A Model Compound of Actinomycin. Conformation of Dimethyl Actinocynilbis(L-threonate)†

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ABSTRACT: The conformation of dimethyl actinocynilbis(L-threonate), a model compound of actinomycin, has been derived from an analysis of infrared (ir), nuclear magnetic resonance (nmr), and preliminary X-ray data. Around the phenoxazone group the structure presents features different from those of the natural occurring antibiotic, which account for significant differences in ir, nmr, and circular dichroism (CD) spectra. Both the compounds interact with DNA by a similar intercalation mechanism, but the model compound lacks biological activity. As a result of this analysis, evidence was found of some structural features, besides the ability of intercalation, which control the antibiotic properties of actinomycin.

The stereochemistry of the interaction between actinomycin and DNA is being widely studied by different approaches (Reich and Goldberg, 1964; Müller and Crothers, 1968; Sobell *et al.*, 1971). The study of model compounds of actinomycin is relevant to the molecular basis of its biological activity and may give useful suggestions for the design of synthetic analogs with similar or improved antibiotic properties.

A few years ago we started an investigation of model compounds of actinomycin in their ability to interact with DNA. Dimethyl actinocynilbiscarboxylate (AMD1)[1] and dimethyl actinocynilbis(L-threonate) (AMD2) were studied and the modification of their visible absorption spectra in DNA complexes was found to be similar to that observed in the complexes between DNA and actinomycin. On the basis of this evidence an intercalation mechanism was proposed (Ascoli *et al.*, 1968; Ascoli and Savino, 1968).

Some kinetic and hydrodynamic studies were also reported on complexes between DNA and very simple actinomycin-related compounds, namely, 2-amino-3-phenoxazone, 2,7-diamino-3-phenoxazone, and actinomine, which also bind with DNA through an intercalation mechanism (Müller and Crothers, 1968); but, none of these compounds show biological activity.

In this paper the molecular structure of AMD2 is derived from the analysis of nuclear magnetic resonance (nmr), infrared spectra (ir), and preliminary X-ray data and, bearing in mind their structural features, a comparison is attempted between optical and biological properties of this model compound and the naturally occurring antibiotic.

Experimental Section

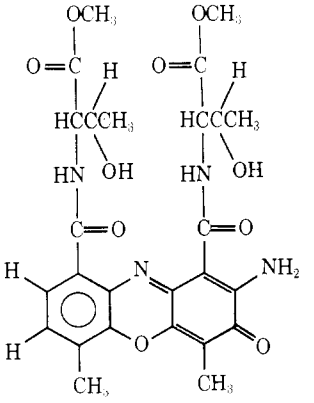
Dimethyl actinocynilbis(L-threonate) was prepared from 2-methoxytoluic acid (Aldrich) through six steps (Brockmann and Muxfeldt, 1958; Mauger and Wade, 1965; Weinstein *et al.*, 1962). Nuclear magnetic resonance spectra were measured by using a Varian 100 spectrometer, in deuterated dimethylsulfoxide or chloroform. Infrared spectra were measured with a Beckmann IR9 spectrophotometer, in deuterated chloroform. Circular dichroism (CD) measurements in acetonitrile were carried out using a Cary 61 dichrograph. The spectra did not exhibit concentration dependence, therefore labile association is ruled out.

Single crystal X-ray diffraction data were collected on $hk0$ and $h01$ reciprocal sections using a Weissenberger camera and CuK radiation. The crystal cell parameters were determined using a precession camera. The space group is the monoclinic $P2_1$ with the following parameters: $a = 8.10$ Å, $b = 30.20$ Å, $c = 11.55$ Å, $\beta = 101.5°$, $d_{theor} = 1.22$, $d_{exp} = 1.22$ (determined in a gradient of KI aqueous solution), and $Z = 4$ corresponding to two molecules per asymmetric unit.

The solubility of AMD2 in DNA solution and in water was determined by equilibrating fine crystals with calf thymus DNA (Sigma) or with doubly distilled water for 3 days at 25° by gentle shaking in the dark (Ascoli *et al.*, 1968). After centrifugation at 60,000*g* for 1 hr in a Spinco LHV ultracentrifuge, or filtration through sintered-glass G4, the solutions were extracted with chloroform and the concentration in the organic layer was determined from the absorption at 444 nm (ϵ 27,800).

