SUPPORTING INFORMATION:

Development of a split esterase for protein-protein interaction dependent small molecule activation

Krysten A. Jones[1], Kaitlin Kentala[1], Michael W. Beck[1], Weiwei An[2], Alexander Lippert[2], Jared C. Lewis[3], Bryan C. Dickinson[1]*

[1]Department of Chemistry, The University of Chicago, Chicago, IL 60637

[2]Department of Chemistry, Center for Drug Discovery, Design, and Delivery (CD4), Center for Global Health Impact (CGHI), Southern Methodist University, Dallas, TX 75275-0314

[3]Department of Chemistry, Indiana University, Bloomington, IN 47405

*Corresponding author. Email address: Dickinson@uchicago.edu (Bryan C. Dickinson)

Methods

General synthetic procedures.

All reactions were performed in dried glassware under an atmosphere of dry N_2. Silica gel P60 (SiliCycle) was used for column chromatography, and Analytical Chromatography TLC Silica gel 60 F_{254} (Merck Millipore, Darmstadt, Germany) or SiliCycle 60 F_{254} silica gel (precoated sheets, 0.25 mm thick) was used for analytical thin layer chromatography. Plates were visualized by fluorescence quenching under UV light or by staining with iodine. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), EMD Millipore (Billerica, MA), Oakwood Chemical (West Columbia, SC), TCI (Tokyo, Japan) and used without further purification. ^{1}H NMR and ^{13}C NMR spectra for characterization of new compounds and monitoring reactions were collected in $CDCl_3$ (Cambridge Isotope Laboratories, Cambridge, MA or Sigma-Aldrich) on a JEOL 500 MHz spectrometer in the Department of Chemistry at Southern Methodist University or using a 500 MHz Bruker Avance II+ spectrometer with 5 mm QNP probe at the Department of Chemistry NMR Facility at the University of Chicago. All chemical shifts are reported in the standard notation of parts per million using the peak of residual proton signals of the deuterated solvent as an internal reference. Coupling constant units are in Hertz (Hz) Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; dt, doublet of triplets. High resolution mass spectroscopy was performed on a Shimadzu IT-TOF (ESI source) at the University of Texas, Arlington or Agilent 6224 TOF High Resolution Accurate Mass Spectrometer (HRA-MS, combination of APCI and ESI) at the Department of Chemistry Mass Spectrometry Facility at the University of Chicago. Low resolution mass spectral analyses and liquid chromatography analysis of SN-38-CM_2 were carried out on an Advion Expression L mass spectrometer (Ithaca, NY) coupled with an Agilent 1220 Infinity LC System (Santa Clara, CA). Synthesis of fluorescein-CM_2 was carried out using literature procedures[1].

(3-(((1*r*,3*r*,5*R*,7*S*)-adamantan-2-ylidene)(methoxy)methyl)-2-chloro-6-((*E*)-2-cyanovinyl)phenoxy)methyl 1-methylcyclopropane-1-carboxylate (1).

Chloromethyl 1-methylcyclopropanecarboxyate[1] (116.3 mg, 0.7832 mmol, 1.5 equiv) was added to the dry, N_2 filled flask, then dissolved with 2.1 mL anhydrous acetone. NaI (128.6 mg, 0.8580 mmol, 1.7 equiv) was added to the solvent and the mixture was stirred for 24 h at ambient temperature. The reaction was concentrated under reduced pressure. Purification by silica column chromatography (CH_2Cl_2) yielded a pale yellow oil. (*E*)-3-(4-(((1*r*,3*r*,5*R*,7*S*)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-hydroxyphenyl)acrylonitrile[2, 3] (184.4 mg, 0.5182 mmol, 1.0 equiv) was dissolved with 2.0 mL anhydrous DMF in an dry, N_2 filled flask and anhydrous *N*,*N*-

diisopropylethylamine (DIPEA, 0.19 mL, 1.1 mmol, 2.1 equiv) was added. Iodomethyl 1-methylcyclopropane-1-carboxylate was dissolved with 3.0 mL anhydrous DMF and added to the flask. The reaction mixture was stirred for 21 h at ambient temperature. The reaction was concentrated under reduced pressure. Purification by silica column chromatography (1:20 EtOAc/Hexane) yielded **1** as a white solid (107.4 mg, 44%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (d, 1H, *J* = 16.6 Hz), 7.36 (d, 1H, *J* = 8.1 Hz), 7.11 (d, 1H, *J* = 8.1 Hz), 5.95 (d, 1H, *J* = 16.6 Hz), 5.70 (m, 2H), 3.29 (s, 1H), 3.25 (s, 1H), 2.04 (s, 1H), 1.94–1.63 (m, 12H), 1.28 (s, 3H), 1.23 (m, 2H), 0.77 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 175.07, 152.02, 144.76, 139.33, 139.04, 133.30, 129.21, 128.62, 128.54, 123.91, 117.83, 98.59, 89.32, 57.44, 39.19, 39.03, 38.61, 38.56, 36.96, 32.91, 31.59, 29.73, 28.29, 28.11, 19.10, 18.54, 17.60; HRMS calcd for $C_{27}H_{30}ClNO_4$ ($M+Na^+$) 490.1756, found 490.1755.

(2-chloro-6-((*E*)-2-cyanovinyl)-3-((1*r*,3*r*,5*r*,7*r*)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)phenoxy)methyl 1-methylcyclopropane-1-carboxylate (**Chemilum-CM**). Enol ether **1** (107.4 mg, 0.2295 mmol, 1.0 equiv) was dissolved with 5 mL THF at 0 °C in a two-neck flask and Rose bengal (12.4 mg, 0.0122 mmol, 0.050 equiv) was added to the solvent. O_2 was bubbled through the solvent when illuminated with a 120W light bulb (Home Depot, Dallas, TX). The reaction was monitored by TLC. After 3 h 15 min, the mixture was concentrated under reduced pressure. Purification by silica column chromatography (1:20 EtOAc/Hexane) yielded chemi-CM_2 as a white solid (76.9 mg, 67%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.97 (d, 1H, *J* = 8.6 Hz), 7.66 (d, 1H, *J* = 16.7 Hz), 7.52 (d, 1H, *J* = 8.6 Hz), 6.03 (d, 1H, *J* = 17.2 Hz), 5.67 (dd, 2H), 3.21 (s, 3H), 3.01 (s, 1H), 1.96 (s, 1H), 1.85–1.58 (m, 12H), 1.28 (s, 3H), 1.23 (m, 2H), 0.79 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 175.11, 152.70, 144.42, 136.53, 130.51, 129.80, 127.19, 124.13, 117.63, 111.63, 99.99, 96.48, 89.32, 49.87, 36.57, 33.99, 33.66, 32.67, 32.24, 31.62, 31.58, 29.82, 26.17, 25.83, 19.18, 18.59, 17.74; HRMS calcd for $C_{27}H_{30}ClNO_6$ ($M+Na^+$) 522.1654, found 522.1654.

[(19S)-10,19-diethyl-7-[(1-methylcyclopropane carbonyloxy)methoxy]-14,18-dioxo-17-oxa-3,13-diazapentacyclo[11.8.0.0^{2,11}.0^{4,9}.0^{15,20}]henicosa-1(21),2,4(9),5,7,10,15(20)-heptaen-19-yl]oxy}methyl 1-methylcyclopropane-1-carboxylate (SN-38-CM_2) In a 250 mL round bottom flask equipped with a magnetic stir bar, freshly prepared Chloromethyl 1-methylcyclopropanecarboxyate[1] (1.5141 g, 10.2 mmol, 20 equiv) was dissolved in 50 mL dry DMF. SN-38 (0.2002 g, 0.51 mmol, 1 equiv) was then added followed by the sequential addition of sodium iodide (5.3476 g, 35.7 mmol, 70 equiv) and potassium carbonate (0.3522 g, 2.5 mmol, 4.9 equiv). The resulting solution allowed to stir at room temperature. After 5 h, when side

products began to appear as measured by LC-MS, the solution was diluted with EtOAc, filtered through a pad of celite, and concentrated under vacuum on a rotary evaporator before drying under high vacuum until the reaction mixture was completely solid. The resulting solid was then suspended in EtOAc and filtered through another pad of celite. The resulting mixture was concentrated before purifying via silica column chromatography (0% to 100% EtOAc in Hexanes) and purifying via silica column chromatography again (0% to 5% MeOH in DCM) yielding 12.3 mg (3.9 % yield) as a light yellow solid. Purity was determined by LC-MS. R_f (5% MeOH in DCM) = 0.47. HRA-MS(+) Calculated for $C_{34}H_{36}N_2O_9$ [M^+]: 616.2421; found 616.2431. ^{1}H-NMR (500 MHz; $CDCl_3$) δ: 8.23 – 8.17 (m, 1H), 7.64 – 7.60 (m, 1H), 7.55 – 7.52 (m, 1H), 5.94 (s, 1H), 5.77 (d, *J* = 15 Hz, 1H), 5.30 (m, 4H), 5.12 (s, 1H), 3.71 (s, 1H), 3.35 (s, 1H), 3.16 (m, 1H), 1.90 (m, 2H), 1.63 (s, 1H), 1.52 (m, 2H), 1.40 (m, 3H), 1.34 (s, 2H), 1.30 (m, 1H), 1.25 (s, 1H), 1.04 (m, 3H), 0.94 (m, 1H) 0.76 (m, 1H). ^{13}C-NMR (126 MHz; $CDCl_3$): 174.7, 174.1, 157.8, 156.1, 151.9, 150.4, 150.2, 147.5, 147.1, 145.4, 132.6, 132.2, 127.6, 127.4, 125.7, 122.6, 118.7, 114.7, 105.8, 98.2, 97.9, 85.4, 73.0, 66.5, 49.6, 31.9, 31.8, 23.4, 19.6, 19.3, 18.0, 14.2, 14.0, 8.0.

Cloning.

All plasmids were constructed by Gibson assembly from PCR products generated using Phusion Polymerase or Q5 DNA Polymerase (NEB). The plasmids were sequenced by the University of Chicago Comprehensive Cancer Center DNA Sequencing and Genotyping Facility. Maps for each plasmid are shown in **Supplementary Fig. S1**. All plasmids are described in **Supplementary Table S1**, which includes links to fully annotated sequence maps. Full vector sequences are also available upon request. The original GPI-anchor plasmid[4] was a gift from Evan W. Miller (UC Berkeley).

Esterase screening in *E. coli*.

BL21 cells were transformed by heat shock with either BS2 esterase, PLE esterase, or TEV protease. Single colonies were grown to saturation overnight at 37 °C and then each well of a 96-well deep well plate containing 0.185 mL of LB with antibiotics, 10 mM arabinose, and 50 μM fluorescein-CM_2 was inoculated with 15 μL of the overnight culture. After growth with shaking for 4 h at 37 °C, the cells were pelleted and the LB was removed. The cells were then resuspended in 0.2 mL PBS and transferred to a 96-well black wall, clear bottom plate (Nunc), and fluorescence intensities (λ_{ex} 485/20 nm, λ_{em} 528/20 nm) and OD_{600} were measured on a Synergy Neo2 Hybrid Multi-Mode Reader (BioTek). The data were analyzed by dividing the emission values by the background-corrected OD_{600} value. All values were then normalized to the emission of cells expressing TEV protease, which was assigned an arbitrary value of 1 to allow for values from

each fluorescence plot to be compared to each other. All experiments were performed in three technical replicates.

Cut site screening in *E. coli*.

BL21 cells were transformed by heat shock with either $BS2_N$-linker-FRB, FKBP-linker-$BS2_C$, or cotransformed with both. Single colonies were grown to saturation overnight at 37 °C and then each well of a 96-well deep well plate containing 0.185 mL of LB with antibiotics, 0.1 mM IPTG, and 0.5 mM fluorescein-CM_2 was inoculated with 15 µL of the overnight culture. As indicated, 20 µM rapamycin or 500 µM abscisic acid was added per well. After growth with shaking for 4 h at 37 °C, the cells were pelleted and the LB was removed. The cells were then resuspended in 0.2 mL PBS and transferred to a 96-well black wall, clear bottom plate (Nunc), and fluorescence intensities (λ_{ex} 485/20 nm, λ_{em} 528/20 nm) and OD_{600} were measured on a Synergy Neo2 Hybrid Multi-Mode Reader (BioTek). The data were analyzed by dividing the emission values by the background-corrected OD_{600} value. All values were then normalized to the emission of non-transformed BL21 cells incubated with fluorescein-CM_2 as indicated above, which was assigned an arbitrary value of 1 to allow for values from each fluorescence plot to be compared to each other. All experiments were performed in at least two technical replicates.

Split esterase assay in *E. coli* cell lysate.

BL21 cells were cotransformed by heat shock with $BS2_N$-linker-FRB and FKBP-linker-$BS2_C$. Single colonies were grown to saturation overnight at 37 °C and then each well of a 24-well deep well plate containing 3 mL of LB with antibiotics, was inoculated with 30 µL of the overnight culture. When cells reached an OD_{600} of 0.7-0.8, 0.1 mM IPTG and 20 µM rapamycin was added and then cultured at 30 °C for 16 h. Cells were collected by centrifugation and the supernatant was removed. Cell pellets were either stored at -20 °C for 5 days prior to lysis or freshly lysed in 0.3 mL lysis buffer (50 mM sodium phosphate buffer, 0.5% (vol/vol) Tween, pH 7.4). Lysed bacterial suspensions were incubated with 0.5 mM fluorescein-CM_2 and 1 µM rapamycin for 30 minutes at room temperature with rocking prior to visualization. A portion (8 µL) of this mixture was then spotted on filter paper (Whatman Grade) and imaged on a ChemiDoc Imaging System (Bio-Rad) at the University of Chicago BioPhysics Core Facility. All experiments were performed in three technical replicates.

Mammalian cell culture and plasmid transfection.

HEK293T (ATCC), MDA-MB-231 (courtesy of Harikrishna Nakshatri, Indiana University), MDA-MB-453 (courtesy of the Greene laboratory, UChicago), HT-29, RAW264.7 (courtesy of the Esser-Kahn laboratory, UChicago) cells were cultured in DMEM (L-glutamine, high glucose, sodium

pyruvate, phenol red; obtained from Corning) supplemented with 10% (vol/vol) fetal bovine serum (FBS, Gemini Benchmark), and 1% (vol/vol) penicillin/streptomycin (Gibco/Life Technologies). Jurkat (ATCC), PC-3, and THP-1 (courtesy of the Esser-Kahn laboratory, UChicago) were cultured in RPMI1640 (L-glutamine, phenol red; obtained from Corning) supplemented with 10% FBS and 1% penicillin/streptomycin. HEPG2 cells (courtesy of the Krishnan laboratory, UChicago) were cultured in DMEM Glutamax (high glucose, sodium pyruvate, phenol red; obtained from ThermoFisher Scientific) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained in a 5% CO_2, water-saturated incubator at 37 °C. Transient transfections were performed using cationic lipid formulations (Lipofectamine 2000 or Lipofectamine 3000; Invitrogen) following the manufacturer's protocol. HEK293T cells are listed in the database of commonly misidentified cell lines maintained by ICLAC (http://iclac.org/databases/cross-contaminations/). We obtained fresh cells from ATCC or early passage aliquots from the Cellular Screening Center, University of Chicago, which were frozen down at an early passage (passage 5) in individual aliquots. The cells were then used for less than 25 passages for all experiments. Multiple biological replicates were performed with cells from different passages and freshly thawed aliquots. There was no testing for mycoplasma infection or further authentication because early passage cells were used for all experiments.

