absorption spectra of *enol* (**1a**) and *keto* tautomer (**1b**) at the B3LYP/6-31+G(d) level in (CH_2Cl_2). The spectra were generated by using TD-SCF method, after the molecular geometry was optimized at the B3LYP/6-31+G(d) level. The calculated absorption maximum for the *enol* form **1a** (λ_{abs}=449 nm) closely matched the experimental value (λ_{abs}=447 nm) in CH_2CH_2 solvent. The calculated absorption for the *keto* form **1b** (λ_{abs}=580 nm) was red-shifted drastically by ~130 nm to a longer wavelength. The results supported the assumption that ESIPT event was responsible for the large Stokes' shift from **1**, as the *keto* tautomer **1b** was only generated in the excited state.

Determination of pK_a for Compound 1:

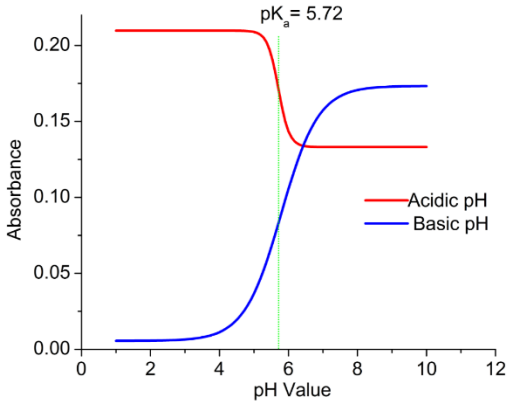


Figure S14(a): Boltzmann's fitting for the plot of absorbance at λ_1 (acidic pH) and at λ_2 (basic pH) at different pH for the compound **1**. The pK_a from the plot is 5.72.

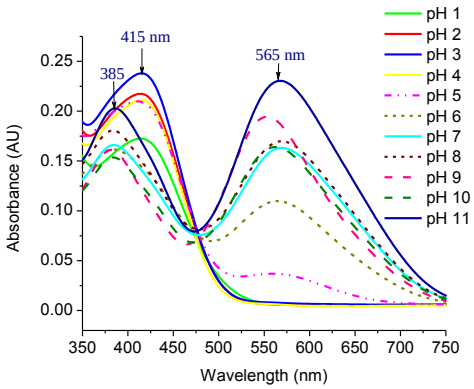


Figure S14(b)**.** Absorbance spectra of compound **1** in water at different pH.

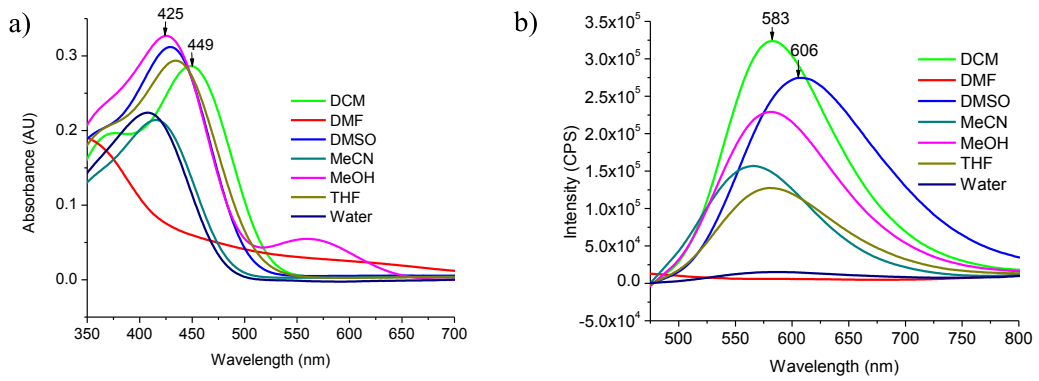


Figure S15: (a) UV- absorption spectra and (b) fluorescence emission spectra of compound **3** in different solvents.(Concentration of the solution 10 μM). The emission spectra were acquired by exciting the solution at 449 nm in DCM and at 425 nm in other solvents.

