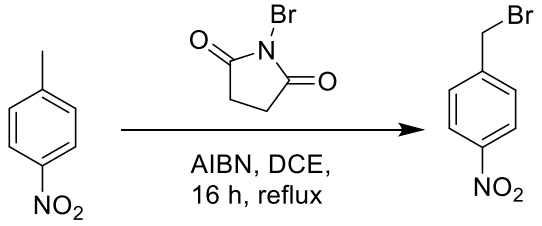
Materials and Methods

All reagents were obtained from commercial vendors and used as received unless otherwise stated. NMR spectra were measured on Agilent 400 MHz or Bruker 500 MHz spectrometers. ^{1}H and ^{13}C NMR chemical shifts are reported in ppm relative to internal solvent resonances. Solvents were used as received unless otherwise noted. Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. Thin-layer chromatography (TLC) was performed on glass-backed silica plates and visualized by UV unless otherwise stated. High-resolution mass spectra were taken on an Agilent Technologies 6230 TOF LC/MS mass spectrometer. LCMS experiments were performed on a Waters Acquity UPLC system equipped with a Kinetex EVO C18-functionalized silica column (C18 – 100 Å, 100 x 4.6 mm, H16-445733, 570-0069), diode array detector, and ESI mass spectrometer.

Cell studies were conducted on an adherent H9C2 line of rat embryonic cardiomyocytes (ATCC, Manassas, VA, USA). Cultures were grown in Dulbecco's Modified Eagle Medium (DMEM, VWR, Radnor, PA), supplemented with 10 % fetal bovine serum (FBS, VWR, Radnor, PA). Cells were cultured at 37 °C in 5 % CO_2-air. The cultures were passaged after 70–80 % confluence was achieved. Cells were rinsed with PBS solution, and then released with trypsin and EDTA solution (VWR, Radnor, PA). The suspension of released cells was centrifuged at 1000 rpm for 5 min.

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were conducted under the approval of the Virginia Tech Institutional Animal Care and Use Committee (IACUC; #19-134) and the Virginia-Maryland College of Veterinary Medicine.

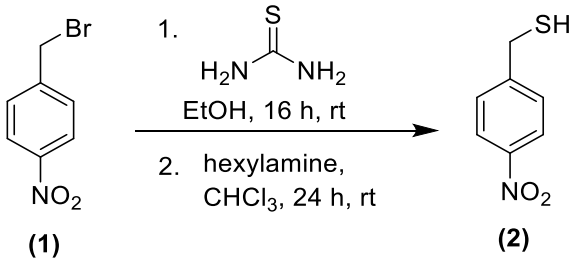
Synthesis of 4-(nitrobenzyl) bromide (**1**)



(1)

A flame-dried, 2-neck round bottom flask equipped with a septum and condenser was charged with 4-nitrotoluene (6.45 g, 47.0 mmol), dry 1,2-dichloroethane (150 mL), and *N*-bromosuccinimide (9.21 g, 51.7 mmol) under N_2 flow. The mixture was stirred until homogeneous, yielding a clear, light yellow solution. Azobisisobutyronitrile (AIBN) (0.386 g, 2.35 mmol) was added in one portion under N_2 flow, and the reaction mixture was degassed with N_2 for 30 minutes. The reaction was then heated to reflux under N_2 flow. Reaction progress was monitored by TLC (CH_2Cl_2) until starting material was consumed (~16 h). The reaction mixture was cooled to rt and washed successively with saturated $NaHCO_3$ (2 x 50 mL) and brine (50 mL). The organic layer was separated, dried over Na_2SO_4, and then concentrated via rotary evaporation to yield a brown, waxy solid. This crude product was then further purified via column chromatography, eluting with 1:1 CH_2Cl_2:hexanes. (Rf = 0.4, visualized with UV) The final product was isolated as white solid (8.90 g, 4.10 mmol, 82% yield). The product may also be recrystallized from boiling ethanol to reach higher purity. ^{1}H NMR ($CDCl_3$): δ 8.16 (d, 2H) 7.49 (d, 2H), 4.43 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 169.2, 149.7, 138.4, 129.6, 34.9. These chemical shifts match literature precedence.[1]

Synthesis of 4-(nitrobenzyl) thiol (**2**)



A two-neck round bottom flask equipped with a hose-barb adaptor and a septum was charged with compound **1** (4.00 g, 18.5 mmol), EtOH (50 mL), and a stirbar. The resulting suspension was bubbled with N_2 for 15 min. Thiourea (2.11 g, 27.8 mmol) was then added in one portion under N_2, and the reaction mixture was stirred at rt. The solids dissolved slowly over the course of several hours to give a clear, light orange solution. Reaction progress was monitored by TLC (CH_2Cl_2) until starting material was consumed (16 h). The reaction mixture was then concentrated via rotary evaporation, and the thiouronium intermediate was subsequently suspended in CH_2Cl_2 (100 mL) in the same two-neck round bottom flask. The suspension was degassed with N_2 for 30 min. Hexylamine (2.06 g, 2.7 mL, 20.4 mmol) was then injected. The resulting suspension slowly dissolved over the course of 4 h, yielding a bright yellow solution. After stirring for 24 h, this solution was washed with 1N HCl (50 mL) and brine (50 mL). The organic layer was separated and dried over Na_2SO_4, and concentrated via rotary evaporation to yield a viscous, yellow oil with a rancid smell. The product was then further purified by silica gel chromatography, eluting with 25% EtOAc:hexanes, yielding a white solid (2.6 g, 75% yield) ^{1}H NMR ($CDCl_3$): δ 8.15 (d, 2H) 7.50 (d, 2H), 3.82 (d, 2H), 1.84 (t, 1H). ^{13}C NMR ($CDCl_3$): δ 148.6, 129.1, 124.1, 28.5. These chemical shifts match literature precedence.[2]

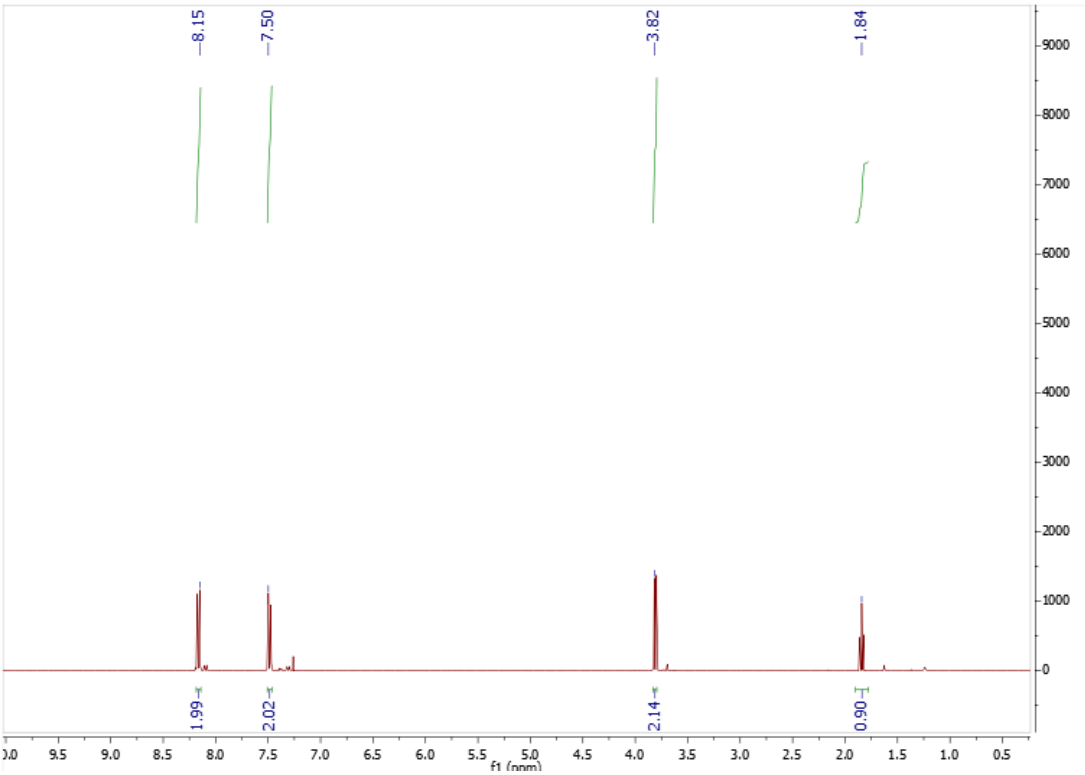


Figure S1. ^{1}H NMR spectrum of 4-(nitrobenzyl) thiol (2).

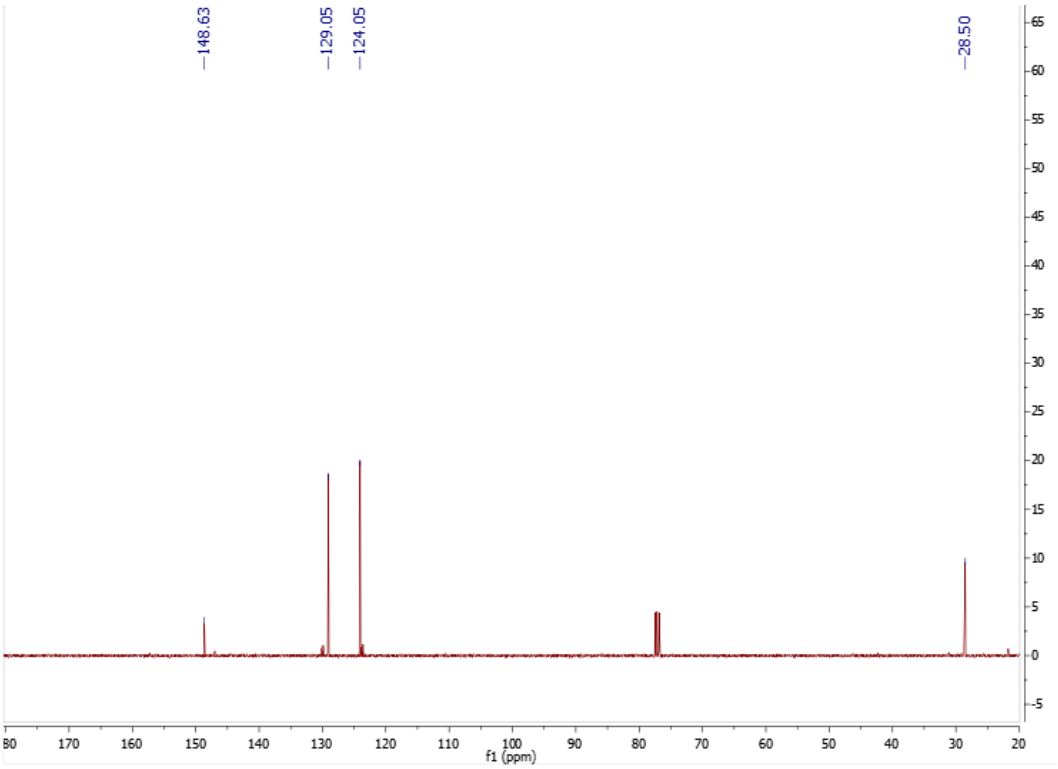
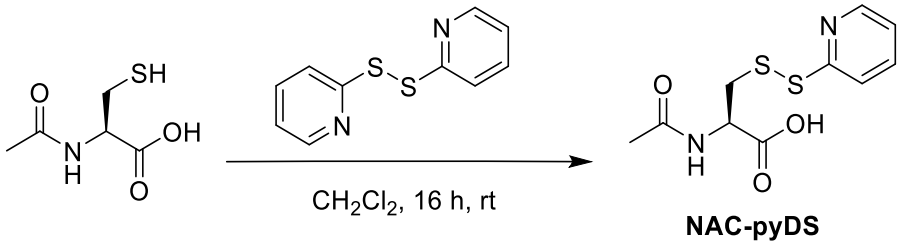


Figure S2. ^{13}C NMR spectrum of 4-(nitrobenzyl) thiol (2).

