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Proton Nuclear Overhauser Effect Study of the Structure of an Actinomycin D Complex with a Self-Complementary Tetranucleoside Triphosphate†

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ABSTRACT: Saturation transfer and nuclear Overhauser effect (NOE) techniques have been used to assign some resonances of nonexchangeable protons in the NMR spectrum of the complex formed between actinomycin D and the self-complementary tetranucleoside triphosphate d(A-G-C-T). Intermolecular NOEs suggest that the drug chromophore intercalates between the two G-C base pairs of the nucleotide double helix, while the pentapeptide lactone rings fill the minor groove. Binding-induced distortions of helix geometry are discussed.

The interaction between actinomycin D (Figure 1) and nucleic acids is probably the most studied of all antitumor compounds (Remers, 1978). Understanding its high selectivity for G-C-rich double-stranded DNA not only has offered the possibility of increasing its therapeutic usefulness but also may have relevance as a model of the contact between proteins and polynucleotides.

The drug binds by intercalation (Muller & Crothers, 1968), but its preference for G-C-rich DNA was accounted for by deductions made from the X-ray crystal structure of its complex with deoxyguanosine (Jain & Sobell, 1972). This relatively simple complex is stabilized by two hydrogen bonds between guanines parallel to the phenoxazone chromophore of the drug and L-threonine in the side chains. These interactions in combination with the overall shape of the actinomycin molecule were used to construct a detailed model of the complex with DNA generally consistent with other experimental data (Sobell & Jain, 1972; Sobell et al., 1977). It has, however, not been possible to examine at high resolution a model system closely resembling the DNA-bound drug; the crystalline complex with d(G-C) (Takusagawa et al., 1982) does not contain a double-helical nucleotide fragment.

NMR spectroscopy has been extensively used in attempts to characterize the binding site and mode of action of actinomycin D. Krugh & Neely (1973a) established that the drug has two binding sites for deoxyguanosine and 5′-dGMP. The same authors (Krugh & Neely, 1973b) studied its interactions with deoxydinucleotides and once again found two binding sites for G-containing species. The G-C sequence was particularly

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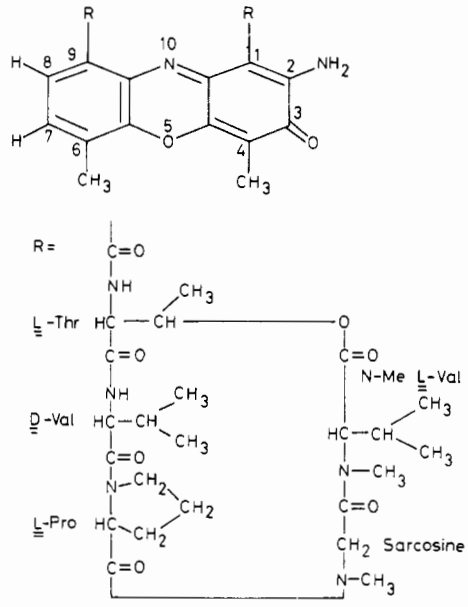

FIGURE 1: Structural formula of actinomycin D.

strongly bound. Patel (1974a) found that actinomycin binding to d(pGpC) caused a downfield shift in the ^{31}P resonance of the diester phosphate, an observation assumed to support the proposal of intercalation between the G-C and C-G base pairs. The guanine amino protons also shifted downfield, a fact which was explained by their participation in hydrogen bonding. Patel (1974b) also characterized the kinetics of exchange of the drug with d(A-T-G-C-A-T). Krugh & Chen (1975) examined the chemical shifts of actinomycin in the presence of an excess of a number of nucleotide species and found that they were invariable over a large range of drug concentrations. Early et al. (1976) and Patel (1976) investigated actinomycin complexes with nucleotide oligomers containing guanine–cytosine base pairs. On the basis of greater upfield shifts of guanine than cytosine ring protons, the former proposed that the actinomycin chromophore overlapped more extensively with the purine base. Patel observed that actinomycin binding abolished the 2-fold symmetry of a duplex, as revealed by the ^{31}P NMR spectrum. Recently Patel et al. (1981) found that actinomycin binding induced upfield shifts of exchangeable cytosine imino protons of the dodecamer d(C-G-C-G-A-A-T-T-C-G-C-G).

The nuclear Overhauser effect (NOE) refers to the change in integrated intensity of a resonance when another resonance which contributes dipole–dipole relaxation to the first is saturated (Noggle & Schirmer, 1971). The magnitude of the observed NOE is a function of the inverse sixth power of the distance between the irradiated and observed nuclei and as such may be used as a sensitive probe of molecular conformations. The NOE has been successfully employed to extract qualitative and quantitative information about internuclear distances in a number of large molecules (Tropp & Redfield, 1981; Alma et al., 1981; Williamson & Williams, 1981).

We have used NOEs to characterize the binding site of actinomycin D with the tetranucleoside triphosphate d(A-G-C-T), a sequence selected for efficient binding of the drug to a single site. Under the conditions employed in this study, in which the drug is in slow exchange on the NMR time scale between free and bound forms, NOEs can locate resolvable protons a short distance (<5 Å) apart. When these juxtaposed nuclei are considered in conjunction with chemical shifts induced by binding, a fairly detailed picture of the interaction emerges.

Experimental Procedures

Nucleotide Synthesis. d(A-G-C-T) was prepared by the solution triester method (Stawinski et al., 1977). It was purified by ion-exchange column chromatography (DEAE-cellulose–triethylammonium bicarbonate) and reversed phase high-pressure liquid chromatography (HPLC) (spherisorb O.D.S.–0.1 M ammonium acetate–acetonitrile). The ammonium salt produced in this way was desalted by gel filtration (Sephadex G-10).

Actinomycin D was obtained from Böhringer, Mannheim, West Germany.

NMR Spectroscopy. The tetranucleoside triphosphate (1.0 mg) was dissolved in 0.4 mL of deuterated 50 mM phosphate buffer containing 100 mM NaCl and giving a pH meter reading of 7.0. It was lyophilized and redissolved in D_2O in a 5-mm o.d. NMR tube twice and finally made up with 0.4 mL of 99.98 atom % D_2O (Sigma). For spectra of the complex 2.4 mg of the nucleotide was used, and carefully weighed amounts of actinomycin D were added to yield drug: tetramer molar ratios of 1:4 and 1:2.

