

# Red-emitting pyrene-benzothiazolium: Unexpected selectivity to lysosomes for real-time cell imaging without alkalinizing effect

Chathura S. Abeywickrama<sup>a</sup>, Kaveesha J Wijesinghe<sup>c</sup>, Robert V. Stahelin<sup>d</sup> and Yi Pang<sup>a,b\*</sup>

<sup>a</sup> Department of Chemistry & <sup>b</sup>Maurice Morton Institute of Polymer Science, University of Akron, Akron, Ohio 44325, USA.

<sup>c</sup> Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana, USA.

<sup>d</sup> Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, Indiana, 47907, USA.

## Supplementary Information

---

\*Corresponding Author

## Materials and Methods

All chemicals for synthesis were purchased from Sigma-Aldrich and Fisher Scientifics. All molecular biology grade reagents for cell culture and fluorescent confocal microscopy were purchased from Sigma-Aldrich, Fisher scientific, and Abcam. UV-vis studies were carried out in Hewlett Packard-8453 diode array spectrophotometer at 25°C. Fluorescence studies were carried out in HORIBA Fluoromax-4 spectrofluorometer. Fluorescence confocal images were obtained from Zeiss LSM 710 confocal microscope. Cell viability assays were carried out by using Spectramax® M5e multimode microplate reader. *Cercopithecus aethiops* (African green monkey kidney cells) Kidney cells (COS-7), Adeno-carcinomic human alveolar basal epithelial cells (A549), Human hepatocyte-derived carcinoma cells (Huh 7.5) and Human embryonic kidney cells 293 (HEK 293) were used for cell studies.

**General procedure for synthesis (2).** To pyrene-1-carboxaldehyde **4** (1.1 mmol) in 15ml of methanol, appropriate benzothiazolium salt **5** (1 mmol) was added and the solution was stirred at room temperature for 5 minutes. Then pyridine 0.2 ml was added, and solution was heated up and stirred at 65° C for 24 hours. After completion of the reaction, the mixture was cooled down to room temperature and concentrated under the vacuum. To resulting brown color solid ethyl acetate (10 ml) was added and the solution was let settled for 30 minutes. Then brown color solid was collected by vacuum filtration and washed with ethyl acetate (10 ml) 3 times. The desired products **2a – 2d** was collected as a fine dark brown powder with 68 -76% yield.

**Characterization of 2a.** Was collected as a brown color powder with 68% yield.  $^1\text{H}$  NMR (300 MHz in DMSO)  $\delta$  9.14 (d, 1H)  $\delta$  8.83 (dd 2H),  $\delta$  8.47 (m, 5H),  $\delta$  8.40 (m, 4H),  $\delta$  8.29 (t, 1H),  $\delta$  7.85 (dd, 2H), and  $\delta$  4.43 (s, 3H).  $^{13}\text{C}$  NMR (400 MHz in DMSO)  $\delta$ : 171.82, 149.29, 144.61, 142.48, 137.59, 134.20, 131.22, 130.35, 129.95, 129.95, 129.92, 128.94, 128.53, 128.53, 127.84, 127.43, 127.41, 127.16, 126.16, 125.92, 124.61, 124.46, 123.08, 117.33, 115.88 and 36.79. HRMS (ESI) found (m/z) for  $[\text{M}^+]$  376.1173, 377.1453 and 378.1674. Calculated (m/z) were 376.1160, 377.1160 and 378.1160.

**Characterization of 2b.** Was collected as a dark brown color powder with 72% yield.  $^1\text{H}$  NMR (300 MHz in DMSO)  $\delta$  9.21 (d, 1H),  $\delta$  8.97 (d 1H),  $\delta$  8.83 (d 1H),  $\delta$  8.39 (m, 5H),  $\delta$  8.28 (m, 4H),  $\delta$  8.16 (t, 1H),  $\delta$  7.85 (dd, 2H),  $\delta$  5.05 (q 2H), and  $\delta$  1.53 (t, 3H).  $^{13}\text{C}$  NMR (400 MHz in DMSO)  $\delta$ : 171.25, 145.11, 141.32, 134.25, 131.16, 130.73, 130.51, 130.35, 130.07, 129.95, 128.93, 128.91, 127.61, 127.42, 127.36, 127.32, 127.16, 126.35, 125.85, 124.80, 124.38, 123.90, 122.96, 117.10, 114.92, 46.07 and 14.91. HRMS (ESI) found (m/z) for  $[\text{M}^+]$  390.1345, 391.1317 and 392.1312. Calculated (m/z) were 390.1317, 391.1317 and 392.1317.

**Characterization of 2c.** Was collected as a brown color powder with 74% yield.  $^1\text{H}$  NMR (300 MHz in DMSO)  $\delta$  9.20 (d, 1H),  $\delta$  8.96 (d 1H),  $\delta$  8.85 (d 1H),  $\delta$  8.47 (m, 5H),  $\delta$  8.36 (m, 4H),  $\delta$  8.17 (t, 1H),  $\delta$  7.87 (dd, 2H),  $\delta$  5.00 (q 2H),  $\delta$  1.96 (m 2H), and  $\delta$  1.04 (t, 3H).  $^{13}\text{C}$  NMR (400 MHz in DMSO)  $\delta$ : 171.81, 145.14, 141.75, 134.25, 130.73, 130.52, 130.36, 129.95, 129.95, 128.95, 127.84, 127.64, 127.44, 127.38, 127.17, 127.17, 126.37, 125.91, 125.87, 124.76, 124.39, 123.91, 122.99, 117.38, 115.25, 50.65, 22.84 and 10.78. HRMS (ESI) found (m/z) for  $[\text{M}^+]$  404.1479, 405.1765 and 406.1914. Calculated (m/z) were 404.1473, 405.1473 and 406.1473.

**Characterization of 2d.** Was collected as a brown color powder with 76% yield. <sup>1</sup>H NMR (300 MHz in DMSO) δ 9.28 (d, 1H), δ 8.96 (dd 2H), δ 8.47 (m, 7H), δ 8.24 (m, 3H), δ 7.85 (dd, 2H), δ 7.42 (m, 5H) and δ 6.43 (s 2H). <sup>13</sup>C NMR (400 MHz in DMSO) δ: 173.04, 145.91, 141.76, 134.45, 134.40, 130.90, 130.51, 130.18, 130.18, 130.02, 130.02, 130.02, 129.60, 129.03, 128.96, 128.96, 128.96, 128.91, 127.83, 127.57, 127.53, 127.42, 127.41, 127.38, 127.26, 126.24, 125.91, 124.98, 124.39, 117.50, 115.19 and 51.81. HRMS (ESI) found (m/z) for [M<sup>+</sup>] 452.1421, 453.1673 and 454.1780. Calculated (m/z) were 452.1473, 453.1473 and 454.1473.

**Fluorescence quantum yield calculation.** The fluorescence quantum yields ( $\phi_{fl}$ ) for compounds were calculated by using Rhodamine 6G as the standard. Where  $\phi_{fl}$  Rhodamine 6G is 0.95 in ethanol. The following equation was used for calculation quantum yields for compounds **2a** and **2d** at 490 nm.

$$(\phi_{fl})_{\text{sample}} = \phi_{\text{ref}} \times (A_{\text{ref}}/A_{\text{sample}}) \times (I_{\text{sample}}) / (I_{\text{ref}}) \times (\eta_{\text{sample}})^2 / (\eta_{\text{ref}})^2$$

A = absorbance

I = Integrated fluorescence intensity

$\eta$  = refractive index of the solvent

**Cell Culture.** COS-7 and HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 1-% FBS and 1% Penstrep at 37° C in a 5% CO<sub>2</sub> humidified incubator. A549 and Huh 7.5 cells were maintained in Roswell Park Memorial Institute medium (RPMI) (Invitrogen) containing 1-% FBS and 1% Penstrep at 37° C in a 5% CO<sub>2</sub> humidified

incubator. The cells were grown to 70 to 80% confluence before passing or transfection. Live cell imaging plates prepared by plating cells in MatTek chambered cell culture plates.

**Cell treatments with fluorescent probes.** Probes **2a – 2d**, LysoTracker® Red DND-99, LysoTracker® Green DND-26 and MitoTracker® Green FM solutions were made in DMSO. For live cell imaging cells were treated with 100 - 500 nM probes **2a – 2d** (final concentration) in PBS for 30 minutes at 37° C. During co-localization studies, cells were treated with 70 nM LysoTracker® Red or LysoTracker® Green. Final concentration of the MitoTracker® Green was maintained at 200 nM concentration for co-localization studies. Final DMSO percentage in cell media was < 0.25% (V/V). For initial cell studies probes (**2a – 2d**) treated cells were used for fluorescent confocal imaging without further washing. During colocalization studies with LysoTracker® or MitoTracker® probes, cells were washed with 1x PBS for 3 times.

**Fluorescence confocal microscopy.** Cells were imaged by using Zeiss LSM 710 fluorescence confocal microscope with an oil 63 x 1.4 numerical aperture objective. LysoTracker® Green DND-26 and MitoTracker® Green -FM were excited with 488 nm laser and emission was collected from 500 nm to 580 nm range. Probes **2a – 2d** and LysoTracker® Red DND-99 were excited with 561 nm laser and emission was collected from 585 nm to 700 nm range. Co-localization experiment results were further analyzed by using Zeiss LSM software and Mander's overlap coefficient was calculated by ImageJ (NIH) software. Mander's overlap coefficient values greater than 0.6 confirms an acceptable significant co-localization of the two fluorophores.

**Long-term imaging ability assessment of the probes.** Zeiss LSM 710 fluorescence confocal microscope with identical settings were used for photostability comparison of the probes (561

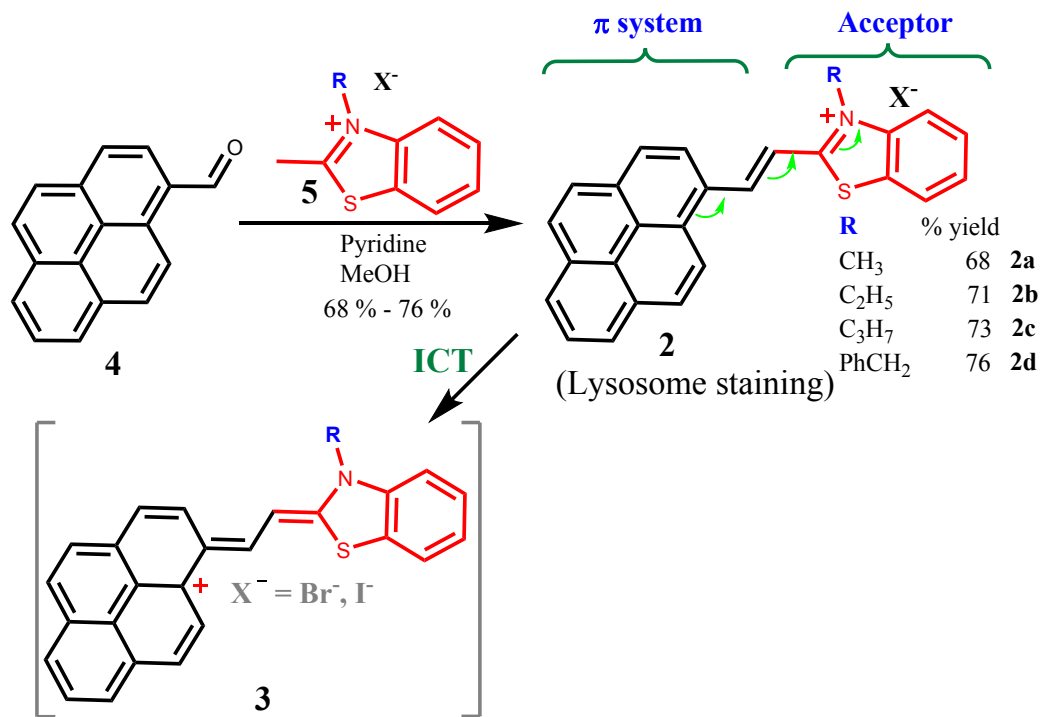
*nm (15 mW) laser line; Laser power percentage 1.0; Digital zoom = 1; Pinhole = 1AU; Master Gain = 500; Digital offset = 0*). Two different imaging plates with A549 cells were incubated with probe **2b** (500 nM) and LysoTracker® Red DND-99 (70 nm) for 30 minutes separately (Probes were washed with 3 x (1X) PBS after 30 minutes. Fluorescence confocal microscopy images were obtained at 30 minute intervals (Identical fluorescence microscopy settings were used during the experiments). ImageJ (NIH) software was used to analyze the obtained images and average relative fluorescence intensity vs plotted as a function of time.

**Photostability assessment of the probes.** Zeiss LSM 710 fluorescence confocal microscope with identical settings were used for photostability comparison of the probes (*561 nm (15 mW) laser line; Laser power percentage 3.0; Digital zoom = 1; Pinhole = 1AU; Master Gain = 700; Digital offset = 0*). Probe **2b** (100 nM), **2d** (100 nM) and LysoTracker Red (100 nM) were used for time stability experiments. A549 cells were incubated with probes for 30 minutes and continuously irradiated with 561 nm laser. Confocal images of the stained cells were obtained within 10 seconds intervals over a period of 8 minutes. ImageJ software was used for calculating average fluorescence intensity of the of the images and a plot was obtained for % intensity of the fluorescence recovered as a function of time.

**Cell viability testing.** For cell viability screening, COS-7 cells were seeded in Corning™ 96 well clear bottom, tissue culture treated opaque microplate. Approximately about 10000 cells were plated in each well (cell density =  $1 \times 10^5$  cells/ml). 24 hours after seeding cells were treated with increasing concentrations of probes (**2a – 2d**) dye for 1 hour at 37°C. Cell toxicity assays were performed using CellTiter-Glo® Luminescent cell viability assay kit (Promega) according

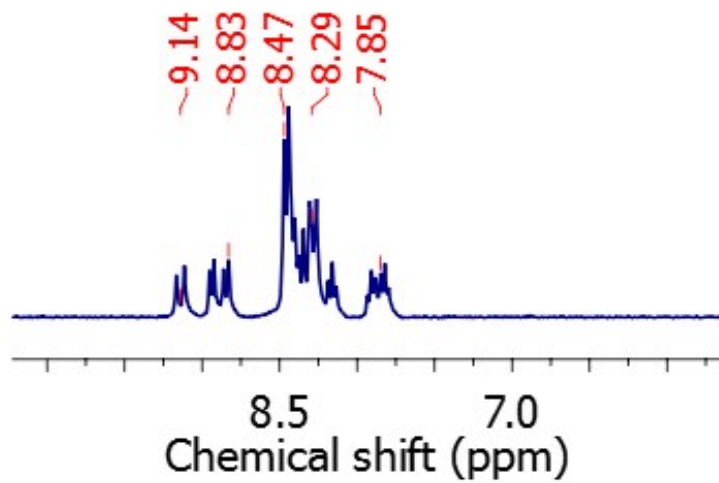
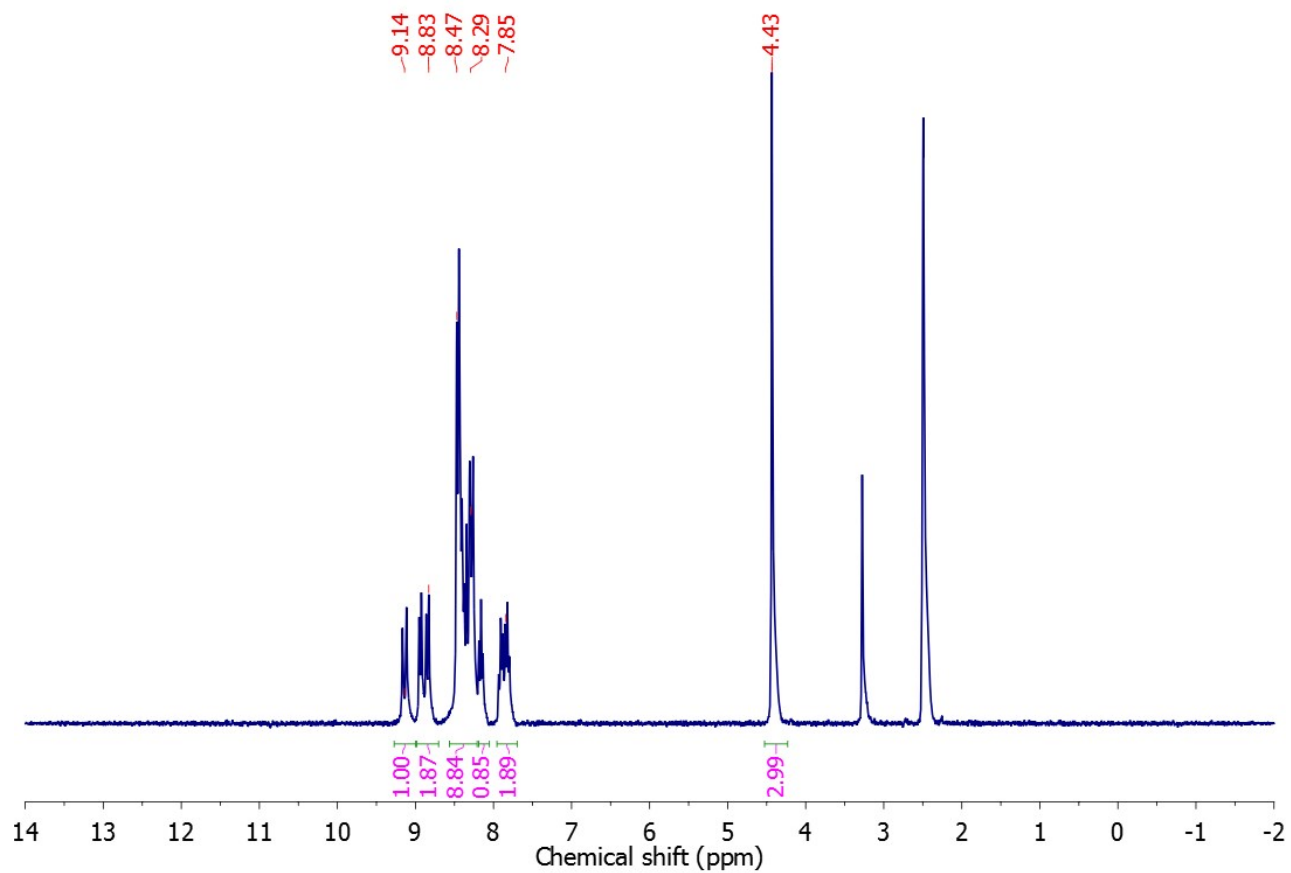
manufactures protocol. Luminescence readings were recorded using Spectramax® 5e microplate reader by using default Spectramax® software installed for CellTiter Glow viability test protocol. LC<sub>50</sub> value for probes were calculated by using dose-response formula in Origin8 software.

**Lipid vesicle (MLV) preparation.** Large multilamellar vesicles (MLV) were prepared (0.2 – 1 μm diameter range) by using Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) in chloroform solution according to the previously reported protocol.<sup>53</sup> MLV composition was PC:PE (80 : 20). Appropriate amounts from PC and PE were mixed in to a vial maintaining total lipid concentration to 1 mM. Lipids were hydrated with resuspension buffer (25 mM HEPES, pH 7.4, containing 150 mM KCl) and incubated at 37°C for 15 min to facilitate hydration of lipids to form while vigorously vortexing multilamellar vesicles (MLVs). These lipid solutions were then incubated with 10 μM probes **2b** and **2d** for 15 minutes. A control experiment was carried out by incubating **2b** and **2d** (10 μM) in HEPES buffer (pH 7.4) for 15 minutes. MLV solutions and controls were then transferred in to a 96 well opaque bottom assay plate and fluorescence emissions were recorded using Spectramax® 5e microplate reader by using 530 nm excitation.



**Figure S1.** Synthesis of probe **2**. And arrows indicate intramolecular charge transfer (ICT) from pyrene to the benzothiazolium unit.





