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# Bio-orthogonal “click-and-release” donation of caged carbonyl sulfide (COS) and hydrogen sulfide (H<sub>2</sub>S)<sup>†</sup>

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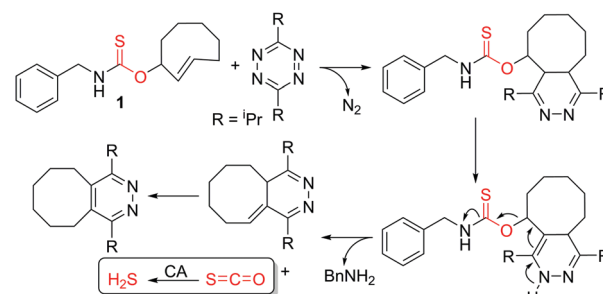
**Hydrogen sulfide (H<sub>2</sub>S) is an important biomolecule with high therapeutic potential. Here we leverage the inverse-electron demand Diels–Alder (IEDDA) click reaction between a thiocarbamate-functionalized *trans*-cyclooctene and a tetrazine to deliver carbonyl sulfide (COS), which is quickly converted to H<sub>2</sub>S by the ubiquitous enzyme carbonic anhydrase (CA), thus providing a new strategy for bio-orthogonal COS/H<sub>2</sub>S donation.**

With the recent addition of hydrogen sulfide (H<sub>2</sub>S) to the list of biologically-relevant gasotransmitters,<sup>1</sup> significant efforts have focused on developing H<sub>2</sub>S donors as powerful research, and potentially therapeutic, tools.<sup>2,3</sup> Endogenous H<sub>2</sub>S production occurs primarily from cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopyruvate transferase (3-MST), and the slow production of H<sub>2</sub>S exerts protective effects throughout the body.<sup>1</sup> Although convenient, inorganic sulfide salts (NaSH and Na<sub>2</sub>S) provide a large, instantaneous bolus of H<sub>2</sub>S, and sulfide oxidation often occurs rapidly after administration.<sup>4</sup> These limitations suggest that more efficacious donors should either more closely mimic slower enzymatic production rates or be stable until triggered to release H<sub>2</sub>S in response to specific stimuli. Available synthetic slow-release donors have already made major impacts in H<sub>2</sub>S research, and several small molecule H<sub>2</sub>S donors have already entered clinical trials.<sup>5</sup> Despite this promise, providing temporal control over H<sub>2</sub>S release remains a major challenge, and there is significant interest in developing synthetic H<sub>2</sub>S donors that are activated by well-defined triggering mechanisms that enable on-demand H<sub>2</sub>S release.

Aligned with this need, we recently pioneered the use of carbonyl sulfide (COS)-releasing molecules as a strategy to access responsive

H<sub>2</sub>S donors. We demonstrated that self-immolative thiocarbamates can be triggered to decompose and release COS, which is rapidly converted to H<sub>2</sub>S by the ubiquitous enzyme carbonic anhydrase (CA).<sup>6</sup> Analogous to the broad applications of self-immolative carbamates as delivery platforms for prodrugs, fluorophores, and other biologically-relevant payloads, thiocarbamates provide a highly tunable platform on which the triggering mechanism can be engineered to initiate self-immolation and COS release by specific analytes of interest. Since our initial report on caged COS/H<sub>2</sub>S release, passive H<sub>2</sub>S donation from small molecule and polymeric *N*-thiocarboxyanhydrides<sup>7</sup> as well as responsive ROS-triggered donors that provide protection against cellular oxidative stress have been reported.<sup>8</sup> Missing from current COS/H<sub>2</sub>S donor technologies are platforms activated by bio-orthogonal triggers to allow precise temporal control for H<sub>2</sub>S release. Motivated by this need, we report here the first example of bio-orthogonal activation of COS/H<sub>2</sub>S release through adaptation of the well-developed inverse-electron demand Diels–Alder (IEDDA) click reaction to release COS/H<sub>2</sub>S (Scheme 1).

