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Self-Immolative Thiocarbamates Provide Access to Triggered H_2S Donors and Analyte Replacement Fluorescent Probes

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Supporting Information

ABSTRACT: Hydrogen sulfide (H_2S) is an important biological signaling molecule, and chemical tools for H_2S delivery and detection have emerged as important investigative methods. Key challenges in these fields include developing donors that are triggered to release H_2S in response to stimuli and developing probes that do not irreversibly consume H_2S. Here we report a new strategy for H_2S donation based on self-immolation of benzyl thiocarbamates to release carbonyl sulfide, which is rapidly converted to H_2S by carbonic anhydrase. We leverage this chemistry to develop easily modifiable donors that can be triggered to release H_2S. We also demonstrate that this approach can be coupled with common H_2S-sensing motifs to generate scaffolds which, upon reaction with H_2S, generate a fluorescence response and also release caged H_2S, thus addressing challenges of analyte homeostasis in reaction-based probes.

The advent of chemical tools to probe and manipulate biochemical processes has revolutionized biological investigations.[1] Spawning from initial investigations into fluorescent proteins,[2] small molecule fluorescent reporters now comprise a key pillar of investigative chemical biology with a remarkable diversity of fluorescent tagging and measurement technologies.[3] Recent years have witnessed a significant expansion of sensor development to include imaging tools for transition-metal, alkali, and alkali earth ions.[4] Many of these sensors can provide real-time, quantitative measurements of ion fluxes due to the reversible interaction of the sensor with the analyte, thus enabling imaging of the dynamic process of metal ion trafficking associated with signaling events ranging from Ca^{2+} sparks during muscle contraction[5] to Zn^{2+} fluxes during mammalian egg fertilization.[6] Complementing these tools are small molecule donors that release caged analytes at controllable rates.[7] Such platforms provide powerful methods to control levels of specific analytes, including pro-drugs, metal ions, or small reactive sulfur, oxygen, and nitrogen species (RSONS), in different biological contexts.

In the last two decades, RSONS have emerged as important bioinorganic molecules involved in myriad biological processes, many of which have been elucidated by utilizing chemical tools for small molecule detection and delivery. RSONS are involved in the complex cellular redox landscape and are often involved in oxidative stress responses, immune responses, signaling pathways and other emerging roles.[8] For example, NO, HNO, and $ONOO^-$ play important roles ranging from smooth muscle relaxation to immune response[9] and are largely intertwined with reactive oxygen species, such as O_2^- and H_2O_2, which have been implicated in oxidative stress responses and aging mechanisms.[10] Similarly, reactive sulfur species, such as H_2S, hydropolysulfides ($HS_{n>1}^-$), and persulfides (RSSH), have recently garnered interest as important signaling molecules with roles in long-term potentiation and cardiovascular health.[11] By contrast to their metal ion counterparts, RSONS are often fleeting and often react irreversibly with cellular targets. This heightened reactivity has provided chemists with significant challenges in developing constructs that can release these molecules under controlled conditions, but have also provided different strategies to devise chemical tools for their detection by engineering reactive groups onto sensing platforms that react selectively albeit irreversibly with the analyte of interest.[12]

Although small molecule donors and reaction-based probes have provided significant insights into RSONS biology, key needs remain. For example, engineering donors with precise but modifiable triggers to enable analyte release in response to specific stimuli and developing reaction-based probes that do not irreversibly consume the analyte would enable new insights. Motivated by these needs we report here a new caged H_2S releasing strategy and provide proof-of-concept applications in both small-molecule donor and reaction-based probe design. By leveraging triggerable self-immolative thiocarbamates, we demonstrate access to H_2S donors that can be triggered by external stimuli (Figure 1a) and address common issues of analyte consumption in reaction-based fluorescent probes (Figure 1b) by developing analyte-replacement reaction-based platforms (Figure 1c).

