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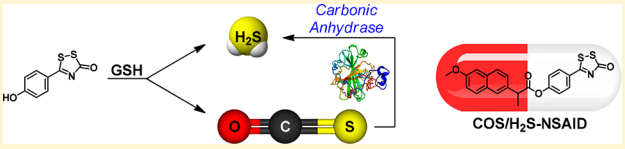
Cyclic Sulfenyl Thiocarbamates Release Carbonyl Sulfide and Hydrogen Sulfide Independently in Thiol-Promoted Pathways

Yu Zhao, Andrea K. Steiger, and Michael D. Pluth*

Department of Chemistry and Biochemistry, Institute of Molecular Biology, Materials Science Institute, University of Oregon, Eugene, Oregon 97403, United States

Supporting Information

ABSTRACT: Hydrogen sulfide (H_2S) is an important signaling molecule that provides protective activities in a variety of physiological and pathological processes. Among the different types of H_2S donor compounds, thioamides have attracted attention due to prior conjugation to nonsteroidal anti-inflammatory drugs (NSAIDs) to access H_2S-NSAID hybrids with significantly reduced toxicity, but the mechanism of H_2S release from thioamides remains unclear. Herein, we reported the synthesis and evaluation of a class of thioamide-derived sulfenyl thiocarbamates (**SulfenylTCMs**) that function as a new class of H_2S donors. These compounds are efficiently activated by cellular thiols to release carbonyl sulfide (COS), which is quickly converted to H_2S by carbonic anhydrase (CA). In addition, through mechanistic investigations, we establish that COS-independent H_2S release pathways are also operative. In contrast to the parent thioamide-based donors, the **SulfenylTCMs** exhibit excellent H_2S releasing efficiencies of up to 90% and operate through mechanistically well-defined pathways. In addition, we demonstrate that the sulfenyl thiocarbamate group is readily attached to common NSAIDs, such as naproxen, to generate **YZ-597** as an efficient H_2S-NSAID hybrid, which we demonstrate releases H_2S in cellular environments. Taken together, this new class of H_2S donor motifs provides an important platform for new donor development.



INTRODUCTION

Gasotransmitters, such as nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S), are small gaseous signaling molecules that are produced endogenously and transmit chemical signals within the organism, tissues, and cells by acting on specific targets.[1−3] H_2S, which is the youngest member of the gasotransmitter family, is generated from cysteine (Cys) and homocysteine (Hcy) by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and cysteine aminotransferase (CAT)/3-mercaptopyruvate sulfur transferase (3-MST), which work either individually or in concert to regulate H_2S levels under physiological conditions.[4−6] Once generated, H_2S plays important roles in a variety of physiological and pathological events.[7−10]

To deliver H_2S in complex environments, numerous H_2S releasing agents (H_2S donors) have been developed. These donors function as important chemical tools to both mimic H_2S biosynthesis and also to investigate H_2S chemistry and biology in contextually rich environments. Although sodium sulfide (Na_2S) and sodium hydrosulfide (NaSH) are the most commonly used sources of exogenous H_2S, both of these compounds release H_2S instantly and spontaneously in aqueous media, making the controlled release of sulfide unfeasible.[11,12] Ideal H_2S donors should only release sulfide upon activation and deliver H_2S with slower, but controllable kinetics. In response to this need, chemists have developed different types of H_2S donors in the past decade, which can be activated by different triggers, such as hydrolysis,[13−15] cellular thiols,[16−26] light,[27−31] pH modulation,[32,33] and enzymes[34,35] (Figure 1).[36−43] Among these donors, aryl thioamides have attracted attention due to their synthetic simplicity and ease of incorporation into common pharmaceutical compounds.[24] For example, when compared to regular nonsteroidal anti-inflammatory drugs (NSAIDs), thioamide-coupled NSAIDs, such as **ATB-346**, have retained promising anti-inflammatory activities while significantly reducing side GI damage,

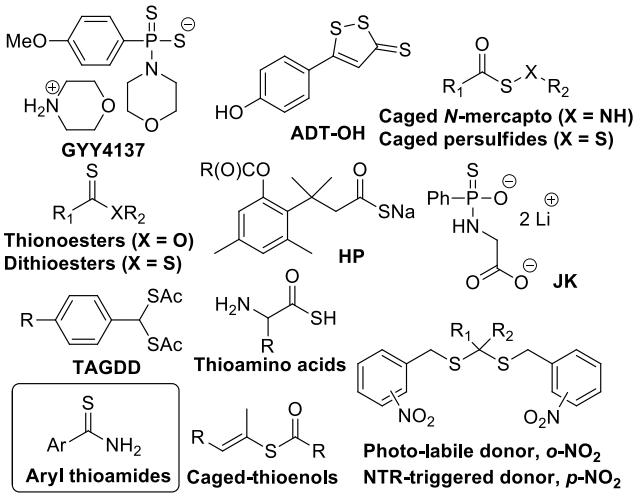


Figure 1. Selected synthetic H_2S donors.

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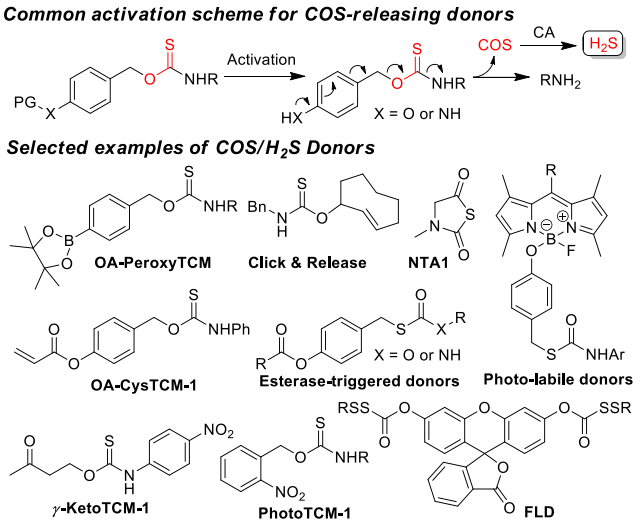
suggesting potential applications of these donors as H_2S-related therapeutics.[38,44] Although thioamides provide promising donor motifs, the H_2S releasing efficiency of thioamide-based donors remains at relatively low levels (typically 1−2%), and the detailed mechanism of H_2S remains unclear (Figure 1 and Scheme 2, top).

