

Supplementary Information

Chemoproteomic profiling reveals cellular targets of nitro-fatty acids

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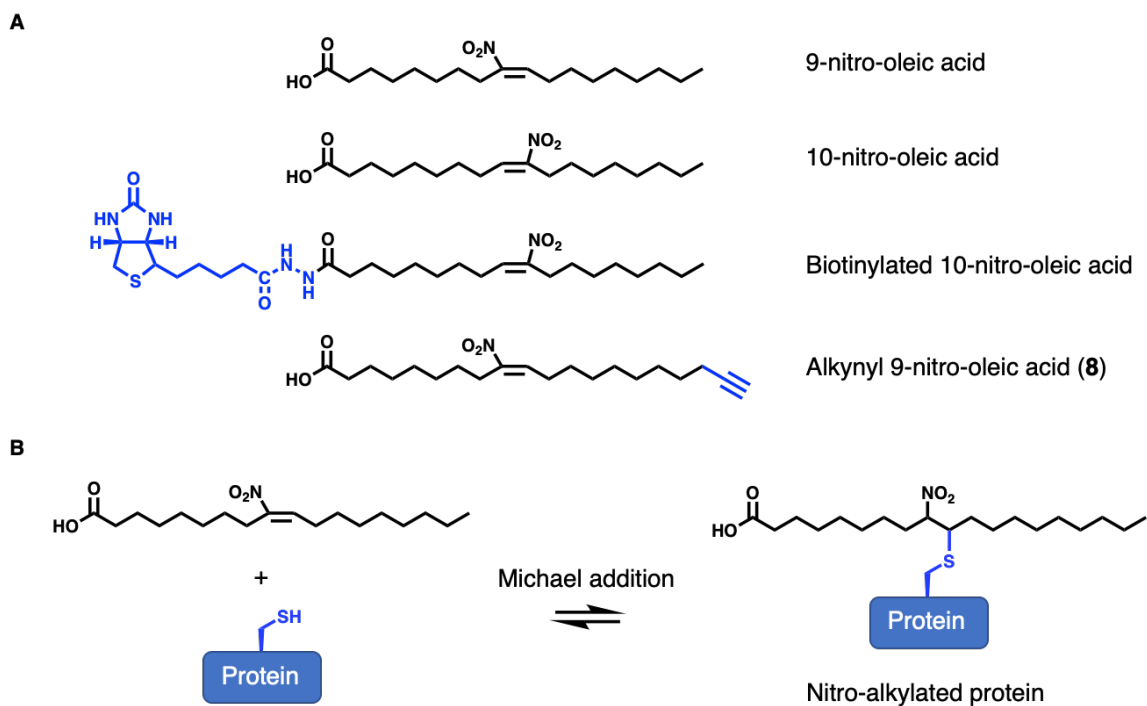
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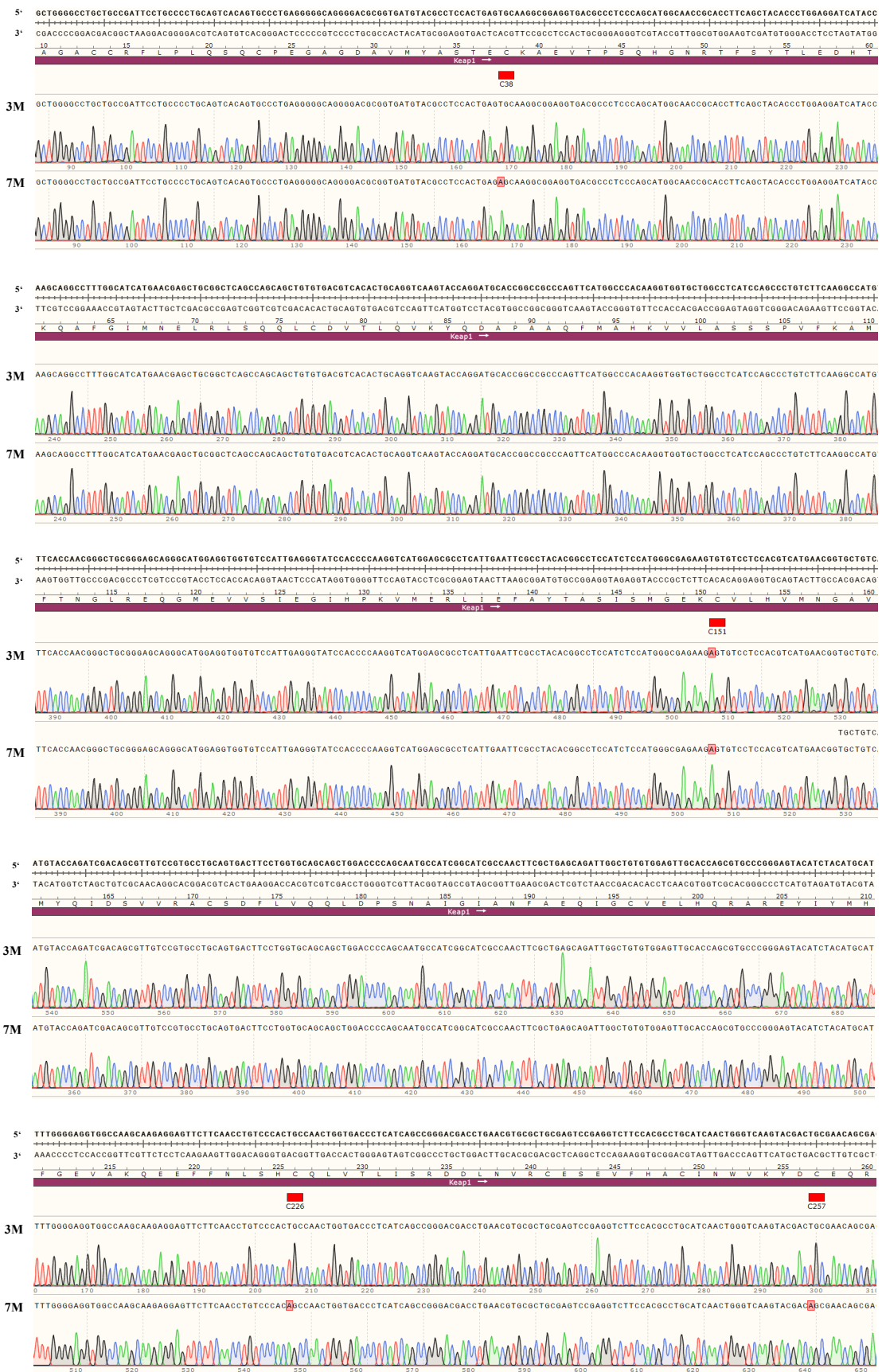
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Supplementary Figures



