DNA Binding and Alkylation by the "Left Half" of Azinomycin B[†]

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ABSTRACT: Azinomycin B (also known as carzinophilin A) contains two electrophilic functional groups—an epoxide and an aziridine residue—that react with nucleophilic sites in duplex DNA to form cross-links at 5′-dGNT and 5′-dGNC sequences. Although the aziridine residue of azinomycin is undoubtedly required for cross-link formation, analogues containing an intact epoxide group but no aziridine residue retain significant biological activity. Azinomycin epoxide analogues (e.g., **5** and **6**) are of interest due to their potent biological activity and because there is evidence that azinomycin may decompose in vivo to yield such compounds. To investigate the chemical events underlying the toxicity of azinomycin epoxides, DNA binding and alkylation by synthetic analogues of azinomycin B (**6**, **8**, and **9**) that comprise the naphthalene-containing "left half" of the antibiotic have been investigated. The epoxide-containing analogue of azinomycin (**6**) efficiently alkylates guanosine residues in duplex DNA. DNA alkylation by **6** is facilitated by noncovalent binding of the compound to the double helix. The results of UV−vis absorbance, fluorescence spectroscopy, DNA winding, viscometry, and equilibrium dialysis experiments indicate that the naphthalene group of azinomycin binds to DNA via intercalation. Equilibrium dialysis experiments provide an estimated binding constant of $(1.3 \pm 0.3) \times 10^3$ M^{-1} for the association of a nonalkylating azinomycin analogue (**9**) with duplex DNA. The DNA-binding and alkylating properties of the azinomycin epoxide **6** provide a basis for understanding the cytotoxicity of azinomycin analogues which contain an epoxide residue but no aziridine group and may provide insight into the mechanisms by which azinomycin forms interstrand DNA cross-links.

Compounds that form covalent DNA cross-links often display interesting medicinal, carcinogenic, or toxic properties (*1*−*8*). The natural product azinomycin B (**1**, also known as carzinophilin A) contains electrophilic epoxide and aziridine groups that react with nucleophilic sites in duplex DNA to form interstrand cross-links (**4a**, Scheme 1) (*9*−*12*). Because azinomycin possesses a unique chemical structure and potent cytotoxic and antitumor activities, the mechanisms by which this agent reacts with DNA are of interest.

Azinomycin forms interstrand DNA cross-links (**4a**) at the sequences (*11*)

5′-d**G**NT	5′-d**G**NC
3′-dCN**A**	3′-dCN**G**

Studies utilizing sequencing gel electrophoresis (*11*), 7-deazapurine-containing deoxyoligonucleotides (*11*), chemical analysis of an isolated dG-dA cross-link (*12*), and molecular modeling (*13*) have provided evidence that the azinomycin-mediated interstrand cross-link spans the N7 positions of two purine (dA or dG) residues on opposing strands of the DNA duplex.

While the aziridine group of azinomycin is undoubtedly required for the formation of DNA cross-links, azinomycin analogues containing an intact epoxide group but no aziridine residue retain significant biological activity (*14*−*16*).[1] Epoxides such as **5a**, **5b**, and **6** are of special interest because they may be formed in vivo by decomposition of azinomycin and may contribute to the overall biological activity of this natural product. Compound **5a** results from spontaneous hydrolysis of azinomycin in buffered aqueous solution (*17*). Likewise, reaction of endogenous cellular thiols with azinomycin is expected (*15*) to produce analogous aziridine-ring-opened compounds (**5b**). The epoxide **6** has been isolated from culture broths of the azinomycin-producing organism *Streptomyces griseofuscus* (*18*) and is thought to stem from spontaneous hydrolysis of the antibiotic (*15*).

To better understand the chemical events underlying the biological activity of azinomycin epoxides, and to gain insight into the mechanisms of DNA cross-linking by the intact natural product, we have characterized noncovalent DNA binding and DNA alkylation by the naphthalene epoxide (**6**) that corresponds to the "left half" of azinomycin B. We find that compound **6** efficiently alkylates DNA and that the reaction with DNA is promoted by binding of the compound's naphthalene residue to the double helix. Interestingly, the 3-methoxy-5-methylnaphthalene group of azinomycin represents a uniquely effective example of a small, uncharged DNA intercalator. Our results show that the epoxide residue of azinomycin can alkylate DNA without

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[1] It was initially reported (*14*) that compound **4** was inactive against bacterial and cancer cell lines; however, more recent work (*15*, *16*) has shown that this compound possesses potent cytotoxicity (e.g., IC_{50} against P388 murine leukemia = 0.0012 μg/mL) comparable to agents such as cisplatin and mitomycin C.

