Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Material

Simple and Effective Strategy for Labeling Cysteine Sulfenic Acid in Proteins By Utilization of ß-ketoesters as Cleavable Probes

Jiang Qian, Revati Wani, Chananat Klomsiri, Leslie B. Poole, Allen W. Tsang,

and Cristina M. Furdui

General

^{1}H NMR, ^{13}C NMR (300 MHz) spectra were recorded in $CDCl_3$ on a Bruker DPX-300. Synthesized compound was analyzed by Electrospray Ionization (ESI) mass spectrometry (ESI-MS) on a Thermo LTQ instrument. ESI-TOF MS analysis was applied to monitor AhpC labeling by the reagents described here. An Agilent MSD TOF system was used for these studies.

Experimental

Synthesis of but-3-yn-1-yl 3-oxobutanoate (2)

Methyl acetoacetate (1 g, 8.6 mmol) was dissolved in 40 mL dry toluene. Boric acid (53 mg, 0.86 mmol) and 3-butyn-1-ol (1 g, 14.3 mmol) were added to the methyl acetoacetate/toluene solution under a N_2 atmosphere and the mixture was refluxed for 24 hr using a Dean-Stark condenser. Solvents were then removed under reduced pressure. The crude product was purified by flash column chromatography (SiO_2, hexane/ethylacetate: 15/1) to obtain compound **2** (950 mg, 73% yield), a pale yellow to colorless liquid. ^{1}H NMR 300 MHz (ppm, $CDCl_3$): 4.21 (2H, t J = 6.8 Hz), 3.48 (2H, s), 2.59-2.54 (2H, dt J = 6.8, 2.7 Hz), 2.29 (3H, s), 2.00 (1H, t J=2.7). 13C NMR ($CDCl_3$, 75 MHz): 200.22, 166.83, 79.70, 70.12, 62.99, 49.92, 30.16, 18.89. ESI-MS: $(M+H)^+$ = m/z 155.18 (calculated MW of $C_8H_{10}O_3$: 154.16 Da).

Generation of C165S AhpC-SOH protein

The C165S AhpC was reduced in 10 mM DTT at room temperature for 30 min and the DTT was then removed by passing the protein solution through a Bio-Gel spin column equilibrated with ddH_2O. The protein concentration was measured based on absorbance at 280 nm using the extinction coefficient 24,300 ($M^{-1}cm^{-1}$). Typically, 200-400 μM C165S AhpC protein was oxidized to –SOH by incubating with 2 equivalents H_2O_2 for 1 min at r.t. with mixing. The excess H_2O_2 was removed using Bio-Gel spin columns equilibrated with ddH_2O.

C165S AhpC-SOH labeling with 1 and reaction with NH_2OH

The C165S AhpC-SOH protein generated as described above was diluted to 50 μM in 50 mM Bis-tris-citric acid buffer (pH 7.4) containing 5 mM (final concentration) of reagent **1**. After 3 h incubation with mixing, 30 μL aliquot of reaction mixture was passed through a Bio-Gel column equilibrated with 0.1% formic acid for ESI-TOF MS analysis. For the hydroxylamine cleavage reactions, labeling of C165S AhpC-SOH with **1** was conducted in 50 mM Bis-tris-citric acid buffers (pH 8.5) for 4 h. The covalent C165S AhpC-**1** adduct was incubated with 50 mM - 1 M hydroxylamine (stock 1N hydroxylamine, pH 8.0-8.5, adjusted by adding 2 N NaOH or 1 N ammonium bicarbonate) for 1 h at 37°C before passing through the Bio-Gel column for ESI-TOF MS analysis.

Control experiments using AhpC-SH, AhpC-$SO_{2/3}H$, and WT AhpC proteins

Reduced form of C165S AhpC (C165S AhpC-SH), hyperoxidized protein (C165S AhpC-SO_2H and –SO_3H) and WT AhpC (disulfide crosslinked dimer, WT AhpC-S-S-AhpC) in 50 mM Bis-tris-citric acid buffers (pH 7.4) were incubated with **1** (5 mM) for 1.5 h at r.t., pass through the Bio-Gel spin column and analyzed by ESI-TOF MS. C165S AhpC-SO_2H and –SO_3H were generated by incubating C165S AhpC-SH (250 μM) in ddH_2O with 20 equivalents H_2O_2 for 1h at r.t. with mixing. The excess H_2O_2 was removed using Bio-Gel spin columns and protein concentration was determined based on absorbance at 280 nm using the extinction coefficient 24,300 ($M^{-1}cm^{-1}$).

