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A Novel Esterase-sensitive Prodrug Approach for Controllable Delivery of Persulfide Species

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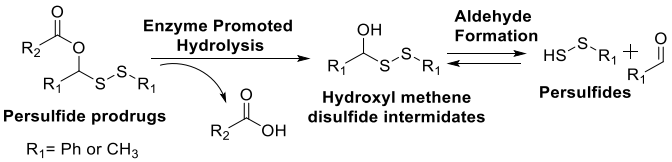
Abstract: A new strategy to deliver a well-defined persulfide species in a biological medium is described herein. Under near physiological conditions, the persulfide prodrug can be activated by an esterase to generate a "hydroxyl methyl persulfide" intermediate, which rapidly collapses to form a defined persulfide. Such persulfide prodrugs can be used either as chemical tools to study persulfide chemistry and biology or for future development as H_2S-based therapeutic reagents. Using the persulfide prodrugs developed in this study, the reactivity between *S*-methyl methanethiosulfonate (MMTS) with persulfide was unambiguously demonstrated. In addition, a representative prodrug exhibited potent cardioprotective effects in a murine model of myocardial ischemia-reperfusion (MI/R) injury with a bell shape therapeutic profile.

The physiologic and potentially therapeutic roles of hydrogen sulfide (H_2S) and related sulfur species are well accepted.[1] However, the detailed mechanism(s) of action for these sulfur species is far from clear. Further, even the question of the "active" sulfur species is not always clear for a given biological response. To complicate this further, different donors of sulfur species without defined chemistry are often used in various studies, leading to difficulty in result interpretation and comparison. For example, polysulfides and garlic-derived sulfur species are often used in biological studies.[2] The chemistry of such sulfur donors is not well defined; consequently, it is not always clear what the "active" species is and the relative ratio of the various species. To advance the field of sulfur biology, it is important to devise chemical strategies that allow for the precise production of various specific sulfur species for mechanistic studies at the molecular level and for understanding the biology. Several labs have made significant contributions in developing prodrugs of hydrogen sulfide[3] and COS[4] as well as sulfur donors at various oxidation states.[5] Along this line, one molecular pathway is known to play an important role in sulfur-mediated signaling, *S*-sulfhydration.[6] Clearly, this is not the kind of chemistry that can be achieved with hydrogen sulfide *per se.* It would require sulfur species at the oxidation state of a persulfide or polysulfide. Indeed, perthiol species have drawn growing attention owing to its potentially dominant roles in H_2S-related signaling pathways.[1g, 7] There are already many studies that explore persulfide chemistry in biology. For example, it was reported that persulfide species such as glutathione persulfide (GSSH) have much stronger "reducing" ability for ferric-cytochrome *c* than H_2S and GSH.[8] Such results are puzzling in terms of the redox chemistry because chemically it is hard to understand how a persulfide species can be stronger reducing agents than sulfide. Such findings suggest that more work is needed at the molecular level to elucidate the mechanism of action. Other examples include the findings that reactive sulfur species (RSS) such as sulfane sulfurs or polysulfides are more effective in *S*-sulfhydration than H_2S.[1g, 9] There is a clear need for investigation of persulfide chemistry and chemical biology in this field.[7a] However, there are two major challenges that face the chemistry field. The first one is a lack of good persulfide precursors/prodrugs that allow for easy and reliable access to persulfides as research tools; and the second one is the limited availability of 'easy to use' detection methods for protein *S*-sulfhydration.[7a] There has been reported work to address the second issue.[1g, 6, 10] We herein focus on developing novel and easy to handle persulfide prodrugs to address the first issue.

The difficulty in developing persulfide prodrugs comes from the unstable nature of persulfide species, which can rapidly decompose to disulfides, polysulfides, elemental sulfur and H_2S.[11] This is especially true if there is an exposed free sulfhydryl group (-SH). To achieve controlled delivery of persulfide in biological studies, the free sulfhydryl group has to be protected, and then regenerated when needed. One class of existing persulfide precursors contains the acyl disulfide group.[3a, 10c] They were cleverly designed to release the persulfides through nucleophilic attack by a thiol group. However, the release of persulfide relies on the addition of an excess amount of thiol in solution.[3a, 10d] Therefore, there is a mix of various sulfur species present at any given time. An approach to directly achieve "pure" persulfide species under physiological conditions without using other sulfide or thiol species is needed.

