Esterase-Activated Perthiocarbonate Persulfide Donors Provide Insights into Persulfide Persistence and Stability

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ABSTRACT

Persulfides (RSSH) are important reactive sulfur species (RSS) that are intertwined with the biological functions of hydrogen sulfide (H_2S). The direct study of persulfides is difficult, however, due to their both nucleophilic and electrophilic character, which leads to the generation of an equilibrium of different (RSS). To investigate the effects of persulfides directly, especially in biological systems, persulfide donors are needed to generate persulfides *in situ*. Here we report the synthesis of esterase-activated perthiocarbonate persulfide donors and investigate the effects of structural modifications on persulfide release. Although steric bulk at the ester did not significantly alter persulfide release kinetics, increased steric bulk of the thiol increased persulfide release rate. In addition, we found that the steric bulk and identity of the thiol significantly impacts persulfide persistence. Further mechanistic investigations into different competing reaction pathways from perthiocarbonates revealed that multiple RSS can be delivered (i.e. H_2S, COS, or RSSH) depending on persulfide donor structure and activator identity.

INTRODUCTION

Important sulfur-containing biomolecules are found across all kingdoms of life. Although biological thiols, such as glutathione and cysteine, are well known bio-active sulfur species, recent studies have revealed the crucial role of other reactive sulfur species (RSS) in biological systems. Specifically, endogenously-produced H_2S has been discovered to regulate essential cellular processes like cell signaling and function, leading to the classification of H_2S as a gasotransmitter alongside CO and NO.[1, 2] H_2S exerts many physiological effects, such as cytoprotection, anti-inflammation, and vasodilation, and for this reason H_2S is currently under investigation as a therapeutic for various disease pathologies.[3-5] H_2S cannot react directly with thiols, but many functions and actions of H_2S are proposed to be mediated by persulfides (RSSH) and related oxidized sulfur species.[6-8] H_2S can generate persulfides upon reaction with organic polysulfides (RSSR, RSSSR, etc.), oxidized thiols such as sulfenic acids (RSOH), or through O_2-mediated radical pathways.[9-13] Further, the oxidative posttranslational modification of cysteine by H_2S occurs through the reaction of H_2S and an oxidant with cysteine residues to form cysteine persulfide, providing cytoprotection against oxidative damage.[8, 12, 14, 15] This conversion of thiols to persulfides, known as *S*-persulfidation or *S*-sulfhydration, protects cysteine from irreversible oxidation.[16] For example, the loss of persulfidated cysteine residues is suspected to be involved in various neurodegenerative diseases including Alzheimer's and Parkinson's disease.[17-20] Persulfides can be produced by both enzymatic and nonenzymatic processes. For example, 3-mercaptopyruvate sulfurtransferase, cystathionine β-synthase, and cystathionine γ-lyase all can generate persulfides, and persulfides have also been found as important intermediates in enzyme function.[14, 21-23] Additionally, micromolar levels of persulfides have been found in cells, tissues, and plasma, exemplifying the importance of persulfides in biological systems.[6, 14, 24] Overall, there

is a close relationship between H_2S and persulfides, but the inherent reactivity of these RSS makes dissecting biological outcomes from H_2S, persulfides, or other RSS difficult.

Persulfides are more nucleophilic when compared to the parent thiols due to the alpha effect, but can be electrophilic at both sulfur atoms.[25-27] Generally, the pK_a of persulfides is 1–3 pK_a units lower than the corresponding thiol analogues, meaning that the majority of persulfides are deprotonated at physiological pH. For example, glutathione has a pK_a of 8.49, whereas glutathione persulfide has a pK_a of 5.45.[28] Due to this heightened reactivity, isolated persulfides are generally unstable and are readily converted to other RSS, such as H_2S, thiols, polysulfides, and elemental sulfur.[25, 29] This reactivity makes it difficult to probe or deliver persulfides directly. One solution to this challenge is to develop and use readily-modifiable small molecule persulfide donors to generate persulfide species *in situ* and to use these systems to further investigate persulfides. Although many H_2S-releasing donors have been reported previously and have increased the fundamental understanding of H_2S in biology, significantly fewer persulfide donors have been developed. The current library of persulfide donors include synthetic polysulfides as well as persulfide donor scaffolds that release persulfides when activated by hydrogen peroxide, enzymes, base, and light, but these scaffolds often lack simple modification methods that allow insights into how structural and electronic effects impact persulfide release rate.[30-38]

Perthiocarbonates, also referred to as sulfenyl thiocarbonates, react with thiols to release carbonyl sulfide (COS) and an alcohol payload (Figure 1a).[39, 40] We recently used this reactivity to develop thiol-activated COS-releasing molecules that simultaneously release a fluorescent dye as the payload. In addition to the electrophilic disulfide motif that can react with thiols, perthiocarbonates also contain a cleavable ester that has been proposed to release persulfides upon activation by a base.[40-42] The Toscano group recently reported a perthiocarbonate-based persulfide

donor using base-mediated hydrolysis of this ester motif (Figure 1b).[43] The electronic effects of ester substitution were investigated by using esters with *p*-substituted phenols, which revealed a mild substituent effect where electron withdrawing groups increased persulfide release rate and electron donating groups decreased persulfide release rate. Furthering our understanding of different pathways in which perthiocarbonates generate RSS, we report here the use of enzymatic esterase cleavage to provide controllable persulfide release (Figure 1c). Furthermore, our investigations into the structural factors that influence persulfide release rates reveal new insights into the stability and persistence of persulfides in the presence of biological nucleophiles.

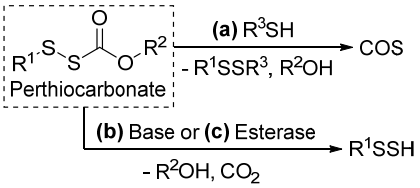

Figure 1. Perthiocarbonate reaction pathways when activated by (a) thiols, (b) base, or (c) esterase.

RESULTS AND DISCUSSION

Perthiocarbonates can be modified at either the ester (R^1) or thiol (R^2) position (Figure 2). To better understand the effect of steric bulk on persulfide release rate and persulfide stability, we prepared persulfide donors **1**–**9**. To simplify these systems, a methyl ester at R^1 or benzyl thiol at R^2 were used as controls when modifying the opposite position, allowing for direct comparisons across a series. For example, persulfide donors **1**–**3** vary by the ester substituent (R^1), and **1** and **4**–**7** vary by the thiol substituent (R^2). Persulfide donors **7**–**9** are based on *N*-acetyl-L-cysteine, *N*-acetyl-L-cysteine methyl ester, and penicillamine derivatives, respectively.

