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Geminal-dithiol-based precursors for reactive sulfur species†

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Caged *gem*-dithiols have been developed as the donors of reactive sulfur species (RSS), but the chemistry of free *gem*-dithiols as RSS donors has not been well understood. Herein, we report the study of a free *gem*-dithiol, 1,3-diphenylpropane-2,2-dithiol, as the precursor for several RSS. It releases H_2S under physiological conditions and can be converted to a mono-*S*-nitrosothiol, which serves as a NO donor. Furthermore, it can be converted to 3,3-dibenzyldithiirane, which is an active sulfur transfer reagent and can induce *S*-persulfidation.
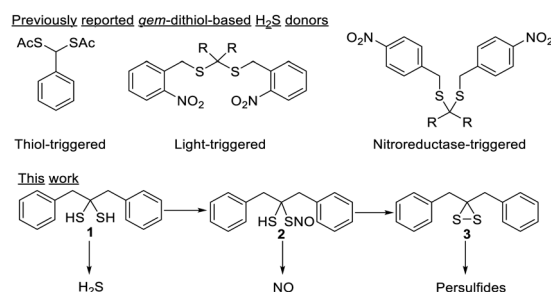
Reactive sulfur species (RSS) are important regulating molecules in biological systems. These include thiols (RSH), hydrogen sulfide (H_2S), persulfides (RSSH), polysulfides (RSS_nSR), and post-translational modification products on protein thiols such as *S*-nitrosothiol (RSNO) and sulfenic acid (RSOH).[1–3] Many RSS are fleeting species that exist in low concentrations, while exhibiting a variety of biological functions such as anti-oxidation and vasodilation. It is therefore important to develop chemicals (*e.g.* donors) that can controllably deliver RSS under physiological conditions. These compounds are useful for understanding the properties and functions of RSS, and have therapeutic potentials. In the past several years, many such donors have been developed, including molecules releasing H_2S, H_2S_n, and RSSH.[4–6] These compounds have greatly advanced the understanding of RSS.

Among the reported H_2S donors, *gem*-dithiol-based compounds are particularly interesting. Free *gem*-dithiols are normally unstable under physiological conditions and quickly decompose to release H_2S. Therefore, bio-compatible protecting groups have been introduced to cage the –SH groups of *gem*-dithiols to create the desired H_2S donors (Scheme 1).[7–9] Interestingly, some free *gem*-dithiol compounds were synthesized and characterized back in the 1950's.[10] We wondered if these relatively stable *gem*-dithiols could be directly used as RSS

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donors. Herein, we report the study of a representative free *gem*-dithiol, 1,3-diphenylpropane-2,2-dithiol (**1**), and its derivatives. Their preparation, stability, reactivity, and ability to serve as RSS donors were studied.

While several *gem*-dithiols (such as methanedithiol, 2,2-propanethiol, cyclohexanedithiol, phenylmethanedithiol) have been reported before, they are unstable and volatile liquids with pungent smells, making them unsuitable for our studies. 1,3-Diphenyl-propane-2,2-dithiol (**1**), first reported by Carmack in 1959,[11] was prepared *via* treating ketone **4** with H_2S (g) and HCl, with thioketone **5** as an intermediate (Scheme 2). This method allowed us to obtain **1** in the gram scale with 65% yield. Compound **1** is a colorless solid with a faint smell of mercaptan. It was found to be stable at room temperature in its neat form or in organic solvents, with no NMR-observable decomposition after storage for days. In water-containing solutions (for example 20% D_2O/CD_3CN), **1** also exhibited good stability, with no decomposition over 12 hours (Fig. S1, ESI†). However, the decomposition of **1** was noted in buffer-containing solutions (such as acetonitrile with 20% pH 7.4 PBS buffer). NMR studies demonstrated that thioketone **5** (in its enethiol form **6**) was the main decomposition product (Fig. S1, ESI†).

