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Studies on the Mechanism of Action of Azinomycin B: Definition of Regioselectivity and Sequence Selectivity of DNA Cross-Link Formation and Clarification of the Role of the Naphthoate

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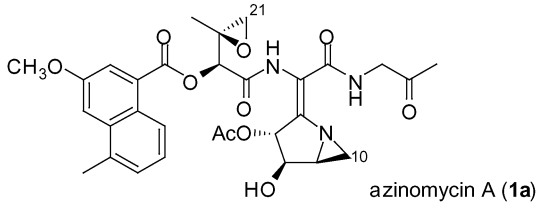
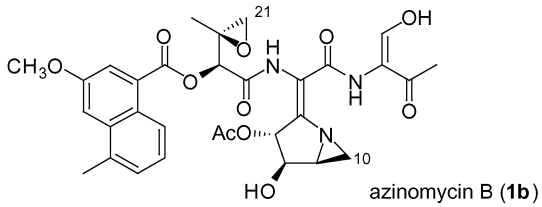
Abstract: Evaluation of the sequence selectivity, noncovalent association, and orientation of the DNA cross-linking agent azinomycin B on its duplex DNA receptor is described. A strong correlation between sequence nucleophilicity and cross-linking yield was observed, and steric effects due to the thymine C5-methyl group were identified. Detailed studies on the role of the azinomycin naphthoate using viscometry, fluorescence contact energy transfer, and DNA unwinding assays point to a nonintercalative binding mode for this group. A kinetic assay for agent regioselectivity was used to determine the orientation of binding and covalent cross-link formation.

1. Introduction

New prototypes of antitumor agents generate tremendous scientific interest in the synthetic organic and medicinal chemistry communities, and the DNA cross-linking agents azinomycins A (**1a**) and B (**1b**) are typical in this regard.[1,2]



azinomycin A (**1a**)



azinomycin B (**1b**)

These naturally occurring agents were isolated in 1986 from cultures of *Streptomyces griseofuscus* S42227[3] and possess an intricately functionalized structure containing the unusual aziridino[1,2-*a*]pyrrolidine ring system. They exhibit potent in vitro cytotoxic activity and promising in vivo antitumor activity against P388 leukemia in mice that is comparable to mitomycin C.[4]

Evaluation of the azinomycins has been hampered by chemical instability and poor availability from natural sources. Information on the biological activity of these agents is limited to the original 1987 report.[4] We recently reported the first total synthesis of a member of this natural product family,[5] and we now disclose our studies that begin to clarify the biological mechanism of action of these agents.

The azinomycins are among a small group of molecules that interact with duplex DNA in the major groove. Azinomycin B forms a covalent interstrand cross-link[6] via the N7 positions of suitably disposed purine bases in the duplex DNA sequence 5′-d(PuNPy)-3′, via the electrophilic C10 and C21 carbons, but the structural origin of the sequence selectivity and binding affinity have yet to be defined. Early studies by Lown and Majumdar[7] provided the original demonstration that azinomycin

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(2) (a) Hartley, J. A.; Hazrati, A.; Kelland, L. R.; Khanim, R.; Shipman, M.; Suzenet, F.; Walker, L. F. *Angew. Chem., Int. Ed.* **2000**, *112*, 3609. (b) Hartley, J. A.; Hazrati, A.; Khanim, R. Hodgkinson, T. J.; Shipman, M.; Suzenet, F.; Kelland, L. R. *Chem. Commun.* **2000**, 2325. (c) Hodgkinson, T. J.; Kelland, L. R. Shipman, M.; Suzenet, F. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 239.

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(4) Ishizeki, S.; Ohtsuka, M.; Irinoda, K.; Kukita, K.; Nagaoka, K.; Nakashima, T. *J. Antibiot.* **1987**, *40*, 60. In vitro cytotoxicity: $IC_{50} = 0.07$ μg/mL (**1a**) and 0.11 μg/mL (**1b**) against L5178Y cells. In vivo antitumor activity: 193% ILS at 16 μg/kg **1b** (3/7 survivors) against P388 leukemia; 161% ILS at 32 μg/kg **1b** (5/8 survivors) against Erlich carcinoma. In the same system, mitomycin C exhibited a 204% ILS at 1 mg/kg against P388 leukemia.

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B (née carzinophilin)[8] covalently cross-links native DNA without prior activation. A more recent, preliminary study on azinomycin/DNA interactions by Armstrong and co-workers[9] demonstrated cross-link formation between the agent and N7 of dG and N7 of dG or dA within the major groove of DNA. These results were confirmed recently by Saito and co-workers,[10] who proposed the orientational binding mode of azinomycin B to its triplet base-pair receptor, which we confirmed in a series of computational studies,[11−13] and now established experimentally.

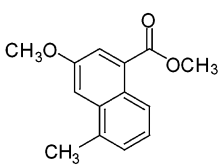
In none of these studies was a clear role for the naphthoic acid defined. Computational work was consistent with either an intercalative or nonintercalative binding model.[12,13] Experimental work on partial structures by Zang and Gates[14] indicated that azinomycin fragments bearing the naphthoate were weak DNA binding agents (10^3 M^{-1}), and these workers proposed an intercalative binding mode.

We now provide our initial results evaluating the covalent and noncovalent interactions of both azinomycin B with duplex DNA. Herein, we provide evidence that more clearly defines the regioselectivity of the agent in the cross-linked lesion, delineates the sequence selectivity of cross-link formation, and demonstrates a lack of effective intercalation by the naphthoate group of the agents.

2. Materials and Methods

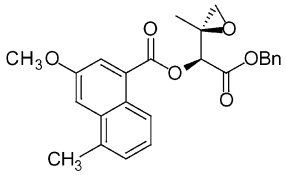
Azinomycin B was obtained from fermentation broths of *Streptomyces* sp. (NRRL 15902) using growth and isolation conditions similar to those originally published. Samples were stored in Et_2O or toluene solution at −80 °C to prevent decomposition; samples degraded significantly after several months, and best results were obtained with freshly isolated samples.

Methyl 3-Methoxy-5-methyl-1-naphthoate (4). A solution of diazomethane in ether was prepared by the addition of 1-methyl-3-nitro-1-nitrosoguanidine (201.5 mg, 1.157 mmol) to a mixture of 40% aqueous NaOH (20 mL) and ether (10 mL). A stirred solution of **2** (50 mg, 0.23 mmol) in THF (5 mL) at room temperature was treated with the diazomethane solution. The reaction mixture was quenched by the addition of 1 N aqueous HCl (2 mL), and the mixture was washed with saturated aqueous NaCl (2 × 2 mL). The organic layer was dried (Na_2SO_4), the solvent was removed, and the residue was purified by flash chromatography (2 × 5 cm silica, 15% ethyl acetate/hexane) to afford pure **4** (52.3 mg, 99%) as a crystalline solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.58 (dd, J = 6.2, 3.7 Hz, 1H), 7.78 (d, J = 2.6 Hz, 1H), 7.42 (d, J = 2.6 Hz, 1H), 7.32 (m, 2H), 4.00 (s, 3H), 3.94 (s, 3H), 2.64 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.3, 156.4, 134.8, 133.5, 129.9, 128.0, 127.2, 125.3, 124.3, 121.8, 108.4, 56.0, 52.7, 20.5; UV (MeOH/H_2O, 1:1) λ_{max} 240 (ϵ 34 000), 298 (ϵ 6300), 338 nm (ϵ 7400); HRMS (ESI), m/z 253.0822 (calcd for $C_{14}H_{14}O_3$ + Na: 253.0841).