C165S AhpC-SOH labeling with dimedone and 2

pH dependence. The C165S AhpC-SOH protein was immediately aliquot to appropriate buffers: 50 mM Bis-tris-citric acid pH 7.4, 8.5, and 9.5, and 25 mM critic acid-Na_2HPO_4 buffers pH 5.5, and 6.5. Final concentrations of 50 μM protein and 5 mM labeling reagents (dimedone or **2**) were used for all reactions. Dimedone stock was made in 0.5 M Bis-tris and DMSO (v:v=1:1) to pre-buffer its acidity around 6.5 before adding it to the reaction buffers. Final DMSO concentration during labeling was 0.5%. At the end of the incubation time (4 h for data in Fig. 1 – pH 7.4 and 8.5; 90 min for data in Fig. S5A – pH 5.5 - 9.5), 30 μL reaction mixture at each pH was passed through a Bio-Gel spin column and analyzed by ESI-TOF MS. Data were fit using SigmaPlot 11.0 and the user defined equation below to obtain the pKa:

$$Y = \frac{Limit_1 + Limit_2 * 10^{(pH-pKa)}}{10^{(pH-pKa)} + 1}$$

Kinetic studies. The C165S AhpC-SOH was incubated with increasing concentrations of **2** (0.2, 0.5, 1, 5, and 10 mM) for 2 h in 50 mM Bis-tris-citric acid (pH 7.4) at r.t. The reaction mixture (30 μL) was passed through a Bio-Gel spin column and analyzed by ESI-TOF MS. Data were fit to a hyperbolic equation to determine $K_{0.5}$ using SigmaPlot 11.0.

Click reaction to add the biotin tag to C165S AhpC adduct with 2 (AhpC-2)

C165S AhpC-SOH protein was first labeled by **2** at pH 9.5 for 1h and passed successively through three Bio-Gel spin columns equilibrated with PBS to remove the unreacted **2**. Near 100% labeling of AhpC was confirmed by ESI-TOF MS. The C165S AhpC-**2** adduct concentration was measured at 280 nm using the extinction coefficient 24,300 ($M^{-1}cm^{-1}$) and then diluted in PBS to a final concentration of 50 μM. Click reaction was conducted by adding the following reagents in order: biotin-OEG_{11}-azide (1 mM, final, from 50 mM stock in DMSO), TCEP (2 mM, final, from 100 mM stock freshly made in H_2O), TBTA (0.1 mM, final, from 1.7 mM stock made in 1:4 DMSO/t-butanol) and $CuSO_4$ (1 mM, final, from 50 mM stock in H_2O). The mixture was incubated at r.t. for 2 h and then 30 μL of reaction mixture was passed through a Bio-Gel column equilibrated with

0.1% formic acid for ESI-TOF MS analysis. The biotin moiety was removed by hydroxylamine treatment as described above and the reaction products were analyzed by ESI-TOF MS.

Labeling of –SOH proteins in NIH 3T3 cell lysates with 2

pH dependence. NIH 3T3 cells were cultured to 95% confluence in complete media (DMEM High Glucose (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin & streptomycin (Invitrogen)). Cells were washed with PBS three times and lysed using 5 mM of **2** in lysis buffer (50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% Glycerol, 1% Triton X-100, 25 mM NaF,10 μM $ZnCl_2$, supplemented with protease and phosphatase inhibitor tablets (Roche)). Lysis buffer was made in 50 mM citric acid/Na_2HPO_4 when labeling was carried out at pH 5.5 and 7.4, and in 50 mM $NaHCO_3$/Na_2CO_3 for labeling at pH 9.5. Lysates were incubated for 2 h at r.t. and then quenched by adding cold MeOH/$CHCl_3$ [1] to remove excess **2**.

Selectivity studies. Cells were lysed using lysis buffer (pH 7.4) supplemented with **2** (5 mM) as described above. The labeling was allowed to proceed at r.t. for 2 h before quenching with MeOH/$CHCl_3$. Protein pellets precipitated from 200 μL lysate were resuspended in 50 μL 1% SDS in PBS, sonicated for 30 s and finally heated at 95 °C for 5 mins. Protein concentration was measured using Bio-Rad protein assay (SDS concentration in the assay 0.001%) and normalized to the same concentration. Click reaction was performed using the solubilized lysate proteins according to the procedures described above. After 2 h incubation, 15 μL reaction aliquot from each sample was mixed with 5 μL sample buffer (with ß-mecaptoethanol) for SDS-PAGE and Western blot analysis. Biotinylated proteins were probed using Streptavidin-HRP. In control experiments, lysates were pre-reduced with 10 mM TCEP at pH 7.4 for 1 h at r.t. before the addition of **2**; lysates prepared in the absence of **2** with or without click reaction were also included in the SDS-PAGE and Western blot analysis.