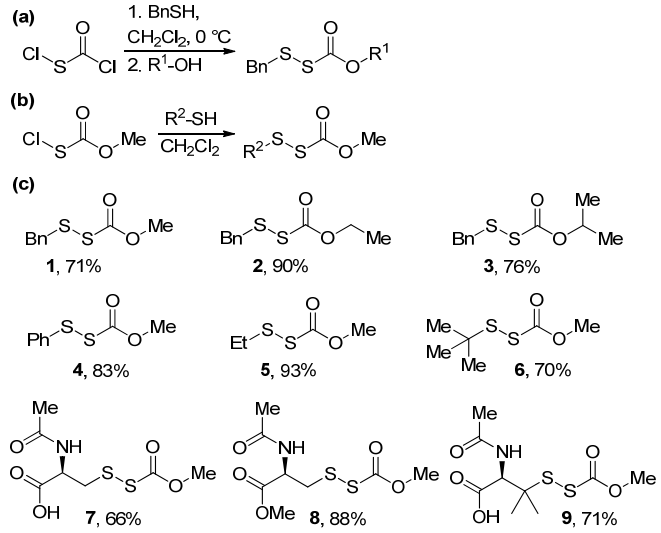

Figure 2. General conditions for preparing persulfide donors **1**–**9** starting from either (a) chlorocarbonylsulfenyl chloride or (b) methoxycarbonylsulfenyl chloride. (c) Persulfide donors synthesized.

Persulfide donors **1**–**3** were synthesized by treating benzyl mercaptan with chlorocarbonylsulfenyl chloride followed by addition of the corresponding alcohol (Figure 2a). Persulfide donors **4**–**9** were prepared in moderate to good yields (66–93%) by treating methoxycarbonylsulfenyl chloride with the desired thiol (Figure 2b). In general, these synthetic routes are simple, efficient, and require inexpensive starting materials to enable various substitutions on either side of the perthiocarbonate. All perthiocarbonate compounds were characterized by ^{1}H and ^{13}C{^{1}H} NMR spectroscopy and mass spectrometry.

Having prepared a small library of persulfide donors, we next measured persulfide release rates. Each persulfide donor (100 μM) was treated with porcine liver esterase (PLE, 1.0 U mL^{-1}) in PBS buffer (pH 7.4, 10 mM) containing 10% (v/v) MeCN at 25 °C. MeCN was added to increase the solubility of the more hydrophobic donors. We used a *p*-nitrophenyl acetate esterase activity assay to confirm that PLE maintained esterase activity with 10% MeCN co-solvent relative to

esterase activity in PBS buffer (Figure S1). Persulfide release was quantified by trapping the released persulfide with an excess of electrophilic 1-fluoro-2,4-dinitrobenzene (FDNB, 4 mM) to form a highly absorptive RSS-DNB adduct (Figure 3a).[35] Although persulfide trapping can be difficult due to the reactivity of the disulfide product formed, use of FDNB is a well-established method to accurately trap persulfides without trapping of similar nucleophiles such as thiols or alcohols.[44] The formation of the RSS-DNB adduct was monitored over 60 minutes by removing reaction aliquots (500 μL) and diluting with MeCN (500 μL) to both ensure solubility of the final trapped product and stop PLE activity prior to HPLC analysis. We prepared and isolated RSS-DNB adducts **10**–**16** independently to confirm product formation from retention times and to generate calibration curves for each RSS-DNB adduct to ensure accurate quantification (Figure 3, Figure S2). Although the slope and absorbances correlated with the calibration curves of the RSS-DNB adducts do not vary greatly by R^2-group identity, slight changes in the calibration curves are accounted for and ensures accurate quantification.

In addition to esterase activation, we also investigated whether persulfide release from these perthiocarbonate-based persulfide donors could be activated by base-mediated hydrolysis. When activated by PLE, persulfide donor **1** released 40.1 μM of persulfides after 60 minutes. To confirm that the persulfide release observed was due to esterase-mediated hydrolysis, the FDNB trapping assay was repeated with PLE that has been pre-incubated with the esterase inhibitor Parathion (10 μM).[45] No persulfide production was observed over the course of three hours, which confirmed that PLE was mediating the observed persulfide release (Figure S3). Having verified that esterase-mediated hydrolysis was occurring, we next investigated the non-enzymatic hydrolysis of these persulfide donors by the FDNB trapping assay under neutral and basic conditions. At pH 7.4, neither persulfide release nor persulfide donor degradation was observed

from persulfide donor **1** over three hours (Figure S3–S4). At pH 9.0, however, slow persulfide release was observed, although it only reached a maximum concentration of 16.1 μM after three hours (Figure S3). Although hydrolysis and resultant persulfide release increases with pH, the persulfides donors release persulfides significantly faster when activated by PLE. We also note that persulfide release has been reported previously from similar penicillamine-derived persulfide donors when activated by thiols through nucleophilic attack at the carbonyl carbon of the perthiocarbonate.[43] We further investigated this reactivity with persulfide donor **6** due to the similar bulkiness at the thiol position to penicillamine. We carried out the FDNB trapping assay in the presence of *N*-acetyl-L-cysteine (NAC, 500 μM) and did not observe any persulfide release over three hours (Figure S5). Based on these results, persulfide donors **1**–**9** likely release COS or other RSS rather than persulfides when activated by thiols. With these control experiments completed, the persulfide release rates from persulfide donors **1**–**9** when activated by PLE was measured.

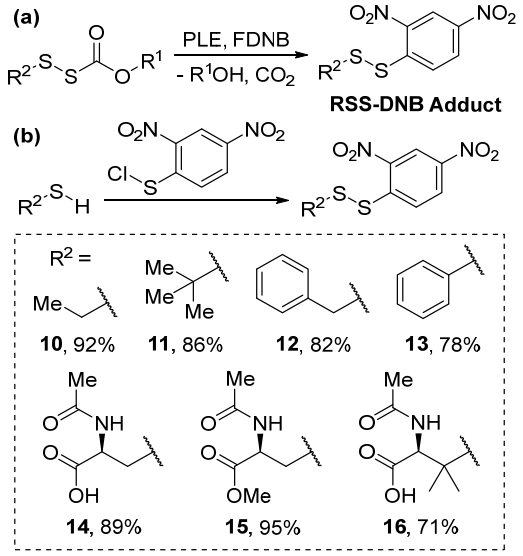

Figure 3. (a) Persulfide trapping assay with FDNB. (b) Synthetic route to prepare RSS-DNB adducts **10**–**16**.

To determine how structural modifications impact persulfide release from perthiocarbonate-based persulfide donors **1–9**, we fit the measured persulfide release curves from the FDNB trapping assay to a pseudo first-order equation to obtain k_{obs} (Figure 4, Table 1). According to the Jones model of the PLE active site, the alkoxy group of the perthiocarbonate does not enter the active site.[46] Based on this model, increasing steric bulk at the ester for persulfide donors **1**–**3** should not impact persulfide release rate since the R^2 position of these donors are all benzyl groups. As expected, steric bulk on the ester of **1**–**3** had a negligible impact on the rate of persulfide release. As seen in Table 1, both $t_{1/2}$ and k_{obs} of persulfide donors **1**–**3** are statistically different from one another and are not correlated with steric bulk. Interestingly, we found that the persulfides trapped by FDNB of **1**–**3** increased with increasing steric bulk and electron donation at the ester position, suggesting that R^1 identity has an effect on persulfide release other than persulfide release kinetics. Based on these data, the ester group identity has a minor effect on persulfide release rate from the scaffold when activated by esterase likely through altering esterase binding.

