Supplementary Information for

A Mitochondrial-Targeted Activity-Based Sensing Probe for Ratiometric Imaging of Formaldehyde Reveals Key Regulators of the Mitochondrial One-Carbon Pool

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General Methods. Reactions that involved the use of moisture- or air-sensitive reagents were run under an N_2 atmosphere with solvents dried and purified using a solvent purification system (JC Meyer), stored under an argon atmosphere. A Biotage® Isolara™ One, equipped with Biotage® Sfär Silica D Duo 60 µm columns, was used for normal phase purification purposes. For reversed phase, HPLC purification, an Agilent 1260 Infinity II binary LC system was used along with an Agilent Zorbax SB-C18 column with 5 µm particle size, inner diameter of 9.4 mm and length of 250 mm (Part number: 880975-202). ^{1}H NMR and ^{13}C NMR spectra were collected in $CDCl_3$ or MeOD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C on Bruker AVQ-400 (^{1}H) and AV-600 with ^{13}C operating frequency of 126 MHz at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard δ notation of parts per million with the residual solvent peak set to 7.26 ($CDCl_3$) or 3.31 (MeOD) for ^{1}H and 77.16 ($CDCl_3$) and 49.1 (MeOD) for ^{13}C. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; p, pentet; m, multiplet; dd, doublet of doublets; td, triplet of doublets. High resolution mass spectral analyses (ESI-MS) were carried out using an Agilent 6230 TOF LC/MS equipped with a ZORBAX Eclipse Plus C18 RRHD column at the University of California, Berkeley.

Spectroscopic Assays and Selectivity Tests

Spectroscopic Assays. Fluorescence spectra were recorded using a Photon Technology International Quanta Master 4 L-format scan spectrofluorometer equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photocounting/analog photomultiplier detection unit, and MD5020 motor driver. Samples for emission measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4-mL volume, Starna).

Selectivity Tests. 999 µL of a 10 µM solution of **MitoRFAP-2** in PBS (10 mM, pH 7.4) was prepared by diluting a 10 mM DMSO stock solution of **MitoRFAP-2** into prewarmed PBS (37 °C) in a 1-cm × 1-cm quartz cuvette. The analyte of interest was added (1µL) to the cuvette for a concentration of analyte of 100 µM (unless otherwise specified) the vial was then inverted several times, and a t = 0 spectrum was acquired. The cuvette was placed in a 37 °C water bath between each time point. Spectra were taken at t = 0, 30, 60, 90, and 120 min, and the cuvette was returned to water bath between each measurement.

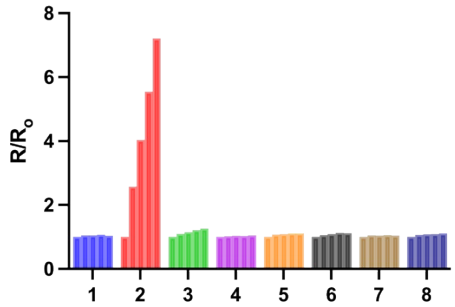


Figure S1. Selectivity Assay of MitoRFAP-2 at Elevated pH. Ratiometric response of **MitoRFAP-2** (10 µM) exposed to several biological analytes (100 µM, unless otherwise noted) in PBS (10mM, pH 7.8) at 37°C; measurements were made at 0, 30, 60, 90, and 120 min. (1) PBS;

(2) FA; (3) methylglyoxal; (4) acetaldehyde; (5) glucose, 1 mM; (6) pyruvate; (7) glutathione; (8) H_2O_2.

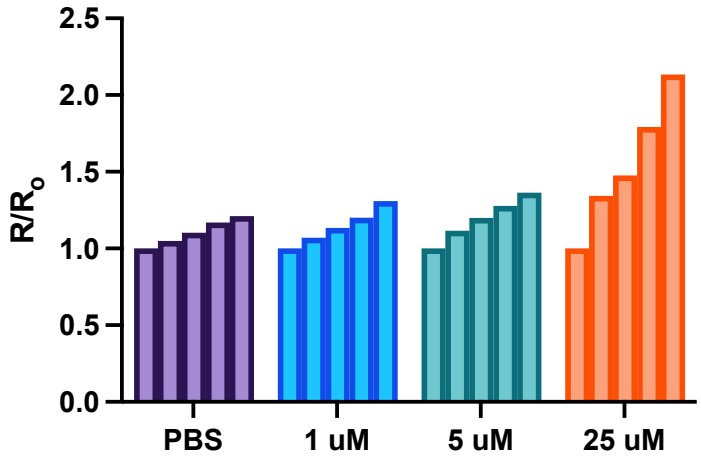


Figure S2. Limits of FA detection with MitoRFAP-2. Ratiometric responses of **MitoRFAP-2** (2 μM) exposed to several lower concentrations of FA (0 μM, 1 μM, 5 μM, and 25 μM) in PBS (10mM, pH 7.8) at 37°C; measurements were made at 0, 30, 60, 90, and 120 min.

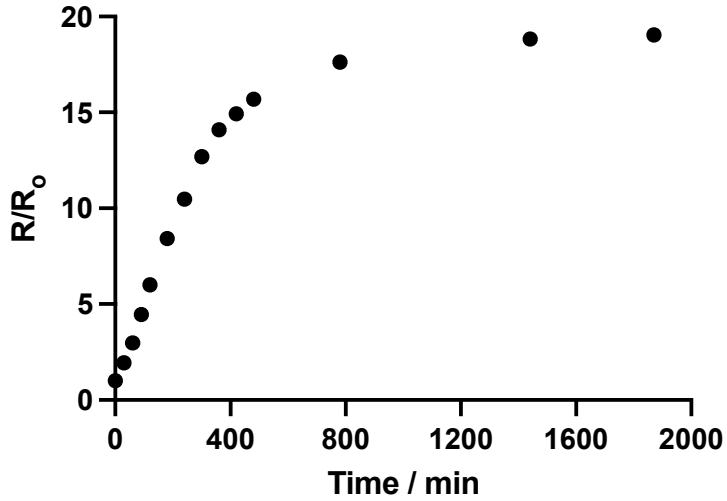


Figure S3. Extended time course of MitoRFAP-2 response to FA. Assay was performed in PBS (10 mM, pH 7.4) at 37°C. Change in excitation ratio (λ_{em} = 510 nm) at λ_{ex} = 470 nm (product) and λ_{ex} = 420 nm (reactant) of **MitoRFAP-2** (10 μM) in response to FA (100 μM) shown at 0, 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, 780, 1440, 1870 min.

Cell Culture and Imaging Procedures

Cell Culture. Cells were maintained by the UC Berkeley Tissue Culture Facility. **HEK 293T** cells (ATCC, cat. no. CRL-3216) were maintained as a monolayer in exponential growth at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Seradigm), 1% glutamax (Gibco), and 1%

non-essential amino acids (NEAA, Gibco). **HeLa** cells (ATCC, cat. no. CCL-2) were maintained as a monolayer in exponential growth at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Seradigm). **MCF-7** cells (MCF-7 – ATCC, cat. no. HTB-22) were maintained as a monolayer in exponential growth at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Seradigm), 1% glutamax (Gibco), 1% non-essential amino acids (NEAA, Gibco) and 1% sodium pyruvate. **ZR-75-1** cells (ATCC, cat. no. CRL-1500) were maintained as a monolayer in exponential growth at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Seradigm), and 1% glutamax (Gibco). **SK-BR-3** cells (ATCC, cat. no. HTB-30) were maintained as a monolayer in exponential growth at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Seradigm), and 1% glutamax (Gibco). **SNU-182** cells (ATCC, cat. no. CRL-2235) were maintained as a monolayer in exponential growth at 37 °C in a 5% CO_2 atmosphere in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum (FBS, Seradigm). **T-47D** cells (ATCC, cat. no. HTB-133) were maintained as a monolayer in exponential growth at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Seradigm). **HCT 116** wildtype cells and **HCT 116** ADH5 and SHMT1/2 knockout cells were received from Prof. K. J. Patel (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10191432/) with permission from Prof. J. D. Rabinowitz. Cells were cultured in DMEM, high glucose, GlutaMAX™ Supplement with 10% FBS. **HCT 116** cell lines were cultured with 1X Hypoxanthine/Thymidine (HT) Supplement (Gibco) to support cell proliferation. One or two days before imaging, cells were passaged and plated on 8-well chamber slide coated with poly L-lysine (50 mg/mL, Sigma, St. Louis, MO), this excludes the experiments in HCT-116 cells, which were not poly L-lysine treated. "HBSS" used in cell culture experiments was Hank's Balanced Salt Solution with calcium chloride and magnesium chloride (14025076, Gibco), all buffers were warmed to 37 °C before application to cells.

General Imaging Procedures. All images were obtained on a Zeiss 880 LSM (laser scanning microscope) using Zen black software. Images for λ_{ex} = 488 nm quantification were obtained by excited with a 488 nm argon laser, emission measured with a META detector between 509-601 nm. Images for λ_{ex} = 405 nm quantification were obtained via excitation with a 405 nm diode laser, emission measured with a META detector between 509-601 nm. Images with λ_{ex} = 633 nm (MitoTracker™, LysoTracker™) were obtained via excitation with a 633 nm HeNe laser, emission measured between 645-701 nm.

Colocalization Imaging Assays. Cells were grown to approximately 70% under conditions mentioned above in an 8-well imaging chamber (Lab-Tek II Chambered Coverglass w/cover, cat. #155409) treated with poly L-lysine (P4707, Sigma). Cells were treated with **MitoRFAP-2** (2 μM) and MitoTracker™ Deep Red (100 nM) or LysoTracker™ Deep Red (50 nM) in HBSS for 30 min at 37 °C in a 5% CO_2 atmosphere. Solution was replaced with HBSS before imaging, 63x oil immersion. Each sample, n, represents quantification of the colocalization of the two dyes in a single HeLa cell.

Imaging Exogenous Formaldehyde (FA) Addition to HEK-293T and HeLa Cells. Cells were grown to approximately 70% under conditions mentioned above in an 8-well imaging chamber treated with (Lab-Tek II Chambered Coverglass w/cover, cat. #155409) treated with poly L-lysine. Cells were treated with **MitoRFAP-2** (2 μM) for 30 min at 37 °C in a 5% CO_2 atmosphere, followed by replacement with HBSS, or HBSS with 125 μM, 250 μM or 500 μM formaldehyde (freshly prepared from commercial 37 wt. % FA) and incubation for 1h at 37 °C in a 5% CO_2 atmosphere. Cells were then imaged, 20x objective. Each sample, n, represents quantification of the λ_{ex} = 488 nm/λ_{ex} = 405 nm ratio in a single well of cells.

Imaging Mitochondrial Formaldehyde (FA) Pools in HCT-116 Cells with Genetic Modulation of Mitochondrial FA-Generating and FA-Scavenging Enzymes. Cells were grown to approximately 70% under conditions mentioned above in an 8-well imaging chamber (Lab-Tek II Chambered Coverglass w/cover, cat. #155409). Cells were treated with **MitoRFAP-2** (2 μM) for 30 min at 37 °C in a 5% CO_2 atmosphere. Followed by replacement with DMEM + 10% FBS and incubation for 1 h at 37 °C in a 5% CO_2 atmosphere. Cells were then imaged, 20x objective. Each sample, n, represents quantification of the λ_{ex} = 488 nm/λ_{ex} = 405 nm ratio in a single well of cells, for this experiment values were collected from several separate biological replicates, a maximum of n = 4 technical replicates were collected in a single biological replicate.

