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MOLECULAR MECHANISM OF BINDING OF PYRROLO(1,4)BENZODIAZEPINE ANTITUMOUR AGENTS TO DEOXYRIBONUCLEIC ACID—

ANTHRAMYCIN AND TOMAYMYCIN*

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Abstract—The synthesis of 3,3-dimethyl-4-oxo-3,4-dihydroquinoline (**16**), 3,3-dimethyl-4-oxo-2-methoxy-1,2,3,4-tetrahydroquinoline (**17**), and of 11*a*-*S*-pyrrolo(1,4)benzodiazepine (**21**) as models to study the mechanism of action of the pyrrolo(1,4)benzodiazepine antitumour antibiotics is described. Both **16** and **21** readily add nucleophiles to the imine bond but only **21**, like the parent antibiotics, readily produces covalent attachment to DNA. The extent of binding of the pyrrolo(1,4)benzodiazepine antibiotics to DNA, measured by suppression of ethidium fluorescence, is proportional to the antibiotic concentration and is partly reversed by a heat-denaturation–renaturation cycle. The extent of binding of the pyrrolo(1,4)benzodiazepines to DNA is also promoted by lower pH (range 4.7 to 9) and higher temperatures (range 0–51°), and the DNA–antibiotic complex is stable to dialysis. There is no evidence that these antibiotics intercalate into DNA, assayed by calf thymus topoisomerase, but they are more reactive toward relaxed PM2-DNA than to supercoiled DNA. Examination of DNA binding of the antitumour antibiotics and their analogues to DNAs of different base composition and separately in conjunction with sequence specific binding agents showed little base preference for the binding. Reaction of the pyrrolo(1,4)benzodiazepines with DNA produces neither depurination, assayed with endonuclease VI, nor strand scission. A free or potential carbinolamine or imine function at the 10, 11 positions in a benzo(1,4)diazepine nucleus is an absolute requirement for DNA binding or for reaction with nucleophiles. These results with the native antibiotics and their analogues, in particular the *N*-acetyl compound **7** favor a molecular mechanism of action by acid-promoted addition of biological nucleophiles to the 10, 11 conjugated imine closely analogous to that proposed for the antitumour agent maytansine.

Anthramycin and tomaymycin belong to the pyrrolo(1,4)benzodiazepine class of antitumor agents. They have been isolated from *Streptomyces refuineus* var. *thermotolerans* [1, 3] and *Streptomyces achromogenes* var. *tomaymyceticus* [4, 5] respectively. Anthramycin was shown to have antibiotic, antitumor, antiprotozoal and chemosterilant activity against houseflies. Tomaymycin has been shown to have antitumor, antiviral and antibiotic activities. Other known members of this class of antibiotics are sibiromycin [6–8] and neothramycins A and B [9, 10]. Another antibiotic, dextrochrisin of unknown structure has also been included in this group [11]. The total synthesis of anthramycin [12] and neothramycins [11] has been reported. The structures of these antibiotics are shown in Fig. 1.

A structural feature common to all these antibiotics is the pyrrolo(1,4)benzodiazepine nucleus. They all contain a carbinolamine function at position 10, 11 ("imine" in the case of neothramycins), a phenolic hydroxy group and an unsaturated side chain on the pyrrole ring. The pyrrole ring can have various degrees of unsaturation. Thus, although these antibiotics lack any of the structural features responsible for tight binding to DNA, they react specifically with it to form nearly irreversible complexes. Several attempts have been made to establish the molecular mechanism of this binding between DNA and anthramycin [13–22], tomaymycin [21–23] and sibiromycin [24–28], and also to correlate the biological activity of anthramycin and several analogues and derivatives with their structures [29, 30]. In spite of these studies, the exact nature of this binding and the functional group requirements of these antibiotics for reaction with DNA remained speculative. We decided to examine the reactions of anthramycin, tomaymycin and other structurally related compounds with different DNAs by the ethidium fluorescence assay [31, 32] with a view to elucidating the molecular mechanism of binding of pyrrolo(1,4)benzodiazepine antibiotics with DNA and to establish the functional group requirements of these compounds for reaction with DNA. Recently, these techniques have been used to examine the interaction of certain antitumor antibiotics with nucleic acids [33–37].

* Studies related to antitumour antibiotics, Part XVI.

† Anthramycin and tomaymycin are most commonly supplied as the 11-methyl ethers, in which form they are more stable. The terms anthramycin and tomaymycin will be used in this paper to designate the methyl ethers.

MATERIALS AND METHODS

Anthramycin† was kindly supplied by Dr. Harry B. Wood of the National Cancer Institute, Bethesda, MD.

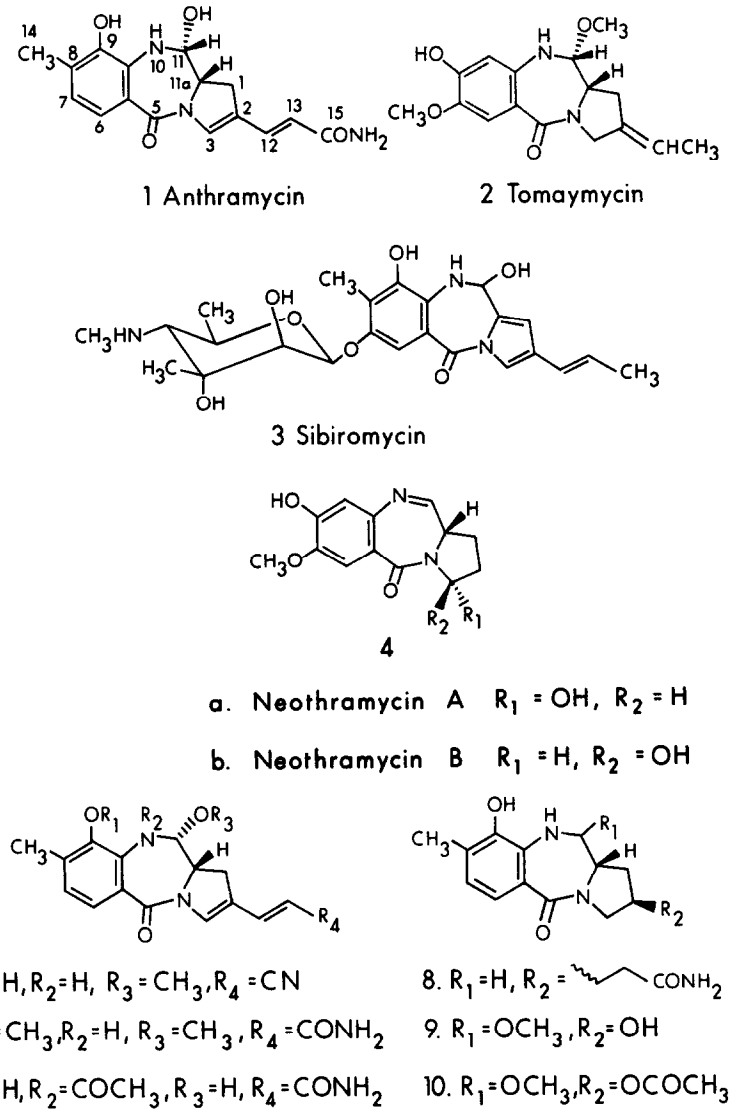

Fig. 1. Structures of the pyrrolo(1,4)benzodiazepine antitumor antibiotics, derivatives and analogues.

