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Possible chemical mechanisms underlying the antitumor activity of *S*-deoxyleinamycin

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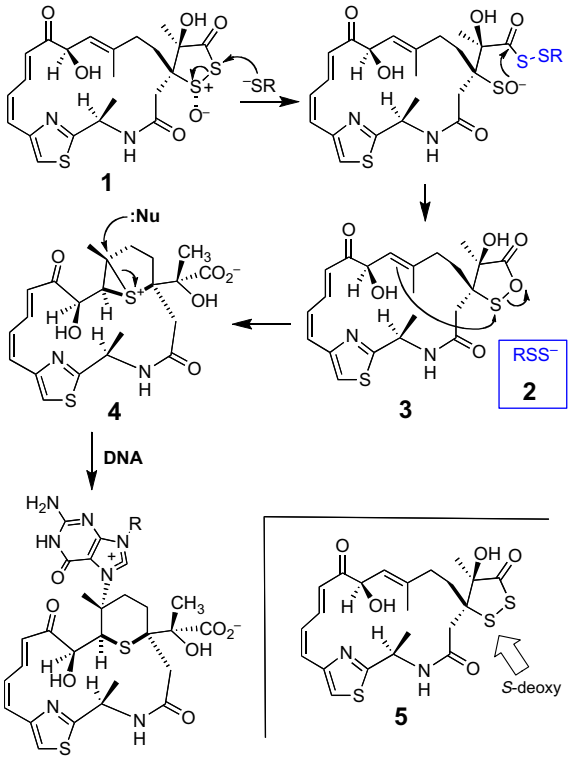
Abstract—Though less potent than the parent natural product leinamycin, *S*-deoxyleinamycin displays activity against human cancer cell lines that is comparable to many clinically used agents. The results reported here suggest that the 1,2-dithiolan-3-one heterocycle found in *S*-deoxyleinamycin reacts with thiols to generate a persulfide intermediate (RSS^-) that could deliver biologically active polysulfides, hydrogen sulfide, and reactive oxygen species ($O_2^{\cdot -}$, H_2O_2, and $HO^{\cdot}$) to the interior of cells.

Leinamycin (**1**) is a structurally interesting natural product that displays impressive, nanomolar IC_{50}-values against human cancer cell lines.[1–5] Reaction of thiols with leinamycin leads to ejection of a persulfide intermediate (**2**) that generates cell-killing reactive oxygen species (Scheme 1).[6–9] In addition, the 1,2-oxathiolan-5-one derivative (**3**) formed in this reaction undergoes further rearrangement to an episulfonium ion (**4**) that efficiently alkylates guanine residues in duplex DNA.[6,10] The resulting 7-alkylguanine residues undergo rapid depurination to generate a burst of cytotoxic abasic sites in duplex DNA.[11–13] The reaction with thiols may be central to the potent biological activity of leinamycin because cells contain millimolar concentrations of the thiol-containing tripeptide glutathione[14] that can trigger the release of cell-killing reactive intermediates from this natural product.

In light of the central role played by leinamycin's sulfoxide oxygen in the generation of cytotoxic reactive intermediates (Scheme 1), it is not surprising that *S*-deoxyleinamycin (**5**) is markedly less cytotoxic than leinamycin. For example, **5** displays an IC_{50} value of 4 μM against HeLa S3 cells, whereas that for leinamycin is 27 nM.[3] Despite its diminished cytotoxicity relative to leinamycin, it is important to note that the activity of **5** is comparable to some clinically used anticancer drugs.[15] Accordingly, studies of **5** have the potential to reveal new chemical processes by which organic molecules can elicit anticancer activity.



Scheme 1.

Keywords: Antitumor agent; Leinamycin; DNA-damage; Hydrogen sulfide; Polysulfide; Thiol-triggered; Strand cleavage; Superoxide radical.
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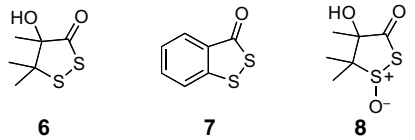
Previous work has shown that **5** does not cause thiol-triggered DNA alkylation;[3] however, our previous experience with related sulfur heterocycles[16–19] led us to consider the possibility that reactions of thiols with the 1,2-dithiolan-3-one heterocycle in **5** might lead to the generation of superoxide radical ($O_2^{\cdot-}$). Under physiological conditions, superoxide radical decomposes to hydrogen peroxide and, ultimately, hydroxyl radical as shown in the (unbalanced) Eqs. 4 and 5, where M represents a transition metal such as iron or copper.[20] Intracellular production of superoxide radical has a variety of deleterious consequences including oxidative damage to DNA, proteins, and lipids and cytotoxicity.[20–28]