The decomposition of compound **1** in buffer solutions prompted us to examine its ability to release H_2S. A Unisense H_2S microsensor was used to measure H_2S production from the solutions of **1** (50 μM) in different buffers (pH 5, 6, 7, 8). Fig. 1a shows that **1** released H_2S in a pH-dependent manner. At pHs
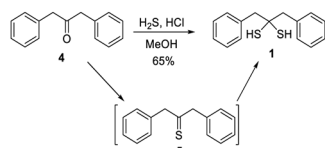

Scheme 1 *gem*-dithiol-based RSS donors.

Scheme 2 The synthesis of free *gem*-dithiol **1**.

Scheme 3 The mechanism of H_2S release from **1**.

6 and 7, $\sim$50 μM H_2S was produced after 150 min, while only $\sim$30 μM H_2S was produced at pH 5. These results suggest that under these pHs, roughly one equivalent of H_2S could be produced from **1**, while slightly more than one equivalent of H_2S ($\sim$68 μM) was produced at pH 8.

To further validate H_2S release from **1**, a gas trapping experiment was performed. Briefly, in sealed vials, the solutions of **1** (1.5 mM) in different buffers (pH 5 and 7.4) were placed with a trapping vial containing 10% zinc acetate solution under room temperature for 3 hours. H_2S generated from **1** would diffuse into the trapping vial to form zinc sulfide, which was then treated with the methylene blue cocktail. The concentration of H_2S was analyzed by UV-vis absorbance at 670 nm. As shown in Fig. 1b, a strong absorbance peak at 670 nm was observed, demonstrating the generation of H_2S from **1**. These results suggested the following mechanism: in aqueous solutions, **1** undergoes elimination to produce H_2S (likely in the form of HS^-) and thioketone **5** (the major form is enethiol **6**) (Scheme 3). This occurs both in acidic and basic pHs but basic conditions might be more efficient. Aliphatic thioketones are known to be unstable and can form complex decomposition products including oligomers and ketones.[12] Nonetheless, it is possible for **5** to undergo hydrolysis and release H_2S, forming ketone **4** as a product. Indeed, **4** was obtained when **1** or **6** was incubated in buffers, with 19% and

18% yields, respectively. This finding suggests that thioketones like **5** could release H_2S in buffers, but the efficiency is low.

Some thiocarbonyl compounds like arylthioamides are known H_2S donors, but their H_2S releasing mechanisms are still unclear.[13] The interactions between thiocarbonyls with certain biomolecules (such as cysteine and GSH) may be responsible for their H_2S release. We next wondered if **5**/**6** could release more H_2S under biomimicking environments. Freshly prepared solutions of **6** (10 mM) were incubated with cysteine (20 mM), HeLa cell lysate, or human plasma for 12 hours. The H_2S released was measured by the H_2S trapping method. As shown in Fig. 2, a notable increase of UV-vis absorbance was detected at 670 nm under these conditions. In contrast, only very weak H_2S production was detected when **6** was incubated in buffer alone. These results indicated biologically-relevant conditions could promote H_2S release from **6**. However, it should be noted that even under these biologically-relevant conditions, the H_2S released from **6** was still significantly less than that of the positive control (5 mM Na_2S), suggesting that it may not be an effective H_2S donor. This adds to the speculation that the bioactivities of thiocarbonyl-based H_2S donors may not come from H_2S, but rather from the thiocarbonyl group itself.

S-Nitrosothiols (SNOs) are one of the key molecules involved in nitric oxide (NO) signal transduction and possess unique chemical and biological properties.[14] Since we had *gem*-dithiol **1** in hand, we tested its nitrosation in the hopes of preparing the *gem*-bis-SNO compound. Interestingly, we were only able to access the mono-SNO compound **2** (in 90% purity, *via* nitrosation of **1** with organic nitrites at low temperature, −20 °C). SNO **2** showed the characteristic green color of tertiary SNO compounds with an obvious UV-vis absorbance peak at
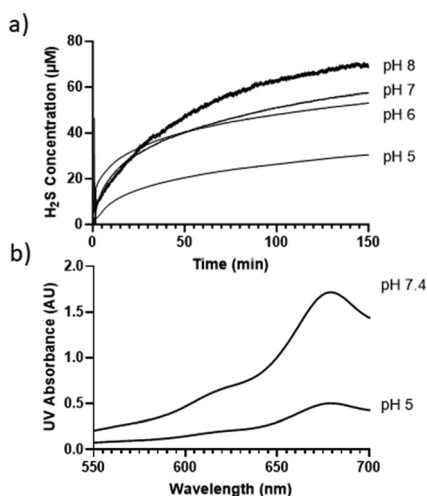

