Chemical Synthesis and Characterization

General Methods for Chemical Synthesis and Characterization

Chemical reagents and solvents (dry) were purchased from commercial suppliers and used without further purification. References to previously synthesized compounds are provided along with characterization data. Thin layer chromatography (TLC) (Silicycle, F254, 250 μm) and preparative thin layer chromatography (PTLC) (Silicycle, F254, 1000 μm) was performed on glass backed plates pre-coated with silica gel which were visualized by fluorescence quenching under UV light. Flash column chromatography was performed on Silicycle Silica Flash F60 (230–400 Mesh) using a forced flow of air at 0.5–1.0 bar. NMR spectra were measured on a Bruker AVB-400 MHz, 101 MHz and a Bruker AV-500 MHz, 126 MHz. NMR spectra measured on a Bruker AVII-900 MHz, 226 MHz, equipped with a TCI cryoprobe accessory, were performed by Dr. Jeffrey Pelton (QB3). Chemical shifts are expressed in parts per million (ppm) and are referenced to Chloroform-*d* (7.26 ppm, 77.16 ppm), Methanol-d_4 (3.31 ppm, 49.00 ppm), or Dimethyl Sulfoxide-d_6 (2.50 ppm, 39.52 ppm). Coupling constants are reported as Hertz (Hz). Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet. High-resolution ESI mass spectra (HR-ESI-MS) were measured by the QB3/Chemistry mass spectrometry service at University of California, Berkeley. High performance liquid chromatography (HPLC) and low resolution ESI Mass Spectrometry were performed on an Agilent Infinity 1200 analytical instrument coupled to an Advion CMS-L ESI mass spectrometer. The column used for the analytical HPLC was a Phenomenex Luna 5 μm C18(2) (4.6 mm I.D. × 75 mm) with a flow rate of 1.0 mL/min. The mobile phases were MQ-H2O with 0.05% trifluoroacetic acid (eluent A) and HPLC grade acetonitrile with 0.05% trifluoroacetic acid (eluent B). Signals were monitored at 254, 545, and 690 nm over 7.5 min with a gradient of 10-100% eluent B unless otherwise noted. Ultra-high performance liquid chromatography (UHPLC) for purification of final compounds was performed using a Waters Acquity Autopurification system equipped with a Phenomenex Luna 10 μm C18(2) column (21.2 mm I.D. x 250 mm) with a flow rate of 30.0 mL/min, made available by the Catalysis Facility of Lawrence Berkeley National Laboratory (Berkeley, CA). The mobile phases were MQ-H2O with 0.05% trifluoroacetic acid (eluent A) and HPLC grade acetonitrile with 0.05% trifluoroacetic acid (eluent B). Signals were monitored at 254, 545, and 690 nm over 20 min with a gradient of 10-100% eluent B, unless otherwise noted.

Spectroscopic Studies

All dyes were dissolved at a concentration of 1 mM in 1:1 MeOH:ACN to generate stock solutions for analysis. These stock solutions were then diluted (1:1000) in the indicated solvent for each spectroscopic analysis. UV-Vis absorbance and fluorescence spectra were recorded using a Shimadzu 2501 Spectrophotometer (Shimadzu) and a Quantamaster Master 4 L-format scanning spectrofluorometer (Photon Technologies International). The fluorometer is equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver. Samples were measured in 1-cm path length quartz cuvettes (Starna Cells).

Extinction coefficients were determined from the absorbance value of a 1 uM dye solution at the absorbance maximum for each dye. The reported extinction coefficients are the average of 3 independent measurements. Relative quantum yields (Φ_{fl}) were calculated by comparison to Rhodamine 101 (Φ_{fl} = 1.0 in MeOH)[1] or Cy5.5 (Φ_{fl} = 0.23 in PBS, pH 7.4)[2] standards.[3] Stock solutions of standards were prepared in DMSO (0.25-1.25 mM) and diluted with appropriate solvent (1:1000 dilution). Absorption and emission (Rhodamine 101: excitation = 576 nm, Cy5.5: excitation = 680 nm) spectra were taken at 5 concentrations. The absorption value at the excitation wavelength was plotted against the integration of the area of fluorescence curve (Rhodamine 101: 586-700 nm, Cy5.5: 690-900 nm). Absorption and emission spectra were taken at the same wavelengths of the indicated standard for each dye at 5 concentrations in a TBS (50 mM Tris-HCl, pH 7.4, 0.1 M NaCl) solution containing 0.1 % (w/w) SDS. The slope of the linear best fit between the absorption value at the excitation wavelength and total fluorescence

area was used to calculate the relative Φ_{fl} by the equation $\Phi_{fl}(X) = \Phi_{fl}(R)(S_X/S_R)(\eta_X/\eta_R)^2$, where S_R and S_X are the slopes of the reference compound and unknown, respectively, and η is the refractive index of the solution. The refractive indices of the PBS and TBS buffers were both assumed to be 1.333.

For long term storage, stock solutions of VoltageFluors were prepared in 1:1 MeOH:ACN (1 mM) and then aliquoted, dried, and stored as solids at -20 °C. Prior to use, aliquots were re-dissolved to 1 mM in DMSO and stored at room temperature away from ambient light until they were used up.

Cell Culture

All animal procedures were approved by the UC Berkeley Animal Care and Use Committees (Approval Number AUP-2016-06-8845-2) and conformed to the NIH Guide for the Care and Use of Laboratory Animals and the Public Health Policy.

HEK cell / immortalized cell line culture

HEK293T cells were acquired from the UC Berkeley Cell Culture Facility and were verified by STR (short tandem repeat) profiling. Cells were routinely checked for mycoplasma contamination. HEK293T cells were maintained in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 4.5 g/L D-glucose, 10% fetal bovine serum (FBS, Seradigm), and 2 mM GlutaMAX (Gibco). Cells were passaged every few days into fresh media following dissociation with 0.05% Trypsin-EDTA (Gibco). All cells were discarded after 30 passages. For imaging experiments, 12 mm and 25 mm glass coverslips (Electron Microscopy Sciences) were prepared by acid washing (1 M HCl, approx. 5 hours), followed by three overnight washes in ethanol and three overnight washes in water. Coverslips were sterilized by heating at 150°C for 2-4 hours. To facilitate cell attachment, sterilized coverslips were coated with Poly-D-Lysine (PDL; 1 mg/mL; Sigma-Aldrich) for 1-24 hours at 37°C, followed by two washes with water and two washes with phosphate-buffered saline. For general imaging, cells were seeded onto prepared coverslips in complete DMEM at a 42,000 cells/cm^2 and used approximately 24 hours after plating. For electrophysiology, cells were seeded at a density of 21,000 cells/cm^2 in low glucose DMEM (1 g/L glucose, 10% FBS, 1 mM pyruvate, 2 mM GlutaMAX) and used approximately 16 hours after plating.

Neuron culture

Hippocampi were dissected from embryonic day 18 Sprague Dawley rats (Charles River Laboratory) in cold sterile HBSS (zero Ca^{2+}, zero Mg^{2+}). All dissection products were supplied by Invitrogen, unless otherwise stated. Hippocampal tissue was treated with trypsin (2.5%) for 15 min at 37 °C. The tissue was triturated using fire polished Pasteur pipettes, in minimum essential media (MEM) supplemented with 5% fetal bovine serum (FBS; Thermo Scientific), 2% B-27, 2% 1 M D-glucose (Fisher Scientific), and 1% glutamax. The dissociated cells were plated onto 12 mm diameter coverslips (Electron Microscopy Sciences, prepared as above) at a density of 27,000 cells per coverslip in MEM supplemented media. Neurons were maintained at 37 °C in a humidified incubator with 5% CO2. At 1 day in vitro (DIV) half of the MEM supplemented media was removed and replaced with Neurobasal media containing 2% B-27 supplement and 1% glutamax. Functional imaging was performed on 14-17 DIV neurons.

Cellular Loading of VoltageFluors

For cellular loading in HEK293T, VoltageFluor aliquots dissolved in DMSO (1 mM) were diluted to the indicated concentration in HBSS (Gibco). HEK293T cells were incubated in the VF-HBSS solution for 20 minutes in a humidified incubator at 37°C. Cells were then transferred to fresh HBSS for imaging. Hippocampal neurons were loaded with VoltageFluor at the indicated concentration in HBSS and incubated in the VF-HBSS solution for 20 minutes in a humidified incubator at 37°C. For imaging spontaneous activity, the hippocampal neurons were then

transferred to fresh HBSS for imaging. For imaging evoked activity and photobleaching, the hippocampal neurons were then transferred to a HBSS solution containing the synaptic blockers 10 μM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; Santa Cruz Biotechnology) and 25 μM D-2-Amino-5-phosphonopentanoic acid (APV; Sigma-Aldrich) to prevent recurrent activity. All cellular imaging was conducted under ambient atmosphere; no imaging samples were used for longer than half an hour.

Fluorescence microscopy

Epifluorescence microscopy

For imaging experiments, excitation light for the epifluorescence intensity image was generated either by a Spectra-X light engine red LED (Lumencor, 631/28 nm bandpass filter, 77.2 mW/mm^2) or a M660L4 mounted LED (Thorlabs, 650/60 nm bandpass filter, 51.1 mW/mm^2). Light was collected with an emission filter (732/68 nm or 775/140 nm bandpass filter) after passing through a dichroic mirror (654 or 700 nm longpass). Fluorescence images were acquired on an upright AxioExaminer Z-1 (Zeiss), controlled with μManager (V1.4, open-source, Open Imaging)[4], which was equipped with a 20x water immersion objective (W-Plan-Apo 20x/1.0 water objective; Zeiss) and an OrcaFlash4.0 sCMOS camera (Hamamatsu, pixel size: 6.5x6.5 μm^2). More detailed imaging information for each fluorescence intensity application is expanded below.

Membrane Staining and Voltage Sensitivity in HEK293T Cells

For membrane staining, images (2048x2048 px^2, pixel size: 0.325x0.325 μm^2) were collected with 1x1 binning and 100 ms exposure times. For voltage sensitivity experiments, images (100x100 px^2, pixel size: 1.3x1.3 μm^2) were collected continuously with 4x4 binning and constant LED illumination at a sampling rate of 0.5 kHz.

Evoked and Spontaneous Activity in Dissociated Rat Hippocampal Neurons

For evoked activity imaging, images (512x100 px^2, pixel size: 1.3x1.3 μm^2) were collected continuously with 4x4 binning and constant M660L4 mounted LED illumination at a sampling rate of 0.5 kHz. Extracellular field stimulation was delivered by a Grass Stimulator connected to a recording chamber containing two platinum electrodes (Warner), with triggering provided through a Digidata 1440A digitizer and pCLAMP 10 software (Molecular Devices). Action potentials were triggered by 1 ms 80 V field potentials delivered at 5 Hz. For spontaneous activity imaging, images (512x100 px^2, pixel size: 1.3x1.3 μm^2) were collected continuously with 4x4 binning and constant M660L4 mounted LED illumination at a sampling rate of 0.5 kHz.

Photostability in Dissociated Rat Hippocampal Neurons

For photostability experiments, images (512x100 px^2, pixel size: 1.3x1.3 μm^2) were collected with 4x4 binning and 100 ms exposure times every 20 seconds for 10 minutes with constant illumination of a M660L4 mounted LED or a Spectra-X light engine red LED (51.1 mW/mm^2).

Image analysis

Voltage Sensitivity in HEK293T Cells (%ΔF/F)

Analysis of voltage sensitivity in HEK293T cells was performed using ImageJ (FIJI). Briefly, a region of interest (ROI) encompassing the cell body was selected and the average fluorescence intensity was calculated for each frame. For background subtraction, a ROI encompassing a region without cells was selected and the average pixel intensity was calculated for each frame. The average of the background trace was used to subtract background signal from the fluorescence intensity trace. A linear bleaching curve was then fit to the background-subtracted trace and subtracted from the background-subtracted trace to yield a bleach corrected trace. ΔF/F values were calculated by subtracting the median value of fluorescence when the cell is held at -60 mV from the bleach corrected trace and

the dividing the resulting ΔF trace by the median value of fluorescence when the cell is held at -60 mV. Voltage sensitivity was then determined by plotting ΔF/F values vs the applied voltage steps and finding the slope of a linear best-fit. Signal-to-noise ratios were calculated by dividing the average ΔF/F value of the 40 mV voltage step by the standard deviation of the ΔF/F trace when the cell is held at -60 mV for 10 frames directly prior to the 40 mV voltage step.

Evoked and Spontaneous Activity in Dissociated Rat Hippocampal Neurons

For analysis of evoked and spontaneous voltage responses in dissociated rat hippocampal neurons, regions of interest (ROIs) encompassing cell bodies were drawn in ImageJ (FIJI) and the mean fluorescence intensities for each frame were extracted. For background subtraction, a ROI encompassing a region without cells was selected and the mean fluorescence intensities for each frame were extracted. ΔF/F values were calculated in the following manner. First, a mean background value was calculated from the background trace and subtracted from all raw fluorescence frames, bypassing the noise amplification which arises from subtracting background for each frame, to give a background corrected trace. A least squares regression was then fit to the background corrected trace. A bleaching curve, derived from the slope of the regression, was then subtracted from the background corrected trace to correct for photobleaching and yield a bleach-corrected trace. The median of the bleach-corrected trace was subtracted from each timepoint of the bleach-corrected trace to yield a ΔF trace. The ΔF trace was then divided by the median of the bleach-corrected trace to give a ΔF/F trace. No averaging has been applied to any voltage traces.

Photostability in Dissociated Rat Hippocampal Neurons

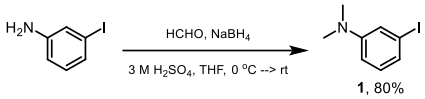
For analysis of photostability in dissociated rat hippocampal neurons, regions of interest (ROIs) encompassing cell bodies were drawn in ImageJ (FIJI) and the mean fluorescence intensities for each frame were extracted. For background subtraction, a ROI encompassing a region without cells was selected and the mean fluorescence intensities for each frame were extracted. The background fluorescence intensity was subtracted for each frame to yield background subtracted fluorescence values. The background subtracted fluorescence values were then normalized to the maximum fluorescence value for each neuron. Bleaching rate was then determined by plotting the normalized fluorescence values for each cell versus time and finding the slope of a linear best-fit.

Electrophysiology

For electrophysiological experiments in HEK293T, pipettes were pulled from borosilicate glass with filament (Sutter Instruments, BF150-86-10) with a P-97 pipette puller (Sutter Instruments) to a resistance of 4-7 MΩ. Pipettes were filled with an internal solution (composition, in mM): 125 potassium gluconate, 10 HEPES, 10 KCl, 5 NaCl, 2 ATP disodium salt, 1 EGTA, 0.3 GTP sodium salt (pH 7.25, 285 mOsm). Pipettes were positioned with an MP-225 micromanipulator (Sutter Instruments). Electrophysiological recordings were obtained with an Axopatch 200B amplifier (Molecular Devices) at room temperature. The signals were digitized with a Digidata 1440A, sampled at 50 kHz, filtered at 5 kHz, and recorded with pCLAMP 10 software (Molecular Devices).