To further diversify available H_2S donor platforms and also to develop mechanistically well-defined donors, our group has recently reported new strategies to access H_2S donors through the intermediate release of carbonyl sulfide (COS). In our initial approach, COS was caged in a self-immolative thiocarbamate system. Upon removal of the protecting group, the cascade decomposition of the thiocarbamate released COS, which is quickly hydrolyzed to H_2S by the ubiquitous mammalian enzyme carbonic anhydrase (CA) with an associated rate constant of 2.2×10^4 M^{-1} s^{-1} (for bovine CA II) (Scheme 1, top).[45−47] Following our initial report, our

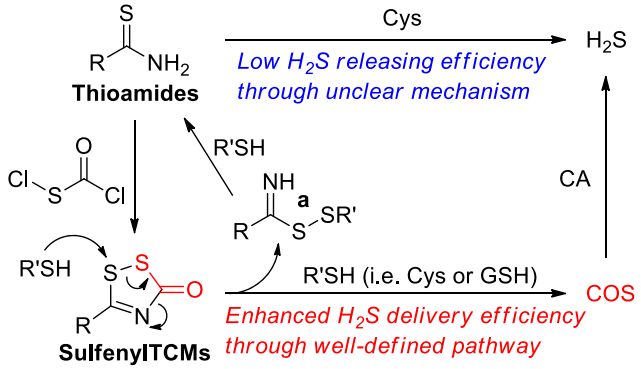
Scheme 1. Examples of COS-Based H_2S Donors



group, as well as others,[48] have developed a series of COS-based H_2S donors that can be triggered through different mechanisms, such as cellular reactive oxygen species (ROS),[49−51] esterases,[46,52,53] nucleophiles,[54] light,[55−57] click chemistry,[58] and Cys.[59] More recently, we have broadened our approach to include different activation strategies and core motifs to develop colorimetric[60] and fluorescent[61] COS-based H_2S donors, such as **γ-KetoTCM-1** and **FLD**, which released COS/H_2S with a concomitant change in optical readout (Scheme 1, bottom).

Advancing from this prior work, we envisioned that hybrid COS-releasing constructs based on thioamide cores could be used to leverage the increased H_2S-releasing efficiency from COS donors with the beneficial properties of thioamides, such as synthetic simplicity and ease of incorporation into common pharmaceutical compounds. Here, we report the design, synthesis, evaluation, and mechanistic investigation of thioamide-derived cyclic sulfenyl thiocarbamates (**SulfenylTCMs**, also known as 1,2,4-dithiazolin-3-ones).[62,63] The **SulfenylTCMs** are stable in aqueous solutions but are activated by cellular thiols to cleave the cyclic disulfide and release COS, which is quickly converted to H_2S by CA. The resultant iminodisulfide intermediate then reacts further with cellular thiols to generate a thioamide product and release H_2S by different pathways (Scheme 2, bottom).

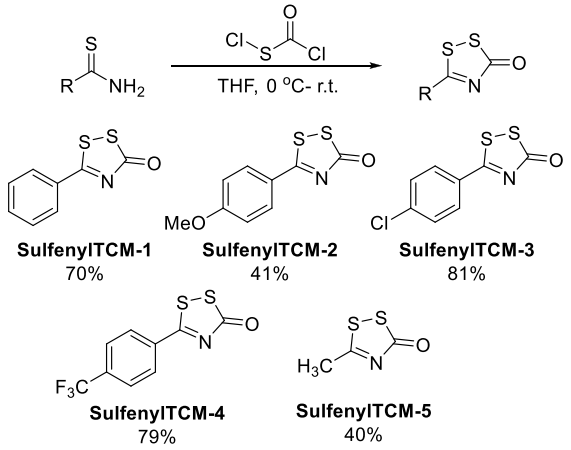
Scheme 2. (Top) Cys-Activated H_2S Release from Thioamides; and (Bottom) Proposed Thiol-Triggered COS/H_2S Release from SulfenylTCMs



RESULTS AND DISCUSSION

Donor Synthesis. To test our hypothesis that cyclic **sulfenylTCMs** can function as thiol-activated COS/H_2S donors, we first prepared **sulfenylTCMs** by treating corresponding thioamides with chlorocarbonylsulfenyl chloride (Scheme 3). Briefly, chlorocarbonylsulfenyl chloride (2.0

Scheme 3. Synthesis of Sulfenyl Thiocarbamates



equiv) was added to anhydrous THF containing the desired thioamide (1.0 equiv) at 0 °C. The resultant solution was stirred at room temperature until the completion of the reaction as indicated by TLC. The reaction solution was then concentrated, and the **sulfenylTCM** was isolated and purified by flash column chromatography. Five donors with aryl (**SulfenylTCM-1−4**) or alkyl (**SulfenylTCM-5**) substituents were prepared, and **SulfenylTCM-1** was selected as the model donor for COS/H_2S releasing and mechanistic evaluations.

GSH-Activated COS/H_2S Release from SulfenylTCM-1. To evaluate thiol-activated H_2S delivery from the donor motifs, we used the colorimetric methylene blue (MB) assay to monitor H_2S production from **SulfenylTCM-1** (25 μM) in the presence of GSH (0−1000 μM) in PBS buffer (pH 7.4, 10 mM) containing cellularly relevant concentrations of CA (25 μg/mL). GSH was used as the model thiol trigger due to its cellular abundance (typically 5−10 mM) and high nucleophil-

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icity. The MB assay was chosen to measure H_2S production because it has been widely used to detect H_2S from a variety of H_2S donors. Treating **SulfenylTCM-1** in PBS in the absence of GSH failed to provide a detectable H_2S signal, indicating negligible spontaneous H_2S delivery from **SulfenylTCM-1**. In the presence of GSH, however, **SulfenylTCM-1** exhibited a dose-dependent COS/H_2S release response (Figure 2). These results demonstrate that **SulfenylTCM-1** is activated by GSH in aqueous buffer, and that the resultant COS is quickly converted to H_2S by CA.

