Design, Synthesis, and Evaluation of a Novel Sequence-Selective Epoxide-Containing DNA Cross-Linking Agent Based on the Pyrrolo[2,1-*c*][1,4]benzodiazepine System

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Synthetic routes have been investigated to prepare a novel C8-epoxide-functionalized pyrrolo-[2,1-*c*][1,4]benzodiazepine **6** as a potential sequence-selective DNA cross-linking agent (Wilson et al. *Tetrahedron Lett.* **1995**, *36*, 6333−6336). A successful synthesis was accomplished via a 10-step route involving a pro-N10-Fmoc cleavage method that should have general applicability to other pyrrolobenzodiazepine (PBD) molecules containing acid- or nucleophile-sensitive groups. During the course of this work, a one-pot reductive cyclization procedure for the synthesis of PBD N10-C11 imines from nitro dimethyl acetals was also discovered, although this method results in C11a racemization which can reduce DNA binding affinity and cytotoxicity. The target epoxide **6** was shown by thermal denaturation studies to have a significantly higher DNA-binding affinity than the parent DC-81 (**3**) or the C8-propenoxy-PBD (**15**), which is structurally similar but lacks the epoxide moiety. The time course of effects upon thermal denaturation indicated a rapid initial binding phase followed by a slower phase consistent with the stepwise cross-linking of DNA observed for a difunctional agent. This was confirmed by an electrophoretic assay which demonstrated efficient induction of interstrand cross-links in plasmid DNA at concentrations >1 μM. Higher levels of interstrand cross-linking were observed at 24 h compared to 6 h incubation. A *Taq* polymerase stop assay indicated a preference for binding to guanine-rich sequences as predicted for bis-alkylation in the minor groove of DNA by epoxide and imine moieties. The pattern of stop sites could be partly rationalized by molecular modeling studies which suggested low-energy models to account for the observed binding behavior. The epoxide PBD **6** was shown to have significant cytotoxicity (45−60 nM) in the A2780, CH1, and CH1cis^R human ovarian carcinoma cell lines and an IC_{50} of 0.2 μM in A2780cis^R. The significant activity of **6** in the cisplatin-resistant CH1cis^R cell line (IC_{50} = 47 nM) gave a resistance factor of 0.8 compared to the parent cell line, demonstrating no cross-resistance with the major groove cross-linking agent cisplatin.

Introduction

There is interest in discovering and developing small molecules capable of binding to DNA in a highly sequence-selective manner.[2] Such agents, with the ability to target and then down-regulate or ablate individual genes, have potential use as drugs for the therapy of genetic-based diseases including cancer,[3] as diagnostic agents, and as research tools for use in target validation and functional genomics studies. The pyrrolo-[2,1-*c*][1,4]benzodiazepine (PBD) family of antitumor antibiotics was first discovered in 1965 when Leimgruber and co-workers isolated anthramycin (**1**) from *Streptomyces refuineus*[4] (Figure 1). Other compounds such as tomaymycin (**2**) and DC-81 (**3**) were later isolated.[5] The PBDs are unique in exerting their activity through covalent binding of the N10-C11 imine functionality to the exocyclic C2-amino group of guanine within the minor groove of duplex DNA.[5,6] The (*S*)-configuration at the chiral C11a position has been shown to be essential for binding activity, as it provides the PBD molecule with the right-handed twist necessary for fit within the host minor groove.[5] Footprinting-type techniques have established that the PBDs span three base pairs of DNA with a rank order of preference for Pu-**G**-Pu > Pu-**G**-Py ~ Py-**G**-Pu > Py-**G**-Py motifs (Pu = purine, Py = pyrimidine).[6] The cytotoxicity of these agents is thought to be due to their ability to inhibit cellular processes such as replication and transcription. Using a bacteriophage T7 RNA polymerase model system, it has been demonstrated that the PBDs can inhibit in vitro transcription in a highly sequence-selective manner.[7] In addition, a direct correlation has been observed between the ability of a series of PBDs to inhibit the action of the endonuclease enzyme *Bam*HI in vitro and their cytotoxicity across a number of cell lines.[8]

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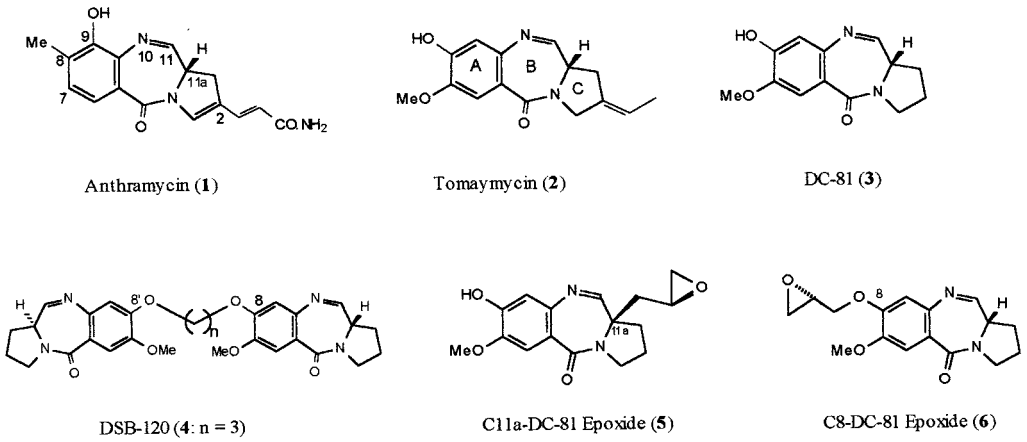


Figure 1. Structures of the PBD monomers (**1**−**3**), the PBD dimer DSB-120 (**4**: $n = 3$), and the C11a (**5**) and C8 (**6**) PBD-epoxides.

In an attempt to extend the number of base pairs spanned by these molecules, PBD "dimers" have been synthesized comprising two PBD units joined through either their C7[9] or C8[10,11] positions via flexible linkers. Suggs and co-workers have produced C7/C7′-linked compounds joined through alkanediyldisulfide or alkanediyldioxy linkers (some including nitrogen heteroatoms) which have been shown to cross-link DNA.[9] Thurston and co-workers have produced a series of C8/C8′-alkanediyldioxy-bridged PBD dimers with linkers of varying lengths (e.g., **4**: $n = 3-6$) that are isohelical with the minor groove of DNA and provide a direct correlation between DNA interstrand cross-linking efficiency and cytotoxicity across a number of cell lines.[11] With the aim of producing a PBD monomer with DNA interstrand cross-linking ability, Confalone and co-workers[12] synthesized a PBD analogue with an epoxide functionality incorporated at the C11a position (**5**). Although DNA-binding data were not reported for this compound, structure−activity considerations suggest[5,13] that attachment of the epoxide moiety to the C11a position may sterically hinder the fit of the PBD within the minor groove as well as interfere with nucleophilic attack of a guanine C2-NH_2 at the C11 position.

On this basis, we designed a new guanine-specific PBD-based cross-linking agent by attaching the second alkylating functionality at the C8 position of the PBD to overcome any potential problems with steric hindrance. Although other types of alkylating moieties were considered, the epoxide group was chosen as the second alkylating moiety, because epoxides have an established base selectivity for guanines, interacting at either the N7 or C2-NH_2 position in the major and minor grooves, respectively.[14] For example, a preference for interaction with the N7 position of guanine in the major groove is shared by the epoxide-containing cross-linking antitumor antibiotic carzinophilin (azinomycin),[15] certain non-bay-region diol epoxides of the benzo[*a*]pyrenes,[16] the activated epoxide-containing form of aflatoxin B_1,[14] non-groove-specific compounds such as the 1,2-epoxyalkanes,[17] and the cross-linking agent diepoxybutane.[18] In contrast, other polycyclic aromatic hydrocarbon diol epoxides derived from benzo[*a*]pyrene[19,20] and benzo[*c*]-phenanthrene[21] have a preference for the exocyclic C2-NH_2 groups of guanines in the minor groove. Therefore, to design a bifunctional PBD molecule (i.e., **6**) with the potential to cross-link two separated guanine C2-NH_2 groups within the minor groove, the epoxide moiety was chosen to complement the guanine-specific PBD nucleus. It was decided to join the epoxide through the C8 position of the PBD in order to retain isohelicity with the contour of the minor groove of the host DNA molecule.[5]

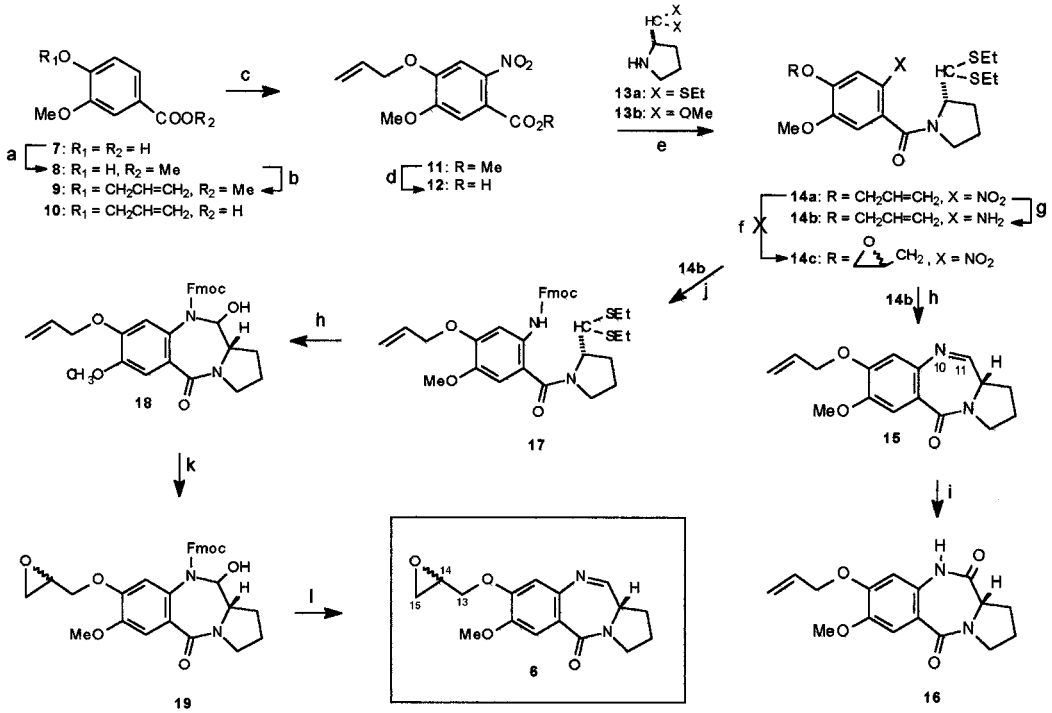
Results and Discussion

Synthesis. Numerous synthetic routes to the pyrrolobenzodiazepine ring system have been investigated since Leimgruber reported the first synthesis of anthramycin,[22] and these have been reviewed.[23] The main problem associated with the synthesis of PBDs relates to generation of the relatively labile N10-C11 imine moiety which is usually introduced at the final step of the synthetic pathway.[23] However, synthesis of the target molecule **6** was further complicated by the presence of the C8-epoxide moiety, which is also chemically reactive. Therefore, a number of different strategies were investigated and are described below.

1. A-Ring Fragment (Scheme 1). As epoxides can be readily synthesized from alkenes, the 4-alkenoxynitrobenzoic acid **12** was initially targeted as a suitable A-ring fragment and epoxide precursor. Vanillic acid (**7**) was first converted to the corresponding methyl ester **8** and then alkylated with NaH and allyl bromide to introduce the propenoxy group (**9**). Facile nitration at the C6 position afforded **11** in >90% yield, and the required allyl ether **12** was obtained by hydrolysis. Alternatively, vanillic acid (**7**) could be directly alkylated to afford **10**, which could then be nitrated to **12** with $SnCl_4$ and fuming HNO_3 at −25 °C. Although the first route requires two additional steps, it results in a higher yield of **12** (73% compared to 55%) and a shorter overall reaction time.

