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Communication

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Toward Direct Protein S-Persulfidation: A Prodrug Approach that Directly Delivers Hydrogen Persulfide

Bingchen Yu, [#] Yueqin Zheng, [#,*] Zhengnan Yuan, Shanshan Li, He Zhu, Ladie Kimberly De La Cruz, Jun Zhang, Kaili Ji, Siming Wang and Binghe Wang*

Department of Chemistry and Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30303 United States

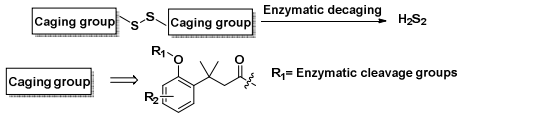
Supporting Information Placeholder

ABSTRACT: A general strategy of delivering hydrogen persulfide (H_2S_2) is described herein. Esterase- and phosphatase-sensitive H_2S_2 prodrugs with tunable release rates have been synthesized. Their utility is validated in examining protein S-persulfidation. With this unique approach of directly delivering H_2S_2, our findings reaffirmed that S-persulfidation leads to decreased activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This new approach complements available prodrugs/donors that directly deliver a single species including hydrogen sulfide, perthiol, and COS, and will be very useful as part of the toolbox in delineating the mechanisms of sulfur signaling.

Hydrogen sulfide (H_2S) plays roles in physiological and pathological processes and has promising therapeutic potential.[1-10] Recent studies suggest that endogenous hydrogen polysulfides (H_2S_n, n≥2) and perthiol (RS_nH, n≥2) have similar physiological effects as H_2S, but with greater potency in some cases.[11-15] For example, hydrogen polysulfides were found to be able to induce Ca^{2+} influx by activating transient receptor potential (TRP)A1 channels in rat astrocytes and are 320 times more potent than H_2S.[16] Protein S-persulfidation (sometimes referred to as S-sulfhydration), in which the thiol group of cysteine (-SH) in protein is converted to a perthiol group (-SSH), has proven to be a major signaling pathway involving sulfur.[17-20] Many enzymes can go through this process, leading to significant changes in activity.[19,21,15,22,17,23] Chemically, H_2S cannot simply "persulfidate" a thiol group. Therefore, either some other oxidation reaction(s) needs to happen or the H_2S source contains sulfur species at higher oxidation states.[15,24] Thus, it is likely that hydrogen polysulfide and/or perthiol derived from H_2S are the actual dominant species in S-persulfidation.[25-28] H_2S and perthiol can be produced enzymatically.[11,12,29] The facts that hydrogen polysulfide and perthiol can lead to protein S-persulfidation and there are enzymatic pathways to produce such species lend further support to the possibility that hydrogen polysulfide and perthiol are the actual signaling molecules in certain processes.[29]

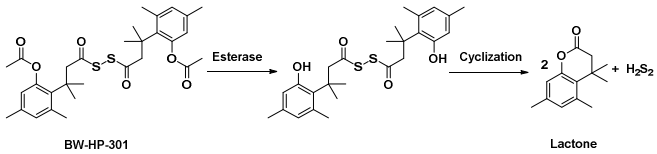
Given the significant role of hydrogen polysulfide and perthiol in protein S-persulfidation signaling, there is a need to prepare prodrugs for generating hydrogen polysulfide as single species without perturbing cellular redox chemistry. Several labs have reported beautiful work in preparing prodrugs for H_2S,[30-34] perthiol,[35] and COS.[31,36-39] What are missing in the toolbox are prodrugs for H_2S_2 as a single or at least as a dominant species. Herein, we described our work in this regard.

Scheme 1. The design concept of H_2S_2 prodrugs.



The need of developing H_2S_2 prodrugs derives from its unstable nature.[40] We used the "trimethyl lock"-facilitated lactonization system as the caging group for prodrug preparation (Scheme 1).[41] Specifically, H_2S_2 is caged as two thiolacid groups linked by a disulfide bond (Scheme 1). A masked phenol hydroxyl group serves as a latent nucleophile for initiation of H_2S_2 release through lactonization.

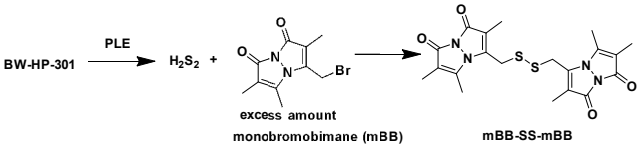
Scheme 2. Mechanism of esterase-triggered H_2S_2 release.



