Supporting Information
for
Strained Cycloalkynes as New Protein Sulfenic Acid Traps

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General Considerations

All chemicals were purchased from Sigma Aldrich Chemical Company and used as received. TLC was performed on Sorbent polyester-backed Silica G plates with UV254 indicator. Visualization was accomplished with UV light unless otherwise indicated. Solvents for extraction and purification were of technical grade and used as received. Liquid chromatography–mass spectrometry (LC-MS) solvents were HPLC grade. For small molecule experiments, ESI-MS was performed on an Agilent 100 Series LC/MSD ion trap. $^1$H and $^{13}$C NMR spectra were recorded using a Bruker Avance 300 MHz NMR spectrometer or Bruker 500 MHz NMR spectrometer. Chemical shifts are given in ppm (δ); multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). UV–vis spectroscopy was performed on a Cary 50 UV-vis spectrophotometer.

Thermally generated organic-soluble cysteine-derived sulfenic acid + 1

A solution of diethyl 2-2-(((R)-3-((tert-butoxy)-2-((tert-butoxycarbonyl)amino)-3-oxopropyl)sulfinyl)propan-2-yl)malonate (150 mg, 0.30 mmol) and bicyclo[6.1.0]non-4-yn-9-ylmethanol (1, 12.5 mg, 0.083 mmol) in DCM (2 mL) was heated to 35 °C and stirred for 12 hours. The resulting solution was concentrated in vacuo and purified by column chromatography on silica gel (EtOAc:MeOH, 99:1) to afford diastereomeric tert-butyl (tert-butoxycarbonyl)(9-(hydroxymethyl)bicyclo[6.1.0]non-4-yn-4-yl)sulfinyl)-D-alaninate (2, 31 mg, 84%) as a colorless oil. Rf 0.5 (EtOAc:MeOH, 19:1, KMnO₄). ESI-MS cale of C$_{22}$H$_{37}$NO$_{6}$S [M+H]$^+$: 444.2; found 444.5. $^1$H NMR (300 MHz, CDCl$_3$): δ 6.44 (q, $J = 6.8$ Hz, 1H), 5.75 (br s, 0.5H), 5.50 (br s, 0.5H), 4.59 – 4.28 (m, 1H), 3.69 (d, $J = 7.5$ Hz, 2H), 3.20 (br s, 1H), 3.04 – 2.82 (m, 1H), 2.67 – 1.98 (m, 7H), 1.96 – 0.80 (m, 27H), $^{13}$C NMR (75 MHz, CDCl$_3$): δ 169.71, 169.43, 169.37, 169.43, 155.60, 155.37, 143.94, 143.80, 143.19, 136.32, 135.47, 135.07, 134.10, 83.32, 83.27, 83.08, 83.03, 80.42, 80.28, 77.56, 59.92, 59.88, 54.52, 53.87, 51.43, 50.88, 28.57, 28.23, 28.21, 28.02, 27.82, 27.54, 27.51, 24.35, 24.29, 24.22, 24.11, 24.07, 23.55, 23.51, 23.35, 23.30, 22.85, 21.65, 21.62, 21.37, 19.52, 19.25, 19.18, 19.13, 19.03, 18.84, 18.80.
Figure S1. Proton NMR of 2
A sealed tube was charged with DCM (2 mL), diethyl 2-((R)-3-((tert-butoxycarbonyl)amino)-3-oxopropyl)sulfinyl)propan-2-yl)malonate (175 mg, 0.35 mmol) and (E)-cyclooctene (78 mg, 0.71 mmol). The tube was heated to 40 °C and stirred for 12 hours. The resulting solution was concentrated in vacuo and purified by column chromatography on silica gel (EtOAc:hexanes, 1:2) to afford diasteromeric tert-butyl (tert-butoxycarbonyl)(cyclooctylsulfinyl)-D-alaninate (100 mg, 70%) as a colorless oil. Rf 0.4 (EtOAc:hexanes, 1:1, I2). ESI-MS calcd for C_{20}H_{37}NO_{5}S [M +Na]^+: 426.2; found 426.5. ^1H
NMR (CDCl₃, 500 MHz): δ 5.80 (d, J = 8.4 Hz, 1H), 5.61 (d, J = 6.3 Hz, 1H), 4.55 (td, J = 7.9, 3.5 Hz, 1H), 4.47 (q, J = 5.7 Hz, 1H), 3.26 (dd, J = 13.2, 5.8 Hz, 1H), 3.16 (dd, J = 13.0, 7.6 Hz, 1H), 2.96 (dd, J = 13.2, 4.9 Hz, 1H), 2.90 (dd, J = 13.2, 3.7 Hz, 1H), 2.84 – 2.74 (m, 1H), 2.06 – 1.94 (m, 2H), 1.94 – 1.70 (m, 4H), 1.70 – 1.57 (m, 5H), 1.49 – 1.34 (m, 29H). ¹³C NMR (126 MHz, CDCl₃) δ 169.74, 169.35, 155.67, 155.45, 83.38, 83.04, 80.41, 80.24, 60.54, 59.80, 51.48, 50.99, 50.83, 49.75, 28.58, 28.24, 28.22, 26.68, 26.66, 26.63, 26.23, 26.21, 26.17, 26.05, 26.02, 26.00, 25.87, 25.72.

