Supporting Information for

Synthesis of Sulfonated Carbofluoresceins for Voltage Imaging

Gloria Ortiz,[‡] Pei Liu,[‡] Su Htet Htet Naing,[‡] Vikram R. Muller,[‡] and Evan W. Miller[‡§†]*

Departments of [‡]Chemistry and [§]Molecular & Cell Biology and [†]Helen Wills Neuroscience Institute. University of California, Berkeley, California 94720, United States.

DOI: 10.1021/jacs.9b01261

Methods

Chemical synthesis and characterization

Chemical reagents and solvents (dry) were purchased from commercial suppliers and used without further purification. Compounds **15, 24, 25, 34, 35,** and iodomethyl 1-methylcyclopropanecarboxylate were prepared according to the literature procedures.[1-6] All reactions were carried out in flame-dried flasks sealed with septa and conducted under a nitrogen atmosphere. Thin layer chromatography (TLC) (silica gel, F254, 250 μm) was performed on precoated TLC glass plates 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 recorded on a Bruker AVB-400 MHz and a Bruker AV-600 MHz spectrometer, or at the QB3 Central California 900 MHz NMR Facility. Chemical shifts (δ) 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 (ESI EI) 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. Semi-preparative HPLC was performed on a Perkin Elmer Series 200 HPLC using a Phenomonex Luna 5 μm C18(2) (150 x 10 mm) column with a flow rate of 5.0 mL/min. Preparative HPLC was conducted on Waters Acquity Autopurification system, prep UHPLC-MS (ESI) equipped with a Waters XBridge BEH 5 μm C18 column (19 mm x 250 mm) and run with a flow rate of 30 mL/min. In all cases, the mobile phases were MQ-H_2O with 0.05% trifluoroacetic acid (eluent A) and HPLC grade MeCN with 0.05% trifluoroacetic acid (eluent B). For analytical HPLC, signals were monitored at 254, 380, and 450 nm over 10 min, with a gradient of 10 to 100% eluent B for 6 min, then held at 100% B for 4 min. For semi-preparative HPLC the mobile phase was ramped from 10 to 100% eluent B over 20 min. For preparative HPLC, signals were monitored at 254 over 20 min with a gradient of 10 to 100% eluent B.

Spectroscopic studies

UV-Vis absorbance and fluorescence spectra were recorded using a 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).

The maximum absorption wavelength (λ_{max}), maximum emission wavelength (λ_{em}), and extinction coefficient (ε) were taken in HBSS (zero Ca^{2+}, zero Mg^{2+}, no phenol red, pH 7.4) using stock solutions of

carbofluorescein dyes in DMSO (0.5-1 mM); the reported value for ε is an average (n = 3). The pK_a values were determined in buffers containing 150 mM NaCl and 10 mM buffer. The following buffer systems were used: citrate (pH 4.0–6.2); phosphate (pH 5.8– 8.0); tris (pH 7.8–9.0). Absorbance values at the λ_{max} were recorded of buffer solutions containing 250 nM fluorophore (n = 3) and fitted to a sigmoidal dose response curve using GraphPad Prism software.

In vitro PLE reactions and characterization

Commercially purified pig liver esterase (PLE, MW = 168 kDa) was obtained from Sigma-Aldrich (E2884) as a suspension in 3.2 M $(NH_4)_2SO_4$, and was diluted from a stock solution of 28.1 mg/mL to appropriate concentrations in HBSS (pH 7.4). For the enzymatic reaction, 2 μL carboVF-EX dyes in DMSO (final concentration of 50 μM) were incubated with 1 μL PLE (final concentration of 0.7025 mg/mL) and 1 μL Pluronic F-127 (20% w/v in DMSO) in 40 μL HBSS at 37 °C for ~ 2 h. The enzymatic products were characterized by spectroscopy and HPLC and compared with the starting materials. Absorbance was collected from 300 nm to 700 nm. For emission scans, the samples were excited at 565 nm and emission was collected from 570 nm to 800 nm. Excitation scans were collected by monitoring emission at 565 nm when exciting from 300 to 610 nm. To obtain the fluorescence turn-on over time, the dyes in the enzymatic reaction were diluted to 0.5 μM in 1 mL HBSS in a cuvette and the absorbance and emission spectra were recorded with and without PLE.

Enzyme kinetics experiments were performed in black, clear-bottom, 96-well polystyrene microplates from Corning. Plates were read from the bottom on a Molecular Devices SpectraMax Paradigm Multi-Mode detection platform plate reader (λ_{ex} 560 nm, λ_{em} 610 nm). All the measurements were done at 37 °C. Enzyme (168 ng/mL) was added to the substrates at t = 5 min and more data were collected for another 5 min. To obtain the kinetic constants, the data were fit to the Michaelis-Menten equation $v = V_{max}[S]/ K_M+[S]$ (GraphPad Prism).

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 maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 4.5 g/L D-glucose, 10% fetal bovine serum (FBS; Thermo Scientific) and 1% GlutaMax (Invitrogen) at 37 °C in a humidified incubator with 5% CO_2. Cells were passaged and plated in DMEM (as above) at a density of 750,000 cells per well in a 6-well plate. Transfection of plasmids was carried out using Lipofectamine 3000 (Invitrogen) ~18-24 h after plating. The cells were split again 48 h after transfection and plated onto 12 mm glass coverslips pre-coated with Poly-D-Lysine (PDL; 1 mg/ml; Sigma-Aldrich) at a density of 75,000 cells per coverslip in DMEM supplemented with 1 g/L D-glucose, 10% FBS and 1% GlutaMax. Imaging was performed 12-18 h after plating.

Hippocampi were dissected from embryonic day 19 Sprague Dawley rats (Charles River Laboratory) in cold, sterile HBSS (zero Ca^{2+}, zero Mg^{2+}, phenol red). 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% FBS, 2% B-27, 2% 1M dextrose (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 25-30,000 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 plasmids was carried out using Lipofectamine 3000 (without P3000 reagent) at 6-7 DIV. Imaging was performed on mature neurons 13-16 DIV.

Unless stated otherwise, for loading of HEK cells and hippocampal neurons, DMSO stock solutions of carboVF dyes (1 mM) were diluted directly into HBSS to working concentrations. CarboVF-EX dyes (1 mM) were diluted first with 1:1 Pluronic F-127 (20% w/v in DMSO) and then further diluted in HBSS to working concentrations. For HEK cells and neurons, the typical working concentration was 500 nM. HEK cells were incubated for 15 mins with carboVF dyes or 30 minutes with carboVF-EX dyes at 37 °C before exchanging dye/HBSS for HBSS without any dye. Neurons were treated identically, unless specified. All imaging was performed in HBSS at room temperature.

Epifluorescence microscopy

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). Images were acquired with a W-Plan-Apo 20x/1.0 water objective (20x; Zeiss). Images were focused onto either an OrcaFlash4.0 sCMOS camera (sCMOS; Hamamatsu) or an eVolve 128 EMCCD camera (EMCCD; Photometrix). For carboVF images, the excitation light was delivered from a LED at 575/35 nm and emission was collected with a triple emission filter (473/22, 543/19, 648/98 nm) after passing through a triple dichroic mirror (475/30, 540/25, 642/96 nm). For EGFP images, the excitation light was delivered from a LED 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).

Image analysis

For image intensity measurements, regions of interest were drawn around cells or neuronal cell bodies and the mean fluorescence was calculated in ImageJ (FIJI, NIH). Background fluorescence was subtracted by measuring the fluorescence where no cells grew. The fold turn-on was calculated by taking the ratio of transfected cells fluorescence and untransfected cells fluorescence, both background subtracted. At least 15-20 cells were quantified for each coverslip and 4 coverslips were examined to get the average fold turn-on. See Figure S8 for an example of regions of interest.

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 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. $\Delta F/F$ values were calculated by first subtracting a mean background value from all raw fluorescence frames, to give a background subtracted trace (bkgsub). A baseline fluorescence value (Fbase) is calculated from the median of all the frames, and subtracted from each timepoint of the bkgsub trace to yield a ΔF trace. The ΔF was then divided by Fbase to give $\Delta F/F$ traces. No averaging has been applied to any voltage traces.

Electrophysiology/Imaging parameters

For electrophysiological experiments, pipettes were pulled from borosilicate glass (Sutter Instruments, BF150-86-10), with a resistance of 4–6 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 a 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 hyper- and de- polarizing steps applied from -100 to +100 mV in 20 mV increments.

Extracellular field stimulation was delivered by a SD9 Grass Stimulator connected to a recording chamber containing two platinum electrodes (Warner), with triggering provided through the same Digidata 1332A digitizer and pCLAMP 9 software (Molecular Devices) that ran the electrophysiology. Action potentials were triggered by 1 ms 60 V field potentials delivered at 5 Hz. To prevent recurrent activity, the HBBS bath solution was supplemented with 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 DL-2-Amino-5-phosphonopentanoic acid (APV; Sigma-Aldrich). For both evoked action potentials and spontaneous activity, images were binned 4x4 to allow sampling rates of 0.5 kHz and 2500 frames (5 s) were acquired for each recording.

Plasmid construction

All plasmids were constructed using Gibson Assembly. We engineered a cell-surface pig liver esterase (PLE) by removing the ER retention signal from the original PLE sequence, adding an IgK leader sequence on the N-terminal and including a HA tag followed by a GPI anchor signal (DAF) on the C-terminal. To track PLE-positive cells during imaging, we added a nuclear-targeted EGFP (NLS-EGFP) down stream of PLE, separated by an internal ribosome entry site (IRES) sequence. For mammalian cell expression, pcDNA3 vector was selected for cloning and a cytomegalovirus (CMV) promoter was used for expressing in HEK cells while a human synapsin promoter (Syn) and a regulatory element from the woodchuck hepatitis virus (WPRE) were used for cultured neuron expression. All constructs were confirmed by sequencing.

The following sequences were used (5' to 3').

IgK

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTGAC

PLE (minus ER retention signal)

ATGGTGTGGCTGCTGCCTCTGGTGCTGACCAGCCTGGCCAGCAGCGCCACCTGGGCCGGCCAGCCCGCCAGCCCTCC
CGTGGTGGACACCGCCCAGGGCAGGGTGCTGGGCAAGTACGTGAGCCTGGAGGGCCTGGCCCAGCCCGTGGCCGTGT
TCCTGGGCGTGCCCTTCGCCAAGCCTCCCTTGGGCAGCCTGAGGTTCGCTCCTCCTCAGCCTGCTGAGCCCTGGAGC
TTCGTGAAGAACACCACCAGCTACCCTCCCATGTGCTGCCAGGATCCCGTGGTGGAGCAGATGACCAGCGACCTGTT
CACCAACGGCAAGGAGAGGCTGACCCTGGAGTTCAGCGAGGACTGCCTGTACCTGAACATCTACACACCCGCCGACC
TGACCAAGAGAGGCAGGCTGCCCGTGATGGTGTGGATCCACGGCGGCGGCCTGGTGCTGGGCGGCGCTCCCATGTAC
GACGGCGTGGTGCTGGCCGCCCACGAGAACGTGGTGGTGGTGGCCATCCAGTACAGGCTGGGCATCTGGGGCTTCTT
CAGCACCGGCGACGAGCACAGCAGgGGCAACTGGGGCCACCTGGACCAGGTGGCCGCCCTGCACTGGGTGCAGGAGA
ACATCGCCAACTTCGGCGGCGATCCCGGCAGCGTGACCATCTTCGGCGAGAGCGCCGGCGGCGAGAGCGTGAGCGTG
CTGGTGCTGAGCCCTCTGGCCAAGAACCTGTTCCACAGGGCCATCAGCGAGAGCGGCGTGGCCCTGACCGTGGCCCT
GGTGAGGAAGGACATGAAGGCCGCCGCCAAGCAGATCGCCGTGCTGGCCGGCTGCAAGACCACCACCAGCGCCGTGT
TCGTGCACTGCCTGAGGCAGAAGAGCGAGGACGAGCTGCTGGACCTGACCCTGAAGATGAAGTTCCTGACCCTGGAC
TTCCACGGCGACCAGAGGGAGAGCCATCCCTTCCTGCCCACCGTGGTGGACGGCGTGCTGCTGCCCAAGATGCCCGA
GGAGATCCTGGCCGAGAAGGACTTCAACACCGTGCCCTACATCGTGGGCATCAACAAGCAGGAGTTCGGCTGGCTGC
TGCCCACTATGATGGGCTTCCCTCTGAGCGAGGGCAAGTTGGACCAGAAGACCGCCACCAGCCTGCTGTGGAAGAGC
TATCCCATCGCCAACATTCCCGAGGAGCTGACACCCGTGGCCACCGACAAGTACCTGGGCGGCACCGACGATCCCGT
GAAGAAGAAGGACCTGTTCCTGGACCTGATGGGCGACGTGGTGTTCGGCGTGCCCAGCGTGACCGTGGCCAGGCAGC
ACAGGGACaCCGGCGCTCCCACCTACATGTACGAGTTCCAGTACAGGCCCAGCTTCAGCAGCGACAAGAAGCCCAAG
TCCGTGATCGGCGACCACGGCGACGAGATCTTCAGCGTGTTCGGCTTCCCTCTGCTGAAGGGCGACGCTCCCGAGGA
GGAGGTGAGCCTGAGCAAGACCGTGATGAAGTTCTGGGCCAACTTCGCCAGGAGCGGCAATCCCAACGGCGAGGGCC

TGCCTCACTGGCCCATGTACGACCAGGAGGAGGGCTACCTGCAGATCGGCGTGAACACCCAGGCCGCCAAGAGGCTG
AAGGGCGAGGAGGTGGCCTTCTGGAACGACCTGCTGAGCAAGGAGGCCGCCAAGAAGCCTCCTAAGATCAAG

HA
TATCCATATGATGTTCCAGATTATGCT

DAF
CCAAATAAAGGAAGTGGAACCACTTCAGGTACTACCCGTCTTCTATCTGGGCACACGTGTTTCACGTTGACAGGTTT
GCTTGGGACGCTAGTAACCATGGGCTTGCTGACTTAG

NLS-EGFP
ATGGTGCCCAAGAAGAAGAGGAAAGTCAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCT
GGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCC
TGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAG
TGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGA
GCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGA
ACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAAC
AGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGA
GGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACA
ACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTC
GTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA

IRES
GCCCCTCTCCCTCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCTATATG
TTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCC
TAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTT
CTTGAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGC
CAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGA
AAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGCACCCCATTGTATGGGAT
CTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAAACGTCTAGGCCCCCCGAACCAC
GGGGACGTGGTTTTCCTTTGAAAAACACGATGATAATATGGCCACA

WPRE
GCTTATCGATAATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTTAACTATGTTGCTCCTTTTA
CGCTATGTGGATACGCTGCTTTAATGCCTTTGTATCATGCTATTGCTTCCCGTATGGCTTTCATTTTCTCCTCCTTG
TATAAATCCTGGTTGCTGTCTCTTTATGAGGAGTTGTGGCCCGTTGTCAGGCAACGTGGCGTGGTGTGCACTGTGTT
TGCTGACGCAACCCCCACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCCGGGACTTTCGCTTTCCCCCTCC
CTATTGCCACGGCGGAACTCATCGCCGCCTGCCTTGCCCGCTGCTGGACAGGGGCTCGGCTGTTGGGCACTGACAAT
TCCGTGGTGTTGTCGGGGAAATCATCGTCCTTTCCTTGGCTGCTCGCCTGTGTTGCCACCTGGATTCTGCGCGGGAC
GTCCTTCTGCTACGTCCCTTCGGCCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCTGCTGCCGGCTCTGCGGCCTC
TTCCGCGTCTTCGCCTTCGCCCTCAGACGAGTCGGATCTCCCTTTGGGCCGCCTCCCCGCATCGATACCG

CMV promoter
GACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGC
GTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTA
TGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACTTGG
CAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTAT
GCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGAT
GCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACG
TCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAA
ATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCT

Synapsin Promoter
GTGTCTAGACTGCAGAGGGCCCTGCGTATGAGTGCAAGTGGGTTTTAGGACCAGGATGAGGCGGGGTGGGGGTGCCT
ACCTGACGACCGACCCCGACCCACTGGACAAGCACCCAACCCCCATTCCCCAAATTGCGCATCCCCTATCAGAGAGG
GGGAGGGGAAACAGGATGCGGCGAGGCGCGTGCGCACTGCCAGCTTCAGCACCGCGGACAGTGCCTTCGCCCCCGCC

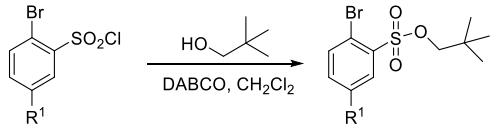
TGGCGGCGCGCGCCACCGCCGCCTCAGCACTGAAGGCGCGCTGACGTCACTCGCCGGTCCCCCGCAAACTCCCCTTC
CCGGCCACCTTGGTCGCGTCCGCGCCGCCGCCGGCCCAGCCGGACCGCACCACGCGAGGCGCGAGATAGGGGGGCAC
GGGCGCGACCATCTGCGCTGCGGCGCCGGCGACTCAGCGCTGCCTCAGTCTGCGGTGGGCAGCGGAGGAGTCGTGTC
GTGCCTGAGAGCGCAGTCGAGA

Immunohistochemistry

In order to detect the expression of cell-surface PLE, HEK cells were fixed at room temperature with 4% paraformaldehyde in PBS for 10 min. Permeabilization was not conducted to make sure that antibody only binds to epitopes on the cell surface. The fixed cells were then blocked with 5% w/v bovine serum albumin (BSA; Sigma Aldrich) in PBS for 1 h. Anti-HA primary antibody (CST, Rabbit IgG) was incubated at 4 °C overnight, followed by AlexaFluor 647 anti-rabbit secondary antibody (Life Technologies) and Hoechst 33342 (Thermo Fischer) at room temperature for 2 h. All antibodies were used at 1:1000 dilution.

Supporting Schemes

Scheme S1. Synthesis of sulfonates **16** and **17**



34: R^1 = H
35: R^1 = Br

16: R^1 = H 46%
17: R^1 = Br 64%

Supporting Figures

Figure S1. pH titrations of sulfonated carbofluorescein dyes.

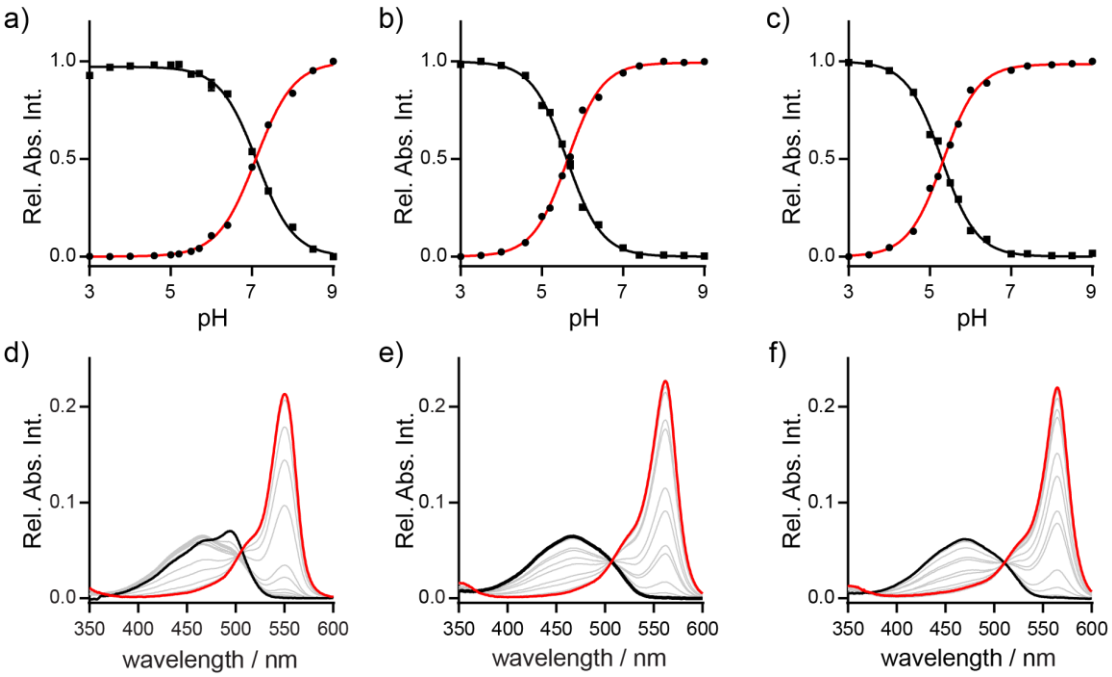


Figure S1. pH titrations of sulfonated carbofluorescein dyes. Upper row: Plots of relative absorbance intensity at the respective λ_{max} vs. pH for sulfonated carbofluorescein derivatives a) **18** (H), b) **20** (F), and c) **22** (Cl). Lower row: Plots of relative absorbance intensity vs. wavelength for d) **18** (H), e) **20** (F), and f) **22** (Cl). Red line indicates spectrum acquired at pH = 9; black line is pH = 3. Grey lines are determined at pH 8.5, 7.4, 7.0, 6.4, 6.0, 5.7, 5.5, 5.2, 4.6, 4.0 and 3.5. Error bars are ± S.E.M. for n = 3 independent determinations. If not visible, error bars are smaller than the marker.

Figure S2. Spectroscopic properties of carboVF dyes.

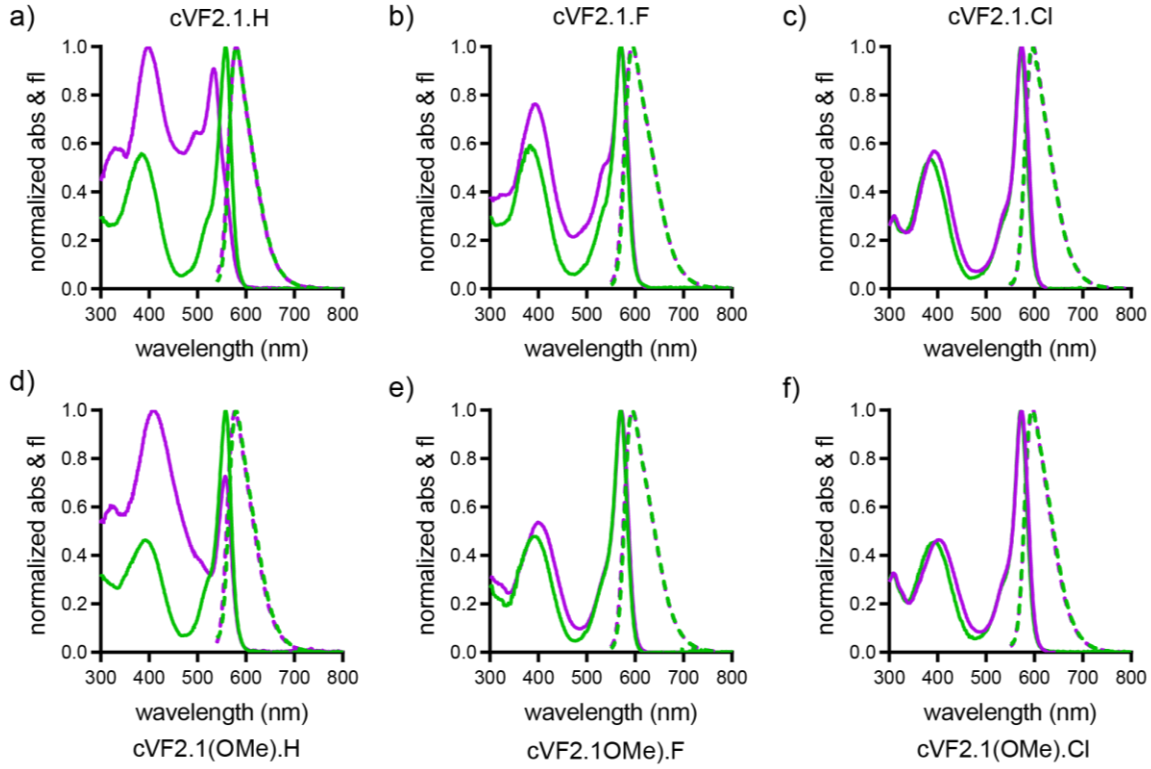


Figure S2. Spectroscopic properties of carboVF (cVF) dyes. Normalized absorbance (solid lines) and emission (dashed lines) spectra of cVF dyes a) cVF2.1.H, b) cVF2.1.F, c) cVF2.1.Cl, d) cVF2.1(OMe).H), e) cVF2.1(OMe).F, and f) cVF2.1.Cl in HBSS with 0.01% SDS (purple lines) or in 1:9 H_2O/MeOH with 0.1 M NaOH (green lines).

Figure S3. Cellular localization and brightness of carboVF dyes.

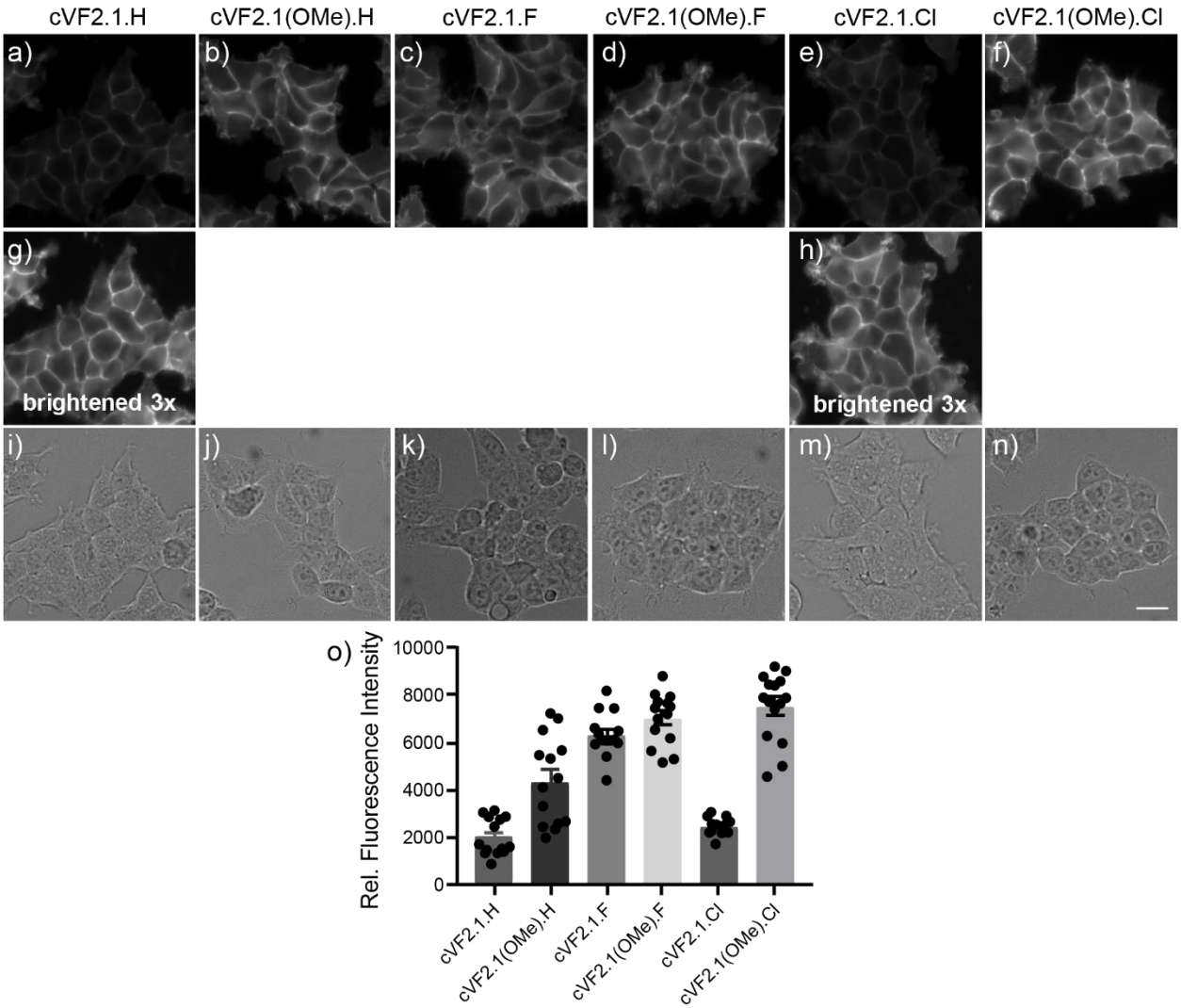


Figure S3. Cellular localization and brightness of carboVF dyes. a-f) HEK cells were stained with carboVF dyes (500 nM, 15 mins) then washed with fresh HBSS. Images are normalized to cVF2.1(OMe).Cl (panel f) to enable comparison of relative brightness of the carboVF dyes. g-h) Images of cVF2.1.H and cVF2.1.Cl were brightened 3-fold to enable visual inspection of cellular localization. i-n) Differential interference contrast (DIC) images of the HEK cells in a-f). Scale bar is 20 μm. o) Comparison of the relative brightness of carboVF dyes loaded in HEK cells (n = 3 coverslips). Each individual point represents the average brightness of HEK cells in a single image. Error bars are ± S.E.M.

Figure S4. Voltage sensitivity of carboVF dyes.

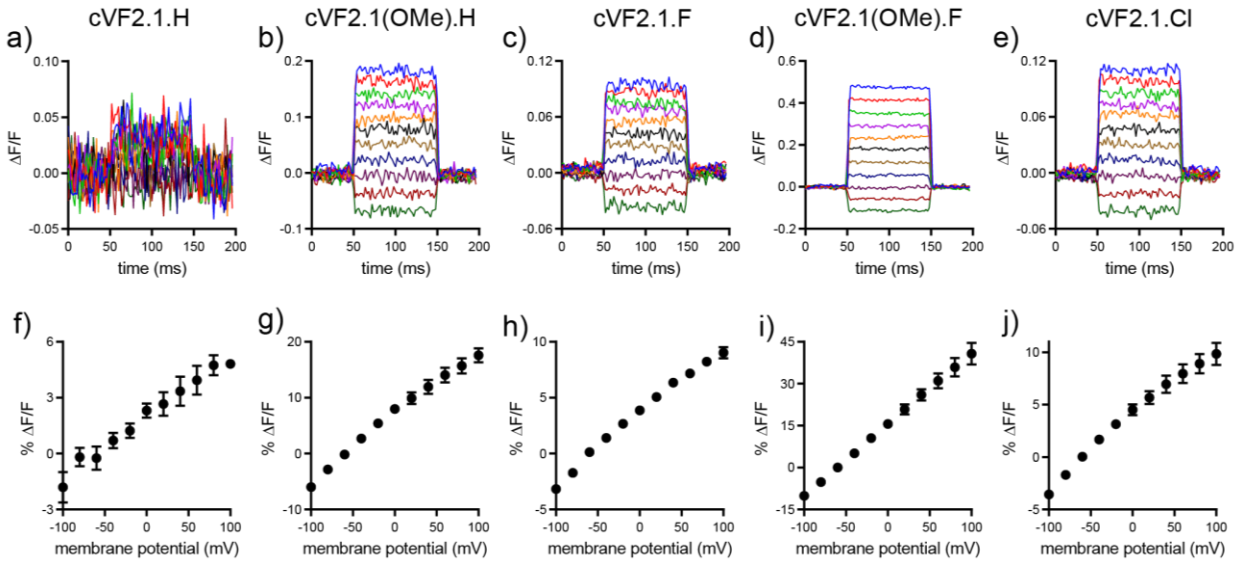


Figure S4. Voltage sensitivity of carboVF dyes. cVF2.1.H (a,f); cVF2.1(OMe).H (b,g); cVF2.1.F (c,h); cVF2.1(OMe).F (d,i); and cVF2.1.Cl (e,j). Upper row: The fractional change in fluorescence is plotted vs. time for HEK cells held at -60 mV and stepped to 100 ms hyper- and depolarizing steps (±100 mV, 20 mV increments) under whole-cell voltage-clamp conditions. Lower row: Plots of % ΔF/F vs. final membrane potential (mV) for n = 5-6 HEK cells for each carboVF dye. Error bars are ± S.D.

Figure S5. Field stimulation of neurons stained with carboVF2.1(OMe).Cl.

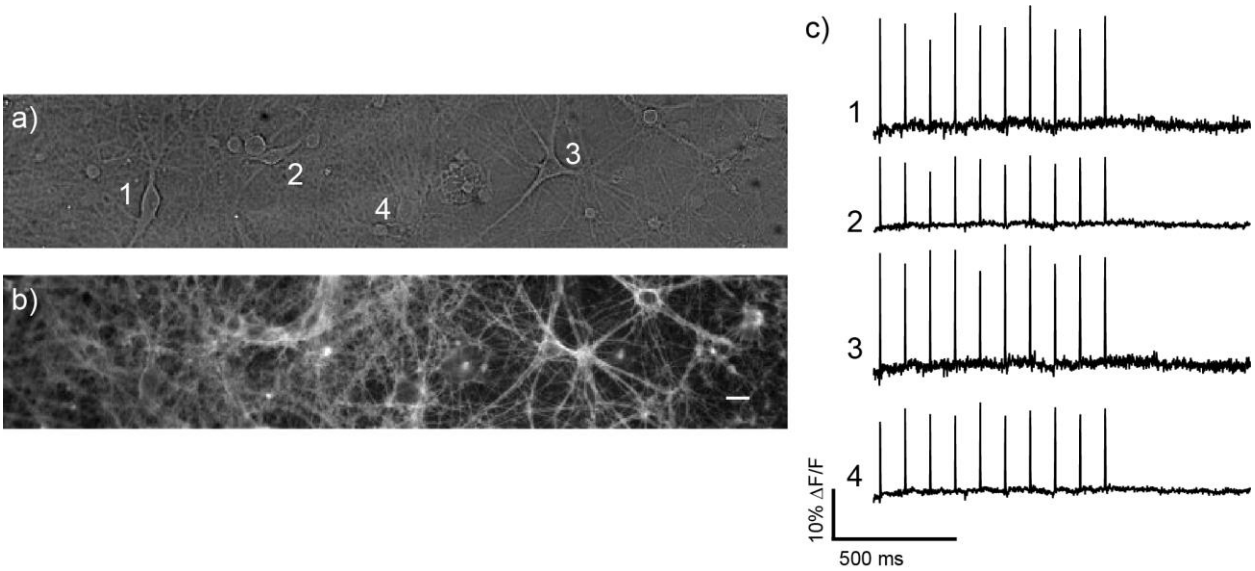


Figure S5. Field stimulation of neurons stained with carboVF2.1(OMe).Cl. Rat hippocampal neurons (DIC, a) were imaged with 500 nM carboVF2.1(OMe).Cl (fluorescence, b)**.** c) Electrode-evoked activity from the neurons in a-b) is shown as ΔF/F traces. Scale bar is 20 μm.

Figure S6. Spectroscopic characterization of carboVF-EX 1 and 2

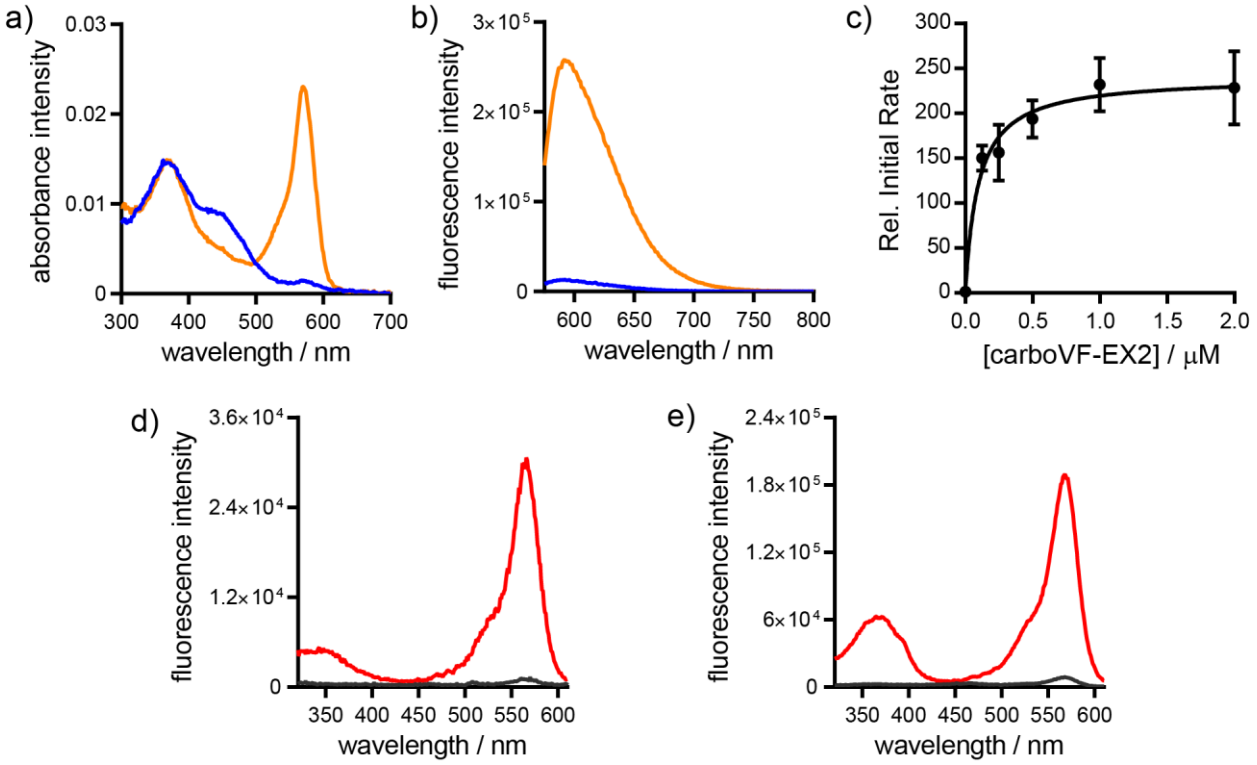


Figure S6. Spectroscopic characterization of carboVF-EX 1 and 2 *in vitro*. a) UV-vis absorption and b) fluorescence emission spectra of carboVF-EX 2 in the absence (blue lines) or presence (orange lines) of PLE (0.7 mg/mL, 2 h). c) Plot of relative initial rate vs substrate concentration for the reaction of PLE (168 ng/mL) with carboVF-EX 2. Data points are the mean ± S.E.M. for n = 8 independent measurements. The solid line is the best fit to the Michaelis-Menten equation (Graphpad). d-e) Excitation spectra of d) carboVF-EX 1 and e) carboVF-EX 2 (both at 500 nM, HBSS) in the presence of PLE (red lines) or absence (black lines) of PLE. Excitation wavelength was from 320 to 610 nm and emission was monitored at 620 nm.

