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EXPERIMENTAL AND COMPUTER GRAPHICS SIMULATION ANALYSES OF THE DNA INTERACTION OF 1,8-BIS-(2-DIETHYLAMINOETHYLAMINO)-ANTHRACENE-9,10-DIONE, A COMPOUND MODELLED ON DOXORUBICIN

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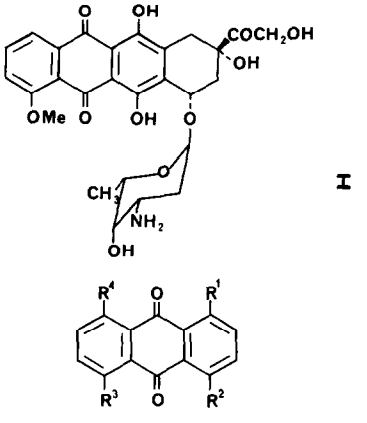
Abstract—The crystal structure of the anthraquinone derivative 1,8-bis-(2-diethylaminoethylamino)-anthracene-9,10-dione has been established. This compound was prepared as a potential DNA-intercalating agent based on the proven intercalators doxorubicin and mitoxantrone. Its DNA-binding properties have been examined experimentally by spectroscopic, thermal denaturation and ccc-DNA unwinding techniques: the results are consistent with an intercalative mode of binding to DNA. Computer graphics simulation of the intercalative docking of this compound into the self-complementary dimer of d(CpG) has provided a minimum energy geometrical arrangement for the bound drug in the intercalation site comparable to that for proflavine when intercalated into the same d(CpG) model system. Entry of the compound into the site can only occur via the major groove.

The anthracyclines, particularly doxorubicin (**I**), are pre-eminent among current antitumour drugs [1]; this has prompted the search for less toxic (especially less cardiotoxic) analogues [2, 3]. Most work has concentrated on tetracyclic compounds, either development of routes for total synthesis of the natural compounds [4], or the preparation of semisynthetic analogues [5]. Consideration of the biological effects of anthracyclines as a basis for development of new analogues may also be rewarding. Inhibition of nucleic acid synthesis has long been known as a major effect of these compounds [6] and this action is thought to be mediated via intercalation of the aromatic ring system into nuclear DNA [7, 8]. Based on the early intercalation models (which have subsequently been revised [9, 10]), several tricyclic anthraquinone derivatives were prepared as totally synthetic potential intercalating analogues of doxorubicin [11, 12]. Coincidentally, compound **II** (see Scheme 1) was identified as a potential antitumour agent in a random screening programme at NCI. Subsequent preparation of further anthraquinones [13, 14] led to mitoxantrone (**III**) which is now in Phase II clinical trials as an antitumour agent [15, 16]. Studies to date ([17–19]; M. A. Hardman and J. R. Brown, unpublished results) indicate that mitoxantrone and similar substituted anthraquinones interact with DNA by intercalation.

As the initial part of a detailed study of the DNA-binding properties of substituted anthraquinones, we have prepared compound **IV** and examined its crystal and molecular structure and its DNA-binding properties in solution. We have also carried out a computerized molecular model building study of the interaction of **IV** with a self-complementary deoxydinucleoside phosphate. This has served to define a possible geometry for the interaction of **IV** with DNA.

MATERIALS AND METHODS

Materials. Ethidium bromide, doxorubicin hydrochloride and calf thymus DNA (Type I) were from Sigma. PM-2 DNA was from Boehringer Mannheim. Solutions were prepared in 0.05 M NaCl.



I

II $R^1 = R^2 = NHCH_2CH_2NHCH_2CH_2OH$, $R^3 = R^4 = H$
III $R^1 = R^2 = NHCH_2CH_2NHCH_2CH_2OH$, $R^3 = R^4 = OH$
IV $R^1 = R^4 = NHCH_2CH_2N(C_2H_5)_2$, $R^2 = R^3 = H$

Scheme 1.

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0.008 M Tris Cl buffer, pH 7.4. 1,8-Bis-(2-diethylaminoethylamino)-anthracene-9,10-dione, (**IV**) was prepared from 1,8-dichloroanthraquinone (Aldrich Chemical Co.) which had been recrystallized from toluene, and N,N-diethylaminoethylenediamine (Fluorochem, Glossop) by the method of Double and Brown [12]. C 71.3%, H 8.3%, N 12.26% (calculated for $C_{26}H_{36}N_4O_2$, C 71.52%, H 8.3%, N12.83%).^{1}H NMR in $CDCl_3$, 1.2 ppm (12H, *t*, 4 × CH_3), 2.5 ppm (12H, *m*, 6 × N-CH_2), 3.25 ppm (4H, *m*, NH-CH_2) and 7.0–7.5 ppm (6H, *m*, aromatic). UV λ_{max}(EtOH), 280 nm ($\varepsilon = 9.59 \times 10^3$) and 550 nm ($\varepsilon = 7.85 \times 10^3$). The free base was used for X-ray crystallography and the hydrochloride salt was prepared for DNA-binding studies.