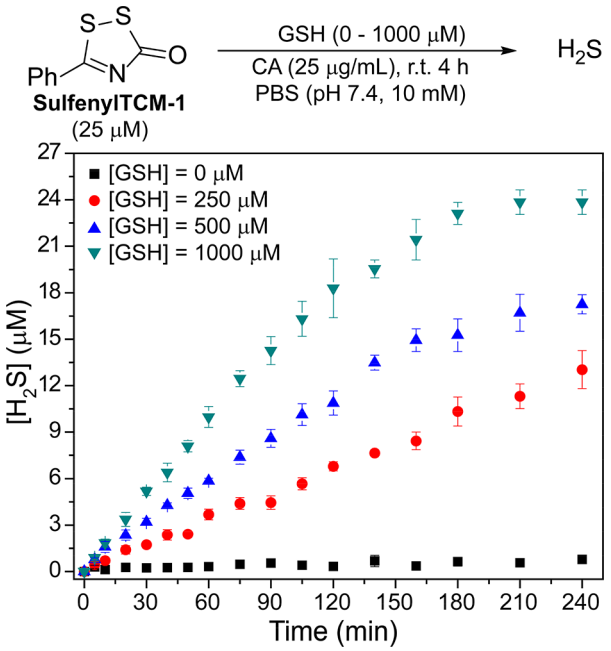

Figure 2. COS/H_2S release from **SulfenylTCM-1** (25 μM) in the presence of 0 μM (black), 250 μM (red), 500 μM (blue), and 1000 μM (green) GSH. The experiments were performed in triplicate, and results are expressed as mean ± SD (n = 3).

Thiol-Activated COS/H_2S Release from SulfenylTCM-1. Because disulfide bonds are readily cleaved by thiol species, we anticipated that **SulfenylTCM-1** should be triggered by not only GSH, but also by other cellular thiols, such as Cys, Hcy, *N*-acetylcysteine (NAC), and penicillamine (PEN). To test this hypothesis, we treated **SulfenylTCM-1** (25 μM) with each thiol trigger (500 μM) in PBS buffer (pH 7.4, 10 mM) containing CA (25 μg/mL) and monitored H_2S generation using the MB assay. As expected, a time-dependent COS/H_2S release was observed in the presence of Cys, Hcy, or NAC, indicating a successful activation of **SulfenylTCM-1**. In comparison, H_2S delivery was significantly reduced (~10% COS/H_2S release) in the presence of PEN, presumably due to the increased steric bulk and resultant decrease in nucleophilicity, which prohibited its reaction with **SulfenylTCM-1** (Figure 3). Taken together, these studies demonstrated that **SulfenylTCM-1** can be activated by a variety of cellular thiol species, such as GSH, Cys, Hcy, NAC, and PEN. In addition, COS/H_2S delivery from **SulfenylTCM-1** can be controlled by using different triggers due to their different reactivities toward **SulfenylTCM-1**.

Effects of Cellular Nucleophiles on COS/H_2S Release from SulfenylTCM-1. To investigate whether COS/H_2S release can be triggered by other species, **SulfenylTCM-1** (25

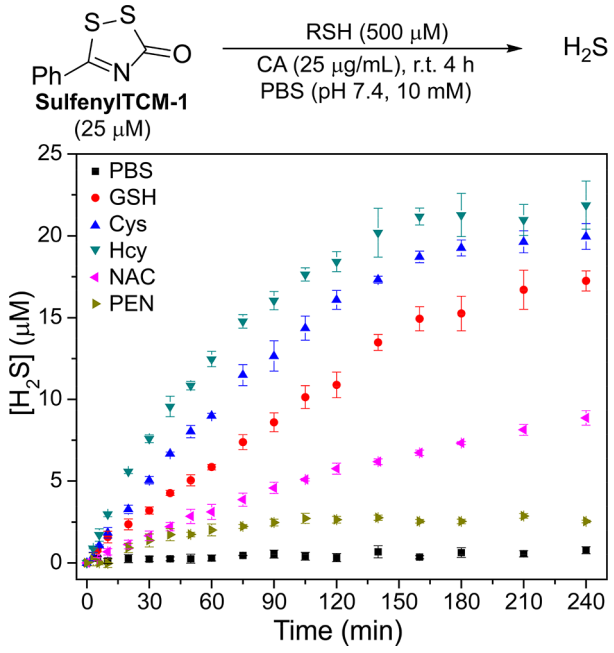

Figure 3. Thiol-dependent (500 μM) COS/H_2S release from **SulfenylTCM-1** (25 μM). The experiments were performed in triplicate, and results are expressed as mean ± SD (n = 3).

μM) was treated with biologically relevant species (500 μM), including oxidized glutathione (GSSG), lysine (Lys), serine (Ser), glycine (Gly), thiosulfate ($S_2O_3^{2-}$), sulfite (SO_3^{2-}), and sulfate (SO_4^{2-}), in PBS buffer (pH 7.4, 10 mM) containing CA (25 μg/mL), and COS/H_2S release was monitored using the MB assay. As compared to GSH-induced donor activation, which led to 70% H_2S production during a 4-h reaction period, none of the above species triggered **SulfenylTCM-1** (Figure 4). These studies confirmed that **SulfenylTCM-1** is stable

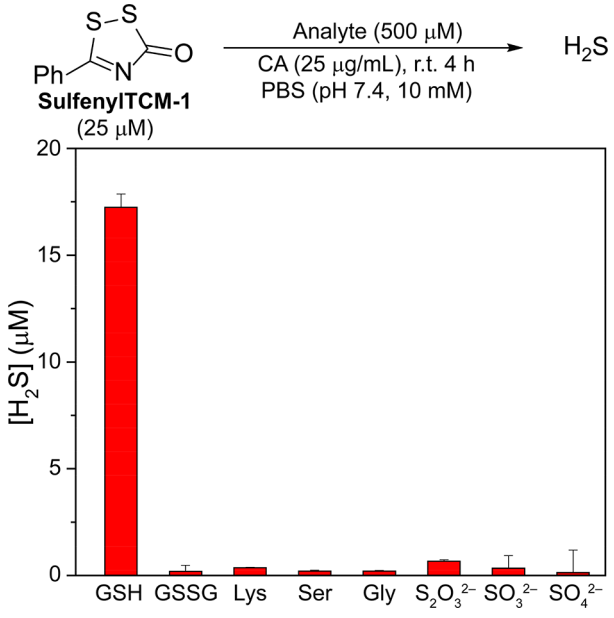

Figure 4. COS/H_2S release from **SulfenylTCM-1** (25 μM) in the presence of cellular nucleophiles (500 μM). H_2S concentration was measured after 4-h incubation. The experiments were performed in triplicate, and the results were expressed as mean ± SD (n = 3).