Figure S7. HPLC analysis of PLE reaction with carboVF-EX 1 and 2

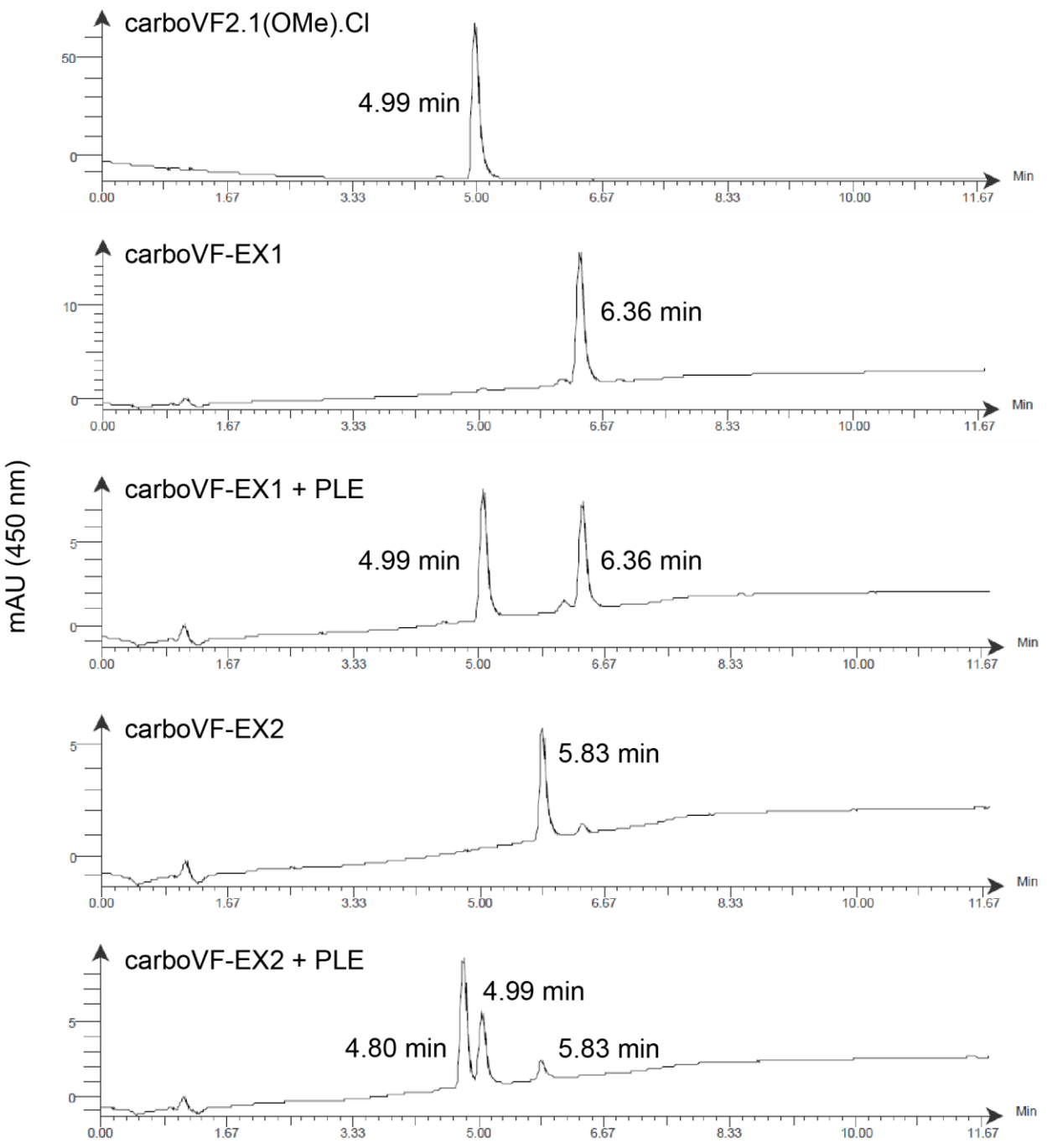


Figure S7. HPLC analysis of PLE reaction with carboVF-EX 1 and 2. Note: the reaction of carboVF-EX2 + PLE leads to carboVF2.1(OMe).Cl (4.99 min) and the intermediate in which only the sulfonate is esterified (4.80 min).[8]

Figure S8. Immunocytochemistry of cell-surface porcine liver esterase (PLE) in HEK cells.

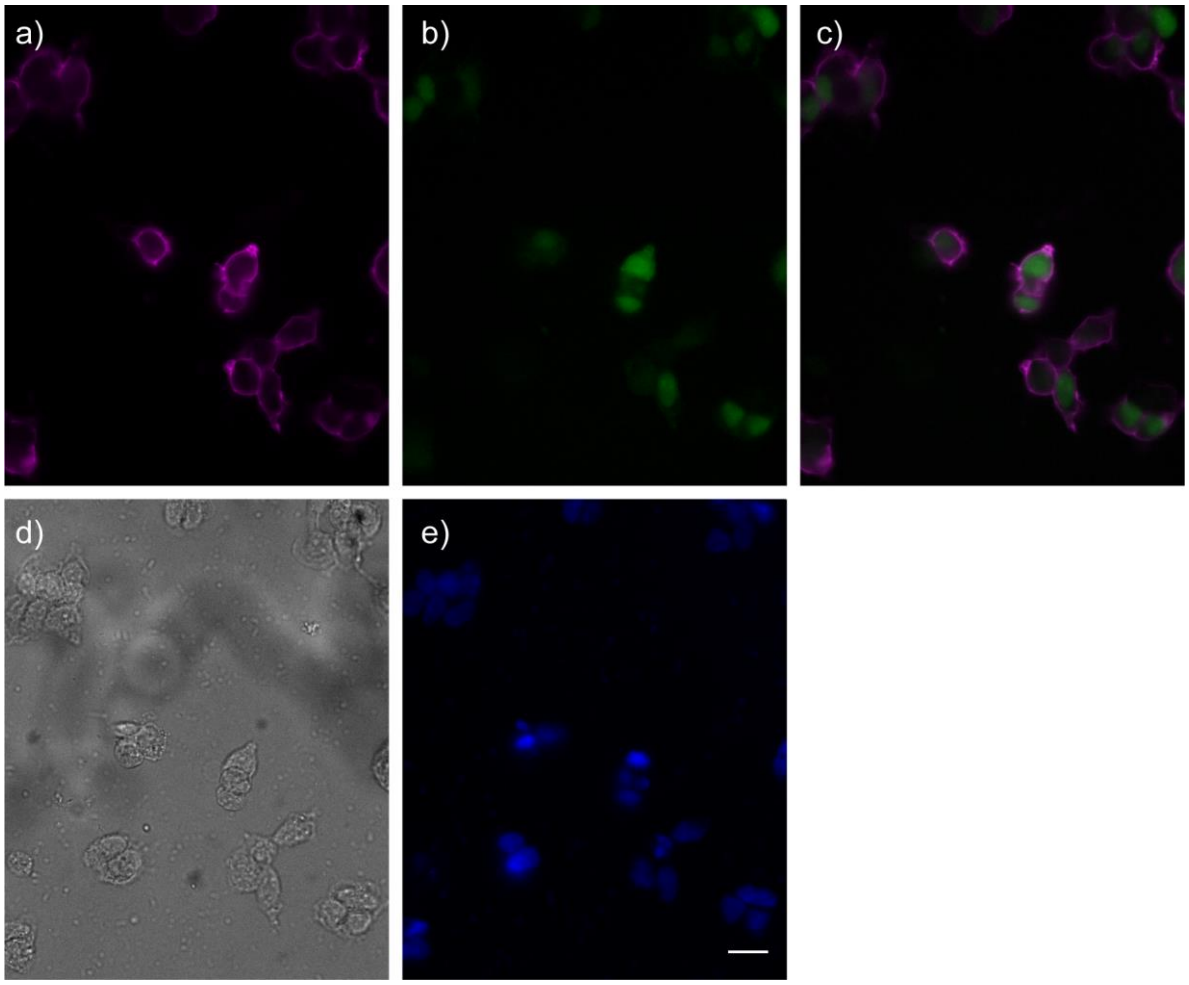


Figure S8. Immunocytochemistry of cell-surface PLE in HEK cells. a) Anti-HA immunofluorescence shows membrane-associated staining that suggests cell-surface PLE expression. b) PLE expression is indicated by nuclear EGFP. c) Merged images of anti-HA staining and EGFP. d) DIC images of fixed cells. e) Nuclei staining by Hoechst 33342. Scale bar is 20 μm.

Figure S9. Quantification of carboVF-EX 1 fluorogenic response in HEK cells.

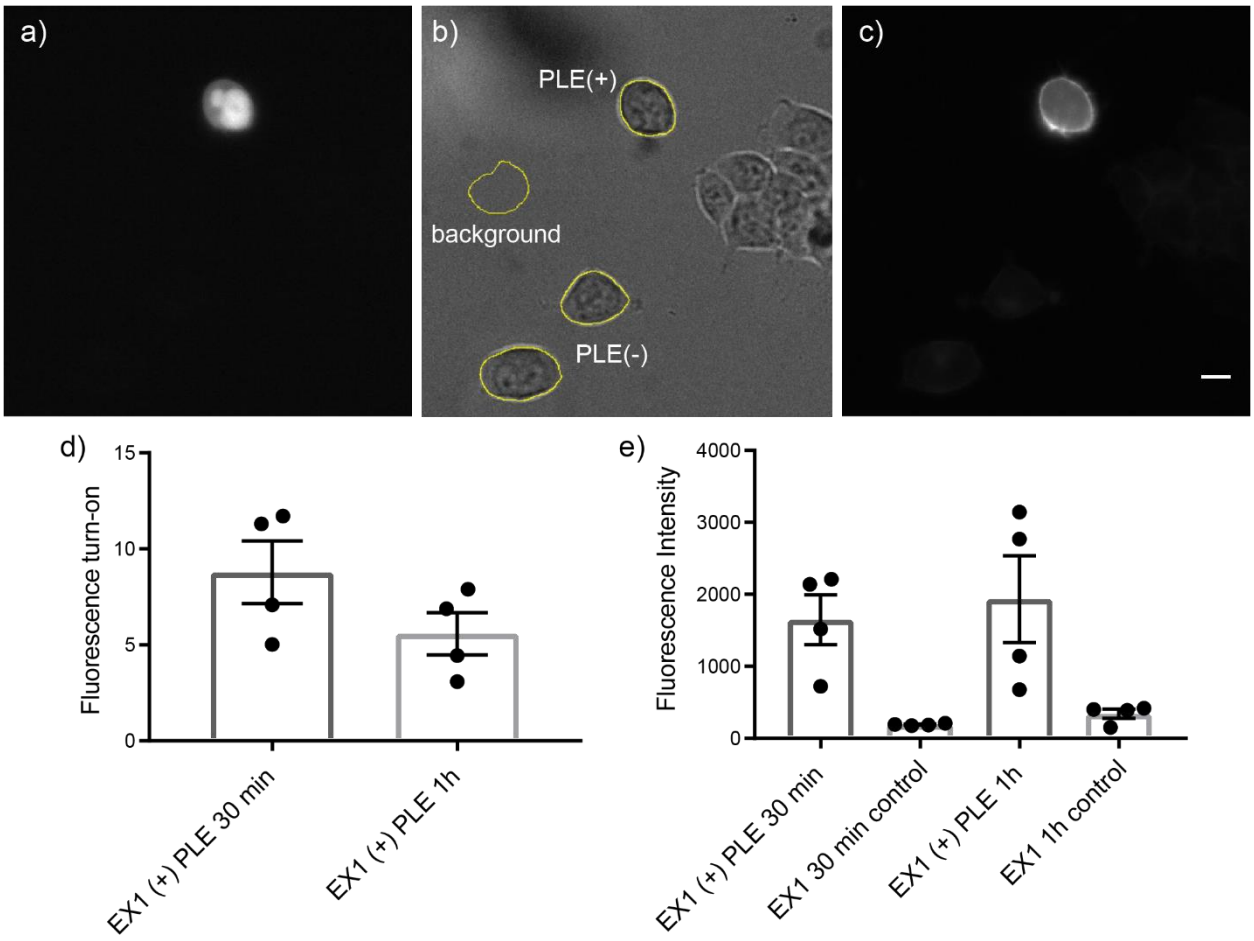


Figure S9. Quantification of contrast between PLE-expressing (DAF targeted) and untransfected HEK cells stained with carboVF-EX 1. HEK cells transfected with PLE were incubated with 500 nM carboVF-EX 1. a) Fluorescence image of PLE expressing HEK cells marked by EGFP. b) DIC image showing regions of interest used to calculate the mean fluorescence in cells. c) Fluorescence image of carboVF-EX 1 showing PLE-dependent fluorescence in HEK cells. Scale bar is 10 μm. d) Contrast (fold turn-on) between control and transfected cells and e) measured 16-bit grey values. Data are mean grey values ± S.E.M. for 4 independent coverslips of cells. Each coverslip comprises 6 fields of view with regions of interest made up of PLE(+) and PLE(-) cells (10-20 PLE (+) cells and 10-20 PLE (-) cells per coverslip).

Figure S10. Fluorogenic labeling of specific neurons with carboVF-EX 1.

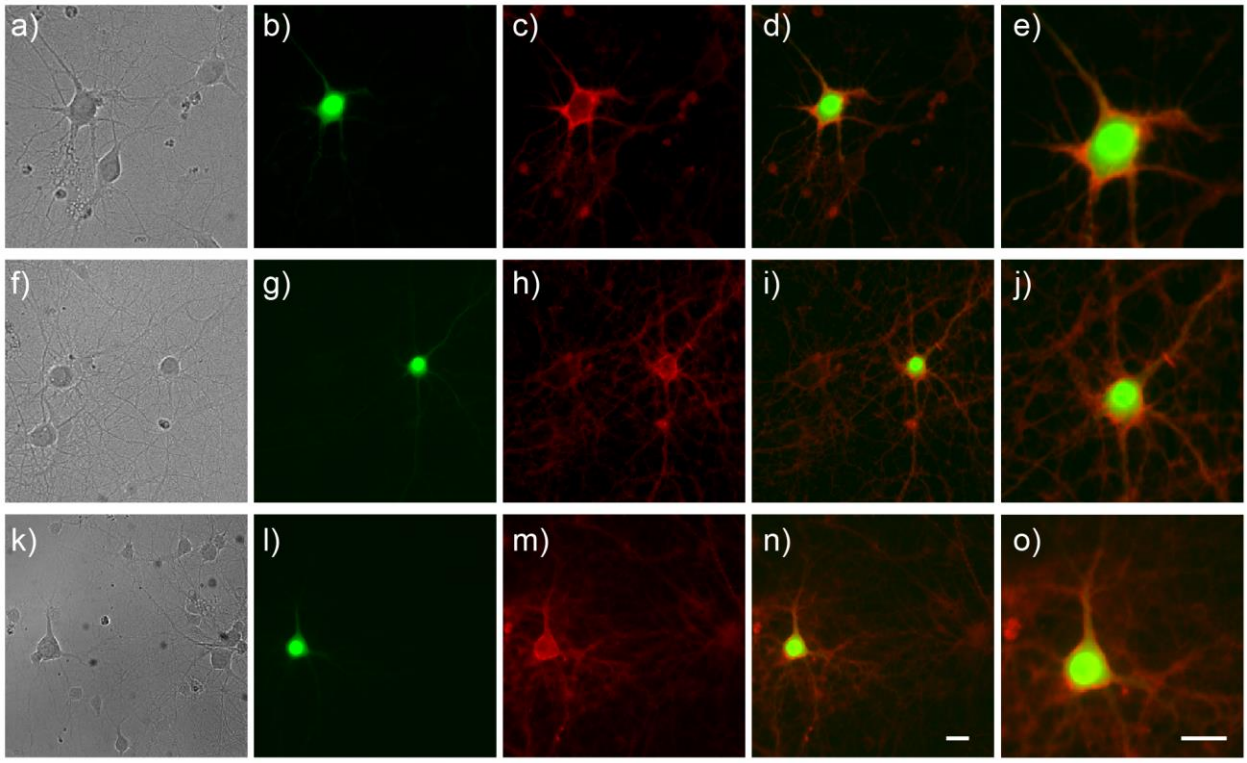


Figure S10. Fluorogenic labeling of specific neurons with carboVF-EX 1. a,f,k) Live cell, DIC image of neurons from different coverslips. b,g,l) PLE-expressing neurons are indicated by nuclear EGFP fluorescence. c,h,m) Fluorescence images of PLE-expressing neurons stained with 500 nM carboVF-EX 1. d,i,n) Overlay of carboVF-EX 1 and EGFP. e,j,o) Enlarged images of neurons in panels d, i, and n to show nuclear GFP and membrane-localized carboVF-EX 1. Scale bars are 20 μm.

Figure S11. Quantification of carboVF-EX 1 fluorogenic response in hippocampal neurons.

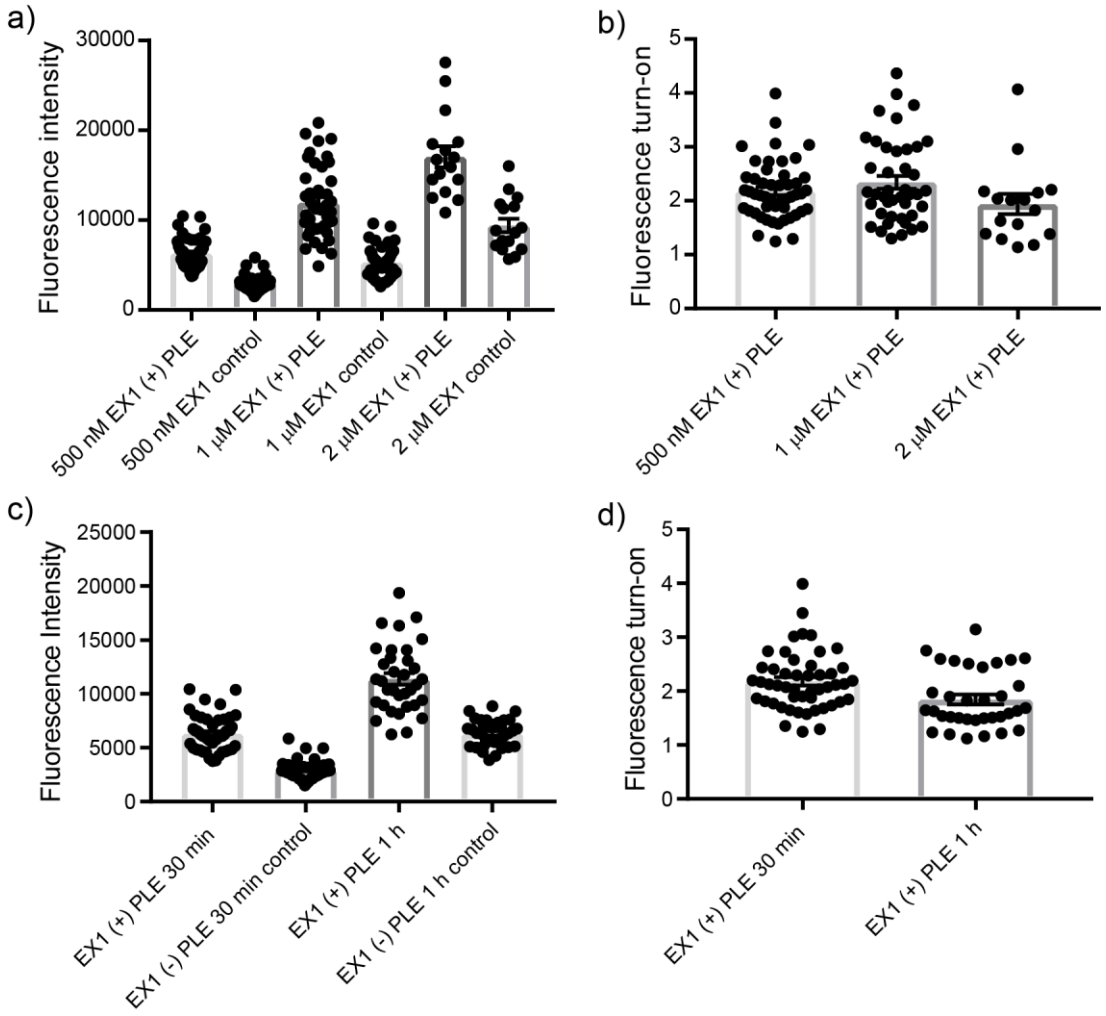


Figure S11. Quantification of the fluorescence turn-on in neurons stained with carboVF-EX 1. a) Comparison of the fluorescence intensity (pixel grey values, 16-bit scale) of neurons loaded with 500 nM, 1 µM, or 2 µM carboVF-EX 1 for 30 mins in PLE-expressing neurons. b) Comparison of the contrast (fold turn-on) for PLE-expressing neurons. c) Comparison of the fluorescence intensity or d) the contrast of neurons expressing PLE and loaded with 500 nM carboVF-EX 1 for 30 mins or 1 h. Each point in a-d) represents an individual neuron. Error bars are ± S.E.M.

Figure S12. Activity profiling with carboVF-EX 1 in neurons.

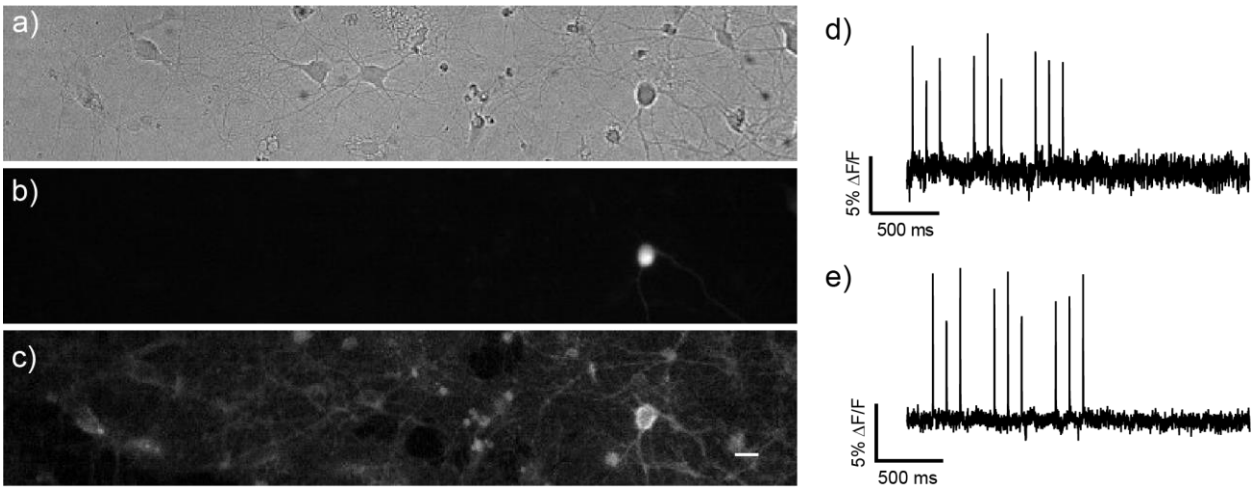


Figure S12. Field stimulation of neurons expressing PLE and stained with carboVF-EX 1. a) DIC Image of neurons sparsely transfected with PLE and stained with 500 nM carboVF-EX 1. b) EGFP fluorescence image of neurons from panel a. c) Fluorescence image indicating the selective uncaging of carboVF-EX 1 in the presence of PLE. Scale bar is 20 μm. d-e) Field stimulation of neurons expressing PLE and stained with d) 500 nM carboVF-EX 1 and e) 1 μM carboVF-EX 1.

Supporting Tables

Table S1. Properties of carbofluorescein fluorophores

compound	λ_{max}[a] / nm	λ_{em}[a] / nm	ε[a] / M^{-1} cm^{-1}	Φ[a,b]	pK_a[a]	h[a]
18	550	576	80,000	0.63	7.11 ± 0.02	0.92 ± 0.01
20	562	593	81,000	0.34	5.63 ± 0.02	1.02 ± 0.04
22	567	593	106,000	0.44	5.31 ± 0.02	0.99 ± 0.01

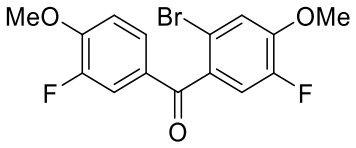
[a] Measured in HBSS (pH 7.4). [b] Relative measurement of quantum yield of fluorescence, referenced to Rhodamine B and Rhodamine 101.

Table S2. Properties of carboVF2.1(OMe).Cl and carboVF-EX dyes

				contrast[c]			% ΔF/F		SNR
dye	Φ [a]	K_m[b]	K_{cat}/K_m[b]	in vitro[d]	HEK[e]	neurons[e]	HEK[f]	neurons[g]	neurons
cVF **31**	0.012	---	---	---	---	---	31 ± 2	13.9 ± 1.4	28 ± 1.9
cVF-EX1	0.001	6.4 ± 1.4 μM	1.3 x 10^5 M^{-1} s^{-1}	45	8.9 ± 0.7	2.4 ± 0.08	18 ± 3	8.4 ± 0.4	12 ± 0.8
cVF-EX2	0.011	0.16 ± 0.08 μM	4.9 x 10^5 M^{-1} s^{-1}	21	---	---	---	---	---

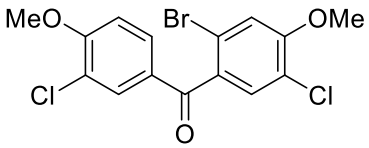
--- = not determined or not applicable. [a] Quantum yield of fluorescence. Measured in HBSS (pH 7.4). [b] Determined by reaction of dye with purified PLE in HBSS (pH 7.4). [c] Ratio of cVF-EX1 or cVF-EX2 fluorescence in PLE-expressing cells (or PLE-containing buffer, for *in vitro*) to cells not expressing PLE. [d] After 2 hours in buffer, with or without PLE. [e] After 30 min using 500 nM cVF-EX 1 at 37 °C. [f] Per 100 mV depolarization, in HEK cells. [g] Per action potential evoked by field stimulation.

Synthesis of Carbofluorescein dyes



(3) (2-bromo-5-fluoro-4-methoxyphenyl)(3-fluoro-4-methoxyphenyl)methanone:

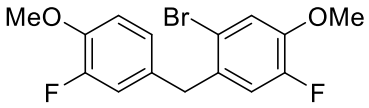
To a suspension of 3-fluoro-4-methoxybenzoic acid (0.50 g, 2.94 mmol) in anhydrous CH_2Cl_2 (2.0 mL) under nitrogen was added oxalyl chloride (0.60 g, 0.38 mL, 4.70 mmol, 1.6 eq), then anhydrous DMF (1 drop). The reaction mixture was stirred until a homogenous solution was observed. The solution was concentrated, azeotroped with CH_2Cl_2 (2x), and further dried under high vacuum. The resulting solid was dissolved in anhydrous CH_2Cl_2 (4 mL) and transferred to a flame-dried 2-neck round bottom flask. To this flask was added 5-bromo-2-fluoroanisole (0.60 g, 2.94 mmol, 1.0 eq) and it was cooled to 0 °C under nitrogen. Aluminum trichloride (0.53 g, 3.97 mmol, 1.35 eq) was added and the reaction was allowed to warm to room temperature and stirred for 20 h. The reaction was then diluted with CH_2Cl_2, poured onto ice water, and extracted with CH_2Cl_2 (3x). The organic extracts were combined, washed with water and sat. $NaHCO_3$, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (2-20% EtOAc/hexanes, linear gradient) to give **3** (0.44 g, 43%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, *J* = 11.6 Hz, 1H), 7.56 (d, *J* = 8.7 Hz, 1H), 7.23 (d, *J* = 7.4 Hz, 1H), 7.14 (d, *J* = 10.6 Hz, 1H), 7.02 (t, *J* = 8.3 Hz, 1H), 4.00 (s, 3H), 3.98 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 192.1, 152.6 (d, $^2J_{CF}$ = 10.8 Hz), 152.0 (d, $^1J_{CF}$ = 248.6 Hz), 151.1 (d, $^1J_{CF}$ = 250.0 Hz), 149.5 (d, $^2J_{CF}$ = 11.3 Hz), 132.3 (d, $^3J_{CF}$ = 4.9 Hz), 129.3 (d, $^3J_{CF}$ = 5.4 Hz), 128.1 (d, $^4J_{CF}$ = 3.2 Hz), 118.0, 117.3 (d, $^2J_{CF}$ = 19.0 Hz), 117.0 (d, $^2J_{CF}$ = 20.7 Hz), 114.5 (d, $^3J_{CF}$ = 3.8 Hz), δ 112.37 (d, ^3J = 1.5 Hz), 56.6, 56.4; HRMS (EI) calcd for $C_{15}H_{11}BrF_2O_3$ $[M\cdot]^+$ 355.9860, found 355.9861.



(4) (2-bromo-5-chloro-4-methoxyphenyl)(3-chloro-4-methoxyphenyl)methanone:

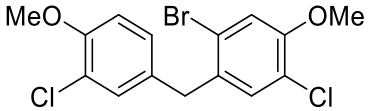
To a suspension of 3-chloro-4-methoxybenzoic acid (1.23 g, 6.58 mmol, 1.0 eq) in anhydrous CH_2Cl_2 (4.5 mL) under nitrogen was added oxalyl chloride (1.34 g, 0.9 mL, 10.5 mmol, 1.6 eq), then anhydrous DMF (1 drop). The reaction mixture was stirred until a homogenous solution was observed. The solution was concentrated, azeotroped with CH_2Cl_2 (2x), and dried under high vacuum. The resulting solid was taken up in anhydrous CH_2Cl_2 (9.1 mL) and transferred to a flame-dried 2-neck round bottom flask. To this flask was added 5-bromo-2-chloroanisole (1.46 g, 6.58 mmol, 1.0 eq) and it was cooled to 0 °C. Aluminum trichloride (1.18 g, 8.88 mmol, 1.35 eq) was added and the reaction was allowed to warm to room temperature and stirred for 20 h. The reaction was then diluted with CH_2Cl_2, poured onto ice water, and extracted with CH_2Cl_2 (3x). The organic extracts were combined, washed with water and sat. $NaHCO_3$, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (2-20% EtOAc/hexanes, linear gradient) to give **4** (1.12 g, 44%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, *J* = 2.2 Hz, 1H), 7.70 (dd, *J* = 8.6, 2.2 Hz, 1H), 7.40 (s, 1H), 7.20 (s, 1H),

7.00 (d, J = 8.7 Hz, 1H), 4.01 (s, 3H), 4.00 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 192.0, 159.4, 156.5, 132.9, 132.1, 131.0, 130.6, 129.7, 123.1, 122.0, 118.7, 116.7, 111.4, 56.7, 56.5; HRMS (EI) calcd for $C_{15}H_{11}BrCl_2O_3$ $[M\cdot]^+$ 387.9269, found 387.9273.



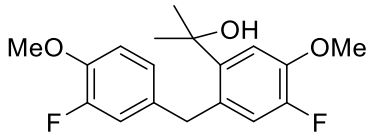
(5) 1-bromo-4-fluoro-2-(3-fluoro-4-methoxybenzyl)-5-methoxybenzene:

Trifluoroacetic acid (15 mL) was cooled to 0 °C under nitrogen. Sodium borohydride (0.78 g, 20.5 mmol, 6.8 eq) was added portionwise until it was almost completely dissolved. A solution of **3** (1.08 g, 3.04 mmol) in CH_2Cl_2 (9 mL) was added within 30 minutes and the reaction was stirred at room temperature for 18 h, after which a second portion of sodium borohydride (0.39 g, 10.3 mmol, 3.4 eq) was added at 0 °C and the reaction stirred for another 18 h at room temperature. It was then quenched with an excess of water under ice bath cooling and sodium hydroxide pellets were added with vigorous stirring until the solution showed a basic pH. The reaction mixture was extracted with EtOAc (3x) and the combined organic extracts were washed with water and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (5-10% EtOAc/hexanes, linear gradient; dry load on silica) to provide **5** as a white solid (0.87 g, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.19 (d, J = 8.0 Hz, 1H), 6.96 – 6.91 (m, 3H), 6.89 (d, J = 11.9 Hz, 1H), 3.97 (s, 2H), 3.91 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 153.2 (d, $^1J_{CF}$ = 245.9 Hz), 150.7 (d, $^1J_{CF}$ = 246.9 Hz), 146.7 (d, $^2J_{CF}$ = 11.6 Hz), 146.2 (d, $^2J_{CF}$ = 10.6 Hz), 132.7 (d, $^3J_{CF}$ = 5.8 Hz), 132.2 (d, $^3J_{CF}$ = 6.1 Hz), 124.4 (d, $^4J_{CF}$ = 3.5 Hz), 118.0, 117.7 (d, ^2J = 14.0 Hz), 117.7, 116.5 (d, ^2J = 18.4 Hz), 113.5 (d, ^3J = 1.7 Hz), 56.5, 56.3, 40.0; HRMS (EI) calcd for $C_{15}H_{13}BrF_2O_2$ $[M\cdot]^+$ 342.0067, found 342.0067.



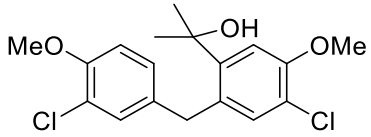
(6) 1-bromo-4-chloro-2-(3-chloro-4-methoxybenzyl)-5-methoxybenzene:

Trifluoroacetic acid (28 mL) was cooled to 0 °C under nitrogen. Sodium borohydride (1.45 g, 38.2 mmol, 6.8 eq) was added portionwise until it was almost completely dissolved. A solution of **4** (2.21 g, 5.65 mmol) in CH_2Cl_2 (17 mL) was added within 30 minutes and the reaction was stirred at room temperature for 18 h, after which a second portion of sodium borohydride (0.72 g, 19.1 mmol, 3.4 eq) was added at 0 °C and the reaction stirred for another 18 h at room temperature. It was then quenched with an excess of water under ice bath cooling and sodium hydroxide pellets were added with vigorous stirring until the solution showed a basic pH. The reaction mixture was extracted with EtOAc (3x) and the combined organic extracts were washed with water and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (5-10% EtOAc/hexanes, linear gradient; dry load on silica) to provide **6** as a white solid (1.71 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 (d, J = 2.1 Hz, 1H), 7.16 (s, 2H), 7.06 (dd, J = 8.4, 2.1 Hz, 1H), 6.89 (d, J = 8.4 Hz, 1H), 3.97 (s, 2H), 3.92 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.0, 153.6, 133.0, 132.4, 131.7, 130.5, 128.0, 122.6, 122.4, 121.8, 116.5, 112.1, 56.5, 56.2, 39.7. HRMS (EI) calcd for $C_{15}H_{13}BrCl_2O_2$ $[M\cdot]^+$ 373.9476, found 373.9481.



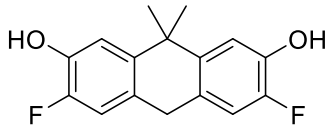
(7) 2-(4-fluoro-2-(3-fluoro-4-methoxybenzyl)-5-methoxyphenyl)propan-2-ol:

A solution of **5** (1.13 g, 3.29 mmol) in anhydrous THF (13 mL) was cooled to -78 °C under nitrogen. *n*-Butyllithium (1.6 M in hexanes, 4.1 mL, 6.59 mmol, 2 eq) was added dropwise via syringe, and the reaction stirred at -78 °C for 15 mins. Anhydrous acetone (0.72 mL, 9.88 mmol, 3 eq) was then added dropwise, and the reaction was stirred at -78 °C for 30 mins. The dry ice bath was removed, and the reaction was stirred at room temperature for 1 h. It was subsequently quenched with sat. NH_4Cl, diluted with water, and extracted with EtOAc (3x). The combined organic extracts were dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (10-40% EtOAc/hexanes, linear gradient) to yield **7** as a colorless oil (0.81 g, 76%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.15 (d, *J* = 8.9 Hz, 1H), 6.92 – 6.81 (m, 3H), 6.79 (d, *J* = 12.6 Hz, 1H), 4.26 (s, 2H), 3.92 (s, 3H), 3.88 (s, 3H), 1.67 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.4 (d, $^1J_{CF}$ = 245.4 Hz), 151.0 (d, $^1J_{CF}$ = 245.3 Hz), 145.7 (d, $^2J_{CF}$ = 10.8 Hz), 145.0 (d, $^2J_{CF}$ = 10.3 Hz), 142.2 (d, $^4J_{CF}$ = 3.5 Hz), 135.2 (d, $^3J_{CF}$ = 5.8 Hz), 131.2 (d, $^3J_{CF}$ = 5.5 Hz), 124.2 (d, $^3J_{CF}$ = 3.4 Hz), 119.9 (d, $^2J_{CF}$ = 18.0 Hz), 116.4 (d, $^2J_{CF}$ = 18.3 Hz), 113.4 (d, $^3J_{CF}$ = 1.9 Hz), 111.9 (d, $^4J_{CF}$ = 1.9 Hz), 73.5, 56.5, 56.3, 37.8, 31.9; HRMS (ESI) calcd for $C_{18}H_{19}F_2O_3$ $[M-H]^-$ 321.1381, found 321.2197. The NMR and HRMS agreed with reported values.[7]



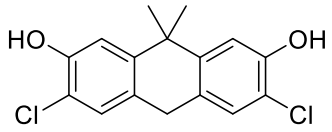
(8) 2-(4-chloro-2-(3-chloro-4-methoxybenzyl)-5-methoxyphenyl)propan-2-ol:

A solution of **6** (0.96 g, 2.55 mmol) in anhydrous THF (10 mL) was cooled to -78 °C under nitrogen. *n*-Butyllithium (1.6 M in hexanes, 3.2 mL, 5.10 mmol, 2 eq) was added dropwise via syringe, and the reaction stirred at -78 °C for 15 mins. Anhydrous acetone (0.94 mL, 12.8 mmol, 5 eq) was then added dropwise, and the reaction was stirred at -78 °C for 30 mins. The dry ice bath was removed, and the reaction was stirred at room temperature for 1 h. It was subsequently quenched with sat. NH_4Cl, diluted with water, and extracted with EtOAc (3x). The combined organic extracts were dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (10-40% EtOAc/hexanes, linear gradient) to yield **8** as a colorless oil (0.57 g, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.14 – 7.11 (m, 2H), 7.07 (s, 1H), 6.96 (dd, *J* = 8.4, 2.2 Hz, 1H), 6.86 (d, *J* = 8.4 Hz, 1H), 4.24 (s, 2H), 3.94 (s, 3H), 3.90 (s, 3H), 1.67 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 153.2, 152.9, 146.0, 135.2, 133.9, 131.0, 130.3, 127.9, 122.3, 120.8, 112.1, 110.1, 73.6, 56.2, 37.6, 31.8; HRMS (ESI) calcd for $C_{18}H_{21}Cl_2O_3$ $[M+H]^+$ 355.0862, found 355.0489.



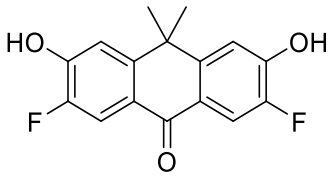
(9) 3,6-difluoro-9,9-dimethyl-9,10-dihydroanthracene-2,7-diol:

A solution of alcohol **7** (1.65 g, 5.12 mmol) in CH_2Cl_2 (39 mL) under nitrogen was cooled to 0 °C, and BBr_3 (1.0 M in CH_2Cl_2, 18.4 mL, 18.4 mmol, 3.6 eq) was added dropwise. The reaction was warmed to room temperature and stirred for 3 h. It was then carefully quenched with water and stirred for 30 mins. The reaction mixture was neutralized with sat. $NaHCO_3$ and extracted with CH_2Cl_2 (2x) and EtOAc (2x). The organic extracts were combined, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (10-50% EtOAc/hexanes, linear gradient; dry load on silica) to provide 1.24 g (87%) of **9** as an air sensitive orange-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.56 (s, 2H), 7.09 (d, J = 8.9 Hz, 2H), 7.02 (d, J = 11.6 Hz, 2H), 3.81 (s, 2H), 1.44 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 149.4 (d, $^1J_{CF}$ = 239.4 Hz), 143.2 (d, $^2J_{CF}$ = 12.1 Hz), 140.8 (d, $^4J_{CF}$ = 3.3 Hz), 127.0 (d, $^3J_{CF}$ = 6.1 Hz), 115.1 (d, $^2J_{CF}$ = 17.9 Hz), 114.4 (d, $^3J_{CF}$ = 2.9 Hz), 38.6, 33.2, 29.42; HRMS (EI) calcd for $C_{16}H_{14}F_2O_2$ $[M\cdot]^+$ 276.0962, found 276.0965. The NMR and HRMS agreed with reported values.[7]



(10) 3,6-dichloro-9,9-dimethyl-9,10-dihydroanthracene-2,7-diol:

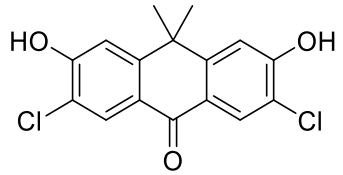
A solution of alcohol **8** (2.03 g, 5.71 mmol) in CH_2Cl_2 (44 mL) under nitrogen was cooled to 0 °C, and BBr_3 (1.0 M in CH_2Cl_2, 20.6 mL, 20.6 mmol, 3.6 eq) was added dropwise. The reaction was warmed to room temperature and stirred for 3 h. It was then carefully quenched with water and stirred for 30 mins. The reaction mixture was neutralized with sat. $NaHCO_3$ and extracted with CH_2Cl_2 (2x) and EtOAc (2x). The organic extracts were combined, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (10-50% EtOAc/hexanes, linear gradient; dry load on silica) to provide 1.53 g (86%) of **10** as an air sensitive orange-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.87 (s, 2H), 7.21 (s, 2H), 7.12 (s, 2H), 3.82 (s, 2H), 1.44 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 151.7, 144.5, 128.8, 127.9, 117.3, 113.1, 39.1, 32.8, 28.8; HRMS (EI) calcd for $C_{16}H_{14}Cl_2O_2$ $[M\cdot]^+$ 308.0371, found 308.0370.