DCF assay

NIH 3T3 cells cultured in 60-mm dishes were serum starved overnight and incubated with 10 mM dimedone, reagent **2** or 1% DMSO individually for 2 h. 5-(and-6)-carboxy-2′,7′-dichloro-

dihydrofluorescein diacetate (carboxy-H2DCFDA) (100 μM; Invitrogen) was added to cells at room temperature after the incubation. The cells were then immediately washed twice with PBS and visualized with Arcturus PixCell II laser capture microscope using 20x objective.

Cell membrane permeability assay

SCC-61 cells were cultured to 95% confluence in complete media (DMEM/F12 High Glucose (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% penicillin & streptomycin (Invitrogen)). Cells were washed with HBSS buffer (pH 7.4) three times and incubated with 10 mM probe **2** in HBSS at r.t. for 2 h. Cells incubated in HBSS without **2** were included as controls. After the incubation, cells were carefully washed by HBSS three times to remove excess **2** and lysed with the lysis buffer described above except in the absence of **2**. Lysates were incubated at r.t. for additional 2 h to ensure complete labeling prior to the addition of cold MeOH/$CHCl_3$ for protein precipitation. The denatured proteins were resolubilized in 1% SDS (in PBS) for the click reaction, which was then followed by Western-blot analysis as described above.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to test the SCC-61 cell viability when incubated with dimedone or **2** for 1 h. SCC-61 cells were seeded in a 96-well plate at a density of 5,000 per well in 50 μL and incubated overnight. Dimedone or **2** (50 μL) was added to each well at final concentrations of 1, 2.5, 5, 10, and 20 mM. Plates were then incubated for 1 h at 37 °C in a humidified atmosphere with 5% CO_2. MTT labeling reagent (Roche; 5 mg/ml in PBS, 10 μL) was added to each well, and the plate was incubated for 4 h at 37 °C. The solubilization solution (100 μL) was then added to each well for overnight incubation at 37 °C. The following day, absorbance was measured at 570 nm and all experiments were repeated twice. The resulting curves were fitted using SigmaPlot software to a sigmoidal dose-response equation to determine the IC50. The IC50 represents the concentration of chemical probe causing a 50% reduction in cell viability.