The IEDDA reaction between a *trans*-cyclooctene (TCO) and a tetrazine is a proven platform for bio-orthogonal click reactions in living systems.<sup>9–11</sup> In addition to providing an important bio-compatible bond-forming tool, the IEDDA reaction has also been adapted for targeted drug release by using functionalized benzylic carbamates, which can be triggered to undergo self-immolative



**Scheme 1** IEDDA reaction of thiocarbamate-functionalized TCO **1** with tetrazine to generate COS/H<sub>2</sub>S.

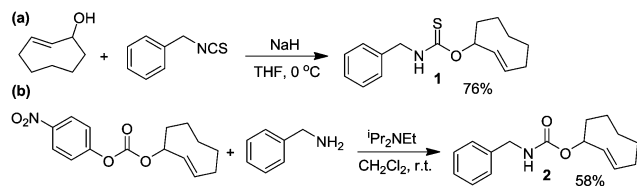
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**Scheme 2** Synthesis of thiocarbamate-functionalized COS/H<sub>2</sub>S donor TCO **1** and the associated carbamate-functionalized control compound TCO **2**.

decomposition following the click reaction and subsequently release the attached drug, as well as CO<sub>2</sub> as a byproduct.<sup>12–14</sup> We envisioned that a similar strategy could be leveraged to develop a fully bio-orthogonal COS/H<sub>2</sub>S releasing platform by using a benzylic thiocarbamate-functionalized TCO (Scheme 1). The initial IEDDA click reaction would generate the thiocarbamate-functionalized dihydropyridazine, which after tautomerization, deprotonation, and rearomatization can extrude COS, BnNH<sub>2</sub>, and the cyclooctylpyridazine product. To test this hypothesis, we prepared TCO **1** by treating (*E*)-cyclooct-2-enol with benzyl isothiocyanate in the presence of NaH.<sup>13</sup> In parallel, we prepared the analogous carbamate-functionalized TCO **2**, which undergoes the same IEDDA reaction but releases CO<sub>2</sub> rather than COS (Scheme 2). Both TCO **1** and **2** are isolated as the axial isomer, which is estimated to be significantly more reactive than the analogous equatorial isomer.<sup>15</sup> Importantly, this design strategy provides simple synthetic access to both the thiocarbamate donor and key carbamate control compounds. Additionally, click-and-release CO donors were recently reported utilizing an intramolecular Diels–Alder reaction, thus supporting the validity of our approach for accessing bio-orthogonal gasotransmitter release.<sup>16</sup>

To confirm that the IEDDA reaction would initiate self-immolative decomposition of the thiocarbamate moiety, we monitored the reaction of **1** and **2** equiv. of bis-isopropyl-1,2,4,5-tetrazine in wet methanol-*d*<sub>4</sub> by <sup>1</sup>H NMR spectroscopy (Fig. 1a). Within 5 minutes of tetrazine addition, we observed disappearance of the alkene peaks (5.3–5.7 ppm), indicative of cycloaddition. New resonances corresponding to BnNH<sub>2</sub> were subsequently observed at 4.25 ppm, while **1** continues to decompose over 24 hours. Due to the complexity of this reaction and the different potential intermediates that could be formed *en route* to COS extrusion, we also monitored product formation by mass spectrometry. Consistent with our design hypothesis we observed the re-aromatized IEDDA product (*M* + *H*<sup>+</sup> 247.239), BnNH<sub>2</sub> (*M* + *H*<sup>+</sup> 108.091), and COS (*M* + *H*<sup>+</sup> 61.044) (Fig. 1b and c) using direct analysis in real time mass spectrometry (DART-MS). Taken together, these data indicate that addition of the tetrazine to **1** results in the expected click reaction and initiates self-immolation of the thiocarbamate moiety, thus producing COS.

Having confirmed the fidelity of the IEDDA reaction, we next investigated click-and-release H<sub>2</sub>S-donation from this system in aqueous buffer at physiological pH (PBS, pH 7.4) using an H<sub>2</sub>S-selective electrode (Fig. 2). Non-enzymatic background hydrolysis of COS to H<sub>2</sub>S is very slow at physiological pH, but is rapid in the presence of carbonic anhydrase (CA). Using biologically-relevant CA concentrations (25 μg mL<sup>−1</sup>) we first monitored TCO **1** alone and



**Fig. 1** (a) <sup>1</sup>H NMR spectra of the reaction of **1** and tetrazine (b) ESI-MS of reaction products, confirming self-immolation. (c) MS confirmation of COS formation.