(11) 2,7-difluoro-3,6-dihydroxy-10,10-dimethylanthracen-9(10*H*)-one:

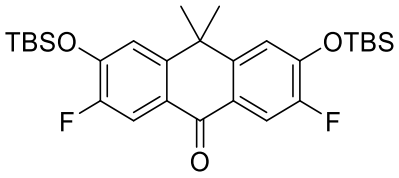
To a solution of phenol **9** (1.08 g, 3.92 mmol) in CH_2Cl_2 (33 mL) and dioxane (17 mL) was added water (4 mL). The mixture was cooled to 0 °C, and DDQ (2.67 g, 11.8 mmol, 3 eq) was added. The ice bath was removed and the reaction was stirred at room temperature overnight. The crude mixture was deposited onto Celite and concentrated *in vacuo*. Flash chromatography (10-100% EtOAc/hexanes, dry load with Celite) afforded 1.02 g (89%) of **11** as an off-white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.96 (s, 2H), 7.77 (d, J = 11.6 Hz, 2H), 7.30 (d, J = 8.1 Hz, 2H), 1.59 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 179.6, 150.7(d,

$^{2}J_{CF}$ = 12.8 Hz), 150.7 (d, $^{1}J_{CF}$ = 243.6 Hz), 148.7 (d, $^{4}J_{CF}$ = 2.9 Hz), 122.3 (d,^{3} J_{CF} = 4.9 Hz), 116.0 (d, $^{3}J_{CF}$ = 2.5 Hz), 113.6 (d, $^{2}J_{CF}$ = 18.3 Hz), 37.6, 33.2; HRMS (ESI) calcd for $C_{16}H_{11}F_2O_3$ [M-H]$^-$ 289.0682, 289.0678. The NMR and HRMS agreed with reported values.[7]



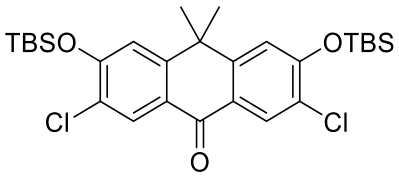
(12) 2,7-dichloro-3,6-dihydroxy-10,10-dimethylanthracen-9(10*H*)-one:

To a solution of phenol **10** (1.38 g, 4.46 mmol) in CH_2Cl_2 (37 mL) and dioxane (19 mL) was added water (5 mL). The mixture was cooled to 0 °C, and DDQ (3.04 g, 13.4 mmol, 3 eq) was added. The ice bath was removed and the reaction was stirred at room temperature overnight. The crude mixture was deposited onto Celite and concentrated *in vacuo*. Flash chromatography (10-100% EtOAc/hexanes, dry load with Celite) afforded 1.19 g (83%) of **12** as an off-white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.25 (s, 2H), 8.04 (s, 2H), 7.29 (s, 2H), 1.60 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 179.0, 158.1, 151.3, 128.8, 122.8, 120.4, 114.5, 37.7, 33.2; HRMS (ESI) calcd for $C_{16}H_{11}Cl_2O_3$ [M-H]$^-$ 321.0091, found 321.0085.



(13) 3,6-bis((*tert*-butyldimethylsilyl)oxy)-2,7-difluoro-10,10-dimethylanthracen-9(10*H*)-one:

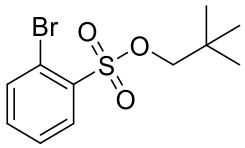
Phenol **11** (0.94 g, 3.23 mmol) was taken up in anhydrous DMF (22 mL) and imidazole (0.66 g, 9.69 mmol, 3 eq) and TBSCl (1.46 g, 9.69 mmol, 3 eq) were added. The reaction was stirred at room temperature for 3 h. It was then diluted with water and extracted with EtOAc (2x). The combined organic extracts were washed with water and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Flash chromatography on silica gel (0-10% EtOAc/hexanes, linear gradient) afforded 1.49 g (89%) of **13** as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, *J* = 11.1 Hz, 2H), 7.15 (d, *J* = 7.7 Hz, 2H), 1.69 (s, 6H), 1.07 (s, 18H), 0.30 (d, *J* = 1.3 Hz, 12H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 180.6, 153.1 (d, $^{1}J_{CF}$ = 246.6 Hz), 148.5 (d, $^{2}J_{CF}$ = 13.4 Hz), 147.6 (d, $^{4}J_{CF}$ = 3.0 Hz), 124.8 (d, $^{3}J_{CF}$ = 5.4 Hz), 120.0 (d, $^{3}J_{CF}$ = 1.9 Hz), 114.5 (d, $^{2}J_{CF}$ = 19.5 Hz), 37.3, 33.1, 25.6, 18.4, -4.6, -4.6; HRMS (EI) calcd for $C_{28}H_{40}F_2O_3Si_2$ [M·]$^+$ 518.2484, 518.2485. The NMR and HRMS agreed with reported values.[7]



(14) 3,6-bis((*tert*-butyldimethylsilyl)oxy)-2,7-dichloro-10,10-dimethylanthracen-9(10*H*)-one:

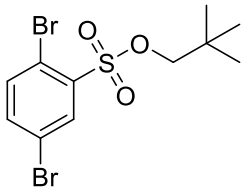
Phenol **12** (1.00 g, 3.09 mmol) was taken up in anhydrous DMF (21 mL) and imidazole (0.63 g, 9.28 mmol, 3 eq) and TBSCl (1.40 g, 9.28 mmol, 3 eq) were added. The reaction was stirred at room temperature for 3 h. It was then diluted with water and extracted with EtOAc (2x). The combined organic extracts were washed with water and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Flash

chromatography on silica gel (0-10% EtOAc/hexanes, linear gradient) afforded 1.5 g (88%) of **14** as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.37 (s, 2H), 7.11 (s, 2H), 1.69 (s, 6H), 1.10 (s, 18H), 0.34 (s, 12H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 180.1, 156.0, 150.2, 129.7, 125.6, 124.8, 118.1, 37.3, 33.0, 25.6, 18.5, -4.2; HRMS (EI) calcd for $C_{28}H_{40}Cl_2O_3Si_2$ $[M\cdot]^+$ 550.1893, found 550.1885.



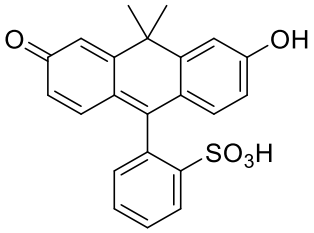
(16) neopentyl 2-bromobenzenesulfonate:

A solution of 2-bromobenzenesulfonyl chloride **34**[1] (1.00 g, 3.91 mmol) and neopentyl alcohol (0.86 g, 9.78 mmol, 2.5 eq) in CH_2Cl_2 (16 mL) was cooled to 0 °C, and 1,4-Diazabicyclo[2.2.2]octane (1.10 g, 9.78 mmol, 2.5 eq) was added. The reaction was stirred at room temperature for 30 min. The reaction mixture was then filtered, washed several times with CH_2Cl_2, and concentrated under reduced pressure. The crude residue was purified by flash chromatography on silica gel (0-10% EtOAc/hexanes, linear gradient) to afford **16** (0.55 g, 46%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 – 8.09 (m, 1H), 7.84 – 7.77 (m, 1H), 7.56 – 7.46 (m, 2H), 3.75 (s, 2H), 0.98 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 135.8, 135.7, 134.6, 132.1, 127.6, 120.8, 80.5, 31.7, 26.2; HRMS (EI) calcd for $C_{11}H_{15}BrO_3S$ $[M\cdot]^+$ 305.9925, found 305.9928.



(17) neopentyl 2,5-dibromobenzenesulfonate:

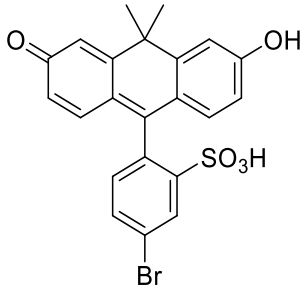
A solution of 2,5-dibromobenzenesulfonyl chloride **35**[2] (5.84 g, 17.5 mmol) and neopentyl alcohol (3.85 g, 43.7 mmol, 2.5 eq) in CH_2Cl_2 (70 mL) was cooled to 0 °C, and 1,4-Diazabicyclo[2.2.2]octane (4.90 g, 43.7 mmol, 2.5 eq) was added. The reaction was stirred at room temperature for 30 min. The reaction mixture was then filtered, washed several times with CH_2Cl_2, and concentrated under reduced pressure. The crude residue was purified by flash chromatography on silica gel (0-10% EtOAc/hexanes, linear gradient) to afford **17** (4.30 g, 64%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, J = 2.3 Hz, 1H), 7.68 (d, J = 8.4 Hz, 1H), 7.62 (dd, J = 8.4, 2.3 Hz, 1H), 3.80 (s, 2H), 1.01 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 137.5, 137.5, 136.9, 134.7, 121.4, 119.5, 81.0, 31.8, 26.1; HRMS (EI) calcd for $C_{11}H_{14}Br_2O_3S$ $[M\cdot]^+$ 383.9030, found 383.9032.



(18) 2-(6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)benzenesulfonic acid:

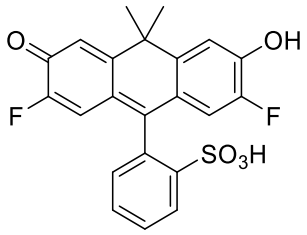
A solution of neopentyl 2-bromobenzenesulfonate **16** (96 mg, 0.31 mmol, 3 eq) in anhydrous CH_2Cl_2 (2.4 mL) under nitrogen was cooled to -16 °C. *n*-Butyllithium (1.6 M in hexanes, 0.19 mL, 0.31 mmol, 3 eq)

was added slowly via syringe, and the reaction was stirred at -16 °C for 15 min. Ketone **15**[3] (50 mg, 0.10 mmol) in anhydrous CH_2Cl_2 (2 mL) was then added dropwise. The reaction was allowed to gradually warm to room temperature overnight. It was then quenched with MeOH, acidified with TFA, and concentrated *in vacuo*. The crude residue was dissolved in minimal 1:4 DMSO/MeCN and purified by preparative HPLC to afford 13 mg (32%) of **18** as an orange solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.94 (d, *J* = 7.7 Hz, 1H), 7.57 (td, *J* = 7.6 Hz, 1H), 7.50 (td, *J* = 7.4 Hz, 1H), 7.13 (m, 3H), 6.97 (d, *J* = 9.1 Hz, 2H), 6.58 (dd, *J* = 9.1, 1.8 Hz, 2H), 1.70 (s, 3H), 1.57 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.2, 156.7, 156.7, 147.3, 139.1, 132.83, 130.0, 129.1, 128.7, 128.1, 123.9, 118.9, 117.2, 41.0, 35.0, 30.1; LRMS (ESI) calcd for $C_{22}H_{19}O_5S$ $[M+H]^+$ 395.1, found 395.1; HRMS (ESI) calcd for $C_{22}H_{17}O_5S$ $[M-H]^-$ 393.0802, found 393.0799.



(19) 5-bromo-2-(6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)benzenesulfonic acid:

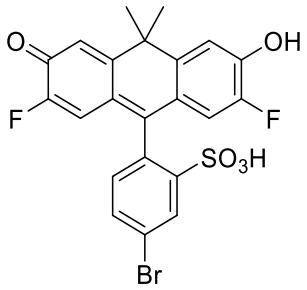
A solution of **17** (120 mg, 0.31 mmol, 3 eq) in anhydrous CH_2Cl_2 (2.4 mL) under nitrogen was cooled to -16 °C. *n*-Butyllithium (1.6 M in hexanes, 0.19 mL, 0.31 mmol, 3 eq) was added slowly via syringe, and the reaction was stirred at -16 °C for 15 min. Ketone **15**[3] (50 mg, 0.10 mmol) in anhydrous CH_2Cl_2 (2.1 mL) was then added dropwise. The reaction was allowed to gradually warm to room temperature overnight. The reaction mixture was quenched with MeOH and acidified with TFA. It was then diluted with brine and extracted with 1:5 iPrOH/CH_2Cl_2 (3x). The combined organic extracts were dried with sodium sulfate, filtered, concentrated *in vacuo*. The crude residue was purified by silica gel chromatography (2-20% MeOH/CH_2Cl_2, linear gradient; dry load on silica) to afford 10 mg (20%) of **19** as an orange solid. ^{1}H NMR (600 MHz, CD_3OD) δ 8.27 (d, *J* = 2.3 Hz, 1H), 7.77 (dd, *J* = 8.1, 2.2 Hz, 1H), 7.13 (d, *J* = 8.1 Hz, 1H), 6.96 (s, 2H), 6.92 (d, *J* = 9.2 Hz, 2H), 6.44 (d, *J* = 9.2 Hz, 2H), 1.70 (s, 3H), 1.64 (s, 3H); ^{13}C NMR (151 MHz, CD_3OD) δ 156.2, 155.2, 145.9, 137.3, 133.0, 132.5, 132.3, 130.6, 122.9, 122.0, 119.1, 117.7, 115.8, 39.9, 33.7, 30.2; HRMS (ESI) calcd for $C_{22}H_{16}BrO_5S$ $[M-H]^-$ 470.9907, found 470.9897.



(20) 2-(2,7-difluoro-6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)benzenesulfonic acid:

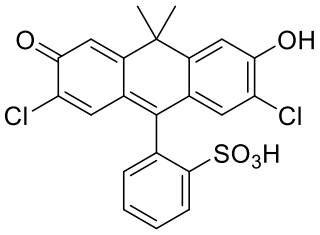
A solution of **16** (96 mg, 0.31 mmol, 3 eq) in anhydrous CH_2Cl_2 (2.4 mL) under nitrogen was cooled to -16 °C. *n*-Butyllithium (1.6 M in hexanes, 0.19 mL, 0.31 mmol, 3 eq) was added slowly via syringe, and the reaction was stirred at -16 °C for 15 min. Ketone **13** (50 mg, 0.10 mmol) in anhydrous CH_2Cl_2 (2.1 mL) was then added dropwise. The reaction was allowed to gradually warm to room temperature overnight. It

was then quenched with MeOH, acidified with TFA, and concentrated *in vacuo*. The crude residue was dissolved in minimal 1:4 DMSO/MeCN and purified by preparative HPLC to afford 23 mg (56%) of **20** as a dark green solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.97 (d, J = 7.7 Hz, 1H), 7.54 (dt, J = 24.6, 7.4 Hz, 2H), 7.14 (d, J = 7.3 Hz, 1H), 7.04 (d, J = 8.3 Hz, 2H), 6.41 (d, J = 12.8 Hz, 2H), 1.64 (s, 3H), 1.53 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.3, 155.5, 151.3 (d, ^{1}J = 248.8 Hz), 151.0, 147.1, 132.9, 130.2, 129.2, 129.1, 128.1, 123.6 (d, $^{3}J_{CF}$ = 6.4 Hz), 119.9, 118.5 (d, $^{2}J_{CF}$ = 18.6 Hz), 39.8, 35.0, 30.9; LRMS (ESI) calcd for $C_{22}H_{17}F_2O_5S$ $[M+H]^+$ 431.1, found 431.0; HRMS (ESI) calcd for $C_{22}H_{15}F_2O_5S$ $[M-H]^-$ 429.0614, found 429.0610.



(21) 5-bromo-2-(2,7-difluoro-6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)benzenesulfonic acid:

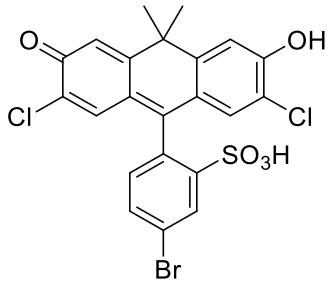
A solution of **17** (120 mg, 0.31 mmol, 3 eq) in anhydrous CH_2Cl_2 (2.4 mL) under nitrogen was cooled to -16 °C. *n*-Butyllithium (1.6 M in hexanes, 0.19 mL, 0.31 mmol, 3 eq) was added slowly via syringe, and the reaction was stirred at -16 °C for 15 min. Ketone **13** (50 mg, 0.10 mmol) in anhydrous CH_2Cl_2 (2.1 mL) was then added dropwise. The reaction was allowed to gradually warm to room temperature overnight. The reaction mixture was quenched with MeOH and acidified with TFA. It was then diluted with brine and extracted with 1:5 iPrOH/CH_2Cl_2 (3x). The combined organic extracts were dried with sodium sulfate, filtered, concentrated *in vacuo*. The crude residue was purified by silica gel chromatography (2-20% MeOH/CH_2Cl_2, linear gradient; dry load on silica) to afford 33 mg (67%) of **21** as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.33 (d, J = 1.9 Hz, 1H), 7.83 (dd, J = 8.1, 2.0 Hz, 1H), 7.20 (d, J = 8.1 Hz, 1H), 7.10 (d, J = 8.0 Hz, 2H), 6.59 (d, J = 12.1 Hz, 2H), 1.70 (s, 3H), 1.65 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 163.9, 155.6, 152.3, 151.7 (d, $^{1}J_{CF}$ = 249.4 Hz), 145.7, 133.0, 132.4, 132.1, 130.7, 123.3 (d, $^{3}J_{CF}$ = 6.6 Hz), 122.7, 119.5, 118.4 (d, $^{2}J_{CF}$ = 19.2 Hz), 39.8, 33.7, 30.6; HRMS (ESI) calcd for $C_{22}H_{14}BrF_2O_5S$ $[M-H]^-$ 506.9719, found 506.9712.



(22) 2-(2,7-dichloro-6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)benzenesulfonic acid:

A solution of **16** (84 mg, 0.27 mmol, 3 eq) in anhydrous CH_2Cl_2 (2.1 mL) under nitrogen and cooled to -16 °C. *n*-Butyllithium (1.6 M in hexanes, 0.17 mL, 0.27 mmol, 3 eq) was added slowly via syringe, and the reaction was stirred at -16 °C for 15 min. Ketone **14** (50 mg, 0.09 mmol) in anhydrous CH_2Cl_2 (1.8 mL) was then added dropwise. The reaction mixture was allowed to gradually warm to room temperature

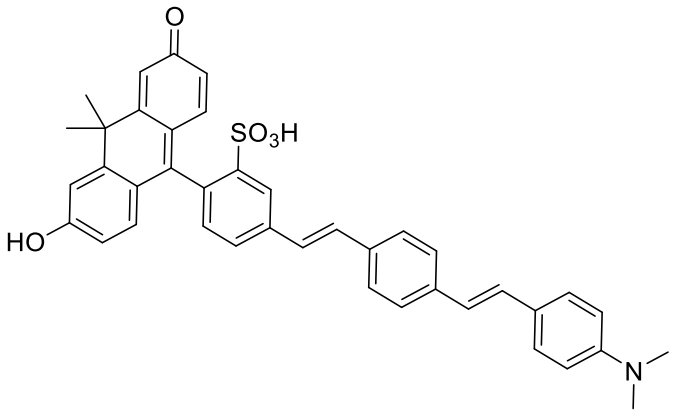
overnight. It was then quenched with MeOH, acidified with TFA, and concentrated *in vacuo*. The crude residue was dissolved in minimal 1:4 DMSO/MeCN and purified by preparative HPLC to afford **22** (23 mg, 55%) as a dark green solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.97 (d, J = 7.7 Hz, 1H), 7.56 (dt, J = 24.0, 7.4 Hz, 2H), 7.16 (d, J = 7.3 Hz, 1H), 7.05 (s, 2H), 6.80 (s, 2H), 1.65 (s, 3H), 1.53 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.1, 152.5, 147.1, 134.9, 132.5, 130.3, 129.3, 129.2, 128.1, 124.3, 124.0, 118.8, 35.2, 30.1; LRMS (ESI) calcd for $C_{22}H_{17}Cl_2O_5S$ $[M+H]^+$ 463.0, found 463.0 ; HRMS (ESI) calcd for $C_{22}H_{15}Cl_2O_5S$ $[M-H]^-$ 461.0023, found 461.0022.



(23) 5-bromo-2-(2,7-dichloro-6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)benzenesulfonic acid:

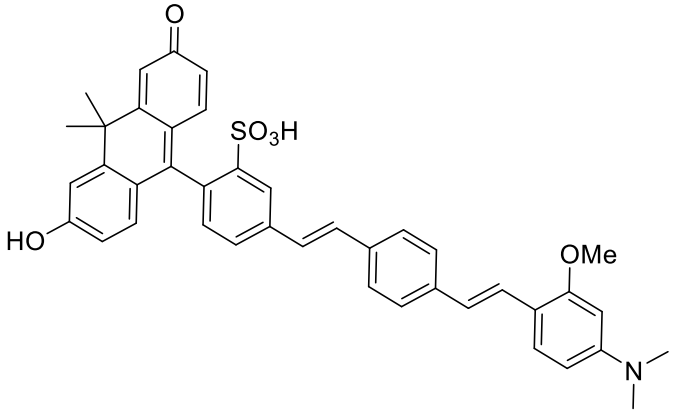
A solution of **17** (120 mg, 0.31 mmol, 3 eq) in anhydrous CH_2Cl_2 (2.4 mL) under nitrogen and cooled to -16 °C. *n*-Butyllithium (1.6 M in hexanes, 0.19 mL, 0.31 mmol, 3 eq) was added slowly via syringe, and the reaction was stirred at -16 °C for 15 min. Ketone **14** (50 mg, 0.10 mmol) in anhydrous CH_2Cl_2 (2.1 mL) was then added dropwise. The reaction was allowed to gradually warm to room temperature overnight. The reaction mixture was quenched with MeOH and acidified with TFA. It was then diluted with brine and extracted with 1:5 iPrOH/CH_2Cl_2 (3x). The combined organic extracts were dried with sodium sulfate, filtered, concentrated *in vacuo*. Flash column chromatography on silica gel (2-20% MeOH/CH_2Cl_2, linear gradient; dry load on silica) afforded **23** (59 mg, 60%) as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.36 (d, J = 2.0 Hz, 1H), 7.82 (dd, J = 8.1, 2.0 Hz, 1H), 7.19 (d, J = 8.1 Hz, 1H), 7.09 (s, 2H), 6.99 (s, 2H), 1.71 (s, 3H), 1.64 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 168.5, 154.4, 153.5, 145.9, 135.0, 132.9, 132.2, 132.0, 130.8, 124.6, 123.8, 122.8, 118.5, 39.7, 33.9, 30.0; HRMS (ESI) calcd for $C_{22}H_{14}BrCl_2O_5S$ $[M-H]^-$ 538.9128, found 538.9123.

Synthesis of Carbofluorescein VoltageFluors



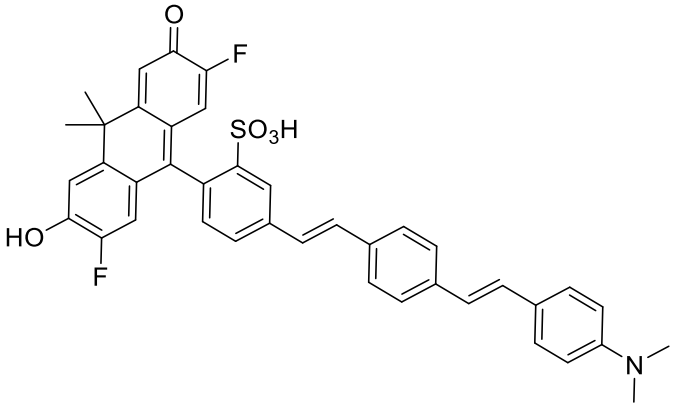
(26) 5-((*E*)-4-((*E*)-4-(dimethylamino)styryl)styryl)-2-(6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)benzenesulfonic acid:

A vial was charged with **19** (19 mg, 40.2 μmol), styrene **24**[4] (10 mg, 40.2 μmol, 1.0 eq), $Pd(OAc)_2$ (4.5 mg, 20.1 μmol, 0.5 eq), and $P(o\text{-}tol)_3$ (12 mg, 40.2 μmol, 1.0 eq). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (0.4 mL) was added and the vial was evacuated/backfilled again with nitrogen (3x). Anhydrous Et_3N (0.2 mL) was added and the reaction was sealed and stirred at 75 °C for 24 h. It was then cooled, diluted with MeOH, and acidified with cold concentrated HCl. After filtration, the solvent was removed under reduced pressure. The crude residue was dissolved in minimal 1:4 DMSO/MeCN and purified by preparative HPLC to afford **26** (8 mg, 31%) as a dark red solid. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.12 (s, 1H), 7.72 (d, *J* = 7.0 Hz, 1H), 7.66 (d, *J* = 8.0 Hz, 2H), 7.57 (d, *J* = 8.0 Hz, 2H), 7.47 (d, *J* = 8.5 Hz, 2H), 7.42 (d, *J* = 16.4 Hz, 1H), 7.35 (d, *J* = 16.3 Hz, 1H), 7.20 (d, *J* = 16.3 Hz, 1H), 7.11 (d, *J* = 7.7 Hz, 1H), 7.04 – 6.99 (m, 3H), 6.95 (d, *J* = 9.1 Hz, 2H), 6.78 (d, *J* = 7.9 Hz, 2H), 6.49 (d, *J* = 8.6 Hz, 2H), 2.96 (s, 6H), 1.68 (s, 3H), 1.55 (s, 3H); ^{13}C NMR (226 MHz, DMSO-d_6) δ 155.0, 149.6, 147.4, 137.9, 137.5, 137.4, 135.3, 131.8, 130.3, 129.3, 128.8, 127.6, 127.1, 126.8, 126.2, 125.7, 125.6, 123.5, 123.3, 118.8, 117.1, 112.6, 40.2, 40.1, 34.6, 29.8; LRMS (ESI) calcd for $C_{40}H_{36}NO_5S$ $[M+H]^+$ 642.2, found 642.5; HRMS (ESI) calcd for $C_{40}H_{34}NO_5S$ $[M-H]^-$ 640.2163, found 640.2147.



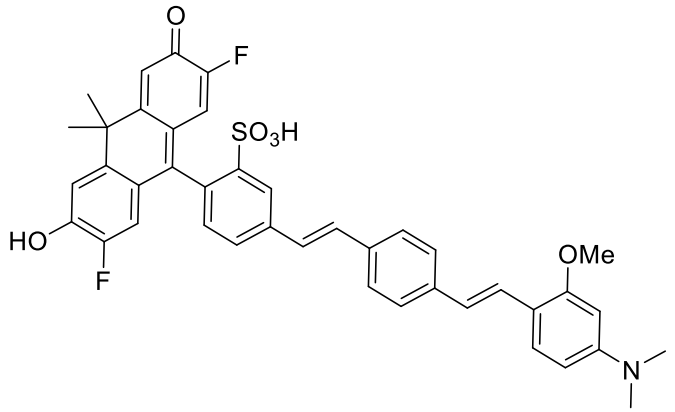
(27) 5-((*E*)-4-((*E*)-4-(dimethylamino)-2-methoxystyryl)styryl)-2-(6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)benzenesulfonic acid:

A vial was charged with **19** (15 mg, 31.8 μmol), styrene **25**[5] (8.9 mg, 31.8 μmol, 1.0 eq), $Pd(OAc)_2$ (3.6 mg, 15.9 μmol, 0.5 eq), and $P(o\text{-}tol)_3$ (9.7 mg, 31.8 μmol, 1.0 eq). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (0.4 mL) was added and the vial was evacuated/backfilled again with nitrogen (3x). Anhydrous Et_3N (0.2 mL) was added and the reaction was sealed and stirred at 75 °C for 24 h. It was then cooled, diluted with MeOH, and acidified with cold concentrated HCl. After filtration, the solvent was removed under reduced pressure. The crude residue was dissolved in minimal 1:4 DMSO/MeCN and purified by preparative HPLC, concentrated, and triturated with MeOH to afford 5 mg (24%) of **27** as a dark red solid. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.13 (s, 1H), 7.77 (d, *J* = 7.3 Hz, 1H), 7.67 (d, *J* = 7.9 Hz, 2H), 7.54 (d, *J* = 8.2 Hz, 3H), 7.44 (d, *J* = 16.4 Hz, 1H), 7.41 – 7.37 (m, 2H), 7.23 (s, 2H), 7.19 (d, *J* = 7.6 Hz, 1H), 7.17 (d, *J* = 9.1 Hz, 2H), 7.05 (d, *J* = 16.4 Hz, 1H), 6.72 (d, *J* = 9.2 Hz, 2H), 6.45 (d, *J* = 6.7 Hz, 1H), 6.41 (s, 1H), 3.89 (s, 3H), 3.01 (s, 6H), 1.74 (s, 3H), 1.60 (s, 3H); ^{13}C NMR (226 MHz, DMSO-d_6) δ 171.5, 157.8, 157.6, 150.6, 147.4, 139.7, 138.1, 138.0, 135.1, 131.0, 129.9, 129.7, 127.3, 127.2, 126.6, 126.1, 125.6, 125.6, 123.9, 123.7, 123.4, 118.2, 118.1, 116.4, 105.6, 96.2, 55.4, 41.0, 40.5, 34.3, 29.8; LRMS (ESI) calcd for $C_{41}H_{38}NO_6S$ $[M+H]^+$ 672.2, found 672.5; HRMS (ESI) calcd for $C_{41}H_{36}NO_6S$ $[M-H]^-$ 670.2269, found 670.2269.



(28) 2-(2,7-difluoro-6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)-5-((*E*)-4-((*E*)-4-(dimethylamino)styryl)styryl)benzenesulfonic acid:

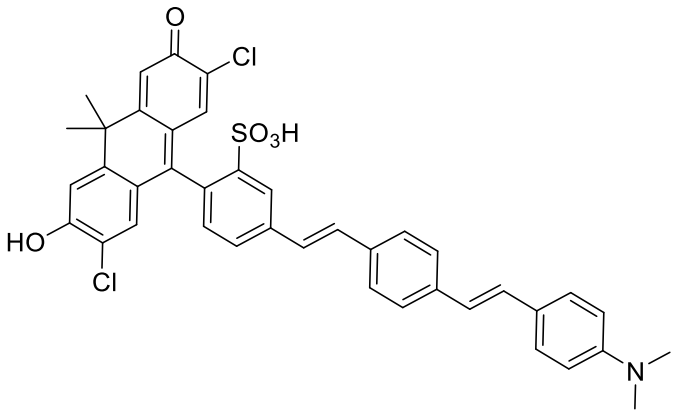
A vial was charged with **21** (27 mg, 53.0 μmol), styrene **24**[4] (13 mg, 53.0 μmol, 1.0 eq), $Pd(OAc)_2$ (5.9 mg, 26.5 μmol, 0.5 eq), and $P(o\text{-}tol)_3$ (16 mg, 53.0 μmol, 1.0 eq). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (0.6 mL) was added and the vial was evacuated/backfilled again with nitrogen (3x). Anhydrous Et_3N (0.3 mL) was added and the reaction was sealed and stirred at 75 °C for 24 h. It was then cooled, diluted with MeOH, and acidified with cold concentrated HCl. After filtration, the solvent was removed under reduced pressure. The crude residue was dissolved in minimal 1:4 DMSO/MeCN and purified by preparative HPLC to afford **28** (10 mg, 28%) as an orange solid. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.14 (d, *J* = 1.8 Hz, 1H), 7.75 (d, *J* = 7.4 Hz, 1H), 7.67 (d, *J* = 7.8 Hz, 2H), 7.58 (d, *J* = 7.8 Hz, 2H), 7.49 (d, *J* = 8.1 Hz, 2H), 7.43 (d, *J* = 16.4 Hz, 1H), 7.37 (d, *J* = 16.4 Hz, 1H), 7.21 (d, *J* = 16.3 Hz, 1H), 7.14 (d, *J* = 7.7 Hz, 1H), 7.07 – 7.01 (m, 3H), 6.81 (s, 2H), 6.48 (d, *J* = 12.7 Hz, 2H), 2.96 (s, 6H), 1.64 (s, 3H), 1.53 (s, 3H); ^{13}C NMR (226 MHz, DMSO-d_6) δ 155.0, 151.4, 150.3, 147.2, 137.6, 137.5, 135.3, 130.8 (d, *J* = 254.9 Hz), 129.5, 128.7, 127.6, 127.1, 126.8, 126.3, 126.1, 125.6, 123.2, 118.0 (d, *J* = 18.7 Hz), 112.7, 39.9, 39.8, 34.5, 30.6; LRMS (ESI) calcd for $C_{40}H_{34}F_2NO_5S$ $[M+H]^+$ 678.2, found 677.8; HRMS (ESI) calcd for $C_{40}H_{32}F_2NO_5S$ $[M-H]^-$ 676.1975, found 676.1956.



(29) 2-(2,7-difluoro-6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)-5-((*E*)-4-((*E*)-4-(dimethylamino)-2-methoxystyryl)styryl)benzenesulfonic acid:

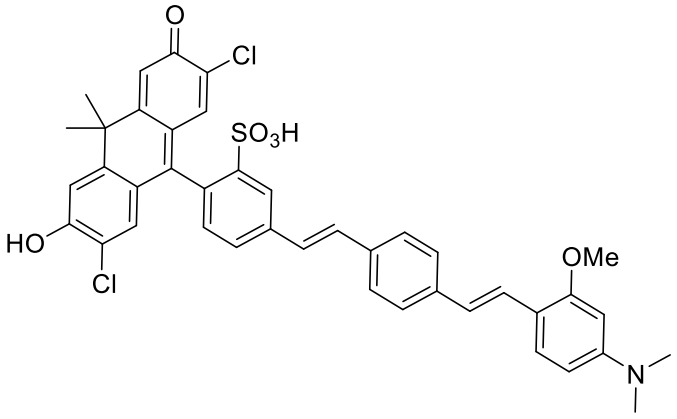
A vial was charged with **21** (34 mg, 66.8 μmol), styrene **25**[5] (19 mg, 66.8 μmol, 1.0 eq), $Pd(OAc)_2$ (7.5 mg, 33.4 μmol, 0.5 eq), and $P(o\text{-}tol)_3$ (20 mg, 66.8 μmol, 1.0 eq). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (0.7 mL) was added and the vial was evacuated/backfilled again with

nitrogen (3x). Anhydrous Et_3N (0.4 mL) was added and the reaction was sealed and stirred at 75 °C for 24 h. It was then cooled, diluted with MeOH, and acidified with cold concentrated HCl. After filtration, the solvent was removed under reduced pressure. The crude residue was dissolved in minimal 1:4 DMSO/MeCN and purified by preparative HPLC to afford 16 mg (34%) of **29** as an orange solid. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.14 (s, 1H), 7.75 (d, *J* = 8.7 Hz, 1H), 7.66 (d, *J* = 7.8 Hz, 2H), 7.57 – 7.52 (m, 3H), 7.42 (d, *J* = 16.4 Hz, 1H), 7.40 – 7.35 (m, 2H), 7.14 (d, *J* = 7.7 Hz, 1H), 7.09 – 7.02 (m, 3H), 6.49 (d, *J* = 12.8 Hz, 4H), 3.89 (s, 3H), 3.01 (s, 6H), 1.64 (s, 3H), 1.53 (s, 3H); ^{13}C NMR (226 MHz, DMSO-d_6) δ 157.7, 155.0, 151.4, 150.3, 147.2, 137.9, 137.6, 135.2, 130.8 (d, *J* = 253.4 Hz), 129.5, 127.3, 127.2, 126.8, 126.2, 126.1, 125.7, 123.2, 123.2, 119.4, 118.0 (d, *J* = 18.4 Hz), 55.4, 40.4, 39.8, 34.5, 30.6; LRMS (ESI) calcd for $C_{41}H_{36}F_2NO_6S$ $[M+H]^+$ 708.2, found 708.2; HRMS (ESI) calcd for $C_{41}H_{34}F_2NO_6S$ $[M-H]^-$ 706.2080, found 706.2067.



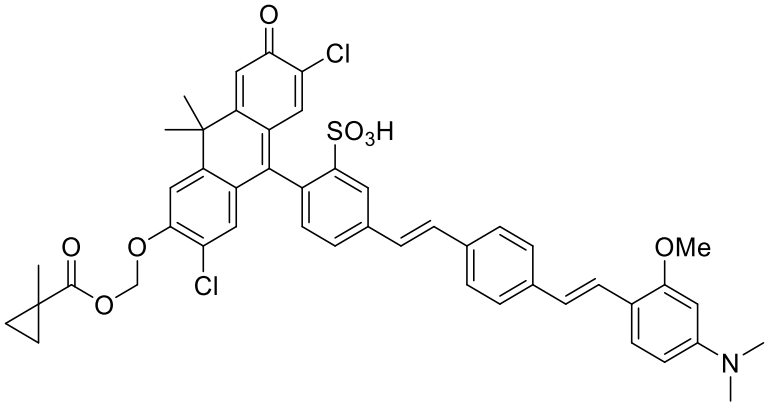
(30) 2-(2,7-dichloro-6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)-5-((*E*)-4-((*E*)-4-(dimethylamino)styryl)styryl)benzenesulfonic acid:

A vial was charged with **23** (20 mg, 36.9 μmol), styrene **24**[4] (9.2 mg, 36.9 μmol, 1.0 eq), $Pd(OAc)_2$ (4.1 mg, 18.4 μmol, 0.5 eq), and P(o-tol)$_3$ (11 mg, 36.9 μmol, 1.0 eq). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (0.4 mL) was added and the vial was evacuated/backfilled again with nitrogen (3x). Anhydrous Et_3N (0.2 mL) was added and the reaction was sealed and stirred at 75 °C for 24 h. It was then cooled, diluted with MeOH, and acidified with TFA. After filtration, the solvent was removed under reduced pressure. The crude residue was dissolved in minimal 1:4 DMSO/MeCN and purified by preparative HPLC to afford **30** (13 mg, 50%) as an orange solid. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.14 (s, 1H), 7.76 (d, *J* = 7.8 Hz, 1H), 7.67 (d, *J* = 7.7 Hz, 2H), 7.58 (d, *J* = 7.8 Hz, 2H), 7.48 (d, *J* = 8.2 Hz, 2H), 7.43 (d, *J* = 16.4 Hz, 1H), 7.38 (d, *J* = 16.4 Hz, 1H), 7.21 (d, *J* = 16.3 Hz, 1H), 7.16 (d, *J* = 7.7 Hz, 1H), 7.04 (m, 3H), 6.87 (s, 2H), 6.78 (d, *J* = 8.0 Hz, 2H), 2.96 (s, 6H), 1.65 (s, 3H), 1.53 (s, 3H); ^{13}C NMR (226 MHz, DMSO-d_6) δ 154.7, 149.6, 147.2, 137.7, 137.6, 135.2, 131.0, 130.4, 129.7, 128.8, 127.6, 127.1, 126.8, 126.2, 126.2, 125.6, 123.8, 123.5, 118.1, 112.7, 112.6, 112.6, 40.2, 39.9, 34.7, 29.8; LRMS (ESI) calcd for $C_{40}H_{34}Cl_2NO_5S$ $[M+H]^+$ 710.2, found 710.0; HRMS (ESI) calcd for $C_{40}H_{32}Cl_2NO_5S$ $[M-H]^-$ 708.1384, found 708.1372.