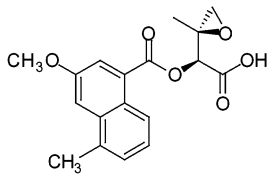
4

Benzyl (2*S*,3*S*)-3,4-Epoxy-2-(3-methoxy-5-methyl-1-naphthoyloxy)-3-methylbutanoate. A stirred solution of benzyl (2*S*,3*S*)-3,4-epoxy-2-hydroxy-3-methylbutanoate[15] (255.0 mg, 1.15 mmol), Et_3N (0.25 mL, 1.72 mmol), and DMAP (4-(*N*,*N*-dimethylamino)pyridine) (14.0 mg, 0.11 mmol) in dry CH_2Cl_2 (15 mL) at 0 °C was treated with a solution of 3-methoxy-5-methyl-1-naphthoyl chloride (320.0 mg, 1.38 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred at this temperature for 3 h before being quenched by the addition of water (50 mL). The organic layer was separated and the aqueous layer was extracted with dichloromethane (3 × 25 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. Purification of the residue by flash chromatography (2 × 10 cm silica, 20% ethyl acetate/hexane) afforded the desired compound (367 mg, 76%) as a colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60 (dd, J = 6.2, 3.7 Hz, 1H), 7.90 (d, J = 2.6 Hz, 1H), 7.51 (d, J = 2.6 Hz, 1H), 7.38 (m, 2H), 7.35 (m, 5H), 5.29 (d, J = 12.0 Hz, 1H), 5.21 (d, J = 12.0 Hz, 1H), 5.19 (s, 1H), 3.91 (s, 3H), 2.95 (d, J = 4.6 Hz, 1H), 2.65 (d, J = 4.6 Hz, 1H), 2.60 (s, 3H), 1.42 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.3, 166.0, 134.9, 133.9, 133.5, 131.2, 130.6, 128.4, 128.3, 128.2, 128.1, 127.8, 126.2, 125.5, 125.4, 124.3, 75.2, 67.2, 55.0, 51.7, 17.8; HRMS (ESI), m/z 443.1471 (calcd for $C_{25}H_{24}O_6$ + Na: 443.1471).



benzyl (2*S*,3*S*)-3,4-epoxy-2-(3-methoxy-5-methyl-1-naphthoyloxy)-3-methylbutanoate

(2*S*,3*S*)-3,4-Epoxy-2-(3-methoxy-5-methyl-1-naphthoyloxy)-3-methylbutanoic Acid. A solution of the above benzyl ester (368 mg, 0.88 mmol) in dry methanol (50 mL) was treated with 10% palladium on carbon (54 mg) and the resulting suspension was stirred under hydrogen (1 atm) for 2 h at room temperature. The reaction mixture was filtered through a pad of Celite, and the filtrate was concentrated in vacuo to afford the corresponding crude acid as a colorless solid, which was used without further purification: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61 (dd, J = 6.2, 3.7 Hz, 1H), 7.89 (d, J = 2.5 Hz, 1H), 7.49 (d, J = 2.5 Hz, 1H), 7.38 (m, 2H), 5.32 (s, 1H), 3.91 (s, 3H), 2.95 (d, J = 4.6 Hz, 1H), 2.65 (d, J = 4.6 Hz, 1H), 2.67 (s, 3H), 1.42 (s, 3H).



(2*S*,3*S*)-3,4-epoxy-2-(3-methoxy-5-methyl-1-naphthoyloxy)-3-methylbutanoic acid

(2*S*,3*S*)-3,4-Epoxy-2-(3-methoxy-5-methyl-1-naphthoyloxy)-3-methylbutanamide (3). A solution of the above crude acid (43 mg, 0.13 mmol) in tetrahydrofuran (THF) (5 mL) at 0 °C was treated with triethylamine (0.05 mL, 0.33 mmol) and ethyl chloroformate (31 mg, 0.26 μmol). After 15 min at 0 °C, aqueous 30% ammonium hydroxide (0.05 mL, 0.35 mmol) was added, and the reaction mixture was stirred for 30 min at 0 °C before being diluted with EtOAc and filtered through

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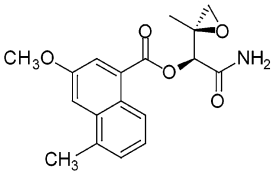
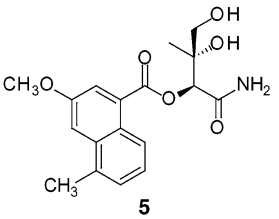
a pad of silica gel. Evaporation of the solvent and purification of the residue by flash chromatography (2 × 10 cm silica, 50% ethyl acetate/hexane) afforded **3** (37.9 mg, 93%) as a colorless solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60 (dd, J = 6.2, 3.6 Hz, 1H), 7.88 (d, J = 2.5 Hz, 1H), 7.45 (d, J = 2.5 Hz, 1H), 7.32 (m, 2H), 6.18 (br s, 1H), 5.96 (br s, 1H), 5.20 (s, 1H), 3.94 (s, 3H), 3.00 (d, J = 4.6 Hz, 1H), 2.76 (d, J = 4.6 Hz, 1H), 2.64 (s, 3H), 1.52 (s, 3H);^{13}C NMR (100 MHz, $CDCl_3$) δ 169.3, 166.0, 156.2, 134.9, 133.6, 128.5, 128.2, 127.3, 125.6, 124.2, 122.5, 108.8, 76.3, 62.9, 56.3, 53.7, 20.5, 18.0; HRMS (ESI), m/z 352.1150 (calcd for $C_{18}H_{19}NO_5$ + Na: 352.1161).



3

(2*S*,3*R*)-3,4-Dihydroxy-2-(3-methoxy-5-methyl-1-naphthoyloxy)-3-methylbutanamide (5). A solution of amide **3** (20 mg, 0.06 mmol) in a 6:1 mixture of THF/H_2O (7 mL) was treated with concentrated aqueous perchloric acid (1 drop), and the reaction mixture was warmed at reflux for 15 min. The mixture was diluted with EtOAc and washed with saturated aqueous solution of $NaHCO_3$ (5 mL) and saturated aqueous NaCl (3 × 3 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated. Purification of the residue by flash chromatography (2 × 5 silica, 50% ethyl acetate/hexane) afforded **5** (10 mg, 50%) as a colorless solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.66 (dd, J = 6.2, 3.6 Hz, 1H), 8.02 (d, J = 2.5 Hz, 1H), 7.57 (d, J = 2.5 Hz, 1H), 7.43 (m, 2H), 5.84 (s, 1H), 4.46 (d, J = 9.9 Hz, 1H), 4.31 (d, J = 9.9 Hz, 1H), 4.03 (s, 3H), 2.73 (s, 3H), 1.59 (s, 3H);^{13}C NMR (100 MHz, $CDCl_3$) δ 169.3, 166.0, 156.2, 134.9, 133.6, 128.5, 128.2, 127.3, 125.6, 124.2, 122.5, 108.8, 76.3, 62.9, 56.3, 53.7, 20.5, 18.0; HRMS (ESI), m/z 370.1363 (calcd for $C_{18}H_{21}NO_6$ + Na: 370.1369).



5

All polyacrylamide gel images were recorded using a Storm phosphorimager from Molecular Dynamics. Both the vertical adjustable slab gel kit (Model ASG-250) for polyacrylamide gel electrophoresis and the agarose gel electrophoresis kit (Model SGE-014-02) were purchased from CBS Scientific. The topoisomerase I DNA unwinding kit was a gift from TopoGen, Inc. of Columbus, OH. The *Bam*H1 and T4 ligase enzymes for the T4 ligase unwinding experiments were from New England Biolabs (NEB). Calf thymus DNA, sheared herring sperm DNA, and supercoiled pB*R*322 were purchased from Sigma, Promega, and NEB, respectively. Other oligodeoxynucleotides used were synthesized using an Applied Biosystems 392 DNA/RNA synthesizer.

Relative viscosities were calculated using the equation $\eta = (t - t_0)/t_0$, where t = flow time of the DNA solution and t_0 = flow time of the buffer, using a Cannon-Manning semimicro (no. 50) viscometer (total volume = 440 μL) immersed in a room temperature water bath. Flow times ranged from 268 s for buffer to 1123 s for ethidium bromide. Values of $(\eta/\eta_0)^{1/3}$, where η_0 = relative viscosity of blank DNA solution, were plotted against the binding ratio r. Approximately 0.8 mM agent per base-pair DNA solution in BPE buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA (EDTA = ethylenediaminetetraacetic acid), pH 7.0) was used. DNA solutions were titrated with the test compounds by adding small volumes of concentrated aqueous solutions. After each addition, the solution was mixed with a gentle stream of air. Each mixture was equilibrated for 10 min at room temperature.

