Chemical Proteomics Identifies SLC25A20 as a Functional Target of the Ingenol Class of Actinic Keratosis Drugs

Christopher G. Parker,[1]* Christian A. Kuttruff,[2] Andrea Galmozzi,[1] Lars Jørgensen,[3] Chien-Hung Yeh,[4] Dan Hermanson[1], Yujia Wang[1], Marta Artola,[2] Steven J. McKerrall,[2] Christopher M. Josyln,[1] Bjarne Nørremark,[3] Georg Dünstl,[3] Jakob Felding,[3], Enrique Saez,[1] Phil S. Baran,[2]* Benjamin F. Cravatt[1]*

[1]Department of Molecular Medicine, The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA, [2]Department of Chemistry, The Scripps Research Institute, La Jolla, CA [3] Research & Development, LEO Pharma, Denmark

*Correspondence: Christopher G. Parker (cparker@scripps.edu), Phil S Baran (pbaran@scripps.edu), and Benjamin F Cravatt, III (cravatt@scripps.edu)

Supporting Information

Supplemental Figures

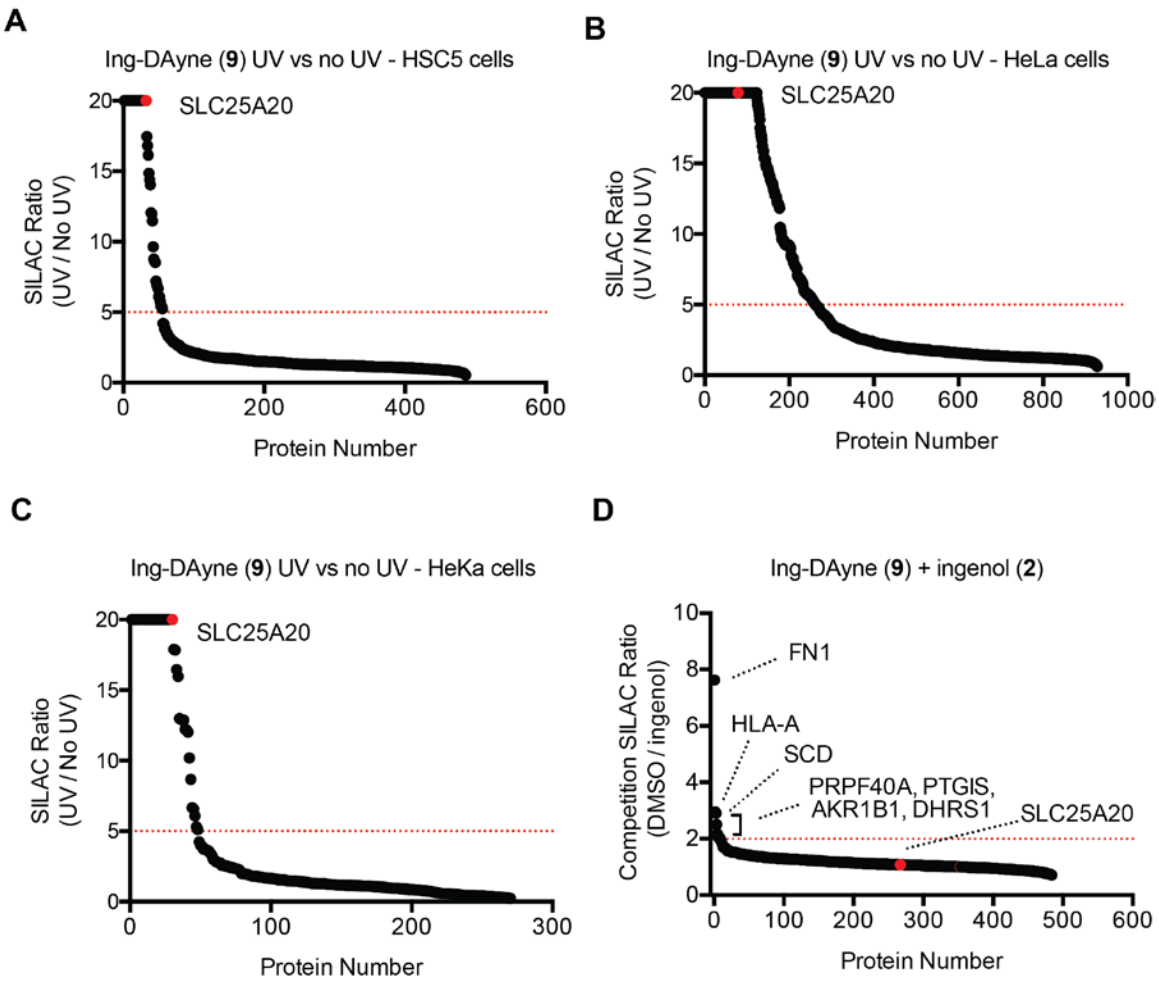


Figure S1. Quantitative MS-based proteomics showing UV-dependent enrichment of proteins by Ing-DAyne and competition of a subset of these proteins by ingenol. (A-C) UV-dependent enrichment of proteins by the Ing-DAyne probe in (A) HSC-5 cells, (B) HeLa cells, and (C) primary human keratinocytes (HeKa), where a cut-off of a five-fold enrichment over no-UV control experiments was used to designate substantially enriched targets of the probe. (D) Competition plot showing proteins for which enrichment by the Ing-DAyne probe (10 μM) was substantially blocked (> three-fold, red dotted line) by ingenol (100 μM) in HSC5 cells. All data represent the average from at least two biological replicates.

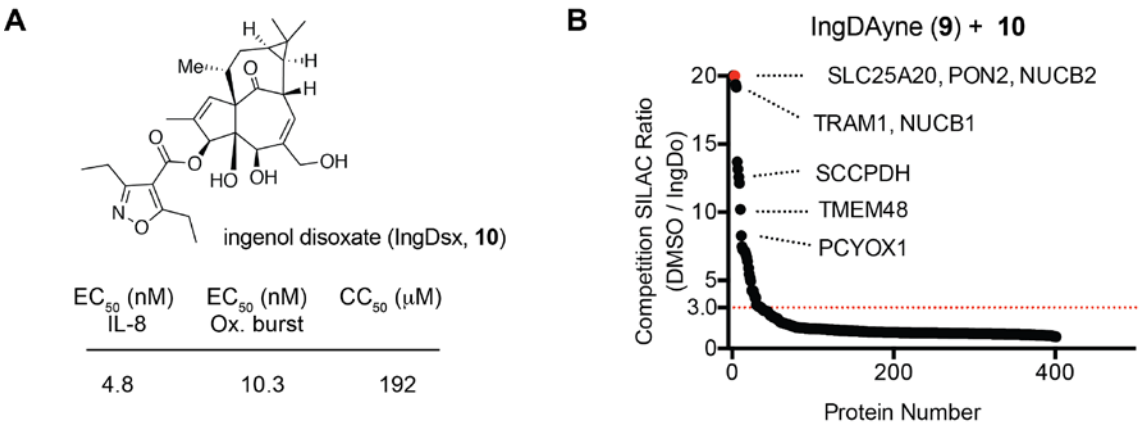


Figure S2. Target profiling of ingenol analog IngDsx (10). (A) Structure of ingenol disoxate IngDsx, and corresponding biological activity. (B) Competition plot showing proteins for which enrichment by the Ing-DAyne probe (10 μM) was substantially blocked (> three-fold, red dotted line) by IngDsx (100 μM) in HSC5 cells. Named proteins are representative targets that were also competitively blocked by IngMb treatment (see Figure 3C). Data represent the average from two biological replicates. See Table S1 for complete target lists.

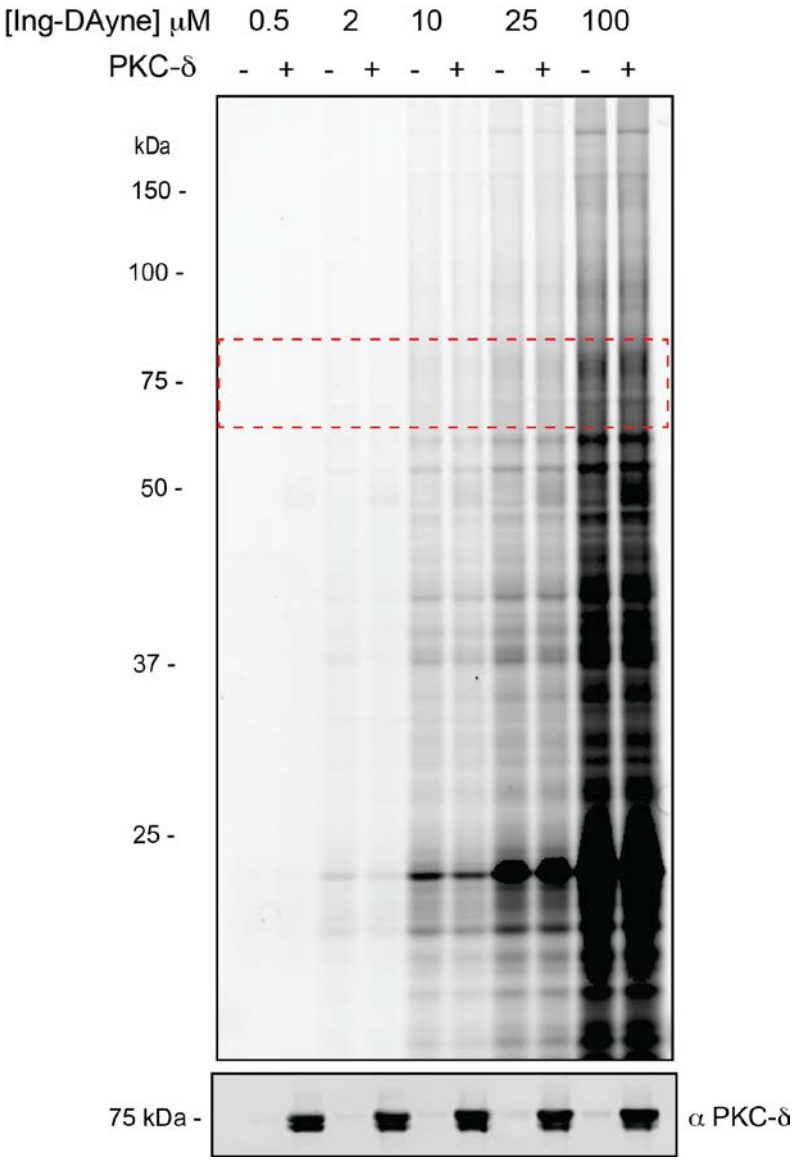


Figure S3. Gel-based profiling of human PKC-δ-transfected cells treated with Ing-DAyne. Upper image**,** fluorescent gel of HEK293T cells transfected with human PKC-δ (+) or a mock vector (-) followed by treatment with the Ing-DAyne probe at the indicated concentrations. Dotted red box indicates MW range where predicted Ing-DAyne labeling of PKC-δ should occur, as indicated by western blot (lower image).

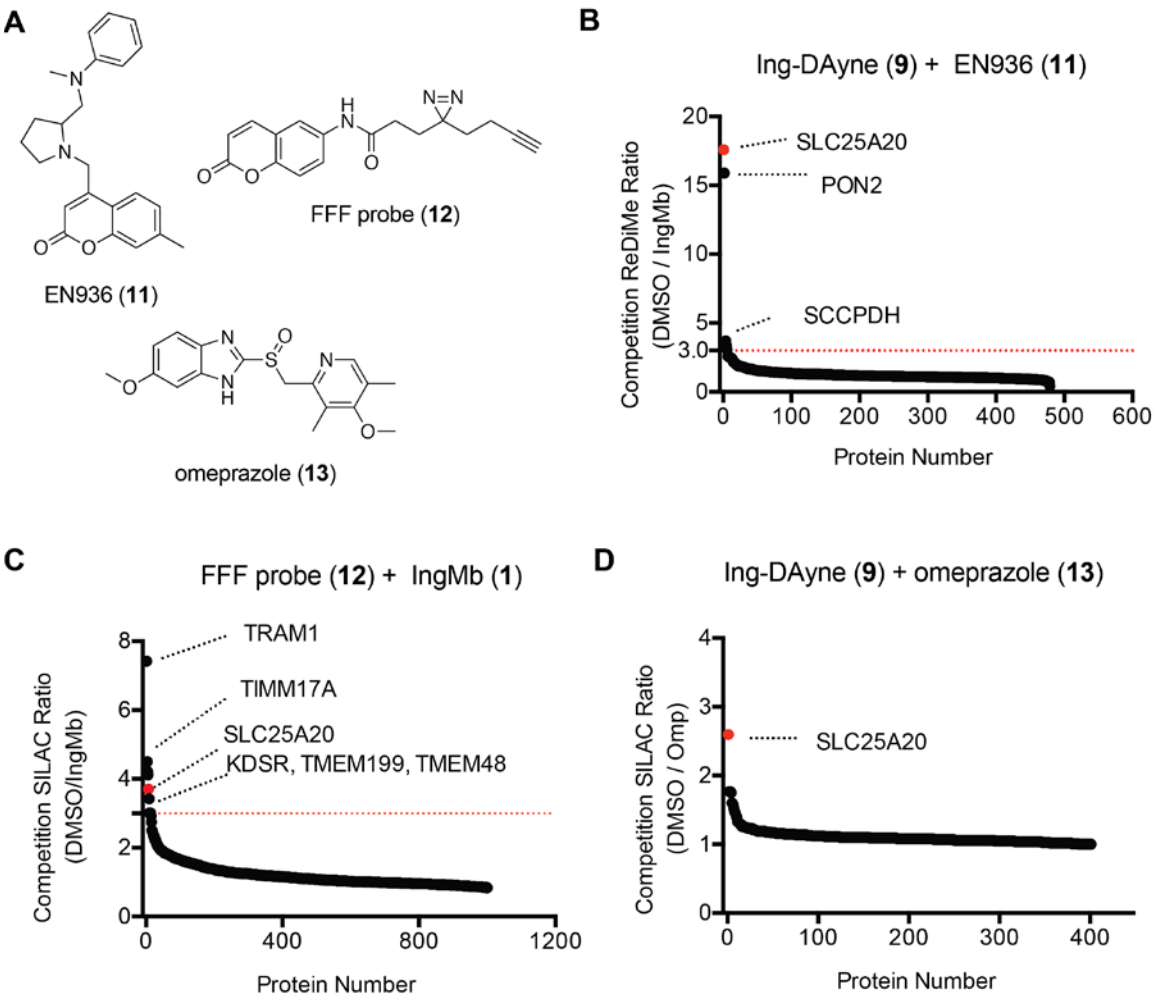
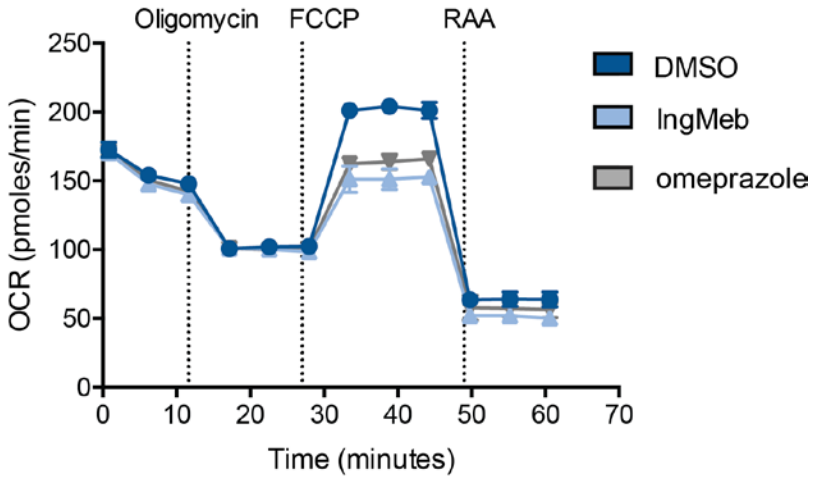


Figure S4. Competition of Ing-DAyne labeling of proteins with other SLC25A20 inhibitors. (A) Structures of reported SLC25A20 ligands. (B) Competition plot showing proteins for which enrichment by the Ing-DAyne probe (10 μM) was substantially blocked (> three-fold, red dotted line) by EN936 (160 μM) in HSC5 cells. (C) Competition plot showing proteins for which enrichment by the FFF probe **12** (20 μM) was substantially blocked (> three-fold, red dotted line) by IngMeb (100 μM) in HSC5 cells. For (B, C), data represent the average from two biological replicates. (D) Competition plot showing Ing-DAyne probe (10 μM) enrichment of SLC25A20 was significantly blocked by omeprazole **13** (100 μM). For (B-D), data represent the average from two biological replicates.