Figure S3. Proton NMR of 5
Figure S4. Carbon NMR of 5

Treatment of Fries Acid with 1 to yield 3

A solution of Fries Acid (15 mg, 0.058 mmol), and bicyclo[6.1.0]non-4-yn-9-ylmethanol\(^2\) (1, 8.7 mg, 0.058 mmol) in MeCN (10 mL) was stirred at rt for 20 minutes.\(^3\) The resulting solution was concentrated *in vacuo* to yield diastereomeric 1-((9-(hydroxymethyl)bicyclo[6.1.0]non-4-en-4-yl)sulfinyl)anthracene-9,10-dione (3) which required no further purification (23.6 mg, 99%) as an orange solid. \(R_f\) 0.4 (EtOAc:MeOH, 19:1). ESI-MS calcd for C\(_{24}\)H\(_{22}\)O\(_4\)S [M +H]\(^+\): 407.1; found 407.4. \(^1\)H NMR (500 MHz, CDCl\(_3\)): \(\delta\) 8.72 (ddd, \(J = 25.9, 7.8, 1.3\) Hz, 2H), 8.48 (ddd, \(J =\)
10.4, 7.7, 1.3 Hz, 2H), 8.33 – 8.26 (m, 2H), 8.26 – 8.19 (m, 2H), 8.03 (dt, J = 10.0, 7.8 Hz, 2H), 7.88 – 7.74 (m, 4H), 7.13 – 7.03 (m, 2H), 3.58 (d, J = 7.4 Hz, 0.5H), 3.55 (d, J = 7.4 Hz, 0.5H) 3.52 – 3.40 (m, 3H), 2.60 (q, J = 5.5 Hz, 0.5H), 2.57 (q, J = 5.5 Hz, 0.5H), 2.49 – 2.34 (m, 4H), 2.34 – 2.22 (m, 2H), 2.15 – 2.02 (m, 2H), 1.93 (dq, J = 14.6, 5.4 Hz, 1H), 1.82 (dq, J = 14.4, 5.5 Hz, 1H), 1.59 – 1.39 (m, 5H), 1.39 – 1.28 (m, 2H), 1.23 – 0.93 (m, 5H), 0.77 (p, J = 16.6 Hz, 1H), 0.67 – 0.56 (m, 1H), 0.05 – -0.01 (m, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 183.15, 182.87, 182.67, 182.60, 148.58, 148.24, 144.94, 144.06, 141.30, 140.41, 135.08, 135.04, 134.91, 134.88, 134.86, 134.44, 134.38, 133.32, 132.78, 131.72, 131.67, 131.22, 131.14, 129.99, 129.84, 127.70, 127.68, 127.64, 127.53, 60.05, 59.87, 28.87, 27.47, 24.44, 23.74, 23.48, 23.43, 23.20, 21.91, 21.65, 21.08, 19.80, 19.59, 18.62, 17.56.