Fluorescence spectra were determined using a FluoroMax-2 fluorimeter from ISA, Inc. Concentrations of DNA in BPE buffer were calculated from A_{260} values. Control experiments without DNA were performed by combining 2 μL of test compound in DMSO (dimethyl sulfoxide) and 180 μL BPE buffer. DNA stock solution (2 μL) was added, and the mixture was incubated 5 min at room temperature.

Topoisomerase I unwinding assays were performed by incubating a mixture of 2 μL of Topo I buffer (10 mM Tris-HCl, 5% glycerol, 0.1 mM spermidine, 1 mM EDTA, 0.15 M NaCl, 1% BSA, pH 7.0), 0.5 ng pHOT1 supercoiled DNA, 12 units topoisomerase I, and double distilled water (18 μL total volume) at 37 °C for 1 h. The relaxed plasmid DNA topoisomers were treated with 2 μL of a DMSO solution of the test compound, and the mixture was incubated at 37 °C for 30 min. The mixture was treated with 5 μL of 1% SDS and 4 μL of proteinase K (50 μg/mL) solutions, and was incubated at 56 °C. After 15−20 min, 2 μL of 10× loading dye (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol) was added to the mixture, which was mixed with an equal volume of 1:1 $CHCl_3$/*i*-AmOH. The aqueous layer was loaded onto a 1% agarose gel (12 × 12.5 cm) cast in TAE buffer (40 mM Tris, 1 mM EDTA, and 20 mM glacial acetic acid). The gel was run at 120 V for 4.5 h, stained with ethidium bromide (0.5 μg/mL) for 2 h, and viewed with a Biorad Gel Doc 2000 transilluminator. Two important control reactions were performed. The test compound was added simultaneously with Topo I to verify that the enzyme is active in the presence of the compound being tested, and the test compound was added in the absence of the enzyme to establish whether the compound damages DNA.

DNA unwinding assays with T4 ligase were performed by linearization of 1 μg of pB*R*322 supercoiled plasmid DNA with 3 units of *Bam*H1 at 37 °C for 1 h in a buffer system consisting of 150 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 100 μg/mL bovine serum albumin (BSA), pH 7.9. The total reaction volume was 50 μL. The DNA was extracted with an equal volume of 1:1 $CHCl_3$/phenol, and the aqueous layer was separated and mixed with three volumes of cold ethanol. The mixture was incubated in a dry ice/acetone bath for 30 min, and the DNA was pelleted by centrifugation at 10000 rpm for 20 min. The DNA was dried, dissolved in T4 ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/mL BSA, pH 7.5), and incubated with the test compound (added in DMSO) for 10 min at room temperature. The total reaction volume was 100 μL (10% DMSO). T4 Ligase (400 units) was added, and the mixture was incubated at 12 °C for 2 h, and mixed with an equal volume of 1:1 $CHCl_3$/phenol mixture to extract the test compound. The aqueous layer was collected and mixed with three volumes of cold ethanol to precipitate the DNA. The mixture was incubated in a dry ice/acetone bath for 30 min, and the DNA was pelleted by centrifugation at 10000 rpm for 20 min. The dried pellet was dissolved in 3 μL loading dye (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol) and loaded onto a 1% agarose gel cast in TAE buffer. The gel was run at 120 V for 4.5 h and was stained with ethidium bromide (0.5 μg/mL) for 2 h. Control experiments similar to those used in Topo I assays were performed to eliminate concerns analogous to those with the topoismerase I assay.

Cross-linking reactions were performed with 15-mer oligodeoxynucleotides. One strand of the duplex was 5′-^{32}P end-labeled and an aliquot with activity of 10^4 cpm was added to a solution containing 0.1 nmol of the unlabeled strand, 0.1 nmol of the complement, 10 μL of 10 mM Tris-HCl (pH 7.5), and 1 μL of 50 mM NaCl. The resulting mixture was dried and redissolved in 10 μL double distilled water to make 10 μM DNA duplex solution in 10 mM Tris-HCl buffer containing 5 mM NaCl. The DNA solution was annealed and cooled to 8 °C. In a separate microcentrifuge tube, the desired volume of a 2 mM toluene stock solution of azinomycin B was evaporated. The DNA solution was added to the azinomycin B, which was incubated at 8 °C

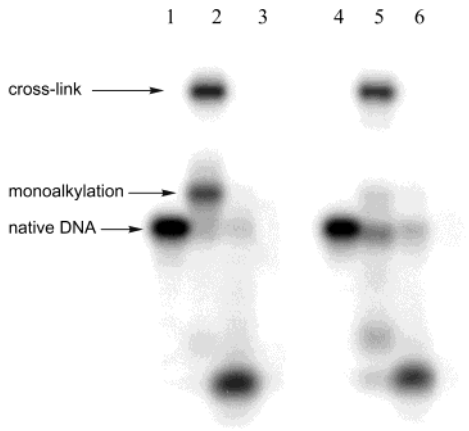


Figure 1. DNA cross-linking by azinomycin B (**1b**). Reaction conditions: 10 mM Tris-HCl (pH 7.5), 5 mM NaCl, 10 μL volume, 8 °C, 20 h, 1 μM duplex DNA, 120 μM **1b**. Sequence used: 5′-d(TATTAT**GCC**TATTAT)-3′ (**A**)/3′-d(ATAATA**CGG**ATAATA)-5′ (**B**). Lanes 1−3, 5′ end-labeled strand is **B**. Lanes 4−6, 5′ end-labeled strand is **A**. Lane 1, control; lane 2, DNA + **1b**; lane 3, DNA + **1b** and then piperidine; lane 4, control; lane 5, DNA + **1b**; lane 6, DNA + **1b** and then piperidine.

for 24 h and then was treated with 11 μL of 20 mM NaOH solution at 37 °C for 45 min to form the formamidinopyrimidine (FAPY) base in order to minimize spontaneous depurination. The sample was desalted using a Sephadex G-25 column and evaporated. The residue was dissolved in 3 μL loading dye and loaded onto a 20% polyacrylamide gel (28 × 16 cm). The gel was run at 20 W for 70 min, and cross-linking was quantified by phosphorimagery.

Kinetic experiments were performed using a duplex consisting of different sized strands, with both strands 5′-^{32}P end-labeled. Labeled oligodeoxynucleotides (10^4 cpm each strand) were combined with 10 pmol each of the unlabeled strands, 10 μL of 10 mM Tris-HCl (pH 7.5), and 1 μL of 50 mM NaCl. The mixture was dried in a speed vacuum and dissolved in 10 μL of double distilled water to reconstitute a 1 μM solution of the duplex DNA in 10 mM Tris-HCl buffer and 5 mM NaCl. The DNA was annealed and reacted with dried azinomycin B prepared as above. The reaction was allowed to proceed at 8 °C for the specified time and was quenched by the addition of 5 μL of 10 mM NaSEt. Parallel reactions requiring different reaction times were run simultaneously. At the termination of all the reactions, they were each desalted using a Sephadex G-25 column and dried. Each sample was dissolved in 3 μL loading dye and loaded onto a 20% polyacrylamide gel (28 × 16 cm). The gel was run at 20 W for 70 min and viewed using a phosphorimager.

3. Results and Discussion

Sequence Selectivity of Cross-Link Formation. We began our evaluation of mechanism of action of azinomycin B with a study of the sequence dependence of cross-link formation, using the recognition sequence 5′-d(GCC)-3′. Initial interstrand DNA cross-link assays were performed using the following 15-mer duplex containing a single binding site embedded within an unreactive A−T sequence.

