Electronic Supplementary Material (ESI) for Chemical Communications

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_2O, 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 $C_7H_{10}O_2S$: 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 $C_7H_{11}NO_2S$: 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 µL, 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_2O. 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_2O_2 under aerobic conditions for 1 min at r.t. with mixing. The unreacted H_2O_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_2O 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_2H) 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_2O_2 at r.t., the protein was passed through a Bio-Gel spin column equilibrated with H_2O to remove excess H_2O_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_2HPO_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_2O_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_2O_2 (0, 5, 10, 50, 100 mM) in citric acid/Na_2HPO_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_2O_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_2HPO4 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_2O_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 ß-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 critric acid/Na_2HPO_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 critric acid/Na_2HPO_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_2CO_3 buffer (pH 10.0). The eluted proteins were mixed with 15 µL of 4x sample buffer (with ß-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 d_4-MeOD) comes from the low integration for the ene proton in the 1HNMR 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 speculated [2]. 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

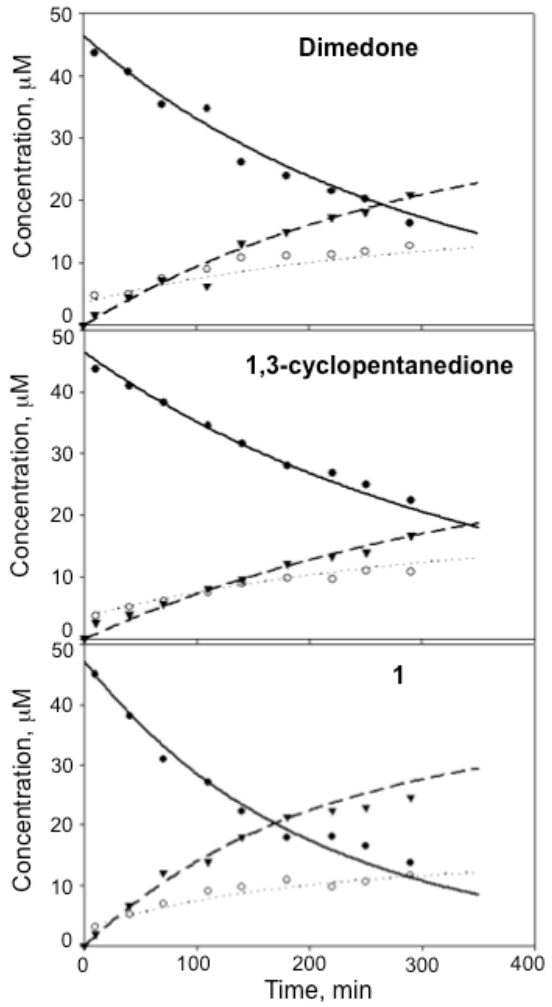
and free thiol groups from proteins and small molecules like GSH in cell lysate, since the equilibrium between thiol and thiolate will be shifted towards thiol state at lower pH. This in turn, will increase the amount of -SOH available for labeling with the probes described. 2. The low pH will prevent postlysis oxidation, which can lead to erroneous experimental results.

Stability of the sulfanyl group in 1 under DTT and H_2O_2 treatment conditions

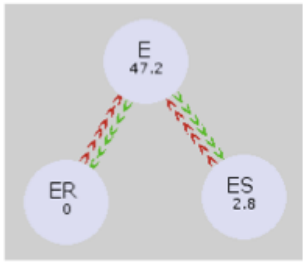
The potential retro-Michael addition of the sulfanyl group in **1** was investigated by treating AhpC-**1** adduct with DTT. Data in Fig. S4A showed unchanged mass profile of AhpC-**1** before and after DTT treatment. This is consistent with earlier results, which showed the stability of the thioether 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 cysteines in Grb2 SH2 domain using NEM[4].

Oxidation to sulfoxide and sulfone by H_2O_2 (or air) is another potential chemical modification of the sulfanyl group in **1** and **2**. To investigate this possiblity, AhpC-**1** or AhpC-1,3-cyclopentadione were incubated with varying concentrations of H_2O_2 for 1 h or overnight. The results showed that the sulfanyl group in **1** was fairly resistant to oxidation at 10 mM H_2O_2 (1 h at room temperature, Fig. S5B, 3rd spectrum). When the concentration of H_2O_2 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 H_2O_2 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 H_2O_2

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_2O_2 concentrations below 1 mM and for maximum 1 hr. Therefore, we conclude that these chemical probes are inert to H_2O_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.



● AhpC (SN + SOH)
▼ AhpC adduct
○ AhpC (SO_2H)



E – AhpC (SN + SOH), 47.2 μM
ES – AhpC (SO_2H), 2.8 μM
ER – AhpC adduct with dimedone, **1**, or 1,3-cyclopentanedione

	k', min^{-1}	
	AhpC (SO_2H)	AhpC adduct
dimedone	1.7×10^{-3}	2.4×10^{-3}
1,3-cyclopentanedione	1.2×10^{-3}	1.8×10^{-3}
1	2.2×10^{-3}	3.6×10^{-3}

Fig S1: Kinetic analysis of AhpC-SOH reaction with dimedone, 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 $–SO_2H$ during labeling reaction. Both AhpC-SO_2H and AhpC-adduct formation were assumed irreversible.