Imaging Pharmacological Activation of Alcohol Dehydrogenase 2 E487K Mutant (ALDH2*2) in SNU-182 Cells. Cells were grown to approximately 60% under conditions mentioned above in an 8-well imaging chamber (Lab-Tek II Chambered Coverglass w/cover, cat. #155409) treated with poly L-lysine. Cells were treated with Alda-1 (50 μM, 0.5% DMSO) in RPMI1640 + 10% FBS for 24 h. Cells were then treated with **MitoRFAP-2** (2 μM) with vehicle (0.5% DMSO) or Alda-1 (50 μM, 0.5% DMSO) in HBSS for 1 h before imaging, 20x objective. Each sample, n, represents quantification of the λ_{ex} = 488 nm/λ_{ex} = 405 nm ratio in a single well of cells, for this experiment values were collected from three separate biological replicates, using cells of a different passage number, n = 4 technical replicates were collected in a single experiment, each from a unique well of cells.

Imaging Activation of WT ALDH2 in HeLa Cells. Cells were grown to approximately 50% under conditions mentioned above in an 8-well imaging chamber (Lab-Tek II Chambered Coverglass w/cover, cat. #155409) treated with poly L-lysine. Cells were treated with Alda-1 (50 μM, 0.5% DMSO) in DMEM + 10% FBS for 24 h. Cells were then treated with **MitoRFAP-2** (2 μM) with vehicle (0.5% DMSO) or Alda-1 (50 μM, 0.5% DMSO) in HBSS for 1 h before imaging, 20x objective. Each sample, n, represents quantification of the λ_{ex} = 488 nm/λ_{ex} = 405 nm ratio in a single well of cells, for this experiment values were collected from three separate biological replicates, using cells of a different passage number, n = 4 technical replicates were collected in a single experiment, each from a unique well of cells.

Image Processing. FIJI (National Institute of Health) was used for all quantification of imaging data. Cell regions to be quantified were selected by setting a threshold based on the max intensity of the Z-projection slices after a Gaussian blur filter (sigma=1) was applied. Then “Create Mask” and “Create Selection” functions were used to select the area to be quantified based on the determined threshold. The mean fluorescence was then quantified for each Z-slice using the “Multi-Measure” function. The average fluorescence intensity was then calculated by taking the

slice with the highest mean intensity and averaging it with the slice above and the slice below. The calculation of the λ_{ex} = 488 nm/λ_{ex} = 405 nm ratio was then conducted by dividing the average emission at λ_{ex} = 488 nm by the average emission at λ_{ex} = 405 nm. Statistical comparison between treatments was conducted by an unpaired t test in Prism9 (GraphPad). Pearson correlation coefficients were calculated by taking images, splitting the channels into individual tabs, highlighting individual cells with the "Freehand Selections" tool in the green channel (**MitoRFAP-2**), then using the "Coloc 2" tool, selecting the green and red channel and then setting the ROI to the region drawn in the green channel (Threshold regression-Costes, PSF=3.0, Costes randomization=10).

Flow Cytometry Procedures

Flow Cytometry Assays. Cell viability experiments and basal breast cancer formaldehyde profiling experiments were conducted using a Attune NxT Flow Cytometer by Life Technologies. For ratiometric fluorescence profiling, two different channels were used to analyze fluorescence of **MitoRFAP-2**, VL1 (λ_{ex} = 405 nm, emission filter 450/40) and BL1 (λ_{ex} = 488 nm, emission filter 530/30). MCF7, T-47D, ZR-75-D and SK-BR-3 cells were grown to approximately 70% confluency in a 12-well plate treated with poly L-lysine. Cells were treated with **MitoRFAP-2** (2 μM) for 30 min in HBSS, followed by an incubation in DMEM + 10% FBS for 1 h. Cells were incubated for five min in PBS at room temp and pipetted to suspend, followed by flow analysis. For each replicate, 10,000 cells were analyzed and median values for each channel were used to determine fluorescence ratio (λ_{ex} = 488 nm/405 nm). Three replicates, individual wells from a 12-well plate, were conducted for each cell type.

Assessing Cell Viability by Flow Cytometry. All cells were grown to approximately 70% confluency in a 12-well plate treated with poly L-lysine. Cells were not treated (left in growth media), treated with vehicle, or treated with **MitoRFAP-2** (2 μM) in HBSS for 30 min followed by collection via trypsinization. Cells were spun down, then resuspended in PBS with Sytox® Red (5 nM) and incubated for 30 min at room temp before analysis by flow cytometer. The RL1 (λ_{ex} = 645 nm, emission filter 670/14) channel was used to analyze Sytox® Red fluorescence. The gain was adjusted to center the "live cell" peak around an intensity value of 10^2, cells with an intensity value above 10^3 were considered dead. Three technical replicates, n=1 is an individual well from a 12-well plate, were conducted and quantified for each group.

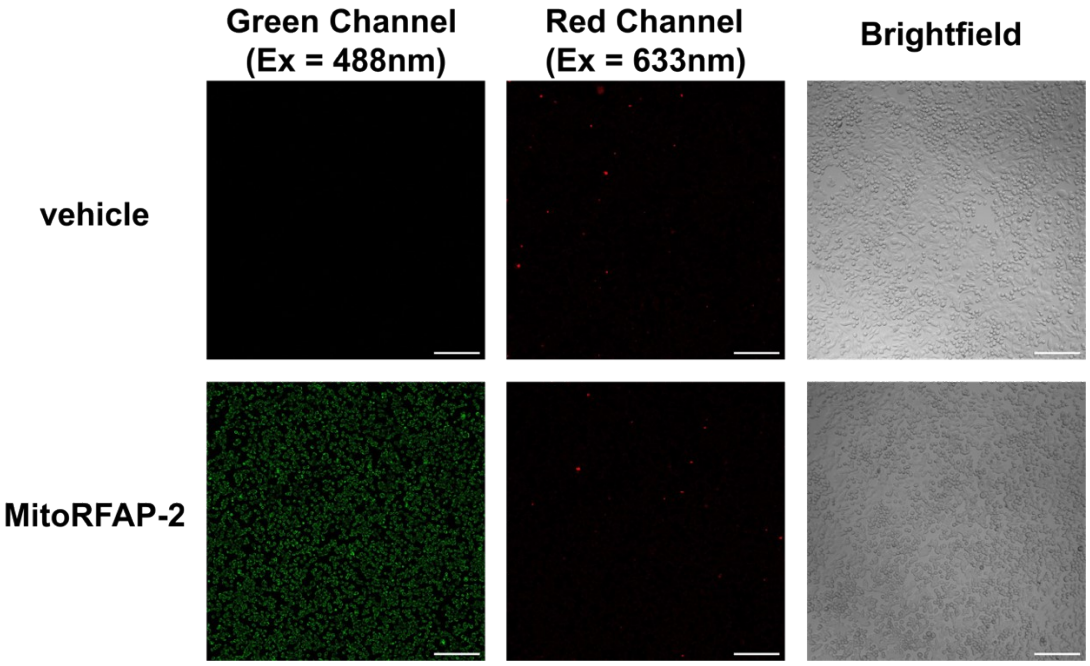


Figure S4. Cell Viability Assays. HeLa cells were treated with vehicle or **MitoRFAP-2** (2 μM) in HBSS for 30 min followed by replacement with HBSS containing Sytox® Red (5 nM) and incubated for 15 min before imaging, an increase in permeability of Sytox® Red signifies cell death. 10x objective, scale bar represents 200 μm in all images.

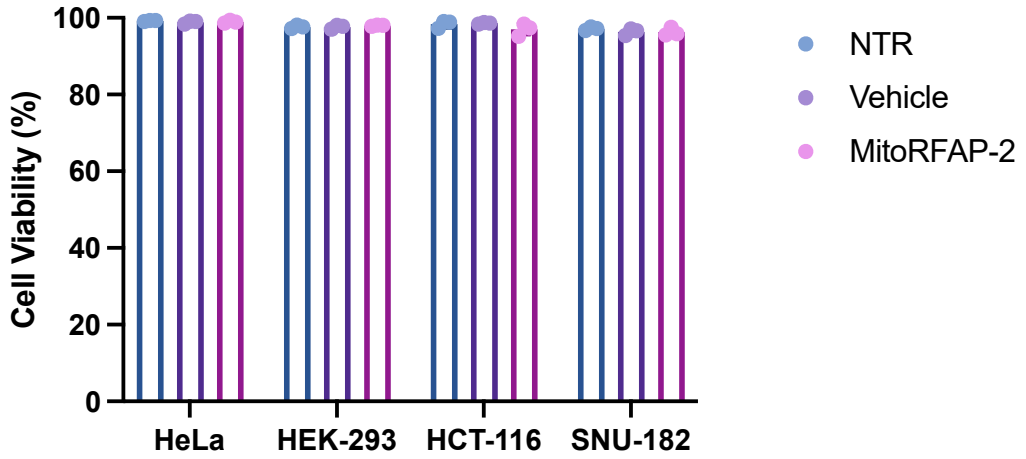


Figure S5. Cell Viability Assays Across Multiple Lines. Cells plated in a 12-well polystyrene culture plate (Corning) were either not treated (NTR, remained in DMEM + FBS during staining) or treated with vehicle (DMSO) or **MitoRFAP-2** (2 μM) in HBSS for 30 min. Cells were then trypsinized, spun down and resuspended in PBS (10 mM, pH 7.4) containing Sytox® Red (5 nM), followed by incubation for 30 min at room temp before flow cytometry analysis. In the **SNU-182** samples, the Mito-RFAP-2 treated cells were also treated with Alda-1 (50 μM) for 24h before staining, the same treatment as the ALDH2 activation experiments. Error bars denote SEM, n = 3.

Co-Staining Studies

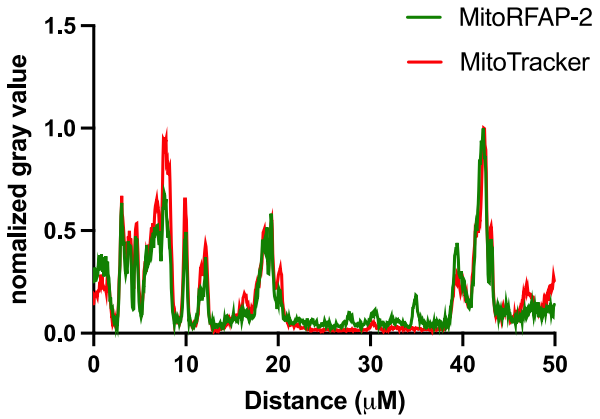


Figure S6. Profile Analysis of a Single HeLa Cell in a Dual-Dye Co-Staining Experiment. HeLa cells were stained with **MitoRFAP-2** (2 μM) and MitoTracker Deep Red (100 nM) in HBSS for 30 min followed by replacement with fresh buffer. The profile was analyzed in FIJI by using the line tool, drawing a line through the center of a single HeLa cell and using the "Profile Plot" tool. Here is shown the normalized intensity profile of the signals of both dyes.

