Active-Site-Directed Reductive Alkylation of Xanthine Oxidase by Imidazo[4,5-*g*]quinazoline-4,9-diones Functionalized with a Leaving Group†

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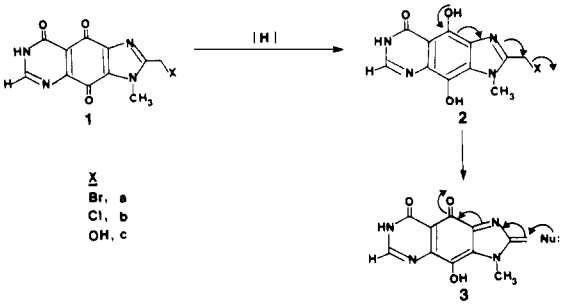
ABSTRACT: A new class of purine antimetabolites, directed toward xanthine oxidase, was designed by employing some of the features found in the bioreductive alkylator mitomycin C. The design involved functionalizing the purine-like imidazo[4,5-*g*]quinazoline ring system as a quinone (4,9-dione) bearing a 2α leaving group. Due to the presence of the electron-deficient quinone ring, the leaving group cannot participate in alkylation reactions. Reduction to the hydroquinone (4,9-dihydroxy) derivative, however, permits elimination of the leaving group to afford an alkylating quinone methide. In spite of the electronic differences, both quinone and hydroquinone derivatives of the imidazo[4,5-*g*]quinazoline system are able to enter the purine-utilizing active site of the enzyme. Thus, the hypoxanthine-like quinone derivative [2-(bromomethyl)-3-methylimidazo[4,5-*g*]quinazoline-4,8,9(3*H*,7*H*)-trione] and its hydroquinone derivative can act as reducing substrates for the enzyme, resulting in conversion to the xanthane-like 6-oxo derivatives. Hydrolysis studies described herein indicate that the hypoxanthine-like hydroquinone derivative eliminates HBr to afford an extended quinone methide species. The observed alkylation of the enzyme by this derivative may thus pertain to quinone methide generation and nucleophile trapping during enzymatic oxidation at the 6-position. Enzymatic studies indicate that the hypoxanthine-like quinone is an oxidizing suicide substrate for the enzyme. Thus, the reduced enzyme transfers electrons to this quinone, and the resulting hydroquinone inactivates the enzyme. As with mitomycin C, reduction and quinone methide formation are necessary for alkylation by the title quinone. This system is therefore an example of a purine active-site-directed reductive alkylator. It is concluded that reductive alkylators of other purine-utilizing enzymes may be designed by functionalizing the imidazo[4,5-*g*]quinazoline system as described above. This assessment is based on the substrate tolerance of many purine-utilizing enzymes for this dimensionally altered form of the purine ring [Leonard, N. J. (1982) *Acc. Chem. Res. 18*, 128]. The utility of these reductive alkylators may lie in their selective activation in low-potential tumor cells, perhaps with reduced xanthine oxidase acting as the activating enzyme.

Reductive alkylation pertains to alkylation by an agent, usually a quinone, only after it has undergone one- or two-electron reduction. Typically, these quinones are substituted with a leaving group, which becomes activated as such when the system is reduced. Since tumor cells possess a low reduction potential (Kennedy et al., 1980a), reductive alkylators are of interest as selective antitumor agents. A well-known example of a reductive alkylator directed toward DNA is the antitumor agent mitomycin C (Schwartz et al., 1963; Iyer & Szybalski, 1964; Kennedy et al., 1980a,b; Tomasz et al., 1983). Either one- or two-electron reduction of the quinone ring activates the aziridine and carbamate groups of mitomycin C, leading to cross-linking of DNA (Lin et al., 1972; Moore, 1977; Tomasz & Lipman, 1981; Peterson & Fisher, 1986; Andrews et al., 1986). In addition to mitomycin C, many other naturally occurring quinones have the structural features necessary for reductive alkylation (Moore & Czerniak, 1981). As yet unreported, however, are reductive alkylators directed toward purine-utilizing enzymes rather than DNA. These enzymes are crucial to the tumor cell (Weber, 1977), and their reductive alkylation is desirable in terms of selective antitumor therapy. Described herein is a reductive alkylator directed toward the purine active site of xanthine oxidase. As is required for successful reductive alkylation, the quinone form is stable in aqueous buffer and does not affect xanthine–oxygen reductase activity. The fully reduced form, on the other hand, irreversibly inactivates the enzyme at low concentrations. Although xanthine oxidase is a catabolic enzyme, this reductive alkylator could see use as an antitumor agent. Some purine antitumor agents are degraded by the enzyme (Elion et al., 1963), and reductive alkylation of the enzyme may potentiate these agents in tumor cells. Another implication of this study is that reductive alkylators of other purine-utilizing enzymes may be designed by the approach described below.

The reductive alkylator design involves functionalizing a substrate of the enzyme as a quinone with a leaving group placed so as to permit quinone methide formation upon two-electron reduction. A substrate of the enzyme to which these structural features could be added is the imidazo[4,5-*g*]-quinazoline system (Lee et al., 1986). The functionalization of this substrate as a reductive alkylator is shown in eq 1. The



(1)

leaving group of **1** is inactive due to the presence of the

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electron-deficient quinone ring as well as the N(3)-methyl group, which prevents leaving-group elimination utilizing the N(3) anion. Reduction of **1** to **2** results in a 1,6-elimination of the leaving group to afford the reactive quinone methide species **3**. This species is likely responsible for the irreversible inactivation of the enzyme observed when **1a** and **1b** are reduced to the corresponding hydroquinone forms. Hydrolysis studies described in this paper indicate that **3** actually forms in solution and selectively traps strong nucleophiles. Enzyme inhibition studies indicate that the reductive alkylator **1a** is an oxidizing suicide substrate of the enzyme. Under reducing conditions, the enzyme reduces **1a** to **2a**, thereby causing its irreversible inactivation.

The success in the design of purine-like reductive alkylators of xanthine oxidase suggests that the design could be extended to other purine-utilizing enzymes. Like xanthine oxidase, many of these enzymes tolerate dimensionally altered substrates (Leonard et al., 1976; Leonard, 1982) and thus could tolerate the reductive alkylators described above.