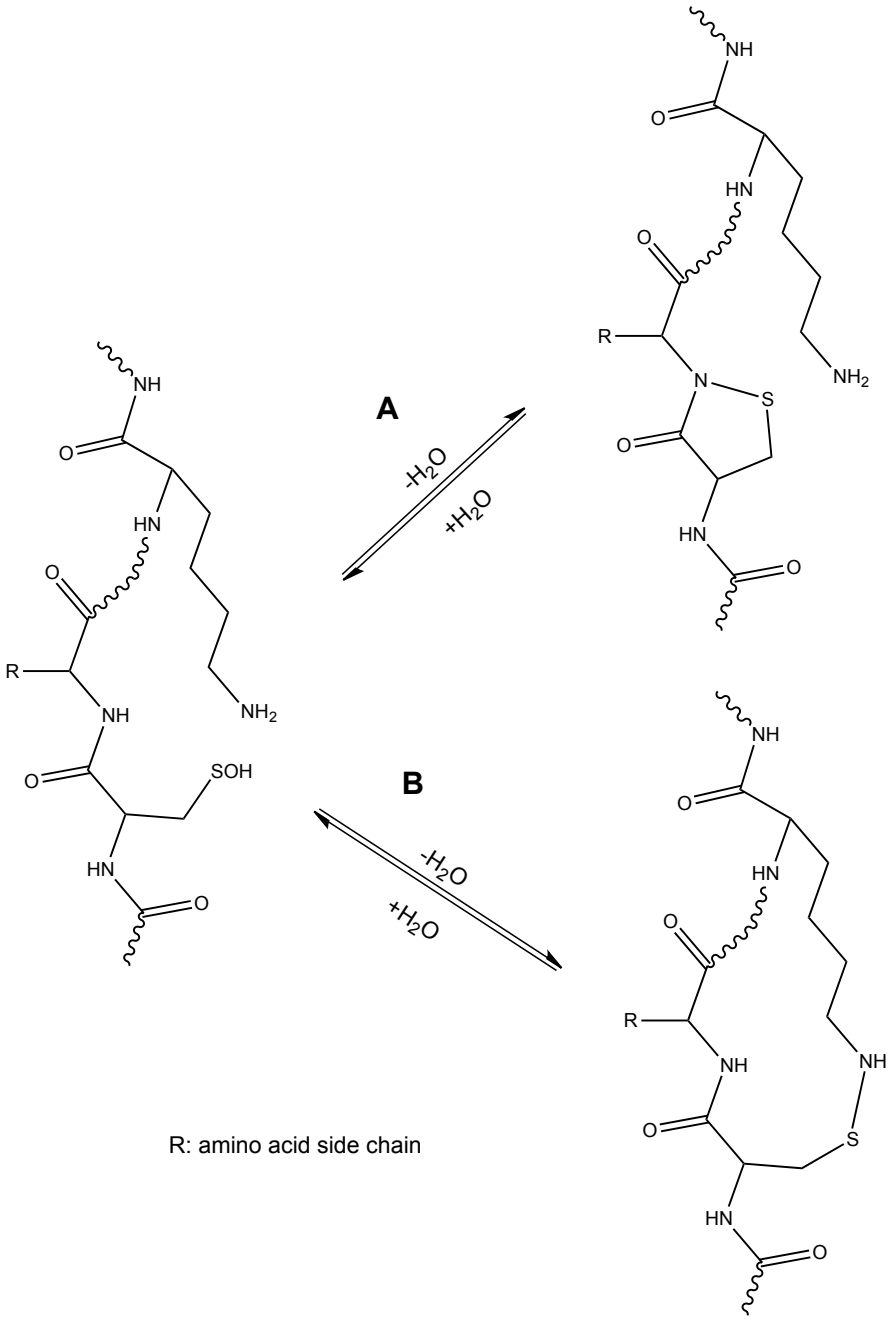
LC-MS/MS analysis of C165S AhpC labeled by 3-methyl-5-isoxazolone

C165S AhpC labeled with 3-methyl-5-isoxazolone was digested using AspN and the resulting peptides were analyzed on a nano-liquid chromatography (LC) system (Dionex Ultimate3000) coupled to a Thermo ESI LTQ mass spectrometer. Peptides were separated using a 60-min gradient of buffer A (0.1% formic acid/97% water/3% acetonitrile, v/v/v) and buffer B (0.1% formic acid/20% water/80% acetonitrile, v/v/v) at a flow rate of 200 nL/min. The LTQ MS was operated in data-dependent acquisition mode using Xcalibur v2.2 (Thermo). After a survey MS scan in the mass range m/z 300–2000, the five most intense precursor ions were isolated and subjected to fragmentation by collision-induced dissociation (CID). The normalized collision energy was set at 35% with activation Q value being 0.25 and dynamic exclusion of 100 s. The acquired raw data were processed using BioWorks software v3.3 (Thermo).

ESI-TOF MS Analysis

ESI-TOF-MS analysis was performed on Agilent MSD TOF system in positive ion mode with the following settings: capillary voltage (VCap) 3500 V, nebulizer gas 30 psig, drying gas 5.0 L min^{-1}; fragmentor 140 V; gas temperature 325°C. The samples were injected at a flow rate of 20 µL min^{-1}, using a syringe pump (KD Scientific). The averaged MS spectra were deconvoluted using the Agilent MassHunter Workstation Software v B.01.03.

[1] D. Wessel, U. I. Flugge, *Anal Biochem* **1984**, *138*, 141-143.



Scheme S1. Formation of a sulfenamide (sulfenyl amide) from cysteine sulfenic acid after loss of H_2O. Electrophilic sulfur atom in -SOH may react with –NH- from the protein backbone (next amino acid example shown in path **A**) or amino acid side chain (e.g. lysine as in path **B**).