6 **7** **8**

To begin these studies, we synthesized 4-hydroxy-4,5,5-trimethyl-1,2-dithiolan-3-one (**6**) using a modified version of the route described by Pattenden and Shuker.[29] We used a plasmid DNA-cleavage assay[30–33] to characterize the ability of **6** to produce superoxide radical. Superoxide radical causes DNA cleavage via a cascade of reactions shown in Eqs. 4 and 5, in which hydroxyl radical is the ultimate DNA-cleaving agent.[20,34] Hydroxyl radical generates direct strand breaks via abstraction of hydrogen atoms from the 2′-deoxyribose backbone of DNA.[35–40] In the assay used here, single-strand cleavage converts supercoiled double-stranded plasmid (form I) into the open circular form (II). The two forms of plasmid DNA are then separated by agarose gel electrophoresis and visualized by staining with a DNA-binding dye such as ethidium bromide.[30–33]

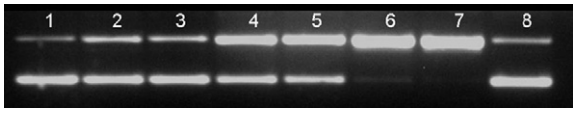


Figure 1. Thiol-dependent DNA cleavage by various concentrations of 1,2-dithiolan 3-one (**6**). Supercoiled pGL2 basic DNA (76 μM bp) was incubated for 12 h at 37 °C with various concentrations of **6** and 15 equiv of 2-mercaptoethanol in sodium phosphate buffer (50 mM, pH 7) containing 10% acetonitrile by volume. Solutions were prepared using glass distilled, deionized water and 99+% pure sodium phosphate salts. Reactions consisted of 20 μL final volumes and were conducted under a headspace of air in sealed 500 μL microcentrifuge tubes. Agarose gel electrophoresis was performed as described previously.[7] The upper band in the gel is nicked form II DNA and the lower band is uncut, form I DNA. The values 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 $S = -\ln f_1$, where f_1 is the fraction of plasmid in a given lane that is present as uncut, form I DNA.[41] Values reported here represent the average of at least three experiments, and the standard error in these measurements is less than 2%. Lane 1, DNA alone (0.16); lane 2, 500 μM **6** alone (0.46); lane 3, 10 μM **6** + 150 μM thiol (0.31); lane 4, 50 μM **6** + 750 μM thiol (0.63); lane 5, 100 μM **6** + 1.5 mM thiol (0.84); lane 6, 250 μM **6** + 3.75 mM thiol (2.83); lane 7, 500 μM **6** + 7.5 mM thiol (4.54); lane 8, 7.5 mM thiol alone (0.21).

We find that incubation of micromolar concentrations of **6** with supercoiled double-stranded plasmid DNA in sodium phosphate buffer (50 mM, pH 7, containing 10% CH_3CN) along with 15 equiv of thiol (2-mercaptoethanol) leads to the generation of single-strand breaks (Fig. 1). Under these reaction conditions, compound **6** alone (no thiol) or thiol alone (without **6**) elicits relatively small amounts of strand cleavage. Other thiols, including the biological thiol glutathione, similarly trigger DNA strand cleavage by **6** (data not shown).[42] For comparison, we note that the structurally related molecule **7** generates relatively weak thiol-triggered DNA cleavage under these conditions. On the other hand, the leinamycin analogue **8** displays superior thiol-dependent DNA-cleaving properties (Table 1). Both of these results are in line with previous reports regarding thiol-triggered DNA-cleaving properties of **7** and **8**.[7]

The direct strand breaks detected in these assays are consistent with the anticipated generation of reactive oxygen species (Eqs. 1–5). To further investigate the role of $O_2^{\cdot-}$, H_2O_2, and $HO^{\cdot}$ in thiol-triggered strand cleavage by **6**, we performed a series of cleavage assays in the presence of additives that interact with various species shown in Eqs. 4 and 5.[20] We find that thiol-triggered DNA cleavage by **6** is inhibited by the classic hydroxyl radical scavengers methanol, ethanol, and DMSO (Table 1). Addition of the hydrogen peroxide-destroying enzyme catalase also inhibits strand cleavage. Likewise, DNA cleavage is effectively prevented by the presence of the chelators of adventitious trace metals, diethylenetriaminepentaacetic acid (DETAPAC) and desferal, which inhibit the trace metal-dependent Fenton reaction (Eq. 5).[20] Interestingly, addition of superoxide dismutase (SOD) significantly increases the yield of DNA strand breaks. SOD catalyzes the disproportionation

