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Nuclear Magnetic Resonance Study on the Interaction of Aclacinomycin-A with a Deoxyribo-hexanucleotide Pentaphosphate $d(CCTAGG)_2$ in Aqueous Solution

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The interaction of aclacinomycin-A (ACM) with a deoxyribo-hexanucleotide pentaphosphate $d(CCTAGG)_2$ in aqueous solution was studied by ^1H, ^{31}P nuclear magnetic resonance techniques and by visible absorption spectroscopy. From the visible absorption spectra, ACM was found to be bound by polynucleotides with binding constants of the order of 10^6, in the order poly(dA-dT) > poly(dC) + poly(dG) > poly(dA-dG) + poly(dT-dC). With gradual addition of ACM to d(CCTAGG), two ^{31}P resonances newly appeared in the lower field region as compared with d(CCTAGG) in the non-complex state, while the ^{31}P resonance assignable to the TpA portion in the non-complexed state decreased in intensity. From these results, the chromophore of ACM is expected to be intercalated in the TpA portion of the oligo-deoxyribonucleic acid duplex. The line widths of the exchangeable proton resonances of complex in H_2O were examined as a function of the temperature. The results suggested that ACM stabilizes the helix of $d(CCTAGG)_2$ at a region around the intercalation site.

Keywords——aclacinomycin; DNA; NMR; interaction

Aclacinomycin-A (ACM) (Fig. 1), isolated from a culture of *Streptomyces galilaeus* MA 144-M1, has antitumor activity against various experimental tumors with low cardiac toxicity.[1] This activity is considered to be related with an intercalation of the molecule between adjacent nucleotides in the deoxyribonucleic acid (DNA) strand,[2,3] although it has also been suggested that the inhibition is produced mainly by a direct interaction of the drug

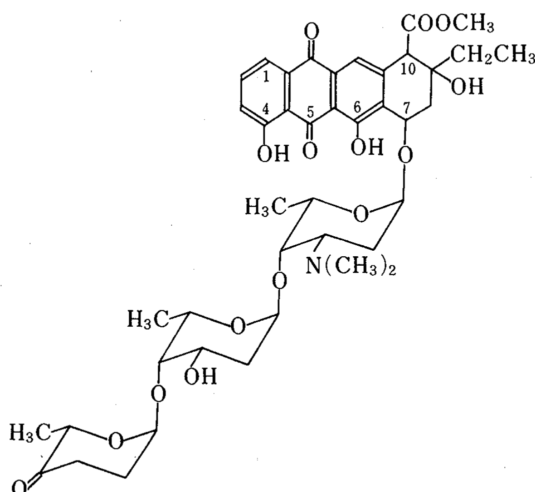

Fig. 1. Structure of Aclacinomycin-A

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with enzymes.[4,5] Direct proof that the chromophore of an anthracycline antibiotic can be intercalated between DNA bases was provided by Quigley *et al.* in their crystallographic study of a daunomycin–d$(CGTACG)_2$ complex.[6]

For studying such an intercalation in aqueous solution, on the other hand, the nuclear magnetic resonance (NMR) technique would be powerful. There have been some studies on the interactions of daunomycin with poly (dA-dT),[7,8] with oligonucleotides containing m^5C and G,[9,10] and with d$(pTpA)_3$[11] by the use of ^{1}H-NMR techniques. Here, however, ^{31}P-NMR would be advantageous because signals are given only by the nucleotides, and the problem of overlap of the resonances of the oligonucleotide and the drug can be avoided. The recent development of the two-dimensional (2D)-NMR method has made the assignments of ^{31}P resonances of oligonucleotides possible.[12] We have previously succeeded in assigning the ^{31}P resonances of d(CCTAGG).[13] An examination of the interaction of ACM with d$(CCTAGG)_2$ by the ^{31}P-NMR technique is presented in this paper. The NMR study of the exchangeable protons in H_2O solution, where the overlap of the signals is not severe, is also expected to give information on the flexibility and stability of the DNA helix. On the basis of the results, we discuss the stability and conformation of the ACM–d(CCTAGG) complex.

Experimental

Materials——Deoxiribo-hexanucleotide pentaphosphate d(CCTAGG) was synthesized and purified according to the method given in the previous paper.[13] Poly(dA-dT). poly(dC)–poly(dG), and poly(dA-dG)–poly(dT-dC) were purchased from Pharmacia P-L Biochemicals. ACM was a kind gift from Prof. H. Umezawa (Institute of Microbial Chemistry). For the ^{31}P-NMR observation, the sample was dissolved in 0.5 ml of 99.95% D_2O. For the NMR observation of exchangeable protons, the sample was dissolved in 0.5 ml of 90% H_2O + 10% D_2O. Each sample solution contained 0.1 M NaCl.

