Structural Basis for the Deoxyribonucleic Acid Affinity of Bleomycins†

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ABSTRACT: The role of the bithiazole moiety of bleomycin in the interaction of the antibiotic with DNA has been studied by the use of synthetic bithiazole derivatives. The DNA affinity of individual C-terminal (bithiazole) analogues of bleomycin was measured in terms of the ability of these species to block the binding of bleomycin to DNA, as judged by diminution of the DNA degradation that attends bleomycin binding. DNA degradation was monitored both by release of [^{3}H]thymine from radiolabeled PM-2 DNA and by alteration of bleomycin-treated DNA oligomers of defined sequence derived from *Escherichia coli* plasmid pLJ3. It was found that the affinity of the bithiazole derivatives for DNA depended on the presence of the bithiazole moiety itself but more importantly on the number and spacing of positively charged groups; 2′-(2-aminoethyl)-2,4′-bithiazole-4-[3-[(4-aminobutyl)amino]propyl]carboxamide (**14**), having three positively charged groups at neutral pH, was a reasonably effective inhibitor of DNA degradation by bleomycin. Consistent with the importance of the spacing of the positively charged groups, tetrapeptide S (**12**) was found to be significantly less inhibitory toward DNA degradation by bleomycin than tripeptide S, in spite of their equal number of positively charged groups and the greater structural similarity of the former to bleomycin A_2. Bleomycin is known to cleave DNA perferentially at certain sequences. It was shown that the inhibitors employed in this study diminished DNA cleavage proportionately at each cleavage site; no alteration was observed in the specificity of cleavage. A number of the bithiazole analogues employed as inhibitors of bleomycin-mediated DNA degradation were also utilized in fluorescence quenching experiments with calf thymus DNA. Consistent with the belief that these species inhibit bleomycin degradation by competitive binding to the DNA substrate, the best inhibitors exhibited the greatest fluorescence quenching upon admixture of DNA.

The bleomycins are a family of glycopeptide-derived antibiotics used clinically for the treatment of certain malignancies (Umezawa, 1973, 1976, 1978; Hecht, 1979). These antibiotics have been shown to mediate a number of biochemical effects (Suzuki et al., 1968; Terasima et al., 1970; Miyaki et al., 1971; Yamaki et al., 1971; Umezawa, 1975; Kuo & Hsu, 1978; Hecht, 1979); their anticancer activity is believed to be related to their ability to bind to DNA and effect strand scission (Suzuki et al., 1970; Terasima et al., 1970; Haidle, 1971; Saunders et al., 1975; Umezawa, 1975; Hecht, 1979). Studies employing cell-free systems have demonstrated that bleomycin binds to a variety of metals, including Fe(II), and that Fe(II)–bleomycin forms a redox-active species with O_2 that can degrade DNA (Ishida & Takahashi, 1975; Horwitz et al., 1979).

Bleomycin reacts with nucleic acids in a specific way. The molecule binds to double-stranded DNA with an apparent equilibrium constant of about 1.2×10^5 M^{-1} at pH 8.4 (Chien et al., 1977). Bleomycin does not degrade RNA (Suzuki et al., 1970; Muller et al., 1972), and single-stranded DNA is not a good substrate for the drug (Shirakawa et al., 1971; Takeshita et al., 1978). Incubation of Fe(II)–bleomycin with double-stranded DNA leads to extensive DNA degradation. Such damage can be detected as release of free bases (Haidle et al., 1972; Muller et al., 1972; Ishida & Takahashi, 1975; Povirk et al., 1978; Sausville et al., 1978b) and as single- and double-stranded breaks in duplex DNA (Suzuki et al., 1968; Nagai et al., 1969; Shirakawa et al., 1971). At high concentrations, bleomycin released all four bases from DNA; preferred release was observed for thymine > cytosine > adenosine > guanine (Povirk et al., 1978; Sausville et al., 1978b). When utilized at lower concentrations, bleomycin exhibited significant base sequence specificity. D'Andrea & Haseltine (1978) and Takeshita et al. (1978) demonstrated that under conditions of limited reaction, there was a marked preference for scission at GT and GC sequences. Moreover, the treatment of DNA (e.g., PM-2 DNA) with limiting amounts of bleomycin has been shown to result in strand scission at discrete sites considerably smaller in number than those possessing these preferred sequences of DNA bases (Haidle et al., 1979).

The molecular basis of bleomycin–DNA interaction, especially as it relates to specificity of binding, is poorly understood.

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§ Trainee of National Institutes of Health National Research Service Award 2T32-CA09172-07.

∥ Fellow of the Economic Development Agency of Puerto Rico. This study was carried out as part of the doctoral program of L.R., in absentia from Massachusetts Institute of Technology.

⊥ This study was carried out as part of the doctoral program of M.D.L., in absentia from Massachusetts Institute of Technology.

+ National Institutes of Health Career Development Awardee, 1975–1980.

0006-2960/82/0421-3711$01.25/0

Previous studies by Povirk et al. (1979, 1981) have suggested that the region of the bleomycin molecule containing the bithiazole moiety is important for interaction with DNA (Murakami et al., 1976). A C-terminal fragment of bleomycin containing the bithiazole moiety has been shown to have an affinity for calf thymus DNA similar to that of bleomycin itself, on the basis of the extent of fluorescence quenching of the bithiazoles upon admixture of the DNA (Chien et al., 1977; Povirk et al., 1979).

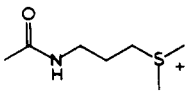
In the present study, we investigated further the role of the bithiazole moiety of bleomycin in DNA binding. DNA fragments of defined sequence were utilized as substrates for bleomycin, and a number of chemically prepared bithiazole derivatives were tested for their ability to inhibit the interaction of bleomycin with DNA. The results of these experiments support the concept that the bithiazole moiety plays an important role in DNA binding. The results also indicate that both the C-terminal substituent of bleomycin and the spacing and number of positively charged groups are important in DNA–bleomycin interaction.

Experimental Procedures

Blenoxane, the clinically used mixture of bleomycins, was obtained from Bristol Laboratories through the courtesy of Drs. Stanley Crooke and William Bradner. It was fractionated as described (Chien et al., 1977) to provide bleomycins A_2 and B_2. The fluorescence measurements were carried out on an SLM-1800 spectrofluorometer at an excitation wavelength of 290 nm (16-nm slit width).

Potential Inhibitors. Compounds **6** and **8** were purchased from Aldrich Chemical Co. Compound **13** was isolated after partial hydrolysis of bleomycin B_2 (Muraoka et al., 1972). The syntheses of tripeptide S (**11**) and tetrapeptide S (**12**) were carried out as described (Levin et al., 1980); the preparations of inhibitors **1**–**5**, **7**, **9**, **10**, and **14** are described below.

(3-Acetamidopropyl)dimethylsulfonium Methosulfate (**1**).



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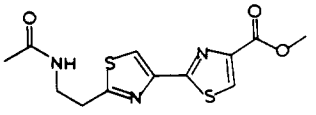

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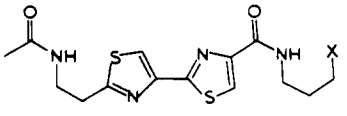
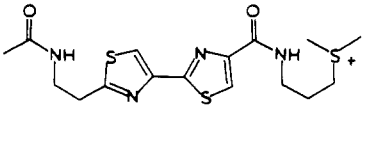


3, X = Br

4, X = SCH_3



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To a solution of 64.2 mg (0.44 mmol) of *N*-acetyl-3-(methylthio)propylamine in 3 mL of CH_2Cl_2 was added 41 μL (0.43 mmol) of dimethyl sulfate. The reaction mixture was stirred overnight at room temperature and then extracted with three 2-mL portions of water. The aqueous phase was back-extracted with several portions of ethyl acetate and then concentrated under diminished pressure to afford (3-acetamidopropyl)dimethylsulfonium methosulfate (**1**) as a clear oil: yield 19.6 mg (16%); ^1H NMR [D_2O, ext $(CH_3)_4Si$] δ 2.0 (s, 3 H), 2.0 (m, 2 H), 2.82 (s, 6 H), 3.2 (m, 4 H), and 3.65 (s, 3 H).