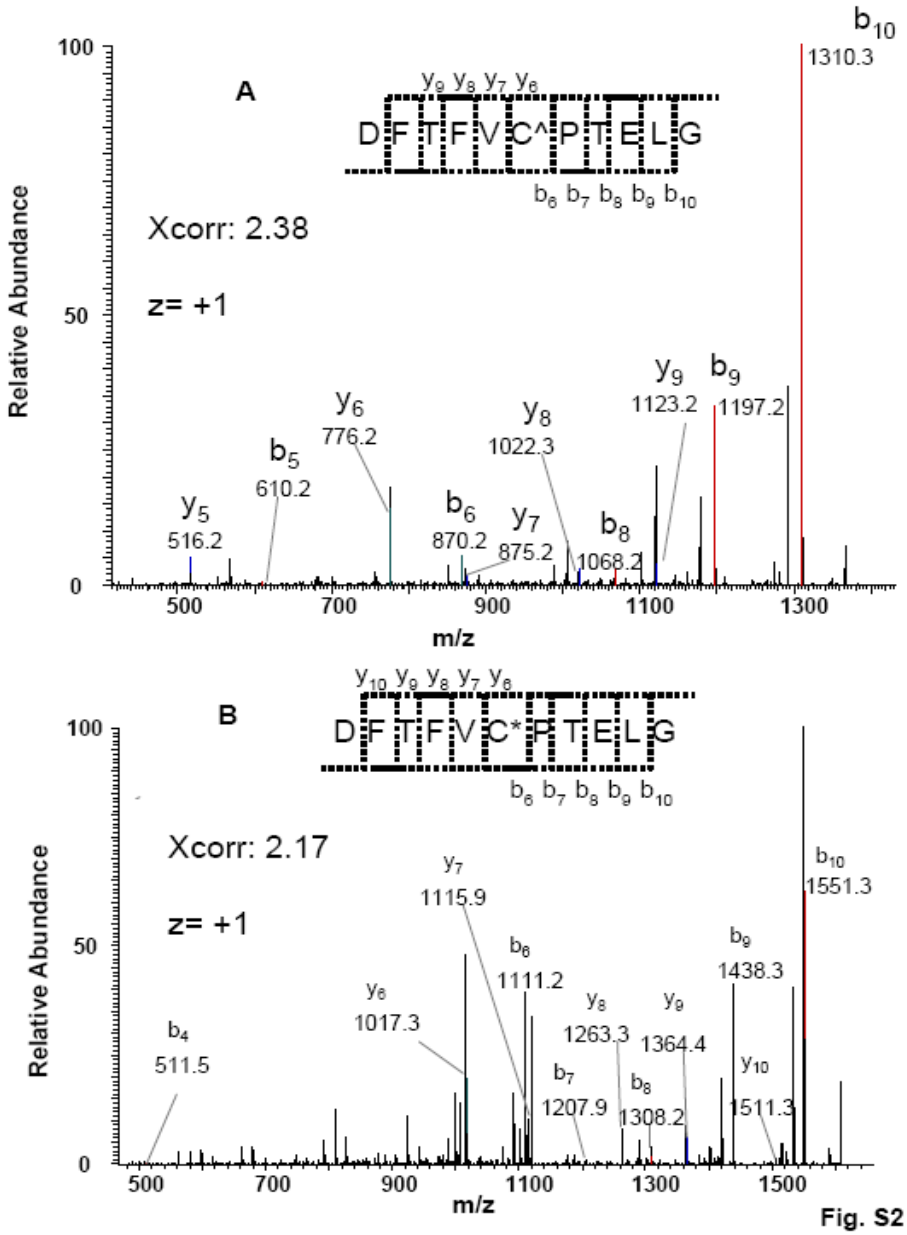


Fig. S2: Positive ion LC-MS/MS spectra of C46 containing peptide in C165S AhpC labeled by **1** (**A**) and **2** (**B**). Single charged precursor ions at m/z 1386.39 and 1627.35 were isolated in separated analyses and fragmented to generate MS^2 spectra (**A**) and (**B**), respectively. The series of b and y ions confirm the sequence of the AhpC peptide and C46 modification by **1** or **2** as indicated by ^ **(A)** and * **(B)** in the sequence.

