# Studies of Pyrrolo[1,2-*a*]benzimidazolequinone DT-Diaphorase Substrate Activity, Topoisomerase II Inhibition Activity, and DNA Reductive Alkylation

Edward B. Skibo,* Samantha Gordon, Laura Bess, Romesh Boruah, and Matthew J. Heileman

*Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287-1604*

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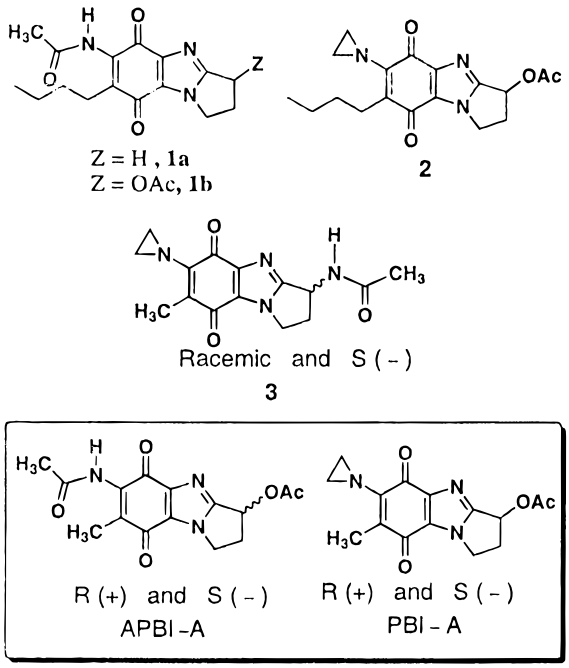
The influence of structure on DT-diaphorase substrate activity, topoisomerase II inhibition activity, and DNA reductive alkylation was studied for the 6-aziridinylpyrrolo[1,2-*a*]benzimidazolequinones (PBIs) and the 6-acetamidopyrrolo[1,2-*a*]benzimidazolequinones (APBIs). The PBIs are reductively activated by DT-diaphorase and alkylate the phosphate backbone of DNA via major groove interactions, while the APBIs are reductively inactivated by this enzyme since only the quinone form inhibits topoisomerase II. Bulk at the 7-position (butyl instead of methyl) significantly decreases $k_{cat}/K_m$ for DT-diaphorase reductase activity for both PBIs and APBIs. As a result, a 7-butyl PBI has little cytotoxicity while the 7-butyl APBI has enhanced cytotoxicity. The type of 3-substituent and the configuration of the 3-position of the PBIs and APBIs influence DT-diaphorase substrate activity to a lesser degree. Bulk at the 7-position (butyl instead of methyl) had an adverse effect on APBI inhibition of topoisomerase II while the configuration of the 3-position had either an adverse or positive effect on inhibition of this enzyme. The configuration of the 3-position, when substituted with a hydrogen bond donor, influences the PBI reductive alkylation of DNA homopolymers. The rationale for this observation is that the *R* or *S* stereoisomers will determine if the 3-substituent points in the 3′ or 5′ direction and thereby influence the hydrogen-bonding interactions. The above findings were used to rationalize the relative cytotoxicity of various PBI and APBI derivatives.

The pyrrolo[1,2-*a*]benzimidazolequinone-based antitumor agents have been investigated in this laboratory for some time.[1−11] These agents possess a 7-methyl substituent and either a 6-acetamido group (APBIs) or a 6-aziridinyl group (PBIs), inset of Chart 1. The PBIs require two-electron reductive activation by DT-diaphorase as evident by the strong correlation between cytotoxicity and cellular levels of this enzyme.[9] The reduced PBI then alkylates DNA at the phosphate backbone.[11] In contrast, the APBIs are inactivated by two-electron reduction and therefore show a strong inverse correlation with DT-diaphorase levels (the highest inverse correlation of 20 000 compounds in the National Cancer Institute's archives).[9] The unchanged (oxidized) APBI, rather than the reduced form, acts as an inhibitor of the first step of topoisomerase II−mediated relaxation of supercoiled DNA.[4] The findings presented above prompted a study of the structural requirements for pyrrolo[1,2-*a*]benzimidazolequinone−DT-diaphorase substrate activity and topoisomerase II inhibition as well as a study of how structural changes influence the level of cytotoxicity.

Presented herein is such a study of the compounds **1**−**3** shown in Chart 1 along with a comparison of results with those obtained with previously reported PBIs and APBIs, inset of Chart 1. These previously reported PBIs and APBIs are designated as found in previous publications (i.e. APBI-A and PBI-A for the 3-acetate derivatives). The effects of the following three parameters were addressed in the study: steric bulk at the 7-position, the bulk of the 3-substituent, and the configuration of the 3-stereocenter. In addition, the ester in the 3-position was substituted with an amide group. It was anticipated that the bulk of the 7-substituent would have the greatest effect on the specificity

**Chart 1**




($k_{cat}/K_m$) for DT-diaphorase reductase activity. Indeed, the 7-butyl APBI derivatives **1a,b** were slowly reduced by the enzyme, resulting in enhanced cytotoxicity compared to the 7-methyl analogues. The 7-butyl PBI **2** was likewise slowly reduced by the enzyme, resulting in a complete loss of cytotoxicity. The bulk of the 3-substituent and the configuration of the 3-stereocenter has a less dramatic effect on DT-diaphorase reductase activity. An inverse correlation between the $k_{cat}/K_m$ for DT-diaphorase reductase activity and the level of cytotoxicity of the APBIs is consistent with reductive inactivation by the enzyme. In contrast, the cytotoxicity

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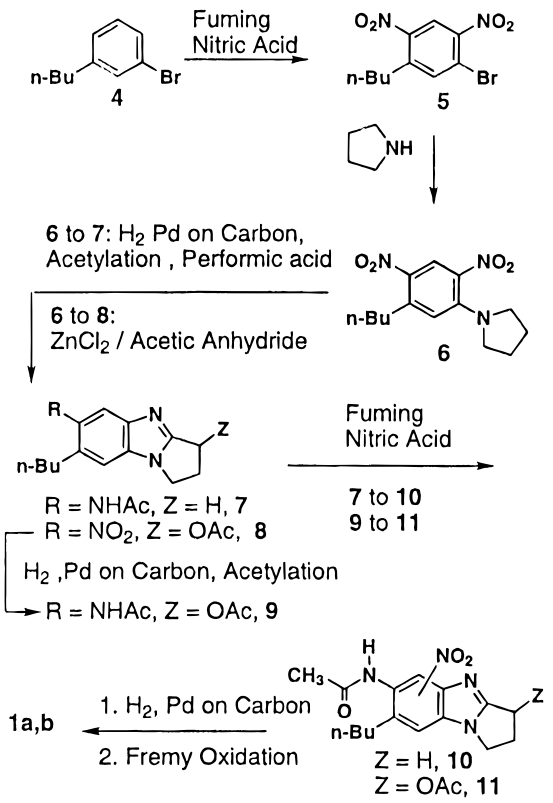
**Scheme 1**




of the PBIs directly correlates with the $k_{cat}/K_m$ for DT-diaphorase reductase activity, consistent with the reductive alkylation mechanism. The level of topoisomerase II inhibition was affected by the configuration of the 3-stereocenter of the APBI and the 7-substituent. As a result of this study, conclusions have been drawn with respect to the design of new APBI-based topoisomerase II inhibitors.

## Chemistry

The *n*-butyl derivatives **1** and **2** were prepared starting with the known compound **4**, Schemes 1 and 2. The synthetic steps shown in Scheme 1 are identical to steps previously carried out in this laboratory for the preparation of 7-methyl analogues.[2] The synthetic steps in Scheme 2 were likewise uneventful except for the preparation of **13** from **4**. Nitration of **4** afforded a mixture of **12** and two other mononitro isomers which could not be separated. The mixture was then treated with pyrrolidine to afford a mixture from which **13** was readily isolated. Verification of structure was made by the conversion of **13** to **14**, a transformation which requires ortho nitro and pyrrolidino substituents.

The racemic and *S*-(−) forms of **3** were prepared from the racemic and *S*-(−) forms of **18**, which were previously prepared in this laboratory,[1] Scheme 3. The synthetic steps leading to **3** did not result in racemization, as verified by chiral HPLC; see the Experimental Section.

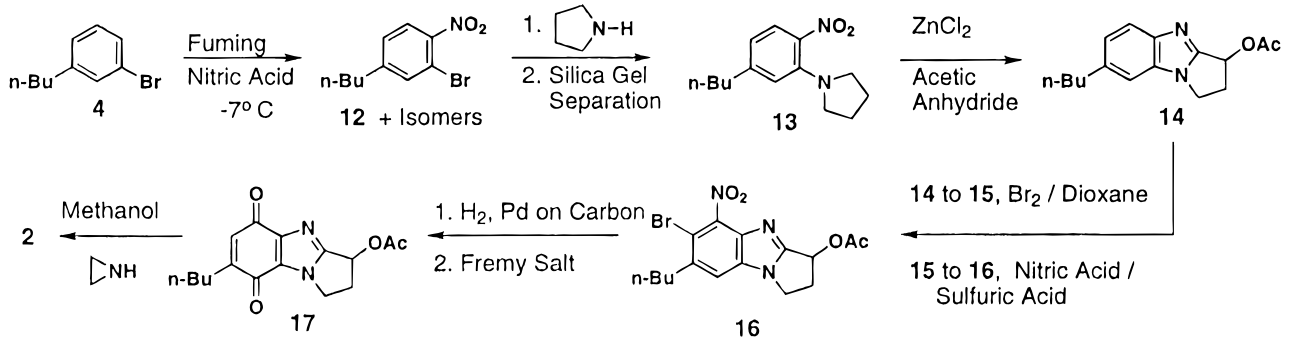
The *R*-(+) and *S*-(−) forms of APBI-A, inset of Chart 1, have been prepared from the *R* and *S* forms of the amino acid **26** (Scheme 4), employing an internal Phillips reaction.[12] Similarly, an internal Phillips reaction was employed in the unambiguous preparation of *R*-(+)- and *S*-(−)-PBI-A, also shown in the inset of Chart 1. Illustrated in Scheme 4 is the preparation of the enantiomers of **23**, which were readily converted to PBI-A by employing the synthetic sequence[2] illustrated in Scheme 3, starting with **19**. A nucleophilic aromatic substitution reaction of **25** with either enantiomer of **26** afforded *R*- or *S*-**27**. Catalytic reduction of the nitro group of **27** followed by acid-catalyzed condensation of the carboxylic acid group with the ortho diamine resulting from reduction (the Phillips reaction[12]) afforded *R*-(+)-**24** or *S*-(−)-**24**. Acetylation of the enantiomers of **24** afforded the enantiomers of intermediate **23**. Unfortunately, the yields of **23** from the amino acid incorporation method were too low, and therefore the enzymatic resolution of the enantiomers of **23** had to be employed for bulk synthesis. With the pure enantiomers of **23** and **24** in hand, optical rotations and retention times on chiral HPLC were employed to follow the course of enzymatic reduction and measure enantiomeric excesses (ee %). The acyl transfer enzyme *Candida antarctica* "B" lipase was observed to hydrolyse the *R*-(+) form of **23** much more rapidly than the *S*-(−) form. Treatment of racemic **23** with the lipase for 2 h afforded *R*-(+)-**24** (98% ee) along with an enantiomeric mixture of **23**. A 24 h reaction time afforded *S*-(−)-**23** (98% ee) along with an enantiomeric mixture of **24**.