Synthesis of **NAC-pyDS**



A round bottom flask was charged with *N*-acetylcysteine (7.0 g, 42.9 mmol), CH_2Cl_2 (200 mL), and a stirbar to give a suspension. 2,2'-Dipyridyl disulfide (18.9 g, 85.8 mmol) was added in one portion resulting in bright yellow suspension. The reaction mixture gradually dissolved over the course of several hours. The reaction mixture was stirred at rt (16 h), and reaction progress was monitored by TLC (EtOAc). The resulting yellow solution was concentrated via rotary evaporation, yielding a bright yellow solid. This crude product was then suspended in cold acetone, stirred for 30 min to dissolve any thiopyridone byproducts, and filtered, rinsing with copious amounts of cold acetone. This yielded a faintly yellow powder (8.4 g, 70% yield). ^{1}H NMR (DMSO-d_6): δ 12.97 (s, 1H), 8.46 (m, 1H), 8.42 (d, *J* = 7.89 Hz, 1H), 7.81 (m, 1H), 7.75 (m, 1H), 7.24 (m, 1H), 4.47 (m, 1H), 3.15 (m, 2H), 1.86 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 171.88, 169.49, 158.77, 149.69, 137.86, 121.33, 119.34, 51.39, 39.93, 22.44. Spectroscopic data agree with literature precedence.[3]

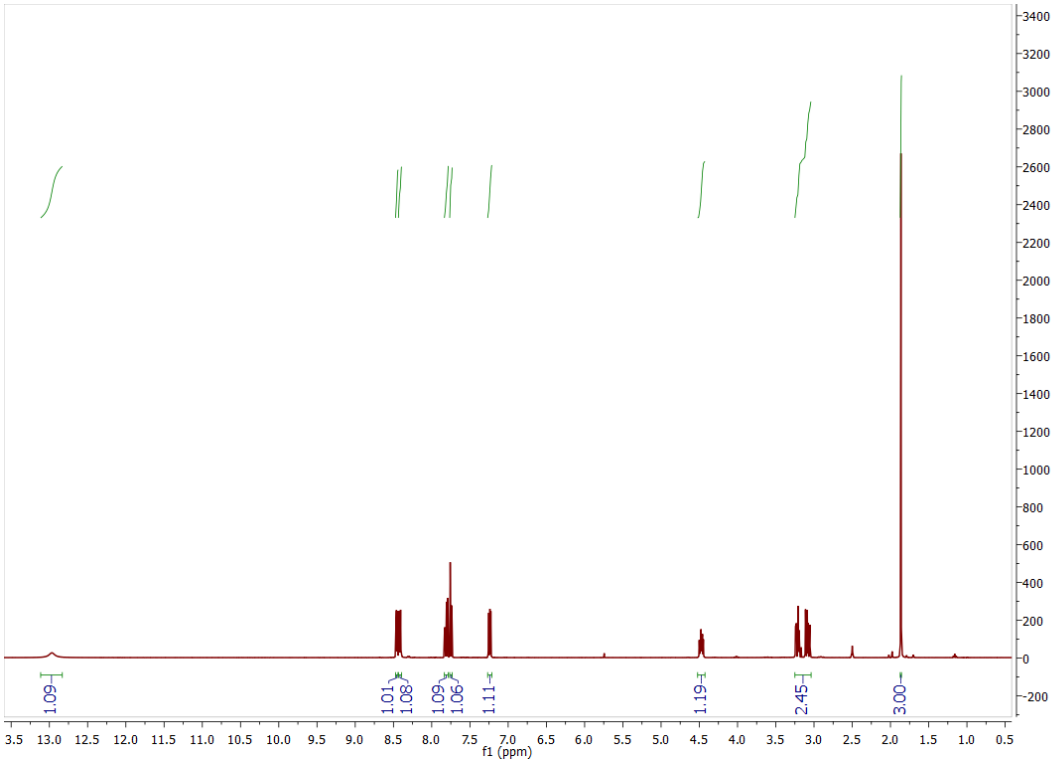


Figure S3. ^{1}H NMR spectrum of **NAC-pyDS**.

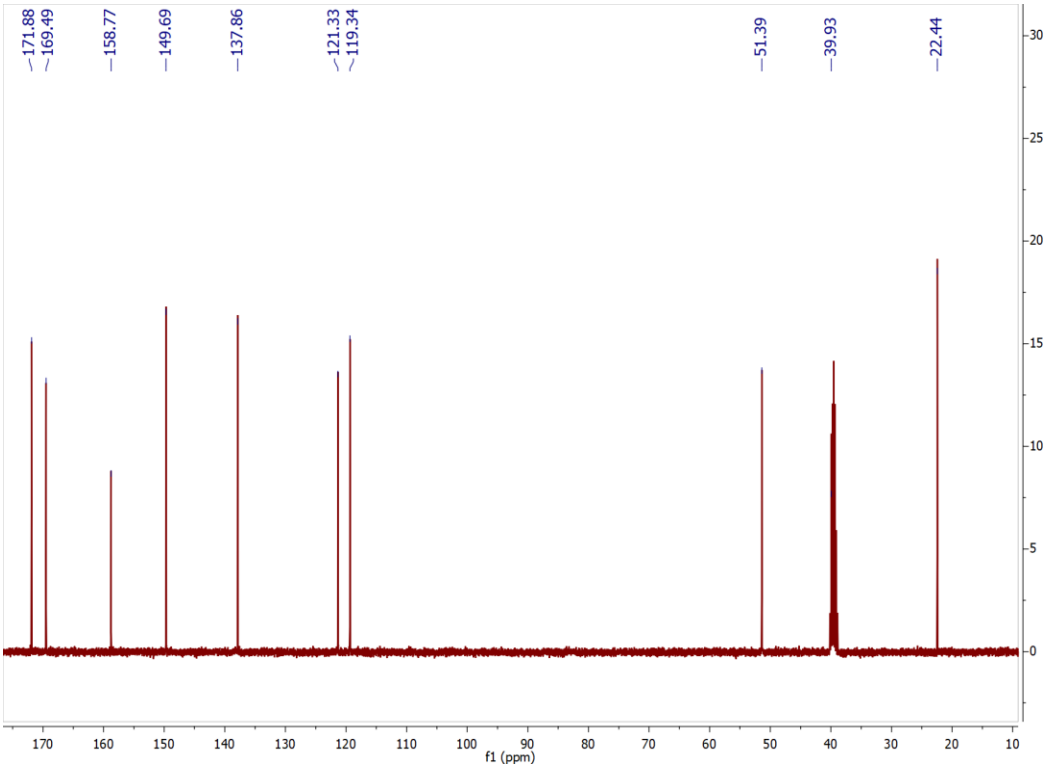
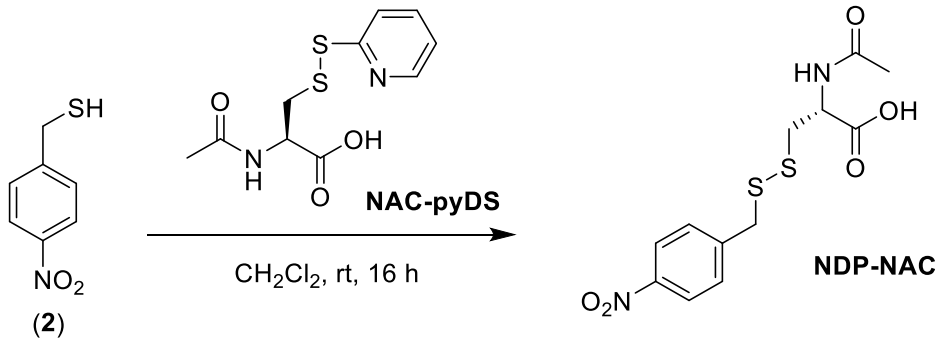


Figure S4. ^{13}C NMR spectrum of **NAC-pyDS**.

Synthesis of **NDP-NAC**



A roundbottom flask was charged with compound **2** (2.12 g, 12.5 mmol), CH_2Cl_2 (50 mL), **NAC-pyDS** (3.41 g, 12.5 mmol), and a stir bar, resulting in a light yellow suspension. The reaction mixture gradually became homogeneous, yielding a bright yellow solution over the course of 16 h. The reaction mixture was then filtered to remove some small particulates and placed in the freezer overnight. The solid that was formed was recovered by filtration and rinsed with cold CH_2Cl_2, resulting in airy, white crystals. (2.30 g, 56 % yield). ^1H NMR (DMSO-d_6) δ 8.29 (d, 1H), 8.20 (d, 2H) 7.61 (d, 2H), 4.44 (m, 1H), 4.11 (m, 2H), 2.92 (m, 1H) 2.75 (m, 1H), 1.85 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 172.4, 169.9, 147.1, 146.4, 130.9, 124.0, 51.5, 40.9, 39.7, , 22.8. HRMS (ESI-TOF) calcd. for $C_{12}H_{14}N_2O_5S_2$ $[M-H]^-$ 329.0266, found 329.0270.

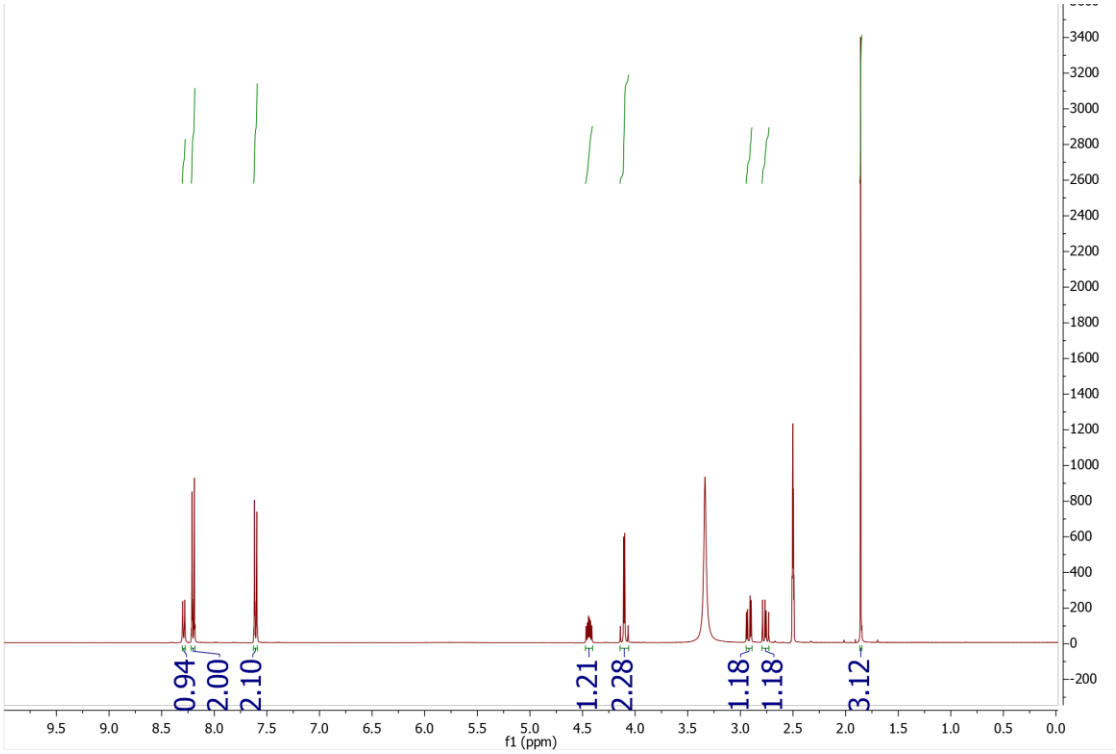


Figure S5. ^{1}H NMR spectrum of **NDP-NAC**.