(31) 2-(2,7-dichloro-6-hydroxy-10,10-dimethyl-3-oxo-3,10-dihydroanthracen-9-yl)-5-((*E*)-4-((*E*)-4-(dimethylamino)-2-methoxystyryl)styryl)benzenesulfonic acid:

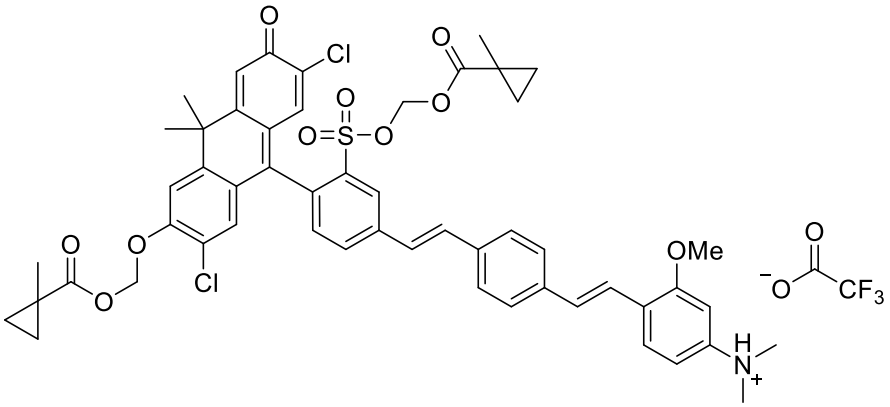
A vial was charged with **23** (59 mg, 109 μmol), styrene **25**[5] (33 mg, 120 μmol, 1.1 eq), $Pd(OAc)_2$ (9.8 mg, 43.5 μmol, 0.4 eq), and $P(o\text{-}tol)_3$ (26 mg, 87 μmol, 0.8 eq). The vial was sealed and evacuated/backfilled with nitrogen (3x). Anhydrous DMF (1.2 mL) was added and the vial was evacuated/backfilled again with nitrogen (3x). Anhydrous Et_3N (0.6 mL) was added and the reaction was sealed and stirred at 75 °C for 24 h. It was then cooled, diluted with MeOH, and acidified with TFA. After filtration, the solvent was removed under reduced pressure. The crude residue was dissolved in minimal 1:4 DMSO/MeCN and purified by preparative HPLC to afford 43 mg (53%) of **31** as an orange solid. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.14 (s, 1H), 7.76 (d, *J* = 7.2 Hz, 1H), 7.67 (d, *J* = 7.8 Hz, 2H), 7.59-7.52 (m, 4H), 7.43 (d, *J* = 16.4 Hz, 1H), 7.40 – 7.36 (m, 2H), 7.17 (d, *J* = 7.7 Hz, 1H), 7.10-7.04 (m, 3H), 6.87 (s, 2H), 6.54 (s, 2H), 3.89 (s, 3H), 3.03 (s, 6H), 1.65 (s, 3H), 1.54 (s, 3H); ^{13}C NMR (226 MHz, DMSO-d_6) δ 157.7, 154.6, 152.0, 147.2, 137.9, 137.7, 135.3, 134.5, 131.0, 130.4, 129.6, 127.3, 127.2, 126.8, 126.2, 126.2, 125.6, 123.8, 123.1, 118.4, 55.5, 39.9, 39.8, 34.7, 29.8; LRMS (ESI) calcd for $C_{41}H_{36}Cl_2NO_6S$ $[M+H]^+$ 740.2, found 740.2; HRMS (ESI) calcd for $C_{41}H_{34}Cl_2NO_6S$ $[M-H]^-$ 738.1489, found 738.1480.



(32) 2-(2,7-dichloro-10,10-dimethyl-6-(((1-methylcyclopropane-1-carbonyl)oxy)methoxy)-3-oxo-3,10-dihydroanthracen-9-yl)-5-((*E*)-4-((*E*)-4-(dimethylamino)-2-methoxystyryl)styryl)benzenesulfonic acid:

To a solution of **31** (25 mg, 0.034 mmol) in anhydrous DMF was added anhydrous diisopropylethylamine (26.5 uL, 0.152 mmol, 4.5 equiv), followed by iodomethyl 1-methylcyclopropanecarboxylate[6] (1.0 M solution in DMF, 33.8 uL, 0.034 mmol, 1.0 eq). The reaction was stirred under nitrogen and monitored by

LCMS. After one hour approximately 50% of the starting material had been consumed. An additional 0.75 equivalents of iodomethyl 1-methylcyclopropanecarboxylate were added. One hour later, an additional 0.2 equivalents of iodomethyl 1-methylcyclopropanecarboxylate were added. The reaction was then neutralized with acetic acid and diluted with 2 mL of MeCN. The diluted reaction was purified by semi-preparative HPLC to obtain 21 mg (74%) of **32** as a light orange solid. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.14 (s, 1H), 7.77 (d, J = 7.8 Hz, 1H), 7.66 (d, J = 8.0 Hz, 2H), 7.60 (s, 1H), 7.55 – 7.48 (m, 3H), 7.44 – 7.35 (m, 3H), 7.20 (d, J = 7.7 Hz, 1H), 7.05 (s, 1H), 7.02 (d, J = 16.4 Hz, 1H), 6.85 (s, 1H), 6.77 (s, 1H), 6.39 (s, 1H), 6.34 (s, 1H), 6.00 (s, 2H), 3.88 (s, 3H), 2.98 (s, 6H), 1.72 (s, 3H), 1.60 (s, 3H), 1.25 (s, 3H), 1.15 (ddd, J = 24.5, 9.6, 3.1 Hz, 2H), 0.82 (d, J = 3.2 Hz, 2H); ^{13}C NMR (226 MHz, DMSO-d_6) δ 177.6, 173.8, 157.8, 155.8, 153.4, 153.4, 147.8, 147.3, 138.2, 137.9, 136.6, 135.0, 131.7, 130.7, 130.4, 129.8, 129.5, 127.6, 127.2, 127.2, 126.6, 126.3, 126.1, 125.5, 123.9, 123.5, 123.5, 120.1, 113.3, 84.9, 55.3, 34.2, 29.9, 18.6, 18.3, 16.7, 16.7; LRMS (ESI) calcd for $C_{47}H_{44}Cl_2NO_8S$ $[M+H]^+$ 852.2, found 852.4; HRMS (ESI) calcd for $C_{47}H_{42}Cl_2NO_8S$ $[M-H]^-$ 850.2014, found 850.1996.

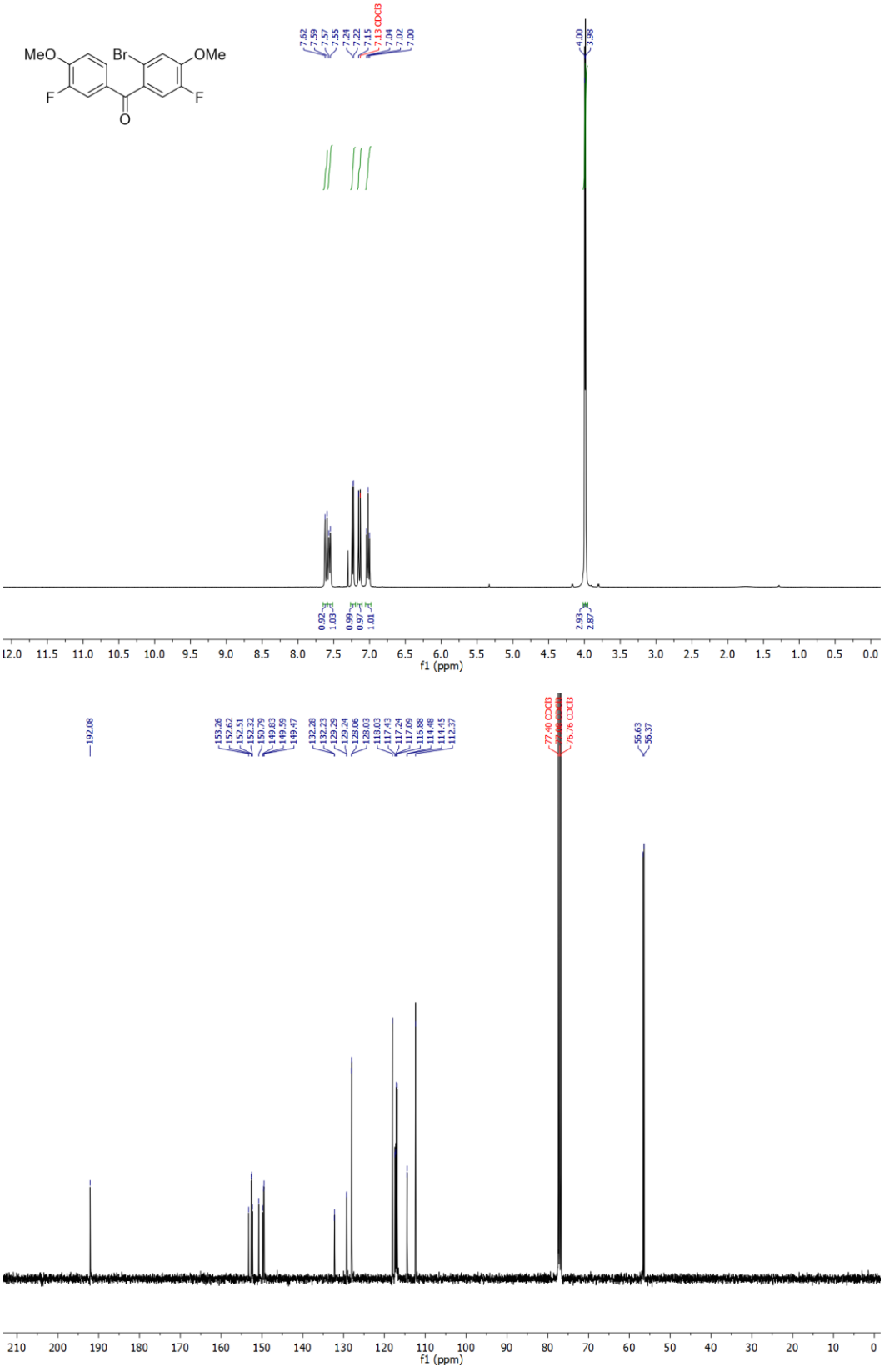


(33) (((2-(2,7-dichloro-10,10-dimethyl-6-(((1-methylcyclopropane-1-carbonyl)oxy)methoxy)-3-oxo-3,10-dihydroanthracen-9-yl)-5-((*E*)-4-((*E*)-4-(dimethylamino)-2-methoxystyryl)styryl)phenyl)sulfonyl)oxy)methyl 1-methylcyclopropane-1-carboxylate:

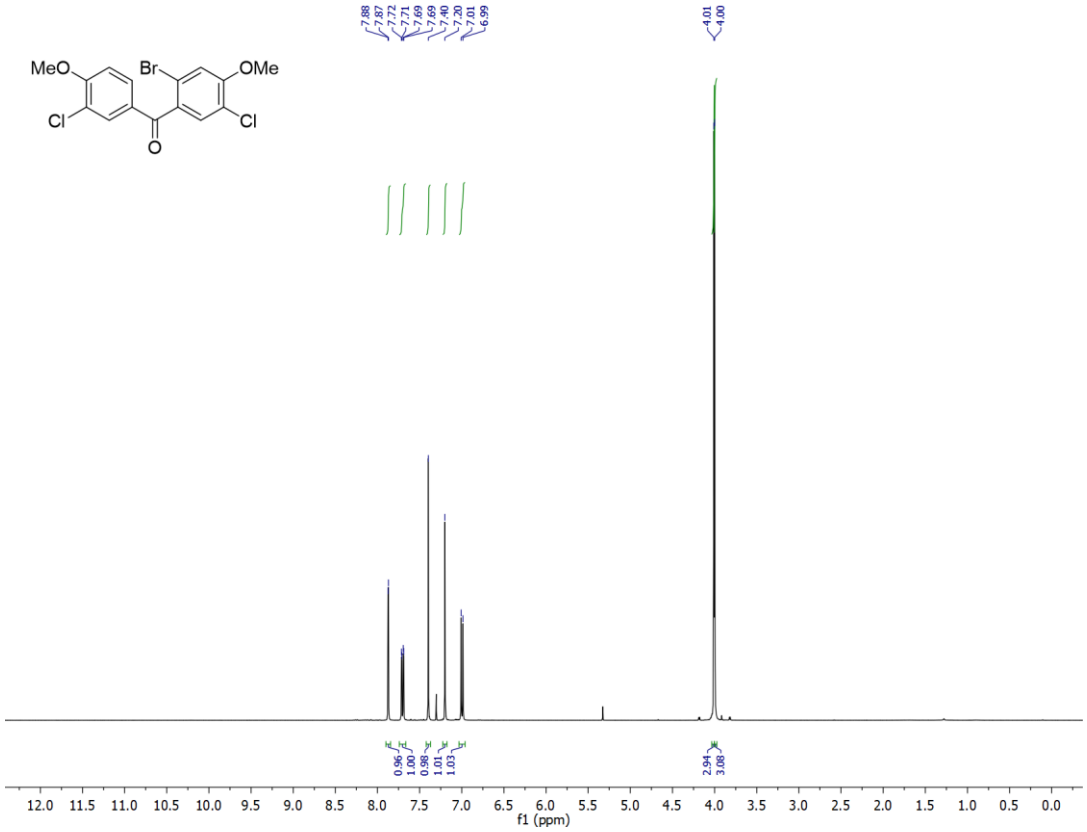
To a solution of **31** (11 mg, 0.015 mmol) in anhydrous DMF was added anhydrous diisopropylethylamine (16 uL, 0.089 mmol, 6 equiv), followed by iodomethyl 1-methylcyclopropanecarboxylate[6] (5.0 M solution in DMF, 18 uL, 0.089 mmol, 6 equiv). The reaction was stirred under nitrogen and monitored by LCMS. After two hours, approximately 50% of the starting material had been consumed. An additional 6 equivalents of iodomethyl 1-methylcyclopropanecarboxylate were added. One hour later, an additional 6 equivalents of anhydrous diisopropylethylamine and iodomethyl 1-methylcyclopropanecarboxylate were added. The reaction was then neutralized with acetic acid and diluted with 2 mL of MeCN. The diluted reaction was purified by semi-preparative HPLC to obtain 5 mg (31%) of **33** as a light orange solid. ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.16 (d, J = 6.3 Hz, 1H), 7.92 (d, J = 8.5 Hz, 1H), 7.79 (d, J = 7.8 Hz, 1H), 7.74 (d, J = 7.8 Hz, 2H), 7.66 (d, J = 7.8 Hz, 2H), 7.60 (s, 1H), 7.50 (m, 2H), 7.45 (s, 1H), 7.42 (d, J = 16.3 Hz, 1H), 7.22 (d, J = 7.7 Hz, 1H), 7.05 (s, 1H), 6.85 (s, 1H), 6.77 (s, 1H), 6.57 (s, 2H), 6.31 (s, 1H), 6.00 (s, 2H), 5.77 (s, 1H), 3.98 (s, 3H), 3.67 (s, 6H), 1.72 (s, 3H), 1.60 (s, 3H), 1.25 (s, 3H), 1.23 (s, 2H), 1.17 – 1.12 (m, 5H), 0.98 (m, 2H), 0.82 (m, 2H), 0.79 (m, 2H); ^{13}C NMR (226 MHz, DMSO-d_6) δ 177.5, 173.8, 172.4, 156.9, 155.8, 153.4, 153.3, 147.9, 147.3, 142.5, 137.7, 136.8, 136.6, 136.5, 131.7, 131.4, 131.0, 130.4, 129.5, 127.7, 127.7, 127.5, 127.3, 127.2, 127.0, 126.4, 125.7, 123.9, 123.5, 120.9, 120.1, 113.7, 113.3, 105.5, 84.8, 84.0, 56.6, 51.2, 40.4, 39.9, 34.2, 29.9, 18.6, 18.3, 18.3, 18.0, 17.0, 16.7, 16.6; LRMS (ESI) calcd for $C_{53}H_{52}Cl_2NO_{10}S$ $[M+H]^+$ 964.3, found 964.9; HRMS (ESI) calcd for $C_{53}H_{51}Cl_2NO_{10}S$ $[M+Na]^+$ 986.2503, found 986.2522.

