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FULL PAPER

Dithiasuccinoyl-Caged Amines Enables COS/H_2S Release Lacking Electrophilic Byproducts

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Abstract: The enzymatic conversion of carbonyl sulfide (COS) to hydrogen sulfide (H_2S) by carbonic anhydrase has been used to develop self-immolating thiocarbamates as COS-based H_2S donors to further elucidate the impact of reactive sulfur species in biology. The high modularity of this approach has provided a library of COS-based H_2S donors that can be activated by specific stimuli. A common limitation, however, is that many such donors result in the intermediate formation of an electrophilic quinone methide byproduct during donor activation. As a mild alternative, we demonstrate here that dithiasuccinoyl groups can function as COS/H_2S donor motifs and that these groups release two equivalents of COS/H_2S and uncage an amine payload under physiologically relevant conditions. Additionally, we demonstrate that COS/H_2S release from this donor motif can be altered by electronic modulation and alkyl substitution. These insights are further supported by DFT investigations, which reveal that aryl and alkyl thiocarbamates release COS with significantly different activation energies.

Introduction

Despite being a malodorous gas,[1] hydrogen sulfide (H_2S) is an important biological signaling molecule often referred to as a gasotransmitter alongside carbon monoxide and nitric oxide.[2] H_2S-mediated signaling is important in several physiological processes including vasodilation,[3] neurotransmission,[4] and inflammation.[5] These findings have led researchers to propose the use of H_2S as a potential therapeutic agent for a variety of conditions and pathologies.[6] Toward this goal, researchers have relied heavily on the use of NaSH and Na_2S as sources of H_2S due to ease of handling and commercial availability; however, H_2S release from these salts is considerably different relative to enzymatic H_2S generation.[7] To better mimic endogenous H_2S production, methods of generating H_2S at controlled rates under physiologically-relevant conditions are needed,[8] and the development of small molecule "H_2S donors" is an active research area aimed at addressing this need.[9] Such compounds generate H_2S by passive hydrolysis[10] or activation in the presence of specific stimuli including light,[11] biological thiols,[12] and cellular enzymes including esterases.[13]

Recently, an alternative approach to H_2S generation has utilized the hydrolysis of carbonyl sulfide (COS) by carbonic anhydrase (CA), a ubiquitous metalloenzyme.[14] Existing in Nature as the most abundant sulfur-containing gas in the atmosphere,[15] COS is rapidly converted to H_2S and carbon dioxide (CO_2) in the presence of bovine carbonic anhydrase II (k_{cat}/K_m = 2.2 x 10^4 M^{-1} s^{-1}).[16] In our initial approach, we were inspired by self-immolative carbamates, which release CO_2 as a byproduct upon activation,[17] and developed analogous self-immolative thiocarbamates that function as tunable COS-based H_2S donors (Figure 1a).[18]

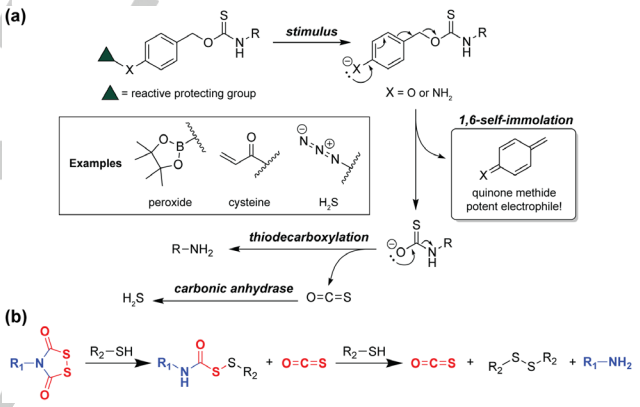

Figure 1. (a) Generalized reaction mechanism for COS release from self-immolative thiocarbamates and subsequent hydrolysis of COS to H_2S by carbonic anhydrase. (b) Overall reaction scheme of COS release from dithiasuccinoyl groups in the presence of thiols.