All NMR spectra were obtained on a Bruker WH-400 NMR spectrometer operating in the Fourier transform mode. NOE and saturation transfer difference spectra were obtained by alternately acquiring eight on-resonance and off-resonance irradiated spectra, using a decoupler attenuation of 15 dB and a 90° pulse width of 7.5 μs. A delay time of 3 s was inserted between each accumulation to allow the reattainment of a steady state. On-resonance irradiated free induction decays (fids) were then subtracted from the off-resonance fid by using an interfaced Aspect computer, and the resultant difference fid Fourier transformed to yield a difference spectrum. Irradiation times were varied depending on the correlation time or exchange rate in the sample under study, and a short delay of 2 ms was inserted between termination of irradiation and acquisition. The magnitude of NOEs was established from difference spectra by dividing the intensity of the observed peak by that of the irradiated peak. In the case of the free nucleotide, NOEs were only used for assignment purposes and have not been quantified. NOEs in the complex were measured after 512 accumulations per spectrum and are judged accurate to within 3%, except for NOEs involving irradiation of methyl groups, which have an accuracy of 1%. Chemical shifts are measured in ppm downfield of internal sodium 4,4-dimethyl-4-silapentanesulfonate.

Models. Model building was performed with Nicholson framework models and CPK space-filling models.

Results

Assignment of the NMR Spectrum of d(A-G-C-T). The spectrum of d(A-G-C-T) at 0 °C is shown in Figure 2. The temperature dependence of the aromatic and anomeric proton chemical shifts can be used to monitor the state of association of the self-complementary tetramer, upfield shifts generally accompanying duplex formation. Chemical shift and spin coupling arguments can be used to assign some of the aromatic resonances. At temperatures above the extreme motional narrowing limit, the cytidine H-6 and H-5 show doublet structure while the thymine H-6 is broadened by four-bond coupling to the methyl protons on C-5. In the single-stranded state (above 30 °C) adenine H-8 resonates to lower field than the corresponding guanine proton, while adenine H-2 shows a particularly long T_1. The simple criteria on which these assignments are based, however, are inadequate in dealing with the anomeric resonances.

At 0 °C the aromatic chemical shifts suggest that the tetramer exists primarily as a duplex. Resonances are con-

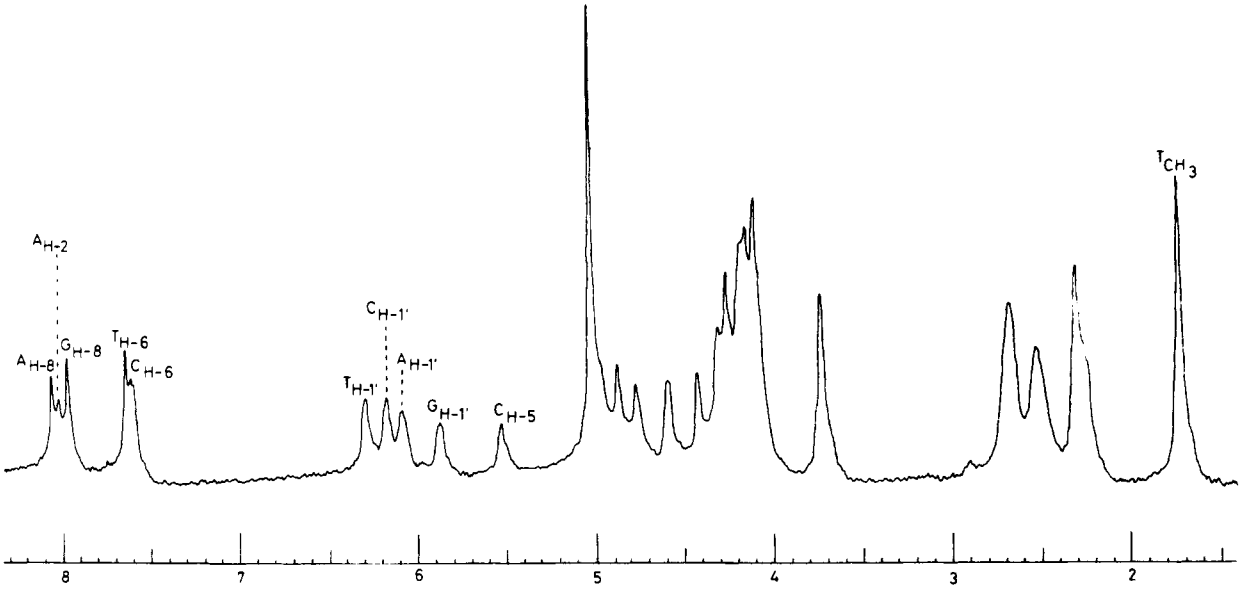

FIGURE 2: 400-MHz proton NMR spectrum of 2.4 mg of d(A-G-C-T) in D_2O at 0 °C, resulting from 100 fids.