Methyl 2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-carboxylate (**2**). A suspension of 4.94 g (16.9 mmol) of 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylate in 200 mL of methanol at 0 °C was saturated with hydrogen chloride over a period of 2 h. The reaction vessel was sealed and maintained at room temperature for 48 h. The reaction mixture was concentrated under diminished pressure and the residue was crystallized from 150 mL of hot methanol, affording 3.79 g of brown needles. Recrystallization from methanol (decolorization) afforded methyl 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylate (hydrochloride) as colorless needles: yield 3.02 g (58%); mp 231–232 °C [lit. (Zee-Cheng & Cheng, 1970) mp 236–237 °C]; ^1H NMR[1] [$CDCl_3$ plus 2 drops of dimethyl-d_6 sulfoxide, $(CH_3)_4Si$] δ 1.93 (s, 3 H), 3.2 (m, 2 H), 3.6 (m, 2 H), 3.92 (s, 3 H), 7.5 (br, 1 H), 8.02 (s, 1 H), and 8.21 (s, 1 H).

To a stirred solution of 1.20 g (3.91 mmol) of the bithiazole methyl ester in 25 mL of water was added sufficient 2 N NaOH to raise the pH to 10. Acetic anhydride (0.9 mL, 9.5 mmol) and 5 mL of 2 N NaOH were added alternately in small portions over a period of 15 min such that the pH was maintained at ~10. After the addition was complete, the solution was stirred at room temperature for 0.5 h, during which time the pH dropped to 4.7. Filtration afforded a white solid that was crystallized from methanol, affording methyl 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-carboxylate (**2**) as colorless needles: yield 0.85 g (70%); mp 176–177 °C; ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 1.99 (s, 3 H), 3.1–3.9 (m, 4 H), 3.95 (s, 3 H), 6.4 (br s, 1 H), 7.98 (s, 1 H), and 8.13 (s, 1 H); silica gel TLC (10:1 $CHCl_3$–CH_3OH) R_f 0.80.

The same compound was obtained by initial N-acetylation of 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylate (see procedure below), followed by esterification. However, the material obtained by this sequence was less pure than that prepared as described above.

2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-carboxylic Acid. A stirred suspension of 2.96 g (10.1 mmol) of 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylate in 60 mL of water was treated with 10 mL of 2 N NaOH, and the resulting solution was then treated alternately with small portions of acetic anhydride and 2 N sodium hydroxide over a period of 20 min such that the pH was maintained between 10 and 12. After a total 2.87 mL of acetic anhydride and 27 mL of 2 N NaOH had been added, the reaction mixture was stirred at room temperature for 1 h, during which time the pH was maintained at 12. The solution was acidified to pH 1 by the addition of 27 mL of 2 N hydrochloric acid and then refrigerated. Filtration afforded off-white plates of 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-carboxylic acid: yield 2.74 g (91%); mp 225–226 °C dec; ^1H NMR (D_2O–NaOD, DSS) δ 2.00 (s, 3 H), 3.0–3.7 (m, 4 H), 7.90 (s, 1 H), and 7.93 (s, 1 H).

2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-(3-bromopropyl)carboxamide (**3**). A solution containing 1.76 g (5.9 mmol) of 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-carboxylic acid and 0.61 g (0.84 mL; 6.0 mmol) of triethylamine in 18 mL of CH_2Cl_2 was stirred at 0 °C (ice bath) for 15 min and then treated with 0.88 g (0.54 mL; 7.4 mmol) of thionyl chloride. The reaction mixture was stirred and cooled for an additional

[1] Abbreviations: NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; CM, carboxymethyl; dATP, 2′-deoxyadenosine 5′-triphosphate; TTP, thymidine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

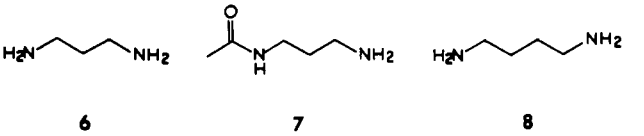
30 min. The reaction mixture was then treated with 180 mg (1.48 mmol) of 4-(dimethylamino)pyridine, 1.56 g (7.13 mmol) of (3-bromopropyl)ammonium bromide, and 2.44 g (3.36 mL; 24.1 mmol) of triethylamine and stirred at 0 °C for 10 min. The cooling bath was removed and the reaction mixture was treated with 10 mL of CH_2Cl_2 to facilitate stirring, which was continued for an additional 10 min. The reaction mixture was diluted to 250 mL with CH_2Cl_2 and then extracted successively with 200-mL portions of water, 0.2 N hydrochloric acid, and 0.1 N sodium bicarbonate. The organic phase was dried (Na_2SO_4) and concentrated to afford 1.9 g of an off-white solid that crystallized as colorless needles from C_2H_5OH–H_2O: yield 1.59 g (64%); mp 153–155 °C; ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 2.02 (s, 3 H), 2.17 (m, 2 H), 3.2–3.9 (m, 8 H), 6.5 (br, 1 H), 7.6 (br, 1 H), 7.93 (s, 1 H), and 8.15 (s, 1 H); silica gel TLC (1:1 CH_3OH–EtOAc) R_f 0.77.

2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-[3-(methylthio)propyl]carboxamide (**4**). To a suspension of 42.1 mg (0.141 mmol) of 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-carboxylic acid in 4 mL of dry CH_2Cl_2 at 0 °C (ice bath) was added 20 μL (0.143 mmol) of triethylamine. The reaction mixture was stirred under N_2 for 10 min, and the resulting solution was treated with 13 μL (0.176 mmol) of thionyl chloride. A white precipitate formed slowly and stirring was continued as the ice melted. After 2 h, 19 μL (0.164 mmol) of (methylthio)-propylamine and 60 μL (0.43 mmol) of triethylamine were added. The reaction mixture was stirred at 25 °C for an additional 3 h, and the solution was then diluted with 5 mL of CH_2Cl_2 and extracted successively with water, 0.1 N hydrochloric acid, and again with water. The aqueous extracts were each back-extracted with CH_2Cl_2, and the combined organic extract was extracted twice with saturated sodium bicarbonate solution, dried (Na_2SO_4), and concentrated under diminished pressure to afford 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-[3-(methylthio)propyl]carboxamide (**4**) as a pale yellow glass (29 mg) that deposited off-white crystals from aqueous ethanol: yield 16 mg (29%); mp 125–126 °C; ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 1.8 (m, 2 H), 1.95 (s, 3 H), 2.1 (s, 3 H), 2.6 (m, 2 H), 3.2 (m, 2 H), 3.6 (m, 4 H), 6.25 (br, 1 H), 7.5 (br, 1 H), 7.8 (s, 1 H), and 8.05 (s, 1 H); silica gel TLC (25:1 $CHCl_3$–CH_3OH) R_f 0.27.

2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-[3-(dimethylsulfonio)propyl]carboxamide Acetate (**5**). A solution of 43 mg (0.103 mmol) of 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-(3-bromopropyl)carboxamide in 12 mL of acetone was concentrated to about 5 mL under diminished pressure, which effected partial precipitation of the bithiazole. The reaction mixture was treated with 56 mg (0.373 mmol) of sodium iodide and stirred at 25 °C for 15 h. The resulting solution was concentrated to about 1 mL and stirred for an additional 3 days. The reaction mixture was concentrated to dryness, and the residue was partitioned between CH_2Cl_2 and water. The organic phase was washed with three portions of water, and the combined aqueous extract was back-extracted with CH_2Cl_2. The combined CH_2Cl_2 extract was concentrated to dryness, and the solid residue was crystallized from hot ethanol, affording 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-(3-iodopropyl)carboxamide: yield 32 mg (67%); mp 158–160 °C.

An anhydrous solution of 32 mg (0.69 mmol) of the iodide, 18.2 mg (0.65 mmol) of silver tosylate, and 1.2 mL of dimethyl sulfide in 3 mL of acetic acid was heated at reflux in the dark. After 6 h, the reaction mixture was worked up in the dark by concentration to a yellow solid, which was dissolved in water, filtered through a Celite pad, and applied to a column (0.9 × 40 cm) of CM-Sephadex C-25 (acetate form). The column was washed with 30 mL of water and then with a linear gradient of acetic acid (400-mL total volume; 0–0.5 M; 4.5-mL fractions) at a flow rate of 20 mL/h. The appropriate fractions were pooled and concentrated, affording 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-[3-(dimethylsulfonio)-propyl]carboxamide (**5**) as a colorless glass: yield 20 mg (67%); ^1H NMR [D_2O, ext $(CH_3)_4Si$] δ 1.95 (s, 3 H), 2.0 (s, $^-$OAc), 2.1 (m, 2 H), 2.85 (s, 6 H), 3.0–3.6 (m, 8 H), 7.92 (s, 1 H), and 8.10 (s, 1 H).

