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Thionoesters: A Native Chemical Ligation-Inspired Approach to Cysteine-Triggered H_2S Donors

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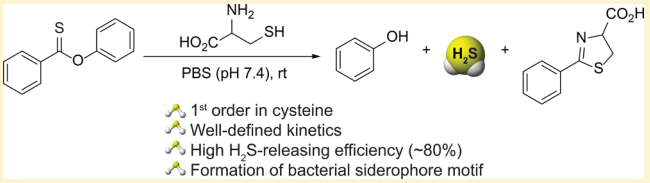
Supporting Information

ABSTRACT: Native chemical ligation (NCL) is a simple, widely used, and powerful synthetic tool to ligate *N*-terminal cysteine residues and *C*-terminal α-thioesters via a thermodynamically stable amide bond. Building on this well-established reactivity, as well as advancing our interests in the chemical biology of reactive sulfur species including hydrogen sulfide (H_2S), we hypothesized that thionoesters, which are constitutional isomers of thioesters, would undergo a similar NCL reaction in the presence of cysteine to release H_2S under physiological conditions. Herein, we report mechanistic and kinetic investigations into cysteine-mediated H_2S release from thionoesters. We found that this reaction proceeds with high H_2S-releasing efficiency (~80%) and with a rate constant (9.1 ± 0.3 M^{-1} s^{-1}) comparable to that for copper-catalyzed azide−alkyne cycloadditions (CuAAC). Additionally, we found that the final product of the reaction of cysteine with thionoesters results in the formation of a stable dihydrothiazole, which is an iron-binding motif commonly found in siderophores produced by bacteria during periods of nutrient deprivation.



INTRODUCTION

Hydrogen sulfide (H_2S) is now recognized as an important biological signaling molecule[1] that is produced endogenously, cell membrane permeable, and reactive toward cellular and/or molecular targets.[2] The endogenous production of H_2S stems primarily from catabolism of cysteine and homocysteine by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST).[3] Recently, increasing interest has focused on harnessing H_2S as a potential therapeutic agent[4] based on its role in vasodilation,[5] neurotransmission,[6] and angiogenesis.[7] Although the majority of prior reports have used sodium hydrosulfide (NaSH) or sodium sulfide (Na_2S) as sources of H_2S, the addition of these salts to a buffer leads to an almost instantaneous increase in H_2S concentration, which is in stark contrast to the slow, gradual endogenous production of H_2S.[8] In efforts to provide more physiologically relevant rates of H_2S release, researchers have developed different types of H_2S-releasing molecules (Figure 1a).[9−11] For example, Lawesson's Reagent and related derivatives[12,13] have been used as hydrolysis-activated H_2S donors that function at physiological pH, and dithiolethiones, such as ADT−OH, have been conjugated to nonsteroidal anti-inflammatory drugs (NSAIDs) to access H_2S prodrug conjugates.[14] More recently, "triggered-release" scaffolds have also been reported, including those activated by light[15] and enzymatic activation.[16] In addition, recent work has demonstrated that carbonyl sulfide (COS)-releasing scaffolds can also function as H_2S donors via the rapid conversion of the released COS to H_2S by carbonic anhydrase.[17]

Drawing parallels to the enzymatic conversion of cysteine or homocysteine to H_2S, a number of H_2S donor motifs have been developed that are activated by thiols, such as cysteine and reduced glutathione (GSH) (Figure 1b). Polysulfides, such

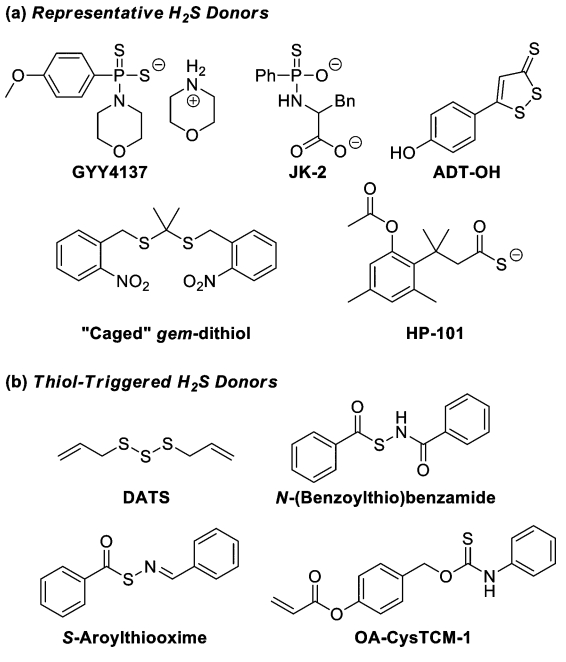


Figure 1. (a) Representative examples of common synthetic, small-molecule H_2S donors; (b) selected small-molecule, thiol-triggered H_2S donors.