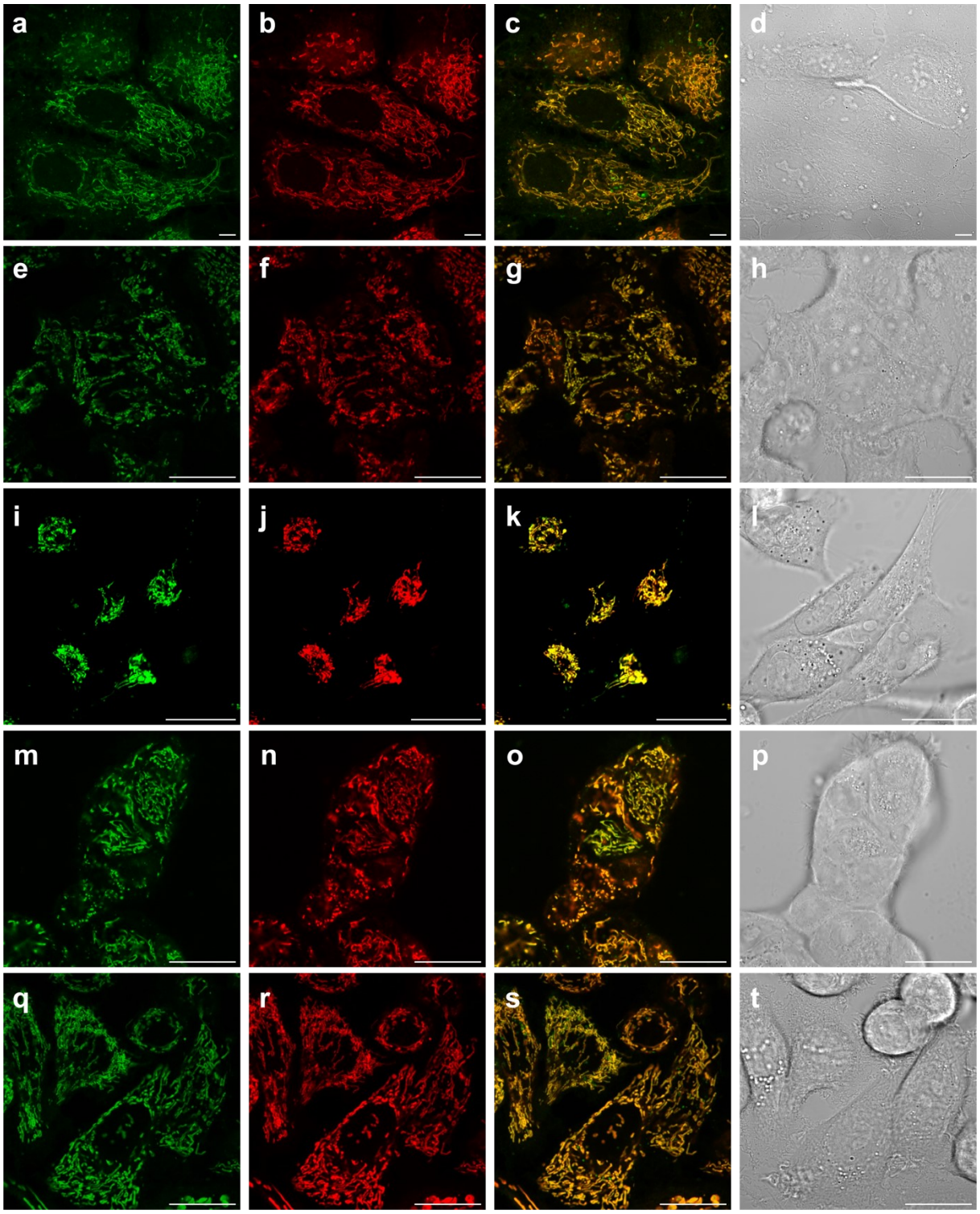


Figure S7. Co-Staining Experiments in Several Cell Lines with MitoRFAP-2. MitoRFAP-2 (2 μM) at λ_{ex} = 488 nm (green channel), MitoTracker™ Deep Red (100 nM) at λ_{ex} = 633 nm (red channel), overlay of λ_{ex} = 488 nm and 633 nm channels and bright-field image of (**a-d**) SNU-182, (**e-h**) T-47D, (**i-l**) MDA-MB-231, (**m-p**) ZR-75-1, and (**q-t**) SK-BR-3 cells (a dose of 50nM MitoTracker™ Deep Red was used for this image). Scale bar represents 20 μm in all images.

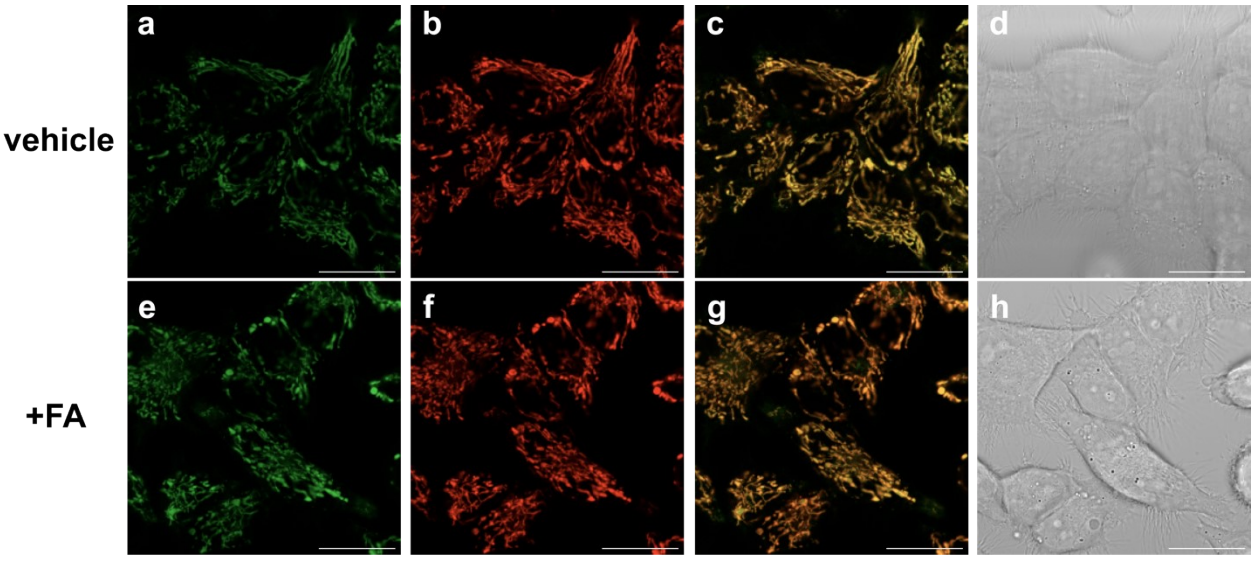


Figure S8. Co-Staining Experiments in HeLa Cells with MitoRFAP-2 with Exogenous Addition of FA. MitoRFAP-2 (2 μM) at λ_{ex} = 488 nm (green channel), MitoTracker™ Deep Red (100 nM) at λ_{ex} = 633 nm (red channel), overlay of λ_{ex} = 488 nm and 633 nm channels and bright-field image of cells stained for 30 min followed by (**a-d**) vehicle treatment for 30 min (**e-h**) treatment with 125 μM FA for 30 min. Scale bar represents 20 μm in all images.

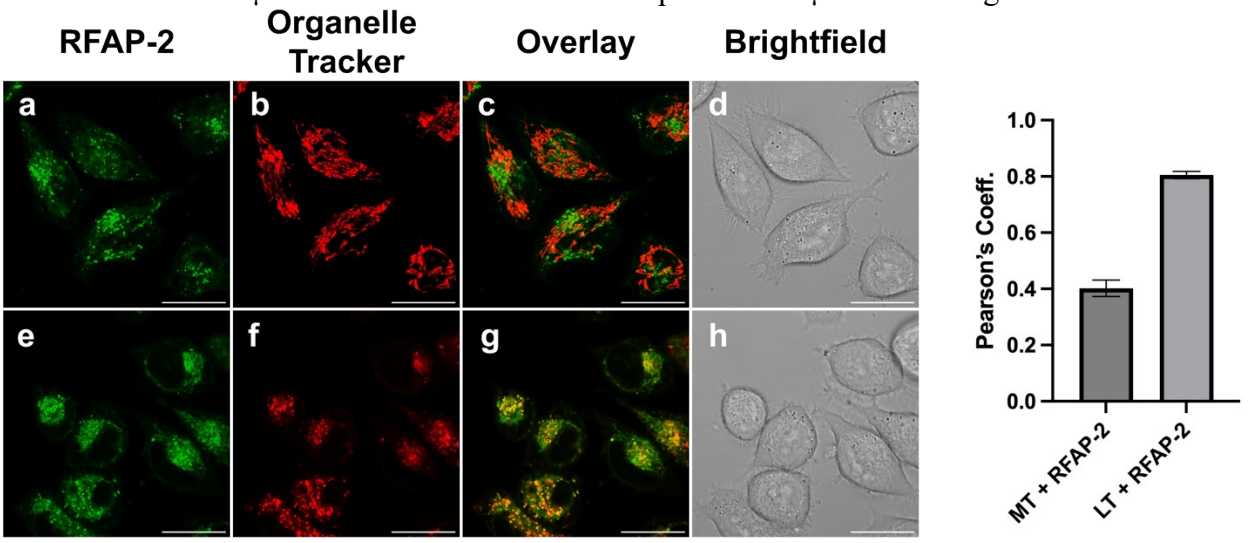


Figure S9. Co-Staining Experiments in HeLa with RFAP-2. RFAP-2 has modest overlap with mitochondrial tracking and strong overlap with lysosomal tracking dyes in HeLa cells. HeLa cells stained for 30 min in HBSS with (a) **RFAP-2** (10 μM) at λ_{ex} = 488 nm (b) MitoTracker™ Deep Red (100 nM) at λ_{ex} = 633 nm (c) overlay of λ_{ex} = 488 nm and 633 nm channels (d) brightfield image of the cells in (a-c). HeLa cells stained for 30 min in HBSS with (e) **RFAP-2** (10 μM) at λ_{ex} = 488 nm (f) LysoTracker™ Deep Red (50 nM) at λ_{ex} = 633 nm (g) overlay of λ_{ex} = 488 nm and 633 nm channels (h) bright-field image of the cells in (e-g). (i) Quantification of the Pearson correlation coefficient of ten individual cells for **RFAP-2** with MitoTracker™ Deep Red and LysoTracker™ Deep Red, scale bar represents 20 μm in all images; error bars denote SEM, n = 10.

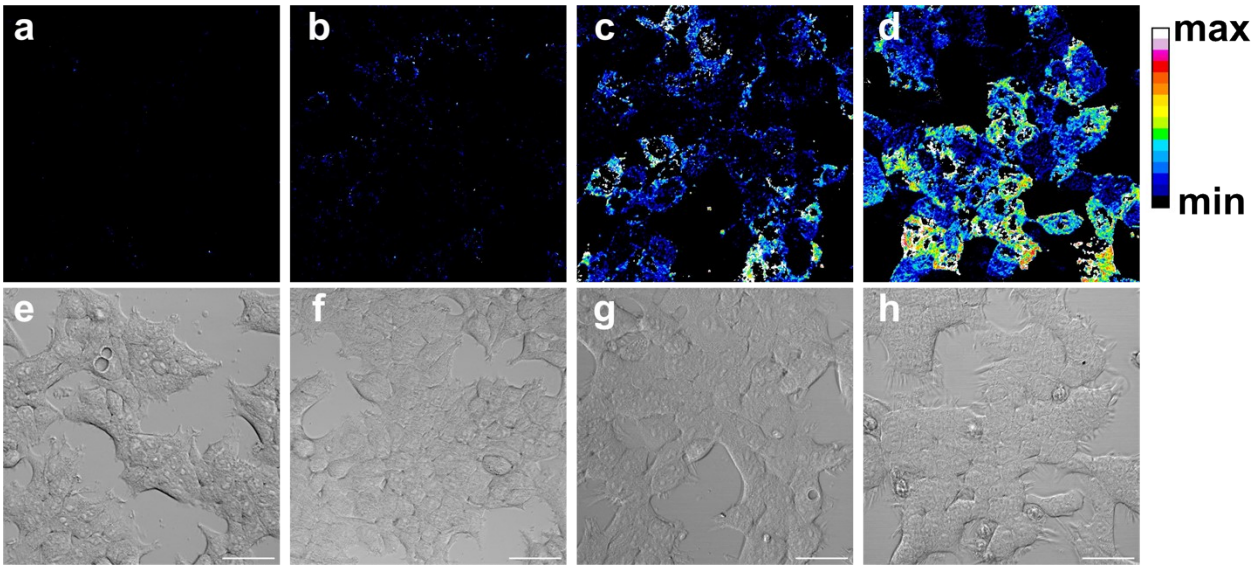


Figure S10. MitoRFAP-2 detects exogenous additions of formaldehyde (FA) in live cells, plotted as a ratiometric heat map. HEK293T cells were stained with **MitoRFAP-2** (2 μM) for 30 min in HBSS, followed by incubation in HBSS (**a**), 125 μM (**b**), 250uM (**c**) or 500 μM FA (**d**) in HBSS for 1 h and respective brightfield images (**e-h**). White pseudo-colouring represents the highest λ_{ex} = 488 nm/λ_{ex} = 405 nm ratio, while black pseudo-colouring represents the lowest.