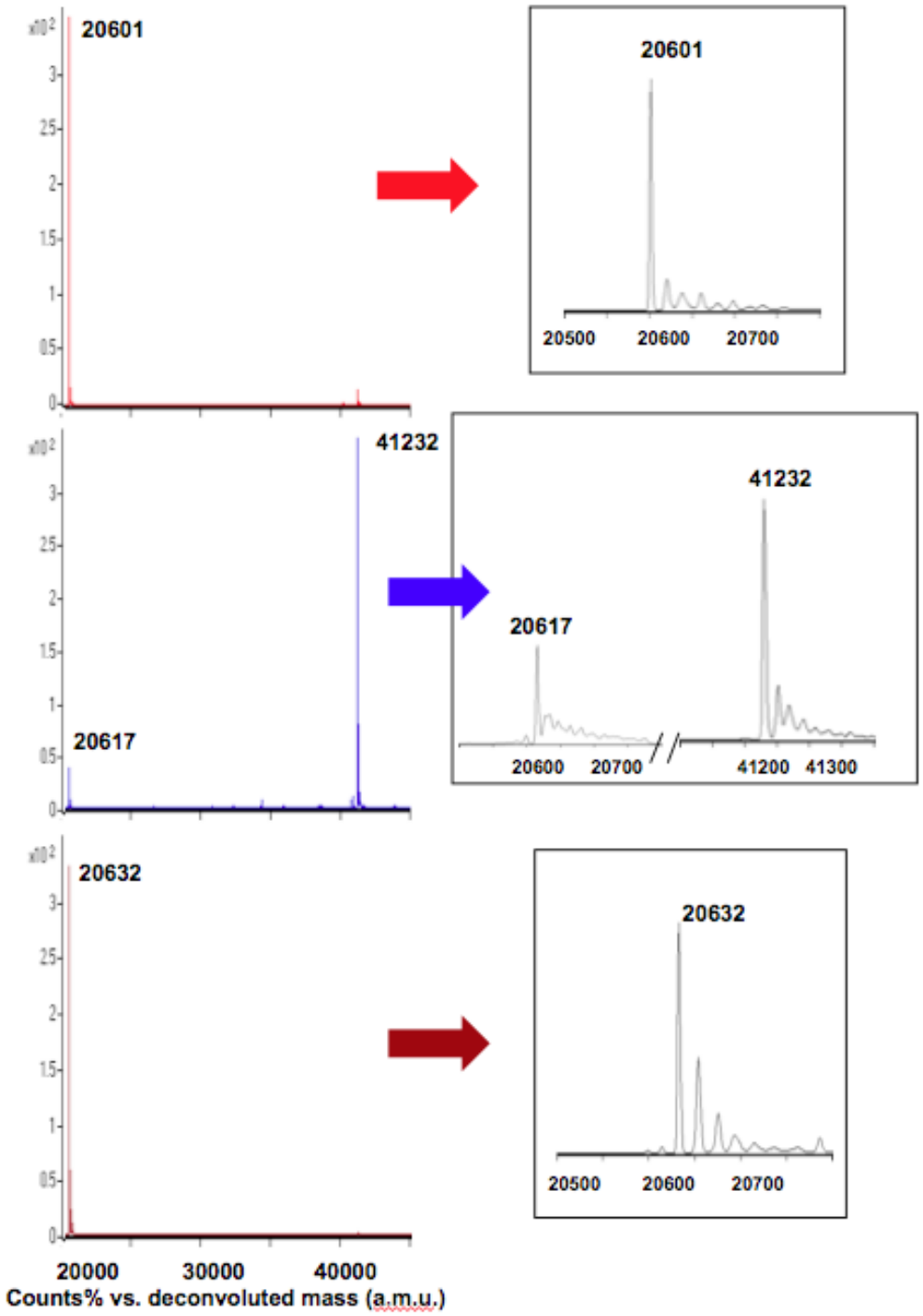


Fig. S3: ESI-TOF MS spectra from control experiments showing that **1** does not react with C165S AhpC-SH (top), WT AhpC-S-S-AhpC (middle) and C165S AhpC-SO_2H (bottom). **1** (5 mM) was incubated with AhpC containing SH, SO_2H or SS at pH 5.5 for 1.5 h at room temperature. The mass (a.m.u.) at 20,600 (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_2H species, respectively.