3-Acetamidopropylamine (**7**). Freshly distilled 1,3-diaminopropane (0.88 g, 11.9 mmol) was dissolved in 25 mL of methanol and treated with 21.6 g (21.1 mmol) of acetic anhydride. The reaction mixture was maintained at room temperature for 2 h and then concentrated under diminished pressure to afford an oil. This oil was dissolved in 200 mL of water and applied to a Bio-Rex 70 column (2.3 × 30 cm, H^+ form); the column was washed with a linear gradient of acetic acid (2-L total volume; 0–0.5 M HOAc; 12.5-mL fractions). This procedure served to separate the desired product from unreacted 1,3-diaminopropane. The appropriate fractions (60–85) were pooled and concentrated and then codistilled with portions of water and finally with 0.1 N HCl to afford a solid residue. This material provided colorless microcrystals of 3-acetamidopropylamine from ethanol: yield 880 mg (48%); mp 155–157 °C; silica gel TLC (2:1 CH_3OH–15 N NH_4OH) R_f 0.67.



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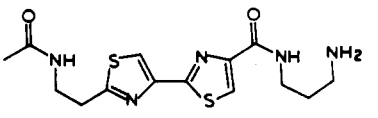

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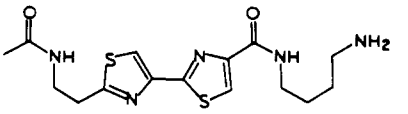

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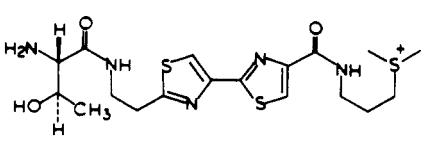
2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-(3-aminopropyl)-carboxamide (**9**). To a suspension of 47 mg (0.16 mmol) of 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-carboxylic acid in 4 mL of methylene chloride at 0 °C (N_2) was added 17 mg (23 μL, 0.17 mmol) of triethylamine and 23 mg (14 μL, 0.19 mmol) of thionyl chloride. The solution was stirred at 0 → 25 °C for 2 h and then treated with 60 μL of triethylamine and 11.5 mg (13 μL, 0.16 mmol) of 1,3-diaminopropane. A white precipitate formed immediately; stirring was continued overnight. The reaction mixture was concentrated under diminished pressure, and the brown residue was dissolved in 0.1 N HCl and applied to a (0.9 × 42 cm) column of Dowex 50 (200–400 mesh, H^+ form). After being washed with 30 mL of water, the column was eluted with a linear gradient of ammonium hydroxide (400-mL total volume; 0–2 N; 4-mL fractions) at a flow rate of 19 mL/h. The appropriate fractions were combined and concentrated to afford a white solid, which was converted to the hydrochloride by treatment with 0.1 N HCl and subsequent concentration under diminished pressure.

2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-(3-aminopropyl)-carboxamide hydrochloride (**9**) was obtained as colorless crystals from aqueous methanol–ethyl acetate: yield 47 mg (76); mp 216–218 °C; ^{1}H NMR [D_2O, ext $(CH_3)_4Si$] δ 1.93 (m + s, 5 H), 2.9–3.6 (m, 8 H), 7.90 (s, 1 H), and 7.96 (s, 1 H); silica gel TLC (1:1 $CHCl_3$–CH_3OH) R_f 0.27.

2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-(4-aminobutyl)-carboxamide (**10**). A solution of 1.0 g (3.2 mmol) of methyl 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-carboxylate (**2**) in 9.7 g (0.11 mol) of 1,4-diaminobutane was heated under N_2 at 85 °C for 3.5 h. Excess diamine was removed from the reaction mixture under diminished pressure. The residue was purified by chromatography on a (45-g) silica gel column; elution was with 200 mL of $CHCl_3$ and then 200 mL of 1:1 $CHCl_3$–CH_3OH. Subsequent washing with CH_3OH effected elution of the desired product, which was converted to the respective hydrochloride. The hydrochloride of 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-(4-aminobutyl)carboxamide (**10**) was deposited as colorless crystals from ethanol–petroleum ether: yield 1.05 g (81%); mp 171–172 °C; ^{1}H NMR [D_2O, ext $(CH_3)_4Si$] δ 1.78 (m, 4 H), 2.01 (s, 3 H), 2.9–3.9 (m, 8 H), 7.90 (s, 1 H), and 8.03 (s, 1 H).

2′-(2-Aminoethyl)-2,4′-bithiazole-4-[3-[(4-aminobutyl)-amino]propyl]carboxamide (**14**). A solution of 43 mg (90.2



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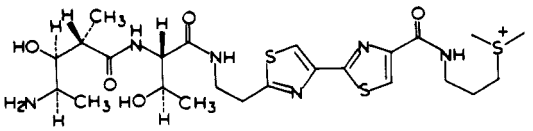

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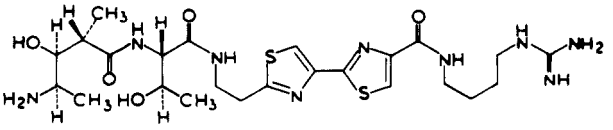

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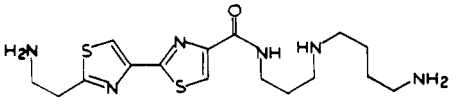

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μmol) of 2′-[2-(trifluoroacetamido)ethyl]-2,4′-bithiazole-4-(3-bromopropyl)carboxamide (Levin et al., 1980) in 1.0 mL (0.88 g, 10 mmol) of 1,4-diaminobutane was maintained at room temperature overnight. Excess diamine was removed under diminished pressure, and the residue was dissolved in water and applied to a (0.9 × 40 cm) column of Dowex 50 (200–400 mesh, H^+ form). The column was washed with a linear gradient of ammonium hydroxide (1.5-L total volume; 0–9 N; 4-mL fractions) at a flow rate of 18 mL/h. The column was then washed with 400 mL of water and finally with a linear gradient of hydrochloric acid (750-mL total volume; 0–9 N). The desired product eluted at a HCl concentration of 6.8 M; concentration of the appropriate fractions under diminished pressure afforded 2′-(2-aminoethyl)-2,4′-bithiazole-4-[3-[(4-aminobutyl)amino]propyl]carboxamide hydrochloride (**14**) as a colorless glass: yield 1050 A_{292} units (75%); mass spectrum *m/e* 382 (M^+), 365, 324, 238, 83, and 69. Bithiazole **14** could not be crystallized, but a solid sample was obtained by lyophilization of an aqueous solution.

DNA Substrates. ^{3}H-Labeled PM-2 DNA (21 × 10^6 cpm/μmol; containing radiolabeled thymine) was prepared as described (Espejo & Canelo, 1968). Calf thymus DNA was purchased from Calbiochem. Specific DNA fragments of the lactose operon region were isolated as described (Haseltine et al., 1980) and labeled by enzymatic extension of the 3′ termini with the Klenow DNA polymerase (Klenow & Henningsen, 1970) in the presence of [α-^{32}P]dATP (2800 Ci/mmol) and [α-^{32}P]TTP (Sanger & Coulsen, 1975). Approximate cpm (Cerenkov) values were determined on a Beckman LS7000.

Bleomycin-Mediated Degradation of [^{3}H]DNA. ^{3}H-Labeled PM-2 DNA (21 × 10^6 cpm/μmol; radiolabeled in thymine) was employed as a substrate for degradation. The reaction (350-μL total volume) was carried out in 50 mM sodium cacodylate, pH 7.0, containing 2.6 μM PM-2 DNA (1.77 × 10^4 cpm) and the potential inhibitor of interest (5 mM concentration, except as noted otherwise). The reaction was initiated by successive additions of bleomycin A_2 and $Fe(NH_4)_2(SO_4)_2$ to final concentrations of 2.6 and 25 μM, respectively. The reaction mixture was incubated at 37 °C, and 50-μL aliquots were removed at predetermined time intervals and applied to glass-fiber disks that had been presoaked with 30 mM thymine and 10% trichloroacetic acid. The dried disks were washed thoroughly with 5% trichloroacetic acid, dried, and used for determination of radioactivity.

