

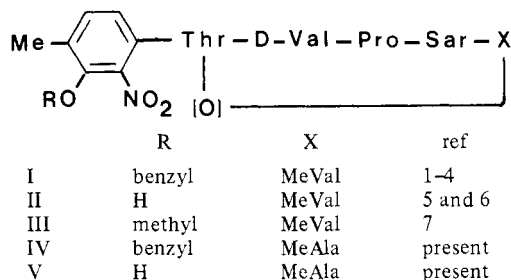
Conformation and Dimerization of Actinomycin-Related Peptide Lactones in Solution and in the Solid State

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Abstract: Actinomycin-related peptide lactone derivatives with the amino acid sequences Thr-D-Val-Pro-Sar-MeVal and Thr-D-Val-Pro-Sar-MeAla and the corresponding actinomycins (actinomycin D and its novel 5,5'-MeAla analogue) were synthesized by a new route. The solution properties of these compounds were studied by proton NMR in chloroform and acetone solutions. In dry chloroform, two conformers, A and C, are found, the ratio of which was concentration-dependent and results from dimerization of the A conformer. An X-ray crystallographic investigation of a Thr-D-Val-Pro-Sar-MeAla derivative indicated that hydrogen-bond associated pairs of A conformers, with a juxtapositional geometry remarkably similar to that known for actinomycin, were present in the solid state.

The pentapeptide lactone I was first described as an intermediate in a total synthesis^{1,2} of actinomycin D. Other syntheses of I^{3,4} and of the related II^{5,6} and III⁷ have also been reported.

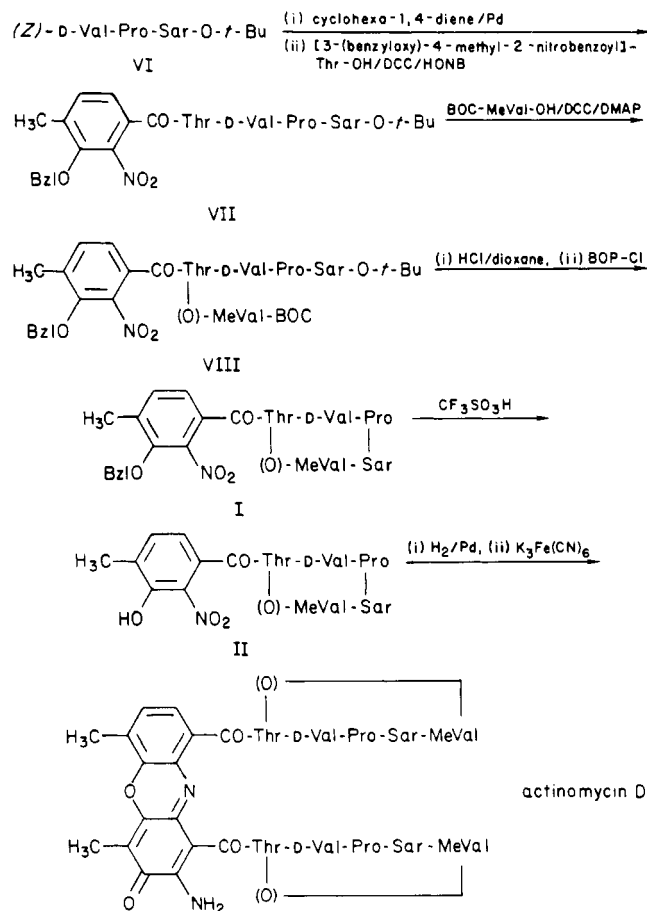


These compounds are of interest for comparison of their conformational characteristics as independent molecules with those observed within the actinomycin molecule in which they are held in close proximity. In the latter situation, an altered conformation should reflect interpeptide interaction and provide information on the nature of such interaction.

On the basis of proton and ¹³C NMR studies, Lackner proposed that in moist chloroform, I and III adopt a conformation (designated C) completely different from that of the peptide units of actinomycin.^{7,8} In acetone, however, another conformation (designated A) generated NMR parameters closely paralleling those produced by the actinomycin peptide units.⁹ X-ray crystallographic studies of complexes of actinomycin D with deoxyguanosine¹⁰ and d(GpC)¹¹ have revealed a conformation featuring cis Val-Pro and Pro-Sar peptide bonds and where each valyl amino H atom is bonded to the valyl O atom of the opposed peptide unit.

The rationale for a similar conformation of actinomycin in solution has been reviewed.⁹ The original purpose of the present study was to compare the conformation and biological activity of actinomycin D with an analog having both *N*-methylvaline residues replaced by *N*-methylalanines. This replacement occurs

Scheme I. Synthesis of Peptide Lactones I and II and Actinomycin D (For Abbreviations, See Text)



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naturally in the actinomycin Z complex, components of which differ from actinomycin D in several amino acid sites, including the replacement of one of the two *N*-methylvalines by *N*-methylalanine.^{12,13} Comparisons of the biological activities of two of these actinomycins (*Z*₁ and *Z*₅) with actinomycin D have been reported.¹⁴ However, the more interesting aspect of the present study evolved from unexpected observations of the self-

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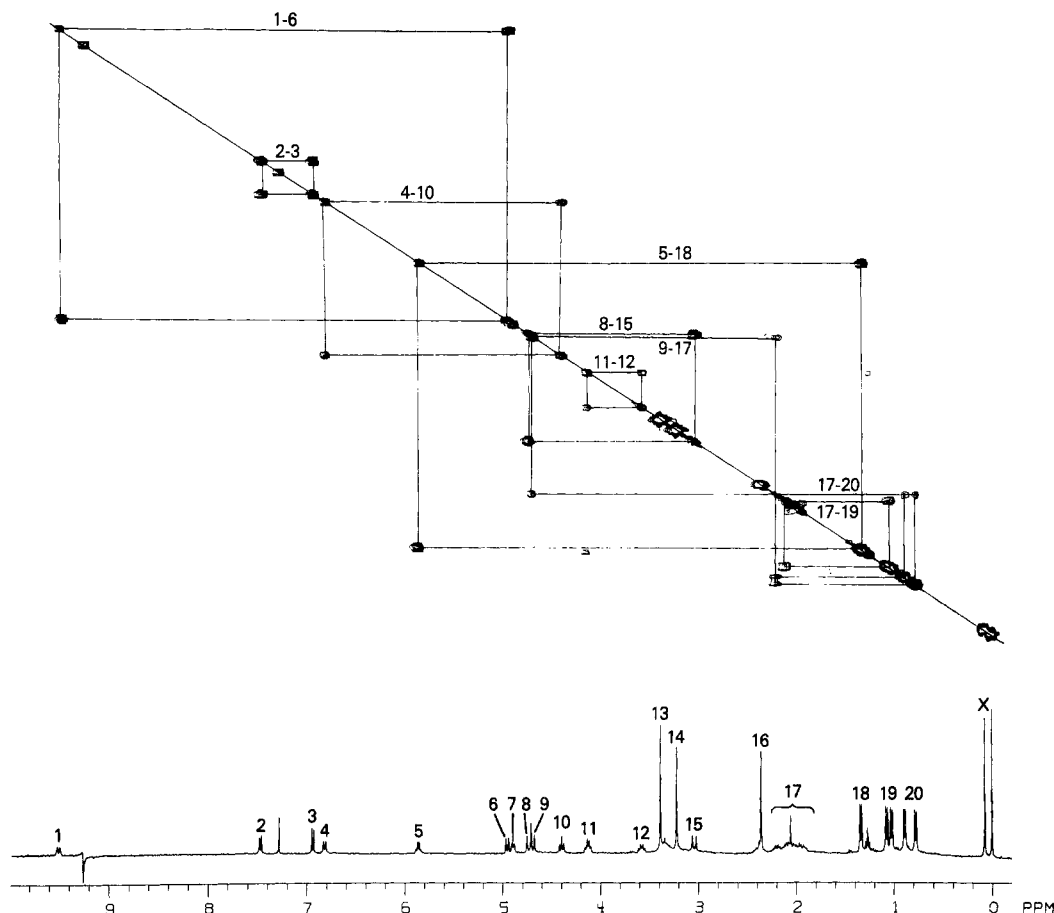


