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# Oxidative Coupling of Mithramycin and Hydroquinone Catalyzed by Copper Oxidases and Benzoquinone. Implications for the Mechanism of Action of Aureolic Acid Antibiotics

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**Abstract**—The copper oxidases human ceruloplasmin and *Polyporous anceps* laccase catalyze the oxidative coupling of mithramycin (**1**) and its aglycone chromomycinone (**2**) with *p*-hydroquinone to form new mithramycin–hydroquinone (**3**) and chromomycinone–hydroquinone adducts (**4**), respectively. Similar adducts could be formed by the nonenzymatic mimic of this reaction using benzoquinone and these aureolic acids in buffer solutions. FABMS of **3** indicated that the hydroquinone moiety was attached to the aureolic acid aglycone. Acid hydrolysis of **3** yielded a compound with the same chromatographic and spectroscopic characteristics as **4**. Structure elucidation of **4** by NMR and MS revealed that the hydroquinone was attached to the C-5 position of the aglycone. NMR evidence indicated that **4** consisted of a mixture of *ortho*-substituted biphenyl rotamers. The mechanism of the copper oxidase catalyzed adduct formation reaction is presumed to involve radical formation through hydrogen removal at the 8-phenolic position, radical isomerization, and coupling with semiquinone radical also formed during enzymatic and nonenzymatic incubations. Identification of the covalent-hydroquinone adduct provides evidence that aureolic acid antibiotics can be metabolically converted to reactive radical intermediates, and it establishes the C-5 position of aureolic acid as an enzymatically reactive site. Unlike mithramycin, the mithramycin–hydroquinone adduct was inactive in the *in vivo* P388 leukemic antitumor test system.

## Introduction

The aureolic acids[1,2] are a group of antitumor antibiotics produced by various strains of *Streptomyces*. Structurally, they contain oxygenated tetrahydroanthracene aglycones with a highly oxygenated pentanyl side chain, linked to two sets of dideoxy oligosaccharides. Clinically significant members of this group are mithramycin (Plicamycin®), chromomycin $A_3$ (Toyomycin®) and olivomycin A. These compounds are broadly active against experimental and human tumors,[3,4] but the clinical uses of mithramycin (**1**) are limited to the treatment of testicular tumors, and hypercalcemia and hypercalciuria associated with malignancy.[5] Dose limiting toxicities of mithramycin are manifested by a hemorrhagic syndrome, renal necrosis and hepatic toxicity.[6,7] The exact mechanism(s) of action of aureolic acids has not been established. However, they inhibit RNA synthesis and gene expression.[8,9] Aureolic acid antibiotics appear to bind as dimers at G–C regions in the minor groove of DNA.[10] They also inhibit osteoclastic bone resorption[11] and fibrin monomer aggregation.[12]

The complex chemistry of aureolic acids has impeded their total synthesis[13,14] and limited structure–activity relationship studies.[15,16] As an initial step towards the biosynthetic production of analogs, our group recently described the pathway of carbon in the biogenesis of these natural products.[17,18] It is surprising that during the nearly 40 years that this group of antibiotics has been known, their possible biotransformations with drug metabolizing enzymes has never been evaluated. Enzymatic biotransformations often result in the generation of reactive intermediates and products that are responsible for biological activities and/or toxicities of many groups of compounds.[19,20] Studies using radiolabeled mithramycin[21] have demonstrated that after administration, the antibiotic becomes localized mainly in plasma, kidney and liver. This distribution pattern appears to correlate with the toxicities of these compounds, and the deposition tissues are well-known for their content of drug metabolizing enzymes. Human ceruloplasmin, and laccase are copper oxidases, and are well-known for their abilities to catalyze biotransformations of phenolic and non-phenolic substrates.[22–28] In this paper, we report the oxidation of mithramycin (**1**) (Figure 1) and its aglycone chromomycinone (**2**) by human serum ceruloplasmin and *Polyporous anceps* laccase, and by a chemical mimic of these enzymes to reactive intermediates that form covalent adducts with hydroquinone.

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This manuscript is dedicated to Bryan Jones in honor of his 60th birthday.

