Supporting Information

Novel Photocrosslinking Chemical Probes Utilized for High-Resolution Spatial Transcriptomics

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Table of Contents **Pages**

Spectroscopy and Fluorescence Imaging Methods

Cell line culture conditions

HEK293T, HeLa, and MDA-MB-231-LM2 cells were cultured in DMEM (Dulbecco's Modification of Eagle's medium) containing high glucose (4.5 g/L) and L-glutamine (Corning, Cat#: 10-017-CM) supplemented with 10% heat inactivated fetal bovine serum (FBS), (Sigma-Aldrich, Cat# 12306C-500ML), 1% penicillin and streptomycin and grown at 37oC, 5% CO2. The HEK293T and HeLa cells were purchased from ATCC. The MDA-MB-231-LM2 cells were purchased from Memorial Sloan Kettering Cancer Center (MSKCC) and developed in the lab of Joan Massagué.

BSA (Bovine serum albumin) tagging *in vitro* and in-gel fluorescence imaging

2 µL of photocaged probe (CrossSeq Probe, 10 mM stock in DMSO) was added to 198 µL of BSA in PBS solution (2 µg/µL) in 1.5 mL amber tubes to protect the probe from ambient light. The mixture was transferred to a 24-well plate and then irradiated in a UV crosslinker (Fisher Scientific Fisherbrand, Cat# 13-245-221) with 368 nm bulbs for the designated time (see below for power output by time). The mixture was transferred to fresh amber 1.5 mL microcentrifuge tubes. Protein was precipitated by adding 800 µL of ice-cold methanol and then stored at -80°C for 1 h minimum or overnight. Protein was collected by centrifugation (20 min, 21,130 RCF, 4 °C). The pellet was washed with 1 mL of ice-cold methanol, vortexed, and centrifuged (3 min, 15,000 RPM, 4°C) three times to remove any unbound CrossSeq probe. The supernatant was removed, and the protein pellet allowed to air dry at room temperature for 15 min. The protein was resuspended in 200 µL of cell lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl) to ~2 µg/µL concentration. An equal amount of loading buffer was added to the purified protein and the sample heated at 95 °C for 5 min. Samples were loaded (8 µg protein) onto 4-20% Mini-PROTEAN TGX gel (Bio-Rad, Cat# 4561094), run at 200 V for 25 min using Tris/Glycine/SDS running buffer (Bio-Rad, Cat# 1610772). Fluorescent gel imaging was done with a ChemiDoc MP imaging system (Bio-Rad) and the contrast was adjusted here after imaging. The gel was incubated with PageBlue™ Protein Staining Solution (Thermo Scientific, catalog# 24620) for 30 min and washed with deionized water 3 times for 1 hour each, then left in deionized water overnight while gently mixing to remove all excess dye. Protein loading control imaging was done with the same imaging system and contrast was adjusted here after imaging.

UV power output by time (µJ):

- 300 sec = 1,123,914
- 120 sec = 462,902
- 60 sec = 219,962
- 30 sec = 108,656
- 10 sec = 33,628

Multiplex on HeLa or HEK293T in navigator mode

HeLa or HEK293T cells were cultured to confluency on an Ibidi 8-well µ-slide (Cat#80826) then washed once with PBS and fixed on ice with glyoxal solution for 20 minutes.[1] Fixative was then removed, and cells were washed three times with PBS. On a Leica Stellaris 8 confocal microscope with a 20X water objective in navigation mode, square regions of interest (ROI) were defined in the same field of view window and then were activated sequentially within one well. ROIs were activated by removing PBS from cells and adding 100 µL of 1 µM CrossSeq sulforhodamine probe (RP) solution to cells, then activated ROI 1 with 100% 405 nm laser for 3 minutes. Then the probe was removed, and cells were washed three times with PBS. Next, 100 µL of 5 µM CrossSeq coumarin probe (CP) solution was added to cells, and ROI 2 was activated with 1% 405 nm laser for 30 seconds. Then the probe was removed, and cells were washed three times with PBS. Finally, 100 µL of 200 µM CrossSeq fluorescein probe (FP) solution was added to the cells, and

ROI 3 was activated with 10% 405 nm laser for 2 minutes. Again, the probe was removed, and cells were washed three times with PBS. All ROIs were visualized simultaneously on three different detectors using the 405 nm laser for the CP ROI, the 492 nm laser for the FP ROI, and the 578 nm laser for the RP. Activated ROIs were still visible 1 week later after storing cells at 4°C in PBS protected from light.

Solution preparation for multiplex tagging

FP = 4 µL 50 mM DMSO into 996µL 10% FBS/DMEM. RP = Prepared a 500 µM working probe solution by adding 10 µL of 50 mM RP stock in DMSO to 990 µL 10% FBS/DMEM, then added 2 µL 500 µM RP solution into 998 µL 10% FBS/DMEM for 1 µM RP final solution. CP = Prepared a 500 µM working probe solution by adding 10 µL of 50 mM CP stock in DMSO to 990 µL 10% FBS/DMEM, then added 10 µL 500 µM CP solution into 990 µL 10% FBS/DMEM for 5 µM CP final solution.

TOC/Abstract graphic - UCI on HEK cells tagged in FRAP mode

HEK cells were cultured to confluency on an Ibidi 8-well µ-slide (Cat#80826) then washed 1X with PBS and fixed on ice with glyoxal solution for 20 minutes [5]. Fixative was removed and cells were washed three times with PBS. On a Leica Stellaris 8 confocal microscope with a 20X water objective in FRAP mode, the UCI regions of interest (ROI) were defined in the same field of view window. The ROI letters were activated by removing PBS and adding 100 µL of 1 µM CrossSeq sulforhodamine probe (RP) solution to cells ROI 1 was then activated with 90% 405 nm laser for 3 minutes. Then the probe was removed, and cells were washed three times with PBS. Next, 100 µL of 5 µM CrossSeq coumarin probe (CP) solution was added to cells, and ROI 2 was activated with 5% 405 nm laser for 30 seconds. Then the probe was removed, and cells were washed three times with PBS. Finally, 100 µL of 200 µM CrossSeq fluorescein probe (FP) solution was added to the cells, and ROI 3 was activated with 10% 405 nm laser for 2 minutes. Again, the probe was removed, and cells were washed three times with PBS. All ROIs were visualized simultaneously on three different detectors using the 405 nm laser for the CP ROI, the 492 nm laser for the FP ROI, and the 578 nm laser for the RP. Activated ROIs were still visible 1 week later after storing cells at 4°C in PBS protected from light.

HeLa multiplex and flow

HeLa cells were cultured to confluency on an Ibidi 8-well µ-slide (Cat#80826) then washed once with PBS and fixed on ice with glyoxal solution for 20 minutes [5]. Fixative was removed and cells were washed three times with PBS. On a Leica Stellaris 8 confocal microscope with 20X water objective in navigation mode, three rectangle regions of interest (ROI) were defined in a single well occupying ~4% surface area of the total well each. ROIs were activated by removing PBS from cells and adding 100 µL of 1 µM CrossSeq sulforhodamine probe (RP) solution to cells, then activated ROI 1 with 100% 405 nm laser for 24 minutes. Then the probe was removed, and cells were washed three times with PBS. Next, 100 µL of 5 µM CrossSeq coumarin probe (CP) solution was added to cells, and ROI 2 was activated with 1% 405 nm laser for 4 minutes. Then the probe was removed, and cells were washed three times with PBS. Finally, 100 µL of 200 µM CrossSeq fluorescein probe (FP) solution was added to the cells, and ROI 3 was activated with 10% 405 nm laser for 16 minutes. Again, the probe was removed, and cells were washed three times with PBS. The cells were then lifted by adding 250 µL accumax (Millipore Sigma, Cat# SCR006) and incubating at 37 C for 15 minutes. The cells were resuspended in accumax and analyzed on an Agilent Novocyte flow cytometer. Lasers and filters used to visualize tagging are as follows. CrossSeq fluorescein was excited with the 488 laser and visualize through the FITC channel 530/30 nm. CrossSeq coumarin was excited with the 405 laser and visualize through the Pacific Blue channel 445/45 nm. CrossSeq sulforhodamine was excited with the 561 laser and visualize through the PE-Texas Red channel 615/24 nm.

MDA-LM2 scratch assay

Plated $1.2x10^6$ MDA-MB-231-LM2 cells, passage number 51, in a 1-well slide (Lab-TeK II Chambered Coverglass w/ Cover, Cat# 155360) and cultured for three days to reach 100%. Six slides were plated in total, with two biological replicates per chch timepoint: 0, 24,48 hours. All open plate manipulation was done in an RNase-free biosafety cabinet. Once RNAzol was added, moved to the bench for processing. Serum starved all 6 samples with DMEM media only for two hours to limit cell proliferation after creating the scratch wound.

A 200 µL pipette tip was used to scratch a straight line through the length of each of the 6 samples. The media was then aspirated, and the scratch wound edge was marked with a permanent marker. The cells were then washed once with 2 mL of 5% serum with 1% Pen/Strep DMEM media, then 3.5 mL of the same media composition was added to the T24 and T48 samples. To the T0 samples, the media was aspirated, and cells were washed once with 2 mL of PBS. The cells were then fixed with 2 mL glyoxal + 10 mM EDTA and incubated on ice for 20 minutes, then washed twice with 2 mL PBS. To sample 2, 2 mL of PBS with 100X RNase inhibitor (Promega RNasin Plus RNase Inhibitor, Cat# N2615) was added for a 1-hour incubation on ice with the plate lid secured shut with parafilm. Sample 1 was left in PBS, sealed with parafilm, and then mapped with the Leica Stellaris 8 confocal microscope LAS X software navigator using a 10X air objective. A 60-square, field of view ROI was defined along the scratch wound.

Once the mapping was complete, the sample was brought back to the biosafety cabinet, and 1.5 mL of 10 µM CrossSeq coumarin probe solution with 100X RNase inhibitor (R.I.) was added and mixed gently to cover the well. The plate was then secured shut with parafilm and the 60-square ROI was activated on the confocal microscope for 60 minutes total. The ROI was activated with a 10X air objective, 5% 405 nm laser power, with a 0.2-micron z-stack in frame mode with frame averaging = 16 giving activation of 55 seconds per ROI in navigator mode.

After activation, the probe was removed from the sample and then washed twice with PBS. 2 mL of PBS with 100X R.I. was then added to the activated sample, the plate was wrapped again in parafilm to secure the cover shut, and the sample was imaged using a 5X air objective to visualize the tagged ROI. Then activation procedure was repeated for sample 2.

After activation of sample 2, 1.5 mL of accumax (Millipore Sigma, Cat# SCR006) was added to both samples and mixed to cover the surface. The plates were then secured with parafilm and incubated at 37ºC for 15 minutes to allow the cells to lift. A pipette was then used to gently lift any cells that had not detached already, and the samples were then added to a 1.5 mL RNase-free microcentrifuge tube each. The samples were then spun down in a microcentrifuge at 700 g for 3 minutes at 4°C. Aspirated supernatant down to the 500 µL mark, then the samples were gently resuspended and transferred to a 5 mL FACS tube (Labcon, Cat# 3336-335-000-9) for sorting.

Cells were then sorted by their coumarin labeled tag into tagged and non-tagged populations per sample on a sterile BD FACSAria Fusion Sorter. Sorted samples were collected in FACS tubes containing 500 µL accumax per tube. After sorting, the solution was agitated and transferred to a 1.5 mL microcentrifuge tube then spun down at 15,000 RPM x 5 minutes at 4°C. Samples were then placed on ice while washing the FACS collection tube with 300 µL of accumax to collect any residual cells. Wash volume was added to the samples accordingly. Vortexed samples well then spun them down at max speed 15,000 RPM at 4°C in a microcentrifuge. Removed supernatant and added 100 µL RNAzol to samples. Homogenized samples well by trituration and then vortexing.

Stored samples in RNAzol at -80°C until all sample time points were collected. The 24 and 48-hour time point samples were processed as previously described to tag the cells within the scratch wound after migration. All previously frozen samples in RNAzol were then thawed on ice and processed using the Zymo Direct-zol MicroPrep RNA extraction kit (Cat# R2060) according to instructions. RNA quantity per sample was measured using a Thermo Scientific Nanodrop 2000 and samples were then submitted to the UCI genomics sequencing core. The core determined

the samples' RIN and DV200 scores using an Agilent 2100 Bioanalyzer Eukaryote Total RNA Pico assay. Then the RNA samples were prepared accordingly for RNA sequencing using the Takara SMARTer Stranded Total RNA-Seq Version 3 – Pico Input Mammalian Kit (Cat# 634485). Samples were then sequenced on an Illumina NovaSeq 6000 using an S4 flowcell with paired-end reads with a read length of 100 bases.

Immunofluorescence validation assay

MDA-MB-231-LM2 cells were seeded at 0.3 x 10^6 cells into an ibiTreated 35 mm µ-dish (ibidi, Cat# 81156) and grown for two days to ~85-90% confluency. One plate was seeded per timepoint (0, 24, and 48 hours) per antibody type for a set of 3 plates total per antibody. Cells were then serum starved with DMEM media only for two hours to limit cell proliferation after creating the scratch wound. A 200 µL pipette tip was used to scratch a straight line through the diameter of the optical portion of the plate for all samples. The media was then aspirated, and the scratch wound edge was marked with a permanent marker. The cells were then washed once with 1 mL of 5% serum with 1% Pen/Strep DMEM media, and then 2 mL of the same media composition was added to the T24 and T48 samples. To the T0 samples, the media was aspirated, and cells were washed once with 2 mL of PBS. The cells were then fixed with 1 mL of 3% glyoxal + 10 mM EDTA and incubated on ice for 20 minutes, then washed three times with 2 mL PBS/0.1% (v/v) Tween 20 (PBS-T). Cells were blocked with 5% BSA (w/v) in PBS-T for 30 minutes at room temperature. The blocking solution was then removed, and primary antibody was diluted into antibody dilution buffer (1% BSA (w/v) in PBS-T) and then added directly to the cells without washing. Cells were incubated in primary antibody (see table below for antibodies and dilutions) overnight at 4°C. Cells were then washed four times with PBS-T and incubated with fluorophore-conjugated secondary antibody in antibody dilution buffer for 1 hour at room temperature protected from light. Cells were then washed three times with PBS-T and incubated in Hoechst 33342 nuclear stain at a 1:1000 dilution in PBS-T for 10 minutes at room temperature protected from light. A final wash was performed with PBS-T, then cells were stored in PBS and imaged using a Leica Stellaris 8 confocal microscope. Fresh primary antibodies and secondary antibodies were used for each plate per time point.

Bioinformatics Analysis and Methods

Sequencing reads were quality-checked using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed using Trimmomatic 0.39 (10.1093/bioinformatics/btu170). Illumina adapters were removed, leading and trailing low-quality bases (<15) were cut, reads were trimmed when quality in a 4 basepair sliding window dropped below 30, and reads shorter than 100 basepairs were dropped. Trimmed reads were pseudoaligned using kallisto 0.48.0 (10.1038/nbt.3519) to a GENCODE v29 reference transcriptome. Differential expression analysis was performed using DESeq2 1.31.16 (10.1186/s13059-014-0550-8). GO term analysis was performed using statistical overrepresentation tests against Biological Process GO terms through PANTHER 17.0 (0.1002/pro.4218). GO term network analysis was performed using the EnrichmentMap 3.3.4 plugin (10.1371/journal.pone.0013984) in Cytoscape 3.8.2 (10.1101/gr.1239303).

Chemical Materials and Methods

Chemical synthesis

Unless stated otherwise, reactions were conducted in oven-dried glassware under an atmosphere of argon. Tetrahydrofuran (THF), 1,2-dimethoxyethane (DME), dimethylformamide (DMF), toluene, dichloromethane (CH_2Cl_2), methanol (MeOH) and triethylamine were dried by passage through activated alumina. All other commercial reagents were purchased and used as received unless otherwise noted. Reaction temperatures were controlled using a temperature modulator, and unless stated otherwise, reactions were performed at room temperature (rt, approximately 23 °C). Thin-layer chromatography (TLC) was conducted with Supelco 1.05715.0001 silica gel 60 F254 pre-coated plates (0.25 mm), and visualized by exposure to UV light (254 nm) or by *p*-anisaldehyde, ceric ammonium molybdate (CAM), or potassium permanganate ($KMnO_4$) staining. Automated column chromatography was performed with the indicated solvents on a Teledyne ISCO CombiflashRf+ with Luknova's SuperSep HP silica or Redisep Rf gold silica (spherical, 25 µm, 70Å). ^{1}H NMR spectra were recorded with one of two NMR instruments as indicated by 400 or 600 MHz and are reported relative to residual solvent signals. UCI's Department of Pharmaceutical Sciences NMR Instrument is a Bruker AvanceNeo Ascend 400. UCI's Department of Chemistry NMR Instrument is a Bruker Avance 600. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz), and integration. Multiplicity is defined as follows: singlet (s), doublet (d), triplet (t), quartet (q), quintet (quin). Dd (doublet of doublets), triplet of doublets (td), doublet of triplets (dt), multiplet (m). ^{13}C NMR spectra were recorded at 100 or 150 MHz as specified. Chemical shift data for ^{13}C NMR spectra are reported relative to the residual solvent signal. High-resolution mass spectra were obtained from UCI's Department of Chemistry Mass Spectrometry Facility on a Waters Micromass LCT Premier high-resolution mass spectrometer with electrospray ionization (ESI). Abbreviations commonly used: IPA (isopropyl alcohol), Hex (hexanes), DMAP (4-dimethylaminopyridine); For others, see JOC Standard Abbreviations and Acronyms.

UV-Vis and fluorescence spectroscopy

CrossTag probes were prepared as stock solutions in DMSO and diluted such that the DMSO concentration did not exceed 1% (vol/vol). Spectroscopy was performed using 1-cm path length, 0.580-mL quartz microcuvettes from Starna Cells. All measurements were carried out at ambient temperature. Absorption spectra were collected on a Cary Model 300 UV-Vis Spectrophotometer. Fluorescence spectra were recorded on a Cary Eclipse Fluorescence Spectrophotometer. The spectral properties of all final compounds (**3**, **4**, **7**, **8**, **11**, and **12**) are summarized in **Table S1**.

Extinction Coefficient Determination

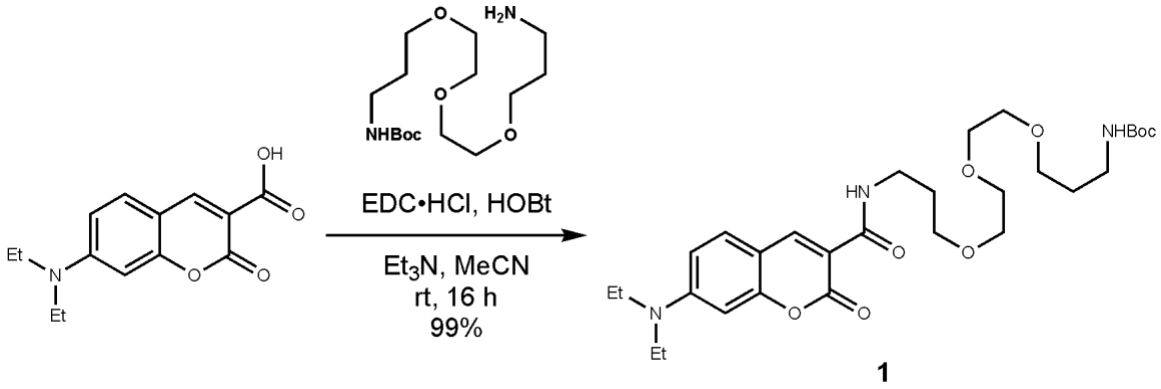
Extinction coefficients were determined for all final compounds in appropriates solvents as detailed in the caption of **Table S1**. Four independent stocks of each compound were prepared from purified solid. The extinction coefficients were calculated using Beer's law ($\varepsilon = A/(c \bullet l)$; ε = extinction coefficient, A = absorbance, c = concentration, l = pathlength). Reported values for ε are averages of n = 3.

Quantum Yield Determination

The absolute fluorescence quantum yields (φ) of the CrossTag probes were determined using the comparative method of Williams *et al*.[2] The probes were assigned to one of three categories based off their parent fluorophore (ie. coumarin, fluorescein, or rhodamine). Standard solutions and test samples were measured at the same excitation wavelength with identical instrument parameters. Measurements were carried out using dilute samples within the linear range of the instrument. Solvents and excitation wavelengths used were based off the chosen standards.

Coumarin 1, fluorescein, and rhodamine B were chosen as the standards for the coumarin, fluorescein, and rhodamine probes, respectively.[3,4,5] The coumarin probes were dissolved in EtOH. The fluorescein probes were dissolved in 0.1M NaOH. The rhodamine probes were dissolved in water. Our procedure follows UCI's "Guide to Recording Fluorescence Quantum Yields" online.[6] For each final compound, the fluorescence spectrum of five solutions of increasing concentration were recorded as well as a blank without changing the slit width. Total fluorescence was calculated by numerical integration of the area under the curve. Plotting the integrated fluorescence intensity v. absorbance generates a straight line that intercepts at 0. The slope (m) of this graph is proportional to the quantum yield (ϕ) of the compound. The quantum yield can then be calculated from: $\phi_x = \phi_{st}(\frac{m_x}{m_{st}})(\frac{\eta_x^2}{\eta_{st}^2})$ where st = standard, x = test compound, η= refractive index. Reported values are averages of n=3.

