(b) **Identification of Nonvolatile Products.** A sample of **2a-c** (9.8 mg, 0.05 mmol) in 0.1 M phosphate buffer (10 mL) was allowed to decompose for 12 h at 37 °C. The aqueous solution was lyophilized and the residue was subjected to CIMS using isobutane as reagent gas; the CIMS of their decomposition products are presented in Table III. After CIMS spectra, the residue was extracted with $CDCl_3$ and the extract examined by NMR. This allowed both the identification (by comparison with authentic samples) and quantitation of cyclohexyloxazoline *N*-cyclohexylcarbamate and 2-hydroxyethylcyclohexylurea.

(2) **In the Presence of Saturated Sodium Chloride.** (a) **Identification of Volatile Products.** A sample of **2a-c** or **4a** (0.1 mmol) in saturated sodium halide (4 mL) in 0.1 M phosphate buffer was allowed to decompose in 5-mL capacity Reacti-vials as above at 37 °C for 12 h. The 1-mL head space was immediately evacuated by a syringe, and the gaseous contents after 12 h were analyzed by GC-MS. Immediately after the removal of the gaseous contents, 0.5 mL of dichloromethane was injected into the vial and shaken thoroughly, and the dichloromethane solution (2 μL) was injected for GC-MS analysis. The retention time and mass spectra of each compound in different reaction conditions are given in Table II.

(b) **Identification of Nonvolatile Products.** After the identification of volatile products, the reaction mixture was extracted with hot acetonitrile and the organic phase was concentrated. The products were identified as ureas and oxazolines by TLC on reverse-phase precoated silica plates using a water/acetonitrile mixture (1:9-2:8) and their characteristic mass spectra.

Fluorescence Determination of Alkylation of PM2-CCC-DNA by (2-Haloethyl)nitrosourea Intermediates. All fluorescence measurements were performed on a G. K. Turner and Associates Model 430 spectrofluorometer equipped with a cooling fan to minimize fluctuations in the xenon lamp source. Wavelength calibration was performed as described in the manual for the instrument; 1-cm^2 cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength was 500 nm. The 100X scale of medium sensitivity was generally used, and water was circulated between the cell compartment and a thermally regulated bath at 22 °C. The method has been described in detail elsewhere.[38] The basis of the method is that while PM2-CCC (covalently closed circular) DNA returns to register after heating to 96 °C and cooling, owing to topological constraints, and therefore shows no change in the fluoroscence of intercalated ethidium, alkylated PM2-CCC-DNA shows a decrease in fluorescence under these conditions because of thermally induced cleavage at the site of alkylation. The ratio of the decrease in fluorescence (after heat denaturation and rapid cooling) to that of control is a measure of the extent of alkylation.

Acknowledgment. This investigation was supported by Grant 1-R01-CA21488-01 awarded by the National Cancer Institute, DHEW, and by a grant from the Alberta Provincial Cancer Hospitals Board. We thank Dr. A. Hogg and his colleagues for extensive mass spectral studies.

Synthesis and DNA Binding of [3-[2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-carboxamido]propyl]dimethylsulfonium Chloride, a Fragment of Bleomycin A_2

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[3-[2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-carboxamido]propyl]dimethylsulfonium chloride (1), the acetyl derivative of the cationic terminal dipeptide of bleomycin A_2, has been synthesized and its binding to DNA and poly(dA-dT) has been studied by proton NMR and fluorescence spectroscopy. The spectral perturbations which occur upon binding of the compound to either nucleic acid indicate that that portion of bleomycin which binds to the nucleic acid can, for the most part, be mimicked by the fragment. The data are discussed in terms of the structure of the drug and the drug-nucleic acid complex.

The antitumor antibiotic bleomycin A_2 (Figure 1) is believed to exert its biological effect(s) by causing the degradation of DNA.[1] The putative mechanism of action involves at least two processes (which may occur stepwise or simultaneously): (i) association of the drug with DNA and (ii) generation of free radicals by the drug, acting in the form of a metal ion complex.[1] It is not known if the radicals are the species responsible for DNA degradation or if the bleomycin molecule also plays a role. Bleomycin A_2 appears to possess two distinct regions, each of which is responsible for one of the functions: a cationic terminus containing an aromatic group for the binding and a metal-ion binding site localized around the pyrimidine moiety necessary for the degradation of the nucleic acid.[1-3] Figure 1 shows the possible ligands involved in the complexation of the metal ions. As a part of ongoing studies on structure-activity relationships in the bleomycin family of drugs, we have synthesized a fragment of bleomycin A_2 which is the acetyl derivative of the cationic terminal "dipeptide" and which contains the presumed DNA binding sites. We have studied the interaction of this derivative with calf thymus DNA and poly(dA-dT) using proton NMR and fluorescence spectroscopy. If the activity of bleomycin A_2 can, in fact, be explained on the basis of a simple bifunctional model in which the two portions of the molecule act more or less independently, the cationic fragment should show all, or nearly all, of the spectral perturbations seen in the binding of the intact drug.

The binding of intact bleomycin A_2 and tripeptide S, the terminal fragment containing threonine (Figure 1), to DNA has been studied using fluorescence[2] and NMR spectroscopy.[2,4] The fluorescence experiments suggested that tripeptide S bound in a manner very similar to the intact

(1) See recent reviews in "Bleomycin: Chemical, Biochemical, and Biological Aspects"; Hecht, S. M., Ed, Springer-Verlag, New York, 1979.

(2) Chien, M.; Grollman, A. P.; Horwitz, S. B. *Biochemistry* **1977**, *16*, 3641-3647.

(3) See, for example, (a) Takeshita, M.; Grollman, A. P. In ref 1, pp 207-221. (b) Kasai, H.; Naganawa, H.; Takita, T.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 1316-1320.

(4) (a) Chen, D. M.; Sakai, T. T.; Glickson, J. D.; Patel, D. J. *Biochem. Biophys. Res. Commun.* **1980**, *92*, 197-205. (b) Pillai, R. P.; Lenkinski, R. E.; Sakai, T. T.; Geckle, J. M.; Krishna, N. R.; Glickson, J. D. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 341-349.

