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Redox-Activated, Hypoxia-Selective DNA Cleavage by Quinoxaline 1,4-di-*N*-Oxide

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Abstract—Quinoxaline 1,4-dioxide (**4**) is the historical prototype for modern heterocyclic *N*-oxide antitumor agents such as 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine, **1**) and 3-amino-2-quinoxalinecarbonitrile 1,4-dioxide (**11**). Early experiments in bacterial cell lines suggested that enzymatic, single-electron reduction of quinoxaline 1,4-dioxides under low-oxygen (hypoxic) conditions leads to DNA damage. Here the ability of quinoxaline 1,4-dioxide to cleave DNA has been explicitly characterized using in vitro assays. The hypoxia-selective DNA-cleaving properties of **4** reported here may provide a chemical basis for understanding the cytotoxic and mutagenic activities of various quinoxaline 1,4-dioxide antibiotics.

Introduction

Several heterocyclic *N*-oxides have recently attracted attention as potential antitumor agents.[1–4] Most prominent among these compounds is 3-amino-1,2,4-benzotriazine 1,4-dioxide (**1**, tirapazamine, tirazone, SR4233, WIN59075), a heterocyclic di-*N*-oxide that is selectively toxic to oxygen-poor (hypoxic) tumor cells.[1,5] This clinically promising antitumor agent is enzymatically reduced in vivo to yield a radical intermediate (**2**) that causes cytotoxic DNA strand breaks (as shown in Scheme 1).[6–13] It remains uncertain whether DNA cleavage is mediated directly by the drug radical **2** or by hydroxyl radical derived from homolytic fragmentation of **2** (Scheme 1), though recent studies have provided support for the involvement of hydroxyl radical.[9,14] The selective toxicity of **1** toward hypoxic cells appears to stem from the rapid deactivation of the reductively activated form of the drug (**2**) by reaction with molecular oxygen (Scheme 1).[3,15] Recent studies have identified a dual role for tirapazamine in DNA damage.[10–12] In addition to initiating the formation of DNA radicals, tirapazamine can transfer oxygen atoms from its *N*-oxide functional groups to these radicals, converting them to base-labile strand cleavage sites.[10,11]

Although compound **1** is the first redox-activated heterocyclic *N*-oxide to find clinical use in humans, a review of the literature reveals that the historical prototype for this general class of medicinal agents is probably quinoxaline 1,4-di-*N*-oxide (**4**). The antibiotic activity of quinoxaline 1,4-dioxides was first reported in 1943.[16] In the early 1970's, it was noted that the antibiotic activity of **4**, like that observed much later for **1**, is enhanced under hypoxic conditions.[17] Subsequent studies indicated that DNA may be an important biological target for the quinoxaline 1,4-dioxides. Based in part upon the examination of the toxicity of quinoxaline 1,4-dioxide against *Escherichia coli* KL399 cells compared to that in DNA repair deficient strains, Suter and coworkers concluded that redox-activated **4** "modifies the integrity and structure" of cellular DNA under hypoxic conditions.[18] Consistent with its suspected ability to damage DNA, the compound was found to be mutagenic in bacteria and yeast and is carcinogenic in rats.[19–21] The mutagenicity of **4** is enhanced under hypoxic conditions.[22] The groundbreaking biological experiments of Suter et al. showed that the compound is rapidly reduced in vivo to yield radical intermediates and they suggested that the hypoxia-selective antibiotic activity of **4** involves enzymatic reduction of the compound to a crucial oxygen-sensitive radical intermediate (**5**, Scheme 2).[18]

In order to better understand the biological action of quinoxaline 1,4-dioxides and to determine whether the chemical properties of this compound are analogous to the more extensively studied di-*N*-oxides such as

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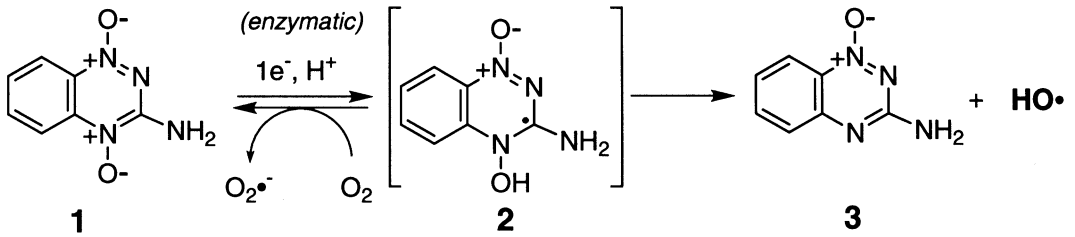
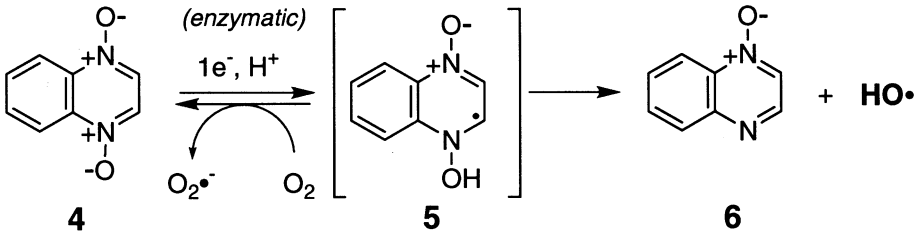


Scheme 1.