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as the commonly used diallyl trisulfide (DATS)[18] or more recently reported synthetic trisulfides[19] and tetrasulfides,[20] release H_2S in the presence of thiols via an intermediate persulfide. Building in complexity, Xian and co-workers have reported thiol-triggered H_2S donors based on protected *N*-mercaptan[21] or persulfide[22] platforms. Similarly, Matson and co-workers reported *S*-aroylthiooxime compounds,[23] which generate a thiol-reactive intermediate thiooxime. Thiol-mediated H_2S release from arylthioamides[24] and aryl isothiocyanates[25] has also been reported, although the mechanisms of H_2S release remains uninvestigated and low releasing efficiencies (~2% and 3%, respectively) are observed. To the best of our knowledge, the only reported cysteine-selective H_2S donor utilizes the established reactivity of acrylate Michael acceptors toward cysteine,[26] to subsequently trigger the generation of COS, which is quickly converted to H_2S by carbonic anhydrase.[27]

To further the development of thiol-triggered H_2S donors, we were inspired by the well-established chemistry of native chemical ligation due to the high biological compatibility and presence of a sulfur atom. Native chemical ligation is the chemoselective reaction between a thioester and an *N*-terminal cysteine residue to generate a new amide bond.[28] This reaction has been applied extensively in the field of protein synthesis, including in the semisynthesis of a potassium channel protein.[29] The mechanism of this important ligation reaction begins by the nucleophilic addition of a cysteine sulfhydryl group to form an intermediate thioester, which then undergoes a rapid S to N acyl transfer to generate the more thermodynamically stable amide product (Figure 2).[30] Despite

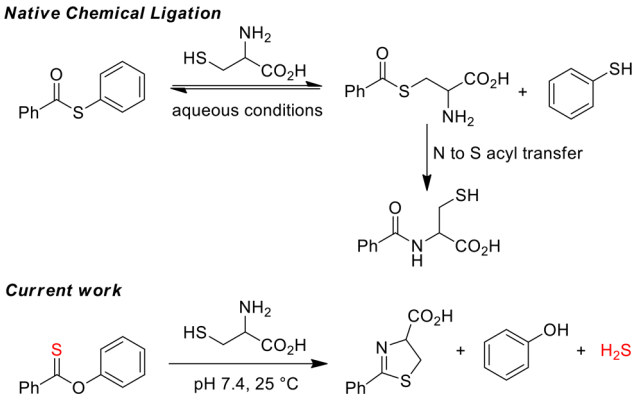

Figure 2. Generalized reaction scheme for native chemical ligation and release of H_2S upon addition of cysteine to a *bis*(phenyl) thionoester.

the broad use of thioesters as activated coupling partners for native chemical ligation, to the best of our knowledge there have not been investigations into similar reactions with thionoesters, which are a constitutional isomer of thioesters. Building from our interest in the chemistry of reactive sulfur species,[31−33] we hypothesized that thionoesters would undergo a similar reaction pathway in the presence of cysteine, but would also generate H_2S during the S to N acyl transfer step of the reaction. Such reactivity would not only provide access to new H_2S-releasing motifs but also provide insights into new mechanisms of chemical ligation that could be accessed by simple interchange of oxygen and sulfur atoms in a reactive electrophile. Additionally, such platforms are also attractive because they mimic the enzymatic conversion of cysteine to H_2S. Herein, we present a mechanistic and kinetic investigation of thionoesters with cysteine and related species and also demonstrate that thionoesters function as cysteine-selective H_2S donors that proceed through a native chemical ligation-type mechanism.