A



B

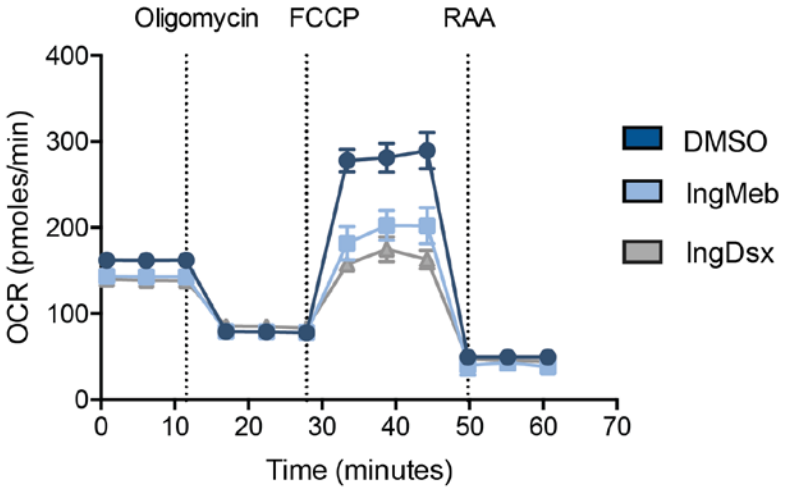


Figure S5. Full OCR plots of human cells treated with IngMeb, omeprazole or IngDsx. (A) IngMeb, omeprazole, and (B) IngDsx (100 µM) reduce exogenous fatty acid oxidation in HSC-5 cells. Oxygen consumption rate (OCR) of HSC-5 cells pre-treated for 40 min with indicated compounds and then provided exogenous palmitate. Data represent average values ± SD; n = 5-6 per group. Oligomycin is an inhibitor of ATP synthase; FCCP = carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone is an ionophore uncoupling reagent that collapses mitochondrial membrane potential, allowing maximal respiration; RAA = rotenone and antimycin A are complex I and complex III inhibitors that block mitochondrial respiration, enabling the calculation of non-mitochondrial respiration.

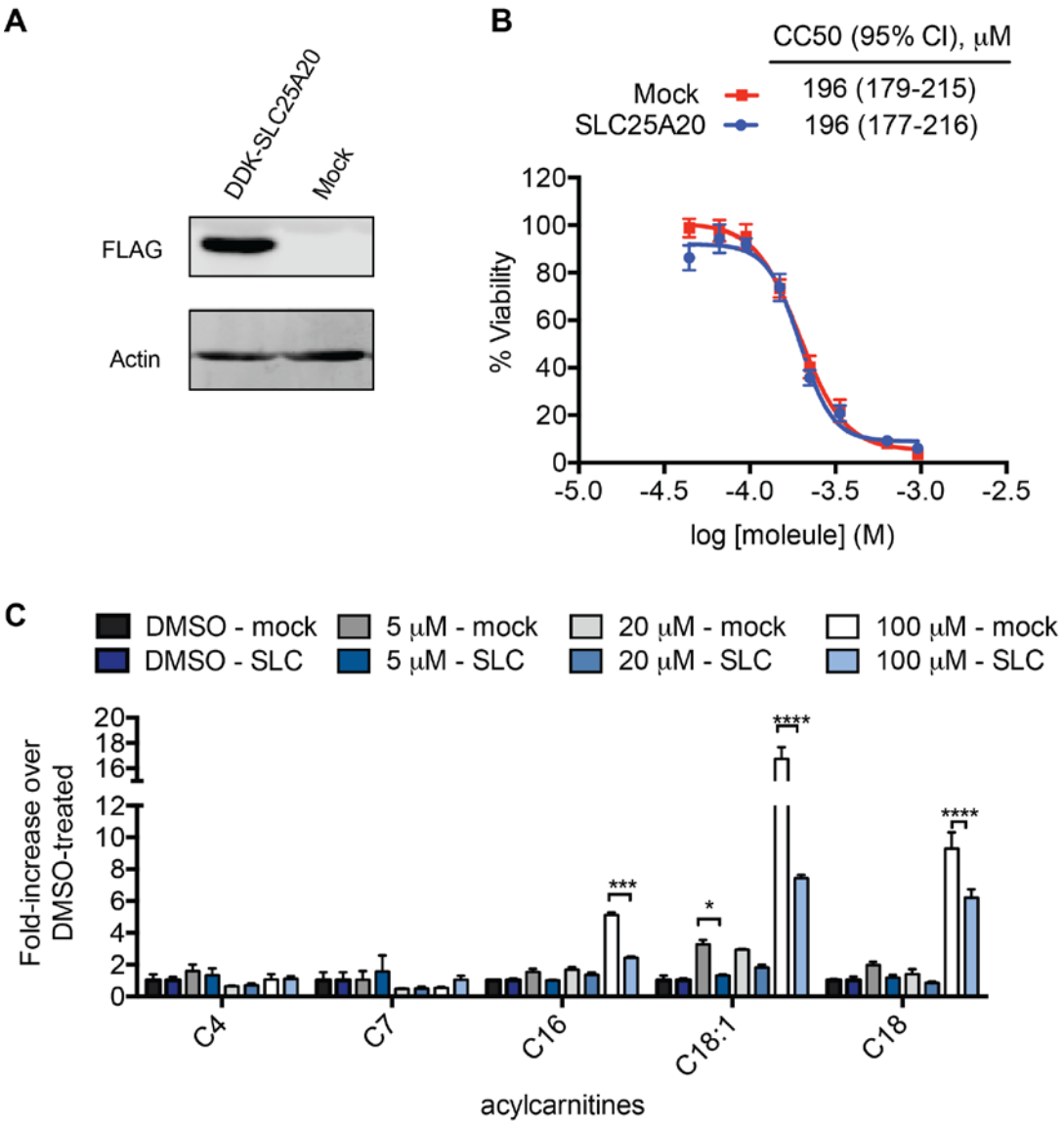


Figure S6. Effects of SLC25A20 overexpression on InMeb activity in cells. (A) Human DDK-tagged SLC25A20 recombinantly expressed by transient transfection in HeLa cells. Empty vector used as 'mock' control. (B) Expression of SLC25A20 in HeLa cells has minimal effects on IngMeb acute cell cytotoxicity. Assay described in Figure 1 and Supplemental Information, reproduced three independent times in triplicate. Representative data shown as average values ± SD; n = 3 per group. (C) Expression of SLC25A20 (SLC) in HeLa cells reduces the IngMeb-mediated buildup long-chain acylcarnitines. Data represent average values ± SD; n = 3 per group; *p < 0.05, ***p < 0.001 and ****p < 0.0001 for SLC25A20-overexpressing versus mock groups.

Cell Lines

HEK293T (ATCC) and HeLa (ATCC) cells were maintained in high-glucose DMEM (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM). HSC-5 (human squamous cell carcinoma, Health Science Research Resource Bank, Japan) cells were maintained in high-glucose IMDM (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL). HeKa (primary human keratinocytes, adult, Invitrogen) were grown in serum-free EpiLife medium with HKGS supplement containing 60 µM calcium following supplier's instructions, and were used between passages 2-10. All cell lines were grown at 37 ºC in a humidified 5% CO_2 atmosphere. For SILAC experiments, each cell line was passaged at least six times in either SILAC DMEM or SILAC IMDM, (Thermo), which lack L-lysine and L-arginine, and supplemented with 10% (v/v) dialyzed FBS (Gemini), PSQ (as above), and either [$^{13}C_6$, $^{15}N_2$]- L-lysine and [$^{13}C_6$, $^{15}N_4$]-L-arginine (100 µg/mL each) or L-lysine•HCl and L-arginine•HCl (100 µg/mL each). Heavy and light cells were maintained in parallel and cell aliquots were frozen after six passages in SILAC media and stored in liquid N_2 until needed. Whenever thawed, cells were passaged at least three times before being used in experiments.

Neutrophil oxidative burst assay

Primary human PMN (polymorphonuclear leukocytes) were isolated and purified from fresh buffy coats by sequential sedimentation, density centrifugation and lysis of contaminating erythrocytes. Buffy coats were incubated with 2% methocel for 30-45 min to differentially sediment red blood cells. The leukocyte-rich supernatant was transferred to Lymphoprep^{TM} tubes to remove mononuclear cells by density centrifugation (400xg, 30 min). The pellet was re-suspended and any remaining erythrocytes lysed using 0.2% NaCl for 30 sec before restoring isotonicity by the addition of 1.2% NaCl. This step was repeated until the cell pellet appeared relatively free of red blood cells. Cells were re-suspended in DPBS (Dulbecco's Phosphate Buffered Saline; without Ca2+, Mg2+) and the

concentration adjusted to 1.4x10E6 cells/ml in HBSS (Hanks Balanced Salt solution; with Ca2+, Mg2+) containing 0.1% BSA (Bovine Serum Albumin) and 5mM glucose just prior to assay initiation. Titrated reference and test compounds were pre-mixed with HE (hydroethidine; 10µM final assay concentration) before addition to 96-well plates containing 2.5xE5 cells. Following 40 min incubation at RT, changes in the respiratory burst were estimated by measuring fluorescence at 579 nm (excitation: 485 nm) using an EnVision plate reader.

Test compound titration curves were fitted to a four-parameter sigmoidal curve after normalizing the effect of the test compound to the effect of the positive control (500 nM ingenol mebutate). Relative EC50 is defined as the concentration of test compound producing an effect that is midway between the fitted top and bottom. Absolute EC50 is the concentration of test compound that provokes a response corresponding to 50% of the maximal effect associated with the positive control (500 nM ingenol mebutate). Results represent average of two independent determinations run in duplicate on individual plates.

HeKa cytokine release assay (IL-8)

Primary human epidermal keratinocytes (HeKa) were seeded (10,000 cells/well) in 96-well plates the day before the assay. Test compounds were diluted in DMSO (dimethyl sulfoxide) and further diluted in assay medium and pipetted into wells of 96 well-plates containing HeKa cells. The plates were incubated for 6 hr at 37°C in humidified air with 5% CO2. Plates were centrifuged briefly to spin down cells at 4°C, the supernatant was removed and analyzed by Meso Scale Discovery (MSD) 4-spot cytokine assay (Pro-inflammatory II Ultra Sensitive kit, MSD, MD, USA). The MSD assay employs a sandwich immunoassay format where capture antibodies are coated in a patterned array on the bottom of the wells of a 4-Spot- Multi-MSD plate. Standard samples were incubated in the MULTI-SPOT plates as well, and the cytokine (IL-8) binds to its corresponding capture antibody spot. The cytokine level was quantitated on a

SECTOR™ Imager using a cytokine-specific Detection Antibody labeled with MSD SULFO-TAG™ reagent.

Test compound titration curves were fitted to a four-parameter sigmoidal curve after normalizing the effect of the test compound to the effect of the positive control (150 nM IngMeb). Results represent average of two independent determinations run in duplicate on individual plates.

Human PKC delta (PKCδ) activation assay

The potency and efficacy of test compounds in stimulating human recombinant protein kinase C (PKC) delta isoform was determined by measuring phosphorylation of a PKC substrate peptide using ^{33}P-ATP. Data points for the EC50 determinations were performed in duplicate. The assay was performed at Millipore (Dundee, UK).

Human PKC delta (PKCδ, Millipore cat# 14-504) was diluted in 20 mM HEPES, 0.03% Triton X-100. Amount of enzyme in each assay was 7.25 ng, final assay volume 25 µl. All compounds for testing were diluted to 1 mM in 100% DMSO as an intermediary dilution. The compounds were then diluted further to 50 µM, and then serially diluted in 100% DMSO in semi-logarithmic decrements for 12 points. 0.5 µL of each concentration, in duplicate, was pipetted into a dry 96 well assay plate using a TTP Mosquito. Control and blank wells received 0.5 µL of 100% DMSO instead of compound. This was followed by addition of 14.5 µL of assay mixture, containing appropriately diluted enzyme and 20 mM HEPES pH 7.4, 0.03% Triton X-100, 0.05 mg/mL phosphatidylserine, and 50 µM of the substrate peptide ERMRPRKRQGSVRRRV. The assay was started with the addition of 10 µL ATP containing γ-33P-ATP (specific activity approx. 500 cpm/pmol) to a final assay concentration of 15 µM. The reaction was allowed to proceed at room temperature for 40 minutes before the addition of 5 µL 3% ortho-phosphoric acid. Blank wells were acid blanks, and had 5 µL 3% ortho-phosphoric acid added before the addition of ATP. 10 µL of the stopped reaction products was transferred to a P30 filtermat which was then washed 4 times in 75 mM ortho-

phosphoric acid, and once in methanol before drying. The filter was read by liquid scintillation counting using a Wallac Trilux.

Acute cancer cell cytotoxicity assay

HeLa cells were seeded at 4×10^3 cells/well and incubated overnight in MEM containing 10% FBS at 37 °C in humidified air/CO_2 (95%/5%). Cells were treated with increasing concentrations of test compound for 30 min at 37 °C. Mitochondrial activity as a sign of cell viability was determined subsequently by incubation with the resazurin-based dye formulation PrestoBlue® (Invitrogen) for 30 min. Active mitochondria convert PrestoBlue to a fluorescent dye which reflects cell viability and can be quantified at 615 nm using an EnVision plate reader. Complete loss of mitochondrial activity was taken as a measure of cell death. Cytotoxic concentration (CC50) was calculated as the concentration producing 50% loss of mitochondrial activity.

***In situ* labeling of live cells with photoaffinity probes for gel- and MS-based experiments**

For gel-based experiments, cells were grown in 6-well plates to ~90% confluence at the time of treatment. Cells were carefully washed with Dulbecco's phosphate buffered saline (DPBS) and replenished with fresh serum-free media containing indicated probe, and, if applicable, competitors or DMSO vehicle (1 mL). Following incubation at 37 °C for 30 min, cells were directly exposed to 365 nm light for 10 min at 4 °C using a Stratagene UV Stratalinker 1800. For no UV experiments, cells were incubated at 4 °C for 10 min under ambient light. For MS-based experiments, cell labeling was performed in a similar manner as described above. Modifications to this protocol included using isotopically 'light' and 'heavy' SILAC cells that were grown to near complete confluence prior to treatment in 10 cm plates (using 5 mL of media containing probe +/- test compound). For MS-based experiments using primary keratinocytes, cells were grown in 15 cm plates (using 12 mL of media containing probe +/- test compound), with 'light' and 'heavy' designations corresponding to cells that will

be eventually labeled with isotopically 'light' or 'heavy' formaldehyde. In UV-versus no UV experiments, isotopically light and heavy cells were treated with indicated probe, however, only the light cells were photocrosslinked as described above while the heavy cells were not. In competition type experiments, heavy and light cells were co-treated with the indicated probe and competitor or DMSO, respectively. Following treatments and photocrosslinking for both gel- and MS-based experiments, cells were harvested in cold DPBS by scraping, centrifuged (1,400 *g*, 3 min, 4 °C), and pellets washed with cold DPBS (2X) and then aspirated. Pellets were either directly processed or kept frozen at -80 °C until use.

Preparation of probe-labeled proteome for gel- and MS-based protein analyses

Cells pellets were lysed in cold DPBS (100-500 μL) using a Branson Sonifier probe sonicator (10 pulses, 30% duty cycle, output setting = 4). For experiments requiring cell fractionation into membrane and soluble proteomes, cell lysates were then centrifuged (100,000 x g, 45 min) to provide soluble (supernatant) and membrane (pellet) fractions. Membrane pellets were resuspended in cold DPBS after separation by sonication. Protein concentration was determined using the DC Protein Assay (Bio-Rad) and absorbance read using a Tecan, Infinite F500 plate reader following manufacturer's instructions. For SILAC and ReDiMe experiments, whole cell lysates were adjusted to 1.5 mg/mL, and for SILAC experiments were then mixed in equal proportions (500 μL each) in cold DPBS. For ReDiMe experiments, lysates were kept separate.

Gel-based analysis of crosslinked proteins in cells

Proteomes from treated cells were diluted to 1 mg/mL. To each sample (50 μL), 6 μL of a freshly prepared "click" reagent mixture containing 0.1 mM tris(benzyltriazolylmethyl)amine (TBTA) (3 μL/sample, 1.7 mM in 1:4 DMSO:*t*-ButOH), 1 mM $CuSO_4$ (1 μL/sample, 50 mM in H_2O), 25 μM tetramethylrhodamine (TAMRA) azide (1 μL/sample, 1.25 mM in DMSO), and

freshly prepared 1 mM tris(2-carboxyethyl)phosphine HCl (TCEP) (1 μL/sample, 50 mM in PBS or H_2O) was added to conjugate the fluorophore to probe-labeled proteins. Upon addition of the click mixture, each reaction was immediately mixed by vortexing and then allowed to react at ambient temperature for 1 hr before quenching the reactions with SDS loading buffer (4X stock, 17 μL). Proteins (25 μg total protein loaded per gel lane) were resolved using SDS-PAGE (10% acrylamide) and visualized by in-gel fluorescence on a Bio-Rad ChemiDoc™ MP flatbed fluorescence scanner. Gel fluorescence and imaging was processed using Image Lab (v 5.2.1) software.