## DT-Diaphorase Structure−Activity Studies

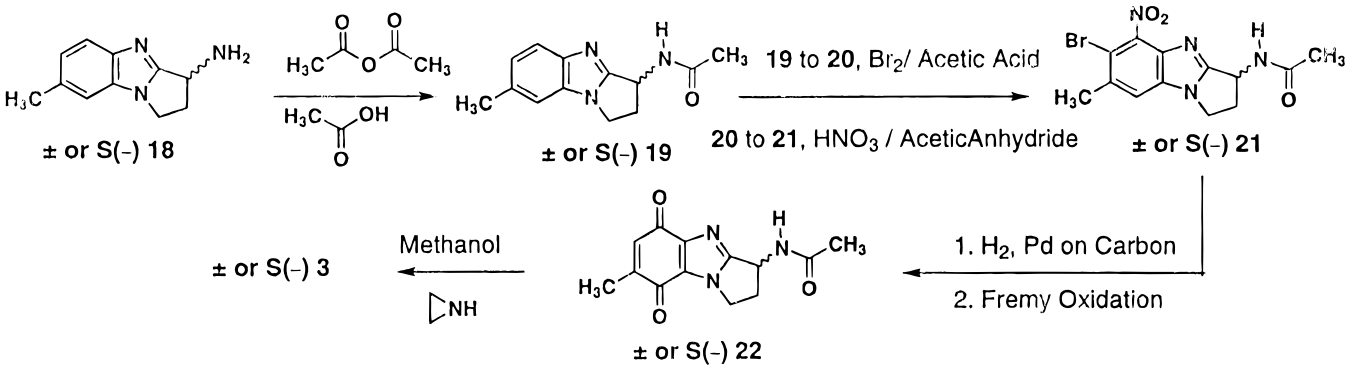
The goal of these studies was to determine the specificity ($k_{cat}/K_m$) of the APBI and PBI quinones for two-electron reduction by rat liver DT-diaphorase. The relative specificity of the quinones would provide insights into both the structure−activity relationship for quinone reduction and the mechanism for cytotoxicity. Since cytotoxicity also pertains to topoisomerase II inhibition (APBIs) or DNA alkylation (PBIs), the influences of structural changes in the APBIs and PBIs on these processes are discussed in the following sections.

Rat liver DT-diaphorase generally reduces quinones much more rapidly than the human enzyme.[13−15] In spite of high activity of the rat liver enzyme, the APBI

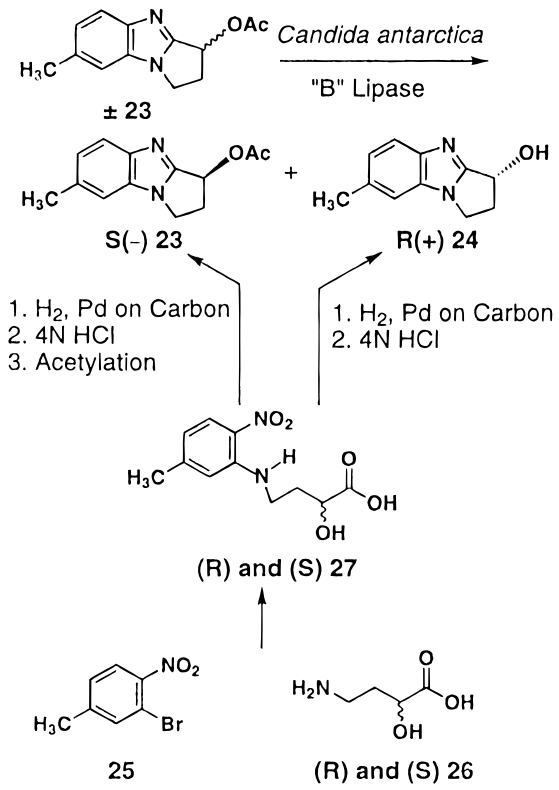
**Scheme 2**



**Scheme 3**




**Scheme 4**




and PBI quinones were found to possess a specificity ($k_{cat}/K_m$) up to 2 orders of magnitude less than those of commonly studied quinones (menadione and DZQ). In order to compensate for the slow rates of reduction, a relatively large concentration of enzyme was present in assay reactions (14.5 nM compared to 0.4−0.8 nM[14]), and the reactions were followed under strict anaerobic conditions in Thunberg cuvettes. Under these conditions, in the presence of excess NADH (0.3 mM), the quinone substrates were completely converted to hydroquinones, permitting the calculation of $\Delta\epsilon$ values at 296 nm for the two-electron reduction. Initial rate measurements and reciprocal (Lineweaver-Burk) plots provided values of $K_m$, $V_{max}$, and $k_{cat}/K_m$ values found inTables 1 and 2. The $k_{cat}/K_m$ values ($M^{-1}$ $s^{-1}$) were calculated from reciprocal plot data and the 14.5 mM of enzyme present in the reactions.

The first structural modification to be discussed is the conversion of 7-methyl to 7-butyl in the APBI (**1** and **2**) and PBI (**3**) quinones. Increasing the bulk at the quinone ring, where hydride transfer occurs from the reduced flavin cofactor, would be expected to decrease the specificity for reduction. Bulk about the quinone may interfere with the stacking interaction between the quinone and the reduced flavin as well as with the hydrogen bonding of tyrosine residues with one of the quinone oxygens. Both of these interactions have been documented to occur when duroquinone binds to the active site.[16]

The (±)-7-*n*-butyl APBI **1b** therefore has a specificity 3-fold less than the 7-methyl analogue (±)-APBI-A, Table 1, while the 7-*n*-butyl PBI (±)-**2** has a specificity 8.7-fold less than (±)-PBI-A, Table 2. The presence of the 7-*n*-butyl group decreases the inhibition of topoisomerase II by **1b**, but this group does not affect DNA reductive alkylation by **3**; see the following sections. Therefore the cytotoxicity differences noted (±)-**2** in Table 2 on changing 7-methyl to 7-*n*-butyl are largely attributed to differences in DT-diaphorase substrate specificity. Since (±)-**2** is reductively activated more slowly than (±) PBI-A, (±)-**2** possesses substantially less cytotoxicity than (±)-PBI-A in both cell lines shown in Table 2. In contrast, (±)-**1b** is reductively inactivated more slowly than (±)-APBI-A but has less inhibitory activity against topoisomerase II. The net effect of the *n*-butyl group appears to increase the cytotoxicity of (±)-**1b** compared to that of (±)-APBI-A, Table 2.

The low substrate specificity of APBIs and PBIs, compared to benzoquinone and naphthoquinone derivatives, is attributed to the steric hindrance of the fused pyrrolo ring.

The next structural modification to be discussed is the 3-substituent. Even though this substituent is far removed from the site of reduction, it can have a substantial effect on the specificity for DT-diaphorase. Previous studies with mitosenes,[17] which are structurally similar to the APBIs and PBIs, revealed that the configuration of the 1-substituent (equivalent to the pyrrolo[1,2-*a*]benzimidazole 3-substituent) influences the specificity for DT-diaphorase. Comparison of the specificities of **1a** and **1b** reveals that the presence of a 3-substituent results in a 4.8-fold decrease in specificity for DT-diaphorase. When the size of the 3-substituent is increased in APBIs, from acetate to propionate to benzoate, the specificity does not change ((±)-APBI-A:(±)-APBI-F:(±)-APBI-G possess $k_{cat}/K_m$ values of 1.73:1.8:1.98, respectively (see Table 1 for structures of these analogues)). The presence of a more polar 3-substituent, the methoxyacetate of (±)-APBI-I, results in a decrease in $k_{cat}/K_m$ to 1.09 $M^{-1}$ $s^{-1}$. This influence of the 3-substituent polarity on $k_{cat}/K_m$ is also apparent in the PBI series; when 3-acetate is changed to 3-acetamido there is a 4.4-fold decrease in specificity. Apparently,

**Table 1.** Parameters for DT-Diaphorase Substrate Activity and Melanoma and Ovarian Cancer $\log_{10}$ $LC_{50}$ (mol/L)Values for APBI Analogues

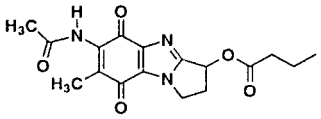
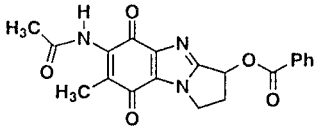
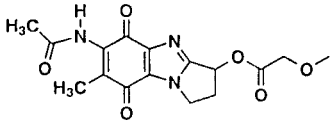
| Structure | $K_m \times 10^5$ | $V_{max}$ (nM $sec^{-1}$) | $K_{cat}/K_m \times 10^{-4}$ | Melanoma $Log_{10}$ $LC_{50}$ | Ovarian $Log_{10}$ $LC_{50}$ |
|---|---|---|---|---|---|
| **1a** | 4.5 | 18.1 | 2.77 | -5.05 | -4.68 |
| **1b** | 18.6 | 15.4 | 0.57 | -5.32 | -5.12 |
| (±)-APBI-A | 15.4 | 38.6 | 1.73 | -5.00 | -4.97 |
| R(+)-APBI-A | 12.0 | 14.6 | 0.84 | -4.97 | -4.66 |
| S(-)-APBI-A | 14.0 | 20.4 | 1.00 | -5.30 | -5.15 |
|  | | | | | |
| (±)-APBI-F | 15.9 | 42.3 | 1.8 | -5.23 | -5.19 |
|  | | | | | |
| (±)-APBI-G | 9.7 | 27.9 | 1.98 | -5.20 | -5.14 |
|  | | | | | |
| (±)-APBI-I | 6.2 | 9.8 | 1.09 | -4.69 | -4.66 |
| R(+)APBI-I | 5.5 | 16.7 | 2.09 | - | - |
| S(-)APBI-I | 20.7 | 28.2 | 0.94 | - | - |

**Table 2.** Parameters for DT-Diaphorase Substrate Activity and Melanoma and Ovarian Cancer $\log_{10}$ $LC_{50}$ (mol/L) Values for PBI Analogues

| structure | $K_m \times 10^5$ | $V_{max}$ (nM $s^{-1}$) | $k_{cat}/K_m \times 10^{-4}$ | melanoma $\log_{10}$ $LC_{50}$ | ovarian $\log_{10}$ $LC_{50}$ |
|---|---|---|---|---|---|
| (±)-PBI-A | 4.5 | 66.5 | 10.2 | $<-7.36$ | $-6.56$ |
| *R*-(+)-PBI-A | 3.9 | 83.4 | 14.7 | $-6.96$ | $-6.12$ |
| *S*-(−)-PBI-A | 5.5 | 62.6 | 7.8 | $-6.08$ | $-5.59$ |
| (±)-**2** | 5.0 | 8.5 | 1.17 | $-4.77$ | $-4.56$ |
| (±)-**3** | 2.5 | 8.3 | 2.3 | $-5.67$ | $-5.65$ |
| *S*-(−)-**3** | 4.5 | 2.2 | 3.4 | $-5.00$ | -4.94 |

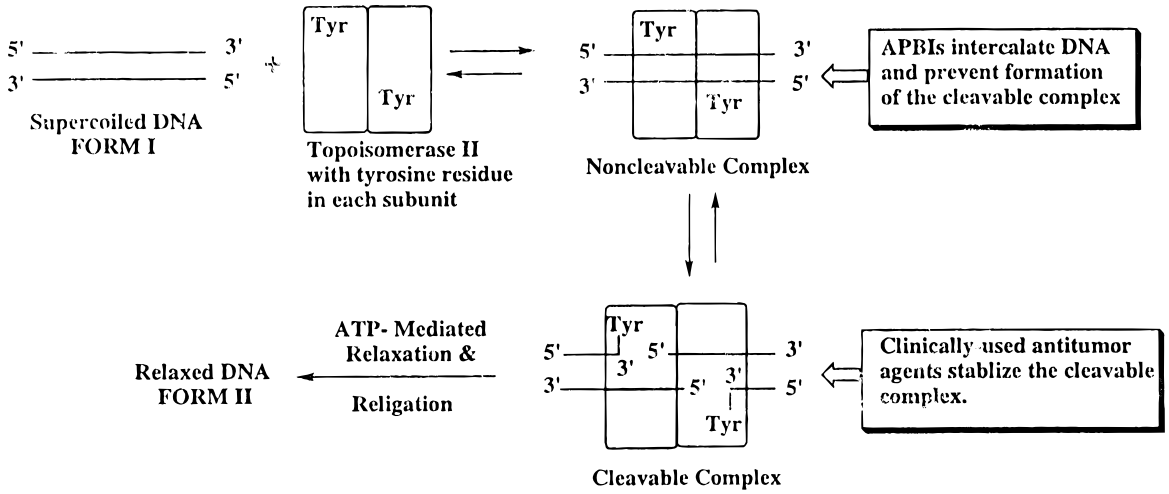
a more polar 3-substituent interferes somewhat with DT-diaphorase substrate specificity.

