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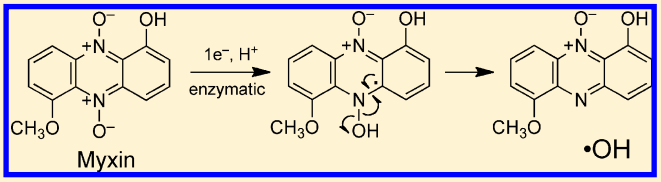
DNA Strand Cleavage by the Phenazine Di-*N*-oxide Natural Product Myxin under Both Aerobic and Anaerobic Conditions

Goutam Chowdhury,[†] Ujjal Sarkar,[†] Susan Pullen,[‡] William R. Wilson,[‡] Anuruddha Rajapakse,[†] Tarra Fuchs-Knotts,[†] and Kent S. Gates*[,†]

[†]Departments of Chemistry and Biochemistry, University of Missouri—Columbia, 125 Chemistry Building, Columbia, Missouri 65211, United States

[‡]Auckland Cancer Society Research Centre, The University of Auckland, Private Bag 92019, Auckland, New Zealand

ABSTRACT: Heterocyclic *N*-oxides are an interesting class of antitumor agents that selectively kill the hypoxic cells found in solid tumors. The hypoxia-selective activity of the lead compound in this class, tirapazamine, stems from its ability to undergo intracellular one-electron reduction to an oxygen-sensitive drug radical intermediate. In the presence of molecular oxygen, the radical intermediate is back-oxidized to the parent molecule. Under hypoxic conditions, the extended lifetime of the drug radical intermediate enables its conversion to a highly cytotoxic DNA-damaging intermediate via a "deoxygenative" mechanism involving the loss of oxygen from one of its *N*-oxide groups. The natural product myxin is a phenazine di-*N*-oxide that displays potent antibiotic activity against a variety of organisms under aerobic conditions. In light of the current view of heterocyclic *N*-oxides as agents that selectively operate under hypoxic conditions, it is striking that myxin was identified from *Sorangium* extracts based upon its antibiotic properties under aerobic conditions. Therefore, we set out to examine the molecular mechanisms underlying the biological activity of myxin. We find that myxin causes bioreductively activated, radical-mediated DNA strand cleavage under both aerobic and anaerobic conditions. Our evidence indicates that strand cleavage occurs via a deoxygenative metabolism. We show that myxin displays potent cytotoxicity against the human colorectal cancer cell line HCT-116 under both aerobic and anaerobic conditions that is comparable to the cell-killing properties of tirapazamine under anaerobic conditions. This work sheds light on the processes by which the naturally occurring aromatic *N*-oxide myxin gains its potent antibiotic properties under aerobic conditions. Furthermore, these studies highlight the general potential for aromatic *N*-oxides to undergo highly cytotoxic deoxygenative metabolism following enzymatic one-electron reduction under aerobic conditions.



INTRODUCTION

Heterocyclic aromatic *N*-oxides are an interesting class of antitumor agents exhibiting bioreductively activated, hypoxia-selective DNA-damaging properties.[1−7] Interest in these compounds has been driven by the fact that they display selective toxicity toward the hypoxic cells found in solid tumors.[8] Tirapazamine (**1**, TPZ, Scheme 1) is the lead compound in this class of drugs and has been examined in a variety of phase I, II, and III clinical trials for the treatment of various cancers.[9−11] Bioreductively activated aromatic *N*-oxides also have the potential to kill *Mycobacterium tuberculosis*[12] and hypoxic bacteria in the gastrointestinal and urinary tracts.[13−16]

The hypoxia-selective cytotoxicity of tirapazamine and other structurally related aromatic *N*-oxides stems from their ability to undergo intracellular one-electron reduction to an oxygen-sensitive drug radical intermediate (**2**, Scheme 1).[17−20] In the presence of molecular oxygen, the radical intermediate is rapidly oxidized to regenerate the parent molecule and an equivalent of superoxide radical.[20,21] In the case of medicinally interesting *N*-oxides such as **1**, this futile cycling process is evidently substantially less toxic than processes that occur under hypoxic conditions. Under hypoxic conditions, the extended lifetime of the drug radical intermediate enables its conversion to highly cytotoxic DNA-damaging intermediates via a "deoxygenative" mechanism involving loss of oxygen from the *N*-oxide group in the 4-position.[22,23] DNA damage is central to the bioactivity of tirapazamine,[24−27] but the exact nature of the reactive intermediate(s) generated following bioreductive activation of tirapazamine remains under investigation. Mechanisms involving homolytic fragmentation to generate the known cell-killing agent hydroxyl radical[28−31] or dehydration to yield a highly oxidizing benzotriazinyl radical[32−36] have been proposed. The mono-*N*-oxide metabolites such as **3** are diagnostic byproducts of the cell-killing, deoxygenative metabolism that can follow one-electron enzymatic activation of tirapazamine.[37]

There are only a handful of natural products that contain aromatic *N*-oxide functional groups.[38−43] The compound, 1-hydroxy-6-methoxyphenazine-N5,N10-dioxide (**7**, Scheme 2), commonly known as myxin, is an interesting example. Myxin was first isolated from *Sorangium* sp. by Peterson et al.[44] This natural product displays potent antimicrobial activity against a wide variety of organisms including *Streptococcus agalactiae*,

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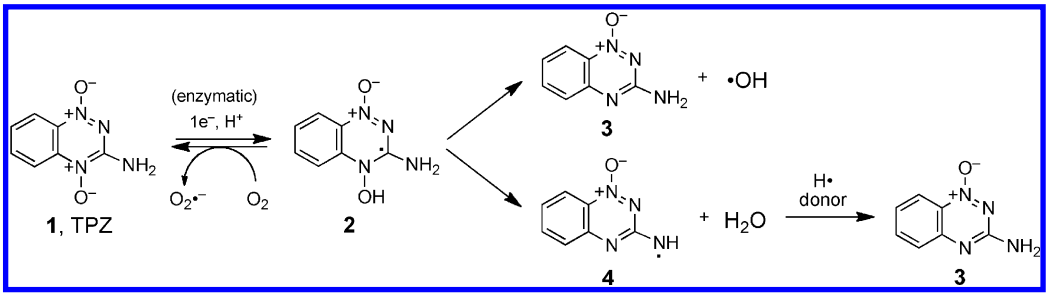
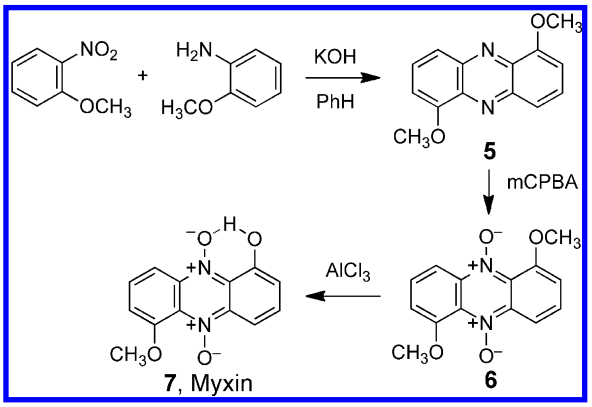
Scheme 1