Mammalian cell fluorescence imaging.

HEK293T cells were plated in a 48-well plate and transfected the next day with 500 ng PLE or BS2 (to test full length esterase activity) or 2 µg $BS2_N$-POI and 500 ng POI-$BS2_C$ (to test split esterase activity) using 1.5 µL of Lipofectamine 2000 following the manufacturer's protocol. For bulk and single cell assays, cells were plated 24 h after transfection in a 96-well black wall, glass bottom plate (Cellvis) pre-treated with 0.1 mg/mL Poly-D-lysine for 2 h. When indicated, 400 nM rapamycin, 10 µM abscisic acid, or a DMSO carrier control was added. After 24 h of culturing, the media was replaced with 10 µM fluorescein-CM_2 in Live Cell Imaging Solution (ThermoFisher Scientific) for bulk cell assays and fluorescence intensities (λ_{ex} 490/20 nm, λ_{em} 545/20 nm) were measured on a Synergy Neo2 Hybrid Multi-Mode Reader every minute for 1 h. For single cell assays, the media was replaced with 10 µM fluorescein-CM_2 and 1 µM Hoechst 33342 in Live Cell Imaging Solution. The cells were incubated for 1 h at room temperature and then imaged on an inverted epifluorescence microscope (Leica DMi8) equipped with a camera (Hamamatsu Orca-Flash 4.0) with either 20x air objective or 63x oil objective (N/A 1.4) and light source (Sutter Lamdba XL, 300 W Xenon) for fluorescein-CM_2 (ET 490/20x, Quad-S, ET 525/36 m), Hoechst 33342 (ET 402/15x, Quad-S, ET 455/50 m), and brightfield using Leica LAS X software. For image analysis of fluorescent emission, low resolution (20x) microscopy images were analyzed in order to obtain more cells in the field and provide a better representation of the cell population. Each

image for a given condition was processed to adjust for maximum brightness and areas containing cells were selected and measured in ImageJ[5]. All experiments were performed in three technical replicates.

Mammalian luciferase assay.

HEK293T cells were plated in a 48-well plate and transfected the next day with 500 ng $Nluc_{11S}$-POI and 500 ng POI-$Nluc_{114}$ using 1.5 µL of Lipofectamine 2000 following the manufacturer's protocol. Cells were plated 24 h after transfection in a 96-well white wall plate. When indicated, 400 nM rapamycin or a DMSO carrier control was added was added. After 24 h, luciferase activity was assayed using the Nano-Glo Live Cell Assay System (Promega) on a Synergy Neo2 Hybrid Multi-Mode Reader. All experiments were performed in three technical replicates.

Inhibitor assay.

HEK293T cells were plated in a 48-well plate and transfected the next day with 2 µg $BS2_N$-tBID and 500 ng Bcl-2-$BS2_C$ or 500 ng $Nluc_{11S}$-tBID and 500 ng Bcl-2-$Nluc_{114}$ using 1.5 µL of Lipofectamine 2000 following the manufacturer's protocol. Cells were plated 24 h after transfection in a 96-well black wall, glass bottom plate pre-treated with 0.1 mg/mL Poly-D-lysine for 2 h for fluorescence imaging or 96-well white wall plate for luminescence imaging. For dual fluorescence and luminescence imaging, cells were plated in a 96-well black wall, glass bottom plate pre-treated with 0.1 mg/mL Poly-D-lysine for 2 h. Then, 1 µM ABT-199 or a DMSO carrier control was added during cell culture for indicated lengths of time prior to imaging. Fluorescence and luminescence imaging were performed as described above. All experiments were performed in three technical replicates.

Extracellular chemiluminescent assay.

HEK293T cells were plated in a 48-well plate and transfected the next day with 500 ng $BS2_N$-FRB-GPI anchor and 500 ng FKBP-$BS2_C$-GPI anchor using 1.5 µL of Lipofectamine 2000 following the manufacturer's protocol. Cells were plated 24 h after transfection in a 96-well white wall plate for luminescence imaging. For DNA titrations, the same procedure was followed with 0.2 – 1000 ng total DNA (1 $BS2_N$-FRB-GPI anchor:1 FKBP-$BS2_C$-GPI anchor) used for transfections. Cells were treated with 400 nM rapamycin or a DMSO control for 24 h. For the rapamycin time course, cells were treated with 400 nM rapamycin or a DMSO control for 0.08 – 6 h. For rapamycin dose response, cells were treated with 0.1 – 400 nM rapamycin or a DMSO control for 6 h. For all experiments, after indicated rapamycin treatment, cells were incubated with 10 µM Chemilum-CM_2 in Live Cell Imaging Solution and luminescence was measured on a

Synergy Neo2 Hybrid Multi-Mode Reader every minute for 1 h. All experiments were performed in three technical replicates.

Extracellular/intracellular luminescence assay.

HEK293T cells were plated in a 48-well plate and transfected the next day with 500 ng $BS2_N$-POI-GPI anchor and 500 ng POI-$BS2_C$-GPI anchor or 500 ng $Nluc_{11S}$-FRB and 500 ng FKBP-$Nluc_{114}$ using 1.5 µL of Lipofectamine 2000 following the manufacturer's protocol. Cells were plated 24 h after transfection in a 96-well white wall plate for luminescence imaging. When indicated, 400 nM rapamycin was added. After 24 h of culturing, the media was replaced with 10 µM Chemilum-CM_2 in Live Cell Imaging Solution and luminescence was measured on a Synergy Neo2 Hybrid Multi-Mode Reader every minute for 1 h. Cells were then gently rinsed in 0.2 mL PBS and subsequently assayed using the Nano-Glo Live Cell Assay System. All experiments were performed in three technical replicates.

Recombinant protein expression and purification.

The pET-BS2 plasmid was transformed into chemically competent E. coli BL21 cells. Cultures (1 L) were grown at 37 °C in LB broth (with 40 µg/mL Kan) to mid log-phase (OD_{600} = 0.6 – 0.8), induced with 0.2 mM IPTG, and incubated at 30 °C for 18 – 20 h. The cells were harvested by centrifugation at 4 °C and resuspended in 50 mM sodium phosphate buffer (pH 7.4), 300 mM NaCl, and 1 mM PMSF. Lysozyme (1 mg) was added and the cells were sonicated and centrifuged at 10000 x *g* for 1 h at 4 °C. BS2 was purified from clarified supernatants using nickel affinity chromatography and dialyzed into 50 mM sodium phosphate buffer (pH 7.4). Prior to storage at -20 °C, 15% glycerol was added to the sample. Final protein concentrations were determined using standard BCA assay or UV spectroscopy. SDS-PAGE was also performed and gels were stained with Coomassie R-250.

BS2 *in vitro* reactions with SN-38-CM_2.

Bioconversions contained purified BS2 (2 µM) and SN-38-CM_2 (1 mM) in reaction buffer (50 mM sodium phosphate buffer, pH 7.4) and were run at room temperature. Aliquots of the *in vitro* reaction were removed over time (5 – 30 min) and quenched 1:1 with methanol. Liquid chromatography analysis was carried out on an Agilent 1220 Infinity LC System.

BS2/SN-38-CM_2 cytotoxicity assay.

MDA-MB-231 or MDA-MB-231 luciferase cells were plated in a 6-well plate and transfected the next day with 2500 ng $BS2_N$-FRB and 2500 ng FKBP-$BS2_C$ or 2500 ng BS2 using 5 µL of P3000 Reagent and 5 µL Lipofectamine 3000 following the manufacturer's protocol. MDA-MB-231 cells

were plated 24 h after transfection in a 96-well white wall plate with MDA-MB-453 luciferase cells (1:1) for luminescence imaging. MDA-MB-231 luciferase cells were plated 24 h after transfection in a 96-well white wall plate. When indicated, 400 nM rapamycin or DMSO control was added. After 12 h of culturing, SN-38-CM_2 (0.1 – 5 μM) was added. A stock solution of D-luciferin (500 μM final) was added after 40 h and luminescence was measured on a Synergy Neo2 Hybrid Multi-Mode Reader. All experiments were performed in three technical replicates.

BS2/SN-38-CM_2 coculture cytotoxicity assay.

MDA-MB-231 cells were plated in a 6-well plate and transfected the next day with 2500 ng $BS2_N$-FRB and 2500 ng FKBP-$BS2_C$ or 2500 ng BS2 using 5 μL of P3000 Reagent and 5 μL Lipofectamine 3000 following the manufacturer's protocol. When indicated, 400 nM rapamycin or DMSO control was added 24 h after transfection. After 12 h of culturing, cells (1×10^5) were suspended in Hydrogel (Sigma, TrueGel3D Hydrogel Kits, SLO-DEXTRAN, PEG crosslinker) and cell culture media according to the manufacture's protocol with SN-38-CM_2 (1 μM) and plated in a 3.5-cm tissue culture plate (Corning). The matrix was solidified at 37 °C for 1 h and MDA-MB-453 luciferase cells were then added (50% confluent) around the hydrogel-cell mix. After 12 h, the cells were rinsed with PBS. A stock solution of D-luciferin (250 μM final) was added after 40 h and luminescence was measured on a Xenogen IVIS 200 at the University of Chicago Optical Imaging Core Facility.

Endogenous esterase screening.

Metastatic cells and liver cells: MDA-MB-231, HT-29, PC-3, and HEPG2 cells were plated in a 96-well plate and transfected the next day with 100 ng BS2 using 0.1 μL P3000 reagent and 0.15 μL Lipofectamine 3000 following the manufacture's protocol. For bulk and single cell assays, cells were plated 24 h after transfection in a 96-well black wall, glass bottom plate pre-treated with 0.1. mg/mL Poly-D-lysine for 2 h. After 24 h of culturing, the media was replaced with Live Cell Imaging Solution with or without 10 μM fluorescein-CM_2 and imaged and quantified as in Mammalian cell fluorescence imaging method.

Immune cells: Jurkat, RAW264.7, and THP-1 cells were plated in a 96-well black wall, glass bottom plate pre-treated with 0.1 mg/mL Poly-D-lysine for 2 h for bulk and single cell assays. For cell activation, ultrapure LPS-EB was incubated at 100 ng/mL for 18 h (RAW264.7), 100 ng/mL for 1 h (Jurkat), and 1 μg/mL for 3 h (THP-1). After culturing in the presence and absence of LPS, the media was replaced with Live Cell Imaging Solution with or without 10 μM fluorescein-CM_2 and imaged and quantified as in Mammalian cell fluorescence imaging method.

Statistical analyses.

Data were analyzed using unpaired two-tailed t-tests (GraphPad Prism 6, GraphPad Software, La Jolla, CA, USA).

Data availability.

All data generated or analyzed during this study are included in the published article (and its supplementary information) or are available from the corresponding author on reasonable request.

Safety statement.

No unexpected or unusually high safety hazards were encountered.

Table S1. List of plasmids used in this work.

Vector Name	Res.	Origin	Purpose	Map*	Benchling Link
KJ89	Chlor	CloDF13	BS2 expression plasmid	a	https://benchling.com/s/seq-iefwLbJz8iB9HIDlaUsY
p9-50	Chlor	CloDF13	PLE expression plasmid	a	https://benchling.com/s/seq-j18JbOR0iSPcjqxbecFF
p1-18	Chlor	CloDF13	TEV expression plasmid	a	https://benchling.com/s/seq-GOEUaX6hsarI55zkuFV3
p9-70	Kan	ColE1	PLE mammalian expression plasmid	b	https://benchling.com/s/seq-ASiFsYMKIQ9AciVPXyRk
KJ128	Kan	ColE1	BS2 mammalian expression plasmid	b	https://benchling.com/s/seq-51TZnY3q5ZfFQi2XXUhS
KJ150	Kan	ColE1	$BS2_N$-linker-FRB expression plasmid	c	https://benchling.com/s/seq-pE2lgWMhm7M4Gf38dBzJ
KJ246	Kan	ColE1	$BS2_N$-linker-ABI expression plasmid	c	https://benchling.com/s/seq-KZyurXJXoeoPALkFmGru
KJ158	Spec	CloDF13	FKBP-linker-$BS2_C$ expression plasmid	d	https://benchling.com/s/seq-I20ugDxo7pzS2F1KZPps
KJ247	Spec	CloDF13	PYL-linker-$BS2_C$ expression plasmid	d	https://benchling.com/s/seq-vWQZoM900bv37RxQkUx9
KJ174	Kan	ColE1	$BS2_N$-linker-ZA mammalian expression plasmid	e	https://benchling.com/s/seq-5PPiZDoazSDpHu9Bar1f
KJ178	Kan	ColE1	$BS2_N$-linker-FRB mammalian expression plasmid	e	https://benchling.com/s/seq-WupOwWWn4LOeeWLtYEIm
KJ242	Kan	ColE1	$BS2_N$-linker-ABI mammalian expression plasmid	e	https://benchling.com/s/seq-M4jnRrFGcwar5tktRqbG
KJ207	Kan	ColE1	$BS2_N$-linker-deadBID mammalian expression plasmid	e	https://benchling.com/s/seq-1ywROLLeq1bu9D5DLUmM
KJ206	Kan	ColE1	$BS2_N$-linker-tBID mammalian expression plasmid	e	https://benchling.com/s/seq-wdtMHBWIkHn99fPFkojE