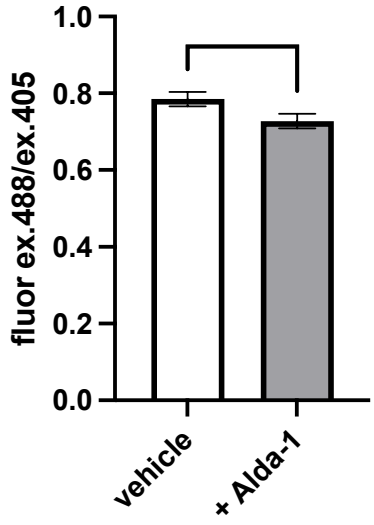


Figure S11. MitoRFAP-2 Imaging of Pharmacological Activation of Alcohol Dehydrogenase 2 (ALDH2) in HeLa Cells. Cells were treated with Alda-1 (50 μM, 0.5% DMSO) or vehicle (0.5% DMSO) in DMEM + 10% FBS for 24 h, followed by **MitoRFAP-2** (2 μM) with vehicle (0.5% DMSO) or Alda-1 (50 μM, 0.5% DMSO) for 1 h before imaging. Quantification of the ratiometric response of **MitoRFAP-2**, λ_{ex} = 488 nm/λ_{ex} = 405 nm; n = 12. Error bars denote SEM; **P* < 0.05.

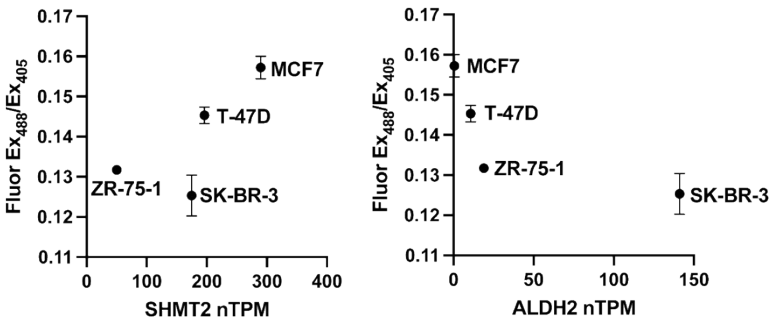


Figure S12. Correlation of Ratiometric MitoRFAP-2 Fluorescence and Expression Levels of Serine Hydroxymethyltransferase 2 (SHMT2) and Aldehyde Dehydrogenase 2 (ALDH2) Across a Panel of Breast Cancer Cell Lines. Relative basal FA signal vs SHMT2 expression in transcripts per million (left) and ALDH2 expression in transcripts per million (right). Error bars denote SEM; n = 3.

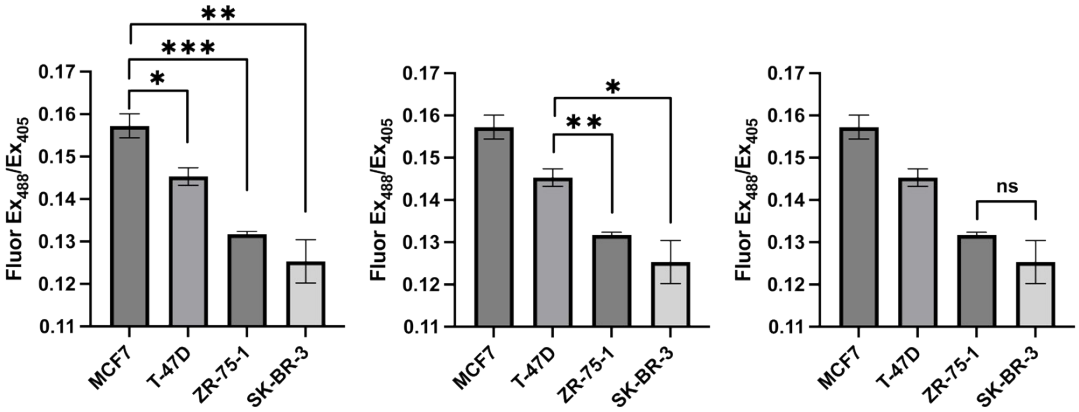
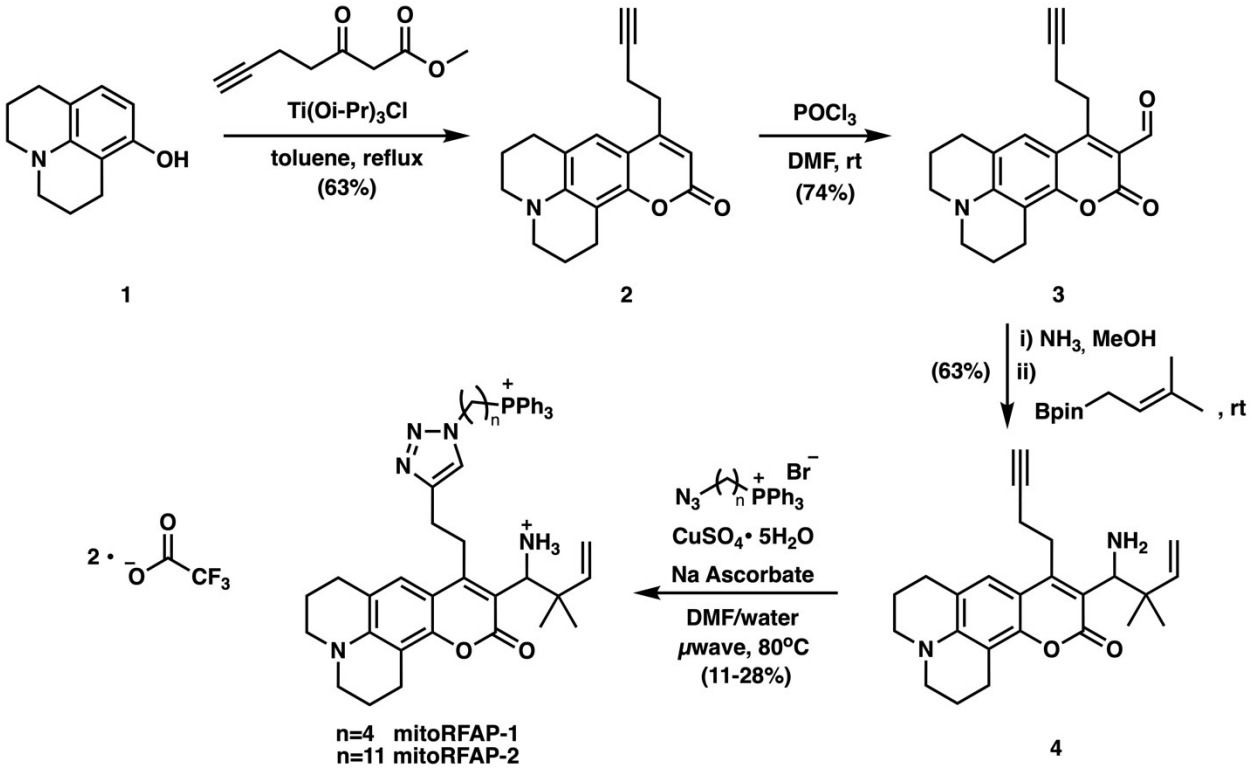


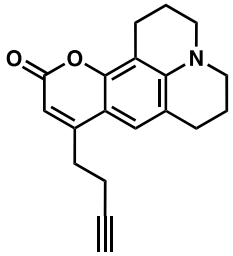
Figure S13. Basal Levels of FA Across a Panel of Breast Cancer Cell Lines. Unpaired t-tests were conducted to test for statistical significance between basal FA signal across several cells lines (right). Error bars denote SEM; n = 3.

Synthetic Chemistry Procedures and Characterization Data

MitoRFAP-1 and **MitoRFAP-2** were prepared via the general sequence below:

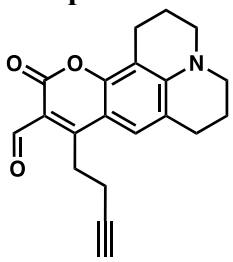


Compound 2



Synthesis was conducted exactly as it was in *Synthesis* **2012**, *44* (13), 2005–2012.

