Structure–Activity Studies of Benzimidazole-Based DNA-Cleaving Agents. Comparison of Benzimidazole, Pyrrolobenzimidazole, and Tetrahydropyridobenzimidazole Analogues

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The synthesis and cytotoxic properties of benzimidazole-based DNA-cleaving agents are presented herein. These agents include pyrrolo[1,2-*a*]benzimidazole (PBI), benzimidazole (BI), and tetrahydropyrido[1,2-*a*]benzimidazole (TPBI) analogues. As a result of these studies, it is concluded that the pyrrolo ring is not necessary for cytotoxicity (PBI is only slightly more cytotoxic than BI) but that homologation of the pyrrolo ring by one carbon results in a system, TPBI, prone to decomposition. Another conclusion is that the 6-aziridinyl derivative of the PBI system is more potent than the 7-aziridinyl derivative. Comparative studies with known antitumor agents revealed that the benzimidazole-based DNA-cleaving agents possess a unique spectrum of activity. Noteworthy observations are the high level of cytotoxicity against melanoma cell lines and the complete absence of activity against leukemia cell lines. The reductive activation and DNA-cleavage properties of the most active analogue (BI-A) are also presented. Reduction of the quinone ring to the hydroquinone results in nucleophile and proton trapping by the aziridinyl group. Documented nucleophiles include water and the oxygen anion of 5′-dAMP. In addition, reduced BI-A reacts with DNA to form a stable adduct, which cleaves at G + A bases upon heating in basic gel-loading solution.

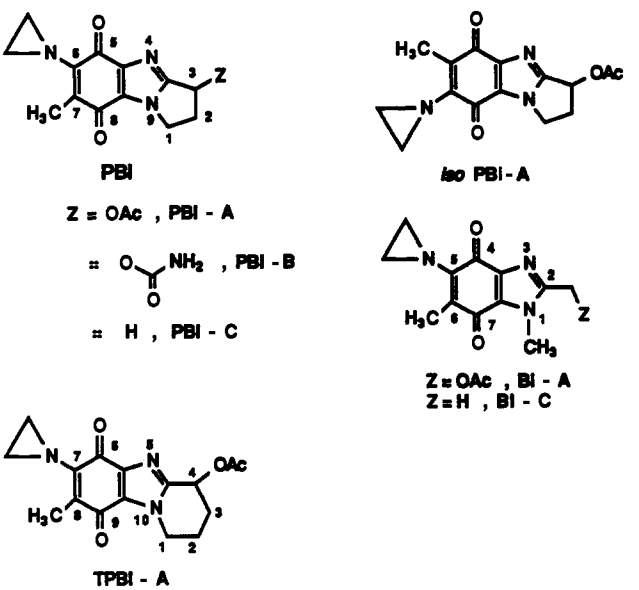
Introduction

The pyrrolo[1,2-*a*]benzimidazoles represent a new class of antitumor agent[2–5] showing activity against a variety of cancers. The mechanism of cytotoxicity involves reductive alkylation of DNA accompanied by cleavage at G and A bases.[6] Presented herein are the results of structure–activity studies wherein the pyrrolo[1,2-*a*]benzimidazole (PBI) ring nucleus was changed to the benzimidazole (BI) and tetrahydropyrido[1,2-*a*]benzimidazole (TPBI) ring variants as well as to an isomeric form (*i*-PBI), Chart 1. As a result of these studies, insights were gained into the influence of the ring nucleus on antitumor activity and DNA cleavage.

The pyrrolo[1,2-*a*]benzimidazoles (PBI, in Chart 1) were initially designed as reductive cross-linkers of DNA related to mitosenes and mitomycins.[7] The leaving group present at the 3-position of the pyrrolo[1,2-*a*]benzimidazole system (PBI-A and PBI-B) would permit formation of an alkylating quinone methide species.[8,9] Structure–activity studies,[3] however, revealed that cleavage of DNA and not cross-linking is involved in the cytotoxic mechanism. This cleavage process is thought to involve DNA alkylation at both G and A bases followed by hydrolytic cleavage. Cleavage mechanisms involving N(7)-purine alkylation and phosphate backbone alkylation have been invoked to explain the G + A cleavage pattern.[6]

In the present study, we show that DNA cleavage and cytotoxic activity are unaffected by the change from PBI to BI. In contrast, the *i*-PBI and TPBI analogues possess diminished cytotoxic activity. We also show that PBI and its analogues possess a range of activity like no other clinically used antitumor agent. Presumably, this feature is due to the reductive cleavage at G and A bases exhibited by these analogues, since the BI-A-mediated cleavage of linear DNA also exhibited a G + A base specificity. Finally, the hydrolytic and nucleophile-trapping chemistry of reduced BI-A is discussed.

Chart 1



Chemistry

The synthesis of the new analogues in Chart 1 is discussed below in conjunction with Schemes 1–4. The synthesis of *i*-PBI-A was carried out in six steps starting with 1, Scheme 1. The Lewis-acid-catalyzed ring closure of 1 to 2 utilized the "*tert* amino effect,"[10] which relies on an internal redox reaction between a nitro group and an ortho-substituted tertiary amine. Previously, the PBI nucleus was prepared in high yield utilizing this effect.[2] Bromination of 2 afforded 3, which was nitrated to afford a mixture of 5- and 8-nitro isomers. One-pot reductive removal of the bromo group and nitro-group reduction, utilizing Pd on carbon and H_2, afforded 5 as a mixture of

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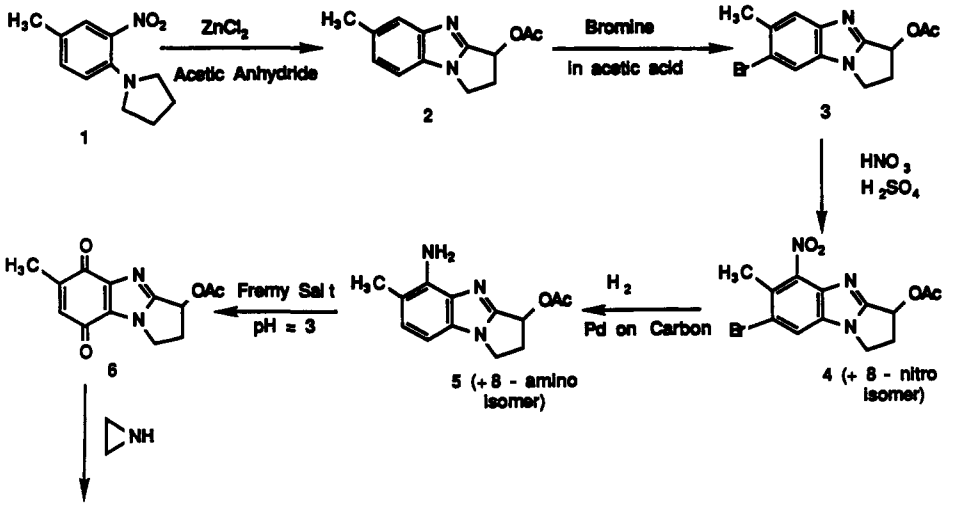
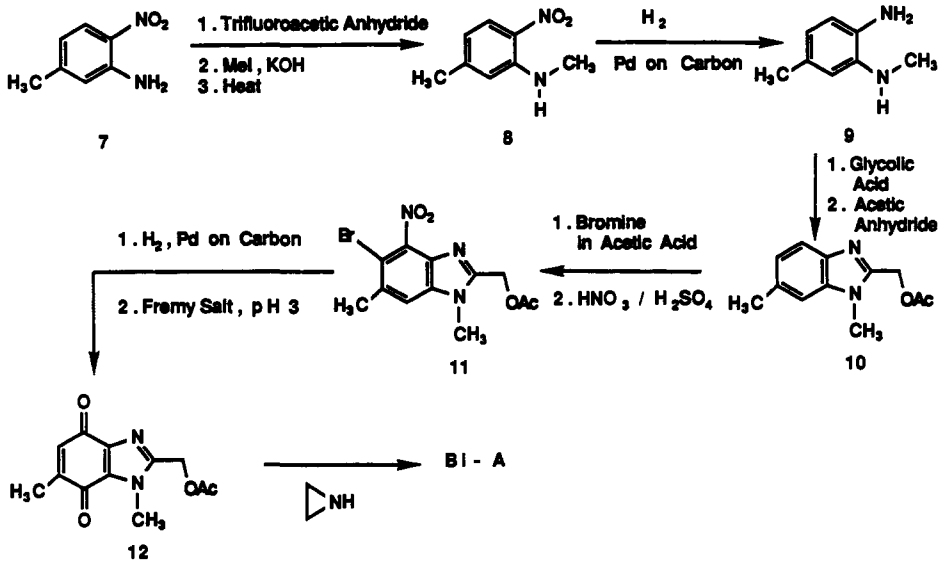
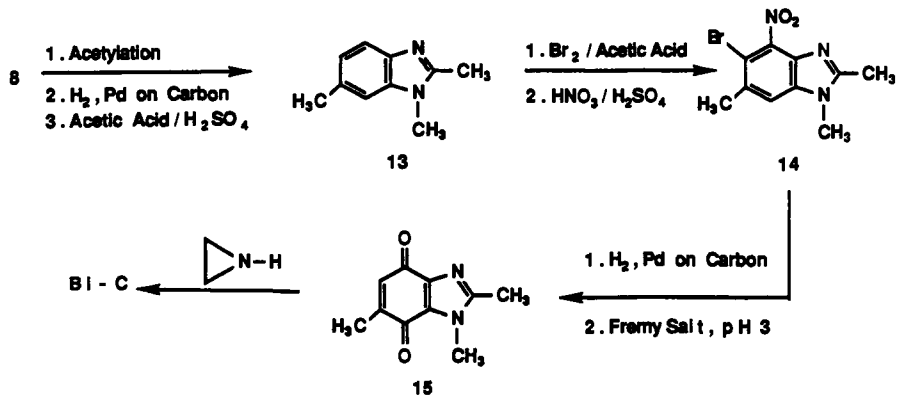
Scheme 1



Scheme 2



Scheme 3

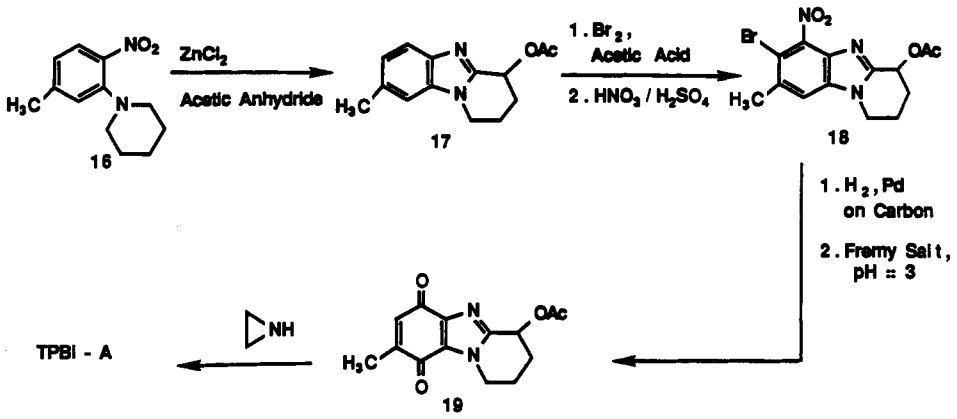


5- and 8-amino isomers. Oxidation of this mixture gave a single quinone, **6**, which was converted to *i*-PBI-A by reductive aziridination and oxidation.

The benzimidazole derivatives BI-A and BI-C were both prepared starting with **8**. Reduction of **8** followed by the Phillips reaction[11] and acetylation of the resulting alcohol afforded the benzimidazole intermediate **10** needed for the preparation of BI-A, Scheme 2. Acetylation of **8** followed by reduction and ring closure afforded the benzimidazole intermediate **13** needed for the preparation of BI-C, Scheme 3. Conversion of the intermediates **10** and **13** to the respective products was carried out utilizing known procedures.[2]

The synthesis of TPBI-A was carried out utilizing procedures previously reported for the synthesis of PBI analogues (Scheme 4).[2] A noteworthy observation is the instability of TPBI-A, even while in the solid form. The red colored TPBI-A becomes a purple solid after a week at room temperature. This solid is polymeric in structure and did not melt or possess a clear mass spectrum. In addition, the solid possesses paramagnetic properties. The PBI analogues similarly decompose in the solid state, but

Scheme 4



only after months at room temperature. In contrast, the BI analogues are stable indefinitely.

Cytotoxic Activity

The cytotoxic properties of the benzimidazole-based aziridinyl quinones described herein are discussed below in conjunction with Tables 1–4. These tables show the log LC_{50} mean graphs obtained by screening drugs against up to 60 human cancer cell lines (LC_{50} is the concentration of drug lethal to 50% of the cancer cells).[12,13] The center line of these graphs is the log of the mean LC_{50} value; bars to the right represent activity greater than the mean (lower log LC_{50} values) while bars to the left represent activity lower than the mean (higher log LC_{50} values). By comparing log LC_{50} mean graphs, rather than LC_{50} values in one or two cell lines, it is possible to make reliable structure–activity correlations.

All of the benzimidazole-based aziridinyl quinones are related to each other as far as the pattern of cytotoxic activity is concerned. Thus, all analogues are completely inactive against leukemia but show varying degrees of activity against lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, and renal cancer cell lines. Of all the cancer types, melanoma is the most sensitive to these quinones (except for cell line M19-MEL, which is nearly always resistant). However, the aziridinyl quinones differ with respect to potency such that the log LC_{50} values vary by as much as 2 orders of magnitude.