KJ208	Kan	ColE1	$BS2_N$-linker-NOXA mammalian expression plasmid	e	https://benchling.com/s/seq-atRLE3HszeiAygxcU85K
KJ176	Kan	ColE1	ZB-linker-$BS2_C$ mammalian expression plasmid	f	https://benchling.com/s/seq-hEK3wVLVoViAdCftONWM
KJ180	Kan	ColE1	FKBP-linker-$BS2_C$ mammalian expression plasmid	f	https://benchling.com/s/seq-itvF3x0l32B9uJ2VNQ5I
KJ243	Kan	ColE1	PYL-linker-$BS2_C$ mammalian expression plasmid	f	https://benchling.com/s/seq-ehAHfJXs6i7dGOQZuWEF
KJ215	Kan	ColE1	(Bcl-2)-linker-$BS2_C$ mammalian expression plasmid	f	https://benchling.com/s/seq-lr5irLU8aaeW7U634C3R
KJ214	Kan	ColE1	(Mcl-1)-linker-$BS2_C$ mammalian expression plasmid	f	https://benchling.com/s/seq-8NIc418kHjasGUEPFIvV
KJ229	Kan	ColE1	$Nluc_{11S}$-linker-FRB mammalian expression plasmid	g	https://benchling.com/s/seq-W8cks59UP7xELm7YFOoC
KJ231	Kan	ColE1	$Nluc_{11S}$-linker-tBID mammalian expression plasmid	g	https://benchling.com/s/seq-m9YI3wtAWPrvH4jDFCO8
KJ230	Kan	ColE1	FKBP-linker-$Nluc_{114}$ mammalian expression plasmid	h	https://benchling.com/s/seq-XYXW8jXkpNhvd6r9Fve0
KJ232	Kan	ColE1	(Bcl-2)-linker-$Nluc_{114}$ mammalian expression plasmid	h	https://benchling.com/s/seq-6PsH8QWPYnbz0qYTkzoN
KJ240	Kan	ColE1	(Mcl-1)-linker-$Nluc_{114}$ mammalian expression plasmid	h	https://benchling.com/s/seq-kXMq76VD3Q7PYt3Llsui
KJ141	Carb	pUC	IgK-BS2-DAF mammalian expression plasmid	i	https://benchling.com/s/seq-P1W4xtabZVduQI07YH0C
KJ182	Carb	pUC	IgK-$BS2_N$-linker-ZA-DAF mammalian expression plasmid	j	https://benchling.com/s/seq-TECKAy2zWpm7cEUcg88P
KJ186	Carb	pUC	IgK-$BS2_N$-linker-FRB-DAF mammalian expression plasmid	j	https://benchling.com/s/seq-m0KkifQj6osisQI7K3Zo
KJ184	Carb	pUC	IgK-ZB-linker-$BS2_C$-DAF mammalian expression plasmid	k	https://benchling.com/s/seq-7IMEIMrznJqcxsiriJZW

KJ248	Carb	pUC	IgK-ZBneg-linker-$BS2_C$-DAF mammalian expression plasmid	k	https://benchling.com/s/seq-L5y9mDosE3A6xzApvMcr
KJ188	Carb	pUC	IgK-FKBP-linker-$BS2_C$-DAF mammalian expression plasmid	k	https://benchling.com/s/seq-PZyMomM636of3SVi9F6E
KJ122	Kan	pBR322	BS2 expression plasmid	l	https://benchling.com/s/seq-Cd1UfTOPGd4vJN4TMYjc

* Vector maps for each construct type shown in Supplementary Figure 1.

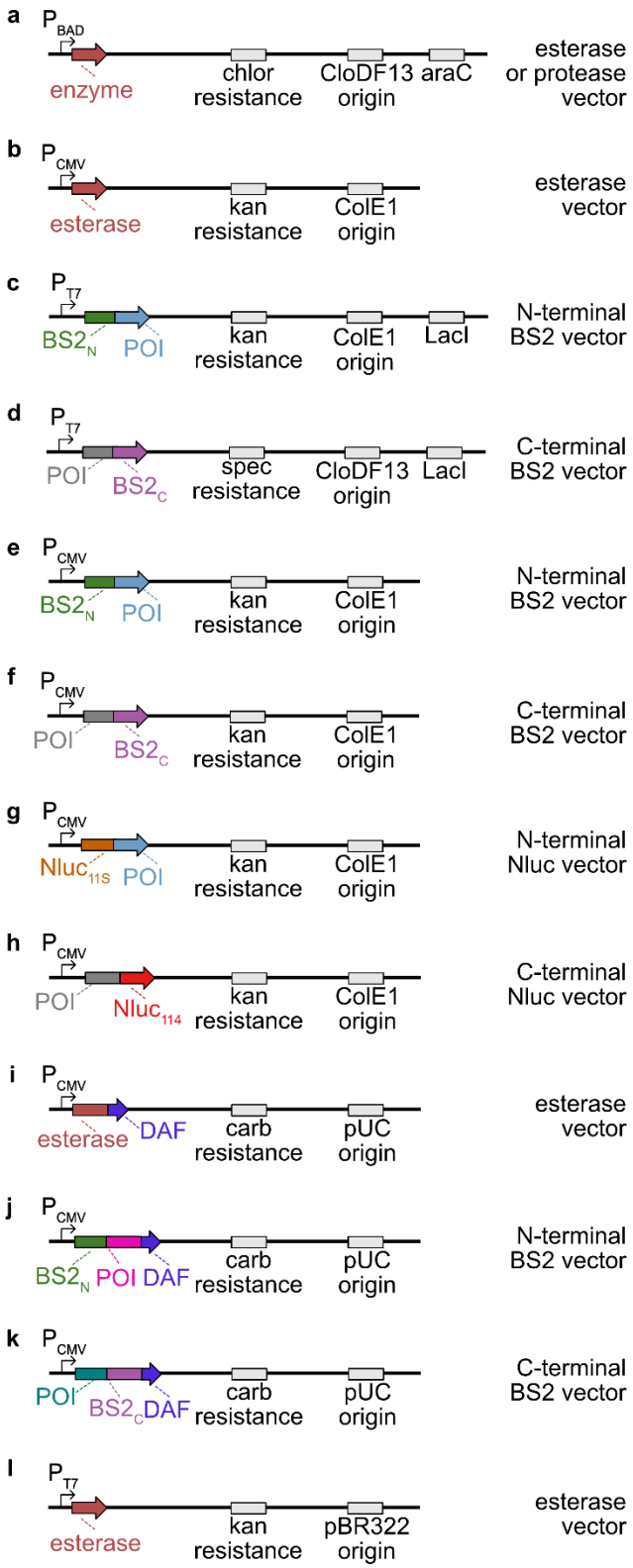


Figure S1. Vector maps for all constructs used in this work. (a-l) Vector maps corresponding to vectors listed in Supplementary Table 1.

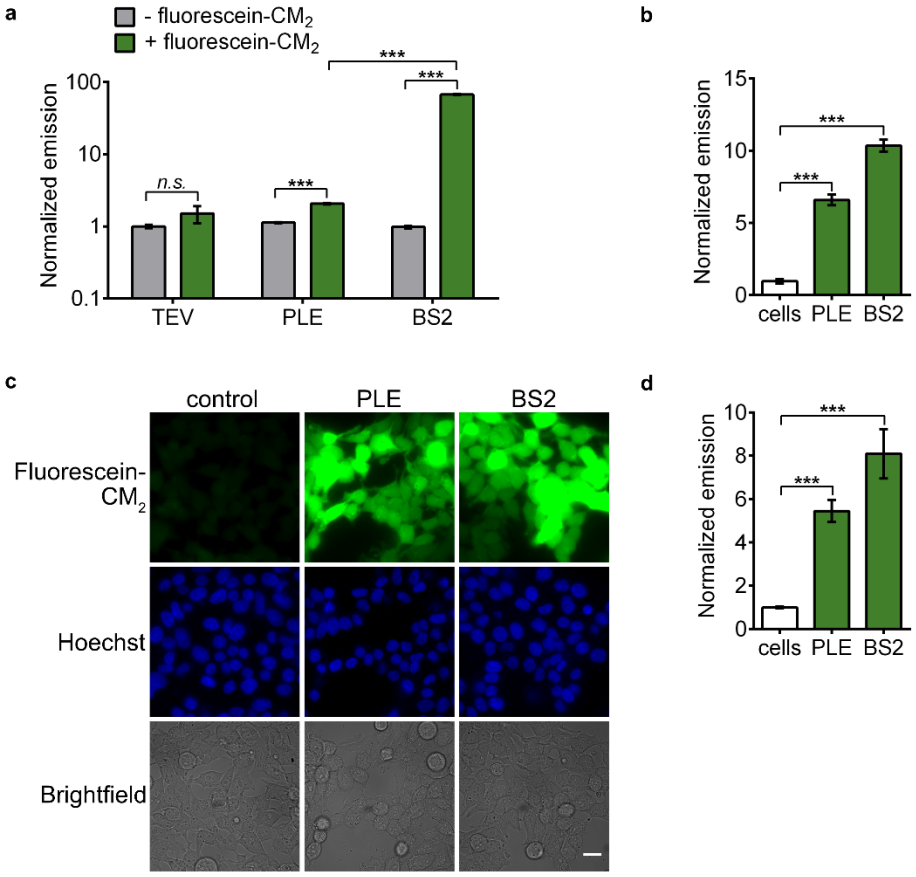


Figure S2. BS2 can efficiently unmask fluorescein-CM$_2$. (**a**) *E. coli* transformed with a control TEV protease, PLE, or BS2 were incubated with IPTG (0.1 mM) in the absence (gray) or presence (green) of fluorescein-CM$_2$ (50 μM) for 4 h. The cells were pelleted and resuspended in PBS prior to imaging. (**b**) HEK293T cells transfected with PLE or BS2 or non-transfected control cells (white) were incubated with fluorescein-CM$_2$ (10 μM) for 1 h (green) and analyzed by plate reader. (**c-d**) HEK293T cells were transfected and treated with fluorescein-CM$_2$ as in (b), loaded with Hoechst 33342 (1 μM) and analyzed by (c) high resolution or (d) low resolution fluorescence microscopy for quantification. HEK293T control cells (white) were similarly imaged. 20 μm scale bars shown. Error bars are the standard deviation for n = 4 replicates. Unpaired t-test; ***P < 0.0001.

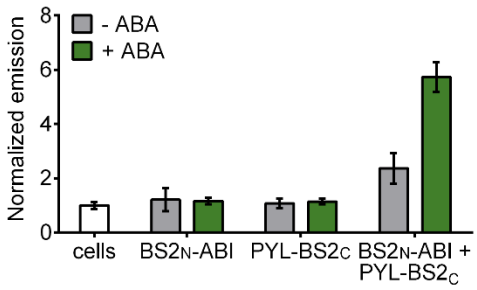


Figure S3. Validation of lead cut site with abscisic acid-inducible dimerization system. *E. coli* transformed with $BS2_N$-fused ABI, PYL-fused $BS2_c$ or both were cultured with IPTG (0.1 mM) in the absence (gray) or presence (green) of ABA with fluorescein-CM_2 (500 μM) for 4 h. The cells were then centrifuged and the pellets were resuspended in PBS prior to imaging. Error bars are the standard deviation for n = 4 replicates.

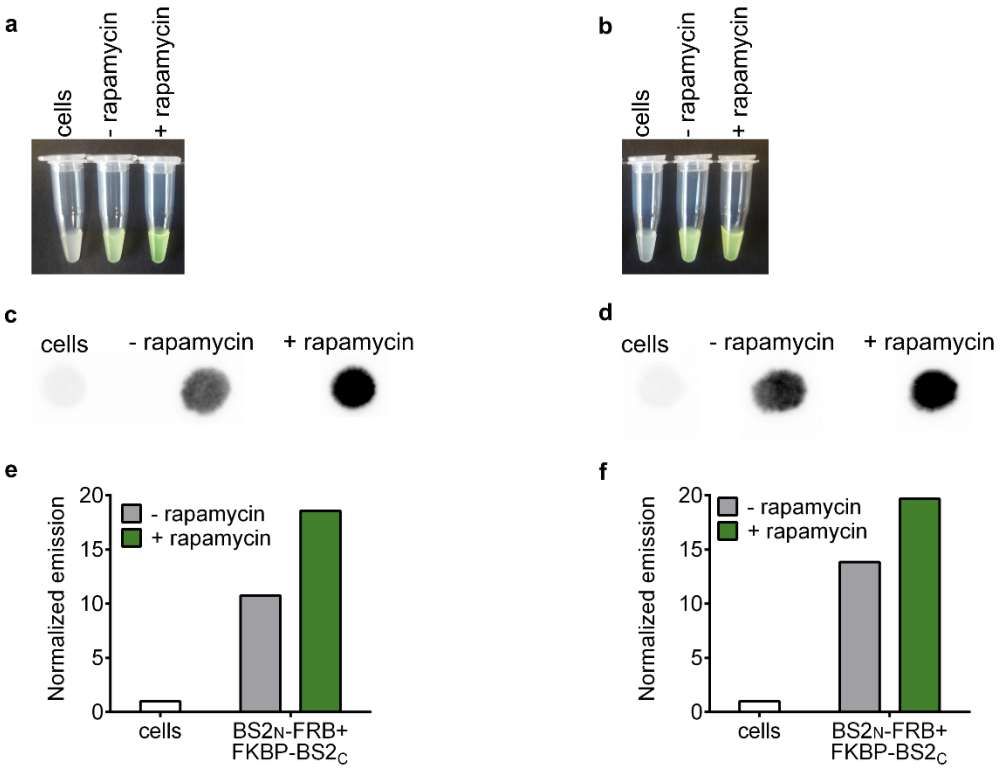


Figure S4. Imaging *E. coli* cell lysate from rapamycin-induced split esterase system. (**a-b**) *E. coli* cells cotransformed with $BS2_N$-fused FRB and FKBP-fused $BS2_C$ or control BL21 cells were induced for 16 h with IPTG (0.1 mM) in the absence and presence of rapamycin (20 μM). The cultures were centrifuged and the pellets were (a) lysed immediately after collection or (b) stored at -20 °C for 5 d prior to lysis. Fluorescein-CM_2 (500 μM) was added to the lysate and incubated for 30 minutes at room temperature prior to imaging with a cell phone camera. (**c-d**) The lysate reaction mixtures from (a-b) were spotted on filter paper and analyzed on a gel imaging system. (**e**) Quantification of the images in (c). (**f**) Quantification of the images in (d).

