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Dithioesters: simple, tunable, cysteine-selective H_2S donors†

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Dithioesters have a rich history in polymer chemistry for RAFT polymerizations and are readily accessible through different synthetic methods. Here we demonstrate that the dithioester functional group is a tunable motif that releases H_2S upon reaction with cysteine and that structural and electronic modifications enable the rate of cysteine-mediated H_2S release to be modified. In addition, we use (bis) phenyl dithioester to carry out kinetic and mechanistic investigations, which demonstrate that the initial attack by cysteine is the rate-limiting step of the reaction. These insights are further supported by complementary DFT calculations. We anticipate that the results from these investigations will allow for the further development of dithioesters as important chemical motifs for studying H_2S chemical biology.

Introduction

Prevalent in the field of polymer chemistry, dithioesters are utilized as chain transfer agents in reversible addition-fragmentation chain transfer (RAFT) polymerizations.[1] Arising from the ability to readily react with propagating radical monomers, the design of thiocarbonyl-containing motifs such as dithioesters, trithiocarbonates, and dithiocarbamates has been well studied to enhance the RAFT polymerization of various monomers.[2,3] We recently demonstrated that related thionoesters, which are structural isomers of thioesters bearing a thiocarbonyl motif, undergo a chemoselective reaction with cysteine to generate a dihydrothiazole and hydrogen sulfide (H_2S).[4] Despite recent advancements in the synthesis of thionoesters,[5] the ability to prepare a diverse library of thionoester-based H_2S donors is limited by the availability of stable, readily-accessible starting materials. However, based on structural similarities between thionoesters and dithioesters, we hypothesized that dithioesters could provide similar reactivity in the presence of cysteine and allow for the development of tunable H_2S donors with precise control over H_2S release rates and efficiencies.

Complementing carbon monoxide and nitric oxide, hydrogen sulfide (H_2S) is now classified as an important biological signaling molecule.[6] Commonly referred to as gasotransmitters, these small, gaseous molecules are produced endogenously, readily permeate cell membranes, and exert physiological responses upon binding to or reacting with cellular and/or molecular targets.[7] The involvement of H_2S-mediated signaling has been observed in a variety of physiological processes such as vasodilation,[8] angiogenesis,[9] and scavenging of reactive oxygen species.[10] In recent years, researchers have become increasingly interested in utilizing H_2S donors for the development of both important research tools and novel therapeutics.[11,12] Towards this goal, researchers have relied heavily on the use of sodium hydrosulfide (NaSH), which is a water-soluble source of H_2S, for preliminary studies. However, a comparison between H_2S production from native enzymes including cystathionine-β-synthase and cystathionine-γ-lyase against exogenous administration of H_2S (*via* NaSH) has revealed stark differences which should be taken into consideration.[13] The dissolution of NaSH in buffered solutions leads to a rapid, almost spontaneous increase in local H_2S concentration,[14] whereas endogenous H_2S production occurs at a slower, at a slower rate. To address this issue, researchers have developed synthetic H_2S donors, which release H_2S either passively *via* hydrolysis or in the presence of a specific analyte at rates comparable to enzymatic H_2S production (Fig. 1).[15–18]

Drawing parallels to the dissolution of NaSH, small molecules derived from dithiole-3-thione[19] and phosphorodithioate[20,21] motifs respectively have been synthesized as hydrolysis-activated H_2S donors, although we note the overall low H_2S-releasing efficiencies from these donors. Towards improving releasing yields and tuning rates of H_2S release, analyte-responsive small molecules have been prepared to release H_2S as a function of pH,[22] in the presence of native enzymes,[23] and upon irradiation with light.[24–26] Initially reported by our group,[27] the conversion of carbonyl sulfide (COS) to H_2S by carbonic anhydrase has been used to access a library of COS/H_2S donors.[28–30] Inspired by the endogenous production