Scheme 2.

tirapazamine (**1**), we have investigated whether compound **4** can serve as a redox-activated DNA-cleaving agent in vitro. The results described here provide the first direct evidence that quinoxaline 1,4-dioxide is a hypoxia-selective, redox-activated DNA-cleaving agent. Our results provide support for the hypothesis[18] that DNA cleavage by **4** requires enzymatic one-electron reduction of the compound to an activated, oxygen-sensitive intermediate (**5**, Scheme 2) and may offer a chemical basis for understanding the cytotoxic and mutagenic activities of this class of compounds.

Results

Redox-activated cleavage of supercoiled plasmid DNA by quinoxaline 1,4-dioxide (4)

The results of previous biological studies of quinoxaline 1,4-di-*N*-oxides,[18] and current understanding of the analogous heterocyclic di-*N*-oxide antitumor agent tirapazamine (**1**),[7–9] suggested that one-electron reductive activation of **4** might initiate DNA cleavage by these compounds under low-oxygen (hypoxic) conditions. In the present studies, we employed a xanthine/xanthine oxidase enzyme system as a one-electron reducing agent for the activation of compound **4**. This enzyme system has been used similarly in previous studies with the heterocyclic *N*-oxide tirapazamine[7,9] and other bioreductively-activated DNA-damaging agents.[23–25] We initially examined the ability of **4** to function as a redox-activated DNA-cleaving agent using a plasmid-based system. In this assay, DNA strand scission is readily measured by observing the conversion of supercoiled (form I) plasmid DNA to the open circular form (form II) resulting from nicking of the DNA backbone. Assays were prepared in an inert atmosphere glove bag and the solutions freeze–pump–thaw degassed or purged with inert gas to remove molecular oxygen. Care was taken to shield samples from excessive exposure to light because **4** and other *N*-oxides are known to be capable of mediating photochemical DNA cleavage.[26,27] We find that **4**, in conjunction with the xanthine/xanthine oxidase system, causes direct single-strand breaks in DNA (Fig. 1). In the absence of the enzymatic reducing system, **4** causes no detectable DNA cleavage. The mono-*N*-oxide (**6**), which is the major metabolite resulting from reductive activation of **4** (see below), does not cleave DNA alone, in the presence of xanthine oxidase, or in the presence of the complete xanthine/xanthine oxidase reducing system. Interestingly, under these reaction conditions, compound **4**, in the presence of xanthine oxidase, but *without* the enzyme substrate xanthine, consistently yields small, but significant amounts of DNA strand cleavage. Although this finding initially seems inconsistent with the mechanism proposed for DNA cleavage in Scheme 2, an explanation consistent with the proposed mechanism is provided in the final section of Results below.

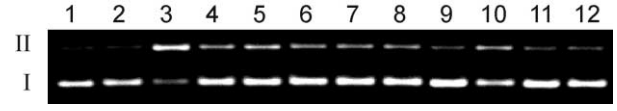
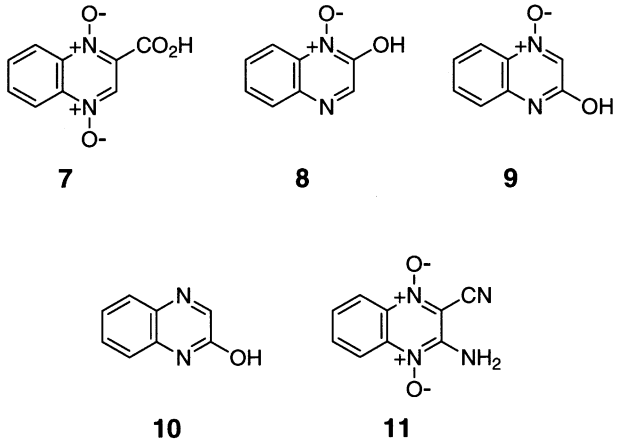