Anthramycin and several analogues were also kindly supplied by Hoffmann-La Roche Inc., Nutley, NJ. Tomaymycin* was a gift from Research Laboratories, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan. Other compounds were prepared as described below. Solutions of the compounds were prepared in dimethylsulfoxide and stored at 0°. Ethidium bromide was purchased from Sigma Chemicals, St. Louis, MO. The λ, PM2-CCC and calf thymus DNAs were obtained as described previously [32]. *Escherichia coli* and *Clostridium perfringens* DNAs were purchased from Sigma. The calf thymus topoisomerase was prepared according to the method of Herrick and Alberts [38]. Endonuclease VI was purified according to the method of Verly and Rassart [39] from *E. coli* BATCC 11303.

Melting points were determined on a Fischer–Johns apparatus and are uncorrected. The i.r. spectra were recorded on a Nicolet 7199 F.T. spectrophotometer, and only the principal sharply defined peaks are reported. The n.m.r. spectra were recorded on Varian A-60 and A-100 analytical spectrometers. The spectra were measured on approximately 10–15% (w/v) solutions in appropriate deuterated solvents with tetramethylsilane as standard. Line positions are reported in parts per million from the reference. Absorption spectra were recorded with a Beckman model DB spectrophotometer. Mass spectra were determined on an Associated Electrical Industries MS-9 double focusing high resolution mass spectrometer. The ionization energy in general was 70 eV. Peak measurements were made by comparison with perfluorotributylamine at a resolving power of 15,000. Kieselgel DF-5 (Camag, Switzerland) and Eastman Kodak precoated sheets were used for thin-layer chromatography. Preparative layer chromatography was performed with 20 × 20 cm, 1 mm thick plates coated with silica gel (E.M. Reagents, Germany). Microanalyses were carried out by Mrs. D. Mahlow of this department.

2-(o-Nitrobenzoyl)isobutyraldehyde (**11**). This compound was prepared by a procedure similar to that reported by Inukai and Yoshizawa [40]. To a stirred solution of 11.13 g (0.06 mole) *o*-nitrobenzoyl chlo-

* See footnote † on p. 2017.

ride in 50 ml benzene [prepared by refluxing 10 g (0.06 mole) *o*-nitrobenzoic acid in 50 ml anhydrous benzene with 7.85 g (0.066 mole) thionyl chloride for 2 hr at room temperature] was added dropwise a solution of 8.46 g (0.06 mole) of 1-morpholinoisobutene [41] in 20 ml of dry benzene, and the mixture was stirred at room temperature for 24 hr. The resulting precipitate was collected and stirred with 50 ml water at room temperature for 2 hr. The mixture was extracted with dichloromethane (3 × 50 ml). The combined extracts were dried (Na_2SO_4) and the solvent was removed *in vacuo*. Chromatography of the resulting oil on florisil and elution with benzene gave 6.00 g (45%) of 2-(*o*-nitrobenzoyl)isobutyraldehyde (**11**). An analytical sample was obtained by distillation under reduced pressure. B.p. 134–136°/0.1 mm. Anal. Calc. for $C_{11}H_{11}NO_4$: C. 59.70; H, 4.98; N, 6.34. Found: C. 59.69; H, 5.09; N, 6.34. P.m.r. [$CDCl_3$]: δ 1.41 (s, 6H, 2 × —C<u>H</u>$_3$); 7.12–8.22 (m, 4H, aromatic protons); 9.60 (s, 1H, —C<u>H</u>O). I.r. ν_{max} (film): 1735 (—CHO); 1696 (>C=O); 1530, 1347 (—NO_2).

Zinc and ammonium chloride reduction of 2-(o-nitrobenzoyl)isobutyraldehyde—nitrone dimer, **14**. A mixture of 1.105 g (5 m-moles) of 2-*o*-nitrobenzoyl)isobutyraldehyde, 300 mg of ammonium chloride, 650 mg of zinc powder, 25 ml of water and 25 ml of methanol was stirred at room temperature for 1 hr. The mixture was filtered and the residue was washed several times with methanol. The combined filtrates were evaporated to dryness *in vacuo*. The residue was extracted with chloroform and filtered again. Removal of the solvent from the filtrate *in vacuo* and crystallization from benzene gave 619 mg (65.5%) of the nitrone dimer, **14**, as colorless crystals. M.p. 239°. Anal. Calc. for $C_{22}H_{22}N_2O_4$ (378.1579): C, 69.84; H, 5.82; N, 7.41. Found: (378.1586, mass spectrum): C, 69.97; H, 5.85; N, 7.41. P.m.r. [$CDCl_3$]: δ 1.33, 1.45 (2s, 12H, 4x —C<u>H</u>$_3$) 5.45 (s, 2H, 2x —C<u>H</u>—N/O); 7.03–8.13 (m, 8H, aromatic protons). I.r. ν_{max} (film): 1682 (>C=O).

