2D NMR Investigation of the Binding of the Anticancer Drug Actinomycin D to Duplexed dATGCGCAT: Conformational Features of the Unique 2:1 Adduct†

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Received April 22, 1988; Revised Manuscript Received June 6, 1988

ABSTRACT: One- and two-dimensional NMR studies on the oligomer $dA_1T_2G_3C_4G_5C_6A_7T_8$, with and without actinomycin D (ActD), were conducted. Analysis of the NMR data, particularly 2D NOE intensities, revealed that the free oligonucleotide is a duplex in a standard right-handed B form. At the ratio of 1 ActD/duplex ($R = 1$), 1D NMR studies indicate that two 1:1 unsymmetric complexes form in unequal proportions with the phenoxazone ring intercalated at a GpC site, in agreement with previous studies [Scott, E. V., Jones, R. L., Banville, D. L., Zon, G., Marzilli, L. G., & Wilson, W. D. (1988) *Biochemistry 27*, 915–923]. The 2D COSY data also confirm this interpretation since eight cytosine H6 to H5 and two ActD H8 to H7 cross-peaks are observed. At $R = 2$, both COSY and NOESY spectra confirm the formation of a unique 2:1 species with C_2 symmetry. The oligomer remains in a right-handed duplex but undergoes extreme conformational changes both at and adjacent to the binding site. The deoxyribose conformation of T_2, C_4, and C_6 shifts from primarily C2′-endo in the free duplex to an increased amount of C3′-endo in the 2:1 complex as revealed by the greater intensity of the base H6 to 3′ NOE cross-peak relative to the intensity of the H6 to H2′ NOE cross-peak. This conformational change widens the minor groove and should help alleviate the steric crowding of the ActD peptides. The orientation of the ActD molecules at $R = 2$ has the quinoid portion of the phenoxazone ring at the G_3pC_4 site and the benzenoid portion of the phenoxazone ring at the G_5pC_6 site on the basis of NOE cross-peaks from ActD H7 and H8 to G_5H8 and C_6H6. All base pairs retain Watson–Crick type H-bonding, unlike echinomycin complexes [e.g., Gao, X., & Patel, D. J. (1988) *Biochemistry 27*, 1744–1751] where Hoogsteen base pairs have been observed. In contrast to previous studies on ActD, we were able to distinguish the two peptide chains. From NOE's between the threonine β protons and GH1′ protons, as well as other evidence, it was clear that the cyclic peptide rings of ActD bind in the minor groove of the DNA with the peptide ring on the quinoid side of the phenoxazone ring pointing toward the end of the duplex and the benzenoid peptide pointing into the center of the duplex. The amino acid protons in the quinoid ring and benzenoid ring have significantly different chemical shifts. However, only the signals of the cyclic peptide pointing toward the center of the duplex and hence toward its equivalent peptide on the other, C_2 related, ActD molecule had unusual shifts. Since these chains are in a crowded region, as evidenced by examination of models, they evidently have undergone a conformational change. The peptide interactions in this close-packed region may be responsible for the unique 2:1 species, for the negative cooperativity in binding, and for the conformational changes in the deoxyribose moieties.

The anticancer agent actinomycin D (ActD),[1] because of its unique structure (Chart I) and DNA binding properties, has been beneficial in the understanding of base pair specific interactions (Muller & Crothers, 1968; Sobell, 1973; Dervan, 1986; Waring, 1981). ActD displays unusually high specificity for binding to GC sequences (Jain & Sobell, 1972; Sobell & Jain, 1972; Sobell, 1980; Wells & Larson, 1970; Chen, 1988), displays cooperative binding to some DNA sequences, and appears to induce long-range structural transitions in adjacent DNA sequences on binding to GC sites (Waring, 1981; Wilson et al., 1986b; Scott et al., 1988, Fox & Waring, 1984). In efforts to understand these effects, a number of NMR studies on oligonucleotides with a single GC binding site at the C_2 axis (Petersheim et al., 1984; Gorenstein & Shah, 1984; Patel et al., 1981; Patel, 1974; Reid et al., 1983) and a 2D NMR study on the 1:1 Act-D–dATGCAT complex (i.e., 1 ActD/self-complementary duplex) have been conducted (Brown et al., 1984).

Recent ^{31}P and imino ^{1}H NMR studies with small self-complementary oligonucleotide duplexes of the type d-$(AT)_nGCGC(AT)_n$ have shown that the addition of ActD to the molar ratio of 1 ActD/duplex forms two unsymmetrical 1:1 ActD–oligonucleotide complexes with the phenoxazone ring intercalated at a 5′GC3′ (Wilson et al., 1986; Scott et al., 1988) site. In both 1:1 complexes, the C_2 symmetry characteristic of self-complementary duplexes is broken. At the molar ratio of 2 ActD/duplex, a unique 2:1 complex with interca-

† This work was supported by NIH Grant GM 29222 to L.G.M. and NSF Grant DBM-8603566 to W.D.W. The 400-MHz spectrometer was obtained with partial support from a Departmental NSF Grant (GSU).

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[1] Abbreviations: ActD, actinomycin D, all sequences written in the 5′→3′ direction; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; 1D, one-dimensional; 2D, two-dimensional; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; COSY, ^{1}H two-dimensional J-correlation NMR; NOESY, ^{1}H two-dimensional NOE correlation NMR; Nmval, L-N-methylvaline; Thr, L-threonine; Dval, D-valine, Pro, L-proline; Sar, sarcosine; R, ratio of ActD/duplex; TSP, 3-(trimethylsilyl)tetradeuterio sodium propionate; benzenoid and quinoid peptide chains refer to the cyclic peptide chains on the benzenoid side and the quinoid side of the phenoxazone ring, respectively.

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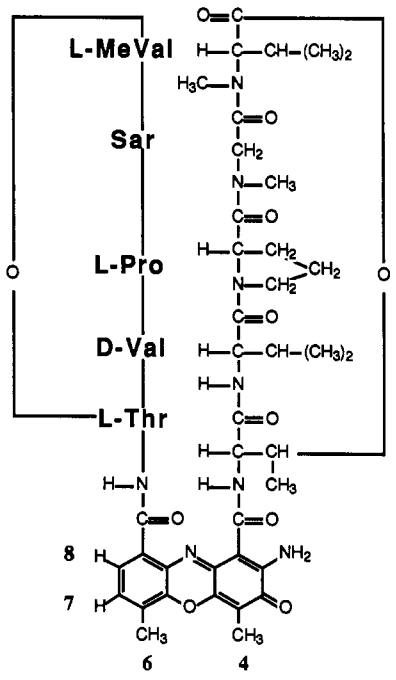
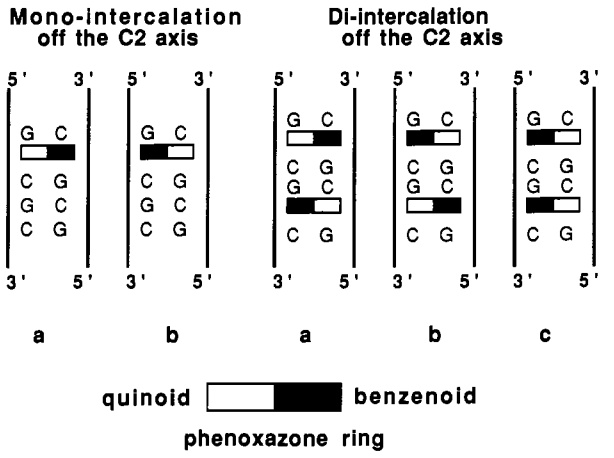
Chart I



Actinomycin D

Chart II



lation at both GC sites is formed, and the complex has C_2 symmetry. Chart II shows the possible 1:1 and 2:1 complexes. In comparison, self-complementary oligonucleotides of the type ···GCXYGC··· (XY = AT and GC) do not form a unique 2:1 complex but rather a mixture of two or more of the three possible 2:1 complexes (Scott et al., 1988; Jones et al., 1988).

To elucidate further the structure of the unique 2:1 ActD–d(AT)$_n$GCGC(AT)$_n$ complexes, 2D NMR studies have been undertaken using the self-complementary oligonucleotide dATGCGCAT. This oligonucleotide was chosen for study because it was the shortest version that exhibited spectra representative of longer strands. The NMR results allow us to probe the reason(s) behind the enhanced stability of the unique duplex by examining (1) the properties of the binding site, (2) the DNA conformation in the complex, and (3) the peptide chain conformation in the complex.

For comparative purposes, we have examined the oligonucleotide itself by 2D NMR methods. This compound is a representative of a class of oligonucleotides that has proved useful in the study of a number of DNA binding agents (Marzilli et al., 1986; Wilson et al., 1986a) as well as ActD.