The change in the position of the aziridinyl group from C(6) to C(7) resulting in *i*-PBI-A is associated with a decrease in overall potency by approximately 1 order of magnitude, Table 1. The mean log LC_{50} values of PBI-A and *i*-PBI-A are −6.61[6] and −5.68, respectively. The diminished activity of *i*-PBI-A compared to that of PBI-A is evident in the log LC_{50} values of the following melanoma lines: LOX IMVI, −7.81 vs <-8; MALME-3M, −6.23 vs −7.26; and SK-MEL-5, −6.65 vs <-8.

Removing the pyrrolo ring from the PBI system to afford the BI system results in a slight decrease in overall potency, Table 2. The mean log LC_{50} of BI-A is −6.22 compared to −6.61 for PBI-A.[6] Against the melanoma line MALME-3M, for example, BI-A possesses a log LC_{50} of −7.18 compared to the value of −7.26 for PBI-A.

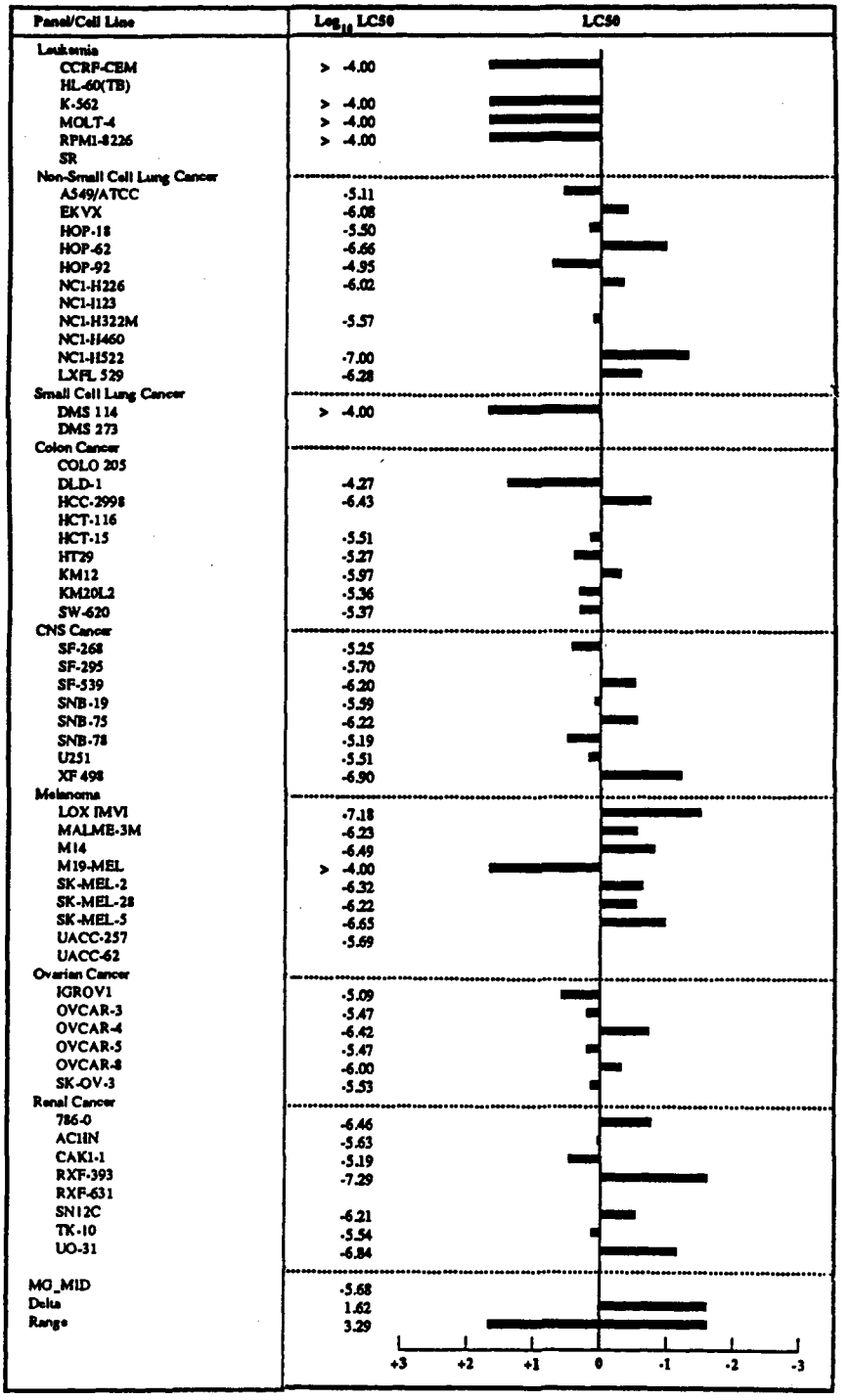
A comparison of Tables 2 and 3 reveals that the change from the 2-α-acetate derivative BI-A to the 2-α-unsubstituted derivative BI-C is accompanied by a significant decrease in potency (mean log LC_{50} values are −6.22 and −5.76 for BI-A and BI-C, respectively). The differences are most apparent in the melanoma cell lines. The acetate group probably contributes to BI-A activity by increasing lipophilicity rather than by acting as a leaving group. In fact, a study of the hydrolytic chemistry of reduced BI-A revealed that acetate elimination does not occur (see Reductive Activation of BI-A). Consistent with this finding, the results of our previous study[3] indicated that the 3-leaving group (analogous to the 2-α group of BI-A) was not essential for PBI cytotoxic activity. A comprehensive study of the influence of the lipophilicity of the 3-substituent on PBI cytotoxic activity is currently underway.

Homologating the pyrrolo ring of PBI-A by one carbon to afford TPBI-A results in a substantial loss in cytotoxic activity (mean log LC_{50} of TPBI-A in Table 4 is −5.04 and that of PBI-A is −6.61). The loss in activity is most apparent in the melanoma cell lines. Against the melanoma cell line MALME-3M, for example, TPBI-A possesses a log LC_{50} of only −5.63 while PBI-A possesses a log LC_{50} of −7.26.

From the foregoing results, the following generalizations concerning the cytotoxicity of the benzimidazole-based aziridinyl quinones can be made. Removal of the pyrrole ring (PBI → BI) has little effect on cytotoxic activity while homologating this ring (PBI-A → TPBI-A) results in a substantial loss in activity. Moving the aziridinyl group from position C(6) to C(7) also results in an activity loss. The loss of cytotoxic activity accompanying pyrrolo-ring homologation is no doubt due to the instability of the resulting analogue. Indeed, solutions of TPBI-A last for only a few hours, and even the solid decomposes over a period of days. The pyrrolo ring is clearly not required for antitumor activity, as is evident from the cytotoxic activity of BI-A. The loss of cytotoxicity seen in the i-PBI-A analogue (compared to PBI-A) may be due to the change in the alkylating center position (see DNA–drug interactions).

The National Cancer Institute has developed the COMPARE program[13] to search for similarities and differences between cancer drugs. Generally, compounds with identical cytotoxicity mechanisms possess nearly identical mean graphs. Thus, excellent correlations have been obtained between anthracycline analogues and between alkylating agents. The log LC_{50} mean graphs of the aziridinyl quinones *i*-PBI-A, TPBI-A, and BI-A were compared with the log LC_{50} mean graphs of known antitumor agents. There are no excellent correlations (correlation coefficients >0.9) obtained from these comparisons. However, the known antitumor agents shown

Table 1.



in Chart 2 appear to possess mechanistic similarities to the aziridinyl quinones.

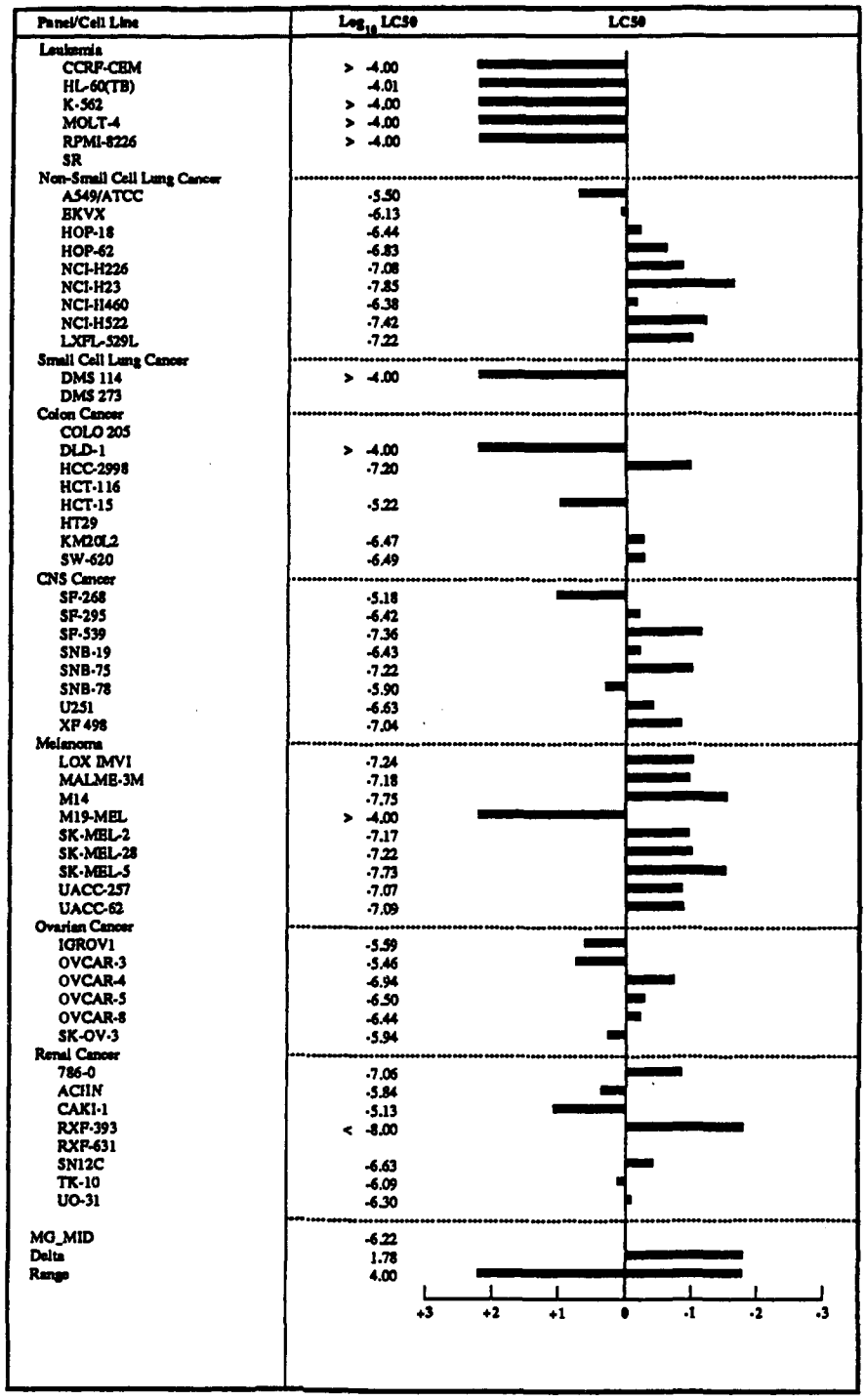
Pyrazoloacridine antitumor activity correlates with TPBI-A and *i*-PBI-A antitumor activity with coefficients of 0.647 and 0.707, respectively. These correlations may be due to the features that pyrazaloacridine has in common with the aziridinyl quinones, such as a reductive activation requirement (i.e., hypoxic selectivity) and DNA-cleavage capability. Bisantrene is another DNA-cleaving agent[15] whose antitumor activity correlates with that of BI-A (correlation coefficient is 0.624). All of the aziridinyl quinone mean graphs correlate to a degree with those of the anthracyclines (correlation coefficient range 0.6–0.7), which are known to cleave DNA upon reductive activation.[16] The aziridinyl quinone mean graphs also correlate well with those of alkylating agents (correlation coefficient range 0.50–0.74) such as piperazinedione[15] and triethylenemelamine (NSC 9706).

In conclusion, the COMPARE study results are consistent with the aziridinyl quinone cytotoxicity mechanism: reduction activation followed by DNA alkylation and cleavage.

Reductive Activation of BI-A

The consequences of 2-electron reduction of BI-A in anaerobic aqueous 0.05 M pH 7.4 tris buffer were studied in the presence and absence of added nucleophiles. In aqueous buffer, reduced BI-A behaves much like a reactive quinone methide species and traps both protons and nucleophiles.[8] In the presence of added nucleotide species

Table 2.