The high modularity of this scaffold has enabled the rapid expansion of this approach by our group[9] as well as others to prepare COS-based H_2S donors activated by various stimuli including acidic pH,[19] esterases,[20] reactive oxygen species,[21] and cysteine.[22] This approach has also been extended to provide oligomeric COS-based H_2S donors.[23] A critical, yet often overlooked component of this approach is the formation of a quinone methide byproduct, which is a potent electrophile and known Michael acceptor in biological systems.[24] Although we have not observed cytotoxicity from this byproduct in our studies, chronic exposure from therapeutic administration of these compounds is likely to induce electrophilic stress leading to long-term cytotoxicity.[25] As an alternative approach, Matson and co-workers have reported both small molecule and polymeric *N*-thiocarboxyanhydrides (NTAs) as COS/H_2S donors, which only result in small peptide byproducts.[26] These donor compounds, however, exhibit a relatively low H_2S-releasing efficiency and lack significant reactivity studies for COS release in response to

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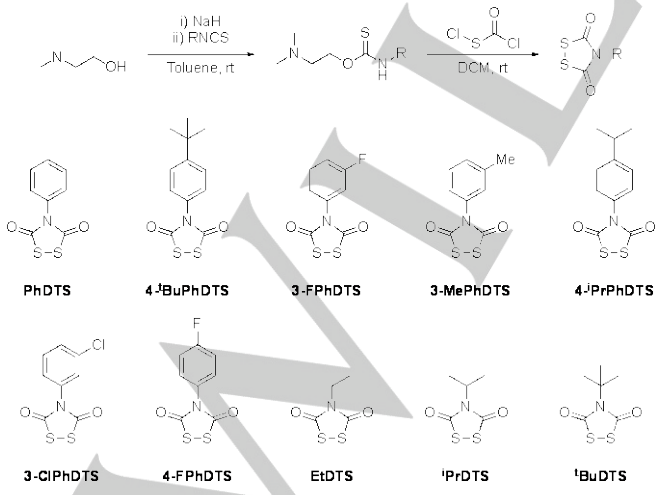
various analytes. To further develop COS-based H_2S donors as both research tools and potential pharmacological agents, alternative donor motifs are needed that lack electrophilic byproducts, maximize COS/H_2S release, and allow for simple tuning of release rates.

To address these needs, we focused on the reactivity of the dithiasuccinoyl (DTS) group, which has been used previously as a protecting group for amines in peptide synthesis.[27] The DTS group is cleaved by thiols, which results in reduction to a symmetric disulfide, two equivalents of COS, and an amine (Figure 1b). We note that previous studies on thiol-mediated reduction of DTS groups examined this reactivity in organic solvents using β-mercaptoethanol as the reductant and quantified reaction kinetics through the use of an amino acid analyzer without direct observation of COS.[28] We envisioned that this reactivity could be harnessed to prepare a library of COS-based H_2S donors that do not release electrophilic byproducts, but that readily release COS/H_2S under physiologically relevant conditions in the presence of CA. Herein, we demonstrate that DTS-caged amines function as versatile COS/H_2S donors activated by biological nucleophiles, including cysteine and reduced glutathione (GSH). Additionally, we use a combination of experimental and computational investigations to demonstrate that the rate of COS/H_2S release can be readily tuned by electronic modulation and subsequent stabilization of the COS-releasing thiocarbamic acid intermediate.

Results and Discussion

To prepare a small library of COS-based H_2S donors with tunable release rates, we treated alkyl and aryl isothiocyanates with *N,N*-dimethylethanolamine in the presence of sodium hydride to generate the desired thiocarbamate intermediate. Subsequent treatment with chlorocarbonylsulfenyl chloride afforded the desired DTS-caged compounds (Scheme 1). The amines chosen for this library included aryl amines with different electron donating/withdrawing properties, as well as alkyl amines with increasing steric bulk.



Scheme 1. Synthesis of DTS-based COS/H_2S donors.