Bleomycin-Mediated Degradation of [3′-^{32}P]DNA. [3′-^{32}P]DNA, approximately 10 000 cpm, was suspended in 100 μL of a 25 mM Tris-HCl buffer, pH 7.4. Fe(II)–blenoxane was added at time zero to a concentration of 5 × 10^{-8} M. After a 10-min incubation at 37 °C, the reactions were terminated by addition of 10 μL of a solution containing 100 mM EDTA, 2 mg/μL tRNA carrier, and 4 M sodium acetate. The inhibition of the Fe(II)–blenoxane reactions by the bithiazole derivatives was determined by first preincubating the [3′-^{32}P]DNA with each potential inhibitor for 10 min at 37 °C. Fe(II)–blenoxane was added as before and incubation was continued for an additional 10 min at 37 °C. Similar reactions that measured the ability of these potential inhibitors to inhibit Fe(II)-mediated [3′-^{32}P]DNA degradation were carried out by replacing the Fe(II)–blenoxane solution with 1 mM $FeSO_4$ and 10 mM β-mercaptoethanol.

For analysis on polyacrylamide gels, the DNA was precipitated with ethanol, washed with 95% cold ethanol, and resuspended in a loading buffer that contained 0.05 N NaOH–7 M urea. The DNA was layered on 8% polyacrylamide–7 M urea denaturing gels that were run at 1000 V. Quantitation of scission products was achieved by measurement of Cerenkov radiation in gel slices. The degree of inhibition of the bleomycin and Fe(II) cleavage reactions by the inhibitors was determined by measuring the fraction of input molecules that migrated at the position of full-length molecules in the treated samples as compared to that of untreated control samples that were analyzed on the same gel.

Results

The bithiazole derivatives required for the present study were prepared by starting from 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylate, the synthesis of which has been described (Zee-Cheng & Cheng, 1970; McGowan et al., 1977). Each of the bleomycin "fragments" studied as a potential inhibitor of bleomycin-mediated DNA degradation was purified chromatographically or by crystallization from the ap-

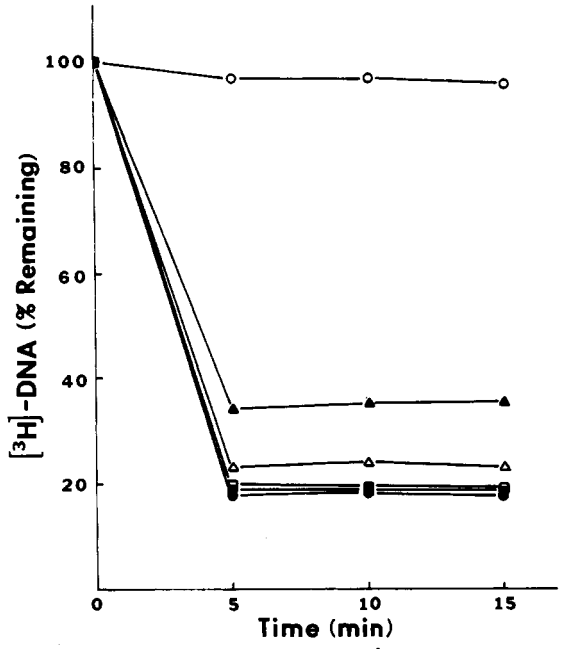

FIGURE 1: Inhibition of bleomycin-mediated [^{3}H]thymine release from PM-2 DNA by **1** (△), **2** (□), **4** (■), and **5** (▲). Bleomycin A_2 was utilized at a concentration of 2.6 μM, while 5 mM concentrations of **1**, **2**, **4**, and **5** were employed. Control experiments were carried out in the absence of added inhibitors (●) and in the absence of bleomycin A_2 (○). The protocol was as described under Experimental Procedures.

propriate solvent(s). The purity of each was verified by melting point, where possible, and by TLC on silica gel.

A sample of ^{3}H-labeled PM-2 DNA radiolabeled in thymine was prepared as described (Espejo & Canelo, 1968) and shown to consist mainly of supercoiled circular DNA by polyacrylamide gel electrophoresis. As shown in Figure 1, under the experimental conditions employed, bleomycin A_2 mediated the release of >80% of the thymine residues present in this DNA, while little degradation was observed in the absence of added bleomycin. Admixture of compound **1** to a reaction mixture containing [^{3}H]DNA and bleomycin A_2 effected only slight inhibition of degradation. Interestingly, compound **5** (which contains all of the structural elements of **1** in addition to a bithiazole) was significantly more effective as an inhibitor. That the increased inhibition observed for **5** was not due to the bithiazole moiety alone may be judged from the results obtained with bithiazole derivatives **2** and **4**, which were devoid of inhibitory activity at all concentrations tested. Thus, both the bithiazole and (positively charged) sulfonium moieties contributed to the inhibitory properties of **5**.

Also investigated was the importance of the number and spacing of positively charged groups. As illustrated in Figure 2, the dication 1,3-diaminopropane (**6**) [pK_as 8.64 and 10.62 (Fasman, 1976)] was a relatively effective inhibitor of bleomycin-mediated DNA degradation when utilized at 5 mM concentration, but its mono N-acetylated analogue (**7**) was not. Remarkably, 1,4-diaminobutane (**8**) was a much more effective inhibitor than **6** or **7**. Thus both the number and spacing of positively charged groups were found to contribute importantly to the inhibitory properties of individual compounds. Also instructive were the results obtained for bithiazole analogues **9** and **10** (derived synthetically from **6** and **8**, respectively). As shown, bithiazole **10** was a slightly better inhibitor than diamine **8**, while bithiazole **9** was slightly less effective than diamine **6**.

Tripeptide S (**11**) has been reported (Chien et al., 1977) to have essentially the same affinity for DNA as bleomycin

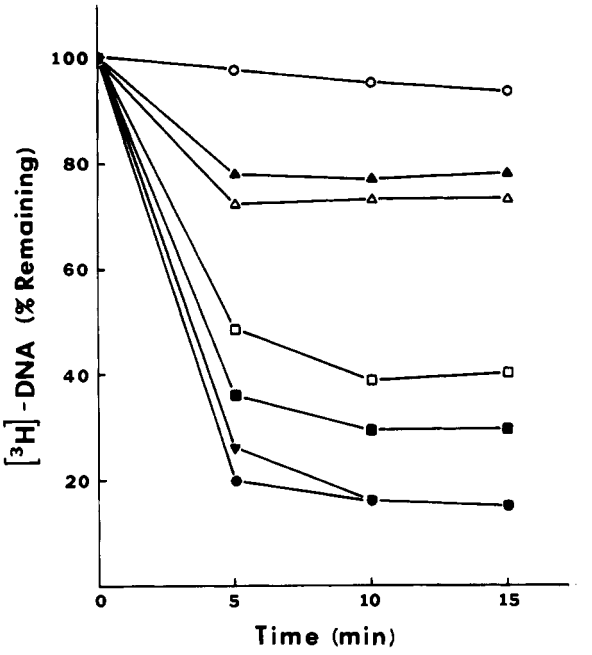

FIGURE 2: Release of [^{3}H]thymine from radiolabeled PM-2 DNA in the presence (●) and absence (○) of bleomycin A_2 and in the presence of bleomycin A_2 and **6** (□), **7** (▼), **8** (△), **9** (■), and **10** (▲). The procedure is described under Experimental Procedures.

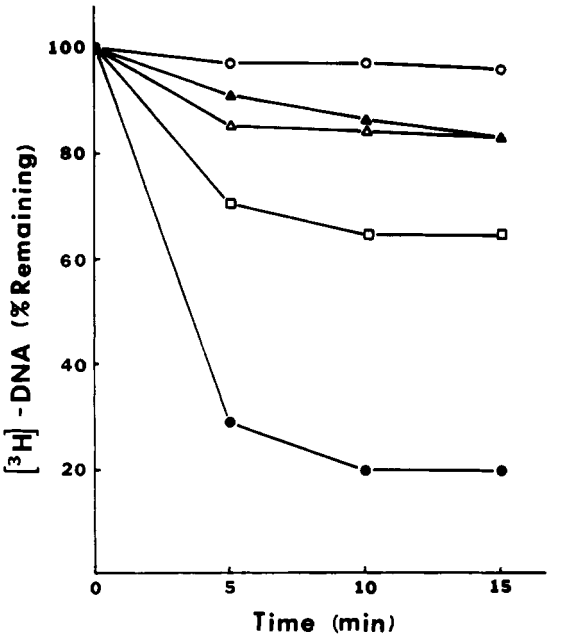

FIGURE 3: Inhibition of bleomycin A_2 mediated (●) DNA degradation by tripeptide S [**11** (△), 4 mM], tetrapeptide S [**12** (□), 5 mM], and **14** [(▲) 4 mM]. The experiment was carried out as described under Experimental Procedures: a control reaction was run in the absence of bleomycin A_2 (○).