X-Ray crystallography. Large prismatic crystals of the free base of **IV** were obtained by slow evaporation from an ethyl acetate solution: they were stable in air. Cell dimensions were obtained by measurement of 25 θ values on a CAD4 diffractometer. These were: $a = 30.910$ (5), $b = 11.951$ (2), $c = 13.623$ (3) Å and $\beta = 96.42(2)°$. The crystals displayed monoclinic symmetry; the space group assignment of C2/c with eight molecules in the unit cell was verified by the structure analysis. The intensities of 4712 reflections were measured with Cu-Kα radiation on the diffractometer, using an $\omega-2\theta$ scan technique. A total of 1837 reflections had significant intensity with $F_0 > 2.5\sigma(F_0)$. The structure was solved by direct methods, and refined by full matrix least-squares techniques. Amine and chromophore hydrogen were located by difference Fourier syntheses; the positions of other hydrogen atoms were calculated by standard geometric considerations. The final reliability index R was 0.081. Atomic co-ordinates are given in Table 1. Listings of bond lengths and angles are available on request (from S.N.).

Table 1. Final atomic positional parameters, with estimated standard deviations in parentheses

Atom	X	Y	Z
O4	0.1779(2)	0.1688(5)	0.0953(4)
O11	0.3495(2)	0.1626(6)	0.2086(6)
N61	0.1898(3)	0.0711(6)	−0.0736(5)
N21	0.1640(2)	0.2899(6)	0.2489(5)
N62	0.0990(3)	0.0334(8)	−0.1046(6)
N22	0.0797(2)	0.3847(7)	0.1633(6)
C3	0.2351(3)	0.2272(7)	0.2123(6)
C4	0.2182(3)	0.1732(7)	0.1181(6)
C12	0.2802(3)	0.2217(7)	0.2423(7)
C2	0.2077(3)	0.2801(7)	0.2739(6)
C5	0.2481(3)	0.1231(7)	0.0541(6)
C1	0.2275(3)	0.3291(8)	0.3619(7)
C10	0.2932(3)	0.1245(7)	0.0830(6)
C11	0.3110(3)	0.1707(8)	0.1789(7)
C6	0.2327(3)	0.0723(7)	−0.0377(6)
C14	0.2717(3)	0.3221(8)	0.3903(7)
C13	0.2978(3)	0.2961(8)	0.3315(7)
C7	0.2637(3)	0.0259(8)	−0.0963(6)
C9	0.3224(3)	0.0803(8)	0.0234(7)
C8	0.3062(3)	0.0301(8)	−0.0642(7)
C61	0.1727(4)	0.0094(9)	−0.1614(7)
C62	0.1252(4)	0.0306(12)	−0.1841(8)
C21	0.1364(3)	0.3853(9)	0.3053(7)
C22	0.0889(3)	0.3419(9)	0.2632(9)
C63	0.0963(6)	−0.0813(13)	−0.0628(11)
C23	0.0403(6)	0.3286(12)	0.1120(13)
C64	0.0832(5)	−0.0806(13)	0.0386(10)
C24	0.0344(6)	0.2227(18)	0.1118(16)
C65	0.0573(5)	0.0812(14)	−0.1315(14)
C66	0.0558(5)	0.2000(16)	−0.1602(14)
C25	0.0704(4)	0.4986(12)	0.1653(11)
C26	0.0689(6)	0.5548(15)	0.0761(19)

Effect of **IV** *on the thermal denaturation of DNA*. An appropriate volume of a solution of the hydrochloride salt of **IV** in double-distilled water and 600 μl of a 2.5×10^{-3}M solution of calf thymus DNA in buffer were mixed and made up to volume with double-distilled water such that the DNA : drug ratio was precisely 10 : 1 (buffer: 0.00288 M Tris Cl, 0.018 M NaCl, pH 7.4). The absorbance of the solution at 260 nm was recorded as the temperature was raised by 0.5°/min over the range 58.0–110.0°. This was repeated for three further samples and the mean *Tm* calculated. The mean *Tm* from four determinations with DNA in the absence of drug was subtracted to give the ΔTm value.