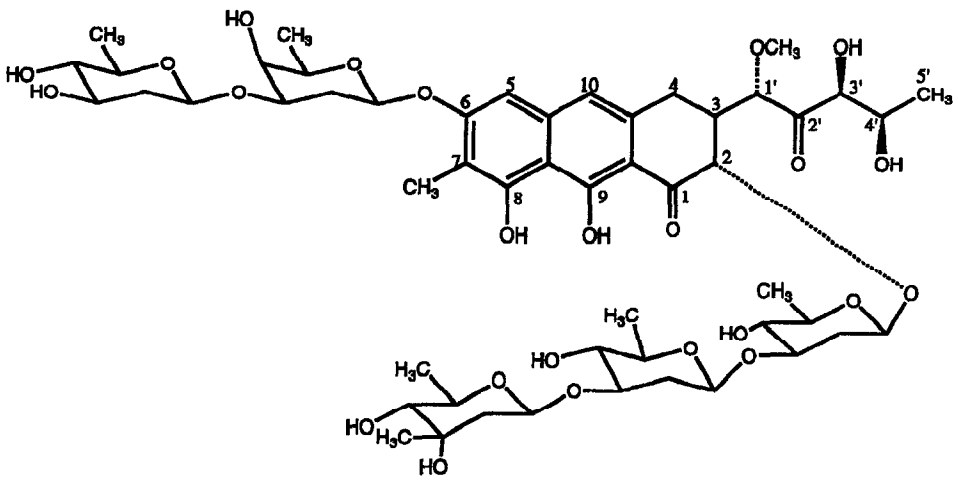
## Results and Discussion

### *Isolation and structure elucidation*

The pathway proposed for the formation of **3** and **4** is depicted in Figure 2. Incubations of **1** with human ceruloplasmin or *Polyporous anceps* laccase in the presence of hydroquinone resulted in approximately 25 % (TLC) conversion to the hydroquinone adduct **3** which was detected as a gold coloured compound at $R_f$ 0.4 on TLC, and at $R_v$ 3.8 mL on reversed phase HPLC. The product **3** was also formed nonenzymatically by reaction of **1** with 1,4-benzoquinone. Benzoquinone was previously shown to serve as a mimic for the copper oxidase catalyzed oxidation of the dimeric Vinca alkaloid vinblastine.[24] Since either the enzyme incubation mixtures, or benzoquinone routinely provided the same mixtures of compounds, benzoquinone could be used to produce sufficient amounts of metabolites **3** and **4** for complete structure elucidation.

The structure of **3** was determined by direct spectral analysis and by chemical degradation to the altered aglycone whose structure was also determined. The FABMS of **3** gave an $[M + Na]^+$ ion at m/z 1215 for a molecular ion with a 108 mass difference versus the $[M + Na]^+$ ion for mithramycin (**1**) at m/z 1107. The mass difference between **1** and **3** indicates the substitution of a H atom on **1** by a hydroquinone moiety ($C_6H_5O_2$) to form **3**. The FABMS fragmentation pattern and acid hydrolysis of **3** indicated that the hydroquinone moiety was attached to the aglycone substructure, and not to a sugar residue of **1**. Acid hydrolysis of **3** to **4** confirmed this result. The base peak in the FABMS of **3** and the molecular ion peak of the acid hydrolysis product (**4**) were both observed at m/z 528, which corresponds to the mass of the aglycone ($C_{21}H_{24}O_9$, mol. wt 420) plus the additional mass of hydroquinone. The exact mass determination of the hydrolysis product corresponds to $C_{27}H_{28}O_{11}$.

Incubations of chromomycinone (**2**) and hydroquinone with human ceruloplasmin resulted in greater than 90 % conversion of **2** to **4**, which was detected as two eluting HPLC peaks with $R_v$ of 3.8 mL and 4.5 mL. Similar results were obtained in the nonenzymatic reaction of **2** with 1,4-benzoquinone. The product (**4**) gave a single spot at $R_f$ 0.35 by regular phase TLC, and two spots at $R_f$ 0.5 and 0.55 by reverse phase TLC. Separation of the two spots by preparative, reverse-phase TLC yielded two substances with essentially identical physical properties, thereby indicating that the two spots are isomeric mixtures. The product (**4**) generated from **2** was chromatographically and spectrally identical with the acid hydrolysis product obtained from **3**. The molecular ion peak at m/z of 528 by HRFABMS corresponded to $C_{27}H_{28}O_{11}$. The 360 MHz $^1H$ NMR spectrum of **4** was similar to that of **2** (Table 1), except for the observed changes in the aromatic region. One of the aromatic singlet signals in the $^1H$-NMR spectrum of chromomycinone (**2**) was absent, indicating that the hydroquinone moiety was attached to the aromatic portion of the aglycone. This could only occur at either C-5 or C-10 of the aglycone, since these are the only carbons in the aureolic acid structure bearing aromatic protons. There were four aromatic signals in the $^1H$ NMR spectrum of the adduct **4**: at δ 6.84 (d, $J$ = 8.7), 6.76 (dd, $J$ = 8.7, 2.9), 6.55 (d, $J$ = 2.9), and 6.46 (s). Three protons were coupled in ABX fashion, indicating *ortho* and *meta* splittings. The number and the coupling pattern of the three new protons suggested that the attachment to the aglycone occurred at one of the non-oxygenated carbons of hydroquinone. The 600 MHz $^1H$ NMR spectrum (Figure 3) of **4** displayed two sets of aromatic signals with relative intensities of 3:2, thereby indicating that **4** actually exists as a mixture of rotameric isomers[29,30]. Atropisomerism is expected for such a compound with an *ortho*-substituted biphenyl substructure. The $^{13}C$-NMR spectrum of **4** was similar to that for **2** except for the appearance of six additional carbon signals, and the substitution of a methine carbon signal at δ 102.0 with a quaternary carbon signal at δ 110.6. Four of the new carbon signals resonated in the aromatic region at δ 121.4, 119.5, 117.1, and 117.5, while the two others resonated at δ 149.5 and δ 151.1 typical for phenolic