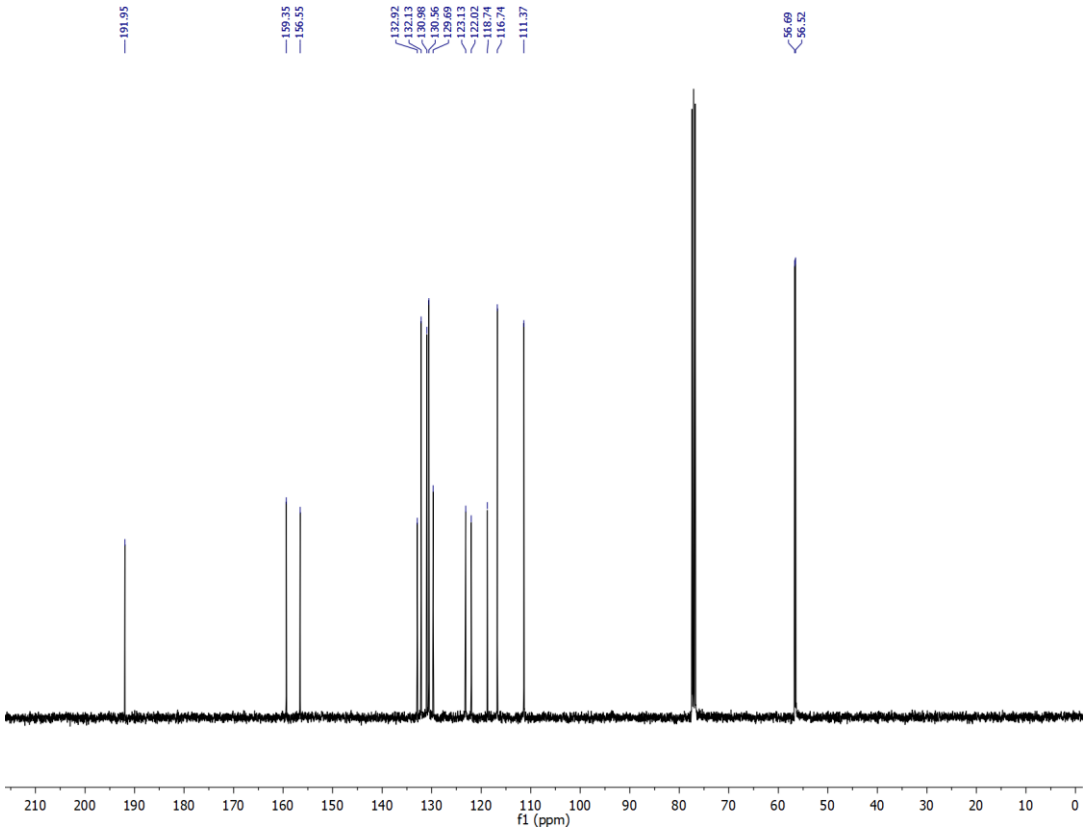
Spectra of Compounds

^{1}H and ^{13}C NMR of compound **3**

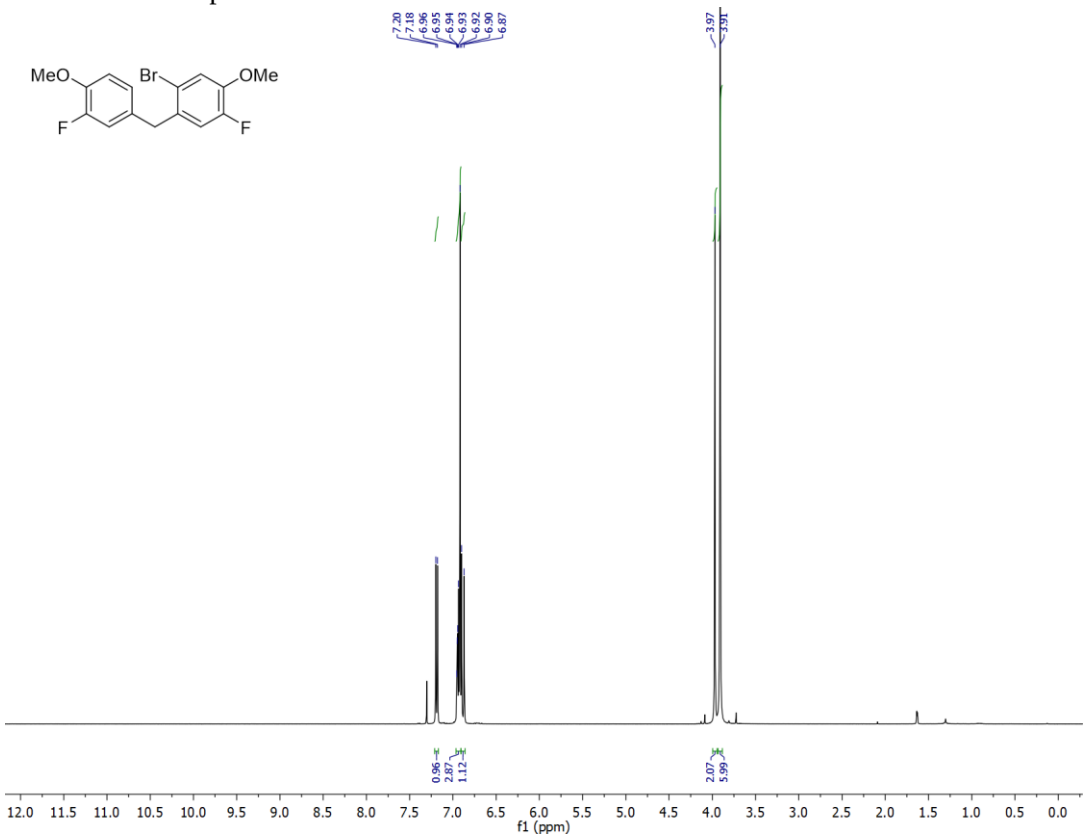


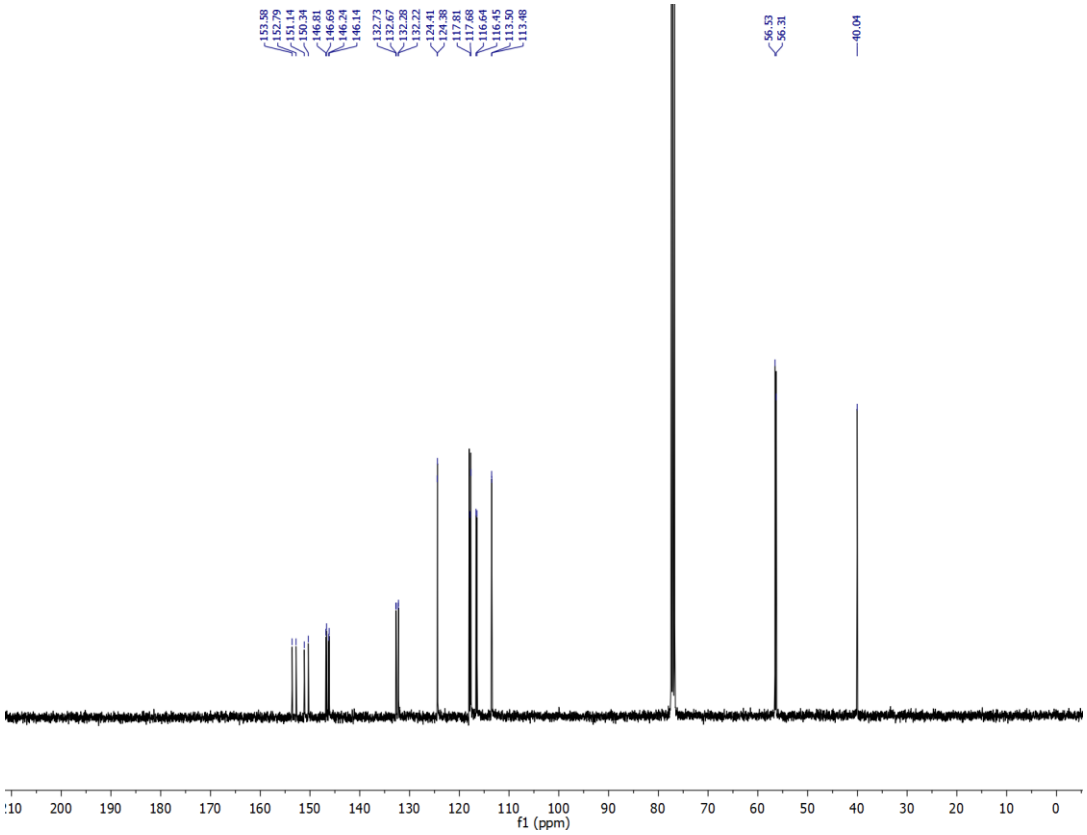
^{1}H and ^{13}C NMR of compound **4**



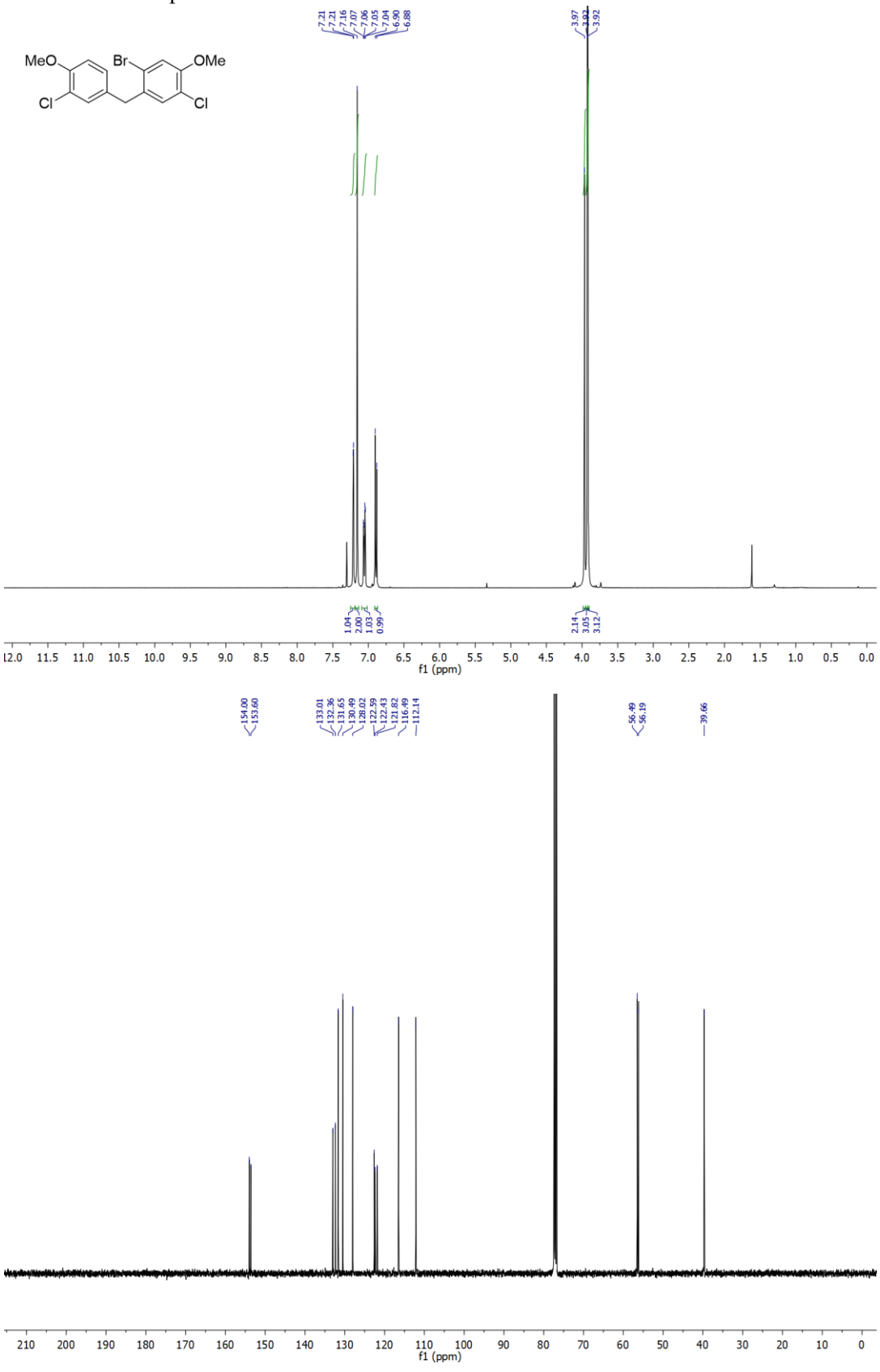


^{1}H and ^{13}C NMR of compound **5**

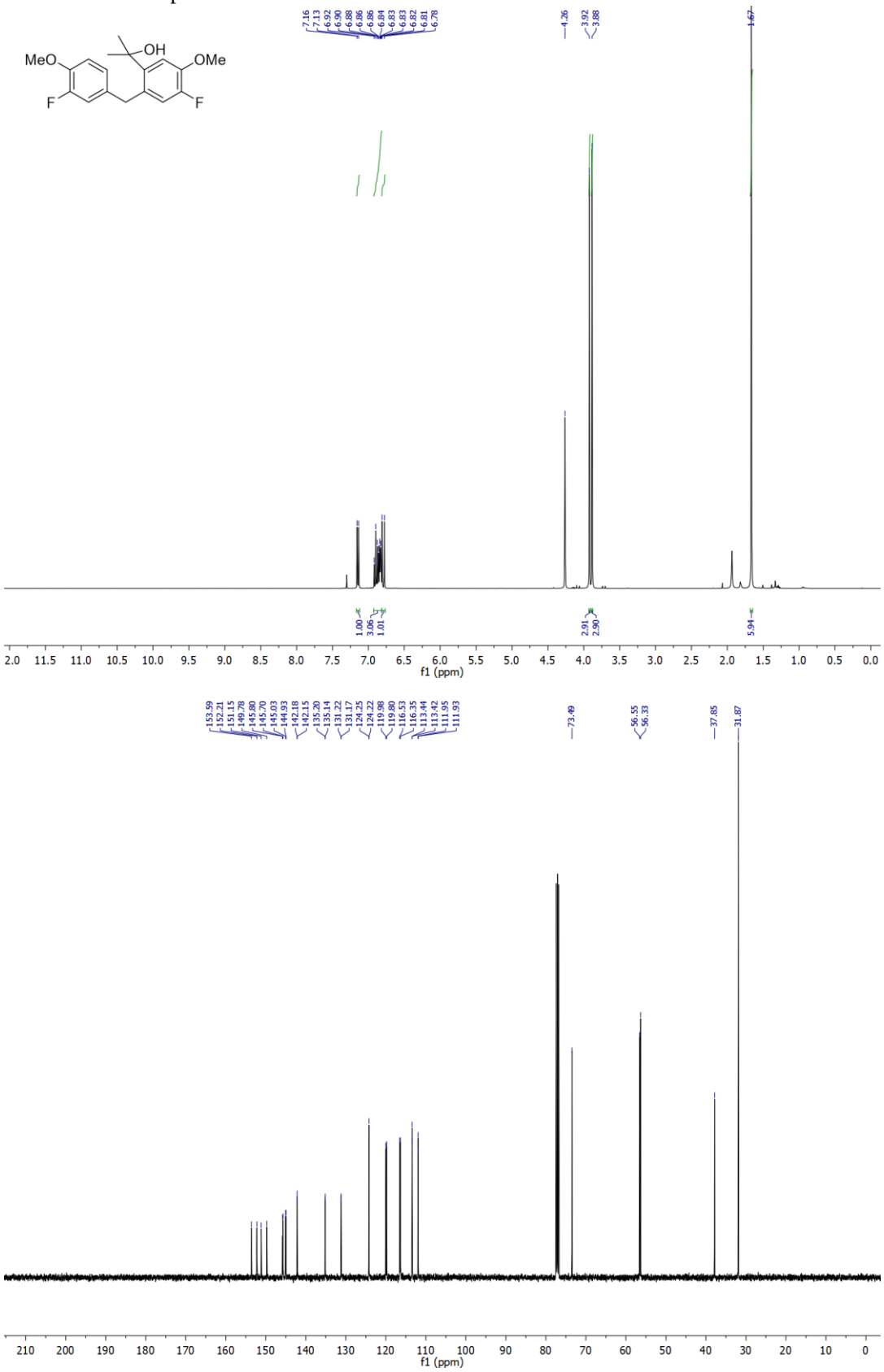




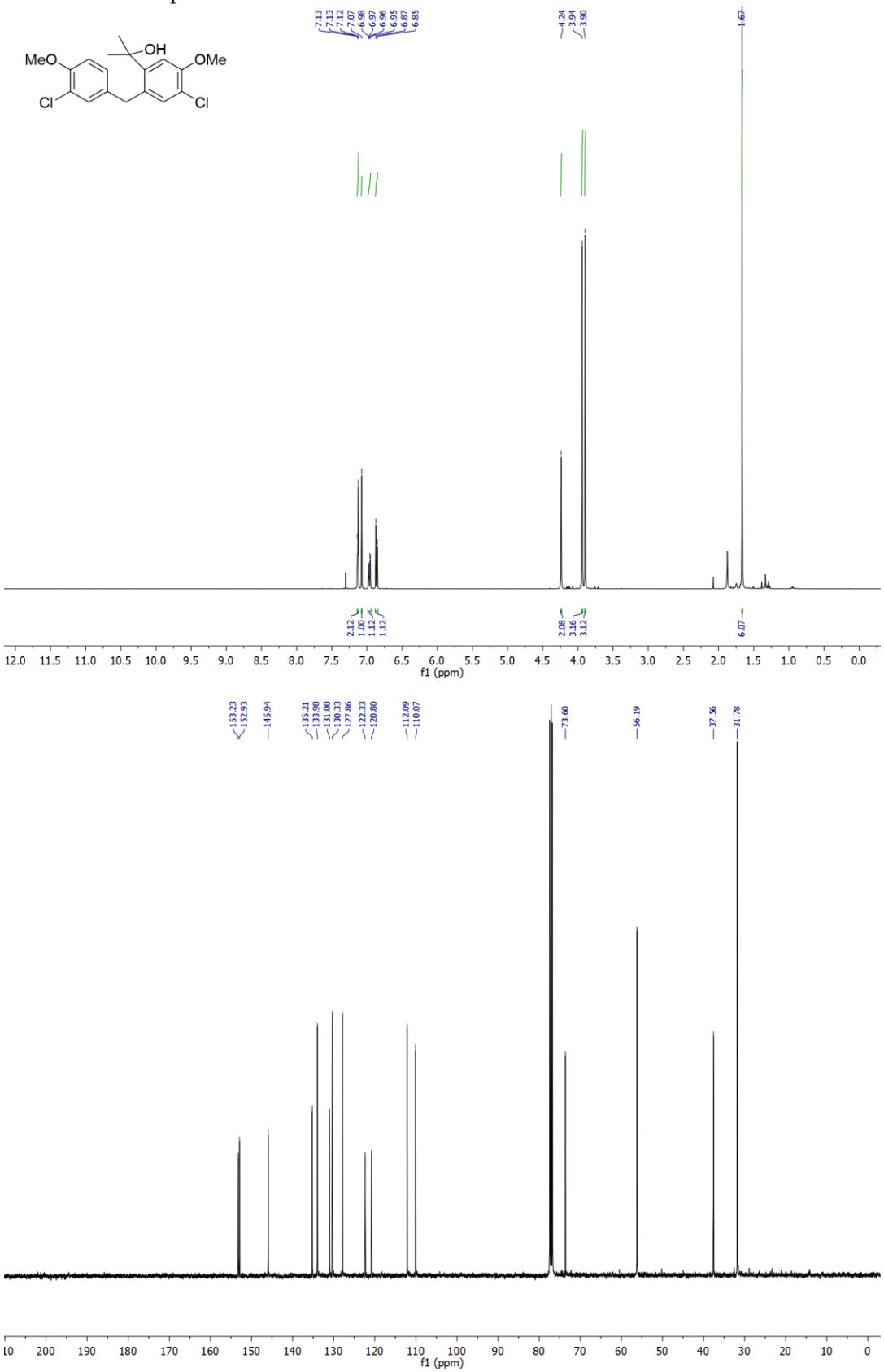
^{1}H and ^{13}C NMR of compound **6**



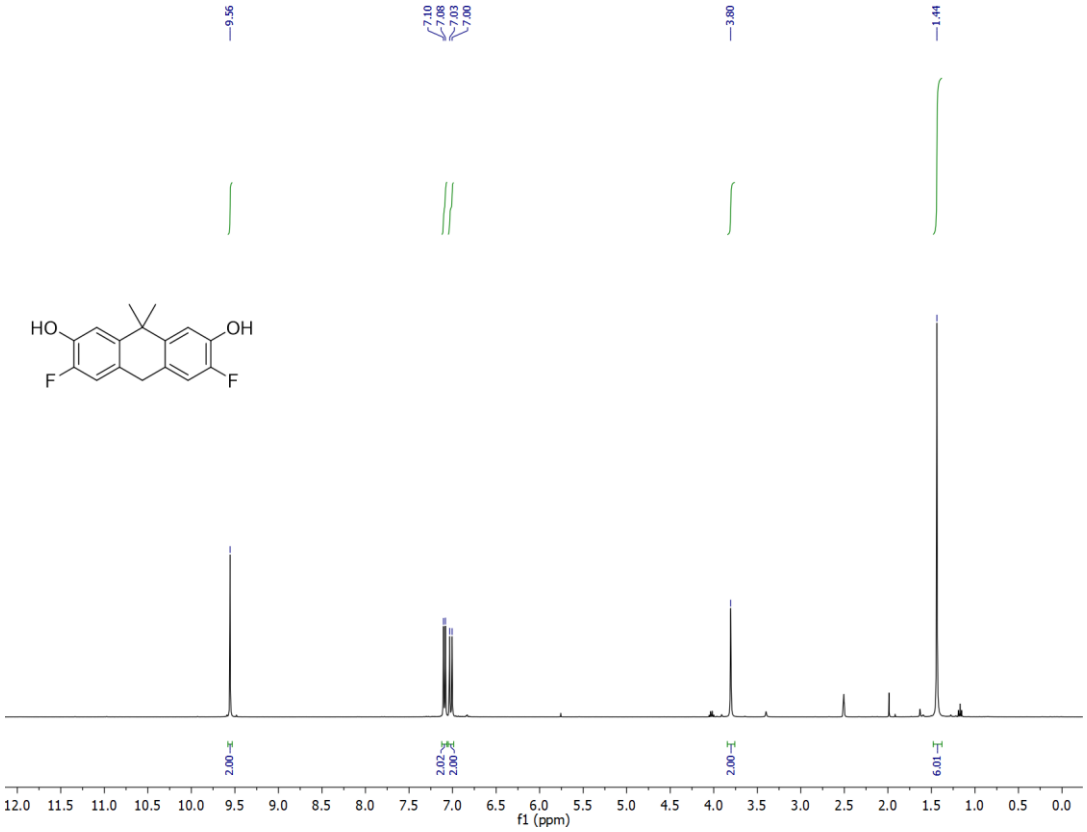
^{1}H and ^{13}C NMR of compound **7**

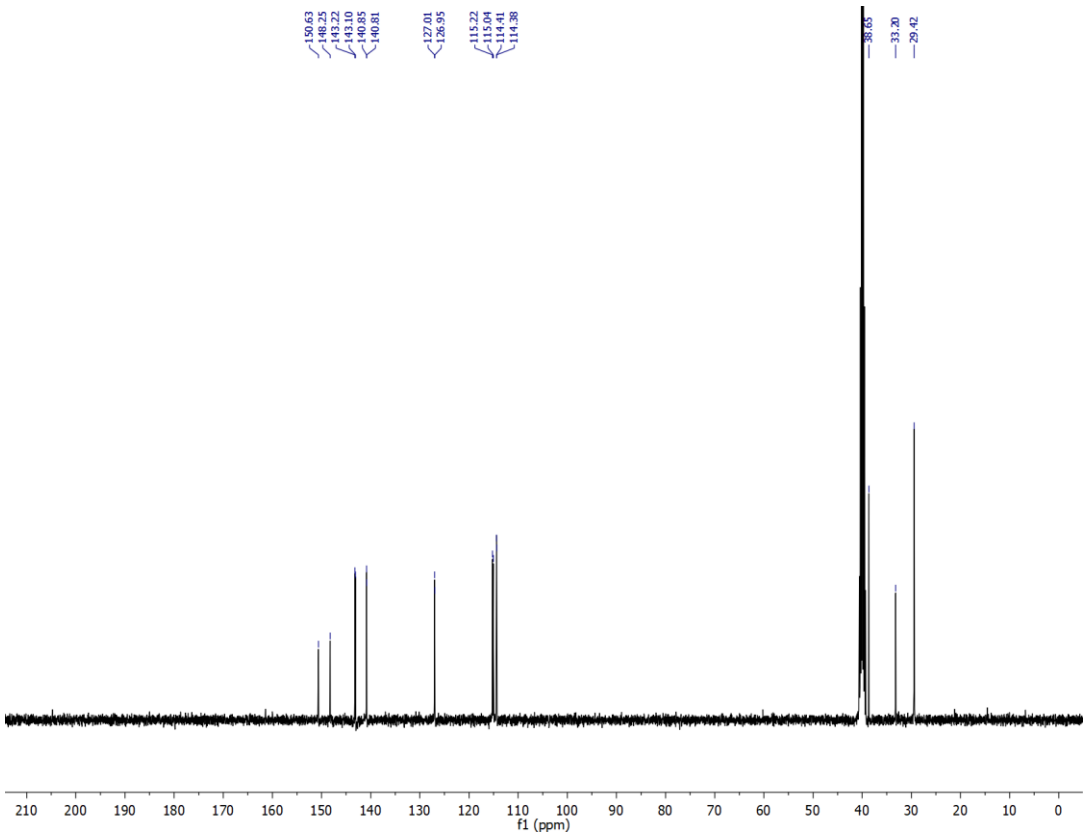


^{1}H and ^{13}C NMR of compound **8**

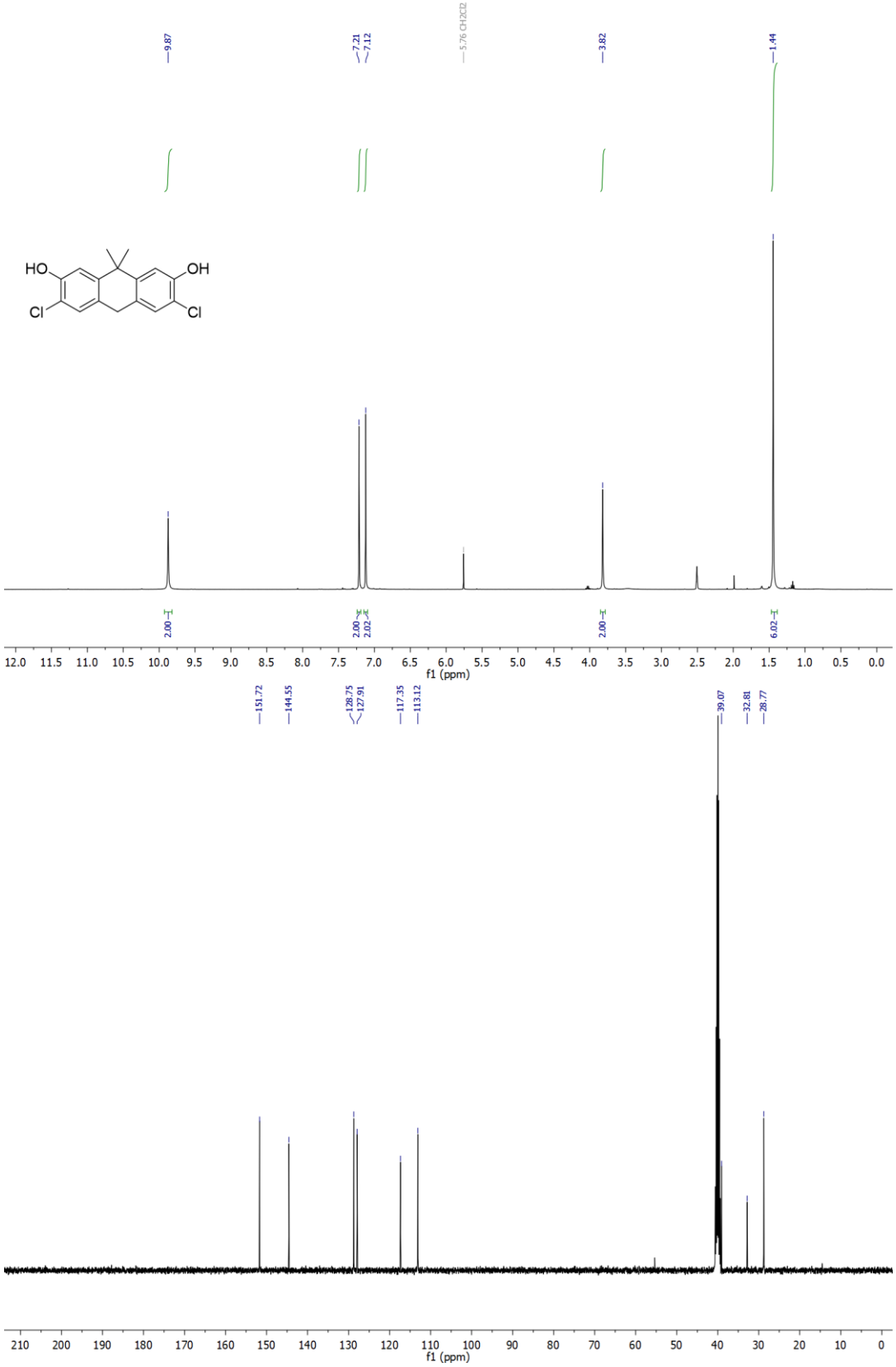


^{1}H and ^{13}C NMR of compound **9**

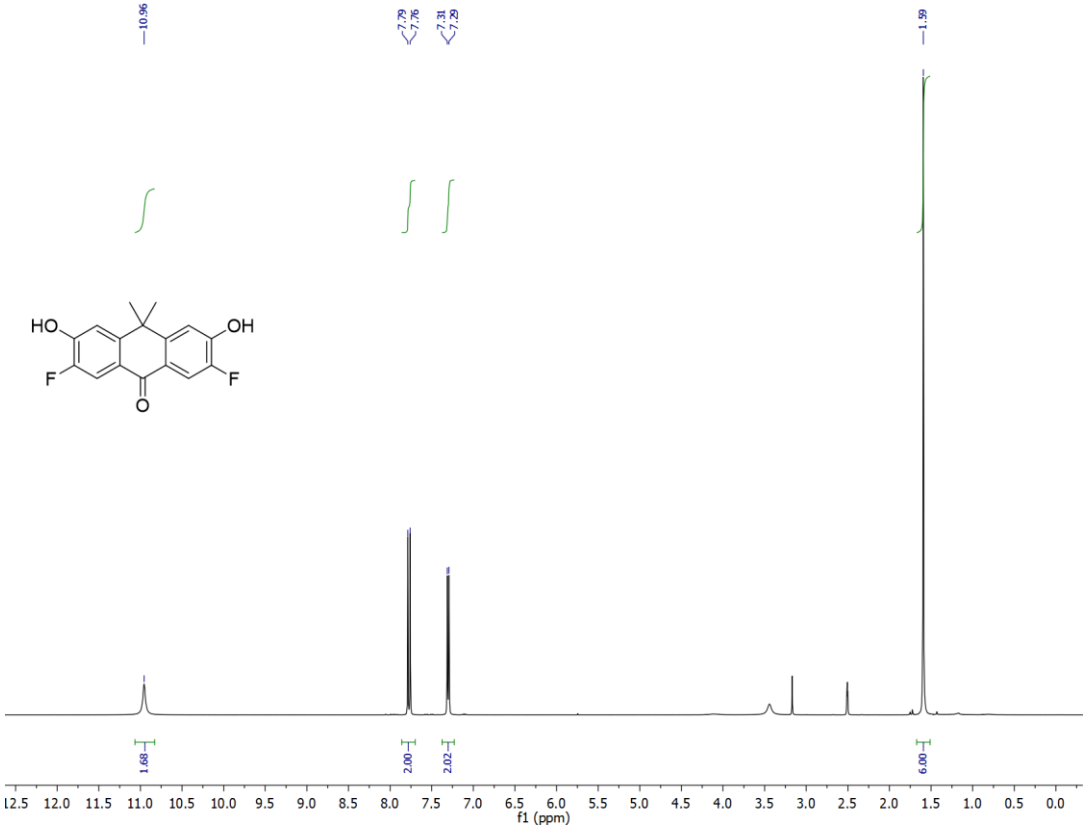


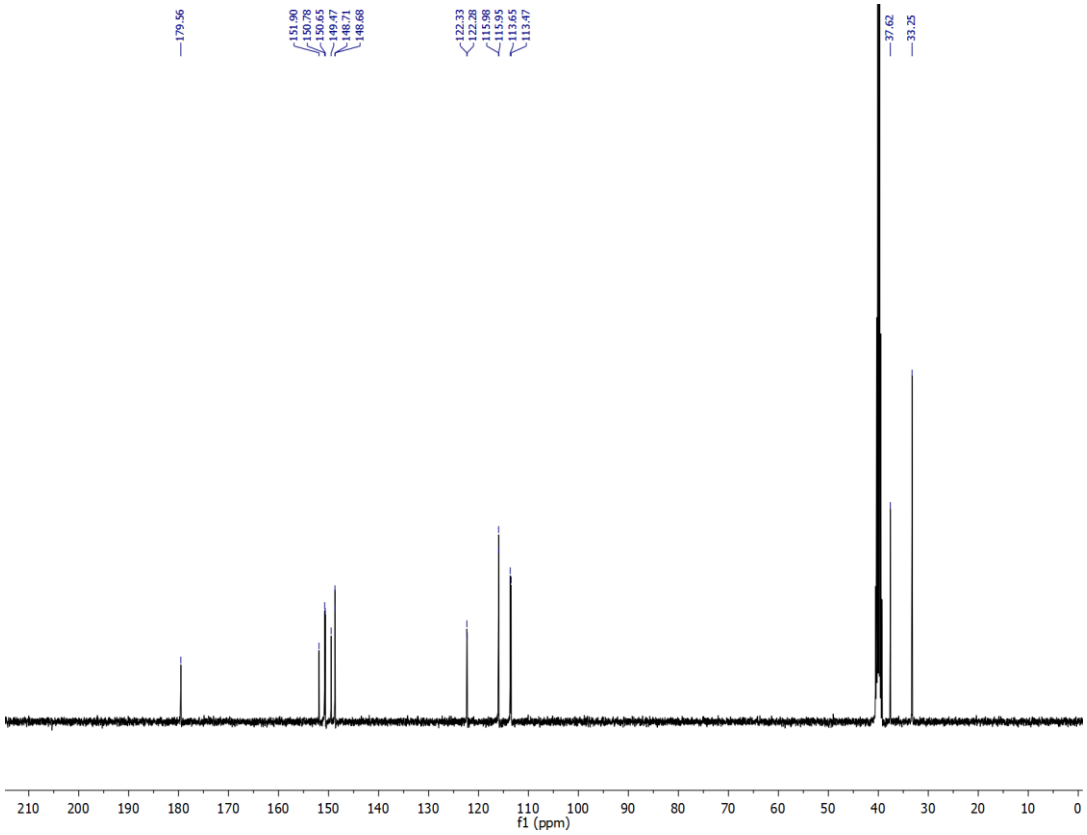


^{1}H and ^{13}C NMR of compound **10**

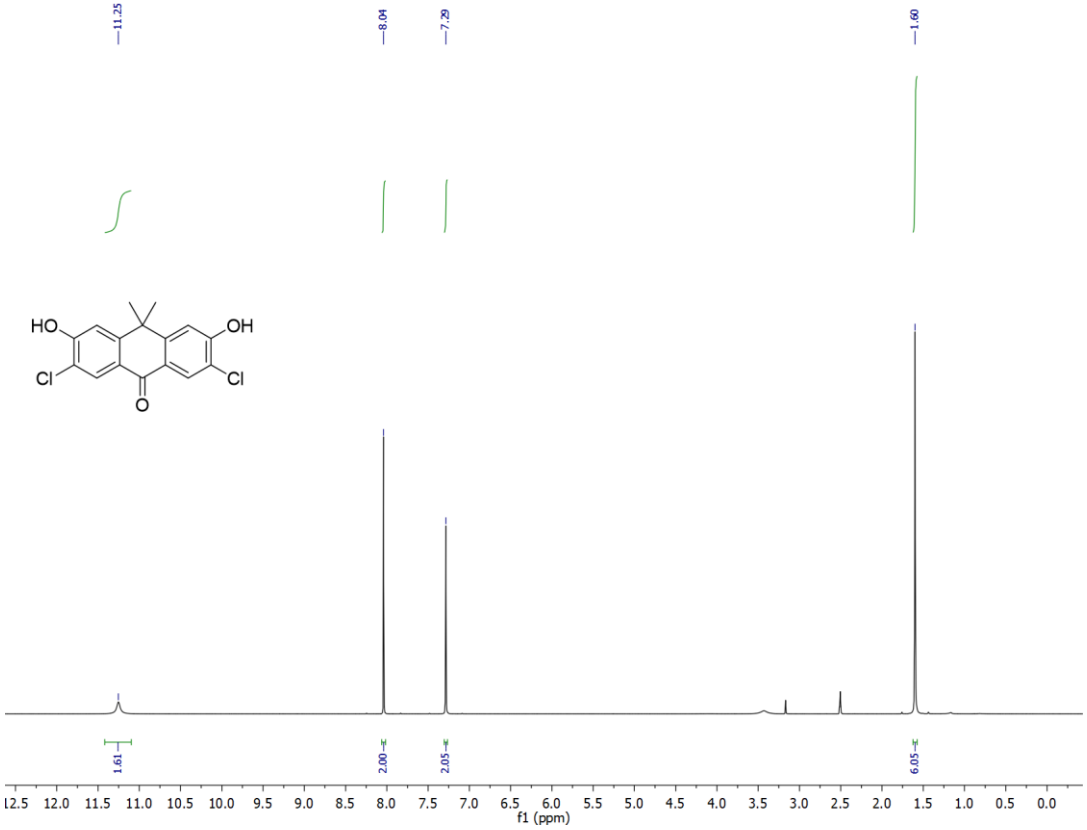


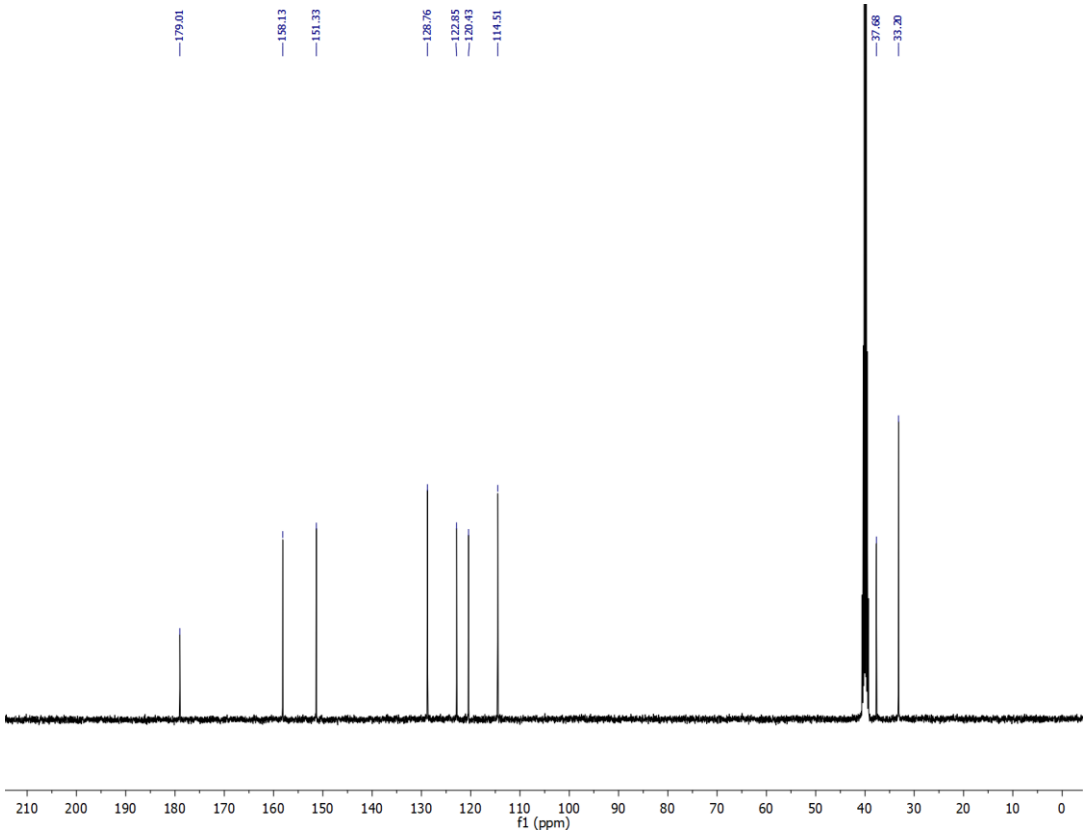
^{1}H and ^{13}C NMR of compound **11**



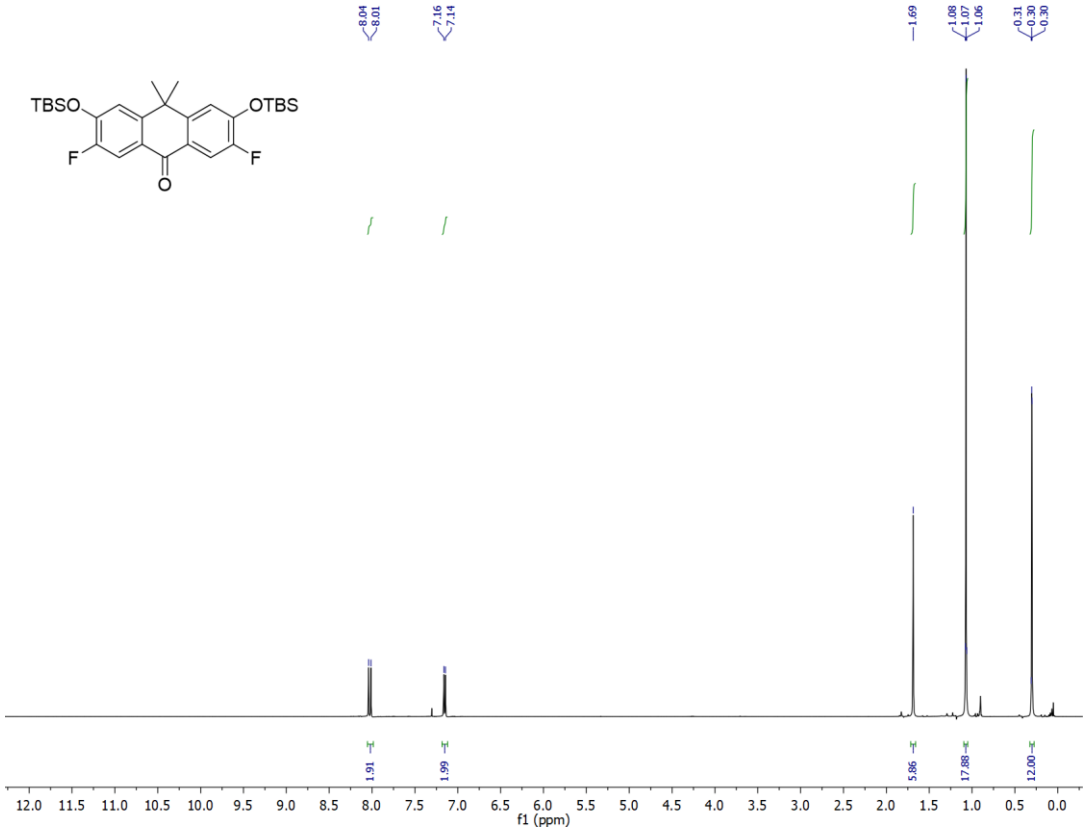


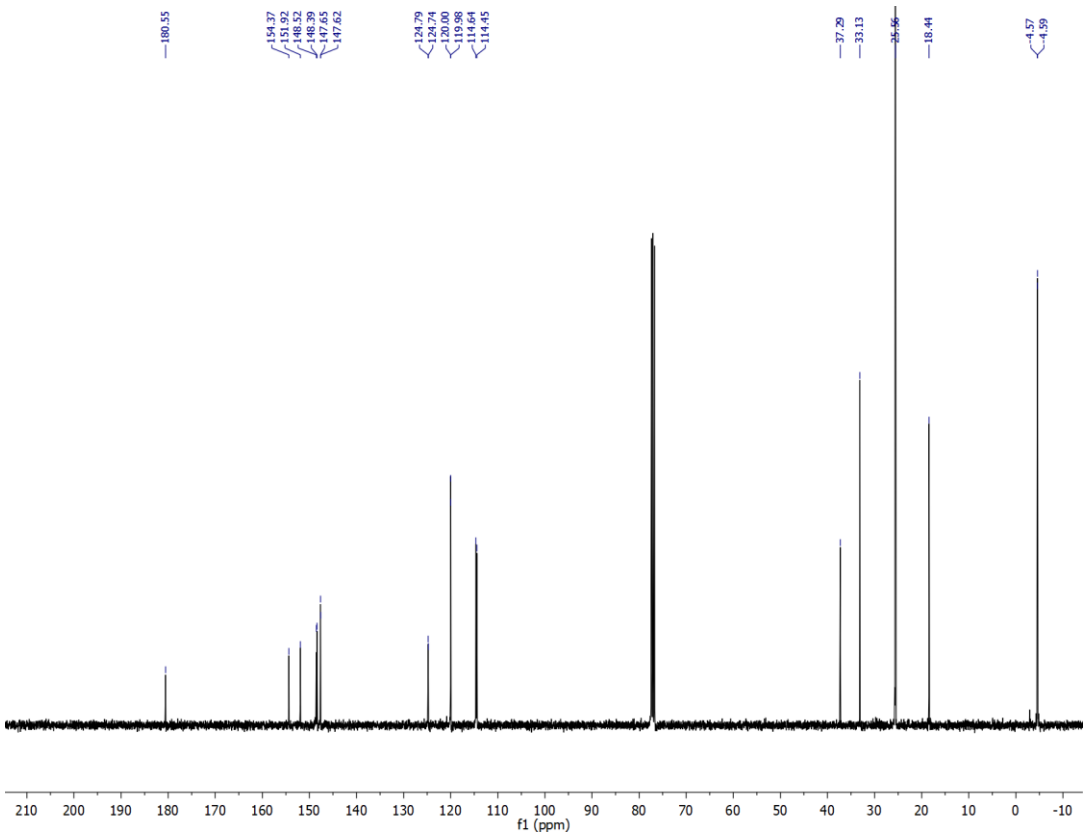
^{1}H and ^{13}C NMR of compound **12**



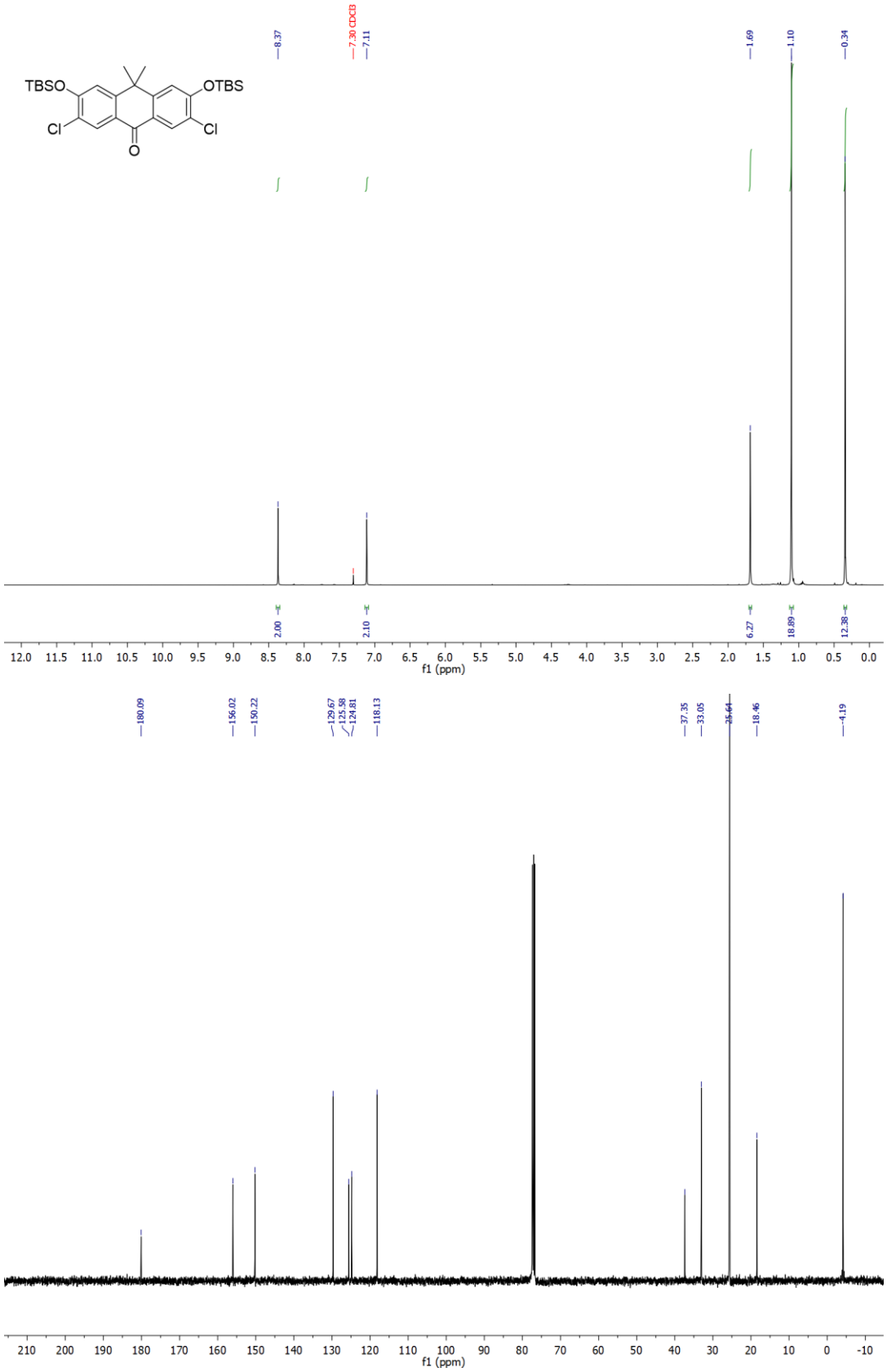


^{1}H and ^{13}C NMR of compound **13**

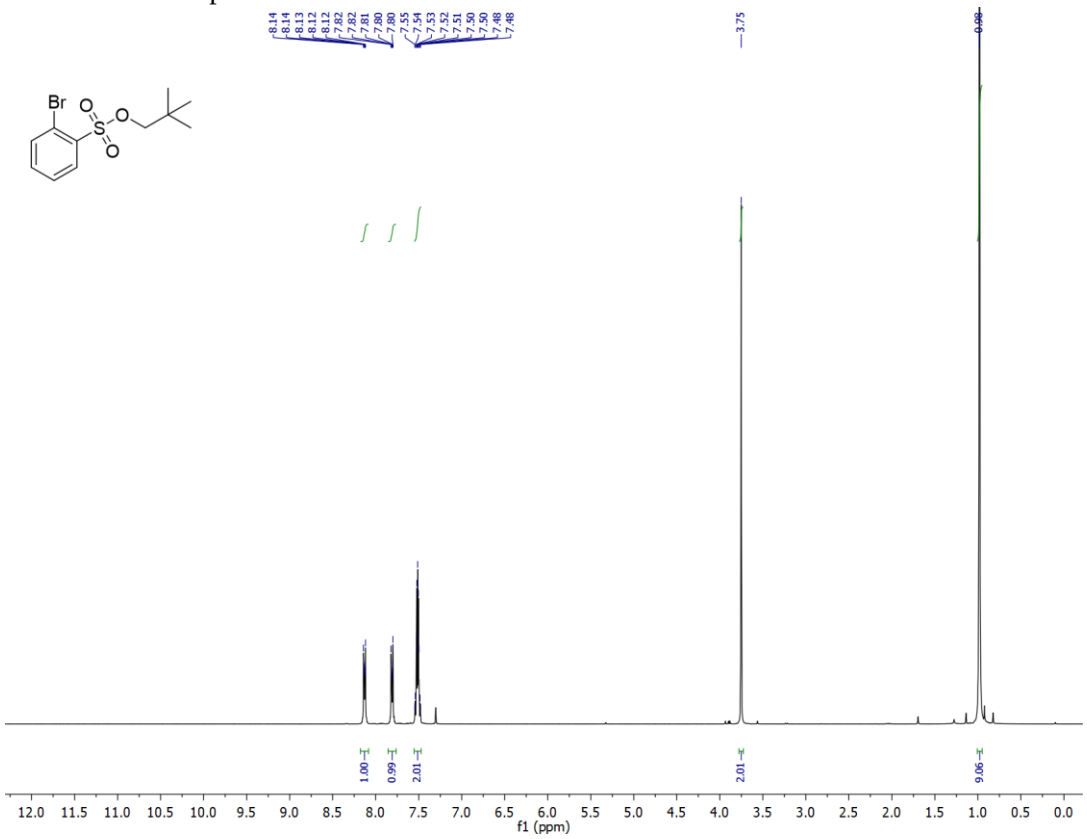


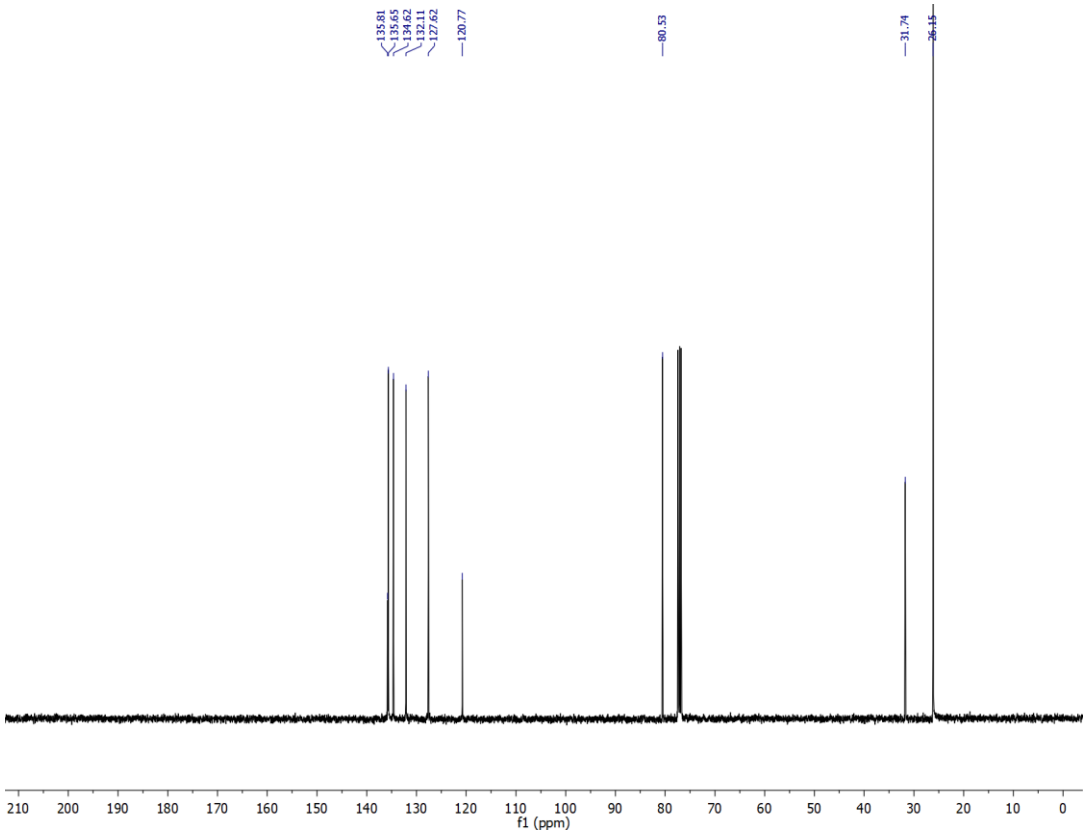


^{1}H and ^{13}C NMR of compound **14**

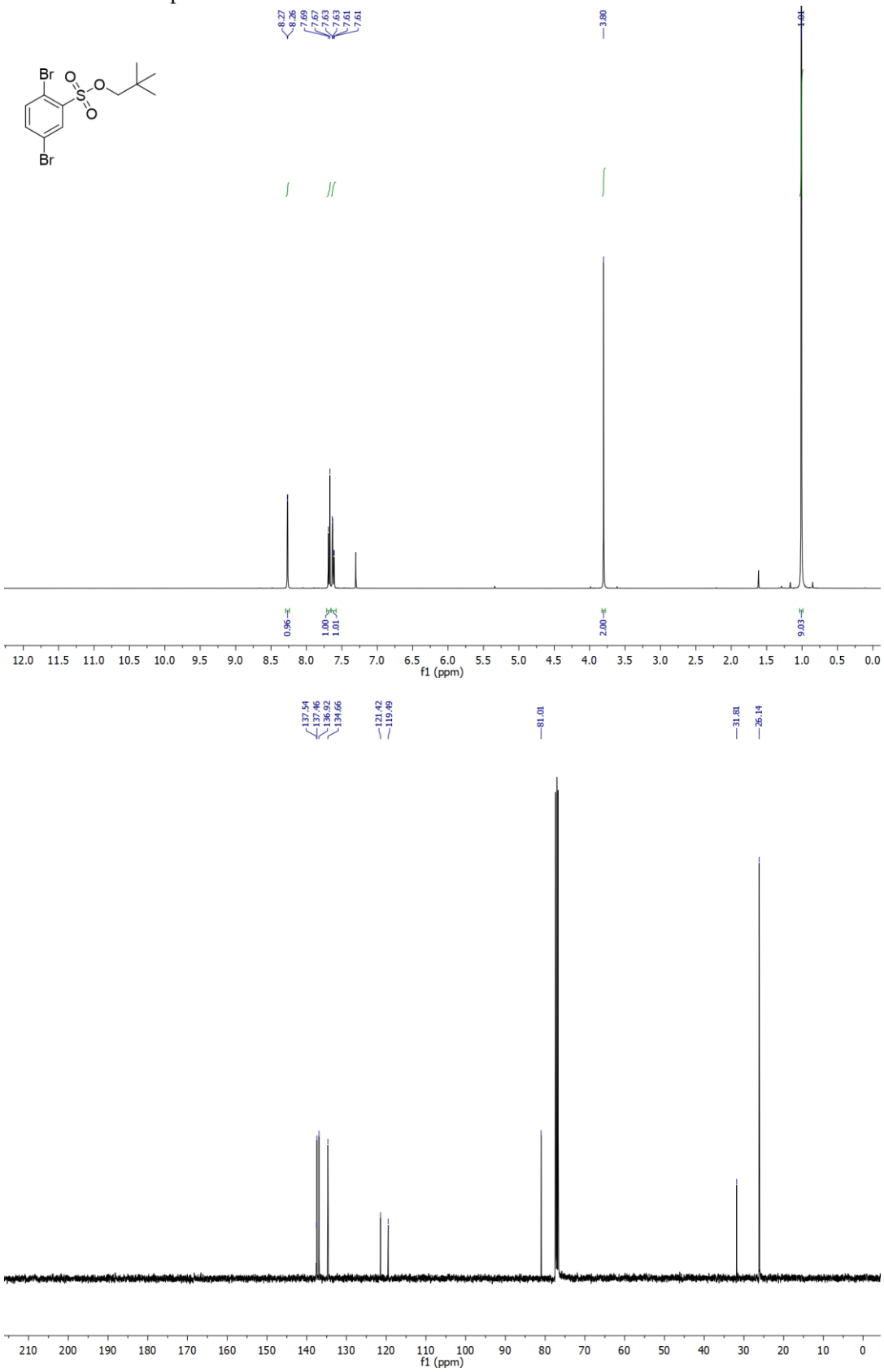


^{1}H and ^{13}C NMR of compound **16**

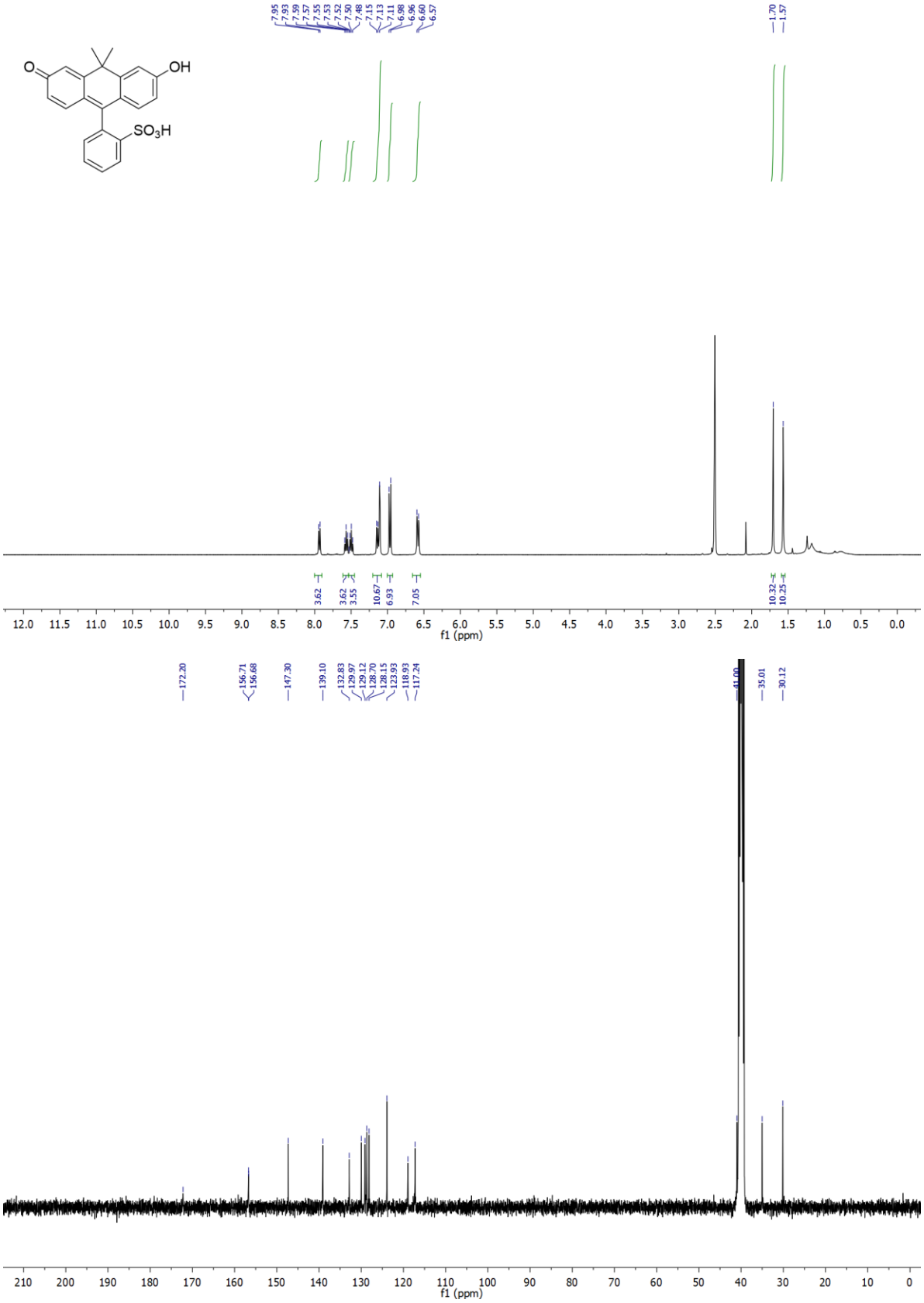




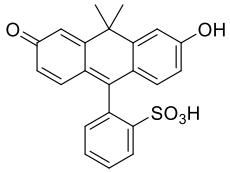
^{1}H and ^{13}C NMR of compound **17**

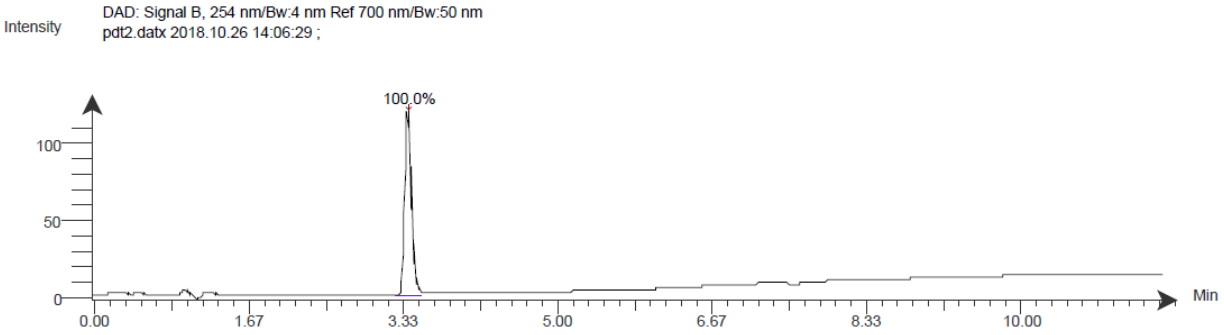


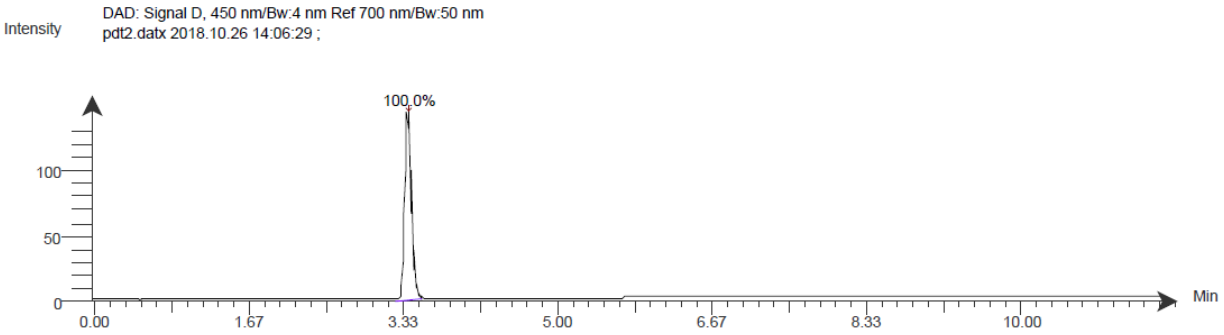
^{1}H and ^{13}C NMR of compound **18**