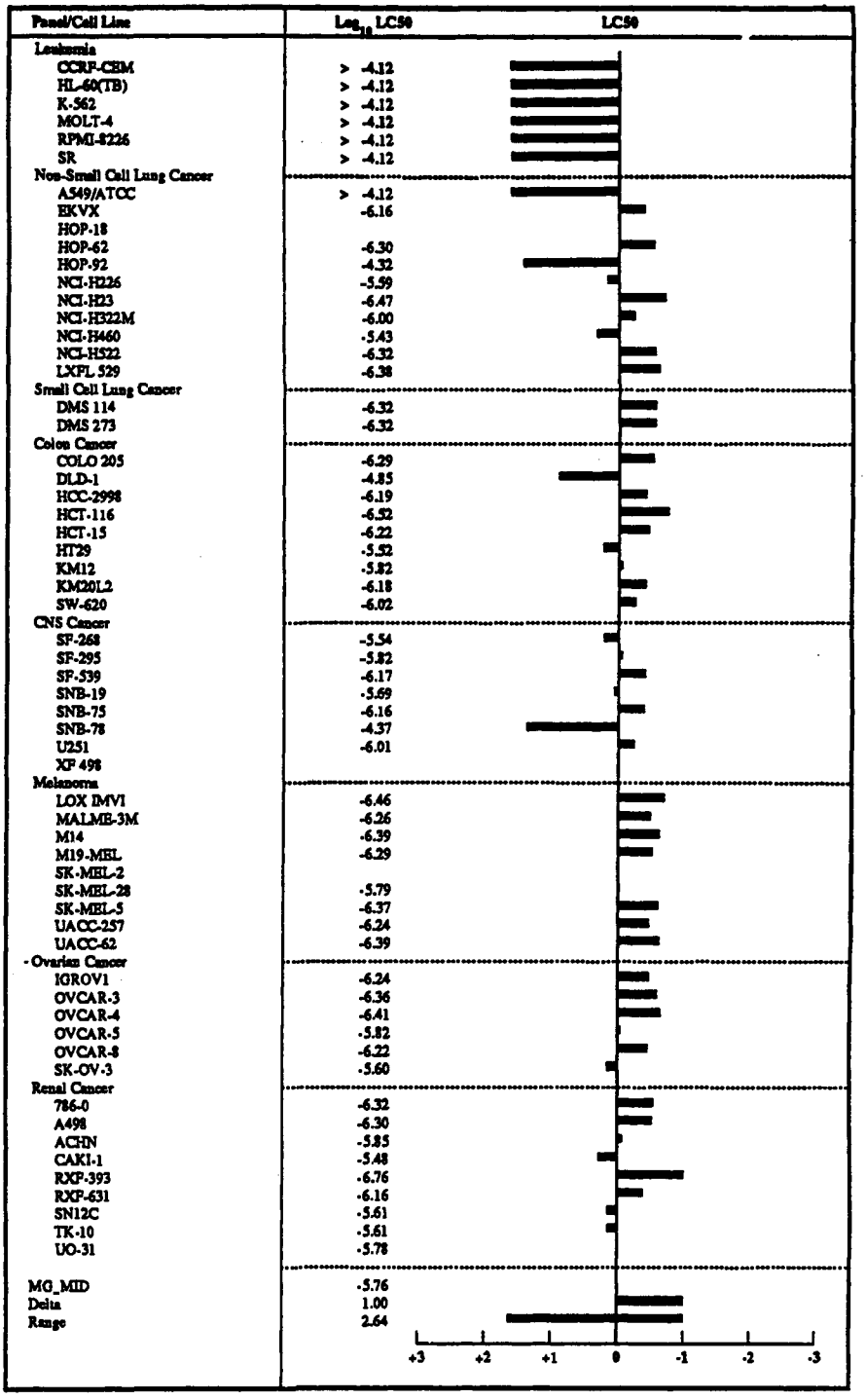


(5′-dAMP and calf thymus DNA), significant amounts of nucleophile trapping by reduced BI-A were observed. These nucleophilic trapping reactions may explain the cytotoxicity of benzimidazole-based aziridinyl quinones.

The hydrolysis of reduced BI-A in anaerobic pH 7.4 buffer is discussed below in conjunction with Scheme 5. Under strict anaerobic conditions, in the absence of added nucleophiles, reduced BI-A traps a proton to afford **20** and **21** and also traps water to afford **22**. The mechanism of proton trapping by the aziridinyl group is illustrated in the inset of Scheme 5. The buildup of the blue quinone **20** was readily apparent during the anaerobic incubation. Upon aerobic workup of the reaction, the oxidation of hydroquinones **21** and **22** occurred to afford a deep blue solution. A preparative reaction of reduced BI-A afforded the following yields of proton- and water-trapping products: **20**, 9.1%; **23**, 37.7%; and **24**, 26.7%. A small amount of deacetylated **23** was detected in the reaction mixture by a mass spectral measurement. The origin of the deacetylated **23** was considered to be from hydrolysis rather than from water trapping by the quinone methide species shown in Scheme 5. The presence of a quinone methide intermediate was dismissed since proton-trapping products (2-methylbenzimidazole analogues) were absent from the reaction mixture. Previous studies in this laboratory showed that a benzimidazole-based quinone methide, structurally similar to the one shown in Scheme 5, is an excellent proton trap.[8]

From the results of the BI-A hydrolysis study, it is apparent that all the nucleophile- and proton-trapping

Table 3.

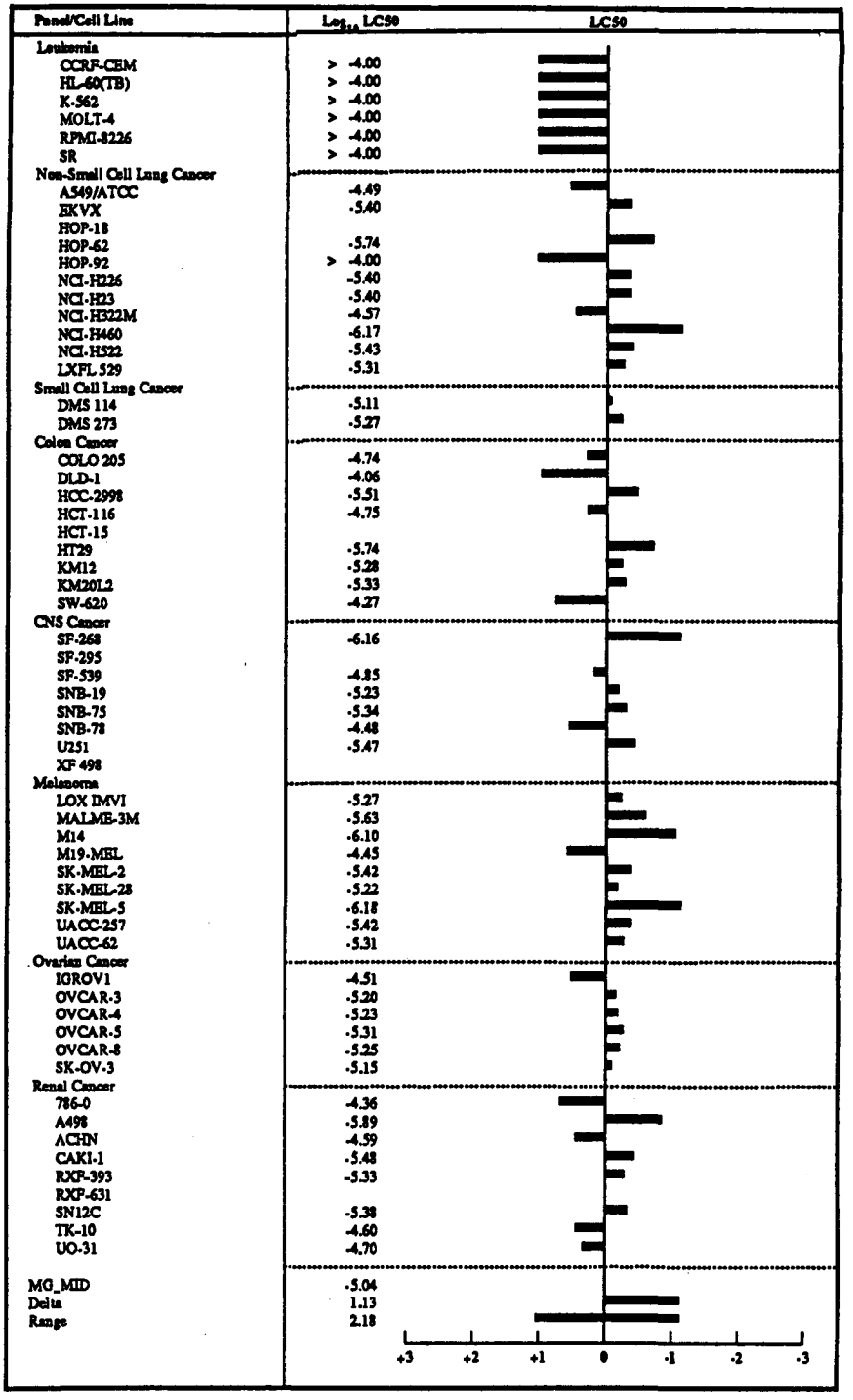


reactions involve the aziridinyl group and that quinone methide formation does not occur. In fact, previous structure–activity studies of the PBI antitumor agents revealed that the aziridinyl group, and not the leaving group, is crucial for antitumor activity.

When 600-bp calf thymus DNA was incubated with reduced BI-A, a blue BI-A–DNA adduct was obtained upon aerobic workup of the reaction. The formation of this adduct occurred at the expense of hydrolysis products, which were obtained in low yield: **20**, 1.9%, **23**, 8%; and **24**, 5.6%. The blue chromophore is the amino quinone moiety formed upon nucleophile trapping by the aziridinyl hydroquinone followed by air oxidation to the quinone. Removal of the chromophore was not possible by precipitating the DNA or by repeated washing with ethanol. Shown in Figure 1 are UV–visible spectra of the blue DNA adduct and the untreated DNA. These findings indicate that the reduced BI-A forms a stable adduct with DNA. Previous studies with the PBI system also provided evidence of a stable blue DNA adduct.[6]

The structures of the BI-A– and PBI–DNA adducts are still under investigation. Evidence suggests that the alkylation site could be at oxygens of the phosphate backbone, although the formation of N(7)-purine adducts has not been entirely dismissed. Indeed, aziridinyl quinones are known to alkylate DNA at the N(7)-center of purine bases.[18] The resulting adducts readily hydrolyze to afford depurinated DNA.[19] In contrast, phosphate oxygen alkylation products are relatively stable to hydrolysis.[20] Heating the BI-A–DNA adduct in pH 7.4 tris buffer at 50

Table 4.



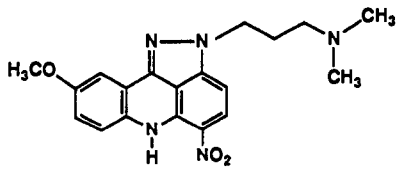
°C over a period of hours afforded quinone hydrolysis products which did not show purine aromatic protons in their ^{1}H NMR spectra. Thus, alkylation of the DNA phosphate backbone by PBIs and BI-A is considered a possibility. In fact, ethylnitrosourea[21] and alkyl methanesulfonates[20] can alkylate oxygens of the DNA phosphate backbone.

DNA alkylation by alkyl methanesulfonates actually can occur at both the N(7)-center and the phosphate backbone. The selectivity of these electrophiles for nucleophilic positions on DNA is thought to be determined by the alkylation mechanism: S_N1-type processes result in phosphate oxygen alkylation whereas S_N2 type processes result in N(7) alkylation.[20] The reaction of reduced BI-A with 5′-dAMP in anerobic buffer afforded the phosphate oxygen alkylation product **25** upon aerobic workup. The only other products obtained from the reaction mixture were formed by hydrolysis of reduced BI-A (**20**, **23**, and **24**). Perhaps the protonated aziridinyl group of reduced BI-A develops some carbocation character in the course of alkylation and therefore shows an S_N1 alkylation product (i.e., phosphate alkylation).

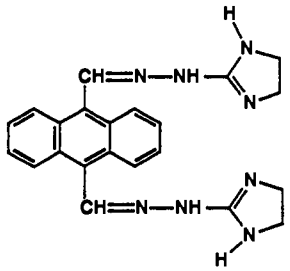
DNA Cleavage by Reduced BI-A

Previous studies showed that the PBIs cleave DNA at G and A base upon reductive activation.[6] Reduced BI-A likewise cleaved DNA at G and A bases as illustrated in Figure 2 with a ^{32}P-3′-end-labeled *Cla*I/*Rsa*I pBR322 restriction fragment. Cleavage was obtained by brief

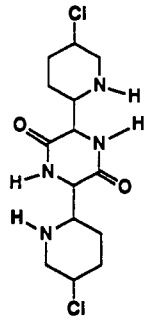
Chart 2



Pyrazoloacridine, NSC 3666140



Bisantrene, NSC 337766



Piperazinedione, NSC 135758

treatment of DNA with reduced BI-A (~30 min) followed by isolation of the treated DNA under aerobic conditions and then heat treatment with basic gel-loading solution (see Experimental Section). This figure also shows that oxidized BI-A and dithionite alone do not cause significant cleavage of DNA. The cleavage results shown in Figure 2 could be interpreted in terms of phosphate backbone or N(7) alkylation.

According to the phosphate backbone alkylation mechanism, trapping of the phosphate oxygen anion by reduced BI-A (Scheme 6) affords a phosphotriester. Aerobic workup of the alkylation reaction then provides a blue DNA adduct (see Figure 1). Finally, hydrolysis of this adduct in base results in cleavage of the DNA at phosphotriester linkages (see Figure 2). Base-catalyzed phosphotriester cleavage of ENU-treated DNA is in fact used in footprinting studies.[21] The G + A specificity is explained by complexation of reduced BI-A in the major groove by hydrogen bonding to G and A bases, resulting in placement of the aziridinyl group proximal to the phosphate backbone. Molecular models with a PBI analogue showed that major-groove binding in this fashion could result in phosphate backbone alkylation.[6] The binding of *i*-PBI-A in the major groove, on the other hand, would not favor alkylation since the aziridinyl group is further away from the phosphate backbone. Perhaps this feature is responsible for the large decrease in cytotoxicity observed on going from the PBI to the *i*-PBI system.

