Temporally Controlled Targeting of 4-Hydroxynonenal to Specific Proteins in Living Cells

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CONTENTS

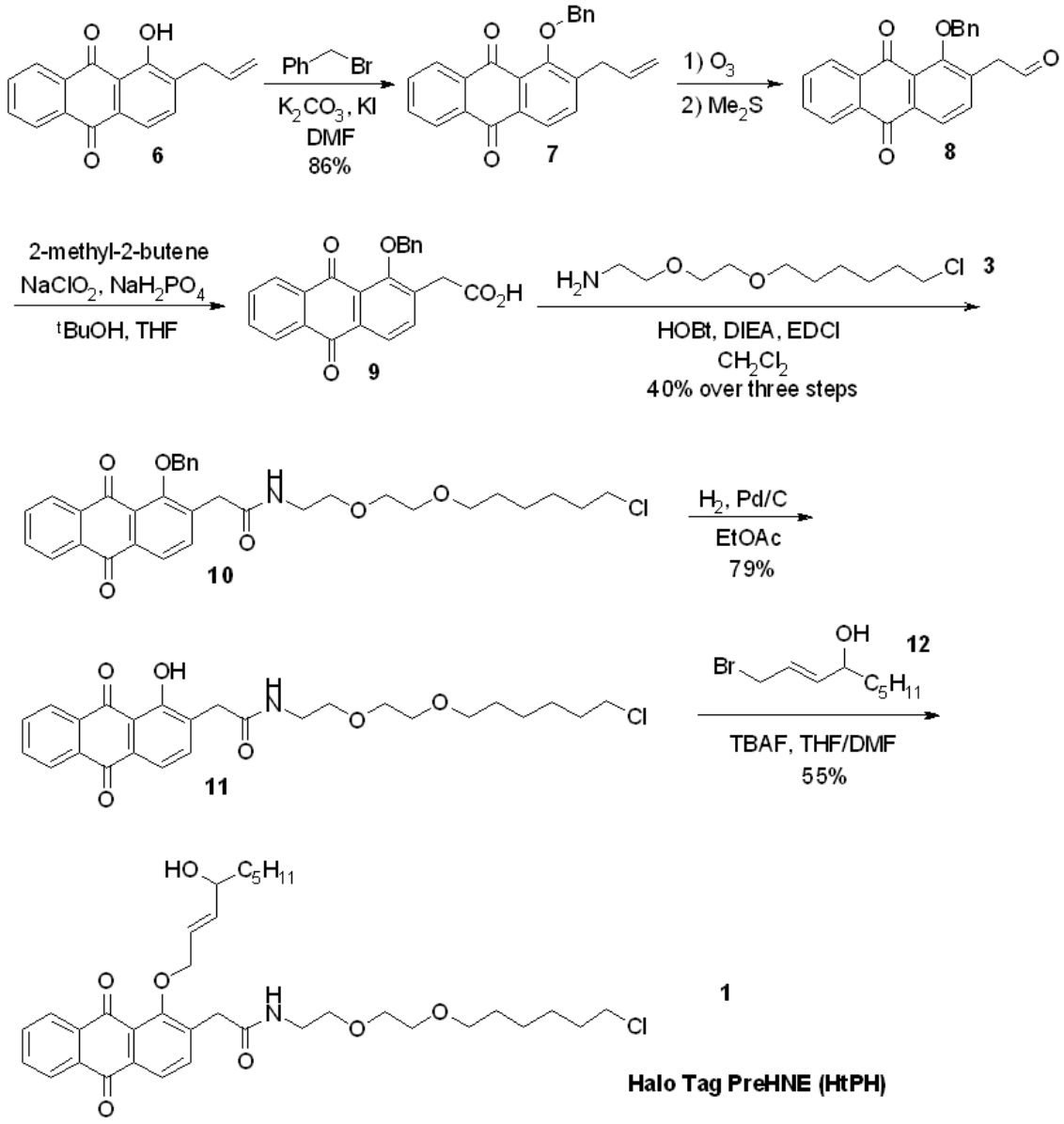
SUPPORTING TEXT

General materials and methods for synthetic protocols

All reactions were performed under an atmosphere of nitrogen or argon in oven- or flame- dried glassware with magnetic stirring. Acetonitrile, ether, tetrahydrofuran, dichloromethane and toluene were purified by distillation. ^{1}H Nuclear Magnetic Resonance (^{1}H-NMR) and ^{13}C Nuclear Magnetic Resonance (^{13}C-NMR) spectra were recorded on Varian INOVA 300, 400, or 600 MHz spectrometer and data are presented as follows: chemical shift in ppm using solvent as an internal standard ($CDCl_3$ at 7.26 ppm for ^{1}H and 77.0 ppm for proton-decoupled ^{13}C), multiplicity (s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet, br = broad), coupling constant (*J*/Hz), integration. NMR spectra were recorded in deuterated chloroform ($CDCl_3$) at room temperature unless otherwise stated. Laser Desorption-Ionization (LDI) mass spectra were acquired on a MALDI Micro MX – TOF mass spectrometer (Waters corporation). Samples dissolved in MeCN were spotted onto the target plate without the use of sample matrix and allowed to air dry. 50-100 spectra, each containing 10 individual laser shots, were summed prior to processing and analysis. The instrument was run in positive-ion and reflectron modes with an acceleration voltage of 20 kV. Mass axis calibration was performed using PET standards (Fluka). Analytical TLC was performed on EM Reagent 0.25 mm silica gel 60-F plates. Visualization was accomplished with UV light and either potassium permanganate stain or ceric ammonium nitrate stain followed by heating. Flash column chromatography was performed on silica gel 60Å (230-400 mesh) from Silicycle Inc. Ozonolysis was performed on Welsbach ozonator according to the manufacturer's guidelines. Unless otherwise noted, all other chemical reagents were obtained from commercial sources and used as received. Double distilled water was from Millipore water purification system. Compounds **1**, **2** and **4** were stored in the dark at –20 ºC, as aliquots in DMSO, and multiple freeze-thaw cycles were avoided. Compound **5** was stored in –80 ºC as one-shot aliquots in DMSO.

Synthesis protocols

Scheme S1. Synthesis of HaloTag PreHNE (HtPH) (1)



To a solution of 2-Allyl-1-hydroxyanthracene-9,10-dione **6** (0.2 g, 0.757 mmol)[1] in DMF (10 mL) was added benzyl bromide (0.27 mL, 2.27 mmol), K_2CO_3 (0.628 g, 4.54 mmol) and KI (0.038 g, 0.227 mmol). The resulting mixture was stirred at 65 ^oC for 1 h, and then cooled to room temperature. Subsequent to dilution with excess water, organic layer was extracted with EtOAc. The organic extracts were washed with water, brine and 1N HCl, dried and concentrated in vacuo to provide **7** as a yellow solid that was

homogeneous by ^{1}H-NMR spectroscopy (0.23 g, 86% yield): ^{1}H-NMR (300 MHz) δ 3.50 (2H, d, *J* = 6.6 Hz), 5.06-5.15 (4H, m), 5.86-5.99 (1H, m), 7.35-7.47 (3H, m), 7.60-7.66 (3H, m), 7.74-7.83 (2H, m), 8.14 (1H, d, *J* = 7.8 Hz), 8.26-8.33 (2H, m).

Compound **7** (0.23 g, 0.65 mmol) was dissolved in CH_2Cl_2 (50 mL) and cooled to –78 ^oC. O_3 was bubbled through the solution for 15 min, and then Me_2S (4.5 mL) was added. The reaction mixture was allowed to warm up to room temperature naturally and stirred for another 10 h. After concentration, the residue was diluted with EtOAc and washed with water. The organic layer was dried and concentrated to afford aldehyde **8** as a yellow solid that was carried forward to the subsequent step without further purification: ^{1}H-NMR spectroscopy (0.23 g, 100% yield): ^{1}H-NMR (300 MHz) δ 3.74 (2H, s), 5.06 (2H, s), 7.38-7.53 (4H, m), 7.61 (1H, d, *J* = 7.4 Hz), 7.80-7.83 (3H, m), 8.18 (1H, d, *J* = 7.2 Hz), 8.28-8.35 (2H, m), 9.63 (1H, s).

Aldehyde **8** (0.23 g, 0.645 mmol) and 2-methyl-2-butene (9 mL) was dissolved in ^{t}BuOH (37.5 mL), cooled to 0 ^oC. NaH_2PO_4•H_2O (0.633 g, 4.59 mmol) and $NaClO_2$ (0.524 g, 5.79 mmol) was dissolved in water (12 mL) and added to the above solution dropwise. The resulting mixture was allowed to warm up to room temperature and stirred overnight. The reaction was quenched with 0.1 N HCl (150 mL) and extracted with EtOAc. The organic extracts were washed with water and brine, and then dried. After filtration, the solution was concentrated to give the corresponding acid **9** as a yellow solid that was carried forward to the following step without further purification: ^{1}H-NMR spectroscopy (0.24 g, 100%): ^{1}H-NMR (300 MHz, CD_3SOCD_3) δ 3.71 (2H, s), 4.97 (2H, s), 7.38-7.46 (3H, m), 7.59 (2H, d, *J* = 7.2 Hz), 7.85 (1H, d, *J* = 7.8 Hz), 7.90-7.93 (2H, m), 8.04 (1H, d, *J* = 8.1 Hz), 8.16-8.23 (2H, m).

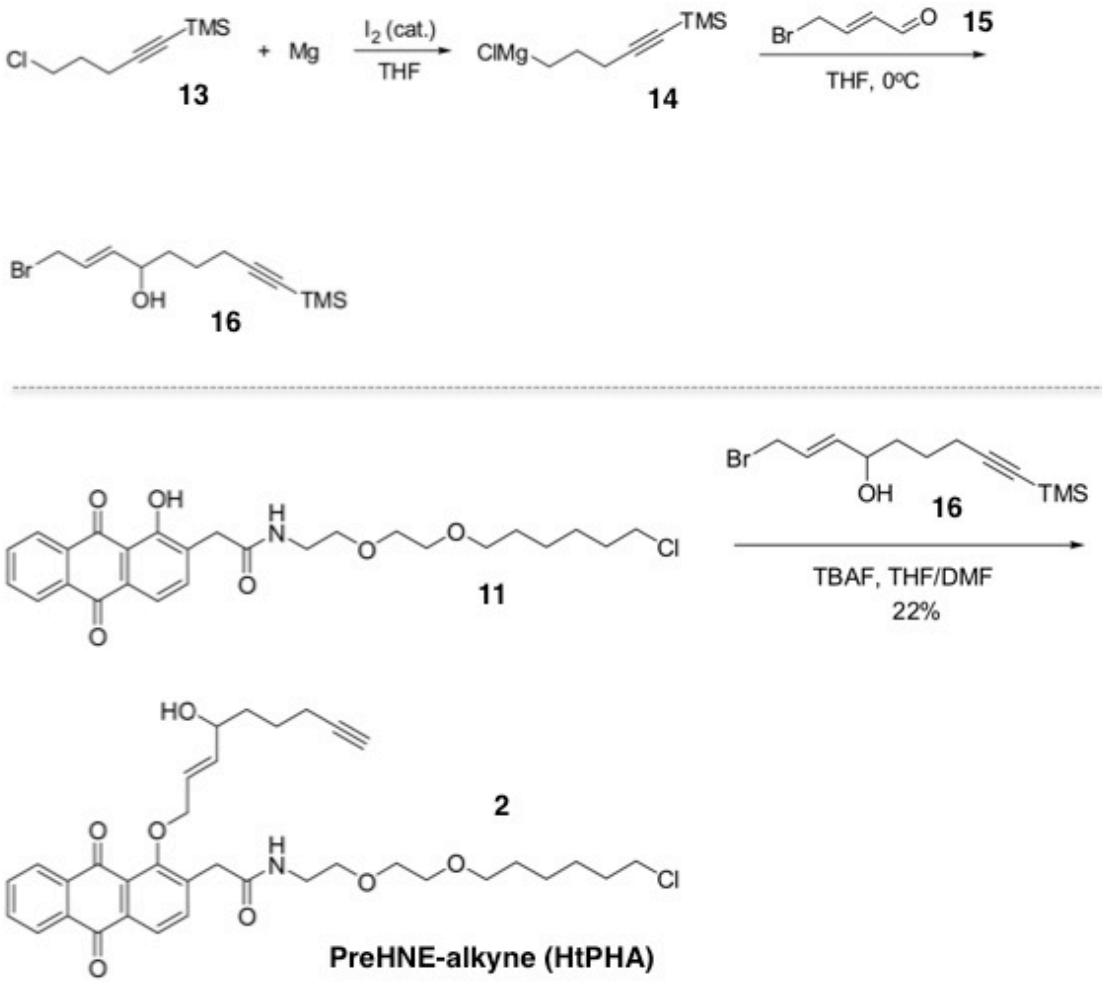
Acid **9** (0.24 g, 0.65 mmol) and 2-(2-(6-chlorohexyloxy)ethoxy)ethanamine (0.144 g, 0.65 mmol) (**3**) (i.e., linker alone) were dissolved in CH_2Cl_2 (15 mL), cooled to 0 ^oC. HOBt (0.131g, 0.78 mmol), DIEA (0.22 mL, 1.95 mmol) and EDCI (0.14 g, 0.91 mmol) were sequentially added. The reaction was warmed to room temperature naturally and stirred overnight. Water was added and then CH_2Cl_2 was added. The organic layers were combined and dried. After concentration, the residue was purified via flash chromatography using Hexanes:EtOAc (1:2 v/v) as eluent to yield amide **10** as a yellow oil that was homogeneous by ^{1}H-NMR spectroscopy (0.16g, 40% yield) over the three

steps (i.e., based on **7**): ^{1}H-NMR (300 MHz) δ 1.23-1.43 (4H, m), 1.46-1.56 (2H, m), 1.65-1.75 (2H, m), 3.33-3.49 (12H, m), 3.55 (2H, s), 5.04 (2H, s), 6.25 (1H, br), 7.34-7.57 (3H, m), 7.56 (2H, d, *J* = 7.2 Hz), 7.69-7.77 (3H, m), 8.07 (1H, d, *J* = 8.1 Hz), 8.17-8.25 (2H, m).