To assess the viability of these compounds as COS/H_2S donors, we examined the release of H_2S from **PhDTS** (25 μM) in the presence of biologically-relevant nucleophiles (500 μM, 20 equiv.) and CA (25 μg/mL) using the methylene blue assay to measure H_2S generation (Figure 2).[29] Our expectation was that this donor functional group would be activated broadly by different nucleophiles rather than one specific biological nucleophile, thus broadening the scope of potential activation pathways.

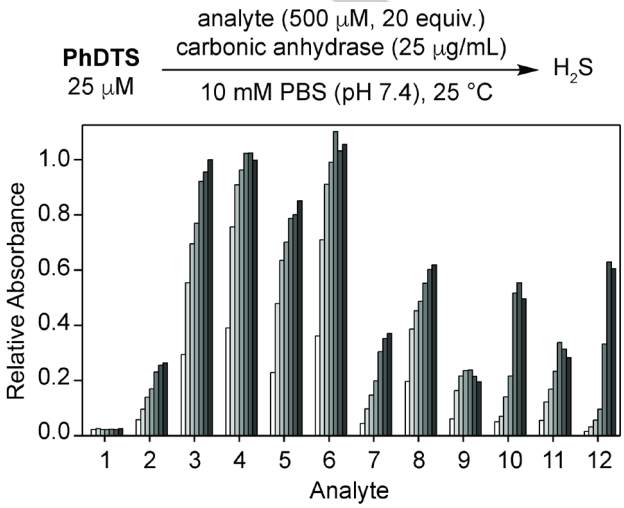


Figure 2. Activation profiles of H_2S release from **PhDTS** (25 μM) in the presence of different potential nucleophiles (500 μM, 20 equiv.) and carbonic anhydrase (25 μg/mL). Data were acquired at 1, 5, 10, 15, 30, 45, and 60 min. Methylene blue absorbance values are relative to the maximum absorbance value obtained from H_2S release in the presence of cysteine (3). Analytes: H_2O with no carbonic anhydrase (1), cysteine with no carbonic anhydrase (2), *N*-acetyl-L-cysteine (4), L-cysteine methyl ester (5), *N*-acetyl-L-cysteine methyl ester (6), homocysteine (7), reduced glutathione (8), serine (9), lysine (10), only carbonic anhydrase (11), *S*-methyl cysteine (12).

In the absence of CA, we did not observe hydrolysis-mediated H_2S release from **PhDTS**. The addition of cysteine to **PhDTS** in the absence of CA resulted in slow, yet gradual H_2S release. We attribute this observation to the hydrolysis of COS at physiological pH, which has been reported previously to be slow.[30] Treatment of **PhDTS** with cysteine in the presence of CA resulted in significant H_2S generation. Using a calibration curve generated with known concentrations of NaSH, we measured 40 μM H_2S generation from 25 μM **PhDTS** in the presence of 500 μM cysteine, which corresponds to an H_2S releasing efficiency of 80% (Figure S45). This observation not only highlights the high efficiency of H_2S release from DTS-caged compounds, but also supports that two equivalents of COS are released per DTS group.

In addition to COS/H_2S release, we also observed the formation of aniline following treatment of **PhDTS** with cysteine by HPLC, which further supports the proposed releasing mechanism (Figure S48). Protection of the amine and/or carboxylate groups on cysteine did not significantly impact the rate or quantity of COS/H_2S release from **PhDTS**, which supports a thiol-mediated releasing pathway. Interestingly, the use of *S*-methyl cysteine resulted in slow, yet considerable H_2S release suggesting a less favorable, thiol-independent reaction pathway. In previous studies, the direct reaction of amines at the carbonyl position of DTS groups has been observed and proposed to result in the

generation of COS and sulfane sulfur.[28] We observed a similar rate of H_2S release in the presence of lysine, which further supports a minor, amine-mediated mechanism of H_2S release. We note an induction phase in the rate of amine activation and find that this reaction is slower relative to the thiol-mediated reduction of DTS groups (Figure S49). Together with the decreased nucleophilicity of amines due to protonation at physiological pH and lower biological concentrations relative to thiols, we expect this mechanism of activation to be negligible in a biological context. In the presence of homocysteine and GSH, we observed lower quantities of COS/H_2S released, which we attribute to the lower nucleophilicity of these thiols as a function of thiolate/thiol speciation at physiological pH.[31] We failed to observe COS/H_2S release in the presence of serine implying that alcohol-mediated mechanisms of activation are not present.