After investigating the effect of ester group identity on persulfide release rate, we next focused on thiol group identity. As the steric bulk on the thiol increases in persulfide donors **1** and **4**–**6**, the rate of persulfide release and persulfide persistence also increases. For example, persulfide donor **6** contains the bulky *tert*-butyl group at the R^2 position and has both a high rate constant (0.17 ± 0.1 min^{-1}) and the highest persulfides trapped by FDNB (70%). This high efficiency of persulfide trapping is likely due to the bulkiness of the *tert*-butyl group, which increases the

persulfide persistence and decreases potential side reactions of the persulfide. One outlier of this trend is persulfide donor **4**, which contains a phenyl group at the thiol position. This aromatic group may be in conjugation with the disulfide of the perthiocarbonate, unlike the other persulfide donors investigated, which could account for the unexpected increase in rate constant (0.23 ± 0.5 min^{-1}) and decrease in percent persulfide trapped (32%) for **4**. In total, increased steric bulk at the R^2 position appears to have a minor effect on persulfide release rate and persistence.

Table 1. Persulfide release from persulfide donors **1**–**6**.

Persulfide Donor	$t_{1/2}$ (min)	k_{obs} (min^{-1})	RSSH Trapping Efficiency (%)
1	5.6 ± 0.9	0.13 ± 0.2	40
2	1.9 ± 0.3	0.37 ± 0.5	57
3	3.3 ± 0.8	0.23 ± 0.6	61
4	3.2 ± 0.7	0.23 ± 0.5	32
5	6.9 ± 0.9	0.10 ± 0.2	36
6	4.2 ± 0.3	0.17 ± 0.1	70

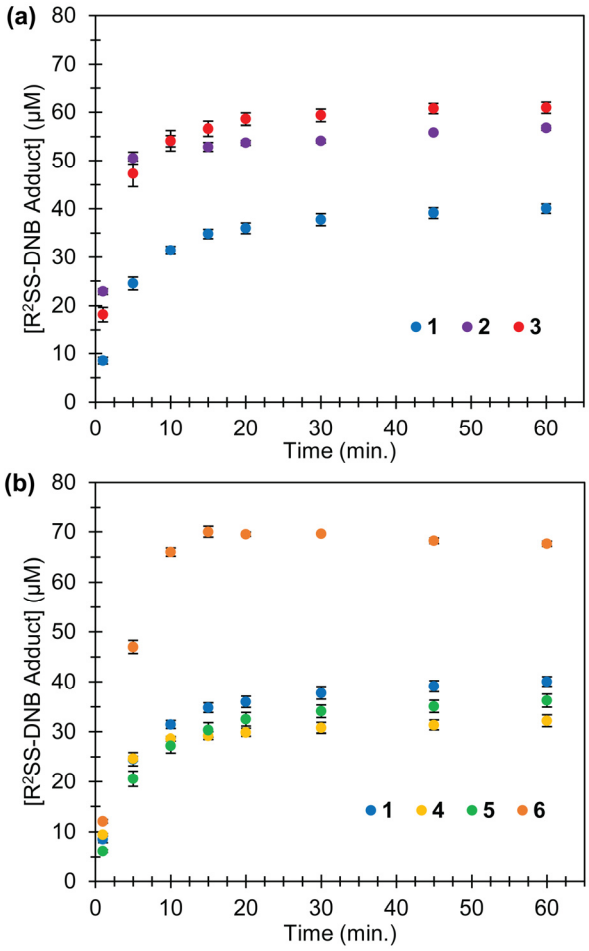

Figure 4. Persulfide release from (a) persulfide donors **1**–**3** and (b) persulfide donors **1** and **4**–**6**. Experiments were performed in triplicate with results expressed in mean ± standard deviation (n = 3).

Persulfide donors **7**–**9** were synthesized to investigate the viability of using our scaffold to release cysteine-derived persulfides, but FDNB trapping studies resulted in negligible trapped persulfides. To determine whether the ester motif of the perthiocarbonate was being cleaved, we monitored the reaction by ^{1}H NMR spectroscopy. Treatment of **7** (10 mM) with PLE (10 U mL^{-1}) failed to generate MeOH, which would be an expected product if the ester were cleaved (Figure S6). Treatment of **7** with *tert*-butoxide (10 mM) to activate persulfide release through hydrolysis,

however, did result in MeOH formation (Figure S7). Additionally, we used a *p*-nitrophenyl acetate assay to determine whether the cysteine-based persulfide donors were acting as PLE inhibitors by completing the assay in the presence of **7** (100 μM). No change in esterase activity was observed when **7** was added, meaning the lack of persulfide release from this donor was not due to alteration of PLE activity by the persulfide donor. Taken together, these results suggest that the esterase cannot cleave the esters in **7**–**9**, leading to low persulfide release. We hypothesized that the bulky and hydrophilic nature of the cysteine-based persulfide donors interferes with the ability of esterase to properly envelop the persulfide donors within the active site and thus prevents the esterase from cleaving the ester.

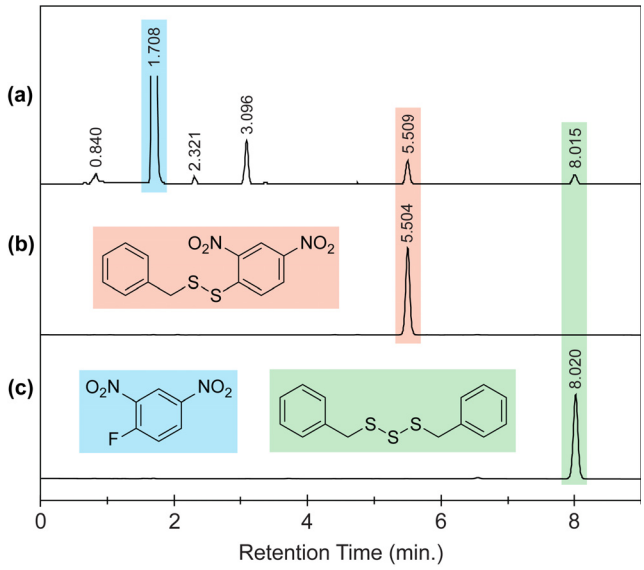

Figure 5. HPLC chromatograms showing (a) FDNB trapping assay results with persulfide donor **1** after 30 minutes, (b) 50 μM BnSS-DNB adduct, and (c) 50 μM benzyl trisulfide. In chromatogram (a) the peak at 3.096 minutes corresponds to residual persulfide donor **1**.