An esterase-sensitive H_2S_2 prodrug, **BW-HP-301** (**301**), was prepared (Scheme 2). **301** is a colorless oil and stable for days at room temperature and months at -20 °C. We studied whether esterase would promote H_2S_2 release from **301** using a H_2S_2 fluorescence probe DSP-3.[42] Thus, **301** was incubated with porcine liver esterase (PLE) at 37 °C in phosphate-buffered saline (PBS, pH = 7.4) for 30 min to fully consume **301**; then 20 μM of DSP-3 was added. Strong fluorescence was observed in experiments with PLE (Figure 1A), indicating the release of H_2S_2. In contrast, negligible fluorescence was detected without PLE. Incubation with 200 μM Na_2S led to negligible fluorescence intensity increase, showing the stability of DSP-3 toward H_2S. To provide further direct evidence of H_2S_2 release, we used monobromobimane (mBB) to trap H_2S_2 in a stable form, mBB-SS-mBB (Scheme 3 and Figure 1B).[43] 68 ± 8 μM of mBB-SS-mBB was detected from 100 μM prodrug **301** in the presence of 10 unit/mL of PLE. The less than 100% conversion could be due to many reasons, including slow reaction kinetics and stability issues for H_2S_2. As a reference point, we also used the same method to trap H_2S_2 from commercially available Na_2S_2. About 71 ± 9 μM of mBB-SS-mBB was detected in 100 μM of Na_2S_2 solution (Figure S2). The fact that the prodrug and Na_2S_2 gave the same results in both

the DSP-3 and mBB assays strongly suggest that the release from the prodrug was nearly 100%. The efficiency of the H_2S_2 trapping reaction was most likely the reason causing the less than 100% conversion to the trapped product, mBB-SS-mBB. It is important to noted that H_2S_2 concentration from the addition of 100 μM Na_2S_2 started decreasing from the moment of dissolution (Figure S2). However, with 100 μM **301**, the H_2S_2 concentration gradually increased with the addition of 2 units/mL PLE and reached a peak concentration at 25 min.

Scheme 3. Direct detection of H_2S_2 by trapping agent mBB.



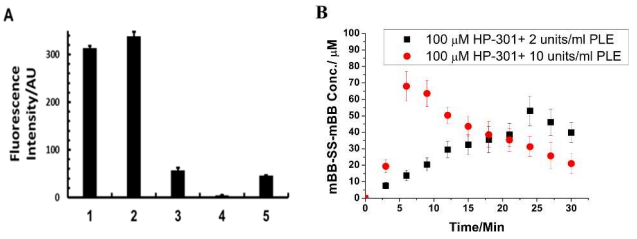


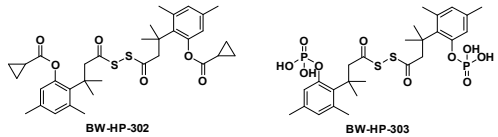
Figure 1. A) Qualitative detection of H_2S_2 release from **301** by DSP-3. **301** was incubated with 2 units/mL PLE in PBS (2% DMSO) for 30 min and then DSP-3 (20 μM) was added. Data were acquired at 515 nm with excitation at 490 nm. n = 3. 1) 100 μM **301** + PLE; 2) 100 μM Na_2S_2 + PLE; 3) PLE; 4) 100 μM **301**; 5) 200 μM Na_2S + PLE. B) Direct detection of H_2S_2 by trapping agent mBB from **301** in PBS (2% DMSO). n = 3.

One would expect the sustained concentration of H_2S_2 to be dependent on the release rate from a prodrug since the generation and consumption of thiol species in the biological system is a dynamic process. Thus, we wanted to prepare prodrugs with varying release rates. Specifically, **BW-HP-302** (**302**) was synthesized (Scheme 4) and its H_2S_2 release ability was assessed (Figure S1). The H_2S_2 release profiles from **301** and **302** are shown in Figure 2B. From 40 μM of the prodrugs, **301** had a peak concentration of 15 μM at around 15 min, while **302** maintained a sustained concentration of about 3 μM. The release profile of **302** was also studied by using mBB. A plateau of 35 ± 7 μM of mBB-SS-mBB was detected from 100 μM of **302** in the presence of 10 units/mL PLE (Figure S2). For 100 μM of the prodrug, the half-life was determined to be 24 min for **301** and 172 min for **302** by HPLC (Table S4). The slower release rate of **302** was presumably due to the bulkier nature of the cyclopropanecarbonyl ester, which hinders esterase-mediated hydrolysis.[44]