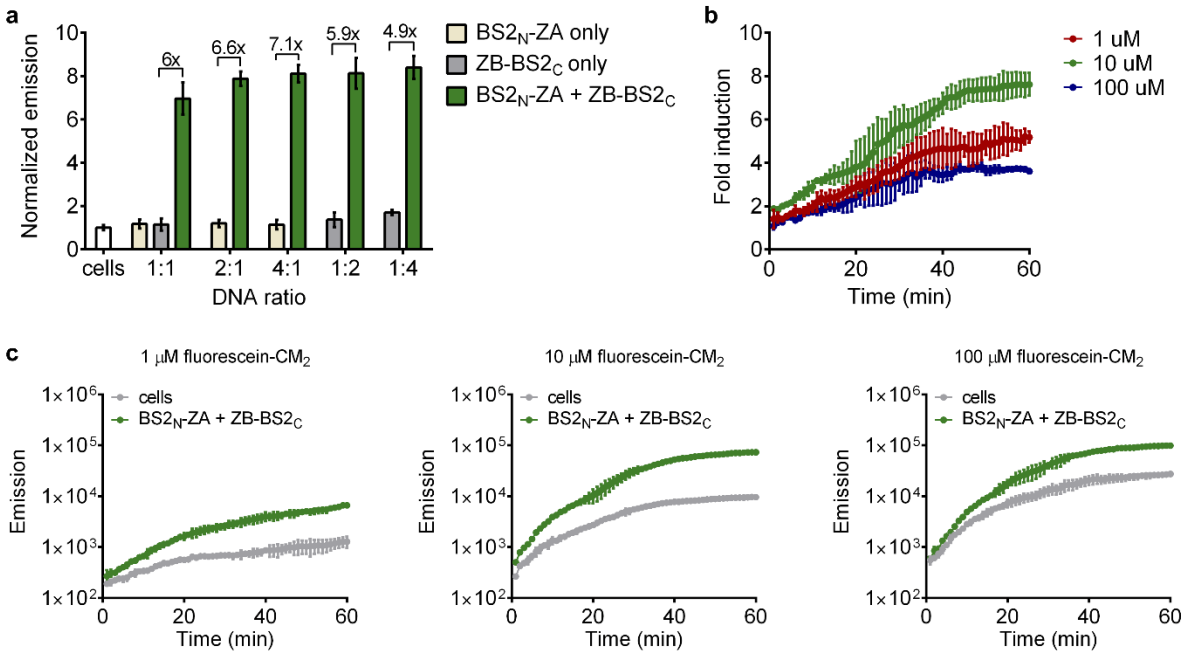


Figure S5. Optimization of split esterase in mammalian cells. (**a**) HEK293T cells were transfected with $BS2_N$-fused ZA (tan), ZB-$BS2_C$ (gray), or cotransfected with both (green) at varying ratios. The transfected cells and control HEK293T cells (white) were incubated with fluorescein-CM_2 (10 μM) and analyzed for fluorescence. (**b**) HEK293T cells cotransfected with $BS2_N$-ZA and ZB-$BS2_C$ (4:1 ratio) or control HEK293T cells were incubated with fluorescein-CM_2 (1-100 μM) and analyzed for fluorescence over time. The fold inductions in emission for cotransfected cells versus control cells are plotted. (**c**) Raw emission values from (b). Error bars are the standard deviation for *n* = 4 replicates.

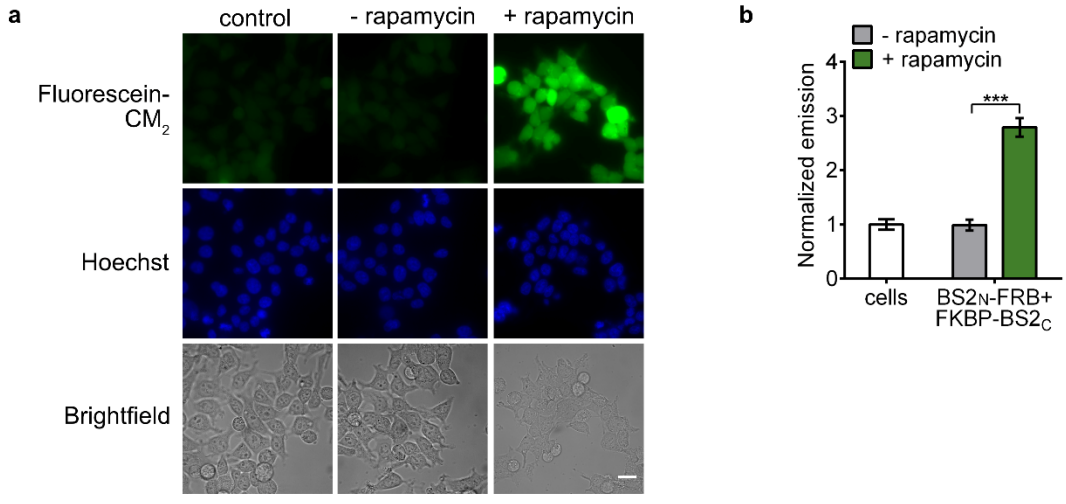


Figure S6. PPI-dependent esterase assembly with rapamycin-inducible dimerization system. (**a**) Complete imaging series from Figure 2b. HEK293T cells cotransfected with BSN_2-fused FRB and FKBP-fused $BS2_c$ were incubated with a DMSO carrier control or rapamycin (400

nM) for 24 h. The cells and control HEK293T cells were loaded with Hoechst 33342 (1 μM), incubated with fluorescein-CM_2 (10 μM), and analyzed by high resolution fluorescence microscopy. (**b**) HEK293T cells cotransfected and treated identically to conditions in (a) were analyzed by low resolution fluorescence microscopy and quantified. 20 μm scale bar shown. Error bars are the standard deviation for n = 4 replicates. Unpaired t-test; ***P < 0.0001.

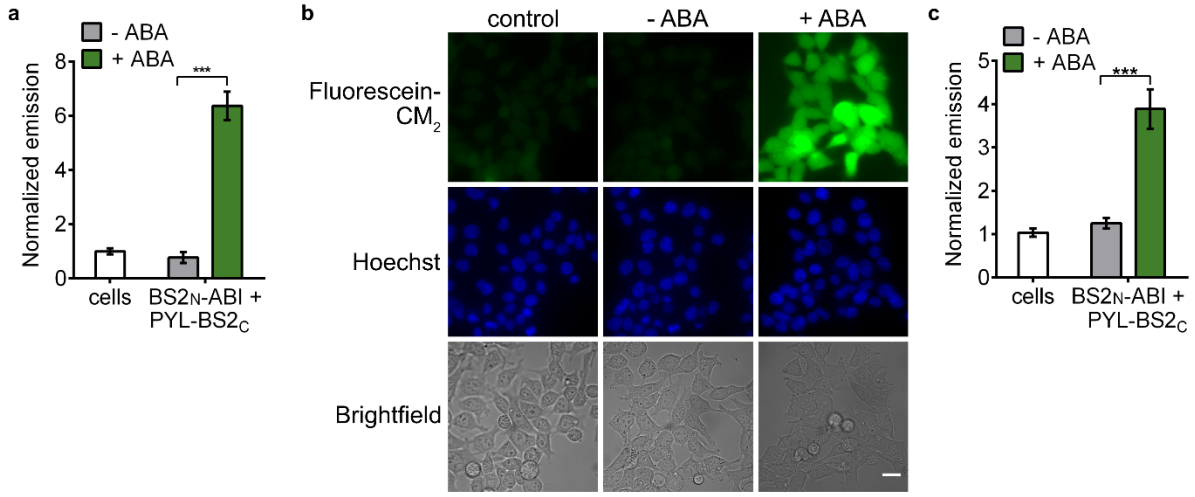


Figure S7. PPI-dependent esterase assembly with ABA-inducible dimerization system. (**a**) HEK293T cells cotransfected with $BS2_N$-fused ABI and PYL-fused $BS2_c$ were incubated with a DMSO carrier control (gray) or ABA (10 μM, green) for 24 h. Fluorescein-CM_2 (10 μM) was added to the cells and control HEK293T cells (white) for 1 h and analyzed by plate reader for fluorescence. (**b**) HEK293T cells cotransfected and treated identically to conditions in (a) were loaded with Hoechst 33342 (1 μM) and analyzed by high resolution fluorescence microscopy. (**c**) HEK293T cells cotransfected and treated identically to conditions in (b) were analyzed by low resolution fluorescence microscopy and quantified. 20 μm scale bar shown. Error bars are the standard deviation for n = 4 replicates. Unpaired t-test; ***P < 0.0001.

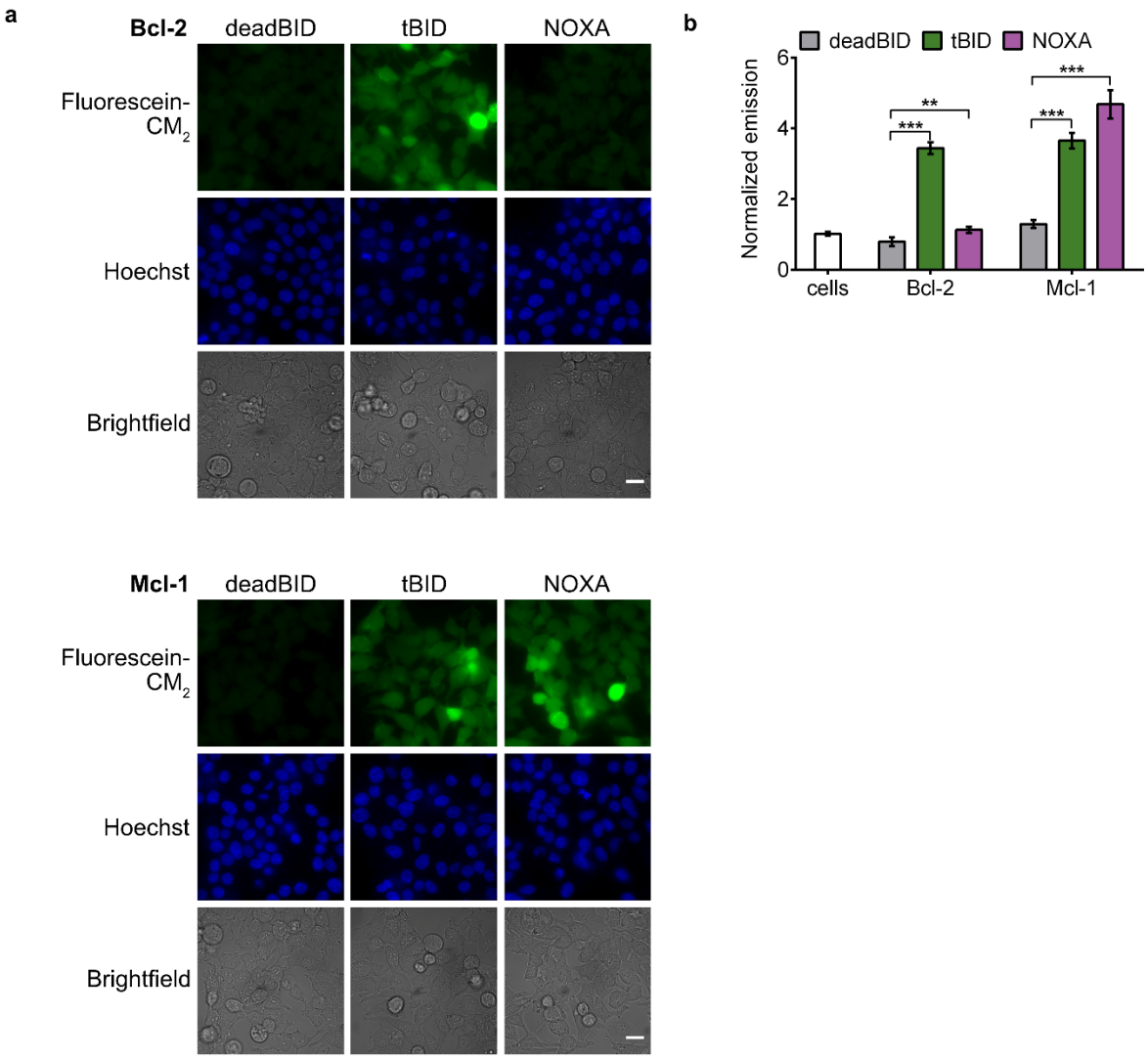


Figure S8. Measuring therapeutically relevant PPIs with split esterase in mammalian cells. (**a**) Complete imaging series from Figure 3c. HEK293T cells cotransfected with the plasmids shown in Figure 3a with $BS2_N$-fused BH3-only protein and Bcl-2-fused $BS2_c$ (top) or Mcl-1-fused $BS2_c$ (bottom). The cells and control HEK293T cells were loaded with Hoechst 33342 (1 μM), incubated with fluorescein-CM_2 (10 μM), and analyzed by high resolution fluorescence microscopy. (**b**) HEK293T cells cotransfected and treated identically to conditions in (a) were analyzed by low resolution fluorescence microscopy and quantified. 20 μm scale bar shown. Error bars are the standard deviation for n = 4 replicates. Unpaired t-test; $^{**}P < 0.001$, $^{***}P < 0.0001$.

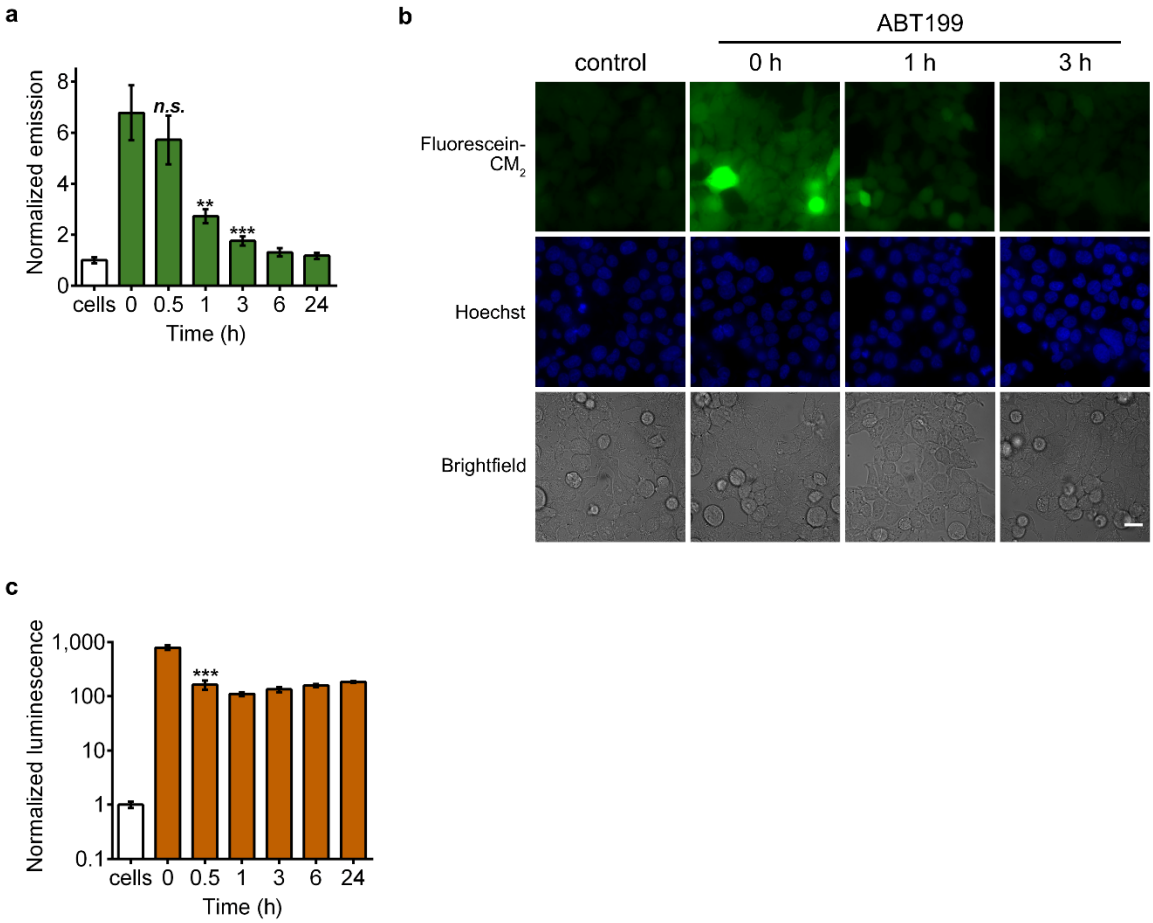


Figure S9. Monitoring pharmacological engagement of PPI inhibitors. (**a**) Complete inhibitor series analyzed by plate reader from Figure 3d. (**b**) HEK293T cells contransfected with $BS2_N$-fused tBID and Bcl-2-fused $BS2_C$ were incubated with a DMSO carrier (0 h) or ABT-199 (1 μM) over time. The cells and control HEK293T cells were loaded with Hoechst 33342 (1 μM), incubated with fluorescein-CM_2 (10 μM), and analyzed by high resolution fluorescence microscopy. (**c**) HEK293T cells cotransfected with $Nluc_{11S}$-fused tBID and Bcl-2-fused $Nluc_{114}$ were incubated with a DMSO carrier (0 h) or ABT-199 (1 μM) over time. The cells (orange) or control HEK293T cells (white) were incubated with furimazine and analzyed for bioluminescence. 20 μm scale bar shown. Error bars are the standard deviation for $n = 4$ replicates. Unpaired t-test; $^{***}P < 0.0001$.

