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Reducing Holomycin Thiosulfonate to its Disulfide with Thiols

Andrew N. Chan,[1,&] Walter J. Wever,[2,&,#] Elisabetta Massolo,[1,‡] Scott E. Allen,[2] and Bo Li[1,*]

[&] These authors contributed equally to this work.
[1] Department of Chemistry, University of North Carolina at Chapel Hill, CB#3290, Chapel Hill, NC 27599 (USA)
[2] Department of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599 (USA)
[#] Present address: Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Center for Life Sciences 11096, 3 Blackfan Circle, Boston, Massachusetts 02215 (USA)
[‡] Present address: a Dipartimento di Chimica, Università degli Studi di Milano, Via Golgi 19, 20133 Milano (Italy)
* Questions should be addressed to boli@email.unc.edu

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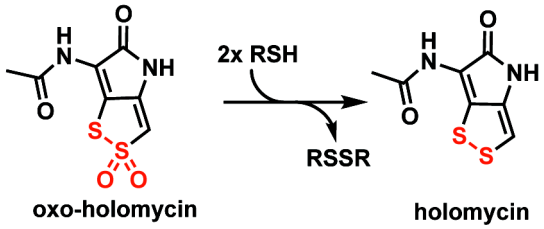


RSH: *N*-acetyl-L-cysteine, glutathione, or protein thiols

Abstract

The dithiolopyrrolone (DTP) natural products contain a unique ene-disulfide that is essential for their antimicrobial and anticancer activities. The ene-disulfide in some DTPs is oxidized to a cyclic thiosulfonate, but it is unknown how the DTP thiosulfonates react with biomolecules. We studied the reactivity of the thiosulfonate derivative of the DTP holomycin, oxo-holomycin, and discovered a unique redox reaction: oxo-holomycin is reduced to its parent disulfide, while oxidizing small molecule and protein thiols to disulfides. Our work reveals that the DTP core is a privileged scaffold that undergoes unusual redox chemistry. The redox chemistry of the DTP natural products may contribute to their mechanism of action.

Introduction

The rise of antibiotic resistance has heightened the pursuit of compounds that display novel mechanisms of action. The dithiolopyrrolone (DTP) family of natural products exhibits broad-spectrum antimicrobial activities and unique chemical structures, thus it is of great interest to understand the molecular mechanism accounting for their biological activities. DTPs are active against both Gram-positive and Gram-negative bacteria, including drug-resistant strains of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Neisseria gonorrhoeae*.[1] They are structurally characterized by a compact bicyclic core containing an ene-disulfide. Examples include holomycin (**1**), thiolutin, xenorhabdin I, and thiomarinol A (**Figure 1**).[1] Thiomarinol A is a hybrid antibiotic featuring the DTP core covalently tethered to the polyketide antibiotic marinolic acid (**Figure 1**).[2-4]

Several cellular effects have been reported for DTPs against bacteria and yeast, including inhibition of transcription, replication, and respiration.[5-8] Only recently have these effects been connected to the DTP ene-disulfide.[9,10] Our work identified a role for holomycin in the intracellular chelation of essential metals and inhibition of zinc metalloenzymes in bacteria.[10] Holomycin is a prodrug and its antimicrobial activity requires intracellular reduction of the ene-disulfide to ene-thiols for metal chelation.[9] A similar mechanism was also demonstrated for the anticancer activity of thiolutin, which involves inhibiting the zinc-dependent protease subunit Rpn11 of the mammalian proteasome.[11] The metal-chelation mechanism of DTPs against bacteria and human cells suggest potential applications in both antimicrobial and anticancer therapy, however, for use as antibiotics the selectivity of DTPs will need to be improved for bacteria over human cells.