Figure 1. Homonuclear correlated 2D spectra (COSY) of II in CDCl_3 (assignments are given in Table I).

associative behavior of the A conformers of the peptide lactones in solution. The question of the structure of the complex naturally arises. Although conformational studies in solution (NMR) and the solid state (X-ray) have been reported on the related cyclic peptide cyclo(Thr-D-Val-Pro-Sar-MeAla),¹⁵ no crystal structure of any of the above peptide lactones has been described previously. We now report the crystal structure of one of these compounds (V) and proton NMR studies of peptide lactones I–V in solution. Parts of this investigation have been reported previously in preliminary form.¹⁶

Synthesis

The peptide lactones I and II and actinomycin D were synthesized according to Scheme I starting with the known protected tripeptide⁵ (Z)-D-Val-Pro-Sar-O-*t*-Bu (VI). Several other total synthesis of actinomycins have been described previously.¹³ The syntheses of peptide lactones IV and V and the new 5,5'-MeAla analog of actinomycin D were effected analogously. For the coupling of [3-(benzyloxy)-4-methyl-2-nitrobenzoyl]-L-threonine¹⁷ with the tripeptide *tert*-butyl ester derived from VI, dicyclohexylcarbodiimide (DCC) was used in conjunction with *N*-hydroxy-5-norbornene-2,3-dicarboxylic acid imide (HONB). The latter reagent has been used to prevent racemization in DCC-mediated coupling reactions,¹⁸ and in this case no allothreonine was detected in hydrolysates of the tetrapeptide derivative VII. 4-(Dimethylamino)pyridine (DMAP) was used as a catalyst¹⁹ in the DCC-mediated esterification step. For the cyclization of the

Table I. Chemical Shifts and Spin-Spin Coupling Constants for II in CDCl_3 (cf. Figure 1)

signal	proton	δ , ppm	coupling const, Hz
1	Thr NH	9.45 ^a	$J_{\text{NH,C}\alpha\text{H}} = 9.8$
2	Ar-H	7.44	$J_{5,6} = 7.8$
3	Ar-H	6.90	$J_{6,5} = J_{3,6} = 7.8$
4	D-Val NH	6.78	$J_{\text{NH,C}\alpha\text{H}} = 8.1$
5	Thr β -H	5.82	$J_{\beta,\text{Me}} = 6.3$
6	Thr α -H	4.92	$J_{\alpha,\beta} \approx 0$
7	Pro α -H	4.85	$J_{\alpha,\beta} = 7.6, 2.2$
8	Sar α -H	4.69	$J_{\alpha,\alpha'} = 14.8$
9	MeVal α -H	4.65	$J_{\alpha,\beta} = 12.1$
10	D-Val α -H	4.35	$J_{\alpha,\beta} = 8.1$
11	Pro δ -H	4.09	complex multiplet
12	Pro δ -H	3.54	complex multiplet
13	Sar N-CH ₃	3.35	singlet
14	MeVal N-CH ₃	3.18	singlet
15	Sar α -H	3.04 ^a	$J_{\alpha,\alpha'} = 14.8$
16	Ar-CH ₃	2.31	singlet
17	D-Val, MeVal, Pro β -, and Pro γ -H	1.9–2.1	obscured
18	Thr CH ₃	1.29	$J_{\text{Me},\beta} = 6.3$
19	D-Val CH ₃	1.07, 1.02	$J_{\text{Me},\beta} = 6.7, 6.7$
20	MeVal C-CH ₃	0.88, 0.77	$J_{\text{Me},\beta} = 6.3, 6.7$

^a Varies somewhat with concentration and temperature.

O-peptide resulting from acidolytic deprotection of VIII, *N,N*-bis(2-oxo-3-oxazolidinyl)phosphorodiamidic chloride (BOP-Cl)²⁰ was employed, resulting in higher yields (ca. 50%) than those obtained with DCC. Characterization of noncrystalline intermediates was undertaken by chemical ionization or Cf-252 plasma desorption mass spectroscopy and proton NMR.

Proton NMR Studies. (a) Peptide Lactones in Chloroform. Spectra of peptide lactones I, II, IV, and V were obtained at 220

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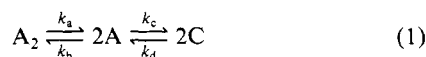
Table II. Chemical Shifts (δ) of Peptide Lactone Protons^a

	CDCl ₃							CD ₃ COCD ₃			
	I(C)	II(C)	IV(C)	V(C)	I(A)	IV(A)	V(A)	I(A)	II(A)	IV(A)	V(A)
Thr NH	9.20	9.45	9.44	8.98	obsc	7.25	7.42	obsc	7.30	7.64	7.55
D-Val NH	6.61	6.78	6.54	obsc	8.08	8.04	8.06	obsc	8.09	8.22	8.14
Pro α-H	obsc	obsc	obsc	obsc	6.30	6.30	6.30	6.40	6.43	6.35	6.35
Thr β-H	5.75	5.82	5.70	5.70	5.25	5.15	5.22	5.30	5.32	5.25	5.25
Sar N-CH ₃	3.40	3.35	3.40	3.37	2.91	2.90	2.89	2.86	2.86	2.86	2.83
MeVal or MeAla N-CH ₃	3.18	3.18	3.20	3.16	3.26	3.24	3.13	3.30	3.25	3.25	3.20
Ar-CH ₃	2.38	2.31	2.39	2.32	2.31	2.31	2.30	2.43	2.32	2.45	2.35

^a Letters A or C in parentheses denote conformer. The concentration of II(A) in CDCl₃ was too low to record data.

MHz and in some cases at 360 MHz. Assignments were aided by the use of two-dimensional homonuclear correlated spectra (COSY);²¹ this is illustrated in the case of II in Figure 1 and Table I. In all cases except that of II, both A and C conformers were observed in dry CDCl₃. Lackner⁷ did not report such conformational heterogeneity, but his spectrum of I was obtained after treating the solution with a drop of D₂O. We observed that addition of a drop of D₂O to a CDCl₃ solution of I eliminated the signals assigned to the A conformer. Chemical shifts of some representative protons in these compounds are shown in Table II; those representing the A conformer in CDCl₃ correlate well with those obtained in CD₃COCD₃. Likewise, $J_{\text{NH,C}\alpha\text{H}}$ values (Hz) for the A conformer of IV in CDCl₃ (Thr = 7.9; D-Val = 5.7) approximate those obtained in CD₃COCD₃ (Thr = 8.4; D-Val = 5.5). NMR spectral parameters (for example, see Table I) assigned to the C conformers of these compounds correlated well with those reported by Lackner⁷ for I and III. The presence of A conformer under these conditions was more pronounced for the compounds having R = benzyl than R = H and more pronounced for the peptides containing *N*-methylalanine than *N*-methylvaline. For example, I and IV had [A]/[C] ratios of 1:2 and 2:1, respectively, at 0.1 M concentration. In the debenzylated series, II contained essentially no A conformer whereas V had an [A]/[C] ratio of 1:1 at 0.2 M concentration. The [A]/[C] ratios were strongly concentration-dependent, higher concentrations favoring the A conformer.

