Supporting Information

Harnessing redox cross-reactivity to profile distinct cysteine modifications

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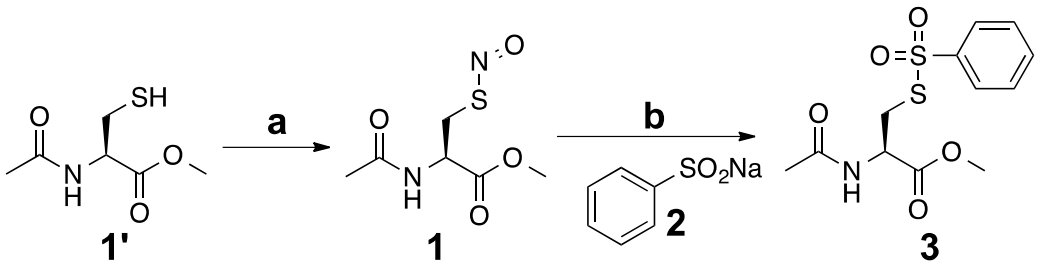
Contents

I. General Procedures and Materials for Experimental Methods

All compounds were purchased from Sigma-Aldrich, unless otherwise noted and were of the highest purity available. NMR analysis was performed using a Varian 400 MHz NMR instrument. NMR integrations and coupling constants were computed using MestreNova. Small molecule high-resolution mass spectrometry was performed using an electrospray Agilent Q-TOF mass spectrometer (accuracy 1-5 ppm) and analyzed using the Agilent MassHunter software suite. Low-resolution mass spectrometry was performed using an electrospray Micromass LCT time-of-flight mass coupled to a HPLC pump with a rheodyne loop injector. Compounds were purified by normal phase flash silica-gel column chromatography or by semi-prep High-Performance Liquid Chromatography (HPLC). HPLC purifications were performed using a Waters semi-preparative 1525 binary pump system coupled to a photodiode array detector, an autosampler, and an automatic fraction collector. Separations were carried out on using the Waters Atlantis prep T3 C_{18} column (10 x 250 mm), in 95/5 water/acetonitrile 0.1% formic acid for 2 minutes, followed by a 40 min gradient increasing the mobile phase to 5/95 water/acetonitrile with 0.1% formic acid. Data were analyzed using the Waters Empower software. Resulting HPLC fractions were lyophilized using a Labconco FreeZone2.5-Plus freeze-drying system. All proteomic experiments were performed after trypsin digestion (MS grade trypsin, Promega), separating the peptides on a Waters nanoAcquity UPLC system fitted with a 5 µM Symmetry C_{18} (180 µm x 20 mm) trap column and a 1.8 µm High Strength Silica (HSS-T3) analytical column (75 µm x 150 mm). Peptides were analyzed on the Waters Synapt G2-S ion mobility time-of-flight mass spectrometer and data processing was performed using the Waters Protein Lynx GLOBAL SERVER software suite coupled with in-house algorithms. Western blots were imaged using the GE Typhoon scanner or the Azure Biosystems C600 imager.

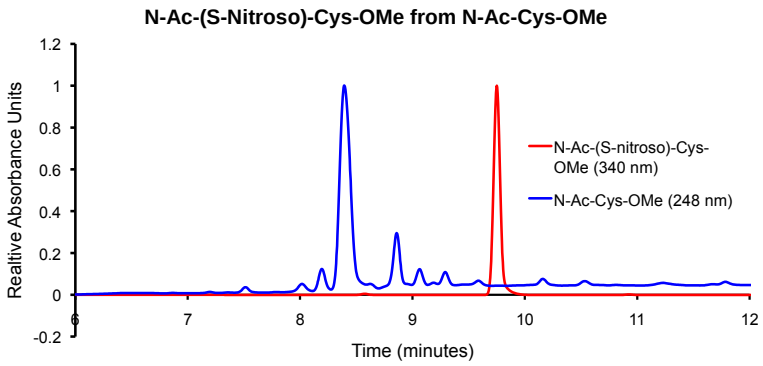
II. Synthetic Protocols and Product Characterization

Synthetic Scheme 1. Synthesis of compound **3**, the thiosulfonate product of reaction between a *N*-acetyl-*S*-nitroso-cysteine methyl ester and phenyl sulfinic acid.



(a) 1N HCl, Methanol, sodium nitrite (b) Deionized water,

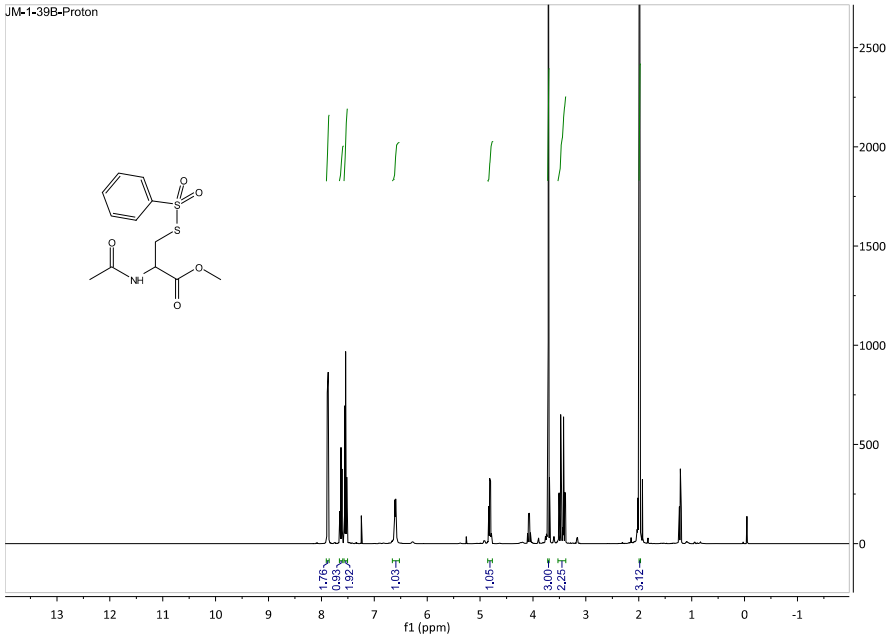
(*R*)-methyl 2-acetamido-3-(nitrosothio)propanoate (1). The nitrosothiol was synthesized based on the method reported earlier[1]. Briefly, an amber round bottom flask was charged with *N*-acetyl-L-cysteine methyl ester (**1'**, 1 eq., 100 mg, 0.56 mmol). The contents were dissolved in methanol (3 mL) and 1 N hydrochloric acid (2 mL), and cooled to 0 °C for 15 minutes. An ice-cold solution of sodium nitrite (1.1 eq., 42.8 mg, 0.62 mmol) in water (1 mL) was then slowly added to the solution of *N*-acetyl-L-cysteine methyl ester in hydrochloric acid and methanol. The reaction mixture was constantly kept in the dark at 0 °C, and allowed to proceed for 30 minutes. HPLC analysis of the crude reaction mixture was performed to monitor completion of the reaction. The crude product was then transferred to an amber separatory funnel and extracted with ethyl acetate (3 x 2 mL). The combined organic extracts were pooled, dried with sodium sulfate (200 mg), filtered and evaporated under vacuum (in the absence of light). This afforded a reddish-pink residue, which was used directly for subsequent reactions within 30 minutes. An HPLC trace of both the starting materials and the *S*-nitroso product is shown below.



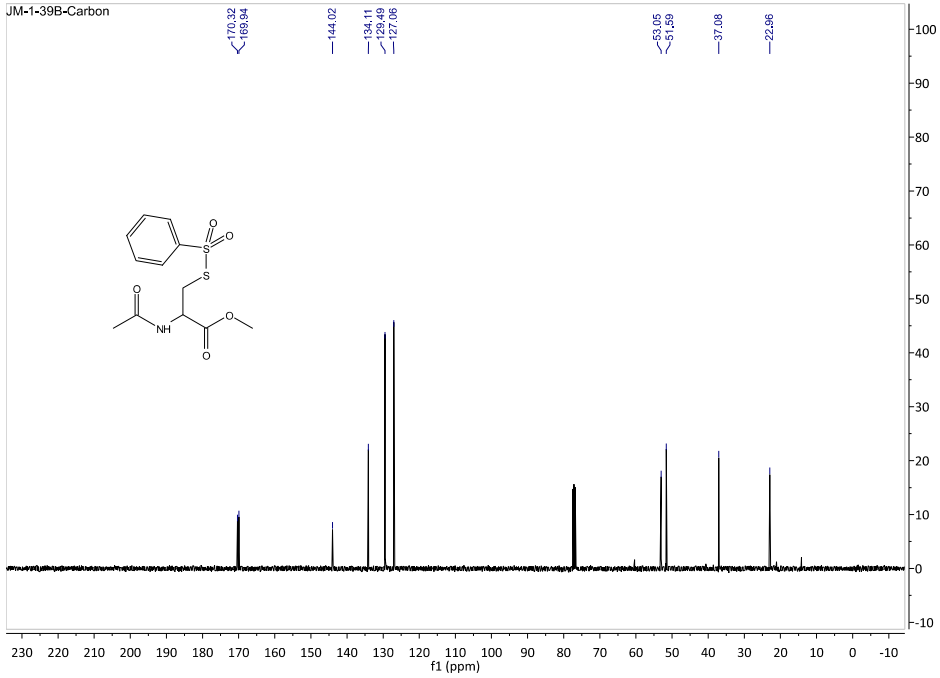
Supplementary Figure A: HPLC trace of *N*-acetyl-cysteine-OMe starting material and the *S*-nitroso product formed upon reaction with sodium nitrite in 1 N HCl and methanol.

(*R*)-methyl 2-acetamido-3-((phenylsulfonyl)thio)propanoate (3). Thiosulfonate compound **3** was synthesized based on a method reported earlier[1, 2]. Briefly, the *N*-acetyl-*S*-nitrosocysteine methyl ester, **1**, obtained in reaction above was dissolved in water followed by addition of sodium benzenesulfinate (**2**, 3 eq., 1.68 mmol, 275.8 mg). The contents were allowed to react for 3 hours at ambient temperature in darkness. The contents were then transferred to a separatory funnel and extracted with ethyl acetate (3 x 10 mL). The organic fractions were pooled, washed with brine (10 mL), dried over sodium sulfate (500 mg), and concentrated under vacuum to afford an oily residue, which was further purified by flash column chromatography (isocratic elution 5% methanol in methylene chloride). This yielded 178 mg (56% over two steps) of thiosulfonate product **3**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 - 7.85 (m, 2H), 7.66 - 7.59 (m, 1H), 7.57 - 7.51 (m, 2H), 6.60 (d, *J* = 7.5 Hz, 1H), 4.81 (dt, *J* = 7.5, 5.1 Hz, 1H), 3.71 (s, 3H), 3.54 - 3.36 (m, 2H), 1.99 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.32, 169.94, 144.02, 134.11, 129.49, 127.06, 53.05, 51.59, 37.08, 22.96. LR-ESI (Pos): *m/z* = 318.04 $[M+H]^+$.

Supplementary Spectra 1 (a) ^{1}H NMR of compound **3**. (b) ^{13}C NMR of compound **3**.

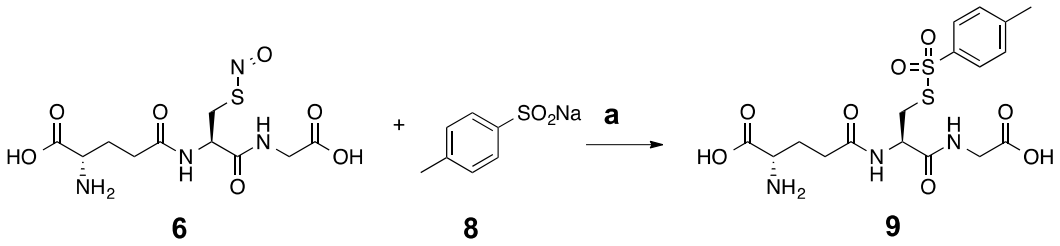


(a) ^{1}H NMR spectrum of (*R*)-methyl 2-acetamido-3-((phenylsulfonyl)thio)propanoate.



(b) ^{13}C NMR spectrum of (*R*)-methyl 2-acetamido-3-((phenylsulfonyl)thio)propanoate.