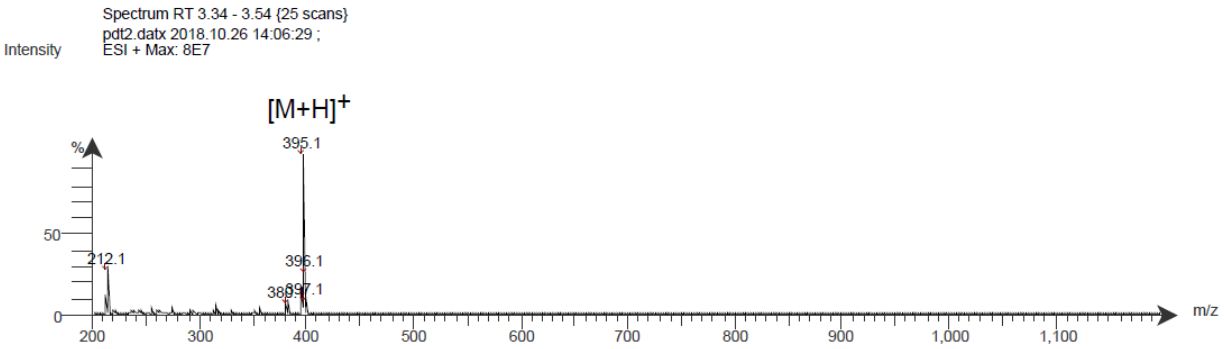


LCMS of compound **18**

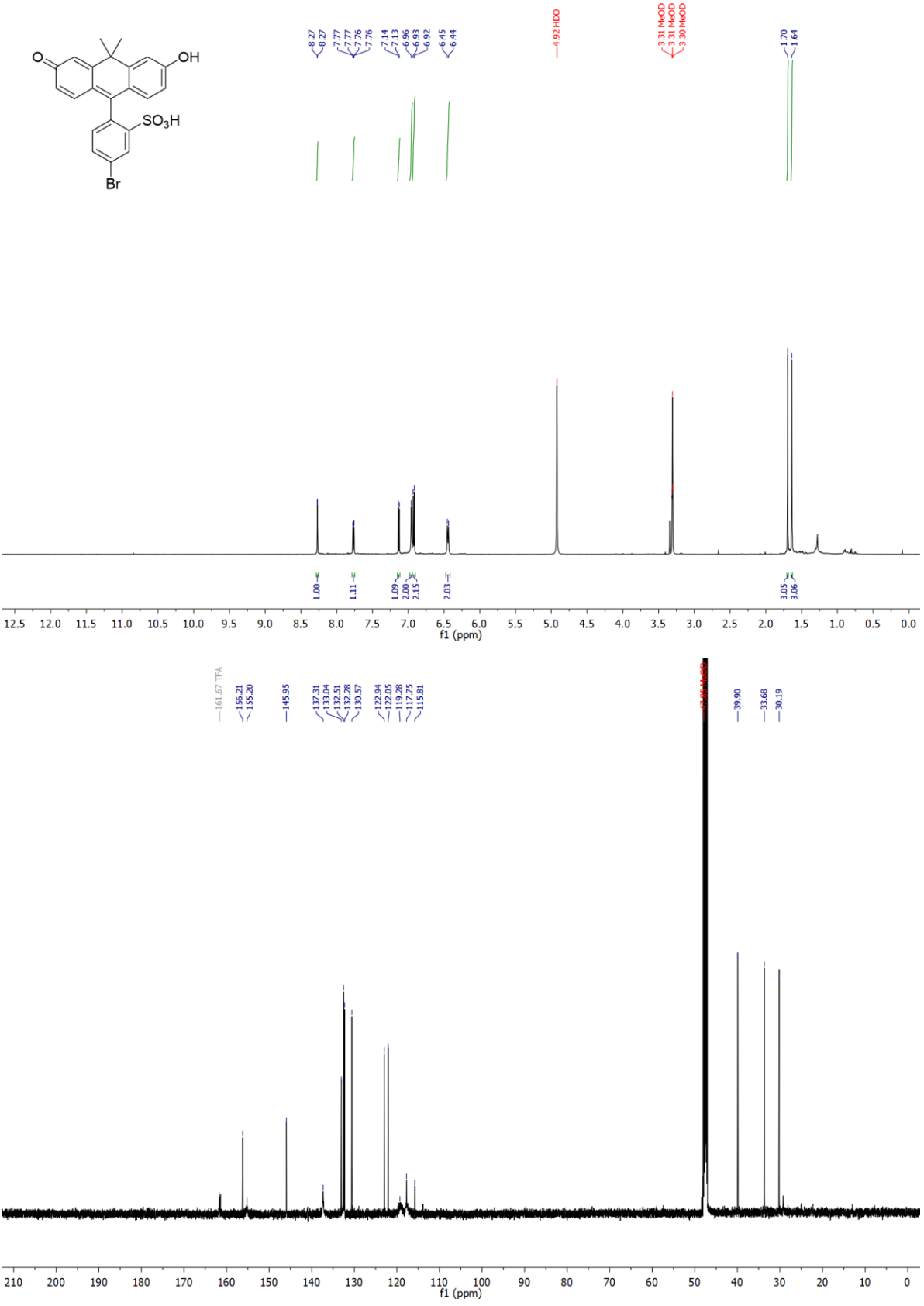




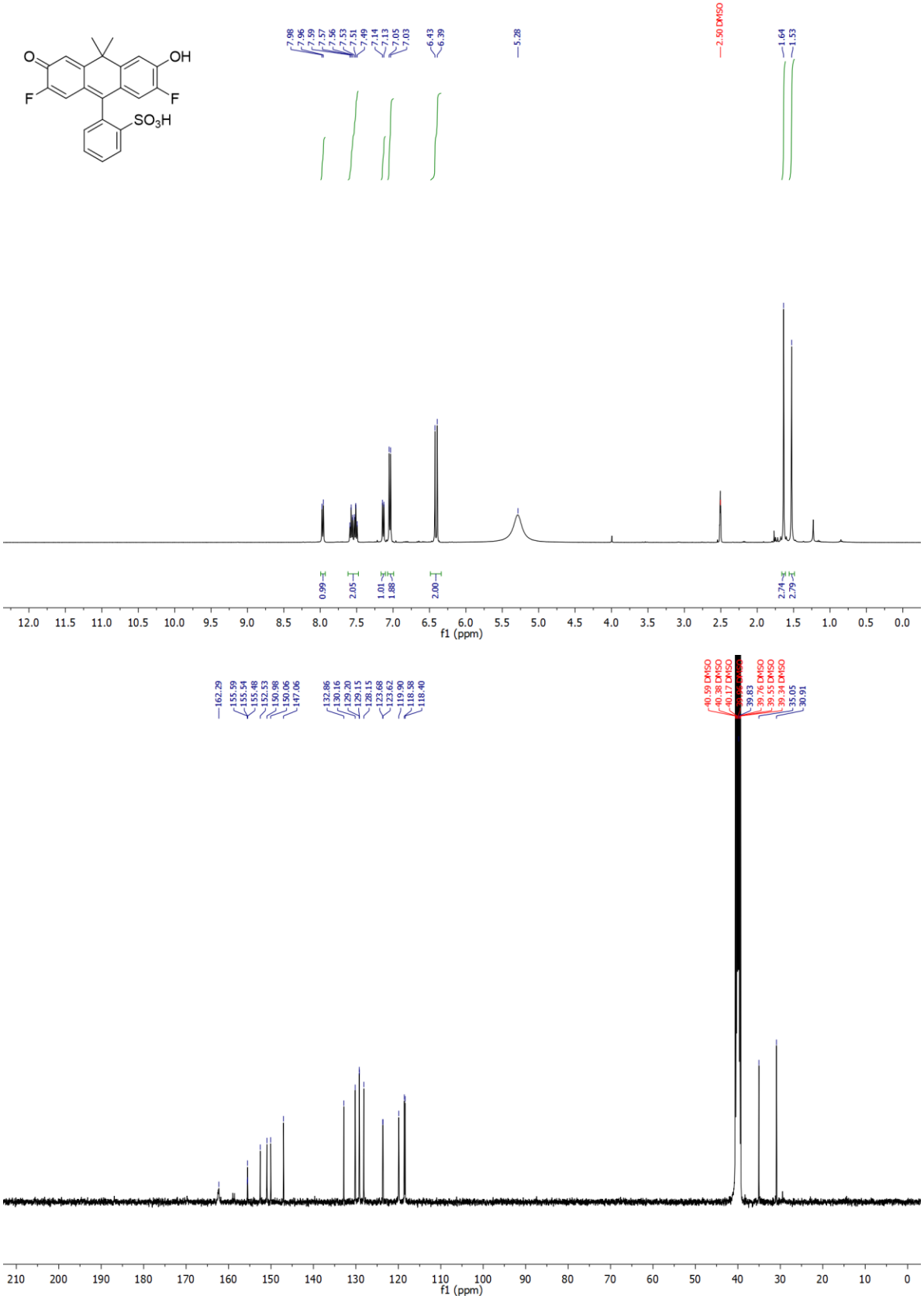




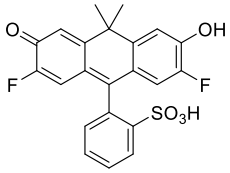
^{1}H and ^{13}C NMR of compound **19**

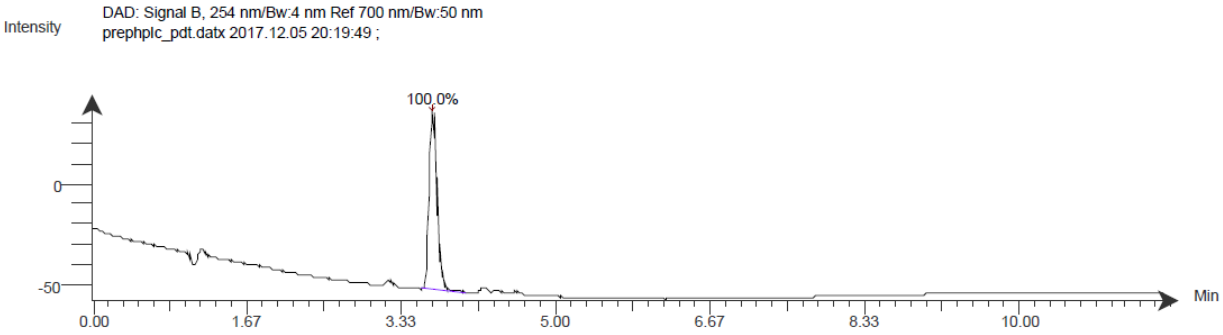


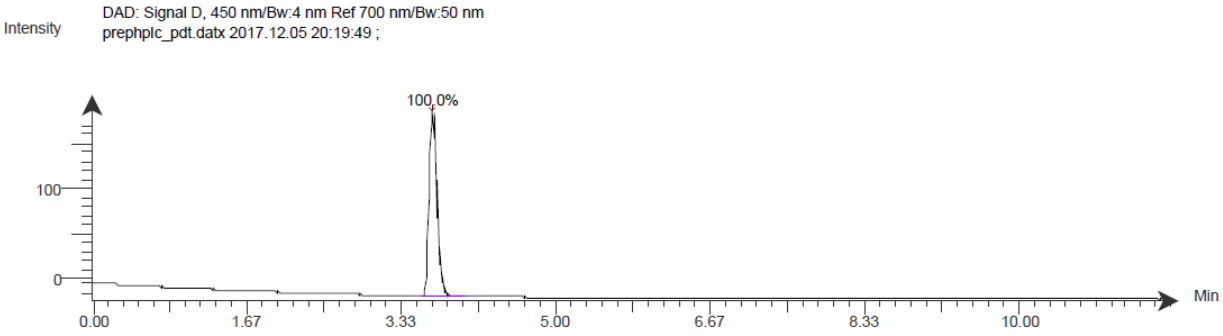
^{1}H and ^{13}C NMR of compound **20**

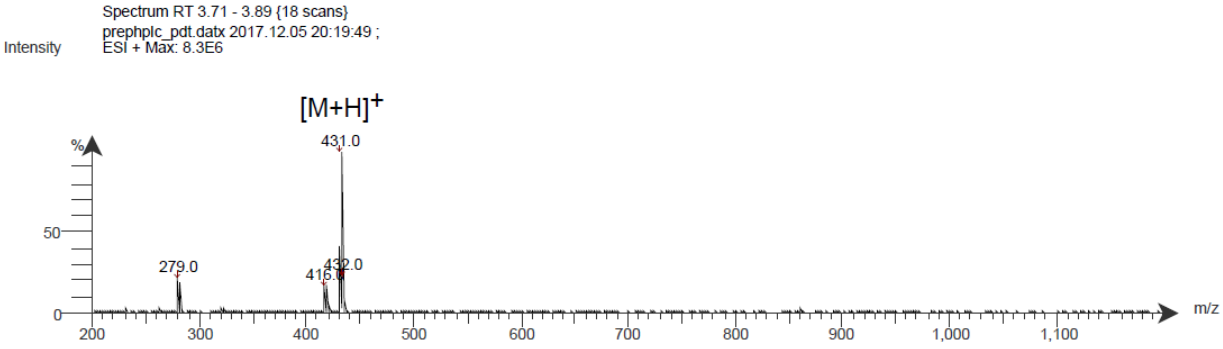


LCMS of compound **20**

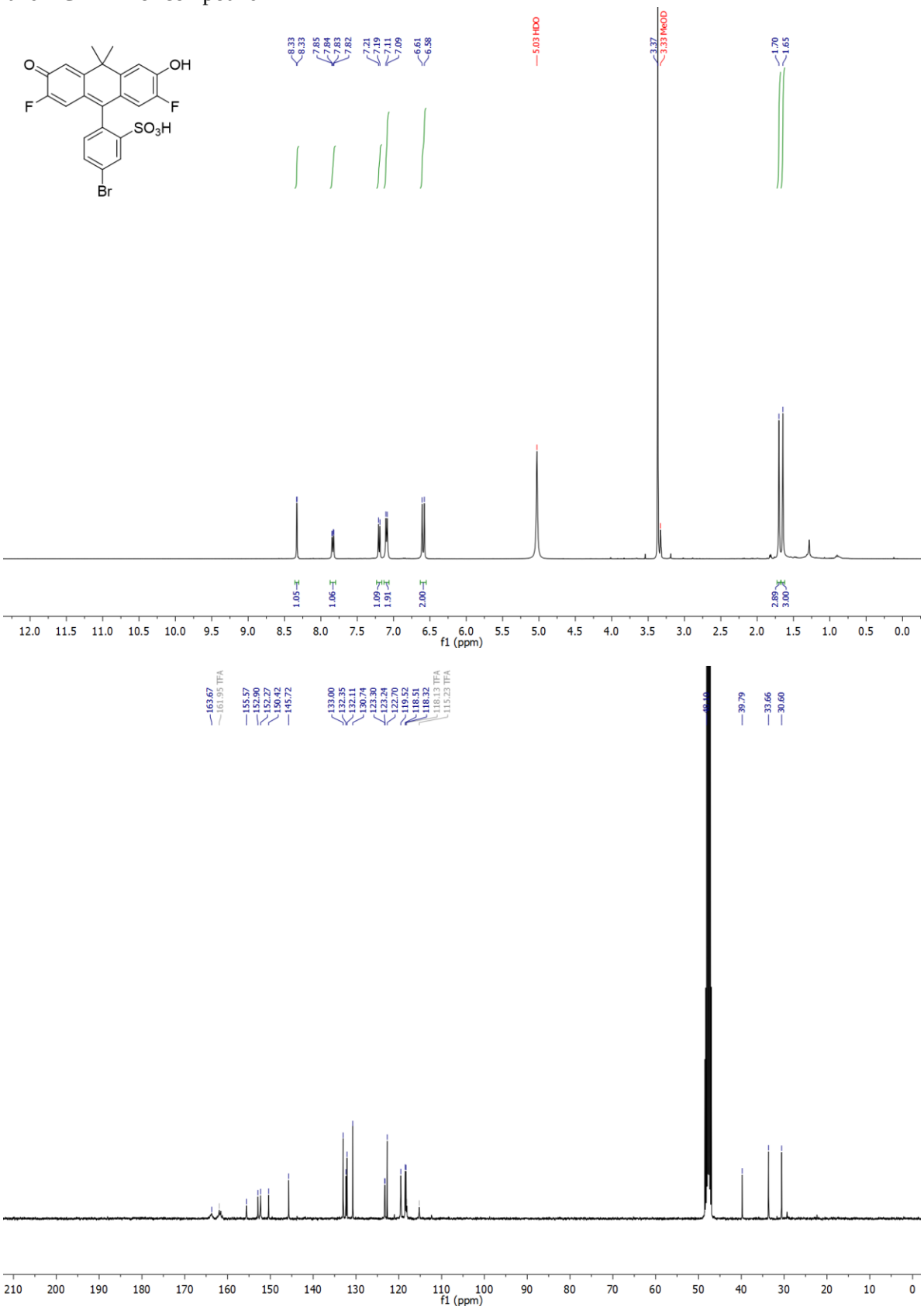




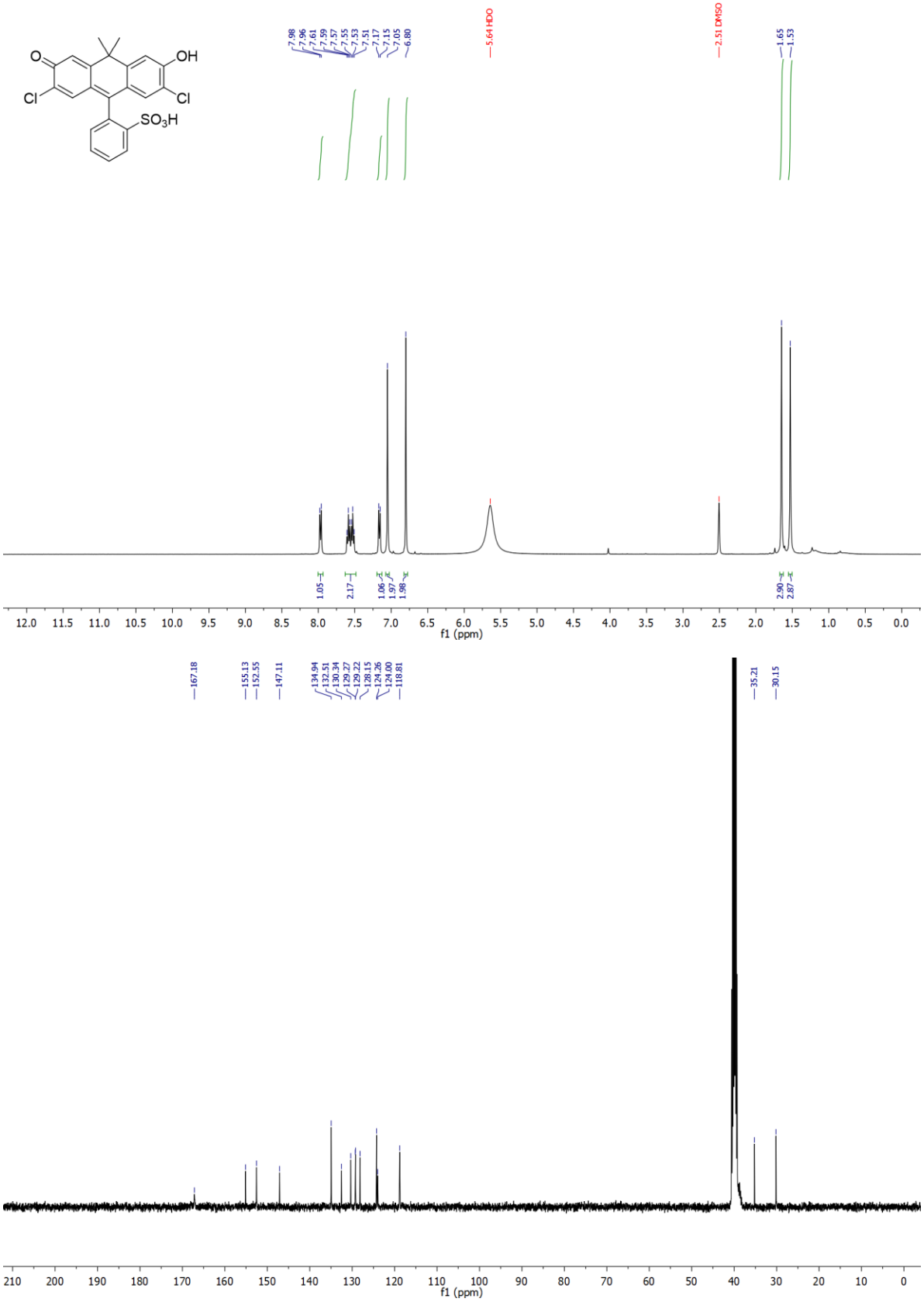




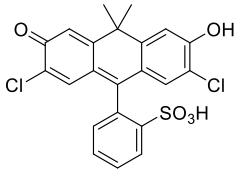
^{1}H and ^{13}C NMR of compound **21**

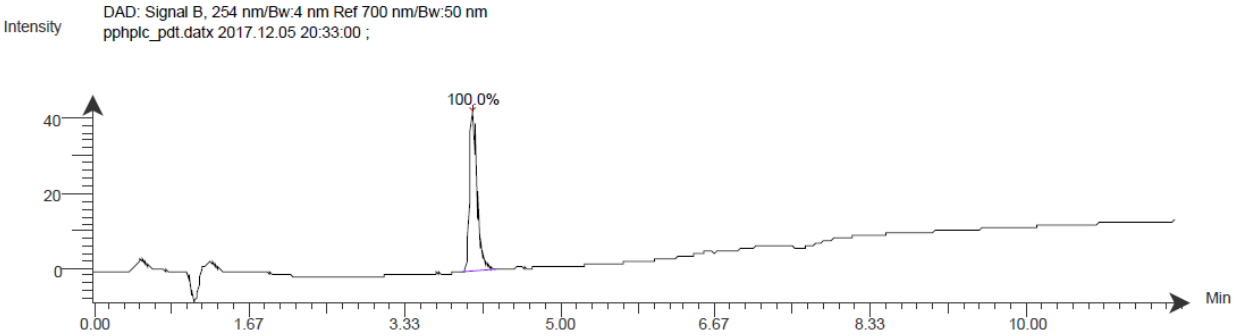


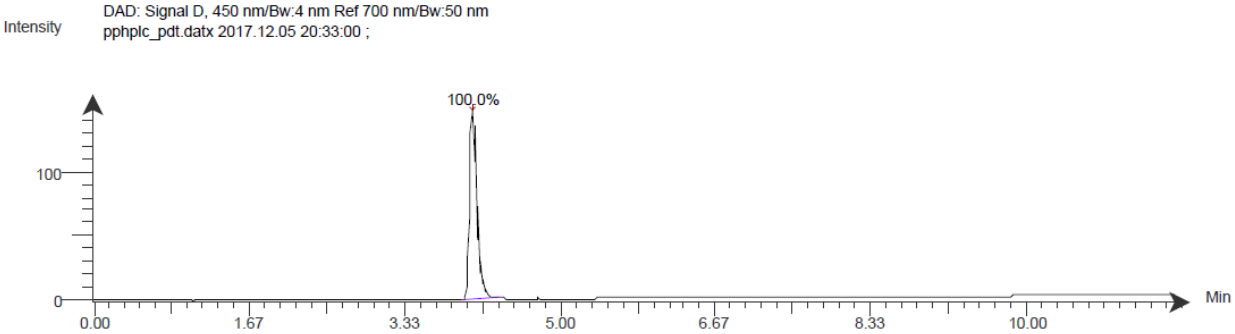
^{1}H and ^{13}C NMR of compound **22**

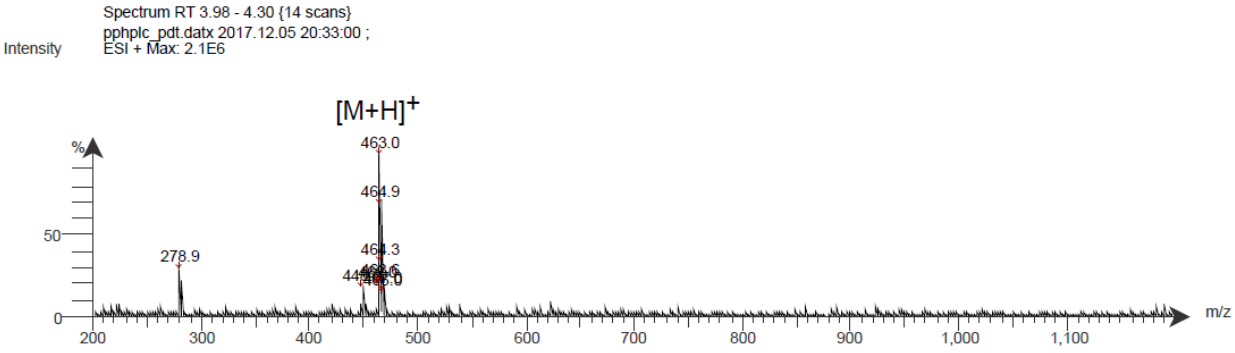


LCMS of compound **22**

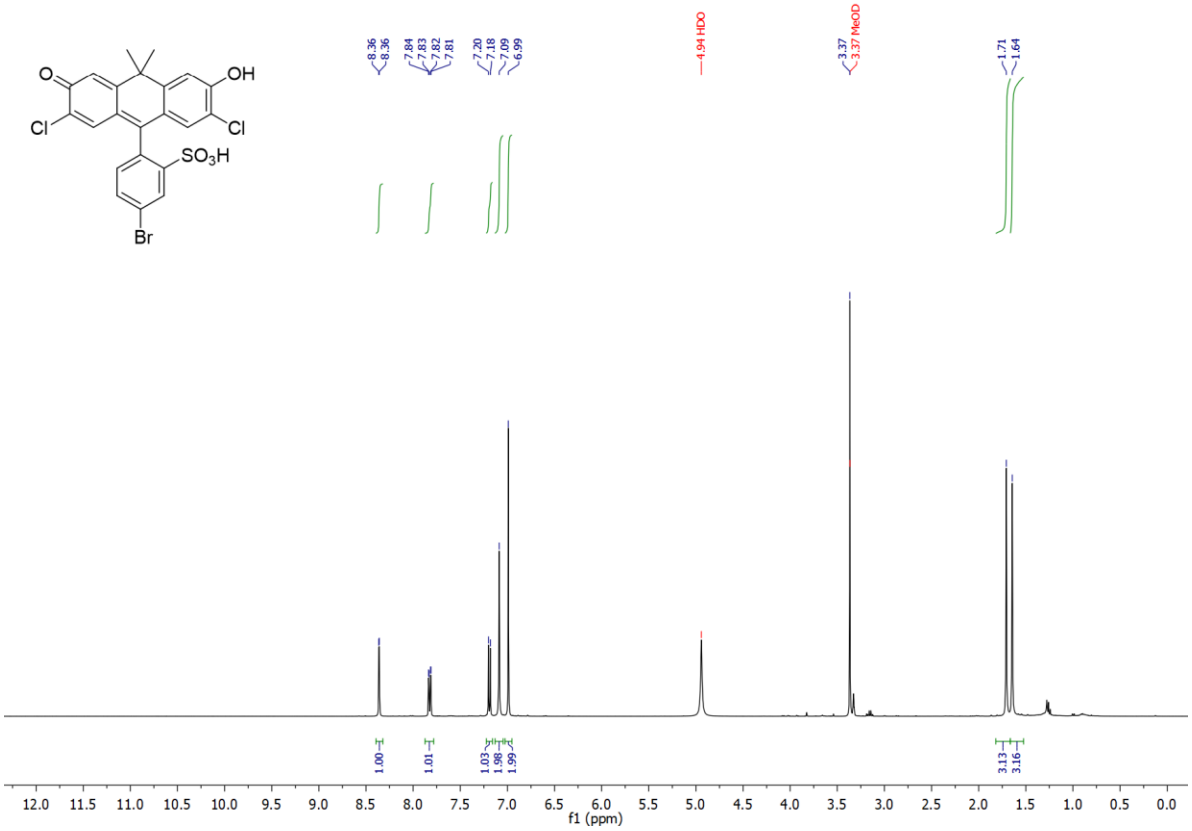


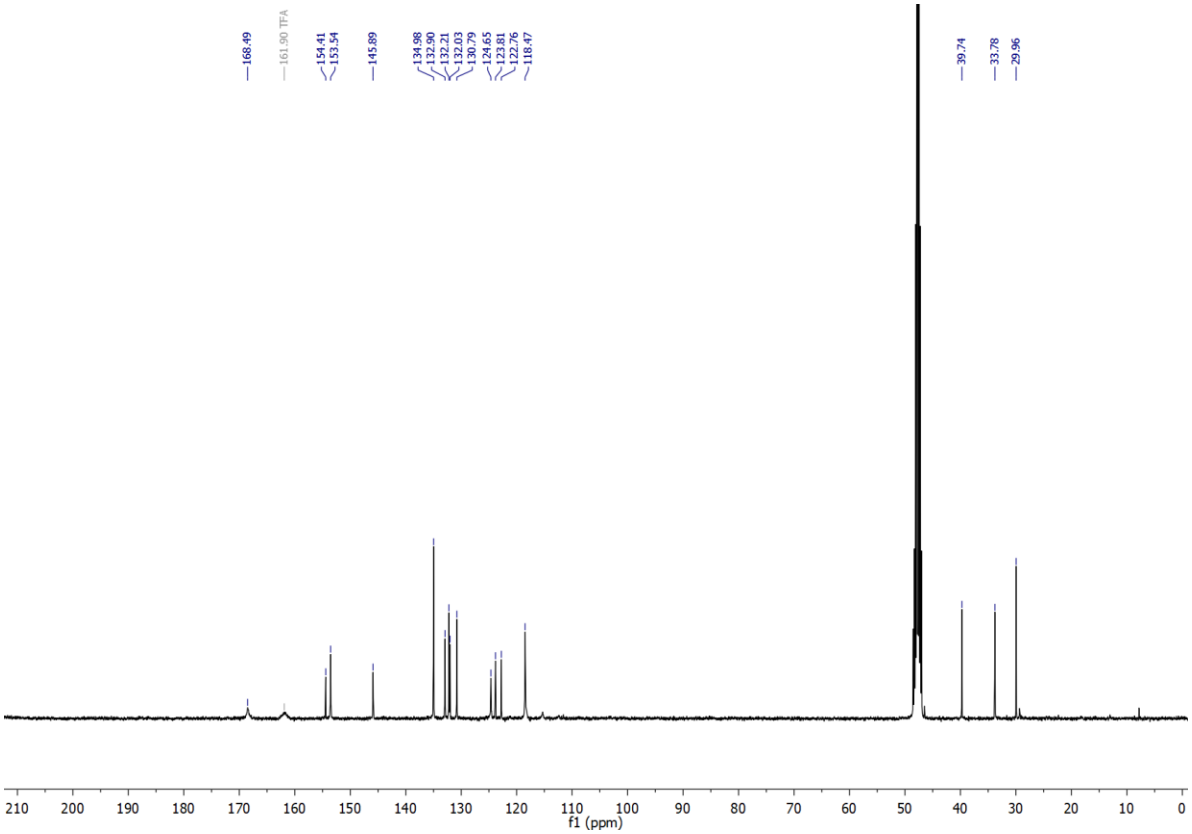




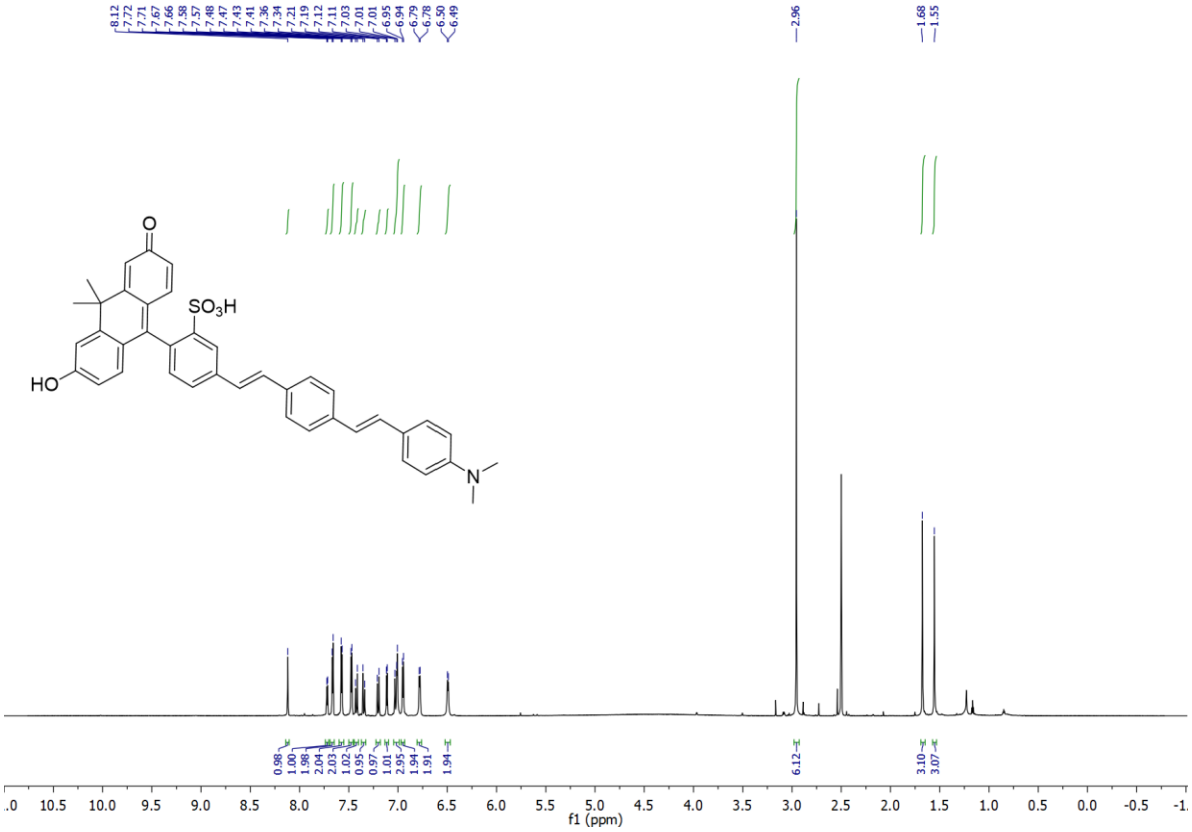


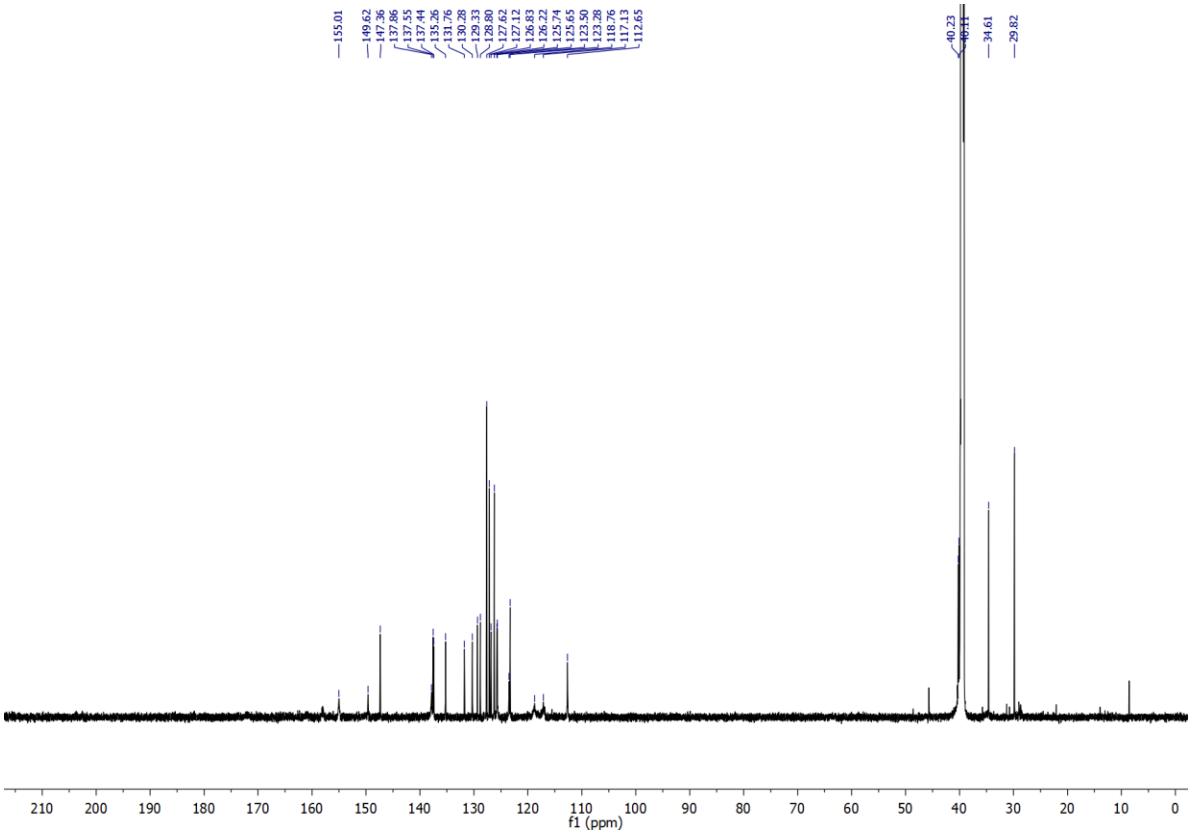
^{1}H and ^{13}C NMR of compound **23**



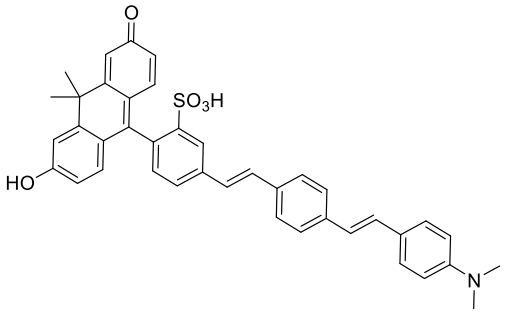


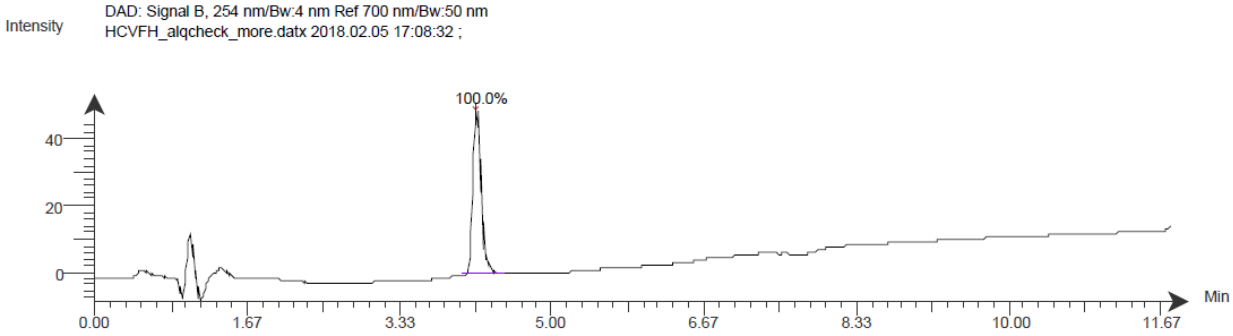
^{1}H and ^{13}C NMR of compound **26**

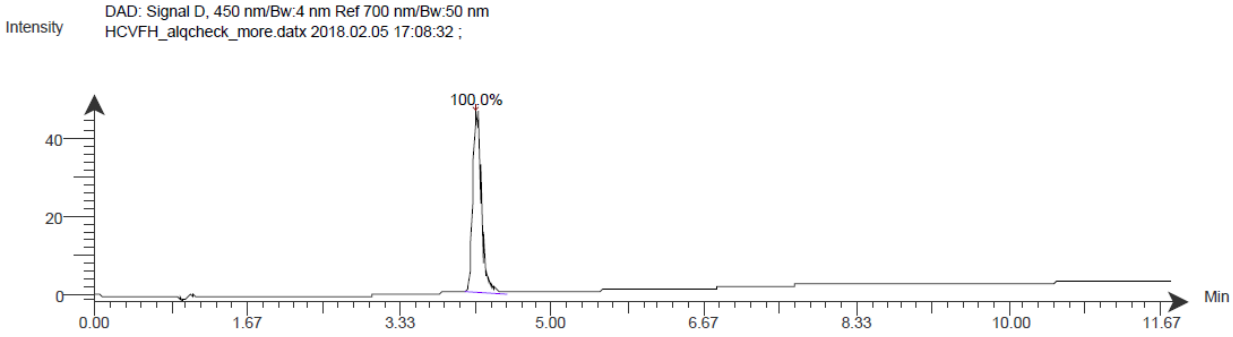


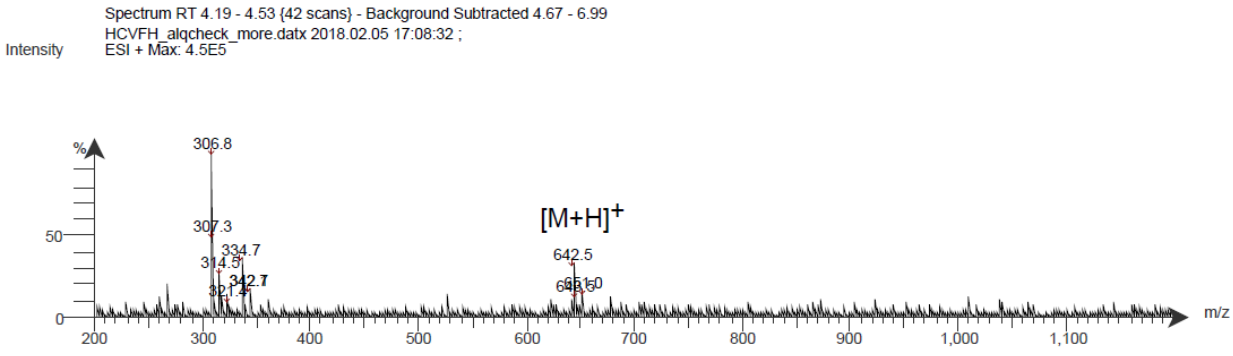


LCMS of compound **26**

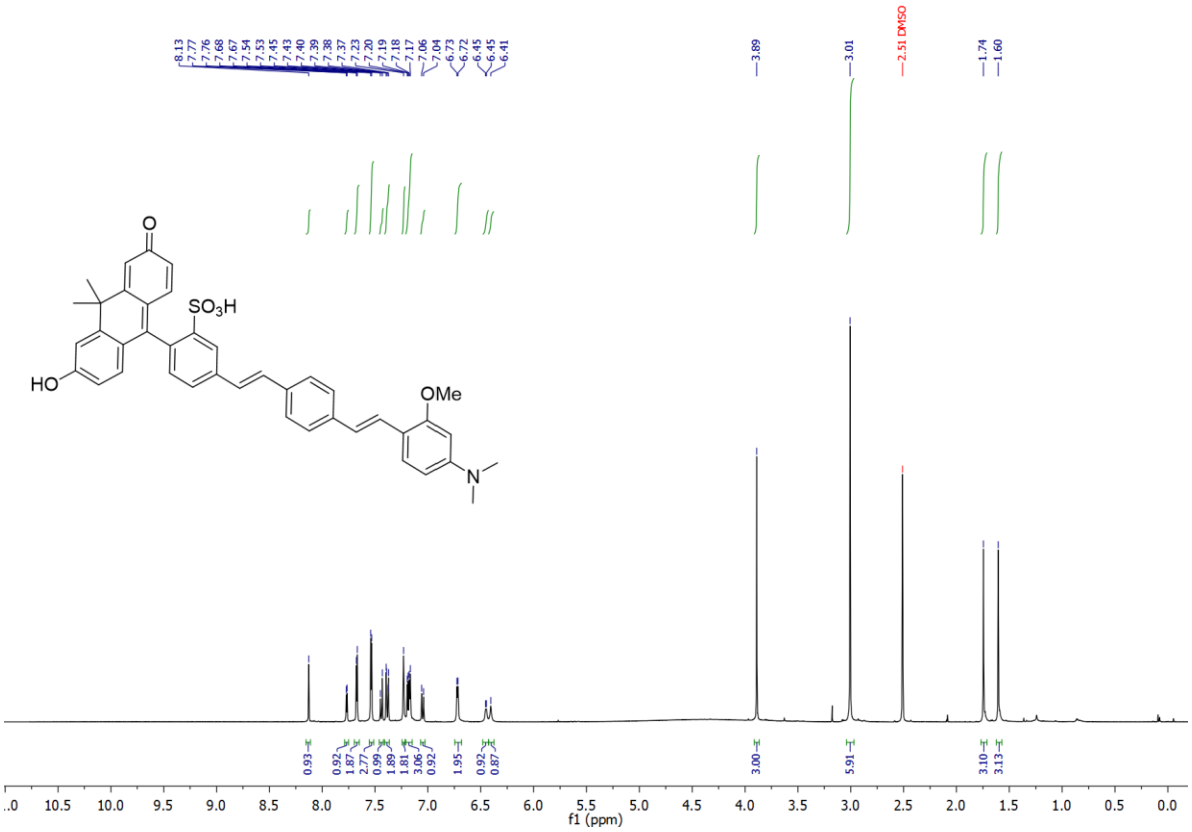


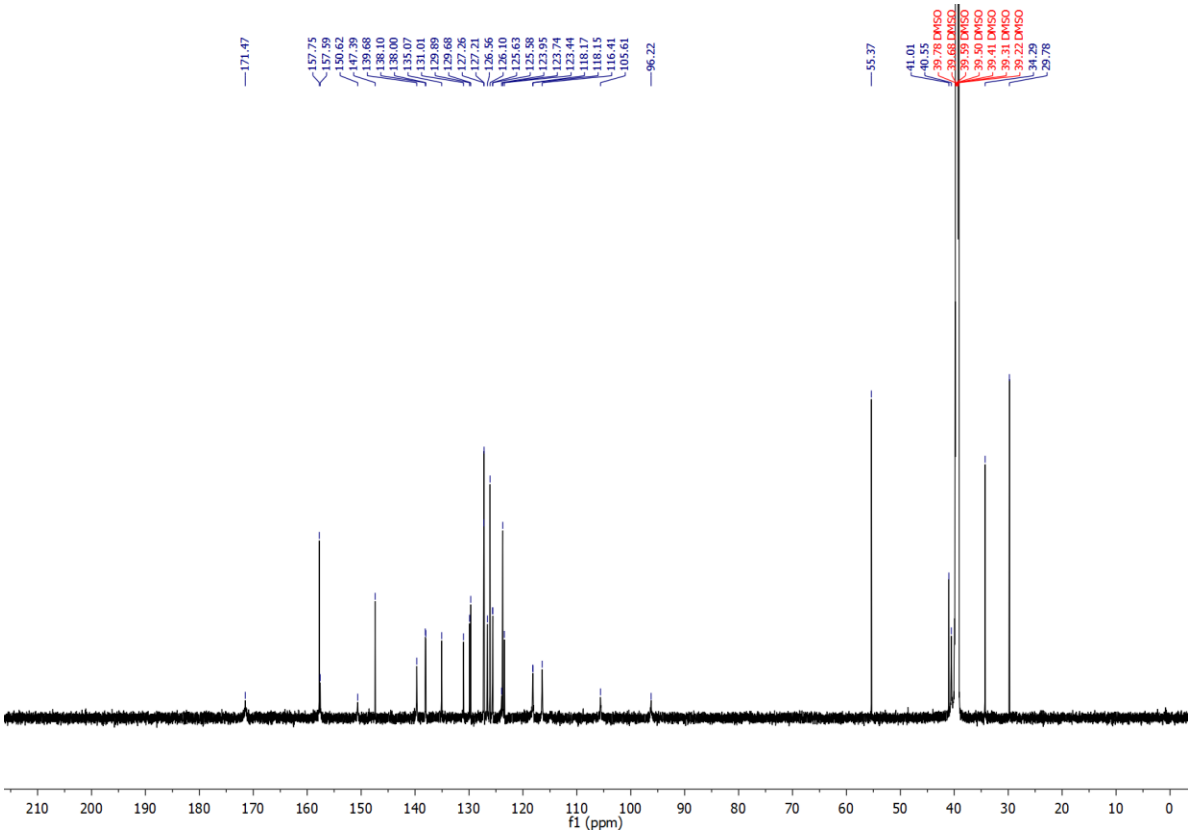




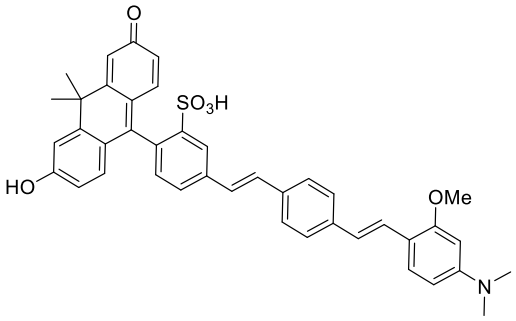


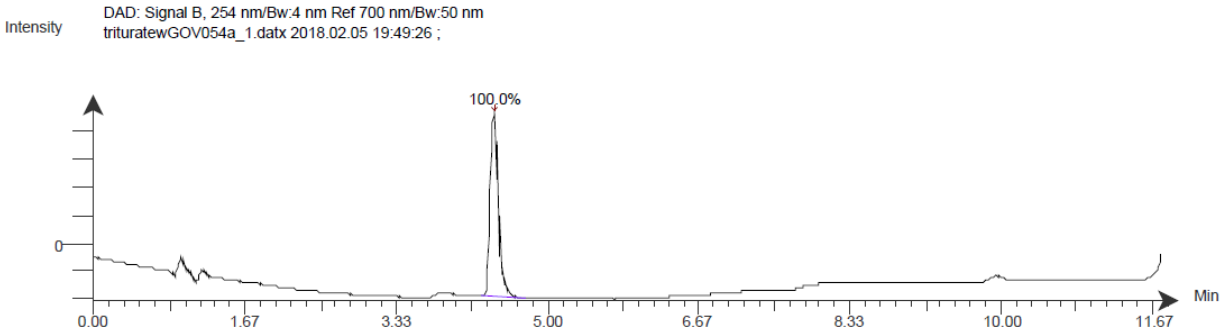
^{1}H and ^{13}C NMR of compound **27**

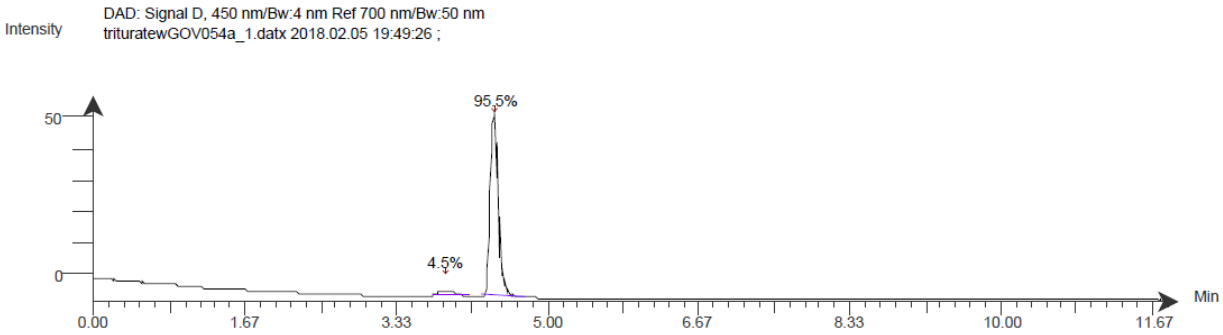


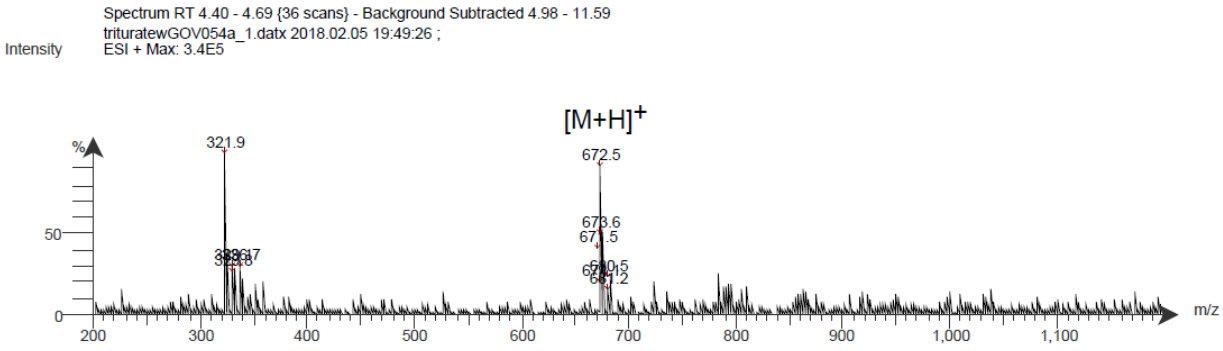


LCMS of compound **27**

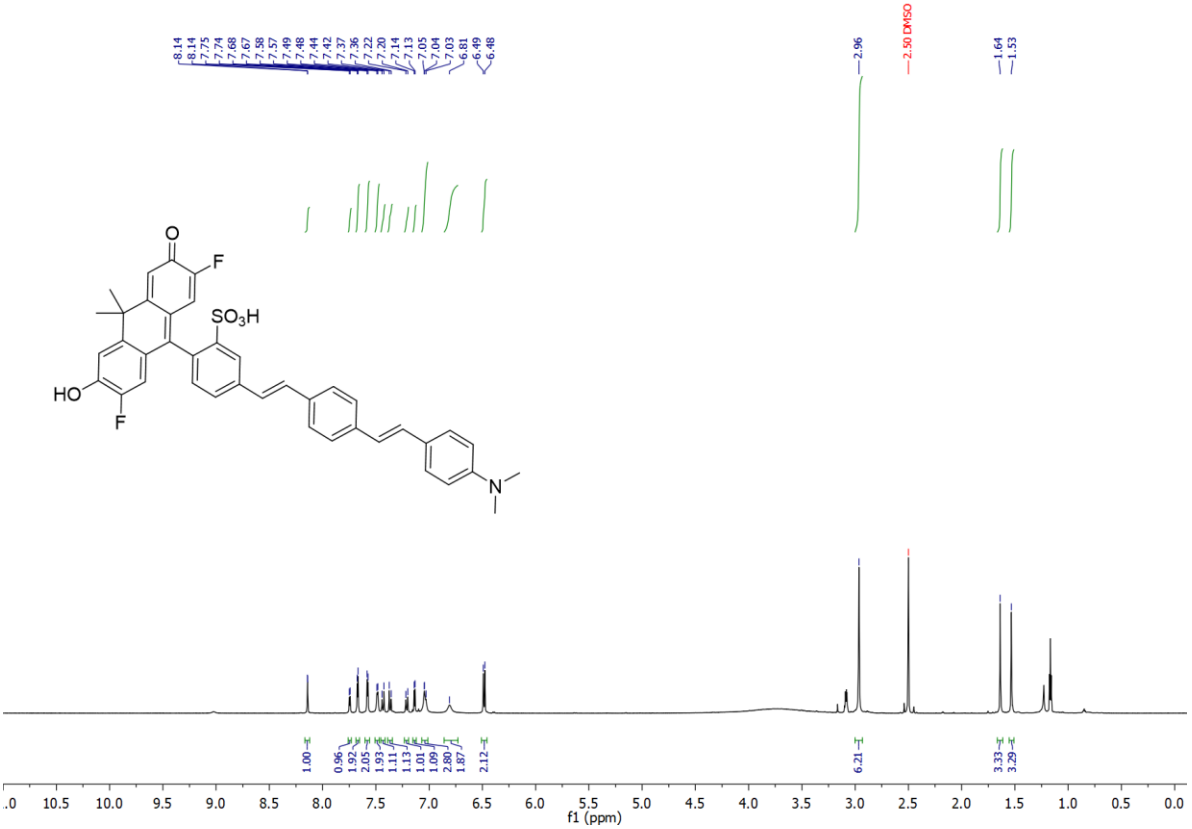