Amide **10** (0.16g, 0.277 mmol) was dissolved in EtOAc (21 mL) in a round-bottom-flask and 10% Pd/C (27.6 mg, 0.0277 mmol) was added. The solution was degassed and refilled with hydrogen gas (1 atm) at room temperature. The resulting mixture was stirred for 1h. After filtration through Celite, the reaction mixture was concentrated to give phenol **11** as a yellow oil that was homogeneous by ^{1}H-NMR spectroscopy (0.1 g, 72% yield): ^{1}H-NMR (300 MHz) δ 1.30-1.49 (4H, m), 1.54-1.63 (2H, m), 1.71-1.78 (2H, m), 3.35-3.59 (12H, m), 3.69 (2H, s), 6.49 (1H, br), 7.72 (1H, d, *J* = 8.1 Hz), 7.81-7.84 (3H, m), 8.29-8.34 (2H, m), 13.5 (1H, s). ^{13}C-NMR (75 MHz) 25.3, 26.6, 29.4, 32.5, 38.3, 29.4, 45.0, 69.5, 69.9, 70.2, 71.2, 76.5, 123.8, 125.9, 126.7, 127.3, 128.4, 128.5, 128.6, 132.5, 33.6, 134.2, 134.6, 134.8, 136.7, 136.9, 138.1, 157.3, 169.6, 182.3, 182.7.

Phenol **11** (0.1 g, 0.2 mmol) and TBAF (0.112 g, 0.4 mmol) were dissolved in THF (2 mL) and DMF (2 mL). Bromide **12** (0.177 g, 0.8 mmol) was added and the resulting mixture was stirred at room temperature for 7.5 h. The reaction was quenched with water and extracted with EtOAc. The organic layer was dried and concentrated under vacuo. The residue was purified via flash chromatography using Hexanes:EtOAc (1:3 v/v) as eluent to afford product **HaloTag PreHNE (HtPH) (1)** as an orange oil that was homogeneous by ^{1}H-NMR spectroscopy (0.069 g, 55% yield): ^{1}H-NMR (400 MHz) δ 0.87 (3H, t, *J* = 6.8 Hz), 1.27-1.58 (14H, m), 1.70-1.77 (2H, m), 2.26 (1H, br), 3.40-3.70 (12H, m), 3.69 (2H, d, *J* = 3.2 Hz), 4.10-4.14 (1H, m), 4.55 (1H, dd, *J* = 6.8, 11.2 Hz), 4.63 (1H, dd, *J* = 6.0, 11.2 Hz), 5.83 (1H, dd, *J* = 6.0, 14.8 Hz), 5.99-6.05 (1H, m), 6.47 (1H, br), 7.74-7.81 (3H, m), 8.12 (1H, d, *J* = 7.2 Hz), 8.24-8.27 (2H, m). ^{13}C-NMR (75 MHz) δ 14.0, 22.6, 25.1, 25.3, 25.6, 29.3, 31.8, 32.5, 36.9, 38.5, 39.4, 45.0, 69.7, 69.9, 70.1, 71.2, 71.7, 74.9, 123.7, 125.4, 125.8, 126.7, 127.2, 132.5, 133.7, 134.2, 134.6, 134.8, 136.6, 138.0, 138.7, 157.3, 169.8, 182.4, 182.8. LRMS (LDI) calc'd for $C_{35}H_{46}ClNO_7$ 627.3 (M^+), found 627.3.

Scheme S2. Synthesis of HaloTag PreHNE-alkyne (HtPHA) (2)



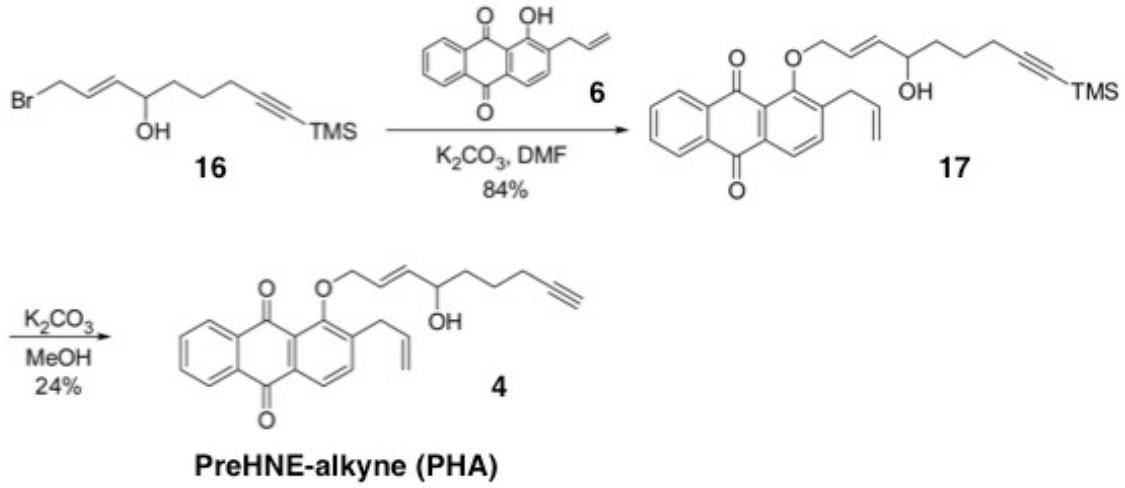
To a mixture of magnesium (0.156 g, 4.76 mmol) in anhydrous THF (2 mL) was added a solution of (5-chloro-1-pentynyl)trimethylsilane **13** (0.1 g, 0.60 mmol) in THF (1 mL) and I_2 (0.01 g, 0.039 mmol) under an atmosphere of nitrogen followed by a further addition of **13** (0.85 mL, 4.76 mmol) in anhydrous THF (8 mL). Additional heating was applied to maintain a temperature of approximately 60 ^{o}C. Upon complete addition, the mixture was refluxed for 2.5 h and cooled to room temperature to afford the corresponding Grignard reagent **14** which was used directly in the following step.

To a stirred solution of (*E*)-4-bromobut-2-enal **15** (0.428 g, 3.17 mmol) in anhydrous THF (10 mL) was added Grignard reagent **14** dropwise at 0 ^{o}C. The mixture was stirred at 0 ^{o}C for 2 h and quenched with saturated aqueous solution of NH_4Cl. The mixture was extracted with Et_2O, dried and concentrated to provide bromide **16** as a yellow oil: ^{1}H-NMR (300 MHz) δ 0.14 (9H, s), 1.55-1.65 (4H, m), 2.24-2.28 (2H, m), 3.96 (2H, d, *J* =

6.9 Hz), 4.15-4.23 (1H, m), 5.75-5.97 (2H, m). The crude bromide **16** was carried forward to the subsequent step without further purification.

To anthraquinone derivative **11** (0.1 g, 0.200 mmol) in THF (2 mL) and DMF (2 mL) was added sequentially TBAF (0.168 g, 0.600 mmol) and bromide **16** (0.17 g, 0.6 mmol). The mixture was stirred at room temperature overnight. Water (10 mL) was added then the reaction extracted with EtOAc. The organic layers were combined, dried and concentrated. The residue was purified via flash chromatography using Hexanes:EtOAc (1:5 v/v) to afford product **HaloTag PreHNE-alkyne (HtPHA) (2)** as a yellow oil that was homogeneous by ^{1}H-NMR spectroscopy (0.03 g, 22% yield): ^{1}H-NMR (400 MHz) δ 1.30-1.36 (2H, m), 1.38-1.44 (2H, m), 1.52-1.63 (6H, m), 1.70-1.77 (2H, m), 1.94 (1H, t, *J* = 2.6 Hz), 2.18-2.22 (2H, m), 3.40-3.59 (12H, m), 3.69 (2H, d, *J* = 3.6 Hz), 4.13-4.18 (1H, m), 4.55 (1H, dd, *J* = 7.2, 12.0 Hz), 4.62 (1H, dd, *J* = 6.0, 12.0 Hz), 5.83 (1H, dd, *J* = 6.0, 15.2 Hz), 6.00-6.07 (1H, m), 6.46 (1H, t, *J* = 5.0 Hz), 7.74 (1H, d, *J* = 8.4 Hz), 7.75-7.79 (2H, m), 8.11 (1H, d, *J* = 8.4 Hz), 8.23-8.26 (2H, m). ^{13}C-NMR (75 MHz) δ 18.3, 24.3, 25.3, 26.6, 29.3, 32.4, 35.8, 38.4, 39.4, 45.0, 68.6, 69.6, 69.9, 70.0, 70.1, 71.2, 74.7, 84.2, 123.7, 125.6, 125.8, 126.7, 127.2, 132.5, 133.7, 134.2, 134.6, 134.8, 136.6, 137.9, 138.2, 157.3, 169.9, 182.4, 182.7. LRMS (LDI) calc'd for $C_{35}H_{42}ClNNaO_7$ 646.3 ($M+Na^+$), found 646.2.

Scheme S3. Synthesis of PreHNE-alkyne (PHA) (4)

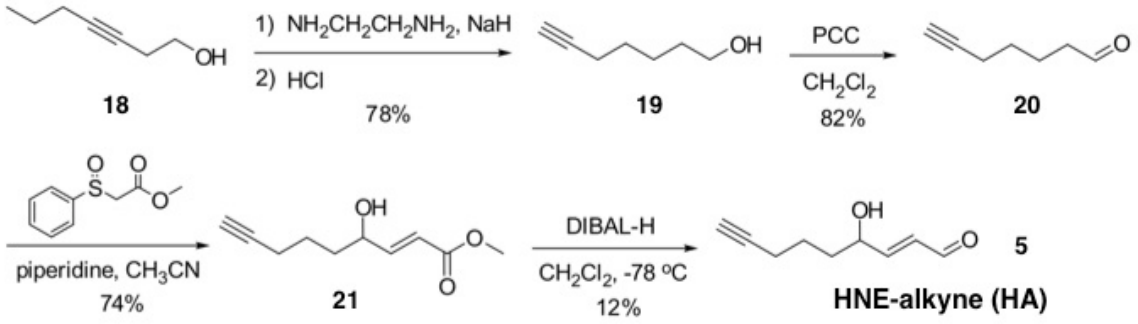


2-Allyl-1-hydroxyanthracene-9,10-dione **6** (0.067 g, 0.253 mmol)[1] was dissolved in DMF (5 mL). K_2CO_3 (0.1 g, 0.759 mmol) was added and the resulting mixture was stirred at 60 ^oC for 10 min. The crude bromide **16** (0.22 g, 0.759 mmol) (Scheme S2) in DMF (2 mL) was added and the heating was continued at 60 ^oC for a further 2 h. Water (20 mL) was added to dilute the mixture that was subsequently extracted with EtOAc (20 mL × 2). The organic layers were combined, dried and concentrated. The residue was purified via flash chromatography using Hexanes/EtOAc (3:1 v/v) as eluent yielding the desired TMS-alkyne product **17** as a yellow oil that was used directly in the following step without further purification. ^{1}H-NMR (300 MHz): δ 0.13 (9H, s), 1.56-1.69 (4H, m), 2.24-2.27 (2H, m), 3.55 (2H, d, *J* = 5.7 Hz), 4.22-4.27 (1H, m), 4.52-4.54 (2H, d, *J* = 5.7 Hz), 5.07-5.16 (2H, m), 5.89-6.14 (3H, m), 7.62 (1H, d, *J* = 8.0 Hz), 7.73-7.79 (2H, m), 8.09 (1H, d, *J* = 8.0 Hz), 8.21-8.27 (2H, m).

A solution of **17** (0.1 g, 0.212 mmol) in MeOH (10 mL) was added K_2CO_3 (0.176 g, 1.27 mmol) and the mixture was stirred at room temperature for 5 h. Water (20 mL) was added and the mixture was extracted with EtOAc (20 mL × 2). The organic layers were combined, dried and concentrated. The residue was purified via flash chromatography using Hexanes/EtOAc (3:1 v/v) as eluent to yield **PreHNE-alkyne** without Halo linker **(PHA) (4)** as a yellow oil that was homogeneous by ^{1}H-NMR spectroscopy (0.02 g, 24% yield): ^{1}H-NMR (300 MHz) δ 1.59-1.69 (4H, m), 1.96 (1H, t, *J* = 2.1 Hz), 2.23-2.26 (2H, m), 3.57 (2H, d, *J* = 6.6 Hz), 4.23-4.29 (1H, m), 4.55 (2H, d, *J* = 5.7 Hz), 5.10-5.18 (2H, m), 5.91-6.16 (3H, m), 7.64 (1H, d, *J* = 7.8 Hz), 7.75-7.79 (2H, m), 8.10-8.12 (1H, d, *J* =

8.1 Hz), 8.24-8.29 (2H, m). ^{13}C-NMR (75 MHz) δ 18.3, 24.3, 34.4, 35.9, 68.6, 71.7, 74.3, 84.2, 117.2, 123.6, 124.7, 126.2, 126.7, 127.2, 131.2, 131.4, 133.5, 134.2, 134.8, 135.6, 135.8, 137.1, 142.7, 157.2, 182.7, 183.1. LRMS (LDI) calc'd for $C_{26}H_{24}O_4$ 400.2 (M^+), found 400.1.

Scheme S4. Synthesis of HNE-alkyne (5)[2]



NaH (2.76 g, 69 mmol) was added to ethylenediamine (26.8 mL, 40 mmol) at 0 °C under an atmosphere of nitrogen. Subsequent to stirring at room temperature for 1 h, the reaction was heated at 60 °C for another hour. The reaction was then cooled to 45 °C and homopropargylic alcohol **18** (2 mL, 16.4 mmol) was added. The mixture was heated to 60 °C for 1 h, then cooled to 0 °C, and 1N HCl (20 mL) was added. The reaction was extracted with Et_2O and the combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified via flash chromatography using Hexanes:EtOAc (2:1 v/v) as eluent yielding alcohol **19** as a yellow oil that was homogeneous by ^{1}H-NMR spectroscopy (1.44 g, 78% yield): ^{1}H-NMR (300 MHz) δ 1.47-1.62 (6H, m), 1.92 (1H, t, J = 2.7 Hz), 2.18-2.23 (2H, m), 3.65 (2H, t, J = 6.3 Hz).

Alcohol **19** (1.44 g, 28.0 mmol) was dissolved in CH_2Cl_2 (40 mL), PCC (5.53 g, 56 mmol) was added and the reaction was stirred at room temperature for 1 h. The mixture was then filtered through Celite and the filtrate was concentrated. Flash chromatography using Hexanes:Et_2O (2:1 v/v) as eluent afforded aldehyde **20** as a colorless oil that was homogeneous by ^{1}H-NMR spectroscopy (1.16 g, 82% yield): ^{1}H-NMR (300 MHz) δ 1.55-1.63 (2H, m), 1.73-1.83 (2H, m), 1.94 (1H, t, J = 2.4 Hz), 2.22 (2H, dt, J = 2.4, 7.2 Hz), 2.45 (2H, dt, J = 1.8, 6.9 Hz), 9.78 (1H, t, J = 1.8 Hz).