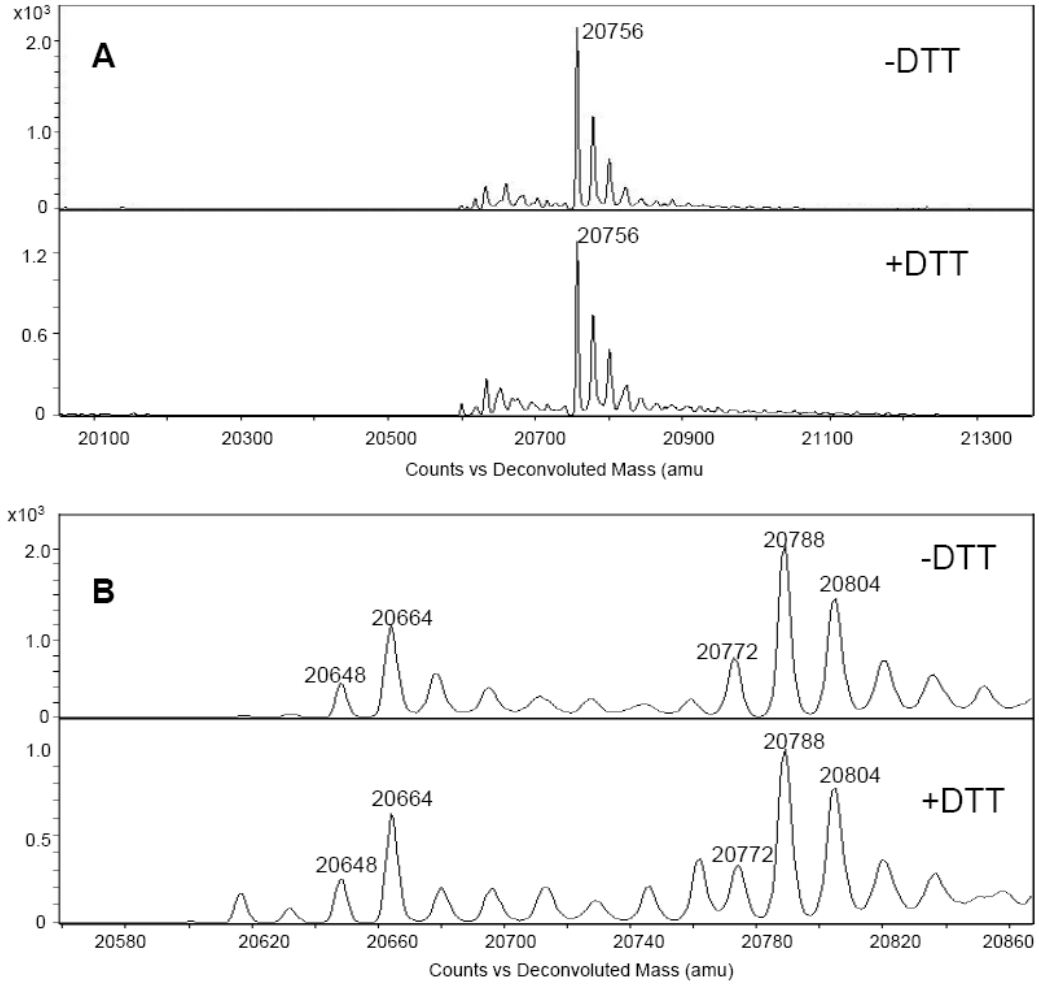


Fig. S4: ESI-TOF MS spectra showing stability of AhpC-**1** and oxidized AhpC-**1** adduct with DTT treatment**.** (**A**) AhpC-**1** was incubated without (upper) or with (bottom) 50 mM DTT for 1h. Sample mixtures were passed through Bio-Gel equilibrated with ammonium bicarbonate for ESI-MS analysis. 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_2O_2 and DTT treatment was performed as in **(A)**. Peaks at 20,648 and 20,664 a.m.u. correspond to AhpC-SO_2H 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.