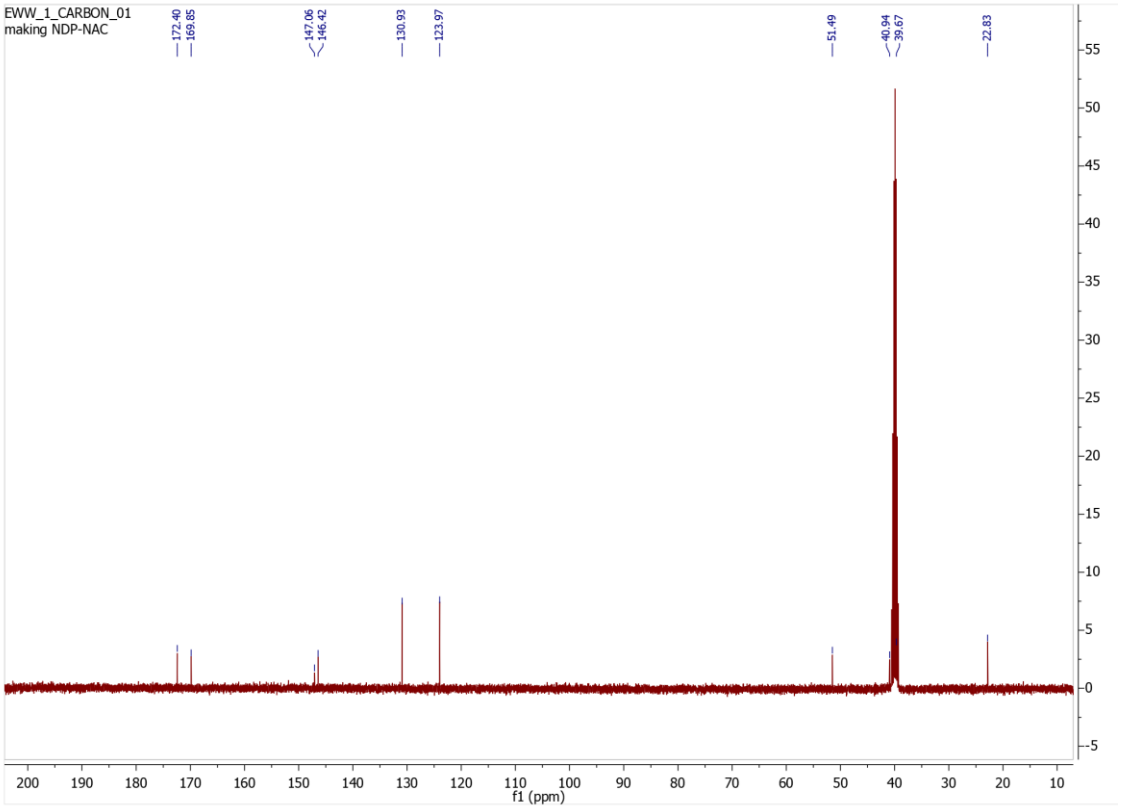
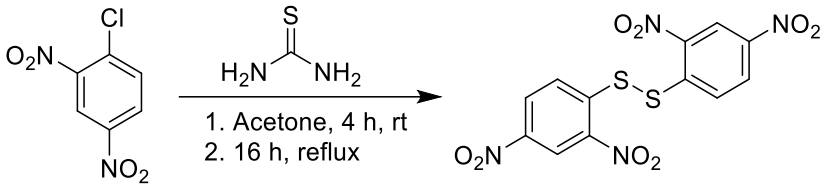


Figure S6. ^{13}C NMR spectrum of **NDP-NAC**.

Synthesis of 2,4-dinitrobenzene disulfide (DNBD)



2,4-Chlorodinitrobenzene (5.00 g, 24.7 mmol) and thiourea (1.88 g, 24.7 mmol) were added to a round bottom flask charged with a stir bar. Acetone (50 mL) was added, and the reaction mixture was allowed to stir for 4 h. The resulting pale yellow solution was refluxed overnight for 16 hours, yielding a bright yellow precipitate. Acetone was removed via rotary evaporation, and the yellow solid was taken up in EtOH (absolute). This slurry was stirred vigorously for 15 minutes while heating to reflux, and then the crude product was recovered by hot filtration. The resulting yellow solid (4.20 g, 85 % yield) was used in the next step of synthesis without further purification. ^{1}H NMR (DMSO-d_6) δ 9.12 (s, 1H), 8.39 (d, *J* = 2.2 Hz, 1H) 7.60 (d, *J* = 2.2 Hz, 1H). ^{13}C NMR (DMSO-d_6) δ 148.7, 146.9, 137.5, 135.3, 128.1, 121.0.

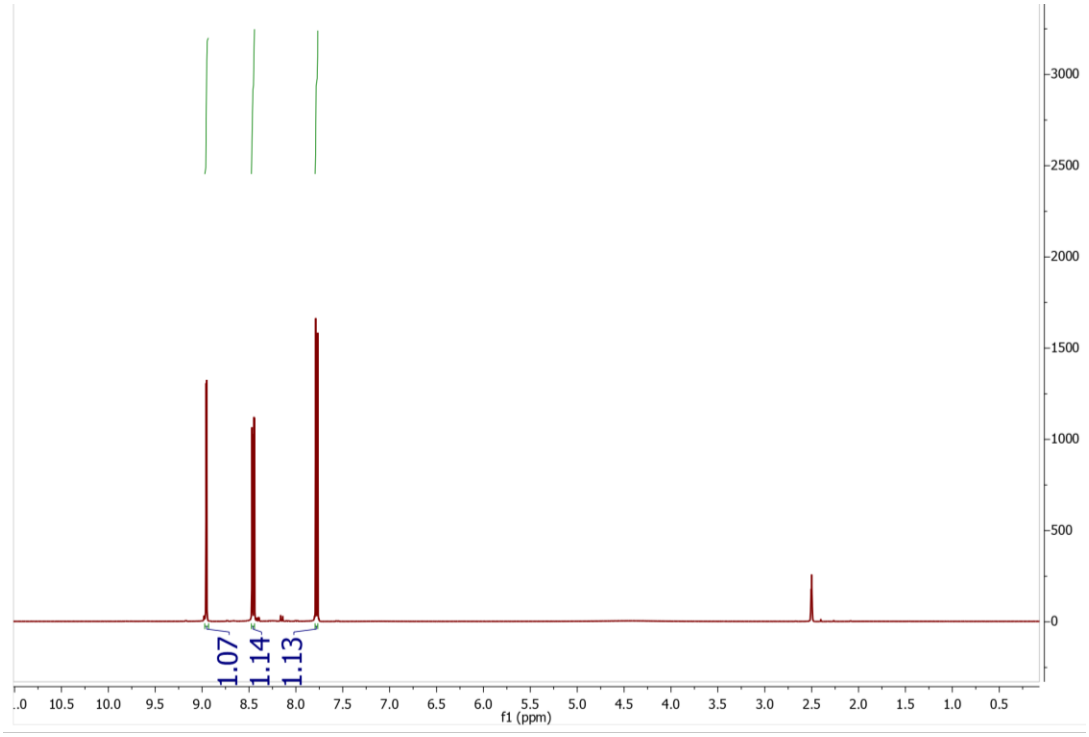


Figure S7. ^{1}H NMR spectrum of DNBD.

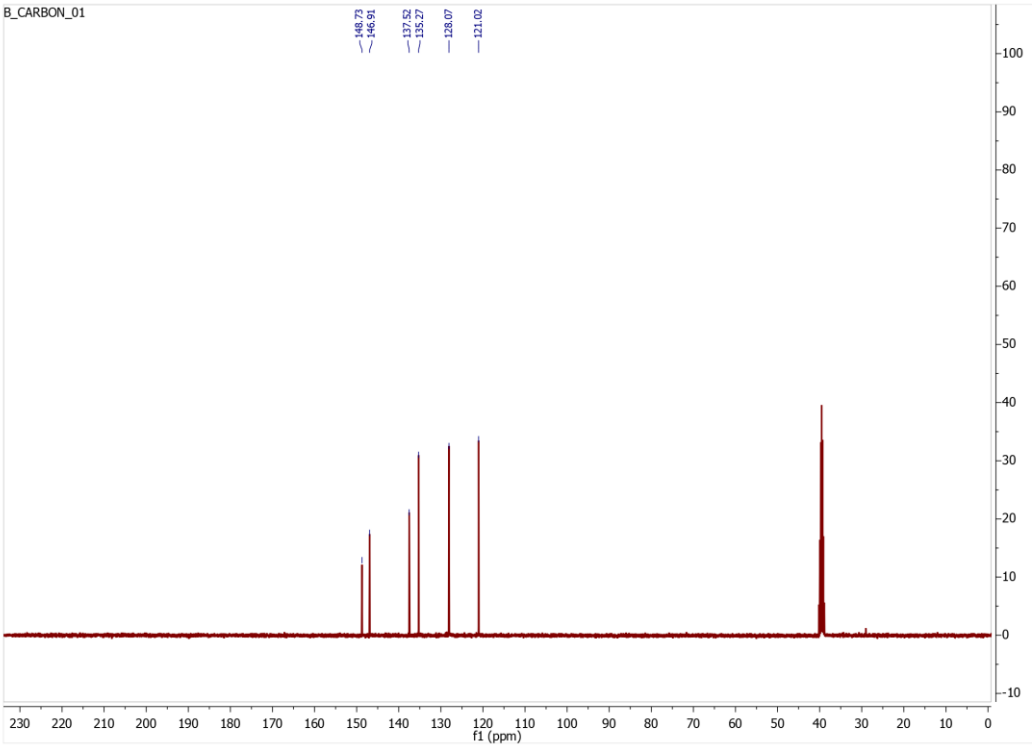
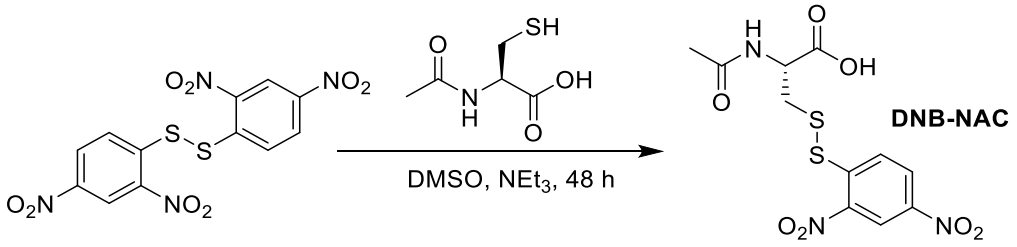


Figure S8. ^{13}C NMR spectrum of DNBD.