Method——NMR spectra were obtained on a JEOL GX-400 spectrometer operating at 399.95 MHz for ^{1}H and 161.70 MHz for ^{31}P. DSS (2,2-dimethyl-2-silapentane-5-sulfonate) for ^{1}H and TMP (trimethyl phosphate) for ^{31}P were used as external references. The exchangeable proton resonances were obrained by the use of the Redfield 2-1-4 pulse sequence[14] and 10000 Hz spectral width was used for the exchangeable ^{1}H-NMR. Visible absorbance spectra were recorded on a Shimadzu UV-260 spectrophotometer.

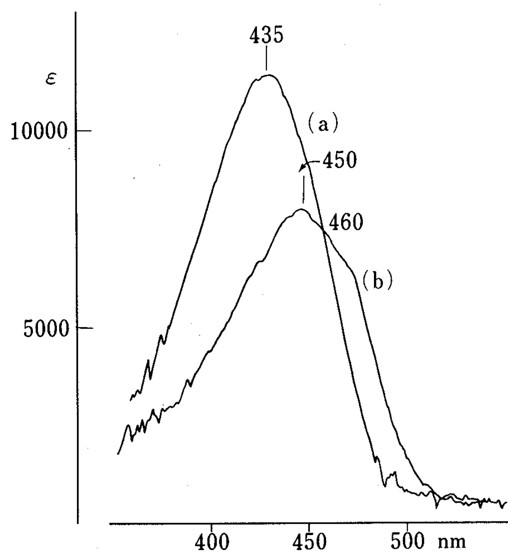

Fig. 2. Visible Absorbance Spectra of (a) ACM and (b) ACM with Excess of Poly(dA-dT) in Aqueous Solution at Room Temperature, 0 M NaCl

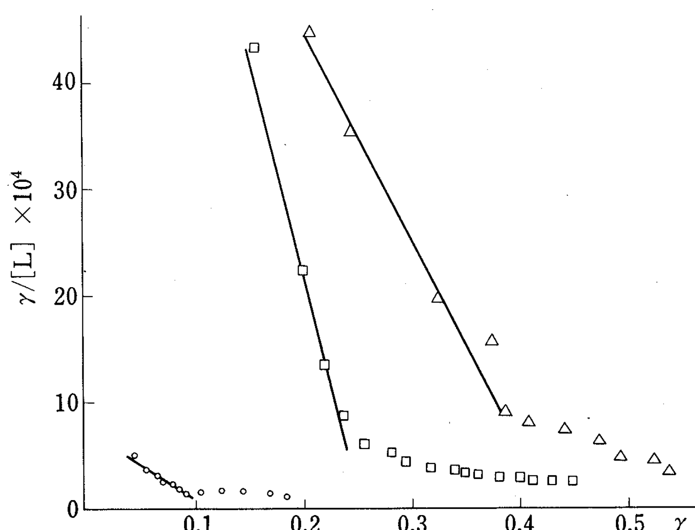

Fig. 3. A Scatchard Plot of the Binding of ACM with □, Poly(dA-dT); △, Poly(dC)–Poly(dG); ○, Poly(dA-dG)–Poly(dT-dC) at Room Temperature

γ means the number of moles of ACM bound per mole of DNA phosphate.

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TABLE I. The Binding Constants (K) of ACM with Polynucleotides (Upper), and the Hypochromicity at 435 nm (Lower)

	$d(CCTAGG)_2$	poly(dA-dT) poly(dA-dT)	poly(dG) poly(dC)	poly(dA-dG) poly(dT-dC)
Binding constant (M^{-1}) $(K)\times 10^{-5}$		38	19	7
Hypochromicity (%)	45	45	33	33