Synthesis

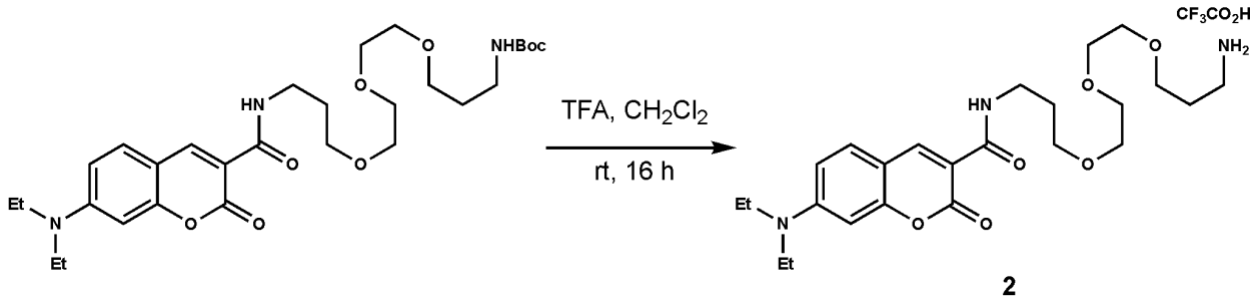


Preparation of *tert*-butyl (1-(7-(diethylamino)-2-oxo-2*H*-chromen-3-yl)-1-oxo-6,9,12-trioxa-2-azapentadecan-15-yl)carbamate (coumarin carbamate; 1): A 100 mL flame-dried round bottom flask was charged with 3-carboxy-7-diethylaminocoumarin (524.9 mg, 2.00 mmol) and HOBt (329.8 mg, 2.44 mmol, 1.2 equiv) in MeCN (20 mL, 0.1M). Then EDC•HCl (469.7 mg, 2.45 mmol, 1.2 equiv) and Et_3N (0.84 mL, 6.00 mmol, 3 equiv) were added. The resulting yellow mixture was stirred for 1 h. The monoboc diamine (770 mg, 2.4 mmol, 1.2 equiv) was added dropwise as a solution in MeCN (2 mL). The reaction was stirred overnight (~16 h) at room temperature. The reaction was diluted with CH_2Cl_2 (50 mL), washed with 1 M HCl (50 mL), and brine (50 mL). The organic layer was then dried over $MgSO_4$, filtered, and concentrated to a brown oil. The crude residue was purified by flash chromatography on a prepacked silica column (1:0 → 95:5 CH_2Cl_2/MeOH) to afford **1** (1.12 g, 99%.) as a yellow oil. R_f = 0.41 (95:5 CH_2Cl_2/MeOH)

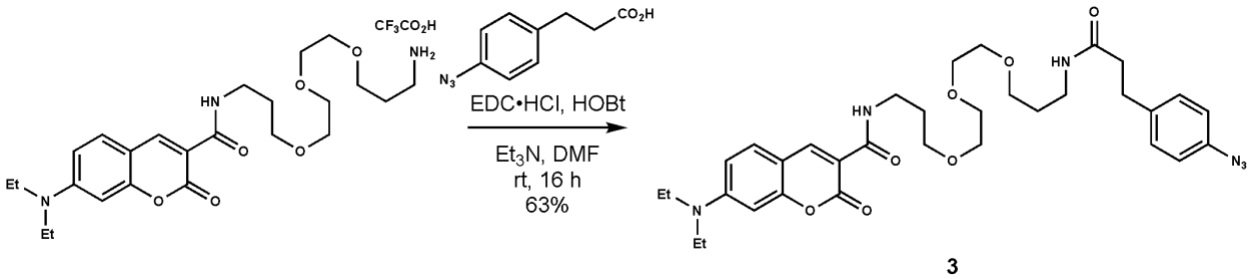
^{1}H NMR (600 MHz, $CDCl_3$) δ 8.76 (s, 1H), 8.51 (d, *J* = 6.5 Hz, 1H), 7.27 (d, *J* = 8.5 Hz, 1H), 6.49 (d, *J* = 7.9 Hz, 1H), 6.31 (s, 1H), 5.13 (s, 1H), 3.63–3.58 (m, 1H), 3.57–3.53 (m, 2H), 3.52–3.47 (m, 4H), 3.46–3.42 (m, 4H), 3.42–3.36 (m, 3H), 3.33–3.25 (m, 4H), 3.11–3.02 (m, 2H), 1.80–1.71 (m, 2H), 1.66–1.56 (m, 2H), 1.28 (s, 9H), 1.08 (t, *J* = 6.5 Hz, 6H)

^{13}C NMR (150 MHz, $CDCl_3$) δ (exists as a mixture of rotamers) 163.07, 163.04, 163.0, 162.5, 162.45, 161.5, 157.5, 156.1, 152.4, 152.38, 147.8, 147.77, 131.0, 110.2, 110.17, 109.9, 109.8, 108.2, 96.4, 78.8, 78.6, 72.64, 72.56, 70.5, 70.4, 70.3, 70.29, 70.1, 69.5, 69.3, 69.1, 61.6, 61.5, 45.0, 38.4, 38.3, 37.05, 37.01, 29.5, 29.4, 28.4, 12.4

HRMS (ESI-TOF) m/z $[M+Na]^+$ calcd for $[C_{29}H_{45}N_3O_8Na]^+$ 586.3104, found 586.3097



Deprotection of coumarin carbamate (2): A round bottom flask was charged with carbamate **1** (376.0 mg, 0.667 mmol) in CH_2Cl_2 (3 mL) and TFA (3 mL, 0.1 M final concentration) was added slowly. The mixture was then stirred at room temperature overnight. The reaction was then concentrated under reduced pressure to afford **2** as a brown oil, which was used without further purification.



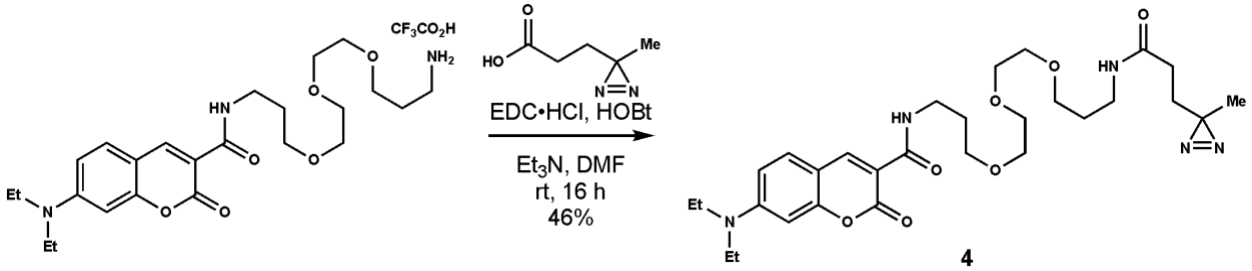
3

Preparation of *N*-(17-(4-azidophenyl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)-7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxamide (coumarin phenyl azide; 3): A flame dried 10 mL round bottom flask was charged with 3-(4-azidophenyl)propionic acid (70.6 mg, 0.369 mmol, 1.1 equiv), HOBt (52.1 mg, 0.385 mmol, 1.1 equiv) and Et_3N (0.24 mL, 1.71 mmol, 5 equiv) in DMF (2.5 mL, 0.1 M). EDC•HCl (73.2 mg, 0.381 mmol, 1.1 equiv) was added. The reaction was then stirred for 1 h at room temperature. Then amine salt **2** (192.6 mg, 0.3335 mmol) in DMF (0.5 mL) was added and the mixture was stirred overnight at room temperature. The reaction was then diluted with CH_2Cl_2 (30 mL) and washed with sat. $NaHCO_3$ (40 mL). The organics were washed with brine (30 mL), dried over $MgSO_4$, filtered, and concentrated to an orange oil. The crude residue was purified by flash chromatography on a prepacked silica column (1:0 → 95:5 → 9:1 CH_2Cl_2/MeOH) to afford **3** (134.1 mg, 63% over two steps) as a light orange oil. R_f = 0.4 (95:5 CH_2Cl_2/MeOH)

^{1}H NMR (600 MHz, $CDCl_3$, exists as a mixture of rotamers) δ 8.88 (t, *J* = 5.6 Hz, 1H), 8.68 (s, 0.4H), 8.65 (s, 0.6H), 7.41 (d, *J* = 8.9 Hz, 0.4H), 7.38 (d, *J* = 8.9 Hz, 0.6H), 7.17 (d, *J* = 8.2 Hz, 2H), 6.90 (d, *J* = 8.2 Hz, 2H), 6.65–6.60 (m, 1H), 6.49–6.46 (m, 1H), 6.44 (s, 1H), 3.70–3.67 (m, 1H), 3.65–3.61 (m, 3H), 3.59–3.56 (m, 2H), 3.55–3.49 (m, 8H), 3.46–3.40 (m, 4H), 3.35–3.31 (m, 2H), 2.92 (t, *J* = 7.7 Hz, 2H), 2.43 (t, *J* = 7.7 Hz, 2H), 1.88 (quin, *J* = 6.6 Hz, 2H), 1.74–1.69 (m, 2H), 1.25–1.21 (m, 6H)

^{13}C NMR (150 MHz, $CDCl_3$, exists as a mixture of rotamers) δ 171.9, 163.2, 163.1, 162.7, 162.65, 157.7, 152.6, 152.5, 148.0, 147.96, 138.1, 137.8, 131.1, 131.09, 129.8, 119.1, 119.0, 110.4, 110.0, 109.9, 108.4, 108.39, 96.6, 96.58, 70.6, 70.5, 70.42, 70.4, 70.1, 70.07, 69.3, 69.2, 45.1, 45.09, 38.3, 38.0, 37.2, 37.15, 31.1, 29.6, 28.8, 12.4

HRMS (ESI-TOF) m/z $[M+Na]^+$ calcd for $[C_{33}H_{44}N_6O_7Na]^+$ 659.3169, found 659.3198



4

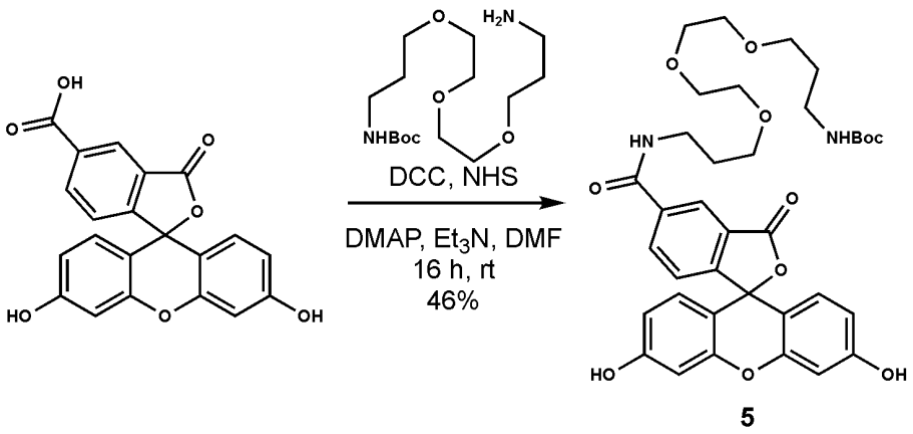
Preparation of 7-(diethylamino)-*N*-(17-(3-methyl-3*H*-diazirin-3-yl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)-2-oxo-2*H*-chromene-3-carboxamide (coumarin diazirine; 4): A flame dried 10 mL round bottom flask was charged with 3-(3-methyl-3H-diazirin-3-yl)propanoic acid (50.4 mg, 0.393 mmol, 1.1 equiv), HOBt (50.7 mg, 0.375 mmol, 1.1 equiv) and Et_3N (0.24 mL, 1.71 mmol, 5 equiv) in DMF (2.5 mL, 0.1 M). EDC•HCl (70.2 mg, 0.366 mmol, 1.1 equiv) was added. The reaction was then stirred for 1 h at room temperature. Amine salt **2** (192.6 mg, 0.3335 mmol) in DMF (0.5 mL) was then added and the mixture was stirred overnight at room temperature. The reaction was diluted with CH_2Cl_2 (30 mL) and washed with sat. $NaHCO_3$ (40 mL). The organics were washed with brine (30 mL), dried over $MgSO_4$, filtered, and concentrated to an orange oil.

The crude residue was purified by flash chromatography on a prepacked silica column (1:0 → 95:5 → 9:1 CH_2Cl_2/MeOH) to afford **4** (88.0 mg, 46% over two steps) as a yellow oil. R_f = 0.61 (95:5 CH_2Cl_2/MeOH)

^{1}H NMR (600 MHz, $CDCl_3$, exists as a mixture of rotamers) δ 8.92–8.85 (m, 1H), 8.69(s, 0.6H), 8.68 (s, 0.4H), 7.43 (d, *J* = 8.9 Hz, 0.6H), 7.41 (d, *J* = 8.9 Hz, 0.4H), 6.63 (dt, *J* = 2.4, 8.7 Hz, 1H), 6.51–6.47 (m, 2H), 3.70–3.68 (m, 2H), 3.67–3.65 (m, 2H), 3.64–3.61 (m, 2H), 3.60–3.55 (m, 6H), 3.55–3.51 (m, 2H), 3.47–3.41 (m, 4H), 3.36 (q, *J* = 6.0 Hz, 2H), 2.0–1.96 (m, 2H), 1.92–1.87 (m, 2H), 1.79–1.72 (m, 4H), 1.26–1.21 (m, 6H), 1.01 (s, 3H)

^{13}C NMR (150 MHz, $CDCl_3$, exists as a mixture of rotamers) δ 171.4, 163.2, 163.1, 162.74, 162.7, 157.66, 157.64, 152.6, 152.5, 148.1, 148.0, 131.2, 131.1, 110.6, 110.4, 110.0, 109.9, 108.44, 108.42, 96.63, 96.6, 70.6, 70.56, 70.44, 70.42, 70.2, 70.17, 69.3, 69.2, 45.1, 45.09, 38.2, 37.2, 37.16, 30.7, 30.2, 29.6, 28.8, 25.6, 20.0, 12.5

HRMS (ESI-TOF) m/z $[M+H]^+$ calcd for $[C_{29}H_{44}N_5O_7]^+$ 574.3241, found 574.3230



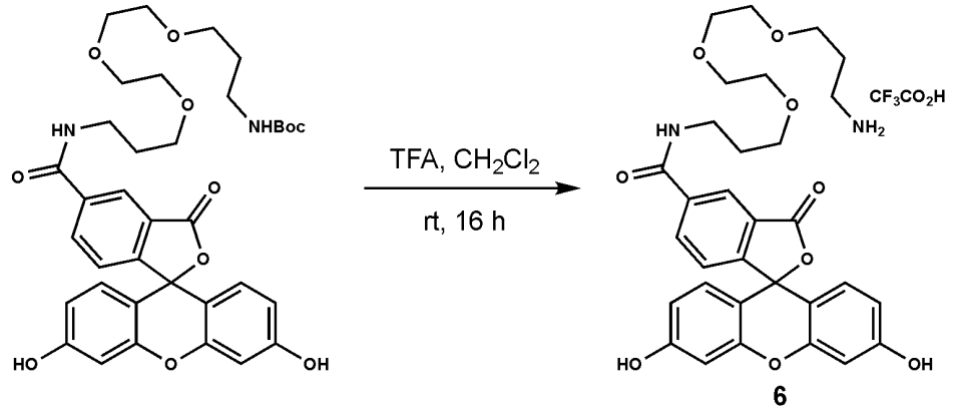
5

Preparation of *tert*-butyl (1-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)-1-oxo-6,9,12-trioxa-2-azapentadecan-15-yl)carbamate (fluorescein carbamate; 5): A 10 mL round bottom flask was charged with 5-carboxyfluorescein (103.2 mg, 0.27 mmol), N-hydroxysuccinimide (39 mg, 0.338 mmol, 1.2 equiv), and DMAP (5 mg, 0.04 mmol, 5 mol %) in DMF (2.5 mL). The mixture was vigorously stirred for 15 minutes or until the solution was homogenous. Then DCC (67.1 mg, 0.325 mmol, 1.2 equiv) was added. The reaction was allowed to stir at room temperature overnight (~16 h). The reaction turned from yellow to red and white precipitate formed. Then monoboc diamine (97.1 mg, 0.303 mmol, 1.1 equiv) and Et_3N (0.08 mL, 0.57 mmol, 2 equiv) were added as a solution in DMF (2.5 mL). The reaction was stirred overnight (~16 h). The reaction was filtered and dry loaded on silica gel (~500 mg). The crude residue was purified by flash chromatography on a prepacked silica column (1:0 → 95:5 → 9:1 CH_2Cl_2 with 1% AcOH/MeOH) to afford **5** (84.2 mg, 46%) as a red-orange oil. R_f = 0.42 (9:1 CH_2Cl_2/MeOH)

^{1}H NMR (600 MHz, CD_3OD) δ 8.43 (s, 1H), 8.21 (dd, *J* = 1.2, 8.0 Hz, 1H), 7.32 (d, *J* = 8.1 Hz, 1H), 6.71 (d, *J* = 2.2 Hz, 2H), 6.63 (s, 1H), 6.62 (s, 1H), 6.56 (dd, *J* = 2.3, 8.7 Hz, 2H), 3.70–3.61 (m, 8H), 3.58–3.53 (m, 4H), 3.48 (t, *J* = 6.2 Hz, 2H), 3.11 (t, *J* = 6.7 Hz, 2H), 1.94 (quin, *J* = 6.3 Hz, 2H), 1.70 (quin, *J* = 5.2 Hz, 2H), 1.43 (s, 9H)

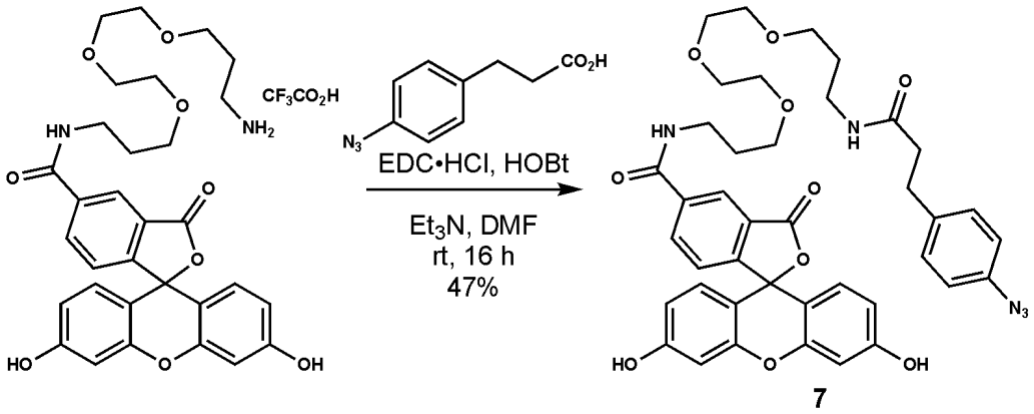
^{13}C NMR (150 MHz, CD_3OD) δ 169.3, 166.9, 160.5, 157.1, 154.7, 152.8, 136.6, 133.9, 128.8, 127.6, 124.5, 123.5, 112.6, 109.7, 109.6, 102.3, 78.5, 70.2, 70.1, 69.9, 69.8, 68.9, 68.5, 37.6, 37.3, 29.5, 28.9, 27.4

HRMS (ESI-TOF) m/z $[M+H]^+$ calcd for $[C_{36}H_{43}N_2O_{11}]^+$ 679.2867, found 679.2855



6

Deprotection of fluorescein carbamate (6): A round bottom flask was charged with carbamate **5** (84.2 mg, 0.124 mmol) in CH_2Cl_2 (0.6 mL) and TFA (0.6 mL, 0.1 M final concentration) was added slowly. The mixture was then stirred at room temperature overnight. The mixture was concentrated under reduced pressure to afford a red oil, which was used without further purification.