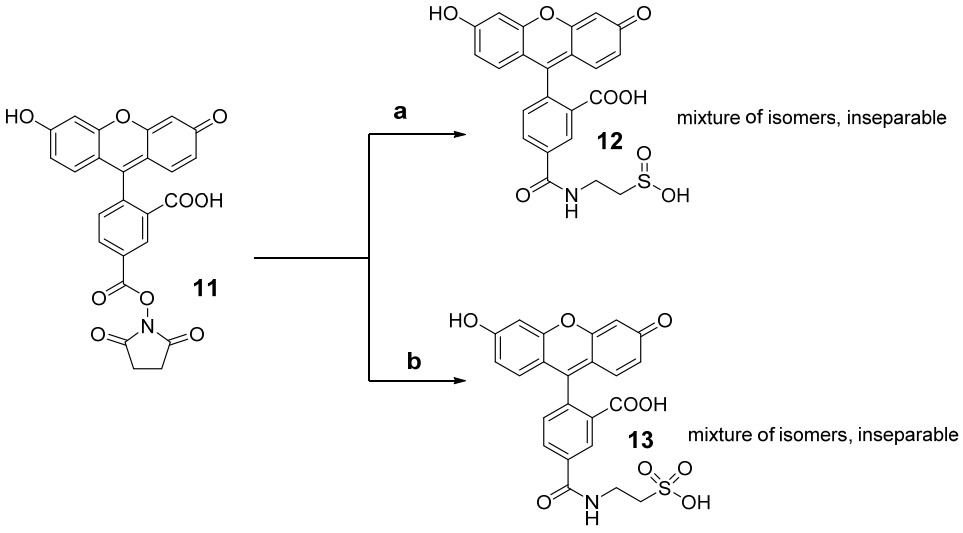
Synthetic Scheme 2. Synthesis of thiosulfonate product **8** from GSNO and 4-methyl-phenyl sulfinic acid.



6 **8** **9**

(*S*)-2-amino-5-(((*R*)-1-((carboxymethyl)amino)-1-oxo-3-(tosylthio)propan-2-yl)amino)-5-oxopentanoic acid (9): Compound **9** was synthesized based on a protocol described above[2]. Briefly, to a cooled solution of *S*-nitroso-glutathione, **6**, (GSNO, Cayman, 1.0 eq., 20 mg, 0.06 mmol) in degassed water, sodium 4-methyl-phenylsulfinate, **8** (3.0 eq., 32 mg, 0.18 mmol) was added allowed to react for 2 hours at ambient temperature in the dark. Compound **9** was purified from the crude reaction by semi-preparative HPLC. ^{1}H NMR (400 MHz, Methanol-d4) δ 7.87 - 7.76 (m, 1H), 7.69 (d, *J* = 8.2 Hz, 1H), 7.43 (d, *J* = 8.2 Hz, 1H), 7.21 (d, *J* = 8.1 Hz, 1H), 4.68 (dd, *J* = 8.1, 5.0 Hz, 1H), 3.90 - 3.84 (m, 2H), 3.79 (t, *J* = 6.3 Hz, 1H), 3.41 (dd, *J* = 14.1, 5.0 Hz, 1H), 3.26 - 3.21 (m, 1H), 2.50 (t, *J* = 7.0 Hz, 2H), 2.44 (s, 3H), 2.35 (s, 2H), 2.17 - 2.07 (m, 2H). HRMS (ESI positive), $[M+H]^+$ Calculated *m/z*: 461.0927, Observed *m/z* = 461.0924.

Synthetic Scheme 3. Synthesis of fluorescein sulfinic acid probe (compound **12**) and the negative control fluorescein sulfonic acid probe (compound **13**).



11 **12** **13**

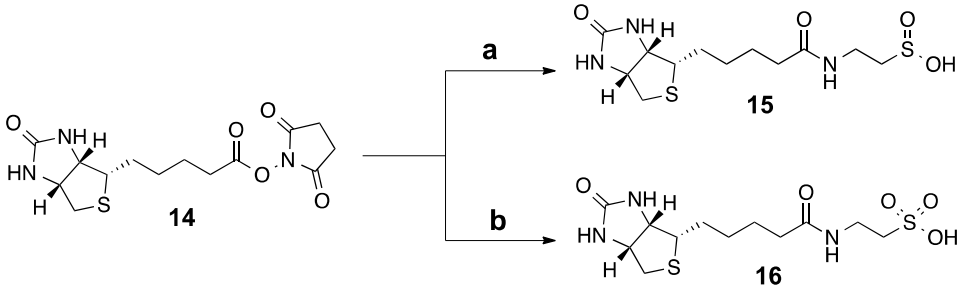
(a) water, THF (1:1), 100μL 0.01N NaOH, Hypotaurine, 4 hours, 0°C, 32%

(b) water, THF (1:1), 100μL 0.01N NaOH, Taurine, 10 hours, 0°C, 54%

2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-((2-sulfinoethyl)carbamoyl)benzoic acid (12). A scintillation vial was charged with a solution of NHS-fluorescein, **11** (Pierce, 1 eq., 7 mg, 0.015 mmol) in degassed THF (1 mL), and the contents were allowed to cool to 0 °C. The atmosphere in the flask was replaced with nitrogen. A solution of hypotaurine (3.0 eq., 4.8 mg, 0.045 mmol) in degassed (sonication, 3 freeze-thaw cycles) 0.01 N sodium hydroxide (100 µL) was then added to the NHS-flourescein. The contents were allowed to react for 4 hours under an atmosphere of nitrogen and progress was monitored by HPLC and mass spectrometry (LR-ESI-Pos). The product, **12**, was purified to homogeneity by semi-preparative HPLC and lyophilized to yield compound **12** as a bright yellow solid in 32% yield. HRMS (ESI Negative), $[M-H]^-$ Calculated: *m/z* = 466.0602, Observed: *m/z* = 466.0570.

2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-((2-sulfoethyl)carbamoyl)benzoic acid (13): A scintillation vial was charged with a solution of NHS-fluorescein, **11**, (Pierce, 1 eq., 15 mg, 0.032 mmol) in THF and the contents were allowed to cool to 0 °C. A solution of taurine (3 eq., 11.9 mg, 0.095 mmol) in 0.01N sodium hydroxide (100 µL) was then added to the NHS-flourescein. The contents were allowed to react for 10 hours and progress was monitored by HPLC and mass spectrometry (LR-ESI-Pos). The product was purified to homogeneity by semi-preparative HPLC and then lyophilized to afford compound **13** as a bright yellow solid in 54% yield. HRMS (ESI Positive), $[M+H]^+$ Calculated: *m/z* = 484.0697, Observed: *m/z* = 484.0693.

Synthetic Scheme 4. Synthesis of biotinylated sulfinic acid probe (**15**) and the negative control biotinylated sulfonic acid probe (**16**).



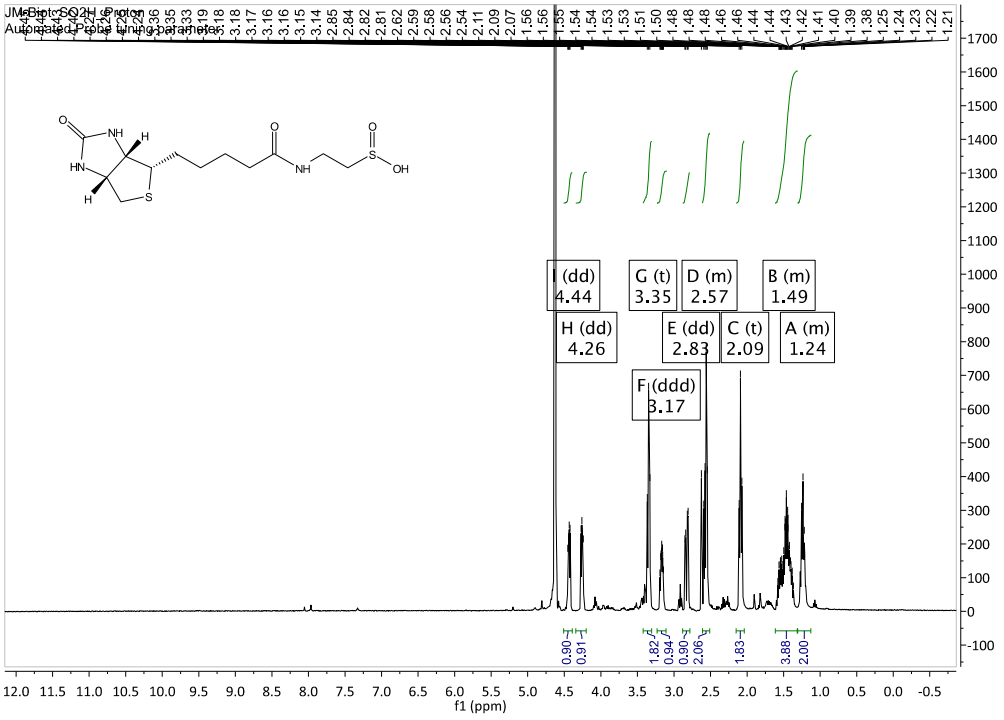
(a) water, THF (1:1), 100µL 0.01N NaOH, Hypotaurine, 4 hours, 0°C, 24%

(b) water, THF (1:1), 100µL 0.01N NaOH, Taurine, 10 hours, 0°C, 43%

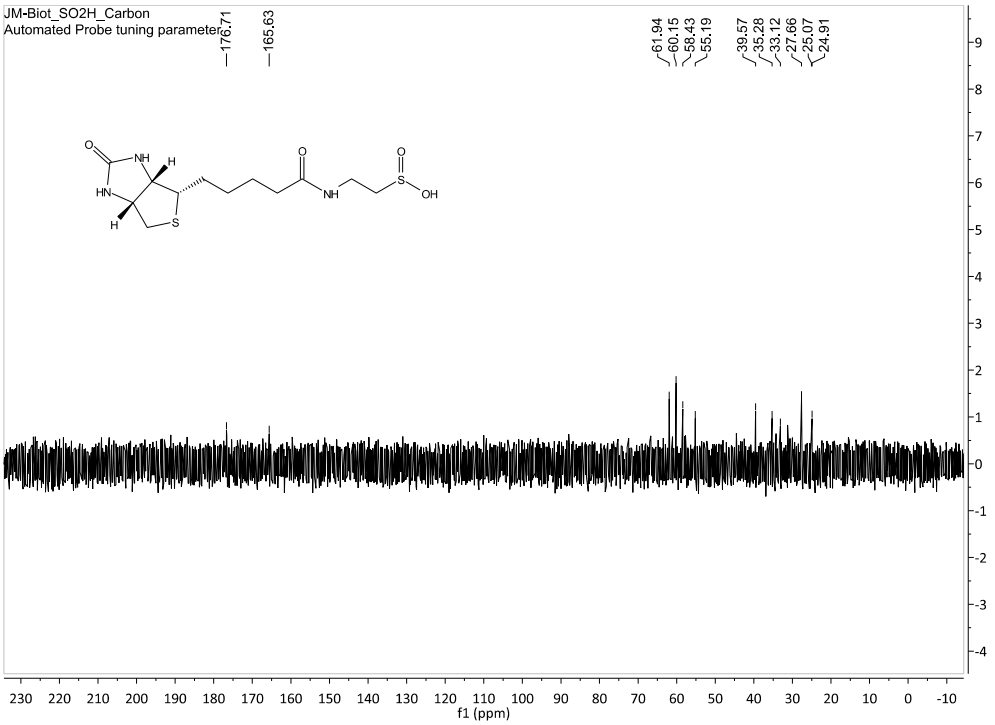
2-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethanesulfinic acid (15). A scintillation vial was charged with a solution of NHS-biotin, **14**, (Pierce, 1.0 eq., 35 mg, 0.1 mmol) in a 1:1 mixture of degassed THF and water

and the contents were allowed to cool to 0 °C. A solution of hypotaurine (3 eq., 34 mg, 0.3 mmol) in degassed (sonication under vacuum followed by three freeze-thaw cycles under vacuum) 0.01 N sodium hydroxide (100 µL) was then added to the NHS-biotin. The contents were allowed to react for 2 hours and the reaction progress was monitored by HPLC and mass spectrometry (LR-ESI-Neg). The product was purified to homogeneity by semi-preparative HPLC and then lyophilized to afford compound **15** as a white solid in 24% yield. ^{1}H NMR (400 MHz, Deuterium Oxide) δ 4.44 (dd, *J* = 7.9, 4.8 Hz, 1H), 4.26 (dd, *J* = 7.9, 4.4 Hz, 1H), 3.35 (t, *J* = 6.5 Hz, 2H), 3.17 (ddd, *J* = 8.8, 5.8, 4.4 Hz, 1H), 2.83 (dd, *J* = 13.0, 4.9 Hz, 1H), 2.68 - 2.45 (m, 2H), 2.09 (t, *J* = 7.2 Hz, 2H), 1.63 - 1.31 (m, 4H), 1.32 - 1.11 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 176.71, 165.63, 61.94, 60.15, 58.43, 55.19, 39.57, 35.28, 33.12, 27.66, 25.07, 24.91. HRMS (ESI Negative), $[M-H]^-$ Calculated: *m/z* = 334.0901, Observed: *m/z* = 334.0896.