Effect of **IV** *on covalently closed circular DNA (PM-2)*. The effect of **IV** on supercoiled DNA was evaluated by a method based on that described by Espejo and Lebowitz [20]. PM-2 DNA was dissolved in pH 7.4 0.05 M Tris buffer containing 0.002 M EDTA, 0.018 M NaCl and 0.02 M Na acetate to give a solution of approximately 100 μg/ml. Appropriate volumes of drug solution (100 μg/ml) were added to eight 5 ml vol. of 1% Agarose Type I (Sigma) in the buffer at 45°, such as to give a range of final concentrations of **IV** from 0.00 to 1.00 μg/ml. Each solution was poured into a 125 mm × 5.8 mm glass tube with one end sealed with nescofilm, and the ends of the resultant gels were trimmed when cool, the nescofilm being replaced with cotton gauze. An aliquot (20 μl) of the PM-2 DNA solution containing **IV** at a 5 : 1 molar ratio of DNA phosphate/**IV** was layered onto the top of the gel, followed by a 20 μl vol. of 20% sucrose and bromophenol blue. Electrophoresis was performed at 45 V (5 mA per tube) at 20–25° for 3–4 hr until the bromophenol blue had migrated to about half the length of the gel. Drug is present throughout the gel and will migrate towards the cathode at about the same rate as bromophenol blue migrates towards the anode, the latter migrating in advance of the DNA. Hence by stopping the electrophoresis when the bromophenol blue has migrated half the length ensures that DNA is still in contact with the drug. The gels were then removed and stained with ethidium bromide (4.0 μg/ml) in the same buffer, then destained overnight with the same buffer and the gels photographed. The critical concentration was determined, this being the concentration at which the ccc-DNA virtually co-migrated with nicked PM-2 DNA in the sample. The procedure was repeated for ethidium bromide and doxorubicin hydrochloride using drug concentrations between 0.00 and 0.20 μg/ml.

Effect of DNA on the spectral properties of **IV**. The spectra of six solutions of **IV** (2.5×10^{-5}M in buffer) containing varying concentrations of calf thymus DNA (so that the DNA: drug ratios varied from 0 to 15 : 1) were recorded superimposed from 380 to 680 nm (about 150 nm below to 150 nm above the

λ_{max} of the unbound drug). For determination of the binding affinity, a spectrophotometric titration method was used. Aliquots (4×40, 12×20 and $7 \times 100 \mu l$)of DNA solution (2.5×10^{-3} M) were added sequentially to each of three samples (3 ml) of drug solution (5×10^{-5} M) in pH 7.4 0.05 M NaCl, 0.008 M Tris Cl buffer, and the absorbance was determined at 550 nm, allowing time for equilibration after each addition before reading the absorbance. For each reading, the molar extinction coefficient of drug (ε_{obs}) was calculated; the fraction of drug bound was then calculated as follows:

$$\text{Fraction of drug bound} = \varepsilon_{free} - \varepsilon_{obs}/\varepsilon_{free} - \varepsilon_{bound}$$

where ε_{free} and ε_{bound} are the molar extinction coefficients for free and totally bound drug at the wavelength utilized. Knowing the fraction bound, c (the molar concentration of free drug) and r (the molar concentration of bound drug/DNA phosphate) were computed. The binding parameter K (the affinity constant) and n (the number of binding sites per DNA phosphate) were then determined by linear regression, at low r values, of the Scatchard plot of r/c vs r (the slope is $-K$ and n is the intercept on the r axis).

Competition studies with **IV** *and ethidium.* (a) Aliquots ($6 \times 10 \mu l$, $5 \times 20 \mu l$, $6 \times 40 \mu l$, $6 \times 100 \mu l$) of a 2×10^{-5} M solution of ethidium bromide in buffer were added sequentially to a 2.0 ml volume of each of (a) 2×10^{-5} M calf thymus DNA in buffer, (b) 2×10^{-5} M calf thymus DNA and 2×10^{-6} M **IV** hydrochloride in buffer, (c) 2×10^{-6} M **IV** hydrochloride in buffer and (d) buffer. The fluorescence of each solution was recorded, before and after each addition, at 596 nm (excitation at 476 nm) at 25°.

(b) The fluorescence polarization values of three samples each of six solutions of 2×10^{-6} M ethidium bromide and 8×10^{-6} M calf thymus DNA containing varying concentrations of **IV** hydrochloride between 0 and 1×10^{-5} M were determined at 596 nm (excitation at 476 nm) at 25°. The usual procedure was followed to correct for spurious polarization due to the instrument. The fluorescence polarization of a solution of 2×10^{-6} M ethidium bromide in buffer was also determined for reference.