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toward common biological nucleophiles and reactive sulfur species, but also highly sensitive toward thiol activation to deliver COS/H_2S.

GSH-Activated COS/H_2S Release from Other SulfenylTCMs. Having evaluated COS/H_2S production from **SulfenylTCM-1**, we next investigated the COS/H_2S releasing efficiency of other **SulfenylTCMs** (**SulfenylTCM-2**−**5**) using GSH as the model trigger. In these experiments, the **SulfenylTCM** donors (25 μM) were incubated with GSH (500 μM) in PBS buffer (pH 7.4, 10 mM) containing CA (25 μg/mL). As expected, all donors were activated by GSH, and COS/H_2S release was measured using the MB assay. Importantly, the rate of COS/H_2S release from these donor motifs was changed by structural modifications, which demonstrates that release rates can be tuned by use of differently substituted thioamide precursors. For example, even though **SulfenylTCM-1**−**3** showed similar COS/H_2S releasing profiles, donors with strong electron-withdrawing groups (i.e., CF_3 in **SulfenylTCM-4**) or small alkyl group (i.e., CH_3 in **SulfenylTCM-5**) provided significantly enhanced COS/H_2S release (Figure 5). During the 4-h time course of these

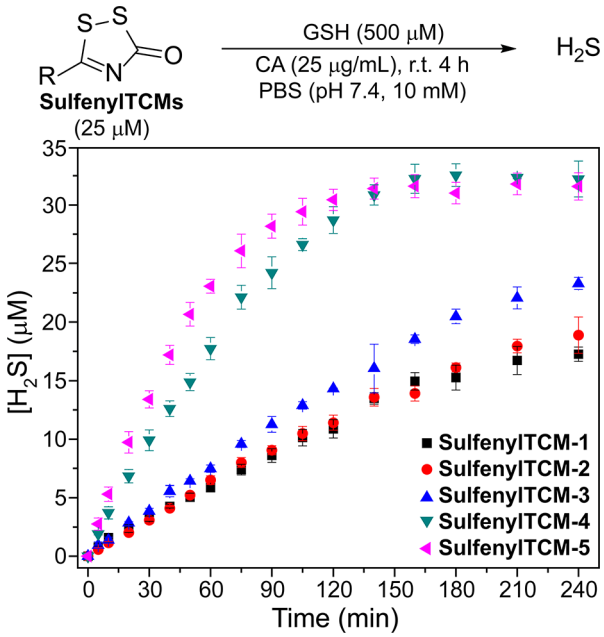

Figure 5. COS/H_2S release from **SulfenylTCM-1**−**5** (25 μM) in the presence of GSH (500 μM) in PBS (pH 7.4, 10 mM) containing CA (25 μg/mL). The experiments were performed in triplicate, and the results are expressed as mean ± SD (n = 3).

experiments, we were surprised to observe over 100% H_2S release from **SulfenylTCM-4** (130%) and **SulfenylTCM-5** (126%). Although the extra H_2S could potentially be attributed to H_2S release from the 4-methoxythiobenzamide and thioacetamide byproducts, the low H_2S releasing efficiency of thioamide-based H_2S donors suggested to us that an alternative COS-independent H_2S releasing pathway was plausible in these **SulfenylTCM** systems.

To determine whether **SulfenylTCM** donors also released H_2S directly through a COS-independent pathway, we treated **SulfenylTCM-1**−**5** (25 μM) with GSH (500 μM) in PBS (pH 7.4, 10 mM) in the absence of CA. For each donor, direct H_2S release was observed using the MB assay, which confirmed the existence of direct H_2S releasing pathway(s) (Figure 6).

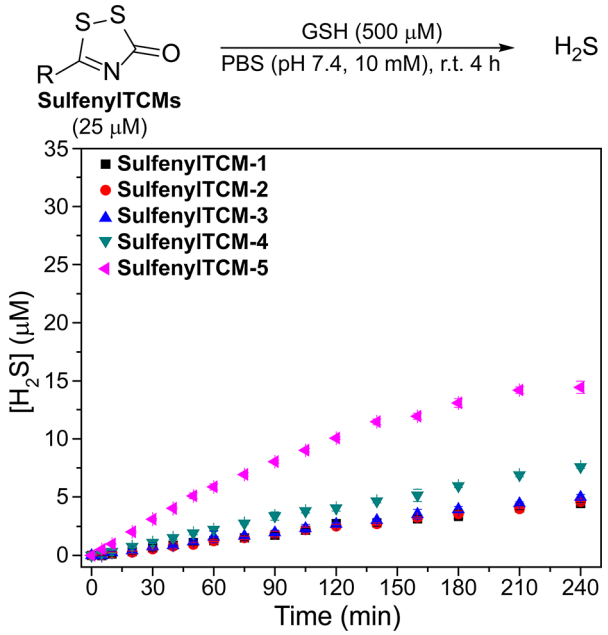

Figure 6. COS-independent H_2S release from **SulfenylTCM-1**−**5** (25 μM) in the presence of GSH (500 μM) in PBS (pH 7.4, 10 mM). The experiments were performed in triplicate, and the results were expressed as mean ± SD (n = 3).

Although certain aryl thioamides have been reported previously as Cys-activated H_2S donors, we did not observe H_2S release from thioamides (25 μM), such as thiobenzamide, in the presence of GSH (500 μM) using the MB assay under our conditions (Figure S1).[24] Taken together, these studies demonstrated that **SulfenylTCM** donors can release H_2S through a COS- and thioamide-independent pathway (vide infra).