Figure 1. Cleavage of supercoiled plasmid DNA (pGL-2 Basic) by quinoxaline 1,4-dioxide (**4**) in the presence of xanthine/xanthine oxidase as an activating system. All reactions contain DNA (600 ng), sodium phosphate buffer (50 mM, pH 7.0), catalase (100 μg/mL), superoxidase dismutase (10 μg/mL) and desferal (1 mM) and were prepared and incubated under anaerobic conditions (except lane 9). After incubation for 15 h at 24 °C the reactions were analyzed by agarose gel electrophoresis. The value in the parenthesis after each lane description represents the mean number of strand breaks per plasmid molecule (S) calculated using the equation $S = -\ln f_I$ where f_I is the fraction of plasmid present as form I.[49] Lane 1, DNA alone (0.23 ± 0.02); lane 2, xanthine (500 μM)/xanthine oxidase (0.4 U/mL) (0.25 ± 0.01); lane 3, **4** (500 ± μM) + xanthine/xanthine oxidase (1.06 ± 0.05); lane 4, **4** + xanthine/xanthine oxidase + methanol (500 mM) (0.39 ± 0.02); lane 5, **4** + xanthine/xanthine oxidase + ethanol (500 mM) (0.45 ± 0.03); lane 6, **4** + xanthine/xanthine oxidase + *t*-butanol (500 mM) (0.41 ± 0.04); lane 7, **4** + xanthine-xanthine oxidase + DMSO (500 mM) (0.35 ± 0.02); lane 8, **4** + xanthine/xanthine oxidase + mannitol (500 mM) (0.34 ± 0.01); lane 9, **4** (500 ± μM) + xanthine/xanthine oxidase + air (0.22 ± 0.02); lane 10, **4** + xanthine oxidase (0.42 ± 0.07); lane 11, **4** only (0.22 ± 0.01); lane 12, **6** (500 μM) + xanthine/xanthine oxidase (0.28 ± 0.02).

Redox-activated DNA cleavage by **4** is significantly inhibited by addition of radical scavenging agents such as ethanol, methanol, mannitol and DMSO (Fig. 1). We have previously shown that these additives do not inhibit reductive metabolism of *N*-oxides by the xanthine/xanthine oxidase enzyme system;[9] thus, the observed inhibition of DNA cleavage is attributed to scavenging of DNA-cleaving radicals by these additives. DNA cleavage by **4** is almost completely inhibited under aerobic conditions.

Side-by-side assays clearly show that redox-activated DNA cleavage by **4** is less efficient than that by the antitumor agent tirapazamine (**1**). Inspection of the data shown in Figure 2 reveals that higher concentrations of **4** are required to achieve DNA-cleavage efficiencies comparable to that afforded by **1**. In addition, it is interesting to note that the naturally-occurring *N*-oxide antibiotic, 2-carboxyquinoxaline 1,4-oxide (**7**)[21] can also function as a redox-activated DNA-cleaving agent with properties analogous to **4** (Fig. 3).



Sequence-specificity of redox-activated DNA cleavage by quinoxaline 1,4-dioxide (4)

In order to further characterize DNA strand scission by **4**, we examined the sequence specificity of redox-activated cleavage of a 5′-^{32}P-labeled 145-base pair restriction fragment under hypoxic conditions. Polyacrylamide sequencing gel analysis reveals that cleavage by **4** occurs at every base with little sequence specificity (Fig. 4). Redox-activated DNA cleavage by **4** closely resembles that observed for the prototypical sequence-independent DNA-cleaving system, iron EDTA (Fig. 4).[28]

Identification of quinoxaline *N*-oxide (6) as a major product resulting from hypoxic metabolism of quinoxaline 1,4-dioxide (4)

Identification of the products resulting from reductive metabolism of **4** under anaerobic conditions is crucial for understanding the chemical mechanism by which this compound effects DNA strand scission. Consistent with the mechanism proposed in Scheme 2, we find that treatment of **4** with xanthine/xanthine oxidase under anaerobic conditions yields the mono-*N*-oxide **6** as the major product (Fig. 5). The identity of the major metabolite **6** was initially ascertained by comparison of its reverse-phase HPLC retention time with authentic synthetic material (by co-injection), and was ultimately confirmed by LC/MS and LC/MS/MS experiments. In keeping with the idea that compound **6** is produced by the same chemical process that causes DNA cleavage, we find that the formation of **6**, like DNA-strand scission, is inhibited under aerobic conditions. Our findings mesh with those of Suter and coworkers[18] who suggested, based on UV–vis data, that **6** was produced by metabolism of **4** in bacterial cells. The reductive metabolism characterized here for compound **4**, resulting in deoxygenation of a heterocyclic *N*-oxide functional group, is analogous to that observed previously for the triazine *N*-oxide, tirapazamine (**1**).