Supplementary Spectra 2: (a) Proton NMR of compound **15** (b) Carbon NMR of compound **15**.



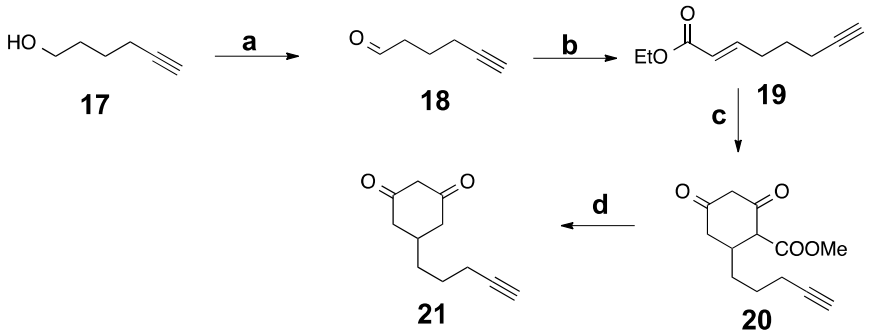
Supplementary Spectrum 2A: ^{1}H NMR of compound **15**.



Supplementary Spectrum 2B. ^{13}C NMR of compound **15**.

2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethanesulfonic acid (16). A scintillation vial was charged with a solution of NHS-biotin, **14**, (Pierce, 10 mg, 0.03 mmol) in a 1:1 mixture of THF and water and the contents were allowed to cool to 0 °C. A solution of taurine (3 eq., 11 mg, 0.09 mmol) in 0.01 N sodium hydroxide (100 µL) was then added to the NHS-biotin. The contents were allowed to react for 2 hours and progress was monitored by HPLC and mass spectrometry (LR-ESI-Pos). The product was purified to homogeneity by semi-preparative HPLC and lyophilized to afford compound **16** as white solid in 43% yield. ^{1}H NMR (400 MHz, Deuterium Oxide) δ 4.44 (dd, *J* = 7.9, 4.8 Hz, 1H), 4.26 (dd, *J* = 8.0, 4.5 Hz, 1H), 3.40 (t, *J* = 6.8 Hz, 2H), 3.24 - 3.07 (m, 1H), 2.91 (t, *J* = 6.7 Hz, 2H), 2.83 (dd, *J* = 13.0, 4.9 Hz, 1H), 2.61 (d, *J* = 13.2 Hz, 1H), 2.10 (t, *J* = 7.4 Hz, 2H), 1.48 (dtt, *J* = 26.3, 14.6, 7.1 Hz, 4H), 1.25 (q, *J* = 7.6 Hz, 2H). HRMS (ESI Positive) $[M+H]^+$ Calculated: *m/z* = 352.0995, Observed: *m/z* = 352.0995.

Synthetic Scheme 5. Synthesis of dimedone alkyne **(21)**:



(a) TEMPO, (diacetoxy iodo) benzene, anhydrous DCM, 4 hours, room temp, 64% (b) triethyl phosphonoacetate, DBU, 12 hours, room temp, 93% (c) anhydrous methanol, sodium methoxide, 32% (d) 20% NaOH, 2 hours, reflux; then H_2SO_4, 99%

5-hexyn-1-al (18): Compound **18** was synthesized as described previously[3]. Briefly, to a stirring solution of (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO, 0.1 eq., 0.48 g, 3 mmol,) and (diacetoxyiodo)benzene (1.1 eq., 10.83 g, 33 mmol,) in anhydrous methylene chloride, 5-hexyn-1-ol (1 eq.,3.37mL, 30 mmol,) was added drop-wise over the course of 30 minutes at ambient temperature and allowed to react for 3 hours. The reaction mixture was then transferred to a separatory funnel and extracted with saturated sodium bicarbonate (2 x 20 mL) and brine (2 x 10 mL). The organic layer was then dried over anhydrous magnesium sulfate (1.5 g) and filtered. The solvent was removed under vacuum using a rotary evaporator the residue was impregnated onto a silica gel column and purified using flash column chromatography (gradient elution using 100% hexanes, 50% hexanes: 50% DCM and 100% DCM) to afford 1.907 g (64%) of compound **18** as a yellowish oil. ^{1}H NMR (400 MHz, Chloroform-d) δ 9.60 (s, 1H), 2.41 (td, *J* = 7.2, 1.3 Hz, 2H), 2.07 (td, *J* = 6.9, 2.6 Hz, 2H), 1.85 (t, *J* = 2.7 Hz, 1H), 1.65 (p, *J* = 7.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.46, 83.08, 69.29, 42.30, 20.66, 17.53.

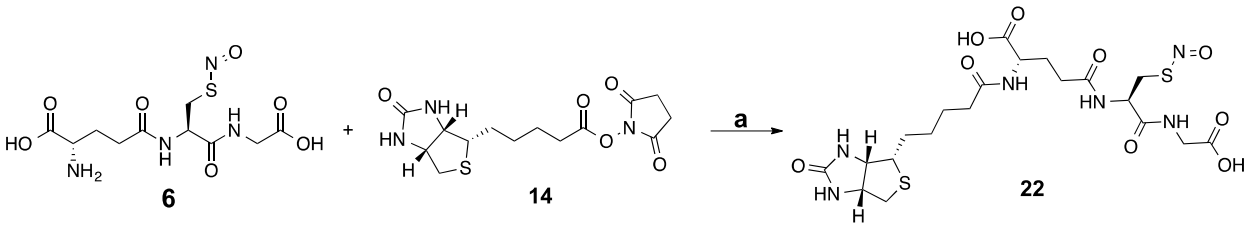
ethyl (E)-oct-2-en-7-ynoate (19): Compound **19** was synthesized by adapting a protocol described earlier[4]. Briefly, to stirring neat 5-hexyn-al (**18**, 1 eq., 0.50 g, 5 mmol,) triethyl phosphonoacetate (1.1 eq., 1.13 mL, 6 mmol,) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 1.5 eq., 1.17 mL, 8 mmol,) were added left for 12 hours at ambient temperature. The resulting reaction mixture was diluted with ethyl acetate, transferred to a separatory funnel, and extracted with 1 M hydrochloric acid (2 x 20 mL) and brine (2 x 20 mL). The organic layer was collected and dried over anhydrous magnesium sulfate (500 mg), filtered, and dried under vacuum. The residue was impregnated onto a silica gel column and purified using flash column

chromatography (isocratic elution using 1:1 hexanes : methylene chloride) to afford 806 mg (93%) of compound **19** as a yellowish oil. ^{1}H NMR (400 MHz, Chloroform-d) δ 6.86 (dtd, *J* = 15.4, 7.0, 1.0 Hz, 1H), 5.78 (dt, *J* = 15.6, 1.4 Hz, 1H), 4.11 (q, *J* = 7.2, 1.0 Hz, 2H), 2.29 - 2.24 (m, 2H), 2.21 (dd, *J* = 17.9, 1.2 Hz, 2H), 1.91 (td, *J* = 2.7, 1.0 Hz, 1H), 1.68 - 1.54 (m, 2H), 1.24 - 1.18 (m, 3H).

Ethyl 2,4-dioxo-6-(pent-4-yn-1-yl)cyclohexane-1-carboxylate (20): Compound **20** was synthesized through minor modifications of a protocol described earlier[5]. Briefly, the atmosphere in a flame-dried round-bottom flask was replaced with dry nitrogen and the flask was allowed to attain ambient temperature. The cooled flask was charged with anhydrous methanol, followed by addition of ethyl (*E*)-oct-2-en-7-ynoate (1 eq., 5 mmol, 880 mg), and ethyl acetoacetate (3 eq., 16 mmol, 2.03 mL) and sodium methoxide (5 eq., 26 mmol, 1.43 g) were added. The mixture was refluxed under nitrogen for 6 hours, dried under vacuum, and extracted with methylene chloride (2 x 20 mL). The aqueous layer was acidified to pH 4 and extracted again with methylene chloride (2 x 20 mL). The organic layer was dried over anhydrous magnesium sulfate (500 mg), filtered, and the solvent removed under reduced pressure. The mixture was then purified by HPLC and lyophilized to yield 55 mg (32%) of compound **20** as a while solid (mixture of diastereomers). ^{1}H NMR (400 MHz, Chloroform-d) δ 3.79 (s, 3H), 3.78 - 3.66 (m, 2H), 3.12 (d, *J* = 9.8 Hz, 1H), 3.08 - 2.98 (m, 1H), 2.53 (d, *J* = 4.2 Hz, 1H), 2.14 (dtd, *J* = 13.5, 6.3, 3.5 Hz, 2H), 1.91 (dt, *J* = 10.4, 2.7 Hz, 2H), 1.46 (d, *J* = 7.3 Hz, 2H), 1.31 - 1.14 (m, 2H). HRMS (ESI positive) $[M+H]^{+}$: Calculated *m/z*: 237.1126, Observed *m/z* = 237.1118

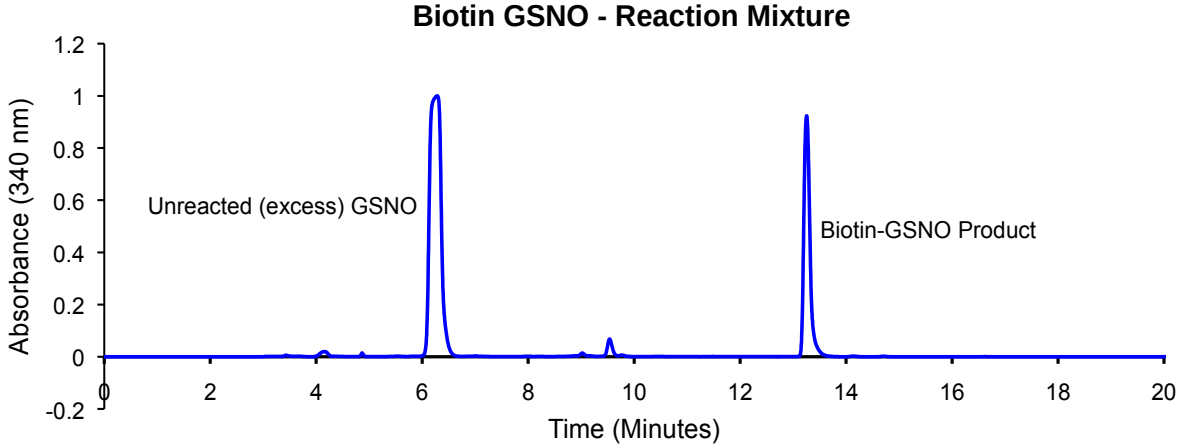
5-(pent-4-yn-1-yl)cyclohexane-1,3-dione (21): A round bottom flask was charged with ethyl 2,4-dioxo-6-(pent-4-yn-1-yl)cyclohexane-1-carboxylate, **20**, and 20 mL of 20% aqueous sodium hydroxide. The contents were allowed to react under reflux for 2 hours, and then cooled to ambient temperature, acidified with concentrated sulfuric acid, and refluxed for two additional hours. Next, the pH was adjusted to pH 4.0 and the reaction extracted with ethyl acetate. The organic layer was dried with magnesium sulfate (500 mg), filtered and the solvent removed under reduced pressure. The reaction mixture was purified by HPLC and lyophilized to yield 99% of compound **21** as a white solid. ^{1}H NMR (400 MHz, Chloroform-d) δ 2.59 - 2.27 (m, 4H), 2.24 - 2.05 (m, 4H), 1.98 - 1.88 (m, 1H), 1.50 (dtt, *J* = 21.4, 12.7, 5.0 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.03, 83.94, 68.60, 57.89, 47.49, 46.21, 37.98, 33.17, 30.27, 25.68, 18.40. HRMS (ESI positive) $[M+H]^{+}$: Calculated *m/z*: 179.1072, Observed *m/z* = 179.1059.

