Supporting Information

Inhibition of NRAS Signaling in Melanoma Through Direct Depalmitoylation Using Amphiphilic Nucleophiles

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Methods

Cell Culture. The WM3000 (NRAS Q61R mutation) and WM2013 (NRAS Q61K mutation) melanoma cell lines were purchased from Rockland Immunochemicals and cultured in Tumor Specialization Media according to the melanoma cell culture protocol. The Tumor Specialization Media was prepared in the laboratory by mixing 400 mL of MCDB 153 (Sigma-Aldrich), 100 mL of Leibovitz's L-15 (Life Technologies), 10 mL of heat-inactivated FBS (Omega Scientific), and 0.42 mL of $CaCl_2$. The Sk-Mel-2 (NRAS Q61K mutation) melanoma cell line was purchased from ATCC and cultured in EMEM with 10% FBS. The Sk-Mel-28 (BRAF V600E mutation) melanoma cell line was generously provided by Dr. Silvio Gutkind at the UCSD Moores Cancer Center and cultured in EMEM with 10% FBS. HeLa S3 cells were obtained from ATCC. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 ºC, 5% CO_2. The DMEM growth media was purchased from Life Technologies, and the EMEM growth media was purchased from Thermo Fisher Scientific.

Plasmid Construction and Cloning. Hs.NRAS was a gift from Dominic Esposito (Addgene plasmid # 83173) and mEGFP-C1 was a gift from Michael Davidson (Addgene plasmid # 54759). The primers 5'-CTT-CGA-ATT-CTG-CAG-TCG-ACA-TGC-CAA-CTT-TGT-ACA-AAA-AAG-3' (NRAS-fwd) and 5'-GAT-CCC-GGG-CCC-GCG-GTA-CCT-TAC-ATC-ACC-ACA-CAT-GG-3' (NRAS-rev) were used for PCR amplification of the NRAS insert and the primers 5'-CTT-TTT-TGT-ACA-AAG-TTG-GCA-TGT-CGA-CTG-CAG-AAT-TCG-AAG-3' (mEGFP-C1-fwd) and 5'-CCA-TGT-GTG-GTG-ATG-TAA-GGT-ACC-GCG-GGC-CCG-GGA-TC-3' (mEGFP-C1-rev) were used for PCR amplification of the mEGFP-C1 vector, which were ligated using Gibson assembly (New England Biolabs), and cloned in DH5α competent *E. coli* cells to produce the final EGFP-NRAS plasmid. The final construct was sequenced by Eton

Bioscience to verify its identity, and the SnapGene software was used for sequence alignment. The final plasmid used for transfection was prepared using a plasmid maxiprep kit (EZgene). pCMV(CAT)T7-SB100 was a gift from Zsuzsanna Izsvak (Addgene plasmid # 34879)[1]. pSBbi-Pur was a gift from Eric Kowarz (Addgene plasmid # 60523)[2]. pSBbi-Pur-EGFP-NRAS was constructed using NEBuilder HiFi Assembly (New England Biolabs). The vector pSBbi-Pur was linearized using SfiI and inserts were prepared by PCR amplification of EGFP-NRAS with the following primers: 5'-CTA-CCC-CAA-GCT-GGC-CTC-TGA-GGC-CAT-GGT-GAG-CAA-GGG-CGA-G-3' (EGFP-NRAS-fwd) and 5'-ATC-CCC-AAG-CTT-GGC-CTG-ACA-GGC-CTT-ACA-TCA-CCA-CAC-ATG-GCA-ATC-3' (EGFP-NRAS-rev). The final construct was sequenced by Eton Bioscience to verify its identity, and the SnapGene software was used for sequence alignment. The final plasmid used for transfection was prepared using a plasmid maxiprep kit (EZgene).

pSBbi-RP was a gift from Eric Kowarz (Addgene plasmid # 60513)[2]. The primers 5'-AGC-TGG-CCT-CTG-AGG-CCA-CCA-TGC-CAA-CTT-TGT-ACA-A-3' (NRAS-Q61R-forward) and 5'-AGC-TTG-GCC-TGA-CAG-GCC-ATT-ACA-TAA-TTA-CAC-ACT-TTG-TCT-T-3' (NRAS-Q61R-reverse), which include the SfiI restriction site, were used for PCR amplification of the NRAS(Q61R)-KRAS4bHVR insert out of a previously created lentiviral transfer plasmid (Supplementary Figure 8). Restriction enzyme digest with SfiI was done for the NRAS(Q61R)-KRAS4bHVR insert and the pSBbi-RP vector. The two samples – insert and vector – were run on an 0.8% agarose gel and extracted using the QIAquick Gel Extraction Kit (Qiagen). The NRAS(Q61R)-KRAS4bHVR insert and pSBbi-RP vector were ligated using the T4 DNA Ligase ligation protocol and transformed into NEB stable DH5α competent *E. coli* cells. The final

construct was sequenced by Eton Bioscience to verify its identity, and the SnapGene software was used for sequence alignment.

Generation of Stable Cell Lines. HeLa S3 cells stably expressing EGFP-NRAS were established using the Sleeping Beauty transposon system with the improved transposase SB100X[1,2]. HeLa S3 cells grown in a 6-well plate were transfected with pSBbi-Pur-EGFP-NRAS and pCMV(CAT)T7-SB100 at a 20:1 ratio [1.5 μg total DNA/well] using Lipofectamine 2000 reagent (Thermo Fisher Scientific) [3 uL/well] according to the manufacturer's protocol. 2 days after transfection, cells were expanded into 6 cm plates with selection media (DMEM; 10% FBS; 2 μg/mL puromycin). Cells were passaged under selection conditions for 2 weeks before experimental use.

A stable transgenic melanoma cell line with the NRAS(Q61R)-KRAS4bHVR was generated using the Sleeping Beauty transposons system[1,2]. The final NRAS(Q61R)-KRAS4bHVR construct was co-transfected with low amounts of the transposase SB100X into the WM3000 human melanoma cell line on 6-well plates using Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer's protocol in Opti-MEM reduced serum medium (Life Technologies). After 2 days, the transfected cells in each well were split onto new 6-well plates for selection with ranging concentrations of puromycin (0 μg/mL, 0.5 μg/mL, 1 μg/mL, and 2 μg/mL) to determine the puromycin kill curve and only select for melanoma cells that were stably transfected with the Sleeping Beauty NRAS(Q61R)-KRAS4bHVR construct.

Live-Cell Imaging of Stable HeLa S3 Cells. HeLa S3 cells stably expressing EGFP-NRAS were plated at 80,000 cells/well in an 8-well Lab-Tek II chamber slide (Thermo Fisher Scientific) and allowed to adhere overnight. The media was exchanged for Opti-MEM reduced serum medium (Life Technologies) before live-cell imaging with different treatment conditions.

100 mM stock solutions of compounds **1** and **2** were prepared by dissolving the solid compounds in DMSO containing 200 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) as a preservative. 100 mM stock solutions of 2-BP was prepared by dissolving the solid compound in DMSO. From these stocks, solutions of 40 μM **1** or **2** (with 80 μM TCEP) or 40 μM 2-BP were prepared in Opti-MEM media. The diluted solutions were added to the indicated final concentration in Opti-MEM within individual wells of the chamber slide right before imaging. The cells were imaged while maintaining 37 ºC, 5% CO_2 in the incubation chamber.

Microscopy. Imaging was performed on an Axio Observer Z1 inverted microscope (Carl Zeiss Microscopy Gmb, Germany) with Yokogawa CSU-X1 spinning disk confocal unit using a 63x, 1.4 NA oil immersion or 20x, 0.8 NA objective to an ORCA-Flash4.0 V2 Digital CMOS camera (Hamamatsu, Japan). For live cell imaging, an incubation chamber with temperature and CO_2 controllers (Okolab Incubation System, H301-K-FRAME stage top chamber) was utilized. Fluorophores were excited with diode lasers (488 nm; 561 nm). Images were acquired using Zen Blue software (Carl Zeiss) and processed using Image J.

S-palmitoylation Acyl-RAC Assay. WM3000 melanoma cells were plated at 2,450,000 cells/dish in 10 cm tissue culture dishes (Genesee Scientific) and allowed to adhere overnight. 100 mM stock solutions of compounds **1** and **2** were prepared by dissolving the solid compounds in DMSO containing 200 mM TCEP as a preservative. 100 mM stock solution of vehicle control was prepared by dissolving TCEP in DMSO. 100 mM stock solution of 2-BP was prepared by dissolving the solid compound in DMSO. From these stocks, solutions of 10 μM **1** or **2** (with 20 μM TCEP), 20 μM vehicle/TCEP, or 10 μM 2-BP were diluted in 6.13 mL of Opti-MEM media and added to the cells. Cells were then incubated at 37 ºC for 2 hours, media was removed, and cells were washed with 1 mL of HBSS. Cells were detached using a cell scraper after the

combined thiol blocking and cell lysis steps of the CAPTUREome S-Palmitoylated Protein Kit (Badrilla, UK) according to the manufacturer's protocol. Input fractions (IF) that consisted of the total proteins and bound fractions (BF) that consisted of the resin-captured S-palmitoylated proteins were ran on a 4–20% SDS-PAGE gel. All of the IF and BF samples were analyzed using western blotting, following the immunoblot protocol below, using the anti-NRAS primary antibody (ab227658) (Abcam). Western blot lanes were analyzed and quantified using ImageJ. Ratios of BF to IF for each of the treatment conditions were calculated and normalized to the control to determine the amount of S-palmitoylated NRAS in each sample. Additionally, BF samples were analyzed using silver staining, following the manufacturer's protocol (Thermo Fisher Scientific).

Western Blots. Cell lysates were prepared from confluent WM3000 and Sk-Mel-28 melanoma cell lines that were treated with six different conditions in Opti-MEM media for 2 hours. Stock solutions of compounds were prepared in DMSO at a concentration of 100 mM as before and diluted to the final concentrations. 10 mM stock solution of binimetinib (BM) was prepared by dissolving the solid compound in DMSO, and a final solution of 12 nM concentration (IC_{50} of BM[3]) was diluted in Opti-MEM media before adding to the cells. After a 2-hour incubation, cells were washed with 1X HBSS and lysed using 0.6 mL of complete RIPA per subconfluent monolayer on a 100 mm cell culture dish. Immediately prior to lysing the cells, 10 μL PMSF solution, 10 μL sodium orthovanadate solution, and 10 μL protease inhibitor cocktail solution per mL of 1X RIPA Lysis buffer were combined to prepare complete RIPA solution (Santa Cruz Biotechnology). The dishes were placed on a shaker for 10 minutes at room temperature. The cell lysates were scraped off the dishes using cell scrapers and pipetted into separate Eppendorf tubes. The tubes were centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was

transferred into new tubes and placed on ice. The reducing agent compatible BCA protein assay kit (Thermo Fisher Scientific) was used to measure protein concentration. Total protein in 1X Laemmli buffer with 10% 2-mercaptoethanol was separated by SDS/PAGE on a 4–20% gradient precast acrylamide gel for 30 minutes at 200 V, transferred to a PVDF membrane for 1 hour at 100 V, and blocked for 1 hour in 5% BSA. Membranes were incubated overnight at 4°C with primary antibodies. Horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling) or anti-mouse IgG (Sigma) were used as secondary antibodies, and membranes were developed using enhanced chemiluminescence (Thermo Fisher Scientific). β-Actin (3700), p-AKT (4060), AKT (4691), p-ERK (4370), ERK (4695), Calnexin (2433), Flotillin-1 (3253), Flotillin-2 (3436), SNAP25 (5308), Gα(i) (5290), and Lyric/Metadherin (14065) primary antibodies were purchased from Cell Signaling Technology. Western blot lanes were analyzed using ImageJ software and normalized to the vehicle/TCEP control.

Cell Viability Assay. The melanoma cell lines were plated at 10,000 cells per well on 96-well plates in Opti-MEM medium. Stock solutions of compounds were prepared in DMSO as before at a concentration of 100 mM and diluted to the final concentrations. The cells were treated with different concentrations of compounds the following day and incubated for 24 hours. Cell viability was assessed using the WST-1 cell proliferation reagent (Sigma-Aldrich) following the manufacturer's instructions. After incubation for 1 hour with the WST-1 reagent, absorbance was measured at 450 nm using a plate reader.