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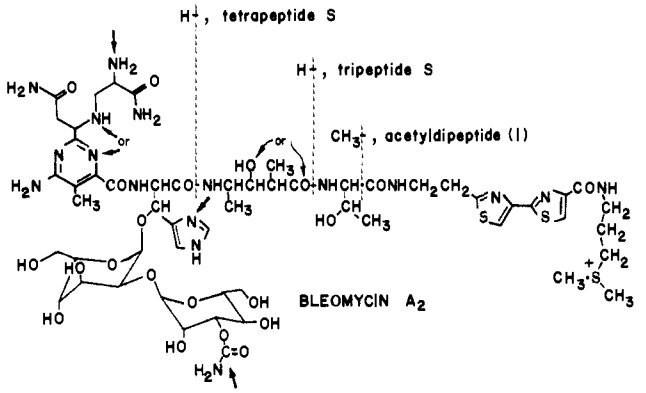


Figure 1. Structures of bleomycin A_2, tripeptide S, tetrapeptide S, and the acetyl dipeptide. Arrows indicate probable ligands for metal ions.[4b]

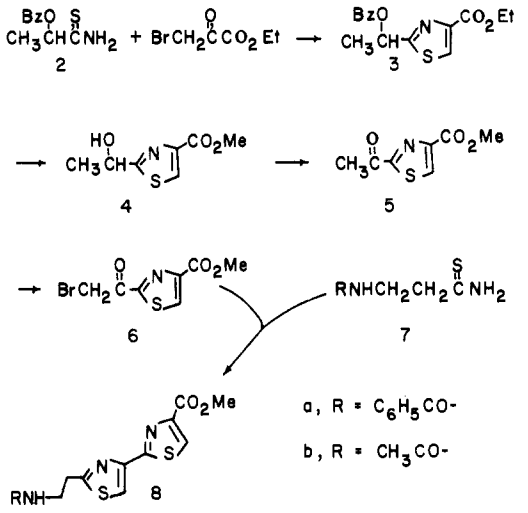
drug. The NMR experiments of Chien et al.[2] on bleomycin A_2 showed that the resonances of the bithiazole aromatic protons and the sulfonium methyl resonances broadened preferentially upon interaction with DNA; however, no NMR data were presented for tripeptide S. Chen et al.[4a] have presented a more detailed proton NMR study on the intact drug using poly(dA-dT) as a model DNA. They noted that the perturbations seen with tripeptide S were grossly similar to those seen with bleomycin A_2.

Although tripeptide S provides a reasonable model for the presumed DNA-binding portion of bleomycin A_2, the presence of the free amino group of the threonine residue makes available another potential site for a secondary or alternative mode of binding with the nucleic acid through the formation of a hydrogen or ionic bond. The threonine hydroxyl group may further help stabilize such a secondary or alternative mode of binding, although it appears to play little or no role in the interaction of intact bleomycin A_2 with poly(dA-dT).[4a] The use of the acetyl derivative 1 avoids such complications.

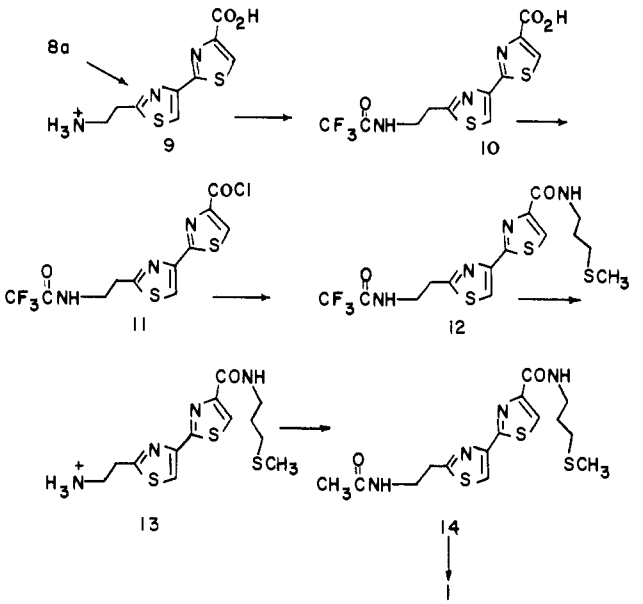
Tripeptide S and the terminal "dipeptide" may be obtained by partial hydrolysis of bleomycin A_2;[5] however, we have found these methods to be unsatisfactory in terms of the yield and the purity of the product obtained and have synthesized the desired compound. Hecht and coworkers have reported the preparation of tripeptide S and tetrapeptide S.[6] Their procedure includes a biomimetic route to 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylic acid starting with β-alanyl-L-cysteine derivatives.[7] Zee-Cheng and Cheng[8] have described a synthesis of the bithiazole amino acid starting with 3-benzamidothiopropionamide and ethyl bromopyruvate. We have adopted an alternative route starting with *O*-benzoylthiolactamide[9] (2) and ethyl bromopyruvate (Schemes I and II) which is of more general utility for our studies.

The condensation of 2 with ethyl bromopyruvate in *N,N*-dimethylformamide containing anhydrous potassium carbonate proceeded smoothly to provide ethyl 2-[1-benzoyloxy)ethyl]thiazole-4-carboxylate (3) in nearly

Scheme I



Scheme II



quantitative yield. Transesterification of 3 with sodium methoxide in methanol gave a 67% yield of methyl 2-(1-hydroxyethyl)thiazole-4-carboxylate (4), which was oxidized to the ketone 5 in 86% yield with activated manganese dioxide in dichloromethane. This oxidation procedure was found superior in all respects to the reported procedure using chromium trioxide.[10] Bromination of 5 was effected in 80% yield using bromine in acetic acid[10] to give bromo ketone 6, together with some dibrominated material and some unchanged 5 as determined by NMR. 3-Benzamidothiopropionamide (7a) and 3-acetamidothiopropionamide (7b) were prepared from the respective nitriles and used in the condensation to form the bithiazole ring system. Both 7a and 7b reacted with 6 in about 70% yield. The use of 7b permitted immediate introduction of the desired acetyl group, and this procedure was adequate for the studies presented here. The use of 7a, while it required the addition of several steps, was felt to be of greater general utility in the synthesis of other possible derivatives of the dipeptide fragment.