Although many excellent NMR studies have been conducted on 1:1 ActD–oligomer complexes, it is important to investigate multiple ActD complexes for several reasons. First, the interaction of ActD with DNA can display either negative (site exclusion) or positive cooperativity depending on the local DNA sequence at the binding site (Walker et al., 1985; Wilson, 1987). Cooperative interactions by their very nature must be investigated with more than one ligand bound to the DNA. Second, if more highly specific binding molecules are to be designed, it is essential that the DNA recognition site be enlarged. This can be accomplished by synthetically linking molecules of ActD (or other molecules with high binding specificity). The synthetic efforts, however, must be directed by physical studies of multiple ligand complexes that indicate the restrictions on closeness of approach and orientation of bound molecules. Third, our understanding of the molecular basis for specificity and for cooperativity in DNA interactions and how that specificity and cooperativity can be varied and/or enhanced is very primitive. High-resolution NMR studies of a well-defined system can provide detailed information about the origins of these effects and how specific binding interactions affect the conformation of adjoining DNA sequences. Finally, both X-ray (Wang et al., 1984) and high-resolution NMR studies (Gao & Patel, 1988) have shown that when the two quinoxaline rings of echinomycin intercalate into oligonucleotide duplexes, some base pairs are converted from Watson–Crick to Hoogsteen type hydrogen bonding. Since two ActD molecules bind to the GC sites in dATGCGCAT, it is important to determine whether base pairs in this complex adopt Watson–Crick or Hoogsteen type configurations. A high-resolution NMR study of the type reported here is the method of choice for distinguishing Watson–Crick from Hoogsteen base pairs in solution (Gao & Patel, 1988).

Experimental Procedures

ActD was purchased from Sigma, and a 1 mM stock solution in water was prepared as previously described (Shafer et al., 1980). NMR solutions were prepared in a phosphate buffer (0.01 M phosphate, 0.10 M NaCl, and 0.1 mM EDTA, pH 7.0). The oligonucleotide dATGCGCAT was synthesized and purified as previously described (Stec et al., 1985). For 2D NMR studies it was dissolved in phosphate buffer to a concentration of 45 mM in bases. The sample was then lyophilized three times from D_2O and taken up in an equal amount of D_2O (99.96% D). Solutions of the ActD–dATGCGCAT complexes at the ratios of 1 ActD/duplex and 2 ActD/duplex were prepared by lyophilizing the appropriate amount of the ActD stock solution and redissolving the solid in the oligonucleotide solution. For 1D NOE studies the sample was lyophilized and brought up in 90% H_2O and 10% D_2O. Higher concentrations of oligomers resulted in line broadening at low temperatures, presumably due to aggregation.

Phase-sensitive NOESY and double quantum filtered COSY data were collected on a Varian VXR 400 NMR spectrometer. One-dimensional NMR studies and the COSY data for the 2:1 complex were recorded on a Nicolet 360-NB NMR spectrometer. Two-dimensional NOESY spectra (mixing times of 75, 250, 500, and 750 ms) were recorded at 15 °C, 40 scans per t_1 increment, 256 t_1 data points, zero filling to 1K in the F_1 dimension, and 1K data points in the F_2 dimension. The sweep width used was 3477 Hz with a repetition delay of 2.5 s. Free induction decays were apodized with a combined exponential–Gaussian function and symmetrized about the diagonal. Chemical shifts were referenced internally to TSP.

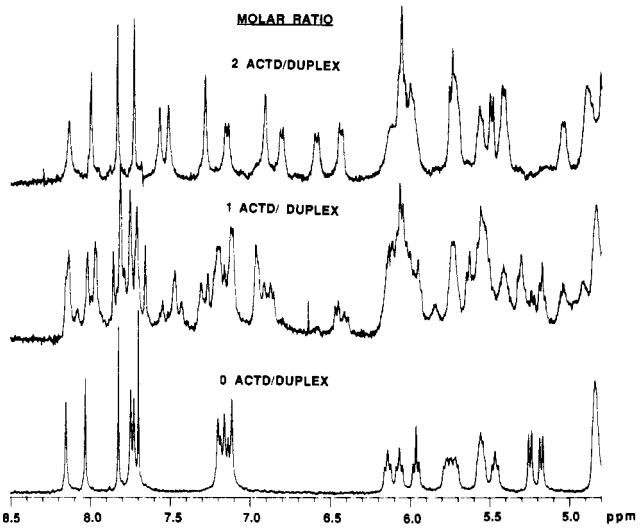


FIGURE 1: (Bottom) Spectrum of free dATGCGCAT in D_2O. (Middle) Spectrum of ActD–dATGCGCAT at the molar ratio of 1 ActD/duplex. At this ratio two 1:1 complexes form. (Top) Spectrum of ActD–dATGCGCAT at the molar ratio of 2 ActD/duplex. A single 2:1 complex with C_2 symmetry forms at this ratio.

RESULTS

Assignment of ^1H NMR Signals of dATGCGCAT. The nonexchangeable proton NMR spectrum of the free oligonucleotide dATGCGCAT in D_2O (Figure 1, bottom spectrum) has been assigned by the use of 2D NMR methods. Assignment of the specific aromatic proton resonances (not including the AH2's and CH5's) was accomplished by using the NOE's of these signals to the anomeric proton signals (Figure 2), and each base shall be referred to according to the following scheme:

$$5'dA_1T_2G_3C_4G_5C_6A_7T_83'$$

Assignments were possible because of the spatial relationship of the H1′ protons to the purine H8 protons and the pyrimidine H6 protons such that a specific H1′ is close enough to its own H8 or H6 to observe an NOE and also close enough to the H8 or H6 of the base 3′ to it to observe an NOE unless the H1′ is on the 3′ terminus (Wüthrich, 1986; Scheek et al., 1983).

The peak for A_1H8 is assigned as the peak at 8.03 ppm because the 5′ base has an NOE cross-peak only to its own anomeric proton resonance. The signal for T_8H6 is also easily assigned as the peak at 7.11 ppm since the 3′ base has an NOE cross-peak to an anomeric H1′ signal that has only one cross-peak to an aromatic CH signal (Figure 2). Taking advantage of these starting points, the connectivities of the anomeric proton signals to the H8 signals of the purines and the H6 signals of the pyrimidines were followed to assign these peaks (Table I). These observed connectivities are consistent with the duplex being in a right-handed B form. Additional proton interactions that also indicate that the duplexed form of the oligonucleotide is in a right-handed B form are observed in the NOESY spectrum between AH8 signals to T-CH_3 signals for 5′AT3′ and GH8 signals to CH5 signals for 5′GC3′.

The AH2's have no significant 2D NOESY cross-peaks in D_2O. The resonance at 7.82 and 7.70 ppm were assigned to A_1H2 and A_7H2, respectively, through 1D NOE's to the T imino proton resonances in H_2O (Wüthrich, 1986; Table I). The C_4H5 and C_6H5 signals at 5.17 and 5.25 ppm, respectively, were assigned from the COSY spectrum through their connectivities to the previously assigned CH6 signals. Assignments of the 2′ and 2″ deoxyribose protons were made by examination of the COSY spectrum from their connectivities

Table I: Assignment of Proton Resonances of dATGCGCAT Uncomplexed (Free) and in the 2:1 ActD–dATGCGCAT Complex (Bound)[a]

$5'A_1T_2G_3$-Q-C_4G_5-B-$C_6A_7T_83'$

proton	free	bound	proton	free	bound
A_1-8	8.03	8.00	G_5-8	7.75	7.51
A_1-2	7.82	7.73	G_5-1′	5.88	5.57
A_1-1′	6.23	6.00	G_5-2′	2.73	2.55[b]
A_1-2′	2.69	2.35	G_5-2″	2.65	2.60[b]
A_1-2″	2.28	2.51	G_5-3′	5.03	
A_1-3′	5.03	4.64	C_6-6	7.15	7.15
T_2-6	7.20	6.91	C_6-5	5.25	5.75
T_2-CH_3	1.24	1.42	C_6-1′	5.63	5.75
T_2-1′	5.71	5.42	C_6-2′	1.96	1.75
T_2-2′	2.17		C_6-2″	2.34	1.84
T_2-2″	2.49		C_6-3′	4.83	4.29
T_2-3′	4.94	4.58	A_7-8	8.15	8.13
G_3-8	7.73	7.57	A_7-2	7.70	7.83
G_3-1′	5.94	5.41	A_7-1′	6.31	6.06
G_3-2′	2.64	2.40	A_7-2′	2.75	2.65
G_3-2″	2.73	2.40	A_7-2″	2.88	2.76
G_3-3′	5.03		A_7-3′	5.03	4.90
C_4-6	7.17	6.59	T_8-6	7.11	7.28
C_4-5	5.17	5.49	T_8-CH_3	1.37	1.55
C_4-1′	5.72	5.72	T_8-1′	6.13	6.06
C_4-2′	2.04	1.64	T_8-2′	2.17	2.09
C_4-2″	2.40	1.64	T_8-2″	2.17	2.09
C_4-3′	4.86	4.28	T_8-3′	4.54	4.40

[a] Experimental conditions discussed under Experimental Procedures. Chemical shift values are given in parts per million. [b] The 2′ and 2″ assignments are unclear.

to the H1′ resonances. These assignments were confirmed from the NOESY spectrum in which cross-peaks from the H1′ signals to their 2′ and 2″ resonances were observed as well as cross-peaks from the base H8 and H6 to their 2′ and 2″ resonances (Wüthrich, 1986). At the mixing time of 75 ms we were able to assign the 2′ and 2″ resonances specifically because we observed intraribose NOE cross-peaks from the H1′ signals to 2″ resonances but not to 2′ resonances. Also, intranucleotide NOE cross-peaks were observed from the base H8 and H6 resonances to the 2′ signals but not to the 2″ signals (Wüthrich, 1986). By examination of the NOESY spectrum, the 3′ deoxyribose protons were assigned by their connectivities to their 2′ and 2″ resonances as well as by their weak intranucleotide NOE's to the base H6 and H8 resonances (Table I). Because of the extreme spectral overlap of the 4′, 5′, and 5″ deoxyribose proton signals between 3.50 and 4.5 ppm, assignments for these were not attempted. There were no unusual NOESY cross-peaks or connectivities to suggest unusual base-pair distortions from the general right-handed B form.