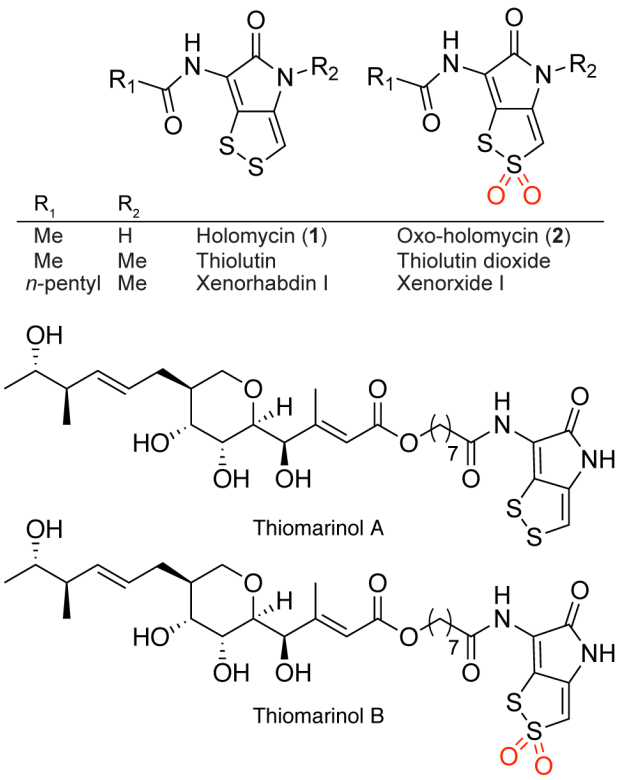


Figure 1. Structures of dithiolopyrrolones and their oxidized thiosulfonate derivatives.

In addition to the ene-disulfide forms of DTPs, the more oxidized thiosulfonate derivatives have also been isolated from bacteria and exhibit a variety of biological activities. A small amount of thiomarinol B featuring a thiosulfonate moiety (**Figure 1**) was isolated from the thiomarinol A producer.[12] The regiochemistry of the oxidation in thiomarinol B was determined by X-ray crystallography.[12] Thiomarinol B inhibits the growth of many infectious bacteria as effectively as the disulfide counterpart thiomarinol A.[13] The thiosulfonate derivatives of thiolutin and xenorhabdins have been isolated as thiolutin dioxide and xenorxides (**Figure 1**), respectively, and possess antibacterial, antimycotic and antineoplastic properties.[14,15]

It is unknown whether the DTP thiosulfonates exhibit the same mechanism of action as the DTP disulfides, given the significant difference in reactivity of disulfides and thiosulfonates. Generally, thiosulfonates are subject to nucleophilic attack on sulfur, resulting in heterolytic cleavage of the sulfur-sulfur bond and generation of a sulfite anion, which can then be trapped with an alkyl halide to form a sulfone.[16-18] Our initial efforts focused on testing physiologically relevant nucleophiles for reactivity with DTP ene-disulfide and thiosulfonate. We report a unique redox reaction—reduction of the DTP thiosulfonate to the parent disulfide by thiols.

Results

We synthesized the thiosulfonate derivative of holomycin—oxo-holomycin (**2**)—as a model compound to investigate the reactivity of DTP thiosulfonates. Using a similar procedure to that of oxidizing thiomarinol A to thiomarinol B,[12] oxo-holomycin was prepared from holomycin using Oxone™ as an oxidant (**Figure S1a, S2–S10**). Oxo-holomycin exhibits identical ^{1}H and ^{13}C NMR shifts as those reported for the DTP thiosulfonate moiety of thiomarinol B (**Table S1, S2** and **Figure S7, S8, S11, S12**),[19] indicating that oxo-holomycin contains the same thiosulfonate moiety as thiomarinol B. This structure is further supported by 2-dimensional (^{1}H, ^{13}C) HSQC and HMBC experiments (**Figure S3–S18**). Thus, oxo-holomycin is a true mimic of the natural DTP thiosulfonates. Holomycin absorbs UV light strongly at 395 nm, resulting in its characteristic yellow color.[20] Oxo-holomycin exhibits a λ_{max} of ~300 nm and reduced yellow color (**Figure S1b**), suggesting that the electronics of the DTP core have been significantly altered.