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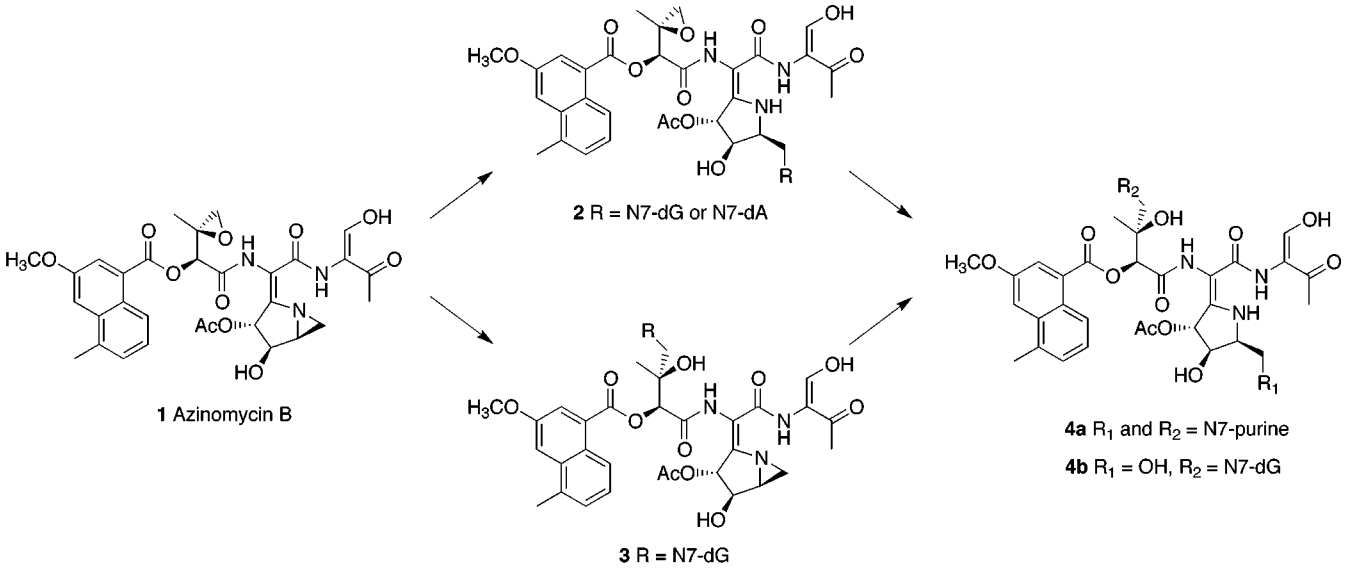
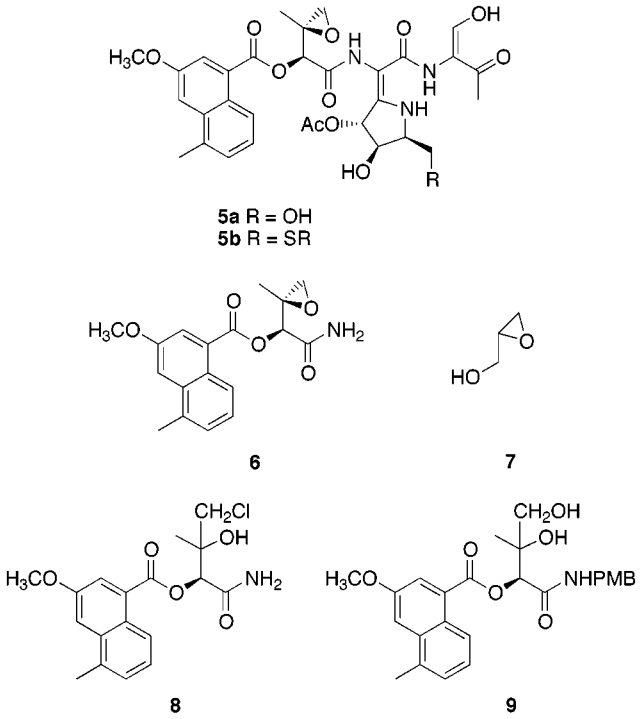
Scheme 1



the need for initial attachment of the antibiotic to DNA through its aziridine residue. This finding suggests that, contrary to previous expectations (*12*, *15*, *19*), interstrand cross-link formation by azinomycin could occur via initial reaction of DNA with the epoxide residue and further implies that the antibiotic may be capable of forming epoxide monoadducts (**3** or **4b**, Scheme 1).



EXPERIMENTAL PROCEDURES

Materials and General Procedures. Reagents were purchased from the following suppliers and were of the highest purity available: supercoiled pBR322 DNA was either prepared using the Qiagen system or purchased from Boehringer Mannheim; herring sperm DNA (catalog no. 223646), *Nhe*I, ammonium persulfate, and *N*,*N*′-bisacrylamide were from Boehringer Mannheim; T4 polynucleotide kinase, *Bam*HI, calf intestinal phosphatase, and T4 DNA ligase were from New England Biolabs; [γ-^{32}P]dATP was from New England Nuclear-Dupont; tris(hydroxymethyl)-aminomethane (Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES),[2] G-10 Sephadex, phenol, and daunomycin were from Sigma Chemical Co.; ethylenediaminetetraacetic acid (EDTA) was from Fluka Chemical Co.; xylene cyanol, bromophenol blue, formamide, acrylamide, tetramethylethylenediamine (TEMED), and ethidium bromide were from United States Biochemical; HPLC-grade solvents (acetonitrile, methanol, ethanol, and chloroform) were from Fisher; glycidol, sodium phosphate, and piperidine were from Aldrich. Compound **6** and its *N*-*p*-methoxybenzyl derivative [2-[[(4-methoxyphenyl)methyl]amino-1-(2-methyloxiranyl)-2-oxoethyl ester]-3-methoxy-5-methyl-1-naphthalenecarboxylic acid] were prepared by Shibuya and Terauchi (*18*).

