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Dual-Reactivity *trans*-Cyclooctenol Probes for Sulfenylation in Live Cells Enable Temporal Control via Bioorthogonal Quenching

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Abstract

Sulfenylation (RSH → RSOH) is a posttranslational protein modification associated with cellular mechanisms for signal transduction and the regulation of reactive oxygen species. Protein sulfenic acids are challenging to identify and study due to their electrophilic and transient nature. Described here are sulfenic acid modifying *trans*-cycloocten-5-ol (SAM-TCO) probes for labeling sulfenic acid functionality in live cells. These probes enable a new mode of capturing sulfenic acids via transannular thioetherification, whereas ‘ordinary’ *trans*-cyclooctenes react only slowly with sulfenic acids. SAM-TCOs combine with sulfenic acid forms of a model peptide and proteins to form stable adducts. Analogously SAM-TCO with the selenenic acid form of a model protein leads to a selenoetherification product. Control experiments illustrate the need for the transannulation process coupled with the activated *trans*-cycloalkene functionality. Bioorthogonal quenching of excess unreacted SAM-TCOs with tetrazines in live cells provides both temporal control and a means of preventing artifacts caused by cellular-lysis. A SAM-TCO biotin conjugate was used to label protein sulfenic acids in live cells, and subsequent quenching by tetrazine prevented further labeling even under harshly oxidizing conditions. A cell-based proteomic study validates the ability of SAM-TCO probes to identify and quantify known sulfenic acid redox proteins as well as targets not captured by dimedone-based probes.

Graphical Abstract

Protein oxidation arising from reactive oxygen species (ROS) is often associated with damaging effects particularly in cancer, aging, cardiovascular, neurodegenerative diseases, and inflammation.¹ ROS are mediators of signal transduction pathways through their ability to modify redox sensor proteins and thus regulate their protein partners and subsequent action.^{2–5} Cysteine targets of ROS are tightly regulated and depend on both their reactivity and cellular location.⁶ For both healthy and diseased tissues there is a continuing need for tools to detect the level of internal oxidative stress and the resulting cellular response.⁷

A central ROS signaling mechanism is the sulfenylation of cysteines to sulfenic acids (R-SOH)—electrophiles capable of reacting with thiolates to form disulfides or with adjacent amide nitrogens to form cyclic sulfenamides.⁸ Several enzymes regulate cellular oxidants through highly reactive sulfenic acids, which are resolved through intramolecular disulfide formation.⁹ The lifetimes of sulfenic acids are further governed by other nucleophiles found in the cell including glutathione (GSH).¹⁰ Due to their low intracellular concentrations, high electrophilicity, and rapid turnover, sulfenic acids have remained difficult to study.¹¹ Thus, the development of chemical probes capable of capturing cellular sulfenic acids *in situ* is critical for their identification within the proteome.

In 1974, dimedone was shown to be a chemoselective probe for sulfenylation.¹² Dimedone and diketone derivatives have since been used to characterize sulfenylation in the proteome.^{7,13} Carroll has reported a small molecule sulfenic acid model that enabled the development of new chemical probes with improved reaction rates and unique profiles of protein targets.^{14–16}

King and co-workers have shown that strained bicyclononyne (BCN) derivatives undergo a concerted 1,3-dipolar cycloaddition reaction to give a sulfoxide product.^{17,18} Tagging by thiolates has also been reported for BCN.^{19,20} Dimedone and BCN derivatives have both been used to covalently modify sulfenic acids in living cells.^{17,21–26} However, these reagents remain active at the point of cell lysis, relying on additives to quench sulfenic acids during work up. Ideally, sulfenic acid probes could instead be fully quenched in live cells, as cell lysis may promote subsequent unwanted redox transformations.^{22,27}

