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Cyclic Thiosulfinates and Cyclic Disulfides Selectively Crosslink Thiols While Avoiding Modification of Lone Thiols

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Supporting Information Placeholder

ABSTRACT: This work addresses the need for chemical tools that can selectively form crosslinks. Contemporary thiol-selective crosslinkers, for example, modify all accessible thiols, but only form crosslinks between a subset. The resulting terminal "dead-end" modifications of lone thiols are toxic, confound crosslinking-based studies of macromolecular structure, and are an undesired—and currently unavoidable—byproduct in polymer synthesis. Using the thiol pair of Cu/Zn-superoxide dismutase (SOD1) we demonstrated that cyclic disulfides—including the drug/nutritional supplement lipoic acid—efficiently crosslinked thiol pairs but avoided dead-end modifications. Thiolate-directed nucleophilic attack upon the cyclic disulfide resulted in thiol-disulfide exchange and ring cleavage. The resulting disulfide-tethered terminal thiolate moiety either directed the reverse reaction, releasing the cyclic disulfide, or participated in oxidative disulfide (crosslink) formation. We hypothesized—and confirmed with density functional theory (DFT) calculations—that mono-*S*-oxo derivatives of cyclic disulfides formed a terminal sulfenic acid upon ring cleavage that obviated the previously rate-limiting step, thiol oxidation, and accelerated the new rate-determining step, ring cleavage. Our calculations suggest that the origin of accelerated ring cleavage is improved frontier molecular orbital overlap in the thiolate-disulfide interchange transition. Five to seven-membered cyclic thiosulfinates were synthesized and efficiently crosslinked up to 10^4-fold faster than their cyclic disulfide precursors; functioned in the presence of biological concentrations of glutathione; and acted as cell-permeable, potent, tolerable, intracellular crosslinkers. This new class of thiol crosslinkers exhibited click-like attributes including, high yields driven by the enthalpies of disulfide and water formation, orthogonality with common functional groups, water-compatibility, and ring strain-dependence.

Thiol-ene reactions are prevalent in applications requiring thiol crosslinking.[1] Synthetic applications of thiol-ene crosslinking reactions include: self-healing polymers,[2] nanogels,[3] thermosetting polymers, hydrogels,[4] and dendrimers.[5] Prevalent biochemical applications of thiol-ene crosslinking include functionalizing or stabilizing biotherapeutics in vitro,[6] and probing high-order protein structure and protein-protein interactions.[7] One shortcoming of these thiol-ene crosslinking tools—in fact all current tools—is that they are not *crosslinking* selective. These tools will form terminal "dead-end" modifications unless two functional groups happen to be within their reach.[8] Dead-end modifications are toxic in vivo; in particular the modification of essential catalytic cysteines (e.g. phosphatases and cysteine [Cys] proteases) and the creation of "non-self" epitopes that increase the risk of an adverse immune response.[9] This inherent toxicity, and poor cell permeability, have stymied in vivo crosslinking. To enable the in vivo use of the crosslinking applications described above, our objective is a chemical tool with improved selectivity for crosslinking thiols (i.e. higher crosslinking efficiency).

In addition, we introduce in vivo thiol crosslinking as a strategy for pharmacological protein stabilization, and a long-sought, non-inhibitory alternative to stabilization with substrate analogues.[10] A number of diseases, including familial Amyotrophic Lateral Sclerosis (fALS), are associated with loss of quaternary structure and protein destabilization (seen with Cu/Zn-superoxide dismutase (SOD1) mutations). Multimer stabilization—exemplified by the substrate/cargo analogue, transthyretin-stabilizing drug tafamidis[11]—is a therapeutic strategy in these diseases. We used thiol-ene crosslinkers in a proof-of-concept

study to demonstrate that crosslinking the thiol pair (Cys $_{111A+B}$, 8 Å apart: PDB 1SPD shown in TOC figure) on adjacent subunits of SOD1 could stabilize fALS-SOD1 variants by up to 40 °C.[7] This approach also rescued the enzymatic activity of inherently inactive fALS SOD1 variants.[7] Through a computational screen of human protein structures, 20 additional multimeric proteins with quaternary structures that could be stabilized by inter-subunit cysteine crosslinking were discovered.