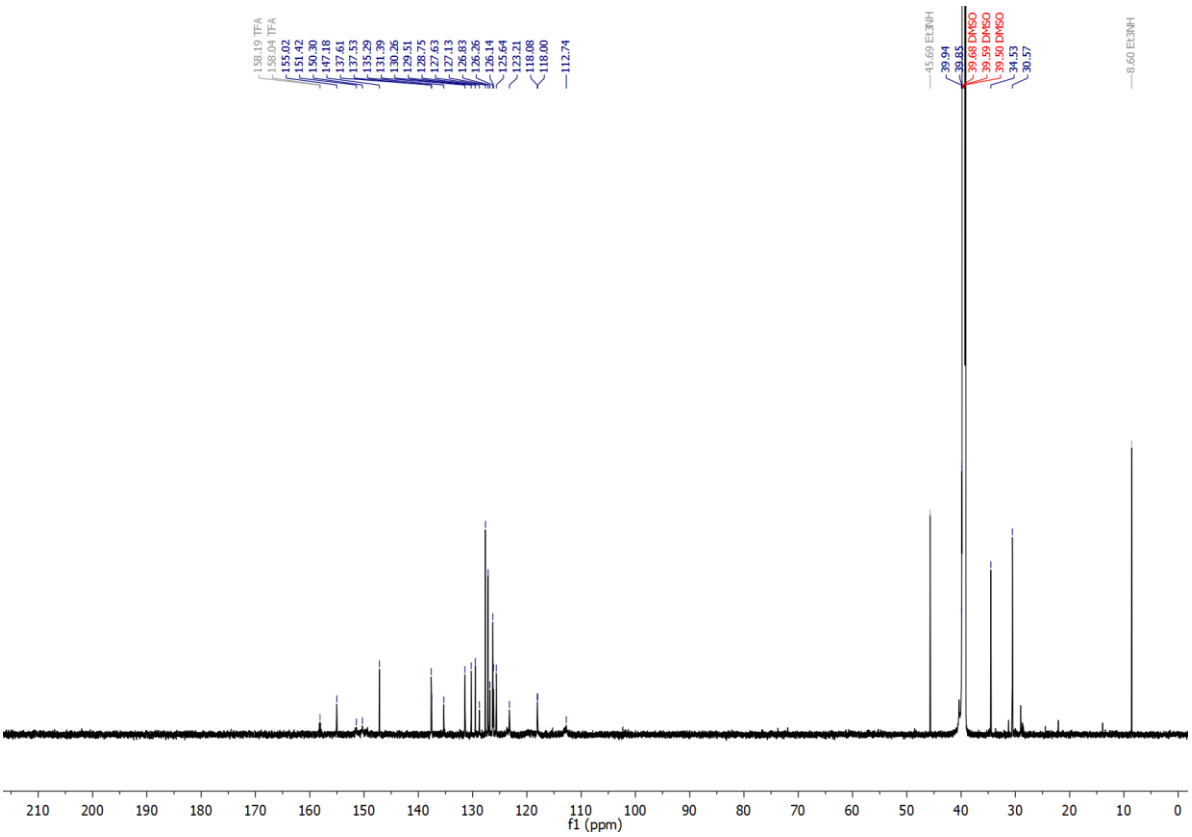




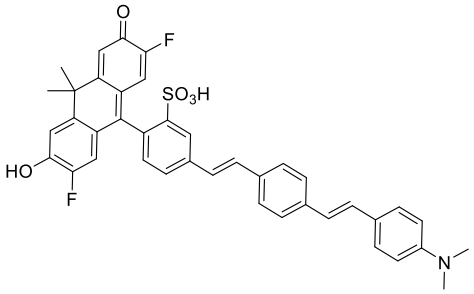


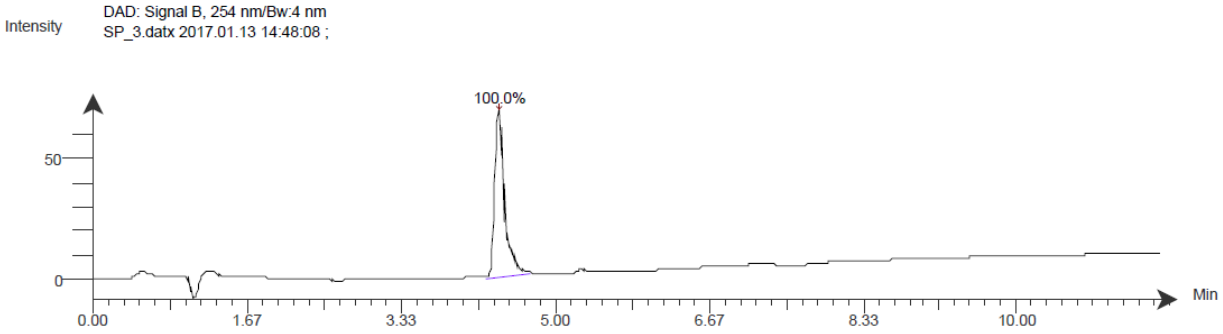
^{1}H and ^{13}C NMR of compound **28**

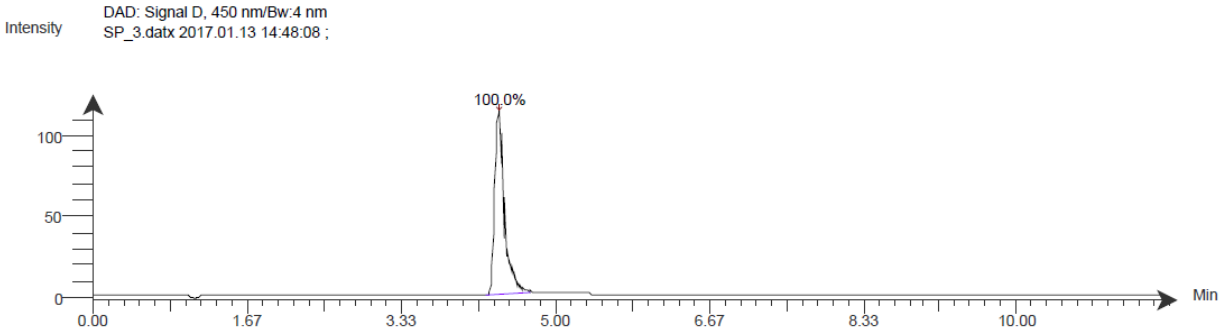


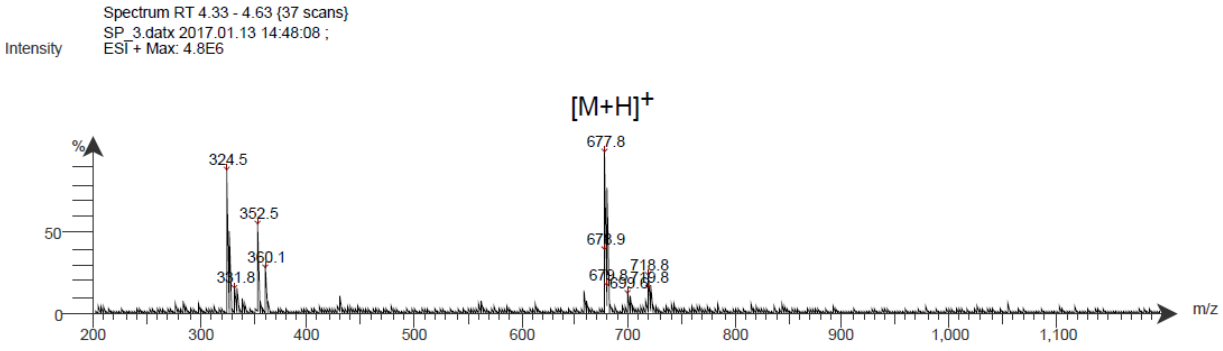


LCMS of compound **28**

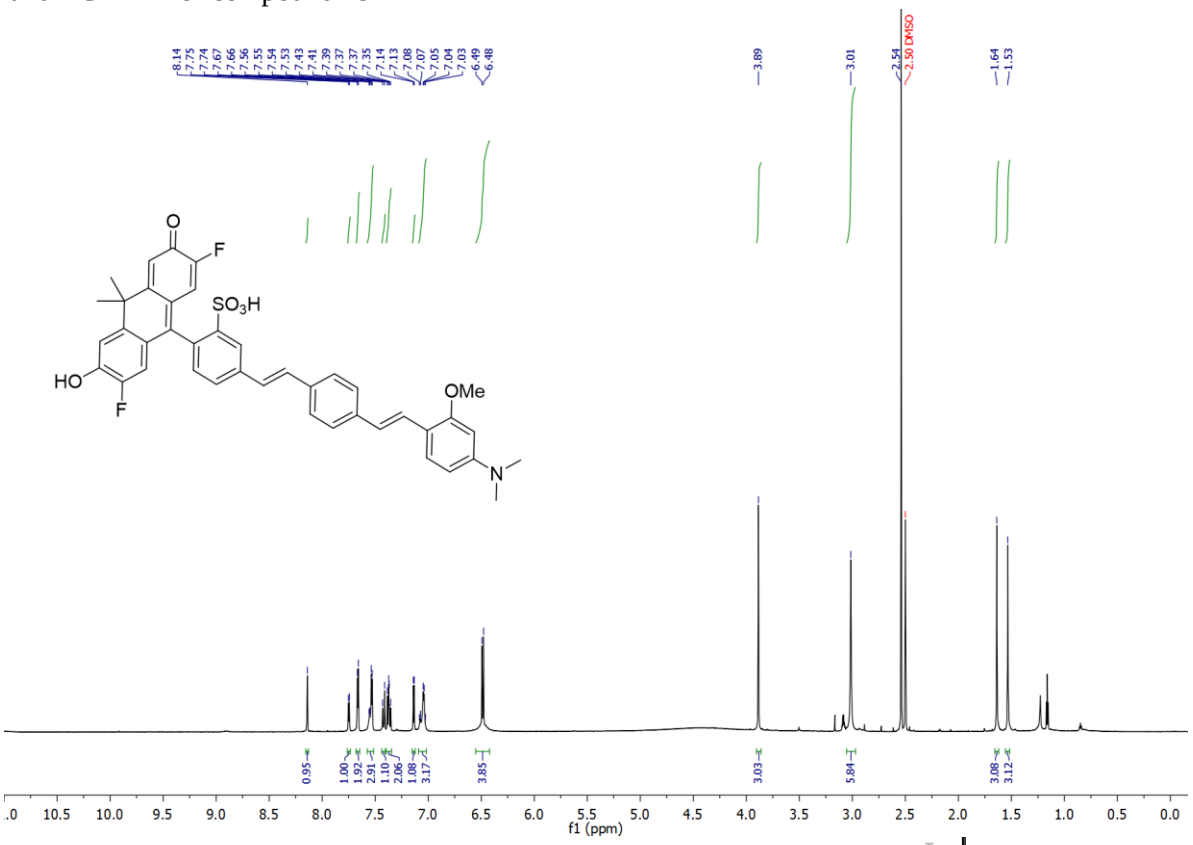


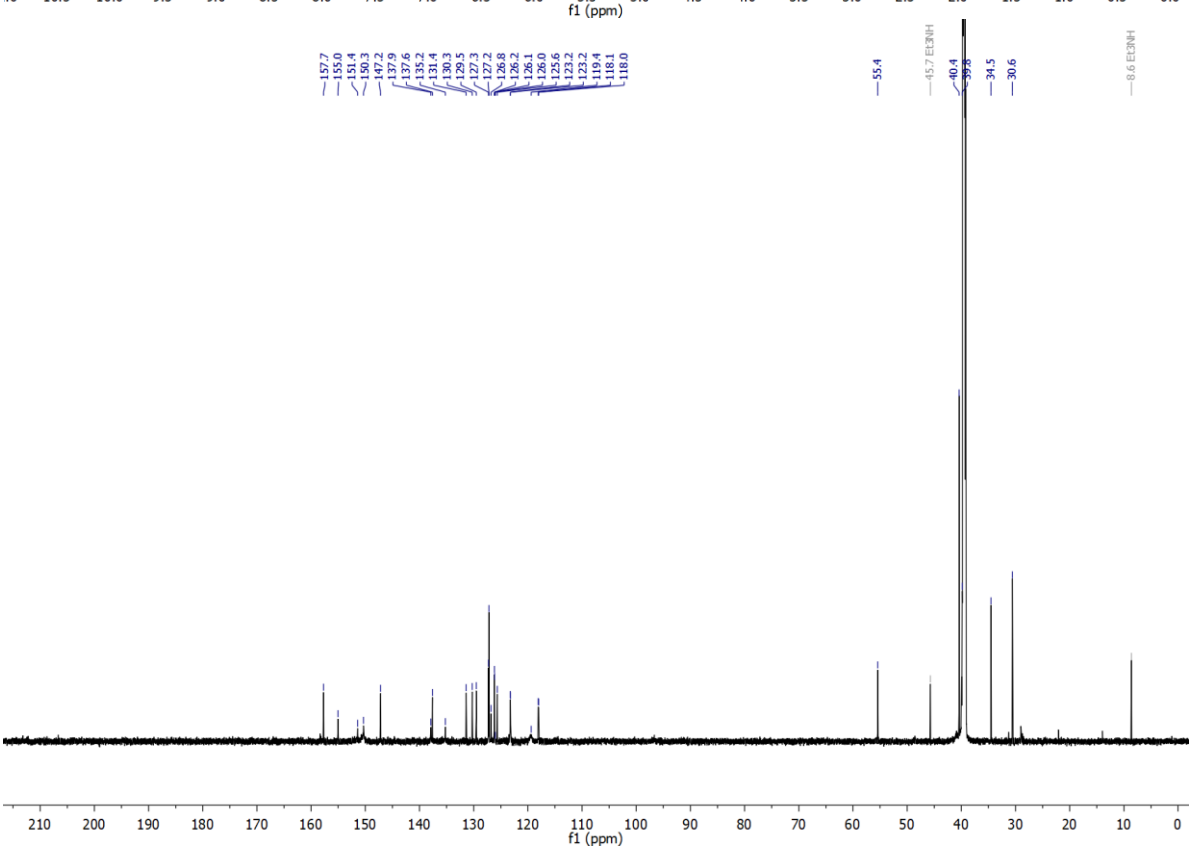




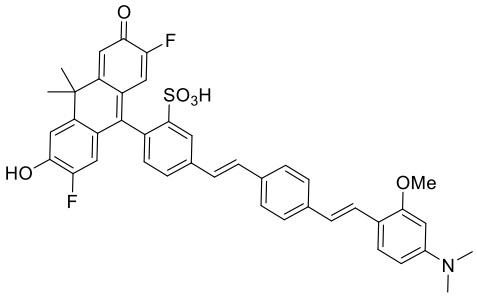


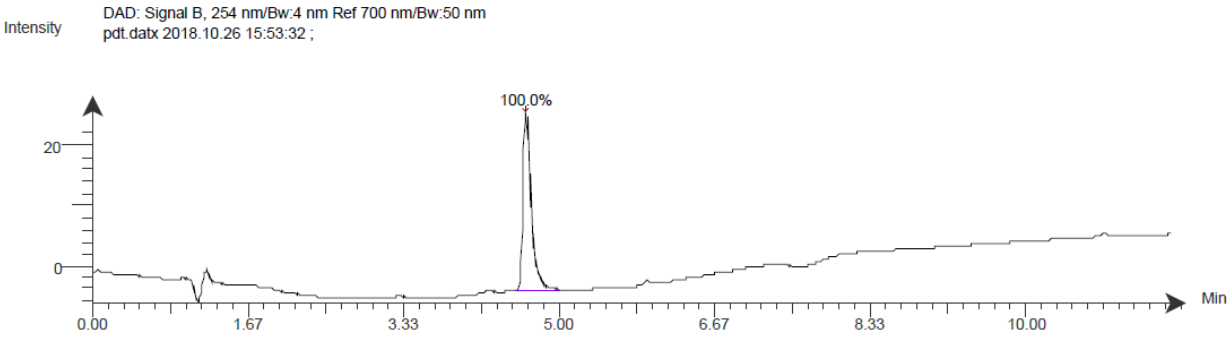
^{1}H and ^{13}C NMR of compound **29**

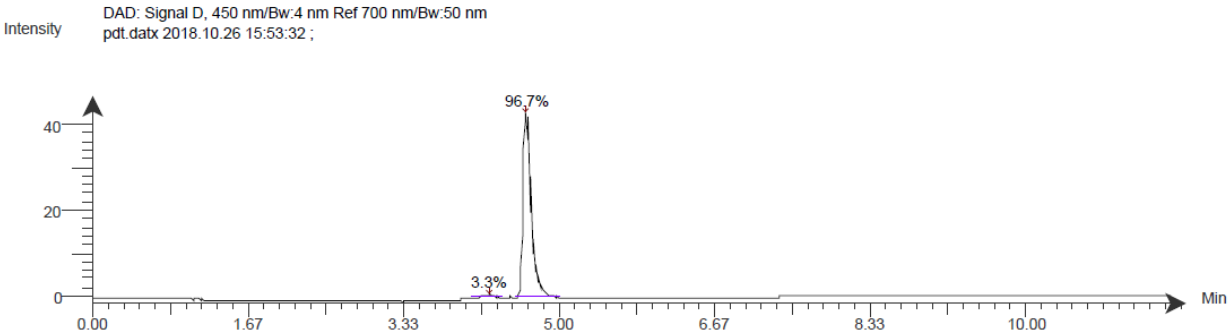


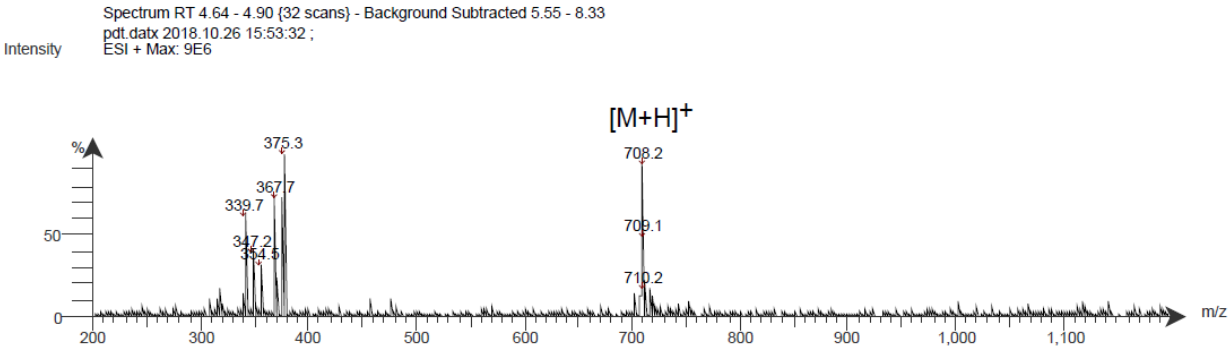


LCMS of compound **29**

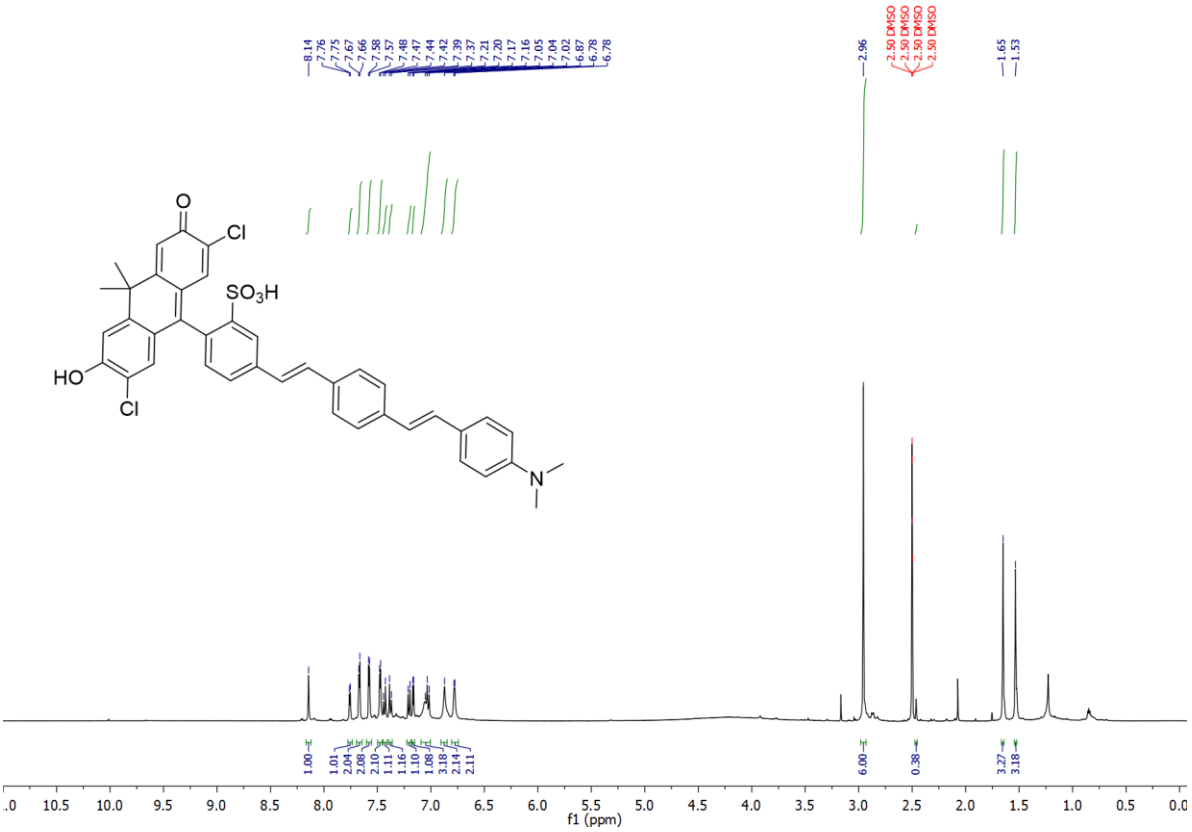


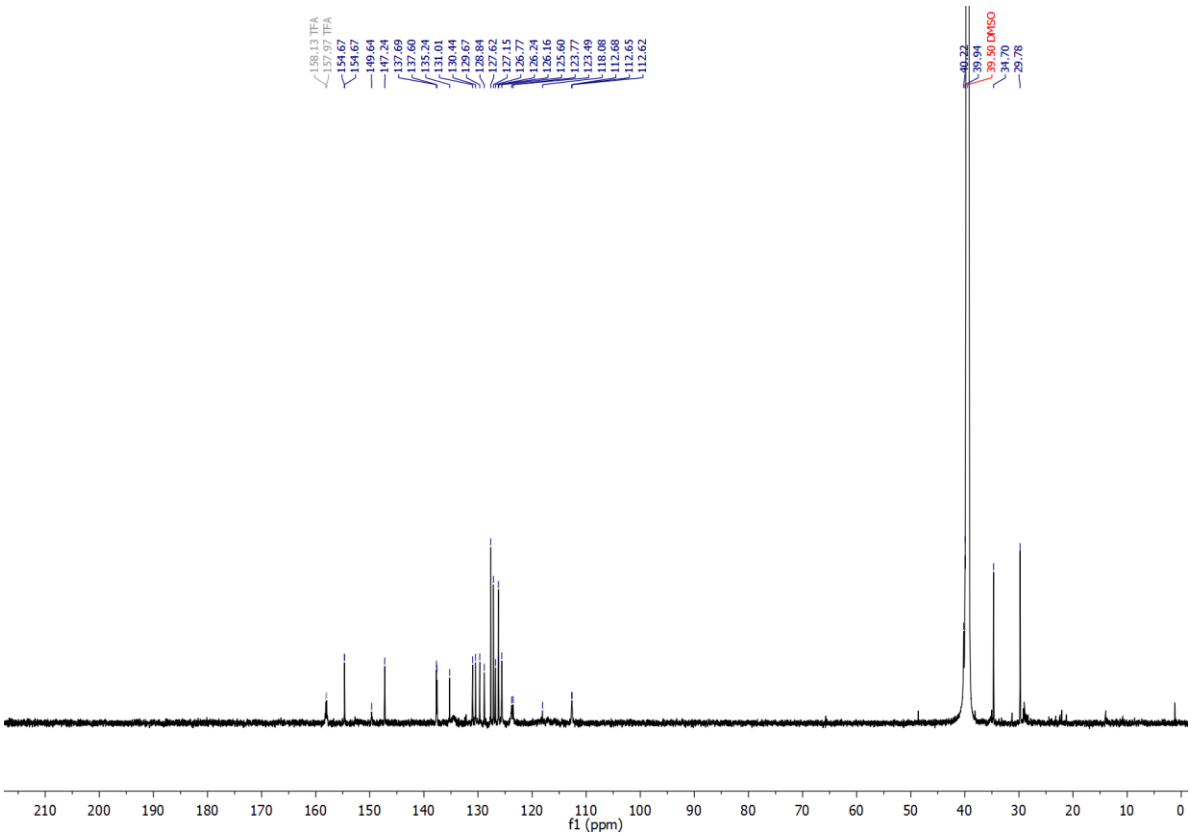




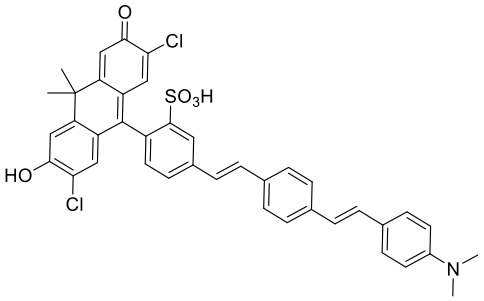


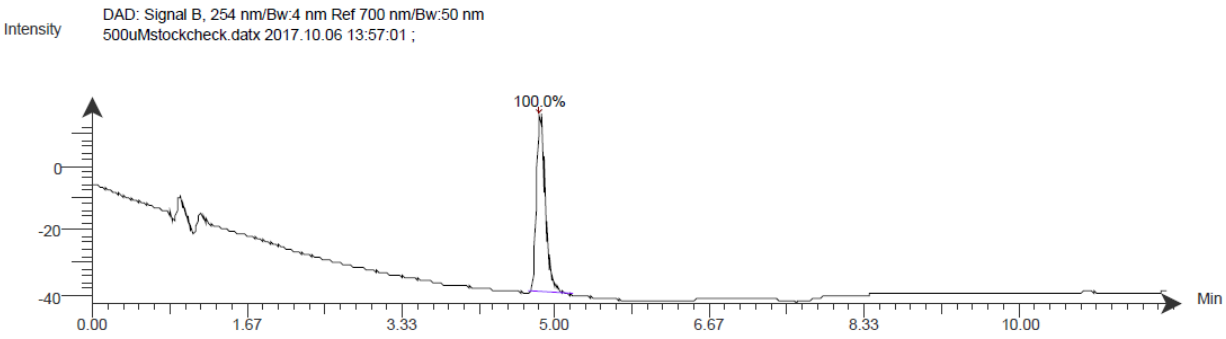
^{1}H and ^{13}C NMR of compound **30**

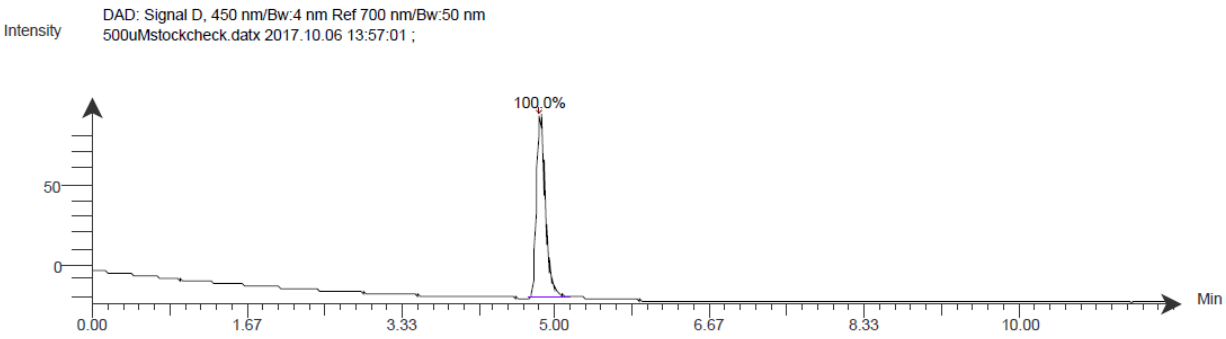


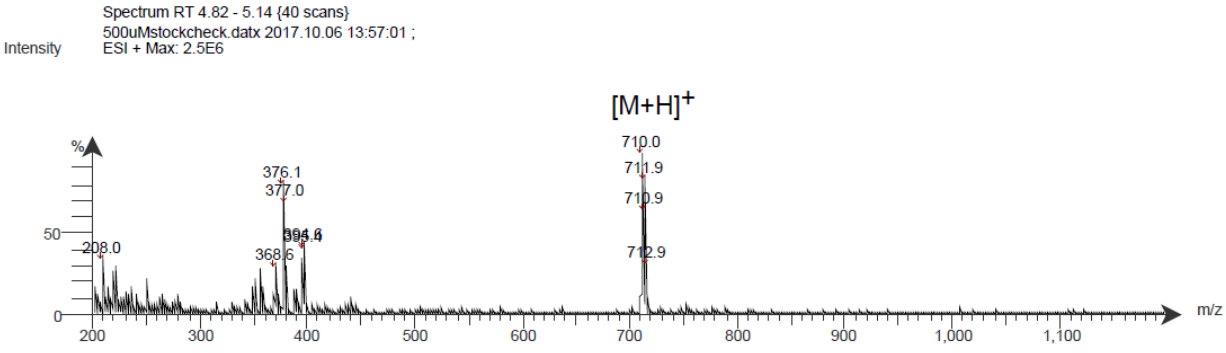


LCMS of compound **30**

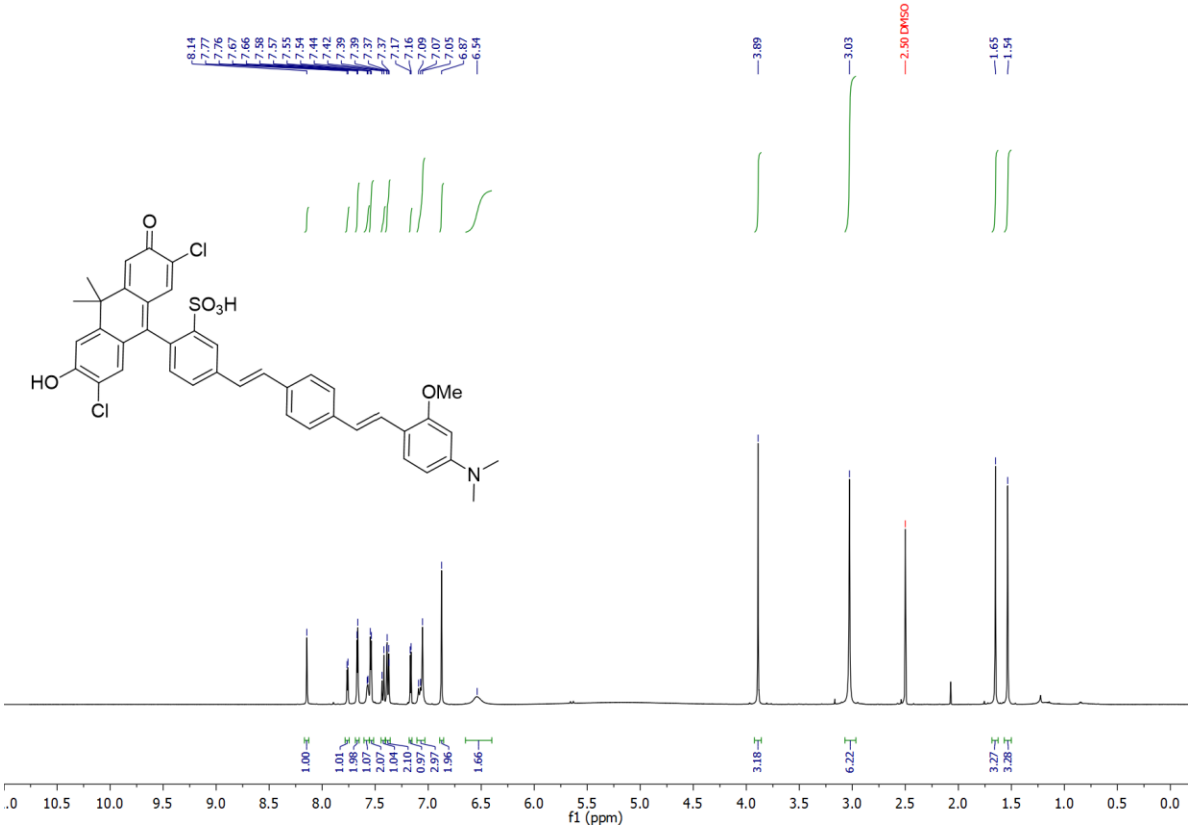


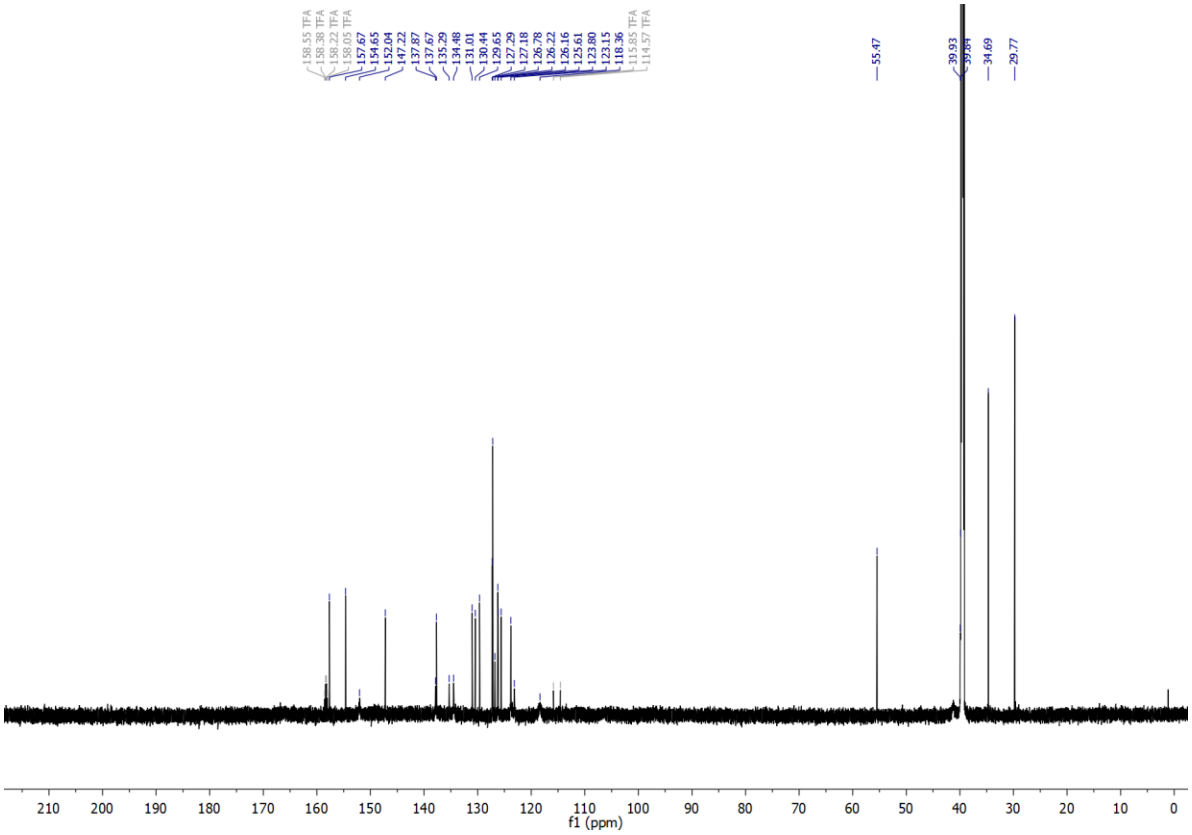




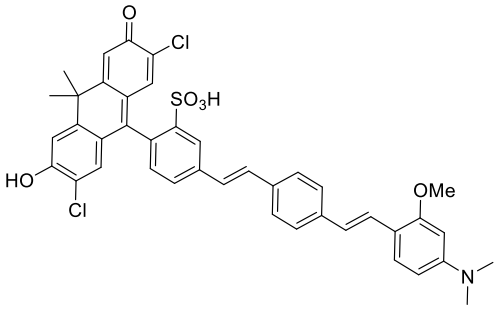


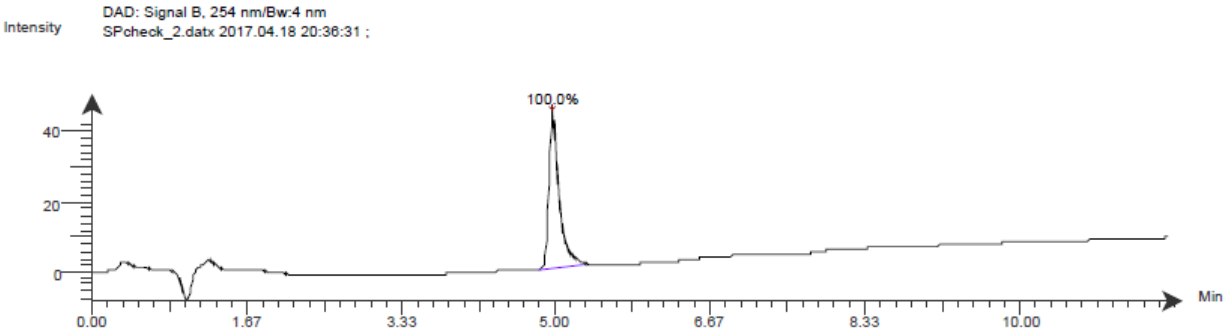
^{1}H and ^{13}C NMR of compound **31**

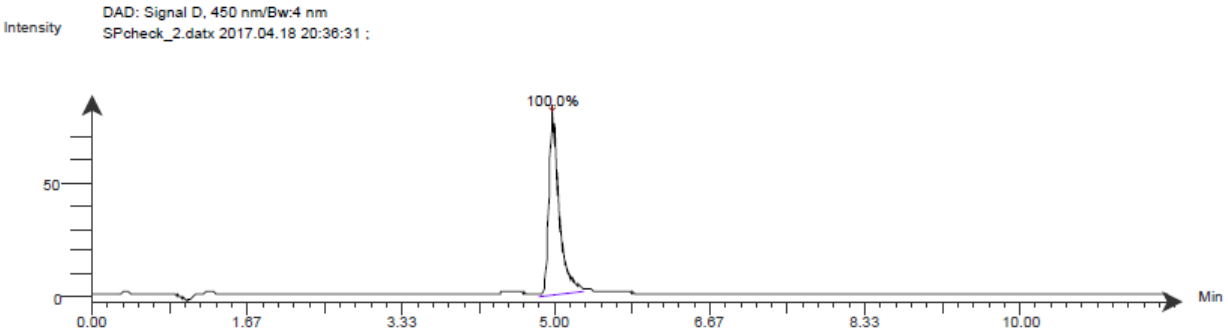


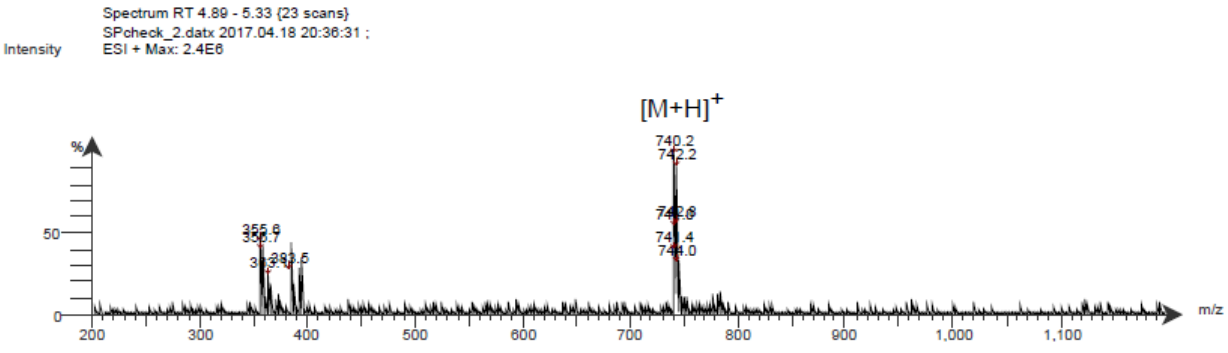


LCMS of compound **31**

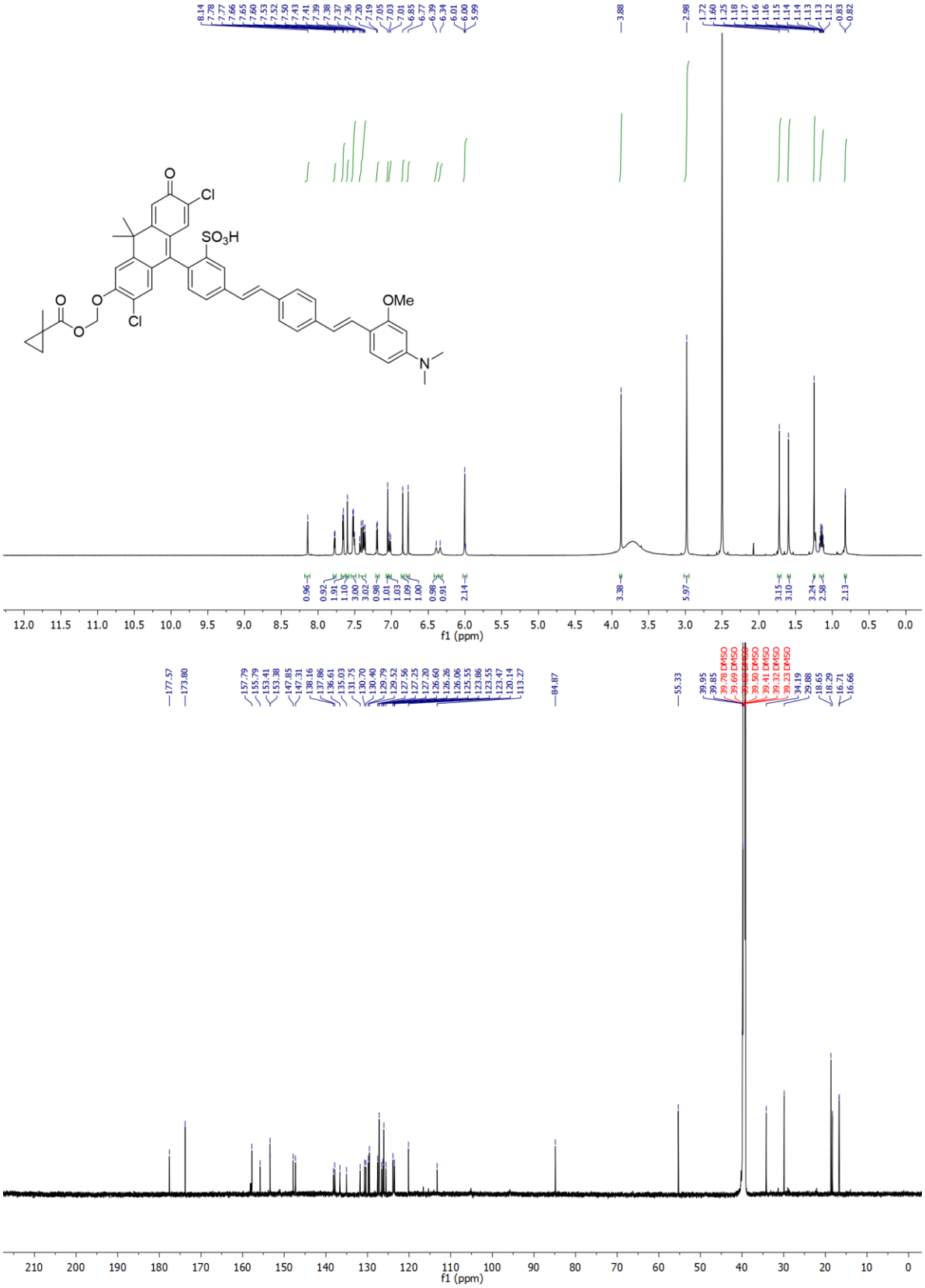




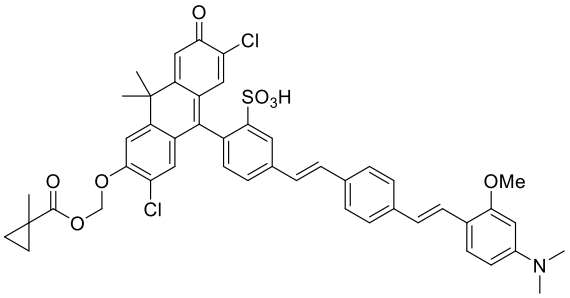


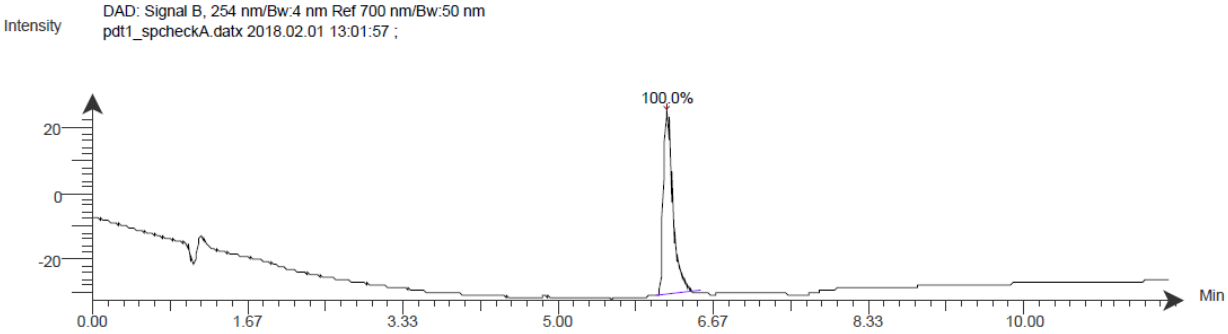


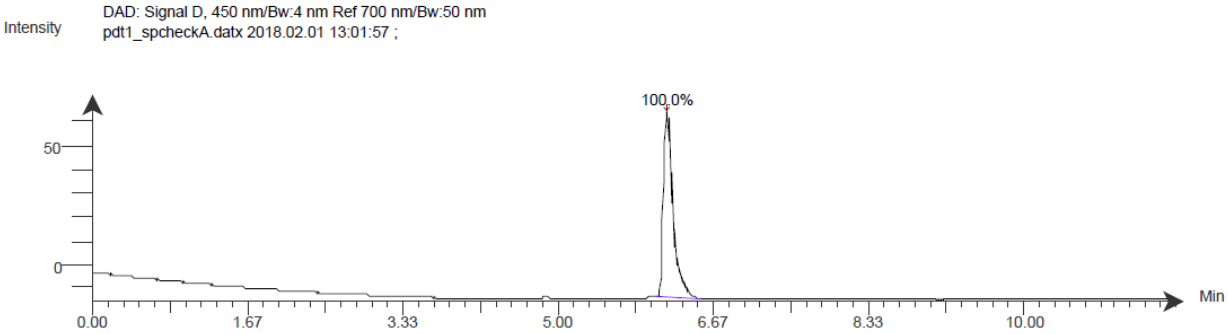
^{1}H and ^{13}C NMR of compound **32**

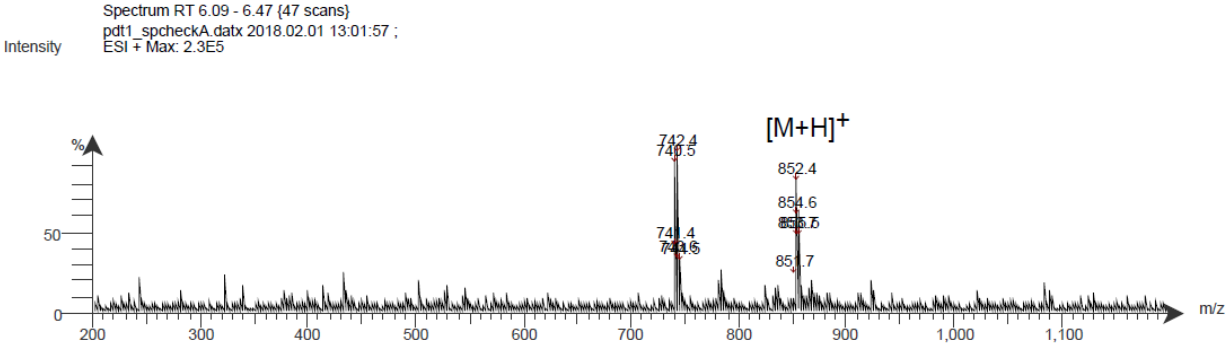


LCMS of compound **32**

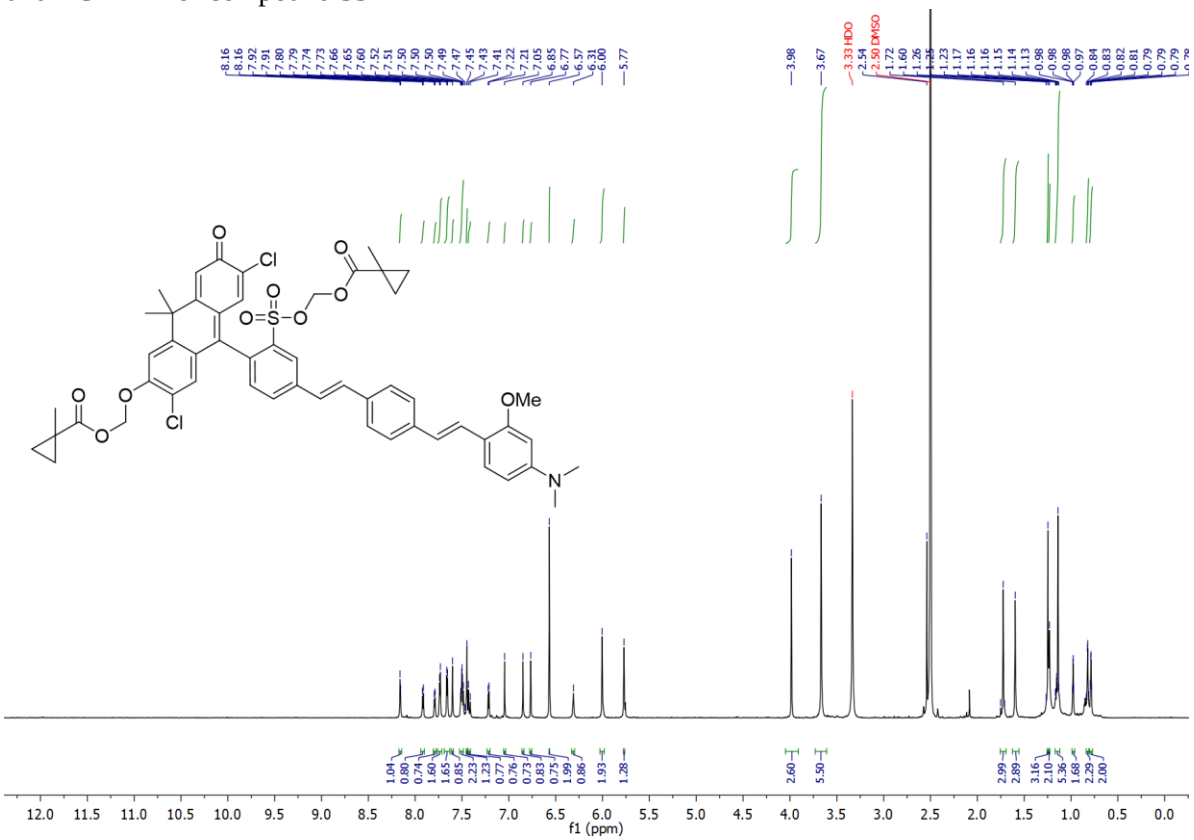


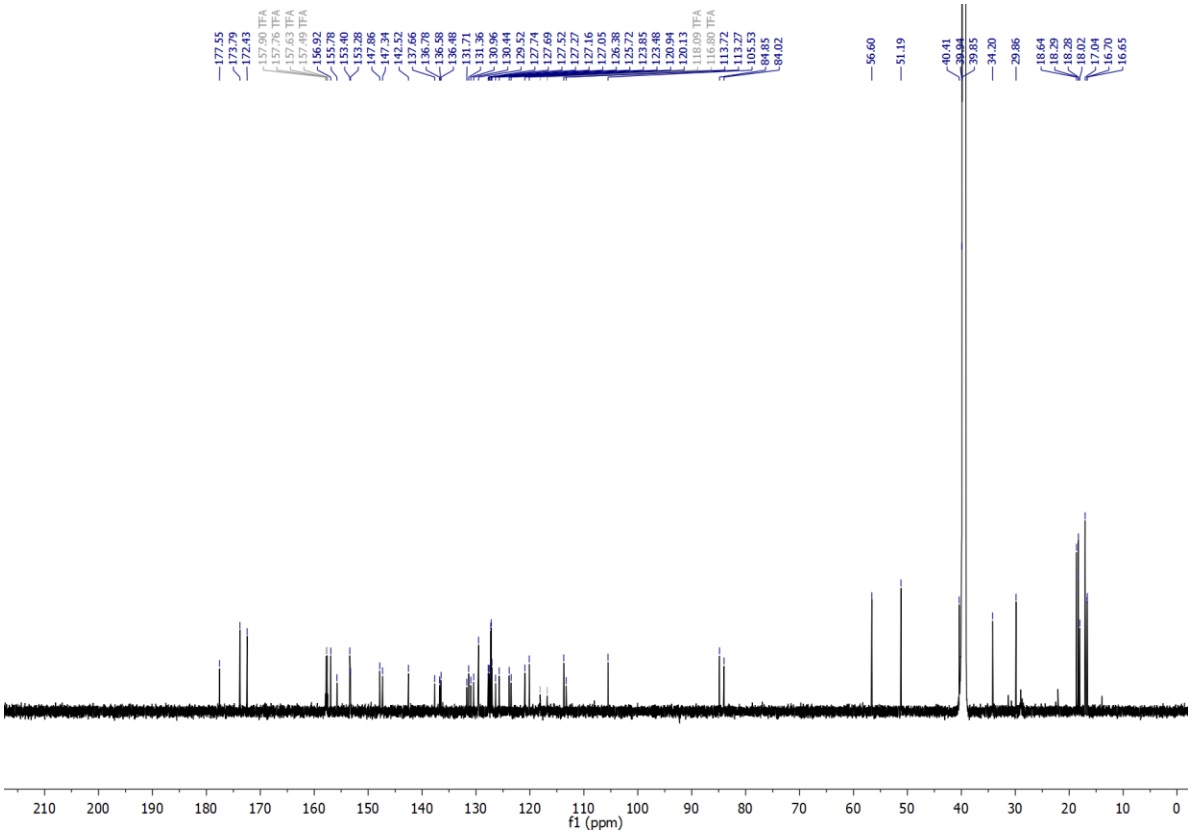