Animal Model. All animal experiments performed in this study were approved and compliant with ethical regulations provided by the University of California San Diego Institutional Animal Care and Use Committee (IACUC). Six-week-old female Nu/Nu nude mice (088) purchased from Charles River Laboratories were given ad lib access to food and water. For subcutaneous

implantation, 1 x 10^6 WM3000 or Sk-Mel-28 cells were spun down and resuspended in 50 ul of 1X PBS plus 50 ul of Matrigel [Mediatech Inc. (Corning)] and kept on ice until ready to use. Cells were implanted subcutaneously into the right flank of anesthetized mice using a 1 cc syringe. Tumor growth was monitored weekly until palpable, at which point a caliper was used and tumor volume was calculated by using the formula V = 1/2(Length x Width^2). For intratumoral injection (n=2), animals were anesthetized with ketamine:xylazine cocktail (17.5 mg/ml ketamine:2.5 mg/ml xylazine, at 0.1 ml/20 g body weight IP) and injected with 50 mg/kg compound 1 (mixed with 2 molar equivalents of TCEP) or with 123.51 mg/kg vehicle/TCEP control (2 molar equivalents) at 0.1 ml/10 g body weight. A volume of 20 μL was injected directly into the tumor in one-minute increments around the whole tumor. For intraperitoneal (IP) treatment, 20 mg/kg compound **1** (mixed with 2 molar equivalents of TCEP) or 49.40 mg/kg vehicle/TCEP control was administered daily for seven days. All compounds were reconstituted in sterile saline solution.

Histology and Immunohistochemistry. Tumors were resected and placed in 10 % formalin for 24 hours, then transferred to 70 % ethanol, paraffin-embedded and sectioned (4 μm thick). All slides were baked at 60 °C for 1 hour to deparaffinized. Once cool, slides were rehydrated through successive alcohols (Xylene 3min, 100% EtOH 3 min (2X), 95% EtOH 3min (2X), 70% EtOH 3 min (2X), diH_2O). Slides were then stained with hematoxylin and eosin (H & E). For immunohistochemistry, slides underwent antigen retrieval by boiling in sodium citrate buffer pH 6.0 for 15 minutes and cooled at room temperature for 30–40 minutes. Pap pen was drawn around the tissue and blocked with 3% H_2O_2 in background punisher (Biocare, cat # BP974) for 10 min, then washed 5 min (2X) in 1X TBST. Slides were protein blocked again with background punisher for another 10 min. Slides were then incubated with cleaved caspase-3

primary antibody (rabbit, Cell Signaling, cat # 9661L, 1:1000), p-ERK primary antibody (rabbit, Cell Signaling cat # 4370, 1:400) in 2.5 % normal horse serum (Vector Labs cat # S-2012), or one drop of Ki67 primary antibody (rabbit, Diagnostic Biosystems, part # RMPD004) at room temperature for 1 hour. Slides were then washed in 1X TBST for 5 min (2X), incubated with anti-rabbit HRP polymer secondary antibody (Biocare, cat # RMR622) for 30 min at room temperature, washed in 1X TBST for 5 min (2X), developed with DAB (Vector Laboratories, cat # SK-4105) for 5 min, and then washed in ice cold diH_2O to stop the reaction. Slides were then dehydrated [50% EtOH, 70% EtOH 3 min (2X), 95% EtOH 3 min (2X), 100% EtOH 3 min (2X), xylene 3 min (2X)] and mount in permount mounting medium (Fisher Scientific, part # SP15500).

Alanine Aminotransferase (ALT) Activity. Serum samples were collected at the end of the study and assayed for ALT levels using the Alanine Aminotransferase Activity Assay Kit according to manufacturer protocol (Sigma-Aldrich, Cat # MAK052).

General Considerations for Synthesis. Commercially available *N*-Boc-*L*-Cys(Trt)-OH, *N*-Ac-*L*-Cys(Trt)-OH, *N*-Boc-*L*-Cys-OH, *N*-Boc-*L*-Ser(^{t}Bu)-OH, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HATU), *N*,*N*-diisopropylethylamine (DIEA), butylamine, octylamine, dodecylamine, hexadecylamine, bromomethylacetate, sodium hydride (NaH), *N*,*N*-dimethylformamide (DMF), trifluoroacetic acid (TFA), triethylsilane (TES) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl) were obtained from Sigma-Aldrich. Deuterated chloroform ($CDCl_3$) and methanol (CD_3OD) were obtained from Cambridge Isotope Laboratories. All reagents obtained from commercial suppliers were used without further purification unless otherwise noted. Analytical thin-layer chromatography was performed on E. Merck silica gel 60 F_{254} plates. Compounds, which were not UV active, were visualized by

dipping the plates in a ninhydrin or potassium permanganate solution and heating. Silica gel flash chromatography was performed using E. Merck silica gel (type 60SDS, 230–400 mesh). Solvent mixtures for chromatography are reported as v/v ratios. HPLC analysis was carried out on an Eclipse Plus C8 analytical column with *Phase A*/*Phase B* gradients [*Phase A*: H_2O with 0.1% formic acid; *Phase B*: MeOH with 0.1% formic acid]. HPLC purification was carried out on Zorbax SB-C18 semipreparative column with *Phase A*/*Phase B* gradients [*Phase A*: H_2O with 0.1% formic acid; *Phase B*: MeOH with 0.1% formic acid]. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian VX-500 MHz or Jeol Delta ECA-500 MHz spectrometers, and were referenced relative to residual proton resonances in $CDCl_3$ (at δ 7.24 ppm) or CD_3OD (at δ 4.87 or 3.31 ppm). ^{1}H NMR splitting patterns are assigned as singlet (s), doublet (d), triplet (t), quartet (q) or pentuplet (p). All first-order splitting patterns were designated on the basis of the appearance of the multiplet. Splitting patterns that could not be readily interpreted are designated as multiplet (m) or broad (br). Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Varian VX-500 MHz or Jeol Delta ECA-500 MHz spectrometers, and were referenced relative to residual proton resonances in $CDCl_3$ (at δ 77.23 ppm) or CD_3OD (at δ 49.15 ppm). Electrospray Ionization-Time of Flight (ESI-TOF) spectra were obtained on an Agilent 6230 Accurate-Mass TOFMS mass spectrometer.

Synthesis of Alkyl Reagents.

H_2N-*L*-Cys-Oct (1) and **H_2N-*L*-Ser-Oct (2)** were synthesized as before[4].

***N*-Boc-*L*-Cys(Trt)-But.** A solution of *N*-Boc-*L*-Cys(Trt)-OH (125.0 mg, 269.6 μmol) in CH_2Cl_2 (2.5 mL) was stirred at 0 ºC for 10 min, and then HATU (112.8 mg, 296.6 μmol) and DIEA (187.9 μL, 1.08 mmol) were successively added. After 10 min stirring at 0 ºC, butylamine (26.7 μL, 269.6 μmol) was added. After 1 h stirring at rt, the reaction mixture was washed with

HCl(5%) (3 × 1 mL) and $NaHCO_3$(sat) (3 × 1 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated, providing a yellow oil, which was purified by flash chromatography (0–4% MeOH in CH_2Cl_2), affording 136.8 mg of *N*-Boc-*L*-Cys(Trt)-But as a white solid [98%, R_f = 0.21 (1% MeOH in CH_2Cl_2)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 7.42 (dd, J_1 = 8.4 Hz, J_2 = 1.4 Hz, 6H, 6 × CH_{Ar}), 7.29 (dd, J_1 = 8.5 Hz, J_2 = 6.8 Hz, 6H, 6 × CH_{Ar}), 7.24–7.19 (m, 3H, 3 × CH_{Ar}), 5.98 (t, J = 6.0 Hz, 1H, 1 × NH), 4.96–4.70 (m, 1H, 1 × NH), 4.00–3.68 (m, 1H, 1 × CH), 3.28–3.08 (m, 2H, 1 × CH_2), 2.78–2.64 (m, 1H, 0.5 × CH_2), 2.56–2.42 (m, 1H, 0.5 × CH_2), 1.46–1.42 (m, 2H, 1 × CH_2), 1.41 (s, 9H, 3 × CH_3), 1.35–1.22 (m, 2H, 1 × CH_2), 0.88 (t, J = 7.3 Hz, 3H, 1 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 170.4, 155.5, 144.5, 129.7, 128.2, 127.0, 80.3, 67.3, 53.7, 39.3, 38.8, 31.5, 28.4, 20.1, 13.8. MS (ESI-TOF) [m/z (%)]: 541 ($[M + Na]^+$, 100).

H_2N-*L*-Cys-But ∪ H_2N-*L*-Cys-But (3').* A solution of *N*-Boc-*L*-Cys(Trt)-But (50.0 mg, 96.5 μmol) in 500 μL of TFA/CH_2Cl_2/TES (225:225:50) was stirred at rt for 30 min. After removal of the solvent, the residue was dried under high vacuum for 3 h. Then, the corresponding residue was diluted in MeOH (250 μL), filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 11.7 mg of the H_2N-*L*-Cys-But ∪ H_2N-*L*-Cys-But (**3'**) as a colorless film [69%, t_R = 7.5 min (Zorbax SB-C18 semipreparative column, 90–10% *Phase A* in *Phase B*, 15 min)]. As a disulfide (RS-SR): t_R = 0.86 min (Eclipse Plus C8 analytical column, 5% *Phase A* in *Phase B*, 5.5 min), or t_R = 4.52 min (Eclipse Plus C8 analytical column, 95–5% *Phase A* in *Phase B*, 5.5 min). ^{1}H NMR (CD_3OD, 500.13 MHz, δ): 4.12–3.94 (m, 2H, 2 × CH), 3.30–3.19 (m, 6H, 2 × CH_2 + 2 × CH), 3.11–2.95 (m, 2H, 2 × CH), 1.63–1.46 (m, 4H, 2 × CH_2), 1.45–1.33 (m, 4H, 2 × CH_2), 0.95 (t, J = 7.3 Hz, 6H, 2 × CH_3). ^{13}C NMR (CD_3OD, 125.77 MHz, δ): 169.9, 53.5, 40.6, 40.5, 32.4, 21.1, 14.1. MS (ESI-TOF) [m/z

(%)]: 351 ([MH]$^+$, 100]. HRMS (ESI-TOF) calculated for [$C_{14}H_{30}N_4O_2S_2$] ([M +Na]$^+$) 373.1702, found 373.1698.

* Air oxidation of the thiol (RSH) causes the disulfide bond (RS-SR) formation.

H_2N-*L*-Cys-But (3). A solution of H_2N-*L*-Cys-But ∪ H_2N-*L*-Cys-But (**3'**, 1.0 mg, 2.9 μmol) in 1.14 mL of a 5 mM solution of TCEP.HCl in H_2O (or DMSO) was stirred at rt for 5 min. Then, it was analyzed by HPLC and/or used directly for the depalmitoylation experiments. As a free thiol (R-SH): t_R = 0.79 min (Eclipse Plus C8 analytical column, 5% *Phase A* in *Phase B*, 5.5 min), or t_R = 3.89 min (Eclipse Plus C8 analytical column, 95–5% *Phase A* in *Phase B*, 5.5 min). MS (ESI-TOF) [m/z (%)]: 177 ([MH]$^+$, 100).

***N*-Boc-*L*-Cys(Trt)-Dodec.** A solution of *N*-Boc-*L*-Cys(Trt)-OH (125.0 mg, 269.6 μmol) in CH_2Cl_2 (2.5 mL) was stirred at 0 ºC for 10 min, and then HATU (112.8 mg, 296.6 μmol) and DIEA (187.9 μL, 1.08 mmol) were successively added. After 10 min stirring at 0 ºC, dodecylamine (50.0 mg, 269.6 μmol) was added. After 1 h stirring at rt, the reaction mixture was washed with HCl(5%) (3 × 1 mL) and $NaHCO_3$(sat) (3 × 1 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated, providing a yellow oil, which was purified by flash chromatography (0–3% MeOH in CH_2Cl_2), affording 163.8 mg of *N*-Boc-*L*-Cys(Trt)-Dodec as a pale yellow oil [96%, R_f = 0.52 (1% MeOH in CH_2Cl_2)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 7.50–7.36 (m, 6H, 6 × CH_{Ar}), 7.28 (dd, J_1 = 8.5 Hz, J_2 = 6.9 Hz, 6H, 6 × CH_{Ar}), 7.24–7.19 (m, 3H, 3 × CH_{Ar}), 5.98 (d, J = 5.8 Hz, 1H, 1 × NH), 4.90–4.71 (m, 1H, 1 × NH), 3.90–3.74 (m, 1H, 1 × CH), 3.24–3.09 (m, 2H, 1 × CH_2), 2.77–2.65 (m, 1H, 0.5 × CH_2), 2.58–2.41 (m, 1H, 0.5 × CH_2), 1.47–1.42 (m, 2H, 1 × CH_2), 1.41 (s, 9H, 3 × CH_3), 1.34–1.14 (m, 18H, 9 × CH_2), 0.87 (t, J = 6.9 Hz, 3H, 1 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 170.3, 155.5, 144.5, 129.7, 128.2,

127.0, 80.3, 67.3, 53.7, 39.6, 38.7, 32.0, 29.8, 29.7, 29.7, 29.7, 29.5, 29.5, 29.4, 28.4, 27.0, 22.8, 14.3. MS (ESI-TOF) [m/z (%)]: 653 ([M +Na]$^+$, 100).