We surveyed drugs to identify mechanisms for selective thiol binding that can be tolerated in vivo. One recent approach to drug design is to attach a soft, sometimes finely "tuned" Cys-selective electrophile[9] to a high-affinity binder.[12-13] Unfortunately, as is often the case, the lack of high-affinity SOD1 binders ruled out this structure-based approach. The other mechanism used by thiolate-selective drugs, disulfide bond formation, is the most prevalent and mature (disulfiram/Antabuse treatments began in 1948).[14] Inactive prodrugs are transformed into thiols, which, after spontaneous oxidation, form long-lived disulfide bonds between the drug metabolite's sulfenic acid and a target protein's cysteine thiolate. Some drugs form disulfides with enzyme active site Cys (e.g. disulfiram[15]) and others with allosteric Cys (e.g. omeprazole/Prilosec,[16] prasugrel/Effiant,[17] *etc.*). Unfortunately, the obvious strategy of binding two of these drugs to create a bifunctional crosslinker would not result in a tool that could avoid dead-end modifications.

Having ruled out crosslinkers composed of existing thiolate-selective warheads, we sought molecules with the ability to minimize dead-end Cys modifications as a starting point for crosslinkers. Cyclic disulfides are the only thiolate-selective scaffold we knew of that can form transient bonds (i.e. can avoid dead-end modifications without the aid of other molecules). Cyclic disulfide chemistry was extensively characterized in a series of publications by the Whitesides' group.[18-20] These studies demonstrated the high effective concentration (EC – i.e. the entropically-driven propensity to remain oxidized and cyclic; specifically the K_{eq} between a dithiol forming a cyclic disulfide and a dialkyl disulfide forming two thiols) of cyclic disulfides results in transient binding to lone thiols. Moreover, the K_{eq} of cyclic disulfide binding to lone thiols (i.e. "K_{eq} dead-end") is highly ring strain-dependent, varying over three orders of magnitude.[18-19] Cyclic disulfide-tethered drug cargos can even be transported across the cell membrane via reversible binding to a transferrin receptor Cys.[21-22] Cyclic disulfides can be tolerated at doses up to 5 g/day/person and have an LD_{50} in the range of ethanol, fructose, and sodium chloride.

We reasoned that cyclic disulfides could crosslink thiol pairs while minimizing dead-end Cys modifications. A reversible S_N2-type attack of a Cys thiolate upon a cyclic disulfide would result in thiolate-disulfide interchange concomitant to ring opening to form a terminal thiolate. If this terminal thiolate was within binding distance of a sulfenate (i.e. oxidized Cys), a crosslink could form by their condensation to a disulfide bond (**Figure 1, Mechanism II**).[23] Otherwise, the cyclic disulfide would be released by the reverse (thiolate-disulfide interchange) reaction (**Figure 1, Mechanism I**). Furthermore, if mono-*S*-oxo cyclic disulfides (cyclic thiosulfinates) were used instead, thiolate oxidation, the slowest step of the crosslinking reaction sequence, would not be required (**Figure 1, Mechanism III**). Instead, thiolate-disulfide interchange with a cyclic thiosulfinate would lead directly to a disulfide-bound terminal sulfenic acid, which would rapidly form a crosslink by condensing with the second, nearby thiolate, releasing water.[23] Thiolate-disulfide interchange proceeds through a linear trisulfide-like intermediate comprised of nucleophilic-(S_n), center-(S_c), and leaving group-(S_l) sulfurs with Brønstead coefficients (β) of ~0.5, -0.3, and -0.7, respectively.[19, 24] The Brønstead coefficients of -0.7 and -0.3 for the leaving group and central sulfur, respectively, and our quantum mechanical calculations (**Figure 1, bottom panel**) imply that the rate of thiolate-disulfide interchange is highly sensitive and inversely proportional to the pK_a of S_l. We used DFT calculations to understand the origins of the nearly 110-fold reactivity increase of **1** towards lone thiolates over **5** and the observed reversibility. We performed a conformational search on the starting materials, transition structures, and intermediates (full computational details are