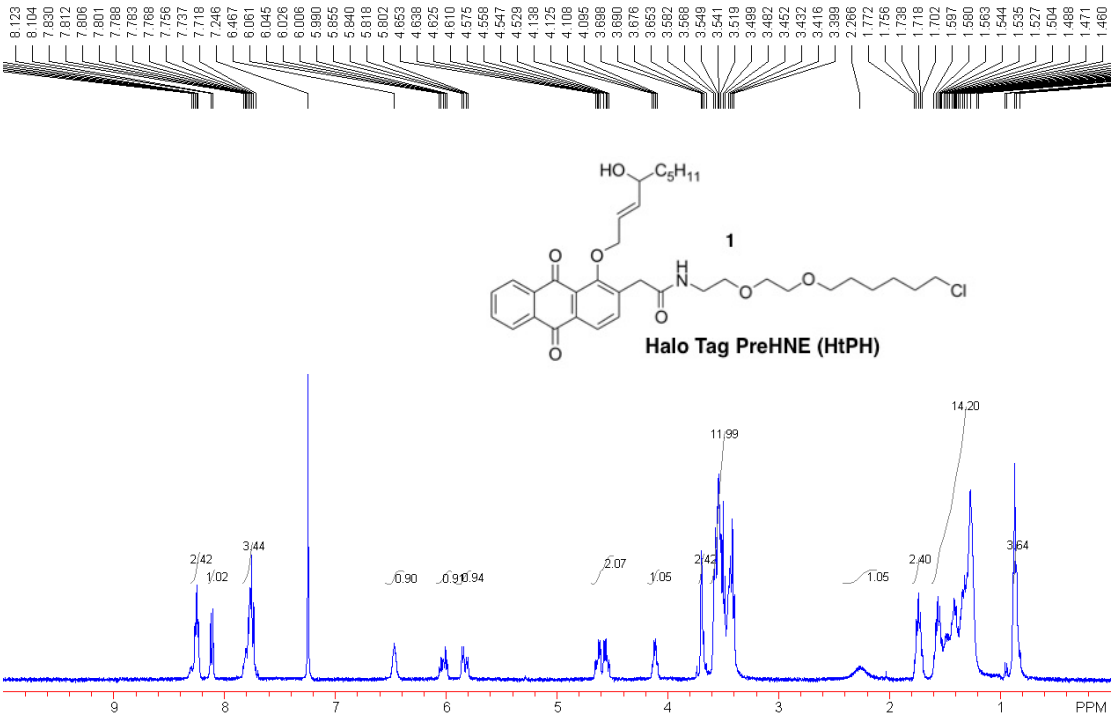
Aldehyde **20** (1.16 g, 10.5 mmol) and piperidine (1.73 mL, 17.6 mmol) were added to a solution of methyl 2-phenylsulfinylacetate (1.74 g, 8.78 mmol) in CH_3CN (40 mL). The reaction was stirred overnight at room temperature at which point aqueous NH_4Cl solution was added and the mixture extracted with CH_2Cl_2. The organic layer was collected, dried and concentrated in vacuo. The residue was purified via flash

chromatography using Hexanes:EtOAc (10:1 v/v) as eluent to afford ester **21** as a yellow oil that was homogeneous by ^{1}H-NMR spectroscopy (1.19 g, 74% yield): ^{1}H-NMR (300 MHz) δ 1.51-1.76 (4H, m), 1.97 (1H, t, *J* = 2.7 Hz), 2.24 (2H, dt, *J* = 2.7, 6.9 Hz), 3.75 (3H, s), 4.36-4.38 (1H, m), 6.05 (1H, dd, *J* = 1.8, 15.9 Hz), 6.95 (1H, dd, *J* = 4.8, 15.6 Hz).

Ester **21** (0.4 g, 2.2 mmol) was dissolved in CH_2Cl_2 (20 mL) and the reaction was cooled to –80 ^{o}C. DIBAL-H (2.0 M in hexane, 4.45 mL, 4.4 mmol) was dissolved in CH_2Cl_2 (20 mL) and added dropwise. Subsequent to 1 h stirring at –80 ^{o}C, the reaction was quenched with 1N HCl (24.7 mL). The reaction was extracted with Et_2O and the organic layer was separated, dried and concentrated in vacuo. Flash chromatography using Hexanes:Et_2O (4:1 v/v) as eluent afforded the desired **HNE-alkyne (HA) (5)**[2] that was homogeneous by ^{1}H-NMR spectroscopy (0.0393 g, 12% yield): ^{1}H-NMR (300 MHz) δ 1.50-1.76 (4H, m), 1.92 (1H, t, *J* = 2.7 Hz), 2.19 (2H, dt, *J* = 2.7, 6.3 Hz), 2.61 (1H, br), 4.39-4.44 (1H, m), 6.25 (H, ddd, *J* = 1.2, 7.8, 15.9 Hz), 6.79 (1H, dd, *J* = 4.5, 15.6 Hz), 9.50 (1H, d, *J* = 7.5 Hz). ^{13}C-NMR (75 MHz) δ 18.1, 24.0, 35.1, 69.1, 70.4, 83.8, 130.7, 159.2, 193.9.

Small molecule characterization data

^{1}H-NMR spectrum of HtPH (1)



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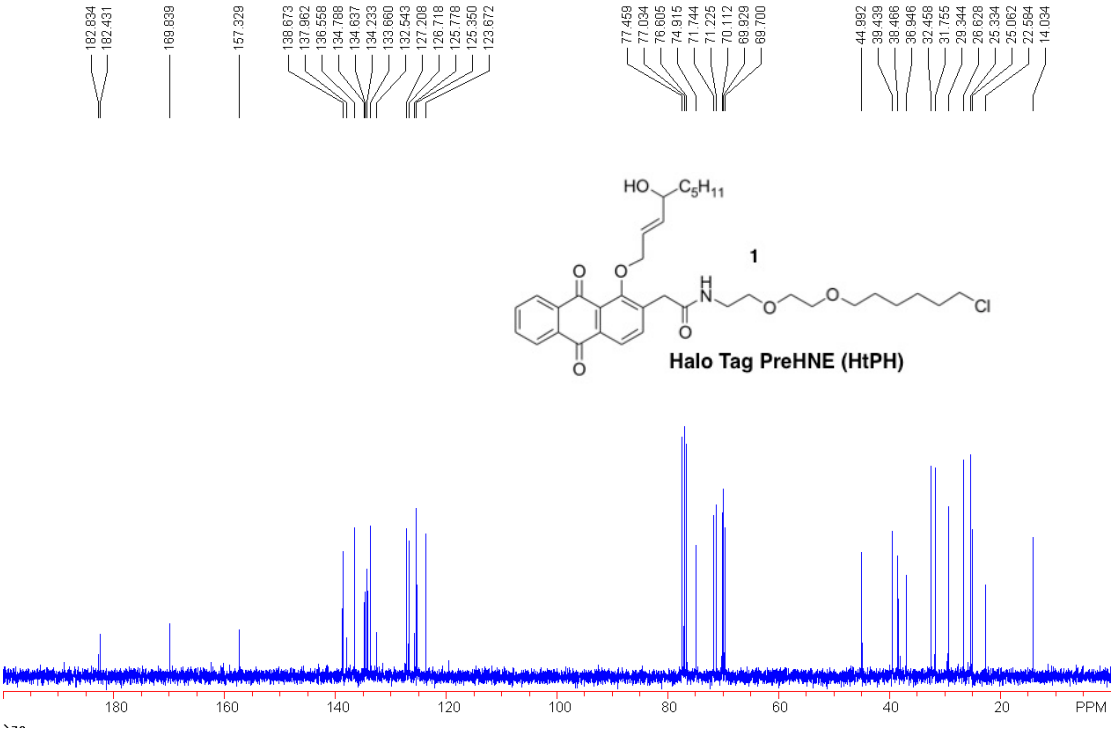

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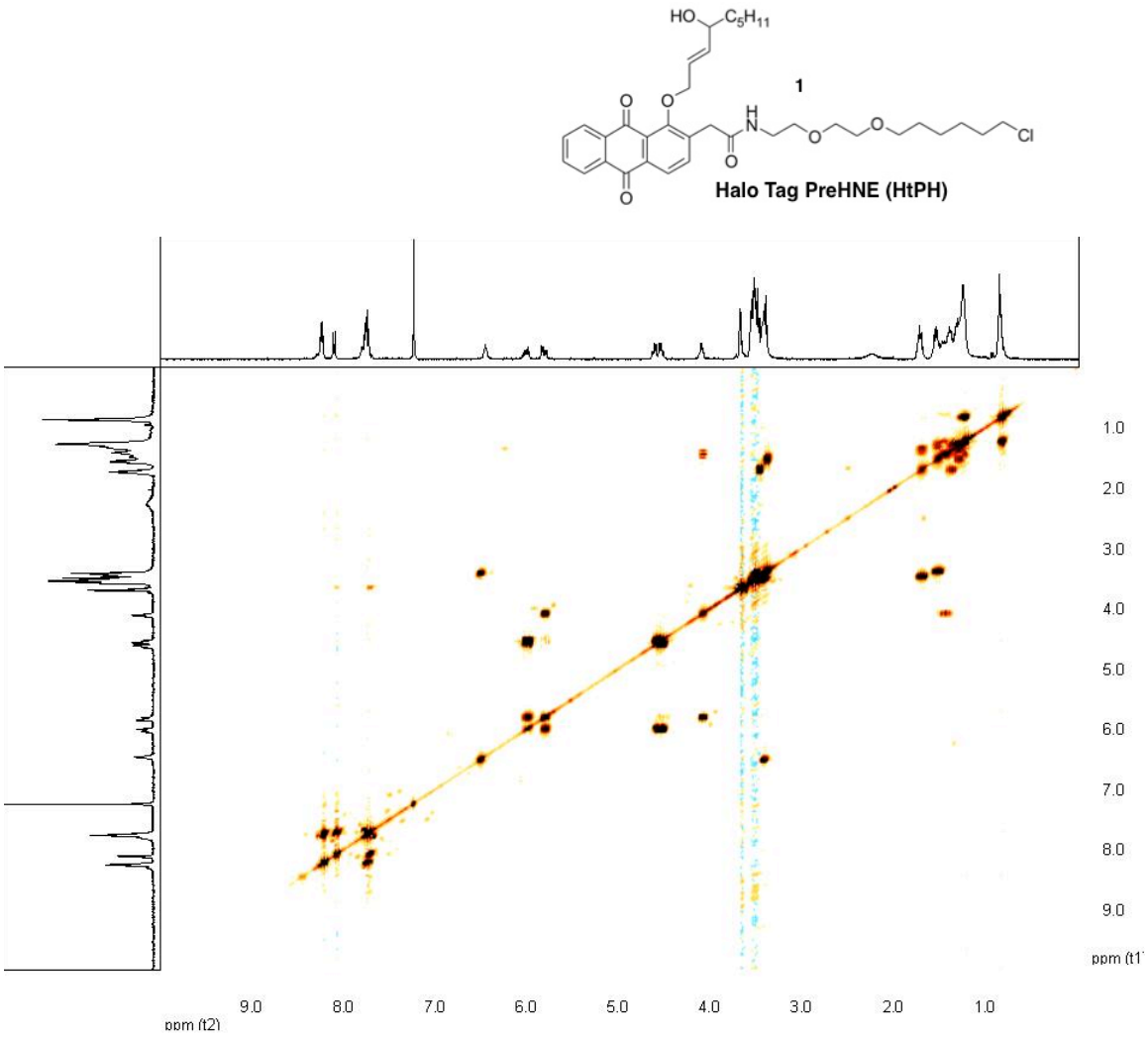
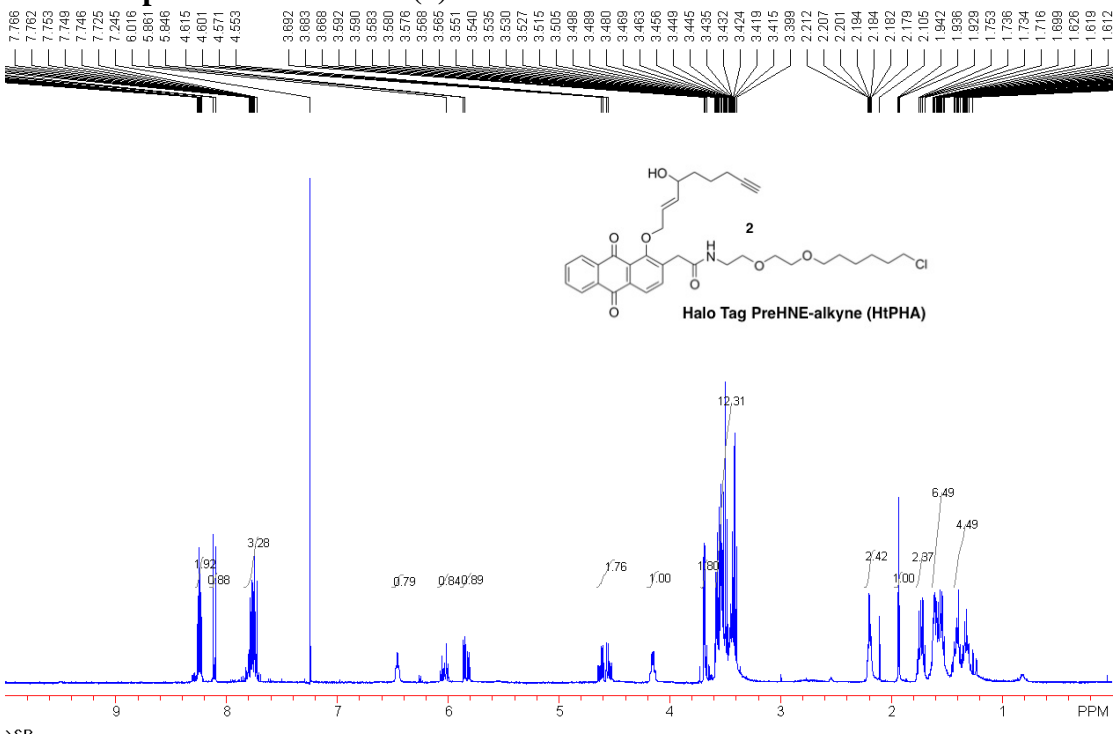
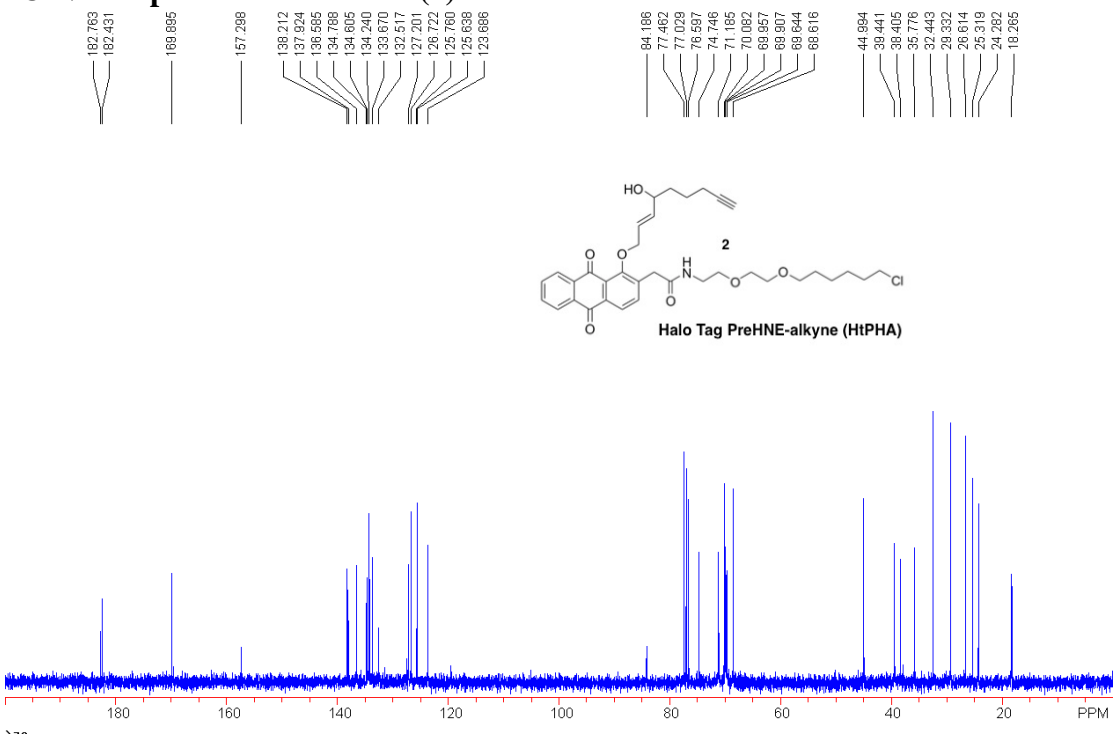