Figure S5. Proton NMR of 3.
Treatment of Fries Acid with tCOT to yield 6

A solution of Fries Acid (10 mg, 0.038 mmol), and (E)-cyclooctene (13 mg, 0.118 mmol) in MeCN (10 mL) in a sealed vial wrapped with foil was stirred for 12 hours. The resulting solution was concentrated \textit{in vacuo} and purified by column chromatography on silica gel (EtOAc:hexanes, 1:2) to afford 1-(cyclooctylsulfinyl)anthracene-9,10-dione (12.5 mg, 90%) as an orange solid. Rf 0.4 (EtOAc:hexanes, 1:1). ESI-MS calcd for C_{22}H_{22}O_{3}S [M +H]^+; 367.1; found 367.4. \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 300 MHz): δ 8.53 (dd, J = 7.9, 1.3 Hz, 1H), 8.46 (dd, J = 7.7, 1.3 Hz, 1H), 8.36 – 8.24 (m, 2H), 8.00 (t, J = 7.8 Hz, 1H), 7.90 – 7.79 (m, 2H), 3.09 (tt, J = 9.9, 3.8 Hz, 1H).
Hz, 1H), 2.66 – 2.48 (m, 1H), 2.28 – 2.11 (m, 1H), 2.02 – 1.02 (m, 16H) \(^{13}\text{C}\) NMR (75 MHz, CDCl\(_3\)) \(\delta\) 184.17, 182.68, 148.93, 135.22, 135.04, 134.86, 134.30, 133.40, 133.05, 131.84, 130.38, 129.61, 127.86, 127.71, 61.98, 31.09, 27.12, 26.66, 26.58, 26.45, 25.57, 22.80.

Figure S7. Proton NMR of 6
Figure S8. Carbon NMR of 6

Synthesis of 4

D-(+)-Biotin (72 mg, 0.29 mmol), hydroxybenzotriazole (7.9 mg, 0.058 mmol), and activated molecular sieves were added to a flask and subjected to three vacuum/argon purge cycles. Anhydrous DMF (2.5 mL) was added and the solution was heated to 60 °C for 30 minutes. The solution was cooled to rt and a solution of dicyclohexylcarbodiimide in DCM (0.32 mL, 0.32 mmol) was added dropwise and stirred for 3 h (solution goes cloudy). Bicyclo[6.1.0]non-4-yn-9-ylmethanol (53 mg, 0.35 mmol) and N,N-dimethylaminopyridine (0.4 mg, 0.003 mmol) were added and the flask was wrapped in aluminum foil, heated to 60 °C for 4 h, then stirred at rt for 24 h.\(^2\) The mixture was filtered and washed with DCM/MeOH (1:1) and the filtrate was concentrated in vacuo. Column chromatography on silica gel (EtOAc:MeOH, 19:1, KMnO\(_4\)) affords 4 (70 mg, 63%) as a colorless oil. R\(_i\) 0.25 (EtOAc:MeOH, 19:1). ESI-MS calcd for
C_{20}H_{28}N_{2}O_{3}S [M +H]^+: 377.18; found 377.2. $^1$H NMR (300 MHz, MeOD) $\delta$ 4.35 (ddd, $J = 7.9$, 5.0, 1.0 Hz, 1H), 4.16 (dd, $J = 7.9$, 4.5 Hz, 1H), 4.05 (d, $J = 8.2$ Hz, 2H), 3.06 (ddd, $J = 8.9$, 5.7, 4.5 Hz, 1H), 2.79 (dd, $J = 12.7$, 5.0 Hz, 1H), 2.57 (d, $J = 12.7$ Hz, 1H), 2.21 (t, $J = 7.2$ Hz, 2H), 2.15 – 1.94 (m, 6H), 1.71 – 1.38 (m, 7H), 1.40 – 1.18 (m, 4H), 0.90 – 0.66 (m, 2H). $^{13}$C NMR (75 MHz, MeOD) $\delta$ 175.79, 166.11, 99.66, 63.62, 63.46, 61.76, 61.64, 57.05, 41.08, 35.04, 30.20, 29.77, 29.53, 26.08, 21.96, 21.44, 18.59.