Catalytic hydrogenation of nitrone dimer, **14**. Two hundred and twenty seven mg (0.6 m-mole) of nitrone dimer, **14**, in 20 ml of methanol was hydrogenated over 200 mg of 5% Pd-$BaCO_3$ at room temperature and atmospheric pressure overnight. Filtration and evaporation gave a residue, which was chromatographed on florisil. Elution with benzene containing 2% methanol gave: (a) 55 mg (26%) of 3,3-dimethyl-4-oxo-1,2,3,4-tetrahydroquinoline (**15**). M.p. 54.5–55° (benzene-hexane). Anal. Calc. for $C_{11}H_{13}NO$ (175.0997): C, 75.40; H, 7.43; N, 8.00. Found (175.1001, mass spectrum): C, 75.12; H, 7.37; N, 7.98. P.m.r. [$CDCl_3$]: δ 1.15 (s, 6H, 2x —C<u>H</u>$_3$); 3.2 (s, 2H, N—C<u>H</u>$_2$—); 6.50–7.87 (m, 4H, aromatic protons). I.r. ν_{max} (film): 3445, 3385 (—NH); 1670 (>C=O). (b) 82 mg (33.5%) of 3,3-dimethyl-4-oxo-2-methoxy-1,2,3,4-tetrahydroquinoline (**17**), containing a small amount of the imine, **16**. Trituration with benzene-hexane resulted in the separation of the methoxy compound as colorless crystals. M.p. 90–107° (dec). Anal. Calc. for $C_{12}H_{15}NO_2$ (205.1103): C, 70.21; H, 7.32; N, 6.83. Found (205.1101, mass spectrum): C, 69.66; H, 7.27; N, 6.72. P.m.r. [$CDCl_3$]: δ 1.22, 1.30 (2s, 6H, 2x —C<u>H</u>$_3$); 3.28 (s, 3H, —OC<u>H</u>$_3$); 4.20, 4.24 (2s, 1H, —C<u>H</u>—N/O); 5.22 bs, 1H, N<u>H</u>); 6.60–7.90 (m, 4H, aromatic protons). I.r. ν_{max} (film): 3326 (NH); 1653 (>C=O). Ultraviolet λ_{max} · (H_2O): 368 (ε = 3,927); 259 (ε = 6,647); 232 (ε = 19,940).

3,3-Dimethyl-4-oxo-3,4-dihydroquinoline. P.m.r. [$CDCl_3$]: δ 1.36 (s, 6H, 2x —C<u>H</u>$_3$); 7.96 (s, 1H, —C<u>H</u>=N). Anal Calc. for $C_{11}H_{11}NO$ (173.0840). Found (173.0839, mass spectrum).

*Dehydrogenation of 3,3-dimethyl-4-oxo-1,2,3,4-tetrahydroquinoline with DDQ.** A mixture of 16 mg (0.09 m-mole) tetrahydroquinoline (**15**), 22.7 mg (0.11 m-mole) DDQ and 3 ml anhydrous benzene was stirred at room temperature overnight under nitrogen. Examination of the reaction mixture by t.l.c. showed complete disappearance of starting material. Chromatography on florisil and elution with benzene–methanol (99: 1) gave 14.9 mg of product which was a mixture of compound **17** and the imine, **16**, in approximately 3: 1 by n.m.r.

Reaction of 3,3-dimethyl-4-oxo-2-methoxy-1,2,3,4-tetrahydroquinoline with thiophenol. A solution of 8 mg (0.039 m-mole) of **17**, 4.3 mg (0.039 m-mole) of thiophenol and a catalytic amount of toluenesulfonic acid in 2 ml of dry dichloromethane was stirred at room temperature overnight. Removal of the solvent *in vacuo* gave compound **18**. P.m.r. [$CDCl_3$]: δ 1.27 (s, 6H, 2x —C<u>H</u>$_3$); 3.50 (bs, 1H, <u>NH</u>); 4.83 (s, 1H, —C<u>H</u>—S—); 6.33–7.97 (m, 9H, aromatic protons).

N-(o-*Nitrobenzoyl*)-L-*2-pyrrolidinemethanol* (**19**). To a stirred solution of 1.011 g (0.01 mole) of L-2-pyrrolidinemethanol and 3 ml of triethylamine in 30 ml of dry dichloromethane at room temperature was added dropwise a solution of 1.855 g (0.01 mole) of *o*-nitrobenzoyl chloride in 20 ml of benzene and then stirred at room temperature for 1.5 hr. The precipitated triethylamine hydrochloride was removed by filtration, and the filtrate was concentrated *in vacuo*. Chromatography of the residual oil on florisil and elution with chloroform containing 3% methanol gave 2.5 g (~100%) of *N*-(*o*-nitrobenzoyl)-L-2-pyrrolidinemethanol (**19**). M.p. 90–92° (benzene-hexane). Anal. Calc. for $C_{12}H_{14}N_2O_4$: C, 57.60; H, 5.60; N, 11.2. Found: C, 57.07; H, 5.57; N, 11.17. P.m.r. [$CDCl_3$]: δ 1.70–2.32 (m, 4H, 2x —C<u>H</u>$_2$—); 3.16–3.34 (m, 2H, N—C<u>H</u>$_2$—); 2.80–3.90 (m, 2H, —C<u>H</u>$_2$—OH); 4.30–4.60 (m, 2H, 1H exchangeable, —<u>OH</u>, N—C<u>H</u>—); 7.32–8.24 (m, 4H, aromatic protons). I.r. ν_{max} (film): 3400 (—OH); 1640 (N—C(=O)—); 1540, 1350 (—NO_2). $[\alpha]_D^{22}$ = −154.7 (C = 1.05, chloroform).

N-(o-*Nitrobenzoyl*)-L-*2-pyrrolidinecarboxaldehyde* (**20**). To a stirred suspension of 324 mg (1.5 m-mole) of pyridinium chlorochromate and 100 mg of anhydrous sodium acetate in 20 ml of dry dichloromethane was added dropwise a solution of 250 mg (1 m-mole) of compound **19**, in 2 ml of dichloromethane, and the mixture was stirred at room temperature overnight. The supernatant liquid was removed by decantation and the residue was extracted several times with dichloromethane. The combined extracts were concentrated *in vacuo*. Chromatography of the residual oil on florisil

* DDQ = 2,3,Dichloro-5,6,dicyano-1,4-benzoquinone.

and elution with chloroform–methanol (49:1) gave 104 mg (42%) of the title compound as an oil. P.m.r. [$CDCl_3$]: δ 1.70–2.5 (m, 4H, 2x —$\underline{CH_2}$—); 3.05–3.50 (m, 2H, N—$\underline{CH_2}$—); 4.30–4.80 (m, 1H, N—$\underline{CH}$—); 7.20–8.30 (m, 4H, aromatic protons); 9.62 (s, 1H, $\underline{CHO}$). I.r. ν_{max} (film); 1732 (—CHO); 1640 ($\rangle$N—C—); 1530, 1348 (—NO_2). $[\alpha]_D^{22}$: −114.9 (c = 0.067, chloroform).