H_2N-*L*-Cys-Dodec ∪ H_2N-*L*-Cys-Dodec (4').* A solution of *N*-Boc-*L*-Cys(Trt)-Dodec (50.0 mg, 79.3 μmol) in 500 μL of TFA/CH_2Cl_2/TES (225:225:50) was stirred at rt for 30 min. After removal of the solvent, the residue was dried under high vacuum for 3 h. Then, the corresponding residue was diluted in MeOH (250 μL), filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 16.8 mg of the H_2N-*L*-Cys-Dodec ∪ H_2N-*L*-Cys-Dodec (**4'**) as a colorless film [73%, t_R = 8.0 min (Zorbax SB-C18 semipreparative column, 50% *Phase A* in *Phase B*, 5 min, and then 5% *Phase A* in *Phase B*, 10 min)]. As a disulfide (RS-SR): t_R = 2.80 min (Eclipse Plus C8 analytical column, 5% *Phase A* in *Phase B*, 5.5 min). ^{1}H NMR (CD_3OD, 500.13 MHz, δ): 4.09–3.96 (m, 2H, 2 × CH), 3.30–3.19 (m, 6H, 2 × CH_2 + 2 × CH), 3.11–2.96 (m, 2H, 2 × CH), 1.63–1.49 (m, 4H, 2 × CH_2), 1.40–1.25 (m, 36H, 18 × CH_2), 0.90 (t, *J* = 6.9 Hz, 6H, 2 × CH_3). ^{13}C NMR (CD_3OD, 125.77 MHz, δ): 170.2, 53.6, 40.9, 40.9, 33.1, 30.9, 30.8, 30.8, 30.5, 30.5, 30.3, 28.1, 23.8, 14.5. MS (ESI-TOF) [m/z (%)]: 575 ([MH]$^+$, 100]. HRMS (ESI-TOF) calculated for [$C_{30}H_{62}N_4O_2S_2Na$] ([M +Na]$^+$) 597.3206, found 597.4209.

* Air oxidation of the thiol (RSH) causes the disulfide bond (RS-SR) formation.

H_2N-*L*-Cys-Dodec (4). A solution of H_2N-*L*-Cys-Dodec ∪ H_2N-*L*-Cys-Dodec (**4'**, 1.0 mg, 1.7 μmol) in 695.7 μL of a 5 mM solution of TCEP.HCl in H_2O (or DMSO) was stirred at rt for 5 min. Then, it was analyzed by HPLC and/or used directly for the depalmitoylation experiments. As a free thiol (R-SH): t_R = 2.67 min (Eclipse Plus C8 analytical column, 5% *Phase A* in *Phase B*, 5.5 min). MS (ESI-TOF) [m/z (%)]: 289 ([MH]$^+$, 100).

***N*-Boc-*L*-Cys(Trt)-Hexadec.** A solution of *N*-Boc-*L*-Cys(Trt)-OH (125.0 mg, 269.6 μmol) in CH_2Cl_2 (2.5 mL) was stirred at 0 ºC for 10 min, and then HATU (112.8 mg, 296.6 μmol) and DIEA (187.9 μL, 1.08 mmol) were successively added. After 10 min stirring at 0 ºC, hexadecylamine (65.1 mg, 269.6 μmol) was added. After 1 h stirring at rt, the reaction mixture was washed with HCl(5%) (3 × 1 mL) and $NaHCO_3$(sat) (3 × 1 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated, providing a yellow oil, which was purified by flash chromatography (0–3% MeOH in CH_2Cl_2), affording 180.7 mg of *N*-Boc-*L*-Cys(Trt)-Hexadec as a pale yellow oil [98%, R_f = 0.63 (1% MeOH in CH_2Cl_2)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 7.45–7.39 (m, 6H, 6 × CH_{Ar}), 7.29 (dd, J_1 = 8.6 Hz, J_2 = 6.9 Hz, 6H, 6 × CH_{Ar}), 7.24–7.18 (m, 3H, 3 × CH_{Ar}), 5.97 (d, J = 5.8 Hz, 1H, 1 × NH), 4.93–4.72 (m, 1H, 1 × NH), 3.92–3.73 (m, 1H, 1 × CH), 3.24–3.07 (m, 2H, 1 × CH_2), 2.78–2.64 (m, 1H, 0.5 × CH_2), 2.57–2.44 (m, 1H, 0.5 × CH_2), 1.47–1.42 (m, 2H, 1 × CH_2), 1.41 (s, 9H, 3 × CH_3), 1.32–1.20 (m, 26H, 13 × CH_2), 0.88 (t, J = 6.9 Hz, 3H, 1 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 170.3, 155.5, 144.5, 129.7, 128.2, 127.0, 80.3, 67.3, 53.7, 39.6, 38.8, 32.1, 29.8, 29.8, 29.8, 29.8, 29.7, 29.7, 29.5, 29.5, 29.4, 28.4, 27.0, 22.8, 14.3. MS (ESI-TOF) [m/z (%)]: 709 ([M +Na]$^+$, 100).

H_2N-*L*-Cys-Hexadec ∪ H_2N-*L*-Cys-Hexadec (5').* A solution of *N*-Boc-*L*-Cys(Trt)-Hexadec (50.0 mg, 72.8 μmol) in 500 μL of TFA/CH_2Cl_2/TES (225:225:50) was stirred at rt for 30 min. After removal of the solvent, the residue was dried under high vacuum for 3 h. Then, the corresponding residue was diluted in MeOH (250 μL), filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 16.3 mg of the H_2N-*L*-Cys-Hexadec ∪ H_2N-*L*-Cys-Hexadec (**5'**) as a colorless film [65%, t_R = 8.9 min (Zorbax SB-C18 semipreparative column, 50% *Phase A* in *Phase B*, 5 min, and then 5% *Phase A* in *Phase B*, 10 min)]. As a disulfide (RS-SR): t_R = 3.13 min (Eclipse Plus C8 analytical column, 5% *Phase A*

in *Phase B*, 5.5 min). ^{1}H NMR (CD_3OD, 500.13 MHz, δ): 4.06–3.95 (m, 2H, 2 × CH), 3.30–3.20 (m, 6H, 2 × CH_2 + 2 × CH), 3.09–2.95 (m, 2H, 2 × CH), 1.63–1.49 (m, 4H, 2 × CH_2), 1.37–1.26 (m, 52H, 26 × CH_2), 0.90 (t, J = 6.8 Hz, 6H, 2 × CH_3). ^{13}C NMR (CD_3OD, 125.77 MHz, δ): 170.4, 53.7, 41.0, 40.9, 33.1, 30.9, 30.8, 30.8, 30.5, 30.5, 30.3, 28.1, 23.8, 14.5. MS (ESI-TOF) [m/z (%)]: 687 ([MH]$^+$, 100]. HRMS (ESI-TOF) calculated for [$C_{38}H_{78}N_4O_2S_2Na$] ([M +Na]$^+$) 709.5458, found 709.5462.

* Air oxidation of the thiol (RSH) causes the disulfide bond (RS-SR) formation.

H_2N-*L*-Cys-Hexadec (5). A solution of H_2N-*L*-Cys-Hexadec ∪ H_2N-*L*-Cys-Hexadec (**5'**, 1.0 mg, 1.5 μmol) in 582.1 μL of a 5 mM solution of TCEP.HCl in H_2O (or DMSO) was stirred at rt for 5 min. Then, it was analyzed by HPLC and/or used directly for the depalmitoylation experiments. As a free thiol (R-SH): t_R = 2.89 min (Eclipse Plus C8 analytical column, 5% *Phase A* in *Phase B*, 5.5 min). MS (ESI-TOF) [m/z (%)]: 345 ([MH]$^+$, 100).

***N*-Ac-*L*-Cys(Trt)-Oct.** A solution of *N*-Ac-*L*-Cys(Trt)-OH (109.3 mg, 269.6 μmol) in CH_2Cl_2 (2.5 mL) was stirred at 0 ºC for 10 min, and then HATU (112.8 mg, 296.6 μmol) and DIEA (187.9 μL, 1.08 mmol) were successively added. After 10 min stirring at 0 ºC, octylamine (44.6 mg, 269.6 μmol) was added. After 1 h stirring at rt, the reaction mixture was washed with HCl(5%) (3 × 1 mL) and $NaHCO_3$(sat) (3 × 1 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated, providing a yellow oil, which was purified by flash chromatography (0–4% MeOH in CH_2Cl_2), affording 129.9 mg of *N*-Ac-*L*-Cys(Trt)-Oct as a white solid [93%, R_f = 0.11 (1% MeOH in CH_2Cl_2)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 7.45–7.40 (m, 6H, 6 × CH_{Ar}), 7.28 (dd, J_1 = 8.5 Hz, J_2 = 6.8 Hz, 6H, 6 × CH_{Ar}), 7.24–7.19 (m, 3H, 3 × CH_{Ar}), 6.04 (d, J = 6.0 Hz, 1H, 1 × NH), 5.95 (d, J = 6.0 Hz, 1H, 1 × NH), 4.07 (dt, J_1 = 7.6 Hz, J_2 = 6.4 Hz, 1H, 1 × CH), 3.23–3.04 (m, 2H, 1 × CH_2), 2.71 (ddd, J_1 = 12.9 Hz, J_2 = 6.8 Hz, J_3 = 1.4 Hz, 1H,

0.5 × CH_2), 2.51 (dd, J_1 = 13.0 Hz, J_2 = 6.0 Hz, 1H, 0.5 × CH_2), 1.88 (s, 3H, 1 × CH_3), 1.42 (t, J = 7.1 Hz, 2H, 1 × CH_2), 1.35–1.13 (m, 10H, 5 × CH_2), 0.86 (t, J = 6.9 Hz, 3H, 1 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 170.2, 170.0, 144.5, 129.7, 128.2, 127.0, 67.3, 52.3, 39.7, 38.8, 31.9, 29.4, 29.3, 29.3, 27.0, 23.3, 22.7, 14.2. MS (ESI-TOF) [m/z (%)]: 539 ([M +Na]$^+$, 100).

N*-Ac-*L*-Cys-Oct (6'). A solution of *N*-Ac-*L*-Cys(Trt)-Oct (50.0 mg, 96.8 μmol) in 500 μL of TFA/CH_2Cl_2/TES (225:225:50) was stirred at rt for 30 min. After removal of the solvent, the residue was dried under high vacuum for 3 h. Then, the corresponding residue was diluted in MeOH (250 μL), filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 15.2 mg of the *N*-Ac-*L*-Cys-Oct (**6'**) as a white solid [57%, t_R = 8.4 min (Zorbax SB-C18 semipreparative column, 50% *Phase A* in *Phase B*, 5 min, and then 5% *Phase A* in *Phase B*, 10 min)]. ^{1}H NMR (CD_3OD, 500.13 MHz, δ): 4.41 (dd, J_1 = 7.0 Hz, J_2 = 6.0 Hz, 1H, 1 × CH), 3.26–3.11 (m, 2H, 1 × CH_2), 2.84 (dd, J_1 = 13.8 Hz, J_2 = 6.0 Hz, 1H, 1 × CH), 2.76 (dd, J_1 = 13.8 Hz, J_2 = 7.1 Hz, 1H, 1 × CH), 2.01 (s, 3H, 1 × CH_3), 1.58–1.44 (m, 2H, 1 × CH_2), 1.37–1.23 (m, 10H, 5 × CH_2), 0.90 (t, J = 6.9 Hz, 3H, 1 × CH_3). ^{13}C NMR (CD_3OD, 125.77 MHz, δ): 173.4, 172.2, 57.3, 40.5, 33.0, 30.4, 30.4, 30.4, 28.0, 26.9, 23.8, 22.5, 14.5. As a free thiol (R-SH): t_R = 2.94 min (Eclipse Plus C8 analytical column, 5% *Phase A* in *Phase B*, 5.5 min). MS (ESI-TOF) [m/z (%)]: 275 ([MH]$^+$, 100). HRMS (ESI-TOF) calculated for [$C_{13}H_{26}N_2O_2SNa$] ([M +Na]$^+$) 297.1607, found 297.1604.