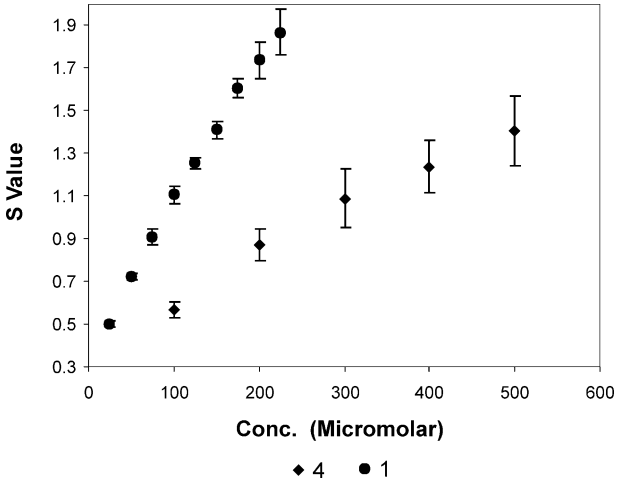


Figure 2. Cleavage of plasmid DNA by various concentrations of **1** or **4** in the presence of xanthine/xanthine oxidase as a reducing system. All assays were prepared under hypoxic conditions as described in the Experimental. Supercoiled plasmid DNA (600 ng) was incubated with **4** (100–500 μM) or **1** (25–225 μM), xanthine (500 μM), xanthine oxidase (0.4 U/mL), catalase (100 μg/mL), superoxidase dismutase (10 μg/mL), sodium phosphate buffer (50 mM, pH 7.0) and desferal (1 mM) under anaerobic conditions at room temperature for 15 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.

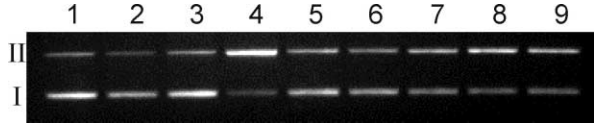


Figure 3. Cleavage of supercoiled plasmid DNA (pGL-2 Basic) by 2-quinoxaline carboxalic acid 1,4-dioxide (**7**) in the presence of xanthine-xanthine oxidase as an activating system. All reactions contain DNA (1 μg), sodium phosphate buffer (50 mM, pH 7.0), catalase (200 μg/mL) and desferal (1 mM) in a total volume of 100 μL and were prepared and incubated under anaerobic conditions. After incubation for 16 h at 37 °C the reactions were analyzed by agarose gel electrophoresis. The value in the parenthesis after each lane description represents the mean number of strand breaks per plasmid molecule (S) calculated using the equation $S = -\ln f_1$ where f_1 is the fraction of plasmid present as form I.[49] Lane 1, DNA alone (0.21 ± 0.01); lane 2, xanthine (500 μM) + xanthine oxidase (0.4 U/mL) (0.22 ± 0.05); lane 3, **7** (500 μM) (0.26 ± 0.03); Lane 4, **7** (500 μM) + xanthine/xanthine oxidase (1.41 ± 0.03); Lane 5, **7** (500 μM) + xanthine/xanthine oxidase + mannitol (100 mM) (0.38 ± 0.004); lane 6, **7** (500 μM) + xanthine/xanthine oxidase + DMSO (100 mM) (0.36 ± 0.02); lane 7, **7** (500μM) + xanthine/xanthine oxidase + ethanol (100 mM) (0.57 ± 0.02); lane 8, **7** (500 μM) + xanthine/xanthine oxidase + methanol (100 mM) (0.75 ± 0.02); lane 9, **7** (500 μM) + xanthine-/xanthine oxidase + *t*-butanol (100 mM) (0.62 ± 0.01).

Incubation of **4** with xanthine/xanthine oxidase under anaerobic conditions affords small amounts of two products in addition to the major product **6**. Further experiments reveal that these minor products are formed by the action of xanthine oxidase on the primary metabolite **6** (no xanthine substrate required). This was demonstrated by the finding that incubation of the mono-*N*-oxide (**6**) with xanthine oxidase under anaerobic conditions produces the two metabolites ($\sim$6 and $\sim$8.5 min retention times in the reverse phase HPLC chromatogram shown in Fig. 5). This result suggests that the mono-*N*-oxide (**6**) can be *oxidized* by xanthine oxidase, presumably undergoing conversion to products such as 2(1*H*)-quinoxalinone 1-oxide (**8**) or 2(1*H*)-quinoxalinone 4-oxide (**9**). Xanthine oxidase is known to catalyze the oxidation of a wide variety of nitrogen heterocycles;[29–31] however, to the best of our knowledge, this is the first example of a heterocyclic *N*-oxide serving as a substrate for this enzyme.