2. Attempted Epoxidation of the Pro-C8-Propenoxy Functionality in the Presence of the Diethyl Thioacetal (Scheme 1). The main difficulty associated with PBD synthesis is formation of the labile N10-C11 carbinolamine−imine functionality to afford PBDs in practical yields.[23] For the targeted epoxide-PBD **6**, this problem was exacerbated by the requirement to include an equally labile epoxide group. Initial studies involved the "thioacetal" route[24] which has the advantage of maintaining stereochemical integrity at the C11a posi-

Scheme 1[a]

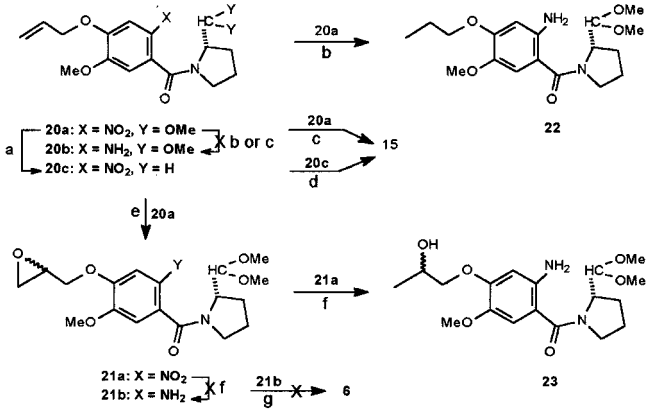


[a] Reagents: (a) MeOH/H_2SO_4/Δ/16 h; (b) DMF/NaH/room temperature/2 h, then allyl bromide/room temperature/18 h; (c) method A, CH_2Cl_2/$SnCl_4$/HNO_3/−25 °C/30 min or method B, HNO_3/0 °C/1 h, room temperature/18 h; (d) THF/aq NaOH/room temperature/24 h; (e) CH_2Cl_2/DCC/room temperature/4 h, then **13a**/room temperature/16 h; (f) $CHCl_3$/3-CPBA/0 °C/8 h; (g) MeOH/$SnCl_2$·$2H_2O$/Δ/3 h; (h) **14b** or **17**/MeCN−H_2O/$HgCl_2$/$CaCO_3$/room temperature/24 h; (i) $CHCl_3$/3-CPBA/0 °C/18 h; (j) **14b**/dioxane/aq Na_2CO_3/Fmoc-Cl/0 °C/4 h, then room temperature/24 h; (k) CH_2Cl_2/3-CPBA/room temperature/72 h; (l) DMF/$Bu_4N^+F^-$/room temperature/15 min.

tion of the PBD. The propenoxy thioacetal **14a** (Scheme 1) was prepared by coupling the nitro acid A-ring (**12**) to the thioacetal C-ring fragment **13a**[23,24] using dicyclohexylcarbodiimide (DCC) in CH_2Cl_2 at room temperature. Although peracids are known to oxidize sulfides to sulfoxides and sulfones at room temperature, selective oxidation of the allylic functionality of **14a** to give **14c** was attempted using 3-chloroperbenzoic acid (3-CPBA) at 0 °C in $CHCl_3$. However, this led to oxidation of both the propenoxy and thioacetal functionalities to different extents, affording a mixture of epoxide/propenoxy sulfoxides and sulfones as evident from mass spectral and NMR data.

3. Attempted Oxidation of the C8-Propenoxy Functionality in the Presence of the N10-C11 Imine (Scheme 1). Even though it is known that 3-CPBA is capable of oxidizing the N10-C11 imine functionalities of PBDs at room temperature to afford the corresponding dilactams[25] (e.g., structure **16** in Scheme 1), it was decided to attempt a selective oxidation of the propene moiety of the fully formed C8-propenoxy-PBD (**15**) by exploiting either temperature control or orthogonal protection of the N10-C11 imine. To produce **15**, the nitro thioacetal **14a** was reduced to the amino thioacetal **14b** with $SnCl_2$·$2H_2O$ in refluxing MeOH and then cyclized (49% from **14b**) by treatment with $HgCl_2$ and $CaCO_3$ in MeCN−H_2O at room temperature for 48 h. However, oxidation of **15** with 1 equiv of 3-CPBA in $CHCl_3$ at 0 °C for 18 h led to selective oxidation of the N10-C11 imine moiety to afford the novel C8-propenoxy-PBD dilactam (**16**) in 70% yield. Attempts to protect the N10-C11 imine functionality of

Scheme 2[a]



[a] Reagents: (a) hydrolysis (H^+); (b) method A, MeOH/10% Pd−C/H_2/1 atm/room temperature/16 h or method B, MeOH/10% Pd−C/1,4-cyclohexadiene/room temperature/18 h or method C, MeOH/10% Pd−$BaCO_3$/H_2/1 atm/room temperature/24 h; (c) MeOH/$SnCl_2$·$2H_2O$/Δ/4 h; (d) MeOH/$SnCl_2$·$2H_2O$/Δ/2 h; (e) $CHCl_3$/3-CPBA/room temperature/96 h or $Cl(CH_2)_2Cl$/3-*tert*-butyl-4-hydroxy-5-methylphenyl sulfide/3-CPBA/Δ/2 h; (f) method A, MeOH/10% Pd−C/H_2/1 atm/room temperature/8 h or method B, MeOH/10% Pd−$BaCO_3$/H_2/1 atm/room temperature/16 h or method C, MeOH/10% Pd−C/1,4-cyclohexadiene/room temperature/18 h or method D, MeOH/$SnCl_2$·$2H_2O$/Δ/30 min; (g) Amberlite resin.

15 as the C11-propoxy ether prior to oxidation of the C8-propenoxy moiety failed, affording only the dilactam **16**.

4. Attempted Reduction of the Aromatic Nitro Functionality in the Presence of the Epoxide (Scheme 2). Due to the problems associated with the above approaches, the use of the dimethyl acetal protecting group was explored to avoid the difficulties

Table 1.[a] Thermal Denaturation Data with CT-DNA at a [PBD]/[DNA] Molar Ratio of 1:5 and in Vitro Cytotoxicity Data in the A2780/A2780cis^R, CH1/CH1cis^R, and SKOV-3 Cell Lines for the PBD Epoxide **6**, C8-*O*-Allyl Precursor **15**, DC-81 (**3**), and DSB-120 (**4**)

	ΔT_m (°C) after incubation for				IC_{50} (μM)[b,c]						
compd	0 h	4 h	18 h	36 h	SKOV-3	A2780	A2780cis^R	*RF*[d]	CH1	CH1cis^R	*RF*[d]
6[e]	0.5	0.8	1.3	1.5	1.3	0.045	0.2	4.4	0.059	0.047	0.8
15	0.4	0.7	0.8	–	3.3	1.05	1.65	1.6	0.16	0.135	0.8
DC-81 (**3**)	0.3	0.5	0.7	0.7	–	–	–	–	0.10	–	–
DSB-120 (**4**)	10.2	13.1	15.1	15.4	0.74	0.0072	0.21	29.2	0.033	0.022	0.7
cisplatin					4.2	0.16	6.1	38.1	0.029	0.17	5.9

[a] Determined for CT-DNA (100 μM) at pH 7.0 with a fixed [PBD]/[DNA] molar ratio of 1:5 (see text). For native CT-DNA, T_m = 67.83 ±0.06 °C. Drug-induced ΔT_m values represent the mean from at least 3 determinations (values ±0.1−0.2 °C). [b] Dose of compound required to inhibit cell growth by 50% compared to untreated cell controls. Cells were incubated with the drug for 96 h at 37 °C. In vitro cytotoxicity data for cisplatin are included for comparative purposes. [c] A2780cis^R and CH1cis^R are cisplatin-resistant counterparts of the A2780 and CH1 cell lines, respectively. [d] Relative resistance factor given by IC_{50}(resistant)/IC_{50}(parent). [e] Evaluated as a diastereomeric mixture due to the racemic epoxide moiety.

relating to sulfur oxidation. For this route, the acetal epoxide **21a** is the key intermediate that can be reduced to the amine **21b** and then cyclized with weakly acidic Amberlite resin[23,26] to afford the required epoxide PBD **6**. Therefore, the propenoxy nitro acid **12** (Scheme 1) was coupled[23] to the pyrrolidine fragment **13b** (Scheme 1), either by initial conversion to its acid chloride or by using DCC, the two methods affording **20a** in overall yields of 53% and 19%, respectively. **20a** was then treated with 3-CPBA in $CHCl_3$ at room temperature. However, even after 96 h reaction was incomplete and workup afforded **21a** in only 10% yield. In an attempt to improve the yield, radical inhibitors such as 3-*tert*-butyl-4-hydroxy-5-methylphenyl sulfide were investigated so that higher temperatures could be used in the presence of the oxidizing agent.[27] This approach succeeded, and use of the sulfide inhibitor with 3-CPBA in $Cl(CH_2)_2Cl$ under reflux for 2 h afforded the novel **21a** in 85% yield.

The penultimate step of this route involved selective reduction of the nitro group of **21a** in the presence of the epoxide functionality to give the amine **21b**. However, hydrogenation for 8 h at 1 atm pressure with 10% Pd−C in MeOH also opened the epoxide to give the novel amino alcohol **23** in good yield. Three milder reduction methods were investigated, including hydrogenation in the presence of a "poisoned" catalyst (10% Pd−$BaCO_3$/MeOH), phase-transfer hydrogenation with 10% Pd−C in 1,4-cyclohexadiene and MeOH at room temperature, and reduction with $SnCl_2 \cdot 2H_2O$ under reflux in MeOH. However, in each case quantitative conversion to **23** occurred within 16 h, 18 h, and 30 min, respectively.

5. Attempted Reduction of the Nitro Functionality Prior to Epoxidation (Scheme 2). Due to the problems encountered with synthesis of the epoxide amino acetal **21b**, reduction of the nitro group of **20a** prior to epoxidation was attempted. However, hydrogenation with either 10% Pd−C or 10% Pd−$BaCO_3$ in MeOH at 1 atm pressure for 16 or 24 h, respectively, or 10% Pd−C in cyclohexadiene and MeOH at room temperature for 18 h resulted in reduction of both the propenoxy and nitro groups to give the novel propanoxy amino acetal **22** rather than **20b**. To avoid reduction of the alkene moiety, $SnCl_2 \cdot 2H_2O$ in refluxing MeOH was investigated next. Surprisingly, instead of forming the expected propenoxy amino acetal **20b**, simultaneous nitroreduction and cleavage of the acetal occurred after 4 h to afford the propenoxy imine **15** in 73% yield. This one-pot reductive cyclization reaction represents a novel method of PBD synthesis, particularly as previous attempts[23,28] to cyclize nitro aldehyde intermediates of type **20c** using a number of different reagents have usually led to low yields of cyclized product due to overreduction to the corresponding secondary amine (e.g., **15** where N10-C11 is $-NH-CH_2-$). Interestingly, in this case, the acetal moiety was shown to be necessary for smooth conversion to the imine, as treatment of the propoxy nitro aldehyde **20c** (prepared by hydrolysis of acetal **20a**) with $SnCl_2 \cdot 2H_2O$ in MeOH for 2 h at reflux caused complete conversion to two new products identified as the C8-propenoxy imine **15** (20% yield) and the secondary amine overreduction product (not isolated).

6. Synthesis of 6 via Epoxidation of the N10-Fmoc-Protected Intermediate 18 (Scheme 1). Due to the problems encountered with the previous synthetic routes, it was decided to utilize the intermediate **14b** in a Fukuyama-type approach.[29,30] It had been reported that the 9-fluorenylmethyloxycarbonyl (Fmoc) group can be used to protect amines in high yields (88−98%)[31] and can then be easily removed by cleavage with $Bu_4N^+F^-$ (TBAF) at room temperature.[32] Other reports suggested that an epoxide group should be relatively stable to F^- ions at this temperature.[33] Therefore, it was decided to attempt epoxidation of the N10-Fmoc intermediate **18** in order to provide the required target molecule **6** after deprotection.

Fmoc protection of the propenoxy amino thioacetal **14b** using 9-fluorenylmethyl chloroformate gave **17** in 88% yield. Cleavage of the diethyl thioacetal group with $HgCl_2$ and $CaCO_3$ in MeCN−H_2O effected ring closure to afford the required N10-Fmoc-protected carbinolamine **18** in 95% yield. The next step involved epoxidation of the C8-propenoxy group with 3-CPBA to afford a diastereomeric mixture of the epoxides (**19**) in 55% yield. Finally, removal of the Fmoc protecting group with TBAF in DMF produced the N10-C11 imine while leaving the epoxide intact, thus affording the target epoxide-PBD **6** in 79% yield.

In summary, these results highlight the advantages of using Fmoc as an N10-protecting group in a Fukuyama-type approach to PBD synthesis; these include rapid N10 deprotection under mild conditions and in high yield. This strategy should be generally applicable to the synthesis of other PBD analogues, particularly those containing acid- or nucleophile-sensitive functional groups.

