Supporting Information for:

Isomerically pure tetramethylrhodamine voltage indicators

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1. General method for chemical synthesis and characterization

Chemical reagents and solvents (dry) were purchased from commercial suppliers and used without further purification. Preparation of phthalaldehydic acids (**3** and **5**) and isomerically pure tetramethyl rhodamines (**6** and **7**) were modified from previously reported procedures.[1-3] Synthesis of 4-(diethylamino)-2-methoxybenzaldehyde **22** and (E)-N,N-dimethyl-4-(4-vinylstyryl)aniline **8** were carried out as previously reported.[4,5] 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 and 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 Bruker AVB-400 MHz, 100 MHz, AVQ-400 MHz, 100 MHz, Bruker AV-600 MHz, 150 MHz. NMR spectra measured on Bruker AVII-900 MHz, 225 MHz, equipped with a TCI cryoprobe accessory, were performed by Dr. Jeffrey Pelton (QB3). Variable temperature NMR experiments were measured on the Bruker AV-600 with the assistance of Hasan Celik. Chemical shifts are expressed in parts per million (ppm) and are referenced to $CDCl_3$ (7.26 ppm, 77.0 ppm) or DMSO (2.50 ppm, 40 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 doublet; m, multiplet. High-resolution 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 Phenomenex Luna 5 μm C18(2) (4.6 mm I.D. × 150 mm) with a flow rate of 1.0 mL/min. The mobile phases were MQ-H_2O with 0.05% formic acid (eluent A) and HPLC grade acetonitrile with 0.05% formic acid (eluent B). Signals were monitored at 254, 340 and 545 nm over 20 min with a gradient of 10-100% eluent B. The column used for semi-preparative HPLC was Phenomenex Luna 5 μm C18(2) (10 mm I.D. x 150 mm) with a flow rate of 5.0 mL/min. The mobile phases were MQ-H_2O with 0.05% formic acid (eluent A) and HPLC grade acetonitrile with 0.05% formic acid (eluent B). Signals were monitored at 254 over 20 min with a gradient of 10-100% eluent B.

2. Spectroscopic studies

Stock solutions of RhoVRs were prepared in DMSO (1-5 mM) and diluted with PBS (10 mM KH_2PO_4, 30 mM $Na_2HPO_4{\cdot}7H_2O$, 1.55 M NaCl, pH 7.2) solution containing 0.10 % (w/w) SDS (1:100-1:1000 dilution). 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).

3. Cell culture

All animal procedures were approved by the UC Berkeley Animal Care and Use Committees and conformed to the NIH Guide for the Care and Use and Laboratory Animals and the Public Health Policy.

Human embryonic kidney 293T (HEK) cells were passaged and plated onto 12 mm glass coverslips pre-coated with Poly-D-Lysine (PDL; 1 mg/ml; Sigma-Aldrich) to provide a confluency of ~15% and 50% for electrophysiology and imaging, respectively. HEK cells were plated and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 4.5 g/L D-glucose, 10% FBS and 1% Glutamax. Transfection of genetic tools was carried out using Lipofectamine 3000 24 h after plating. Imaging was performed 18-24 h following transfection.

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% 1M D-glucose (Fisher Scientific) and 1% glutamax. The dissociated cells were plated onto 12 mm diameter coverslips (Fisher Scientific) pre-treated with PDL (as above) at a density of 30-40,000 cells per coverslip in MEM supplemented media (as above). Neurons were maintained at 37 °C in a humidified incubator with 5 % CO_2. 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. Transfection of genetic tools was carried out using Lipofectamine 3000 at 7 DIV. Functional imaging was performed on mature neurons 13-20 DIV, except electrophysiological experiments which were performed

on 12-15 DIV neurons. Unless stated otherwise, for loading of HEK cells and hippocampal neurons, RhoVRs were diluted in DMSO to 500 μM, and then diluted 1:1000 in HBSS. All imaging experiments were performed in HBSS.

4. DNA constructs

GCaMP6s was a gift from Douglas Kim (Addgene plasmid # 40753) and was driven in mammalian cells by the cytomegalovirus promoter.

5. Imaging parameters

Epifluorescence imaging was performed on an AxioExaminer Z-1 (Zeiss) equipped with a Spectra-X Light engine LED light (Lumencor), controlled with Slidebook (v6, Intelligent Imaging Innovations). Co-incident excitation with multiple LEDs was controlled by Lumencor software triggered through a Digidata 1332A digitizer and pCLAMP 10 software (Molecular Devices). Images were acquired with either a W-Plan-Apo 20x/1.0 water objective (20x; Zeiss) or a W-Plan-Apo 63x/1.0 water objective (63x; Zeiss). Images were focused onto either an OrcaFlash4.0 sCMOS camera (sCMOS; Hamamatsu) or an eVolve 128 EMCCD camera (EMCCD; Photometrix). More detailed imaging information for each experimental application is expanded below.

Confocal imaging was performed with a Zeiss LSM 880 NLO AxioExaminer equipped with a Diode 405 nm laser line, Argon 458, 488, and 514 laser lines, and a DPSS 561 nm laser line. Images were acquired using a W-Plan-Apo 40x/1.0 DIC objective and a Zeiss Airyscan detector.

5a. Multicolor imaging of RhoVR 1 in HEK cells and photostability

eGFP transfected HEK cells were incubated with a HBSS solution (Gibco) containing RhoVR 1 (500 nM) at 37°C for 20 min prior to transfer to fresh HBSS (no dye) for imaging. Microscopic images were acquired with a W-Plan-Apo 20x/1.0 water objective (Zeiss) and OrcaFlash4.0 sCMOS camera (Hamamatsu). For RhoVR 1 images, the excitation light was delivered from a LED (9.72 W/cm^2; 100 ms exposure time) at 542/33 (bandpass) nm and emission was collected with a quadruple emission filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP). For eGFP images, the excitation light was delivered from a LED (5.77 W/cm^2; 20 ms exposure time) at 475/34 nm and emission was collected with a quadruple emission filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP). For photostability experiments HEK cells were incubated separately with RhoVR 1 (500 nM) and VF2.1.Cl (500 nM) in HBSS at 37°C for 20 min and then the dye loading buffer was exchanged for fresh HBSS. Data were acquired with a W-Plan-Apo 63x/1.0 objective (Zeiss) and OracFlash4.0 sCMOS camera (Hamamatsu). Images (pixel size 0.38 μm × 0.38 μm) were taken every 3 sec for 10 min with constant illumination of LED (60 W/cm^2; 10 ms exposure time). For RhoVR images, the excitation light was delivered at 542/33 nm and emission was collected with a quadruple emission filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP). For VF2.1.Cl images, the excitation light was delivered at 475/34 nm and emission was collected with an emission filter (540/50 nm) after passing through a dichroic mirror (510 nm LP). The obtained fluorescence curves (background subtracted) were normalized with the fluorescence intensity at t = 0 and averaged (three different cells of each dye).

5c. Voltage sensitivity in HEK cells

Functional imaging of the RhoVR voltage dyes was performed using a 20x objective paired with image capture from the EMCCD camera at a sampling rate of 0.5 kHz. RhoVRs were excited using the 542 nm LED with an intensity of 9.73 W/cm^2. For initial voltage characterization emission was collection with the QUAD filter and dichroic (see above).

5d. Imaging groups of neurons

Imaging experiments looking at functional responses from many (>5) neurons (Figure 2 and SI Figure 5) required a larger field of view which were obtained using the sCMOS camera with a 20x objective. RhoVR 1 was excited using the 542 nm LED with an intensity of 1.73-3.07 W/cm^2 and emission was collected with a QUAD filter and dichroic (see above). Images were binned 4x4 to allow sampling rates of 0.5 kHz. eGFP was excited by the 475 nm LED with an intensity of 0.82-1.20 W/cm^2 and emission was collected with the same QUAD filter and dichroic.

5e. Dual-View imaging

Dual-view imaging was preformed using a 20x objective paired with the sCMOS camera. RhoVR 1 was excited using the 542 nm LED with a light intensity of 2.40-4.82 W/cm^2 while GCaMP6s was excited simultaneously using a 475 nm LED with a light intensity of 0.82-1.20 W/cm^2. Emission was collected with a QUAD filter and dichroic (see above) used in conjunction with a Dual-View emission splitter (Optical Insights). The Dual-View was equipped with a 585dcxr dichroic (Chroma) and 520/28 nm (Semrock) and 610/75 nm (Chroma) emission filters which separated the GCaMP6s and RhoVR 1 signals.

5f. Cellular localization of rhodamines by confocal microscopy

eGFP transfected HEK cells were incubated with a HBSS solution (Gibco) containing 500 nM of either RhoVR 1, **12**, **18**, **7**, or **23** at 37°C for 20 min prior to transfer to fresh HBSS (no dye) for imaging. Rhodamine derivatives **7** and **23** were excited at 561 nm at 7% laser power while RhoVR 1, **12** and **18** were imaged at 11% laser power. Emission for all rhodamines was collected from 580-695 nm. eGFP was excited at 488 nm and the emission was collected from 500-560 nm.

6. Image analysis

Analysis of voltage sensitivity in HEK cells was performed using ImageJ (FIJI). Briefly, a region of interest (ROI) was selected automatically based on fluorescence intensity and applied as a mask to all image frames. Fluorescence intensity values were calculated at known baseline and voltage step epochs. For analysis of RhoVR 1 voltage responses in neurons, regions of interest encompassing cell bodies (all of approximately the same size) were drawn in ImageJ and the mean fluorescence intensity for each frame extracted. ΔF/F values were calculated by first subtracting a mean background value from all raw fluorescence frames, bypassing the noise amplification which arises from subtracting background for each frame, to give a background subtracted trace (bkgsub). A baseline fluorescence value (F_{base}) is calculated either from the first several (10-20) frames of the experiment for evoked activity, or from the median for spontaneous activity, and was subtracted from each timepoint of the bkgsub trace to yield a ΔF trace. The ΔF was then divided by F_{base} to give ΔF/F traces. No averaging has been applied to any voltage traces.

Movies were created using FIJI according to the following protocol. In FIJI, a substack of the first 70 images were averaged to create a single baseline or "F" image. The full stack was then divided by this image to give a 32-bit floating decimal result, whose scale ranged from 0.92 and 1.18. The images were multiplied by 50,000 (56,000 for Ca image) to give an image that was within the 16-bit range. After this transformation, the scale was between approximately 46,000 and 59,000. The full 16-bit range was applied, a 3D Gaussian blur (1.0 pixels in x, y, and z dimensions) was applied, and the baseline/bleaching was corrected using the built-in ratio method. The images were converted to 8-bit .AVI files.

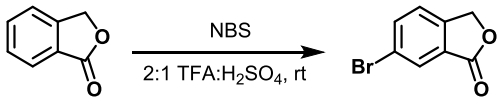
Analysis of cellular localization was performed in FIJI. Briefly, a line segment was drawn across the width of an eGFP transfected HEK cell and the normalized fluorescence intensity plotted against the normalized length of the line segment (0-100). The fluorescence of the interior of the cell was taken to be the average fluorescence from the rhodamine channel across the 20^{th}-80^{th} percentile of the line segment. The edge of the cell was then defined by the point at which the eGFP fluorescence fell below one half of the average fluorescence of the center of the cell. "Edge fluorescence" was then calculated by averaging the intensities of the line segment ± .2 microns from the cell edge in the rhodamine channel.

7. Electrophysiology

For electrophysiological experiments, pipettes were pulled from borosilicate glass (Sutter Instruments, BF150-86-10), with a resistance of 5–8 MΩ, and were filled with an internal solution; 115 mM potassium gluconate, 10 mM BAPTA tetrapotassium salt, 10 mM HEPES, 5 mM NaCl, 10 mM KCl, 2 mM ATP disodium salt, 0.3 mM GTP trisodium salt (pH 7.25, 275 mOsm). Recordings were obtained with an Axopatch 200B amplifier (Molecular Devices) at room temperature. The signals were digitized with Digidata 1440A, sampled at 50 kHz and recorded with pCLAMP 10 software (Molecular Devices) on a PC. Fast capacitance was compensated in the on-cell configuration. For all electrophysiology experiments, recordings were only pursued if series resistance in voltage clamp was less than 30 MΩ. For whole-cell, voltage clamp recordings in HEK 293T cells, cells were held at -60 mV and 100 ms hyper- and de- polarizing steps applied from -100 to +100 mV in 20 mV increments.

8. Synthesis

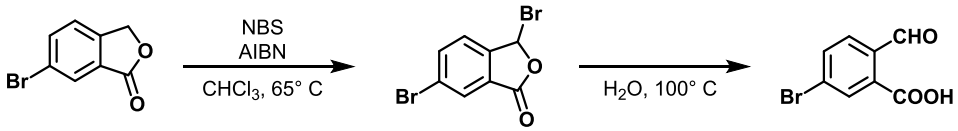
Preparation of Carboxybenzaldehydes:



1 **2**

Synthesis of 6-bromoisobenzofuran-1(3H)-one, 2:

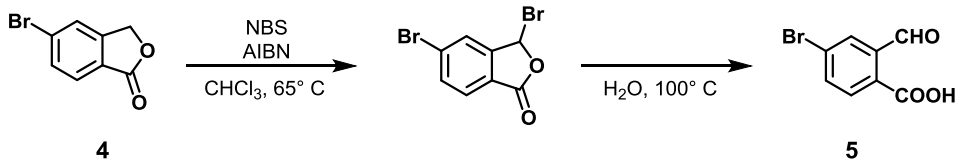
Acetic acid (10 mL) and concentrated sulfuric acid (4.5 mL) were added to **1** (5.00 g, 37.3 mmol). *N*-bromosuccinimide (9.95 g, 55.9 mmol) was added portion-wise over 6 hours and the reaction was stirred at 22 °C for 5 d, after which the reaction was judged complete by NMR. The reaction mixture was poured over 150 mL of ice, then extracted with dichloromethane (2x 200 mL), dried with anhydrous magnesium sulfate and the solvent removed by rotary evaporation. The crude solid was purified by flash chromatography (10-30% EtOAc in hexanes, linear gradient), yielding 4.95 g (23.2 mmol, 62%) of **2** and 2.18 g of a mixture of other isomers. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, *J* = 1.2 Hz, 1H), 7.79 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.39 (d, *J* = 8.1 Hz, 1H), 5.28 (s, 2H).



2 **3**

Synthesis of 5-bromo-2-formylbenzoic acid, 3:

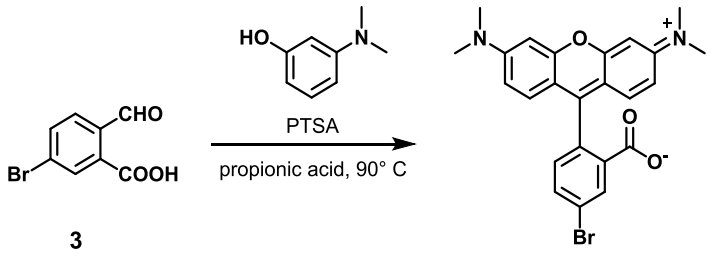
2 (1.50 g, 7.04 mmol) was stirred in $CHCl_3$ (40 mL) with *N*-bromosuccinimide (1.38 g, 7.75 mmol) and AIBN (86 mg, 0.35 mmol) at reflux for 8 h, after which a second portion of *N*-bromosuccinimide (1.38 g, 7.75 mmol) and AIBN (86 mg, 0.35 mmol) was added. The reaction was stirred for a further 16 h, after which the bromination was deemed complete by analysis of the crude reaction mixture by NMR. The precipitated solid was filtered off and the filtrate concentrated *in vacuo*. Water (75 mL) was added to the resulting solid and refluxed for 2 h. The resulting white solid was collected by vacuum filtration and washed with water (50 mL) and hexanes (25 mL), affording **3** as a white solid (1.42 g, 6.21 mmol, 88%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.28 (s, 1H), 8.01 (s, 1H), 7.97 (d, *J* = 8.1 Hz, 1H), 7.64 (d, *J* = 8.3 Hz, 1H), 6.66 (s, 1H).



4 **5**

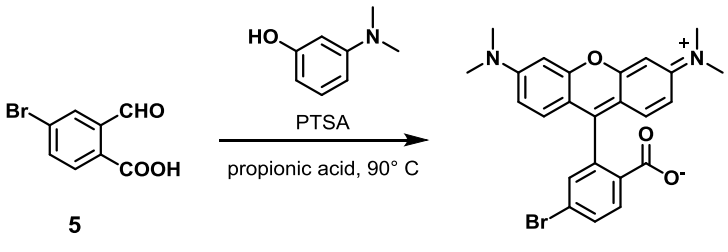
Synthesis of 4-bromo-2-formylbenzoic acid, 5:
4 (1.50 g, 7.04 mmol) was stirred in $CHCl_3$ (40 mL) with *N*-bromosuccinimide (1.38 g, 7.75 mmol) and AIBN (86 mg, 0.35 mmol) at reflux for 8 h, after which a second portion of *N*-bromosuccinimide (1.38 g, 7.75 mmol) and AIBN (86 mg, 0.35 mmol) was added. The reaction was stirred for a further 16 h, after which the bromination was deemed complete by NMR analysis of the crude reaction mixture. The precipitated solid was filtered off and the filtrate concentrated *in vacuo*. Water (75 mL) was added to the resulting solid and refluxed for 2 h. The resulting white solid was collected by vacuum filtration and washed with water (50 mL) and hexanes (25 mL), affording **5** as a white solid (1.27 g, 5.55 mmol, 79%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.29 (s, 1H), 7.93 (d, *J* = 1.6 Hz, 1H), 7.86 (dd, *J* = 8.1, 1.7 Hz, 1H), 7.76 (d, *J* = 8.1 Hz, 1H), 6.65 (s, 1H).

Preparation of Tetramethylrhodamines:



3 **6**

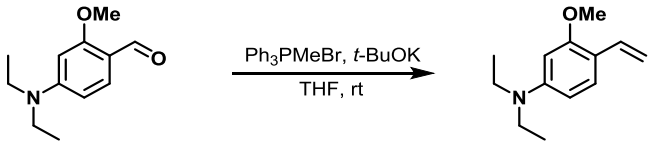
Synthesis of 4'-Br-TMR, 6:
3 (229 mg, 1.00 mmol), 3-dimethylaminophenol (288 mg, 2.01 mmol) and PTSA (35 mg, 0.2 mmol) were stirred in propionic acid (4 mL) at 90 °C for 18 h. The mixture was cooled to 22 °C, then the solvent removed *in vacuo*. The resulting crude residue was purified by flash chromatography (5-10% MeOH in DCM, linear gradient), affording **6** as a purple solid (163 mg, 0.350 mmol, 35%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.15 (s, 1H), 7.73 (d, *J* = 8.4 Hz, 1H), 7.05 (d, *J* = 8.1 Hz, 1H), 6.64 (d, *J* = 8.6 Hz, 2H), 6.49 (s, 2H), 6.43 (d, *J* = 8.0 Hz, 2H), 3.00 (s, 12H); Analytical HPLC retention time 9.67 min; MS (ESI) Exact mass calcd for $C_{24}H_{22}{}^{79}BrN_2O_3^+$ $[M+H]^+$: 465.1, found: 464.9; HR-ESI-MS m/z for $C_{24}H_{22}{}^{79}BrN_2O_3^+$ calcd: 465.0808 found: 465.0806.



5 **7**

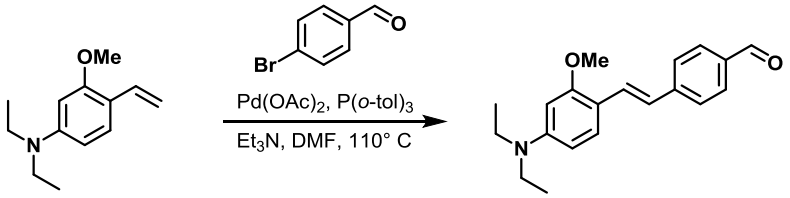
Synthesis of 5'-Br-TMR, 7:
5 (229 mg, 1.00 mmol), 3-dimethylaminophenol (288 mg, 2.01 mmol) and PTSA (35 mg, 0.2 mmol) were stirred in propionic acid (4 mL) at 90 °C for 18 h. The mixture was cooled to 22 °C, then the solvent removed *in vacuo*. The resulting crude residue was purified by flash chromatography (5-10% MeOH in DCM, linear gradient), affording **7** as a purple solid (267 mg, 0.574 mmol, 57%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.85 (d, *J* = 8.2 Hz, 1H), 7.70 (d, *J* = 8.1 Hz, 1H), 7.32 (s, 1H), 6.62 (d, *J* = 8.8 Hz, 2H), 6.48 (d, *J* = 2.7 Hz, 2H), 6.42 (dd, *J* = 8.9, 2.7 Hz, 2H), 2.99 (s, 12H); Analytical HPLC: retention time 9.97 min; MS (ESI) Exact mass calcd for $C_{24}H_{22}{}^{79}BrN_2O_3^+$ $[M+H]^+$: 465.1, found: 465.2; HR-ESI-MS m/z for $C_{24}H_{22}{}^{79}BrN_2O_3^+$ calcd: 465.0808 found: 465.0807.

Synthesis of Methoxy Molecular Wire:



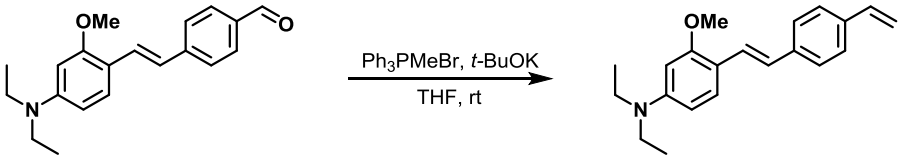
22 **23**

Synthesis of 3-methoxy-N,N-diethyl-4-vinylaniline, 23:
A round-bottom flask was charged with methyltriphenylpohsphonium bromide (1.04 g, 5 mmol) and then evacuated/backfilled with nitrogen (3x). Anhydrous THF (25 mL) was added and the reaction stirred for 15 min, then 1 M potassium *tert*-butoxide (in THF, 10.5 mL, 10.5 mmol) was added via syringe. After stirring for another 15 min, **22** was added and the reaction stirred for 18 h. The solvent was then removed *in vacuo* and the remaining crude material taken up in hexanes and filtered through an alumina plug, washing with hexanes. The combined organics were dried with $MgSO_4$ and the solvent removed *in vacuo*, affording **23** as a pale yellow oil (927 mg, 4.51 mmol, 90%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33 (d, *J* = 8.6 Hz, 1H), 6.93 (dd, *J* = 17.7, 11.1 Hz, 1H), 6.28 (dd, *J* = 8.6, 2.5 Hz, 1H), 6.17 (d, *J* = 2.4 Hz, 1H), 5.53 (dd, *J* = 17.7, 1.8 Hz, 1H), 5.01 (dd, *J* = 11.1, 1.8 Hz, 1H), 3.84 (s, 3H), 3.37 (q, *J* = 7.1 Hz, 4H), 1.18 (t, *J* = 7.1 Hz, 6H); Analytical HPLC retention time 8.54 min; HR-ESI-MS m/z for $C_{13}H_{20}NO^+$ calcd: 206.1539 found: 206.1539.



23 **24**

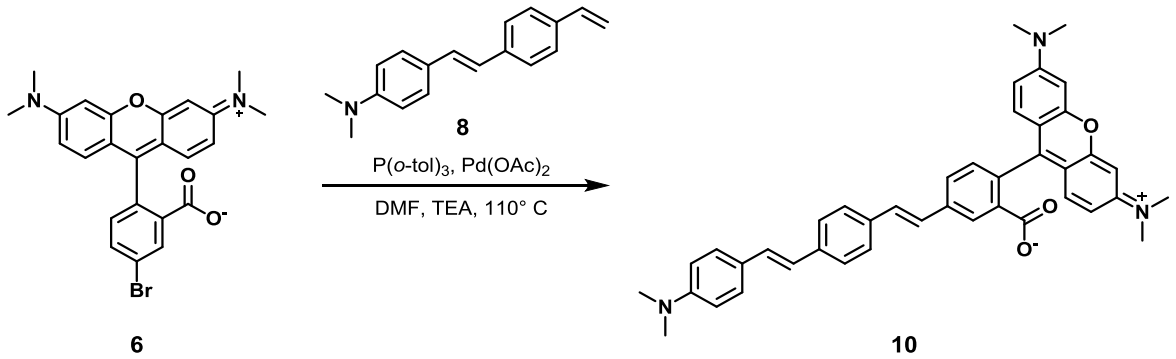
Synthesis of (E)-4-(4-(diethylamino)-2-methoxystyryl)benzaldehyde, 24:
A Schlenk flask was charged with **23** (822 mg, 4.00 mmol), bromobenzaldehyde (815 mg, 4.40 mmol), $Pd(OAc)_2$ (9 mg, 0.04 mmol), and P(*o*-tol)$_3$ (37 mg, 0.12 mmol). The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (8 mL) and anhydrous Et_3N (8 mL) were added via syringe and the reaction stirred at 110 °C for 18 h. The reaction was cooled and the solvent removed *in vacuo*. The remaining residue was purified by flash chromatography (0-20% EtOAc in hexanes, linear gradient) affording **24** as an orange oil (698 mg, 2.26 mmol, 53%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.93 (s, 1H), 7.80 (d, *J* = 8.3 Hz, 2H), 7.63 – 7.420 (m, 4H), 6.95 (d, *J* = 16.4 Hz, 1H), 6.31 (dd, *J* = 8.8, 2.5 Hz, 1H), 6.17 (d, *J* = 2.4 Hz, 1H), 3.89 (s, 3H), 3.40 (q, *J* = 7.0 Hz, 4H), 1.20 (t, *J* = 7.1 Hz, 6H); Analytical HPLC retention time 11.16 min; HR-ESI-MS m/z for $C_{20}H_{24}NO_2^+$ calcd: 310.1802 found: 310.1801.



24 **9**

Synthesis of (E)-N,N-diethyl-3-methoxy-4-(4-vinylstyryl)aniline, 9:
A round-bottom flask was charged with methyltriphenylpohsphonium bromide (643 mg, 1.80 mmol) and then evacuated/backfilled with nitrogen (3x). Anhydrous THF (5 mL) was added and the reaction stirred for 15 min, then 1 M potassium *tert*-butoxide (in THF, 2.4 mL, 2.4 mmol) was added via syringe. After stirring for another 15 min, **24** (371 mg, 1.20 mmol) was added and the reaction stirred for 5 h. The reaction was diluted with hexanes, then the solids removed by vacuum filtration, rinsing with hexanes. The organics were concentrated *in vacuo* and the remaining oil was purified by flash chromatography (10% EtOAc in hexanes, isocratic) affording **9** as a yellow solid (305 mg, 0.992 mmol, 83%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 – 7.32 (m, 6H), 6.91 (d, *J* = 16.4 Hz, 1H), 6.70 (dd, *J* = 17.6, 10.9 Hz, 1H), 6.31 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.19 (d, *J* = 2.4 Hz, 1H), 5.72 (dd, *J* = 17.6, 1.2 Hz, 1H), 5.19 (dd, *J* = 10.8, 1.2 Hz, 1H), 3.88 (s, 3H), 3.39 (q, *J* = 7.1 Hz, 4H), 1.20 (t, *J* = 7.0 Hz, 6H); Analytical HPLC retention time 13.40 min; HR-ESI-MS m/z for $C_{21}H_{20}NO^+$ calcd: 308.2009 found: 308.2007.

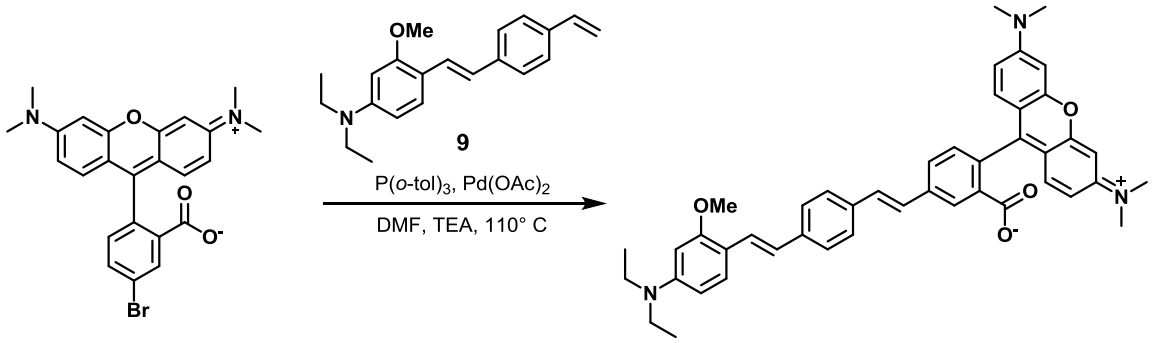
Preparation of RhoVR Voltage Dyes:



6 10

Synthesis of 10:

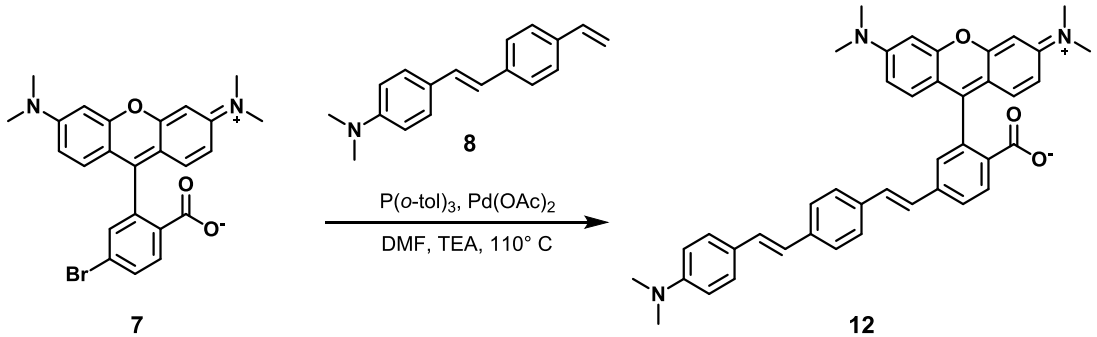
A Schlenk flask was charged with **6** (100 mg, 0.214 mmol), **8** (59.0 mg, 0.236 mmol), $Pd(OAc)_2$ (0.5 mg, 0.02 mmol) and $P(o\text{-tol})_3$ (1.3 mg, 0.043 mmol). The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (3 mL) and anhydrous Et_3N (1.5 mL) were added via syringe and the reaction stirred at 110 °C for 18 h. The reaction was cooled and the solvent removed *in vacuo*. The remaining residue was purified by flash chromatography (5-10% MeOH in DCM, linear gradient) affording **10** as a purple solid (65.0 mg, 0.103 mmol, 48%). ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.17 (s, 1H), 7.99 (d, *J* = 8.0 Hz, 1H), 7.60 (dd, *J* = 55.9, 7.9 Hz, 4H), 7.52 – 7.42 (m, 4H), 7.24 – 7.17 (m, 2H), 7.00 (d, *J* = 16.3 Hz, 1H), 6.73 (d, *J* = 8.3 Hz, 2H), 6.56 (d, *J*= 8.8 Hz, 2H), 6.51 (s, 4H) 2.94 (m, 18H); Analytical HPLC retention time 13.88 min; MS (ESI) Exact mass calcd for $C_{42}H_{41}N_3O_3^+$ $[M+2H]^{2+}$: 317.7, found: 318.3; HR-ESI-MS m/z for $C_{42}H_{40}N_3O_3^+$ calcd: 634.3064 found: 634.3060.