Synthetic Scheme 6. Synthesis of Biotin-(*S*-Nitroso)-Glutathione (Biotin GSNO, **22**)



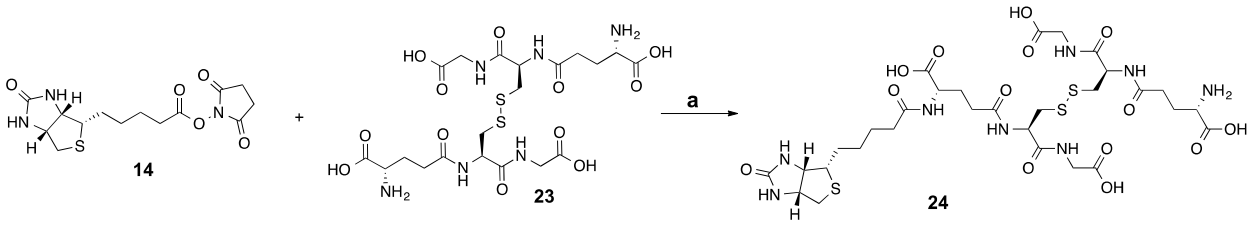
(a) THF, water (1:1), 2mL, triethylamine, 0 °C, 2 hours, dark

Biotin-GSNO. 5-(((*R*)-1-((carboxymethyl)amino)-3-(nitrosothio)-1-oxopropan-2-yl)amino)-5-oxo-2-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)pentanoic acid (22). A 25 mL scintillation vial was charged with 11 mg of Biotin-NHS ester (30 µmol, 1.1 eq), 10 mg *S*-nitroso-glutathione (Cayman, 30 µmol, 1 eq.), and 17 µL triethylamine (119 µmol, 4 eq) in water/THF (2 mL) and stirred in the dark for 2 hours. The mixture was then purified by HPLC in the dark. The purified product (retention time 13 minutes) was lyophilized in the dark to obtain compound **22** in 43% yield. ^{1}H NMR (400 MHz, Deuterium Oxide) δ 4.50 (t, *J* = 6.4 Hz, 1H), 4.44 (dd, *J* = 7.9, 4.9 Hz, 1H), 4.26 (dd, *J* = 7.9, 4.4 Hz, 1H), 4.14 (dd, *J* = 9.4, 4.8 Hz, 1H), 3.92 (s, 1H), 3.79 (s, 3H), 3.17 (dt, *J* = 9.8, 5.2 Hz, 1H), 2.83 (dd, *J* = 13.1, 5.0 Hz, 1H), 2.61 (d, *J* = 13.1 Hz, 1H), 2.17 (dt, *J* = 14.2, 7.2 Hz, 3H), 2.00 (dq, *J* = 13.5, 7.3 Hz, 1H), 1.79 (dq, *J* = 14.7, 7.4 Hz, 1H), 1.49 (dddq, *J* = 29.8, 21.8, 14.5, 7.8, 7.1 Hz, 5H), 1.25 (p, *J* = 7.6 Hz, 3H). HRMS (ESI positive): Calculated *m/z*: $[M+Na]^+$ = 585.1408, Observed *m/z* $[M+Na]^+$ = 585.1407.



Supplementary Figure B. HPLC trace of reaction mixture of biotin-GSNO, compound **16**. Peak at minute 13.4 represents product as confirmed by HRMS and NMR analysis.

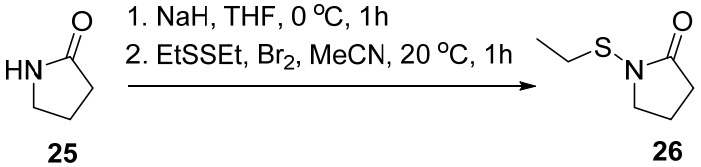
Synthetic Scheme 7. Synthesis of Biotin-glutathione disulfide (Biotin GSSG, **24**)



(a) THF, water (1:1), 2mL, triethylamine, 0 °C, 2 hours, dark

Biotin-GSSG. (2*S*,7*R*,12*R*,17*S*)-2-amino-17-carboxy-7,12-bis((carboxymethyl)carbamoyl)-5,14,19-trioxo-23-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-9,10-dithia-6,13,18-triazatricosanoic acid (24). A 25 mL scintillation vial was charged with 31 mg of Biotin-NHS ester (0.09 mmol, 1.0 eq.) and 556 mg of oxidized glutathione (0.9 mmol, 10 eq.) in water/THF (2 mL) and stirred in the dark for 18 hours. The mixture was then purified by HPLC. The purified product (retention time 8 minutes) was lyophilized to obtain compound **24** in 67% yield. ^{1}H NMR (400 MHz, Methanol-d4) δ 4.79 – 4.74 (m, 3H), 4.48 (dd, *J* = 7.9, 4.7 Hz, 2H), 4.38 (dd, J = 8.9, 5.0 Hz, 2H), 4.30 (dd, J = 7.9, 4.4 Hz, 2H), 3.69 (t, J = 6.2 Hz, 2H), 3.21 (ddd, *J* = 11.0, 4.5, 1.8 Hz, 3H), 3.01 – 2.88 (m, 4H), 2.54 (t, *J* = 7.0 Hz, 2H), 2.40 (t, *J* = 7.5 Hz, 2H), 2.28 (p, *J* = 6.8 Hz, 3H), 2.18 – 2.11 (m, 3H), 1.99 – 1.93 (m, 1H), 1.71 – 1.64 (m, 3H), 1.58 (dd, *J* = 14.5, 6.3 Hz, 2H), 1.45 (t, *J* = 7.5 Hz, 2H). HRMS (ESI positive): Calculated *m/z*: $[M+H]^+$ = 839.2396, Observed *m/z* $[M+H]^+$ = 839.2385.

Synthetic Scheme 8: Synthesis of pyrrolidinone sulfenamide (**26**)



1-(ethylthio)pyrrolidin-2-one (26) To a 100 mL flame-dried two-neck round-bottom flask was added sodium hydride as a 60% suspension in mineral oil (1.337 g, 34 mM, 4.2 eq), which was thoroughly washed with hexanes (3 x 25 mL portions) under nitrogen. This was suspended in 25

mL dry THF and cooled on an ice bath. 2-pyrrolidinone (2.50 mL, 33 mM, 4 eq), was added dropwise to this solution with stirring and allowed to equilibrate for 1 hr with the evolution of gas. In a separate 25 mL flame-dried round-bottom flask, to a solution of ethyl disulfide (1 g, 8.2 mM, 1eq) and dry acetonitrile (10 mL) was added elemental bromine (1.31 g, 8.2 mM, 1 eq), and this was allowed to react for 1 hr. Following this, the solution was added dropwise via cannula to the suspension of sodium pyrrolidinonate, which was accompanied by a loss of color. This was allowed to warm to room temperature, and left to react for one hour. Hexanes (100 mL) was added to the heterogeneous reaction, which was then filtered through a plug of glass wool to remove pyrrolidinate and other salts. The solvent was removed under vacuum, and the crude mixture was purified by flash chromatography on a 0 - 2% methanol gradient in dichloromethane. Fractions were combined, and 1.56 g (66%) was collected as a light brown liquid. ^{1}H NMR (401 MHz, Chloroform-d) δ: 3.58 (t, *J* = 7.1 Hz, 2H), 2.79 (q, *J* = 7.4 Hz, 2H), 2.40 (t, *J* = 8.1Hz, 2H), 2.04 (tt, *J* = 7.8, 6.8Hz, 2H), 1.22 (t, *J* = 7.4Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.87, 77.33, 77.01, 76.69, 52.85, 30.96, 30.24, 18.83, 13.16. HRMS (ESI positive): Calculated *m/z* = [M+H$^+$] = 146.0640, Observed *m/z*: 146.0634.

III. Experimental Methods.

Rate-constant determination. *S*-nitroso-glutathione (GSNO, Cayman) and sodium phenylsulfinate (Sigma-Aldrich) were combined in four different pH buffers: pH 1.0 (0.2 N HCl / KCl buffer), pH 4.0 (0.1 M sodium acetate / acetic acid buffer), pH 7.0 (0.1 M potassium phosphate buffer) and pH 10.0 (0.1 M Sodium bicarbonate / Sodium hydroxide buffer). The purity of *S*-nitrosoglutathione was calculated as 94 ± 1.3% using molar extinction coefficient at 334 nm (ε = 900 M^{-1} cm^{-1}). Assays were performed using Tecan Infinite F500 plate reader monitoring absorbance of 2 mM GSNO at 340 nm over a course of 90 minutes in the presence of varying concentrations of sodium phenylsulfinate. Absorbance data was imported into KaleidaGraph (version 4.02, Synergy Software), and logarithmically fit to the first order exponential decay. The k_{obs} was calculated for each phenylsulfinic acid concentration, and plotted to calculate the rate constant for the overall reaction.

LC-MS quantitation of reaction products and selectivity. Analysis was performed using an Agilent Q-TOF mass spectrometer (1 – 5 ppm accuracy) coupled to a HPLC system and connected to a photodiode array detector. Compounds and reaction mixtures were injected in 5 µL volumes on an Agilent Zorbax Eclipse plus C18 rapid resolution column (2.1 x 50 mm, 1.8 µ), and separated using the 10-minute gradient starting at 5% and increasing to 100% acetonitrile in 8 minutes. A solution of 500 µM GSNO was made in degassed, LC-MS quality water (correcting for the purity of GSNO) and allowed to react with various concentrations of 4-methyl-phenylsulfinic acid for 3 hours at ambient temperature before measurement. To generate standard curves, commercial *N*-hydroxy-4-methylbenzenesulfonamide (Combi-Blocks) and the HPLC purified thiosulfonate product were diluted in LC-MS grade water (pH 6.9) for LC-MS analysis. For selectivity assays, compounds and reaction mixtures were injected in 5 µL volumes on a Waters Atlantis C_{18} rapid resolution column (2.1 x 50 mm, 1.8 µm), and separated using the 15-minute gradient starting at 5% and increasing to 100% acetonitrile in 12 minutes. Standard traces were collected by injecting 5 µL of 200 µM solutions of *N*-acetylcysteine methylester and 2-iodoacetamide. Both compounds were incubated with phenylsulfinic acid in phosphate buffered saline, pH 7.4.

To investigate the stability of *N*-hydroxy sulfonamides, 200 µM solutions of 4-methyl-phenyl-*N*-hydroxysulfonamide were prepared in buffers: pH 1.0 (0.2 N HCl / KCl buffer), pH 4.0 (0.1 M sodium acetate / acetic acid buffer), pH 7.0 (0.1 M potassium phosphate buffer) and pH 10.0 (0.1 M Sodium bicarbonate / Sodium hydroxide buffer). A 5 µL aliquot was injected on the LC-MS system fitted with the Waters Symmetry C_{18} column (3.5 µm, 4.6 x 75 mm) and the analyte

was eluted by gradient elution (5 to 60% acetonitrile over 12 minutes). MS data was collected in ESI negative mode and the integration of the extracted ion chromatogram for *m/z* 186.0303 $[M-H]^-$ was used to compute the loss and assess the stability of 4-methyl-phenyl-*N*-hydroxysulfonamide at various pH values versus time.

Mammalian cell culture. Human 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS, JR Scientific) and 1% (v/v) penicillin-streptomycin-glutamine solution (PSQ, Life Technologies). Cells were harvested at 80% confluence in phosphate buffered saline (PBS) (Life Technologies). For stable isotope labeling with amino acids in cell culture (SILAC), 293T cells were grown in SILAC DMEM (Thermo), 100 µg/mL [$^{13}C_6$,$^{15}N_4$] L- Arginine-HCl and [$^{13}C_6$,$^{15}N_2$] L-Lysine-HCl (Sigma) or L-Arginine-HCl and L-Lysine-HCl (Sigma) for greater than 6 passages.

Handling, use and storage of probes and chemicals. MAHMA NONOate solutions were always freshly prepared immediately before use. Solid aliquots of MAHMA NONOate (Cayman) was stored at -80 °C and removed from the freezer just prior to use. A 1 mM solution of MAHMA NONOate was prepared in cold PBS (pH 7.4) and handled as described[6]. An appropriate volume was then added to the lysate or purified protein and the nitrosation was allowed to proceed for about 10 minutes in the dark. MAHMA NONOate was selected as the preferred NO donor due to its short half-life of 3 minutes at room temperature and the fact that it donates 2 equivalents of NO per mole of MAHMA NONOate. MAHMA NONOate is also extremely convenient to handle and use, owing to its stability to ambient light (unlike GSNO) and the nitrosation reaction with protein thiols proceeds quickly on time scales that are appropriate for the assay.

The biotin-sulfinic acid probe was synthesized and stored in de-gassed water in 100 µL aliquots (10-30mM) at -80 °C. We have observed that the biotin-sulfinic acid probe is stable during the duration of labeling (<1 hour), but does oxidize to the non-reactive sulfonic acid after ~5 hours at room temperature upon exposure to atmospheric oxygen. At -80 °C, the probe is stable for about 3-4 months, provided it has avoided >2 freeze-thaw cycles.

The biotin-GSNO probe was used the same day it was synthesized to minimize decomposition. All manipulations and experiments with biotin-GSNO were performed in the dark. Briefly, after HPLC purification, fractions containing biotin-GSNO were flash frozen in liquid nitrogen and lyophilized for 6 hours followed by reconstitution of probe in degassed, chelex-treated water (LCMS grade) and used immediately for lysate labeling.