DNA-Bonding Activity. 1. Thermal Denaturation Studies. The DNA-binding activities of the PBD com-

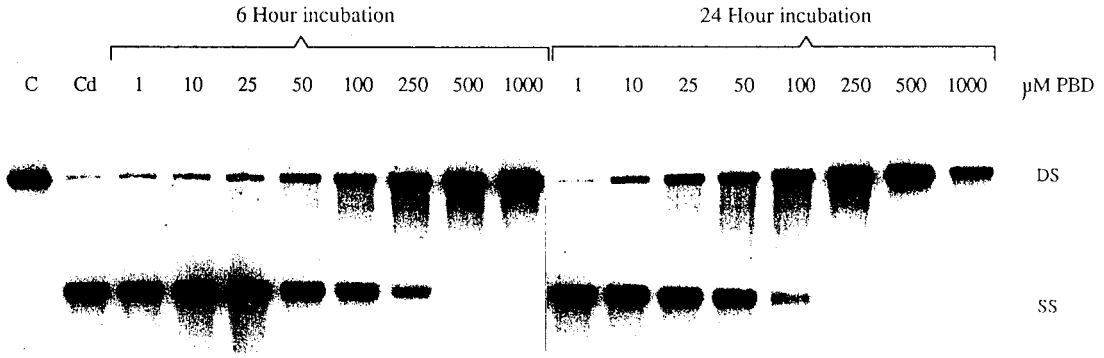


Figure 2. Autoradiograph of a neutral agarose gel showing induction of interstrand DNA cross-links by epoxide-PBD **6** with linear ^{32}P-end-labeled pBR322 DNA. Reactions (6 and 24 h at 37 °C) for concentrations of 1−1000 μM of **6** were in 25 mM triethanolamine/1 mM EDTA, pH 7.2 buffer with 10 ng of DNA in a final volume of 50 μL. Reaction was terminated by the addition of an equal volume of 0.6 M NaOAc, 20 mM EDTA, and 100 mg/mL tRNA, followed by precipitation of the DNA with EtOH. Dried pellets were taken up in strand separation buffer (30% DMSO in 1 mM EDTA), followed by denaturation for 2 min at 90 °C and then immediate chilling in an ice−water bath. Electrophoresis was carried out on submerged horizontal 0.8% agarose gels at 40 V for 16 h with Tris-acetate running buffer. Single-stranded (Cd,SS) and double-stranded (C,DS) controls were included, and both types of DNAs were quantitated by scanning laser densitometry.

pounds produced were first assessed from the stabilization afforded to duplex-form calf thymus DNA toward thermal denaturation.[5,8,10a,11,34] Melting studies (Table 1) show that these agents stabilize the thermal helix → coil transition (T_m) for the CT-DNA duplex at pH 7.0, with the epoxide PBD **6** showing greater activity than either the structurally similar C8-propenoxy-PBD analogue **15** or the less substituted DC-81 (**3**) molecule, both of which monoalkylate DNA. Interestingly, the induced ΔT_m shifts are dependent on the length of incubation at 37 °C, although each compound can effect a similar level of stabilization without significant DNA−drug contact (i.e., 0 h incubation). Time course plots of ΔT_m for **3**, **6**, and **15** are similar for incubation periods up to 4 h (data not shown), although values for both **3** and **15** reach plateau levels after 4−18 h, whereas the ΔT_m for **6** continues to increase up to 36 h, giving an ultimate 3-fold increase in stabilization. In contrast, the symmetric PBD dimer DSB-120 (**4**: $n = 3$) is much more effective at short incubation times although there is also a strong time-dependent component to the binding profile.[10a,11]

For many cross-linking agents, it is known that the formation of DNA interstrand cross-links involves initial covalent reaction of the agent with a nucleophilic site on the first strand to form a monoadduct, followed by a subsequent reaction with a nucleophile on the opposite DNA strand. This "second-arm" reaction is generally slow compared to initial monoadduct formation, resulting in a diagnostic "biphasic" DNA-binding time course in DNA-binding assays. It is also known that the covalent binding of epoxides to DNA can be slow. For example, the 7-(2,3-epoxypropoxy) analogue of actinomycin D has been shown[35] to reversibly intercalate through the minor groove of DNA where the epoxide group covalently binds to guanine, taking up to 6 h for complete reaction. On this basis, the results shown in Table 1 can be interpreted as rapid formation of an initial monoadduct of **6** with DNA involving reaction of the PBD N10-C11 imine with the C2-NH_2 of a guanine base, as suggested by the similar early reaction rates for the monofunctional agents **3** and **15**. This is presumably followed by a slower time-dependent covalent interaction of the epoxide group of DNA-bound **6** to form either inter- or intrastrand DNA cross-links, manifesting as an increasing ΔT_m value after further incubation. The slow second-arm alkylation reaction may also involve an unbonding/bonding-type redistribution of the covalently bound monoadduct to more favorable or competitive cross-linking sites in the host DNA molecule.

2. DNA Cross-Linking Assay. To confirm that the epoxide-PBD **6** causes DNA cross-linking, an agarose gel electrophoresis assay was performed,[36] the results of which are shown in the autoradiograph in Figure 2. The results clearly demonstrate that **6** forms interstrand cross-links with DNA efficiently. However, as with the DNA thermal denaturation studies, the results also reveal the relatively slow nature of the process. Following a 6 h incubation, cross-links are detectable at doses as low as 1−10 μM and increase dose-dependently reaching almost 100% at 500 μM or above. Following a 24 h incubation, the level of cross-linking was higher at each dose, with 100% cross-linking observed by 250 μM. These results clearly demonstrate the formation of interstrand DNA cross-links; however, the presence (and ratio) of intrastrand cross-links and/or epoxide−DNA or imine−DNA monoadducts cannot be determined from this experiment.

3. *Taq* Polymerase Assay. The sequence selectivity of the covalent interaction of **6** with DNA was investigated using a *Taq* polymerase stop assay.[37] The autoradiograph shown in Figure 3 indicates that **6** produces discrete "stop" sites in a concentration-dependent manner. Furthermore, using the sequencing markers to establish base positions, it is clear that the stop sites occur at guanine residues and preferentially within guanine-rich sequences. This result compares favorably with the benzo[*a*]pyrene diol epoxides which also bind to the C2-NH_2 of guanine in the minor groove with a pronounced sequence preference for guanines immediately flanked by other guanines and with G_{3-5} tract sequences binding 3−4-fold more readily than isolated guanines.[19,20] In the case of the benzo[*a*]pyrene diol epoxides, this selectivity can be explained by calculating the molecular electrostatic potential at the N2 position of central guanines within different base triplets[20,38] which suggests that central guanines have the highest potentials. Guanines neighboring one other guanine base have the next highest potentials, and isolated

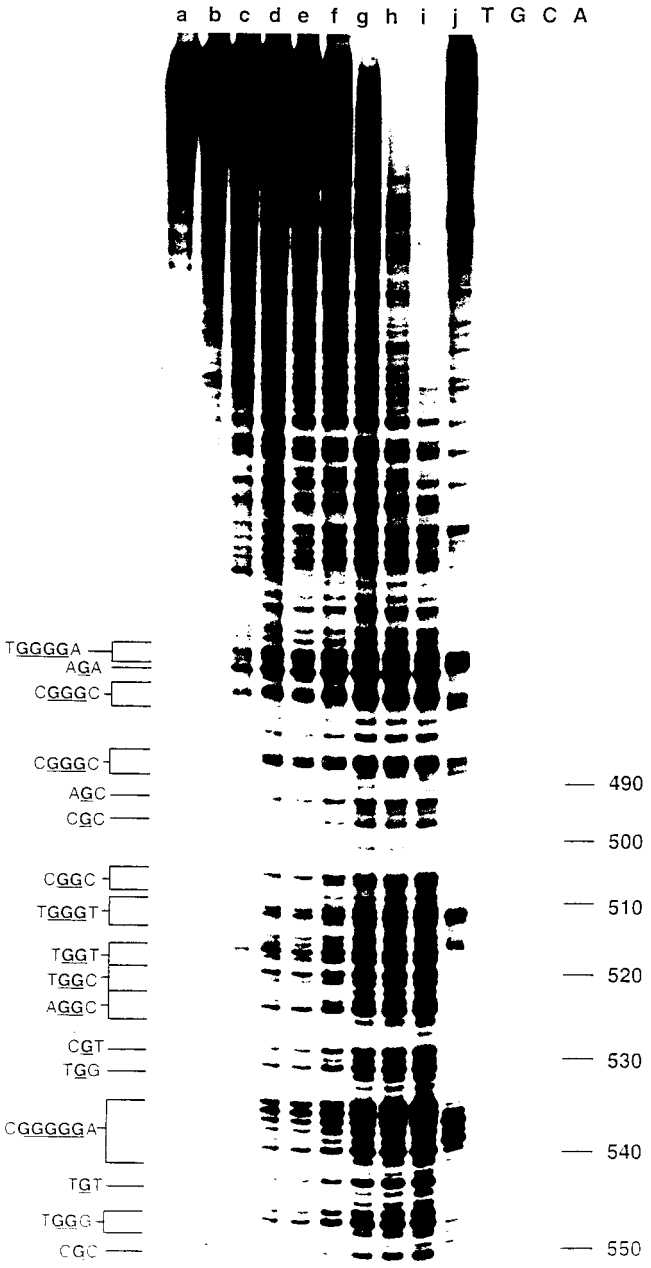


Figure 3. Autoradiograph showing the results of the *Taq* polymerase stop assay for the epoxide-PBD **6**. The agent was incubated at 37 °C with linearized calf thymus DNA for 24 h. Lanes a−i correspond to increasing concentrations of **6** (0, 1, 10, 25, 50, 100, 250, 500, and 1000 μM), and lane j is 10 μM cisplatin after incubation with the DNA at room temperature for 1 h. Lanes T, G, C, and A correspond to thymine, guanine, cytosine, and adenine sequencing lanes, respectively.

guanines are associated with the lowest potentials.

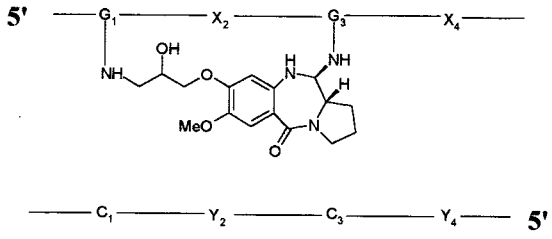
On the basis of the known preference for both epoxide and imine alkylating moieties to target guanine bases, and the minor groove preference and base-pair span of the PBDs, the interstrand adduct of the bifunctional molecule **6** is thought to involve interaction with either two adjacent guanines on opposite strands or an analogous site separated by one spacing base pair [i.e., (**G**/C)(C/**G**) or (**G**/C)N(C/**G**), respectively; N = any other base pair], with the alkylating units bonded to the guanine C2-NH_2 functions. However, a more detailed analysis of the sequence selectivity is restricted by the fact that some of the stop sites shown in Figure 3 may result from intrastrand cross-linked adducts and/or monoadducts. In addition, data are only available from one strand of the DNA duplex host. Despite these limitations, a number of consensus sequences such as 5′-GG, 5′-GC, 5′-CGG, and 5′-GGG are clearly discernible.

4. Molecular Modeling. Modeling experiments to rationalize the interstrand cross-linking results started with the assumption that **6** bonds to separated guanines in the minor groove due to the known base preference of imine and epoxide moieties and the known groove preference of the PBDs.[5] Preliminary studies of the interaction of **6** with model DNA duplex sequences suggested that upon bonding of the PBD N10-C11 imine to a guanine C2-NH_2 in the minor groove (e.g., G_3 in Figure 4a), the unopened pendant epoxide moiety should be approximately positioned between the neighboring base pair (i.e., X_2·Y_2) and the next adjacent base pair (i.e., G_1·C_1). Assuming that this could lead to either intra- or interstrand cross-links with either of the two base pairs implicated, four distinct models were envisaged (Figure 4a−d), each with the benzenoid A-ring of **6** oriented toward the 5′-end of the PBD-modified strand with (*S*)-stereochemistry at the C11 position of the PBD (designated "5S"). Four equivalent models were also examined (not shown) with the adducts in the reverse orientation with the A-rings orientated toward the 3′-end of the covalently modified strand and with C11(*S*) stereochemistry (designated "3S"). Models with the C11-(*R*)-configuration were not considered, as this configuration is known to be of significantly higher energy for structures based on the DC-81 (**3**) core.[5] However, as both (*R*)- and (*S*)-stereochemistries are possible for the C8 chiral epoxide residue, 16 models in total were examined for DNA−**6** cross-link formation.

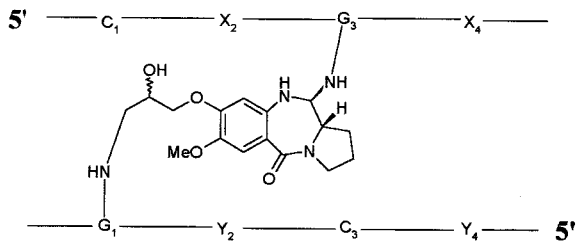
Molecular mechanics (MM) and dynamics (MD) simulations using the CHARMm force field[39] were carried out on these models, and the results are collected in Table 2. The 9-mer duplex DNA sequences for the simulations (see Table 2) used a core 5′-A**G**A motif (the preferred PBD binding sequence[5]) wherever possible (i.e., not in the case of the nonadjacent models [e.g., Figure 4a,b]) and were terminated with flanking G·C base pairs to improve helical stability (see Experimental Section). The results shown in Table 2 indicate three energetically favored models. The lowest-energy model (depicted in Figure 5, $E_{\text{interaction}} = -22.1$ kcal mol^{-1}) involves an interstrand cross-link between adjacent guanines on opposite strands (i.e., model 4d), with (3*S*)-orientation of the PBD and (*S*)-stereochemistry for the epoxide. The next most favored models also involve interstrand cross-links but to nonadjacent guanines separated by one base pair (i.e., model 4b) and with (5*S*)-alignment of the PBD and (*R*)- or (*S*)-stereochemistries for the epoxide (−18.8 and −19.3 kcal mol^{-1}, respectively). A second adjacent-base interstrand cross-linking model (Figure 4d) is the next preferred model (−17.3 kcal mol^{-1}) with (3*S*)-orientation of the PBD and an (*R*)-configuration for the epoxide. The first intrastrand cross-link model involves nonadjacent guanines (model 4a, (3*S*)-PBD, (*R*)-epoxide) and has an energy of −15.6 kcal mol^{-1}.