Table 1. Effect of additives on thiol-mediated DNA cleavage by compound **6**[a]

Reaction/additive	% Nicked, form II DNA	*S*-value[b]
DNA Alone	13.5	0.14 ± 0.01
6 alone (150 μM, no thiol)	18.2	0.2 ± 0.01
Std rxn: **6** (150 μM) + thiol (2.25 mM)	73.1	1.32 ± 0.16
Std rxn + additive		
Methanol (1 M)	27.5	0.32 ± 0.05
Ethanol (1 M)	19.0	0.21 ± 0.02
Desferal (10 mM)	25.9	0.30 ± 0.01
DETAPAC (10 mM)	19.0	0.21 ± 0.02
DMSO (1 M)	15.5	0.17 ± 0.02
Catalase (100 μg/mL)	19.3	0.21 ± 0.02
SOD (100 μg/mL)	100	—
SOD (50 μg/mL)	100	—
7 (150 μM) + thiol (2.25 mM)	33.1	0.40 ± 0.01
8 (150 μM) + thiol (2.25 mM)	92.5	2.58 ± 0.12

[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: $S = -\ln f_1$, where f_1 is the fraction of uncut, form I DNA remaining, where % form $I = 100 - \%$ form II, and the experiments were conducted in a manner such that only form I and II DNA were present (no form III observed).

of $O_2^{\cdot-}$ to H_2O_2 and O_2.[20] This catalytic process may increase the yield of H_2O_2, thus increasing the yields of strand cleavage. In addition, the reaction catalyzed by SOD increases the concentration of dissolved oxygen (O_2) in the assays, an effect that may facilitate both the generation of additional $O_2^{\cdot-}$ and oxygen-dependent production of DNA strand breaks.[35–40,43,44] Other possible origins of the SOD effect are discussed below. Overall, the evidence clearly indicates that the reaction of thiol with **6** leads to the generation of the reactive oxygen species $O_2^{\cdot-}$, H_2O_2, and $HO^{\cdot}$.

$$RSSH \rightleftharpoons RSS^- + H^+ \quad (1)$$

$$RSS^- + M^{(n+1)+} \rightarrow RSS^{\cdot} + M^{n+} \quad (2)$$

$$M^{n+} + O_2 \rightarrow M^{(n+1)+} + O_2^{\cdot-} \quad (3)$$

$$O_2^{\cdot-} + HO_2^{\cdot} \rightarrow H_2O_2 + O_2 \quad (4)$$

$$H_2O_2 + M^{n+} \rightarrow HO^{\cdot} + HO^- + M^{(n+1)+} \quad (5)$$

$$RSS^{\cdot} + RS^- \rightarrow RSSSR^{\cdot-} \quad (6)$$

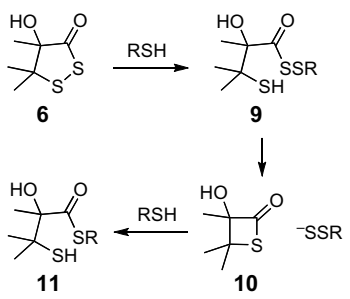
$$RSSSR^{\cdot-} + O_2 \rightarrow RSSSR + O_2^{\cdot-} \quad (7)$$

$$RSH + RSSSR \rightarrow RSS^- + RSSR \quad (8)$$

$$RSSH + RSH \rightarrow RSSR + H_2S \quad (9)$$

$$HS^- + O_2 \xrightarrow{SOD} S^{\circ} + H_2O_2 \quad (10)$$

To gain insight into the chemical nature of the processes that might be responsible for the generation of superoxide radical ($O_2^{\cdot-}$) in these experiments, we examined the reaction of thiols with **6**. Incubation of **6** with 2-mercaptoethanol (5 equiv) in sodium phosphate buffer (50 mM, pH 7, containing 20% CH_3CN) led to the generation of the thioester **11** (54%, Scheme 2). In addition, we observed the production of disulfide and polysulfides (RSSR, RSSSR, and RSSSSR).[45]



Scheme 2.