Catalytic hydrogenation of N-(o-*nitrobenzoyl*)-L-*2-pyrrolidine carboxaldehyde over Pd-C. Pyrrolo(1,4)benzodiazepine* **22**. Eighty mg (0.32 m-mole) nitroaldehyde, **20**, in 10 ml methanol was hydrogenated at room temperature and atmospheric pressure over 20 mg of 5% Pd-C overnight. Filtration and evaporation gave a colorless foam. Preparative layer chromatography on silica gel (benzene–methanol = 19:1) and crystallization from benzene gave 39 mg (60%) of compound **22**. M.p. 173–175°. Anal. Calc. for $C_{12}H_{14}N_2O$ (202.1106): C, 71.29; H, 6.93; N, 13.87. Found (202.1101, mass spectrum): C, 71.24; H, 7.02; N, 13.92. P.m.r. [$CDCl_3$]: δ 1.52–2.36 (m, 4H, 2x —$\underline{CH_2}$—); 3.20–4.02 (m, 5H, 2x —N—$\underline{CH_2}$—, N—$\underline{CH}$—); 4.32 (bs, 1H, NH); 6.50–8.28 (m, 4H, aromatic protons). I.r. ν_{max} (film): 3320 (—NH); 1617, 1593 (N—$\overset{O}{\overset{\|}{C}}$—). $[\alpha]_D^{22} = +311.6$ (c = 0.35, chloroform).

Catalytic hydrogenation of N-(o-*nitrobenzoyl*)-L-*2-pyrrolidine carboxaldehyde over Pd-BaCO₃. Pyrrolo(1,4)benzodiazepine* **21**. Hydrogenation of 90 mg (0.36 m-mole) of **20** in 10 ml of methanol at room temperature and atmospheric pressure over 40 mg of 5% Pd-$BaCO_3$ for 17 hr and work-up by preparative layer chromatography on silica gel (benzene–methanol, 19:1) gave 6 mg (8.5%) of **21** as a colorless hygroscopic foam. Anal. Calc. for $C_{12}H_{12}N_2O$ (200.0949). Found (200.0947, mass spectrum). P.m.r. [$CDCl_3$]: δ 1.90–2.50 (m, 4H, 2x —$\underline{CH_2}$—); 3.40–4.0 (m, 3H, N—$\underline{CH_2}$—, N—$\underline{CH}$—); 7.77 (d, 1H, J = 4.5 Hz, —N=$\underline{CH}$—); 7.20–8.10 (m, 4H, aromatic protons). I.r. ν_{max} (film): 1630 (N—$\overset{O}{\overset{\|}{C}}$—). Ultraviolet λ_{max} (water): 298 (ε = 1,860); 217 (13,660). $[\alpha]_D^{22} = +319$ (c = 0.147, chloroform).

Catalytic hydrogenation of **21** *on Pd-C.* A solution of 4.4 mg of **21** in 2 ml of methanol was hydrogenated at room temperature and atmospheric pressure over 3 mg of 5% Pd-C overnight. Work-up gave 4.3 mg of product, which was identical to **22** by t.l.c. and i.r. spectrum.

Reaction of pyrrolo(1,4)benzodiazepine **21** *with thiophenol.* A solution of 8.2 mg (0.041 m-mole) of **21**, 4.5 mg (0.041 m-mole) of thiophenol and a catalytic amount of toluenesulfonic acid in 2 ml of dry dichloromethane was stirred at room temperature overnight. Removal of the solvent *in vacuo* gave compound **23**. P.m.r. [$CDCl_3$]: δ 1.8–2.45 (m, 4H, 2x —$\underline{CH_2}$—); 3.5–3.9 (m, 3H, N—$\underline{CH_2}$, N—$\underline{CH}$—); 4.83 (d, 1H, J = 11 Hz, —$\underline{CH}$—S—); 6.5–8.0 (m, 9H, aromatic protons).

Fluorescence determination of reaction of PM2-CCC-DNA with anthramycin, tomaymycin and analogues and pH and temperature dependence. All measurements were performed on a G. K. Turner and Associates model 430 spectrofluorometer equipped with a cooling fan to reduce fluctuations in the xenon lamp source. Wavelength-calibration was performed as described in the manual for the instrument. One-centimeter-square cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength was 600 nm. The 30 × and 100 × scale of medium sensitivity was generally used, and water was circulated between the cell compartment and a thermally regulated bath at 22°.

The reaction mixtures were buffered to the appropriate pH with potassium phosphate, pH 4.7, 5.8, 7.2, 8 and 9. The reactions were carried out in a total volume of 100 μl at the appropriate temperature (0, 24, 37 and 51°). The reaction solution contained approximately 1.60 A_{260} units of PM2-CCC-DNA (87% CCC, 13% OC), 50 mM buffer, e.g. 0.5 mM anthramycin and 10% (v/v) dimethylsulfoxide. At intervals 10-μl aliquots were withdrawn and added to 2 ml of assay solution which contained 20 mM potassium phosphate, pH 11.8. 0.2 mM EDTA and 0.5 μg/ml of ethidium bromide [31]. The fluorescence was measured using a blank without added sample. The solution was then heat denatured at 96° on a Temp Blok for 4 min and cooled rapidly in an ice-bath and then in a thermostated water-bath at 22° for 5 min and the fluorescence was read again. The percentage decrease in fluorescence of the sample to that of the control was taken as a measure of the extent of reaction. In a control experiment it was shown that none of the components interfered with the ethidium fluorescence.

Reactions with other DNAs were also carried out in a similar fashion. Anthramycin and tomaymycin were also tested for evidence of covalent cross-linking of λ-DNA.

Fluorescence assay for detecting intercalation by anthramycin into DNA base pairs. The reaction was carried out on a 110 μl scale. The reaction mixture contained 10 mM Tris–hydrochloride buffer, pH 8, 1 mM EDTA, 0.2 M sodium chloride, approximately 1.60 A_{260} units of PM2-CCC-DNA (87% CCC, 13% OC), 0.5 mM anthramycin and 5% (v/v) dimethylsulfoxide. The control did not contain any anthramycin. Both the sample and control were incubated at 37° for 3 hr, at which point an assay indicated a 39.5 per cent decrease in ethidium fluorescence. Five-μl aliquots of topoisomerase were added both to the sample and control and incubated at 37°. Ten-μl aliquots were withdrawn at intervals and assayed as before. For both the sample and the control, the fluorescence decreased by 30–35 per cent. The basis of the assay is that intercalation into DNA base-pairs is accompanied by relaxation of supercoiled PM2-CCC-DNA to a non-supercoiled form. Topoisomerase also relaxes supercoiled PM2-CCC-DNA by a nicking-closing mechanism which produces a 30 per cent decrease in ethidium fluorescence because of differences in topological constraints of the two forms of DNA.