Enzyme-sensitive prodrugs have been widely used in drug delivery.[12] We are interested in designing esterase-sensitive persulfide prodrugs, which are stable under physiological conditions and can efficiently generate persulfide in the presence of an enzymatic trigger. Specifically, similar to the idea of using "hemiacetal" as an unstable intermediate in prodrug design,[13] we were interested in exploiting the "hydroxyl methyl disulfide" (HOCHRSSR) analog as a key intermediate in our design. In this design, an ester group is introduced to mask the hydroxyl group, leading to a stable precursor. Activation of this prodrug thus relies on the cleavage of this ester bond, resulting in an unstable HOCHRSSR intermediate, which would collapse to give an aldehyde and a persulfide (Scheme 1).



Scheme 1. General design of persulfide prodrugs, and their release mechanism.

To test our design, persulfide prodrug **1** (**P1, BW-HP-201**,Scheme 2) was synthesized by a one-step reaction between 1,2-dibenzyldisulfane and propionic acid using $KMnO_4$ as the

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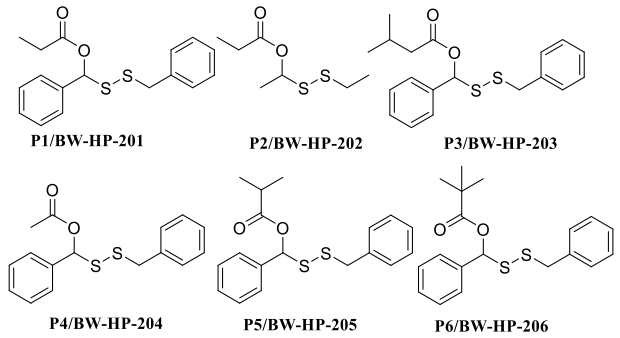
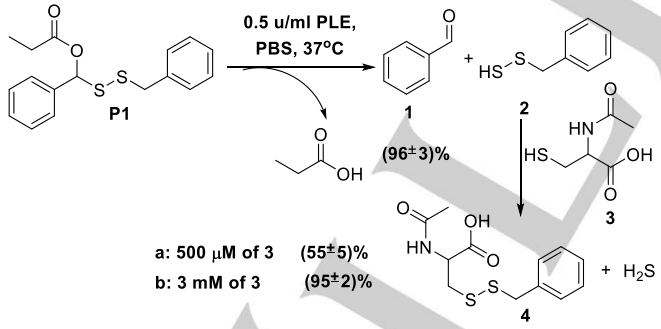
oxidant by following a similar literature procedure.[14] **P1** is a colorless oil without the characteristic smell of sulfide such as benzyl mercaptan and is stable at room temperature for 5 days and at -20 °C for 3 months. **P2~6** (BW-HP-202-206) were also synthesized using the same procedures.



Scheme 2. Structures of persulfide prodrugs.

We first studied the ability for **P1** to undergo the intended reaction by monitoring the formation of both benzyl persulfide and benzaldehyde by HPLC. It turned out that the benzyl persulfide (compound **2**) (Scheme 3a) was not sufficiently stable for detection on an HPLC time scale. This further affirms the rationale for designing these prodrugs, i.e., without the protection of the terminal sulfhydryl group, the persulfide species would not be stable enough for long-term storage. For detection purpose, we therefore, trapped benzyl persulfide using *N*-acetyl-L-cysteine (compound **3**) to give compound **4**, which is stable for HPLC detection. Specifically, 100 μM **P1** was treated with porcine liver esterase (0.5 u/ml, PLE) for 10 min at 37 °C in the phosphate buffered saline (PBS, pH= 7.4) containing 500 μM compound **3** (Scheme 3a). The results show that the amount of benzaldehyde **1** detected was the equivalent of 96% conversion. However, only 55% recovery of disulfide **4** were detected under such conditions, clearly indicating that the trapping reaction was not fast enough on the time scale of the study. By increasing the concentration of **3** to 3 mM, we were able to detect about 95% recovery of disulfide **4** (Scheme 3a). Such results clearly indicated that the release reaction occurred as designed.