Gel-based analysis of *S*-nitrosation and *S*-sulfination. Cells and lysates were protected from light and kept on ice throughout the labeling protocol, except when noted. For *S*-nitrosation studies, 293T cell pellets were lysed by sonication (4 °C, dark, 10% duty cycle, 10 seconds) in 6 M urea / PBS buffer containing 50 mM iodoacetamide to alkylate free thiols. Protein concentrations were determined using the Bio-Rad DC assay, and diluted to 3 mg / mL in 6 M urea / PBS. Lysates were then treated with the specific perturbant (such as ascorbate, hypotaurine, etc), followed by incubation with 500 μM biotin-hypotaurine or fluorescein-hypotaurine for 45 minutes at room temperature. For *S*-sulfination studies, 293T cell pellets were lysed in 6 M urea / PBS, and incubated with 1 mM dithiothreitol for 15 minutes in the dark, followed by 50 mM iodoacetamide at room temperature for 30 minutes. To demonstrate peroxide sensitivity, the samples were treated with 15 mM hydrogen peroxide for 30 minutes at 37 °C, precipitated with chloroform:methanol, washed with cold methanol, and re-solubilized in 6 M urea / PBS. Next, lysates were incubated with ~900 μM biotin-GSNO for 45 minutes in the dark, followed by chloroform:methanol precipitation, and re-solubilized in 6 M urea / PBS. TCEP reduction was demonstrated by incubating samples for 15 min with 15 mM TCEP. Tissues were collected from 4 month-old C57/B6 mice and frozen immediately in liquid nitrogen, and later dounce homogenized on ice in aqueous 6 M urea / PBS supplemented with 50 mM iodoacetamide. The homogenate was cleared by centrifugation at 5000*g* for 5 minutes, and the supernatant was processed as described above. For gel-based analysis, samples were mixed with non-reducing SDS-PAGE loading buffer and analyzed without boiling. Gels were transferred to 0.45 μm polyvinylidine difluoride membrane (Immobilon-P, Millipore) and blocked with 5% bovine serum albumin (BSA, Fisher) in Tris buffered saline-Tween 20 buffer (TBS-T, pH 7.4) for 2 hours at room temperature. After blocking with 5% BSA, the membrane was probed with streptavidin-DyLight 633 (Thermo, 50 μg/L, 2.5% BSA, 0.02% NaN_3, TBS-T, pH 7.4) for 1 hour at room temperature and washed with TBS-T. For GAPDH detection, blots were probed with the anti-GAPDH mouse monoclonal antibody (mAb 6C5, Calbiochem, 1 μg/mL, 2.5% BSA, 0.02% NaN_3, TBS-T, pH 7.0), washed and probed with a secondary Alexa Fluor 532 nm goat-anti-mouse antibody conjugate (IgG H +L, Life Technologies, 2 μg / mL antibody, 0.06% NaN_3, TBS-T) for 1 hour at room temperature. Fluorescein and streptavidin blots were analyzed using a GE typhoon scanner at appropriate wavelengths (488/526 for fluorescein and 633/670 for Cy5-Streptavidin).

To detect *S*-sulfination with biotin-GSNO, HEK293T cell were lysed in 6 M urea / PBS containing 2% SDS. Disulfides were reduced with DTT (10 mM, 30 minutes) followed by iodoacetamide alkylation (40 mM, 45 minutes, dark). The excess unreacted DTT and

iodoacetamide were removed by chloroform/methanol precipitation, and pellet was resuspended in 6 M urea / PBS containing 2% SDS and 10 mM iodoacetamide. Two aliquots of 20 μL each were taken from the resuspended lysate and treated with either buffer or competing agent (1mM *S*-Me-glutathione or dimedone). Next, biotin-GSNO or biotin-GSSG was added and left for 30 minutes, followed by non-reducing SDS-PAGE and western blot analysis probing with streptavidin DyLight 800 using the Azure c600 Biosystems imager.

Purification, labeling, and preparation of purified proteins for LC-MS analysis. Human GAPDH and DJ-1 cDNAs were amplified from 293T cDNA, cloned into the bacterial 6-His expression vector pET45b, and transformed into BL21 E.coli. Bacteria were grown in LB media at 37 °C to an OD600 of 0.6, and induced with 0.4 mM IPTG for 4 additional hours at 37 °C. After lysozyme treatment and sonication, the cleared lysate was incubated with Talon resin (Clontech), and loaded on a gravity column. After sufficient washing, the purified recombinant protein was eluted with imidazole and dialyzed into PBS, typically yielding 10-15 mg / L of culture. Each protein was separately diluted to 0.2 mg / mL in PBS and treated with 20 mM iodoacetamide for 30 minutes in the dark. GAPDH was incubated with 100 μM biotin-SO_2H for 30 minutes. DJ-1 was treated with 2 mM hydrogen peroxide for 20 minutes at 37 °C to promote Cys106 sulfinic acid formation, and then mixed with freshly synthesized *N*-acetyl-(*S*-Nitroso)-Cys-OMe (~1 mM) for 30 minutes in the dark at room temperature. After labeling, samples were dried using a Savant SPD1010 concentrator (Thermo) and reconstituted in 200 μL of 2 M urea in 25 mM ammonium bicarbonate buffer with 2 μL of 100 mM $CaCl_2$. The reconstituted sampled incubated with mass spectrometry grade Trypsin (Promega, 1 μL of 0.5 mg / mL) for 6 hours at 37 °C with agitation. After trypsin digestion, additional salts were removed using a Waters Oasis HLB μElution plates (30 μm) following the manufacturer's protocol. The eluted peptide samples were dried and reconstituted in 1-D LC-MS buffer (0.1% formic acid in 3% acetonitrile/water with 5 fmol / μL *Saccharomyces cerevisiae* alcohol dehydrogenase (Uniprot accession: P00330, Waters).

Enrichment of *S*-nitrosated proteins for LC-MS analysis. Cells and lysates were protected from light and kept on ice throughout the labeling protocol. SILAC labeled and unlabeled cells were lysed in the presence of 50 mM iodoacetamide in aqueous 6 M urea / PBS, and diluted to to 3 mg / mL. Approximately 1.2 mg total lysate was collected for each biological replicate. Next, "light" and "heavy" lysates were treated with 400 μM of the biotin-SO_2H probe, and the other "light" and "heavy" lysates were treated with 400 μM of the biotin-SO_3H probe. Samples were

incubated for 45 minutes in the dark, and quenched by chloroform-methanol precipitation to remove excess probe and iodoacetamide. The isolated protein pellet was washed 3 times with cold methanol and re-solubilized in 500 µL of 6 M urea / 25 mM ammonium bicarbonate (37 °C, 10 min). Protein concentrations were re-measured and normalized to the lowest concentration obtained across the 8 samples. The “light” and “heavy” samples were mixed in a 1:1 ratio (vol / vol), matching a “light” lysate labeled with the biotin-SO_2H probe with a “heavy” lysate labeled with the biotin-SO_3H probe and vice-versa, yielding a total of four independent biological replicates. The paired samples were combined and transferred to a 15 mL conical tube containing 280 µL of 10% SDS. After brief heating (50 °C, 2 min), samples were diluted with 5.5 mL PBS containing 100 µL of a 50% streptavidin slurry (Millipore). After 2 hours on a rotary mixer at room temperature, the beads were washed 3-times with 3 mL of 1% SDS in PBS, and 7-times with 3 mL of PBS. The resin was then transferred to a 1.5 mL screw-top conical tube in 200 µL of 2 M urea / 25 mM ammonium bicarbonate / 1 mM aqueous calcium chloride with 2 µg of trypsin, and allowed to digest at 37 °C with agitation for 6-8 hours. The supernatant was pooled with two additional 150 µL washes and cleaned up using an Oasis HLB µElution plates (30 µm). The eluted peptide sample was dried and reconstituted in final 2D-LC-MS buffer (20 mM ammonium formate, pH 10.0 buffer with 5 fmol / µL *Saccharomyces cerevisiae* alcohol dehydrogenase (Waters)). For site of labeling studies, resin-bound tryptic peptides were resuspended in 500 µL of 0.1 M phosphate buffer (pH 7.0) containing 30 mM *N*-ethyl-maleimide. Neutralized TCEP (pH 7.0) was added to the sample to achieve a final concentration of 5 mM, and incubated on a rotary mixer for 30 minutes. The eluent was purified using the Waters Oasis HLB µElution plate and reconstituted in 1-D LC-MS buffer.

Enrichment of *S*-sulfinated proteins for LC-MS analysis. Cell lysates were prepared from 293T cells as described above in 6 M urea / PBS, followed by addition of 2 mM DTT for 15 minutes, and 50 mM iodoacetamide for 45 minutes. Next, “light” and “heavy” lysates were treated with 900 µM of freshly-prepared (within 15 hours) biotin-GSNO probe, and the matching “light” and “heavy” lysates were treated with 900 µM of biotin (Sigma-Aldrich) for 45 minutes in the dark, followed by chloroform/methanol precipitation. The isolated protein pellet was washed 3-times with cold methanol and re-solubilized in 500 µL of aqueous 6 M urea (37 °C, 10 min). Protein concentrations re-measured and normalized to the lowest concentration across the 8 samples. The “light” and “heavy” samples were mixed in a 1:1 ratio (vol / vol), matching a “light” lysate labeled with the biotin-GSNO probe with a “heavy” lysate labeled with biotin and vice-versa, yielding a total of four independent biological replicates. The samples were incubated

with streptavidin beads, washed, digested with trypsin, and reconstituted in 1D LC-MS buffer (3% acetonitrile in LC-MS grade water, 0.1% formic acid, and 10 fmol/µL *Saccharomyces cerevisiae* alcohol dehydrogenase). For site-specific analysis, beads linked to modified peptides were resuspended in 500µL of 0.1 M phosphate buffer (pH 7.0). Neutralized TCEP (5 mM) was added to the sample and incubated on a rotary mixer for 30 minutes. The eluent was purified using an Oasis HLB µElution plates and reconstituted in 1-D LC-MS buffer.

LC-MS Proteomic analysis. In-line liquid chromatography of tryptic peptides was performed on the Waters 2D-NanoAcquity chromatography system fitted with a X-BRIDGE BEH130 C18 5 µM (300 µM x 50 mm) peptide separation technology fractioning column (column chemistry: 1,2-bis(siloxyethane) [-$O_3SiCH_2CH_2SiO_3$-]), a 5 µM Symmetry C18 (180 µm x 20 mm) trap column and a 1.8 µm High Strength Silica (HSS-T3) analytical column (75 µm x 150 mm). For *S*-nitrosation studies, tryptic peptides were first loaded onto the fractioning column (20 mM ammonium formate, pH 10.0) followed by fractionation by sequential gradients that gradually increase in the organic component (10.4%, 14%, 16.7%, 20.4% and 50% acetonitrile). In each step, a fraction of the peptides are eluted to the trapping column, and delivered to the analytical column (2nd dimension) for separation (7% acetonitrile to 85% acetonitrile over 40 minutes). The 2nd dimension uses 0.1% formic acid in water and 0.1% formic acid in acetonitrile as the solvents. During trapping stage, the basic buffers are diluted 1:10 with the acidic solvents to capture the peptide fraction on the trapping column, transferred to the analytical column, and separated over a reverse phase gradient for direct electrospray ionization to the instrument source. For *S*-sulfination studies, tryptic digests were injected to a 1D Waters NanoAcquity UPLC system equipped with a 5 µM Symmetry C18 (180 µm x 20 mm) trap column and a 1.8 µm High Strength Silica (HSS-T3) analytical column (75 µm x 150 mm). Tryptic peptides were loaded onto the trap column over 3 minutes, followed by analytical separation over a 110 minute gradient (3% acetonitrile to 40% acetonitrile over 92 minutes). For unenriched proteome analysis**,** lysates from two different biological replicates were diluted to 3 mg / mL, reduced with 20 mM dithiothreitol (DTT), alkylated with 50 mM iodoacetamide, and digested with trypsin. Tryptic digests were injected to a 1D Waters NanoAcquity UPLC system equipped with a 5 µM Symmetry C18 (180 µm x 20 mm) trap column and a 1.8 µm High Strength Silica (HSS-T3) analytical column (75 µm x 150 mm). Tryptic peptides were separated over a 120-minute gradient in which the concentration of the organic phase gradually increased from 7% to 35% over 85 minutes. Peptides were analyzed using a Waters Synapt G2S HDMS time-of-flight mass spectrometer using ion mobility separation and data independent fragmentation

algorithms. Briefly, the instrument was tuned using the following parameters. The quadruple mass analyzer was manually set for mass 500, 600 and 700. The sampling cone was adjusted to 32 eV and the nano flow gas was set to flow at 0.2 bar. The purge gas was set to flow at 50 L/h and the source temperature was set at 70 °C. For all measurements, the mass spectrometer was operated in V-mode (resolution mode) with a resolving power of at least 20,000 FWHM (full width at half maximum) in positive-mode ESI for 400 *m/z*. The time-of-flight analyzer of the mass spectrometer was calibrated with a 100 fmol / µL solution of [Glu1]-Fibrinopeptide B from *m/z* 50 to 1250 to within 0.7 ppm. The data were post-acquisition lock mass corrected using the doubly charged monoisotopic ion of [Glu1]-Fibrinopeptide B (*m/z* = 785.8426), which was collected at a frequency of 30 seconds during the runs. Accurate mass LC-MS data were collected in DIA modes of analysis using MS^E in combination with on-line IMS. For IMS, the wave height was set as 40 V and IMS wave velocity as 600 m/s. The spectral acquisition time in each mode was 0.5 s. In low-energy MS mode, data were collected without applying collision energy in the trap or the transfer stage. A collision energy (CE) ramp from 16 eV to 60 eV during each 0.5 s-integration was used as standard setting for the elevated energy MS scan in the H/$UDMS^E$ mode. For data collected in MS^E and $HDMS^E$ mode (purified protein samples), a standard CE ramp of 19 to 45 eV was applied for the elevated energy scans. For complex proteomic samples (enriched proteomics, unenriched proteomics and sites of labeling samples), the $UDMS^E$ approach was employed to gain better depth of proteome coverage. Optimal drift-time-dependent CE values were obtained by maximal number of proteins obtained using a tryptic digest human cells as described[7]. Briefly, the following CE settings were obtained after optimization and employed throughout the studies in the elevated energy scan: (i) ion-mobility bins 0 – 20: CE of 117 eV, (ii) ion-mobility bins 20 – 110: CE of 17 eV to 56 eV, (iii) ion-mobility bins 110 – 200: 56 to 72 eV.