In the presence of CA, but absence of any added nucleophiles, we did observe slight H_2S production. We hypothesized that this release could be due to coordination of **PhDTS** to the Zn^{2+} center in CA, which would facilitate hydrolysis by carbonyl activation. To probe this reactivity, we pre-incubated CA with the CA inhibitor acetazolamide (5 μM) and measured H_2S release from **PhDTS** (Figure S46). Under these conditions, we failed to observe H_2S generation, which supports the hypothesis of a minor CA/Zn^{2+}-mediated hydrolysis mechanism. Alternatively, this could also be due to minor background DTS hydrolysis followed by COS conversion to H_2S by CA. Further experiments using a catalytic amount of $Zn(OAc)_2$ (5 μM) did not result in COS/H_2S release from **PhDTS** thus highlighting the need for the protein microenvironment present in CA for activation of **PhDTS** (Figure S47).[32] Similar to the reactivity with amines, the rate of CA/Zn^{2+}-mediated hydrolysis is slower than the thiol-mediated reduction. Taken together, these results demonstrate the validity of **PhDTS** and related compounds to serve as COS/H_2S donors under physiologically-relevant conditions in the presence of thiols and CA.

With a small library of DTS-based donors in hand, we next examined the effect of the amine payload on COS/H_2S release using each donor (25 μM) in the presence of cysteine (500 μM, 20 equiv.) and CA (25 μg/mL) at physiological pH (Figure 3). We hypothesized that DTS-caging of functionalized anilines would allow COS/H_2S release rates to be tuned based on prior work aimed at solvent-dependent linear free energy relationship investigations into the phosphine-mediated sulfur extrusion from DTS.[33] Additionally, we expected that the caging of alkyl amines would lead to stabilization of the COS-releasing thiocarbamic acid intermediate and subsequently decrease the rate of H_2S release relative to that observed for DTS-caged anilines. In the presence of cysteine and CA, we observed varying rates and quantities of H_2S release from the reported aryl-based DTS compounds with **3-MePhDTS** and **4-^{t}BuPhDTS** displaying the fastest and slowest rates of H_2S release, respectively (Figure 3a). The releasing curves from the DTS-caged anilines did not fit cleanly to first-order exponential decay, which we attribute to competing COS-releasing and DTS consumption pathways, such as direct thiol activation versus CA-mediated activation. Additionally, previous work has reported the formation of isothiocyanates from sufficiently acidic thiocarbamates, which likely further complicates the rates of release from DTS-caged anilines containing electron-withdrawing groups.[34] Overall, the functionalization of caged anilines directly alters rates of COS/H_2S release from DTS-based donors, and the ability to control tuning of these releasing kinetics merits future investigation. By contrast, we observed the caging of alkyl amines resulted in significantly slower rates of H_2S release relative to DTS-caged anilines with **^{t}BuDTS** and **EtDTS** displaying the fastest and slowest rates of H_2S release, respectively (Figure 3b). We reasoned that inductively donating alkyl amines likely stabilize the COS-releasing thiocarbamic intermediates leading to a decrease in H_2S-releasing kinetics.