The most energetically preferred model (Figure 4d, (3*S*)-PBD, (*S*)-epoxide, −22.1 kcal mol^{-1}) implicates a

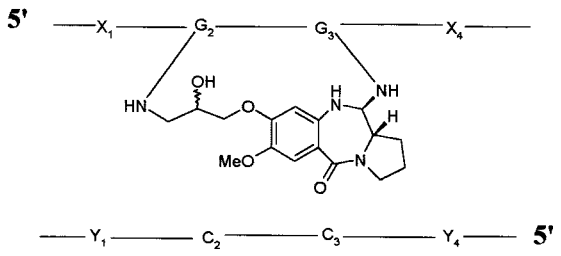
(a) Intrastrand cross-linking model (non-adjacent base pairs):



(b) Interstrand cross-linking model (non-adjacent base pairs):



(c) Intrastrand cross-linking model (adjacent base pairs):



(d) Interstrand cross-linking model (adjacent base pairs):

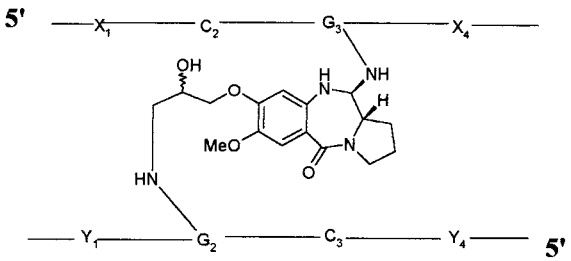


Figure 4. Possible models for covalent interaction between the epoxide-PBD **6** and the 9-mer DNA duplexes shown in Table 2 (column 1), with the difunctional molecule bound to nonadjacent (a or b) or adjacent (c or d) guanine bases, leading to either intrastrand (a or c) or interstrand (b or d) DNA cross-links. The PBD is in the favored C11(*S*)-configuration for all models shown, with the aromatic A-ring oriented toward the 5′-end ("5*S*") of the covalently modified strand. The epoxide moiety may have either the (*R*)- or (*S*)-configuration prior to ring opening.

5′-**G**C sequence. In support of this model, analysis of the stop sites shown in Figure 3 reveals that 8 of the 17 marked sites include 5′-**G**C sequences. The next-favored models (Figure 4b, (5*S*)-PBD, (*R*/*S*)-epoxide) require a 5′-CN**G** sequence, and 4 of the 17 observed stop sites include this motif. Although the modeling suggests that intrastrand cross-linking is less energetically favorable, potential stop sites relating to the sequence requirements for both of the intrastrand models (i.e., **4a**: 5′-**G**N**G** and **4c**: 5′-**GG**) can also be found in Figure 3. Therefore, until further experimental data become available, the modeling results can be used only in a predictive sense.

5. In Vitro Cytotoxicity of the Epoxide-PBD 6. The cytotoxic potencies of the epoxide-PBD **6**, the C8-propenoxy-PBD (**15**), and DC-81 (**3**) were determined in five cell lines, and the results are shown in Table 1. Data for the cross-linking agents DSB-120 (**4**: $n = 3$) and cisplatin (which forms intra- and interstrand cross-links in the major groove of DNA) are included for comparison. Using the median of the cytotoxicity values for each compound across the five cell lines, the DNA cross-linking epoxide PBD **6** (median = 0.059 μM) is ~18-fold more cytotoxic than **15** (median = 1.05 μM) which is only capable of monoalkylating DNA. The median IC_{50} for **6** is only slightly higher than that for the established interstrand minor groove cross-linking agent DSB-120 (median = 0.033 μM), also supporting an interstrand cross-linking mechanism of action. A2780 (followed closely by CH1cis^R) is the most sensitive cell line, in which **6** (IC_{50} = 0.045 μM) is ~23-fold more cytotoxic than **15**, suggesting that these cells may be more prone to the effects of cross-linking, as with DSB-120 which is significantly more cytotoxic (IC_{50} = 0.0072 μM) in A2780 compared to the other four cell lines. Furthermore, the significant potency of **6** in both the cisplatin-resistant cell line CH1cis^R and its parent CH1 (giving a resistance factor of 0.8) indicates a lack of cross-resistance with cisplatin. As with previous studies on series of PBD compounds,[11] the higher cytotoxicity of **6** compared to **15** is reflected in the thermal denaturation data for these compounds (see Table 1). Finally, it is important to note that **6** was evaluated as a mixture of diastereomers due to the chirality of the epoxide moiety. Although the molecular modeling studies suggest that, in some cases, there is little difference in binding energy between DNA adducts with either the (*R*)- or (*S*)-epoxide configuration, it is possible that greater cytotoxicity may be realized with single diastereomers of **6**.

Conclusions

The first example (**6**) of a pyrrolo[2,1-*c*][1,4]benzodiazepine molecule with an epoxide moiety at the C8 position has been synthesized via a novel pro-N10 deprotection procedure utilizing a variation of a method originally pioneered by Fukuyama and co-workers.[30] The procedure adopted here differed in two key aspects: (i) the B-ring of **6** was closed via cleavage of a thioacetal functionality rather than through an oxidative cyclization process, and (ii) the N10-Alloc protecting group originally used by Fukuyama was replaced with a Fmoc moiety which offers significant advantages including rapid deprotective cleavage under mild conditions. Therefore, this new strategy should have general applicability to the synthesis of other PBD analogues, particularly those containing acid- or nucleophile-sensitive functional groups.

On the basis of the results of the electrophoretic assay shown in Figure 2, **6** induces interstrand DNA cross-links at low micromolar concentrations, a result supported by both the DNA thermal denaturation studies and the enhanced cytotoxicity profile compared to

Table 2. Interaction Energies of the Epoxide-PBD **6** with the 9-mer DNA Duplex Sequences (shown in column 1)[a]

sequence of 9-mer duplex (5′)	bonding site of PBD	bonding site of epoxide	PBD orientation	epoxide configuration	cross-linking mode	DNA−PBD model Figure	$E_{interacton}$ (kcal mol^{-1})
GCGAG^{5}AG^{7}CG	G^5	G^7	3*S*	*R*	intra	**4a**	−15.6
GCGAG^{5}AC^{7}CG	G^5	G^7	3*S*	*R*	inter	**4b**	−12.0
GCGAG^{5}AG^{7}CG	G^5	G^7	3*S*	*S*	intra	**4a**	−10.0
GCGAG^{5}AC^{7}CG	G^5	G^7	3*S*	*S*	inter	**4b**	−14.4
GCG^{3}AG^{5}AGCG	G^5	G^3	5*S*	*R*	intra	**4a**	−15.4
GCC^{3}AG^{5}AGCG	G^5	G^3	5*S*	*R*	inter	**4b**	−18.8
GCG^{3}AG^{5}AGCG	G^5	G^3	5*S*	*S*	intra	**4a**	−6.0
GCC^{3}AG^{5}AGCG	G^5	G^3	5*S*	*S*	inter	**4b**	−19.3
GCGAG^{5}G^{6}GCG	G^5	G^6	3*S*	*R*	intra	**4c**	+2.6
GCGAG^{5}C^{6}GCG	G^5	G^6	3*S*	*R*	inter	**4d**	−17.3
GCGAG^{5}G^{6}GCG	G^5	G^6	3*S*	*S*	intra	**4c**	−0.5
GCGAG^{5}C^{6}GCG	G^5	G^6	3*S*	*S*	inter	**4d**	−22.1
GCGG^{4}G^{5}AGCG	G^5	G^4	5*S*	*R*	intra	**4c**	−5.0
GCGC^{4}G^{5}AGCG	G^5	G^4	5*S*	*R*	inter	**4d**	−10.2
GCGG^{4}G^{5}AGCG	G^5	G^4	5*S*	*S*	intra	**4c**	−8.9
GCGC^{4}G^{5}AGCG	G^5	G^4	5*S*	*S*	inter	**4d**	−11.1

[a] The results of 16 molecular mechanics/dynamics simulations are shown (column 8), with the molecule bonding either across adjacent G·C base pairs (i.e., models 4c or d) or those one base pair removed (i.e., models 4a or b) (columns 2, 3). All models have the C11 position of the PBD in the (*S*)-configuration, with **6** orientated toward either the 5′ (5*S*) or 3′ (3*S*) end of the covalently modified strand (column 4). The epoxide moiety was modeled in both the (*R*)- and (*S*)-configurations (column 5), both of which could give rise to intra- or interstrand DNA cross-links (column 6).

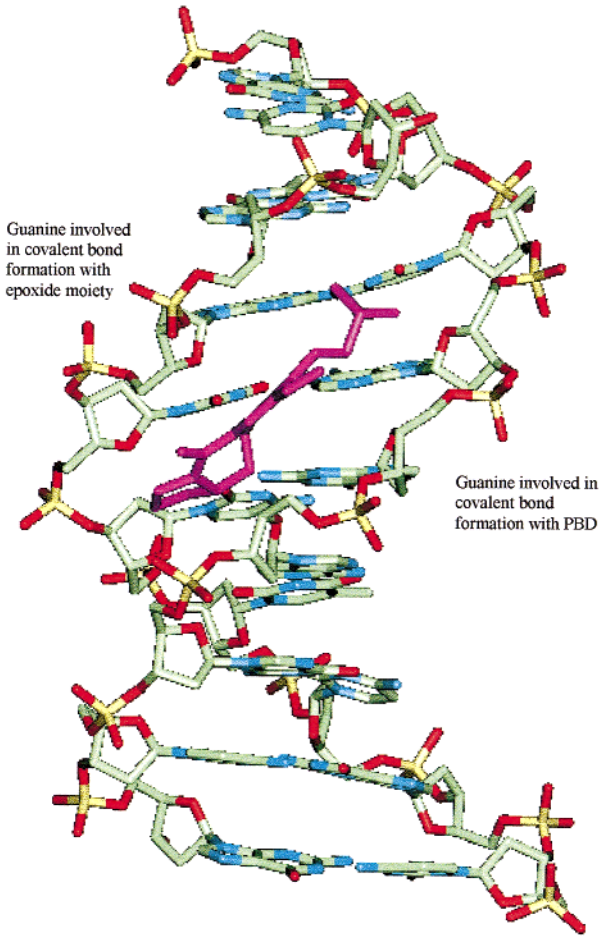


Figure 5. Energy-minimized representation of the adduct of epoxide-PBD **6** in the minor groove of the d(GCCA**G**AGCG)·d(CGCTCT**G**GC) duplex according to the interstrand cross-linking model shown in Figure 4b. The epoxide and N10-C11 imine functionalities are covalently bound to the exocyclic C2-NH_2 groups of guanines on opposite strands separated by one base pair. The molecule is oriented with the epoxide moiety toward the 5′-end of the covalently modified strand.

monofunctional PBD analogues of similar structure. Furthermore, the results of the *Taq* polymerase assay (Figure 3) demonstrate that **6** produces concentration-dependent sequence-selective covalent adducts in guanine-rich regions of DNA. Molecular modeling studies have suggested low-energy models for such adducts where **6** spans either two adjacent [i.e., (GC)·(CG)] base pairs or an analogous arrangement with a further spanned base pair [i.e., (GC)·N·(GC)] with the electrophilic epoxide and imine functionalities alkylating guanine residues on opposite strands. However, from the data available so far, it is not possible to rule out the formation of intrastrand cross-links and/or monoalkylated adducts, even though the molecular modeling studies suggest that interstrand cross-linking leads to the most energetically favored adducts.

The epoxide PBD **6** has significant cytotoxicity across the five cell lines examined, with activity in the 45−60 nM range in the A2780, CH1, and CH1cis^R cell lines. The similar potency in both the cisplatin-resistant cell line CH1cis^R and the parent CH1 (resistance factor = 0.8) indicates that **6** is not cross-resistant with cisplatin. This suggests that, as is the case with the symmetric PBD dimers [e.g., DSB-120 (**4**: $n = 3$)],[10,11] the bifunctional epoxide PBD **6** may have potential application in the treatment of certain cisplatin-resistant tumors. Future studies will include the synthesis of **6** in its diastereomerically discrete forms, each of which may possess greater cytotoxicity and DNA cross-linking efficiency than the racemic mixture examined here, and may provide more interpretable patterns of stop sites in the *Taq* polymerase assay.

Experimental Section

Synthetic Chemistry. Melting points (mp) were determined on a Gallenkamp P1384 digital melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded using a Perkin-Elmer 297 spectrophotometer. All ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solution (unless stated otherwise) using a JEOL GSX 270 MHz FT-NMR instrument operating at 20 ± 1 °C. Chemical shifts are reported in parts per million (δ) downfield from Me_4Si. Spin multiplicities are described as: s (singlet), bs (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), or m (multiplet). Mass spectra (MS) were recorded using a JEOL JMS-DX 303 GC-mass spectrometer (EI mode: 70 eV, source 117−147 °C).

Accurate molecular masses (HRMS) were determined by peak matching using perfluorokerosene (PFK) as an internal mass marker. Column chromatography was performed using chromatography grade silica gel 400 (Aldrich). Thin-layer chromatography (TLC) was performed using GF254 silica gel including fluorescent indicator on glass plates. All solvents and reagents, unless otherwise stated, were purchased from Aldrich and were used as supplied. Anhydrous solvents were prepared by distillation under dry N_2 in the presence of the appropriate drying agent and were stored over 4-Å molecular sieves or sodium wire. Intermediates and final products did not provide useful combustion analysis data as they were either oils and/or were found to be prone to either oxidation (e.g., amines such as **14b**, **20b**, **21b**, **22**, and **23**) and/or water absorption. Therefore, high resolution mass spectrometry data were obtained throughout the study, and many intermediates were used directly in subsequent steps.