Results

(1) Visible Absorbance Spectra

Before the NMR examination, the binding constants of ACM with polynucleotides were preliminarily determined. Three kinds of polynucleotides, poly(dA-dT), poly(dC)–poly(dG), and poly(dA-dG)–poly(dT-dC), were chosen because these three provide three different types of intercalation sites between adjacent base pairs which are involved in $d(CCTAGG)_2$. Figure 2 shows the visible absorbance spectra of free ACM (a) and ACM bound with excess of poly(dA-dT) (b). The binding of ACM to poly(dA-dT) results in a red shift of the absorbance maximum from 435 to 450 nm and a hypochromism at 435 nm. ACM was added stepwise to an excess of this polynucleotide. The result of this absorbance titration examination is presented in the form of a Scatchard plot[15] in Fig. 3 (□). The figure includes the results for the other two polynucleotides. From the slope of the linear parts in this plot, the binding constants (K) of ACM with the polynucleotides can be obtained. These results are summarized in Table I. The hypochromicity of ACM bound to d(CCTAGG) is also given in Table I. This table indicates that the binding of ACM with poly(dA-dT) has the highest constant and the same hypochromicity as in the case of $d(CCTAGG)_2$. Thus it is suggested that ACM prefers the TpA portion among the three possible intercalation sites in $d(CCTAGG)_2$.

(2) ^{31}P-NMR

Figure 4 shows the 161.7 MHz ^{31}P-NMR spectra of d(CCTAGG) (11 °C) in the presence of various amounts of ACM. The assignments of the resonances of d(CCTAGG) in the absence of ACM were made in the previous paper,[13] and are given at the top of Fig. 4. As ACM is added to d(CCTAGG), two resonances appear in the 1 and 1.5 ppm lower field region than those of d(CCTAGG) itself. These large downfield shifts were also observed in the case of actinomycin-D–DNA complex by Patel and his coworkers.[16,17] It is suggested that the drug is intercalated between adjacent bases. The two newly observed resonances (see Fig. 4) are assignable to the two phosphodiester linkages belonging to the partner strands facing the chromophore ring of ACM. The chemical shifts of these two ^{31}P's may well be different (-3.2 and -2.5 ppm), because the ACM molecule is asymmetric and therefore the ACM–d(CCTAGG) complex is also asymmetric.

The spectral feature in the upfield region (around -4 ppm), on the other hand, is expected to be a simple spectral sum of phosphorus atoms of free d(CCTAGG) and the complex d(CCTAGG)+ACM because the exchange between the free and complex states would be slow in comparison with the NMR time scale. Actually, as may be seen in Fig. 4, the signal intensity of TpA phosphorus of free d(CCTAGG) decreases markedly as ACM is added, while the others remain almost unchanged. It is concluded that the newly observed lower field resonances (-3.2 and -2.5 ppm) are assignable to TpA phosphorus in the complex state.

On the basis of the experimental results given in section (1) and (2), it is strongly

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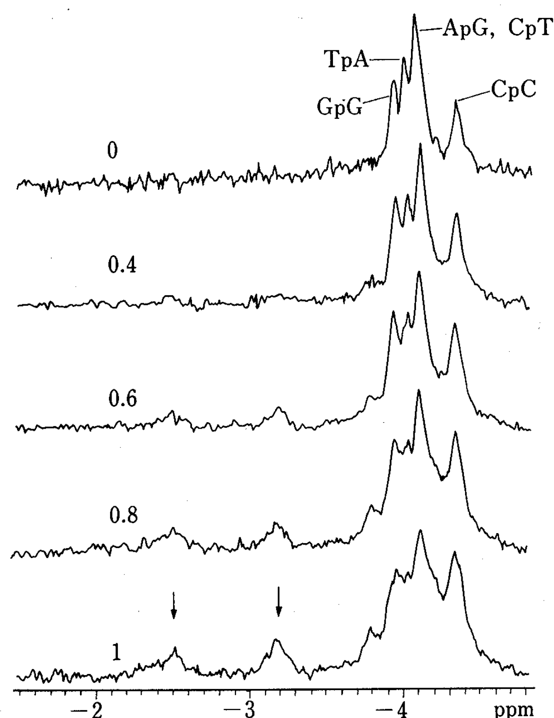

Fig. 4. The 161.7 MHz ^{31}P-NMR Spectra of d(CCTAGG) (Top) and d(CCTAGG) Plus Various Amounts of ACM

The figures at the left side indicate the molar ratio of added ACM *versus* d(CCTAGG) double-strand (the concentration of the latter is 700 OD/ml).