Development of analyte-replacement sensing platforms requires two important components: a versatile H_2S donation motif that releases H_2S in response to a specific trigger, and a method to couple this caged donor to a sensing platform. As a proof-of-concept design toward this objective, we chose to use H_2S-mediated azide reduction for our sensing platform, which has emerged as the most common method for H_2S detection and exhibits high selectivity for H_2S over other RSONS (Figure 2a).[13] Although a number of H_2S-donating motifs have been reported,[14] none of these fit the design requirement of our approach. To develop an H_2S-donating motif compatible with

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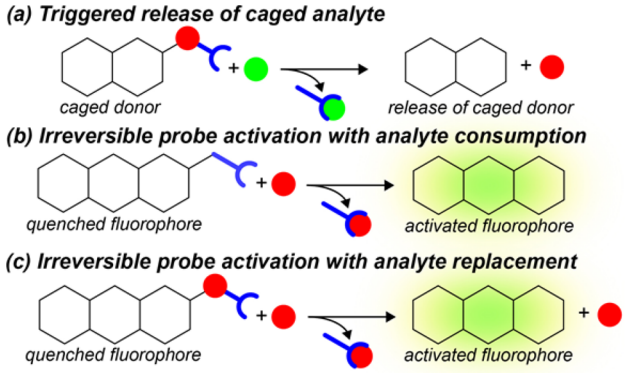

Figure 1. (a) Caged donors triggered by different stimuli. (b) Reaction-based probes typically consume the target analyte. (c) Analyte-replacement reaction-based probes enabled by incorporation of caged analytes into reaction-based motifs.

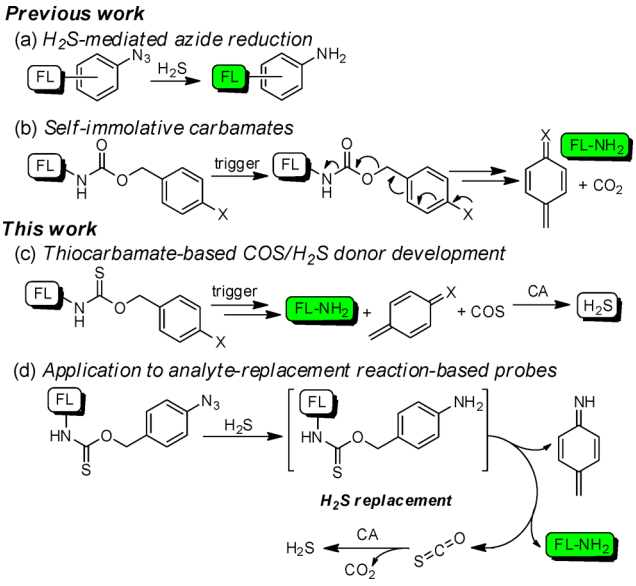

Figure 2. Established strategies for (a) H_2S-mediated azide reduction and (b) self-immolative carbamates to deliver an amine-bound fluorophore. Incorporation of self-immolative thiocarbamates enables access to (c) triggered H_2S donors and (d) analyte replacement probes.

our design requirements, we reasoned that common strategies in drug and fluorophore release, namely the self-immolative cascade decomposition of para-functionalized benzyl carbamates (Figure 2b),[15−17] could be modified to enable triggered H_2S release. Because self-immolative carbamates release an amine-containing payload and extrude CO_2 as a byproduct, we reasoned that replacing the carbonyl oxygen with a sulfur atom to generate a thiocarbamate would result in carbonyl sulfide (COS) release (Figure 2c). In a biological environment, COS is quickly hydrolyzed to H_2S and CO_2 by carbonic anhydrase (CA), which is a ubiquitous enzyme in plant and mammalian cells.[18] The second byproduct of the thiocarbamate self-immolation is a reactive quinone methide, which rapidly rearomatizes upon reaction with nucleophiles, such as water or cysteine.[19] On the basis of the requirements outlined above, we expected that a quenched fluorophore could be functionalized with a *p*-azidobenzylthiocarbamate to enable H_2S-mediated azide reduction to form the transient aryl amine intermediate, which would subsequently undergo the self-immolative cascade reaction to extrude COS/H_2S and liberate the fluorophore to access an analyte-replacement sensing motif (Figure 2d).