**Figure S2.1**  $^1\text{H}$  NMR spectra of **2a** (300 MHz in DMSO- $d_6$ )

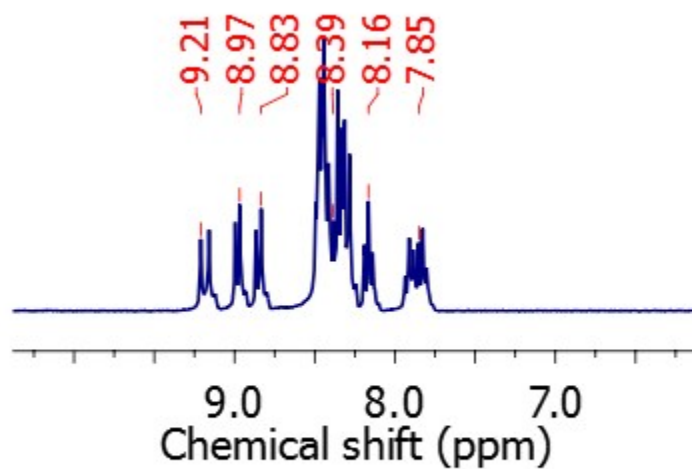
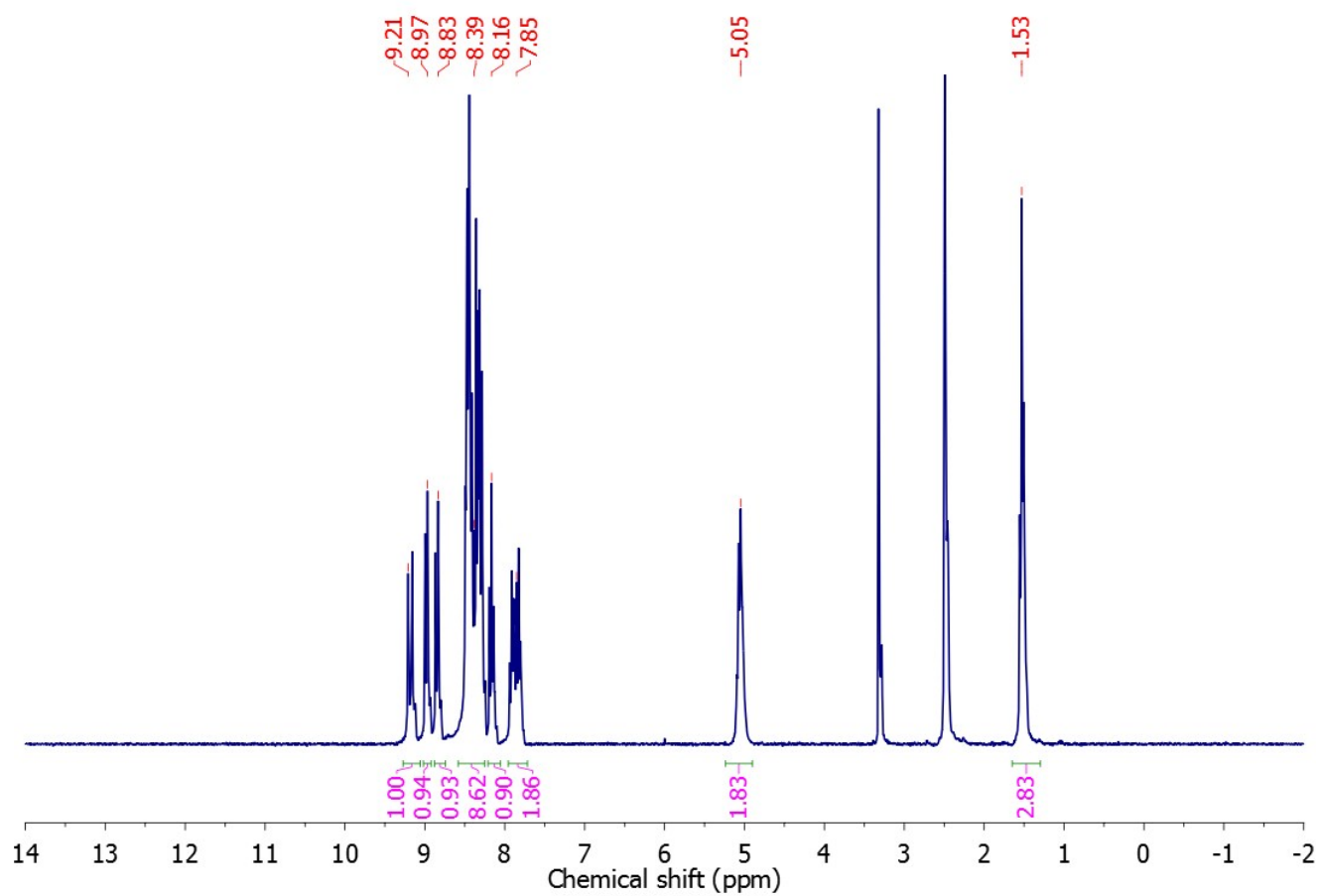
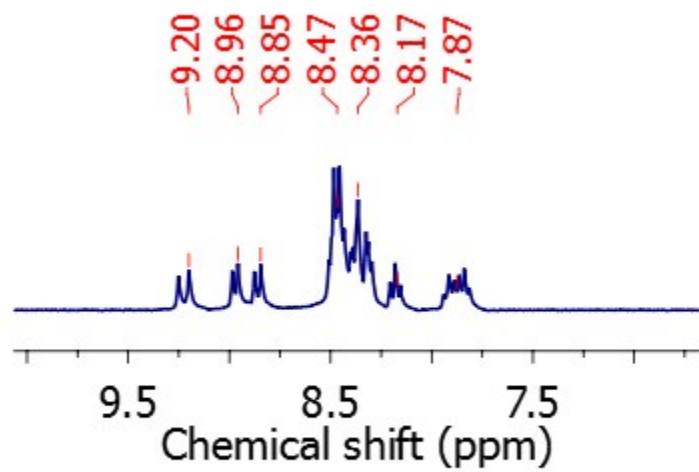
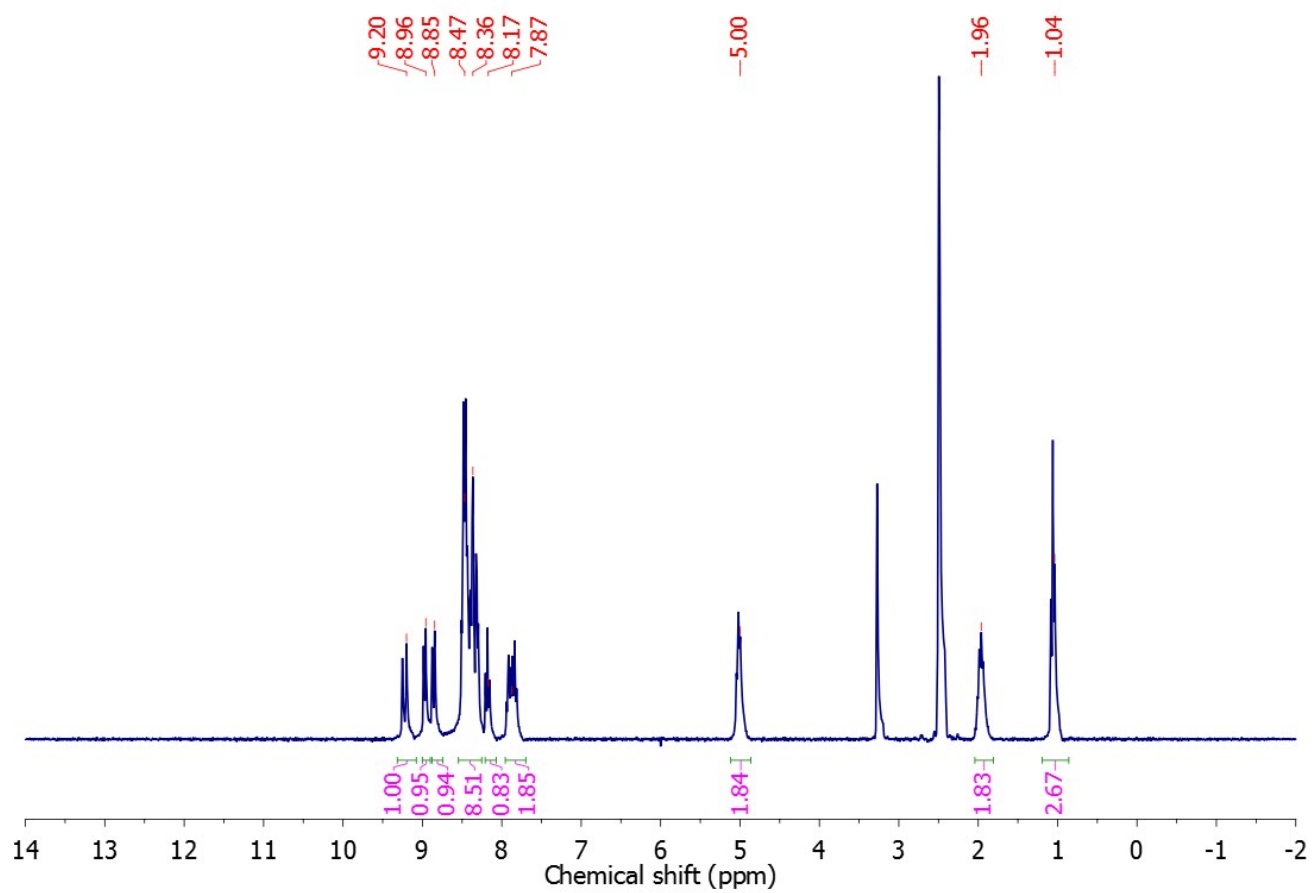


Figure S2.2  $^1\text{H}$  NMR spectra of **2b** (300 MHz in DMSO- $d_6$ )



**Figure S2.3**  $^1\text{H}$  NMR spectra of **2c** (300 MHz in DMSO- $d_6$ )

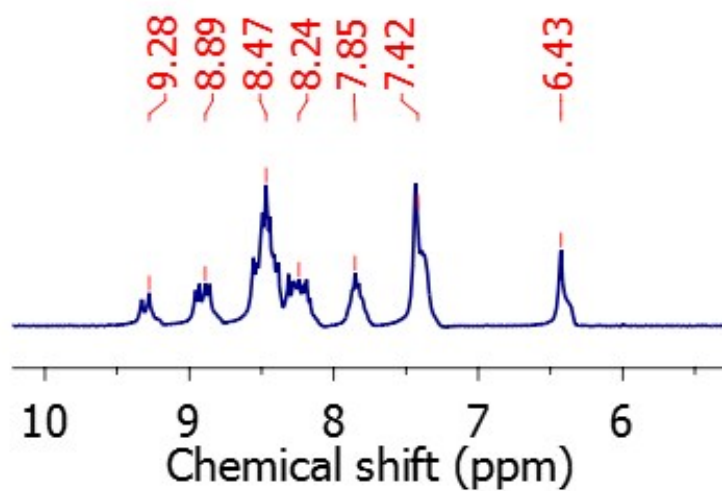
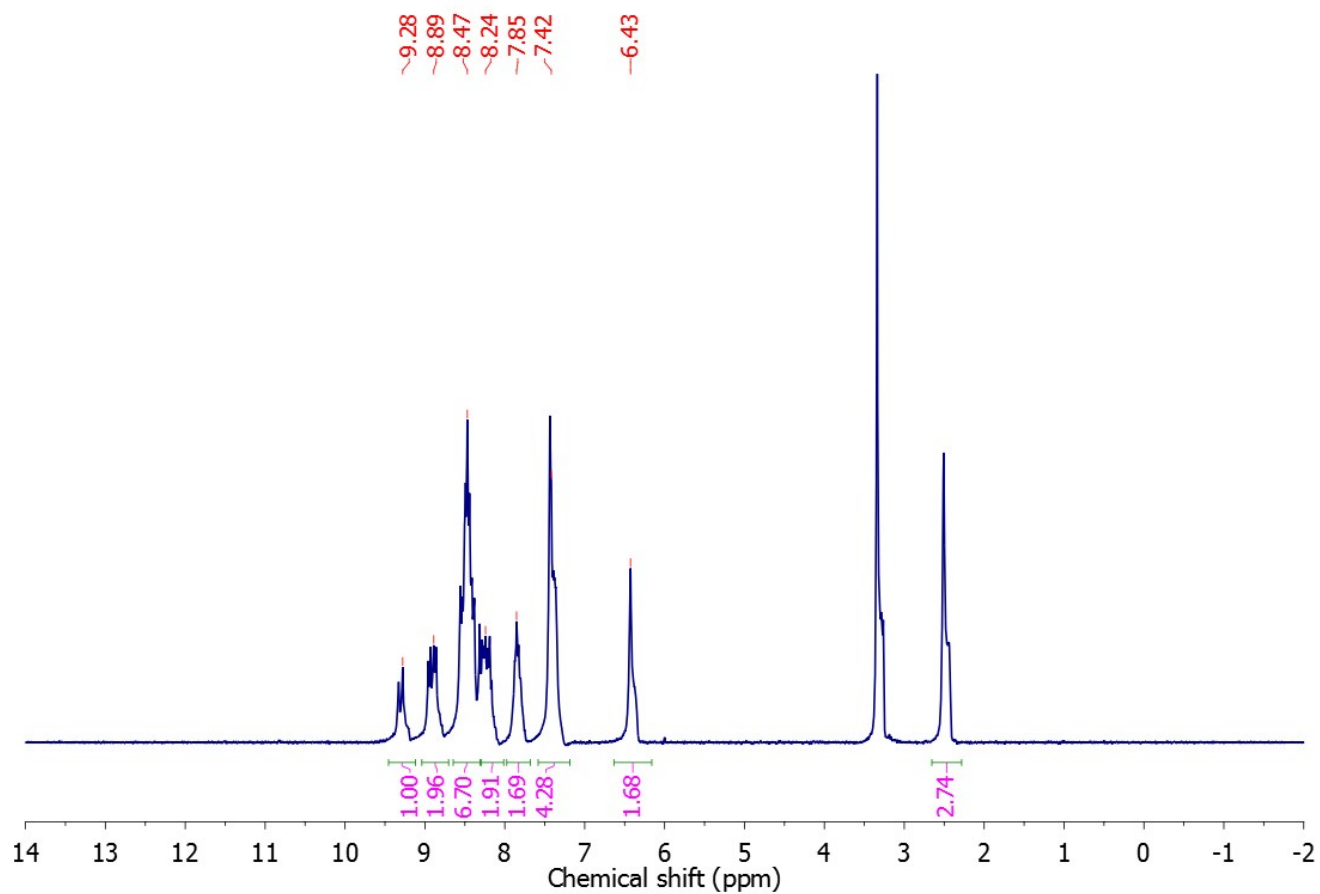
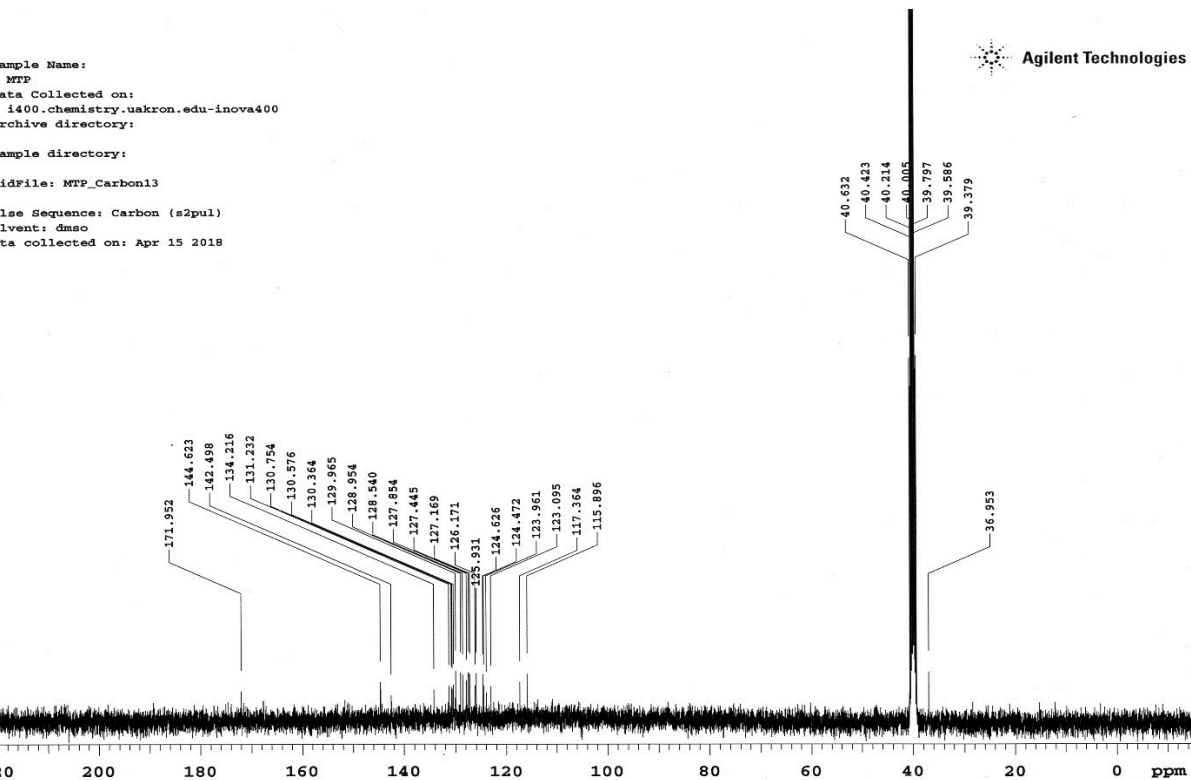
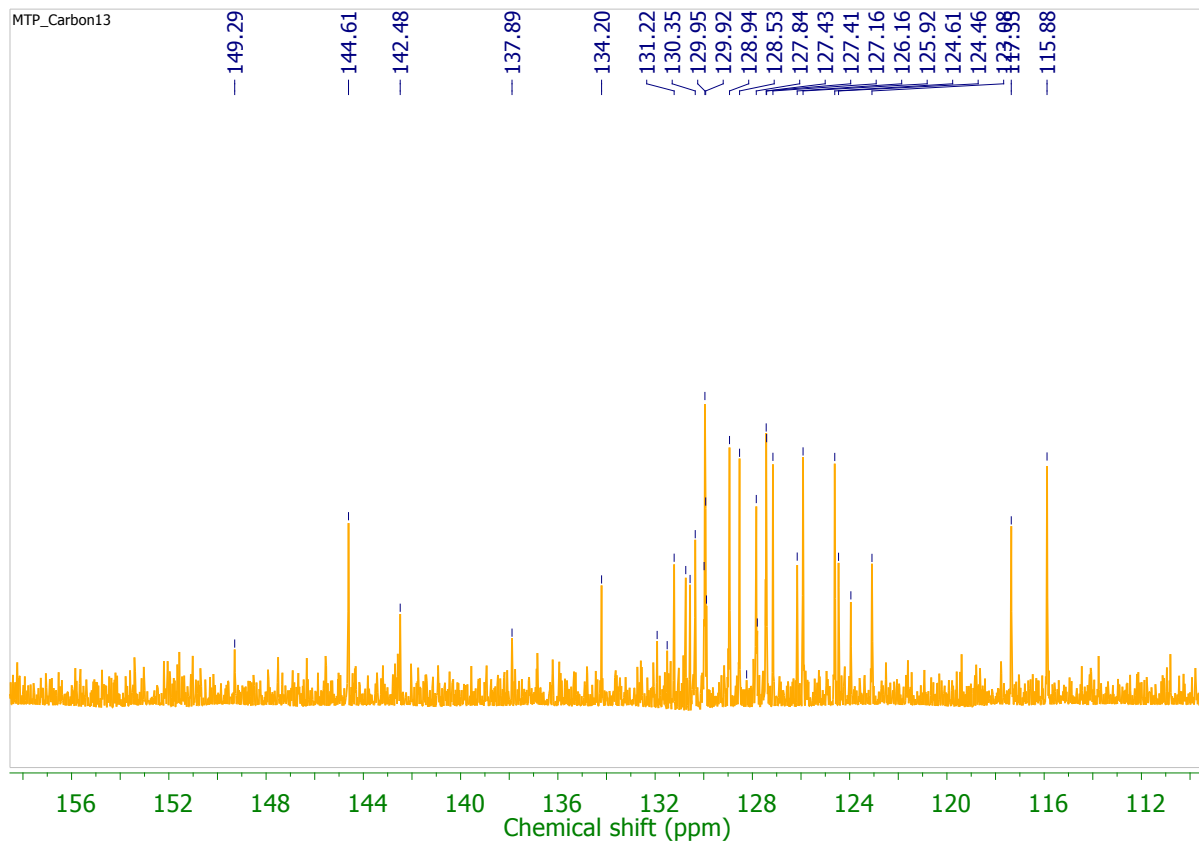


Figure S2.4  $^1\text{H}$  NMR spectra of **2d** (300 MHz in DMSO- $d_6$ )

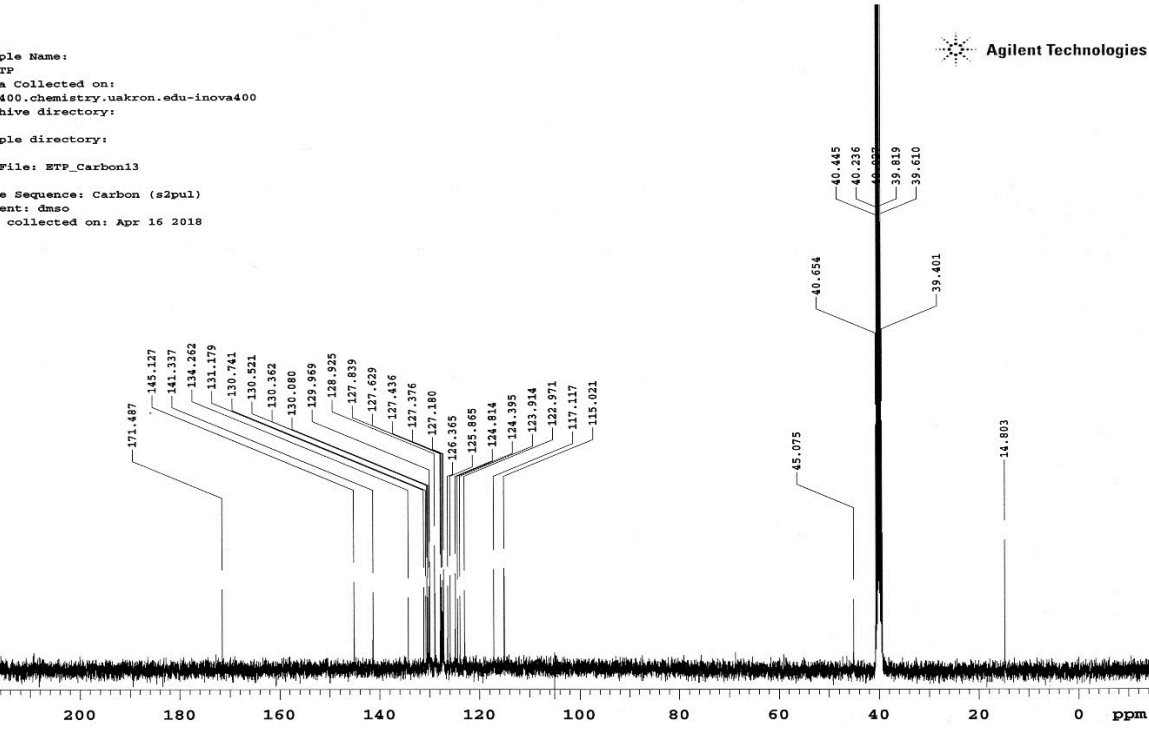
Sample Name:  
MTP  
Data Collected on:  
1400.chemistry.uakron.edu-inova400  
Archive directory:  
Sample directory:  
Raw File: MTP\_Carbon13  
Pulse Sequence: Carbon (s2pul)  
Solvent: dmsd  
Data collected on: Apr 15 2018