The blocked ester 8a was hydrolyzed to the bithiazole acid 9 in 94% yield with 6 N HCl.[7] The amino group was

(5) Takita, T.; Muraoka, Y.; Maeda, K.; Umezawa, H. Proceedings of the 8th Symposium on Peptide Chemistry, Osaka, Japan, 1970, pp 179–183.
(6) Levin, M. D.; Subrahamanian, K.; Katz, H.; Smith, M. B.; Burlett, D. J.; Hecht, S. M. *J. Am. Chem. Soc.* **1980**, *102*, 1452–1453.
(7) McGowan, D. A.; Jordis, U.; Minster, D. K.; Hecht, S. M. *J. Am. Chem. Soc.* **1977**, *99*, 8078–8079.
(8) Zee-Cheng, K. Y.; Cheng, C. C. *J. Heterocycl. Chem.* **1970**, *78* 1439–1440.
(9) Olin, J. F.; Johnson, T. B. *Recl. Trav. Chim. Pays-Bas* **1931**, *50*, 72–76.
(10) Brookes, P.; Clark, R. J.; Fuller, A. T.; Mijovic, M. P. V., Walker, J. *J. Chem. Soc.* **1960**, 916–924.

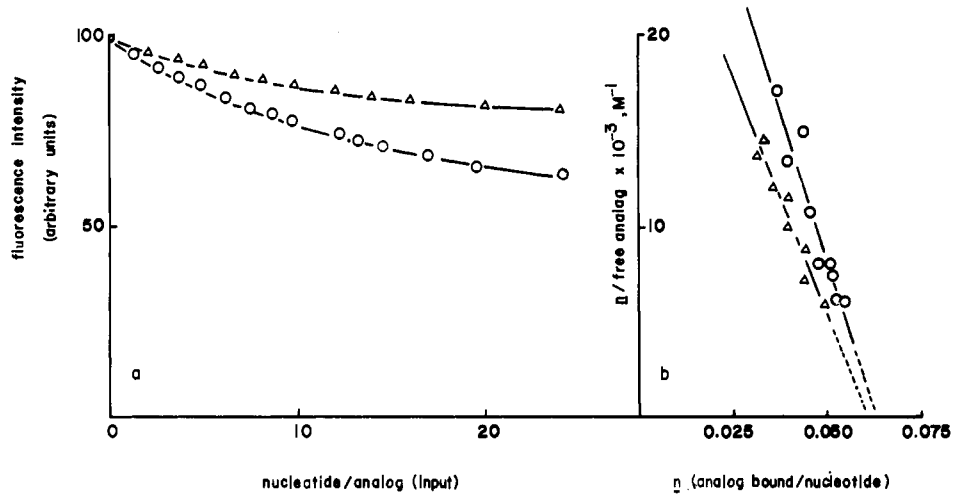


Figure 2. (a) Effect of DNA (○) and poly(dA-dT) (△) on the fluorescence intensity of the acetyl dipeptide (2×10^{-5} M). Conditons: 0.01 M sodium cacodylate, pH 7.0–0.1 mM EDTA, 25 °C; λ_{ex} = 300 nm (slit width 20 nm), λ_{em} = 354 nm (slit width 5 nm). (b) Scatchard plots of the data (a) for DNA (○) and poly(dA-dT) (△).

trifluoroacetylated with trifluoroacetic anhydride in pyridine to give **10**, which was converted to the acid chloride **11** by treatment with thionyl chloride. The acid chloride was reacted with an excess of 3-(methylthio)propylamine in dioxane to form **12** in 89% yield. Compound **12** was deblocked with ammonium hydroxide in methanol to **13**, which was acetylated to **14** using acetic anhydride in pyridine. Conversion of **8b** to **14** could also be achieved in one step by treating the ester with the neat amine at elevated temperature. Extractive workup provided the crystalline amide, identical with material prepared by the other route, in 93% yield.

Methylation of **14** with methyl iodide in methanol at 70 °C (sealed tube) gave the iodide salt of **1**. This material was hygroscopic and tended to discolor and was converted to the chloride salt by ion-exchange chromatography. The product was obtained as a stable amorphous solid after lyophilization.

Results and Discussion

Fluorescence Studies. The interaction of bleomycin A_2 and of tripeptide S with nucleic acids can be monitored by the decrease in fluorescence of the bithiazole system which occurs upon binding of the compounds.[2] A similar decrease was observed with the acetyl dipeptide using calf thymus DNA (Figure 2a). The fluorescence and ultraviolet absorption properties of **1** are essentially the same as those of bleomycin A_2. As found with the intact drug and tripeptide S, the decrease in fluorescence was very sensitive to the ionic strength of the medium, and high ionic strengths inhibited the loss of fluorescence. At low ionic strength (10 mM), a substantial (~40%) decrease was noted in the presence of DNA. Similar effects were noted with poly(dA-dT), except that the decrease in fluorescence was considerably less (~20%), even at low ionic strength.

By monitoring the decrease in fluorescence as a function of the ratio of nucleic acid–phosphate to acetyl dipeptide (Figure 2a) and analyzing the data as described by Chien et al.[2] (see Experimental Section), it was estimated that one molecule of **1** was bound per approximately eight base pairs (or 16 nucleotide residues) of DNA at an ionic strength of 10 mM (Figure 2b). The apparent binding constant is 7.5×10^5 M^{-1}. Similar results were obtained with poly(dA-dT), which indicated the same number of binding sites and an apparent binding constant of approximately 5×10^5 M^{-1}. As the ionic strength was raised, fewer molecules were bound and the value of the apparent binding constant decreased. These data are essentially in agreement with the observations made on the binding of bleomycin A_2 and tripeptide S to DNA monitored in the same manner.[2] Interestingly, Huang et al.[11] have reported that there appear to be two types of quenching of bithiazole fluorescence in the binding of bleomycins and the related talisomycins and their metal complexes to DNA: a sodium chloride sensitive process and one that is insensitive to sodium chloride. In addition, they noted that the addition of salt reduced the apparent binding constant while the stoichiometry was unchanged. The reason(s) for this discrepancy is unclear, although the 2 mM ionic strength used by these workers undoubtedly allowed the observation of many processes not seen at higher ionic strengths.