Fig. 1 (a) H_2S release profiles of **1** (50 μM) in different buffers. (b) Methylene blue UV-vis absorbance spectra of H_2S released from **1** as measured by the gas trapping method (1.5 mM **1** was incubated in buffers for 3 hours. H_2S gas was trapped by zinc acetate and measured by the methylene blue method).
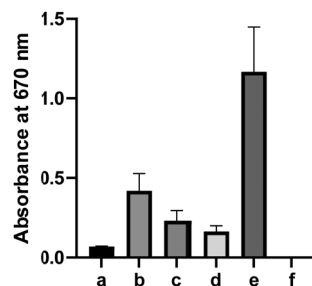

Fig. 2 H_2S release from **6** measured by the gas trapping method. **6** (10 mM) or Na_2S (5 mM) was incubated in PBS buffer (pH 7.4) under various conditions. H_2S gas was trapped by zinc acetate and measured by the methylene blue method. (a) **6** only; (b) **6** with 20% HeLa cell lysate; (c) **6** with 10% plasma; (d) **6** with 20 mM cysteine; (e) Na_2S only; (f) blank.
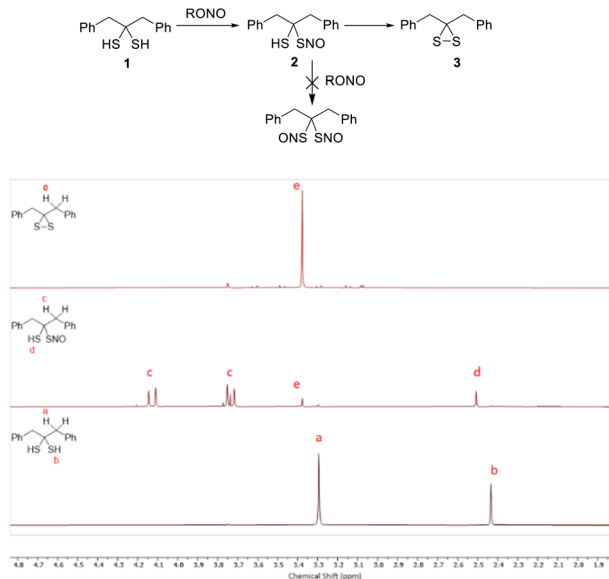
Fig. 3 Nitrosation of **1** and ^{1}H-NMR spectra of compounds **1**, **2**, and **3**.

618 nm (Fig. S2 (ESI†), ε = 26 M^{-1}cm^{-1} in CHCl$_3$). It also exhibited decent stability in solutions as determined by clean NMR spectra (Fig. 3). Attempts to further nitrosate **2** were unsuccessful, and this may be explained by the hydrogen bonding between the –SH and NO group of **2**. SH-based hydrogen bonding is known to stabilize certain structures.[15] DFT calculations were carried out to estimate the H-bond energy (see ESI†). Nevertheless, the solution of **2** could degrade in a few hours to form dithiirane **3**.

