3-Amino-1,2,4-benzotriazine 4-Oxide: Characterization of a New Metabolite Arising from Bioreductive Processing of the Antitumor Agent 3-Amino-1,2,4-benzotriazine 1,4-Dioxide (Tirapazamine)

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Tirapazamine (**1**) is a promising antitumor agent that selectively causes DNA damage in hypoxic tumor cells, following one-electron bioreductive activation. Surprisingly, after more than 10 years of study, the products arising from bioreductive metabolism of tirapazamine have not been completely characterized. The two previously characterized metabolites are 3-amino-1,2,4-benzotriazine 1-oxide (**3**) and 3-amino-1,2,4-benzotriazine (**5**). In this work, 3-amino-1,2,4-benzotriazine 4-oxide (**4**) is identified for the first time as a product resulting from one-electron activation of the antitumor agent tirapazamine by the enzymes xanthine/xanthine oxidase and NADPH:cytochrome P450 oxidoreductase. As part of this work, the novel *N*-oxide (**4**) was unambiguously synthesized and characterized using NMR spectroscopy, UV−vis spectroscopy, LC/MS, and X-ray crystallography. Under conditions where the parent drug tirapazamine is enzymatically activated, the metabolite **4** is produced but readily undergoes further reduction to the benzotriazine (**5**). Thus, under circumstances where extensive reductive metabolism occurs, the yield of the 4-oxide (**4**) decreases. In contrast, the isomeric two-electron reduction product 3-amino-1,2,4-benzotriazine 1-oxide (**3**) does not readily undergo enzymatic reduction and, therefore, is found as a major bioreductive metabolite under all conditions. Finally, the ability of the 4-oxide metabolite (**4**) to participate in tirapazamine-mediated DNA damage is considered.

Introduction

Tirapazamine (**1**) selectively kills the oxygen-poor (hypoxic) cells found in solid tumors and is currently undergoing phase I, II, and III clinical trials for the treatment of various cancers.[1] Tirapazamine undergoes in vivo enzymatic reduction to its radical form (**2**)[2−5] and, in the absence of molecular oxygen, the resulting drug radical, or its fragmentation product hydroxyl radical (Scheme 1), causes cytotoxic DNA damage.[5−12] Tirapazamine is relatively nontoxic to normally oxygenated cells because the activated form of the drug (**2**) is rapidly quenched by reaction with O_2.[13,14]

Although tirapazamine has been studied for over 10 years, the products stemming from bioreductive activation of this drug have not been completely characterized. In a recent review,[15] it was noted that, in many experiments, between 30 and 60% of the starting drug is left unaccounted for by the known metabolites, 3-amino-1,2,4-benzotriazine 1-oxide (**3**) and 3-amino-1,2,4-benzotriazine (**5**).[16,17] It is important to fully characterize the metabolites stemming from bioreductive activation of tirapazamine because these products may provide insight into the chemical events underlying drug action. In addition, all metabolites should be examined for their biological activity.

Here we report the identification and characterization of a new tirapazamine metabolite, 3-amino-1,2,4-benzotriazine 4-oxide (**4**). This compound is produced from in vitro one-electron reductive activation of the antitumor agent tirapazamine by the enzymes xanthine/xanthine oxidase and NADPH:cytochrome P450 oxidoreductase. Under conditions where the parent drug tirapazamine is enzymatically activated, the metabolite **4** is produced and then readily undergoes further reduction to the benzotriazine **5**. Thus, the yield of the 4-oxide metabolite (**4**) varies depending upon reaction conditions and, under circumstances where more extensive reductive metabo-

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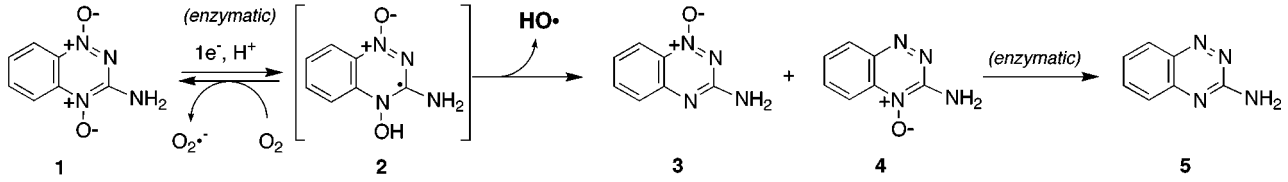
Scheme 1



lism occurs, the yield of the 4-oxide (**4**) decreases. As part of this work we have performed the first unambiguous synthesis and characterization of the heterocyclic *N*-oxide **4**. Finally, the ability of this metabolite to contribute to DNA damage by the antitumor agent tirapazamine is considered.

Experimental Section

Chemicals were purchased from the following suppliers and were of the highest purity available: potassium acetate and glycerol, Sigma Chemical Co.; tris(hydroxy)aminomethane, xanthine, cyanamide, 2-nitroaniline, *tert*-butyl peracetate (in mineral spirits), acetic anhydride, sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydrodisulfite (sodium dithionite), thin-layer chromatography plates (silica gel, 250 μm thickness, 60 Å pore size), Aldrich Chemical Co.; hexane, ethyl acetate, HPLC grade acetonitrile, methanol and 2-propanol, hydrochloric acid, glacial acetic acid, 30% hydrogen peroxide, sodium hydroxide, chromatographic silica gel-G type 60A, grade 633, Fisher; ethidium bromide, xanthine oxidase (cat. no. 110442), catalase (cat. no. 106828), superoxide dismutase (cat. no. 567680), pB*R*322 and pUC19, Roche Molecular Biochemicals; SeaKem LE agarose, FMC bioproducts; ethylenediaminetetraacetic acid, sodium dodecyl sulfate, United States Biochemical Corp.; chloroform-*d* (w/0.05% v/v TMS), dimethyl-d_6 sulfoxide, Cambridge Isotope Laboratories Inc. and desferal was a generous gift from CIBA. Ethyl acetate and hexane were distilled before use. Pyrex brand standard borosilicate glass tubing was purchased from Ace Glass. Water for HPLC and reactions was distilled, deionized, and glass redistilled.