ActD–dATGCGCAT Complex, R = 1. At the ratio of 1 ActD/duplex ($R = 1$), two 1:1 unsymmetrical complexes can form in which the phenoxazone ring intercalates at a GpC site (Chart II). Previous ^1H and ^{31}P NMR results have suggested that both 1:1 complexes form but in unequal ratios (Scott et al., 1988). The D_2O spectrum at the 1:1 ratio is quite complex (Figure 1, middle spectrum). The COSY spectrum at this ratio shows additional evidence for the existence of the two 1:1 complexes in solution (Figure 3). Box b of Figure 3 shows the CH6 to CH5 NOE cross-peaks. If C_2 symmetry is removed as in Chart I, a cross-peak is expected for each of the four C's in each 1:1 species. The COSY spectrum has the expected eight well-resolved CH6 to CH5 cross-peaks. Also, upon examination of box a in Figure 3, two cross-peaks for ActD H8 to H7 are seen, corresponding to an ActD connectivity for each 1:1 species. The intensity differences of the COSY cross-peaks for the ActD and the C's confirm our

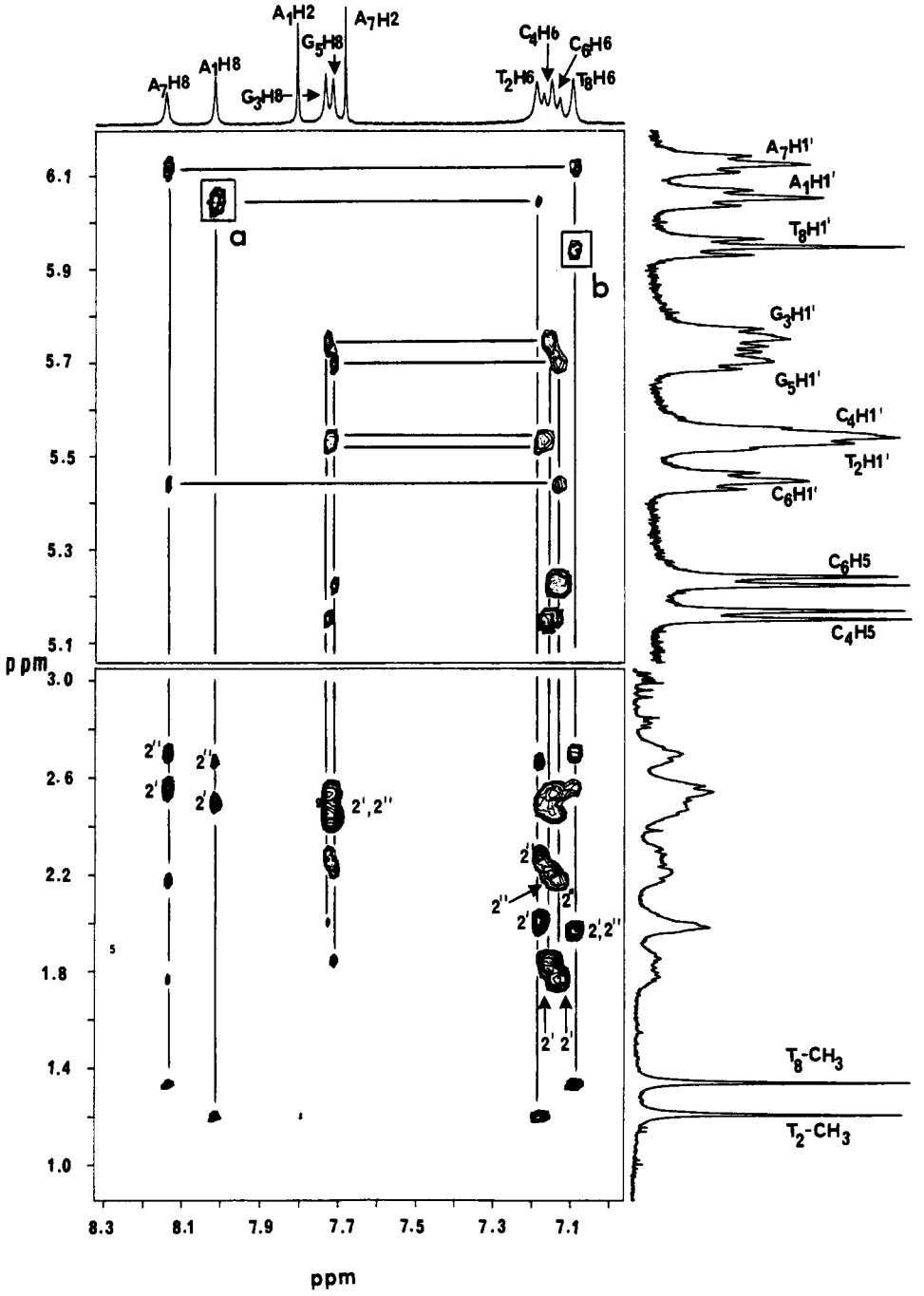


FIGURE 2: Expansion of the contour plot of the NOESY spectrum (mixing time of 250 ms) of the free oligonucleotide $dA_1T_2G_3C_4G_5C_6A_7T_8$. The boxed cross-peaks are for the following connectivities: (a) A_1H8 to its H1′; (b) T_8H6 to its H1′. The lines show the connectivities of the base proton resonances to the H1′, 2′, and 2″ resonances. Also observed are the connectivities of base proton resonances to the T-CH_3 resonances.

previous results that the two 1:1 complexes are present in unequal ratios (Scott et al., 1988). The intensity difference can also be seen in the 1D spectrum (Figure 1, middle spectrum). Compare, for example, the peaks for the ActD H7 signals at ca. 6.4 ppm that have been assigned by analogy to chemical shift patterns of the intercalated species by Brown et al. (1984) and from the COSY spectrum of the 2:1 complex (Figure 4).

Assignment of ^{1}H NMR of ActD–dATGCGCAT Complex, R = 2. We found in previous imino proton and ^{31}P NMR studies (Wilson et al., 1986; Scott et al., 1988) that a unique 2:1 ActD–dATGCGCAT complex is formed in which an ActD is intercalated between each GC site, and this is confirmed by the nonexchangeable proton spectrum in Figure 1. Because the 2:1 complex has C_2 symmetry, only structure a or structure b of the 2:1 models in Chart II is possible, and they can be distinguished by 2D NMR methods. NOESY spectra on the 2:1 complex were first collected with mixing times of 75 and 250 ms, identical experimental conditions used for the free oligonucleotide. NOESY spectra at the mixing times of 500 and 750 ms were also collected to provide information on long-range connectivities not observed at lower values. Because of possible spin diffusion contributions, these latter spectra were not used for conformational considerations.

The assignment of the spectrum of the R = 2 ActD–dATGCGCAT complex (Figure 1, top spectrum) was initiated by examining the aromatic region from 8.2 to 6.3 ppm. In this region, we expected to find two signals for the AH8's, AH2's, GH8's, TH6's, and CH6's and one signal for the ActD H8 as well as one signal for the ActD H7. Because of signal