A_2 itself, and it was, therefore, of interest to compare tripeptide S with other species as potential inhibitors of DNA degradation by bleomycin. As shown in Figure 3, tripeptide S was significantly more effective than tetrapeptide S (**12**) in inhibiting bleomycin-mediated DNA degradation and almost as effective as **14** (which contains three positively charged groups at neutral pH in addition to the bithiazole moiety).

In a second series of experiments, we investigated the ability of the bithiazole compounds to inhibit bleomycin-mediated strand scission. DNA molecules of defined sequence, labeled at one terminus, were used as substrates in these experiments. The DNA substrates were fragments of *Escherichia coli*

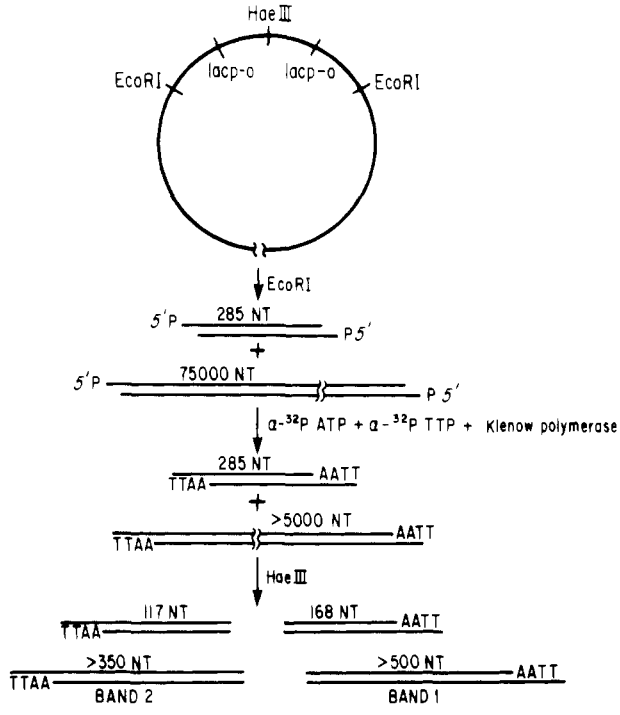

FIGURE 4: Schematic representation of isolation of 3'-end-labeled DNA fragments of *lac p-o* region.

plasmid pLJ3; they were purified by cleavage of the plasmid with restriction enzyme *Eco*RI. Cleavage with the enzyme produced two fragments (Figure 4), the termini of which contain a four nucleotide long 5' single-stranded end. The termini were labeled by incubation of the mixture of fragments with [α-^{32}P]dATP and [α-^{32}P]TTP in the presence of the Klenow fragment of *E. coli* DNA polymerase I (Klenow & Henningsen, 1970). This reaction resulted in labeling of the 3' termini of each fragment. The DNA was then incubated with the restriction endonuclease *Hae*III, a reaction that yielded four DNA fragments, each labeled at a single terminus (Figure 5). The DNA sequences of the fragments denoted *lac* operon 117 and 168 and fragment 2 have been determined previously (Haseltine et al., 1980). The use of such substrates permits analysis of cleavage events at specific sequences. The experimental design permitted us to ask whether inhibition of bleomycin activity by the bithiazole analogues occurred equally at all sites.

To establish the optimal concentration range for the bleomycin reaction, we incubated a 3'-end-labeled substrate with bleomycin over the concentration range 10^{-9}–10^{-4} M. Equimolar concentrations of a freshly prepared solution of Fe(II) were added to the bleomycin solutions immediately prior to DNA treatment, and reactions were carried out open to the atmosphere at 37 °C for 30 min. After treatment with bleomycin A_2, the DNA was heat denatured and layered on high-resolution polyacrylamide gels of the types used for DNA sequence analysis (Maxam & Gilbert, 1977). DNA molecules that differ in length from one another by a single nucleotide are resolved cleanly in this gel system; the shorter a DNA fragment, the more rapid its electrophoretic mobility. The length distribution of the ^{32}P-end-labeled cleavage products was determined by autoradiography.

Figure 6 illustrates the results of this experiment. No significant cleavage occurred in reactions that contained 1×10^{-9} or 1×10^{-8} M bleomycin A_2. However, significant breakage of the labeled DNA strand did occur in all reactions that contained Fe(II)–bleomycin concentrations of at least 5×10^{-8} M. The extent of strand scission in these reactions was determined by measurement of the amount of ^{32}P in gel slices containing the full-length molecule as well as the shorter cleavage products. The extent of the cleavage reaction is expressed as the percent of the total radioactivity in a lane that migrates more rapidly than the untreated control sample. The percent of strand scission as a function of bleomycin A_2 concentration is shown in Figure 7. In these experiments strand scission reached a plateau at about 80% cleavage at a concentration of about 10^{-5} M bleomycin A_2. This was in good agreement with the observed extent of bleomycin-mediated thymine release from ^{3}H-labeled PM-2 DNA (Figures 1–3). Incubation of the DNA with concentrations of bleomycin of 10^{-4} and 10^{-3} M did not result in additional strand breakage. This result suggests that in these reactions the extent of strand scission is limited by another component in the reaction. Conceivably, dissolved oxygen may be limiting in these reactions, as bleomycin-mediated DNA breakage is known to be oxygen dependent (Ishida & Takahasi, 1975; Sausville et

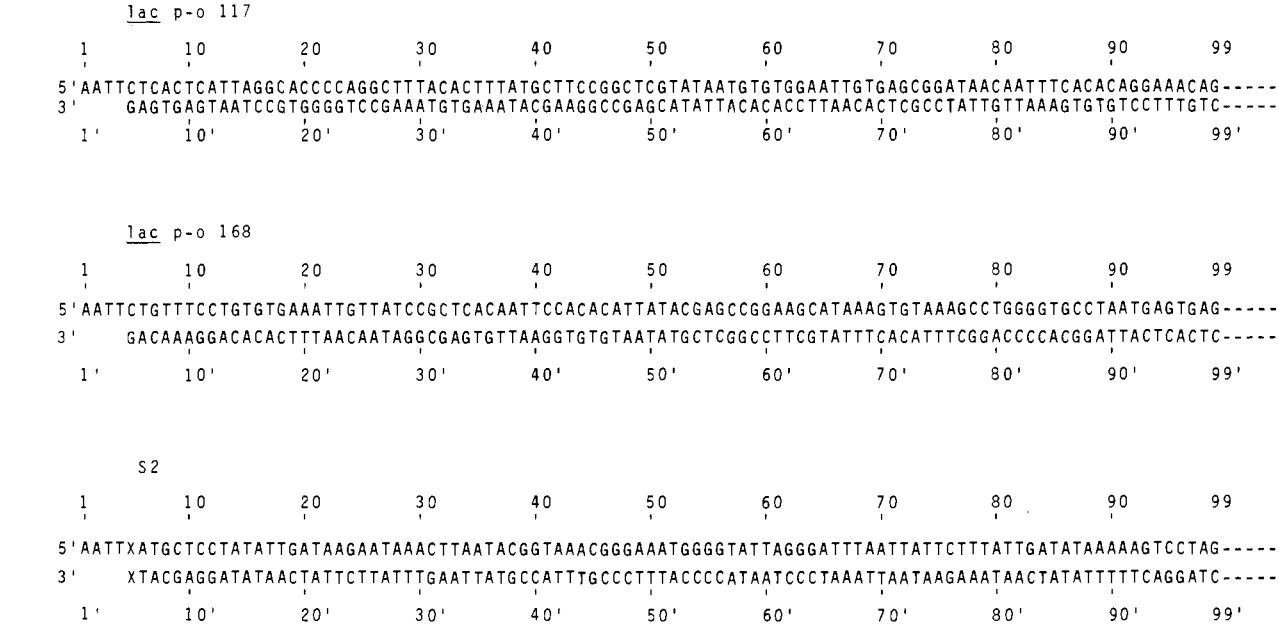

FIGURE 5: Sequences of three DNA substrates obtained from pLJ3 plasmid.