Results and Discussion

Conformational Studies. AMD2 may be considered a model



compound of actinomycin in which the pentapeptide lactones were interrupted at the level of the L-threonine residues directly connected to phenoxazone. In Figure 1 the ir spectrum

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[1] Abbreviations used are: AMD1, dimethyl actinocynilbiscarboxylate; AMD2, dimethyl actinocynilbis(L-threonate).

TABLE I: Observed Frequencies and Assignments of Actinomycin and AMD2.

Actinomycin		AMD2	
Frequency (cm^{-1})	Assignment	Frequency (cm^{-1})	Assignment
3457, 3391, 3267, 3221	NH_2 stretch (phenoxazone), amide A[a]	3612	Free OH stretch
		3413, 3255, 3200 sh	Bonded OH stretch, amide A[a] NH_2 stretch (phenoxazone)
3055	Amide B[a]	2980, 2955, 2935, 2855	CH stretch
2970, 2935, 2875, 2855	CH stretch		
		1745	Ester C=O stretch
1745, 1715 sh	Ester C=O stretch	1658, 1642	Free and bonded C=O stretch (phenoxazone)[b]
1685, 1670 sh, 1663, 1646, 1630	Amide I[a], C=O stretch (phenoxazone)[b]	1616, 1608	Amide I[a]
		1573	C=N and C=C (phenoxazone)[b]
1580	C=N and C=C (phenoxazone)[b]	1526	Amide II[a]
1513	Amide II[a]		

[a] Bellamy (1958). [b] Musso and Matties (1957).

of AMD2 is shown around the spectral regions of OH, NH, and CO stretching modes. For the sake of comparison, the spectrum of actinomycin is reported in the same figure. The observed frequencies and assignments are listed in Table I.

As can be observed the frequency (1745 cm^{-1}) and the integral intensity of methyl ester of AMD2, as well as the lactone of actinomycin >C=O stretching bands, seem to be unperturbed, whereas the amide CO band of AMD2 is shifted to a lower frequency (~1615 cm^{-1}) than in actinomycin. Incidentally, five bands can be recognized in the amide I region of actinomycin which account for the structural differences in the amide groups. These findings suggest that, whereas the ester groups of AMD2, as well as the lactone groups in actinomycin, are not involved in hydrogen bonding, the >C=O amide group of AMD2 are hydrogen bonded and possibly conjugated with the aromatic chromophore.

The absence of a free OH band at approximately 3600 cm^{-1} (only a weak band is present at 3612 cm^{-1}) indicates that the OH groups of the threonine residues are hydrogen bonded. In Figure 2 the nmr spectrum of AMD2 in $CDCl_3$ is shown, and the assignment of the signals is reported. The strong splitting of 3.7 ppm of the two NH amide signals, and the unusually low field 10.75 ppm which characterizes one of these signals is particularly interesting. These features are not present in actinomycin nmr spectrum where only a splitting of 0.6 ppm and normal values (7.78, and 7.17 ppm) (Conti and De Santis, 1970; Arison and Moogsteen, 1970) of the chemical shift were observed. These results could be

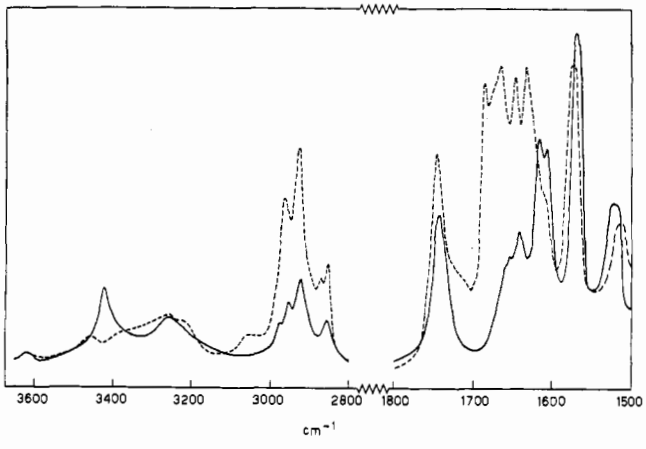

FIGURE 1: Infrared spectra of AMD2 (——) and actinomycin (- - -) in $CDCl_3$.