The 3-substituent of the APBIs interacts with the active site of topoisomerase II as well as with the active site of DT-diaphorase. Therefore correlations between $k_{cat}/K_m$ for DT-diaphorase substrate activity and cytotoxicity are not clear-cut. For example, the analogue (±)-APBI-I has a low specificity for the enzyme and low cytotoxicity. Recall that low substrate specificity for the enzyme resulted in high cytotoxicity for other APBI analogues (see beginning of this section).

The 3-substituent of the PBIs is known to influence the degree and base-pair specificity of DNA bioreductive alkylation.[9,11] The 3-acetate and 3-acetamido PBI substituents have identical influence on DNA bioreductive alkylation (see following sections). Therefore differences in cytotoxicity must pertain to differences in lipophilicity and the specificity for DT-diaphorase. A 16-fold decrease in cytotoxicity against the melanoma cell lines was observed when the racemic 3-acetate (PBI-A) was compared to the less lipophilic carbamate analogue.[9] The observed 48-fold decrease in cytotoxicity against the melanoma cell lines when the racemic 3-acetate (PBI-A) is changed to the racemic 3-acetamido ((±)-**3**) analogue must in part be due to the decreased specificity for DT-diaphorase.

The influence of the configuration of the 3-stereocenter on the substrate specificity for DT-diaphorase will now be considered. It was expected that one

**Scheme 5**




stereoisomer would have higher specificity for the enzyme than the other while the specificity of the racemate would be in between those of the stereoisomers. Both APBI-I and PBI-A fit this expectation with the *S* enantiomer possessing a lower specificity for DT-diaphorase than the *R* enantiomer. The *S* enantiomer of **3** possesses a higher specificity for the enzyme than the racemate (the *R* enantiomer of **3** has not yet been synthesized).

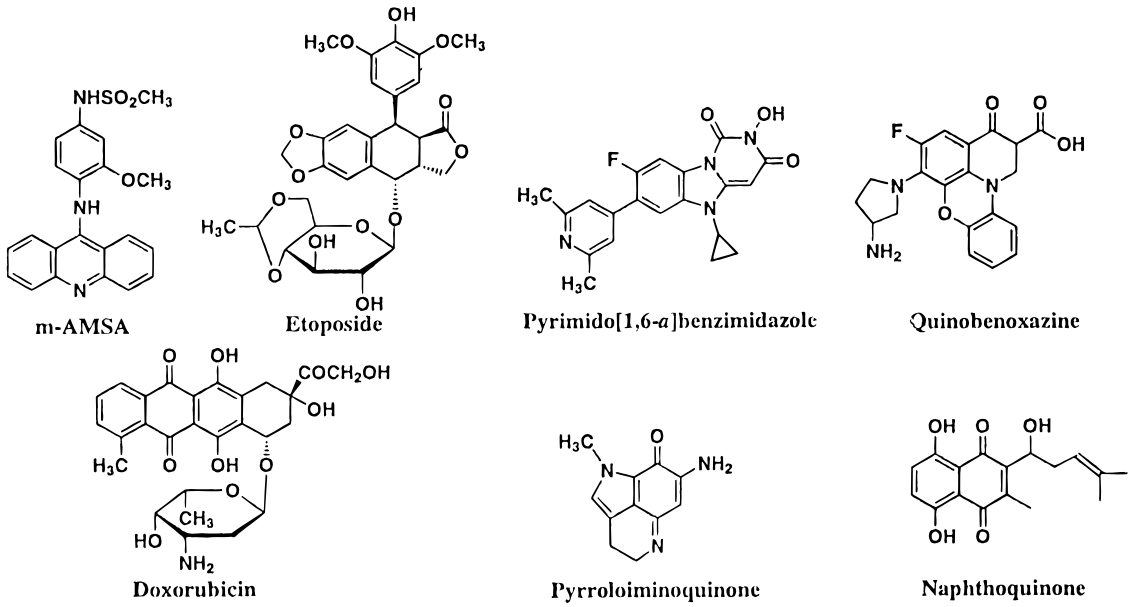
An unusual finding was noted for the APBI-A stereoisomers: The *R*-(+) and *S*-(−) enantiomers have nearly equal specificity for the enzyme while the racemate has a higher specificity for the enzyme than either enantiomer. An explanation for this observation is the presence of two substrates bound to the DT-diaphorase dimer resulting in either *RR*, *SS*, or *RS* forms of substrate−enzyme complex. The active sites of the DT-diaphorase dimer are located next to each other,[18] resulting in the stereocenters of the substrates "seeing each other". Therefore the use of pure enantiomers as substrates will result in an *RR* or *SS* complex, while the racemate will afford a diastereomeric *RS* complex which must be associated with a higher $k_{cat}/K_m$ than the enantiomeric forms.

In conclusion, the greatest effect of structural changes on the specificity for DT-diaphorase are substitution of a large group (*n*-butyl) on the quinone ring and the presence of a polar (methoxyacetate or acetamido) substituent in the 3-position. The configuration of the 3-position and the size of 3-substituents have small effects on specificity for the enzyme.

## Topoisomerase Inhibition Studies

Topoisomerases I and II cause relaxation of supercoiled DNA by single or double strand cleavage respectively to afford an enzyme−DNA complex followed by religation of the cleaved strands[19] after relaxation, Scheme 5. The activity of the topoisomerases is important in cell division, and therefore many types of cancer cells possess elevated levels of these enzymes.[20] Most topoisomerase II inhibitors of clinical value act at the religation step by stabilizing the enzyme−DNA complex formed upon double strand cleavage, the cleavable complex, Scheme 5. Examples of clinically used agents which stabilize the cleavable complex include *m*-AMSA, etoposide, and doxorubicin,[21−24] Scheme 6. Both *m*-AMSA and doxorubicin are intercalative inhibitors while etopside operates by a nonintercalative mechanism. In

**Scheme 6**



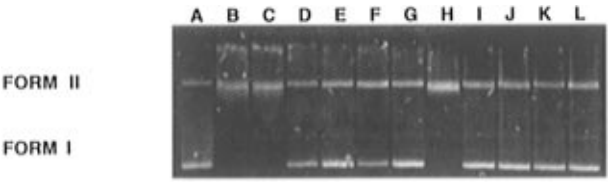



**Figure 1.** Effect of APBI-A and APBI-G on the relaxation of SV-40 supercoiled DNA by *Drosophila* topoisomerase II. A 1.3% agarose gel run in 0.045 M Tris·borate buffer with 1 mM EDTA was employed to assay the relaxation reactions. The topoisomerase relaxation reactions were carried out with 0.25 $\mu$g of SV-40 supercoiled (form I) DNA and10 units of topoisomerase II as described in the Experimental Section: lane A, control without enzyme; lane B, control with enzyme; lane C, 0.23 mM APBI-A; lane D, 0.48 mM APBI-A; lane E, 0.69 mM APBI-A; lane F, 0.954 mM APBI-A; lane G, 1.15 mM APBI-A; lane H, 0.15 mM APBI-G; lane I, 0.31 mM APBI-G; lane J, 0.45 mM APBI-G; lane K, 0.63 mM APBI-G; lane L, 0.75 mM APBI-G.

addition, Scheme 6 shows relatively new compounds which also stabilize this complex: the pyrimido[1,6-*a*]-benzimidazole,[25] the pyrrolo imino quinone,[26] and the naphthoquinone.[27] In contrast to the other agents above, the quinobenoxazine[28] acts at the noncleavable complex and inhibits the double-strand cleavage step leading to the cleavable complex.

The APBI topoisomerase II inhibitors have structural similarities to some of the compounds in Scheme 6, particularly the pyrimidobenzimidazole, pyrrolo imino quinone, and naphthoquinone analogues. Previous work showed that the APBIs intercalate DNA and prevent the noncleavable complex from proceeding to the cleavable complex and eventual formation of relaxed (form I) DNA, Scheme 5.[4,10] Shown in Figure 1 are inhibition assays of APBI derivatives bearing an acetate (APBI-A) and a benzoate (APBI-G) in the 3-position. These assays show that the supercoiled DNA (form I) builds up without apparent formation of linear DNA (form III) in the presence of 20 units of topoisomerase II. The use of such high concentrations of enzyme is required to see the linear DNA since this DNA is formed by proteinase digestion of the cleavable complex consisting of supercoiled DNA/APBI/topoisomerase II. The current view of APBI inhibition is that the APBI can slow the formation of the cleavable complex. Even the clinically used topoisomerase II inhibitors are thought to slow the formation of the cleavable complex as well as stabilize this complex; see discussion in ref 28.

To follow is a discussion of the structural features which influence the APBI mediated formation of the cleavable complex. The results shown in Figure 1 reveal that the size of the 3-substituent (acetate vs benzoate) does not influence the degree of inhibition. Even the absence of a 3-substituent in the APBI still resulted in inhibition of cleavable complex formation. In the presence of 10−20 units of topoisomerase II, the APBIs studied so far inhibit formation of cleavable complex at or above 0.3−0.5 mM concentrations. However, the *n*-butyl analogue (**1b**) is much less active than APBI-A, and only concentrations of **1b** greater than 1 mM caused inhibition of topoisomerase II. The *n*-butyl group very likely interferes with the intercalation of DNA, which is important in the inhibition of topoisomerase II. The substituent at the 3-position could interact with topoisomerase II while the benzimidazolequinone intercalates DNA. Therefore the configuration of the 3-position should influence topoisomerase II inhibition. The results provided below indicate that configuration does have a small effect on inhibition. The results for *R*-(+)- and *S*-(−)-APBI-I indicate that the *R*-(+) enantiomer inhibits relaxation at 4-fold lower concentrations than the *S*-(−) enantiomer. In contrast, the *S*-(−) form of APBI-A inhibits at 5-fold lower concentrations than the *R*-(+) enantiomer. The differences between APBI-A and APBI-I cited above may result from the hydrophobic/hydrophilic interactions between acetate and methoxyacetate and the enzyme.

The results of the DT-diaphorase and topoisomerase II studies suggest that *S*-(−)-APBI-A should be a good antitumor agent. This enantiomer has a specificity for DT-diaphorase lower than that of the racemate and is therefore more slowly inactivated by reduction. This feature and the higher activity of the *S*-(−) enantiomer against topoisomerase II likely account for its higher cytotoxicity than either the *R*-(+) enantiomer or the racemate against melanoma and ovarian cancer cell lines, Table 1. Currently *S*-(−)-APBI-A is undergoing *in vivo* screening at the National Cancer Institute.

## DNA Alkylation Studies

The PBIs are reductive alkylating agents of the DNA phosphate backbone.[5] Extensive structure−activity studies of the PBIs have been carried in this laboratory,[6,7,9−11] leading to the conclusions that the 4-nitrogen is required for the alkylation reaction[7] and that the 3-position influences the base-pair specificity.[9] The fused pyrrolo ring is not important for the alkylation reaction, and this ring can either be removed or homologated to a tetrahydropyrido ring.[6] These findings are consistent with the major groove binding model[4,5] wherein the reduced PBI hydrogen bonds to the major groove by utilizing the hydroquinone hydroxyl, the 4-nitrogen, and the 3-substituent. The fused pyrrolo ring points away from the major groove, and therefore this group does not influence the alkylation reaction. In the model, the 7-*n*-butyl substituent would also point away from the major groove and not influence the alkylation of DNA. In order to compare the 7-*n*-butyl (**2**) and the 7-methyl (PBI-A) analogues with respect to DNA alkylation, both compounds in their reduced forms were reacted with 600 bp calf thymus DNA; see the Experimental Section. The percent incorporation of PBI into DNA, based on one alkylation per base pair representing 100% alkylation, was calculated from absorbance measurements at 550 nm ($\epsilon = 785\ m^{-1}\ cm^{-1}$) for the PBI chromophore; see ref 11 for details. As expected from the major groove binding model, **2** and PBI-A reductively alkylated DNA to nearly the same degree, 10% and 13%, respectively. This observation suggests that the low cytotoxicity of **2** is largely due to the slow reductive activation by DT-diaphorase; see DT-Diaphorase Structure−Activity Studies.