Scheme 2



Staphylococcus aureus, *E. coli*, *Pseudomonas aeruginosa*, and *Candida albicans*, with minimum inhibitory concentrations in the range of 1−20 μM.[45,46] Several structurally related synthetic phenazine di-*N*-oxides that display bioreductively activated cytotoxicity under aerobic conditions have been identified.[3,47] Several lines of evidence from early studies indicated that interactions with DNA may be important in the biological activity of myxin. Myxin inhibits both DNA and RNA synthesis and causes extensive degradation of cellular DNA in *E. coli*.[48,49] In *Salmonella*, myxin causes frameshift mutations.[50] Myxin also was found to noncovalently associate with DNA via intercalation.[51] It was also noted that myxin undergoes reduction in *E. coli* to biologically inactive products, though structural characterization of the metabolites was not presented in this early work.[46,52]

In light of the current view of heterocyclic *N*-oxides as agents that selectively operate under hypoxic conditions, it is striking that myxin was identified from *Sorangium* extracts based upon its antibiotic properties under aerobic conditions. Therefore, we set out to examine potential molecular mechanisms underlying the biological activity of myxin. Our evidence suggests that myxin causes bioreductively activated, radical-mediated DNA strand cleavage under aerobic conditions via a deoxygenative mechanism analogous to that characterized for tirapazamine under anaerobic conditions.

MATERIALS AND METHODS

Materials. Materials were obtained from the following suppliers and were of the highest purity available. Unless otherwise mentioned, all chemicals were from Aldrich Chemical Co. (Milwaukee, WI); sodium acetate, NADPH, desferal, and NADPH/cytochromeP450 reductase, Sigma Chemical Co. (St. Louis, MO); HPLC grade solvents acetonitrile, methanol, ethanol, *t*-butanol, ethyl acetate, hexane, and acetic acid, Fisher (Pittsburgh, PA); xanthine oxidase, catalase, SOD, and ethidium bromide, Roche Molecular Biochemicals (Indianapolis, IN); Seakem ME agarose, FMC; ethanol, McCormick Distillation Co. (Brookfield, CN); and bromophenol blue, United States Biochemicals (Cleveland, OH).

Synthesis of 1,6-Dimethoxyphenazine (5). This compound was prepared previously by Pachter et al., and we used a slight modification of the published approach.[53] To a solution of benzene (180 mL) was added *o*-anisidine (28.4 mL, 0.25 mol), *o*-nitroanisole (55.8 mL, 0.45 mol), and powdered potassium hydroxide (70 g; 1.25 mol). The mixture was heated at reflux for 6.5 h with vigorous stirring (not under inert gas). Upon cooling, the dark-brown benzene solution was decanted off, and water (500 mL) was added to the remaining solid in the reaction flask. After breaking up the chunky residue with a spatula or glass rod, the solid was collected and washed twice with ethanol (250 mL) to give a yellow crystalline solid. The original benzene solution from above was diluted with additional benzene and extracted with 15% HCl. The aqueous layer was then made basic, and the resulting yellow solid was collected and washed with ethanol. The yellow crystalline solids from above were combined and recrystallized from hot ethanol to give **5** (8.7 g, 14%): R_f = 0.51 (100% ethyl acetate). ^{1}H NMR ($CDCl_3$) δ 7.98 (d, 2H), 7.74 (t, 2H), 7.08 (d, 2H), 4.18 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 155.6, 143.7, 137.6, 130.8, 111.7, 107.6, 57.2.

Synthesis of 1,6-Dimethoxyphenazine-N5,N10-dioxide (6). The preparation of this compound was achieved using a modification of the route described by Weigele et al.[54] To a solution of methylene chloride (25 mL) was added **5** (120 mg, 0.5 mmol) and *m*-CPBA (0.5 g, Aldrich, 77% max). The mixture was stirred for 2 h. Removal of solvent by rotary evaporation, followed by column chromatography on silica gel eluted with 100% ethyl acetate, afforded **6** (18 mg; 13%) as a red solid: R_f = 0.06 (100% ethyl acetate). ^{1}H NMR ($CDCl_3$) δ 8.32 (d, 2H), 7.63 (t, 2H), 7.08 (d, 2H), 4.09 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 153.6, 139.5, 130.5, 112.1, 110.2, 57.2. Spectral data matched that reported by Weigele et al.[54]

Synthesis of 1-Hydroxy-6-methoxyphenazine (8). Aluminum chloride (410 mg) was added to a solution of **5** (151 mg, 0.62 mmol) in methylene chloride (50 mL) at room temperature. The reaction mixture was stirred at room temperature for 20 min and quenched by the addition of 2 N HCl (100 mL). The resulting mixture was extracted with methylene chloride (5 × 100 mL), the combined organic extracts dried over sodium sulfate, and then evaporated under reduced pressure. Column chromatography on silica gel eluted with 1:1 ethyl acetate/hexane gave **8** (25 mg, 17%) as a yellow solid: R_f = 0.67 (100% ethyl acetate). ^{1}H NMR ($CDCl_3$) δ 8.17 (s, 1H), 7.92 (d, 1H), 7.74 (m, 3H), 7.24 (d, 1H), 7.06 (d, 1H), 4.17 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 155.1, 151.3, 142.4, 141.8, 137.4, 134.5, 131.2, 130.3, 120.8, 120.4, 109.1, 106.7, 56.4. HRMS (ESI) calcd for $C_{14}H_{13}N_2O_4$ $[M + H^+]^+$ 273.0875 found 273.0876.