6 11

Synthesis of 11:

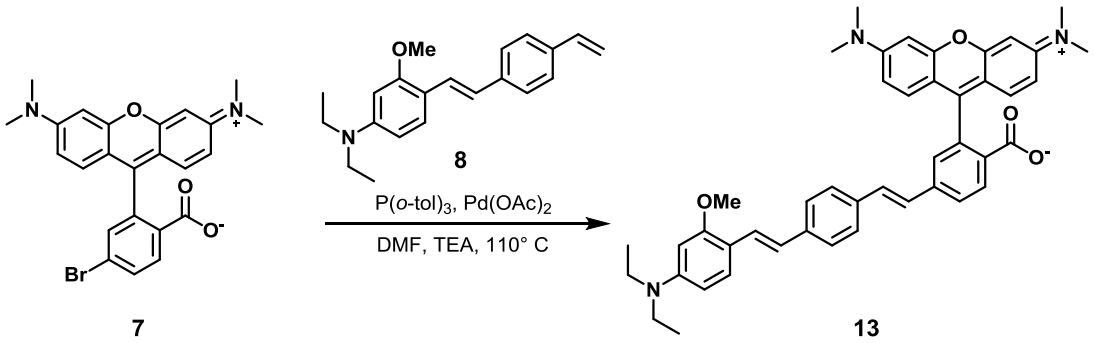
A Schlenk flask was charged with **6** (100 mg, 0.214 mmol), **9** (73.0 mg, 0.236 mmol), $Pd(OAc)_2$ (0.5 mg, 0.02 mmol) and $P(o\text{-tol})_3$ (1.3 mg, 0.043 mmol). The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (3 mL) and anhydrous Et_3N (1.5 mL) were added via syringe and the reaction stirred at 110 °C for 18 h. The reaction was cooled and the solvent removed *in vacuo*. The remaining residue was purified by flash chromatography (5-10% MeOH in DCM, linear gradient) affording **11** as a purple solid (62 mg, 0.089 mmol, 41%). ^{1}H NMR (400 MHz, $CDCl_{3z}$) δ 8.11 (d, *J* = 1.6 Hz, 1H), 7.75 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.51 (s, 4H), 7.47 (d, *J* = 4.0 Hz, 1H), 7.44 (d, *J* = 11.8 Hz, 1H), 7.21 (d, *J* = 12.1 Hz, 2H), 7.14 (d, *J* = 8.1 Hz, 1H), 6.94 (d, *J* = 16.4 Hz, 1H), 6.66 (d, *J* = 8.8 Hz, 2H), 6.49 (d, *J* = 2.6 Hz, 2H), 6.40 (dd, *J* = 8.9, 2.6 Hz, 2H), 6.32 (dd, *J* = 8.7, 2.4 Hz, 1H), 6.20 (d, *J* = 2.4 Hz, 1H), 3.89 (s, 3H), 3.40 (q, *J* = 7.0 Hz, 4H), 2.98 (s, 12H), 1.20 (t, *J* = 7.1 Hz, 6H); Analytical HPLC retention time 12.96 min; Exact mass calcd for $C_{45}H_{46}N_3O_4^+$ $[M+H]^+$: 692.3, found: 692.5; HR-ESI-MS m/z for $C_{45}H_{46}N_3O_4^+$ calcd: 692.3488 found: 692.3470.



7 12

Synthesis of 12:

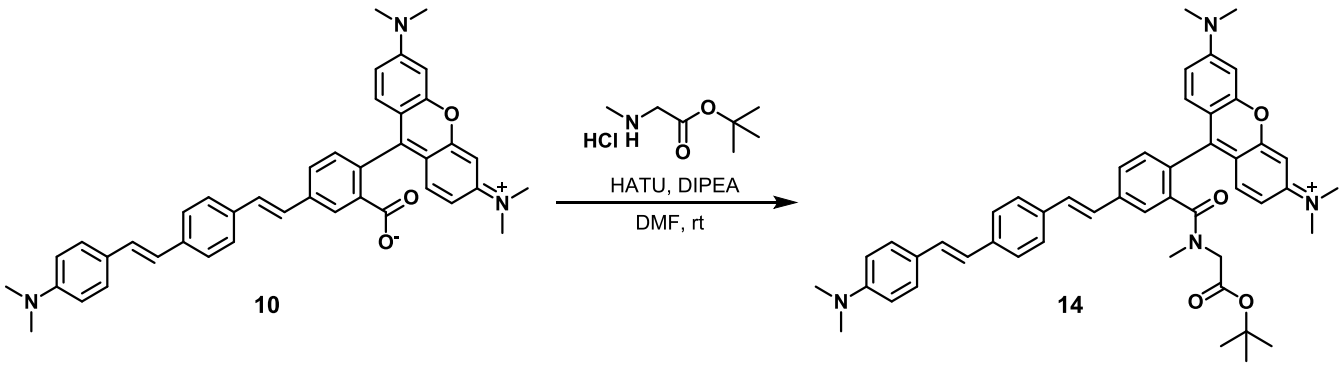
A Schlenk flask was charged with **7** (100 mg, 0.214 mmol), **8** (59.0 mg, 0.236 mmol), $Pd(OAc)_2$ (0.5 mg, 0.02 mmol) and P(*o*-tol)$_3$ (1.3 mg, 0.043 mmol). The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (3 mL) and Et_3N (1.5 mL) were added via syringe and the reaction stirred at 110 °C for 18 h. The reaction was cooled and the solvent removed *in vacuo*. The remaining residue was purified by flash chromatography (5-10% MeOH in DCM, linear gradient) affording **12** as a purple solid (91.2 mg, 0.144 mmol, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 8.0 Hz, 1H), 7.52 (d, *J* = 8.0 Hz, 1H), 7.21 (s, 4H), 7.16 (d, *J* = 8.5 Hz, 2H), 7.07 (s, 1H), 6.87 (m, 3H), 6.65 (d, *J* = 16.3 Hz, 1H), 6.48 (dd, *J* = 12.7, 8.7 Hz, 4H), 6.32 – 6.21 (m, 4H), 2.80 (s, 12H), 2.75 (s, 6H); Analytical HPLC retention time 13.54 min; MS (ESI) Exact mass calcd for $C_{42}H_{40}N_3O_3^+$ $[M+H]^+$: 634.3, found: 634.4; HR-ESI-MS m/z for $C_{42}H_{40}N_3O_3^+$ calcd: 634.3064 found: 634.3055.



Synthesis of 13:

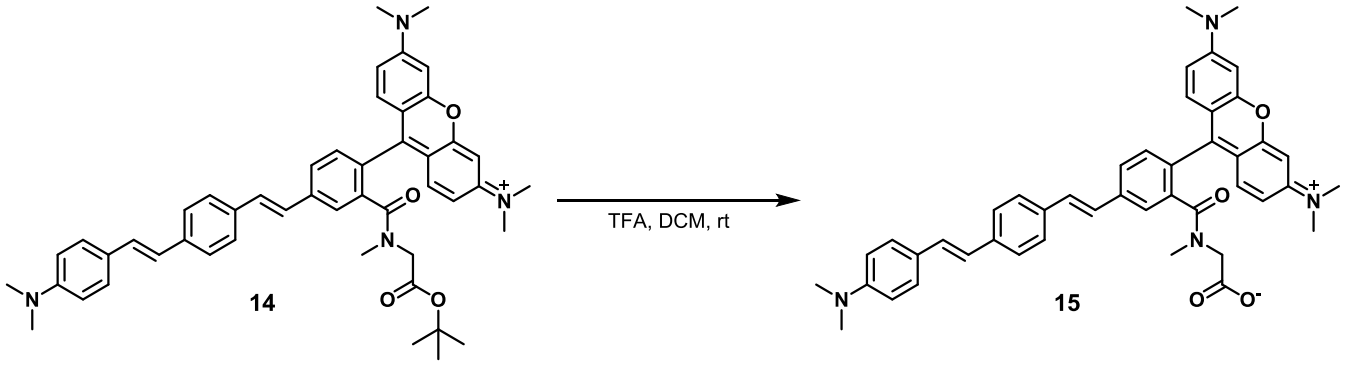
A Schlenk flask was charged with **7** (100 mg, 0.214 mmol), **8** (73.0 mg, 0.236 mmol), $Pd(OAc)_2$ (0.5 mg, 0.02 mmol) and P(*o*-tol)$_3$ (1.3 mg, 0.043 mmol). The flask was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (3 mL) and anhydrous Et_3N (1.5 mL) were added via syringe and the reaction stirred at 110 °C for 18 h. The reaction was cooled and the solvent removed *in vacuo*. The remaining residue was purified by flash chromatography (5-10% MeOH in DCM, linear gradient) affording **13** as a purple solid (75 mg, 0.11 mmol, 47%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.91 (dd, *J* =18.1, 8.3 Hz, 2H), 7.53 (d, *J* = 8.3 Hz, 2H), 7.46 – 7.38 (m, 5H), 7.32 (dd, *J* = 16.4, 6.8 Hz, 2H), 6.92 (d, *J* = 16.4 Hz, 1H), 6.60 – 6.48 (m, 6H), 6.28 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.20 (d, *J* = 2.4 Hz, 1H), 3.83 (s, 3H), 3.37 (q, *J* = 6.9 Hz, 4H), 2.94 (s, 12H), 1.12 (d, *J* = 7.0 Hz, 6H); Analytical HPLC retention time 12.16 min; Exact mass calcd for $C_{45}H_{46}N_3O_4^+$ $[M+H]^+$: 692.3, found: 692.4; HR-ESI-MS m/z for $C_{45}H_{46}N_3O_4^+$ calcd: 692.3483 found: 692.3479.

Synthesis of RhoVR.Sarc Voltage Dyes:

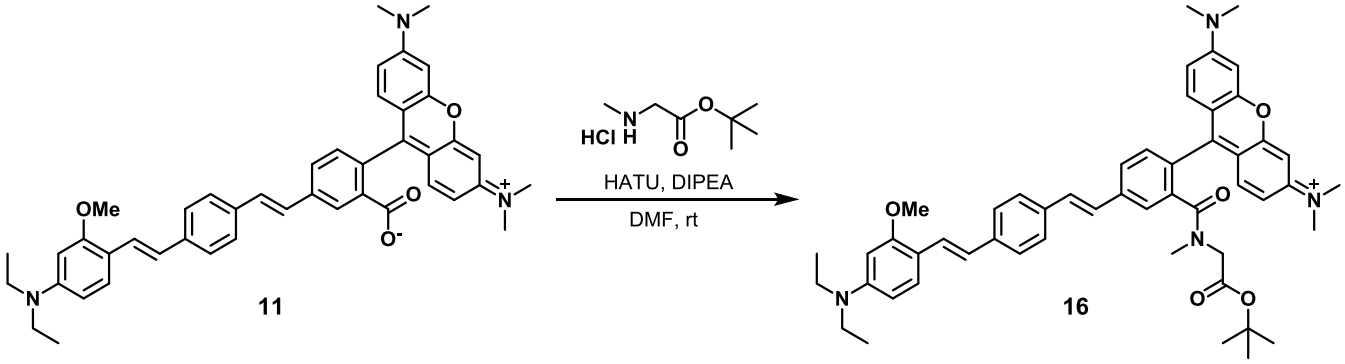


Synthesis of 14:

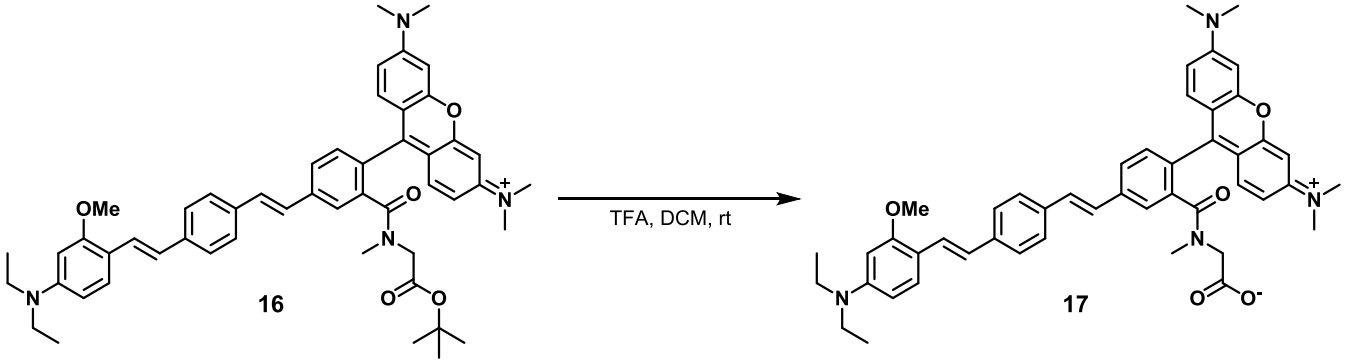
A vial was charged with **10** (14.0 mg, 22.1 μmol), N-methyl sarcosine *t*-Bu ester hydrochloride (5.0 mg, 28 μmol), and HATU (8.4 mg, 22 μmol). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (2 mL) and anhydrous diisopropylethylamine (5.8 μL, 28 μmol) were added and the vial flushed with nitrogen, sealed, and stirred at 22 °C for 18 h. The solvent was removed *in vacuo* and the remaining residue was purified by flash chromatography (2.5% MeOH in DCM) affording **14** as a purple solid **(**13.5 mg, 17.7 μmol, 80%). ^{1}H NMR (only major rotamer peaks reported, 600 MHz, $CDCl_3$) δ 7.78 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.73 (d, *J* = 1.6 Hz, 1H), 7.53 (q, *J* = 8.3 Hz, 4H), 7.50 (s, 1H), 7.44 (d, *J* = 8.7 Hz, 2H), 7.37 (m, 3H), 7.29 (d, *J* = 16.2 Hz, 1H), 7.19 (d, *J* = 16.3 Hz, 1H), 7.11 (d, *J* = 16.2 Hz, 1H), 6.95 – 6.92 (m, 3H), 6.80 (d, *J* = 2.6 Hz, 2H), 6.73 (d, *J* = 8.7 Hz, 2H), 3.78 (s, 2H), 3.30 (s, 12H), 3.00 (s, 6H), 2.80 (s, 3H), 1.33 (s, 9H); Analytical HPLC retention time 14.83 min; HR-ESI-MS m/z for $C_{49}H_{53}N_4O_4^+$ calcd: 761.4061 found: 761.4055.



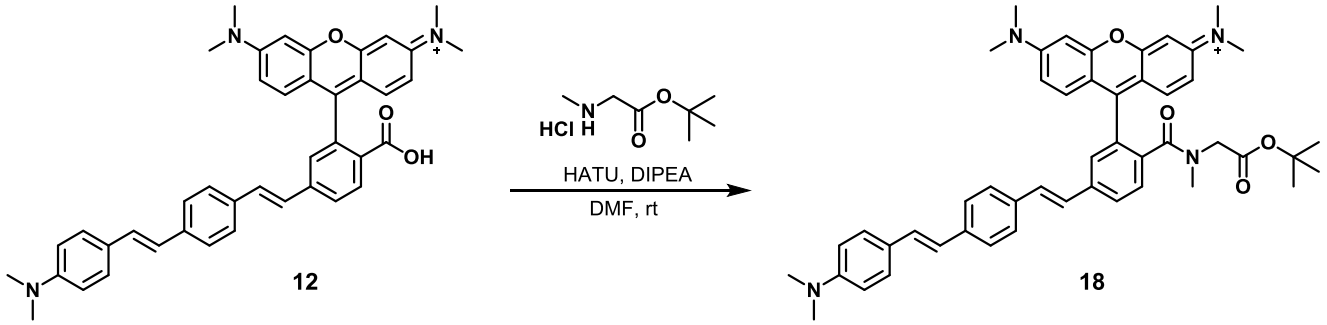
Synthesis of 15:
To a solution of **14** (7.0 mg, 9.2 μmol) in DCM (1 mL) was added trifluoroacetic acid (1 mL). The reaction was stirred at 22 °C for 1 h, then the solvent removed under a stream of nitrogen. The remaining crude solid was purified by semi-preparative HPLC, affording **15** as a purple solid (1.5 mg, 2.1 μmol, 23%). Analytical HPLC retention time 12.83 min; HR-ESI-MS m/z for $C_{45}H_{45}N_4O_4^+$ calcd: 705.3435 found: 705.3441.



Synthesis of 16:
A vial was charged with **11** (20.0 mg, 28.9 μmol), N-methyl sarcosine *t*-Bu ester hydrochloride (6.0 mg, 33 μmol), and HATU (11.0 mg, 28.9 μmol). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (4 mL) and anhydrous diisopropylethylamine (5.0 μL, 29 μmol) were added and the vial flushed with nitrogen, sealed, and stirred at 22 °C for 18 h. The solvent was removed *in vacuo* and the remaining residue was purified by flash chromatography (2.5% MeOH in DCM) affording **16** as a purple solid (21.4 mg, 26.1 μmol, 91%). LC-MS showed this material to be approximately 95% pure. This material was used without further purification for further reactions. ^{1}H NMR (only major rotamer peaks reported, 600 MHz, $CDCl_3$) δ 7.77 (dd, *J* = 8.0, 1.7 Hz, 1H), 7.71 (d, *J* = 1.8 Hz, 1H), 7.51 (s, 4H), 7.47 – 7.43 (m, 2H), 7.36 (d, *J* = 9.7 Hz, 3H), 7.28 (d, *J* = 7.5 Hz, 1H), 7.17 (d, *J* = 16.2 Hz, 1H), 6.95 – 6.91 (m, 3H), 6.78 (d, *J* = 2.6 Hz, 2H), 6.32 (dd, *J* = 8.7, 2.4 Hz, 1H), 6.20 (d, *J* = 2.5 Hz, 1H), 3.90 (s, 3H), 3.78 (s, 2H), 3.40 (q, *J* = 7.1 Hz, 4H), 3.29 (s, 12H), 2.80 (s, 3H), 1.33 (s, 9H), 1.21 (t, *J* = 7.0 Hz, 6H); Analytical HPLC retention time 13.61 min; HR-ESI-MS m/z for $C_{52}H_{59}N_4O_5^+$ calcd: 819.4485 found: 819.4490.

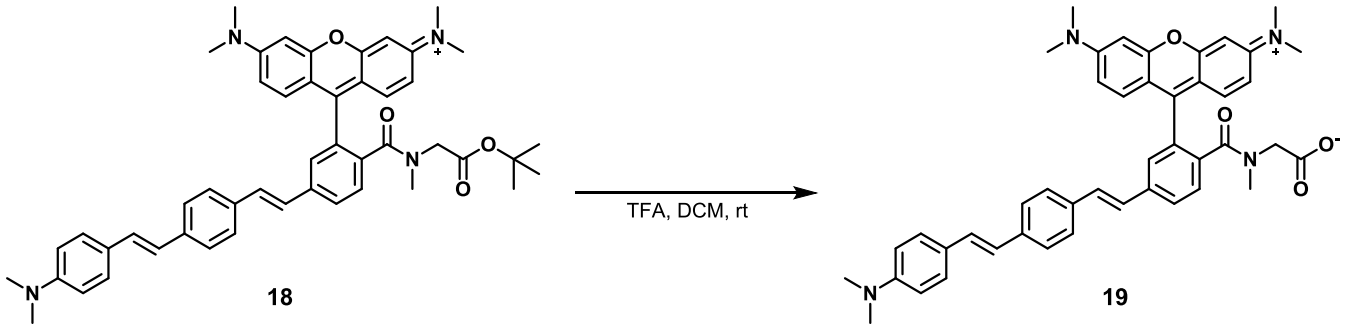


Synthesis 17:
To a solution of **16** (7.0 mg, 8.5 μmol) in DCM (1 mL) was added trifluoroacetic acid (1 mL). The reaction was stirred at 22 °C for 1 h, then the solvent removed under a stream of nitrogen. The remaining crude solid was purified by semi-preparative HPLC, affording **17** as a purple solid (1.2 mg, 1.6 μmol, 18%). Analytical HPLC retention time 11.65 min; HR-ESI-MS m/z for $C_{48}H_{51}N_4O_5^+$ calcd: 763.3859 found: 763.3849.



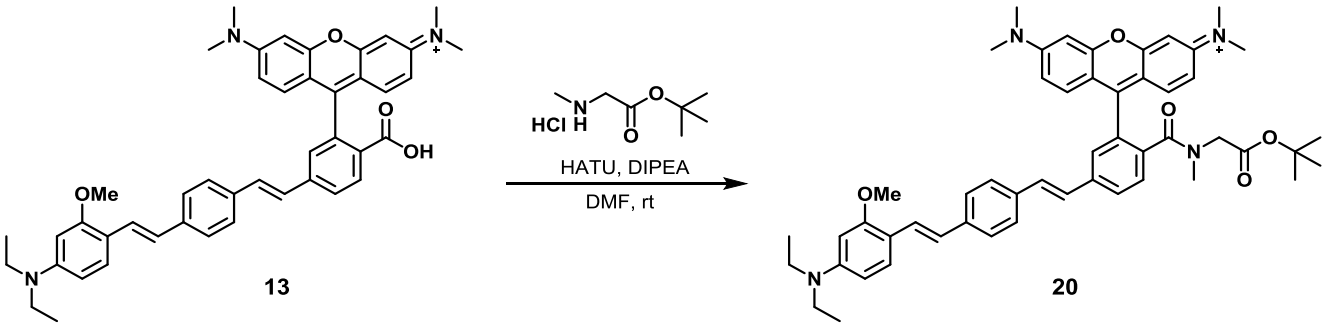
Synthesis of 18:

A vial was charged with **12** (53.0 mg, 83.5 μmol), N-methyl sarcosine *t*-Bu ester hydrochloride (16.7 mg, 91.8 μmol), and HATU (31.8 mg, 83.5 μmol). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (4 mL) and anhydrous diisopropylethylamine (15.0 μL, 86 μmol) were added and the vial flushed with nitrogen, sealed, and stirred at 22 °C for 18 h. The solvent was removed *in vacuo* and the remaining residue was purified by flash chromatography (2.5% MeOH in DCM) affording **18** as a purple solid (46.0 mg, 60.4 μmol, 72%). LCMS showed this material to be approximately 95% pure. This material was used without further purification for further reactions. ^{1}H NMR (only major rotamer peaks reported, 600 MHz, $CDCl_3$) δ 7.77 (d, *J* = 8.2 Hz, 1H), 7.60 (d, *J* = 8.0 Hz, 1H), 7.46 (m, 5H), 7.41 (d, *J* = 8.3 Hz, 2H), 7.33 (d, *J* = 9.5 Hz, 2H), 7.23 – 7.11 (m, 2H), 7.06 (d, *J* = 16.2 Hz, 1H), 6.93 (dd, *J* = 9.7, 2.5 Hz, 2H), 6.88 (d, *J* = 16.2 Hz, 1H), 6.77 (d, *J* = 2.5 Hz, 2H), 6.71 (d, *J* = 8.6 Hz, 2H), 3.78 (s, 2H), 3.29 (s, 12H), 2.99 (s, 6H), 2.86 (s, 3H), 1.32 (s, 9H), 1.25 (s, 3H); Analytical HPLC retention time 14.51 min; HR-ESI-MS m/z for $C_{49}H_{53}N_4O_4^+$ calcd: 761.4061 found: 761.4051.



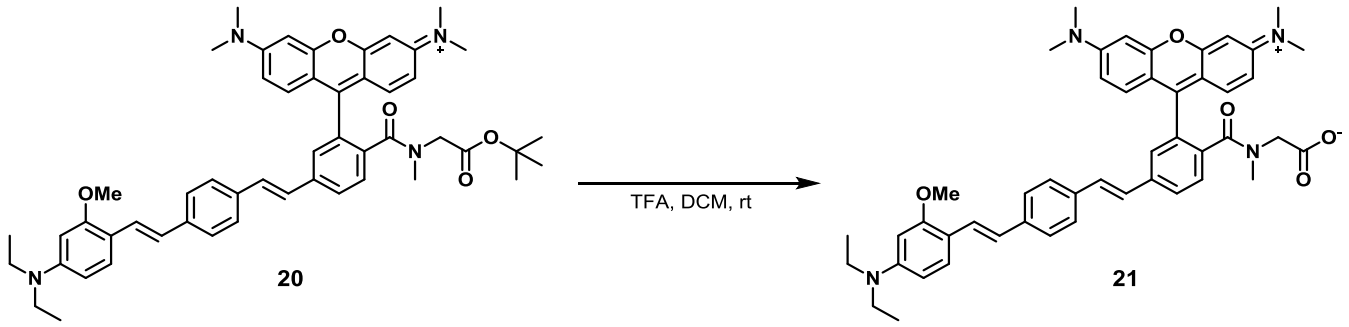
Synthesis of Carboxy RhoVR 1, 19:

To a solution of **18** (7.5 mg, 9.9 μmol) in DCM (1 mL) was added trifluoroacetic acid (1 mL). The reaction was stirred at 22 °C for 1 h, then the solvent removed under a stream of nitrogen. The remaining crude solid was purified by semi-preparative HPLC affording **19** as a purple solid (1.5 mg, 2.1 μmol, 21%). Analytical HPLC retention time 12.48 min; HR-ESI-MS m/z for $C_{44}H_{41}N_4O_4^+$ calcd: 705.3435 found: 705.3431.



Synthesis of RhoVR 1 N-methyl sarcosine *t*-Bu ester, 20:

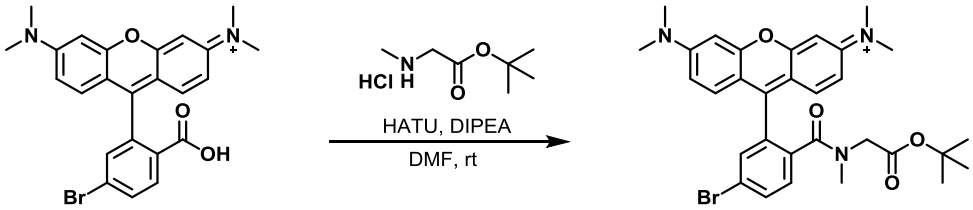
A vial was charged with **13** (35 mg, 51 μmol), N-methyl sarcosine *t*-Bu ester hydrochloride (14 mg, 77 μmol), and HATU (20 mg, 53 μmol). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (2 mL) and anhydrous diisopropylethylamine (16 μL, 76 μmol) were added and the vial flushed with nitrogen, sealed, and stirred at 22 °C for 18 h. The solvent was removed *in vacuo* and the remaining residue was purified by flash chromatography (2.5% MeOH in DCM) affording **20** as a purple solid (29 mg, 35.4 μmol, 70%). ^{1}H NMR (only major rotamer peaks reported, 600 MHz, DMSO-d_6) δ 7.96 (d, *J* = 8.2 Hz, 1H), 7.79 (s, 1H), 7.62 (d, *J* = 8.0 Hz, 1H), 7.56 (d, *J* = 8.0 Hz, 2H), 7.52 – 7.41 (m, 4H), 7.40 – 7.24 (m, 4H), 7.12 (dd, *J* = 9.8, 2.6 Hz, 2H), 7.00 – 6.89 (m, 3H), 6.29 (d, *J* = 10.3 Hz, 1H), 6.21 (s, 1H), 3.84 (s, 3H), 3.76 (s, 2H), 3.38 (q, *J* = 7.0 Hz, 4H), 3.33 (s, 12H), 2.81 (s, 3H), 1.25 (s, 9H), 1.12 (t, *J* = 7.0 Hz, 6H); Analytical HPLC retention time 13.40 min; HR-ESI-MS m/z for $C_{52}H_{59}N_4O_5^+$ calcd: 819.4480 found: 819.4484.



20 21

Synthesis of RhoVR 1, 21:

To a solution of **20** (16.4 mg, 20.0 μmol) in DCM (1 mL) was added trifluoroacetic acid (1 mL). The reaction was stirred at 22 °C for 1 h, then the solvent removed under a stream of nitrogen. The remaining crude solid was purified by semi-preparative HPLC affording **21** as a purple solid (1.6 mg, 2.1 μmol, 10%). Analytical HPLC retention time 11.52 min; HR-ESI-MS m/z for $C_{52}H_{59}N_4O_5^+$ calcd: 763.3854 found: 763.3856.



7 22

Synthesis of 22:

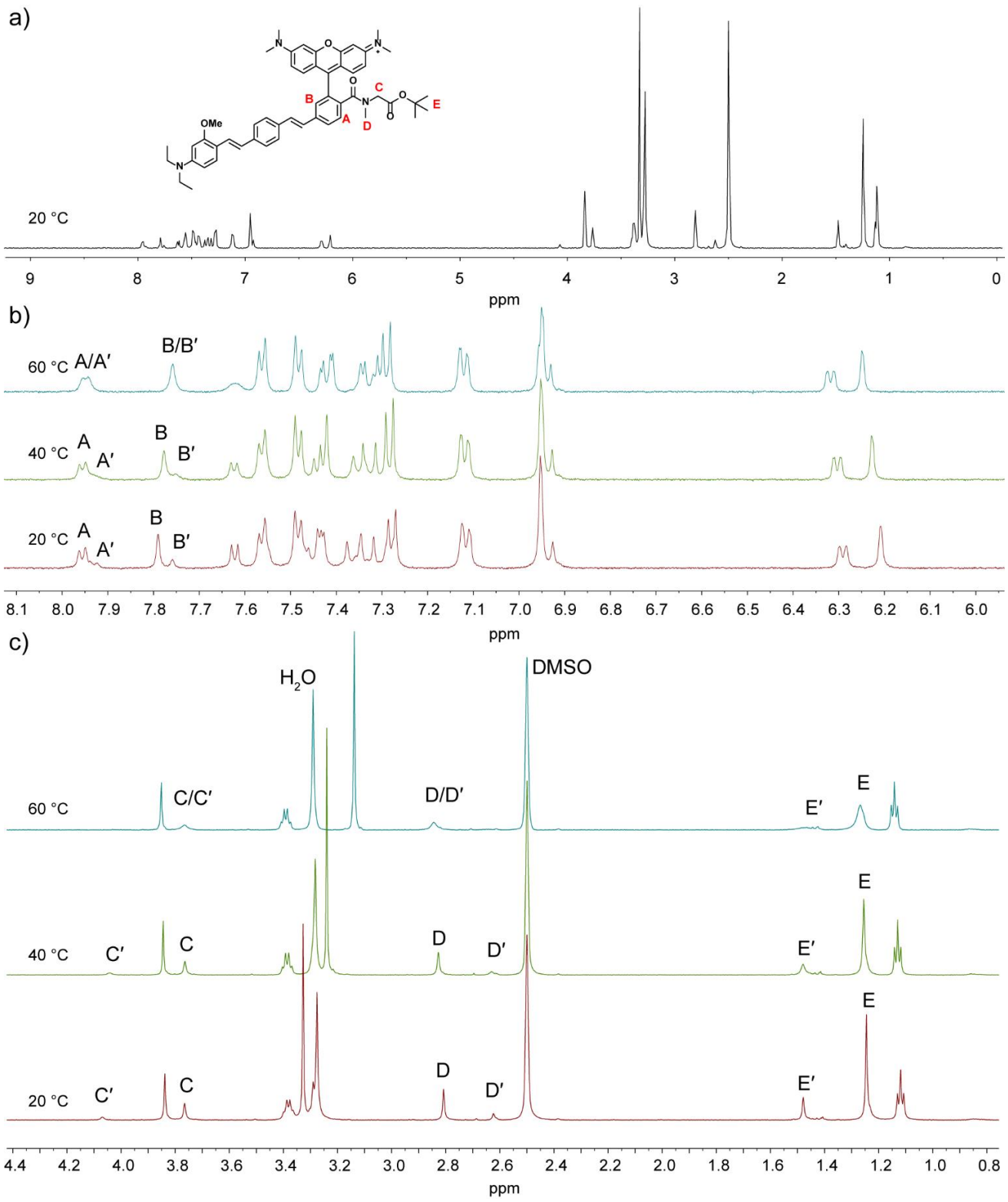
A vial was charged with **7** (20.0 mg, 42.9 μmol), N-methyl sarcosine *t*-Bu ester hydrochloride (9.7 mg, 54 μmol), and HATU (16.3 mg, 42.9 μmol). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (1 mL) and anhydrous diisopropylethylamine (12 μL, 57 μmol) were added and the vial flushed with nitrogen, sealed, and stirred at 22 °C for 18 h. The solvent was removed *in vacuo* and the remaining residue was purified by PTLC (5% MeOH in DCM) affording **22** as a purple solid (16.0 mg, 27.0 μmol, 63%). LCMS showed this material to be approximately 98% pure. This material was used without further purification for further reactions. ^{1}H NMR (only major rotamer peaks reported, ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.98 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.86 (d, *J* = 2.0 Hz, 1H), 7.57 (d, *J* = 8.3 Hz, 1H), 7.23 (d, *J* = 9.5 Hz, 2H), 7.09 (dd, *J* = 9.6, 2.4 Hz, 2H), 6.93 (d, *J* = 2.5 Hz, 2H), 3.74 (s, 2H), 3.27 (s, 12H), 2.74 (s, 3H), 1.23 (s, 9H).; Analytical HPLC retention time 13.56 min; HR-ESI-MS m/z for $C_{31}H_{35}N_3O_4{}^{79}Br_1^+$ calcd: 592.1805 found: 592.1797.