**Figure S2.5**  $^{13}\text{C}$  NMR spectra of **2a** (400 MHz in DMSO- $d_6$ )

Sample Name:  
Sample Collected on:  
400.chemistry.uakron.edu-inova400  
Sample directory:  
Sample directory:  
File: ETP\_Carbon13  
Sequence: Carbon (s2pul)  
Solvent: dmsc  
Collected on: Apr 16 2018



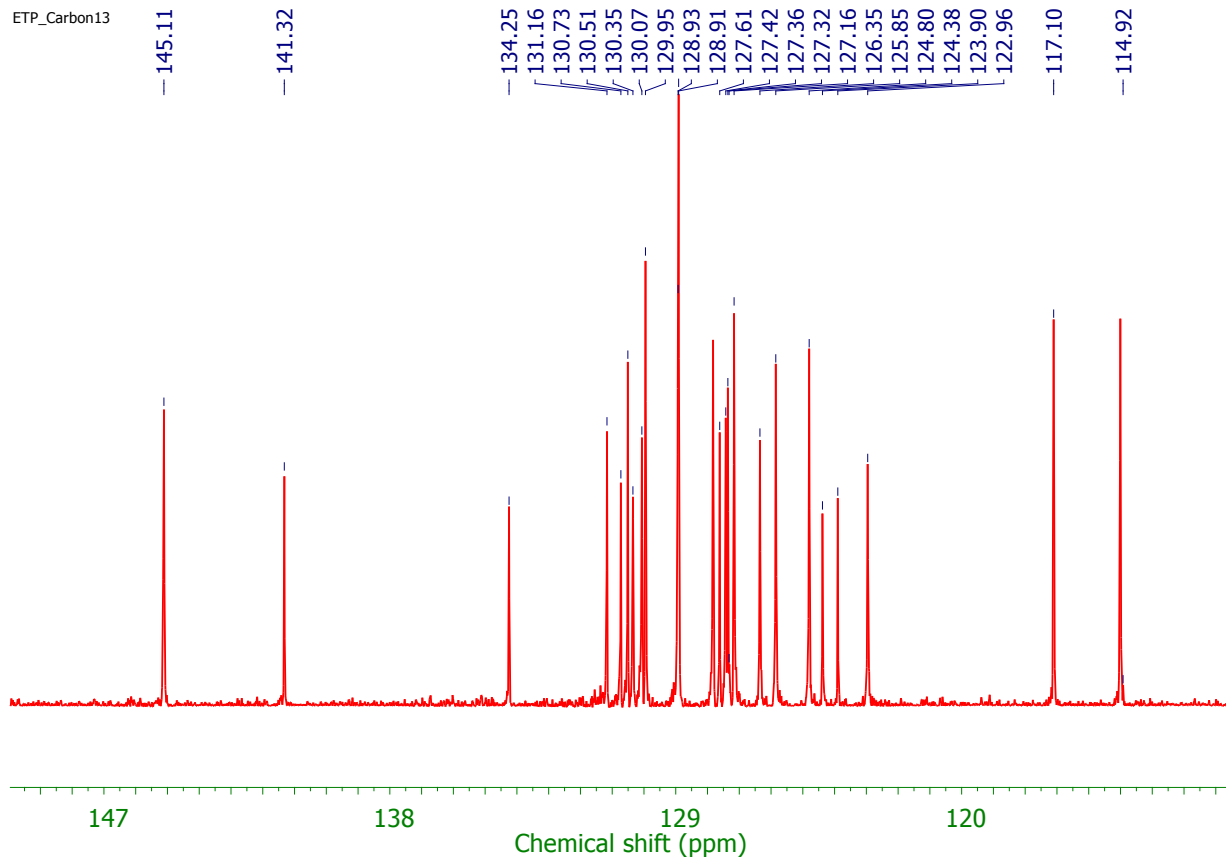
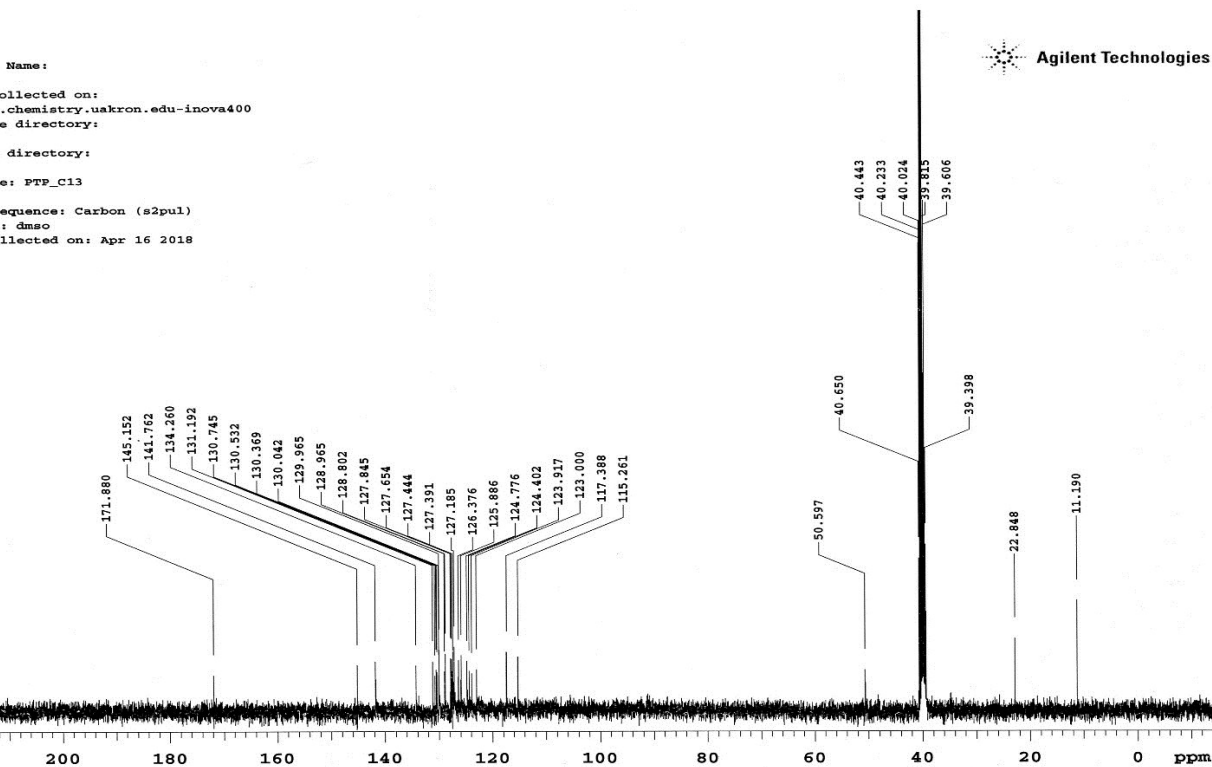
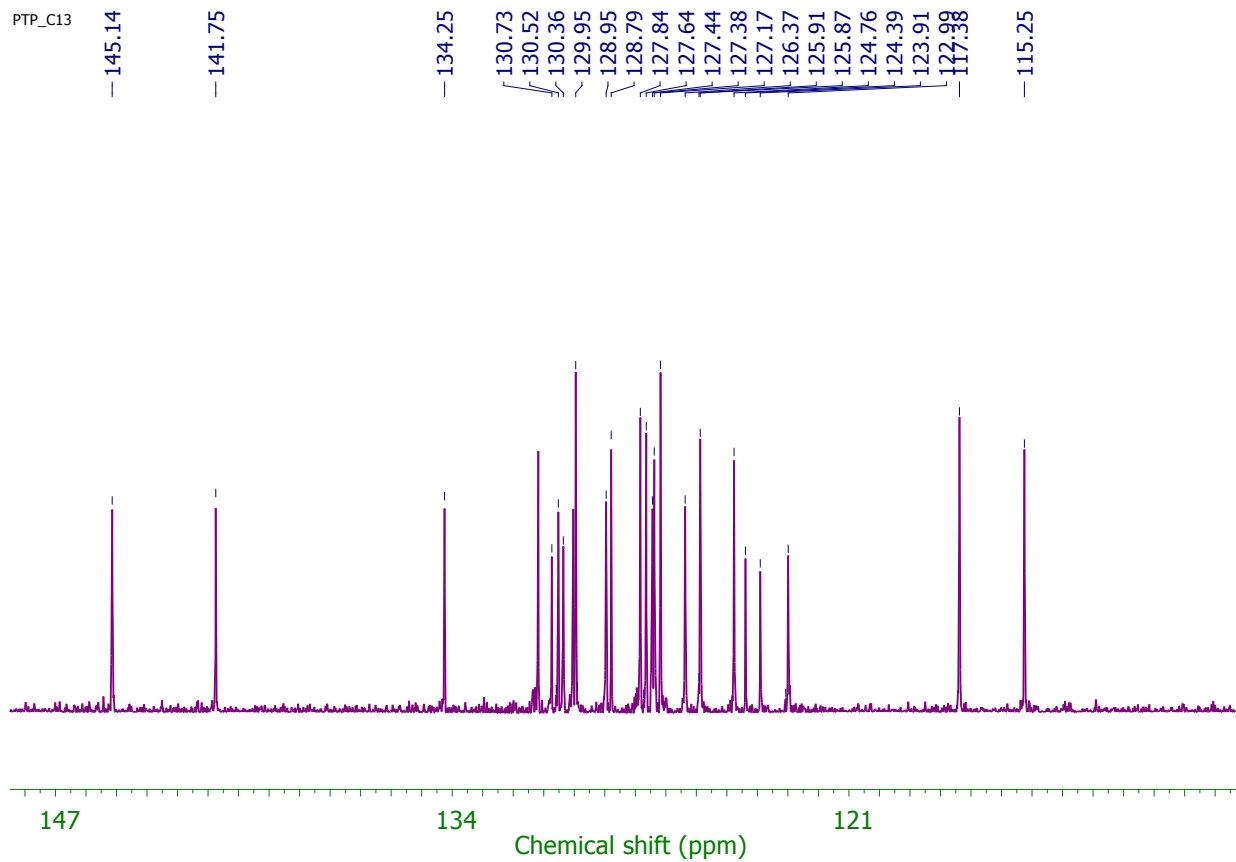


Figure S2.6  $^{13}\text{C}$  NMR spectra of **2b** (400 MHz in DMSO- $d_6$ )



Name:  
Collected on:  
chemistry.uakron.edu-inova400  
e directory:  
directory:  
e: PTP\_C13  
Sequence: Carbon (s2pul)  
: dmsc  
llected on: Apr 16 2018



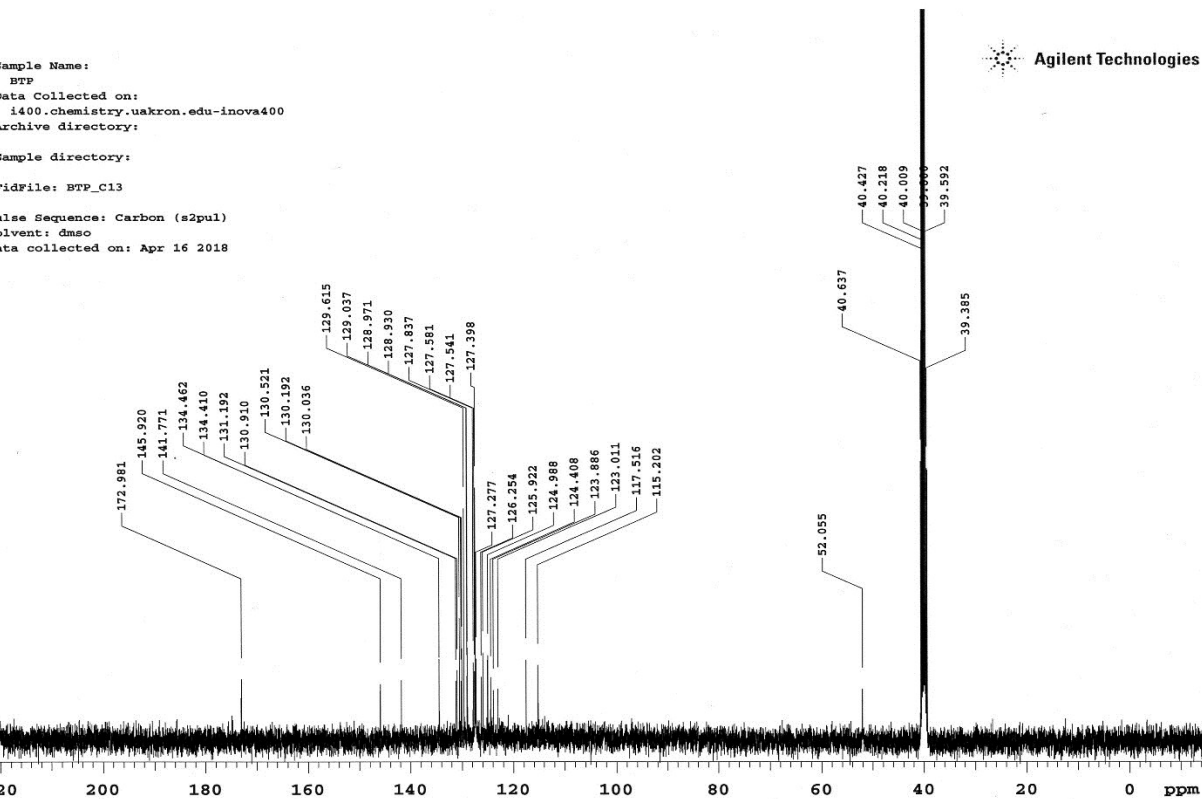


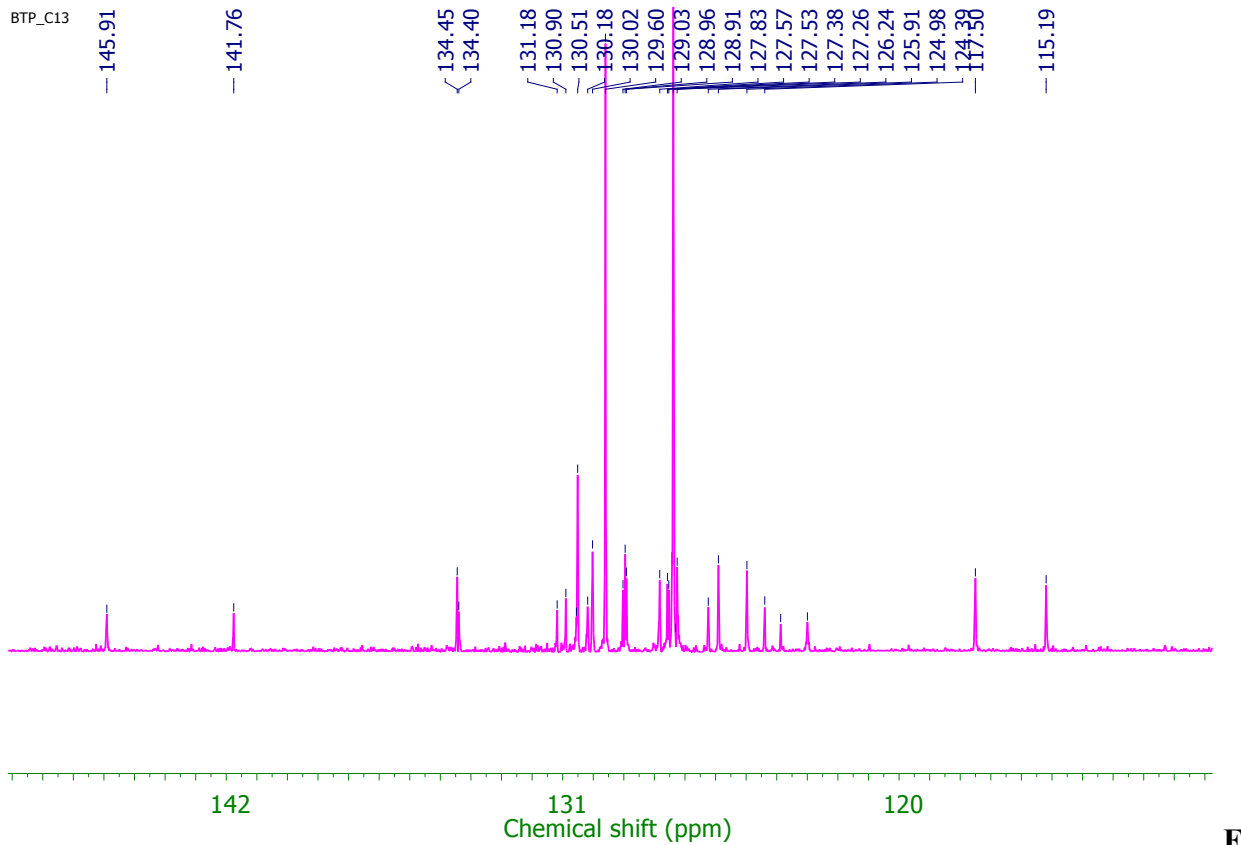
**Figure S2.7**  $^{13}\text{C}$  NMR spectra of **2c** (400 MHz in DMSO-d<sub>6</sub>)

Sample Name:  
BTP  
Data Collected on:  
1400.chemistry.usakron.edu-inova400  
Archive directory:

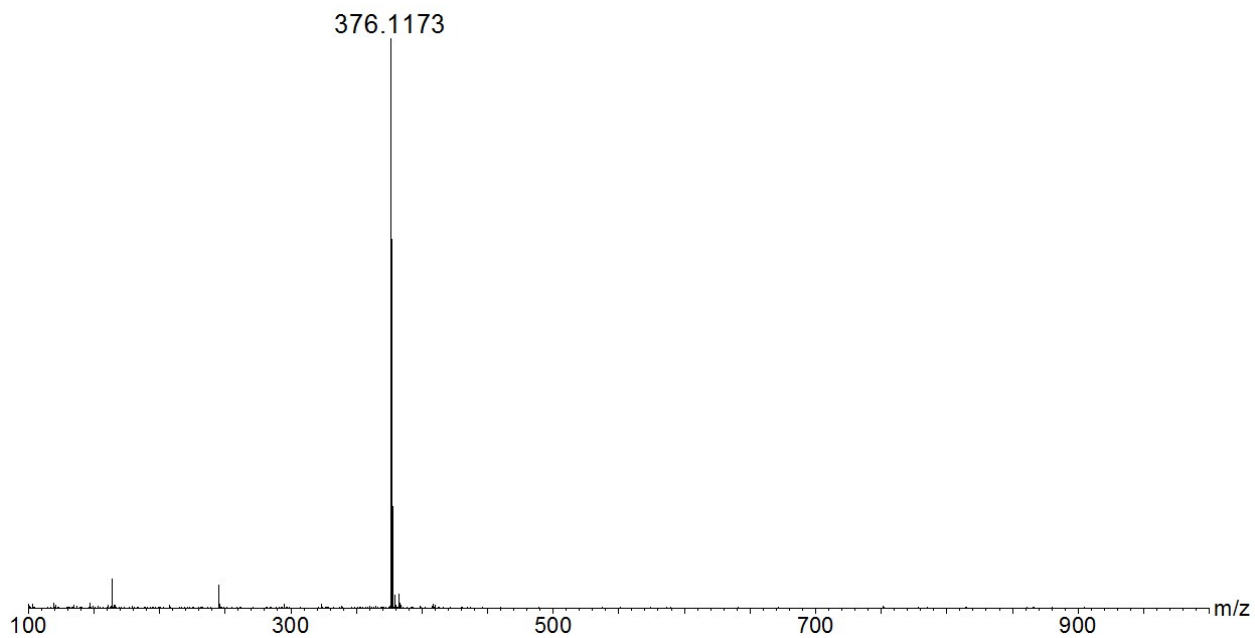
Sample directory:  
Filename: BTP\_C13

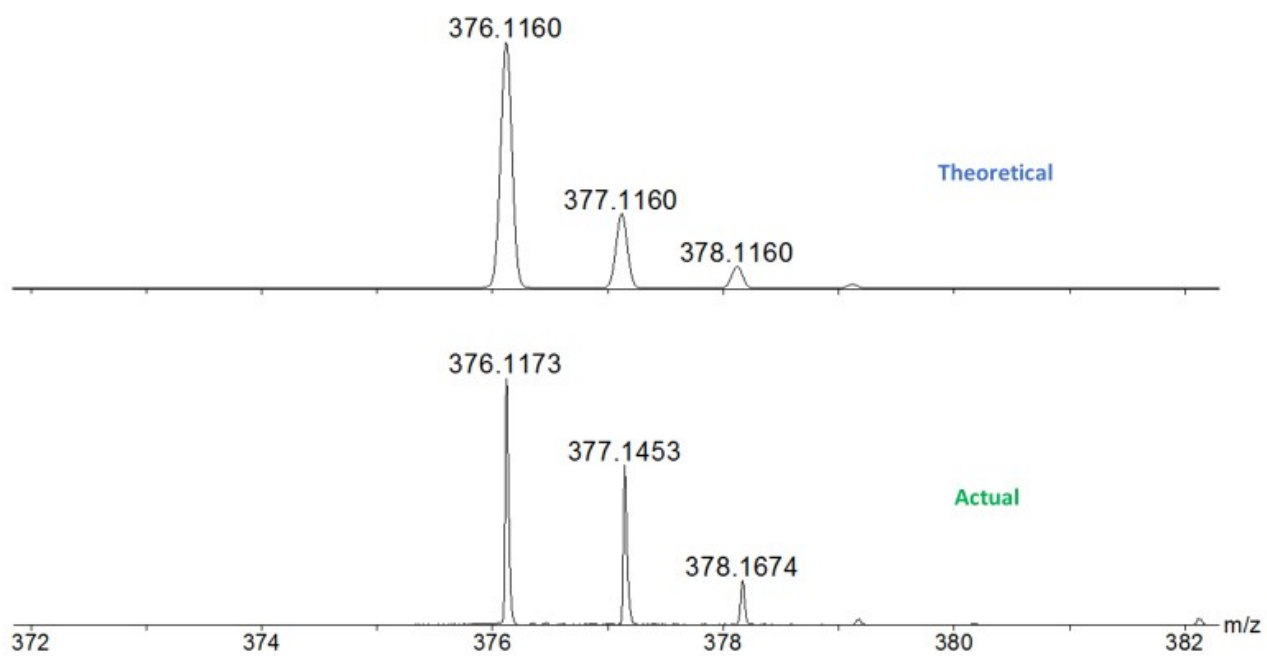
File Name: BTP\_C13  
Pulse Sequence: Carbon (s2pul)  
Solvent: dmsc  
Data collected on: Apr 16 2018