We next investigated whether other RSS were formed from the persulfide donors, which

could account for the non-quantitative persulfide trapping by FDNB. We further analyzed the FDNB trapping assay results and found polysulfide formation occurring alongside RSS-DNB adduct formation. Specifically, in the case of **1**, we observed benzyl trisulfide (BnSSSBn) formation during the FDNB trapping experiments (Figure 5). The trisulfide can be formed by the direct reaction of two equivalents of benzyl persulfide to release H_2S, which has been demonstrated previously using isolated BnSSH (Figure 6a).[29] To confirm H_2S release, we used the methylene blue (MB) H_2S assay. We first measured H_2S release in the presence of FDNB (4 mM) and PLE (1.0 U mL^{-1}), and only minimal H_2S release from **1** (100 μM) was observed. This outcome was expected because trisulfide formation and concomitant H_2S release should only be a minor side reaction in the presence of excess highly electrophilic FDNB trapping agent. In the absence of FDNB, we expected that more H_2S would be observed; however, we still observed minimal H_2S release from **1** (Figure S8). Because consumption of **1** is still observed in the absence of FDNB, we hypothesized the operable reaction pathway to generate the trisulfide does not release H_2S directly. One pathway that meets these requirements is persulfide attack at the α-sulfur atom of the perthiocarbonate to release COS and generate a trisulfide. Nucleophilic attack at this position of the perthiocarbonate has been observed previously in the thiol-mediated release of COS from perthiocarbonates.[39] To test whether **1** was releasing COS rather than H_2S, we added carbonic anhydrase (CA, 50 μg mL^{-1}) to the MB assay to convert any generated COS to H_2S. Under these reaction conditions, we observed significant H_2S release reaching a maximum of 8.5 μM after 90 minutes (Figure S8). Based on these results, persulfide donor **1** can also release COS to form the trisulfide, likely stemming from persulfide attack on the α-sulfur atom of the perthiocarbonate (Figure 6b).

To test whether steric bulk at the thiol can be used to influence reactivity of the

perthiocarbonates, we used **6** to investigate if the COS/trisulfide releasing pathway was also present even when the α-sulfur atom was sterically blocked. Similar to **1**, minimal H_2S release was observed in the presence of FDNB and PLE for persulfide donor **6**. In the absence of FDNB, however, significant H_2S release was observed in the absence of CA and reached a maximum of 9.3 μM after 45 minutes (Figure S8). Based on these results, we attribute H_2S release from **6** to preferential persulfide attack at the β-sulfur atom of the perthiocarbonate due to the bulky *tert*-butyl group at the thiol position (Figure 6c). To support the proposed reaction pathway, we also performed a methylene blue assay with a decreased concentration of PLE (0.1 U mL^{-1}) since PLE levels should directly affect H_2S release. Under these conditions, H_2S release was decreased as expected, with only 5.3 μM H_2S observed after 90 minutes (Figure S9). Furthermore, the reactivity between **6** (10 mM) and PLE (10 U mL^{-1}) was monitored by $^{13}C\{^1H\}$ NMR spectroscopy over 12 hours and clearly shows the appearance of the expected ^{t}BuSH byproduct (Figure S10), which also supporting the pathway proposed in Figure 6c. Overall, these experimental results show that nucleophilic attack on the perthiocarbonate can be controlled by altering thiol group identity, where **1** has preferential attack at the α-sulfur atom and **6** has preferential attack at the β-sulfur atom. Based on these results, the steric bulk next to the perthiocarbonate thiol as well as the identity of the nucleophile determines whether perthiocarbonates function as H_2S, COS, or persulfide donors.

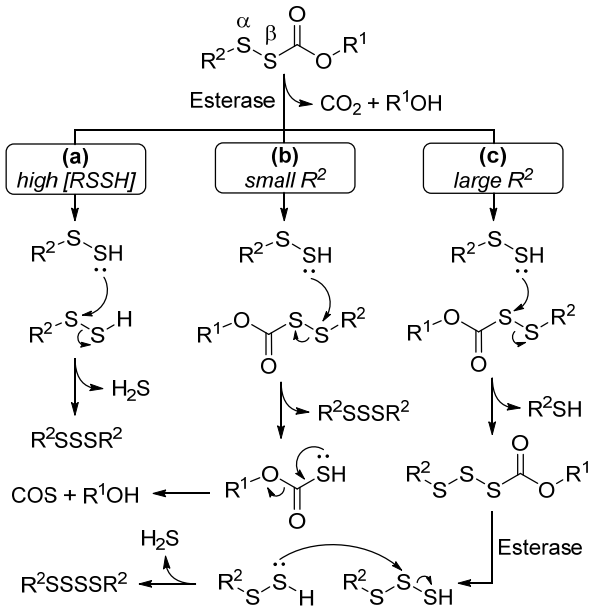

Figure 6. Alternate reaction pathways of persulfides produced by perthiocarbonate-based persulfide donors. All compounds are shown in the neutral state for simplicity. Pathways (a), (b), and (c) are likely most prevalent for high persulfides concentrations, small R^2 groups, and larger R^2 groups, respectively.

In general, the detection and delivery of persulfides is challenging because persulfides can function as both nucleophiles and electrophiles, which can lead to complex reaction pathways that generate RSS mixtures rather than one distinct product. Furthermore, the persulfide persistence, meaning how long a persulfide exists in solution before reacting with other species, remains an under-investigated and underappreciated challenge. Fundamental properties, such as the pK_a and steric bulk at the α-sulfur atom of the persulfide, should all contribute to persulfide persistence. Because persulfide donors **1**–**6** do not result in quantitative persulfide trapping, we suspected that persulfide persistence may be a contributing factor. To determine directly whether the steric bulk of R^2 impacts persulfide persistence, we next measured *in situ* persulfide concentration over time when persulfides were not trapped with FDNB directly after release. We modified the FDNB

trapping assay so that FDNB was not present during the persulfide-generating reaction, but rather was added after aliquots were removed from the reaction mixture. This approach effectively tests the concentration of persulfides within the reaction vial at specific time points. Under these conditions, more reactive (or less persistent) persulfides will react more quickly to form other RSS upon generation, resulting in lower measured persulfide levels and equating to a lower persulfide persistence. Persulfide donors (100 μM) were treated with PLE (1.0 U mL^{-1}) in 10% MeCN in PBS buffer (pH 7.4, 10 mM) at 25 °C. At each time point, reaction aliquots were removed, diluted in acetonitrile containing FDNB (8 mM), and filtered before analysis by HPLC. For compounds **1** and **5**, HPLC analysis did not show any formation of the RSS-DNB adducts, meaning that persulfides generated in solution were consumed prior to FDNB addition (Figure 7). In contrast, persulfides were trapped from **6** with the total trapped persulfides peaking at 23.7 μM for a trapping efficiency of 47% after 5 minutes. Further, for persulfide donor **6** there is an initial increase in RSSH trapped before a decrease observed after 5 minutes. This trend is likely due to polysulfide concentration being high enough to initiate further reaction with persulfides only after 5 minutes. This observed difference in trapped persulfide levels suggests that *t*BuSSH released from **6** is significantly more persistent than persulfides released from **1** and **5**. We attribute the greater persistence of *t*BuSSH to the increased steric bulk of the *tert*-butyl group, which blocks nucleophilic attack at the α-sulfur.