The effect of ionic strength on the binding of **1** to nucleic acids indicates that its interaction, as well as those of the intact antibiotic and tripeptide S, with DNA is at least partially electrostatic in nature. However, the effect of ionic strength on nucleic acid structure and how structural changes in DNA which occur in response to changes in ionic strength influence drug binding must also be considered. At least moderate (50–100 mM) ionic strengths are usually necessary for the maintenance of a double-helical structure, particularly with poly(dA-dT). Therefore, the fluorescence changes seen at low ionic strengths may reflect interaction of the drug (and its fragments) with a denatured, or partially denatured, form of the nucleic acid. Such interactions may involve stacking of the bithiazole rings with the bases of the partially open DNA helix. It is not possible on the basis of these data to determine whether this binding involves an intercalative interaction which results in elongation of the nucleic acid helix or whether the drug wedges in between the base pairs which occur at bends in a "kinked" DNA.[12]

NMR Studies. Proton NMR provides a convenient, nondestructive method of observing the interaction between **1** and DNA, and poly(dA-dT) serves as a model DNA whose NMR spectral features have been well-characterized. As mentioned previously, Chen et al.[4a] have recently reported results on the binding of intact bleomycin

(11) Huang, C.-H.; Galvan, L.; Crooke, S. T. *Biochemistry* **1980**, *19*, 1761–1767.
(12) See, for example, Patel, D. J. *Acc. Chem. Res.* **1979**, *12*, 118–125.

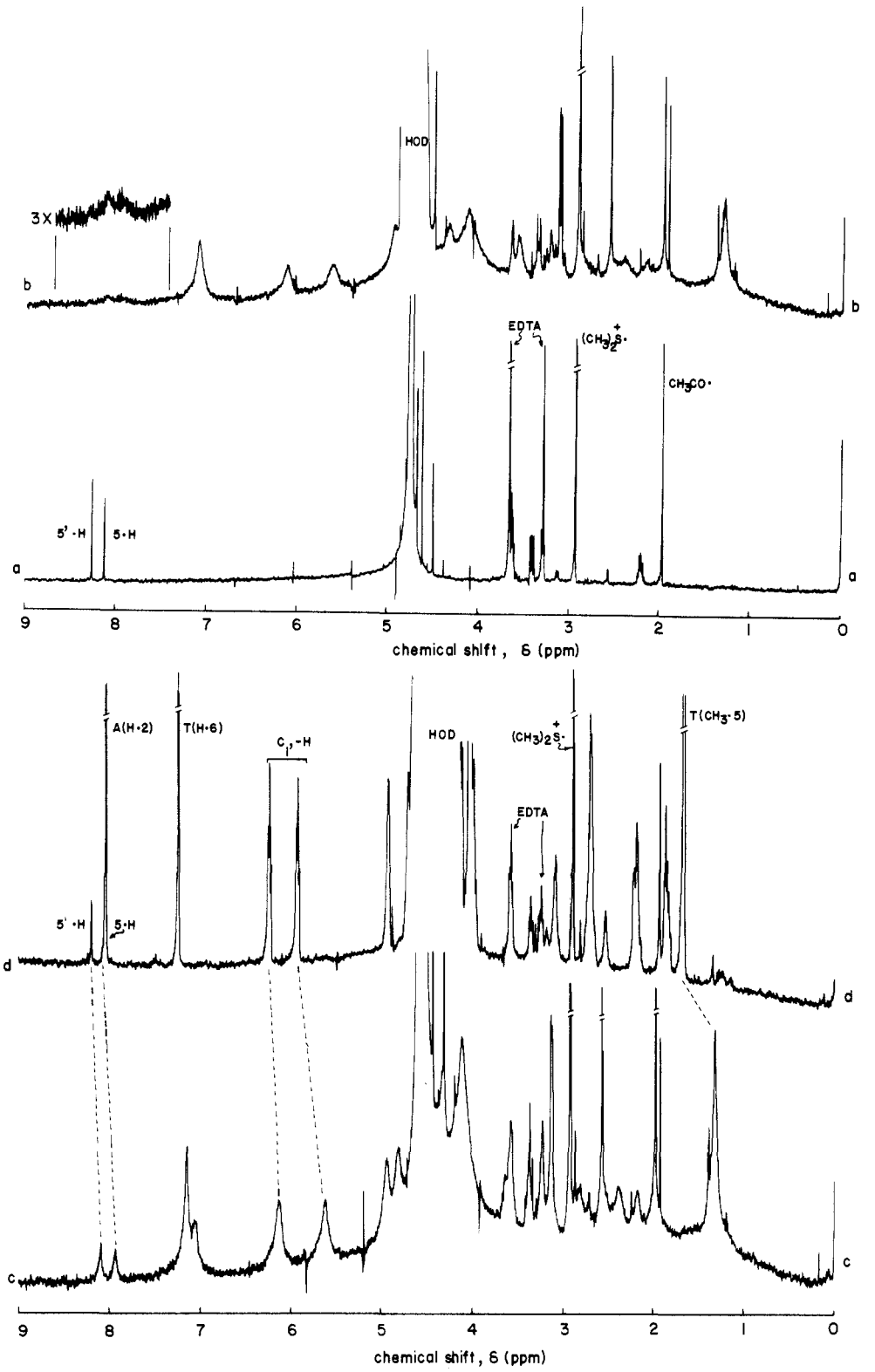


Figure 3. Proton NMR spectra of the acetyl dipeptide (1.2 mM) (a) free at 30 °C and in the presence of 10 mM poly(dA-dT) at (b) 30 °C, (c) 50 °C, and (d) 75 °C. The buffer is 0.1 M sodium phosphate–1 mM EDTA in D_2O, pH (meter reading) 6.8. The assignments of the resonances are noted in panel a for the acetyl dipeptide and in panel d for the denatured polynucleotide.

A_2 to poly(dA-dT) and those data provide the basis for comparison.