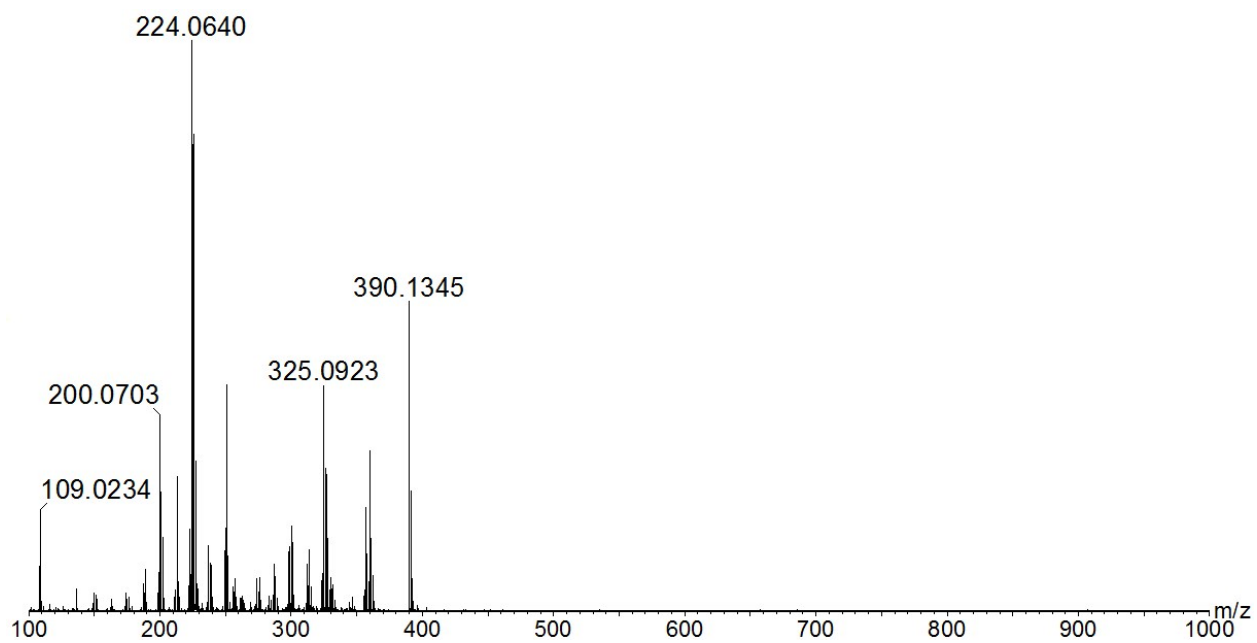


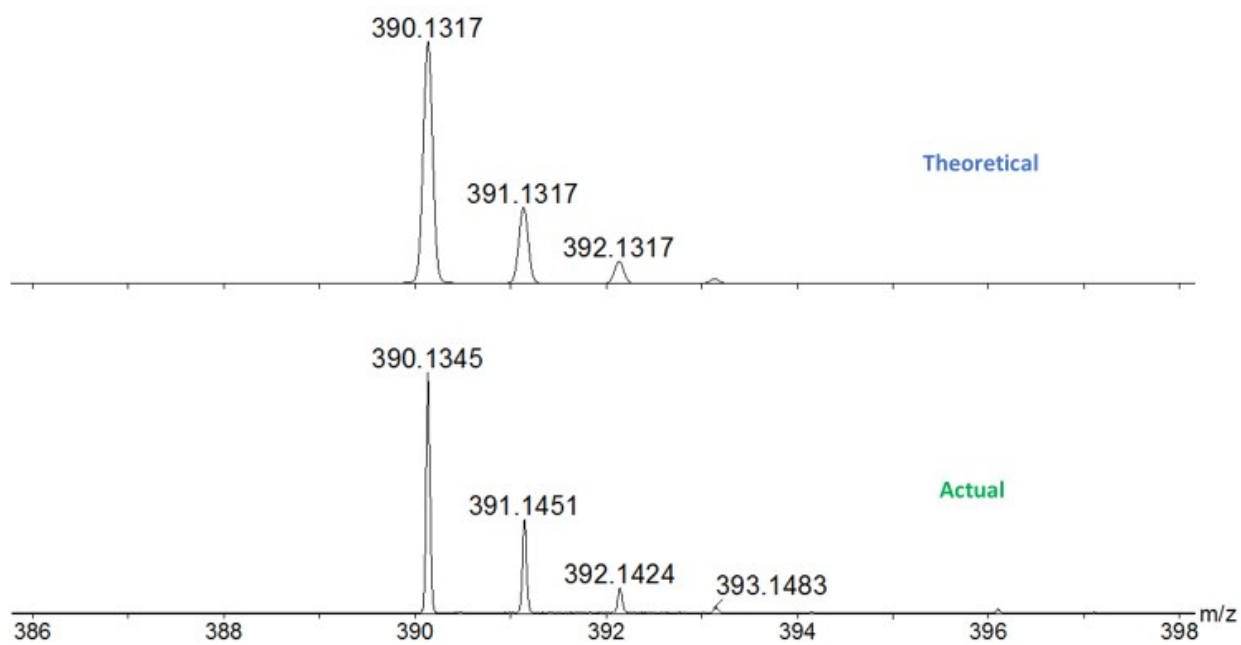
**figure S2.8**  $^{13}\text{C}$  NMR spectra of **2d** (400 MHz in DMSO- $d_6$ )