Methyl 4-Hydroxy-3-methoxybenzoate (8). Concentrated H_2SO_4 (16.56 g, 0.16 mol, 0.5 equiv) was added dropwise to a stirred solution of vanillic acid (**7**) (50.44 g, 0.30 mol) in MeOH (250 mL) at room temperature, and the mixture was then heated at reflux for 16 h. The solvent was removed in vacuo and the residue dissolved in EtOAc (200 mL). This solution was washed with saturated $NaHCO_3$ (3 × 100 mL), water (2 × 100 mL), and brine (100 mL), then dried ($MgSO_4$), and evaporated in vacuo to give ester **8** as a beige solid (50.94 g, 93%): mp 69−70 °C; IR (Nujol) *ν* 3536 (OH), 2924, 2854, 1699 (C═O), 1600 (C═C), 1512, 1462, 1376, 1284, 1220, 1188, 1113, 1026, 984, 872, 768; ^{1}H NMR *δ* 3.89 (s, 3H, H-8), 3.91 (s, 3H, H-9), 6.31 (bs, 1H, H-10), 6.94 (d, 1H, J = 8.3 Hz, H-5), 7.55 (d, 1H, J = 1.8 Hz, H-2), 7.63 (dd, 1H, J = 1.8 & 8.3 Hz, H-6); ^{13}C NMR *δ* 52.0 (C-8), 56.1 (C-9), 111.8 (C-2), 114.2 (C-5), 122.2 (C-1), 124.2 (C-6), 146.3 (C-3), 150.12 (C-4), 167.0 (C-7); MS *m*/*z* (relative intensity) 182 ([*M*]$^+$, 74), 167 (7), 151 (100), 139 (4), 123 (17), 108 (7), 77 (3), 65 (4), 51 (6). HRMS: calcd for $C_9H_{10}O_4$ (182.0579), found 182.0583.

Methyl 3-Methoxy-4-(2-propenoxy)benzoate (9). A solution of **8** (25 g, 137 mmol) in dry DMF (50 mL) was added dropwise to a stirred suspension of NaH (5 g, 208 mmol, 1.5 equiv) in dry DMF (200 mL) at 0 °C under a N_2 blanket, and stirring was continued for 2 h at room temperature. Allyl bromide (33.2 g, 274 mmol, 2 equiv) was added dropwise at 0 °C, and stirring continued for 18 h at room temperature. Brine (300 mL) and EtOAc (200 mL) were added to the mixture with shaking and the phases allowed to separate. The aqueous phase was extracted with EtOAc (2 × 200 mL) and the combined organic phase was washed with brine (2 × 200 mL), then dried ($MgSO_4$), and evaporated in vacuo. Toluene (100 mL) was added to the residue and the mixture evaporated in vacuo. This process was repeated a further three times. The resulting waxy solid was triturated with hexane (20 mL) and filtered, and this process was repeated five times. The residue was dried for 16 h at 50 °C to give allyl ether **9** as a white solid (28.06 g, 92%): mp 54−55 °C; IR (Nujol) *ν* 2924, 2854, 1718 (C═O), 1588 (C═C), 1508, 1460, 1377, 1284, 1217, 1170, 1128, 998, 947, 870, 759; ^{1}H NMR *δ* 3.89 (s, 3H, H-8), 3.93 (s, 3H, H-9), 4.65−4.68 (m, 2H, H-10), 5.27−5.49 (m, 2H, H-12), 6.01−6.15 (m, 1H, H-11), 6.88 (d, 1H, J = 8.4 Hz, H-5), 7.56 (d, 1H, J = 2.0 Hz, H-2), 7.64 (dd, 1H, J = 2.0 & 8.4 Hz, H-6); ^{13}C NMR *δ* 52.0 (C-8), 56.0 (C-9), 69.7 (C-10), 111.9 (C-2), 112.2 (C-5), 118.5 (C-12), 122.8 (C-1), 123.4 (C-6), 132.5 (C-11), 148.9 (C-3), 151.9 (C-4), 166.9 (C-7); MS *m*/*z* (relative intensity) 222 ([*M*]$^+$, 100), 207 (3), 191 (17), 181 (98), 163 (8), 153 (11), 149 (20), 121 (19), 107 (4), 94 (9), 79 (12), 65 (4), 59 (14), 51 (9).

Methyl 5-Methoxy-2-nitro-4-(2-propenoxy)benzoate (11). Allyl ether **9** (4.4 g, 19.8 mmol) was added slowly over 15 min to a stirred solution of HNO_3 (70% w/w, 47.22 g, 0.52 mol) at 0 °C. The mixture was stirred at 0 °C for 1 h and then allowed to return to room temperature over 45 min, before pouring onto ice and stirring at room temperature for 18 h. The reaction mixture was then extracted with EtOAc (3 × 50 mL), and the extracts were washed with saturated aqueous $NaHCO_3$ (5 × 100 mL), water (200 mL), and brine (100 mL) and then dried ($MgSO_4$). Solvent removal in vacuo gave nitro ester **11** as a yellow solid (4.92 g, 93%): mp 82−83 °C; IR (Nujol) *ν* 2924, 2854, 1724 (C═O), 1606 (C═C), 1540 (NO_2), 1458, 1377, 1295, 1212, 1138, 985, 870, 814; ^{1}H NMR *δ* 3.91 (s, 3H, H-8), 3.98 (s, 3H, H-9), 4.68−4.71 (m, 2H, H-10), 5.36−5.50 (m, 2H, H-12), 5.99−6.14 (m, 1H, H-11), 7.08 (s, 1H, H-6), 7.47 (s, 1H, H-3); ^{13}C NMR *δ* 53.3 (C-8), 56.7 (C-9), 70.3 (C-10), 108.5 (C-3), 110.9 (C-6), 119.5 (C-12), 121.8 (C-1), 131.5 (C-11), 140.9 (C-2), 149.2 (C-5), 152.8 (C-4), 166.4 (C-7); MS *m*/*z* (relative intensity) 267 ([*M*]$^+$, 100), 236 (24), 226 (67), 210 (13), 195 (11), 181 (10), 165 (14), 121 (30), 109 (11), 93 (11), 77 (9), 65 (8), 59 (22), 51 (5). HRMS: calcd for $C_{12}H_{13}O_6N$ (267.0743), found 267.0817.

5-Methoxy-2-nitro-4-(2-propenoxy)benzoic Acid (12). A mixture of ester **11** (2.2 g, 8.2 mmol) and aqueous NaOH (25 mL of 1 M) in THF (25 mL) was stirred at room temperature for 24 h. The THF was evaporated in vacuo and the aqueous layer extracted with EtOAc (3 × 20 mL) which was then discarded. The aqueous phase was acidified to pH 1 (concentrated HCl) and extracted with EtOAc (3 × 25 mL). The combined organic layer was washed with water (4 × 20 mL) and brine (20 mL), dried ($MgSO_4$), and evaporated in vacuo to give the nitrobenzoic acid **12** as a yellow solid (1.98 g, 95%): mp 118−119 °C; IR (Nujol) v 3287−2010, 1684 (C═O), 1597 (C═C), 1539 (NO_2), 1459, 1377, 1275, 1217, 919, 870, 760, 722; ^{1}H NMR ($CDCl_3$−DMSO-d_6) *δ* 3.97 (s, 3H, H-8), 4.68−4.70 (m, 2H, H-9), 5.35−5.49 (m, 2H, H-11), 5.99−6.14 (m, 1H, H-10), 7.16 (s, 1H, H-6), 7.41 (s, 1H, H-3); ^{13}C NMR ($CDCl_3$−DMSO-d_6) *δ* 56.5 (C-8), 70.1 (C-9), 108.3 (C-3), 111.1 (C-6), 119.3 (C-11), 122.6 (C-1), 131.6 (C-10), 141.1 (C-2), 149.0 (C-5), 152.5 (C-4), 167.3 (C-7); MS *m*/*z* (relative intensity) 253 ([*M*]$^+$, 97), 236 (5), 212 (20), 208 (45), 195 (30), 167 (41), 121 (63), 111 (10), 95 (14), 77 (11), 65 (7), 51 (18), 41 (100). HRMS: calcd for $C_{11}H_{11}O_6N$ (253.0586), found 253.0603.

(2*S*)-*N*-(5-Methoxy-2-nitro-4-[2-propenoxy]benzoyl)-pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (14a). 1,3-Dicyclohexylcarbodiimide (815 mg, 4 mmol) was added to a solution of benzoic acid **12** (1 g, 4 mmol) in dry CH_2Cl_2 (50 mL), and the mixture was stirred for 4 h at room temperature under a N_2 atmosphere. A solution of thioacetal **13a**[23,24] (811 mg, 4 mmol) in dry CH_2Cl_2 (10 mL) was then added and the mixture stirred for 16 h at room temperature. The mixture was filtered, and the filtrate evaporated in vacuo to afford the coupled benzamide thioacetal **14a** as a pale yellow oil (1.73 g, 99%): IR (neat) *ν* 2924, 2854, 1634 (C═O), 1580 (C═C), 1524 (NO_2), 1462, 1377, 1328 (NO_2), 1276, 1215, 1060, 981, 850, 825, 761, 668; ^{1}H NMR *δ* 1.31−1.39 (m, 6H, H-18 & H-20), 1.62−2.36 (m, 4H, H-9 & H-10), 2.59−2.87 (m, 4H, H-17 & H-19), 3.19−3.34 (m, 2H, H-8), 3.96 (s, 3H, H-12), 4.42−4.46 (m, 1H, H-11), 4.69−4.71 (m, 2H, H-13), 4.94 (d, 1H, J = 3.9 Hz, H-16), 5.36−5.51 (m, 2H, H-15), 6.01−6.15 (m, 1H, H-14), 6.84 (s, 1H, H-6), 7.69 (s, 1H, H-3); ^{13}C NMR *δ* 15.0 & 15.1 (C-18 & C-20), 24.9 (C-9), 26.3 & 27.2 (C-17 & C-19), 26.6 (C-10), 50.2 (C-8), 52.8 (C-16), 56.5 (C-12), 61.0 (C-11), 70.2 (C-13), 108.7 (C-3), 109.3 (C-6), 119.4 (C-15), 128.3 (C-1), 131.6 (C-14), 137.2 (C-2), 147.8 (C-5), 154.4 (C-4), 166.5 (C-7); MS *m*/*z* (relative intensity) 440 ([*M*]$^+$, 9), 411 (5), 379 (3), 351 (8), 334 (2), 305 (44), 252 (7), 236 (100), 195 (6), 174 (3), 135 (38), 121 (8), 70 (8). HRMS: calcd for $C_{20}H_{28}O_5N_2S_2$ (440.1440), found 440.1509.

(2*S*)-*N*-(2-Amino-5-methoxy-4-[2-propenoxy]benzoyl)-pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (14b). A solution of nitro thioacetal **14a** (1 g, 2.3 mmol) and $SnCl_2$·$2H_2O$ (2.56 g, 11.4 mmol, 5 equiv) in MeOH (100 mL) was refluxed for 3 h. After solvent removal in vacuo and digestion in $CHCl_3$ (300 mL), saturated aqueous $NaHCO_3$ (300 mL) was added and the mixture was stirred for 16 h at room temperature. The mixture was then filtered through Celite and the organic layer separated. The aqueous layer was extracted with $CHCl_3$ (2 × 100 mL) and the combined organic layers were washed with water (3 × 200 mL), dried ($MgSO_4$), and evaporated in vacuo to give the amine **14b** as a yellow oil (746 mg, 80%): IR (neat) *ν* 3440 (NH_2), 2923, 2854, 1701 (C═O), 1614 (C═C), 1501, 1451, 1402, 1381, 1340, 1255, 1228, 1191, 1168, 1100, 1030, 998, 870, 820, 755, 701; ^{1}H NMR *δ* 1.12−1.37 (m, 6H, H-19 & H-21), 1.67−2.31 (m, 4H, H-9 & H-10), 2.65−2.74

(m, 4H, H-18 & H-20), 3.59−3.71 (m, 2H, H-8), 3.79 (s, 3H, H-12), 4.57−4.69 (m, 4H, H-11, H-13 & H-17), 5.28−5.44 (m, 2H, H-15), 5.99−6.14 (m, 1H, H-14), 6.26 (s, 1H, H-3), 6.83 (s, 1H, H-6); ^{13}C NMR δ 14.9 & 15.2 (C-19 & C-21), 25.2 (C-9), 26.5 & 27.2 (C-18 & C-20), 26.6 (C-10), 51.6 (C-8), 53.2 (C-17), 56.8 (C-12), 60.9 (C-11), 69.5 (C-13), 102.1 (C-3), 110.3 (C-1), 112.8 (C-6), 118.2 (C-15), 132.8 (C-14), 141.1 (C-2), 147.4 (C-5), 150.9 (C-4), 169.8 (C-7); MS *m*/*z* (relative intensity) 410 ([*M*]$^+$, 57), 381 (8), 330 (6), 275 (36), 248 (10), 206 (100), 191 (14), 180 (8), 166 (17), 135 (15), 107 (4), 94 (6), 83 (3), 70 (8), 56 (3). HRMS: calcd for $C_{20}H_{30}O_3N_2S_2$ (410.1698), found 410.1739.