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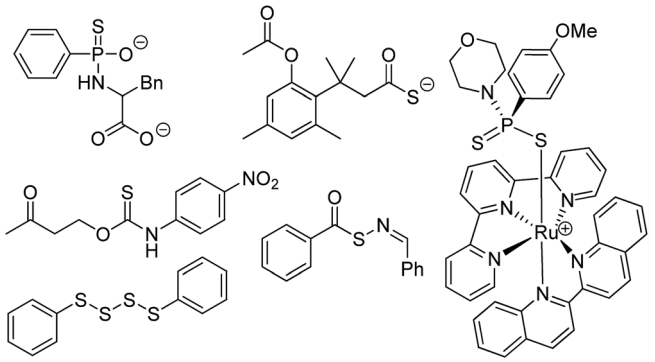
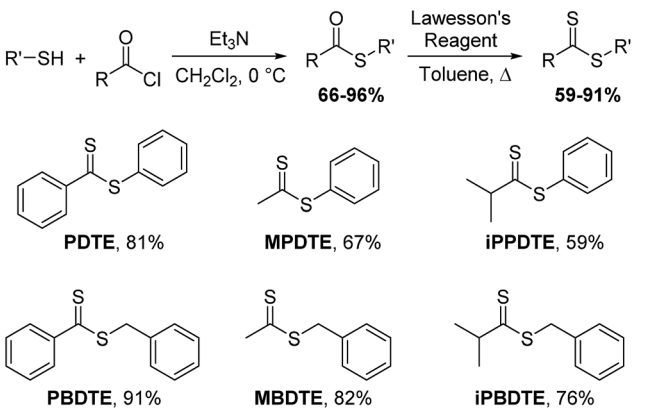


Fig. 1 Selected examples of synthetic H_2S donors.

of H_2S *via* enzymatic conversion of cysteine, a number of thiol-triggered H_2S donors have been reported. Abundant in nature, organic polysulfides[31] have been shown to release H_2S in the presence of biological thiols including cysteine[32] and reduced glutathione (GSH).[33] The generation of persulfides, an important class of biologically-relevant reactive sulfur species,[34] *via* thiol-mediated reduction has been used to prepare a series of synthetic H_2S donors with potent protective effects against myocardial-ischemia reperfusion injury.[25] Similarly, the generation of *N*-mercaptan species in the presence of thiols has been demonstrated as a viable platform for H_2S delivery with tunable releasing kinetics.[35–37] Under physiological conditions, thionoesters react rapidly with cysteine *via* a native chemical ligation-like mechanism to afford an equivalent of phenol, H_2S, and a cysteine-derived dihydrothiazole (Fig. 2a). Although dithioesters have been used to prepare polymeric H_2S donors,[38,39] direct use of this motif as an H_2S donor scaffold has not been reported. Herein, we present our findings on the development of dithioesters as novel, cysteine-triggered H_2S donors (Fig. 2b). We demonstrate the release of H_2S in the presence of cysteine from a representative (bis)phenyl dithioester with a comprehensive kinetic analysis. Additionally, we demonstrate the ability to tune the rate of H_2S release from dithioesters *via* simple alkyl functionalization.

Results and discussion

Building upon our previous work, we hypothesized that the intermediate dithioester formed during the chemoselective reaction of cysteine with thionoesters could provide a platform for future investigations leading to the development of novel, cysteine-triggered H_2S donors. Unfortunately, the functionalization of thionoester-based H_2S donors is hindered by a limited number of stable, isolable alkyl chlorothionoformates. By contrast, the enhanced reactivity of Lawesson's reagent toward thioesters over esters allows for convenient functionalization and synthetic accessibility of various dithioesters.[40] To prepare different dithioesters, we treated the desired thiol with the desired acid chloride in the presence of triethylamine followed by thionation with Lawesson's reagent to access to a library of dithioesters with good to excellent yields (Scheme 1). The modularity of this synthetic route readily allows for the introduction of various functional groups and can be expanded upon to include payloads of interest including fluorophores and known therapeutics for the development of H_2S-releasing prodrugs. In the interest of accessing tunable H_2S donors, we hypothesized modulation of thiocarbonyl electrophilicity and



Scheme 1 General synthesis of dithioesters.