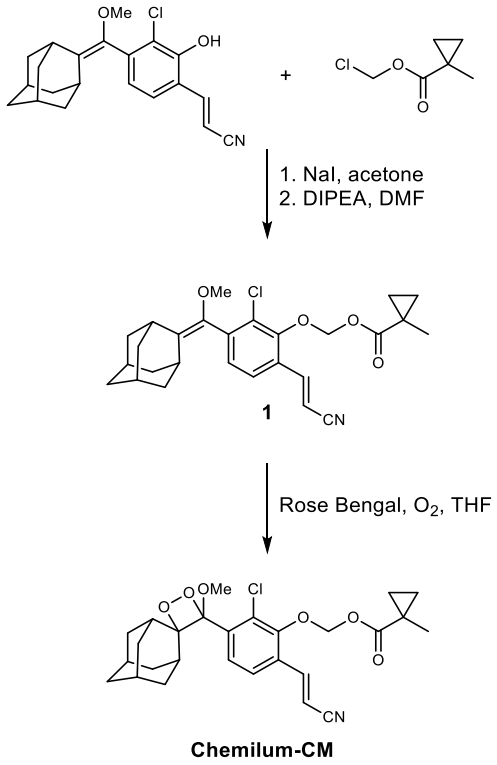


Figure S10: Synthetic scheme of Chemilum-CM.

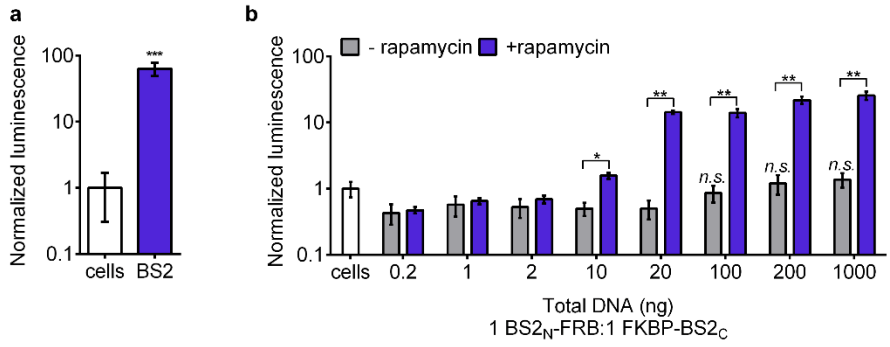


Figure S11. Cell surface localized esterase. (**a**) HEK293T cells transfected with GPI-anchored full length-BS2 esterase (blue) or HEK293T control cells (white) were incubated with Chemilum-CM_2 (10 μM) and analyzed for luminescence. (**b**) HEK293T cells co-transfected with $BS2_N$-fused FRB and FKBP-fused $BS2_C$ with GPI anchors were treated with a DMSO carrier control (gray) or rapamycin (blue) for 24 h. The cells or HEK293T control cells (white) were incubated with Chemilum-CM (10 μM) and analyzed for luminescence. Error bars are the standard deviation for $n = 4$ replicates. Unpaired t-test; *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$.

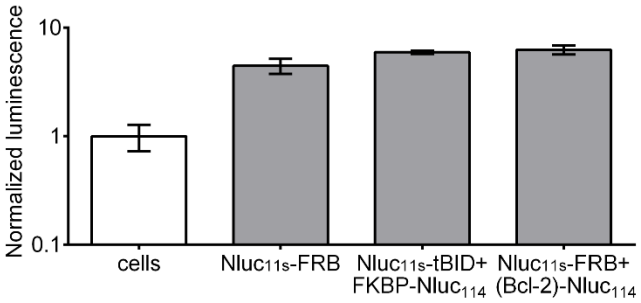


Figure S12. Imaging of Nanobit controls. HEK293T cells were transfected with $Nluc_{11S}$-fused FRB or cotransfected with $Nluc_{11S}$-fused tBID and FKBP-fused $Nluc_{114}$ or $Nluc_{11S}$-fused FRB and Bcl-2-fused $Nluc_{114}$. The cells (gray) or HEK293T control cells (white) were incubated with furimazine and analyzed for bioluminescence. Error bars are the standard deviation for n = 4 replicates.

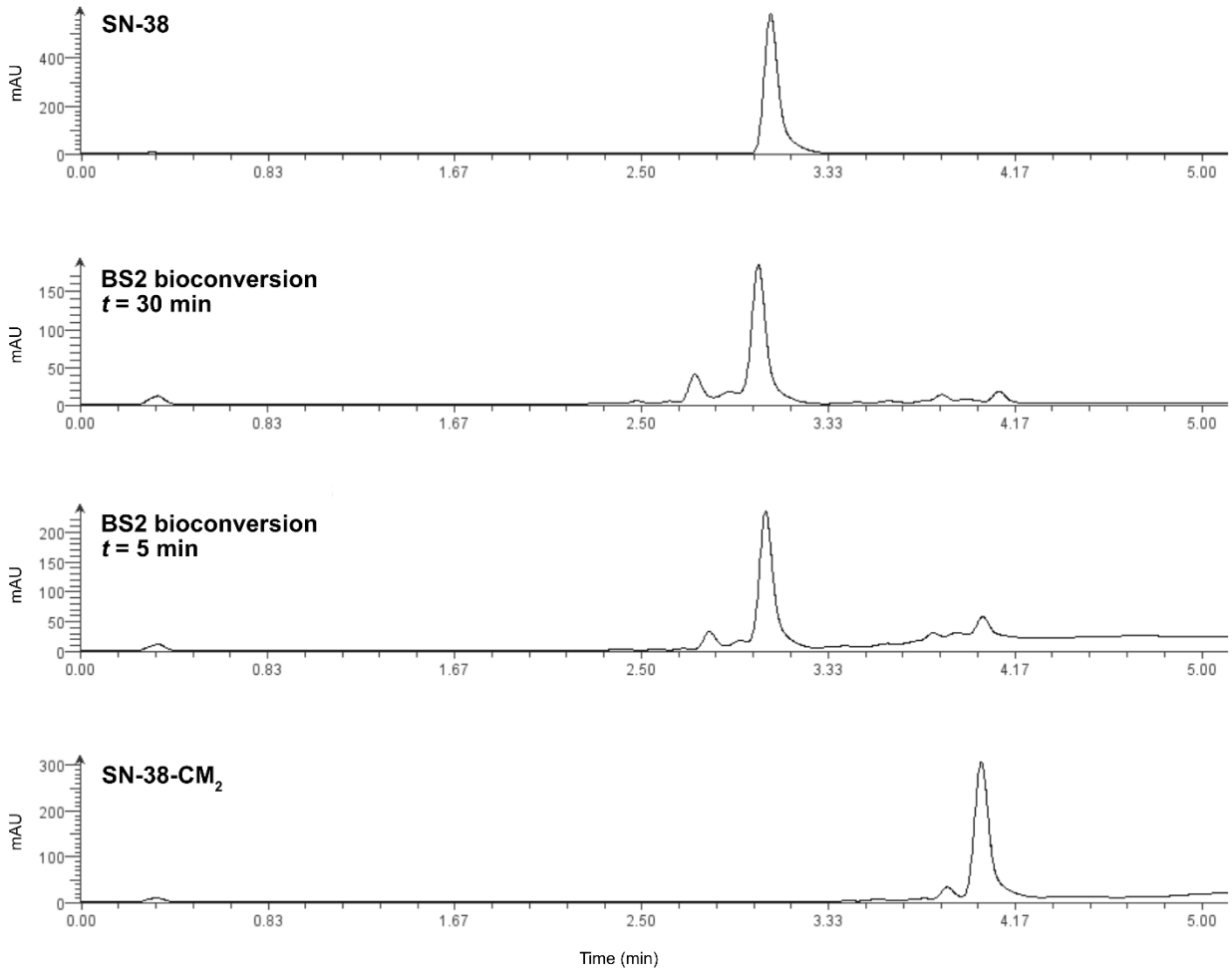


Figure S13. Bioconversion of SN-38-CM_2 to SN-38 using BS2. Reaction of SN-38-CM_2 and BS2 monitored by LC. LC traces showing elution times for bioconversion of SN-38-CM_2 to SN-38 using BS2, SN-38-CM_2, and the commercially available SN-38.

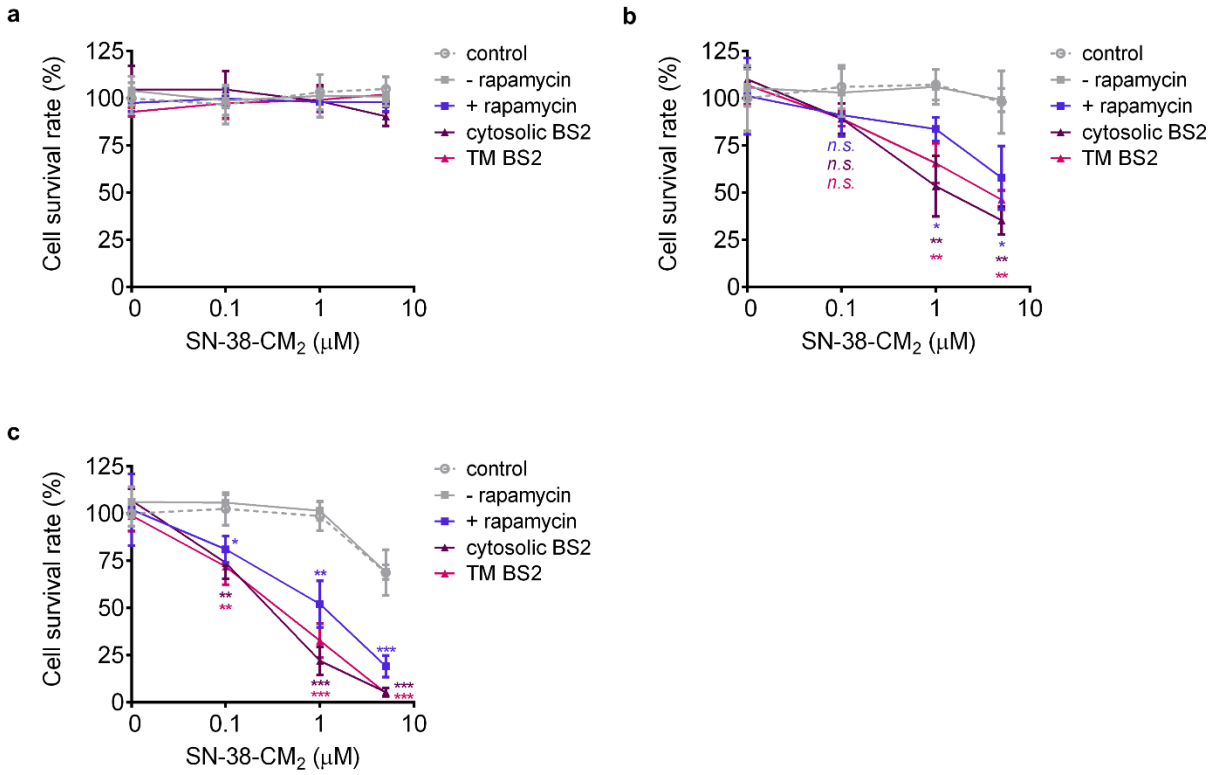


Figure S14. Monitoring esterase mediated cytotoxicity in mammalian cells over time. MDA-MB-231 luciferase cells were transfected with GPI anchored $BS2_N$-fused FRB and FKBP-fused $BS2_C$, cytosolic full length BS2 (purple), or GPI anchored full length BS2 (pink). $BS2_N$-fused FRB and FKBP-fused $BS2_C$ cells were treated with a DMSO carrier control (gray) or rapamycin (blue) for 12 h. The transfected cells or control cells (gray circle, dashed) were then incubated with SN-38-CM_2 for (**a**) 1 h, (**b**) 3 h, or (**c**) 6 h. The cells were then rinsed and cultured for 40 h prior to addition of D-luciferin and imaging. Error bars are the standard deviation for n = 4 replicates. Unpaired t-test; *P < 0.01, **P < 0.001, ***P < 0.0001.