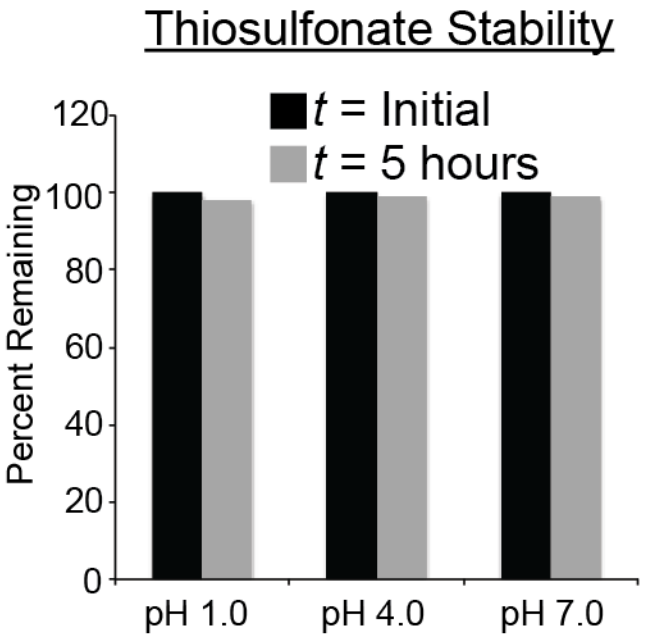
Mass spectrometry data processing and SILAC quantification. LC-MS spectra were collected in continuum mode and searched using the ProteinLynx Global SERVER version 3.0.2 (Waters) with the reviewed human reference proteome (UniProtKB downloaded on 2014-03-01). Precursor- and fragment-ion mass tolerances were automatically determined by PLGS 3.0.2 during database searching. Identified peptides met the following search criteria: (i) trypsin as digestion enzyme, (ii) a maximum of one missed cleavage, (iii) lysine (+8) or arginine (+10) defined as fixed modifier reagent groups, (iv) carbamidomethyl cysteine as a fixed modification and methionine oxidation as the variable modification, (v) a minimum of two identified fragment ions per peptide and a minimum of five fragments per protein, and (vi) at least two identified

peptides per protein. The false discovery rate (FDR) for peptide and protein identification was set at 4% using a reversed database. Using in-house Python scripts, all data from technical and biological replicates were merged, removing in-source fragments, unlabeled peptides not containing an arginine or lysine, and any precursors greater than ± 5 ppm in mass difference from the calculated theoretical mass. Data was sorted to match SILAC peptides with their corresponding “Heavy” or “Light” partners. Peptides without a pair were labeled as “potential uniques” and further analyzed using in-house Python scripts to determine if the protein they represented is truly unique in a given dataset. Additional custom scripts were used to calculate the average SILAC ratio, the estimated top3 abundance, number of SILAC peptides pairs identified for each protein, the SILAC ratio standard deviation, and the number of times the protein was identified across all replicates. Enriched datasets were filtered to include only proteins with a SILAC ratio ≥ 3, ≥3 quantified SILAC peptide pairs, and detection in at least 3 experiments). Proteins that met these criteria were identified as being *S*-nitrosated or *S*-sulfinated.

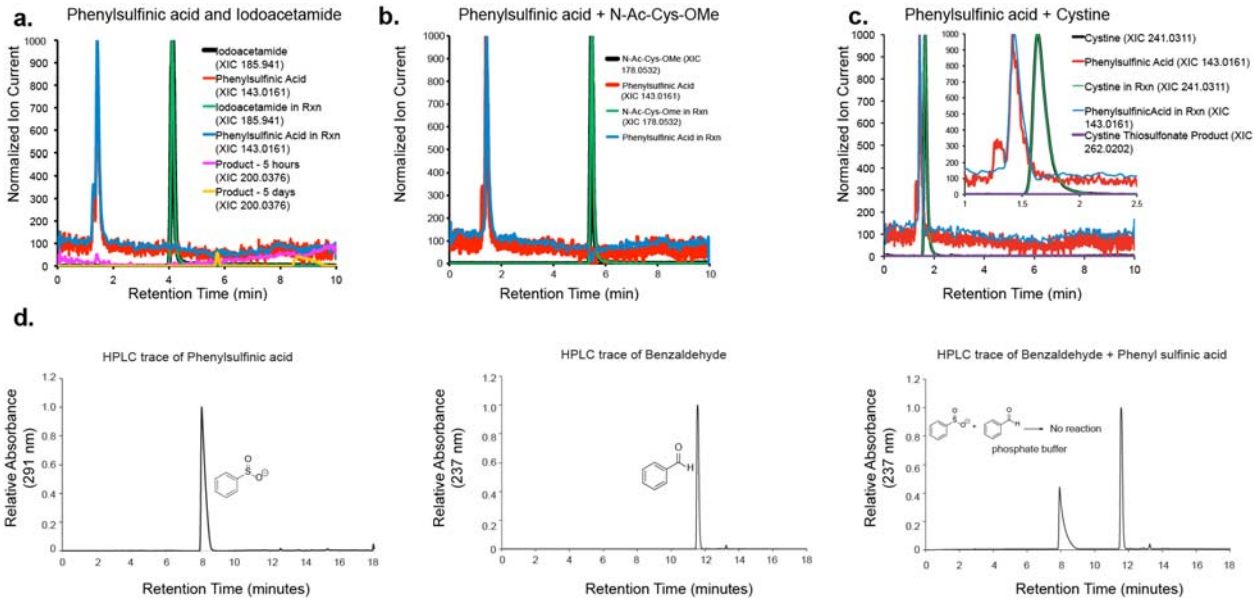
Estimation of stoichiometry of *S*-nitrosation and *S*-sulfination. Absolute quantitation of proteins under the DIA approach is performed using the three most intense peptides for each protein. This summed intensity signal correlates with abundance. Using a known concentration of internal standard (10 fmol / μL of yeast alcohol dehydrogenase, P00330, (Waters Corp.)) in our case) added to all samples, the abundance of all proteins in the sample can be calculated[8]. We used Waters PLGS 3.0.2 to perform the data analysis. For every protein, the intensity of the three most intense peptides were summed in both the enriched and the unenriched samples was obtained. A simple ratio of these summed intensities allows for the quantitation of relative protein abundance in each condition and thus addresses stoichiometry of the modification. Using this approach, we compared our probe-enriched data to the unenriched data, arriving at a relative stoichiometry. The stoichiometry estimation is affected by the number of sites of modification, the relative stability of the modification, and many other factors. The overall goal is that if a protein has very low abundance, but is highly enriched by either probe, then it is likely a protein with enhanced susceptibility to oxidation.

III. Supplementary Figures

Supplementary Figure 1: **Thiosulfonates are stable in aqueous buffers.** The thiosulfonate (**3**) is stable in pH 1, 4 and 7 for over 5 hours. 5 mM solutions of the thiosulfonate (**3**), were prepared in buffers at pH 1, 4, 7 and 10. The mixtures were injected on the HPLC and absorbance of product peak (11.7 minutes, 283 nm) were measured immediately upon preparing the solution and after 5 hours of standing at room temperature. The figure below shows that the thiosulfonate product is stable in buffers of pH 1, 4 and 7 for over 5 hours. Spontaneous hydrolysis of the product was observed at pH 10.0.

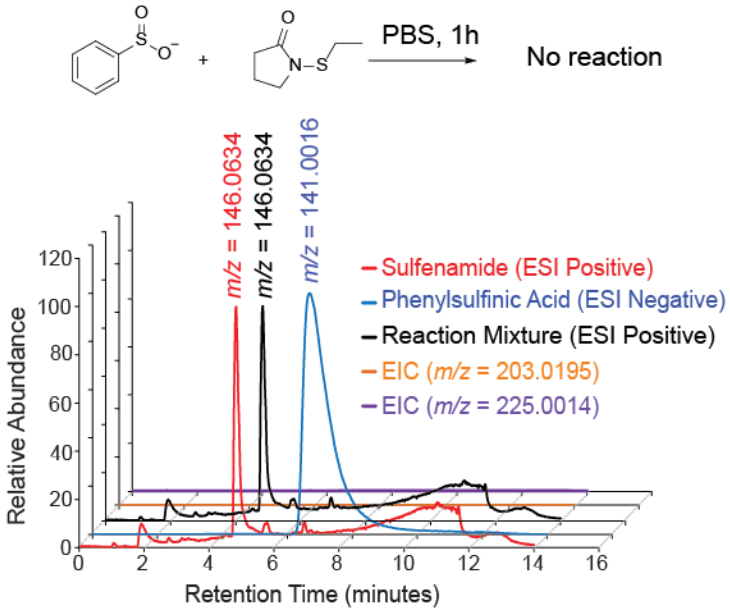


Supplementary Figure 2. Sulfinic acids do not react with iodoacetamide, cysteine, cystine, or benzaldehyde in phosphate buffer. (a) Phenylsulfinic acid does not react with iodoacetamide. A 10 mM solution of phenylsulfinic acid was allowed to react with a 20 mM solution of iodoacetamide in potassium phosphate buffer pH 7.4 for 5 hours. After 5 hours, the reaction mixture was injected on an LC-MS and separated using gradient elution (5% ACN to 95% ACN over 15 minutes). Absence of additional peaks was interpreted as no reaction between the sulfinic acid and iodoacetamide. (b) For cysteine, cystine and benzaldehyde cross reactivity, a 2 mM solution of phenylsulfinic acid was allowed to react with 20 mM solutions of *N*-acetyl-cysteine-methyl ester, cystine, or benzaldehyde. The reaction mixtures for (b) and (c) were analyzed by high-resolution LC-MS, confirming no observed reduction in reactants and no observed products. The reaction displayed in (d) was analyzed by HPLC using a photodiode array detector, and confirm that no new product peaks appear.