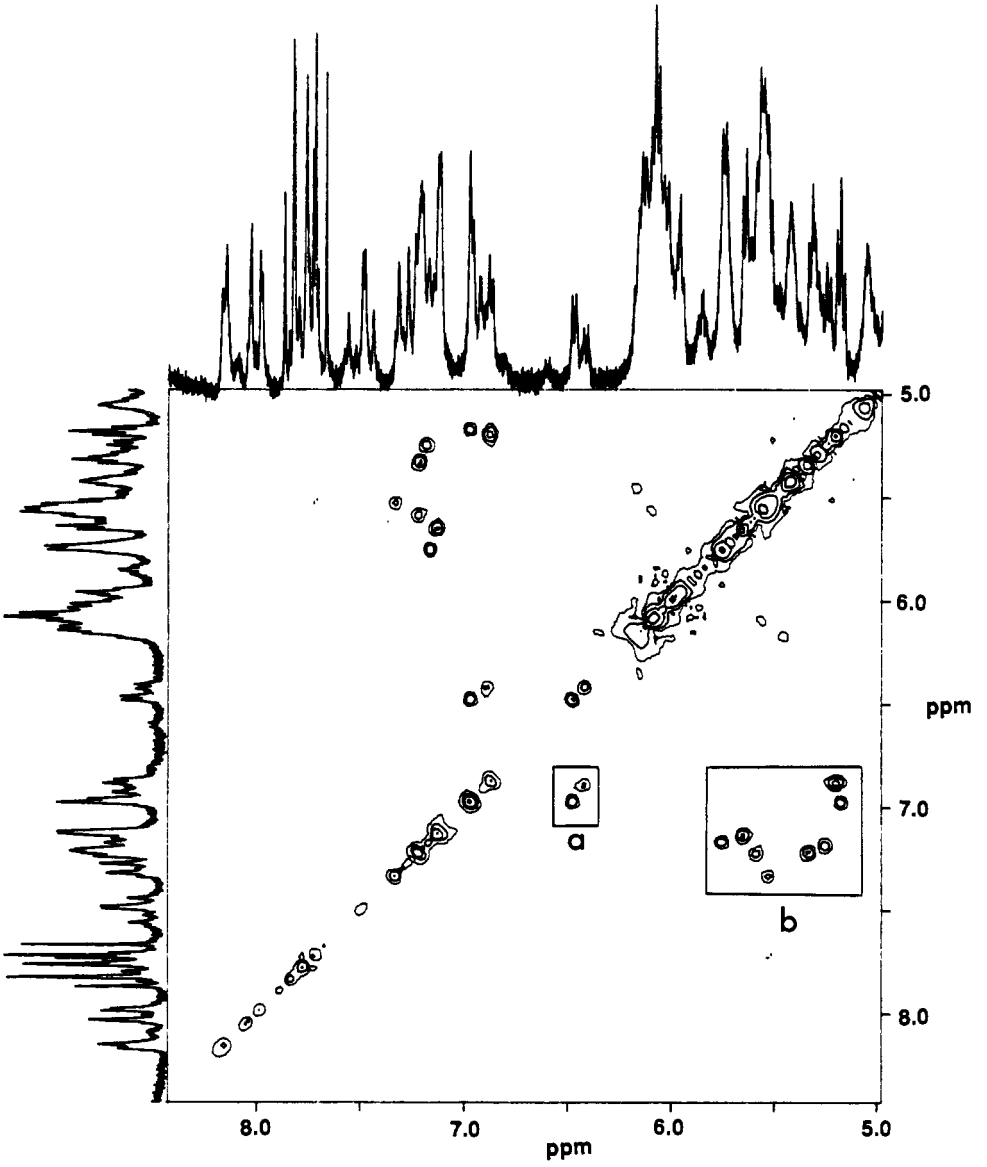


FIGURE 3: Expansion of the COSY spectrum of ActD–dATGCGCAT at the molar ratio of 1 ActD/duplex. At this ratio, two 1:1 complexes are present. This fact is evidenced by the two ActD H8 to H7 cross-peaks in box a (one cross-peak for each 1:1 species) and the eight CH6 to CH5 cross-peaks in box b (four cross-peaks for each 1:1 species).

overlap, assignments were much more difficult to make than for the free oligonucleotide. We first classified the signals as to specific type without assigning them to a particular base in the sequence.

Only ActD H8, ActD H7, and the two CH6 signals have connectivities in the COSY spectrum in the 8.2–6.3 ppm region (Figure 4). Of these signals, ActD H8 is coupled to ActD H7 (Figure 4, box a), and these are identified (peaks at 6.80 and 6.44 ppm, respectively) through their similar chemical shifts, which are similar to the value reported for the overlapping peaks in the free drug. The other two signals at 7.15 and 6.59 ppm must be for the CH6's. The COSY spectrum allows us to identify the signals for the CH5's. These CH6 signals at 6.59 and 7.15 ppm and CH5 signals at 5.49 and 5.75 ppm have shifts characteristic for such protons.

The remaining signals in the 8.2–6.3 ppm region are singlets that can be assigned by examining the NOESY spectrum (mixing time 250 ms, Figure 5). The two AH2 signals were easily identified since they have no NOE's to any anomeric protons at 250 ms. As in the free nucleotide, the AH2's were assigned by 1D NOE's through the imino proton resonances (Table I). These NOE results indicate that all four A-T base pairs in the 2:1 complex are Watson–Crick and not Hoogsteen base pairs. The TH6 resonances were recognized by their strong NOE's to methyl resonances, whereas the AH8 resonances were recognized by their weak NOE's to these same methyl resonances. The remaining two resonances must be attributed to the GH8's.

At this stage of the assignment, each aromatic CH signal had been identified as to type but had not been assigned to a specific base. In the NOESY (250 ms) spectrum (Figure 5), all of the H8 and H6 signals have an NOE to only one anomeric proton (Figure 5), unlike the free oligonucleotide. This complication made specific assignments difficult. The connectivity could be either inter- or intra-nucleotide. Each base H8 or H6 resonance has an NOE to a different H1′ resonance, and all eight of the expected H1′ resonances are observed. Furthermore, the A_1H8 resonance will have an NOE only to its own H1′ resonance since it does not have a nucleotide on its 5′ end. Therefore, all the base proton to H1′ NOE's are intranucleotide and not internucleotide. Additional support for these assignments is provided by the observation of an internucleotide NOE from the T_2H6 signal to the A_1H1′ signal at the higher mixing time of 500 ms.

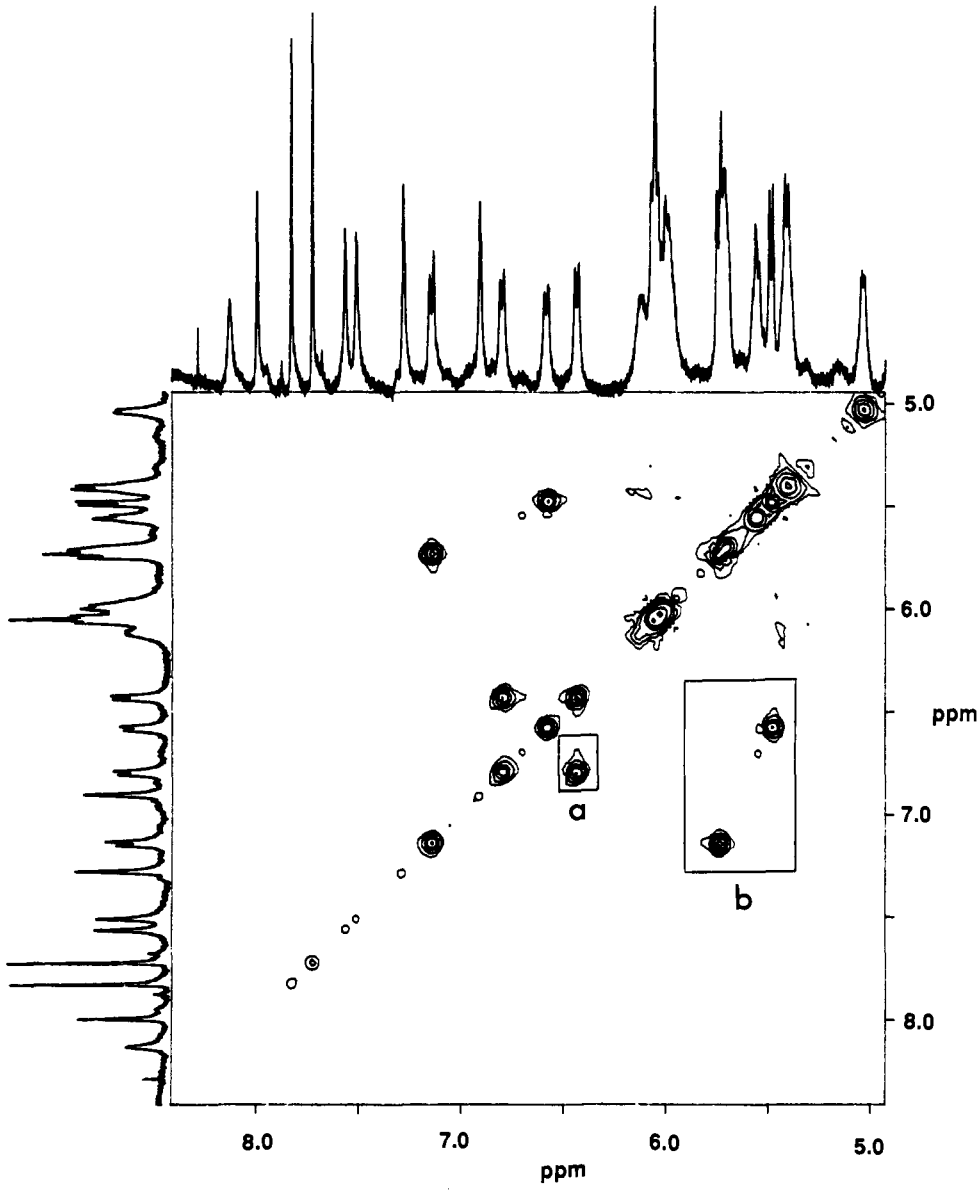


FIGURE 4: Expansion of the COSY spectrum of ActD–dATGCGCAT at the molar ratio of 2 ActD/duplex. Only one 2:1 species is formed, and C_2 symmetry of the duplex is restored as shown by only one ActD H8 to H7 cross-peak (box a) and only two CH6 to CH5 cross-peaks (box b).

Unlike the free oligonucleotide, the signals could not be readily assigned to a specific base because of the lack of the internucleotide NOE's to H1′. However, it is possible to group signals. For example, the GH8 resonance at 7.51 ppm and the CH6 resonance at 7.15 ppm both have NOE's to the ActD H8 and H7 signals, showing them to be close to the benzenoid side of the phenoxazone ring of the ActD and to be adjacent bases (benzenoid-GC as G-B-C). By process of elimination the other GH8 resonance at 7.57 ppm and CH6 resonance at 6.59 ppm are close to the quinoid side of the phenoxazone ring and adjacent to each other (quinoid-GC as G-Q-C). Recognition of which A was adjacent to which T was accomplished from the weak NOE's observed between the AH8 signals to the signals of the methyl groups on the adjacent T. At this point we know the GC and AT groupings but not the location of these dinucleotides in the strand.