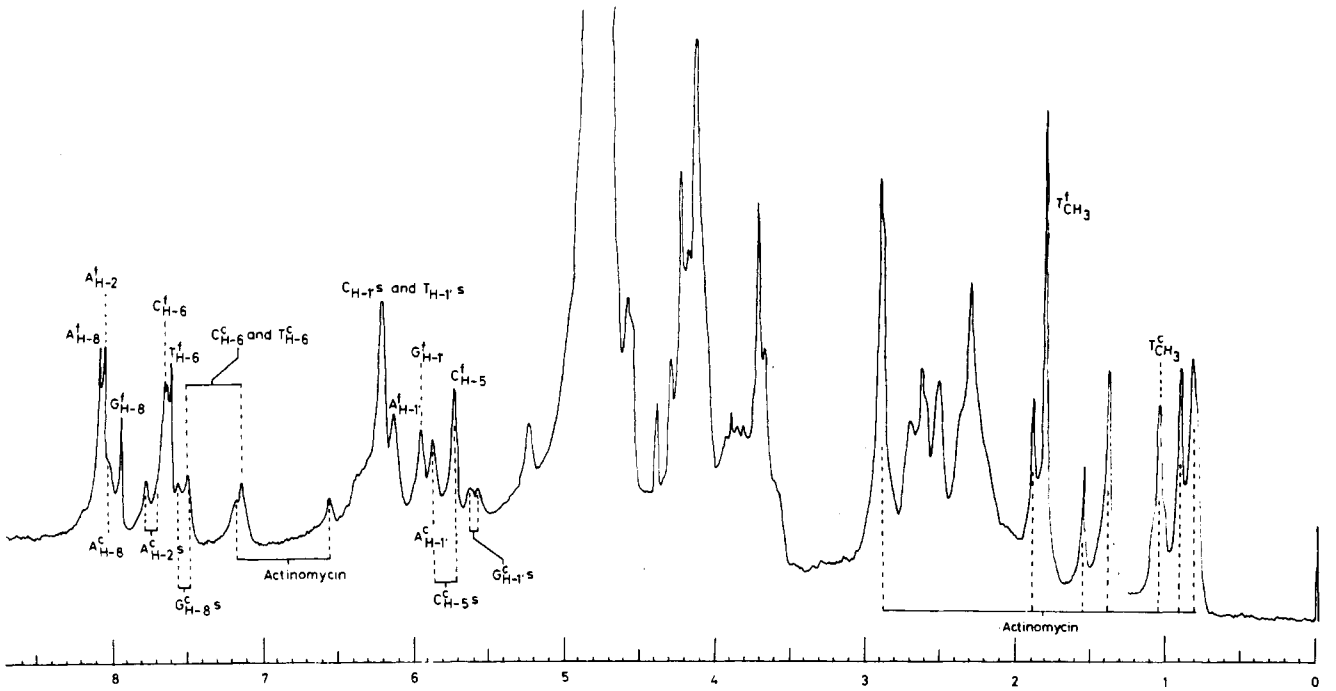

FIGURE 3: 400-MHz proton NMR spectrum of a mixture of actinomycin D and d(A-G-C-T) in relative concentrations corresponding to a 2:1 ratio of helix to drug. The temperature is 30 °C. Superscripts f and c identify resonances in the free nucleotide and the complex, respectively.

siderably broadened by an increased rotational correlation time. Under such conditions, negative NOEs can be of use in assigning the anomeric proton region of nucleotide oligomers of known sequence (Reid et al., 1983), and these are summarized in Table I. When A_{H-8} is irradiated for 0.5 s, an NOE is observed to only one of the four anomeric signals, which must correspond to $A_{H-1'}$. Irradiation of G_{H-8} causes NOEs to two anomeric protons, one of which at 6.08 ppm has already been indicated as $A_{H-1'}$. Therefore the other resonance at 5.87 ppm is $G_{H-1'}$. Separate irradiation of T_{H-6} and C_{H-6} is difficult, but when they are saturated simultaneously, NOEs are seen to the anomeric proton resonances at 6.23 and 6.17 ppm which must be $T_{H-1'}$ and $C_{H-1'}$, and to $G_{H-1'}$. $C_{H-1'}$ and $T_{H-1'}$ may be distinguished by the fact that an NOE to the former alone is expected when the thymine methyl resonance is irradiated. Thus the signal at 6.17 ppm is attributed to $C_{H-1'}$. These assignments are all confirmed by the pattern of NOEs resulting from irradiation of the anomeric resonances.

Assignment of Bound Nucleotide and Actinomycin Resonances. Addition of actinomycin up to a final concentration equivalent to one-quarter of that of the nucleotide strands (i.e., a 2:1 helix:drug ratio) at 30 °C produces the spectrum shown in Figure 3. Peaks due to the free oligonucleotide may be identified by comparison with the spectrum of the free tetramer at the same temperature in the absence of drug. Signals due to the drug–duplex complex are generally broader due to the slower tumbling rate of the complex. Below about 15 °C, the correlation time for tumbling of the complex becomes so long that signals due to the complex are severely broadened and hence obscured. Above 35 °C, signals due to free and bound duplex begin to coalesce as the exchange rate between free and bound states becomes intermediate.

When a signal assigned to the free oligonucleotide is irradiated, the saturation may be transferred by a chemical ex-

Table I: NOEs Observed in the Free Tetramer,[a] between Protons of the Bases and Anomeric Protons

	proton obsd									
proton irr	A_{H-8}	G_{H-8}	T_{H-6}	C_{H-6}	$T_{H-1'}$	$C_{H-1'}$	$A_{H-1'}$	$G_{H-1'}$	C_{H-5}	T_{CH_3}
A_{H-8}							X			
G_{H-8}							X	X	X	
T_{H-6}, C_{H-6}[b]					X	X		X		X
$T_{H-1'}$			X							
$C_{H-1'}$			X	X						X
$A_{H-1'}$	X	X								
$G_{H-1'}$		X		X						
C_{H-5}				X				X		X
T_{CH_3}			X	X		X				

[a] Peaks to which NOEs were observed after 0.5-s irradiation are marked with an X. All NOEs were negative. [b] Separate irradiation of T_{H-6} and C_{H-6} was impossible, although NOEs to these resonances could be readily distinguished.

Table II: Percentage NOEs in the Actinomycin–d(A-G-C-T) Complex[a]

	protons to which NOEs are observed (%)																						
protons irradiated	A_{H-8}[s]	A_{H-2}	$A_{H-2'}$	G_{H-8}[q]	G_{H-8},[b] C_{H-6}[s]	P_{H-8}	T_{H-6}[s]	P_{H-7}	$C_{H-1'}$[s]	$T_{H-1'}$[s]	C_{H-5},[b] $A_{H-1'}$[s]	C_{H-5}[q]	$G_{H-1'}$[q]	$G_{H-1'}$[b]	N-CH_3 of L-Val	N-CH_3 of sarcosine	P_{CH_3}-6	P_{CH_3}-4	Thr_{CH_3}	T_{CH_3}[s], L-Val_{CH_3}	D-Val_{CH_3}	L-Val_{CH_3}, D-Val_{CH_3}
A_{H-8}[s]											10												
A_{H-2}															15					5			
$A_{H-2'}$											(7)[b]		(2)		(30)					(3)			
G_{H-8}[q]																							
G_{H-8},[b] C_{H-6}[s]						7		8	7		17	6		7			5	4	8	23			
P_{H-8}					23			20	8		9			13									
T_{H-6}[s]									13	9										30			
P_{H-7}					26	29			4		12						41						
$C_{H-1'}$[s]					7										[22]								
$T_{H-1'}$[s]							11								[47]								
C_{H-5},[b] $A_{H-1'}$[s]	7				19	4	7	7									6						
C_{H-5}[q]					23													4					
$G_{H-1'}$[q]				13											16				3				
$G_{H-1'}$[b]					9	5									48				21				
N-CH_3 of L-Val / N-CH_3 of sarcosine		(5)	(5)								(3)		[(2)]						(6)	(8)	(9)	(14)	
P_{CH_3}-6					7			6			4							8					
P_{CH_3}-4				2								1	2				15						
Thr_{CH_3}									2		5		[11]		6								
T_{CH_3}[s], L-Val_{CH_3}									1		1	1										14	
D-Val_{CH_3}															~2	11							large
L-Val_{CH_3}, D-Val_{CH_3}															~5	13			2	4	large		

[a] A positive percentage represents a negative NOE, observed after 200-ms irradiation. [b] Where two or more resonances are distinct but too close to be separately irradiated, this is conveyed by parentheses. [c] Where resonances are so close that it is impossible to resolve the NOE transmitted to one from that transmitted to the other, this is conveyed by brackets.