RESULTS AND DISCUSSION

To prepare a model thionoester system, we treated phenyl chlorothionoformate with phenylmagnesium bromide at −78 °C in anhydrous THF to yield *O*-phenyl benzothioate (**DPTE**).[34] Despite previous reports,[35] we found that treatment of phenyl benzoate with Lawesson's reagent required extended reaction times and afforded undesirable yields, which is consistent with the predicted decrease in reactivity of esters toward Lawesson's reagent.[36] The structure and purity of **DPTE** were confirmed by NMR spectroscopy and HPLC (see Supporting Information). To determine whether thionoesters are a viable platform for H_2S release, we added 25 μM **DPTE** to buffered aqueous solutions (10 mM PBS, pH 7.4) containing varying concentrations of cysteine (25−500 μM) and monitored H_2S generation using the spectrophotometric methylene blue assay[37] (Figure 3a). Consistent with our design

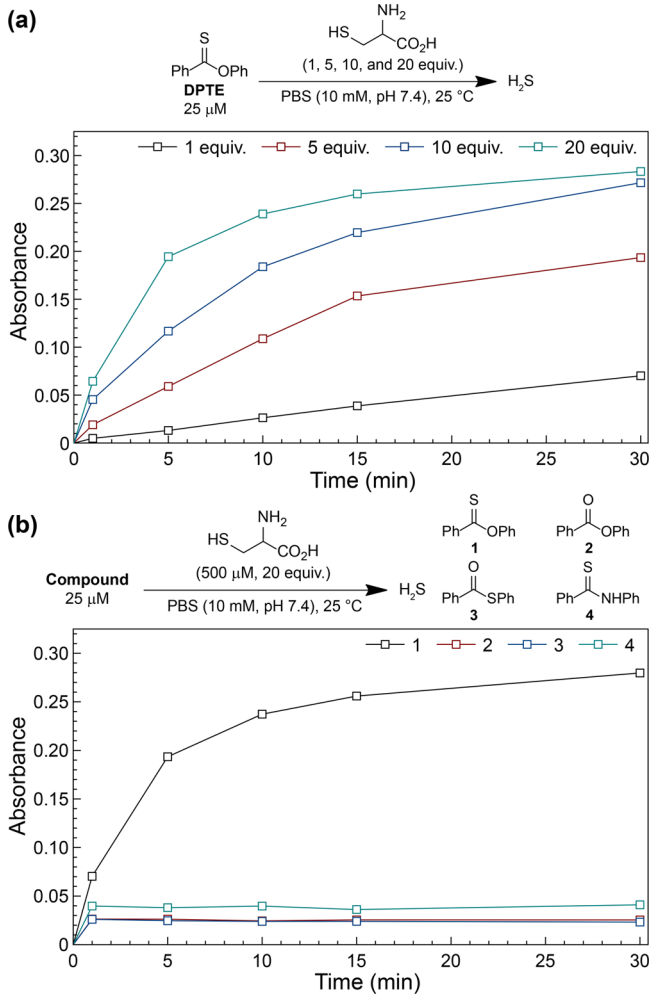

Figure 3. (a) Release of H_2S from **DPTE** in the presence of increasing cysteine concentrations (25, 125, 250, and 500 μM) in 10 mM PBS, pH 7.4 at 25 °C. (b) Lack of H_2S release from structurally related compounds (25 μM) in the presence of cysteine (500 μM, 20 equiv).

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hypothesis, we observed an increase in H_2S release from **DPTE** at higher cysteine concentrations, suggesting that thionoesters are a viable platform for cysteine-triggered H_2S donation.

To assess the H_2S-releasing efficiency from thionoesters, we used a methylene blue calibration curve to quantify the H_2S release (Figure S4). We measured that 20 μM of H_2S was released from a 25 μM solution of **DPTE** in the presence of 500 μM cysteine (20 equiv), which corresponds to a releasing efficiency of 80%. In addition to the thionoester system, we also investigated H_2S release from structurally related diphenyl ester (**2**) and diphenyl thioester (**3**) compounds under our conditions (Figure 3b). As expected, neither of these compounds released H_2S when treated with excess cysteine. Similarly, a representative secondary thioamide (**4**) failed to release H_2S in the presence of cysteine, suggesting the release of H_2S occurs exclusively from the thionoester moiety in the presence of cysteine.