Assay for inhibition of reaction between anthramycin and DNA by sequence specific reagents. Netropsin, distamycin, chromomycin A_3 and olivomycin. The reactions were performed on a 100 μl scale. The reaction mixtures contained 50 mM phosphate buffer, pH 5.8, 1.60 A_{260} units of PM2-CCC-DNA (87% CCC, 13%

OC), 1 mM magnesium chloride for chromomycin A_3 and olivomycin and the appropriate concentrations of netropsin, distamycin, chromomycin A_3 and olivomycin. The mixtures were incubated at 37° for 10 min. To one cuvette, 10 μl of a 5 mM solution of anthramycin in dimethylsulfoxide was added and to the other 10 μl of dimethylsulfoxide. The solutions were incubated at 37° and assayed at intervals as described before.

Effect of intercalated ethidium on the reaction of anthramycin with PM2-CCC-DNA. The reactions were carried out on a 100 μl scale. The reaction mixtures contained 0.05 M potassium phosphate buffer, pH 5.8, 1.60 A_{260} units of PM2-CCC-DNA (87% CCC, 13% OC), 30 μg/ml of ethidium bromide and 0.5 mM anthramycin. The control did not contain anthramycin. The solutions were prepared by allowing the ethidium bromide to equilibrate with the buffered DNA for 5–10 min before the addition of the antibiotic. Solutions were stoppered and protected from light to prevent possible cleavage of the DNA by ethidium [42]. Ten-μl aliquots were withdrawn at intervals and analyzed by the pH 11.8 ethidium fluorescence assay.

Endonuclease VI assay for possible depurination of PM2-CCC-DNA on reaction with anthramycin. Reaction was carried out on a 100 μl scale. PM2-CCC-DNA (87% CCC, 13% OC) at a final concentration of 1.60 A_{260} units in 50 mM potassium phosphate buffer, pH 7.0, was incubated at 37° for 3 hr with 10 μl of 10 mM anthramycin in dimethylsulfoxide. The pH 8 ethidium fluorescence assay showed a 53 per cent drop in fluorescence compared to the control. A 10-μl aliquot of endonuclease VI solution was then added and incubated at 37°. Ten-μl aliquots were withdrawn at intervals and analyzed by the pH 8 ethidium fluorescence assay.

RESULTS

The reaction of anthramycin with DNA results in a decrease in ethidium fluorescence. At the concentrations used, none of the compounds in these studies either quenches or enhances the ethidium fluorescence before or after alkaline heat-denaturation. The behaviour of tomaymycin and other reactive analogues was similar. However, unlike other alkylating agents, alkaline denaturation of PM2-CCC-DNA reacted with pyrrolo(1,4)benzodiazepine compounds did not result in strand scission to any significant extent. Instead, some of the bound drug molecules dissociated from the DNA resulting in a slight enhancement of ethidium fluorescence. This behaviour was more pronounced at lower pH values than at pH 11.8. Therefore, the before-heat-denaturation decrease in ethidium fluorescence was taken as an indication of reaction with DNA, and the extent of drop in fluorescence was taken as a measure of the extent of reaction.

Reaction of anthramycin with PM2-CCC-DNA (87% CCC, 13% OC) at pH 5.8 at a concentration of 0.5 mM results in a 63 per cent drop in ethidium fluorescence in 90 min. On heating at 96° for 4 min at pH 11.8, the fluorescence increases from the before-heat value (63→48.5 per cent). Thus, approximately 23 per cent of bound anthramycin dissociates from the DNA on heating. Adjusting the pH of this solution to 7.0 and again heating at 96° for 4 min results in a further increase in the fluorescence so that the decrease

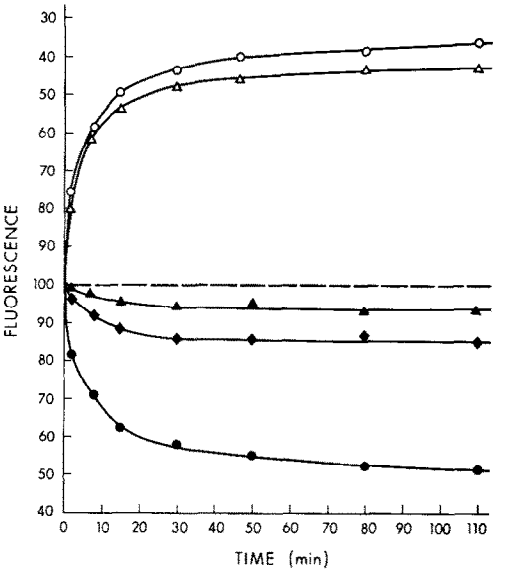

Fig. 2. Alkylation of PM2-CCC-DNA by anthramycin and the effect of pH on the DNA–drug complex. Reactions were performed at 37° in 50 mM potassium phosphate buffer, pH 5.8, and contained 1.60 A_{260} units/ml of PM2-CCC-DNA (87% CCC) and 0.5 mM anthramycin. The symbols (○), (●) and (◆) represent, respectively, before-heat fluorescence (pH 11.8), after-heat fluorescence (pH 11.8), and after-heat fluorescence after adjusting the pH to 7.0 from 11.8 and heating at 96° for 4 min. Symbols (△) and (▲) represent before- and after-heat denaturation fluorescence readings assayed at pH 7.2 respectively; (– – – –) control.

in fluorescence from that of the control is only 15 per cent. Similarly, assay at pH 7.2 shows a 57.5 per cent decrease in fluorescence, which on denaturation by heating at 96° for 4 min changes to approximately 6 per cent (Fig. 2).

The rate of reaction of anthramycin (and other compounds examined in these studies) with DNA is dependent on the concentration of anthramycin. Thus, at anthramycin concentrations of 1 mM, 0.5 mM and 25 μM at 37° and pH 5.8, the decreases in ethidium fluorescence after 5 min of reaction with constant concentration of PM2-CCC-DNA are 45, 38 and 8 per cent respectively.

The pH and temperature have a great influence on the rate of reaction between pyrrolo(1,4)benzodiazepine antibiotics and DNA, at least within the pH range of 4.7 to 9 and temperature range of 0–51°. The lower the pH, the higher is the reaction rate. Thus, with anthramycin, for example, at a concentration of 0.5 mM and 37° the decreases in fluorescence after 90 min of reaction with PM2-CCC-DNA are 73, 63, 45, 25 and 13 per cent at pH values 4.7, 5.8, 7.2, 8 and 9 respectively. Similarly, at a concentration of 0.5 mM and pH 5.8, the values after 90 min are 71, 63, 55 and 28 per cent at 51, 37, 24 and 0° respectively.

Neither anthramycin, nor tomaymycin or any of the analogues and derivatives that we have examined, cross-link DNA. Covalent cross-linking of λ-DNA would have been apparent since a cross-link would serve as a nucleation point for renaturation after heating at 96° and rapid cooling [31, 33, 37]. In all cases, the after-heat fluorescence equalled that of the control.