Materials and Methods

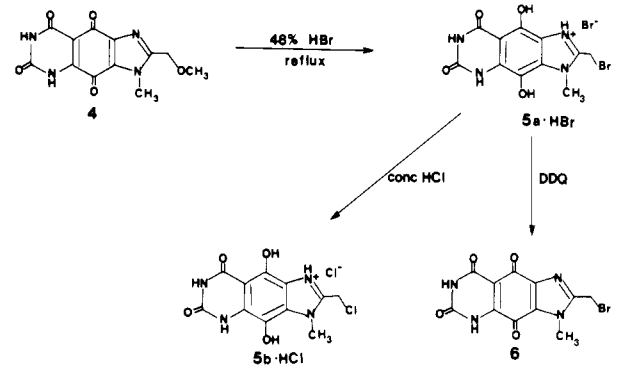
All kinetic measurements were carried out on either a Perkin-Elmer 559 spectrometer or a Perkin-Elmer Lambda-3 spectrometer in which the cell holder had been maintained at 30.0 ± 0.2 °C by circulating thermostated water. UV–vis spectra were obtained on the above spectrometers, employing aqueous buffer as solvent. Uncorrected melting points were determined with a Mel-Temp apparatus. Elemental analyses were performed by MicAnal Laboratories, Tucson, AZ. Thin-layer chromatography (TLC) was run on Merck silica gel 60 (F_{254}) plates. IR spectra were taken as KBr pellets with a Nicolet MX-1 FTIR spectrometer; only the wavelengths of intense bands are tabulated. ^{1}H NMR spectra were taken on a Bruker WH-90 spectrometer. Mass measurements were carried out in the electron-impact mode with a Varian MAT 200 spectrometer. Measurements of pH were made with a Radiometer PHM84 pH meter equipped with a Radiometer GK2401C combination electrode.

Buttermilk xanthine oxidase grade III, FAD, and xanthine were purchased from Sigma Chemical Co. The inorganic salts used in the preparation of buffer solutions were analytical reagent grade obtained from Mallinkrodt and were used as such. The buffer solutions were prepared in doubly glass-distilled water. Anaerobic studies were carried out under argon, which had been passed through an Oxiclear filter to remove trace amounts of oxygen.

The preparations of **1a** and **2a** were carried out as previously described (Lee et al., 1986). The preparation of **2b**·HCl was carried out by treating **2a** with concentrated HCl; the corresponding quinone **1b** was not prepared. Refluxing a mixture of **2a** in aqueous ethanol provided **2c**, which was oxidized to **1c** by treatment with dichlorodicyanobenzoquinone (DDQ). The xanthine-like derivatives shown in Scheme I were also prepared to assess their inhibitory activity. Treatment of **4** (Lee et al., 1986) with 48% HBr results in the replacement of methoxy with bromo as well as the HBr-mediated reduction of the quinone ring, resulting in the hydrobromide salt of **5a**. The hydrochloride salt of the 2α-chloro derivative (**5b**·HCl) was prepared by incubating **5a**·HBr in concentrated HCl/dimethyl sulfoxide for 5 min at room temperature. Oxidation of hydroquinone **5a**·HBr to **6** was carried out by treatment with DDQ. Details of the preparation of new compounds and their physical properties are provided below.

2-(Chloromethyl)-4,9-dihydroxy-3-methylimidazo[4,5-g]-quinazolin-8(3H,7H)-one Hydrochloride (**2b**·*HCl*). A mixture of 35 mg (0.086 mmol) of **2a**·HBr and 4 mL of concentrated

Scheme I



HCl was stirred for 10 h at room temperature. The completed reaction was diluted with 15 mL of 100% ethanol and cooled in an ice bath. Pale yellow crystals of **2b**·HCl formed immediately. Yield upon filtration, washing with ethanol, and drying was 25 mg (83%): ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 7.99 [1 H, s, C(6) H], 5.09 (2 H, s, chloromethyl), 4.14 [3 H, s, N(3) methyl]; mass spectrum (EI) *m/e* 280 (P^+). Anal. Calcd for $C_{11}H_9ClN_4O_3$·HCl·H_2O: C, 39.42; H, 3.58; N, 16.72. Found: C, 39.39; H, 2.89; N, 16.09. Although the mass spectrum and ^{1}H NMR indicate pure **2b**·HCl, the H and N analyses deviate significantly from the theoretical values. Due to the instability of this compound, purification by recrystallization could not be carried out.

2-(Hydroxymethyl)-3-methylimidazo[4,5-g]quinazoline-4,8,9(3H,7H)-trione (**1c**) *and Its Hydroquinone Derivative* **2c**. A mixture of 50 mg (0.018 mmol) of **2a**·HBr in 20 mL of ethanol (95%) was heated at reflux for 1 h. Cooling to 0 °C afforded the 2α-hydroxyhydroquinone derivative **2c** as the HBr salt. Recrystallization was carried out by dissolving this salt in 100% ethanol and prompting crystallization by addition of hexane: yield 40 mg (64%); ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 8.07 [1 H, s, C(6) H], 4.89 (2 H, s, hydroxymethyl), 4.16 [3 H, s, N(3) methyl]; mass spectrum (EI) *m/e* 262 (P^+).

A suspension of **2c**·HBr (20 mg, 0.076 mmol) in 5 mL of dry methanol was combined with 20 mg (0.088 mmol) of DDQ. The reaction mixture was stirred for 20 min at room temperature, resulting in the crystallization of analytically pure **1c**. The yellow solid was filtered and washed with methanol: yield upon drying 10 mg (70%); TLC [1-butanol–acetic acid–water (5:3:2)] R_f 0.23; IR (KBr) 3451, 1708, 1688, 1541, 1471, 1412, 1254, 1106, 1069 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 8.55 [1 H, s, C(6) H], 5.72 (1 H, t, J = 5.6 Hz, hydroxyl), 4.65 (2 H, d, J = 5.6 Hz, hydroxymethyl), 3.97 [3 H, s, N(3) methyl]; mass spectrum (EI) *m/e* 260 (P^+). Anal. Calcd for $C_{11}H_8H_4O_4$·0.7H_2O: C, 48.44; H, 3.45; N, 20.53. Found: C, 48.25; H, 3.45; N, 20.53.

2-(Bromomethyl)-4,9-dihydroxy-3-methylimidazo[4,5-g]-quinazoline-6,8(3H,5H,7H)-dione Hydrobromide (**5a**·*HBr*). A mixture of **4** (50 mg, 0.17 mmol) in 20 mL of 48% HBr was heated at reflux for 3.5 h and then chilled in a refrigerator overnight. The crystalline solid that formed was filtered, washed with ethyl acetate, and dried; the yield of **5a**·HBr was 63 mg (87%). The product was sufficiently pure to carry on to the next synthetic step or use in enzymatic studies. Attempted recrystallization resulted in decomposition. Physical properties: decomposition point >250 °C; TLC [1-butanol–acetic acid–water (5:2:3)] R_f 0.12; IR (KBr) 3533, 3449, 3161, 2995, 2947, 2851, 1704, 1643, 1627, 1445, 1259 cm^{-1}; ^{1}H NMR (dimethyl-d_6 sulfoxide) δ 4.93 (2 H, s, bromomethyl), 4.03 [3 H, s, N(3) methyl]. Anal. Calcd for $C_{11}H_9BrN_4O_4$·HBr·0.5H_2O: C, 30.63; H, 2.57; N, 12.98.

Found: C, 30.57; H, 2.50; N, 12.56.