***** HPLC-ELSD, NMR and MS showed that the product obtained was the thiol *N*-Ac-*L*-Cys-Oct (**6**) and not the expected disulfide *N*-Ac-*L*-Cys-Oct ∪ N-Ac-*L*-Cys-Oct (**6'**).

***N*-Ac-*L*-Cys-Oct (6)** A solution of the possible formed *N*-Ac-*L*-Cys-Oct ∪ *N*-Ac-*L*-Cys-Oct (**6'**, 1.0 mg, 1.8 μmol) in 731.5 μL of a 5 mM solution of TCEP.HCl in H_2O (or DMSO) was

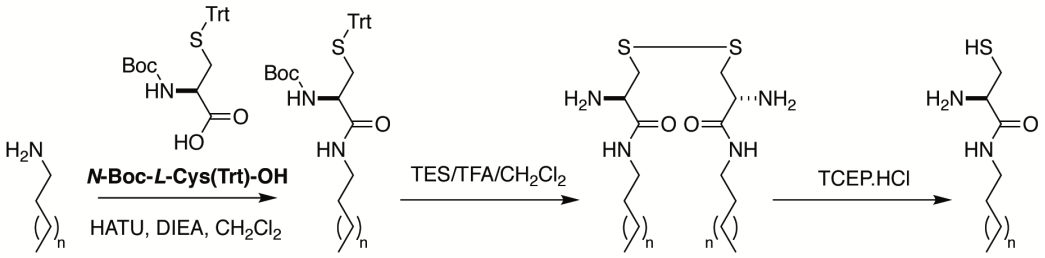
stirred at rt for 5 min. Then, it was analyzed by HPLC and/or used directly for the depalmitoylation experiments. As a free thiol (R-SH): t_R = 2.94 min (Eclipse Plus C8 analytical column, 5% *Phase A* in *Phase B*, 5.5 min). MS (ESI-TOF) [m/z (%)]: 275 ($[MH]^+$, 100). HRMS (ESI-TOF) calculated for [$C_{13}H_{26}N_2O_2SNa$] ($[M+Na]^+$) 297.1607, found 297.1604.

* As we mentioned above, HPLC-ELSD, NMR and MS showed that the product obtained was *N*-Ac-*L*-Cys-Oct (**6**). However, air oxidation of the thiol (RSH) over time could cause the disulfide bond (RS-SR) formation. Therefore, the product was treated with TCEP.HCl to be completely sure that we have the desired depalmitoylating agent (**6**).

Supplementary Schemes

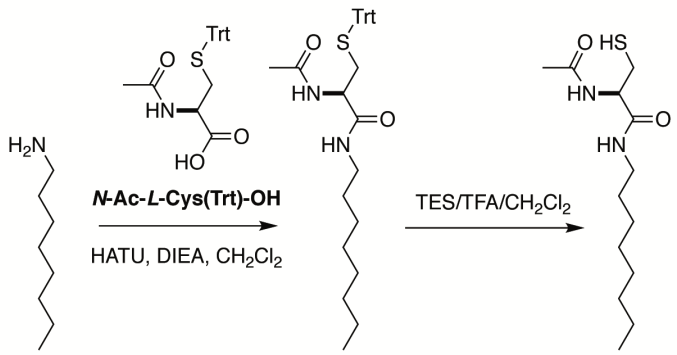
Supplementary Scheme 1. Synthesis of alkyl cysteine derivatives. **(a)** Synthesis of H_2N-*L*-Cys-But (**3**), H_2N-*L*-Cys-Oct (**1**), H_2N-*L*-Cys-Dodec (**4**) and H_2N-*L*-Cys-Hexadec (**5**). **(b)** Synthesis *N*-Ac-*L*-Cys-Oct (**6**). **(c)** Synthesis of H_2N-*L*-Ser-Oct (**2**). [ButA: butylamine, OctA: octylamine, DodecA: dodecylamine, HexadecA: hexadecylamine].

a



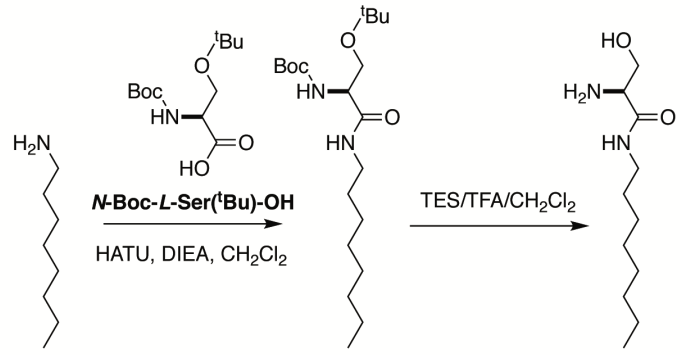
ButA, n = 1 — ***N*-Boc-*L*-Cys(Trt)-But**, n = 1 — **3'**, n = 1 — **3**, n = 1
OctA, n = 5 — ***N*-Boc-*L*-Cys(Trt)-Oct**, n = 5 — **1'**, n = 5 — **1**, n = 5
DodecA, n = 9 — ***N*-Boc-*L*-Cys(Trt)-Dodec**, n = 9 — **4'**, n = 9 — **4**, n = 9
HexadecA, n = 13 — ***N*-Boc-*L*-Cys(Trt)-Hexadec**, n = 13 — **5'**, n = 13 — **5**, n = 13

b



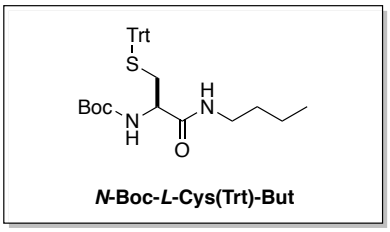
OctA — ***N*-Ac-*L*-Cys(Trt)-Oct** — **6**

c

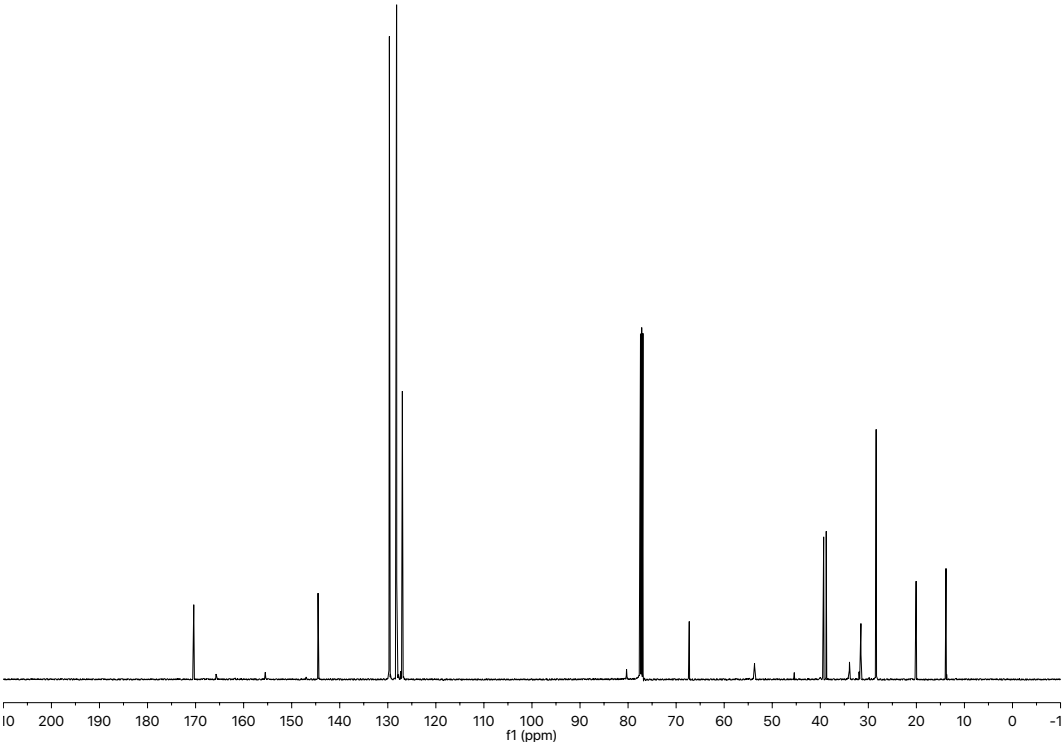


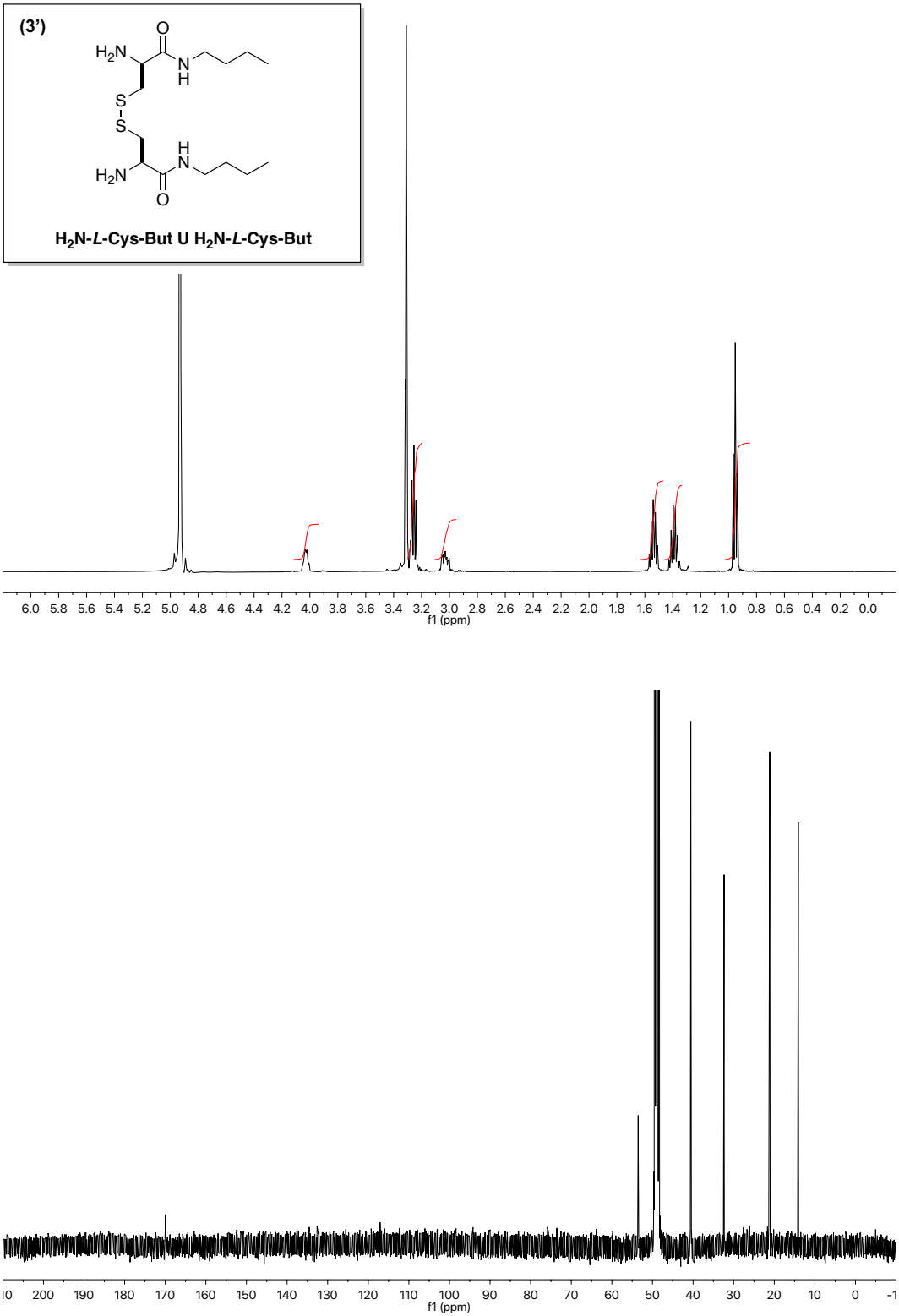
OctA — ***N*-Boc-*L*-Ser(ᵗBu)-Oct** — **2**