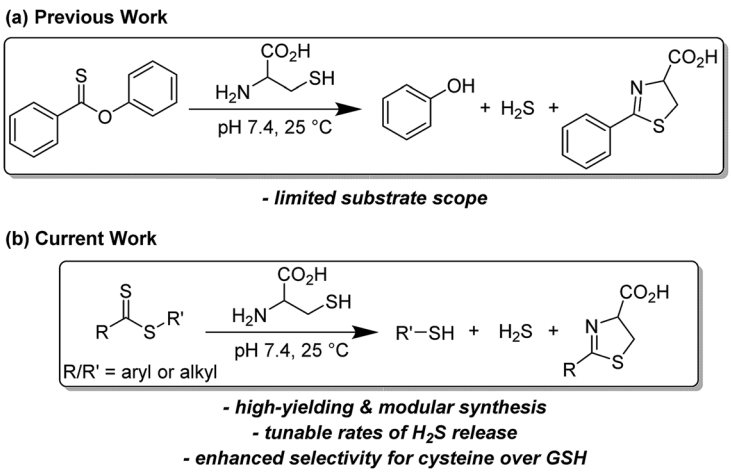


Fig. 2 (a) Cysteine-triggered H_2S release from thionoesters. (b) Cysteine-triggered H_2S release from dithioesters.

thiolate leaving group ability would directly alter the rates of cysteine-triggered H_2S release from dithioesters.

With synthesized dithioesters in hand, we began our studies by examining the reactivity of **PDTE** (25 μM) as a representative dithioester towards various concentrations of cysteine (250, 500, 1000, and 1250 μM) and monitoring the release of H_2S *via* the spectrophotometric methylene blue assay, which allows for H_2S quantification (Fig. 3a).[41] Consistent with our hypothesis, we observed increasing amounts of H_2S released with increasing cysteine concentrations from **PDTE**. To quantify the H_2S-releasing efficiency, we used a methylene blue calibration curve generated with NaSH (see ESI†) and we found 25 μM **PDTE** released approximately 17 μM H_2S after 1 h, which corresponds to a releasing efficiency of ~68%. Under identical conditions, we note a slight decrease in H_2S-releasing efficiency between dithioesters and thionoesters (80%), although both of these constructs are efficient H_2S-releasing motifs. To quantify the rate of H_2S release, we were able to fit these releasing curves and obtain pseudo-first order rate constants (k_{obs}). A plot of log[Cys] *versus* log(k_{obs}) provided a linear plot with slope near one, which suggests the overall reaction is first order in cysteine and proceeds *via* a mechanism similar to the reaction of thionoesters with cysteine (Fig. 3b). A plot of [Cys] *versus* k_{obs} yielded a second-order rate constant (k_2) of 1.8 ± 0.1 M^{-1} s^{-1} (Fig. 3c), which is approximately five times slower relative to cysteine-triggered H_2S from thionoesters (9.1 ± 0.3 M^{-1} s^{-1}). This difference in H_2S-releasing kinetics prompted us to further investigate the kinetics of cysteine-triggered H_2S release from dithioesters and elucidate potential mechanistic differences between both classes of H_2S donors.