22 23

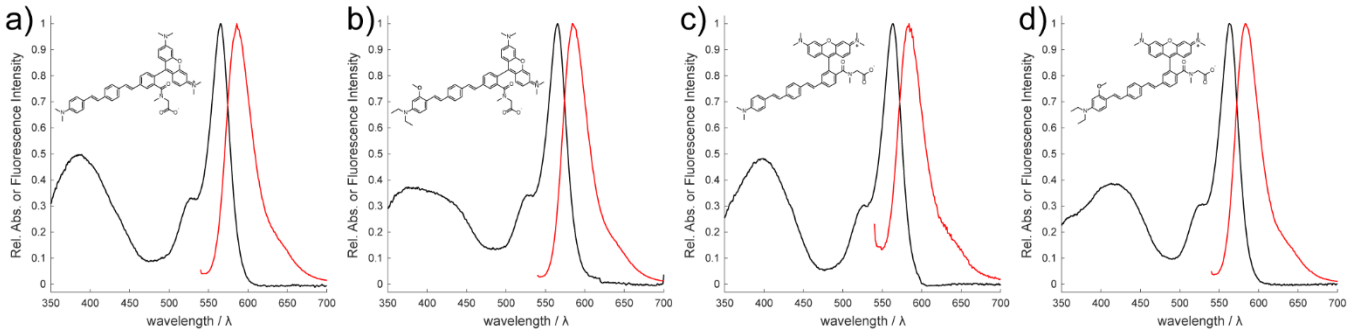
Synthesis of 23:

To a solution of **22** (10.0 mg, 16.9 μmol) in DCM (1 mL) was added trifluoroacetic acid (1 mL). The reaction was stirred at 22 °C for 2 h, then the solvent removed under a stream of nitrogen. The remaining crude solid was purified by PTLC (7.5% MeOH in DCM) affording **23** as a purple solid (4.5 mg, 8.4 μmol, 50%). Analytical HPLC retention time 10.92 min; HR-ESI-MS m/z for $C_{27}H_{27}N_3O_4{}^{79}Br_1^+$ calcd: 536.1179 found: 536.1176.

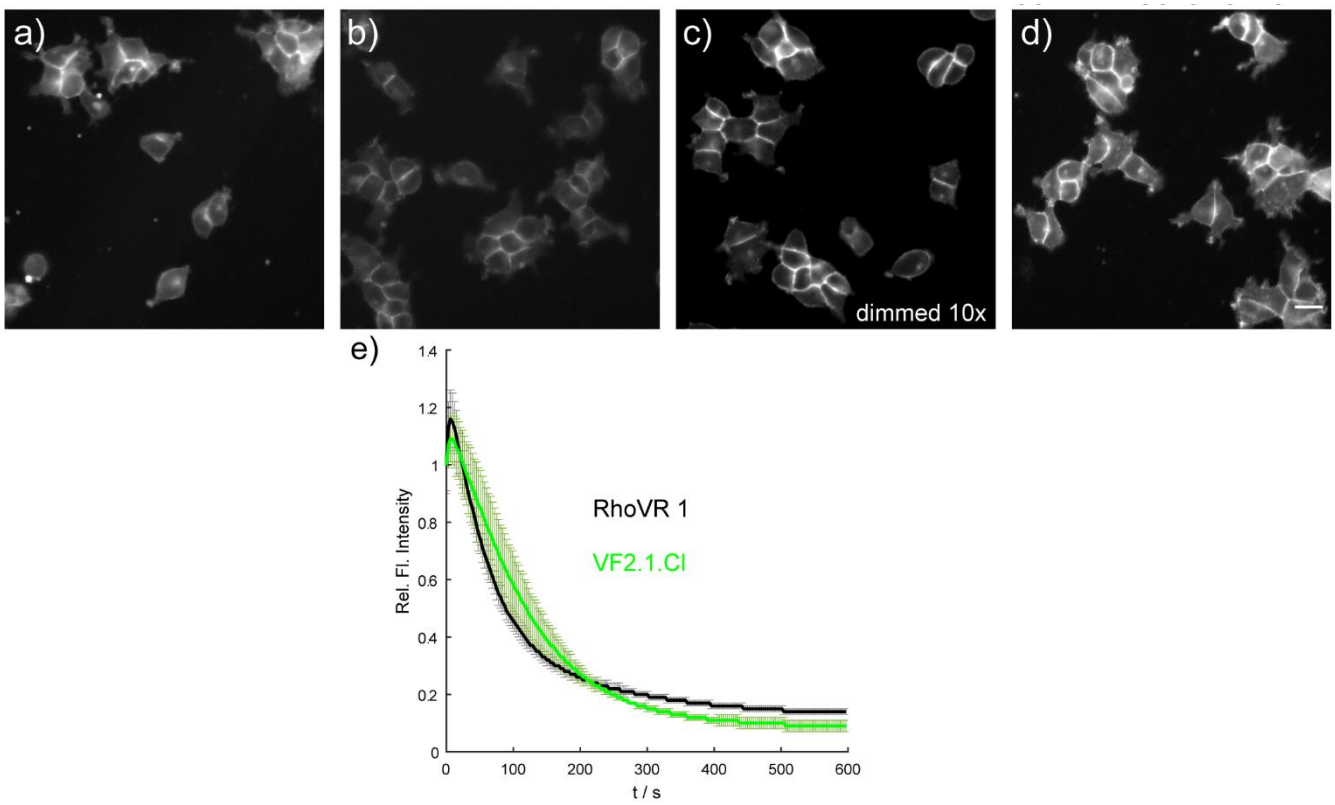
9. Supporting Figures



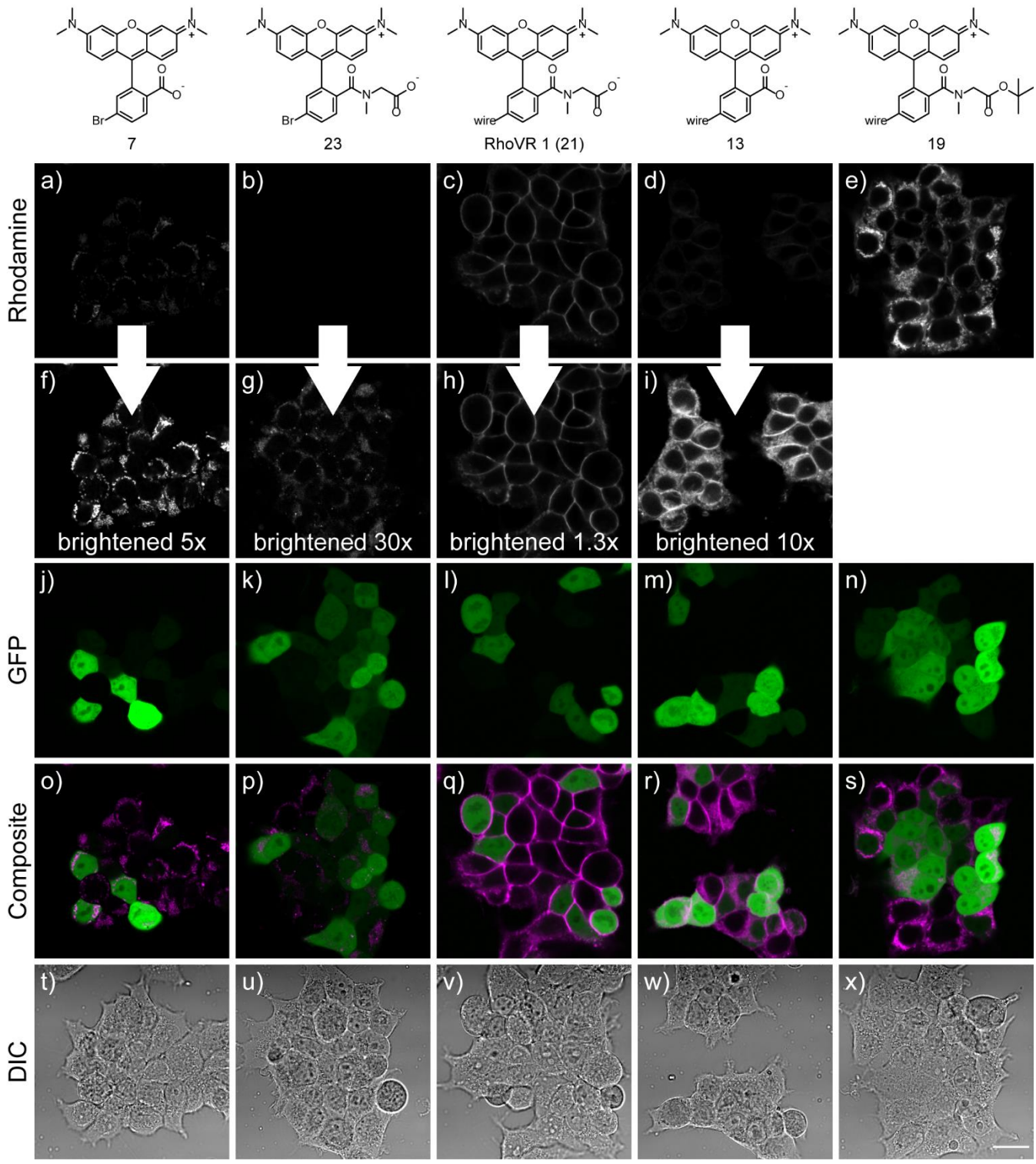
SI Figure 1. Variable temperature NMR study of compound 20. a) Full spectrum of **20** at 20 °C in d_6-DMSO. b) Expanded aromatic region. The prime peaks correlate to the minor rotamer. A/A' and B/B' peaks are split due to their proximity to the amide bond, while more distant protons undergo minor changes in δ. c) Expanded alkyl region. The prime peaks correlate to the minor rotamer. Unlabeled peaks correlate to the distant molecular wire protons, which display only minor changes in δ. Integration reveals the ratio of the major to minor rotamers is approximately 4:1 at 20° C.



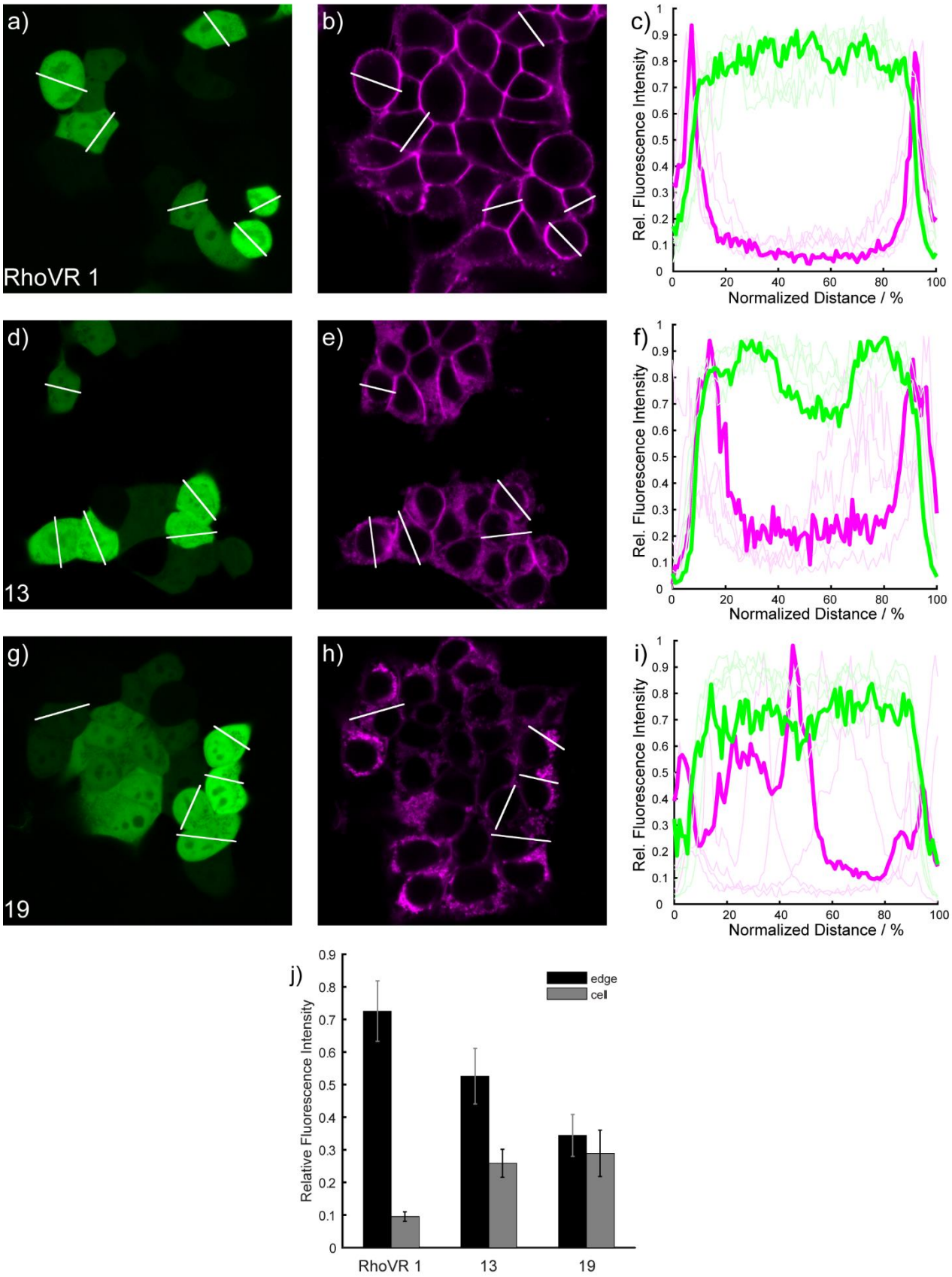
SI Figure 2. Normalized absorbance and emission profiles of **15** (a), **17** (b), **19** (c), **21** (RhoVR 1) (d). All spectra were acquired at a dye concentration of 1 μM in PBS with 0.1% SDS.



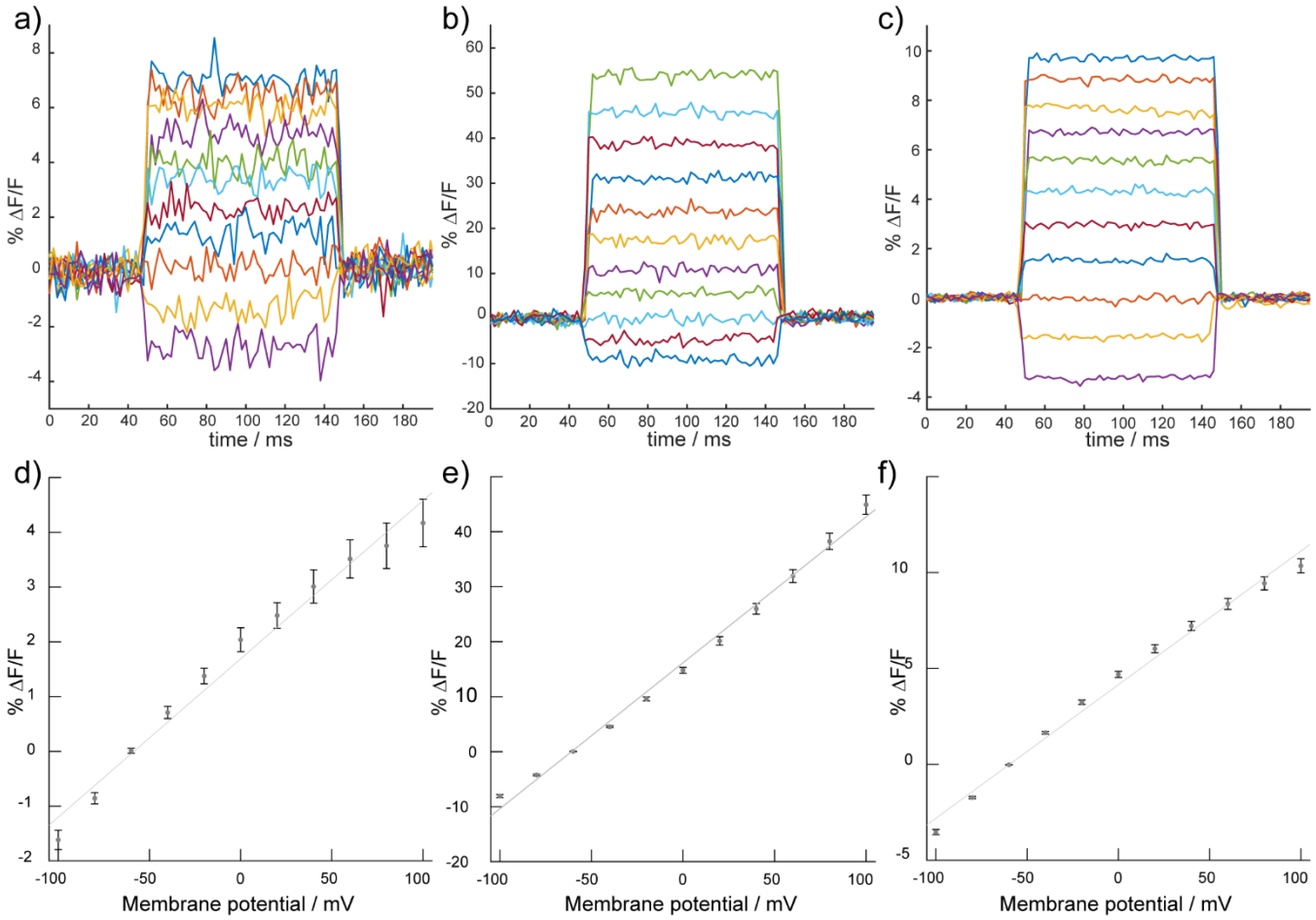
SI Figure 3. Cellular localization and photostability of RhoVR derivatives. Widefield fluorescence microscopy images of RhoVRs (500 nM) in HEK cells. **15** (a), **17** (b), **19** (c), **21** (RhoVR 1) (d). All acquisition and processing parameters are identical, to permit a comparison of loading efficacy and cellular brightness. The exception is **19** (panel c), which has been dimmed 10x (grey values in each pixel divided by 10) so that the displayed values are similar to panels a, b, and d. e) Relative photostability of RhoVR 1 (black) and VF2.1.Cl (green). Plot shows the relative fluorescence intensity decay under high-powered illumination (illumination intensities matched at 60 W/cm^2). Error bars are ±S.E.M. for 3 separate cells. There is no significant difference between the relative fluorescence values of RhoVR 1 and VF2.1.Cl prior to 300 seconds ($p > 0.05$, two-tailed Student's t-test). After 300 seconds, RhoVR 1 is significantly brighter than VF2.1.Cl ($p < 0.05$, two-tailed Student's t-test).



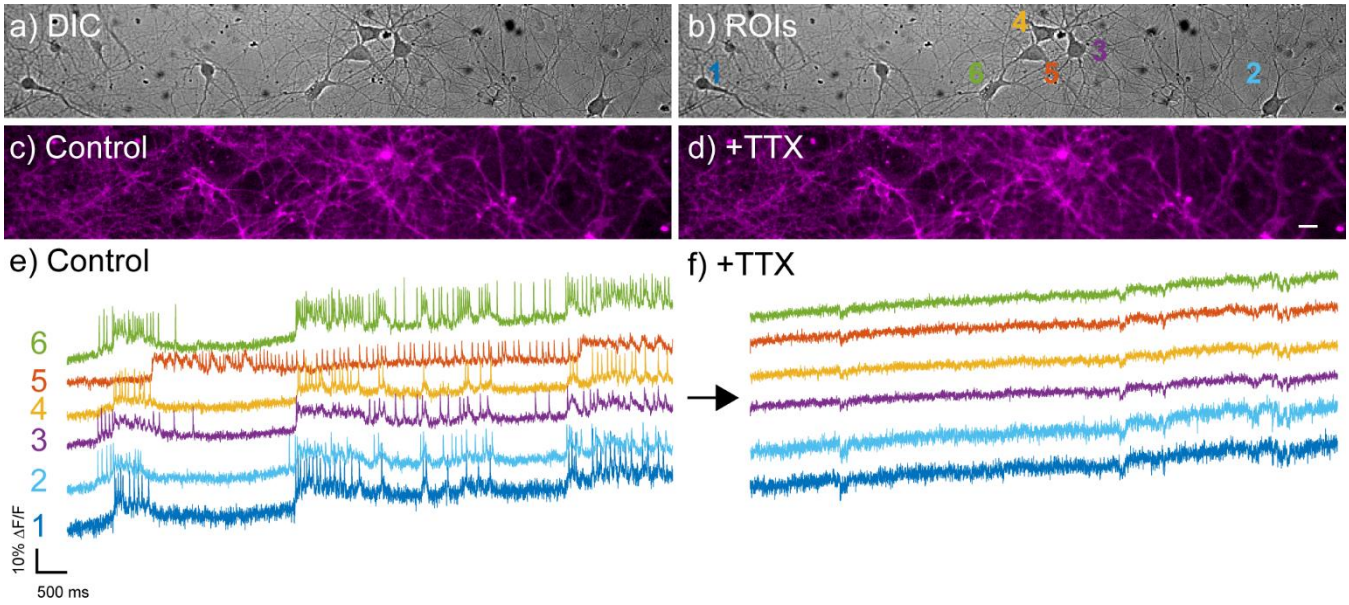
SI Figure 4. Analysis of cellular localization of rhodamines by confocal microscopy. The indicated rhodamine derivatives were loaded in HEK cells (500 nM, then washed with fresh HBSS) expressing cytosolic eGFP. a-e) All display settings are identical to enable comparison of rhodamine loading efficiency. f-i) Images of compounds **7**, **23**, **21**, and **13** were brightened by the indicated amount to enable visual inspection of cellular localization. j-n) Images showing eGFP fluorescence (all display settings identical). o-s) Composite images showing localization of rhodamines and eGFP. Each image's display settings are individually optimized to facilitate comparison of eGFP and rhodamine staining. t-x) Transmitted light images. Scale bar is 20 μm.


a)
b)
c)
RhoVR 1
Rel. Fluorescence Intensity
Normalized Distance / %
d)
e)
f)
13
Rel. Fluorescence Intensity
Normalized Distance / %
g)
h)
i)
19
Rel. Fluorescence Intensity
Normalized Distance / %
j)
edge
cell
Relative Fluorescence Intensity
RhoVR 1
13
19

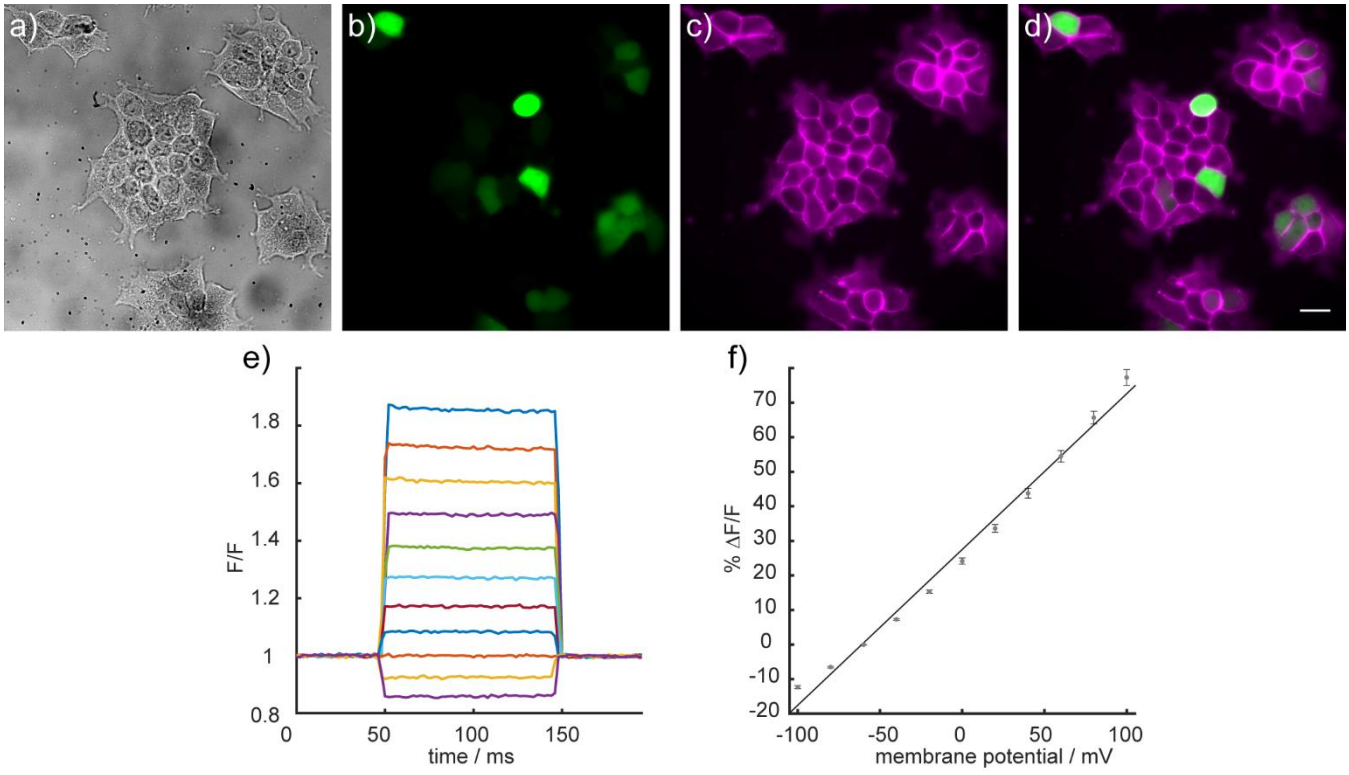
SI Figure 5. Quantification of RhoVR 1 and rhodamine cellular localization. The fluorescence intensity of rhodamine signal was measured relative to cytosolic eGFP by measuring the pixel intensity across a line segment spanning individual HEK cells. Images are shown for RhoVR 1 (compound **21**, a-b), compound **13** (free carboxylate, no sarcosine, d-e), and compound **19** (*t*-Bu ester of RhoVR 1, g-h). Plots c, f, and i show the normalized fluorescence intensity for eGFP (green) or rhodamine derivative (magenta) vs. the normalized widths of the white line segments in the fluorescence images on the left. Thick traces represent the average of 5-6 cells for each condition. Light traces represent individual intensity profiles. Plot j depicts the relative fluorescence intensity of rhodamine derivatives at the edge of the cell ("edge", black bars) vs. the cytosol ("cell", grey bars). Error bars are ± S.E.M.



SI Figure 6. Voltage sensitivity of RhoVR derivatives. **15** (a,d); **17** (b,e); and **19** (c,f). (upper row) The fractional change in fluorescence is plotted vs. time for 100 ms hyper- and depolarizing steps (±100 mV, 20 mV increments) from a holding potential of -60 mV for a single HEK cells under whole-cell voltage-clamp mode. (lower row) A plot of % ΔF/F vs. final membrane potential (mV), summarizing data from 9 separate cells, reveals a voltage sensitivity of approximately 47% per 100 mV. Error bars are ±S.E.M.

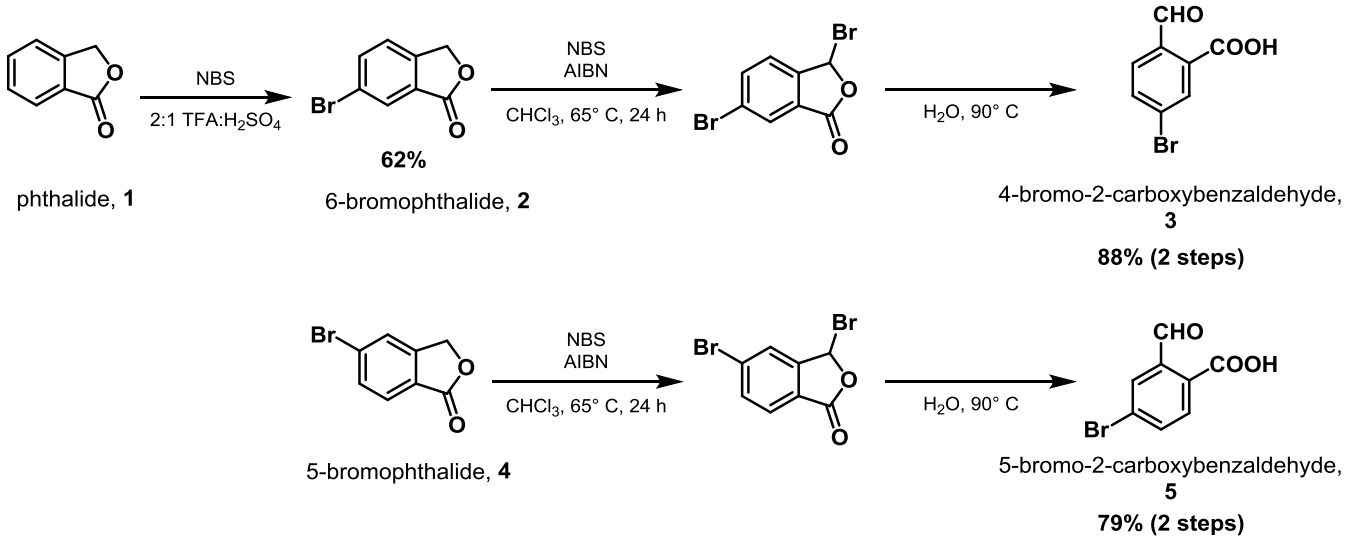


SI Figure 7. TTX treatment abolishes neuronal activity as measured by RhoVR 1. (a) Transmitted light/DIC image of dissociated rat hippocampal neurons stained with RhoVR 1 (500 nM) and imaged at 500 Hz. (b) Regions of interest (ROIs) around the indicated cell bodies were examined before (c) and after (d) addition of TTX (1 μm). The relative change in fluorescence vs. time is plotted for the control (e) and TTX-treated neurons (f). TTX treatment causes a clear loss of activity, as measured by RhOVR 1 fluorescence. Image scale bar is 20 μm.

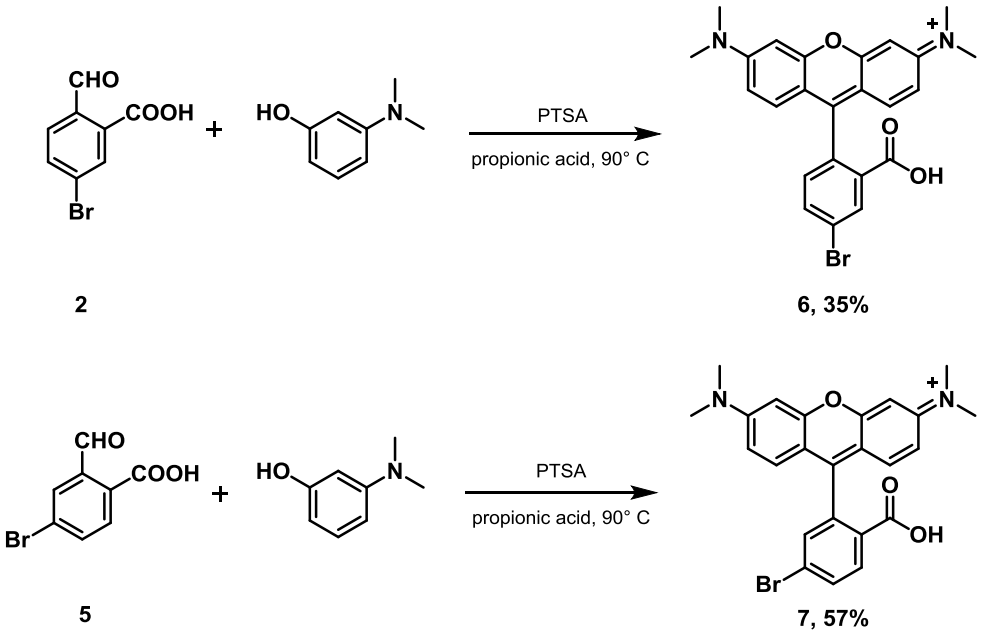


SI Figure 8. RhoVR 1 and eGFP display minimal excitation and emission cross-talk. a) DIC image of HEK cells expressing eGFP and stained with 500 nM RhoVR 1. b) eGFP and c) RhoVR 1 fluorescence are collected in separate channels. Panel d shows an overlay of the images from panels b and c. HEK cells that are positive for cytosolic eGFP (panel b; GFP+) display similar levels of cytosolic fluorescence in the RhoVR 1 channel (panels c and d). Scale bar is 20 μm. e) Voltage sensitivity of a single GFP+, RhoVR 1-stained HEK cells. f) Plots of ΔF/F vs mV reveal a 45±1.3% ΔF/F per 100 mV, which is comparable to the voltage-sensitivity in the absence of GFP. Error bars are ±S.E.M for n = 5 cells.