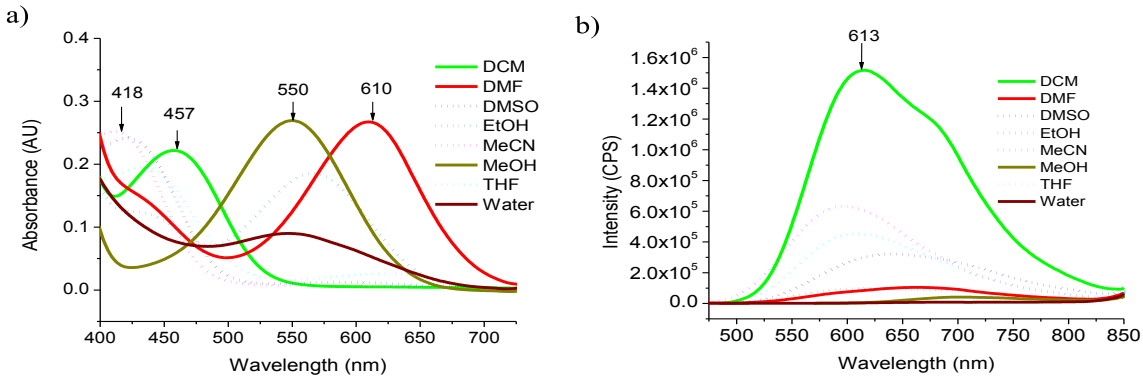


Figure S16: (a) UV-vis absorption spectra and (b) fluorescence emission spectra of compound **8** in different solvents (concentration 10 μM). Excitation wavelengths (DCM 457 nm, DMF 450 nm, DMSO 450 nm, EtOH 457 nm, MeCN 418 nm, MeOH 550 nm, THF 450 nm and Water 450 nm for all solvents except in DCM which was excited at 447 nm.

4. Quantum Yield Calculation:

The quantum yield of compounds were calculated with reference to rhodamine 6G (Φ= 0.95 in ethanol). Fluorescence emission of compounds in different solvents and rhodamine 6G in ethanol were measured under identical conditions of slits while **1** and **3** were excited at absorption maximum λ_{abs}. The quantum yield was calculated using the relation:

$$\Phi_s = \Phi_r . \frac{I_s}{I_r} . \frac{A_r}{A_s} . \frac{{\eta_s}^2}{{\eta_r}^2}$$

where, subscript r and s represent reference and samples respectively, Φ = Quantum yield, I = Integrated area of fluorescence emission, A= Absorbance at $\lambda_{excitation}$ and η = Refractive index of solvents.

5. Studies in Biological Cells

Cell culture. Normal human lung fibroblasts (NHLF) (CC-2512) were obtained from LONZA (Walkersville, MD). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA), 100 units/ml penicillin-streptomycin, 2 mM L-glutamine (Invitrogen) and maintained at 37°C in a humidified 5% CO2 environment. Cells from passage 6 to 8 were used.

Cell Staining. NHLF cells were cultured on MatTek glass bottom dishes to confluency (150,000). Cells were loaded with LysoTracker (1 μM) (Life Technologies, Carlsbad, CA) for 1 hour in growth medium, washed 3 times with PBS and resuspended in Live Cell Imaging Solution (Life Technologies).

CI-50 of Compound **1:**

MTT Assay. Cytotoxicity was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells (Normal Human Lung Fibroblasts) 1×10^3 per well were seeded in a 96-well plate in DMEM media with 10% FBS and incubated overnight at 37^0 C with 5% CO_2. The cells were then treated with a series of dilution of compound **1** (0-1 mM) (n=8). MTT was used at a final concentration of 0.5 mg/ml per well followed by incubation at 37^0 C for 2 hours. The insoluble tertrazolium salt was dissolved by addition of (150µL) DMSO. The absorbance was detected using Epoch Bio-Tek microplate reader at 570nm to calculate the IC_{50} value.

Table 1: MTT Assay data for cell viability measurement.

Concentration (mM)	Absorbance	Stdev	SEM	Log Abs
0	0.276	0.069025875	0.024404332	-0.559090918
0.000976563	0.2725	0.068448103	0.024200059	-0.564633493
0.001953125	0.249	0.0595483	0.021053503	-0.603800653
0.00390625	0.178875	0.037498333	0.013257663	-0.747450353
0.0078125	0.13125	0.024034202	0.008497374	-0.881900688
0.015625	0.10925	0.014772078	0.005222718	-0.961578554
0.03125	0.06325	0.00795972	0.002814186	-1.19893947
0.0625	0.050125	0.003603074	0.001273879	-1.299945614
0.125	0.04975	0.005035588	0.001780349	-1.303206915
0.25	0.0475	0.004035556	0.001426785	-1.32330639
0.5	0.048375	0.003461523	0.001223833	-1.315379022
1	0.046375	0.002825269	0.000998883	-1.333716077
				CI50=31.25µM