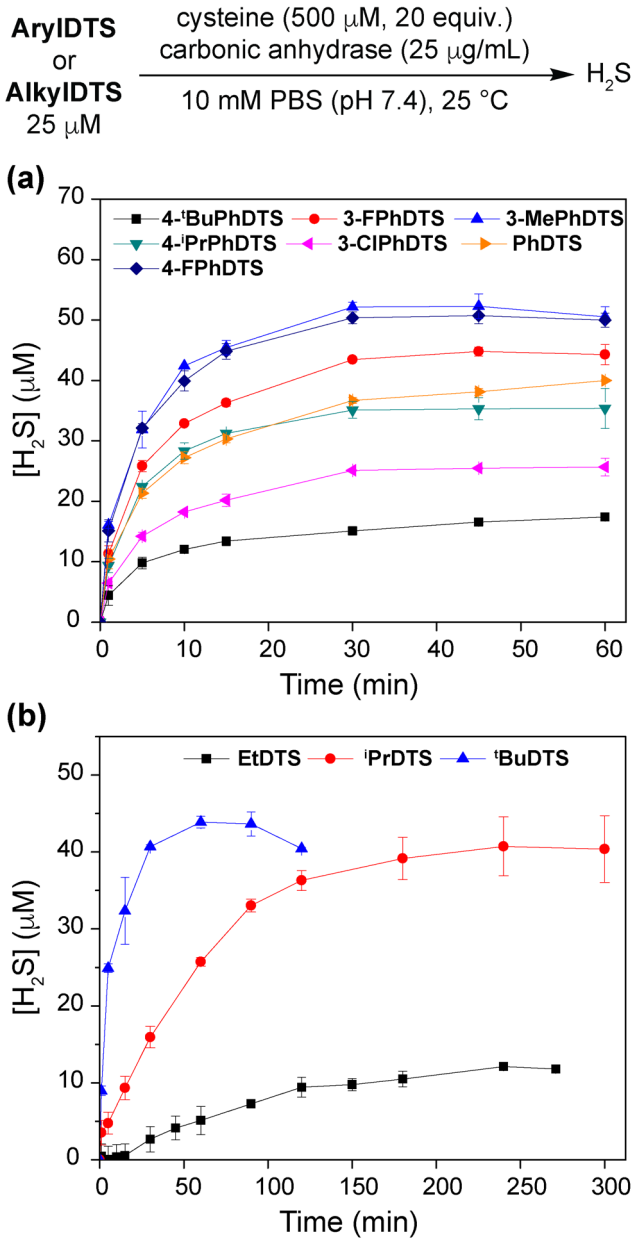


Figure 3. (a) H_2S release from aryl-based DTS compounds. (b) H_2S release from alkyl-based DTS compounds.

To further investigate the differences between aryl and alkyl amine substitution, we used density functional theory (DFT) to examine the potential energy surface for COS release from **PhDTS** and **AlkylDTS** compounds. In these systems, we used methyl thiol (**MeSH**) to simplify possible protonation states of non-participating functional groups during the reaction. Calculations

were performed using Gaussian 09 at the B3LYP/6-311++G(d,p) level of theory applying the IEF-PCM water solvation model (Figure 4).