Figure S9. Proton NMR of 4
Stock solutions of Fries Acid (3 mM) in MeCN and 1 (30 mM) in MeCN were prepared. The Fries Acid stock (0.1 mL) was added to MeCN (0.8 mL) in a 1 mL UV-vis cuvette and lastly an aliquot of the stock solution of 1 (0.1 mL) was added, followed by a quick shake of the cuvette. The cuvette was immediately loaded into a Cary 50 UV-vis spectrophotometer and measurements began. This experiment was repeated 1x at identical concentrations and 2x at half the 1 concentrations (0.05 mL of 1 and 0.85 mL of MeCN). UV-vis data was recorded at 453 nm with 6 second intervals and averaged at each time point. SigmaPlot was utilized to plot data using exponential decay with the equation: \( f = y_0 + a e^{-bx} \).
Stock solutions of Fries Acid (3 mM) and \( \mathbf{1} \) (30 mM) were prepared in MeCN. The Fries Acid stock (0.1 mL) was added to MeCN (0.3 mL) and ammonium bicarbonate buffer (0.5 mL, 50 mM, pH 7.5) in a 1 mL UV-vis cuvette, then an aliquot of the stock solution of \( \mathbf{1} \) (0.1 mL) was added, followed by a quick shake of the cuvette. The cuvette was immediately loaded into a Cary 50 UV-vis spectrophotometer and measurements began. This experiment was repeated 1x at identical concentrations and 2x at half the concentration of \( \mathbf{1} \) (0.05 mL of \( \mathbf{1} \), 0.35 mL MeCN, and 0.5 mL buffer). UV-vis data was recorded at 453 nm with 6 second intervals and averaged at each time point. SigmaPlot was utilized to plot data using exponential decay with the equation: \( f = y_0 + a(e^{-bx}) \).
**UV-Vis Kinetics; Fries Acid + tCOT**

Stock solutions of Fries Acid (3 mM) and tCOT (60 mM) were prepared in MeCN. The Fries Acid stock (0.1 mL) was added to MeCN (0.8 mL) in a 1 mL UV-vis cuvette, then tCOT (0.1 mL) was added, followed by a quick shake of the cuvette. The cuvette was immediately loaded into a Cary 50 UV-vis spectrophotometer and measurements began. UV-vis data was recorded at 453 nm with 60 second intervals. SigmaPlot was utilized to plot data using exponential decay with the equation: \( f = y_0 + a(e^{-bx}) \).

![Figure S13. UV-Vis Kinetics; Fries Acid + tCOT](image)

### Reactivity of Fries Acid with Dimedone

Fries acid and NaOH were combined at an equimolar concentration of 0.3 mM (15% MeCN:85% H2O), resulting in a characteristic peak at 675 nm corresponding to the Fries acid anion. Addition of 1 equivalent (0.3 mM) of dimedone results in an immediate decrease of the anion peak and a simultaneous increase of an absorbance peak at ~460 nm. Addition of another equivalent (0.3 mM) of NaOH results in the immediate reformation of the Fries acid anion peak at 675 nm.

| ko \( s^{-1} \) | Conc. | \( k \) \( M^{-1} s^{-1} \) |
| --- | --- | --- |
| 0.0006 | 0.06 | 0.01 |

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For comparison, solutions of Fries acid (0.3 mM) with AcOH (3.0 mM) and Fries acid (0.3 mM) with dimedone (3.0 mM) were prepared (15% MeCN:85% H₂O, Figure S15). Additionally, the incubation of Fries acid anion with dimedone (10 mM each) for 2 h provided a bright orange-red solution with small amounts of an orange precipitate. Analysis of this solution by ESI-MS and NMR showed no sign of adduct formation. The precipitate is believed to be the thiosulfinate formed by the condensation of two sulfenic acids. Accurate identification of this precipitate was prevented by its insolubility in common solvents. The high similarity between peaks, facile reversibility, and the lack of NMR and MS evidence of adduct formation strongly suggest that the dominant reaction between Fries acid and dimedone is an acid/base reaction.
Figure S15. Fries Acid + Dimedone or AcOH

Reactivity of BCN with various Small Molecule Sulfur Oxoforms, General Procedure

A stock solution of BCN (133.3 mM) was prepared with HPLC grade MeCN. Additional stocks (133.3 mM) of reduced glutathione, s-nitrosoglutathione, oxidized glutathione, phenylsulfinic acid sodium salt, and phenylsulfonic acid were prepared with 50:50 MeCN:ammonium bicarbonate buffer (50 mM, pH 7.5). The BCN stock solution was combined with each oxoform stock separately to final concentrations of BCN (10 mM) and oxoform (10 mM) in 50:50 MeCN:ammonium bicarbonate buffer (50 mM, pH 7.5). The solutions were analyzed by ESI-MS (positive ion mode) for disappearance of BCN and sulfur oxoform at both 1 h and 24 h. No change in ESI-MS was observed. UV-Vis tracking of s-nitrosoglutathione at 335 nm showed no decrease over a 24h period. Additionally, the PhSO₂Na and PhSO₃H solutions were concentrated in vacuo and determined to be unchanged via ¹H NMR.
Stability of BCN-Alkenylsulfoxide to Common Reducing Agents, General Procedure