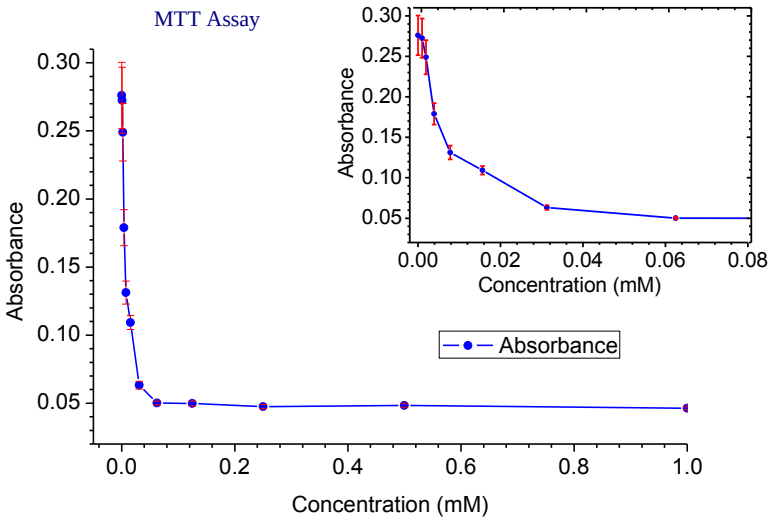


Figure S17: Plot of absorbance vs concentration for MTT assay. The inset shows the expanded region at low concentration.

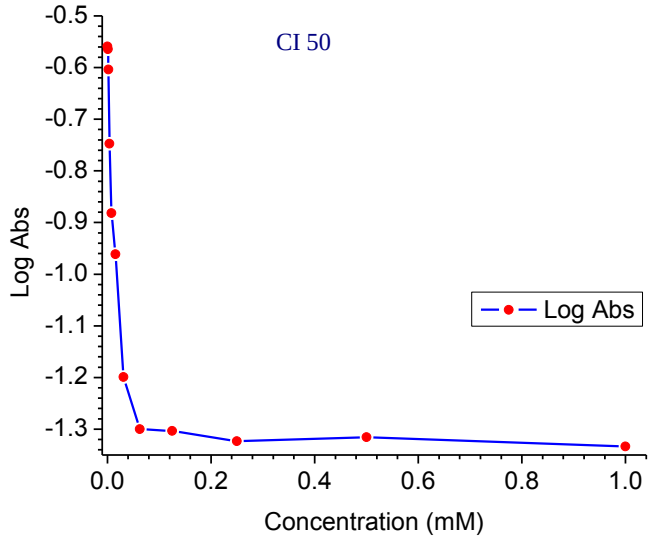


Figure S18: Plot of Log(Absorbance) vs concentration.

Cell images for Compound 1:

Imaging was performed on a Nikon A1 confocal system with a 100x Plan Apo λ, NA=1.45 oil objective with both GaAsP dectectors and high sensitivity low noise PMTs for detection. The excitation used for compound **1** was 405 nm with 680-720 nm filter for emission. The LysoTracker

was excited using 577 nm and with a 590/50 nm bandpass filter used for emission. All imaging was done in an Okolab Bold Cage Incubator at 37°C, and images were processed using NIS Elements or Image J Pro imaging software.

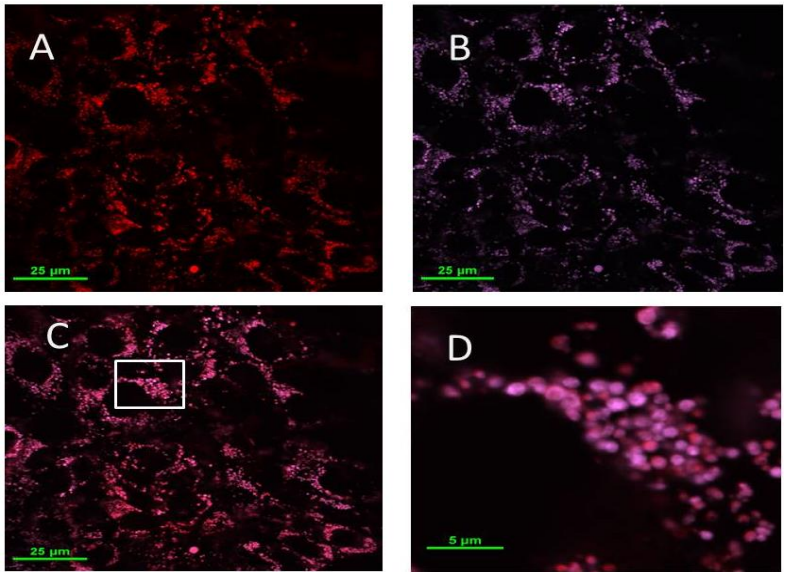


Figure S19: Images of a mouse endothelial cell (mec) with (A) LysoTracker, (B) Compound **1**, (C) overlap in 100x and (D) Enclosed portion of 'C' digitally enhanced by 526x. Excitation for compound **1** is 405 nm with 680-720 nm filters for emission, and the LysoTracker has an excitation/emission of 577/590.