The interaction of 1 with poly(dA-dT), as in the case of bleomycin A_2, appears to be at least moderately fast on the NMR time scale, since only a single resonance is observed for each chemically distinct hydrogen of the acetyl dipeptide in the presence of limiting or excess concentrations of poly(dA-dT). As found with bleomycin A_2, the bithiazole aromatic proton resonances of 1 were the most sensitive to the interaction with poly(dA-dT). At 30 °C, with an excess of poly(dA-dT)-nucleotide residues (nucleotide/1 = 8.5) under which conditions all of the acetyl dipeptide is essentially bound, these resonances broadened to such an extent that determination of their chemical shifts was difficult (Figure 3b). As the temperature was raised to 50 °C, these resonances sharpened somewhat and tracked to higher field (Figure 3c). As the temperature was raised beyond the thermal denaturation temperature

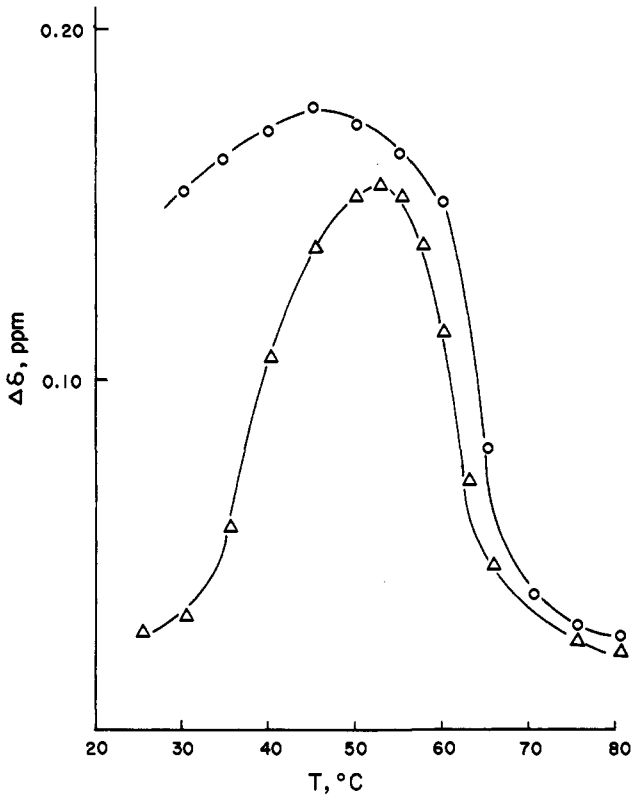


Figure 4. Effect of temperature of the chemical shift of the 5-H resonance of the acetyl dipeptide (1.2 mM) in the presence of 10 mM poly(dA-dT) (O). The data have been corrected for temperature effects on the free acetyl dipeptide. A positive value of $\Delta\delta$ denotes an upfield shift. The data for the analogous resonance of bleomycin A_2, plotted in the same manner, are shown for comparison (Δ).

of the poly(dA-dT) to 75 °C (T_m = 60 °C), the resonances of the poly(dA-dT) sharpened dramatically and the spectrum of the acetyl dipeptide in the presence of the nucleic acid was essentially that of the free acetyl dipeptide at the same temperature. This indicated that the analogue was dissociated from the denatured polynucleotide.

A maximum complexation shift of the order of 0.2 ppm was observed at a temperature of about 50 °C (Figure 4). Higher temperatures and, to some extent, lower temperatures, caused the resonances to track back downfield. This maximum complexation occurs some 10 °C below the T_m of poly(dA-dT); however, some "breathing" of the helix is believed to occur at these temperatures. At 50 °C, small complexation shifts (~0.05 ppm) were also observed for the protons next to the sulfonium group and for the methylene protons next to the acetamido function. The bithiazole resonances of the acetyl dipeptide did exhibit complexation shifts at temperatures below 50 °C which were larger than those observed with the intact drug at the same temperatures (Figure 4).

These data are in general agreement with the observations of Chen et al.[4a] The broadening of the resonances is indicative of immobilization of the bithiazole system due to binding of the molecule to the nucleic acid. The upfield shifts suggest that the protons on the rings are experiencing the ring current effects of the nucleic acid base pairs or bases.

The temperature data show that the interaction of both the intact drug and the acetyl dipeptide is maximal at a temperature at which the poly(dA-dT) structure is partially denatured or "loosened". The persistence of complexation shifts for the fragment at temperatures below 50 °C indicates that steric factors may be important in the binding process. The fragment, by virtue of its smaller size, encounters less hindrance to binding than does the intact drug molecule.

The relatively small magnitude of the complexation shifts argues against a full intercalation mechanism for the binding of the fragment, since such interactions are generally characterized by shifts of the order of 1 ppm.[13] However, uncertainty as to the magnitude of complexation shifts which might be encountered for a bithiazole system under various degrees of intercalation made it impossible to completely rule out a full intercalative mechanism, and Povirk et al.[14] have cited evidence for intercalation of the bithiazole rings of bleomycin.

The NMR experiments reported here were conducted at an ionic strength of 100 mM. Unlike the situation found with the fluorescence experiments, binding clearly occurs at the higher ionic strengths according to NMR measurements. Binding is enhanced (as indicated by an increase in complexation shifts) at lower ionic strengths, although the maximum shift appears to occur by an ionic strength of 50 mM (T. E. Booth and J. D. Glickson, unpublished results) and lower values of ionic strength do not markedly increase the shift. This difference between the fluorescence and NMR experiments may be due to the higher concentrations of drug and nucleic acid used in the NMR studies. Experiments are underway to try to understand the reason(s) for this difference.

Conclusion

The observation that bleomycin A_2 and the acetyl dipeptide behave similarly supports a bifunctional model for bleomycin A_2 in which the two parts of the bleomycin molecule act relatively independently. The sensitivity of the binding process to ionic strength and to temperature and the effects of these variables on the structure of the nucleic acids are consistent with the preferential interaction of bleomycin A_2 with a partially or locally denatured form of DNA. Such interactions may play a role in the biological activity of the bleomycins.

Experimental Section

Melting points were determined on a Laboratory Devices Mel-Temp apparatus and are uncorrected. Infrared spectra were obtained on a Perkin-Elmer Model 283 spectrophotometer. Ultraviolet spectral measurements were made on a Beckman Model 25 spectrophotometer and fluorescence experiments were performed on a Perkin-Elmer MPF-3 fluorimeter. Proton NMR measurements were made on either a Bruker HX-90 or WH-400 spectrometer. Chemical shifts are referenced to internal tetramethylsilane (organic solvents) or sodium 4,4-dimethyl-2,2,3,3-tetradeuterio-4-silapentanoate (aqueous solvents). Thin-layer chromatography was run on precoated silica gel F_{254} plates (0.25-mm thickness, Eastman Kodak, Rochester, NY). All analytical samples were found to be homogeneous upon chromatography using ethyl acetate, ethyl acetate–petroleum ether (bp 35–60 °C) (4:1, v/v) or chloroform–methanol (3:1, v/v) as solvent systems. Combustion analyses were performed by Altantic Microlabs, Atlanta, GA. All commercially obtained chemicals were reagent grade.