Compound 3



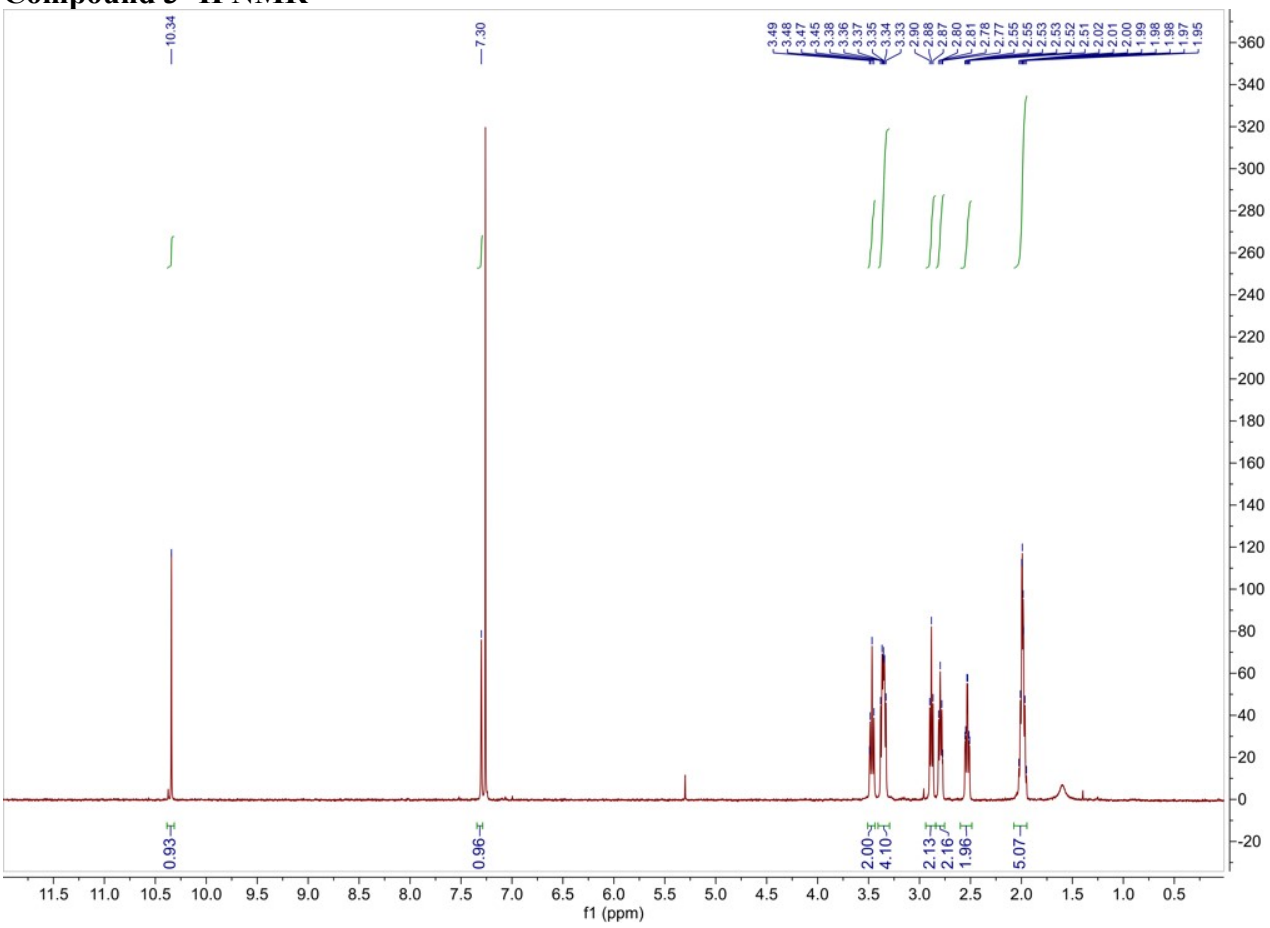
Synthesis was based on a procedure described in *Chem. Sci.* **2017***, 8 (11),* 7588–7592. A flame dried round bottom flask was charged with **Compound 2** (1 equiv., 290 mg, 0.989 mmol) followed by dry DMF (2 mL). $POCl_3$ (3 equiv., 285 μL, 2.97 mmol) was then added and the reaction was stirred at room temp for 1 h. At this time the reaction was diluted with DCM (20 mL) and quenched with sat. aq. $NaHCO_3$ (10 mL, until bubbling ceased). The aqueous layer was extracted with DCM (3 x 20 mL) and combined organics were washed with brine (3 x 15 mL), dried over anhydrous sodium sulfate and concentrated. The residue was purified by flash chromatography (0 to 5% MeOH in DCM), to furnish an orange solid (234 mg, 0.728 mmol, 74%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.34 (s, 1H), 7.30 (s, 1H), 3.47 (t, *J* = 7.5 Hz, 2H), 3.36 (dt, *J* = 9.2, 5.7 Hz, 4H), 2.89 (d, *J* = 6.4 Hz, 2H), 2.80 (t, *J* = 6.3 Hz, 2H), 2.53 (td, *J* = 7.6, 2.6 Hz, 2H), 2.12 – 1.80 (m, 5H).

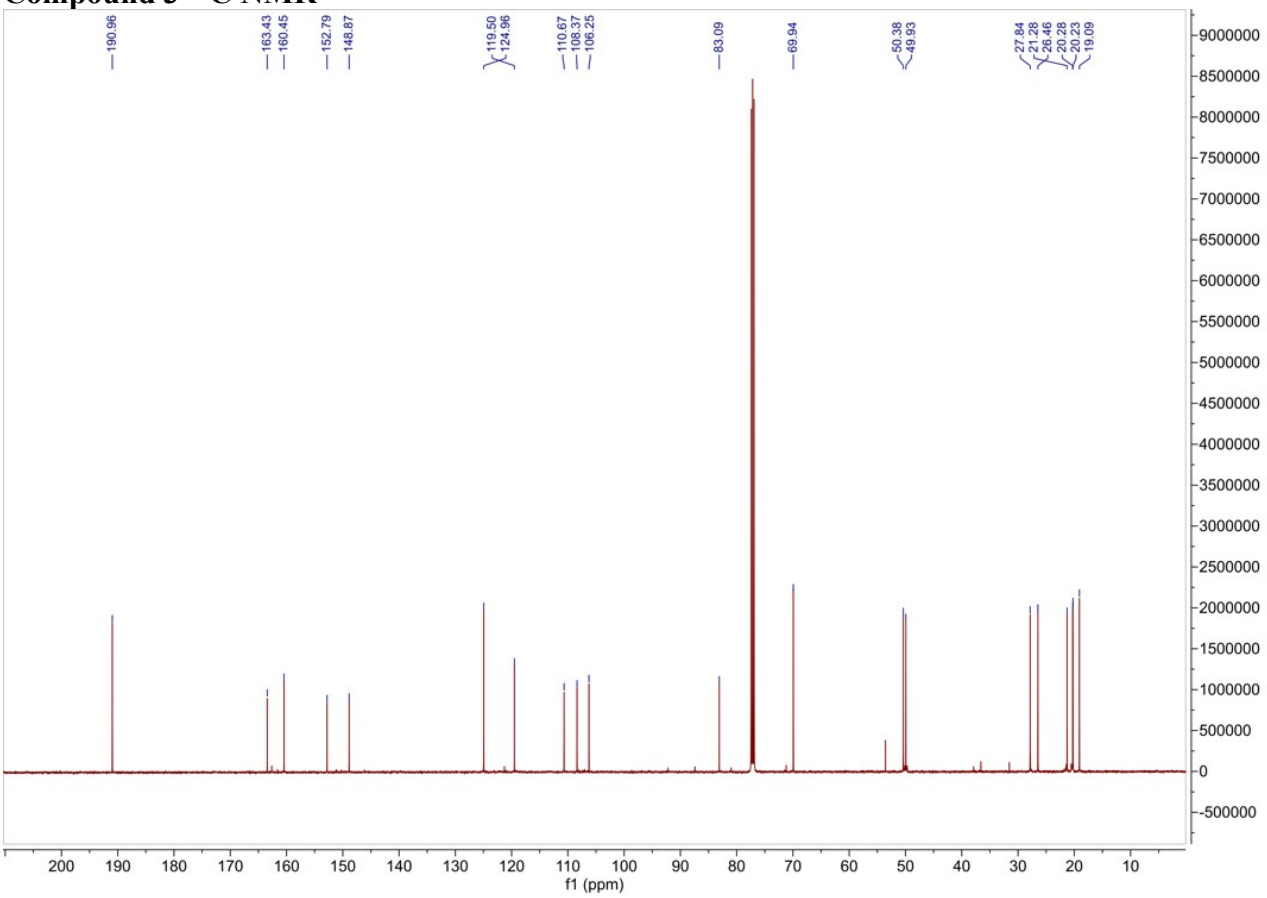
^{13}C NMR (126 MHz, $CDCl_3$) δ 190.96, 163.43, 160.45, 152.79, 148.87, 124.96, 119.50, 110.67, 108.37, 106.25, 83.09, 69.94, 50.38, 49.93, 27.84, 26.46, 21.28, 20.28, 20.23, 19.09.

HRMS $[M+H]^+$: Expected 322.1438, Found 322.1457

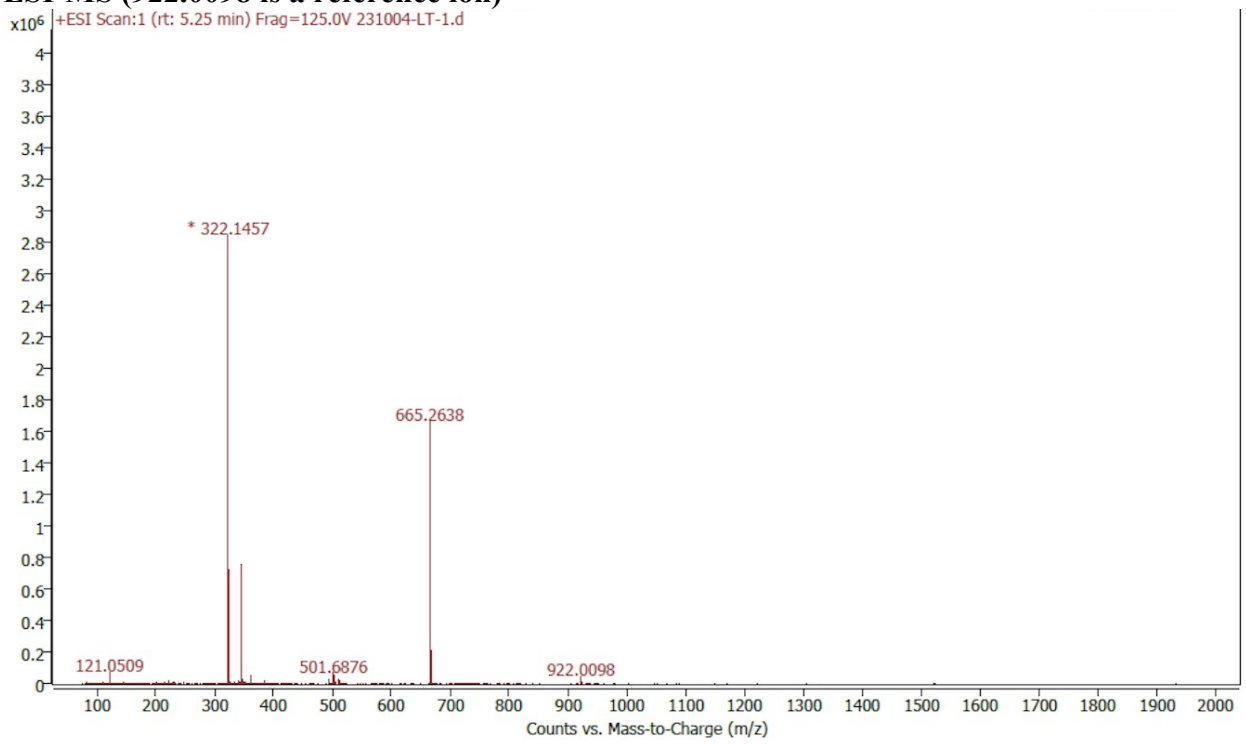
Compound 3 ^{1}H NMR



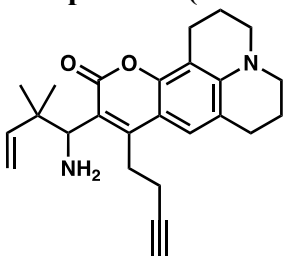
Compound 3 ^{13}C NMR



ESI-MS (922.0098 is a reference ion)



Compound 4 (RFAP-alkyne)