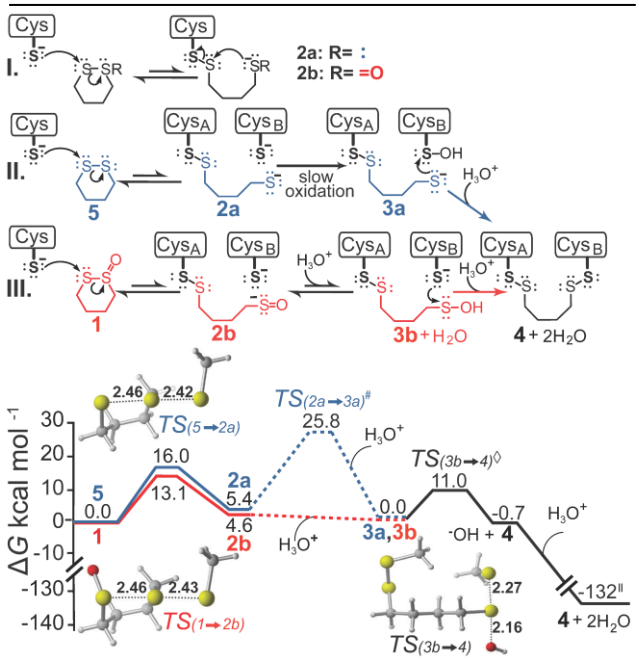

Figure 1. Proposed mechanism of thiol crosslinking using cyclic disulfides (blue) and cyclic thiosulfinates (red). (Top) Formation of the first disulfide bond is reversible. Dead-end modification is minimized by entropically favorable ring closure (**I**). Crosslinking proceeds through condensation of Cys_A, and a sulfenic acid derived from either rate limiting S-oxidation of thiolate$_B$ (**II**) or a cyclic thiosulfinate (**III**). (Bottom) A potential energy surface for the non-enzymatic reaction according to the mechanism proposed in the top panel. The free energy values are computed using M06-2X/6-311+G(d,p) IEF-PCM^{H2O}//M06-2X/6-31+G(d,p) IEF-PCM^{H2O}. #The dotted blue line indicates an activation barrier derived from the zeroth-order half-life kinetics of the oxidation of **5**. Details on energy value of structures 3a and 3b are given in **SI Section II, O**. ‖-132 kcal mol^{-1} experimentally-derived enthalpy from reported values of heat of formation of water and experimentally determined bond energy of dimethyl disulfide.[25-26] ◊The activation barrier for **TS(3a→4)** is 0.5 kcal mol^{-1} higher than **TS(3b→4)** (11.5 kcal mol^{-1}) **(Figure S10)**. Transition structures for the nucleophilic addition of MeS$^-$ to **1** and **5** are shown. The bond lengths and energy values are reported in Å and kcal mol^{-1}, respectively.

provided in the **Section II-part N** of the SI). We represented a Cys thiolate as methyl thiolate to reduce the conformational search space and computation time.

The 2.9 kcal mol^{-1} lower activation free energy of **TS(1→2b)** vs. **TS(5→2a)** is due to more favorable frontier molecular orbital interactions in the transition state. The σ* orbital (**Figure S11**) of **1** is 0.21 eV lower in energy than that of **5**, thus lowering the energy of **TS(1→2b)**. The ring-opening step for **1** and **5** are endergonic (ΔG = 4.6 and 5.4 kcal mol^{-1}, respectively) and reversible, consistent with experiments. The formation of the crosslinked product and water is thermodynamically favored, over 130 kcal mol^{-1} lower in free energy than the reactants (**1** and **5**).[25-26] Upon ring-opening of 1,2-dithiane, **2a** is slowly oxidized ($t_{1/2}$ = 10 days) to **3a**, which is rate-determining and affords the final crosslinked product. The QM results highlight two major implications for the low pK_a sulfenic acid group. First, the nucleophilic attack on the non-oxo-thiosulfinate *S*, which releases sulfenic acid, is >10-fold faster and therefore more likely than the attack on the more electrophilic sulfinyl sulfur. Second, in addition to eliminating the need for rate-limiting thiol oxidation, thiosulfinates, through the sulfenate intermediate generated, also increase the rate of thiolate-disulfide interchange.