**1**

**Figure 1.** Mithramycin (**1**).

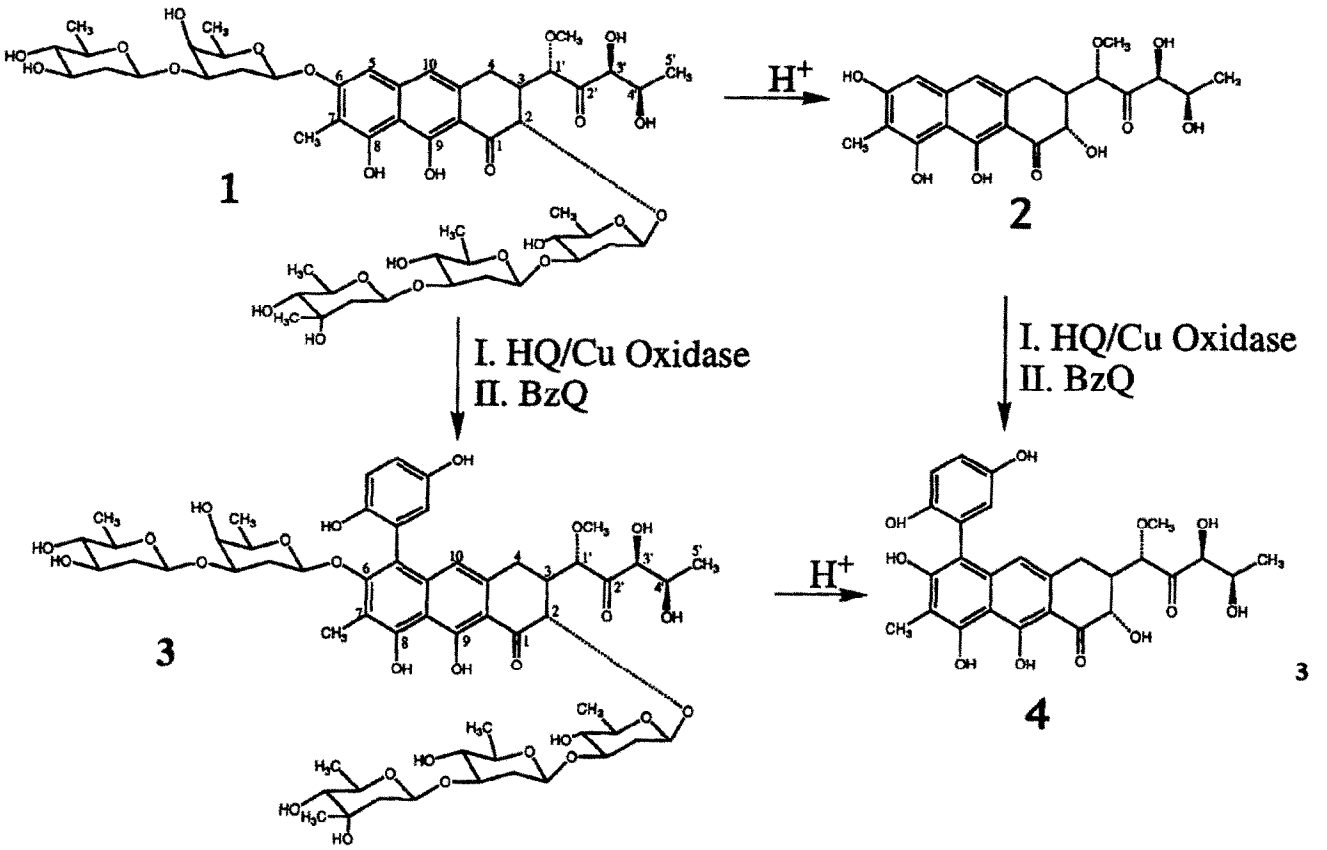



**Figure 2.** Hydroquinone adducts of mithramycin (**1**) and of the aglycone, chromomycinone (**2**).

carbons. HMBC and HMQC spectral analyses were used to assign the point of linkage of the hydroquinone moiety to C-5 in **4**. By HMQC, the lone aromatic singlet (δ 6.45) was shown to be directly bonded to the C-10 carbon which resonates at δ 115.1. This assignment was further confirmed by HMBC (Table 1, Figure 4) in which H-10 (δ 6.45) displayed three bond correlations to C-4 (δ 27.8) and C-5 (δ 110.6). The H-4 signals (δ 2.97, 2.57) also exhibited three bond correlations to C-10 (δ 115.1). Such correlations cannot be demonstrated for the C-5 position in such an experiment. The H-6" signal (δ 6.56) on the hydroquinone moiety and H-10 (δ 6.45) of chromomycinone also showed strong three bond connectivities to C-5 at δ 110.6, thereby establishing this as the only possible point of linkage between the two substructures.

*Mechanism of copper oxidase catalyzed couplings of mithramycin and chromomycinone with hydroquinone*

Ceruloplasmin and laccase belong to a family of enzymes known as blue copper oxidases.[27,28] These enzymes catalyze oxidations of aromatic amines, phenolics, ascorbate and Fe(II) by a one-electron abstraction mechanism that is ultimately coupled to the reduction of molecular oxygen to water.[22–28] Oxidation of ferrous iron by ceruloplasmin is known as ferroxidase activity, and it is one of the most important known functions of the copper protein. Other typical substrates include biogenic amines such as epinephrine, 5-hydroxyindoles, and compounds like the phenothiazines. The oxidation of phenols involves abstraction of an electron and a proton from the hydroxyl group of phenolic substrates to generate phenoxy radicals,[31,32] which can react nonenzymatically to form various products.[33] Quinones are versatile oxidizing agents and are routinely used for oxidations of phenolic compounds.[34] We investigated the nonenzymatic formation of the adducts by the reaction of the aureolic acids and benzoquinone since it would likely involve a similar reaction mechanism, and because we had successfully used this approach to produce larger quantities of vinca alkaloid oxidation products also formed by the copper oxidases.[24]