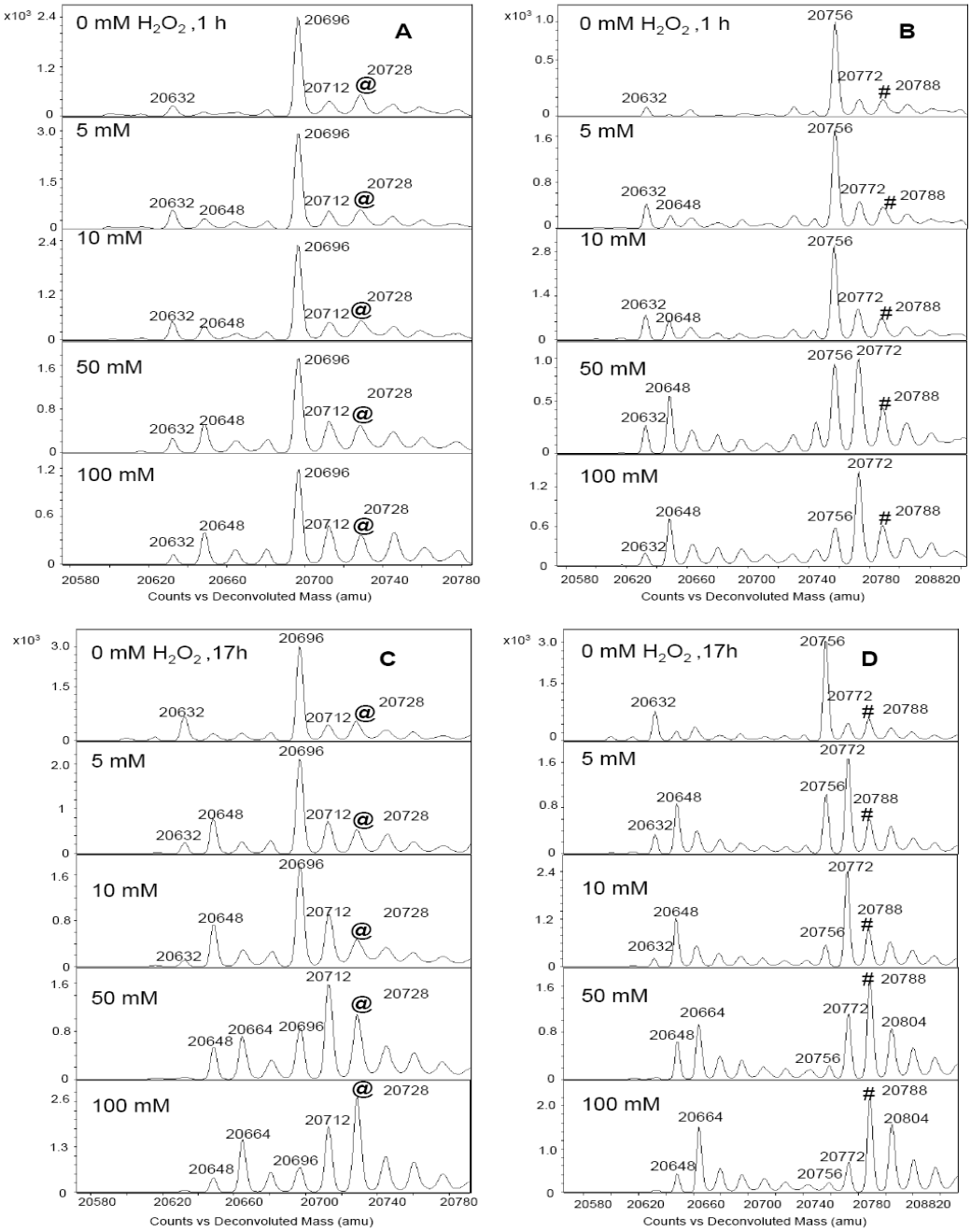


Fig. S5: ESI-TOF MS spectra of AhpC labeled by 1,3-cyclopentanedione (**A, C**) or **1** (**B, D**) that were treated by various concentrations of H_2O_2 for 1 hr (**A, B**) or 17 h (**C, D**). (**A**) Peaks at 20,632, 20,648, 20,696, 20,712, and 20,728 (**@**) a.m.u correspond to AhpC-SO_2H, AhpC-SO_3H, AhpC-1,3-cyclopentanedione adduct, and oxidized AhpC-1,3-cyclopentanedione 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_2H, AhpC-SO_3H, AhpC-**1** adduct, and oxidized AhpC-**1** adduct (one oxygen and two oxygens), respectively.

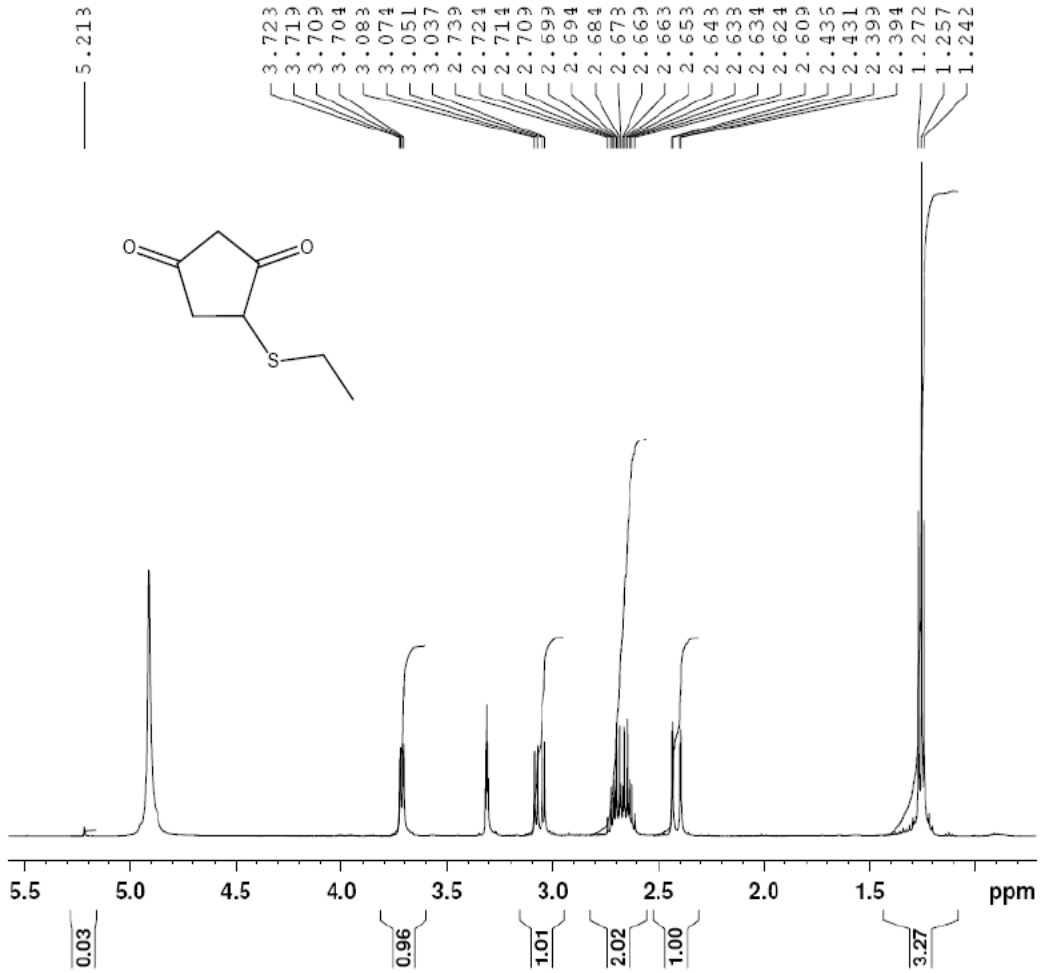


Fig S6: ^{1}H NMR spectrum of 4-(ethylthio)cyclopentane-1,3-dione.