Analysis of the reaction mixture by LC/MS confirms that the compound eluting at $\sim$6 min has a mass consistent with the structures **8** and **9** (*m*/*z* 204, $M+H+CH_3CN$; ESI, positive ion mode). It remains unclear as to which regioisomer is produced. The compound eluting at $\sim$8.5 min was identified in LC/MS and LC/MS/MS experiments as 2-hydroxyquinoxaline (**10**) by comparison with authentic material. These observations are best explained by a scenario in which xanthine oxidase oxidizes **6** to the 2(1*H*)-quinoxalinone oxide (**8** or **9**) and the reduced form of the enzyme generated in this initial reaction can subsequently reduce the 2(1*H*)-quinoxalinone oxide (**8** or **9**) to 2-hydroxyquinoxaline (**10**). The xanthine oxidase-mediated isomerization of **6** to **10** likely occurs via two separate reactions (not in a single enzyme-catalyzed event where the same molecule is oxidized and subsequently reduced at the active site). The stepwise nature of this transformation is supported by the finding that, when molecular oxygen is present as an alternate electron acceptor (aerobic conditions), the 2(1*H*)-quinoxalinone oxide metabolite **8**/**9** is still produced, but its subsequent reduction to 2-hydroxyquinoxaline (**10**) is inhibited. Evidence that the metabolite **8**, **9** and **10** do not play a significant role in DNA damage by **4** is provided by the observation that incubation of **6** (which produces these compounds) with the xanthine/xanthine oxidase system does not yield significant amounts of DNA damage (Fig. 1).

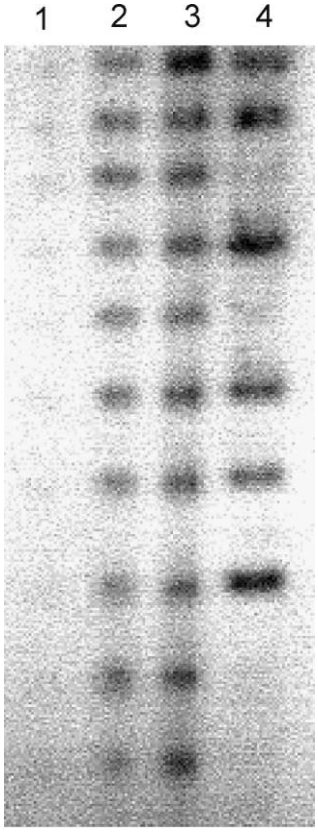


Figure 4. Cleavage of a 5′-^{32}P-labeled DNA fragment by reductively-activated quinoxaline 1,4-dioxide (**4**). In a typical assay, a 145-base pair, 5′-labeled restriction fragment ($\sim$100,000 cpm) in a deoxygenated solution of phosphate buffer (20 mM, pH 7), desferal (0.4 mM) and catalase (0.2 units/mL) was treated with quinoxaline 1,4-dioxide (**4**, 5.5 mM), xanthine (35 μM) and xanthine oxidase (1.8 units/mL), followed by 20% denaturing polyacrylamide sequencing gel analysis as described in the Experimental. Lane 1, DNA alone; lane 2, DNA + **4** + xanthine/xanthine oxidase; lane 3, DNA + Fe/EDTA cleaving system; lane 4, Maxam–Gilbert G-reaction.

Discussion

Our work provides direct evidence that quinoxaline 1,4-dioxide (**4**) is a redox-activated, hypoxia-selective DNA-cleaving agent. In conjunction with the xanthine/xanthine oxidase one-electron reducing system, compound **4** cleaves DNA under hypoxic conditions. Redox-activated DNA cleavage by **4** is completely inhibited under aerobic conditions. Hypoxic metabolism of **4** by xanthine/xanthine oxidase produces the two-electron reduced mono-*N*-oxide **6** as the major product. The metabolite **6** does not show significant DNA-cleaving activity either alone or in the presence of the xanthine/xanthine oxidase system. The dependence of DNA cleavage on reductive activation, combined with the observed inactivity of the two-electron reduced metabolite **6**, strongly supports the notion[18] that the one-electron reduced species (**5**) is a necessary intermediate in this DNA-damage process.

The action of **4** on DNA in the assays reported here yields direct strand breaks, consistent with the involvement of radical species.[32,33] In contrast, alkylating agents typically yield alkaline-labile lesions. In addition, strand scission is inhibited by commonly used radical scavenging agents such as methanol, ethanol, mannitol and dimethylsulfoxide.[34] The observation that DNA cleavage occurs with almost no sequence specificity is also consistent with a radical-mediated cleavage process involving hydrogen atom abstraction from the deoxyribose backbone of DNA.[32,33] Inhibition of DNA cleavage under aerobic conditions probably results from molecular oxygen-mediated oxidation of the activated species (**5**) back to the starting material (**4**) (Scheme 2).

