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Reaction of Thiols with 7-Methylbenzopentathiepin

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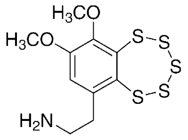
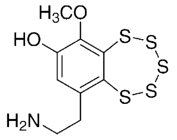
Abstract—Polysulfides typically react readily with thiols, thus, reactions of endogenous cellular thiols with the polysulfide linkage in naturally-occuring pentathiepin cytotoxins are likely to be an important aspect of their biological chemistry. Here, it is reported that the reaction of thiols with the pentathiepin ring system initially produces a complex mixture of polysulfides that further decomposes in the presence of excess thiol to yield the corresponding 1,2-benzenedithiol with concomitant production of H_2S and dimerized thiol. In this reaction, a single molecule of the pentathiepin consumes approximately six equivalents of thiol. The reaction of thiols with the pentathiepin ring system is faster than the analogous reaction involving typical di- and trisulfides.

The naturally-occurring antibiotics varacin (**1**), lissoclinotoxin A (**2**), and 5-(methylthio)varacin (**3**) possess an unusual cyclic polysulfide (pentathiepin) ring system that is required for biological activity.[1–3] The observed hypersensitivity of DNA-repair deficient cell lines to the cytotoxic action of varacin provided early evidence that this compound may derive its activity through DNA damage.[1] Consistent with this observation, subsequent studies demonstrated that 7-methylbenzopentathiepin[4] and the natural product varacin[5] react with thiols to generate DNA-cleaving oxygen radicals under physiologically-relevant conditions. The attack of thiols on polysulfides is typically facile[6–8] and cells contain high concentrations of thiols (1–10 mM glutathione)[9] so it is reasonable to expect that reactions of endogenous thiols with the polysulfide linkage in the benzopentathiepin antibiotics might be of fundamental relevance to the biological action of these compounds. For this reason, we investigated reactions of thiol with 7-methyl-1,2,3,4,5-benzopentathiepin (**4**), a simple synthetic compound that contains the crucial cyclic polysulfur ring system found in the natural products varacin, lissoclinotoxin A and 5-(methylthio)varacin.[10]

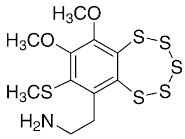
The compound 7-methylbenzopentathiepin **4** was prepared by two different routes involving treatment of 3,4-dimercaptotoluene (**8**) with either sulfur monochloride (S_2Cl_2) or elemental sulfur (S_8) and triethylamine in chloroform.[11,12] We examined the reaction of **4** with both moderate amounts (3 equiv) and a large excess of thiol (100 equiv). The reaction of **4** (40 mM) with 2-mercaptoethanol (3 equiv) in chloroform containing a trace of triethylamine (0.2 equiv) at room temperature initially affords a bright yellow-orange solution that is indicative of polysulfide anions.[7,13] The color fades over the course of 15 min and reverse-phase HPLC analysis reveals a complex spectrum of products that elute as a series of evenly spaced peaks characteristic of polysulfide mixtures (Fig. 1).[14] Such mixtures are known to be generated in the reaction of thiols with acyclic polysulfides.[6–8]



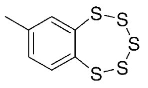
1, Varacin



2, Lissoclinotoxin A



3, 5-(Methylthio)varacin



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LC/MS analysis of the reaction mixture confirms that the products correspond to various polysulfur species (**5**, **6** and **7**, Scheme 1). The products eluting at early times (9–27 min) are polysulfides (di-, tri-, tetra-, penta-,

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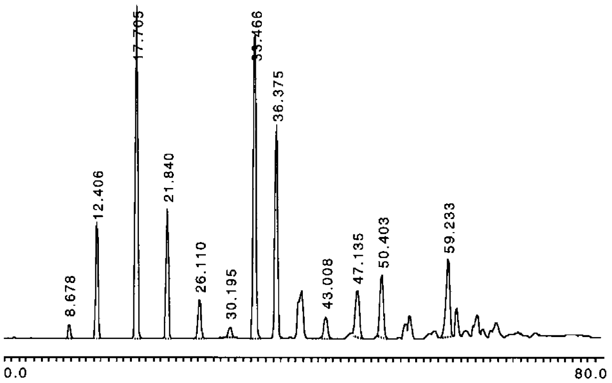