Sequencing Gel Analysis of DNA Alkylation by ***6***. A 5′-^{32}P-labeled 145 base pair DNA fragment was prepared by digesting plasmid DNA (pBR322) with *Bam*HI followed by treatment with calf intestinal phosphatase. The linearized plasmid was 5′-labeled using [γ-^{32}P]dATP and T4 polynucleotide kinase and then digested with *Nhe*I. The resulting 5′-^{32}P-labeled 145 base pair DNA fragment was isolated by excising the appropriate band from a 5% nondenaturing polyacrylamide gel (*20*).

In a typical alkylation reaction, ~120 000 cpm of labeled DNA and 5 μg of herring sperm carrier DNA in sodium phosphate buffer (10 mM, pH 7, 20 μL final volume) were incubated with **6** for 2 h at 37 °C. Following incubation, the DNA was ethanol precipitated (*20*) and washed with 70% ethanol−water (0.2 mL), and the resulting pellet was redissolved in aqueous piperidine (100 μL of a 0.2 M solution) and incubated at 90 °C for 30 min. The resulting solution was evaporated in a SpeedVac concentrator at 37 °C and then redissolved in water and evaporated (2×). The resulting DNA fragments were dissolved in formamide loading buffer (*20*) and loaded onto a denaturing polyacrylamide gel (20%, 19:1 cross-linked, containing 8 M urea).

[2] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TEMED, tetramethylethylenediamine; DMSO, dimethyl sulfoxide.

The gel was electrophoresed at 600 V for 35 min and then 1500 V for 6 h (until the xylene cyanol dye had moved 15 cm from the origin). The resolved DNA fragments in the gel were visualized by autoradiography using Fuji film (catalog no. 04-441-96) or by phosphorimager analysis.

*Preparation of the Ring-Opened Azinomycin Analogue **8***. 2-[Amino-1-(2-methyloxiranyl)-2-oxoethyl ester]-3-methoxy-5-methyl-1-naphthalenecarboxylic acid (**6**, 1 mg) was dissolved in a mixture of CD_3CN (0.5 mL), DMSO-d_6 (0.5 mL), and 1 M HCl (in D_2O, 0.5 mL). The reaction was allowed to stand at room temperature for 1.5 h. The solution was then frozen in dry ice and lyophilized in a SpeedVac concentrator with warming at 37 °C to yield **8** as a white powder: ^{1}H NMR (CD_3CN/DMSO-d_6/D_2O, 1:1:1) δ 1.31 (s, 3H), 2.61 (s, 3H), 3.67 (q, $J = 9.0$ Hz, 2H), 3.91 (s, 3H), 5.21 (s, 2H), 7.35 (s, 2H), 7.55 (s, 1H), 7.77 (s, 1H), 8.40 (d, $J = 7.8$ Hz, 1H); MS (EI) calcd for $C_{18}H_{20}ClNO_5$ 365.1, found 365.1. The reaction of hydrochloric acid with epoxides is known to yield predominantly the less substituted alkyl chloride (*21*). In addition, attack of various nucleophiles on azinomycin analogues occurs at the less substituted position of the epoxide residue (*12*, *15*, *18*). The product of this reaction was used without further purification and was dissolved in DMSO for T4 DNA ligase and fluorescence experiments and in MeOH for UV absorption experiments.

*Preparation of Hydrolyzed Azinomycin Analogue **9***. 2-[[(4-Methoxyphenyl)methyl]amino-1-(2-methyloxiranyl)-2-oxoethyl ester]-3-methoxy-5-methyl-1-naphthalenecarboxylic acid (the *N*-*p*-methoxybenzyl derivative of **6**, 5 mg, 11 mmol) was dissolved in 1:1 THF:H_2O (10 mL), followed by addition of *p*-toluenesulfonic acid (35 mg, 15 equiv). The reaction was stirred at room temperature for 3 days, warmed at 40 °C for 2 days, and then warmed at 50 °C for 1 day. The mixture was then rotary evaporated (aspirator vacuum) for 15 min to remove THF, the resulting aqueous mixture extracted with chloroform (10 mL), and the chloroform layer dried over anhydrous sodium sulfate. The product was purified by flash column chromatography on silica gel (eluted with hexane:ethyl acetate:chloroform, 10:2:1, followed by 100% ethyl acetate) to provide **9** (3.1 mg, 58% yield) as a white powder (~2:1 mixture of diastereomers): $R_f = 0.28$ (40:60 hexane:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.48 (s, 3H), 2.63 (s, 3H), 3.45 (q, $J = 5.9$ Hz, 1H), 3.62 (q, $J = 5.9$ Hz, 1H), 3.70 (s, 3H), 3.89 (s, 3H), 4.38 (d, $J = 5.8$ Hz, 2H), 5.60 (s, 1H), 6.60 (br, 1H), 6.76 (d, $J = 7.0$ Hz, 2H), 7.11 (d, $J = 8.5$ Hz, 2H), 7.28 (d, $J = 7.0$ Hz, 2H), 7.42 (s, 1H), 7.77 (s, 1H), 8.54 (d, $J = 8.3$ Hz, 1H); HRMS (FAB) calcd for $C_{26}H_{29}O_7NaN$ ($M + Na^+$) 490.1841, found 490.1840.

