Communication

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Vinayak S. Khodade, and John P. Toscano

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Development of *S*-Substituted Thioisothioureas as Efficient Hydropersulfide Precursors

Vinayak S. Khodade and John P. Toscano*

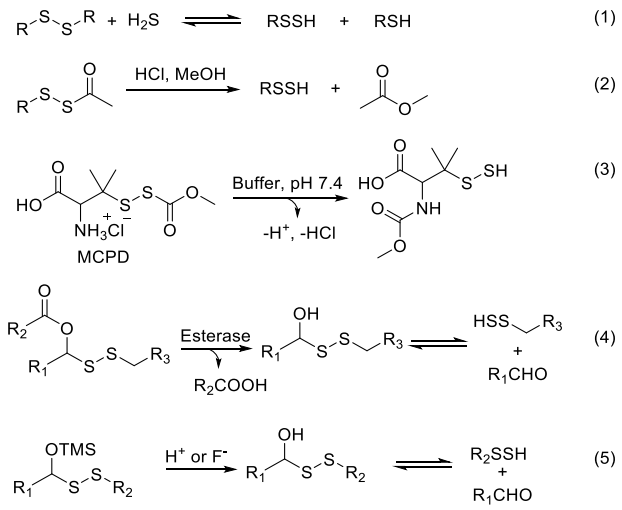
Department of Chemistry, Johns Hopkins University, Baltimore, MD 21218, USA

Supporting Information Placeholder

ABSTRACT: Due to their inherent instability, hydropersulfides (RSSH) must be generated *in situ* using precursors, but very few physiologically useful RSSH precursors have been developed to date. In this work, we report the design, synthesis, and evaluation of novel *S*-substituted thiosiothioureas as RSSH precursors. These water-soluble precursors show efficient and controllable release of RSSH under physiological conditions.

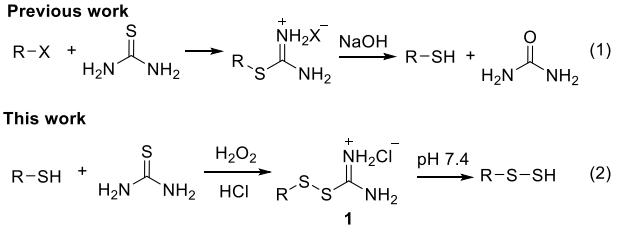
Hydrogen sulfide (H_2S) is a biologically important signaling molecule that exerts diverse roles in a myriad of physiological processes.[1-3] However, recent reports indicate that much of H_2S biological activity may be attributed to hydropersulfides (RSSH) and/or polysulfides (RSS_nR).[4-11] RSSH have been detected at significant levels in tissues, cells, and plasma and play a regulatory role in redox biology.[6, 12-13] RSSH are also excellent hydrogen transfer agents towards a variety of radicals.[14-15] Despite increasing evidence of the role of RSSH in redox signaling, the fundamental chemistry and biological roles of RSSH are still poorly understood. This deficiency is partly due to the unstable nature of RSSH. Although sterically hindered hydropersulfides are isolable in organic solvents,[16-17] they rapidly convert to a variety of products including trisulfides, tetrasulfides, elemental sulfur, and H_2S in aqueous solution.[17-19] Because of this inherent reactivity, RSSH are typically generated *in situ*. Previously reported generation strategies are summarized in Scheme 1.[18, 20-26] To advance our understanding of RSSH roles in biology, the development of new precursors that can cleanly and reliably produce RSSH under biological conditions is critical.