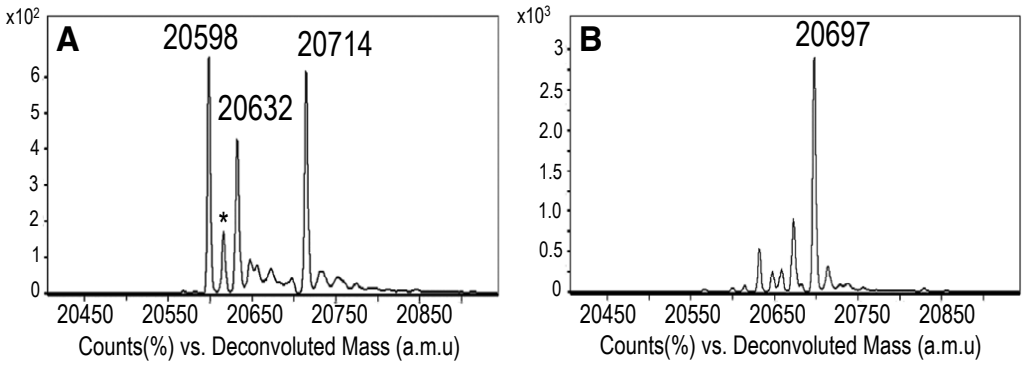


Fig. S1. ESI-TOF MS spectra of C165S AhpC-SOH reaction with **1** (**A**) and of C165S AhpC labeled with 3-methyl-5-isoxazolone (**B**). **A**. Peaks at 20,598, 20,616 (*), 20,632 a.m.u. correspond to sulfenamide (SN), sulfenic acid (-SOH), and sulfinic acid (-SO_2H) formation in C165S AhpC, respectively. Labeled adduct peak was observed at a.m.u. 20,714. (AhpC: 50 μM, labeling reagent **1**: 5 mM; buffer: 50 mM Bis-tris-citric acid pH 7.4; reaction time: 3h). **B**. Disappearance of adduct peak at a.m.u. 20,714 (in **A**) and appearance of a new peak at 20,697 a.m.u. indicates nearly 100% completion of C165S AhpC-**1** adduct cleavage with hydroxylamine treatment. Note: the spectrum in **B** was generated using C165S AhpC labeled with **1** at pH 8.5 to achieve nearly complete labeling prior to hydroxylamine treatment.