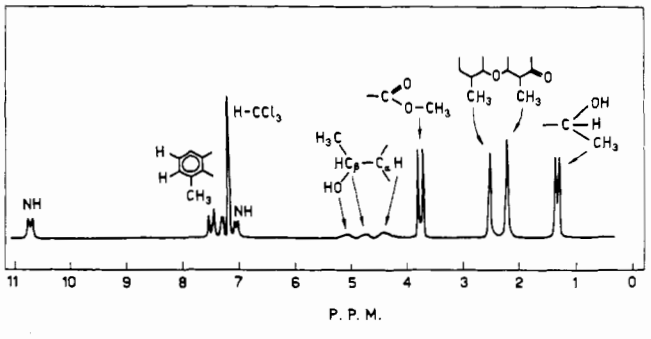

FIGURE 2: Nuclear magnetic resonance spectrum of AMD2 in $CDCl_3$-saturated solution with the assignment of various resonances.

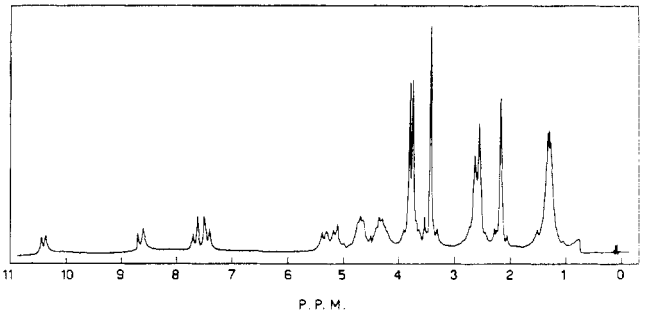

FIGURE 3: Nuclear magnetic resonance spectrum of AMD2 in Me_2SO-d_6.

explained assuming that the amide group connected to the quinone system lies in the same plane of phenoxazone and forms a chelate hydrogen bond, in which the lone pair of the aromatic nitrogen atom is involved. An additional hydrogen bond is also formed between the CO amide group and the NH_2 group of phenoxazone. Therefore both the chelate hydrogen-bond shift and the paramagnetic ring current shift from the aromatic polycycle, could account for the low field of the NH signal. This seems to be the case also when the nmr spectrum is recorded in dimethyl sulfoxide solution as shown in Figure 3, where one signal of NH group retains the same unusual chemical shift at 10.55 ppm, whereas the other is shifted to a lower field (8.59 ppm) of about 1.5 ppm. This result supports the above explanation and suggests that high-field NH groups are easily accessible to the solvent. On the contrary, recent investigations into the molecular structure of actinomycin have shown that the corresponding peptide groups, connecting the phenoxazone to the pentapeptide lactones, are out of the aromatic chromophore plane (Sobell *et al.*, 1971; De Santis *et al.*, 1972).

It is worthwhile noting that in the case of AMD2 in spite of the differences in the chemical shifts, the $J_{C_\alpha H-NH}$ coupling constants are very close to each other in the two solvents investigated (7.8 and 8.0 Hz in Me_2SO, 8.3 and 7.8 ppm in $CDCl_3$). The same consideration holds for the OH signals which show different chemical shifts, but very similar $J_{C_\beta H-OH}$ coupling constants.

Finally, the need to account simultaneously for (a) the formation of hydrogen bonds where the two OH and the CO amide groups are involved, as indicated by ir data, (b) the close values of the coupling constants, and (c) the coplanarity of the amide group with the phenoxazone, suggests, on the basis of simple stereochemical considerations, the scheme of the molecular structure illustrated in Figure 4. In this structure the two chemically equivalent threonine methyl ester residues are conformationally equivalent and related by simple translation in the molecule. This symmetry is imposed by the fact that the coplanarity of one amide group excludes the presence of a dyad axis, which would have related the two amino acid residues in the structure, as it was shown to occur in actinomycin. This conformation can account not only for the differences in the chemical shifts of OH and NH protons, and the similarities of the coupling constants but also for the splitting of the $-OCH_3$ signal. In spite of the local conformational equivalence of both threonine methyl ester residues, all these protons experience differ-