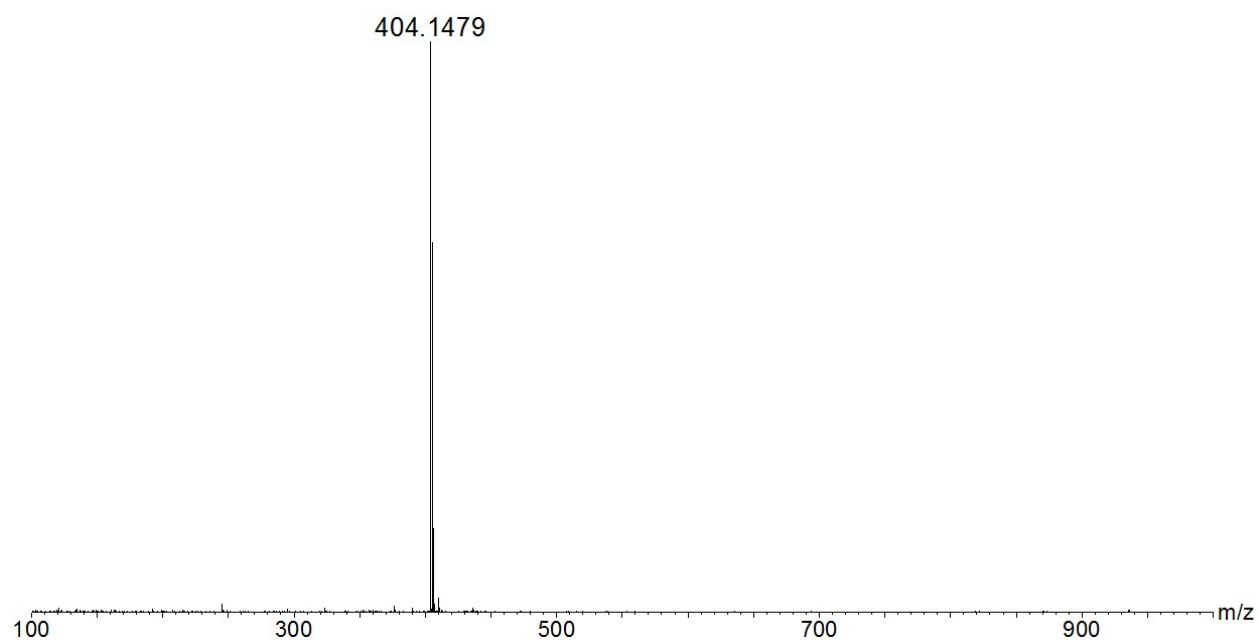


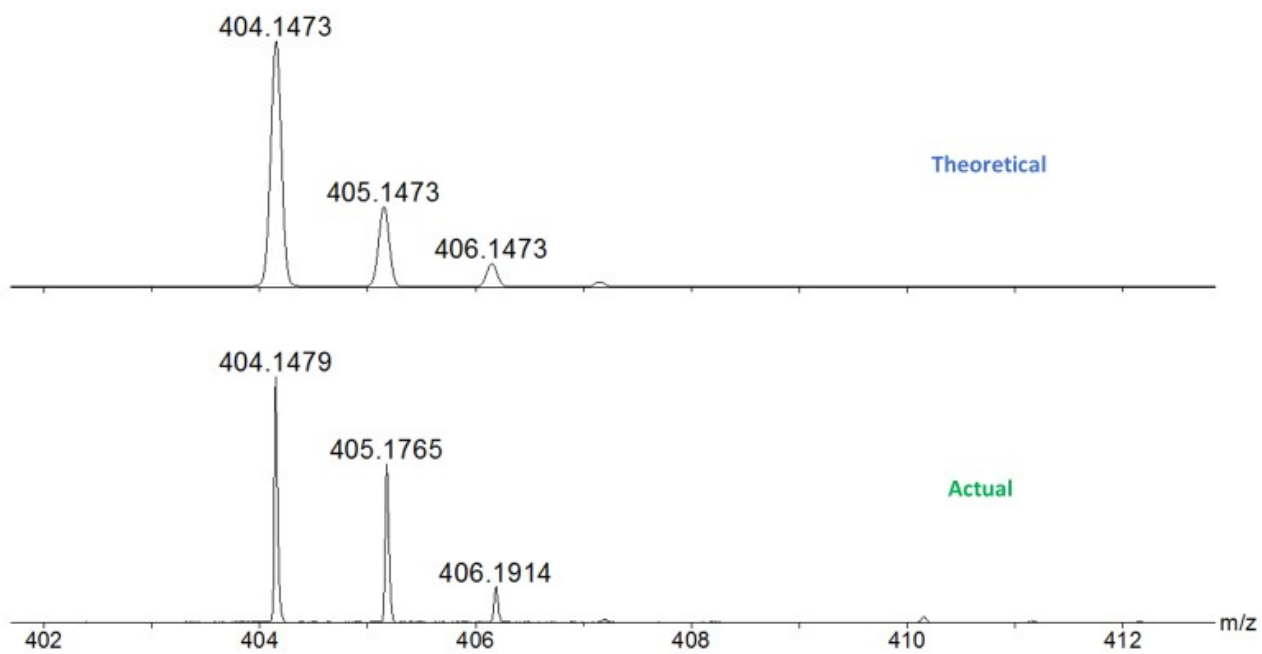
**Figure S3.1** Mass spectra of 2a



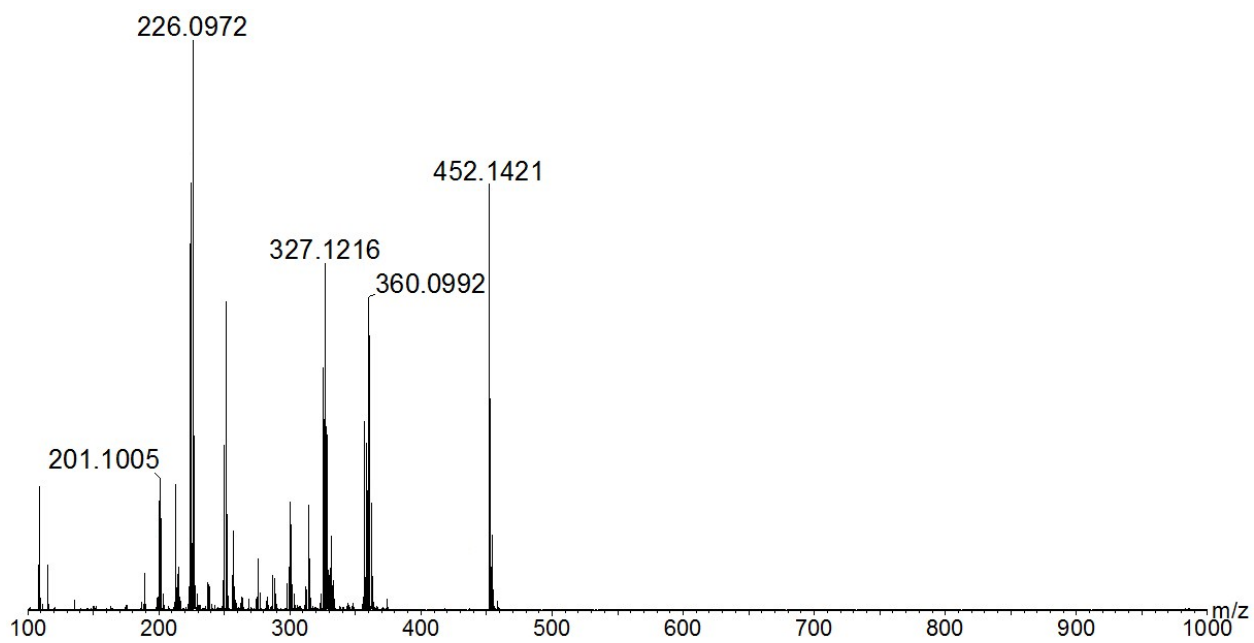


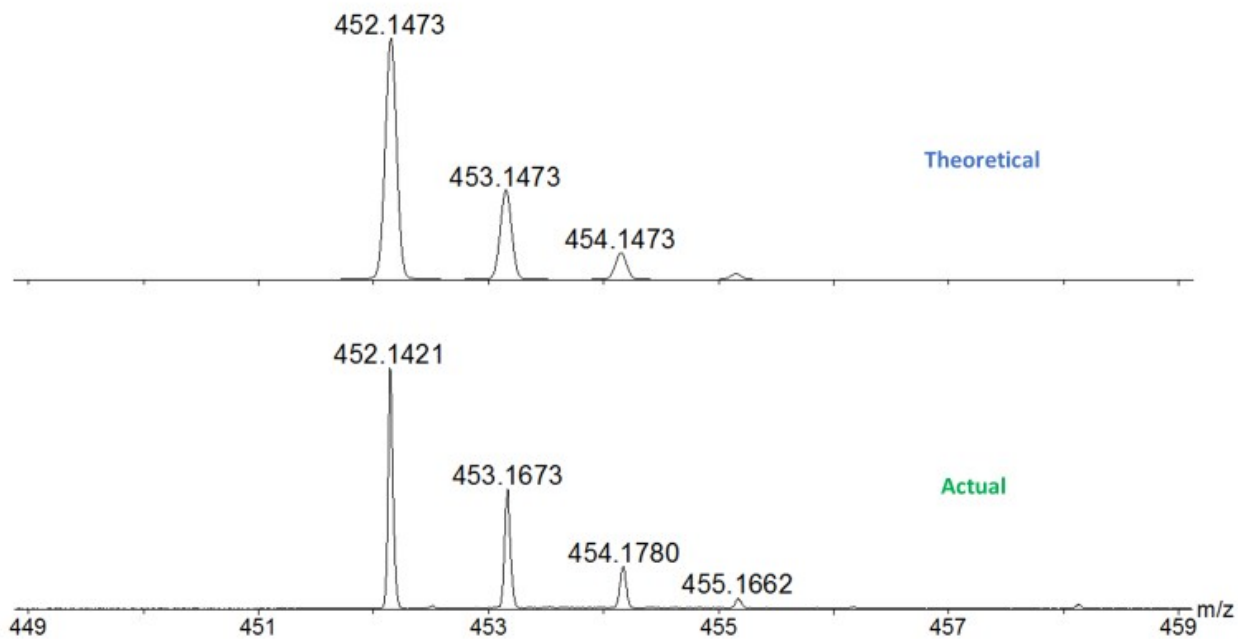
**Figure S3.2** Mass spectra of **2b**





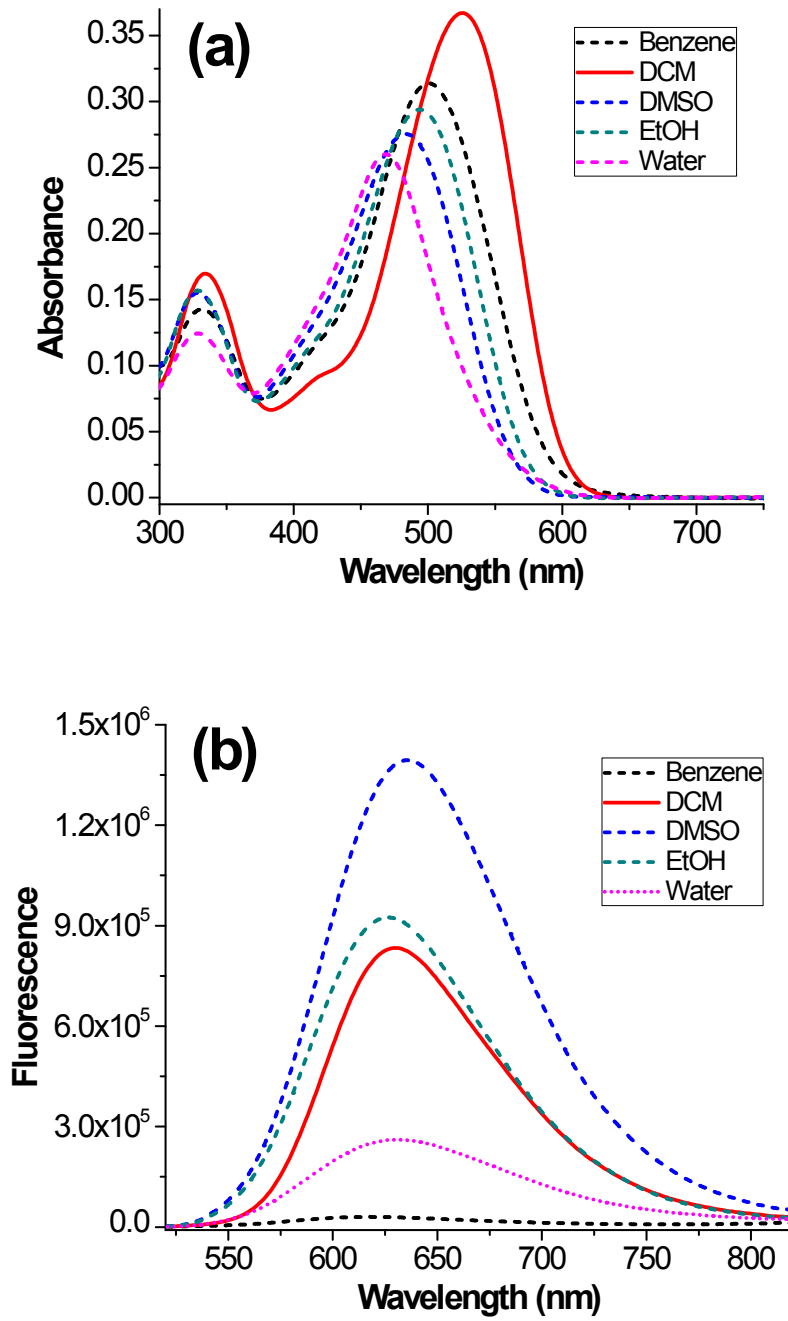
**Figure S3.3** Mass spectra of 2c



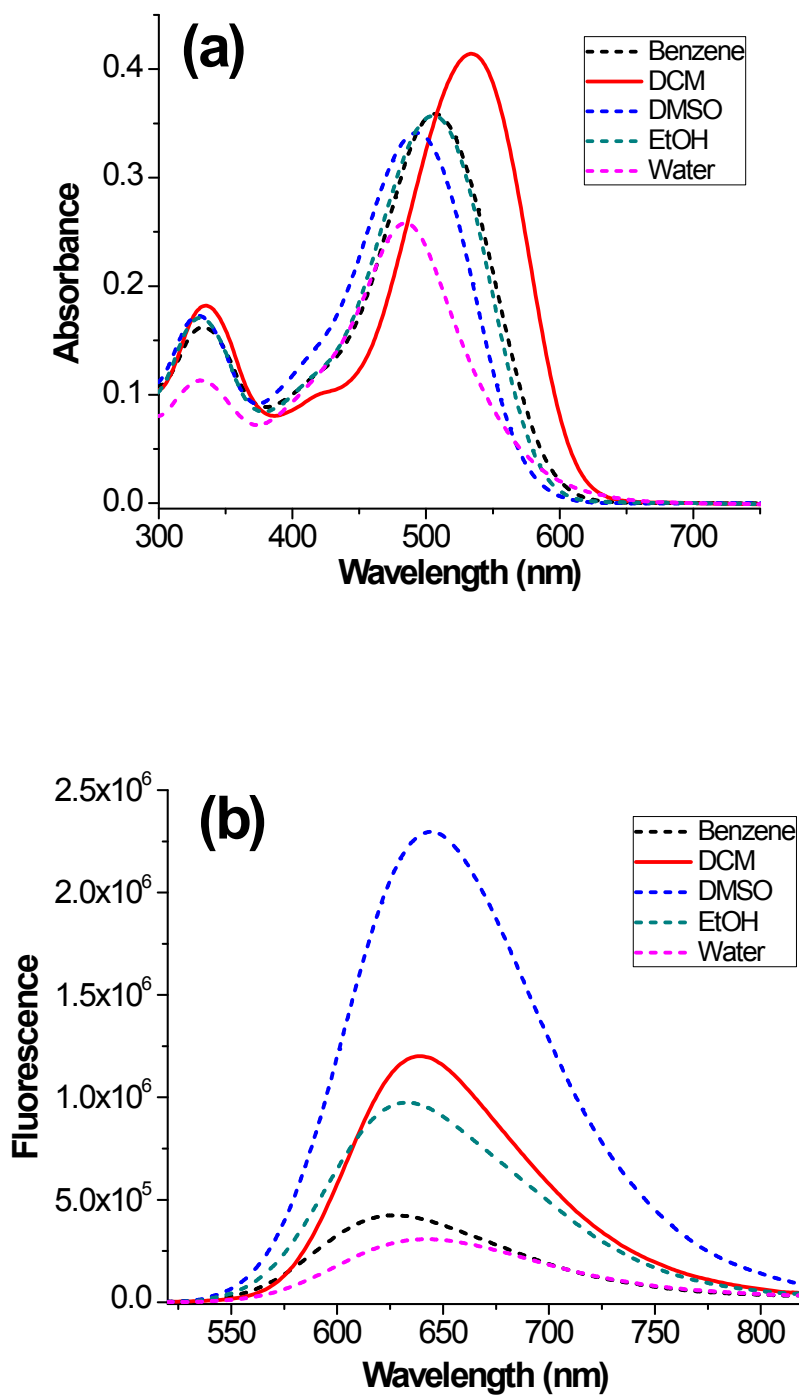


**Figure S3.4** Mass spectra of **2d**

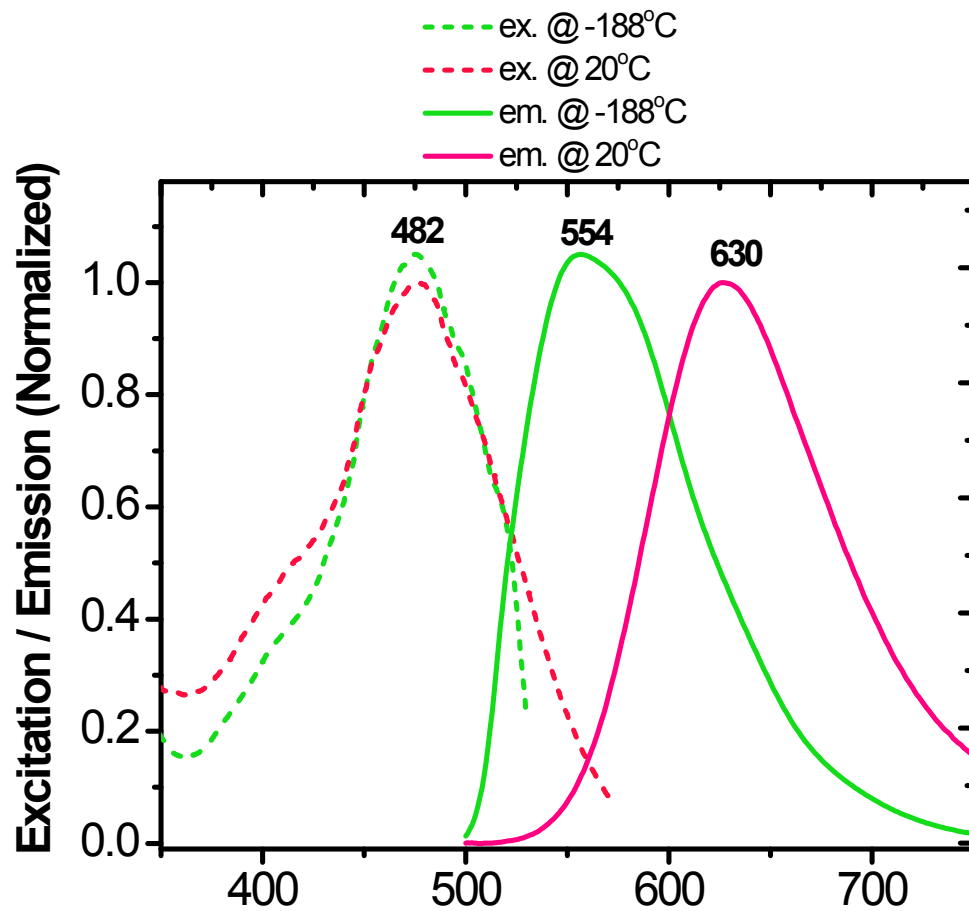




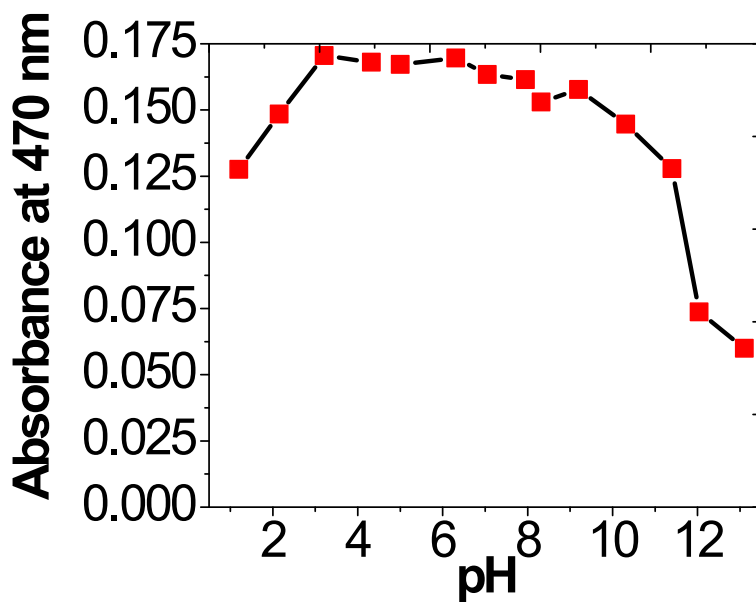
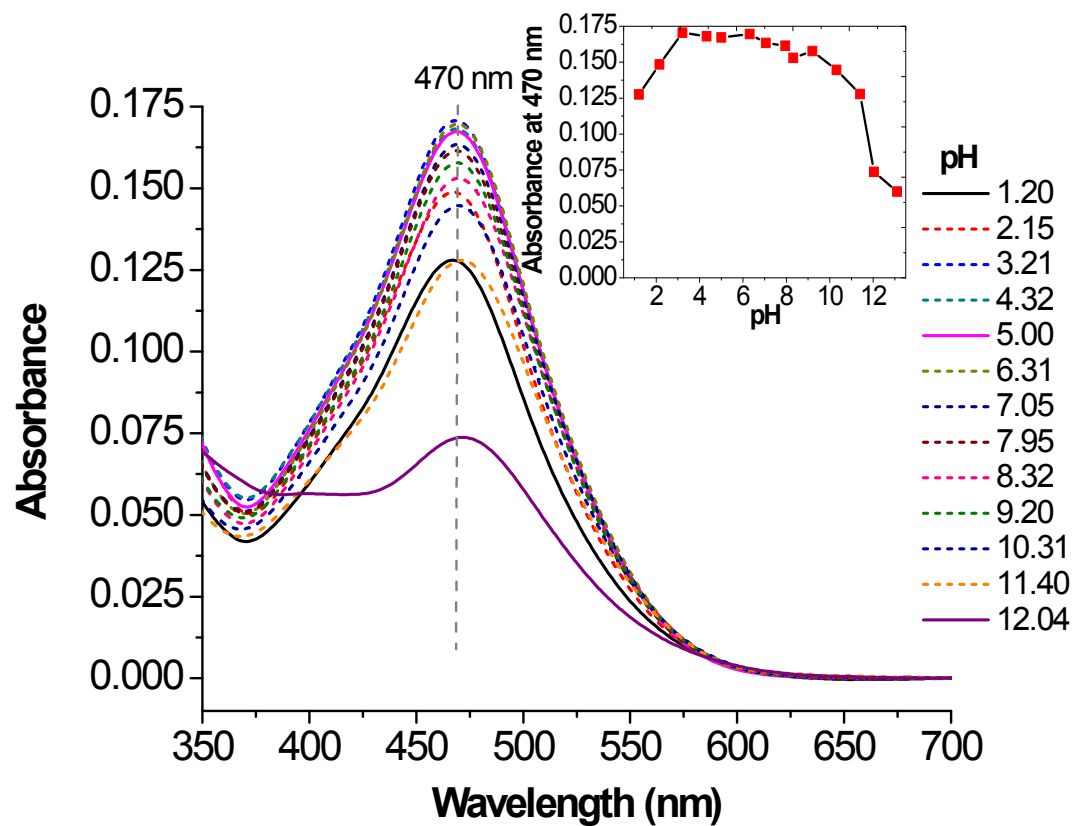
**Figure S4.1** Absorbance (a) and fluorescence emission (b) spectra obtained for **2b** ( $1 \times 10^{-5}$  M) in different solvents at room temperature



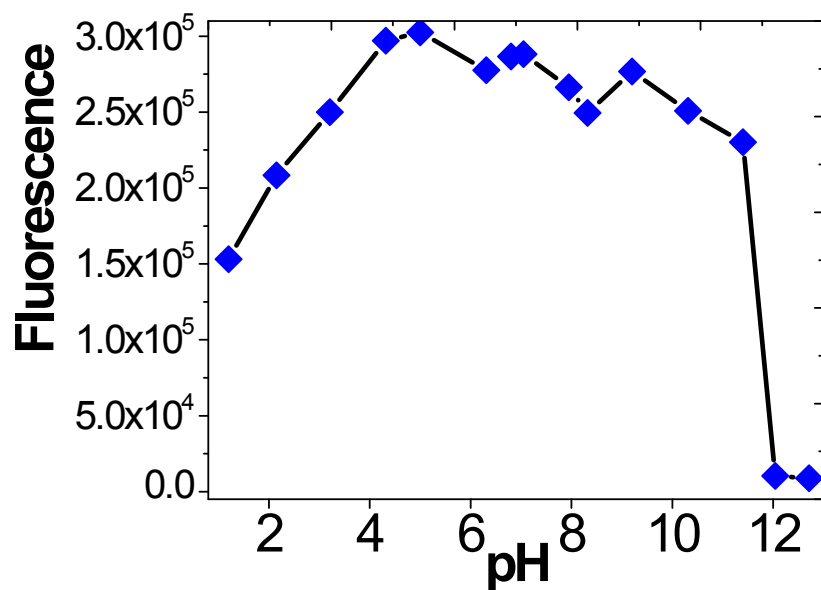
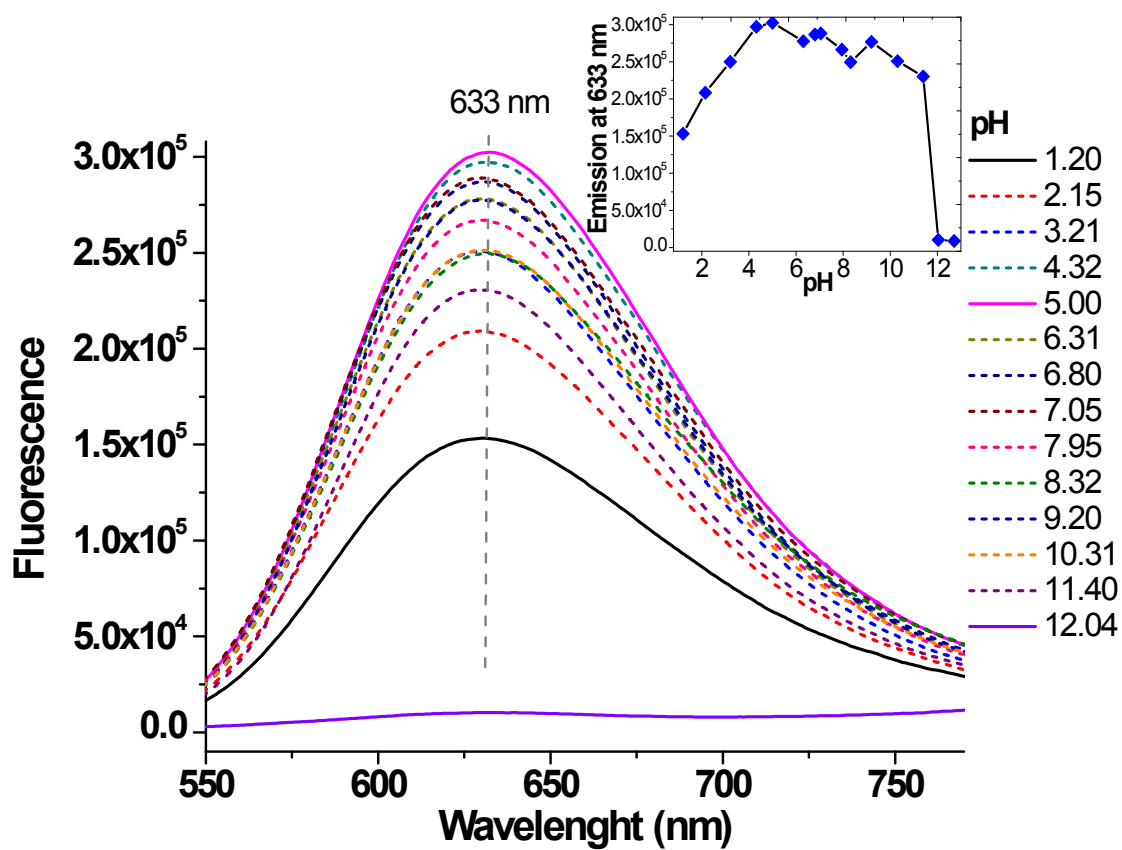
**Figure S4.2** Absorbance (a) and fluorescence emission (b) spectra obtained for **2d** ( $1 \times 10^{-5}$  M) in different solvents at room temperature.



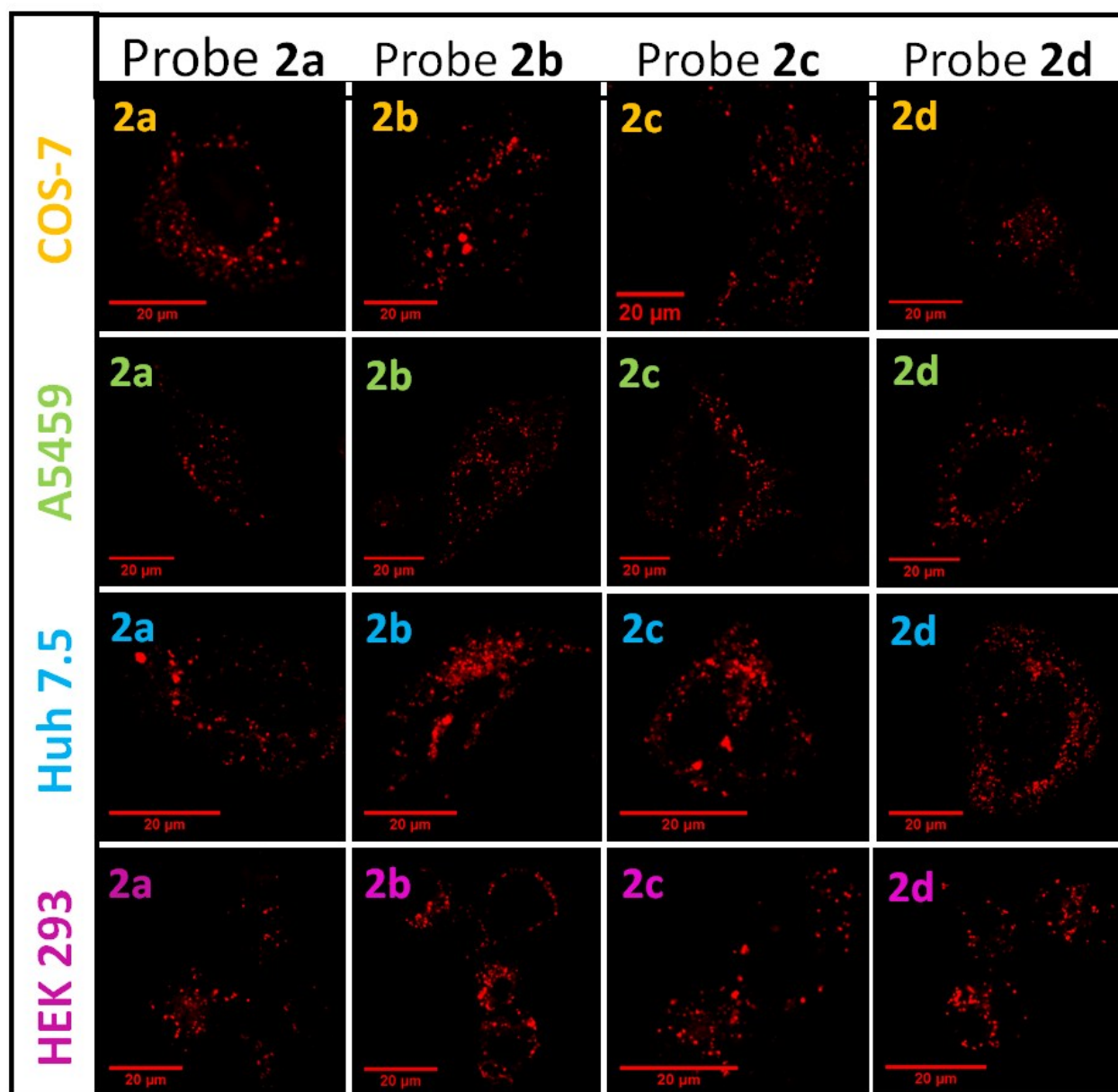
**Figure S5.** Normalized excitation and fluorescence emission spectra obtained for probe **2b** ( $1 \times 10^{-6}$  M in Ethanol) at different temperature conditions.



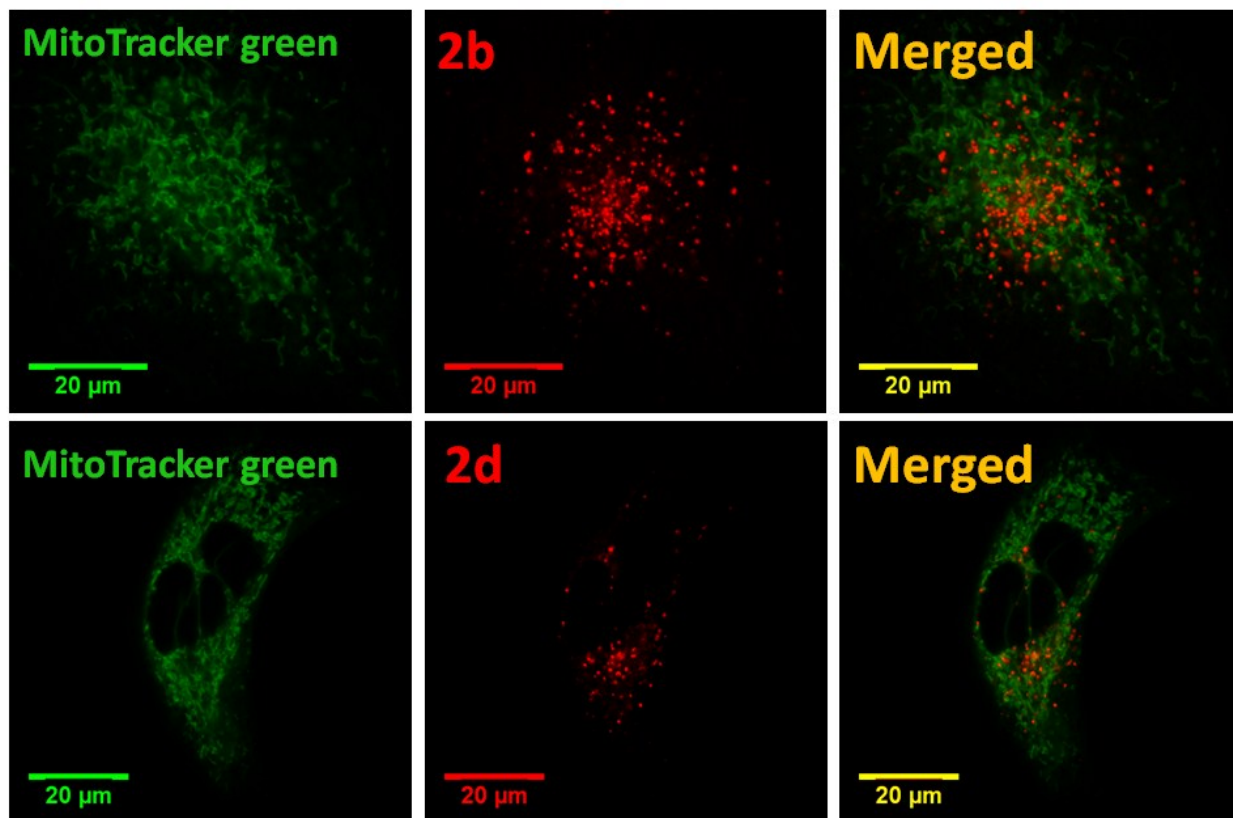
**Figure S6.1.** Absorbance spectra obtained for **2d** ( $1 \times 10^{-5}$  M) in different pH conditions at room temperature.



