Supporting Material

Simple synthesis of 1, 3-cyclopentanedione derived probes for labeling sulfenic acid proteins

General

All reactions were conducted under an atmosphere of dry nitrogen. Chemicals including ethanethiol, cyclopent-4-ene-1,3-dione, 2-aminoethanethiol, N,N-diisopropylethylamine were purchased from Sigma-Aldrich. Biotin-NHS was purchased from Cayman Chemicals. All the reagents were used without further purifications. $^1$H NMR(300 MHz and 500 MHz) and $^{13}$C NMR (125.76 MHz) spectra were recorded in $d_4$-MeOD or $d_6$-DMSO on a Bruker DRX-500 or DPX-300. Synthesized compounds were analyzed by Electrospray Ionization (ESI) mass spectrometry (ESI-MS) on a Thermo LTQ instrument. Compound 2 was also analyzed by liquid chromatography-high resolution mass spectrometry (LC-HRMS) on a Thermo Orbitrap LTQ under positive mode with a gradient from 5% A (95% H$_2$O, 0.1% formic acid) to 60% B (95% MeOH, 0.1% formic acid) over 10 min. 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 and characterization of 4-(ethylthio)cyclopentane-1,3-dione (1)

Ethanethiol (3.36 g, 54 mmol, 4 mL) was added to cyclopent-4-ene-1,3-dione (50 mg, 0.5 mmol) under a N$_2$ atmosphere and the mixture was stirred at room temperature for 2 days at which time the ethanethiol was removed under reduced pressure. The crude product was purified by flash column chromatography (SiO$_2$, acetone) to obtain 4-(ethylthio)cyclopentane-1,3-dione (74 mg, 90% yield) a pale yellow solid. Rf = 0.2 (Acetone); $^1$H NMR 500 MHz (ppm, $d_4$-MeOD): 5.22 (1H,
s), 3.71 (1H, dd J = 7.1, 2.5 Hz), 3.06 (1H, dd J = 18.4, 7.1 Hz), 2.70 (1H, dq J = 13.8, 7.5 Hz),
2.64 (1H, dq J = 13.8, 7.5 Hz), 2.41 (1H, dd J = 18.5, 2.4 Hz), 1.26 (3H, t J = 7.5). Enol OH
exchanges with the water and the ene proton exchanges with the D in MeOD resulting in a low
integration. \(^{13}\)C NMR (d\(_4\)-MeOD, 125.76 MHz): 201.16, 198.36, 105.51 (1C, t, J(13C-2H) = 26
Hz), 44.95, 41.09, 25.34, 14.88. ESI-MS: (M-H\(^-\) = m/z 157.18 (calculated MW of C\(_7\)H\(_{10}\)O\(_2\)S:
158.21 Da).

Synthesis and characterization of biotin-cyclopentane-1,3-dione (2)

Preparation of 4-((2-aminoethyl)thio)cyclopentane-1,3-dione (Int)

A solution of cyclopent-4-ene-1,3-dione (150 mg, 1.6 mmol) in anhydrous DMF (0.5 mL) was
added dropwise over 15 min to a heated solution of 2-aminoethanethiol in DMF (148 mg, 1.9
mmol). This mixture was stirred at room temperature (r.t.) overnight and concentrated under
reduced pressure. The crude product was purified by flash column chromatography (SiO\(_2\), eluent:
5/2 CHCl\(_3\)/MeOH) as pale yellow foamy solid with 89% yield. ESI-MS: (M+H\(^+\) = m/z 174.4
(calculated MW of C\(_7\)H\(_{11}\)NO\(_2\)S: 173.2 Da). The compound was then used as starting material for
synthesis of 2 (biotin-cyclopentane-1,3-dione), as described below.