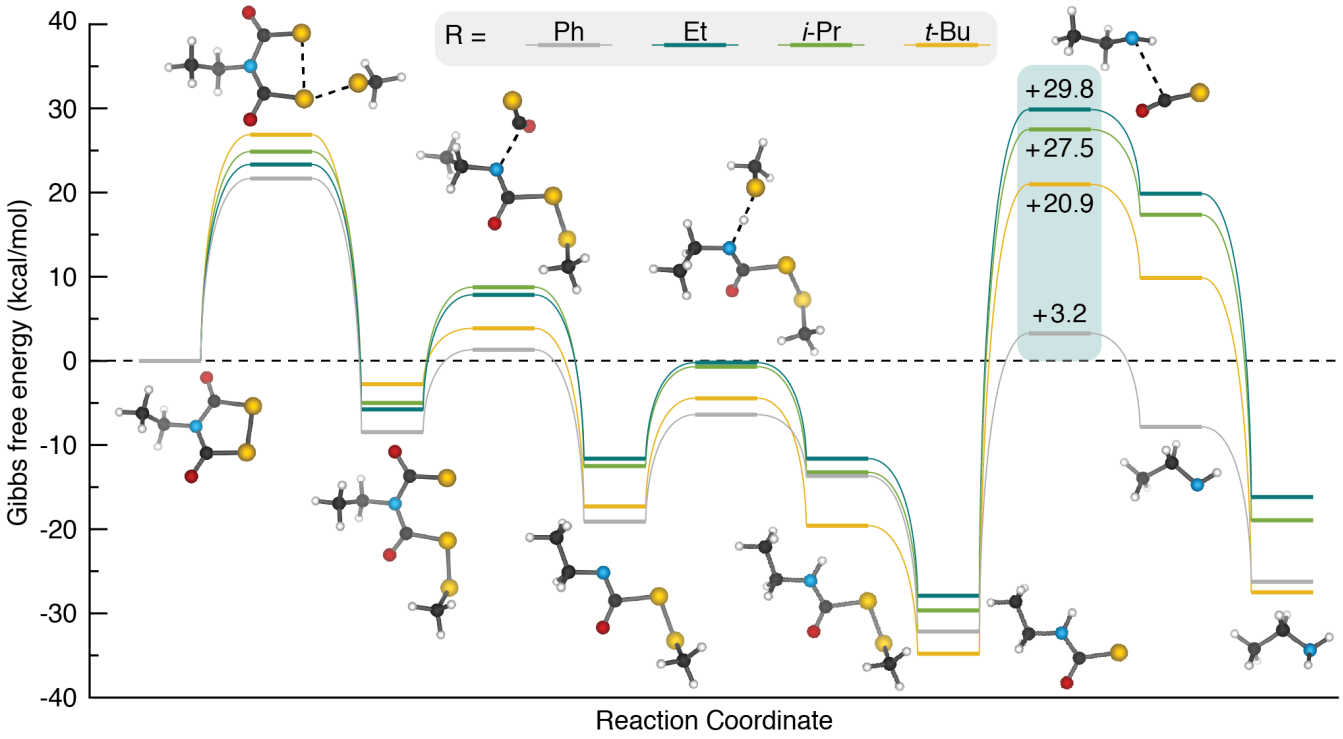


Figure 4. Potential energy surface for COS release from **PhDTS** and **AlkylDTS** compounds. Calculations were performed using Gaussian 09 at the B3LYP/6-311++G(d,p) level of theory applying the IEF-PCM water solvation model. **MeSH** was used as the thiol nucleophile to simplify accessible protonation states of non-participating functional groups on the thiol nucleophile.

For **PhDTS**, we found that the initial nucleophilic attack by the thiolate was the highest barrier (21.7 kcal/mol) on the reaction coordinate and dethiocarboxylation of the thiocarbamic acid intermediate was only moderately endothermic (+3.2 kcal/mol) with respect to the starting materials. By contrast, although the **AlkylDTS** compounds showed similar activation barriers for the initial attack by thiolate (23.5 – 26.8 kcal/mol), the activation barrier for the final dethiocarboxylation varied significantly as a function of the alkyl group. The highest activation barrier for dethiocarboxylation was found for the **EtDTS** compound (+29.8 kcal/mol), but this barrier decreased with the increasing donating ability of **iPrDTS** (+27.5 kcal/mol), and **tBuDTS** (+20.9 kcal/mol); all of which were competitive with the activation barriers for initial thiol attack on the DTS motif. These relative energetic barriers are consistent with the observed rates of COS/H_2S release from the **AlkylDTS** compounds. Moreover, these results suggest that the inductive contributions, rather than the steric bulk differences of the alkyl substituents, have a larger impact on the release of COS from thiocarbamic acid intermediates. Taken together, the combination of experimental and computational data demonstrates the ability to tune H_2S/COS release from this scaffold by simple structural modifications. Moreover, these results provide guidance for controlling the COS/H_2S release rate from any donor motifs that proceed through a thiocarbamic acid intermediate prior to COS extrusion.

Conclusion

We demonstrated the use of DTS-based compounds to serve as COS/H_2S donors in the presence of thiols without the formation of electrophilic byproducts. Reactivity studies using **PhDTS** as a model compound were used to investigate COS/H_2S release as a function of biological nucleophiles and thiol identity. By modifying the structure of the amine payloads, we also demonstrated that the rate of COS/H_2S release from DTS-based donors can be modified by simple structural modifications. The results from DFT calculations has shed light on the impact of amine identity on COS release from thiocarbamic acids and provides a foundation to guide future work on this reactive intermediate. Specifically, this work directly elaborates on the observed reactivity differences between aryl and alkyl thiocarbamates as COS-releasing motifs, which provides fundamental information upon which to expand the utility of these donors. The simple synthetic conditions and unique reactivity of this donor scaffold would readily allow for the incorporation amine-based payloads including fluorophores[35] and known therapeutics[36] to provide COS-based H_2S fluorescent donors and prodrugs.