With the relatively stable SNO **2** in hand, we wondered if its conversion to **3** could release NO. To this end, an indirect NO trapping and detection experiment was performed. The *in situ* generated **2** was incubated in buffers under different pHs in a sealed 20 mL vial. NO generated in the solution was diffused into an Eppendorf vial containing NO red, an NO-specific fluorescent probe.[16] As shown in Fig. 4, the formation of NO was noted in a pH-dependent manner in all conditions. In CHCl$_3$ and in more acidic buffers (pH 3 and 5), NO released from **2** was comparable to the positive control, a pyrrolidine-based NONOate. Under pH 7.4, 9, and 10, less NO production
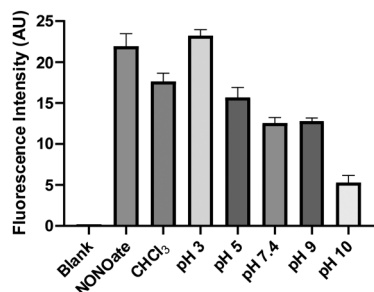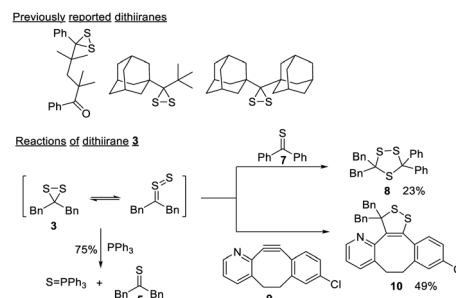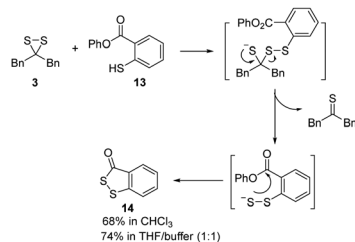
from **2** was observed. The formation of NO from **2** suggested a homolytic S–N bond cleavage process with a thiyl radical intermediate. This was confirmed by TEMPO-9-Ac, a thiyl radical fluorescent probe.[17] When **2** was incubated with TEMPO-9-AC in 10% THF/PBS, significant fluorescence turn on at 432 nm was observed (Fig. S3, ESI†) while other more stable SNOs (trityl SNO and GSNO) gave much weaker fluorescence. It is interesting to note that NO formation from **2** at higher pH (10) decreased. The results suggested that the conversion of **2** to **3** could also proceed *via* an anionic pathway to form nitroxyl (HNO). We attempted to trap HNO using GSH,[18] but were unable to obtain the corresponding sulfinamide product. Nevertheless, HNO generation was still possible. These results suggest **2** could be considered as an interesting NO (or HNO) donor.

Dithiiranes are the smallest cyclic disulfides.[19] The S–S bond of dithiiranes is forced to adopt a "cisoid" geometry, resulting in maximum lone pair interactions and a weak S–S bond.[20] Due to this property, most dithiiranes are unstable and can only exist as reaction intermediates.[21,22] A few isolable dithiiranes[23–25] have been reported (Scheme 4), and they are derived from highly sterically hindered substrates. To verify the compound obtained in our reactions was indeed a dithiirane, we studied its properties and reactions. **3** could be generated from the direct reaction between **1** and an excess of *t*-butyl nitrite. It was isolated as a yellow liquid ($\sim$90% purity) once the solvent was removed. **3** showed a UV-vis absorbance at 441 nm (Fig. S4, ESI†), which corresponds to the $n\pi \rightarrow \sigma^*$ transition of the dithiirane ring and is similar to the dithiirane reported before (at 452 nm).[24] In its neat form, **3** was found to be unstable at room temperature and could degrade completely in 6 hours. When it was dissolved in organic solvents, it appeared to be more stable, but still decomposed in $\sim$4 days. The degradation products were complex, with thioketone **5** being one of the identified compounds, and this matched the properties of reported dithiiranes.[26] The treatment of **3** with PPh$_3$ or Et$_3$N resulted in more rapid formation of **5**. In the case of PPh$_3$, triphenyl phosphine sulfide (Ph$_3$P = S) was isolated in 75% yield (Scheme 4). The reaction between **3** and PPh$_3$ indicated that dithiiranes could be effective sulfur transfer reagents while the corresponding thioketones appeared to lack this reactivity.