Preparation of labeled proteome for MS-based analysis – SILAC

Profiling experiments were adapted methods previously reported.[1] To the combined mixture of heavy and light proteomes (1.5 mg) in 1 mL DPBS, a mixture of TBTA (60 μL/sample, 1.7 mM in 1:4 DMSO:t-BuOH), CuSO4 (20 μL/sample, 50 mM in H2O), TCEP (20 μL/sample, 50 mM in DPBS) and Biotin-N_3 (10μL/sample, 10 mM in DMSO) was added and each sample was rotated at room temperature. After 1 hr, the mixture was transferred to a 15 mL falcon tube and a cold 4:1 mixture (2.5 mL) of methanol (MeOH)/chloroform ($CHCl_3$) was added followed by cold PBS (1 mL) on ice. The resulting cloudy mixture was centrifuged (5,000 x *g*, 10 min, 4°C) to fractionate the protein interphase from the organic and aqueous solvent layers. After washing the protein disc carefully with cold 1:1 MeOH:$CHCl_3$ (3 x 1 mL) followed by sonication in cold 4:1 MeOH:$CHCl_3$ (3 mL) to ensure click reagents were efficiently removed, the remaining precipitate was pelleted by centrifugation (5,000 x *g*, 10 min, 4°C). The pellet was aspirated and resuspended in a freshly-prepared solution of proteomics-grade urea (500 μL, 6 M in DPBS) containing 10 μL of 10% SDS and then dissolved by sonication. Disulfides were reduced by adding 50 μL of a 1:1 mixture containing TCEP (200 mM in DPBS) pre-neutralized with potassium carbonate (600 mM DPBS) for 30 min at 37°C. Reduced thiols were then alkylated by addition of iodoacetamide (70 μL of 400 mM in DPBS) for 30 min at ambient temperature protected from light. To each solution, 130 μL of 10% SDS (in DPBS) was added

and then diluted to ~0.2% SDS with DPBS (5.5 mL) and incubated with pre-equilibrated streptavidin agarose resin (100 µL 1:1 slurry, Pierce) for 1.5 hr at ambient temperature on a rotator. The streptavidin beads were collected by centrifugation (1,400 *g*, 1–2 min) and sequentially washed with 0.2% SDS in DPBS (1 x 5 mL), detergent-free DPBS (2 x 5 mL), and H_2O (2 x 5 mL) to remove unbound protein, excess detergent, and small molecules. The resin was transferred to a Protein LoBind tube (Eppendorf) and bound proteins were digested on-bead overnight at 37°C in ~200 µL total volume containing sequencing grade porcine trypsin (2 µg, Promega) in the presence of urea (2 M in DPBS) and $CaCl_2$ (1 mM). The proteolyzed supernatant was transferred to a fresh Protein LoBind tube, acidified with formic acid (5% final) and stored at –20°C until analyzed.

Preparation of labeled proteome from primary keratinocytes for MS-based analysis – Reductive dimethylation (ReDiMe)

Profiling experiments were adapted methods previously reported.[2,3] HeKa cells and lysates were prepared as described above and samples to be isotopically labeled with either 'light' or 'heavy' formaldehyde were kept separate at 500 µL 1.5mg/mL solutions in DPBS. To the separated lysates, a mixture of TBTA (30 µL/sample, 1.7 mM in 1:4 DMSO:t-BuOH), CuSO4 (10 µL/sample, 50 mM in H2O), TCEP (10 µL/sample, 50 mM in DPBS) and Biotin-N_3 (5µL/sample, 10 mM in DMSO) was added and each sample was rotated at room temperature. After 1 hr, the mixture was transferred to a 15 mL falcon tube and a cold 4:1 mixture (2.5 mL) of methanol (MeOH)/chloroform ($CHCl_3$) and centrifuged (5,000 x *g*, 10 min, 4°C) to pellet the proteome. Pellet was aspirated and resuspended in cold 1:1 MeOH:$CHCl_3$ (3 mL) followed by sonication and the remaining precipitate was pelleted by centrifugation (5,000 x *g*, 10 min, 4°C) and washed once more with 4:1 MeOH:$CHCl_3$ (3 mL) to ensure click reagents were efficiently removed. The pellet was aspirated and resuspended in a freshly-prepared solution of proteomics-grade urea (500 µL, 6 M in DPBS) containing 10 µL of 10% SDS and then dissolved by sonication. Disulfides were reduced by adding 50 µL of a 1:1

mixture containing TCEP (200 mM in DPBS) pre-neutralized with potassium carbonate (600 mM DPBS) for 30 min at 37°C. Reduced thiols were then alkylated by addition of iodoacetamide (70 µL of 400 mM in DPBS) for 30 min at ambient temperature protected from light. To each solution, 130 µL of 10% SDS (in DPBS) was added and then diluted to ~0.2% SDS with DPBS (5.5 mL) and incubated with pre-equilibrated streptavidin agarose resin (100 µL 1:1 slurry, Pierce) for 1.5 hr at ambient temperature on a rotator. The streptavidin beads were collected by centrifugation (1,400 *g*, 1–2 min) and sequentially washed with 0.2% SDS in DPBS (1 x 5 mL), detergent-free DPBS (2 x 5 mL), and H_2O (2 x 5 mL) to remove unbound protein, excess detergent, and small molecules. The resin was transferred to a Protein LoBind tube (Eppendorf) and bound proteins were digested on-bead overnight at 37°C in ~200 µL total volume containing sequencing grade porcine trypsin (2 µg, Promega) in the presence of urea (2 M in 100mM triethylammonium bicarbonate, TEAB) and $CaCl_2$ (1 mM). Designated isotopically 'light' and 'heavy' digests were treated with 8 µL of 4% light formaldehyde ($C^{12}H_2O$, in water, made from 37% stock, Aldrich, 252549) or heavy formaldehyde ($C^{13}D_2O$, in water, made from 20% stock, Aldrich, 596388), respectively, followed by 8 µL of 0.6M sodium cyanoborohydride (in water, Aldrich) and incubated for 1 hr at room temperature while shaking. Reactions were quenched by the addition of 32 µL of NH_4OH (1% in water) to each sample, followed by 16 µL of formic acid (5% final). Corresponding heavy and light resin/supernatant were combined and centrifuged (3 min, 1500rpm), and supernatant was transferred to a fresh LoBind tube. Resin was washed once with water (100 µL) and washings were separated from the resin and added to the previously separated supernatant. Solutions can be stored at stored at -20°C until analyzed.

Multidimensional liquid chromatography-tandem mass spectrometry (LC/LC-MS/MS) analysis of tryptic digests

Peptides from tryptic digests were pressure loaded onto a 250 µm (inner diameter) fused silica capillary column packed with C18 resin (4 cm, Aqua 5 µm,

Phenomenex). Samples were analyzed using an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) coupled to an Agilent 1200 series quaternary pump. Peptides were eluted by two-dimensional separation on a column with a 5 µm tip [100 µm fused silica, packed with C18 (10 cm) and strong cation exchange (SCX) resin (4 cm, Phenomenex)] using a five-step 'MudPIT' protocol [4] that involves 0%, 25%, 50%, 80% and 100% salt bumps of ammonium acetate (NH_4OAc; 500 mM) to elute peptides stepwise from the SCX to the C18 resin followed by an increasing gradient of acetonitrile in each step (5%–100% buffer B in buffer A; buffer A: 95% H_2O, 5% acetonitrile, 0.1% formic acid; buffer B: 5% H_2O, 95% acetonitrile, 0.1% formic acid). The flow rate through the column was 0.25 µl/min and the voltage applied to the nano-LC electrospray ionization source was 2.5 kV. Spectra were collected in a data-dependent acquisition mode such that each scan cycle involved a single high-resolution full MS spectrum of parent ions (MS1 scan from 400–1800 *m/z*) collected in the orbitrap coupled to 30 CID-induced fragmentation (MS2) scans in the ion trap of the 30 most abundant parent ions from the MS1 scan. Dynamic exclusion (repeat count of 1, exclusion duration of 20 s). Parent ions with unassigned or +1 charge states by the instrument were excluded for fragmentation. All other parameters were left at default values.

Peptide and protein identification and quantification

From each of the five .raw files (one for each salt 'bump') generated by the instrument (Xcalibur software), the MS2 spectra for all fragmented parent ions (.ms2 file) were extracted using RAW Xtract (version 1.9.9.2; 2004 release). Each .ms2 file was searched using the ProLuCID algorithm against a reverse-concatenated, nonredundant (gene-centric) database of the human proteome (Uniprot release –11/05/2012) or mouse proteome (11/05/2012) and filtered using DTASelect 2.0 within the Integrated Proteomics Pipeline (IP2) software. All cysteine residues were specified with a static modification for carbamidomethylation (+57.0215 Da) and one oxidized methionine residue per peptide (if found) was allowed as a variable oxidation (+15.9949 Da). In addition,

peptides were required to have at least one tryptic terminus. For ReDiMe samples, each dataset was simultaneously searched for both light and heavy isotopologues of the same peptide by specifying the mass shift of light and heavy residues as static modifications; for the ‘light’ search, static modifications on lysine and N-termini (+28.0313 *m/z*) were specified; for the ‘heavy’ search, static modifications on lysine (+34.06312 *m/z*) and N-termini were specified (+34.06312 *m/z*). For SILAC samples, each dataset was simultaneously searched for both light and heavy isotopologues of the same peptide by specifying the mass shift of heavy residues as static modifications on lysine (+8.0142 Da) and arginine (+10.0082 Da) in a coupled ‘heavy’ search. The precursor ion mass tolerance for a minimum envelope of three isotopic peaks was set to 50 ppm, the minimum peptide length was six residues, the false-positive rate was set at 1% or lower and at least 2 peptides of a protein must be detected in order to be advanced to the next step of analysis.

Heavy and light parent ion chromatograms associated with successfully identified peptides were extracted and compared using in-house software (CIMAGE) as previously described [5]. Briefly, extracted MS1 ion chromatograms (± 10 ppm error tolerance of predicted *m/z*) from both 'light' and 'heavy' target peptide masses (m/z) were generated using a retention time window (± 10 min) centered on the time when the peptide ion was selected for MS/MS fragmentation (minimum 3 MS1’s per peak), and subsequently identified. Next, the ratio of the peak areas under the light and heavy signals (signal-to-noise ratio > 2.5) was calculated. Computational filters used to ensure that the correct peak-pair was used for quantification include a co-elution correlation score filter ($R^2 \geq 0.8$), removing target peptides with bad co-elution profile, and an 'envelope correlation score' filter ($R^2 > 0.8$) that eliminates target peptides whose predicted pattern of the isotopic envelope distribution does not match the experimentally observed high-resolution MS1 spectrum. In addition, peptides detected as ‘singletons,’ where only the heavy ion of a peptide pair was identified, but that cleared all other filtering parameters, are given a default assigned ratio of ‘20,’ which is

defined as any measured ratio that is ≥ 20 and is the maximum ratio reported here. Unprocessed raw data for multiple examples of each experimental type provided in **Table S1.**

Proteomic analysis of probe-labeled proteins by mass spectrometry

Median SILAC/ReDiMe ratios were filtered to ensure that each protein ratio was resultant from three or more unique and quantified peptides and that the combined peptide ratios possessed a standard deviation of less than 10. SILAC/ReDiMe ratios meeting these criteria were then averaged across replicate datasets from the same cells and treatment conditions; if the median ratios were greater than 60% of the mean, the ratio was assigned the lowest median ratio. All MS-based experiments were performed in two more biological replicates. Serum-derived/contaminant Keratin, Albumin, Trypsin, Hornerin and Statherin proteins manually removed from protein lists.

In order to be classified as a probe target, proteins must (1) comply with the above criteria and (2) be enriched greater than 5-fold in UV versus no-UV experiments. Targets fulfilling these criteria are shown in **Table S1** along with representative raw data sets. For competition experiments, in order to be designated a target of the competitor (e.g. ingenol mebutate), proteins (1) must be designated probe targets, as described above and (2) competed greater than 3-fold (competition SILAC/ReDiMe ratio >3) unless otherwise noted. Targets fulfilling these criteria are shown in **Table S1** along with representative raw data sets.

Cloning and transient overexpression of proteins in HEK293T cells

Unless otherwise noted, cDNAs were cloned from hORFeome library using Gateway cloning technology. Genes of interest were cloned from hORFeome entry vectors into a pRK5 expression vector with a C-terminal FLAG tag following standard Gateway protocols. Human SLC25A20 in a pCMV6-Entry vector with a C-terminal FLAG tag was purchased from Origene. Empty pCMV-Entry vector was used as ‘mock’ control for experiments with SLC25A20. Human PRKCD

cDNA in pCMV-SPORT6 vector was purchased from GE Dharmacon (Accession BC043350, Clone ID 5539909). Empty pCMV-SPORT6 vector was used as 'mock' control for experiments with PRKCD. To recombinantly overexpress proteins used in *in situ* treatments, HEK293T cells were grown to 40-60% confluency under standard growth conditions in 6-well (for gel-based experiments) or 10 cm tissue culture plates (for MS-based experiments) and transiently transfected with 0.5-3 μg of desired construct (6-well plates) or 5 μg (10 cm plates) using polyethyleneimine 'MAX' (MW 40,000, PEI; Polysciences, Inc.). 'Mock' transfected cells were transfected with a vector containing METAP2 for 48 hr.

For cytotoxicity experiments with HeLa transfected cells, cell transfected as described above, for 24 hours. Cell then harvested and seeded into 96-well plates overnight and treated as described above.

Western blot analysis

After scanning for fluorescence, proteins were transferred to a nitrocellulose membrane in Towbin buffer, the membrane was blocked for ~1 hr at ambient temperature with 5% nonfat dry milk (w/v) or 5% BSA in Tris-buffered saline with Tween 20 (TBST) and incubated with primary antibodies in the same solution overnight at 4ºC. Blots were washed (3 × 5 min, TBST), incubated with secondary antibodies (IRDye 800CW) in milk or BSA for 1 hr at ambient temperature, washed (3 × 5 min, TBST), rinsed in water and visualized on a LICOR Odyssey Scanner. Anti-FLAG (Sigma-Aldrich, F1804), IRDye 800CW anti-rabbit (LICOR, 925-32211), IRDye 800CW anti-mouse (LICOR, 925-32210), rabbit anti-PKC delta polyclonal (ThermoFisher, PA5-13744), rabbit anti-beta-actin (Cell Signaling Technology, 4970).

LCMS analysis of acylcarnitines in HSC-5 cells

Profiling experiments were adapted methods previously reported.[1] HSC-5 cells were seeded in 10 cm plates and grown to ~90% confluence. For analogous

experiments in SLC25A20 or mock transfected HeLa cells, cells in 10 cm plates transfected first as described above and then continued following this procedure. Media was aspirated, cells were washed carefully with DPBS (3mL) and resuspended in freshly-prepared serum-free IMDM media containing test compound(s) or vehicle. After incubation at 37 ºC for 3 hr, the media was removed and cells were washed with cold DPBS (2 x 3mL). Cells were scraped in 4 mL cold DPBS, transferred to a falcon tube and centrifuged at 2000 rpm for 8 min, and resuspended in 1mL cold DPBS. Cells were lysed using a probe sonicator, and 1 mL of lysates normalized to 1.5mg/mL was transferred to 2-dram glass vials. MeCN (3 mL) containing acyl carnitine internal standard mix (Cambridge Isotope Laboratories) was added to lysates and vigorously vortexed. Internal standards include $^{2}H_{9}$ carnitine (2.28 nmol); $^{2}H_{3}$ acetyl carnitine (C2, 570 pmol); $^{2}H_{3}$ propionyl carnitine (C3, 120 pmol); $^{2}H_{3}$ butryl carnitine (C4, 120 pmol); $^{2}H_{9}$ isovaleryl carnitine (C5, 120 pmol); $^{2}H_{3}$ octanoyl carnitine (C8, 120 pmol); $^{2}H_{9}$ myristoyl carnitine (C14, 120 pmol); $^{2}H_{3}$ palmitoyl carnitine (C16, 240 pmol). Samples were centrifuged at 1000 rpm for 5 min to pellet insoluble precipitate, and the remaining eluent carefully transferred to fresh 2-dram vials to avoid disturbing the precipitate. The eluent was concentrated under a stream of N_2, and samples were stored at -80ºC until analysis. Directly prior to analysis, samples were reconstituted in 500 uL of MeCN:H2O (1:1, v/v) and analyzed by LC/MS/MS. The indicated acyl carnitines were quantified by measuring the area under the peak relative to an internal standard ($^{2}H_{3}$ palmitoyl carnitine for C16, C18 and C18:1; $^{2}H_{9}$ myristoyl carnitine for C12 and C14; $^{2}H_{3}$ octanoyl carnitine for C8 and C7; $^{2}H_{3}$ butyryl carnitine for C4).

Extracts were analyzed in positive mode using an Agilent 6460 Triple Quadrupole LC/MS system and LC separation was performed on a Kinetex 5 µm C18 100A, 50 x 4.6 mm column. Mobile phase A was composed of 95:5:0.1 H_2O/methanol/formic acid and mobile phase B was composed of 60:35:5:0.1 isopropyl alcohol/methanol/H_2O/formic acid. Following injection (15 µL), samples were eluted with an initial constant flow rate of 100 µL/min in 100% mobile phase

A (0-5 min) and further eluted using the following gradient: Mobile phase A (100%, 400 μL/min), 5-7 min; B increased linearly to 100% (400 μL/min), 7-30 min and held constant 30-38 min (with increased flow of 500 μL/min); B decreased immediately to 0%, and held constant 38-42min; B increased linearly to 100%, 42-46 min and held constant 46-50 min; B decreased linearly to 0%, 50-54 min and held constant 54-57 min. To minimize carryover, LC solvents were cycled between 100% Mobile Phase A and 100% Mobile Phase B over 5 min after each run. The following MS parameters were used to measure the indicated metabolites by MRM (precursor ion, product ion): C12 (344.2, 85.1), C14 (372.3, 85.1), C16 (400.3, 85.1), C18:1 (426.3, 85.1), C18 (428.3, 85.1), C4 (232.1, 85.1), C8 (288.1, 85.1), C7 (274.1, 85.1), D3 acetyl (207.1, 85.1), D3 butyryl (235.1, 85.1), D3 octanoyl (291.1, 85.1), D3 palmitoyl (403.3, 85.1), D3 propionyl (221.1, 85.1), D9 isovaleryl (255.1, 85.1), and D9 myristoyl (381.3, 85.1).