UV−Vis Spectroscopy. Absorption spectra were collected using a Hewlett-Packard 8452A diode array spectrophotometer. The effect of duplex DNA on the absorbance spectra of **6** and **8** was measured by adding 4 μL aliquots of a herring sperm DNA stock solution (4 mM bp in water) to 800 μL of a solution containing the compound (20 μM in 50 mM sodium phosphate, pH 7, containing 10% methanol) in a quartz cuvette. Each addition of herring sperm DNA was accompanied by addition of an appropriate amount of a concentrated stock of **6** or **8** in methanol to keep the ligand concentration constant at 20 μM throughout the course of the experiment. Before the spectra of **6** or **8** at each DNA concentration were recorded, the spectrophotometer was blanked on an identical solution containing all components except the DNA-binding ligand.

Fluorescence Experiments. Changes in the fluorescence of **9** caused by the addition of duplex DNA were measured using an SLM AMINCO Model 8100 spectrofluorometer. Fluorescence excitation and emission wavelengths were 347 and 429 nm, respectively. The wavelength of the emission maximum for **9** did not change upon addition of DNA. To a solution of **9** (1−200 nM) in buffer (50 mM sodium phosphate, pH 7, containing 10% DMSO) were added aliquots of a solution of herring sperm DNA (0.25−5 mM bp in water). Fluorescence readings were corrected for dilution. The fluorescence of appropriate blank solutions (containing the same concentrations of DNA used in the above experiments but lacking **9**) was measured and subtracted from the fluorescence readings of solutions containing **9** and DNA. Control experiments showed that the fluorescence signals for the compounds under study do not change over the course of a typical experiment in either the presence or absence of DNA and that the fluorescence signals are linear over the concentrations used in these experiments (0.1−10 nM).

T4 Ligase DNA-Winding Assay. In a typical assay, linearized pBR322 DNA (0.5 μg, linearized using *Bam*HI) was incubated with varying concentrations (2−100 μM in the case of **8**; 20 nM−10 μM in the case of daunomycin) of the DNA-binding ligand and T4 DNA ligase (100 units) in T4 ligase buffer (NEB) at 16 °C for 2 h in a final volume of 200 μL containing 10% DMSO by volume. The reaction was then extracted with 150 μL of phenol:chloroform (1:1), the organic layer removed, and the DNA in the aqueous layer ethanol precipitated (*20*). The DNA pellet was dried in a SpeedVac concentrator without heat for 2 min and then redissolved in 20 μL of water and 5 μL of sucrose loading buffer (*20*) containing bromophenol blue (0.25%). The solution was loaded on a 0.9% agarose gel, and the DNA topoisomers were separated by electrophoresis at 45 V for 16 h. The gel was stained by soaking in an aqueous solution of ethidium bromide (0.66 μg/mL) for 8 h. The DNA bands were visualized by UV_{254} transillumination and the gel was documented using an IS-1000 digital imaging system.

Viscosity Studies. DNA fragments for use in viscometry experiments were prepared as follows: In a 15 mL conical polycarbonate centrifuge tube, herring sperm DNA (20 mg) was suspended in Tris-HCl buffer (10 mL, 5 mM, pH 7, containing 1 mM NaCl and 0.5 mM EDTA), the tube immersed in a Fisher Scientific FS-60 sonicating bath, and the solution sonicated for 30 min, cooled to 4 °C for 10 min, and then sonicated for an additional 30 min. The DNA solutions were passed through a 0.45 μm filter prior to use. Viscosity measurements were performed using an Ostwald-type flow viscometer (2 mL capacity) at room temperature. Changes in the viscosity of a DNA solution (800 μM bp, pH 7, 5 mM Tris-HCl, 1 mM NaCl, and 0.5 mM EDTA) caused by addition of increasing amounts of **6** (0−360 μM final concentrations, added as a concentrated 10 mM stock solution in acetonitrile) were detected by measuring the resulting changes in flow time of the solution through the viscometer. Each addition of **6** was accompanied by addition of an appropriate amount of DNA from a concentrated (1.6 mM bp) solution in order to maintain a constant DNA concentration (800 μM bp) throughout the experiment.

Following each addition of **6** and DNA, the solution was mixed by passing a stream of air through the capillary end of the viscometer so as to cause bubbling through the solution reservoir for 2−3 min. Each measurement reported is the average of three to four runs. Flow times for buffer, buffered DNA solutions, and DNA plus compound ranged from 88 to 94 s. Control experiments showed that addition of a "blank" acetonitrile solution to the buffered DNA solution does not yield a significant increase in viscosity relative to those caused by addition of acetonitrile solutions containing **6**. Relative viscosities of solutions were obtained from the equation $\eta = (t - t_0)/t_0$, where t is the flow time of the DNA−drug solution and t_0 is the flow time of the DNA−buffer solution. Viscosity data were analyzed as described by Cohen and Eisenberg (*22*). Apparent DNA fragment length (L/L_0) was calculated as $(\eta/\eta_0)^{1/3}$ and plotted versus r, where r represents the ratio of ligand to DNA base pairs.