Synthesis of 3-Amino-1,2,4-benzotriazine 1-Oxide (3). Compound **3** was prepared by a modification of previous methods.[18,19] 2-Nitroaniline (2.48 g; 18 mmol) and cyanamide (1.51 g; 36 mmol) were heated with stirring at 100 °C until a deep red melt formed. The reaction was cooled to room temperature, and cold concentrated hydrochloric acid (6.5 mL, 78 mmol) was added dropwise. The reaction was then warmed to 100 °C and stirred for 20 min, then cooled again to room temperature. To the cooled reaction mixture NaOH was added 6.5 mL of a 16 M aqueous solution in 0.5 mL portions over approximately 15 min. The resulting cloudy yellow-orange mixture was warmed to 100 °C and stirred for 2 h. Addition of water (~25 mL) produced a yellow precipitate. The suspension was allowed to cool for 10 min, collected by vacuum filtration, and then washed with water followed by ethyl acetate to remove unreacted 2-nitroaniline (until the wash changes from orange to light yellow). The bright yellow solid collected from the filtration (1.07 g, 39% yield) was recrystallized from 2-propanol to yield **3**: (R_f 0.6 in 100% ethyl acetate; a fluorescent yellow-green spot under long wave UV). ^{1}H NMR ($CDCl_3$) δ 8.31 (dd, J = 8.7, 0.9 Hz, 1H), 7.75 (ddd, J = 7.7, 6.9, 1.4 Hz, 1H), 7.61 (dd, J = 7.6, 1.0 Hz, 1H), 7.37 (ddd, J = 8.5, 7.0, 1.3 Hz, 1H), 5.13 (bs, 2H); ^{1}H NMR (DMSO-d_6) δ 8.14 (dd, J = 8.6, 1.3 Hz, 1H), 7.77 (ddd, J = 8.4, 6.9, 1.4 Hz, 1H), 7.52 (dd, J = 7.8, 0.5 Hz, 1H), 7.33 (ddd, J = 8.5, 7.0, 1.3 Hz, 2H), 7.31 (bs, 2H); ^{13}C NMR (DMSO-d_6) δ 160.2, 148.8, 135.7, 129.9, 125.8, 124.6, 119.8; MS (EI) *m*/*z* 162 (100), 118 (12), 91 (27), 90 (18); MS (ESI) *m*/*z*, 163 (100), 133 (12), 92 (20).

Synthesis of 3-Amino-1,2,4-benzotriazine 1,4-Dioxide (1). Compound **1** was prepared by a minor modification of Mason and Tennant's route.[18] Compound **3** (250 mg, 1.49 mmol) was suspended in glacial acetic acid (12 mL) and heated to 50 °C. To this suspension was added 30% hydrogen peroxide (8 mL), and the resulting mixture was stirred in the dark for 10 h. Between two and four additional aliquots of acetic acid (2 mL) and 30% hydrogen peroxide (2 mL) were added over the next 24 h until starting material was largely consumed (as judged by TLC). The reaction was lyophilized to dryness (behind a blast shield), and the resulting burnt orange solid purified by flash-column chromatography on silica gel (eluted with 75:25 ethyl acetate:hexane, followed by 100% ethyl acetate, followed by 25:75 2-propanol:ethyl acetate) to yield **1** (40−60%). The resulting red solid can be recrystallized from 2-propanol or ethanol to yield red plates: R_f = 0.3 (15:85 2-propanol:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 8.45 (d, J = 9.6 Hz, 1H), 8.10 (t, J = 7.9 Hz, 2H), 7.82 (d, J = 8.4 Hz, 1H), 7.68 (t, J = 7.8 Hz, 1H), 6.84 (bs, 2H); ^{1}H NMR (DMSO- d_6) δ 8.21 (dd, J = 8.3, 0.6 Hz, 1H), 8.15 (dd, J = 8.3, 0.7 Hz, 1H), 8.03 (bs, 2H), 7.94 (ddd, J = 7.1, 7.1, 1.3 Hz, 1H), 7.57 (ddd, J = 8.6, 7.1, 1.3 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 151.4, 138.4, 135.4, 130.6, 126.9, 121.1, 117.0. MS (EI) *m*/*z* 178 (100), 162 (14), 136 (34), 78 (16), 77 (10), 76 (17).

Synthesis of 3-Amino-1,2,4-benzotriazine (5). The benzotriazine **5** was prepared via a modification of Mason and Tennant's procedure.[18] To a solution of **3** (32 mg, 0.22 mmol) in 70% ethanol−water (5 mL) was added sodium dithionite (75 mg, 0.44 mmol). The resulting suspension was refluxed for 30 min, an additional aliquot of sodium dithionite (38 mg, 0.22 mmol) added, and the suspension refluxed for another 30 min. One final addition of sodium dithionite (14 mg, 0.11 mmol) was added and the solution refluxed for another 30 min at which time all starting material was consumed (as judged by TLC). The solvent was removed by rotary evaporation with warming and the resulting pale yellow residue purified by column chromatography on silica gel (eluted with ethyl acetate) to yield 16 mg (57%) of **5**: R_f = 0.77 (100% ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 8.32 (dd, J = 8.4, 0.7 Hz, 1H), 7.79 (ddd, J = 7.6, 7.7, 1.4 Hz, 1H), 7.62 (dd, J = 1.2, 6.7 Hz, 1H), 7.51 (ddd, J = 7.6, 6.9, 1.2 Hz, 1H), 5.61 (bs, 1H); ^{1}H NMR (DMSO-d_6) δ 8.17 (dd, J = 8.4, 1.4 Hz, 1H), 7.78 (ddd, J = 7.4, 6.6, 1.5 Hz, 1H), 7.59 (sb, 2H), 7.52 (dd, J = 8.6, 0.6 Hz, 1H), 7.45 (ddd, J = 8.3, 6.9, 1.2 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 160.5, 142.1, 141.9, 135.6, 129.4, 125.8, 124.7; MS (EI) *m*/*z* 146 (41), 118 (100), 91 (46), 76 (20).