In an effort to broaden the tunability of H_2S_2 release, a phosphatase-sensitive H_2S_2 prodrug, **BW-HP-303** (**303**), was synthesized (Scheme 4). Alkaline phosphatase (ALP) is widely used for prodrug activation.[45-47] Phosphatase-dependent H_2S_2 release was examined by DSP-3 (Figure 2A). Incubation of **303** with 10 units/mL ALP led to strong fluorescence, demonstrating H_2S_2 release. In the absence of phosphatase, almost no H_2S_2 was detected, indicating the chemical stability of the prodrug. A peak concentration of 65 ± 8 μM of mBB-SS-mBB was achieved from 100 μM **303** in the presence of 10 units/mL ALP. Such result is similar to that of 100 μM **301** and Na_2S_2, demonstrating the efficient H_2S_2 release from **303** (Figure S2). From 40 μM **303**, a peak concentration of 10 μM of H_2S_2 was detected (Figure 2B) with a half-life of 28 min in the presence of 2 units/mL ALP (Table S4).

To assess the utility of the prodrug in delivering H_2S_2 in biological systems, cells were co-treated with 100 μM **301** and PLE or 100 μM **303** and ALP (Figure S9), respectively. Strong fluorescence was observed, indicating H_2S_2 release. No obvious cytotoxicity was found at up to 100 μM of the prodrugs or lactone on H9c2 cells after 24 and 48 h incubation (Figure S5,6).

Scheme 4. Structures of 302 and 303



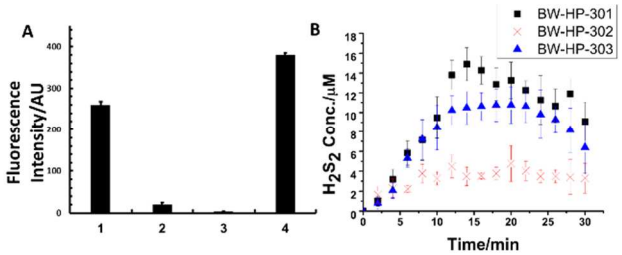


Figure 2. A) Qualitative assessment of H_2S_2 release from **303** by DSP-3. The concentration of DSP-3 is 20 μM and ALP is 10 units/mL in PBS (1% MeOH). n = 3. 1) 100 μM **303** + ALP; 2) 100 μM **303**; 3) ALP; 4) ALP + 100 μM Na_2S_2. B) H_2S_2 releasing profile form 40 μM **301** and **302** with 1 unit/mL PLE in PBS (2% DMSO) and **303** with 2 units/mL ALP at 37 °C in PBS (2% MeOH). 20 μM DSP-3 was used. n = 3.

S-persulfidation is a major sulfur signaling pathway.[17,19] We then examined the S-persulfidation efficiency of the prodrugs on GAPDH using a tag-switch assay.[48] Incubation with 100 μM **301** and 10 units/mL PLE at 37℃ led to a significant increase in GAPDH S-persulfidation level (Figure 3B and C, line 1) compared to the untreated group (line 5). In contrast, incubation with 200 μM H_2S (line 4) failed to elevate GAPDH S-persulfidation level. Protein samples were also treated with 100 μM Na_2S_2 or 100 μM H_2O_2 followed by 100 μM H_2S (line 2, 3) to mimic the two endogenous S-persulfidation process (Figure S10).[15,48,49] These two methods each led to a significant increase of GAPDH S-persulfidation level. Such results further affirm that H_2S itself is incapable of protein S-persulfidation. Previously H_2S has been shown to abolish S-persulfidation by itself.[50] The roles that H_2S plays in S-persulfidation and signaling are dependent on ROS and cellular redox environment. However, **301** was able to induce S-persulfidation independent of ROS. These H_2S_2 donors are important research tools to conduct S-persulfidation without perturbing the redox balance. Even with data suggesting H_2S_2 being the dominant species released from **301**, we can't exclude the possibility that other polysulfide derived from H_2S_2 degradation may also play a role in S-persulfidation because of the unstable nature of H_2S_2.