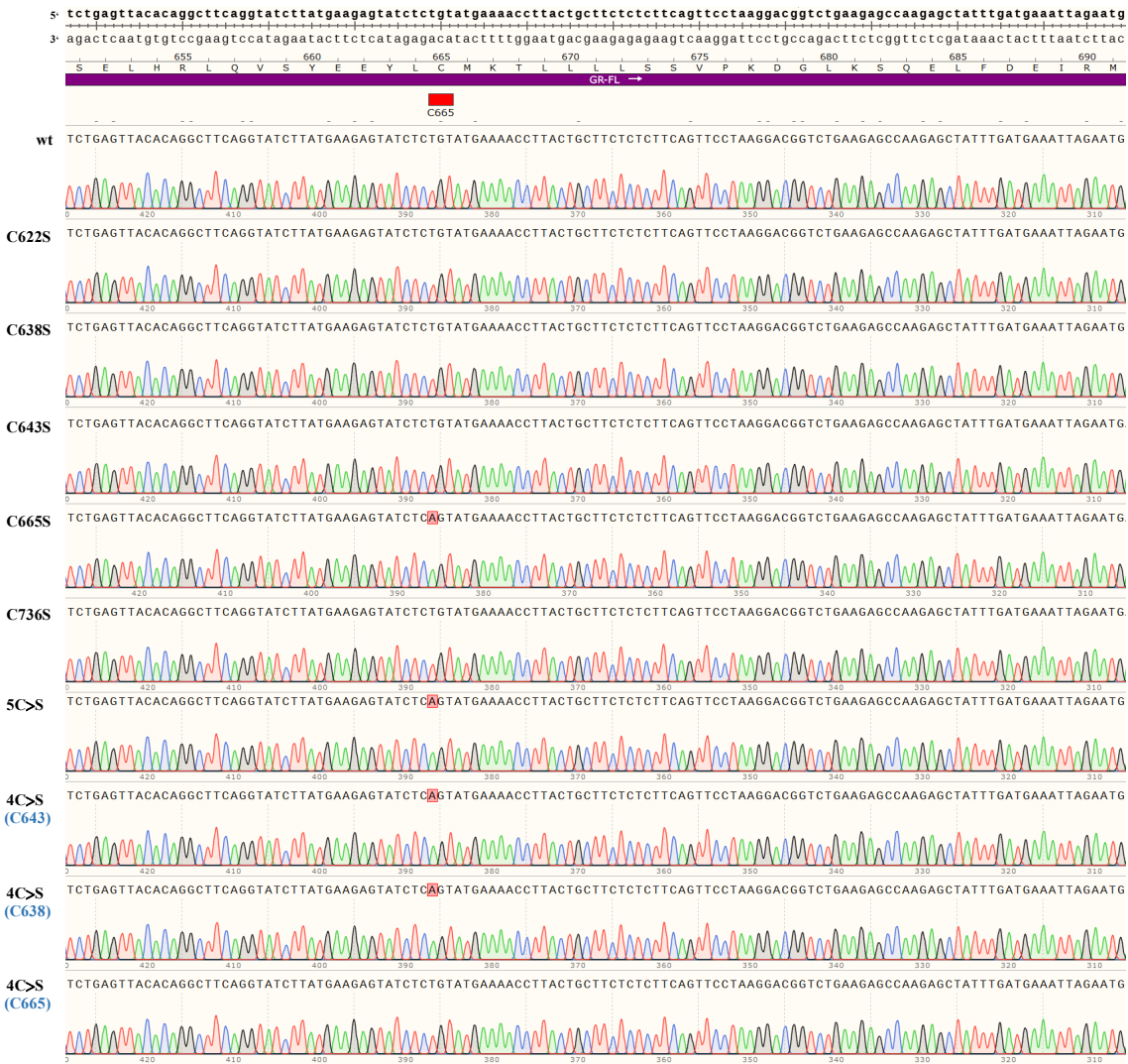
Supplementary Figure 1. Protein nitro-alkylation is a reversible protein modification. (A) Chemical structures of selected positional isomers of nitro-oleic acids, 9-nitro-oleate and 10-nitro-oleate. Also shown are the chemical structures of nitro-oleate probes for the detection of nitro-alkylated proteins, including the widely used biotinylated nitro-oleate [1], and the alkynyl-9-nitro-oleate probe **8** from this study. **(B)** Nitro-fatty acids form reversible protein adducts with the thiols of cysteine residues of target proteins via Michael addition.





Supplementary Figure 2. Sanger sequencing traces confirming the presence of indicated mutations in 3M and 7M KEAP1 mutants. 3M: C151S, C273W, C288E. 7M: C38S, C151S, C226S, C257S, C273W, C288E, C489S.