Synthesis of 1-Hydroxy-6-methoxyphenazine-N5,N10-dioxide (7). To a solution of **6** (23 mg, 0.08 mmol) in methylene chloride (10 mL) was added aluminum chloride (50 mL). The mixture was stirred for 1.5 h. After the reaction was complete, 2 N HCl (40 mL) was added to the mixture, and a color change from blue to red-orange occurred. The aqueous layer was then extracted with methylene chloride (8 × 30 mL) and the combined organic extracts dried by rotary evaporation. Column chromatography over silica gel eluted with 100% ethyl acetate, followed by 4:1 ethyl acetate/hexane, afforded **7** (5 mg, 24%): R_f = 0.58 (100% ethyl acetate). ^{1}H NMR ($CDCl_3$) δ 14.59

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(s, 1H), 8.25 (d, 1H), 8.02 (d, 1H), 7.68 (t, 1H), 7.63 (t, 1H), 7.12 (d, 1H), 7.09 (d, 1H), 4.09 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 154.5, 154.4, 139.4, 136.6, 133.1, 132.4, 130.6, 126.6, 115.6, 111.3, 110.4, 109.6, 58.0. The material was pure as judged by NMR, TLC, and HPLC. Spectral data matched that in the published literature.[54,55]

Synthesis of 1-Hydroxy-6-methoxyphenazine-N10-oxide (9) and 1-Hydroxy-6-methoxyphenazine-N5,N10-dioxide (7) by Oxidation of 1-Hydroxy-6-methoxyphenazine (8). To a solution of **8** (45 mg, 0.2 mmol) in methylene chloride (10 mL), was added *m*-CPBA (250 mg) and the resulting mixture stirred at room temperature for 12 h. The solvent was then removed under reduced pressure, and column chromatography on silica gel eluted with 4:1 ethyl acetate/hexane gave **9** (5 mg, 10%) as the first eluting compound: R_f = 0.65 (100% ethyl acetate). ^{1}H NMR ($CDCl_3$) δ 13.68 (s, 1H), 8.17 (d, 1H), 7.85 (d, 1H), 7.71 (q, 2H), 7.11 (d, 2H), 4.19 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 156.4, 152.7, 145.9, 139.6, 134.2, 133.1, 132, 125.7, 120.8, 113.6, 110.4, 108.8, 57.8. The second eluting compound was myxin, **7** (4 mg, 8%).

Synthesis of *t*-Butyldiphenylsilyl-Protected 1-Hydroxy-6-methoxyphenazine (10). To a solution of **8** (23 mg, 0.1 mmol) in methylene chloride (5 mL) was added imidazole (34 mg, 0.5 mmol) and the mixture allowed to stir at room temperature for 10 min. To the solution *t*-butyldiphenylsilyl chloride (TBDPS-Cl, 41 μL, 0.15 mmol) was added and the reaction stirred for another 4 h. The reaction mixture was extracted with a saturated aqueous solution of sodium chloride (10 mL). The aqueous layer was back-extracted with methylene chloride (3 × 10 mL), the organic fractions pooled, dried over sodium sulfate, and evaporated under reduced pressure. Finally, column chromatography on silica gel eluted with 2:1 hexane/ethyl acetate gave **10** (22 mg, 47%): R_f = 0.82 (100% ethyl acetate). ^{1}H NMR ($CDCl_3$) δ 7.95 (d, 1H), 7.84 (d, 4H), 7.64 (t, 1H), 7.54 (m, 2H), 7.63−7.38 (m, 6H), 7.09 (d, 1H), 7.03 (d, 1H), 4.16 (s, 3H), 1.27 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 154.7, 151.6, 142.9, 142.8, 138.1, 136.4, 135.3, 133.3, 130.1, 129.5, 129.4, 127.4, 122.6, 121.8, 116.3, 106.4, 56.2, 26.7, 20.0.

Synthesis of 1-Hydroxy-6-methoxyphenazine-N5-oxide (12). To a solution of **10** (22 mg, 0.047 mmol) in methylene chloride (5 mL) was added *m*-CPBA (17 mg) and the mixture stirred for 5 h. Additional *m*-CPBA (17 mg) was added to the reaction mixture and stirred for another 10 h. Tetrabutylammonium fluoride (TBAF, 1 M solution in THF, 50 μL, 0.05 mmol) was added to the reaction mixture and allowed to stir for 3 h. The reaction was quenched by the addition of saturated aqueous solution of sodium chloride (20 mL) and extracted with methylene chloride (3 × 50 mL). The organic extracts were dried over sodium sulfate and evaporated under reduced pressure. Column chromatography on silica gel eluted with a 1:1 ethyl acetate/hexane gave **12** (6 mg, 55%) as a pale violet solid: R_f = 0.51 (100% ethyl acetate). ^{1}H NMR ($CDCl_3$) δ 8.09 (bs, 1H), 8.07 (d, 1H), 7.74 (d, 1H), 7.65 (t, 1H), 7.59 (t, 1H), 7.20 (d, 1H), 7.00 (d, 1H), 4.08 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 154.0, 153.2, 146.3, 137.8, 137.2, 131.45, 131.43, 130.2, 122.8, 111.3, 110.4, 108.7, 57.7. HRMS (ESI) calcd for $C_{13}H_{11}N_2O_3$ $[M + H^+]^+$ 243.0770; found, 243.0771.

Cleavage of Supercoiled Plasmid DNA. Typical DNA cleavage reactions contained supercoiled plasmid DNA (750 ng, pGL2-basic), **1** (25−225 μM) or **7** (50−250 μM), xanthine (500 μM), xanthine oxidase (0.4 U/mL), catalase (100 μg/mL), superoxide dismutase (10 μg/mL), sodium phosphate buffer (50 mM, pH 7.0), and desferal (1 mM). In the case of the anaerobic reactions, individual components, with the exception of DNA and enzyme, were degassed prior to use by three freeze−pump−thaw cycles in Pyrex tubes and then sealed under vacuum. Sealed tubes were scored, transferred to a glovebag purged with argon, and opened. The degassed solutions were used to prepare individual reactions mixtures (final volume 30 μL). Reactions were initiated by the addition of xanthine oxidase, wrapped with aluminum foil to prevent exposure to light, and incubated for 16 h in the glovebag at room temperature (24 °C). Following incubation, the reactions were stopped by the addition of 3 μL of 50% glycerol loading buffer, and the resulting mixture loaded onto 0.9% agarose gel. The gel was electrophoresed for approximately 3 h at 80 V in 1 × Tris-acetate-EDTA (TAE) buffer and then stained in a solution of aqueous ethidium bromide (0.3 μg/mL) for 1−2 h. DNA was visualized by UV-transillumination and quantitated by digital imaging. The values reported are not corrected for differential staining of form I and form II DNA by ethidium bromide.[56] DNA cleavage assays containing radical scavengers were performed as described above with the exception that radical scavengers like methanol, ethanol, *t*-butanol, DMSO, or mannitol (500 mM) were added to the reaction mixture before the addition of xanthine oxidase. Aerobic reactions were performed as described above, with the exception that the solutions were not degassed, and the incubations were performed under air.