Electrophysiology was performed in the whole cell voltage clamp configuration. After gigaseal formation and break-in, recordings were only pursued if series resistance in voltage clamp was less than 30 MΩ and the recording maintained a 30:1 ratio of membrane resistance to access resistance throughout all voltage steps. No series resistance compensation was applied. Fast capacitance was compensated in the cell attached configuration.

Detailed Synthetic Procedures



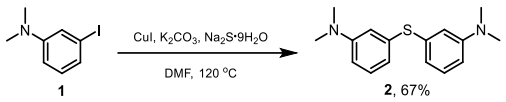
3-iodo-N,N-dimethylaniline, 1:

Formaldehyde (37% solution, 5.1 mL) and 3 M sulfuric acid (13.7 mL) were added to THF (60 mL) and the reaction mixture was stirred at 0 °C for 10 min. 3-Iodoaniline (5.00 g, 22.8 mmol) was then added dropwise and the reaction mixture was stirred at 0 °C for 10 min. Finally, sodium borohydride (3.45 g, 91.3 mmol) was added slowly to the reaction mixture at 0 °C. The reaction mixture was then allowed to warm to room temperature and stirred for 1 h, after which saturated sodium bicarbonate solution (90 mL) was added to quench the reaction. The reaction mixture was extracted with dichloromethane (3 x 25 mL), dried with anhydrous sodium sulfate, filtered, and concentrated to dryness *in vacuo*. The crude residue was purified by flash chromatography (100% EtOAc, isocratic), yielding **1** as a brown oil (4.53 g, 80%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.05 – 6.99 (m, 2H), 6.93 (t, *J* = 8.2 Hz, 1H), 6.67 (dd, *J* = 8.3, 2.6 Hz, 1H), 2.93 (s, 6H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 151.70, 130.53, 125.34, 121.23, 111.70, 95.69, 40.47.

HRMS (ESI+) Calculated for $C_8H_{11}N_1I_1$ $[M+H]^+$ 247.9931; Found 247.9931.



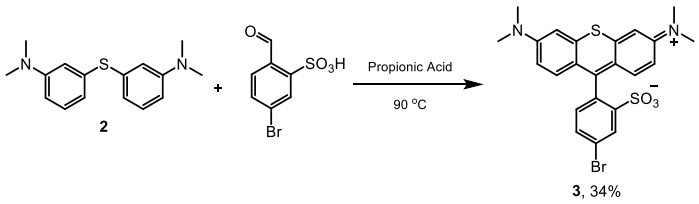
3,3'-thiobis(N,N-dimethylaniline), 2:

1 (4.53 g, 18.3 mmol) was added to a flame-dried round-bottom flask followed by copper(I) iodide (350 mg, 1.83 mmol), potassium carbonate (2.54 g, 18.3 mmol), and sodium sulfide nonahydrate (2.64 g, 11.0 mmol). The round-bottom flask was then evacuated and backfilled with nitrogen (3x). Anhydrous DMF (45 mL) was then added to the round-bottom flask and the reaction mixture was stirred at 120 °C for 3 days. The reaction mixture was then cooled, and the solvent was removed *in vacuo*. The crude mixture was re-dissolved in ethyl acetate, filtered, and the solvent was removed *in vacuo*. The crude residue was then purified by flash chromatography (1:9 EtOAc:Hexanes, isocratic), yielding **2** as a purple oily solid (1.66 g, 67%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.15 (t, *J* = 8.0 Hz, 2H), 6.77 (s, 2H), 6.70 (dd, *J* = 6.8, 1.7 Hz, 2H), 6.60 (dd, *J* = 8.4, 2.6 Hz, 2H), 2.91 (s, 12H).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 151.15, 136.55, 129.68, 119.16, 114.89, 111.30, 40.63.

HRMS (ESI+) Calculated for $C_{16}H_{21}N_2S_1$ $[M+H]^+$ 273.1420; Found 273.1421.



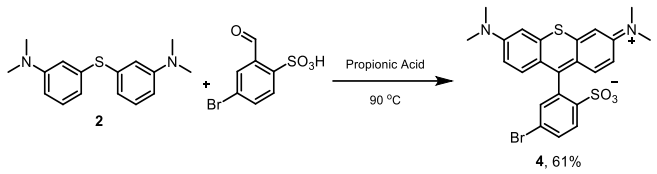
5-bromo-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-thioxanthen-9-yl)benzenesulfonate, 3:

4-bromo-2-sulfobenzaldehyde (100 mg, 0.367 mmol, synthesized as previously reported[5]) and **2** (105 mg, 0.367 mmol) were added to a flame-dried round-bottom flask and then dissolved in propionic acid (1.5 mL). The round-bottom flask was sealed, and the reaction mixture was stirred at 90 °C for 16 h. The reaction mixture was then cooled, and the propionic acid was removed *in vacuo*. The crude residue was then run through a celite plug (1:1 DCM:MeOH, isocratic gradient) and the solvent was removed *in vacuo*. The crude residue was then re-dissolved in minimal 1:1 DCM:MeOH and precipitated out in ether. The precipitate was then recrystallized in ethanol, yielding **3** as a dark purple solid (64 mg, 34%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.10 (d, *J* = 2.1 Hz, 1H), 7.76 (dd, *J* = 8.1, 2.2 Hz, 1H), 7.36 (d, *J* = 2.4 Hz, 2H), 7.16 – 7.06 (m, 5H), 3.24 (s, 12H).

^{13}C NMR (226 MHz, DMSO-d_6) δ 159.82, 153.19, 148.79, 142.94, 136.77, 131.89, 131.69, 131.54, 130.32, 122.13, 118.83, 114.97, 105.23, 40.20.

HRMS (ESI+) Calculated for $C_{23}H_{22}O_3N_2Br_1S_2$ $[M+H]^+$ 517.0250; Found 517.0246.



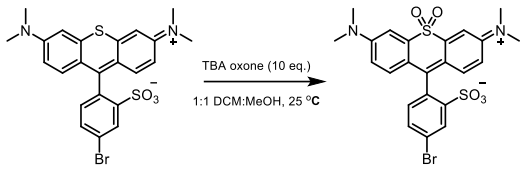
Synthesis of meta-bromo sulfide rhodamine, 4:

5-bromo-2-sulfobenzaldehyde (100 mg, 0.367 mmol, synthesized as previously reported[5]) and **2** (105 mg, 0.367 mmol) were added to a flame-dried round-bottom flask and then dissolved in propionic acid (1.5 mL). The round-bottom flask was sealed, and the reaction mixture was stirred at 90 °C for 16 h. The reaction mixture was then cooled, and the propionic acid was removed *in vacuo*. The crude residue was then run through a celite plug (1:1 DCM:MeOH, isocratic gradient) and the solvent was removed *in vacuo*. The crude residue was then re-dissolved in minimal 1:1 DCM:MeOH and precipitated out in ether. The precipitate was then washed with cold ethanol, yielding **4** as a dark purple solid (115 mg, 61%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.92 (d, J = 8.5 Hz, 1H), 7.82 (dd, J = 8.5, 2.2 Hz, 1H), 7.44 (d, J = 2.0 Hz, 1H), 7.36 (d, J = 2.2 Hz, 2H), 7.21 – 7.08 (m, 4H), 3.25 (s, 12H).

^{13}C NMR (226 MHz, DMSO-d_6) δ 159.30, 153.17, 146.21, 143.03, 136.74, 134.70, 131.97, 131.70, 129.91, 121.74, 118.84, 115.06, 105.19, 40.19.

HRMS (ESI+) Calculated for $C_{23}H_{22}O_3N_2Br_1S_2$ $[M+H]^+$ 517.0250; Found 517.0250.



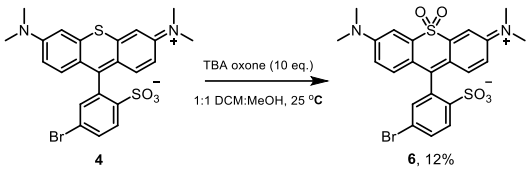
Synthesis of para-bromo sulfone rhodamine, 5:

3 (100 mg, 0.193 mmol) was added to a flame-dried round-bottom flask and dissolved in 40 mL of 1:1 DCM:MeOH. The tetrabutylammonium salt of oxone (3,153 mg, 1.93 mmol) was then added to the mixture. The flask was then sealed and stirred at 25 °C for 16 h. Sodium bisulfite (402 mg, 3.86 mmol) dissolved in 20 mL of water was then added to the mixture to quench residual oxone. The mixture was then diluted with 100 mL of brine and 100 mL of 1:1 DCM:^{i}PrOH. The layers were allowed to separate, and the organic layer was collected. The aqueous layer was then extracted two more times with 100 mL of 1:1 DCM:^{i}PrOH. The organic layer was then dried with anhydrous sodium sulfate, filtered, and concentrated to dryness *in vacuo*. The residue was then first purified by flash chromatography (0-7.5% Methanol in DCM, gradient) and then further purified by preparative HPLC (10-100% acetonitrile in water with 0.05% TFA, gradient), yielding **5** as an iridescent dark green solid (8 mg, 8%).

^{1}H NMR (500 MHz, 1:1 Chloroform-*d*:Methanol-d_4) δ 8.34 (d, *J* = 2.0 Hz, 1H), 7.73 (dd, *J* = 8.1, 2.0 Hz, 1H), 7.61 (d, *J* = 2.7 Hz, 2H), 7.14 – 7.09 (m, 3H), 6.81 (dd, *J* = 9.6, 2.7 Hz, 2H), 3.42 (s, 12H).

^{13}C NMR (226 MHz, 1:1 Chloroform-*d*:Methanol-d_4) δ 160.61, 156.96, 147.45, 145.23, 141.37, 133.91, 132.20, 131.59, 130.42, 124.78, 120.65, 115.95, 112.42, 41.75.

HRMS (ESI+) Calculated for $C_{23}H_{21}O_5N_2Br_1Na_1S_2$ $[M+Na]^+$ 570.9967; Found 570.9966.



Synthesis of meta-bromo sulfone rhodamine, 6:

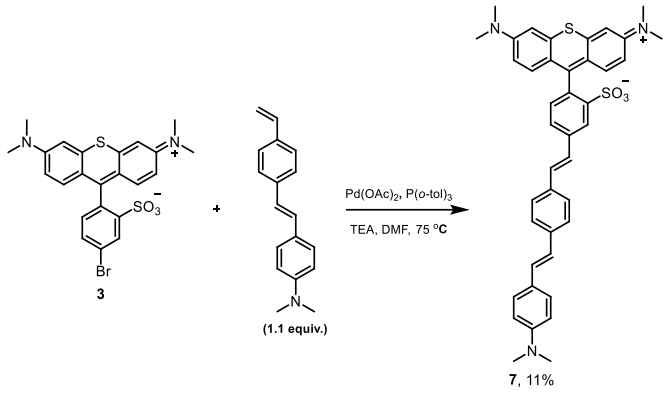
4 (25 mg, 0.048 mmol) was added to a flame-dried round-bottom flask and dissolved in 10 mL of 1:1 DCM:MeOH. The tetrabutylammonium salt of oxone (783 mg, 0.48 mmol) was then added to the mixture. The flask was then sealed and stirred at 25 °C for 7 days. Sodium bisulfite (100 mg, 0.96 mmol) dissolved in 5 mL of water was then added to the mixture to quench residual oxone. The mixture was then diluted with 25 mL of brine and 25 mL of 1:1 DCM:^{i}PrOH. The layers were allowed to separate, and the organic layer was collected. The aqueous layer was then extracted two more times with 25 mL of 1:1 DCM:^{i}PrOH. The organic layer was then dried with anhydrous sodium sulfate, filtered, and concentrated to dryness *in vacuo*. The residue was then first purified by preparative HPLC (10-100% acetonitrile in water with 0.05% TFA, gradient), and then further purified by preparative TLC (7.5% Methanol in DCM, isocratic), yielding **6** as an iridescent dark green solid (3 mg, 12%).

^{1}H NMR (400 MHz, 1:1 Chloroform-*d*:Methanol-d_4) δ 8.06 (d, *J* = 8.4 Hz, 1H), 7.81 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.64 (d, *J* = 2.6 Hz, 2H), 7.41 (d, *J* = 1.9 Hz, 1H), 7.11 (d, *J* = 9.5 Hz, 2H), 6.85 (dd, *J* = 9.6, 2.6 Hz, 2H), 3.43 (s, 12H).

^{13}C NMR (226 MHz, 1:1 Chloroform-*d*:Methanol-d_4) δ 159.81, 156.85, 145.10, 144.81, 141.31, 134.46, 133.90, 132.76, 130.92, 124.91, 120.52, 115.95, 112.39, 41.77.

HRMS (ESI+) Calculated for $C_{23}H_{22}O_5N_2Br_1S_2$ $[M+H]^+$ 549.0148; Found 549.0147.

Authors' Note: The solubility of the voltage reporter compounds (**7-10**) is quite poor in both DMSO and other common solvent combinations (methanol with either chloroform or acetonitrile). Therefore, it was challenging to prepare NMR samples of the following compounds at sufficient concentrations to obtain spectra with high signal-to-noise ratios. The compounds p-SRhoVR (**7**) and p-SuRhoVR (**9**) were particularly insoluble and tended to precipitate out of solution on the timescale of a ^{13}C experiment. Therefore, we were unable to obtain ^{13}C spectra for both of these compounds. In order to more fully demonstrate the identity and purity of the novel fluorescent compounds synthesized in this work (**3-10**), we have included high-resolution mass spectrometry data along with UV-Vis absorbance traces from the liquid chromatography traces of each purified compound in addition to our spectral data. All dyes have a dye content of >95%, as judged by HPLC.

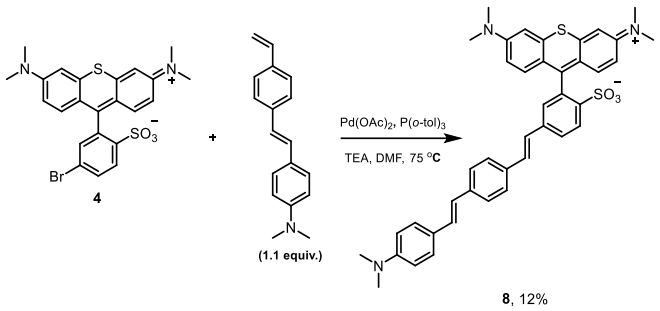


Synthesis of p-SRhoVR, 7:

3 (100 mg, 0.193 mmol), molecular wire (53 mg, 0.213 mmol, synthesized as previously reported[6]), palladium acetate (11 mg, 0.048 mmol), and tris(o-tolyl)phosphine (29 mg, 0.097 mmol) were added to a flame-dried Schlenk flask which was evacuated and backfilled with nitrogen (3x). Anhydrous DMF (2 mL) and triethylamine (1 mL) were added under nitrogen, the Schlenk flask was sealed, and the reaction mixture was stirred at 75 °C for 16 h. The reaction mixture was then cooled, and the solvent was removed *in vacuo*. The crude residue was then purified by flash chromatography (7.5% MeOH in DCM, isocratic gradient), yielding **7** as a dark purple solid (14 mg, 11%).