NMR Spectra



***N*-Boc-*L*-Cys(Trt)-But**




(3')
H2N
O
N
H
S
S
H2N
H
N
O
H2N-L-Cys-But U H2N-L-Cys-But
6.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4 0.2 0.0
f1 (ppm)
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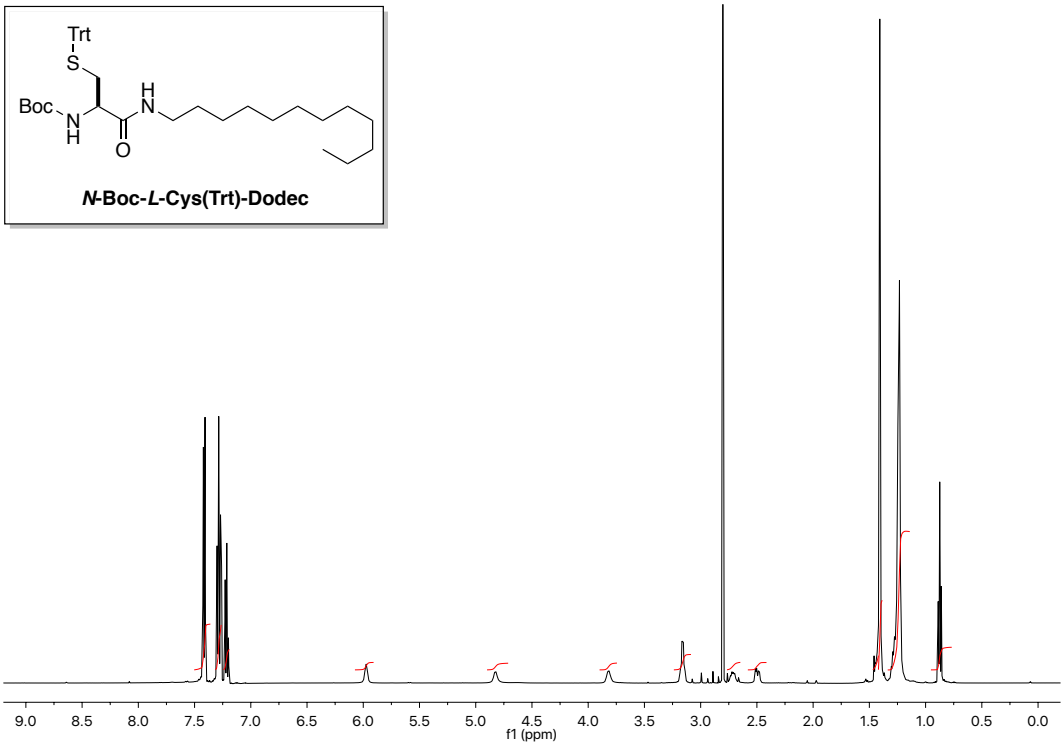
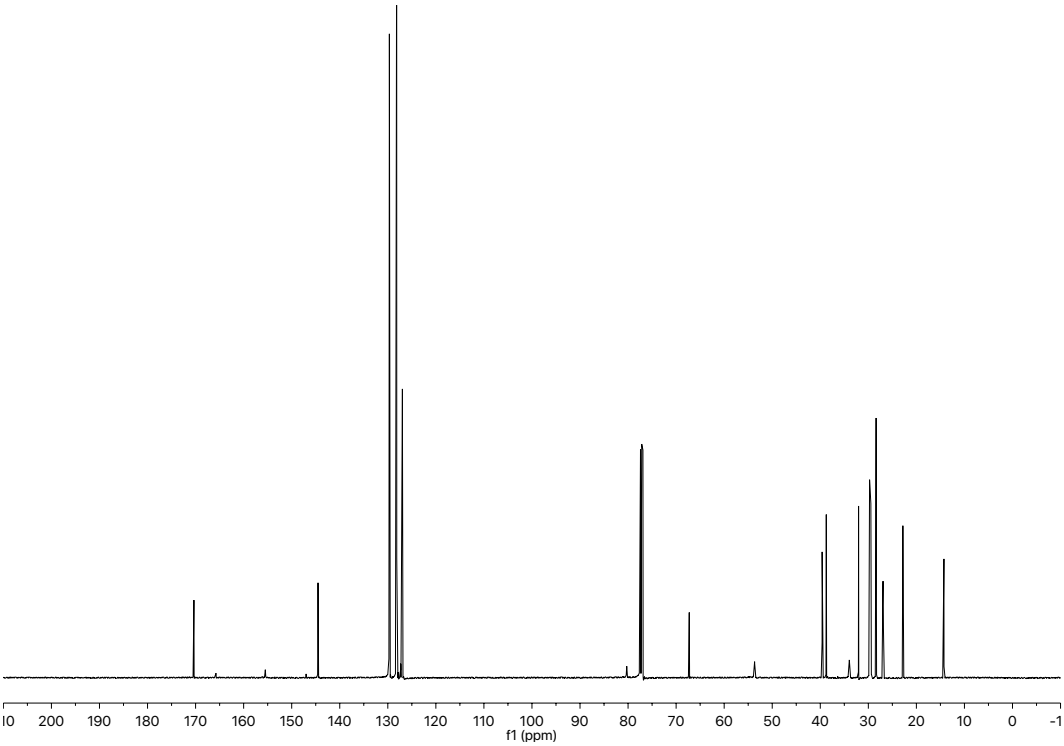
f1 (ppm)


Trt
S
Boc
N
H
H
N
O
N-Boc-L-Cys(Trt)-Dodec
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
f1 (ppm)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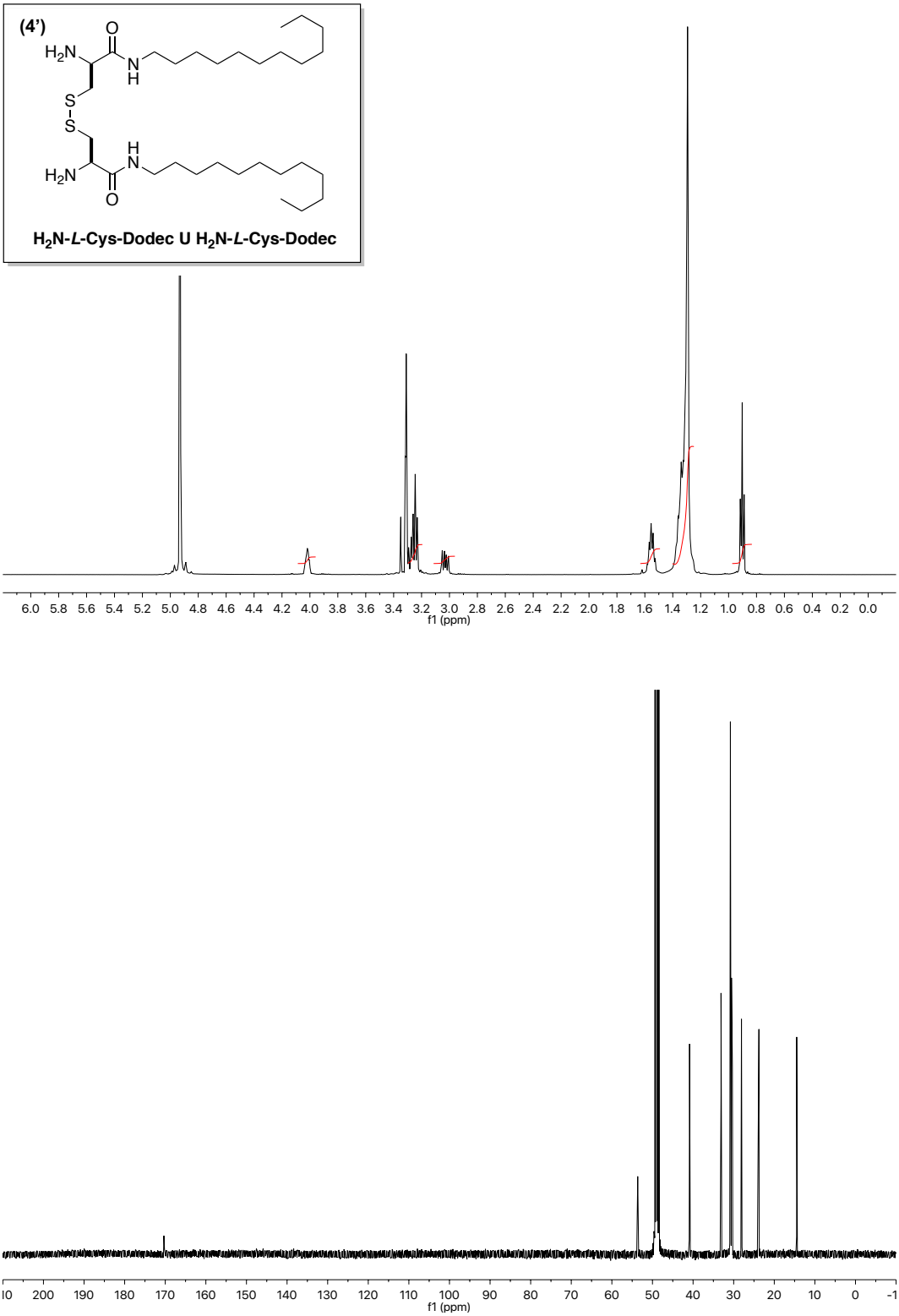
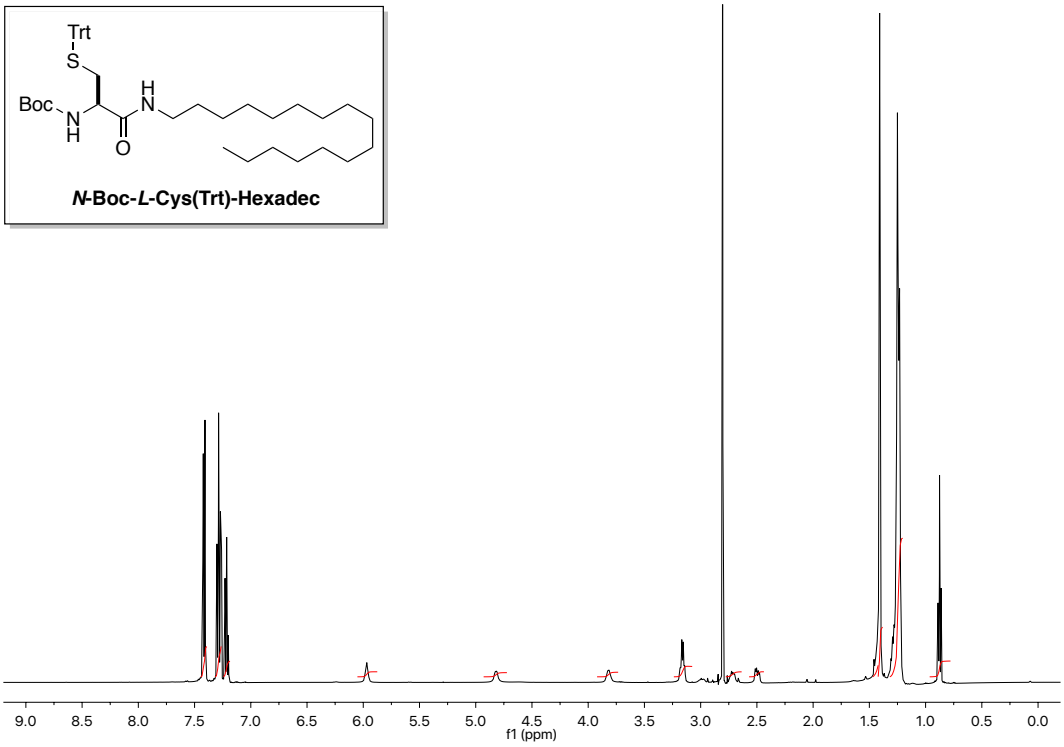
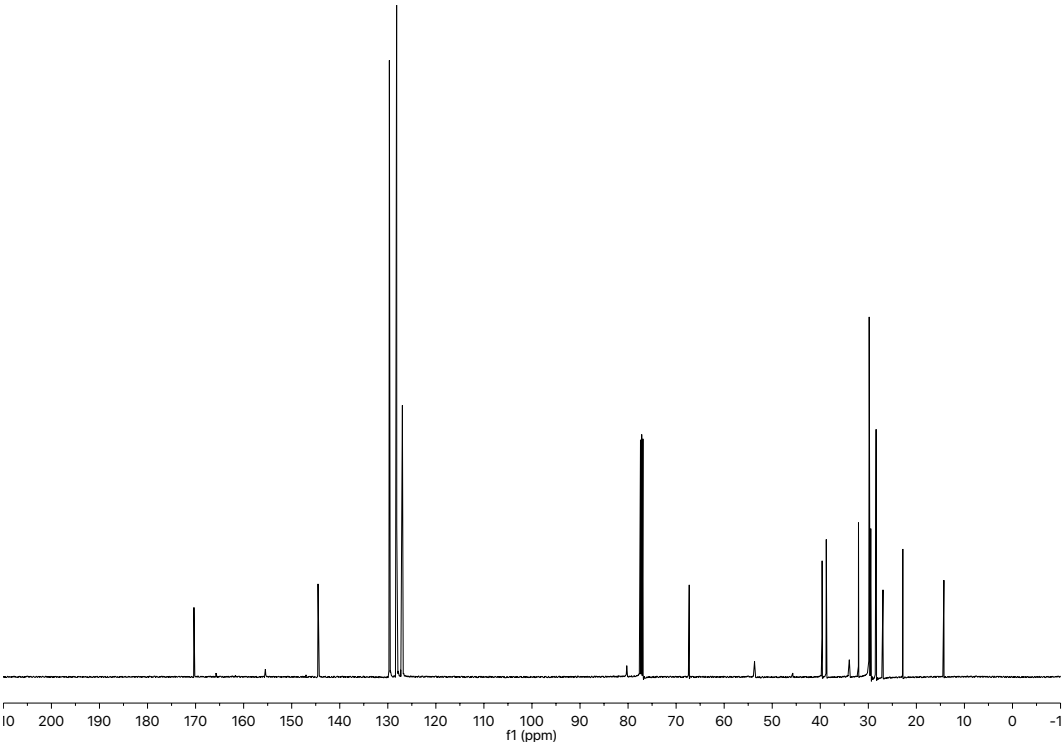