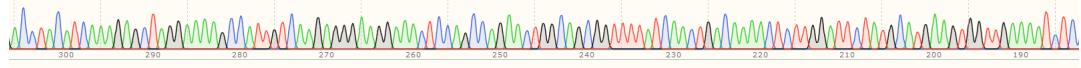


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 3' tggatgtagtttctcgatccttttcggtaacagttctcccttcccttgaggtcggctctgaccgctcgccaaaatagttgactgttttgagaacctaaagatcgtaacctcaccacacttttagag
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 GR-FL →

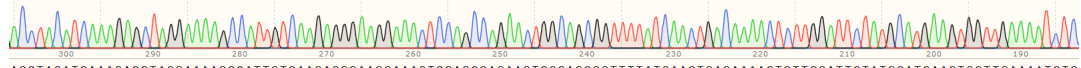
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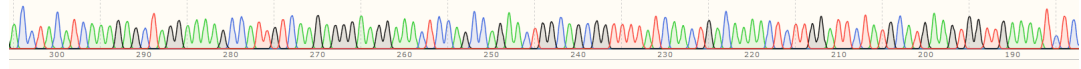
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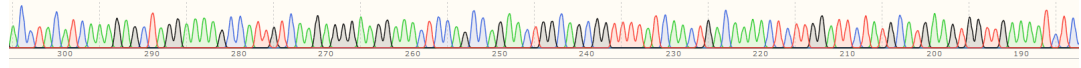
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C736S ACCTACATCAAAGAGCTAGGAAAAGCCATTGTCAAGAGGGAAGGAACTCCAGCCAGAACTGGCAGCGGTTTTATCAACTGACAAAACCTTTGGATTCTATGCATGAAGTGGTTGAAAATCTC



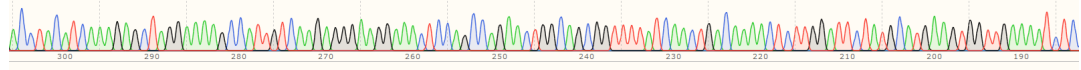
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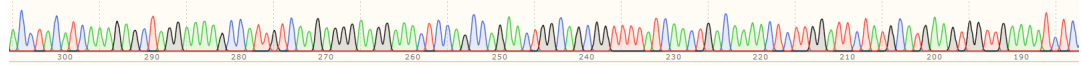
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4C>S (C638) ACCTACATCAAAGAGCTAGGAAAAGCCATTGTCAAGAGGGAAGGAACTCCAGCCAGAACTGGCAGCGGTTTTATCAACTGACAAAACCTTTGGATTCTATGCATGAAGTGGTTGAAAATCTC