Characterization of Products Arising from in Vitro Metabolism of Myxin. In a typical assay, a solution of myxin (**7**, 250 μM), desferal (1 mM), and xanthine (250 μM) in sodium phosphate buffer was mixed with xanthine oxidase (0.4 U/mL). The assays were incubated for 3 h at room temperature. After incubation, the reaction mixture was extracted with methylene chloride (2 × 100 μL) and then analyzed by normal phase HPLC employing a Rainin Microsorb-MV amino column (100 Å sphere size, 5 mm pore size, 25 cm length, 4.6 mm i.d.) eluted with an isocratic mobile phase of 70% ethyl acetate and 30% heptane (each solvent containing 0.5% acetic acid), at a flow rate of 1 mL/min. The elution of products from the column was monitored by UV absorbance at 284 nm. The identity of the major metabolites was determined by comparison of the retention time to that of authentic standards and by coinjection with the authentic synthetic samples.

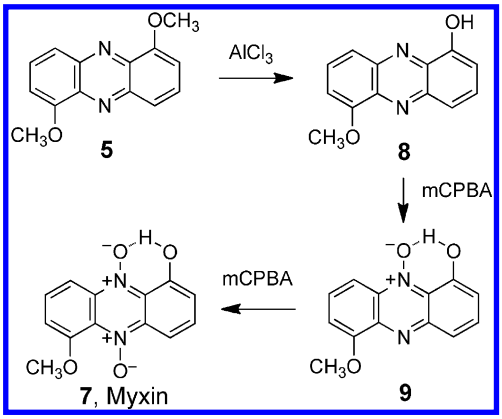
Cytotoxicity Assay. The colon carcinoma line HCT116, sourced from ATCC, was grown in AlphaMEM plus 5% fetal calf serum (FCS) from frozen stocks confirmed to be mycoplasma-free by PCR-ELISA (Roche Diagnostics). Late log-phase cultures were harvested by trypsinization and centrifuged to prepare cell pellets for parallel exposure to compounds under aerobic and anaerobic conditions. For the latter, the cell pellet was transferred to a Pd catalyst anaerobic chamber (Bactron II, Shellab) with an atmosphere of 5% H_2/5%CO_2/90% N_2. The pellet was resuspended in Alpha MEM with 10% FCS plus 10 mM added glucose and 0.2 mM 2-deoxycytidine. The medium and plasticware were previously equilibrated in the chamber for ≥3 days to remove residual oxygen. Cells were plated in 96-well plates (1000 cells/well) and allowed to attach at 37 °C for 2 h before the addition of tirapazamine (**1**) or myxin (**7**) from DMSO stocks (final DMSO concentration <0.5%) by making 3-fold serial dilutions across the plate in duplicate wells. Plates were incubated for a further 4 h, removed from the chamber, and washed 3× with fresh medium (in parallel with plates exposed under oxic conditions). After growth in a CO_2 incubator for a further 5 days, total cellular protein was determined by sulforhodamine B staining[57] using a ELx808 plate reader (BioTek Instruments). IC_{50} values (concentration for 50% inhibition relative to 8 controls on the same plate) were interpolated by four-parameter logistic regression (KC4 v3.4 software).

RESULTS

Synthesis of Myxin. Preparation of the myxin precursor 1,6-dimethoxyphenazine (**5**) was carried out using the method of Patcher and Kloetzel, involving a modified version of the Wohl-Aue reaction.[53,58] Briefly, 2-nitroanisole and 2-anisidine were dissolved in benzene and heated at reflux in the presence of KOH (Scheme 2). A black tar was obtained which, upon repeated washing with ethanol, gave yellow crystals of 1,6-dimethoxyphenazine. The resulting 1,6-dimethoxyphenazine was then oxidized by *meta*-chloroperbenzoic acid (*m*-CPBA) to give the 1,6-dimethoxyphenazine-N5,N10-dioxide (**6**). Myxin (**7**) was prepared by the method of Weigele and Liemgruber involving partial demethylation of **6** by $AlCl_3$.[54] The yield of this reaction was low due to the formation of the 1,6-dihydroxy compound as a side product (the dihydroxy compound is a different natural product, known as iodinin.[59]).

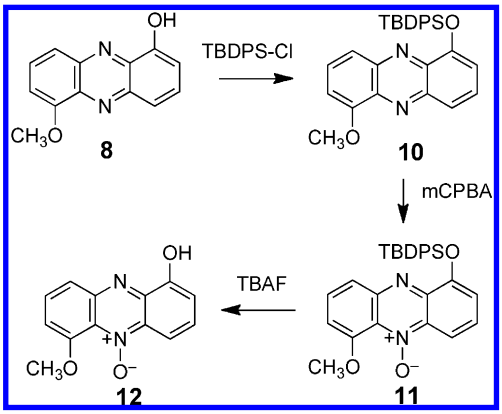
Myxin and its metabolites also were synthesized by an alternative route (Scheme 3). This method involved partial

Scheme 3



demethylation of 1,6-dimethoxyphenazine by $AlCl_3$ to give 1-hydroxy-6-methoxyphenazine (**8**) in the first step. Compound **8** was then oxidized by *m*-CPBA to give the mono-*N*-oxide **9** and myxin (**7**). Although two mono-*N*-oxide isomers are possible from the partial oxidation of **8**, the reaction gave **9** as the sole mono-*N*-oxide product. The position of *N*-oxidation in **9** was indicated by a diagnostic chemical shift for the phenolic proton that appears as a sharp singlet at approximately 13 ppm in $CDCl_3$, presumably due to hydrogen bonding with the oxygen of the *N*-oxide group.[54] The fact that the "down" 5-oxide **12** was not observed in the oxidation of **8** suggested that the 1-hydroxy group may direct *N*-oxidation to the N10 position. We wished to prepare the 5-*N*-oxide analogue for use as a standard for the *in vitro* metabolism experiments described below. To enable selective oxidation at N5, the hydroxyl group in **8** was protected with a *t*-butyldiphenylsilyl (TBDPS) group (Scheme 4). This was intended to mask the directing effect of

Scheme 4



the hydroxyl group and also was expected to sterically hinder oxidation at the N10 position. Accordingly, oxidation of the TBDPS-protected compound **10** by *m*-CPBA gave the 5-*N*-oxide **11** as the major product. Finally, deprotection of the hydroxyl group by tetrabutylammonium fluoride (TBAF) gave compound **12** in 26% overall yield. In contrast to the NMR of **9**, the phenolic proton in **12** appears as a broad singlet at about 8 ppm versus tetramethylsilane (TMS).