5′-d(TATTAT***GCC***TATTAT)-3′

3′-d(ATAATA**CGG***G*ATAATA)-5′

In our preliminary evaluation using this d(GCC·CGG) binding triplet, we found that azinomycin B afforded exceptionally high levels of formation of the cross-linked product, oftentimes in excess of 90% as measured by densitometry (Figure 1). The site of alkylation of the end-labeled strand was confirmed by Maxam−Gilbert sequencing to be the italicized dG bases (G-lane data not shown). When the 3′-CGG-5′ strand was 5′ end-labeled and the duplex was reacted with azinomycin B (lanes 1−3), two electrophoretically distinct species were formed (lane 2) that were of lower mobility than the starting oligonucleotide: (1) a band directly above the starting DNA that corresponded to monoalkylation by the drug; (2) a band corresponding to a cross-linked duplex at much lower mobility. Lane 3 is the fragmented oligomer resulting from piperidine treatment of the cross-linked duplex and demonstrates that the DNA is site-specifically alkylated—at a single guanine—both in the monoalkylated intermediate and in the cross-linked product. When the complementary 5′-GCC-3′ strand was end-labeled, only a trace of monoalkylated species was formed, and the cross-linked duplex was by far the major product of the reaction. This study served to define the strand that reacted in the initial monoalkylation reaction (the 3′-CG*G*-5′ strand) and provided evidence on which strand underwent subsequent cross-link formation (the 5′-*G*CC-3′ strand) in addition to providing clear definition of which bases in the DNA are alkylated.

Table 1. Sequence Selectivity of Azinomycin B[a]

	triplet sequence	cross-link (%)	mono (%)	starting DNA (%)
1	5′-GCC-3′	77	5	18
	3′-CGG-5′	52	48	0
2	5′-GTC-3′	18	12	70
	3′-CAG-5′	30	23	46
3	5′-GCT-3′	24	37	39
	3′-CGA-5′	24	16	28
4	5′-GTT-3′	3	19	73
	3′-CAA-5′	4	0	96
5	5′-ATC-3′	0	0	98
	3′-TAG-5′	0	21	77
6	5′-ACC-3′	2	7	91
	3′-TGG-5′	5	95	0

[a] Reaction conditions: 10 mM Tris-HCl (pH 7.5), 5 mM NaCl, 10 μL reaction volume, 20 h, 8 °C, 10 μM duplex DNA, and 400 μM azinomycin B. Numbers opposite sequences refer to yields when that strand of the duplex DNA was 5′ end-labeled with ^{32}P.

In a full investigation of the sequence selectivity of covalent cross-linking by azinomycin B (**1b**), we used DNA 15-mer duplexes with the general sequence shown (Py = pyrimidine; Pu = purine). The triplet binding sequence contained 5′-disposed reactive purines (*Pu*) flanked by a nonnucleophilic T-residue. Cross-linking experiments were performed with each DNA strand alternately end-labeled with ^{32}P phosphate.

5′-d(TATTAT***Pu*PyPy**ATTATT)-3′

3′-d(ATAATA**PyPu*Pu***TAATAA)-5′

Results from these studies are presented in Table 1, and the yields for the various species formed in the reaction of azinomycin B with the targeted DNA were determined by densitometry of denaturing polyacrylamide gels using a phosphorimager. Analysis of the sequence selectivity data was complicated by a competing, facile, and sequence-dependent depurination of the cross-linked duplex, and the column labeled "mono" contains the combined percentage of monoalkylated and depurinated oligomer. In all cases, Maxim−Gilbert sequencing confirmed the site of alkylation.

Cross-link formation with azinomycin B was optimal between two guanine bases when the intervening base pair was G·C (Table 1, entry 1). With the d(GGC·CCG) sequence, yields for

cross-link formation were reproducibly 75−80%, and oftentimes in excess of 90%. The lower level of cross-link formation observed and the accompanying increase in monoalklation when the 3′-CGG-5′ strand was 5′ end-labeled are due to selective depurination of the guanine in the 5′-GCC-3′ containing strand. The 5′-GCC-3′ containing strand underwent essentially no monoalkylation compared to the more nucleophilic 3′-CGG-5′ strand, as expected from a consideration of sequence-dependent nucleophilicity.[16]

Cross-linking yields dropped dramatically when the intervening base pair between the reactive guanines was A·T (Table 1, entry 2). This result is expected based on the higher degree nucleophilicity of the 5′-dG in GG sequences compared to AG and because of steric hindrance of cross-link formation by the C5-methyl group of the thymine that is 5′ to one of the reactive guanines.[17] In this case, there is still a greater degree of monoalkylation of the more nucleophilic 3′-CAG-5′ strand than of the 5′-GTC-3′ strand, but the overall level of cross-link formation is reduced.

Cross-linking between G and A was modest to minimal within a variety of sequence contexts (Table 1, entries 3−6), contrasting previous descriptions of some of these sequences being effectively cross-linked by azinomycin B. With the d(GCT·CGA) sequence that has been reported recently by Saito and co-workers[10] and earlier by Armstrong and co-workers[9] to be efficiently cross-linked by azinomycin B, the cross-linking yield was 24% with either strand end-labeled (Table 1, entry 3), which is comparable to that observed by Saito and co-workers (32%). In this entry, for the CGA-containing strand, yields do not sum to 100% because a measurably significant amount of radioactivity was nonspecifically localized in the gel. When the intervening base pair is changed to T·A (Table 1, entry 4), cross-link formation is essentially abolished, and only minimal levels of monoalkylation of the guanine in the 5′-GTT-3′ strand was observed.[17]

With sequences containing the more reactive 3′-TGG-5′ and 3′-TAG-5′ sequences (Table 1, entries 5 and 6), only monoalkylation of the reactive guanine bases was observed with little or no cross-link formation, and yields were proportional to guanine nucleophilicity.[16]

Making a comparison of our results (Table 1) with those of an earlier study by Armstrong and co-workers[9] is difficult because the earlier data are qualitative. Nevertheless, the two sequences that underwent cross-linking most effectively in our work (GCC·CGG and GCT·CGA) were the same as the best sequences from the Armstrong study. While we observed significant differences in the yield of cross-link formation between these two sequences where Armstrong and co-workers did not, there are subtle flanking sequence differences between the oligodeoxynucleotide sequences used in the two studies (there is an A·T transversion adjacent to the purine of the PyPuPu-containing strand).

Role of the Naphthoate. Given the structural similarity between the azinomycin and neocarzinostatin naphthoate,[18] we had antiticipated that the drugs would share a common, intercalative mode of association with duplex DNA. In experiments designed to define the intercalative ability of the azinomycins, we have examined the interaction of the natural agent **1b** and the partial structures **2**,[19] **3**, **4**, and **5** with duplex DNA.

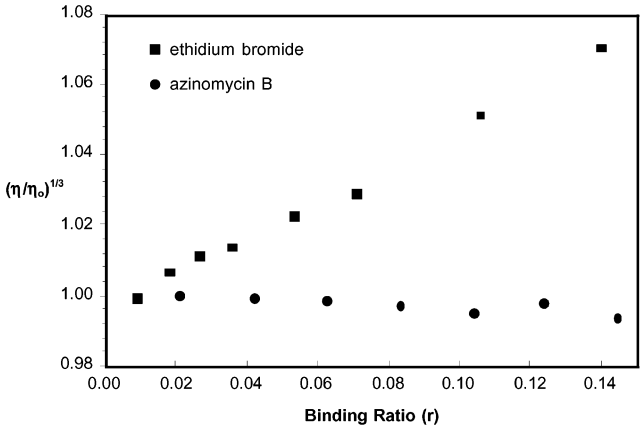
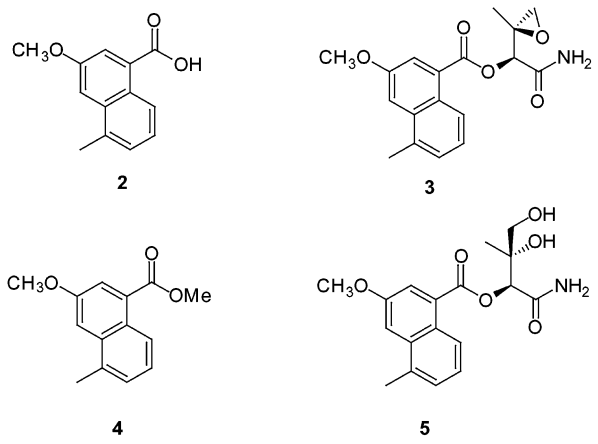


Figure 2. Plot of relative specific viscosity $(\eta/\eta_0)^{1/3}$ of calf thymus DNA versus binding ratio (per base pair) for the indicated compounds. Approximately 0.8 mM agent per base-pair DNA solution in BPE buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.0) was used.