Calf thymus DNA (Sigma Chemical Co., St. Louis, MO) and poly(dA-dT) (Collaborative Research, Waltham, MA or P-L Biochemicals, Milwaukee WI) were purified by exhaustive dialysis against 0.1 M sodium phosphate, pH 7.0–1 mM EDTA. Concentrations of DNA and poly(dA-dT) were determined using nucleotide molar absorptivity values of 6.4×10^3 and 6.6×10^3 M^{-1} cm^{-1}, respectively. Acetyl dipeptide concentrations were

(13) Krugh, T. R.; Wittlin, F. N.; Cramer, S. P. *Biopolymers* **1975**, *14*, 197–210.

(14) Povirk, L. F.; Hogan, M.; Dattagupta, N. *Biochemistry* **1979**, *18*, 96–101.

measured using a molar absorptivity of 1.5×10^4 M^{-1} cm^{-1}.

For fluorescence quenching experiments, all solutions were made up in 10 mM sodium cacodylate, pH 7.0–0.1 mM EDTA. Titrations were carried out by the sequential addition of aliquots of a concentrated (6 mM) solution of poly(dA-dT) containing 2 $\times 10^{-5}$ M acetyl dipeptide to a solution of 2×10^{-5} M acetyl dipeptide. In this manner, the acetyl dipeptide remained constant and only the ratio of nucleotide to acetyl dipeptide changed. Fluorescence intensity measurements were made after each addition using an excitation wavelength of 300 nm (slit width 20 nm) and monitoring the emission at 354 nm (slit width 5 nm). The saturation curve obtained in this manner was analyzed by the method of Chien et al.[2] The fraction f of acetyl dipeptide bound at a given fluorescence reading F is given by $f = (F_0 - F)/(F_0 - F_b)$, where F_0 and F_b are the fluorescence intensities of the free (no nucleic acid) and bound (saturating nucleic acid) acetyl dipeptide, respectively. From the known input concentration of acetyl dipeptide, the bound and free concentrations of the fragment may be calculated. The nucleic acid concentration at any given point may be calculated knowing the initial volume of acetyl dipeptide solution and the amount and concentration of the poly(dA-dT) added. Analysis was accomplished using a Scatchard plot of n vs. $n/[\text{acetyl dipeptide}]_{free}$, where n is the concentration of bound acetyl dipeptide divided by the total poly(dA-dT) concentration at the point in question.

Proton NMR experiments involving poly(dA-dT) were performed on the Bruker WH-400 spectrometer operating in the pulse Fourier transform mode using 5 mm (o.d.) spinning sample tubes. Temperatures were determined using the separation of ethylene glycol resonances.[4a] Chemical shifts are referenced to internal sodium 4,4-dimethyl-2,2,3,3-tetradeuterio-4-silapentanoate. All experiments were carried out in deuterium oxide (99.8% d, Stohler Isotope Chemicals, Waltham, MA) containing 0.1 M sodium phosphate–1 mM EDTA, pH (meter reading) 6.8. All spectra were obtained after collecting 512 transients.

Ethyl 2-[1-(Benzoyloxy)ethyl]thiazole-4-carboxylate (3). This compound was prepared from *O*-benzoylthiolactamide[9] (**2**) and ethyl bromopyruvate by a modification of the procedure of Brookes et al.[10] *N,N*-Dimethylformamide replaced ethanol as the solvent, and anhydrous potassium carbonate was used as an acid scavenger instead of calcium carbonate. The product was obtained was an oil in nearly quantitative yield.

Methyl 2-(1-Hydroxyethyl)thiazole-4-carboxylate (4). A solution of **3** in absolute methanol was treated with 0.5 equiv of sodium methoxide. After standing at room temperature for 18 h, the solution was neutralized with Dowex 50X8 (H^+ form), and the resin was filtered and washed with methanol. The filtrate and washings were combined and evaporated to an oil from which methyl benzoate was removed by distillation (10–15 mmHg, bath temperature 75 °C). The residue crystallized on standing at 4 °C. The crude crystals were collected and washed with carbon tetrachloride. The filtrate and washings gave three additional crops for a total yield of 67% (19 g), mp 90–93.5 °C. Recrystallization from ethyl acetate gave an analytical sample: mp 92–93.5 °C (lit.[10] mp 89–92 °C); IR 3240 (OH), 1730 (ester C=O) cm^{-1}; NMR ($CDCl_3$) δ 8.12 (s, 1, H-5), 5.21 (q, 1, CH_3CH), 3.90 (s, 3, OCH_3), 1.64 (d, 3, CH_3CH).

Methyl 2-Acetylthiazole-4-carboxylate (5). A solution of **4** (23.2 g, 0.124 mol) in 600 mL of dichloromethane was refluxed for 3 h with 72 g of activated manganese dioxide prepared by the procedure of Morton and co-workers.[15] The mixture was filtered through a Celite pad and the cake was washed thoroughly with dichloromethane. The filtrate and washings were combined and evaporated to dryness in vacuo, giving 19.7 g (86%) of **5**: mp 104–105.5 °C (lit.[10] mp 78–80 °C). Recrystallization from ethyl acetate–hexane did not change the melting point: IR (Nujol) 1730 (ester C=O), 1700 (ketone C=O) cm^{-1}; NMR ($CDCl_3$) δ 8.43 (s, 1, H-5), 3.97 (s, 3, OCH_3), 2.77 (s, 3, CH_3CO).

Methyl 2-(2-Bromoacetyl)thiazole-4-carboxylate (6). A solution of **5** in acetic acid was treated with bromine as described by Brookes et al.[10] NMR spectra of the product showed it to be an 8:1:1 mixture of the mono-, di-, and unbrominated ketones. This mixture was satisfactory for use in subsequent reactions without further purification.

3-Benzamidothiopropionamide (7a). Reaction of benzamidopropionitrile[16] with hydrogen sulfide using the general procedure of Gilbert et al.[17] gave **7a** in 86% yield, mp 162–165 °C (lit.[18] mp 160–162 °C).