This aspect was studied in more detail in the case of I by measuring [A]/[C] at several concentrations with use of the *N*-methyl singlets. The interpretation of the observations is that the A conformation is predominantly dimeric in solution. We visualize this by first writing the equilibrium between the A and C conformers as



The relevant rate constants are k_a , k_b , k_c , and k_d . Equilibria between the various forms give

$$[A]^2/[A_2] = k_a/k_b = K_1; [C]/[A] = k_c/k_d = K_2 \quad (2)$$

$$[C]^2/[A_2] = K_1 K_2^2$$

If we take the total amount of A conformation in solution (normalized as the monomeric form) to be {A}, we have

$$\{A\} = [A] + 2[A_2] \quad (3)$$

Substituting for [A] and [A₂] from eq 2, we obtain

$$\{A\} = [C]/K_2 + 2[C]^2/(K_1 K_2^2) \quad (4)$$

$$\{A\}/[C] = 1/K_2 + 2[C]/(K_1 K_2^2) \quad (5)$$

Equation 5 shows that the {A}/[C] ratio will be a linear function of [C]. Knowing {A}/[C] from the NMR spectra and hence [A] from the initial makeup of the solution, one can calculate [C] and therefore plot {A}/[C] vs. [C] as shown in Figure 2. The straight-line behavior with an intercept which is zero within the experimental error provides strong evidence for the existence of

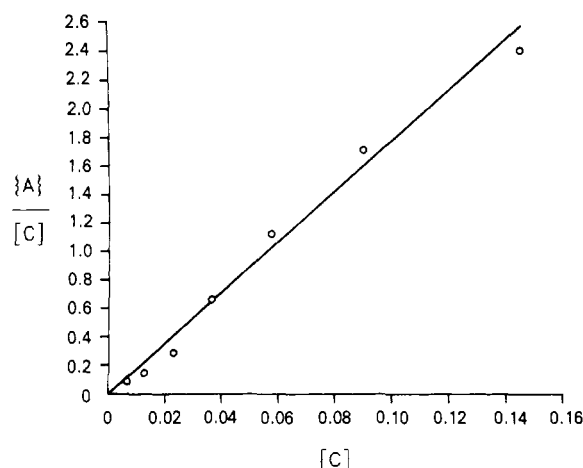


Figure 2. Plot of the {A}/[C] conformeric ratio of I against the molar concentration of the C conformer in CDCl₃.

Table III. Comparison of Chemical Shifts (δ) of Representative Protons in Actinomycin D and Its 5,5'-MeAla Analogue^a

solv, actinomycin	CDCl ₃		C ₆ D ₆ /CDCl ₃ (1:1)	
	D	analogue	D	analogue
D-Val NH	8.12 (5.7)	8.15 (5.5)	8.52	8.43
	7.98 (5.9)	7.99 (5.9)	8.28	8.21
Thr NH	7.80 (6.6)	7.74 (6.5)	7.93	7.96
	7.17 (6.8)	7.21 (6.7)	7.40	7.45
H-8	7.65	7.66	7.60	7.63
H-7	7.38	7.37	7.01	7.02
Pro α -H	6.03	6.02	6.18	6.16
	5.98	5.94	6.05	6.09
N-CH ₃	2.91	2.93	2.76	2.73
	2.90	2.91	2.75	2.73
	2.87	2.86	2.48 ^b	2.23 ^c
	2.87	2.86	2.36 ^b	2.17 ^c
6-CH ₃	2.55	2.55	2.21	2.29
4-CH ₃	2.24	2.23	1.95	1.98
Thr CH ₃	1.26	1.26	1.42, 1.39	1.46, 1.43

^a Figures in parentheses represent $J_{\text{NH,C}\alpha\text{H}}$ values. ^b N-CH₃ protons of *N*-methylvaline. ^c N-CH₃ protons of *N*-methylalanine.

the A conformer as a dimer, an observation consistent with the occurrence of a dimer in the solid state. (The actual least-squares values are -0.02 (6) and 8.8 (5) mol⁻¹ for the intercept and slope, respectively.) The equilibrium constant for the A₂ = 2C reaction, [C]²/[A₂], is found to be 0.114 (4) mol.

(b) Peptide Lactones in Acetone. Spectra of the peptide lactones I, II, IV, and V in CD₃COCD₃ resembled those reported⁹ for III and were attributed to the A conformation. Chemical shifts of some representative protons are given in Table II. No conformational heterogeneity was observed provided the solvent was dry, but in moist acetone, as reported previously,⁹ mixtures of A and C conformers were observed.

(c) Actinomycins. Spectra of natural and synthetic actinomycin D in CDCl₃ were indistinguishable from each other and from those reported in the literature.²² The 5,5'-MeAla analogue produced

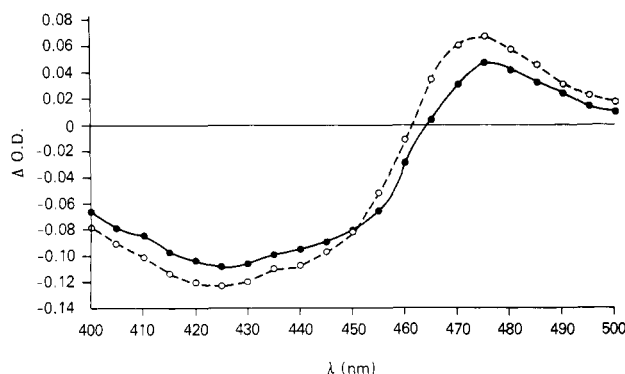
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Table IV. Possible Hydrogen Bonds in Crystals of the Peptide Lactone. The Subscripts Designate the First and Second Molecules. Distances Are Given in Å and Angles in deg

	donor	acceptor	D-A	H...A	D-H...A	symmetry operation ^a
1	water ^b	O(Sar) ₁	3.10 (1)			2 _{1b}
2	water	O(D-Val) ₂	3.16 (1)			2 _{1b}
3	O(08) ₁	O(1)(Etac)	2.985 (8)	2.45 (8)	129 (8)	2 _{1b}
4	O(08) ₁	O(Thr) ₂	2.957 (7)	2.41 (8)	131 (8)	1
5	N(Thr) ₂	O(1)(Etac)	3.086 (7)	2.10 (6)	174 (6)	2 _{1b}
6	N(D-Val) ₁	O(D-Val) ₂	2.938 (7)	2.04 (6)	170 (6)	2 _{1a}
7	N(D-Val) ₂	O(D-Val) ₁	2.900 (7)	2.05 (6)	161 (6)	2 _{1a}
8	O(08) ₂	O(Pro) ₁	2.953 (8)	2.30 (8)	112 (5)	2 _{1c}
9	O(08) ₂	N(09) ₂	2.848 (8)	2.44 (7)	97 (4)	1
10	O(08) ₂	O(12) ₂	2.713 (9)	2.04 (7)	112 (5)	1
11	N(Thr) ₁	O(Thr) ₁	2.609 (7)	2.20 (7)	107 (5)	1

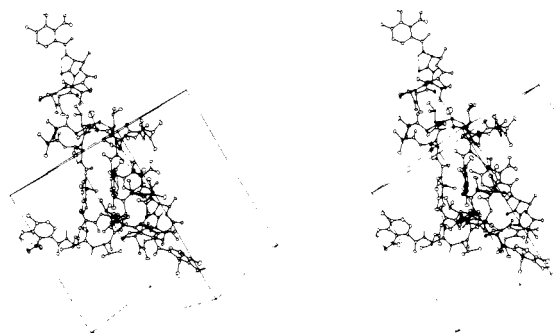
^a The symbols 2_{1a}, 2_{1b}, and 2_{1c} represent the 2-fold screw axis operations parallel to *a*, *b*, and *c*, respectively. ^b The H atoms attached to the O atom of the water molecule were not found.

**Figure 3.** Difference spectra for the actinomycins with calf thymus DNA in 0.01 M phosphate buffer, pH 7.00. Actinomycin D (synthetic): dashed line; 5,5'-methylalanylactinomycin D: solid line. Concentrations of actinomycins and DNA, 26 and 38 μg/mL, respectively.

spectra which resemble those of actinomycin D even more closely than those of a series of analogs in which variations have been introduced in the proline sites²³ (see Table III). The only differences were predictably attributable to the replacement of *N*-methylvaline by *N*-methylalanine. For example, doublets at δ 1.32 and 1.37, not present in spectra of actinomycin D, were assigned to the *N*-methylalanyl C(methyl) protons. Although the benzene shifts of the *N*-methylalanyl N(methyl) protons were even larger (*c* vs. *b*, Table III) than those of the corresponding *N*-methylvaline protons of actinomycin D, this is presumably due to their greater steric accessibility. In other words, no evidence for any peptide backbone conformational difference between these actinomycins is apparent.