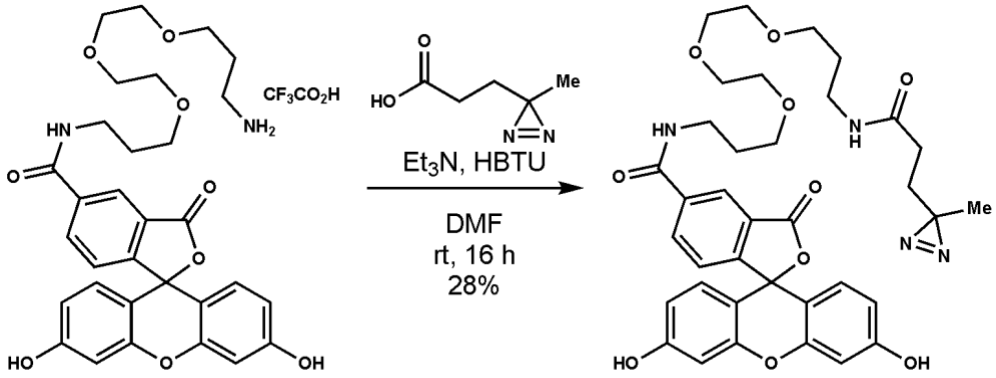


7

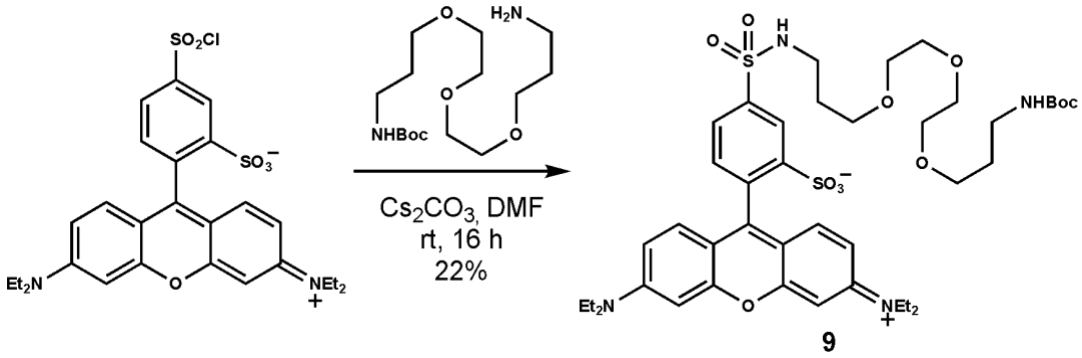
Preparation of *N*-(17-(4-azidophenyl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)-3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamide (fluorescein phenyl azide; 7): A flame dried 10 mL round bottom flask was charged with 3-(4-azidophenyl)propionic acid (27.4 mg, 0.14 mmol, 1.1 equiv), HOBt (19.4 mg, 0.14 mmol, 1.1 equiv) and Et_3N (0.09 mL, 0.64 mmol, 5 equiv) in DMF (1.0 mL, 0.1 M). EDC•HCl (29.4 mg, 0.15 mmol, 1.1 equiv) was added. The reaction was then stirred for 1 h at room temperature. Crude amine salt **6** (86 mg, 0.124 mmol) in DMF (0.5 mL) was then added and the mixture was stirred overnight at room temperature. The reaction was diluted with CH_2Cl_2 (30 mL) and washed with sat. $NaHCO_3$ (40 mL). The organics were washed with brine (30 mL), dried over $MgSO_4$, filtered, and concentrated to an orange oil. The crude residue was dry loaded on silica gel (~300 mg). The crude residue was purified by flash chromatography on a prepacked silica column (1:0 → 95:5 → 9:1 → 4:1 CH_2Cl_2/MeOH) to afford **7** (43.4 mg, 47% over two steps) as an orange oil. R_f = 0.44 (95:5 CH_2Cl_2/MeOH)

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.43 (s, 1H), 8.20 (dd, *J* = 1.4, 8.0 Hz, 1H), 7.30 (d, *J* = 8.1 Hz, 1H), 7.21 (d, *J* = 8.2 Hz, 2H), 6.94 (d, *J* = 8.4 Hz, 2H), 6.71 (d, *J* = 2.2 Hz, 2H), 6.61 (s, 1H), 6.60 (s, 1H), 6.55 (dd, *J* = 2.3, 8.7 Hz, 2H), 3.67–3.59 (m, 8H), 3.54 (t, *J* = 6.7 Hz, 2H), 3.53–3.49 (m, 2H), 3.38–3.34 (m, 2H), 3.19 (t, *J* = 6.8 Hz, 2H), 2.88 (t, *J* = 7.5 Hz, 2H), 2.45 (t, *J* = 7.5 Hz, 2H), 1.92 (quin, *J* = 6.3 Hz, 2H), 1.65 (quin, *J* = 6.5 Hz, 2H)

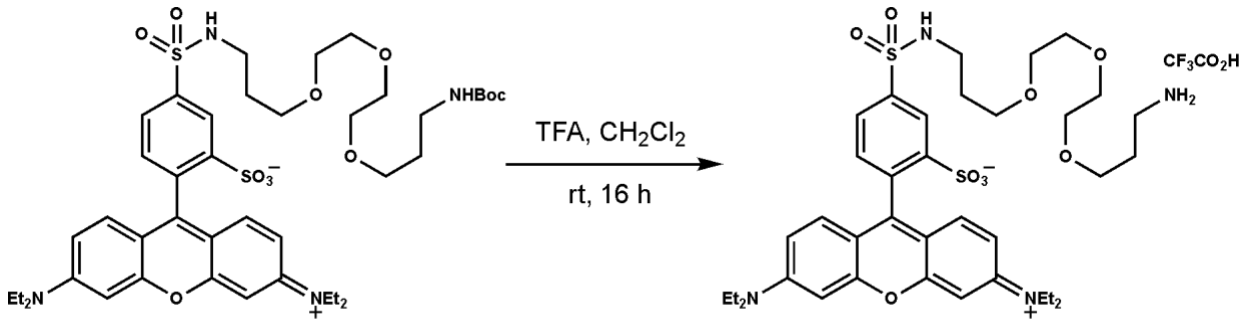
^{13}C NMR (150 MHz, $CDCl_3$) δ 173.5, 169.1, 166.8, 160.1, 152.7, 137.9, 137.7, 136.6, 134.1, 129.6, 128.8, 127.2, 124.4, 123.5, 118.6, 112.4, 109.5, 102.3, 70.13, 70.1, 69.9, 69.8, 68.9, 68.4, 37.6, 37.4, 36.3, 30.8, 28.94, 28.9
HRMS (ESI-TOF) m/z $[M+H]^+$ calcd for $[C_{40}H_{42}N_5O_{10}]^+$ 752.2932, found 752.2929



Preparation of 3',6'-dihydroxy-*N*-(17-(3-methyl-3*H*-diazirin-3-yl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamide (fluorescein diazirine; 8): A 20mL scintillation vial was charged with crude amine **6** (55 mg, 0.08 mmol) and 3-(3-methyldiazirin-3-yl)propanoic acid (15 mg, 0.12 mmol, 1.5 equiv) in DMF (2.0 mL, 0.05 M). Et_3N (81 mg, 0.8 mmol, 10 eqiv) and HBTU (45 mg, 0.12 mmol, 1.5 equiv) were added. The reaction was then stirred overnight at room temperature. The reaction was diluted with CH_2Cl_2 and washed with 1 M HCl. The aqueous layer was then washed with CH_2Cl_2 (4 x 20 mL). The combined organics were dried over $MgSO_4$, filtered, and concentrated to an orange oil. The crude residue was dry loaded on celite. The crude residue was purified by flash chromatography on a prepacked silica column (1:0 □ 95:5 □ 9:1 CH_2Cl_2/MeOH with 0.1% TFA) to afford **8** (15 mg, 28% over two steps) as a yellow oil. R_f = 0.35 (95:5 CH_2Cl_2/MeOH with 0.1% TFA)
^{1}H NMR (600 MHz, MeOD) δ 8.44 (s, 1H), 8.21 (dd, *J* = 8.01, 1.58 Hz, 1H), 7.32 (d, *J* = 8.01 Hz, 1H), 6.73 (s, 2H), 6.65 (d, *J* = 8.70 Hz, 2H), 6.58 (d, *J* = 8.66 Hz, 2H), 3.66 (dd, *J* = 5.87, 2.71 Hz, 2H), 3.63 (td, *J* = 5.90, 2.49 Hz, 6H), 3.58 – 3.52 (m, 4H), 3.49 (t, *J* = 6.17 Hz, 2H), 3.23 (t, *J* = 6.85 Hz, 2H), 2.07 – 2.03 (m, 2H), 1.93 (p, *J* = 6.36 Hz, 2H), 1.73 (p, *J* = 6.53 Hz, 2H), 1.66 – 1.62 (m, 2H), 0.98 (s, 3H)
^{13}C NMR (150 MHz, MeOD) δ 170.5, 168.2, 154.3, 138.00, 136.4, 130.3, 128.7, 125.9, 125.9, 125.0, 114.0, 111.1, 103.6, 71.5, 71.5, 71.3, 71.2, 70.3, 69.8, 39.0, 37.8, 31.5, 31.4, 30.3, 26.3, 19.7
HRMS (ESI-TOF) m/z $[M+Na]^+$ calcd for $[C_{36}H_{40}N_4O_{10}]^+$ 711.2641, found 711.2636

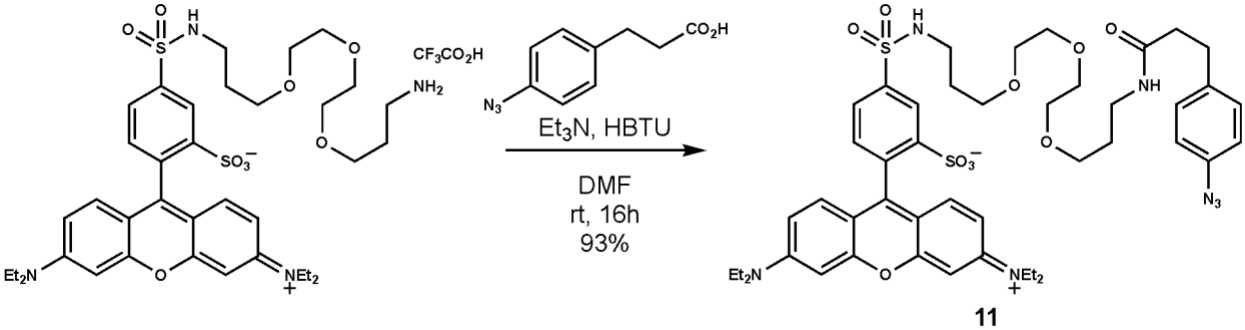


Preparation of 2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)-5-(*N*-(2,2-dimethyl-4-oxo-3,9,12,15-tetraoxa-5-azaoctadecan-18-yl)sulfamoyl)benzenesulfonate (rhodamine sulfonamide; 9): A 25 mL round bottom flask was charged with a solution of Lissamine Rhodamine B Sulfonyl Chloride (256.4 mg, 0.44 mmol) in DMF (8 mL, 0.05 M). Then monoboc diamine (148.2 mg, 0.46 mmol, 1.1 equiv) and Cs_2CO_3 (722.1 mg, 2.21 mmol, 10 equiv) were added. The mixture was stirred at room temperature overnight (~16 h). The mixture was diluted with CH_2Cl_2 (50 mL) and washed with sat. $NaHCO_3$ (200 mL) and brine (50 mL). The organic layer was then concentrated and dry loaded on silica gel (~1.0 g). The crude residue was purified by flash chromatography on a prepacked silica column (1:0 → 95:5 → 9:1 CH_2Cl_2/MeOH) to afford **9** (83.6 mg, 22%) as a deep red solid. R_f = 0.31 (95:5 CH_2Cl_2/MeOH)
^{1}H NMR (400 MHz, MeOD) δ ^{1}H NMR (400 MHz, MeOD) δ 8.65 (s, 1H), 8.11 (d, *J* = 8.06 Hz, 1H), 7.52 (d, *J* = 7.97 Hz, 1H), 7.12 (d, *J* = 9.45 Hz, 2H), 7.01 (d, *J* = 9.56 Hz, 2H), 6.94 (s, 2H), 3.67 (dt, *J* = 12.30, 5.95 Hz, 12H), 3.62 – 3.53 (m, 6H), 3.50 (t, *J* = 6.10 Hz, 2H), 3.16 – 3.06 (m, 4H), 1.80 (q, *J* = 6.29 Hz, 2H), 1.74 – 1.65 (m, 3H), 1.42 (s, 8H), 1.30 (t, *J* = 6.91 Hz, 14H)
^{13}C NMR (150 MHz, $CDCl_3$) δ 159.3, 158.1, 156.3, 155.7, 148.3, 142.2, 133.7, 129.8, 127.4, 127.2, 114.5, 113.6, 95.7, 70.7, 70.4, 69.8, 69.6, 46.0, 42.3, 38.6, 29.8, 29.1, 28.6, 12.7
HRMS (ESI-TOF) m/z $[M+H]^+$ calcd for $[C_{42}H_{61}N_4O_{11}S_2]^+$ 861.3778, found 861.3799



10

Deprotection of rhodamine sulfonamide (10): A round bottom flask was charged with sulfonamide **10** (83.6 mg, 0.097 mmol) in CH_2Cl_2 (1 mL). TFA (1 mL, 0.05 M final concentration) was added slowly. The reaction was stirred at room temperature overnight. The reaction was then concentrated under reduced pressure to afford a dark red oil, which was used without further purification.



11

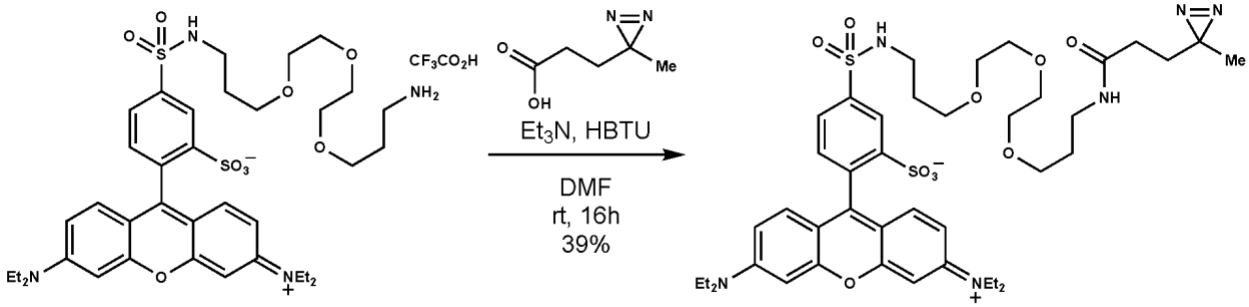
Preparation of 5-(*N*-(17-(4-azidophenyl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)sulfamoyl)-2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzenesulfonate (rhodamine phenyl azide; 11): To a solution of crude **10** (84.9 mg, 0.097 mmol) and 3-(4-azidophenyl)propionic acid (29.6 mg, 0.154 mmol, 1.5 equiv) in DMF (2 mL, 0.05 M), Et_3N (0.07

mL, 0.485 mmol, 5 equiv) was added followed by HBTU (56.7 mg, 0.149 mmol, 1.5 equiv). The reaction was then stirred at room temperature overnight (~16 h). The reaction was diluted with CH_2Cl_2 (40 mL) and washed with 1 M HCl (40 mL). The aqueous layer was washed with CH_2Cl_2 (4 x 20 mL). The combined organics were dried over $MgSO_4$, filtered, and concentrated. The crude residue was dry loaded on silica gel (~300 mg). The crude residue was purified by flash chromatography on a prepacked silica column (1:0 → 95:5 → 9:1 → 4:1 CH_2Cl_2/MeOH) to afford **11** (84.2 mg, 93% over two steps) as a dark red solid. R_f = 0.55 (95:5 CH_2Cl_2/MeOH)

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.74 (s, 1H), 8.77 (s, 1H), 7.99 (d, *J* = 7.8 Hz, 1H), 7.22–7.15 (m, 2H), 7.13–7.06 (m, 2H), 6.82–6.71 (m, 4H), 6.62 (s, 2H), 3.66–3.36 (m, 18H), 3.21–3.13 (m, 2H), 3.12–3.01 (m, 4H), 2.77 (t, *J* = 7.2 Hz, 2H), 2.41–2.27 (m, 2H), 1.80–1.71 (m, 2H), 1.69–1.59 (m, 2H), 1.32–1.19 (m, 12H)

^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7, 158.1, 157.8, 155.5, 147.1, 142.4, 138.4, 137.3, 133.5, 133.2, 130.1, 130.0, 127.7, 127.1, 118.8, 114.2, 114.1, 113.7, 95.7, 70.2, 70.17, 69.8, 69.3, 69.1, 45.9, 45.8, 41.3, 37.8, 36.8, 31.2, 29.7, 29.0, 28.9, 12.6, 8.6

HRMS (ESI-TOF) m/z $[M+Na]^+$ calcd for $[C_{46}H_{59}N_7O_{10}S_2Na]^+$ 956.3663, found 956.3682



Preparation of 2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)-5-(*N*-(17-(3-methyl-3*H*-diazirin-3-yl)-15-oxo-4,7,10-trioxa-14-azaheptadecyl)sulfamoyl)benzenesulfonate (rhodamine diazirine; 12): To a solution of crude **10** (91 mg, 0.104 mmol) and 3-(3-methyl-3H-diazirin-3-yl)propanoic acid (26.7 mg, 0.208 mmol, 2 equiv) in DMF (2 mL, 0.05 M), Et_3N (0.15 mL, 1.07 mmol, 10 equiv) was added followed by HBTU (67.6 mg, 0.178 mmol, 1.5 equiv). The reaction was then stirred at room temperature overnight (~16 h). The reaction was diluted with CH_2Cl_2 (40 mL) and washed with 1 M HCl (40 mL). The aqueous layer was washed with CH_2Cl_2 (4 x 20 mL). The combined organics were dried over $MgSO_4$, filtered, and concentrated. The crude residue was dry loaded on silica gel (~300 mg). The crude residue was purified by flash chromatography on a prepacked silica column (1:0 → 95:5 → 9:1 CH_2Cl_2/MeOH) to afford **12** (34.9 mg, 39% over two steps) as a dark purple solid. R_f = 0.52 (9:1 CH_2Cl_2/MeOH)

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.67 (d, *J* = 1.4 Hz, 1H), 8.13 (dd, *J* = 1.6, 7.9 Hz, 1H), 7.54 (d, *J* = 7.9 Hz, 1H), 7.14 (s, 1H), 7.13 (s, 1H), 7.03 (dd, *J* = 2.2, 9.5 Hz, 2H), 6.95 (d, *J* = 2.2 Hz, 2H), 3.78–3.58 (m, 18H), 3.57 (t, *J* = 6.0 Hz, 2H), 3.53 (t, *J* = 6.2 Hz, 2H), 3.24 (t, *J* = 6.7 Hz, 2H), 3.14 (t, *J* = 6.7 Hz, 2H), 2.06 (t, *J* = 7.7 Hz, 2H), 1.81 (quin, *J* = 6.3 Hz, 2H), 1.76 (quin, *J* = 6.5 Hz, 2H), 1.64 (t, *J* = 7.7 Hz, 2H), 1.32 (t, *J* = 7.1 Hz, 12H), 1.00 (s, 3H)

^{13}C NMR (150 MHz, $CDCl_3$) δ 173.0, 158.0, 156.4, 155.8, 145.9, 142.5, 134.0, 132.3, 131.1, 127.9, 126.3, 113.9, 113.7, 95.6, 70.2, 70.1, 69.9, 69.8, 68.4, 68.0, 45.4, 40.2, 36.6, 36.4, 30.1, 30.0, 29.98, 29.5, 29.0, 25.0, 18.4, 11.5

HRMS (ESI-TOF) m/z $[M+Na]^+$ calcd for $[C_{42}H_{58}N_6O_{10}S_2Na]^+$ 893.3553, found 893.3561

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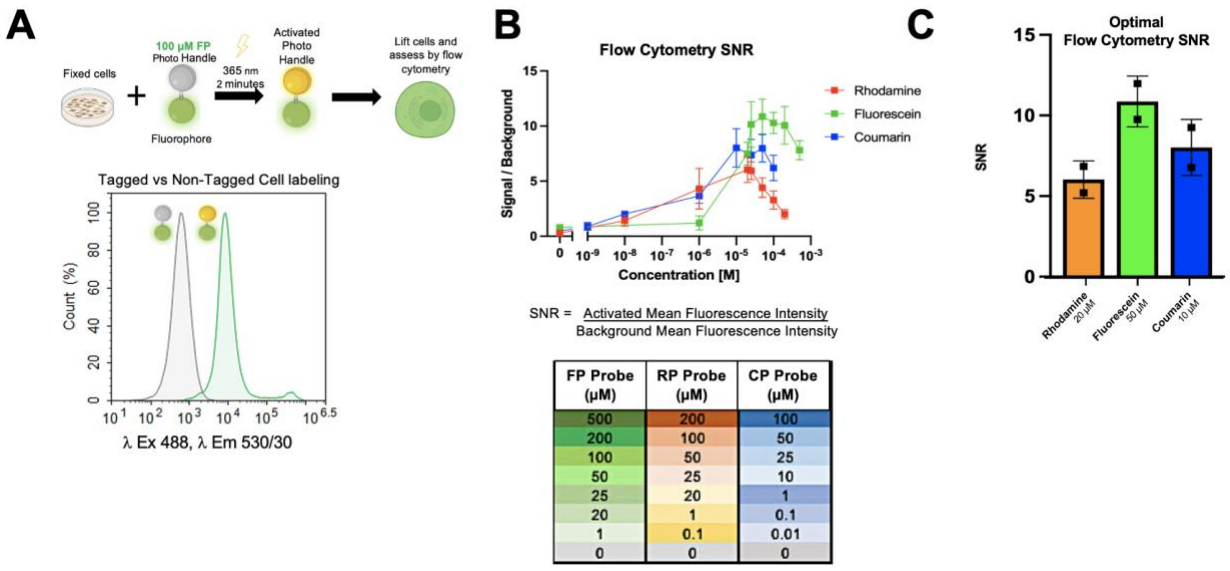
Supplementary Figures and Tables

Table S1. Properties of CrossTag Fluorophores

Probe	λ_{abs} (nm)	ε ($M^{-1}cm^{-1}$)	λ_{em} (nm)	φ
CD	375	328,000	463	0.10
CP	375	152,000	463	0.11
FD	496	287,000	521	0.85
FP	496	508,000	521	0.87
RD	514	191,000	589	0.26
RP	514	227,000	589	0.09

CrossTag probes were prepared as stock solutions in DMSO and diluted so that the final DMSO concentration did not exceed 1% (vol/vol). Coumarin probe solutions were diluted with 100% EtOH. Fluorescein probes solutions were diluted to 0.1M NaOH. Rhodamine probe solutions were diluted with water. Emission wavelengths were recorded using their respective maximum excitation wavelengths.