To further investigate the selectivity of H_2S release from thionoesters, we treated **DPTE** with other biologically relevant nucleophiles (Figure 4).[38] In the absence of any added

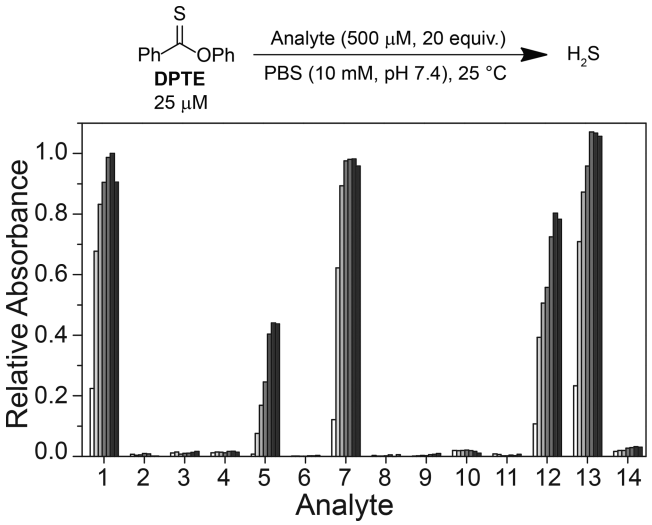


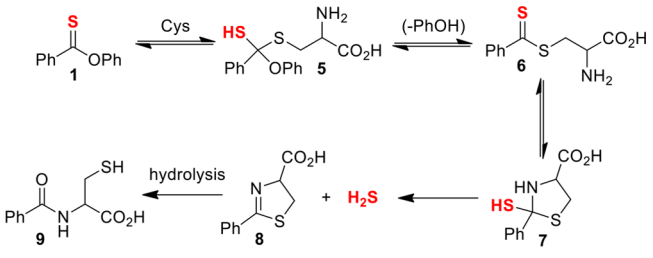
Figure 4. Selectivity of H_2S release from **DPTE** in the presence of different analytes. Data were acquired at 1, 5, 10, 15, 30, 45, and 60 min. Methylene blue absorbance values are relative to the maximum absorbance value obtained from H_2S release in the presence of cysteine (1). Analytes: H_2O/PBS buffer (2), serine (3), lysine (4), L-homocysteine (5), DL-penicillamine (6), L-cysteine methyl ester hydrochloride (7), *N*-acetyl-L-cysteine (8), *N*-acetyl-L-cysteine methyl ester (9), *S*-methyl-L-cysteine (10), GSH (11), cysteine + GSH (12), cysteine + lysine (13), PLE (1.0 U/mL) (14).

nucleophiles, no hydrolysis-mediated H_2S release was observed from **DPTE** at physiological pH, although we note prior reports show that thionoesters are hydrolyzed under basic conditions to afford the corresponding thioacid and alcohol.[39] Treatment of **DPTE** with serine or lysine, chosen as representative alcohol- and amine-based nucleophiles respectively, did not result in H_2S release, although prior reports suggest that amines can react with thionoesters to yield thioamides via displacement of the corresponding alcohols.[40] To investigate this potential side reactivity, cysteine-triggered (500 μM) H_2S release from **DPTE** (25 μM) was measured in the presence of lysine (500 μM) and we observed no change in H_2S-releasing efficiency. We also investigated the reactivity of **DPTE** with thiol-based nucleophiles. Treatment of **DPTE** with homocysteine also resulted in H_2S release, although at a slower rate than from treatment with cysteine. This observation is consistent with a larger, less favorable transition state required for an intramolecular S to N acyl transfer in the homocysteine system in comparison with the cysteine system. Alternatively, the reduced rate may be reflective of the significant pK_a difference between cysteine (pK_a $\approx$ 8.5) and homocysteine (pK_a $\approx$ 10),[41] meaning that, under physiological conditions, the effective concentration of cysteine thiolate is much greater than homocysteine thiolate (~10% vs ~0.03%). Surprisingly, treatment of **DPTE** with penicillamine did not result in H_2S release. We anticipated that geminal methyl groups would help to preorganize the intermediate dithioester generated after nucleophilic attack and would result in faster H_2S release.[42] However, the geminal methyl groups also likely significantly reduce the nucleophilicity of the thiol moiety due to steric congestion, which would subsequently disfavor the initial nucleophilic attack on the thionoester.