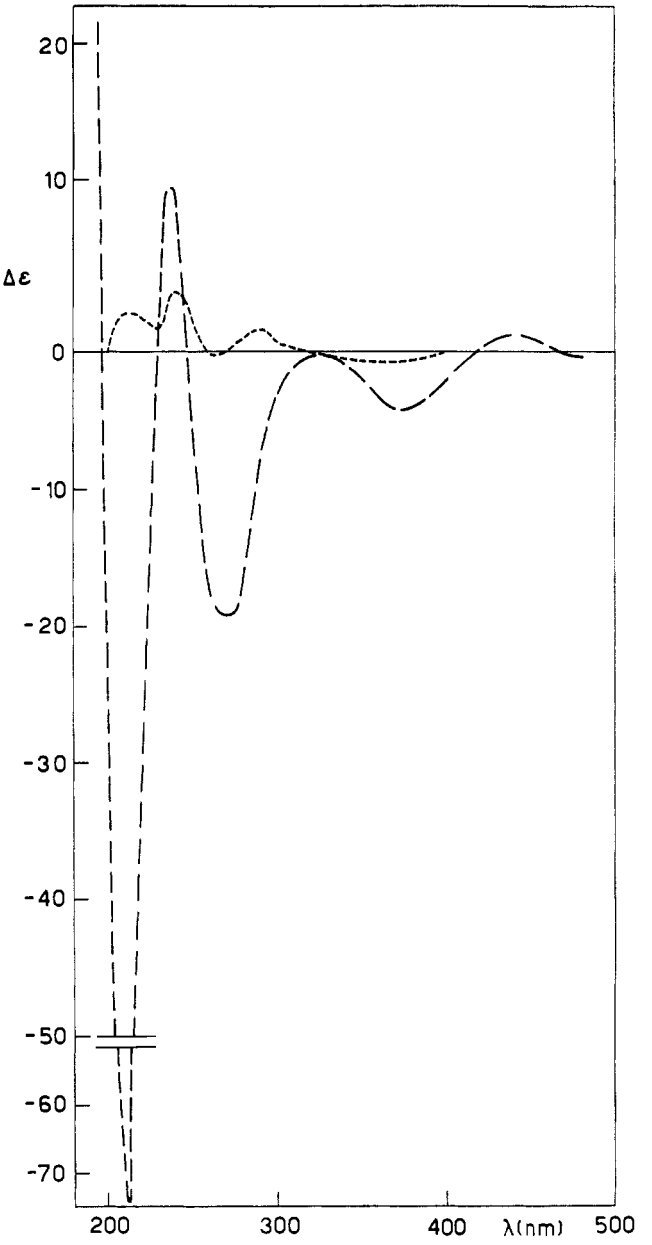

FIGURE 5: Circular dichroism of AMD2 (- - - -) and actinomycin (— — — —) in CH_3CN.

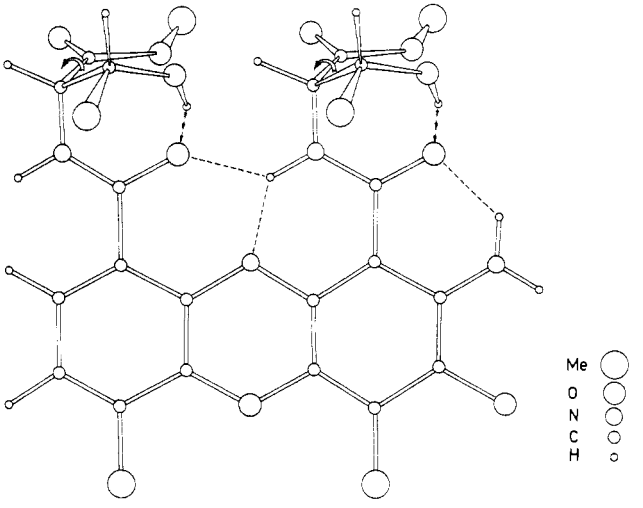

FIGURE 4: Scheme of the molecular conformation of AMD2.