FP Probe (µM)	RP Probe (µM)	CP Probe (µM)
500	200	100
200	100	50
100	50	25
50	25	10
25	20	1
20	1	0.1
1	0.1	0.01
0	0	0

Figure S1. Titration and activation of CrossSeq probes on fixed cells to define the functional operating range and signal-to-noise ratio of tagged cells by flow cytometry analysis. The signal-to-noise ratio (SNR) of activated bound probe to background signal at varying concentrations was evaluated on fixed cells by flow cytometry, as shown in schematic A. HEK293T cells were grown to confluency in a 12-well plate, fixed with glyoxal on ice for 20 minutes, washed three times with PBS, then incubated with each CrossTag probe over a range of concentrations from 0.01 µM to 500 µM, as shown in B. Cells were either activated in a probe solution by 365 nm irradiation in a Fisher Scientific UV Crosslinker for 2 minutes or incubated with a probe for the same time to establish a background signal. Then the cells were washed three times with PBS to remove the unbound probe, lifted with 500 µL of accumax at 37°C for 15 minutes and flowed in the same solution to evaluate for fluorescent SNR by flow cytometry. Full SNR titration curves are shown in B and optimal concentrations selected for use moving forward are shown in C. From this analysis, a maximum SNR of 10:1, 8:1, and 6:1 was defined at the lowest possible concentration using these activation conditions for the fluorescein phenyl azide (FP), coumarin phenyl azide (CP), and rhodamine phenyl azide (RP) probes, respectively.

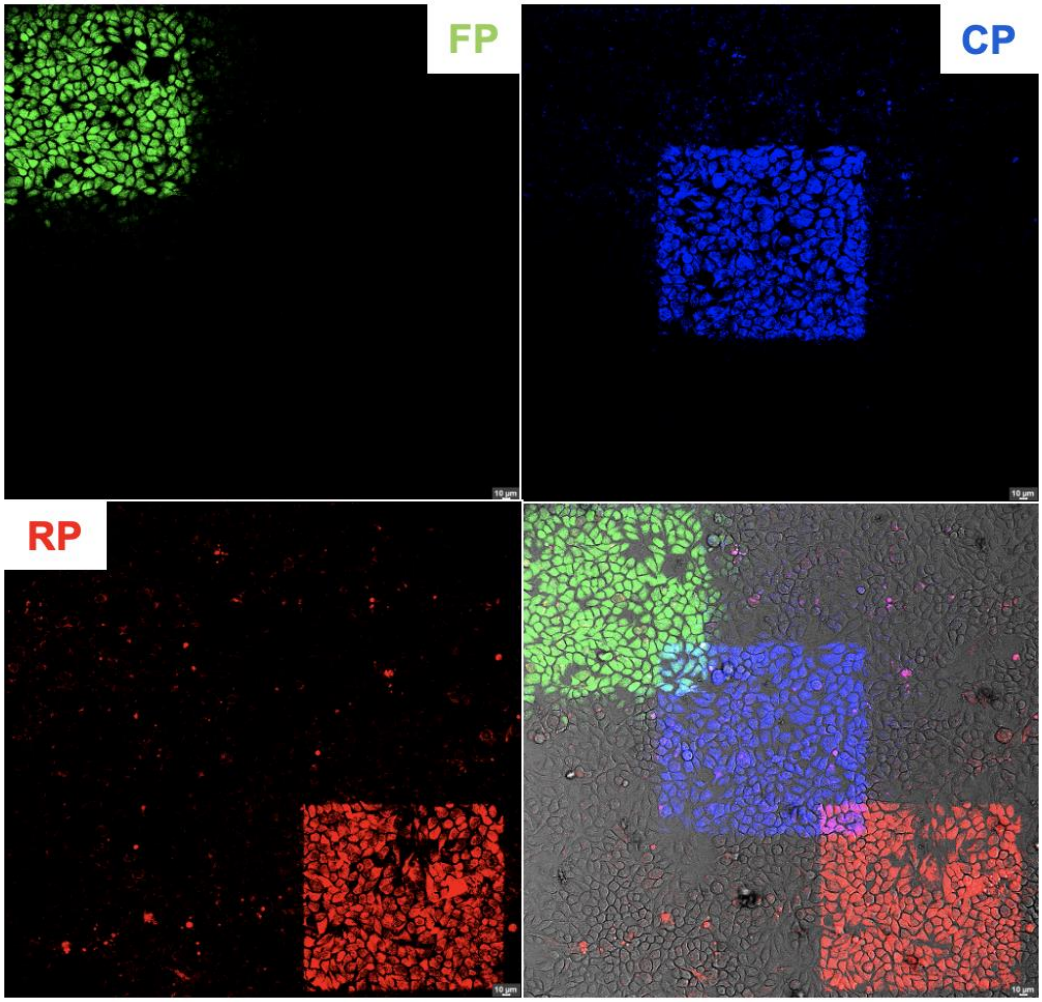


Figure S2. Example of multiplex tagging on fixed HEK293T cells in navigator mode. Method for tagging outlined in methods section above.

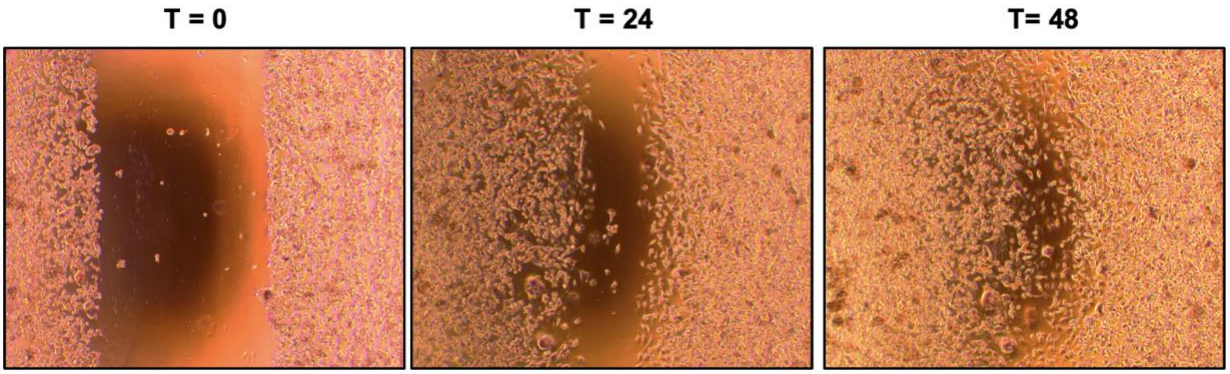


Figure S3. MDA-LM2 48 Hr gap closure tracking. 0.5 x 10^6 MDA-LM2 cells were plated in one well of a 6-well plate and grown until confluency. The cells were then serum starved in DMEM media only for two hours to limit proliferation. Then a 200 µL pipette tip was used to scratch a straight line through the length of the sample. The media was then aspirated, and the scratch wound edge was marked with a permanent marker. The cells were then washed once with 5% FBS/DMEM with 1% Pen/Strep media, then the same media composition was added and cells were monitored over time until gap closure using an inverted microscope.

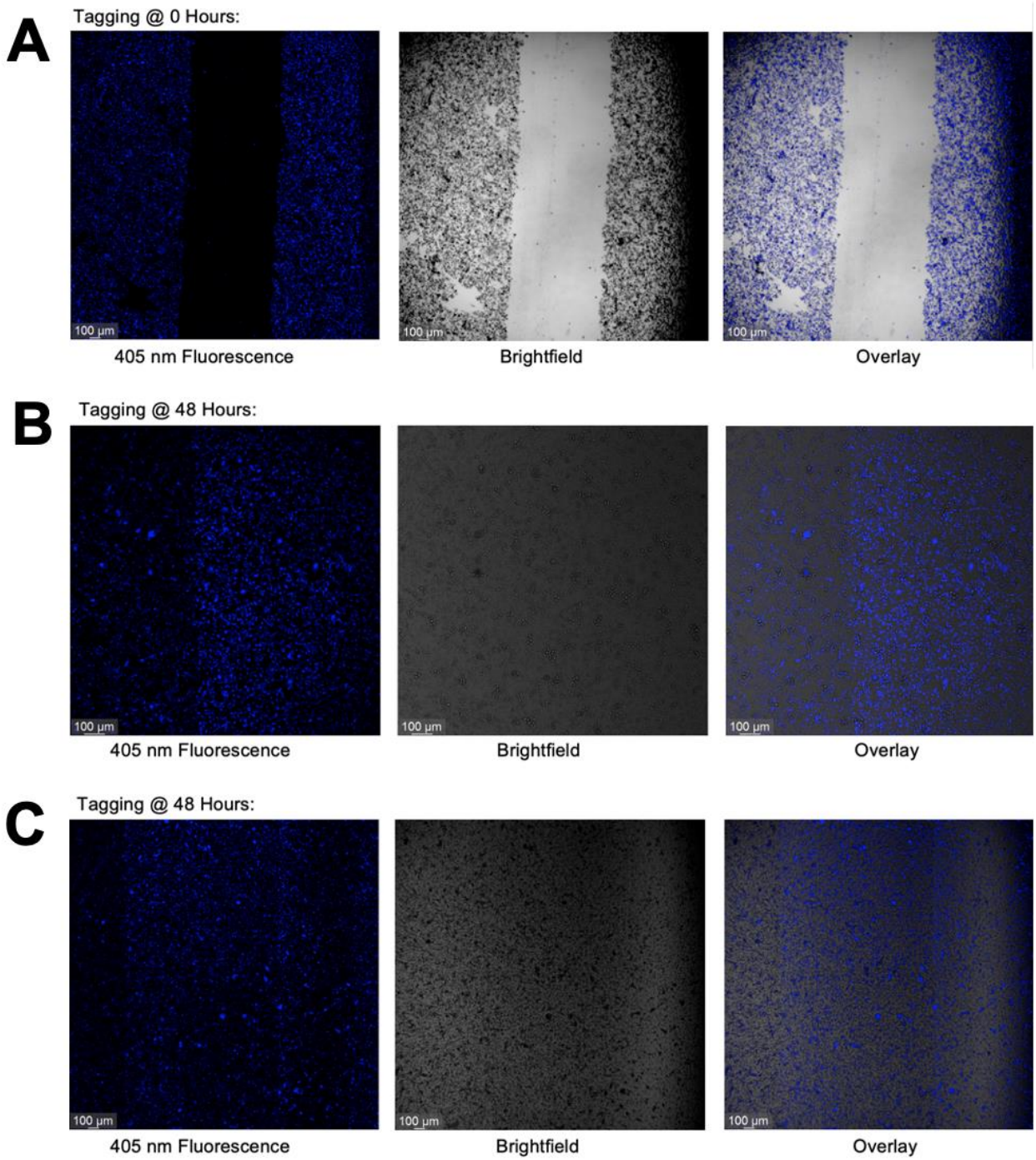


Figure S4. MDA-LM2 scratch assay time course experiment images acquired after CrossSeq CP probe activation. Images of 0 hour and 48 hour scratch samples acquired after scratch wound tagging with CrossSeq CP probe on the confocal microscope as described in the methods above. A) 0 hour sample images acquired with 5X air objective. B) 48 hour sample images acquired with 10X air objective. C) 48 hour sample images acquired with 5X air objective.

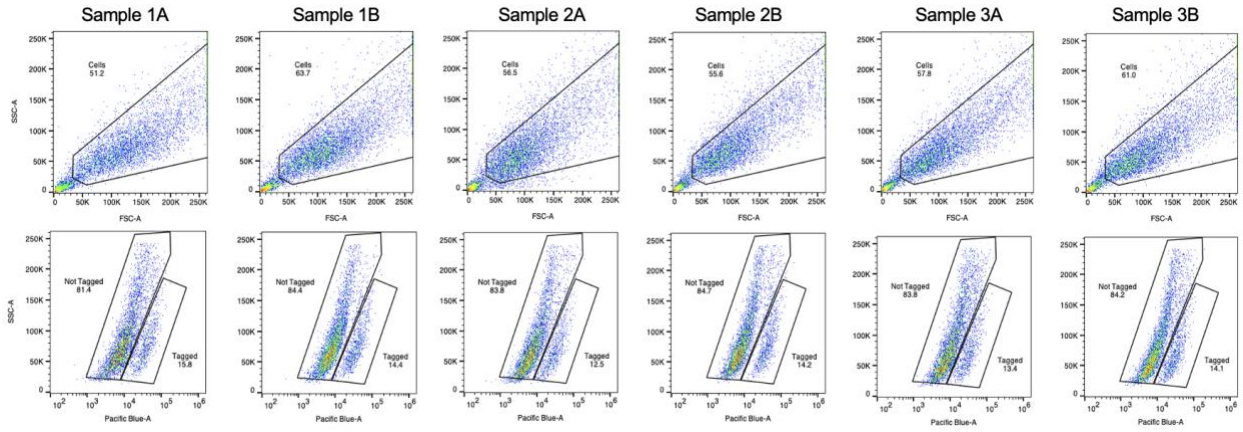
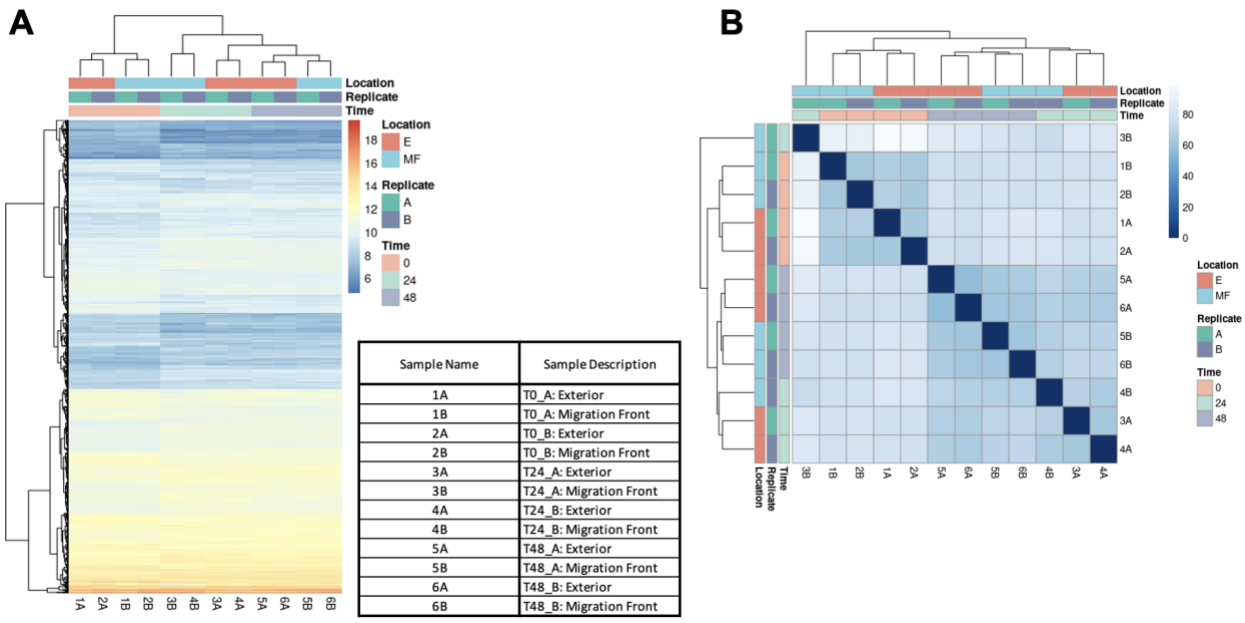


Figure S5. CrossSeq CP probe tagged MDA-LM2 scratch assay samples sorted by flow cytometry. Scratch assay samples sorted by their coumarin labeled tag into tagged and non-tagged populations per sample on a sterile BD FACSAria Fusion Sorter. 10,000 events were recorded per sample at the beginning of sorting.



Sample Name	Sample Description
1A	T0_A: Exterior
1B	T0_A: Migration Front
2A	T0_B: Exterior
2B	T0_B: Migration Front
3A	T24_A: Exterior
3B	T24_A: Migration Front
4A	T24_B: Exterior
4B	T24_B: Migration Front
5A	T48_A: Exterior
5B	T48_A: Migration Front
6A	T48_B: Exterior
6B	T48_B: Migration Front

Figure S6. RNA-seq analysis results of differentially expressed genes per time point. A) RNA-seq unsupervised cluster heatmap of both migration front and exterior region, per time point, for each biological replicate. B) Total RNA expression matrix analyzed per sample by distance of both migration front and exterior region, per time point, for each biological replicate.

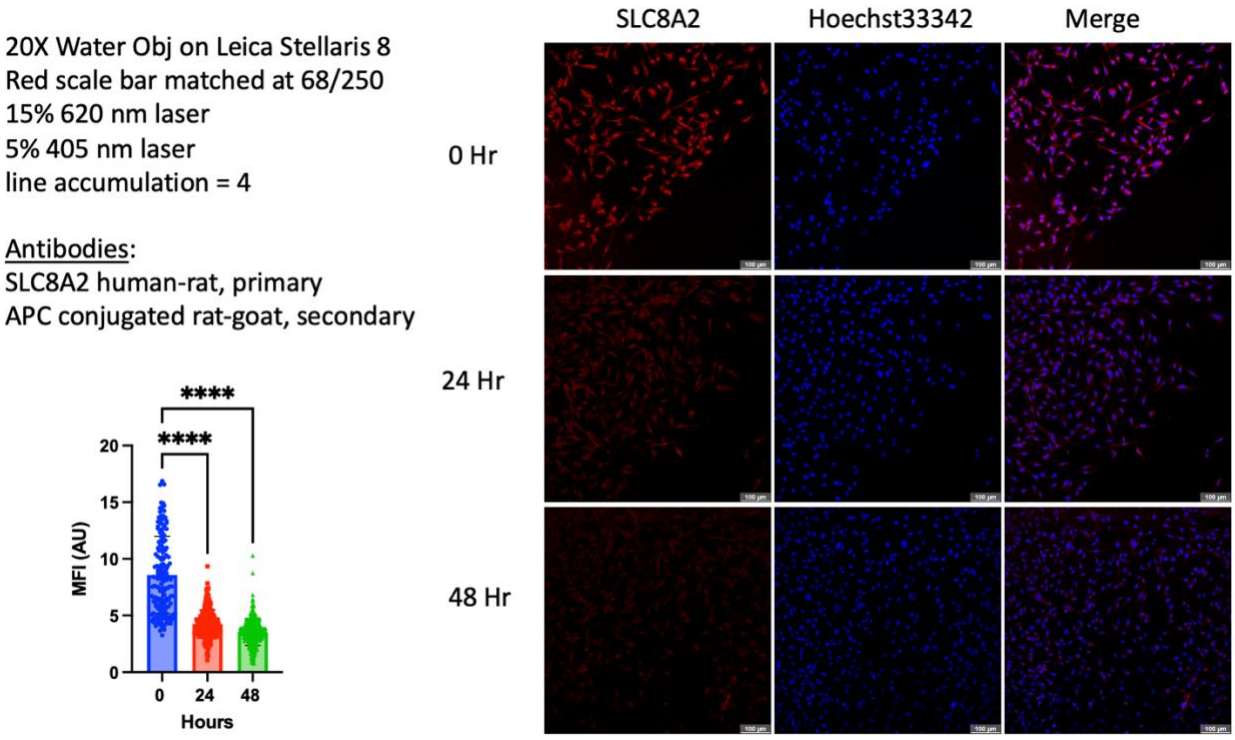


Figure S7. Immunofluorescence validation of RNA-seq changes in SLC8A2 gene expression within MDA-LM2 migration front comparison over time. Comparison of the mean fluorescence intensity of the analyzed protein expression within cells at the migration front across time points. Images acquired with a 20X water objective. Significance was calculated using one-way ANOVA with multiple comparisons (**** represents P < 0.0001).

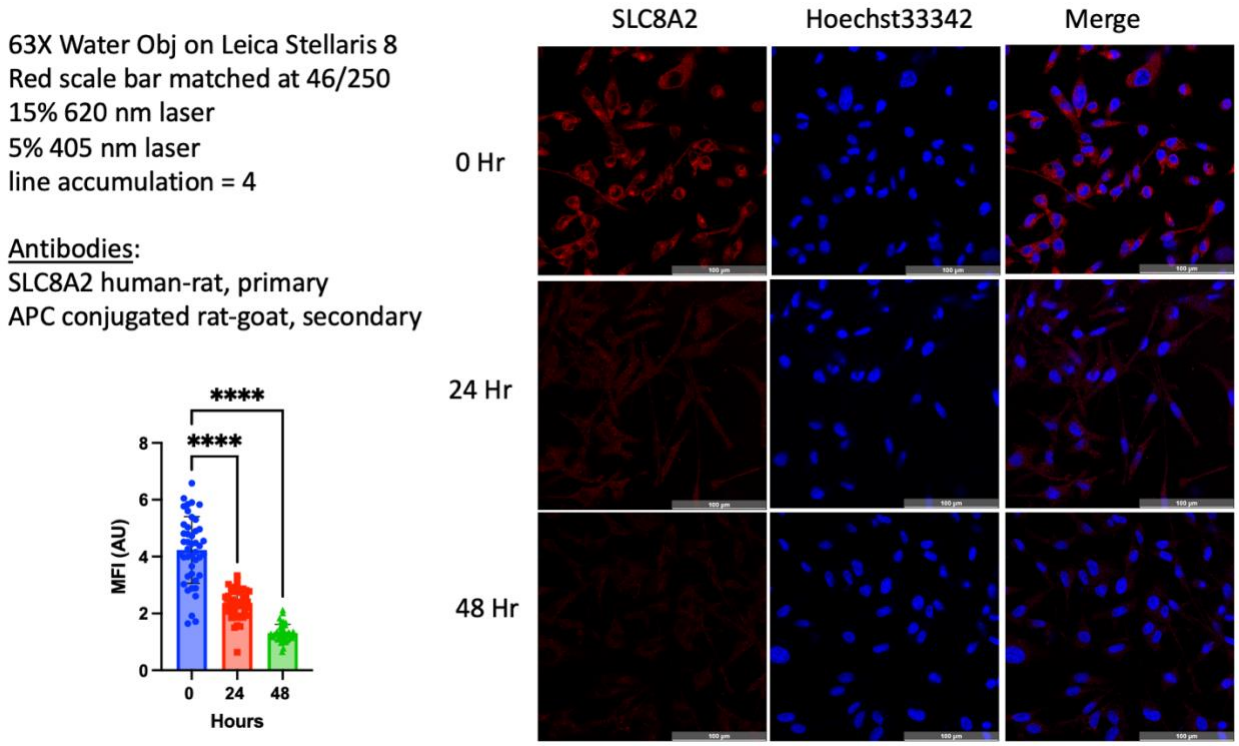


Figure S8. Immunofluorescence validation of RNA-seq changes in SLC8A2 gene expression within MDA-LM2 migration front comparison over time. Comparison of the mean fluorescence intensity of the analyzed protein expression within cells at the migration front across time points. Images acquired with a 63X water objective. Significance was calculated using one-way ANOVA with multiple comparisons (**** represents P < 0.0001).