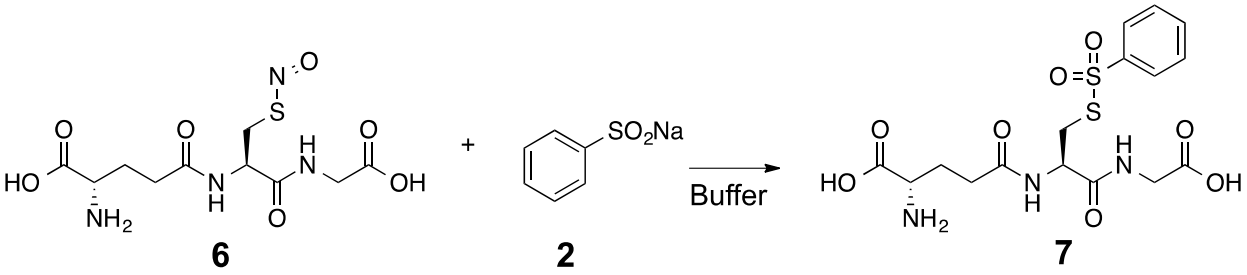


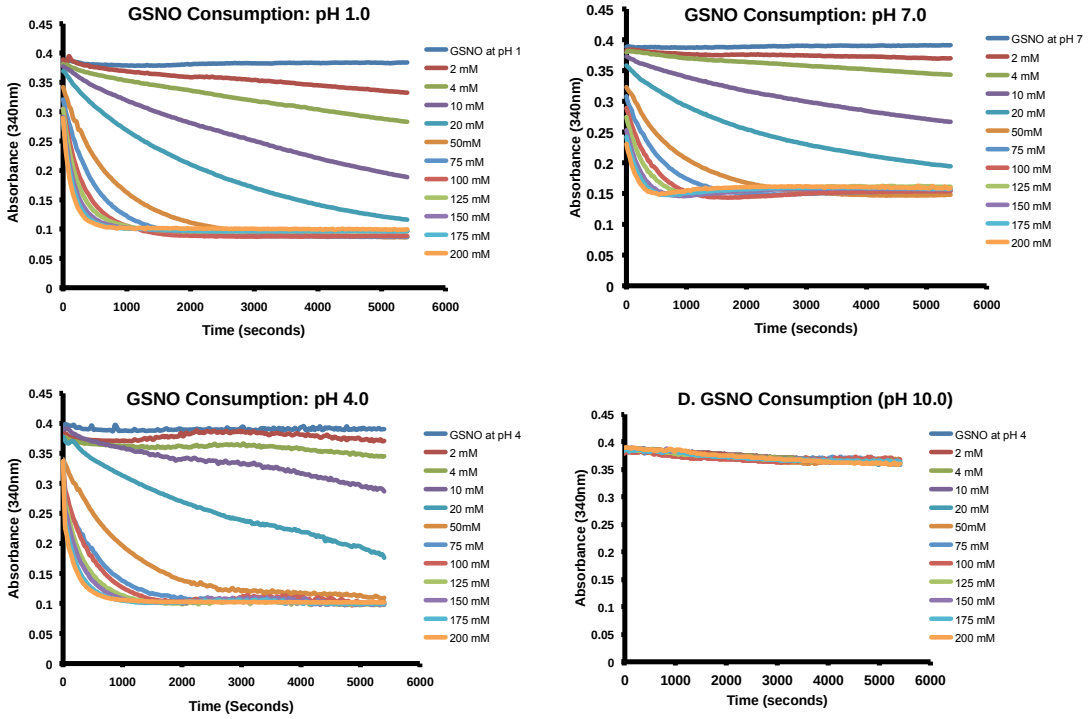
Supplementary Figure 3. **Sulfinic acids do not react with sulfenamides.**

The pyrrolidinone sulfenamide **(26)** was reacted with phenylsulfinic acid in PBS (pH 7.4) for 1 hour. The reaction mixture was injected for LC-MS analysis. No peaks were obtained for the putative thiosulfonate product (*S*-ethyl benzenesulfonothioate, *m/z* = 203.0195) or its sodium adduct (*m/z* = 225.0014) demonstrating no reaction between phenylsulfinic acid and sulfenamide under these conditions. The figure below shows the normalized relative abundances of the sulfenamide, phenylsulfinic acid, the reaction mixture and the extracted ion chromatograms for the putative products.



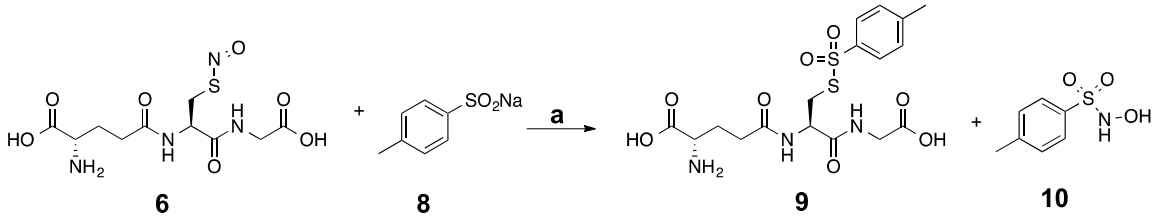
Supplementary Figure 4. GSNO is consumed by increasing concentrations of sodium phenylsulfinate. (a) A 2 mM solution of *S*-nitrosoglutathione (GSNO, Cayman) was treated with increasing concentrations of phenylsulfinic acid, and the absorbance of GSNO was monitored over a period of 90 minutes at 340 nm using a plate reader (Tecan Infinite F500) in varying pH buffers. For more details, please refer the “rate constant determination” description in the experimental methods section (page 15 of this document).





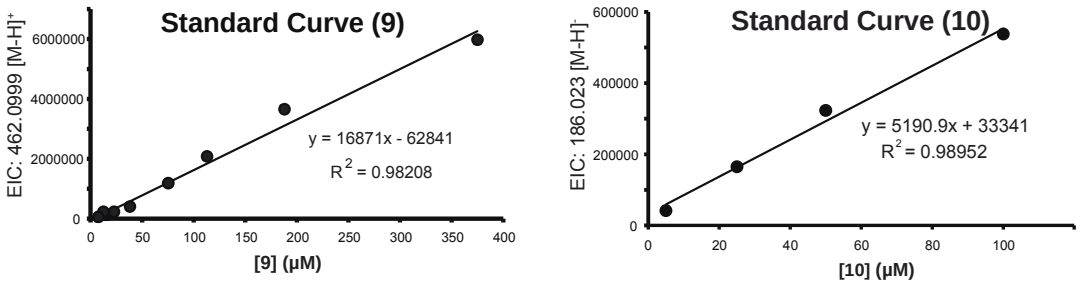
Supplementary Figure 5. Determination of reaction efficiency and quantification of products and by-products. (a) Reaction between GSNO and 4-Me-phenylsulfinic acid. The products **9** and **10** were quantified by LC-MS. **(b)** LC-MS standard curves were generated from the HPLC purified thiosulfonate product (**9**) and commercial 4-Me-piloty's acid (**10**). (**c**) Raw LC-MS traces (total ion chromatographs, TIC) of standards and reaction (1:50; **6**:**8**) identified 6 species. **(d)** Individual LC-MS TIC traces are presented. **(e)** Stability of 4-methyl-phenyl-*N*-hydroxysulfonamide in various buffers over time. For additional experimental details, please refer to the "LC-MS quantitation of reaction products and selectivity" section in the experimental methods (page 15 of this document).

a

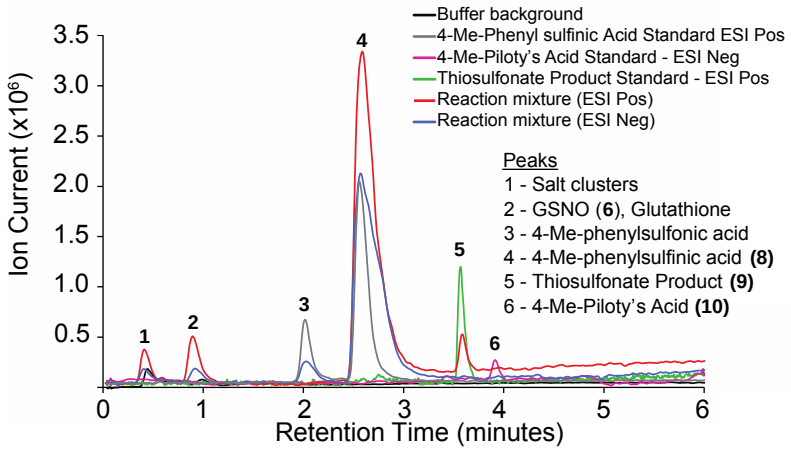


(a) Water, 0 °C – room temperature, 2 hours, dark

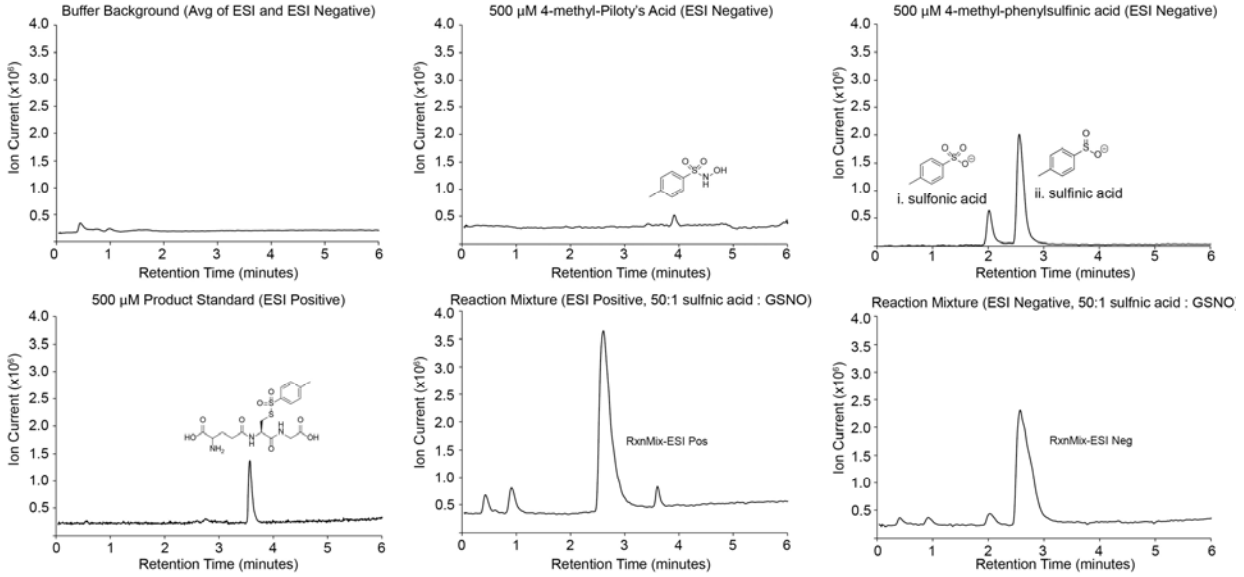
b



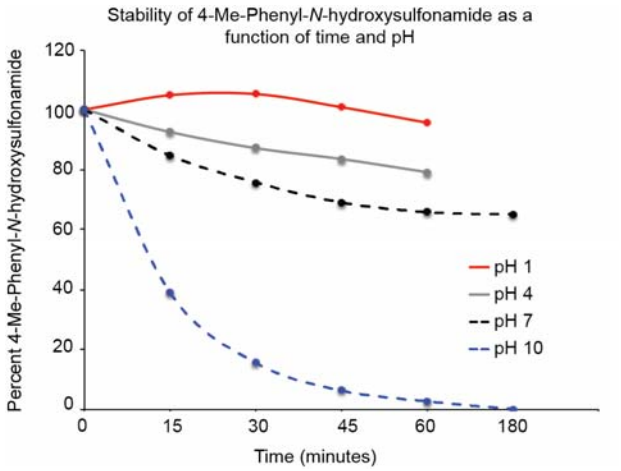
c.



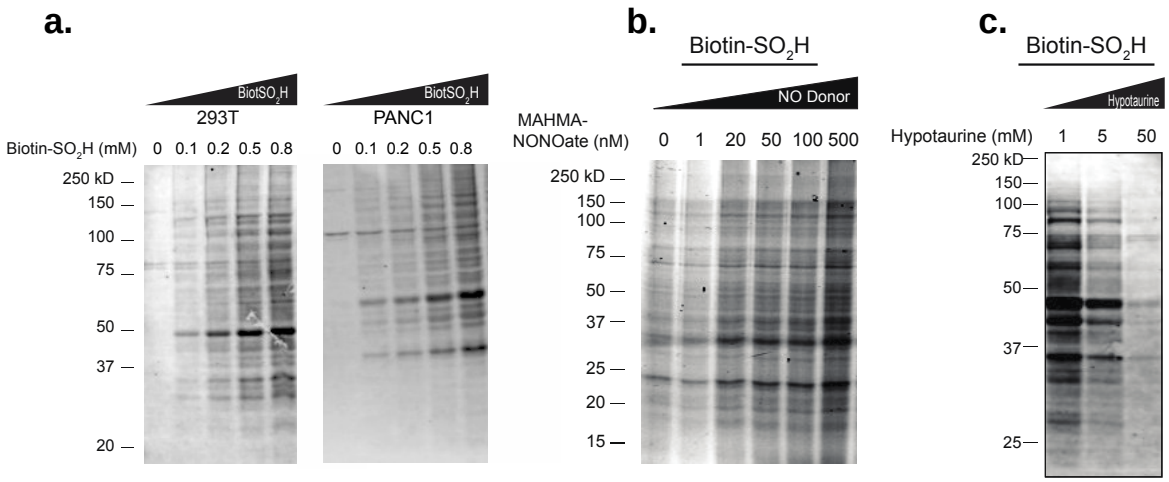
d.