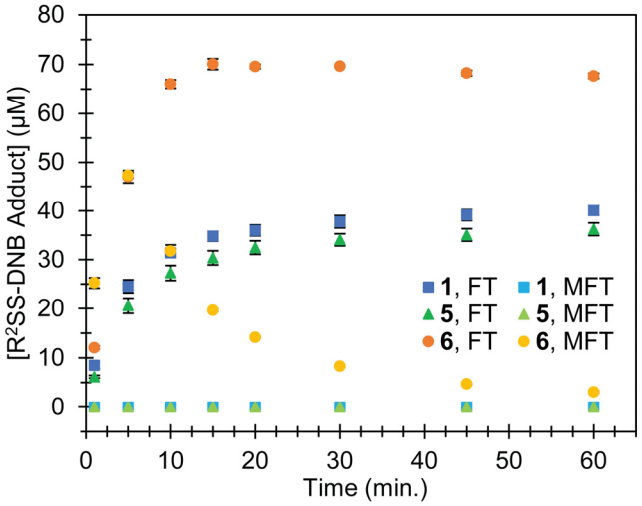

Figure 7. Investigation of persistence of persulfides released from donors **1** and 5–**6** by use of the FDNB trapping assay (FT) or the modified FDNB trapping assay (MFT). Experiments were performed in triplicate with results expressed in mean ± standard deviation (n = 3).

CONCLUSIONS

Perthiocarbonates can be cleaved by esterase to release persulfides, which were quantified by trapping with FDNB. We found that modification of the steric bulk of the ester (R^1) had little effect on persulfide release, whereas increased steric bulk at the thiol (R^2) generally led to an increase in persulfide release rate. Cysteine-based perthiocarbonates were also prepared but did not release appreciable persulfide due to incompatible interactions with the esterase. Investigations into alternate reaction pathways of the perthiocarbonate-based persulfide donors revealed that the thiol position (R^2 group) size directly influences what RSS are generated from perthiocarbonates. Perthiocarbonates release COS when a non-bulky group is at the thiol position, but release H_2S when a bulky group is at the thiol position. Furthermore, we used the perthiocarbonate-based donors to study persulfide persistence. These investigations showed that increased steric bulk at the R^2 position significantly increases persulfide persistence by blocking nucleophilic attack on

the α-sulfur. Based on these results, perthiocarbonates can function as either H_2S, COS, or persulfide donors depending on the triggering mechanism and biological environment, which would likely result in the delivery of multiple RSS in complex environments. A benefit of these esterase-activated persulfide donors over other persulfide donors, such as those activated by base-mediated hydrolysis, is the ability to halt persulfide production by destruction of the esterase by addition of organic solvent, addition of an inhibitor, or other means. Overall, these persulfide donors can be used to investigate persulfide fundamental properties or apply persulfides to specific biological isolates like enzymes to further understand the complex chemistry of persulfides.

METHODS

Methods and Materials:

Reagents were purchased from Sigma-Aldrich, Tokyo Chemical Industry (TCI), Fisher Scientific, Combi-Blocks, and VWR and used directly as received. Silica gel (SiliaFlash F60, Silicycle, 230−400 mesh) was used for column chromatography. Deuterated solvents were purchased from Cambridge Isotope Laboratories. ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were recorded on Bruker 500 MHz NMR instruments at the indicated frequencies. Chemical shifts are reported in ppm relative to residual protic solvent resonances. Mass spectrometric measurements were performed by the University of Illinois, Urbana Champaign MS facility or on a Xevo Waters ESI LC/MS instrument. Methylene blue absorbance was monitored by a Cary 60 UV-Vis spectrometer. FDNB trapping assay analysis was conducted on an Agilent 1260 Infinity II HPLC.

*General Procedure for Synthesis of Persulfide Donors **1** and **4**–**9**:*

Methoxycarbonylsulfenyl chloride (2.2 mmol, 1.1 equiv.) was added to 10 mL CH_2Cl_2 containing the thiol starting material (2.0 mmol, 1.0 equiv.). The reaction mixture was stirred at room temperature until the completion of the reaction was indicated by TLC (about 1 hour). The reaction was quenched by adding water (10 mL) and then aqueous solution was extracted with CH_2Cl_2 (3 × 10 mL). The organic layers were combined, washed with brine, dried over $MgSO_4$, and then evaporated under vacuum. The product was then isolated after purification by column chromatography.

*General Procedure for Synthesis of Persulfide Donors **2** and **3**:*

The thiol starting material (2.0 mmol, 1.0 equiv.) was added to 10 mL CH_2Cl_2 and then cooled to 0 ºC in an ice bath under nitrogen. Chlorocarbonylsulfenyl chloride was added slowly (2.2 mmol, 1.1 equiv.). The reaction mixture was stirred at 0 ºC until the completion of the reaction was indicated by TLC (about 1 hour). Next, 15 mL of the alcohol starting material was added and the reaction was allowed to warm to room temperature over an hour. The reaction was quenched by adding water (10 mL) and then the aqueous solution was extracted with CH_2Cl_2 (3 × 10 mL). The organic layers were combined, washed with brine, dried over $MgSO_4$, and then evaporated under vacuum. The product was then isolated after purification by column chromatography.

Persulfide Donor **1** was prepared by reacting methoxycarbonylsulfenyl chloride (403 μL, 4.43 mmol) with benzyl mercaptan (471 μL, 4.01 mmol) using the general synthetic procedure described above. Compound **1** was isolated as clear oil by column chromatography using ethyl acetate/hexanes (1/20, v/v) as the eluent (611 mg, 71.0% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 7.34 (m, 5H), 4.08 (s, 2H), 3.80 (s, 3H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO-d_6) δ (ppm):

168.9, 136.6, 129.9, 128.9, 128.1, 56.3, 42.6. HRMS m/z $[M]^+$ calcd. for $[C_9H_{10}O_2S_2]^+$ 214.0122; found 214.0114.