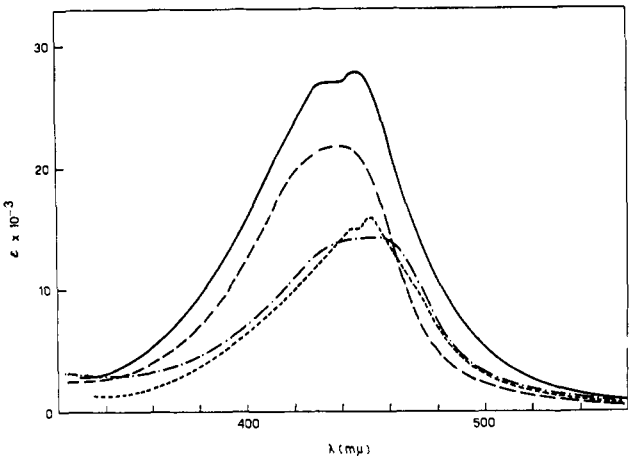

FIGURE 6: Absorption spectra in water and in DNA solution of AMD2 (—, - - -) and actinomycin (— —, –·–).

ent chemical environments as is apparent from Figure 4. Furthermore the proposed structure fits the ir findings consistently.

We are at present trying the crystal structure analysis of AMD2 crystallized from chloroform. The interpretation of the Patterson projection on *hk*0 plane and the fact that the asymmetric unit cell contains a pair of molecules, as well as the parameters of the crystal cell, indicate that a dimer is present, stabilized by a pair of equivalent hydrogen bonds between the NH and CO groups of the phenoxazone. This also seems to be the case in concentrated chloroform solutions where the ir and nmr spectra were measured, as suggested by the presence of a large band at about 3250 cm^{-1} in the ir spectrum.

In Figure 5 circular dichroism spectra of AMD2 and actinomycin in CH_3CN are shown in the spectral region 500–200 nm. As may be observed the rotatory strengths corresponding to AMD2, are much more low than those of actinomycin. This is consistent with our explanation (Ascoli *et al.*, 1970, 1972) of the origin of the main contribution of the CD bands in actinomycin, as arising from the dissymmetry of the chromophore due to the noncoplanarity of phenoxazone and the two amide groups directly connected with it. In fact, in the case of AMD2 the coplanarity of these groups considerably reduces the ellipticities of the CD bands.

Interaction with DNA. The possibility of interaction of AMD2 with DNA was investigated. On the basis of solubilization experiments tried with AMD2 in DNA solutions, evidence can be obtained of a strong change in the spectral properties corresponding to the electronic transitions of phenoxazone system, which indicate the formation of a complex between DNA and AMD2.

The absorption maximum at 440 nm shifts to a longer wavelength by about 10 nm with a 30% decrease in the extinction. As may be observed in Figure 6 the changes in the absorption spectra of AMD2 and actinomycin measured in water solutions and in the DNA complexes show similar features.

Recent experiments (Müller and Crothers, 1968; Ascoli *et al.*, 1968; Sobell *et al.*, 1971) have now confirmed that actinomycin interacts with DNA by intercalation of the phenoxazone moiety between two base pairs; the similarity of the optical properties of AMD2–DNA complex with those of actinomycin may be taken as a proof of a similar mechanism of intercalation.

In contrast, AMD2 lacks biological activity, as shown by the absence of inhibition in the transcription reaction "*in vitro*" (Ascoli and Savino, 1968). This behavior is common to other simpler actinomycin-related compounds investigated by Crothers and Müller (Müller and Crothers, 1968).

Other structural features are then required to assure the biological activity in actinomycin model compounds. Recent findings (Sobell *et al.*, 1971; De Santis, 1972) have in fact suggested that the interaction of actinomycin with DNA is stabilized by a pair of hydrogen bonds between suitably oriented CO groups of L-threonine residues and the guanine amino group on DNA. It follows that the design of synthetic analogs of actinomycin must be such as to obtain molecular structures capable of stabilizing all these interactions. Work is in progress in our laboratory on these lines.

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