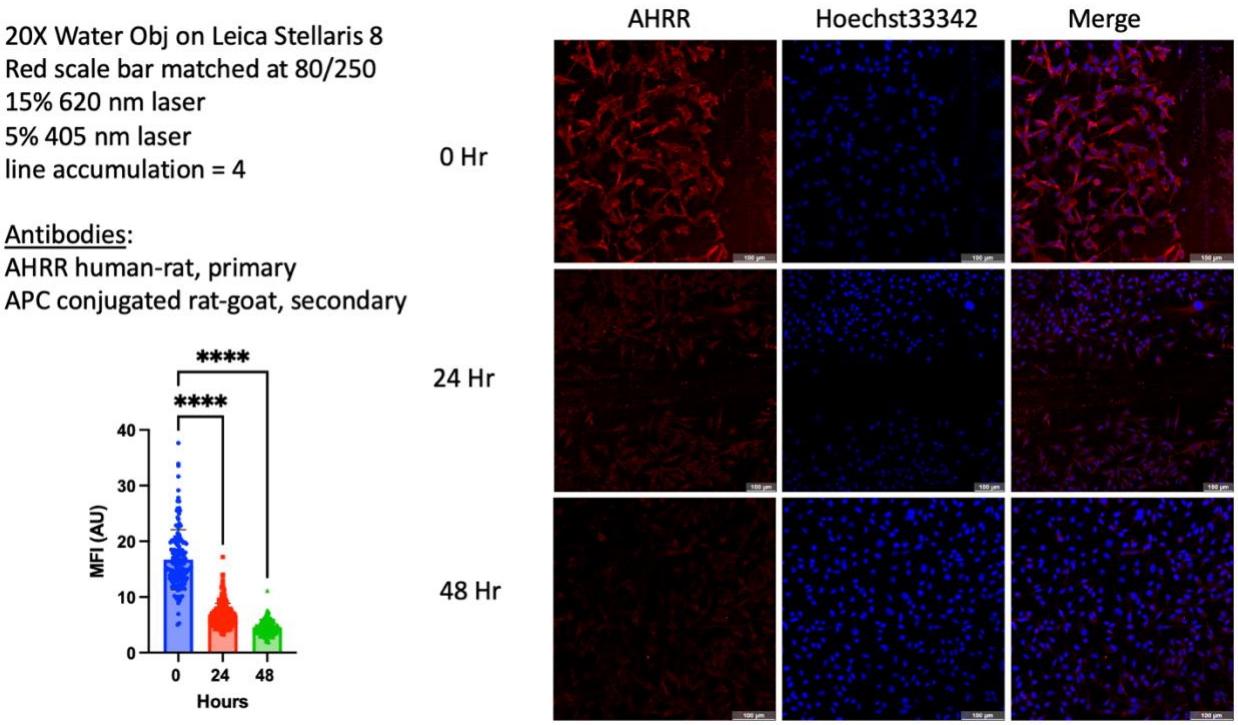


Figure S9. Immunofluorescence validation of RNA-seq changes in AHRR gene expression within MDA-LM2 migration front comparison over time. Comparison of the mean fluorescence intensity of the analyzed protein expression within cells at the migration front across time points. Images acquired with a 20X water objective. Significance was calculated using one-way ANOVA with multiple comparisons (**** represents $P < 0.0001$).

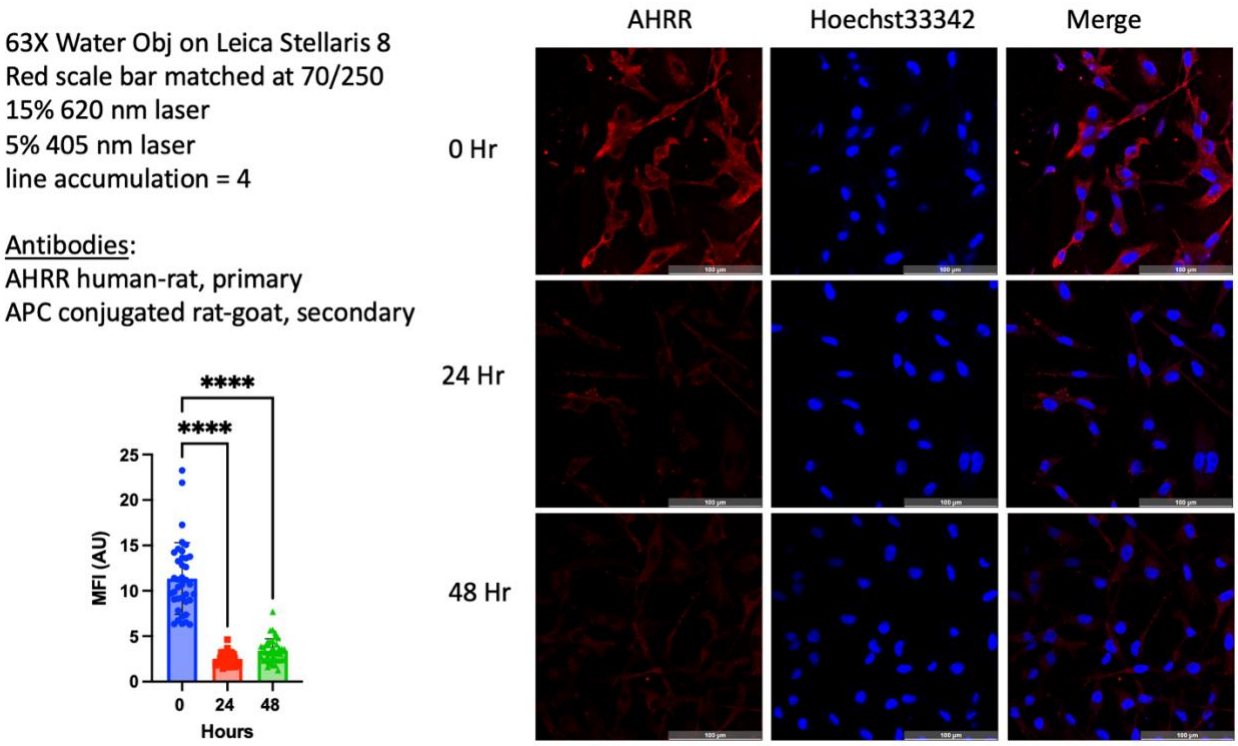


Figure S10. Immunofluorescence validation of RNA-seq changes in AHRR gene expression within MDA-LM2 migration front comparison over time. Comparison of the mean fluorescence intensity of the analyzed protein expression within cells at the migration front across time points. Images acquired with a 63X water objective. Significance was calculated using one-way ANOVA with multiple comparisons (**** represents P < 0.0001).

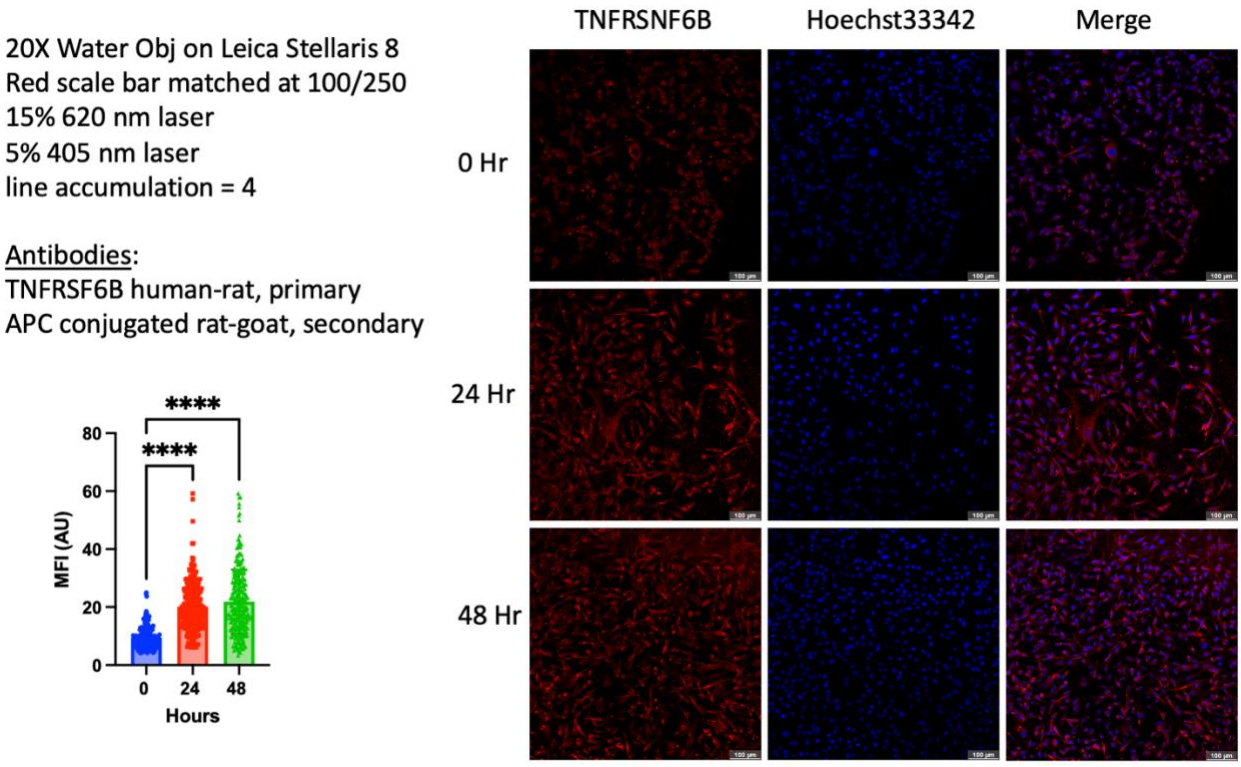


Figure S11. Immunofluorescence validation of RNA-seq changes in TNFRSNF6B gene expression within MDA-LM2 migration front comparison over time. Comparison of the mean fluorescence intensity of the analyzed protein expression within cells at the migration front across time points. Images acquired with a 20X water objective. Significance was calculated using one-way ANOVA with multiple comparisons (**** represents $P < 0.0001$).

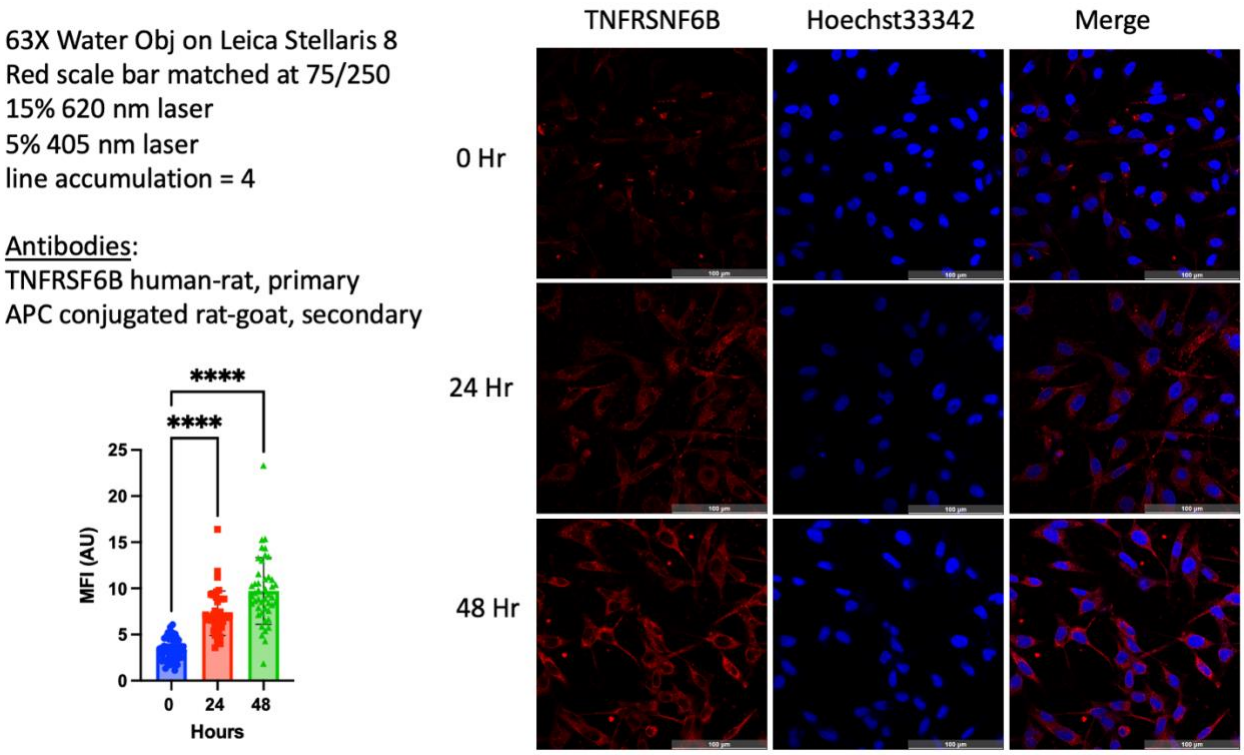
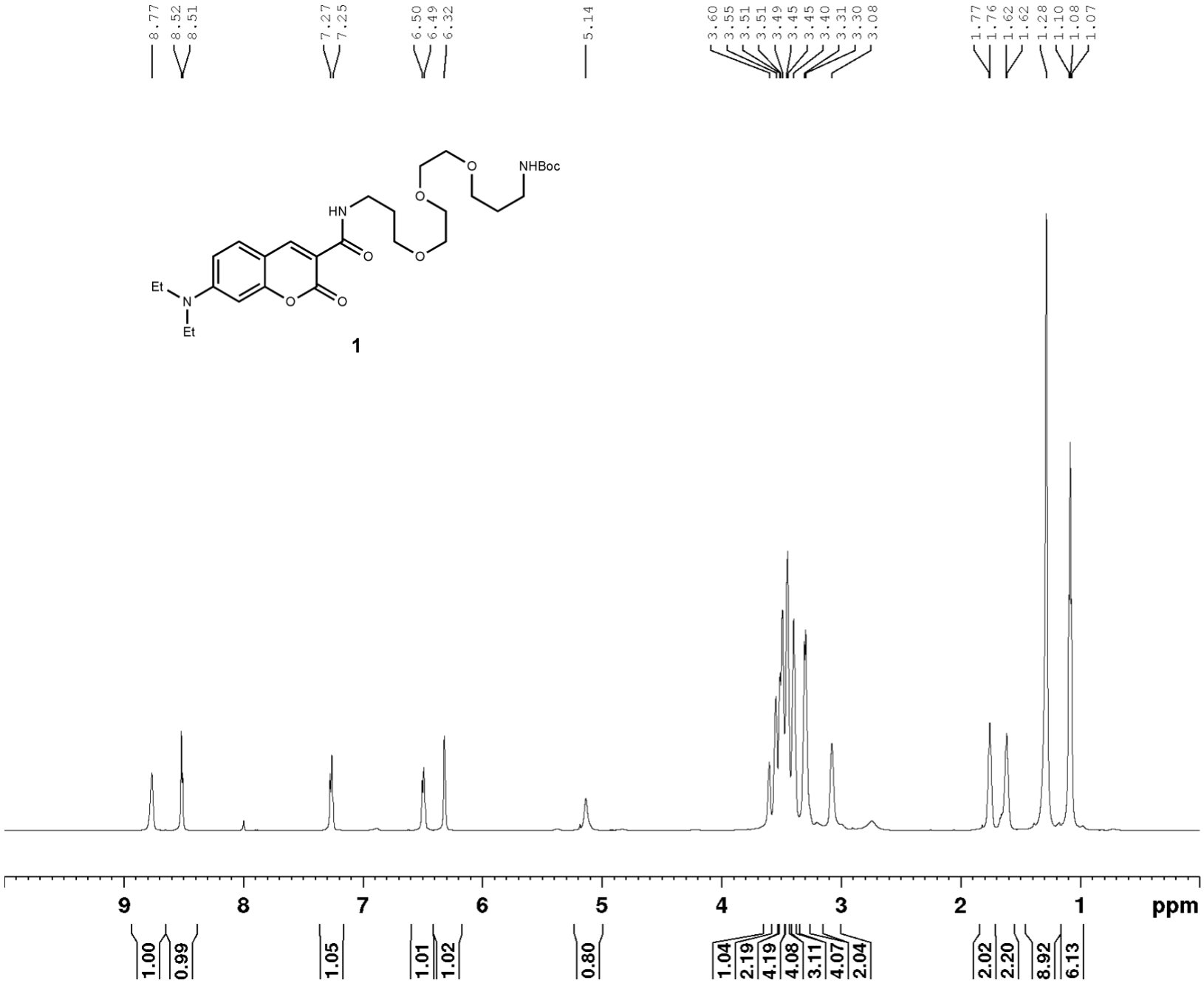


Figure S12. Immunofluorescence validation of RNA-seq changes in TNFRSNF6B gene expression within MDA-LM2 migration front comparison over time. Comparison of the mean fluorescence intensity of the analyzed protein expression within cells at the migration front across time points. Images acquired with a 63X water objective. Significance was calculated using one-way ANOVA with multiple comparisons (**** represents P < 0.0001).

Table S2. Immunofluorescence Antibodies

Antibody	Manufacturer	Part Number	Dilution
SLC8A2, Rabbit/IgG	Bioss through Thermofisher Scientific	BS-1997R	1:100
TNFRSF6B	Antibodies Online Inc.	ABIN484183	1:100
AHRR	Antibodies Online Inc.	ABIN7144661	1:100
Goat Anti-Rabbit IgG H&L (APC) preadsorbed	Abcam	ab130805	1:5000

NMR Spectra



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TD	98074
SOLVENT	CDCl3
NS	16
DS	2
SWH	9615.385
FIDRES	0.098042
AQ	5.0998478
RG	8
DW	52.000
DE	14.23
TE	298.2
D1	0.10000000
TD0	1

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SFO1	600.1342009
NUC1	1H
P1	9.50
PLW1	30.00000000

F2 - Processing paramet

SI	65536
SF	600.1300342
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SSB	0
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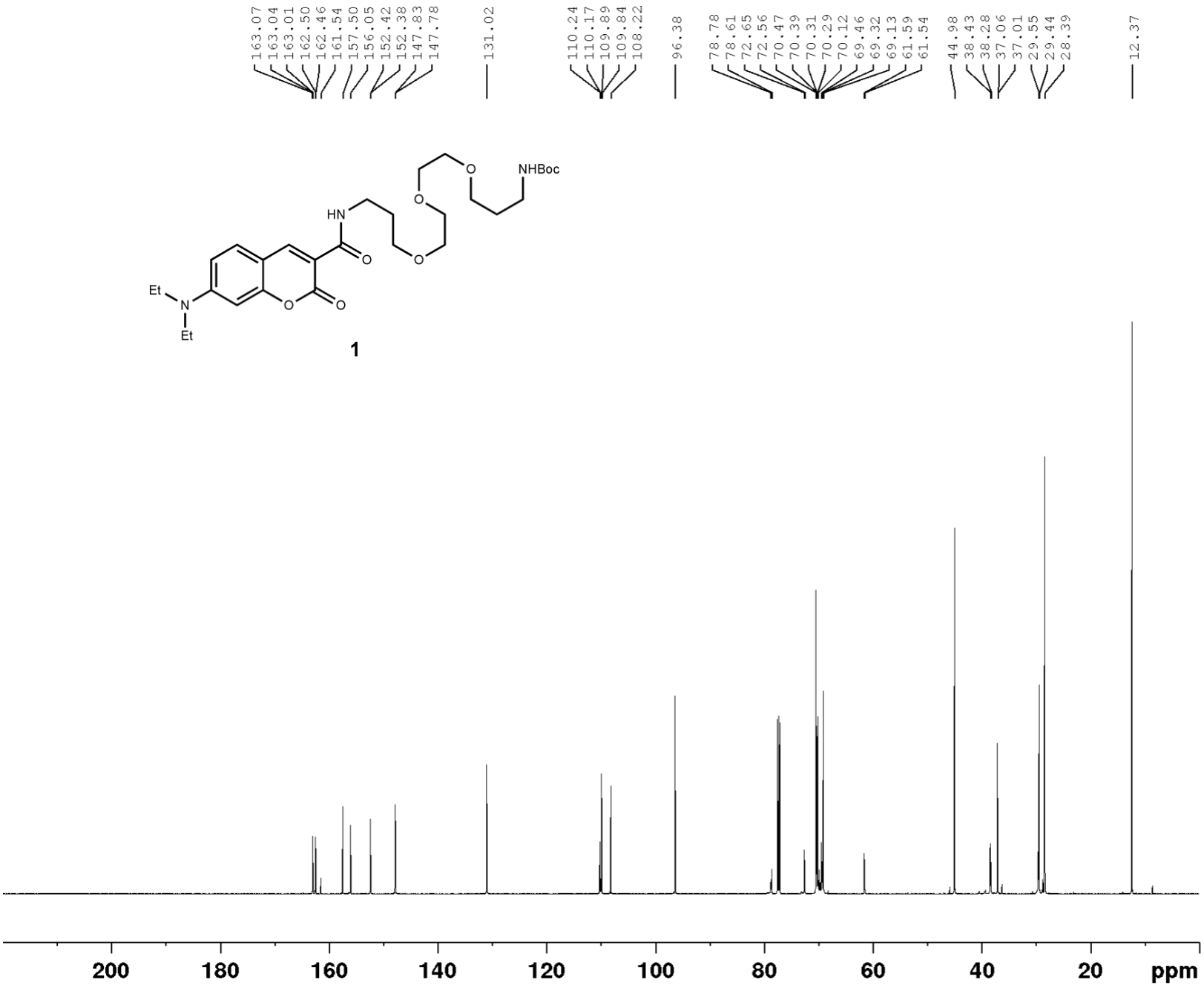