The formation of polysulfides may provide a key to understanding the thiol-dependent production of ROS in the reaction of thiols with **6**. This result is informative because equilibrating mixtures of polysulfides have previously been observed as characteristic byproducts generated in the thiol-triggered production of reactive oxygen species ($O_2^{\cdot-}$, H_2O_2, and $HO^{\cdot}$) by the leinamycin analogue **8**.[6–9] Polysulfides result from the oxidation of a persulfide intermediate (RSS^-) that is released from **8** by its reaction with thiols.[6–9] Analogous to the autooxidation of thiols,[46] this reaction likely involves metal-mediated oxidation of the sulfur anion (Eq. 2). Importantly, in aerobic solution, this process generates $O_2^{\cdot-}$ (Eqs. 2 and 3). The resulting persulfide radical ($RSS^{\cdot}$), again in analogy with mechanisms established for the oxidation of thiols,[47–49] may react with thiolate (RS^-) to generate the polysulfide radical anion (Eq. 6). Oxidation of such a radical anion is anticipated[47–49] to generate a polysulfide along with superoxide radical (Eq. 7). The polysulfide byproducts then undergo further reaction with thiol to regenerate a persulfide (RSS^-, Eq. 8) and additional superoxide radical. Via this series of reactions, small amounts of persulfide intermediates have the potential to act as catalysts that convert substantial amounts of thiol into disulfide and reactive oxygen species.

Persulfides also are expected to react with thiols to release hydrogen sulfide (H_2S, Eq. 9).[17,50] Indeed, we qualitatively detected H_2S produced in the reaction of 2-mercaptoethanol with **6**. Hydrogen sulfide was determined as the characteristic lead sulfide precipitate resulting from its reaction with lead acetate.[51] Neither **6** alone or thiol alone generated significant quantities of H_2S under the conditions employed here. It seems likely that thiol-mediated generation of H_2S contributes to the generation of reactive oxygen species and to the biological activities of polysulfides and persulfides.[52–57]

The presence of H_2S in our DNA-cleavage assays may contribute to the substantial increase in the yield of strand breaks observed upon addition of SOD (Table 1). Typically, SOD catalyzes the disproportionation of two molecules of $O_2^{\cdot-}$ to H_2O_2 and O_2.[20] However, it has been reported that this enzyme can act as an HS^-:O_2 oxidoreductase that converts HS^- and O_2 into H_2O_2 and S° (Eq. 10, where S° is defined as elemental sulfur and related species in which sulfur is bonded to sulfur).[58] Under the reaction conditions employed here, elemental sulfur, in the form S_8, is expected[17,59] to react with thiol to generate additional persulfides (RS_xS^-) that, in turn, generate additional superoxide radical. In addition, Cu, Zn-SOD has the potential to act as an oxidase that converts thiols in the assay mixture to disulfide and H_2O_2.[60,61] Together the thiol- and H_2S-oxidase properties[58,60] of Cu, Zn-SOD could explain the increases in strand cleavage observed upon addition of SOD to the assays described here.

Several different mechanisms can be formulated to explain the products identified in the reaction of **6** with thiol. We favor the pathway shown in Scheme 1 involving initial attack of thiol on the sulfur atom adjacent to the carbonyl group in **6** to yield the ring-opened 3-mercaptoperthioester **9**. Intramolecular cyclization of the

thiol residue in **9** onto the carbonyl group will generate the thiolactone **10** and eject a persulfide ($HOCH_2CH_2SS^-$). The persulfide goes on to generate reactive oxygen species, H_2S, and polysulfides as discussed above. Finally, reaction of excess thiol with the thiolactone **10** is expected to yield the observed product **11**. Consistent with this proposed mechanism, the thiolactone (**10**) was observed by thin layer chromatography as an intermediate in the reaction of **6** with 2 equiv of thiol. In addition, we found that reaction of an authentic, synthetic sample of thiolactone (**10**, prepared by the route of Pattenden and coworkers)[29] with 2-mercaptoethanol does, in fact, generate **11** under the conditions used for this model reaction.[62]