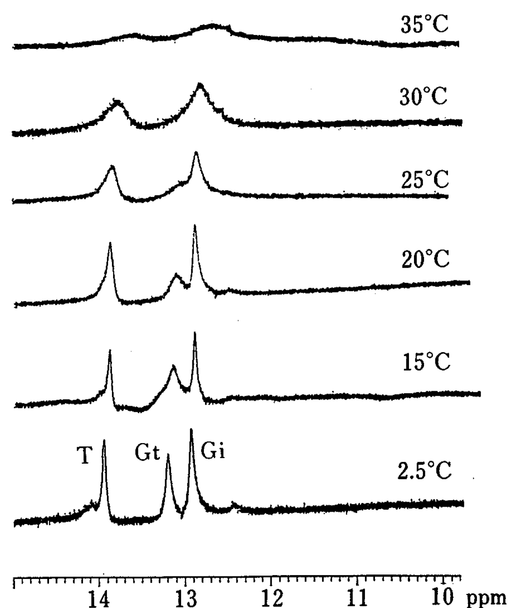

Fig. 5. The 399.95 MHz ^{1}H-NMR Exchangeable Imino Proton Spectra of d(CCTAGG) (700 OD/ml) in H_2O as a Function of Temperature

T denotes the imino proton, Gi the internal guanine proton, and Gt the terminal guanine proton.

suggested that ACM is intercalated at the T(3′–5′)A sites.

(3) Exchangeable ^{1}H-NMR in H_2O

Figure 5 shows the 400 MHz ^{1}H-NMR exchangeable imino proton spectra of $d(CCTAGG)_2$ in H_2O as a function of the temperature. The assignments were achieved on the basis of the general rule that G-N1 imino protons resonate in the 13.0—13.5 ppm region while T-N3 imino protons resonate at lower field than 13.5 ppm; further, the terminal imino proton resonance is broadened out at lower temperature than its melting temperature due to "fraying".[16] The assignments are given on the bottom spectrum of Fig. 5. The "melting" temperature of $d(CCTAGG)_2$ at this concentration (700 OD/ml) is about 35 °C (see Fig. 5). The two chromophore OH proton resonances of ACM (positions 4 and 6, see Fig. 1) were observed at 12.0 and 12.7 ppm from tetramethylsilane in chloroform at 25 °C; these signals were not observed in H_2O even at 2.5 °C due to a rapid exchange with water. Figure 6 shows the 400 MHz ^{1}H-NMR exchangeable proton resonances in the mixture of ACM with $d(CCTAGG)_2$ (molar ratio 1 : 1) as a function of the temperature. Eight resonances were observed at 27 °C. They are designated a—h in Fig. 6. Six of the eight resonances can be assigned to imino protons of $d(CCTAGG)_2$ complexed with ACM, while the other two can be assigned to the chromophore OH protons of complexed ACM, which are considered to exchange slowly with water.[8] On increasing the temperature, the resonances (d) and (f) (Fig. 6) are broadened, and disappear at 35 °C; these can be assigned to the terminal imino protons. The resonance (c) disappears at 45 °C, and the resonance (b) at 50 °C. The other four resonances are observable even at 50 °C.

To fit such observations, a model of the ACM–$d(CCTAGG)_2$ complex has been constructed as shown in Fig. 7 on the basis of the structure actually found in the daunomycin–

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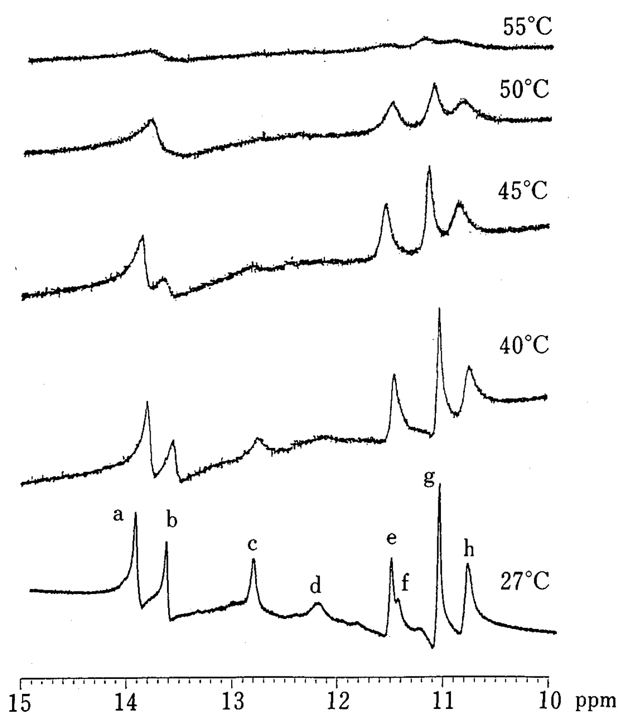