Assignment of signals to specific bases was accomplished from the NOESY experiment at 500 ms (supplementary material, Figure S1) in which some interbase NOE's were observed. An NOE from the TH6 resonance at 6.91 ppm and the T-CH_3 resonance at 1.42 ppm to the GH8 resonance at 7.57 ppm assigns this resonance to the 5′ATGC end since only the G_3H8 of the two G's present can possibly have an NOE to a TH6 signal by the following reasoning: In the duplex form, there are two possibilities for observing an NOE from a GH8 resonance to a TH6 resonance, i.e., T_2 to G_3, an *intrastrand* connectivity, or T_8 to G_3, an *interstrand* connectivity. From the scheme below one can see that G_3 (underlined) is the only G with an H8 close enough to a TH6 to produce an NOE cross-peak. An interstrand connectivity to a TH6 can be ruled out since G_3 is too far from the T_8 on its complement to permit observation of an NOE.

5′dATGCGCAT3′

3′dTACGCGTA5′

These connectivities resulted in assignment of the 5′ATGC signals of the strand, and by process of elimination, the 3′GCAT signals were assigned (Table I, bound). Since the GH8 signal at 7.57 ppm is Q-GH8, the following order is now known:

$$5'dA_1T_2G_3\text{-}Q\text{-}C_4G_5\text{-}B\text{-}C_6A_7T_83'$$

Assignment of the H1′ resonances was accomplished by examining the NOESY and COSY spectra (Table I). NOE's

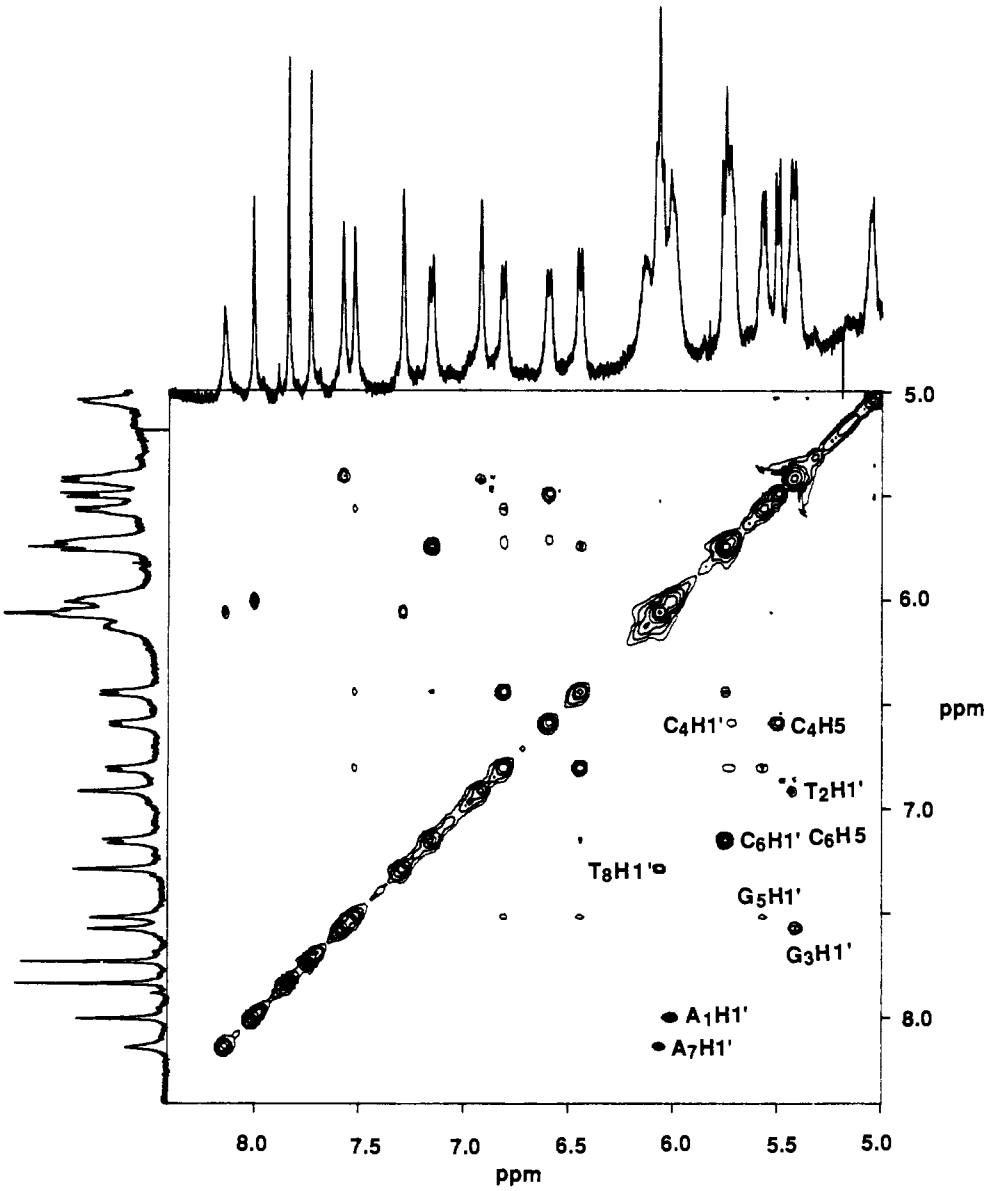


FIGURE 5: Expansion of the NOESY spectrum (mixing time of 250 ms) of the 2:1 complex. Labels for base proton to H1′ connectivities are shown. Notice that each base proton resonance has an NOE to only one H1′ resonance.

were observed from the assigned H8 and H6 signals to the corresponding H1′ signals as discussed above. COSY cross-peaks from the anomeric signals were then used to assign most of the 2′ and 2″ resonances (Figure S2, Table I). Since some of these connectivities were weak, we examined the NOESY cross-peaks between these signals to confirm the COSY connectivities and to finish the assignment for most of the 2′ and 2″ signals (Figure S3, Table I). We were not able to assign the 2′ and 2″ resonances for T_2, and we were not able to distinguish between the 2′ and 2″ resonances of G_5. Assignments for the deoxyribose 3′ resonances were made from the NOESY connectivities observed to the base protons, except for G_3 and G_5. Overlap of the 4′, 5′, and 5″ deoxyribose signals prevented direct assignment in this region.

The sugar conformation for each nucleotide in the 2:1 complex was determined by comparing the relative NOE intensities of the deoxyribose 2′, 2″, and 3′ resonances to their base proton resonances. In the C2′-endo conformation the base proton will have a stronger NOE to the 2′ and 2″ sugar resonances than to the 3′ resonance. In the C3′-endo conformation, the 3′ deoxyribose proton has the closest intranucleotide distance to the base proton (~3.0 Å) and will have a stronger intranucleotide NOE than will the 2′ or 2″ resonances. Although in a normal right-handed A form duplex, the closest proton to an H8 or H6 is the H2′ of the 5′ nucleotide (Wüthrich, 1986), we are fairly certain that we do not observe this internucleotide connectivity since the base protons do not have NOE's to the H1's of their 5′ nucleotides.

It appears that the nucleotides A_1, G_3, G_5, A_7, and T_8 remain primarily in the C2′-endo conformation since they have stronger NOE cross-peaks from their H8 or H6 signals to their 2′ and 2″ signals than to their 3′ signals (Wüthrich, 1986; Figure S3). The T_2 and C_4 nucleotides appear to have shifted to a higher population of the C3′-endo conformation because their H6 signals have stronger NOE's to their deoxyribose 3′ signals than to their 2′ and/or 2″ signals. The C_6 nucleotide has similar NOE intensities for H6 to the 2′, 2″, and 3′ signals and may have an unusual sugar conformation or an equilibrium between populations of the C2′- and C3′-endo forms.

There are aromatic proton resonances for the ActD H8 and H7 at 6.80 and 6.44 ppm. We were able to assign the signal at 6.44 ppm to the H7 proton since it had a strong NOE to the methyl resonance at 1.85 ppm, assigned to the ActD 6-CH_3 proton. By process of elimination, the resonance at 6.80 ppm

Table II: Actinomycin D Proton Assignments[a]

proton	free[b]	ATGCGCAT 2:1	ATGCAT 1:1[c]
H8	7.5	6.80	7.09
H7	7.5	6.44	6.60
6-CH_3	2.50	1.85	1.97
4-CH_3	2.04	1.47	1.62
Thr β	5.35	5.04 (Q), 4.86 (B)	5.23
Thr γ	1.38	1.22 (Q), 1.23 (B)	1.40
Dval α	3.60	3.45 (Q), 3.33 (B)	
Dval β	2.15	1.99 (Q and B)	
Dval γ	0.85, 1.10	0.66 (Q, and B), 0.90 (Q), 0.91 (B)	
Pro α	6.35	6.13 (Q), 5.97 (B)	6.13
Nmval α	3.35	2.76 (Q), 3.00 (B)	
Nmval β	2.55	2.35 (Q), 2.30 (B)	
Nmval γ	0.82, 0.99	0.69 (Q), 0.78 (Q), 0.63 (B), 0.81 (B)	
Nmval N-CH_3	3.08	2.83 (Q), 2.76 (B)	
Sar N-CH_3	2.94	2.78, 2.71	

[a] Experimental conditions are discussed under Experimental Procedures. Q represents signals of protons on the peptide ring on the quinoid side of the phenoxazone ring and B the signals of protons on the peptide ring on the benzenoid side of the phenoxazone ring. Chemical shift values are given in parts per million. [b] Values were taken from Angerman et al. (1972) and Brown et al. (1986). [c] Values were taken from Brown et al. (1986).

is assigned to the ActD H8 proton. We were also able to assign the signal at 1.47 ppm to the ActD 4-CH_3 from its NOE to the ActD 6-CH_3 resonance.