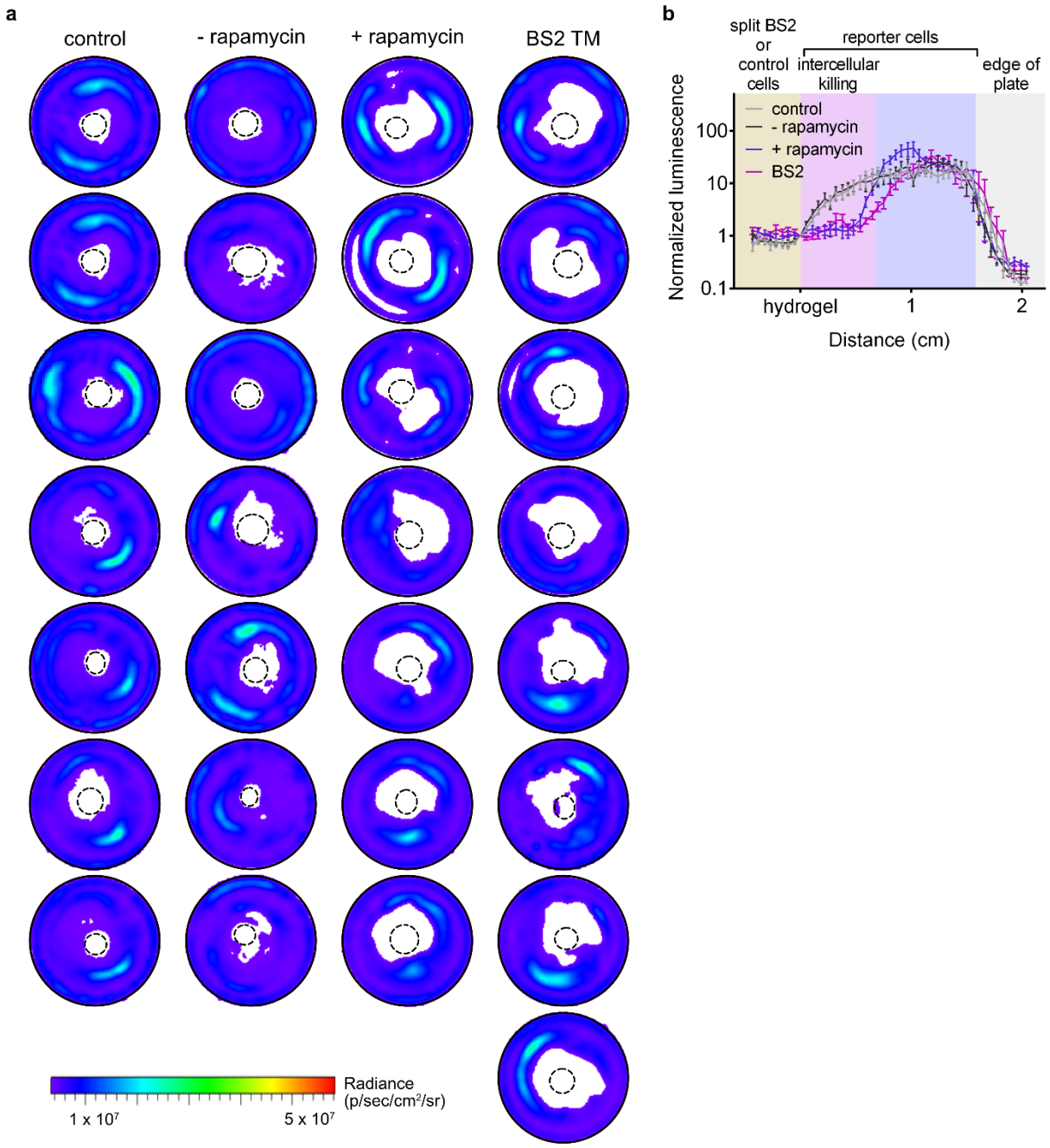


Figure S15. Coculture assays. (**a**) Complete coculture cytoxicity assays from Figure 5e and with GPI anchored BS2 expressing MDA-MB-231 cells. (**b**)Quantification of light emission from bioluminescence images of cocultures. Photon counts along a 2 cm line in the direction of most observed cell killing from the hydrogel embedded cells (hydrogel) to the edge of each dish are plotted. Error bars are the standard error of the mean for *n* = 8 replicates.

	$BS2_N$	**$BS2_c$**
Fusion proteins	FRB, ABI +/- MBP	FKBP, PYL +/- MBP
Induction conditions	50 – 500 µM IPTG	50 – 500 µM IPTG
Expression temperature	16, 30, 37 °C	16, 30, 37 °C
Expression length	8 – 18 h	8 – 18 h
Media conditions	LB, 2xYT, Autoinduction media	LB, 2xYT, Autoinduction media

Figure S16. Preliminary conditions for split BS2 expression. Initial conditions tested for expression of split BS2 in *E. coli*.

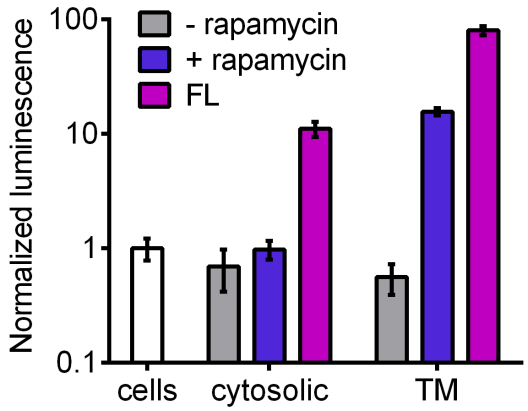


Figure S17. HEK293T cells were transfected with cytosolic or GPI-anchored full length-BS2 esterase (pink) or co-transfected with cytosolic or GPI-anchored $BS2_N$-fused FRB and FKBP-fused $BS2_C$. Split esterase expressing cells were treated with a DMSO carrier control (gray) or rapamycin (blue) for 24 h. The cells or HEK293T control cells (white) were incubated with Chemilum-CM_2 (10 µM) and analyzed for luminescence.

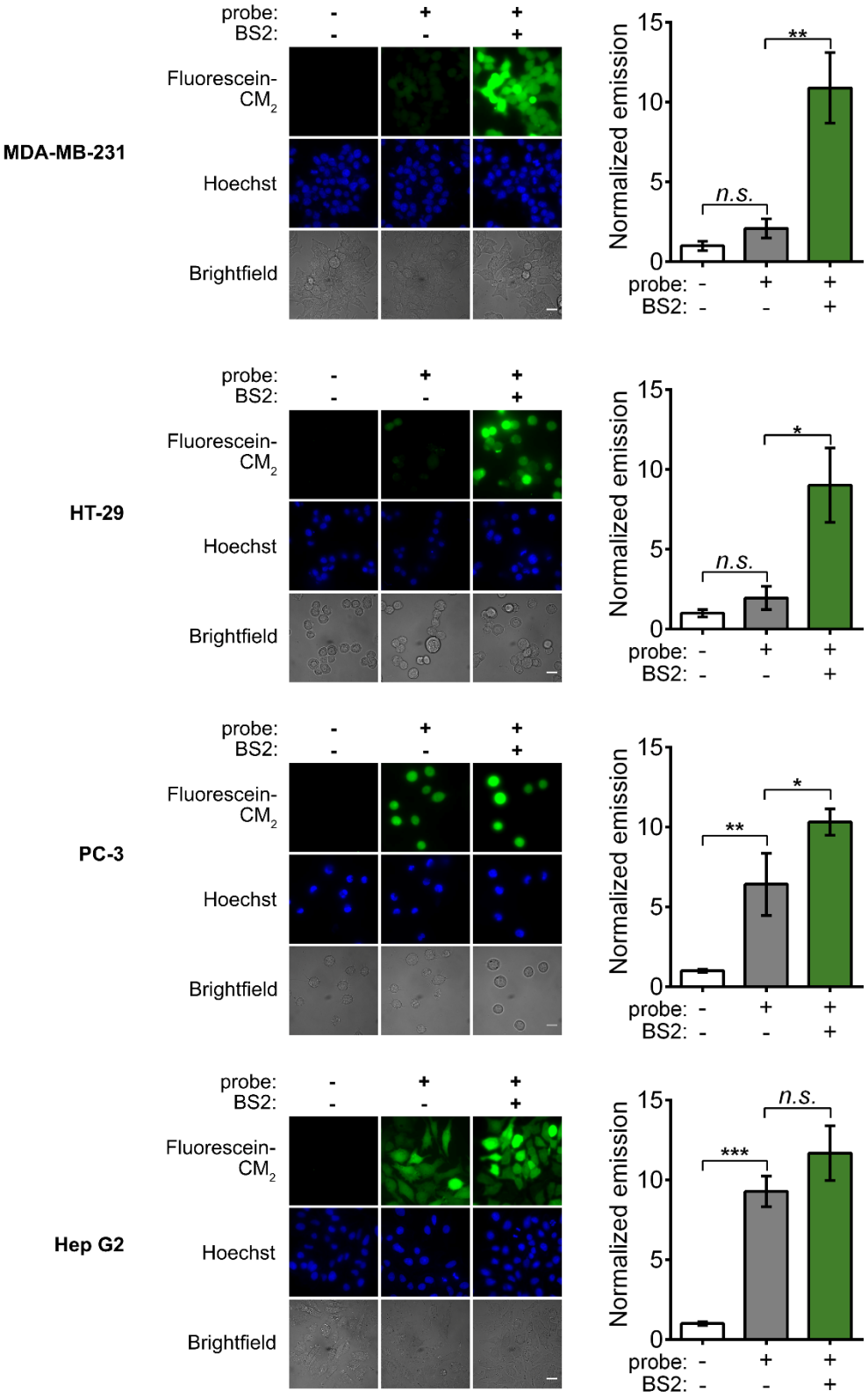


Figure S18. MDA-MB-231, HT-29, PC-3, and Hep G2 cells were transfected with BS2. The cells and non-transfected cells were loaded with Hoechst 33342 (1 μM), incubated in the presence and absence of fluorescein-CM_2 (10 μM), and analyzed by high resolution fluorescence microscopy

(left) or plate reader (right) for emission. 20 μm scale bars shown. Error bars are the standard deviation for $n = 4$ replicates. Unpaired t-test; *$P < 0.01$, **$P < 0.001$ ***$P < 0.0001$.

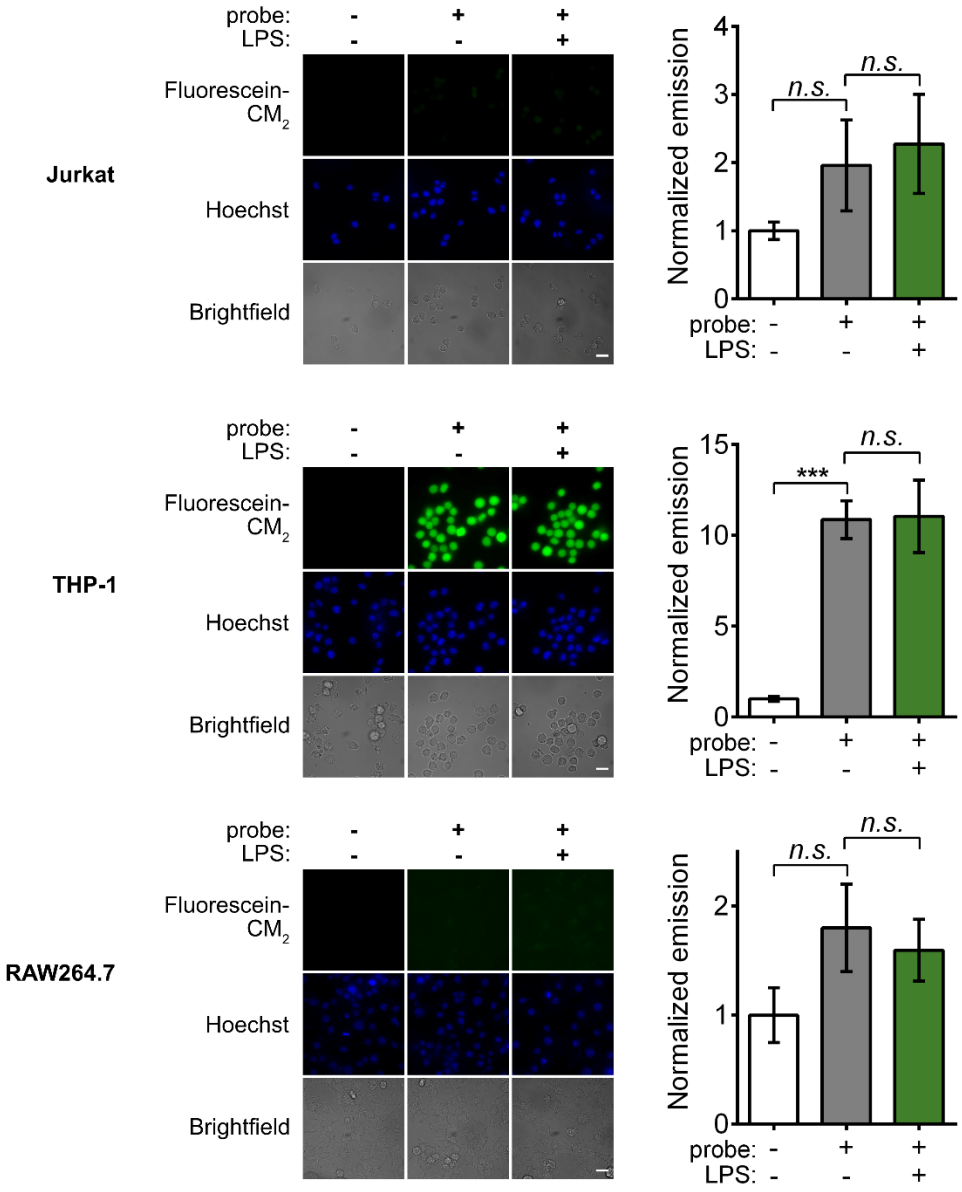
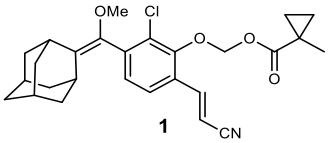


Figure S19. Jurkat, THP-1, and RAW264.7 cells were cultured in the presence or absence of LPS for 3-18 h. Media was replaced with Hoechst 33342 (1 μM) in the presence or absence of fluorescein-CM_2 and analyzed by high resolution fluorescence microscopy (left) or plate reader (right) for emission. 20 μm scale bars shown. Error bars are the standard deviation for $n = 4$ replicates. Unpaired t-test; *$P < 0.01$, **$P < 0.001$ ***$P < 0.0001$.

Supplementary Note 1. Chemical characterization data for 1.