Synthesis of 3-Amino-1,2,4-benzotriazine 2-Oxide (6). Hydrogen peroxide (30%, 3.5 mL) was added to a solution of **5** (100 mg, 0.68 mmol) in glacial acetic acid (10 mL). Upon addition of hydrogen peroxide, compound **5** dissolves and the resulting solution was stirred for 24 h. The solvent was then removed by lyophilization (behind a blast shield) and the resulting brownish-yellow residue purified by flash column chromatography on silica gel (eluted with 1:1 ethyl acetate: hexane) to give 69 mg of **6** (63%): R_f = 0.87 (100% ethyl acetate); ^{1}H NMR ($CDCl_3$) 7.78 (d, J = 8.2 Hz, 1H), 7.66−7.58 (m, 2H), 7.51 (t, J = 7.1 Hz, 1H), 6.23 (bs, 2H) ^{1}H NMR (DMSO-d_6) δ 8.15 (bs, 2H), 7.65−7.41 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 149.1, 135.5, 133.8, 130.8, 125.8, 124.2, 123.9. MS (EI) *m*/*z*, 162 (61), 133 (11), 132 (100), 118 (13), 117 (33), 105 (81), 91 (23), 90 (64), 78 (23). Crystals of **6** suitable for X-ray diffraction were obtained by slow evaporation of a 1:1 ethyl acetate−hexane solution. Crystal data for **6**: monoclinic, space group $P2_1/n$, a = 5.07771(6) Å, b = 18.681(2) Å, c =

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7.8231(10) Å, $\beta = 104.719(2)°$, $V = 717.62(15)$ Å^3, $\rho_{calcd} = 1.501$ mg·m^{-3}, Mo Kα radiation ($\lambda = 0.71070$ Å) for $Z = 4$. Intensity data were collected on a Bruker SMART system at 173 K. Least squares refinement based on 1588 reflections with $I_{net} > 2.0\sigma$(Inet) (out of 4447 total reflections) gave a final $R = 0.0418$. All crystallographic calculations were conducted using SHELX 97.

Synthesis of 3-Amino-1,2,4-benzotriazine 4-Oxide (4) via Oxidation of the Corresponding Benzotriazine (5) with Hydrogen Peroxide. To a stirred solution of the benzotriazine (**5**, 500 mg, 3.1 mmol) in glacial acetic acid (25 mL) was added 30% hydrogen peroxide (8 mL), and the resulting solution then stirred, protected from the light at 50 °C for 2 h. The reaction mixture (containing starting material, 2-oxide **6**, 1-oxide **3**, 4-oxide **4**, di-*N*-oxide **1**, and an additional uncharacterized product with an $R_f = 0.36$ in 100% ethyl acetate, blue fluorescent TLC spot under long wave UV) was lyophilized (behind a blast shield) to produce an orange-yellow solid. This solid was dissolved in ethyl acetate, mixed with silica gel (3.5 g), and then evaporated under reduced pressure. This silica gel mixture was then placed on the top of a silica gel column (88 g) packed in ethyl acetate. Elution of the column with ethyl acetate followed by 15:85 2-propanol:ethyl acetate provided ∼50 mg of impure **4**. A second step of column chromatography of this material on silica gel (eluted with 75:25 ethyl acetate:hexane, followed by 100% ethyl acetate) afforded 40 mg (7% overall yield) of **4** as a mustard yellow powder: $R_f = 0.49$ (100% ethyl acetate, green fluorescent TLC spot under long wave UV$_{366}$); ^{1}H NMR ($CDCl_3$) δ 8.38 (dd, $J =$ 5.9, 1.2 Hz, 1H), 8.35 (dd, $J = 8.3$, 1.2 Hz, 1H), 7.88 (ddd, $J =$ 7.9, 7.8, 1.2 Hz, 1H), 7.65 (ddd, $J = 7.8$, 7.7, 1.2 Hz, 1H), 6.5 (bs, H2); ^{1}H NMR (DMSO-d_6) δ 8.30 (d, $J = 8.6$ Hz, 1H), 8.2 (bs, 2H), 8.16 (d, $J = 8.4$ Hz, 1H), 7.91 (ddd, $J = 8.6$, 7.1, 1.2 Hz, 1H), 7.63 (ddd, $J = 8.4$, 7.0, 1.2 Hz, 1H) ^{13}C NMR (DMSO-d_6) δ 154.0, 142.7, 135.01, 129.6, 127.2, 127.1, 115.9; MS (EI) *m*/*z* 162 (100), 146 (3), 117 (13), 116 (12), 90 (33), 77 (12); MS (ESI) *m*/*z* 163.3 (20), 162.7 (33), 118.4 (27), 117.9 (100), 91.4 (32), 90.6 (38); UV−vis λ_{max} 208 ($\epsilon = 9200$), 256 ($\epsilon = 27000$), 326 ($\epsilon = 3400$), and 462 ($\epsilon = 3600$) nm.

Synthesis of 3-Amino-1,2,4-benzotriazine 4-Oxide (4) by Reaction of 3-Amino-1,2,4-benzotriazine 1,4-Dioxide (1) with *tert*-Butyl Peracetate. To a solution of **1** (50 mg, 0.28 mmol) in water (5 mL) was added *tert*-butyl peracetate (268 μL, 50 wt % in mineral spirits, 0.84 mmol), and the stirred mixture was refluxed in a 105−112 °C oil bath for 21 h. An additional aliquot (50 μL, 0.15 mmol) of *tert*-butyl peracetate was added and the reaction refluxed for 29 h. A final aliquot of *tert*-butyl peracetate (50 μL, 0.15 mmol) was added to the reddish-orange mixture and refluxing continued for 16 h. The reaction was cooled and transferred to a test tube. The flask was rinsed with 2-propanol and the combined solution centrifuged to remove a small amount (6 mg) of reddish-black solid which had formed on the sides of the reaction flask. The orange-red supernatant was lyophilized (behind a blast shield) to produce a reddish-orange solid. The final reaction mixture contained significant amounts of **4**, **3**, 3-amino-7-methyl-1,2,4-benzotriazine 1-oxide, and starting material (**1**). Repeated flash column chromatography as described above gave pure **4** (1.7 mg, 3.7%).

Synthesis of 3-Amino-1,2,4-benzotriazine 4-Oxide (4) by Reaction of 3-Amino-1,2,4-benzotriazine (5) with *m*-Chloroperbenzoic Acid. The benzotriazine (**5**, 50 mg, 0.3 mmol) and *m*-chloroperbenzoic acid (196 mg, Aldrich 57−86% pure) were dissolved in methylene chloride (5 mL) with stirring at room temperature. After 2.5 h, the wine red solution was evaporated under reduced pressure. Column chromatography on silica gel (eluted with 75:25 ethyl acetate:hexane, followed by 100% ethyl acetate) afforded 2 mg (4%) of **4**. The reaction also affords significant amounts of **1**, **3**, **6**, and an additional uncharacterized product ($R_f = 0.36$ in 100% ethyl acetate).