(11a*S*)-7-Methoxy-8-(2-propenoxy)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (15). A suspension of amino thioacetal **14b** (110 mg, 0.27 mmol), $HgCl_2$ (160 mg, 0.59 mmol, 2.2 equiv), and $CaCO_3$ (67 mg, 0.67 mmol, 2.5 equiv) in MeCN−H_2O (4:1 v/v, 5.5 mL) was stirred for 24 h at room temperature. The mixture was filtered through Celite and the filtrate evaporated in vacuo to give a dark yellow oil which was purified by gradient chromatography (100% EtOAc, followed by 100:0→95:5 v/v $CHCl_3$−MeOH) to afford the title compound **15** as a pale yellow oil (47 mg, 61%): IR (neat) *v* 2923, 2854, 1630 (C=O), 1508 (C=C), 1451, 1263, 1150, 1015, 780, 755, 715; ^{1}H NMR δ 2.02−2.11 (m, 2H, H-2), 2.29−2.37 (m, 2H, H-1), 3.53−3.87 (m, 3H, H-3 & H-11a), 3.96 (s, 3H, H-12), 4.60−4.72 (m, 2H, H-13), 5.31−5.47 (m, 2H, H-15), 6.02−6.16 (m, 1H, H-14), 6.82 (s, 1H, H-6), 7.52 (s, 1H, H-9), 7.68 (d, 1H, J = 4.4 Hz, H-11); ^{13}C NMR δ 24.2 (C-2), 29.6 (C-1), 46.6 (C-3), 53.7 (C-11a), 56.1 (C-12), 69.7 (C-13), 110.8 (C-6), 111.5 (C-9), 118.7 (C-15), 120.4 (C-5a), 132.3 (C-14), 140.4 (C-9a), 147.7 (C-7), 150.2 (C-8), 162.4 (C-11), 164.6 (C-5); MS *m*/*z* (relative intensity) 286 ([*M*]$^+$, 100), 271 (18), 245 (14), 230 (11), 217 (49), 203 (11), 176 (13), 160 (3), 121 (5), 93 (9), 70 (11). HRMS: calcd for $C_{16}H_{18}O_3N_2$ (286.1317), found 286.1292.

(11a*S*)-1,2,3,10,11,11a-Hexahydro-7-methoxy-8-(2-propenoxy)-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5,11-dione (16). A solution of 3-chloroperoxybenzoic acid (50−60% w/w, 60 mg, 0.17 mmol) and **15** (50 mg, 0.17 mmol) in $CHCl_3$ (10 mL) was stirred for 18 h at 0 °C, after which TLC ($CHCl_3$−MeOH, 95:5 v/v) indicated that reaction was complete. The solvent was evaporated in vacuo and the residue purified by gradient chromatography (100:0→85:15 v/v $CHCl_3$−MeOH) to afford the dilactam **16** as a yellow solid (36 mg, 70%): mp 114−115 °C; IR (Nujol) *v* 3500 (NH), 2923, 2854, 1693 & 1653 (C=O), 1614 (C=C), 1500, 1462, 1377, 1257, 1217, 1018, 722; ^{1}H NMR δ 1.92−2.36 (m, 4H, H-1 & H-2), 3.45−3.92 (m, 3H, H-3 & H-11a), 3.93 (s, 3H, H-12), 4.66−4.76 (m, 2H, H-13), 5.33−5.52 (m, 2H, H-15), 6.03−6.18 (m, 1H, H-14), 6.56 (s, 1H, H-9), 7.51 (s, 1H, H-6), 9.70 (bs, 1H, H-10); ^{13}C NMR δ 29.7 (C-2), 32.1 (C-1), 46.7 (C-3), 56.3 (C-12), 56.4 (C-11a), 69.8 (C-13), 105.5 (C-9), 108.0 (C-6), 113.5 (C-5a), 119.0 (C-15), 132.0 (C-14), 144.4 (C-9a), 148.9 (C-7), 153.8 (C-8), 158.5 (C-11), 160.3 (C-5); MS *m*/*z* (relative intensity) 302 ([*M*]$^+$, 64), 272 (100), 257 (7), 231 (47), 217 (17), 203 (51), 192 (9), 156 (33), 139 (25), 111 (9), 91 (5), 70 (32). HRMS: calcd for $C_{16}H_{18}N_2O_4$ (302.1267), found 302.1227.

(2*S*)-*N*-(2-[9-Fluorenylmethoxycarbonyl]amino-5-methoxy-4-[2-propenoxy]benzoyl)pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (17). 9-Fluorenylmethyl chloroformate (584 mg, 2.26 mmol, 2.4 equiv) in dioxane (2.4 mL) was added dropwise to a stirred solution of **14b** (387 mg, 0.94 mmol) and aqueous Na_2CO_3 (2.5 mL of 10% w/v) in dioxane (1.2 mL) at 0 °C. Stirring was continued for 4 h at 0 °C then for 24 h at room temperature, after which water (2 mL) was added and the solvent removed in vacuo. Gradient chromatography (20:80→50:50 v/v EtOAc−hexane) of the residue afforded the Fmoc-protected thioacetal **17** as a yellow oil (523 mg, 88%): IR (neat) *v* 3432 (NH), 2926, 2855, 1730 (C=O), 1598 (C=C), 1522, 1450, 1403, 1333, 1263, 1203, 1117, 1029, 914, 869, 757; ^{1}H NMR δ 1.18−1.43 (m, 6H, H-34 & H-36), 1.68−2.27 (m, 4H, H-9 & H-10), 2.62−2.79 (m, 4H, H-33 & H-35), 3.44−3.65 (m, 2H, H-8), 3.85 (s, 3H, H-12), 4.26−4.77 (m, 7H, H-11, H-13, H-18, H-19 & H-32), 5.29−5.48 (m, 2H, H-15), 6.02−6.14 (m, 1H, H-14), 6.92 (s, 1H, H-6), 7.31−7.79 (m, 8H, H-21−24 & H-27−30), 7.87 (s, 1H, H-3), 8.99 (bs, 1H, H-16); ^{13}C NMR δ 14.9 & 15.2 (C-34 & C-36), 25.3 (C-9), 26.5 & 27.3 (C-33 & C-35), 26.8 (C-10), 47.1 (C-19), 52.2 (C-8), 53.2 (C-32), 56.4 (C-12), 61.3 (C-11), 67.1 (C-18), 69.8 (C-13), 105.5 (C-3), 111.7 (C-6), 118.8 (C-15), 120.1 (C-1), 120.7, 125.2, 127.1, & 127.8 (C-21−24 & C-27−30), 132.4 (C-14), 141.2 (C-2), 141.3 (C-25 & C-26), 143.7 (C-20 & C-31), 143.8 (C-5), 150.2 (C-4), 153.8 (C-17), 168.9 (C-7); MS *m*/*z* (relative intensity) 395 (9), 366 (5), 260 (43), 191 (100), 149 (8), 122 (6).

10-(9-Fluorenylmethoxycarbonyl)-1,2,3,10,11,11a-hexahydro-11-hydroxy-7-methoxy-8-(2-propenoxy)-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (18). A solution of thioacetal **17** (316 mg, 0.50 mmol), $HgCl_2$ (298 mg, 1.10 mmol, 2.2 equiv), and $CaCO_3$ (125 mg, 1.25 mmol, 2.5 equiv) in MeCN−H_2O (6 mL of 4:1 v/v) was stirred for 24 h at room temperature. The mixture was filtered through Celite and the filtrate evaporated in vacuo to give a dark yellow oil. Purification by column chromatography ($CHCl_3$) afforded benzodiazepinone **18** as a pale yellow solid (250 mg, 95%): mp 75−76 °C; IR (Nujol) *v* 3458 (OH), 2923, 2854, 1709 (C=O), 1603 (C=C), 1520, 1453, 1330, 1216, 915, 758; ^{1}H NMR ($CDCl_3$−CD_3OD) δ 1.61−2.12 (m, 4H, H-1 & H-2), 3.37−3.62 (m, 2H, H-3), 3.90 (s, 3H, H-12), 4.00−4.69 (m, 7H, H-11a, H-13, H-17, H-18 & H-31), 5.23−5.45 (m, 2H, H-15), 5.63 (d, 1H, J = 9.5 Hz, H-11), 6.00−6.14 (m, 1H, H-14), 6.74 (s, 1H, H-6), 6.83−7.69 (m, 9H, H-9, H-20−23 & H-26−29); ^{13}C NMR ($CDCl_3$−CD_3OD) δ 23.1 (C-2), 28.7 (C-1), 46.6 (C-3), 46.9 (C-18), 56.3 (C-12), 60.1 (C-11a), 68.5 (C-17), 70.0 (C-13), 85.8 (C-11), 111.2 (C-9), 114.7 (C-6), 118.5 (C-5a), 118.7 (C-15), 120.0, 120.5, 125.1, 125.4, 127.1, 127.2, 127.8 & 127.9 (C-20−23 & C-26−29), 132.4 (C-14), 141.3 (C-9a), 143.2 (C-24 & C-25), 143.5 (C-19 & C-30), 143.8 (C-7), 150.3 (C-8), 154.1 (C-16), 167.6 (C-5); MS *m*/*z* (relative intensity) 330 (13), 315 (20), 300 (25), 285 (56), 261 (18), 239 (36), 191 (100), 178 (44), 151 (26), 119 (44), 97 (37), 85 (44), 71 (63), 57 (91).

(11a*S*)-8-(2,3-Epoxypropoxy)-10-(9-fluorenylmethoxycarbonyl)-1,2,3,10,11,11a-hexahydro-11-hydroxy-7-methoxy-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (19). A solution of allyl ether **18** (160 mg, 0.30 mmol) and 3-chloroperoxybenzoic acid (50−60% w/w, 149 mg, 0.43 mmol, 1.4 equiv) in CH_2Cl_2 (10 mL) was stirred for 72 h at room temperature. The reaction mixture was then evaporated in vacuo and the residue purified by gradient chromatography (100:0→96:4 v/v $CHCl_3$−MeOH) to give epoxide **19** as a yellow oil (90 mg, 55%): IR (neat) *v* 3459 (OH), 3040, 2926, 2855, 1711 (C=O), 1605 (C=C), 1520, 1455, 1340, 1217, 1060, 990, 914, 810, 755; ^{1}H NMR δ 1.83−2.17 (m, 4H, H-1 & H-2), 2.69−2.74 (m, 1H, H-15), 2.83−2.87 (m, 1H, H-15), 3.34−3.36 (m, 1H, H-14), 3.38−3.87 (m, 2H, H-3), 3.91 (s, 3H, H-12), 3.99−4.44 (m, 7H, H-11a, H-13, H-17, H-18 & H-31), 5.64 (d, 1H, J = 9.5 Hz, H-11), 6.75 (s, 1H, H-6), 6.84−7.70 (m, 9H, H-9, H-20−23 & H-26−29); ^{13}C NMR δ 23.0 (C-2), 28.7 (C-1), 44.7 (C-15), 46.5 (C-3), 46.6 (C-18), 49.9 (C-14), 56.3 (C-12), 60.1 (C-11a), 68.5 (C-17), 70.0 (C-13), 86.0 (C-11), 111.2 (C-9), 114.8 (C-6), 118.4 (C-5a), 120.0, 125.0, 125.4, 127.0, 127.1, 127.7 & 127.9 (C-20−23 & C-26−29), 141.2 (C-9a), 143.2 (C-24 & C-25), 143.5 (C-19 & C-30), 143.7 (C-7), 149.9 (C-8), 154.1 (C-16), 166.9 (C-5); MS *m*/*z* (relative intensity) 307 (8), 279 (19), 251 (8), 202 (4), 167 (3), 149 (100), 94 (3), 71 (5), 57 (8).

(11a*S*)-8-(2,3-Epoxypropoxy)-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (6). A solution of Fmoc-protected derivative **19** (70 mg, 0.13 mmol) and Bu_4N^+ F^- (1 M solution in THF; 289 mg, 0.32 mmol, 2.5 equiv) in dry DMF (5 mL) was stirred for 15 min at room temperature. The reaction mixture was then quenched with MeOH (5 mL) and evaporated in vacuo, after which the residue was dissolved in EtOAc (25 mL) and extracted with water (3 × 25 mL) and the organic layer discarded. Brine (20 mL) was added to the aqueous extract, which was then extracted with $CHCl_3$ (3 × 25 mL). The combined organic phase was dried ($MgSO_4$) and the solvent evaporated in vacuo. Gradient chromatography (EtOAc, followed by 100:0→94:6 v/v $CHCl_3$−MeOH) provided the title epoxide PBD **6** as a pale yellow oil

(31 mg, 79%): IR (neat) ν 3040, 2924, 2854, 1628 (C=O), 1512 (C=C), 1452, 1270, 1215, 1015, 870, 813, 780, 755; ^{1}H NMR δ 2.02−2.11 (m, 2H, H-2), 2.28−2.37 (m, 2H, H-1), 2.75−2.80 (m, 1H, H-15), 2.90−2.94 (m, 1H, H-15), 3.40−3.43 (m, 1H, H-14), 3.53−3.87 (m, 3H, H-3 & H-11a), 3.95 (s, 3H, -OCH_3), 3.99−4.22 & 4.25−4.38 (m, 2H, H-13), 6.85 (s, 1H, H-9), 7.53 (s, 1H, H-6), 7.67 (d, 1H, J = 4.4 Hz, H-11); ^{13}C NMR δ 24.1 & 24.2 (C-2), 29.6 & 29.7 (C-1), 44.9 (C-15), 46.7 (C-3), 49.8 & 49.9 (C-14), 53.7 (C-11a), 56.1 & 56.2 (C-12), 69.6 & 70.1 (C-13), 111.0 & 111.1 (C-6), 111.8 (C-9), 121.0 (arom.), 140.5 (arom.), 147.8 (arom.), 150.2 (arom.), 162.6 (C-11), 164.5 (C-5); MS m/z (relative intensity) 302 ([M]$^+$, 100), 273 (5), 259 (4), 245 (16), 217 (3), 70 (9), 57 (8). HRMS: calcd for $C_{16}H_{18}N_2O_4$ (302.1266), found 302.1209.