DNA Cleavage by Myxin under Aerobic and Anaerobic Conditions. We used a plasmid-based assay to assess the ability of the myxin to cause bioreductively activated DNA strand cleavage. This assay exploits the fact that oxidative DNA strand cleavage causes conversion of the supercoiled plasmid to the open circular form.[60−63] These two forms of plasmid DNA can be separated using agarose gel electrophoresis, followed by staining with the DNA-binding dye ethidium bromide. In these studies, we used xanthine/xanthine oxidase for reductive activation of **7** and other compounds. This enzyme system has been used for the activation of tirapazamine in a number of *in vitro* studies.[13,20,28−30,64−66] For reactions carried out under hypoxic conditions, molecular oxygen was removed from the solutions by freeze−pump−thaw degassing and the assay mixtures prepared and incubated in an inert atmosphere glovebag. Unless otherwise noted, catalase, superoxide dismutase, and desferal were added to prevent strand cleavage stemming from the conversion of molecular oxygen to the superoxide radical as described previously.[29,65] To prevent the potential photocleavage of DNA mediated by these compounds,[67,68] the reactions were shielded from light using foil.

Myxin was found to induce DNA cleavage in the presence of the xanthine/xanthine oxidase enzyme system under anaerobic conditions (Figure 1). The efficiency of DNA cleavage of myxin

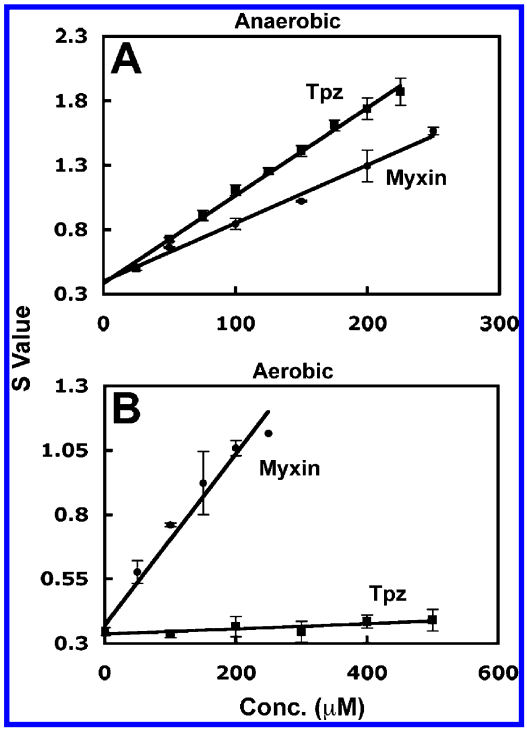


Figure 1. Efficiency of DNA cleavage of tirapazamine (**1**, TPZ) and myxin (**7**) under anaerobic or aerobic conditions in the presence of the xanthine/xanthine oxidase system. Supercoiled plasmid DNA (750 ng) was incubated with **1** or **7**, xanthine (500 μM), xanthine oxidase (0.4 U/mL), catalase (100 μg/mL), superoxide dismutase (10 μg/mL), sodium phosphate buffer (50 mM, pH 7.0), and desferal (1 mM) under anaerobic or aerobic conditions at room temperature for 16 h, followed by agarose gel electrophoretic analysis. Strand breaks per plasmid DNA molecule (S) was calculated using the equation $S = -\ln f_1$, where f_1 is the fraction of plasmid present as form I.

is comparable to that generated by the medicinally relevant *N*-oxide, tirapazamine, under anaerobic conditions (Figure 1A). Surprisingly, when DNA cleavage was performed under aerobic conditions, myxin retained substantial DNA-cleaving power (Figure 1B). In contrast, tirapazamine generates little or no strand cleavage under aerobic conditions (Figure 1B).[29]

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Control reactions showed that xanthine + myxin (no enzyme), myxin alone, or xanthine oxidase + xanthine (no myxin) did not induce significant strand cleavage under these conditions (Figure 2).

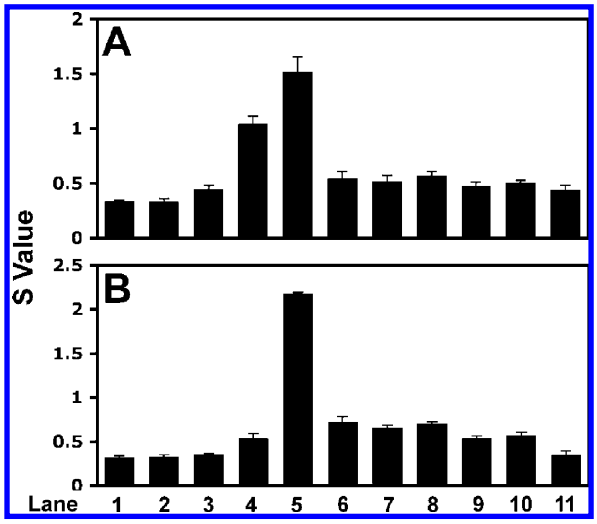

Figure 2. DNA cleavage by myxin (7, 250 μM) under anaerobic (A) and aerobic conditions (B) in the presence of various radical scavengers. Xanthine/xanthine oxidase was used as a one-electron reducing agent to activate the compounds. Supercoiled plasmid DNA (750 ng) was incubated with 7, xanthine (500 μM), xanthine oxidase (0.4 U/mL), catalase (100 μg/mL), superoxide dismutase (10 μg/mL), sodium phosphate buffer (50 mM, pH 7.0), and desferal (1 mM) under anaerobic (panel A) or aerobic conditions (panel B) in the presence of various radical scavengers (500 mM) at room temperature for 16 h, followed by analysis using agarose gel electrophoresis. Lane 1, DNA alone; lane 2, DNA + xanthine/xanthine oxidase; lane 3, DNA + 7 + xanthine; lane 4, DNA + 7 + XO; lane 5, DNA + 7 + X/XO; lanes 6–10, DNA + 7 + X/XO + methanol, ethanol, *t*-butanol, DMSO (500 mM), and mannitol; and lane 11, DNA + 7. Strand breaks per plasmid DNA molecule (S) was calculated using the equation $S = -\ln f_1$, where f_1 is the fraction of plasmid present as form I.