trans-Cyclooctenes (TCOs) are useful reagents that engage in rapid bioorthogonal reactions with tetrazines. At sub-millimolar concentrations, TCO does not covalently modify thiols²⁸ and displays little background labeling in the cell.²⁹ While TCO and other olefins can form covalent adducts with sulfenic acids, their reaction rates are modest.^{17,27} We postulated that TCO may rapidly form thiiranium ions in the cellular environment, but without an internal nucleophile the reaction could be reversible.^{30,31} Accordingly, we designed probes based on *trans*-cycloocten-5-ol (**1**) that could rapidly irreversibly capture thiiranium ion intermediates *via* intramolecular thioetherification.^{32,33} We reasoned that the olefinic strain of *trans*-cyclooctene **1** would make it particularly adept to form a thiiranium ion (**2**), and that the ring system would position a hydroxyl nucleophile for subsequent transannular attack (Fig 1).^{32,34} We show that these sulfenic acid modifying *trans*-cycloocten-5-ol (SAM-TCO) probes function for the rapid, selective, and stable capture of sulfenic acids in live cells. Unique to this approach, bioorthogonal tetrazine ligation can quench SAM-TCO reagents in live cells with temporal control, thereby minimizing concerns about redox changes resulting from cell lysis.

We initially tested the ability of SAM-TCO **1** to react with selenium electrophile *N*-phenylselenophthalimide (*N*-PSP). Compound **1** combined with *N*-PSP at room temperature to give selenoether **3** in 82% yield (Fig 2). Diol **6**, which has axial and equatorial hydroxyls, gives [3.3.1]oxabicycle **7** in 88% yield. Non-observance of [4.2.1]oxabicycle **8** illustrates the significance of the axial hydroxyl group for efficient capture of the seleniranium ion. Consistent with these observations, compound **4** with an equatorial hydroxyl substituent requires elevated temperatures to react with *N*-PSP and after forming a seleniranium ion (**4a**), must undergo ring flip prior to transannular attack at C-2 of **4b** to give [4.2.1] bicycle **5**.

We also investigated the ability of SAM-TCOs to react with Carroll's dipeptide cyclic sulfenamide (**9**), which is capable of *in situ* generation of sulfenic acid in 2:1 PBS:ACN.¹⁵ Reaction of dimedone (**10**) with **9** forms **11** with a second order rate constant of $11.8 \text{ M}^{-1}\text{s}^{-1}$.¹⁵ In competition, axial alcohol **1** (300 μM) and dimedone (300 μM) were pre-mixed and then added to a limiting solution of **9** (30 μM). LC/MS analysis for formation of **11** and **12** showed that **1** reacted 6.5 times faster than **10** at pH 7.4. Equatorial alcohol **4** is less reactive than **1**, but reacts with a rate similar to dimedone. SAM-TCOs also retain reactivity at low pH as is relevant to tumor microenvironments of pH 5.8–7.6.³⁵ At pH 5.5, **1** is 11 times faster than dimedone, which is known not to be pH dependent.³⁶

Compound **1** (1 mM) does not label glutathione (0.2 mM), S-nitrosoglutathione (0.2 mM) or glutathione disulfide (0.2 mM). Compounds **14** and **15** gave no labeling with **9**, establishing that the *trans*-cycloalkene and the axial nucleophile are both critical to the efficacy of SAM-TCOs. Thioether **12** (25 μM) is very stable toward strong reductants (250 μM DTT, GSH or TCEP) after 72 h at room temperature in pH 7.4 PBS (Fig S-4–S-6).^{17,37}

Several proteins with catalytic cysteine residues have been used for *in vitro* studies of sulfenic acid probes.³⁶ We chose to study a C84S C94S mutant of free methionine sulfoxide reductase (fRMSr, **16**), which produces stable sulfenic acid **17** upon selective oxidation by methionine sulfoxide.³⁶ Purified fRMSr-SOH **17** was combined with excess SAM-TCO **18**

to give thioether **20** (Fig 3A–C). Because SAM-TCOs can also undergo rapid bioorthogonal cycloadditions with tetrazines, **18** can be fully quenched at defined timepoints with excess tetrazine **19**. After quenching with **19**, samples were reduced by DTT converting any remaining **17** to **16**. As fRMs **16** and thioether fRMs **20** have equal ionization efficiencies (Fig S-11), ESI-MS could be used to calculate the second order rate constant (k_2) of $750 \pm 150 \text{ M}^{-1}\text{s}^{-1}$ in PBS at 25 °C (Fig 3D).^{7,36} Site-specific alkylation of **17** at C118 was verified by digestion and tandem MS (Fig 3E). In competition, SAM-TCO **1** and dimedone **10** were combined with **17** under pseudo-first order conditions; analysis by LC/MS determined that dimedone was outcompeted by **1** (Fig S-15). In contrast to the observed selectivity with fRMs, glyceraldehyde 3-phosphate dehydrogenase was 35% labeled by dimedone (5 mM) in H₂O₂ (0.2 mM), but was not tagged by **1** (Fig S-35). This probe-based selectivity is consistent with prior observations by Carroll that structurally divergent probes target different protein sulfenic acids.¹⁴