Previous studies showed that the 3-carbamate PBI (PBI-B) reductively alkylated the DNA phosphate to a greater degree than any PBI bearing a 3-ester substituent.[4,9] This property of the 3-carbamate is attributed to a hydrogen-bonding interaction in the major groove not present with esters. Similarly, the 3-acetamido PBI (±)-**3** alkylates calf thymus DNA to a greater degree than PBI-A, 24% compared to 10%. If the 3-substituent interacts in the major groove of DNA, the configuration

**Table 3.** Percentages of PBI Incorporation into Various DNAs

| DNA | (±)-**3** | *S*-(−)-**3** | *R*-(+)-PBI-A | *S*-(−)-PBI-A |
|---|---|---|---|---|
| poly(dG) poly(dC) | 8.2 | 12.1 | | |
| poly(dA) poly(dT) | 41.5 | 32.7 | 5.9 | 8.5 |
| poly(dA-dT) poly(dT-dA) | 28.0 | 38.0 | | |

of the 3-position should influence base-pair specificity because the *R*- or *S* stereoisomers will determine if the 3-substituent points in the 3′ or 5′ direction. This possibility is discussed below in conjunction with Table 3.

Shown in Table 3 are DNA incorporation percentages for racemic and *S*-(−)-**3** as well as for *R*-(+)- and *S*-(−)-PBI-A. Racemic **3** and its *S*-(−) enantiomer show up to a 5-fold preference for A-T base pairs over G-C base pairs. The explanation for this preference is hydrogen bonding of the amide N-H with a thymine carbonyl. Preferences for A-T are also seen in the 3-carbamate[4,9] as well as the 3-ureido (unpublished result) derivative. When only an ester is present in the 3-position, as in PBI-A, the amount of DNA reductive alkylation decreases and there is equal preference for A-T and G-C base pairs [see PAGEs (polyacrylamide gels) on page 3052 of ref 5]. The configuration of the 3-stereocenter influences base-pair specificity very little for PBI-A and **3** (at G-C base pairs). In these cases there are only small differences in small percentages for incorporation into DNA, Table 3. In the case of **3**, reductive alkylation of poly(dA)·poly(dT) and the alternating polymer poly(dA-dT)·poly(dT-dA) show a definite enantiomeric selectivity. Racemic **3** reductively alkylates poly(dA)·poly(dT) to a greater degree than the *S*-(−) enantiomer, which suggests that the *R*-(+) enantiomer shows a preference for this DNA. In contrast, the *S*-(−) enantiomer of **3** alkylates an alternating polymer to a greater degree than the *R*-(+) enantiomer. The higher cytotoxicity of (±)-**3** against melanoma and ovarian cancer cell lines (Table 2) compared to the *S*-(−) enantiomer could be due to the greater selectivity of the *R*-(+) enantiomer for a run of A-T base pairs. A-T rich DNA is known to be the target of other antitumor agents such as CC-1065[29] and the distamyin and netropsin[30] based systems.

## Conclusions

The influence of APBI and PBI structure on DT-diaphorase substrate specificity, topoisomerase II inhibition, and DNA reductive alkylation was investigated to determine the structural requirements for cytotoxicity. The particular structural elements of interest include the 7-substituent, the 3-substituent, and the configuration of the 3-position. The conclusions of this investigation are of value in the design of new APBI and PBI antitumor agents.

The change from 7-methyl to 7-butyl has a profound effect on both DT-diaphorase substrate specificity and topoisomerase II inhibition. The bulk of the 7-butyl substituent significantly slows the enzymatic reduction of both APBI and PBI systems. The slow reduction should enhance the cytotoxicity of APBIs which are inactivated by reduction. However, the 7-butyl substituent decreases the ability of APBIs to inhibit topoisomerase II. The net result of these opposing effects of the 7-butyl substituent is an increase in cytotoxicity for **1b** compared to the 7-methyl analogue APBI-A. The *n*-butyl substituent does not influence the reductive alkylation of DNA by **2**. Therefore the loss of cytotoxicity exhibited by **2**, compared to the 7-methyl analogue PBI-A, is due to the slow reductive activation. The conclusion is that the 7-butyl substituent confers some cytotoxic advantage in the APBIs.

The bulk of the APBI 3-substituent does not affect the specificity for DT-diaphorase or topoisomerase II inhibition. Previous studies showed that the lipophilicity of the 3-substituent is important in APBI cytotoxicity.[9] Given the absence of a significant effect of the 3-substituent on the enzyme systems studied, future APBI analogues will bear 3-substituents of appropriate lipophilicity. Previous studies showed that a hydrogen-bonding donor (carbamate) at the 3-position of a PBI resulted in reductive alkylation predominately at AT base pairs.[9,11] Consistent with this finding, the 3-acetamido PBI derivative **3** exhibited up to a 5-fold preference for AT base pairs over the 3-acetate derivative (PBI-A). Furthermore, **3** and the carbamate derivative reductively alkylate DNA to a greater degree than a 3-ester derivative. It is concluded that PBIs bearing a 3-substituent with hydrogen bond donor capability will be efficient reductive alkylating agents of DNA and show high A-T base pair specificity.

The configuration of the 3-position is important in the cytotoxicity of both the PBIs and APBIs because the configuration influences the interaction with both DT-diaphorase and topoisomerase II. The high cytotoxicity of *S*-(−)-APBI-A, compared to the *R*-(+) enantiomer and racemate, is due to the low specificity of the *S*-(−) enantiomer for reductive inactivation by DT-diaphorase as well as the more potent inhibition of topoisomerase II by this enantiomner. The high cytotoxicity of *R*-(+)-PBI-A, compared to its enantiomer, is due to the high specificity of the *R*-(+) enantiomer for reductive activation by DT-diaphorase.

It is concluded that the configuration of the 3-position is important in PBI and APBI cytotoxicity, and therefore the resolution of enantiomers will be a part of future analogue development.

The configuration of the 3-position does not influence the reductive alkylation of DNA by PBIs if an ester substituent is present at this position. However, the studies with (±)-**3** and its *S*-(−) enantiomer suggest that configuration at the 3-position becomes important in DNA reductive alkylation if a hydrogen bond donor is present. The results of this study suggests that *R*-(+)-**3** has a high selectivity for A-T base pairs and therefore has greater cytotoxicity than the *S*-(−) enantiomer.

## Experimental Section

All solutions and buffers for kinetic, DNA, and electrophoresis studies used doubly distilled water. All analytically pure compounds were dried under high vacuum in a drying pistol over refluxing toluene. Elemental analyses were run at Atlantic Microlab, Inc., Norcross, GA. All TLCs were performed on silica gel plates using a variety of solvents and a fluorescent indicator for visualization. IR spectra were taken as thin films and the strongest absorbances reported. $^1$H NMR spectra were obtained from a 300 MHz spectrometer. All chemical shifts are reported relative to TMS. Optical rotations were obtained from a Perkin-Elmer polarimeter employing a 10-cm quartz cell, 0.2 g/dL enantiomer in methanol. Enamtiomeric purity was determined using optical rotations and

chiral HPLC, see Chiral High-Pressure Liquid Chromatography in this section. Purified topoisomerase II (*Drosophila melanogaster*) was purchased from United States Biochemical; activity: 20 units/mL. SV-40 DNA was purchased from Gibco BRL (Life Technologies). NADH and reagents employed in affinity column preparation were purchased from Sigma. *Candida antarctica* "B" lipase (Altus 13) was purchased from Altus Biologics, Inc.

**5-Butyl-2,4-dinitro-1-*N*-pyrrolidinobenzene (6).** The synthesis of **6** was carried out in the two-step process described below.

To 10 mL of fuming nitric acid, chilled in an ice bath, was added 1.99 g (9.33 mmol) of 1-bromo-5-butylbenzene (**4**) over a period of 20 min. The mixture was stirred at room temperature for 14 h and then poured over ice. The resulting mixture was extracted five times with 10 mL portions of chloroform. The extracts were combined and dried ($Na_2SO_4$), and the solvent was removed by evaporation. The product (**5**) was isolated as an orange oil, which was carried on to the next step.

A mixture of 3.30 mL (39.4 mmol) of pyrrolidine, 20 mL of ethanol, and crude **5** obtained above were refluxed for 3 h. The ethanol was then removed by evaporation. The residue was dissolved in chloroform and washed five times with 25 mL portions of 1 N HCl. The organic layer was dried ($Na_2SO_4$) and the chloroform removed by evaporation. The residue was placed on a silica gel flash column, and the product was eluted with hexane/chloroform (80:20). Evaporation of the eluant afforded a yellow residue. Recrystallization from hexane produced fine yellow crystals of **6**: 1.51 g (55%) yield from **4**; mp 93.5−94 °C; TLC (chloroform/hexane, 70:30) $R_f = 0.54$; IR (KBr pellet) 2986, 2957, 2928, 2872, 2859, 1607, 1566, 1503, 1464, 1370, 1315, 1285 $cm^{-1}$; $^1H$ NMR (dimethyl sulfoxide-$d_6$) $\delta$ 8.54 and 6.99 (2H, 2s, C(3) and C(6) aromatic protons), 3.28 (4 H, m, pyrrolidine protons next to nitrogen), 2.97 (2H, dd, $J \approx 7.6$ Hz, C(1′)), 1.94 (4H, m, other pyrrolidine protons), 1.54 (2 H, quintet, $J = 7.3$ Hz, C(2′), 1.38 (2H, sextet, $J = 7.5$ Hz, C(3′)), 0.92 (3 H, t, $J = 7.3$ Hz C(4′)); mass spectrum (EI mode), $m/z$ 293 ($M^+$). Anal. ($C_{14}H_{19}N_3O_4$) C, H, N.

**6-Acetamido-7-butyl-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]benzimidazole (7)** was prepared from **6** according to the following two-step procedure: A suspension of 1.0 g (3.41 m mol) of **6** and 0.24 g of 5% Pd on charcoal in 100 mL of methanol was shaken under 50 psi of $H_2$ for 3.5 h. The mixture was then filtered through Celite. The filtrate was added to a solution of 10 mL of acetic anhydride and 5 mL of acetic acid. This solution was stirred at room temperature for 2.5 h, and then the solvent was evaporated *in vacuo*. The oily residue was dried under high vacuum and triturated with ether to afford 2,4-diacetamido-5-butyl-2-*N*-pyrrolidinobenzene. Yield of crude product, suitable for the next step, was 0.973 g (90%). An analytical sample was prepared by recrystallization from chloroform: mp 179 °C dec; TLC (chloroform/methanol, 85:15) $R_f = 0.60$; IR (KBr pellet) 3250, 2957, 2932, 2870, 2816, 1651, 1527, 1420, 1370, 1281 $cm^{-1}$; $^1H$ NMR (dimethyl sulfoxide-$d_6$) $\delta$ 9.11 and 9.07 (2H, 2s, amide protons), 7.14 and 6.63 (2H, 2s, aromatic), 3.13 (4H, t, $J = 6.12$ Hz pyrrolidine protons next to nitrogen), 2.45 (2H, t, $J = 7.63$ Hz, C(1′)), 2.00 and 1.97 (6H, 2s, amide methyls), 1.85 (4H, m, other pyrrolidine methylenes), 1.45 (2H, quintet, $J = 7.71$ Hz, C(2′), 1.29 (2H, sextet, $J = 7.37$ Hz, C(3′)), 0.88 (3H, t, $J = 7.22$ Hz C(4′)); mass spectrum (EI mode), $m/z$ 317 ($M^+$), 274 ($M^+$ − acetyl), 259 ($M^+$ − acetamido).