To test the crosslinking activity of cyclic disulfides and cyclic thiosulfinates, 1,2-dithiane and 1,2-dithiane-1-oxide were synthesized and incubated with SOD1, a homodimeric protein containing a solvent accessible thiol pair (Cys_{111A+B}, 8 Å apart) on adjacent subunits. The reaction was monitored using a mass spectrometry (MS) assay that uses a combination of increased voltage within the region of hypersonic gas expansion and brief treatment with 10% formic acid to create exclusively monomeric SOD1 or covalently crosslinked SOD1 dimer. Formic acid also quenches the reaction, transforming any reactive thiolates to unreactive thiols. As a result, if no reaction occurs, only apo SOD1 monomer is detected (**Figure 2, top**). Consistent with our hypothesized mechanism: 1) 1,2-dithiane-1-oxide, but not 1,2-dithiane, resulted in rapid and complete dithiolate crosslinking (half-life ~2-3 min) of SOD1 (**Figures 2, middle and Figure S1**); 2) no binding to single Cys residues (SOD1 has free Cys_{111} and Cys_6) was observed in any sample with either compound; 3) No crosslinking was observed without the loss of the oxygen from the *S*-oxo of 1,2-dithiane-1-oxide; 4) No crosslinking was observed when incubating 1,2-dithiane-1-oxide with $C_{111}S$ SOD1 (**Figure S2**). Given sufficient time for thiolates to be oxidized to sulfenic acid (which occurs on the order of days-weeks), even 1,2-dithiane was expected to crosslink SOD1. After 72 h of incubation with 1,2-dithiane, 11% of SOD1 had formed the expected covalent dimer (**Figure S3**). Comparable results were observed from the incubation of SOD1 with 1,2-dithiepane and 1,2-dithiepane-1-oxide (**Figure S12**).

To demonstrate the utility of 1,2-dithaine-1-oxide as a cell penetrating, dithiol pair crosslinker, the crosslinking reaction was examined both in two widely used human cell lines (Hep G2 and HeLa), and with purified SOD1 in the presence of competing reduced glutathione or DTT. Hep G2 and HeLa cells both contain approximately 5 mM glutathione.[27] Hep G2 cells (**Figure 2, bottom)** and HeLa cells (**Figure S4**) incubated with various concentrations of 1,2-dithaine-1-oxide for 30 min showed an EC_{50} of ~5 μM in western blots, confirming that cellular conditions do not prohibit crosslinking. Cell viability was not affected by 1,2-dithiane-1-oxide concentrations that were 50-fold higher than the EC_{50}, and the LC_{50} of 1,2-dithiane-1-oxide was approximately 200-fold greater than its EC_{50} (**Figure S5**). Consistent with the cellular studies, crosslinking of purified