There was no evidence of intercalation between base-

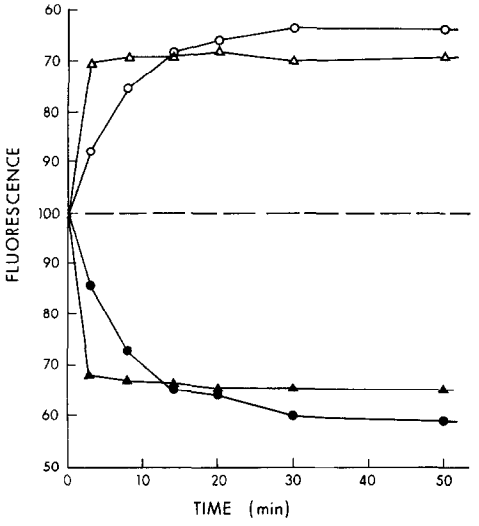

Fig. 3. Relaxation of native negatively supercoiled and anthramycin-bound PM2-CCC-DNA by calf thymus topoisomerase. Reactions were performed in 0.2 M NaCl containing 10 mM Tris–hydrochloride buffer, pH 8, 1 mM EDTA, 1.60 A_{260} units/ml of PM2-CCC-DNA (87% CCC) and 0.5 mM anthramycin. The solutions were incubated at 37° for 3 hr before adding the topoisomerase. The before-heat denaturation fluorescence readings are shown as open symbols and the closed symbols are fluorescence readings after denaturation at 96° and rapid cooling; (△) native PM2-CCC-DNA, (○) anthramycin-bound DNA, and (– – – –) control.

pairs in DNA by anthramycin. Supercoiled PM2-CCC-DNA, reacted with anthramycin, is relaxed to the same extent as native PM2-CCC-DNA by calf thymus topoisomerase but at a slower rate (Fig. 3). It was also found that reaction of anthramycin and tomaymycin causes a 30 per cent greater decrease in fluorescence compared with the control for relaxed PM2-CCC-DNA than for supercoiled DNA under comparable conditions. Whether this is due to greater reactivity of the relaxed form or to some other factor is not known at this stage.

The effect of sequence specific reagents on the reaction of anthramycin with PM2-CCC-DNA was investigated. Thus, olivomycin, chromomycin A_3, netropsin and distamycin all inhibited the reaction at 37° and pH 5.8. Olivomycin and chromomycin A_3, which in the presence of Mg^{2+} ions bind specifically to G + C rich regions in the DNA, inhibited reaction by 67.5 and 61 per cent at concentrations of 0.75 mg/ml and 0.5 mg/ml respectively. Mg^{2+} ions at the concentrations used did not have any effect on the reaction. Netropsin and distamycin, which are specific for A + T rich regions, inhibited the reaction by 71.5 and 92 per cent at concentrations of 0.3 mg/ml and 0.33 mg/ml respectively. Similarly, intercalated ethidium bromide also inhibited the reaction by 32 per cent at a concentration of 30 μg/ml.

Next, the reactions of anthramycin and the analogue **9** with DNAs of different G + C content were examined. Thus, reaction of anthramycin at a concentration of 25 μM, at 37° and pH 4.7, caused ethidium fluorescence decreases by 64, 55 and 58 per cent for *C. perfringens* (30% G + C), calf-thymus (40% G + C) and *E. coli* (50% G + C) DNAs respectively. Similarly, the reaction of **9** at a concentration of 4 mM under the above conditions produced fluorescence drops of 40, 46 and 42.5 per cent, respectively, for the above DNAs.

The reaction of anthramycin with DNA is not accompanied by elimination of either purine or pyrimidine bases. Depurination would have been revealed by treatment of supercoiled PM2-CCC-DNA, reacted with anthramycin with endonuclease VI. This enzyme cleaves apurinic PM2-CCC-DNA and thereby converts it to open circular DNA which results in a characteristic rise in ethidium fluorescence before heat-denaturation when measured at pH 8.0.

The DNA–anthramycin complex is remarkably stable to dialysis. Dialysis of PM2-CCC-DNA, reacted with anthramycin against 20 mM phosphate buffer, pH 7.2, at 4° for 44 hr, resulted in a loss of ~4.5 per cent of bound drug molecules. Similarly, dialysis of anthramycin-reacted calf-thymus DNA against 15 mM sodium citrate buffer, pH 7.0, for 45 hr at 4° caused only a loss of ~3.5 per cent drug molecules from the DNA. Inter-

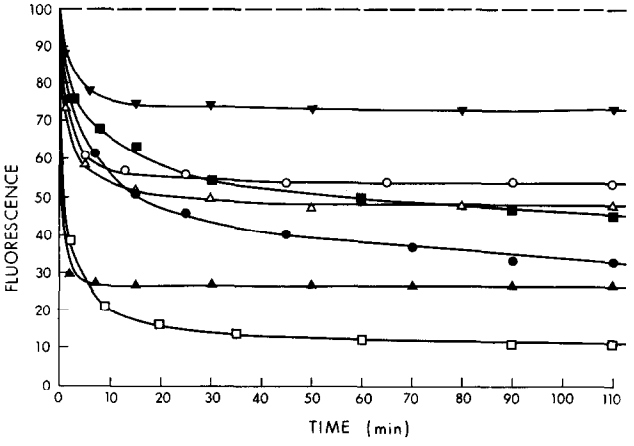

Fig. 4. Alkylation of calf-thymus DNA by anthramycin, tomaymycin and analogues. Reactions were performed at 37° in 50 mM potassium phosphate buffer, pH 4.7, except for anthramycin (pH 5.8) and contained 2.05 A_{260} units/ml of calf thymus DNA. Additional components were (□) 0.5 mM anthramycin, (▲) 2 mM tomaymycin, (●) 2 mM **6**, (△) 4 mM **10**, (■) 1 mM **5**, (○) 4 mM **9**, (▼) 4 mM **21**, and (– – – –) control.

estingly, incubation of the dialyzed solution at 37° resulted in a further dissociation of drug molecules from both PM2-CCC and calf-thymus DNAs, as evidenced by an increase in ethidium fluorescence compared to the control. (Approximately 22 and 19 per cent of anthramycin dissociated from PM2-CCC and calf-thymus DNAs, respectively, after 48 hr.)

To establish the structural requirements of the pyrrolo(1,4)benzodiazepine antibiotics for reaction with DNA, the reactions of several derivatives and analogues (Fig. 1) with different DNAs were studied under various conditions. Compound **5**, in which the acrylamide side chain in anthramycin is replaced by an acrylonitrile group, reacted with DNA. Similarly, the 9-methyl derivative **6** also was reactive. On the other hand, the *N*-acetyl derivative **7** and the saturated compound **8** in which the carbinolamine function is no longer present did not react with any DNA. However, compounds **9** and **10**, although they lack the unsaturated side chain, did react with DNA. The kinetic plots for the reaction of the above compounds with calf-thymus DNA are shown in Fig. 4.