We also investigated whether different cysteine derivatives could generate H_2S release from **DPTE** to further understand the requirements for H_2S release from thionoesters. Treatment of **DPTE** with cysteine methyl ester did not affect H_2S production, suggesting that the carboxylic acid is not required for H_2S generation. By contrast, treatment of **DPTE** with *N*-acetylcysteine, *N*-acetylcysteine methyl ester, or *S*-methylcysteine completely abolished H_2S release, highlighting the requirement of a 2-aminoethanethiol moiety for productive H_2S release. Consistent with these results, treatment of **DPTE** with GSH, the most abundant biological thiol, did not generate H_2S, which is consistent with the requirement of a pendant amine to generate H_2S release. Despite the lack of H_2S release, we anticipated that GSH would still attack **DPTE** to form an intermediate dithioester, which should still be sufficiently electrophilic to react with cysteine to generate H_2S. To test this hypothesis, we treated **DPTE** (25 μM) with GSH (1 mM) and cysteine (500 μM) and observed a reduced rate of H_2S release. These results suggest that the competitive, nonproductive, addition of GSH to the thionoester is reversible, and that the thionoester moiety can still react with Cys in the presence of GSH to release H_2S. Adding to the selectivity investigations, treatment of **DPTE** with porcine liver esterase (PLE) failed to generate H_2S; however, we cannot rule out consumption of the thionoester moiety by PLE or other native enzymes. Taken together, these results demonstrate the high selectivity of the thionoester moiety toward cysteine and homocysteine for H_2S release.

Building from the selectivity studies, as well as from the established mechanism of native chemical ligation, we proposed a mechanism for cysteine-mediated H_2S release from thionoesters (Scheme 1). Initial nucleophilic addition by

Scheme 1. Proposed Mechanism of H_2S Release from DPTE in the Presence of Cysteine



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cysteine on **1** generates tetrahedral intermediate **5**, which collapses to form dithioester intermediate **6** and extrude 1 equiv of phenol. Similar to native chemical ligation, subsequent nucleophilic attack by the pendant amine on the thiocarbonyl leads to the formation of substituted thiazolidine **7**. Loss of H_2S, by either direct extrusion of HS^- or solvent-assisted extrusion of H_2S, results in formation of dihydrothiazole **8**, which could be further hydrolyzed to form *N*-benzoyl-cysteine (**9**).

As a first step toward investigating our proposed mechanism, we determined the reaction order in cysteine by treating **DPTE** (25 μM) with varying concentrations of cysteine under pseudo-first-order conditions at 25 °C and measuring H_2S release using the methylene blue assay (Figure 5). As expected,

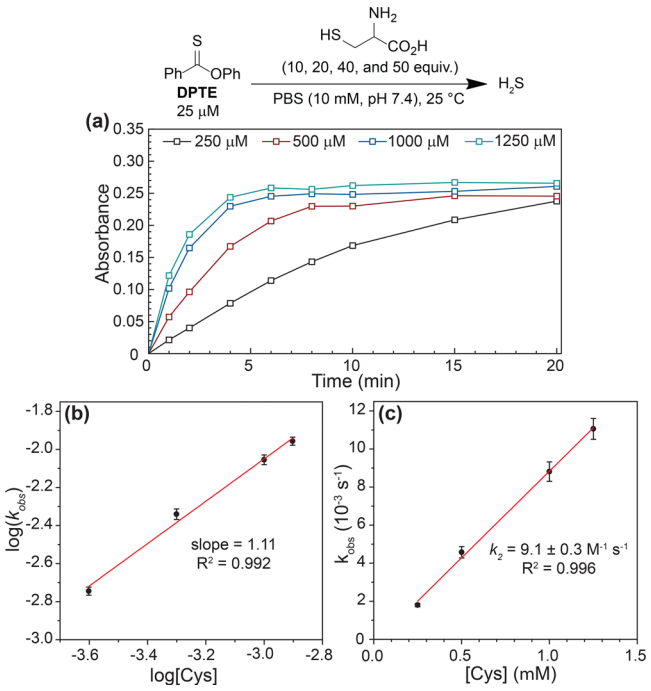

Figure 5. (a) H_2S release by **DPTE** in the presence of increasing cysteine concentrations (250, 500, 1000, and 1250 μM). (b) Plot of $\log(k_{obs})$ vs $\log([Cys])$ for **DPTE**. (c) Plot of k_{obs} vs [Cys].