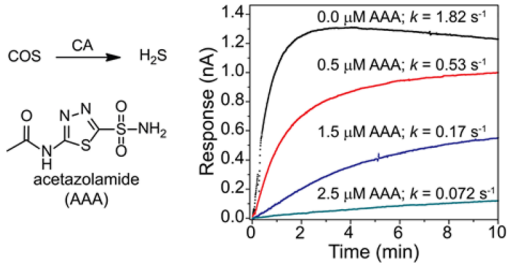

Figure 3. Conversion of COS to H_2S by CA with varying concentrations of CA inhibitor AAA in PBS buffer, pH 7.4.

To confirm that the released COS could generate H_2S, we first established that independently prepared COS could be efficiently hydrolyzed to H_2S by CA. Upon addition of COS to deoxygenated aqueous buffer (PBS, 1 mM CTAB, pH 7.4) containing CA from bovine erythrocytes, we observed rapid H_2S production using an H_2S-responsive electrode. In the absence of CA, negligible current was observed from COS alone (Figure S4).[20] We also observed a dose-dependent reduction in H_2S production upon addition of the CA inhibitor acetazolamide (AAA),[21] which confirmed the enzymatic hydrolysis of COS by CA (Figure 3).

We next prepared model thiocarbamates to confirm that the proposed decomposition cascade to release COS occurs efficiently and to demonstrate the biological compatibility of this donor motif. We incorporated an azide in the para position of the benzylthiocarbamate to function as the H_2S-responsive trigger for self-immolation and COS release. To facilitate NMR identification of the products, we first prepared thiocarbamate **1** with a *p*-fluoroaniline payload and the corresponding carbamate **2** as a control compound (Figure 4a−c). Although **2** should undergo the same self-immolative decomposition upon azide reduction, it releases CO_2 rather than COS and thus should not donate H_2S upon reaction with CA. To monitor the reactivity of the model compounds under controlled reaction conditions, we used tris(2-carboxyethyl)phosphine (TCEP) to trigger self-immolation, due to its near-instantaneous reduction of azides. In each case, NMR spectroscopy was used to monitor the reaction. Consistent with our design hypothesis, we observed the disappearance of the benzylic peak, loss of the thiocarbonyl carbon peak, and formation of new resonances upon self-immolation by NMR spectroscopy (Figures S1−S3). All such changes were observed within 5 min of TCEP addition, confirming the rapid self-immolation of the scaffold upon reduction, and were consistent with COS release from the thiocarbamate scaffold upon azide reduction.

Having confirmed that CA rapidly catalyzes COS hydrolysis, we next investigated the H_2S-donating ability of model compounds **1** and **2** under identical conditions. Monitoring thiocarbamate **1** in buffer containing CA did not result in H_2S formation, confirming that the thiocarbamates do not react directly with CA and that aryl azides are stable in the presence of CA (Figure S5). Upon injection of TCEP, however, rapid release of H_2S was observed, indicating that azide reduction to an amine is essential to trigger self-immolation and COS release. Additionally, repeating the experiment with added AAA significantly reduced the rate of H_2S production, confirming that uninhibited CA is required for significant H_2S production

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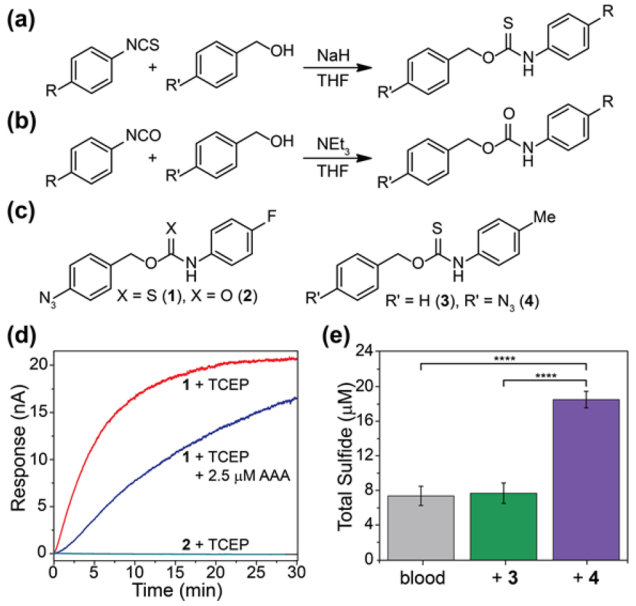

Figure 4. (a,b) Synthesis of model thiocarbamates and carbamates. (c) Model compounds. (d) H_2S release from **1** after reduction by TCEP in the presence of CA, under identical conditions with the addition of AAA (2.5 μM,), and from carbamate **2**. (e) Quantification of total sulfide in whole mouse blood after treatment 25 μM **3** and **4** after 30 min of incubation time in the presence of excess TCEP.