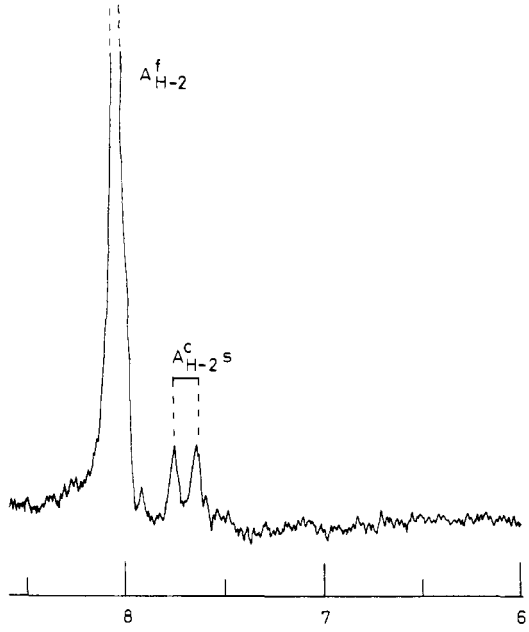

FIGURE 4: Saturation transfer difference spectrum resulting from irradiation at 30 °C of the free A_{H-2} signals. The nondegeneracy of the bound A_{H-2} protons is clearly seen.

change mechanism to corresponding signals in the complex, causing a diminution in the integrated intensity of these peaks. If spectra are acquired in which a free nucleotide resonance is irradiated in alternation with irradiation at a control frequency not corresponding to any free or bound peak, transfer of saturation may be observed by subtracting the former from the latter to produce a difference spectrum. A typical saturation transfer difference spectrum is shown in Figure 4. The technique may be complicated by the generation of negative NOEs either within the free nucleotide or within the complex from inadvertent irradiation of overlapping bound resonances. Chemical exchange saturation transfer is accelerated by an increase in temperature whereas the rate of NOE buildup, which is proportional to the molecular rotational correlation time, is lowered. When irradiations are performed at two different temperatures, the two effects may be resolved.

When the signal due to free A_{H-8} is irradiated for 1 s at 30 °C, equal saturation transfer is observed to two bound resonances, one at 7.79 ppm and the other to a peak obscured in the normal spectrum by free C_{H-6}. The width of the pulse used to saturate free A_{H-8}, however, causes considerable saturation of free A_{H-2}, and when this latter peak is separately irradiated, a greater reduction in intensity of the same two bound peaks is observed than accompanied free A_{H-8} irradiation. Moreover, when the complex resonance at 7.79 ppm is irradiated, only free A_{H-2} is affected. If the experiment is repeated at 23 °C, although the free A_{H-8} and A_{H-2} signals overlap, a reduction in saturation transfer rate clearly indicates a chemical exchange mechanism. The observation of two distinct bound A_{H-2} signals is due to the abolition of the 2-fold symmetry of the free helix on interaction with the asymmetric phenoxazone moiety of the drug. The identity of the bound A_{H-8} signals is not definitely established by this technique, but the broad signal to slightly higher field of free A_{H-8} and A_{H-2}, which is too close to these latter signals to be unperturbed when they are saturated, is tentatively ascribed to the A_{H-8} resonances in the complex.

Saturation of the free G_{H-8} at 30 °C establishes that the two signals at 7.58 and 7.52 ppm are due to bound G_{H-8}s. Once again the phenoxazone chromophore has rendered the two G_{H-8} protons chemically nonequivalent. Separate irradiation of C_{H-6} and T_{H-6} is not possible, but when they are simultaneously saturated, the peaks at 7.52 and 7.15 ppm are reduced, although the signal at 7.58 ppm is unaffected. Saturation transfer is also observed to free A_{H-2} due to saturation of the bound A_{H-2} resonance coincident with free C_{H-6}. Negative NOEs are observed to free C_{H-5} and T_{CH_3}. These results suggest that bound C_{H-6} and T_{H-6} contribute to the signals at 7.52 and 7.15 ppm, although assignment of either signal to a specific base type is not possible by this technique.

At 30 °C the free $C_{H-1'}$ and $T_{H-1'}$ signals are coincident. When they are simultaneously irradiated no effect ascribable to saturation transfer is observed elsewhere in the spectrum, suggesting that actinomycin binding does not significantly perturb the pyrimidinyl anomeric proton chemical shifts. However, irradiation of free $A_{H-1'}$ causes a clear transfer of saturation to a complex resonance at 5.88 ppm, while similar treatment of the free $G_{H-1'}$ peak identifies the two signals at 5.63 and 5.58 ppm with these protons in the bound state. The two guanosyl anomeric positions are rendered nonequivalent by the drug chromophore. Irradiation at free C_{H-5} identifies the signal at 5.68 ppm with this proton in the bound state, although further addition of drug shows that bound C_{H-5} also contributes to the resonance which is irradiated. Thus the C_{H-5} sites are also differentially perturbed by interaction with the drug. The thymine methyl groups shift to high field on complexation, and their broad resonance contributes to the peak at 1.03 ppm.

When more actinomycin is added, the signals ascribed to the free nucleotide diminish in intensity and finally disappear at a tetramer to drug ratio of 2:1. The spectrum of the complex at 23 °C is shown in Figure 5. The methyl groups on the drug chromophore give rise to the two relatively sharp signals at 1.83 and 1.50. Their absolute identity will be established by NOE experiments. The threonyl methyl groups on each pentapeptide ring produce the peak at 1.36 ppm. The valine methyl groups give rise to three distinct signals of relative intensity (from low to high field) 1:1:2. The signals at 2.95 and 2.88 ppm are due to the two N-methylated amide linkages. The protons directly attached to the aromatic system of the drug resonate at 7.10 and 6.50 ppm and can be assigned to specific positions by use of NOEs. The identity of the broad, apparently structured peak at 6.38 ppm is not immediately obvious. Peptide signals in the region of the spectrum obscured by the sugar proton resonances have not been assigned.