^{1}H-NMR spectrum of HtPHA (2)



^{13}C-NMR spectrum of HtPHA (2)



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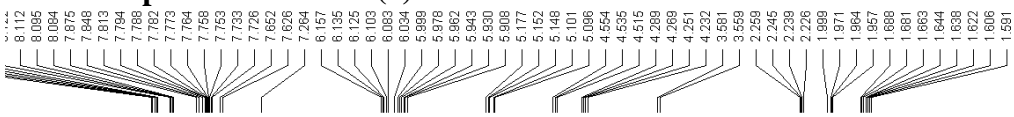

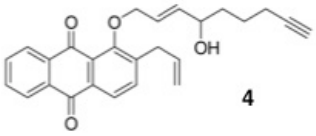

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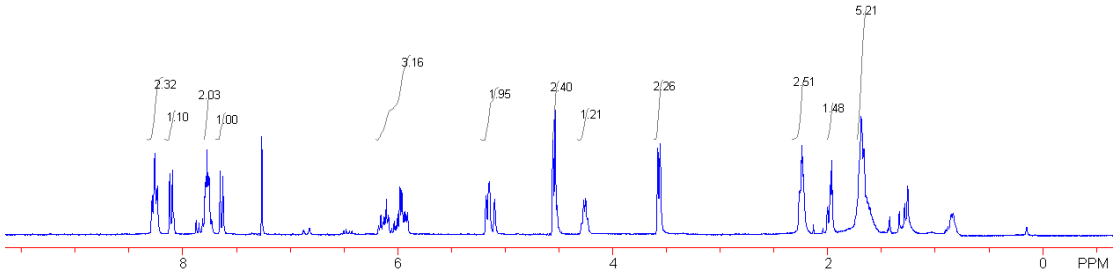
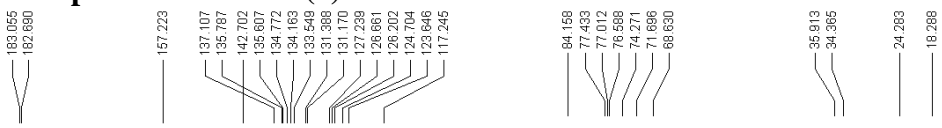
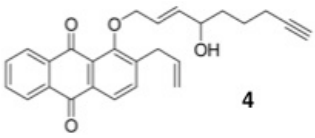
PreHNE-alkyne (PHA)



^{13}C-NMR spectrum of PHA (4)





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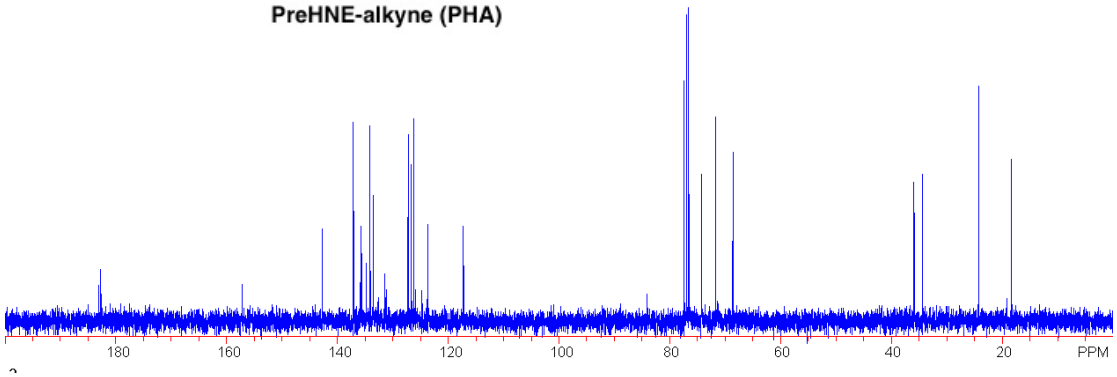
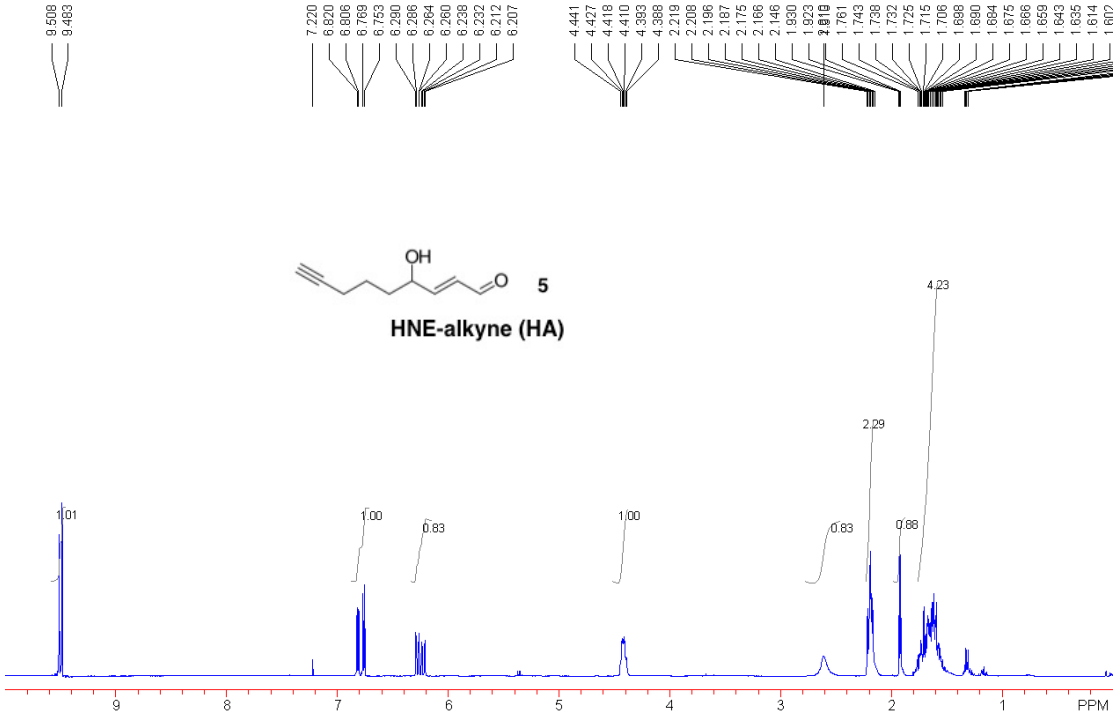
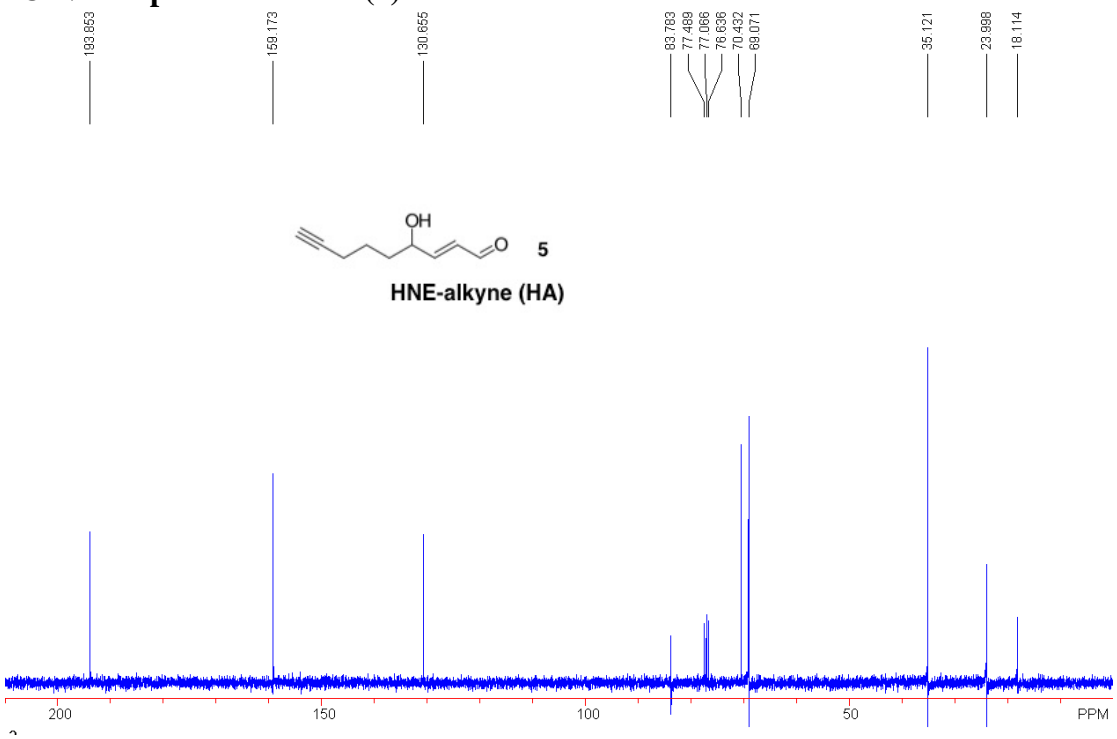
PreHNE-alkyne (PHA)



^{1}H-NMR spectrum of HA (5)



^{13}C-NMR spectrum of HA (5)



General materials and methods for biochemical protocols

All reagents were from Sigma unless otherwise noted. Cy5-azide was from lumiprobe (B3030). G5 Ubiquitin isopeptidase inhibitor I and HNE were from Santa Cruz. HaloTag® TMR Ligand was (G8251) from Promega. All primers were from IDT. Fusion HotStartII polymerase was from Thermo and all the restriction enzymes were from NEB. phrGFP-Keap1 and pcDNA3 GFP-PTEN mammalian expression plasmids (28025 and 10759, respectively) and pRK793 Tev protease (S219V mutant) bacterial expression plasmid (8827) were from Addgene. The latter was a kind gift of Jeffrey Boucher, Brandeis University, MA. pET28a empty vector was a kind gift of Professor Lizbeth Hedstrom, Brandeis University, MA. pMIR_DsRed_IRES_His_6H1 construct[3] was previously made. COS-1 and HEK-293 cells were from American Type Culture Collection (ATCC). 1X PBS (Dulbecco's phosphate-buffered saline), 1X TrypLE™ Express (stable trypsin-like enzyme with phenol red), 1X DMEM, and 100X penicillin-streptomycin were from Invitrogen. Fetal bovine serum (FBS) (100 nm-triple filtered, SH30071.03) was from Hyclone. Serum-compatible broad-spectrum transfection reagent TransIT-2020 was from Mirus Bio LLC. Olympus CKX31 and Zeiss 510 meta confocal microscope systems were used, respectively, for light and confocal fluorescence microscopy. Fluorescence-activated cell sorting (FACS) was performed on a Beckton Dickinson FACSCalibur flow cytometer and FACS data analysis was carried out using FlowJo (version 7.6.4). In gel fluorescence analysis and imaging of the Coomassie-stained gels were performed on Bio-Rad ChemiDoc-MP imaging system. Densitometric quantitation was made by Bio-Rad Image Lab software (v 4.1). Cy5 excitation source was red epi illumination and emission filter used was 695/55 filter. Light exposure experiments were performed with the use of a hand-held UV lamp (Fisher, S45157, 365 nm, longwave 4 W tube). The lamp was positioned such that the lamp cover screen was at a distance of 1–2 cm directly above either the monolayer cell culture or solutions containing HNE precursors. His_6-Tev-S219V was recombinantly expressed and purified from *E. coli* using TALON affinity chromatography (clontech).

UV-Vis analysis of photoinducible HNE release (Figure S1)

Photoinducible HNE release from an analogous anthraquinone-based platform in an aqueous organic solvent mixture has been previously characterized by the use of NMR.[1] The time course of HNE release was monitored on a Shimadzu UV-2600 UV-Visible spectrophotometer equipped with peltier temperature controller. HaloTag PreHNE and HaloTag PreHNE-alkyne ligands (HtPH and HtPHA) (**1** and **2**, respectively) were diluted to a final concentration of 150 μM in 20 mM Hepes (pH=7.6) in a 1 cm quartz cuvette, and incubated under a 365 nm, 4 W hand-held lamp at 37 ºC for indicated periods prior to spectral data acquisition at 37 ºC. An authentic sample of HNE (Santa Cruz Biotech) under identical conditions yielded λ_{max} at 228 nm, suggesting a time-dependent release of HNE from photolysis of HtPH (**1**) and HtPHA (**2**) (Figure S1). Interference from absorbance changes in anthraquinone moiety precluded us from absolute quantitation of liberated HNE (Figure S1a). The use of NMR was limited by the compounds' solubility in neat D_2O at the concentrations required for reliable quantitation under a short reaction time. Nevertheless, UV-Vis analysis showed saturation of the rise in absorbance was reached within 30 min at 37 °C for both HtPH and HtPHA (Figure S1b).