^{1}H NMR (900 MHz, 1:1 Methanol-d_4:Acetonitrile-d_3) δ 8.56 (s, 1H), 8.39 (d, J = 1.9 Hz, 1H), 8.13 (s, 1H), 7.97 (d, J = 8.0 Hz, 1H), 7.89 (d, J = 8.1 Hz, 1H), 7.86 (dd, J = 7.8, 1.9 Hz, 1H), 7.66 (d, J = 8.2 Hz, 1H), 7.58 (d, J = 8.3 Hz, 1H), 7.48 (d, J = 8.7 Hz, 1H), 7.46 – 7.39 (m, 4H), 7.28 – 7.25 (m, 2H), 7.23 (d, J = 7.7 Hz, 1H), 7.19 (d, J = 16.3 Hz, 1H), 7.05 (dd, J = 9.8, 2.7 Hz, 2H), 6.81 (d, J = 8.7 Hz, 2H), 3.35 (s, 12H), 3.27 (s, 6H).

HRMS (ESI+) Calculated for $C_{41}H_{40}O_3N_3S_2$ $[M+H]^+$ 686.2506; Found 686.2501.



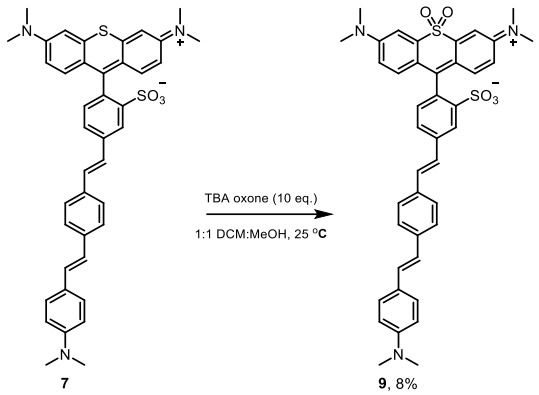
Synthesis of m-SRhoVR, 8:

4 (100 mg, 0.193 mmol), molecular wire (53 mg, 0.213 mmol, synthesized as previously reported[6]), palladium acetate (11 mg, 0.048 mmol), and tris(o-tolyl)phosphine (29 mg, 0.097 mmol) were added to a flame-dried Schlenk flask which was evacuated and backfilled with nitrogen (3x). Anhydrous DMF (8 mL) and triethylamine (4 mL) were added under nitrogen, the Schlenk flask was sealed, and the reaction mixture was stirred at 75 °C for 16 h. The reaction mixture was then cooled, and the solvent was removed *in vacuo*. The crude residue was then run through a celite plug (1:1 DCM:MeOH, isocratic gradient) and the solvents were removed *in vacuo*. The crude residue was then re-precipitated in ether and then purified by flash chromatography (7.5% MeOH in DCM, isocratic gradient), yielding **8** as a dark purple solid (16 mg, 12%).

^{1}H NMR (900 MHz, 3:1 Methanol-d_4:Chloroform-*d*) δ 8.16 (d, *J* = 8.4 Hz, 1H), 7.72 (dd, *J* = 5.7, 3.3 Hz, 2H), 7.60 (dd, *J* = 5.7, 3.3 Hz, 1H), 7.44 – 7.37 (m, 6H), 7.29 (s, 1H), 7.19 – 7.14 (m, 2H), 7.12 (s, 1H), 7.05 (d, *J* = 16.5 Hz, 1H), 7.02 – 6.94 (m, 3H), 6.88 (d, *J* = 16.1 Hz, 1H), 6.73 (d, *J* = 8.9 Hz, 2H), 3.25 (s, 12H), 2.96 (s, 6H).

^{13}C NMR ^{13}C NMR (226 MHz, 3:1 Methanol-d_4:Chloroform-*d*) δ 169.07, 154.67, 145.38, 140.49, 139.43, 138.13, 133.18, 132.29, 132.09, 130.08, 129.64, 129.15, 128.61, 128.42, 127.94, 127.85, 127.05, 126.76, 124.46, 120.73, 115.87, 113.52, 105.78, 40.75, 40.64.

HRMS (ESI+) Calculated for $C_{41}H_{40}O_3N_3S_2$ $[M+H]^+$ 686.2506; Found 686.2494.

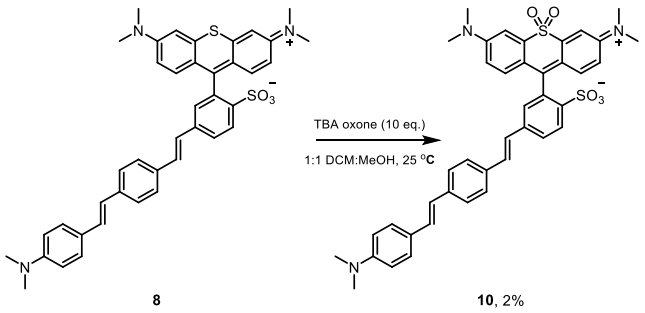


Synthesis of p-SuRhoVR, 9:

7 (6 mg, 0.0087 mmol) was dissolved in 2.4 mL of 1:1 dichloromethane:methanol. Tertbutylammonium oxone (143 mg, 0.087 mmol) was then added and the mixture was sealed and stirred at 25 °C for 6 days. The solvent was then removed *in vacuo* and the crude residue was triturated with ethyl acetate followed by brine containing 0.03 M sodium bisulfite. The crude residue was then further purified by preparative HPLC (10-100% acetonitrile in water with 0.05% TFA, gradient), yielding **9** as an iridescent dark green solid (0.5 mg, 8%).

^{1}H NMR (900 MHz, 3:1 Methanol-d_4:Chloroform-d) δ 8.36 – 8.33 (m, 1H), 7.91 (s, 1H), 7.68 – 7.66 (m, 3H), 7.58 (d, J = 7.8 Hz, 2H), 7.52 (d, J = 7.9 Hz, 2H), 7.43 (d, J = 8.4 Hz, 2H), 7.23 (d, J = 7.8 Hz, 1H), 7.20 (d, J = 9.7 Hz, 2H), 7.18 (d, J = 9.6 Hz, 1H), 7.12 (d, J = 16.3 Hz, 1H), 6.95 (d, J = 16.3 Hz, 1H), 6.89 (dd, J = 9.7, 2.9 Hz, 2H), 6.76 (d, J = 8.5 Hz, 2H), 3.43 (s, 12H), 2.98 (s, 6H).

HRMS (ESI+) Calculated for $C_{41}H_{40}O_5N_3S_2$ $[M+H]^+$ 718.2404; Found 718.2403.



Synthesis of m-SuRhoVR, 10:

8 (16 mg, 0.023 mmol) was dissolved in 6.4 mL of 1:1 dichloromethane:methanol. Tertbutylammonium oxone (381 mg, 0.233 mmol) was then added and the mixture was sealed and stirred at 25 °C for 6 days. The solvent was then removed *in vacuo* and the crude residue was triturated with ethyl acetate followed by brine containing 0.03 M sodium bisulfite. The crude residue was then further purified by preparative HPLC (10-100% acetonitrile in water with 0.05% TFA, gradient), yielding **10** as an iridescent dark green solid (0.4 mg, 2%).

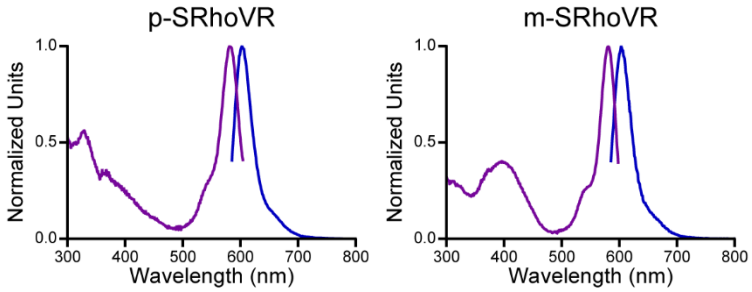
^{1}H NMR (900 MHz, 3:1 Methanol-d_4:Chloroform-*d*) δ 8.14 (d, *J* = 8.5 Hz, 1H), 7.84 (d, *J* = 8.3 Hz, 1H), 7.67 (s, 2H), 7.52 – 7.44 (m, 4H), 7.42 – 7.37 (m, 3H), 7.27 (d, *J* = 16.3 Hz, 1H), 7.22 – 7.16 (m, 3H), 7.08 (d, *J* = 16.1 Hz, 1H), 6.93 – 6.85 (m, 3H), 6.74 (d, *J* = 7.8 Hz, 2H), 3.43 (s, 12H), 2.96 (s, 6H).

^{13}C NMR (226 MHz, 3:1 Methanol-d_4:Chloroform-*d*) δ 157.05, 145.39, 141.68, 139.65, 136.01, 132.74, 130.21, 129.73, 128.47, 128.36, 128.16, 128.07, 127.13, 126.96, 126.15, 124.60, 124.52, 120.92, 116.02, 113.58, 112.36, 112.31, 107.59, 41.72, 40.76.

HRMS (ESI+) Calculated for $C_{41}H_{40}O_5N_3S_2$ $[M+H]^+$ 718.2404; Found 718.2402.

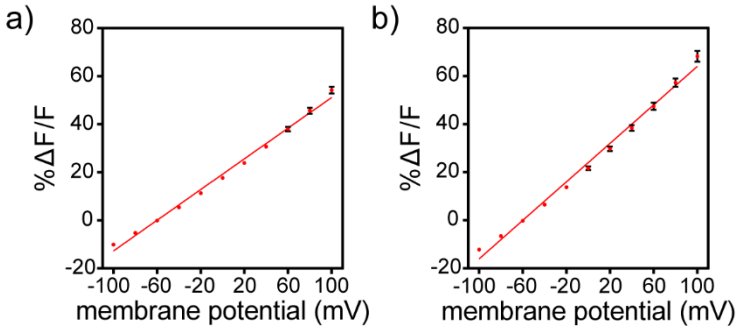
Supporting Figures

Figure S1. Optical properties of S-RhoVRs.



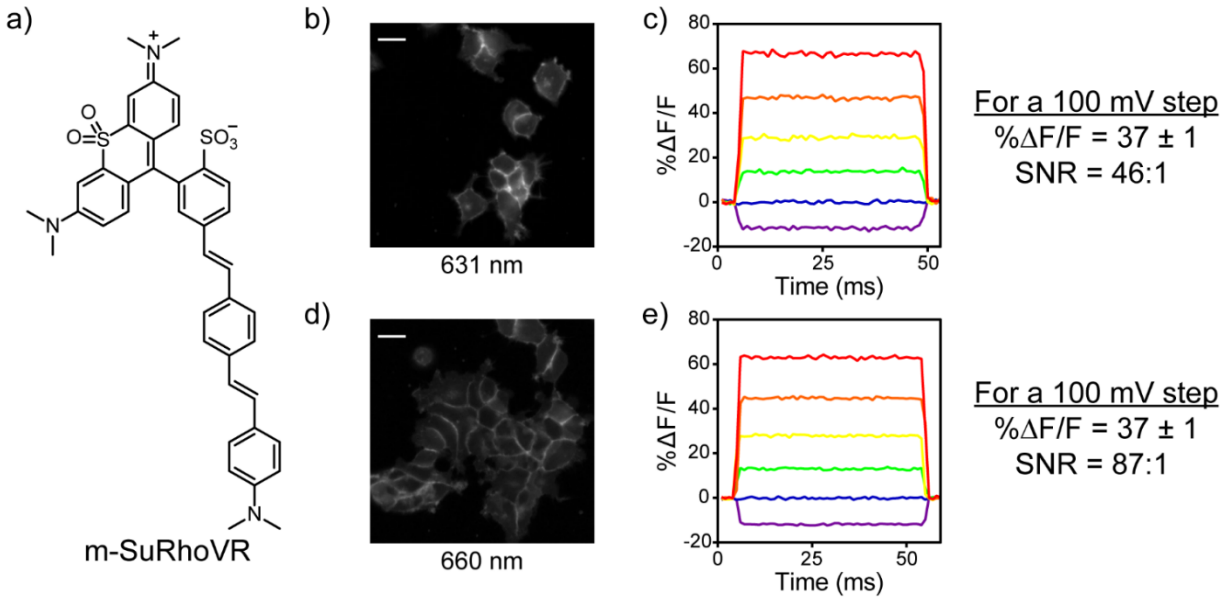
Absorbance (purple) and Emission (blue) Spectra of SRhoVRs. All spectra were obtained at 1 μM dye concentration in a TBS (50 mM Tris-HCl, pH 7.4, 0.1 M NaCl) solution containing 0.1 % (w/w) SDS. All spectra were normalized to their respective maxima.

Figure S2. Voltage sensitivity of Su-RhoVRs in HEK cells.



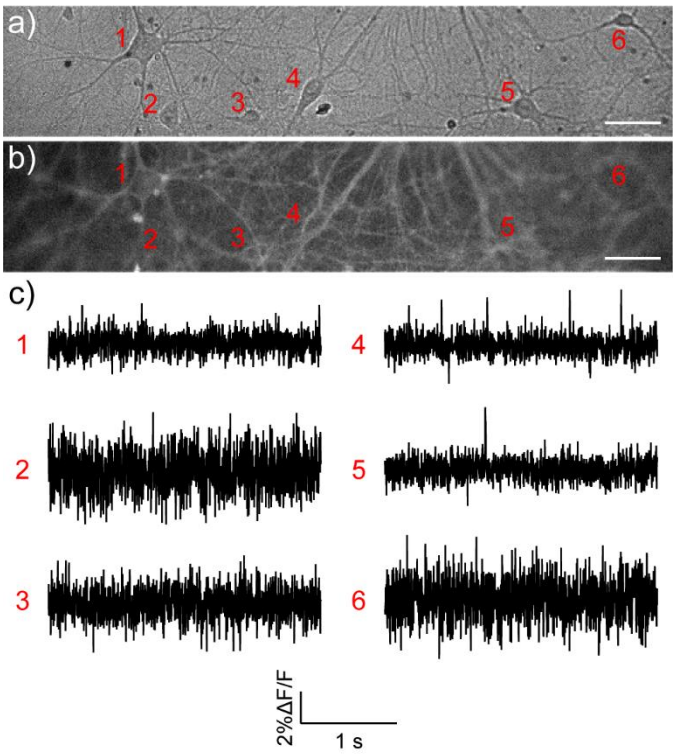
Plots of %ΔF/F vs. membrane potential for voltage-clamped HEK cells stained with 1 μM p-SuRhoVR (**a**) or 1 μM m-SuRhoVR (**b**). The cells were excited with 631 nm excitation light. Voltage sensitivity was determined by plotting %ΔF/F values vs. applied voltage steps for each cell and finding the slope of a linear best-fit. Error bars reflect SEM and when not visible are smaller than the marker indicating the average %ΔF/F value for a given potential. For p-SuRhoVR, n=5 HEK cells; For m-SuRhoVR, n=4 HEK cells.