Viscometry is the classic assay for DNA intercalation. Under the assumption that duplex DNA is a rigid rodlike structure, hydrodynamic properties such as viscosity are highly dependent upon changes in length.[20] In the classic intercalation model where ligands lengthen the DNA helix, viscometry provides a sensitive way of determining the intercalation between the stacked base pairs of DNA. We determined the effect of incremental levels of azinomycin B (**1b**) and naphthoic acid (**2**) on the viscosity of solutions of calf thymus DNA (Figure 2).

Adding incremental levels of azinomycin B to a solution of calf thymus DNA failed to measurably affect the viscosity of the solution. This is in marked contrast to the pronounced increase in the viscosity when a solution calf thymus DNA was titrated with the intercalator ethidium bromide[21] (Figure 2). These results contradict the report by Gates and co-workers[14] in which they demonstrated an increasing trend in the relative viscosity of a solution of sheared herring sperm DNA when

(16) Sugiyama, H.; Saito, I. *J. Am. Chem. Soc*. **1996**, *118*, 7063.
(17) For a similar report of steric hindrance of cross-link formation due to the C5-methyl group of thymine, see: Coleman, R. S.; Pires, R. M. *Nucleic Acids Res.* **1997**, *25*, 4771.
(18) Gao, X.; Stassinopoulos, A.; Gu, J.; Goldberg, I. H. *Bioorg. Med. Chem.* **1995**, *3*, 795.
(19) Shishido, K.; Omodani, T.; Shibuya, M. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2053.
(20) Cohen, G.; Eisenberg, H. K. *Biopolymers* **1969**, *8*, 45.
(21) For essentially identical data comparing ethidium bromide with the minor groove binder Hoechst 33258, see Figure 5 in ref 23.

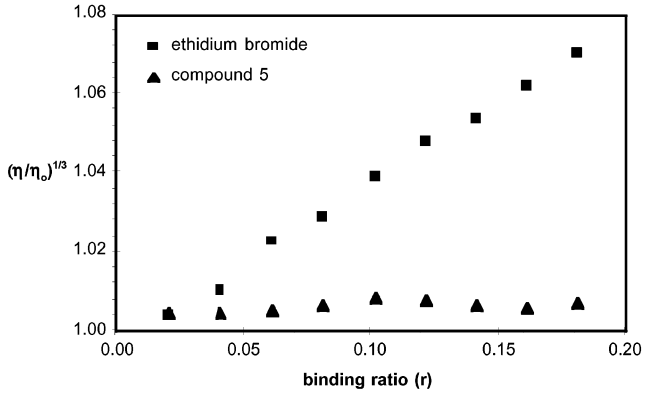


Figure 3. Plot of relative specific viscosity $(\eta/\eta_0)^{1/3}$ of sheared herring sperm DNA versus binding ratio (per base pair) for the indicated compounds. Approximately 0.8 mM agent per base-pair DNA solution in BPE buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.0) was used.

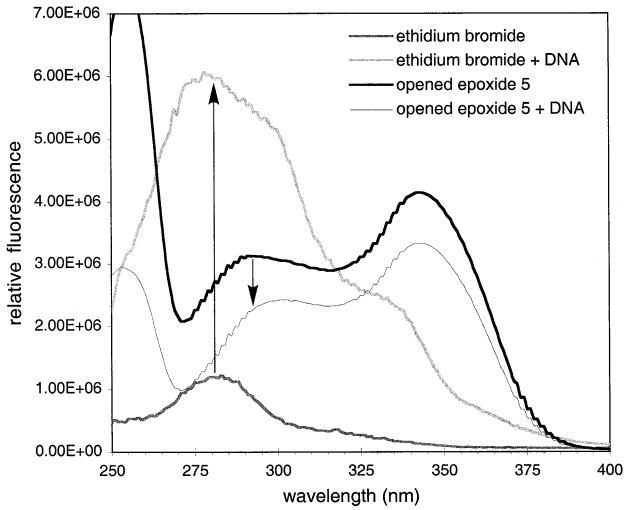


Figure 4. Excitation spectra of opened epoxide **5** and ethidium bromide in the absence and presence of sheared herring sperm DNA in BPE buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.0). Emission was measured (*y*-axis, arbitrary units) at 636 nm for ethidium bromide and 435 nm for **5** as a function of excitation wavelength (*x*-axis).

treated with epoxyamide **3**, a compound that interacts with DNA covalently.

Because shorter strands of DNA are generally more sensitive to length changes resulting from intercalation, we employed sheared herring sperm DNA (average length of 300−1000 bp) in a second set of viscosity experiments. The model compound **5** was investigated (Figure 3). This compound failed to show an increase in the relative viscosity of the DNA sample, indicating a lack of intercalative binding. These results contradict the report by Zang and Gates[14] in which they demonstrated an increasing trend in the relative viscosity of a solution of sheared herring sperm DNA when treated with a compound analogous to **5**. However, there are significant differences in the experimental designs of the two studies, and a comparative interpretation is made even more difficult because of the extremely low dynamic range of their viscometry measurements and their lack of either positive or negative controls.

Fluorescence contact energy transfer[22] from DNA bases to a bound ligand is the most definitive method to confirm intercalation in duplex DNA.[23] Light absorbed by the DNA bases is transferred to a bound fluorophore under specific geometric and spectroscopic conditions: (1) there must be spectral overlap between the donor and acceptor systems (i.e., they both must absorb in the same region of the spectrum); (2) the donor−acceptor distance must be appropriate, as energy transfer exhibits a distance dependence of r^{-6} (4−7 Å in DNA);[24] (3) the donor and acceptor dipoles must be suitably aligned for efficient energy transfer. For a molecule bound to duplex DNA, these three conditions are met simultaneously only when the acceptor is bound intercalatively. In the case of drugs bound along the grooves or on the surface of the DNA duplex, the larger drug-to-DNA distance and the poor orbital overlap prohibits energy transfer. Experimentally, an increase in fluorescence quantum yield of an intercalated molecule is observed when the DNA is irradiated.

$$h\nu \rightarrow \underset{\text{(donor)}}{\text{DNA}} \rightarrow \underset{\text{(acceptor)}}{\text{agent}} \rightarrow h\nu$$

In the present studies, three different nucleic acid systems were examined: calf thymus DNA, sheared herring sperm DNA (300−1000 bp), and the 15-mer oligomer shown containing the known binding site 5′-d(GGC)-3′.

5′-d(ATAATAGGCATAATA)-3′
3′-d(TATTATCCGTATTAT)-5′

In the excitation spectra shown in Figure 4 with ethidium bromide (10 μM)−a well-characterized DNA intercalator[25] with binding constants between 10^4−10^5 M^{-1}−in the presence of sheared herring sperm DNA, there is a 6-fold enhancement of ethidium fluorescence measured at 636 nm when a solution of DNA (50 μM base pairs) is added. Conversely, there is a decrease in naphthalene fluorescence measured at 435 nm when DNA (50 μM base pairs) is added to a solution of opened epoxide **5** (10 μM). This reduction in fluorescence quantum yield is due to screening of the fluorophore by the DNA absorbance. Essentially identical excitation spectra were obtained with an excess of agent (10 μM **5** and 2 μM DNA base pairs; data not shown). Similar results were obtained for naphthoic acid **2** and methyl naphthoate **4**, using sheared herring sperm and calf thymus DNA. In each case, either there was no change or a decrease was observed in the measured fluorescence intensity when DNA was added to a solution of the agent (data not shown).