Computerized model building. The system chosen to represent an intercalated fragment of double-stranded DNA was the self-complementary dimer of the deoxydinucleoside phosphate d(CpG). The crystal and molecular structure of a d(CpG) : proflavine intercalated complex [21] provided the starting set of co-ordinates for the analysis. To date, accurate experimental data on intercalation geometry is only available at the dinucleoside level, from crystal-structure analyses of a number of drug complexes. Analysis of these data has revealed that the dinucleoside backbone conformation is remarkably invariant in all these structures [22–24]. We therefore feel confident in using the d(CpG) system, with the important provision that the relationship of dinucleoside complexes to polymeric nucleic acid ones has not been determined.

Calculations were performed on an interactive computer graphics system, using the MOLEC system of programs [25]. The geometry of potential complexes between d(CpG) and compound **IV** was examined by intermolecular molecular mechanics calculations. The energy of a particular arrangement was calculated as the sum of terms representing non-bonded (N-B), electrostatic (E-S) and hydrogen-bonded potential energies. The d(CpG) geometry was held invariant throughout the study, and the chromophore of the drug was restricted to translational and rotational movements in the plane parallel to and midway between the d(CpG) base pairs.

Geometric and energy searches were performed with the free base **IV**, with the species protonated at the side-chain nitrogen atoms N22 and N62 and with the main chromophore of **IV** alone; in the second case, the additional proton positions were calculated by standard geometric considerations. Partial charge densities for the drug structures and d(CpG) were calculated with the *ab initio* GAUSSIAN 76 system.

RESULTS AND DISCUSSION

Crystallographic analysis

Figures 1 and 2 show views of the free base **IV** as determined in the crystal structure. The anthraquinone ring system itself is almost entirely coplanar, with an angle between the two outer rings of 4.0°. The anthraquinone moiety has effective mirror symmetry although this is not utilized by the space group. The amino protons on N21 and N61 form two strong intramolecular hydrogen bonds with the carbonyl oxygen atom O4. The O4 . . . HN21 and O4 . . . HN61 distances are 1.98 and 1.88 Å respectively, and the O4 . . . HN21–N21, O4 . . . HN61–N61 hydrogen-bond angles are 119.4° and 129.9°. The effect of these hydrogen bonds is to force the N21–C21 and N61–C61 bonds to be approximately coplanar with the ring system. Thus, the torsion angles Cl–C2–N21–C21 and C7–C6–N61–C61 have values of 5.5° and 10.2°.

The overall effect of these intramolecular hydrogen bonds is to somewhat increase the effective planar area of the molecule, as shown in Fig. 2. The

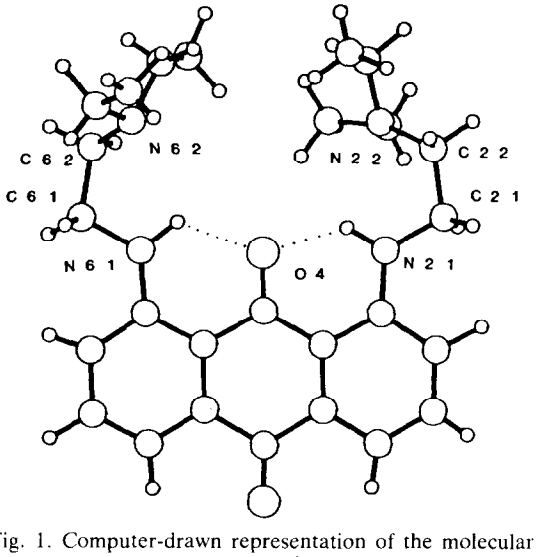

Fig. 1. Computer-drawn representation of the molecular structure of **IV**.

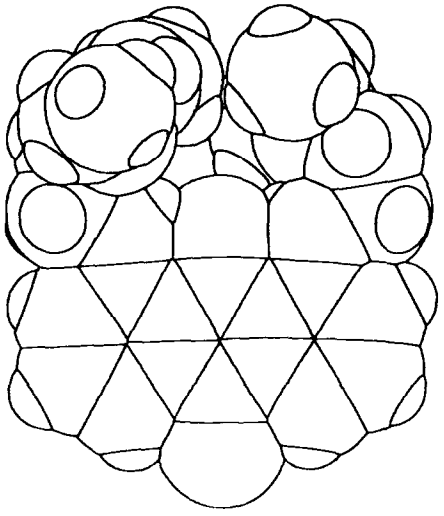

Fig. 2. The molecular structure of **IV**, with atoms drawn with their van der Waals radii.

two side-chains adopt somewhat differing conformations (Table 2), thus ensuring that the molecule as a whole does not display mirror symmetry. Figure 2 shows that the side-chains project into opposing directions—this is possibly partly attributable to otherwise unacceptable steric interactions were both side-chains to protrude on the same side of the molecule.