In the absence of oxidation, the hydroquinone form of the adduct can facilitate backbone cleavage by internal nucleophilic attack, Scheme 6. It is known that the hydroxyethyl phosphotriester of DNA[22] and even amine-containing phosphodiesters[23] are hydrolytically labile under mild conditions due to internal nucleophilic attack. This mechanism could account for the cytotoxicity observed in cell lines (Tables 1–4). Cellular reductive activation[24] would be followed by phosphodiester backbone alkylation and cleavage. In fact, PBI-A treatment of myeloma cells resulted in DNA single-strand cleavage.[3]

The classic N(7) alkylation DNA-cleavage process (i.e., the Maxam and Gilbert G ladder[25]) also seems to be a reasonable mechanism for PBI- and BI-mediated DNA cleavage. Other aziridinyl quinones are known to alkylate DNA at N(7) and bring about G + A cleavage.[18] Unlike the N(7)-alkylating agents, however, the PBI- and BI-DNA adducts do not release the purine adducts upon heating. Indeed, this property has been used in the past to distinguish between N(7) and phosphate alkylation products.[20] Therefore PBI- and BI-DNA adducts are considered to be mainly phosphotriesters, although the formation of some N(7) alkylation products has not been rigorously excluded.

Conclusions

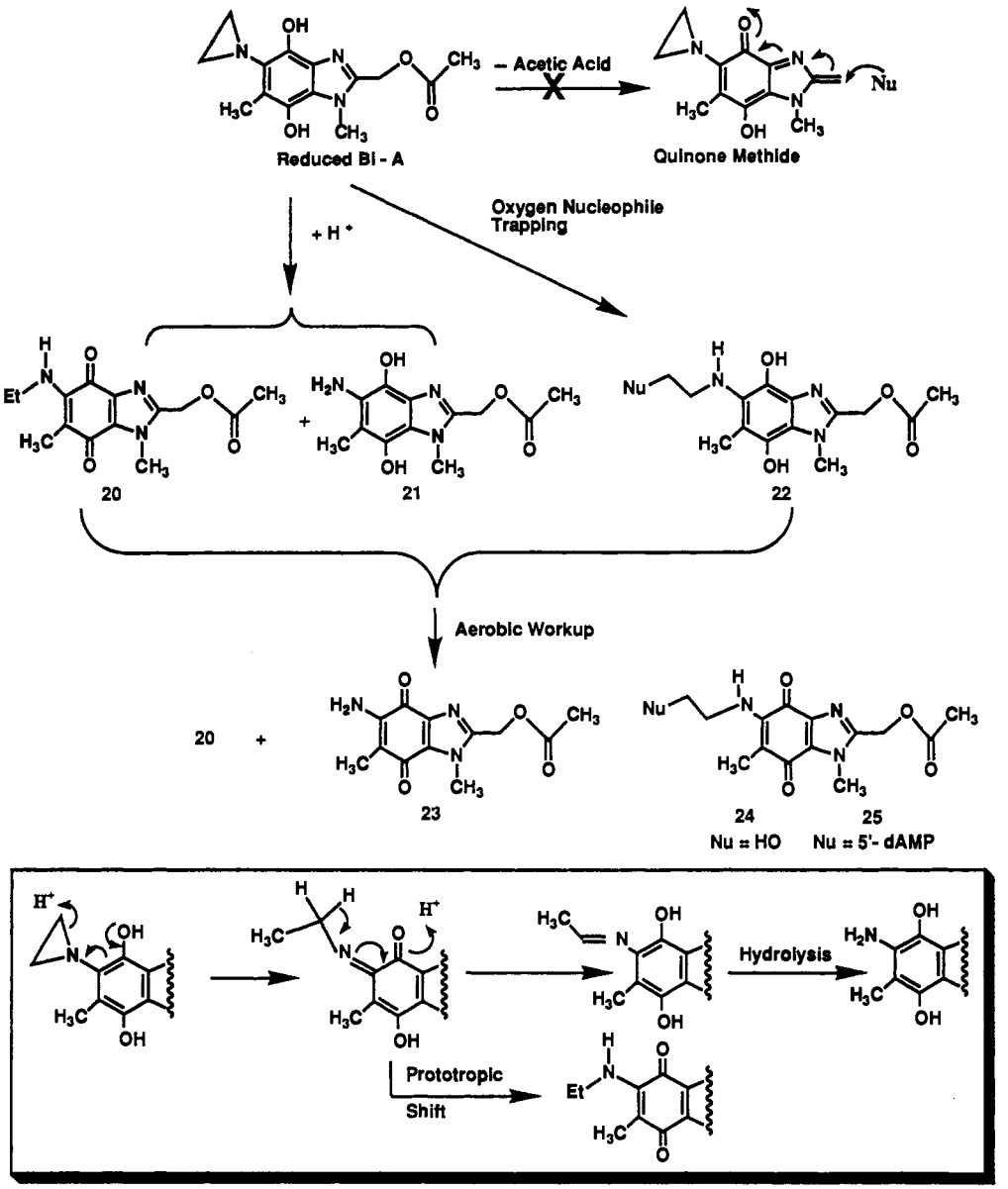
The cytotoxicity of benzimidazole-based DNA-cleaving agents was compared in 60 cancer cell lines. The pyrrolo ring is not essential for cytotoxic activity and thus the benzimidazole (BI) and pyrrolobenzimidazole (PBI) analogues have similar levels of potency. Homologating the pyrrolo ring by one carbon to afford the tetrahydropyrido (TPBI) analogue reduces cytotoxic potency by 2 orders of magnitude on the average. This effect may be due to the instability of the TPBI system brought about by ring strain. Moving the aziridinyl group from the 6- to the 7-position to afford the *i*-PBI system decreases the cytotoxic potency by 1 order of magnitude on the average. This effect could be due to the greater distance of the aziridinyl-alkylating center from DNA nucleophiles in the *i*-PBI–DNA complex.

COMPARE studies revealed that the benzimidazole-based DNA-cleaving agents are unlike clinically used antitumor agents with respect to their cytotoxicity profile. This observation could be due to a unique mechanism of cytotoxicity (phosphate backbone alkylation) exerted by these agents. A recently discovered aziridinyl quinone cytotoxic agent EO9 (NSC 382459)[26] in fact has a similar cytotoxicity profile to the benzimidazole-based DNA-cleaving agents. Since EO9 possesses some structural similarities to the agents discussed herein, both classes of antitumor agents may have a similar cytotoxicity mechanism.

In order to assess the reactivity of benzimidazole-based DNA-cleaving agents, the hydrolytic chemistry of reduced BI-A was studied in aqueous buffer. All the reactivity centers around the aziridinyl ring, which can trap a proton or a nucleophile. It was demonstrated that sonicated calf thymus DNA and The phosphate oxygen anion of 5′-dAMP can act as nucleophiles in this reaction. These nucleophilic reactions could be involved in DNA cleavage as well as in the cytotoxicity exhibited by the benzimidazole-based systems presented herein.

Finally, it was demonstrated that BI-A cleaves DNA at G and A bases upon reduction. Postulated cleavage mechanisms include N(7) alkylation followed by depurination/phosphate backbone cleavage and phosphate alkylation followed by hydrolytic phosphate backbone cleavage. The hydrolytic stability of the BI-A–DNA adduct and the absence of depurination products suggest that phosphate

Scheme 5



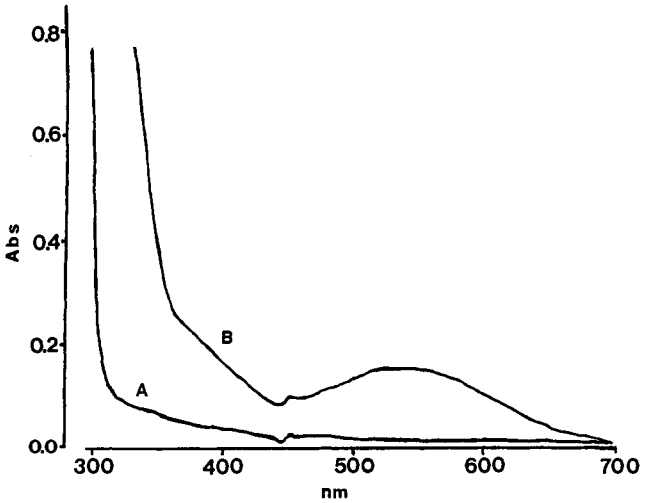


Figure 1. Absorbance (Abs) versus wavelength (nm) plots for sonicated calf thymus DNA (A) and for the same DNA treated with reduced BI-A (B).

alkylation could be occurring. The N(7) alkylation mechanism has not been rigorously excluded, however. Currently, this research group is investigating the DNA adduct structure, employing ^{31}P and ^{1}H NMR spectroscopic studies. Our findings will be reported in the coming months.

Experimental Section

All analytically pure compounds were dried under high vacuum in a drying pistol heated with refluxing methanol. Some compounds contained water of crystallization that was determined from the elemental analyses found. Elemental analyses were run at Atlantic Microlab, Inc., Norcross, GA. Uncorrected melting and decomposition points were determined with a Mel-Temp apparatus. All TLC was run with silica gel plates with fluorescent indicator, employing a variety of solvents. IR spectra were taken as KBr pellets or thin films; the strongest IR absorbances are reported. ^{1}H NMR spectra were obtained on a 300-MHz spectrometer, and chemical shifts are reported relative to TMS.

Preparation of New Compounds and Their Physical Properties. 4-(*N*-Pyrrolidino)-5-nitrotoluene (1). A mixture consisting of 17.3 g (80 mmol) of 4-bromo-5-nitrotoluene and 17 g (240 mmol) of pyrrolidine was heated at reflux for 3 h. The cooled reaction mixture was poured over 400 g of cracked ice, and the resulting mixture was extracted three times with 200-mL portions of chloroform. The dried extracts (Na_2SO_4) were concentrated to an oily residue, which was placed on a silica gel flash column. The product was eluted with hexane/chloroform

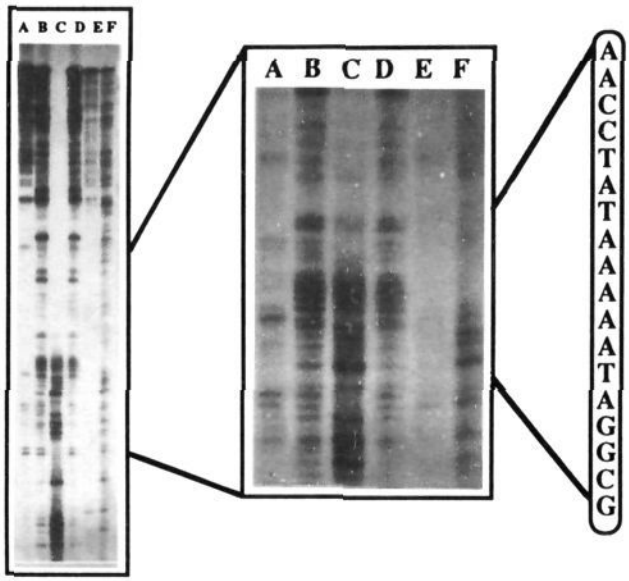


Figure 2. Autoradiogram of an 8% polyacrylamide/7 M urea slab gel (far left) of the *Cla*I/*Rsa*I restriction fragment of pBR322 which had been subjected to the following treatments: (A) treatment with 4.5 mM dithionite under anaerobic conditions, (b) treatment with dithionite-reduced 0.97 mM BI-A, (C) *treatment with dithionite-reduced 1.93 mM BI-A (dithionite-*reducing agent was added twice—at the beginning of the incubation and after 18 min of incubation, resulting in more DNA cleavage), (D) treatment with dithionite-reduced 3.8 mM BI-A, and (E) treatment with aerobic 3.8 mM BI-A. The above treatments were carried out in 0.05 M pH 7.4 tris buffer in the presence of the end-labeled restriction fragment (10 000 cpm) and ~1 ng of cold DNA (pBr322). The reaction was deaerated with argon followed by the addition of 1 μL of a 34 mM dithionite solution in the buffer mentioned above. The reaction mixture was incubated at 37 °C for 30 min, and the DNA pellet was isolated by precipitation and centrifugation and then denatured by heating in a basic gel-loading buffer (for details, see ref 6). In the center is a blowup of part of the autoradiogram and to the right is a partial sequence.

(50:50). Evaporation of the eluant afforded an orange oil: 15.2 g (92%); TLC (chloroform) R_f = 0.61; IR (thin film on NaCl) 2690, 2600, 1626, 1553, 1522, 1496, 1367, 1336, 1269, 1181 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.56 (1 H, s, C(6)-proton), 7.195 and 6.83 (2 H, ABX system, J_{ortho} = 8.64 Hz, J_{meta} = 2.1 Hz, J_{para} = 0 Hz, C(2)- and C(3)-protons), 3.19 (4 H, m, pyrrolidine methylene adjacent to nitrogen), 2.28 (3 H, s, methyl), 1.97 (4 H, m, other pyrrolidine methylenes). Anal. ($C_{11}H_{14}N_2O_2 \cdot 1^1/_3H_2O$) C, H, N.