Biological Activities. (a) Binding of the actinomycins to calf thymus DNA was assessed by difference spectroscopy.²⁴ Comparison of synthetic actinomycin D with its 5,5'-MeAla analogue is shown in Figure 3; the difference spectrum of the former was almost superimposable on that of natural actinomycin. The data show that the analogue binds somewhat less strongly than actinomycin D. (b) Antimicrobial activities were measured by the agar diffusion-well method.²⁵ Minimal inhibitory concentrations (in μg/mL) vs. *B. subtilis* 558 and *M. luteus* PCI were as follows: actinomycin D (natural), 0.19 and 0.39 (these were sequential dilutions and are within experimental error of each other); actinomycin D (synthetic), 0.19 and 0.19; 5,5'-(methylalanyl)actinomycin D, 3.13 and 3.13. The antimicrobial activity of the analogue is approximately an order of magnitude lower than that of actinomycin D.

Hydrogen Bonding in the Solid State. Details of the very extensive hydrogen bonding are given in Table IV. Bonds 3 and

**Figure 4.** Packing diagram showing the environment of a hydrogen-bonded dimer in the crystal. Although a unit cell is delineated, not all the molecules in it are shown. The origin is at the top center of the cell, the *a* axis extends upwards to the right, the *c* axis downward to the left, and the *b* axis downwards to the right.

4 represent a bifurcated bond and bonds 8–10 a trifurcated bond (such bonds seem to be quite well established; a recent compilation for amino acids²⁶ list three). The donor–H...acceptor angles are appropriate, being roughly 120° and 109°, respectively. Bonds 6 and 7 link the molecules into the hydrogen-bonded dimer which is the basic packing unit. There is some evidence of intramolecular hydrogen bonding, but it probably does not occur across the rings since the possible bond indicated as 11 is doubtful because of the unfavorable donor–H...acceptor angle, although the contact distance is quite small. Interatomic distances are never a reliable indicator of hydrogen bonds, and it might be noted that in the second molecule where the threonine N atom is indicated as forming a hydrogen bond to the solvent, the corresponding intramolecular N...O distance is very similar. The remainder of the intramolecular bonding is somewhat complex since O(08)₁ of the protecting group appears to form a bifurcated bond along the *a* axis to O(Thr)₁ and also to O(1) of the ethyl acetate. The corresponding O atom in the second molecule, O(08)₂, may be involved in a trifurcated bond, stabilizing the conformation of the protecting group and also providing some intermolecular attraction along the *b* axis. Overall, there is a pattern of reasonably strongly linked hydrogen-bonded dimers with evidence of considerable interaction along the *a* and *b* axes. The largest difference in an ω conformational angle is that between the two values for Sar-Pro, and the deviation may be caused by the involvement of only one of the O atoms in the trifurcated bond.

The details of bonds 1 and 2 are somewhat indefinite since difference maps did not show the water H atoms (not surprising in view of the high O thermal parameters), but the nonbonded distances are reasonably small and the water molecule both links the dimers and fills a hole along the *a* axis.

Proline Conformation and Disorder. It is interesting that still another instance of a disordered proline conformation in a peptide is shown by the present structure. A previous investigation of a similar peptide¹⁵ also reported a two-molecule asymmetric unit

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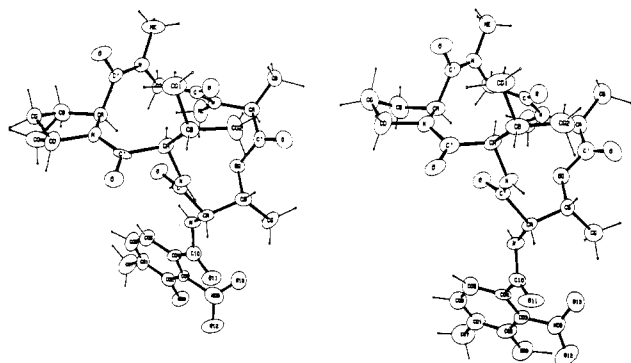


Figure 5. Crystal conformations of the two independent molecules (note: not a stereodiagram).

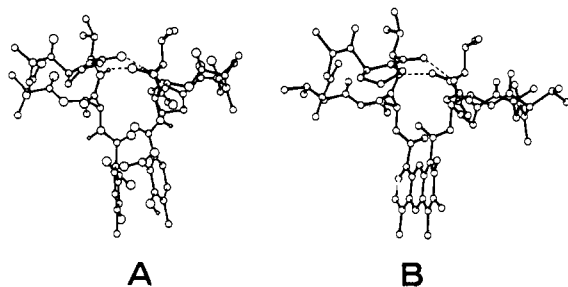
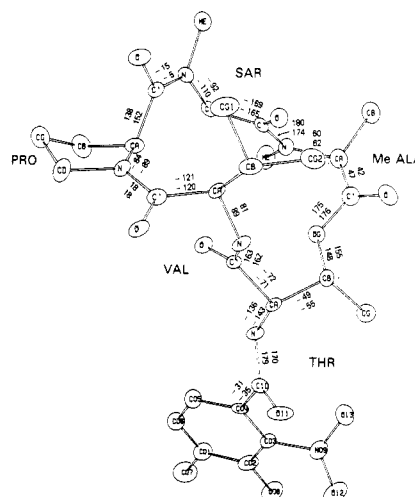


Figure 6. Local 2-fold axial situation in the crystal of the peptide lactone (A) compared with that in the actinomycin D complex of Jain and Sobell¹⁰ (B).

with one proline disordered and the other ordered. Examination of packing diagrams (Figure 4) and intermolecular distances discloses that C γ for the first molecule in either of its two sites is approximately at a van der Waals distance²⁷ from any other atom but the corresponding atom in the other molecule and its associated H atoms are essentially locked in by the ethyl acetate molecule. The difference in packing is not dramatic, but obviously, given the occurrence of such disorder, only small energy differences are sufficient to stabilize the proline conformation.

Solid-State Conformation and Dimensions. The bond lengths and angles are given in Table V and the crystal conformations of the two independent molecules are shown in Figure 5. The lengths and angles in the residues show considerable individual variation, but, within one standard deviation, the mean values do not differ from those quoted by Benedetti²⁸ or from those in the previous study¹⁵ of an actinomycin-related peptide. A packing diagram with indication of hydrogen bonds is given as Figure 4. It can be seen that the two molecules of the asymmetric unit are related by a local approximate 2-fold axis, and this situation is indicated in Figure 6. A program, written to investigate the local symmetry, indicated that all atoms in the two molecules could be related by a 2-fold axis with the largest deviations, involving the aromatic ring of the protecting group, being 1.2–1.8 Å. If the atoms of the protecting groups are disregarded, all heavy atoms lie within 1 Å of the positions required by a 2-fold axis. The similarity of the two-molecule unit to that shown by actinomycin D in its complex with deoxyguanosine¹⁰ (Figure 6) is quite remarkable. The present structure reproduces most of the features including conformation and hydrogen bonds, and the protecting groups have a similar role to the aromatic group of actinomycin. In the actinomycin complex, as in the present crystal structure, there is no crystallographic requirement that the two peptide units have the same conformation. The conformational angles for the two peptide lactones are shown in Figure 7, and the observed conformation is essentially that observed in actinomycin¹⁰ and designated the A' conformation by Lackner. The ϕ - ψ plot, given as Figure 8 shows the small differences between the independent



indicates, its presence does not prevent crystallization of the A conformer from ethyl acetate. The manner in which this stabilization might occur was considered with the aid of a space-filling (CPK) molecular model of the C conformation proposed by Lackner.⁹ It was found that the interatomic distances would readily allow a hydrogen-bonded water bridge between the carbonyl O atoms of D-valine and N-methylvaline. It is hoped that it will be possible to obtain a suitable crystal to investigate this speculative structure by X-ray crystallography. The present crystallographic investigation reveals a hydrogen-bonded interaction between peptide lactone molecules in the A conformation analogous to that present within the actinomycin molecule. This interaction produces juxtapositional geometry closely resembling that of actinomycin, despite the absence of the covalent clamping provided in the latter case by the chromophore. The results of the NMR observations in chloroform solution can be explained by the presence of a similar dimeric structure. This tendency to dimerization could accelerate the oxidative condensation of two molecules of a 3-hydroxy-4-methylanthraniloyl peptide lactone which represents the final step in the synthesis (and, probably, biosynthesis³⁰) of an actinomycin. Evidence that this is the case will be presented in a future publication.