Mechanistic Investigations on COS/H_2S Release from SulfenylTCM-1. To further investigate the operative direct H_2S release mechanism from **SulfenylTCMs**, we next conducted NMR experiments to determine which intermediates and products were formed during the course of the reaction in the absence of CA. We first confirmed the stability of **SulfenylTCM-1** in DMSO-d_6/D_2O (9:1) over the course of the standard experiments (Figure S3). Next, we prepared a DMSO-d_6/D_2O (9:1) solution containing **SulfenylTCM-1** (10 mM) and 5 equiv of benzyl mercaptan (BnSH, 50 mM) and monitored the reaction by NMR spectroscopy. This experiment showed that the reaction was complete with full consumption of **SulfenylTCM-1** within 30 min. The two major products of the reaction were thiobenzamide and benzyl disulfide (BnSSBn), which were confirmed by comparison to authentic samples (Figures 7 and S4). In related experiments using substoichiometric amounts of BnSH (0.1−0.5 equiv), we also observed complete **SulfenylTCM-1** consumption, which indicated that thiols also serve as promotors for **SulfenylTCM** activation (Figures S5 and S6). This observation is consistent with a mechanism in which activation of **SulfenylTCM-1** by BnSH generates reactive thiol intermediates, which would further react with the donor motif to release COS/H_2S. In addition, we also treated **SulfenylTCM-1** (5.0 mM) with GSH (10.0 mM) in DMSO/PBS (pH 7.4, 10 mM) (1:1) for 4 h. Thiobenzamide was then isolated as the final product (yield 98%), which is consistent with our NMR study using BnSH as a model trigger (Figure S7).

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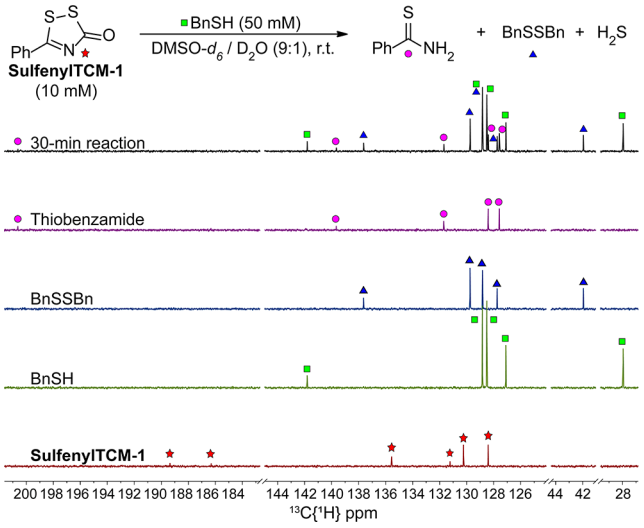
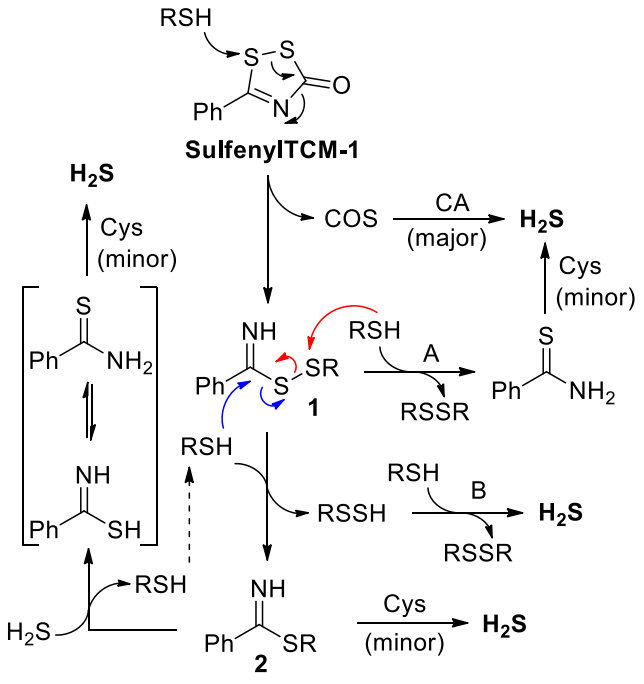


Figure 7. $^{13}C\{^1H\}$ NMR spectra of the reaction between **SulfenylTCM-1** (red ★) and BnSH (green ■). Thiobenzamide (purple ●) and BnSSBn (blue ▲) were identified as major products using authentic samples.

On the basis of the MB measurements and NMR experiments, three main observations drive the mechanistic requirements of donor activation: (1) **SulfenylTCM** donors can be activated by thiols to release COS, which functions as an H_2S precursor in the presence of CA; (2) **SulfenylTCM** donors can also directly release H_2S in the absence of CA; and (3) thiobenzamide and disulfide (RSSR) are the two major products when treating **SulfenylTCM-1** with either sub-stoichiometric or excess RSH (0.1−5 equiv), such as BnSH. According to these results, we proposed donor activation and H_2S releasing pathways in Scheme 4. Briefly, thiols (RSH) can react with the **SulfenylTCM** to cleave the disulfide bond,

Scheme 4. Proposed Mechanism of Thiol-Triggered COS/H_2S Release from SulfenylTCM-1



which results in a dethiocarboxylation to release COS, which is converted to H_2S by CA (COS-dependent pathway). This step also generates iminodisulfide intermediate **1**, which can undergro thiol/disulfide exchange to generate RSSR and thiobenzamide (pathway A). A thiol can also react with iminodisulfide **1** at the electrophilic imine carbon to yield iminothioether **2** and a persulfide (RSSH), which can react further with thiols to generate H_2S (pathway B, COS-independent H_2S releasing pathway). Adding further complexity to this reactivity, the generated H_2S or persulfides could also likely intercept **1** to yield the thiobenzamide product and generate an additional persulfide or polysulfide, respectively. In addition, any persulfide intermediates can also likely react with the donor motif directly to propagate this chain of reactions to yield H_2S.