6-Methyl-3-acetoxy-2,3-dihydro-1*H*-pyrrolo[1,2-α]benzimidazole (2). A mixture consisting of 4.05 g (19.66 mmol) of 1, 2.72 g (20 mmol) of anhydrous $ZnCl_2$, and 20 mL of acetic *anhydride was stirred at 90–100 °C for 5 h (or until the starting* material was no longer seen by TLC). The excess acetic anhydride was evaporated *in vacuo*, and the black residue was dissolved in 100 mL of chloroform. The chloroform solution was washed with water and dried over sodium sulfate. Purification by silica gel chromatography, using chloroform/hexane (90:10) as the eluant, afforded the product as a white-colored solid: 2.11 g (46%); mp 203–205 °C; TLC (chloroform/methanol, 90:10 R_f = 0.65; IR (KBr pellet) 1746, 1537, 1494, 1371, 1290, 1266, 1248, 1081, 1052, 800 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.58 (1 H, br s, C(5)-proton), 7.33 and 7.03 (2 H, ABX system, J_{ortho} = 8.2 Hz, $J_{meta} \simeq$ 0 Hz, J_{para} = 0 Hz, C(7)- and C(8)-protons), 6.18 (1 H, dd, J = 7.4 Hz, J = 3.21 Hz, C(3)-proton), 4.25 and 4.12 (2 H, 2 m, C(1)-diastereomeric methylene), 3.18 and 2.65 (2 H, 2 m, C(2)-diastereomeric methylene), 2.48 (3 H, s, C(5)-methyl), 2.13 (3 H, s, acetate methyl); mass spectrum (EI mode) m/z 230 (M^+), 187 (M^+ – acetyl). Anal. ($C_{13}H_{14}N_2O_2$) C, H, N.

7-Bromo-6-methyl-3-acetoxy-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]benzimidazole (3). To a solution of 1.45 g (6.33 mmol) of 2 in 70 mL of glacial acetic acid, heated at 100 °C, was added 333 μL of bromine in 3 mL of glacial acetic acid. After the addition, the reaction mixture was heated at 100-110 °C for 4 h. The cooled reaction mixture was diluted with 40 mL of water and then neutralized to pH 6.5 with aqueous sodium bicarbonate. The product crystallized from the solution as a white solid; the yield upon drying the collected solid was 1.65 g (84%). Recrystallization from chloroform/hexane afforded analytically pure material: mp 196 °C; TLC (chloroform/methanol, 90:10) R_f = 0.64; IR (KBr pellet) 1748, 1535, 1443, 1432, 1371, 1289, 1249, 1082, 1054, 850 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.65 and 7.60 (2 H, 2 s, aromatic protons), 6.17 (1 H, dd, J = 7.5 Hz, J = 3.3 Hz, C(3)-proton coupled with C(2)-methylene), 4.25 and 4.11 (2 H, 2 m, C(1)-diastereomeric methylene), 3.19 and 2.67 (2 H, 2 m, C(2)-diastereomeric methylene), 2.51 (3 H, s, C(6)-methyl), 2.13 (3 H, s, acetate methyl); mass spectrum (EI mode) m/z 308 and 310 (M^+, ^{79}Br and ^{81}Br), *265 and 267 (M^+ – acetyl).* Anal. ($C_{13}H_{13}BrN_2O_2$) C, H, N.

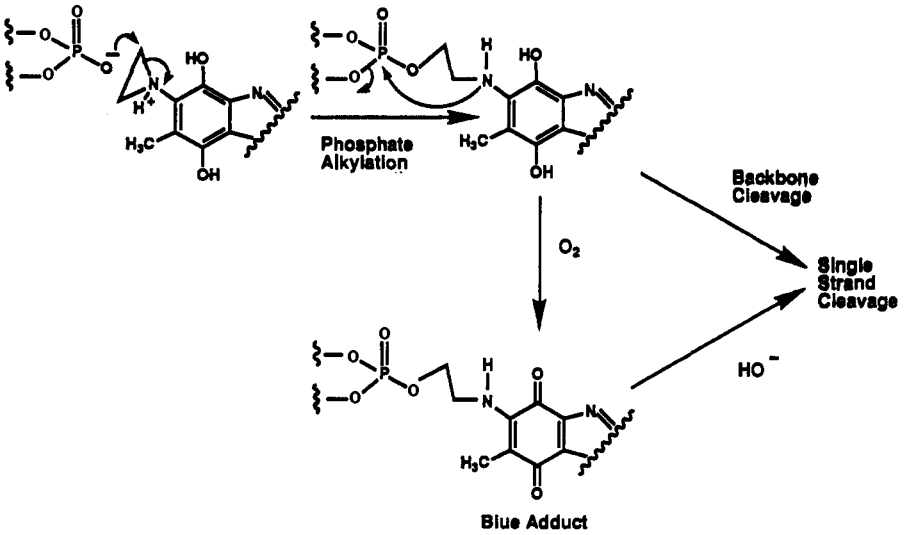
6-Methyl-5(and 8)-amino-3-acetoxy-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]benzimidazole (5) was prepared by the following 2-step procedure. To a mixture of 9 mL of fuming nitric acid and 1 mL of concentrated sulfuric acid, chilled in an acetone–dry ice bath, was added 1 g (3.23 mmol) of 3. The reaction mixture was stirred in an acetone–dry ice bath for 15 min and then allowed to warm to room temperature over a 30-min period. The reaction mixture was poured over 100 g of cracked ice and the resulting mixture adjusted to pH 7 with aqueous sodium bicarbonate. The mixture was then extracted three times with 50-mL portions of chloroform. Drying the combined chloroform extracts (Na_2SO_4) followed by concentration afforded 4 as a yellow solid. Recrystallization was carried out from chloroform/hexane: 945 mg (82%); mass spectrum (EI mode) m/z 353 and 355 (M^+, ^{79}Br and ^{81}Br); ^{1}H NMR showed the presence of two compounds.

A suspension of 350 mg (0.98 mmol) of the product obtained above in 100 mL of methanol was shaken under 50 psi of H_2 in the presence of 50 mg of 5% Pd on carbon for 8 h. The reaction mixture was filtered through Celite into a flask containing a few drops of 1 N HCl. Evaporation of the filtrate to dryness afford the dihydrochloride salt of the products. Recrystallization was carried out from ethyl acetate/methanol: 250 mg (81%); mass spectrum (EI mode) m/z 245 (M^+).

6-Methyl-3-acetoxy-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]benzimidazole-5,8-dione (6). To a suspension of 240 mg (0.75 mmol) of 5 in 10 mL of water containing 100 mg of monobasic potassium phosphate was added a solution of 1.5 g of Fremy's salt in 50 mL of water containing 500 mg of monobasic potassium phosphate. The reaction mixture was stirred at room temperature for 2 h, and then, it was extracted five times with 30-mL portions of chloroform. The dried extracts (Na_2SO_4) were concentrated to a brown residue, which was flash chromatographed on a silica gel column, employing chloroform as the eluant. Evaporation of the eluants afforded the pure product, which was recrystallized from ethyl acetate: 100 mg (51%); mp 136 °C; TLC (chloroform/methanol, 90:10) R_f = 0.59; IR (KBr pellet) 1738, 1675, 1665, 1655, 1514, 1375, 1269, 1228, 1027, 980 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.49 (1 H, q, J = 1.6 Hz, C(7)-proton split by methyl), 6.10 (1 *h, dd, J = 7.65 Hz, J = 3.09 Hz, C(3)-proton), 4.35 (2 H, m,* C(1)-diastereomeric methylene), 3.17 and 2.65 (2 H, 2 m, C(2)-diastereomeric methylene), 2.15 (3 H, d, J = 1.6 Hz, ((6)-methyl), 2.13 (3 H, s, acetate methyl); mass spectrum (EI mode) m/z 260 (M^+), 217 (M^+ – acetyl). Anal. ($C_{13}H_{12}BrN_2O_4$) C, H, N.

7-(*N*-Aziridinyl)-6-methyl-3-acetoxy-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]benzimidazole-5,8-dione (*i*-PBI-A). To a solution of 50 mg (0.19 mmol) of 6 in 5 mL of dry methanol was added 200 μL of ethylenimine. The reaction mixture was stirred at room temperature for 4 h. The solvent was then removed *in vacuo*, and the red-colored residue was flash chromatographed on silica gel, using chloroform as the eluant. The purified product was recrystallized with chloroform/hexane: 24 mg (41%); mp 75–76 °C; TLC (chloroform/methanol, 90:10) R_f = 0.51; IR (KBr pellet) 1746, 1668, 1649, 1528, 1377, 1343, 1247, 1228, 1144, 1080 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.06 (1 H, dd, J = 7.6 Hz, J = 2.94, C(3)-proton), 4.32 (2 H, m, C(1)-diastereomeric methylene), 3.16 and 2.63 (2 H, 2 m, C(2)-diastereomeric methylene), 2.31 (4 H, s, aziridine protons), 2.12 and 2.09 (6 H, 2 s, C(6)-methyl and acetate methyl); mass spectrum (EI mode) m/z 301 (M^+), 258

Scheme 6



(M^+ -acetyl), 241 (M^+ - acetic acid). Anal. ($C_{15}H_{15}N_3O_4 \cdot 0.25 H_2O$) C, H, N.

3-(Methylamino)-4-nitrotoluene (8). To a solution of 2.84 g (18.68 mmol) of 7 in 20 mL of trifluoroacetic acid was added 3 mL of trifluoroacetic anhydride. The reaction mixture was stirred at room temperature for 1 h and then poured over cracked ice. Collection of the resulting precipitate by filtration followed by washing with water and drying *in vacuo* gave the N-trifluoroacetylated derivative of 7.

The entire amount of the trifluoroacetylated product was added to a mixture consisting of 3.4 mL of methyl iodide, 3.08 g of KOH, and 100 mL of acetone. The reaction mixture was refluxed for 8 h, and then, the liquor was decanted from the solids while still hot. Concentration of the liquor afforded a red oil, which was triturated with 50 mL of benzene. The resulting solid was filtered off and discarded. The filtrate was concentrated again to an oil, which was combined with 25 mL of hexane. The mixture was chilled to afford a red-colored crystalline mass. Chromatography was carried out on silica gel, using 80:20 chloroform/hexane, to obtain the pure N-methylated compound: 1.84 g (60%) based on 7; mp 69 °C; TLC (chloroform) R_f = 0.4; IR (KBr pellet) 3380, 1626, 1584, 1502, 1406, 1339, 1332, 1266, 1225, 1212, 1186 cm^{-1}; ^1H NMR (dimethyl sulfoxide-d_6) δ 8.17 (1 H, br q, amine proton), 7.96 and 6.51 (2 H, ABX system, J_{ortho} = 8.7 Hz, J_{meta} = 1.68 Hz, J_{para} = 0 Hz, C(5)- and C(6)-proton), 6.79 (1 H, s, C(2)-proton), 2.95 (3 H, d, J = 5.4 Hz, N-CH_3), 2.32 (3 H, s, methyl); mass spectrum (EI mode) m/z 166 (M^+). Anal. ($C_8H_{10}N_2O_2$) C, H, N.

3-(Methylamino)-4-aminotoluene (9). A solution of 1 g (6.02 mmol) of 8 in 100 mL of methanol was shaken under 50 psi in the presence of 200 mg of 5% Pd on carbon for 1.5 h. The catalyst was removed by filtration of the reaction mixture through Celite, and then, 1 mL of concentrated HCl was added to the filtrate. The filtrate was evaporated *in vacuo* to a white residue, which was recrystallized from methanol/ethyl acetate to afford 9 as the dihydrochloride salt: 0.94 g (75%); mp 193 °C dec; TLC (chloroform/methanol, 80:20) R_f = 0.57; IR (KBr pellet) 3400, 2923, 2892, 2817, 1637, 1525 cm^{-1}; ^1H NMR (dimethyl sulfoxide-d_6) δ 7.06 and 6.59 (2 H, ABX system, J_{ortho} = 8.2 Hz, $J_{meta} \simeq 0$ Hz, J_{para} = 0 Hz, C(5)- and C(6)-protons), 6.66 (1 H, br s, C(2)-proton), 2.74 (3 H, s, N-CH_3), 2.52 (3 H, s, methyl); mass spectrum (EI mode) m/z 136 (M^+), 121 ($M^+ - CH_3$).

2-(Acetoxymethyl)-1,6-dimethylbenzimidazole (10) was prepared by the following 2-step procedure. 2-(Hydroxymethyl)-1,6-dimethylbenzimidazole was prepared by refluxing for 4 h a mixture consisting of 7.0 g (0.0335 mol) of the dihydrochloride salt of 9, 12 g (0.134 mol) of 85% glycolic acid, and 50 mL of 4 N hydrochloric acid. The reaction mixture was then cooled to room temperature and the pH adusted to 6.5 with sodium bicarbonate, resulting in crystallization of the crude benzimidazole product, 6.6-g (~100%) yield. A pure sample was prepared by dissolution of the compound in a small amount of chloroform followed by the addition of hexane: mp 183–185 °C; TLC (chloroform/methanol, 90:10) R_f = 0.28; IR (KBr pellet) 3436, 3205, 3147, 1477, 1399, 1335, 1221, 1040, 864, 813 cm^{-1}; ^1H NMR (dimethyl sulfoxide-d_6) δ 7.01 and 7.46 (2 H, ABX, J_{ortho} = 8.16 Hz, J_{meta} = 1.5 Hz, J_{para} = 0 Hz, C(4)- and C(5)-aromatic protons), 7.32 (1 H, br s, C(7)-aromatic proton), 4.68 (2 H, s, hydroxymethyl), 3.78 (3 H, s, N(1)-methyl), 2.44 (3 H, s, C(6)-methyl); mass spectrum (EI mode) m/z 176 (M^+), 159 (M^+ - OH).