Oxygen Consumption Rate Measurements

Palmitate-BSA oxidation measurements were performed using a Seahorse XFe96 Extracellular Flux Analyzer. Briefly, HSC5 cells were plated at 4.0×10^4 cells/well and incubated for 24 hr in a 37°C, 5% CO_2 incubator. One hour prior to the XF assay, media was changed to 1X Krebs-Henseleit buffer (111 mM NaCl, 4.7 mM KCl, 2 mM $MgSO_4$, 1.2 mM Na_2HPO_4, pH 7.4) with 2.5 mM glucose, 0.5 mM carnitine, and 5 mM HEPES. 20 min after media exchange, cells were treated with either vehicle (DMSO) or test compounds at indicated concentrations. After 40 min, cells were given palmitate:BSA (667 μM and 167 μM respectively) or BSA alone and the XF assay was started. Perturbation compounds (oligomycin 4 μM, FCCP 4 μM, RAA 2 μM) were prepared in 1X KH buffer and injected from the reagent ports automatically onto wells.

Quantification and Statistical Analysis

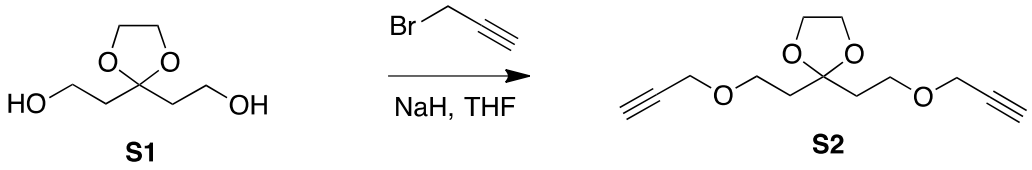
All data fitting and statistical analysis performed using GraphPad Prism version 6.0 for Mac OS X, Graphpad Software, La Jolla California USA, www.graphpad.com. Statistical values, including the exact *n* and statistical

significance are reported in the Figure Legends. One-way ANOVA was performed to test for any difference in the indicated treatments (groups), and p-values for each pair-wise comparison were computed using unpaired Student's *t*-test. These p-values were adjusted for multiple comparisons using Dunnet's method.

Chemistry Materials

Purchased starting materials were used as received unless otherwise noted. All moisture sensitive reactions were performed in an inert, dry atmosphere of nitrogen in flame dried glassware. Reagent grade solvents were used for extractions and flash chromatography. Dry diethyl ether (Et_2O), dichloromethane (CH_2Cl_2), acetonitrile (CH_3CN), toluene (PhMe), tetrahydrofuran (THF), methanol (MeOH), and triethylamine (Et_3N) were obtained by passing these previously degassed solvents through activated alumina columns. Reaction progress was checked by analytical thin-layer chromatography (TLC, Merck silica gel 60 F-254 plates). The plates were monitored either with UV illumination, or by charring with anisaldehyde (2.5% *p*-anisaldehyde, 1% AcOH, 3.5% H_2SO_4 (conc.) in 95% EtOH) or ninhydrin (0.3% ninhydrin (w/v), 97:3 EtOH-AcOH) stains. Flash column chromatography was performed using silica gel (F60, 40-63um, 60A). Preparative thin layer chromotography (PTLC) was carried out using glass backed PTLC plates 1000-2000 µm thickness (Analtech). The solvent compositions reported for all chromatographic separations are on a volume/volume (v/v) basis. ^{1}H-NMR spectra were recorded at either 400, 500 or 600 MHz and are reported in parts per million (ppm) on the δ scale relative to $CDCl_3$ (δ 7.26) as an internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), and integration. ^{13}C-NMR spectra were recorded at either 100 or 125 MHz and are reported in parts per million (ppm) on the δ scale relative to $CDCl_3$ (δ 77.00). Mass spectrometry data were collected

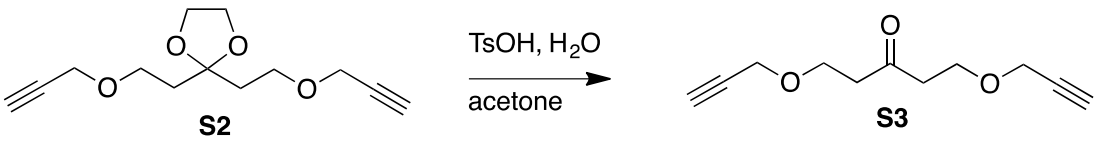
on a HP1100 single-quadrupole instrument (ESI; low resolution) or an Agilent ESI-TOF instrument (HRMS).



S2: To a flame dried flask was added **S1** (622 mg, 3.84 mmol), followed by THF (20 mL) and NaH (60% dispersion in mineral oil, 352 mg, 9.6 mmol, 2.5 equiv.) and resulting solution was stirred for 30 minutes under an atmosphere of nitrogen, after which propargyl bromide (80% solution in toluene, 3.0 mL, 2.4 g, 9.6 mmol, 2.5 equiv.) was added. Resulting solution was stirred for 14 hours at room temperature and was carefully quenched with the addition of sat. NH_4Cl (30 mL). The mixture was extracted with EtOAc (3 x 30 mL) and organic layers were combined, dried over Na_2SO_4, filtered and concentrated. The brown residue was purified by column chromatography (100% Hexanes → 10:1 Hexanes:EtOAc → 4:1 Hexanes:EtOAc) to yield **S2** as a yellow oil (620 mg, 2.6 mmol, 68%). MS (ESI+) *m/z* calc'd for $C_{13}H_{19}O_4$ [M+H]+ 239.3, found 239.4.

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.12 (d, *J* = 2.4 Hz, 4H), 3.93 (s, 4H), 3.62 (t, *J* = 7.0 Hz, 4H), 2.42 (t, *J* = 2.4 Hz, 2H), 1.97 (t, *J* = 7.0 Hz, 4H).

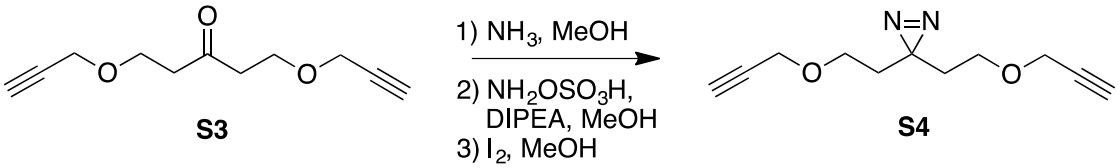
^{13}C NMR (126 MHz, $CDCl_3$): δ 109.72, 80.20, 74.72, 66.18, 65.25, 58.50, 37.63.



S3: To a solution of **S2** (562 mg, 2.36 mmol) in acetone/water (15:1, 10 mL), added *p*-toluenesulfonic acid monohydrate (184 mg, 0.94 mmol, 0.4 equiv.). The resulting solution was stirred for 16 hours at room temperature and was quenched with the addition of sat. NH_4Cl (5 mL). The mixture was extracted with EtOAc (3 x 15 mL) and organic layers were combined, dried over Na_2SO_4, filtered and concentrated. The yellow oil was purified by column chromatography (100% Hexanes → 9:1 Hexanes:EtOAc → 4:1 Hexanes:EtOAc) to yield **S3** as a yellow oil (406 mg, 2.09 mmol, 92%). MS (ESI+) *m/z* calc'd for $C_{11}H_{15}O_3$ [M+H]+ 195.2, found 195.2.

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.15 (d, *J* = 2.4 Hz, 4H), 3.80 (t, *J* = 6.2 Hz, 4H), 2.75 (t, *J* = 6.2 Hz, 4H), 2.44 (t, *J* = 2.4 Hz, 2H).

^{13}C NMR (126 MHz, $CDCl_3$): δ 207.05, 79.90, 75.00, 65.08, 58.67, 43.43.

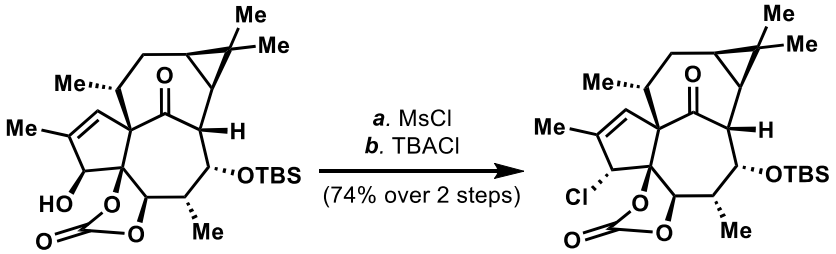


S4: To **S3** (200 mg, 1.03 mmol) in a round bottom flask, added 7N NH_3 in MeOH (10 mL) at 0 ^oC under an atmosphere of nitrogen, and stirred for 3 hours at 0 ^oC. Added hydroxylamine-*O*-sulfinic acid (163 mg, 144 mmol, 1.4 equiv.) as a solution in anhydrous MeOH (2 mL) dropwise at 0 ^oC and resulting solution was stirred an additional hour at 0 ^oC for and then allowed to warm to room temperature over an additional 14 hours. The resulting suspension was evaporated to dryness and resuspended in MeOH (2 mL) and white solid was filtered off, washing several times with MeOH. The combined filtrate was evaporated and subsequently resuspended in anhydrous MeOH (8 mL) and cooled to 0 ^oC. To chilled solution, diisopropylethylamine (400 μL) was added, followed by iodine (portion-wise, flask protected from light) until a dark brown color persisted for more than 30 minutes, indicating completed oxidation of diaziridine. The solution was then diluted with EtOAc (20 mL) and washed with

aq. 1N HCl (30 mL), saturated $Na_2S_2O_3$ (3 X 50 mL, or until organic phase is clarified), and brine (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The brown residue was purified by column chromatography (100% Hexanes → 20:1 Hexanes:EtOAc → 10:1 Hexanes:EtOAc → 7:1 Hexanes:EtOAc) to yield **S4** as a clear oil (60 mg, 0.29 mmol, 28%). MS (ESI+) *m/z* calc'd for $C_{11}H_{15}N_2O_2$ [M+H]+ 207.2, found 207.3.

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.19 (dd, *J* = 2.4, 0.6 Hz, 4H), 3.45 (td, *J* = 6.4, 0.7 Hz, 4H), 2.51 (td, *J* = 2.4, 0.6 Hz, 2H), 1.78 (td, *J* = 6.4, 0.7 Hz, 4H).

^{13}C NMR (126 MHz, $CDCl_3$): δ 79.85, 74.96, 64.95, 58.54, 33.77, 26.13.



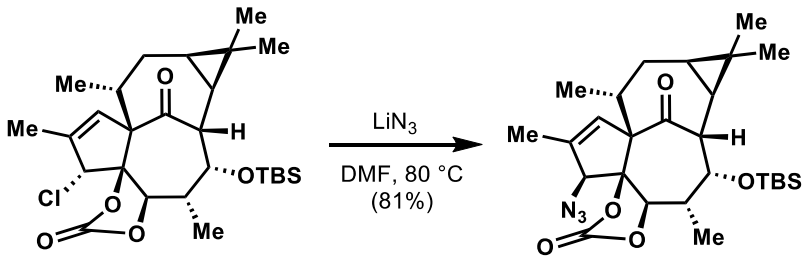
S5: To a solution of **3** (117 mg, 0.238 mmol, 1.0 equiv.) in DCM (8 mL) at 0 °C was added triethylamine (36 mg, 50 µL, 0.358 mmol, 1.5 equiv.) and methanesulfonyl chloride (41 mg, 28 µL, 0.358 mmol, 1.5 equiv.). The reaction mixture was stirred at 0 °C for 1 h and was subsequently quenched by the addition of aq. $NaHCO_3$ (8 mL). The organic layer was separated; the aq. layer was extracted once more with DCM (8 mL), and the combined organic phases were dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude mesylate was dissolved in DMF (8 mL) and TBACl (198 mg, 0.714 mmol, 3 equiv.) was added. The reaction mixture was heated to 75 °C. After 5 h, water (8 mL) was added. The resulting mixture was extracted with EtOAc (4 x 6 mL), and the combined organic layers were dried over sodium sulfate, filtered and evaporated under reduced pressure. The crude material was purified by silica gel column chromatography (column packed in DCM, then hex/EtOAc 25:1 to 15:1) to afford compound **S5** (89 mg, 74%).

HRMS (*m/z*): calc. for $C_{27}H_{41}ClO_5Si$ $[M+H]^+$= 509.2484; found, 509.2504.

$[\alpha]_D$= +14.5° (c = 1.0, $CHCl_3$).

^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 6.04 (br s, 1 H), 5.54 (d, *J*=3.95 Hz, 1 H), 5.35 (s, 1 H), 4.26 (dd, *J*=7.23, 4.60 Hz, 1 H), 2.84 - 2.93 (m, 1 H), 2.41 (br dd, *J*=6.58, 3.51 Hz, 1 H), 2.32 (dd, *J*=10.96, 4.38 Hz, 1 H), 2.01 - 2.09 (m, 1 H), 1.83 - 1.89 (m, 1 H), 1.82 (s, 3 H), 1.55 (s, 1 H), 1.10 (s, 3 H), 1.07 (s, 3 H), 1.00 - 1.05 (m, 1 H), 0.95 (br s, 11 H), 0.80 (d, *J*=8.33 Hz, 3 H), 0.67 - 0.73 (m, 1 H), 0.12 (s, 3 H), 0.07 (s, 3 H).

^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 203.85, 134.83, 127.59, 94.47, 85.92, 73.86, 71.80, 66.71, 49.17, 40.60, 37.95, 30.27, 28.36, 25.76, 25.20, 23.25, 21.97, 18.26, 15.62, 15.16, 14.76, 10.57, -4.89, -4.97.



S5 **4**

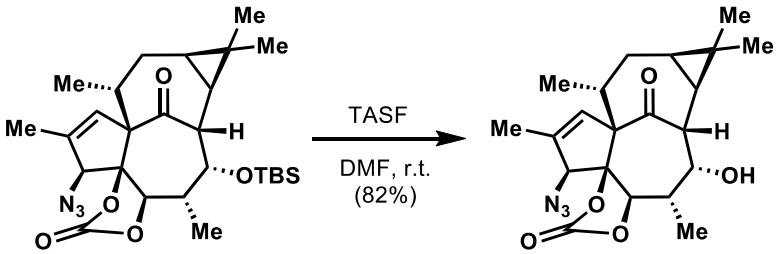
4: To a solution of **S5** (80 mg, 0.098 mmol, 1.0 equiv.) in DMF (5 mL) was added an aq. solution of lithium azide (20%wt, 15 µL, 0.295 mmol, 3.0 equiv.), the tube was sealed with a plastic cap and the reaction mixture was heated to 80 °C. After 1 h, another portion of lithium azide (20%wt, 15 µL, 0.295 mmol, 3.0 equiv.) was added and the reaction mixture was continued to be heated at 80 °C. Over the next 8 h, several additional portions of lithium azide (60µL in total) were added until TLC indicated complete consumption of starting material. The reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3 x 20 mL). The combined organic layers were dried over sodium sulfate, filtered and concentrated in vacuo. The crude product was purified by silica gel chromatography (column packed in DCM, then hex/EtOAc 50:1 → 20:1 → 10:1) to afford **4** (41 mg, 81%).

HRMS (*m/z*): calc. for $C_{27}H_{41}N_3O_5Si$ $[M+H]^+$= 516.2894; found, 516.2899.

$[\alpha]_D$= +131° (c = 1.0, $CHCl_3$)

^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 6.05 (br s, 1 H), 4.78 (d, *J*=4.82 Hz, 1 H), 4.21 (dd, *J*=7.45, 4.38 Hz, 1 H), 3.85 (s, 1 H), 2.84 (quind, *J*=7.78, 7.78, 7.78, 7.78, 4.82 Hz, 1 H), 2.36 - 2.45 (m, 2 H), 1.87 - 1.92 (m, 2 H), 1.85 (s, 3 H), 1.24 - 1.62 (m, 1 H), 1.11 (s, 3 H), 1.05 (s, 3 H), 0.99 (d, *J*=7.02 Hz, 3 H), 0.94 (s, 9 H), 0.82 (d, *J*=7.89 Hz, 3 H), 0.71 (q, *J*=8.33 Hz, 1 H), 0.11 (s, 3 H), 0.06 (s, 3 H).

^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 204.91, 152.10, 133.54, 131.61, 92.23, 91.60, 79.74, 74.71, 66.94, 49.52, 39.73, 39.10, 30.73, 29.68, 28.42, 25.73, 24.50, 23.11, 22.26, 18.22, 15.10, 10.34, -4.86, -4.93.



4 **S6**

S6: Compound **4** (45 mg, 0.087 mmol, 1.0 equiv.) was dissolved in anhydrous DMF (3 mL) and a solution of TASF (48 mg, 0.174 mmol, 2.0 equiv.) in anhydrous DMF (1 mL) was added. The reaction mixture was stirred at rt for 22 h, before water (5 mL) was added. The mixture was extracted with EtOAc (3 x 10 mL) and the combined organic layers were washed with an aq. solution of LiCl (15%wt), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (column packed in DCM, then hex/EtOAc 10:1 → 5:1 → 2:1) to afford **S6** (29 mg, 82%).