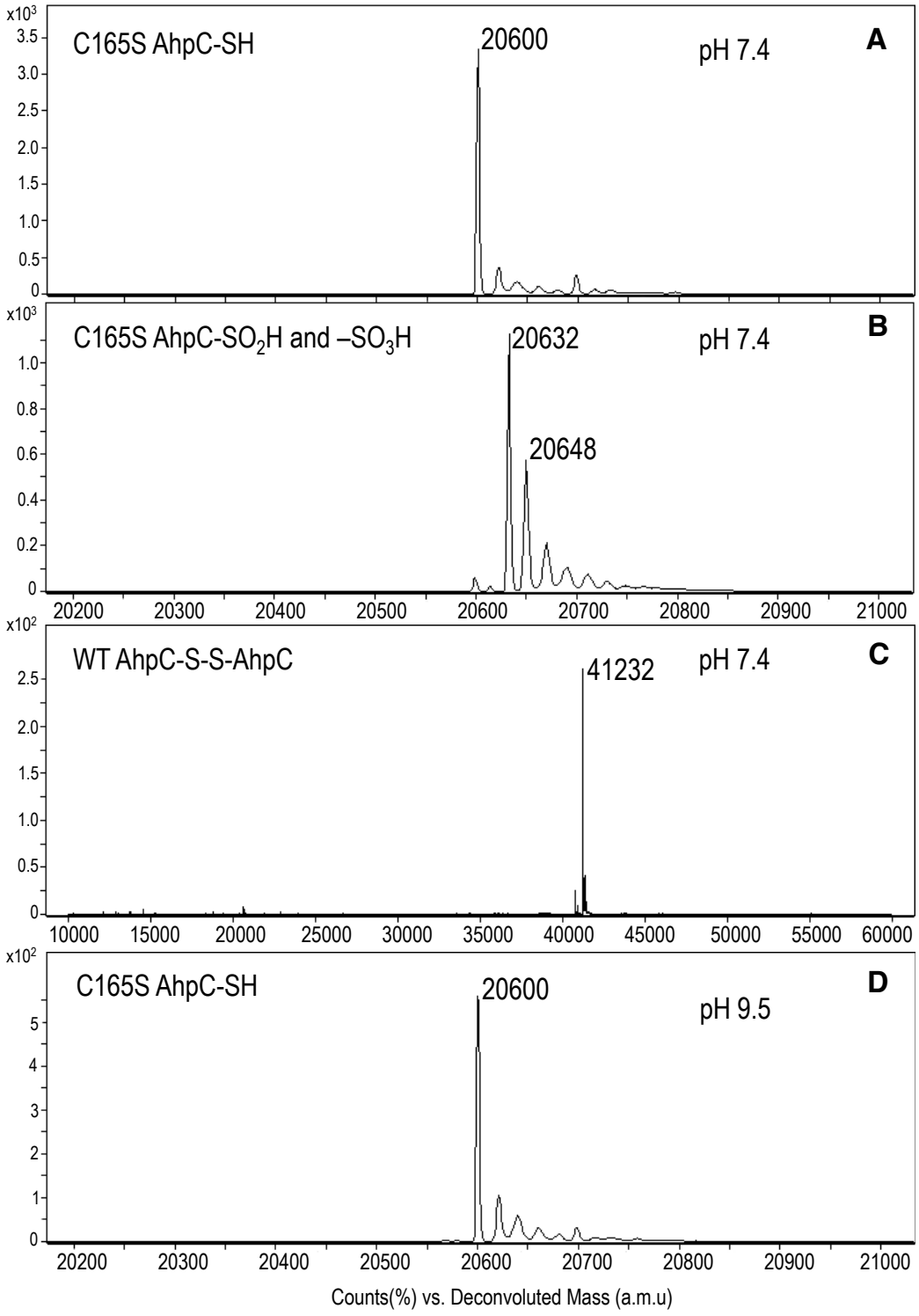


Fig. S2: ESI-TOF MS spectra from control experiments showing that **1** does not react with C165S AhpC-SH (**A**), AhpC-$SO_{2/3}H$ (**B**), or WT AhpC-S-S-AhpC (**C**) at pH 7.4. The lack of reaction of **2** with C165S AhpC-SH at pH 9.5 is shown in (**D**). **1** or **2** (5 mM) was incubated with AhpC containing -SH, -$SO_{2/3}H$ or –S-S- for 1.5 h at room temperature. The mass (a.m.u.) at 20,600 (top), 20,632/20648 (middle) and 41,232 (bottom) correspond to C165S AhpC-SH, AhpC- $SO_{2/3}H$ and WT AhpC-S-S-AhpC species, respectively.