To a suspension of 1.79 g (10.16 mmol) of the benzimidazole product in 100 mL of dry methylene chloride was added 1.06 mL (12 mmol) of acetic anhydride and 0.96 mL (12 mmol) of pyridine. The resulting mixture was refluxed for 9 h. The solvents were then evaporated *in vacuo*, and the solid residue (10) was recrystallized from chloroform/hexane: 2.17 g (98%); mp 104–106 °C; TLC (chloroform/methanol, 90:10) R_f = 0.66; IR (KBr pellet) 1749, 1479, 1373, 1249, 1031, 809 cm^{-1}; ^1H NMR (dimethyl sulfoxide-d_6) δ 7.06 and 7.51 (2 H, ABX, J_{ortho} = 8.16 Hz, J_{meta} = 1.56 Hz, J_{para} = 0 Hz, C(4)- and C(5)-aromatic protons), 7.38 (1 H, br s, C(7)-aromatic proton), 5.32 (2 H, s, acetoxymethyl methylene), 3.70 (3 H, s, N(1)-methyl), 2.46 (3 H, s, C(6)-methyl), 2.1 (3 H, s, acetate methyl); mass spectrum (EI mode) m/z 218 (M^+), 175 (M^+ - acetyl). Anal. ($C_{12}H_{14}N_2O_2$) C, H, N.

2-(Acetoxymethyl)-5-bromo-1,6-dimethyl-4-nitrobenzimidazole (11) was prepared by the following 2-step procedure.

To a solution of 1.6 g (7.33 mmol) of 10 in 70 mL of glacial acetic acid, heated at 100 °C, was added 0.384 mL (7.5 mmol) of bromine in 3mL of glacial acetic acid. After the addition, the reaction mixture was heated at 100–110 °C for 4 h. The cooled reaction mixture was neutralized to pH 6.5 with aqueous sodium bicarbonate, and the brominated 10 crystallized from the solution as white crystals. The yield upon drying of the collected solid was 1.85 g (85%). Recrystallization from chloroform/hexane afforded the analytically pure material: m.p. 122–125 °C; TLC (chloroform/methanol, 90:10) R_f = 0.75; IR (KBr pellet) 1743, 1476, 1406, 1376, 1256, 1245, 1224, 1253, 1030, 843 cm^{-1}; ^1H NMR (dimethyl sulfoxide-d_6) δ 7.61 and 7.84 (2 H, 2 s, C(4)- and C(7)-aromatic protons), 5.30 (2 H, s, acetoxymethyl methylene), 3.77 (3 H, s, N(1)-methyl), 2.46 (3 H, s, C(6)-methyl), 2.08 (3 H, s, acetate methyl); mass spectrum (EI mode) m/z 296 and 298 (M^+, ^{79}Br and ^{81}Br), 253 and 255 (M^+ - acetyl).

To a mixture of 20 mL of (9:1) fuming nitric acid and sulfuric acid, cooled in a dry ice–acetone bath, was added 1.85 g (6.22 mmol) of the brominated 10 portionwise. After 10 min, the reaction mixture was removed from the ice bath, stirred for 15 min at room temperature, and then poured into a mixture consisting of 200 g of ice and 150 mL of chloroform. Saturated aqueous sodium bicarbonate was added to the above mixture with vigorous stirring until the pH was neutral. The chloroform layer was then separated, and the aqueous layer was extracted two times with 100-mL portions of chloroform. Drying the combined chloroform extracts (Na_2SO_4), concentration *in vacuo* to a small volume, and then adding hexane afforded the light-yellow-colored product: 1.52 g (71%); m.p. 185 °C; TLC

(chloroform/methanol, 90:10 R_f = 0.54; IR (KBr pellet) 1751, 1533, 1478, 1380, 1371, 1366, 1252, 1219, 1037, 1013 cm^{-1}; ^{1}H NMR (dimethyl sulfoxide-d_6) δ 7.97 (1 H, s, aromatic proton), 5.35 (2 H, s, acetoxymethyl methylene), 3.86 (3 H, s, N(1)-methyl), 2.57 (3 H, s, C(6)-methyl), 2.11 (3 H, s, acetate methyl); mass spectrum (EI mode) m/z 341 and 343 (M^+, ^{79}Br and ^{81}Br), 298 and 300 (M^+ - acetyl), 281 and 283 (M^+ - acetic acid). Anal. ($C_{12}H_{12}BrN_3O_4$) C, H, N.

2-(Acetoxymethyl)-1,6-dimethylbenzimidazole-4,7(1*H*)-dione (12) was prepared by the following 2-step procedure.

A suspension of 254 mg (0.92 mmol) of 11 in 20 mL of methanol containing 50 mg of 5% Pd on charcoal was shaken under 50 psi of H_2 for 8 h. The reaction mixture was then filtered through Celite into a flask containing 1 mL of 1 N HCl. Evaporation of the filtrate *in vacuo* afforded the dihydrochloride salt of 4-amino-2-(acetoxymethyl)-1,6-dimethylbenzimidazole, 155 mg (55%). Recrystallization was carried out from ethyl acetate/methanol: mp > 360 °C; TLC (chloroform/methanol, 80:20) R_f = 0.61; IR (KBr pellet) 3388, 3306, 3201, 1763, 1644, 1499, 1459, 1395, 1223, 1075 cm^{-1}; ^{1}H NMR (dimethyl sulfoxide-d_6) δ 6.91 and 6.62 (2 H, 2s, aromatic protons), 5.51 (2 H, s, acetoxymethyl methylene), 3.81 (3 H, s, N(1)-methyl), 2.37 (3 H, s, C(6)-methyl), 2.18 (3 H, s, acetate methyl); mass spectrum (EI mode) m/z 233 (M^+), 190 (M^+ - acetyl).

To a suspension of 132 mg (0.43 mmol) of the amine dihydrochloride salt obtained above in 5 mL of water containing 20 mg of monobasic potassium phosphate was added a solution of 500 mg of Fremy's salt in 40 mL of water containing 200 mg of monobasic potassium phosphate. The reaction mixture was stirred at room temperature for 3 h and then extracted three times with 25-mL portions of chloroform. The dried extracts (Na_2SO_4) were concentrated to give a yellow-brown solid, which was flash chromatographed on silica gel, using chloroform as the eluant. The product was recrystallized from chloroform/hexane: 45 mg (42%), mp 109–110 °C; TLC (chloroform/methanol, 90:10) R_f = 0.59; IR (KBr pellet) 1744, 1688, 1678, 1658, 1515, 1483, 1384, 1250, 1178, 1032 cm^{-1}; ^{1}H NMR (dimethyl sulfoxide-d_6) δ 6.60 (1 H, q, J = 1.59 Hz, C(5)-proton), 5.24 (2 H, s, acetoxymethyl methylene), 3.92 (3 H, s, N(1)-methyl), 2.08 (3 H, s, acetate methyl), 2.02 (3 H, d, J = 1.56 Hz, C(6)-methyl); mass spectrum (EI mode) m/z 248 (M^+), 205 (M^+ - acetyl). Anal. ($C_{12}H_{12}N_2O_4$) C, H, N.

2-(Acetoxymethyl)-5-(*N*-aziridinyl)-1,6-dimethylbenzimidazole-4,7(1*H*)-dione (BI-A). To a solution of 50 mg (0.20 mmol) of 12 in 4 mL of dry methanol, chilled at 0 °C, was added 200 μL of ethylenimine. After being stirred at 0 °C for 15 min, the reaction mixture was stirred at room temperature for 3 h. The solvent was then removed *in vacuo*, and the brick-red residue was flash chromatographed on silica gel, using chloroform as the eluant. The purified product was recrystallized from chloroform/hexane: 10.6 mg (18%); mp 153–155 °C; TLC (chloroform/methanol, 90:10) R_f = 0.61; IR (KBr pellet) 1763, 1751, 1678, 1635, 1529, 1375, 1338, 1304, 1222, 1212 cm^{-1}; ^{1}H NMR (dimethyl sulfoxide-d_6) δ 5.23 (2 H, s, acetoxymethyl methylene), 3.98 (3 H, s, N(1)-methyl), 2.47 (4 H, s, aziridine protons), 2.10 and 2.07 (6 H, 2s, C(6)-methyl and acetate methyl); mass spectrum (EI mode) m/z 289 (M^+), 246 (M^+ - acetyl). Anal. ($C_{14}H_{15}N_3O_4$) C, H, N.

1,2,6-Trimethylbenzimidazole (13) was prepared by the following 3-step procedure. To a solution of 3.2 g (19.27 mmol) of 8, in 200 mL of dry dichloromethane containing 1.7 mL (21.2 mmol) of pyridine, was added 10 mL of acetyl chloride. The reaction mixture was refluxed for 8 h and then evaporated to dryness. Trituration of the yellowish oil with chloroform/hexane afforded crystalline 3-(*N*-methylacetamido)-4-nitrotoluene in 3.4 g (81%): m.p. 39–40 °C; TLC (chloroform/methanol, 90:10) R_f = 0.58; IR (KBr pellet) 1664, 1602, 1590, 1519, 1485, 1422, 1378, 1340, 1315, 835 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.96 and 7.34 (2 H, ABX system, J_{ortho} = 8.34 Hz, $J_{meta} \simeq$ 0 Hz, J_{para} = 0 Hz, C(4- and C(5-aromatic protons), 7.18 (1 H, br s, C(2)-proton), 3.20 (3 H, s, N-CH_3), 2.48 (3 H, s, methyl), 1.81 (3 H, s, acetamido methyl); mass spectrum (EI mode) m/z 208 (M^+), 166 (M^+ - ketene).

A suspension of 1.12 g (5.38 mmol) of the above acetylation product, in 200 mL of methanol containing 100 mg of 5% Pd on charcoal, was shaken under 50 psi of hydrogen for 4 h. The reaction was filtered through Celite, and the filter cake was washed with methanol. The solvent was completely evaporated, and the white colored solid was crystallized from ethyl acetate/hexane to afford 920 mg (96%) of 4-amino-3-(*N*-methylacetamido)toluene: m.p. 112 °C; TLC (chloroform/methanol, 85:15) R_f = 0.64; IR (KBr pellet) 3421, 3346, 3240, 1644, 1632, 1614, 1578, 1516, 1382, 813 cm^{-1}; ^{1}H NMR (dimethyl sulfoxide-d_6) δ 6.86 and 6.68 (2 H, ABX system, J_{ortho} = 8.04 Hz, $J_{meta} \simeq$ 0 Hz, $J_{p\ ara}$ = 0 Hz, C(5- and C(6-protons), 6.79 (1 H, s, C(2-proton), 4.92 (2 H, br s, NH_2 protons), 2.99 (3 H, s, N-methyl), 2.14 (3 H, s, ring methyl), 1.68 (3 H, s, acetamido methyl); mass spectrum (EI mode) m/z 178 (M^+), 161 (M^+ - OH).

To a solution of 950 mg (5.33 mmol) of the product obtained above in 20 mL of acetic acid was added 3 drops of concentrated H_2SO_4, and the reaction mixture was stirred at 110 °C for 6 h. The reaction mixture was then cooled to room temperature and the pH adjusted to 7 with aqueous $NaHCO_3$. Extraction of the neutralized mixture three times with 50-mL portions of chloroform, drying of the combined extracts (Na_2SO_4), and then concentration *in vacuo* afforded a yellow oil. Trituration of the oil with a chloroform/hexane mixture (1:9) afforded pure 13: 780 mg (91%); m.p. 109 °C; TLC (ethyl acetate/methanol, 90:10) R_f = 0.3; IR (KBr pellet) 2915, 1523, 1478, 1447, 1435, 1399, 1327, 1283, 1240, 808 cm^{-1}; ^{1}H NMR (dimethyl sulfoxide-d_6) δ 7.44 and 6.98 (2 H, ABX system, J_{ortho} = 8.1 Hz, $J_{meta} \simeq$ 0 Hz, J_{para} = 0 Hz, C(4)- and C(5)-protons), 7.24 (1 H, br s, C(7)-proton), 3.67 (3 H, s, N(1)-methyl), 2.48 and 2.42 (6 H, 2 s, C(2)- and C(6)-methyls); mass spectrum (EI mode) m/z 160 (M^+), 145 (M^+ - methyl). Anal. ($C_{10}H_{12}N_2$) C, H, N.

5-Bromo-1,2,6-trimethyl-4-nitrobenzimidazole (14) was prepared by the following 2-step procedure. To a solution of 2.1 g (13.12 mmol) of 13 in 40 mL of acetic acid heated at 100 °C was added 717 μL of a bromine solution in 5 mL of acetic acid. After the addition, the reaction mixture was heated at 110 °C for 4 h. The cooled reaction mixture was diluted with 20 mL of water and the pH adjusted to 6.5 with aqueous sodium bicarbonate. The product crystallized out from solution as white crystals; the yield of the brominated 13 was 2.6 g (86%). An analytical sample was prepared by recrystallization from chloroform/hexane: m.p. 135–136 °C; TLC (ethyl acetate/methanol, 90:10) R_f = 0.25; IR (KBr pellet) 2926, 1516, 1477, 1446, 1399, 1365, 1315, 971, 884, 836 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.86 and 7.15 (2 H, 2 s, C(4)- and C(7)-aromatic protons), 3.69 (3 H, s, N(1)-methyl), 2.60 and 2.52 (6 H, 2 s, C(2)- and C(6)-methyls); mass spectrum (EI mode) m/z 238 and 240 (M^+, ^{79}Br and ^{81}Br).