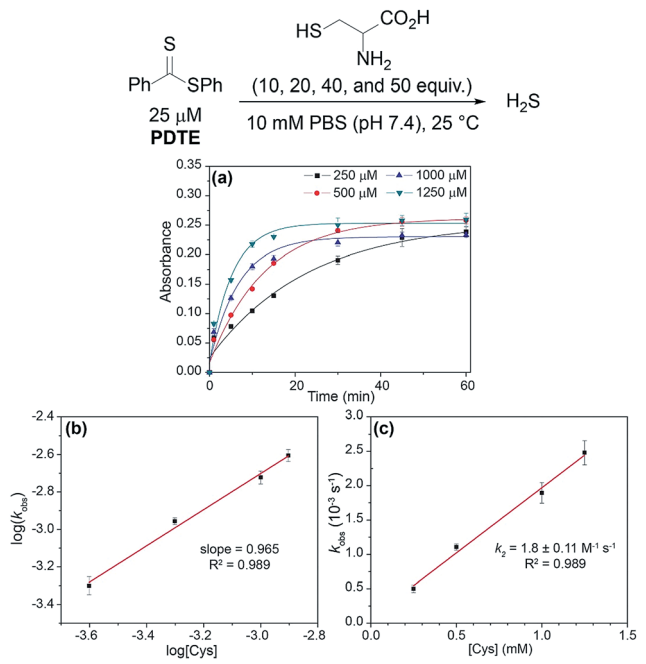


Fig. 3 (a) Release of H_2S from **PDTE** (25 μM) in the presence of increasing cysteine concentrations (250, 500, 1000, and 1250 μM). (b) Plot of log(k_{obs}) *vs.* log([Cys]). (c) Plot of k_{obs} *vs.* [Cys].

To further probe the reactivity of dithioesters with respect to H_2S release, we next investigated the effect of cysteine derivatives and related thiol-based nucleophiles on H_2S release from **PDTE** (Fig. 4). In the absence of nucleophiles, we did not observe H_2S release under hydrolytic conditions. Although the conversion of a thiocarbonyl to the corresponding carbonyl is thermodynamically favorable with an enthalpic gain of ~43 kcal mol^{-1} when comparing C=S *versus* C=O bond strengths, the hydrolysis of dithioesters is a slow process and can be considered negligible when considering the rate of cysteine-triggered H_2S release.[42,43] Further supporting a similar mechanism of cysteine-triggered H_2S release between thionoesters and dithioesters, masking of either the thiol or amine moieties in cysteine completely abolished H_2S release from **PDTE**. Additionally, the use of cysteine methyl ester did not affect H_2S release when compared to H_2S release in the presence of cysteine. To assess the effect of cysteine analogues on H_2S release, we measured H_2S release in the presence of homocysteine and penicillamine. Interestingly, we observed a significant reduction in the H_2S release rate in the presence of homocysteine relative to cysteine-triggered H_2S release. In the presence of penicillamine, we failed to observe significant H_2S release, which we attribute to a reduction in nucleophilicity due to the presence of geminal methyl groups.

We next examined the release of H_2S from **PDTE** in the presence of reduced glutathione (GSH), which is the most abundant biological thiol to determine the effect of competitive thiols on H_2S release. In the presence of 500 μM GSH, we did not observe significant H_2S release, but also could not rule out that transthioesterification by GSH, which would result in consumption of the dithioester moiety with a lack of H_2S release. Considering the nucleophilicity of the departing thiophenol, we hypothesized that the reversibility of

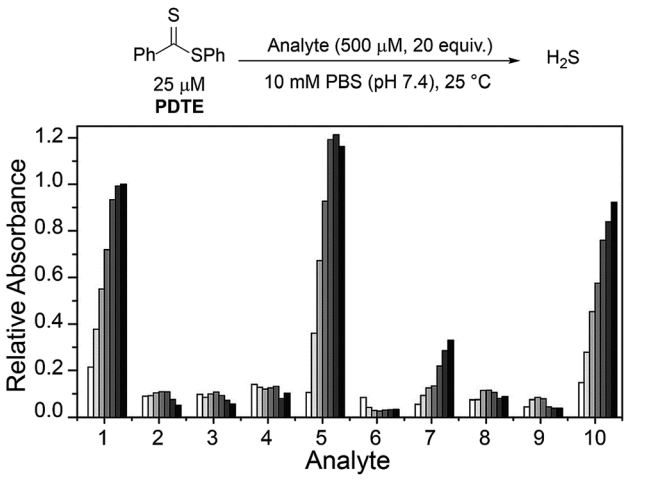


Fig. 4 Selectivity of H_2S release from **PDTE** in the presence of different analytes. Data were acquired at 1, 5, 10, 15, 30, 45, and 60 min. Methylene blue absorbance values are relative to the maximum absorbance value obtained from H_2S release in the presence of cysteine (1). Analytes: H_2O (2), *N*-acetyl-L-cysteine (3), *S*-methyl-L-cysteine (4), L-cysteine methyl ester hydrochloride (5), *N*-acetyl-L-cysteine methyl ester (6), DL-homocysteine (7), DL-penicillamine (8), GSH (9), cysteine + 1 mM GSH (10).