Scheme 3a. Benzyl persulfide was release from **P1** and trapped by **3**

Besides using HPLC to detect the formation of **1** and **4**, we also used the standard methylene blue (MB) method to detect the H_2S release from **P1** (Figure 1) in the presence of **3**. The results showed no obvious H_2S generation in the absence of PLE, and only less than 5 μM H_2S (5%) were formed in the presence of 500 μM compound **3,** suggesting the possibility of a small percentage of the prodrug to undergo sulfur exchange. However, with the addition of 0.5 u/ml PLE at 37 °C in PBS **P1** generated about 23 μM, 33 μM and 45 μM H_2S in the presence of 0 μM, 500 μM and 3 mM compound **3,** respectively. Compared with H_2S release, the formation of benzaldehyde was not affected by compound **3** (Figure 1). More than 90% benzaldehyde was detected in 2 min in PLE-containing solution with or without compound **3**, and less than 5% benzaldehyde was detected in the solution with 500 μM compound **3** without the addition of PLE. Such results indicated that the prodrugs were most effectively activated by an esterase, which controlled the rate-determining step.

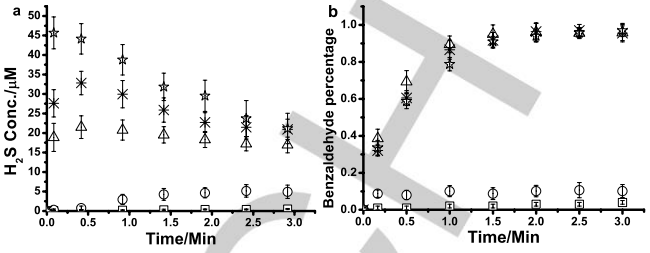


Figure 1. **P1** hydrolysis at 37 °C in PBS (2% DMSO). □:100 μM **P1**; ○:100 μM **P1** + 500 μM Compound **3;** △ :100 μM **P1** + PLE; ✳:100 μM **P1** + 500 μM Compound **3** + PLE; ☆ 100 μM **P1** + 3 mM Compound **3** + PLE. a) H_2S release detection by MB method; b) Benzaldehyde formation detection by HPLC.

For small molecule bioregulators (SMBs), release rate is a very important factor in determining the sustained effective concentration and thus biological effect. Donors at the same concentration could lead to different biological effects when the release rates are different. Our earlier work on H_2S prodrugs demonstrated that varying the acyl moiety allows for tuning the esterase-catalyzed hydrolysis rates.[15] Thus we reasoned that similarly designed persulfide prodrugs should also show different release profiles. Therefore, we next examined release kinetics. Specifically, we treated all these precursors (100 μM) with 0.5 u/ml PLE in PBS containing 500 μM compound **3** at 37 °C. Aldehyde formation was monitored by HPLC (Table 1), and H_2S release was tested by the MB method (Figure S5).

Table 1. Total percentage of aldehyde formation (A%) and 50% aldehyde formation time ($t_{1/2}$)

Compound	P1	P2	P3	P4	P5	P6
A%	96±3	Δ	91±4	97±3	97±2	89±6
$t_{1/2}$ (s)	25±5	12±6*	85±9	17±6	29±6	145±12

Δ: not detectable because of low boiling point of acetaldehyde;*: 50% **P2** remaining at the time of sampling. n= 3 p=0.95.