FIGURE 6: DNA degradation by bleomycin A_2. The 168-base-pair 3′-end-labeled [^{32}P]DNA (10000 cpm, 1 mg of DNA/reaction) was incubated with equimolar Fe(II)–bleomycin A_2 or Fe(II)–blenoxane over the concentration range 10^{-9}–10^{-4} M for 10 min at 37 °C. The reactions were terminated by addition of 10 mM EDTA and analyzed on a denaturing 8% polyacrylamide gel. Concentrations of 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M Fe(II)–bleomycin A_2 were analyzed in lanes 3–8, respectively. Lane 2 contained 10^{-4} M Fe(II)–blenoxane. Lane 1 contained an untreated 168-base-pair 3′-end-labeled [^{32}P]DNA.

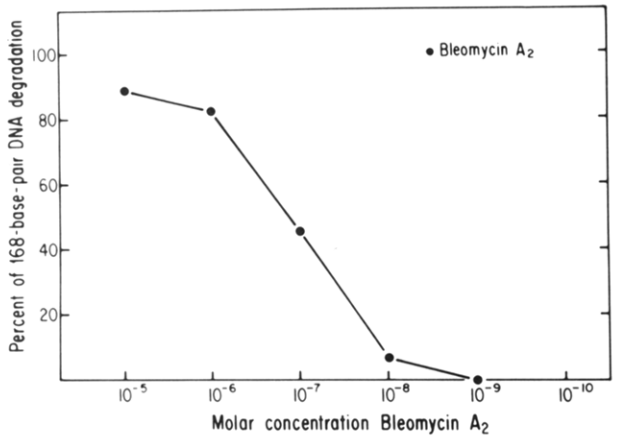

FIGURE 7: DNA degradation as a function of bleomycin A_2 concentration. The 168-base-pair 3′-end-labeled [^{32}P]DNA was incubated at 37 °C in the presence of several concentrations of Fe(II)–bleomycin A_2 for 10 min. Each reaction was terminated with 10 mM EDTA; the DNA was ethanol precipitated and loaded onto an 8% polyacrylamide gel. Quantitation of scission products was effected by measurement of Cerenkov radiation in gel slices. The extent of DNA breakage is expressed as the fraction of radiation that migrated more rapidly than intact, full-length molecules.

al., 1976, 1978a; Lown & Sim, 1977; Povirk, 1979). On the basis of these experiments, a 5×10^{-8} M Fe(II)–bleomycin concentration was selected for the inhibition studies. This concentration of Fe(II)–bleomycin (either a single species or the clinical mixture) resulted in about 50% strand scission of the 168-nucleotide-long substrate; the reaction was complete within 10 min.

To examine the ability of the synthetic bithiazole analogues to inhibit the strand scission reactions, we incubated the compounds with DNA for 10 min at 37 °C prior to the addition of bleomycin. The first and last samples prepared in each experiment contained no inhibitor. This control was

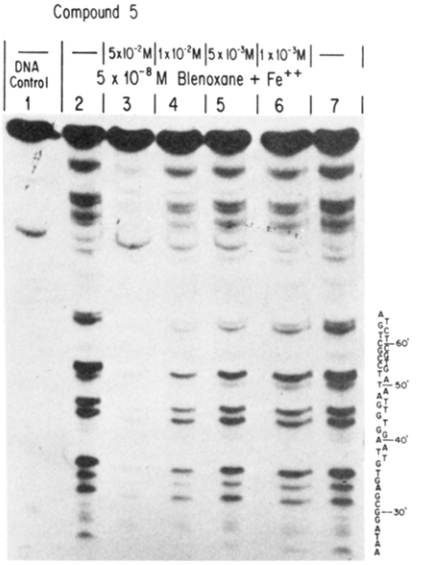

FIGURE 8: Inhibition of bleomycin degradation of DNA. The 168-base-pair 3′-end-labeled [^{32}P]DNA was incubated with concentrations of bithiazole analogue **5** ranging from 10^{-3} to 5×10^{-2} M at 37 °C for 10 min prior to the addition of 5×10^{-8} M bleomycin and continued incubation for 10 min. The reaction products were analyzed on a denaturing 8% polyacrylamide gel. Concentrations of 5×10^{-2}, 10^{-2}, 5×10^{-3}, and 10^{-3} M bithiazole **5** were in lanes 3, 4, 5, and 6, respectively. No bithiazole **5** was added to lane 2 or 7. Lane 1 contained untreated 168-base-pair 3′-end-labeled [^{32}P]DNA.

Table I: Concentrations of Bithiazole Derivatives Needed to Inhibit 168-Base-Pair 3′-End-Labeled [^{32}P]DNA Degradation by 5×10^{-8} M Fe(II)–Blenoxane

bithiazole	concentration (M)	
	100% inhibition	50% inhibition
2	*a*	2.8×10^{-2}
3	*a*	2.7×10^{-2}
9	*a*	2.4×10^{-2}
5	3.8×10^{-2}	6.0×10^{-3}
13	1.0×10^{-2}	2.0×10^{-4}
12	5.0×10^{-3}	3.0×10^{-5}
14	5.0×10^{-3}	6.0×10^{-6}

[a] Not reached at any tested concentration.

necessary as Fe(II)–bleomycin was found to be labile and is known to be subject to autodegradation (Sausville et al., 1976). For all of the experiments reported here, the extent of strand scission in the first sample was the same as that in the last. The effects of **5**, **13**, and **14** on the scission products are illustrated in the autoradiographs of the gels in Figures 8, 9, and 10, respectively. The data for **2**, **3**, **5**, **9**, **12**, **13**, and **14** are presented quantitatively in Figure 11, in which the extent of strand scission is plotted as a function of inhibitor concentration. These data are also summarized in Table I as the concentrations of the inhibitor required for 100% and for 50% inhibition of the DNA cleavage reaction. It is evident that the compounds varied markedly in the effective concentrations at which half-maximal inhibition was achieved. For example, half-maximal inhibition for **14** was obtained at a 6.0×10^{-6} M concentration, while a 6.0×10^{-3} M concentration of **5** was required to effect inhibition to the same extent.

For some of the compounds, such as **2**, **3**, and **9**, the concentrations required to affect DNA strand scission were very high, suggesting that the observed inhibition might have been due to some nonspecific process, e.g., quenching of the "activated species" resulting from combination of Fe(II), bleomycin, and O_2. To test this possibility, we utilized some of the bithiazole derivatives as potential inhibitors of Fe-

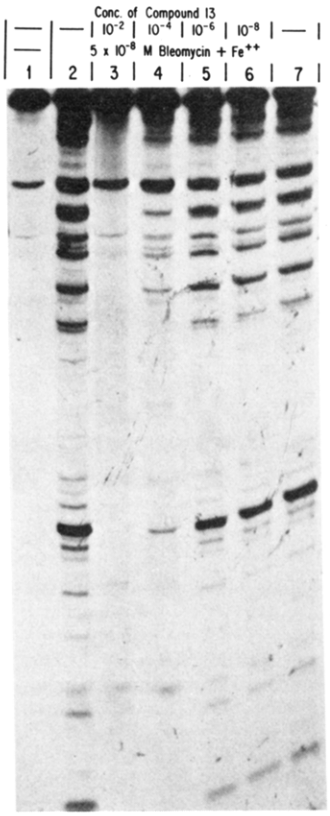

FIGURE 9: Inhibition of bleomycin degradation of DNA by **13**. The 168-base-pair 3′-end-labeled DNA was incubated at 37 °C with analogue **13** at concentrations of 10^{-8}–10^{-2} M for 10 min prior to the addition of 5×10^{-8} M bleomycin and continued incubation for 10 min. The reaction products were analyzed on a denaturing 8% polyacrylamide gel. Concentrations of 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} M bithiazole **13** were added to lanes 3, 4, 5, and 6, respectively. No bithiazole **13** was added to lane 2 or 7. Lane 1 contained untreated 168-base-pair 3′-end-labeled [^{32}P]DNA.

(II)-mediated DNA cleavage (D'Andrea & Haseltine, 1978), a process that probably involves the intermediate generation of ·OH (Haber & Weiss, 1934). As shown in Figure 12, at a 4×10^{-3} M concentration **2** inhibited 90% of Fe(II)-mediated DNA breakage, consistent with the suggestion that inhibition observed at high concentrations of the bithiazole derivatives may not be due to inhibition of bleomycin–DNA interaction but rather to nonspecific quenching of the activated species actually responsible for DNA degradation. It should be noted, however, that such inhibition was not observed at concentrations of bithiazole derivatives below 1×10^{-3} M. For example, Figure 13 shows that the degradation of DNA by Fe(II) was not inhibited by **14** over the concentration range 1×10^{-5}–1×10^{-3} M.