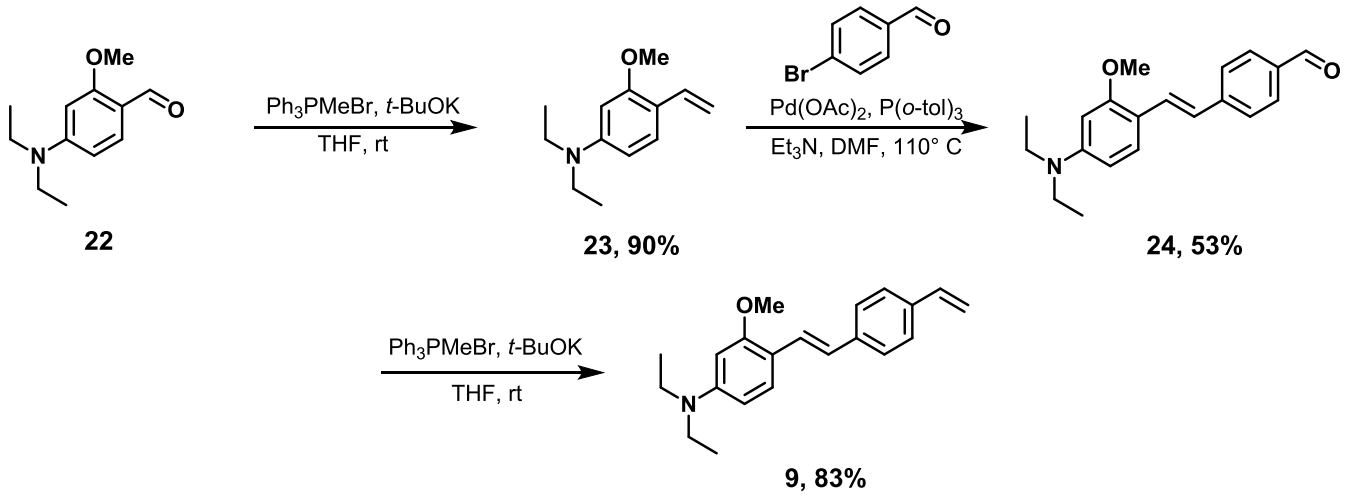
SI Scheme 1. Synthesis of 2-carboxybenzaldehydes.



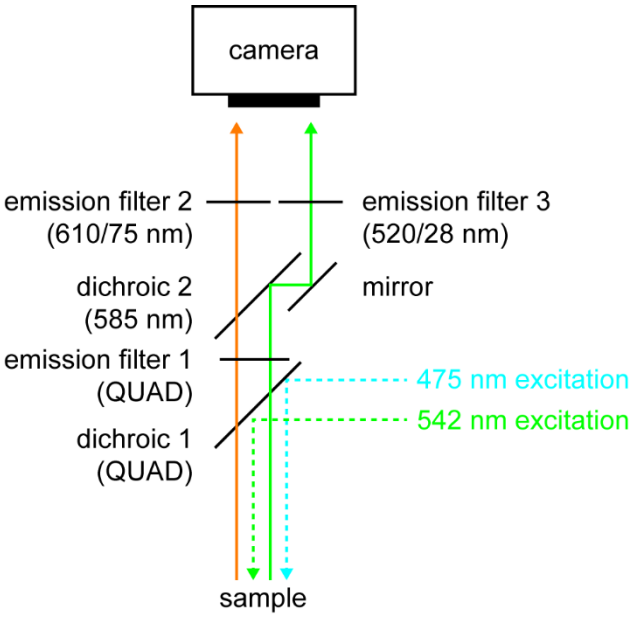
SI Scheme 2. Synthesis of 4'- and 5'-Br-TMR.



Scheme 3. Synthesis of styrene 9.

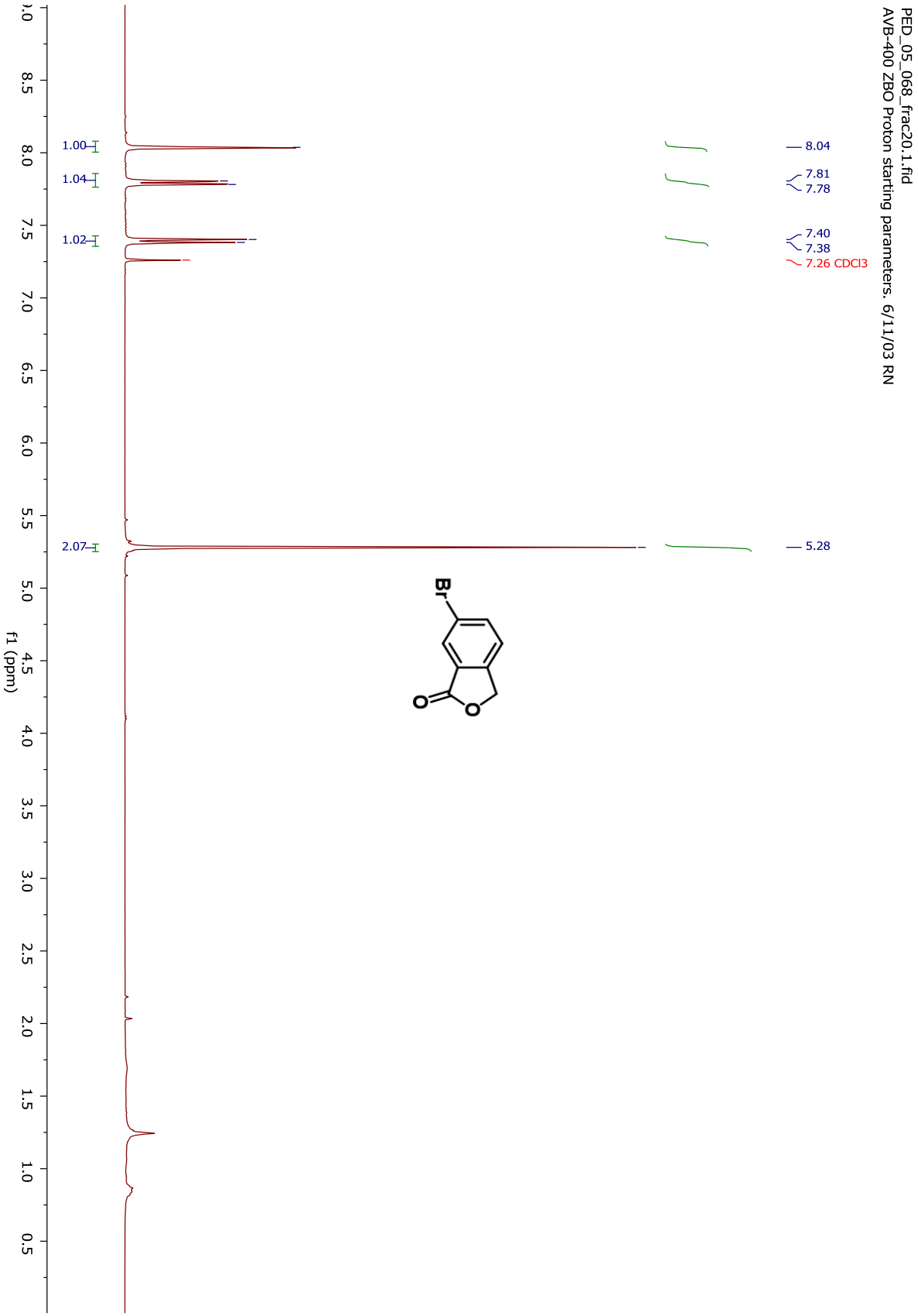


SI Scheme 4. Optical path for two-color voltage and Ca^{2+} imaging.

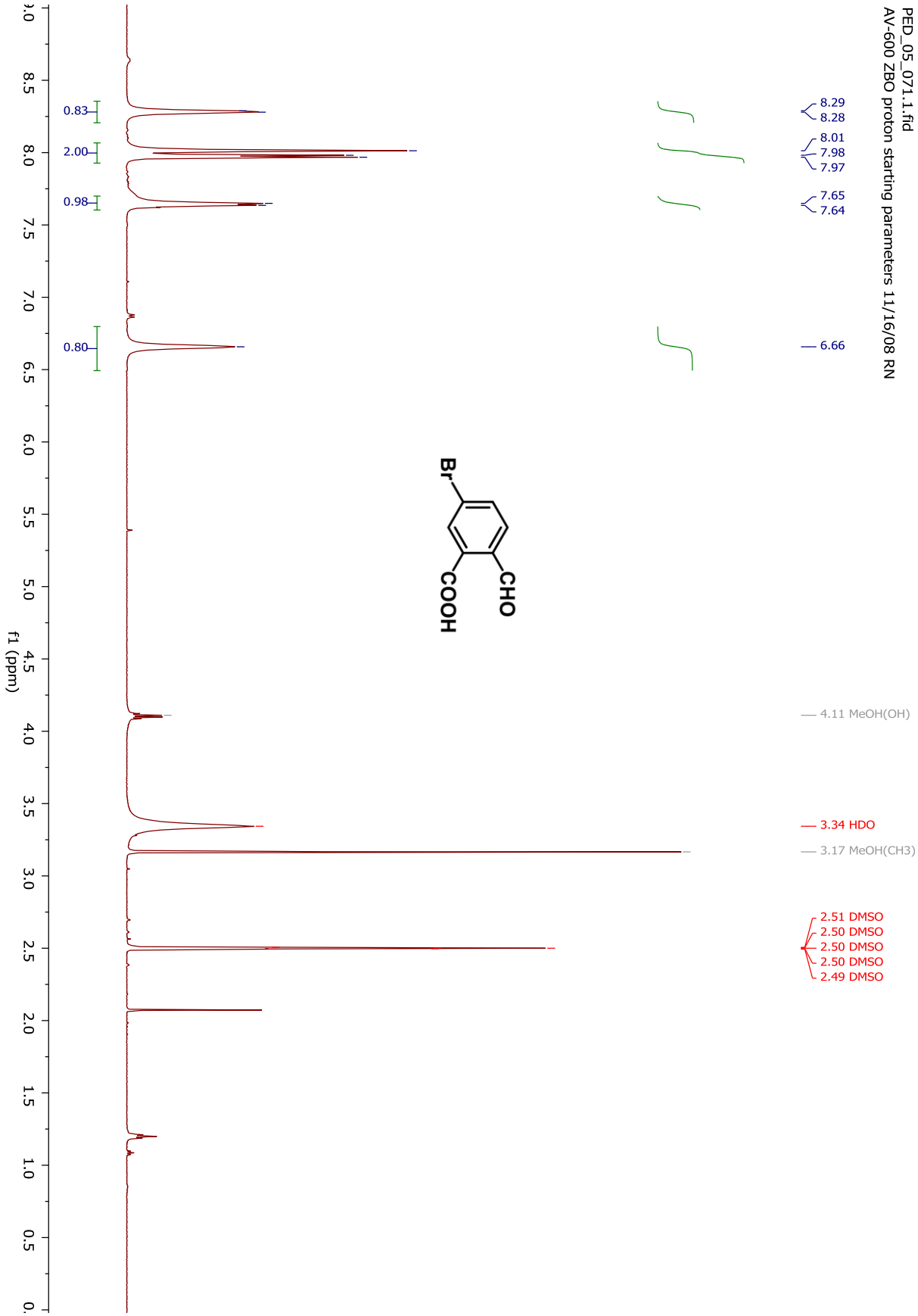


Cyan excitation: 475/34. Green excitation: 542/33. Dichroic 1: "QUAD", 432/38, 509/22, 586/40, 654LP. Emission filter 1: 430/32, 508/14, 586/30, 708/98. Dichroic 2 (DualView): 585LP. Emission filter 2 (DualView): 610/75 or 593/40. Emission filter 3 (DualView): 520/28. All values in nm.

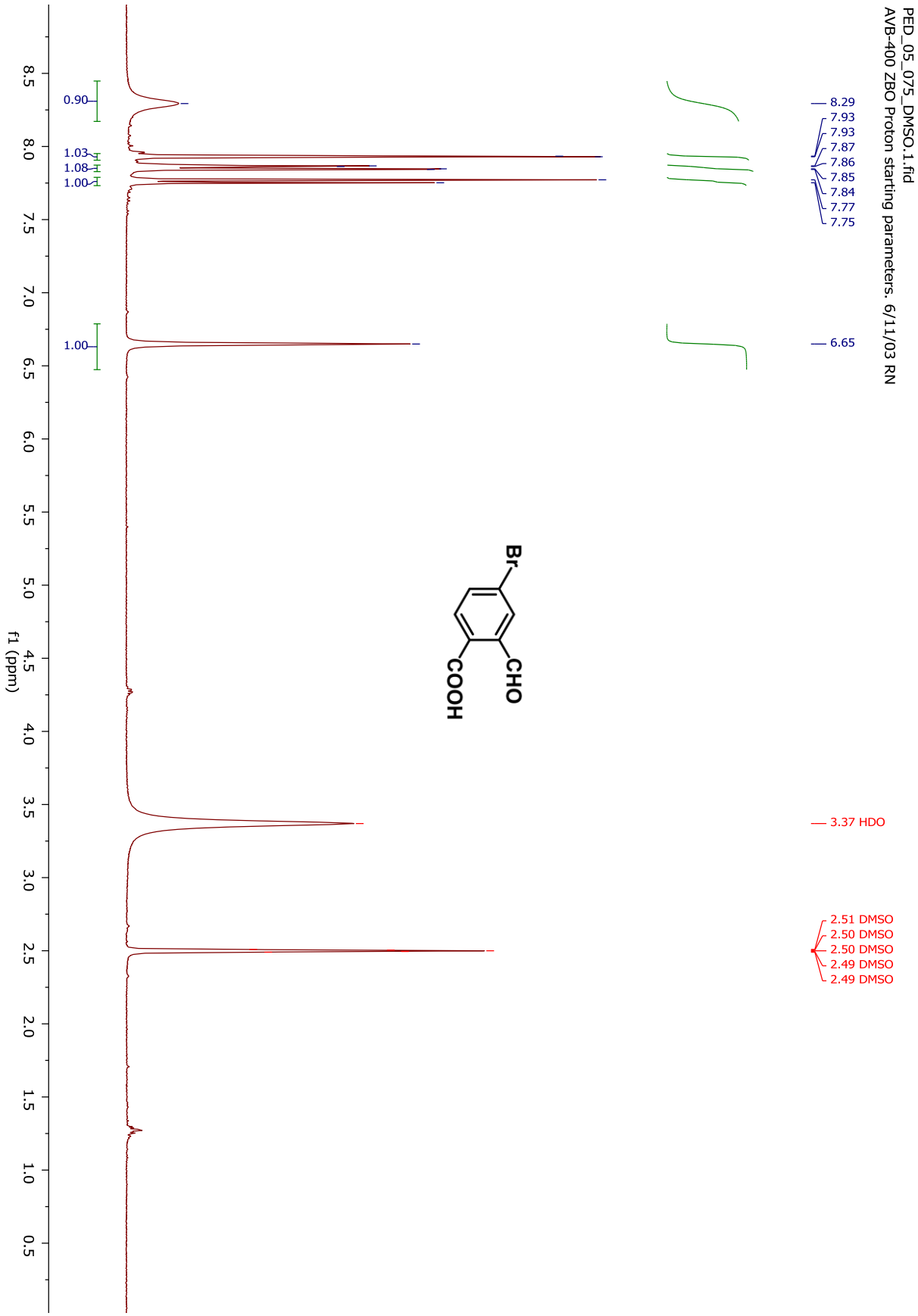
10. Spectra



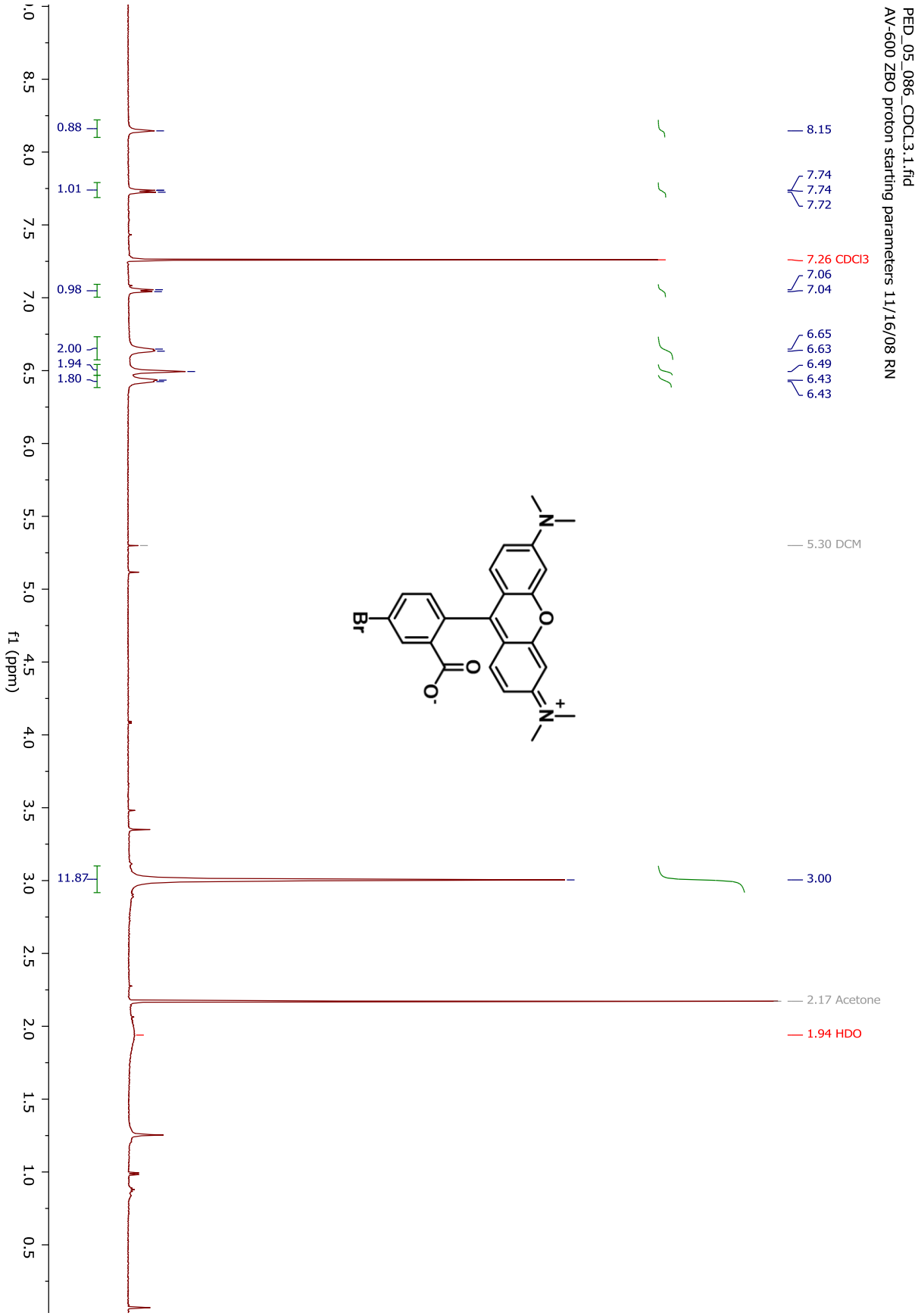
SI Spectrum 1. ^{1}H NMR of bromophthalide **2**



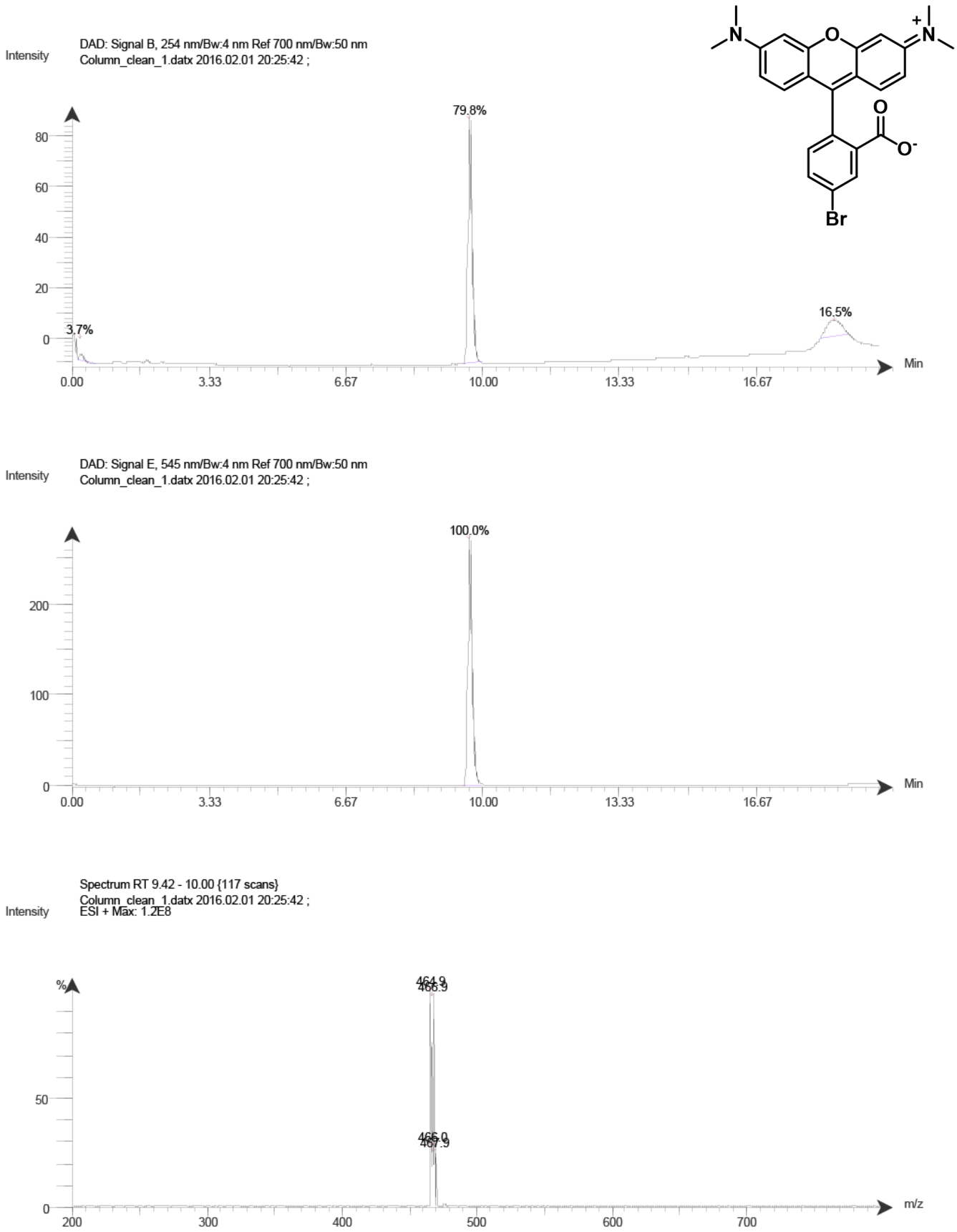
SI Spectrum 2. ^{1}H NMR of 5-bromo-2-formylbenzoic acid, **3**



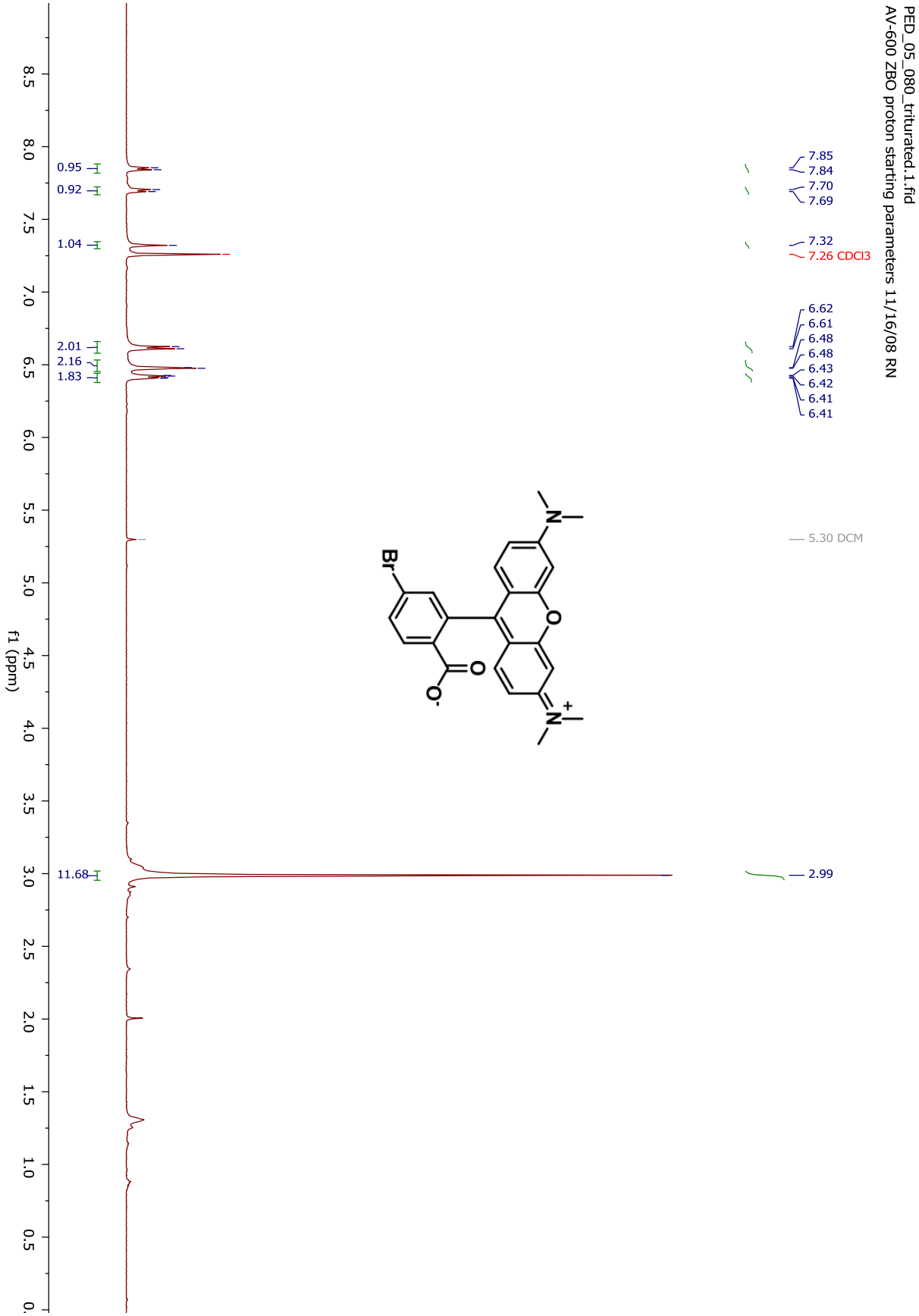
SI Spectrum 3. ^{1}H NMR of 4-bromo-2-formylbenzoic acid, **5**