In our investigations, we did not observe iminothioether **2** directly in our NMR experiments (Figures 7 and S4−S6), but we suspected that **2** would likely react directly with H_2S to generate a thiol, which would further react with **SulfenylTCM-1**. This general reaction scheme supports the role of thiols acting as a promotor for **SulfenylTCM** activation (Figures S5 and S6). To test our hypothesis, we synthesized iminothioether **2** and treated it (10 mM) with NaSH (100 mM) in DMSO-d_6/D_2O (9:1). NMR experiments showed the full consumption of **2** by H_2S within 10 min, and the formation of thiobenzamide and thiol as the final products (Figure S8), which is consistent with proposed reaction pathway B. This reactivity suggests that H_2S generated in the system may go on to propagate donor activation. Furthermore, this reactivity is consistent with the recently reported Cys-triggered H_2S donation from imino-thioether derivatives.[25] Moreover, our studies here provide useful mechanistic insights on internal H_2S scavenging by iminothioether donor motifs, which also helps to explain the low H_2S releasing efficiency observed in the recently reported iminothioether donor systems.[25]

Generation of a COS-Hybrid Naproxen as a New COS/H_2S-Releasing NSAID. To demonstrate the generality of sulfenyl thiocarbamate incorporation into biologically active platforms, we envisioned that this donor motif could be readily incorporated into compounds used for anti-inflammatory activity, such as nonsteroidal anti-inflammatory drugs (NSAIDs). One of the major limitation of NSAIDs is the potential gastrointestinal (GI) and cardiovascular toxicity.[64,65] Recent efforts to improve the therapeutic profile and reduce the GI toxicity of these drugs have included the generation of a series of H_2S-releasing hybrid NSAIDs (H_2S-NSAIDs), many of which include thioamides (Figure 8a−c).[38,66,67] For

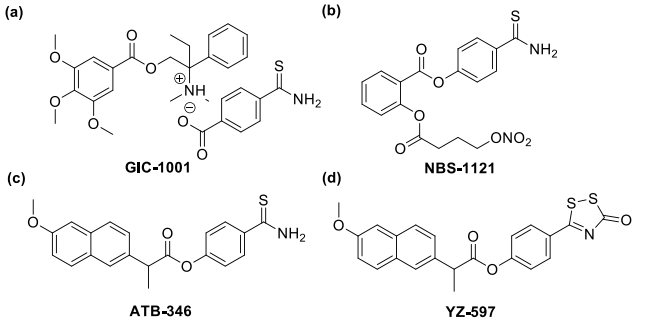


Figure 8. Representative thioamide-containing H_2S-NSAIDs, including (a) **GIC-1001**, (b) **NBS-1121**, (c) **ATB-346**, and (d) COS/H_2S-NSAID **YZ-597**.

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example, one of the most successful H_2S-NSAIDs that progressed into clinical development is **ATB-346**, a naproxen derivative coupled with thiobenzamide as an H_2S releasing moiety. This hybrid H_2S-NSAID exhibited anti-inflammatory activities similar to those of naproxen but with significantly reduced GI damage.[38,44,68] Aligned with this high therapeutic potential, **ATB-346** has advanced to two clinical trials in 2014[69] and 2017.[70] One limitation of **ATB-346**, much like other thioamide-based donors, is the poorly understood H_2S releasing mechanism and the relatively low efficiency of H_2S release, which was reported to be only 10−20 μM H_2S from 1 mM of the drug motif.[38] We viewed that developing related H_2S-releasing NSAIDs with more efficient and mechanistically understood donor motifs could provide a useful platform for further leveraging these hybrid H_2S donor/drug system.

To investigate whether cyclic sulfenyl thiocarbamates could be used to enhance H_2S release from such systems, we prepared **YZ-597** from **ATB-346** (Figure 8d). Our expectation, based on our work described above, was that this compound would be activated much more efficiently than the thioamide-based parent H_2S-NSAID conjugate. Consistent with this hypothesis, when we incubated **YZ-597** (25 μM) in the presence of GSH (500 μM) in PBS buffer (pH 7.4, 10 mM) containing CA (25 μg/mL) and 1 mM CTAB to help solubilize the components, we observed rapid and efficient H_2S release (90%). Although H_2S release from **ATB-346** has been reported to be favored in the presence of reducing agents, such as Cys and GSH or in the presence of biological materials, we did not observed efficient H_2S release from **ATB-346** under our experimental conditions (Figure 9).[68] These data suggest that **YZ-597** may have more accessible H_2S releasing pathways than the thioamide-derived **ATB-346** motif.

Having confirmed H_2S release from **YZ-597** in the presence of GSH, we next investigated whether this conjugate could release H_2S efficiently in live cells. We first incubated HeLa cells with FBS-free DMEM containing the H_2S-responsive fluorescent probe **SF7-AM** (5 μM), and after washing then

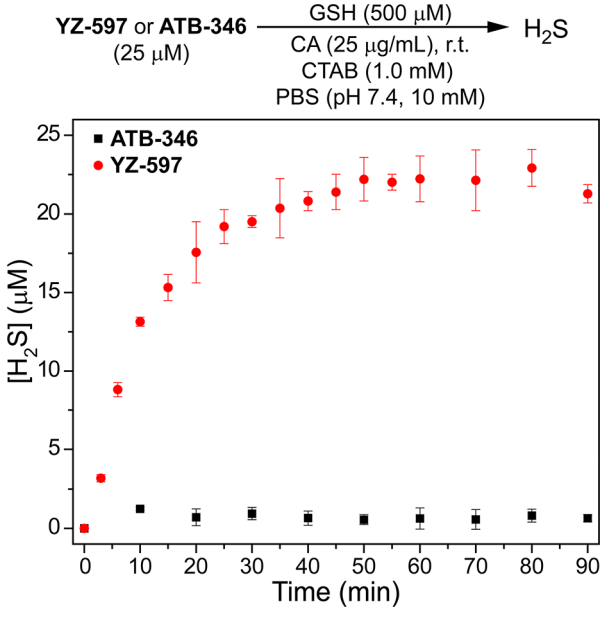

Figure 9. GSH (500 μM)-triggered H_2S release from **YZ-597** or **ATB-346** (25 μM). The experiments were performed in triplicate, and the results are expressed as mean ± SD (n = 3).

added either **YZ-597** (50 μM) or vehicle.[71] As shown in Figure 10, the HeLa cells displayed negligible H_2S-derived fluorescence in the absence of **YZ-597**, suggesting minimal endogenous H_2S levels. In comparison, cells treated with **YZ-597** revealed a strong fluorescent signal, indicating that **YZ-597** was activated to release H_2S in a cellular environment. We also treated HeLa cells with the donor motif **SulfenylTCM-1** under identical conditions and observed similar fluorescence enhancement (Figure S9). Taken together, we view that **YZ-597** and related sulfenyl thiocarbamate motifs can provide a useful platform for investigating the action of H_2S-hybrid NSAIDs and related pharmacologically active compounds.