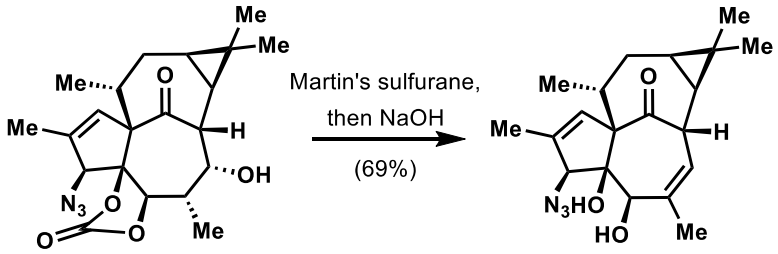
HRMS (*m/z*): calc. for $C_{21}H_{27}N_3O_5$ $[M+H]^+$= 402.2023; found, 402.2023.

$[\alpha]_D$= +151° (c = 1.0, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 5.90 (s, 1 H), 4.71 (d, *J*=5.26 Hz, 1 H), 4.13 (ddd, *J*=10.38, 7.16, 3.51 Hz, 1 H), 3.85 (s, 1 H), 2.86 (br d, *J*=10.23 Hz, 2 H), 2.69 (br d, *J*=8.77 Hz, 1 H), 2.43 - 2.52 (m, 1 H), 1.90 - 1.96 (m, 2 H), 1.89 (s, 3

H), 1.14 (s, 3 H), 1.07 (s, 3 H), 1.00 (d, *J*=6.72 Hz, 3 H), 0.93 (d, *J*=7.89 Hz, 3 H), 0.71 - 0.78 (m, 1 H).

^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 209.71, 151.97, 135.19, 129.45, 92.01, 91.79, 78.83, 74.00, 68.10, 48.01, 40.02, 38.37, 30.85, 29.67, 28.66, 22.85, 22.32, 17.06, 15.01, 14.90, 11.33.



S6 **5**

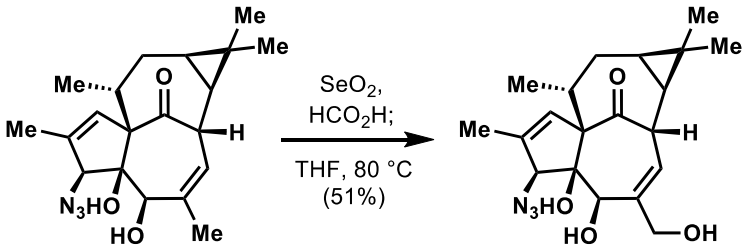
5: To a solution of **S6** (20 mg, 0.05 mmol, 1.0 equiv.) in anhydrous chloroform (0.7 mL) was added a solution of Martin's sulfurane in anhydrous chloroform (80 mg in 0.5 mL $CHCl_3$) and the resulting reaction mixture was heated to reflux for 1 h. The solvent was removed in vacuo, the brown residue was dissolved in THF (1 mL), aq. sodium hydroxide (10%, 2 mL) was added. and the resulting mixture was stirred at room temperature for 3 h. The reaction mixture was extracted with EtOAc (3 x 5 mL) and chloroform (3 x 5 mL) and the combined organic layers were dried over sodium sulfate, filtered and concentrated in vacuo. The crude product was purified by silica gel column chromatography (column packed in DCM, then hex/EtOAc 20:1 → 10:1 → 5:1) to afford **5** (12 mg, 69%).

HRMS (*m/z*): calc. for $C_{20}H_{27}N_3O_3$ $[M+Na]^+$= 380.1945; found, 380.1949.

$[\alpha]_D$= +102° (c = 1.0, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 6.03 (br s, 1 H), 5.75 (br s, 1 H), 4.49 (br d, *J*=3.51 Hz, 1 H), 3.91 - 4.02 (m, 1 H), 3.57 (br d, *J*=2.63 Hz, 1 H), 3.48 (br d, *J*=9.65 Hz, 1 H), 2.50 - 2.60 (m, 1 H), 2.16 - 2.32 (m, 2 H), 1.90 (br d, *J*=2.63 Hz, 3 H), 1.77 (br s, 4 H), 1.09 (br d, *J*=4.09 Hz, 3 H), 1.05 (br d, *J*=3.80 Hz, 3 H), 0.98 (br dd, *J*=6.72, 3.51 Hz, 3 H), 0.90 (ddd, *J*=11.69, 8.33, 3.65 Hz, 2 H), 0.61 - 0.73 (m, 1 H).

^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 206.57, 137.88, 135.68, 131.46, 123.68, 85.73, 76.45, 74.83, 71.97, 43.60, 38.62, 30.81, 28.40, 24.23, 23.14, 22.68, 21.99, 17.02, 16.27, 15.42.



5 **6**

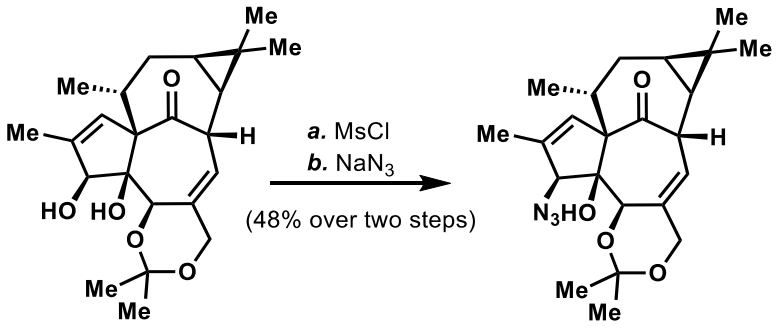
3-(*S*)-azido-3-deoxy-ingenol (**6**): To a solution of **5** (9 mg, 0.025 mmol, 1.0 equiv.) in anhydrous dioxane (600 µL) was added formic acid (300 µL) and selenium dioxide (28 mg, 0.25 mmol, 10 equiv.) and the resulting mixture was heated at 80 °C for 6 h. All volatiles were evaporated in vacuo and the crude material was dissolved in THF (1 mL). Aq. sodium hydroxide (10%, 3 mL) was added and the reaction mixture was stirred at 80 °C for 1 h. The mixture was extracted with EtOAc (5 x 5 mL) and chloroform (5 x 5 mL) and the combined organic layers were dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (column packed in DCM, then hex/EtOAc 10:1 → 5:1 → 1.5:1) to afford **6** (5 mg, 51%).

HRMS (*m/z*): calc. $C_{20}H_{27}N_3O_4$ $[M+Na]^+$= 396.1894; found, 396.1912.

$[\alpha]_D$= +39.7° (c = 1.0, $CHCl_3$).

^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 6.06 (br d, *J*=4.38 Hz, 1 H), 5.98 (s, 1 H), 4.47 (s, 1 H), 4.17 - 4.23 (m, 1 H), 4.09 - 4.16 (m, 2 H), 3.88 (br s, 1 H), 3.72 (br s, 1 H), 2.40 (dt, *J*=7.02, 3.51 Hz, 1 H), 2.25 (ddd, *J*=15.35, 9.65, 2.63 Hz, 1 H), 1.89 (s, 3 H), 1.79 (dt, *J*=15.78, 5.26 Hz, 1 H), 1.10 (s, 3 H), 1.07 (s, 3 H), 0.98 (d, *J*=7.02 Hz, 3 H), 0.88 - 0.95 (m, 2 H), 0.67 - 0.74 (m, 1 H).

^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 206.48, 139.57, 136.08, 130.76, 127.88, 85.85, 75.79, 74.25, 71.91, 67.12, 43.67, 38.34, 30.84, 28.42, 24.26, 22.93, 22.84, 17.09, 16.17, 15.50.

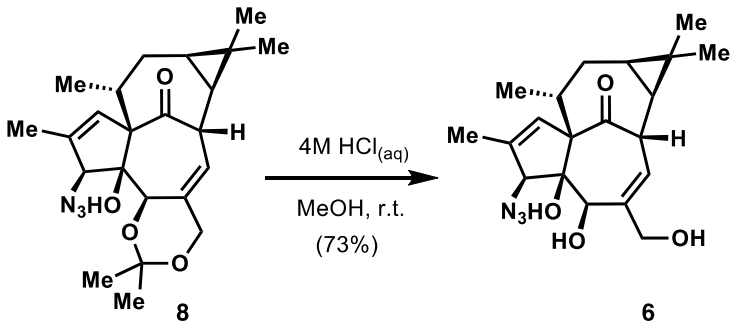


7 8

5,20-Acetonide-3-(S)-azido-3-deoxy-ingenol (8): Ingenol-5,20-acetonide **7** (prepared according to reference 5) (10.00 g, 25.7 mmol) was dissolved in DCM (200 ml) under argon. Triethylamine was added (3.63 g, 35.9 mmol). The clear solution was cooled to 0°C. Methanesulfonyl chloride (3.83 g, 33.5 mmol, 2.59 mL) was added slowly. The internal temperature was kept under 5°C. The reaction was monitored by TLC (eluent heptane/EtOAc 2:1). After 30 min TLC showed that all starting material was consumed. The reaction mixture was poured into a separation funnel with MTBE (500 ml) and saturated aq. $NaHCO_3$ (500 ml). The layers were separated and the organic layer was washed with a 1:1 mixture of water and saturated aq. $NaHCO_3$ (500 ml) and then with brine (500 ml). The organic layer was dried over sodium sulfate, filtered, and concentrated *in vacuo*. The crude mixture was used in the next step without further purification. Crude mesylate (12.0 g, 25.7 mmol) was dissolved in DMF (100 ml). Sodium azide (7.00 g, 108 mmol) was added and the suspension was stirred at 60°C for 45 min. The reaction mixture was cooled on an ice bath and water (~100 ml) was added slowly. The precipitate was collected by filtration, washed with water, and dried *in vacuo*. The solid was dissolved in MTBE and the solution was washed with brine. The organic phase was dried over sodium sulfate, filtered, and concentrated *in vacuo*. The resulting oil was dissolved in MTBE (10 ml) and

heptane (110 ml) was added. Crystallization started and the mixture was kept at 5°C for 16h. The product was filtered to afford **8** as an off-white solid (5.18g, 12.5 mmol, 48% over two steps). Compound could not be ionized.

^{1}H NMR (600 MHz, $CDCl_3$) δ 5.93 (q, J = 1.6 Hz, 1H), 5.81 (dq, J = 1.5, 3.4 Hz, 1H), 4.23 – 4.11 (m, 4H), 3.94 (t, J = 1.6 Hz, 1H), 3.31 (s, 1H), 2.58 – 2.51 (m, 1H), 2.26 (ddd, J = 3.1, 8.8, 15.8 Hz, 1H), 1.83 (d, J = 1.6 Hz, 3H), 1.82 – 1.76 (m, 1H), 1.44 (s, 3H), 1.39 (s, 3H), 1.11 (s, 3H), 1.05 (s, 3H), 0.96 (d, J = 7.1 Hz, 3H), 0.91 (dd, J = 8.4, 11.8 Hz, 1H), 0.70 (td, J = 6.3, 8.6 Hz, 1H).
^{13}C NMR (151 MHz, $CDCl_3$) δ 207.31, 136.22, 135.28, 130.45, 122.78, 100.52, 85.22, 74.17, 73.73, 72.36, 64.15, 43.61, 37.32, 31.11, 28.50, 26.81, 24.05, 23.30, 22.81, 20.91, 17.61, 16.13, 15.56.



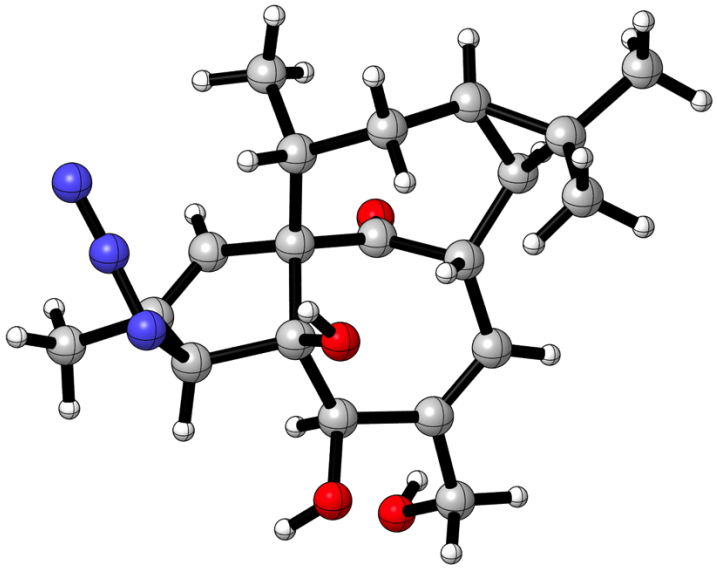
8 6

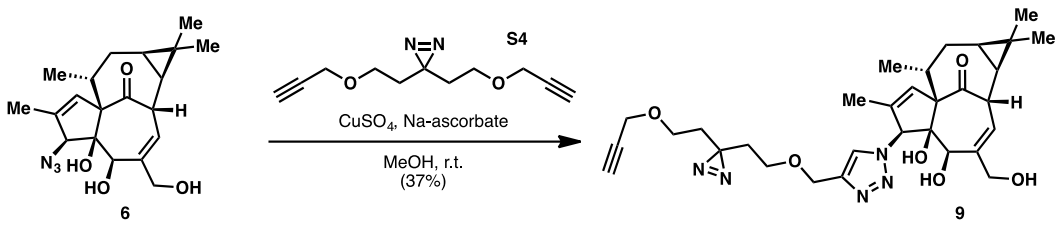
3-(*S*)-azido-3-deoxy-ingenol (6) (*via* semisynthesis): **8** (1.69 g, 4.09 mmol) dissolved in MeOH (30 mL) was added 4 M HCl in water (1.2 mmol, 0.3 mL). The solution was stirred at room temperature for 4 h. To this solution was added saturated aq. $NaHCO_3$ (5 mL) and water (10 ml). The mixture was extracted with MTBE. The combined organic phases were washed with water, brine, dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residual oil was taken up in acetonitrile (5 ml) resulting in a crystalline precipitate which was isolated by filtration (1.17 g, 2.98 mmol, 73 %)
HRMS (m/z): calc. for $C_{20}H_{28}N_3O_4$ $[M+H]^+$ = 374.2074; found, 374.2094.

^{1}H NMR (600 MHz, $CDCl_3$) δ 6.09 – 6.04 (m, 1H), 5.98 (q, J = 1.6 Hz, 1H), 4.47 (s, 1H), 4.20 (d, J = 12.5 Hz, 1H), 4.16 – 4.09 (m, 2H), 3.87 (d, J = 7.6 Hz, 1H), 3.80 (d, J = 7.9 Hz, 1H), 3.72 (d, J = 1.1 Hz, 1H), 2.42 – 2.36 (m, 1H), 2.31 – 2.21 (m, 2H), 1.89 (d, J = 1.6 Hz, 3H), 1.79 (ddd, J = 4.4, 6.4, 15.8 Hz, 1H), 1.10 (s, 3H), 1.06 (s, 3H), 0.98 (d, J = 7.2 Hz, 3H), 0.92 (dd, J = 8.3, 11.9 Hz, 1H), 0.70 (ddd, J = 6.3, 8.3, 9.5 Hz, 1H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 206.51, 139.61, 136.12, 130.76, 127.88, 85.92, 75.87, 74.30, 71.96, 67.13, 43.70, 38.34, 30.89, 28.44, 24.26, 23.00, 22.90, 17.11, 16.15, 15.52.

The structure of **6** was confirmed on the basis of X-ray crystallographic analysis. See Baran350.cif for details.





9: To a solution of **6** (15 mg, 0.04 mmol, 1.0 equiv.), diyne **S4** (42 mg, 0.20 mmol, 5.0 equiv.), $CuSO_4 \cdot 5H_2O$ (2 mg, 8 µmol, 0.2 equiv.) and sodium ascorbate (16 mg, 0.40 mmol, 2.0 equiv.) in MeOH (1.5 mL, 0.025 M). The reaction mixture was stirred at room temperature for 20 h and followed by ether (15 mL) dilution, then washed with brine (5 mL). To the resulting organic layer was concentrated under reduced pressure. Purification of the crude product by flash column chromatography (silica gel, hexanes/EtOAc = 7:3→5:5→3:7→0:10) afforded **9** (8.5 mg, 37%) as a colorless foam.

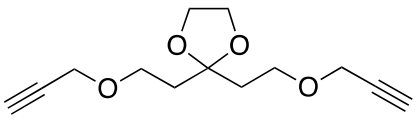
$\mathbf{R_f}$ = 0.65 (EtOAc = 1; anisaldehyde).

HRMS (m/z): calc. for $C_{31}H_{41}N_5O_6$ $[M+H]^+$= 580.3135; found, 580.3146.

$\mathbf{[\alpha]_D}$= +16.6° (c = 0.85, CH_2Cl_2).