Synthesis of persulfide adduct DNB-NAC



2,4-Dinitrobenzene disulfide (500 mg, 1.26 mmol) and NET_3 (0.37 mL, 2.64 mmol) were added to a two-neck round bottom flask charged with a stir bar. DMSO (8 mL) was then added, and the reaction mixture was degassed for 20 min. The reaction mixture was placed under nitrogen flow and stirred until homogeneous, resulting in a yellow solution. NAC (205 mg, 1.26 mmol) was then added under nitrogen flow, and the reaction vessel was sealed and stirred for two d, yielding a bright red solution. The solution was then concentrated, precipitated into CH_2Cl_2 (50 mL), and filtered. The resulting bright orange solid was then redissolved in a minimal amount of methanol (2 mL) and re-precipitated twice more from CH_2Cl_2. The resulting pale orange solid was dissolved in 50 mL 1:1 CH_3CN:H_2O and purified using reverse-phase preparation HPLC. HPLC was performed using an Agilent Technologies 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a fraction collector. Separations were performed using an Agilent PLRP-S column (100 Å, 10 µm, 150 × 25 mm) monitoring at 220 nm. The compound was eluted using a solvent gradient of 2% CH_3CN in H_2O to 100% CH_3CN over 30 min, with 0.1% NH_4OH added to each mobile phase to aid in solubility. A 50 mg injection of crude product yielded 4 mg yield (8%) of a pale yellow powder. ^1H NMR (DMSO-d_6) δ 8.60 (s, 1H), 8.39 (d, 1H) 8.14

(d, 1H), 8.72 (d, 1H), 4.36 (m, 1H) 3.54 (d, 1H), 1.65 (s, 3H). HRMS (ESI-TOF) calcd. for $C_{11}H_{12}N_3O_7S_2$ $[M+H]^+$ 362.0096, found 362.0071

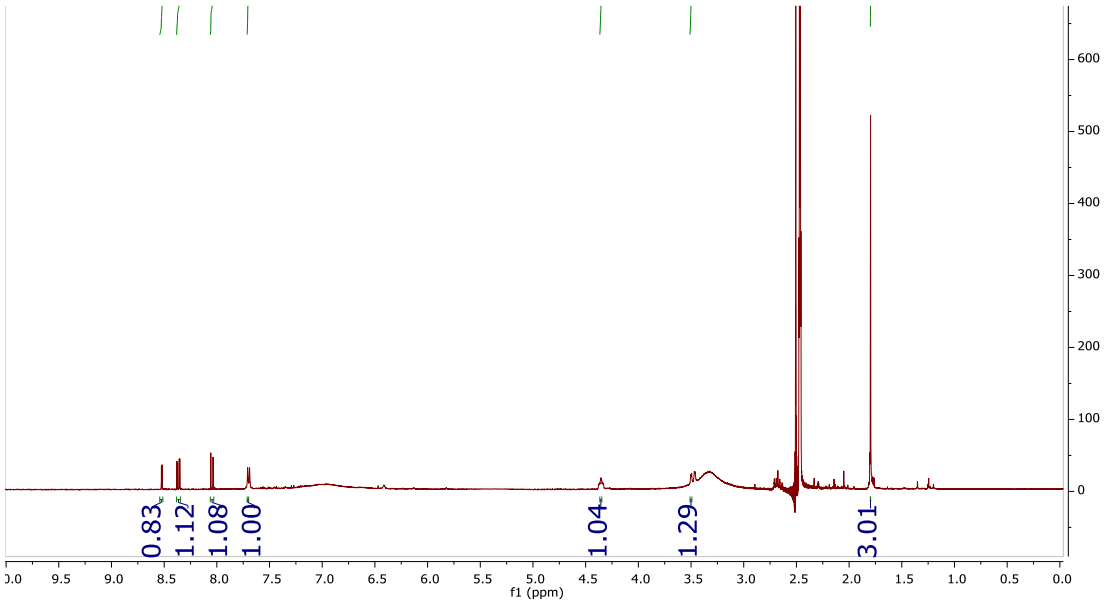


Figure S9. ^1H NMR spectrum of **DNB-NAC**.

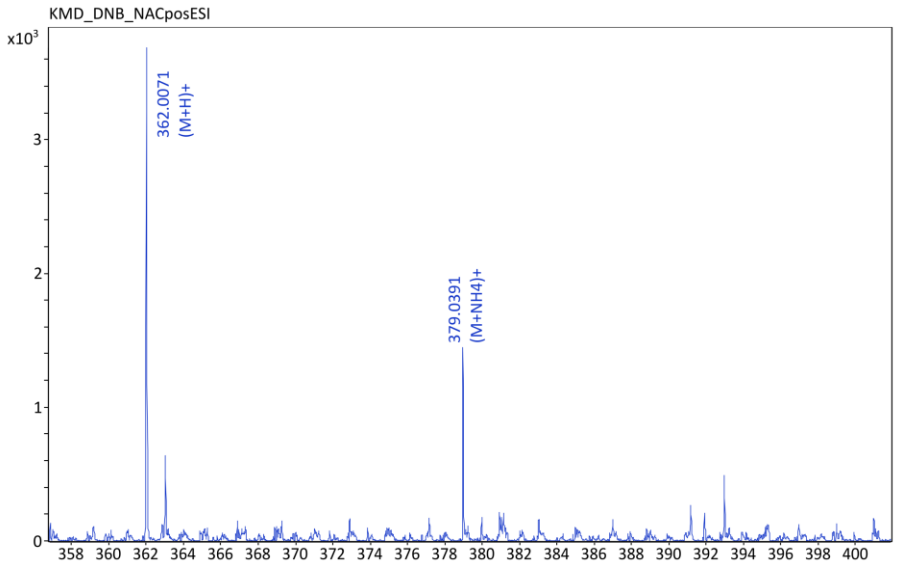
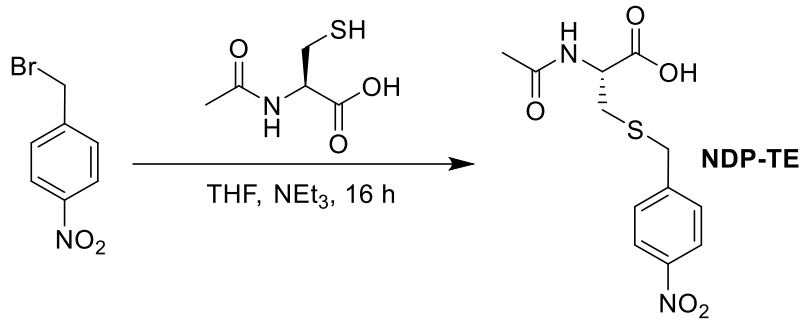


Figure S10. HRMS (ESI-TOF) calcd. For **DNB-NAC**. $C_{11}H_{12}N_3O_7S_2$ $[M+H]^+$ 362.0096, found 362.0071

Synthesis of NDP-NAC-thioether (**NDP-TE**)



To a two-neck round bottom flask charged with a stirbar was added 4-(nitrobenzyl) bromide (1.00 g, 4.63 mmol) and NET_3 (0.97 mL, 6.94 mmol). THF (10 mL) was then added, and the reaction mixture was degassed for 20 min. The reaction mixture was placed under nitrogen flow and stirred until homogeneous, resulting in a yellow solution. **NAC** (906 mg, 5.55 mmol) was then added under nitrogen flow, and the reaction vessel was sealed and stirred for 16 h, yielding a suspension of white triethylamine salts. Reaction progress was monitored by TLC in CH_2Cl_2. The salts were filtered off, and the solvent was removed via rotary evaporation. The resulting off-white solid was then taken up in 1:1 CH_2Cl_2: EtOAc and purified via column chromatography using gradient elution from 1:1 CH_2Cl_2: EtOAc to 100% EtOAc. The product, a colorless, crystalline solid, was isolated in a 73% yield (1.00 g). ^{1}H NMR (DMSO-d_6) δ12.84 (s, 1H), 8.27 (d, *J* = 4.6 Hz, 1H), 8.19 (d, *J* = 2.1 Hz, 2H) 7.55 (d, *J* = 2.1 Hz, 2H), 4.38 (m, 1H), 3.88 (s, 2H), 2.78 (m, 1H), 2.66 (m, 1H), 1.87 (s, 3H). ^{13}C NMR (DMSO-d_6) δ172.11, 169.37, 146.8, 146.43, 130.12, 123.59, 51.65, 34.62, 32.44, 22.37. HRMS (ESI-TOF) calcd. for $C_{12}H_{15}N_2O_5S_1$ $[M+H]^+$ 299.0701, found 299.0704.