2-(Bromomethyl)-3-methylimidazo[4,5-g]quinazoline-4,6,8,9(3H,5H,7H)-tetrone (**6**). A mixture of 64 mg (0.15 mmol) of **5a**·HBr and 41 mg (0.18 mmol) of DDQ in 5 mL of methanol was stirred at room temperature for 1 h. The bright yellow solid that formed during the reaction was filtered off and washed with methanol. Recrystallization was carried out from aqueous dimethylformamide (DMF), yield 30 mg (59%): decomposition point >250 °C; TLC [1-butanol–acetic acid–water (5:2:3)] R_f 0.35; IR (KBr) 3563, 3467, 3037, 2993, 2844, 1732, 1717, 1528, 1412 cm^{-1}; NMR (dimethyl-d_6 sulfoxide) δ 4.92 (2 H, s, bromomethyl), 3.94 [3 H, s, N(3) methyl]. Anal. Calcd for $C_{11}H_7BrN_4O_4 \cdot 1H_2O$: C, 36.98; H, 2.53; N, 15.67. Found: C, 36.59; H, 2.53; N, 15.54.

2-(Chloromethyl)-4,9-dihydroxy-3-methylimidazo[4,5-g]-quinazoline-6,8(3H,5H,7H)-dione Hydrochloride (**5b**·*HCl*). Addition of 2 drops of concentrated HCl to a solution consisting of 30 mg of **2a**·HBr (0.07 mmol) in 4 mL of dimethyl sulfoxide resulted in warming and the crystallization of **5b**·HCl. After the reaction mixture was stirred for 5 min, 5 mL of water was added, resulting in additional crystallization. The product was filtered off, washed with water, and dried in vacuo. The yield of **5b**·HCl was 22 mg (95%): NMR (dimethyl-d_6 sulfoxide) δ 5.04 (2 H, s, chloromethyl), 4.05 [3 H, s, N(3) methyl]. Anal. Calcd for $C_{11}H_9ClN_4O_4 \cdot HCl \cdot 1.25H_2O$: C, 37.15; H, 3.54; N, 15.74. Found: C, 37.08; H, 3.50; N, 15.25.

Preparative Hydrolysis of **2a**. A solution of 20 mg (0.05 mmol) of **2a**·HBr in 0.5 mL of dimethyl sulfoxide was added to 500 mL of pH 7.00, 0.2 M phosphate buffer (μ = 1.0, KCl) under strict anaerobic conditions. The reaction mixture was then stirred for 5 h at 30 °C followed by exposure to the air and evaporation in vacuo to ~50 mL. The concentrated reaction mixture was placed on a 100-mL AG-1-X4 (200–400 mesh) Bio-Rad resin column, which was then washed with 500 mL of distilled water to remove buffer salts. Elution with 0.01 N HCl removed 2,3-dimethylimidazo[4,5-g]quinazoline-4,8,9(3*H*,7*H*)-trione (**7**, Scheme II) from the column. These fractions were concentrated to a small volume and diluted with ethanol, resulting in the crystallization of **7** in 80% yield. Identity is based on comparison of ^1H NMR and mass spectral data with those of authentic **7** (Lee et al., 1986). The ^1H NMR spectrum of the product indicated that a trace amount of **1c** was also present from a comparison of the spectrum with that of authentic material prepared as described above.

Anaerobic Reaction of **7** *with Xanthine Oxidase*. A solution of 7.6 mg (0.031 mmol) of **7** in 2.5 mL of 0.05 M, pH 7.4 phosphate buffer was combined with 2.3 mg of purified xanthine oxidase under strict anaerobic conditions. During the 15-min reaction, the yellow reaction mixture assumed a light tan color and a colorless precipitate began to form. The reaction mixture was opened to the air and immediately acidified with concentrated HCl. The colorless solid was collected by filtration and washed with water and then ethanol: yield of **11**·HCl 4.5 mg (49%); ^1H NMR (dimethyl-d_6 sulfoxide) δ 4.03 [3 H, s, N(3) methyl)], 2.69 [3 H, s, C(2) methyl]; mass spectrum (EI) *m/e* 262 (P^+ for **11**).

Kinetic Studies of Hydrolysis. The hydrolytic studies of **2a** and **5b** were carried out in aqueous buffer at 30.0 ± 0.2 °C under an argon atmosphere with Thunberg cuvettes. A dimethyl sulfoxide stock of the hydrohalide salt of **2a** or **5b** was placed in the top port, and the aqueous buffer was placed in the bottom port. After a stream of purified argon was passed into each port for 30 min, the cuvette was sealed and equilibrated at 30 °C in a thermostated cell holder for 20 min. The ports were then mixed and absorbance vs time data obtained with a UV–vis spectrophotometer. These data were computer fit to a first-order or a two-consecutive, first-order rate law.