The aureolic acids all contain phenolic aglycones which render these compounds potential substrates for copper oxidases and other oxidoreductase enzymes. Oxidation of aureolic acids by copper oxidases could be expected to involve initial hydrogen abstraction from either the C-8 or C-9 hydroxyl groups with the glycoside mithramycin. The presence of two phenolic hydroxyl groups in the aromatic ring of the aglycone, chromomycinone, and the hydrogen bonding of the hydroxyl group at C-9 with the *peri*-ketone functional group at C-1 would indicate the likelihood of initial hydrogen abstraction from either compound at the C-8 hydroxyl group. The mechanism for the oxidative coupling of **1** and **2** with hydroquinone (Figure 5) requires

**Table 1.** $^{1}H$- and $^{13}C$-NMR Assignments for **2** and **4**

| Carbon | 2 | | | 4 | | |
|---|---|---|---|---|---|---|
| | $^{13}C$ (δ) | $^{1}H$ (δ) | $^{1}H$-$^{13}C$ correlations | $^{13}C$ (δ) | $^{1}H$ (δ) | $^{1}H$-$^{13}C$ correlations |
| 1 | 204.2 | ---- | 2, 10 | 204.3 | ---- | 2 |
| 2 | 72.8 | 4.48 d[a] | 1', 4, 3 | 72.7 | 4.47 d[a] | 1', 4 |
| 3 | 44.5 | 2.77 ddd[b] | 1', 2, 4 | 44.3 | 2.77 ddd[b] | 1', 2, 4 |
| 4 | 28.6 | 2.95 dd[c]<br>2.62 dd[d] | 10, 1', 2 ,3 | 27.8 | 2.97 dd[c]<br>2.65 dd[d] | 10, 1', 2 |
| 5 | 102.0 | 6.62 s | 7-Me, 10 | 110.6 | ---- | 10, 6", 7-Me |
| 6 | 161.6 | ---- | 5, 7-Me | 161.9 | ---- | 7-Me |
| 7 | 110.1 | ----- | 5, 7-Me | 110.5 | ----- | 7-Me |
| 8 | 157.0 | ----- | 7-Me, 5, 10 | 156.3 | ----- | 7-Me |
| 9 | 165.2 | ---- | 5, 10 | 158.0 | ---- | ---- |
| 10 | 116.5 | 6.71 s | 5, 4 | 115.1 | 6.46 s | 4 |
| 4a | 136.6 | ---- | 10, 4 | 136 | ---- | 4 |
| 10a | 139.5 | ---- | 10, 5 | 138 | ---- | 10 |
| 8a | 107.0 | ----- | 5, 10 | 107.2 | ----- | 10 |
| 9a | 107.4 | ----- | 10, 4 | 109.9 | ----- | 10, 4 |
| 1' | 83.0 | 4.87 s | 2, 4, 1'-OMe | 82.6 | 4.84 s | 2, 1'-OMe |
| 2' | 211.3 | ----- | 1', 3' | 210.8 | ----- | 1', 3' |
| 3' | 79.2 | 4.34 m | 5' | 78.8 | 4.27 m | 5' |
| 4' | 68.4 | 4.30 m | 5', 3' | 68.2 | 4.24 m | 5', 3' |
| 5' | 20.2 | 1.26 d[e] | 3', 4' | 19.5 | 1.26 d[e] | 3' |
| 1" | ---- | ---- | ---- | 121.4 | ---- | 3" |
| 2" | ---- | ---- | ---- | 149.5 | ---- | 6", 3", 4" |
| 3" | ---- | ---- | ---- | 117.5 | 6.84 d[f] | ---- |
| 4" | ---- | ---- | ---- | 117.1 | 6.76 dd[g] | 6" |
| 5" | ---- | ---- | ----- | 151.1 | ----- | 3", 4", 6" |
| 6" | ----- | ----- | ---- | 119.5 | 6.56 d[h] | 4" |
| 7-Me | 8.41 | 2.13 s | 5 | 8.3 | 2.12 s | ---- |
| 1'-OMe | 59.2 | 3.46 s | 1' | 58.9 | 3.45 s | 1' |

*J* values (Hz); [a]11.7, [b]11.9, 11.2, 2.6, [c]15.9, 11.9, [d]15.9, 11.9, [e]6.2, [f]8.7, [g]8.7, 2.9, [h]2.9.