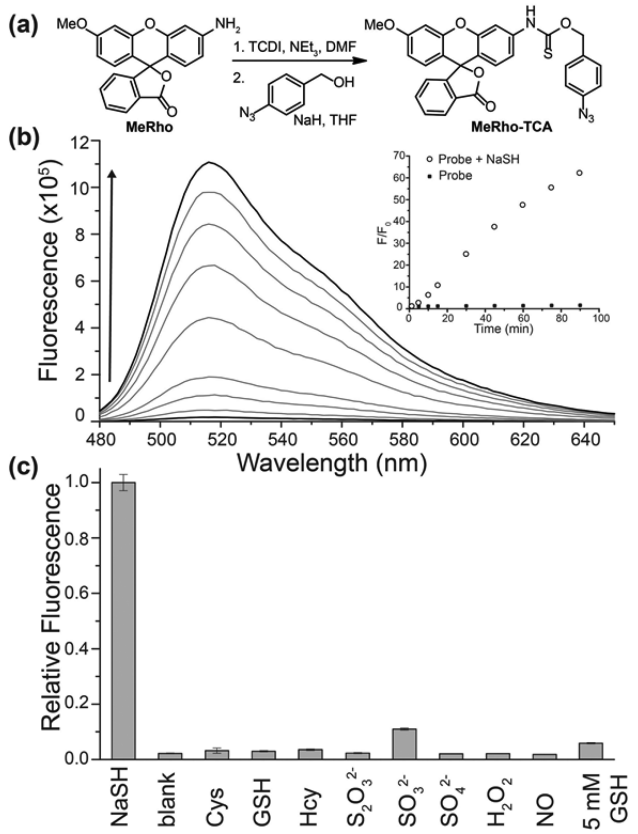

Figure 5. (a) Synthesis of MeRho-TCA. (b) Fluorescence response of MeRho-TCA to H_2S. Inset shows integrated fluorescence over time by comparison to MeRho-TCA in the absence of NaSH. (c) Selectivity of MeRho-TCA for H_2S over other RSONs. Conditions: 5 μM probe, 250 μM RSONs unless noted otherwise, in PBS buffer, 1 mM CTAB, pH 7.4, 37 °C; λ_{ex} = 476 nm, λ_{em} = 480–650 nm.

from the triggered thiocarbamate scaffold (Figure 4d). Under identical conditions, the analogous carbamate (**2**) failed to produce H_2S, confirming that the thiocarbamate is required for H_2S formation. In total, these experiments demonstrate the validity of using thiocarbamates as a triggerable source of H_2S release in aqueous solution, which we expect will prove fruitful for researchers interested in the pharmacological and physiological roles of sulfide-donating molecules.[14]

Expanding on our cuvette-based studies, we also investigated H_2S release from model thiocarbamates in whole mouse blood. Although murine systems provide a convenient model, mice have among the lowest CA levels in mammals, with murine blood only containing about 15% of the CA present in human blood,[22] and thus represent a challenging target for sulfide release mediated by CA. To quantify total sulfide levels, we used the monobromobimane (mBB) method which allows for the analytical measurement of different sulfide pools and is compatible with many types of biological samples.[23] Measurement of the total sulfide, which includes free sulfide as well as bound sulfane-sulfur, revealed background levels of 8 μM, which are higher than total sulfide levels commonly observed in plasma, but are consistent with the high sulfane-sulfur content in red blood cells.[23,24] We prepared thiocarbamate **3**, which lacks the azide trigger, to confirm that the thiocarbamate group was stable in whole blood and did not release COS without activation of the trigger group and compared results obtained with this model compound with azide-functionalized **4**. Total sulfide levels were measured for each compound, as well as the control, after 30 min of incubation with excess TCEP (Figure 4e). Consistent with our expected results, only samples containing donor **4** with the azide trigger increased total sulfide levels in blood ($p \leq 0.0001$). These results establish the stability of the thiocarbamate in biological milieu and confirm that endogenous CA in murine blood, even though significantly lower than in most other biological environments,[22] is sufficient to hydrolyze the COS released from thiocarbamates after the self-immolation cascade is triggered, highlighting the efficacy of this H_2S-releasing strategy in biological environments.