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SWH              36231.883
FIDRES           0.552855
AQ               0.9043968
RG                    2050
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DE                  19.63
TE                  298.1
D1               0.40000001
D11              0.03000000
TD0                       1

======== CHANNEL f1 ====
SFO1           150.9194080
NUC1                   13C
P1                   10.10
PLW1           64.00000000

======== CHANNEL f2 ====
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NUC2                   1H
CPDPRG[2           waltz16
PCPD2                80.00
PLW2           30.00000000
PLW12           0.37154001

F2 - Processing paramet
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SF              150.9028085
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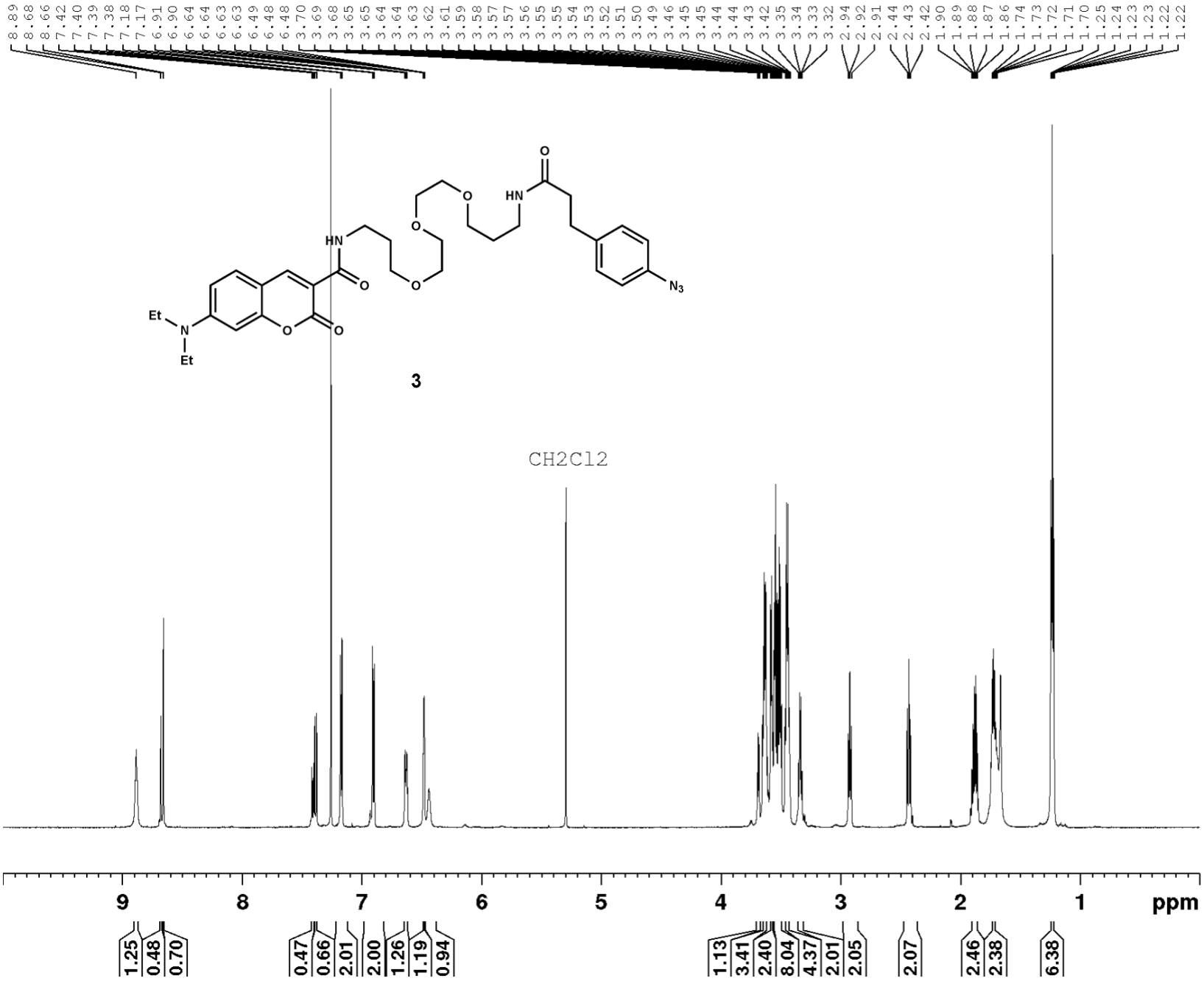


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TD                   98074
SOLVENT              CDCl3
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DS                       2
SWH                9615.385
FIDRES             0.098042
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RG                      10
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DE                   14.23
TE                   298.0
D1                0.10000000
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======== CHANNEL f1 ====
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NUC1                     1H
P1                     9.50
PLW1            30.00000000

F2 - Processing paramet
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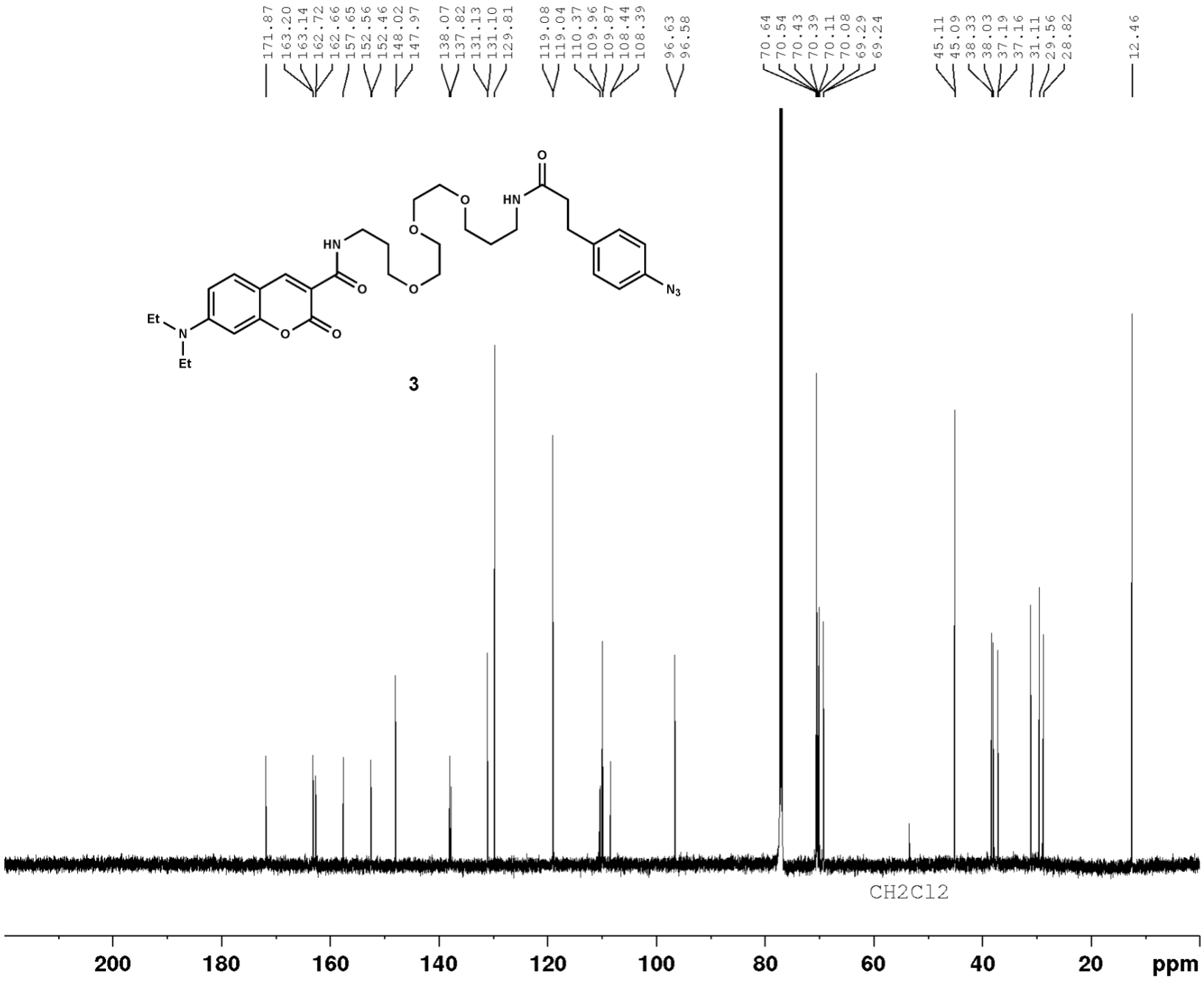


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FIDRES          0.552855
AQ             0.9043968
RG                  2050
DW                13.800
DE                 19.63
TE                 298.0
D1             0.40000001
D11            0.03000000
TD0                    1

======== CHANNEL f1 ====
SFO1          150.9194080
NUC1                  13C
P1                  10.10
PLW1          64.00000000

======== CHANNEL f2 ====
SFO2          600.1330010
NUC2                  1H
CPDPRG[2          waltz16
PCPD2               80.00
PLW2          30.00000000
PLW12          0.37154001

F2 - Processing paramet
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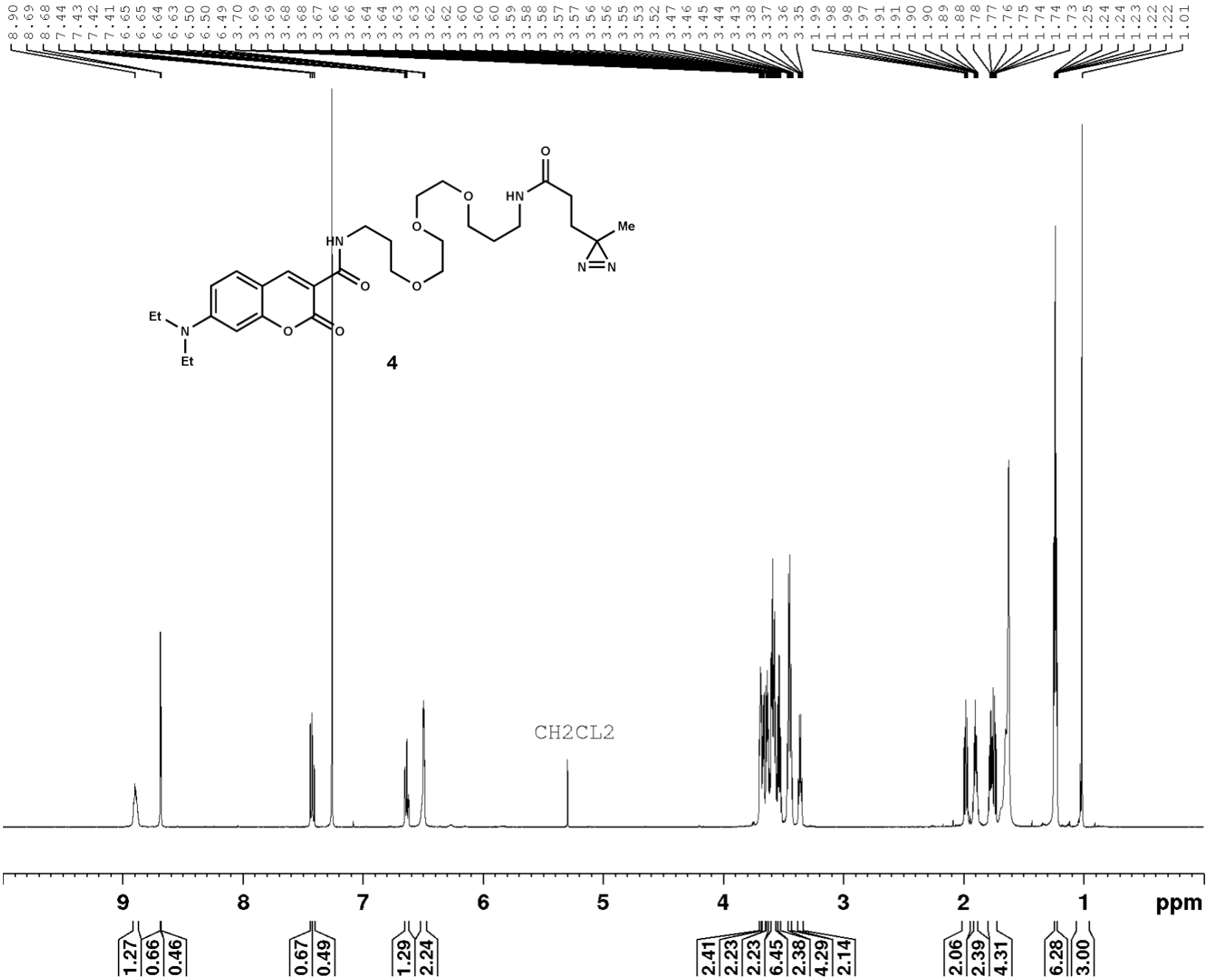


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TD                   98074
SOLVENT              CDCl3
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DS                       2
SWH                9615.385
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AQ                 5.0998478
RG                      10
DW                   52.000
DE                   14.23
TE                   298.0
D1                0.10000000
TD0                      1

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P1                    9.50
PLW1           30.00000000

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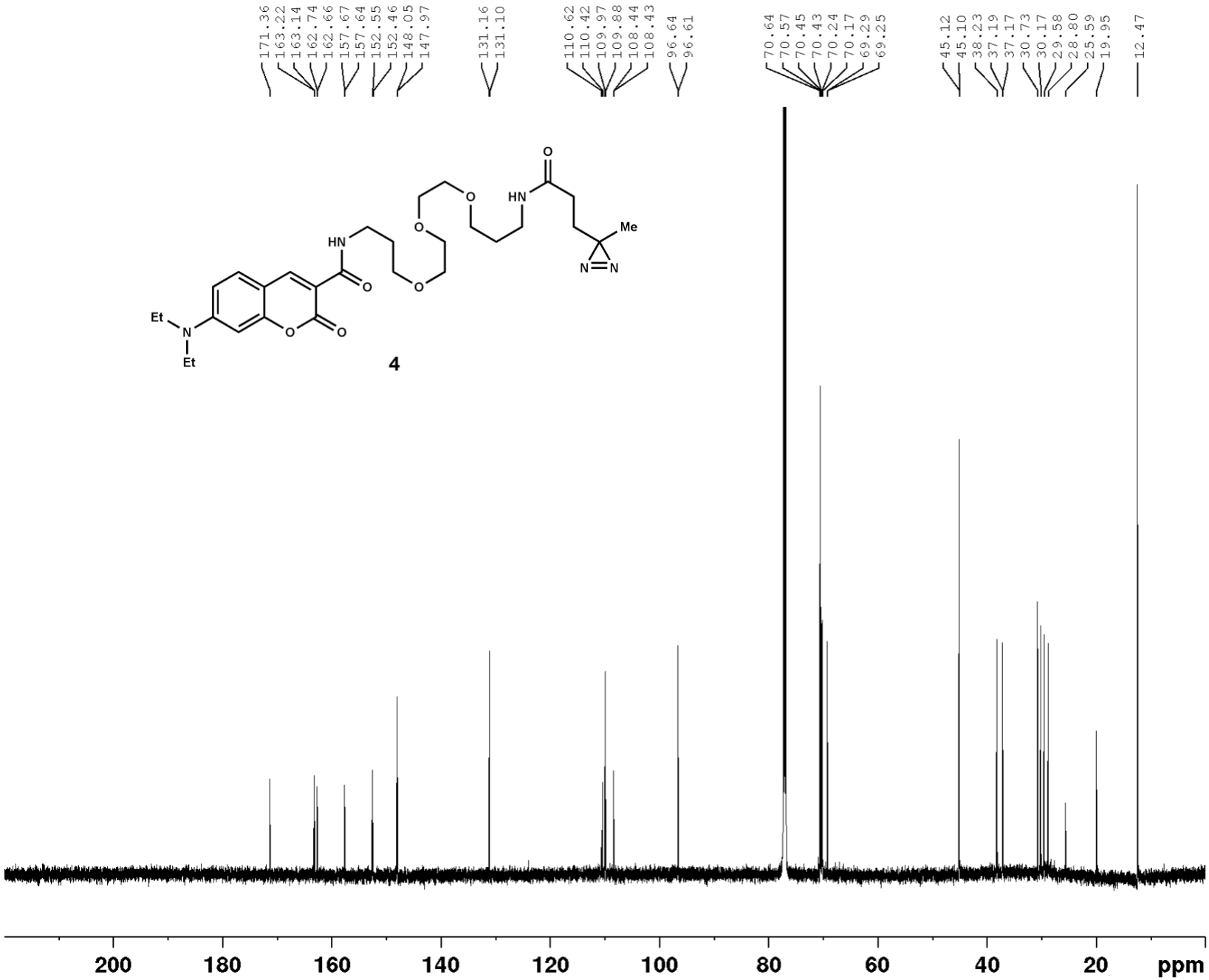


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FIDRES          0.552855
AQ              0.9043968
RG                  2050
DW                13.800
DE                19.63
TE                298.0
D1             0.40000001
D11            0.03000000
TD0                    1

======== CHANNEL f1 ====
SFO1          150.9194080
NUC1                  13C
P1                  10.10
PLW1          64.00000000

======== CHANNEL f2 ====
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NUC2                  1H
CPDPRG[2          waltz16
PCPD2               80.00
PLW2          30.0000000C
PLW12          0.37154001

F2 - Processing paramet
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LB                  1.00
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PC                  1.00
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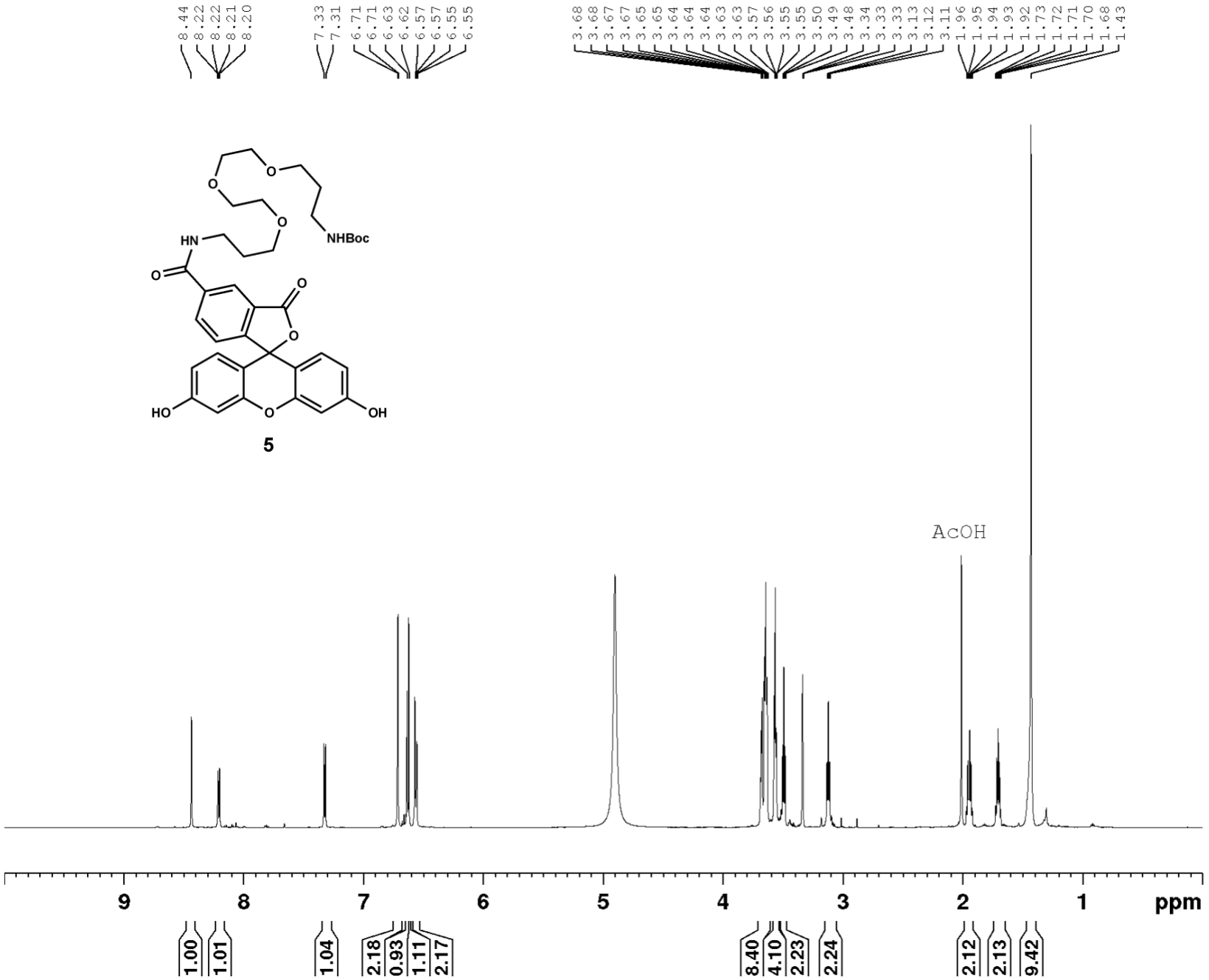