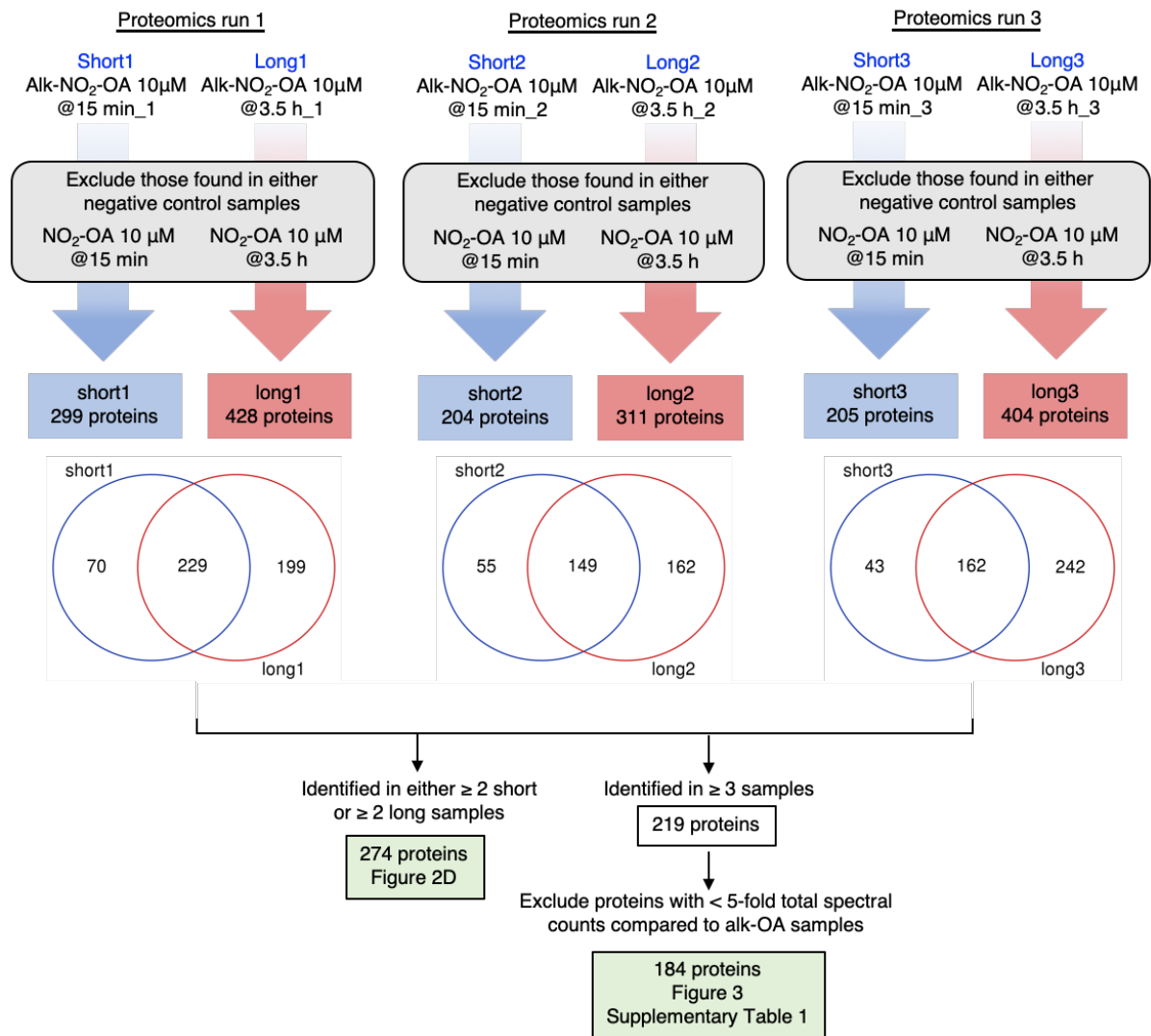


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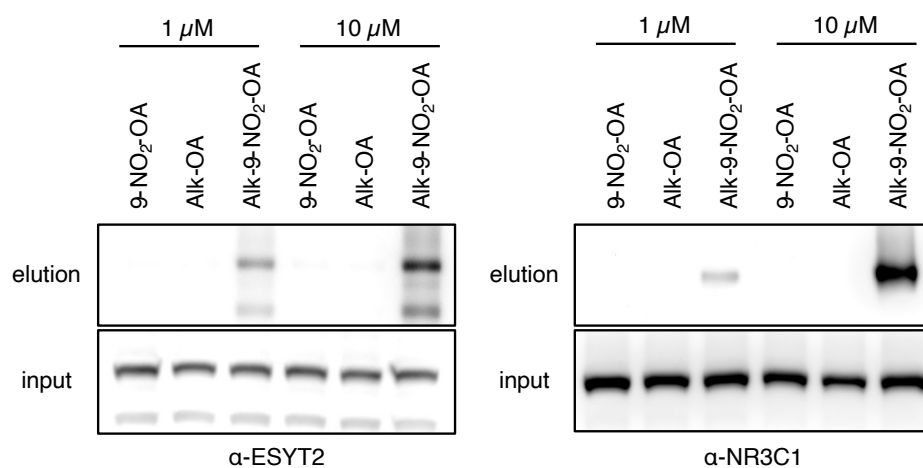




Supplementary Figure 3. Sanger sequencing traces confirming the presence of indicated cysteine to serine mutations in NR3C1-LBD. 5C>S refers to the mutant with all 5 cysteines (C622, C638, C643, C665, C736) in the LBD mutated to serine. 4C>S (Cxxx) refers to mutants with 4 of the 5 cysteines in the LBD mutated to serine, with the indicated cysteine remaining intact.



Supplementary Figure 4. Filter criteria for the identification of nitro-alkylated proteins from THP1 cells. Each proteomics run is an independent biological repeat. For each proteomics run, from the list of proteins obtained in the alk-9-NO₂-OA samples that were treated for 15 min (short) and 3.5 h (long), we excluded proteins that were also identified in either of the two negative control samples (9-NO₂-OA). Filtering for proteins that were either identified in ≥ 2 short or ≥ 2 long samples yielded 274 proteins as represented in Figure 2D. Filtering for proteins that were identified ≥ 3 times in the total of 6 samples yielded and excluding proteins that were similarly enriched using alk-OA yielded 184 proteins. These proteins were used for bioinformatics analyses represented in Figure 3 and listed in Supplementary Table 1.



Supplementary Figure 5. Selective enrichment of selected nitro-alkylated proteins at lower probe concentrations. Lysates from THP1 macrophages treated with the lipids at the indicated concentrations for 15 min were reacted with biotin-azide via CuAAC. Protein samples before NeutrAvidin enrichment (input) and after elution were immunoblotted for the ESYT2 and NR3C1.

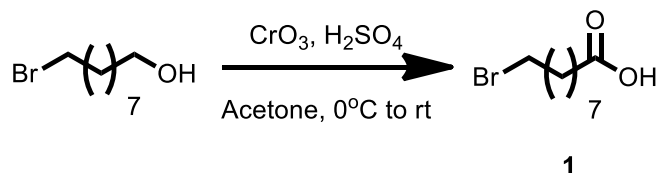
Supplementary Tables Legends

Supplementary Table 1. High-confidence list of nitro-alkylated proteins from THP1 macrophages identified by chemoproteomics study. Proteins are ranked by the total number of peptide spectrum matches (PSMs) identified in alk-9-NO₂-OA samples. (.xlsx)

Supplementary Table 2. Comparative analysis of high confidence nitro-alkylated protein list from this study with protein targets of other indicated electrophilic compounds. Proteins found to be modified by the indicated electrophiles in their respective studies are highlighted in green. IA, iodoacetamide [2]. DMF, dimethyl fumarate [3]. HNE, 4-hydroxy-2-nonenal and ONE, 4-oxo-2-nonenal [4,5]. Sulforaphane [6]. BMK, bromomethyl ketone [7]. Curcumin [8,9]. HD, 2-trans-hexadecenal [10]. CDDO-Im, imidazole derivative of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid [11]. 2BP, 2-bromopalmitate [12,13]. (.xlsx)

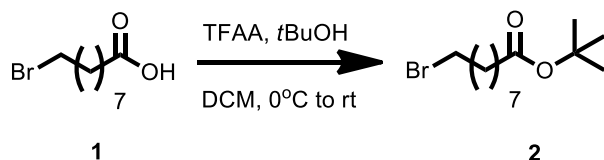
Supplementary Materials and Methods

Chemical synthesis of clickable alk-9-NO₂-OA probe.