From the NOESY and COSY spectra, assignments for most of the amino acid signals in the cyclic peptide were made (Table II). Signals for the Thr γ methyl groups were assigned at 1.23 and 1.22 ppm since each had an NOE to a GH1′ at ca. 5.5 ppm and to a Thr β signal at ca 5.0 ppm (Figure 6). The latter assignment is based on the characteristic downfield shift of Thr β signals in comparison to the more upfield shifted β signals for the valines. These assignments were also rationalized as follows: The Thr residues are the only amino acid residues in the peptide chains expected to be very close to a G at a GpC site since the Thr's are both expected to be hydrogen bonded to a G (Sobell, 1973; Brown et al., 1984). Therefore, the only γ protons close enough to a GH1′ to have an NOE would be on Thr residues. We have also assigned the Thr residues to specific chains, that is, the quinoid or benzenoid peptide chain, by the NOE from its methyl groups to the GH1′ on the quinoid or benzenoid position in the complex. Thus, the first three residues of each chain (Thr, Dval, and Pro) can be recognized through the NOE's from the Thr β signals to the Dval γ signals and then the NOE's from the Dval α and γ signals to the Pro α resonances. Furthermore, the internal consistency of the assignment of the remaining amino acid protons of the quinoid and benzenoid chains in the 2:1 complex supports our initial assignments for the Thr β and γ protons. From the COSY spectrum a connectivity from the Thr β signal for each peptide ring to its Thr γ proton signal was also seen (Figure 7, Table II). The resonances for the Thr α protons were not located, presumably because they were under the water signal.

Assignment of two of the four Dval γ proton signals was made from their NOE's to the previously assigned Thr β resonances (Figure 6, Table II). The COSY spectrum was then used to assign the Dval β resonances by their connectivity to the Dval γ resonances and also to assign the Dval α signals by their connectivities to their Dval β signals (Figure 7). From the NOESY and COSY spectra (Figures 6 and 7) the two other Dval γ signals are readily identified. The remaining methyl signals in the far upfield region of the spectrum are from the Nmval γ protons. Assignment of the Nmval β and Nmval α signals were made by using the COSY spectrum in the same fashion as described for the Dval signals (Figure 7, Table II). The Nmval α signal at 3.00 ppm has a weak NOE to the G_6H1′ resonance and is assigned to the Nmval on the benzenoid peptide chain. This Nmval α resonance also has an observable NOE to its N-CH_3 group at 2.76 ppm.

Table III: Selected NOE's of 2:1 ActD–$dA_1T_2G_3C_4G_5C_6A_7T_8$ Complex[a]

resonance	resonances
A_1H8	A_1H2′, A_1H1′, A_1H2″, A_1H3′, T_2-CH_3
A_1H1′	A_1H2″, A_1H2′, A_1H3′
T_2H6	T_2-CH_3, T_2H3′, Thr γ (Q), T_2H1′, A_1H1′[b]
T_2H1′	T_2-CH_3, T_2H3′
G_3H8	G_3H2′, G_3H2″, G_3H1′, T_2H6[b], T_2-CH_3[b]
G_3H1′	G_3H2″, G_3H2′, Thr γ (Q)[b]
C_4H6	C_4H5, C_4H3′, C_4H1′, Dval γ (B), C_4H2′
C_4H1′	C_4H2′, C_4H2″, C_4H3′, Dval γ (B)
G_5H8	G_5H2′, G_5H2″, G_5H1′, ActD-H8, ActD-H7
G_5H1′	G_5H2″, Thr γ (B), G_5H2′, ActD-H8
C_6H6	C_6H5, C_6H1′, C_6H3′, C_6H2′, ActD-H7, Thr γ (B), ActD-H8[b]
C_6H1′	C_6H3′, C_6H2″, C_6H2′, Nmval α
A_7H8	A_7H2′, A_7H1′, A_7H2″, A_7H3′, T_8-CH_3, T_8H6[b]
A_7H2	Nmval N-CH_3 (Q), Pro α (Q),[b] T_8-CH_3[b]
A_7H1′	A_7H2′, A_7H2″, A_7H3′
T_8H6	T_8H2′, T_8H2″, T_8-CH_3, T_8H1′, T_8H3′
T_8H1′	T_8H2′, T_8H2″, T_8H3′, T_8CH_3
Thr β (B)	Thr γ (B), Dval γ (B)
Thr β (Q)	Thr γ (Q), Dval γ (Q)
Dval α (B)	Dval γ (B), Pro α (B), Dval β (B)
Dval α (Q)	Dval γ (Q), Pro α (Q), Dval β (Q)

[a] Chemical shift values for resonances are listed in Tables I and II. Q is for the quinoid side of the phenoxazone ring, and B is for the benzenoid side of the phenoxazone ring. Signals in right column are in order of strong to weak NOE's. [b] NOE's that were observed at mixing times of 500 ms and higher. All other NOE's were observed at 250 ms.

Location of the Pro α resonances at 6.13 and 5.97 ppm was accomplished by their moderate NOE's to the respective Dval α resonances (Figure 6). The other signals for the N-CH_3 groups in the spectrum have been assigned to the resonances at 2.83, 2.78, 2.76, and 2.71 ppm. We were not able to assign specifically which methyl signals were for Sar or Nmval except for the signal at 2.76 ppm assigned above. However, the signal at 2.83 ppm has an NOE to the A_7H2 and T_2H1′ resonances; this leads us to suspect it to be the signal for the N-CH_3 group of the Nmval on the quinoid cyclic peptide since a similar connectivity was observed by Brown et al. (1985) with dATGCAT. We also examined a model of the complex based on the Sobell crystal structure (Sobell, 1973) and modified it by the major NOE's observed in the NOESY spectra and found that only the N-CH_3 on the Nmval is close enough to A_7H2 to exhibit an NOE. The remaining N-CH_3 signals at 2.78 and 2.71 ppm are for the Sar residues. The only other amino acid signals that have not yet been assigned in the 2:1