Sulfoxide 2 was combined with DTT and TCEP in separate solutions to final concentrations of 10 mM each in 50:50 MeOH:ammonium bicarbonate buffer (50 mM, pH 7.5). The reactions were monitored by TLC for disappearance of 2 at 1 h, 24 h and 72 h. No change in TLC was observed, and the solutions were concentrated in vacuo and analyzed by $^1$H NMR, revealing no change in 2. Additional analysis via ESI-MS showed no change in the MS spectrum, with strong signals (m/z) for 2 at 444.3 [M+H]$^+$ and 466.3 [M+Na]$^+$.

**Generation of AhpC-SOH and AhpC-SO$_2$H**

The C165A mutant of *Salmonella typhimurium* AhpC was overexpressed and purified from *E. coli* as previously described.$^6,7$ Mutant AhpC was pre-reduced with DTT (10 mM) for 30 min at ambient temperature, and DTT was then removed by passing the solution through a Bio-Gel P6 spin column equilibrated with ammonium bicarbonate (50 mM). Protein concentration was determined using the solution absorbance at 280 nm ($\varepsilon = 24,300$ M$^{-1}$ cm$^{-1}$).$^6$ The sulfenic acid species was generated via treatment with 1 equivalent of hydrogen peroxide for 30-45 seconds at room temperature (pH 7-7.5 buffer) with quenching by passage through a Bio-Gel spin column equilibrated with ammonium bicarbonate (50 mM). The sulfinic acid was generated via treatment of AhpC-SH with 2 equivalents of hydrogen peroxide for 45-75 seconds at room temperature with similar removal of unreacted substrate. Formation of each oxidized species was confirmed by ESI-TOF MS.

**Generation of AhpC-SNO and AhpC-S-S-Cys**

C165A AhpC was pre-reduced as detailed above then transnitrosated with freshly prepared S-nitrosocysteine in HEPES/DTPA buffer (25 mM HEPES, 1 mM DTPA, pH 7.7) for 1 h in the dark as previously described.$^8$ Cys-SNO was removed using Bio-Gel spin columns equilibrated with ammonium bicarbonate (50 mM) and formation of AhpC-SNO and the mixed disulfide AhpC-S-S-Cys were confirmed by ESI-TOF MS.
Reactivity of AhpC oxoforms with strained cyclooctynes

Strained cyclooctyne probes were pre-treated with immobilized TCEP for 1 h at room temperature, then spun to concentrate beads and retain supernatant. Freshly prepared AhpC-SOH (40 µM), AhpC-SO₂H (40 µM), or the AhpC-SNO/AhpC-S-S-Cys mixture (40 µM total protein) was incubated at room temperature in the presence of the strained alkyne probe (100 µM) in ammonium bicarbonate buffer (50 mM, pH 7.5). Reactions involving AhpC-SNO were protected from light. Aliquots (40-50 µL) were quenched at various time points by passage through Bio-Gel spin columns pre-equilibrated with either 0.1% formic acid in water or ammonium bicarbonate (50 mM, pH 7.5) for analysis by ESI-TOF MS.

Reactivity of the C165A AhpC-S(O)-4 adduct with H₂O₂ and reductants

C165A AhpC-SOH (40 µM) was freshly prepared as detailed above, then treated with 4 (250 µM) at pH 7.5 for 4 h (r.t., 50 mM NH₄HCO₃) to ensure maximum adduct formation, then incubated overnight at 4°C in the presence of H₂O₂ (750 µM) to convert unreacted protein species to the irreversible oxoforms sulfinic and sulfonic acid. The resulting mixture was passed through a Bio-Gel spin column to remove 4 and H₂O₂ and analyzed by ESI-TOF MS for both the amount of AhpC-S(O)-4 adduct and to demonstrate the stability of the adduct toward excess H₂O₂. AhpC labeled with 4 (40 µM protein) was then treated with reductants DTT, TCEP, β-mercaptoethanol, glutathione, and N-acetyl cysteine (1 mM each) at pH 7.5 (1 h, r.t.). An aliquot from each reaction was analyzed by SDS-PAGE (using non-reducing 4x Laemmli buffer) and Western blotting, while the remainder of each sample was quenched by passage through Bio-Gel spin columns pre-equilibrated with 0.1% formic acid in water for analysis by ESI-TOF MS.