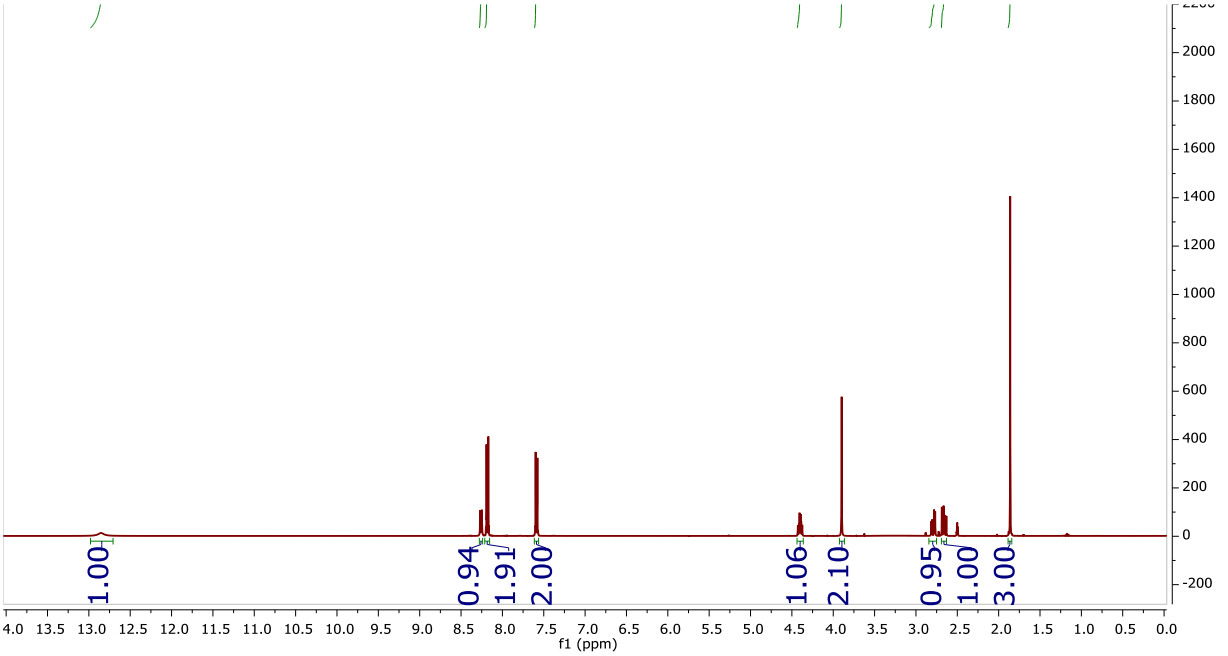


Figure S11. ^{1}H NMR spectrum of **NDP-TE**

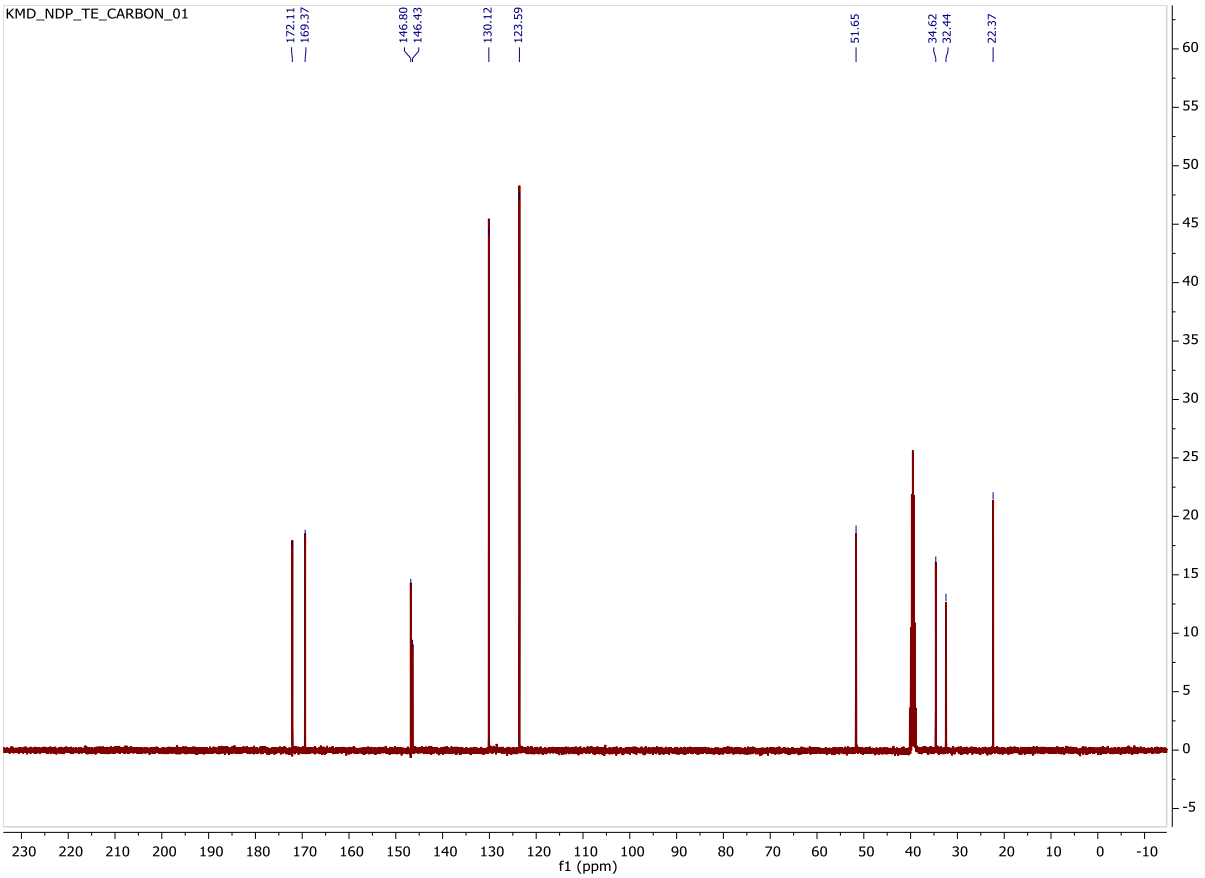
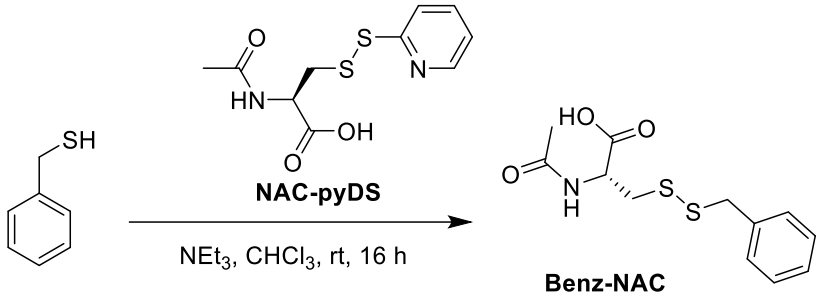


Figure S12. ^{13}C NMR spectrum of **NDP-TE**

Synthesis of benzyl-NAC disulfide (**Benz-NAC**)



A single neck round bottom flask was charged with **NAC-pyDS** (0.7 g, 2.4 mmol) and $CHCl_3$ (5 mL) to form a suspension. Benzyl mercaptan (0.26 mL, 2.0 mmol) and NET_3 (0.42 mL, 3.0 mmol) were added sequentially via syringe. The reaction mixture was stirred for 4 h at rt, monitoring reaction progress by TLC (25 % EtOAc in hexanes, UV visualization). Once complete conversion was observed, the reaction mixture was diluted with $CHCl_3$ (10 mL), washed successively with 1 N HCl (2 x 10 mL) and brine (10 mL), dried over Na_2SO_4, and concentrated by rotary evaporation. The resulting yellow powder was purified by silica gel chromatography eluting with a gradient of 0% to 15 % MeOH in CH_2Cl_2, yielding an off-white solid (0.356 g, 62 % yield). ^{1}H NMR (DMSO-d_6): δ 12.94 (s, 1H), 8.30 (d, J = 4.7, 1H), 7.37-7.23 (m, 5H), 4.46 (m, 1H), 3.97 (m, 2H), 2.99-2.71 (m, 2H), 1.86 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 172.10, 169.39, 137.37, 129.30, 128.44, 127.31, 51.25, 41.92, 39.45, 22.45. HRMS (ESI-TOF) calcd. for $C_{12}H_{16}NO_3S_2$ $[M+H]^+$ 286.0571, found 286.0567. Calcd. for $C_{12}H_{15}NNaO_3S_2$ $[M+Na]^+$ 309.0414, found 309.0419.

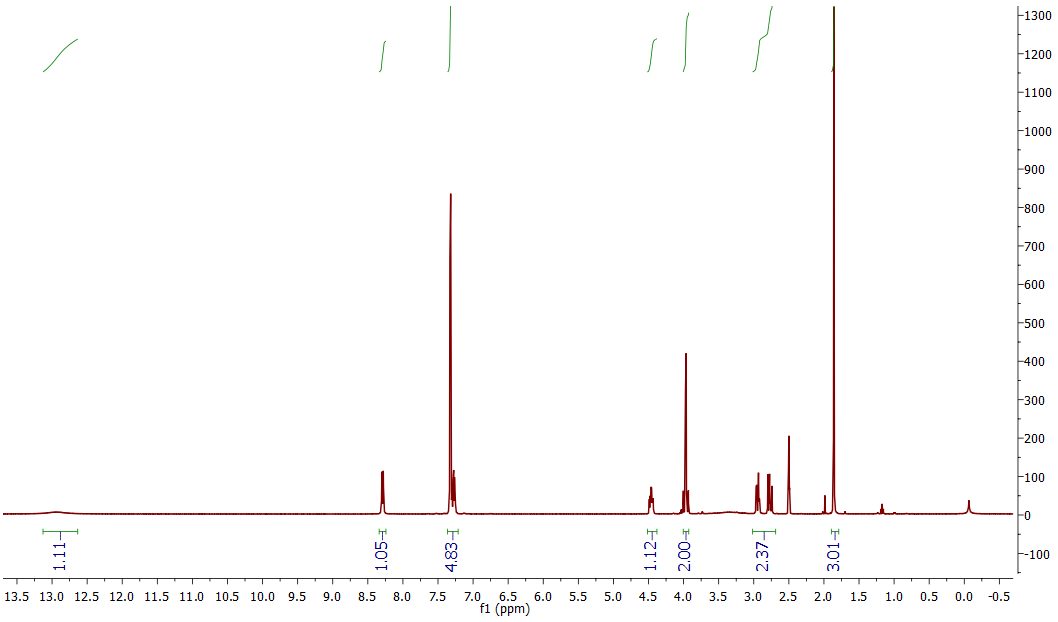


Figure S13. ^{1}H NMR spectrum of **Benz-NAC**

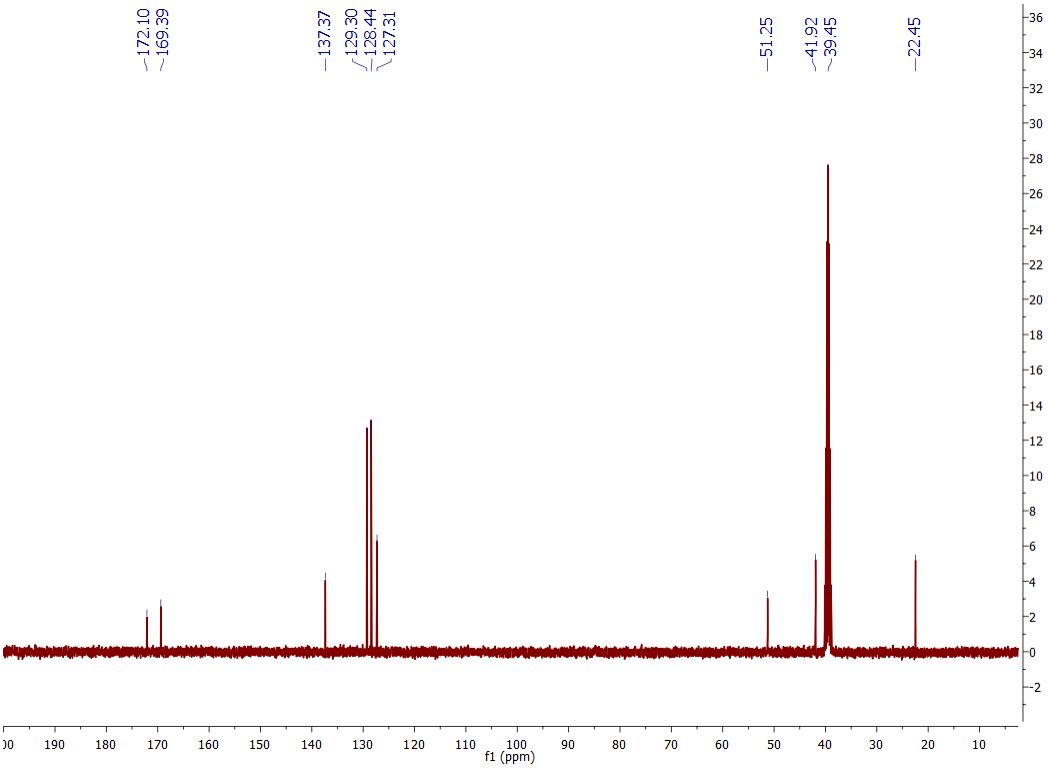


Figure S14. ^{13}C NMR spectrum of **Benz-NAC**

Persulfide Trapping:

Analysis of persulfide release by LCMS was carried out as follows: A one-dram vial was charged with a solution of NDP-NAC (100 µL; 1 mM in HPLC grade CH_3CN), NADH (100 µL, 5 mM in LCMS H_2O), 2,4 -dinitrofluorobenzene (DNFB, 100 µL, 5 mM in HPLC CH_3CN) and diluted with 1X pH 8.0 PBS (made with LCMS H_2O, 0.7 mL) to give a clear, colorless solution. An aliquot (50 µL) was removed and diluted into CH_3CN (0.95 mL) in a vial equipped with a screw cap lid with a rubber septum. After removal of an aliquot to serve as the t=0 timepoint, nitroreductase (10 µL, 1 mg/mL stock) was added to the reaction mixture. The vial was shaken thoroughly, and after 1 min the first aliquot was removed and diluted with CH_3CN (1 mL). This process was repeated every 30 min for 2 h. The aliquots were then cooled in a dry ice/acetone bath until cloudy (~1 min) and centrifuged for 5 min at 2000 rpm. The supernatant was decanted into vials and the (small) pellet that formed (most likely charged impurities, NADH/NAD^+) was discarded. The samples were then analyzed by LCMS as follows: 20 µL injection volume eluting 5 to 95% CH_3CN in water with 10 mM trifluoroacetic acid (TFA) in each mobile phase over the course of 10 min, holding at 95% CH_3CN for 2 min, and then ramping down from 95 to 5% CH_3CN in water over 2 min. This chromatographic cycle was followed by a 5-min column equilibration in 5 % CH_3CN in water. Experiments were run at room temperature (22 °C). The UV detector readout was set to 282 nm. As shown in Figure S11, the peak eluting at 4.0 minutes (NDP-NAC) declines in intensity after addition of nitroreductase. The peak at 6.1 minutes matches the elution time of DNB-NAC under the same LCMS conditions. For these experiments, the pH level during the reaction was 7.8. We did not observe DNFB reduction by *E. coli* nitroreductase under these conditions.