Scheme 1. Methods for Hydropersulfide Generation



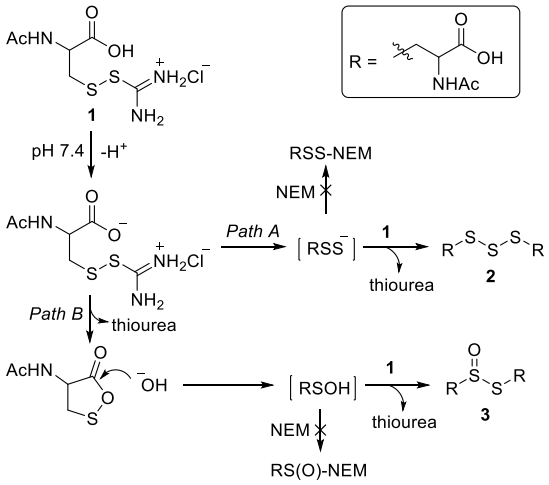
A general strategy for RSSH generation is to substitute the terminal sulfhydryl group with a suitable protecting group. The reaction of alkyl halides with thiourea is a commonly used method for thiol synthesis (Scheme 2, eq. 1).[27] This multistep one-pot process proceeds via the intermediacy of an isothiourea, which is then hydrolyzed under basic condition to produce the desired thiol and urea as a byproduct.[28-29] Based on this reaction, we thought to protect the terminal sulfhydryl group of RSSH in the form of an *S*-alkylthioisothiourea (Scheme 2, eq. 2), which can then potentially be deprotected under physiological condition to produce RSSH. We expected that the more acidic nature of RSSH vs. RSH should facilitate RSSH release.[7] In this design, thiols are used to construct the RSSH precursors in a convenient one-pot process. We also anticipated that different thiol and thiourea substituents could potentially affect the rates of deprotection, thereby offering tunability of RSSH release.

Scheme 2. Design of *S*-Substituted-Thioisothioureas as Hydropersulfide Precursors



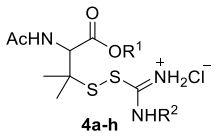
To test this hypothesis, we synthesized precursor **1** (Scheme 2, eq. 2),[30-31] and examined RSSH generation using *N*-ethylmaleimide (NEM) as a trap;[32] see Supporting Information (SI) for synthetic and analytical details. The RSS-NEM adduct was monitored by ultra-performance liquid chromatography–mass spectrometry (UPLC–MS). Incubation of **1** with NEM in pH 7.4 buffer at 37 °C for 30 min led to complete disappearance of **1**, with no evidence of persulfide-NEM adduct formation. Instead, formation of dialkyltrisulfide **2** was observed (SI, Figure S1). Precursor **1** does indeed produce RSSH; however, due to the more electrophilic nature of the precursor itself compared with NEM, RSSH reacts preferentially with **1** to produce trisulfide **2** (Scheme 3, Path A). In addition, we also observe thiosulfinate RS(O)-SR **3** as a major product (SI, Figure S1), suggesting an intramolecular cyclization mechanism and subsequent production of a sulfenic acid (RSOH) intermediate that is also trapped by precursor **1** (Scheme 3, Path B).

Scheme 3. Proposed Mechanism of Trisulfide 2 and Thiosulfinate 3 Formation from Precursor 1



To reduce reaction with the precursor, we examined precursor **4a**, equipped with an inhibiting dimethyl substituent alpha to the disulfide (Figure 1). UPLC-MS analysis shows formation of RSS-NEM **5a** (SI, Figure S12), confirming RSSH generation. In addition, we also observe RS(O)-NEM **6a** formation (SI, Figure S12), analogous to previous obervations,[33-34] indicating that the RSOH-producing reaction (Scheme 4, Path B) remains competitive with the desired production of RSSH (Path A).

Figure 1. Hydropersulfide Precursors 4a-h with Synthetic Yields

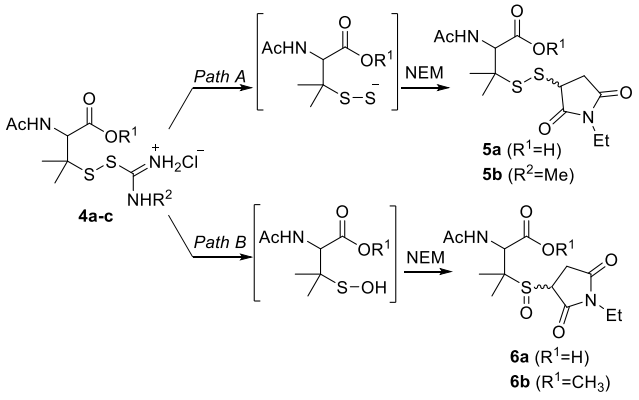


	R^1	R^2	% Yield
4a	H	H	83
4b	H	CH_3	85
4c	CH_3	H	63
4d	H	Ph	87

	R^1	R^2	% Yield
4e	H	4-MeOPh	58
4f	H	2-ClPh	78
4g	H	4-ClPh	53
4h	H	4-CF_3Ph	82

Next, precursor **4b**, equipped with methyl group on the nitrogen of the thiourea moiety, was synthesized to examine the effect of *N*-alkyl substituents on RSSH generation. The RSSH yield was reduced compared with that from **4a** (SI, Figure S18), suggesting that an alkyl substituent on nitrogen disfavors RSSH generation and RSOH generation remains the dominant reaction path.