A mixture of 0.30 g (0.95 mmol) of the diacetamido derivative obtained above, 1.6 mL formic acid, and 0.8 mL of 30% hydrogen peroxide was stirred at 70−75 °C for 1 h. The cooled reaction mixture was then neutralized with sodium bicarbonate. The neutralized solution was extracted with 4 × 50 mL portions of chloroform. The extracts were combined and dried (sodium sulfate), and the solvent was removed *in vacuo*. Trituration of the residue in chloroform with a small volume of hexane afforded crude **7** in 0.16 g (65%) yield. An analytically pure sample was prepared by silica gel flash chromatography eluting with chloroform: mp 177−179 °C; TLC (chloroform/methanol, 80:20) $R_f = 0.62$; IR (KBr pellet) 3250, 2957, 2931, 2870, 1651, 1613, 1528, 1420, 1370, 1281 $cm^{-1}$; $^1H$ NMR (dimethyl sulfoxide-$d_6$) $\delta$ 9.27 (1H, s, amide proton), 7.38 and 7.24 (2H, 2s, aromatic protons), 4.06 (2H, t, $J = 6.94$ Hz, C(3) methylene), 2.91 (2H, t, $J = 7.23$ Hz, C(1) methylene), 2.62 (4H, m, C(2) and C (1′) methylene protons), 2.03 (3H, s, acetate methyl), 1.51 (2H, quintet, $J = 7.74$ Hz, C(2′)), 1.33 (2H, sextet, $J = 7.37$ Hz, C(3′)), 0.90 (3H, t, $J = 7.29$ Hz C(4′)); mass spectrum (EI mode), $m/z$ 271 ($M^+$), 228 ($M^+$ − acetyl). Anal. ($C_{16}H_{21}N_3O \cdot 0.5H_2O$) C, H, N.

**7-Butyl-6-nitro-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]benzimidazol-3-yl Acetate (8).** A mixture containing 0.5 (1.7 mmol) of **6**, 0.45 g (3.30 mmol) of $ZnCl_2$, and 5 mL of acetic anhydride was refluxed at 150 °C for 6 h. The reaction mixture was cooled and combined with 50 mL of water. The mixture was then extracted with 3 × 50 mL portions of chloroform. The extracts were combined, dried ($Na_2SO_4$), and the solvent removed *in vacuo*. The residue was placed on a silica gel flash column, and the product was eluted off with ethyl acetate. Recrystallization with chloroform/hexane afforded pure **8**: 0.119 g (22% yield); mp 98−99 °C; TLC (ethyl acetate) $R_f = 0.35$; IR (KBr pellet) 2974, 2862, 1745, 1629, 1525, 1458, 1334, 1217, 1084, 977 $cm^{-1}$; $^1H$ NMR (dimethyl sulfoxide-$d_6$) $\delta$ 8.23 (1H, s, C(5)), 7.63 (1H, s, C(8)), 6.13 (1H, dd, $J = 7.50$ Hz, $J = 3.75$ Hz, C(3)), 4.28 and 4.17 (2H, 2m, C(1) diastereomeric methylene), 3.11 and 2.57 (2H, 2m, C(2) diastereomeric methylene), 2.89 (2H, t, $J = 7.77$ Hz, C(1′)), 2.04 (3H, s, acetate methyl), 1.55 (2H, quintet, $J = 7.47$ Hz, C(2′), 1.32 (2H, sextet, $J = 7.23$ Hz, C(3′)), 0.87 (3H, t, $J = 7.32$ Hz, C(4′)); mass spectrum (EI mode), $m/z$ 317 ($M^+$), 300 ($M^+$ − OH), 274 ($M^+$ − acetyl), 258 ($M^+$− acetate). Anal. ($C_{16}H_{19}N_3O_4$) C, H, N.

**6-Acetamido-7-butyl-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]benzimidazol-3-yl Acetate (9).** A suspension of 0.394 g (1.24 mmol) of **8** and 79 mg of 5% Pd on charcoal in 60 mL of methanol was shaken under 50 psi of $H_2$ for 3 h. The catalyst was removed by filtering the solution through Celite, and the filtrate was added to a flask containing 20 mL of acetic acid and 4.0 mL of acetic anhydride. The resulting solution was stirred at room temperature for 2 h. The solvent was removed *in vacuo*, and the residue was triturated with ether. Recrystallization with ethyl acetate afforded analytically pure **9**: mp 191 °C; TLC (chloroform/methanol, 80:20) $R_f = 0.66$; IR (KBr pellet) 3244, 2960, 2874, 1743, 1647, 1529, 1371, 1234, 1030 $cm^{-1}$; $^1H$ NMR (dimethyl sulfoxide-$d_6$) $\delta$ 9.28 (1H, s, amide proton), 7.45 and 7.32 (2H, 2s, aromatic protons), 6.06 (1H, dd, $J = 7.62$ Hz, $J = 3.27$ Hz, C(3)), 4.14 (2H, m, C(1)), 3.08 and 2.53 (2H, 2m, C(2)-diastereomeric methylene), 2.61 (2H, t, $J = 7.65$ Hz, C(1′)), 2.03 and 2.00 (6H, 2s, acetamido and acetate methyl), 1.48 (2H, $J = 7.53$ Hz, C(2′)), 1.29 (2H, sextet, $J = 7.35$ Hz, C(3′)), 0.865 (3H, t, $J = 7.29$ Hz, C(4′)); mass spectrum (EI mode), $m/z$ 329 ($M^+$), 286 ($M^+$ − acetyl), 270 ($M^+$ − acetate), 244. Anal. ($C_{18}H_{23}N_3O_3$) C, H, N.

**6-Acetamido-7-butyl-5-nitro-2,3-dihydro-1*H*-pyrrolo-[1,2-*a*]benzimidazole (10).** To a mixture of 4.5 mL of fuming nitric acid and 0.5 mL of concentrated surfuric acid, chilled at 0 °C, was added 0.143 g (0.527 mmol) of **7**. The resulting solution was stirred for 6 min and then poured over 50 mL of ice. The solution was neutralized with sodium bicarbonate and then extracted with 5 × 50 mL portions of chloroform. The extracts were combined and dried (sodium sulfate), and the solvent was evaporated *in vacuo*. Recrystallization was carried out from chloroform/hexane. Purified product was obtained by silica gel flash chromatography employing chloroform/methanol (95:5) as the eluant: 0.110 g (66%) yield; mp 210−211 °C; TLC (chloroform/methanol, 80:20) $R_f = 0.65$; IR (KBr pellet) 3229, 3175, 3007, 2957, 2932, 2870, 1684, 1520, 1450, 1358, 1267 $cm^{-1}$; $^1H$ NMR (dimethyl sulfoxide-$d_6$) $\delta$ 9.68 (1H, br s, amide proton), 7.62 (1H, s, aromatic proton), 4.16 (2H, t, $J = 7.04$ Hz, C(1) methylene), 2.98 (2H, t, $J = 7.62$ Hz, C(3) methylene), 2.65 (4H, m, C(2) and C(1′) methylene), 1.52 (2H, quintet, $J = 7.78$, Hz, C(2′) methylenes), 1.32 (2H, sextet, $J = 7.37$ Hz, C(3′) methylene), 0.90 (3H, t, $J = 7.28$ Hz, C(4′) methylene); mass spectrum (EI mode), $m/z$ 316 ($M^+$), 270 ($M^+$ − $NO_2$), 227 ($M^+$ − $NO_2$ − acetyl). Anal. ($C_{16}H_{20}N_4O_3$) C, H, N.

**6-Acetamido-7-butyl-5-nitro-2,3-dihydro-1*H*-pyrrolo-[1,2-*a*]benzimidazol-3-yl Acetate (11).** To a mixture of 9 mL of fuming nitric acid and 1 mL of concentrated surfuric

acid, chilled at −8 °C, was added 250 mg (0.761 mmol) of **9**. The reaction mixture was stirred for 20 min at ice bath temperature and then poured into a mixture of 50 g of ice and 50 mL of chloroform. The chloroform layer was removed, and the aqueous layer was extracted with 5 × 30 mL portions of chloroform. The extracts were combined and dried ($Na_2SO_4$), and the solvent was removed *in vacuo*. The residue was recrystallized from chloroform and hexane to afford yellow crystals of **11**: 191 mg (67%) yield; mp 199−200 °C; TLC (chloroform/methanol, 95:5) $R_f$ = 0.35; IR (KBr pellet) 3167, 2955, 1749, 1678, 1525, 1460, 1363, 1234, 1084, 1058 $cm^{-1}$; $^1H$ NMR (dimethyl sulfoxide-$d_6$) $\delta$ 9.73 (1H, s, amide proton), 7.72 (1H, s, aromatic proton), 6.11 (1H, dd, $J$ = 7.7 Hz, $J$ = 3.6 Hz, C(3)), 4.29 and 4.18 (2H, 2m, C(1)-diastereomeric methylene), 3.10 and 2.55 (2H, 2m, C(2)-diastereomeric methylene), 2.64 (2H, t, $J$ = 7.4 Hz, C(1′)) 2.05 and 1.97 (6H, 2s, acetamido and acetate protons), 1.49 (2H, quintet, $J$ = 7.29 Hz, C(2′)), 1.29 (2H, sextet, $J$ = 7.35 Hz, C(3′)), 0.866 (3H, t, $J$ = 7.29 Hz, C(4′)); mass spectrum (EI mode), $m/z$ 374 ($M^+$), 356 ($M^+ - H_2O$), 328 ($M^+ - NO_2$), 254 ($M^+ - H_2O$ − ketene − acetic acid). Anal. ($C_{18}H_{22}N_4O_5$) C, H, N.

**6-Acetamido-7-butyl-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]benzimidazole-5,8-dione (1a)**. The synthesis of **1a** was carried out in a two-step process described below.

A suspension of 0.109 g (0.344 mmol) of **10** in 50 mL of methanol with 0.023 g of Pd on carbon was shaken under 50 psi of $H_2$ for 3 h. The solution was filtered through Celite and the solvent evaporated *in vacuo*. After being washed through the Celite, a solution of 0.085 g of potassium phosphate monobasic in 10 mL of water was added to the residue. To this mixture was added another solution consisting of 0.2 g of potassium phosphate monobasic and 0.5 g of Fremy salt in 45 mL of water. The resulting purple solution was stirred at room temperature for 4 h and then extracted with 6 × 50 mL portions of chloroform. The extracts were combined and dried (sodium sulfate), and the solvent was evaporated *in vacuo*. The concentrated oil was then flash chromatographed, employing silica gel with ethyl acetate/methanol (90:10) as eluant. Pure product was obtained by recrystallization from chloroform/hexane: 0.0462 g (44%) yield; mp 176 °C dec; TLC (chloroform/methanol, 80:20) $R_f$ = 0.67; IR (KBr pellet) 3237, 3173, 3136, 2996, 2957, 2932, 2870, 1653, 1518, 1267 $cm^{-1}$; $^1H$ NMR (dimethyl sulfoxide-$d_6$) $\delta$ 9.46 (1H, br s, amide proton), 4.17 (2H, t, $J$ = 7.12 Hz, C(1) methylene), 2.86 (2H, t, $J$ = 7.50 Hz, C(3) methylene), 2.61 (2H, m, C(2) methylene), 2.36 (2H, t, $J$ = 7.4 Hz, C(1′) methylene), 1.3 (4H, m, C(2′) and C(3′) methylenes), 0.856 (3H, t, $J$ = 7.1 Hz, C(4′) methylene); mass spectrum (EI mode), $m/z$ 301 ($M^+$). Anal. ($C_{16}H_{19}N_3O_3$) C, H, N.