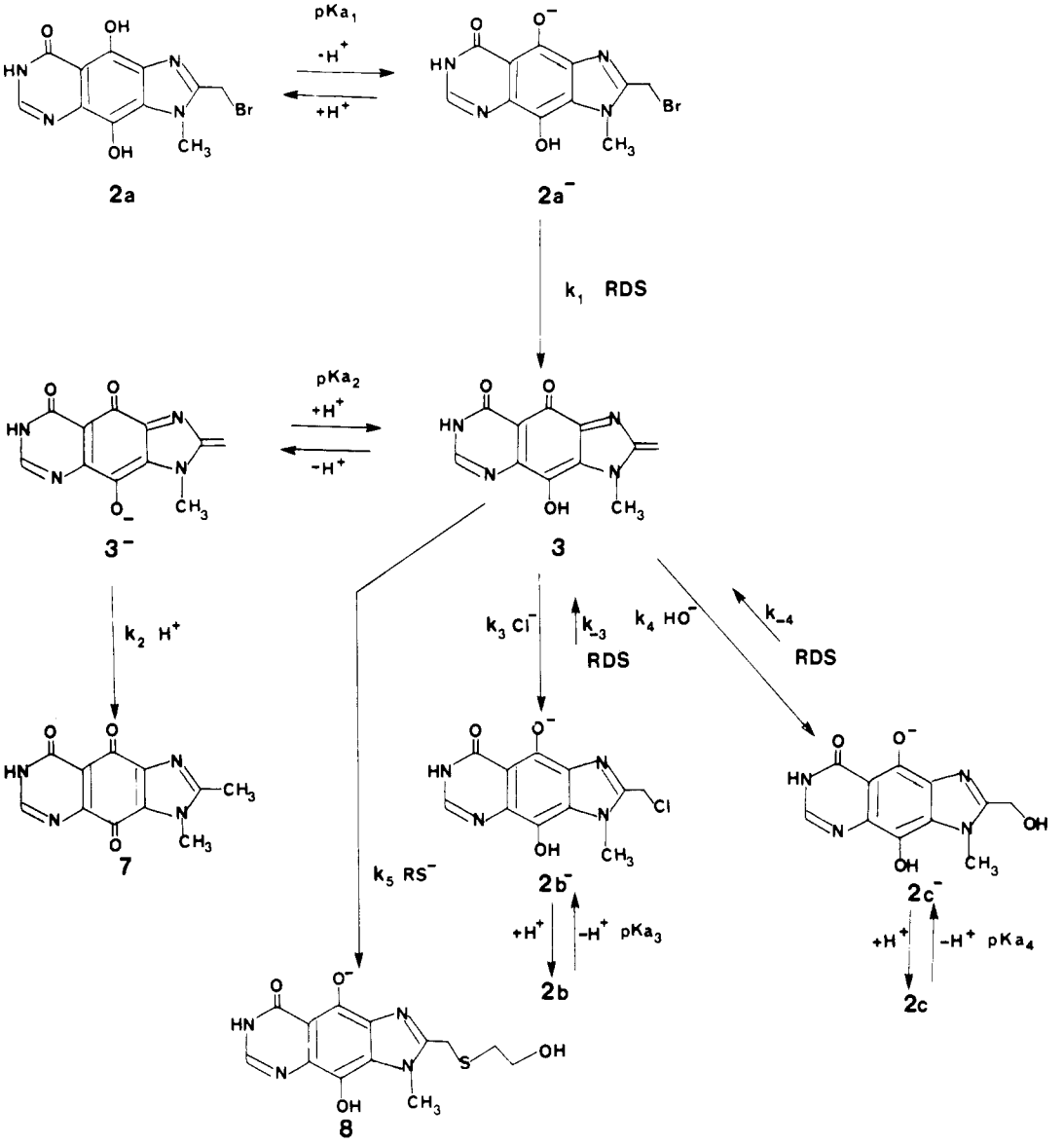
Kinetic Studies of Enzyme Inhibition. Inhibition studies were carried out in 0.05 M, pH 7.40 phosphate buffer (μ = 0.1, KCl) containing 22 μM ethylenediaminetetraacetic acid (EDTA). The approximate molarity of enzyme was determined from an absorbance measurement at 450 nm and the ϵ_{450} value of 37 800 M^{-1} cm^{-1} per enzyme-bound FAD (Massey et al., 1969). The xanthine oxidase employed, grade III (Sigma), is chromatographically pure and is reported to contain 10 units/mL. Stock solutions of the hydrohalide salts of hydroquinones **2a–c** and **5a,b**, as well as quinones **1a** and **6**, were prepared in dimethyl sulfoxide distilled from sodium hydroxide. The hydroquinones decompose slowly in this solvent, and stock solutions were always prepared fresh for an inhibition study. The inhibition studies consisted of either anaerobic or aerobic incubation of enzyme with the potential inhibitor for 1 h followed by a determination of remaining xanthine–oxygen reductase activity. Anaerobic incubations were carried out in Thunberg cuvettes degassed with argon (loc. cit., Kinetic Studies of Hydrolysis). After an incubation, the mixture was aerated if anaerobic and uric acid formation followed at 290 nm in the presence of 6 μM xanthine. The low concentration of xanthine employed permits convenient velocity measurements (OD s^{-1}) over a wide range of enzyme concentrations. Also, this xanthine concentration is well below that which results in significant velocity decreases due to substrate inhibition. Comparisons of enzyme activities with controls permitted assessments to be made concerning the reversible and irreversible nature of inhibition (see Figure 2).

Electrochemical Studies. The two-electron reduction potentials (E_m) for quinones **1a** and **10** were measured in 0.2 M, pH 7.00 phosphate buffer (μ = 1.0, $NaClO_4$) at room temperature under a nitrogen atmosphere. Measurements were carried out by conventional cyclic voltammetry with a modified Princeton Applied Research Model 174 polarographic analyzer as previously described (Skibo & Bruice, 1983). The working electrode was a carbon paste consisting of 3 g of 325-mesh graphite and 2 mL of paraffin oil (McCreery et al., 1974; Kissinger et al., 1973). Scan speeds were from 100 to 200 mV s^{-1}; at these speeds hydrolysis of the hydroquinone **2a** formed during the scan was insignificant. Cyclic voltammograms for both **1a** and **10** are highly symmetric (α ~0.5), permitting an accurate determination of the anodic ($E_{p,a}$) and cathodic ($E_{p,c}$) potentials. The midpoint potential (E_m) was determined from the average of $E_{p,a}$ and $E_{p,c}$.

Results and Discussion

Hydrolysis Studies. Queries posed at the outset of any alkylation study deal with the rates and mechanism of hydrolysis of the alkylating agents, the type of reactive species formed, and the selectivity of this species for nucleophiles. The Answers to these questions have a bearing on the length of incubation times as well as the specificity of the alkylator for the target. The results of hydrolysis studies are discussed below in conjunction with Schemes II and III. The mechanism shown in Scheme II for the hydrolysis of the hypoxanthine-like alkylator **2a** strongly resembles that proposed for the hydrolysis of its benzimidazole analogue (Skibo, 1986a). An important feature of this mechanism is the formation of a quinone methide species (**3**), which can act as an effective nucleophile trap. Hydrolysis of the corresponding xanthine-like derivative **5b** likewise occurs via a quinone methide species (**9**) (Scheme III). This quinone methide species readily ketonizes without substantial nucleophilic trapping, however.

Scheme II



The hydrolysis of **2a** was studied in anaerobic aqueous buffers with μ = 1.0 (KCl) at 30.0 ± 0.2 °C. At the pH values of these buffers, **2a** as well as suspected intermediates exists in anionic and neutral forms. The total concentration of these species is expressed in the following way: $[\mathbf{2a}_T] = [\mathbf{2a}] + [\mathbf{2a}^-]$. UV–vis spectra of nearly completed reactions, in which $[\mathbf{2a}_T]$ was initially 5×10^{-5} M, indicate that quinone **7** is the major product. A preparative reaction, worked up under aerobic conditions, provided an 80% yield of **7** as well as a trace amount of **1c** (see Material and Methods). Absorbance (258 nm) vs time (seconds) plots of $\mathbf{2a}_T$ hydrolysis indicate that **7** forms by a three-consecutive, first-order process. The third first-order process is extremely slow and was not always followed to completion. The first and second first-order processes are relatively fast, however. Fitting absorbance vs time data for these processes to a two-exponential equation (Alcock et al., 1970) provided the corresponding first-order rate constants. These rate constants were determined over the pH range 5–9 and are independent of total buffer concentration. Found in Figure 1 are k_{obsd} values for the first (plot A) and second (plot B) kinetic phases plotted as the log vs pH.

The mechanism of the first phase of hydrolysis is postulated to be rate-determining formation of quinone methide **3** at k_1 s^{-1} from $\mathbf{2a}^-$. In rapid steps, **3** is trapped by Cl^- and HO^- to afford $\mathbf{2b}_T$ and $\mathbf{2c}_T$, respectively. In a competing process, ketonization of $\mathbf{3}^-$ to **7** occurs by protonation at the exocyclic methylene center. Both nucleophilic trapping and ketonization have been documented for the quinone methides derived from benzimidazole (Skibo, 1986a) and naturally occurring antitumor quinones (Ramakrishnan & Fisher, 1983; 1986; Kleyer & Koch, 1983, 1984; Brand & Fisher, 1986; Peterson & Fisher, 1986).