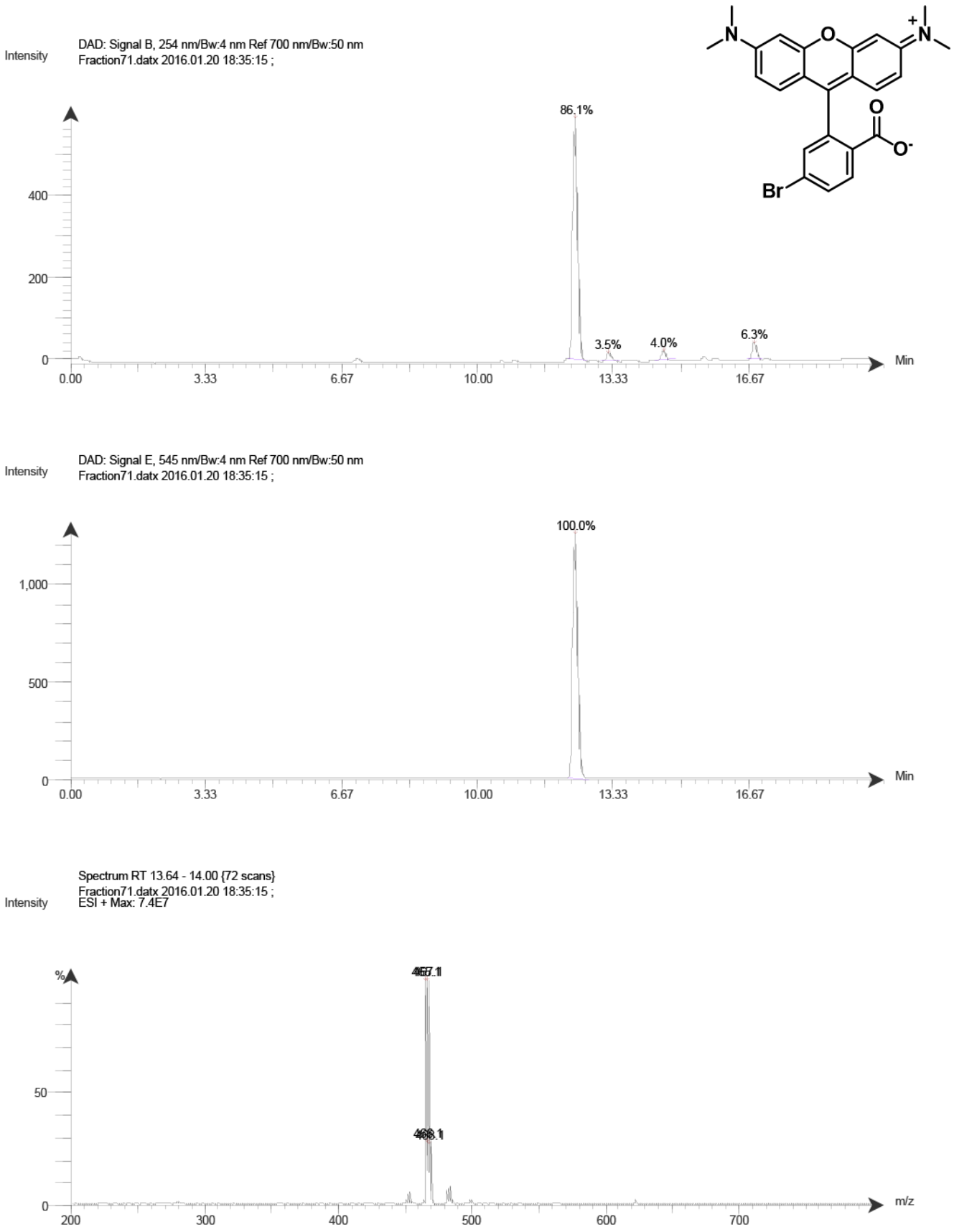
SI Spectrum 4. ^{1}H NMR of **6**



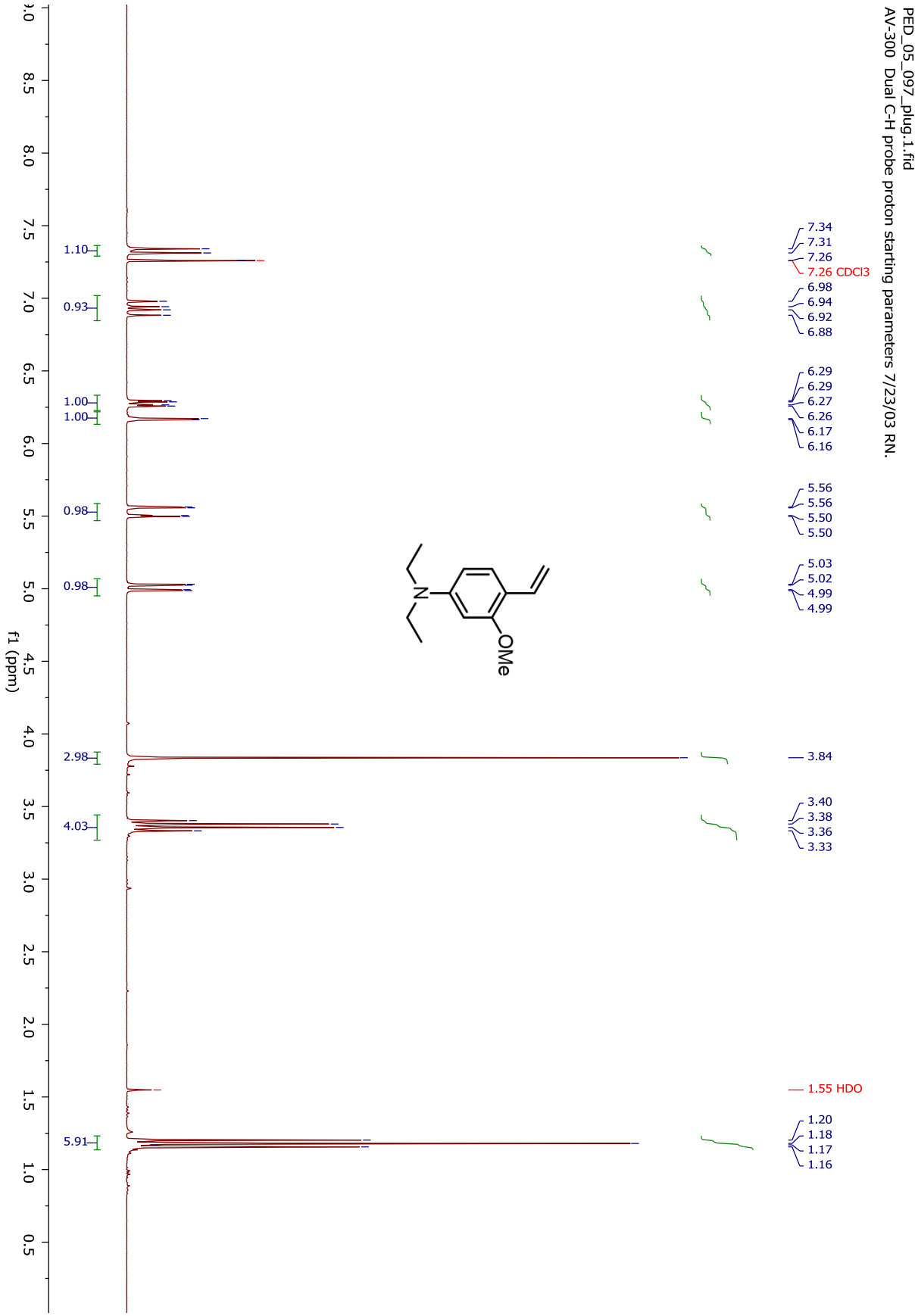
SI Spectrum 5. LC-MS of **6**



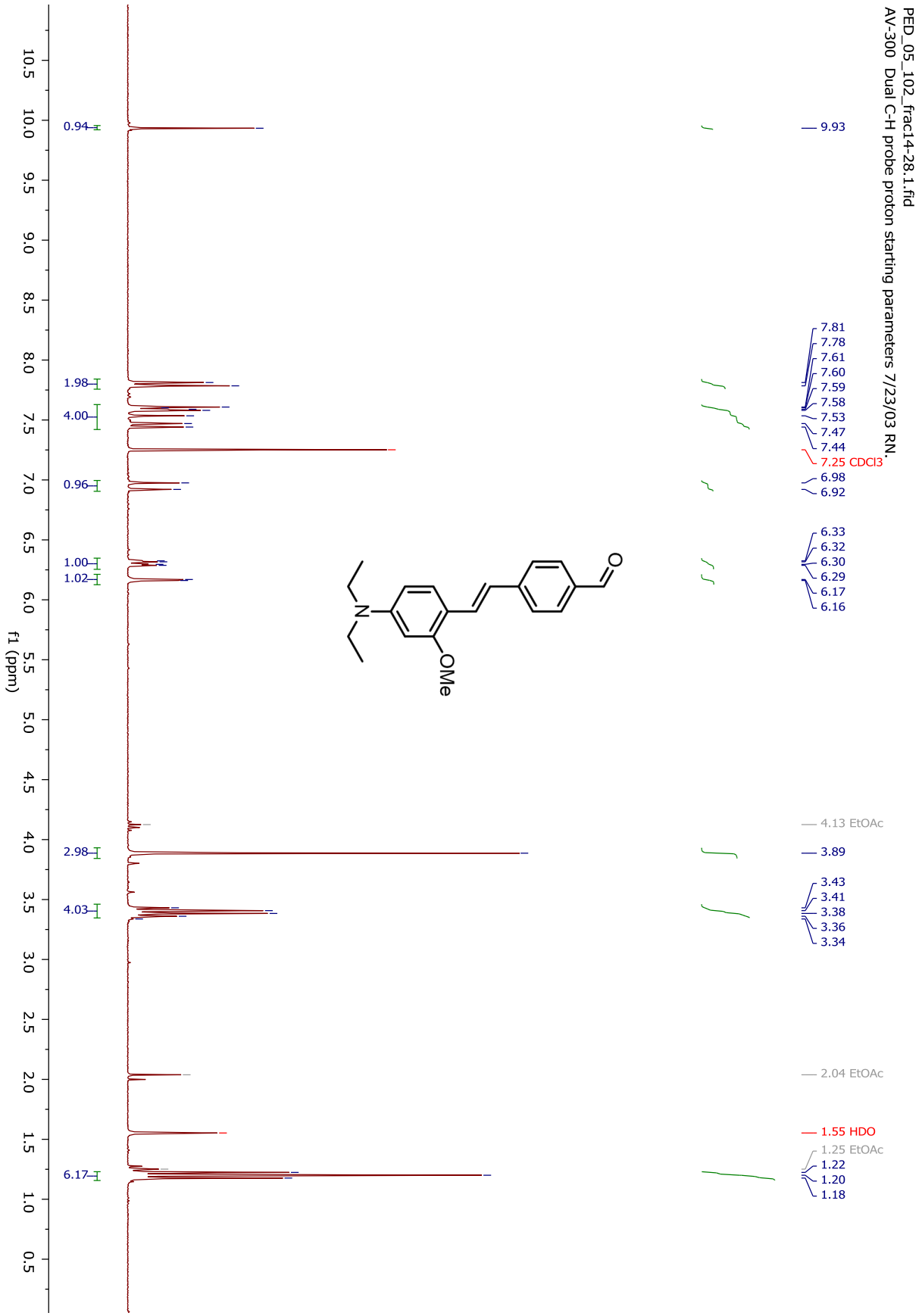
SI Spectrum 6. ^{1}H NMR of **7**



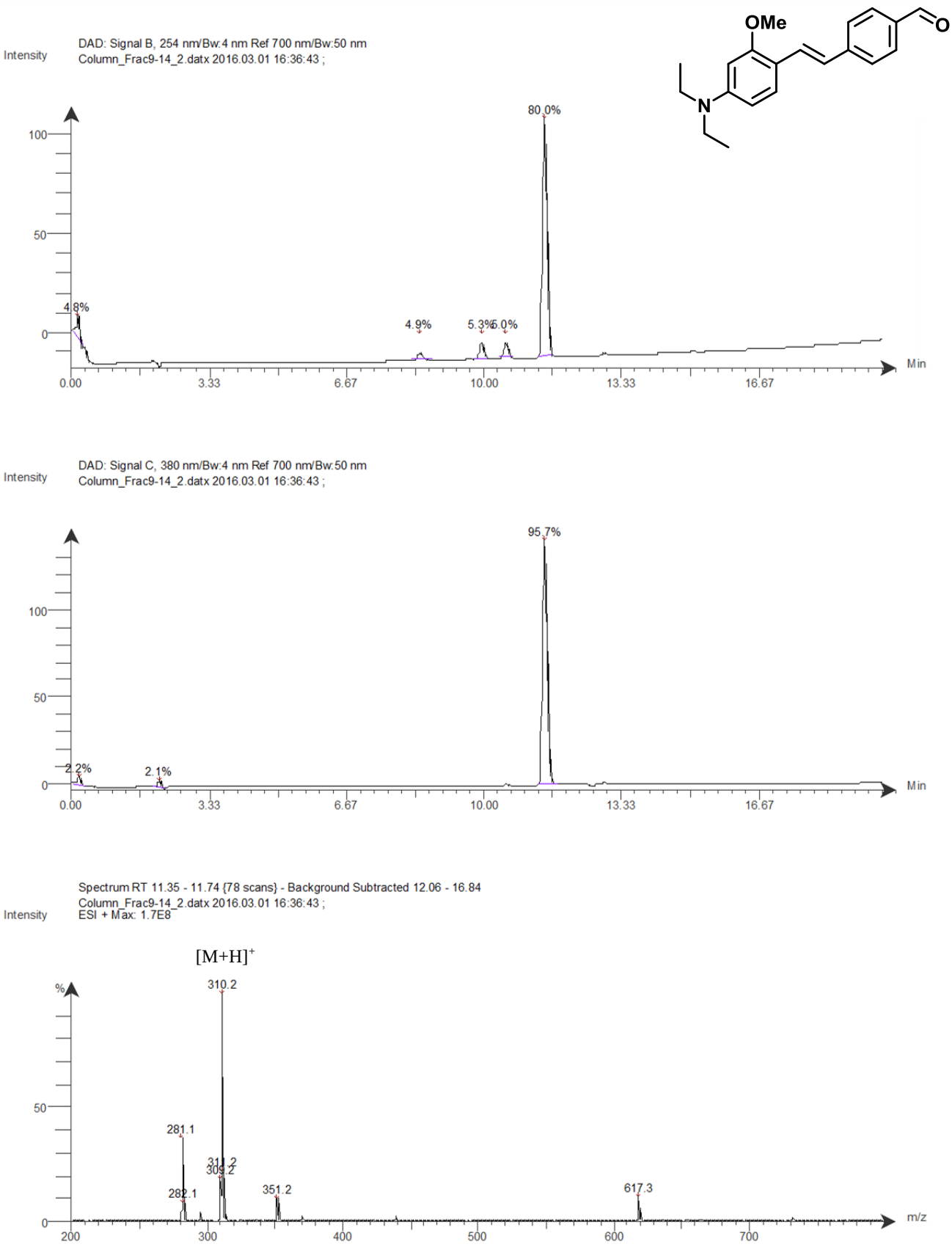
SI Spectrum 7. LC-MS of **7**



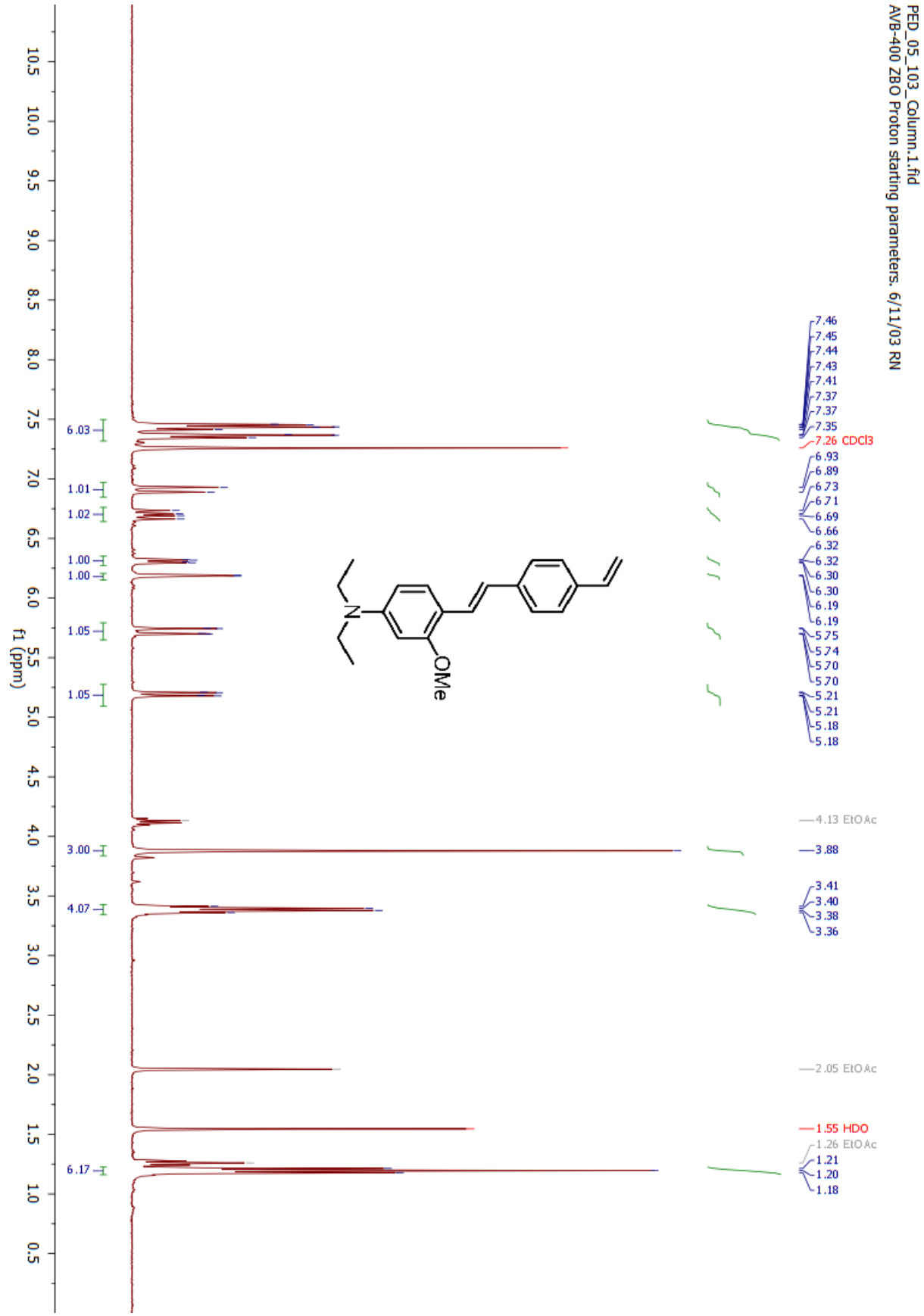
SI Spectrum 8. ^{1}H NMR of **23**



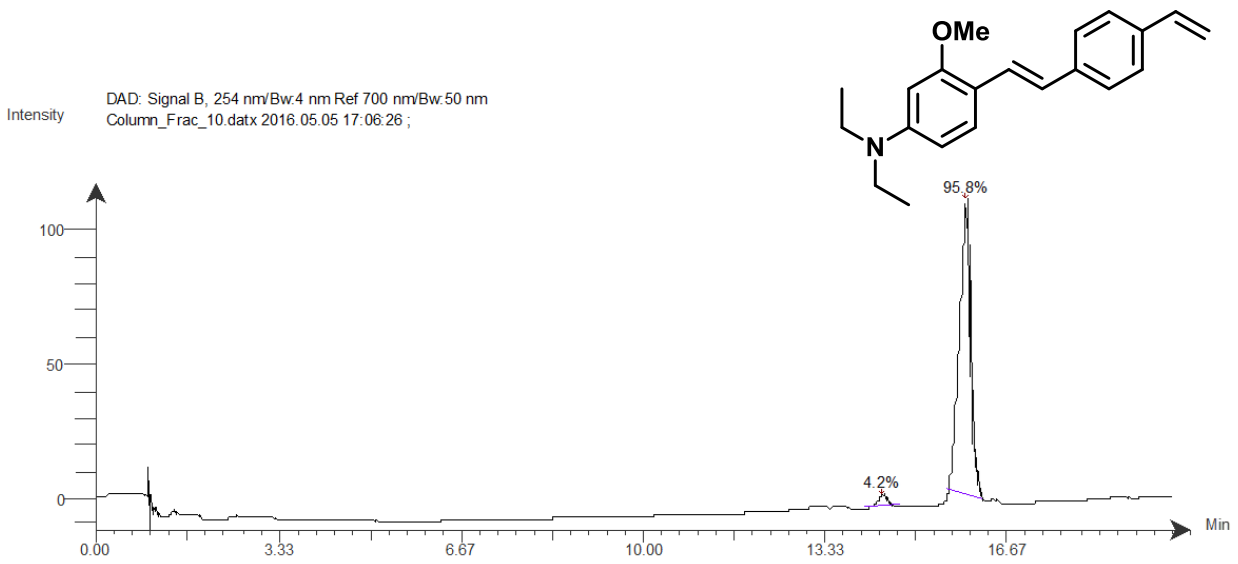
SI Spectrum 9. ^{1}H NMR of **24**

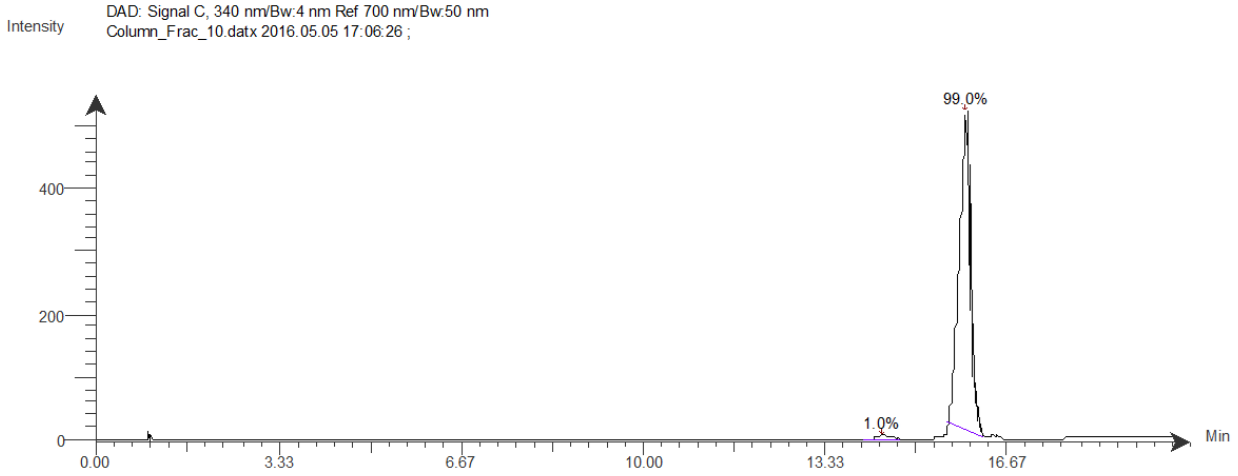


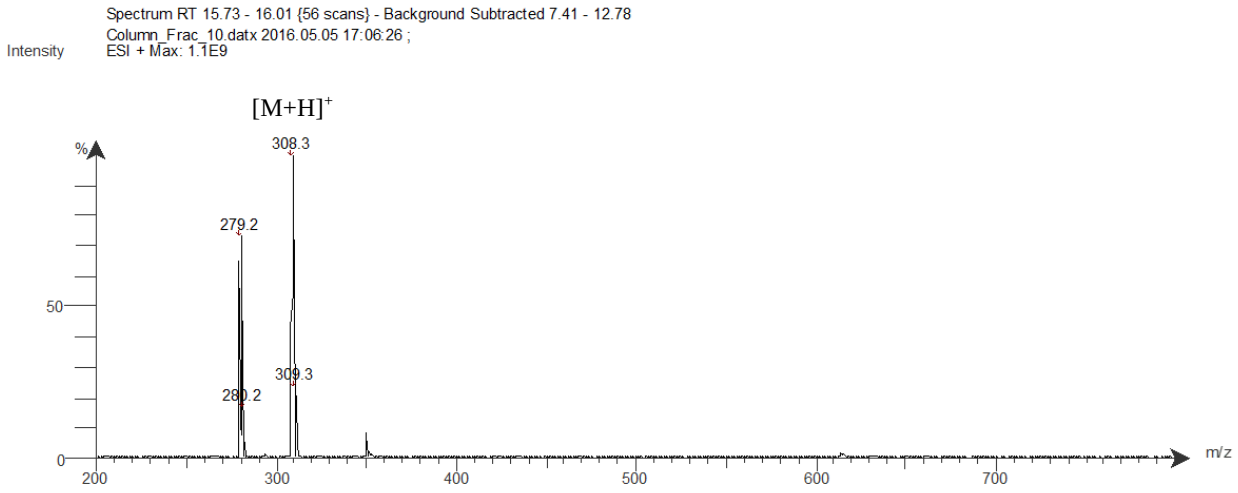
SI Spectrum 10. LC-MS of **24**



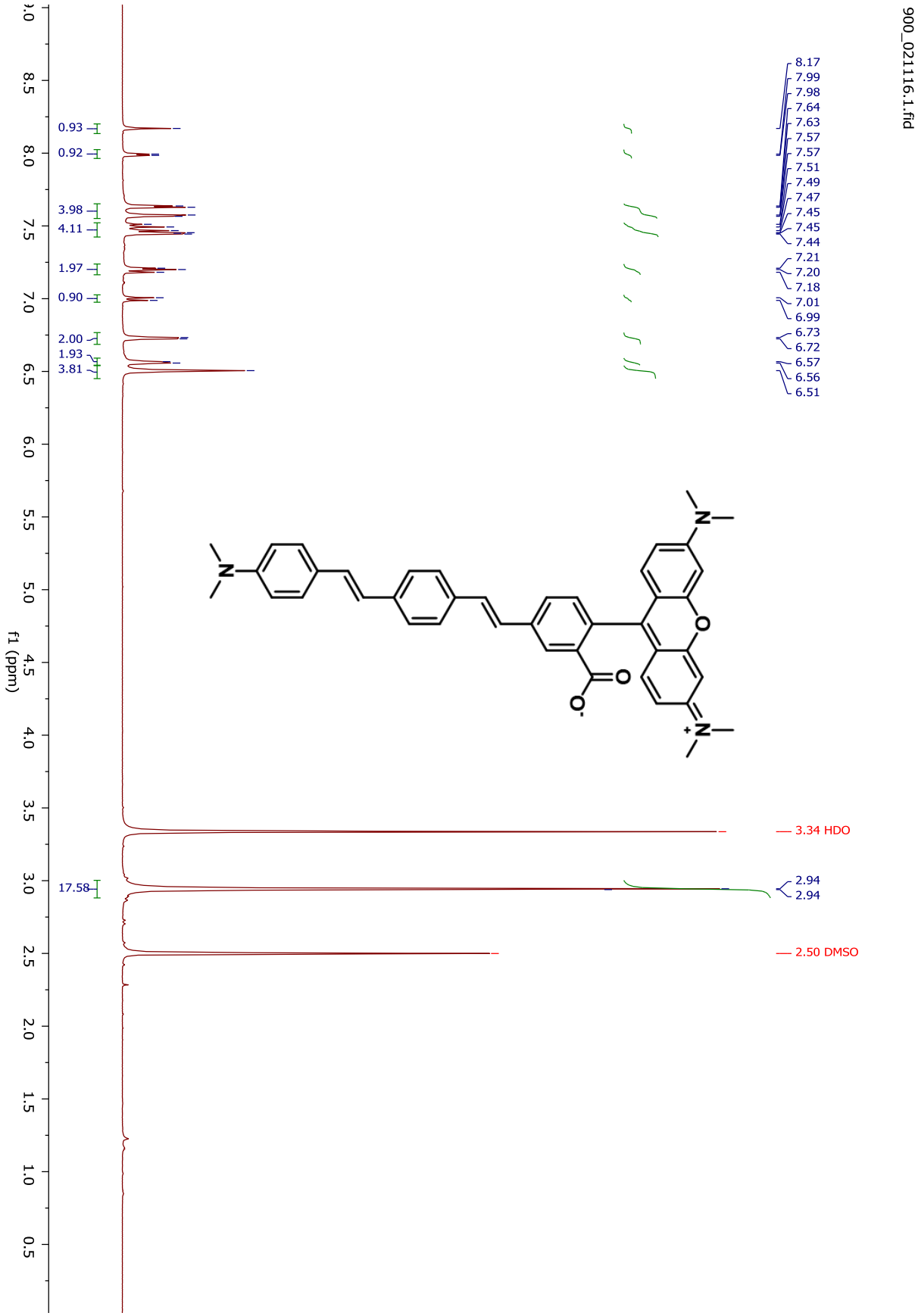
SI Spectrum 11. ^{1}H NMR of **9**



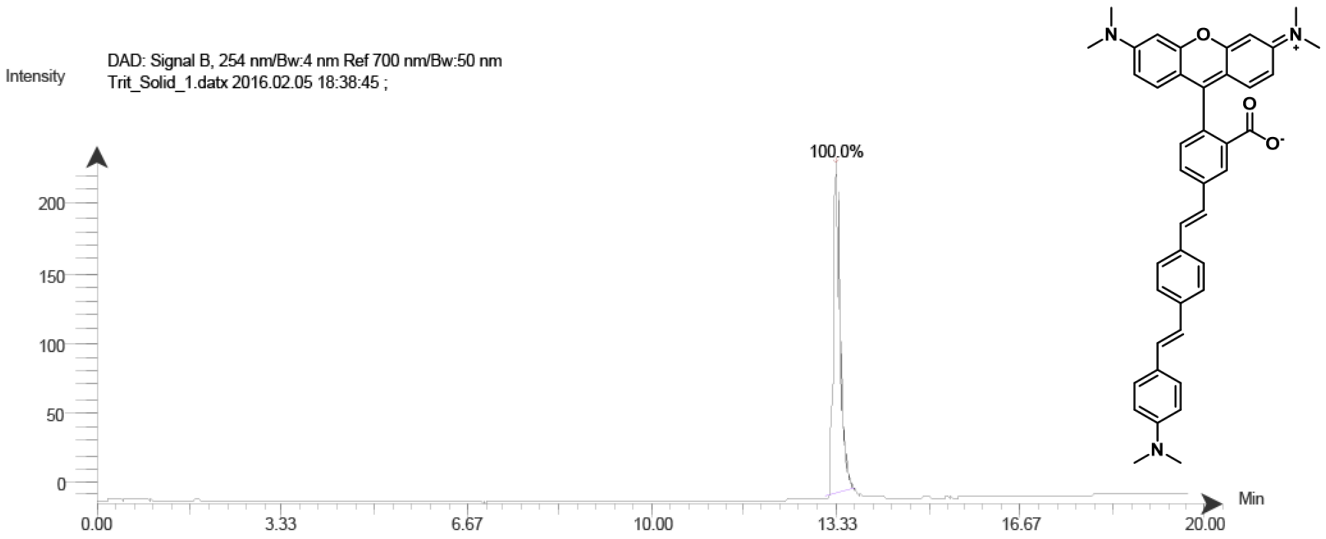


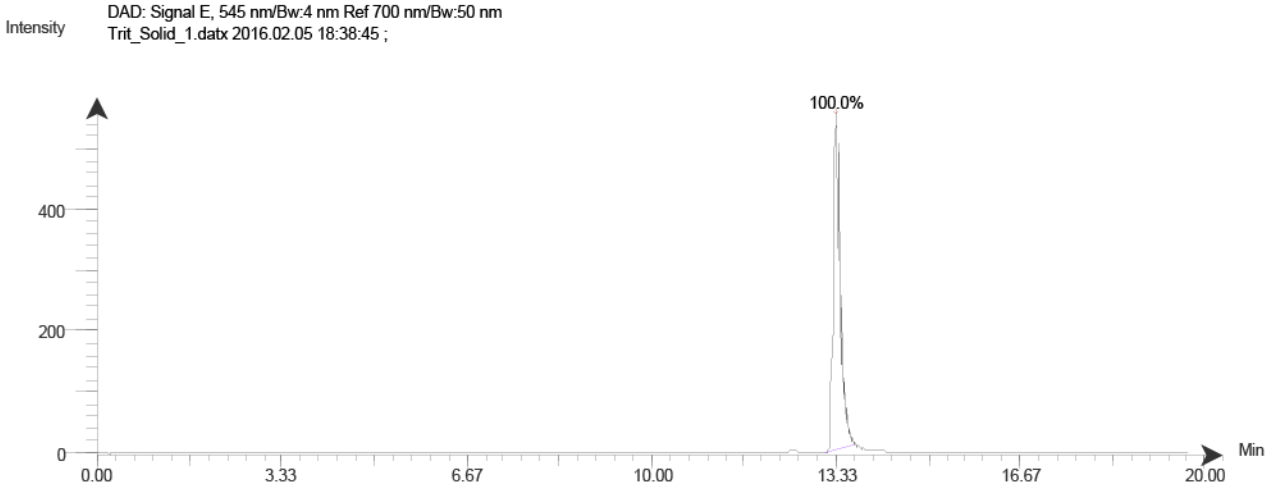


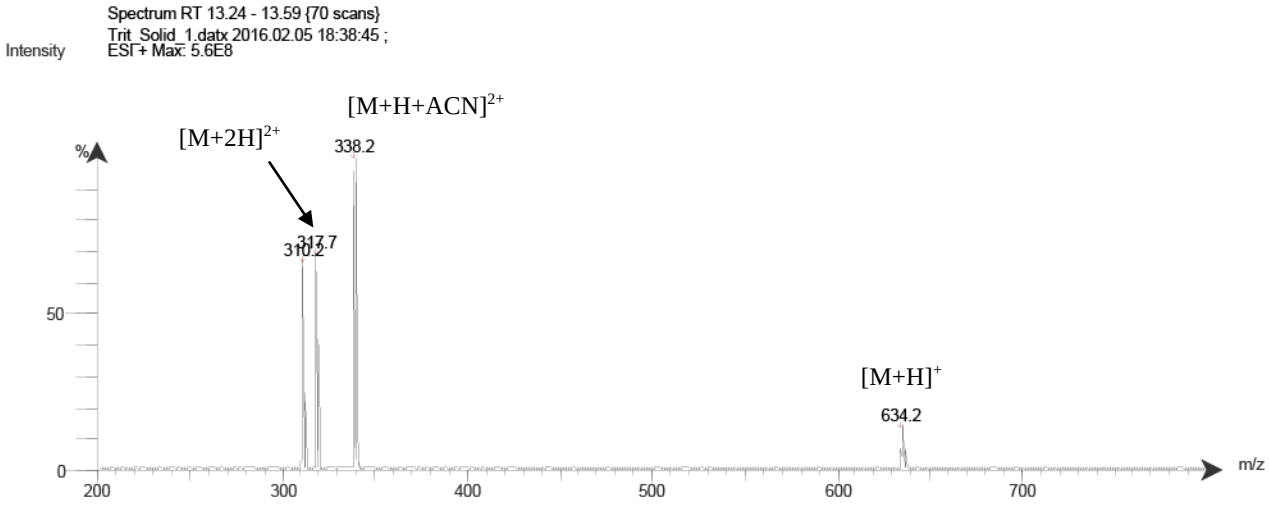
SI Spectrum 12. LC-MS of **9**



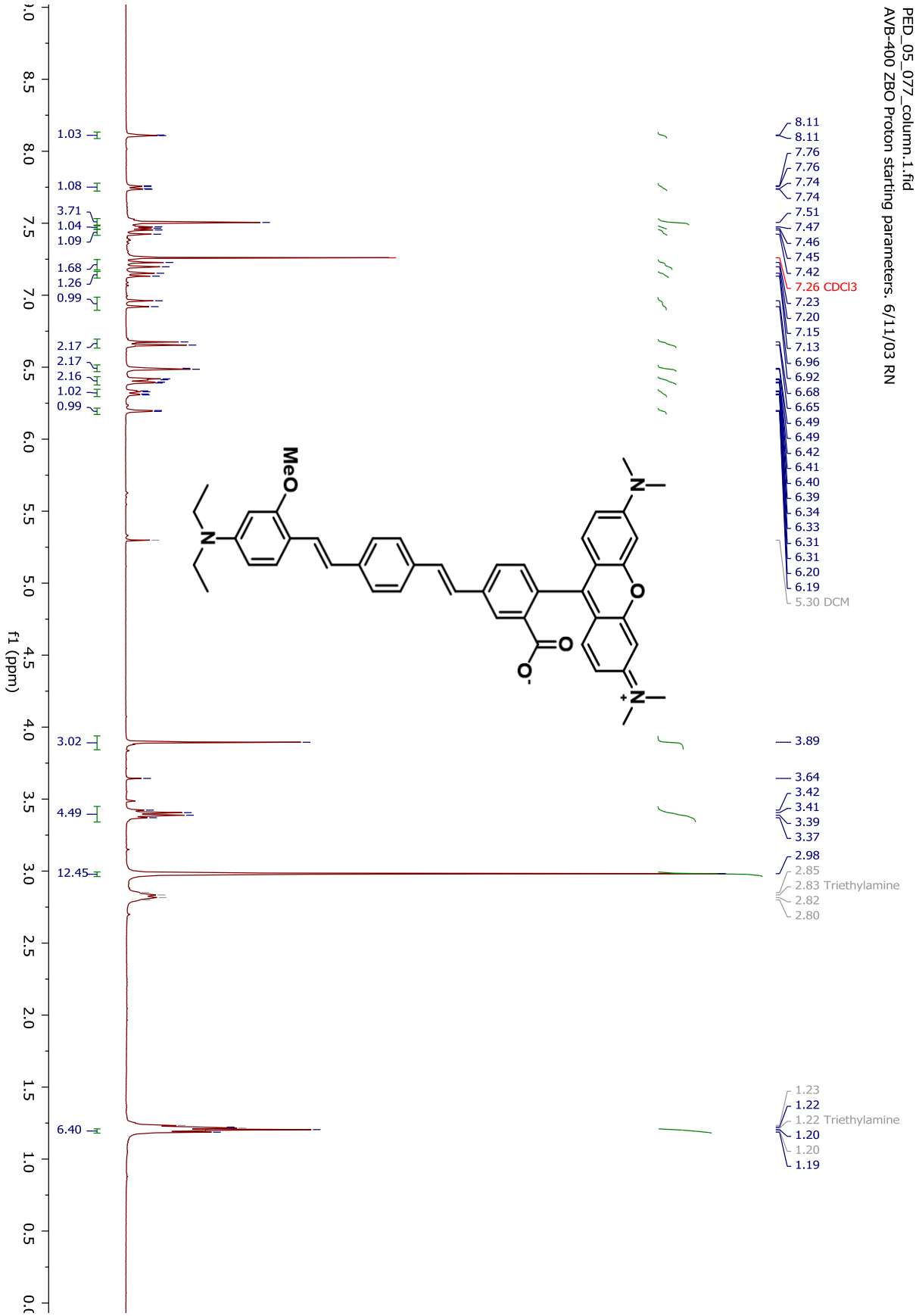
SI Spectrum 13. ^{1}H NMR of **10**



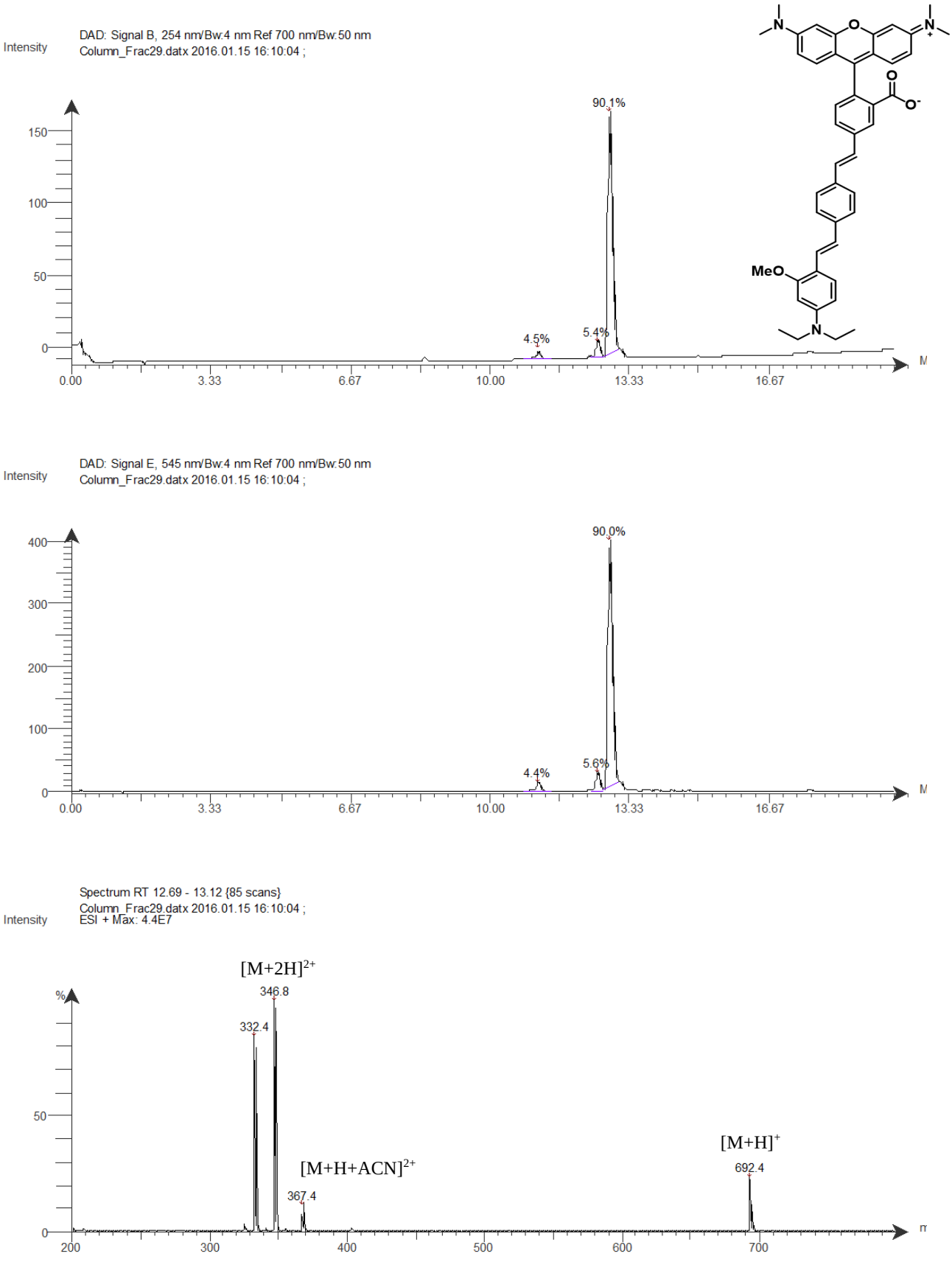