**Fig. 2** H<sub>2</sub>S release profiles from TCO **1** (50 μM) with 5–25 equiv. of tetrazine in the presence of CA (25 μg mL<sup>−1</sup>) in buffer (PBS, pH 7.4).

confirmed that H<sub>2</sub>S is not released spontaneously in the presence of CA. As anticipated, the bis-isopropyl-1,2,4,5-tetrazine alone also failed to produce an H<sub>2</sub>S response. We next monitored H<sub>2</sub>S release from TCO **1** (50 μM) with varying concentrations of tetrazine (5–25 equiv.) and observed increased H<sub>2</sub>S production in the presence of excess tetrazine. Using a calibration curve, we measured 12 μM H<sub>2</sub>S release from 50 μM TCO **1** with 25 equiv. of tetrazine, resulting in an H<sub>2</sub>S release efficiency of approximately 25%. As additional confirmation of the importance of CA for H<sub>2</sub>S formation, we performed identical reactions in the presence of acetazolamide (AAA, 2.5 μM), a known CA inhibitor. Furthermore, use of the control compound TCO **2** in the presence of excess tetrazine failed to generate COS/H<sub>2</sub>S. Together, these data confirm that the IEDDA click reaction is necessary to generate COS, and that uninhibited CA is required for efficient conversion of this released COS to H<sub>2</sub>S at physiological pH.

To demonstrate the basic biological compatibility of the reaction, we also investigated H<sub>2</sub>S release from TCO **1** (50 μM) with the tetrazine (500 μM) in complex media (Fig. 3). For these experiments, we chose to use whole sheep and bovine blood due





Fig. 3  $\text{H}_2\text{S}$  release profiles from TCO **1** ( $50\ \mu\text{M}$ ) with 10 equiv. of tetrazine in whole bovine blood (red), whole sheep blood (blue), and sheep plasma (grey), diluted 1:1 with buffer (PBS, pH 7.4).

to the presence of CA. Using sheep blood and plasma, diluted 1:1 in PBS (pH 7.4) with no additional CA added, a similar  $\text{H}_2\text{S}$  release profile was observed using an  $\text{H}_2\text{S}$ -selective electrode. Additionally,  $\text{H}_2\text{S}$  production was also observed in diluted whole bovine blood, although the process was slower. These experiments confirm that bio-orthogonal click-and-release strategy has significant potential within a biological environment and endogenous CA levels are sufficient to allow for  $\text{H}_2\text{S}$  donation from the released COS. Additionally, we confirmed the cellular compatibility of TCO **1** using the CCK-8 cell viability assay, which indicated that concentrations up to  $100\ \mu\text{M}$  of TCO **1** are not cytotoxic in N2A neuroblastoma cells (see ESI†).

In an effort to expand this strategy to a cellular environment, we attempted to obtain cell images using a variety of fluorescent probes for  $\text{H}_2\text{S}$ , including HSN2, WSP-5, and SF7-Am.<sup>17–19</sup> Unfortunately, we found that the click-and-release reaction was not compatible with these current fluorescent detection strategies for  $\text{H}_2\text{S}$ . This observation was confirmed in cuvette-based fluorimetry studies as well, in which no fluorescent turn-on was observed after several hours despite the production of  $\text{H}_2\text{S}$ , as confirmed by  $\text{H}_2\text{S}$ -electrode experiments. Although unexpected, this outcome may be due to slower and/or less-efficient COS/ $\text{H}_2\text{S}$  release from this first-generation IEDDA platform than from previously reported COS/ $\text{H}_2\text{S}$  donors. In a closed system, it is also possible that the tetrazine may also scavenge the generated  $\text{H}_2\text{S}$ , as evidenced by a recent report demonstrating that  $\text{H}_2\text{S}$  can partially reduce dialkoxy tetrazines to the dihydrotetrazine.<sup>20</sup> Therefore, future investigations into the differential reactivity of  $\text{H}_2\text{S}$  with substituted tetrazines appears warranted, both to increase the biocompatibility in this system and also to increase the initial efficiency of the IEDDA click reaction.<sup>21</sup> For example, a recent report highlighted that the efficiency of the IEDDA reaction can be improved through strategic choice of the tetrazine. These, as well as other modifications to the thiocarbamate scaffold are expected to provide much more efficient  $\text{H}_2\text{S}$  release from future click-and-release scaffolds.