It is evident that replacement of both of the N-methylvaline residues of actinomycin D by N-methylalanine drastically reduces the biological activity. This difference is not explained on conformational grounds, since the NMR studies described here reveal a conformational conformity between the two compounds. The explanation presumably lies in the altered shape of the peptide periphery since the isopropyl side chains of the N-methylvaline residues are replaced by less bulky methyl groups. According to a proposed stereochemical model³¹ for the actinomycin-DNA complex, the N-methylvalyl side chains are located where hydrophobic interaction with the deoxyribose units may play a role in binding. Replacement of only one N-methylvaline residue by N-methylalanine is not as deactivating, to judge from the biological activities reported¹⁴ for actinomycins Z₁ and Z₅. However, such a comparison is complicated by the presence in the latter actinomycins of additional amino acid variations.¹³

Experimental Section

Elementary analyses were provided by Galbraith Laboratories, Inc., Knoxville, TN.

Amino acid analyses were obtained on a Beckmann Model 121 MB amino acid analyzer for threonine, valine, and proline. The N-methyl-amino acids, which were not measurable by this procedure, were assayed relative to valine by gas-liquid chromatography (on a Shimadzu Model 4BM equipped with flame ionization detectors) after derivatization of the hydrolyzate amino acids as their N-formyl methyl esters. An OV225 column (3% on Gas Chrom Q, 100–120 mesh) was employed at 120 °C with a 4 °C/min temperature program (method A) or an EGA column (0.5% on Chromosorb W, 60–80 mesh) at 100 °C with a 4 °C/min temperature program (method B). Retention times (min) were as follows. Method A, Sar, 7.1; MeVal, 8.3; Val, 9.2; Pro, 14.7. Method B, Sar, 9.8; MeAla, 10.5; Val, 13.0; Pro, 17.9. Molar ratios of amino acids were obtained from peak area ratios in comparison with standard mixtures. The molar ratios reported here are normalized to a total of 4.00 for all the amino acids in an actinomycin except threonine; low values for this amino acid in actinomycin D have been reported previously³² and attributed to partial destruction during hydrolysis.

Chemical ionization mass spectra (CIMS) were obtained on a Finnigan 1015 mass spectrometer with ammonia or methane as specified. Cf-252 plasma desorption mass spectrometry (PDMS)³³ was performed on an instrument constructed by Dr. R. Macfarlane (Texas A & M University). Samples were applied to an aluminized mylar film by the electrospray technique and spectra were acquired for 20 min. H and Na were used as internal mass calibrants.

Proton NMR spectra were obtained on a Varian HR220 in the CW mode and on a Nicolet NT-360WB in the Fourier transform mode. The two-dimensional homonuclear correlated spectra (COSY) were obtained by using the usual two-pulse sequence and processed by using a "sine bell"

function.³⁴ The connectivities were obtained by inspection directly from the 2D contour plots.

The concentration dependence of the conformeric A/C ratio was studied in the case of I by obtaining spectra at 24 °C at seven different concentrations in CDCl₃ ranging from 0.0074 to 0.49 M. The A/C ratio at each concentration was measured by comparing peak heights of the sarcosine N-CH₃ singlet in A (at δ 2.91) with that of N-methylvaline in C (at δ 3.18); the peak widths were equal. From these data, [A]/[C] and hence [C] were determined for each concentration and plotted (Figure 2).

Difference spectra (UV-visible) of actinomycins with calf thymus DNA were measured on a Beckman DB spectrophotometer. Optical densities of actinomycin solutions were subtracted from those measured for solutions of similar concentration containing DNA (see Figure 3).

Antimicrobial activities of actinomycins were obtained by the agar well-diffusion method²⁵ through the courtesy of Dr. J. W. Westley of Hoffmann-La Roche Inc. Samples were dissolved in acetonitrile/dimethyl sulfoxide/methanol (1:2:1) prior to dilution. The agar used was "Antibiotic Medium No. 1" (Seed Agar, Baltimore Biologicals Inc.)

[3-(Benzyloxy)-4-methyl-2-nitrobenzoyl]-L-threonyl-D-valyl-L-prolyl-sarcosine *tert*-Butyl Ester (VII). A solution of carbobenzoxy-D-valyl-L-prolylsarcosine *tert*-butyl ester⁵ (VI) (2.03 g, 4.27 mmol) and 1,4-cyclohexadiene³⁵ (5.0 mL) in ethanol (40 mL) was stirred under nitrogen with 10% palladium/charcoal (250 mg) until TLC indicated the absence of VI. After filtration and evaporation, the residue was dissolved in dimethylformamide (15 mL) and [3-(benzyloxy)-4-methyl-2-nitrobenzoyl]-L-threonine¹⁷ (2.00 g, 5.15 mmol) was added. After stirring and cooling to -5 °C, a solution of HONB¹⁸ (1.97 g) in dimethylformamide (5 mL) was added followed by DCC (2.04 g, 9.89 mmol). After 48 h, the solution was filtered and evaporated in vacuo and the residue was subjected to flash chromatography³⁶ on silica gel with 3% ethanol/ethyl acetate to afford VII as an amorphous solid, yield 2.23 g (73%). CIMS with ammonia [ions at m/z 712 (M + 1, rel intensity 0.55) and 729 (M + 18, rel intensity 0.12)] indicate M = 711 (C₃₆H₄₉N₅O₁₀ requires M = 711.81); NMR (CDCl₃) δ 0.95 and 1.00 (d and d, D-Val CH₃), 1.24 (d, Thr CH₃), 1.44 (s, *t*-Bu), 2.37 (s, Ar-CH₃), 3.13 (s, N-CH₃); other signals were complicated by conformational heterogeneity.

N-[3-(Benzyloxy)-4-methyl-2-nitrobenzoyl]-O-(*tert*-butoxycarbonyl-N-methyl-L-valyl)-L-threonyl-D-valyl-L-prolylsarcosine *tert*-Butyl Ester (VIII). A solution of VII (1.17 g, 1.26 mmol) and *tert*-butoxycarbonyl-N-methyl-L-valine³⁷ (430 mg, 1.86 mmol) in dichloromethane (10 mL) was stirred at 0 °C prior to addition of DMAP (102 mg) and a solution of DCC (420 mg, 2.04 mmol) in dichloromethane (4 mL). After 16 h at 26 °C, the solution was filtered and evaporated and the residue subjected to flash chromatography on silica gel with ethyl acetate/chloroform (2:1) to afford VIII as an amorphous solid, yield 1.35 g (89%); PDMS peaks at 924.39 (M - H)⁺, 926.30 (M + H)⁺, 948.24 (M + Na)⁺, indicate M = 925 (C₄₇H₆₈N₆O₁₃ requires M = 925.09); NMR (CDCl₃) δ 1.32 (d, Thr CH₃), 1.43 and 1.45 (s and s, *t*-Bu), 2.38 (s, Ar-CH₃); other protons gave multiple signals due to conformational heterogeneity.

N-[3-(Benzyloxy)-4-methyl-2-nitrobenzoyl]-O-(*tert*-butoxycarbonyl-N-methyl-L-alanyl)-L-threonyl-D-valyl-L-prolylsarcosine *tert*-Butyl Ester (IX). This was prepared by the same procedure as VIII from VI (3.20 g, 4.50 mmol) and *tert*-butoxycarbonyl-N-methyl-L-alanine³⁷ (1.50 g, 7.38 mmol) and purified by flash chromatography as before to afford IX as an amorphous solid, yield 3.27 g (81%); PDMS peaks at 896.31 (M - H)⁺, 898.32 (M + H)⁺, 920.02 (M + Na)⁺, indicate M = 897 (C₄₅H₆₄N₆O₁₃ requires M = 897.03); NMR (CDCl₃) δ 1.40 and 1.44 (s and s, *tert*-butyl), 2.38 (s, Ar-CH₃); other protons gave multiple signals due to conformational heterogeneity.