NOE Studies on the Actinomycin–d(A-G-C-T) Complex. The size of the drug–helix complex causes broadening of its NMR spectrum due to its long rotational correlation time. Under such conditions of slow molecular tumbling, spin diffusion is considerable and can complicate NOE measurements (Kalk & Berendsen, 1976) unless irradiation times are short enough to allow only first-order effects to be observed (Wagner & Wüthrich, 1979). Table II lists NOEs resulting from 200-ms irradiations. Some representative NOE difference spectra are shown in Figure 6. Effects were observed to methylene and methine resonances, but as their assignments are not at all straightforward, they have not been included in the results.

When the lower field (7.10 ppm) single-proton resonance of the phenoxazone chromophore is irradiated, a strong NOE is observed to the higher field signal of this moiety (6.50 ppm). When the resonance at 6.50 ppm is saturated, strong NOEs are seen to the signals at 7.10 and 1.83 ppm, whereas irradiation of the chromophore methyl group signal at 1.83 ppm causes NOEs to the single proton resonance at 6.50 ppm and

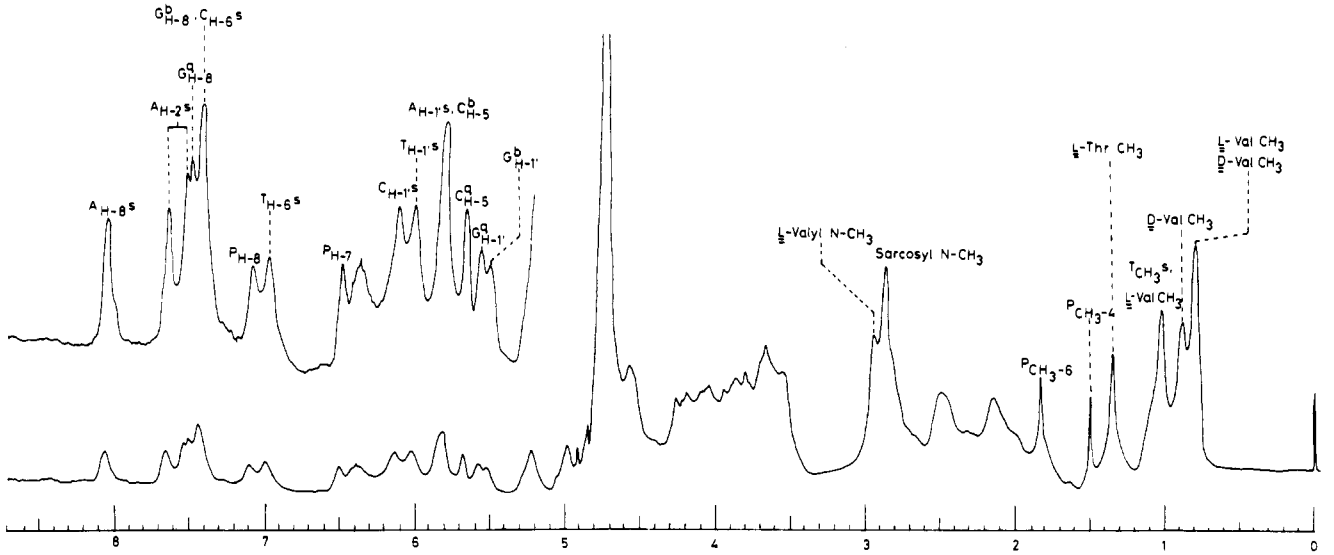

FIGURE 5: 400-MHz proton NMR spectrum of a mixture of actinomycin D and d(A-G-C-T) in relative concentrations corresponding to a 1:1 drug:helix ratio at 23 °C.

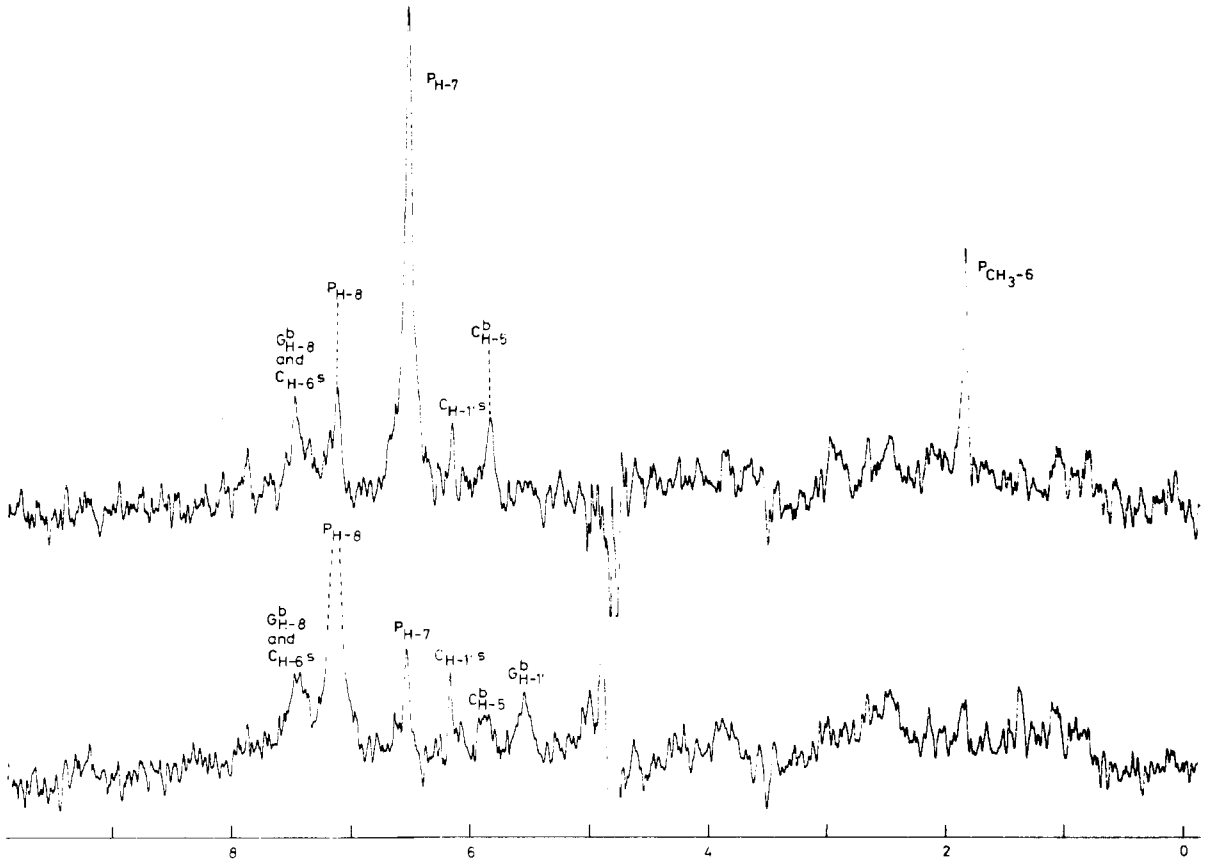

FIGURE 6: Some typical NOE difference spectra. The bottom spectrum results from a 200-ms irradiation of the P_{H-8} signal and the top one from a similar irradiation at the P_{H-7} resonance.

to the other chromophore methyl group. This evidence establishes the signal at 7.10 ppm as the proton on the phenoxazone 8-position (hereafter referred to as P_{H-8}), that at 6.50 ppm as P_{H-7}, and the methyl signals at 1.83 and 1.50 ppm as P_{CH_3-6} and P_{CH_3-4}, respectively.