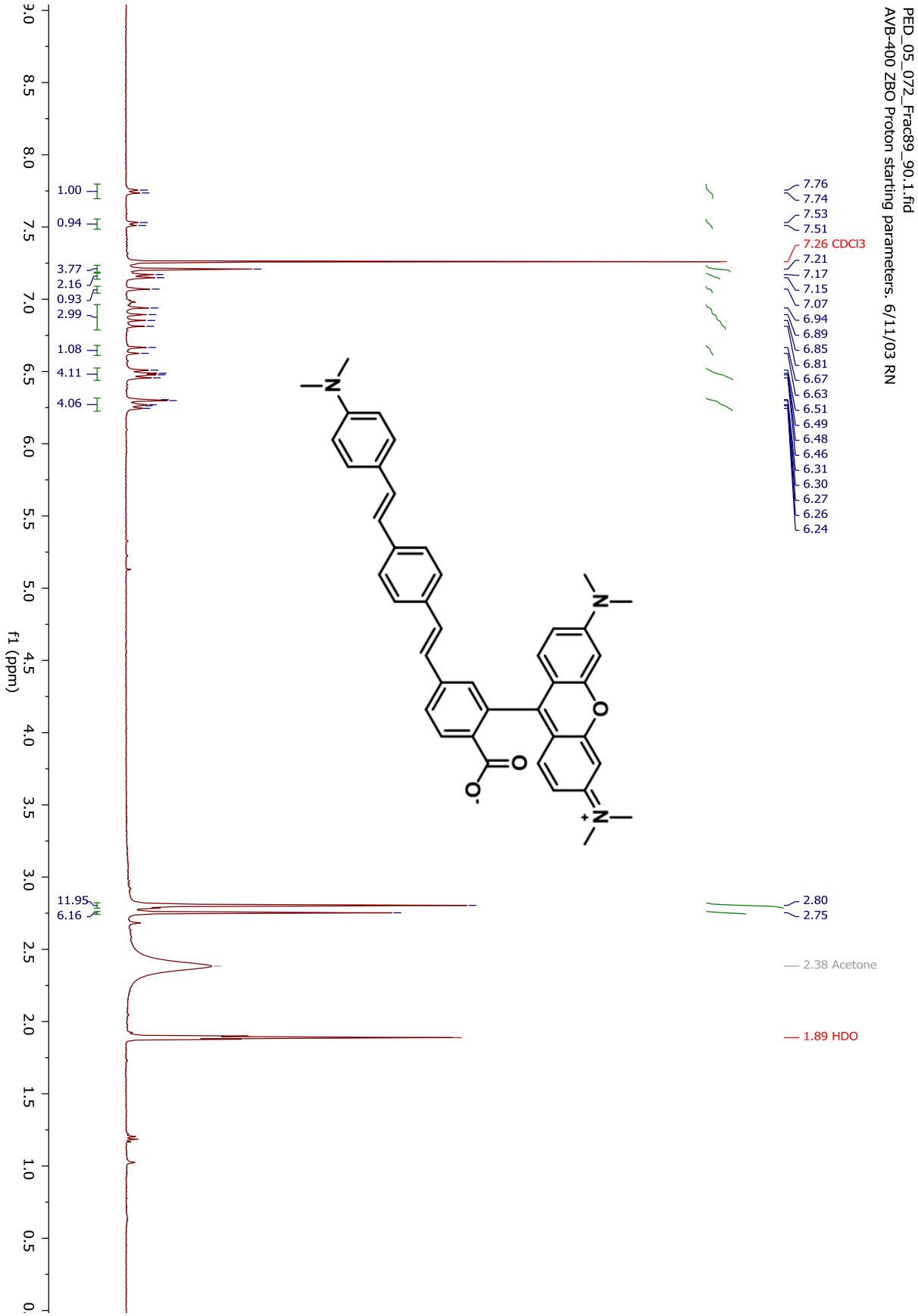
SI Spectrum 14. LC-MS of **10**



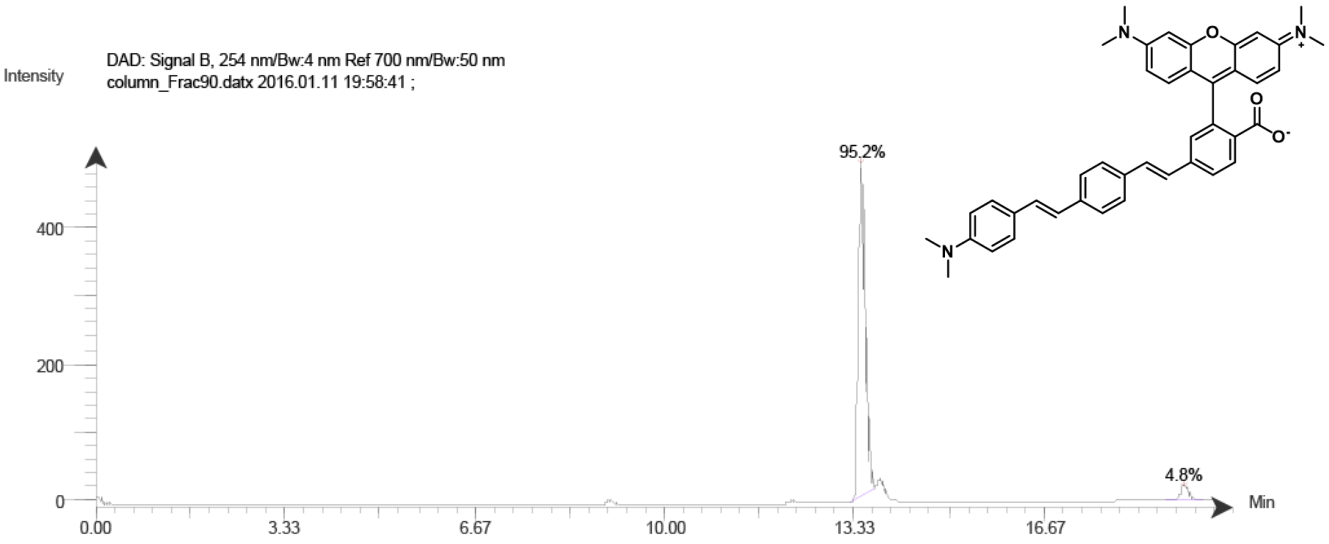
SI Spectrum 15. ^{1}H NMR of **11**

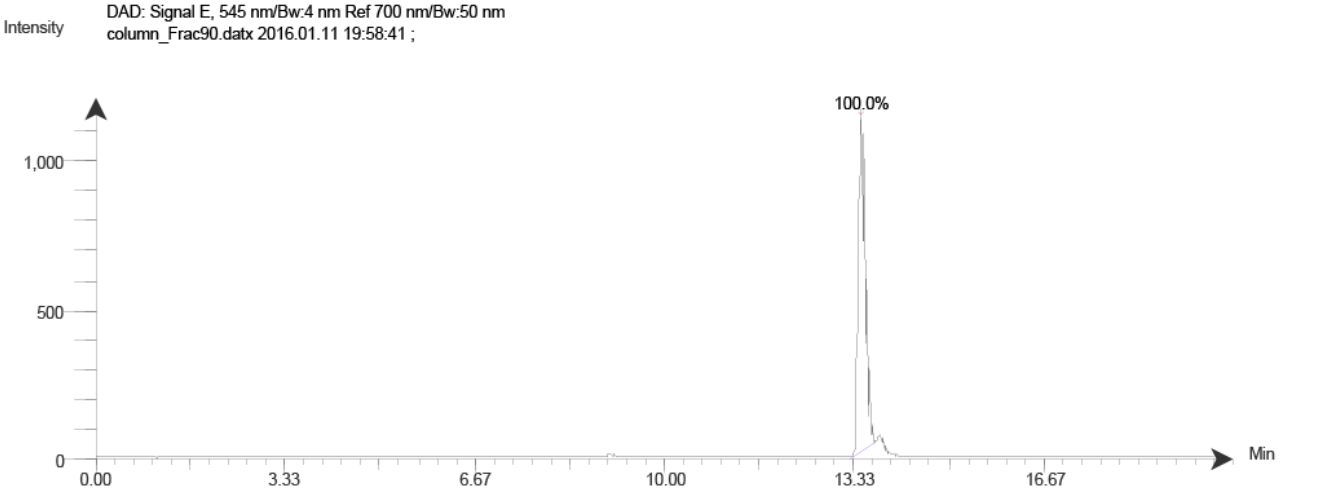


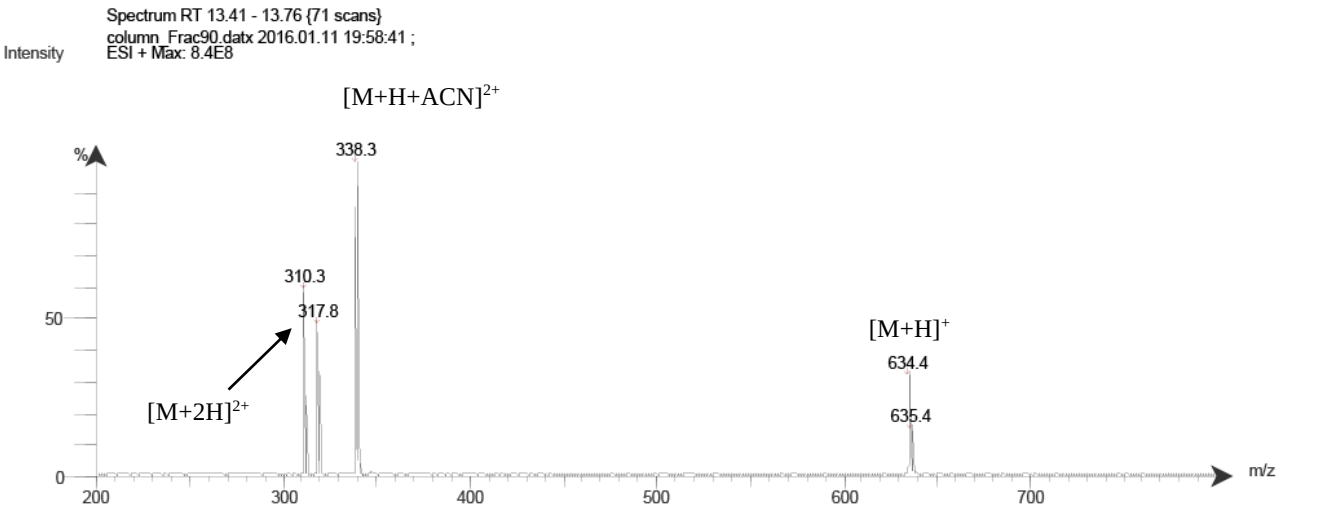
SI Spectrum 16. LC-MS of **11**



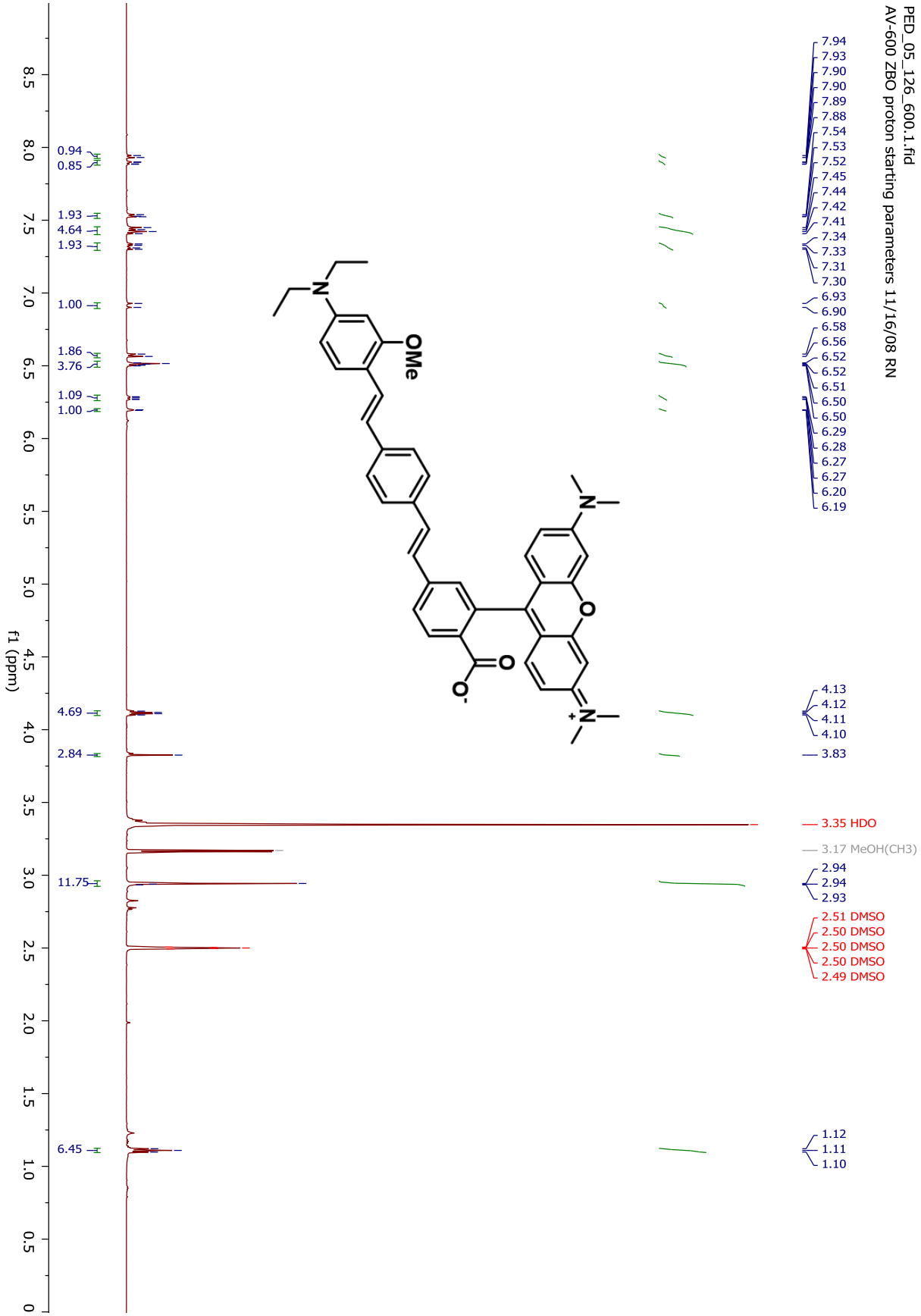
SI Spectrum 17. ^{1}H NMR of **12**



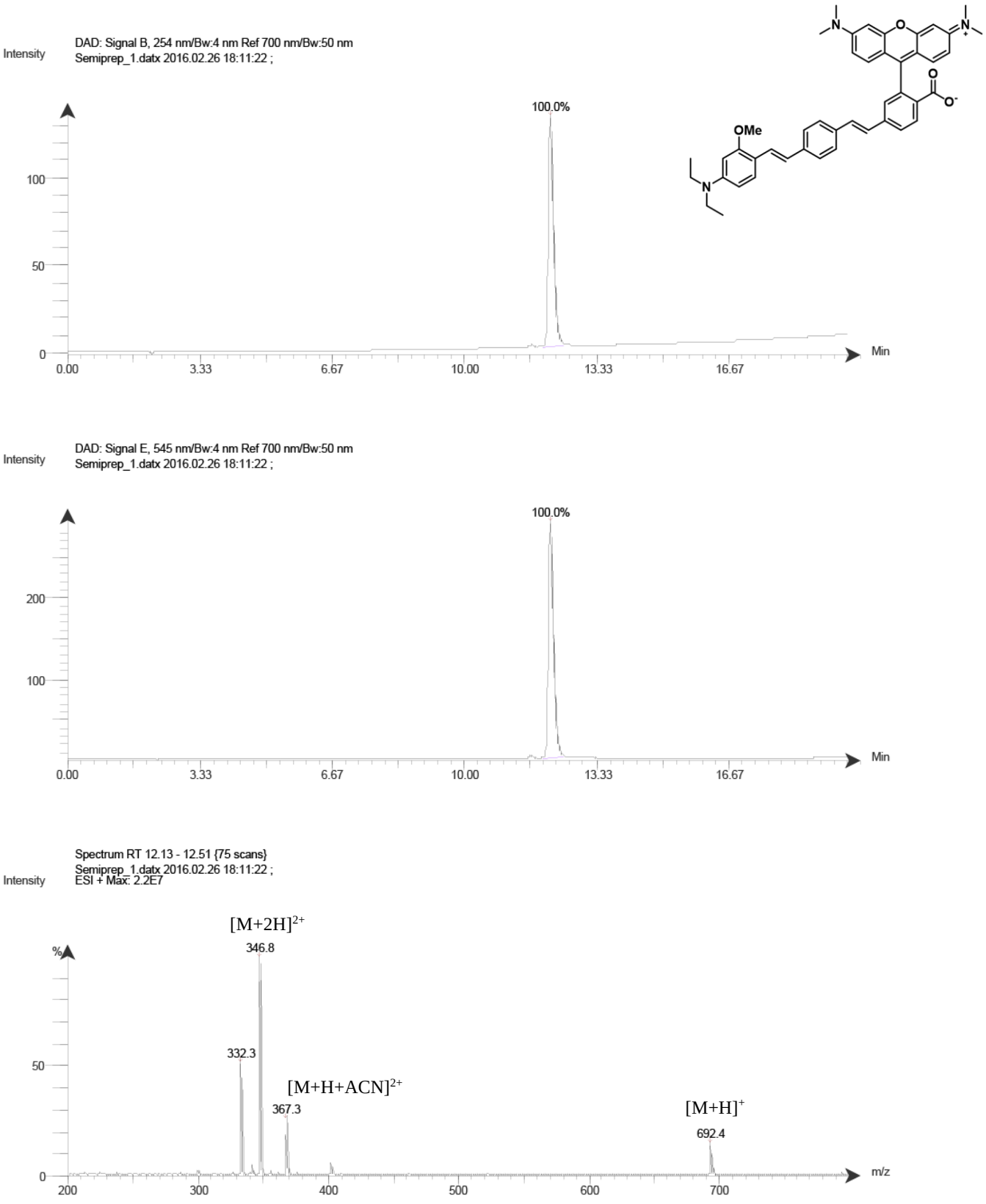




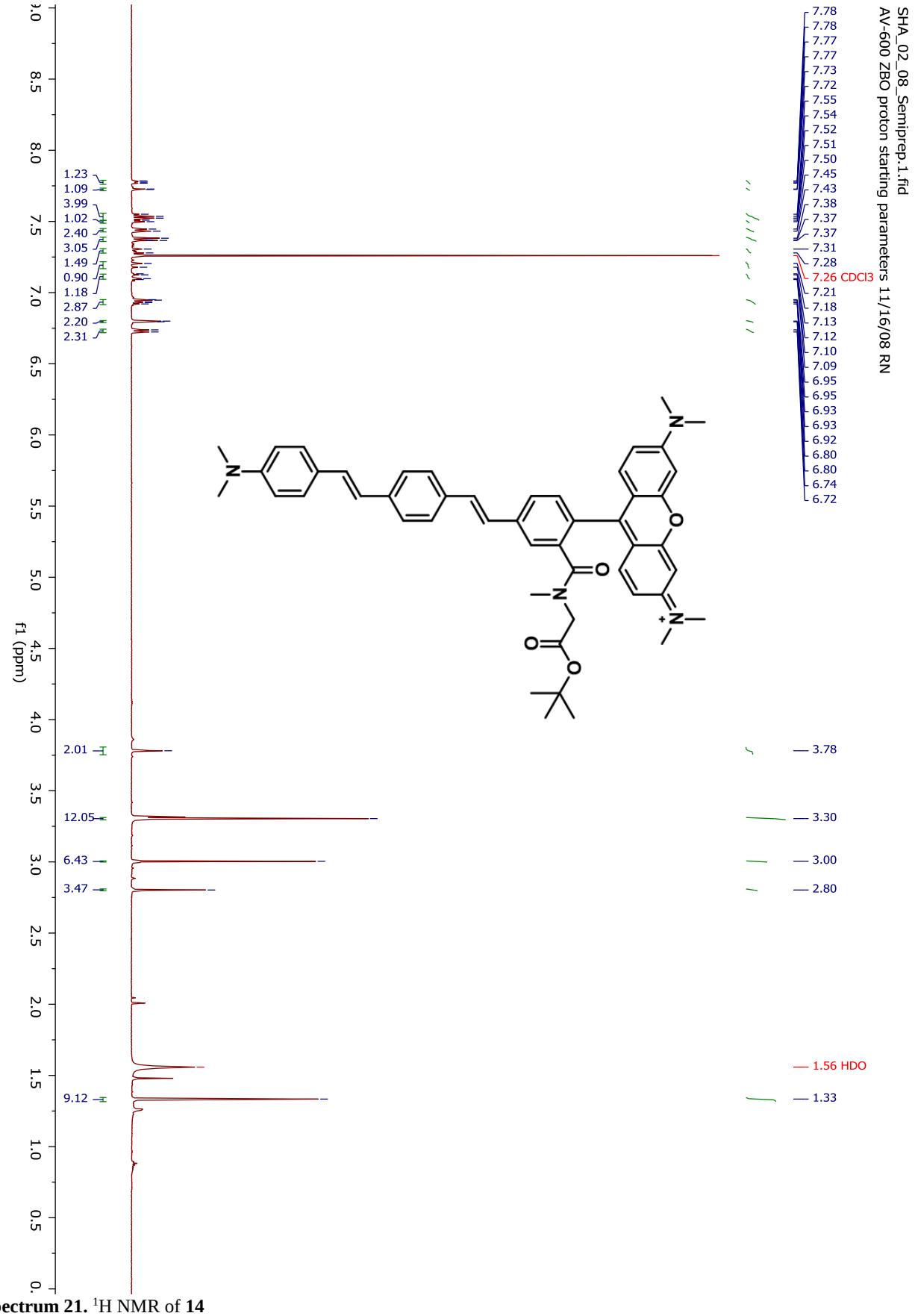
SI Spectrum 18. LC-MS of **12**



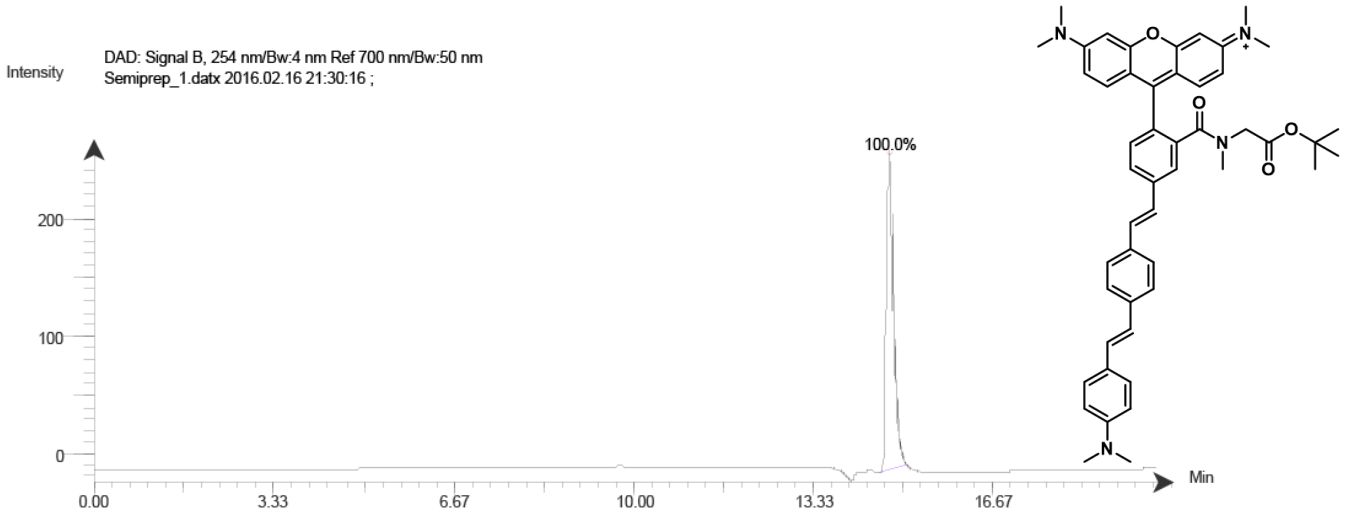
SI Spectrum 19. ^{1}H NMR of **13**

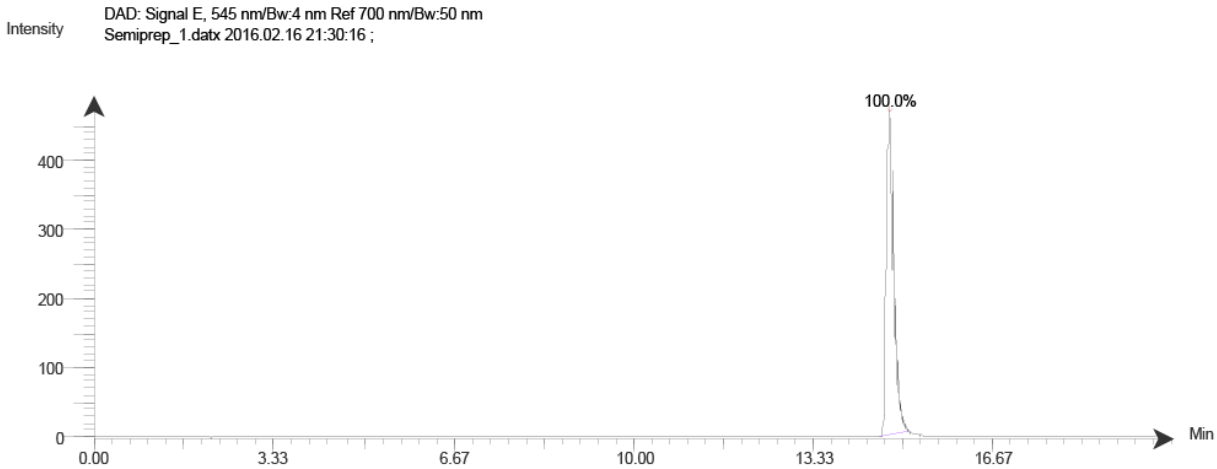


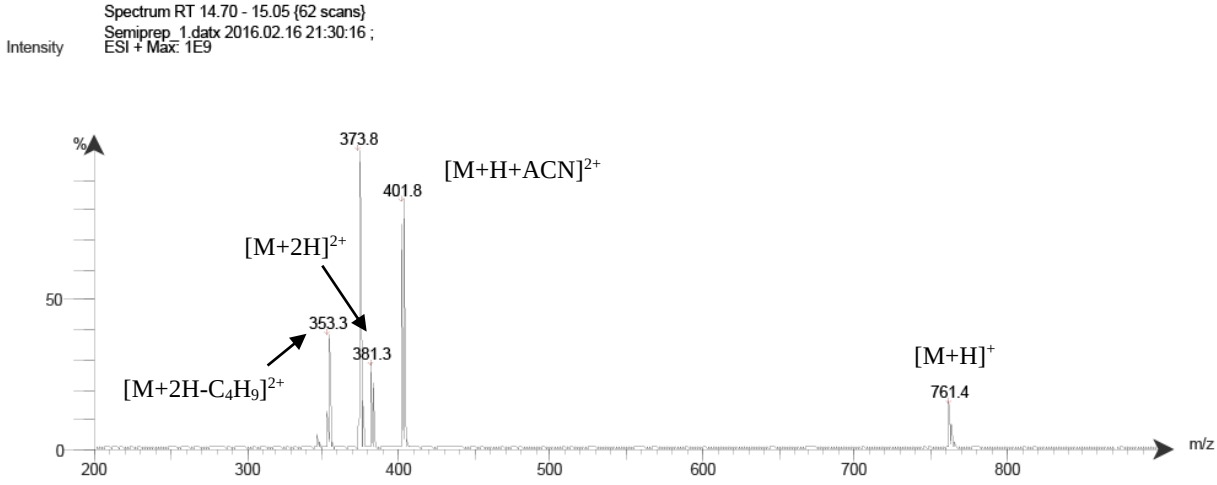
SI Spectrum 20. LC-MS of **13**



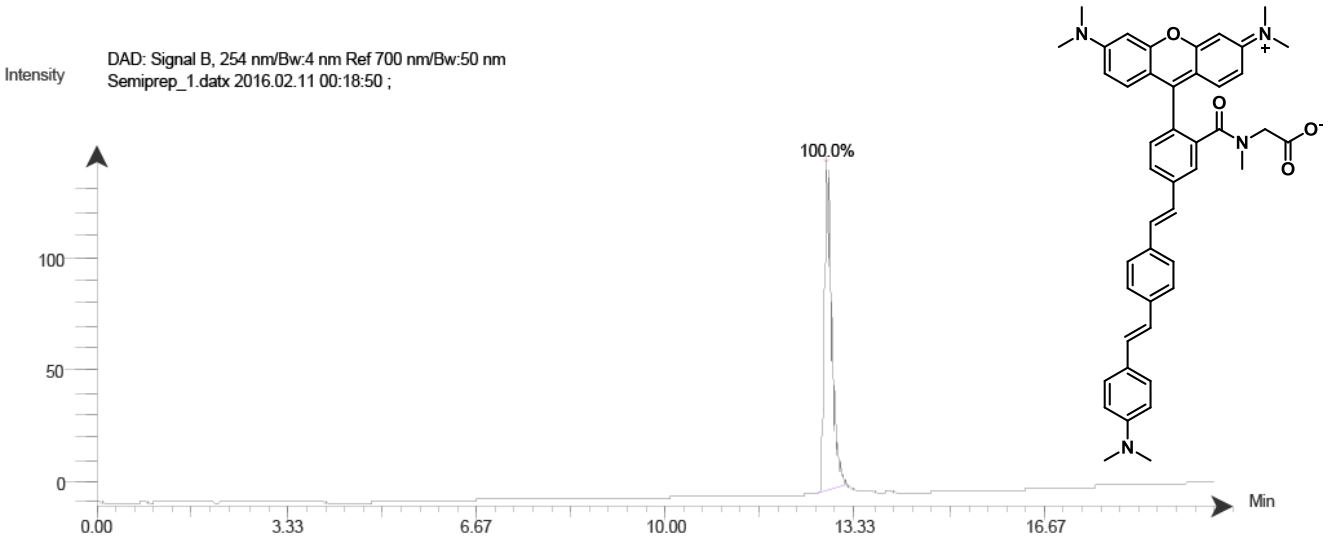
SI Spectrum 21. ^{1}H NMR of **14**

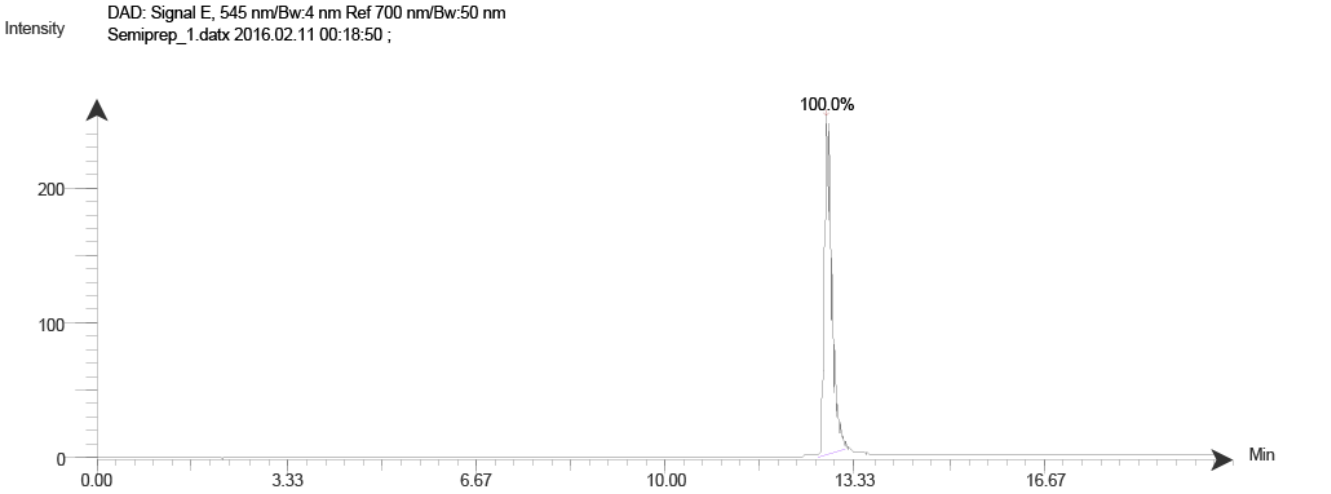


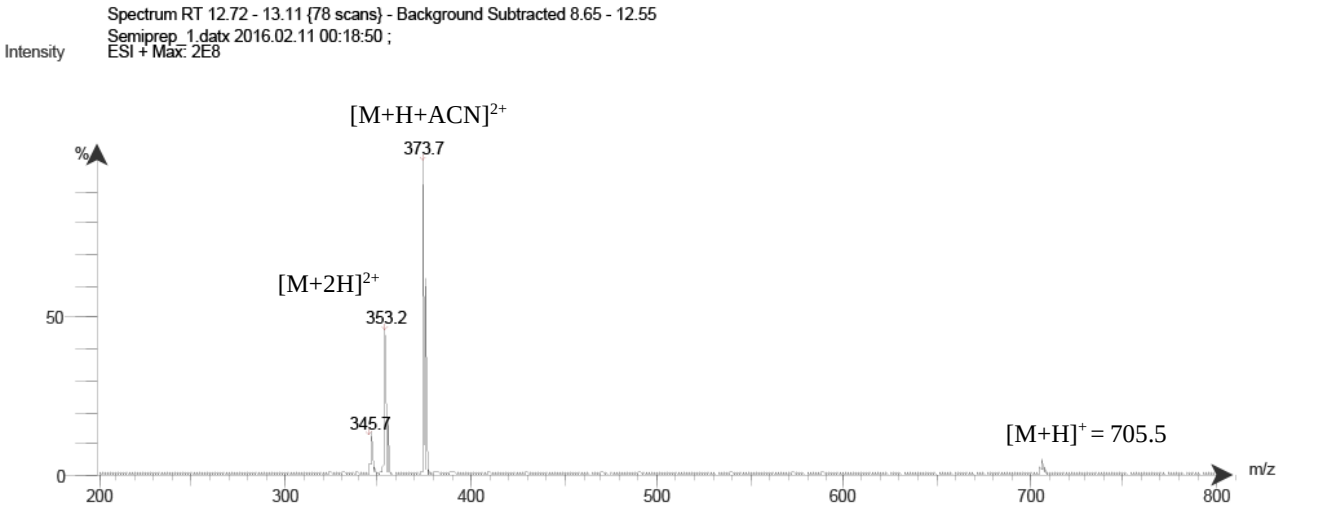




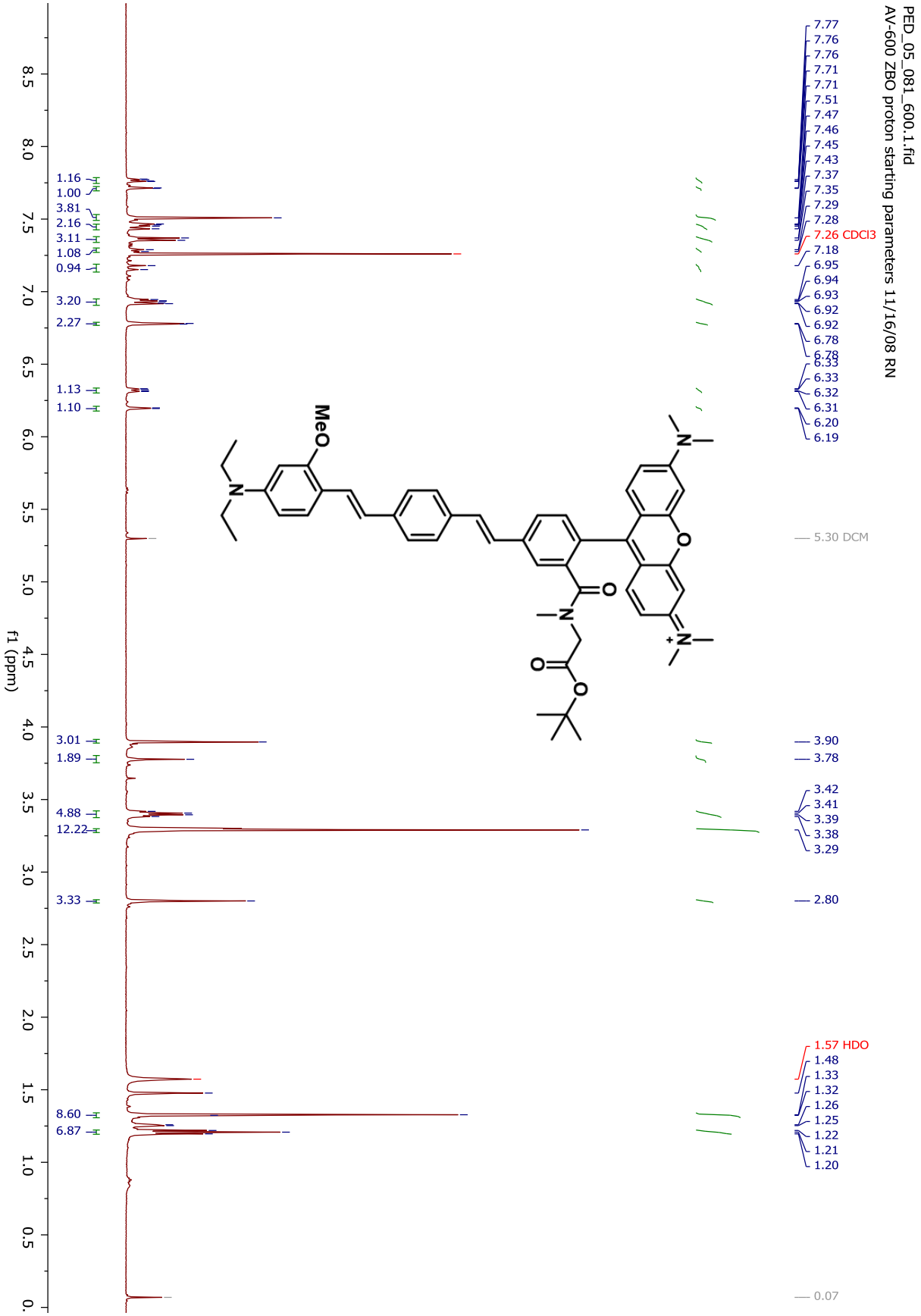
SI Spectrum 22. LC-MS of **14**



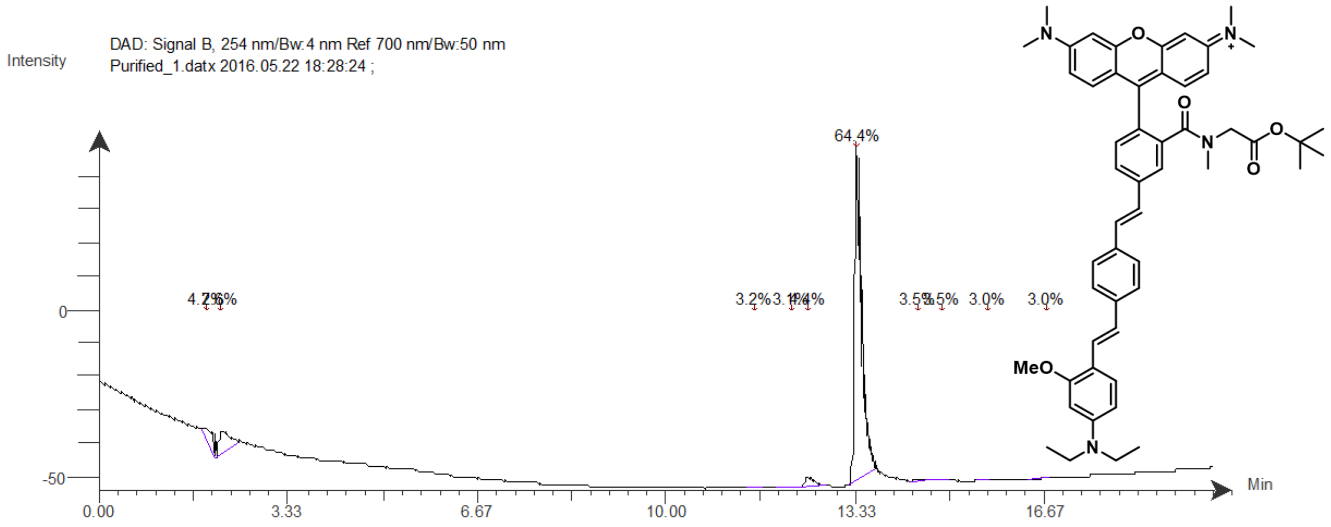


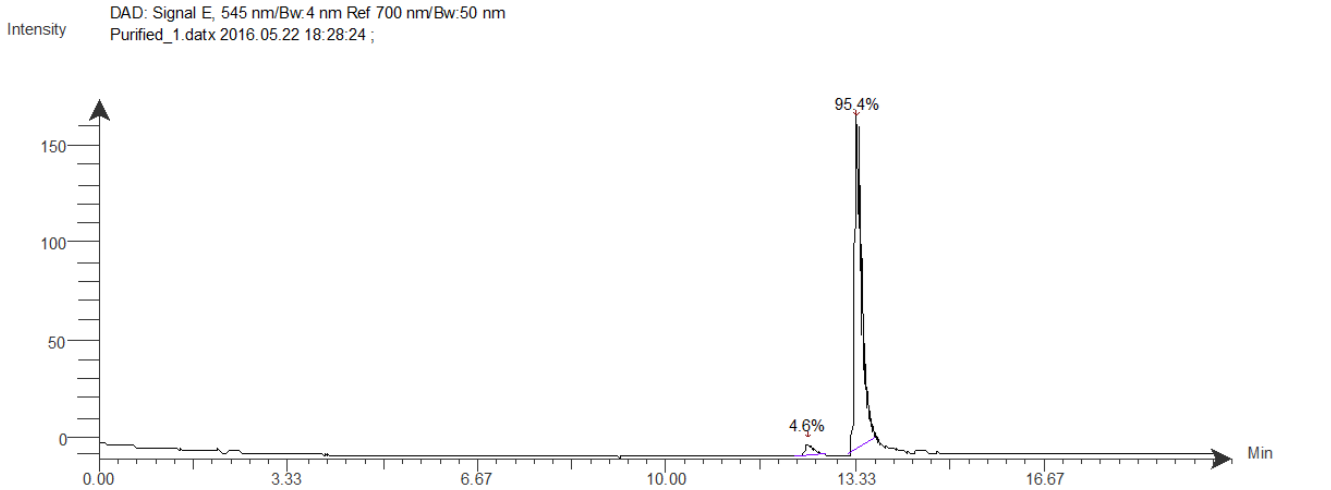