we observed that increased cysteine concentrations led to increased rates of H_2S production. The resultant releasing curves were fit to obtain pseudo-first-order rate constants (k_{obs}), and plotting log[Cys] versus log[k_{obs}] confirmed a first-order dependence in cysteine, which is consistent with our proposed mechanism. Additionally, the obtained k_{obs} values were plotted against Cys concentrations to obtain a second-order rate constant of 9.1 ± 0.3 M^{-1} s^{-1} for the reaction. In comparison to other known reactivities, the rate of cysteine-triggered H_2S release from **DPTE** is comparable to the rate (10−100 M^{-1} s^{-1}) of copper(I)-catalyzed azide−alkyne cycloadditions (CuAAC), a classic example of a "click reaction."[43]

To further evaluate our proposed mechanism, we sought to identify the rate-determining step in cysteine-triggered release of H_2S from thionoesters. In native chemical ligation, the initial nucleophilic attack by thiols to form intermediate thioesters is reversible and has been utilized to enhance the reactivity of alkyl thioesters for native chemical ligation. However, in the presence of cysteine, the transthioesterification resulting from nucleophilic attack of the sulfhydryl group on the thioester is thought to be rate-limiting due to the rapid and irreversible subsequent S to N acyl transfer to form the more thermodynamically stable amide bond.[30] In the thionoester system, the initial attack by a thiol on **DPTE** results in extrusion of phenol, which is a much weaker nucleophile than a thiol and should not attack the generated dithioester intermediate. If other thiols are present in solution, then it is likely that they could attack the dithioester intermediate in a transdithioesterification reaction. This thiol exchange is supported by the observed reduced rate of H_2S generation from **DPTE** in the presence of competing thiols, suggesting that the initial nucleophilic attack on dithioesters is reversible.

Using similar pseudo-first-order conditions as those used for the cysteine order dependence investigations (25 μM **DPTE**, 500 μM cysteine), we performed an Eyring analysis to determine the activation parameters for the reaction in an effort to further understand the amount of disorder in the rate-limiting transition state for the reaction (Figure 6). Our

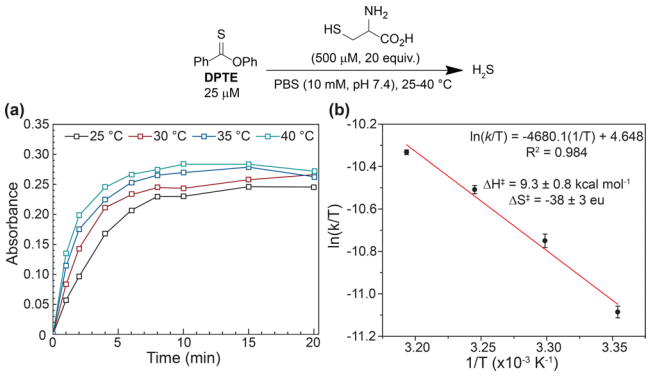

Figure 6. (a) Effect of temperature on rate of H_2S release from **DPTE** (25 μM) in the presence of cysteine (500 μM, 20 equiv). (b) Eyring analysis of H_2S release from **DPTE**.

expectation was that if initial thiol addition is the rate-limiting step, then we would observe a negative entropy of activation ($\Delta S^{\ddagger}$) of approximately −20 eu, which is typical for a bimolecular reaction. In contrast, if the intramolecular S to N thioacyl transfer to form the substituted thiazolidine is the rate-limiting step, then we would expect a larger, more negative $\Delta S^{\ddagger}$ due to the highly ordered structure required for the intramolecular cyclization. Under our experimental conditions, we observed $\Delta S^{\ddagger} = -38 \pm 3$ eu, which is most consistent with intramolecular cyclization being the rate-determining step of the reaction.