While the exact identity of the DNA-cleaving radical resulting from redox activation of **4** remains uncertain, there are two obvious possibilities to consider. First, the

quinoxaline 1,4-dioxide radical (**5**) may *directly* abstract hydrogen atoms from the DNA backbone, followed by elimination of water to yield the metabolite **6**. Alternatively, the quinoxaline 1,4-dioxide radical (**5**) may fragment to form the known DNA-cleaving agent hydroxyl radical and the metabolite **6** (as shown in Scheme 2). Analogous to our previous discussion of DNA cleavage by tirapazamine,[9] we favor the mechanism involving production of hydroxyl radical for several reasons. First, fragmentation reactions of this type are thermodynamically favorable and are well precedented in the chemical literature.[35–40] In addition, for the specific case of redox-activated DNA damage by **4**, the involvement of hydroxyl radical receives further circumstantial support from early experiments[18] showing that the systems involved in the repair of DNA damage by this agent in *E. coli* are the same as those required to repair damage by X-rays (hydroxyl radical is the principle DNA-damaging agent produced by X-rays).[41,42] Finally, the lack of sequence specificity observed for redox-activated DNA cleavage by **4** in our experiments (Fig. 3) is consistent with involvement of a small, highly reactive species such as hydroxyl radical. The observed cleavage by **4** is similar in nature to that by the iron–EDTA system (lane 3, Fig. 4) that is known to generate hydroxyl radical (or an oxidant of very similar reactivity).[28]

The finding that quinoxaline *N*-oxide (**6**) can be oxidized by xanthine oxidase offers a reasonable explanation for the small amounts of DNA cleavage produced in the control lane containing xanthine oxidase and **4** (Fig. 1). The di-*N*-oxide **4** (like many *N*-oxides)[43,44] is prone to thermal or photochemical *N*-deoxygenation. Thus, traces of compound **6** formed spontaneously in assay mixtures will be oxidized by xanthine oxidase and the reduced enzyme will then reductively activate a molecule of **4**, which, in turn, decomposes to yield another equivalent of **6**. These reactions yield a cycle, in which the product resulting from the reductive activation of **4** can serve as 'fuel' for further xanthine oxidase-mediated activation of **4**. This is a slow process relative to activation of **4** by xanthine/xanthine oxidase (data not shown), but given sufficiently long incubation times, the spontaneous generation of traces of the mono-*N*-oxide **6** from **4** can ultimately lead to significant DNA cleavage in assays containing only **4** and xanthine oxidase (no xanthine).

The hypoxia-selective antibiotic and mutagenic activity of quinoxaline 1,4-dioxides has been known for over 20 years. Our studies provide some additional understanding of the chemical processes that may be responsible for the biological activities of these compounds. Recently, several substituted quinoxaline 1,4-dioxides such as **11** with promising hypoxia-selective antitumor activity (comparable to tirapazamine, **1**) have been reported. Although the ability of these compounds to damage DNA has not been investigated, the results presented here for the parent quinoxaline 1,4-dioxide heterocycle **4** and for the carboxy-substituted analogue **7** clearly suggest that substituted di-*N*-oxides such as **11** can act as redox-activated, hypoxia-selective DNA-damaging agents under physiologically relevant conditions.[4] We are currently conducting further investigations on the DNA-damaging properties of substituted quinoxaline 1,4-dioxides.