Besides these intramolecular effects, NOEs are also observed from the drug to protons previously assigned to the bound form of the nucleotide duplex. Thus on irradiation of P_{H-8} a considerable effects is observed on the signal corresponding to overlap of one of the guanine C-8 protons with either the cytosine or thymine C-6 protons. Also affected are two anomeric signals, one corresponding to the higher field of the two $G_{H-1'}$ protons and the other to either $C_{H-1'}$ or $T_{H-1'}$ in the complex. The latter signals cannot be distinguished by saturation transfer since they are coincident in the free form and insufficiently perturbed by complexation. Similarly, saturation of P_{H-7} significantly perturbs the same large peak corresponding to one G_{H-8} and the C_{H-6} or T_{H-6} protons, and either the $C_{H-1'}$ or $T_{H-1'}$ signals. The composite peak at 5.82 ppm previously ascribed to the $A_{H-1'}$ protons and one C_{H-5} is also reduced in intensity. Both of these overlapping signals show a diminution when P_{CH_3-6} is irradiated. However, irradiation of P_{CH_3-4} affects the lower field G_{H-8}, the lower field of the two resolved $G_{H-1'}$ resonances, and the C_{H-5} furnishing the

single proton resonance at 5.68 ppm.

These results allow some deductions to be made about the actinomycin binding site. The fact that both sugar and base resonances of deoxyguanosine are perturbed by irradiation of chromophore protons shows that these moieties are in close proximity in the complex. Furthermore, both the higher field G_{H-8} and $G_{H-1'}$ are adjacent to the benzenoid portion of the phenoxazone, while the lower field G_{H-8} and $G_{H-1'}$ are close to the quinoid. The former are identified in subsequent discussion by a superscript "b" and the latter by a superscript "q". The identity of the other protons contributing to the peak containing $G_{H-8}{}^{b}$ is established by the considerable reduction in intensity experienced by this peak when either of the two C_{H-5} signals is saturated. They are clearly due to the two C_{H-6} protons, while the peak at 7.00 ppm which shows a strong NOE to the T_{CH_3} resonance may be ascribed to the T_{H-6} protons. The large reduction in intensity of the composite peak at 7.43 ppm when either P_{H-8} or P_{H-7} is irradiated may be explained by the transmission of NOEs to both $G_{H-8}{}^{b}$ and $C_{H-6}{}^{b}$.

Thus the cytidylyl residue is clearly also close to the chromophore in the complex. The higher field of the two C_{H-5}s to which an NOE is observed from P_{CH_3-6} is thus identified as $C_{H-5}{}^{q}$, while the other C_{H-5} overlapping with the $A_{H-1'}$s is $C_{H-5}{}^{b}$. It is apparent that the guanosyl and cytidylyl residues of the nucleotide are most directly involved in the binding of the phenoxazone portion of the drug.

Intranucleotide NOEs provide useful information about the structure of the complex. Irradiation of the A_{H-8} resonances results in an NOE transmitted to the adenosyl anomeric protons, while these latter and the $G_{H-1'}$ signals are also affected by irradiation of the G_{H-8} resonances. The observation of intraresidue NOEs such as those between the G_{H-8} and $A_{H-1'}$ protons confirms that this part of the duplex has retained a helical conformation in the complex broadly similar to its unbound conformation. In the free helix, NOEs are observed between the C_{H-6} and $G_{H-1'}$ protons. When $G_{H-1'}{}^{b}$ is irradiated, NOEs *are* seen to the peak corresponding to $G_{H-8}{}^{b}$ and the C_{H-6} protons, although overlap of the signals makes it difficult to resolve a possible effect on the latter from the expected NOE to the guanosyl H-8. However, $G_{H-1'}{}^{q}$ irradiation produces a clear NOE to $G_{H-8}{}^{q}$ but not to the signal corresponding to the C_{H-6} protons. Thus, at least in the case of the residues adjacent to the quinoid side of the chromophore, the conformation which placed $G_{H-1'}$ and C_{H-6} is close proximity in the free duplex has been altered by complexation to move these protons further apart.

It has previously been mentioned that distinction between the $C_{H-1'}$ and $T_{H-1'}$ protons, which are only slightly perturbed by drug binding, is difficult. However, when the anomeric signal at 6.02 ppm is irradiated, an NOE is observed only to the peak at 7.00 ppm previously ascribed to the T_{H-6} protons on the grounds of a strong NOE between this resonance and that of the thymine methyls. In the free helix the thymidyl anomeric proton is close to only a single aromatic proton, namely, thymine H-6. When the anomeric signal at 6.13 ppm is saturated, however, an NOE is seen to the C_{H-6} resonance, suggesting that the former resonance is due to the $C_{H-1'}$ protons. In the free duplex an NOE may be seen between the $C_{H-1'}$ and T_{CH_3} and T_{H-6} resonances, but these NOEs are abolished by actinomycin binding. Similarly NOEs between C_{H-5} and T_{CH_3} in the free helix are not observed in the complex. These observations suggest that a conformational change has taken place on drug binding which alters the relative orientation of the cytidyl and thymidyl residues and has the effect of increasing the distance between the 5, 6, and 1′ substituents of the two nucleotides.

Other intermolecular NOEs are observed which serve to clarify the mode of interaction further. When either of the two separate A_{H-2} signals is irradiated, a distinct effect is observed to only one of the two *N*-methyl groups of the cyclic pentapeptide backbone, as well as weaker effects to the peak corresponding to overlap of the thymine methyls and a pair of actinomycin valyl methyl groups. Other nucleotide protons which show NOEs to identifiable nuclei on the pentapeptide backbone of the drug are the anomerics. The $T_{H-1'}$ and $C_{H-1'}$ protons show NOEs to both of the drug *N*-methyl groups. NOEs are also observed between the $G_{H-1'}$ protons and the same *N*-methyl group which interacted with the A_{H-2} protons, and from this methyl group to the $A_{H-1'}$ protons. Irradiation of the threonyl methyl groups strongly affects both $G_{H-1'}$ resonances and has a weaker influence on the $A_{H-1'}$ and $C_{H-1'}$ resonances. The Thr_{CH_3} signals are likewise affected when the $G_{H-1'}$ resonances are saturated, but any NOE from the adenosyl or cytidylyl anomerics is too weak to be resolved.