Electrospray ionization time-of-flight mass spectrometry

ESI-TOF MS analyses were performed on an Agilent 6120 MSD-TOF system operating in positive ion mode with the following settings: capillary voltage of 3500 V, nebulizer gas pressure of 30 psig, drying gas flow of 5 L/min, fragmentor voltage of 175 V, skimmer voltage of 65 V, and gas temperature of 325°C. Samples were introduced via direct infusion at a flow rate of 20 µL/min using a syringe pump (KD Scientific). Mass spectra were averaged and deconvoluted using the Agilent MassHunter Workstation software v B.02.00.

nanoLC-MS/MS analysis

Digestions were performed at pH 7.5-8.0 using Trypsin Gold (Promega) overnight at 37°C. The resulting peptides were analyzed on a Dionex UltiMate3000 splitless nanoLC system coupled to a Thermo Orbitrap Velos Pro high-resolution mass spectrometer. Peptides were separated using a gradient of buffer A (0.1% formic acid/2% acetonitrile/98% water) and buffer B (0.1% formic
acid/20% water/80% acetonitrile) over 60 minutes (2 to 85% B) at a flow rate of 300 nL/min with the column held at 35°C. Eluant was introduced to the mass spectrometer via positive nanospray ESI with the following settings: capillary temperature 200°C, spray voltage 1.8 kV, spray current 100 mA. The mass spectrometer was operated in data-dependent acquisition mode using Xcalibur v. 2.1 (Thermo). After a full scan (150-2000 m/z range) at high resolution (60,000), the top 15 most intense precursor ions were isolated and fragmented using collision-induced dissociation (CID). Dynamic exclusion was enabled with a repeat duration of 30 seconds and an exclusion duration of 9.5 seconds. The normalized collision energy was set at 35%, activation Q at 0.25, and activation time at 10 ms. Acquired raw data were processed using Proteome Discoverer v 1.4 (Thermo).

**General SDS-PAGE and Western blot procedures**

Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (0.45 µm, BioRad). Blocking was achieved with 5% (w/v) BSA (5% nonfat milk for AhpC detection) in TBS buffer containing 0.1% Tween 20 (TBS-T). Protein biotinylation was detected by overnight incubation with anti-biotin HRP (Cell Signaling Technologies) diluted 1:1000-1:2000 in 5% (w/v) BSA in TBS-T. AhpC content was detected using anti-AhpC (1:2000) in 5% (w/v) non-fat milk in TBS-T. B-actin levels were detected using anti-β-actin (1:10,000) (Cell Signaling Technologies). The secondary antibody used for detection of AhpC and β-actin was anti-rabbit HRP (1:2000-1:5000 in 1% BSA, TBS-T) (Cell Signaling Technologies). Western blots were developed using Western Lightning Plus ECL (PerkinElmer) or WesternBright ECL (Bioexpress) reagents followed by exposure to autoradiography film (BioExpress).