To a mixture of Int (80 mg, 0.46 mmol) and DIPEA (81 \(\mu\)L, 0.46 mmol) in anhydrous DMF (5 mL)
was added biotin-NHS (172 mg, 0.50 mmol) at r.t.. The mixture was stirred overnight at r.t. and
then the solvents were removed under reduced pressure. The crude product was purified by flash
column chromatography (SiO\(_2\), eluent: 5/1 DCM/MeOH) to give light yellow waxy solid with an
yield of 65%. Rf = 0.13 (DCM/MeOH 5/2); \(^1\)H NMR 500 MHz (ppm, d\(_6\)-DMSO); 7.99 (1 H, dd J =
5.4, 5.2 Hz), 6.41 (1H, s), 6.35 (1H, s), 5.07 (1 H, s), 4.30 (1H, dd J= 5.7, 7.7 Hz), 4.13 (1H, m),
3.63 (1H, dd J= 3.4, 7.9 Hz), 3.31 (1H, dt J = 13.0, 6.8 Hz), 3.22 (1H, dt J = 13.0, 6.9 Hz), 3.10
(1H, m), 2.91 (1H, dd J= 18.1, 7.7 Hz), 2.81 (1H, dt J= 12.6, 5.2 Hz), 2.74 (1H, dt J = 18.7, 6.9
Hz), 2.60 (1H, dt J = 18.7, 7.1 Hz), 2.57 (1H, d J = 12.6 Hz), 2.22 (1H, dd J = 17.9, 2.5 Hz), 2.06
(2H, q J = 7.0 Hz), 1.61 (1H, m), 1.56-1.40 (3H, m), 1.38-1.78 (2H, m). Enol OH was not
observed due the exchange with water in solvent. \(^{13}\)C NMR (d\(_6\)-DMSO, 125.76 MHz): 172.21,
172.03, 103.80, 61.02, 59.84, 43.39, 39.62, 38.50, 37.32, 35.14, 29.76, 28.16, 28.00, 25.22. * 

found in 2D $^1$H-$^{13}$C gHMQC. ESI-HRMS: (M+H)$^+$ = m/z 400.1325 (calculated exact mass of C17H25N3O4S2: 399.1287)

**Monitoring of C165S AhpC-SOH labeling with 1, dimedone and 1,3-cyclopentanedione**

The C165S mutant of AhpC from *Salmonella typhimurium* in 50 mM Tris buffer was reduced in 10 mM DTT at room temperature for 30 min and the DTT was removed by passing the protein solution through a Bio-Gel spin column equilibrated with H$_2$O. The protein concentration was measured at 280 nm using extinction coefficient 24,300 (M$^{-1}$cm$^{-1}$). The C165S AhpC protein was oxidized by 2 equivalent H$_2$O$_2$ under aerobic conditions for 1 min at r.t. with mixing. The unreacted H$_2$O$_2$ was removed by Bio-Gel spin column. The C165S AhpC-SOH protein was then immediately aliquoted to 50 mM Bis-tris-citric acid buffer (pH 5.5) containing dimedone, 1,3-cyclopentadione or compound 1 respectively, to reach a final concentration of 50 µM protein and 5 mM labeling reagent. The labeling reagent stocks (0.5 M) used in this study were all made in 0.5 M Bis-tris in 50% DMSO. 30 µL reaction mixture was passed through a Bio-Gel column equilibrated with 0.003% formic acid in H$_2$O to quench the reaction at the following time points (min): 10, 40, 70, 110, 140, 180, 220, and 250. The flow through containing labeled and unlabeled C165S AhpC was then analyzed by ESI-TOF MS. In parallel control experiments, 5 mM 1 was incubated with the reduced form of C165S AhpC (C165S AhpC-SH), hyperoxidized protein (C165S AhpC-SO$_2$H) and WT AhpC (disulfide crosslinked dimer, WT AhpC-S-S-AhpC), respectively, for 1.5 h at r.t., and analyzed as described above.

**pH and concentration dependence studies of AhpC labeling with 1**

After 1 min oxidation of C165S AhpC using 2 equivalents of H$_2$O$_2$ at r.t., the protein was passed through a Bio-Gel spin column equilibrated with H$_2$O to remove excess H$_2$O$_2$. This process was necessary to minimize sequential oxidation of C165S AhpC during the next experimental steps. C165S AhpC-SOH protein was equally distributed into 25 mM critic acid-Na$_2$HPO$_4$ buffer at different pH (3.5, 4.5, 5.5, and 6.5) to yield 5 mM 1, final concentration. Aliquots of reaction
mixture were removed at a reaction time of 40 min, passed through a Bio-Gel column and analyzed by ESI-TOF MS as described above. For the concentration dependence experiments, C165S AhpC-SOH was mixed with various concentrations of 1 (0.5, 1, 2, 5, and 10 mM) in 50 mM Bis-tris-citric acid buffer (pH 5.5). After 85 min, the reaction mixture (30 µL) was passed through a Bio-Gel column and analyzed by ESI-TOF MS.