In conclusion, we find that the reaction of thiols with the 1,2-dithiolan-3-one heterocycle found in *S*-deoxyleinamycin leads to the generation of reactive oxygen species. We propose that this occurs via thiol-triggered release of a persulfide intermediate (RSS^-). This is analogous to the previously characterized release of persulfides from leinamycin and its synthetic analogues,[6–9] although the overall production of DNA-cleaving oxygen radicals is less efficient in the case of **6** than it is for **8**. Nonetheless, the thiol-mediated generation of oxidative stress by compound **6** provides a plausible molecular basis for the substantial activity of *S*-deoxyleinamycin against cancer cell lines, as it is well known that intracellular generation of reactive oxygen species can have cytotoxic consequences.[20–28] Furthermore, agents capable of thiol-triggered release of persulfides can display potent biological activity.[1,9,16] Of course, the current study does not rule out the possibility that functional groups other than the 1,2-dithiolan-3-one moiety may contribute to the cytotoxicity of *S*-deoxyleinamycin. For example, the α, β, γ, δ-unsaturated carbonyl residue has the potential to react with various cellular nucleophiles. Alternatively, it is possible that metabolism in mammalian cell lines converts *S*-deoxyleinamycin into leinamycin via an enzymatic sulfoxidation process analogous to that proposed for the final step in the biosynthesis of the natural product.[63,64] Finally, it is of general interest in the fields of medicinal chemistry and toxicology to identify molecules that generate reactive intermediates selectively upon entering the thiol-rich environment inside cells.[65] Drawing inspiration from *S*-deoxyleinamycin, we find that the 1,2-dithiolan-3-one heterocycle may provide a new strategy for delivery of biologically active polysulfides, hydrogen sulfide, and reactive oxygen species to the interior of cells.

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45. To a stirred solution of compound **6** (20 mg, 0.112 mmol) in water (410 μL), and acetonitrile (450 μL) was added sodium phosphate buffer (1350 μL, 500 mM, pH 7.5) and 2-mercaptoethanol (40 μL) and the resulting colorless solution was stirred at 25 °C (final concentrations: **6**, 50 mM; buffer, 300 mM; thiol, 250 mM; acetonitrile, 20% by volume). The reaction was allowed to stir for 1 h as the disappearance of the starting material was monitored using TLC (1:1 hexane/EtOAc). The product was extracted into diethyl ether (3 × 5 mL) and the combined organic extracts dried over anhydrous sodium sulfate, filtered, and evaporated to yield a yellow oil, which was then purified by a flash column chromatography (7:3 hexane/EtOAc) to yield compound **9** (13.4 mg, 54%) as colorless oil. $R_f = 0.25$ (7:3 hexane/EtOAc). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.46 (3H, s), 1.48 (3H, s), 1.53 (3H, s), 1.86 (1H, t, OH), 1.97 (1H, s), 3.05 (2H, m), 3.35 (1H, s), 3.77 (2H, m); ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ 205.76, 84.12, 61.69, 52.37, 31.68, 28.54, 27.33, 21.90. MS (ESI) $[M+H]^+$ 224.90. Detection of polysulfides in the reaction of **6** with 2-mercaptoethanol was carried out as follows: **6** was incubated (10 μL of a 10 mM stock in CH_3CN) at 25 °C in a mixture of sodium phosphate buffer (50 μL, 500 mM, pH 7), water (333 μL), and acetonitrile (90 μL). To this was added 2-mercaptoethanol (17 μL of a 30 mM stock) as the final component. The mixture (final concentrations: **6**, 200 μM; buffer, 50 mM, pH 7; thiol, 1 mM; acetonitrile, 20% by volume) was vortex mixed and analyzed by reverse phase HPLC using a previously reported method.[8,9,16] An authentic sample of polysulfides was generated by reaction of thiol with *3H*-1,2-benzodithiol-3-one 1-oxide.[6,8]
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61. Control experiments show that, under the experimental conditions employed here, addition of SOD (100 μg/mL) increases strand cleavage by 2-mercaptoethanol (5 mM) by 45% (an additional 0.59 breaks per plasmid) and that by H_2S (~0.5 mM) by 29% (0.34 breaks per plasmid). Addition of SOD alone does not generate any strand breaks above background.
62. An alternative pathway to the thiolactone **10** involving generation and subsequent cyclization of a 3-mercaptothioacid derivative can be envisioned. This pathway seems unlikely because 3-mercaptothioacids are stable, isolable molecules that are not observed to generate a thiolactone.[29] On a separate note, the inability of **7** to generate strand cleavage may be explained by the inaccessibility and/or instability of the corresponding thiolactone in the benzo series: Mitra, K.; Gates, K. S. *Tetrahedron Lett.* **1995**, *36*, 1391; Mitra, K.; Pohl, M. E.; MacGillivray, L. R.; Barnes, C. L.; Gates, K. S. *J. Org. Chem.* **1998**, *62*, 9361.
63. Along these lines, it is worth noting that, in a control there is no evidence that **6** converts spontaneously to **8** under the reaction conditions employed for these studies. Specifically, the reaction of thiol with **6** is complete inside of 1 h. Within this timeframe, in the absence of thiol, there is no detectable oxidative or hydrolytic dcomposition of **6**.
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