Similar to sulfenic acids, selenenic acids also play a role in signal transduction.³⁸ As shown in Fig 2, transannular selenoetherification of SAM-TCO's by *N*-PSP is facile. Demonstrating selenoetherification in a protein system, selenocysteine-fRMs (C84S C94S C118U) **21** can be oxidized using methionine sulfoxide and the resulting selenenic acid can be quantitatively captured in the presence **18** to give adduct **22** (Fig 3F).

We next explored the intracellular capture of sulfenic acids in live cells using **SAM-TCO biotin**—a probe that can be bioorthogonally quenched in live cells using a tetrazine. This ensures that changes resulting from lysis are not falsely attributed to native sulfenic acid labeling.³⁹ Cell permeability of **SAM-TCO biotin** was assessed by observance of dose-dependent labeling (50–500 μM) in LNCaP prostate cancer cells. Live cells were combined with **SAM-TCO biotin** for 2 h at 37 °C, and subsequently quenched by tetrazine **19** (5 mM). Cell lysis, SDS-PAGE, and western blot revealed prominent labeling beyond native biotinylation (Fig 4B). Specificity of the labeling reaction was demonstrated by two controls: incubating **SAM-TCO biotin** (100 μM) in the presence of **18** (5 mM), followed by tetrazine quenching; wherein, no labeling was observed by western blot (Fig S-37). The necessity for an internal hydroxyl group to capture low abundant sulfenic acids was assessed in live cells. No labeling was observed with **TCO-biotin** under conditions that are successful for **SAM-TCO biotin** (Fig 4C). Additionally, the toxicity of SAM-TCO **18** toward LNCaP cells was shown to be low (LC₅₀ >20 mM, 24 h viability assay, Fig S39).

Evidence for the cell permeability of **SAM-TCO-biotin** was validated by its ability to undergo tetrazine ligation in live cells. Here, HEK293T cells expressing a cytosolic HaloTag fusion protein were first labeled with a tetrazine-HaloTag ligand. Confocal microscopy showed that conjugation with SAM-TCO-biotin quenched the protein for subsequent reaction with a fluorescent TAMRA-TCO reagent (Fig S36).

We next confirmed that tetrazine **19** is capable of quenching in live cells. LNCaP cells were lifted and labeled by **SAM-TCO biotin** (500 μM) at 37 °C. After 30 minutes, the sample was split and labeled **A–D** (Fig 4D). Sample **A** was quenched with tetrazine **19** (5 mM); sample **B** with **19** (5 mM) for 10 seconds, followed by H₂O₂ (1 mM); sample **C** with H₂O₂ (1 mM), and sample **D** was unmodified. After an additional 30 minutes, samples **C** and **D**

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were treated with **19** (5 mM). Cells were then processed, lysed, and analyzed by western blot (Fig 4D). Samples **C** and **D** displayed the highest intensity signals as they were quenched after 60 minutes instead of 30 minutes, with **C** being much more intense and displaying new bands because of the added H₂O₂. Samples **A** and **B**, which were quenched by **19** after 30 minutes, displayed equally low band intensity, demonstrating that **SAM-TCO biotin** had been quenched by the tetrazine prior to the addition of H₂O₂ for sample **B**. Overall, the quenched samples display equal and lower signal intensities than unquenched samples, confirming that SAM-TCO probes can be intracellularly quenched with temporal control. Cell lysis has been previously reasoned to cause oxidative stress^{11,22,27} and therefore an active probe could potentially lead to labeling artifacts observed through cell lysis mechanisms. Labeling by **SAM-TCO biotin** in live and lysed cells was compared by briefly incubating the probe for 30 minutes at 4°C under each condition. Overall labeling is higher under lysis conditions in comparison to live cell labeling (Fig S-38).