[3-(Benzyloxy)-4-methyl-2-nitrobenzoyl]-L-threonyl-D-valyl-L-prolyl-sarcosyl-N-methyl-L-valine Lactone (I). A solution of VIII (1.91 g, 2.06 mmol) in 4 N HCl/dioxane (30 mL) was protected from moisture at 22 °C for 2 h and then evaporated in vacuo. The residue was dissolved in water (30 mL) and the pH adjusted to 7.0 with aqueous sodium hydroxide. After evaporation in vacuo, the dry residue was dissolved in dichloromethane (500 mL). Diisopropylethylamine (0.35 mL) was added followed by a solution of BOP-Cl²⁰ (640 mg, 2.51 mmol) in dichloromethane (100 mL). After 3 days at 22 °C, additional diisopropylethylamine (0.17 mL) and BOP-Cl (320 mg) were added. After stirring a further 2 days, the solution was evaporated and the residue dissolved in ethyl acetate (50 mL) to form a cloudy solution which was filtered

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Table V. Dimensions of the Molecules: Heavier Atom Bond Lengths (Å) and Angles (deg)^a

<i>i</i>	1 (Pro)	2 (Sar)	3 (MeAla)	4 (Thr)	5 (D-Val)
Lengths					
C'(i)-O(i)	1.215 (7)	1.220 (8)	1.185 (8)	1.234 (8)	1.269 (8)
	1.236 (8)	1.235 (7)	1.192 (8)	1.217 (8)	1.250 (8)
C'(i)-C ^α (i)	1.522 (9)	1.522 (10)	1.551 (10)	1.523 (9)	1.552 (9)
	1.526 (9)	1.528 (10)	1.503 (11)	1.528 (9)	1.502 (9)
C ^β (i)-O ^β (i)				1.464 (8)	
				1.470 (9)	
C ^α (i)-N(i)	1.476 (8)	1.451 (9)	1.484 (8)	1.456 (8)	1.458 (8)
	1.485 (8)	1.463 (9)	1.483 (9)	1.454 (8)	1.464 (8)
C ^β (i)-C ^γ (i)				1.520 (11)	1.538 (11), 1.548 (12)
				1.510 (11)	1.561 (12), 1.522 (13)
C ^γ (i)-C ^δ (i)	1.45 (2), 1.48 (2)				
	1.512 (13)				
C ^γ (i)-C ^δ (i)	1.45 (2), 1.55 (2)				
	1.521 (14)				
C ^δ (i)-N(i)	1.481 (8)				
	1.483 (10)				
C ^α (i)-C ^β (i)	1.526 (10)		1.520 (11)	1.514 (10)	1.525 (10)
	1.542 (12)		1.514 (13)	1.544 (10)	1.570 (9)
N(i)-Me(i)		1.456 (13)	1.451 (11)		
		1.470 (11)	1.467 (10)		
N(i)-C'(i-1)	1.323 (8)	1.348 (8)	1.342 (7)		1.344 (8)
	1.345 (8)	1.334 (8)	1.334 (8)		1.354 (8)
O ^β (i)-C'(i-1)				1.337 (7)	
				1.349 (8)	
Protecting Group					
N(4)-C10	1.343 (9)	1.364 (8)	C01-C06	1.374 (10)	1.381 (11)
C01-C07	1.489 (13)	1.550 (13)	C02-C01	1.391 (10)	1.395 (11)
C02-O08	1.361 (8)	1.348 (9)	C03-C02	1.392 (9)	1.391 (10)
C03-N09	1.494 (8)	1.432 (8)	C04-C03	1.366 (8)	1.408 (9)
C04-C05	1.380 (10)	1.375 (10)	C06-C05	1.388 (11)	1.379 (11)
N09-O12	1.225 (7)	1.213 (8)	N09-O13	1.216 (7)	1.230 (8)
C10-C04	1.505 (9)	1.516 (9)	C10-O11	1.227 (8)	1.214 (8)
Ethyl Acetate					
O1-C1	1.182 (9)		C1-C2	1.472 (12)	
C1-O2	1.310 (9)		O2-C3	1.476 (33)	
O2-C3'	1.529 (33)		C3-C4	1.496 (31)	
C3'-C4	1.279 (34)				
<i>i</i>	1 (Pro)	2 (Sar)	3 (MeAla)	4 (Thr)	5 (D-Val)
Angles					
C'(i)-C ^α (i)-C ^β (i)	109.0 (5)		110.6 (6)	109.2 (5)	109.7 (5)
	110.7 (6)		112.1 (7)	108.3 (5)	109.3 (5)
C'(i)-C ^α (i)-N(i)		111.3 (5)	110.9 (5)	106.2 (5)	109.6 (5)
		113.4 (5)	114.6 (6)	110.7 (5)	111.4 (5)
C'(i-1)-N(i)-C ^α (i)	125.6 (5)				
	126.8 (5)				
C ^α (i)-C ^β (i)-C ^γ (i)				116.6 (6)	
				113.9 (6)	
C ^α (i)-C ^β (i)-C ^γ (i)					111.7, 110.1 (6)
					110.5, 111.4 (6)
C ^α (i)-C ^β (i)-O ^β (i)				103.4 (5)	
				106.7 (5)	
C ^α (i)-N(i)-C'(i-1)		128.0 (5)	118.6 (5)		120.7 (5)
		125.0 (5)	118.3 (5)		120.3 (5)
C ^α (i)-N(i)-Me(i)		116.2 (6)	116.4 (5)		
		113.7 (5)	117.8 (6)		
C ^β (i)-C ^α (i)-N(i)			115.2 (6)	112.9 (6)	111.0 (5)
			112.5 (7)	115.2 (5)	107.1 (5)
C ^β (i)-O ^β (i)-C'(i-1)				115.7 (4)	
				115.1 (5)	
C ^δ (i)-C ^γ (i)-C ^β (i)	106, 102 (1)				
	103.0 (7)				
C ^δ (i)-N(i)-C'(i-1)	120.4 (5)				
	120.2 (6)				
C ^δ (i)-N(i)-C ^α (i)	112.3 (5)				
	111.5 (6)				
C ^γ (i)-C ^δ (i)-C ^α (i)	107, 109 (1)				
	104.8 (6)				
C ^γ ¹ (i)-C ^β (i)-C ^γ ² (i)					109.6 (6)
					109.5 (7)
C ^γ (i)-C ^β (i)-O ^β (i)				107.3 (6)	
				108.8 (6)	
			120.7 (6)	123.9 (6)	
C ^γ (i)-C ^δ (i)-N(i)	104, 102 (1)				
	104.4 (7)				

Table V (Continued)

<i>i</i>	1 (Pro)	2 (Sar)	3 (MeAla)	4 (Thr)	5 (D-Val)
Me(<i>i</i>)-N(<i>i</i>)-C'(<i>i</i> - 1)			115.7 (6)	124.7 (5)	
N(<i>i</i> + 1)-C'(<i>i</i>)-C ^α (<i>i</i>)	118.9 (5)	117.8 (5)		116.2 (5)	120.6 (5)
	118.9 (5)	117.6 (5)		111.0 (5)	117.5 (5)
N(<i>i</i> + 1)-C'(<i>i</i>)-O(<i>i</i>)	121.6 (5)	121.2 (5)		122.8 (5)	121.7 (5)
	121.6 (6)	122.8 (6)		125.9 (6)	120.6 (6)
N(<i>i</i>)-C ^α (<i>i</i>)-C'(<i>i</i>)	111.1 (5)				
	112.4 (5)				
N(<i>i</i>)-C ^α (<i>i</i>)-C ^β (<i>i</i>)	102.0 (5)				
	102.0 (5)				
O(<i>i</i>)-C'(<i>i</i>)-C ^α (<i>i</i>)	119.4 (5)	121.0 (5)	123.8 (5)	120.9 (5)	117.5 (5)
	119.2 (5)	119.6 (6)	125.1 (6)	122.9 (6)	121.9 (5)
O ^β (<i>i</i> + 1)-C'(<i>i</i>)-C ^α (<i>i</i>)			110.6 (5)		
			111.0 (6)		
O ^β (<i>i</i> + 1)-C'(<i>i</i>)-O(<i>i</i>)			125.3 (6)		
			123.7 (6)		
Protecting Group					
C ^α (4)-N(4)-C10	122.9 (5)	120.0 (5)	C02-C01-C06	118.8 (6)	118.4 (7)
C02-C01-C07	120.2 (7)	119.0 (8)	C07-C01-C06	121.0 (7)	122.7 (8)
C03-C02-C01	117.5 (6)	119.7 (6)	C03-C02-C08	118.2 (6)	123.4 (7)
O08-C02-C01	124.3 (6)	116.7 (6)	C04-C03-C02	124.8 (6)	120.6 (6)
C04-C03-N09	121.0 (5)	119.6 (6)	N09-C03-C02	114.0 (5)	119.8 (6)
C03-C04-C05	116.4 (6)	119.2 (6)	C10-C04-C03	120.5 (6)	121.5 (6)
C10-C04-C05	123.1 (5)	119.1 (6)	C04-C05-C06	120.7 (6)	119.5 (7)
C01-C06-C05	121.7 (7)	122.5 (7)	N(4)-C10-C04	114.9 (6)	115.8 (5)
N(4)-C10-O11	122.6 (6)	122.6 (6)	O11-C10-C04	122.5 (6)	121.6 (5)
C03-N09-O12	119.0 (5)	117.7 (6)	C03-N09-O13	117.2 (5)	119.9 (6)
O12-N09-O13	123.7 (6)	122.4 (6)			
Ethyl Acetate					
O1-C1-C2	125.7 (7)	O1-C1-O2	123.8 (7)	C2-C1-O2	110.5 (7)
C1-O2-C3	124, 111 (1)	O2-C3-C4	105, 114 (2)		