As a final step of characterizing the proposed mechanism, we performed a preparative scale reaction and isolated the reaction products. In addition to recovered starting material, we isolated a cysteine-derived dihydrothiazole (**CysDHT**) rather than *N*-benzoyl-L-cysteine as the major product of the reaction (Figure 7). These results suggest that the dihydrothiazole is stable under aqueous conditions and is not further hydrolyzed to *N*-benzoyl-L-cysteine. To further confirm the formation of **CysDHT** from **DPTE**, we synthesized an authentic sample of **CysDHT** and used HPLC to monitor the reaction progress. We treated a 100 μM solution of **DTPE** with 20 equiv of L-cysteine methyl ester and observed nearly complete conversion to phenol and **CysDHT** within 1 h. Using known concentrations of phenol and **CysDHT** to construct an HPLC calibration curve, we measured that the concentrations

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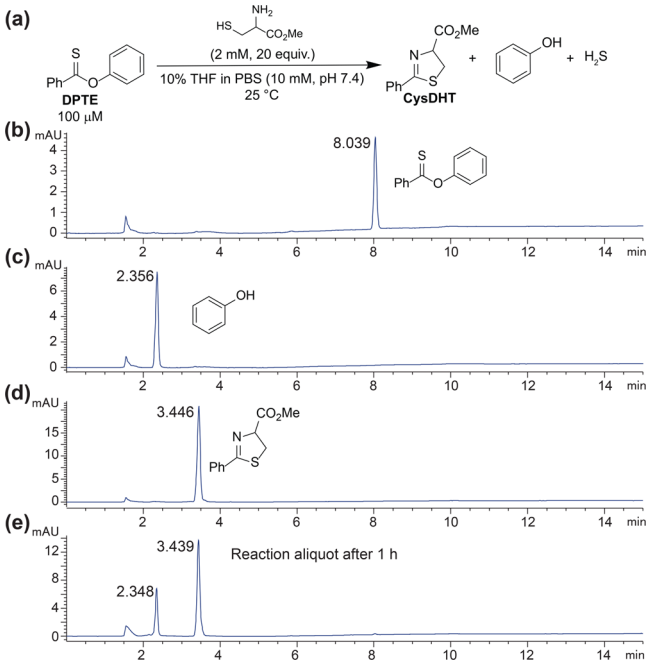

Figure 7. (a) Reaction conditions; (b) 100 μM DPTE in PBS (10 mM, pH 7.4) with 10% THF; (c) 100 μM PhOH in PBS (10 mM, pH 7.4) with 10% THF; (d) 100 μM CysDHT in PBS (10 mM, pH 7.4) with 10% THF; (e) reaction aliquot after 1 h.

of phenol and **CysDHT** after 1 h were approximately 76 μM and 64 μM, respectively, which supports the high H_2S-releasing efficiency of thionoesters.

Although we were initially surprised by the inherent stability of the dihydrothiazole product, we note that biological formation of the dihydrothiazole moiety is a known post-translation modification of cysteine residues in bacteria.[44] For example, the cyclodehydration of internal cysteine residues results in formation of Fe(III)-coordinating dihydrothiazole, which is commonly found in sideophores,[45] such as yersiniabactin[46] and pyochelin.[47] Additionally, adjacent dihydrothiazole moieties can be oxidized to a *bis*(thiazole), and the planarity of this motif allows for intercalation of DNA as seen in bleomycin.[48] Taken together, these observations highlight the biological significance of the dihydrothiazole motif and provides new areas of investigation using this established reactivity.

CONCLUSION

By investigating the reactivity of **DPTE** with cysteine, we not only demonstrated the inherent reactivity of thionoesters toward cysteine in a native chemical ligation-type mechanism but also demonstrated that this functional group provides a novel platform for highly efficient H_2S donation. We demonstrated that this reaction occurs at rates similar to those for the commonly used Cu(II)-mediated azide/alkyne click reaction, with a second-order rate constant of 9.1 ± 0.3 M^{-1} s^{-1}. Our mechanistic investigations suggest that, in comparison to native chemical ligation, the rate-determining step has been shunted from the addition of cysteine to the intramolecular S to N thioacyl transfer. Taken together, these investigations demonstrate that thionoesters are a novel, cysteine-triggered H_2S releasing scaffold. Additionally, the high selectivity of **DPTE** toward cysteine warrants future exploration into the thionoester functional group for cysteine-selective reactive probes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b07268.

Experimental procedures, NMR spectra, HPLC data (PDF)

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Notes

The authors declare no competing financial interest.

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