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Generation of reactive oxygen species by a persulfide (BnSSH)

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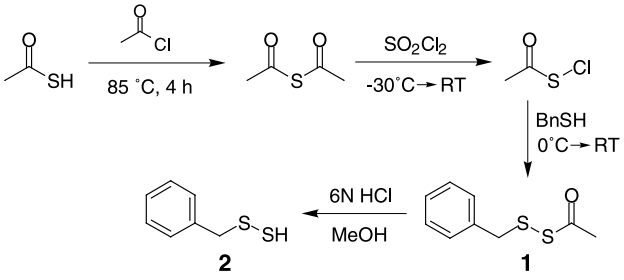
Abstract—Hydropersulfides (RS_xSH) have been implicated as important intermediates in the cell-killing action of the anticancer natural products leinamycin and varacin. It has been suggested that persulfides can mediate the conversion of molecular oxygen to reactive oxygen species ($O_2^{\bullet-}$, H_2O_2, and $HO^{\bullet}$). Here, experiments with synthetic benzyl hydrodisulfide (BnSSH) provide direct evidence that persulfides readily decompose to produce reactive oxygen species under physiologically relevant conditions.

Hydropersulfides (RS_xSH) have been implicated as key intermediates in the cell-killing action of the anticancer natural products leinamycin and varacin.[1–6] In each case, unstable persulfide intermediates are released by reaction of cellular thiols with the parent compound.[3,5–7] It has been suggested that hydropersulfides generate reactive oxygen species under biologically relevant conditions via the sequence of reactions shown in the (unbalanced) Eq. 1, where M^{n+} is a transition metal such as Fe^{2+}.[3,5] DNA damage caused by reactive oxygen species generated in this manner may contribute to the cytotoxic properties of leinamycin and varacin.[4,8]

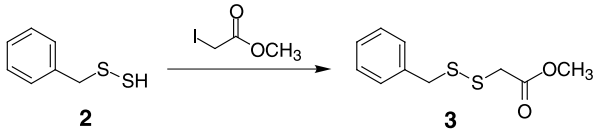
$$RSS^- + O_2 \rightarrow O_2^{\bullet-} \rightarrow H_2O_2 + M^{n+} \rightarrow HO^{\bullet} + M^{(n+1)+} \quad (1)$$

To better understand the biological properties of hydropersulfide-producing natural products such as varacin and leinamycin, we set out to prepare a synthetic persulfide and characterize its ability to produce DNA-damaging reactive oxygen species. Our preparation of benzyl hydrodisulfide (**2**) followed the general route described previously by Derbesy and Harpp for the synthesis of *t*-butyl hydrodisulfide (Scheme 1).[9] Thus, acetyl sulfenyl chloride, prepared from thioacetic anhydride and sulfuryl chloride, was reacted with benzyl mercaptan to afford acetyl benzyl disulfide (**1**, Scheme 1).[10] Treatment of this compound with 6 N HCl results in removal of the acetyl protecting group to afford benzyl hydrodisulfide (**2**).[11] The hydrodisulfide **2** was characterized by NMR, mass spectroscopy, and by derivatization with methyl iodoacetate to yield **3** (Scheme 2).



Scheme 1.

We employed a plasmid-based assay to characterize the DNA-cleaving properties of benzyl hydrodisulfide (**2**). In this assay, single-strand cleavage converts supercoiled double-stranded plasmid (form I) into the open circular form (II). The two forms of plasmid DNA were separated using agarose gel electrophoresis and visualized by staining with ethidium bromide.[12] We found that



Scheme 2.

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incubation of micromolar concentrations of **2** with supercoiled double-stranded plasmid DNA leads to the generation of single-strand breaks (Fig. 1). In contrast, benzyl mercaptan (BnSH) induces little or no strand cleavage under these conditions (data not shown).

To shed light on the chemical mechanism of DNA damage by **2**, we performed cleavage assays in the presence of various additives that are known to have an effect on strand breaks arising from the reactions shown in Eqs. 2–6. We find (Table 1) that DNA cleavage by **2** is inhibited by the radical scavengers methanol, ethanol, and DMSO, by the hydrogen peroxide-destroying enzyme catalase, and by the chelators of adventitious trace metals, diethylenetriaminepentaacetic acid (DETAPAC) and desferal, which are known to inhibit the metal-dependent Fenton reaction (Eq. 6).[13] Addition of the enzyme superoxide dismutase (SOD), which catalyzes the disproportionation of superoxide radicals to hydrogen peroxide (Eq. 5) and molecular oxygen does not inhibit DNA cleavage. Other instances where SOD does not inhibit DNA strand cleavage stemming from superoxide radical production have been reported.[3,5,12,14–16] Overall, the results indicate that benzyl hydrodisulfide causes DNA strand cleavage via the cascade of reactions shown in Eqs. 2–6, in which hydroxyl radical is produced as the ultimate DNA-cleaving agent. Hydroxyl radical is a well-known DNA-cleaving agent.[17] DNA damage caused by **2** shows the same mechanistic signature seen for the hydropersulfide-generating natural products leinamycin and varacin.[3,5,12,18] Perthiyl radicals (Eq. 3) may combine with molecular oxygen to yield perthioperoxyl and sulfonyl radical intermediates;[19] however, our findings provide no indication that such species contribute to DNA strand cleavage by **2**.