^a It should be noted that when *i* is 1, *i* - 1 is 5 and when *i* is 5, *i* + 1 is 1. When two values are given separated by commas, they refer to either the two disordered sites of proline or to the two C^γ atoms of D-valine.

through celite and evaporated. The residue was treated with ethanol (10 mL), and a precipitate was filtered off. After evaporation of the filtrate flash chromatography of the residue on silica gel with 10% ethanol/ethyl acetate afforded I as an amorphous solid, yield 717 mg (46%): CIMS with methane [ions at *m/z* 751 (*M* + 1, rel intensity 1.00), 780 (*M* + 29, rel intensity 0.037), and 791 (*M* + 41, rel intensity 0.026)] indicated *M* = 750 (C₃₈H₅₀N₆O₁₀ requires *M* = 750.85); NMR see Table II.

[3-(Benzyloxy)-4-methyl-2-nitrobenzoyl]-L-threonyl-D-valyl-L-prolyl-sarcosyl-N-methyl-L-alanine Lactone (IV). This was prepared by the same procedure as I from IX (1.75 g, 1.95 mmol) using 600 + 300 mg of BOP-Cl for cyclization. After flash chromatography on silica gel with 10% ethanol/ethyl acetate, IV was obtained as an amorphous solid, yield 719 mg (51%): CIMS (a) with methane [ion at *m/z* 723 (*M* + 1, rel intensity 1.00)] and (b) with ammonia [ion at *m/z* 723 (*M* + 1, rel intensity 0.34)] indicated *M* = 722 (C₃₆H₄₆N₆O₁₀ requires *M* = 722.79); NMR see Table II.

(3-Hydroxy-4-methyl-2-nitrobenzoyl)-L-threonyl-D-valyl-L-prolyl-sarcosyl-N-methyl-L-valine Lactone (II). A solution of I (432 mg, 0.575 mmol) in dichloromethane (8 mL) was treated with trifluoromethanesulfonic acid³⁸ (0.9 mL) at 23 °C for 10 min with stirring. Water (100 mL) was added, and the solution was extracted 3 times with ethyl acetate. The extracts were washed with water and extracted with aqueous sodium bicarbonate; the resulting orange solution, after acidification with hydrochloric acid, was extracted 3 times with ethyl acetate. The extracts were dried (Na₂SO₄) and evaporated to afford II as pale yellow prisms, mp 196–198 °C; yield 358 mg (94%): CIMS with methane [ions at *m/z* 661 (*M* + 1, rel intensity 1.00), 689 (*M* + 29, rel intensity 0.12), and 701 (*M* + 41, rel intensity 0.029)] indicated *M* = 660 (C₃₁H₄₄N₆O₁₀ requires *M* = 660.72); NMR see Table I and Figure 1.

(3-Hydroxy-4-methyl-2-nitrobenzoyl)-L-threonyl-D-valyl-L-prolyl-sarcosyl-N-methyl-L-alanine Lactone (V). This was prepared by the same procedure as II from IV (179 mg, 0.248 mmol) using 0.4 mL of trifluoromethanesulfonic acid, to afford V as pale yellow prisms from chloroform; mp 183–186 °C; yield, 146 mg (93%). Anal. (C₂₉H₄₀N₆O₁₀·1/4CHCl₃) C, H, N. CIMS with methane [ions at *m/z* 633 (*M* + 1, rel intensity 0.72), 662 (*M* + 29, rel intensity 0.082), and 674 (*M* + 41, rel intensity 0.021)] indicated *M* = 632 (C₂₉H₄₀N₆O₁₀ requires *M* = 632.67); NMR see Table II.

Actinomycin D. A solution of II (124 mg, 0.188 mmol) in 1:1 methanol/ethyl acetate (20 mL) was hydrogenated over 10% palladium/

charcoal (50 mg) at atmospheric pressure for 4 h. After filtration through Celite, the filtrate was evaporated, and a solution of the residue in methanol (25 mL) was added to a stirred solution of potassium ferricyanide (180 mg) in 0.067 M phosphate buffer (pH 7.12, 25 mL). After stirring at 23 °C for 10 min, the solution was diluted with water (75 mL) and extracted 3 times with ethyl acetate. The extracts were washed with water, dried (Na₂SO₄), and evaporated. The residue was chromatographed on a column of acid-washed alumina³⁹ with chloroform and crystallized from ethanol/chloroform as red prisms, mp 246–247 °C, yield, 68 mg (58%). Anal. (C₄₂H₅₆N₁₂O₁₆) C, H, N. PDMS peaks at 1254.63 (*M* - H)⁻, 1256.18 (*M* + H)⁺, and 1278.36 (*M* + Na)⁺ indicate *M* = 1255 (required *M* = 1255.43); amino acid analysis Thr, 0.61; Val, 0.97; Pro, 1.08; Sar, 1.09; MeVal, 0.87; NMR (CDCl₃) identical in every detail with that of natural actinomycin D.

5,5'-Methylalanylactinomycin D. A solution of V ((245 mg, 0.387 mmol) in methanol (20 mL) was hydrogenated over 10% palladium/charcoal (80 mg) at atmospheric pressure for 3 h. After filtration through Celite, the solution was diluted to 50 mL with methanol and added to a stirred solution of potassium ferricyanide (310 mg) in 0.067 M phosphate buffer (pH 7.12, 50 mL). After 15 min, the solution was diluted with water (150 mL) and extracted 3 times with ethyl acetate. The extracts were washed with water, dried (Na₂SO₄), and evaporated. The residue was chromatographed on a column of acid-washed alumina³⁹ with chloroform and crystallized from ethanol/chloroform as red prisms: mp 247–249 °C; yield, 165 mg (71%). Anal. (C₃₈H₅₈N₁₂O₁₆) C, H, N. PDMS peaks at 1198.57 (*M* - H)⁻, 1200.32 (*M* + H)⁺, and 1222.50 (*M* + Na)⁺ indicate *M* = 1199 (required *M* = 1199.33); amino acid analysis Thr, 1.05; Val, 1.11; Pro, 0.95; Sar, 0.96; MeAla, 0.98; NMR (CDCl₃ and 1:1 C₆D₆/CDCl₃) see Table III.