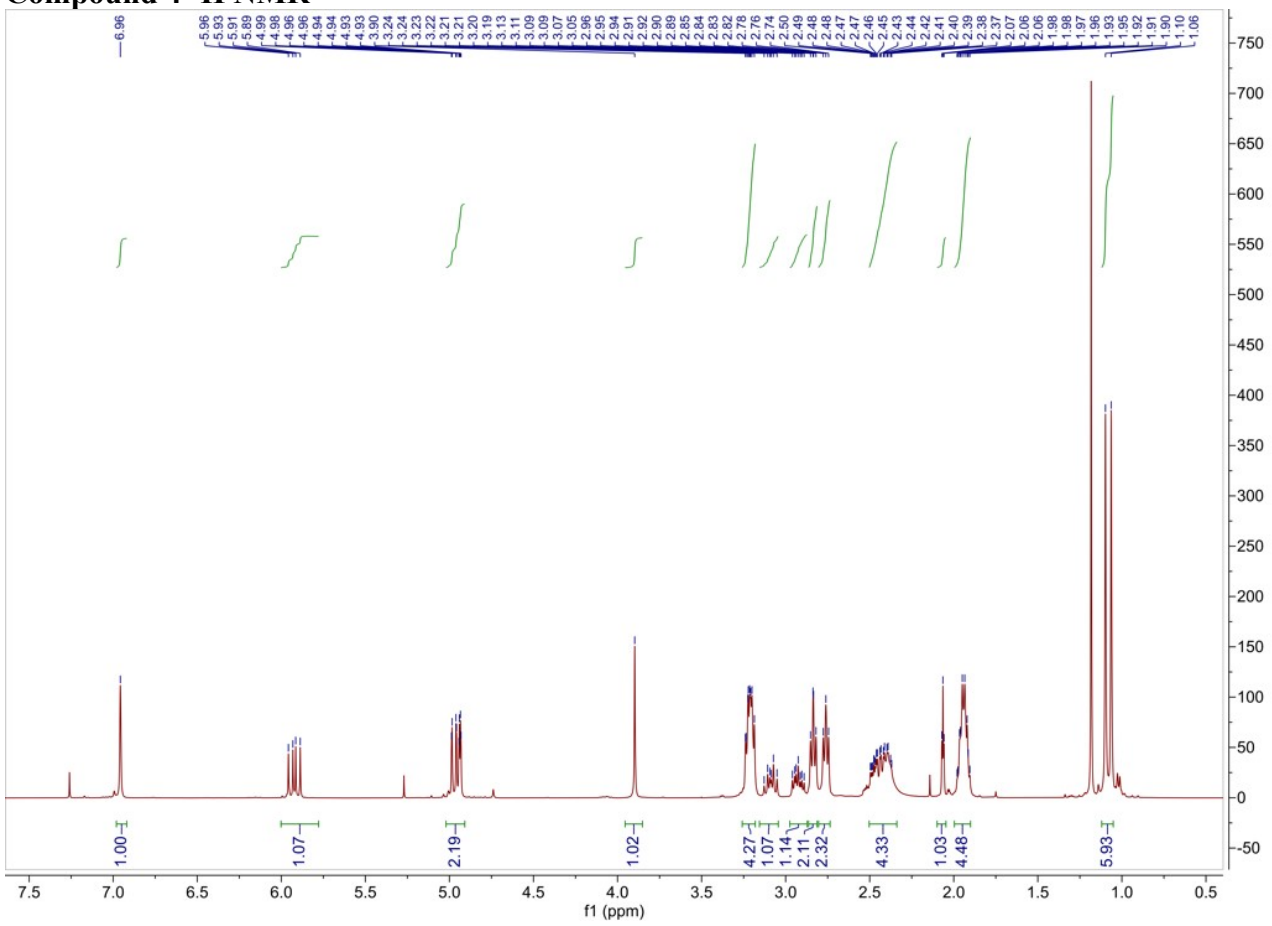
The synthesis of this compound was based on a general procedure described in *Chem. Sci.* **2017**, *8* (5), 4073–4081. **Compound 3** (1 equiv., 15 mg, 0.0467 mmol) was dissolved in dry DCM (0.40 mL) and MeOH (0.85 mL) under N_2 atmosphere. NH_3 (7N in MeOH, 10 equiv., 67 μL, 0.467 mmol) was added and the reaction was stirred at room temperature for 30min. At this time, 3-methyl-2-butenylboronic acid pinacol acid (2 equiv., 21 μL, 0.0934 mmol) was added and the reaction was stirred overnight at room temp. The crude reaction was concentrated and purified by flash chromatography (0 to 5% MeOH in DCM with 0.2% NH_4OH), to furnish an orange solid (11.5 mg, 0.0294 mmol, 63%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.96 (s, 1H), 5.92 (dd, *J* = 17.4, 10.8 Hz, 1H), 5.00 – 4.91 (m, 2H), 3.90(s, 1H), 3.22 (m, 4H), 3.13 – 3.04 (m, 1H), 2.92 (m, 1H), 2.87 – 2.81 (t, *J* = 6.4 Hz, 2H), 2.76 (t, *J* = 6.4 Hz, 2H), 2.55 – 2.35 (m, 4H), 2.06 (t, *J* = 2.6 Hz, 1H), 1.99 – 1.86 (m, 4H), 1.08 (d, *J* = 13.7 Hz, 6H).

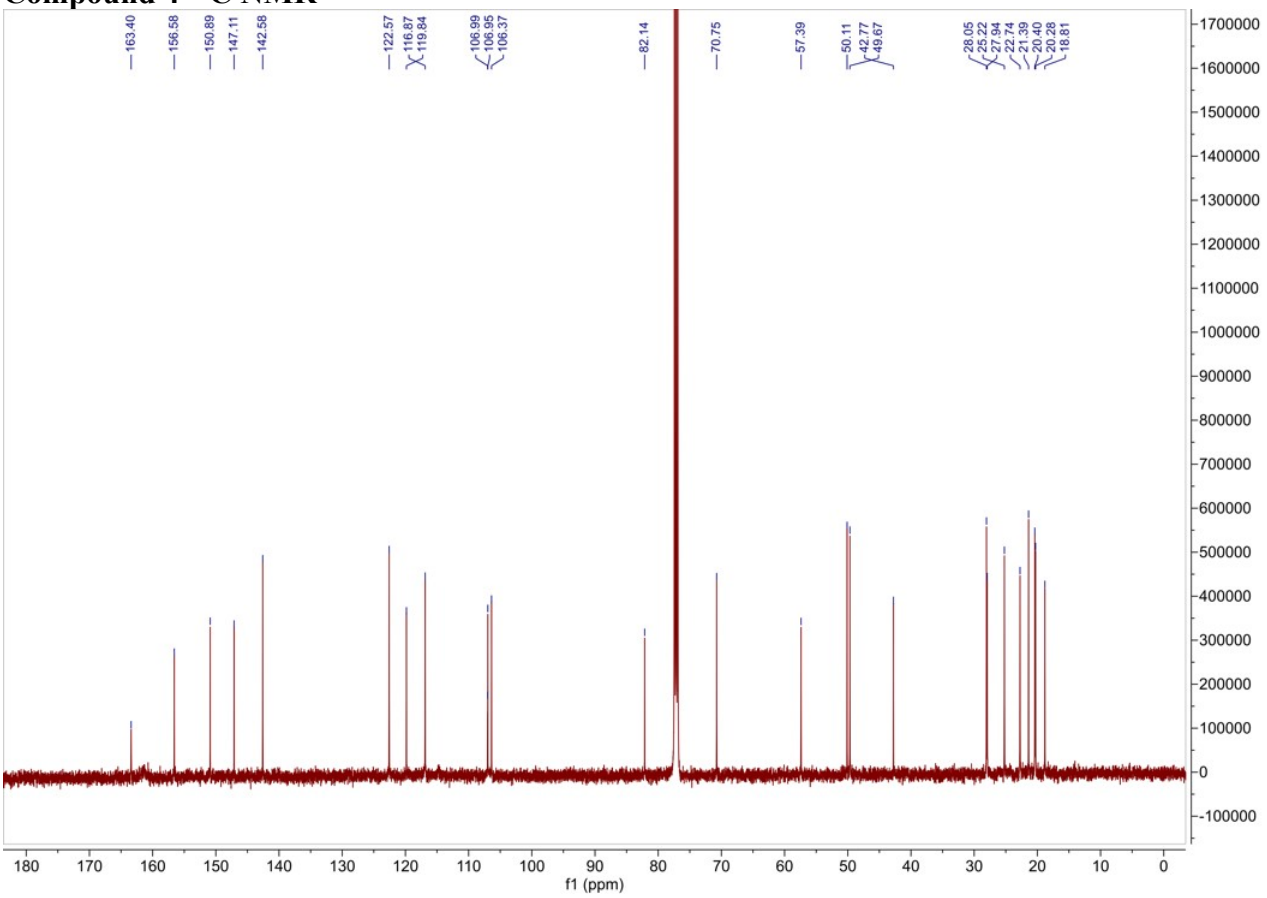
^{13}C NMR (126 MHz, $CDCl_3$) δ 163.40, 156.58, 150.89, 147.11, 142.58, 122.57, 119.84, 116.87, 106.99, 106.95, 106.37, 82.14, 70.75, 57.39, 50.11, 49.67, 42.77, 28.05, 27.94, 25.22, 22.74, 21.39, 20.40, 20.28, 18.81.

HRMS $[M+H]^+$: Expected 391.2380, Found 391.2413

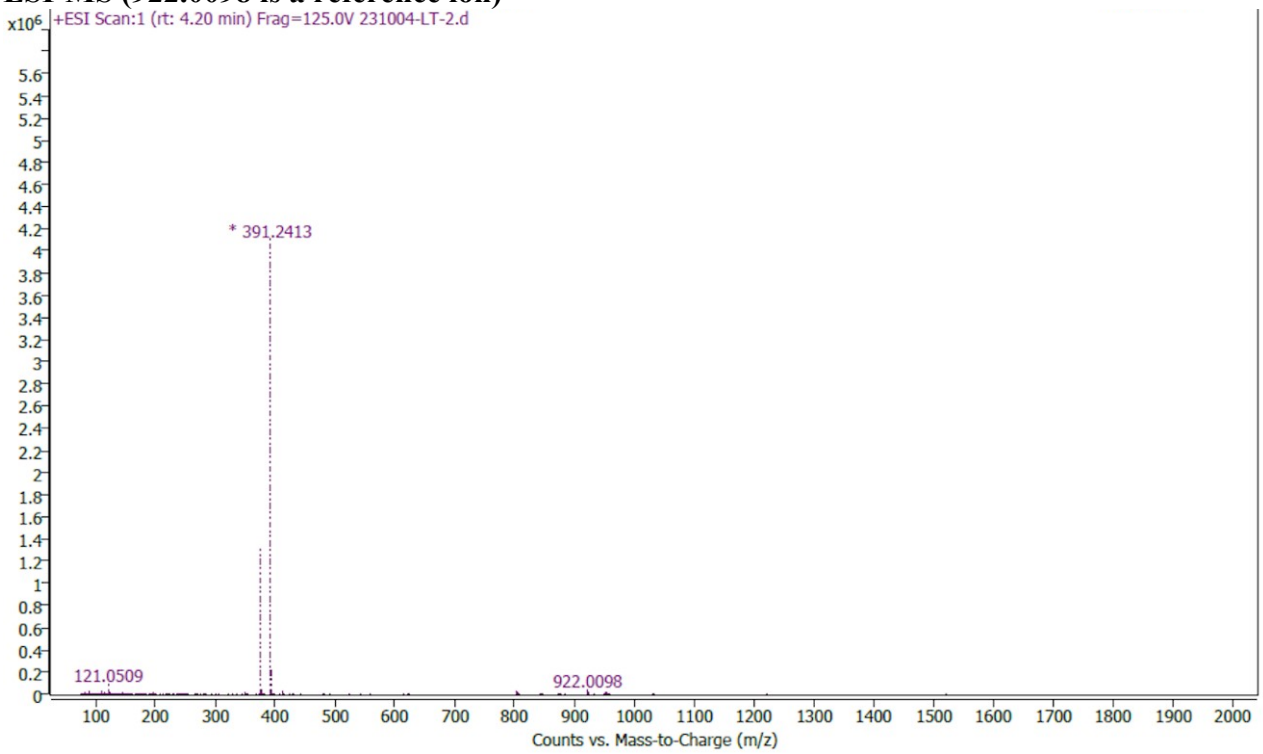
Compound 4 ^{1}H NMR



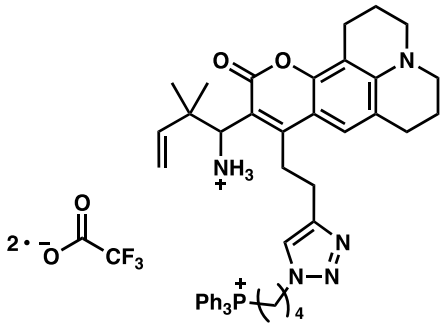
Compound 4 ^{13}C NMR



ESI-MS (922.0098 is a reference ion)