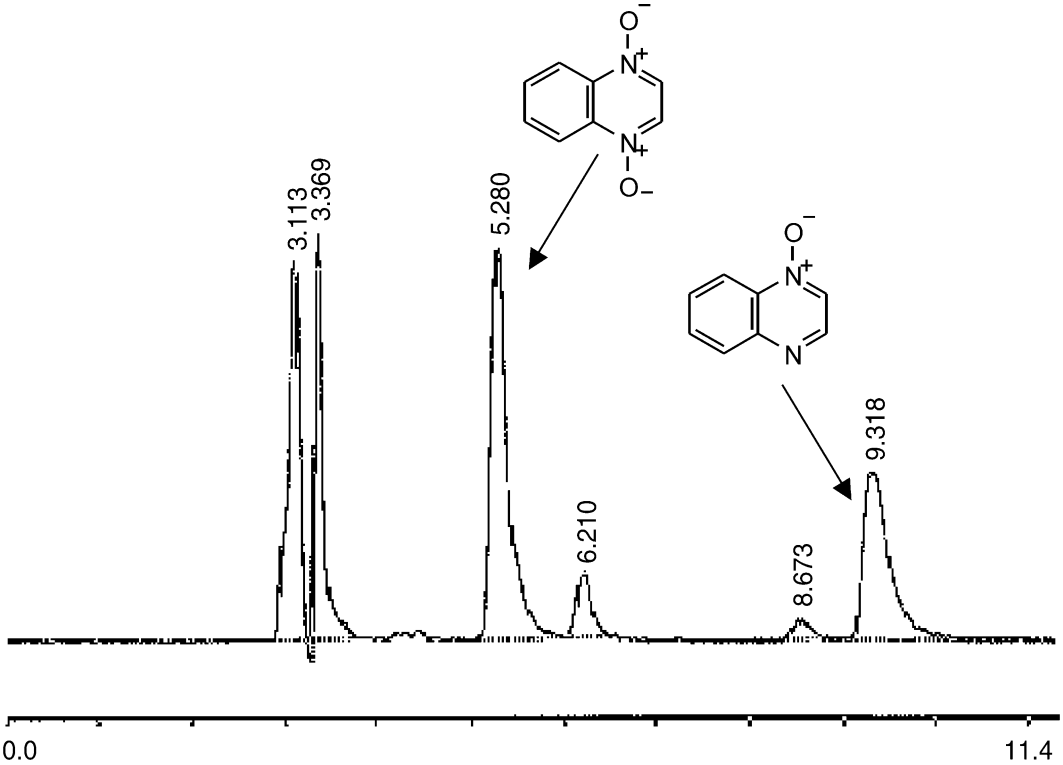


Figure 5. HPLC analysis of the products formed by xanthine/xanthine oxidase-mediated metabolism of quinoxaline 1,4-dioxide. The figure depicts C18-reverse phase HPLC analysis of the products formed during hypoxic metabolism of quinoxaline 1,4 dioxide (**4**, 1 mM) in 50 mM sodium phosphate buffer (pH 7.0) containing 500 μM xanthine, 0.2 mg/mL catalase, and 0.4 units/mL xanthine oxidase as described in the Experimental.

Experimental

Materials

Materials were purchased from the following suppliers and were of the highest purity available: xanthine, mannitol, DMSO, sodium phosphate, and quinoxaline, 2-hydroxyquinoxaline, Aldrich Chemical Co. (Milwaukee, WI); sodium acetate, tris(hydroxymethyl)aminomethane (Tris), glycerol, and *N*,*N*′-methylenebisacrylamide, Sigma Chemical Co. (St Louis, MO); hydrogen peroxide, HPLC grade solvents (dichloromethane, acetonitrile, ethyl acetate, dimethylformamide, methanol) and *t*-butanol, Fisher (Pittsburgh, PA); T4 polynucleotide kinase, calf intestinal phosphatase, DNA polymerase I, EcoR I, Nhe I, New England Biolabs (Beverly, MA); 2′-deoxyadenosine triphosphate, Pharmacia (Piscataway, NJ); xanthine oxidase, catalase, pBR322 plasmid DNA, acrylamide, and ethidium bromide, Roche Molecular Biochemicals (Indianapolis, IN); Seakem ME agarose, FMC; ethanol; McCormick Distilling Co. (Brookfiled, CN); urea, xylene cyanol, bromophenol blue, and sodium dodecyl sulfate (SDS), United States Biochemical (Cleveland, OH); desferal was a generous gift from Ciba-Geigy Co.; 5′-[γ-^{32}P]dATP, New England Nuclear-DuPont (Wilmington, DE). Compounds **4** and **6** were prepared via the oxidation of quinoxaline with trioxalorhenium using the general method of Sharpless and coworkers.[45] Compound **7** was prepared by selenium dioxide-mediated oxidation of 2-methylquinoxaline 1,4-dioxide following the method of Elina and Magidson.[46] The starting 2-methylquinoxaline 1,4-dioxide for this synthesis was prepared by the reaction of benzofuroxan with acetone.[47]

Cleavage of supercoiled plasmid DNA

In a typical assay, supercoiled plasmid DNA (600 ng) was incubated with **4** (100–500 μM) or **1** (25–225 μM), xanthine (500 μM), xanthine oxidase (0.4 U/mL), catalase (100 μg/mL), superoxidase dismutase (10 μg/mL), sodium phosphate buffer (50 mM, pH 7.0) and desferal (1 mM) in a total volume of 30 μL. A stock solution of xanthine was prepared by dissolving the compound in 40% NaOH. Individual components of the DNA cleavage reactions, except DNA and the enzymes, 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 glove bag purged with argon, opened and degassed solutions used to prepare individual reactions. Enzymes and DNA were diluted with degassed water in the glove bag to prepare working stock solutions. Reactions were initiated by adding xanthine oxidase and the microentrifuge tube was then wrapped with aluminum foil to prevent exposure to light and the mixture incubated in the glove bag for 15 h at room temperature (24 °C). Following incubation, the reactions were stopped by adding 3 μL of 50% glycerol loading buffer and the resulting mixture loaded onto a 0.9% agarose gel. The gel was electrophoresed for approximately 3 h at 80 V in 1×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 using an Alpha Innotech IS-1000 digital imaging system. The values reported are not corrected for differential staining of form I and form II DNA by ethidium bromide. 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 μM) were added to the reaction mixture before addition of xanthine oxidase. Superoxide dismutase and catalase were added to all assay mixtures to minimize background DNA damage that might be caused by the conversion of remaining traces of molecular oxygen into superoxide radical.