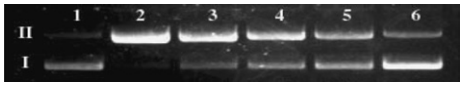

Figure 1. DNA cleavage by benzyl hydrodisulfide (**2**). Supercoiled pBR322 DNA (38 μM bp) was incubated for 12 h at 37 °C with various concentrations of **2** in sodium phosphate buffer (50 mM, pH 7) containing 10% acetonitrile (by volume). Reaction conditions (e.g., incubation times) were selected, in part, to allow comparison with our previous results.[3] Solutions were prepared using glass distilled, deionized water and 99+% pure sodium phosphate salts (no transition metals were added to the reactions). Reactions were 20 μL final volume and were conducted under a headspace of air in sealed 1.5 mL Eppendorf tubes. Agarose gel electrophoresis was performed as described previously.[12] The numbers in parentheses following the description of each lane below indicates the *S*-value (mean number of strand breaks per plasmid molecule) for each lane and was calculated using the equation $S = -\ln f_I$, where f_I is the fraction of plasmid in a given lane that is present as uncut, form I DNA. Values reported here represent the average of at least two experiments, and the standard error in these measurements is less than 2%. Lane 1, DNA alone (0.2); lane 2, 1 mM **2** (3.4); lane 3, 500 μM **2** (1.9); lane 4, 100 μM **2** (1.2); lane 5, 50 μM **2** (0.9); lane 6, 10 μM **2** (0.3).

Table 1. Effect of additives on DNA cleavage by compound **2**[a].

Reaction/additive	% Nicked, form II DNA	*S*-value[b]
DNA alone	36	0.44
Std rxn: BnSSH, **2** (100 μM)	99	4.6
Std rxn + additive:		
Methanol (1 M)	44	0.57
Ethanol (1 M)	53	0.76
DMSO (1.4 M)	26	0.29
Catalase (100 μg/mL)	45	0.59
SOD (100 μg/mL)	100	—
Desferal (5 mM)	50	0.50
DETAPAC (10 mM)	40	0.69

[a] Reactions were carried out as described in the legend of Figure 1.
[b] The *S*-value is the mean number of strand breaks per plasmid molecule and is calculated using the equation: $S = -\ln f_I$, where f_I is the fraction of uncut, form I DNA remaining.

$$RSSH \rightleftharpoons RSS^- + H^+ \quad (2)$$

$$RSS^- + M^{(n+1)+} \rightarrow RSS^{\bullet} + M^{n+} \quad (3)$$

$$M^{n+} + O_2 \rightarrow M^{(n+1)+} + O_2^{\bullet -} \quad (4)$$

$$O_2^{\bullet -} + HO_2^{\bullet} \rightarrow H_2O_2 + O_2 \quad (5)$$

$$H_2O_2 + M^{n+} \rightarrow HO^{\bullet} + HO^- + M^{(n+1)+} \quad (6)$$

Analogous to the autooxidation of thiols,[20–22] it is likely that the production of superoxide radical by BnSSH proceeds via initial oxidation of the persulfide anion ($BnSS^-$) by adventitious traces of transition metals present in the aqueous buffer (Eq. 3), followed by reaction of the reduced metal ion with molecular oxygen to produce superoxide (Eq. 4). The persulfide radical formed as part of this process is thought to be a relatively stable species.[19,23–25] In addition, it is important that hydrodisulfides are markedly more acidic than the corresponding thiol, meaning that a larger fraction exists in the reactive, anionic form.[23,26] Indeed, our NMR and HPLC experiments show that **2** is oxidized within minutes (<5 min) to a mixture of di-, tri-, tetra-, and pentasulfides in aerobic sodium phosphate buffer (pH 7) at room temperature. In contrast, benzyl mercaptan is oxidized slowly under these conditions (~7% oxidized after 6 days). Consistent with the expected[27] role for transition metals in persulfide oxidation, we find that **2** oxidizes relatively slowly in an aerobic solution of $CDCl_3$ (71% oxidized after 12 h) and that oxidation is markedly accelerated by addition of catalytic amounts of $FeCl_3$ to the solution (76% oxidized after 6.5 h).

$$GSSH + GSH + O_2 \rightarrow GSSSG + O_2^{\bullet -} \quad (7)$$

$$GSSSG + GSH \rightarrow GSSH + GSSG \quad (8)$$

Importantly, in the presence of physiological concentrations of thiol,[28] small amounts of persulfide have the potential to yield significant levels of oxidative stress (production of reactive oxygen species *and* depletion of

cellular thiols) via the redox-cycle shown in (unbalanced) Eqs. 7 and 8, where persulfide serves as a *catalyst* for the thiol-mediated conversion of molecular oxygen to superoxide radical.[29–31] Studies are currently underway to characterize the catalytic properties of persulfides.

In summary, our work with synthetic benzyl hydrodisulfide shows that the persulfide functional group readily decomposes to yield reduced oxygen species under physiologically relevant conditions.[32–40] The cell-killing properties of reactive oxygen species are well known.[13,41–44] Overall, the findings reported here may afford a deeper understanding of the mechanisms behind the biological activity of leinamycin and host of polysulfide-containing natural products such as varacin,[4] lissoclinotoxin A,[45] diallyl trisulfide,[46–48] bis(2-hydroxyethyl) trisulfide,[49] leptosin,[50] and sirodesmin,[51] that yield persulfide anions upon reaction with cellular thiols.[6]

Acknowledgment

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References and notes

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