Scheme 4. Proposed Mechanism of Precursors 4a-c Decomposition in the Presence of NEM



To prevent sulfenic acid generation from a carboxylic acid-mediated intramolecular cyclization, we examined precursor **4c** in which the carboxylic acid was masked as methyl ester. Although decomposition of **4c** in the presence of NEM produced no evidence of RS(O)-NEM **6b** formation, RSS-NEM adduct **5b** is formed in less than 50% yield with other products also observed (SI, Figure S23). These additional non-RSSH producing reaction pathways for **4c** are under further investigation.

Next, we examined precursor **4d** in which the thiourea is substituted with a phenyl group. Analysis of RSSH generation from **4d** in the presence of NEM shows clean conversion of **4d** to the RSS-NEM adduct **5a** with no evidence of the sulfenic acid-derived product **6a**. Based on the mechanism of isothiourea hydrolysis which produces alkyl thiol and urea (Scheme 2, eq. 1),[28-29] we expected *N*-phenylurea to be formed in high yield. However, UPLC-MS analysis showed no evidence for *N*-phenylurea, indicating that hydrolysis is not the operative mechanism for RSSH generation. Instead, phenyl cyanamide is observed in 93% yield (SI, Figures S27-30), indicating an elimination mechanism for RSSH generation (Scheme 5).[31, 35]

To gain additional insight into the mechanism of RSSH formation, **4d** was analyzed by UPLC-MS in acetonitrile. Here, we observe a peak at 0.66 min (Figure 2a, bottom trace). Immediately following introduction of **4d** to pH 7.4 buffer this peak disappears with concomitant appearance of a new peak at 4.2 min, which is assigned to the neutral form of **4d** (**4d**-H^+; see SI for experimental details, Figures S32-34). This result suggests that precursor **4d** rapidly undergoes neutralization at pH 7.4 to form an intermediate **4d**-H^+, which then decomposes to release RSSH and phenyl cyanamide. The ability of **4d** to release RSSH in the presence of thiols (likely to be present under physiological conditions) was also examined (SI). We observe formation of the RSSH-derived trisulfide as the major product. The anticipated disulfide product formed from reaction of **4d** with thiol is also detected, but only in minor amounts, confirming the ability of **4d** to release RSSH even in the presence of excess thiol (SI, Figures S67-73). RSSH generation from **4d** was also confirmed using iodoacetamide (IAM),[32, 36] another commonly used RSSH trap (SI, Figures S35-37). Together, these results confirm the improved ability of **4d** to produce RSSH.

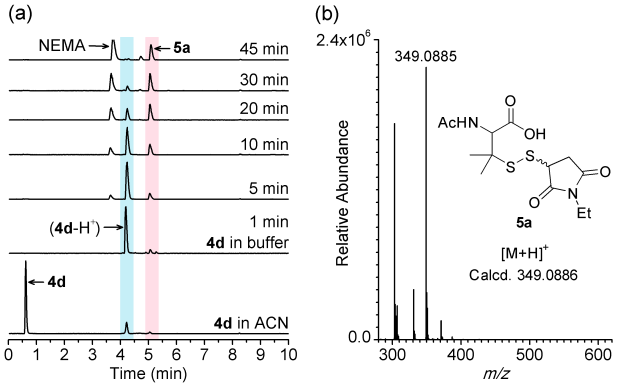


Figure 2. (a) UPLC-MS chromatograms of RSSH generation from **4d** (200 μM) in the presence of NEM (4 mM) incubated in pH 7.4 ammonium bicarbonate (100 mM) with the metal chelator DTPA (100 μM) at 37 ˚C. A peak at 3.7 min corresponding to *N*-ethylmaleamic acid (NEMA), derived from NEM hydrolysis, was observed under these conditions (see SI); (b) MS spectrum (ESI+ mode) of the product eluting at 5.06 min corresponding to **5a** with *m/z* 349.0885 (expected *m/z* 349.0886).