Experimental Section

Synthesis Materials and Methods

Reagents were purchased from Sigma-Aldrich, Tokyo Chemical Industry, or VWR and used directly as received. Deuterated solvents were purchased from Cambridge Isotope Laboratories and used as received. ^{1}H, ^{13}C{^{1}H}, and ^{19}F NMR spectra were recorded on a Bruker 500 MHz instrument. Chemical shifts were reported relative to residual protic solvent resonances. MS data was collected on a Xevo G2-XS QTof (Waters) instrument. Silica gel (SiliaFlash F60, Silicycle, 230-500 mesh) was used for column chromatography. All air-free manipulations were performed under an inert atmosphere using standard Schlenk technique.

Synthesis

General Procedure for the Synthesis of Thiocarbamates: This procedure has been modified from a previous report.[33] In a flame-dried round bottom flask under nitrogen, sodium hydride (1.25 equiv.) and *N*,*N*-dimethylethanolamine were added to anhydrous toluene (20 mL). After stirring briefly until gas evolution ceased, the desired isothiocyanate (1.0 equiv.) was added dropwise (if liquid) or in a single portion (if solid). The reaction was stirred at room temperature for 3 h under nitrogen. The reaction was quenched with deionized H_2O (30 mL) and extracted with ethyl acetate (3 x 15 mL). The combined organic extractions were washed with brine (1 x 20 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The desired product was obtained following purification by column chromatography. All NMR data for these compounds was obtained at 60 °C due to hindered rotation of thiocarbamates at room temperature. We note the alkyl thiocarbamates displayed hindered rotation at 60 °C giving rise to two sets of peaks corresponding to the *E* and *Z* isomers.

General Procedure for the Synthesis of Dithiasuccinoyls: This procedure has been modified from a previous report.[33] To a flame-dried round bottom flask under N_2, chlorocarbonylsulfenyl chloride (1.0 equiv.) was added to anhydrous DCM (20 mL). In a separate vial, the desired thiocarbamate (1.0 equiv.) was dissolved in anhydrous DCM (1 mL) and added dropwise to the reaction. The reaction mixture was stirred at room temperature for 1 h. The reaction was quenched with 1 M HCl (15 mL) and the organic layer separated. The organic layer was washed with deionized water (2 x 20 mL), brine (1 x 20 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The desired product obtained by purification *via* preparative thin layer chromatography.

H_2S Detection Materials and Methods

Phosphate buffered saline (PBS) tablets (1X, CalBioChem) were used to prepare buffered solutions (140 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4) in deionized water. Buffer solutions were sparged with nitrogen to remove dissolved oxygen and stored in an Innovative Atmosphere nitrogen-filled glovebox. Donor stock solutions (in acetonitrile) were prepared inside a nitrogen-filled glovebox immediately before use. Trigger stock solutions (in PBS) were freshly prepared in an N2-filled glovebox immediately before use. CA stock solutions (in PBS) were freshly prepared in a nitrogen-filled glovebox immediately before use.

General Procedure for Measuring H_2S Release via Methylene Blue Assay (MBA)