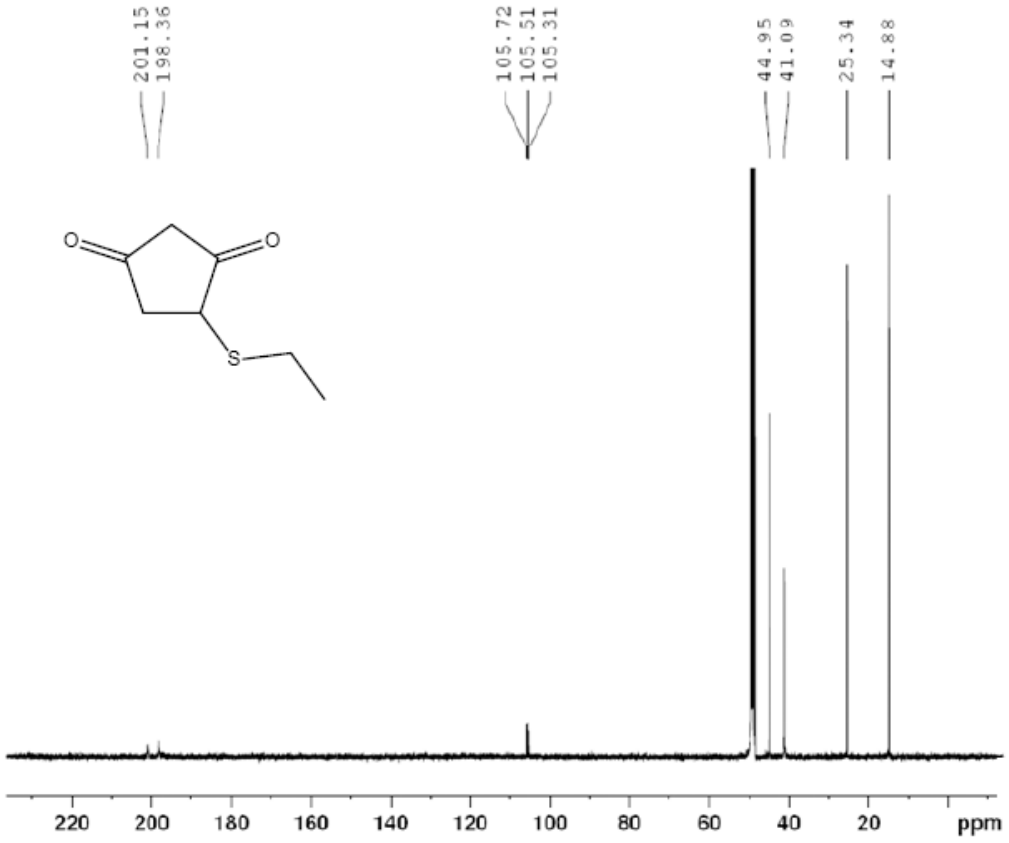


Fig S7: ^{13}C NMR spectrum of 4-(ethylthio)cyclopentane-1,3-dione.

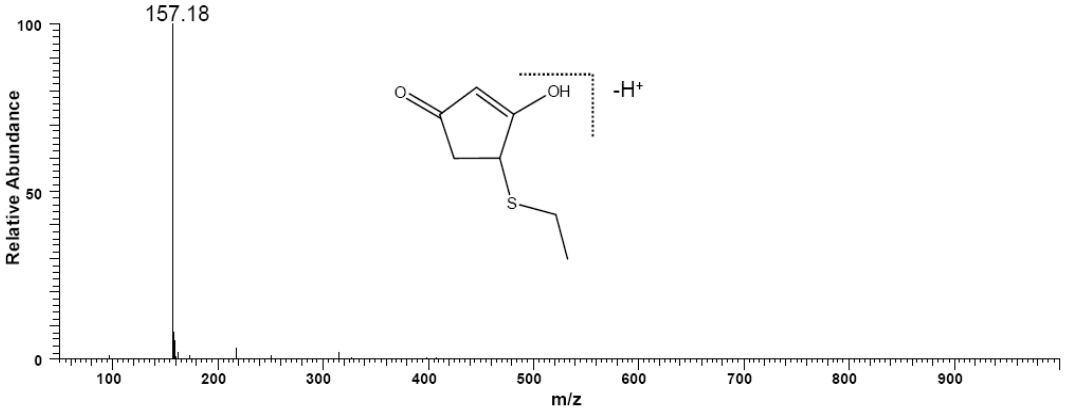


Fig S8: Negative ESI-MS of 4-(ethylthio)cyclopentane-1,3-dione.