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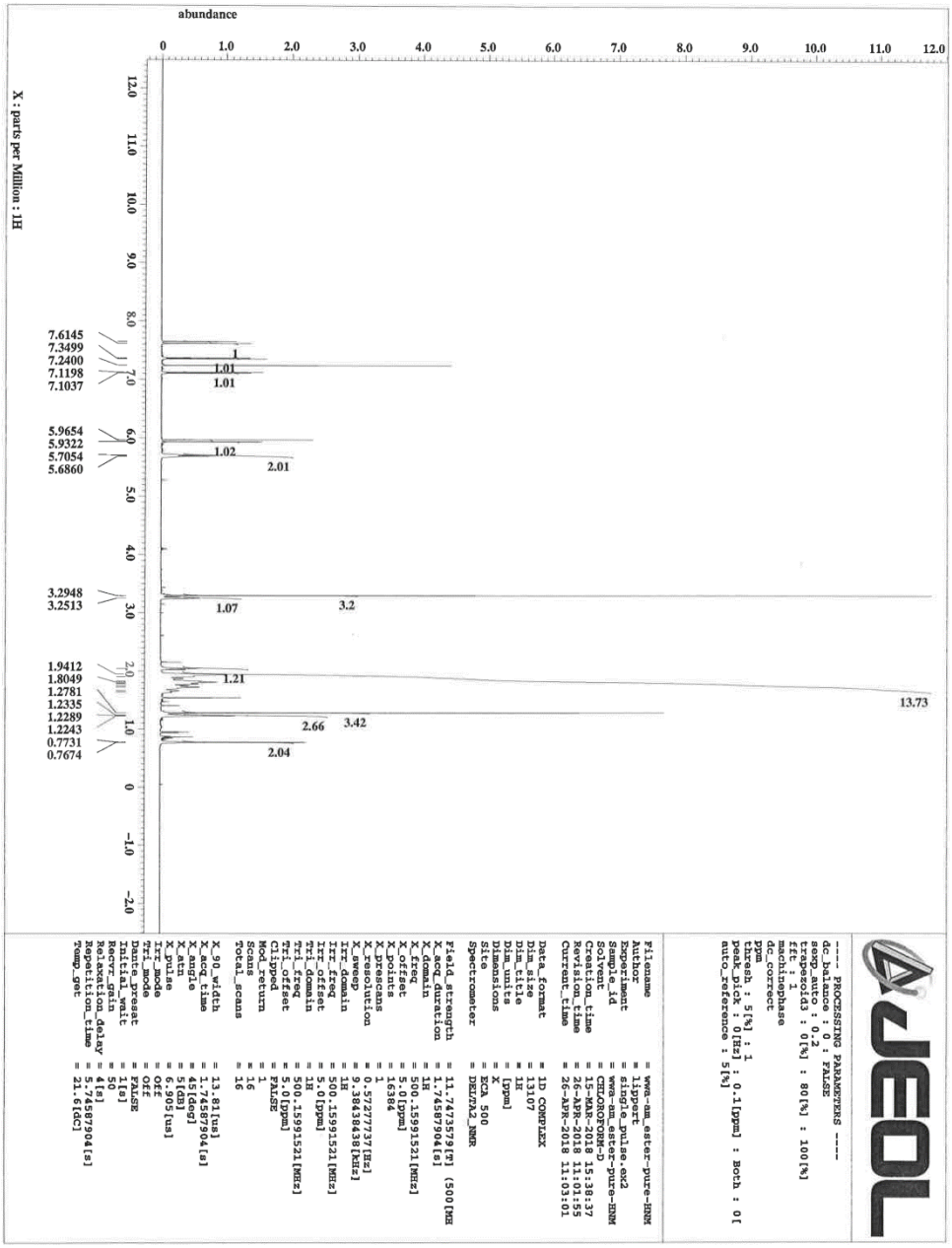
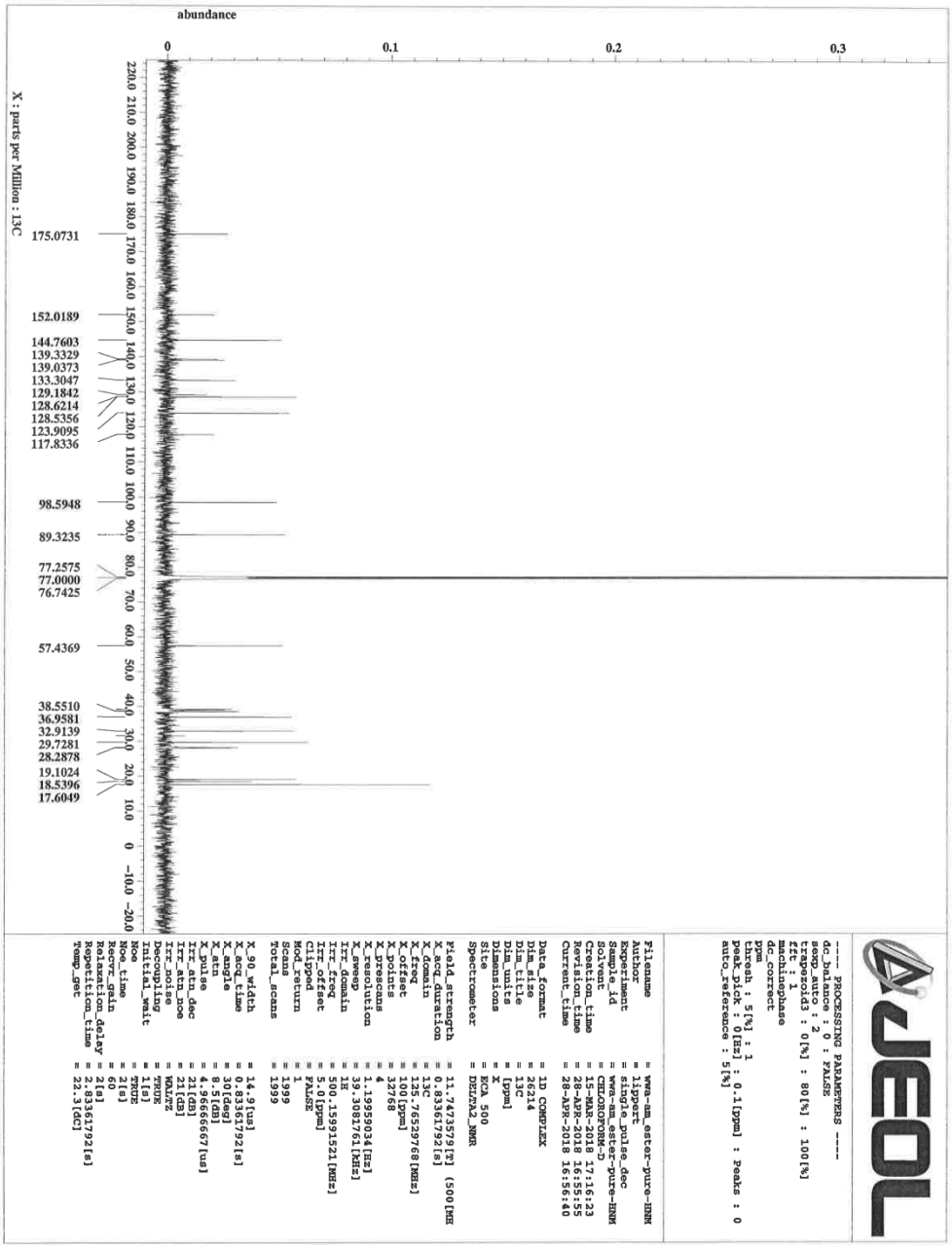


^{1}H NMR of 1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JEOL
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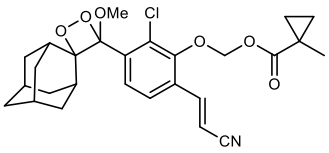
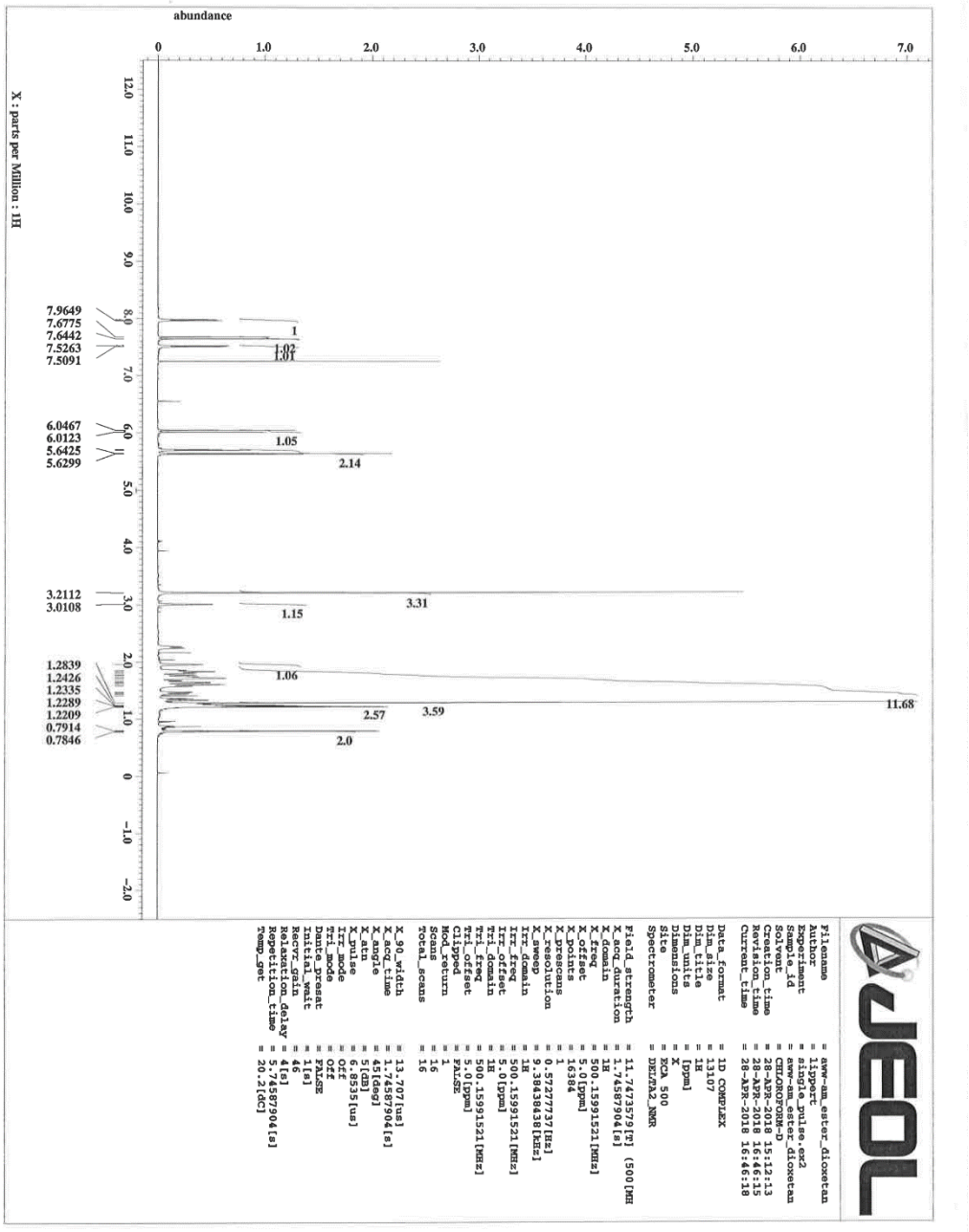
^{13}C NMR of 1

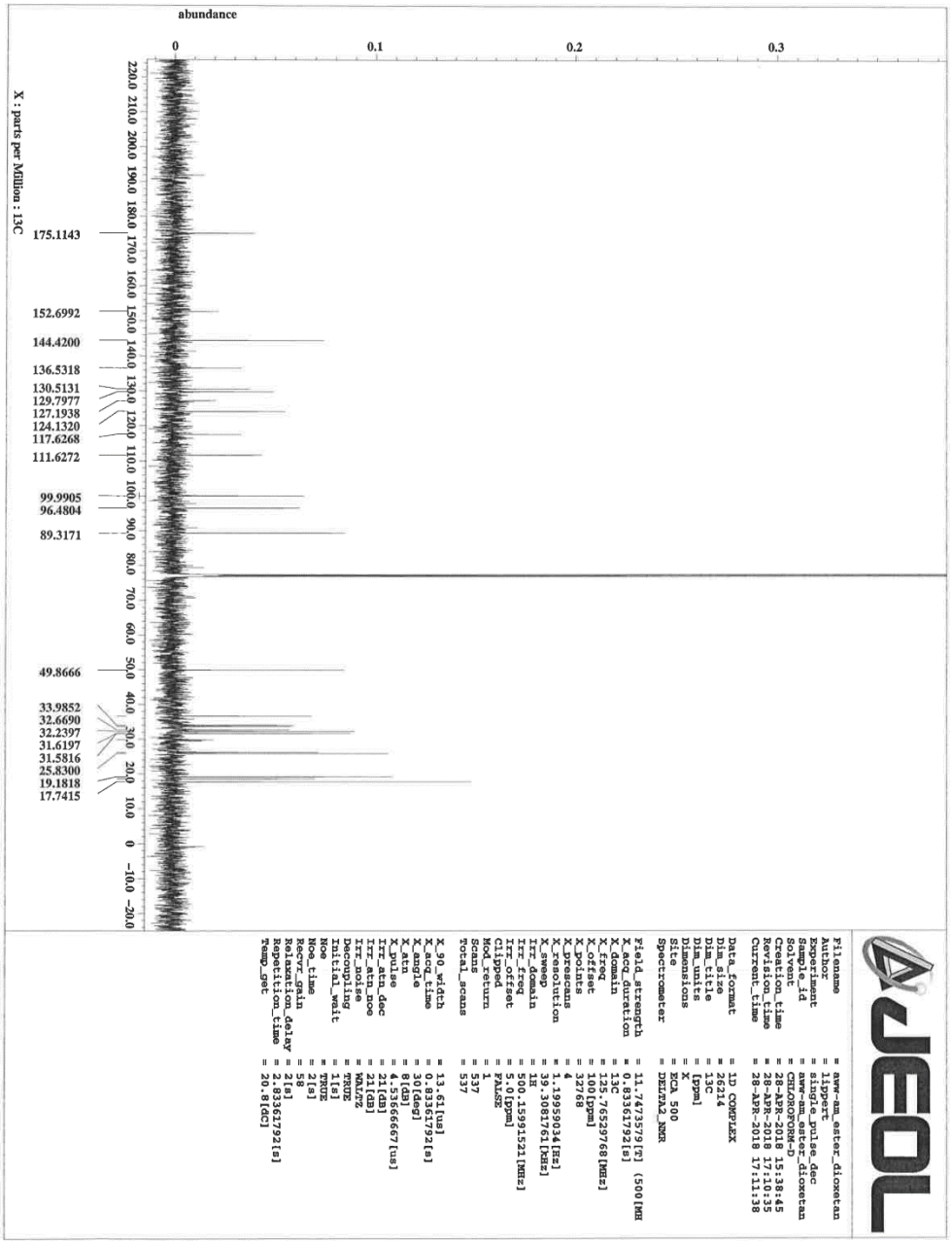
Supplementary Note 2. Chemical characterization data for Chemilum-CM.