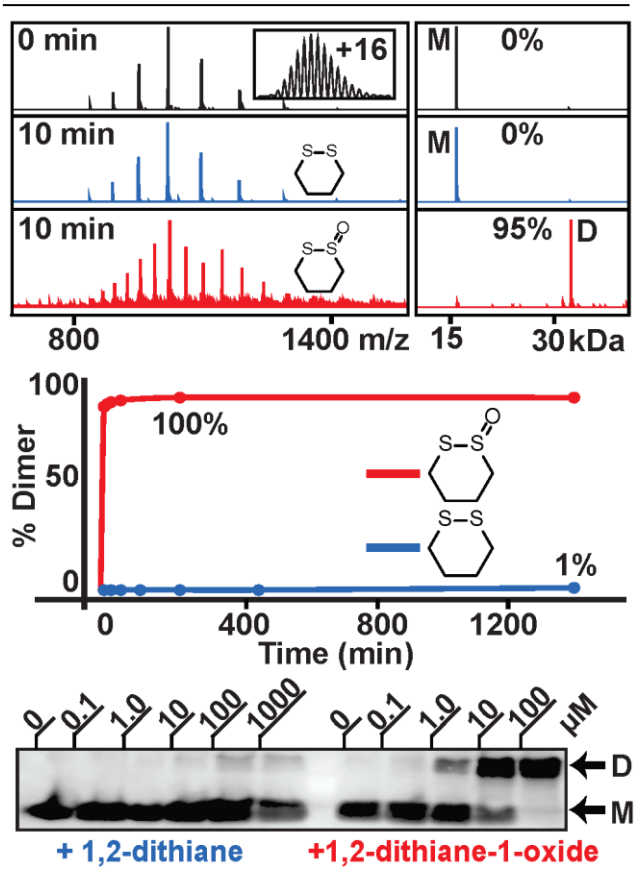

Figure 2. Thiol crosslinking by cyclic thiosulfinates kinetically stabilizes the SOD1 dimer in vitro and in cells. Representative raw (Top left) and deconvoluted (Top right) mass spectra used for calculating crosslinking rates (Middle). The 31,808 Da molecular mass of the crosslinked dimer (**D**) supports the mechanism proposed in **Figure 1** (e.g. two SOD1 monomers [2 x 15,844 Da (**M**)] + 1,2-dithiane-1-oxide [136 Da] – oxygen [16 Da]). After 10 min of incubation 1,2-dithiane-1-oxide and 1,2-dithiane crosslinked 95% and 0% of SOD1, respectively. Consistent with the rate of cyclic disulfide crosslinkers being limited by thiol oxidation, 1,2-dithiane crosslinks only 11% of SOD1 after three days (**Figure S3**). (Bottom) Western blot of SOD1 from Hep G2 cells incubated with various concentrations of compounds for 30 min. EC_{50}s for 1,2-dithiane-1-oxide crosslinking were 1-5 μM in Hep G2 and HELA (**Figure S4**) cells.

SOD proceeded to completion in the presence of 10:1 ratio of glutathione:1,2-dithiane-1-oxide (**Figure S8**), and even in the presence of equimolar concentrations of the reducing agent dithiothreitol (DTT) (**Figure S9**). The rate of crosslinking was decreased in the presence of competing reductants, presumably due to reversible thiolate-disulfide interchange between reductants and 1,2-dithiane-1-oxide (no glutathionyl or DTT adducts with 1,2-dithiane-1-oxide or SOD1 were observed). These results confirm the utility of these crosslinkers even in presence of modest amounts of additional reducing agents.

Cyclic disulfides[28] and their derivitives (e.g. dithiolene thiones)[29] have been used therapeutically and many of their targets are known. However, the binding mechanism of these drugs, including that of the nutritional supplement and diabetic complication treatment, α-lipoic acid (ALA), have not been characterized.[30] To broaden the applicability of cyclic disulfide

mediated crosslinking and explore a potential MOA, ALA was purchased and β-lipoic acid (BLA) was synthesized and assayed as above. Compared to ALA, BLA crosslinked SOD1 in cells and crosslinked 30% more SOD1 in vitro (**Figure S6 and S7**). Notably, the terminal carboxylic acid on ALA and BLA presents an opportunity for functionalization.[31]

Critical features of cyclic thiosulfinate reactivity include: 1) toxic binding to single thiolates is reversible through thiolate-disulfide interchange but thiol pair crosslinking is not; 2) the leaving group is expended upon crosslinking; 3) crosslinking proceeds in water and is driven by the considerable bond enthalpies of S-S bond and water formation (-132 kcal mol^{-1}), resulting in high yields; 4) Reactive functional groups, including carboxylates, amines, and disulfides, are avoided; 5) Cyclic disulfide S-S bond strength and reactivity has a strain-dependence that greatly exceeds that of rings composed of period-two elements;[18] and 6) competing reductants are tolerated and crosslinking can occurs in cells.

In summary, cyclic disulfide reactivity, including reversible binding to lone thiols, is predictable and highly tunable. Cyclic thiosulfinate crosslinkers have potential as: 1) A less toxic alternative to Cys specific di-ene crosslinkers and phenylarsine oxide crosslinkers, which can both react with monothiols,[32] 2) Probes for proteinaceous Cys-dithiolates, which perform essential in vivo functions and often serve as metal and metallocofactor ligands,[33-35] 3) Inter-functional group distance measurement tools, 4) Biocompatible templates for higher order structures in polymer synthesis, and 5) Cellular thiol pair crosslinkers.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website (link) at DOI (#####, with link). Experimental procedures and characterization data for reactions and products (PDF link).

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Notes

The authors declare no competing financial interests.

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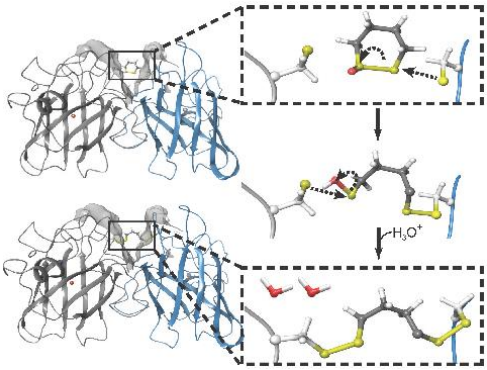

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