Solution binding studies

On interaction with DNA, **IV** was shown to undergo bathochromic (11 nm) and hypochromic (29% reduction in ε) shifts. Spectrophotometric titration at the λ_{max} of unbound drug yielded binding parameters (estimated by Scatchard plot) of $K = 1.710 \times 10^6\ M^{-1}$ and $n = 0.20$. Electrophoresis of PM-2 DNA in the presence of **IV** showed that, as with ethidium and doxorubicin, the rate of migration of PM-2 DNA decreased as drug concentration increased (i.e. the DNA was unwound) down to a minimum when the DNA almost co-migrated with the nicked PM-2 present in the PM-2 DNA sample. As drug concentration was increased further, rate of migration increased progressively (i.e. DNA was supercoiled in the opposite way).

Figure 3 shows a typical electrophoretic run. The concentration of drug at which the DNA almost co-migrated with the nicked DNA (the critical concentration) was found to be 0.16 μg/ml (4.058×10^{-7} M) for ethidium, 0.16 μg/ml (2.759×10^{-7} M) for doxorubicin and 0.40 μg/ml (7.874×10^{-7} M) for **IV**. To calculate the degree of unwinding due to each bound molecule of drug, it is necessary to know the superhelical density of the PM-2 DNA and the number of drug molecules bound per DNA phosphate under the conditions when the supercoils in the PM-2 DNA are totally unwound. The latter was calculated from the Scatchard equation:

$$r = \frac{Kcn}{1 + Kc} \tag{1}$$

K and n have already been determined (see above) and substituting c', the 'critical concentration' of drug, into the equation yields the value of r', the number or drug molecules/DNA phosphate which gives full relaxation of the supercoiled DNA. The method of calculating the unwinding angle was that used by Espejo and Lebowitz [20] and DeLeys and Jackson [26], and is as follows:

$$\sigma_0 = \frac{-10\phi r'}{180} \tag{2}$$

where σ_0 is the superhelical density of the PM-2 DNA and ϕ is the unwinding angle. The superhelical density for PM-2 DNA quoted by Espejo and Lebowitz is 0.042 based on an unwinding angle of 12° for ethidium; it is now accepted that the unwinding angle for ethidium is 26° and recalculation gave 0.0911 as the superhelical density for PM-2 DNA. Equation (2) thus becomes:

$$-\phi = \frac{1.638}{r'} \tag{3}$$

Using the K and n values above, the unwinding angle for each molecule of **IV** was calculated to be 14.3°. As well as unwinding the helix of DNA, **IV** also causes stabilization of the helix. The *Tm* of calf thymus DNA in the absence of **IV**, in pH 7.4 0.018 M NaCl, 0.00288 M Tris buffer, was found to be 71.2 ± 0.1° and in the presence of **IV** (at a 10 : 1 DNA to drug ratio) was 80.7 ± 0.1°, giving a ΔTm of 9.5° compared to a value of 16.25° for doxorubicin under the same conditions [27]. This confirms that the interaction of **IV** with DNA is weaker than that of doxorubicin with DNA.

The spectral shifts shown by **IV** in the presence of DNA, the magnitude of the K and n values for binding of **IV** to DNA, the significant stabilization of DNA to thermal denaturation in the presence of **IV**, and the unwinding of ccc-DNA in the presence of **IV** are all indicative of an intercalative mechanism of binding of **IV** to DNA ([18, 27]; M. A. Hardman and J. R. Brown, unpublished results). Taken individually, none of these observations is definitive proof of intercalation but all the effects are typical of those seen with anthracyclines proven to inter-

Table 2. Side-chain torsion angles, in °

C6–N61–C61–C61	−176.7	C2–N21–C21–C22	−174.6
N61–C61–C61–N61	−41.5	N21–C21–C22–N22	−66.0
C61–C61–N62–C63	−69.0	C21–C22–N22–C23	−157.3
C61–C61–N62–C65	165.1	C21–C22–N22–C25	−85.0
C62–N62–C63–C64	−160.4	C22–N22–C23–C24	−47.8
C62–N62–C65–C66	−64.5	C22–N22–C25–C26	168.4