Equilibrium Dialysis. A solution (200 μL) of **9** (1 μM) and herring sperm DNA (0.17 mM bp) in sodium phosphate buffer (50 mM, pH 7.0, containing 100 mM NaCl) was placed in a mini dialysis unit (Slide-A-Lyzer, Pierce Chemical Co., Rockford, IL; molecular weight cutoff 3500) and dialyzed for 48 h at room temperature with gentle stirring against an identical solution (5 mL) of **9** (containing no DNA) in a covered beaker. After 48 h of equilibration, the concentration of drug in the DNA-containing compartment and in the dialysate was determined. Aliquots (100 μL) from both the DNA-containing compartment and the dialysate were filtered by centrifugation through Amicon Microcon filters (MWCO 3000; Millipore Corp., Bedford, MA), and the filtrate (80 μL) was diluted to 3 mL with ultrapure deionized water. The concentrations of **9** were determined using fluorescence spectroscopy (excitation and emission wavelengths were 347 and 429 nm, respectively) by comparison to calibration curves. Readings were corrected to account for background fluorescence contributed by Amicon filtration (by subtraction of fluorescence of an Amicon-filtered buffer solution). Amounts of DNA-bound **9** were measured by calculating the increased amounts of ligand in the DNA-containing compartment relative to a control experiment in which the dialysis unit contained no DNA. The binding constant (K_b) was calculated using the equation:

$$K_b = [\text{ligand}\cdot\text{DNA}]/([\text{ligand}][\text{DNA}])$$

where [ligand], [DNA], and [ligand·DNA] are concentrations of free ligand, free DNA, and DNA-bound ligand, respectively. At the low ratios of [ligand·DNA]:[DNA] in these experiments, nearest neighbor exclusion can be ignored.

RESULTS

*DNA Alkylation by Azinomycin Epoxide **6***. We investigated the reaction of **6** with a 5′-^{32}P-labeled 145 base pair restriction fragment. Sequencing gel analysis reveals that **6** generates base-labile lesions selectively at guanosine residues in double-stranded DNA (Figure 1). This result strongly suggests that **6** alkylates DNA at the N7 position of guanosine to generate an alkylguanosine lesion as previously seen for intact azinomycin (*11*, *12*). No significant alkylation of adenosine residues is seen in our experiments. The observed alkylation of guanosine residues is not highly sequence dependent; alkylation is seen at every guanosine residue in the DNA fragment.

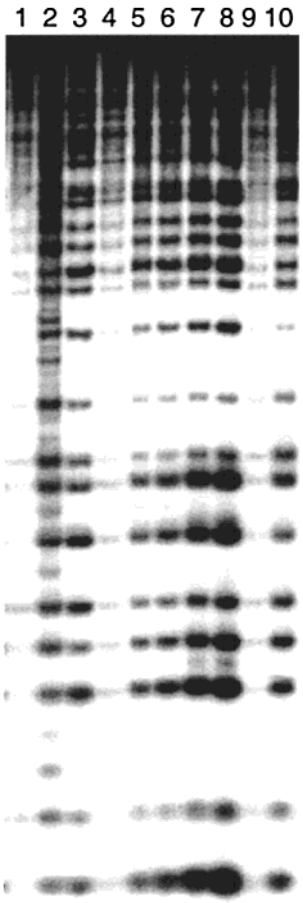


FIGURE 1: Alkylation of duplex DNA by azinomycin analogue **6** and glycidol (**7**). Reactions and 20% denaturing polyacrylamide gel electrophoresis were performed as described in Experimental Procedures. Lanes: 1, DNA alone; 2, Maxam−Gilbert A + G reaction; 3, Maxam−Gilbert G reaction; 4−8, DNA + **6** (0.25, 0.5, 1.0, 2.5, and 5.0 μM); 9 and 10, glycidol (**7**, 1.0 μM and 50 mM).

We compared the DNA-alkylating ability of **6** with that of a simple epoxide, glycidol (**7**), which is known to alkylate the N7 position of guanines in DNA (*23*). Under our assay conditions, ~50 000 times higher concentrations of **7** are required to achieve alkylation yields comparable to that generated by the azinomycin epoxide **6** (Figure 1, lane 6 versus lane 10; lanes 4 and 9 also appear similar but are each comparable to the control lane 1 and, thus, depict essentially no DNA alkylation by either of the two epoxides). The reactivity of the epoxide residue in **6** may be somewhat greater than that of **7** due to intramolecular catalysis of the ring-opening reaction by the neighboring amide functional group (*2*); however, it seemed likely that the markedly superior DNA-alkylating ability of **6** stems largely from noncovalent association of the molecule with DNA. Consistent with the notion that **6** binds noncovalently to DNA, we found that addition of the DNA intercalator ethidium bromide to our assays inhibited DNA alkylation by **6** (data not shown). Thus, we were motivated to further investigate the ability of this azinomycin analogue to noncovalently associate with DNA.

*Preparation of Ring-Opened Azinomycin Analogue **8** and Hydrolyzed Analogue **9***. We suspected that the naphthalene

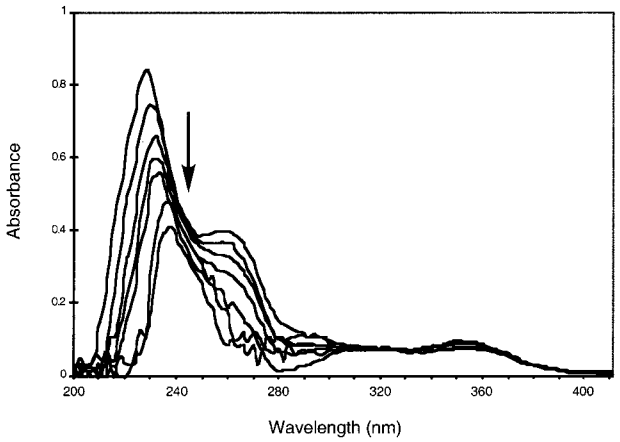


FIGURE 2: UV−vis spectra of **6** in the presence of increasing concentrations of duplex DNA. The UV−vis spectrum of **6** (20 μM) was recorded in the presence of 0, 40, 80, 120, 160, 200, and 240 μM (bp) DNA as described in Experimental Procedures.

residue of **6** associates noncovalently with DNA via intercalation (*24*) between the stacked base pairs of the double helix. Others have previously suggested that the naphthalene residue of azinomycin may intercalate with DNA (*25*, *26*). In the following sections, we describe the results of spectroscopic, DNA-winding, and hydrodynamic experiments that provide the first strong support for this hypothesis. To minimize covalent DNA modification during experiments designed to study noncovalent DNA interactions, we prepared and utilized analogues of compound **6** in which the epoxide ring was destroyed.