In the presence and absence of the 15-mer duplex shown above containing the known recognition sequence 5′-GCC-3′, an identical result was obtained (Figure 5). Using different agent/base pair ratios (1:3, 1:1, and 5:1) to determine the behavior of the fluorescence excitation spectra when an excess amount of DNA was added to the compound and vice versa, the fluorescence intensities for both emission (data not shown) and excitation spectra of azinomycin B were insignificantly altered upon the addition of DNA. Identical results were obtained using calf thymus DNA (data not shown). With each DNA system,

(22) LePeck, J. B.; Paoletti, C. *J. Mol. Biol.* **1967**, *27*, 87.
(23) Suh, D.; Chaires, J. B. *Bioorg. Med. Chem.* **1995**, *3*, 723.
(24) Förster, T. *Ann. Phys.* **1948**, *2*, 55.
(25) Wilson, W. D.; Krishnamoorthy, C. R.; Wang, Y.-H.; Smithy, J. C. *Biopolymers* **1985**, *24*, 1941. Bresloff, J. L.; Crothers, D. M. *Biochemistry* **1981**, *20*, 3547.

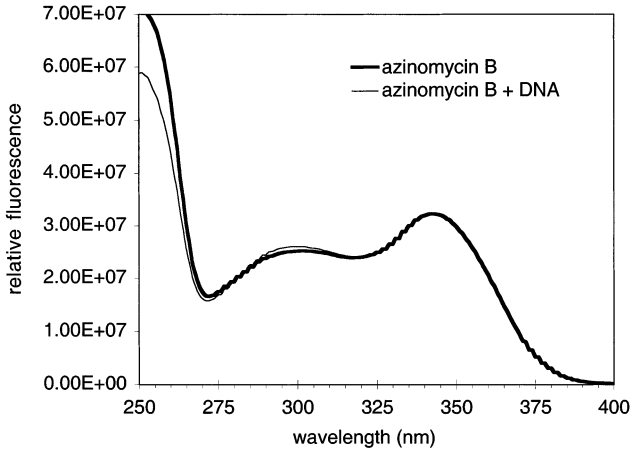


Figure 5. Excitation spectra of azinomycin B (**1b**; 40 μM) in the absence and presence of 15-mer duplex DNA (40 μM in base pairs) in BPE buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.0). Emission was measured (*y*-axis, arbitrary units) at 435 nm as a function of excitation wavelength (*x*-axis). For clarity, the positive ethidium bromide control is not shown.

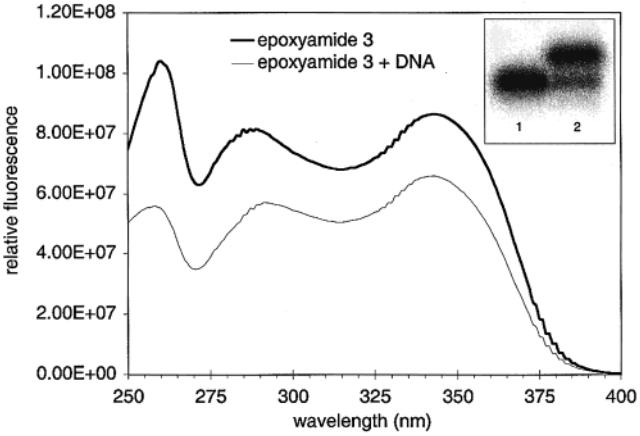


Figure 6. Excitation spectra of epoxyamide **3** (100 μM) in the absence and presence of 15-mer duplex DNA (5 μM) in BPE buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.0). The drug + DNA spectrum was obtained after 20 h incubation of **3** and the duplex at 8 °C. Emission was measured (*y*-axis, arbitrary units) at 435 nm as a function of excitation wavelength (*x*-axis). Denaturing polyacrylamide gel electrophoresis insert: lane 1, 5′ ^{32}P end-labeled DNA; lane 2:, 5′ ^{32}P end-labeled DNA + drug after incubation.

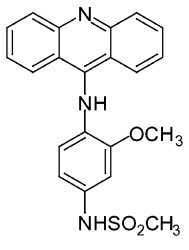
ethidium bromide was used as a positive control, and a large enhancement of fluorescence intensity was observed upon the addition of duplex DNA (cf., Figure 4). The Barton $Ru(phen)_3^{2+}$ (phen = 1,10-phenanthroline) major groove binder[26] was used as a negative control (data not shown).

Epoxyamide partial structure **3** is highly effective at covalent modification of duplex DNA, alkylating guanine bases in the GCC·CGG receptor with yields as high as 86%.[27] The excitation spectrum of **3** (Figure 6) showed no enhancement upon the addition of 15-mer duplex DNA; the observed decrease in fluorescence intensity is due to the DNA absorbance shielding the naphthoate absorbance. In this experiment, the drug was incubated with the duplex DNA for 20 h at 8 °C. Polyacrylamide gel electrophoresis confirmed that epoxyamide **3** had alkylated the DNA duplex in ≥75% yield, thereby localizing the drug irreversibly onto its DNA receptor. The gel electrophoresis autoradiogram insert (Figure 6) shows the conversion of native DNA to alkylated DNA at the end of this incubation period.

In the interpretation of negative results such as those obtained in the fluorescence contact energy transfer experiments, it is reasonable to ask whether the experimental design was appropriate for detecting potentially weak binding. There is no information on the threshold of agent binding for this experiment to yield positive results, but in a recent paper Viola and coworkers observed a strong positive enhancement of agent fluorescence in the presence of DNA using a furocoumarin with a $K_{app} = 3.3 \times 10^4$. The DNA/agent ratio in the Viola work was 10:1.[28] In the results shown in Figure 5, the DNA/agent ratio was 0.067, and the agent is covalently bound to the DNA duplex. In Figure 6, gel electrophoresis was used to confirm that the agent was covalently bound to the duplex, making discussions of agent/DNA ratios and binding constants irrelevant in this case.

In three separate systems (**1b**, which forms covalent cross-links; opened epoxide **5**, which can only interact with DNA noncovalently; and epoxyamide **3**, which has been demonstrated to effectively alkylate both single and double-stranded DNA),[27] using three different forms of duplex DNA (sheared herring sperm; calf thymus; and a synthetic 15-mer), we observed no enhancement of agent fluorescence upon the addition of DNA under conditions where ethidium bromide exhibited a strong, positive enhancement of fluorescence.

To confirm the negative results obtained with viscometry and fluorescence spectroscopy, we examined two enzyme-based assays for intercalation. Topoisomerase I unwinds supercoiled DNA. The topoisomerase assay for intercalation is based on the fact that intercalating agents unwind the DNA double helix, and with relaxed plasmid DNA as a substrate, the unwinding caused by a bound intercalating agent introduces positive superhelical twists in the DNA structure. Hence, the plasmid behaves as if it were positively supercoiled. Treatment of the intercalator−DNA complex with topoisomerase I (topo I) repairs the extra superhelical twists in the DNA structure, resulting in an apparently relaxed plasmid that in reality is negatively supercoiled because of the presence of the intercalator. Extraction of the intercalator from the helix causes the formerly relaxed DNA to negatively supercoil. Intercalating agents thus promote the conversion of relaxed plasmid DNA to the negatively supercoiled form in the presence of topo I. This is manifested in the electrophoretic analysis as a shifting of the relaxed topoisomeric ladder to the more highly mobile, negatively supercoiled form of the plasmid. The known intercalating agent 4′-(acridin-9-ylamino)-3′-methoxymethanesulfonalinide (*m*-AMSA) is used as a positive control.



m-AMSA

(26) For a recent review, see: Kane-Maguire, N. A. P.; Wheeler J. F. *Coord. Chem. Rev.* **2001**, *211*, 145, and references therein.
(27) Coleman, R. S.; Burk, C. H.; Navarro, A.; Brueggemeier, R. W.; Diaz-Cruz, E. S. *Org. Lett.* **2002**, *4*, 3545.