Figure S3. Cellular Characterization of m-SuRhoVR with 631 nm and 660 nm excitation light.



a) Chemical structure of m-SuRhoVR. Widefield fluorescence images of HEK cells stained with 1 μM m-SuRhoVR and excited with either 631 nm (**b**) or 660 nm (**d**) light. Representative concatenated traces from single patched HEK cells stained with 1 μM m-SuRhoVR and excited with either 631 nm (**c**) or 660 nm (**e**) light which show the percent change in fluorescence over time as the holding potential is changed from +100 mV (red) to -100 mV (purple) in 40 mV increments. The average %ΔF/F value and signal-to-noise ratio (SNR) for a 100 mV step are shown for each excitation light beside the concatenated traces. For 631 nm, n=4 HEK cells; for 660 nm, n=3 HEK cells. Scale bars are 20 μm.

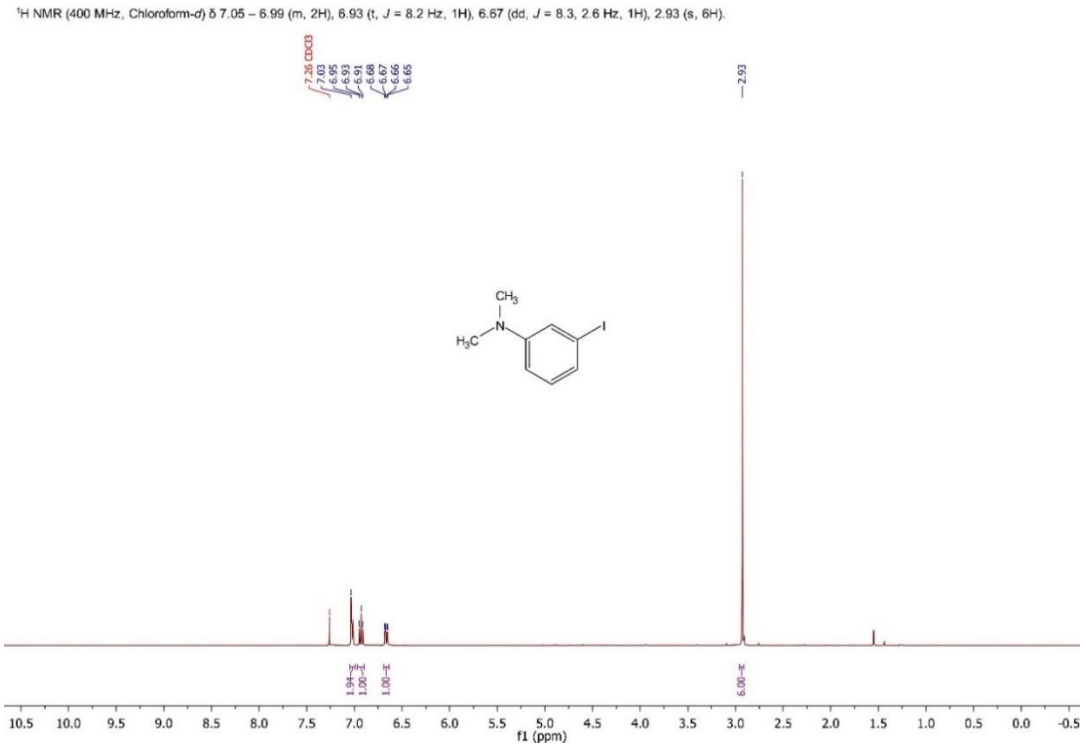
Figure S4. Monitoring Spontaneous Neuronal Activity with m-SuRhoVR.



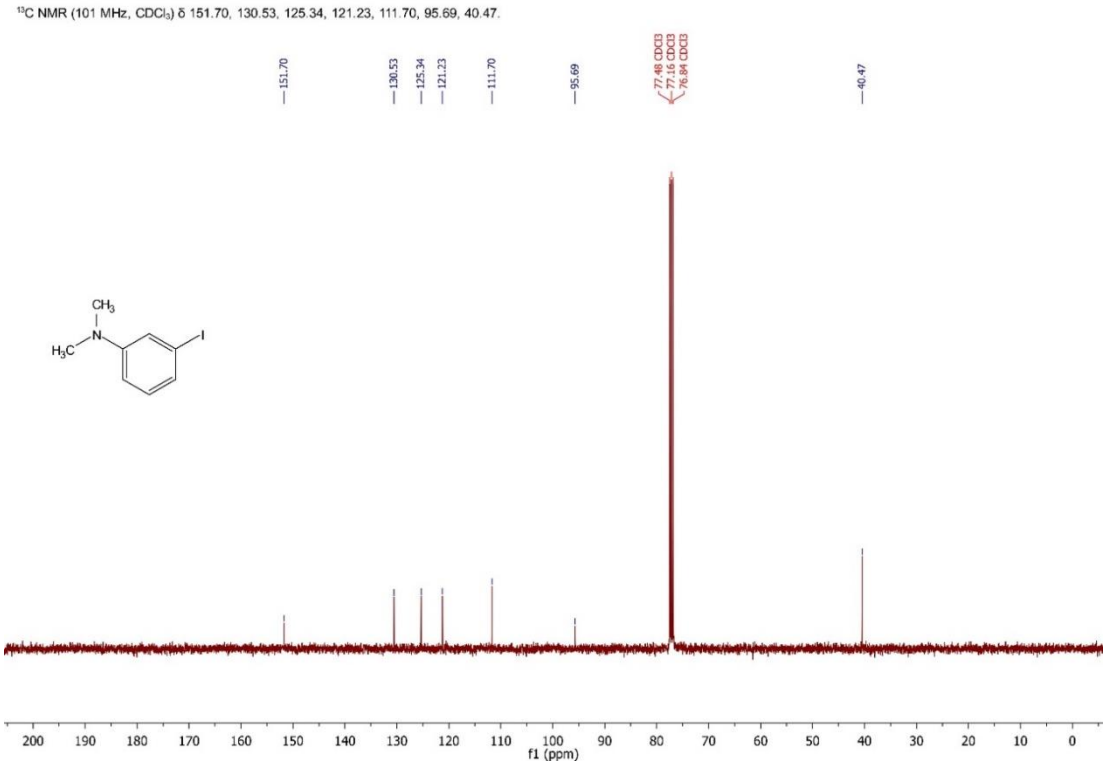
a) a) Brightfield and **b)** epifluorescence images of dissociated rat hippocampal neurons stained with 500 nM m-SuRhoVR. **c)** ΔF/F traces from the labeled neurons showing spontaneous neuronal activity. A 660 nm LED was used for excitation light. Scale bars are 10 μm.

Supporting Spectra

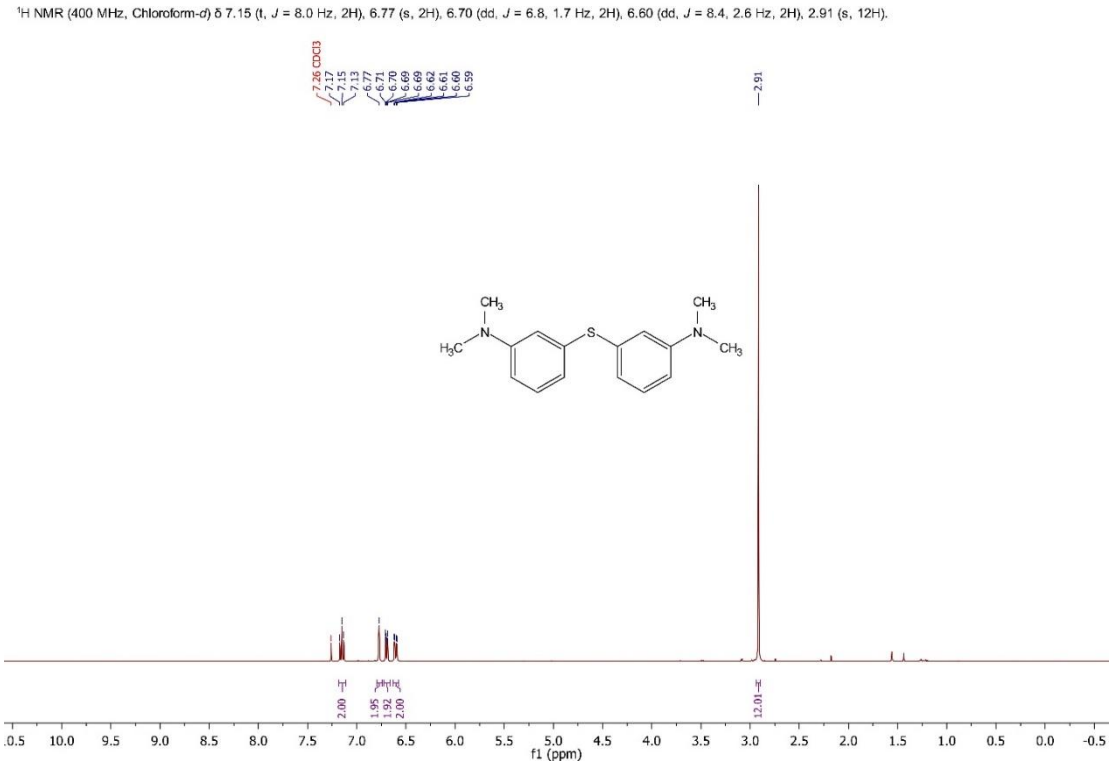
Spectrum S1. ^{1}H NMR spectrum of 3-iodo-N,N-dimethylaniline, 1:



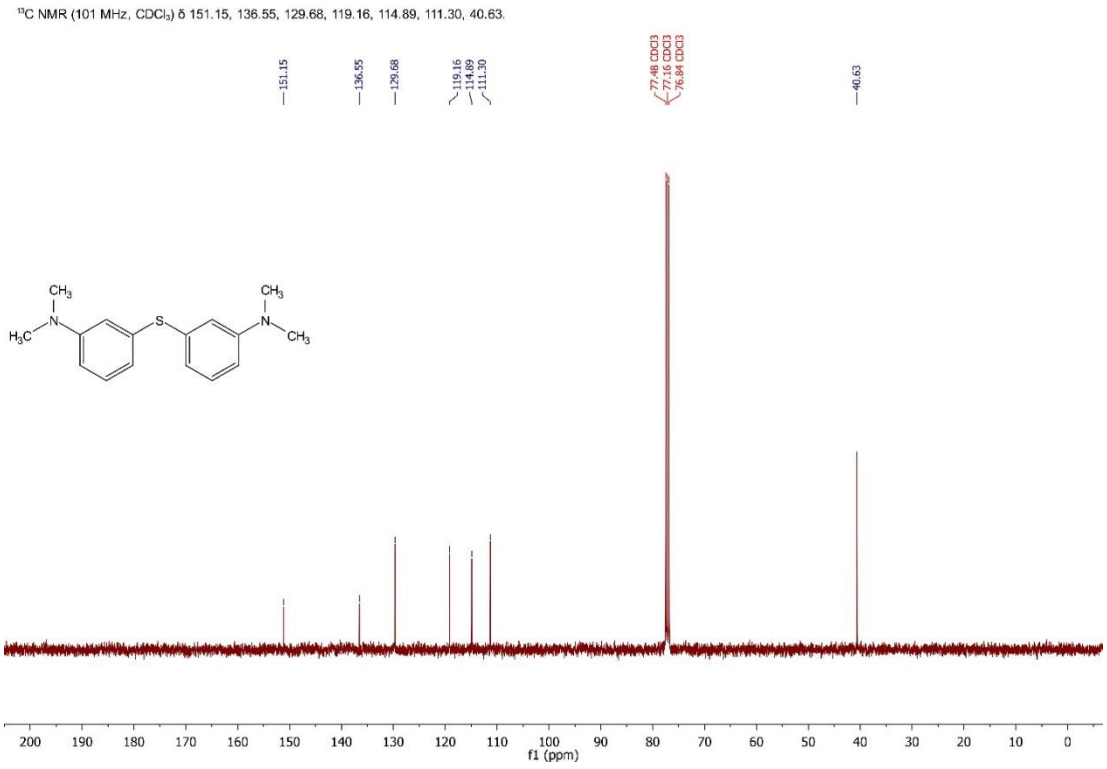
Spectrum S2. ^{13}C NMR spectrum of 3-iodo-N,N-dimethylaniline, 1:



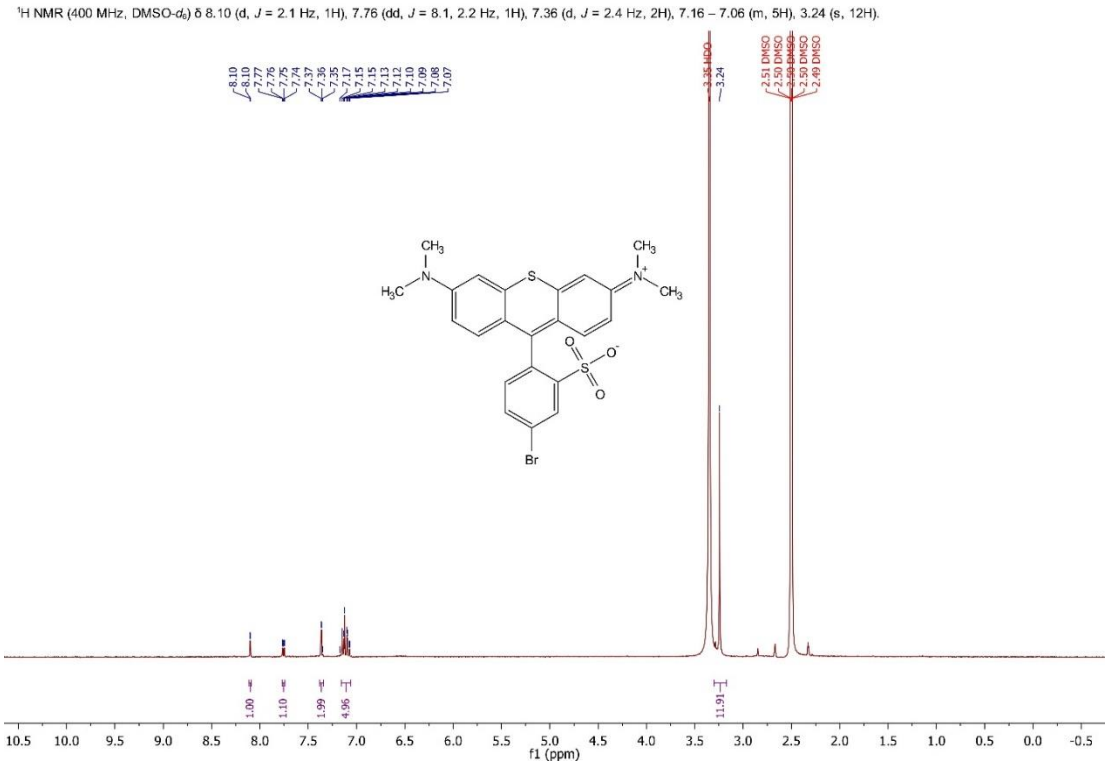
Spectrum S3. ^{1}H NMR spectrum of 3,3'-thiobis(N,N-dimethylaniline), 2:



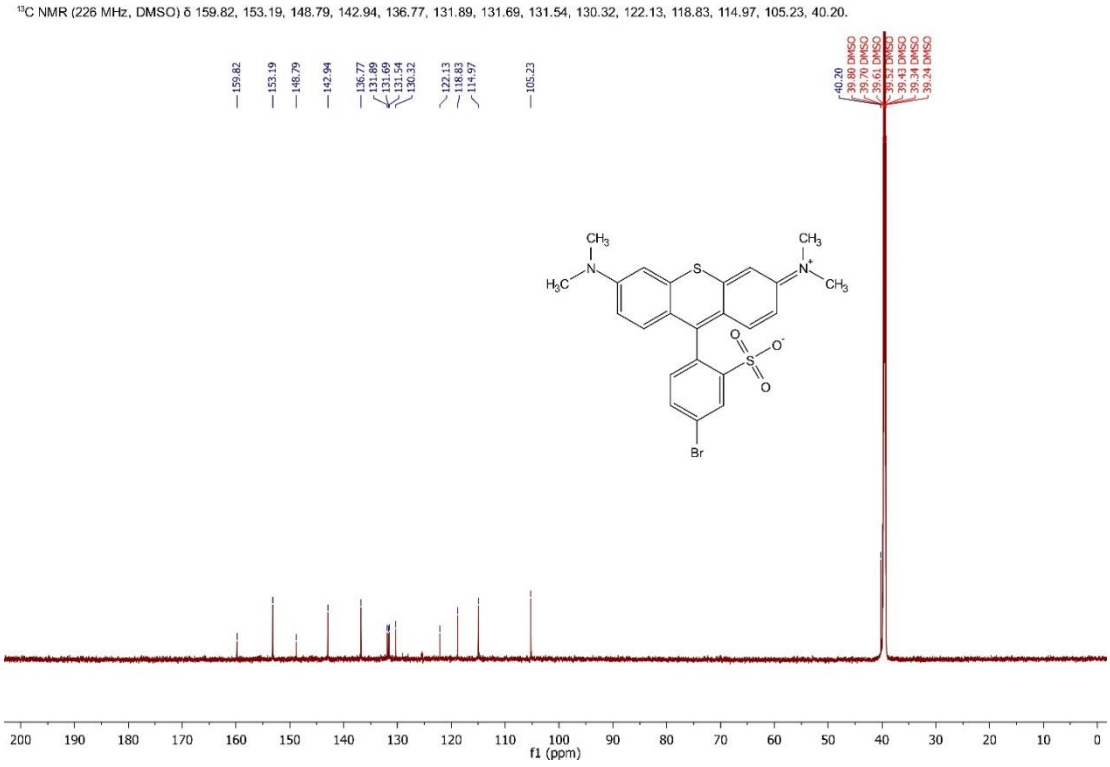
Spectrum S4. ^{13}C NMR spectrum of 3,3'-thiobis(N,N-dimethylaniline), 2:



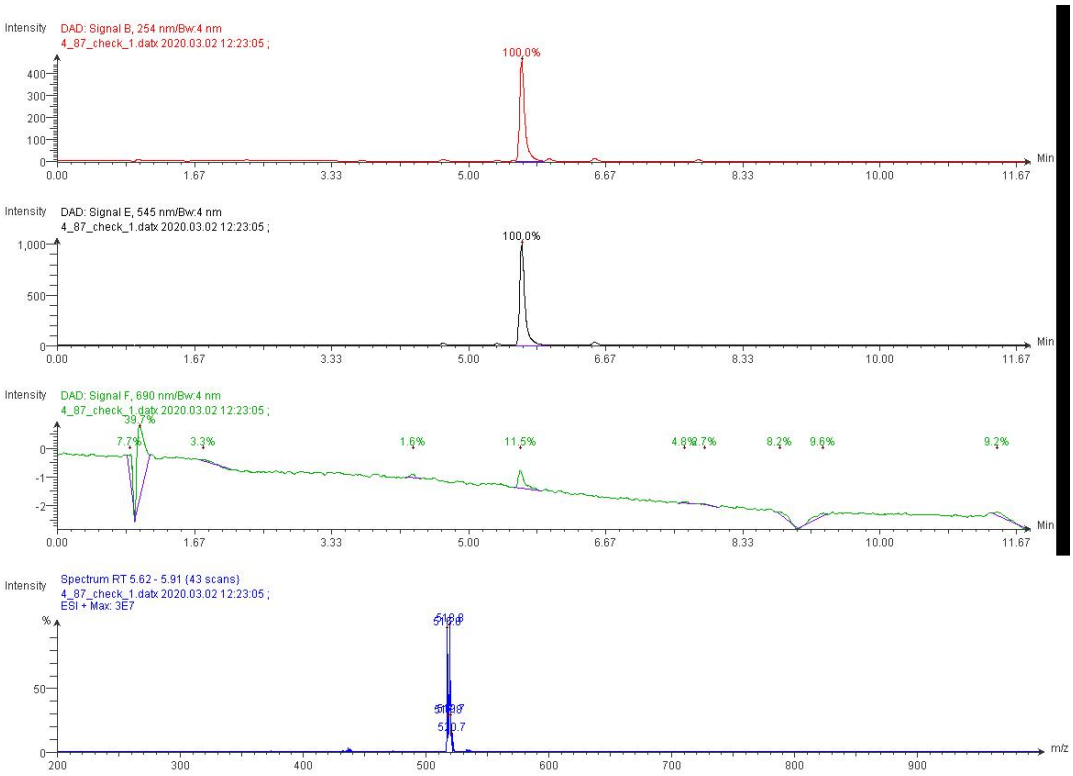
Spectrum S5. ^{1}H NMR spectrum of para-bromo sulfide rhodamine, 3:



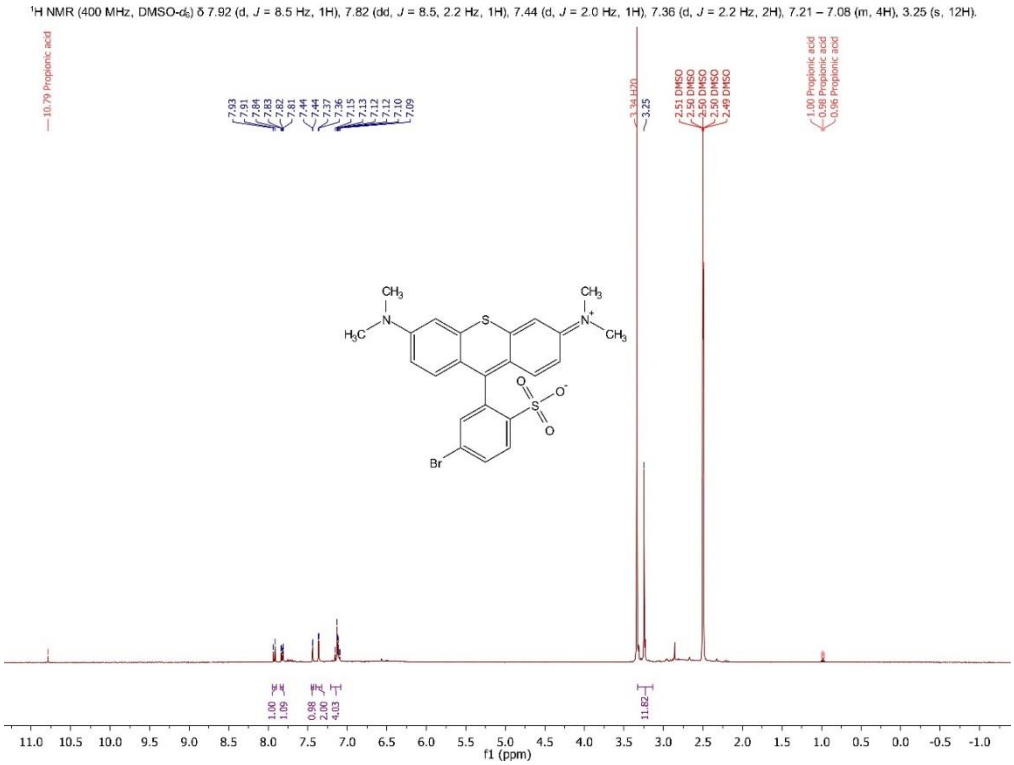
Spectrum S6. ^{13}C NMR spectrum of para-bromo sulfide rhodamine, 3:



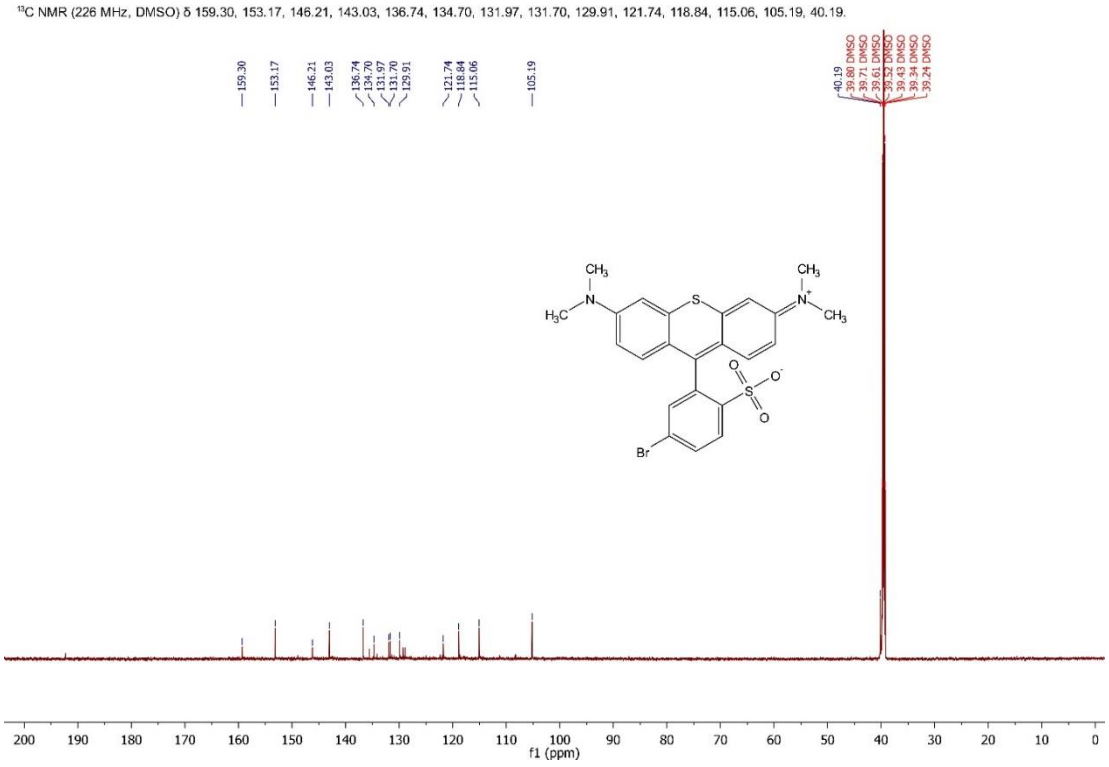
Spectrum S7. LC-MS of para-bromo sulfide rhodamine, 3:



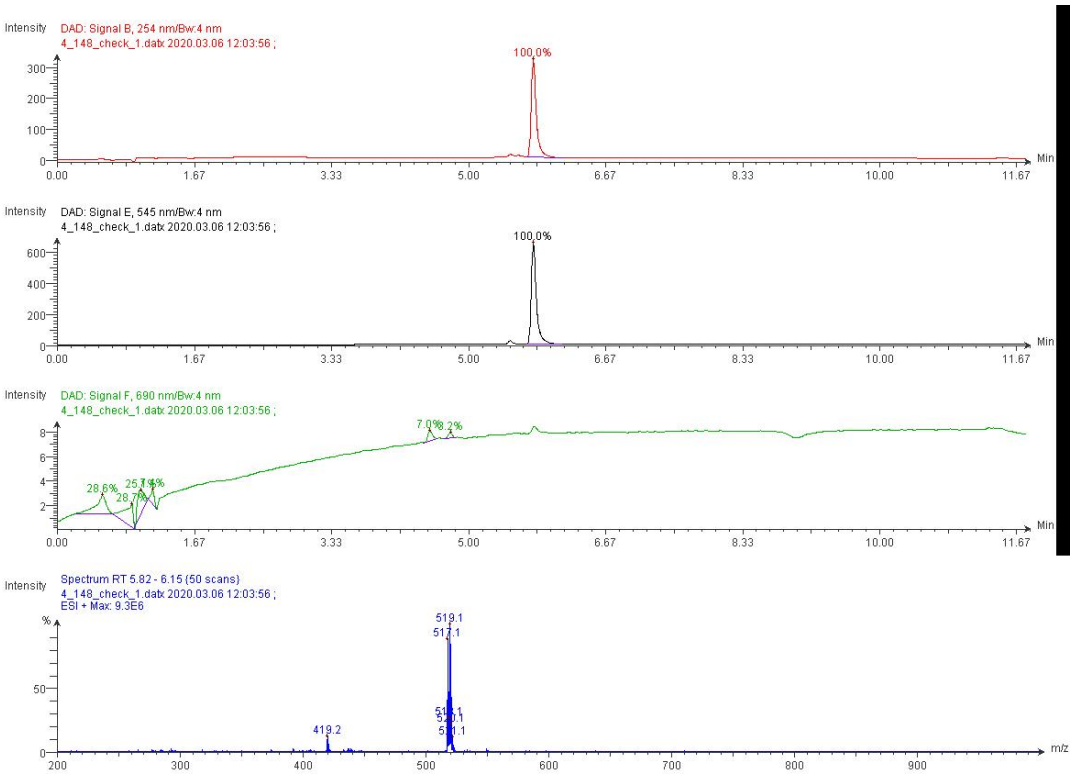
Spectrum S8. ^{1}H NMR spectrum of meta-bromo sulfide rhodamine, 4:



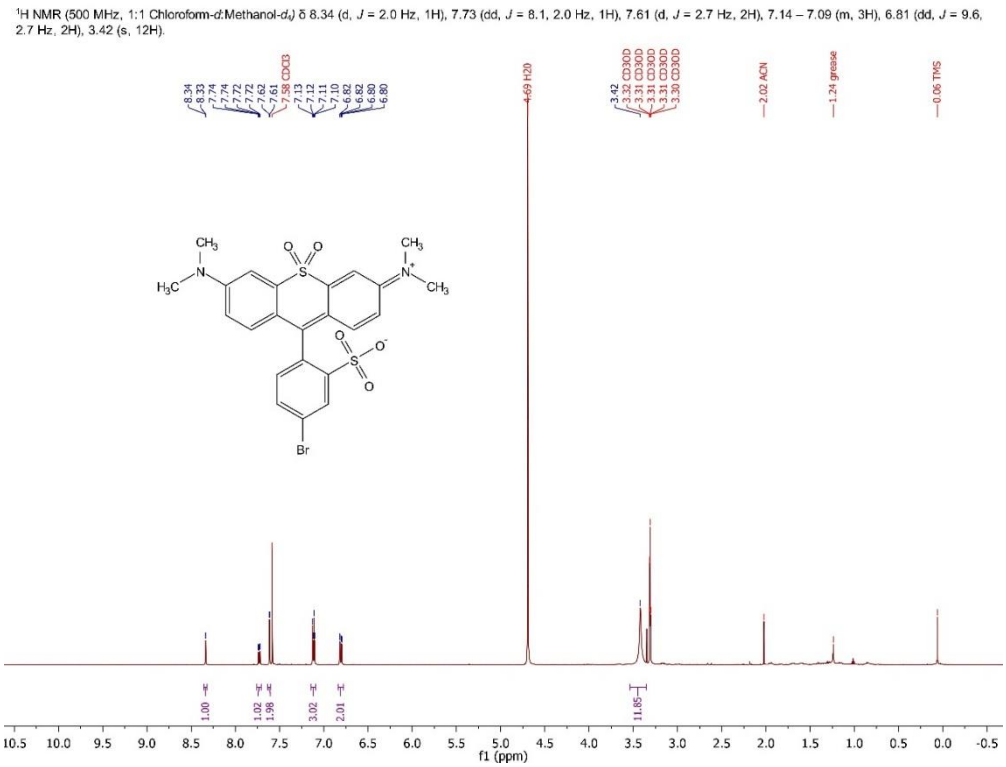
Spectrum S9. ^{13}C NMR spectrum of meta-bromo sulfide rhodamine, 4:



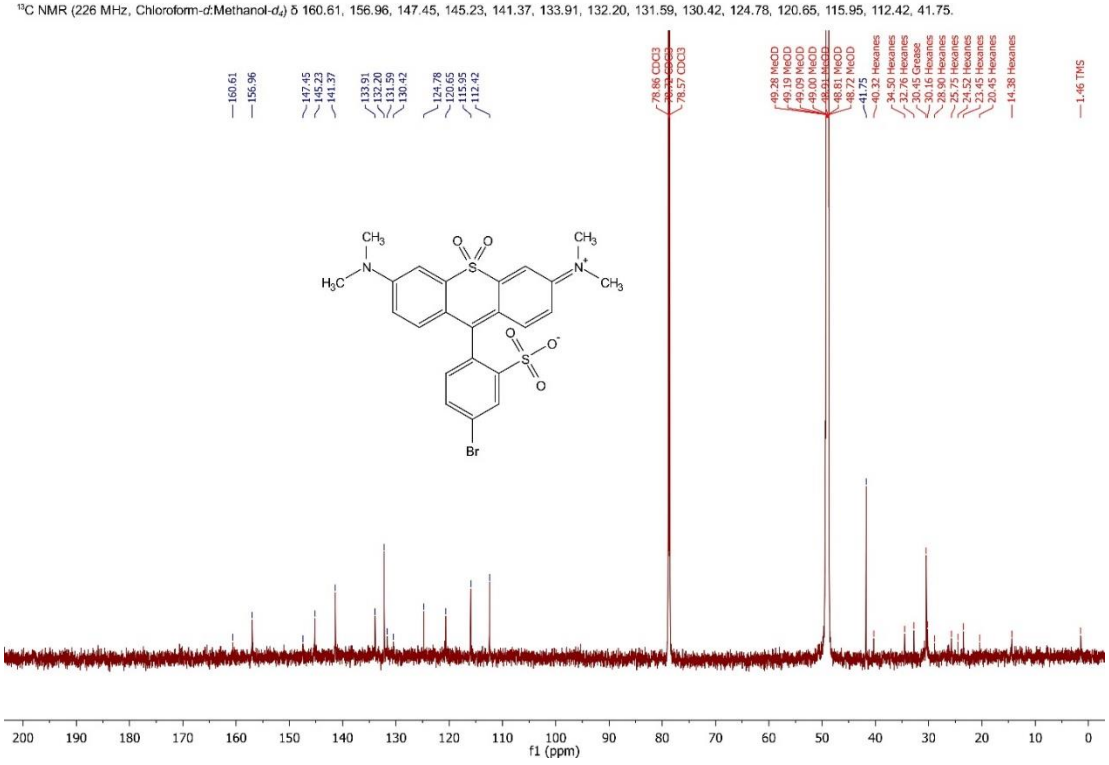
Spectrum S10. LC-MS of meta-bromo sulfide rhodamine, 4:



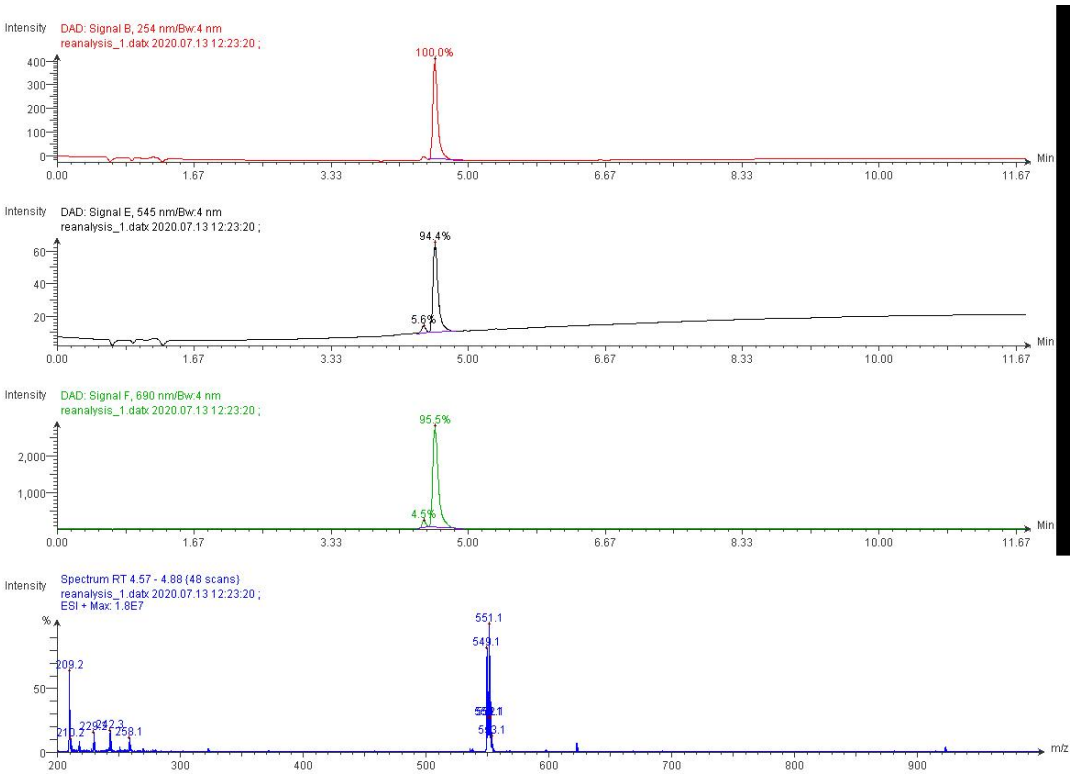
Spectrum S11. ^{1}H NMR spectrum of para-bromo sulfone rhodamine, 5:



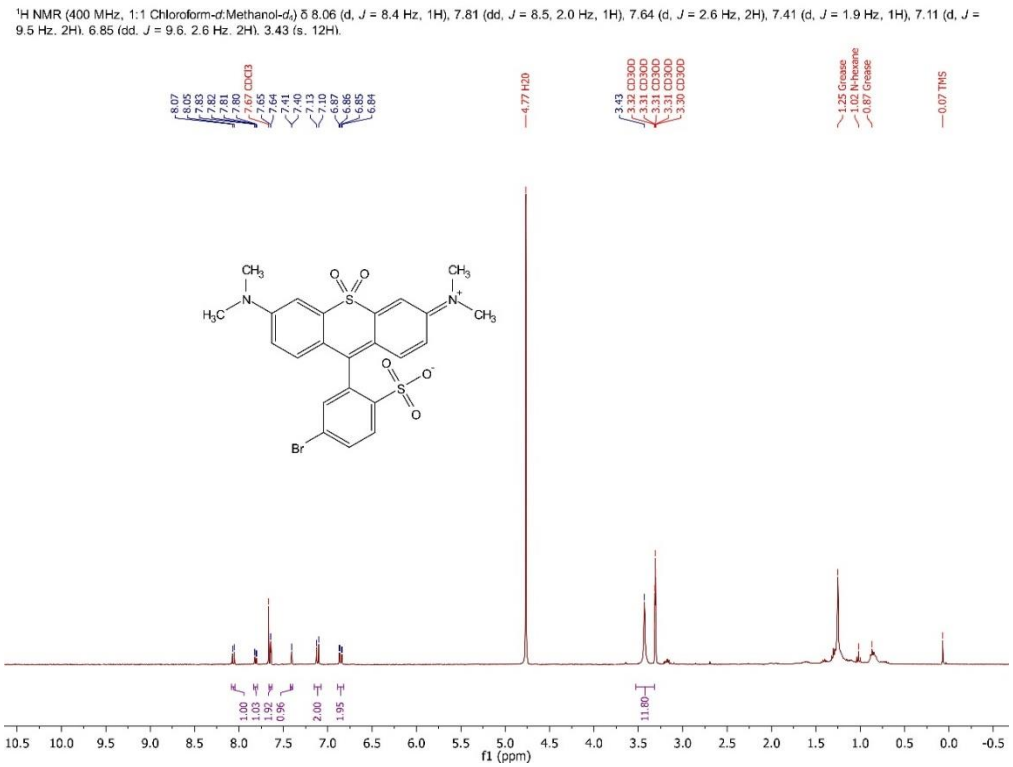
Spectrum S12. ^{13}C NMR spectrum of para-bromo sulfone rhodamine, 5:



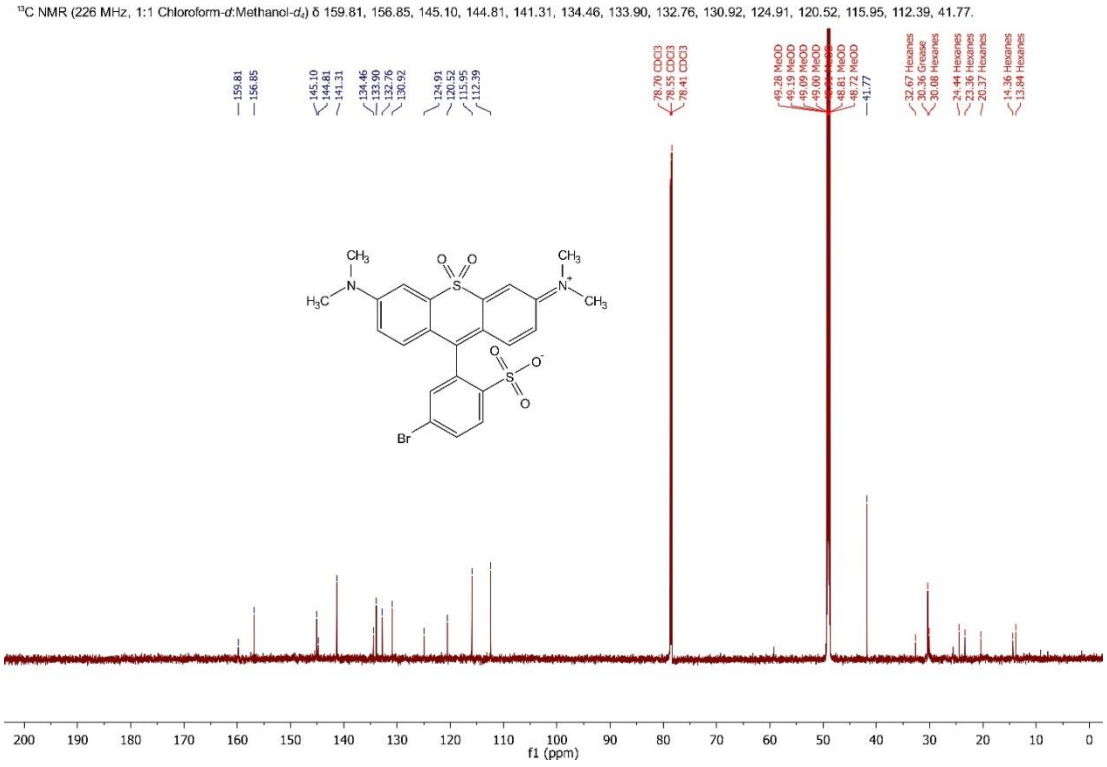
Spectrum S13. LC-MS of para-bromo sulfone rhodamine, 5:



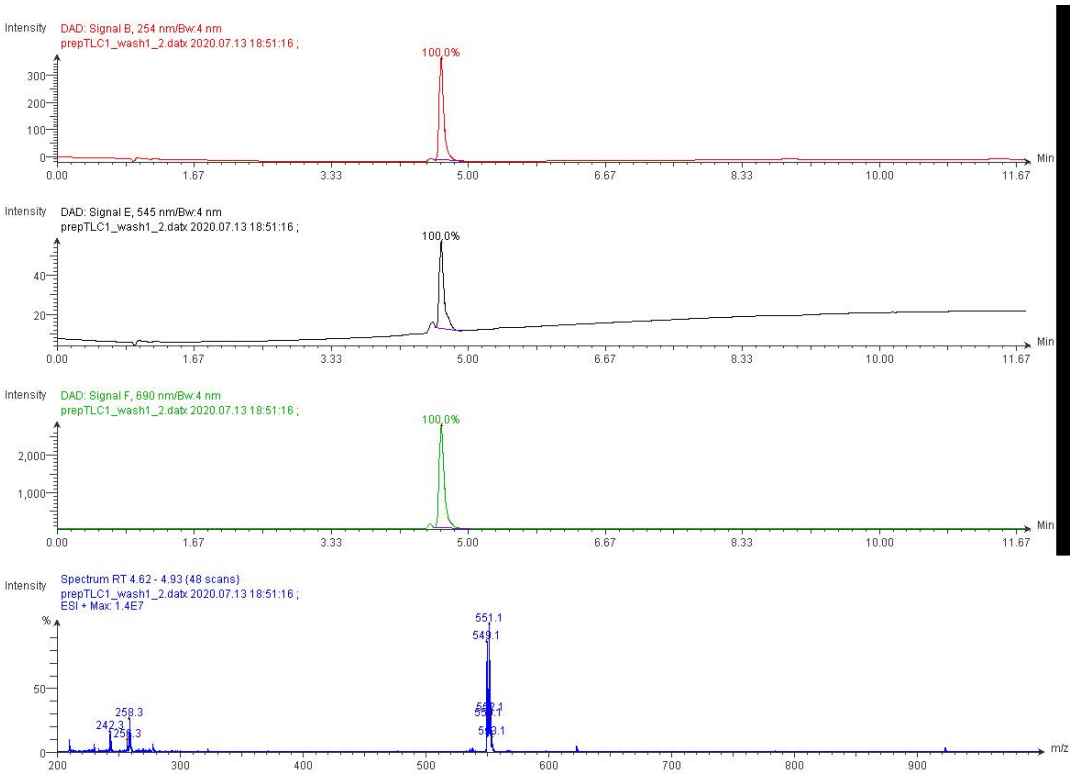
Spectrum S14. ^{1}H NMR spectrum of meta-bromo sulfone rhodamine, 6:



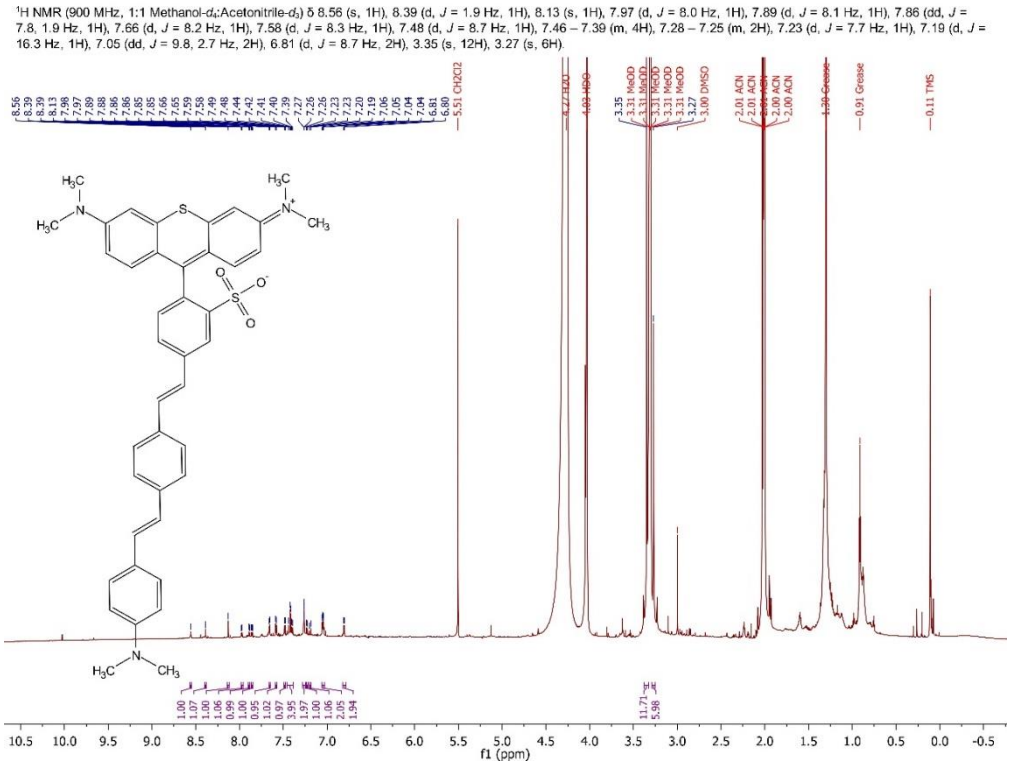
Spectrum S15. ^{13}C NMR spectrum of meta-bromo sulfone rhodamine, 6:



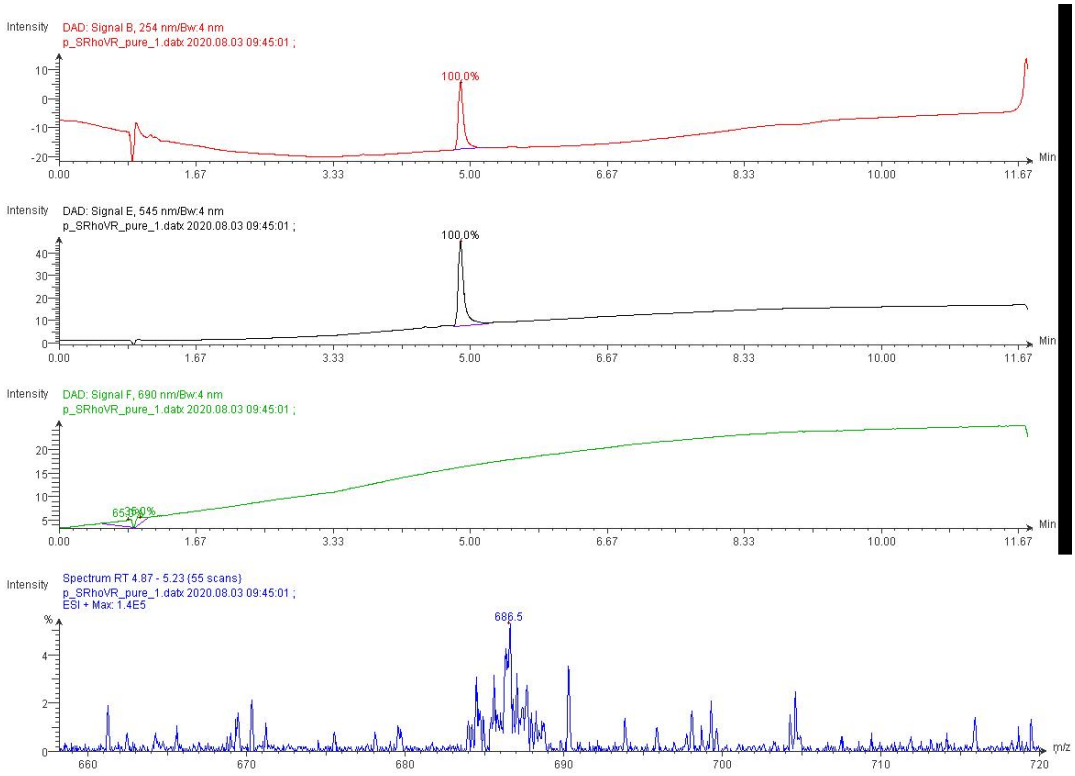
Spectrum S16. LC-MS of meta-bromo sulfone rhodamine, 6:



Spectrum S17. ^{1}H NMR spectrum of p-SRhoVR, 7

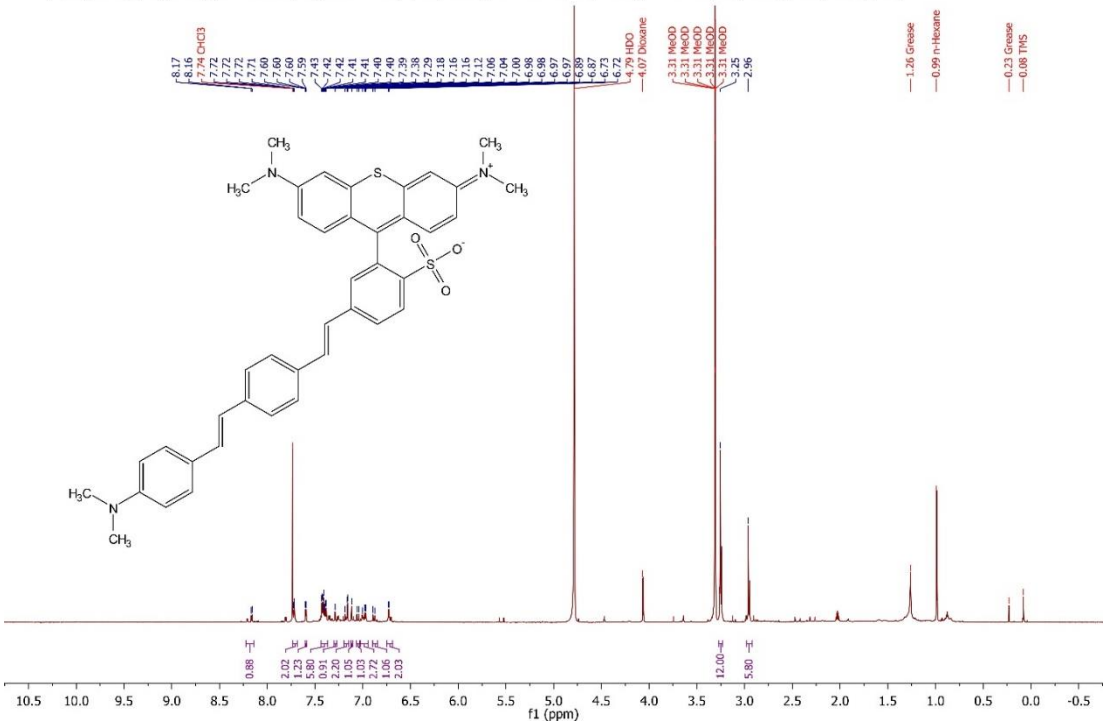


Spectrum S18. LC-MS of p-SRhoVR, 7



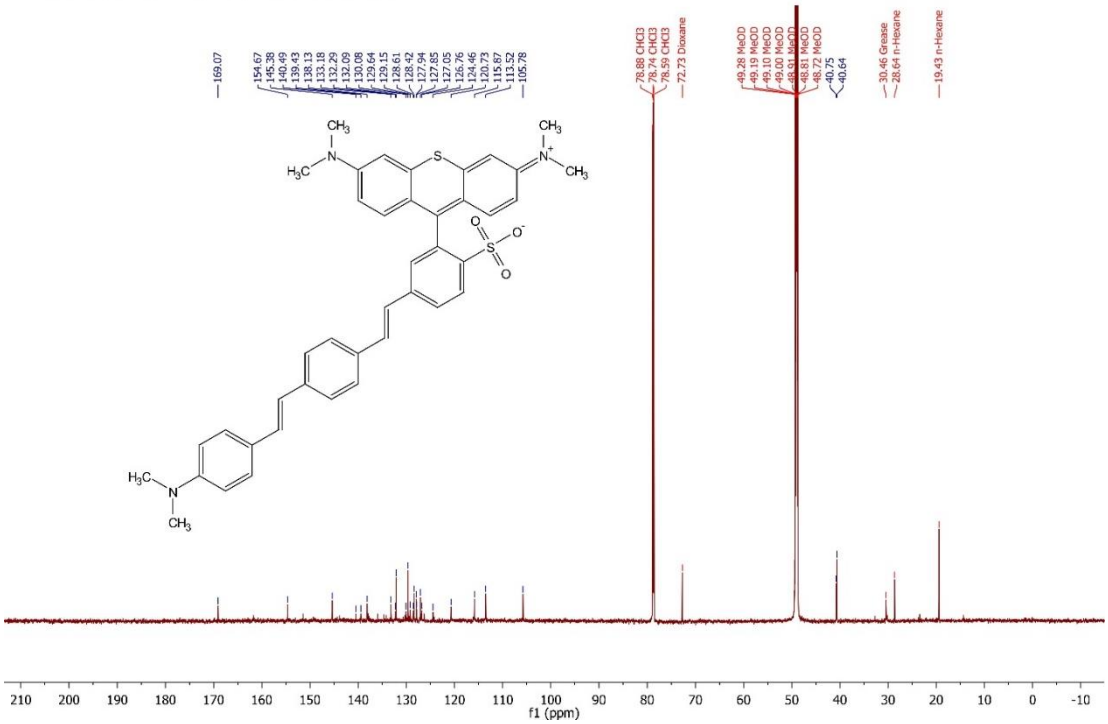
Spectrum S19. ^{1}H NMR spectrum of m-SRhoVR, 8:

^{1}H NMR (900 MHz, 3:1 Methanol-d_4:Chloroform-d) δ 8.16 (d, J = 8.4 Hz, 1H), 7.72 (dd, J = 5.7, 3.3 Hz, 2H), 7.60 (dd, J = 5.7, 3.3 Hz, 1H), 7.44 – 7.37 (m, 6H), 7.29 (s, 1H), 7.19 – 7.14 (m, 2H), 7.12 (s, 1H), 7.05 (d, J = 16.5 Hz, 1H), 7.02 – 6.94 (m, 3H), 6.88 (d, J = 16.1 Hz, 1H), 6.73 (d, J = 8.9 Hz, 2H), 3.25 (s, 12H), 2.96 (s, 6H).

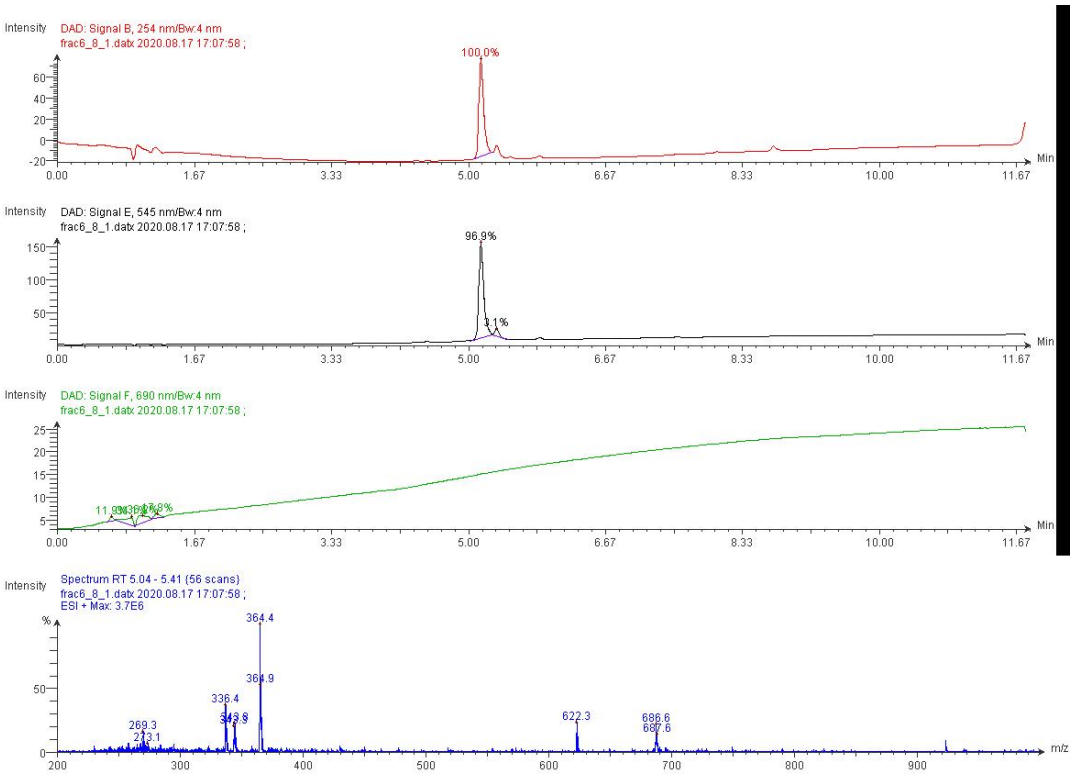


Spectrum S20. ^{13}C NMR Spectrum of m-SRhoVR, 8:

^{13}C NMR (226 MHz, 3:1 Methanol-d_4:Chloroform-d) δ 169.07, 154.67, 145.38, 140.49, 139.43, 138.13, 133.18, 132.29, 132.09, 130.08, 129.64, 129.15, 128.61, 128.42, 127.94, 127.85, 127.05, 126.76, 124.46, 120.73, 115.87, 113.52, 105.78, 40.75, 40.64.