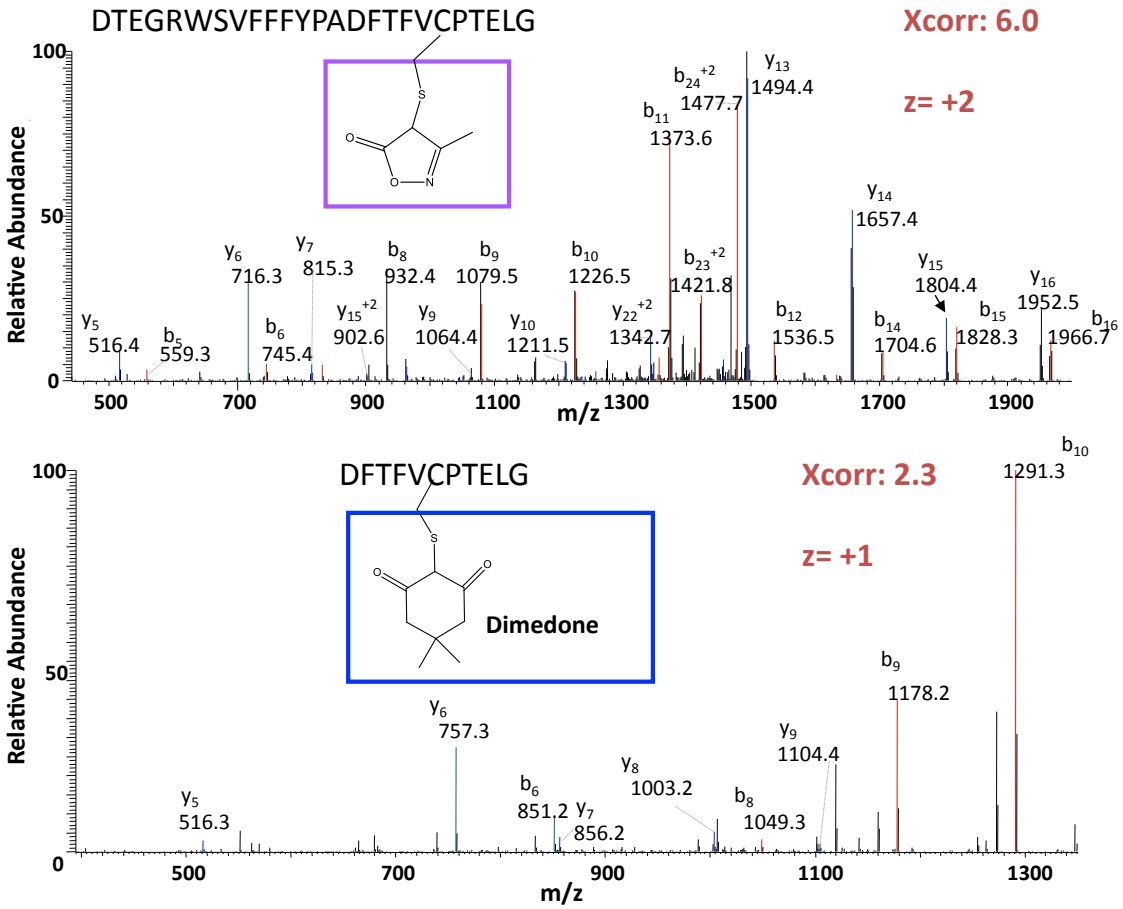


Fig. S3. Positive ion LC-MS/MS spectra of C46 containing peptides in C165S AhpC labeled by 3-methyl-5-isoxazolone after NH_2OH cleavage (**top**), and dimedone (**bottom**). The series of b and y ions confirm the sequence of the AhpC peptide and C46 modification.