Cleavage of a 5′-^{32}P-labeled 145-base pair restriction fragment by reductively-activated quinoxaline 1,4-dioxide

A 5′-labeled 145-base pair BamH I-Nhe I fragment of pBR322 was prepared using standard techniques.[48] Cleavage of this restriction fragment by quinoxaline 1,4-dioxide was carried out in Pyrex^{TM} glass tubes, freeze–pump–thaw degassed (3×) and sealed with a propane torch. In a typical cleavage reaction, a degassed solution (final volume 48 μL) containing the 5′-labeled restriction fragment (~1 million cpm), quinoxaline 1,4-dioxide (5.5 mM), phosphate buffer (20 mM, pH 7), desferal (0.4 mM), xanthine (35 μM) was freeze–pump–thaw degassed (3×) in a Pyrex tube, torch sealed and transferred to an inert atmosphere glove box. Reactions were initiated by breaking the Pyrex tubes open in the glove box, followed by addition of an argon-degassed solution of xanthine oxidase and catalase. The final concentrations of xanthine oxidase and catalase were 1.8 and 0.2 units/mL, respectively. Reactions were transferred to 0.5-dram glass screw-top vials and incubated overnight at 37 °C. Reactions were ethanol precipitated, briefly dried under vacuum, and redissolved in formamide loading buffer. Reactions were then heated for 5 min at 90 °C and cooled immediately in ice water for 5 min. The resulting solution was then loaded onto a 20% denaturing polyacrylamide gel (acrylamide/bisacrylamide 19:1, 0.4 mm thick, containing 7.5 M urea) in aliquots containing 100,000 cpm. The gel was electrophoresed at 800 V for 30 min, and then at 1800 V for 2 h in 1× TBE buffer. Following electrophoresis, radioactivity on the gel was visualized using Fuji RX X-ray film or phosphorimage analysis. Note: these gel electrophoresis conditions employed here do not resolve the expected phosphate and phosphoglycolate end products expected from oxidative damage by hydroxyl radical.[28] Comparison reactions in which the DNA fragment was cleaved using an iron–EDTA system were performed as described by Pogozelski and Tullius.[28]

Detection of the products formed by xanthine/xanthine oxidase-mediated metabolism of quinoxaline 1,4-dioxide using HPLC

A typical anaerobic reduction experiment contained 1 mM quinoxaline 1,4 dioxide (**4**) 50 mM sodium phosphate buffer (pH 7.0), 500 μM xanthine, 0.2 mg/mL catalase, and 0.4 units/mL xanthine oxidase. Solutions were degassed and reactions performed under inert atmosphere as described above for the plasmid assays. Reactions were

incubated for 24 h at 37 °C for 24 and analyzed by HPLC on a Microsorb^{TM}-MV C-18 reverse-phase analytical column (100 Å spheres, 5 μM pore size, 25 cm length, 4.6 mm id, Varian) eluted with an isocratic solvent mixture composed of 74% water, 25% methanol, and 1% acetic acid at a flow rate of 0.9 mL/min. Products were observed by monitoring absorbance at 240 nm. The identities of the deoxygenated product (**6**) and 2-hydroxyquinoxaline (**10**) were initially determined by comparison of their retention times to that of authentic standards and confirmed by coinjection experiments.

LC/MS analysis of the products formed by xanthine/xanthine oxidase-mediated metabolism of quinoxaline 1,4-dioxide

Reactions were performed as described above for HPLC analysis. LC/MS and LC/MS/MS experiments were carried out on a Finnigan TSQ 7000 triple quadrupole instrument interfaced to a ThermoSeparations liquid chromatograph (TSP4000). HPLC separation was performed as described above except using an isocratic solvent mixture composed of 75% water, 25% acetonitrile, and 0.1% phosphoric acid at a flow rate of 1.5 mL/min. Positive ion electrospray was used as the means of ionization and collision-induced dissociation (CID) involved argon gas (~2 mtorr). Other instrument settings included a capillary voltage of 4.5 kV, a capillary temperature of 350 °C, and a source temperature of 75 °C.

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