Synthesis of 3-Acetamido-1,2,4-benzotriazine 4-Oxide (7). The 4-oxide (**4**, 8 mg, 0.05 mmol) was dissolved in neat acetic anhydride (0.7 mL, 7.4 mmol). After stirring at 45−50 °C for 3 h nearly all starting material was consumed as judged by TLC. The acetic anhydride was removed by warming under high vacuum (∼1 mmHg) and the resulting residue purified by flash column chromatography on silica gel (eluted with ethyl acetate) to yield 9 mg (90%) of **7** as a yellow powder: $R_f = 0.5$ (100% ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 9.54 (bs, 1H), 8.49 (d, $J = 8.5$ Hz, 1H), 8.41 (d, $J = 8.4$ Hz, 1H), 7.96 (ddd, $J =$ 8.6, 7.6, 1.3 Hz, 1H), 7.83 (ddd, $J = 8.5$, 7.7, 1.4 Hz, 1H), 2.74 (s, 3H); ^{1}H NMR (DMSO-d_6) δ 10.96 (bs, 1H), 8.50 (d, $J = 7.7$ Hz, 1H), 8.35 (d, $J = 8.4$ Hz, 1H), 8.08 (d, $J = 7.5$ Hz, 1H), 7.97 (t, $J = 7.5$ Hz, 1H).^{13}C NMR (DMSO-d_6) δ 169.1, 148.1, 146.0, 135.9, 134.8, 131.5, 129.9, 117.6, 24.4. Crystals of **7** suitable for X-ray diffraction were obtained by slow evaporation of 2-propanol. Crystal data for **7**: monoclinic, space group $P2_1/n$, $a = 4.0178(4)$ Å, $b = 15.5538(14)$ Å, $c = 14.0228(13)$ Å, $\beta = 93.004(2)°$, $V = 875.11(14)$ Å^{3,} $\rho_{calcd} = 1.542$ mg·m^{-3}, $\theta_{range} = 1.96-27.11°$, Mo Kα radiation ($\lambda = 0.71070$ Å) for $Z = 4$. Intensity data were collected on a Bruker SMART system at 173 K. Least squares refinement based on 1916 reflections with $I_{net} > 2.0\sigma$(Inet) (out of 5427 total reflections) gave a final $R = 0.0496$. All crystallographic calculations were conducted using SHELX 97.

Characterization of Products Arising from In Vitro Metabolism of *N*-Oxides (1). In a typical assay, a solution of Tris buffer (pH 7, 25 mM, final volume 150 μL) containing the appropriate *N*-oxide (250 μM) and xanthine in a Pyrex tube (i.d. 3.0 mm, o.d. 5.0 mm, sealed at one end) was degassed by three freeze−pump−thaw cycles and then sealed under vacuum. The stock solution of xanthine (5 mM) was prepared by dissolving the compound in 40% NaOH. Sealed tubes were transferred to an inert atmosphere glovebox, scored, opened, and transferred to eppendorf tubes. To these solutions xanthine oxidase (degassed in the glovebox by bubbling with argon) was added, and the assays were incubated in the glovebox protected from the light for 1 h at room temperature. Xanthine oxidase was removed from the assay mixtures by centrifugation (Brinkmann tabletop centrifuge, @10000 rpm) through Amicon Microcon (YM3) filters. The filtrate was analyzed by HPLC, employing a C-18 reverse-phase Rainin Microsorb-MV column (100 Å sphere size, 5 μm pore size, 25 cm length, 4.6 mm i.d.) eluted with an isocratic mobile phase composed of 1% acetic acid, 25% methanol, and 74% water at a flow rate of 0.9 mL/min. The products were monitored by UV absorbance (254 nm). The 4-oxide produced by in vitro metabolism of tirapazamine was shown to comigrate with authentic synthetic 4-oxide under three different HPLC conditions: the conditions described above, as well as eluction of a C-18 reverse-phase Rainin Microsorb-MV column column or a Rainin Microsorb-MV Phenyl column (100 Å sphere size, 5 μm pore size, 25 cm length, 4.6 mm i.d.) with an isocratic mobile phase of 1% acetic acid, 5% acetonitrile, 94% water. Concentrations of various components of the reaction mixtures were determined from peak area or peak height using calibration curves prepared by injection of known amounts of authentic products.

LC/MS and LC/MS/MS Analysis of the Products Resulting From In Vitro Metabolism of Tirapazamine (1). Samples were prepared as described above, but on 1 mL scale employing larger Pyrex tubes (i.d. 6.0 mm) for freeze−pump−thaw-degassing. Samples were passed through Amicon filters prior to 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 was performed on a C-18 reverse-phase Rainin Microsorb-MV column (100 Å sphere size, 5 μm pore size, 25 cm length, 4.6 mm i.d.) at a flow rate of 1 mL/min eluted with an isocratic mobile phase composed of 20% methanol, 10% acetonitrile, 70% water. 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.

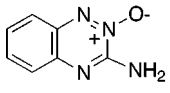
Investigation of the DNA-Cleaving Properties of the 3-Amino-1,2,4-benzotriazine 4-Oxide Metabolite (4). All assays were performed under anaerobic conditions and were carried out in an inert atmosphere glovebox. In a typical assay,

a solution of sodium phosphate buffer (pH 7, 25 mM, final volume 70 μL) containing the appropriate *N*-oxide (250 μM), xanthine (250−500 μM), DNA (supercoiled pB*R*322 or pUC19, 0.6 μg), and desferal (1 mM) in a Pyrex tube (i.d. 3.0 mm, o.d. 5.0 mm, sealed at one end) was degassed by three freeze−pump−thaw cycles and then sealed under vacuum. Sealed tubes were transferred to an inert atmosphere glovebox, scored, opened, and transferred to eppendorf tubes. To these solutions were added superoxide dismutase (700 ng), catalase (7 μg), and xanthine oxidase (0.04 units, each degassed in the glovebox by bubbling with argon) and the assays incubated in the glovebox protected from the light for 1 h at room temperature. Following incubation, loading buffer (5 μL of 50% glycerol loading buffer containing 0.1% bromophenol blue, 150 mM EDTA, 1% SDS in 2 M Tris, 1 M acetate, pH 8) was added to the reactions and the resulting mixture loaded immediately onto a 0.9% agarose gel containing 0.87 μg/mL ethidium bromide. The gel was electrophoresed for approximately 4 h at 80 V in TAE buffer (40 mM tris, 20 mM acetate, 1 mM EDTA, pH 8). DNA in the gel was visualized by UV-transillumination, the gel image recorded, and amounts of DNA in each band quantitated using an Alpha Innotech IS-1000 digital imaging system. The values reported are uncorrected for differential ethidium staining of form I and II DNA.