The above studies implicated the carbinolamine function at position 10, 11 as the group responsible for binding to DNA. To test this hypothesis two analogues were synthesized (Figs. 5 and 6), and their reactions with DNA studied. The quinoline derivative **17** did not react with DNA, as shown by ethidium fluorescence and u.v. studies. Interestingly, the imine **16** readily reacted with methanol to form **17**. Compound **17** also reacted with thiophenol to form the adduct **18**. On the other hand, the pyrrolo(1,4)benzodiazepine **21** did react with DNA as shown by ethidium fluorescence assay (Fig. 4). It also reacted with thiophenol to form the adduct **23**. The u.v. absorption maximum of **21** was too close to that of DNA to establish any change in the u.v. spectrum on reaction with DNA, but the saturated compound **22** was unreactive. In summary, all pyrrolo(1,4)benzodiazepine compounds with a free carbinolamine, imine or α-methoxyamine function at the 10, 11-position did react with DNA.

DISCUSSION

The unusual stability of the anthramycin–DNA complex along with the modest size of the molecule

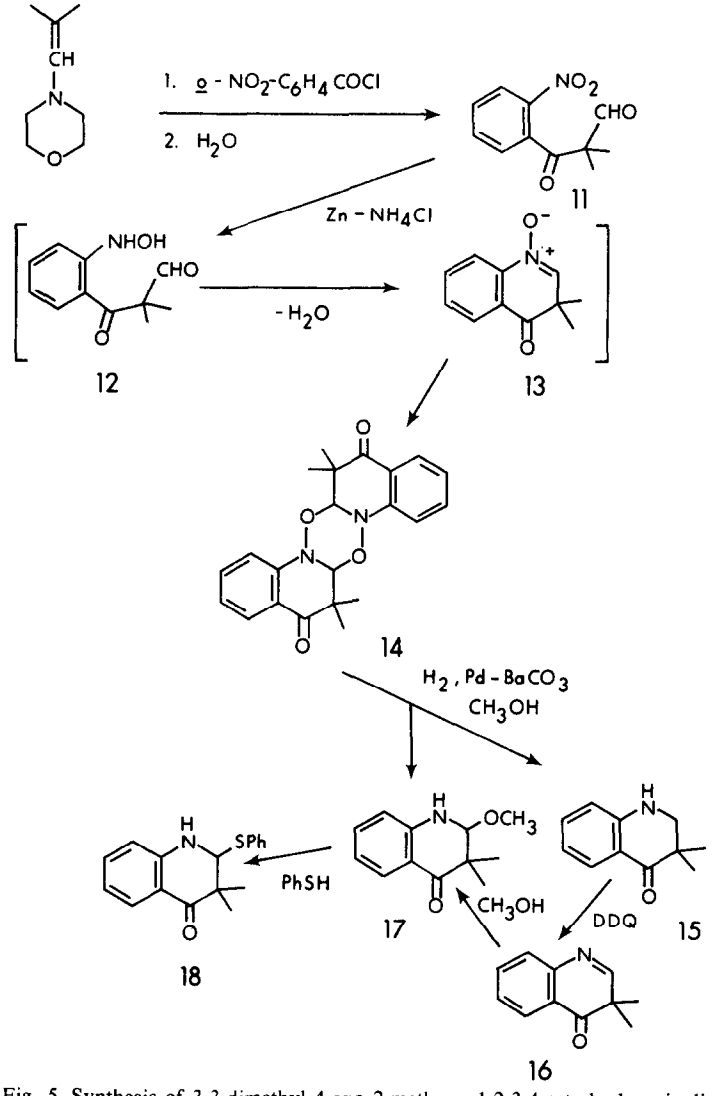

Fig. 5. Synthesis of 3,3-dimethyl-4-oxo-2-methoxy-1,2,3,4-tetrahydroquinoline.

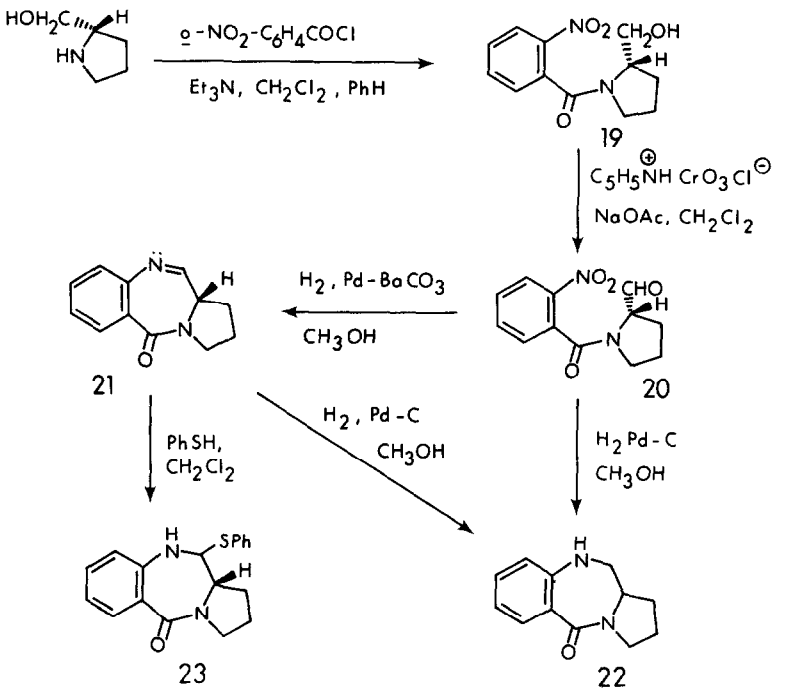

Fig. 6. Synthesis of pyrrolo(1,4)benzodiazepine.

compared with other DNA-binding antibiotics, has been attributed to the covalent nature of the binding [16]. However, unlike other known alkylating agents, like dimethylsulfate and aziridinoquinones [24, 43], the alkylated site is not susceptible to detectable alkaline cleavage. Instead, even at pH 11.8, some of the bound drug molecules dissociate from the DNA strands, as shown by increased fluorescence after heat rather than before heat. These results are consistent with a reversible alkylation of DNA by anthramycin. Tomaymycin and other reactive structurally related compounds behaved in a similar fashion although all of them were less reactive toward DNA than anthramycin itself.