Having confirmed the viability of triggered H_2S release with the model compounds, we next applied this design to incorporate a fluorophore to access an H_2S-responsive fluorescent probe that releases H_2S upon H_2S detection. Our primary goal was to demonstrate that the thiocarbamate group could be appended to common fluorophore motifs and efficiently quench the fluorescence. We chose to use the methylrhodol (MeRho)[25] fluorophore due to its single fluorogenic amine, which could be readily converted into the desired thiocarbamate. Since the azide-functionalized scaffold would be triggered by H_2S to release both MeRho and COS, this would function as a fluorescent H_2S probe that would replenish sulfide through the release of COS. To access the desired scaffold, we treated MeRho with thiocarbonyldiimidazole (TCDI) and NEt_3 in DMF to afford methylrhodol isothiocyanate (MeRho-NCS) in 60% yield. Subsequent treatment with 4-azidobenzyl alcohol and NaH afforded the methylrhodol thiocarbamate azide (MeRho-TCA) in 35% yield (Figure 5a). We note that one benefit of this simple synthetic route is that almost any fluorophore containing a fluorogenic nitrogen can be functionalized with the benzylazide thiocarbamate group, thus providing access to a diverse library of fluorophores.

With a sulfide-replenishing H_2S probe in hand, we investigated the fluorescence response upon addition of sulfide. Treatment of MeRho-TCA with 50 equiv of NaSH in aqueous buffer (PBS, 1

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mM CTAB, pH 7.4) resulted in a 65-fold fluorescence turn-on over 90 min (Figure 5b). Additionally, we confirmed that the MeRho-TCA scaffold was selective for HS^- over other RSONS, by measuring the fluorescence response to Cys, GSH, Hcy, $S_2O_3^{2-}$, SO_3^{2-}, SO_4^{2-}, H_2O_2, and NO (Figure 5c). As expected, the MeRho-TCA scaffold exhibited excellent selectivity for sulfide over other RSONs, demonstrating that the thiocarbamate linker group did not erode the selectivity of the azide trigger and also establishing that the MeRho-TCA scaffold can function as a viable H_2S reporter. Because MeRho-TCA releases H_2S upon reaction with H_2S, we note that one consequence of this analyte replacement approach is that the resultant fluorescence response is not directly proportional to the initial H_2S concentration. Additionally, in isolated systems, 2 equiv of HS^- are required for complete azide reduction, suggesting that the first-generation analyte-replacement scaffolds only replace one-half of the consumed sulfide.[26] It is also possible, however, that in biological media 1 equiv of a thiol may play a role in H_2S-mediated azide reduction, which remains a question for future investigations. In the present system, preliminary mechanistic investigations indicate that H_2S-mediated azide reduction is the rate-limiting step of the self-immolative process and that the subsequent release of COS and hydrolysis by CA to form H_2S is rapid. Taken together, these data highlight the potential of this strategy to access analyte-replacement, reaction-based fluorescent scaffolds.

In summary, we have outlined and demonstrated a new strategy for triggered H_2S release based on self-immolative thiocarbamates. Importantly, this strategy provides solutions to key challenges associated with both H_2S delivery and detection. Thiocarbamate-based H_2S donors provide a new, versatile, and readily modifiable platform for developing new H_2S donor motifs that can be triggered by endogenous or biorthogonal triggers. Similarly, this same H_2S donation strategy can be coupled to fluorescent probe development to access reaction-based fluorescence reporters that replace the analyte that has been consumed by the detection event. In a broader context, we expect that the self-immolative thiocarbamate donors will find significant utility as a potential platform for academic and potentially therapeutic H_2S donors. Moreover, we anticipate that similar strategies can be applied to provide access to other analyte-replacement reaction-based sensing motifs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b03780.

Experimental details, H_2S release profiles, spectra (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors have filed a provisional patent on thiocarbamate-based and other COS-related donation strategies, including those outlined within this manuscript.

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