Treatment of **6** with HCl provides a simple route to an epoxide-ring-opened derivative that is a poor DNA-alkylating agent. The reaction of **6** with hydrochloric acid (1 N) in an acetonitrile/DMSO solvent mixture results in epoxide ring opening to yield the halohydrin **8**. Compound **8** does not efficiently form covalent attachments to duplex DNA during the typical time frame of the DNA-binding experiments described here. This was demonstrated by the observation that the characteristic fluorescence signal of DNA-bound **8** (vide infra) resulting from incubation of the compound with DNA (4 h, 20 nM **8**, 10 μM bp) diminishes upon dialysis (16 h). Thus, the data indicate that this compound can effectively be used to probe noncovalent DNA binding by the naphthalene fragment found in azinomycin. In addition, a nonalkylating, hydrolyzed azinomycin analogue (**9**) was prepared by warming a solution of 2-[[(4-methoxyphenyl)-methyl]amino-1-(2-methyloxiranyl)-2-oxoethyl ester]-3-methoxy-5-methyl-1-naphthalenecarboxylic acid (the *N*-*p*-methoxybenzyl analogue of **6**) in THF/water (1:1) with *p*-toluenesulfonic acid (15 equiv) for 6 days.

UV−Vis Experiments. The UV−vis spectra of organic ligands are often perturbed when the molecule associates with DNA (*27*−*30*). DNA intercalation typically causes a red shift and a decrease in the extinction coefficient at the absorbance maximum for the ligand (*27*−*30*). We find that titration of **6** and **8** with herring sperm DNA results in a progressive red shift in the absorbance maximum of the naphthalene residue (Figure 2). The 20 nm red shift and 45% decrease in absorbance observed for **6** are comparable to those seen previously for the binding of other intercalators to DNA (*27*−*30*).

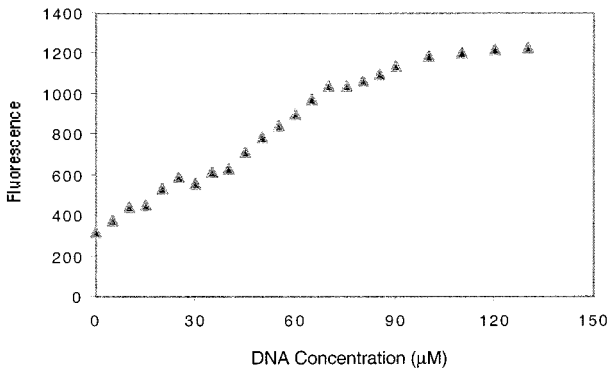


FIGURE 3: Increase in the fluorescence signal of **9** upon addition of duplex DNA. Fluorescence emission intensity (λ_{ex} = 347 nm, λ_{em} = 429 nm) of **9** was measured in the presence of increasing concentrations of duplex DNA as described in Experimental Procedures. The results of a single representative experiment are shown.

Fluorescence Experiments. Noncovalent association of organic compounds with DNA can result in either increases or decreases in the fluorescence signal intensity for the ligand (*27*−*30*). We find that addition of double-stranded DNA to **9** results in a concentration-dependent enhancement of the fluorescence emission signal intensity for the naphthalene residue (Figure 3). Approximately a 3-fold increase in fluorescence intensity is observed at saturating DNA concentrations. This result closely matches the fluorescence increase reported previously by Lown and co-workers for DNA binding by carzinophilin (*25*).

T4 Ligase DNA-Winding Assay. Intercalative binding of ligands to duplex DNA causes unwinding of the double helix (*27*, *29*, *31*). Such ligand-induced changes in the winding state of duplex DNA can be detected using a T4 DNA ligase circularization assay (*32*−*34*). In this assay, the ligand is added to a solution of linearized plasmid DNA which undergoes unwinding of the double helix if the compound intercalates. The DNA fragment (with the ligand bound) is then circularized using T4 DNA ligase and the ligand removed by extraction. Upon removal of the ligand, the normal twist of the helix is restored and the circularly constrained DNA adopts a superhelically twisted topology in order to accommodate the increased helical tension. The resulting topoisomers are then separated and visualized by agarose gel electrophoresis. The distribution of topoisomers obtained reflects the extent to which the small molecule was bound to the duplex as well as the degree of unwinding caused by each binding event (*31*).

Using the T4 DNA ligase assay, we find that **8** yields a concentration-dependent shift in the topoisomer distribution relative to control reactions containing no ligand (Figure 4). The effects of **8** in this assay were compared to the known intercalating ligand daunomycin ($K = 1.4 \times 10^5$ M^{-1}) (*31*). We find that between 200- and 500-fold higher concentrations of **8** are required to obtain winding effects comparable to those caused by daunomycin (Figure 4, lane 5 versus lane 9).