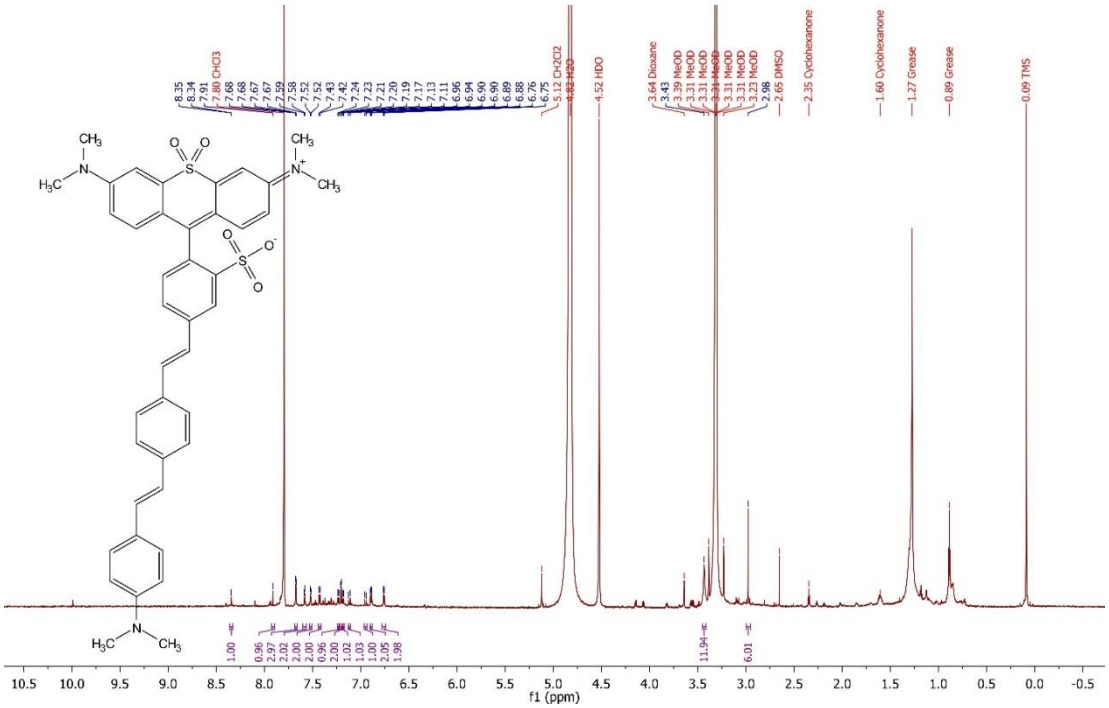


Spectrum S21. LC-MS of m-SRhoVR, 8:

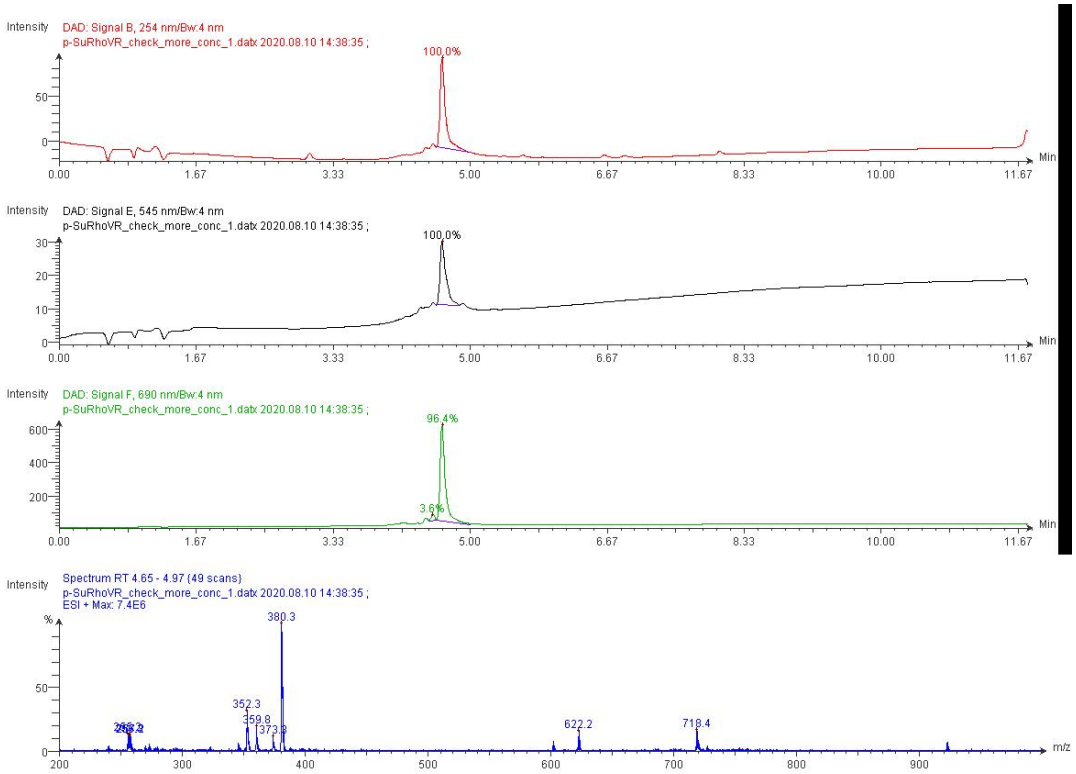


Spectrum S22. ^{1}H NMR spectrum of p-SuRhoVR, 9:

^{1}H NMR (900 MHz, 3:1 Methanol-d_4:Chloroform-d) δ 8.36 – 8.33 (m, 1H), 7.91 (s, 1H), 7.68 – 7.66 (m, 3H), 7.58 (d, J = 7.8 Hz, 2H), 7.52 (d, J = 7.9 Hz, 2H), 7.43 (d, J = 8.4 Hz, 2H), 7.23 (d, J = 7.8 Hz, 1H), 7.20 (d, J = 9.7 Hz, 2H), 7.18 (d, J = 9.6 Hz, 1H), 7.12 (d, J = 16.3 Hz, 1H), 6.95 (d, J = 16.3 Hz, 1H), 6.89 (dd, J = 9.7, 2.9 Hz, 2H), 6.76 (d, J = 8.5 Hz, 2H), 3.43 (s, 12H), 2.98 (s, 6H).

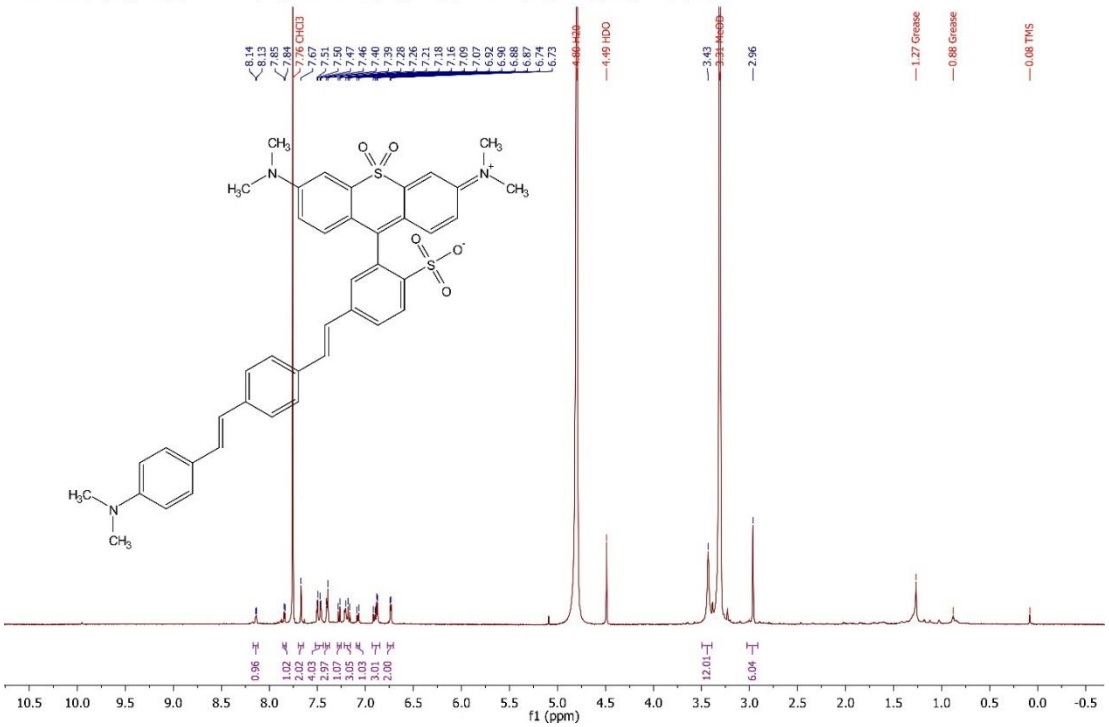


Spectrum S23. LC-MS of p-SuRhoVR, 9:



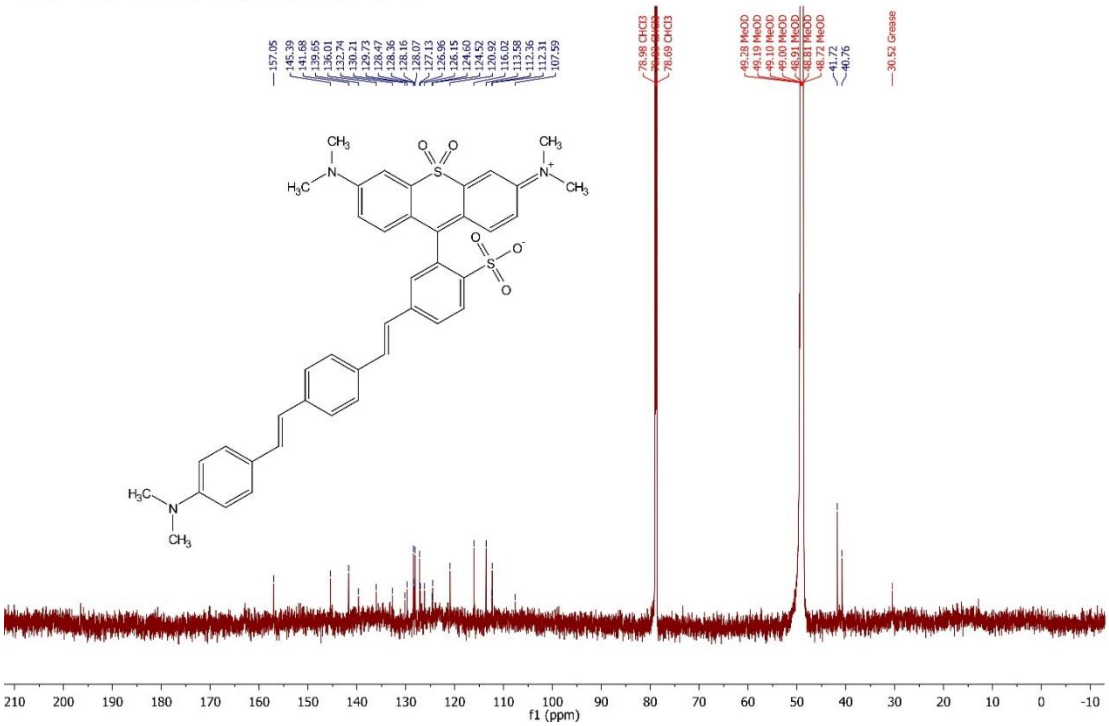
Spectrum S24. ^{1}H NMR spectrum of m-SuRhoVR, 10:

^{1}H NMR (900 MHz, 3:1 Methanol-d_4:Chloroform-d) δ 8.14 (d, J = 8.5 Hz, 1H), 7.84 (d, J = 8.3 Hz, 1H), 7.67 (s, 2H), 7.52 – 7.44 (m, 4H), 7.42 – 7.37 (m, 3H), 7.27 (d, J = 16.3 Hz, 1H), 7.22 – 7.16 (m, 3H), 7.08 (d, J = 16.1 Hz, 1H), 6.93 – 6.85 (m, 3H), 6.74 (d, J = 7.8 Hz, 2H), 3.43 (s, 12H), 2.96 (s, 6H).

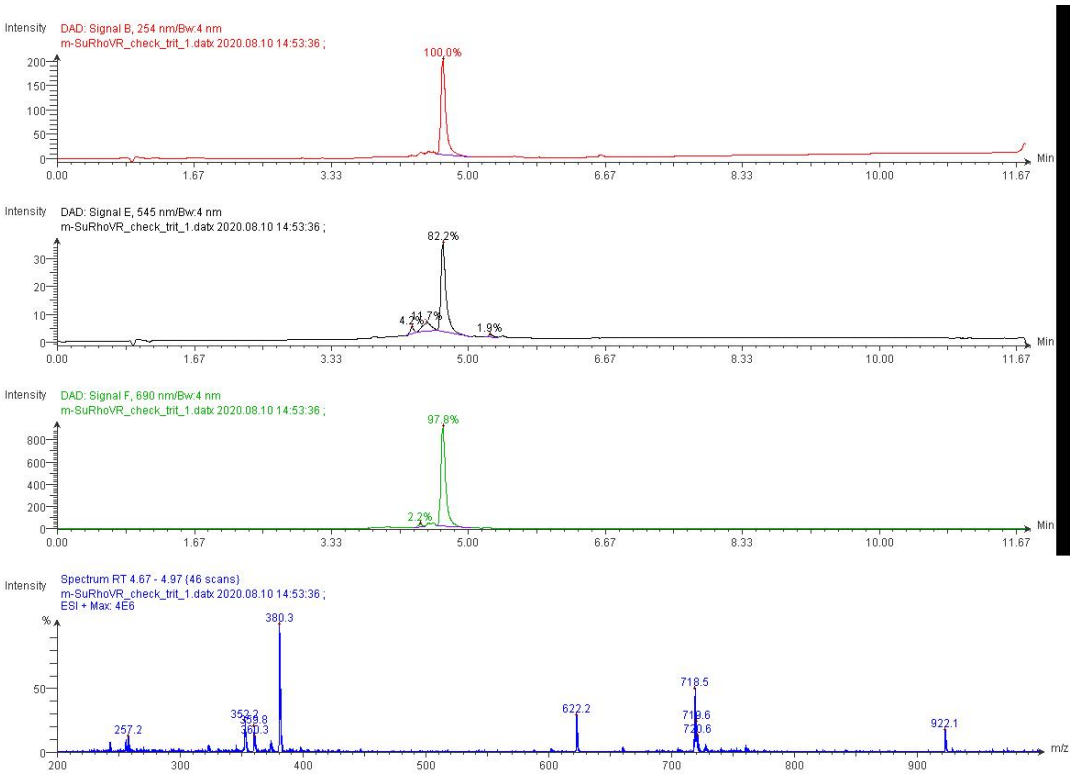


Spectrum S25. ^{13}C NMR spectrum of m-SuRhoVR, 10:

^{13}C NMR (226 MHz, 3:1 Methanol-d_4:Chloroform-d) δ 157.05, 145.39, 141.68, 139.65, 136.01, 132.74, 130.21, 129.73, 128.47, 128.36, 128.16, 128.07, 127.13, 126.96, 126.15, 124.60, 124.52, 120.92, 116.02, 113.58, 112.36, 112.31, 107.59, 41.72, 40.76.



Spectrum S26. LC-MS of m-SuRhoVR, 10:



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