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Communication

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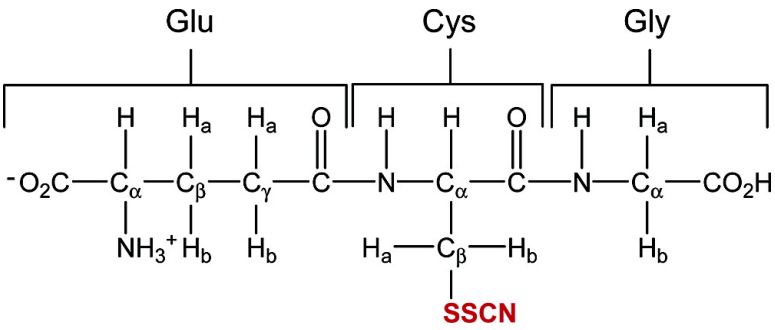

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Reactive Sulfur Species: Aqueous Chemistry of Sulfenyl Thiocyanates

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The familiar roles of thiols in cellular oxidant defense systems are altered when reactive conjugates are formed. These so-called reactive sulfur species (RSS)[1,2] can exhibit beneficial or deleterious biochemistry. While most RSS that have been studied to date are organic compounds, it has been suggested that the inorganic RSS hypothiocyanite ($OSCN^-$), an antimicrobial agent that is produced by myeloperoxidase,[3,4] eosinophil peroxidase,[5−8] lactoperoxidase,[9] and salivary peroxidase,[10] may target key protein sulfhydryl groups in pathogens, perhaps vis-à-vis sulfenyl thiocyanates (RSSCN). Sulfenyl thiocyanates are relatively esoteric compounds due to their general instability. Nonetheless, despite the aforementioned implication of RSSCN intermediates in the nonimmune defense system,[5,11] and similar proposed intermediates in the synthesis of polypeptides,[12] there exist no bona fide examples of such compounds in aqueous solution. We demonstrate herein that the equilibrium mixture $OSCN^- \rightleftarrows HOSCN \rightleftarrows (SCN)_2 \rightleftarrows (SCN)_3^-$,[13−15] hereafter collectively referred to as "hypothiocyanite", reacts with cysteine derivatives to yield sulfenyl thiocyanates. *These products represent the first organic species to be characterized that are derived from hypothiocyanite in water and the first water-soluble sulfenyl thiocyanates*.

Following our realization that *t*-BuSSCN[16] can be synthesized from $(SCN)_2$ in organic solvents and that it is sufficiently stable to permit isolation, we prepared the tertiary sulfenyl thiocyanate derivative of penicillamine (Scheme 1, PENSCN) in situ at low pH (eq 1, RSH = PEN; in this and subsequent formulas the sequence of addition of the reagents to acidified solutions is the order in which they appear in the equation).

$$\mathrm{RSH} + (\mathrm{SCN})_2 \xrightarrow{\mathrm{pH}=0} \mathrm{RSSCN} + \mathrm{H}^+ + \mathrm{SCN}^- \qquad (1)$$

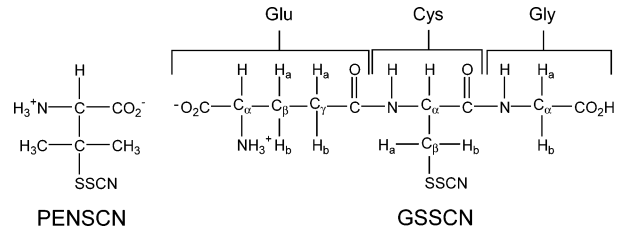
The thiocyanogen (10 mM, synthesized from $Pb(SCN)_2 + Br_2$ in CCl_4)[17] was extracted into 1 M HCl solutions of PEN (5 mM). Details concerning the procedures that were employed to effect the reactions we report in this communication are described in the SI. In planning our experiments, we incorrectly assumed a tertiary thiol was required to confer stability on RSSCN compounds. In fact, the glutathione (GSH) analogues can be synthesized under similar conditions (Scheme 1, eq 1). PENSCN and GSSCN were identified in situ by characteristic NMR, IR, and ES-MS spectra.