To a mixture of 5.4 mL of fuming nitric acid and 0.6 mL of H_2SO_4, cooled at -20 °C, was added 600 mg (2.5 mmol) of the brominated 13 portionwise. The reaction mixture was then stirred at -20 °C for 15 min and then taken out of the ice bath and stirred at room temperature for another 30 min. The reaction mixture was poured into a mixture consisting of 200 g of ice and 50 mL of chloroform followed immediately by neutralization with aqueous $NaHCO_3$. After separation of the chloroform layer, the aqueous layer was extracted three times with 50-mL portions of $CHCl_3$. The combined extracts were dried (Na_2SO_4) and then evaporated *in vacuo* to give the product as a yellow-colored solid. Recrystallization of the product was carried out by dissolution in a minimum amount of chloroform followed by the addition of hexane: 435 mg (61%); m.p. 201 °C dec; TLC (chloroform/methanol, 85:15) R_f = 0.66; IR (KBr pellet) 1532, 1513, 1475, 1440, 1398, 1384, 1329, 1317, 890, 813 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.29 (1 H, s, C(7)-aromatic proton), 3.73 (3 H, s, N(1)-methyl), 2.62 and 2.58 (6 H, 2 s, C(2)- and C(6)-methyls); mass spectrum (EI mode) m/z 283 and 285 (M^+, ^{79}Br and ^{81}Br). Anal. ($C_{10}H_{10}BrN_3O_2$) C, H, N.

1,2,6-Trimethylbenzimidazole-4,7(1*H*)-dione (15) was prepared by the following 2-step procedure. A suspension of 250 mg (0.88 mmol) of 14 in 100 mL of methanol containing 40 mg of 5% Pd on charcoal was shaken under 50 psi of H_2 for 8 h. The reaction mixture was filtered through Celite into a flask containing 1 mL of concentrated HCl. The reaction solvent was completely evaporated, and the white-colored residue was dissolved in a minimum amount of methanol and then crystallized by adding ethyl acetate: 210 mg (96%) of the dihydrochloride salt of 4-amino-1,2,6-trimethylbenzimidazole; mp 191 °C dec; TLC (chloroform/methanol, 80:20) R_f = 0.35 (KBr pellet) 2836, 2762,

2560, 1630, 1545, 1504, 1459, 1422, 1244, 1030; ^{1}H NMR (dimethyl sulfoxide-d_6) δ 6.80 and 6.52 (2 H, 2 s, aromatic protons), 3.79 (3 H, s, N(1)-methyl), 2.75 (3 H, s, C(2)-methyl), 2.34 (3 H, s, C(6)-methyl); mass spectrum (EI mode) m/z 175 (M^+), 160 (M^+ – methyl).

To a a suspension of 108 mg (0.43 mmol) of the amine dihydrochloride salt in 5 mL of water, containing 50 mg of monobasic potassium phosphate, was added a solution of 500 mg of Fremy's salt in 25 mL of water containing 100 mg of monobasic potassium phosphate. The reaction mixture was stirred at room temperature for 3.5 h and then extracted three times with 50-mL portions of chloroform. The combined extracts were dried (Na_2SO_4) and then concentrated to afford a yellow–brown residue, which was chromatographed over silica gel, using chloroform as the eluant. The yellow product was isolated from the column fractions and recrystallized from chloroform/hexane: 49 mg (58%); m.p. 190 °C dec; TLC (chloroform/methanol, 85:15) R_f = 0.52; IR (KBr pellet) 1665, 1654, 1610, 1535, 1518, 1477, 1422, 1376, 1178, 951 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.47 (1 H, q, J = 1.52 Hz, C(5)-proton coupled to C(6)-methyl), 3.91 (3 H, s, N(1)-methyl), 2.51 (3 H, s, C(2)-methyl), 2.09 (3 H, br s, C(6)-methyl); mass spectrum (EI mode) m/z 190 (M^+). Anal. ($C_{10}H_{10}N_2O_2$) C, H, N.

5-(*N*-Aziridinyl)-1,2,6-trimethylbenzimidazole-4,7(1*H*)-dione (BI-C). To a solution of 34 mg (0.17 mmol) of 15 in 5 mL of dry methanol was added 0.3 mL of ethylenimine. The reaction mixture was stirred at room temperature for 3.5 h during which time the reaction was monitored by TLC. The solvent was then evaporated *in vacuo*, and the red residue was flash chromatographed on silica gel, using methanol/chloroform (1:99) as the eluant. The purified product was recrystallized with chloroform/hexane: 11 mg (28%); m.p. 163–165 °C; TLC (chloroform/methanol, 80:20) R_f = 0.56; IR (KBr pellet) 1674, 1632, 1584, 1532, 1474, 1374, 1336, 1303, 1145, 971 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.87 (3 H, s, N(1)-methyl), 2.46 (3 H, s, C(2)-methyl), 2.33 (4 H, s, aziridinyl protons), 2.06 (3 H, s, C(6)-methyl); mass spectrum (EI mode) m/z 231 (M^+), 216 (M^+ – methyl). Anal. ($C_{12}H_{13}N_3O_2 \cdot {}^1/_6H_2O$) C, H, N; N: calcd, 17.93; found, 17.43.

3-(*N*-Piperdino)-4-nitrotoluene (16). A mixture of 10.0 g (46.3 mmol) of 3-bromo-4-nitrotoluene and 15.0 mL (151.7 mmol) of piperidine was heated at 100 °C for 3 h. The cooled reaction mixture was combined with 200 mL of water and the resulting mixture extracted four times with 50-mL portions of chloroform. The combined extracts were washed three times with 20-mL portions of 1 N HCl and once with 20 mL of water. The dried extracts (Na_2SO_4) were concentrated to an oil and purified by silica gel chromatography, employing chloroform as the eluant. Evaporation of the product fractions afforded pure 16 as an oil: 10 g (98%); TLC (chloroform) R_f = 0.46; IR (film on NaCl) 2938, 2855, 2810, 1605, 1578, 1512, 1451, 1381, 1341, 1300, 1242, 812 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.72 and 6.76 (2 H, ABX, J_{ortho} = 8.31 Hz, J_{meta} = 1.5 Hz, $J_{para} \simeq$ 0 Hz, C(5)- and C(6)-aromatic protons), 6.90 (1 H, br s, C(2)-aromatic proton), 3.01 (4 H, t, piperidine protons adjacent to N), 2.36 (3 H, s, methyl), 1.73 (4 H, m, piperidine protons), 1.59 (2 H, m, piperidine protons); mass spectrum (EI mode) m/z 206 (M^+). Anal. ($C_{12}H_{16}N_2O_2 \cdot 0.2H_2O$) C, H, N.

8-Methyl-4-acetoxy-1,2,3–,4-tetrahydropyrido[1,2-*a*]benzimidazole (17). A solution consisting of 8.5 g (38.6 mmol) of 16, 5.3 g (38.8 mmol) of $ZnCl_2$, and 39 mL of acetic anhydride was refluxed for 18 h. The cooled reaction mixture was poured over 200 g of ice, neutralized with sodium bicarbonate, and then extracted three times with 50-mL portions of ethyl acetate. The dried extracts (Na_2SO_4) were concentrated and purified by silica gel chromatography, employing chloroform/acetone (50:50) as the eluant. The product fractions were concentrated to dryness and recrystallized from chloroform/hexane: 3.99 g (42%); mp 149–150.5 °C; TLC (chloroform/methanol, 90:10) R_f = 0.62; IR (KBr pellet) 2963, 2905, 2675, 2610, 2558, 1740, 1532, 1234, 1061, 866 cm^{-1}; ^{1}H NMR (dimethyl sulfoxide-d_6) δ 7.50 and 7.06 (2 H, ABX, J_{ortho} = 8.22 Hz, J_{meta} = 1.47 Hz, $J_{para} \simeq$ 0 Hz, C(6)- and C(7)-aromatic protons), 7.33 (1 H, br s, C(9)-aromatic proton), 6.08 (1 H, dd, J = 3.9 Hz, J = 3.7 Hz, C(4)), 4.21 and 4.00 (2 H, 2m, C(1)-diastereomeric methylene), 2.45 (3 H, s, C(8)-methyl), 2.08 (3 H, s, acetate methyl), 2.04–2.22 (4 H, br, m, C(2)- and C(3)-diastereomeric methylene); mass spectrum (EI mode) m/z 244 (M^+), 201 (M^+ – acetyl). Anal. ($C_{13}H_{14}N_2O_2$) C, H, N.

7-Bromo-4-acetoxy-8-methyl-6-nitro-1,2,3,4-tetrahydropyrido[1,2-*a*]benzimidazole (18) was prepared by the following 2-step procedure. The solution of 0.502 g (2.05 mmol) of 17 in 7 mL of acetic acid, heated at 100 °C, was added to 3 mL of 0.72 M bromine in acetic acid. The reaction mixture was heated at 100 °C for 5 h and then cooled to room temperature. Dilution of the cooled reaction mixture with 50 mL of water was followed by neutralization with aqueous bicarbonate resulted in crystallization of the pure product, 0.4491-g (75%) yield. An analytical sample was obtained by recrystallizing from chloroform/hexane: mp 186–189 °C dec; TLC (chloroform/methanol, 90:10) R_f = 0.68; IR (KBr pellet) 2982, 2953, 1738, 1487, 1449, 1425, 1373, 1314, 1240, 1208, 1022, 964, 854 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.99 and 7.23 (2 H, 2s, C(6)- and C(9)-aromatic protons), 6.20 (1 H, dd, J = 5.0 Hz, J = 4.9 Hz, C(4)-proton), 4.16 and 4.03 (2 H, 2 m, C(1)-diastereomeric methylene), 2.54 (3 H, s, C(8)-methyl), 2.16 (3 H, s, acetate methyl), 2.1–2.4 (4 H, m, C(2)- and C(3)-diastereomeric methylenes); mass spectrum (EI mode) m/z 322 and 324 (M^+, ^{79}Br and ^{81}Br), 279 and 281 (M^+ – acetyl), 263 and 265 (M^+ – acetic acid).

To 10 mL of fuming nitric acid/concentrated sulfuric acid (9:1), chilled at 0 °C, was added 0.520 g (1.61 mmol) of the brominated 17. The resulting mixture was stirred at 0–10 °C for 10 min and then poured into 40 mL of crushed ice. The aqueous mixture was neutralized with sodium bicarbonate and then extracted four times with 50-mL portions of chloroform. The extracts were combined and dried (Na_2SO_4) and then evaporated to a solid, which was recrystallized from chloroform/hexane: 0.407 g (69%); mp 245.5–247.5 °C; TLC (chloroform/methanol, 90:10) R_f = 0.63; IR (KBr pellet) 2971, 2947, 2922, 1742, 1534, 1447, 1375, 1314, 1223, 1067, 1045, 862 cm^{-1}; ^{1}H NMR (dimethyl sulfoxide-d_6) δ 7.91 (1 H, s, C(9)-aromatic proton), 6.09 (1 H, dd, J = 5.01 Hz, J = 4.11 Hz, C(4)-proton), 4.2 7 and 4.11 (2 H, 2m, C(1)-diastereomeric methylene), 2.57 (3 H, s, C(8)-methyl), 2.09 (3 H, s, acetate methyl), 2.0–2.3 (4 H, m, C(2)- and C(3)-diastereomeric methylenes); mass spectrum (EI mode) m/z 367 and 369 (M^+, ^{79}Br and ^{81}Br), 324 and 326 (M^+ – acetyl), 307 and 309 (M^+ – acetic acid). Anal. ($C_{14}H_{14}N_3O_4 \cdot 0.5H_2O$) C, H, N.

4-Acetoxy-8-methyl-1,2,3,4-tetrahydropyrido[1,2-*a*]benzimidazole-6,9-dione (19) was prepared by the following 2-step procedure. A suspension of 0.653 g (1.774 mmol) of 18 and 0.1 g of Pd on carbon in 200 mL of methanol was shaken under 50 psi of H_2 for 40 h. The reaction mixture was then filtered through Celite and the filtrate evaporated to dryness. Both the Celite filter cake and the evaporation residue were saved for the following step.

A solution of 2.89 g of monobasic potassium phosphate in 80 mL of water was washed through the Celite filter cake and then combined with the evaporation residue. To this solution was added a solution consisting of 2.6 g of Fremy salt and 3.3 g of monobasic potassium phosphate in 60 mL of water. The resulting reaction mixture was stirred for 5 h at room temperature and then extracted 10 times with 80-mL portions of chloroform. The combined extracts were dried (Na_2SO_4) and concentrated to a solid, which was purified on a silica gel chromatography column, employing chloroform as the eluant. Recrystallization of the purified product was carried out with chloroform/hexane: 0.306 g (63%); mp 159–160 °C; TLC (chloroform/methanol, 90:10) R_f = 0.64; IR (KBr pellet) 3441, 2963, 1744, 1657, 1375, 1236, 1177, 1161, 928 cm^{-1}; ^{1}H NMR (dimethyl sulfoxide-d_6) δ 6.60 (1 H, q, J = 1.62 Hz, C(7)-proton), 6.00 (1 H, dd, J = 4.80 Hz, J = 3.27 Hz, C(4)-proton), 4.40 and 4.19 (2 H, 2m, C(1)-diastereomeric methylene), 2.07 (3 H, s, acetate methyl), 2.01 (3 H, d, J = 1.68 Hz, C(8)-methyl), 2.2-1.9 (4 H, m, C(2)- and C(3)-diastereomeric methylenes); mass spectrum (EI mode) m/z 274 (M^+), 231 (M^+ – acetyl). Anal. ($C_{14}H_{14}N_2O_4$) C, H, N.