UV-vis experiments were conducted to examine the reactivity of oxo-holomycin in the presence of biologically relevant nucleophiles. Oxo-holomycin was separately treated with *N*-acetyl-L-cysteine (NAC, **3**) as a model thiol or glycine methyl ester as a model amine for nucleophilic addition. These reactions were assayed and monitored for changes in UV absorbance, which would indicate changes to the DTP chromophore. To probe the effect of acidic or basic conditions on reactivity, each reaction was examined at pH 4.5, 7.0, and 8.2, using a 50 mM sodium phosphate buffer at room temperature. Incubation of 0.1 mM oxo-holomycin with 10 equivalents (eq.) of glycine methyl ester or in buffer alone did not alter the UV absorbance over 14 hours (**Table S3**), indicating that oxo-holomycin does not react with amine nucleophiles or water. Treatment of oxo-holomycin with 10 eq. of NAC resulted in an increased absorbance at 395 nm, the signature λ_{max} for holomycin (**Figure 2**). Liquid-chromatography coupled high-resolution mass spectrometry (LC-HRMS) comparison with a synthetic standard confirmed that the product is holomycin (**Figure S19**). This reaction is accelerated at acidic pH, though a moderate amount of reactivity can be observed at neutral pH (**Table S3**). In contrast, holomycin shows no significant UV shifts when incubated with water, amine or thiol nucleophiles at the examined pH values (**Table S4**), suggesting that the reactivity with thiols is unique to the thiosulfonate

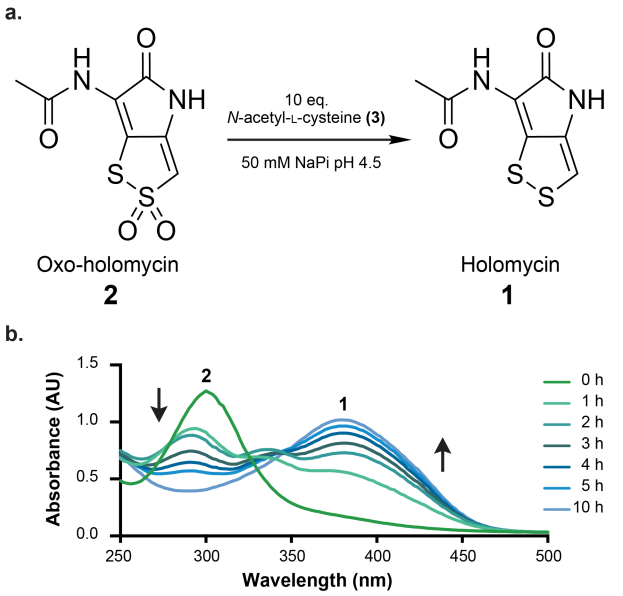


Figure 2. Oxo-holomycin is converted to holomycin by *N*-acetyl-L-cysteine under acidic conditions. This reaction is time dependent as shown by the change in UV spectrum over time: the absorbance at 300 nm for oxo-holomycin decreases and the absorbance at 395 nm for holomycin increases.