Figure 1. Reverse-phase HPLC trace of the product mixture resulting from the reaction of 2-mercaptoethanol (3 equiv) with 7-methylbenzopentathiepin.

and hexasulfides, **7**, $x = 0$–4) derived from the thiol used in the reaction (2-mercaptoethanol), while the products with longer retention times (30–50 min) largely correspond to aromatic polysulfides such as **5** and **6**.[15]

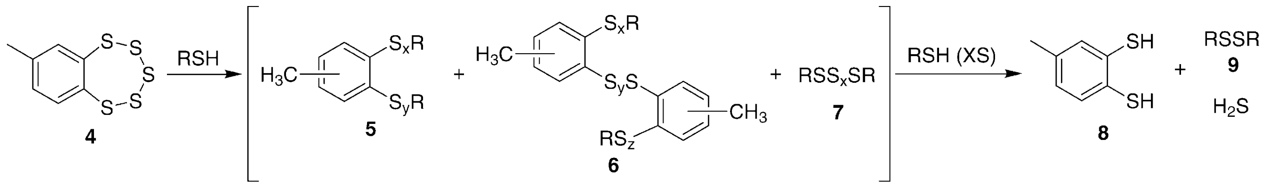
When a large excess of thiol is used, a less complex product mixture is obtained. Reaction of compound **4** with 100 equivalents thiol in chloroform (containing 0.2 equiv of triethylamine) for 1 h yields **8**, **9**, and hydrogen sulfide as major products (Scheme 1). The aromatic dithiol, 3,4-dimercaptotoluene (**8**), was isolated in 80% yield as its bis(methylthioether) derivative following workup of the reaction mixture with methyl iodide. In the reaction of **4** with 100 equivalents thiol, approximately 3 equivalents of hydrogen sulfide are released,[16] presumably via the well precedented, general reaction shown in Scheme 2.[6–8,17] In addition, HPLC analysis reveals that 6.0 ± 0.5 equivalents of the thiol are converted to the corresponding disulfide (**9**) in this reaction.

Reaction of thiol with the pentathiepin heterocycle is quite rapid under physiologically relevant conditions. The half-life of **4** (6.25 μM) in the presence of the GSH (188 μM) in buffered aqueous solution (50 mM NaP_i, pH 7.0, containing 30% acetonitrile) at 25 °C is less than 1 min.[18] This corresponds to an apparent second-order rate constant of at least $60\,M^{-1}\,s^{-1}$ for the reaction of thiol with **4**. For purposes of comparison, we determined the rate constant for the reaction of the biological thiol glutathione (GSH) with the acyclic trisulfide, benzyl trisulfide (BnSSSBn) under similar conditions. The pseudo-first order rate constants for the reaction of GSH (1.25, 1.88, and 2.5 mM) with benzyltrisulfide (BnSSSBn, 25 μM) in pH 7.0 sodium phosphate (50 mM) containing 30% acetonitrile at 25 °C are 8.6 ± 0.2, 12.6 ± 0.2, and $15.1 \pm 0.7\,h^{-1}$, respectively. The slope from a plot of these pseudo-first order rate constants versus glutathione concentration yields an apparent second-order rate constant of $1.77 \pm 0.06\,M^{-1}\,s^{-1}$ for the reaction of GSH with benzyl trisulfide under these conditions. This value closely matches that previously reported by Myers and coworkers for the reaction of the trisulfide residue found in calicheamicin with glutathione in the presence of DNA.[8] As an additional point of reference, we note that the published rate constant for reaction of GSH with glutathione disulfide (GSSG) is $0.41\,M^{-1}\,s^{-1}$.[19] Overall, the fast reaction between GSH and **4** observed here is consistent with recent computational studies indicating a very low activation barrier for the attack of a sulfur nucleophile (HS^-) on the pentathiepin system.[10]