Persulfide donor **2** was prepared by reacting chlorocarbonylsulfenyl chloride (136 μL, 1.49 mmol) with benzyl mercaptan (189 μL, 1.61 mmol) using the general synthetic procedure described above. Compound **2** was isolated as clear oil by column chromatography using dichloromethane/hexanes (1/4, v/v) as the eluent (331 mg, 90.2% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 7.33 (m, 5H), 4.26 (m, 2H), 4.07 (s, 2H), 1.22 (t, J = 7.09 Hz, 3H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO-d_6) δ (ppm): 168.1, 136.6, 129.9, 128.9, 128.1, 65.8, 42.6, 14.5. HRMS m/z $[M + Na]^+$ calcd. for $[C_{10}H_{12}O_2S_2Na]^+$ 251.0171; found 251.0169.

Persulfide donor **3** was prepared by reacting chlorocarbonylsulfenyl chloride (136 μL, 1.49 1.49 mmol) with benzyl mercaptan (189 μL, 1.61 mmol) using the general synthetic procedure described above. Compound **3** was isolated as clear oil by column chromatography using dichloromethane/hexanes (1/4, v/v) as the eluent (297 mg, 76.2% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 7.32 (m, 5H), 5.01 (m, 1H), 4.06 (s, 2H), 1.24 (d, J = 6.26 Hz, 6H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO-d_6) δ (ppm): 167.5, 136.6, 129.9, 128.9, 128.0, 74.5, 42.5, 22.0. HRMS m/z $[M + Na]^+$ calcd. for $[C_{11}H_{14}O_2S_2Na]^+$ 265.0327; found 265.0329.

Persulfide donor **4** was prepared by reacting methoxycarbonylsulfenyl chloride (182 μL, 2.00 mmol) with phenyl mercaptan (185 μL, 1.82 mmol) using the general synthetic procedure described above. Compound **4** was isolated as a clear oil by column chromatography using ethyl acetate/hexanes (1/20, v/v) as the eluent (302 mg, 82.9% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 7.57 (d, J = 8.04 Hz, 2H), 7.43 (t, J = 7.45 Hz, 2H), 7.38 (m, 1H), 3.91 (s, 3H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO-d_6) δ (ppm): 168.5, 135.2, 130.0, 129.8, 129.1, 56.9. HRMS m/z $[M]^+$ calcd. for $[C_8H_8O_2S_2]^+$ 199.9966; found 199.9964.

Persulfide donor **5** was prepared by reacting methoxycarbonylsulfenyl chloride (239 μL, 2.00 mmol) with ethyl mercaptan (221 μL, 2.20 mmol) using the general synthetic procedure described above. Compound **5** was isolated as a clear oil by column chromatography using ethyl acetate/hexanes (1/20, v/v) as the eluent (284 mg, 93.4% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 3.87 (s, 3H), 2.82 (m, 2H), 1.25 (t, J = 7.27 Hz, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 169.5, 56.3, 32.2, 14.3. HRMS m/z $[M]^+$ calcd. for $[C_4H_8O_2S_2]^+$ 151.9966; found 151.9971.

Persulfide donor **6** was prepared by reacting methoxycarbonylsulfenyl chloride (1.99 mL mg, 4.02 mol) with *tert*-butyl mercaptan (1.44 mL, 4.43 mol) using the general synthetic procedure described above. Compound **6** was isolated as clear oil by column chromatography using ethyl acetate/hexanes (1/20, v/v) as the eluent (2.09 g, 69.6% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 3.86 (s, 3H), 1.30 (s, 9H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 169.5, 56.7, 49.1, 29.5. HRMS m/z $[M]^+$ calcd. for $[C_6H_{12}O_2S_2]^+$ 180.0279; found 180.0277.

Persulfide donor **7** was prepared by reacting methoxycarbonylsulfenyl chloride (199.0 μL, 2.20 mmol) with *N*-acetyl-L-cysteine (326 mg, 2.00 mmol) using the general synthetic procedure described above. Compound **7** was isolated as white solid by column chromatography using 10% methanol, 40% DCM, and 50% hexane as the eluent (369 mg, 66.2% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 12.98 (s, 1H), 8.31 (d, J = 8.01 Hz, 1H), 4.49 (m, 1H), 3.89 (s, 3H), 3.20 (dd; J = 4.88, 13.80 Hz; 1H), 3.08 (dd; J = 8.71, 13.81 Hz; 1H), 1.87 (s, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 172.1, 169.8, 169.1, 56.5, 51.6, 22.8. HRMS m/z $[M + H]^+$ calcd. for $[C_7H_{12}NO_5S_2]^+$ 254.0151; found 254.0153.

Persulfide donor **8** was prepared by reacting methoxycarbonylsulfenyl chloride (199 μL, 2.20 mmol) with *N*-acetyl-L-cysteine methyl ester (354 mg, 2.00 mmol) using the general synthetic

procedure described above. Compound **8** was isolated as white solid by column chromatography using 5% methanol, 40% dichloromethane, and 55% hexane as the eluent (464 mg, 87.7% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.47 (d, J = 7.78 Hz, 1H), 4.53 (m, 1H), 3.87 (s, 3H), 3.65 (s, 3H), 3.20 (dd; J = 5.16, 13.93 Hz; 1H), 3.10 (dd; J = 8.59, 13.94 Hz; 1H), 1.87 (s, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 171.2, 169.9, 169.0, 56.5, 52.7, 51.6, 22.8. HRMS m/z $[M + H]^+$ calcd. for $[C_8H_{14}NO_5S_2]^+$ 268.0308; found 268.0312.

Persulfide donor **9** was prepared by reacting methoxycarbonylsulfenyl chloride (131 μL, 1.10 mmol) with *N*-acetyl-D-penicillamine (191 mg, 1.00 mmol) using the general synthetic procedure described above. Compound **9** was isolated as white solid by column chromatography using first column had 1:4:5 methanol:dichloromethane:hexane as the solvent while the second column had 5% ethyl acetate in hexane as the eluent (76.3 mg, 27.9% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 12.97 (s, 1H), 8.23 (d, J = 9.22 Hz, 1H), 4.51 (d, J = 9.30 Hz, 1H), 3.85 (s, 3H), 1.91 (s, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 171.4, 169.9, 169.2, 58.3, 56.4, 53.3, 25.3, 23.7, 22.8. HRMS m/z $[M + H]^+$ calcd. for $[C_9H_{16}NO_5S_2]^+$ 282.0464; found 282.0464.

*General procedure for synthesis of RSSH-DNB adducts **10–16**:*

2,4-Dinitrophenylsuelfenyl chloride (1.5 mmol, 1.0 equiv.) was added to 10 mL CH_2Cl_2 and stirred momentarily. Next, the thiol starting material (1.5 mmol, 1.0 equiv.) was added dropwise, then this solution was stirred for 1 hour until the completion of the reaction was indicated by TLC. The reaction was quenched by adding water (10 mL) and then the aqueous solution was extracted with CH_2Cl_2 (3 × 10 mL). The organic layers were combined, washed with brine, dried over $MgSO_4$, and then evaporated under vacuum. The final product was then isolated after purification by column chromatography as a yellow solid.