Viscometry Experiments. The results of the spectroscopic and DNA-winding experiments described above clearly indicate that the naphthalene residue found in **6**, **8**, and **9** associates with DNA. While the results of these experiments are consistent with the expected intercalative mode of binding

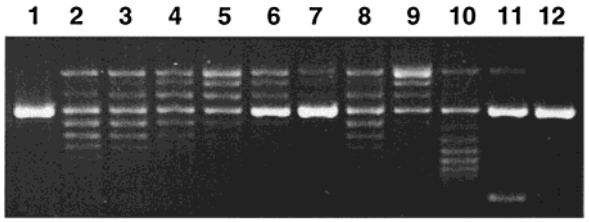


FIGURE 4: Changes in DNA winding induced by **8** and daunomycin measured by T4 DNA ligase assay. The assay was performed as described in Experimental Procedures. Lanes: 1, marker lane containing linearized DNA; 2, control T4 ligase assay containing no DNA-binding ligand; 3−7, T4 ligase assay containing **8** (2, 10, 20, 50, and 100 μM); 8−12, T4 ligase assay containing daunomycin (0.02, 0.1, 0.5, 2.0, and 10 μM).

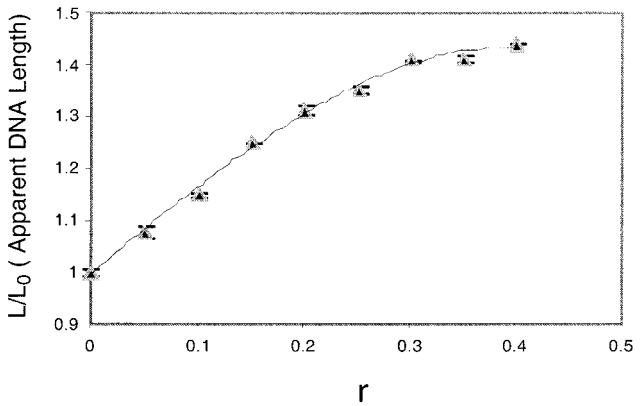


FIGURE 5: Increase in the viscosity of a duplex DNA-containing solution caused by the addition of increasing amounts of **6**. The values L/L_0 were calculated as described in Experimental Procedures. The value *r* represents the moles of drug per mole of DNA bp. Error bars represent the standard error from at least three measurements.

for the naphthalene residue, they do not *rigorously establish* the mode of DNA binding because nonintercalative binding can produce similar results in each of these assays (*27*, *29*, *31*).

Hydrodynamic methods such as viscosity or sedimentation measurements can provide rigorous evidence for intercalative DNA binding. Suh and Chaires recently noted that "viscosity provides a simple, theoretically sound means of distinguishing DNA binding mode" (*27*). The separation of base pairs that accompanies intercalative binding to the DNA duplex causes an increase in the length of DNA fragments. This increase in fragment length, in turn, causes a characteristic increase in the viscosity of DNA-containing solutions (*24*, *35*). Other methods such as electric linear dichroism can similarly provide evidence for intercalation.

We find that addition of increasing amounts of **6** to a buffered aqueous solution containing short fragments (~100−200 bp) of double-stranded DNA results in a progressive increase in the viscosity of the solution (Figure 5). This result provides strong evidence that **6** associates with double-stranded DNA via intercalation. Viscosity experiments utilized the DNA-alkylating compound **6**. Importantly, increases in the viscosity of DNA solutions resulting from the addition of **6** can be attributed to intercalation of the molecule into the DNA duplex and *not* to DNA alkylation by the molecule. Previous work has shown that DNA alkylation leads to a *decrease* in the viscosity of duplex DNA-containing solutions (*36*); thus, DNA alkylation by **6** cannot explain the viscosity increases observed in our experiments. Importantly, the nonalkylating azinomycin analogue **9** also binds to DNA via intercalation as indicated by the increase in viscosity resulting from the addition of this compound to DNA solutions.

Determination of Binding Constant by Equilibrium Dialysis. Equilibrium dialysis experiments utilizing the nonalkylating azinomycin analogue **9** yield a binding constant of $(1.3 \pm 0.3) \times 10^3$ M^{-1} for the association of this molecule with herring sperm DNA in sodium phosphate buffer (50 mM, pH 7.0, containing 100 mM NaCl). After 48 h equilibration, the concentrations of **9** in the DNA-containing compartment of the dialysis apparatus and in the dialysate were determined using fluorescence spectroscopy (excitation and emission wavelengths were 347 and 429 nm, respectively). Amounts of DNA-bound **9** were measured by calculating the increased amounts of ligand in the DNA-containing compartment relative to a control experiment in which the dialysis unit did not contain DNA. The binding constant (K_b) was calculated using the equation:

$$K_b = [\text{ligand}\cdot\text{DNA}]/([\text{ligand}][\text{DNA}])$$

where [ligand], [DNA], and [ligand·DNA] are concentrations of free ligand, free DNA, and DNA-bound ligand, respectively.