Scintillation vials containing 20 mL of 10 mM PBS (pH 7.4) were prepared in a nitrogen-filled glovebox. To these solutions, 20 µL of 500 mM analyte stock solution and 50 µL of 10 mg/mL CA were added for final concentrations of 500 µM and 25 µg/mL respectively. While stirring, solutions were allowed to thermally equilibrate in heating block set at 25 °C for approximately 20-30 min. Immediately prior to donor addition, 0.5 mL solutions of methylene blue cocktail were prepared in disposable 1.5 mL cuvettes. The methylene blue cocktail solution contains: 200 µL of 30 mM $FeCl_3$ in 1.2 M HCl, 200 µL of 20 mM *N*,*N*-dimethyl-*p*-phenylene diamine in 7.2 M HCl, and 100 µL of 1% (w/v) $Zn(OAc)_2$. To begin an experiment, 20 µL of 25 mM donor stock solution was added for a final concentration of 25 µM. At set time points after the addition of donor, 500 µL reaction aliquots were added to the methylene blue cocktail solutions and incubated for 1 h at room temperature shielded from light. Absorbance values at 670 nm were measured 1 h after addition of reaction aliquot. Each experiment was performed in quadruplicate unless stated otherwise. UV-Vis spectra were acquired on an Agilent Cary 60 UV-Vis spectrophotometer equipped with a Quantum Northwest TC-1 temperature controller set at 25 ± 0.05 °C.

Computational Methods

All structures were initially constructed, and optimized using the UFF force field as implemented, in Avogadro.[37] The resultant structures were further optimized using the unrestricted hybrid GGA functional, B3LYP, as implemented in Gaussian 09,[38] with a triple zeta basis set that includes diffuse and polarization functions on heavy atoms, 6-311+G*. A pseudosolvent polarizable continuum model for water was used to account for solvation effects. Attacking thiols were modeled as methyl thiol to reduce computational expense.

Transition state searches were carried out at the same level of theory as ground state structures. First, a potential energy surface scan of the active reaction coordinate was used to obtain a good starting point for the ultimate transition state search algorithm. Vibrational analysis confirmed a single imaginary frequency corresponding to the direction of bond formation or breaking for each activated complex. No transition state was found for thiol disulfide exchange. This could indicate a barrierless transition, or a shallow potential energy surface with a loose transition state. Biologically relevant thiols, such as cysteine, whose attack may be sterically hindered unlike the methyl thiol employed in this model, may experience a more defined transition state and activation barrier.

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Keywords: hydrogen sulfide • carbonyl sulfide • reactive sulfur species

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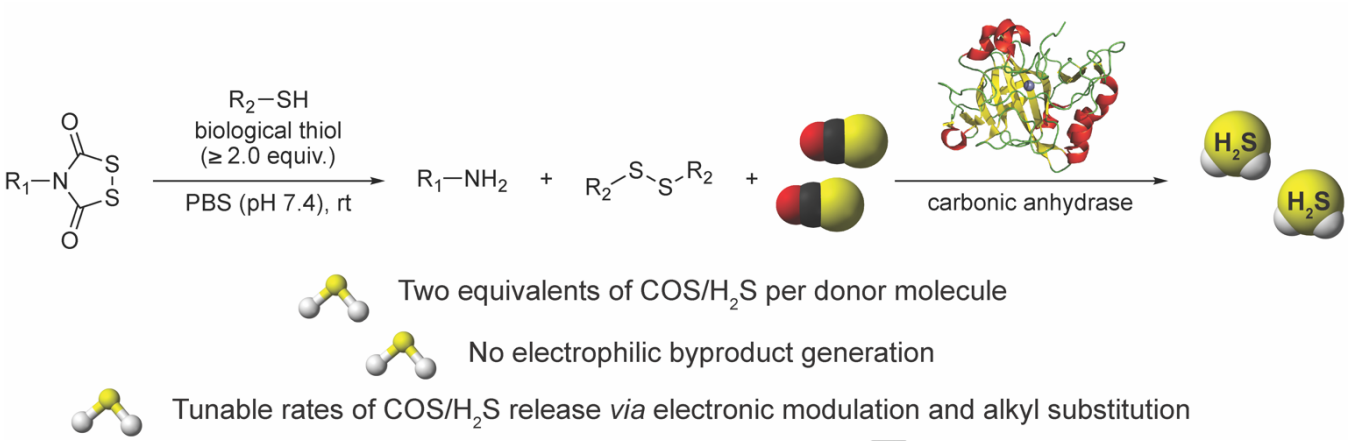
Entry for the Table of Contents



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