The results demonstrated that all the prodrugs were sensitive to PLE with hydrolysis half-lives ranging from 12 to 145 s. As a consequence of their respective release rates, the peak concentrations and sustained concentrations are also different. For example, H_2S level released from **P1** reached a peak concentration of 33 μM within 30 s and then decreased slowly. On the other hand, the slowest prodrug **P6** showed a gradual increase in H_2S concentration, reaching a maximum of 15 μM after 3 min (Figure S5).

The above studies clearly demonstrated the chemical feasibility of the prodrug activation and allow for studies of reaction kinetics. However, such studies used benzaldehyde and hydrogen sulfide as surrogates for product detection. Next, it was important to demonstrate persulfide formation without the added the thiol species, **3**. In the field of designing detection methods for protein *S*-sulfhydration, a key step is developing trapping reagents, which could efficiently trap the persulfide under physiological conditions.[7a] For this study, we used dinitrofluorobenzene (DNFB, Compound **5**), which was known to trap persulfides (Scheme 3b).[16] Specifically, we incubated 100 μM of the prodrugs with 1u/ml PLE at 37 °C for 10 s and then added 4 mM of DNFB to the mixture. The resulting solution was further incubated for another 30 min. Then the formation of disulfide compound **6** was analyzed by HPLC (Table 2). We were able to trap 70%-82% of the persulfide released from the prodrugs. There could be several reasons for the incomplete "conversion" to **6**. First, it is possible that the reaction rate between the released persulfide and **5** is not high enough for 100% conversion.

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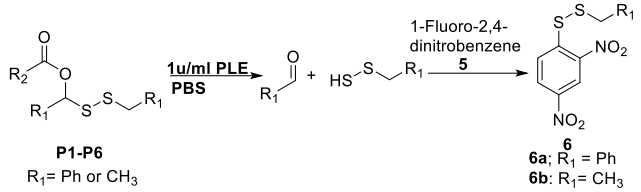
However, the fact that all prodrugs gave similar percentages of **6** (Table 2) suggests that this is not controlled by reaction kinetics at the trapping step because the various prodrugs gave substantially different peak concentrations, and trapping yields are not correlated with release rates. The second possible reason is the competition between trapping and disproportionation reaction of the persulfide released. We tend to think that the latter was the reason for the less than 100% conversion to **6**. To confirm this, we incubate 100 μM of the prodrug with 1u/ml PLE and 4 mM DNFB at r.t., and found that the trapping yields increased to 82%-93%. Clearly, at lower temperature (room temperature vs. 37 °C), the disproportion reaction was slower, allowing for the improved trapping efficiency. The results demonstrated that the prodrugs could efficiently afford persulfide for further studies.

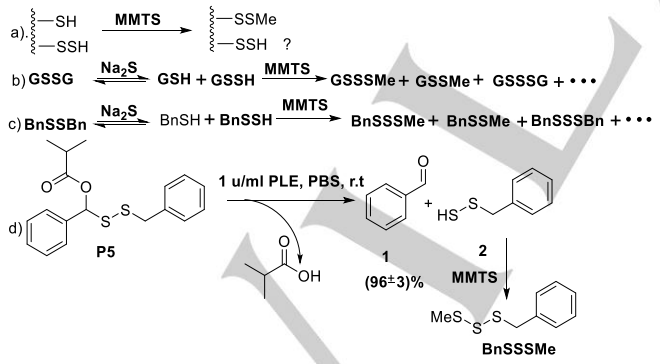


Scheme 3b. Persulfide released from precursors and trapped by DNFB

Table 2. Persulfide released from precursors and trapped by DNFB (A: trapping yield at 37 °C, B: trapping yield at r.t. n= 3, p=0.95)

Prodrugs	P1	P2	P3	P4	P5	P6
A (%)	79±5	70±4	71±5	80±4	82±5	78±4
B (%)	87±5	80±6	81±5	88±6	93±5	86±5

As a research tool, the system can provide relatively "pure" persulfide, which is very unique and important in the field of protein *S*-sulfhydration. In 2009, sulfide signaling through *S*-sulfhydration was first demonstrated.[6] A modified biotin switch technique, using *S*-methyl methanethiosulfonate (MMTS) as an alkylating reagent, was used to identify a large number of proteins that may undergo *S*-sulfhydration. The underlying chemical mechanism was based on the assumption that MMTS would selectivity react with thiol groups (RSH), but not persulfide group (RSSH) (Scheme 4a).