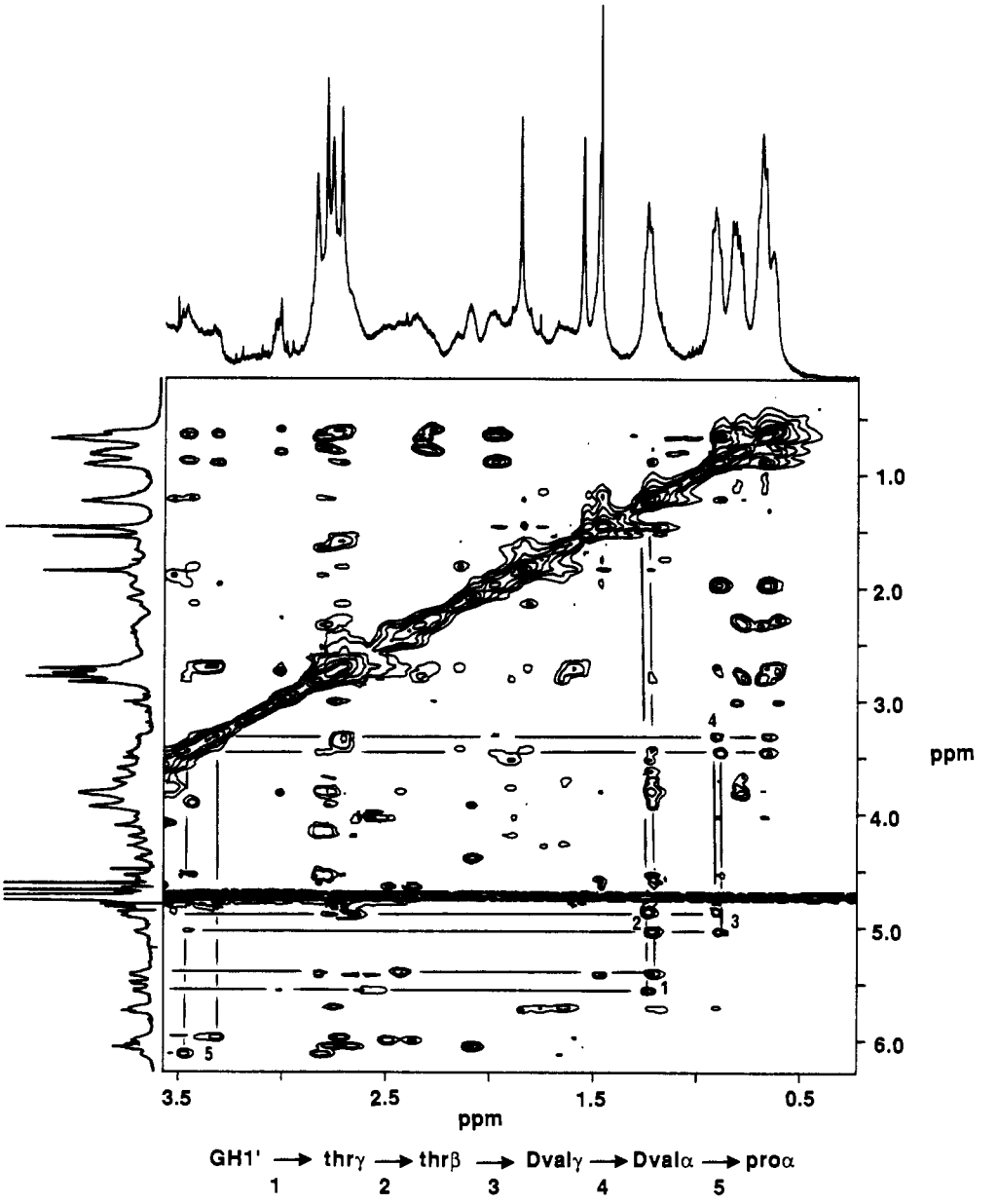


FIGURE 6: Expansion of the NOESY spectrum (mixing time of 250 ms) of the 2:1 complex illustrating the connectivities of some of the protons on the amino acid residues, as shown in the scheme below the spectrum. This spectrum clearly demonstrates that the amino acid residues on each chain can be distinguished.

complex are Pro β, γ, and δ and also Sar α.

Discussion

The $d(AT)_nGCGC(AT)_n$ series of oligomers is a hybrid representative for the two commonly compared synthetic polynucleotides poly[d(G-C)]·poly[d(G-C)] and poly[d(A-T)]·poly[d(A-T)]. We have previously examined the interaction of these oligomers with porphyrins (Marzilli et al., 1986) and found that some porphyrins appear to bind preferentially at 5′CG3′ sites (Marzilli et al., 1986; unpublished studies). Gibbs et al. (1988) have suggested recently that the binding selectivity may be peculiar to the sequence in the oligonucleotide. However, our 2D NMR analysis of the free duplexed oligonucleotide shows it to be in a standard right-handed B form. This conclusion was drawn from characteristic NOESY cross-peaks from base to sugar and among sugar protons. There were no unusual cross-peaks in the NOESY spectra that evidenced any unusual DNA conformations. This finding agrees with our previous observations with nonselective intercalators (Wilson et al., 1986a) that this type of oligomer has no unusual structural features which could account for selective 5′CG3′ binding by porphyrins (Marzilli et al., 1986).

Previous imino proton and ^{31}P NMR studies have shown that the ActD phenoxazone ring intercalates at the GpC sites of dATGCGCAT as well as in longer versions of this oligonucleotide containing more flanking A-T base pairs (Scott et al., 1988). The COSY spectrum at the ratio of 1 ActD/duplex strongly supports our previous conclusions for two different 1:1 complexes (Wilson et al., 1986; Scott et al., 1988). The eight CH6 to CH5 cross-peaks demonstrate that each 1:1 complex has four distinct cytosines. The two ActD H8 to H7 cross-peaks indicate that the ActD phenoxazone ring is in a different orientation for each 1:1 species.

At the ratio of 2 ActD/duplex, formation of a single 2:1 complex with C_2 symmetry simplifies the spectrum greatly in comparison to the 1:1 spectrum (Figure 1). This simplification is especially noticeable in the spectral region downfield of the HDO resonance (4.8 ppm). Our 2D NMR data allow us to conclude unambiguously that the orientation of the phenoxazone ring in the 2:1 complex with dATGCGCAT has the

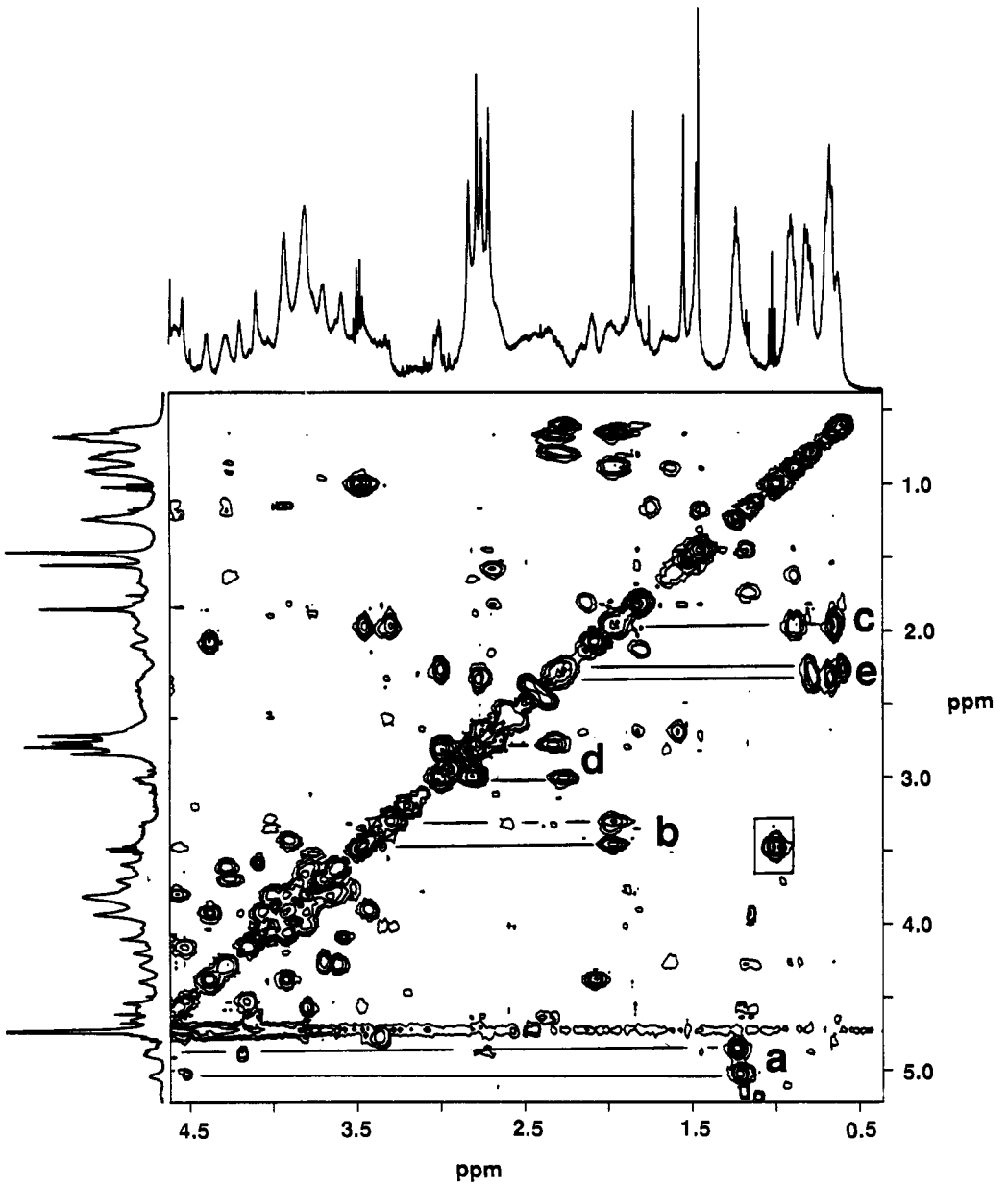


FIGURE 7: Expansion of the COSY spectrum of the 2:1 complex showing connectivities (a) Thr β to Thr γ, (b) Dval α to Dval β, (c) Dval β to Dval γ, (d) Nmval α to Nmval β, and (e) Nmval β to Nmval γ. The boxed region contains the cross-peak for the ethanol multiplets, a minor impurity in this particular solution.

quinoid side of the intercalated chromophore located between the GpC site on the 5′ side of the sequence with its pentapeptide lactone ring binding in the minor groove, pointing toward the end of the duplex. The benzenoid side of the phenoxazone ring is located between the GpC site on the 3′ side of the sequence, and its peptide ring is pointing toward the center of the complex. These results confirm our proposed model based on 1D imino proton spectra (Scott et al., 1988). This GpC selectivity of binding in the 2:1 complex still appears to involve Thr–G H-bonds as in the 1:1 Sobell model.

Previously, we suggested that the intercalation of the ActD can cause long-range conformational distortions in DNA strands. This was apparent from ^{31}P NMR studies in which several phosphorus signals were shifted downfield of the main signals by intercalation of ActD (Wilson et al., 1986; Scott et al., 1988). The 2D NMR data to be discussed next strongly support this model and indicate what type of conformational changes occur to accommodate the two ActD bound to dATGCGCAT.