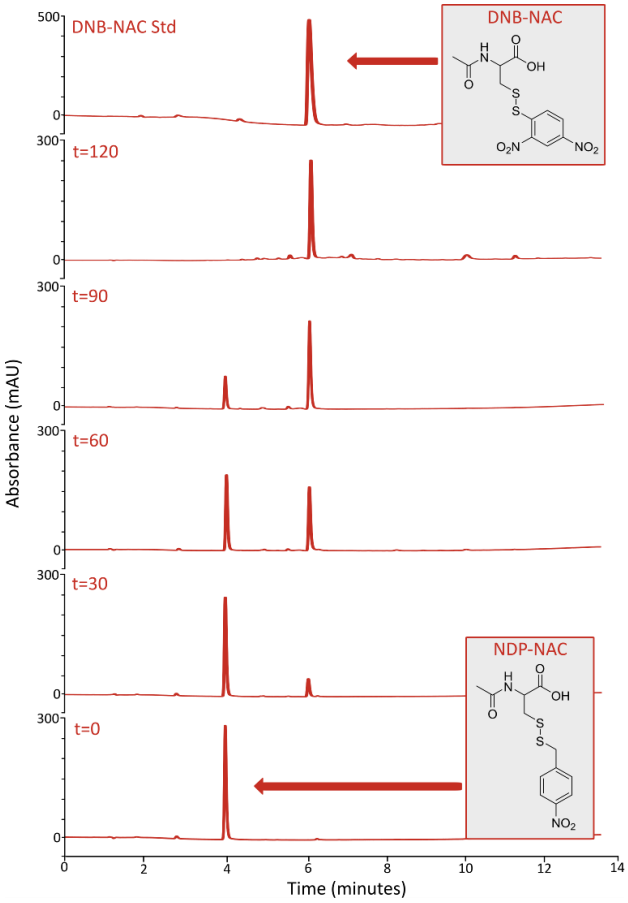


Figure S15. Chromatogram series depicting persulfide trapping from NDP-NAC in response to nitroreductase. The peak eluting at 4.0 minutes corresponds to NDP-NAC, whereas the peak that increases in intensity over time (6.1 minutes) corresponds to the persulfide adduct DNB-NAC standard.

^{1}H NMR *p*-ABA Release Kinetics:

NDP-NAC (1.0 mg) and NADH (5 equiv) were dissolved in 200 µL DMSO-d_6. pD 8.0 dPB (733 µL) was added, and a first ^{1}H NMR time point was collected. Nitroreductase (20 µg dissolved in 67 µL pD 8.0 dPB) was then added, the NMR tube was capped, and subsequent ^{1}H NMR scans were performed at intervals described in Figure 2. Experiments were conducted at room temperature (22 °C) and the pD level during the reaction was pD = 8.1.

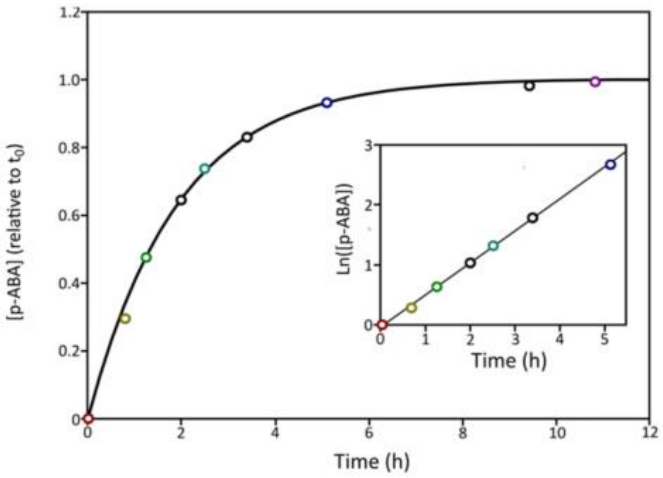


Figure S16. Pseudo-first order plot of ^{1}H NMR release kinetics depicting pABA release from NDP-NAC.

Thiol-disulfide exchange control experiments:

NDP-NAC (2 mg/mL) and Cys (2 eq) were dissolved in 1:4 DMSO-d_6:pD 8.0 dPB or 1:4 DMSO-d_6:pD 2.5 dPB. Under both mildly basic and strongly acidic conditions, no substantial changes to the ^{1}H NMR spectra were observed after 24 h at rt. However, Cys oxidation to cystine was observed.

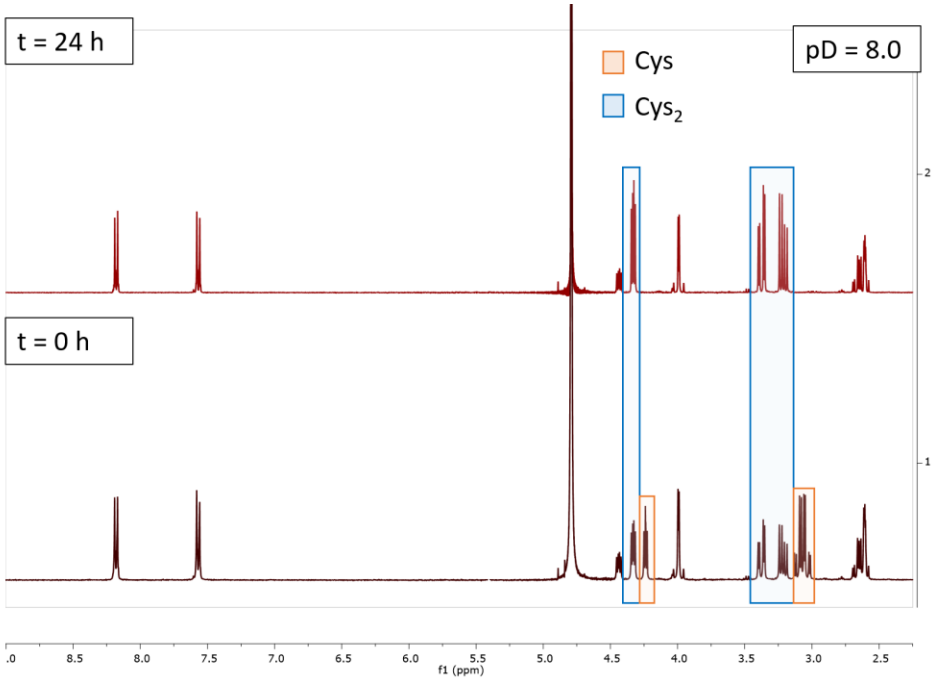


Figure S17. Thiol-disulfide exchange control experiment utilizing NDP-NAC and Cys at pD 8.0. No change in signals attributed to NDP-NAC (not boxed) were observed, although oxidation of Cys to cystine (Cys_2) was noted (boxed peaks).

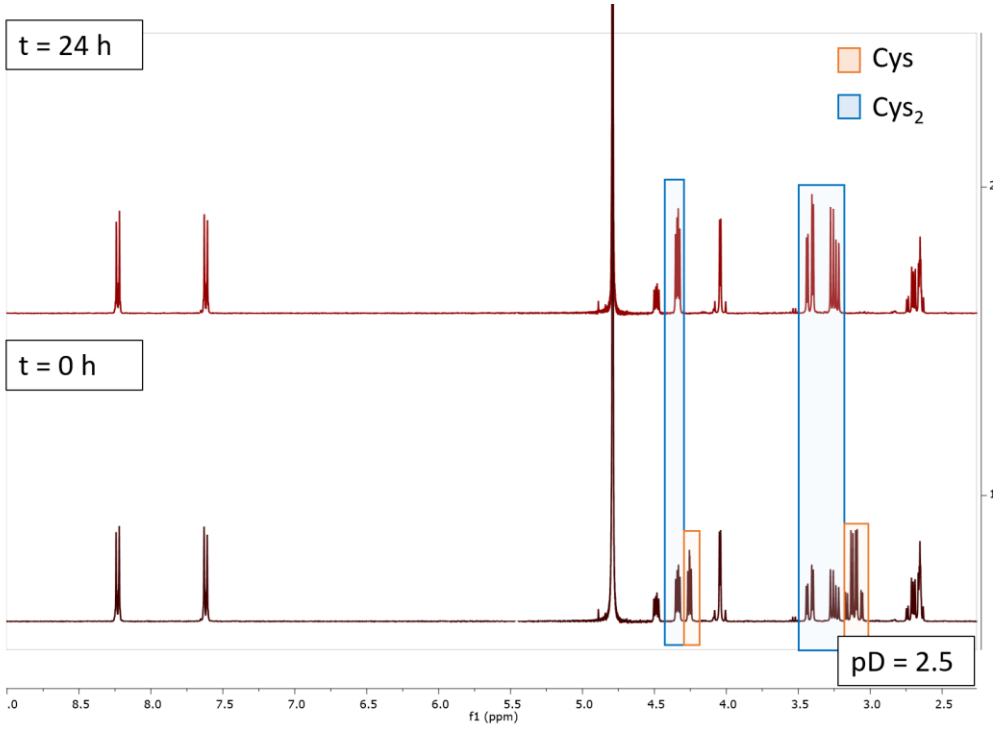


Figure S18. Thiol-disulfide exchange control experiment utilizing NDP-NAC and Cys at pD 2.5. No change in signals attributed to NDP-NAC (not boxed) were observed, although oxidation of Cys to cystine (Cys_2) was noted (boxed peaks).

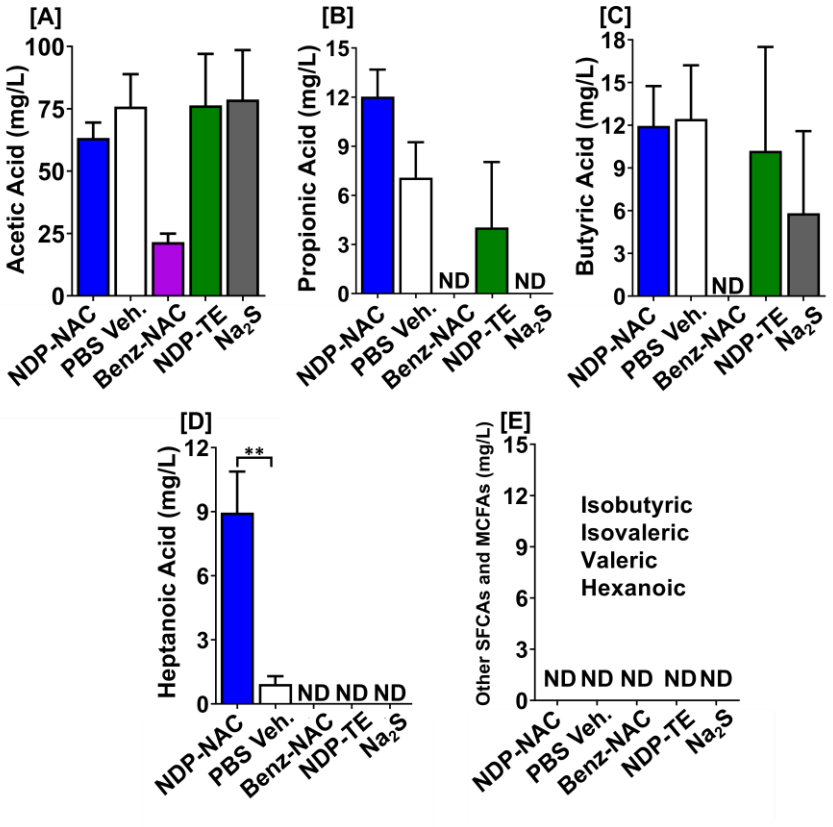


Figure S19. Short and medium chain fatty acid profiles (SCFA and MCFA) of mouse excrement after daily treatment with NDP-NAC or indicated control. ** designates $p < 0.01$ for comparisons between treatment groups indicated with brackets. Isobutyric, isovaleric, valeric, and hexanoic acid concentrations were below the level of detection, which is typical for these types of experiments. ND = not detected (below limit of detection, LOD). Acetic acid LOD = 2 mg/mL, LOD = 1 mg/mL for all other SCFA/MCFAs. Statistical analysis was performed using ANOVA with Tukey's *post-hoc* test.