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DE 14.23
TE 298.0
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======== CHANNEL f1 ====
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NUC1 1H
P1 9.50
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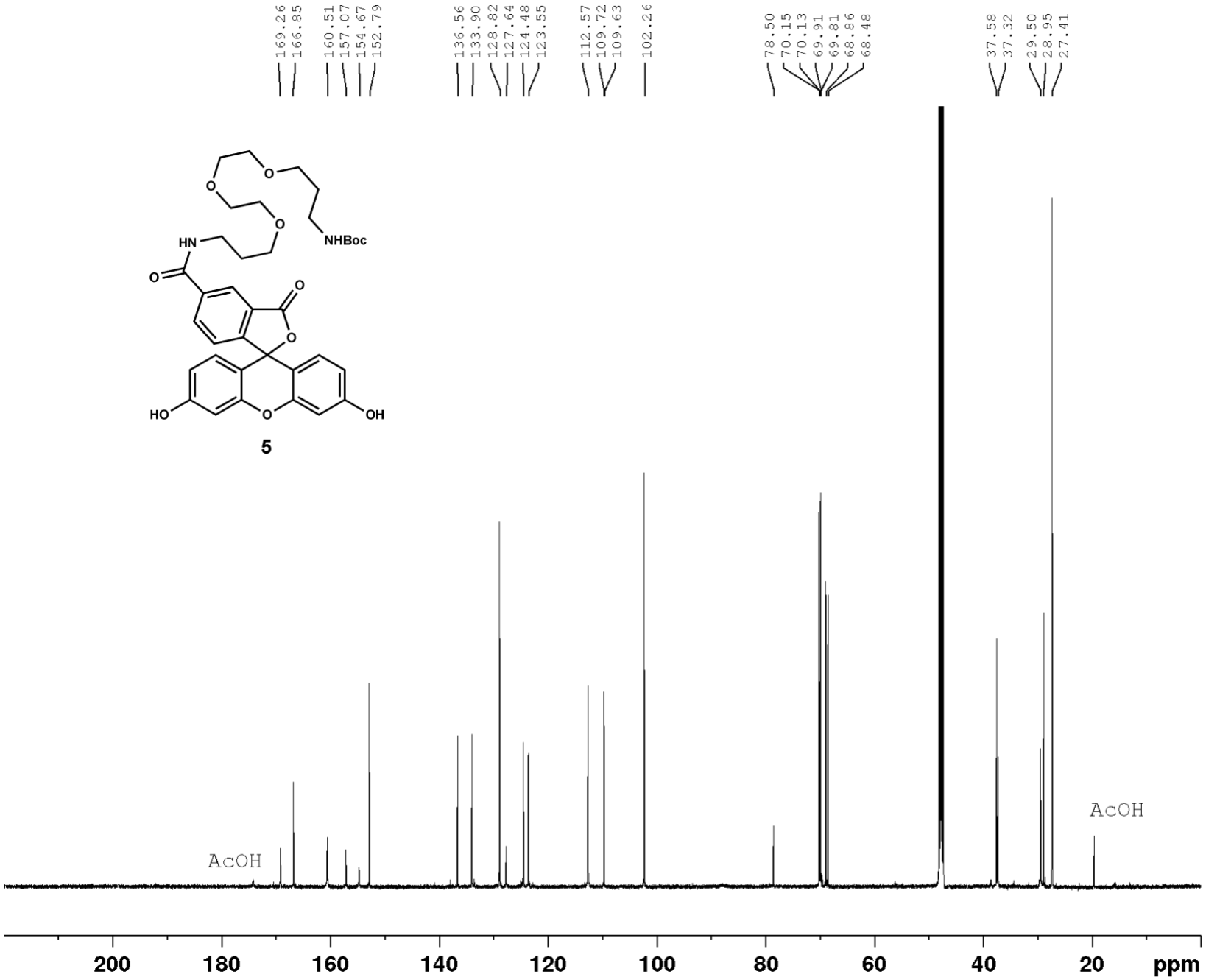


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DS                      4
SWH              36231.883
FIDRES           0.552855
AQ               0.9043968
RG                   2050
DW                 13.800
DE                 19.63
TE                 298.0
D1               0.40000001
D11              0.03000000
TD0                     1

======== CHANNEL f1 ====
SFO1            150.9194080
NUC1                   13C
P1                   10.10
PLW1            64.00000000

======== CHANNEL f2 ====
SFO2            600.1330010
NUC2                   1H
CPDPRG[2           waltz16
PCPD2                80.00
PLW2            30.00000000
PLW12            0.37154001

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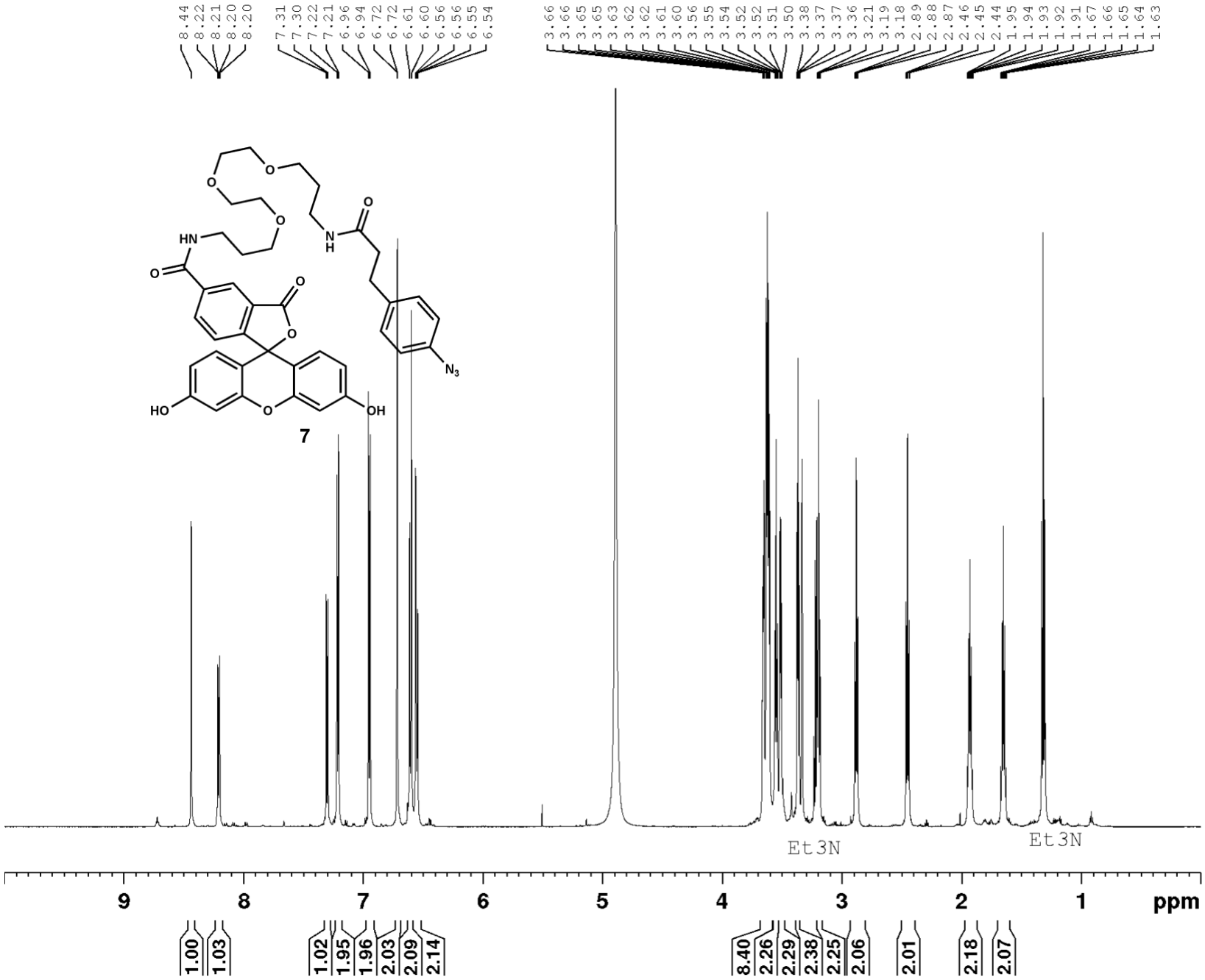


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DS                       2
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FIDRES            0.098042
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RG                      10
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DE                   14.23
TE                   298.0
D1              0.10000000
TD0                       1

======== CHANNEL f1 ====
SFO1           600.1342009
NUC1                    1H
P1                    9.50
PLW1           30.0000000C

F2 - Processing paramet
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WDW                     EM
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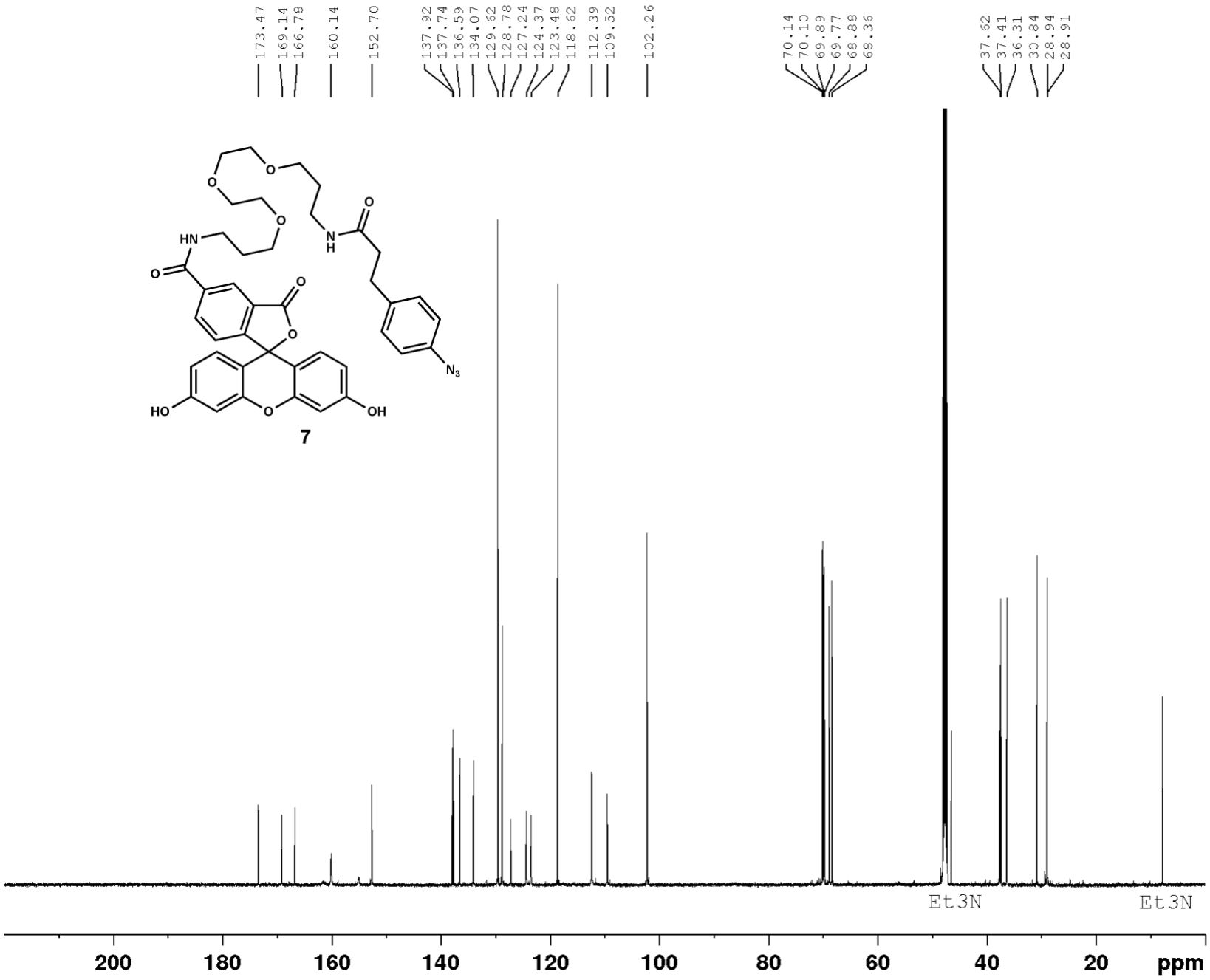


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TD                 65536
SOLVENT              MeOD
NS                   1200
DS                      4
SWH             36231.883
FIDRES           0.552855
AQ              0.9043968
RG                   2050
DW                 13.800
DE                 19.63
TE                 298.1
D1              0.40000001
D11             0.03000000
TD0                     1

======== CHANNEL f1 ====
SFO1           150.9194080
NUC1                   13C
P1                   10.10
PLW1           64.00000000

======== CHANNEL f2 ====
SFO2           600.1330010
NUC2                   1H
CPDPRG[2          waltz16
PCPD2                80.00
PLW2           30.00000000
PLW12           0.37154001

F2 - Processing paramet
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WDW                     EM
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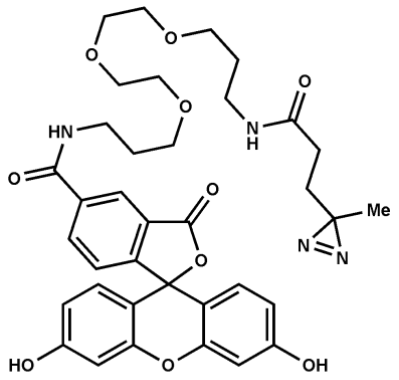

8

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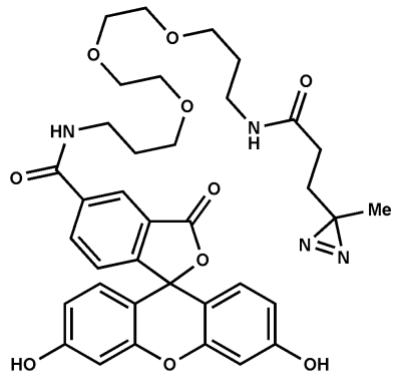

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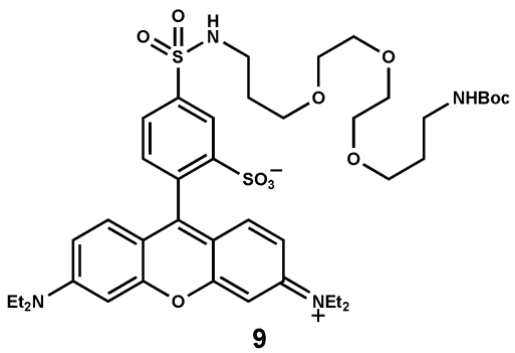

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NS	16
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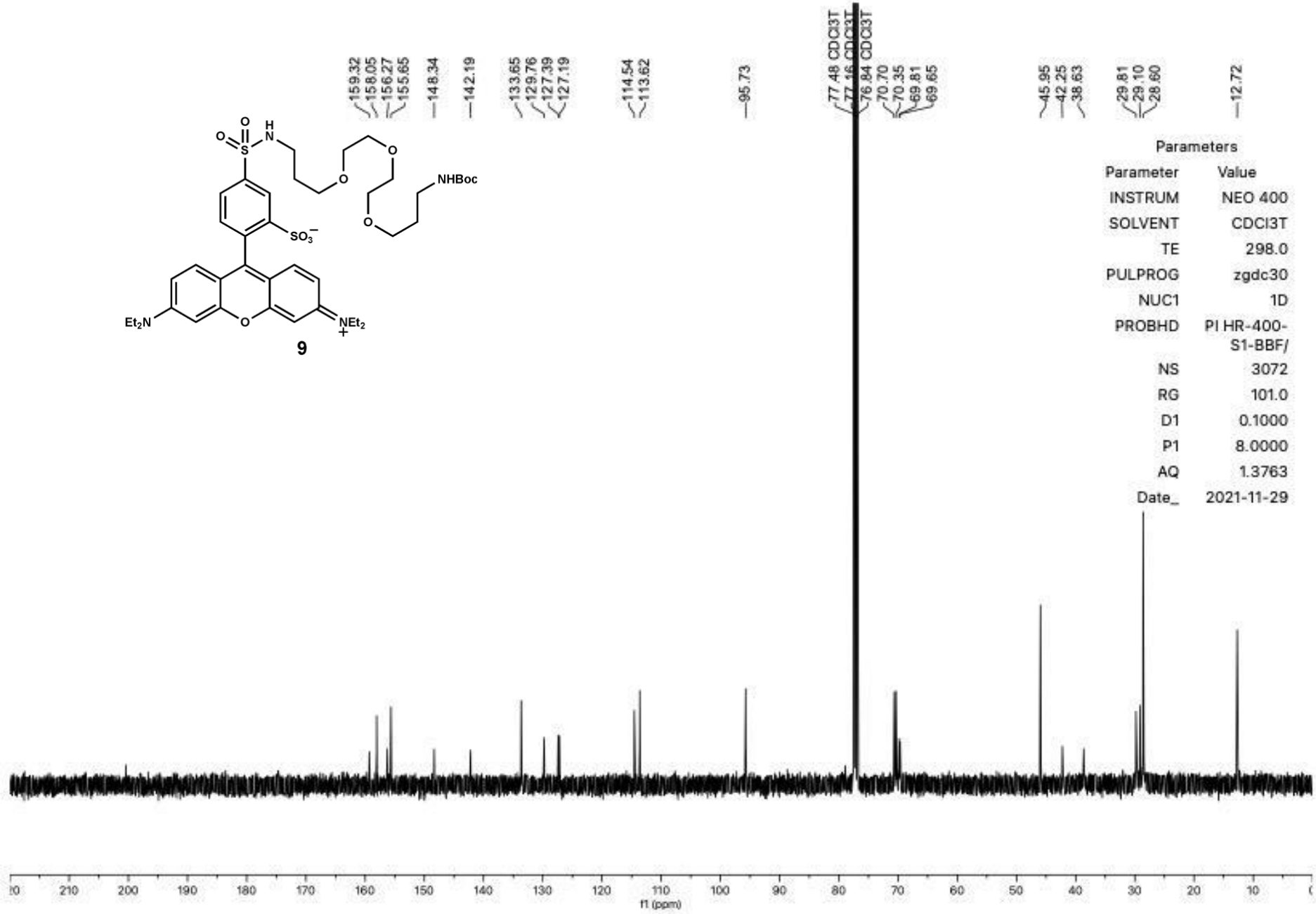
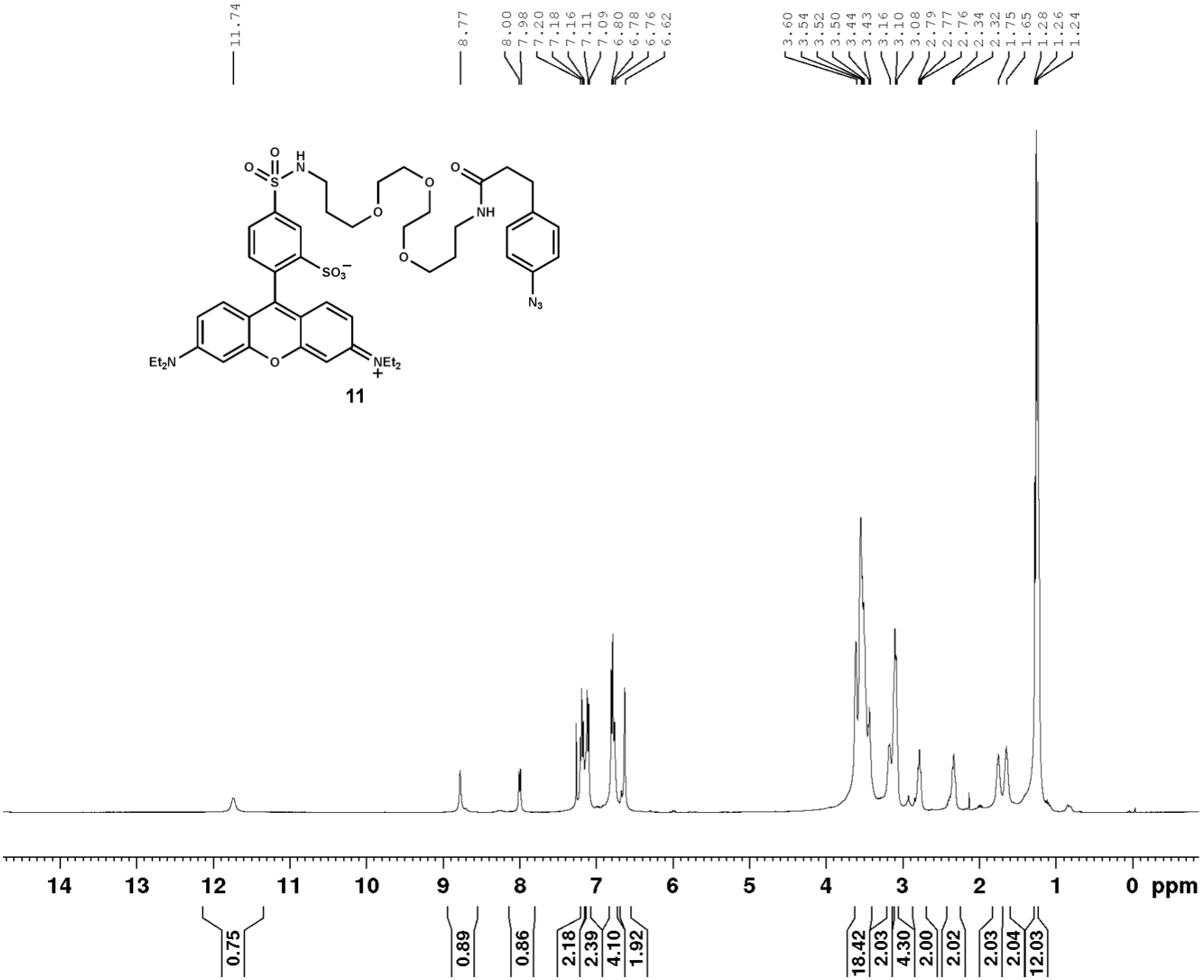
AQ	5.2429
Date_	2021-11-29