**H$_2$O$_2$ treatment of AhpC-1 and AhpC-1,3-cyclopentanedione adducts; Reaction of non-oxidized and oxidized adducts with thiols (DTT)**

AhpC protein (100 µM) labeled with 1,3-cyclopentanedione or 1 was incubated with H$_2$O$_2$ (0, 5, 10, 50, 100 mM) in citric acid/Na$_2$HPO$_4$ buffer (pH 7.5) for 1 hr or 17 h. The reaction was quenched by passing the mixture through a Bio-Gel column equilibrated with 0.003% formic acid. The collected flow-through was then analyzed by ESI-TOF MS, as described above. In a parallel experiment, H$_2$O$_2$ treated (100 mM, 17 h incubation) or untreated AhpC adducts were incubated with 50 mM DTT at pH 7.5 for 1 hr, followed by Bio-Gel column treatment and MS analysis.

**Labeling of oxidized AhpC and NIH 3T3 cell lysates with 2**

Experimental procedures for labeling of C165S AhpC-SOH with 2 were the same as described above for 1. The reaction mixture contained 2 (5 mM) and 50 µM C165S AhpC-SOH protein in 50 mM Bis-tris-citric acid buffer (pH 5.5). The reaction was allowed to proceed for 4 hours and labeled C165S AhpC was analyzed by ESI-TOF MS, as described above.

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 lysis buffer pH 7.5 (50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% Glycerol, 1% Triton X-100, 25 mM NaF, 10 µM ZnCl$_2$, in citric acid/Na$_2$HPO$_4$ buffer supplemented with protease and phosphatase inhibitor tablets (Roche)). One μL of 1 M TCEP was added to 97 μL cell lysate, followed by addition of 2 μL of DTT free, pre-reduced AhpC protein (stock, 8.8 mg/mL). The reduction was continued at r.t for 1 h before adding 2 (final concentration, 1 mM). After 1 min, 0.7 µL 1 M citric acid was added
to 49 μL of the pre-reduced lysate to lower the pH to 5.5 and labeling was allowed to proceed at r.t. for additional 1 h. The same amount of AhpC protein was also spiked in non-TCEP treated lysate before the addition of 2. As controls, lysates without AhpC or without 2 were also included. Three different concentrations of H$_2$O$_2$ (50, 100, 500 μM, final) were used to oxidize cell lysates in the presence of 2 and for 1 min before lowering the pH to 5.5. Untreated samples were kept at r.t. under aerobic conditions for 1 hr. Labeling was quenched by adding 15 μL 4x sample buffer (with β-mecaptoethanol). Labeled cell lysates were then separated by SDS-PAGE and probed by Western blot for incorporation of 2 using streptavidin-HRP. The blot was then stripped and re-probed for β-actin and AhpC. Enrichment in proteins labeled with 2 was performed as follows: Equal aliquot of 200 μL sample (300 μg) under each experiment condition above was used for captavidin pull-down. Labeling with 2 (including control lysate without 2) was quenched by adding equal volume of 0.2 % SDS containing 10 mM TCEP (in citric acid/Na$_2$HPO$_4$ buffer, pH 5.5). Each sample was mixed with 50 μL pre-washed captavidin beads (Invitrogen) at 4°C for overnight. The following day, the beads were washed with 1 mL citric acid/Na$_2$HPO$_4$ buffer at pH 4.5 and 5.5 twice, respectively. The beads were finally washed by 1 mL PBS. Labeled proteins were eluted with 50 μL NaHCO$_3$/Na$_2$CO$_3$ buffer (pH 10.0). The eluted proteins were mixed with 15 μL of 4x sample buffer (with β-mecaptoethanol) for SDS-PAGE and Western blot with anti-PLCγ1 antibody.

**LC-MS/MS analysis of AhpC labeled by 1 or 2**

C165S AhpC labeled by 1 or 2 was digested using AspN and the resulting peptides were analyzed on a nano-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). Peptides were filtered by cross-correlation score (XCorr), i.e. 2.0 for singly charged peptide ions, 2.5 for doubly charged peptide ions and 3.0 for the triply charged ions.