H9C2 Cell Viability Assays:

For eukaryote experiments, H9C2 cells were plated at a density of 5000 cells per well in a volume of 180 µL serum-containing media per well in a 96-well plate and cultured for 24 h before treatment. Cell viability data was analyzed using a BioTek Synergy Mx plate reader (BioTek, Winooski, VT). Cell viability assays were performed by using Cell counting kit 8 (CCK-8, Dojindo, Rockville, Md.). H9C2 cardiomyocytes in a 96-well plate (n = 5 for each group) were treated with various concentrations of NDP-NAC and allowed to age 4 h in serum-containing media. After incubation with NDP-NAC or controls for an additional 4 h, the cells were then washed with 1X PBS buffer three times. After washing, fresh DMEM (100 µL) without FBS and 10 v/v % CCK-8 solution was added, and the cells were incubated for 3 h. Absorbance was recorded then at 450 and 750 nm.

Bacterial Cell Viability Assays:

Escherichia coli, Listeria monocytogenes, and Staphylococcus aureus were acquired and validated from the American Type Culture Collection and cultured following standard procedures defined by the vendor. Sub-cultures were streaked on nutrient agar containing either 0, 1, 10, or 100 µg/ml of NDP-NAC. Percent viability was determined by colony forming unit assessments.

Ultra High Performance Liquid Chromatography with Tandem Mass Spectrometry (UPLC-MS/MS) analysis of NDP-NAC in mouse plasma

Mouse plasma samples were run at the VA-MD College of Veterinary Medicine Analytical Research Laboratory by McAlister Council-Troche. Plasma concentrations of NDP-NAC and *p*-aminobenzyl alcohol (pABA) were determined by UPLC-MS/MS after protein precipitation with 5% (v/v) formic acid (FA) in CH_3CN.

NDP-NAC, pABA, and NAC-pyDS were used as reference standards, with NAC-pyDS being used as the internal standard (IS). Stock solutions of both compounds were initially made up in CH_3CN and then separately diluted in CH_3CN to their final standard concentrations.

Plasma samples were prepared by combining 100 µL of plasma, 20 µL of NAC-pyDS (200 ng/mL in CH_3CN), and 300 µL of CH_3CN with 5% FA in 0.6 mL microcentrifuge tubes. The protein-precipitated samples were briefly shaken and then vortexed for 30 sec before being centrifuged (Eppendorf Microcentrifuge Model 5415R) at 16,100 g for 10 min. The resulting supernatant solutions were then transferred to fresh 4 mL glass vials and dried under nitrogen at 40 °C. The dried samples were then reconstituted in 200 µL of 10/90/1 CH_3CN/H_2O/FA, and then vortexed for 5 min to completely dissolve the sample residue. The reconstituted samples were then transferred to fresh 0.6 mL microcentrifuge tubes and centrifuged for another 10 min at 16,100 g. The supernatants were then transferred to 2 mL amber vials with low volume inserts before being placed in the refrigerated autosampler of the UPLC-MS/MS for analysis.

Sample extracts were subjected to chromatographic separation performed on a Waters H-Class UPLC system with a Phenyl column (Waters Acquity UPLC BEH Phenyl, 100 mm length x 2.1 mm ID x 1.7 µm) and matching guard column (Waters Acquity UPLC BEH Phenyl VanGuard Pre-Column, 5 mm length x 2.1 mm ID x 1.7 µm) maintained at 40 °C. 5 µL sample was injected onto the column using a refrigerated autosampler maintained at 8 °C. Mobile phase A consisted of 1% (v/v) FA in H_2O, and mobile phase B consisted of 1% (v/v) FA in CH_3CN. The mobile phase was delivered to the UPLC column at a flow rate of 0.4 mL/min. The gradient elution is shown below in Table S1.

Time (min)	%A (1%FA in H_2O)	%B (1%FA in CH_3CN)
0.00	98	2
0.25	98	2
3.25	2	98
3.75	2	98
3.76	98	2
5.75	98	2

Table S1. UPLC gradient method used for the chromatographic separation of NDP-NAC, pAB, and NAC-pyDS.

In order to keep the MS clean, the divert valve was used to transfer the column effluent to the MS from 0.5 to 3.75 min. From 0 to 0.5 and 3.75 to 6.0 minutes, all the column effluent was transferred to waste. The retention times of pABA, NDP-pyDS, and NDP-NAC were approximately 0.75, 3.17, and 3.18 minutes, respectively. The UPLC column effluent was pumped directly without any split into a triple-quadrupole mass spectrometer (Waters Xevo TQD) equipped with a Z Spray ionization source which was operated in positive-ion electrospray mode (ESI+) using multiple reaction monitoring (MRM). The parent and product ion transitions for the compounds of interest are shown in Table S2.

Analyte	Parent Ion (amu)	Product Ion (amu)	Cone Energy (V)	Collision Energy (eV)	Quant/Qual Transition
pABA	124.0 $[M+H]^+$	94.0	36	14	Quantifier
	124.0 $[M+H]^+$	89.0	36	20	Qualifier 1
	124.0 $[M+H]^+$	77.0	36	22	Qualifier 2
NDP-NAC	331.1 $[M+H]^+$	162.0	22	12	Quantifier
	331.1 $[M+H]^+$	116.0	22	30	Qualifier 1
	331.1 $[M+H]^+$	199.9	22	22	Qualifier 2
NAC-pyDS (IS)	286.0 $[M+H]^+$	162.1	20	10	Quantifier
	286.0 $[M+H]^+$	91.0	20	38	Qualifier 1
	286.0 $[M+H]^+$	116.0	20	32	Qualifier 2

Table S2. MRM transitions and specific mass spectrometry tuning parameters for the quantification of pAB and NDP-NAC.

Commercial software (MassLynx) was used to analyze the data. Tuning was performed on each analyte by direct infusion of standard solution (0.1 ng/µL) at a rate of 10 µL/min. Mass spectrometer parameters used for the detection of pABA, NDP-NAC, and NAC-pyDS are shown in Table S3.

Parameter	Value
Capillary (kV)	3.40
Cone (V)	20
RF (V)	2.50
Extractor (V)	3.00
Source Temperature (°C)	150
Desolvation Temperature (°C)	600
Cone Gas Flow (L/Hr)	10
Desolvation Gas Flow (L/Hr)	1000

Table S3. Mass spectrometer tuning parameters for the detection of pABA, NDP-NAC, and NAC-pyDS.

A six-point calibration curve made up in blank mouse plasma was prepared in the same manner as the samples but was spiked with a range of approximately 2 to 95 ng/mL plasma for both pABA and NDP-NAC.

A linear calibration curve was constructed for both compounds of interest using the MassLynx software to determine analyte concentration in samples based on the sample/IS ratio. The coefficient of determination (R^2) for all curves was >0.99, and all standard values were within ±10% of the expected range. *The system had a limit of detection (LOD) of approximately 0.06 ng NDP-NAC/mL plasma and approximately 0.1 ng* p*ABA/mL plasma, as determined by the signal-to-noise ratio, and the limit of quantification (LOQ), determined by the lowest concentration on a linear regression line of the calibration curve, was 2 ng/mL plasma for both analytes*. The calibration curves used in the NDP-NAC and pABA analyses are shown in Figures S14 and S15, respectively. **Neither NDP-NAC nor pABA were detected in any of the six mouse plasma samples that were tested.**

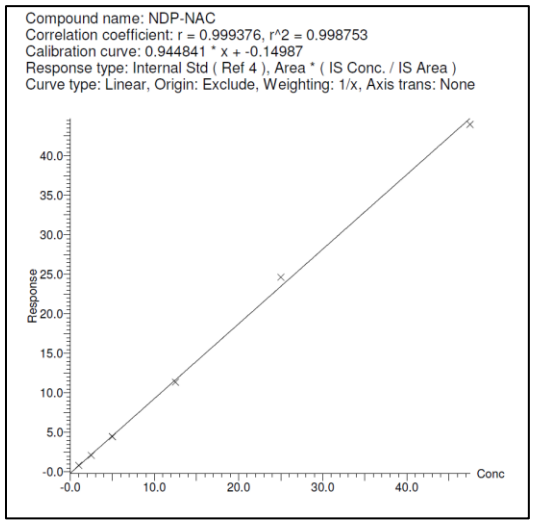


Figure S20. Linear calibration curve used in the analysis of NDP-NAC in mouse plasma. It should be noted that the concentration range on the x-axis is in ng NDP-NAC / mL solution. This is easily converted to plasma concentrations by multiplying by the reconstitution volume (0.2 mL) and dividing by the plasma volume (0.1 mL).

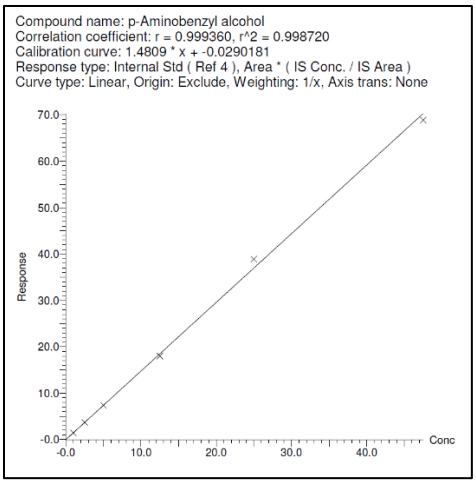


Figure S21. Linear calibration curve used in the analysis of pABA in mouse plasma. It should be noted that the concentration range on the x-axis is in ng pABA/mL solution. This is easily converted to plasma concentrations by multiplying by the reconstitution volume (0.2 mL) and dividing by the plasma volume (0.1 mL).

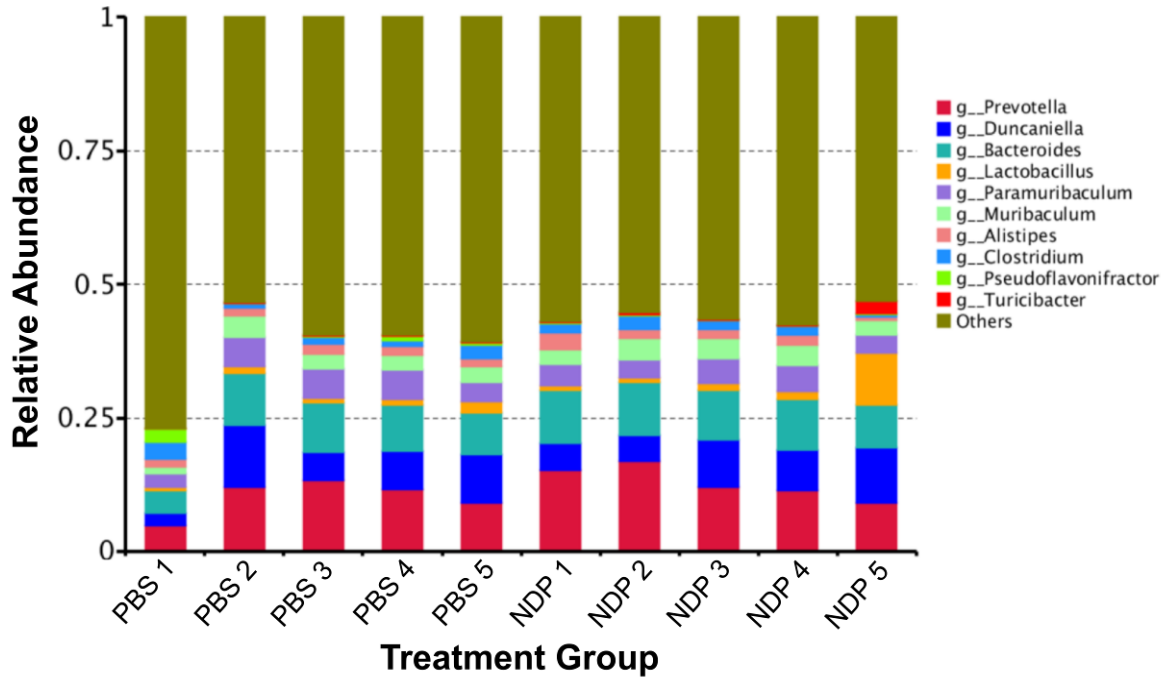


Figure S22. Stacked bar graph depicting overall bacterial populations after treatment with NDP-NAC/vehicle controls and collection and processing of feces. No significant difference in general populations was observed.