The results reported here indicate that the pentathiepin ring system is likely to react rapidly with cellular thiols under physiological conditions. The initial reaction of thiol with the pentathiepin ring system generates a complex mixture of polysulfides. It is important to note that, while the reactions described here were performed in organic solvent ($CHCl_3$), a similarly rapid reaction that yields analogous product mixtures is seen in aqueous buffered solution and when the biological thiol, glutathione, is used (data not shown). The observed products are completely consistent with the previous proposal[4] that thiol-triggered DNA cleavage by **4** proceeds via production of highly reducing polysulfide anion intermediates (RSS_xS^-)[13,20,21] that convert molecular oxygen to superoxide radical, thereby initiating the well-known cascade of chemical reactions involving reduced oxygen species[22] that ultimately yields DNA-cleaving radicals as shown in the (unbalanced) eq 1.[23]

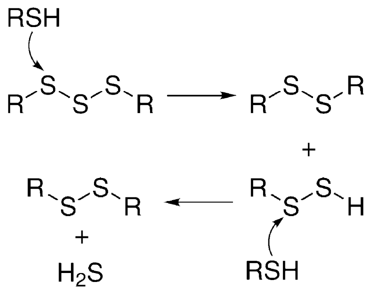
$$\begin{aligned} RSS_xS^- + O_2 &\rightarrow O_2^{\bullet -} \rightarrow H_2O_2 + M^{n+} \\ &\rightarrow HO^\bullet + M^{(n+1)+} \end{aligned} \tag{1}$$

When excess thiol is present, sulfur atoms are stripped from the pentathiepin ring system to afford the aromatic dithiol **8**. Concomitant with this process approximately 6 equivalents of thiol are converted to the corresponding disulfide and hydrogen sulfide is produced. The pentathiepin-mediated conversion of thiols to disulfides described here could play a role in the cytotoxic action of these antibiotics. Depletion of cellular thiols, in conjunction with production of reactive oxygen species ($O_2^{\bullet -}$, H_2O_2, $HO^\bullet$),[4] can result in oxidative stress[9,24] and initiation of programmed cell death (apoptosis).[25–28] In addition, modification of protein



x,y,z = 1-4; R = CH_2CH_2OH

Scheme 1.



Scheme 2.

thiol groups could play a role in the biological activity of the pentathiepin antibiotics. For example, the previously observed inhibition of protein kinase C by pentathiepin-containing natural products[2] may involve modification of critical cysteine residues on the enzyme.[29,30] Finally, our findings raise the possibility that hydrogen sulfide generated in the reaction of endogenous thiols with pentathiepin antibiotics could contribute to the production of oxygen radicals[31] and to the overall cytotoxicity[32] of these compounds.