Chemilum-CM

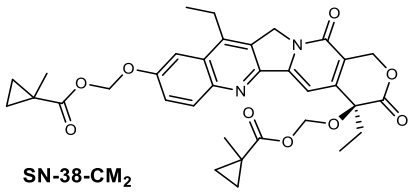


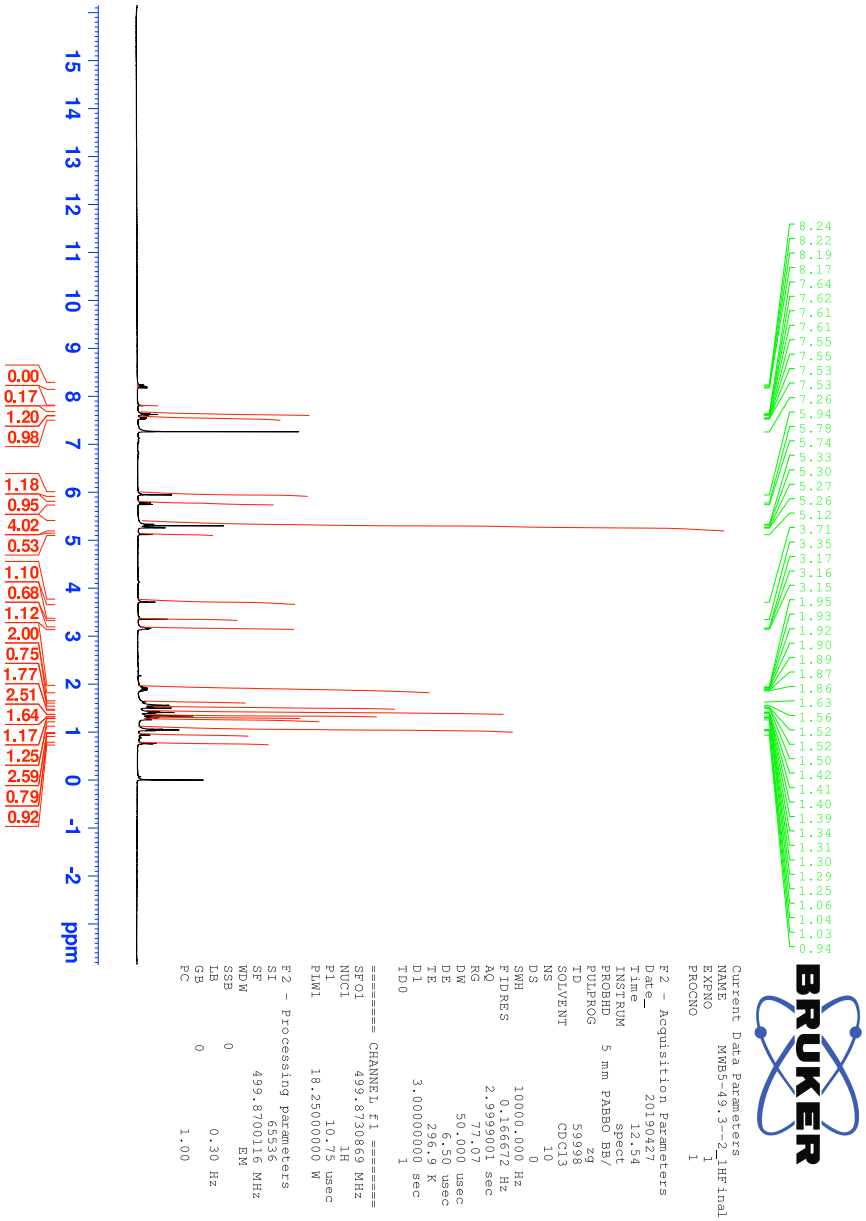
^{1}H NMR of Chemilum-CM

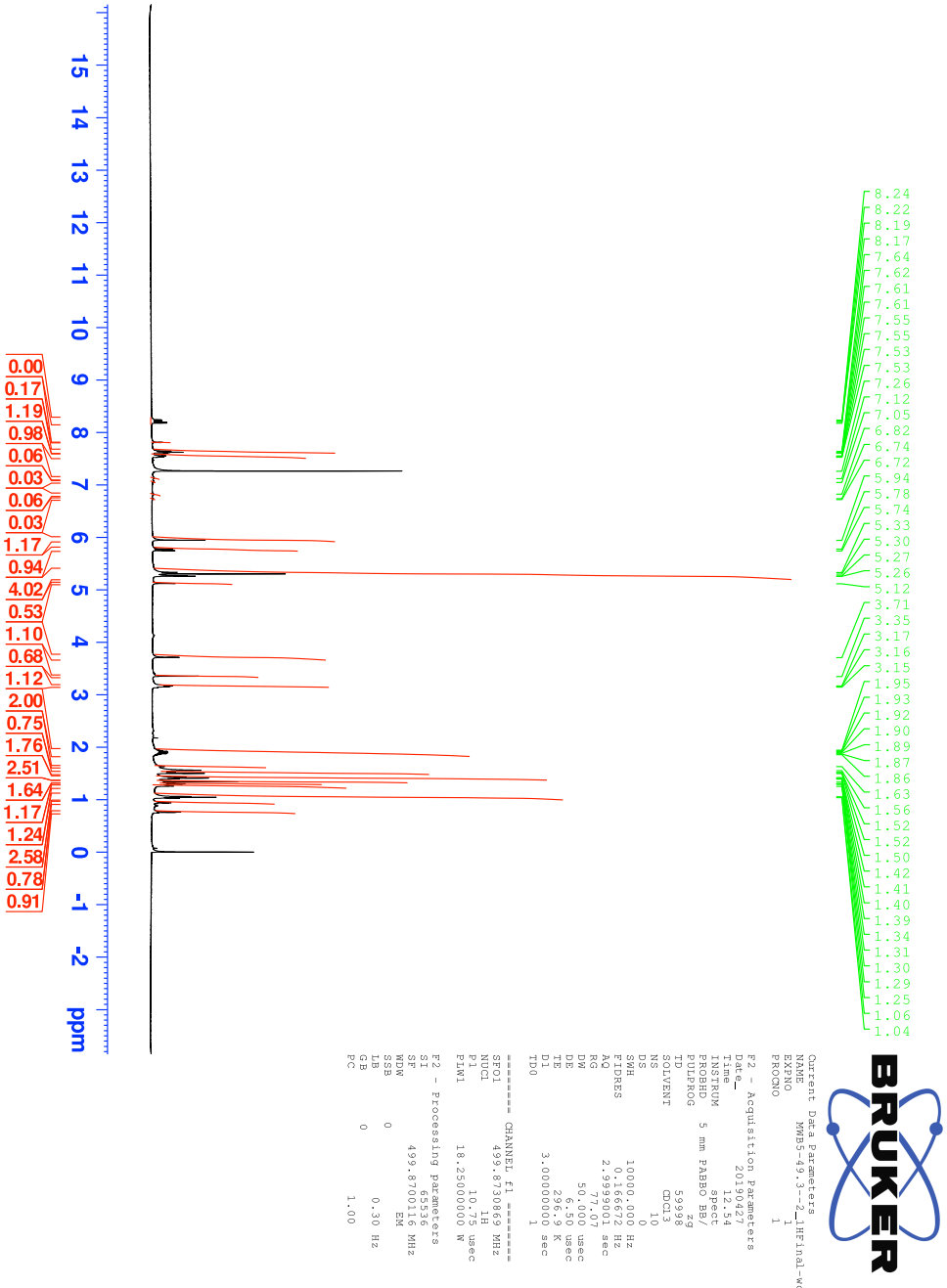


^{13}C NMR of Chemilum-CM

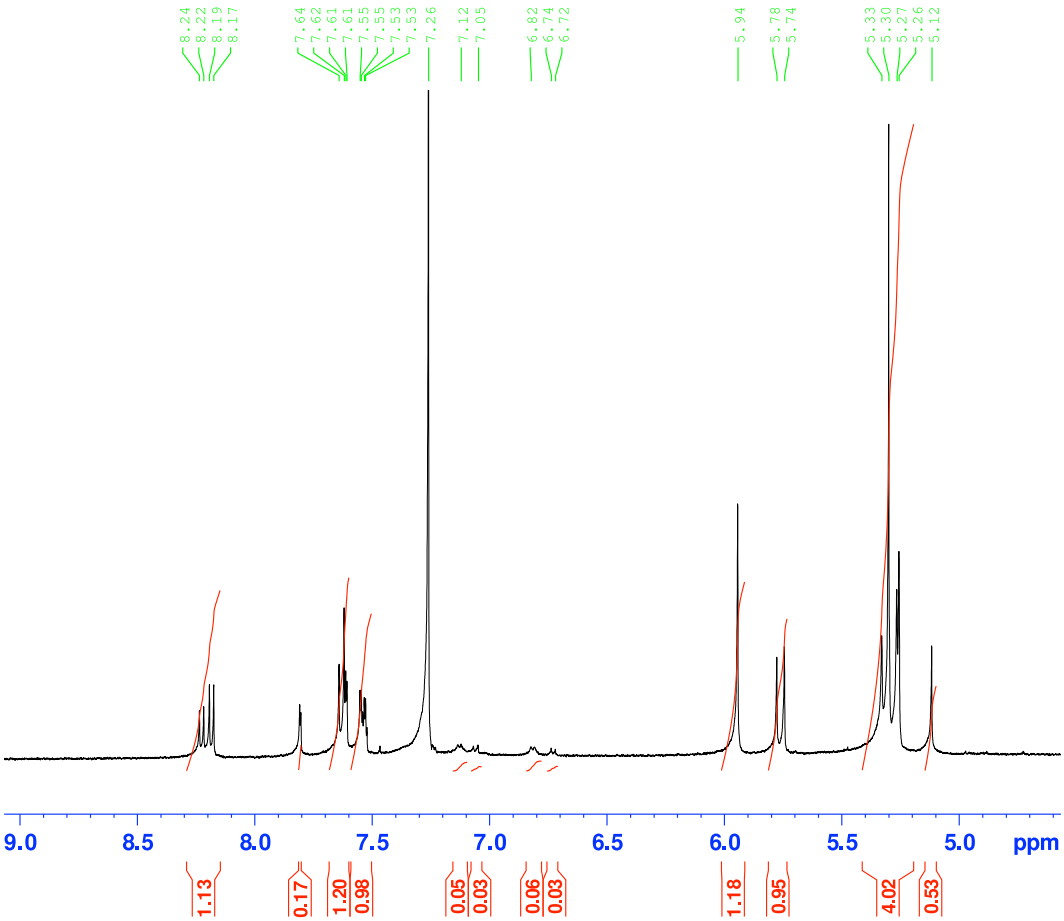
Supplementary Note 3. Chemical characterization data for SN-38-CM$_2$.



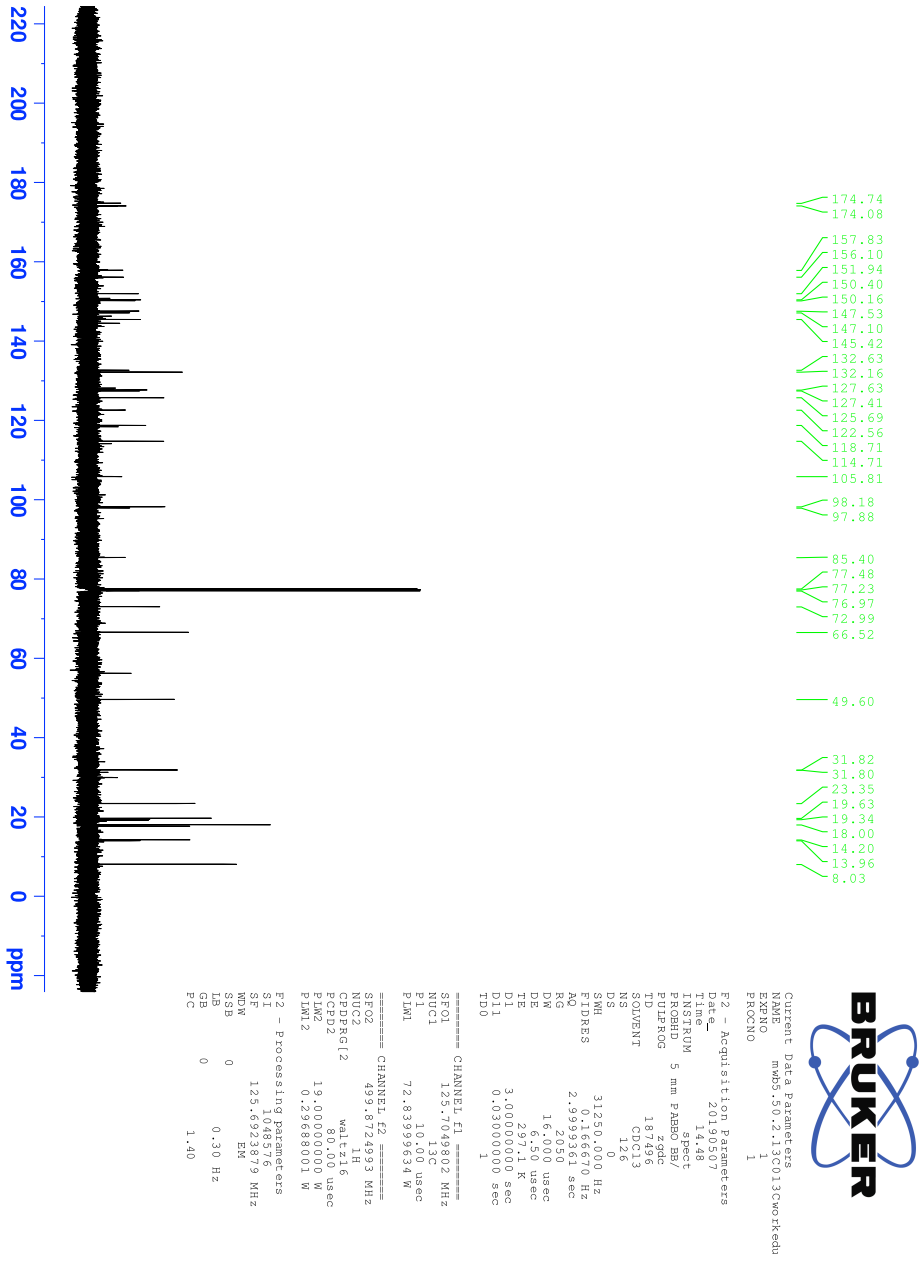




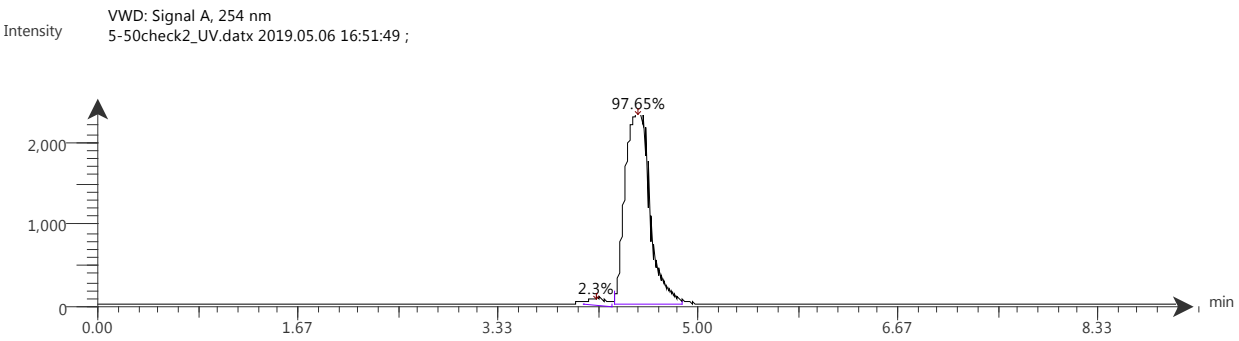
^{1}H NMR of SN-38-CM$_2$



^{1}H NMR of Aromatic Region of SN-38-CM$_2$



^{13}C NMR of SN-38-CM$_2$



LC UV (254 nm) Trace of SN-38-CM$_2$

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