To better understand the mechanism of DNA strand cleavage by myxin under both aerobic and anaerobic conditions, the plasmid-based DNA-cleavage assay was performed in the presence of various radical scavengers (Figure 2). DNA strand cleavage by tirapazamine and structurally related analogues has been shown to proceed via a radical mechanism, and addition of radical scavengers such as methanol, ethanol, *t*-butanol, mannitol, and DMSO can quench bioreductively activated DNA strand cleavage by these drugs.[13,29,30,65,66] Here, we find that addition of the radical scavenging agents methanol, ethanol, *t*-butanol, mannitol, or DMSO significantly decreased bioreductively activated strand cleavage caused by myxin under both aerobic and anaerobic conditions (Figure 2).

Interestingly, myxin causes DNA strand scission in the presence of xanthine oxidase, even in the *absence* of the enzyme's usual electron source, xanthine (Figure 2A, lane 4). Under anaerobic conditions, the xanthine-free strand cleavage yields are clearly above background, although they are markedly less than those resulting from the process that is driven by the complete xanthine/xanthine oxidase enzyme system. A similar phenomenon was observed previously for quinoxaline 1,4-di-*N*-oxide.[13] In that case, oxidation of the quinoxaline heterocycle by xanthine oxidase generated reduced enzyme that, in turn, carried out reductive activation of the 1,4-di-*N*-oxide. Thus, some heterocyclic *N*-oxides are able to serve as substrates for xanthine oxidase, in effect fueling their own reductive activation. Xanthine oxidase is able to oxidize a variety of heterocyclic nitrogen substrates,[69–71] and it appears that myxin, or adventitious traces of the mono-*N*-oxides derivatives, can serve this role. The xanthine oxidase-catalyzed "self-fueled activation" of myxin is more effective under anaerobic conditions than it is under aerobic conditions presumably because, under aerobic conditions, molecular oxygen competes with the *N*-oxide to accept electrons from the reduced enzyme.[72] This enzyme-mediated, self-fueled activation process was well characterized in the case of quinoxaline di-*N*-oxide[13] but has not been studied in detail here.

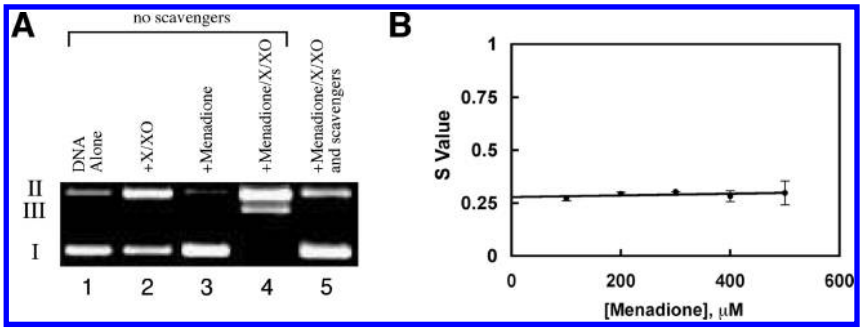

Figure 3. Aerobic DNA cleavage caused by redox cycling of menadione in the presence of xanthine/xanthine oxidase as an activating system can be quenched by the presence of superoxide dismutase, catalase, and desferal. (A) Treatment of supercoiled plasmid DNA with menadione and xanthine/xanthine oxidase. Numbers in parentheses following each lane description are the number of strand breaks, S, measured under each condition, where $S = -\ln f_1$, where f_1 is the fraction of plasmid present as form I.[84] For lane 4, containing form III DNA, the number of strand breaks was calculated using the equation form I + form II = $[1 - S(2\,h + 1)/(2\,L)]^{S/2}$, where h is the maximum distance between breaks on opposing strands that can yield linearized plasmid via spontaneous melting of the resulting sticky ends (16 bp), and L is the total number of base pairs in the plasmid (5598, pGL2-basic).[84] Lane 1, supercoiled plasmid DNA (750 ng) (0.1 ± 0.1); lane 2, DNA + xanthine (500 μM) and xanthine oxidase (0.4 U/mL) (1.0 ± 0.1); lane 3, DNA + menadione (250 μM) (0.02 ± 0.01); lane 4, DNA + menadione (250 μM) + xanthine (500 μM) and xanthine oxidase (0.4 U/mL) (7.6 ± 5.3); lane 5, DNA + menadione (250 μM) + xanthine (500 μM) and xanthine oxidase (0.4 U/mL) + desferal (1 mM), SOD (10 μg/mL), and catalase (100 μg/mL) (0.3 ± 0.1). Assays were conducted in sodium phosphate buffer (pH 7.0, 50 mM) and were incubated at room temperature for 16 h under aerobic conditions. (B) No significant strand cleavage above background was generated by the incubation of DNA + menadione (0–500 μM) + xanthine (500 μM) and xanthine oxidase (0.4 U/mL) in the presence of desferal (1 mM), SOD (10 μg/mL), and catalase (100 μg/mL) in sodium phosphate buffer (pH 7.0, 50 mM) incubated at room temperature for 16 h under aerobic conditions.