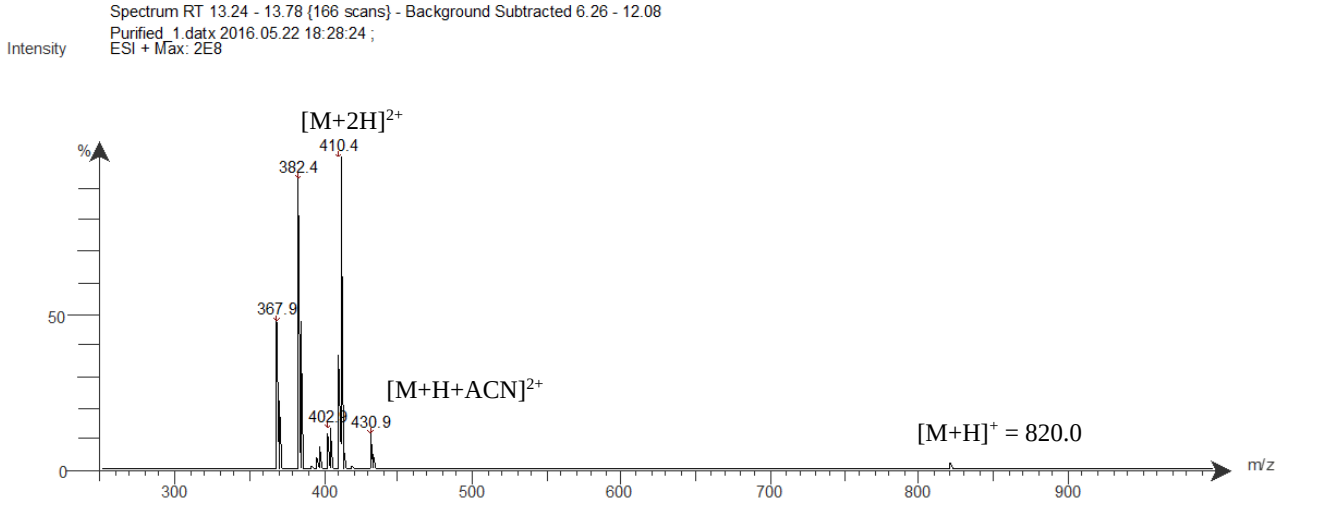
SI Spectrum 23. LC-MS of **15**



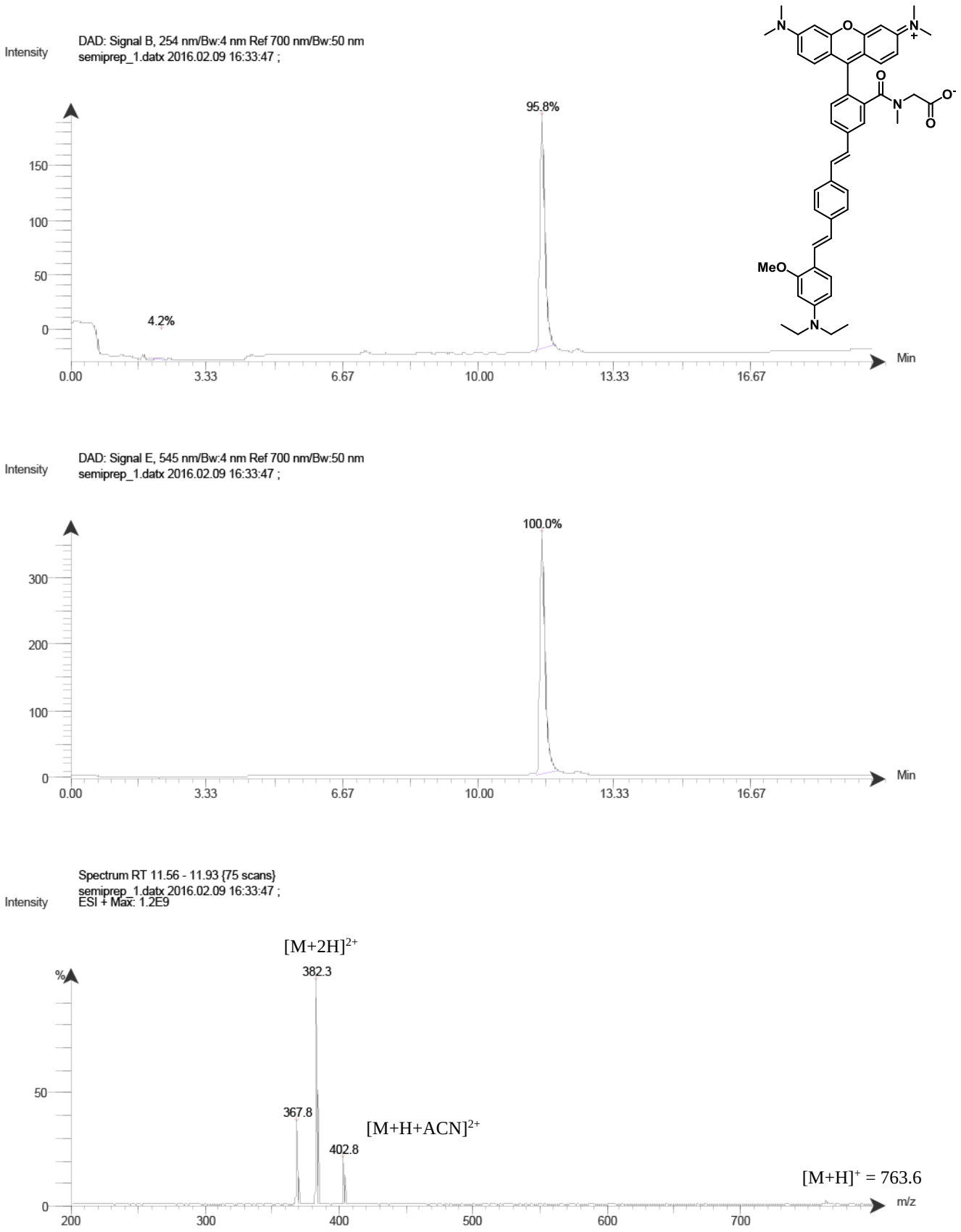
SI Spectrum 24. ^{1}H NMR of **16**



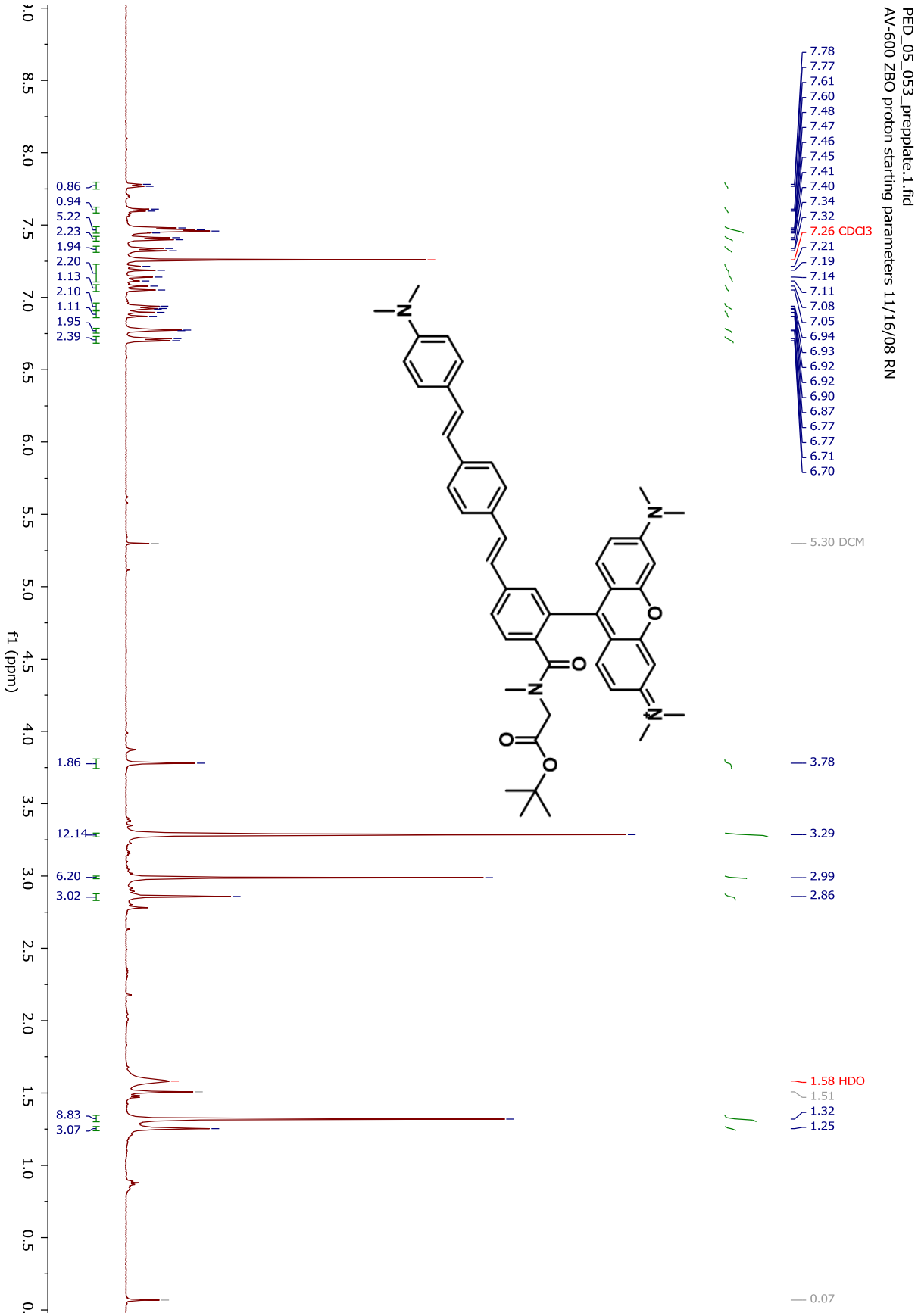




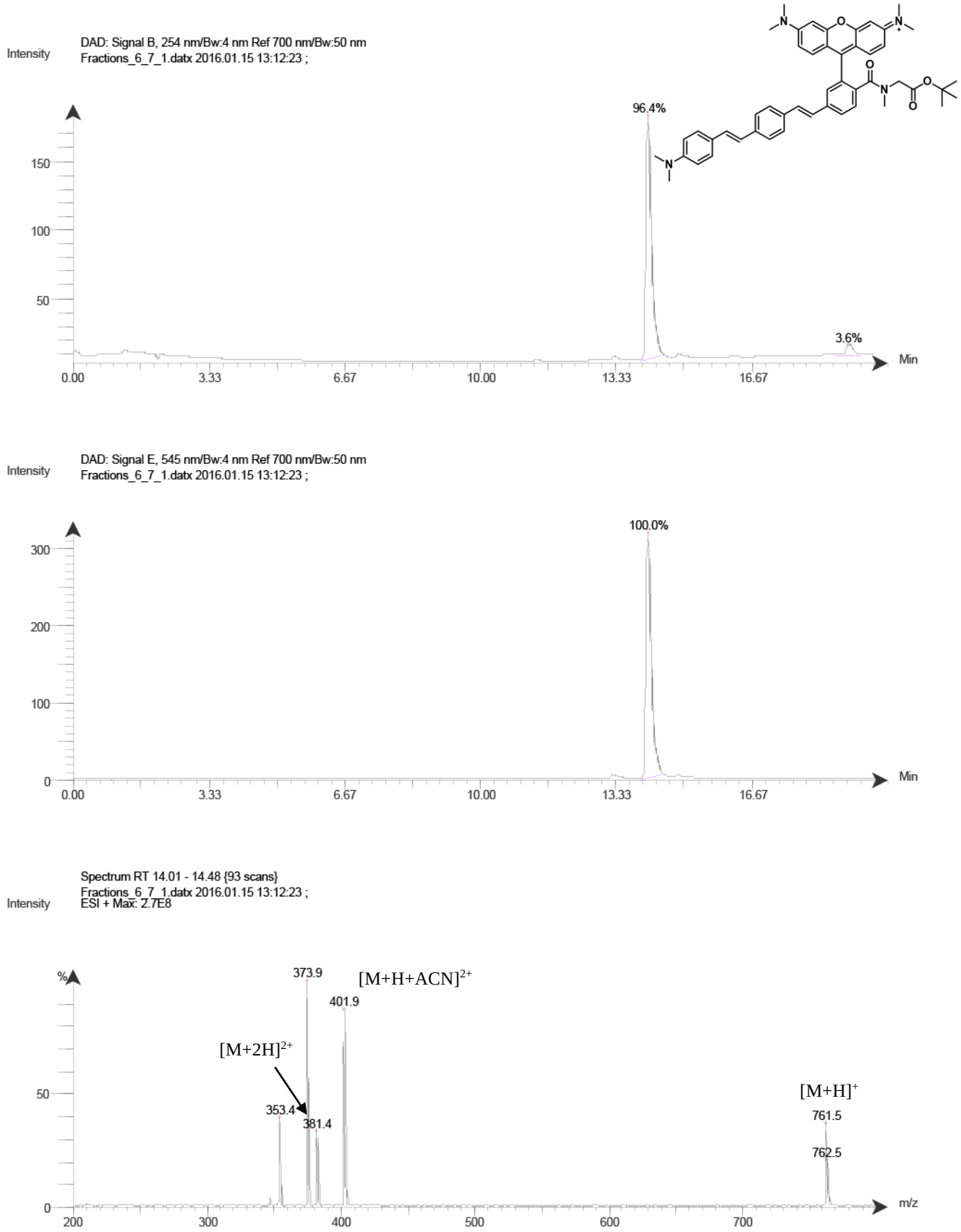
SI Spectrum 25. LC-MS of **16**



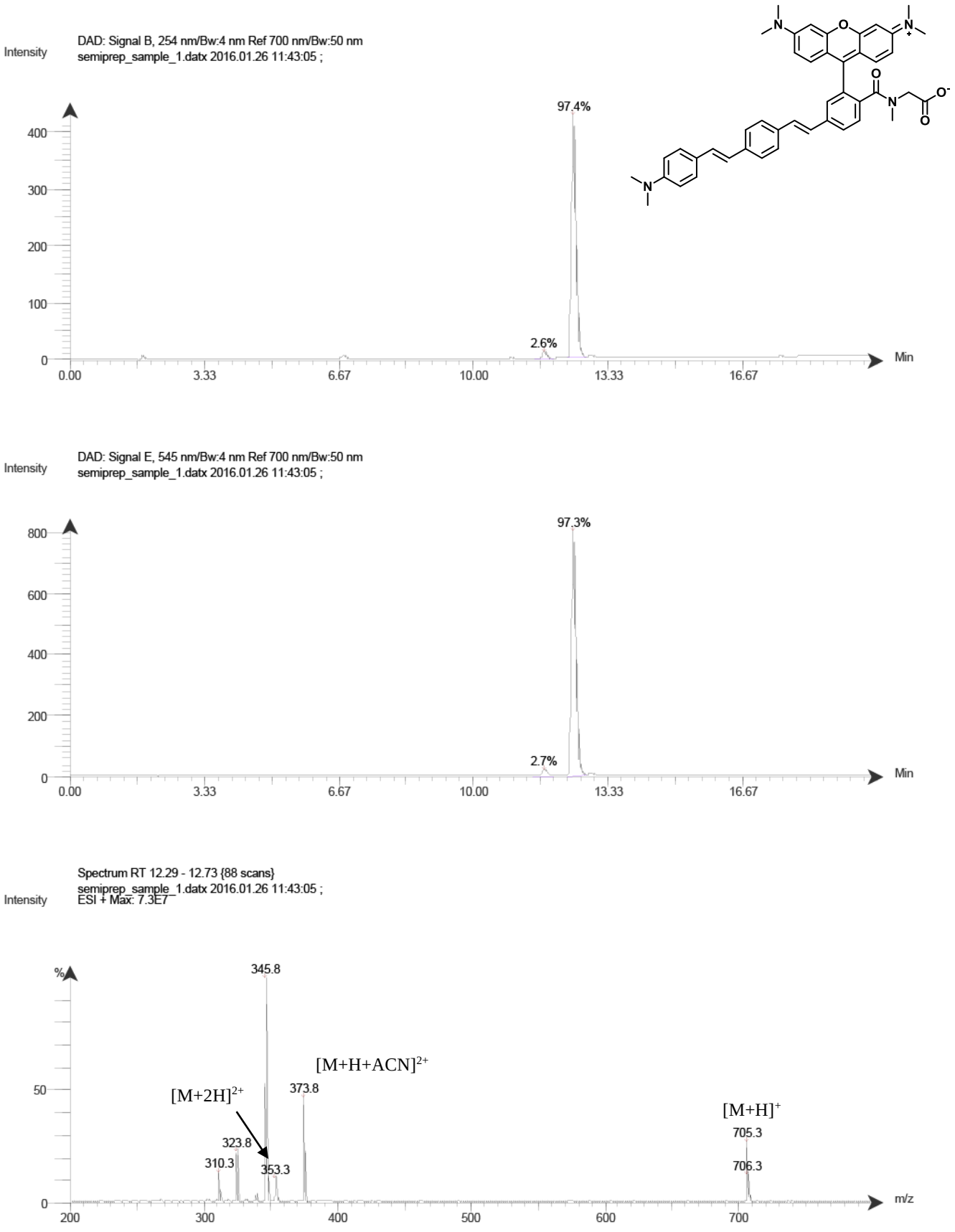
SI Spectrum 26. LC-MS of **17**



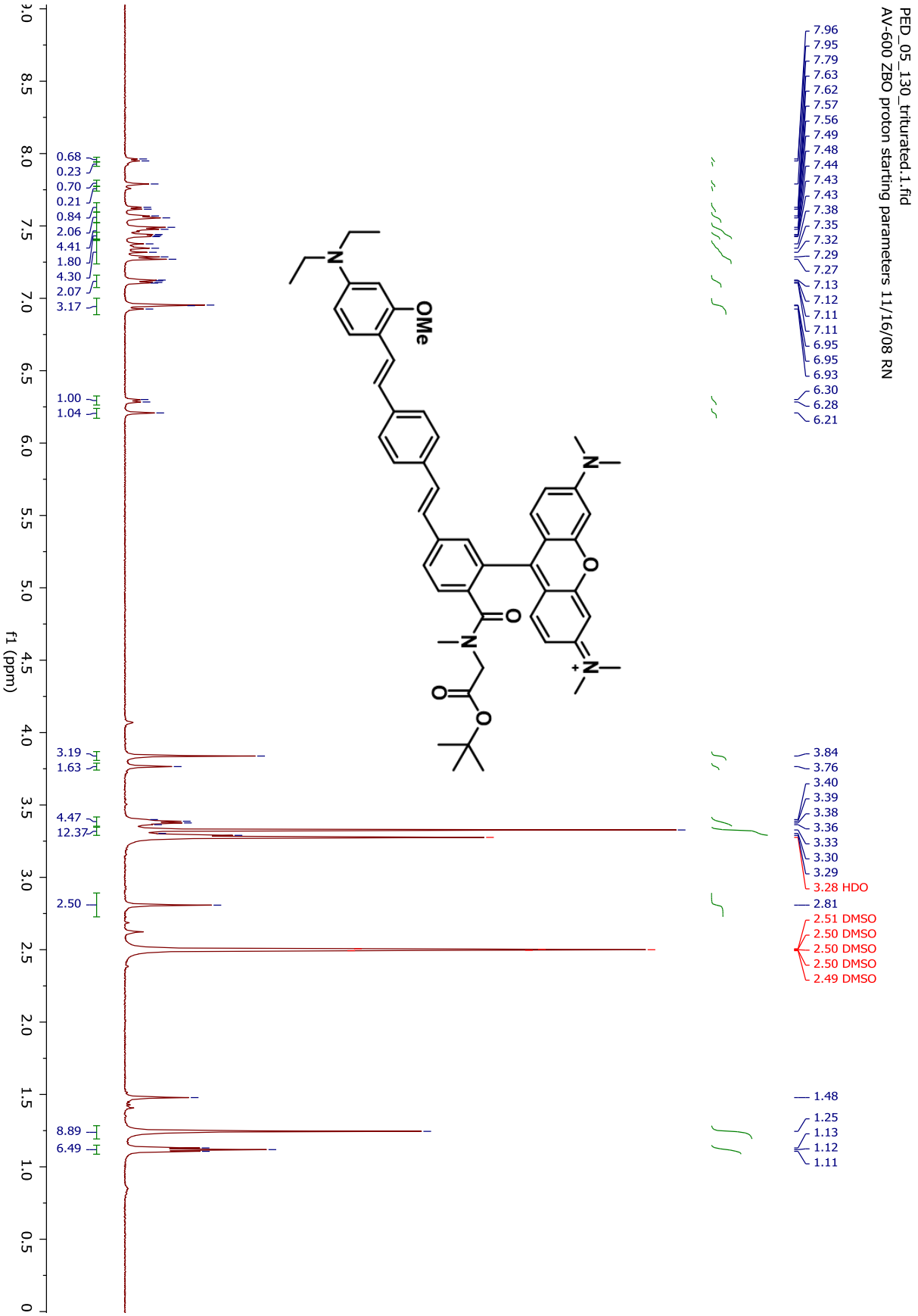
SI Spectrum 27. ^{1}H NMR of **18**



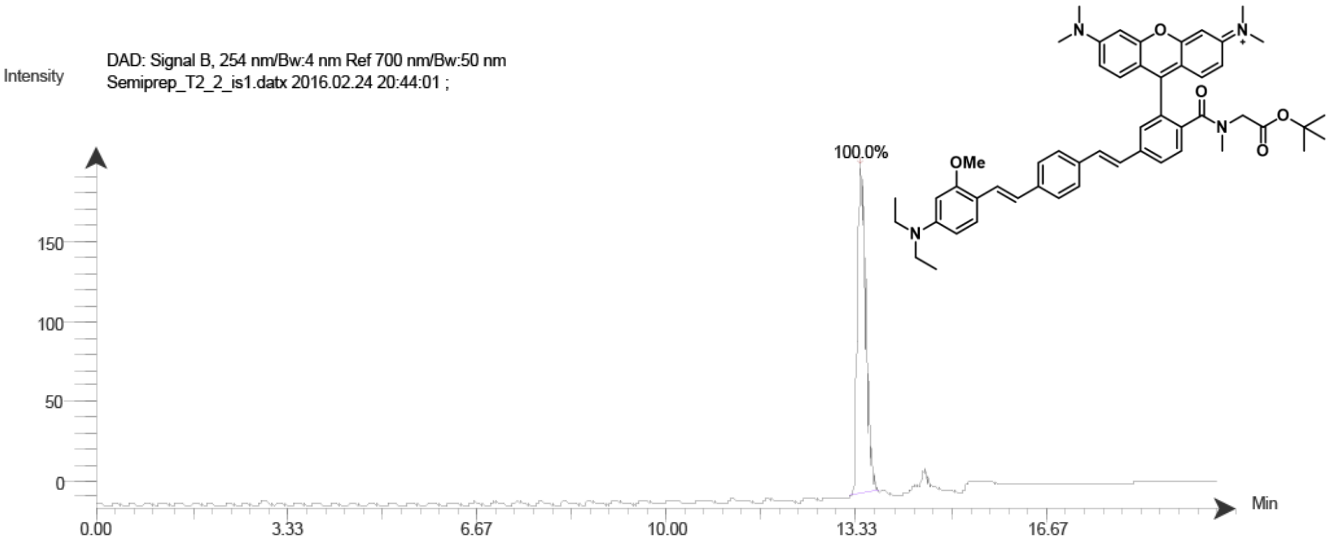
SI Spectrum 28. LC-MS of **18**

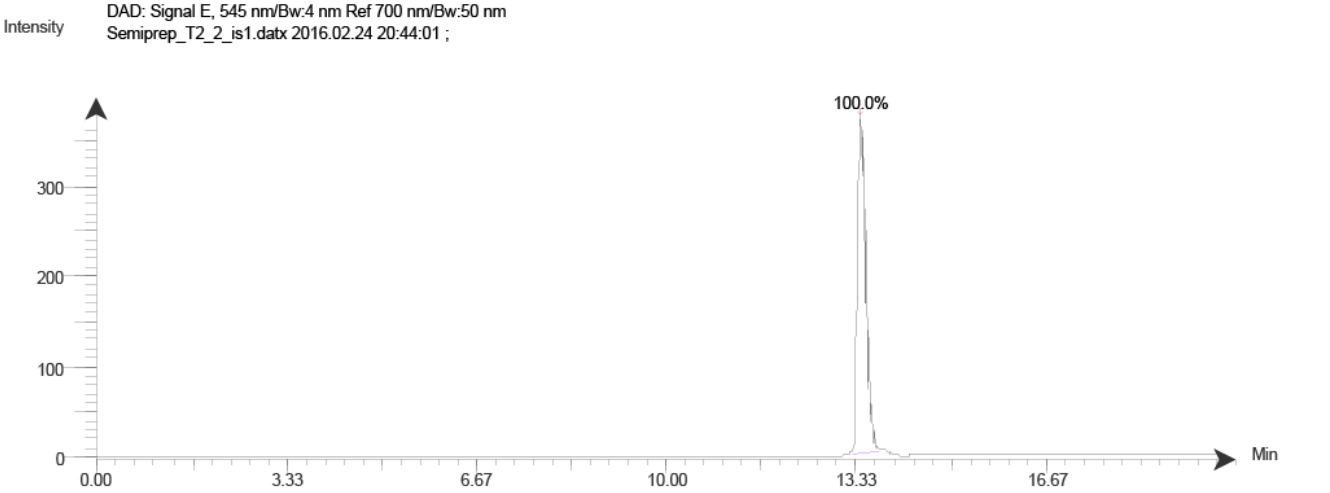


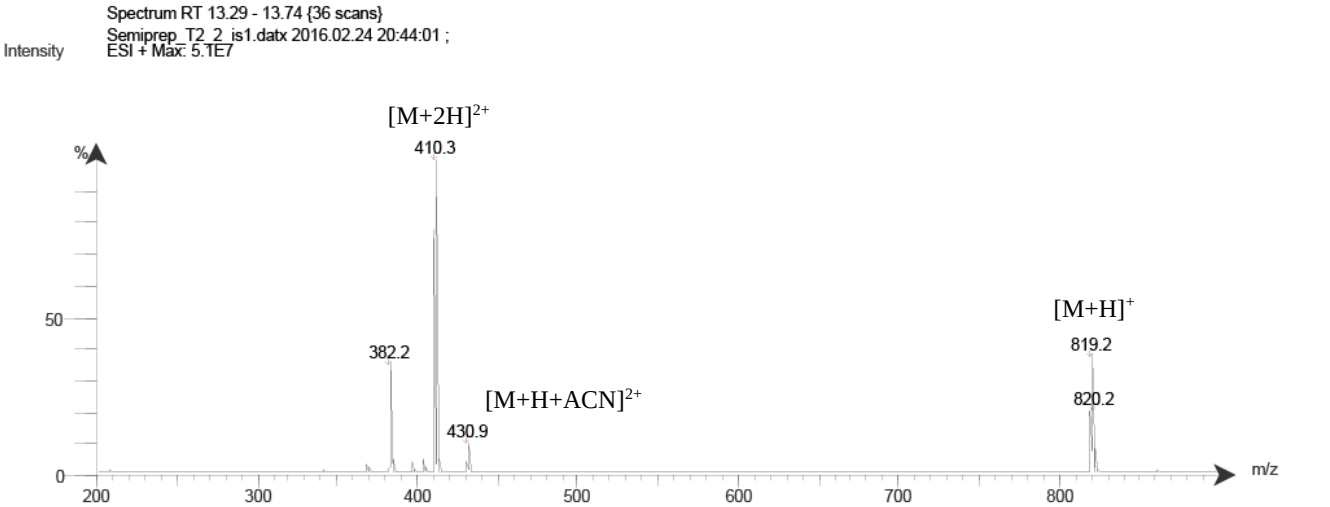
SI Spectrum 29. LC-MS of **19**



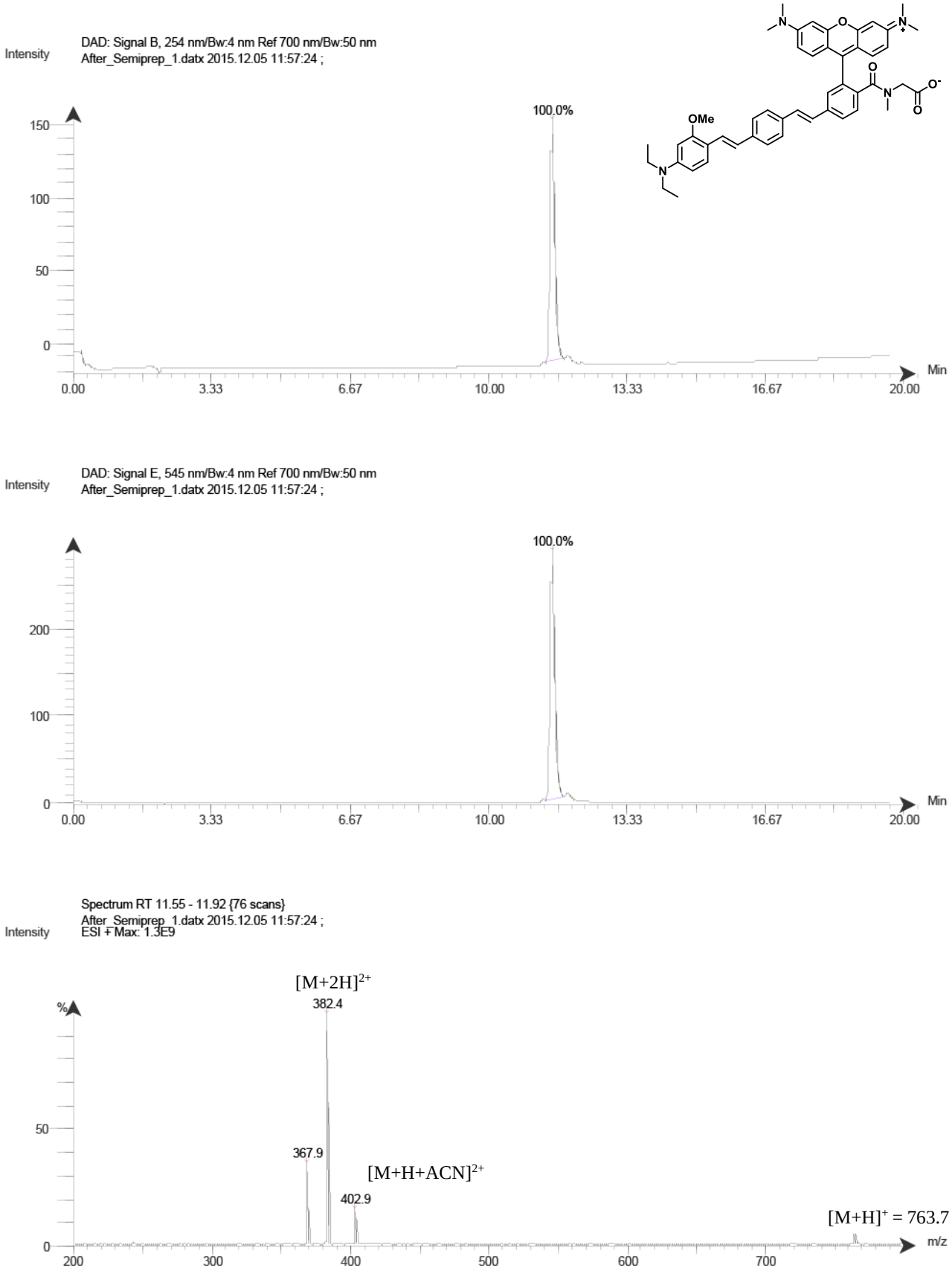
SI Spectrum 30. ^{1}H NMR of **20**



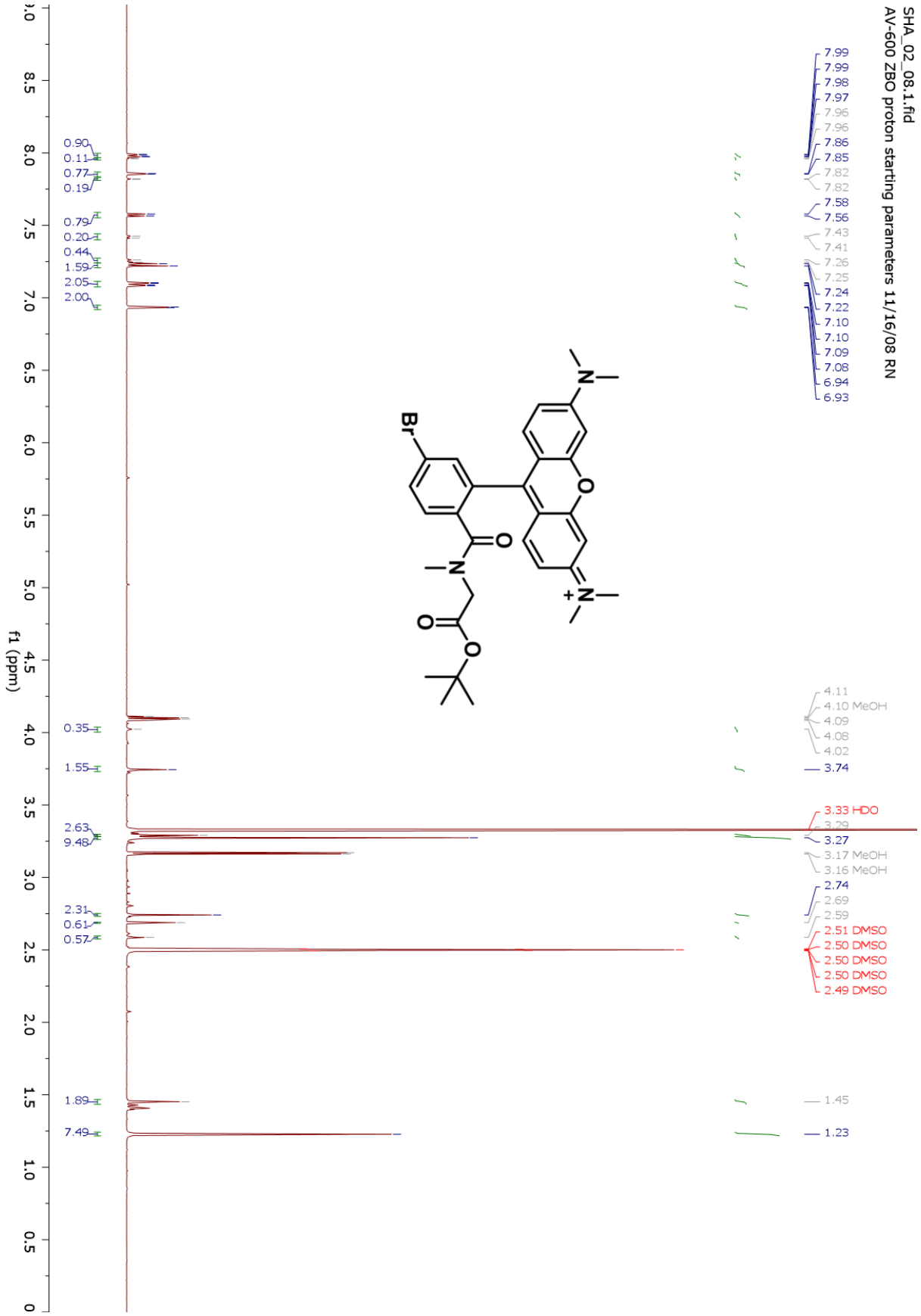




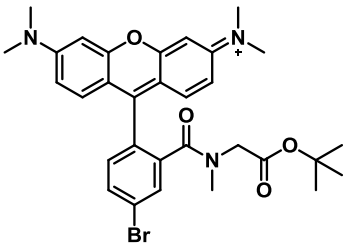
SI Spectrum 31. LC-MS of **20**

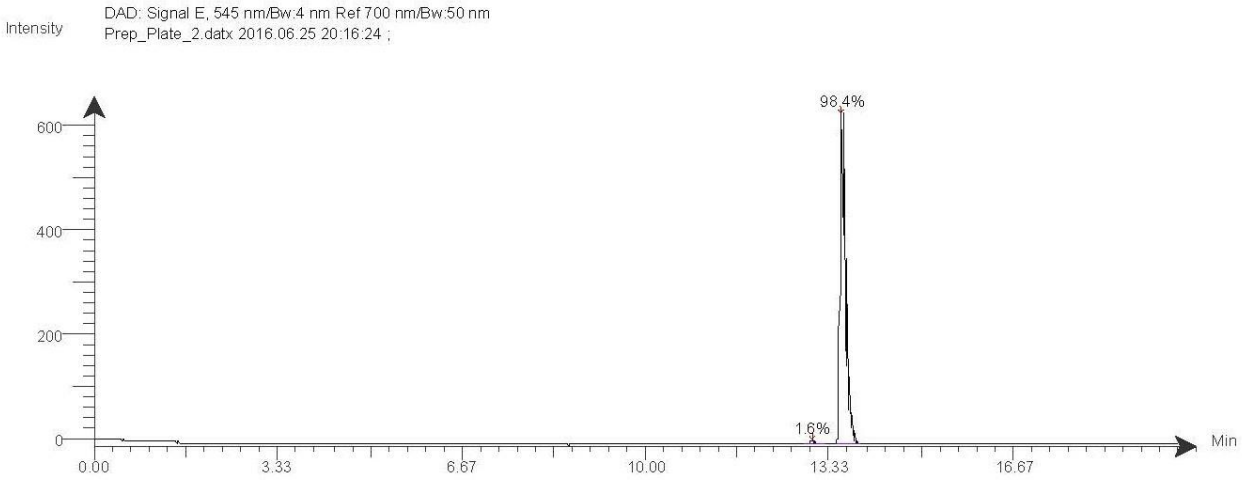


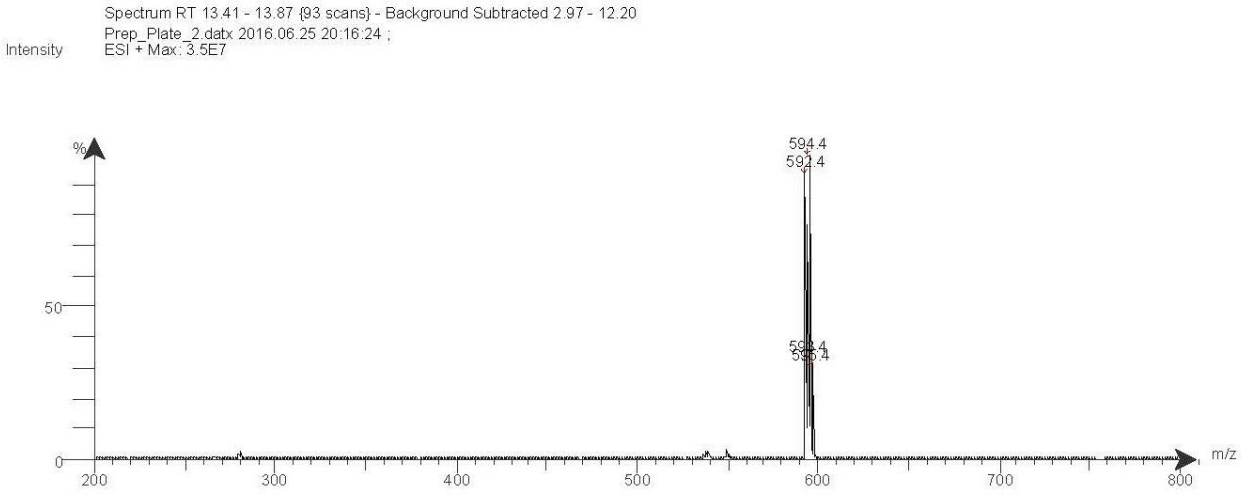
SI Spectrum 32. LC-MS of **21**