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15. Separations were conducted using a C$_{18}$ Rainin Dynamax Microsorb column (100 Å sphere size, 5 µm pore size, 25 mm length, 10 mm id) eluted with a linear gradient of 100:0 to 61.5:38.5 water/acetonitrile over 40 min, followed by a linear gradient of 61.5:38.5 to 0:100 water/acetonitrile over 20 min and a final elution with 0:100 water/acetonitrile for 20 min. LC/MS and LC/MS/MS experiments were carried out using a Thermoseparations liquid chromatograph interfaced to a Finnigan TSQ 7000 triple quadrupole instrument operated in negative ion electrospray mode. Components of the mixture were separated by C$_{18}$ reverse-phase chromatography as described above, except at a flow rate of 0.8 mL/min and with NH$_4$OAc (0.01%) added to the mobile phase. Instrument settings included a capillary temperature of 350 °C and capillary voltage of 2.5 kV. LC/MS analysis of the mixture shown in Figure 1 is as follows: 8.6 min, corresponds to 2-mercaptoethanol (identified by retention time and coinjection of authentic material); 12.4 min, *m*/*z* 212.9, corresponds to [M + OAc]$^-$ for **7**, $x = 0$; 17.7 min, *m*/*z* 244.9, corresponds to [M + OAc]$^-$ for **7**, $x = 1$; 21.8 min, *m*/*z* 276.9, corresponds to [M + OAc]$^-$ for **7**, $x = 2$; 26.1 min, *m*/*z* 309.0, corresponds to [M + OAc]$^-$ for **7**, $x = 3$; 30.2 min, *m*/*z* 341.0, corresponds to [M + OAc]$^-$ for **7**, $x = 4$ (the retention times of all preceeding peaks correspond to that of authentic samples prepared as described in ref 23); 33.5 min, *m*/*z* 367.1, corresponds to [M + OAc]$^-$ for **5** $x = 2$, $y = 2$; *m*/*z* 36.4 min, *m*/*z* 399.1, corresponds to [M + OAc]$^-$ for **5** $x = 2$, $y = 3$; 38 and 39 min, *m*/*z* 431.2 corresponds to [M + OAc]$^-$ for either **5** $x = 3$, $y = 3$ or **5** $x = 2$, $y = 4$; 43.0 min, *m*/*z* 463.2, corresponds to [M + OAc]$^-$ for **5** $x = 3$, $y = 4$; 47.1 min, *m*/*z* 521.4, corresponds to [M + OAc]$^-$ for **6** $x,y,z = 2$; 50.4 min, *m*/*z* 553.3, corresponds to [M + OAc]$^-$ for **6** $x,y = 2$, $z = 3$, or **6** $x,z = 2$, $y = 3$; 59.2 min, corresponds to starting material **4**. For information regarding the formation of anionic adducts (e.g., [M + OAc]$^-$) in negative ion electrospray mass spectroscopy, see: Cai, Y.; Cole, R. B. *Anal. Chem.* **2002**, *74*, 985. Kamel, A. M.; Brown, P. R.; Munson, B. *Anal. Chem.* **1999**, *71*, 5481.
16. In the reaction of 100 equiv of 2-mercaptoethanol with **4**, the aromatic dithiol product (**8**) was characterized as its dimethylthioether derivative using the following protocol: to a solution of **4** (21.5 mg, 0.086 mmol) in chloroform (1 mL) was added 2-mercaptoethanol (603 µL, 8.6 mmol, 100 equiv) and triethylamine (2.4 µL, 0.017 mmol, 0.2 equiv). The reaction was agitated on a vortex mixer for 5 min and allowed to stir for 30 min at 25 °C. The mixture was evaporated to dryness under reduced pressure to yield a pale yellow oil that was mixed with hexane (3 mL) to yield a biphasic mixture. The hexane layer (top layer) containing the 3,4-dimercaptotoluene was separated from the bottom layer (containing 2-mercaptoethanol) and the hexane extract was evaporated under reduced pressure to yield a pale yellow oil. The oil was redissolved in benzene (1 mL) and the resulting solution mixed with an aqueous solution of NaOH (100 µL of a 2.7-mM solution in water), followed by methyl iodide (14 µL, 0.224 mmol), and the phase-transfer catalyst Aliquat-336 (80 µL, 0.175 mmol). The reaction mixture was stirred for 1 h at 50 °C and then extracted with ether (3 × 2 mL). The extract was dried over sodium sulfate, filtered, and evaporated under reduced pressure. The resulting colorless oil was subjected to flash column chromatography on silica gel eluted with 5:1 hexane/ethyl acetate to yield the derivatized product, 3,4-bis(methylthio)toluene as a white crystalline solid ($R_f = 0.67$, 12.9 mg, 80% yield). Spectral data for this material matched with that described in the literature: Root, M. J.; Sullivan, B. P.; Meyer, T. J.; Deutsch, E. *Inorg. Chem.* **1985**, *24*, 2731. Release of hydrogen sulfide in this reaction was measured as follows: to a solution of **4** (300 mg, 1.2 mmol) in chloroform (1 mL) was added 2-mercaptoethanol (8.42 mL, 120 mmol, 100 equiv) and triethylamine (33 µL, 0.24 mmol, 0.2 equiv). A stream of nitrogen gas was bubbled into the reaction mixture and the effluent passed through solution of lead acetate (350 mg in 5 mL water, 0.184 mM) to yield a black precipitate (PbS). Control reactions utilizing **4** alone or thiol alone did not produce a black precipitate. The black precipitate was

collected, dried under vacuum, and weighed (795 mg). Qualitative analysis tests support the identity of the black solid as lead sulfide. For example, the black precipitate was converted to a white solid ($PbSO_4$) upon boiling in concentrated nitric acid. Simlarly, boiling in H_2O_2 (3%) converted the black precipitate into white $PbSO_4$; see: Vogel, A. I. *Vogel's Textbook of Macro and Semimicro Quantitative Inorganic Analysis;* Orient Longman Ltd, London: 1985, 195. The disulfide of 2-mercaptoethanol was quantitatively measured using HPLC.

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