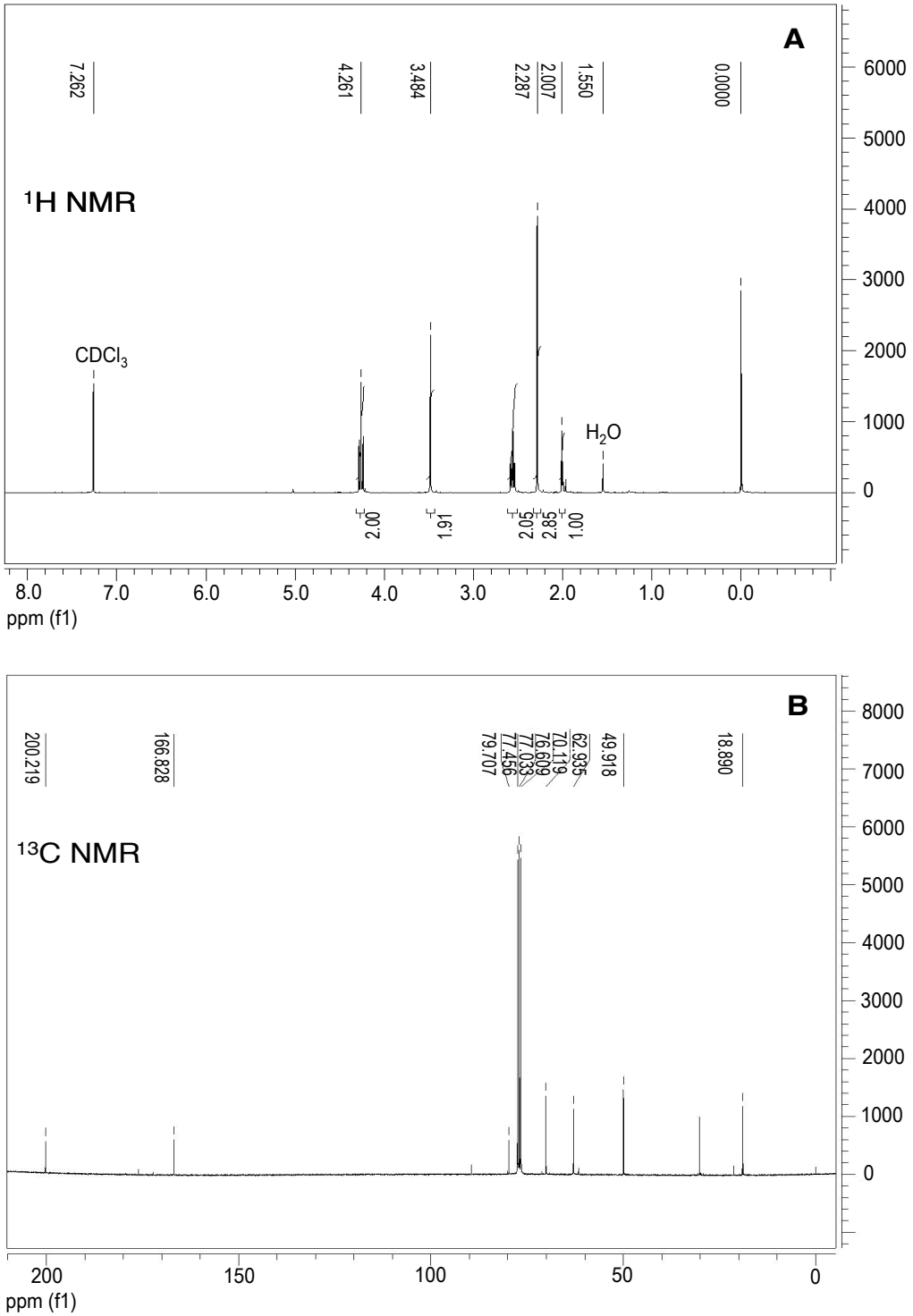


Fig. S4. ^{1}H NMR spectrum (**A**) and ^{13} CNMR spectrum (**B**) of but-3-yn-1-yl 3-oxobutanoate (**2**).

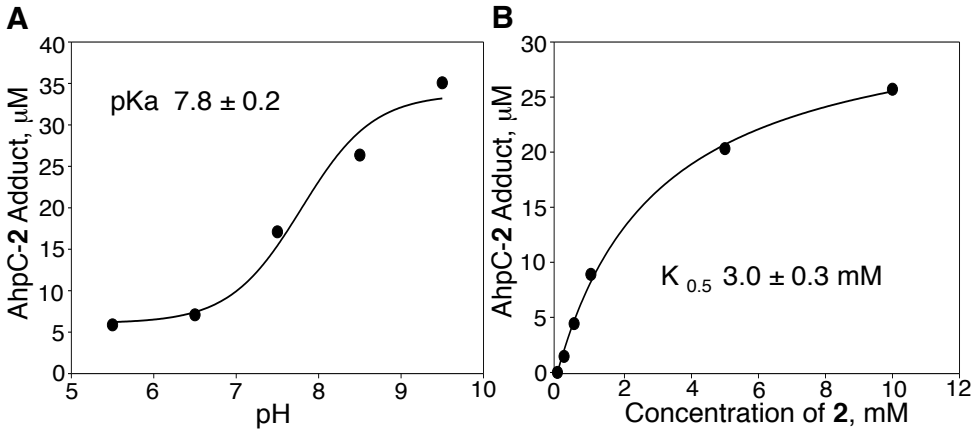


Fig. S5: Plots of C165S AhpC-**2** adduct formation as function of pH (**A**) and concentration of labeling probe **2** (**B**). The concentration of C165S AhpC-SOH was 50 mM in both (**A**) and (**B**); the concentration of **2** was 5 mM in (A) and varied between 0 and 10 mM in (B). The reaction was monitored for 90 min at r.t. in (**A**) and 120 min at pH 7.4 in (**B**). The relative adduct formation was calculated based on its abundance among the total ion abundances of C165S AhpC-SN, -SOH, -SO2H and -**2**.

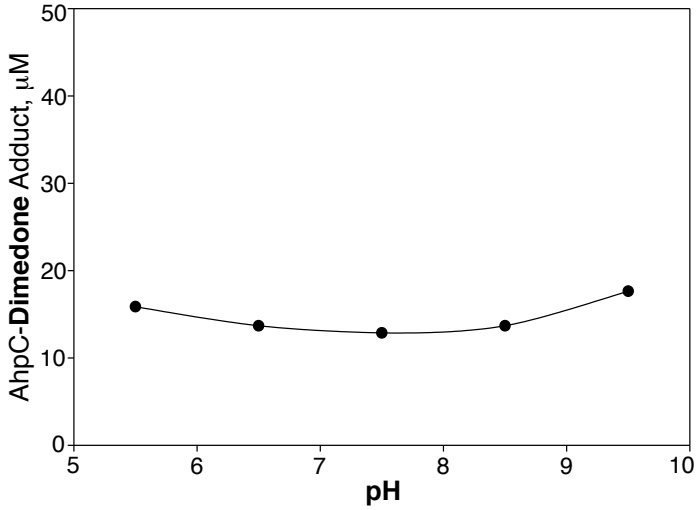


Fig. S6: Plot of C165S AhpC-dimedone adduct formation as function of pH. The concentration of C165S AhpC-SOH was 50 μM; the concentration of dimedone was 5 mM in 50 mM Bis-tris-citric acid buffer (pH 7.4, 8.5, or 9.5) or in 25 mM critic acid-Na_2HPO_4 buffers (pH 5.5, or 6.5). After 90 min reaction at r.t. the mixture was passed through a Bio-gel column equilibrated by 0.1% formic acid for ESI-MS. The relative adduct formation was calculated based on its abundance among the total ion abundances of C165S AhpC-SN, SOH, -SO_2H and dimedone.