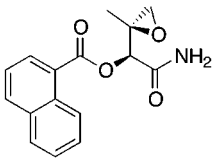
DISCUSSION

We find that the azinomycin epoxide **6** efficiently alkylates guanine residues in double-stranded DNA. Although this is the first report characterizing DNA alkylation by an azinomycin-derived epoxide, this result is easily understood in the context of existing literature. Simple epoxides such as glycidol (**7**) and diepoxybutane are known to alkylate duplex DNA at the N7 position of guanosine residues (*6*, *37*, *38*). Furthermore, efficient alkylation at the N7 position of guanosine in DNA has been well characterized for a number of epoxides that are appended to DNA intercalators. Examples of such systems include aflatoxin epoxides (*39*−*41*), the pluramycins (*42*, *43*), kapurimycin (*44*), and psorospermin (*45*). Shipman and co-workers previously suggested that the cytotoxicity of compound **6** and other structurally related azinomycin epoxides may stem from DNA alkylation by the epoxide residue (*16*).

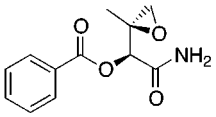
Efficient alkylation of guanosine by compound **6** is driven by noncovalent association of the compound with duplex DNA. It is well known that the efficiency of reactions with DNA can be enhanced by tethering alkylating agents to a DNA-binding moiety (*46*−*50*). The results of our UV−vis, fluorescence, DNA-winding, and viscometry experiments show the naphthalene residue binds to DNA by intercalation. These findings are noteworthy because there are few examples of DNA intercalation by such a small, uncharged aromatic system (*51*−*64*).[3] A binding constant of $(1.3 \pm 0.3) \times 10^3$ M^{-1} for the association of **9** with duplex DNA was obtained from equilibrium dialysis experiments. This binding

[3] DNA binding by other small, "nonclassical" intercalators has been characterized (*51*−*64*); however, these compounds are typically part of bisintercalating systems or possess positively charged functional groups that presumably provide favorable electrostatic interactions (*65*, *66*) with the sugar−phosphate backbone of DNA.

constant is smaller than that for some other intercalators of similar aromatic surface area, such as quinine ($\sim 10^4$ M^{-1}) and neocarzinostatin (2.5×10^5 M^{-1}) (*52*, *53*, *58*, *59*, *61*); however, these molecules contain functional groups (e.g., charged amino residues and hydrophobic groups) in addition to the aromatic intercalating moiety that contribute favorable binding interactions with DNA (*65*−*68*). In fact, Schneider and co-workers studied a variety of unsubstituted quinolines and naphthalenes and found that these compounds do not bind to DNA via intercalation unless they are appended with positively charged groups (*61*). Similarly, it is reasonable to speculate that the diminished biological activity of azinomycin analogues such as **10** and **11** (relative to that of **6**) may be the result of ineffective DNA binding by these compounds (*16*). Given that unsubstituted naphthalenes do not typically bind to DNA by intercalation, it appears that the substituents on the naphthalene ring of azinomycin play a crucial role in DNA binding. Thus, in the 3-methoxy-5-methylnaphthalene group of azinomycin, nature has provided a uniquely effective small, uncharged intercalator that accurately positions an appended electrophile in the major groove of DNA near the N7 position of guanosine residues. This naphthalene system is readily accessible by chemical synthesis (*18*, *69*, *70*) and may offer bioorganic and medicinal chemists a small, easily derivatized DNA-binding agent for the delivery of reactive species to the major groove of DNA.



10



11

It is noteworthy that the 3-fold increase in fluorescence observed upon addition of saturating DNA concentrations to a solution of **9** mirrors that previously reported by Lown and co-workers for carzinophilin (*25*). This similarity in fluorescence properties suggests that the azinomycin B/carzinophilin fragments **6**, **8**, and **9** studied here may bind to DNA in a manner similar to that of the intact natural product. To the extent that **6**, **8**, and **9** serve as accurate models for the DNA binding and reactivity of intact azinomycin B, the work described here may offer insights regarding the mechanisms of DNA modification by azinomycin. For example, our work may shed some light on the question of whether azinomycin cross-links always form via initial reaction of DNA at the aziridine group of the antibiotic. The aziridine residue of azinomycin appears to be significantly more reactive than the epoxide moiety (*15*, *17*, *26*), and others have suggested that azinomycin-mediated cross-link formation proceeds via initial reaction of DNA with the aziridine group to yield **2**, followed by reaction at the epoxide group to produce cross-linked DNA (**4a**, as in the upper pathway of Scheme 1) (*12*, *15*, *19*). However, the results described here indicate that DNA alkylation by the epoxide residue does not necessarily require initial reaction of DNA with the aziridine group and, thus, presents the formal possibility that, under some conditions or at some DNA sequences, cross-link formation could occur by initial reaction of guanosine residues at the epoxide of azinomycin to yield **3**, followed by a (probably rapid) reaction of an adenosine or guanosine residue on the opposing DNA strand with the aziridine residue to afford cross-linked DNA (**4a**, as in the lower pathway of Scheme 1). In addition, our work suggests that azinomycin may form epoxide monoadducts (i.e., **3** or **4b**, Scheme 1) at some guanosine residues in duplex DNA.

Finally, the finding that **6** efficiently alkylates guanine residues in DNA provides a chemical basis for understanding the previously reported (*16*, *18*) cytotoxicity of epoxide-containing analogues of azinomycin. Alkylation of guanosines in cellular DNA is known to have cytotoxic effects (*42*−*45*, *48*, *71*). Given the chemical lability of the aziridine residue in azinomycin, it is reasonable to expect that significant amounts of compounds such as **5a** and **5b** are formed in vivo and may contribute to the overall biological activity of this antibiotic.

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