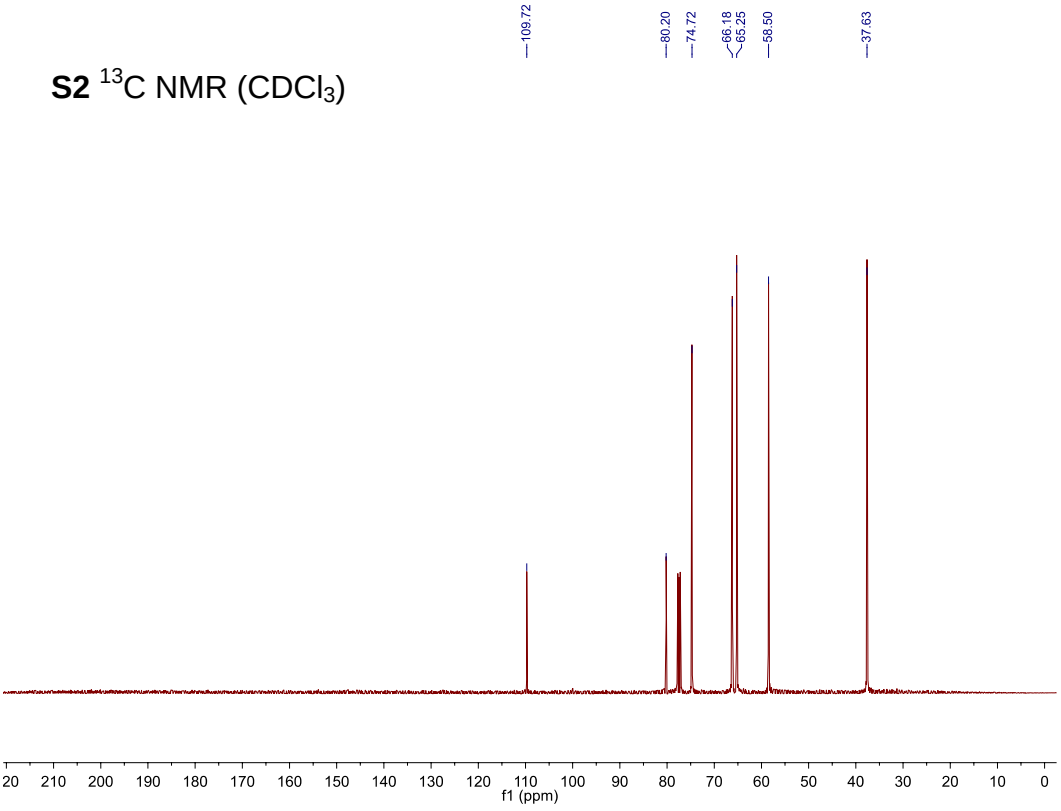
^{1}H NMR (600 MHz, $CDCl_3$) δ 7.59 (s, 1H), 6.23 (s, 1H), 6.06 (d, *J* = 4.7 Hz, 1H), 5.49 (s, 1H), 4.68 – 4.53 (m, 2H), 4.31 (d, *J* = 12.0 Hz, 1H), 4.20 – 4.12 (m, 3H), 4.09 (d, *J* = 2.4 Hz, 2H), 4.00 – 4.93 (m, 1H), 3.36 – 3.32 (m, 5H), 2.43 (t, *J* = 2.4 Hz, 1H), 2.10 – 2.04 (m, 1H), 1.78 (s, 3H), 1.72 (td, *J* = 6.2, 2.1 Hz, 2H), 1.66 (t, *J* = 6.4 Hz, 3H), 1.26 – 1.24 (m, 1H), 1.05 (d, *J* = 7.2 Hz, 3H), 1.02 (s, 3H), 0.97 (s, 3H), 0.90 (dd, *J* = 11.9, 8.2 Hz, 1H), 0.66 (td, *J* = 9.1, 6.2 Hz, 1H).

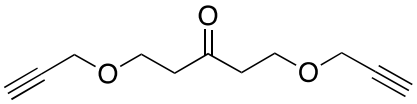
^{13}C NMR (151 MHz, $CDCl_3$) δ 205.6, 144.8, 139.0, 133.4, 133.1, 129.0, 122.9, 110.0, 85.8, 79.4, 74.7, 73.4, 72.5, 67.1, 65.0, 64.5, 64.5, 58.1, 43.7, 37.8, 33.4, 33.2, 31.3, 28.3, 25.9, 24.3, 22.9, 22.8, 16.9, 15.5, 15.4.



S2 ^{1}H NMR ($CDCl_3$)

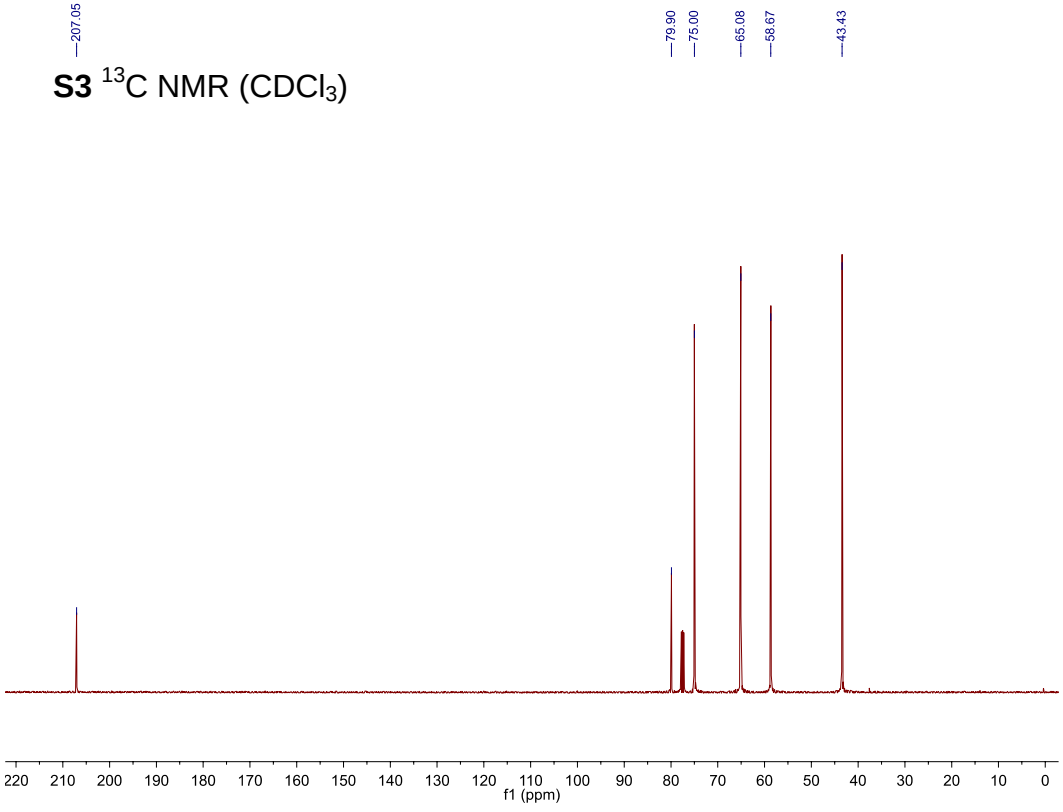
S2 ^{13}C NMR ($CDCl_3$)

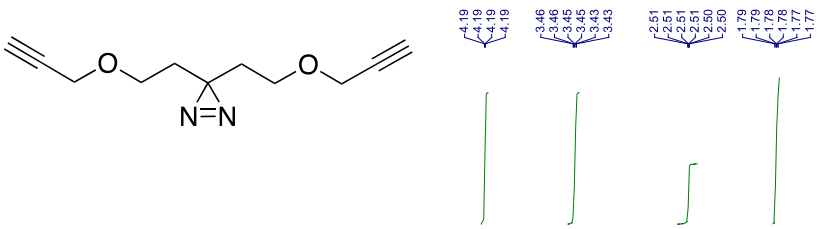




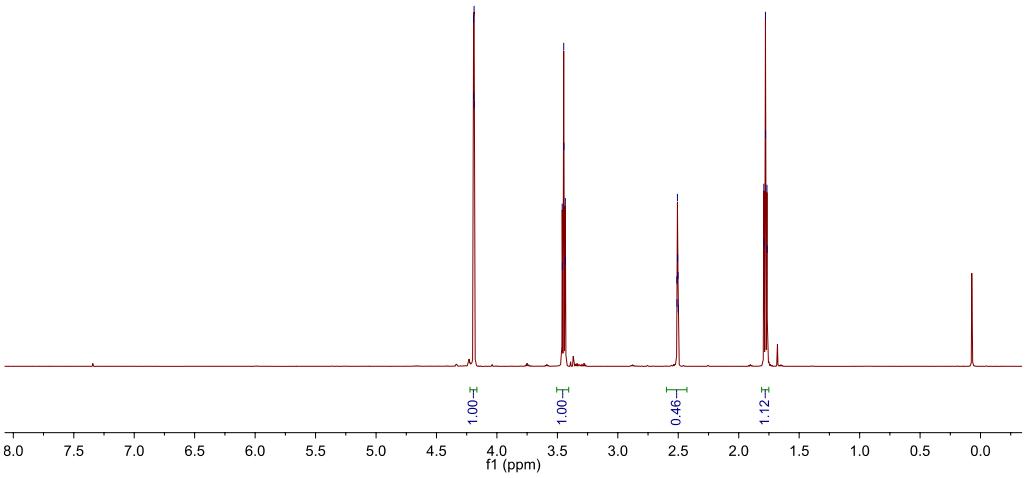
S3 ^{1}H NMR ($CDCl_3$)

S3 ^{13}C NMR ($CDCl_3$)

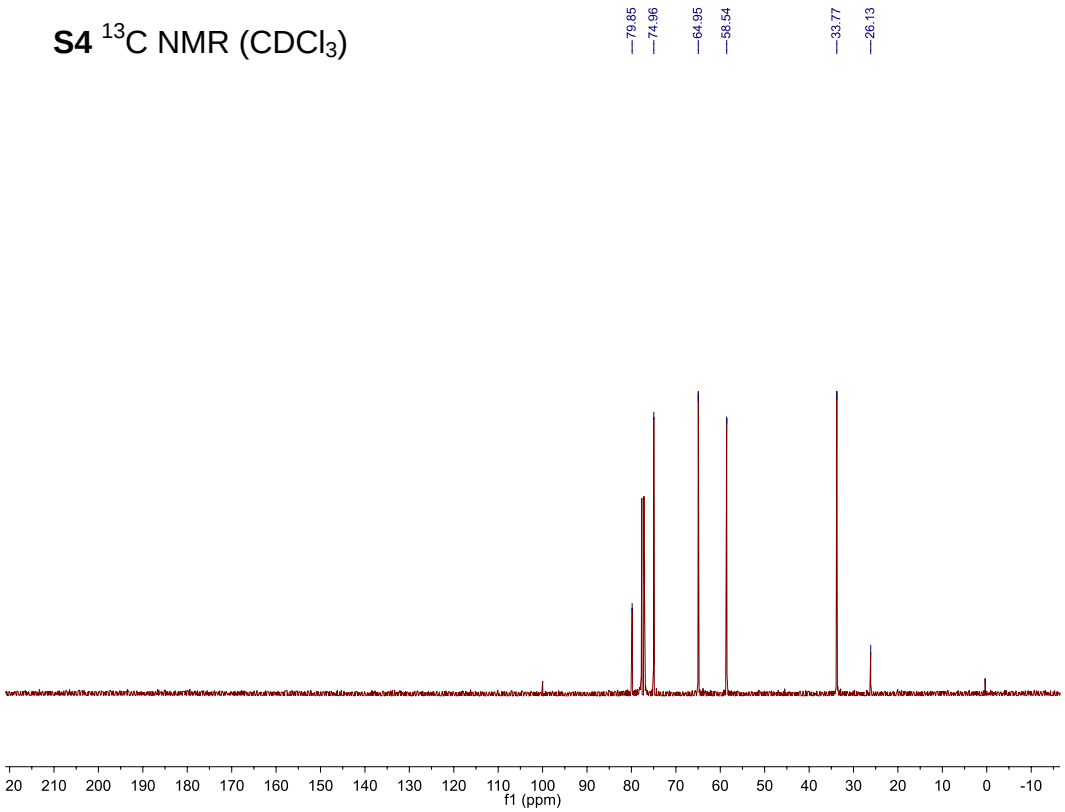


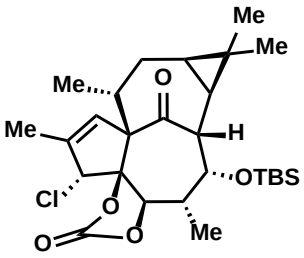


S4 ^{1}H NMR ($CDCl_3$)



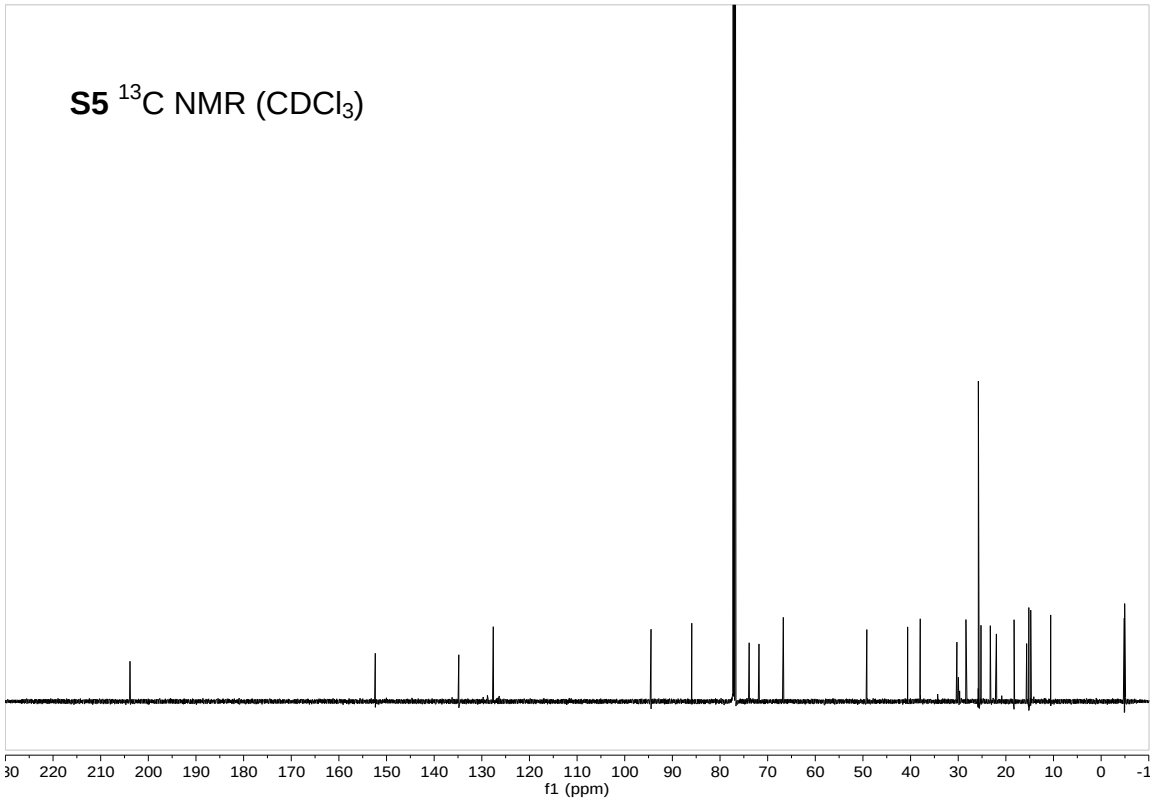
S4 ^{13}C NMR ($CDCl_3$)