**Labeling of protein SOH in lysate using 4**

Human squamous cell carcinoma cells (SCC-61) were cultured in DMEM/F12 medium supplemented with 10% FBS (Invitrogen) at 37°C and 5% CO₂. Cells were lysed with modified RIPA (mRIPA) buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 15 mM NaCl, 1 mM EDTA, 1 mM NaF, supplemented with protease and phosphatase inhibitor tablets (Roche)) and incubated on ice for 1 h followed by centrifugation at 13,000 rpm for 10 min. The supernatant was retained, and as needed, passed through Bio-Gel spin columns pre-equilibrated with PBS. Immobilized TCEP resin (Thermo; 0.4 mL of suspension) was placed in a clean tube and pre-washed twice with 0.5 mL of 100 mM NH₄HCO₃ then once with 0.5 mL of 50 mM citrate bis-Tris propane pH 8.5. Washes consisted of resuspending resin by vortexing, spinning to collect resin, and removing supernatant. Neutralization of TCEP resin was confirmed by monitoring pH of supernatant. Lysate flow-through in PBS was combined (460 µL of 1.24 mg/mL) and added to the pre-washed TCEP resin. The mixture was rotated end-over-end at r.t. for 45 min, and beads were then removed by filtration. The reduced protein solution was spiked with C165A AhpC (9 µg) for loading control and aliquotted in 60 µg fractions. Lysates were
treated with 100 µM of 4 and the following H₂O₂ concentrations: 2.5, 5, 10, 20, 50, and 500 µM. A control sample containing 60 µg of lysate was treated with 5 mM TCEP to prevent protein oxidation then 100 µM of 4 was immediately added. Two additional controls included untreated lysate and lysate incubated with 100 µM of iodoacetyl-LC-biotin (Thermo). Final reaction volumes were 0.1 mL, and all samples were incubated for 30 min at r.t. in the dark. Reactions were quenched via the addition of 4x Laemmli buffer containing 10% (v/v) 2-mercaptoethanol.

Labeling of protein SOH at lysis with 4

SCC-61 cells were cultured as detailed above. One hour before treatment, media was removed and replaced with media devoid of FBS. Media was removed, cells were washed twice with ice cold PBS, then lysed with mRIPA (0.2 mL) in the presence of TCEP (10 mM) or 4 (0.1 mM) and incubated for 30 min on ice. Compound 4 was then added to the TCEP-treated lysates to a final concentration of 0.1 mM. DMSO was present at 0.2% (v/v) and all reaction times were limited to 30 min on ice (all incubations with 4 were performed simultaneously). Each treatment was performed in triplicate. Lysates were clarified via centrifugation at 13,000 rpm for 10 min at 4°C and excess probe was removed as described above. Samples were normalized and replicates analyzed individually by reducing SDS-PAGE and Western blotting for protein biotinylation and β-actin levels (loading control).

Cell viability assay

Cell viability was determined using SCC-61 cells and a modified MTT assay. Briefly, 50,000 cells per well were plated in 24-well plates and incubated overnight. Cells were then treated with either dimedone (0.1 to 3.0 mM) or 4 (0.01 to 1 mM) for 48-72 h. The SOH probes were added as solutions in DMSO to the media such that the final concentration of DMSO was < 0.3%. At the end of the incubation period, MTT was added and the cultures were incubated for 4 h at 37°C. The dark crystals formed were dissolved by the addition of an equal volume of sodium dodecyl sulfate/dimethylformamide (SDS/DMF) extraction buffer. Plates were then incubated overnight at 37°C. A 100 µL aliquot of each soluble fraction was transferred into a 96-well microplate, and the absorbance at 570 nm was measured. The mean absorbance of the biological replicates (3 replicates for 4; 4 replicates for dimedone) was calculated and survival data is reported relative to untreated control cells.
Labeling of protein SOH in live cells using 4 and DCP-Bio1