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Carbon-nucleophilic probes have been used for the chemoproteomic profiling of sulfenylation in cell lysates.⁷ As shown in Fig 5, transannular thioetherification represents a mechanistically distinct tool for the chemoproteomic identification and quantification of cellular sulfenic acids. HEK293T cell lysate was combined with 5 mM SAM-TCO-alkyne (**23**) for 120 minutes with or without added H₂O₂.⁴⁰ Intact proteins were then subjected to CuAAC using a bifunctional azide-biotin with an acid-cleavable DADPS⁴¹ linker followed by enzymatic digestion. Peptides were enriched via streptavidin, released from the resin by acid, and then tandem mass tag (TMT) labeled for quantitative LC-MS/MS analysis (Figure 5B). A total of 640 sites of sulfenylation were identified (63% quantified) on 477 protein targets using **23**. Of the targets identified by **23**, 411 (64%) were also identified using an analogous dimedone-based probe (DYN2⁴²; Figure S42). The top quantified peptide hits, which are quantitatively compared in the heatmap in Fig 5C, include well-known sulfenic acid redox proteins such as peroxiredoxin 6. Additionally, 229 (36%) of the identified peptides were unique to SAM-TCO **23**, (Fig S42). These proteomic data further illustrate the selective and complementary reactivity of SAM-TCO probes for protein sulfenylation. Future efforts will be directed to develop a proteomic workflow that will enable sulfenic acid profiling by SAM-TCO probes in living cells.

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In summary, SAM-TCOs are cell permeable and bioorthogonal reagents dependent upon an intramolecular nucleophile for transannular thioetherification of sulfenic acid species. SAM-TCO selectively labels a model peptide sulfenic acid, and also labels the sulfenic acid form of the protein fRMsR with an *in vitro* rate constant of 750 M⁻¹s⁻¹. We have demonstrated the capture of sulfenic acid species in live cells using a biotinylated SAM-TCO probe. Further, SAM-TCO probes can also engage in bioorthogonal Diels-Alder reactions with tetrazines, providing a temporally-controlled method for quenching the activity of the probes in living cells. A cell-based proteomic study validates the ability of SAM-TCO probes to identify and quantify known sulfenic acid redox proteins as well as targets not captured by dimedone-based probes. We anticipate these advantages will facilitate identification of native sulfenic acid species present in living systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SAM-TCO probes for transannular thioetherification can be quenched by tetrazine ligation in live cells with temporal precision.

Figure 2.
Labeling Studies of SAM-TCO with Electrophilic Selenium and Sulfur Model Compounds.

Figure 3.

(A) Oxidation of fRMSr (**16**) gives a stable sulfenic acid **17** that can be captured by SAM-TCO **18**. Kinetics were followed by bioorthogonal quenching with **19** at discrete timepoints. (B,C) Deconvoluted ESI-MS spectra of **16** and the reaction mixture of **17** and **18** after 300 s, demonstrating conversion to **20**. (D) Second order rate constant (k_2) for the reaction of **17** with **18** was determined under pseudo-first order conditions. (E) Tandem MS analysis of GluC digested fMSR shows that labeling by **18** is site-specific. (F) Deconvoluted ESI-MS spectra of **21** (before) and **22** (after) quantitative capture with **18**.

Figure 4.

(A) Live cell labeling with **SAM-TCO biotin**, illustrating use of **19** to quench the probe prior to cell lysis and western blot analysis. (B) Live LNCaPs were labeled with increasing amounts of TCO-Biotin (0 – 500 μ M) under native conditions and analyzed by Western blot using HRP-Streptavidin chemiluminescence and GAPDH as a loading control. (C) Comparison of SAM-TCO biotin 100 μ M and TCO biotin 100 μ M labeling in live cells. (D) Temporal control for quenching of SAM-TCO biotin in live cells with **19** was assessed by the introduction of H₂O₂ after bioorthogonal quenching (Lane B) and prior to quenching (Lane C) with **19**. Lane A is a control for quenching with **19** without additional H₂O₂ and Lane D is a control for increased labeling time.

Figure 5. SAM-TCO probe **5** labeling of target proteins in HEK293T cell lysate. (A) Workflow for proteomic analysis. (B) Tandem MS analysis of peroxiredoxin-6 (PRDX6) as a representative example of SAM-TCO labeled proteins that were identified and quantified. (C) Heatmap illustrating the top quantified peptide hits that were labeled by **23**, with comparison to DYn2.