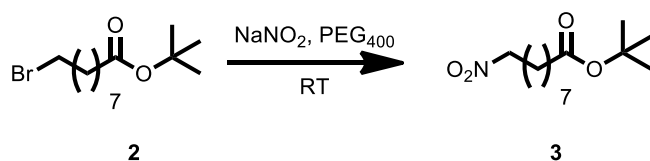


9-bromononanoic acid 1: To the solution of 2.5 M Jones' reagent (30 mL, 76.18 mmol, 1.7 eq.) in 10 mL of acetone was added 9-bromononanol (10 g, 44.81 mmol) in acetone (10 mL) at 0 °C. After reaction was completed, the solution mixture was added water (100 mL) and Et₂O (100 mL). After phase separation, the aqueous layer was extracted with Et₂O (50 mL x 3). The combined organic layer was washed with 1.0 N NaOH (30 mL x 3). The aqueous layers were combined and washed with DCM (30 mL x 2) to remove nonpolar impurities. The aqueous layer was acidified to pH 2 with conc. HCl and extracted with Et₂O (30 mL x 3). The combined organic layers were washed with brine (50 mL x 2), dried over MgSO₄, filtered, and concentrated in vacuo to provide 9-bromononanoic acid **1** (6.68 g, 63%). ¹H NMR (CDCl₃, 400 MHz): δ 3.38 (2H, t, *J* = 9.2 Hz), 2.33 (2H, *J* = 10 Hz), 1.88-1.78 (2H, m), 1.64-1.57 (2H, m), 1.42-1.40 (2H, m), 1.38-1.30 (6H, m); ¹³C NMR (CDCl₃, 176 MHz): δ 180.51, 34.20, 34.04, 32.88, 29.14, 29.02, 28.65, 28.19, 24.71 [14].

Jone's reagent preparation: Dissolve CrO₃ (25 g, 0.25 mol) in H₂O (75 mL) then was added conc. H₂SO₄ (25 mL) slowly at 0 °C. The concentration of the solution prepared by this procedure is 2.5 M.

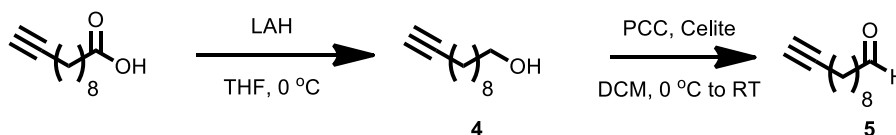


Ester 2: To a solution of 9-bromononanoic acid **1** (208 mg, 0.88 mmol) in DCM (4.4 mL) was added dropwise TFAA (405 mg, 1.93 mmol, 2.2 eq.) at 0 °C. The resulting mixture was stirred for 2.5 h before addition *t*BuOH (228 mg, 3.07 mmol, 3.5 eq.) at 0 °C. The resulting mixture was slowly warmed to RT. After reaction was completed, resulting mixture was added 10 mL of NaHCO₃ (sat. aq) and extracted with Et₂O (10 mL x 3). The combined organic layers were washed with brine, dried over MgSO₄, filtered, and concentrated in vacuo to give ester **2** (238 mg, 93%). ¹H NMR (400 MHz, CDCl₃): δ 3.38 (2H, t, *J* = 7.2 Hz), 2.18 (2H, s, *J* = 7.6 Hz), 1.86-1.79 (2H, m), 1.59-1.52 (overlap with water residual, 2H, m), 1.43-1.38 (11H, m), 1.29 (6H, br); ¹³C NMR (176 MHz, CDCl₃): δ 173.20, 79.91, 35.59, 33.93, 32.85, 29.16, 29.02, 28.65, 28.19, 28.17, 25.09 [15].



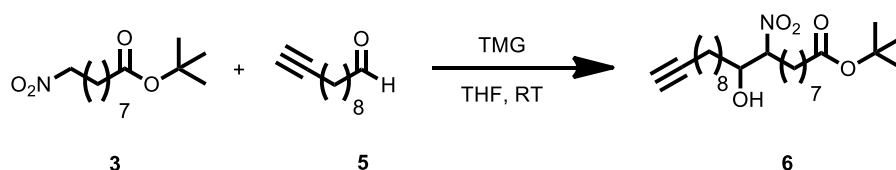
tert-butyl 9-nitrononanoate 3: To the flask was added NaNO₂ (424 mg, 6.15 mmol, 3.0 eq.) and PEG-400 (4.0 mL). The resulting mixture was stirred for 3 h at RT before addition ester **2**

(600 mg, 2.05 mmol). After the reaction was completed, resulting mixture was added 40 mL of water and extracted with Et₂O (20 mL x 3). The combined organic layers were washed with brine, dried over MgSO₄, filtered, and concentrated in vacuo. The residue was purified by flash chromatography (10% EtOAc/hexane, R_f=0.33) to yield the *tert*-butyl 9-nitrononanoate **3** (234 mg, 44%). ¹H NMR (400 MHz, CDCl₃): δ 4.351 (2H, t, *J* = 7.2 Hz), 2.18 (2H, t, *J* = 7.6), 1.99-1.94 (2H, m), 1.58-1.52 (2H, m), 1.42 (9H, s), 1.37-1.29 (8H, m); ¹³C NMR (176 MHz, CDCl₃): δ 172.96, 79.72, 75.47, 35.28, 28.73, 28.66, 28.49, 27.90, 27.16, 25.96, 24.77; HRMS (ESI): calculated for C₁₃H₂₅NNaO₄ [M+Na⁺] 282.1676, found 282.1690.