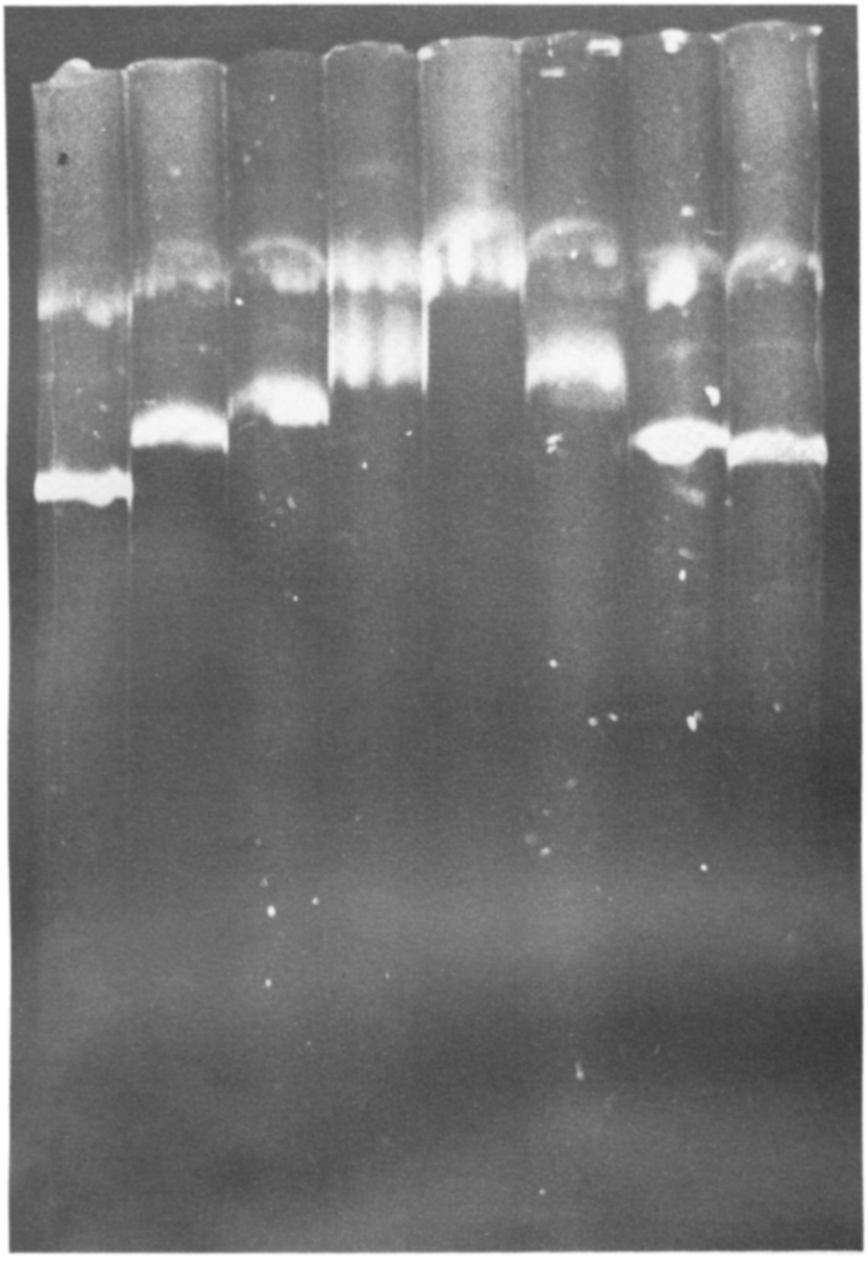

Fig. 3. Gel electrophoresis of ccc-DNA (PM-2) in the presence of varying concentrations of **IV**, from left to right, 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.8 and 1.0 μg/ml.

calate into DNA [27] and also with proven intercalating anthraquinones such as mitoxantrone [18]. When considered together, the data give strong evidence of an intercalative interaction of **IV** with DNA. To delineate further the mechanism of the interaction, the ability of **IV** to displace intercalated ethidium from DNA was investigated by two methods. In the first method, ethidium was added to a solution of calf thymus DNA and **IV** (10 : 1 ratio) and the fluorescence enhancement compared with that on binding to DNA in the absence of **IV**. The results (Fig. 4) show that fluorescence enhancement, and hence DNA-binding, of ethidium is reduced when **IV** is also bound to DNA. (Controls showed that there was no fluorescence due to **IV** at the wavelengths used and that **IV** had no effect on the fluorescence of ethidium in the absence of DNA.) To confirm that the intercalation of ethidium is reduced in the presence of **IV**, the polarization of ethidium fluorescence on irradiation with polarized light was monitored. In the absence of DNA, the fluorescence was depolarized ($P = 0.04$) due to rapid rotational movement in solution but when ethidium was intercalated the fluorescence was polarized ($P = 0.26$). In the presence of DNA and **IV**, the increase in polarization was less than with just DNA: this can only occur if there is exclusion of ethidium from DNA intercalation sites due to the presence of **IV**.