Results and Discussion

Synthesis and Characterization of 3-Amino-1,2,4-benzotriazine 4-Oxide (4). During the course of previous studies on the antitumor agent tirapazamine,[6,20,21] we examined the products resulting from in vitro reductive metabolism of this drug. In these earlier studies, we observed the expected[15−17] two-electron reduction product 3-amino-1,2,4-benzotriazine 1-oxide (**3**) and the expected four-electron reduction product 3-amino-1,2,4-benzotriazine (**5**). In addition, we were intrigued to observe significant amounts of an uncharacterized product under some conditions.[21] We suspected that the unknown product might be the "alternate" two-electron reduction product, 3-amino-1,2,4-benzotriazine 4-oxide (**4**). Thus, we prepared the putative 4-oxide metabolite (**4**) and undertook experiments to determine whether this compound is, in fact, produced by bioreductive metabolism of the antitumor agent tirapazamine.

Interestingly, although tirapazamine (**1**) and its corresponding 2-oxide (**6**) have been known in the chemical



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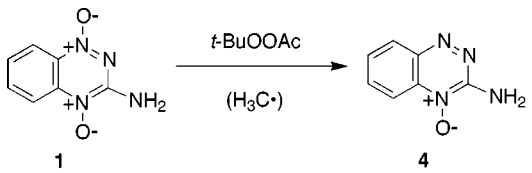
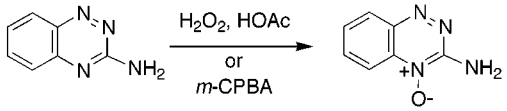
literature for over 40 years,[22] and the corresponding 1-oxide (**3**) and benzotriazine (**5**) analogues known for nearly 90 years,[23] the 4-oxide analogue (**4**) has not previously been characterized. In 1957, Robbins and coworkers claimed[22] to isolate the 4-oxide (**4**) from the treatment of 3-amino-1,2,4-benzotriazine (**5**) with hydrogen peroxide at room temperature; however, based on NMR data, the structure of this product was later called into question and reassigned as the 2-oxide (**6**).[18] Thus, when we began this work, no unambiguous synthesis

Scheme 2



1 **4**

Scheme 3



5 **4**

existed for the putative tirapazamine metabolite 3-amino-1,2,4-benzotriazine 4-oxide (**4**).

We prepared the suspected tirapazamine metabolite 3-amino-1,2,4-benzotriazine 4-oxide (**4**) by three independent routes and fully characterized the compound by NMR spectroscopy, UV−vis spectroscopy, LC/MS, and X-ray crystallography. Treatment of tirapazamine with methyl radical ($H_3C\bullet$) generated by thermolysis of *tert*-butyl peracetate[24,25] in water, followed by separation of the resulting products by flash column chromatography, affords the 4-oxide (**4**) in low yield (4%) (Scheme 2). An alternate approach involving oxidation of 3-amino-1,2,4-benzotriazine (**5**) with *m*-CPBA also provides a 4% yield of the 4-oxide (Scheme 3). The most convenient route involves treatment of the benzotriazine (**5**) with 30% hydrogen peroxide in glacial acetic acid at 50 °C to afford the 4-oxide in 7% yield (Scheme 3). The yields of **4** in these reactions are low because the compound is produced as a relatively minor component of complex mixtures that include the mono-*N*-oxides **3** and **6**, the benzotriazine **5**, and tirapazamine (**1**).

The ^{1}H and ^{13}C NMR spectra of the 4-oxide (**4**) are clearly distinct from that of the 1-oxide (**3**) and 2-oxide (**6**) isomers. Consistent with the trends observed in the proton NMR's of tirapazamine (**1**) and its 1-oxide analogue (**3**),[18] the proton on the 5-position of the aromatic ring in **4** (in the peri-position, adjacent to the *N*-oxide oxygen) is shifted downfield relative to the same proton in the benzotriazine derivative (**5**). Similarly, the mass spectrum (EI) of the 4-oxide is distinct from that of the isomeric mono-*N*-oxides **3** and **6**. In addition to the molecular ion peak at *m*/*z* 162, the 4-oxide shows characteristic fragmentation products at *m*/*z* 134 (loss of N_2) and *m*/*z* 117 (loss of OH and N_2). In contrast, the 1-oxide (**3**) shows a characteristic fragmentation product at *m*/*z* 118 (loss of N_2O) and the 2-oxide (**6**) fragments with loss of NO to yield a peak at *m*/*z* 132. ESI-MS/MS of the 4-oxide yields a characteristic fragment ion corresponding to $[M + H - N_2 - OH]^+$ at *m*/*z* 118, whereas the 1-oxide produces a major fragment corresponding to $[M + H - NO]^+$ at *m*/*z* 133. Initial confirmation of the structure of the synthetic 4-oxide was provided by exposing the compound to reaction conditions designed to yield characteristic products. For example, this compound can be oxidized to tirapazamine (**1**) by treatment with

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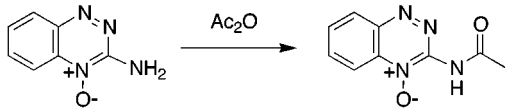
Scheme 4



4 7

hydrogen peroxide in acetic acid[26] and is reduced to the corresponding benzotriazine **5** by treatment with sodium dithionite (data not shown).[18]