Evidence for the formation of $\mathbf{3}_T$ (=**3** + $\mathbf{3}^-$) was obtained from the pH–rate law for the first phase of hydrolysis and from nucleophile trapping studies employing 2-hydroxyethyl mercaptide. The disappearance of $\mathbf{2a}_T$ (and the appearance of **7**) obeys the general rate law

$$k_{obsd} = kK_a/(a_H + K_a) \qquad (2)$$

where k is a first-order rate constant, K_a is an acid dissociation constant, and a_H is the proton activity determined with a pH meter. This rate law indicates that the rate-determining step for the first phase of $\mathbf{2a}_T$ hydrolysis occurs from an anionic species formed by acid dissociation. Thus, the rate-determining step (RDS) is considered to be loss of bromide from $\mathbf{2a}^-$ at k_1 s^{-1} (Scheme I). Computer fitting of the k_{obsd} values for the first kinetic phase of $\mathbf{2a}_T$ hydrolysis to eq 2 provided the solid line shown in plot A of Figure 1. Interpreting the parameters

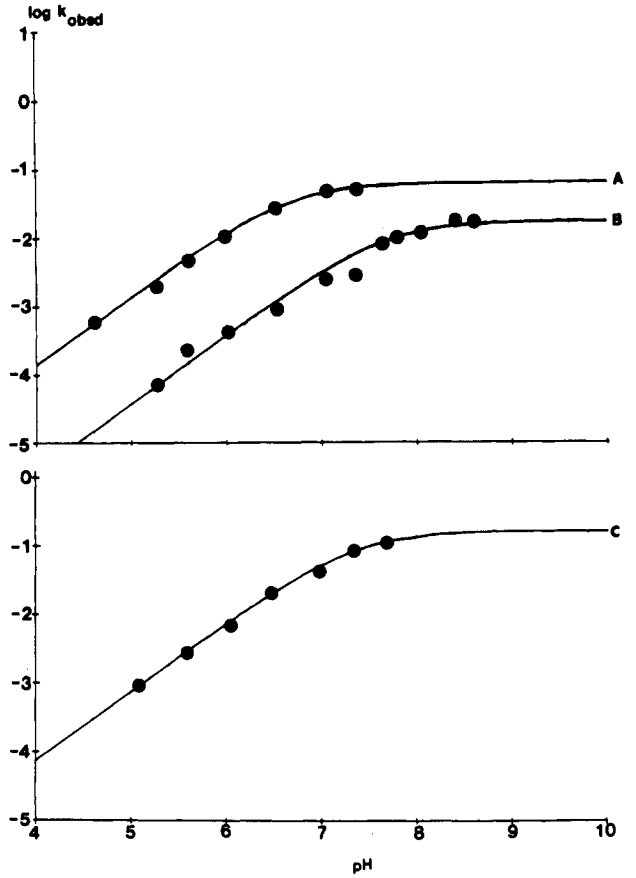
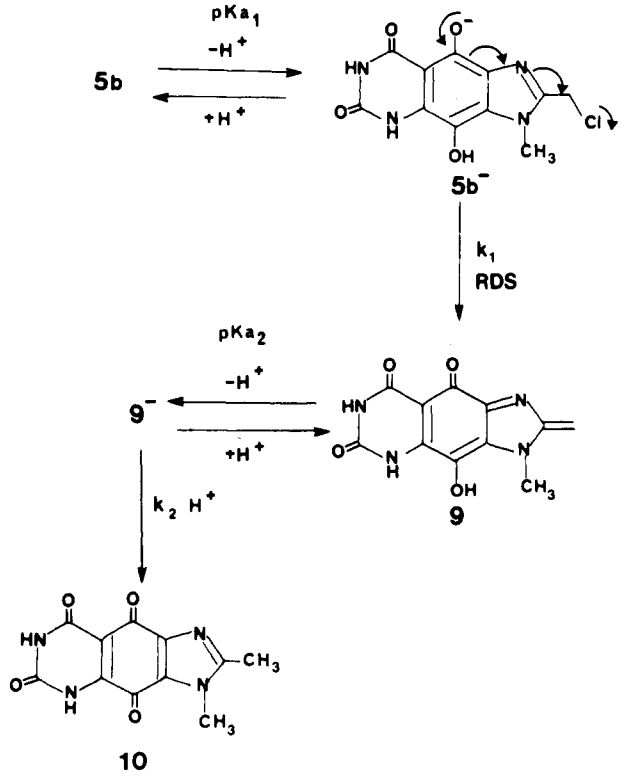


FIGURE 1: Plots of log k_{obsd} vs pH for the first (A) and second (B) phases of **2a** hydrolysis and the single phase of **5b** hydrolysis (C) obtained in anaerobic aqueous buffer (μ = 1.0, KCl) at 30.0 ± 0.2 °C.

Scheme III



obtained from the computer fit in terms of the postulated mechanism provides k_1 = 0.063 s^{-1} and pK_{a1} = 6.65. Previous studies with benzimidazole (Skibo, 1986a) and imidazo[4,5-*g*]quinazoline hydroquinones (Lee et al., 1986) indicate that the 9-hydroxyl group of **2a** ionizes first as shown in Scheme II. Measurement of this pK_a value for **2b** (pK_{a3} in Scheme II) directly (vide infra) suggests that the kinetic value of pK_{a1} actually represents the thermodynamic value.

Addition of a 10–100× excess of 2-(hydroxyethyl)mercaptan (pK_a = 9.5) over [$\mathbf{2a}_T$] = 5 × 10^{-5} M had no effect on the k_{obsd} value for the first phase of hydrolysis. Under these conditions, **7** is not formed and the second and third phases of hydrolysis are not observed. These findings are consistent with rate-determining formation of **3**, which traps 2-hydroxyethyl mercaptide in a fast step (k_5, Scheme II). As was concluded in a previous study (Skibo, 1986a), a species formed from the hydroquinone anion ($\mathbf{2a}^-$) able to trap nucleophiles as well as ketonize is taken to be a quinone methide.