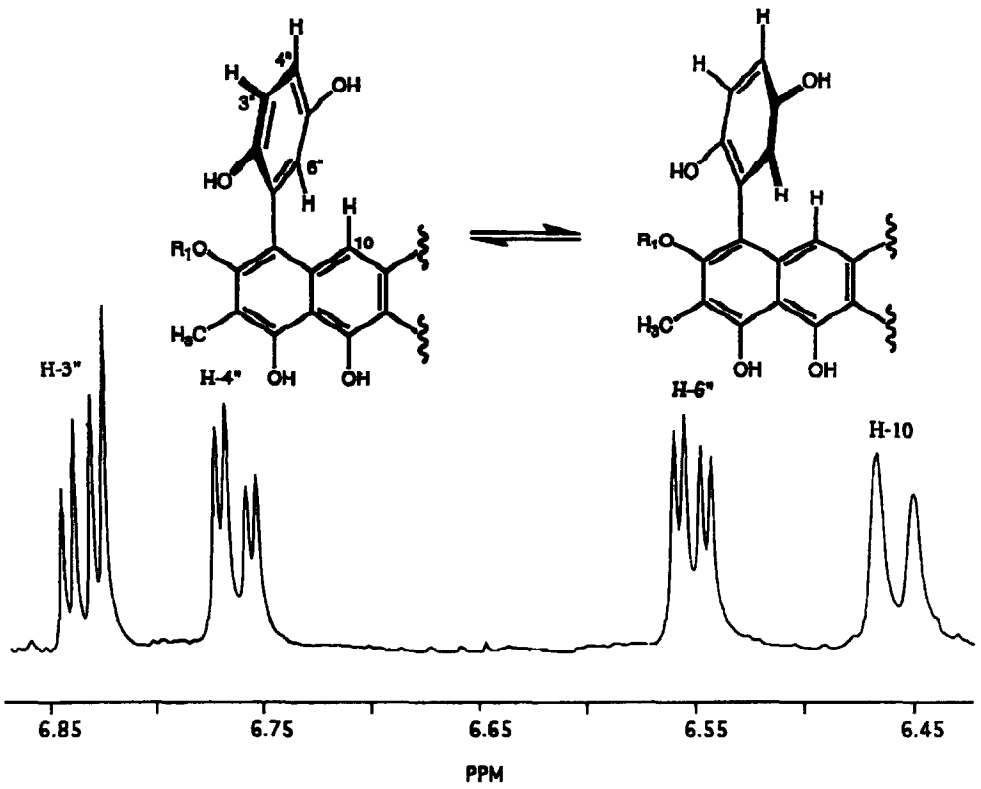
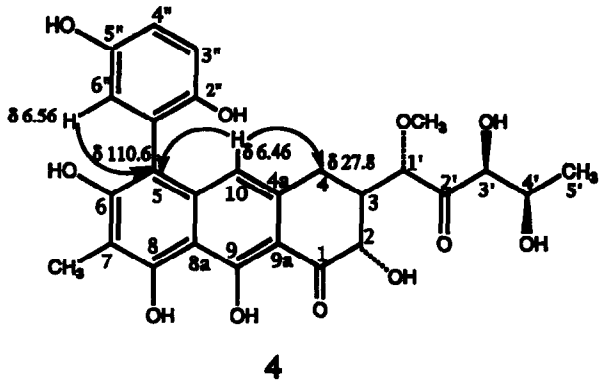



**Figure 3.** The aromatic region of the 600 MHz $^1$H-NMR spectrum, of **4** and the structures of possible atropisomers.



**4**

**Figure 4.** Selected three bond ($^1$H–X–X–$^{13}$C) correlations observed in the HMBC spectrum of **4**.

subsequent isomerization of the aureolic acid C-8 phenoxy radical to a C-5 carbon centered radical. Oxidation of hydroquinone by copper oxidases to a semiquinone radical[31–33] leads to the ultimate coupling of the two radical species to form **3** or **4**.

An alternative mechanism that cannot be ruled out is the initial oxidation of hydroquinone to semiquinone radical and subsequent hydrogen abstraction from the C-8 hydroxyl group of **1** or **2**. In this scheme, coupling occurs between the semiquinone radical and the isomerized C-5 carbon-centered radical to form **3** or **4**.

*Implications for the mechanism(s) of action of aureolic acid antibiotics*

Enzymatic activation to free radical species has been suggested to play a major role in the cytotoxicity displayed by anticancer agents.[35–38] The identification of the hydroquinone adducts **3** and **4** implicates the possible involvement of aureolic acid phenoxy and carbon centered radicals in the aureolic acid antibiotics mechanism of action. The hydroquinone adducts **3** and **4** may be regarded as the products of 'trapped' radical species formed during the course of copper oxidase and benzoquinone-mediated oxidations of mithramycin (**1**) and its aglycone chromomycinone (**2**). The unambiguous location of the adduct at position C-5 of the antitumor antibiotic enables the identification of this position for the first time as a metabolic and chemical 'hot spot' for the generation of free radical species of possible importance in aureolic acids mechanism(s) of action and toxicity.

Interestingly, an earlier study had suggested that aureolic acids may be reductively activated by ferredoxin reductase to cause free radical damage to deoxyribose.[39] Ferredoxin reductase and other flavoproteins are known to catalyze one-electron reductions of quinone containing antitumor agents.[37] However, since mithramycin lacks a classical quinone moiety in its structure, the mechanism for aureolic acid radical generation by this route is not clear. The hydroquinone adduct **3** showed no antitumor activity over a dosage range of 8–125 μg/kg in the *in vivo* P388 leukemic antitumor test system. It is possible that the lack of antitumor activity by **3** is due to the blockage of the C-5 position, that either prevents the generation of a reactive carbon centered radical at that position, or prevents binding of the hydroquinone–mithramycin adduct (**3**) to DNA.[10]