Scheme 4. Persulfides react with MMTS

However, later studies suggested that this assumption on the lack of reactivity between MMTS with RSSH was questionable (Scheme 4a).[10d] One reported work in studying the reactivity between MMTS with RSSH used a mixture of GSSG and sodium sulfide (Scheme 4b). In doing so, a small peak of GSSSMe was indeed detected. However, the origin of this "trisulfide" peak could be interpreted in more than one way due to complexity of the mixture formed in the process of preparing GSSH (Scheme 4b). To further probe the reactivity between MMTS with RSSH, we conducted the reaction between MMTS and the persulfide released from **P5**. Specifically, 100 μM of the prodrug was incubated with 1u/ml PLE and 4 mM MMTS at r.t. for 30 min (Scheme 4d). Then LC-MS was applied to analyze product formation. For comparison, we also incubated 4 mM of MMTS with 1 mM dibenzyl disulfide BnSSBn and 1 mM of Na_2S (Scheme 4c). The results are shown in Figure 2a and 2b. The reaction in 4c led to BnSSMe as the major product, and only a small amount of BnSSSMe was formed. Such results are quite similar to the product formation pattern in the reported reaction between MMTS with GSSG and Na_2S.[10d] However in the reaction of MMTS with benzyl persulfide released from **P5** (Scheme 4d, Figure 2b), BnSSSMe clearly formed as the dominant product, and other possible sulfide products were only observed in minute quantities. The results here clearly demonstrated that MMTS could react with persulfide. By taking advantage of the unique property of our persulfide prodrug system, we reconfirmed that MMTS can efficiently react with persulfide.

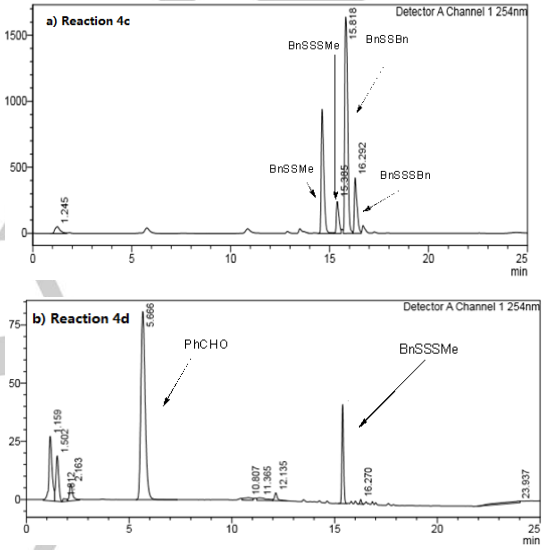


Figure 2. a) HPLC trace of BnSSBn reacting with Na_2S and MMTS. b) HPLC trace of esterase mediate **P5** hydrolysis and persulfide trapping by MMTS.

Besides using the prodrugs as chemical tools to study persulfide chemistry under near physiological conditions, we also examined their biological effects. We first assessed the cytotoxicity of these prodrugs using the H9c2 cell line. All prodrugs showed no obvious toxicity at up to 100 μM after 24 h of incubation (Figure S2 and S3). Various sulfur species have been shown to exhibit protective effects in heart myocardial infarction reperfusion (MI/R) injury studies.[1f, 3a, 17] As a test of the biological relevance of the prodrugs designed, we selected **P2**, which has a relative good water solubility and low toxicity of side product (acetaldehyde), as a representative for examination of its protective effect in a murine model of MI/R injury. The compounds were tested in a mouse heart ischemia reperfusion injury model. Mice were subjected to 45 min of ischemia induced by left coronary artery (LCA) occlusion followed by 24 h of reperfusion of **P2** (0, 12.5, 50, 100, 500 μg/kg) or vehicle, administered by intracardiac injection at the time of reperfusion. The LCA was re-occluded at 24h of reperfusion at the same position. Then Evens Blue dye was injected through right common carotid artery to delineate the area-at-risk (AAR). Due to the occlusion of LCA, AAR did not receive blood flow nor the Evens Blue dye, while the remainder of the heart was stained blue. Therefore, AAR were the non-blue regions in the mid-ventricular slice (Figure 3a). The infarct size (INF) was stained white by triphenyltetrazolium chloride solution. Thus, the white regions in the mid-ventricular slice represent INF (Figure 3a). Left ventricle (LV) is the sum of blue and non-blue regions. All of the animal groups displayed