3-Acetamidothiopropionamide (7b). Using the same procedure described for **7a**, **7b** was prepared from 3-acetamidopropionitrile in 97% yield; recrystallization from acetone–petroleum ether (bp 35–60 °C) gave an analytical sample: mp 103–104.5 °C; IR (Nujol) 3300, 3260 (NH_2), 1630 (amide C=O), 1560 (amide II) cm^{-1}; NMR ($CDCl_3$) δ 7.61 (s, 1, NH_aH_b), 7.44 (s, 1, NH_aH_b), 6.20 (s, 1, NH), 3.66 (q, 2, NCH_2), 2.88 (t, 2, CH_2), 1.98 (s, 3, CH_3CO). Anal. ($C_5H_{10}N_2OS$) C, H, N.

Methyl 2′-(2-Benzamidoethyl)-2,4′-bithiazole-4-carboxylate (8a). A mixture of crude **6** (5.8 g, 22 mmol) and **7a** (4.57 g, 21.9 mmol) in 12 mL of freshly distilled *N,N*-dimethylformamide was heated at 70 °C for 1.5 h. Crystallization occurred upon standing at room temperature. The solid was filtered and washed with anhydrous ether and dried in vacuo, giving **8a** as a light yellow solid: yield 5.1 g (62%); mp 173.5–176 °C. A portion was recrystallized from ethanol to give an analytical sample: mp 179.5–180.5 °C; IR (Nujol) 3310 (NH), 1730 (ester C=O), 1645 (amide C=O), 1535 (amide II) cm^{-1}; NMR ($CDCl_3$) δ 8.21 (s, 1, H-5), 8.06 (s, 1, H-5′), 7.82 (m, 2, *o*-C_6H_6), 7.50 (m, 1, *p*-C_6H_6), 7.44 (m, 2, *m*-C_6H_6), 7.27 (s, 1, NH), 3.98 (s, 3, OCH_3), 3.95 (q, 2, NCH_2), 3.55 (t, 2, CH_2). Anal. ($C_{17}H_{15}N_3O_3S_2$) C, H, N.

Methyl 2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-carboxylate (8b). This was prepared in the same manner as described for **8a**, with the exception that the reaction was allowed to proceed for 3 h. After cooling, the solid product was filtered, washed with water, and dried in vacuo to give 4.1 g of **8b**, mp 173–175 °C. The filtrate was concentrated to yield a second crop (0.4 g): mp 170–173 °C; total yield 72%. Crystallization of a portion from ethyl acetate–petroleum ether (bp 35–60 °C) gave an analytical sample: mp 178–179 °C; IR (Nujol) 3410 (NH), 1730 (ester C=O), 1640 (amide C=O), 1545 (amide II) cm^{-1}; NMR ($CDCl_3$) δ 8.20 (s, 1, H-5), 8.06 (s, 1, H-5′), 6.23 (s, 1, NH), 3.98 (s, 3, OCH_3), 3.74 (q, 2, NCH_2), 3.24 (t, 2, CH_2), 2.01 (s, 3, CH_3CO). Anal. ($C_{12}H_{13}N_3O_3S_2$) C, H, N.

2′-(2-Aminoethyl)-2,4′-bithiazole-4-carboxylic Acid Hydrochloride (9). This was prepared by hydrolysis of **8a** as described by Zee-Cheng and Cheng[8] for the corresponding ethyl ester. The product was obtained as a white solid in 94% yield, mp 238–243 °C dec. Recrystallization from ethanol gave a white solid, mp 241–242 °C dec (lit.[8] mp 268–270 °C dec); IR (Nujol) 1700 (acid C=O), 1600 ($-NH_3^+$) cm^{-1}; NMR (D_2O) δ 8.34 (s, 1, H-5), 8.06 (s, 1, *H*-5′), 3.50 (m, 4, CH_2CH_2).

2′-[2-(Trifluoroacetamido)ethyl]-2,4′-bithiazole-4-carboxylic Acid (10). Pyridine (1 mL) was added to a suspension of **9** (1.7 g, 5.5 mmol) in 10 mL of trifluoroacetic anhydride, and the mixture was stirred at room temperature for 4 h. The resulting solution was poured into an ice–water mixture (100 mL) and the solid was filtered, washed with water, and dried in vacuo: yield 1.82 g (94%); mp 241.5–244.5 °C. Recrystallization from water gave an analytical sample, mp 245–246 °C; IR (Nujol) 3310 (NH), 1705, 1695 (amide C=O), 1680 (acid C=O), 1555 (amide II) cm^{-1}; NMR (Me_2SO-d_6) δ 9.60 (t, 1, NH), 8.48 (s, 1, H-5), 8.24 (s, 1, H-5′), 3.61 (m, 2, NCH_2), 3.30 (t, 2, CH_2). Anal. ($C_{11}H_8F_3N_4O_3S_2$) C, H, N.

Methyl 3-[2′-[2-(Trifluoroacetamido)ethyl]-2,4′-bithiazole-4-carboxamido]propyl Sulfide (12). A mixture of **10** (1.3 g, 3.7 mmol) and 15 mL of thionyl chloride was refluxed for 2 h. Removal of thionyl chloride in vacuo gave a quantitative yield of the crude acid chloride **11**, which was used without further purification. A suspension of **11** (1.15 g, 3.1 mmol) in 10 mL of anhydrous dioxane was treated with a solution of 3-(methylthio)propylamine (1.10 g, 10.4 mmol) in 5 mL of anhydrous dioxane, and the mixture was stirred at room temperature for 2 h. After this time, the solution was poured into 50 mL of water, and

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the resulting precipitate was filtered, washed with cold water, and dried in vacuo: yield 1.21 g (89%); mp 135–138 °C. Recrystallization from ethanol–water gave an analytical sample: mp 129–131 °C; IR (Nujol) 3300 (NH), 1710 (amide C=O), 1675 (amide C=O), 1650, 1540 (amide II) cm^{-1}; NMR ($CDCl_3$) δ 8.13 (s, 1, H-5), 7.91 (s, 1, H-5′), 7.86 (br s, 1, $NHCH_2CH_2CH_2$), 7.56 (br t, 1, $NHCH_2CH_2CH_2$), 3.88 (q, 2, $NHCH_2CH_2$), 3.59 (q, 2, $NHCH_2CH_2CH_2$), 3.30 (t, 2, $NHCH_2CH_2$), 2.61 (t, 2, $NHCH_2CH_2CH_2$), 2.13 (s, 3, CH_3S), 1.96 (p, 2, $CH_2CH_2CH_2$). Anal. ($C_{15}H_{17}F_3N_4O_2S_3$) C, H, N.