It has been a subject of debate as to whether S-persulfidation leads to increased or decreased activity of GAPDH.[17,23] To address this point, GAPDH was treated with the exact conditions that was used in the S-persulfidation assay above. Then enzyme activity was determined. As shown in Figured 3B and D, GAPDH treated with **301**, Na_2S_2, or H_2S and H_2O_2 together each showed elevated pro-

tein S-persulfidation levels and decreased enzyme activity compared with untreated groups. Meanwhile, H_2S alone failed to affect enzyme activity. GAPDH activity also showed a concentration dependent decrease in response to **301** with a half inhibition concentration of approximately 1 μM (Figure 3E). Maximal inhibition was achieved with 5 μM of the **301** with 17 ± 1% catalytic activity remaining. Further

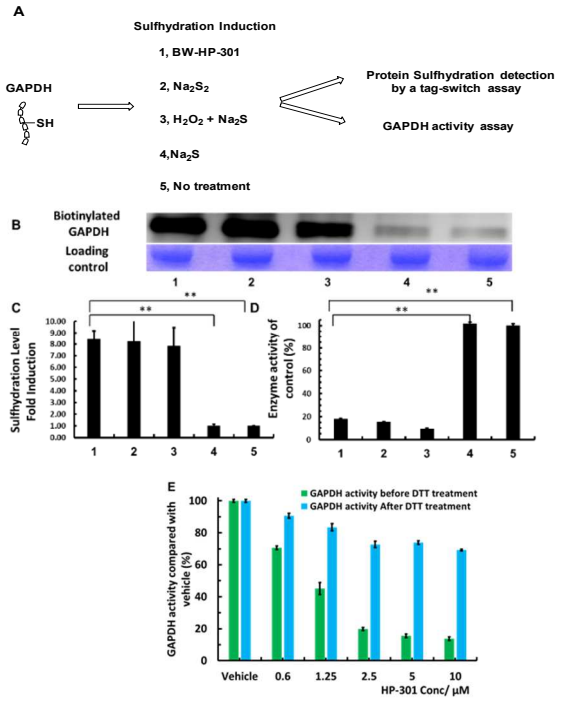


Figure 3, A: Work flow of GAPDH S-persulfidation process; B and C: GAPDH S-persulfidation level assay. GAPDH (2 mg/mL) was subjected to different treatments and analyzed by the protein S-persulfidation switch tag assay; D: GADPH activity assay; All groups have 10 units/mL PLE and 2% DMSO. (1) 100 μM **301**; (2) 100 μM Na_2S_2; (3) 100 μM H_2O_2+ 100 μM Na_2S; (4) 200 μM Na_2S; (5) PLE alone; GADPH was subjected to various treatment at 37 ℃ for 0.5 h, then its S-persulfidation level and activity was determined. E: Concentration dependent inhibition of GAPDH activity by **301**. 2 μg/mL GAPDH was incubated with various concentration of **301** at 37 ℃ for 0.5 h with 10 units/ mL PLE, after which the enzyme activity was determined. Then each group was incubated with 2 mM DTT at r.t. for 2 h, after which the GAPDH activity was measured. Values are means ± SEM. n = 3, **P < 0.01.

increase in prodrug concentration to 10 μM did not decrease the enzyme activity further. After treatment with **301**, GAPDH was treated with 2 mM DTT at r.t. for 2 h, which would reduce the S-persulfidation product to free the thiol group again. Indeed, enzyme activity was restored to 75-95% of its original activity by DTT treatment. Considering the important roles of GAPDH,[51,52] the above results suggest an important role for H_2S_2 in energy metabolism, proliferation and redox balance.

In conclusion, we provide a general strategy to H_2S_2 prodrugs with well-defined release mechanism. Secondly, controllable release patterns and tunable release rates have been achieved. Such a H_2S_2 donor can directly induce protein S-persulfidation, leading to significant enzyme activity changes. This novel series of H_2S_2 prodrugs should be important research tools for future studies.

ASSOCIATED CONTENT

Supporting Information

Experiment details and supplementary figures and table

AUTHOR INFORMATION

Author Contributions

#These authors contributed equally and the names are listed alphabetically.

Corresponding Author

Binghe Wang: Wang@gsu.edu;
Yueqin Zheng: yzheng6@gsu.edu

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