similar area-at-risk per left ventricle (AAR/LV), suggesting that the surgical procedure produced the same degree of ischemic damage. Compared with vehicle-treated mice, those receiving the prodrug displayed significant reductions in infarct size per area-at-risk (INF/AAR) at the dosage of 50 or 100 μg/kg (Figure 3b). It is well-known that sulfide's protective effects are bell-shaped with regard to dosage.[3c, 18] It is important to note that indeed the prodrug's protective effect had an optimal concentration of 50-100 μg/kg. Substantially lower (12.5 μg/kg) or higher (500 μg/kg) doses showed no protective effects. Moreover, circulating cardiac troponin I levels, a marker for acute myocardial infarction, paralleled the results of infarction area measurements (Figure 3c). In addition to that, we also validated the sulfane sulfur production from the prodrug *in vivo*. As shown in Figure 3d, administration of **P2** led to a significant increase of sulfane sulfur in blood. Such results strongly suggest that persulfide prodrug indeed serves as a persulfide donor.

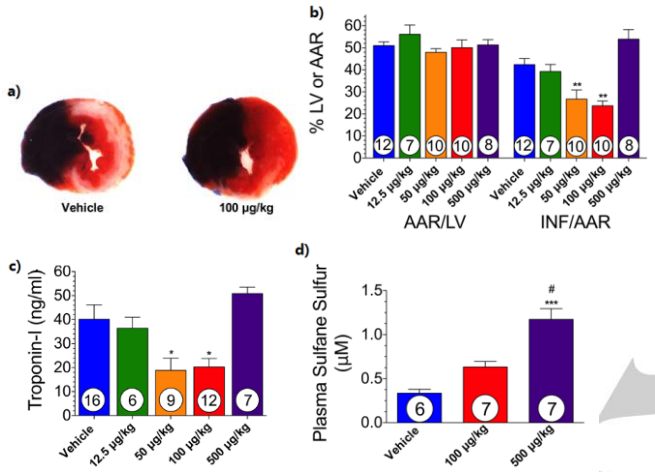


Figure 3. a) Representative photomicrographs of a mid-ventricular slice after MI/R and stained with Evan's blue and 2,3,5-triphenyltetrazolium chloride for both vehicle- and prodrug-(**P2**) treated hearts. b) AAR/LV and INF/AAR for **P2** treated or Vehicle treated mice. c) Circulating troponin I level for **P2** treated, or Vehicle treated mice. d) Plasma sulfane sulfur levels at 5 minutes post intracardiac injection. Values are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 the vehicle group, #P < 0.01 vs the 100 μg/kg of **P2** treated group.

In summary, we have developed a series of persulfide prodrugs with controllable release rates. These persulfide prodrugs release persulfide through an esterase-mediated hydrolysis mechanism. In the presence of the PLE, the prodrugs efficiently released persulfides under near physiological conditions. Using the prodrug, we reaffirmed the reactivity between persulfide and MMTS. The protective effects of **P2** in a murine model of MI/R injury have also been demonstrated. All the studies above demonstrate that this novel type of persulfide prodrugs not only can be used as research tools, but also are possible therapeutic agents.

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Keywords: Persulfide prodrugs • H_2S • Persulfide reactivity • bell shape therapeutic profile

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