The chloro and hydroxy derivatives ($\mathbf{2b}_T$ and $\mathbf{2c}_T$, respectively) formed during the first phase of hydrolysis are slowly converted to **7**. The second kinetic phase is attributed to the slow loss of chloride from $\mathbf{2b}^-$ at k_{-3} s^{-1} to afford $\mathbf{3}_T$, which then ketonizes to **7**. The conclusions that $\mathbf{2b}_T$ forms during the first kinetic phase and is converted to **7** during the second kinetic phase were arrived at after consideration of the following observations: (i) holding ionic strength constant with perchlorate instead of chloride results in the absence of a second kinetic phase for $\mathbf{2a}_T$ hydrolysis and (ii) the observed second-phase rates constants for $\mathbf{2a}_T$ hydrolysis were reproduced by following the conversion of authentic $\mathbf{2b}_T$ to **7**. Computer fitting of the k_{obsd} values for the second kinetic phase of $\mathbf{2a}_T$ hydrolysis to eq 2 provided the solid line shown in plot B of Figure 1. The parameters obtained from the computer fit are k_{-3} = 0.017 s^{-1} and pK_{a3} = 7.65. The apparent first-order rate constant (k in eq 2) obtained from the fit is actually a complex mixture of terms due to the presence of a back reaction ($Cl^- + \mathbf{3} \rightarrow \mathbf{2b}^-$) and the presence of $\mathbf{3}_T$ on the path leading to **7** (Skibo, 1986a). The apparent first-order rate constant is approximately equal to k_{-3}, however. Consistent with the postulated mechanism, the kinetically obtained value of pK_{a3} (7.6) is approximately equal to the thermodynamic value (8.2) determined by spectrophotometric means with Thunberg cuvettes [see Skibo and Bruice (1983) for the method used].

The third kinetic phase is attributed to the slow loss of hydroxide from $\mathbf{2c}^-$. Because hydroxide is a much poorer leaving group than chloride, this phase of hydrolysis occurs over a longer time than the second phase. Evidence for the formation of **2c** was obtained from product studies, which provided the oxidized form **1c** (loc. cit.).

Since the hydrolysis of the xanthine-like derivative bearing a bromo leaving group (**5a**) is quite rapid, the chloro derivative **5b** was studied instead. The postulated mechanism derived from these studies is found in Scheme III.

The study of $\mathbf{5b}_T$ hydrolysis was carried out under the same conditions employed for the study of **2a** hydrolysis. Absorbance (258 nm) vs time (seconds) plots indicated that hydrolysis proceeds by a simple first-order process. A UV–vis spectrum of the final product is consistent with quantitative formation of **10** [see Lee et al. (1986) for UV–vis spectral data of **10**]. Computer fitting of the pH–rate data for $\mathbf{5b}_T$ hydrolysis in plot C of Figure 1 to eq 2 provided the solid line shown in this figure. Thus, hydrolysis must pertain to the rate-determining loss of chloride from $\mathbf{5b}^-$ (Scheme III). Interpreting the kinetic parameters obtained from the fit in terms of this mechanism provides k_1 = 0.17 s^{-1} and pK_{a1} = 7.35. The intermediate resulting from the rate-determining step must be converted to **10** and is considered to be quinone methide $\mathbf{9}_T$ (**9** + $\mathbf{9}^-$). In contrast to quinone methide $\mathbf{3}_T$, this

quinone methide ketonizes without substantial trapping by hydroxide. Thus $\mathbf{5b}_T$ is converted to **10** by a simple first-order process rather than by a two-consecutive, first-order process. The latter process would be observed is the hydroxide-trapped product is slowly re-forming $\mathbf{9}_T$ (Scheme II). The different reactivity pattern of $\mathbf{3}_T$ and $\mathbf{9}_T$ could be explained by the relative potentials of the quinones (**7** and **10**, respectively) resulting from ketonization. The basis of this explanation is that the more stable product would be favored in a competing reaction (nucleophile trapping vs ketonization). Quinone **7** possesses an E_m value of +70 mV (NHE) in μ = 1.0 (perchlorate), 0.2 M phosphate buffer held at pH 7.00, whereas quinone **10** possesses an E_m value of 39 mV (NHE) in the same buffer. Thus, $\mathbf{3}_T$ is more susceptible to nucleophilic trapping than $\mathbf{9}_T$ since a relatively high-potential quinone (**7**) results from its ketonization.

The results of the hydrolysis studies indicate that **2a** and **2b** should be alkylating agents since the resulting quinone methide **3** is an efficient nucleophile trap. In contrast, **5b** should not act as an alkylating agent since the resulting quinone methide **9** readily ketonizes. Indeed, enzyme inhibition studies revealed that **2a** and **2b** alkylate the enzyme, whereas **5a** and **5b** do not. The rapid rates of quinone methide formation and decomposition did not permit a study of the rate of enzyme inactivation by **2a** and **2b**, however. Incubation studies described in the next section were carried out within the time frame of the first two phases of **2a** hydrolysis, about 1 h.

A necessary feature of a reductive alkylating system is the absence of nucleophilic substitution when the alkylator is in the quinone form. A hydrolysis study of **1a** revealed that this compound is stable in neutral aqueous buffer at room temperature. Even recrystallization of **1a** from hot aqueous DMF did not result in appreciable hydrolysis (see Materials and Methods).

Enzyme–Quinone Interactions. The imidazo[4,5-*g*]-quinazoline-4,9-dione system can interact with the enzyme both as a reducing and as an oxidizing substrate to afford 6-oxidized and 4,9-dihydroxy (hydroquinone) products, respectively. Both interactions were studied by employing purified buttermilk xanthine oxidase in 0.05 M, pH 7.40 phosphate buffer (μ = 0.1, KCl) containing 22 μM EDTA and held at 30.0 ± 0.2 °C.

The ability of imidazo[4,5-*g*]quinazoline-4,9-diones to act as reducing substrates was previously documented in this laboratory (Lee et al., 1986). An important question in the present study dealt with the ability of imidazo[4,5-*g*]-quinazoline-4,9-dione derivatives bearing a leaving group to alkylate the enzyme. Ideally, the 4,9-dione (quinone) derivative is not an alkylating agent, whereas the 4,9-dihydroxy (hydroquinone) derivative can form an alkylating quinone methide species. It was observed that **1a** is enzymatically converted to the 6-oxidized derivative **6** at k_{cat} = 2000 min^{-1} with a K_m value of 3.4 × 10^{-5} M. The specificity (k_{cat}/K_m) of **1a** of the enzyme is 33% that of the substrate hypoxanthine. To determine if irreversible inactivation of the enzyme occurs during oxidation, 60 μM **1a** was incubated with various concentrations of enzyme in aerobic buffer and the remaining enzyme activity measured. Found in Figure 2 is the resulting plot (plot B) of velocity for xanthine–oxygen reductase activity vs [xanthine oxidase]. The zero intercept of this plot indicates that irreversible inactivation is not occurring during the treatment with **1a**. The lower slope of plot B, compared to the control plot (plot A), indicates that there is noncompetitive inhibition of the enzyme. This inhibition is due to the presence