Fig. 6. The 399.95 MHz ^{1}H-NMR Exchangeable Proton Resonances in a Mixture of ACM with d(CCTAGG)$_2$ (Molar Ratio 1 : 1) in H_2O as a Function of the Temperature

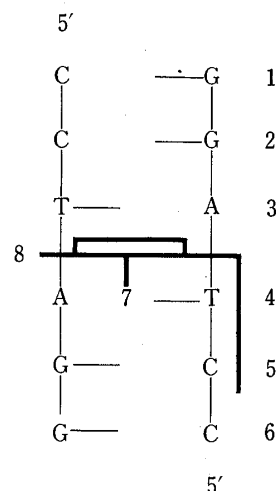

Fig. 7. A Proposed Model of the ACM–d(CCTAGG)$_2$ Complex

The ACM moiety is shown with thick lines. The numbers from 1 to 6 designate the base-pairs in d(CCTAGG)$_2$ and also the imino proton on each of them, while numbers 7 and 8 designate the chromophore OH protons at positions 6 and 4 of ACM, respectively.

Table II. The Proposed Assignments of the Exchangeable Protons on the Basis of the Model Given in Fig. 8 at 27 °C

	G1	G2	T3	T4	G5	G6	ACM7	ACM8
ppm	11.4	12.8	11.5	13.9	13.6	12.2	11.0	10.75

G, guanosine; T, thymidine; ACM7, position-6 OH; ACM8, position-4 OH. The numbering of the base pairs is given in Fig. 8.

d(CGTACG)$_2$ complex. In Fig. 5 the internal G-N1 and T-N3 imino proton signals broaden at the same temperature. However, in the ACM–d(CCTAGG)$_2$ complex, the T-N3 proton signals are expected to broaden at higher temperature than the G-N1 proton signals because the intercalation of ACM probably stabilizes the helix at this site. Thus, signals (c) and (b) (Fig. 6) are assignable to the internal G-N1 protons (2 and 5 in Fig. 7). The other four resonances are therefore assignable to the two T-N3 imino and two chromophore OH protons (3, 4, 7 and 8 in Fig. 7).

Discussion

In our present series of experiments, it has been found, first of all, that ACM binds with a DNA duplex with a binding constant K of the order of 10^6 at room temperature, and that the binding takes place with ApT or TpA sequences in preference to other sequences in DNA. Similar conclusions were reached by previous investigators using other methods.[3,18] As for daunomycin, it has been reported that the binding shows no sequence preference.[19] On the other hand, adriamycin binds exclusively to poly(dG-dC) and not to poly(dA-dT).[18] It

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remains to be established what factors determine the base-pair preference in the binding of the anthracyclines with DNA.

By the use of a sequence-controlled oligomer, d(CCTAGG), we have obtained a detailed knowledge of a particular example of DNA–ACM interaction. Here, the chromophore of ACM seem to enter only the TpA portion of this duplex. This "intercalation" causes a large downfield shift of the ^{31}P resonance at the TpA portion (as great as 0.8 or 1.5 ppm), and at the same time, a 15 nm red shift of the peak of the chromophore absorption band with a 45% intensity decrease. The sugar portion of ACM seems to reach one of the G·C base pairs adjacent to an A·T pair, so that the duplex becomes extremely asymmetric. This binding causes large shifts in the guanine-1-NH's, thymine-3-NH's, ACM-4-OH, ACM-6-OH proton resonances (their NMR signals are scattered in the 10.7—14.0 ppm range). Such large shifts were not found on forming either the daunomycin–DNA or the actinomycin-D–DNA complex. The binding also causes a marked suppression of the structural fluctuation of the DNA duplex. This suppression seems to take place in a different manner in different portions. Thus, the terminal "fraying" takes place at below 25 °C in the free $d(CCTAGG)_2$ duplex, but only at below 40 °C in the ACM–$d(CCTAGG)_2$ complex. The broadening of the inner guanine-1-NH signal takes place at 35 °C in the free duplex, but only at 45 or 50 °C in the complex. One G-NH (No. 5 in Fig. 7) is located close to the sugar moiety of ACM and can interact with it, whereas the other (No. 2 in Fig. 7) is not. Therefore signal (b), which broadens at higher temperature (50 °C, Fig. 6), is probably assignable to No. 5 (Fig. 7), and signal (c) to No. 2. The broadening of the thimine-3-NH signals taken place at 35 °C in the free duplex, but only at 55 °C in the complex. A nuclear Overhauser effect experiment is necessary for the complete assignment of the exchangeable protons. Such an experiment is in progress and will be described in a subsequent report.

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