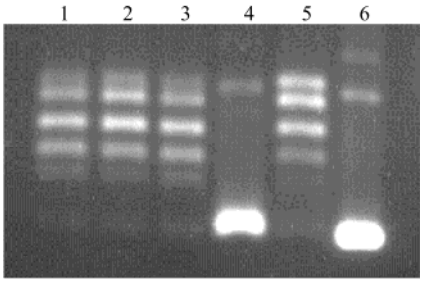


Figure 7. DNA unwinding experiment with naphthoic acid **2** and topo I. Lanes 1−3, 1000, 500, 100 μM **2**, respectively; lane 4, 100 μM *m*-AMSA; lane 5, relaxed plasmid DNA; lane 6, supercoiled plasmid DNA.

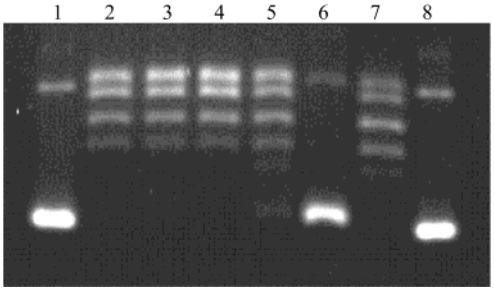


Figure 8. DNA unwinding experiment with methyl naphthoate **4** and topo I. Lane 1, control (supercoiled plasmid DNA + 1000 μM **4** without topo I); lane 2, 1000 μM **4** and topo I added simultaneously; lanes 3−5, 1000, 500, 100 μM **4**, respectively, added to plasmid DNA preincubated with topo I; lane 6, 100 μM *m*-AMSA added to plasmid DNA preincubated with topo I; lane 7, relaxed plasmid DNA; lane 8, supercoiled plasmid DNA.

Unwinding assay results obtained with compounds **2** and **4** are shown in Figures 7 and 8, respectively. Lanes 5 and 7 (Figures 7 and 8, respectively) show the behavior of relaxed plasmid DNA in 1% agarose gel. The ladder of bands represents the topoisomeric forms (topoisomers) of the plasmid. The distribution of the topoisomers shifts when an intercalating agent is added, as was observed with the intercalator *m*-AMSA (lanes 4 and 6, Figures 7 and 8, respectively). Compounds **2** and **4** were examined at concentrations as high as 1000 μM. In agreement with the results obtained in viscosity and fluorescence experiments, the compounds showed little or no ability to induce electrophoretic shifts in plasmid DNA mobility, indicating no or an extremely weak ability to bind to DNA intercalatively. While this is not surprising for a compound such as **2** that bears a negative charge at neutral pH, the identical lack of shifts was observed the corresponding methyl ester. In particular, with methyl naphthoate **4** (Figure 8), there was no detectable shift in DNA mobility with concentrations of **4** as high as 1 mM. This experiment is based on a concentration-dependent shift in topoisomer mobility, and an examination of lanes 1−3 in Figure 7 or lanes 2−5 in Figure 8 leads to the conclusion that such a shift does not occur.

We also performed a DNA unwinding experiment with T4 ligase. Similar to the topoisomerase I experiments, this assay makes use of the ability of intercalating agents to unwind double stranded DNA. Linearized plasmid DNA is treated with an intercalating agent followed by the enzyme T4 ligase, which links the two ends of the duplex to re-form the closed circular plasmid. If the agent under examination binds to duplex DNA intercalatively, then upon ligation and subsequent removal of the agent, the closed circular DNA will be underwound and hence negatively supercoiled. Such DNA will migrate more rapidly upon agarose gel electrophoresis compared to linearized DNA ligated in the absence of an intercalating agent.

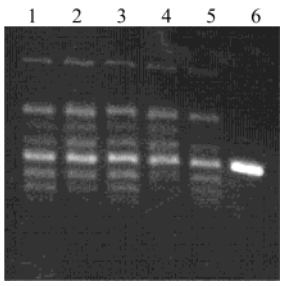


Figure 9. DNA unwinding experiment with **2** and T4 ligase. Lanes 1−4, 1000, 100, 50, and 10 μM **2**, respectively; lane 5, linearized DNA + T4 ligase; lane 6, linearized DNA.

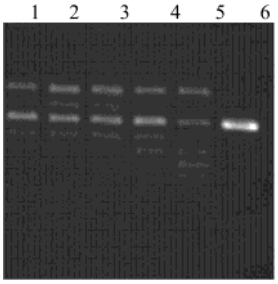


Figure 10. DNA unwinding experiment with **4** and T4 ligase. Lanes 1−4, 1000, 100, 50, and 10 μM **4**, respectively; lane 5, linearized DNA + T4 ligase; lane 6, linearized DNA.

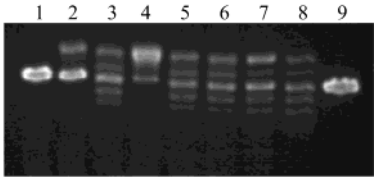


Figure 11. DNA unwinding experiment with **5** and T4 ligase. Lane 1, linearized DNA + 1000 μM **5**; lanes 2, 1000 μM **5** and T4 ligase added simultaneously to linearized DNA; lane 3, 100 μM **5** and T4 ligase added simultaneously to linearized DNA; lanes 4−7, 1000, 100, 50, and 10 μM **5**, respectively; lane 8, linearized DNA + T4 ligase; lane 9, linearized DNA.

As shown in Figure 9 (lanes 1−4), ligation of linearized DNA in the presence of **2** did not promote the production of negatively supercoiled DNA even at very high concentrations (1000 μM). Similar results were obtained with compound **4** (Figure 10, lanes 1−4). With **5**, products with slower mobility started to appear at a compound concentration of 1000 μM (Figure 11, lane 1). We interpreted this result as a sign of inhibition of the activity of the enzyme because the product resembles nicked or incompletely ligated DNA. Inhibition of the activity of the enzyme was confirmed on lane 2 (Figure 11), where the enzyme was added simultaneously with **5** to the linearized DNA substrate. The inhibition is manifested by the small change in the amount of the linearized DNA starting material during the ligation reaction and the formation of a small amount of nicked or incompletely ligated DNA observed just above the band of the DNA substrate.

Orientation of the Agent. Definition of the orientation of binding of azinomycin B to the target sequence d(GGC)·(CCG) was determined using a gel electrophoresis-based assay that differentiated kinetically between the initial monoalkylation and subsequent cross-link formation. Using a short 15-mer strand **6**

(28) Viola, G.; Uriarte, E.; Gia, O.; Moro, S. *Farmaco* **2000**, *55*, 276.