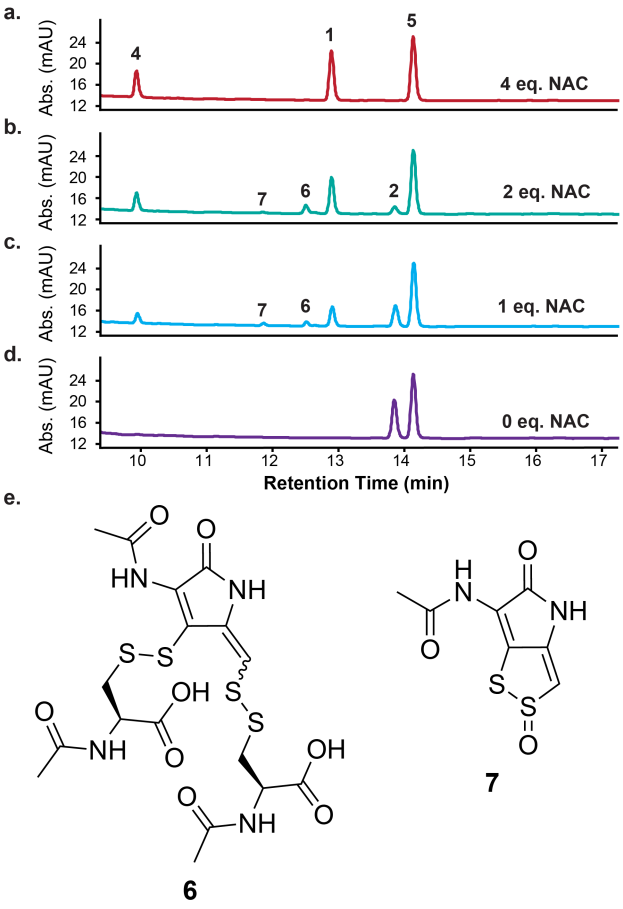


Figure 3. Conversion of oxo-holomycin to holomycin under different equivalents of *N*-acetyl-L-cysteine (NAC). The total wavelength chromatograms from LC-HRMS analysis are shown for oxo-holomycin incubated with (**a**) 4 eq. of NAC, (**b**) 2 eq. of NAC, (**c**) 1 eq. of NAC, and (**d**) no NAC. Full traces are shown in **Figure S21**. (**e**) Proposed structures of the reaction intermediates **(6)** and **(7)**.

form. Oxo-holomycin is also converted to holomycin by glutathione (**Figure S20**). Thus, the thiosulfonate of oxo-holomycin can be converted to a disulfide using biologically relevant thiol nucleophiles under mild, aqueous conditions.

We conducted LC-HRMS experiments to determine the stoichiometry of thiol consumed in converting oxo-holomycin to holomycin and to identify potential reaction intermediates. Incubation of oxo-holomycin with 4 eq. of NAC results in complete conversion to holomycin with *N,N'*-diacetyl-L-cystine (**4**) as the major by-product (**Figure 3a, S21**). *N*-acetyl-L-phenylalanine (**5**) was used as an internal standard for LC-HRMS analysis. Incubation of oxo-holomycin with 1 or 2 eq. of NAC resulted in incomplete consumption of oxo-holomycin and accumulation of a major intermediate (**6**, **Figure 3b, 3c, S21**). This intermediate displays a mass and a UV profile that correspond to a bis-disulfide between reduced holomycin and two NAC molecules (**6, Figure 3e, S22**). A holomycin thiosulfinate intermediate (**7**) was also identified (**Figure 3b, 3c, 3e, S21, S22**). In the absence of NAC, the amount of oxo-holomycin is unchanged, confirming that oxo-holomycin is stable under the assay conditions (**Figure 3D, S21**). Using standards for holomycin, oxo-holomycin, NAC, and *N,N'*-diacetyl-L-cystine, we quantified the amount of *N,N'*-diacetyl-L-cystine and holomycin products (**Figure S23**). In the presence of 4 eq. of NAC, 2.52 ± 0.02 eq. (average ± SD) of NAC is oxidized per 1 eq. of oxo-holomycin (**Figure S21**, **Table S5, S6**).

We monitored the timecourse for the reaction of oxo-holomycin with 4 eq. of NAC. LC-HRMS experiments revealed that the bis-disulfide intermediate **6** forms rapidly and is consumed over time, coinciding with the formation of holomycin and *N,N'*-diacetyl-L-cystine (**Figure S24**). The holomycin thiosulfinate **7** appears to be an early stage intermediate (**Figure S24**). Bis-disulfides similar to **6** have been shown to form from reduction of thiosulfinates with thiols.[17] Therefore, **6** may arise from a similar reaction with **7** and NAC reacts directly with oxo-holomycin.