SCC-61 cells were cultured in 6-well plates as detailed above. One hour before treatment, media was removed and replaced with media devoid of FBS. For treatment, media was removed, and each well treated in triplicate with 1 mL media containing various concentrations of 4 (25 µM, 50 µM, 100 µM) or DCP-Bio1 (25 µM, 100 µM, 1 mM). DMSO was present at a final concentration of 0.2% (v/v). The cells were then incubated for 30 min at 37°C and 5% CO₂. The media was removed, the cells washed twice with ice cold PBS, then lysed with mRIPA buffer 1 h on ice followed by centrifugation at 13,000 rpm for 10 min at 4°C. Excess probe was removed using Bio-Gel spin columns pre-equilibrated with PBS, samples were normalized, and replicates analyzed individually. Proteins were separated by reducing SDS-PAGE and analyzed by Western blotting for protein biotinylation and β-actin levels (loading control).
Figure S16. Treatment of C165A AhpC-SO$_2$H with 4. A: Untreated sample of C165A AhpC-SO$_2$H (40 μM, 20616 amu) at pH 7.5. B-C: Incubation of AhpC-SO$_2$H (40 μM) with 4 (100 μM) at r.t. and pH 7.5. B: 10 min, C: 60 min. A small amount of AhpC-SOH/alkyne adduct is observed at 20976.5 amu, likely resulting from the minor SN and SOH species observed in Panel A prior to treatment.
Figure S17. Treatment of C165A AhpC-SNO and C165A AhpC-S-S-Cys with 4. A: Formation of C165A AhpC-SNO (20612.7 amu) and C165A AhpC-S-S-Cys (20702.9 amu). B: Incubation of AhpC-SNO and AhpC-S-S-Cys (40 µM total protein) with 4 (100 µM) for 80 min at r.t. (in dark) and pH 7.5. C: Untreated sample of AhpC-SNO and AhpC-S-S-Cys (40 µM total protein) at 80 min at r.t. (in dark) and pH 7.5.
Figure S18. Treatment of C165A AhpC-SH with 4. **A**: Untreated sample of C165A AhpC-SH (40 μM, 20584.6 amu) at pH 7.5. **B**: Incubation of AhpC-SH (40 μM) with 4 (100 μM) and TCEP (5 mM) for 60 min at r.t. and pH 7.5.

Figure S19. Western blot analysis in non-reducing conditions of C165A AhpC cysteine oxoforms (40 μM) incubated with 4 (100 μM) for 1 h at r.t. and pH 7.5.
Figure S20. Polymeric addition to AhpC-SOH with high concentration of 4; ESI-TOF MS analysis of the reaction of C165A AhpC-SOH (40 µM) and 4 (5 mM) at 15 min (rt in 50 mM NH₄HCO₃, pH 7.5).

Figure S21. Positive ion MS² (CID) spectrum of the Cys-containing peptide of C165A AhpC resulting from the reaction of AhpC-SOH (40 µM) with 1 (100 µM) at pH 7.5. Protein species were digested with trypsin and the resulting peptides separated by nanoLC.
Figure S22. Positive ion MS$^2$ (CID) spectrum of the Cys-containing peptide of C165A AhpC resulting from the reaction of AhpC-SOH (40 µM) with 4 (100 µM) at pH 7.5. Protein species were digested with trypsin and the resulting peptides separated by nanoLC.
Figure S23. Stability of the C165A AhpC-S(O)-4 adduct (40 μM) toward biochemical reductants (1 mM) as judged by ESI-TOF MS. Reactions were performed in 50 mM NH₄HCO₃ (pH 7.5) for 1 h at r.t. A: Untreated control of C165A AhpC-S(O)-4 generated via the reaction of C165A AhpC-SOH (40 μM) with 4 (250 μM) (4 h, pH 7.5, r.t.) followed by treatment with H₂O₂ (750 μM) overnight (4°C). H₂O₂ and 4 were removed, and the protein species were treated with B: 1 mM DTT. C: 1 mM TCEP. D: 1 mM β-mercaptoethanol. E: 1 mM reduced glutathione. F: 1 mM N-acetyl cysteine. The species at 20,616 amu is C165A AhpC-SO₂H, and the species marked with an asterisk (*) at 20,632.9 amu is C165A AhpC-SO₃H. Percent abundance values for the C165A AhpC-S(O)-4 adduct were determined using the ion abundance relative to the sum of ion abundance values for all relevant protein species observed in each mass spectrum.
Figure S24. Western blot analysis of AhpC-S(O)-4 adduct stability in the presence of reductants: DTT, TCEP, β-mercaptoethanol, glutathione, and N-acetyl cysteine (1 mM each, 1 h at r.t.). Reducants were not removed before SDS-PAGE and Laemmli buffer without additional reductant was used to prepare samples.

Figure S25. Labeling of endogenous protein SOH using 4 -/+ TCEP. Where indicated, SCC-61 cells were lysed in mRIPA buffer containing TCEP (10 mM) for 30 min on ice, then treated with 4 (0.1 mM) for an additional 30 min on ice (n = 3). For the TCEP-untreated samples, cells were lysed in mRIPA buffer containing 4 (0.1 mM) for 30 min on ice (n = 3). DMSO was present at 0.2% (v/v) in all samples. Lysates were clarified by centrifugation at 4°C, then reactions quenched by the removal of unreacted TCEP and 4.

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