MitoRFAP-1



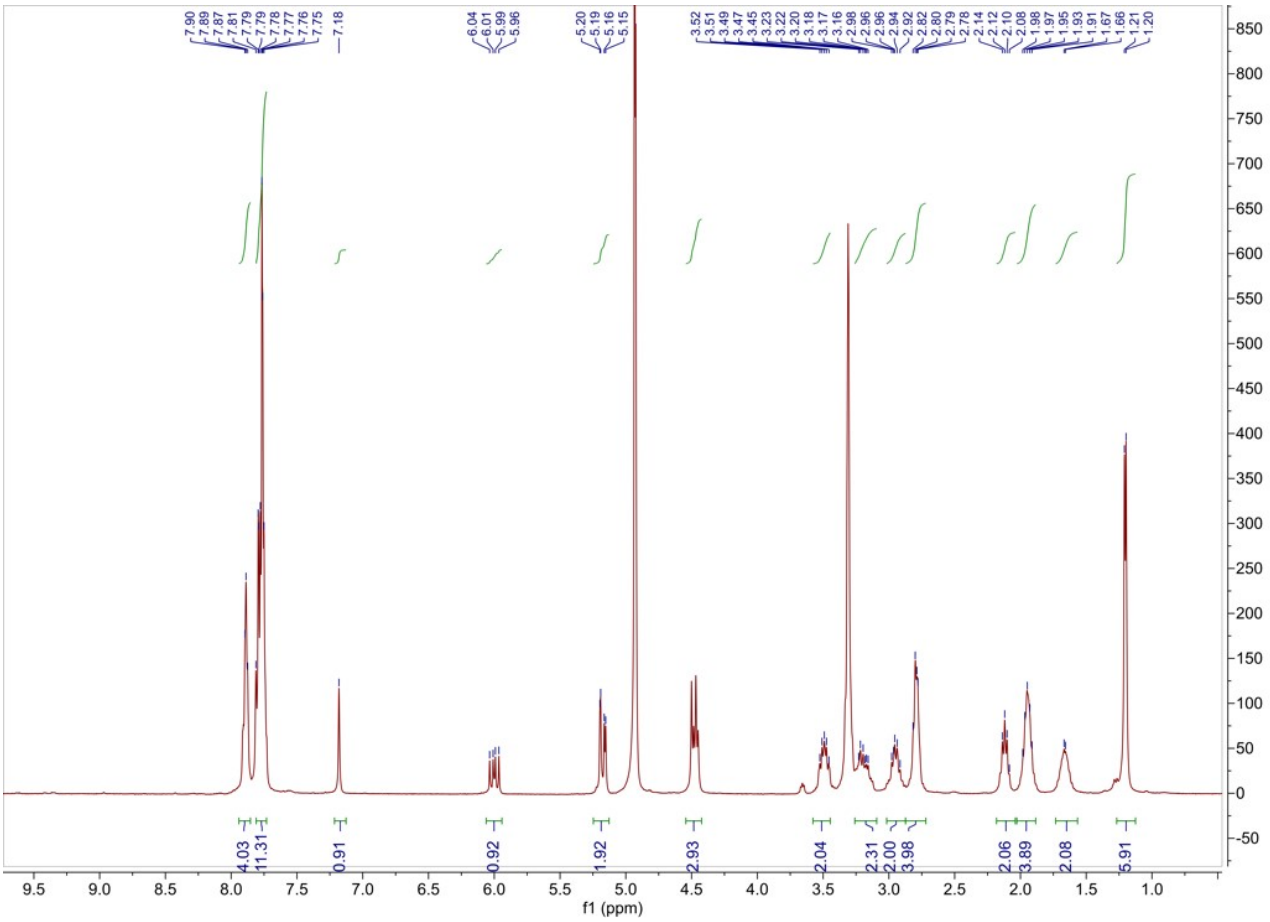
RFAP-alkyne (1 equiv., 19.0 mg, 0.0487 mmol) and 4-(azidobutyl)triphenylphosphonium bromide (1.2 equiv., 25.7 mg, 0.0584 mmol) were added to a vial under N_2 and the vial was sealed, DMF (0.8 mL) was then added. $CuSO_4 \bullet 5H_2O$ (20 mol%, 2.4 mg, 0.0097 mmol) dissolved in H_2O (0.2 mL) was added to the vial followed by sodium ascorbate (20 mol%, 1.9 mg, 0.0097 mmol) dissolved in H_2O (0.2 mL). The reaction was performed in a microwave reactor for 1 h at 80 °C and then allowed to cool to room temperature. The solvent was then removed by rotary evaporation and residue was suspended in MeOH (2 mL) and filtered through cotton. The resulting solution was purified via reverse phase HPLC (25 to 50% ACN in H_2O + 0.1% TFA) to yield an orange solid (13 mg, 0.014 mmol, 28%).

^{1}H NMR (400 MHz, MeOD) δ 7.89 (m, 4H), 7.77 (m, 11H), 7.18 (s, 1H), 6.00 (dd, *J* = 17.5, 10.6 Hz, 2H), 5.18 (m, 2H), 4.48 (m, 3H), 3.50 (td, *J* = 12.1, 5.6 Hz, 2H), 3.26 – 3.11 (m, 2H), 2.96 (m, 2H), 2.88 – 2.68 (m, 4H), 2.11 (m, 2H), 1.95 (p, *J* = 6.6 Hz, 4H), 1.66 (d, *J* = 4.4 Hz, 2H), 1.20 (d, *J* = 5.1 Hz, 6H).

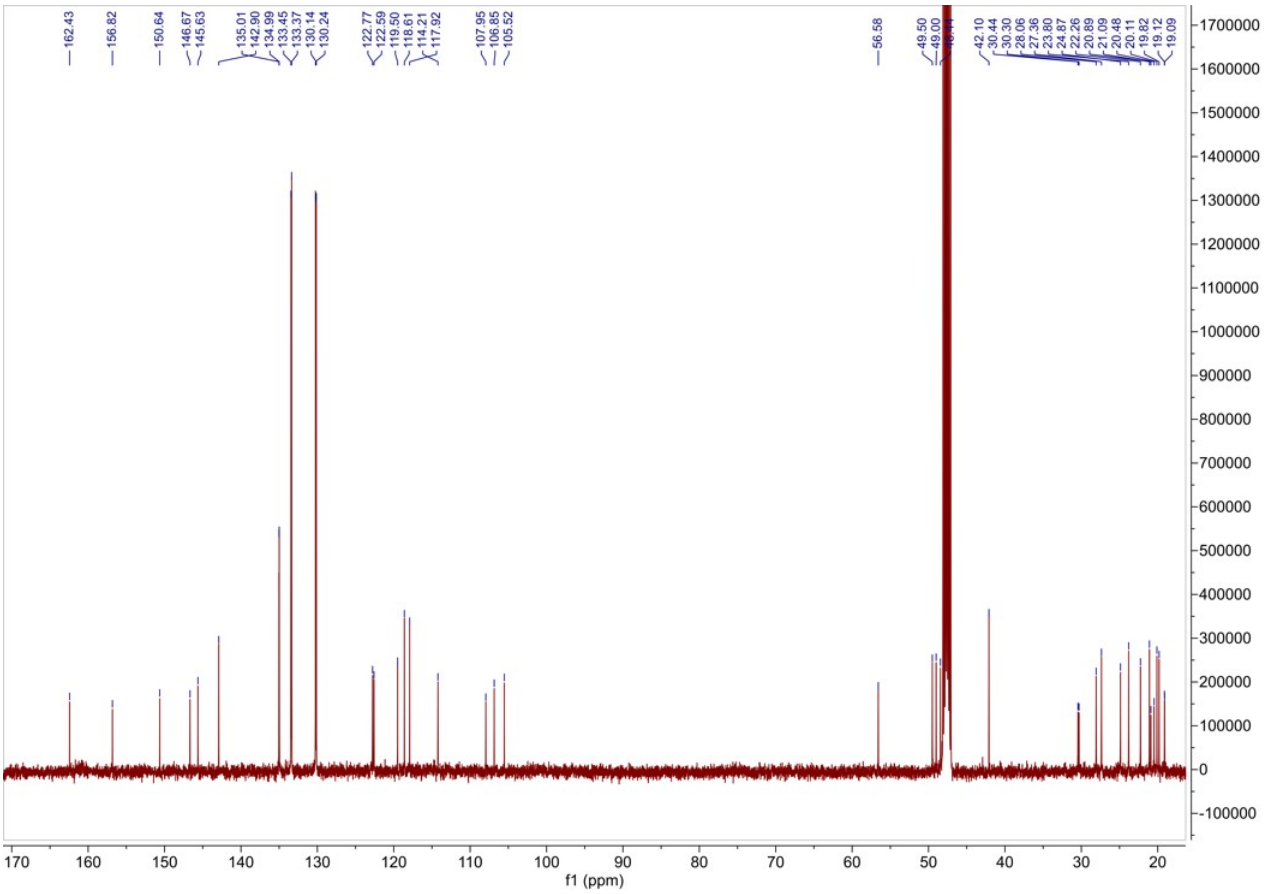
^{13}C NMR (126 MHz, MeOD) δ 162.43, 156.82, 150.64, 146.67, 145.63, 142.90, 135.01, 134.99, 133.45, 133.37, 130.24, 130.14, 122.77, 122.59, 119.50, 118.61, 117.92, 114.21, 107.95, 106.85, 105.52, 56.58, 49.50, 49.00, 48.44, 42.10, 30.44, 30.30, 28.06, 27.36, 24.87, 23.80, 22.26, 21.09, 20.89, 20.48, 20.11, 19.82, 19.12, 19.09.