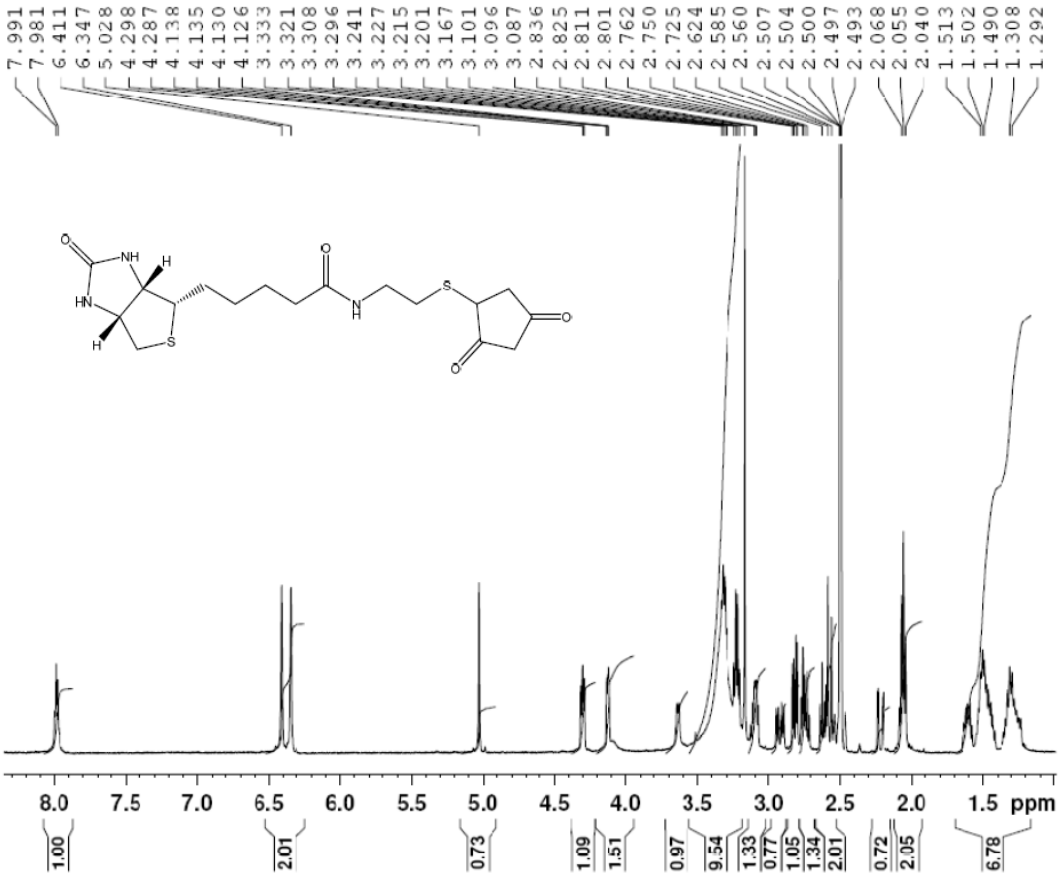


Fig S9: ^{1}H NMR spectrum of biotin-cyclopentane-1,3-dione

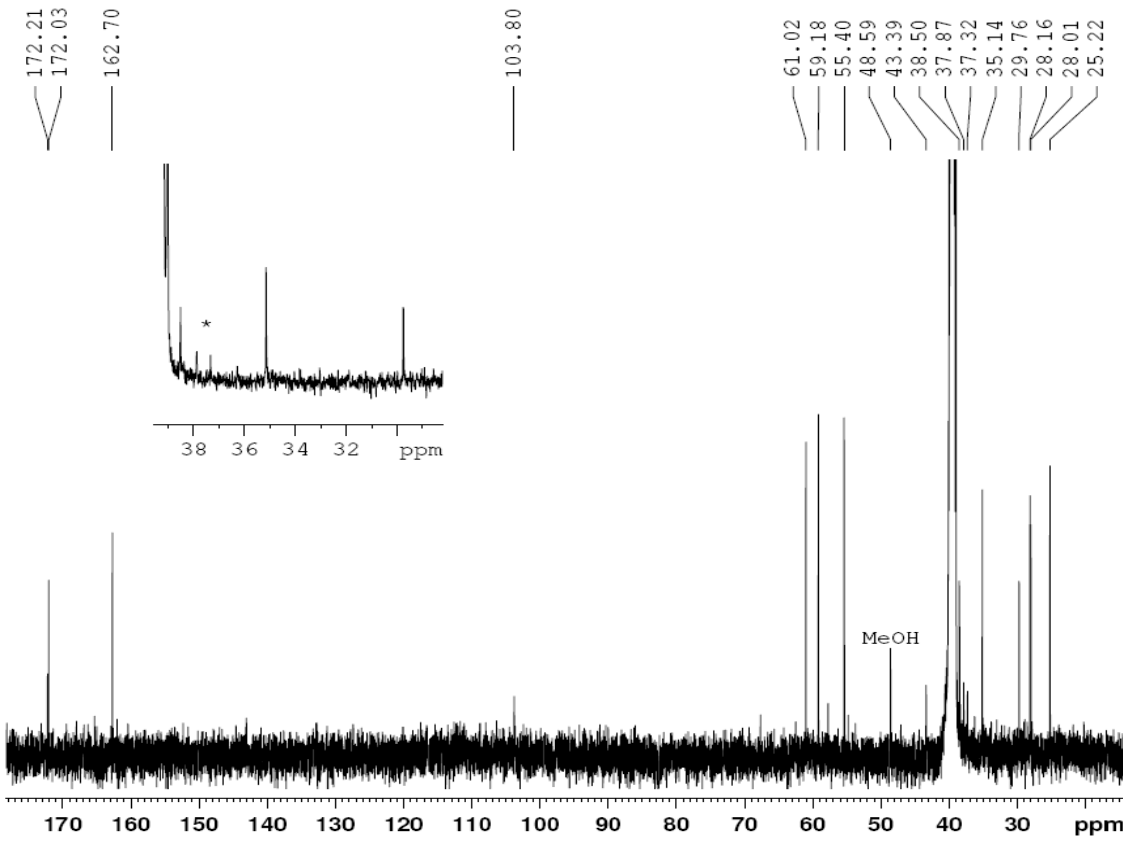


Fig S10: ^{13}C NMR spectrum of biotin-cyclopentane-1,3-dione. The small impurity is shown as (*) in *Inset*.

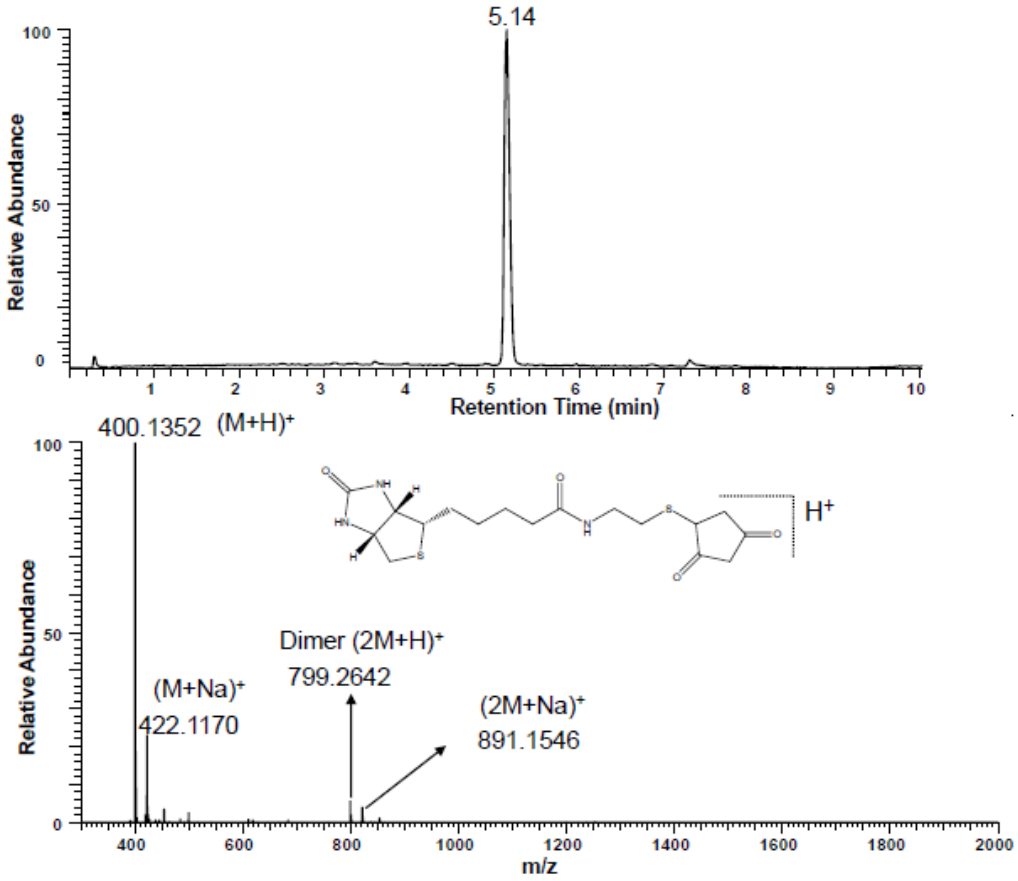


Fig S11: LC-HRMS of biotin-cyclopentane-1,3-dione (compound **2**). Top – LC chromatogram; Bottom – HRMS spectrum.

1. F. M. Wong, J. R. Keeffe and W. M. Wu, *Tetrahedron Lett.*, 2002, **43**, 3561-3564.
2. K. G. Reddie and K. S. Carroll, *Curr. Opin. Chem. Biol.*, 2008, **12**, 746-754.
3. D. Lin, S. Saleh and D. C. Liebler, *Chem. Res. Toxicol.*, 2008, **21**, 2361-2369.
4. M. E. B. Smith, F. F. Schumacher, C. P. Ryan, L. M. Tedaldi, D. Papaioannou, G. Waksman, S. Caddick and J. R. Baker, *J. Am. Chem. Soc.*, 2010, **132**, 1960-1965.