Bleomycin cleaves DNA preferentially at specific sequences (D'Andrea & Haseltine, 1978; Takeshita et al., 1978). Inspection of Figures 8–10 suggests that inhibition of the cleavage reaction by the bithiazole analogues occurs equally at all possible cleavage sites. This observation was substantiated by quantitative measurement of the extent of inhibition of the reaction at each specific site. For these measurements, the amount of radioactivity at a specific site was measured. The fraction of the cleavage products at each site decreased proportionally in these experiments (data not shown). No preferential inhibition of specific cleavage events was observed.

Several of the bithiazole analogues tested as inhibitors of DNA degradation were also utilized in fluorescence quenching experiments. As shown in Table II, admixture of calf thymus DNA to solutions of the bithiazole derivatives caused

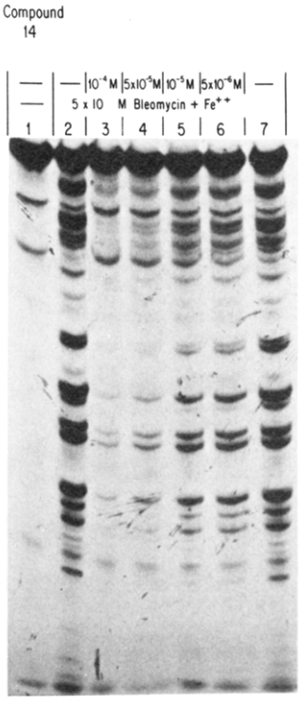

FIGURE 10: Inhibition of bleomycin degradation of DNA by **14**. The 168-base-pair 3′-end-labeled DNA was incubated at 37 °C with concentrations of bithiazole **14** of 5×10^{-6} to 10^{-4} M for 10 min prior to the addition of 5×10^{-8} M bleomycin and continued incubation for 10 min. The reaction products were analyzed on a denaturing 8% polyacrylamide gel. Concentrations of 10^{-4}, 5×10^{-5}, 10^{-5}, and 5×10^{-6} M bithiazole **14** were added in lanes 3, 4, 5, and 6, respectively. No bithiazole **14** was added to lane 2 or 7. Lane 1 contained untreated 168-base-pair 3′-end-labeled DNA.

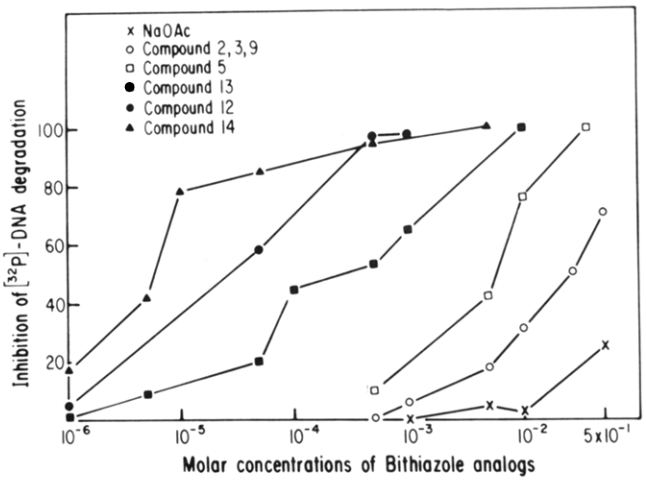

FIGURE 11: Inhibition of DNA degradation by several bithiazole analogues. The 168-base-pair 3′-end-labeled [^{32}P]DNA was incubated in the presence of 5×10^{-8} M Fe(II)–bleomycin and varying concentrations of inhibitors **2**, **3**, and **9** (○), **5** (□), **12** (●), **13** (■), and **14** (▲). Quantitation of scission products was by measurement of Cerenkov radiation in gel slices. The extent of inhibition of the bleomycin cleavage reaction was determined by measurement of the fraction of total radioactivity that migrated more rapidly than the untreated DNA in each lane. Sodium acetate was employed as a control "inhibitor" and found to have little effect on bleomycin-mediated DNA degradation.

quenching of bithiazole fluorescence excitation, presumably reflecting an association of the bithiazole derivatives with the added DNA. Interestingly, the best inhibitors of bleomycin-mediated DNA degradation exhibited the greatest degree of fluorescence quenching upon addition of DNA, consistent with the interpretation that inhibition was due to the association

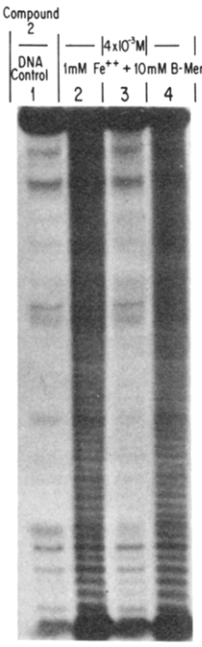

FIGURE 12: Inhibition of Fe(II)-mediated DNA cleavage by **2**. The 117-base-pair 3′-end-labeled [^{32}P]DNA was incubated at 37 °C with 4×10^{-3} M bithiazole **2** for 10 min prior to the addition of 1 mM ferrous sulfate and 10 mM β-mercaptoethanol, followed by continued incubation for 10 min. The reaction products were analyzed on a denaturing 8% polyacrylamide gel. A concentration of 4×10^{-3} M bithiazole **2** was contained in lane 3. Lanes 2 and 4 contained no bithiazole **2**. Lane 1 contained untreated 117-base-pair 3′-end-labeled [^{32}P]DNA.

Table II: Fluorescence Quenching of Bithiazole Derivatives by Calf Thymus DNA[a]

bithiazole	fluorescence intensity[b] −DNA	fluorescence intensity[b] +DNA	% fluorescence quenching
14	58	7.6	87
11	68.4	11.7	83
12	69.5	16	77
10	77	23.5	69
4	54	17.2	68
2	83.6	28.0	67
5	68.5	23.5	66
9	49.3	17.7	64

[a] Fluorescence measurements were made at 25 °C in 15 mM Tris-HCl, pH 7.5, with individual bithiazole derivatives at a concentration of 22 μM in the presence or absence of 570 μM calf thymus DNA. Fluorescence excitation and emission were recorded at 290 and 352 nm, respectively. [b] Arbitrary units.

of the individual analogues with DNA.

Discussion

Since the antineoplastic properties of bleomycin are thought to be based at the level of bleomycin–DNA interaction, the molecular nature of DNA binding and cleavage by bleomycin is of considerable importance. Mechanistically, bleomycin-mediated strand scission of DNA has several interesting features, including the induction of both single- and double-strand breaks, sequence specificity, a lack of proportionality between the amount of bleomycin utilized and the number of resulting DNA nicks, and the probability that at least two chemical mechanisms must operate in parallel to account for the observed reaction products (Hecht, 1979; Giloni et al., 1981).

Utilized as a probe of bleomycin–DNA interaction were a number of compounds structurally related to the C terminus of bleomycin. These were employed to block bleomycin-mediated DNA degradation in an effort to (i) define the structural features in bleomycin that promote DNA binding, (ii) determine whether the affinity of bleomycin for DNA is specified entirely by the tripeptide S portion of the antibiotic, and (iii) determine whether the nature of bleomycin binding to DNA is altered as increasing numbers of molecules are bound.

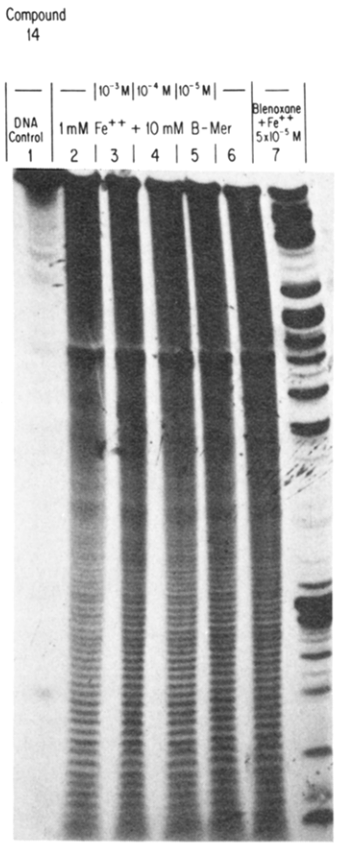

FIGURE 13: Test of inhibition by **14** of Fe(II)-mediated DNA breakage. Band 2 (3′-end-labeled) DNA was incubated at 37 °C for 10 min prior to the addition of 1 mM ferrous sulfate and 10 mM β-mercaptoethanol, followed by continued incubation for 10 min. The reaction products were analyzed on a denaturing 8% polyacrylamide gel. Concentrations of 10^{-3}, 10^{-4}, and 10^{-5} M bithiazole **14** were contained in lanes 3, 4, and 5, respectively. Lanes 2 and 6 contained no bithiazole **14**. Lane 1 contained untreated band 2 3′-end-labeled DNA.