Next, we used ^{1}H NMR spectroscopy to monitor the kinetics of RSSH generation from **4d** by trapping with NEM in 10% D_2O, pH 7.4 buffer containing DMSO as an internal standard at 37 °C. An increase in the peak intensity attributed to one of the diasterotopic protons of the succinimide ring of the two product diastereomers (H_b, δ 2.99-2.91 ppm) was observed (Figure 3a). The other diasterotopic proton (H_c, δ 3.30-3.21 ppm) overlaps

with *N*-ethylmaleamic acid (NEMA), a product derived from NEM hydrolysis (SI, Figure S56).[37] Similarly, the intensity of two methyl group protons of precursor **4d** (δ 1.44 and 1.41 ppm) decreased as four methyl group protons of the two diastereomers of **5a** (H_a, δ 1.40, 1.37 ppm and 1.40, 1.34 ppm) increased, supporting the clean conversion of **4d** to **5a**. The ^{1}H NMR peak assignments for **5a** are consistent with the RSS-NEM adduct formed during *S*-methoxycarbonyl penicillamine disulfide (MCPD) decomposition (Scheme 1, eq. 3) in the presence of NEM (SI, Figure S57). The half-life of precursor **4d** at 37 °C is 5.6 min with a 96% yield of RSS-NEM adduct **5a** (Figure 3b).

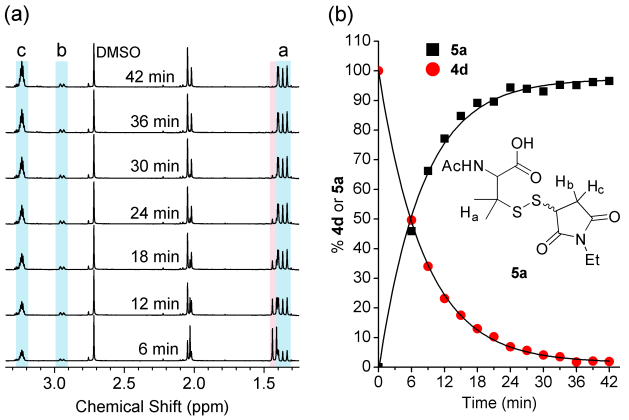


Figure 3. (a) Representative stacked ^{1}H NMR spectra showing the decomposition of precursor **4d** (1 mM) with NEM (20 mM) in 10% D_2O, phosphate buffer (PB), pH 7.4 at 37°C to produce RSS-NEM adduct **5a**. Precursor **4d** was introduced to a solution of NEM in PB at *t*=0 min and following equilibration and shimming, the first ^{1}H NMR spectrum was obtained after 6 min; (b) The kinetics of decomposition of **4d** (circles represent the -CH_3 protons (H_a) of the precursor) and formation of **5a** (squares represent the diasterotopic protons (H_b) of the succinimide ring of the two product diastereomers). The curves are calculated best fits to a single-exponential function (k = 0.12 min^{-1} and $t_{1/2}$ = 5.6 min for each fit).

Table 1. Hydropersulfide yields and half-lives for Precursors 4d-h

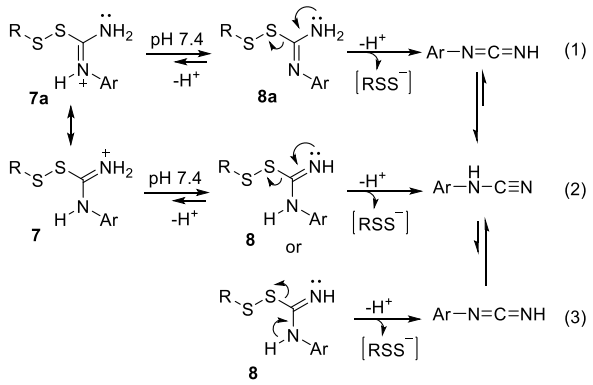
Precursor	R^1	R^2	Hydropersulfide Yield (%)[a]	$t_{1/2}$ (min)[b]
4d	H	Ph	96 ± 1	5.6 ± 0.3[c]
4e	H	4-MeOPh	75 ± 2	5.0 ± 0.5
4f	H	2-ClPh	95 ± 2	17.3 ± 0.4
4g	H	4-ClPh	91 ± 1	9.3 ± 0.6
4h	H	4-CF_3Ph	95 ± 2[d]	6.8 ± 0.2[d]

[a]RSSH yield was calculated based upon % precursor consumed. [b]RSSH precursors (1 mM) were incubated in the presence of NEM (20 mM) in pH 7.4 PB containing 10% D_2O at 37 °C. Reported data represent averages ± SD (n = 3). [c]The half-life of **4d** is 4.3 min in pH 7.4 buffer with 20% MeOD. [d]Reduced aqueous solubility required this experiment to be carried out in pH 7.4 buffer with 20% MeOD.