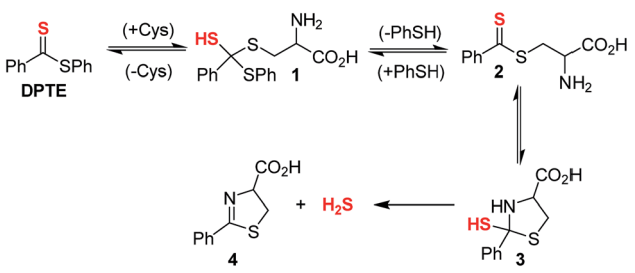
transthioesterification is likely to be more efficient for dithioesters than for thionoesters, which should result in enhanced selectivity for cysteine over GSH. To test this hypothesis, we measured H_2S release from **PDTE** in the presence of 500 μM cysteine and 1 mM GSH. In support of the expected enhanced reversibility, we observed minimal change on the cysteine-triggered H_2S release from **PDTE** even in the presence of excess GSH. This presents a considerable advancement over the thionoester scaffold, which yielded a decreased H_2S-releasing efficiency in the presence of GSH and cysteine. Taken together, these results demonstrate the selectivity of the dithioester scaffold for cysteine over other thiol-based nucleophiles including GSH and we proposed the following mechanism of H_2S release (Scheme 2).

Initial nucleophilic attack by cysteine on **PDTE** generates tetrahedral intermediate **1**, which collapses upon thiocarbonyl formation and extrusion of thiophenol to yield intermediate **2**. Nucleophilic attack by the pendant amine generates intermediate **3**, which extrudes H_2S upon formation of dihydrothiazole **4**. Based on the negligible loss in H_2S-releasing efficiency in the presence of excess GSH, the generation of **1** and **2** is likely highly reversible and could provide enhanced selectivity of the dithioester moiety for cysteine. In the mechanism of cysteine-triggered release of H_2S from thionoesters, the poor nucleophilicity of the departing alcohol likely impedes formation of the initial thionoester. In our proposed mechanism of H_2S release from dithioesters, the departing thiolate bears enhanced nucleophilicity and likely reacts with the intermediate dithioester to regenerate the initial dithioester effectively reversing cysteine addition. Additionally, the enhanced reversibility of transthioesterification supports the considerably slower kinetics between dithioesters and thionoesters. Taken together, we hypothesized this enhanced reversibility would result in the initial nucleophilic attack by cysteine being the rate-determining step of cysteine-triggered H_2S release from dithioesters.

To confirm the formation of a cysteine-based dihydrothiazole, **PDTE** (100 μM) was treated with L-cysteine methyl ester (2 mM, 20 equiv.) and a reaction aliquot after 1 h was subjected to HPLC analysis (see ESI†). In agreement with our proposed mechanism, the HPLC analysis revealed the formation of the expected dihydrothiazole in ~61% yield, which is consistent with the H_2S-releasing efficiency of **PDTE** as measured *via* the methylene blue assay. To gain insights on the rate-determining step, we measured the effect of temperature on the rate of H_2S release from **PDTE** (25 μM) in the presence of cysteine (500 μM, 20 equiv.) (Fig. 5). If nucleophilic attack by cysteine on **PDTE** is the rate-determining step of the reaction, we would expect to observe an entropy of activation ($\Delta S^{\ddagger}$) of approximately −20 eu, which would be characteristic of a bimolecular reaction.[44,45]