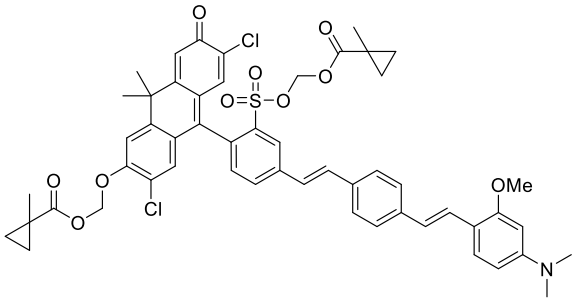


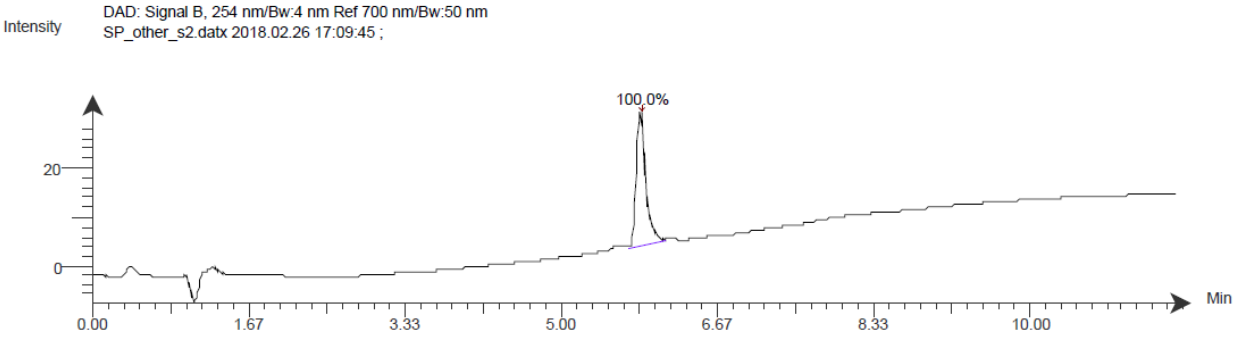
^{1}H and ^{13}C NMR of compound **33**

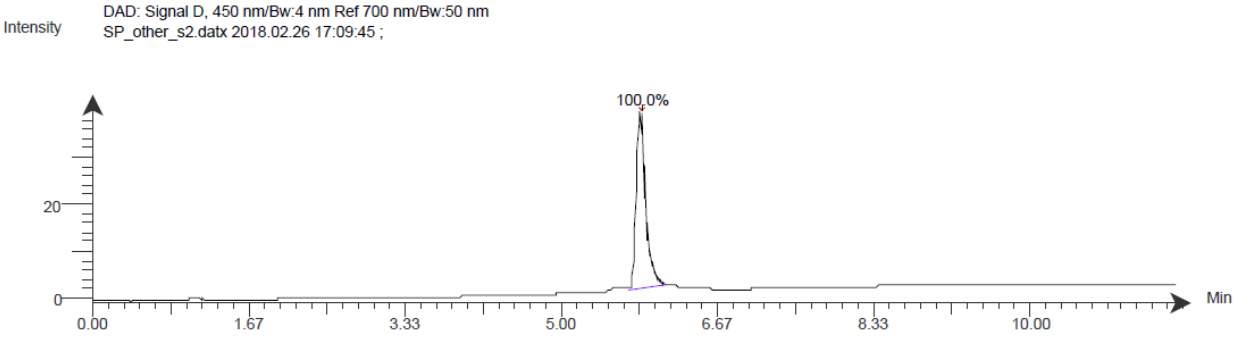


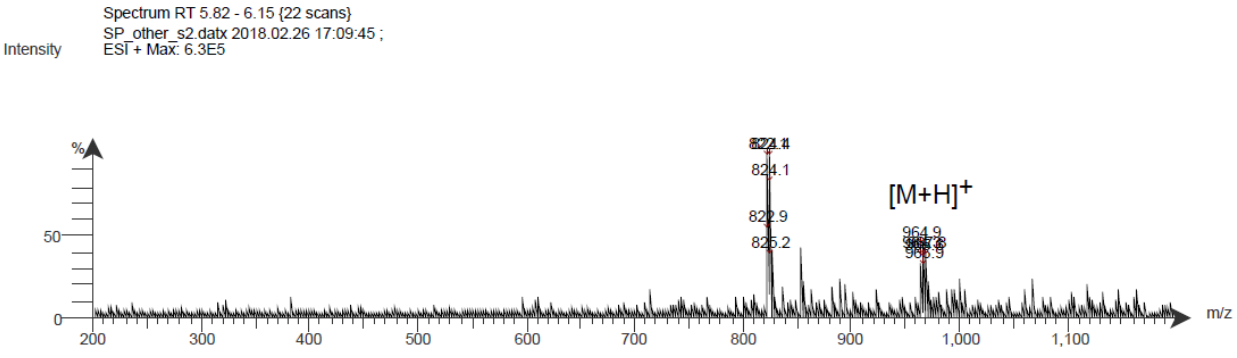


LCMS of compound **33**









References

1. Murugesan, N.; Gu, Z.; Stein, P. D.; Bisaha, S.; Spergel, S.; Girotra, R.; Lee, V. G.; Lloyd, J.; Misra, R. N.; Schmidt, J.; Mathur, A.; Stratton, L.; Kelly, Y. F.; Bird, E.; Waldron, T.; Liu, E. C.; Zhang, R.; Lee, H.; Serafino, R.; Abboa-Offei, B.; Mathers, P.; Giancarli, M.; Seymour, A. A.; Webb, M. L.; Hunt, J. T. *J. Med. Chem*. **1998**, *41*, 5198.
2. Nakamura, T.; Yonesu, K.; Mizuno, Y.; Suzuki, C.; Sakata, Y.; Takuwa, Y.; Nara, F.; Satoh, S. Bioorg. *Med. Chem*. **2007**, *15*, 3548.
3. Grimm, J. B.; Sung, A. J.; Legant, W. R.; Hulamm, P.; Matlosz, S. M.; Betzig, E.; Lavis, L. D. *ACS Chem. Biol.* **2013,** *8*, 1303.
4. Miller, E. W.; Lin, J. Y.; Frady, P.; Steinbach, P. A.; Kristan, W. B.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *109*, 2114.
5. Woodford, C. R.; Frady, P.; Smith, R. S.; Morey, B.; Canzi, G.; Palida, S. F.; Araneda, R. C.; Kristan, W. B.; Kubiak, C. P.; Miller, E. W.; Tsien, R. Y. *J. Am. Chem. Soc.* **2015**, *137*, 1817.
6. Tian, L.; Wysocki, L. M.; Arnold, A. C.; Hu, A.; Ravichandran, B.; Sternson, S. M.; Looger, L. L.; Lavis, L. D. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 4756.
7. Grimm, J. B.; Gruber, T. D.; Ortiz, G.; Brown, T. A.; Lavis, L. D. *Bioconjug. Chem.* **2016,** *27*, 474.
8. Liu, P.; Grenier, V.; Hong, W.; Muller, V. R.; Miller, E. W. *J. Am. Chem. Soc.* **2017,** *139*, 17334.