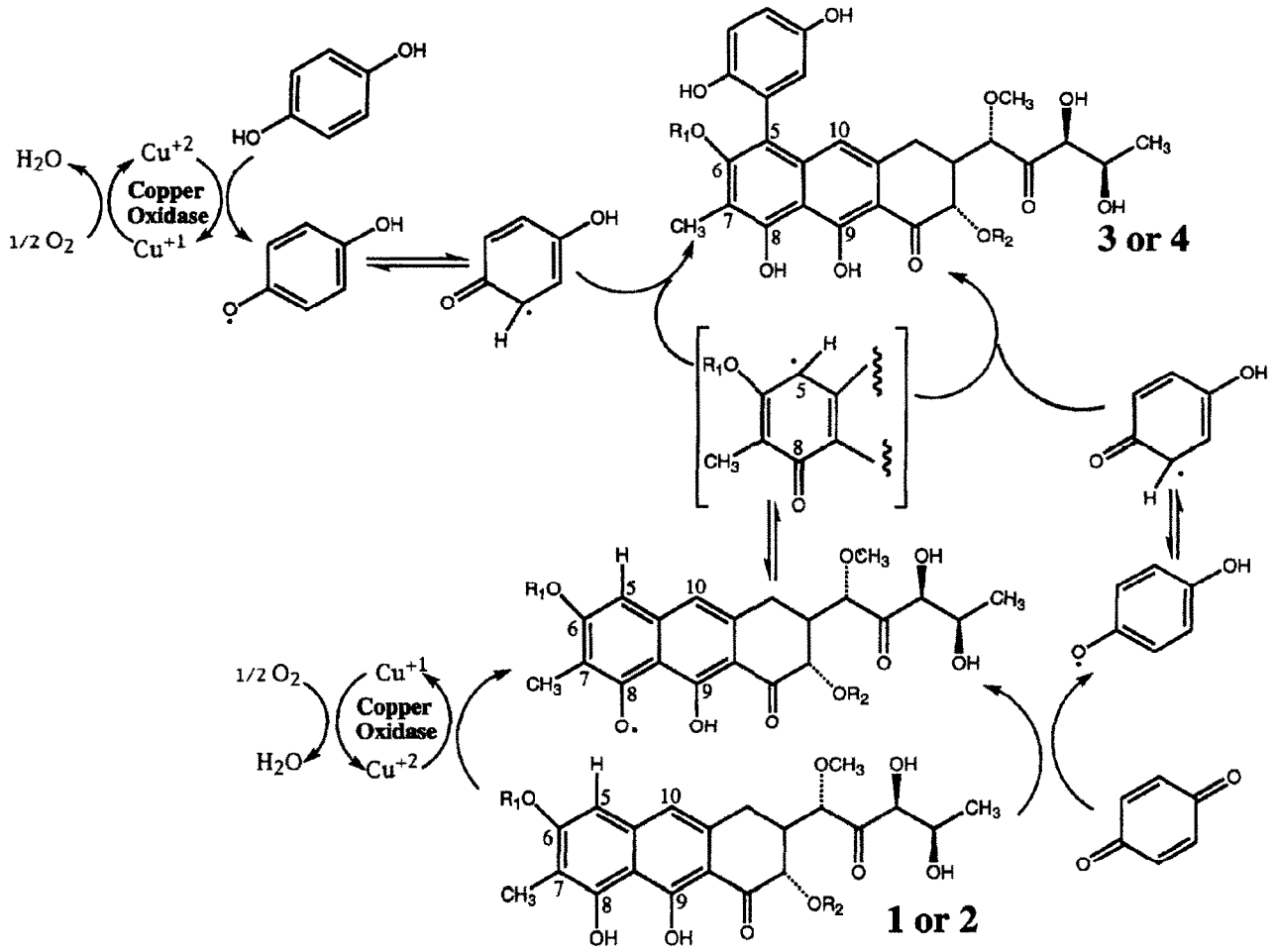



**Figure 5.** Proposed mechanism for oxidative coupling of aureolic acids and hydroquinone catalyzed by copper oxidases and benzoquinone.

## Experimental

NMR experiments were performed at the University of Iowa, High Field NMR core facility. $^{1}H$ and $^{13}C$ NMR spectra were obtained using a Bruker WM-360 spectrometer at 360.13 and 90.7 MHz respectively, with a 5 mm broadband probe and DISN85 software program. NMR spectra were processed on a PCNMR workstation. HMBC and HMQC NMR experiments[40,41] were performed with a Bruker AMX 600 spectrometer operating at 600.14 and 150.92 MHz for $^{1}H$- and $^{13}C$- nuclei respectively, with a 5 mm inverse probe and UXNMR-900330 software. Some routine $^{1}H$ NMR spectra were also obtained on the AMX 600 spectrometer. All NMR samples were dissolved in acetone-$d_6$ (100 %) and the residual acetone peaks at 2.04 ppm, and 29.8 or 206 ppm were used as $^{1}H$ and $^{13}C$ NMR reference peaks, respectively. NMR chemical shifts ($\delta$) are reported in parts per million (ppm) and the coupling constants ($J$ values) are in Hertz (Hz). NMR abbreviations include singlets (s), doublets (d), doublets of doublets (dd), doublets of doublets of doublets (ddd), and multiplets (m).

FABMS was performed at the University of Iowa, High Resolution Mass Spectrometry core facility using a VG Analytical ZAB instrument with 3-nitrobenzyl alcohol (3-NBA) or thioglycerol as ionizing matrices, or at the Midwest Center for Mass Spectrometry at the University of Nebraska–Lincoln using 3-NBA or dithiothreitol/dithioerythritol (DTT/DTE) matrices.

Melting points were determined with a Thomas Hoover Unimelt capillary melting point apparatus.

### *Materials*

Mithramycin (**1**) was obtained from the National Cancer Institute, and as a gift from Pfizer Inc., Groton, CT, and was completely characterized by $^{1}H$ and $^{13}C$ NMR and MS before use.[17,18] Chromomycinone (**2**) was prepared by acid hydrolysis of **1**.[42] Laccase (0.6 units/mL) was isolated from cultures of *Polyporous anceps* using our methods.[43] Human ceruloplasmin (4655 units/mL, 5 % solution in 0.25 M sodium chloride/0.05 M sodium acetate, pH 7.0) was purchased from Sigma (St Louis, MO). The 'oxidase' activity of laccase and ceruloplasmin were determined as previously reported.[43,44] Hydroquinone and 1,4-benzoquinone were also purchased from Sigma. NMR solvents were purchased from Aldrich Chemical Co.