Upon measuring the rates of H_2S release at different temperatures, we constructed an Eyring plot using the obtained k_{obs} values, which afforded $\Delta S^{\ddagger} = -23 \pm 1$ eu. The observed $\Delta S^{\ddagger}$ supports our mechanistic hypothesis and is consistent with the initial addition of cysteine to the dithioester to generate **1** being the rate-determining step of cysteine-triggered H_2S release from dithioesters. In the reaction of thionoesters with cysteine, we interpreted an experimentally-determined $\Delta S^{\ddagger} = -38 \pm 3$ eu to suggest the intramolecular cyclization as the rate determining step. In comparison between both mechanisms, by simply altering the nucleophilicity of the leaving group (*i.e.* alcohol *vs.* thiol) we have shunted the rate-determining step of the reaction. Additionally, our results suggest that cysteine-triggered H_2S release from dithioesters and native chemical ligation share a similar rate-determining step.

To complement our experimental results, we used density functional theory (DFT) to examine the potential energy surface for H_2S release from dithioesters. Because **PDTE** was used for our experimental mechanistic investigations, we chose to investigate the reaction of **PDTE** with cysteine thiolate using Gaussian 09 at the B3LYP/6-311++G(d,p) level of theory applying the IEF-PCM water solvation model. We found that the initial nucleophilic attack by cysteine thiolate on **PDTE** resulted in an activation barrier of 19.1 kcal mol^{-1}, which was the highest barrier on the reaction coordinate and qualitatively agrees with the experimentally-observed $\Delta H^{\ddagger}$ of 14.4 kcal mol^{-1}. The resultant transthioesterified cysteine adduct is 3.7 kcal mol^{-1} more stable than the **PDTE** starting material, and subsequently undergoes an intramolecular S to N thioacyl transfer reaction with an associated barrier of 8.9 kcal mol^{-1}, resulting in the final and more thermodynamically-favorable dihydrothiazole product (Fig. 6).



Scheme 2 Proposed mechanism for release of H_2S from **PDTE** in the presence of cysteine.

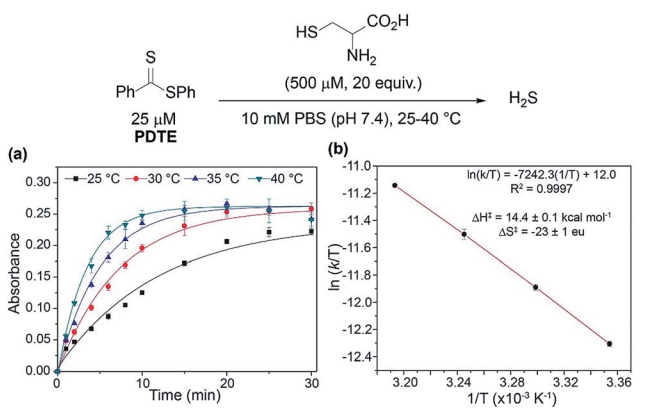


Fig. 5 (a) Effect of temperature on rate of H_2S release from **PDTE** (25 μM) in the presence of cysteine (500 μM, 20 equiv.). (b) Eyring analysis of cysteine-triggered H_2S release from **PDTE**.

The ease of synthesis of dithioester compounds also enabled further investigation into the effect of alkyl functionalization on H_2S release. We hypothesized that the electrophilicity at the thiocarbonyl position could be modified directly by introducing various alkyl, and thus alter the rate of H_2S release. Additionally, we hypothesized that modification of the departing thiolate nucleophilicity would also alter the rate of H_2S release from dithioesters. We anticipated the introduction of inductively donating alkyl groups, such as methyl and isopropyl, would decrease the thiocarbonyl electrophilicity and resulting in decreased rates of H_2S release. Additionally, we anticipated that replacement of thiophenol with benzyl mercaptan would result in decreased rates of H_2S release due to the enhanced nucleophilicity of benzyl mercaptan over thiophenol. To test our hypotheses, we measured H_2S release from our library of alkyl functionalized dithioesters (25 μM) in the presence of cysteine (500 μM, 20 equiv.) (Fig. 7).