3-[2′-(2-Aminoethyl)-2,4′-bithiazole-4-carboxamido]propyl Methyl Sulfide Hydrochloride (13). To a solution of **12** (1.0 g, 2.3 mmol) in 25 mL of methanol was added 25 mL of concentrated ammonium hydroxide and the solution was allowed to stand for 18 h at room temperature. Removal of solvent in vacuo gave a residue which was extracted with ether (2 × 25 mL) and then taken to dryness. The residue (1 g) was recrystallized from ethanolic HCl to give an analytical sample (0.7 g, 85%): mp 209–210 °C; IR (Nujol) 3460 ($-NH_3^+$), 3315 (amide NH), 1640 (amide C=O), 1540 (amide II) cm^{-1}; NMR (D_2O) δ 8.14 (s, 1, H-5), 8.09 (s, 1, H-5′), 3.50 (m, 6, $NHCH_2CH_2$, $NHCH_2CH_2CH_2$), 2.63 (t, 2, $NHCH_2CH_2CH_2$), 2.13 (s, 3, CH_3S), 1.94 (p, 2, $CH_2CH_2CH_2$). Anal. ($C_{13}H_{19}ClN_4OS_3$) C, H, N.

3-[2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-carboxamido]propyl Methyl Sulfide (14). Method A. A solution of **13** (100 mg, 0.26 mmol) in 3 mL of acetic anhydride containing 0.5 mL of pyridine was stirred at room temperature for 18 h. The solvent was removed in vacuo and the residue was washed with 5 mL of water. The crude product was dried in vacuo and the residue was washed with 5 mL of water. The crude product was dried in vacuo, giving 93 mg (91%) of **14**, mp 132–133 °C. Recrystallization from ethanol gave an analytical sample: mp 135–137 °C; IR (Nujol) 3340, 3280 (NH), 1675, 1640 (amide C=O), 1545 (amide II) cm^{-1}; NMR ($CDCl_3$) δ 8.10 (s, 1, H-5), 7.87 (s, 1, H-5′), 7.53 (br s, 1, $NHCH_2CH_2CH_2$), 6.23 (br s, 1, $NHCH_2CH_2$), 3.75 (q, 2, $NHCH_2CH_2$), 3.59 (q, 2, $NHCH_2CH_2CH_2$), 3.25 (t, 2, $NHCH_2CH_2$), 2.61 (t, 2, $CH_2CH_2CH_2$), 2.13 (s, 3, CH_3S), 2.00 (s, 3, CH_3CO), 1.96 (m, 2, $CH_2CH_2CH_2$). Anal. ($C_{15}H_{20}N_4S_3O_2$) C, H, N.

Method B. A solution of **8b** (1.50 g, 4.82 mmol) in 4 mL of 3-(methylthio)propylamine was heated at 80 °C for 16 h. The reaction mixture was poured into 50 mL of water, and the resultant solid was filtered and dried in vacuo to give 1.72 g (93%) of crude **14**, mp 133–135 °C. Recrystallization from ethanol gave an analytical sample identical in all respects with material prepared by method A.

[3-[2′-(2-Acetamidoethyl)-2,4′-bithiazole-4-carboxamido]propyl]dimethylsulfonium Chloride (1). A solution of **14** (260 mg, 0.68 mmol) in 2 mL of methyl iodide containing 0.5 mL of methanol was heated in a sealed tube at 70 °C for 20 h. Evaporation of the solvent gave the iodide salt as a hygroscopic yellow solid. This was converted to the chloride salt by passage of an aqueous solution through a column (1 × 5 cm) of Dowex 1X8 (chloride form). Lyophilization of the eluate gave 290 mg (98%) of **1** in analytical pure form: NMR (D_2O) δ 7.94 (s, 1, H-5), 7.78 (s, 1, H-5′), 3.46 (t, 2, NCH_2CH_2), 3.41 (t, 2, $NCH_2CH_2CH_2$), 3.33 (t, 2, NCH_2CH_2), 3.04 (t, 2, CH_2S), 2.88 [s, 6, $S^+(CH_3)_2$], 2.09 (p, 2, $CH_2CH_2CH_2$), 1.88 (s, 3, CH_3CO). Anal. ($C_{16}H_{23}ClN_4O_2S_3 \cdot 2H_2O$) C, H, N.

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Esters of Isoguvacine as Potential Prodrugs

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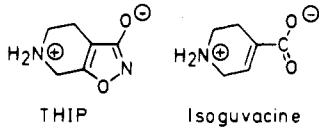
The syntheses of the methyl ester, butyl ester, (ethoxycarbonyl)methyl ester, and 11 (acyloxy)methyl esters of the potent γ-aminobutyric acid agonist isoguvacine (1,2,3,6-tetrahydropyridine-4-carboxylic acid) are described. The chemical stability of the esters and their in vitro rates of hydrolysis under approximately physiological conditions by nonspecific esterases from human serum were examined. A selected number of the esters were tested for antagonism of convulsions induced by bicuculline, isoniazide, and by electroschock. While the compounds showed only weak activities in the bicuculline and isoniazide tests, a good correlation between in vitro rates of enzymatic hydrolysis and the time of onset of the antagonism of the electroshock-induced convulsions could be found.

Decreased functions of the central γAbu (γ-aminobutyric acid) neutrotransmitter system contribute to the pathogenesis of certain psychiatric and neurological disorders.[1,2] Consequently, γAbu stimulation or replacement therapies may be relevant for the treatment of these diseases,[3] the γAbu-metabolizing enzyme (γAbu-T),[4] the γAbu uptake systems,[5,6] and, in particular, the γAbu receptors[7] being of primary interest as pharmacological sites of attack.

A variety of γAbu analogues with γAbu agonist activity have been developed.[8,9] Among these compounds, the potent γAbu agonists THIP (4,5,6,7-tetrahydroisoxazolo-



THIP Isoguvacine

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