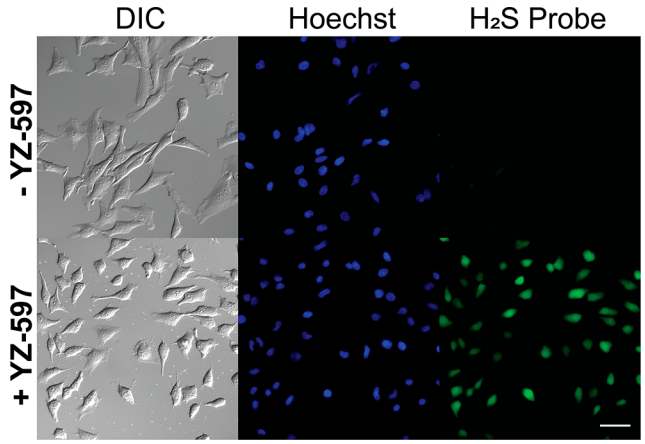

Figure 10. H_2S delivery from **YZ-597** in HeLa cells. HeLa cells were treated with **SF7-AM** (5 μM) and Hoechst (10 μg/mL) for 5 min. After removal of extracellular SF7-AM and Hoechst, cells were incubated in FBS-free DMEM in the absence (top row) or presence (bottom row) of **YZ-597** (50 μM) for 30 min. Cells were then washed and imaged in PBS. Scale bar: 50 μm.

CONCLUSIONS

Thioamide-derived sulfenyl thiocarbamates function as thiol-activated COS/H_2S donors. These compounds can be activated by cellular thiol species, such as GSH, Cys, Hcy, and NAC, to deliver H_2S. We demonstrated that the H_2S-releasing efficiency of **SulfenylTCMs** is significantly enhanced in comparison to the parent thioamide compounds. Moreover, COS and H_2S release from **SulfenylTCM** compounds is mechanistically well-defined, which provides the ability to tune release parameters. Leveraging this design platform, we also prepared the COS/H_2S-NSAID, **YZ-597**, which is built off of the **ATB-346** platform, and demonstrated the efficient H_2S release both in vitro and in live cells from **YZ-597**. In addition, we anticipate that **YZ-597** and related compounds will also serve as promising chemical tools to provide insights into the relationship between H_2S release and efficacy of H_2S-hybrid compounds. Further investigations into the activity of **YZ-597** and related H_2S hybrids are currently ongoing in our laboratory.

EXPERIMENTAL SECTION

Materials and Methods. Reagents were purchased from Sigma-Aldrich, Tokyo Chemical Industry (TCI), Fisher Scientific, and VWR and used directly as received. Carbonic anhydrase (CA) from bovine erythrocytes was purchased from Sigma-Aldrich (C2624). Silica gel (SiliaFlash F60, Silicycle, 230−400 mesh) was used for column chromatography. Deuterated solvents were purchased from Cam-

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bridge Isotope Laboratories (Tewksbury, MA). ^{1}H, ^{19}F, and ^{13}C{^{1}H} NMR spectra were recorded on a Bruker 500 MHz NMR instrument at the indicated frequencies. Chemical shifts are reported in ppm relative to residual protic solvent resonances. Mass spectrometric measurements were performed by the University of Illinois, Urbana–Champaign MS facility, or on a Xevo Waters ESI LC/MS instrument. Methylene blue absorbances were mesured on a UV–vis spectrometer (Cary 100, Agilent Technologies, Santa Clara, CA) in PBS buffer. **SF7-AM**,[71] **ATB-346**[72] and iminothioether **2**[25] were synthesized by following the literature report. HeLa cells were purchased from ATCC (Manassas, VA). Cell imaging experiments were performed on a Leica DMi8 fluorescence microscope, equipped with an Andor Zyla 4.2+ sCMOS detector.

Synthesis. *SulfenylTCM-1*. Thiobenzamide (135 mg, 1.00 mmol) was dissolved in anhydrous THF (15 mL) followed by the addition of chlorocarbonylsulfenyl chloride (262 mg, 2.00 mmol) at 0 °C. The resultant solution was stirred at 0 °C for 10 min, after which the ice bath was removed, and the reaction mixture was stirred at rt for 1 h. The reaction solution was then concentrated under vacuum, and the crude product was purified by column chromatography. **SulfenylTCM-1** was isolated as yellow solid (70%). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.16 (d, J = 10.0 Hz, 2H), 7.80 (d, J = 10.0 Hz, 1H), 7.66 (t, J = 10.0 Hz, 2H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 189.3, 186.1, 135.5, 131.4, 130.3, 128.5. IR (cm^{-1}): 1680, 1655, 1592, 1501, 1479, 1445, 1306, 1235, 1086, 1065, 923, 766, 683, 669. HRMS m/z [M + H]$^+$ calcd for [$C_8H_6NOS_2$]$^+$ 195.9891; found 195.9891.

***SulfenylTCM-2*.** This was prepared from 4-methoxythiobenzmide by following the procedure described above (47 mg, 41% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.14 (d, J = 10.0 Hz, 2H), 7.17 (d, J = 10.0 Hz, 2H), 3.91 (s, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 188.2, 185.8, 165.3, 130.9, 123.9, 115.6, 56.4. IR (cm^{-1}): 2963, 2835, 1668, 1597, 1574, 1521, 1484, 1421, 1308, 1245, 1168, 1078, 1023, 925, 828, 666. HRMS m/z [M + H]$^+$ calcd for [$C_9H_8NO_2S_2$]$^+$ 225.9996; found 225.9999.

***SulfenylTCM-3*.** This was prepared from 4-chlorothiobenzmide by following the procedure described above (91 mg, 81% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.17 (d, J = 10.0 Hz, 2H), 7.73 (d, J = 10.0 Hz, 2H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 188.1, 186.0, 140.3, 130.3, 130.2. IR (cm^{-1}): 3068, 3033, 1660, 1590, 1504, 1477, 1400, 1306, 1281, 1239, 1178, 1008, 827, 666. HRMS m/z [M + H]$^+$ calcd for [$C_8H_5ClNOS_2$]$^+$ 229.9505; found 229.9497.