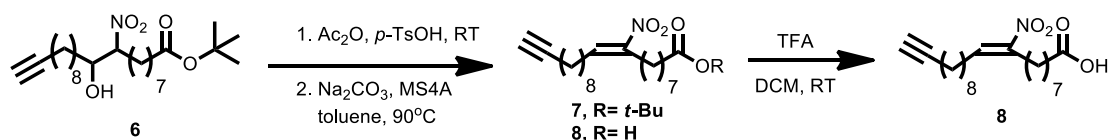


To a solution of LAH (603 mg, 15.09 mmol) in THF (40 mL) was added dropwise undec-10-ynoic acid (1338 mg, 7.95 mmol) in THF (10 mL) at 0 °C. After the reaction was completed, resulting mixture was added 10 mL of water at 0 °C and then adjusted to pH 3-5 using conc. HCl. The aqueous layer was extracted with Et₂O (100 mL x 3). The combined organic layers were washed with brine, dried over MgSO₄, filtered, and concentrated in vacuo to give crude alcohol **4**. The crude alcohol **4** was used for next reaction without further purification. ¹H NMR (400 MHz, CDCl₃): δ 3.64 (t, *J* = 6.6 Hz, 2H), 3.49 (s, 1H), 2.18 (dt, *J* = 7.1, 2.6 Hz, 2H), 1.94 (t, *J* = 2.7 Hz, 1H), 1.63 – 1.47 (m, 4H), 1.46 – 1.21 (m, 10H); ¹³C NMR (100 MHz, CDCl₃): δ 84.92, 68.20, 63.20, 32.91, 29.57, 29.49, 29.16, 28.85, 28.60, 25.84, 18.53.

Alcohol **4** was dissolved in DCM (40 mL) followed by addition of PCC (2.23 g, 10.34 mmol) and celite (2.6 g) at 0 °C. After the reaction was completed, resulting mixture was filtered by celite and concentrated in vacuo. The residue was purified by flash chromatography (10%, EtOAc/hexane, R_f=0.53) to yield the aldehyde **5** (759 mg, 57% in two steps).[16] ¹H NMR (400 MHz, CDCl₃): δ 9.74 (1H, t, *J* = 1.6 Hz), 2.40 (2H, td, *J* = 7.36, 1.84 Hz), 2.16 (2H, td, *J* = 7.0, 2.64 Hz), 1.92 (1H, t, *J* = 2.64 Hz), 1.64-1.59 (3H, m), 1.53-1.46 (2H, m), 1.40-1.35 (2H, m), 1.33-1.26 (5H, m); ¹³C NMR (100 MHz, CDCl₃): δ 203.163, 84.901, 68.324, 44.092, 29.407, 29.285, 29.071, 28.842, 28.621, 22.247, 18.579 [16].