The structure of the synthetically prepared 4-oxide (**4**) was ultimately confirmed by X-ray crystallography. The 4-oxide readily crystallizes from 2-propanol, but the resulting material did not yield a high-resolution X-ray structure due to disorder in the crystals. The acetylated derivative 3-acetamido-1,2,4-benzotriazine 4-oxide (**7**) was prepared by treatment of **4** with neat acetic anhydride (Scheme 4) in the hopes that this analogue would yield a high quality X-ray structure. As an interesting aside, we find that acetylation of the exocyclic amino group in **4** is quite efficient relative to the same reaction on the 1-oxide isomer (**3**). This finding is reminiscent of the previous observation that 2-aminopyridine *N*-oxide undergoes *N*-acetylation much more rapidly than 3-aminopyridine *N*-oxide.[27] In this earlier study, neighboring group assistance involving initial acetylation of the *N*-oxide oxygen, followed by intramolecular acetyl-group transfer to the adjacent amino group was invoked to explain the relatively rapid *N*-acetylation of 2-aminopyridine *N*-oxide. Happily, **7** crystallizes from 2-propanol, and the resulting crystals provided a high-resolution X-ray structure which confirms that this compound is the acetylated 4-oxide derivative (see Supporting Information for X-ray data). The structure of the 2-oxide (**6**) was also unambiguously confirmed by X-ray analysis.

Characterization of 3-Amino-1,2,4-benzotriazine 4-Oxide (4) Produced by Enzymatic Activation of Tirapazamine (1). The identity of the enzymes responsible for in vivo reductive activation of tirapazamine remains under investigation.[15] NADPH:cytochrome P450 reductase is thought to play a major role,[15] but it has also been suggested that DNA damage by the drug may be primarily triggered by an as yet uncharacterized reductase located in the cell nucleus.[28]

In the experiments described here, we have primarily utilized the xanthine/xanthine oxidase enzyme system to investigate reductive metabolism of tirapazamine. Although xanthine oxidase may not play a central role in the in vivo metabolism of tirapazamine, this enzyme system provides a reasonable model system for studying the reductive activation of tirapazamine. In support of this notion, the xanthine/xanthine oxidase system is able to initiate DNA cleavage by the drug[6,8,29] and generates the same primary tirapazamine metabolites (i.e., **3** and **5**) that are produced by other in vitro and in vivo systems.[7,8,16,17,30]

Reverse-phase HPLC analysis of the products resulting from in vitro metabolism of tirapazamine by the xanthine/xanthine oxidase enzyme system under hypoxic conditions reveals the presence of xanthine and uric acid (eluting at ~3 min), the well-known 1-oxide and benzotriazine metabolites (**3** and **5**), and two additional previously uncharacterized compounds eluting from the HPLC at approximately 8 and 10 min (Figure 1). No significant metabolism of tirapazamine is observed under aerobic conditions, indicating that all of the products seen under hypoxic conditions are formed via the oxygen-sensitive radical **2** (Scheme 1).

The identity of the unknown tirapazamine metabolite eluting at ~8 min in our HPLC analysis was established by several experiments. Consistent with our initial suspicion that the compound was the heterocyclic mono-*N*-oxide **4**, we found that the substance decomposes upon treatment of the crude reaction mixture with sodium dithionite, a reagent known to readily reduce *N*-oxides. Evidence for the exact identity of the unknown metabolite was provided by the observation that the HPLC retention time of this compound matches that of authentic, synthetically prepared 4-oxide (**4**) under several different HPLC conditions. Coinjection of authentic material with the reaction mixture obtained from in vitro metabolism of **1** under anaerobic conditions conclusively showed that the ~8 min peak comigrates with the 4-oxide (**4**). LC/MS analysis of the reaction mixture provided confirmation that the new tirapazamine metabolite observed in our HPLC traces is 3-amino-1,2,4-benzotriazine 4-oxide (**4**). LC/ESI-MS of the 8 min peak yielded an ion corresponding to $[M + H + CH_3CN]^+$ at *m*/*z* 204. Fragmentation of this ion was examined by LC/ESI-MS/MS using CID. In this experiment, we observed the characteristic fragment ion of the 4-oxide corresponding to $[M + H - N_2 - OH]^+$ at *m*/*z* 118. The LC/MS properties of the 8 min metabolite are identical to those of authentic, synthetically prepared 4-oxide (**4**). Additional studies showed that the 4-oxide metabolite (**4**) is also produced (along with the other expected metabolites **3** and **5**) by in vitro metabolism of tirapazamine by NADPH:cytochrome P450 oxidoreductase under hypoxic conditions (data not shown). The identity of the metabolite eluting at ~10 min remains under investigation.

Yield of 3-Amino-1,2,4-benzotriazine 4-Oxide (4) from in Vitro Metabolism of Tirapazamine under Various Conditions. In vitro metabolism of tirapazamine (**1**) by xanthine/xanthine oxidase produces significant quantities of 3-amino-1,2,4-benzotriazine 4-oxide (**4**). For example, incubation of tirapazamine (250 μM) with xanthine oxidase (0.04 units) and xanthine (250 μM) under hypoxic conditions in aqueous buffer (tris, pH 7) yields a 1:1.5:6 ratio of **4**, **5**, and **3**, respectively. Under these conditions, significant amounts (15−30%) of tirapazamine remain unmetabolized. Interestingly, under conditions where greater amounts of tirapazamine are consumed (e.g., when higher concentrations of xanthine are employed), yields of the 4-oxide metabolite *decrease* (Figure 2). This finding can be explained by the observation that the 4-oxide (**4**) produced by metabolism of tirapazamine readily undergoes further xanthine/xanthine oxidase-mediated reduction to 3-amino-1,2,4-benzotriazine (**5**). In contrast, the 1-oxide (**3**) is relatively resistant to further reduction by xanthine/xanthine oxidase. For instance, treatment of the 1-oxide (**3**) and the 4-oxide (**4**) (250 μM) with xanthine oxidase (0.04 units) and xanthine (250 μM) under anaerobic conditions in tris buffer (pH 7) affords 5% and 74% yields of 3-amino-1,2,4-

(26) This result does not provide unambiguous evidence that the product is the 4-oxide (**4**) because, in the parent benzotriazine system, the 2-oxide is also known[22] to isomerize during oxidation to afford the 1,4-dioxide.