Adduct **10** was prepared by reacting 2,4-dinitrophenylsulfenyl chloride (352 mg, 1.50 mmol) with ethyl mercaptan (86.3 μL, 1.50 mmol) using the general synthetic procedure described above. Compound **10** was isolated as yellow solid by column chromatography using ethyl acetate/hexanes (1/9, v/v) as the eluent (371 mg, 92.2% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.91 (d, J = 2.50, 1H), 8.62 (dd; J = 2.52, 8.97 Hz; 1H), 8.51 (d, J = 9.00 Hz, 1H), 2.92 (m, 2H), 1.27 (t, J = 7.39 Hz, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 145.8, 145.4, 145.2, 129.3, 128.6, 122.0, 32.3, 14.5. LRMS m/z $[M + H]^+$ calcd. for $[C_8H_9N_2O_4S_2]^+$ 260.9998; found 261.0070.

Adduct **11** was prepared by reacting 2,4-dinitrophenylsulfenyl chloride (352 mg, 1.50 mmol) with *tert*-butyl mercaptan (169 μL, 1.50 mmol) using the general synthetic procedure described above. Compound **11** was isolated as a light-yellow solid by column chromatography using ethyl acetate/hexanes (1/9, v/v) as the eluent (370 mg, 85.5% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.90 (s, 1H), 8.58 (m, 2H), 1.35 (s, 9H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 145.8, 145.3, 139.8, 128.2, 121.8, 51.5, 29.9. LRMS m/z $[M + H]^+$ calcd. for $[C_{10}H_{13}N_2O_4S_2]^+$ 289.0311; found 289.0367.

Adduct **12** was prepared by reacting 2,4-dinitrophenylsulfenyl chloride (379 mg, 1.61 mmol) with benzyl mercaptan (189 μL, 1.61 mmol) using the general synthetic procedure described above. Compound **12** was isolated as yellow solid by column chromatography using ethyl acetate/hexanes (1/9, v/v) as the eluent (416 mg, 81.7% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.83 (d, J = 2.44 Hz, 1H), 8.45 (dd; J = 2.47, 8.97 Hz; 1H), 8.26 (d, J = 8.98 Hz, 1H), 7.31 (d, J = 6.58 Hz, 2H), 7.24 (m, 3H), 4.16 (s, 2H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 145.6, 145.2, 144.8, 136.3, 130.0, 129.5, 129.0, 128.1, 121.7, 42.2. LRMS m/z $[M + H]^+$ calcd. for $[C_{13}H_{11}N_2O_4S_2]^+$ 323.0155; found 323.0264.

Adduct **13** was prepared by reacting 2,4-dinitrophenylsulfenyl chloride (352 mg, 1.50 mmol) with phenyl mercaptan (153 μL, 1.50 mmol) using the general synthetic procedure described above. Compound **13** was isolated as yellow solid by column chromatography using ethyl acetate/hexanes (1/9, v/v) as the eluent (362 mg, 78.4% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.93 (d, J = 2.49 Hz, 1H), 8.62 (dd; J = 2.47, 8.99 Hz; 1H), 8.46 (d, J = 8.98 Hz, 1H), 7.60 (d, J = 8.02 Hz, 2H), 7.35 (m, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 146.2, 145.2, 143.8, 133.9, 130.2, 129.0, 122.1. LRMS m/z $[M + H]^+$ calcd. for $[C_{12}H_9N_2O_4S_2]^+$ 308.9998; found 309.0074.

Adduct **14** was prepared by reacting 2,4-dinitrophenylsulfenyl chloride (352 mg, 1.50 mmol) with *N*-acetyl-L-cysteine (245 mg, 1.50 mmol) using the general synthetic procedure described above. Compound **14** was isolated as a pale-yellow solid by column chromatography using a gradient of 10-60% ethyl acetate/hexanes as the eluent (337 mg, 88.7% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 13.01 (s, 1H), 8.91 (d, J = 2.43 Hz, 1H), 8.61 (d; J = 2.46, 9.00 Hz; 1H), 8.51 (d, J = 8.98 Hz, 1H), 8.46 (d, J = 8.02 Hz, 1H), 4.47 (m, 1H), 3.27 (dd; J = 4.54, 13.76 Hz; 1H), 3.16 (dd; J = 9.03, 13.71 Hz; 1H), 1.87 (s, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 172.0, 170.0, 145.9, 145.3, 144.9, 129.4, 128.6, 129.4, 128.6, 121.9, 51.8, 22.9. HRMS m/z $[M + H]^+$ calcd. for $[C_{11}H_{12}N_3O_7S_2]^+$ 362.0111; found 362.0107.

Adduct **15** was prepared by reacting 2,4-dinitrophenylsulfenyl chloride (352 mg, 1.50 mmol) with *N*-acetyl-L-cysteine methyl ester (266 mg, 1.50 mmol) using the general synthetic procedure described above. Compound **15** was isolated as a pale-yellow solid by column chromatography using a gradient of 10-60% ethyl acetate/hexanes as the eluent (378 mg, 95.4% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.91 (d, J = 2.43 Hz, 1H), 8.61 (dd; J = 2.48, 8.98 Hz; 1H), 8.56 (d, J = 7.87 Hz, 1H), 8.49 (d, J = 8.99 Hz, 1H), 4.55 (m, 1H), 3.62 (s, 3H), 3.25 (dd;

J = 4.81, 13.89 Hz; 1H), 3.15 (dd; *J* = 8.93, 13.86 Hz; 1H), 1.88 (s, 3H). $^{13}C\{^{1}H\}$ NMR (125 MHz, DMSO-d_6) δ (ppm): 171.0, 170.0, 145.9, 145.3, 144.7, 129.4, 128.7, 122.0, 52.7, 51.7, 22.8. HRMS m/z $[M + H]^+$ calcd. for $[C_{12}H_{14}N_3O_7S_2]^+$ 376.0268; found 376.0271.

Adduct **16** was prepared by reacting 2,4-dinitrophenylsulfenyl chloride (176 mg, 0.75 mmol) with *N*-acetyl-D-penicillamine (112 mg, 0.75 mmol) using the general synthetic procedure described above. Compound **16** was isolated as a yellow solid by column chromatography using a gradient of 10-60% ethyl acetate/hexanes as the eluent (206 mg, 70.5% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.90 (s, 1H), 8.56 (s, 2H), 8.37 (d, *J* = 9.25 Hz, 1H), 4.58 (d, *J* = 9.16 Hz, 1H), 1.92 (s, 3H), 1.37 (s, 6H). $^{13}C\{^{1}H\}$ NMR (125 MHz, DMSO-d_6) δ (ppm): 171.3, 170.0, 145.8, 145.7, 145.4, 130.1, 128.2, 121.8, 58.7, 55.4, 25.6, 24.0, 22.8. HRMS m/z $[M + H]^+$ calcd. for $[C_{13}H_{16}N_3O_7S_2]^+$ 390.0424; found 390.0421.