While the yields are high for reactions 1, the order of addition of the reagents is critical. Thus, if $(SCN)_2$ (10 mM) is first extracted into 1 M HCl and GSH (5 mM) is added immediately thereafter, a 1:1 mixture of disulfide and unreacted GSH is observed (eq 2).

$$(\mathrm{SCN})_2 + \mathrm{GSH} \xrightarrow{\mathrm{pH}=0} \mathrm{GSSG} \qquad (2)$$

It appears that the thiocyanating electrophile is short-lived in reaction 2. Remarkably, the longevity of this species can be increased substantially by adding SCN^-. Thus, when SCN^- (150 mM) is added to 1 M HCl, followed by $(SCN)_2$ (15 mM), GSH (5 mM) can be added 30 min later to produce quantitative yields of GSSCN (eq 3).

Scheme 1



$$\mathrm{SCN}^- + (\mathrm{SCN})_2 + \mathrm{GSH} \xrightarrow{\mathrm{pH}=0} \mathrm{GSSCN} \qquad (3)$$

We interpret the product distributions of experiments 1−3 in terms of equilibria 4 and 5 and reaction 6:

$$(\mathrm{SCN})_2 + \mathrm{H_2O} \rightleftharpoons \mathrm{SCN}^- + \mathrm{HOSCN} + \mathrm{H}+ \qquad (4)$$

$$(\mathrm{SCN})_2 + \mathrm{SCN}^- \rightleftharpoons (\mathrm{SCN})_3^- \qquad (5)$$

$$2\ \mathrm{HOSCN} \rightarrow \mathrm{SCN}^- + \mathrm{O_2SCN}^- + 2\ \mathrm{H}^+ \qquad (6)$$

We have performed preliminary stopped-flow kinetics measurements that demonstrate equilibria 4 and 5 are much faster than the kinetics of reaction 1 (RSH = GSH). Accordingly, we believe the higher yield of GSSCN at higher $[SCN^-]$ is due to the effect of SCN^- driving equilibrium 4 to the left, thereby diminishing the [HOSCN] and the rate of disproportionation 6. Cyanosulfite (O_2SCN^-) is generally believed to be a short-lived species in water (eq 6) so that, presumably, it eventually decomposes to give SO_4^{2-} and HNCO. The latter species, isocyanic acid, further reacts in 1 M HCl to give CO_2 and NH_4^+ in a reaction sequence that ultimately depletes the thiocyanating electrophile. It is clear that SCN^- has the effect of increasing the longevity of the thiocyanating electrophile, since the amount of GSSCN from reactions such as 3 with more modest concentrations of SCN^- ($[SCN^-]$ = 50 mM, $[(SCN)_2]$ = 15 mM, [GSH] = 5 mM in 1 M HCl) vary with the time that passes before addition of GSH (GSSCN = 100, 69, 52, 47, and 14% and GSSG = 0, 14, 25, 33, 71% for t = 1, 5, 10, 15, 30 min, respectively).

The aforementioned experiments indicate $(SCN)_2$, $(SCN)_3^-$, HOSCN, and/or $OSCN^-$ are the thiocyanating electrophiles. Thiocyanogen is certainly capable of producing sulfenyl thiocyanates in organic media, but what about $OSCN^-$ and HOSCN in aqueous solution? This is an important issue because eq 4 lies to the right under physiological conditions and only ca. 2% of the $OSCN^-$ is protonated at pH = 7. Since even small amounts of SCN^- will drive eq 4 to a mixture of $(SCN)_2$ and $(SCN)_3^-$ at pH = 0, conditions under which the hypothiocyanite equilibrium mixture

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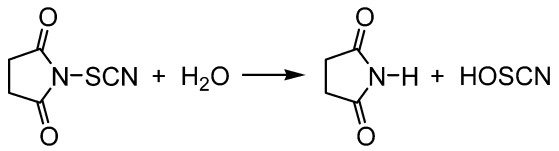
is relatively stable and can be studied, it is necessary to synthesize HOSCN in the absence of SCN^- to address this issue. We believe we have achieved such a reaction using the electrophilic thiocyanating agent, *N*-thiocyanatosuccinimide (NTS), a compound that has been proposed as an intermediate but heretofore never isolated.[18] We have developed a procedure for synthesizing NTS, apparently the first chemical species with an N−SCN bond to be structurally characterized by X-ray crystallography (SI). We have observed that NTS reacts with *t*-BuSH in organic solvents to yield *t*-BuSSCN. We have also observed that NTS dissolves in water to produce succinimide and what we assume to be initially $OSCN^-$/HOSCN (eq 7). Hydrolysis of NTS affords the first method of generating $OSCN^-$ at neutral pH in the absence of excess SCN^-.