Construction of IRES-based bicistronic mammalian expression plasmid encoding DsRed and Halo–Keap1 genes (pMIR-DsRed-IRES-HaloKeap1) (Figure S2)

Ligase-free PCR cloning was used to construct the titled plasmid using the set of primers shown in **Table S1**. HaloKeap1 fusion gene containing Tev cleavage site was first built into a pET28a vector to obtain the plasmid, pET28a_HaloKeap1 (**Table S1A**). In order to achieve this, human Keap1 gene was cloned out from the commercially available plasmid, phrGFP-Keap1 (Addgene) using the primers fwd-**1** and rev-**1** shown (**Table S1A**). The resultant PCR product was extended using the shown extended primers fwd-ext-**1** and rev-ext-**1**. These two PCR products served as mega-primers for the subsequent PCR-cloning into the linearized pET28a_Halo vector. The identity of pET28a_HaloKeap1 plasmid was verified by sequencing of the entire gene at the Cornell University Life Science Core Laboratories Center.

Using the plasmid pET28a_HaloKeap1, HaloKeap1 fusion gene containing Tev cleavage site (**Figure S2**) was cloned out using the fwd-**2** and rev-**2** primers shown (**Table S1B**) yielding to the first PCR product. This product was subsequently extended using the fwd and rev extender primers (fwd-ext-**2** and rev-ext-**2**) shown to give the second set of PCR products. A linearized pMIR_DsRed_IRES_His_6H1 plasmid[3] was used as a template in the final PCR-cloning step, wherein the second PCR products above served as mega primers. The identity of the final desired construct pMIR-DsRed_IRES_HaloKeap1 was verified by sequencing of the entire fusion gene at the Cornell University Life Science Core Laboratories Center.

Construction of mammalian expression plasmid encoding Halo–PTEN genes (pMIR-HaloPTEN) (Figure S3)

Ligase-free PCR cloning was used to construct the titled plasmid using the set of primers shown in **Table S2**. HaloPTEN fusion gene containing Tev cleavage site was first built into a pET28a vector to obtain the plasmid, pET28a_HaloPTEN (**Table S2**). In order to achieve this, human PTEN gene was cloned out from the commercially available plasmid, pcDNA3-GFP-PTEN (Addgene) using the primers fwd-**1** and rev-**1** shown (**Table S2A**). The resultant PCR product was extended using the shown extended primers fwd-ext-**1** and rev-ext-**1**. These two PCR products served as mega-primers for the subsequent PCR-cloning into the linearized pET28a_Halo vector. The identity of pET28a_HaloPTEN plasmid was verified by sequencing of the entire gene at the Cornell University Life Science Core Laboratories Center.

Using the plasmid pET28a_PTEN, HaloPTEN fusion gene containing Tev cleavage site (**Figure S3**) was cloned out using the fwd-**2** and rev-**2** primers shown (**Table S2B**) yielding to the first PCR product. This product was subsequently extended using the fwd and rev extender primers (fwd-ext-**2** and rev-ext-**2**) shown to give the second set of PCR products. A linearized pMIR_DsRed_IRES_His_6H1 plasmid[3] was used as a template in the final PCR-cloning step, wherein the second PCR products above served as mega primers. The identity of the final desired construct pMIR-HaloPTEN was verified by sequencing of the entire fusion gene at the Cornell University Life Science Core Laboratories Center.

Cell culture protocol

COS-1 and HEK-293 cells (ATCC) were cultured in DMEM (Invitrogen) supplemented with 10% v/v FBS (Hyclone) in the presence of 1X penicillin-streptomycin antibiotics (Invitrogen 15140-122). All cells were cultivated in adherent culture plates (15 x 65 mm, Corning) in a humidified atmosphere of 5% CO_2 at 37 ºC. COS-1 cells were harvested by trypsinization (Invitrogen) and HEK-293 cells were dislodged by gently washing the plates with complete media.

Cell viability analysis by flow cytometry analysis

Samples originating from treated and untreated COS-1 cells were prepared by resuspending the freshly harvested cell pellets in 1% FBS in 1X PBS with 4 μg/mL propidium iodide (Sigma). Data were collected using a Beckton Dickinson FACSCalibur flow cytometer and analysis was carried out using FlowJo (v 7.6.4). G5 Ubiquitin isopeptidase inhibitor I (Santa Cruz) was used as a positive control for cell death initiation.

Confocal fluorescence microscopy analysis

COS-1 cells were cultivated in glass bottom dishes (D35-20-1.5N, In Vitro Scientific) and imaged using a Zeiss 510 metal confocal fluorescence microscope (63x oil) 24–30 h post transfection. Where applicable, treatment with indicated small molecules was carried out prior to analysis.

Protocol for HaloTag fusion protein-directed targeting with HNE(-alkyne) in living mammalian cells

Optimized transfection was achieved using serum-compatible Mirus TransIT-2020 transfection reagent with the use of complete media. Transfection was typically performed at 50-60% confluency according to the Mirus optimized protocol. pMIR_DsRed_IRES_HaloKeap1 and pMIR_HaloPTEN plasmids were purified by

Maxiprep kit (Qiagen). All steps post transfection were handled under dim light or in the dark. Subsequent to transfection (24–30 h), cells were rinsed (x2) with serum-free media and treated with one of the following: HtPH (25–50 μM) (compound **1**, Scheme S1), HtPHA (25–50 μM) (compound **2**, Scheme S2), PHA (50 μM) (compound **4**, Scheme S3), HaloTag® TMR (5 μM), HA (25 μM) (compound **5**, Scheme S4) or DMSO for 2–3 h in serum-free media. In blocking experiments (Figure S4 and S5), samples were first treated with either unsubstituted chloroalkane ligand alone (compound **3**, Scheme S1) (100 μM), or compound **1** (136 μM). Second, cells were rinsed with serum-free media (x2) prior to treatment, respectively, with compound **2** (25–50 μM), or HaloTag® TMR (5 μM). For all samples, at the end of final compound treatment, rinsing (x2) was performed every 30 min over 1.5 h with the serum-free media. For the samples designated for light exposure, plate covers were removed and adhered cells were exposed to 365 nm, 4 W lamp for 20 min at room temperature (at ~1 inch distance from the light source) and reincubated at 37 ºC for a further 5 min prior to harvest. In the experiments where the entire cell was treated with HA (compound **5**), cells were harvested subsequent to 20 min incubation with HA. Cells were then washed thoroughly to remove residual HNE-alkyne in the buffer.

Subsequent to centrifugation (500 x g, 8 min) and washing with 1X PBS (x3), the resultant cell pellets were flash frozen in liq N_2 and subjected to 3 cycles of freeze-thaw in 50 mM Hepes (pH 7.6) and 0.3 mM TCEP. All procedures hereafter were performed at 4 ºC. Debris was removed by centrifugation (18,000 x g, 8 min). Supernatant was made up to final volume of 25 μL containing in final concentrations, 50 mM Hepes (pH 7.6), 0.3 mM TCEP and 0.1 mg/mL His_6-Tev-S219V. The mixture was incubated at 37 ºC for 30 min, and subsequently subjected to click reaction. All steps hereafter were handled in the dark. Briefly, in a final volume of 30 μL, the reaction mixture contained, 50 mM Hepes (pH 7.6), 1.7 mM TCEP, 5% v/v *t*-BuOH, 1 mM $CuSO_4$, 10 μM Cy5azide and Tev-protease-treated lysate above. Subsequent to 30 min incubation at 37 ºC, the reaction was quenched with 4X laemmli buffer that contained 3% BME and further incubated for 5 min at 37 ºC. 30 μL was directly loaded into each well of 10% polyacrylamide gel. Upon completion of gel-electrophoresis, the gel was rinsed with ddH_2O (x2, 5 min) and

analyzed for Cy5 signal on a Bio-Rad ChemiDoc-MP and subsequently coomassie-stained.

Figure S1. Time-dependent HNE-release profile monitored by UV-Vis spectrophotometer. 150 μM **1** or **2** in 20 mM Hepes (pH=7.6) was exposed to 365 nm, 4 W lamp at 37 ºC for indicated periods prior to spectral acquisition at 37 ºC. (a) Representative UV-Vis profile from photolysis of HtPH (**1**). (b) Kinetic profiles for time-dependent release of HNE (●) and HNE-alkyne (▲) from HtPH (**1**) and HtPHA (**2**), respectively (Figure 2). Error bars are S.D. (N = 3). λ_{max}= 228 nm for an authentic sample of HNE (Santa Cruz Biotech) measured under identical conditions.

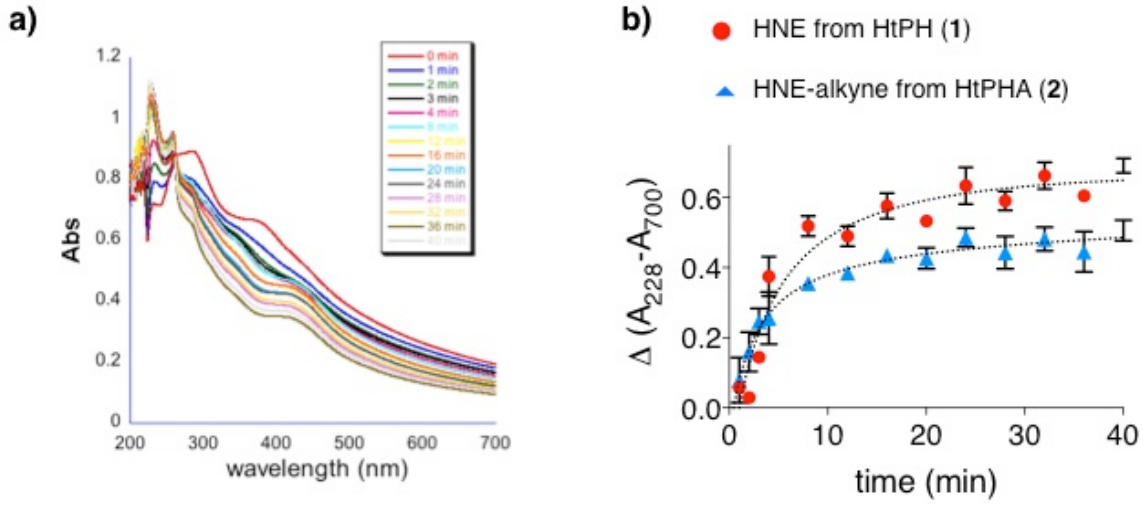
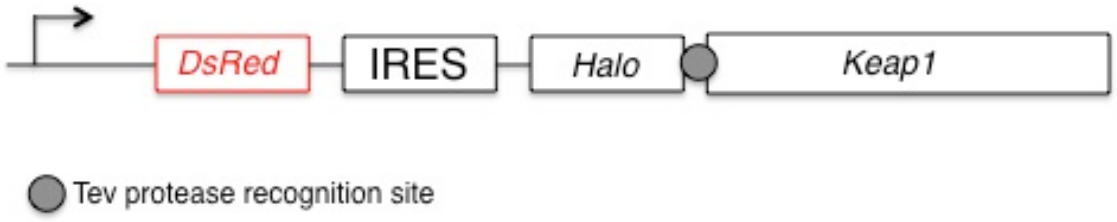


Figure S2. Dual expression construct for DsRed and Halo–Keap1 proteins



Amino acid sequence of Halo–Keap1 fusion protein (blue, Halo; **grey**, Tev recognition sequence; green, human Keap1)

Halo–Keap1, 104 kDa
Keap1, 70 kDa

MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIP
HVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDW
GSALGFHWAKRNPERVKGIAFMEFIRPIPTWDEWPEFARETFQAFRTTDVGRK
LIIDQNVFIEGTLPMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEP
ANIVALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLAKSLPNCKAVDIGP
GLNLLQEDNPDLIGSEIARWLSTLEISGSGENLYFQGSGMQPDPRPSGAGACCR
FLPLQSQCPEGAGDAVMYASTECKAEVTPSQHGNRTFSYTLEDHTKQAFGIMNEL
RLSQQLCDVTLQVKYQDAPAAQFMAHKVVLASSSPVFKAMFTNGLREQGMEVVSI
EGIHPKVMERLIEFAYTASISMGEKCVLHVMNGAVMYQIDSVVRACSDFLVQQLDP
SNAIGIANFAEQIGCVELHQRAREYIYMHFGEVAKQEEFFNLSHCQLVTLISRDDLN
VRCESEVFHACINWVKYDCEQRRFYVQALLRAVRCHSLTPNFLQMQLQKCEILQS
DSRCKDYLVKIFEELTLHKPTQVMPCRAPKVGRLIYTAGGYFRQSLSYLEAYNPSD
GTWLRLADLQVPRSGLAGCVVGGLLYAVGGRNNSPDGNTDSSALDCYNPMTNQ
WSPCAPMSVPRNRIGVGVIDGHIYAVGGSHGCIHHNSVERYEPERDEWHLVAPML
TRRIGVGVAVLNRLLYAVGGFDGTNRLNSAECYYPERNEWRMITAMNTIRSGAGV
CVLHNCIYAAGGYDGQDQLNSVERYDVETETWTFVAPMKHRRSALGITVHQGRIY
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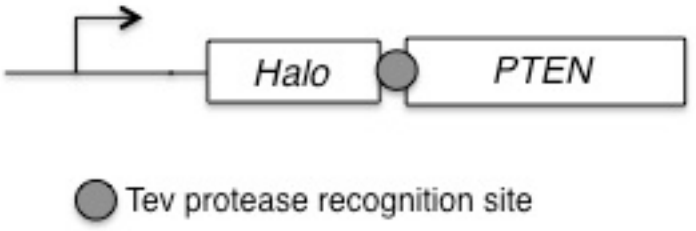
CTC

Figure S3. Expression construct for Halo–PTEN protein



Amino acid sequence of Halo–PTEN fusion protein (blue, Halo; **grey**, Tev recognition sequence; orange, human PTEN)