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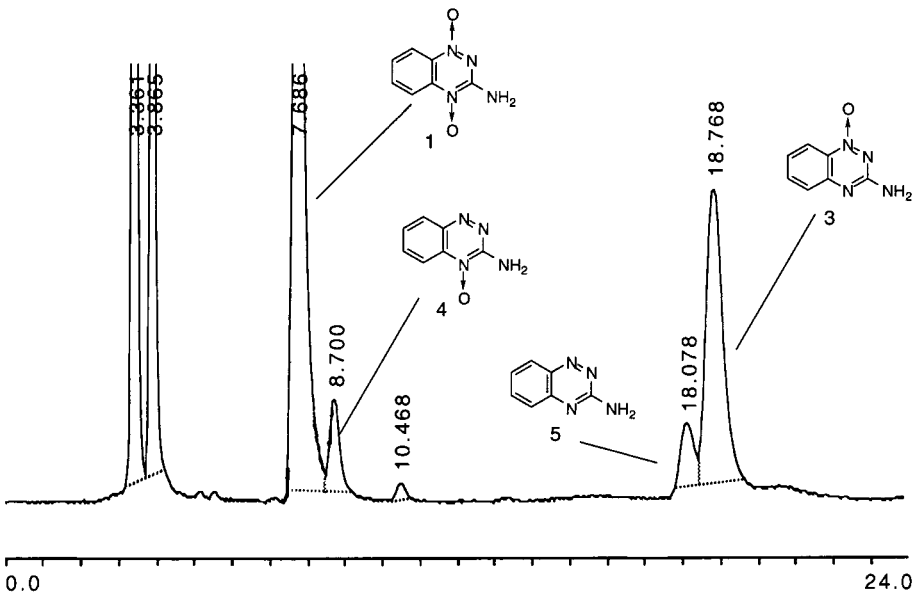

Figure 1. Reverse-phase HPLC analysis of the metabolites resulting from reductive activation of tirapazamine by the xanthine/xanthine oxidase enzyme system.

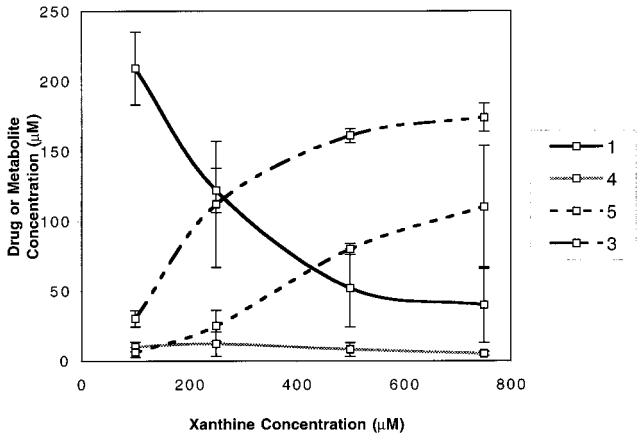

Figure 2. Metabolism of tirapazamine (**1**) by xanthine/xanthine oxidase: yields of 1-oxide (**3**), 4-oxide (**4**), benzotriazine (**5**), and tirapazamine (**1**) as a function of xanthine concentration. Assays were performed as described in the Experimental Seciton.

benzotriazine (**5**), respectively. Unlike the enzymatic reduction of tirapazamine and its 1-oxide analogue (**3**), xanthine/xanthine oxidase-mediated reduction of the 4-oxide (**4**) occurs with reasonable efficiency even under aerobic conditions. Specifically, in analogous experiments under conditions of ambient oxygenation, 8−14% of the 4-oxide (**4**) is consumed, whereas <1% of the 1-oxide (**3**) is converted to benzotriazine (**5**).

In the absence of enzymatic reduction, the 4-oxide metabolite is stable in solution. For example, a sample of the 4-oxide stored in buffered aqueous solution (sodium phosphate, pH 7) at room-temperature protected from light remains unchanged (as judged by ^{1}H NMR and TLC) over the course of eight weeks. However, the 4-oxide, like many *N*-oxides, is photosensitive.[31,32] An aqueous buffered solution of the 4-oxide stored in a clear glass container under ambient fluorescent lab lighting is completely degraded after two weeks (data not shown). It is perhaps worth noting, however, that the unidentified compound eluting at ~10 min in our experiments (Figure 1) is *not* a photolytic breakdown product of the 4-oxide, but is actually a product resulting from bioreductive metabolism.

Investigation of the DNA-Cleaving Properties of the 3-Amino-1,2,4-benzotriazine 4-Oxide Metabolite (4). Tirapazamine derives its biological activity from redox-activated DNA damage; thus, it is important to characterize the ability of the drug and its metabolites to react with DNA. In vitro DNA cleavage by tirapazamine has previously been investigated,[6,8,29] and it was found that, in conjunction with the xanthine/xanthine oxidase enzyme system, under hypoxic conditions, the drug yields significant amounts of single strand breaks.[6] The 1-oxide metabolite **3** causes no detectable DNA strand cleavage alone and only small amounts of redox-activated strand scission.[6] Similarly, previous studies have shown that the benzotriazine metabolite **5** does not cleave DNA alone or in conjunction with enzymatic activation.[6]

The ability of the newly characterized 4-oxide metabolite (**4**) to cleave double-stranded plasmid DNA was investigated using an agarose gel electrophoresis assay. This assay allows a quantitative assessment of spontaneous DNA strand cleavage by measuring the conversion of uncut supercoiled (form I) plasmid DNA to the nicked form (form II). As demonstrated previously,[6,8,29] we find that incubation of tirapazamine with the xanthine/xanthine oxidase enzyme system under hypoxic conditions yields significant amounts of single-strand DNA cleavage (Table 1). In contrast, the 4-oxide (**4**) does not produce significant DNA cleavage under these conditions.[33] Similarly, incubation of the 4-oxide with plasmid

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(33) Similarly, the 4-oxide does not cause significant DNA cleavage when incubated in the presence of the NADPH:cytochrome P450 oxidoreductase reducing system.