(7)

Provided GSH (5 mM) is already present in 1 M HCl, NTS (15 mM) added as a solid produces GSSCN quantitatively (eq 8).

$$\mathrm{GSH} + \mathrm{NTS} \xrightarrow{\mathrm{pH}=0} \mathrm{GSSCN} \qquad (8)$$

It is conceivable that NTS reacts directly with GSH during reaction 8 (rather than proceeding via the hydrolysis of eq 7). However, we note that the hydrolysis of eq 7 and the equilibrium of eq 4 are evidenced by the fact that GSSCN is formed cleanly when NTS (15 mM) is added to a 1 M HCl solution containing SCN^- (150 mM) followed by GSH (5 mM), cf. eq 3. We note that there is no unreacted NTS; instead, succinimide is observed in these experiments prior to addition of GSH.

Turning our attention to the nature of the species that produce GSSG, as was the case for $(SCN)_2$, no GSSCN is produced if NTS is added first, followed by GSH (5 mM), but in this case the principal species remaining is unreacted GSH (6% and 20% GSSG were observed for 5 mM and 10 mM NTS, respectively) (eq 9). Thus, the oxidizing species is shorter lived when NTS is used as a precursor. This is consistent with the predomination of HOSCN in reaction 8 and more facile disproportionation (eq 6).

$$\mathrm{NTS} + \mathrm{GSH} \xrightarrow{\mathrm{pH}=0} \mathrm{N.R.} \qquad (9)$$

Lowering the concentration of HCl to 0.1 M is sufficient to diminish the longevity of the thiocyanating species as evidenced by lower yields of GSSCN and production of GSSG. For example, solutions of SCN^- (150 mM), NTS (15 mM), and GSH (5 mM) in 0.1 M HCl produce mainly GSSG (eq 10).

$$SCN^- + \{(SCN)_2 \text{ or NTS}\} + \mathrm{GSH} \xrightarrow{\mathrm{pH}=1} 70\text{−}80\%\ \mathrm{GSSG} + 20\text{−}30\%\ \mathrm{GSSCN} \qquad (10)$$

However, as was the case in experiments 1 and 8, if GSH is already present, high yields of GSSCN are produced. Thus, when NTS (15 mM) is added to a solution of GSH (5 mM) in 0.1 M HCl, only GSSCN is observed (eq 11).

$$\mathrm{GSH} + \mathrm{NTS} \xrightarrow{\mathrm{pH}=1} \mathrm{GSSCN} \qquad (11)$$

The origin of GSSG is not certain in that it might be produced by one-electron oxidation of GSH by species such as O_2SCN^- (eq 6) or by the condensation of GSSCN and GSH. Solutions of GSSCN (5 mM) in 1 M HCl react cleanly with one molar equivalent of GSH to yield GSSG (eq 12).

$$\mathrm{GSSCN} + \mathrm{GSH} \rightarrow \mathrm{GSSG} + SCN^- + H^+ \qquad (12)$$

Preliminary stopped-flow kinetic measurements demonstrate that reaction 12 is slow relative to reaction 1. Nonetheless, it is conceivable that reaction 12 becomes important if the reactants are depleted before all of the GSH is consumed. After carrying out several dozen measurements under different reaction conditions, we believe the production of GSSG in some cases is the result of a one-electron oxidant such as O_2SCN^- (or other decomposition products of HOSCN or $(SCN)_2$).

Therefore, what is the nature of the thiocyanate electrophile? For NTS, since no unreacted NTS, and instead succinimide, is observed in experiments 7, 9, and 10 prior to addition of GSH, we assume the hydrolysis of eq 7 has occurred. However, we are currently exploring the possibility that NTS reacts directly with GSH during reactions 8 and 11 (rather than proceeding via the hydrolysis of eq 7). When $(SCN)_2$ is the reagent, it is conceivable that HOSCN, $(SCN)_2$, or $(SCN)_3^-$ is the electrophile, or even some combination of these species. We are presently determining the rate law for reaction 3 to establish whether SCN^- facilitates formation of GSSCN or is in fact an inhibitor.

At pH = 0, 1, and 3, the lifetimes of GSSCN are ca. days, hours, and minutes, respectively. The mixture of products that are observed upon raising the pH, including disulfides, are consistent with the formation of intermediate sulfenic acids (RSOH).[19−21] We are currently exploring the product distributions of these reactions.

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Supporting Information Available: Experimental details and X-ray crystal structure data for NTS. This material is available free of charge via the Internet at http://pubs.acs.org.

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