### *Chromatography*

High performance liquid chromatography (HPLC) was performed with a Shimadzu system equipped with an LC-6A dual pump, SCL-6B system controller and CR 501 Chromatopac using an Econosil reverse-phase column (C-

18, 10 mm, 250 x 4.6 mm, Alltech/Applied Sciences). Compounds were eluted isocratically with degassed and filtered solvents at a flow rate of 1 mL/min, while being detected by UV absorption at 280 nm. Solvent system RP-A ($CH_3OH/H_2O/HCOOH$, 60:40:1 v/v/v) was used for **2**, and system RP-B (80:20:1, v/v/v) was used for **1**. Retention volumes ($R_v$) are reported in mL.

Regular phase thin layer chromatography (TLC) consisted of $KH_2PO_4$ plates developed with solvent system A ($CHCl_3/CH_3OH/H_2O$, 90:10:1 v/v/v) for **2** and 75:25:3 (B) for **1**. $KH_2PO_4$ TLC plates were prepared by spreading a 50 % suspension of silica gel 60 $GF_{254}$ (EM Sciences, NJ) in 4 % $KH_2PO_4$ solution on glass plates at 0.5 mm thickness (analytical) or 1 mm thickness (preparative) using a QuikFit (London, UK) spreader. The plates were left to dry for 1 h, and oven-activated by heating at 120 °C for 30 min. Reverse-phase TLC (RP TLC) consisted of 0.25 mm or 1 mm C-18 plates (Merck, RP-$18F_{254}$) and the same solvent systems used in HPLC. The aureolic acids appear as yellow to gold spots on TLC and as UV quenching spots when irradiated with UV light at 254 nm.

For regular phase column chromatography (CC) Baker silica gel for flash column chromatography (J. T. Baker Inc., NJ) was oven dried as a 50 % suspension in 4 % $KH_2PO_4$. The solvent system was the same as for TLC. For reverse-phase CC, silica gel 60 RP-2 (EM Science) was used as adsorbent. CC was performed in J. T. Baker flash glass columns (35 x 2.0 cm) containing 25 g of adsorbent. Columns were usually packed dry, and pressurized at 5–10 psi with $N_2$ gas. Samples of 10 mL were collected using ISCO Retriever II fraction collector operating in the time mode.

*Acid hydrolysis[42] of mithramycin (1) to chromomycinone (2).*

Mithramycin (**1**) (1 g, 0.92 mmol) was dissolved in 50 mL 5 % methanolic HCl, and the solution was held at room temperature for 2 h, until TLC showed that the reaction was complete. Cold water (100 mL) was added to the reaction mixture, which was then extracted three times each with 100 mL EtOAC. The combined extracts were washed with saturated NaCl, dried over anhydrous $Na_2SO_4$ and concentrated to a brown residue. The residue was washed with petroleum ether and precipitated with hexanes to yield 300 mg (0.71 mmol, 78 %) of **2**, which displayed the following physical properties: TLC ($KH_2PO_4$ plates, solvent A) $R_f$ 0.55; TLC (RP-A ), single spot $R_f$ 0.45; HRFAB MS, $[M + H]^+$ $C_{21}H_{25}O_9$, calculated 421.1498, observed 421.1499; $C_{21}H_{24}O_9Na$ $[M + Na]^+$ calculated 443.1318, observed 443.1326; FABMS (DTT/DTE) m/z, $[M + Na]^+$ 443.1, 421.1 $[MH]^+$, 420.1 $[M^+]$. Both the $^1H$ and $^{13}C$ NMR spectra matched literature values.[17,18,45,46] $^1H$ NMR and $^{13}C$ NMR spectral assignments are listed in Table 1. $^1H/^{13}C$ correlations were established by HMQC ($^1H$–$^{13}C$) and HMBC ($^1H$–X–X–$^{13}C$ and $^1H$–X–$^{13}C$) as shown in Table 1.

*Coupling of mithramycin (1) and hydroquinone to form adduct (3) by human serum ceruloplasmin*

Incubations of equimolar concentrations (0.8 μmol) of **1** and hydroquinone with 23 units ceruloplasmin (5 μL) were conducted in 0.5 mL of pH 6.0, 0.1 M NaOAc buffer held at room temperature. Incubations of **1** and hydroquinone, and incubations of hydroquinone or **1** with ceruloplasmin were used as controls. After 30 min, 10 μL samples were withdrawn and injected for HPLC analysis to show peaks for **1** ($R_v$ 5.7 mL), hydroquinone ($R_v$ 2.9 mL), and **3** ($R_v$ 3.8 mL).

*Coupling of mithramycin (1) and hydroquinone to form adduct (3) by* Polyporous anceps *laccase*

Incubations of **1** (1 mg, 0.92 μmol) and hydroquinone (1 mg, 9.1 μmol) with 0.05 units of *P. anceps* laccase were conducted in 3 mL of pH 5.0, 0.2 M $KH_2PO_4$ buffer held at room temperature. Incubations of hydroquinone and **1** with boiled enzyme, and incubations of hydroquinone and laccase without **1** were used as the experimental controls. After 4 h incubation mixtures were acidified to pH 3.0, extracted with 1 mL EtOAC/*i*-PrOH (9:1) to show (TLC, $KH_2PO_4$ plates, solvent B) **1** $R_f$ 0.5, and **3** as a gold spot at $R_f$ 0.4.