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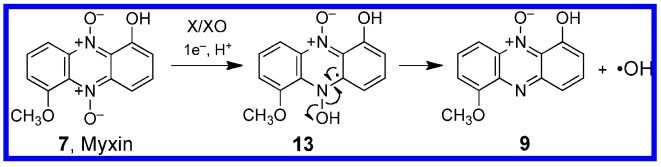
In principle, the DNA strand cleavage observed for myxin under aerobic conditions could result either from deoxygenative metabolism processes or from redox cycling as shown on the left side of Scheme 1 in the context of tirapazamine.[20,21,66] Redox cycling has the potential to generate the superoxide radical, hydrogen peroxide, and, ultimately, the DNA-cleaving hydroxyl radical.[73] However, it is important to reiterate that our assays contained superoxide dismutase, catalase, and desferal that are intended to squelch strand cleavage arising from redox-cycling processes. Our early results suggested that these additives did indeed perform the desired function. Specifically, despite the fact that tirapazamine is known to undergo enzyme-driven redox cycling under aerobic conditions,[20,21,66] little strand cleavage above background was observed in the aerobic tirapazamine reactions (Figure 1). Nonetheless, the possibility remained that myxin might be a more efficient redox cycling agent that overwhelmed the ability of superoxide dismutase, catalase, and desferal to protect against strand cleavage arising via superoxide, hydrogen peroxide, and Fenton-type generation of the hydroxyl radical. To investigate this issue, we examined whether the additives employed in our assays were capable of quenching superoxide-derived strand cleavage by the archetypal redox-cycling agent menadione.[74,75] First, we demonstrated that menadione (250 μM), in the *absence* of the additives superoxide dismutase, catalase, and desferal, but under conditions otherwise identical to those used in the myxin assays, caused complete nicking of the plasmid substrate along with the generation of some extensively cleaved and linearized form III plasmid (Figure 3A, lane 4). The xanthine/xanthine oxidase enzyme system is known to generate strand cleavage via one-electron reduction of O_2 to the superoxide radical;[72] but strand cleavage by the menadione–xanthine/xanthine oxidase combination was more than seven times greater than that generated by the xanthine/xanthine oxidase enzyme system alone (Figure 3A, lanes 2 and 4). We showed that the additives, superoxide dismutase, catalase, and desferal effectively suppress strand cleavage by the menadione–xanthine/xanthine oxidase system (Figure 3A, lane 5 and Figure 3B). These control experiments illustrate that superoxide dismutase, catalase, and desferal effectively quench strand cleavage processes arising via enzyme-driven redox-cycling processes. Taken together, the results indicate that the bioreductively activated strand cleavage observed for myxin under aerobic conditions likely does *not* proceed via redox cycling processes that generate superoxide radical.

Enzymatic Metabolism of Myxin. We anticipated that examination of the products arising from *in vitro* aerobic bioreductive metabolism of myxin would shed light on the chemical mechanisms underlying DNA strand cleavage by the natural product. Specifically, strand cleavage mechanisms proceeding via deoxygenative mechanisms will generate characteristic mono-*N*-oxide metabolites (Scheme 5). However, redox cycling processes will not generate the deoxygenated, reduced metabolites.

Scheme 5



Accordingly, we performed HPLC analysis of the metabolites formed during reductive bioactivation of myxin by xanthine/xanthine oxidase under aerobic conditions (Figure 4). We found that reductive metabolism of myxin by xanthine/xanthine oxidase under aerobic conditions gave **9** as the major metabolite (Scheme 5). The identity of the metabolite was confirmed by coinjection with authentic synthetic standards. Significant amounts of the other possible mono-*N*-oxide, **12** (Scheme 4), and the nor-oxide metabolite **8** were not detected in these experiments. Interestingly, previous work showed that electrochemical reduction of myxin similarly afforded **9** as the sole mono-*N*-oxide product.[76]

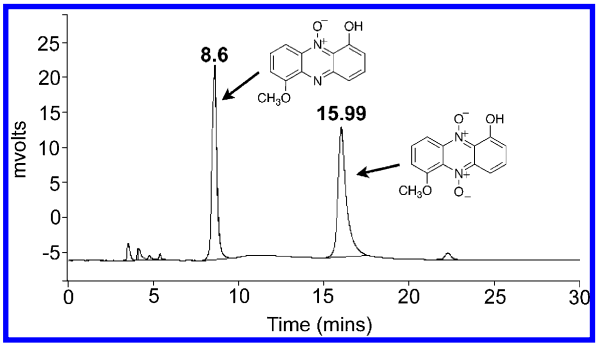


Figure 4. HPLC trace of the products generated by *in vitro* metabolism of myxin (7) in the presence of xanthine/xanthine oxidase under aerobic conditions. In a typical assay, a solution of 7 (250 μM) in sodium phosphate buffer (pH 7.0, 50 mM) was incubated with xanthine (250 μM) and xanthine oxidase (0.4 U/mL) at room temperature for 3 h.

DNA Cleavage by the Deoxygenated Myxin Analogues 8, 9, and 12. The DNA-cleaving properties of the deoxygenated analoguess of myxin were examined using the plasmid-based DNA cleavage assay under aerobic conditions using xanthine/xanthine oxidase as the one-electron activating system (Figure 5). Under these conditions, the mono-*N*-oxide **12** and the nor-oxide metabolite, 1-hydroxy-6-methoxyphena-

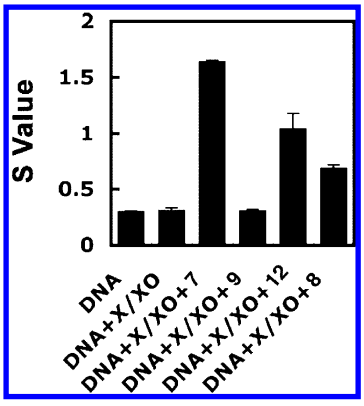


Figure 5. Aerobic DNA cleavage by myxin (7) and its deoxygenated analogues **8**, **9**, and **12** in the presence of xanthine/xanthine oxidase as a one-electron activating system. In a typical assay, a solution of the compound (250 μM), DNA (750 ng), desferal (1 mM), SOD (10 μg/mL), catalase (100 μg/mL), and sodium phosphate buffer (pH 7.0, 50 mM) was incubated with xanthine (500 μM) and xanthine oxidase (0.4 U/mL) at room temperature for 16 h under aerobic conditions. Strand breaks per plasmid DNA molecule (S) was calculated using the equation $S = -\ln f_1$, where f_1 is the fraction of plasmid present as form I.

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zine (**8**), were found to cleave DNA, albeit less effectively than myxin. No detectable cleavage was observed for the major metabolite generated in the bioreductive activation of myxin, the mono-*N*-oxide **9**.