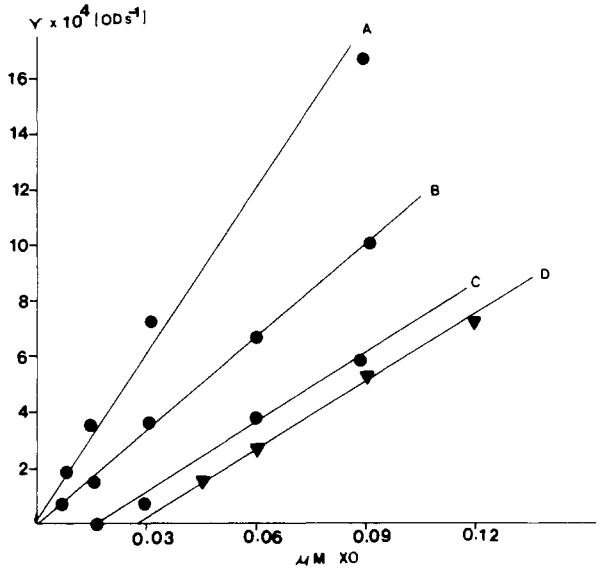


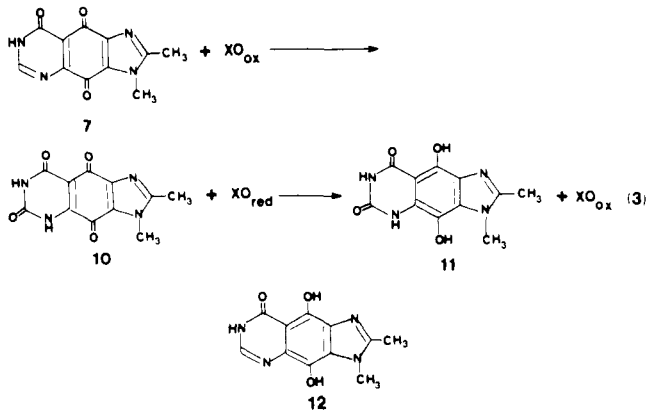
FIGURE 2: Plots of velocities of uric acid formation vs [XO] obtained in 0.05 M, pH 7.40 phosphate (μ = 0.1, KCl) containing 22 μM EDTA at 30 °C: (A) control; (B) after treatment of the enzyme with 60 μM **1a** for 1 h under aerobic conditions; (C) after treatment of the enzyme with 60 μM **1a** for 1 h under anaerobic conditions; (D) after treatment of the enzyme with 60 μM **2a** for 1 h under anaerobic conditions.

of **6**, which is a linear noncompetitive inhibitor (K_i = 2 × 10^{-4} M) of xanthine–oxygen reductase activity (Lineweaver–Burk plot not shown).

The above findings indicate that **1a** and **6** are not alkylating agents, in spite of the 2α-bromo leaving group. This is likely due to the electron-deficient quinone ring, which also protects these compounds from hydrolysis to the 2α-hydroxy forms (loc. cit. Hydrolysis Studies). The substrate activity of **1a** suggests that the reduced form (**2a**) can enter and alkylate the active site. Quinone **6**, on the other hand, cannot enter the active site (on the basis of the linear, noncompetitive inhibition), which suggests that its reduced form **5a** will behave likewise. Consistent with these observations, **1a** proved to be a reductive alkylator, while **6** displayed no activity. Another possible reason for the inactivity of **6** is the rapid ketonization of its reduced from (loc. cit. Hydrolysis Studies).

It has been documented that reduced enzyme can transfer electrons to substrates such as quinones and dyes (Bray, 1976). Indeed, reduced xanthine oxidase may be one of the enzymes involved in reductive activation of mitomycin C (Pan et al., 1984). The studies described below indicate that the imidazo[4,5-*g*]quinazoline-4,9-dione system acts as an efficient oxidizing substrate for the enzyme.

Treatment of **7** with a catalytic amount of enzyme in anaerobic pH 7.4 buffer affords **11** after only 15 min (see Materials and Methods). The UV–vis spectrum of the completed reaction indicated that **11** had formed in quantitative yield (eq 3). The formation of **11** is thought to involve enzymatic oxidation of **7** to **10**, as previously documented (Lee et a., 1986), followed by electron transfer from the reduced enzyme (XO_{red}) to **10**. Alternatively, **7** could also be an electron acceptor; the resulting hydroquinone **12** could then be enzymatically oxidized to **11** (vide infra, Enzyme–Hydroquinone Interactions). To assess the electron-acceptor ability of **10**, the enzymatic oxidation of xanthine (6 × 10^{-6} M) was followed in the presence of various concentrations of **10** under strict anaerobic conditions. The concentration of xanthine employed is near saturation but well below concentrations that result in decreasing velocities due to substrate inhibition (Hofstee, 1955). The catalytic parameters obtained from a

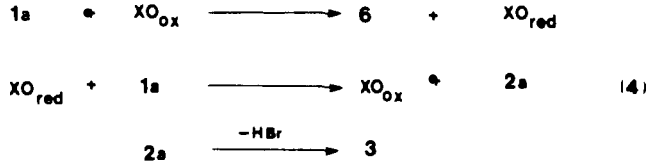


Lineweaver–Burk plot (not shown) are k_{cat} = 245 min^{-1} and K_m = 15 μM. The K_m value obtained above is the apparent dissociation constant of **10** from the enzyme. It is noteworthy that this value is somewhat less than the K_m for O_2 during xanthine–oxygen reductase activity (Olson et al., 1974). Thus, the reduced enzyme will efficiently activate purine-like reductive alkylators, perhaps in low-oxygen-tension tumor cells.

Studies with enzyme possessing an inactivated functional FAD cofactor indicate that this center is not the site of electron transfer to quinone **10**. Inactivation of the cofactor was carried out by C(4a) alkylation with 1-methyl-2-(bromomethyl)-4,7-dimethoxybenzimidazole as previously described (Skibo, 1986b). Although the treated enzyme possesses negligible xanthine–oxygen reductase activity, it is still able to catalyze the conversion of **7** to **11** under anaerobic conditions (eq 3) as well as the conversion of **7** to **10** under aerobic conditions. The aerobic reaction occurs at k_{cat} = 555 min^{-1} with K_m = 2.6 × 10^{-5} M. Catalytic cycling must therefore involve electron transfer from either the molybdenum or Fe/S center to **10** and probably **7**. Studies with the corresponding hydroquinone forms, **11** and **12**, indicate that reoxidation occurs in aerobic aqueous buffer at stopped-flow rates. Thus, the electron acceptor(s) for the enzyme should be rapidly regenerated. Since quinones **6** and **10** do not interact with the purine-binding site (loc. cit. this section), electron transfer probably occurs from the Fe/S centers.

Enzyme–Hydroquinone Interactions. The 4,9-dihydroxyimidazo[4,5-*g*]quinazoline (hydroquinone) analogues, bearing a leaving group in the 2α-position, are able to act both as alkylators of the enzyme and as reducing substrates providing 6-oxidized products. Due to the air sensitivity of the hydroquinone substrates, detailed steady-state studies of substrate oxidation could not be carried out. Alkylation may occur while the hydroquinone substrate is bound to the molybdenum-containing active site, perhaps as a result of quinone methide formation.