The broadness of the drug resonances at the temperatures used in this study obscure any details of spin–spin coupling which might assist in further assignment of the peptide resonances. The dissociation of the complex and resultant precipitation of actinomycin preclude experiments at elevated temperatures. However, irradiation of the threonyl methyl groups enables distinction at least between the two pairs of *N*-methyl groups by causing a distinct NOE to the lower field of the two signals. Examination of molecular models establishes that only the *N*-methyl group of the methylated L-valine is sufficiently close to the threonyl methyl to be affected. This leaves the higher field signal to be assigned to the methyl group of sarcosine. The threonyl methyl groups, however, lie at a considerable distance from any of the other valyl methyls, whose assignment is possible only when intermolecular NOEs are considered and the mode of drug binding has been clarified.

Discussion

The origin of the specificity of actinomycin D for G-C rich DNA has been convincingly demonstrated by two X-ray studies (Jain & Sobell, 1972; Takusagawa et al., 1982). Neither complex, with two molecules of deoxyguanosine and d(G-C), respectively, contained a recognizable helical DNA fragment, but in each case guanine residues, stacked against the phenoxazone chromophore, were held by pairs of hydrogen bonds. These were formed between N-2 and N-3 of guanine and the amide nitrogen and carbonyl oxygen of threonyl residues in the cyclic pentapeptide portion of the drug and may be invoked to account for its specificity. The actinomycin molecule itself was held relatively rigidly by a pair of hydrogen bonds between D-valine residues in each cyclic peptide to achieve an overall shape strikingly adapted to intercalation into a right-handed DNA helix.

Our results are consistent with these predictions on the nature of the complex with DNA. Intercalation between G-C base pairs increases the perpendicular distance between them and so accounts for the abolition of NOEs between adjacent G and C residues seen in the free nucleotide. In the Berman and Neidle structure (Takusagawa et al., 1982), a guanine H-8 is positioned almost perpendicularly above the phenoxazone 7-substituent and is thereby in close proximity to the 6- and 8-positions as well. The cytosine stacked on the opposite side of the phenoxazone from this guanine residue interacts to a much lesser extent with the chromophore, as a consequence of which its 5 and 6 protons are rather more distant from the 6, 7, and 8 phenoxazone substituents. Also at a greater dis-

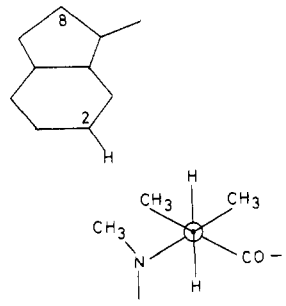

FIGURE 7: A view down from the L-valyl C_β–C_α bond, showing the orientation which places one methyl group in close proximity to A_{H-2}.

tance from these drug protons in the crystal are the guanosyl and cytidylyl anomerics, although the NOE results show that the latter protons are certainly in proximity to P_{H-8}. Also consistent with this crystal structure are the NOEs observed between $G_{H-8}{}^q$ and $C_{H-5}{}^q$ and P_{CH_3-4}, although the NOE from the latter to $G_{H-1'}{}^q$ is unexpected.

The specific hydrogen bonds observed in the solid-state investigations between the guanine moiety of the binding site and the peptide backbone of the drug have certain implications regarding the orientation of the latter with respect to the nucleotide helix. The guanine hydrogen bonding sites, viz., the amino and N-3 groups, project into the minor groove. The two pentapeptide rings of the drug are locked into fairly rigid conformations by inter-ring hydrogen bonds. These specific interactions seem to determine a drug binding mode in which the peptide backbone fits into the helix minor groove somewhat distorted by binding. NOEs between nucleotide substituents and the peptide rings confirm this supposition.

Examination of either of the published structures, and of molecular models, shows that the *N*-methyl groups of the methylated L-valine residues project from the pentapeptide rings in a plane roughly parallel to that containing the phenoxazone group. This projection places the methyl groups in close proximity to the narrow groove substituents of any nucleotide stacked above the guanine ring. This proximity accounts for the NOEs observed between these methyl groups and the A_{H-2} protons, and from the methyls to the $A_{H-1'}$ protons, all of which project into the minor helical groove. NOEs are also observed between the L-valyl *N*-methyl groups and both $G_{H-1'}$ protons, which also project into the narrow groove in such a way as to be close to the methyls. The A_{H-2} protons also show an NOE to one of the valine β-methyl signals. If the L-valine side chain is allowed to adopt a stereochemically favorable orientation in which the α proton is staggered between the two β-methyl groups, molecular models show that one of these groups is in proximity to an A_{H-2} proton (Figure 7). The NOEs may thus be invoked to assign the other valyl β-methyl groups tentatively. The group displaying the NOE to the A_{H-2} resonance (1.03 ppm) also shows a large NOE to the signal at 0.81 ppm which must therefore contain the signal from the other L-valyl β-methyl group. Intensity is also contributed to this peak by a D-valyl methyl and explains the NOE to what must correspond to the other D-valyl signal at 0.89 ppm.

In summary, the NOEs are entirely consistent with a binding mode in which the actinomycin chromophore intercalates between the two G-C base pairs with the peptide backbone situated in the minor groove of the double helix. At this stage, it is in order to try to characterize any changes in helix geometry induced by drug binding by a consideration of the NOEs and chemical shifts. It is immediately apparent that those resonances which shift most are not necessarily those closest to the binding sites, a fact which underlines the doubtful nature of any characterization of binding which depends on induced shifts alone.