**6-Acetamido-7-butyl-2,3-dihydro-5,8-dioxo-1*H*-pyrrolo-[1,2-*a*]benzimidazol-3-yl Acetate (1b).** A suspension of 0.102 g (0.272 mmol) of **11** and 20 mg of 5% Pd on charcoal in 80 mL of methanol was shaken at 50 psi of $H_2$ for 3 h. The solution was then filtered through Celite and concentrated to a residue. A solution containing 20 mL of water and 85 mg of potassium phosphate monobasic was washed through the Celite and then added to the residue. To this solution was added a solution consisting of 45 mL of water, 200 mg of potassium phosphate monobasic, and 500 mg of Fremy salt. The resulting mixture was stirred at room temperature for 5 h, and then it was extracted with 8 × 25 mL portions of chloroform. The combined extracts were dried ($Na_2SO_4$), and the solvent was removed *in vacuo*. Pure product was obtained by silica gel chromatography, employing ethyl acetate/chloroform (80:20) as the eluant. Recrystallization from chloroform and hexane afforded yellow crystals of **1b**: 25 mg (26%) yield; mp 178−179 °C; TLC (chloroform/methanol, 95:5) $R_f$ = 0.55; IR (KBr pellet) 2960, 1745, 1658, 1608, 1521, 1438, 1371, 1319, 1236, 1035 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 7.53 (1H, s, amide proton), 6.06 (1H, dd, $J$ = 7.55 Hz, $J$ = 3.0 Hz, C(3)), 4.24 (2H, m, C(1)), 3.14 and 2.63 (2H, 2m, C(2)-diastereomeric methylene), 2.51 (2H, t, $J$ = 6.93 Hz, C(1′)), 2.21 and 2.08 (6H, 2s, acetamido and acetate methyls), 1.39 (2H, quintet, $J$ = 7.08 Hz, C(2′)), 1.28 (2H, sextet, $J$ = 7.26 Hz, C(3′)), 0.862 (3H, t, $J$ = 7.23 Hz, C(4′)); mass spectrum (EI mode), $m/z$ 359 ($M^+$), 317 ($M^+$ − ketene), 257 ($M^+$ − acetate − ketene). Anal. ($C_{18}H_{21}N_3O_5$) C, H, N.

**4-Butyl-1-nitro-2-*N*-pyrrolidinobenzene (13).** The conversion of **4** to **13** was carried out by a two-step process described below. A total of 9.98 g (0.0468 mol) of **4** was divided in half and added to two 20 mL portions of chilled (−8 °C) fuming nitric acid over a 20 min period. The resulting reaction mixtures were stirred at −5 °C for an additional 10 min, and then they were poured over ice. Both mixtures were extracted with 5 × 50 mL portions of chloroform. All extracts were combined and dried (sodium sulfate), and the solvent was removed *in vacuo*. The mixture of mononitrated isomers of **12** was carried on to the next step without further purification.

A solution of 15 mL of pyrrolidine, 60 mL of ethanol, and the isomers of **12** was refluxed for 3.5 h. The reaction mixture was cooled, and the solvent was removed *in vacuo*. The residue was dissolved in 100 mL of chloroform and extracted with 3 × 40 mL portions of 1 N HCl. The extracts were combined, dried ($Na_2SO_4$), and concentrated to a residue, which was chromatographed on silica gel, employing hexane/chloroform (80:20) as the eluant. Evaporation of the solvent afforded **13** as an oil: 3.03 g (26%) overall yield. An analytically pure sample was obtained by sublimation: TLC (chloroform) $R_f$ = 0.66; IR (KBr pellet) 2957, 2872, 2361, 1616, 1568, 1506, 1361, 1282, 1010, 829 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 7.66, 6.66, and 6.52 (3H, ABX, $J_{ortho}$ = 8.46 Hz, $J_{meta}$ = 1.68 Hz, $J_{para} \approx 0$ Hz, C(6), C(3), and C(5) aromatic protons, respectively), 3.18 (4H, quintet, $J$ = 3.93 Hz, pyrrolidine methylenes adjacent to N), 2.56 (2H, t, $J$ = 7.7 Hz, C(1′)), 1.93 (4H, quintet, $J$ = 3.51 Hz, other pyrrolidine methylenes), 1.56 (2H, m, C(2′)), 1.33 (2H, sextet, $J$ = 7.2 Hz, C(3′)), 0.898 3H, t, $J$ = Hz, C(4′)); mass spectrum (EI mode), $m/z$ 248 ($M^+$), 231 ($M^+$ − OH), 201 ($M^+$ − H − $NO_2$). Anal. ($C_{14}H_{20}N_2O_2$) C, H, N.

**7-Butyl-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]benzimidazol-3-yl Acetate (14).** A mixture consisting of 4.4 g (17.7 mmol) of **14**, 2.5 g (18.3 mmol) of $ZnCl_2$, and 70 mL of acetic anhydride was refluxed at 100−110 °C for 5 h. The reaction mixture was cooled and then combined with 150 mL of water. The resulting solution was extracted with 5 × 100 mL portions of chloroform. The extracts were combined, dried ($Na_2SO_4$), and concentrated *in vacuo*. Chromatography of the residue on a silica gel flash column employing chloroform/methanol (95:5) as the eluant afforded crude **14** in 2.0 g (41%) yield. Recrystallization from chloroform and hexane afforded analytically pure **14**: mp 141−141.5 °C; TLC (chloroform/methanol, 95:5) $R_f$ = 0.38; IR (KBr pellet) 2955, 2931, 2858, 1747, 1622, 1537, 1373, 1249, 1051, 814 $cm^{-1}$; $^1H$ NMR (dimethyl sulfoxide-$d_6$) $\delta$ 7.49, 7.31 and 7.03 (3H, ABX, $J_{ortho}$ = 8.34 Hz, $J_{meta}$ = 1.65 Hz, $J_{para} \approx 0$ Hz, C(5), C(8) and C(6) aromatic protons, respectively), 6.07 (1H, dd, $J$ = 7.56 Hz, $J$ = 3.27 Hz, C(3)), 4.14 (2H, m, C(1)), 3.06 and 2.54 (2H, 2m, C(2) diasteriomeric methylene), 2.03 (3H, s, acetate methyl), 1.57 (2H, quintet, $J$ = 7.59 Hz, C(2′)), 1.28 (2H, sextet, $J$ = 1.53 Hz, C(3′)), 0.863 (3H, t, $J$ = 7.29 Hz, C(4′)); mass spectrum (EI mode), $m/z$ 272 ($M^+$), 229 ($M^+$ − acetyl). Anal. ($C_{16}H_{20}N_2O_2 \cdot 0.75H_2O$) C, H, N.

**6-Bromo-7-butyl-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]benzimidazol-3-yl Acetate (15).** A mixture containing 0.972 g (3.57 mmol) of **14**, 13 mL of dioxane, 1.14 g of $K_2CO_3$, and 2.7 mL of 1 M bromine in dioxane was stirred at room temperature for 30 min. At this time, another 2.6 mL of the 1 M bromine solution was added to the mixture followed by stirring for 50 min at room temperature. The reaction mixture was then combined with 150 mL of water and then extracted with 5 × 100 mL portions of chloroform. The extracts were combined and dried ($Na_2SO_4$), and the solvent was removed *in vacuo*. Pure product was obtained by flash column chromatography on silica gel, employing ethyl acetate/chloroform (50:50) as the eluant. Trituration with hexane produced white crystals of **15**: 1.02 g (64% yield); mp 111−112 °C, TLC (chloroform/ethyl acetate, 50:50) $R_f$ = 0.32; IR (KBr pellet) 2960, 2926, 2858, 1741, 1520, 1454, 1373, 1228, 1037, 858 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 7.94 (1H, s, C(8)), 7.19 (1H, s, C(5)), 6.14 (1H, dd, $J$ = 7.62 Hz, $J$ = 3.33 Hz, C(3)), 4.22 and 4.08 (2H, 2m, C(1) diastereomeric methylene), 3.16 and 2.62 (2H, 2m, C(2) diastereomeric methylene), 2.81 (2H, t, $J$ = 7.71 Hz, C(1′)), 2.09 (3H, s, acetate methyl), 1.61 (2H, m, C(2′)), 1.41 (2H, sextet, $J$ = 7.29 Hz,

C(3′)), 0.934 (3H, t, $J$ = 7.26 Hz, C(4′)); mass spectrum (EI mode), $m/z$ 351 and 352 ($M^+$, $^{79}Br$ and $M^+$, $^{81}Br$), 307 and 309 ($M^+$ − acetyl). Anal. ($C_{16}H_{19}BrN_2O_2 \cdot 0.125H_2O$) C, H, N.

**6-Bromo-7-butyl-5-nitro-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]-benzimidazol-3-yl Acetate (16).** To a mixture of 9 mL of fuming nitric acid and 1 mL of concentrated sulfuric acid chilled to −8 °C was added 256 mg (0.728 mmol) of **15**. The reaction mixture was stirred for 20 min at ice bath temperature. The mixture was then combined with 50 g of ice and 50 mL of chloroform. The resulting mixture was neutralized with sodium bicarbonate and the chloroform layer separated. The aqueous phase was then extracted with 4 × 50 mL portions of chloroform. The extracts were combined and dried ($Na_2SO_4$), and the solvent was removed *in vacuo*. The solid residue was recrystallized from chloroform/hexane to afford pure **16**: 186 mg (64%) yield; mp 101.5−103 °C; TLC (chloroform/methanol, 95:5) $R_f$ = 0.50; IR (KBr pellet) 2957, 2872, 1745, 1537, 1375, 1300, 1226, 1085, 881, 750 $cm^{-1}$; $^1H$ NMR (dimethyl sulfoxide-$d_6$) $\delta$ 7.84 (1H, s, C(8) aromatic proton), 6.11 (1H, dd, $J$ = 7.84 Hz, $J$ = 3.87 Hz, C(3)), 4.29 and 4.18 (2H, 2m, C(1) diastereomeric methylene), 3.09 and 2.57 (2H, 2m, C(2) diastereometric methylene), 2.85 (2H, t, $J$ = 7.53 Hz, C(1′)), 2.06 (3H, s, acetate methyl), 1.57 (2H, m, C(2′)), 1.35 (2H, sextet, $J$ = 7.32 Hz, C(3′)), 0.901 (3H, t, $J$ = 7.23 Hz C(4′)); mass spectrum (EI mode), $m/z$ 395 and 397 ($M^+$, $^{79}Br$ and $M^+$, $^{81}Br$), 352 and 354 ($M^+$ − acetyl), 335 and 337 ($M^+$ − acetic acid). Anal. ($C_{16}H_{18}BrN_3O_4$) C, H, N.

**7-Butyl-2,3-dihydro-5,8-dioxo-1*H*-pyrrolo[1,2-*a*]benzimidazol-3-yl Acetate (17).** A suspension of 225 mg (0.568 mmol) of **16** and 45 mg of 5% Pd on charcoal in 100 mL of methanol was shaken under 50 psi of $H_2$ for 13 h. The solution was then filtered through Celite and concentrated to a dry residue consisting of the 5-amino derivative. A solution consisting of 20 mL of water and 17 mg of potassium phosphate monobasic was washed through Celite and then added to the 5-amino derivative. This solution was combined with a solution consisting of 90 mL of water, 100 mg of potassium phosphate monobasic, and 1 g of Fremy salt. The resulting mixture was stirred at room temperature for 5 h and then extracted with 5 × 50 mL portions of chloroform. The extracts were combined and dried ($Na_2SO_4$), and the solvent was removed *in vacuo*. Pure product was obtained by flash column chromatography on silica gel, employing chloroform as the eluant. Recrystallization with acetone and hexane afforded yellow crystals of **17**: 58 mg (30%) yield; mp 105.5−106 °C; TLC (ethyl acetate/chloroform, 80:20) $R_f$ = 0.37; IR (KBr pellet) 2960, 2931, 1747, 1655, 1525, 1371, 1224, 1153, 1084, 1043 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 6.46 (1H, d, $J$ = 1.47 Hz, C(6) aromatic proton), 6.06 (1H, dd, $J$ = 7.65 Hz, $J$ = 3.15 Hz, C(3)), 4.32 (2H, m, C(1) diastereomeric methylene), 3.14 and 2.63 (2H, 2m, C(2) diastereomeric methylene), 2.44 (2H, t, $J$ = 7.02 Hz, C(1′)), 2.07 (3H, s, acetate methyl), 1.48 (2H, m, C(2′)), 1.37 (2H, sextet, $J$ = 7.23 Hz, C(3′)), 0.907 (3H, t, $J$ = 7.29 Hz, C(4′)); mass spectrum (EI mode), $m/z$ 302 ($M^+$), 259 ($M^+$ − acetyl). Anal. ($C_{16}H_{18}N_2O_4$) C, H, N.