**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.

**Expanded Results and Discussions**

**Mechanism of 1,3-diketone compounds reaction with protein sulfenic acid**

Dimedone, 1,3-cyclohexadione-derived reagents and the 1, 3-cyclopentadione reagents (compound 1 and 2) presented here have very low pKa values (4.34 for dimedone and 5.23 for 1,3-cyclopentadione)¹. These low pKa values indicate that each of these compounds will exist in the enolate form (with some percentage of enol) at neutral pH. Lowering the pH of the reaction mixture will favor the presence of the enol form and suggests that pertinent trapping reaction occurs from the enol (as opposed to the enolate) form of the reagents. Spectroscopic evidence for the enol form (compound 1 in d₄-MeOD) comes from the low integration for the ene proton in the 1H NMR indicating exchange with the deuterium of MeOD. This is further confirmed by the presence of a diagnostic 1:1:1 triplet at 105.53 ppm with J (13C-2H) of ~26Hz (Fig. S7). Thus, it is likely the reaction proceeds through 1,4-addition or direct nucleophilic substitution as has been previously speculated². Further work is needed to completely explain the mechanism and pH dependence of these reactions.

Besides the enhanced reactivity of 1 and 2 at low pH, the use of low pH will be beneficial for other two reasons, however: 1. the low pH would decrease the cross-reaction between sulfenic acid
and free thiol groups from proteins and small molecules like GSH in cell lysate, since the
equilibrium between thiol and thiolate will be shifted towards thiol state at lower pH. This in turn,
will increase the amount of -SOH available for labeling with the probes described. 2. The low pH
will prevent postlysis oxidation, which can lead to erroneous experimental results.

Stability of the sulfanyl group in 1 under DTT and H₂O₂ treatment conditions

The potential retro-Michael addition of the sulfanyl group in 1 was investigated by treating AhpC-1
adduct with DTT. Data in Fig. S4A showed unchanged mass profile of AhpC-1 before and after
DTT treatment. This is consistent with earlier results, which showed the stability of the thioether
bond formed between thiol (from cysteine residues), and N-ethylmaleimide (NEM) analogues in
the presence of reduced glutathione (GSH)³. The retro-Michael addition was observed only in
intact cells when enzymatic reactions were possibly involved. Another example supporting the
probe’s insusceptibility to reduction is from Baker’s group, which also shows irreversible
modification of cysteine in Grb2 SH2 domain using NEM⁴.