To further characterize oxo-holomycin reactivity, we investigated the effect of oxo-holomycin on redox-active cysteines in proteins. Arginine phosphatase YwlE in *Bacillus subtilis* contains two cysteine residues in its active site that are critical for catalysis and sensitive to oxidation.[21] These cysteines are also the only cysteines in the entire protein and intramolecular disulfide formation between them deactivates the enzyme.[21] We used LC/HRMS to directly probe the effect of oxo-holomycin on the oxidation state of YwlE. Treatment of YwlE with oxo-holomycin resulted in a 2 Da decrease in the protein mass, consistent with disulfide formation (**Figure 4a, 4b, S25**). Holomycin was detected as a product of the reaction between YwlE and oxo-holomycin (**Figure S26**), indicating that oxo-holomycin has been reduced to holomycin while oxidizing YwlE. Directly treating YwlE with holomycin also resulted in disulfide formation in YwlE (**Figure 4a, 4c**). However, the reduced, dithiol form of holomycin could not be detected, likely due to rapid re-oxidation to holomycin in air.[20] Oxidation of YwlE by oxo-holomycin and holomycin occurs rapidly at both pH 4.5 and 7.4 (**Figure 4**, **S25**), suggesting that reactions of DTPs with protein cysteines can occur under both acidic and physiological pH. Notably, both holomycin and oxo-holomycin promote selective disulfide formation, while H_2O_2 produces various oxidized forms of sulfur such as sulfenic and sulfinic acids (**Figure S25**);[21] thus, DTPs facilitate disulfide formation while avoiding alternative cysteine oxidation states.

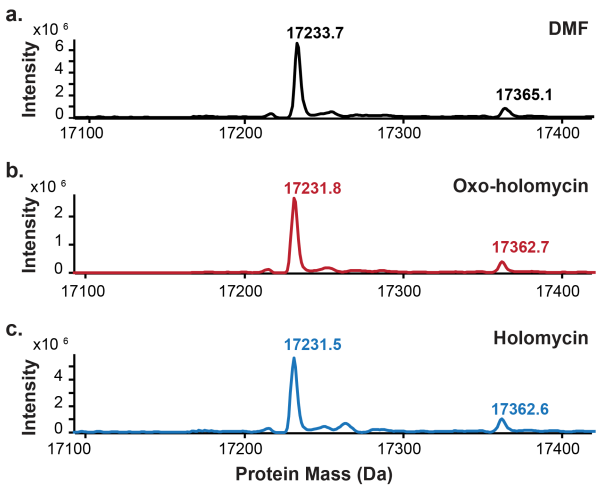
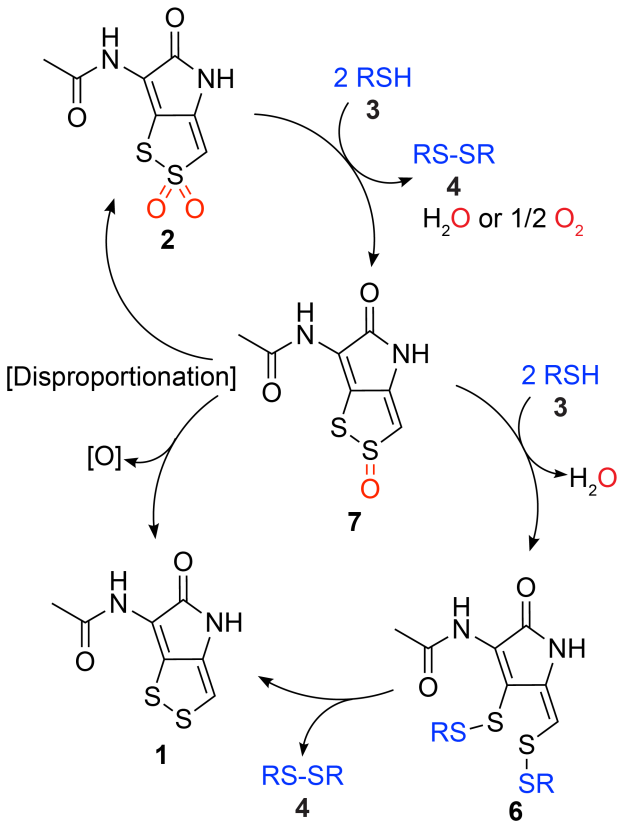


Figure 4. YwlE is oxidized to its disulfide form by oxo-holomycin and holomycin at pH 7.4. The deconvoluted protein spectra are shown for 200 μM YwlE treated with (**a**) DMF control, (**b**) 0.5 mM oxo-holomycin, or (**c**) 2 mM holomycin. YwlE mass: 17233 Da (reduced), 17231 Da (oxidized).