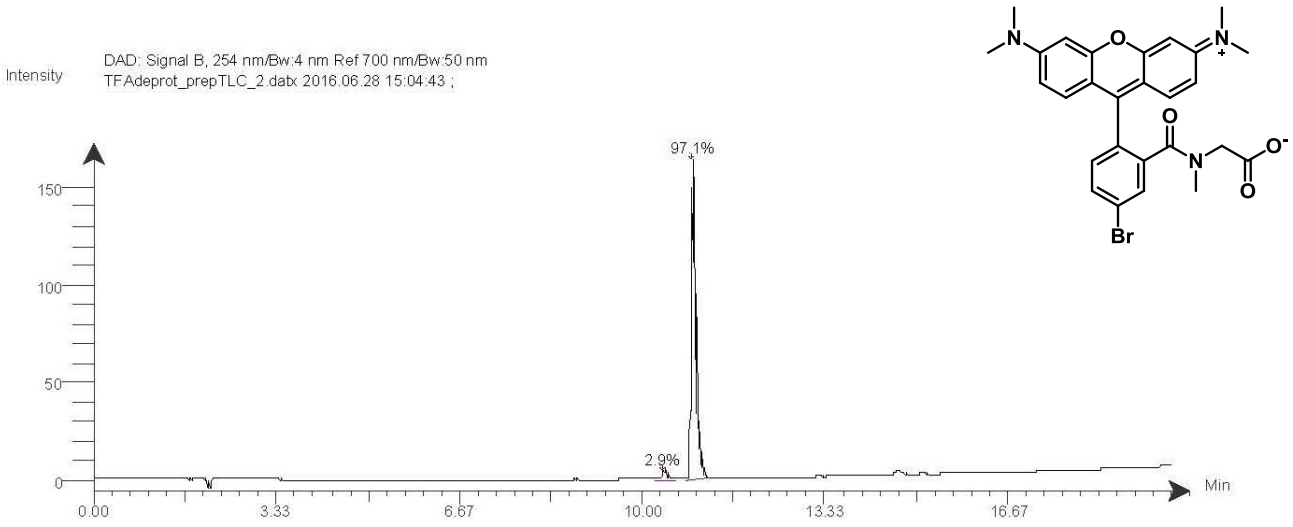
SI Spectrum 33. ^{1}H NMR of **22**

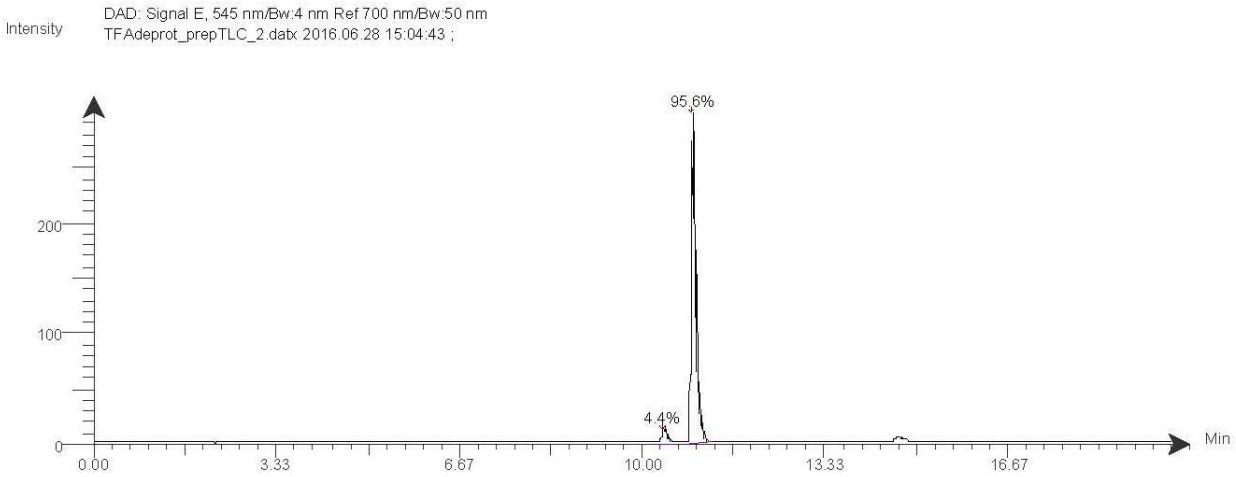


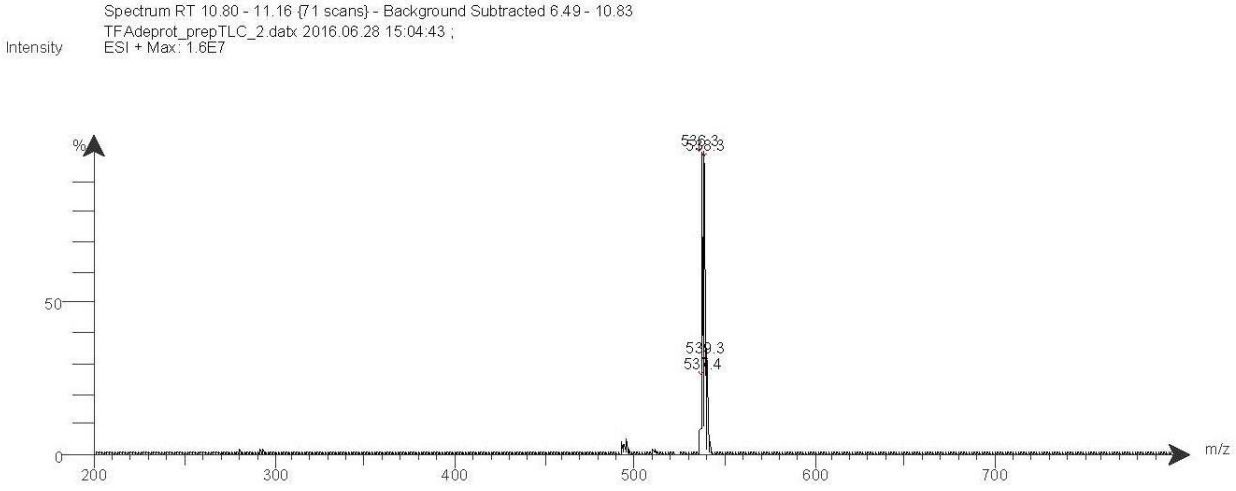




SI Spectrum 34. LC-MS of **22**





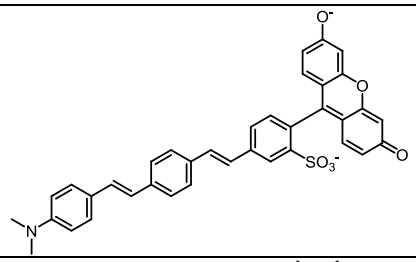
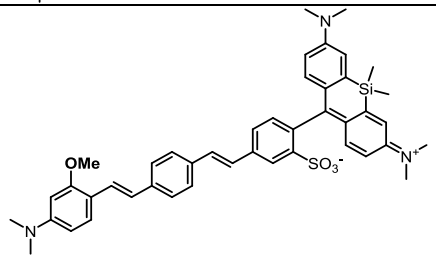


SI Spectrum 35. LC-MS of **23**

11. SI Movie Caption

SI Movie 1. F/F movie of RhoVR 1 and GCaMP6s fluorescence in spontaneously spiking neurons from Figure 3 in the main text. This movie shows the simultaneous recording of voltage and calcium in spontaneously firing hippocampal neurons stained with RhoVR 1 and expressing GCaMP6s.

12. SI Table

Name	Structure	Excitation Maximum	Emission Maximum
VoltageFluor2.1.Cl (VF2.1.Cl)		522 nm	536 nm
BeRST 1		658 nm	681 nm

12. SI References

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(5) Miller, E. W.; Lin, J. Y.; Frady, E. P.; Steinbach, P. A.; Kristan, W. B., Jr.; Tsien, R. Y. *Proc Natl Acad Sci U S A* 2012, *109*, 2114.