Halo–PTEN, 82 kDa
PTEN, 47 kDa

MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIP
HVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDW
GSALGFHWAKRNPERVKGIAFMEFIRPIPTWDEWPEFARETFQAFRTTDVGRK
LIIDQNVFIEGTLPMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEP
ANIVALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLAKSLPNCKAVDIGP
GLNLLQEDNPDLIGSEIARWLSTLEISGSG**ENLYFQG**SGMTAIIKEIVSRNKRRYQ
EDGFDLDLTYIYPNIIAMGFPAERLEGVYRNNIDDVVRFLDSKHKNHYKIYNLCAERH
YDTAKFNCRVAQYPFEDHNPPQLELIKPFCEDLDQWLSEDDNHVAAIHCKAGKGR
TGVMICAYLLHRGKFLKAQEALDFYGEVRTRDKKGVTIPSQRRYVYYYSYLLKNHL
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EIDSICSIERADNDKEYLVLTLTKNDLDKANKDKANRYFSPNFKVKLYFTKTVEEPSN
PEASSSTSVTPDVSDNEPDHYRYSDTTDSDPENEPFDEDQHTQITKV

Figure S4. Keap1 targeted delivery of HNE. COS-1 cells transiently overexpressing DsRed and Halo–Keap1 (Figure S2) were treated with 25 μM HtPHA (**2**) for 2.5 h at 37 ºC. Subsequent to rinsing with serum-free media (x2) every 30 min over 1.5 h, designated plates were exposed to a hand-held 4 W lamp (365 nm) for 30 min at room temperature, and further incubated for 5 min at 37 ºC prior to harvest. Cells were lysed at 4 ºC and lysates were subjected to Tev cleavage for 30 min at 37 ºC followed by click reaction with Cy5-azide for 20 min at 37 ºC. Samples were analyzed by SDS-PAGE. a) Resultant SDS-PAGE with Coomassie staining. b) In-gel fluorescence analysis of the same SDS-PAGE with Cy5 excitation. Lane 1, ladder; Lane 2, negative control from cells not exposed to light; Lane 3, results from cells exposed to light; Lane 4, identical to Lane 3 except that Tev was replaced with buffer; Lane 5, identical to Lane 3 except that PHA (compound **4**, Scheme S3) was used; Lane 6, ladder; Lane 7, halo binding site pre-blocked with unsubstituted chloroalkane ligand alone (compound **3**, Scheme S1) followed by treatment with HtPHA (compound **2**, Scheme S2); Lane 8, independent duplicate of Lane 3; Lane 9, identical to Lane 3 and Lane 8, but performed on non-transfected cells with no halo-fusion protein.

*, Halo–Keap1, 104 kDa
+, Keap1, 70 kDa
x, Halo, 33 kDa

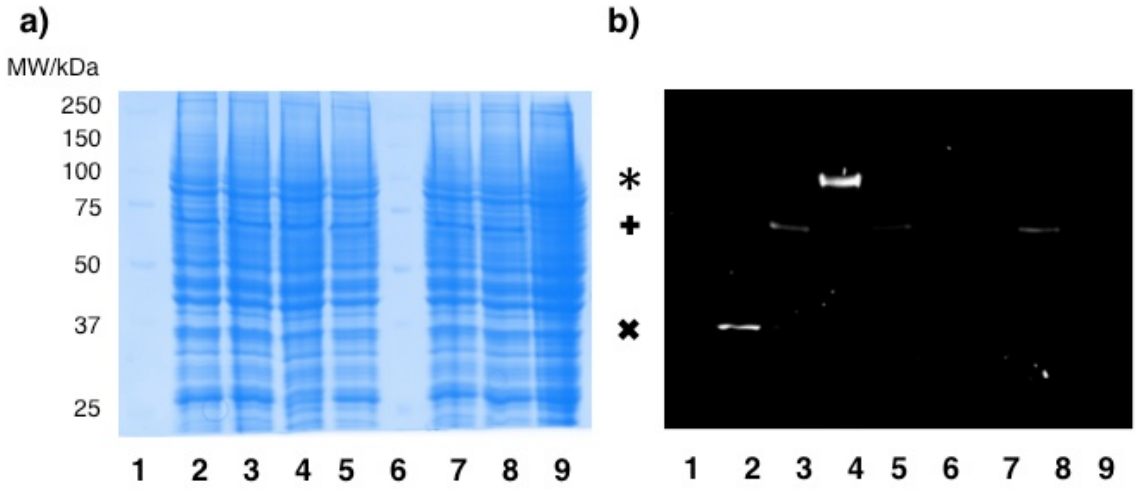


Figure S5. Representative data on PTEN-targeted delivery of HNE. Cultured HEK-293 cells transiently overexpressing Halo-PTEN were treated with 25 μM HtPHA (**2**) for 2.5 h at 37 ºC. Subsequent to rinsing with serum-free media (x2) every 30 min over 1.5 h, designated plates were exposed to a hand-held 4 W lamp (365 nm) for 20 min at room temperature, and further incubated for 5 min at 37 ºC prior to harvest. Cells were lysed at 4 ºC and lysates were subjected to Tev cleavage for 30 min at 37 ºC followed by click reaction with Cy5-azide for 30 min at 37 ºC. Samples were analyzed by SDS-PAGE. a) Resultant SDS-PAGE with Coomassie staining. b) In-gel fluorescence analysis of the same SDS-PAGE with Cy5 excitation. Lane 1, ladder; Lane 2, results from sample where Tev treatment in lysate was replaced with buffer without Tev; Lane 3, results from cells exposed to light; Lane 4, halo binding site on Halo-PTEN was pre-blocked with linker alone (compound **3**, Scheme S1) followed by treatment with HtPHA (compound **2**, Scheme S2); Lane 5, cells were treated with 25 μM HA (compound **5**, Scheme S4) for 20 min; Lane 6, identical to Lane 3 but performed on non-transfected cells with no halo-fusion protein; Lane 7, negative control from cells not exposed to light; Lane 8, independent duplicate of Lane 3; Lane 9, independent duplicate of Lane 4; Lane 10, independent duplicate of Lane 7. From the independent duplicate data sets shown here and from additional duplicates shown only during the manuscript reviewing stage, average Cy5 signal intensity transfer from Halo band before light exposure, to PTEN band after light exposure, is quantitated to be 28 ± 10% (n = 4).

*, Halo–PTEN, 82 kDa
+, PTEN, 47 kDa
x, Halo, 33 kDa

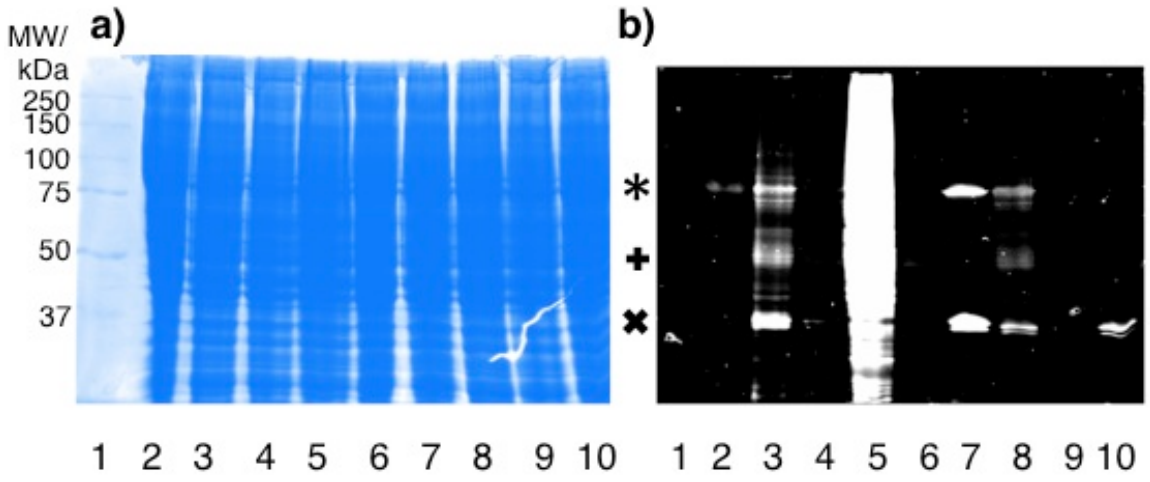


Table S1

(A) Primers for cloning out human Keap1 gene from phrGFP-Keap1 plasmid and dropping into pET28a_Halo vector, to construct pET28a_HaloKeap1 wherein Tev cleavage site exists between Halo and Keap1.

Fwd-**1**:
TCGAGATTTCCGGCTCCGGAGAAAACTTGTATTTCCAGGGCTCAGGGATGCAGCCAGATC

Rev-**1**:
TCAACAGGTACAGTTCTGCTGGTCAAT

Fwd-ext-**1**:
GGACCTGATCGGCAGCGAGATCGCGCGCTGGCTGTCGACGCTCGAGATTTCCGGCTCCGG

Rev-ext-**1**:
CTCAGCTTCCTTTCGGGCTTTGTTATCAACAGGTACAGTTCTGCTGGTCAAT

(B) Primers for cloning out HaloKeap1 gene from pET28a_HaloKeap1, to construct pMIR-DsRed-IRES-HaloKeap1.

Fwd-**2**:
TTTTCCTTTGAAAAACACGATGATAATATGGCCACAACCATGGCAGAAATCGGTACTGGC

Rev-**2**:
TTTAGTACTCTTGAGTCTGGACTTTCTGATCAACAGGTACAGTTCTGCTGGTC

Fwd-ext-**2**:
ACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAATA

Rev-ext-**2**:
AGTTTTAAGGAAAATCCATTATTATTAAAAGTTTAGTACTCTTGAGTCTGGACTTTCTGA

Table S2

(A) Primers for cloning out human PTEN gene from pcDNA3-GFP-PTEN plasmid and dropping into pET28a_Halo vector, to construct pET28a_HaloPTEN wherein Tev cleavage site exists between Halo and PTEN.

Fwd-**1**:
TCGAGATTTCCGGCTCCGGAGAAAACTTGTATTTCCAGGGCTCAGGGATGAC
AGCCATCAT

Rev-**1**:
TCAGACTTTTGTAATTTGTGTATGCTG

Fwd-ext-**1**:
GGACCTGATCGGCAGCGAGATCGCGCGCTGGCTGTCGACGCTCGAGATTTCC
GGCTCCGG

Rev-ext-**1**:
CTCAGCTTCCTTTCGGGCTTTGTTATCAGACTTTTGTAATTTGTGTATGCTG

(B) Primers for cloning out HaloPTEN gene from pET28a_HaloPTEN, to construct pMIR-HaloPTEN.

Fwd-**2**:
TAGTGAACCGTGGATCCACCATGGCAGAAATCGGTACTGGC

Rev-**2**:
AGTGCCAAGCTAGCGGCCTCAGACTTTTGTAATTTGTGTATGCTGAT

Fwd-ext-**2**:
AGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTG
GATCCACC

Rev-ext-**2**:
ACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGC
TAGCGGCC

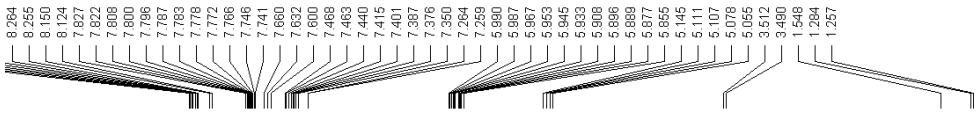
References

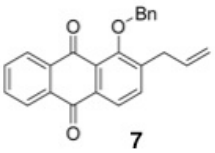
[1]Brinson, R. G.; Jones, P. B. *Org. Lett.* **2004**, *6*, 3767-3770.

[2]Vila, A.; Tallman, K. A.; Jacobs, A. T.; Liebler, D. C.; Porter, N. A.; Marnett, L. J. *Chem. Res. Toxicol.* **2008**, *21*, 432-444.

[3]Aye, Y.; Brignole, E. J.; Long, M. J. C.; Chitturulu, J.; Drennan, C. L.; Asturias, F. J.; Stubbe J. *Chem. Biol.* **2012,** *19*, 799-805.

Appendix: ^{1}H NMR spectra of intermediate products





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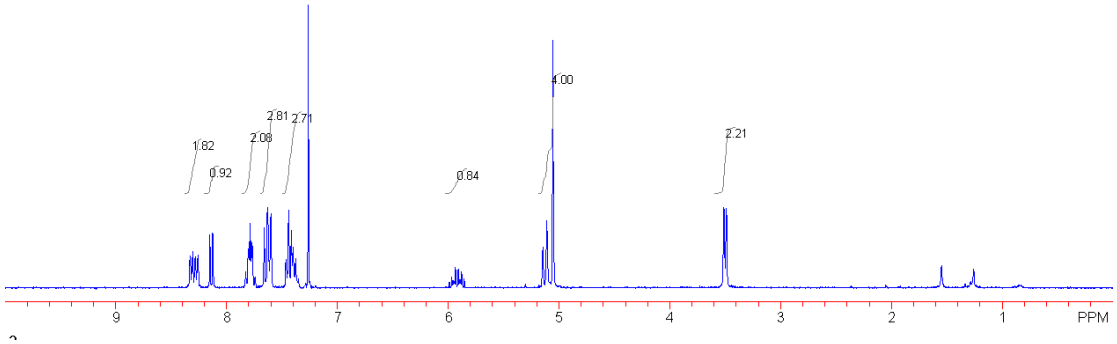