Oxidation to sulfoxide and sulfone by H₂O₂ (or air) is another potential chemical modification of
the sulfanyl group in 1 and 2. To investigate this possibility, AhpC-1 or AhpC-1,3-cyclopentadione
were incubated with varying concentrations of H₂O₂ for 1 h or overnight. The results showed that
the sulfanyl group in 1 was fairly resistant to oxidation at 10 mM H₂O₂ (1 h at room temperature,
Fig. S5B, 3rd spectrum). When the concentration of H₂O₂ was increased to 50 and 100 mM,
however, oxidation of sulfanyl to sulfoxide and sulfone (Fig. S5B, 4th and 5th spectra) was
observed, while control sample (AhpC-1,3-cyclopentadione) that was lacking this sulfanyl group
showed no significant oxidation (Fig. S5A, 4th and 5th spectra). Oxidation of this control sample
was however observed when the incubation with 10 mM H₂O₂ was performed overnight (~ 40%
oxidation) (Fig. S5C, 3rd spectrum). This oxidation could occur at one of the three methionine
residues present in AhpC or at the thiol ether bond between C46 and the 1,3-cyclopentadione.
By contrast, about 90% of AhpC-1 adduct was oxidized under the same conditions (Fig. S5D, 3rd
spectrum) implying that about 50% of the sulfanyl group from probe 1 was oxidized. Sulfoxide
and sulfone oxidation products of AhpC-1 adduct became dominant species at 100 mM H₂O₂.
after overnight incubation (Fig. S5A, 5th spectrum). These oxidized species were shown to be stable in the presence of 50 mM DTT (Fig. S4B). Most biological experiments require treatment of cells with H$_2$O$_2$ concentrations below 1 mM and for maximum 1 hr. Therefore, we conclude that these chemical probes are inert to H$_2$O$_2$ oxidation under experimental conditions most commonly encountered in biology. When oxidized to sulfoxide and sulfone, these species are stable and can be accounted for using mass spectrometry.
**Fig S1**: Kinetic analysis of AhpC-SOH reaction with dimeredone, 1,3-cyclopentanedione and 1. Data were fit using KinTek Global Kinetic Explorer and the simplified reaction scheme shown in order to account for –SOH conversion to –SO₂H during labeling reaction. Both AhpC-SO₂H and AhpC-adduct formation were assumed irreversible.
Fig. S2: Positive ion LC-MS/MS spectra of C46 containing peptide in C165S AhpC labeled by 1 (A) and 2 (B). Single charged precursor ions at m/z 1386.39 and 1627.35 were isolated in separated analyses and fragmented to generate MS^2 spectra (A) and (B), respectively. The series of b and y ions confirm the sequence of the AhpC peptide and C46 modification by 1 or 2 as indicated by ^a (A) and ^a (B) in the sequence.
Fig. S3: ESI-TOF MS spectra from control experiments showing that 1 does not react with C165S AhpC-SH (top), WT AhpC-S-S-AhpC (middle) and C165S AhpC-SO$_2$H (bottom). 1 (5 mM) was incubated with AhpC containing SH, SO$_2$H or SS at pH 5.5 for 1.5 h at room temperature. The mass (a.m.u.) at 20,600 (top), 20,617 (middle), 41,232 (middle) and 20,632 (bottom) correspond to C165S AhpC-SH, WT AhpC, WT AhpC-S-S-AhpC and C165S AhpC-SO$_2$H species, respectively.
Fig. S4: ESI-TOF MS spectra showing stability of AhpC-1 and oxidized AhpC-1 adduct with DTT treatment. (A) AhpC-1 was incubated without (upper) or with (bottom) 50 mM DTT for 1h. Sample mixtures were passed through Bio-Gel equilibrated with ammonium bicarbonate for ESI-MS analysis. The peak at 20,756 a.m.u. corresponding to AhpC-1 adduct shows no mass shift(s) as result of DTT treatment. (B) Oxidation of AhpC-1 adduct was achieved by 17 h incubation with 100 mM H$_2$O$_2$ and DTT treatment was performed as in (A). Peaks at 20,648 and 20,664 a.m.u. correspond to AhpC-SO$_2$H plus one and two oxygens, respectively, and peaks at 20,772, 20,788 and 20,804 a.m.u. correspond to oxidized AhpC-1 adducts with one, two and three oxygens, respectively; one oxygen can be added to the cysteine to generate the sulfonic acid in the first two cases, and/or in all cases oxidation of methionine residue(s) is possible or likely. Addition of DTT did not result in breakdown of oxidized AhpC-1 adducts.
**Fig. S5**: ESI-TOF MS spectra of AhpC labeled by 1,3-cyclopentanediene (A, C) or 1 (B, D) that were treated by various concentrations of H$_2$O$_2$ for 1 hr (A, B) or 17 h (C, D). (A) Peaks at 20,632, 20,648, 20,696, 20,712, and 20,728 (©) a.m.u correspond to AhpC-SO$_2$H, AhpC-SO$_3$H, AhpC-1,3-cyclopentanediene adduct, and oxidized AhpC-1,3-cyclopentanediene adduct (one oxygen and two oxygens), respectively. (B) Peaks at 20,632, 20,648, 20,756, 20,772, and 20,788 (#) a.m.u correspond to AhpC-SO$_2$H, AhpC-SO$_3$H, AhpC-1 adduct, and oxidized AhpC-1 adduct (one oxygen and two oxygens), respectively.
Fig S6: $^1$H NMR spectrum of 4-(ethylthio)cyclopentane-1,3-dione.
**Fig S7:** $^{13}$C NMR spectrum of 4-(ethylthio)cyclopentane-1,3-dione.

**Fig S8:** Negative ESI-MS of 4-(ethylthio)cyclopentane-1,3-dione.
Fig S9: $^1$H NMR spectrum of biotin-cyclopentane-1,3-dione
Fig S10: $^{13}$C NMR spectrum of biotin-cyclopentane-1,3-dione. The small impurity is shown as (*) in Inset.
**Fig S11:** LC-HRMS of biotin-cyclopentane-1,3-dione (compound 2). Top – LC chromatogram; Bottom – HRMS spectrum.