Esterase activity in solvent system:

A freshly prepared *p*-nitrophenyl acetate stock solution (15 μL, 15 mM in acetonitrile) was added to 3 mL of PBS buffer (pH 7.4, 10 mM) in a quartz cuvette. The esterase stock solution (6 μL, 500 U mL^{-1} in PBS buffer) was added to begin the experiment and the absorbance was measured at 405 nm on a UV-Vis spectrophotometer over 15 minutes. This procedure was repeated using 10% (v/v) acetonitrile in PBS buffer and 20% (v/v) acetonitrile in PBS buffer as the solvents to determine esterase activity at varying levels of organic solvents as seen in **Fig. S1**.

Esterase activity with Parathion:

A freshly prepared esterase stock solution (6 μL, 500 U mL^{-1} in PBS buffer) and Parathion stock solution (30.0 μL, 1.0 mM in acetonitrile) was added to 3 mL of PBS buffer (pH 7.4, 10

mM) in a quartz cuvette and incubated for 30 minutes. The *p*-nitrophenyl acetate stock solution (15 μL, 15 mM in acetonitrile) was added to begin the experiment, and the absorbance was measured at 405 nm on a UV-Vis spectrophotometer over 15 minutes as seen in Fig. S1.

General persulfide trapping FDNB assay procedure:

A freshly prepared 1-fluoro-2,4-dinitrobenzene stock solution (20 μL, 4.0 M in acetonitrile) was added to 20 mL of 10% (v/v) acetonitrile in 10 mM PBS buffer (pH 7.4) containing PLE (1.0 U mL^{-1}) in a vial. This solution was then stirred for 30 minutes at 25 °C. A persulfide donor stock solution (20 μL, 100 μM in acetonitrile) was added to begin the experiment and 500 μL aliquots were taken at 1, 5, 10, 15, 20, 30, 45, and 60 minutes and then added to 2 mL GC vials containing 500 μL of acetonitrile. After filtering, these solutions were run on a reverse-phase HPLC. The HPLC method is as follow: Mobile phase A (5% methanol in water) and mobile phase B (acetonitrile); Flow rate of 1 mL/min; Injection volume of 5 μL; Detection wavelength of 250 nm; Column used was InfinityLab Poroshell 120 EC-C18, 4.6 x 100 mm, 2.7 μm, analytical LC column; Gradient method with 40% A from 0 to 5 min, 40% to 10% A from 5 to 10 minutes, 10% to 0% A from 10 to 15 minutes, 0% A from 15 to 17 minutes, 0% to 40% A from 17 to 20 minutes, and then 40% A from 20 to 22 minutes.

*Calibration curves with RSS-DNB adducts **10**–**16**:*

Calibration curves were created by first preparing a 10 mM stock solution of the RSS-DNB adduct in acetonitrile and then serial diluting to obtain 5, 2.5, 1.25, 0.625, and 0.3125 mM solutions. Next 10 μL volume of these solutions were added to vials containing 990 μL of 50% acetonitrile in PBS buffer (pH 7.4, 10 mM) to obtain final RSS-DNB adduct concentrations of

100, 75, 50, 25, 12.5, 6.25, and 3.125 μM. These solutions were filtered and then run on a reverse-phase HPLC using the same method as described above for the FDNB trapping assay. The calibration curves of **10**–**16** are plotted in Fig. S2.

^{1}H NMR spectroscopy experiments monitoring ester cleavage by esterase or tert-butoxide:

To monitor ester cleavage by esterase, a freshly prepared stock solution of FDNB (8.33 μL, 300 mM in acetonitrile-d_3) was added to 442 μL of D_2O along with 16.67 μL of acetonitrile-d_3 and a PLE stock solution (8 μL, 625 U mL^{-1} in D_2O). A stock solution of persulfide donor **7** (25 μL, 200 mM in acetonitrile-d_3) was added, the tube was inverted to mix, and then ^{1}H NMR spectra were taken at t = 5 m, 30 m, 1 h, 2 h, 4 h, 6 h, 10 h, and 18 h.

To monitor ester cleavage by *tert*-butoxide, a freshly prepared stock solution of FDNB (50 μL, 200 mM in THF-d_8) was added to 250 μL of 20 mM KO*t*Bu in THF-d_8 along with 50 μL D_2O and 100 μL THF-d_8. A stock solution of persulfide donor 7 (50 μL, 100 mM in THF-d_8) was added, the tube was inverted to mix, and then ^{1}H NMR spectra were taken at t = 5 m, 30 m, 1 h, 2 h, 4 h, 6 h, 10 h, and 18 h. ^{1}H NMR spectra are shown in Fig. S6 and Fig. S7.

*Methylene blue assay experiments with persulfide donors **1** and **6**:*

Four experiments were completed: (1) MB assay of persulfide donor **1** with PLE, (2) MB assay of persulfide donor **1** with PLE and CA, (3) MB assay of persulfide donor **6** with PLE, and (4) MB assay of persulfide donor **6** with PLE and CA. MB assays were also completed in the presence of FDNB, but this data is omitted due to negligible H_2S being released, as expected. A stock solution of the persulfide donor (20 μL, 100 mM) was added to 20 mL of degassed 10% acetonitrile in PBS buffer (pH 7.4, 10 mM) containing PLE (1.0 U mL^{-1}) as well as CA (50 μg

mL^{-1}) for experiments 2 and 4. At 1, 5, 10, 15, 20, 30, 45, 60, and 90 minutes 500 μL aliquots were taken and placed in cuvettes containing 500 μL of 2:2:1 iron (III) chloride/*N,N*-dimethyl-*p*-phenylenediamine/zinc acetate and then filtered. After 1 hour the absorbance at 670 nm was measured using a UV-Vis spectrophotometer and was converted to H_2S concentration by using an H_2S calibration curve. Quantified H_2S release from persulfide donors **1** and **6** with and without CA present are plotted in Fig. S8.

*^{13}C NMR spectroscopy monitoring the reactivity of persulfide donor **6** and PLE*:

A freshly prepared stock solution of **6** (25 μL, 200 mM in CD_3CN) was added to solution of 442 μL of D_2O and 25 μL of CD_3CN. A stock solution of PLE (8 μL, 625 U mL^{-1} in PBS buffer) was added, the tube was inverted to mix, and then ^1H NMR spectra were taken at t = 1 h, 3 h, 6 h, 12 h. The ^{13}C NMR spectra are shown in Fig. S10.

Supporting Information Available: Persulfide release measurements, donor stability experiments, methylene blue data, NMR spectra.This material is available free of charge via the Internet.

CONFLICTS OF INTEREST

There are no conflicts to declare.

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