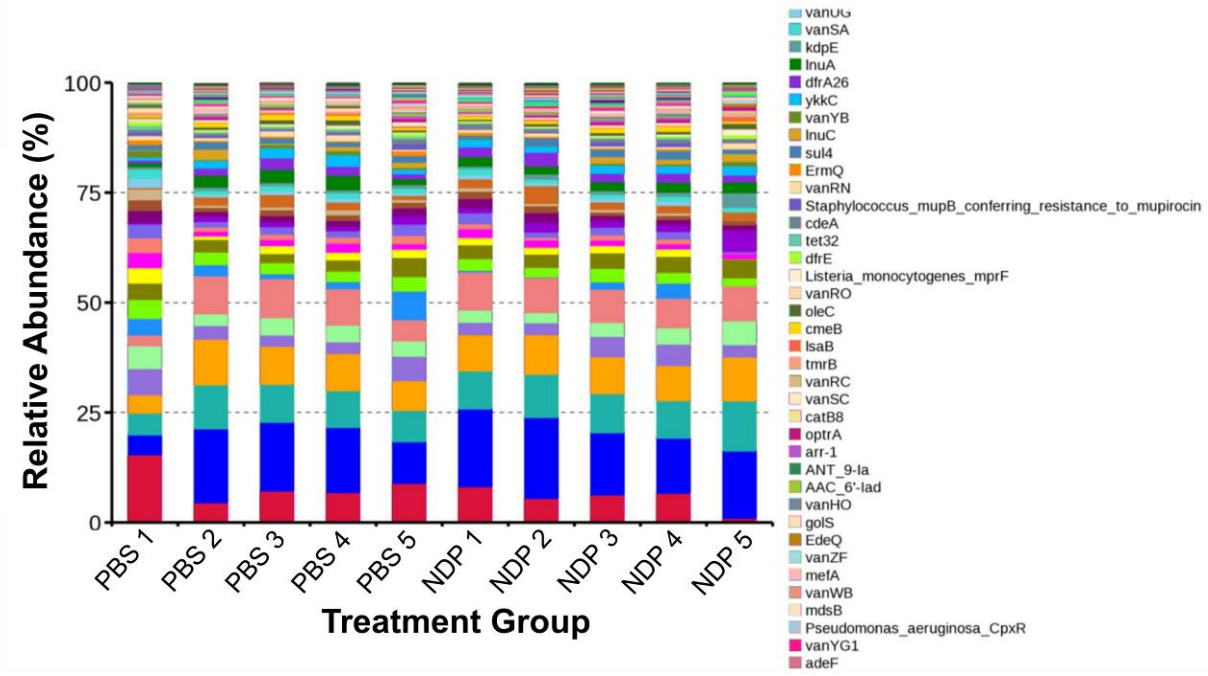


Figure S23. Stacked bar graph showcasing differences in genes associated with antibiotic resistance (ARG) after treatment with NDP-NAC/vehicle controls and collection and processing of feces. No significant difference in ARG observed.

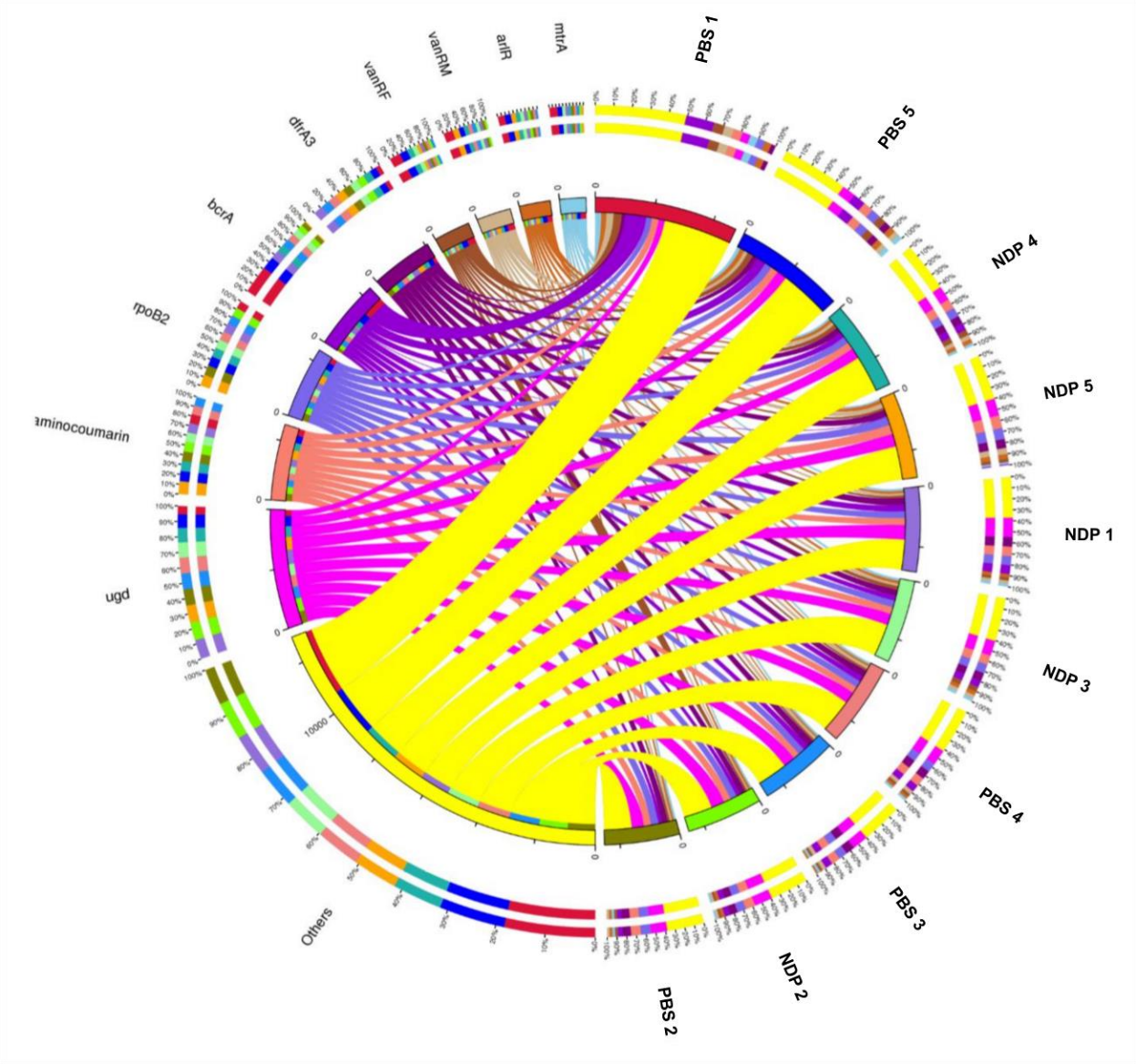


Figure S24. Circle plot showcasing differences in genes associated with antibiotic resistance after treatment with NDP-NAC/vehicle controls and collection and processing of feces. No significant differences in number of ARG observed.

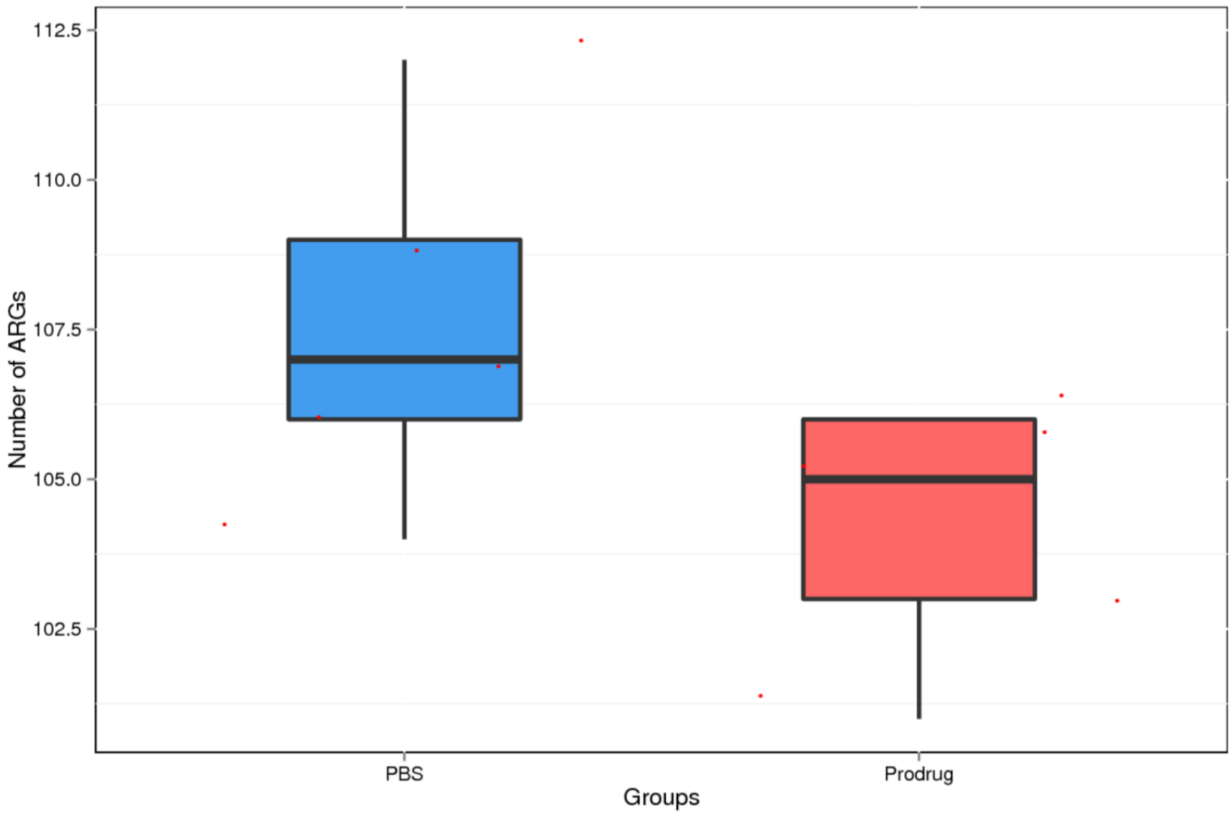


Figure S25. Box plot highlighting average number of ARG in the NDP-NAC treatment group compared to PBS vehicle. ARG in the NDP-NAC treatment group trended downward on average as compared to the PBS vehicle.

References

[1] D. Cantillo, O. de Frutos, J. A. Rincon, C. Mateos, C. O. Kappe, *J. Org. Chem.* **2014**, *79*, 223-229.

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[3] R. S. Navath, B. Wang, S. Kannan, R. Romero, R. M. Kannan, *J. Controlled Release* **2010**, *142*, 447-456.