(2*S*)-*N*-(5-Methoxy-2-nitro-4-[2-propenoxy]benzoyl)-pyrrolidine-2-carboxaldehyde Dimethyl Acetal (20a). Method A: DMF (1 drop) was added to a stirred solution of benzoic acid **12** (88 mg, 0.35 mmol) and oxalyl chloride (53 mg, 0.42 mmol, 1.2 equiv) in dry THF (10 mL), and stirring was continued for 3 h at room temperature. Volatiles were removed in vacuo, and the resulting oil was dissolved in dry THF (5 mL) and then added dropwise over 5 min to a stirred suspension of the pyrrolidine dimethyl acetal **13b** (50 mg, 0.34 mmol), triethylamine (71 mg, 0.70 mmol, 2 equiv), and water (1 drop) at 0 °C. The reaction mixture was warmed to room temperature and then stirred for an additional 16 h. After concentration in vacuo, the residue was purified by gradient chromatography (100:0→95:5 v/v $CHCl_3$−MeOH) to give the coupled nitro acetal **20a** as a yellow oil (113 mg, 85%).

Method B: 1,3-Dicyclohexylcarbodiimide (705 mg, 3.42 mmol) was added to a solution of **12** (866 mg, 3.42 mmol) in dry CH_2Cl_2 (50 mL) and the mixture stirred for 4 h at room temperature under a N_2 atmosphere. A solution of **13b** (497 mg, 3.42 mmol) in dry CH_2Cl_2 (20 mL) was then added, and stirring was continued for 16 h. The reaction mixture was then filtered and the filtrate evaporated in vacuo. Gradient chromatography (50:50→100:0 v/v EtOAc−hexane) afforded nitro acetal **20a** as a pale yellow oil (407 mg, 31%): IR (neat) ν 2939, 1635 (C=O), 1579 (C=C), 1522 (NO_2), 1427, 1336 (NO_2), 1276, 1218, 1180, 1060, 981, 864, 790, 757; ^{1}H NMR δ 1.74−2.22 (m, 4H, H-9 & H-10), 3.11−3.27 (m, 2H, H-8), 3.57 & 3.59 (s, 6H, H-17 & H-18), 3.98 (s, 3H, H-12), 4.41−4.46 (m, 1H, H-11), 4.69−4.71 (m, 2H, H-13), 4.95 (d, 1H, J = 2.4 Hz, H-16), 5.36−5.51 (m, 2H, H-15), 6.01- -6.15 (m, 1H, H-14), 6.76 (s, 1H, H-6), 7.71 (s, 1H, H-3); ^{13}C NMR ($CDCl_3$) δ 24.1 (C-9), 24.7 (C-10), 48.9 (C-8), 56.7 (C-12), 56.8 & 57.8 (C-17 & C-18), 59.0 (C-11), 70.2 (C-13), 104.8 (C-16), 108.7 (C-3), 109.2 (C-6), 119.4 (C-15), 128.3 (C-1), 131.6 (C-14), 137.3 (C-2), 147.9 (C-5), 154.6 (C-4), 166.5 (C-7); MS m/z (relative intensity) 380 ([M]$^+$, 2), 349 (29), 305 (89), 273 (25), 236 (100), 220 (17), 191 (49), 162 (30), 121 (63), 112 (45), 89 (45), 75 (99). HRMS: calcd for $C_{18}H_{24}O_7N_2$ (380.1584), found 380.1525.

(2*S*)-*N*-(4-[2,3-Epoxypropoxy]-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde Dimethyl Acetal (21a). A solution of 3-chloroperoxybenzoic acid (50−60% w/w, 64 mg, 0.18 mmol, 1.4 equiv), nitro acetal **20a** (50 mg, 0.13 mmol), and 3-*tert*-butyl-4-hydroxy-5-methylphenyl sulfide (1 mg, 3 μmol, 0.02 equiv) in $Cl(CH_2)_2Cl$ (10 mL) was refluxed at 85 °C for 2 h, after which TLC (75:25 v/v EtOAc−hexane) indicated that reaction was complete. The mixture was neutralized with an aqueous solution of $NaHCO_3$ (92 mg, 1.10 mmol in 5 mL). The organic layer was separated then washed with water (4 × 20 mL) and dried ($MgSO_4$) and the solvent removed in vacuo to give a yellow oil. Gradient chromatography (50:50→100:0 v/v EtOAc−hexane) gave epoxide acetal **21a** as a yellow oil (44 mg, 85%): IR (neat) ν 3050, 2924, 2854, 1721 & 1632 (C=O), 1579 (C=C), 1522 (NO_2), 1462, 1377 (NO_2), 1276, 1220, 1185, 1060, 980, 862, 791, 756, 722; ^{1}H NMR δ 1.69−2.24 (m, 4H, H-9 & H-10), 2.81 (dd, 1H, J = 2.5 & 4.7 Hz, H-15), 2.96 (dd, 1H, J = 4.0 & 4.5 Hz, H-15), 3.09−3.26 (m, 2H, H-8), 3.41−3.43 (m, 1H, H-14), 3.56 & 3.59 (s, 6H, H-17 & H-18), 3.97 (s, 3H, H-12), 4.01−4.16 (m, 1H, H-13), 4.2−4.33 (m, 1H, H-11), 4.44 (dd, 1H, J = 2.8 & 11.4 Hz, H-13), 4.94 (d, J = 1.7 Hz, H-16), 6.76 (s, 1H, H-6), 7.77 (s, 1H, H-3); ^{13}C NMR ($CDCl_3$) δ 24.1 (C-9), 24.7 (C-10), 44.6 (C-15), 48.9 (C-8), 49.7 (C-14), 56.5 (C-12), 56.6 & 57.7 (C-17 & C-18), 59.0 (C-11), 70.4 (C-13), 104.8 (C-16), 109.0 (C-3), 109.4 (C-6), 128.8 (C-1), 137.3 (C-2), 147.9 (C-5), 154.6 (C-4), 166.7 (C-7); MS m/z (relative intensity) 396 ([M]$^+$, 1), 380 (2), 365 (5), 321 (5), 296 (4), 267 (6), 252 (49), 236 (4), 222 (4), 207 (6), 149 (6), 122 (6), 75 (100), 57 (8). HRMS: calcd for $C_{18}H_{24}O_8N_2$ (396.1533), found 396.1541.

(2*S*)-*N*-(2-Amino-5-methoxy-4-propoxybenzoyl)pyrrolidine-2-carboxaldehyde Dimethyl Acetal (22). Method A: A mixture containing nitro acetal **20a** (350 mg, 0.92 mmol), 10% Pd−C (20 mg), and MeOH (20 mL) was hydrogenated for 16 h at room temperature and 1 atm pressure until TLC (99:1, v/v $CHCl_3$−MeOH) indicated that reaction was complete. The catalyst was removed by filtration through Celite, and the filtrate evaporated in vacuo to afford amino acetal **22** as a yellow oil (311 mg, 96%).

Method B: A mixture of nitro acetal **20a** (300 mg, 0.79 mmol) and 10% Pd−$BaCO_3$ (60 mg) in MeOH (10 mL) was hydrogenated for 24 h at room temperature and 1 atm pressure until TLC (99:1, v/v $CHCl_3$−MeOH) indicated that reduction was complete. The catalyst was removed by filtration through Celite, and the solvent evaporated in vacuo to give **22** as a yellow oil (272 mg, 92%).

Method C: A mixture of nitro acetal **20a** (400 mg, 1.05 mmol), 1,4-cyclohexadiene (8.83 g, 0.11 mol), and 10% Pd−C (20 mg) in MeOH (40 mL) was stirred for 18 h at room temperature until TLC (99:1 v/v $CHCl_3$−MeOH) indicated that reduction was complete. The catalyst was removed by filtration through Celite, and the solvent evaporated in vacuo to furnish propyl ether **22** as a yellow oil (332 mg, 90%): IR (neat) ν 3520 (NH_2), 3477 (OH), 1632 (C=O), 1576 (C=C), 1428, 1277, 1219, 1182, 1062, 980, 865, 755; ^{1}H NMR δ 1.17 (t, 3H, J = 6.8 Hz, H-15), 1.79−2.27 (m, 6H, H-9, H-10 & H-14), 3.51−3.64 (m, 10H, H-8, H-13, H-18 & H-19), 3.81 (s, 3H, H-12), 4.56−4.61 (m, 1H, H-11), 4.66 (d, 1H, J = 2.7 Hz, H-17), 6.32 (s, 1H, H-3), 6.78 (s, 1H, H-6); ^{13}C NMR δ 10.1 (C-15), 24.1 (C-14), 24.4 (C-9), 24.8 (C-10), 50.2 (C-8), 56.7 & 57.7 (C-18 & C-19), 57.0 (C-12), 59.1 (C-11), 75.5 (C-13), 102.1 (C-3), 105.2 (C-17), 110.3 (C-1), 112.7 (C-6), 141.2 (C-2), 146.8 (C-4), 147.9 (C-5), 169.7 (C-7); MS m/z (relative intensity) 352 ([M]$^+$, 1), 321 (36), 290 (21), 277 (92), 234 (8), 208 (100), 203 (4), 165 (31), 144 (7), 121 (48), 82 (28), 75 (89). HRMS: calcd for $C_{18}H_{28}O_5N_2$ (352.1998), found 352.1967.

(2*S*)-*N*-(2-Amino-4-[2-hydroxypropoxy]-5-methoxybenzoyl)pyrrolidine-2-carboxaldehyde Dimethyl Acetal (23). Method A: Epoxide acetal **21a** (40 mg, 0.10 mmol) was dissolved in dry MeOH (10 mL) and 10% Pd−C (20 mg) was added. The mixture was hydrogenated for 8 h at room temperature and 1 atm pressure, after which TLC (97:3 v/v $CHCl_3$−MeOH) indicated that the reaction was complete. The catalyst was removed by filtration through Celite, and the solvent evaporated in vacuo to give the 2-hydroxypropyl ether **23** as a yellow oil (38 mg, 99%).

Method B: A mixture containing **21a** (60 mg, 0.15 mmol) and 10% Pd−$BaCO_3$ (40 mg) in MeOH (10 mL) was hydrogenated for 16 h at room temperature and 1 atm pressure until TLC (97:3 v/v $CHCl_3$−MeOH) indicated that reaction was complete. The catalyst was removed by filtration through Celite, and the solvent evaporated in vacuo to afford **23** as a yellow oil (55 mg, 95%).

Method C: A mixture of **21a** (245 mg, 0.62 mmol), 1,4-cyclohexadiene (5.16 g, 64.4 mmol), and 10% Pd−C (20 mg) in MeOH (25 mL) was stirred for 18 h at room temperature until TLC (97:3 v/v $CHCl_3$−MeOH) indicated that the reaction was complete. The catalyst was removed by filtration through Celite, and the solvent evaporated in vacuo to afford the 2-hydroxypropyl ether acetal derivative **23** as a yellow oil (214 mg, 90%).

Method D: A mixture of **21a** (200 mg, 0.50 mmol) and $SnCl_2 \cdot 2H_2O$ (226 mg, 1.0 mmol, 2 equiv) in MeOH (50 mL) was refluxed at 60 °C for 30 min; then the solvent was removed in vacuo. The residue was purified by gradient chromatography (100:0→97:3 v/v $CHCl_3$−MeOH) to afford **23** as a yellow oil (136 mg, 71%): IR (neat) ν 3542 (OH), 3480 (NH_2), 1726 (C=

O), 1585 (C═C), 1530, 1461, 1376, 1271, 1220, 1053, 976, 865, 720; ^{1}H NMR δ 1.21 (t, 3H, J = 6.6 Hz, H-15), 1.69−2.34 (m, 4H, H-9 & H-10), 3.05−3.32 (m, 2H, H-8), 3.56 & 3.59 (s, 6H, H-19 & H-20), 3.97 (s, 3H, H-12), 4.02−4.20 (m, 3H, H-13 & H-14), 4.40−4.45 (m, 1H, H-11), 4.95 (d, 1H, J = 2.4 Hz, H-18), 6.29 (s, 1H, H-3), 7.30 (s, 1H, H-6); ^{13}C NMR δ 8.5 (C-15), 26.0 (C-9), 26.6 (C-10), 49.8 (C-8), 56.3 (C-12), 56.5 & 56.7 (C-19 & C-20), 59.4 (C-11), 69.8 (C-14), 72.8 (C-13), 104.7 (C-18), 101.4 (C-3), 113.5 (C-6), 118.7 (C-1), 139.3 (C-2), 147.2 (C-5), 153.5 (C-4), 169.9 (C-7); MS m/z (relative intensity) 368 ([M]$^+$, 2), 337 (21), 310 (15), 306 (35), 293 (76), 236 (100), 224 (96), 209 (4), 166 (13), 121 (45), 82 (17), 70 (9). HRMS: calcd for $C_{18}H_{28}O_6N_2$ (368.1947), found 368.1902.

(11a*RS*)-7-Methoxy-8-(2-propenoxy)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (15). A solution of nitro acetal **20a** (200 mg, 0.53 mmol) and $SnCl_2 \cdot 2H_2O$ (593 mg, 2.63 mmol, 5 equiv) in MeOH (50 mL) was refluxed for 4 h. After solvent removal in vacuo the residue was dissolved in $CHCl_3$ (50 mL), then saturated aqueous $NaHCO_3$ solution (50 mL) added, and the mixture stirred for 18 h at room temperature. The reaction mixture was then filtered through Celite and the organic layer separated. The aqueous layer was extracted with $CHCl_3$ (2 × 50 mL) and the combined organic phase washed with water (3 × 50 mL), dried ($MgSO_4$), and evaporated in vacuo. Gradient chromatography (100% EtOAc, then 100:0→94:6 v/v $CHCl_3$−MeOH) gave imine **15** as a yellow oil (111 mg, 73%). Analytical data were identical to those for **15** prepared by the route shown in Scheme 1.