159.32
158.05
156.27
155.65
148.34
142.19
133.65
129.76
127.39
127.19
114.54
113.62
95.73
77.48 CDCl3T
77.16 CDCl3T
76.84 CDCl3T
70.70
70.35
69.81
69.65
45.95
42.25
38.63
29.81
29.10
28.60
12.72
NHBoc
SO3−
Et2N
NEt2
9
Parameters
Parameter Value
INSTRUM NEO 400
SOLVENT CDCl3T
TE 298.0
PULPROG zgdc30
NUC1 1D
PROBHD PI HR-400-S1-BBF/
NS 3072
RG 101.0
D1 0.1000
P1 8.0000
AQ 1.3763
Date_ 2021-11-29
f1 (ppm)




```
Current Data Parameters
NAME                  4-139
EXPNO                     3
PROCNO                    1

F2 - Acquisition Parame
Date_              20210730
Time                 15.53
INSTRUM            NEO 400
PROBHD    Z163739_0122 (
PULPROG               zg30
TD                   65536
SOLVENT              CDCl3
NS                      16
DS                       2
SWH                6250.000
FIDRES            0.190735
AQ                5.2428799
RG                   38.9208
DW                   80.000
DE                     8.64
TE                     298.0
D1                0.10000000
TD0                       1
SFO1           400.1328009
NUC1                    1H
P0                     2.67
P1                     8.00
PLW1          22.47400093

F2 - Processing paramet
SI                    65536
SF             400.1300235
WDW                      EM
SSB       0
LB                     0.30
GB        0
PC                     1.00
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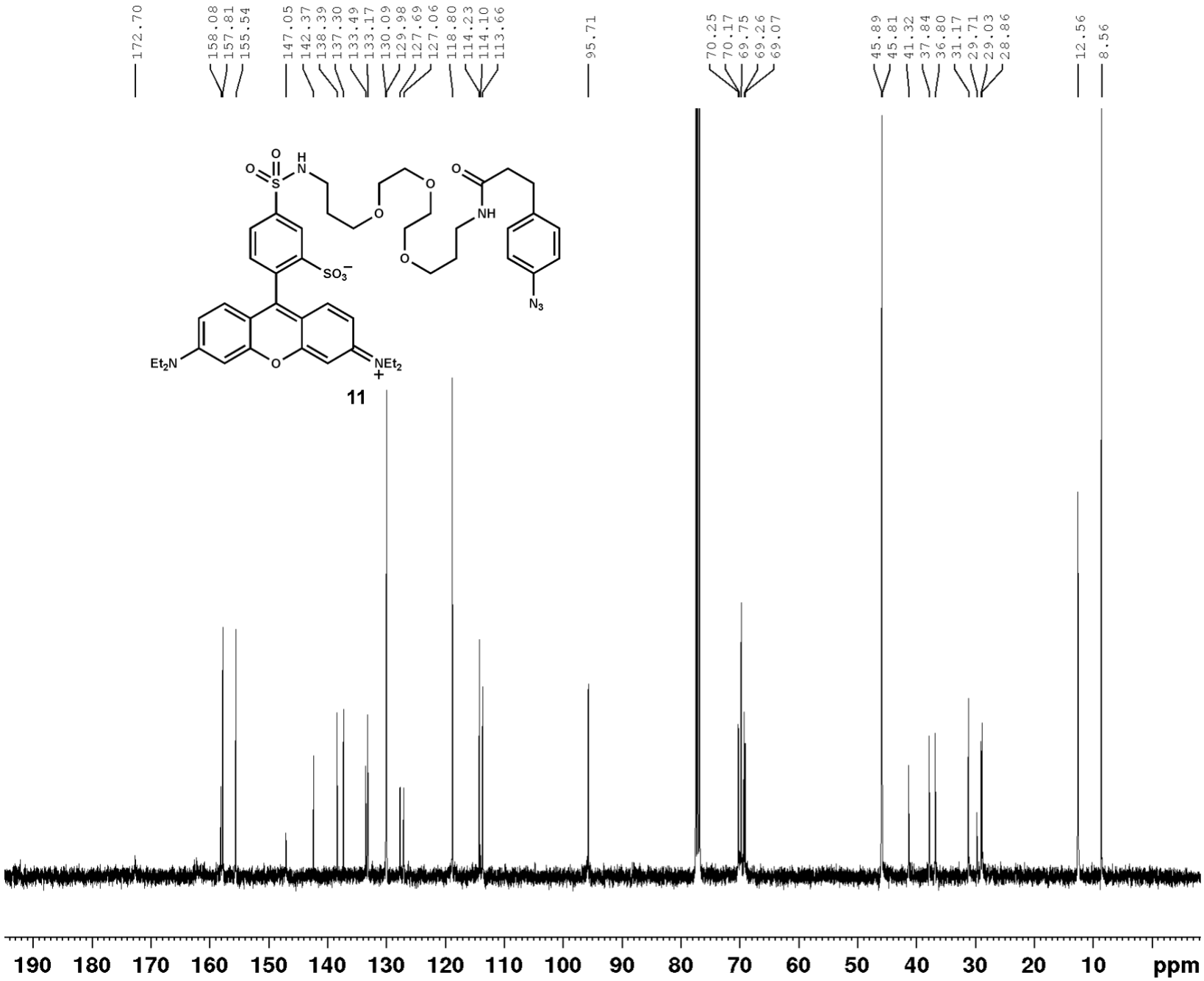


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Current Data Parameters
NAME                  4-139
EXPNO                     2
PROCNO                    1

F2 - Acquisition Parame
Date_              20210730
Time                 16.38
INSTRUM            NEO 400
PROBHD    Z163739_0122 (
PULPROG             zgdc30
TD                   65536
SOLVENT              CDCl3
NS                    1200
DS                       4
SWH              23809.523
FIDRES            0.726609
AQ               1.3762560
RG                     101
DW                  21.000
DE                    6.50
TE                   298.0
D1              0.10000000
D11             0.03000000
TD0                      1
SFO1           100.6238359
NUC1                   13C
P0                    2.67
P1                    8.00
PLW1           92.65799713
SFO2           400.1320007
NUC2                   1H
CPDPRG[2           waltz65
PCPD2                90.00
PLW2           22.47400093
PLW12           0.17757000

F2 - Processing paramet
SI                   65536
SF              100.6127685
WDW                     EM
SSB       0
LB                    1.00
GB        0
PC                    1.40
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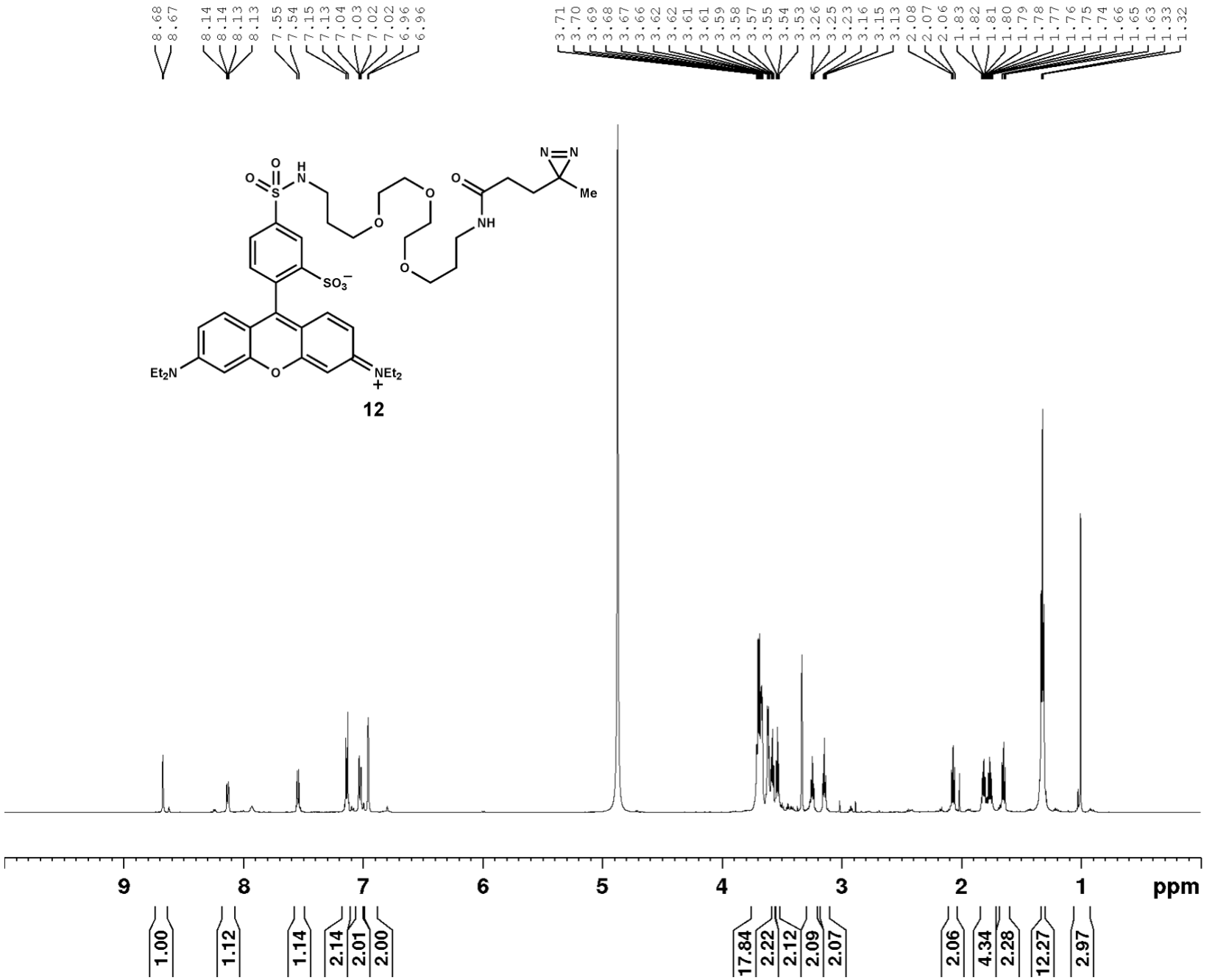
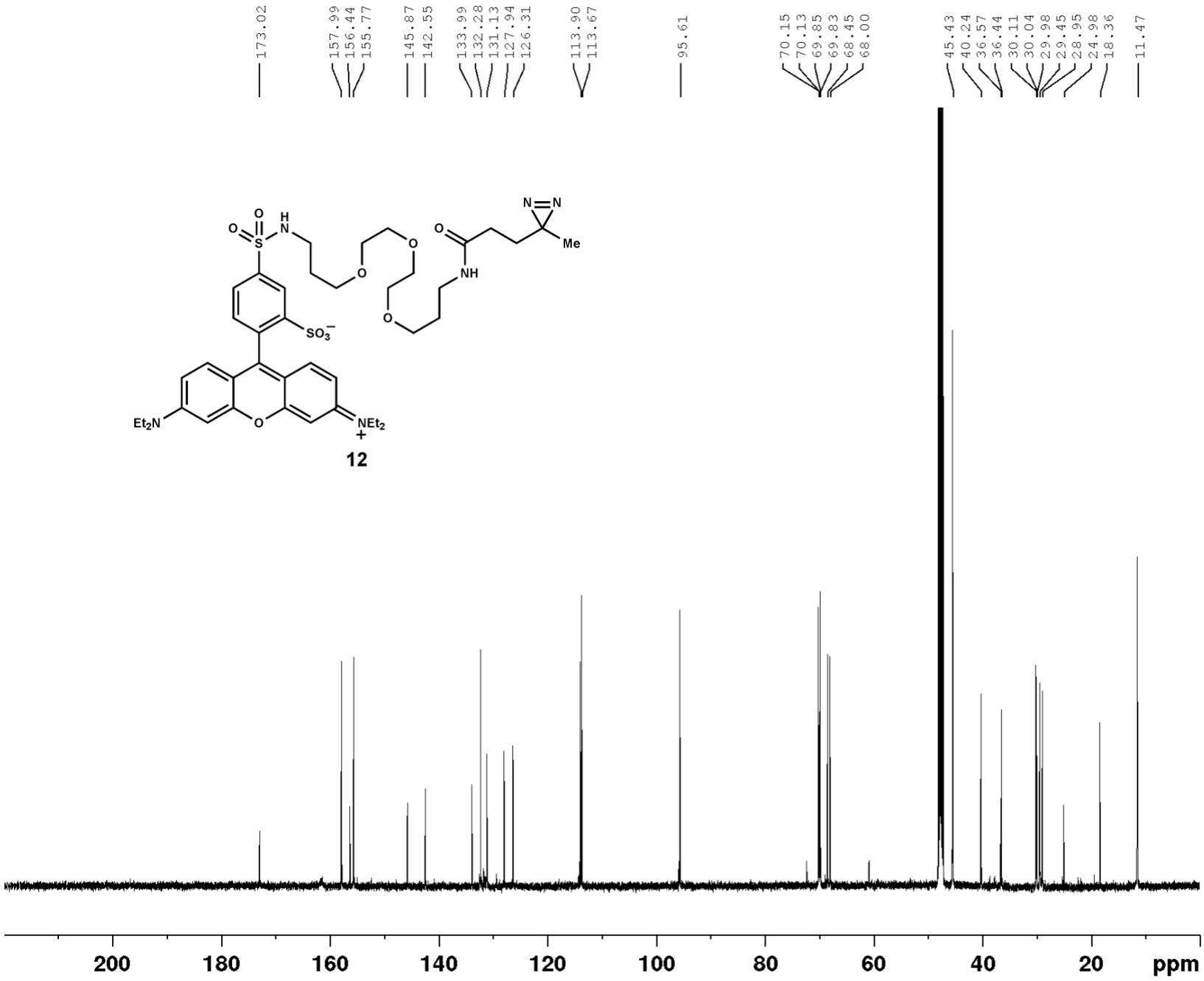


Current Data Parameters
NAME 4-141
EXPNO 3
PROCNO 1

F2 - Acquisition Parame
Date_ 20210829
Time 11.07
INSTRUM av600
PROBHD 5 mm CPBBO BB-
PULPROG zg30
TD 98074
SOLVENT MeOD
NS 16
DS 2
SWH 9615.385
FIDRES 0.098042
AQ 5.0998478
RG 10
DW 52.000
DE 14.23
TE 298.0
D1 0.10000000
TD0 1

======== CHANNEL f1 ====
SFO1 600.1342009
NUC1 1H
P1 9.50
PLW1 30.00000000

F2 - Processing paramet
SI 65536
SF 600.1300000
WDW EM
SSB 0
LB 0.30
GB 0
PC 1.00




```
Current Data Parameters
NAME                  4-141
EXPNO                     4
PROCNO                    1

F2 - Acquisition Parame
Date_              20210829
Time                 11.18
INSTRUM              av600
PROBHD    5 mm CPBBO BB-
PULPROG           zgdc30
TD                  65536
SOLVENT              MeOD
NS                   1033
DS                      4
SWH              36231.883
FIDRES           0.552855
AQ               0.9043968
RG                   2050
DW                  13.800
DE                  19.63
TE                  298.0
D1               0.40000001
D11              0.03000000
TD0                     1

======== CHANNEL f1 ====
SFO1            150.9194080
NUC1                   13C
P1                   10.10
PLW1            64.00000000

======== CHANNEL f2 ====
SFO2            600.1330010
NUC2                   1H
CPDPRG[2           waltz16
PCPD2                80.00
PLW2            30.0000000C
PLW12            0.37154001

F2 - Processing paramet
SI                   65536
SF              150.9028085
WDW                    EM
SSB       0
LB                   1.00
GB        0
PC                   1.00
```

HRMS Spectra

MASS SPECTROMETRY
ACCURATE MASS REQUEST

Date 8/23/21

Submitted by Tyler Allred
Room 4624PRH
Extension

Sample name TKA-4149B
Formula $C_{29}H_{43}N_5O_7$
Monoisotopic exact mass 573.31625
Estimated purity >95%

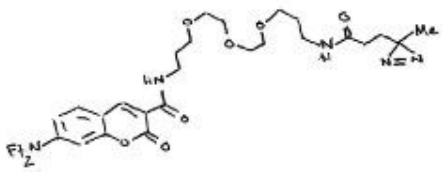
Ionization method
- Facility to choose X
- Electrospray ionization (ESI)
- Chemical ionization (CI)
- Electron ionization (EI)

Suggested solvent
- Methanol X
- Dichloromethane
- Other

Additional information (e.g important fragments expected, GC conditions (attach trace) etc.)

Structure

CD



MASS SPECTROMETRY
ACCURATE MASS REQUEST

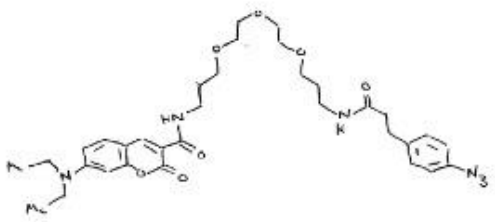
Date F 2/26/21

Submitted by Tyler Allred
Room 4024 FRB
Extension

Sample name TKA-4053C
Formula $C_{33}H_{44}N_6O_7$
Monoisotopic exact mass 636.32715
Estimated purity >90 %

Structure

CP



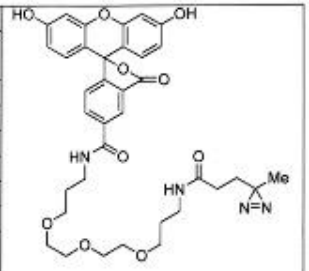
Ionization method
Facility to choose ✓
Electrospray ionization (ESI)
Chemical ionization (CI)
Electron ionization (EI)

Suggested solvent
Methanol ✓
Dichloromethane
Other

Additional information (e.g important fragments expected, GC conditions (attach trace) etc.)

Mass Spectrometry
Accurate Mass Request Form

Date	12/8/21	
Submitted by	Natalie Falco Spitale Lab, Pharmaceutical Sciences	
Room	101 Theory, Room 113	
Extension		
Email	nfalco1@uci.edu	
Sample name	NF-211021	
Formula	$C_{36}H_{40}N_4O_{10}$	
Monoisotopic exact mass	688.27	
Estimated purity	>90%	
Ionization method	Facility to choose ESI CI EI	X
Suggested solvent	Methanol Dichloromethane Other	X
Additional information		



FD

MASS SPECTROMETRY
ACCURATE MASS REQUEST

Date 9/5/21

Submitted by Tyler Allred
Room 4024 FRH
Extension

Sample name TKA-4152C
Formula $C_{40}H_{41}N_5O_{10}$
Monoisotopic exact mass 751.28534
Estimated purity >95%

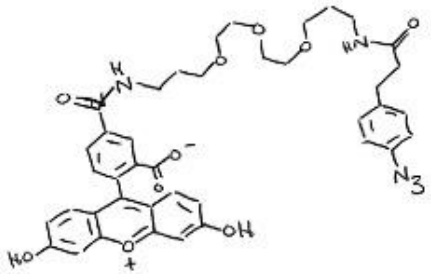
Ionization method
- Facility to choose X
- Electrospray ionization (ESI)
- Chemical ionization (CI)
- Electron ionization (EI)

Suggested solvent
- Methanol X
- Dichloromethane
- Other

Additional information (e.g important fragments expected, GC conditions (attach trace) etc.)

Structure

FP



MASS SPECTROMETRY
ACCURATE MASS REQUEST

Date 9/5/21

Submitted by Tyler Allred
Room 4024 FRH
Extension

Sample name TKA-4141B
Formula $C_{42}H_{58}N_6O_{10}S_2$
Monoisotopic exact mass 870.36588
Estimated purity 95%.

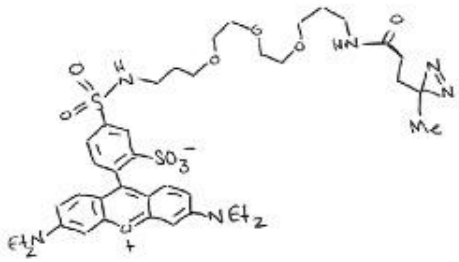
Ionization method
Facility to choose X
Electrospray ionization (ESI)
Chemical ionization (CI)
Electron ionization (EI)

Suggested solvent
Methanol X
Dichloromethane
Other

Additional information (e.g important fragments expected, GC conditions (attach trace) etc.)

Structure

RD



/

MASS SPECTROMETRY
ACCURATE MASS REQUEST

Date 8/11/21

Submitted by Tyler Allred
Room 4024 FRB
Extension

Sample name TKA-41398
Formula $C_{44}H_{59}N_7O_{10}S_2$
Monoisotopic exact mass 933.37618
Estimated purity >95%

Ionization method
- Facility to choose X
- Electrospray ionization (ESI)
- Chemical ionization (CI)
- Electron ionization (EI)

Suggested solvent
- Methanol X
- Dichloromethane
- Other

Additional information (e.g important fragments expected, GC conditions (attach trace) etc.)

Structure

RP