(4')
H2N-L-Cys-Dodec U H2N-L-Cys-Dodec
f1 (ppm)
f1 (ppm)


Trt
S
Boc
N
H
H
N
O
N-Boc-L-Cys(Trt)-Hexadec
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
f1 (ppm)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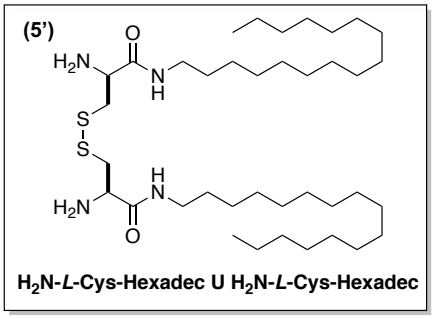
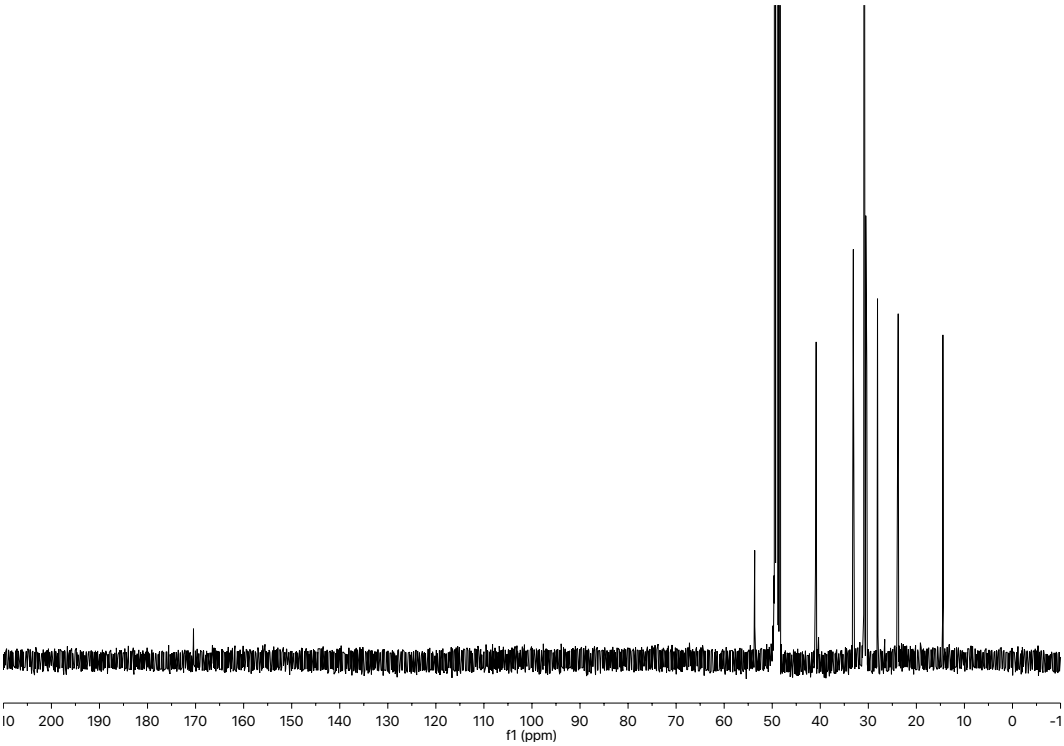
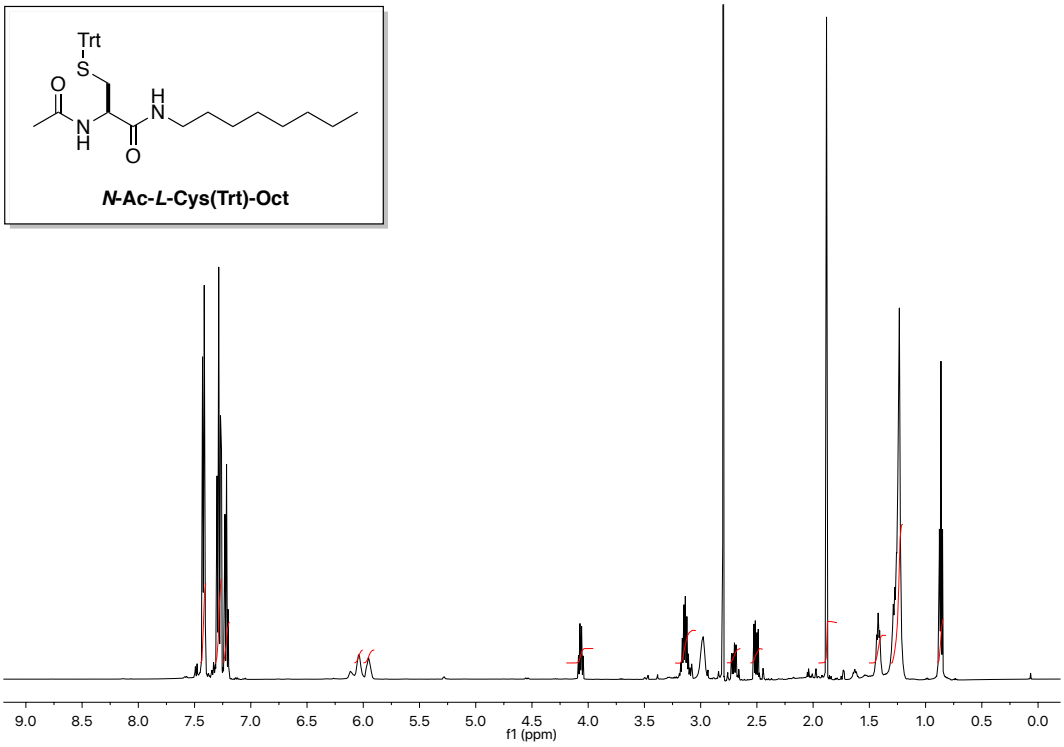
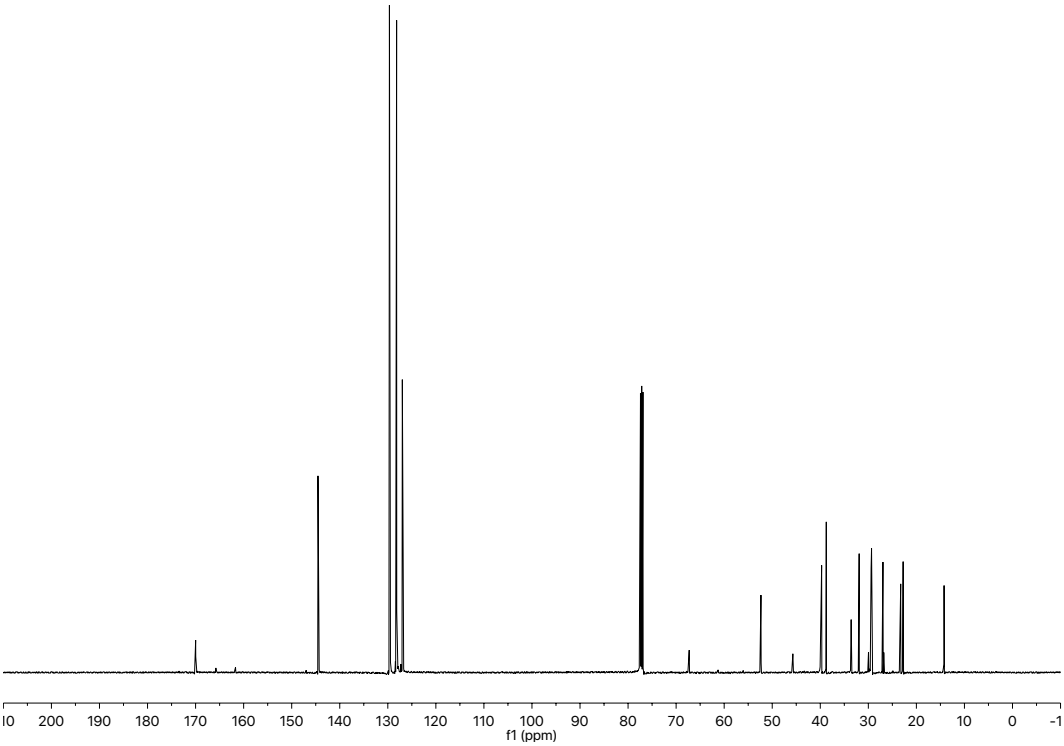
f1 (ppm)