Many of the NOESY and COSY connectivities observed in the free oligonucleotide are not seen or are very weak in the 2:1 complex: e.g., (1) cross-peaks from the H1′ resonances to the 2′ and 2″ resonances in the COSY spectrum and (2) cross-peaks from aromatic CH resonances to H1′ resonances on the 5′ nucleotide in the NOESY spectrum. Brown et al. (1984) also observed a decrease in intensities of some NOE cross-peaks in the 1:1 ActD–dATGCAT complex. Obviously, unwinding of the entire duplex as a result of binding of two ActD molecules to dATGCGCAT has significantly changed the orientation of all nucleotides with respect to each other. Similarly, the binding of the cyclic peptides in the minor groove, as evidenced by NOE's from the minor groove protons, A_7H2 and T_8H1' to the ActD Nmval-NCH_3(Q), as well as NOE's from GH1's to Thr γ's, has induced changes in the sugar conformations of some nucleotides. An equilibrium shift from C2′-endo to C3′-endo is induced in the deoxyribose moieties of T_2, C_4, and C_6. This sugar conformation change allows us to answer the question of how the large peptide rings of ActD can fit into the narrow minor groove of B-form DNA in the center of dATGCGCAT. The change from the C2′-

endo to C3′-endo sugar conformation causes a substantial widening of the minor groove and allows considerably more room for the cyclic peptides. This is another example of the inherent flexibility of DNA and the polymorphic nature of the molecule (Arnott, 1981). We believe the sugar C2′-endo to C3′-endo conformational changes may be a common feature of binding of molecules with bulky side chains that must fit into the minor groove. Previous ideas, based on the dimensions of the DNA grooves from the B-form structure (Saenger, 1984; Arnott, 1981), concerning the size of molecules that could fit into the major or minor groove must be reconsidered.

Another extremely important conformational change, which has been discovered by crystallographic (Wang et al., 1984) and high-resolution NMR (Gao & Patel, 1988) methods in oligonucleotide complexes with two rings of the bisintercalator echinomycin intercalated, is the transition from Watson–Crick to Hoogsteen type base pairing. For example, in the complex dCGTACG with quinoxaline rings of two echinomycin stacked on the ends of the duplex and intercalated at GT and AC sites, the central A-T base pairs are converted to the Hoogsteen arrangement (Wang et al., 1984). Our 2:1 complex with ActD at both GC sites in dATGCGCAT has intercalating rings separated by the same number of base pairs. We do not, however, observe the transition to Hoogsteen base pairs. As suggested by Gao and Patel (1988), the Hoogsteen base pairs in the echinomycin complex may form to improve base stacking with the small quinoxaline ring system of echinomycin. This suggests that the larger phenoxazone ring of ActD can stack better with the Watson–Crick type base-pairing arrangement. It will be interesting to compare the effects of intercalating rings of other size and shape on base-pairing conformations, and we have such studies under way.

The spectrum of the 1:1 ActD–dATGCAT complex contained only one signal for each type of amino acid proton (Brown et al., 1984). Therefore, the benzenoid and the quinoid peptide rings could not be identified or distinguished. *In contrast*, for $R = 2$ ActD–dATGCGCAT, the signal for at least one type of proton in each amino acid could be identified as belonging to either the benzenoid or quinoid peptides, and these could be assigned for all amino acids except Sar. Therefore, the two cyclic peptide chains in the 2:1 complex appear to be in different conformations. Thus, both the oligomer and the antibiotic undergo conformational changes to form the tightly packed 2:1 complex.

The peptide signals from the amino acids on the quinoid side of our 2:1 complex have shifts similar to those observed by Brown et al. (1984). The signals are shifted from the free ActD values by a smaller amount than those for amino acids on the benzenoid side. Thus, while little to no conformational or environmental changes in the cyclic peptides occurred on binding to dATGCAT, the binding of ActD in our 2:1 complex leads to such changes, especially in the peptides on the benzenoid side of the phenoxazone ring that point into the crowded central portion of the complex. Changes in peptide conformation can be explained by the steric crowding of the two benzenoid peptide rings in the center of the complex. This requirement for a change in peptide conformation and the close packing of the central peptide rings could explain the formation of only one (a) of the three possible 2:1 adducts illustrated in Chart II. This crowding may also be responsible for the anticooperativity in the binding that we observe here (Figure 1) and also in longer oligonucleotides with ···GCGC··· sequences (Scott et al., 1988). Positive cooperativity has been observed in ActD binding isotherms, and it is important to establish the molecular basis for this effect (Walker et al., 1985). We have also evaluated interactions of ActD with oligonucleotides with ···GCXYGC··· (XY = AT and GC) sequences (Jones et al., 1988). We have found no evidence of positive cooperativity for interactions in these systems, and it may be necessary to investigate longer oligomers containing more binding sites before positive cooperativity can be observed.

The C_2 symmetry of the unique 2:1 adducts we have discovered may mask interpeptide NOE's that otherwise would help us define the peptide–peptide contacts. Additional insight into the binding as well as more details of structural features requires isolation of crystalline products (studies in progress). Alternatively, a refinement of the present model could be achieved by using higher field NMR data in conjunction with distance-geometry methods, particularly for a non-self-complementary oligonucleotide lacking C_2 symmetry but containing the ···GCGC··· sequence. We hope to begin such studies in the future.

Supplementary Material Available

Figures S1–S3, NOESY and COSY spectra for $R = 2$ ActD–dATGCGCAT (3 pages). Ordering information is given on any current masthead page.

Registry No. ActD, 50-76-0; $dA_1T_2G_3C_4G_5C_6A_7T_8$, 95206-23-8.

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Mischarging *Escherichia coli* tRNA^{Phe} with L-4′-[3-(Trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine, a Photoactivatable Analogue of Phenylalanine†

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Received March 25, 1988; Revised Manuscript Received June 14, 1988

ABSTRACT: The Boc-protected derivative of a photoactivatable, carbene-generating analogue of phenylalanine, L-4′-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine [(Tmd)Phe], was used to acylate 5′-*O*-phosphorylcytidylyl(3′–5′)adenosine (pCpA). A diacyl species was isolated which upon successive treatments with trifluoroacetic acid and 0.01 M HCl yielded a 1:1 mixture of 2′(3′)-*O*-(Tmd)phenylalanyl-pCpA and of its 2′–5′-phosphodiester isomeric form. Adapting a procedure introduced by Hecht's group [Heckler, T. G., Chang, L. H., Zama, Y., Naka, T., Chorghade, M. S., & Hecht, S. M. (1984) *Biochemistry 23*, 1468–1473], brief incubation of a 15 molar excess of this material with *Escherichia coli* tRNA^{Phe}, missing at the acceptor stem the last two nucleotides (pCpA), in the presence of T4 RNA ligase and ATP afforded "chemically misaminoacylated" tRNA^{Phe} in approximately 50% yield. Following chromatographic purification on DEAE-Sephadex A-25, benzoylated DEAE-cellulose, and Bio-Gel P-6, the misaminoacylated tRNA^{Phe} was characterized by (i) urea–polyacrylamide gel electrophoresis, (ii) enzymatic reaminoacylation under homologous conditions following chemical deacylation, and (iii) its ability to stimulate protein synthesis in an in vitro translation system which, through the addition of the phenylalanyl-tRNA synthetase inhibitor phenylalaninyl-AMP, was unable to charge its endogenous tRNA^{Phe}. The data demonstrate that we have prepared a biologically active misaminoacylated tRNA^{Phe}.

T4 RNA ligase mediated coupling of 2′(3′)-O-acylated pCpA derivatives with tRNAs missing the 3′-terminal cytidine and adenosine moieties (tRNA-C_{OH})[1] has in the last years been utilized by Hecht's group to prepare a number of novel "chemically misacylated" tRNAs (Hecht et al., 1978; Heckler et al., 1983, 1984a,b; Roesser et al., 1986). Prebound to the P site of *Escherichia coli* ribosomes, several of these tRNAs were shown to mediate dipeptide formation upon admixture of phenylalanyl-tRNA^{Phe} to the A site (Heckler et al., 1983; Roesser et al., 1986). Since the current methodology for chemical misacylation can be applied only to acyl moieties that do not contain a free amino group (e.g., an N-protected amino acid), these misacylated tRNAs do not function in the ribosomal A site and, hence, cannot be used to incorporate biosynthetically nonnatural analogues of amino acids into polypeptides. In view of the considerable potential such application would have, it was very tempting to search for procedures by which it would be possible to prepare also the "free", N-deprotected aminoacyl-tRNAs.

An approach that could lead to such tRNAs would be to include in Hecht's general scheme a step that generates the free amino group from its protected form. Such deprotection may either follow directly acylation of pCpA (prior to ligation) or, alternatively, be carried out after ligation on the misacylated tRNA itself. In the present paper we describe the successful misaminoacylation of *E. coli* tRNA^{Phe} with an analogue of phenylalanine, (Tmd)Phe. For transient protection of the amino function, the butoxycarbonyl (Boc) group was used and deprotection was done immediately after acylation of pCpA. Evidence is also presented that the resulting

† This work was supported by a research grant from the Swiss National Science Foundation, Bern (3.228-0.85 to J.B.). Dedicated to Prof. G. Semenza on the occasion of his sixtieth birthday.
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[1] Abbreviations: (Tmd)Phe, L-4′-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; tRNA-CC_{OH}, tRNA missing the 3′-terminal adenosine moiety; tRNA-C_{OH}, tRNA missing the 3′-terminal cytidine and adenosine moieties; tRNA^{Phe}, phenylalanyl-specific tRNA; Na^+-Hepes sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/88/0427-7951$01.50/0