Precursor **4e**, equipped with a 4-OMe substituent, was synthesized to study the effect of an electron donating group on the phenyl ring. The half-life of **4e** was found to be 5.0 min (Table 1), suggesting no significant effect on the kinetics of RSSH generation. However, the RSSH yield (75%) was reduced and sulfenic acid-derived product **6a** is now observed (SI, Figures S38 and S58), suggesting that an electron donating group on the phenyl ring disfavors RSSH generation. In contrast, we find that electron withdrawing substituents maintain efficient RSSH generation, with a small effect on the kinetics of RSSH release. For example, 2-Cl substituted precursor **4f** produces RSS-NEM in 95% yield with a 17.3 min half-life, and 4-Cl substituted precursor **4g** produces the NEM adduct in 91% with a 9.3 min half-life. Similarly, 4-CF_3 substituted precursor **4h** produces 95% of RSS-NEM with a half-life of 6.8 min.

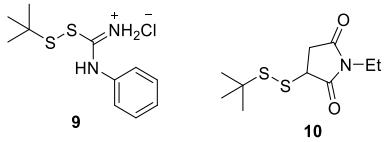
Based on the clean formation of hydropersulfide and aryl cyanamide during decomposition of **4d-h**, we propose the general mechanism shown in Scheme 5. Neutralization of *S*-alkyl-thioisothiourea **7** (or its resonance partner **7a**) occurs at pH 7.4 to produce a neutral species **8** and/or **8a**, which can further undergo an elimination reaction to produce RSSH and aryl cyanamide.[31, 35] The elimination reaction can be initiated by either donation of the imine-nitrogen lone pair of **8** to form aryl cyanamide and RSSH (Scheme 5, eq. 2) or deprotonation of the arylamino-nitrogen proton to form RSSH and an aryl carbodiimide, which can further undergo tautomerization to afford the aryl cyanamide (Scheme 5, eq. 3). Alternately, an elimination reaction can be initiated by donation of the nitrogen lone pair of intermediate **8a** to produce RSSH and aryl carbodiimide (Scheme 5, eq. 1).[38] We have preliminarily examined the decomposition of precursor **4d** at pH 6 and pH 8.5 (SI, Figures S64-S66). Decomposition is substantially slowed at lower pH and increased at higher pH, consistent with the mechanism proposed in Scheme 5. The reduced RSSH yield from methoxy-substituted **4e** suggests that an electron donating group on the phenyl ring disfavors neutralization to form **8**/**8a**, and as a result carboxylate anion attack of the disulfide to produce sulfenic acid can contribute. Slower RSSH generation from **4f**–**h** suggests that an electron withdrawing group on the phenyl ring might cause the nitrogen lone pair (e.g., in **8a**) to be less available for the elimination reaction. In addition, the thermodynamic stability of the aryl cyanamide produced could contribute to the observed rate of RSSH release.

Scheme 5. Proposed Mechanism of Hydropersulfide Generation from *S*-substituted Thioisothioureas



To demonstrate the generality of *S*-substituted thioisothioureas as hydropersulfide precursors, we also examined *tert*-butylthiol-based precursor **9** (Figure 4), which produces a near quantitative yield (97%) of RSS-NEM adduct **10** with a half-life of 17.3 min (SI, Figure S62). Slower RSSH generation observed from **9** compared with **4d** suggests additional influence of the *S*-alkyl substituent on RSSH generation.

Figure 4. Precursor 9 and RSS-NEM adduct 10



In summary, we have designed, synthesized, and evaluated *S*-substituted-thioisothioureas as RSSH precursors. These precursors show efficient and controllable release of RSSH under physiological conditions. We also demonstrated that RSSH generation can be tuned by structural modifications. Importantly, these precursors are found to be stable for several months on the bench-top in the solid state and for a few weeks in D_2O at room temperature (SI, Figure S63). Given the significance of RSSH in redox biology, we hope that these *S*-substituted thioisothioureas will find use as research tools to advance our understanding of RSSH chemical biology.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Experimental details and supplementary figures, S1−S84 (PDF)

AUTHOR INFORMATION

Corresponding Author

jtoscano@jhu.edu

Notes

The authors declare no competing financial interests.

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