Discussion

Based on the identified reaction intermediates, we propose a mechanism for the reduction of holomycin thiosulfonate to holomycin (**Scheme 1**). Holomycin thiosulfonate **2** reacts with NAC and is converted to the thiosulfinate **7**. Further reaction of **7** with NAC produces the bis-disulfide **6** with concurrent release of water. Rearomatization of **6** generates *N,N'*-diacetyl-L-cystine and holomycin. Alternative mechanisms may also exist. The conversion of oxo-holomycin to holomycin involves a net 4-electron reduction and the observation that less than 4 eq. of NAC are oxidized suggests the overall reduction involves latent transformations. For example, the thiosulfinate **7** may decompose to holomycin or undergo disproportionation to generate oxo-holomycin and holomycin.[22-25] We have been unable to discern these side reactions. The overall reaction is accelerated at acidic pH, suggesting that the rate-limiting step is acid catalyzed. The detailed reaction mechanism will be the subject of future studies.

YwlE oxidation by holomycin is likely accompanied with holomycin reduction to its ene-dithiol form. Our previous work showed that metal chelation by holomycin requires holomycin reduction,[10] but the mechanism for this reduction has remained elusive. Holomycin cannot be reduced by NAC or glutathione (**Table S4**), and previously we have shown that the reduction of holomycin requires tris-(2-carboxyethyl)-phosphine or dithiothreitol.[20] This study suggests that holomycin is reduced by protein cysteines.



Scheme 1. Proposed reaction mechanism for the reduction of oxo-holomycin to holomycin. The fate of oxygen remains to be determined.

This newly found reactivity of oxo-holomycin with small molecule and protein thiols may contribute to how oxo-holomycin exerts its action in cells. Oxidation-sensitive cysteine residues play important regulatory roles in protein function,[26] therefore oxo-holomycin may exert its inhibitory mechanism by perturbing protein oxidation states. In addition, reduction of oxo-holomycin to holomycin by glutathione or proteins could converge on the same mechanism of action as holomycin that involves disrupting metal-dependent processes. Consistent with this proposal, thiolutin dioxide, the thiosulfonate derivative of thiolutin, has been described to inhibit neurolysin,[15] a zinc metalloprotease implicated in neurodegenerative diseases.[27]

The reduction of oxo-holomycin to holomycin is different from the typical reactivity of unactivated thiosulfonates with thiols. The sulfur-sulfur bond in oxo-holomycin may be cleaved by a thiol, generating a ring open form containing a disulfide and a sulfinic acid, and the sulfinic acid is further reduced to a sulfenic acid and then to a thiol, eventually reforming the disulfide in holomycin. Reduction of sulfinic acids to thiols is extremely rare in biology and requires an ATP-dependent repair enzyme,[28] whereas seleninic acids can be reduced to selenols with thiols.[29,30] In contrast, while sulfoxides are readily oxidized to sulfones, the oxidation of selenoxides to selenones are much more difficult.[31] The reactivity difference is presumably due to the lower electronegativity and larger size of selenium and weaker bonds compared to sulfur. The reduction chemistry of the DTP sulfinic acid resembles that of a selenic acid and is likely due to the unique electronics of the DTP scaffold.

In summary, we report the unusual reactivity of oxo-holomycin with thiols. Oxo-holomycin is reduced to holomycin while oxidizing thiols to disulfides. Oxo-holomycin oxidizes glutathione and the redox-sensitive enzyme YwlE to disulfides. The interesting redox chemistry of oxo-holomycin may contribute to

its biological activity. Many natural products contain cyclic thiosulfinates and thiosulfonates.[32,33] Our work highlights that understanding the reactivity of the disulfide-containing natural products will likely reveal new redox chemistry relevant to their mode of action.

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Conflicts of interest

There are no conflicts of interest to declare.

Supporting Information

Detailed experimental procedures, supplementary figures and tables.

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