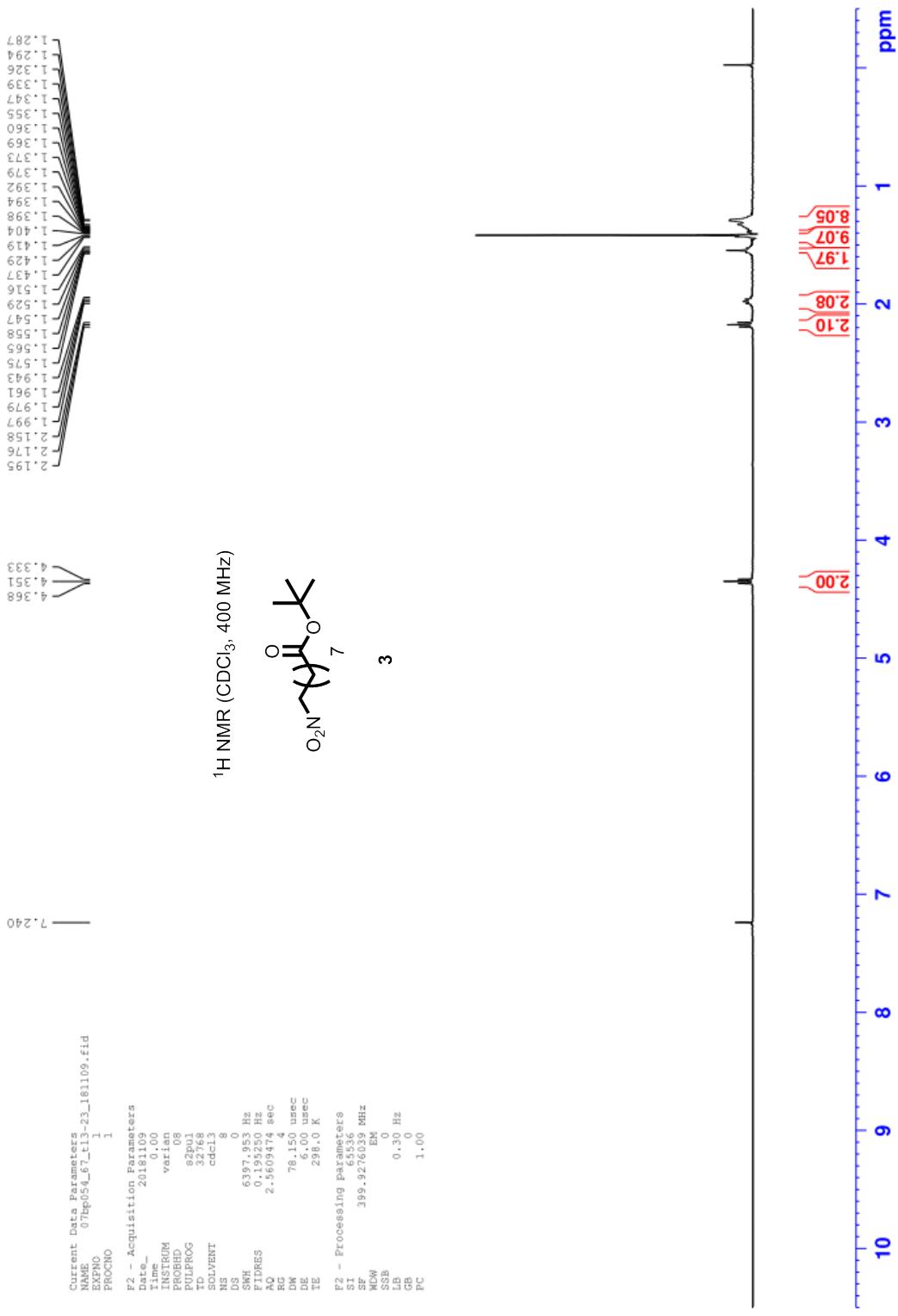


β-Hydroxynitro ester 6: To a solution of *tert*-butyl 9-nitrononanoate **3** (299 mg, 1.15 mmol) and aldehyde **5** (211 mg, 1.27 mmol, 1.1 eq.) in THF (6.0 mL) was added TMG (27 mg, 0.23 mmol, 0.2 eq.) dropwise at RT. After the reaction was completed, solvent was removed by reducing pressure and the residue was purified by flash chromatography (10% EtOAc/hexane, R_f=0.08) to yield the 45:55 mixture of diastereomers β-hydroxynitro ester **6** (332 mg, 68%). The mixture was used for next reaction without further purification.



((E)-9-nitricos-9-en-19-ynoic acid 8: The β -hydroxynitro ester **6** (205 mg, 0.48 mmol) in round bottom flask was added acetic anhydride (3.0 mL) as solvent and a catalytic amount of *p*-toluenesulfonic acid (10 mg). The solution was stirred under N_2 at RT for overnight. After reaction was completed, the solvent was removed by azeotropic removal with toluene (3×20 mL) under reduced pressure. The residual was dissolved in toluene (3 mL) and addition of Na_2CO_3 (51 mg, 0.482 mmol, 1.0 eq.). The solution was heated to 90°C under N_2 for overnight. After the reaction was completed, the solution is cooled to RT and diluted with 10 mL of NH_4Cl (sat. aq) and 10 mL Et_2O . After phase separation, the aqueous layer is extracted with Et_2O (10 mL \times 3). The combined organic layers were washed with brine, dried over MgSO_4 , filtered, and concentrated in vacuo to afford nitroalkene products **7** and **8** as a mixture. The mixture was used for next reaction without further purification.

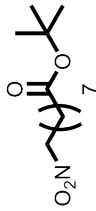
To the above mixture in DCM (3.0 mL) was added TFA (1.0 mL) at RT. After the reaction was completed, the solvent and excess TFA were removed by reducing pressure. The residual was purified by flash chromatography (20% EtOAc/hexane, $R_f=0.42$) to yield the (*E*)-9-nitricos-9-en-19-ynoic acid **8** (69 mg, 41% in three steps). ^1H NMR (400 MHz, CDCl_3): δ 7.08 (1H, t, $J=7.6$ Hz), 2.56 (2H, appear t), 2.35 (2H, t, $J=7.48$), 2.24-2.16 (4H, m), 1.94 (1H, t, $J=2.64$), 1.65-1.61 (3H, m), 1.54-1.44 (8H, m), 1.41-1.25 (11H, m); ^{13}C NMR (100 MHz, CDCl_3): δ 180.0, 151.7, 136.4, 84.6, 68.1, 33.9, 29.2, 29.1, 28.9, 28.84, 28.80, 28.6, 28.4, 28.3, 27.9, 27.8, 26.2, 24.5; HRMS (ESI): calculated for $\text{C}_{20}\text{H}_{32}\text{NO}_4$ [$\text{M}-\text{H}^+$] 350.2337, found 350.2321.