HRMS $[M+H]^+$: Expected 751.4004, Found 750.3942

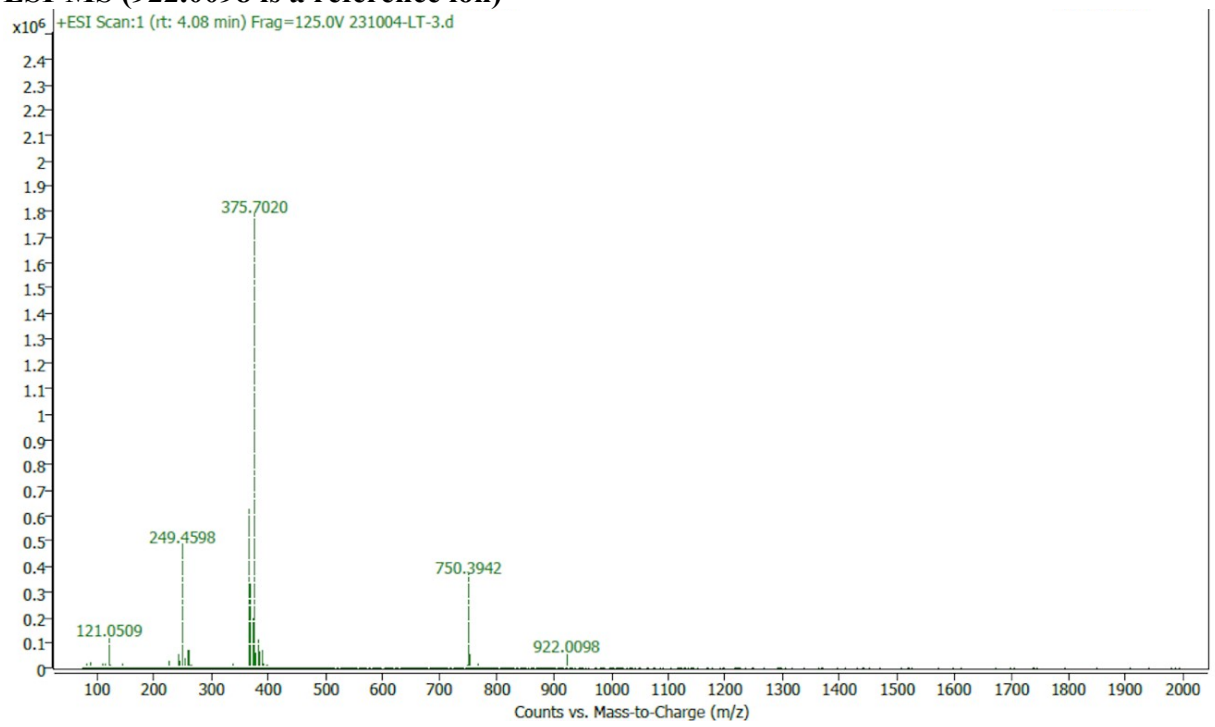
MitoRFAP-1 ^{1}H NMR



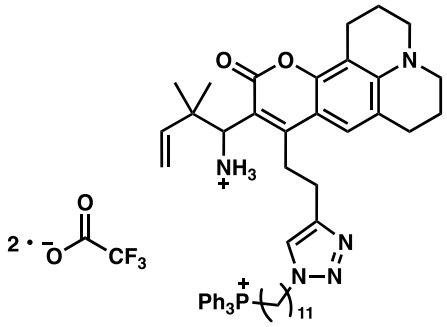
MitoRFAP-1 ^{13}C NMR



ESI-MS (922.0098 is a reference ion)



MitoRFAP-2



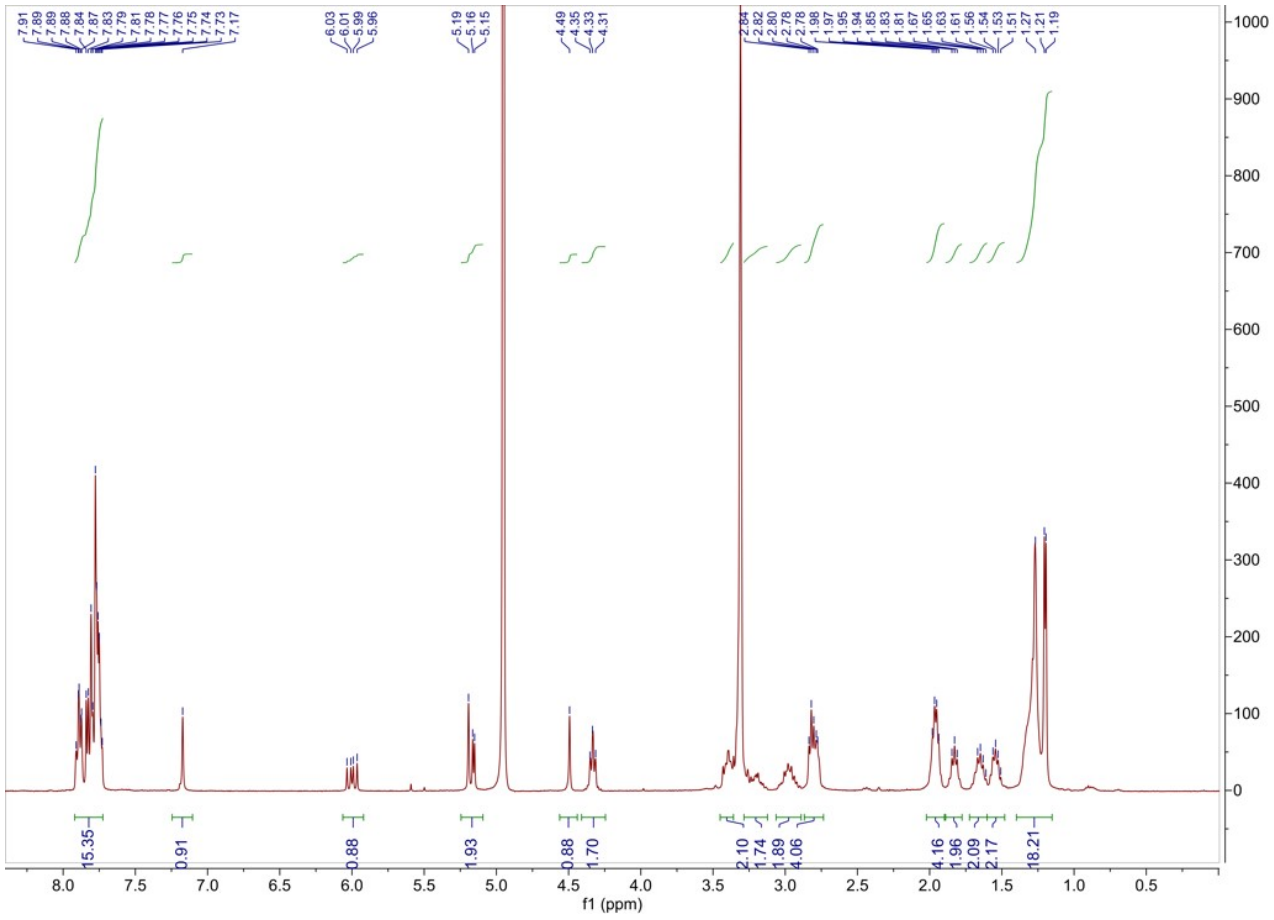
RFAP-alkyne (1 equiv., 80 mg, 0.205 mmol) and 4-(azidoundecyl)triphenylphosphonium bromide (1.1 equiv., 121 mg, 0.225 mmol) were added to a vial under N_2 and the vial was sealed, DMF (1 mL) was then added. $CuSO_4 \bullet 5H_2O$ (20 mol%, 10.2 mgs, 0.0410 mmol) dissolved in H_2O (0.5 mL) was added to the vial followed by sodium ascorbate (20 mol%, 8.1 mg, 0.041 mmol) dissolved in H_2O (0.5 mL). The reaction was stirred overnight under N_2 at room temperature. The solvent was then removed by rotary evaporation and residue was suspended in MeOH (2 mL) and filtered through cotton. The resulting solution was purified via reverse phase HPLC (25 to 50% ACN in H_2O + 0.1% TFA) to yield an orange solid (25 mg, 0.023 mmol, 11%).

^{1}H NMR (400 MHz, MeOD) δ 7.79 (m, 15H), δ 7.89 (s, 1H), 6.00 (dd, *J* = 17.5, 10.6 Hz, 2H), 5.18 (m, 2H), 4.49 (s, 1H), 4.33 (t, *J* = 7.9 Hz, 2H), 3.43-3.37 (m, 2H), 3.25-3.13 (m, 2H), 3.03-2.92 (m, 2H), 2.84-2.78 (m, 4H), 1.98-1.95 (m, 4H), 1.85-1.81 (m, 2H), 1.67-1.63 (m, 2H), 1.56-1.51 (m, 2H), 1.27-1.19 (m, 18H).

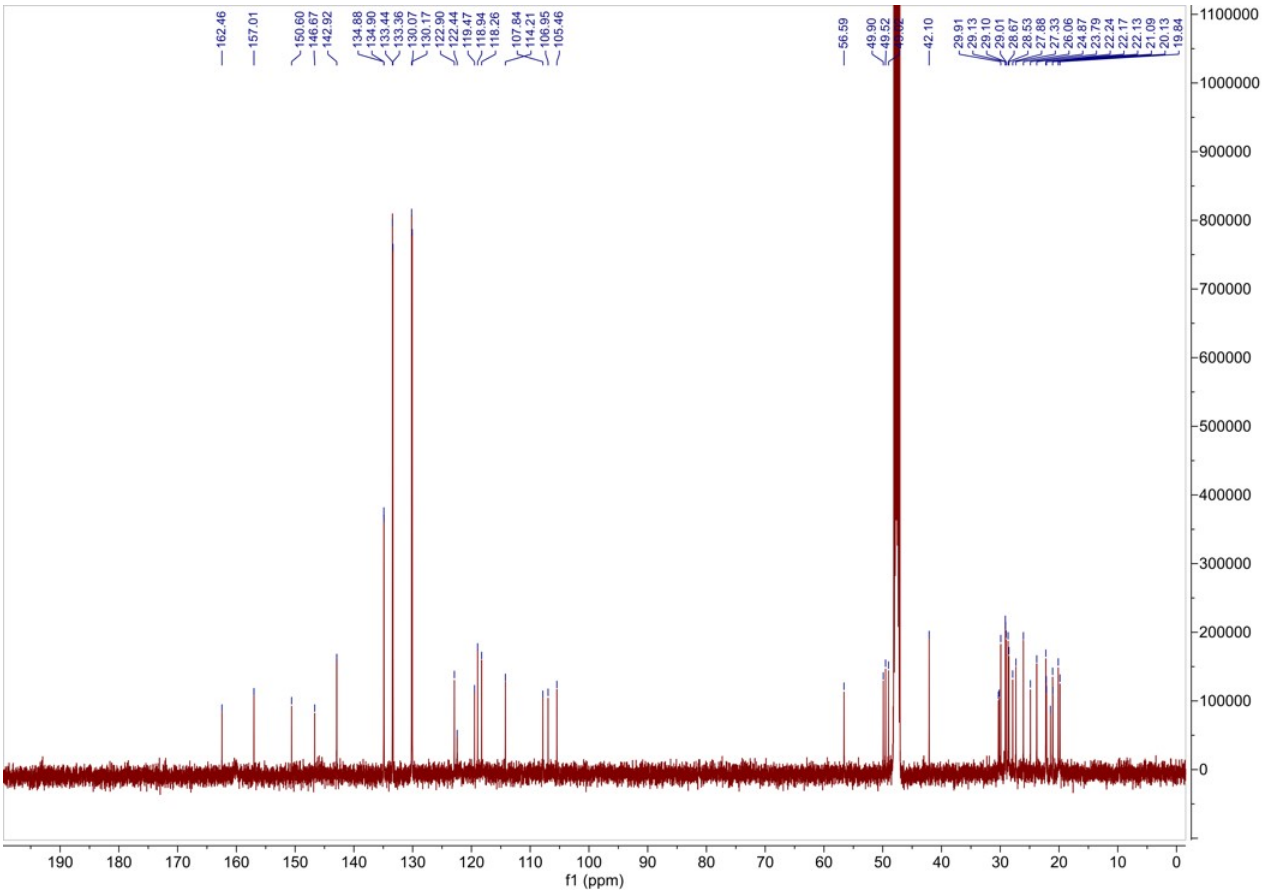
^{13}C NMR (126 MHz, MeOD) δ 162.46, 157.01, 150.60, 146.67, 142.92, 134.90, 134.88, 133.44, 133.36, 130.17, 130.07, 122.90, 122.44, 119.47, 118.94, 118.26, 114.21, 107.84, 106.95, 105.46, 56.59, 49.90, 49.52, 49.02, 42.10, 30.31, 30.18, 29.91, 29.13, 29.10, 29.01, 28.67, 28.53, 27.88, 27.33, 26.06, 24.87, 23.79, 22.24, 22.17, 22.13, 21.46, 21.09, 21.06, 20.13, 19.84.

HRMS $[(M+H)/2]^+$: Expected 424.7550 Found 424.7567

MitoRFAP-2 ^{1}H NMR



MitoRFAP-2 ^{13}C NMR



ESI-MS (922.0098 is a reference ion)