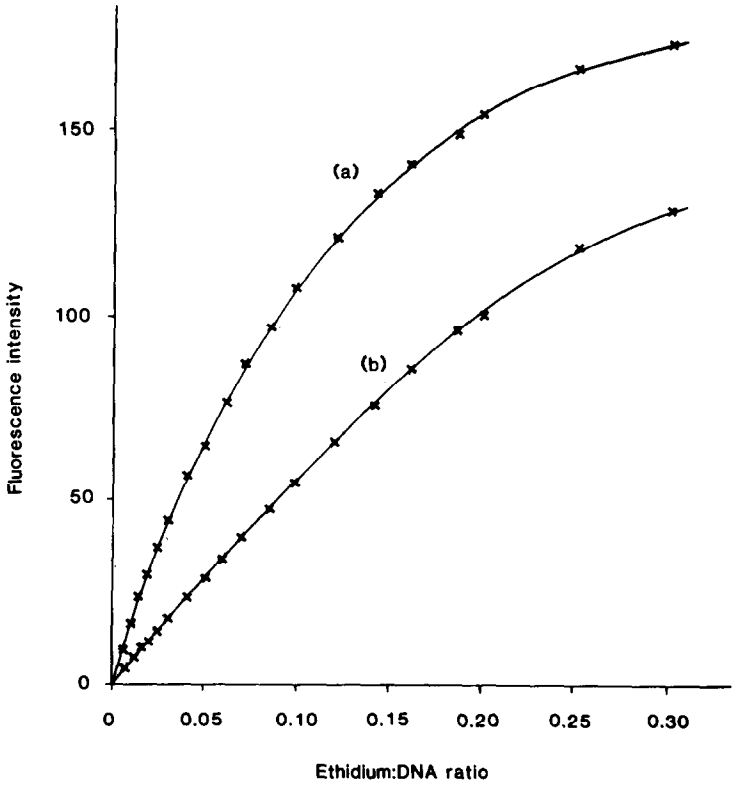

Fig. 4. Fluorescence intensity of ethidium on addition of aliquots of a solution of ethidium to DNA (curve a) and to a 10 : 1 (molar ratio) mixture of DNA and **IV** (curve b).

Under the conditions used, the increase in polarization of ethidium fluorescence was 50% of the expected value when **IV** was present at 3.5×10^{-6} M : 0.7×10^{-6} M doxorubicin was found to be required to achieve the same effect. This again shows that the interaction of **IV** with DNA is qualitatively similar to, but weaker than, the interaction of the proven intercalator doxorubicin. The evidence taken as a whole shows that **IV** has an intercalative interaction with DNA and so modelling of the fit into a DNA intercalation site is validated.

Computerized model building

An initial comparison of major vs minor groove binding for either **IV** or its protonated form to d(CpG) clearly showed that a satisfactory binding site could not be found in the minor groove. This was due to unacceptable close contacts developing between the anthraquinone side chains and the ribose groups thus excluding any interaction between the chromophore and the base pairs.

The results for the free base of **IV** binding in the major groove (Table 3) show that the intermolecular dispersion energy of the chromophore–base pairs dominates the total energy of interaction, and is approximately the same as that of proflavine in the crystal structure of its d(CpG) complex [21]. The anthraquinone chromophore alone gave a very similar result.

In general, it was found that the minimum-energy

Table 3. Energies of interaction with d(CpG), in kcal/mole

	Dispersion contribution	Electrostatic contribution	Total
PF^+–d(CpG) (crystallographic conformation)	−59	−64	−112
PF^+–d(CpG) (minimum energy after translation of 0.6Å towards minor grove)	−63	−64	−127
AQ–d(CpG) (superimposed on PF^+ position)	−65	−12	−77
AQ–d(CpG) (minimum position)	−80	−12	−92
AQ^+–d(CpG) (superimposed on PF^+ position)	−65	−65	−130
AQ^+–d(CpG) (minimum position)	−86	−83	−169
AQm–d(CpG) (superimposed on PF^+ position)	−57	3	−54
AQm–d (CpG) (minimum position)	−60	4	−56

PF^+, protonated proflavine; AQ, free base compound **IV**; AQ^+, protonated compound **IV**; AQm, chromophore only of compound **IV**.