None of the pyrrolo(1,4)benzodiazepine compounds examined in our studies did cross-link DNA. Similar results have been obtained by other workers [16]. This indicates that only one functional group on the molecule is involved in covalent binding to DNA. Further, the possibility of intercalation has been excluded from our studies with topoisomerase and also by physical measurements by other workers [18].

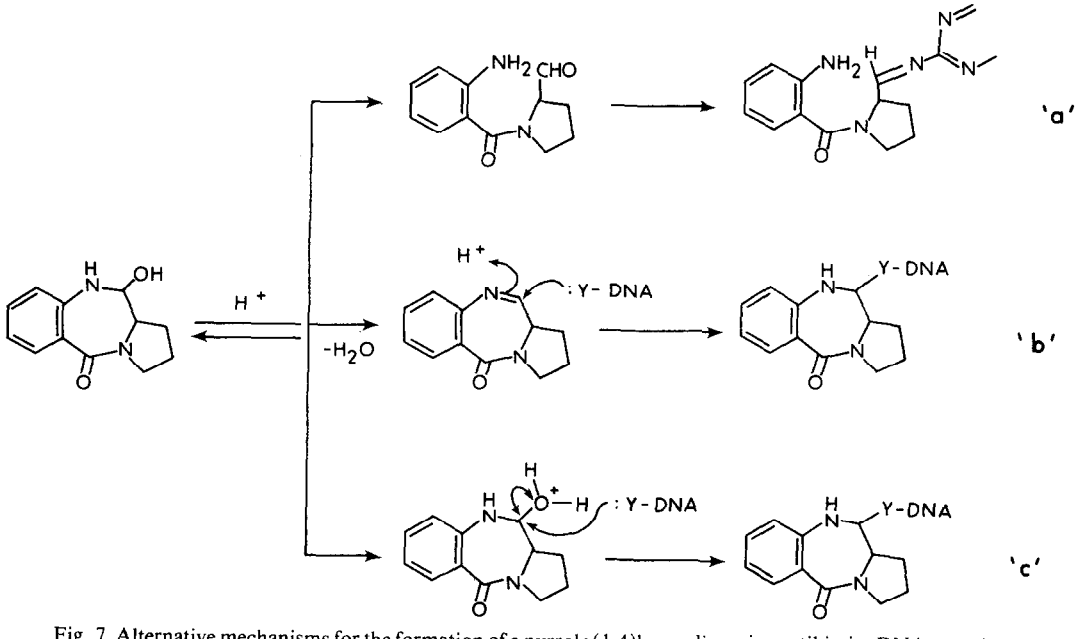

Fig. 7. Alternative mechanisms for the formation of a pyrrolo(1,4)benzodiazepine antibiotic–DNA complex.

Although it has been reported that anthramycin reacts specifically with DNA containing guanine [13, 15, 16, 18] our studies with sequence specific DNA binding agents and DNAs of varying G + C content showed no obvious DNA base specificity.

The pH dependence of the reaction of anthramycin or tomaymycin with DNA and the absence of any interstrand cross-linking in the reaction of these antibiotics with DNA point to the fact that only one acid sensitive functional group on these antibiotics is responsible for covalent binding to DNA. Examination of the structures of these antibiotics reveals that the carbinolamine function at position 10, 11 may be this functional group. Our studies with the analogues also lead to this conclusion. Thus, any compound in which the carbinolamine function is intact reacted with DNA, whereas any modification of this functional group, by oxidation of the hydroxy group to the carbonyl, the presence of an acyl group on the nitrogen, or the conversion of the carbinolamine to the secondary amine, resulted in complete loss of activity. On the other hand, modification or even the absence of the unsaturated side chain and methylation of the phenolic hydroxy group did not make these compounds inactive, although the reactivity was reduced in all cases.

It is interesting to note that, even though the quinoline derivative **17** reacted with nucleophiles like thiophenol, presumably through the imine **16**, it failed to react with DNA. Evidently the benzodiazepine nucleus is an essential requirement for reactivity toward DNA.

It has been reported by Stefanovic [13] that absence of the unsaturated side chain at the 2-position of the pyrrole ring and methylation of the phenolic hydroxy group in anthramycin resulted in inactivity. The absence of any obvious spectral change on reaction with DNA was taken as evidence for inactivity. Similar conclusions have been reached by other workers from studies on the chemosterilant action on houseflies and inhibition of *E. coli* RNA polymerase with anthramycin and other structurally related compounds [29]. By contrast, our studies clearly indicate that compounds **6**, **9** and **10** and also the synthetic analogue **21** in which there is no other "proposed" functional group other than the azomethine function do react with DNA. It is possible that other functional groups may assist in the binding process by bringing together the reactants to the proper configurations by hydrogen bonding or similar interactions.

Hurley *et al.* [21, 22] have considered three different mechanisms for the reaction of pyrrolo(1,4)benzodiazepine antibodics with DNA (Fig. 7). These authors favor mechanism "c" based on the fact that (i) reduction of DNA–antibiotic complexes did not result in any increase in the amount of acid-stable complex, which rules out mechanism "a", and (ii) the imine of sibiromycin (anhydrosibiromycin) is biologically unreactive [8]. Further evidence against mechanism "a" is that the nitroaldehyde, **20**, failed to react with DNA. On the other hand, our study with analogues is not in agreement with this mechanism. The anthramycin derivative **7**, which has a free hydroxy group at the 11 position but has an acetyl group on the nitrogen instead of hydrogen for anthramycin, did not react with DNA. If the proposed Sn_1ca mechanism is operative, **7** should also react with DNA. We consider that, at least in the cases of anthramycin, tomaymycin and neothramycins, mechanism "b" is operative, since the azomethine function conjugated to the carbonyl through the benzene ring can act as a powerful alkylating agent. Such a process can be reversed under acidic conditions or on heating. This mechanism is in close analogy to that proposed for the reaction of maytansine and other carbinolamide analogues with DNA via a reactive azomethine lactone [36]. In both cases, the reaction is faster under acidic pH conditions.

Studies on the role of the pyrrole ring and the carbonyl group at the 5-position in the pyrrole(1,4)benzodiazepine antitumor antibiotics in their reactions with DNA will be published elsewhere.

Since tumor cells are characterized by having a lower pH than normal cells due to their high anaerobic glycolytic rate and production of lactic acid [44, 45], the observed acid-promoted alkylation of biological macromolecules by pyrrolo(1,4)benzodiazepine antitumor agents may contribute to their selectivity.

In conclusion, the above studies serve to delineate the reactions of anthramycin and tomaymycin with nucleic acids and should assist in advancing the understanding of the mode of action of these valuable antitumor agents.

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