Cell Killing Assay under Aerobic and Anaerobic Conditions. Unlike tirapazamine, myxin is able to carry out bioreductively activated strand cleavage under aerobic conditions. The strand cleaving ability of myxin under aerobic conditions is comparable to that of tirapazamine under hypoxic conditions. Together, these findings suggested that myxin should possess cytotoxic activity comparable to that of tirapazamine with the important distinction that myxin should be active under *both* aerobic and anaerobic conditions. The antibiotic properties of myxin have been characterized under aerobic conditions,[45,46] but the effects of oxygen on the cytotoxicity of myxin have not previously been examined. In addition, to the best of our knowledge, the activity of myxin against human cancer cell lines has not been reported. With these things in mind, we examined the activity of myxin against the HCT-116 human colorectal cancer cell line under both aerobic and hypoxic conditions. The activity of myxin was compared to that of the hypoxia-selective agent tirapazamine. We found that the activities of myxin (**7**) and tirapazamine (**1**) against HCT-116 cells were comparable under hypoxic conditions, with IC_{50} values of 4.2 and 1.8 μM, respectively. The activity of myxin was similar under aerobic and anaerobic conditions, with IC_{50} values of 1.8 and 4.2 μM, respectively. The slightly superior activity of myxin under aerobic conditions may reflect the ability of O_2 to capture (or "fix") DNA radical intermediates more effectively than the *N*-oxide itself.[77,78] In contrast, tirapazamine was approximately 30-fold less cytotoxic to the HCT-116 cells under aerobic conditions (Table 1). The metabolite **9** produced by *in vitro* metabolism of myxin showed

Table 1. Aerobic and Anaerobic Cytotoxicity of Tirapazamine (1), Myxin (7), and the Myxin Metabolite 9 against Human Colorectal Cancer Cell Line HCT-116

compd	aerobic IC_{50} (μM)	anaerobic IC_{50} (μM)	hypoxic cytotoxicity ratio
1	58.6 ± 5.5	1.77 ± 0.15	33 ± 2
7	1.75 ± 0.17	4.2 ± 0.6	0.44 ± 0.07
9	60.4 ± 4.6	225 ± 25	0.29 ± 0.05

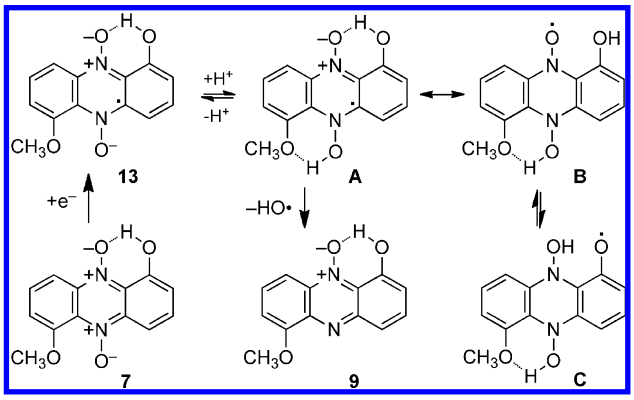
relatively low aerobic cytotoxicity comparable to that of tirapazamine (60.4 ± 4.6 and 58.6 ± 5.5 μM IC_{50} values, respectively, Table 1). The cytotoxicity of **9** under anaerobic conditions was even weaker, showing an IC_{50} value of 225 ± 25 μM. This data suggests that **9** may engage in redox cycling similar to that of tirapazamine under aerobic conditions but that the radical anion is incapable of decomposing to release cytotoxic species (e.g., hydroxyl radical) under either aerobic or hypoxic conditions.

DISCUSSION

The results show that one-electron enzymatic reduction of myxin leads to radical-mediated DNA strand cleavage under both aerobic and anaerobic conditions. Strand cleavage by myxin under both aerobic and hypoxic conditions is comparable to that by the medicinally relevant compound tirapazamine under hypoxic conditions. Likewise, myxin displays activity against a human cancer cell line under both aerobic and hypoxic conditions that is comparable to the hypoxic cytotoxicity of tirapazamine. *In vitro* metabolism studies showed that, under aerobic conditions, myxin undergoes deoxygenative transformation to the mono-*N*-oxide analogue following one-electron reduction in a manner analogous to the anaerobic metabolism of tirapazamine and other hypoxia-selective *N*-oxides.[13,22,23,30,65,66]

We have previously suggested a general mechanism involving the release of hydroxyl radical to account for the bioreductively activated DNA damage caused by hypoxia-selective heterocyclic *N*-oxides (Scheme 5), but the exact nature of the DNA-damaging species remains a subject of ongoing study.[28−36] In the context of tirapazamine, at least, the deoxygenative metabolism stemming from one-electron reduction of aromatic *N*-oxides is substantially more cytotoxic than the redox cycling that occurs under aerobic conditions (hence the hypoxia-selective cytotoxicity of this agent).[66] It is interesting that the deoxygenative metabolism of myxin produces only one of the possible mono-*N*-oxide metabolites (**9**, Schemes 5 and 6). Along these lines, it may be important to note that the

Scheme 6



deoxygenation reaction proceeds via the *protonated* *N*-oxide. Importantly, protonation of the radical anion **13** seems more likely to occur at the lower *N*-oxide (as drawn in Scheme 6) rather than the upper oxide because hydrogen bonding with the neighboring phenol likely renders the upper *N*-oxide of the radical anion much less basic than the lower oxide. By way of supporting analogy, the first pK_a of 1,8-naphthalene diol is 6.6, compared to 9.4 for 1-naphthol.[79] It is also important to consider structural reasons why the deoxygenative metabolism of myxin can proceed in the presence of molecular oxygen. Why is the radical anion **13** stable to molecular oxygen while the radical anions derived from tirapazamine and at least some other phenazine di-*N*-oxides are oxygen-sensitive?[3,17−20,47,80] It is possible that the radical anion **13** possesses some phenoxyl radical character (structure C, Scheme 6) which lends exceptional stability.[81] Phenoxyl radicals do not react rapidly with O_2.[82] Finally, it is interesting to point out that a recent study identified other substituted phenazines with activity against cancer cell lines under aerobic conditions and provided evidence for the release of HO•; however, the mechanisms underlying oxygen sensitivity in this series remain uncertain.[80]

This work may have some broad implications related to the medicinal chemistry and toxicology of drug candidates that contain nitrogen heterocycles. Nitrogen heterocycles are common pharmacophores, and metabolism of these structures in humans can generate aromatic *N*-oxide products.[83] The results presented here highlight the potential for aromatic *N*-

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oxides to undergo highly cytotoxic deoxygenative metabolism under aerobic conditions. Thus, cycles of *N*-oxidation and bioreductively activated one-electron deoxygenation could lead to hepatic or systemic toxicity for some nitrogen heterocycles. Further studies are necessary to elucidate the structural features that enable both one-electron bioreductive activation of aromatic *N*-oxides and their subsequent conversion to cytotoxic reactive intermediates in normal aerobic tissue.

AUTHOR INFORMATION

Corresponding Author

*Phone: (573) 882-6763. Fax: (573) 882-2754. E-mail: gatesk@missouri.edu.

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