In summary, we have reported the first example of COS/ $\text{H}_2\text{S}$  donors activated by a bio-orthogonal trigger, which provides a

significant step toward developing controllable  $\text{H}_2\text{S}$  donors with high temporal resolution. Given the novelty of this bio-orthogonal reaction in the field of sulfide donation, as well as the significant impact that similar click strategies have provided to adjacent fields in chemical biology, we anticipate that future optimization of this system will result in fast and highly targeted methods for  $\text{H}_2\text{S}$  donation.

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## References

- 1 R. Wang, *Physiol. Rev.*, 2012, **92**, 791–896.
- 2 Y. Zhao, T. D. Biggs and M. Xian, *Chem. Commun.*, 2014, **50**, 11788–11805.
- 3 Z. J. Song, M. Y. Ng, Z.-W. Lee, W. Dai, T. Hagen, P. K. Moore, D. Huang, L.-W. Deng and C.-H. Tan, *MedChemComm*, 2014, **5**, 557–570.
- 4 K. R. Olson, *Antioxid. Redox Signaling*, 2012, **17**, 32–44.
- 5 J. L. Wallace and R. Wang, *Nat. Rev. Drug Discovery*, 2015, **14**, 329–345.
- 6 A. K. Steiger, S. Pardue, C. G. Kevil and M. D. Pluth, *J. Am. Chem. Soc.*, 2016, **138**, 7256–7259.
- 7 C. R. Powell, J. C. Foster, B. Okyere, M. H. Theus and J. B. Matson, *J. Am. Chem. Soc.*, 2016, **138**, 13477–13480.
- 8 Y. Zhao and M. D. Pluth, *Angew. Chem., Int. Ed.*, 2016, **55**, 14638–14642.
- 9 M. L. Blackman, M. Royzen and J. M. Fox, *J. Am. Chem. Soc.*, 2008, **130**, 13518–13519.
- 10 N. K. Devaraj, G. M. Thurber, E. J. Keliher, B. Marinelli and R. Weissleder, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 4762–4767.
- 11 R. Rossin, P. R. Verkerk, S. M. van den Bosch, C. M. Vuldres, I. Verel, J. Lub and M. S. Robillard, *Angew. Chem., Int. Ed.*, 2010, **49**, 3375–3378.
- 12 J. M. Mejia Oneto, I. Khan, L. Seebald and M. Royzen, *ACS Central Sci.*, 2016, **2**, 476–482.
- 13 R. M. Versteegen, R. Rossin, W. ten Hoeve, H. M. Janssen and M. S. Robillard, *Angew. Chem., Int. Ed.*, 2013, **52**, 14112–14116.
- 14 R. Rossin, S. M. J. van Duijnhoven, W. ten Hoeve, H. M. Janssen, L. H. J. Kleijn, F. J. M. Hoebe, R. M. Versteegen and M. S. Robillard, *Bioconjugate Chem.*, 2016, **27**, 1697–1706.
- 15 M. T. Taylor, M. L. Blackman, O. Dmitrenko and J. M. Fox, *J. Am. Chem. Soc.*, 2011, **133**, 9646–9649.
- 16 X. Ji, C. Zhou, K. Ji, R. E. Aghoghovbia, Z. Pan, V. Chittavong, B. Ke and B. Wang, *Angew. Chem., Int. Ed.*, 2016, DOI: 10.1002/anie.201608732.
- 17 L. A. Montoya and M. D. Pluth, *Chem. Commun.*, 2012, **48**, 4767–4769.
- 18 B. Peng, W. Chen, C. R. Liu, E. W. Rosser, A. Pacheco, Y. Zhao, H. C. Aguilar and M. Xian, *Chem. – Eur. J.*, 2014, **20**, 1010–1016.
- 19 V. S. Lin, A. R. Lippert and C. J. Chang, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 7131–7135.
- 20 Z. Zhao, L. Cao, T. Zhang, R. Hu, S. Wang, S. Li, Y. Li and G. Yang, *ChemistrySelect*, 2016, **1**, 2581–2585.
- 21 X. Fan, Y. Ge, F. Lin, Y. Yang, G. Zhang, W. S. C. Ngai, Z. Lin, S. Zheng, J. Wang, J. Zhao, J. Li and P. R. Chen, *Angew. Chem., Int. Ed.*, 2016, **55**, 14046–14050.