Enzyme-mediated activation of the reductive alkylator **1a** was apparent when the incubation experiments used to generate plot B of Figure 2 were repeated under strict anaerobic conditions. The resulting data, velocity of uric acid formation vs [xanthine oxidase] obtained after the incubation, are shown in plot C of Figure 2. The observed nonzero value of [xanthine oxidase] when the velocity is zero on this plot indicates that the enzyme is irreversibly inactivated (Segel, 1975). This intercept value and the concentration of **1a** employed (60 μM) indicate that [**1a**]/[xanthine oxidase] must be at least 3300 for complete inactivation.[1] The processes that may lead to inactivation are discussed below in conjunction with eq 4.



As noted earlier, **1a**–oxygen reductase activity affords **6** without concomitant inactivation. In the absence of oxygen, **6** and XO_{red} are still formed, but the excess **1a** must now act as the electron acceptor. The two-electron reduction potential of **1a** (100 mV vs NHE in pH 7.00 buffer) is much greater than that of the electron acceptor **10** (39 mV vs NHE in pH 7.00 buffer) discussed under Enzyme–Quinone Interactions. The potential difference suggests that **1a** is a better electron acceptor for reduced enzyme than **10**. **2a** thus formed is postulated to be the species involved in irreversible inactivation. Consistent with this postulate, incubation with 60 μM **2a** provided the velocity vs [xanthine oxidase] data shown in plot D of Figure 2. These data are nearly identical with those shown in plot C; [**2a**]/[XO] must be at least 2100 for complete inactivation. Incubation studies with the hydroquinone analogue bearing a 2α-chloro substituent (**2b**) also resulted in irreversible inactivation; [**2b**]/[XO] must be at least 2500–3500 for complete inactivation. The 2α-hydroxy derivative **2c**, on the other hand, did not inactivate the enzyme at any concentration, probably due to the poor leaving ability of the hydroxyl group (loc. cit. Hydrolysis Studies).

Anaerobic incubation of the xanthine-like quinone **6** with xanthine-reduced enzyme did not result in alkylation at any concentration. The same results were obtained from incubation studies with the corresponding hydroquinone derivatives **5a** and **5b**. As stated earlier, the lack of alkylation by the xanthine-like reductive alkylators may pertain to rapid hydrolysis and/or inability to bind to the enzyme. These results indicate that the **6** formed in the sequence shown in eq 4 is not participating in enzyme inactivation.

The preparation and study of bulk-inhibited enzyme was not possible due to denaturation at low dilutions of inhibitor. However, the following observations suggest that alkylation occurs at the purine-binding site: (i) the hypoxanthine-like hydroquinones can enter this active site and be oxidized at the 6-position and (ii) the addition of a substrate protects the enzyme from alkylation. Discussions of these observations are provided below.

The reductive half-reaction of xanthine oxidase with hydroquinone **12** was followed at 450 nm under anaerobic conditions in 0.05 M, pH 7.4 phosphate buffer (μ = 0.1, KCl) containing 22 μM EDTA. When 3.3 μM enzyme was mixed with 31 μM **12**, a biphasic absorbance decrease was observed at 450 nm. As was previously noted for xanthine reduction of the enzyme under these conditions (Olson et al., 1974), a biphasic process was observed consisting of fast and relatively slow absorbance decreases. Unlike xanthine reduction, however, these absorbance changes require somewhat longer times for completion: ~6 s for the first phase and ~8 min for the second phase. The transfer of reducing equivalents to the enzyme is considered to involve enzymatic oxidation of **12** resulting in **11** (eq 3). An alternative possibility considered was the transfer of reducing equivalents from the hydroquinone ring of **12**. The absence of a significant absorbance change at 450 nm when the hydroquinone **11** is combined with the enzyme served to dismiss this possibility.

The findings presented above indicate that the alkylator **2a** will also be enzymatically oxidized at the 6-position, resulting

[1] A reviewer has noted that Sigma grade xanthine oxidase is probably 40–60% functional due to loss of the terminal sulfur ligand from the molybdenum center. Consequently, this and other inactivation ratios that follow may actually be somewhat higher.

in the active alkylator **5a**. Hydrolysis of **2a** provides quinone **7**, which would reoxidize the reduced enzyme formed in this reaction (loc. cit., Hydrolysis Studies). The combination of **2a** hydrolysis and its enzymatic oxidation to **5a** may explain the excessive amounts of inhibitor needed for complete enzyme inactivation. If **2a** alkylates the enzyme while bound to the purine-binding site, it should be possible to protect the enzyme by adding a nonalklylating substrate such as the hydroquinone analogue **12**. Indeed, anaerobic incubation of the enzyme in the presence of **2a** and **12** resulted in a shift in the x intercept and plot D in Figure 2 toward zero.

An important question deals with the residue alkylated in the active site. Possibilities include the terminal oxo ligand associated with the molybdenum center (Gutteridge & Bray, 1980; Bray & Gutteridge, 1982; Bray & George, 1985; Skibo et al., 1987) and a phosphoserine residue proximal to the molybdenum center (Davis et al., 1984). If alkylation occurs while **2a** is bound as a substrate, however, neither of these nucleophiles would be near the alkylating center. A more likely possibility is the basic protein residue exploited by Baker and Wood (1968a,b) and Baker et al. (1968) for the design of irreversible inhibitors of the enzyme. Baker's inhibitors are 9-phenylpurine derivatives with an alkylating center attached to the phenyl ring. If these derivatives bind in a fashion similar to natural purine substrates, the alkylating center is in the same general location as the 2α-bromo group of **2a**. Since we have not been able to determine if **2a** alkylation occurs during enzymatic oxidation, a firm assessment of the nucleophile involved cannot be made at the present time. Currently, a reductive alkylator is under study that has the alkylation center located at the 6-position rather than the 2-position of the imidazo[4,5-*g*]quinazoline system. The results of these studies will be presented in a future paper.

Conclusions

This study demonstrates the feasibility of designing purine active-site-directed reductive alkylators by employing some of the structural features found in mitomycin C and other naturally occurring reductive alkylators. Thus, the hypoxanthine-like reductive alkylator **1a** is activated by reduction to its hydroquinone form **2a**. Our studies show that the enzyme can carry out this reduction by electron transfer from the Fe/S centers. Since **2a** eventually alkylates the enzyme, **1a** is considered to be an oxidizing suicide substrate. Hydrolytic studies indicate that **2a** forms a reactive quinone methide species by 1,6-elimination of HBr. Enzymatic studies suggest that **2a** binds to the purine-binding site and is oxidized at its 6-position. Alkylation is thus considered to occur at this center, perhaps as a result of trapping of the quinone methide species by a basic protein residue. Protection studies with a substrate support this interpretation. It is concluded that the reductive alkylator design represented by **1a** could be extended to other purine-utilizing enzymes able to tolerate dimensionally altered purine analogues. The utility of these systems may lie in their selective activation in the low-potential environment of tumor cells.

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