**6-*N*-Aziridinyl-7-butyl-2,3-dihydro-5,8-dioxo-1*H*-pyrrolo-[1,2-*a*]benzimidazol-3-yl Acetate (2).** A solution containing 235 mg (0.777 mmol) of **17** and 16 mL of dry methanol was chilled to 0 °C, at which time 0.85 mL of ethyleneamine was added. The reaction mixture was stirred for 30 min at 0 °C and then stirred at room temperature for 1.3 h. The methanol was evaporated, and the residue was purified by flash chromatography on silica gel, employing chloroform as the eluant. Pure **3** was recrystallized from ethyl acetate and hexane: 136 mg (51%) yield; mp 122−122.5 °C; TLC (chloroform/methanol, 99:1) $R_f$ = 0.21; IR (KBr pellet) 3448, 2958, 2872, 2362, 1749, 1678, 1629, 1520, 1228, 1138 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 6.02 (1H, dd, $J$ = 7.51 Hz, $J$ = 3.83 Hz, C(3)), 4.27 (2H, m, C(1) diastereomeric methylene), 3.11 and 2.61 (2H, 2m, C(2) diastereomeric methylene), 2.55 (2H, t, $J$ = 7.01 Hz, C(1′)), 2.34 (4H, s, aziridine protons), 2.06 (3H, s, acetate methyl), 1.37 (4H, m, C(2′) and C(3′)), 0.914 (3H, t, $J$ = 7.00 Hz, C(4′)); mass spectrum (EI mode), $m/z$ 343 ($M^+$), 300 ($M^+$ − acetyl) 283 ($M^+$ − acetic acid), 240 ($M^+$ − acetic acid − aziridine). Anal. ($C_{18}H_{21}N_3O_4 \cdot 0.125H_2O$) C, H, N.

**(±)- or (*S*)-(−)-3-Acetamido-2,3-dihydro-7-methyl-1*H*-pyrrolo[1,2-*a*] benzimidazole (19).** To a solution of 10 mL of acetic acid and 10 mL of acetic anhydride was added 75 mg (0.401 mmol) of **18**·HCl. The solution was stirred at room temperature for 1.5 h, and the solvent was removed to afford a solid residue, which was neutralized with aqueous sodium bicarbonate. The product was extracted from the aqueous layer with chloroform, and the extracts were dried ($Na_2SO_4$). The dried extracts were concentrated, and the resulting solid was recrystallized with chloroform/hexane to afford a white solid: 78 mg (85%) yield; mp 218-220 °C; TLC (chloroform/methane, 90:10) $R_f$ = 0.40; IR (KBr pellet) 3246, 3069, 2991, 1637, 1562, 1421, 1500 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) 7.50 (1H, d, $J$ = 8.2 Hz, 5-aromatic proton), 7.04 (1H, d, $J$ = 8.3 Hz, 6-aromatic proton), 6.94 (1H, s, 8-aromatic proton), 5.44 (1H, m, 3-methine proton), 4.03−3.93 (2H, m, 1-methylene protons), 2.56−2.50 (1H, m, 2-methylene protons), 2.45 (3H, s, methyl), 2.10 (3H, s, methyl); mass spectrum (EI), $m/z$ 229 ($M^+$), 186 ($M^+$ − $COCH_3$), 171, 158, 145, 133, 116, 104. Rotation of *S*-(−)-**19** in methanol solvent, $[\alpha]^{25}_D$ = −59.6°. Anal. ($C_{13}H_{15}N_3O$) C, H, N.

**(±)- or (*S*)-(−)-3-Acetamido-2,3-dihydro-6-bromo-7-methyl-1*H*-pyrrolo[1,2-*a*]benzimidazole (20).** To a solution of 200 mg (0.87 mmol) of **19** in 10 mL of glacial acetic acid was added 1 mL of 0.8 M bromine in acetic acid, and the resulting mixture was stirred at room temperature for 20 min. The reaction mixture was poured into 200 mL of 0.2 M phosphate buffer (pH = 7.0), and then the aqueous mixture was extracted with 2 × 80 mL portions of ethyl acetate. The extracts were washed with 2 × 30 mL portions of saturated $NaHCO_3$ and dried over $Na_2SO_4$. Removal of solvent gave **20** as a white solid, which was recrystallized from ethyl acetate: 188 mg (77%) yield; mp 249−250 °C; TLC (chloroform/methanol, 90:10) $R_f$ = 0.30; IR (KBr pellet) 3265, 3065, 2982, 1641, 1560, 1523, 1423, 1296 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) 7.81 and 7.08 (2H, 2s, aromatic protons), 7.12 (1H, bs, NH), 5.43 (1H, m, 3-methine proton), 4.17−3.96 (2H, m, 1-methylene protons), 3.32−4.09 (1H, m, 2-methylene proton), 2.60−2.51 (1H, m, 2-methylene proton), 2.46 (3H, s, methyl), 2.09 (3H, s, methyl); mass spectrum (EI), $m/z$ 307, 309 ($M^+$, $Br^{81}$ and $M^+$, $Br^{79}$), 266, 264 ($M^+$ − acetyl), 236, 211, 169, 131. Rotation of *S*-(−)-**20** in methanol solvent, $[\alpha]^{25}_D$ = −57.7°. Anal. ($C_{13}H_{14}BrN_3O$) C, H, N.

**(±)- or (*S*)-(−)-3-Acetamido-2,3-dihydro-6-bromo-7-methyl-5-nitro-1*H*-pyrrolo[1,2-*a*]benzimidazole (21).** To 5 mL of ice-cooled fuming nitric acid was slowly added 220 mg (0.72 mmol) of **20** with stirring followed by 0.5 mL of acetic anhydride. The reaction mixture was stirred at 10−25 °C for 1.5 h and then poured into 200 mL of ice-cold water. The mixture was buffered to pH = 7.0 using saturated $NaHCO_3$ and then extracted with 2 × 30 mL portions of ethyl acetate. The extracts were dried ($Na_2SO_4$), and the solvent was removed to give an oil. Tritration of the oil with *n*-hexane afforded a solid compound which was recrystallized from ethyl acetate, resulting in light yellow crystals: 172 mg (68%) yield; mp 248−250 °C (dec); TLC (chloroform/methanol, 90:10) $R_f$ = 0.30; IR (KBr pellet) 3260, 3065, 2926, 1672, 1535, 1373, 1298 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 8.08 (trace, s, 5-aromatic proton of 8-nitro isomer), 7.66 (1H, d, $J$ = 6 Hz, amide N-H), 7.36 (1H, s, 8-aromatic proton), 5.45−5.39 (1H, m, 3-methine proton), 4.39−4.05 (2H, 2m, 1-methylene protons), 3.34−3.25 (1H, m, 2-methylene proton), 2.75−2.68 (1H, m, 2-methylene proton), 2.55 and 2.04 (6H, 2s, methyl); mass spectrum (E1), $m/z$ 354 ($M^+$, $^{81}Br$) 352 ($M^+$, $^{79}Br$), 292 and 294 ($M^+$ − $NHCOCH_3$), 265, 236, 184, 156. Rotation of *S*-(−)-**21** in methanol solvent, $[\alpha]^{25}_D$ = −42.2°. Anal. ($C_{13}H_{13}BrN_4O_3$) C, H, N.

**(±)- or (*S*)-(−)-3-Acetamido-2,3-dihydro-7-methyl-1*H*-pyrrolo[1,2-*a*]benzimidazole-5,8-dione (22).** A solution of 210 mg (0.60 mmol) of **21** in 100 mL of methanol containing 100 mg of 5% Pd on carbon was shaken under 50 psi of $H_2$ for 4 h. The reaction mixture was filtered through Celite, and the filter cake was washed with 2 × 20 mL methanol. The combined filtrates were concentrated under reduced pressure to afford a brown oily compound. The compound was dissolved in a solution of 1 g of $KH_2PO_4$ in 50 mL water and treated with a solution of 2.0 g (0.75 mmol) of Fremy salt dissolved in

100 mL of water containing 3 g of $KH_2PO_4$. The reaction mixture was stirred for 1 h during which the reaction turned orange yellow. It was extracted with 4 × 25 mL portions of chloroform and dried over $Na_2SO_4$. Removal of solvent gave a yellow solid compound which was purified by flash column chromatography, employing chloroform/methanol (95:5) as the eluant. Recrystallization was carried out with ethyl acetate/hexane: 110 mg (71%) yield; mp 186−88 °C; TLC (chloroform/methanol, 9:1) $R_f$ = 0.30; IR (KBr pellet) 3289, 3041, 1664, 1541, 1481, 1273, 1157, 959 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 7.41 (1H, d, $J$ = 3.33 Hz, NH), 6.43 (1H, q, $J$ = 1.6 Hz, C-7 proton), 5.30 (1H, m, 3-methine proton), 4.36−4.35 (1H, m, 1-methylene proton), 4.20−4.11 (1H, m, 1-methylene proton), 3.26−3.14 (1H, m, 2-methylene proton), 2.73−2.63 (1H, m, 2-methylene proton), 2.05 and 2.04 (6H, 2s, methyls); mass spectrum (E1), $m/z$ 259 ($M^+$), 216 ($M^+$ − acetyl). Anal. ($C_{13}H_{13}N_3O_3$) C, H, N.

**(±)- or (*S*)-(−)-3-Acetamido-7-aziridinyl-2,3-dihydro-7-methyl-1*H*-pyrrolo[1,2-*a*]benzimidazole-5,8-dione (3).** To a solution of 60 mg (0.23 mmol) of **22** in 30 mL of dry methanol was added 0.5 mL of ethyleneamine at 0 °C, and the mixture was stirred for 30 min. The solvent was removed under vacuum to afford a red mass. The product was purified by flash column chromatography, employing chloroform/methanol (95:5) as the eluant. Recrystallization was carried out with chloroform/hexane: 38 mg (55%) yield; mp 238−240 °C dec; TLC (chloroform/methanol, 9:1) $R_f$ = 0.25; IR (KBr pellet) 3294, 2995, 2928, 1686, 1638, 1520, 1309, 1138, 977 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 7.60 (1H, d, $J$ = 7.23 Hz, acetamido NH), 5.31 (1H, m, 3-methine proton), 4.36−4.27 (1H, m, 1-methylene proton), 4.13−4.04 (1H, m, 1-methylene proton), 3.16−3.10 (1H, m, 2-methylene proton), 2.69−2.62 (1H, m, 2-methylene proton), 2.38 (4H, bs, aziridinyl protons), 2.06 and 2.00 (6H, 2s, methyls); mass spectrum (EI), $m/z$ 300 ($M^+$); CD for *S*-(−)-**3** ($c$ = 0.08 in dioxane) at 25 °C $[\theta]_{600} = -10$, $[\theta]_{470} = +2885$, $[\theta]_{400} \approx 0$. Anal. ($C_{15}H_{16}N_4O_3 \cdot 0.25H_2O$) C, H, N.