For instance, the thymine methyl groups, which are unequivocally assigned by saturation transfer, and H-6 proton resonances experience upfield shifts of about 0.7 and 0.6 ppm, respectively. The corresponding cytidine substituents, however, display much smaller drug-induced perturbation. These observations may be explained by a distortion of the helix which is not confined to the widening of the separation between the G-C base pairs, but which affects the orientation of the A-T pairs relative to them as well. The flanking A-T pairs are within the length of helix occupied by the drug molecule and are in close contact as shown by NOEs inter alia between *N*-methyl valine and the A_{H-2} protons. Clearly the 5- and 6-positions of the thymine and 2- and 1′-positions of adenosine have been moved into areas of considerably higher shielding. If the A-T pair is twisted so as to decrease the local winding angle to about 10° at this point, the thymine 5- and 6-positions are moved over the ring-current shielding zone of the cytidine, while A_{H-2} is increasingly shielded by the six-membered ring of guanine. The structure of Takusagawa et al. (1982) shows that the phenoxazone interacts primarily with the guanines of the G-C pairs. This interaction is maximized in our model by reducing the local winding angle between the G-C base pairs while simultaneously moving them apart to a separation of about 7.0 Å to accommodate the chromophore. In this structure, the thymine 5- and 6-positions are oriented roughly perpendicularly above the strong shielding zone of the intercalated moiety. The proposed distortions also help to explain why binding eliminates the NOEs observed between the cytidylyl and thymidyl residues in the free helix. The twisting of the A-T relative to the G-C base pairs moves the 5-, 6-, and 1′-substituents of the two pyrimidines further apart. However, this action also moves G_{H-8} and $A_{H-1'}$ closer together, and indeed strong NOEs are observed between these protons. In Figure 8, some of the observed NOEs are related to the structure of the whole complex.

The NMR results are capable of resolving the asymmetry of the complex which results when interaction of the unsymmetrical chromophore abolishes the 2-fold symmetry of the nucleotide duplex. This was not observed in X-ray studies of the d(G-C) complex where static disorder in the crystal caused the actinomycin to appear symmetrical. Thus any information regarding possible effects of the asymmetry of the chromophore on the mode of binding and resultant nucleotide structure is lost in the solid-state study but is potentially accessible from the NMR results. In this regard it is interesting to note that an NOE is observed from P_{CH_3-4} to $G_{H-1'}{}^q$ but not from P_{CH_3-6} to $G_{H-1'}{}^b$. This observation may have an explanation in the fact that the relative contribution of P_{CH_3-6} to the relaxation of $G_{H-1'}{}^b$ is negligible compared to those from P_{H-7} and P_{H-8}. However, it is interesting to speculate whether this NOE may be due to a somewhat asymmetric chromophore orientation in which the quinoid moiety is less overlapped with the guanine and projects more into the minor groove, bringing P_{CH_3-4} and $G_{H-1'}{}^q$ into proximity. To establish whether this speculation is valid would require reliable determinations of internuclear distances. These are accessible in principle from measurements of NOE buildup rates. However, the large line widths exhibited by the complex make such studies extremely time consuming.

Conclusion

Study of a relatively well-characterized system such as the interaction of actinomycin D with DNA is an appropriate test

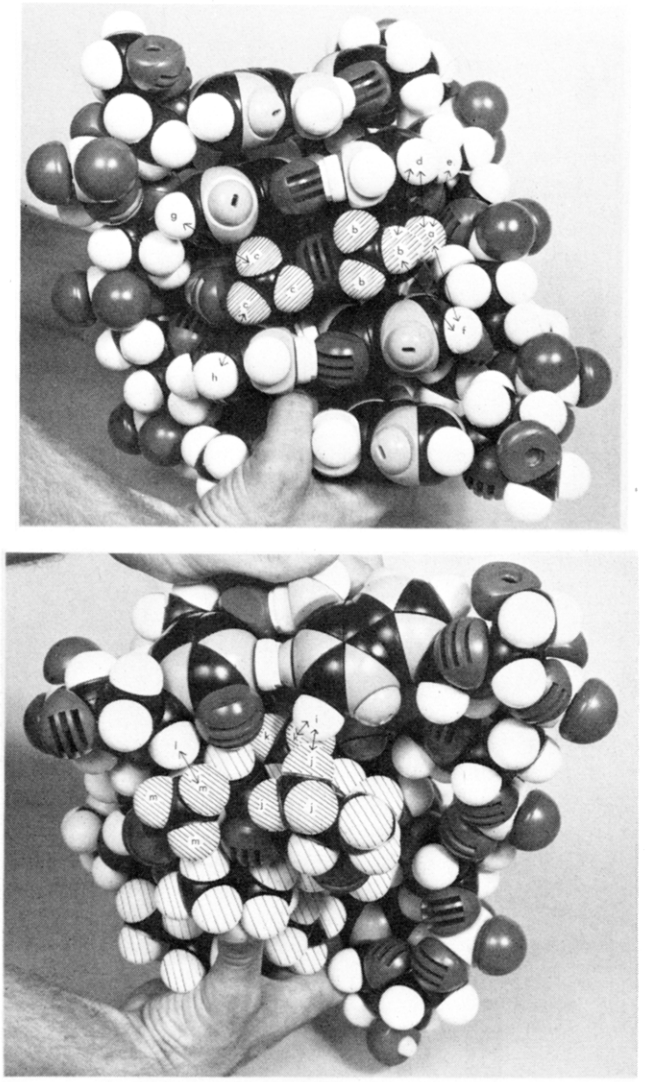

FIGURE 8: Two views of the entire drug–tetramer complex, in which the drug protons are identified by diagonal lines. Arrows connect drug and nucleotide protons between which NOEs are observed. (Top panel) A view perpendicular to the helix axis into the major groove. The identity of the individual groups is as follows: (a) P_{H-7}; (b) P_{CH_3-6}; (c) P_{CH_3-4}; (d) $C_{H-5}{}^{b}$; (e) $C_{H-6}{}^{b}$; (f) $G_{H-8}{}^{b}$; (g) $G_{H-8}{}^{q}$; (h) $C_{H-5}{}^{q}$. (Bottom panel) A view into the minor groove oblique to the helix axis: (i) A_{H-2}; (j) L-Val_{CH_3}; (k) L-Val_{N-CH_3}; (l) $T_{H-1'}$; (m) Sar_{N-CH_3}.

for application of any new structural probe to nucleotide complexes. NOEs in this complex are clearly consistent with intercalation of the drug between the central dinucleotide of the tetramer d(A-G-C-T). They also confirm close contacts in the minor groove surrounding proposed sites of hydrogen bonding which account for specific binding of the drug to G-C sequences in DNA. In addition to these well-described features, interactions are indicated between hydrophobic regions of the nucleotide and the peptide portions of the drug as well as distortion of the helix produced on complex formation. We conclude that NOEs represent a powerful method for studying the structure of DNA–ligand complexes in model systems and that the technique can be usefully applied to the study of DNA–protein interactions.

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Registry No. Actinomycin D–d(A-G-C-T) complex, 84520-46-7.

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