Buffer Background (Avg of ESI and ESI Negative)
500 µM 4-methyl-Piloty's Acid (ESI Negative)
500 µM 4-methyl-phenylsulfinic acid (ESI Negative)
i. sulfonic acid
ii. sulfinic acid
500 µM Product Standard (ESI Positive)
Reaction Mixture (ESI Positive, 50:1 sulfnic acid : GSNO)
RxnMix-ESI Pos
Reaction Mixture (ESI Negative, 50:1 sulfnic acid : GSNO
RxnMix-ESI Neg
Ion Current (x10⁶)
Retention Time (minutes)

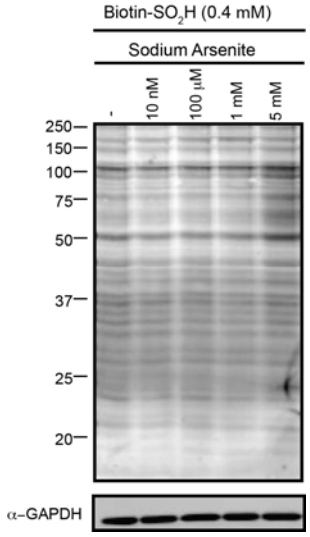
e.


Stability of 4-Me-Phenyl-N-hydroxysulfonamide as a function of time and pH
Percent 4-Me-Phenyl-N-hydroxysulfonamide
Time (minutes)
pH 1
pH 4
pH 7
pH 10

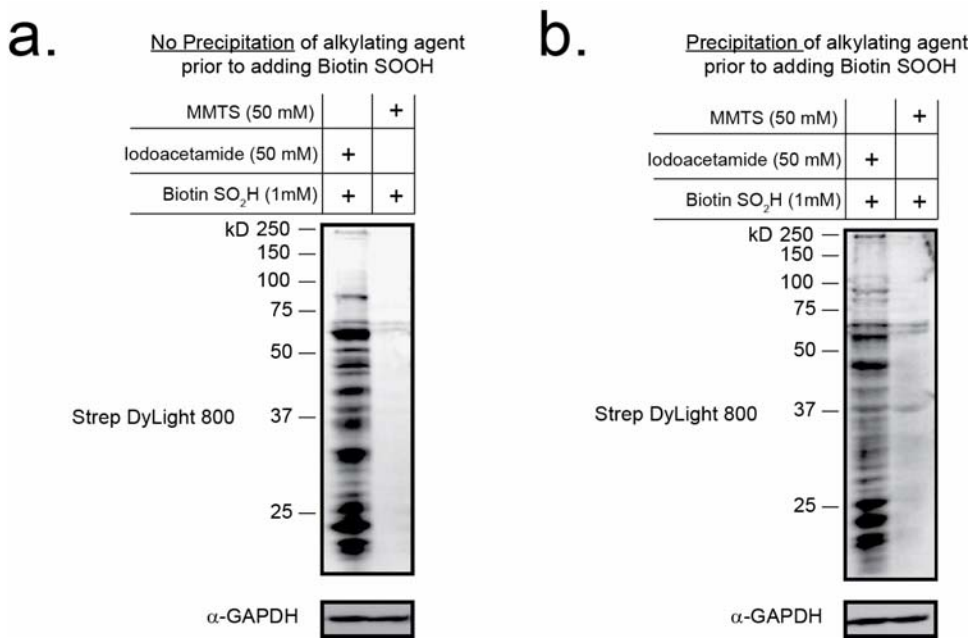
Supplementary Figure 6. Dose dependent labeling of S-nitrosated proteins in cell lysates. (a) *S*-Nitrosated proteins are labeled in a dose dependent manner by biotin-SO_2H. 293T and PANC1 cells were lysed in 6 M urea / PBS and alkylated with 50 mM iodoacetamide for 30 minutes, followed by incubation with increasing concentrations of biotin-SO_2H for 45 minutes. (b) Dose-dependent enhancement of *S*-nitrosation by the nitric oxide donor (MAHMA NONOate). 293T cell lysates were treated with increasing concentrations of nitric oxide donor, MAHMA NONOate for 5 minutes, followed by treatment with 50 mM iodoacetamide for 20 minutes to alkylate free thiols. *S*-nitrosated proteins were detected by incubation with fluorescein-SO_2H for 45 minutes, and separated by non-reducing SDS-PAGE for in-gel fluorescence detection. (c) Hypotaurine competes with biotin-SO_2H. 293T cell lysates were pre-incubated with 5 mM or 50 mM hypotaurine for 30 minutes, followed by treatment with 50 mM iodoacetamide for 30 minutes. The lysate was then labeled with biotin-SO_2H for 30 minutes, separated by SDS-PAGE, and transferred to nitrocellulose for streptavidin detection of endogenous *S*-nitrosation.



Supplementary Figure 7. Sodium arsenite does not affect biotin-SO_2H labeling of *S*-nitrosation. HEK 293T cells were lysed in 6M urea / PBS followed by alkylation of free thiols by 50 mM iodoacetamide (30 minutes, dark). Lysates were then treated with varying amounts of sodium arsenite (which reduces sulfenic acids) for a further 30 minutes. Lysates were then treated with 400 μM biotin-SO_2H for 30 minutes, and analyzed by non-reducing SDS-PAGE and streptavidin blotting. Sodium arsenite is reported to reduce protein sulfenic acids[9]. No reduction in labeling of protein nitrosothiols with arsenite can be interpreted as a selective reaction of the sulfinic acid probe with cellular nitrosothiols, and not with sulfenic acids.



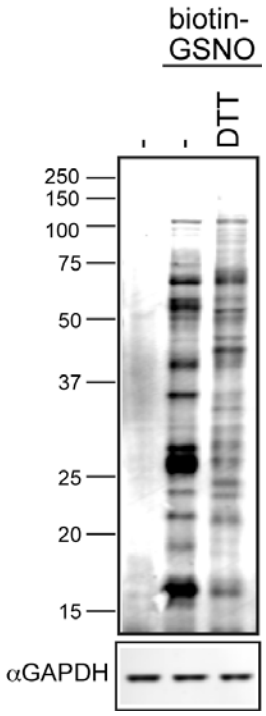
Supplementary Figure 8. MMTS alkylation prevents *S*-nitrosation detection by biotin-SO_2H. HEK293T cells were lysed in 6 M urea / PBS containing 1 mM EDTA and 100 μM neocuproine and normalized to 2 mg / mL. Lysates were either treated with 50 mM iodoacetamide or 50 mM MMTS for 30 minutes in the dark. Next, an aliquot of 20 μL was removed, diluted with 25 μL PBS and treated with biotin-SO_2H (1 mM) for 45 minutes. The remaining lysate was treated with chloroform-methanol to remove any residual iodoacetamide or MMTS by precipitating the proteins. Precipitated proteins were re-suspended in 6M urea / PBS and treated with 1 mM biotin-SO_2H for 45 minutes. Samples were separated by non-reducing SDS-PAGE, transferred to PVDF membrane, and imaged using streptavidin Dylight-800. (a) MMTS treatment eliminates biotin-SO_2H labeling of *S*-nitrosation. (b) Precipitation and removal of excess MMTS does not rescue biotin-SO_2H labeling of *S*-nitrosation.



Supplementary Figure 9. Biotin-SO_2H reacts with human GAPDH and forms a thiosulfonate. Recombinant human GADPH was labeled with biotin-SO_2H for site-specific analysis of *S*-nitrosation by high-resolution mass spectrometry. Briefly, 200 μL of 0.05 mg/mL recombinant and purified GAPDH was denatured in 6 M urea / PBS followed by treatment with 10 μM GSNO for 30 minutes in the dark. This was followed by incubation with 10 mM iodoacetamide for 30 minutes in the dark. *S*-nitrosated residues were labeled with 200 μM biotin-SO_2H for 30 minutes. Following this, the mixture was diluted 3-fold, trypsinized, desalted and analyzed by LC-MS as described in the experimental methods and details section. Since GSNO was added to denatured GAPDH, all three cysteines are detected as thiosulfonate products. This analysis confirms thiosulfonate occurs on proteins and can be detected by mass spectrometry.

Peptide (Site of Modification in bold red)	Modification	Peptide MH^+	Peptide Charge	Peptide *m/z*	Peptide RT (min)	# Frag	Mass Error (ppm)
VPTANVSVVDLT**C^{247}**RLEKPAK	C-SO_2-Ph	2280.2060	3	760.7402	55.85	25	6.22
IISNAS**C^{152}**TTN**C^{156}**LAPLAK	Carbamidomethyl (152 and 156)	1833.9360	2	917.4717	40.99	15	1.97
IISNAS**C^{152}**TTN**C^{156}**LAPLAK	C-SO_2-Ph (152), Carbamidomethyl (156)	1916.9121	2	958.9597	55.82	13	2.88
IISNAS**C^{152}**TTN**C^{156}**LAPLAK	Carbamidomethyl (152), C-SO_2-Ph (156)	1916.9104	2	958.9588	56.71	13	2.01
IISNAS**C^{152}**TTN**C^{156}**LAPLAK	C-SO_2-Ph (152), C-SO_2-Ph (156)	1999.8885	2	1000.4479	79.05	15	3.82

Supplementary Figure 10. Pre-treatment with DTT and iodoacetamide before biotin-GSNO labeling reports *S*-sulfinated proteins. 293T cells were lysed in 6 M urea / PBS, reduced with or without 2 mM DTT for 15 minutes, and alkylated with 50 mM iodoacetamide for 30 minutes in the dark. Lysates were then incubated with biotin-GSNO (~900 μM) for 30 minutes, followed by non-reducing SDS-PAGE separation and Western blot detection. Pre-treatment with DTT reduces disulfides, which are then alkylated with iodoacetamide. This is critical since any free glutathione will exchange with native disulfides, scrambling the resulting thiosulfonates at sites of *S*-sulfination. The observed molecular weight changes are due to reduction and alkylation of native mixed disulfides.



Supplementary Figure 11. *S*-nitroso-*N*-Acetyl-Cysteine methyl ester reacts with oxidized DJ-1 to form a thiosulfonate. Data was collected in MS^E mode.

Protein: Human PARK7 (DJ-1), recombinant

Peptide: GLIAAIC*****AGPTALLAHEIGFGSK

Modification: C* = -SO_2-S-*N*-Ac-Cys-OMe

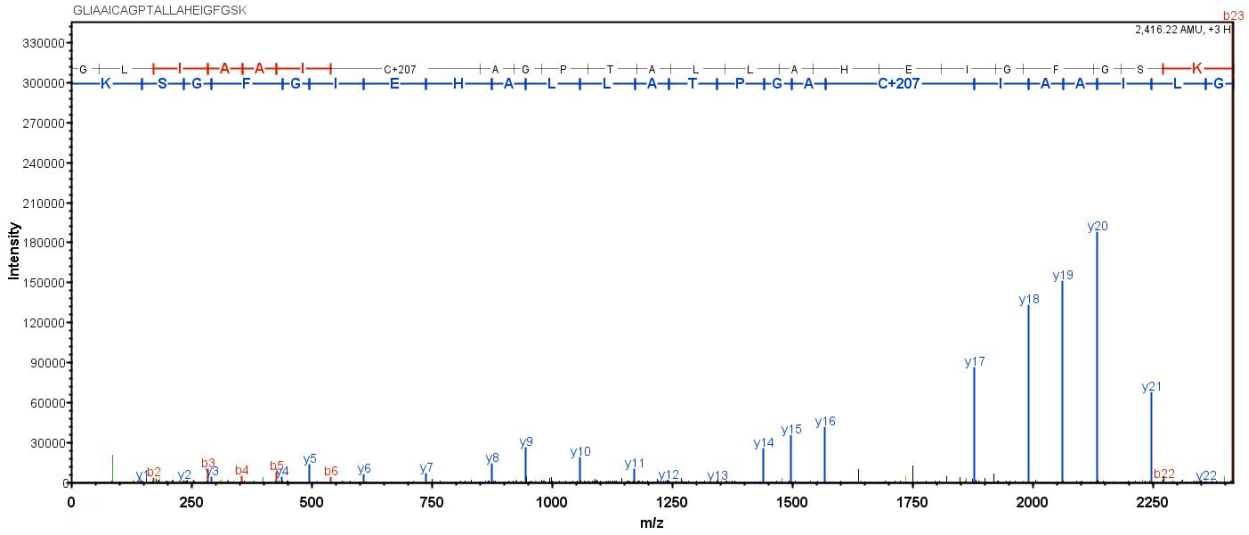
Retention Time: 73.49 minutes

Peptide Charge: +3

Peptide $[M+H]^+$: 2417.2234

Product Ions Observed: b2b3b4b5b6b7b14°b18b18°b22b23y1y2y2°y3y3°y4y5y5°y6y7y8y 9y9°y10y11y12y13y13°y14y15y15°y16y17y18y19y20y21y22y23

Mass Error: 1.2899 ppm



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