H_2N-*L*-Cys-Hexadec U H_2N-*L*-Cys-Hexadec




Trt
S
O
N
H
H
N
O
N-Ac-L-Cys(Trt)-Oct
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
f1 (ppm)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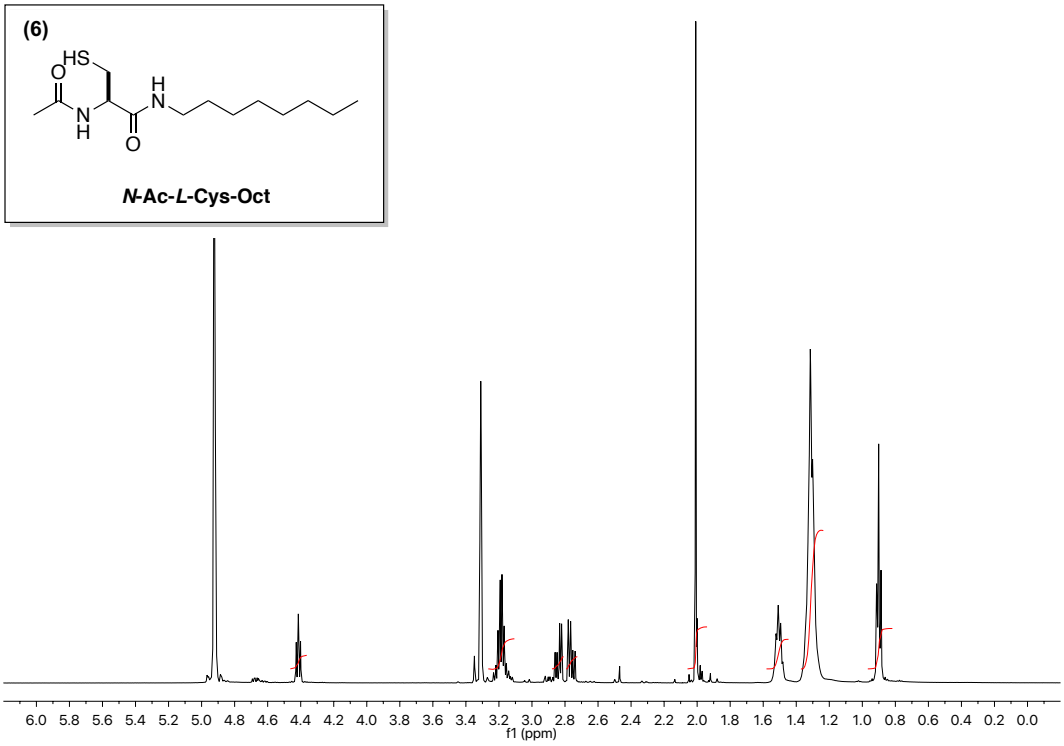
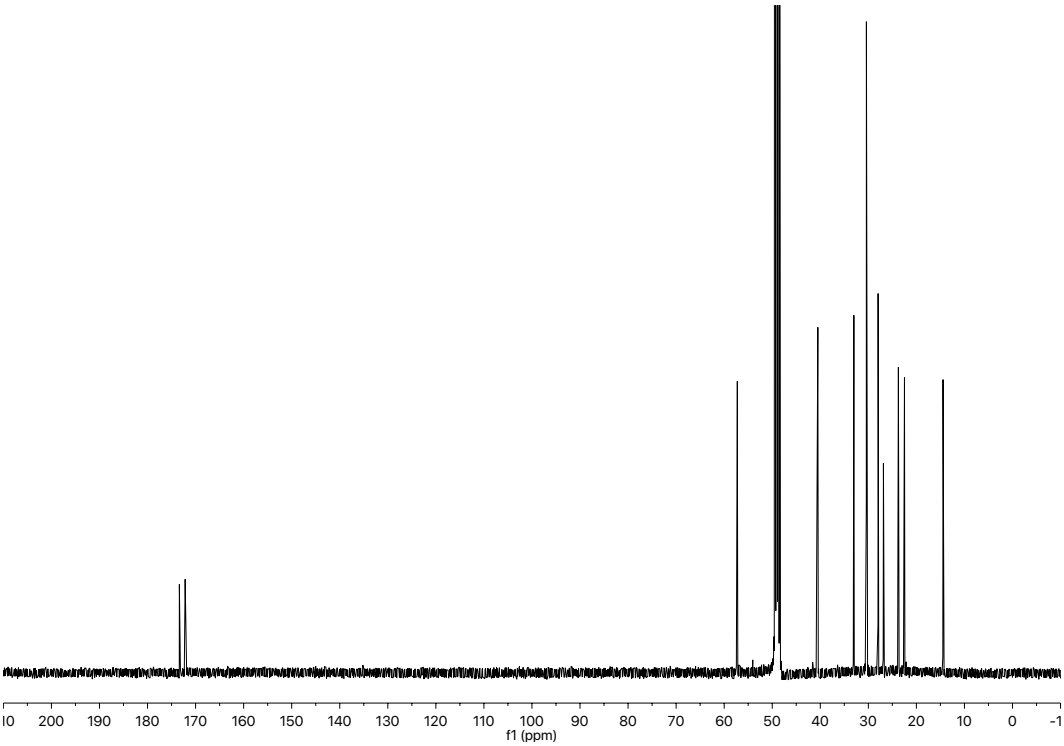
f1 (ppm)


(6)
HS
O
H
N
N
H
O
N-Ac-L-Cys-Oct
6.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4 0.2 0.0
f1 (ppm)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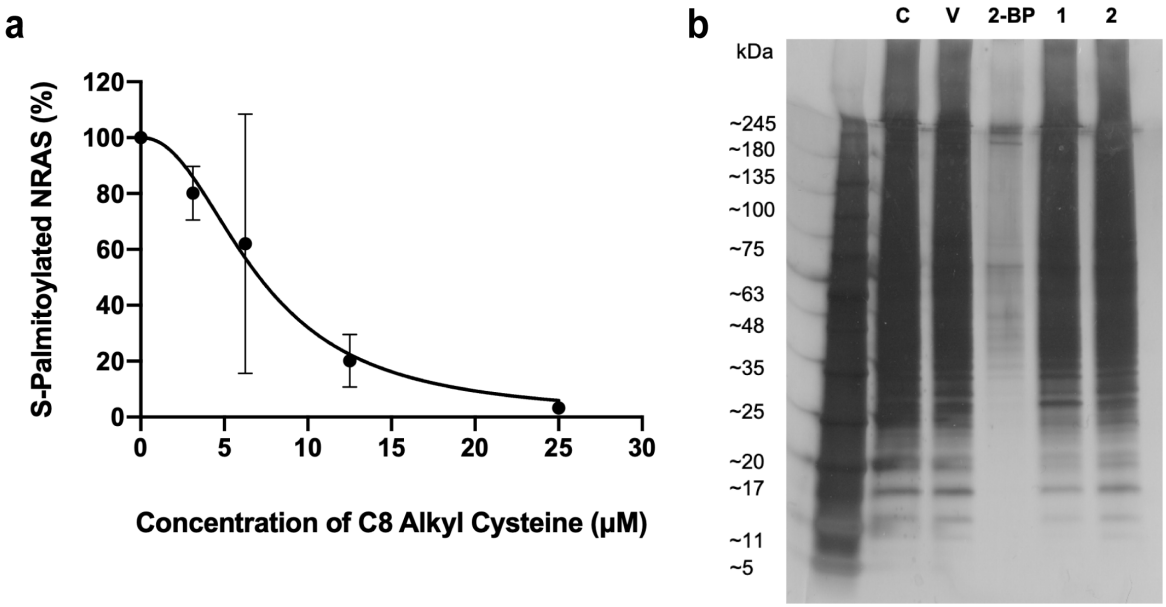
f1 (ppm)

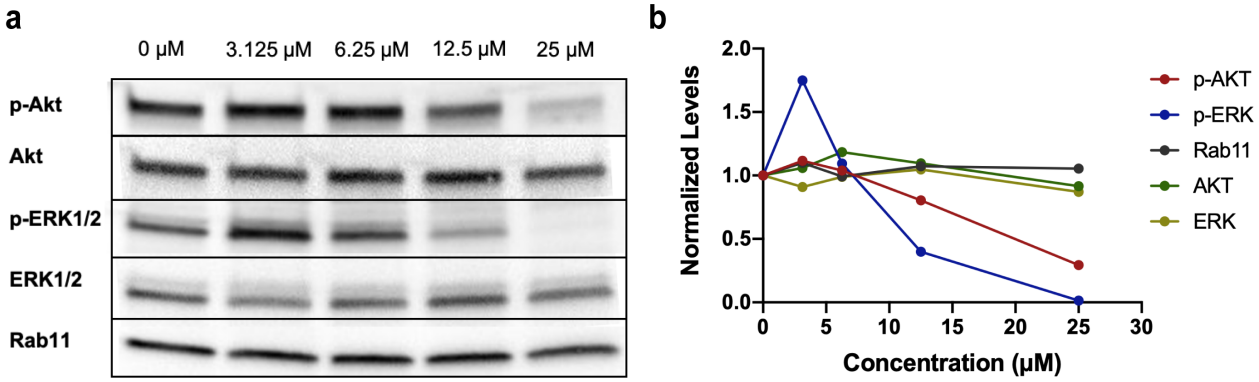
Supplemental Figures

Supplementary Figure 1. Depalmitoylation by C8 Alkyl Cysteine in NRAS-Driven Melanoma Cells. (a) Western blot analysis of WM3000 NRAS-mutated melanoma cells after treatment with increasing concentrations (0–25 μM) of C8 alkyl cysteine (**1**) from Figure 1c. Nonlinear regression (curve fit) with variable slopes was used to determine IC_{50}. Results are the mean ± SEM of three independent experiments. **(b)** Silver staining of S-palmitoylated proteins in WM3000 NRAS-mutated melanoma. Treatment conditions include the untreated control (C), vehicle control (20 μM TCEP in DMSO) (V), 10 μM 2-bromopalmitate (2-BP), 10 μM C8 alkyl cysteine (**1**), and 10 μM C8 alkyl serine (**2**). All samples were from the acyl resin-assisted capture (acyl-RAC) bound fractions (BF) that contain only S-palmitoylated proteins from Figure 1d.

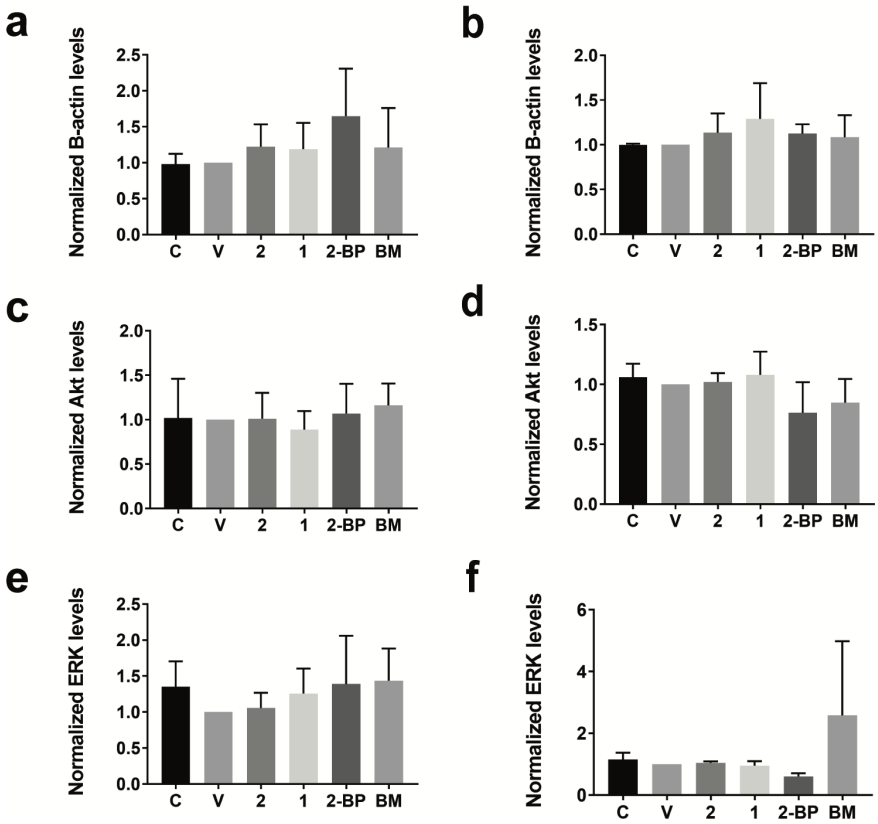


Supplementary Figure 2. Depalmitoylation of Proteins in NRAS-Driven Melanoma Cells. **(a)** Western blot detection of palmitoylated proteins in acyl resin-assisted capture fractions of WM3000 melanoma cells after 2-hour treatment with vehicle (20 μM TCEP in DMSO), 10 μM C8 alkyl cysteine (**1**), 10 μM C8 alkyl serine (**2**), or 10 μM 2-bromopalmitate (2-BP). Input fractions (IF) contain total cellular protein and bound fractions (BF) contain only S-palmitoylated proteins. **(b)** Western blot lanes were analyzed and quantified using ImageJ. Ratios of BF to IF for each treatment condition were calculated and normalized to vehicle to determine the amount of the S-palmitoylated proteins in each sample.

Supplementary Figure 3. C8 Alkyl Cysteine Inhibits Oncogenic RAS Signaling in NRAS-Mutated Melanoma Cells in a Dose-Dependent Manner. (a) Western blot of increasing concentrations of C8 alkyl cysteine (**1**) in the WM3000 melanoma cell line with the NRAS Q61R mutation. **(b)** Western blot lanes for p-AKT, p-ERK, Rab11, AKT, and ERK were analyzed and quantified using ImageJ. Each dose treatment was normalized to the 0 µM control.