The effects of these compounds on the bleomycin–DNA reaction were followed by their ability to inhibit the production of free thymine or single-strand breaks; excellent agreement was obtained between these two assays. For compounds tested by both methods, the inhibitory potentials of the compounds were found to be **14** > **12** > **5** > **2**. Also tested by both methods was **9**, which was found to have activity intermediate between **5** and **2** in both assays.

Several observations illustrate the importance of the number and spacing of positive charges on the inhibitory potential of these compounds. Although **5** corresponds to the C terminus of bleomycin A_2 it has only a single positive charge and is only moderately inhibitory toward bleomycin-mediated DNA degradation (Figure 1; Table I). Most other uncharged or singly charged derivatives, e.g., **1**, **2**, **3**, **4**, and **7**, also failed to act as strong inhibitors of DNA degradation (Figures 1 and 2; Table I).

Interestingly, 1,4-diaminobutane (**8**) was found to be a good inhibitor of bleomycin-induced thymine release from ^{3}H-labeled PM-2 DNA (Figure 2), illustrating the importance of multiple positive charges on the inhibitory potential of individual species. The structurally related bithiazole (**10**) was

slightly more inhibitory than **8**, in spite of the fact that it contains only a single positively charged group at neutral pH. Thus, as also suggested by comparison of the inhibitory activities of **1** and **5** (Figure 1), the bithiazole moiety appears to contribute substantially to the affinity of these compounds for DNA and hence to their ability to inhibit DNA binding by bleomycin. Remarkably, 1,3-diaminopropane (**6**) was markedly less inhibitory toward bleomycin-mediated thymine release from PM-2 DNA than was **8**, as was the bithiazole derivative corresponding to **6** (**9**). That both the number and spacing of positively charged groups contribute to the inhibitory potential of individual compounds can be also appreciated by comparison of **5**, **11**, and **12**. It was, therefore, not surprising to find that bithiazole **14** (having three optimally positioned positive charges) was the most inhibitory derivative tested. Thus, comparison of the inhibitory properties of these compounds suggests strongly that their affinity for DNA (and presumably that of structurally related bleomycins) depends on the number and spacing of positively charged groups and on the bithiazole moiety. Thus, our results are in agreement with those of Huang et al. (1980), who have identified an ionic and nonionic component of bleomycin affinity for DNA, and with those of Kashi et al. (1978), who demonstrated that a positively charged C-terminal substituent facilitated the binding of bleomycin to DNA.

Of particular interest in the context of DNA binding by bleomycin are the results shown in Figure 3. Tripeptide S (**11**) was found to be much more efficient than tetrapeptide S (**12**) in inhibiting bleomycin A_2 mediated degradation of ^{3}H-labeled PM-2 DNA, which presumably reflects the greater affinity of **11** for this DNA. Thus the relative affinities of the two compounds for PM-2 DNA were not in the same order as their structural similarities to bleomycin A_2. Chien et al. (1977) have reported previously that both tripeptide S and bleomycin A_2 have about the same K_a for calf thymus DNA, suggesting that the DNA affinity of bleomycin A_2 may be due entirely to the tripeptide S moiety. While the experiments reported here are not designed to measure directly the affinities of bithiazole derivatives for DNA, they are consistent with an important role for the tripeptide S moiety in bleomycin–DNA interaction. Nonetheless, the relative potencies of bithiazoles **11** and **12** as inhibitors of bleomycin-mediated DNA degradation suggest that the numerical similarities for the measured K_a's of tripeptide S and bleomycin A_2 (Chien et al., 1977) may simply have been fortuitous. In agreement with the published observations of other workers (Kashi et al., 1978; Huang et al., 1980), our results suggest strongly that both the positively charged C and N termini of (metallo)bleomycins should contribute significantly to the DNA affinity of these drugs.

What is the mechanism by which these compounds inhibit bleomycin-induced thymine release and DNA degradation? The data in Tables I and II suggest that DNA–inhibitor binding accounts for inhibition. There is good agreement between the extent of fluorescence quenched by DNA and the inhibitory potential of a given compound. One superficially curious feature of the results is that the inhibitory potentials of individual compounds were not altogether dissimilar in the presence of 2.6×10^{-6} M DNA (Figures 1–3) or 5×10^{-8} M DNA (Figure 11). Aside from the differences in the types of DNA employed in these experiments, it may be noted that this lack of effect of DNA concentration on inhibition does not discount competitive binding as a mechanism of inhibition because DNA concentration was not limiting in either reaction.

No evidence was found for a change in site specificity of bleomycin–DNA degradation during inhibition by bithiazole analogues. As shown in Figure 6, the site specificity of bleomycin–DNA degradation remains constant over a wide concentration range of bleomycin A_2 and is identical with that obtained with the commercial blenoxane preparation. This site specificity was not altered by inhibition with bithiazole analogues (Figures 8–10).

Acknowledgments

We thank Dr. Mohammed Shekhani, Howard Katz, and Steven Rodgers for assistance with preparation of some of the bithiazole analogues and Dr. Paul Lau for preparation of the radiolabeled PM-2 DNA.

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Phosphorylation of Deoxyribonucleic Acid Dependent RNA Polymerase II by Nuclear Protein Kinase NII: Mechanism of Enhanced Ribonucleic Acid Synthesis†

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ABSTRACT: RNA polymerase II was purified from Morris hepatoma 3924A by a series of ion-exchange and affinity column chromatographic fractionations, followed by sucrose gradient centrifugation in the presence of 0.3 M KCl. Purified RNA polymerase II had a specific activity of greater than 400 nmol of UMP incorporated (30 min)$^{-1}$ (mg of protein)$^{-1}$ by using double-stranded DNA as template. The purified enzyme contained five polypeptides (M_r 214 000, 140 000, 33 000, 25 000, and 21 000) that were present in molar quantities and two additional polypeptides (M_r 19 000 and 18 000) that had a combined molar ratio of 1.0. The cyclic AMP independent nuclear protein kinase NII, also purified from hepatoma 3924A, was able to phosphorylate RNA polymerase II polypeptides of M_r 214 000, 140 000, and 21 000. Phosphorylation of the polymerase was accompanied by enhanced transcription of double-stranded DNA, heat-denatured DNA, and poly[d-(A-T)]. The elevation in RNA polymerase activity was dependent upon the presence of hydrolyzable ATP and resulted from an increased number of RNA molecules synthesized in vitro. The average length of RNA chains was not affected by the kinase. Under similar conditions, protein kinase NII also stimulated homologous RNA polymerase I. In contrast to the phosphorylation of polymerase II, modification of polymerase I resulted in an increase in the average size, but not number, of RNA chains synthesized. The specificity of the NII kinase-catalyzed reaction was demonstrated by the inability of another homologous protein kinase, NI, to phosphorylate or activate RNA polymerase II.

Phosphorylation of chromatin proteins has been correlated with enhanced expression of several genes. Both histone and nonhistone nuclear proteins are phosphorylated in response to biological stimuli [see Krebs & Beavo (1979)]. The modification of nonhistone chromatin proteins is most probably catalyzed by nuclear kinases which are cyclic AMP independent. Two major nuclear protein kinases are found in rat liver and are designated NI and NII according to their elution from DEAE-Sephadex (Desjardins et al., 1972). These kinases have distinct reaction properties and preferentially phosphorylate casein and phosvitin in vitro. Protein kinases NI (Thornburg et al., 1978; Rose & Jacob, 1979) and NII (Thornburg & Lindell, 1977; Rose et al., 1981a) have been extensively purified from rat liver and a rat hepatoma. Although phosphorylation of nuclear proteins by endogenous or exogenous nuclear kinase has been studied in a number of

† From the Department of Pharmacology and Specialized Cancer Research Center, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033. *Received January 4, 1982.* Supported by U.S. Public Health Service Grant GM-26740 from the National Institute of General Medical Sciences and Contract CB-14345-39 (awarded to Dr. Wayne Criss, Howard University, Washington, DC) from the National Cancer Institute and by Grant IN-109 from the American Cancer Society. K.M.R. is the recipient of a Research Career Development Award from the National Cancer Institute.

0006-2960/82/0421-3721$01.25/0