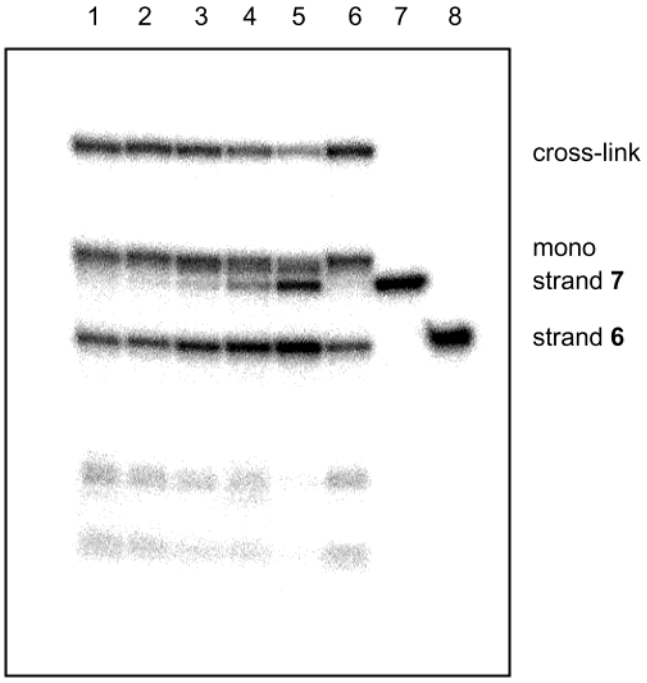


Figure 12. Kinetic assay for regioselective of cross-link formation. Lanes 1−5, 2.0, 1.5, 1.0, 0.5, and 0 h reaction, respectively; lane 6, 24 h reaction; lane 7, single strand **7**; lane 8, single strand **6**.

and long 21-mer strand **7**, we 5′ end-labeled *both* strands with ^{32}P and followed the progression of covalent bond formation from initial monoalkylation to cross-link formation. We worked under the assumptions that the aziridine ring of **1b** is the most reactive electrophile, consistent with observations made during the course of synthetic work, and that the initial covalent bond is formed between one of the DNA dG-N7's and C10 of **1b**. Which strand underwent the initial alkylation was not known.

Following the progression of covalent adduct formation by denaturing polyacrylamide gel electrophoresis (Figure 12), we observed the rapid monoadduct formation with a concomitant decrease in the amount of longer strand **7** (lane 5) accompanied by a small amount of cross-link formation. As the reaction progresses, the long DNA strand **7** reacts completely (lane 1) and with kinetics distinct from that for cross-link formation. Furthermore, the rate of cross-link formation is identical with that for the disappearance of short strand **6**, and these observations suggest that the aziridine alkylates the dG of the 3′-CGG-5′-containing strand as the site of the initial monoalkylation.

We know from our synthetic work that the aziridine of the azinomycin azabicyclic system is exceptionally reactive toward even weak nucleophiles such as bromide or water, whereas the epoxide is not. While it is strictly true that we have no conclusive evidence that the aziridine is more reactive than the epoxide in the drug−DNA complex, it would be extremely unlikely that the large difference in reactivity between the aziridine and epoxide in the native agent would be completely reversed upon binding to DNA.

This work is consistent with the model put forth by Saito and co-workers,[10] who proposed that the aziridine reacts in the initial monoalkylation event, followed by subsequent cross-linking by the epoxide. Detailed molecular modeling studies not only support the proposed mode of cross-link formation[12] but also reproduce accurately the experimentally observed sequence selectivity of azinomycin B that is reported in Table 1.[13]

Conclusion

From the preliminary set of experiments evaluating the sequence selectivity of binding, it would appear that sequence selectivity of cross-link formation is related generally to nucleobase nucleophilicity and specifically to steric effects in the major groove of duplex DNA. There are some subtle sequence effects on agent binding, but at a basic level it appears that the effectiveness of the agent in the formation of covalent cross-links is related more to the nucleophilicity of the DNA sequence rather than to any specific recognition elements. However, this simplistic assessment will certainly evolve, as additional details of the specific contacts between the agent and the DNA receptor are uncovered.

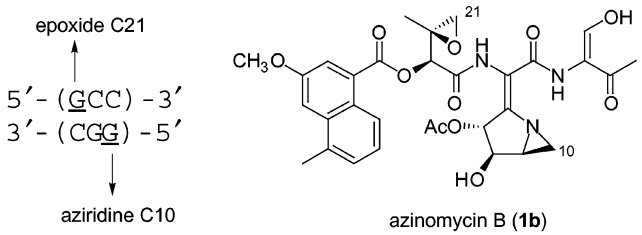
The conclusion that can be drawn from the assays for intercalative binding is that the azinomycin naphthalene does not detectably intercalate within duplex DNA. In both the full and partial structures examined, we could find no evidence for intercalative binding. Given the structural analogy between the azinomycin and neocarzinostatin naphthoate systems, the latter of which has been demonstrated to bind duplex DNA intercalatively,[18] we had anticipated that the two systems would share mechanistic similarity. However, it is not clear whether the neocarzinostatin naphthoate provides binding affinity on its own or whether other structural features of the molecule are driving a weak intercalative binding. Apparently, in the structures examined, including the parent azinomycin molecules, there is no appreciable intercalative binding. These results raise the interesting question of what role this hydrophobic aromatic group plays in the mechanism of action of these antitumor agents. Our experimental results with epoxide-bearing partial structures have shown that the naphthoate is essential for effective covalent interaction of the agents with DNA,[27] so we can draw the limited conclusion that the naphthoate provides an important degree of noncovalent association that may be due simply to hydrophobic interactions with the DNA duplex. It also raises interesting questions in drug design with respect to optimizing noncovalent interactions of these agents with DNA in efforts to increase selectivity and potency.

With respect to the discrepancy between our results and those of Zang and Gates,[14] their conclusion of intercalative binding by the azinomycin naphthoate was based on a single viscometry experiment. (Their remaining experiments are equivocal with respect to intercalative versus nonintercalative binding.) In their viscometry results, using a compound similar to our **4** (compound **9** in their work), they observed a marked increase in the viscosity of solutions of sheared herring sperm DNA (100−200 bp). However, since these workers did not include controls (i.e., a known intercalator or a molecule demonstrated to bind nonintercalatively), and because of the extremely narrow dynamic range for flow times that were observed (88−94 s), we do not feel that this experiment can be unequivocally or reliably interpreted to indicate intercalative binding.

Concerning the T4 DNA ligase assay used by Zang and Gates,[14] wherein unwinding of linearized plasmid DNA in the presence of an intercalating agent is measured, when these workers added increasing concentrations of a compound related to **5** in the presence of T4 DNA ligase, an increase in the linear

form of the plasmid was observed, rather than the expected supercoiled form, as was observed with the intercalator daunomycin. This may be due to inhibition of the ligase enzyme by the drug, but it is does not appear to be the result of intercalation by the agent.

Our results on the regioselectivity of agent−DNA cross-link formation provide evidence that the more nucleophilic purine reacts with the most electrophilic site of the agent. The agent forms a covalent cross-link by an initial reaction of the aziridine C10 with the 5′-G of the 3′-PyPuPu-5′-containing strand in the monoalkylation event with subsequent cross-link formation occurring between the epoxide C21 and the 5′-disposed purine two bases removed on the complementary strand. This conclusion is based on observations made in the course of synthetic studies that the aziridine ring of azabicyclo[3.1.0]hexane systems is exceptionally electrophilic, and readily reacts with weak nucleophiles such as bromide under dilute reaction conditions (CH_2Cl_2, 25 °C).[29] This assumption remains to be proven unequivocally.



azinomycin B (**1b**)

In a series of detailed investigations using Monte Carlo simulations of DNA−drug adducts, we observed a good correlation between experimental data and computational estimations of sequence selectivity.[12,13] The comparison of the conformational properties of the drug−DNA monoadducts and complexes confirmed the most probable mechanism of action involves an initial aziridine and subsequent epoxide alkylation in the orientation proposed. The different hydrogen bonding networks in the monoadducts and in the complexes between the drug and the three-base-pair receptor is the primary reason for the sequence-dependent cross-linking reactivity. In addition, steric hindrance of the major groove exposed methyl group of central thymine-based triplets plays an important role in the lack of the reactivity of those sequences.

The results presented are selected from an extensive set of experiments, and they provide a preliminary picture of our work on the interaction of the azinomycins with duplex DNA. In particular, we have begun to define the origin of the sequence selectivity exhibited by the natural agent, and we have defined the regioselectivity of the covalent cross-link. There appears to be no appreciable intercalative binding of the natural agents or naphthalene-bearing partial structures to duplex DNA. In related work,[27] we have examined the covalent interactions of epoxide-bearing partial structures with duplex DNA, and we have found that the naphthalene is essential for achieving appreciable covalent bond formation. The issue of identifying the role of the aromatic group in noncovalent interactions of the agents with DNA currently remains incompletely resolved.

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