Contrary to our initial hypothesis, the incorporation of inductively-donating alkyl groups led to enhanced rates of H_2S release relative to **PDTE**. To better understand the apparent releasing trends, we considered the stability of the charge-separated thiocarbonyl motif and the effect of different alkyl groups (Fig. 8). In the case of **PDTE**, a charge-separated thiocarbonyl yields a benzylic carbocation which can readily delocalize *via* resonance effectively altering the electrophilicity of the thiocarbonyl *via* delocalization of the carbocation. In the presence of inductively donating groups such as methyl and isopropyl in **MPDTE** and **iPPDTE** respectively, the resulting carbocation is localized to the thiocarbonyl position, which would result in enhanced rates of H_2S release in comparison to **PDTE**. Incorporation of an isopropyl group in **iPPDTE**, however,

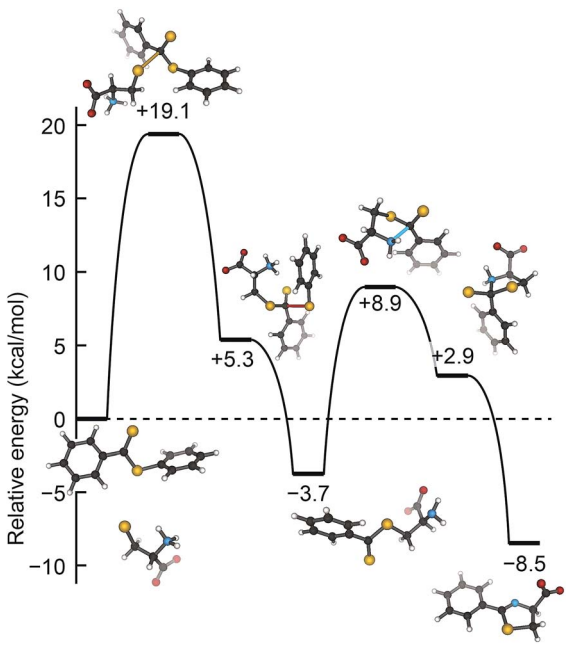


Fig. 6 Potential energy surface for the attack of cysteine thiolate on **PDTE**. Calculations were performed in Gaussian 09 at the B3LYP/6-311++G(d,p) level of theory applying the IEF-PCM water solvation model.

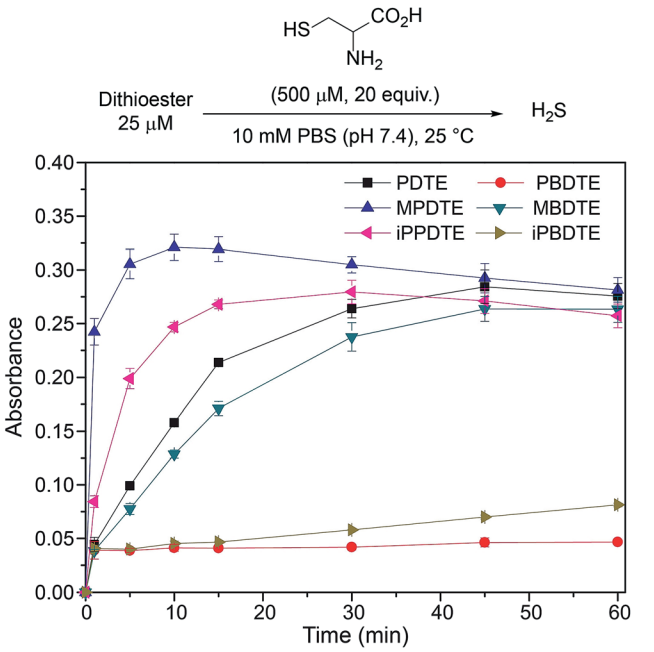


Fig. 7 Effect of alkyl functionalization on the rate of H_2S release from dithioesters.

also introduces the potential for an intermediate 1,2-methyl shift which would partially delocalize the carbocation and hinder H_2S release relative to **MPDTE**. Considering these contributions, we interpret the enhanced release of H_2S from alkyl-functionalized dithioesters as a reflection of altered thiocarbonyl electrophilicity *via* carbocation delocalization. Although this outcome was contrary to our initial hypothesis, these findings demonstrate the ability to tune the rate of H_2S release from dithioesters by simple alkyl substitutions.