Table 1. Investigation of the DNA-Cleaving Properties of 3-Amino-1,2,4-benzotriazine 4-Oxide (4)

reaction[a]	% form I	% form II	S value[b]
anaerobic; DNA alone	72	28	0.33 (±0.15)
anaerobic; DNA + X/XO	71	29	0.34 (±0.11)
anaerobic; 250 μM **1** + X/XO	16	84	1.80 (±0.49)
anaerobic; 250 μM **4** + X/XO	67	33	0.40 (±0.25)
anaerobic; 250 μM **4** + XO	77	23	0.26 (±0.11)
anaerobic; 250 μM **4** alone	70	30	0.35 (±0.13)

[a] Cleavage reactions contained supercoiled pBR322 or pUC19 DNA (37 μM bp) in a solution of sodium phosphate buffer (25 mM, pH 7.0), xanthine (250−500 μM), desferral (1 mM), xanthine oxidase (0.04 U), catalase (7 μg), and superoxide dismutase (700 ng). Reactions solutions were freeze−pump−thaw degassed three times, enzymes were added, and the reactions were incubated for 1 h at 25 °C in an argon-filled glovebox. Reactions and densitometry were performed as described in the Experimental Section. [b] *S* is the mean number of strand breaks per plasmid molecule and is calculated using the equation: $S = -\ln(\%\text{ form I DNA})$.[42]

DNA in the absence of the xanthine/xanthine oxidase system produces no detectable cleavage.[34]

Conclusions

We have unambiguously shown that 3-amino-1,2,4-benzotriazine 4-oxide (**4**) is produced by in vitro reductive metabolism of the antitumor agent tirapazamine. Under conditions where tirapazamine (**1**) is bioreductively activated, the yield of this metabolite is typically moderate because the 4-oxide that is produced readily undergoes further reduction to the benzotriazine analogue (**5**, Scheme 1). On the other hand, the isomeric two-electron reduction product 3-amino-1,2,4-benzotriazine 1-oxide (**3**) is not readily reduced by the xanthine/xanthine oxidase enzyme and, therefore, is found as a major metabolite resulting from bioreductive processing of tirapazamine regardless of the reaction conditions. Together, these results suggest that the 3-amino-1,2,4-benzotriazine (**5**) product which has long been known as a metabolite of tirapazamine may be formed predominantly via reduction of the 4-oxide (**4**).

Although the 4-oxide metabolite (**4**) described here has not previously been characterized, an inspection of the literature indicates that this compound may have previously been seen as a product resulting from the metabolism of tirapazamine under several different conditions.[8,17,30,35] For example, a compound with similar relative retention time on C18-reverse phase HPLC was produced by in vivo metabolism of tirapazamine in CHO cells and in mice.[17,35] Similarly, enzymatic and radiolytic activation of the drug afforded an uncharacterized product—likely the 4-oxide—that migrated between the drug (**1**) and the benzotriazine (**5**) on reverse-phase HPLC.[8,30] We find that the 4-oxide metabolite is produced by hypoxic metabolism of tirapazamine by the NADPH: cytochrome P450 reductase enzyme system as well as the xanthine/xanthine oxidase enzyme system.

Previous researchers who have analyzed the products resulting from metabolism of tirapazamine have noted that significant amounts (30−60%) of the starting mass balance cannot be accounted for in the remaining starting material, the 1-oxide (**3**), and the benzotriazine (**5**) metabolites.[8,9,15,30] Our characterization of the metabolite **4** helps provide a more complete picture of the products formed by enzymatic processing of tirapazamine and may provide some insight regarding the identity of the missing mass balance. Quantitative HPLC analysis of our reactions indicates ~90% of the starting material balance in our reactions can be accounted for in unchanged tirapazamine (**1**), 1-oxide (**3**), 4-oxide (**4**), and benzotriazine (**5**).

There are several chemical pathways by which the 4-oxide metabolite (**4**) can be produced. First, one-electron reduction of tirapazamine to the delocalized radical (**2**) may yield the metabolite **4** via a mechanism analogous to those proposed previously[6,8,16] for the formation of the metabolite **3**. Alternatively, it is possible that the 4-oxide metabolite (**4**) is formed by two sequential one-electron enzymatic reductions of the drug (**1**). Finally, we have previously shown that the 4-oxide (**4**) is formed by the reaction of tirapazamine with carbon-centered radicals.[20] Accordingly, a portion of the observed 4-oxide metabolite in the current studies may arise from reaction of the parent drug, tirapazamine, with carbon-centered radicals that are generated by the action of the reductively activated drug on organic substrates in the reaction mixture. In the reactions reported here, carbon-centered radicals may be derived from the organic buffer, Tris, xanthine oxidase, xanthine, or uric acid. When DNA is present, deoxyribose radicals resulting from the action of the activated drug on the biopolymer will react with the parent drug to produce the 4-oxide metabolite.[20,21]

The 4-oxide metabolite characterized here does not cause efficient DNA strand cleavage either alone or in conjunction with reductive activation by the xanthine/xanthine oxidase enzyme system. The metabolite (**4**) readily undergoes reduction by xanthine/xanthine oxidase; however, we suggest that enzymatic processing of **4** does not produce DNA damage due to the fact that the compound is converted to the benzotriazine (**5**) either via direct two-electron enzymatic reduction or two sequential enzymatic one-electron transfers, rather than by one-electron reduction followed by N−O bond fragmentation to produce hydroxyl radical (analogous to the reaction of **2** shown in Scheme 1). Although **4** does not cleave DNA, this compound may nonetheless play a significant indirect role in facilitating strand cleavage by tirapazamine. We previously demonstrated that the 4-oxide (**4**) efficiently transfers the oxygen from its *N*-oxide functional group to a deoxyribose radical in double-stranded DNA with a rate constant of $3.1 \times 10^7\ M^{-1}\ s^{-1}$, thus resulting in net conversion of the DNA radical into a base-labile strand break.[20] Typically, DNA strand break formation by radicals is inefficient under hypoxic conditions because molecular oxygen is required for the efficient conversion of DNA radicals into strand breaks.[36−4136−42] However, the 4-oxide (**4**) acts as an oxygen surrogate and, thus, may serve to increase the overall efficiency of drug-mediated DNA strand cleavage under hypoxic conditions. Further

(34) Enzyme-activated DNA cleavage by **4** is not above background even when considered at the 50% confidence level.

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investigations of the chemical and biological properties of this newly characterized tirapazamine metabolite are currently underway.

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Supporting Information Available: Complete X-ray data for **6** and **7** and ^{1}H NMR spectra for **1**, **3**, **4**, **5**, **6**, and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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