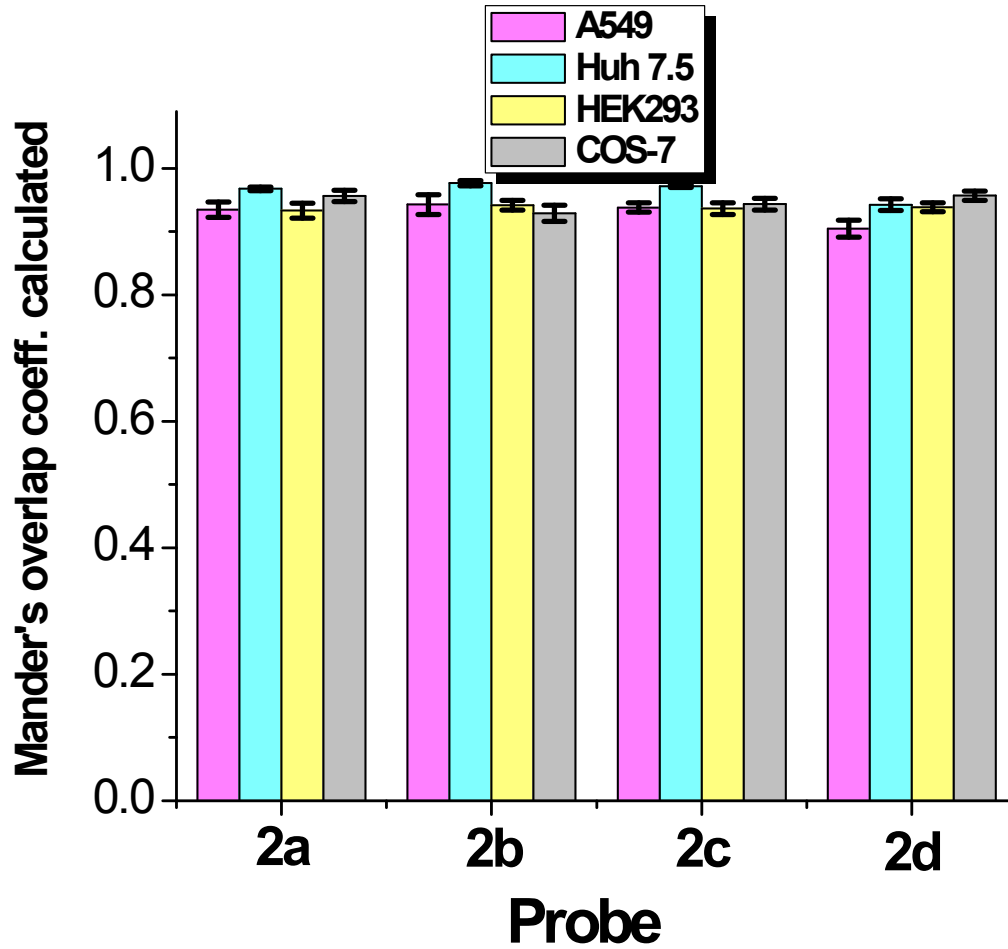
**Figure S6.2.** Fluorescence emission spectra obtained for **2d** ( $1 \times 10^{-5}$  M) in different pH conditions at room temperature.



**Figure S7.** Fluorescent confocal microscopy images of COS-7, A549, Huh 7.5 and HEK 293 cells treated with **2a** – **2d** (500 nM) for 30 minutes. Probes **2a** – **2d** were excited with a 561 nm laser and emission collected in 580 to 720 nm.



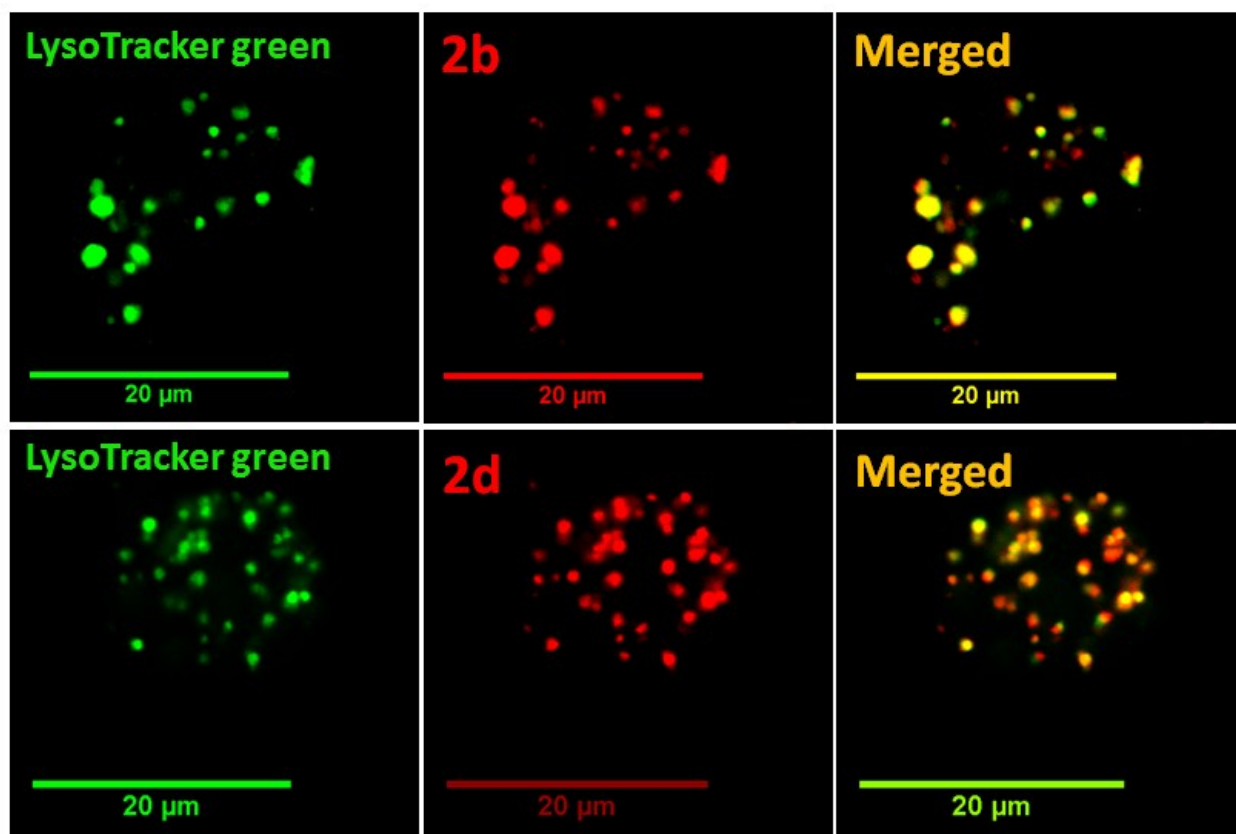
**Figure S8.** Fluorescent confocal microscopy images of A549 cells stained with probes **2b** and **2d** (500 nM) for 30 minutes in the presence of commercial MitoTracker Green DND-26 (200 nM) at 63x magnification using an oil objective. Images from left to right represents the confocal imaging of Mitotracker green (a,d) Probe **2b** (b), **2d** (e) and overlapped images (c,f). Probes **2b** and **2d** were excited with 561 nm laser line and MitoTracker green was excited with 488 nm laser line.



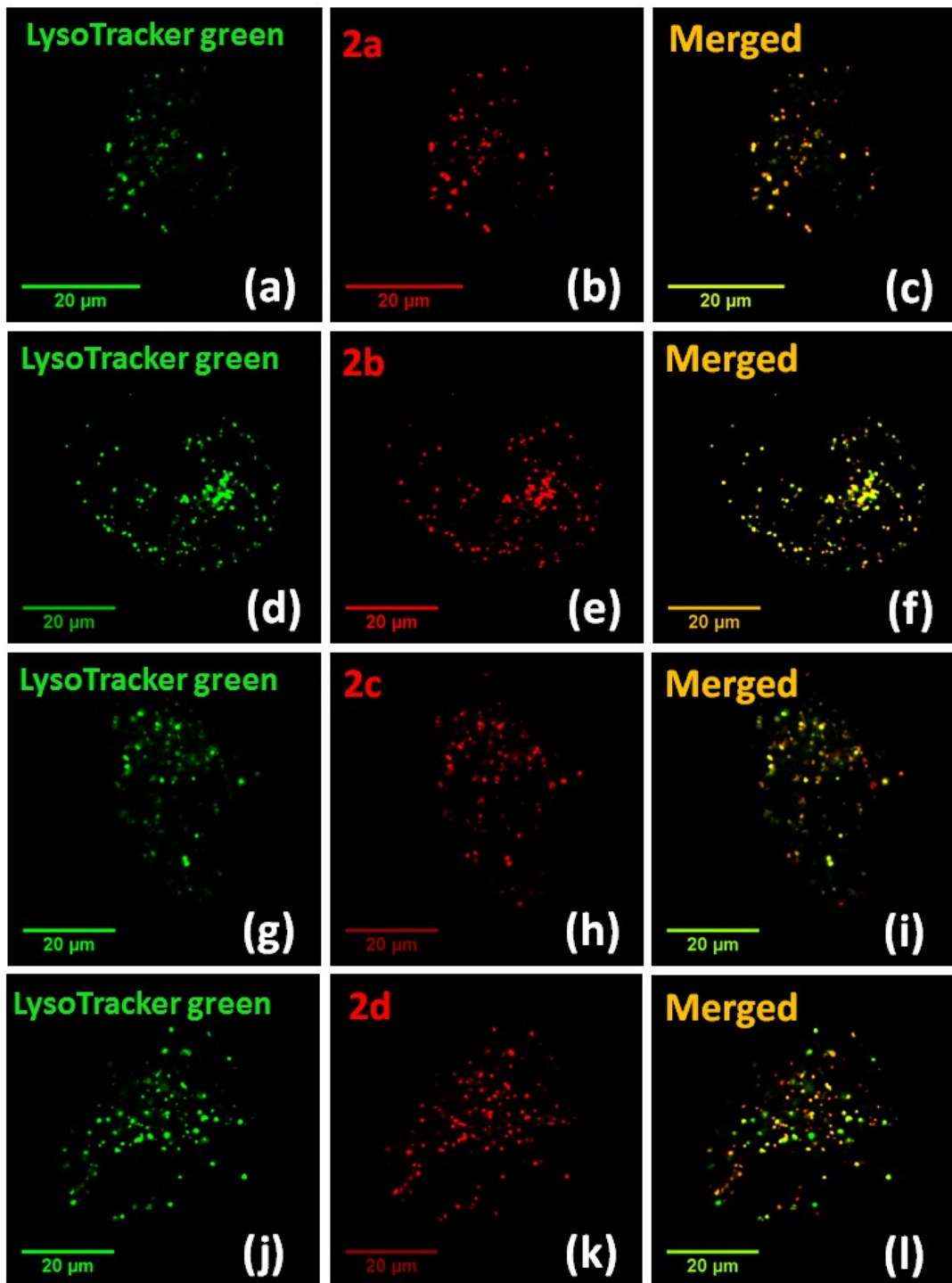
Mander's overlap coefficient calculated								
Cell Type	A549		Huh 7.5		HEK293		COS-7	
Probe	Coeff.	Error +/-	Coeff.	Error +/-	Coeff.	Error +/-	Coeff.	Error +/-
1a	0.9346	0.01203	0.96767	0.00284	0.9332	0.01206	0.9565	0.00901
1b	0.9428	0.01549	0.9766	0.00388	0.9418	0.00788	0.929	0.0125
1c	0.9382	0.00725	0.972	0.00193	0.9366	0.00931	0.9436	0.0092
1d	0.9048	0.01349	0.9426	0.0092	0.9385	0.00693	0.9568	0.00716

**Figure S9.** Mander's correlation coefficient calculation for probes **2a – 2d** for colocalization studies conducted with LysoTracker Green DND-26.

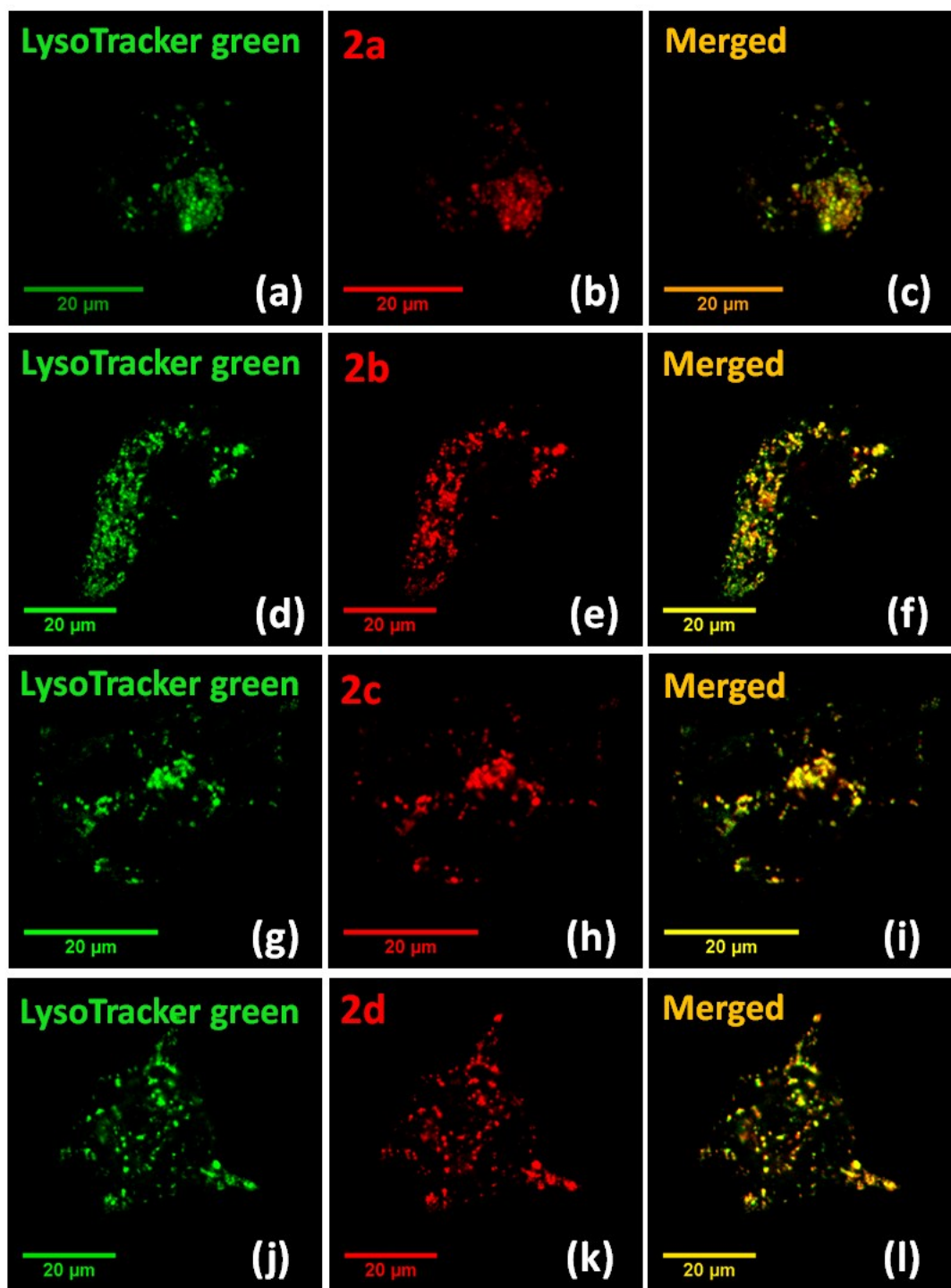




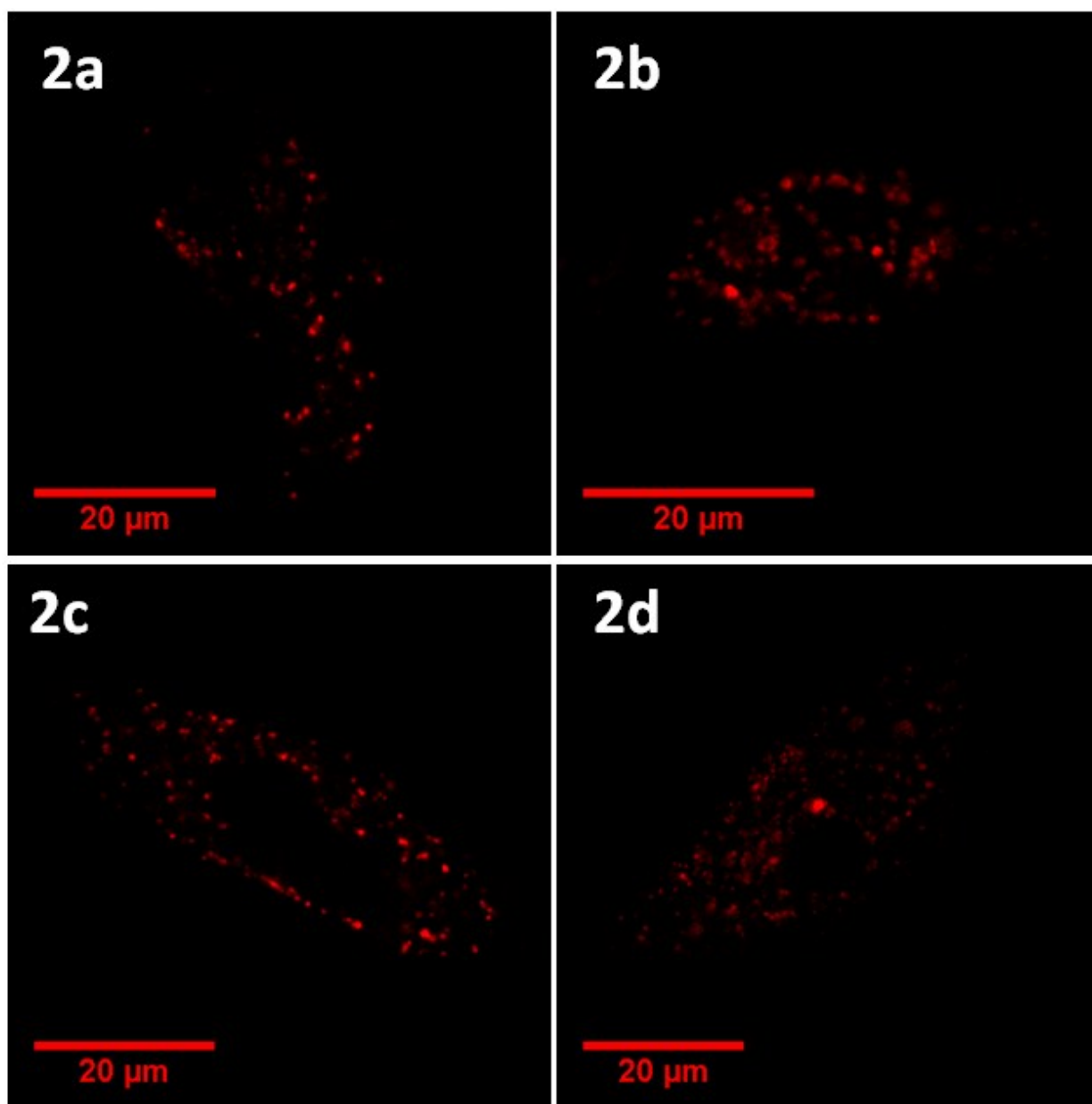
**Figure S10.** Fluorescent confocal microscopy images of HEK 293 cells stained with probes **2b** and **2d** (500 nM) for 30 minutes in the presence of commercial LysoTracker Green DND-26 (70 nm) at 63x magnification using an oil objective. Images from left to right represents the confocal imaging of LysoTracker green (a,d) Probe **2b** (b), **2d** (e) and overlapped images (c,f). Probes **2b** and **2d** were excited with a 561 nm laser line and LysoTracker green was excited with a 488 nm laser line.



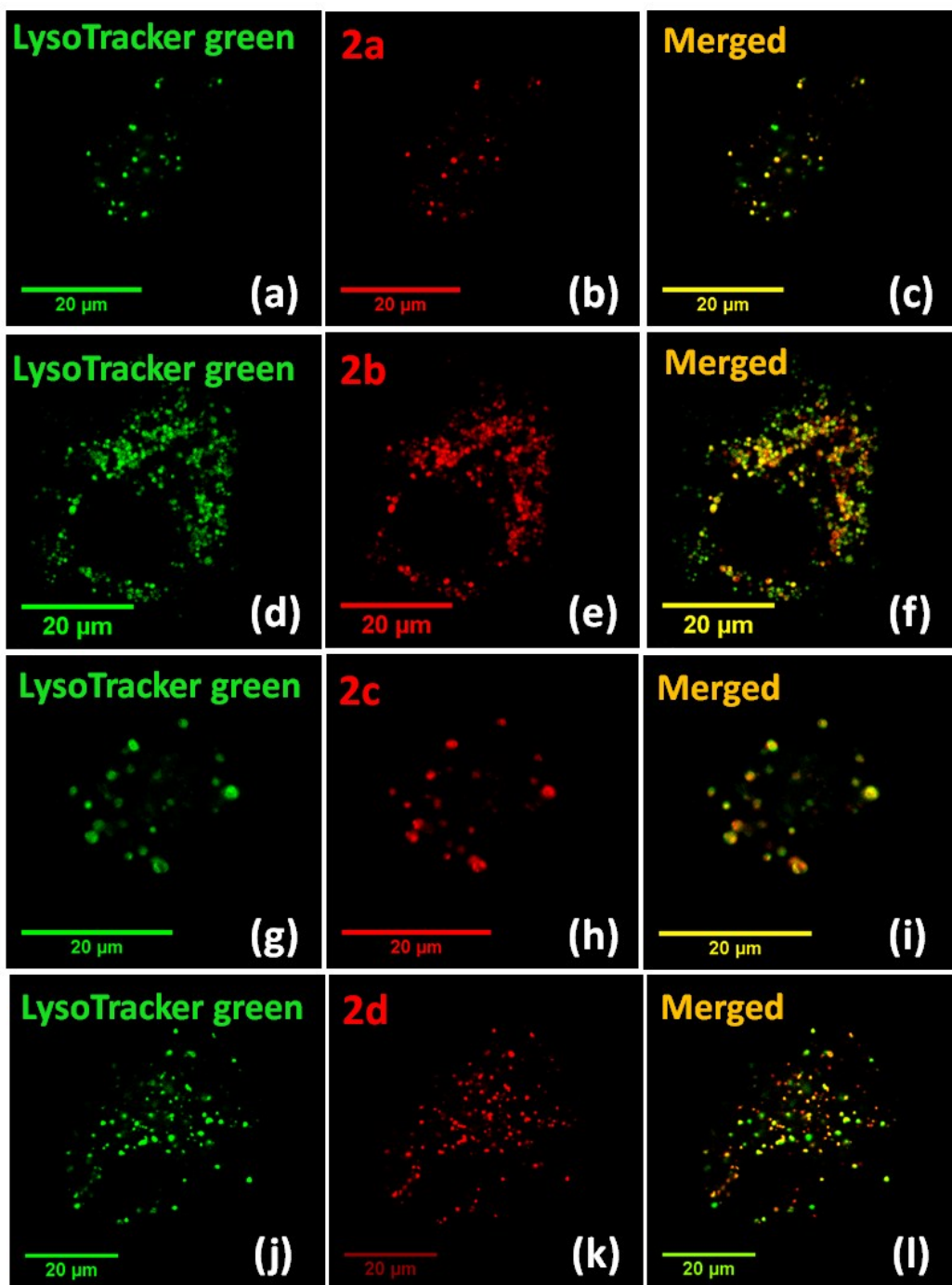
**Figure S11.** Fluorescent confocal microscopy images of A549 cells stained with probes **2a**, **2b**, **2c** and **2d** (500 nM) for 30 minutes in the presence of commercial LysoTracker Green DND-26 (70 nm) at 63x magnification using an oil objective. Images from left to right represents the confocal imaging of LysoTracker green (a,d,g,j) Probe **2a** (b), **2b** (e), **2c** (h) and **2d** (k). Images c, f, i and l represent overlapped images for the probes with LysoTracker green.. Probes **2a** - **2d** were excited with a 561 nm laser line and LysoTracker green was excited with a 488 nm laser line.