***SulfenylTCM-4*.** This was prepared from 4-trifluoromethylthiobenzmide by following the procedure described above (104 mg, 79% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.35 (d, J = 10.0 Hz, 2H), 8.01 (d, J = 10.0 Hz, 2H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 188.0, 186.0, 134.9, 129.4, 127.1, 125.0, 122.9. ^{19}F NMR (470 MHz, DMSO-d_6) δ (ppm): − 61.8. IR (cm^{-1}): 3047, 1661, 1517, 1493, 1408, 1330, 1305, 1190, 1171, 1118, 1064, 1011, 924, 845, 669. HRMS m/z [M + H]$^+$ calcd for [$C_9H_5F_3NOS_2$]$^+$ 263.9765; found 263.9760.

***SulfenylTCM-5*.** This was prepared from thioacetamide by following the procedure described above (35 mg, 40% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 2.79 (s, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 192.9, 187.0, 22.9. IR (cm^{-1}): 1662, 1516, 1494, 1165, 1071, 1000, 668, 628, 589. HRMS m/z [M + H]$^+$ calcd for [$C_3H_4NOS_2$]$^+$ 133.9734; found 133.9735.

YZ-597 was prepared from **ATB-346** by following the procedure described above (105 mg, 87% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.19 (d, J = 5.0 Hz, 2H), 7.88 (s, 2H), 7.86 (s, 1H), 7.54 (d, J = 5.0 Hz, 1H), 7.34 (s, 2H), 7.32 (s, 1H), 7.19 (d, J = 10.0 Hz, 1H), 4.29 (q, J = 5.0 Hz, 1H), 3.88 (s, 3H), 1.63 (d, J = 10.0 Hz, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 188.2, 186.0, 172.8, 157.8, 155.9, 135.3, 134.0, 130.3, 129.7, 129.0, 127.8, 126.7, 126.4, 123.5, 119.4, 106.3, 55.7, 45.0, 18.8. IR (cm^{-1}): 1742, 1677, 1662, 1485, 1412, 1206, 1165, 1135, 1117, 1087, 1028, 893, 853, 844, 814. HRMS m/z [M + H]$^+$ calcd for [$C_{22}H_{18}NO_4S_2$]$^+$ 424.0677; found 424.0657.

H_2S Release from SulfenylTCMs in PBS. A **SulfenylTCM** stock solution (50.0 μL, 10.0 mM in DMSO) was added to 20.0 mL of PBS (pH 7.40, 10.0 mM) containing CA (25.0 μg/mL) in a 25 mL scintillation vial. A thiol stock solution (0.100 M in H_2O) was then added to generate the desired thiol working concentrations as shown in Figures 2, 3, and 5. For H_2S release in the absence of CA, the measurement was set up by following the procedure as described above in PBS with no CA addition (Figure 6). For H_2S release from **YZ-597**, the measurement was set up by following the procedure as described above in PBS containing CA (25.0 μg/mL) and CTAB (1.00 mM) (Figure 9). Next, 0.300 mL aliquots of the reaction mixture were transferred to UV cuvettes containing 0.300 mL of MB cocktail (0.060 mL of zinc acetate (1.00% w/v), 0.120 mL of $FeCl_3$ (30.0 mM in 1.20 M HCl), and 0.120 mL of *N*,*N*-dimethyl-*p*-phenylenediamine (20.0 mM in 7.20 M HCl)) at different time points. The absorbance at 670 nm was then measured after 30 min and was converted to H_2S concentration by using the H_2S calibration curve.

Selectivity Investigations on H_2S Release. A **SulfenylTCM-1** stock solution (50.0 μL, 10.0 mM in DMSO) was added to 20.0 mL of PBS (pH 7.40, 10.0 mM) containing CA (25.0 μg/mL) in a 25 mL scintillation vial. An analyte stock solution (100 μL, 100 mM in H_2O) was then added. The reaction was stirred at rt for 4 h. Next, a 0.300 mL aliquot of the reaction mixture was transferred to UV cuvettes containing 0.300 mL of MB cocktail (0.060 mL of zinc acetate (1.00% w/v), 0.120 mL of $FeCl_3$ (30.0 mM in 1.20 M HCl), and 0.120 mL of *N*,*N*-dimethyl-*p*-phenylenediamine (20.0 mM in 7.20 M HCl)). The absorbance at 670 nm was then measured after 30 min and was converted to H_2S concentration by using the H_2S calibration curve.

Mechanism Investigations of H_2S Release. **SulfenylTCM-1** (0.0200 M) and BnSH (0.100 M) solutions were prepared by adding **SulfenylTCM-1** (3.90 mg) and BnSH (12.4 mg) in DMSO-d_6/D_2O (9:1, 1.00 mL), respectively. Next, **SulfenylTCM-1** (0.0200 M, 0.500 mL) was added to BnSH (0.100 M, 0.500 mL) to reach the concentrations of 0.0100 M of **SulfenylTCM-1** and 0.0500 M of BnSH, respectively. The reaction process was then monitored using a Bruker 500 MHz NMR instrument.

Cellular Imaging of H_2S Release from YZ-597. HeLa cells were plated in poly-D-lysine-coated plates (MatTek) containing 2 mL of DMEM and incubated at 37 °C under 5% CO_2 for 24 h. The confluent cells were washed with PBS and then incubated with **SF7-AM** (5.00 μM) and Hoechst dye (10.0 μg/mL) for 5 min. The cells were then washed with PBS and incubated in FBS-free DMEM in the absence or presence of **YZ-597** (50.0 μM) for 30 min. Prior to imaging, cells were washed with PBS and bathed in 2 mL of PBS. Cell imaging was performed on a Leica DMi8 fluorescent microscope using DIC for bright field and standard DAPI and GFP filter cubes for fluorescence imaging, respectively. The scale bar represents 50 μm.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b06319.

H_2S release data, NMR experiments, cell imaging, and spectra (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*pluth@uoregon.edu

ORCID

Yu Zhao: 0000-0003-1250-9480

Andrea K. Steiger: 0000-0003-1655-7973

Michael D. Pluth: 0000-0003-3604-653X

Notes

The authors declare no competing financial interest.

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