7-(*N*-Aziridinyl)-4-acetoxy-8-methyl-1,2,3,4-tetrahydropyrido[1,2-*a*]benzimidazole-6,9-dione (TPBI-A). To a solution of 0.108 g (0.399 mmol) of 19 in 6 mL of dry methanol chilled at 0 °C was added 1.0 mL of ethylenimine with stirring. The reaction mixture was stirred at 0 °C for 40 min and then at room temperature for 2 h. The solvent was then evaporated and the residue chromatographed on silica gel, employing ethyl acetate as the eluant. The purified product was recrystallized from ethyl

acetate/hexane: 15 mg (12%); mp 135 °C dec; TLC (ethyl acetate) R_f = 0.30; IR (KBr pellet) 3526, 3435, 2955, 2361, 1991, 1933, 1757, 1416, 829, 646 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.08 (1 H, dd, J = 4.53 Hz, J = 4.17 Hz, C(4)-proton), 4.53 and 4.18 (2 H, 2m, C(1)-diastereomeric methylene), 2.35 (4 H, s, aziridine protons), 2.11 (3 H, s, C-(8)-methyl), 2.08 (3 H, s, acetate methyl); mass spectrum (EI mode) m/z 315 (M^+), 272 (M^+ - acetyl). Anal. ($C_{16}H_{17}N_3O_4 \cdot {}^1/_6H_2O$) C, H, N.

Hydrolysis of Reduced BI-A. A 10-mL volume of 0.05 M pH 7.4 tris buffer was purged with argon for 30 min. To the deaerated solution was added 20 mg (6.9×10^{-5} mol) of BI-A in 2 mL of dimethyl sulfoxide and 10 mg of 5% Pd on carbon. The mixture was then purged with argon for 10 min. Hydrogen gas was passed through the deaerated mixture for 15 min, resulting in the formation of a colorless solution. Argon was then passed through the reduced mixture to remove excess hydrogen. Addition of air at this point resulted in near quantitative recovery of BI-A. Therefore, the hydrogenation step itself does not produce any of the observed reaction products. Incubation of the reduced BI-A solution for 18 h at 37 °C resulted in the formation of a blue solution, which was opened to the air and filtered through Celite. The deep blue filtrate was extracted three times with 50-mL portions of chloroform. Drying the extracts (Na_2SO_4) was followed by evaporation to a residue and then silica gel chromatography, using ethyl acetate as the eluant. The order of elution of the products was **20**, **23**, and finally **24**. The yields of each obtained upon evaporation of collected fractions were 1.82 mg (9.1%) of **20**, 6.81 mg (37.7%) of **23**, and 5.63 mg (26.7%) of **24**. Physical properties of these hydrolysis products and the independent synthesis of **20** are provided below.

The aqueous layer which had been extracted with chloroform was added to a 20-g Bakerbond Phenyl (40-μm) reverse-phase column prepared with water. The column was eluted with 500 mL of water to remove all the buffer salts. A single blue product eluted from the column with methanol/water (46:54). The product fraction was concentrated *in vacuo* to afford a small amount of a blue solid. A mass spectrum [m/z 221 (M^+)] indicated that this material was the deacetylated **23**.

2-(Acetoxymethyl)-5-(ethylamino)-1,6-dimethylbenzimidazole-4,7(1*H*)-dione (20). This hydrolysis product was formed in very low yield and an independent synthesis was carried out as follows. The procedure for the conversion of **11** to **12** was carried out as described above except concentrated HCl instead of 1 N HCl was employed to acidify the catalytic reduction reaction mixture. The stronger acid resulted in deacetylation, and therefore, the product obtained from the Fremy oxidation step was the deacetylated **12**. Purification was carried out by flash chromatography on silica gel (chloroform/methanol, 95:5) and recrystallization from chloroform/hexane: 68% yield; mp 167–169 °C; TLC (chloroform/methanol, 90:10) R_f = 0.54; IR (KBr pellet) 2926, 1656, 1616, 1516, 1487, 1332, 1261, 1176, 954, 910 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.51 (1 H, q, J = 1.5 Hz, C(5)-proton split with C(6-)-methyl), 4.84 (2 H, d, J = 6 Hz, hydroxymethyl methylene), 4.02 (3 H, s, N(1)-methyl), 3.29 (1 H, t, J = ~5 Hz, hydroxyl proton split with methylene), 2.10 (3 H, d, J = 1.6 Hz, C(6)-methyl split by C(5)-proton); mass spectrum (EI mode) m/z 206 (M^+), 177 (M^+ - HC≡O), 149. Anal. ($C_9H_{13}N_3O_3$) C, H, N.

To a solution of the deacetylated **12**, 50 mg (0.24 mmol), in 4 mL of methanol was added 90 μL (2.4 mmol) of 70% aqueous ethylamine. The reaction mixture was stirred at 0 °C for 0.5 h, and then, the ice bath was removed and the reaction mixture stirred for 1.5 h. The completed reaction mixture was concentrated *in vacuo* and flashed chromatographed on silica gel (chloroform/methanol, 98:2), resulting in isolation of the crude material (26 mg) consisting of the 5-ethylamino- and 5-hydroxy-substituted quinone derivatives.

A 15-mg portion of the crude material obtained above was dissolved in 15 mL of dry methylene chloride and then combined with 320 μL of pyridine and 14 μl of acetic anhydride. The resulting mixture was stirred for 12 h at room temperature. After the reaction was complete, the mixture was diluted with 15 mL of methylene chloride and then extracted three times with 30-mL portions of water. The dried extracts (Na_2SO_4) were concentrated and flash chromatographed on silica gel, using chloroform as the eluant. The isolated product **20** was recrystallized from ethyl acetate/hexane: ~2.5 mg (~14%); mp 127 °C; TLC (chloroform/methanol, 95:5) R_f = 0.70; IR (KBr pellet) 3327, 1736, 1683, 1620, 1591, 1510, 1255, 1134, 1033, 983 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.81 (1 H, br m, C(5)-amino proton), 5.22 (2 H, s, methylene of acetoxymethyl), 4.01 (3 H, s, N(1)-methyl), 3.61 (2 H, br quint, J = 7.0 Hz, methylene of ethylamino), 2.12 (6 H, s, methyl of acetoxymethyl and C(6)-methyl), 1.28 (3 H, t, J = 7.0 Hz, methyl of ethylamino); mass spectrum (EI mode) m/z 291 (M^+), 231 (M^+ - acetic acid). Anal. ($C_{14}H_{17}N_3O_4 \cdot 0.22H_2O$) C, H, N; N: calcd, 14.23; found, 13.75.

The physical properties of **20** matched those of the material isolated from the hydrolysis reaction.

2-(Acetoxymethyl)-5-amino-1,6-dimethylbenzimidazole-4,7(1*H*)-dione (23). The physical properties of **23** isolated from the hydrolysis reaction mixture are as follows: mp 213–214 °C dec; TLC (ethyl acetate) R_f = 0.40; IR (KBr pellet) 3335, 1743, 1628, 1591, 1520, 1379, 1228, 1211, 1033, 746 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.19 (2 H, s, methylene of acetoxymethyl), 5.0 (2 H, br s, amino) 3.97 (3 H, s, N(1)-methyl), 2.089 (3 H, s, methyl of acetoxymethylene), 1.845 (3 H, s, C(6)-methyl); mass spectrum (EI mode) m/z 263 (M^+), 220 (M^+ - ketene). Anal. ($C_{12}H_{13}N_3O_4 \cdot 0.2H_2O$) C, H, N.

2-(Acetoxymethyl)-5-[(2-hydroxyethyl)amino]-1,6-dimethylbenzimidazole-4,7(1*H*)-dione (24). The physical properties of **24** isolated from the hydrolysis reaction mixture are as follows: mp 75–80 °C dec; TLC (ethyl acetate) R_f = 0.24; IR (KBr pellet) 3344, 2930, 1745, 1680, 1589, 1527, 1232, 1053 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.23 (2 H, s, methylene of acetoxymethyl), 4.00 (3 H, s, N(1)-methyl), 3.86 and 3.73 (4 H, 2t, J = 5.0 Hz, ethylene), 2.12 (3 H, s, methyl of acetoxymethyl), 2.09 (3 H, s, C(6)-methyl); mass spectrum (E I mode) m/z 307 (M^+).

Preparation of the 5′-dAMP Adduct 25. The reaction mixture consisted of the following components: 10 mL of 0.05 M pH 7.4 tris buffer containing 50 mg (1.15×10^{-4} mol) of 5′-dAMP, 17 mg (5.8×10^{-5} mol) of BI-A in 2 mL of dimethyl sulfoxide, and 10 mg of 5% Pd on carbon. The combination of these components, deaeration, and catalytic reduction were carried out as described under Hydrolysis of Reduced BI-A. The anaerobic incubation of the reaction was carried out at 37 °C for 12 h. After the reaction was opened to the air, the catalyst was filtered off utilizing a Celite filter pad and the filterate extracted three times with 50-mL portions of chloroform to remove BI-A hydrolysis products. The aqueous layer containing **25** was placed on a 20-g Bakerbond Phenyl (40-μm) reverse-phase column prepared with water. The column was eluted with 500 mL of water to remove salts. During this elution, a blue band slowly moved down the column. ^{1}H NMR studies of the isolated blue product showed it to be a mixture of **25** and unreacted 5′-dAMP. This mixture was separated on a 10-g Bakerbond Phenyl (40-μm) reverse-phase column employing water as the eluant: 2.71-mg (8%) yield upon lyophilizing the product fraction to dryness; TLC (*n*-butyl alcohol/acetic acid/water, 5:2:3) R_f = 0.54; ^{1}H NMR (dimethyl sulfoxide-d_6) δ 8.42 and 8.13 (2 H, 2s, adenine protons), 7.22 (2 H, br s, adenine amino group), 7.1 (2 H, br m, 3′-OH and 5-NH), 6.35 (1 H, t, J = 6 Hz, C(1′)-proton), 5 .5 (1 H, d, J = 5.8 Hz, hydroxy of hydroxymethyl), 4.55 (2 H, d, J = 5.8 Hz, methylene of hydroxymethyl), 4.42 (1 H, m, C(3′)-proton), 3.93 (1 H, m, C(4′)-proton), 3.91 (3 H, s, N(1)-methyl), 3.8 (4 H, m, ethylene), 3.68 (2 H, m, C(5′)-proton), 2.7 and 2.3 (2 H, 2m, C(2′)-protons), 1.93 (3 H, s, C(6)-methyl). The AMP chemical shifts in this adduct were nearly identical to those of the PBI adduct.[6]

Preparation of the DNA Adduct. The reaction mixture consisted of the following components: 70 mg of sonicated calf thymus DNA dissolved in 25 mL of 0.05 M pH 7.4 tris buffer, 21.5 mg (7.4×10^{-5} mmol) of BI-A dissolved in 2 mL of dimethyl sulfoxide, and 10 mg of 5% Pd on carbon. The combination of these components, deaeration, and catalytic reduction were carried out as described under Hydrolysis of Reduced BI-A. The anaerobic incubation of the reaction was carried out at 37 °C for 24 h. The completed reaction was opened to the air and filtered through Celite to remove the catalyst. The filtrate was extracted three times with 50-mL portions of chloroform to remove hydrolysis products. These products were separated and yields obtained as described under Hydrolysis of Reduced BI-A. The aqueous layer was adjusted to 0.3 M sodium acetate with 3.0 M pH 5.1 sodium acetate stock and then diluted with three volumes of absolute ethanol. This mixture was chilled at −20 °C for 24

h and then centrifuged at 5000g for 15 min. The supernatant was evaporated to dryness and then dissolved in water and placed on a Bakerbond Phenyl (40-μm) reverse-phase column and the blue product (deacetylated **23**) separated. The DNA pellet was washed by suspending in ethanol and centrifuging. Weight of the vacuum-dried DNA pellet was 47 mg, and the yields of the hydrolysis products were 1.9% of **20**, 8% of **23**, 5.6% of **24**, and a trace amount of the deacetylated **23**. There were no indications of purine-containing DNA release products (by ^{1}H NMR) in either the chloroform extracts or the aqueous phase.

DNA Sources, PAGE Materials, and DNA Cleavage. Buffer salts, EDTA, and urea for PAGE were purchased from Sigma and used as obtained. Acrylamide and methylene bisacrylamide were purchased from BioRad in 99.9% pure form. pBR322 DNA, *Cla*I, *Rsa*I, and Klenow fragment were purchased from New England Biolabs. ^{32}P-labeled CTP was purchased from DuPont. Buffers and other solutions were prepared in doubly-distilled water. The *Cla*I/*Rsa*I restriction fragment of pBR322 was parpared using the New England Biolabs protocols provided with the restriction enzymes. The restriction fragment was purified and end-labeled using previously reported procedures.[25] The BI-A-mediated cleavage of the labeled restriction fragment was carried out as previously described for PBI cleavage.[6] Sonicated calf thymus DNA (~600 bp) was prepared as previously described.[27]

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