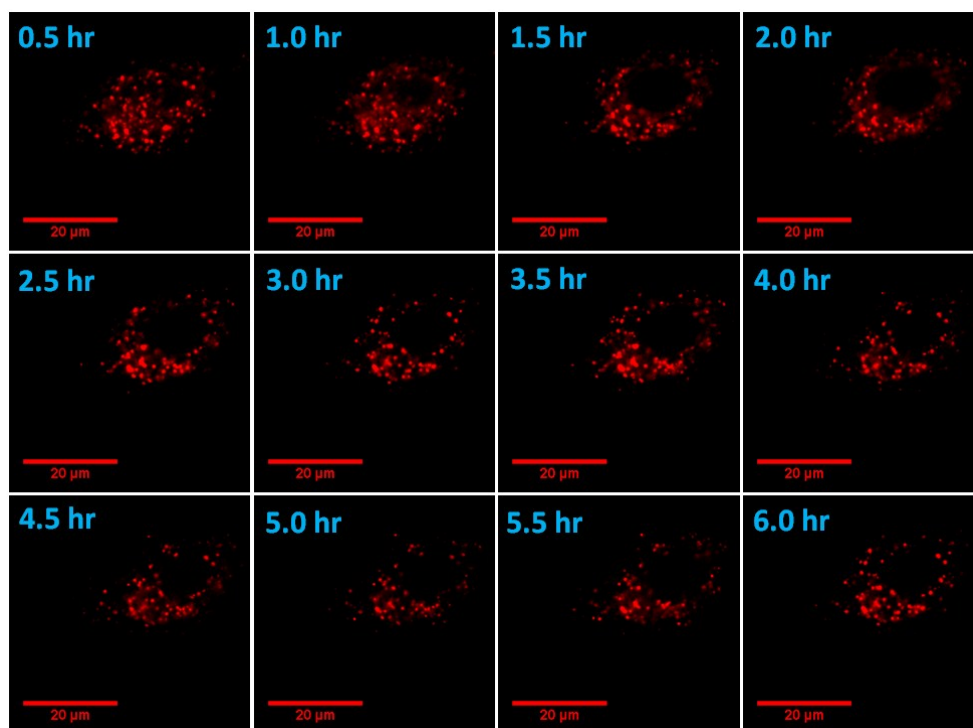
**Figure S12.** Fluorescent confocal microscopy images of Huh 7.5 cells stained with probes **2a**, **2b**, **2c** and **2d** (500 nM) for 30 minutes in the presence of commercial LysoTracker Green DND-26 (70 nm) at 63x magnification using an oil objective. Images from left to right represents the confocal imaging of Lysotracker green (a,d,g,j) Probe **2a** (b), **2b** (e), **2c** (h) and **2d** (k). Images c, f, i and l represent overlapped images for the probes with Lysotracker green. Probes **2a** - **2d** were excited with a 561 nm laser line and LysoTracker green was excited with a 488 nm laser line.



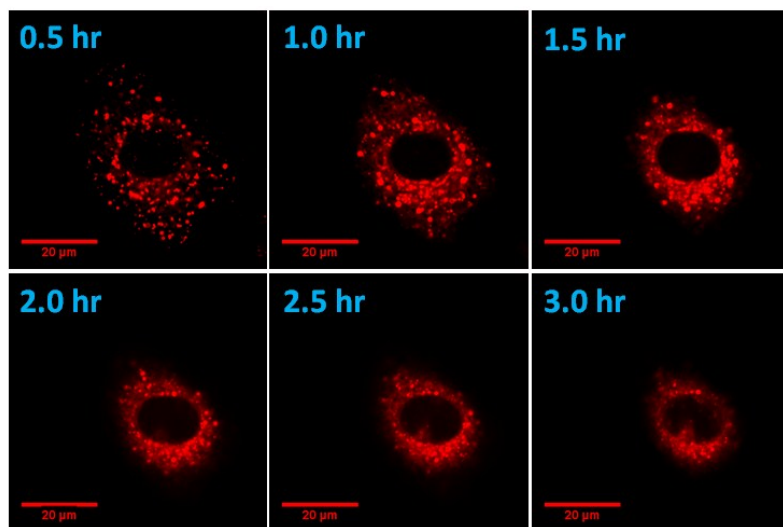
**Figure S15.** Fluorescent confocal microscopy images of **2a** – **2d** (100 nM) treated with COS-7 cells for 30 minutes. Probes **2a** – **2d** were excited with a 561 nm laser.



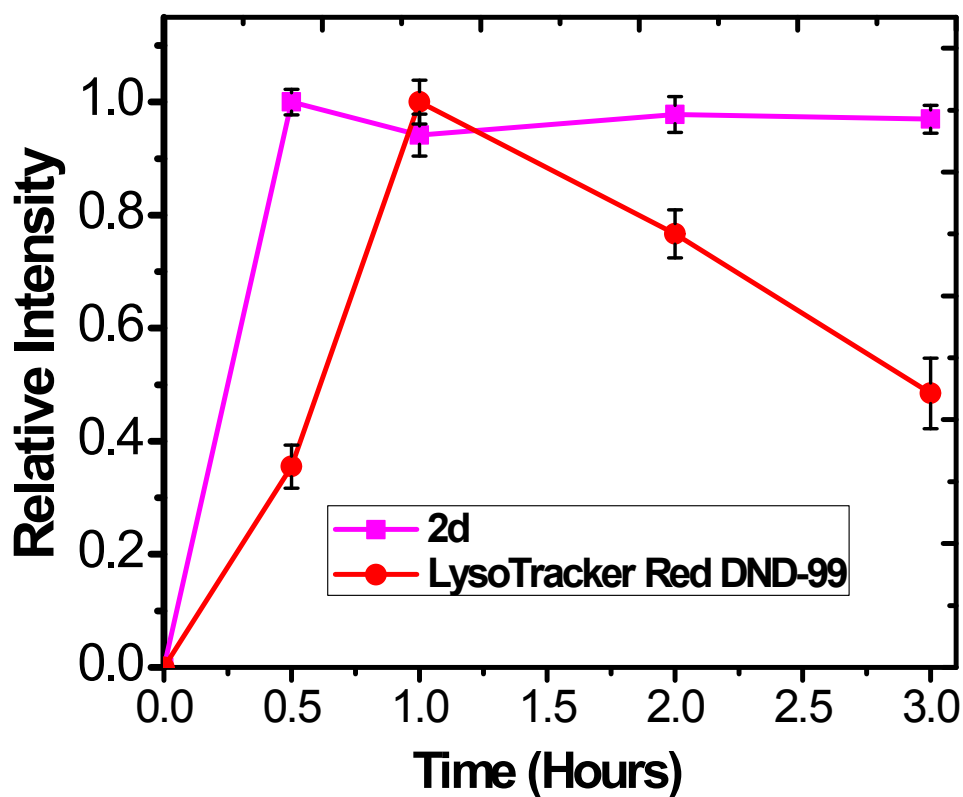
**Figure S16.** Fluorescent confocal microscopy images of COS-7 cells stained with probes **2a**, **2b**, **2c** and **2d** (500 nM) for 30 minutes in the presence of commercial LysoTracker Green DND-26 (70 nm) at 63x magnification using an oil objective. Images from left to right represents the confocal imaging of LysoTracker green (a,d,g,j) Probe **2a** (b), **2b** (e), **2c** (h) and **2d** (k). Images c, f, i and l represents overlapped images for the probes with LysoTracker green. Probes **2a** - **2d** were excited with a 561 nm laser line and LysoTracker green was excited with a 488 nm laser line.



**Figure S18.** Fluorescence microscopy images obtained for probe **2d** in A549 cells over a period of 6 hours. A549 cells were first incubated with **2d** (500 nM) for 30 minutes. Images were obtained every 30 minutes under consistent parameters of the microscope. Probe **2d** was excited with a 561 nm laser.

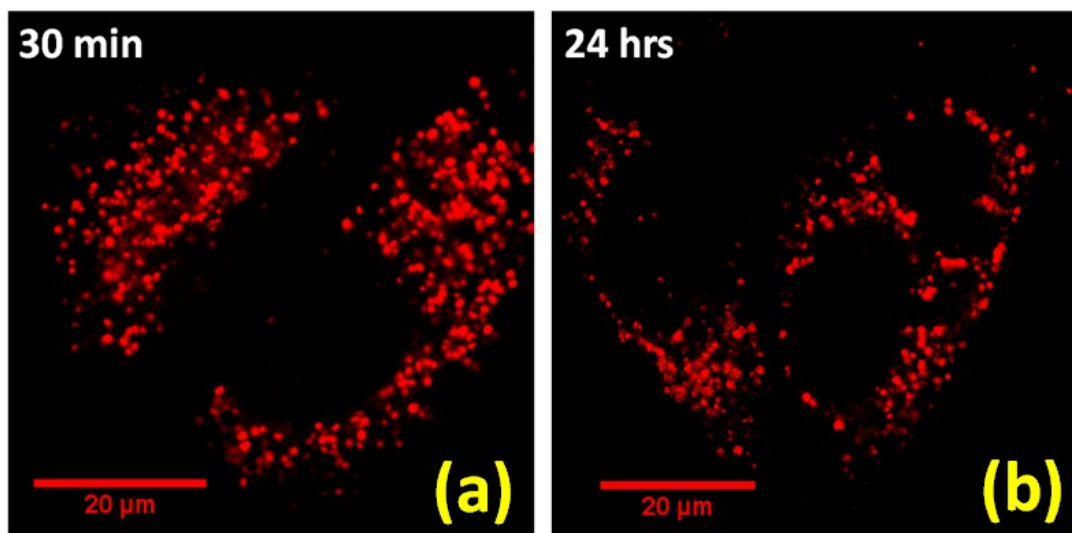


**Figure S19.** Fluorescence microscopy images obtained for LysoTracker® Red DND-99 in A549 cells over a period of 3 hours. A549 cells were first incubated with LysoTracker Red DND-99 (70 nM) for 30 minutes. Images were obtained every 30 minutes intervals under consistent parameters of the microscope. LysoTracker Red was excited with 561 nm laser.



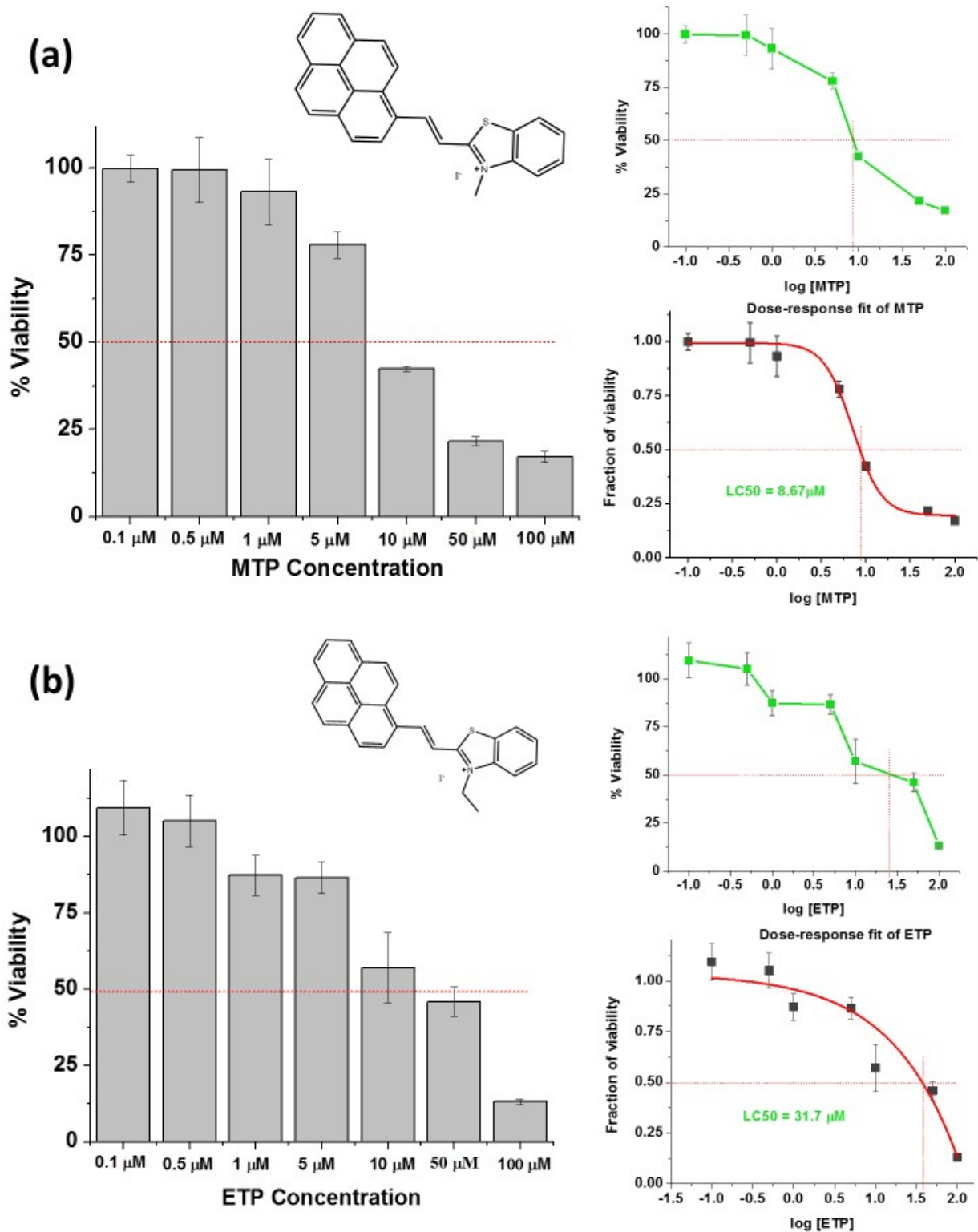
**Figure S20.** Relative average fluorescence intensity obtained from A549 cells stained with probe **2d** (500 nM) and LysoTracker® Red (70 nM) for period of 3 hours. Probe **2d** and LysoTracker® Red were excited with 561 nm laser.



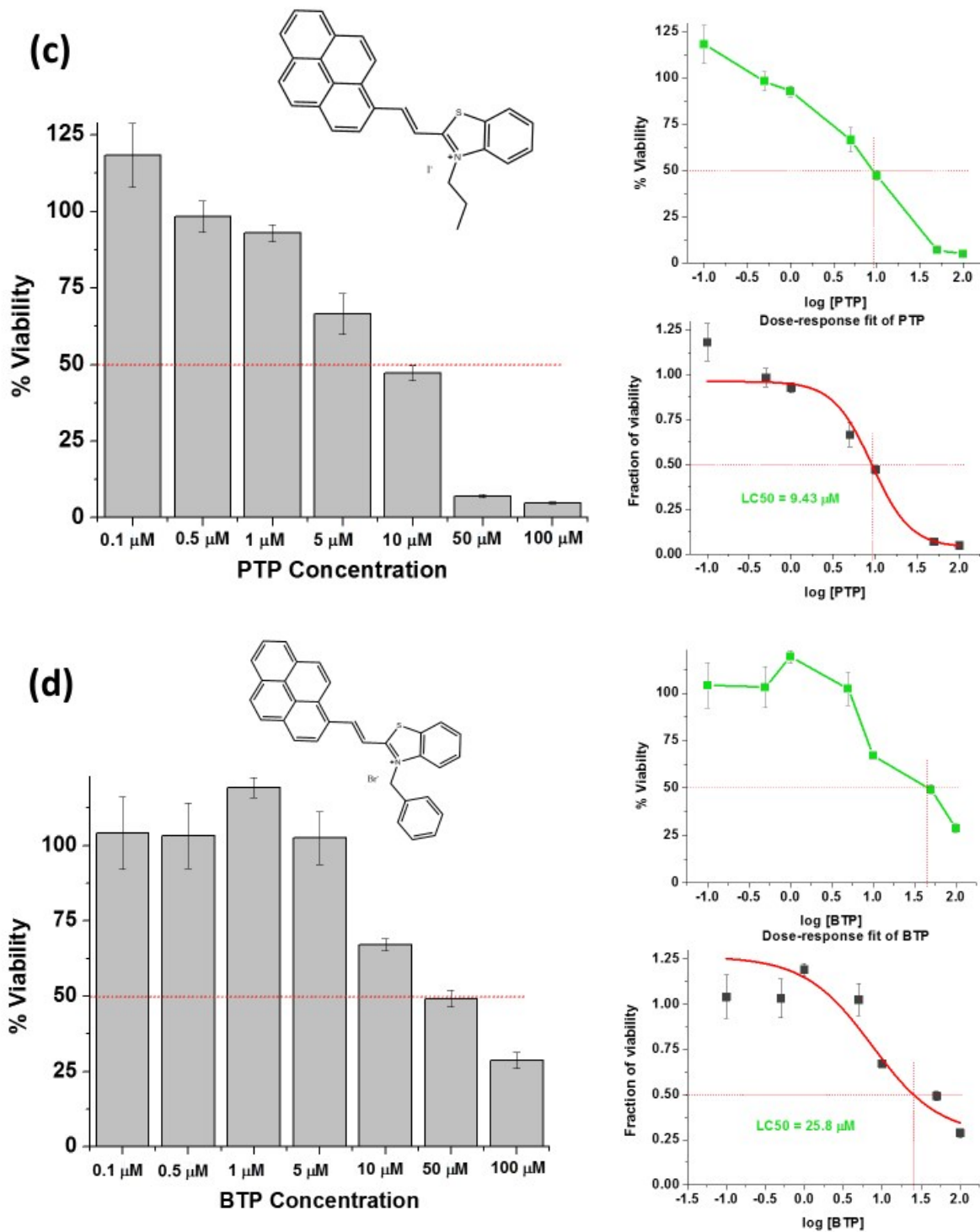


**Figure S21.** Fluorescence confocal images obtained for COS-7 cells incubated with **2d** (500 nM) for 30 minutes. Images were obtained at 30 minutes period and 24-hour period. Probe **2d** was excited with a 561 nm laser. Note that (a) and (b) represents two different cell locations.

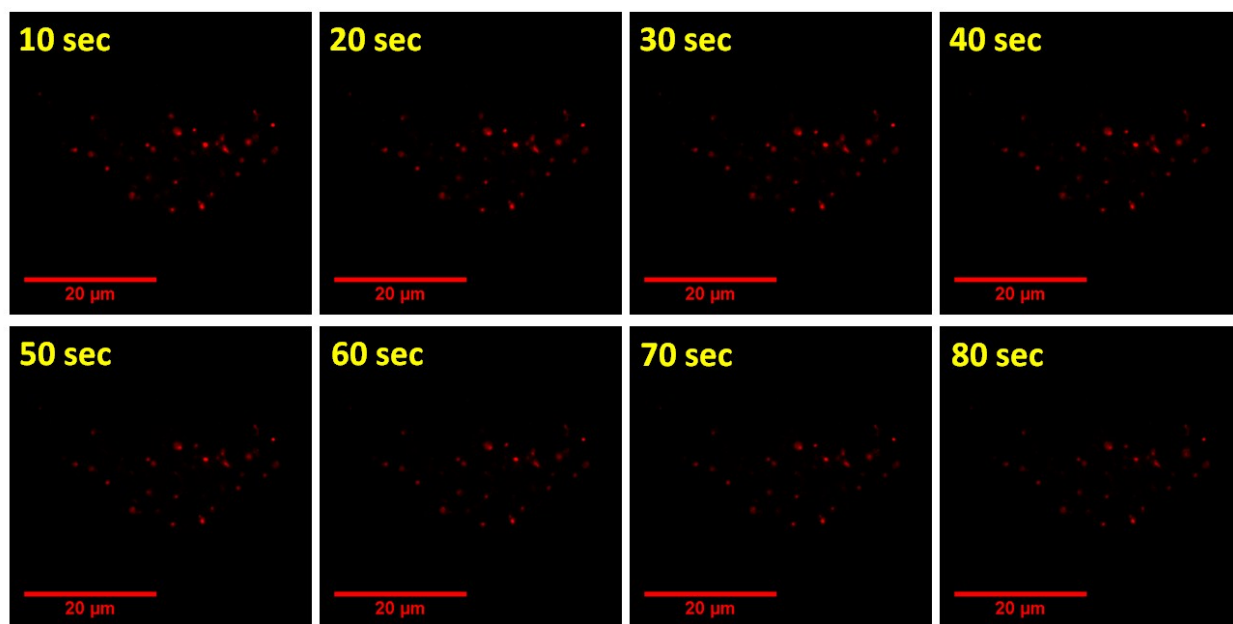




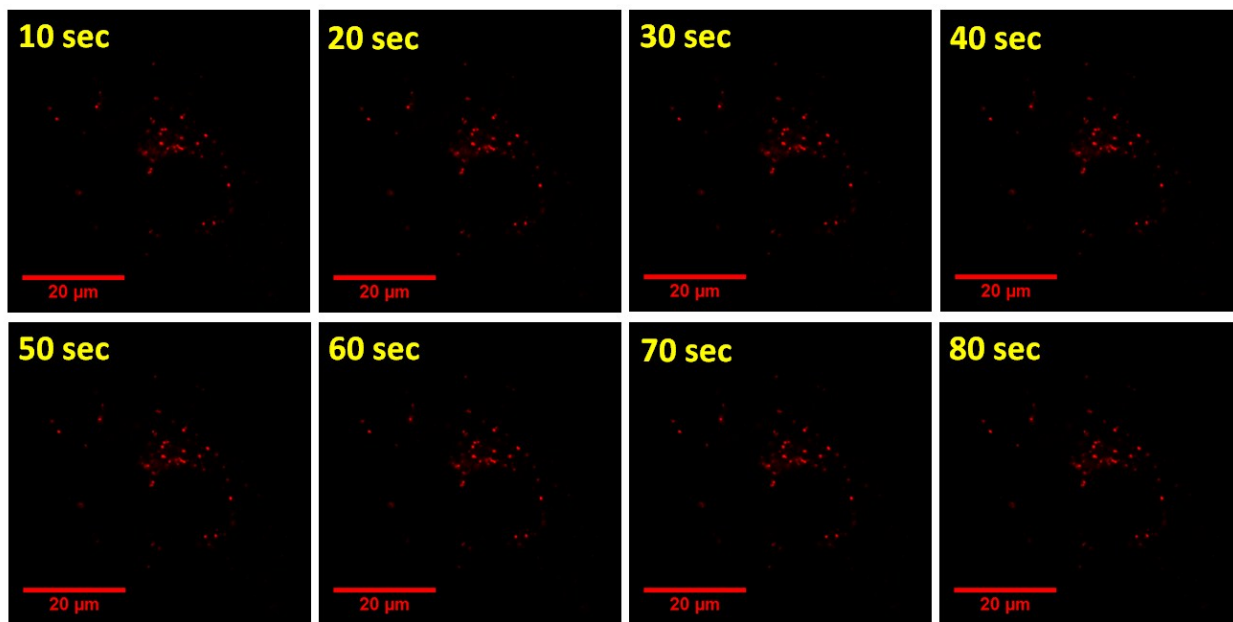
**Figure S22.1** Cell viability results (bar-chart) obtained for **2a** (a) and **2b** (b) by CellTiter Glow® cell viability assay.