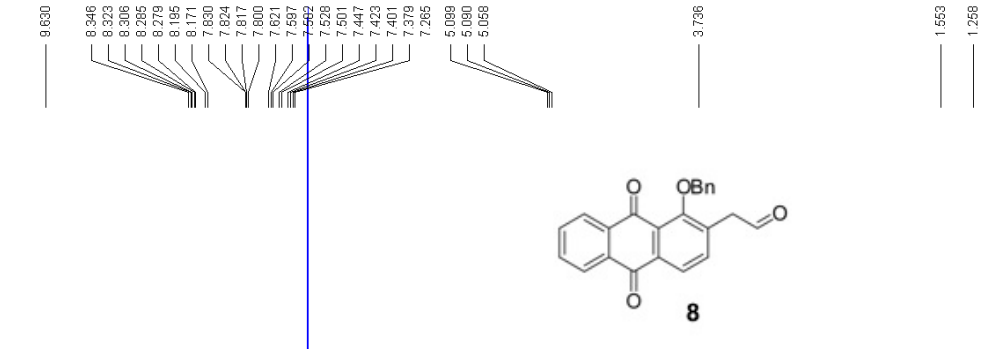

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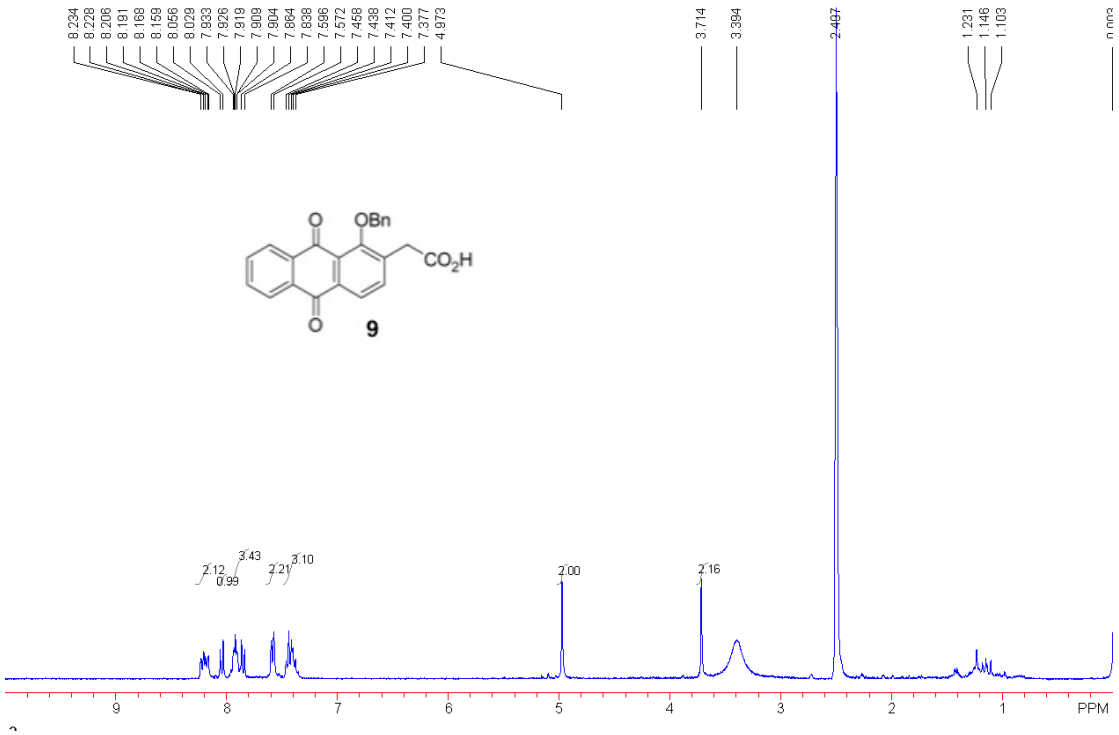
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1.103
OBn
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2.12
0.99
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2.21
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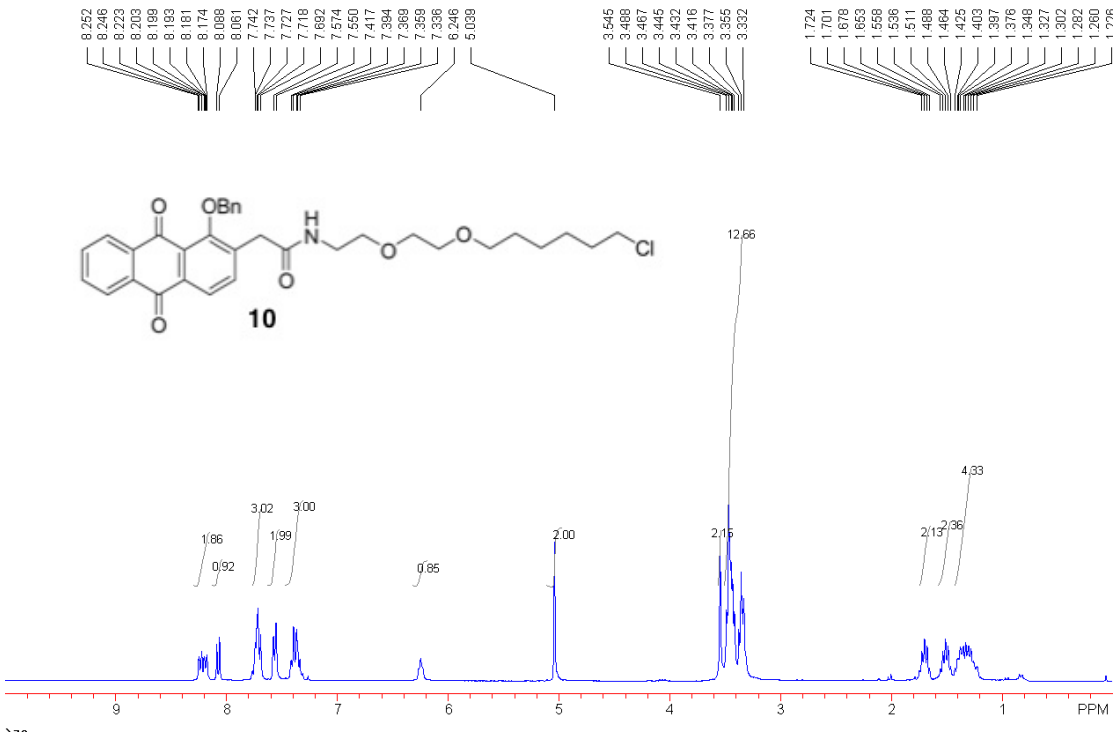
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7.574
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OBn
H
N
O
Cl
10
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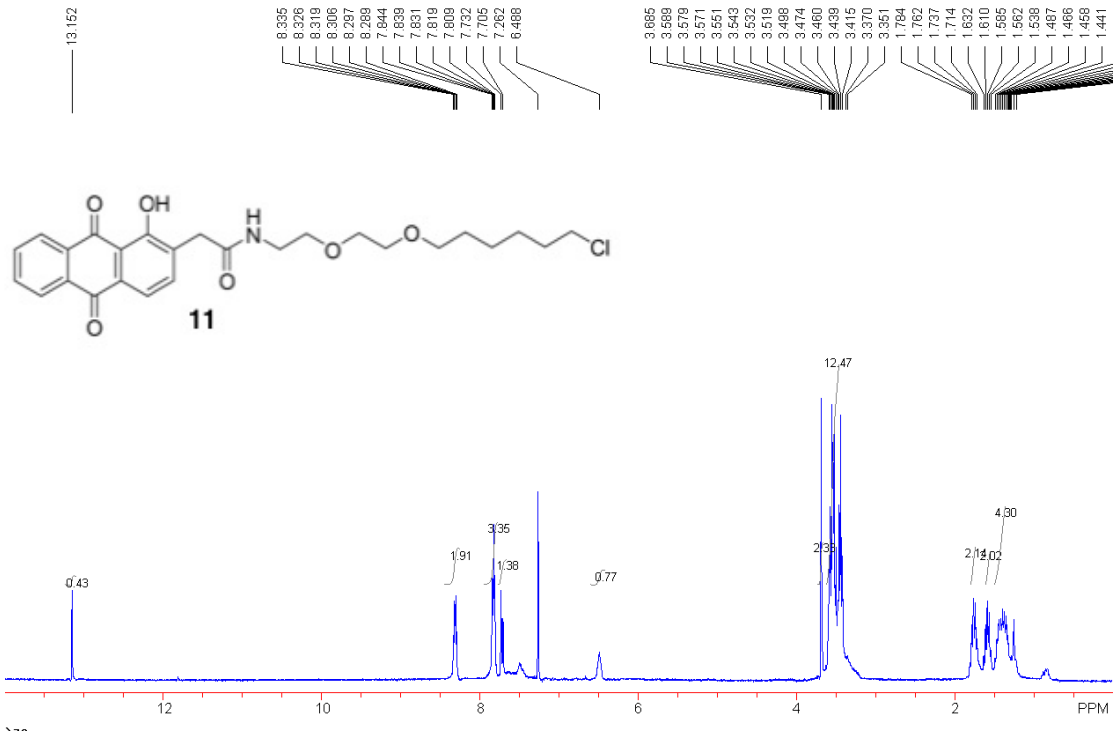
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1.562
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N
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O
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Cl
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2.02
12
10
8
6
4
2
PPM
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5.921
5.903
5.901
5.885
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5.774
5.761
5.759
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4.167
4.151
4.135
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1.491
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1.399
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1.378
1.370
1.364
7.79
OH
12
Br
C5H11
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9
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7
6
5
4
3
2
1
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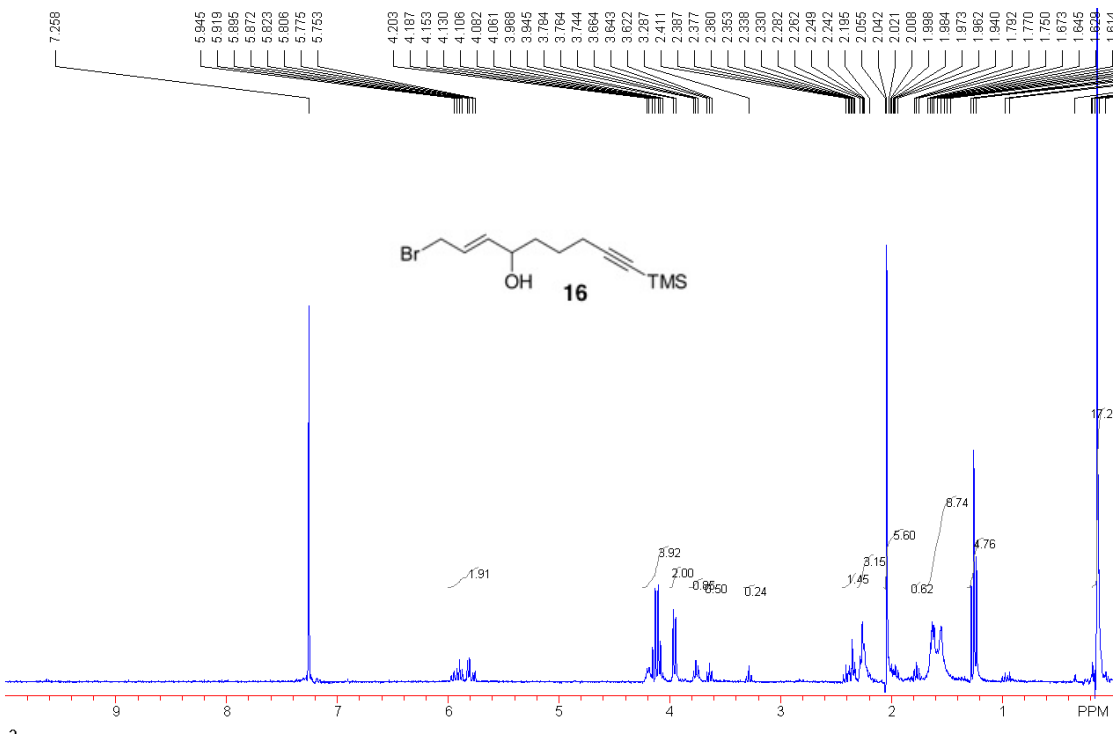
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16
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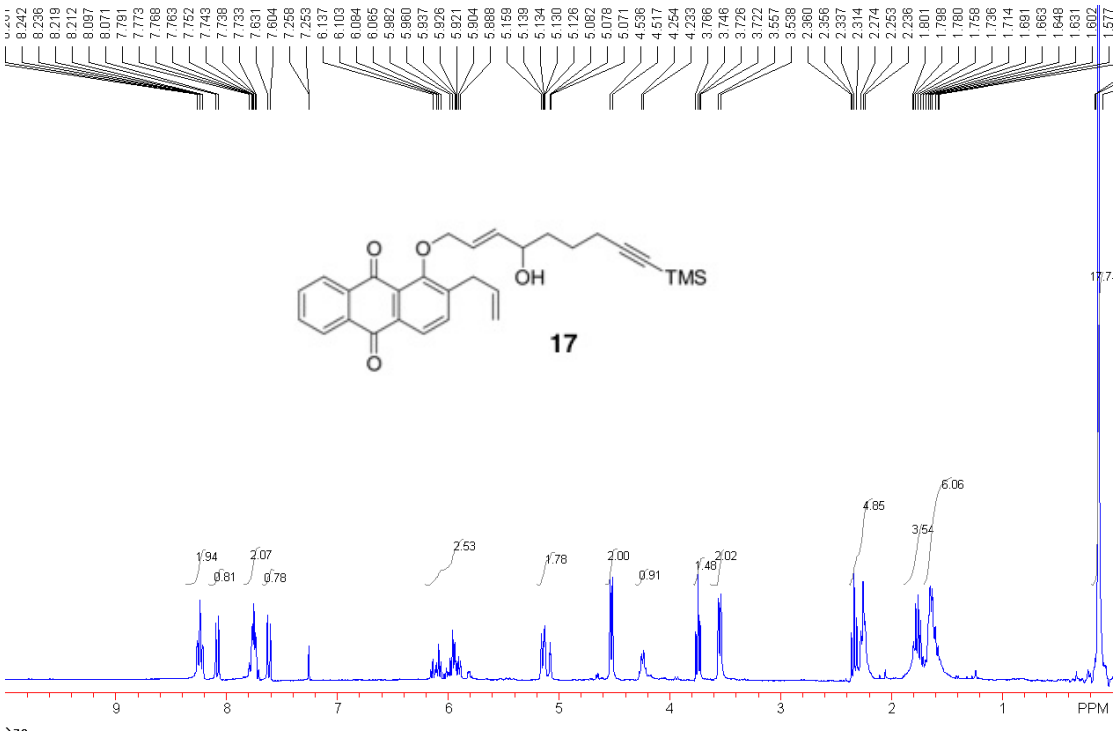
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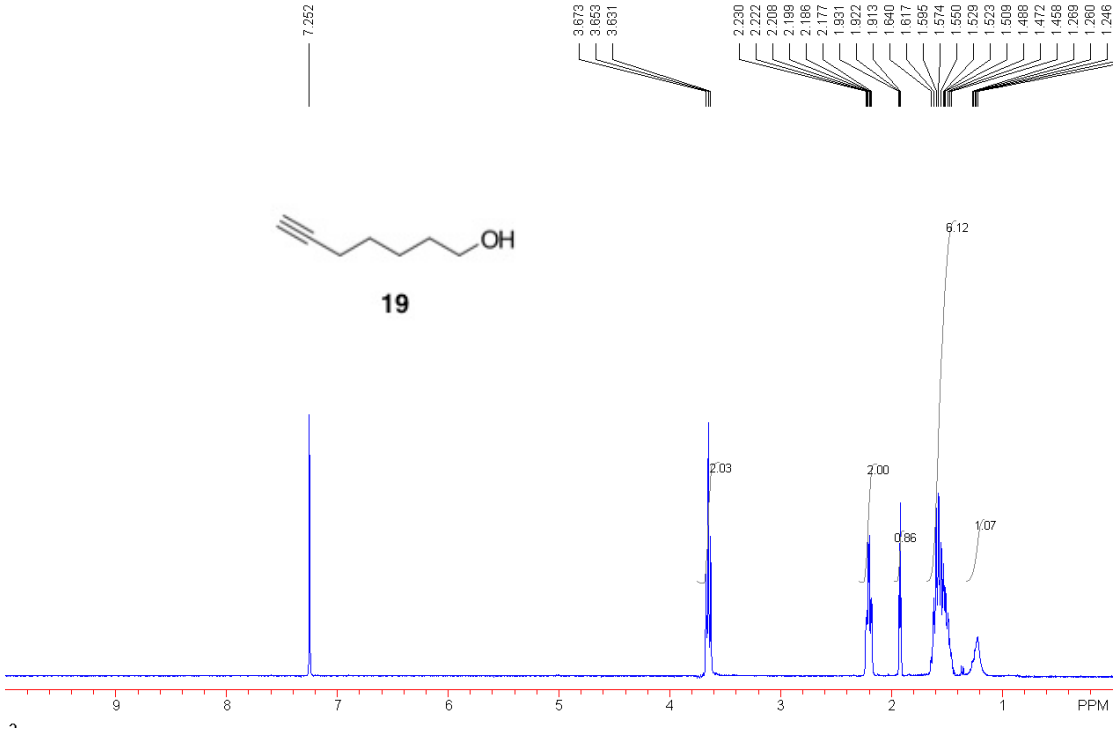
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1.595
1.574
1.550
1.529
1.523
1.508
1.488
1.472
1.458
1.268
1.260
1.246
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6
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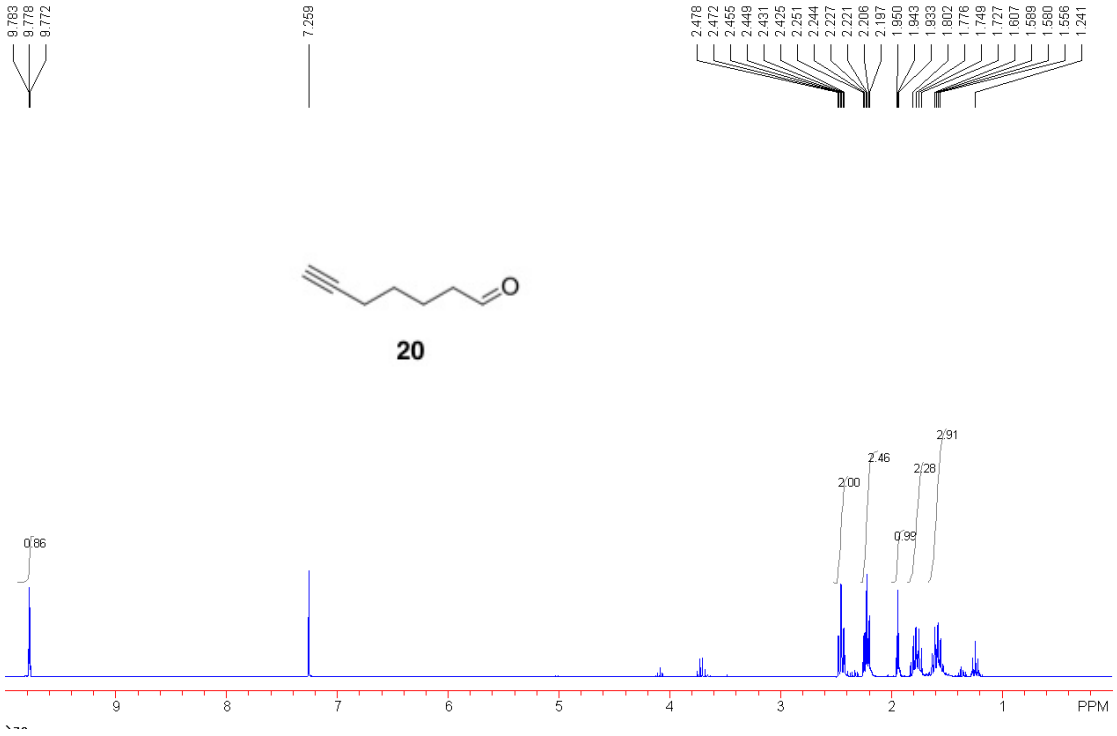
4
3
2
1
PPM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2.221
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2.197
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1.943
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1.776
1.749
1.727
1.607
1.589
1.580
1.556
1.241
O
20
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7
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2
1
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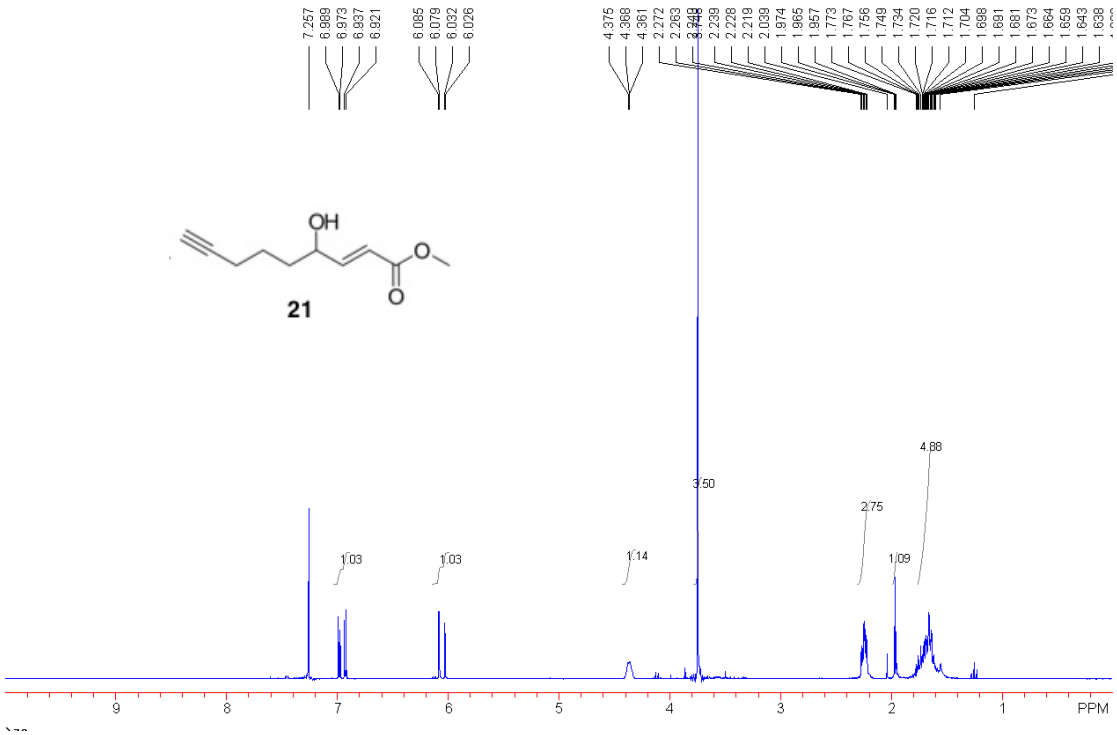
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4.361
2.272
2.263
2.239
2.228
2.219
2.039
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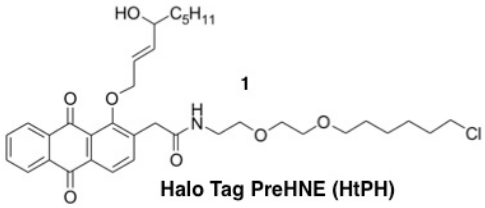
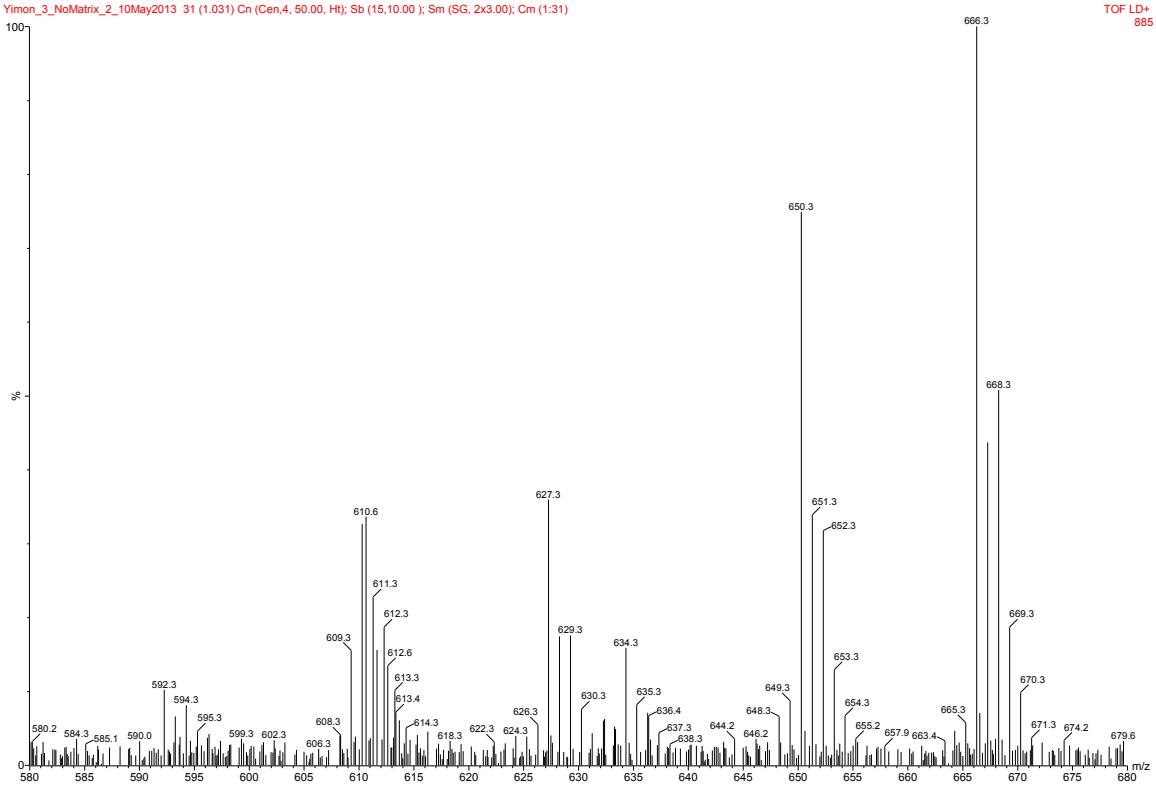
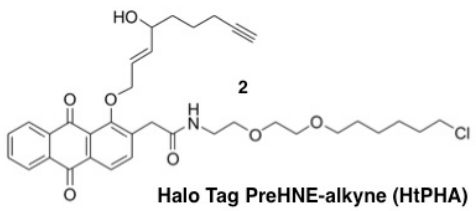
1.965
1.957
1.773
1.767
1.756
1.749
1.734
1.720
1.716
1.712
1.704
1.698
1.681
1.681
1.673
1.664
1.659
1.643
1.638
OH
O
O
21
1.03
1.03
1.14
3.50
2.75
1.09
4.88
9
8
7
6
5
4
3
2
1
PPM