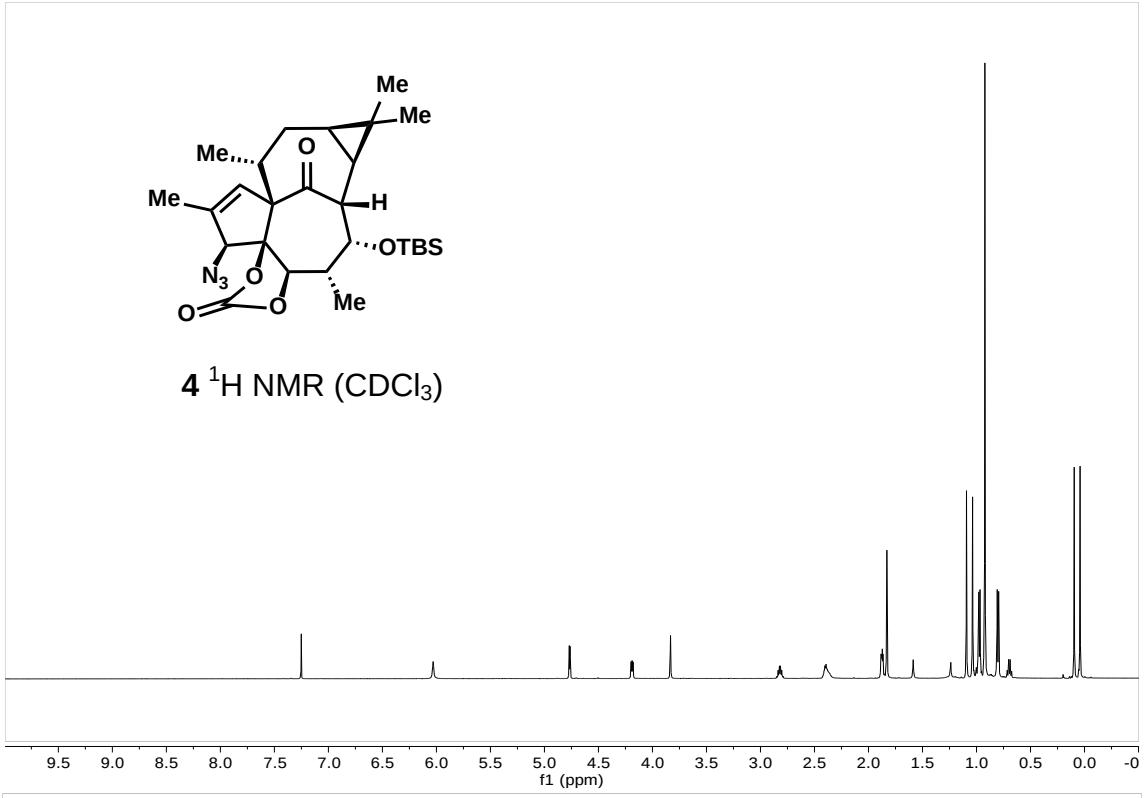


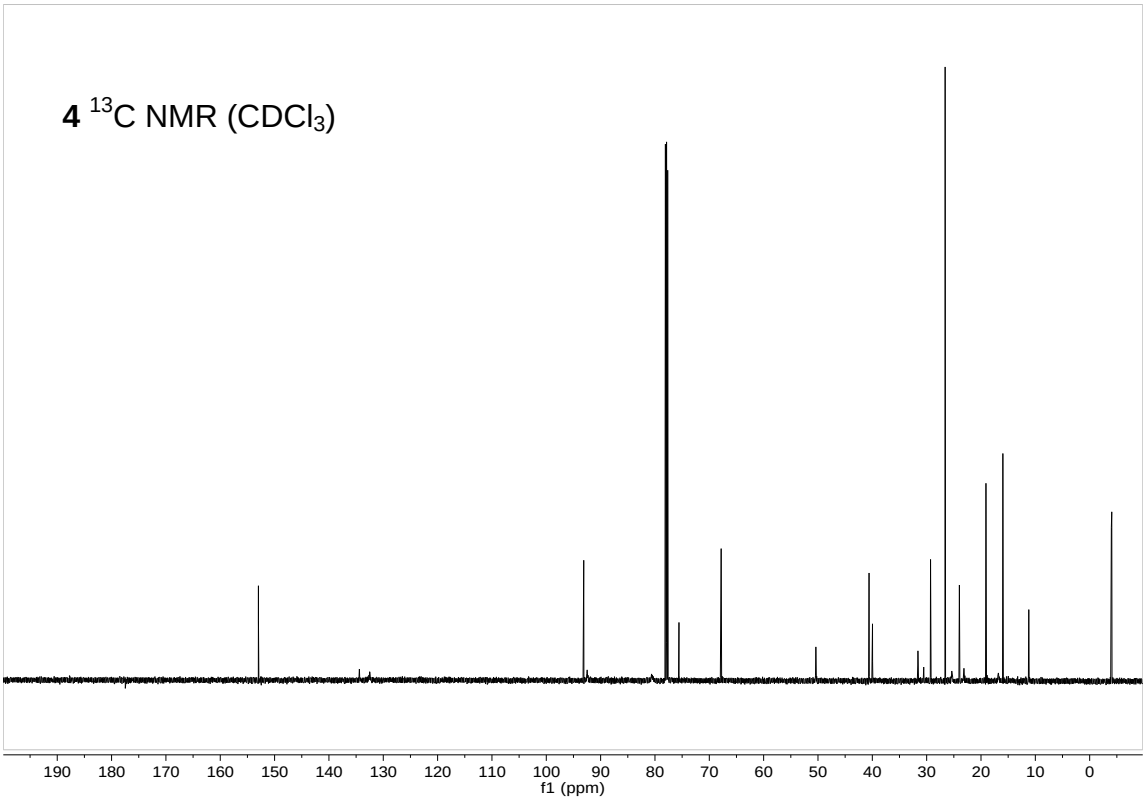


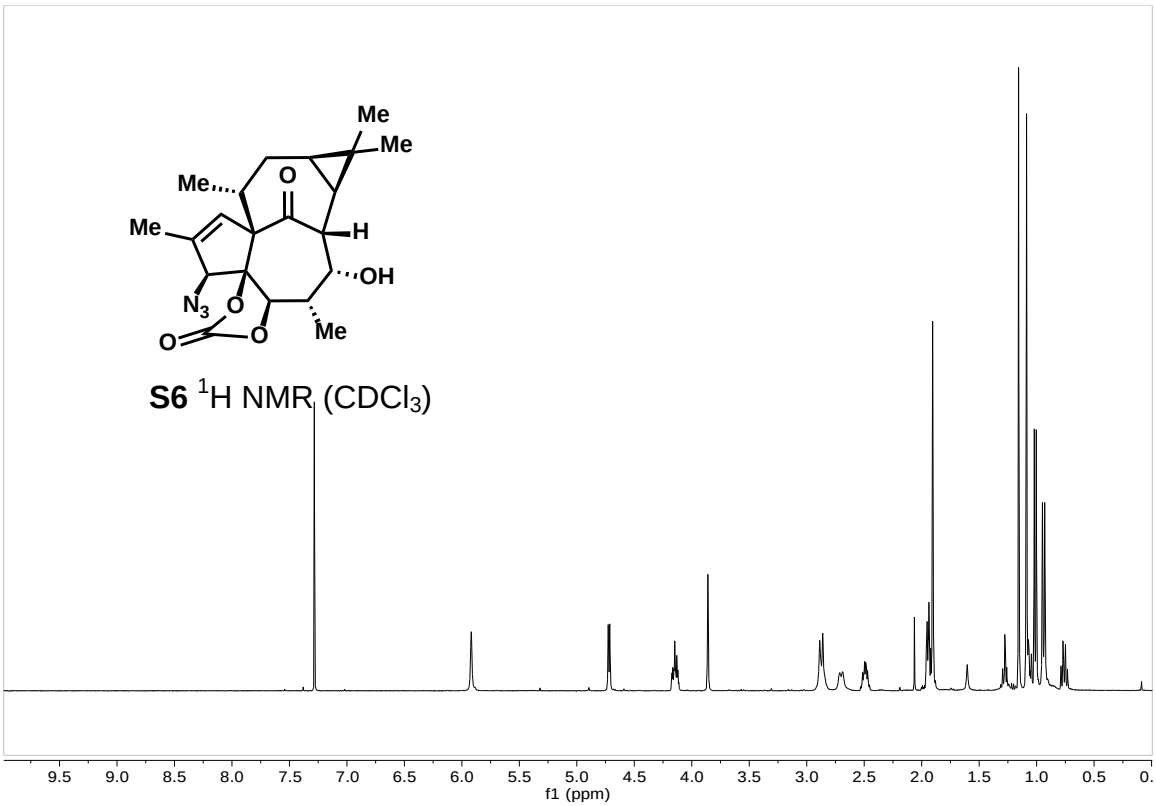
S5 ^{1}H NMR ($CDCl_3$)

S5 ^{13}C NMR ($CDCl_3$)

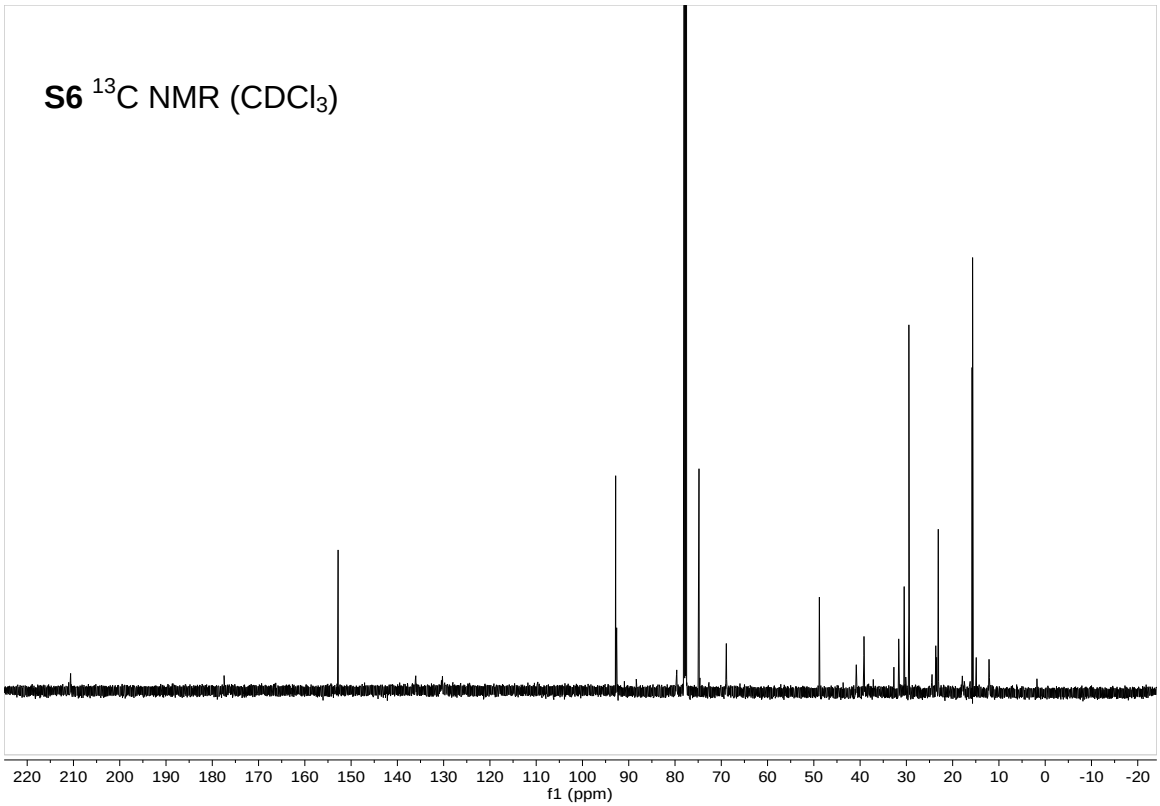



Me
Me
Me
O
Me
H
OTBS
N3
O
O
O
Me
4 1H NMR (CDCl3)
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0
f1 (ppm)

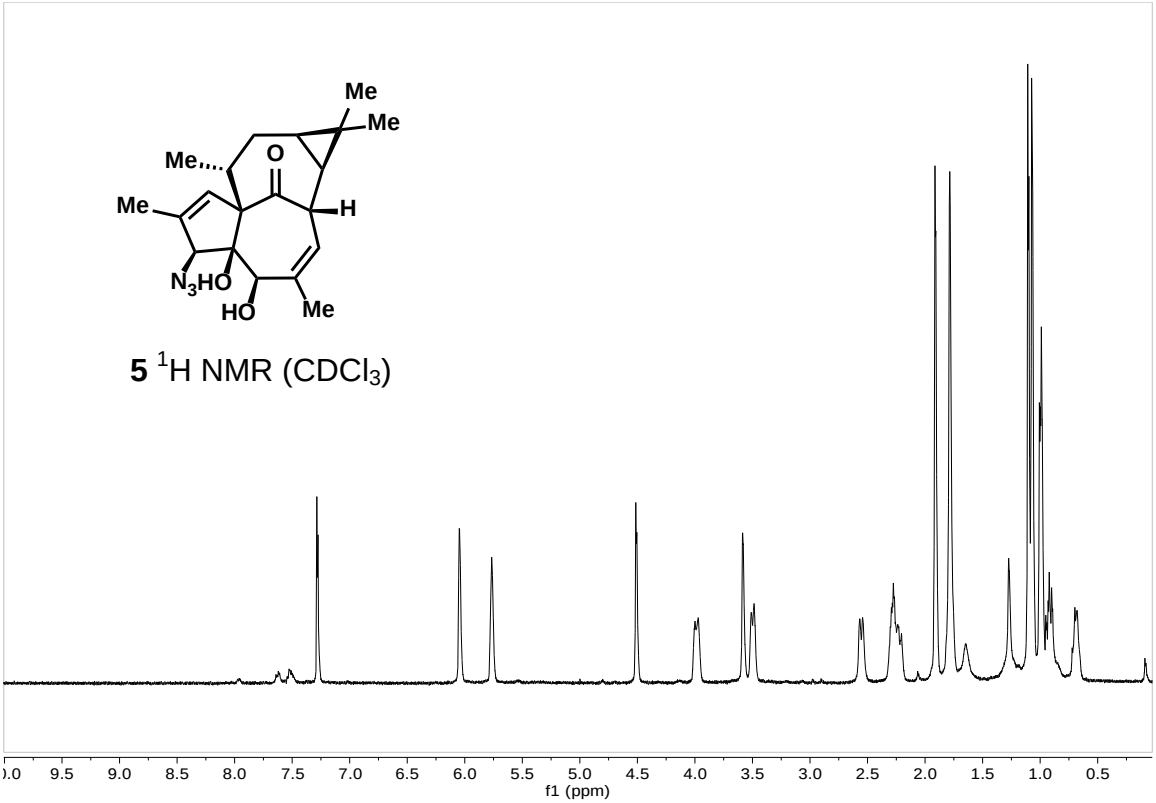

4 13C NMR (CDCl3)
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

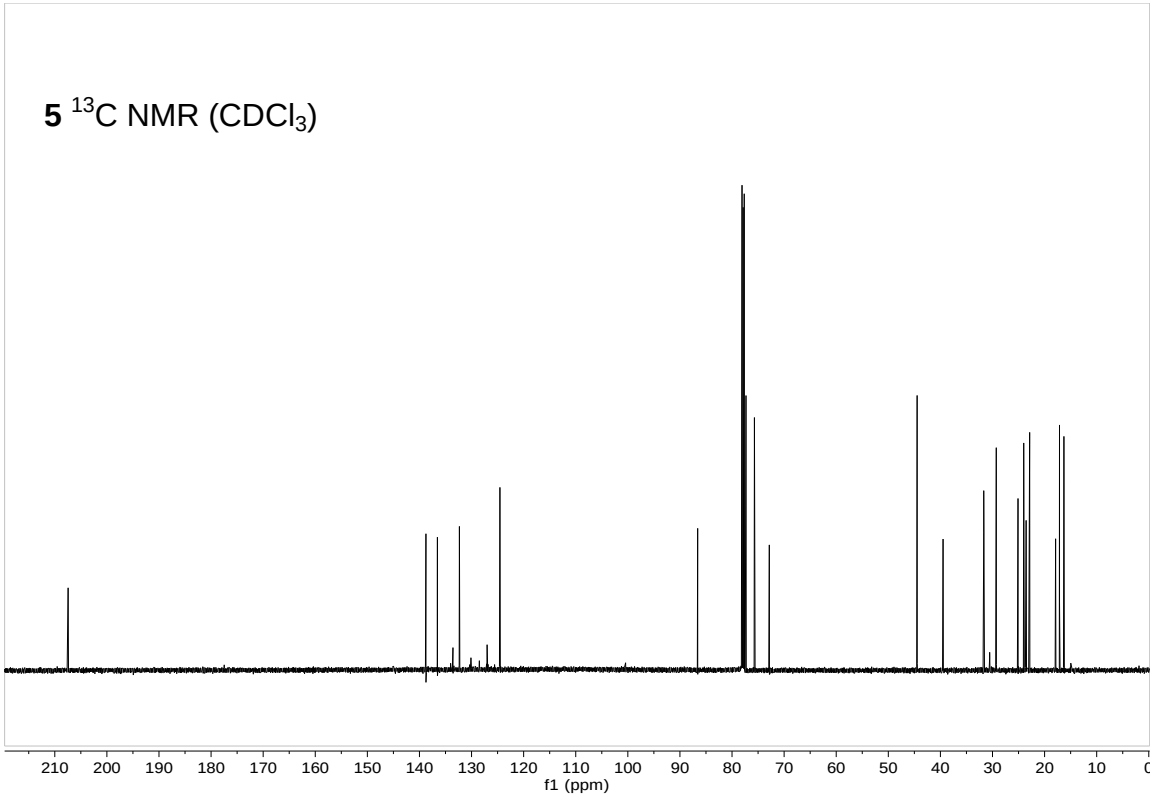


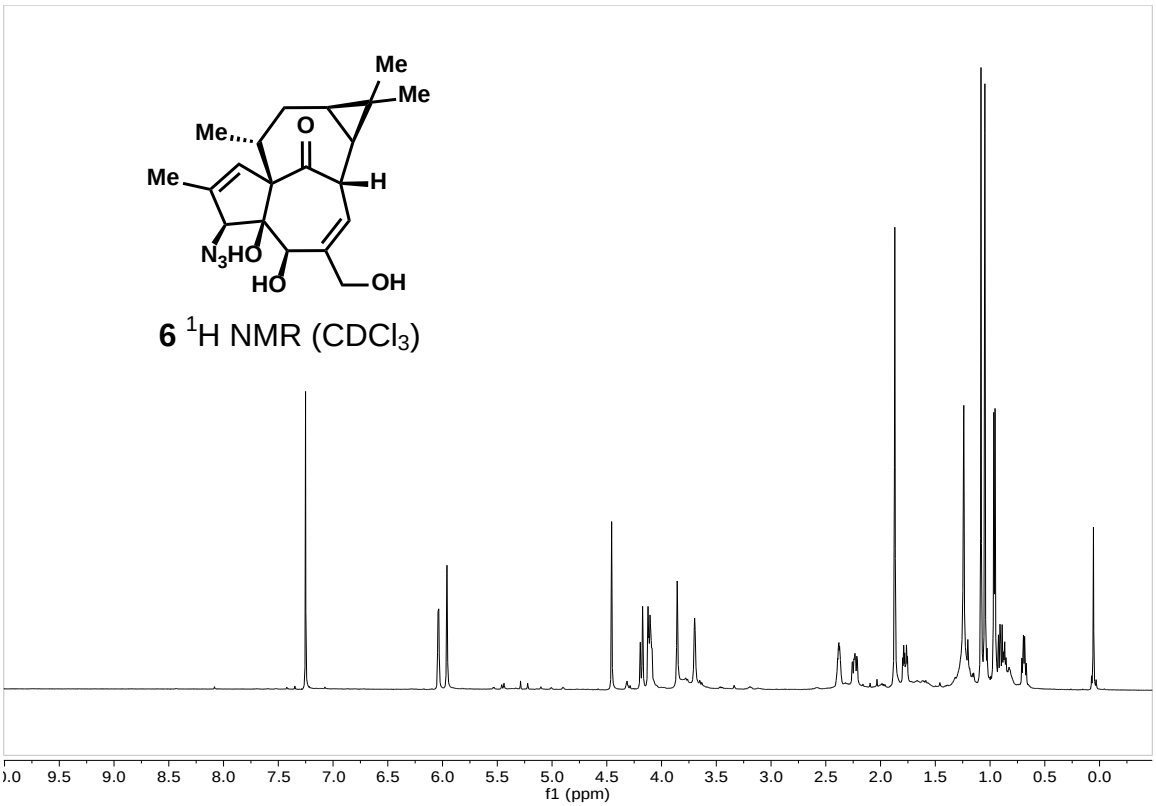
S6 ^{1}H NMR ($CDCl_3$)