**(*R*)- and (*S*)-4-[(5′-Methyl-2′-nitrophenyl)amino]-2-hydroxybutanoic Acid (27).** To a solution of 1.0 g (8.4 mmol) of *R*- or *S*-**26** in 10 mL of 0.84 mM dry sodium ethoxide/ethanol was added 2.0 g (9.3mmol) of **25** in 4 mL of dry ethanol and 10 mL of dry dimethyl formamide. The resulting mixture was refluxed for 16 h. After the mixture was cooled to room temperature, 100 mL of saturated aqueous sodium bicarbonate was added. Extraction of the aqueous solution with 3 × 50 mL portions of chloroform removed unreacted **25**. The aqueous solution was then acidified to pH 3 with 4 N hydrochloric acid, and *R*- or *S*-**27** was removed with 8 × 200 mL portions of chloroform. The extract was dried (sodium sulfate) and concentrated to an orange oil: 1.20 g (56%) yield; TLC (butanol/water/acetic acid, 5:3:2) $R_f$ = 0.74; IR (NaCl thin film) 3366, 1742, 1628, 1576, 1408, 1225, 1161, 752 $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 8.21 (1H, s, amine proton), 7.94, 6.60 and 6.38 (3H, ABX, $J_{ortho}$ = 9.0 Hz, $J_{meta}$ = 1.5 Hz, $J_{para}$ < 0 Hz, aromatic protons), 4.37 (1H, dd, $J$ = 7.2 Hz, $J$ = 4.2 Hz, C(2) methylene), 3.46 (2H, t, $J$ = 7.2 Hz, C(4) methylene), 2.29 (3H, s, methyl), 2.02 and 1.59 (2H, 2m, C(3) diastereomeric methylene). Rotation of **27** in methanol solvent, $[\alpha]^{25}_D = +5.3°$ (*S*) and −5.3° (*R*).

**(*R*)- or (*S*)-3-Hydroxy-7-methyl-2,3-dihydro-1*H*-pyrrolo-[1,2-*a*]benzimidazole (24).** A solution of 1.0 g (3.93 mmol) of *R*-**27** in 20 mL of methanol was shaken under 50 psi of $H_2$ in the presence of 700 mg of 5% Pd on activated carbon for 2 h. The completed reaction was treated with 4 N hydrochloric acid and filtered through Celite. The filtrate was evaporated *in vacuo* to an acid/amine mixture and then combined with 20 mL of 4 N hydrochloric acid. The mixture was neutralized to pH 6 with saturated aqueous sodium bicarbonate, and *R*- or *S*-**27** was removed with 3 × 100 mL of chloroform. The extract was dried (sodium sulfate) and concentrated to a tan residue. The crude product was recrystallized by dissolution in chloroform/methanol (3:2) followed by addition of hexane: 165 mg (19%) overall yield; TLC (chloroform/methanol, 80:20) $R_f$ = 0.62; $^1H$ NMR (dimethyl sulfoxide-$d_6$) 7.43, 7.24, and 6.96 (3H, ABX, $J_{ortho}$ = 8.4 Hz, $J_{meta}$ = 1.2 Hz, $J_{para} \approx 0$ Hz, aromatic protons), 5.77 (1H, d, $J$ = 6.0 Hz, hydroxyl proton), 5.03 (1H, m, C(3) proton), 4.13 and 3.97 (2H, 2m, C(1) diastereomeric methylene), 2.86 and 2.34 (2H, 2m, C(2) diastereomeric methylene), 2.38 (3H, s, methyl). Rotation of **24** in methanol, $[\alpha]^{25}_D = -28°$ (*S*) and +28° (*R*).

**(*R*)- or (*S*)-7-Methyl-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]benzimidazol-3-yl Acetate (23).** To a solution of 180 mg (0.96 mmol) of *R*- or *S*-**24**, dissolved in 10 mL of acetic anhydride, was added 2 mL of glacial acetic acid and 1 mL of water. The resulting mixture was stirred at room temperature for 2 h, after which the solvents were evaporated *in vacuo*. The remaining residue was flash chromatographed on silica gel using chloroform to elute the first fraction and then chloroform/methanol (99:1) to elute *R*- or *S*-**23**. The product was recrystallized by dissolution in a small volume of chloroform followed by addition of hexane: 146 mg (66%) yield; TLC (chloroform/methanol, 80:20) $R_f$ = 0.88; $^1H$ NMR (dimethyl sulfoxide-$d_6$) 7.51, 7.34 and 7.05 (3H, ABX, $J_{ortho}$ = 8.4 Hz, $J_{meta}$ = 1.2 Hz, $J_{para} \approx 0$ Hz, aromatic protons), 6.10 (1H, dd, $J$ = 7.5 Hz, $J$ = 3.3 Hz, C(3) proton), 4.22 and 4.13 (2H, 2m, C(1) diastereomeric methylene), 3.12 and 2.57 (2H, 2m, C(2) diastereomeric methylene), 2.43 (3H, s, 7-methyl), 2.07 (3H, s, acetate methyl). Rotation of **23** in methanol, $[\alpha]^{25}_D = +91°$ **(*R*)** and −90° **(*S*)**.

**Enantioselective Hydrolysis of (±)-23.** A solution of 500 mg of racemic **23** in 4 mL of dimethylformamide was added to a suspension of 300 mg of *Candida antarctica* "B" lipase in 120 mL of pH 7 phosphate buffer. The resulting mixture was stirred at room temperature for 2 h to obtain >97% ee of *S*-**23** or for 24 h to obtain >98% ee of *R*-**24**. The solution was then extracted with 3 × 100 mL of chloroform, and any emulsion formed was filtered through a thin pad of Celite. The extract was dried (sodium sulfate), concentrated to a small volume, and then flash chromatographed on silica gel using chloroform/methanol (95:5) as eluant. The products were recrystallized by dissolution in chloroform/methanol (3:2), followed by addition of hexane. Enantiomeric excess was calculated from optical rotations. $[\alpha]^{25}_D = -88°$ *S*-**23** and +27.5° *R*-**24**.

**Chiral High-Pressure Liquid Chromatography.** All HPLC separations of enantiomers were done on Chirex columns (50 × 3.2 mm) from Phenomenex. The detector was set at 254 mm and sensitivity at 0.5. The flow rate was 0.5 mL/min with the mobile phase consisting of the indicated solvent system. Chirex column no. 3014 was used for compounds **23** and **24**. Measured retention times in minutes were as follows: 2.3, *R*-**24**; 3.0, *S*-**24**; 5.0, *R*-**23**; 6.5, *S*-**23**). The mobile phase was hexane/1,2-dichloroethane/methanol, 99:11:1. Chirex column no. 3017 was used for (*R*)- and (*S*)-APBI-A which were previously prepared in this laboratory;[1] measured retention times in minutes were as follows: 11.2, (*S*)-APBI-A and 12.5, (*R*)-APBI-A. The mobile phase was hexane/1,2-dichloroethane/methanol, 85:10:5. Chirex column no. 3014 was also used for *R*- and *S*-**3**. Measured retention times in minutes were as follows: 34, *S*-**3**; 44, *R*-**3**. The mobile phase was hexane/1,2-dichloroethane, 60:40.

**Alkylation of DNA by Reduced PBIs.** To a mixture of 1−5 mg of DNA (600 bp calf thymus, poly(dA)·poly(dT), poly(dG)·poly(dC), poly(dA-dT)·poly(dT-dA)) in 2.0 mL of Tris buffer, pH 7.4, and 0.1 mg of Pd on carbon was added a one-to-one base-pair equivalent amount of the PBI dissolved in 0.5 mL of dimethyl sulfoxide. The resulting solution was degassed under argon for 30 min, after which the mixture was purged with $H_2$ for 10 min. The solution was then purged with argon for 10 min and placed in a 30 °C bath for 24 h. The reaction was opened to the air, and the catalyst was filtered off. The filtrate was combined with 0.385 mL of sodium acetate and 11 mL of ethanol. The mixture was chilled at −20 °C for 12 h and the DNA pellet collected by centrifuging at 12000*g* for 20 min. The pellet was redissolved in water and then precipitated and centrifuged again. The resulting pellet was suspended in ethanol, centrifuged, and dried.

**Isolation of DT-Diaphorase by Affinity Chromatography.** The azodicoumarol Sepharose 6B column material was prepared according to the procedure by Höjeberg et al.[31] and equilibrated for 24 h in a 2.5 × 13 cm column with a solution of 0.25 M sucrose in 50 mM pH 7.5 Tris·HCl (buffer A). The following steps were carried out in a cold room held at 4 °C. Rat livers (200 g wet weight, Hooded rats) were added to a 0.25 M sucrose solution and homogenized in a Waring

blender at high speed. The homogenate was centrifuged at 36 000 rpm (~105000*g*) for 60 min and a supernatant filtered through several layers of cheese cloth. The supernatant was applied to the column at 90 mL/h, after which the column was eluted with buffer A until no protein was detected by the Bradford assay.[32] To remove non-specifically-bound proteins, a solution of 2 M potassium chloride and 0.25 M sucrose in 50 mM Tris·HCl (pH 8.9) buffer was applied to the column. The flow was then reversed with buffer A at 30 mL/h, and 11.2 mL of a solution of 20 mM NADH in buffer A was applied to remove the bound DT-diaphorase. Fractions with the highest activity were combined and concentrated to 10 mL by ultrafiltration in a Diaflo cell (PM10 filter, 43 mm, $N_2$ = 20 psi). The concentrated solution was applied to a Sephacryl S-200 column (3.2 × 55 cm) equilibrated with buffer A at a flow rate of 30 mL/h. Fractions with a constant protein/flavin ratio ($A_{270}/A_{450}$ = 6.7) were combined. The concentration of the flavin was 8.7 $\mu$M in the combined fractions with a constant protein/flavin ratio.

**Kinetic Studies.** Kinetic studies were carried out in 0.05 M pH 7.4 Tris·HCl buffer, under anaerobic conditions, employing Thunberg cuvettes. A 2 mM stock solution of the appropriate PBI or APBI was prepared in dimethyl sulfoxide (DMSO). To the top port was added the quinone stock, and to the bottom port was added DT-diaphorase and NADH in the Tris buffer. The top and bottom ports were purged with argon for 20 min and equilibrated to 30 °C. The ports were then mixed and the reaction followed at 296 nm for 25 min to obtain initial rates. The concentrations after mixing were 0.3 mM NADH, (1−20) × $10^{-5}$ M quinone, and 14.5 nM (based on flavin) of enzyme active sites. The value of $\Delta\epsilon$ was calculated from the initial and final absorbance values for complete quinone reduction; the usual value for $\epsilon$ is 6000−8000 $M^{-1}$ $cm^{-1}$. The value of $\Delta\epsilon$ was used to calculate $V_{max}$ in M $s^{-1}$. The results were fitted to a Lineweaver−Burke plot from which $k_{cat}/K_m$ values were calculated based on 14.5 nM of active sites.

**Electrophoresis Studies.** The relaxation reactions were assayed in a 0.045 M Tris·borate buffer using a 1.3% agarose gel. The topoisomerase relaxation reactions were carried out with 0.5 $\mu$g of SV-40 supercoiled DNA (form I), 1 $\mu$L of a solution of 20 units of topoisomerase II in 20 $\mu$L of 100 mM tris·Cl, pH 7.9, containing 500 mM NaCl, 500 mM KCl, 50 mM $MgCl_2$, 1 mM EDTA, 150 $\mu$g/mL BSA, and 10 mM ATP. The reactions were run for 45 min at 37 °C and then mixed with 1 $\mu$L of a stop solution consisting of 75 mg of SDS and 3.75 mg of proteinase K in 1.5 mL of water. After incubation for an additional 45 min, the reactions were combined with 4 mL of gel loading solution. The gel was loaded with 5 $\mu$L of gel-loading solution. The gel was loaded with 5 $\mu$L of reaction solution per well and run at 40 V (12 milliamperes) for 13 h after which the gel was washed with 100 $\mu$L of a 1 mg/mL ethidium bromide solution in 700 mL of 0.045 M Tris·borate buffer.

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