*Oxidative coupling of chromomycinone (2) with hydroquinone by human ceruloplasmin*

Incubations of equimolar concentrations (0.8 μmol) of chromomycinone (**2**) and hydroquinone with 23 units of human ceruloplasmin were conducted in 0.5 mL of pH 6.0, 0.1 M NaOAc buffer held at room temperature. Incubations of **2** and hydroquinone, and incubations of hydroquinone or **2** with ceruloplasmin were used as the experimental controls. After 5 min, 10 μL samples were withdrawn and injected for HPLC analysis to show that chromomycinone ($R_v$ 6.6 mL) was replaced by two new peaks with $R_v$ of 3.7 mL and 4.4 mL, and hydroquinone at $R_v$ 2.7 mL. No new peaks were detected in controls. Similar peaks were also detected in the nonenzymatic reaction of **2** with benzoquinone.

*Reaction of chromomycinone (2) and benzoquinone to form 4*

A 10 mL methanolic solution of **2** (50 mg, 0.12 mmol) and 5 mL methanolic solution of 1,4-benzoquinone (25 mg, 0.23 mmol) were mixed with 15 mL of degassed, pH 5.0, 0.2 M $KH_2PO_4$ buffer pH 5.0 and held in the dark at room temperature for 30 min. The reaction mixture was acidified to pH 3.0, and extracted three times with equal volumes of EtOAC, the extracts were concentrated and precipitated with hexanes to give 40 mg of crude **4** (63 % yield). The analytical sample of **4** (20 mg) was obtained by reverse phase CC to show the following properties: TLC ($KH_2PO_4$ plates, solvent A) single $R_f$ 0.35; TLC (RP-A) two spots, $R_f$ 0.5 and 0.55. Preparative TLC (RP-A) gave samples of the two spots each of which contained

a similar 3:2 mixture of spots again at $R_f$ 0.5 and 0.55. HRFAB mass analysis of the two spots gave m/z 528.1639 and 528.1638 for $R_f$ 0.5 and 0.55, respectively, indicating a molecular ion of $C_{27}H_{28}O_{11}$ (calculated 528.1631). The two spots also displayed identical MS and NMR spectral characteristics. FABMS (3-NBA matrix) [m/z] 529.1 ([MH]$^+$); 528.1 ([M$^+$]); $^1$H and $^{13}$C NMR (Table 1).

*Synthesis of hydroquinone adduct (3) by reaction of benzoquinone with mithramycin (1)*

Mithramycin (1) (600 mg, 0.55 mmol) and 1,4-benzoquinone (600 mg, 5.55 mmol) were dissolved in 100 mL of pH 5.0, 0.2 M $KH_2PO_4$ buffer and stirred at room temperature for 75 min. The reaction mixture was acidified to pH 3 and extracted with four equal volumes of EtOAC/*i*-PrOH (9:1). The combined extracts were washed with saturated NaCl, dried over anhydrous $Na_2SO_4$ and concentrated to a dark brown residue. Precipitation with hexanes afforded 522 mg of crude **3**. A 190 mg sample of crude **3** was dissolved in MeOH and purified by preparative TLC ($KH_2PO_4$ plates, 1 mm, solvent B). The yellow bands at $R_f$ 0.4 were combined, washed with EtOH, filtered, and concentrated. Precipitation with hexanes yielded the analytical sample of 59 mg of **3** as a yellowish-brown powder with the following physical properties: TLC ($KH_2PO_4$ plates, solvent B), $R_f$ 0.4; mp (corr.) 148–150 °C; FABMS (thioglycerol), ([m/z] % relative intensity) 1216.3 ([MH + Na]$^+$, 19.3 %), 1215.3 ([M + Na]$^+$, (28.7 %), 1214.7 (10.4 %), 529.9 (30.6 %), 528.9 (78.4), 527.9 ([M – sugars]$^+$, 100 %).

*Acid hydrolysis of mithramycin adduct (3) to the aglycone (4)*

A sample of crude **3** (400 mg, 0.34 mmol) was hydrolyzed in 5 % methanolic HCl as before to obtain 131 mg **4** (73 %). The product was chromatographically and spectroscopically identical to **4** and gave the following physical properties: HRFABMS, calculated for $C_{27}H_{28}O_{11}$, 528.1631, observed 528.1657; FABMS (3-NBA), ([m/z] % relative intensity) 551.1 ([M + Na]$^+$, 6 %); 529.1 ([MH]$^+$, 55 %); 528.1 ([M]$^+$, 100 %). $^1$H NMR identical with **4** (Table 1).

*Determination of* in vivo *P388 antitumor activities of 3 versus 1*

The antitumor activities of mithramycin (**1**) and the hydroquinone adduct (**3**) were evaluated at the National Cancer Institute (NCI), Developmental Therapeutics Program, Division of Cancer Treatment in a standard P388 leukemia assay. Ascitic fluid tumor cells ($10^6$ cells) were transplanted intraperitoneally (ip) into $CDF_1$ male mice. The compounds were administered to six mice ip in water for each dose which ranged from 0.06–1.0 mg/kg for **1** as a positive control, and 0.008–1.0 mg/kg for **3**, daily for 9 days. Tests were evaluated by the ratio of the median survival time (MST) of the treated versus control (% T/C), [% T/C = (MST treated/MST control) x 100]. Compounds were considered toxic if the number of survivors on day 5 were less than 4 out of 6 treated mice. Under these conditions, **3** displayed no P388 activity at any tested dose. At 1 mg/kg, none of the test animals survived at day 5 for **3** while 3 of six test animals survived at the same dose for mithramycin.

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