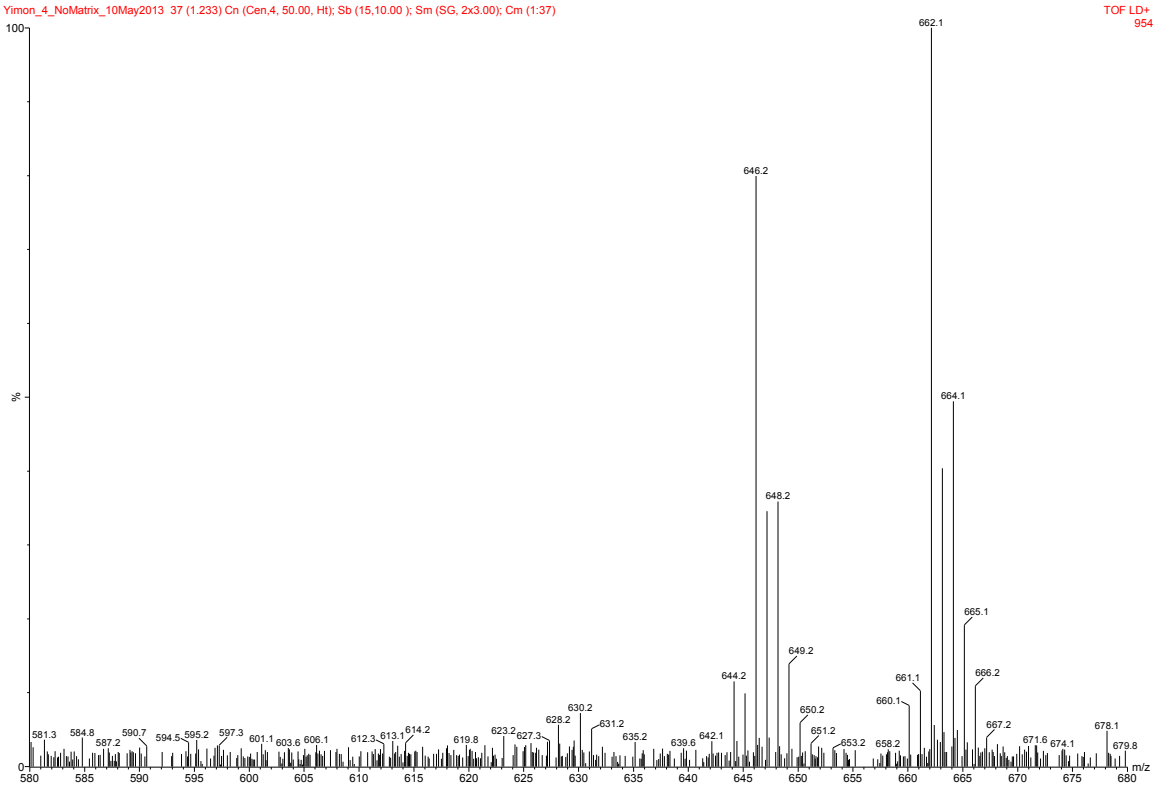
Appendix continued:
LDI (Laser Desorption-Ionization) mass spectra of compounds 1, 2 and 4

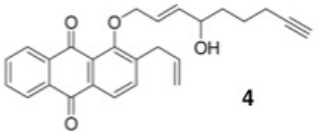






Halo Tag PreHNE-alkyne (HtPHA)





PreHNE-alkyne (PHA)