DNA Binding Studies. 1. Thermal Denaturation. Compounds were subjected to thermal denaturation studies with duplex-form calf thymus DNA (CT-DNA) using an adaptation of a reported procedure.[34] Working solutions in aqueous buffer (10 mM NaH_2PO_4/Na_2HPO_4, 1 mM Na_2EDTA, pH 7.00 ± 0.01) containing CT-DNA (100 μM in phosphate) and the PBD (20 μM) were prepared by addition of concentrated PBD solutions in MeOH to obtain a fixed [PBD]/[DNA] molar ratio of 1:5. The DNA−PBD solutions were incubated at 37 °C for 0, 4, 18, or 36 h prior to analysis. Samples were monitored at 260 nm using a Varian-Cary 1E spectrophotometer fitted with a Peltier heating accessory, and heating was applied at 1 °C min^{-1} in the 40−98 °C range. Optical data were imported into the Origin 5.0 computer package (MicroCal Inc., Northampton, MA) for analysis, and DNA helix → coil transition temperatures (T_m) were obtained from the maxima in the d(A_{260})/dT derivative plots. Results are given as the mean ± SD from three determinations and are corrected for the effects of MeOH cosolute using a linear correction term.[34b] Drug-induced alterations in DNA melting behavior are given by: $\Delta T_m = T_m$(DNA + PBD) − T_m(DNA alone), where the T_m value for the PBD-free CT-DNA is 67.83 ± 0.06 °C (from 50 determinations). The fixed [PBD]/[DNA] ratio used did not result in binding saturation of the host DNA duplex for any compound examined.

2. DNA Cross-Linking Assay. The agarose gel electrophoresis assay[36] was carried out with **6** using pBR322 DNA and T4 polynucleotide kinase (PNK) purchased from Northumbria Biological Ltd. and was used as supplied. Bacterial alkaline phosphatase (BAP) and *Hind*III enzymes were purchased from BRL and used as supplied.

3. Linearization of pBR322 DNA. A mixture of circular pBR322 DNA (10 μg in 20 μL), React 2 aqueous buffer (3 μL [500 mM Tris-HCl, pH 8, 100 mM $MgCl_2$, 500 mM NaCl]), *Hind*III (3 μL, 30 units), and water (4 μL) was incubated at 37 °C for 1 h. Aqueous 4 M NaOAc was added to give a 0.3 M solution, then EtOH (95% v/v, 3 volumes) was added, and the mixture chilled. After centrifugation and removal of supernatant, the pellet was lyophilized.

4. Dephosphorylation of Linearized DNA. A mixture of linearized DNA in water (15 μL), BAP buffer (4 μL [50 mM Tris-HCl, pH 8, 600 mM NaCl]), and BAP (1 μL, 100 units) was incubated at 65 °C for 1 h. After extraction with Tris/EDTA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)/saturated phenol (1:1 v/v), the aqueous layer was collected and the phenolic layer back-extracted with buffer. The combined aqueous phase was extracted twice with $CHCl_3$/isoamyl alcohol (24:1 v/v); then aqueous 4 M NaOAc was added to give a 0.3 M solution, followed by EtOH (3 volumes). After chilling and centrifugation, the supernatant was removed and the pellet lyophilized.

5. 5′-End Labeling. The resultant dephosphorylated DNA was 5′-labeled with [γ-^{32}P]ATP (5000 Ci/mmol; Amersham plc). A mixture of the DNA (10 μg in 30 μL water), Forward Reaction Buffer (9 μL [300 mM Tris-HCl, pH 7.8, 75 mM 2-thioethanol, 50 mM $MgCl_2$, 1.65 μM ATP]), [γ-^{32}P]ATP (3 μL, 30 μCi), and PNK (4 μL, 24 units) was incubated at 37 °C for 30 min. Aqueous NH_4OAc (7.5 M, 0.5 volume) and EtOH (2 volumes) were added, and the mixture was chilled and centrifuged (13 000 rpm/10 min). The supernatant was removed and the pellet resuspended in 20 μL of Tris/EDTA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.3 M NaOAc). After reprecipitation with EtOH (60 μL) and centrifugation, the supernatant was removed and the pellet washed with EtOH (20 μL) and lyophilized. The DNA was then resuspended in sterile double-distilled water at 1 μg/μL. Approximately 10 ng of labeled DNA was used for each experiment.

6. Reaction Protocols. Reactions were performed with epoxide-PBD **6** at concentrations of 1, 10, 25, 50, 100, 250, 500, and 1000 μM in 25 mM triethanolamine/1 mM EDTA at pH 7.2 and 37 °C. In separate experiments, reactions were terminated after either 6 or 24 h by the addition of an equal volume of stop solution (0.6 M NaOAc/20 mM EDTA/100 μg/mL tRNA). After precipitation of the DNA by addition of EtOH (3 volumes) followed by centrifugation and removal of supernatant, the pellet was dried by lyophilization.

7. Electrophoresis. Samples were dissolved in strand separation buffer (10 μL of 30% DMSO/1 mM EDTA/0.04% bromophenol blue/0.04% xylene cylanol), heated at 90 °C for 2 min, and then chilled immediately in an ice−water bath prior to loading. Control nondenaturated samples were dissolved in 6% sucrose/0.04% bromophenol blue (10 μL) and loaded directly. Electrophoresis was performed on a 20-cm horizontal submerged 0.8% agarose gel at 40 V for 16 h using a 40 mM Tris/20 mM HOAc/2 mM EDTA gel running buffer.

8. Autoradiography. Gels were dried at 80 °C between one layer of Whatman 3 mm and one layer of DE81 filter paper within a Bio-Rad model 583 gel drier connected to a vacuum. Autoradiography was performed with Hyperfilm MP (Amersham plc) for 4 h at −70 °C using a DuPont-Cronex lighting-plus intensifying screen.

9. *Taq* DNA Polymerase Assay. The *Taq* DNA polymerase assay[37] was carried out on **6** using pBR322 DNA, T4 polynucleotide kinase (PNK), and the *Hind*III enzyme (Northumbria Biological Ltd.; used as supplied). [γ-^{32}P]ATP (5000 Ci/mmol) and Amplitaq recombinant *Taq* DNA polymerase were obtained from Amersham plc and Perkin-Elmer Cetus, respectively, and used as supplied. Linearization of pBR322 DNA and PBD treatments were carried out as described above.

10. *Taq* Polymerase Conditions. The PBD-bound DNA (0.5 μg), prepared as above, was combined with *Taq* DNA polymerase (1 unit), ^{32}P-5′-end-labeled oligonucleotide primer (5 pmol, 5′-TATGCGACTCCTGCATTAGG-3′), dideoxynucleoside triphosphate mixture (62.5 nM), gelatin (0.01%), $(NH_4)_2SO_4$ (20 μM), and $MgCl_2$ (2.5 μM) in reaction buffer (10 μL, Tris [pH 9, 75 μM]/Tween 20 [0.01%]) to give a total volume of 100 μL. Thermal cycling was then carried out as follows: 94 °C for 1 min (denaturation), 58 °C for 1 min (annealing), and 72 °C for 1 min (primer extension); this cycle was repeated for a total of 30 cycles, with the annealing step being extended by 1 s for each new cycle. The products were then chilled on ice, extracted with $CHCl_3$/isoamyl alcohol (24:1 v/v), precipitated with EtOH (95% v/v, 3 volumes), washed with EtOH (70% v/v, 100 μL), and dried by lyophilization.

11. Electrophoresis. Products were separated on 0.4-mm thick 6% denaturing polyacrylamide at 3000 V and 55 °C for approximately 3 h using a Tris/boric acid/EDTA running buffer. Autoradiography was carried out as described above.

Cytotoxicity Studies. In vitro cytotoxicity was evaluated using the human ovarian carcinoma cell lines SKOV-3, A2780,

and CH1, and the cisplatin-resistant counterparts of the A2780 and CH1 lines (A2780cis^R and CH1cis^R, respectively). Viable cells were seeded in growth medium (160 μL) into 96-well microtiter plates and allowed to attach overnight. The PBDs were dissolved in DMSO (to give a 20 mM concentration in each case) immediately prior to adding to the cells in quadruplicate wells. Final PBD concentrations in the wells ranged from 100 μM to 2.5 nM as follows: 100, 25, 10, 2.5, 1 μM; 250, 100, 25, 10, 2.5 nM. This was achieved by diluting the PBDs in growth medium and then adding 40 μL to the existing well volume of 160 μL to give the final concentrations stated above. After 4 days (96 h) the medium was removed and the remaining cells were fixed using 10% trichloroacetic acid on ice for 30 min. Wells were then washed 3−4 times with tap water and air-dried overnight, and 100 μL of 0.4% sulforhodamine B (dissolved in 1% glacial HOAc) was added to each well. Staining was allowed to continue for 10−15 min; the wells were then washed 3−4 times with 1% acetic acid and air-dried, and Tris base (100 μL of 10 mM) was added to each one. The plates were then shaken and absorbance readings taken at 540 nm using a plate reader. From plots of concentration versus percentage absorbance (compared to 8 untreated wells), IC_{50} values were calculated using the Quattro-Pro software package.

Molecular Modeling Studies. The strategy used for the molecular modeling was developed from recent studies of DNA−PBD adduct formation.[10b,40] Host DNA duplexes for energy calculations were selected with embedded 5′-(**G**/C)A$_{0-1}$-(**G**/C) target sequences flanked by strand-terminating G·C base pairs to prevent strand fraying during MD simulations (Table 2). Additional flanking adenine bases were incorporated, where appropriate, to provide the favored 5′-A**G**A motifs for alkylation by the electrophilic PBD moiety. For each DNA sequence, simulations were initially carried out with covalent PBD adducts formed with C11(*S*)-stereochemistry (see earlier) and alignment of the aromatic A-ring toward either the 5′- or 3′-end of the covalently modified strand. Models were examined with the epoxide in either the bound or unbound states, and in each possible (i.e., *R* or *S*) configuration. This required 8 simulations for the monoadducts and 16 simulations for the cross-linked adducts.

Using the Quanta 4.1 program (Molecular Simulations Inc., San Diego, CA), versions of compound **6** were constructed with each epoxide configuration based on the crystal coordinates of anthramycin methyl ether.[41] ESP charges were then calculated using the MNDO Hamiltonian in MOPAC 6, and coordinates for the DNA duplexes were generated using the nucleic acid sequence builder module in Quanta 4.1. Guanine bases used to form covalent bonds with either the PBD or epoxide moieties were built by deleting the outer-facing exocyclic H22 proton followed by rationalization of the residual charge over the remaining atoms.[10b] A similar procedure was adopted for the loss of a proton at the C11 position of the bound PBD molecule. As nucleophilic ring opening of the epoxide would form a secondary alcohol, this species was also constructed.

The following stepwise protocol using the CHARMm 23 program[39] was adopted to dock and minimize the ligand into the minor groove of DNA: (i) the PBD was manually docked in the minor groove with the C11 atom positioned 1.45 Å from the N2 of G[5] and in the plane of the base, thereby effectively replacing the outer-facing guanine C2-NH$_2$ proton not involved in G·C base pairing. (ii) Powell-type MM minimization for 1000 steps, or to a gradient tolerance change of 0.1 kcal mol^{-1}, was carried out to remove bad contacts from the initial docking. (iii) The system was heated from 0 to 300 K for 20 ps (0.2-fs time step) and the equilibrium assessed by monitoring the temperature every 200 steps and uniformly reassigning velocities from the averaged velocities at that time step if more than ±10 K from the target 300 K. (iv) MD was continued at 300 K for a further 25 ps with a 1-fs time step. The coordinates were saved after every 100 steps to give a total of 500 coordinate sets. (v) Final Powell MM minimization of the rms-averaged snapshots was carried out to a gradient tolerance change of 0.1 kcal mol^{-1}.

Angle constraints of −110° (purines) and −115° (pyrimidines) were applied to the glycosidic torsions in the B-type DNA, using a force constant of 500 kcal mol^{-1} during stages (ii)−(iv). Planar restraints were not required for the bases, although atoms C1′ and P of all terminal bases were fixed during MD stages (iii) and (iv) to prevent the short DNA duplex from unravelling or "fraying". Nonbonded interactions were included up to 11.5 Å; the switching function was employed between 10.5 and 9.5 Å, and a factor of 0.4 was used to dampen 1,4-electrostatic interactions. Hydrogen-bonded contacts were included up to 3.5 Å, with a switching function used between 4.0 and 4.5 Å. Solvent and counterion effects were simulated using a distance-dependent dielectric constant[42] with $\epsilon = cr_{ij}$, where $c = 4.0$ during the MM stages (ii) and (v) and $c = 1.0$ during the MD stages (iii) and (iv). Covalent interaction energies were calculated using $E_{\text{interaction}} = E_{\text{complex}} - [E_{\text{DNA}} + E_{\text{PBD}}]$, where E_{complex} is the energy of the minimized average structure for the DNA−PBD adduct, and the E_{DNA} and E_{PBD} component energies were determined from separate single-point energy calculations on this complex after removal of either the ligand or DNA and replacement of all required protons (i.e., guanine C2-NH$_2$ or H11 for the PBD). In the case where the PBD is covalently bound to the DNA through C11 but the epoxide is in the ring-opened form, the alcohol was built and used instead of the intact epoxide ring.

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