Fig. 4 NO release from **2** measured by NO red. 6.4 mM **2** was incubated with CHCl$_3$ or PBS buffers (containing 10% THF) for 1.5 h. NO was then trapped by NO red (40 μM), and its fluorescence (λ_{em} 593 nm, λ_{ex} 540 nm) was measured.

Scheme 4 The structures of reported dithiiranes and dithiirane **3**'s reactions.

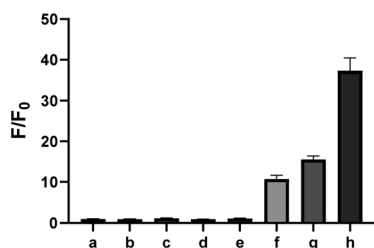Scheme 5 Assessing the sulfur transferring capability of **3** using **13**.

Fig. 5 Fluorescence enhancements of SSP4 (5 μM) with persulfidated papain samples and controls. (a) blank; (b) **3** only; (c) **6** only; (d) papain only; (e) papain + **6**; (f) papain + **3**; (g) papain + DTNB + **1**; (h) papain + DTNB + Na$_2$S; results are represented as the mean ± SEM ($n = 3$).

Dithiiranes can be considered as the isomers of thiocarbonyl *S*-sulfides,[19] which are usually *in situ* generated by the prolonged heating of trithiolanes. These thiocarbonyl *S*-sulfides are known to react with alkynes or thioketones to form cycloaddition products.[27] We thus treated **3** with thiobenzophenone **7** and a strained cyclooctyne **9** (Scheme 4). The corresponding products (trithiolane **8** and the cyclic disulfide **10**) were obtained in modest yields. These results demonstrated that isolated dithiiranes could perform thiocarbonyl *S*-sulfide-mediated 1,3-dipolar cycloadditions under mild conditions.

Given the possibility of **3** being a unique sulfur transfer reagent, we wondered if it could transfer its sulfur to thiols (RSH), which would form persulfides (RSSH). This type of sulfur species plays important roles in redox biology,[1] but currently reliable methods to induce persulfidation in biological systems are still needed. We treated **3** with a model RSH compound **13** in both organic and water-containing solvents (Scheme 5). The desired sulfur transfer product **14** was obtained in good yields. Although **3** was found to be unstable and would degrade to complex products (including **6**) in aqueous solutions, we tested its ability in inducing protein persulfidation. Briefly, papain (a cysteine protease) was treated with **3** in tris buffer for 30 min. The protein was then purified by a PD-10 column to remove excess small molecules and their byproducts. The level of papain persulfidation was analyzed by an established protocol using the fluorescent probe SSP4.[28] As shown in Fig. 5, papain treated with **3** gave significant fluorescence, albeit not as strong as the signal from papain treated with DTNB and Na$_2$S, an authentic method for protein persulfide formation. The combination of compound **1** with DTNB also resulted in considerable fluorescence. Other controls, including papain treated with **6**, did not give detectable fluorescence. The control experiment with only compound **3** was to demonstrate that the PD-10 column could effectively remove small molecules. As such, the signal from papain treated with **3** was not due to the incomplete removal of **3** from the sample. These results suggest that dithiirane compounds have the potential to be developed as useful persulfidation reagents. However, structural modifications to enhance their stability in aqueous solutions will be needed.

In conclusion, we studied the reactions and properties of a stable *gem*-dithiol **1** and its derivatives. **1** can serve as a H$_2$S donor. Its SNO derivative **2** shows good stability. To the best of our knowledge, compound **2** is the first compound with both free thiol and nitrosothiol moieties in the structure, making it a unique precursor for both NO (or HNO) and reactive sulfur species. Dithiirane **3**, which is generated from **2** after NO release, is a sulfur transfer reagent and can be used to induce protein persulfidation. Further structural optimization on these molecules may provide reagents with better stability and specificity (as H$_2$S donors, NO donors, or protein persulfidation reagents), and that is ongoing research in our lab.

Conflicts of interest

There are no conflicts to declare.

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