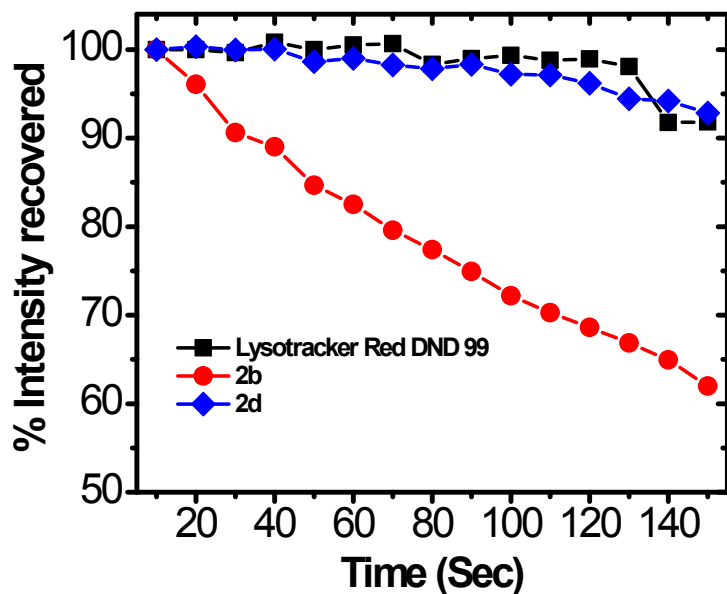
**Figure S22.2** Cell viability results (bar-chart) obtained for **2c** (c) and **2d** (d) by CellTiter Glow® cell viability assay.



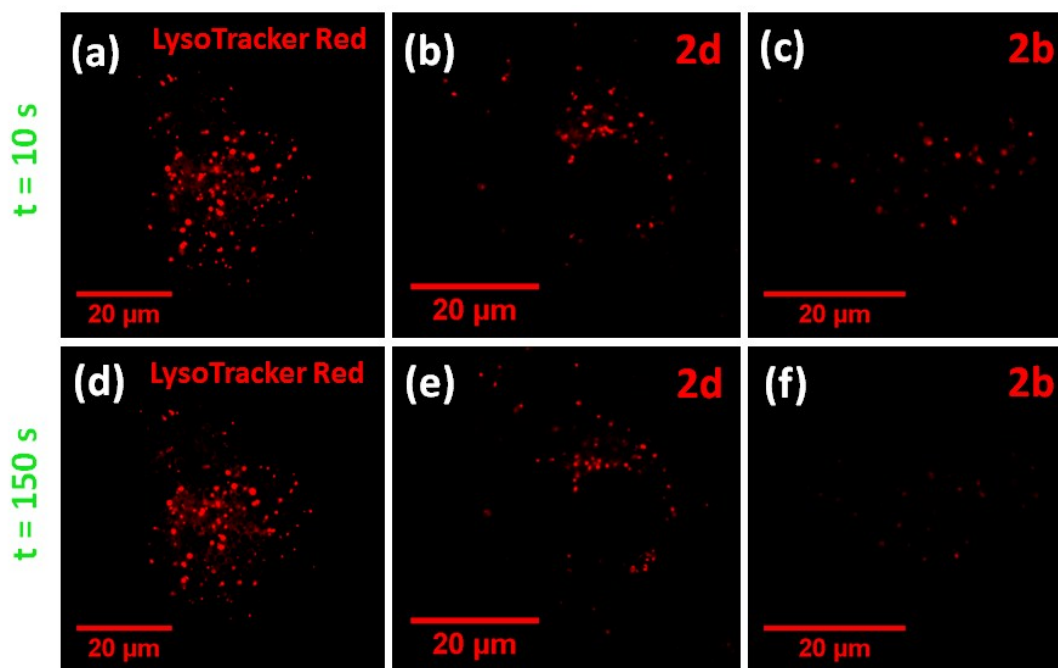
**Figure S23.** Fluorescent Confocal microscope images obtained for probe **2b** (100 nM) in A549 cells during continuous irradiation with a 561 nm laser for 80 seconds. Images were obtained at 10 second intervals.



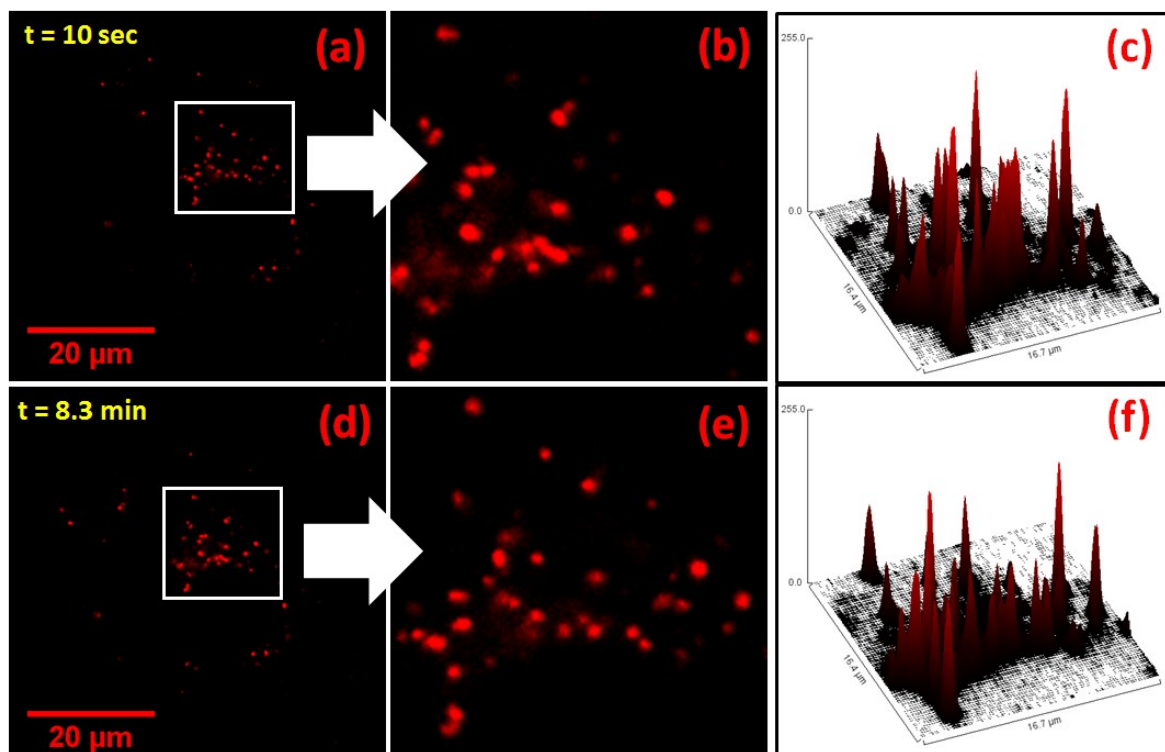
**Figure S24.** Fluorescent Confocal microscope images obtained for probe **2d** (100 nM) in A549 cells during continuous irradiation with a 561 nm laser for 80 seconds. Images were obtained at 10 second intervals



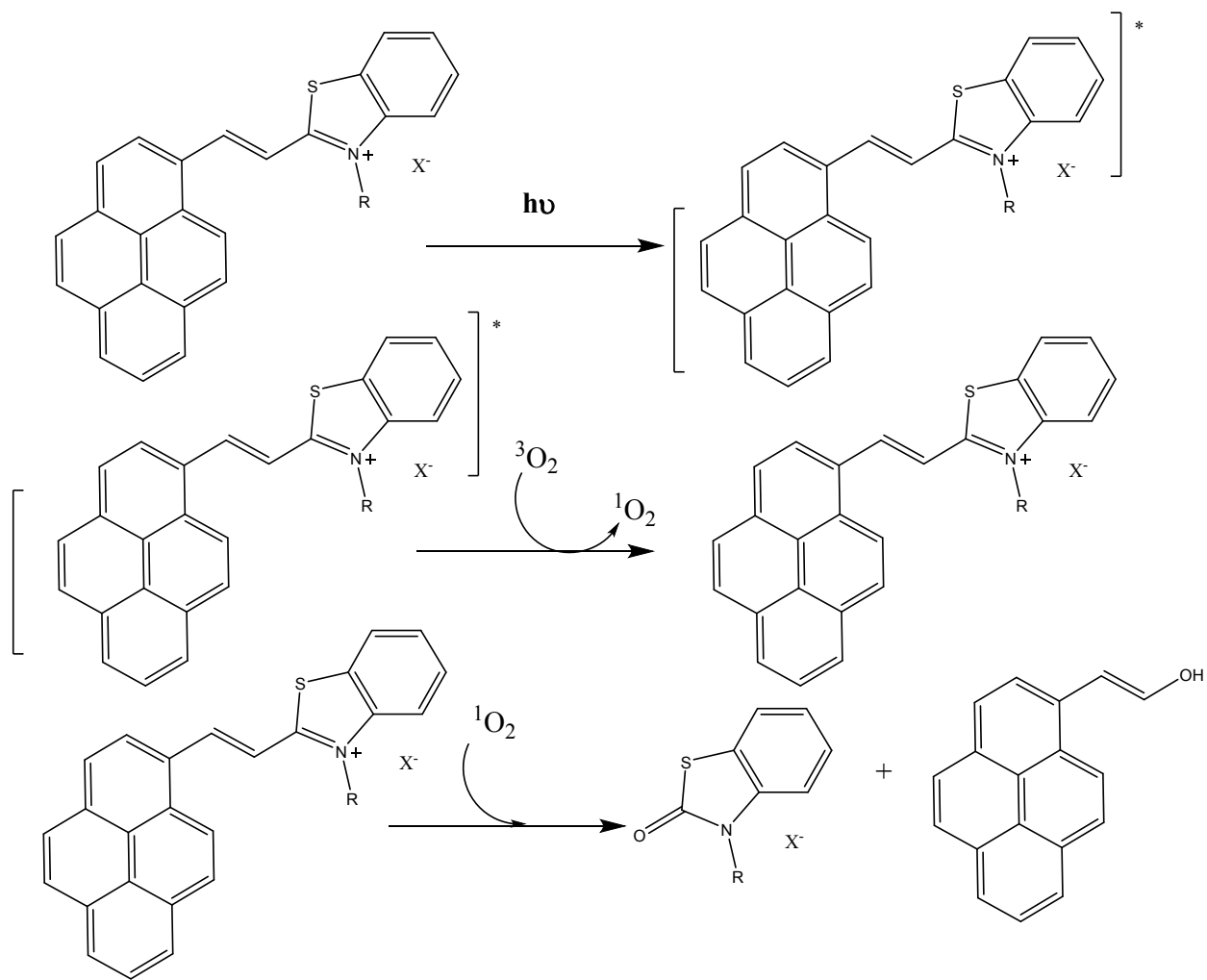
**Figure 25.** The plot of recovered fluorescence intensity versus irradiation time calculated for LysoTracker Red, **2b** and **2d**. All probes were stained in A549 cells, and excited with a 561 nm laser.



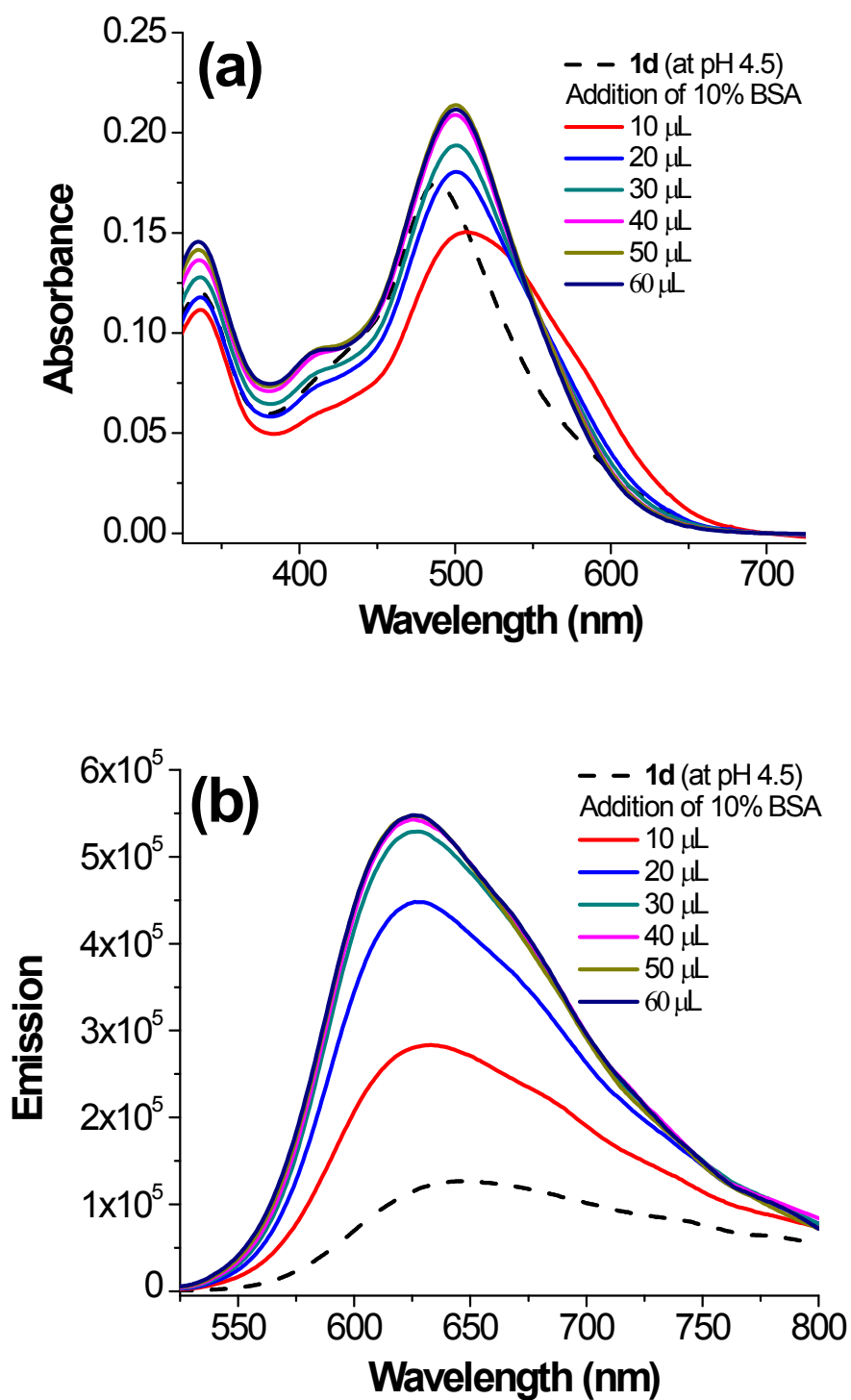
**Figure S26.** Confocal microscope images obtained for LysoTracker<sup>®</sup> Red DND-99 (100 nM, a,d), probe **2d** (100 nM, b,e) and probe **2b** (100 nM, c,e) in A549 cells during continuous irradiation with a 561 nm laser. The top row figures (a- c) show images obtained at  $t = 10$  s intervals (a,b,c) and bottom row figures (d – f) show images obtained at  $t = 150$  s intervals (d,e,f).



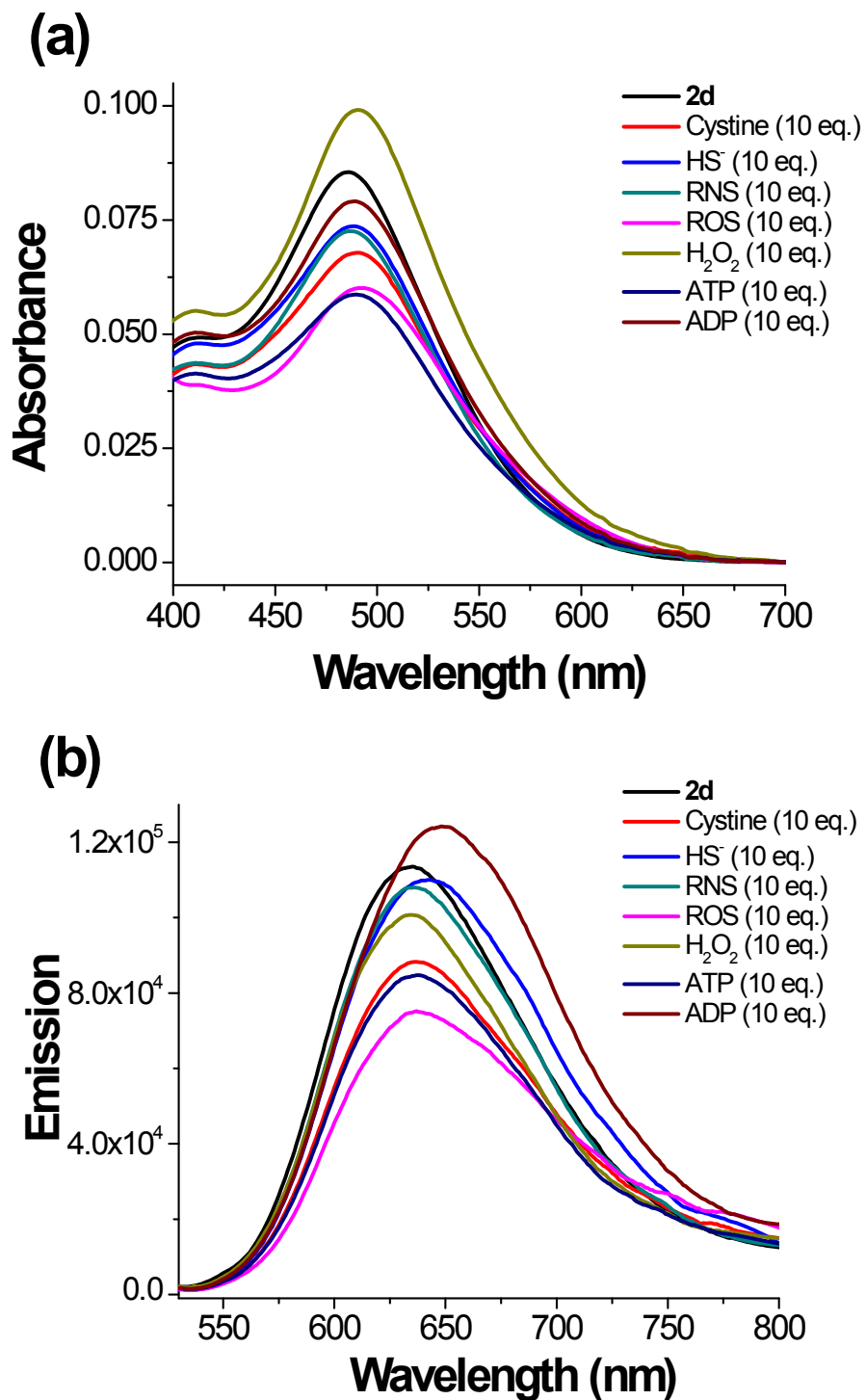
**Figure S27.** (a) - (c) represents the confocal microscope imaging results obtained at  $t = 10$  s intervals and (d) - (f) represents the confocal microscope results obtained at  $t = 8.3$  minutes upon continuous irradiation of A549 cells stained with probe **2d** (100 nM) for 30 minutes. Figures (c) and (f) represents the surface emission plots of the highlighted regions of (a) and (d) obtained for **2d** during  $t = 10$  sec and  $t = 8.3$  min intervals. Probe **2d** was excited with a 561 nm laser line.



**Figure S28.** Possible photodecomposition mechanism for probe 2



**Figure S29.** Absorbance (a) and fluorescence emission (b) observed upon addition of 10% bovine serum albumin (BSA) in to an aqueous solution (pH 4.5) of probe **2d** ( $1 \times 10^{-6}$  M) at room temperature.



**Figure S30.** Absorbance (a) and fluorescence emission (b) observed upon addition upon different reactive species to an aqueous solution of probe **2d** ( $1 \times 10^{-6}$  M) at room temperature.