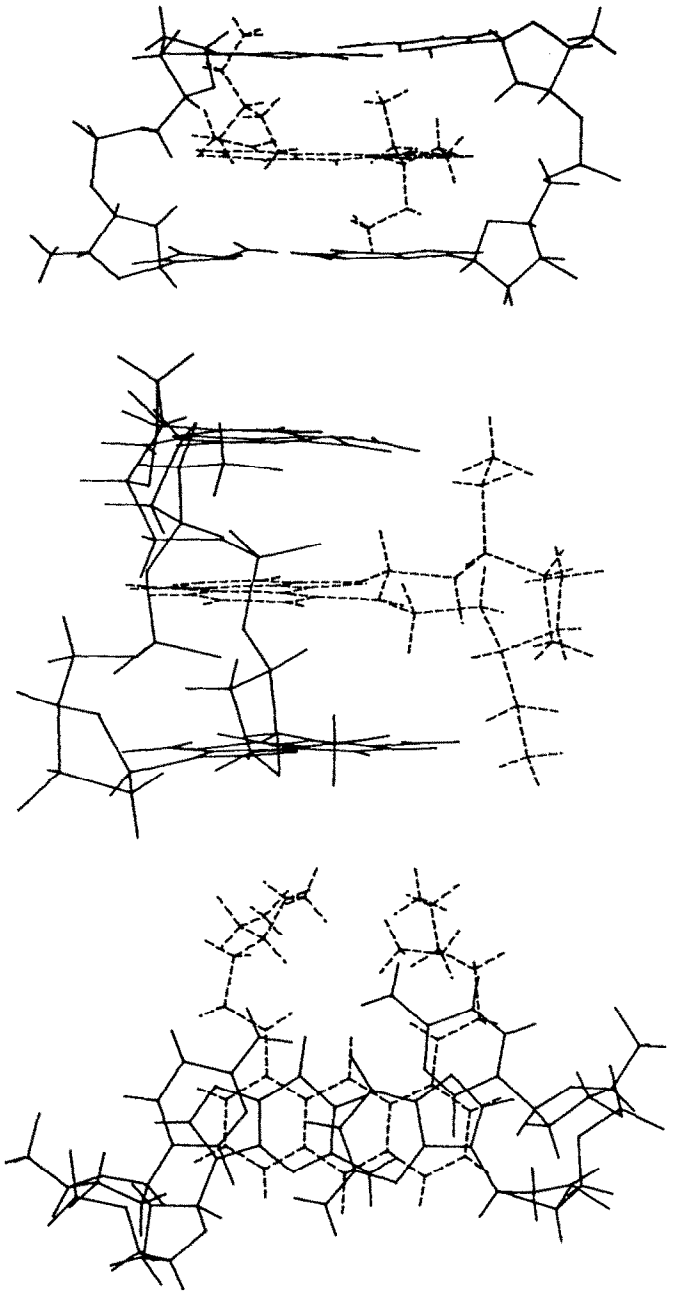

Fig. 5. Three computer-drawn views of the minimum-energy geometry of the complex between d(CpG) and the protonated form of **IV** (shown as dashed lines).

positions (Fig. 4) for compound **IV**, both as free base and protonated forms, were close to the crystallographically-observed position for proflavine [21], as far as the chromophore positions are concerned. These minimum positions could be altered by ± 0.3 Å translations and ± 10° rotations of the molecule, at an energy cost of just 5 kcal/mole. Table 3 shows that protonation of the side-chains in **IV** is indeed important in considerably increasing the strength of the total interaction. This increase arises from electrostatic interactions between the side-chains and the dinucleoside bases, in particular with O6 guanine atoms.

The conformational flexibility of the side-chains in IV was explored with the complex in its minimum-energy position (Fig. 5). There was some flexibility around all except the (N61–C61 and C61–C62) and (N21–C21 and C21–C22) bonds. These four angles are limited in their values to about ± 15° of the crystallographic ones (Table 2). Even without total energy minimization of the side-chain conformation, it is clear that their *N*-ethyl termini have a marked degree of freedom.

There is at present no detailed structural data on the conformation of nucleotide residues at both 3′ and 5′ sides of an intercalation site, either in an

oligo- or a polynucleotide. Thus, it is not possible to state whether in such a system the side-chains of **IV** could interact (by either specific hydrogen bonds or generalized electrostatic forces) with adjacent residues. Nonetheless, examination of Fig. 5 does suggest that the side-chains are probably of insufficient length for such stabilization to occur.

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