Furthering our studies, we also examined the effect of benzyl mercaptan as a leaving group on H_2S release from **MBDTE**, **PBDTE**, and **iPBDTE** respectively. In comparison to thiophenol, benzyl mercaptan is a considerably better nucleophile and likely perturbs the equilibrium of transthioesterification to disfavor the addition of cysteine. In concert with the effect of alkyl groups on thiocarbonyl electrophilicity, we anticipated H_2S release from dithioesters bearing benzyl mercaptan to be dramatically altered relatively to their thiophenol analogue. In agreement with our hypothesis, H_2S release was observed exclusively from **MBDTE**. Considering the lack of carbocation delocalization by the pendant methyl group, this result suggests the thiocarbonyl moiety in **MBDTE** is sufficiently electrophilic to promote H_2S release in the presence of cysteine. Alternatively, we observed effectively no H_2S release from **PBDTE** and relatively slow H_2S release from **iPBDTE**. Considering the effect of alkyl groups on thiocarbonyl electrophilicity *via* carbocation delocalization in concert with the enhanced nucleophilicity of benzyl mercaptan, a significantly hindered rate of release from **iPBDTE** and complete shutdown of H_2S release from **PBDTE** is also in agreement with our hypothesis. Overall, we found the use of benzyl mercaptan decreased overall H_2S-releasing efficiency and yielded hindered rates of release in comparison to

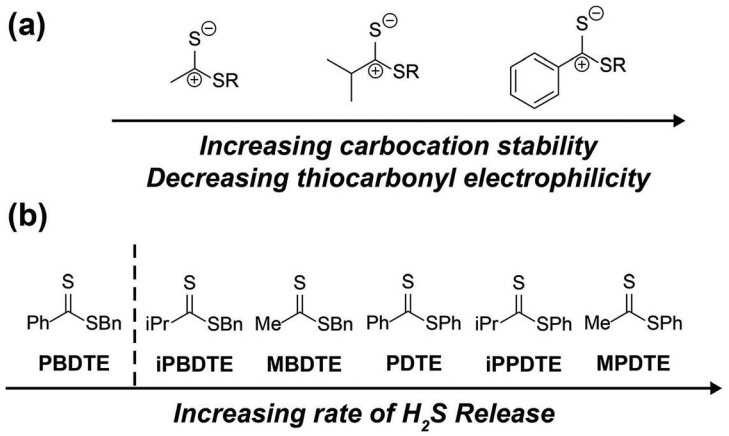


Fig. 8 (a) Effect of pendant groups on thiocarbonyl electrophilicity and carbocation stability. (b) Scale of increasing H_2S releasing rates for reported dithioesters.

thiophenol-based dithioesters. Taken together, these findings demonstrate the rate of H_2S release from dithioesters can be enhanced or decreased as a function of alkyl substitution.

Conclusions

Advancing our previous work on synthetic H_2S donors *via* the chemoselective condensation of thionoesters and cysteine, we have identified that dithioesters are a viable and tunable platform for developing cysteine-triggered H_2S donors. Using **PDTE** as a representative dithioester, we have demonstrated H_2S release in the presence of cysteine proceeds with good efficiency, and that dithioesters provide significant advancements over thionoesters, including ease of functionalization and enhanced selectivity for cysteine over other thiol-based nucleophiles including GSH. Mechanistic investigations, including rate-order analysis and activation parameters support a mechanism in which the initial nucleophilic attack by cysteine on the dithioesters is the rate-determining step of the reaction, which is also supported by computation. Furthermore, we demonstrated that the rate of H_2S release from a small library of dithioesters can be controlled by alkyl substitution. The findings presented herein provide several advancements over thionoesters as cysteine-triggered H_2S donors and provides a foundation for further development of dithioesters for expanding upon the chemical biology of H_2S.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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