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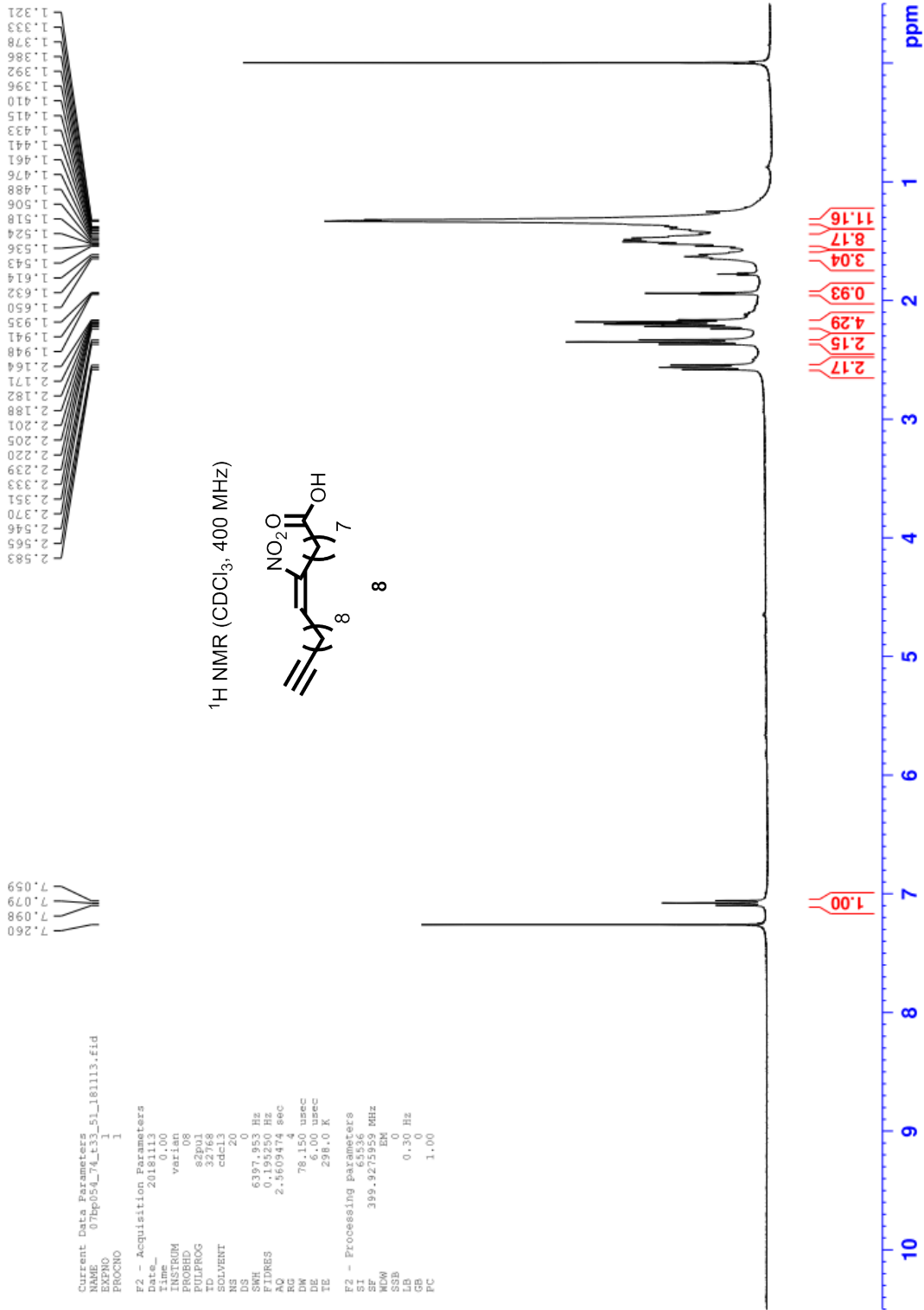
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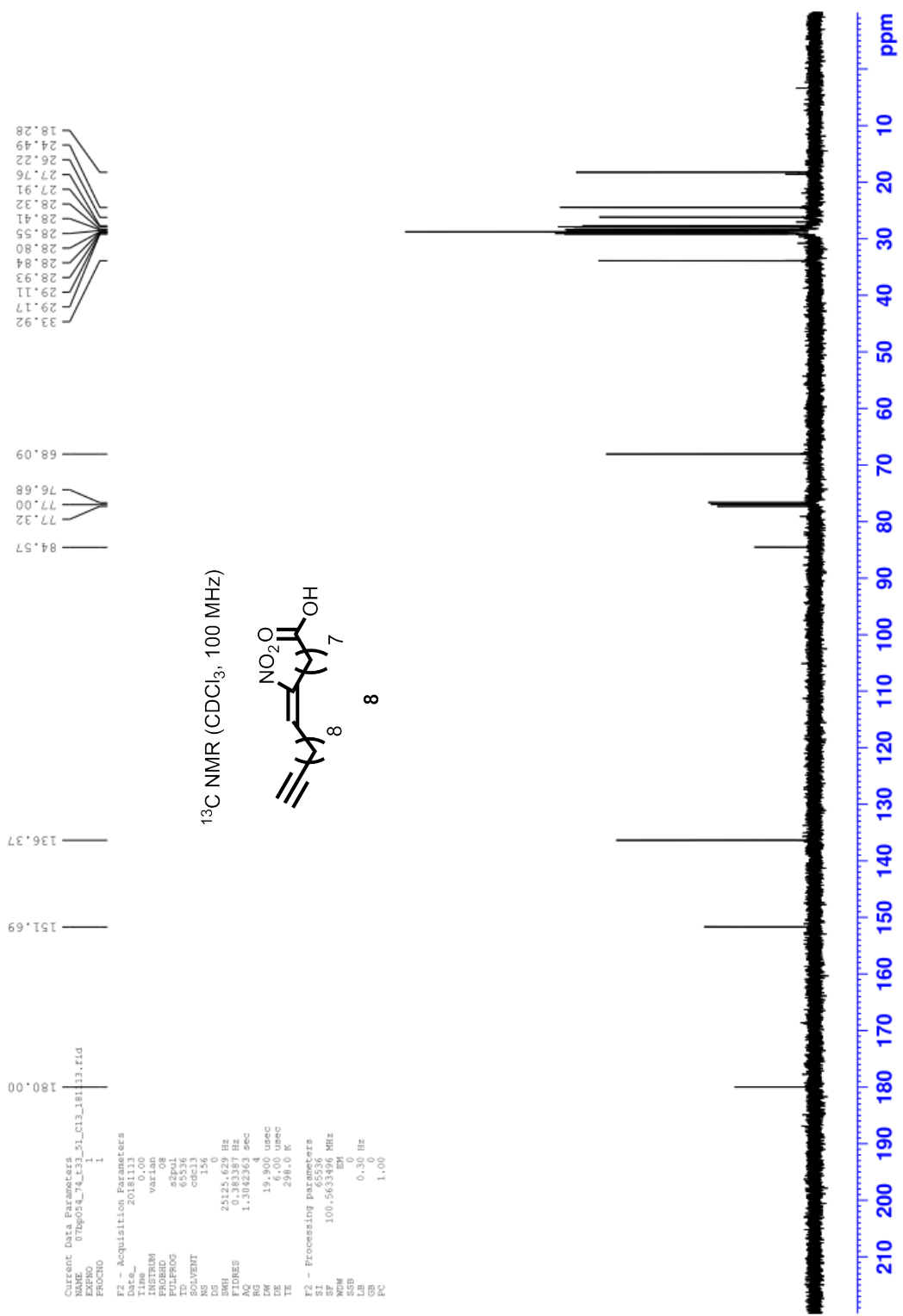
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