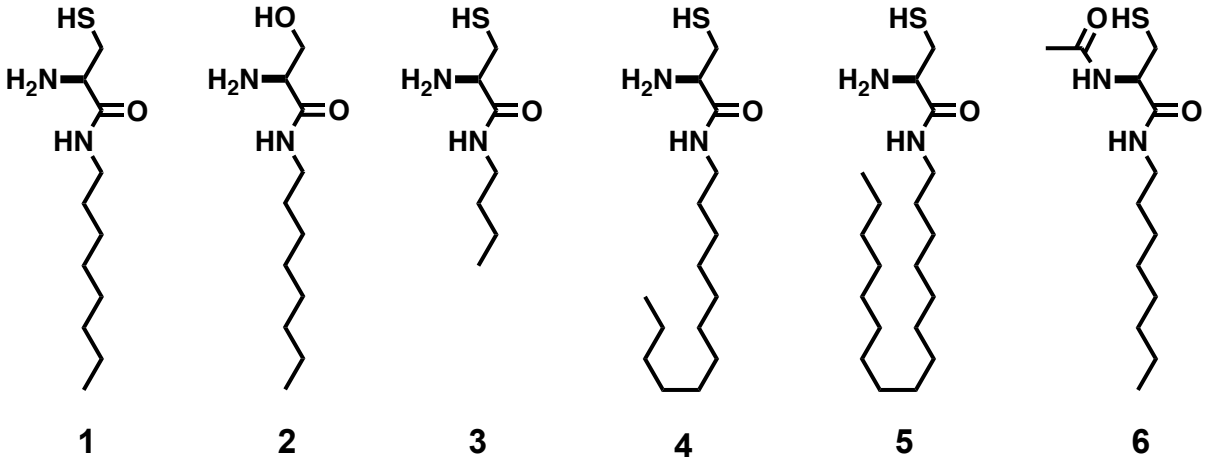


Supplementary Figure 4. Western Blot Protein Levels in NRAS-Mutated Melanoma vs. BRAF-Mutated Melanoma. Treatment conditions include the untreated control (C), vehicle control (20 μM TCEP in DMSO) (V), 10 μM C8 alkyl serine (**2**), 10 μM C8 alkyl cysteine (**1**), 10 μM 2-bromopalmitate (2-BP), and 12 nM binimetinib (BM). There was no significant difference in β-actin levels in the **(a)** WM3000 NRAS-mutated melanoma cells or the **(b)** Sk-Mel-28 BRAF-mutated melanoma cells. There was no significant difference in total AKT levels in the **(c)** WM3000 NRAS-mutated melanoma cells or the **(d)** Sk-Mel-28 BRAF-mutated melanoma cells. There was no significant difference in total ERK levels in the **(e)** WM3000 NRAS-mutated melanoma cells or the **(f)** Sk-Mel-28 BRAF-mutated melanoma cells, excluding the MEK inhibitor binimetinib. Western blot lanes for all protein levels were analyzed and quantified using ImageJ. Each treatment condition was normalized to the vehicle control. Results are the mean ± s.d. of three independent experiments (one-way ANOVA).

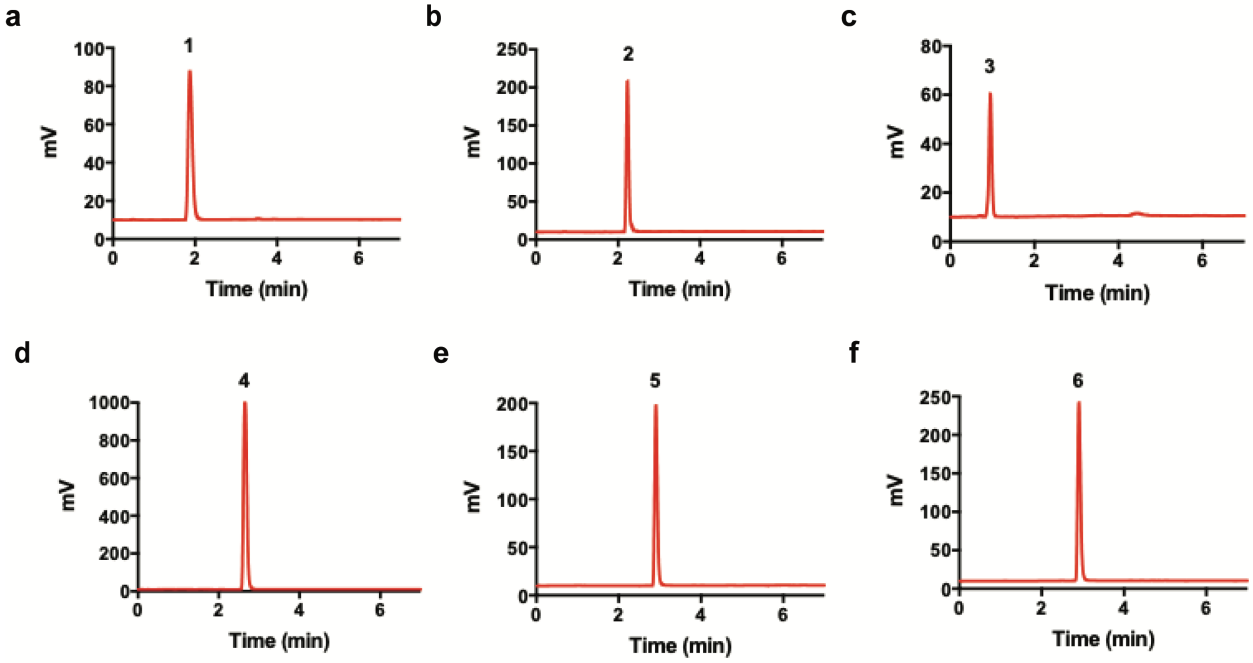


Supplementary Figure 5. Chemical Structures of Alkyl Cysteine Derivatives and Controls.

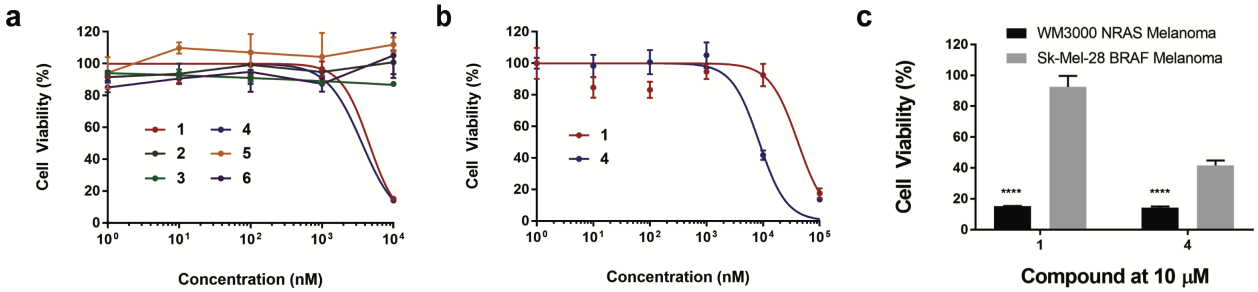
H_2N-*L*-Cys-Oct (**1**), H_2N-*L*-Ser-Oct (**2**), H_2N-*L*-Cys-But (**3**), H_2N-*L*-Cys-Dodec (**4**), H_2N-*L*-Cys-Hexad (**5**), and *N*-Ac-*L*-Cys-Oct (**6**).



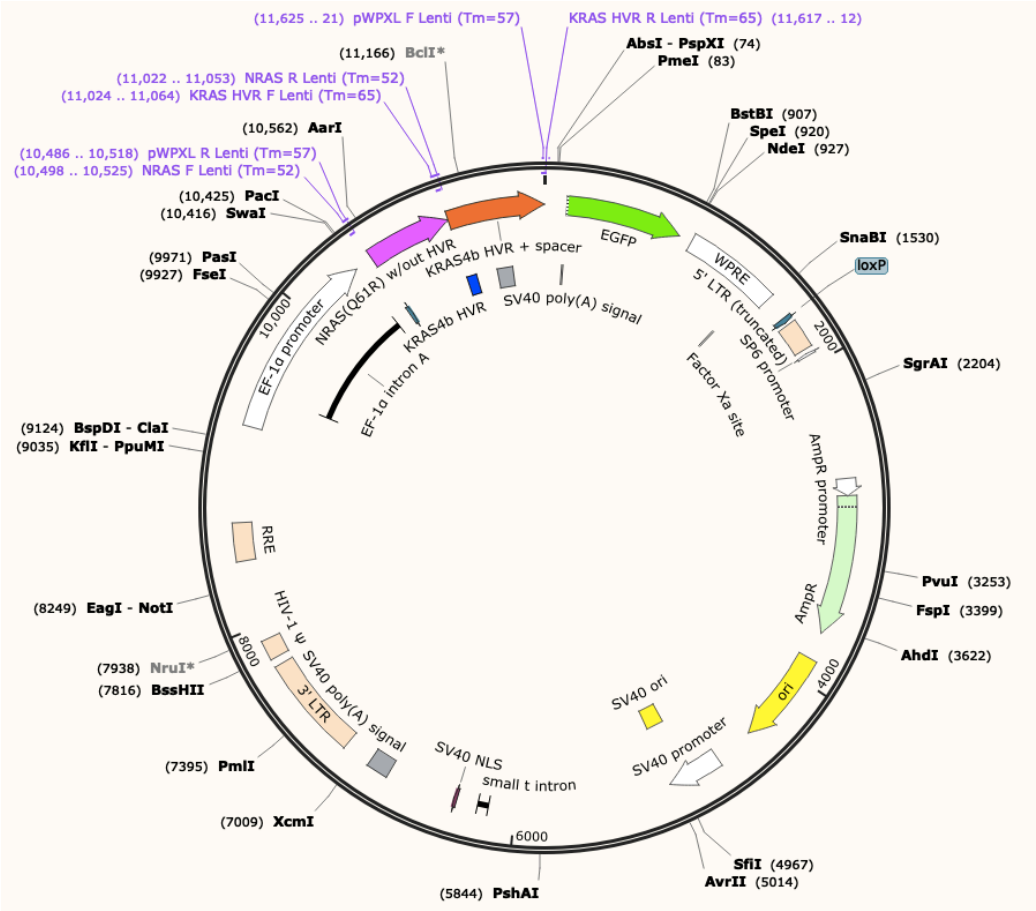
Supplementary Figure 6. HPLC/ELSD Spectrum. (a) H_2N-*L*-Cys-Oct (**1**), **(b)** H_2N-*L*-Ser-Oct (**2**), **(c)** H_2N-*L*-Cys-But (**3**), **(d)** H_2N-*L*-Cys-Dodec (**4**), **(e)** H_2N-*L*-Cys-Hexadec (**5**), **(f)** *N*-Ac-*L*-Cys-Oct (**6**). Retention times (t_R) were verified by mass spectrometry.



Supplementary Figure 7. Cell Viability of Melanoma Cell Lines Rreated with Alkyl Cysteine Derivatives and Controls. (a) WM3000 NRAS-mutated melanoma cells treated with six different compounds including C8 alkyl cysteine (**1**), C8 alkyl serine (**2**), C4 alkyl cysteine (**3**), C12 alkyl cysteine (**4**), C16 alkyl cysteine (**5**), and C8 acylated amine (**6**). **(b)** The control Sk-Mel-28 BRAF-mutated melanoma cells treated with two of the compounds (**1** and **4**) that had effects on the WM3000 melanoma cell line. **(c)** Comparison of treatment with 10 μM of either **1** or **4** between the WM3000 NRAS-mutated melanoma cell line and Sk-Mel-28 BRAF-mutated melanoma cell line from **(a)** and **(b)**. Results are the mean ± s.d. of three experiments. ****p ≤ 0.0001 (two-way ANOVA, multiple comparisons).



Supplementary Figure 8. SnapGene Lentiviral Plasmid Sequence Map. The lentiviral transfer plasmid containing the NRAS(Q61R)-KRAS4bHVR insert was used to PCR out the insert region and clone into the pSBbi-RP vector meant for the generation of a stable cell line using the Sleeping Beauty transposon system.



Supplemental Videos

Supplementary Video 1. Live-Cell Imaging of Stably Transfected EGFP-NRAS HeLa S3 Cells. Cells were treated with 20 μM C8 alkyl cysteine (**1**) and delocalization of NRAS from the plasma membrane was visualized for 98 min. Immediately after, at time point 1:38 according to the video, compound **1** was removed and cells were washed with HBSS then replaced with fresh Opti-MEM media. Frames are 2 min apart and video frame rate is 5 fps.

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