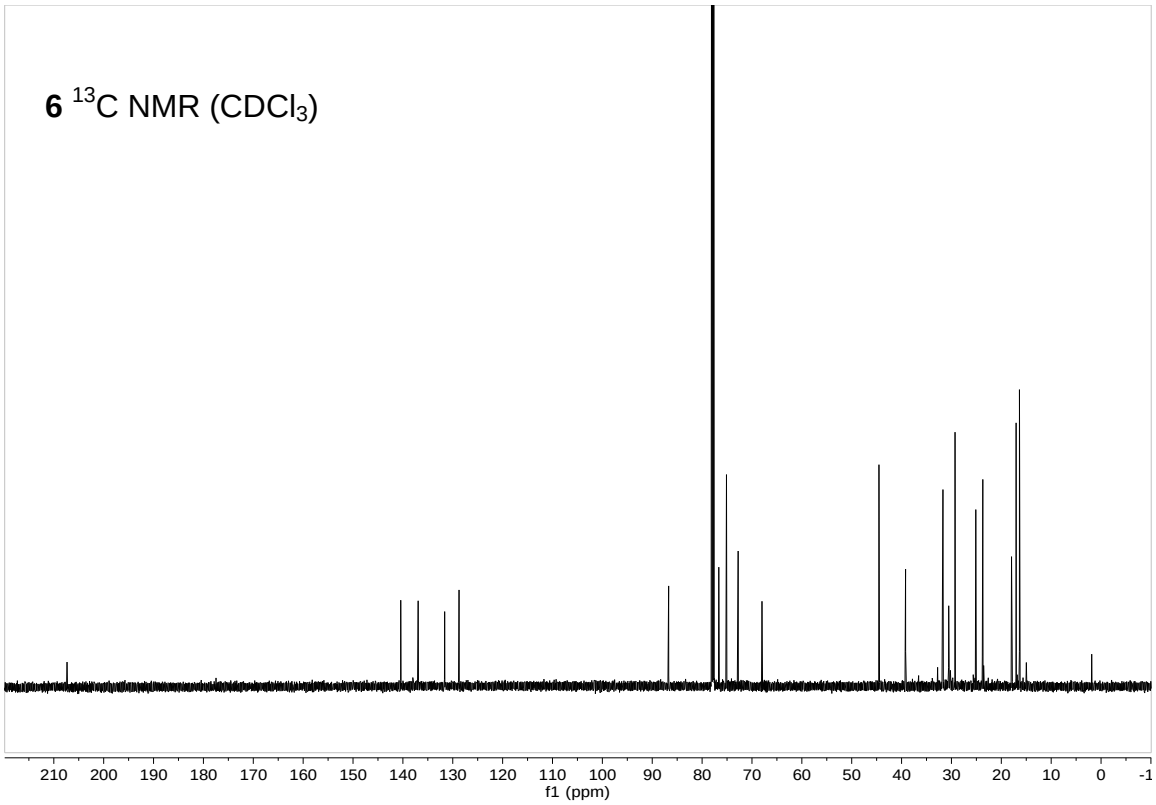


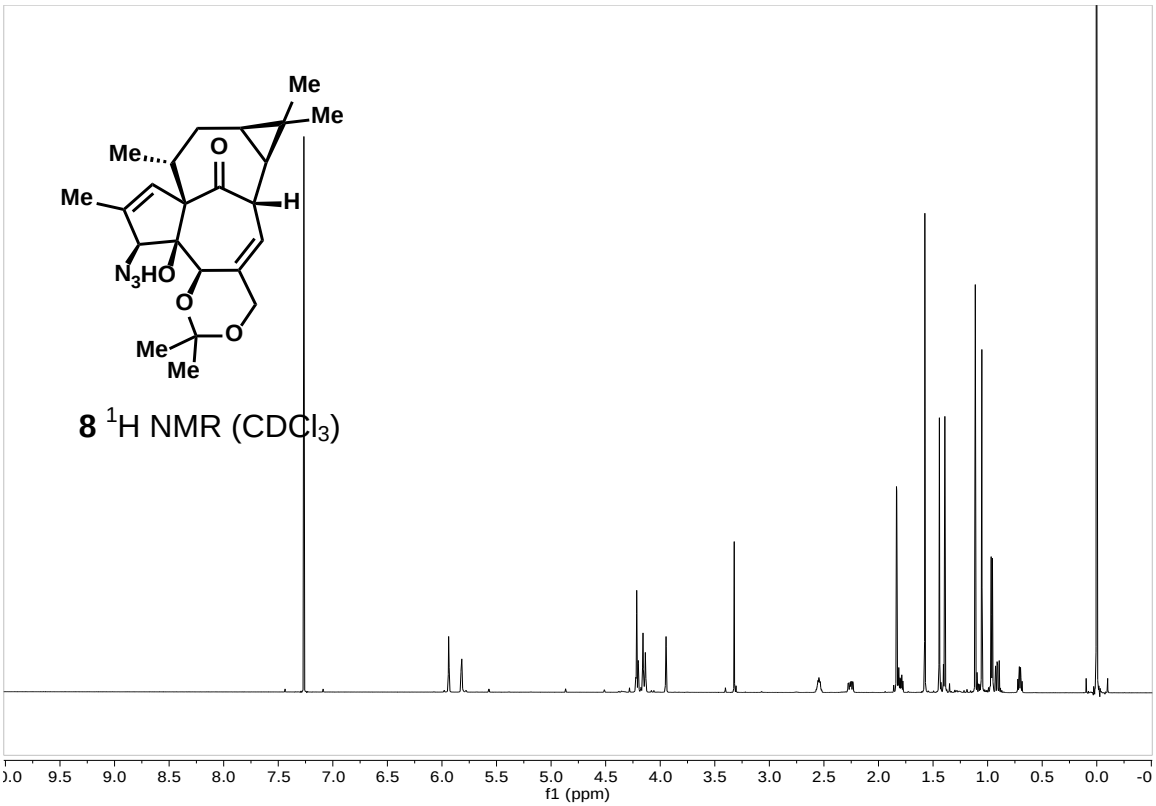
S6 ^{13}C NMR ($CDCl_3$)

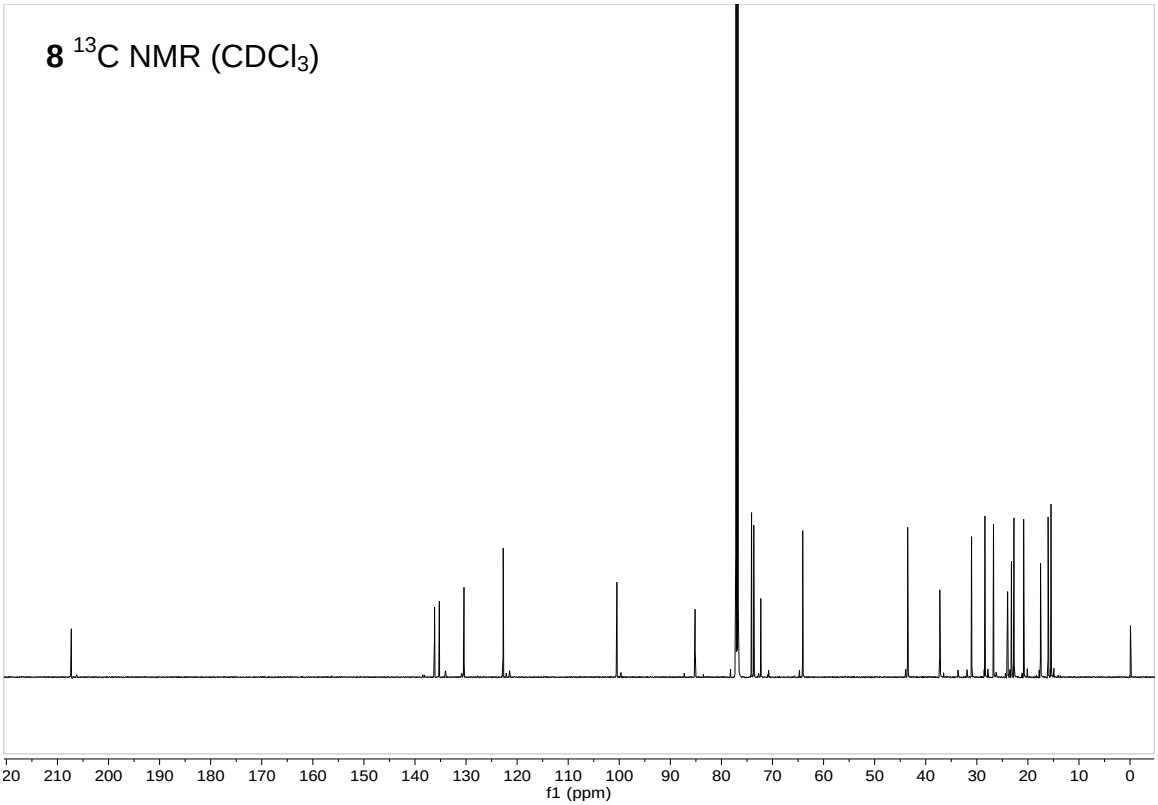

Me
Me
Me
O
Me
H
N3HO
HO
Me
5 1H NMR (CDCl3)
f1 (ppm)

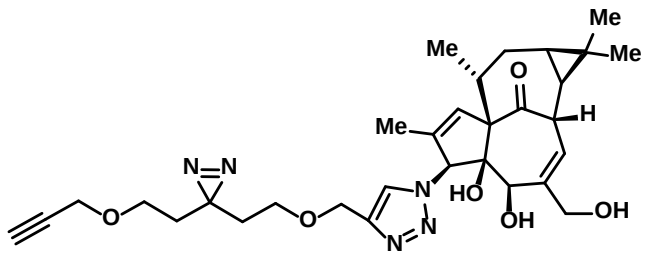

5 13C NMR (CDCl3)
f1 (ppm)


Me
Me
Me
O
Me
H
N3HO
HO
OH
6 1H NMR (CDCl3)
f1 (ppm)

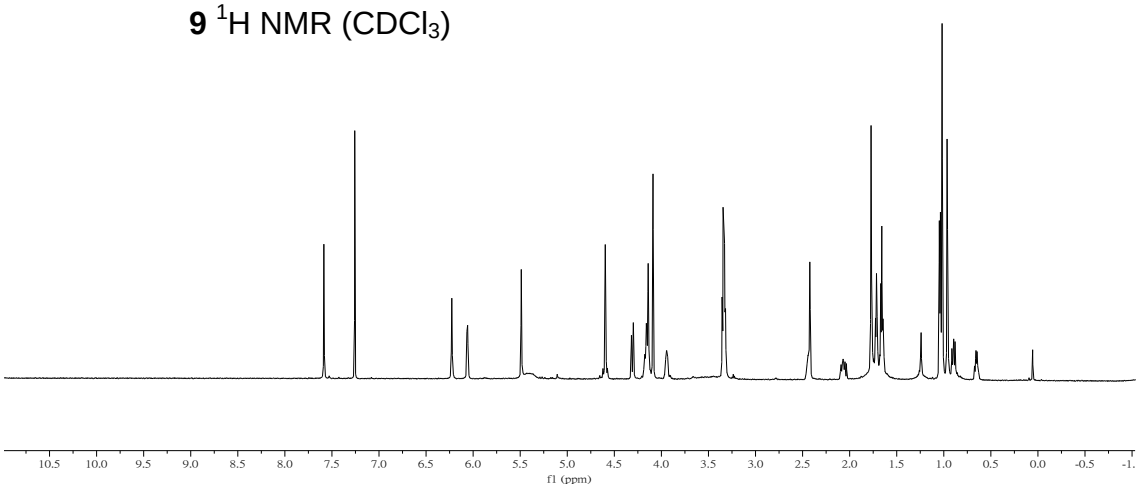

6 13C NMR (CDCl3)
f1 (ppm)


Me
Me
Me
O
Me
H
N3HO
O
O
Me
Me
8 1H NMR (CDCl3)
f1 (ppm)

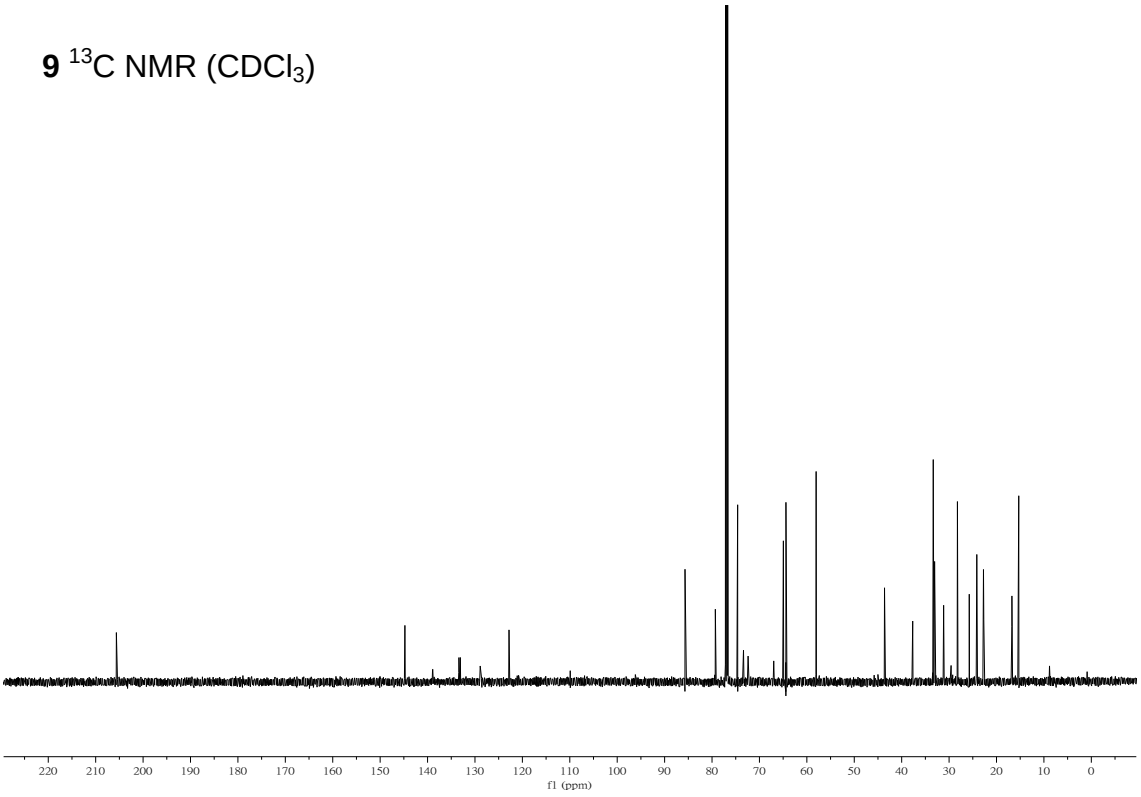

8 13C NMR (CDCl3)
f1 (ppm)



9 ^{1}H NMR ($CDCl_3$)



9 ^{13}C NMR ($CDCl_3$)



References

(1) Parker, C. G.; Galmozzi, A.; Wang, Y.; Correia, B. E.; Sasaki, K.; Joslyn, C. M.; Kim, A. S.; Cavallaro, C. L.; Lawrence, R. M.; Johnson, S. R.et al. Ligand and Target Discovery by Fragment-Based Screening in Human Cells. *Cell* **2017,** *168* (3), 527-541 e529.

(2) Inloes, J. M.; Hsu, K. L.; Dix, M. M.; Viader, A.; Masuda, K.; Takei, T.; Wood, M. R.; Cravatt, B. F. The hereditary spastic paraplegia-related enzyme DDHD2 is a principal brain triglyceride lipase. *P Natl Acad Sci USA* **2014,** *111* (41), 14924-14929.

(3) Boersema, P. J.; Raijmakers, R.; Lemeer, S.; Mohammed, S.; Heck, A. J. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat Protoc* **2009,** *4* (4), 484-494.

(4) Washburn, M. P.; Wolters, D.; Yates, J. R., 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol* **2001,** *19* (3), 242-247.

(5) Weerapana, E.; Wang, C.; Simon, G. M.; Richter, F.; Khare, S.; Dillon, M. B.; Bachovchin, D. A.; Mowen, K.; Baker, D.; Cravatt, B. F. Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* **2010,** *468* (7325), 790-795.