X-ray Crystallographic Work. There appear to be several crystal modifications of the peptide, and they are described here although only one crystal structure was elucidated. Pale-yellow prisms with well-developed pyramidal caps were readily crystallized from ethyl acetate but were unstable when removed from the mother liquor, developing cracks and falling apart within 5 s. A crystal, sealed in a thin glass capillary, proved to be tetragonal: space group *P*4₂2₁ or *P*4₂2₁2 with cell dimensions *a* = 18.505 (2) Å and *c* = 34.431 (3) Å. The space group has eight general positions, and that number of molecules in the unit cell of volume 11790.4 Å³ would correspond to a density of 0.712 g cm⁻³. Since so low a density is unlikely, there might be 16 molecules in the cell or, more

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Table VI. Positional Parameters ($\times 10000$) and U Values ($\times 1000$) for the Heavier Atoms^a

	<i>x</i>	<i>y</i>	<i>z</i>	<i>U</i>		<i>x</i>	<i>y</i>	<i>z</i>	<i>U</i>
Proline (1)					D-Valine (5)				
O	6081 (4)	7130 (2)	-3163 (2)	53 (2)	O	9089 (4)	5945 (2)	-3135 (2)	48 (2)
	9310 (4)	9 (2)	-5502 (2)	60 (2)		5902 (4)	-636 (2)	-5340 (2)	51 (2)
N	7344 (4)	6062 (2)	-3130 (2)	34 (2)	N	8752 (4)	5825 (3)	-4304 (2)	33 (2)
	7174 (4)	73 (3)	-5298 (2)	42 (2)		5666 (4)	-128 (2)	-6437 (2)	36 (3)
C'	5997 (5)	6743 (3)	-3513 (3)	38 (3)	C'	8252 (6)	6078 (3)	-3377 (3)	36 (3)
	8827 (5)	429 (3)	-5723 (3)	40 (3)		6541 (5)	-299 (3)	-5577 (3)	46 (3)
C ^α	6321 (5)	6083 (3)	-3395 (3)	35 (3)	C ^α	8336 (5)	6312 (3)	-3964 (3)	32 (3)
	7760 (6)	609 (3)	-5509 (3)	44 (3)		6643 (5)	-298 (3)	-6178 (3)	34 (3)
C ^β	5612 (6)	5819 (3)	-2963 (3)	57 (3)	C ^β	9024 (6)	6883 (3)	-3983 (3)	38 (3)
	7864 (8)	1017 (4)	-5005 (4)	67 (4)		6925 (6)	-964 (3)	-6375 (3)	43 (4)
C ^δ	7272 (6)	5856 (4)	-2564 (3)	50 (4)	C ^{γ1}	9281 (7)	7070 (5)	-4564 (4)	54 (4)
	7148 (8)	73 (5)	-4703 (4)	68 (4)		7944 (7)	-1192 (5)	-6102 (5)	74 (5)
C ^γ	6170 (16)	5895 (9)	-2441 (8)	6 (1)	C ^{γ2}	8485 (8)	7425 (4)	-3693 (4)	61 (4)
	6242 (19)	5484 (8)	-2563 (10)	7 (1)		7051 (7)	-986 (5)	-6983 (4)	71 (5)
	7919 (7)	569 (5)	-4541 (4)	76 (4)	Protecting Group				
Sarcosine (2)					C01	5816 (6)	2575 (3)	-5279 (3)	54 (3)
O	6833 (4)	6968 (2)	-4849 (2)	50 (2)		1486 (7)	2906 (4)	-6527 (3)	67 (4)
	8238 (4)	388 (2)	-7020 (2)	52 (2)	C02	6662 (5)	2541 (3)	-5624 (3)	41 (3)
N	5551 (4)	6872 (3)	-3989 (2)	44 (2)		1232 (5)	2587 (4)	-6995 (3)	63 (4)
	9254 (4)	769 (2)	-6110 (2)	43 (2)	C03	7382 (5)	3018 (3)	-5604 (3)	40 (3)
C'	6087 (5)	6629 (3)	-4910 (3)	38 (3)		1741 (5)	2041 (3)	-7117 (3)	45 (3)
	8268 (5)	951 (3)	-6959 (3)	42 (3)	C04	7317 (5)	3516 (3)	-5272 (3)	36 (3)
C ^α	5387 (6)	6463 (4)	-4441 (3)	37 (3)		2470 (5)	1790 (3)	-6758 (3)	39 (3)
	8699 (6)	1220 (4)	-6438 (3)	41 (3)	C05	6483 (7)	3530 (4)	-4925 (3)	57 (3)
C(Me)	5208 (11)	7505 (5)	-4060 (4)	78 (1)		2693 (7)	2098 (4)	-6290 (3)	58 (3)
	10318 (8)	648 (5)	-6294 (4)	63 (4)	C06	5744 (7)	3065 (4)	-4932 (3)	65 (4)
Methylalanine (3)						2198 (7)	2647 (4)	-6181 (3)	69 (4)
O	8413 (4)	6625 (2)	-5866 (2)	52 (2)	C07	5012 (9)	2083 (5)	-5283 (5)	82 (1)
	6611 (4)	200 (2)	-8101 (2)	79 (2)		969 (9)	3538 (5)	-6420 (6)	96 (1)
N	5863 (4)	6387 (2)	-5392 (2)	31 (2)	O08	6818 (4)	2081 (3)	-5985 (2)	53 (2)
	7911 (4)	1344 (3)	-7327 (2)	46 (2)		540 (5)	2857 (3)	-7326 (2)	86 (3)
C'	7672 (5)	6330 (3)	-5752 (2)	35 (3)	N09	8227 (5)	2967 (3)	-6013 (2)	51 (3)
	6694 (6)	650 (4)	-7830 (3)	58 (3)		1536 (5)	1736 (3)	-7615 (2)	67 (3)
C ^α	6541 (6)	6542 (3)	-5855 (3)	39 (3)	C10	8159 (6)	3996 (3)	-5271 (3)	49 (3)
	7582 (7)	1097 (4)	-7855 (3)	60 (4)		2950 (5)	1162 (3)	-6853 (3)	46 (3)
C ^β	6516 (7)	7212 (4)	-6028 (4)	59 (4)	O11	9049 (4)	3885 (2)	-5416 (2)	51 (2)
	8482 (8)	849 (5)	-8180 (4)	84 (1)		2421 (4)	714 (2)	-6950 (2)	60 (2)
C(Me)	5064 (8)	5930 (5)	-5488 (4)	70 (4)	O12	8906 (4)	2580 (2)	-5950 (2)	60 (2)
	7864 (10)	2013 (4)	-7248 (4)	72 (1)		645 (5)	1720 (3)	-7771 (3)	108 (3)
Threonine (4)					O13	8173 (4)	3303 (2)	-6402 (2)	73 (2)
O	7196 (3)	5359 (2)	-4417 (2)	46 (2)		2256 (4)	1511 (3)	-7873 (2)	88 (3)
	5805 (4)	900 (2)	-6252 (2)	46 (2)	Ethyl Acetate				
N	7850 (4)	4561 (3)	-5123 (3)	38 (2)	O1	9990 (4)	7586 (2)	-1675 (2)	73 (2)
	4005 (4)	1137 (3)	-6822 (2)	38 (2)	C1	10191 (6)	7234 (4)	-2022 (3)	75 (4)
C'	8116 (5)	5420 (3)	-4545 (3)	36 (3)	O2	10274 (6)	6639 (3)	-1949 (3)	116 (3)
	5393 (5)	470 (3)	-6481 (3)	38 (3)	C2	10395 (9)	7393 (4)	-2587 (4)	10 (1)
C ^α	8574 (5)	5059 (3)	-5012 (3)	34 (3)	C3	10452 (20)	6351 (15)	-1420 (13)	10 (1)
	4539 (5)	554 (3)	-6900 (3)	34 (3)	C3'	9747 (18)	6445 (15)	-1426 (14)	9 (1)
O ^γ	7689 (3)	5754 (2)	-5563 (2)	38 (2)	C4	9640 (9)	5863 (5)	-1376 (5)	15 (1)
	5961 (4)	842 (2)	-7482 (2)	49 (2)	Water				
C ^β	8721 (5)	5487 (3)	-5486 (3)	41 (3)	O	2726 (11)	3363 (4)	-246 (5)	28 (1)
	5020 (6)	461 (4)	-7462 (3)	43 (3)					
C ^γ	9034 (8)	5190 (4)	-6014 (4)	58 (4)					
	4314 (7)	645 (4)	-7919 (4)	58 (4)					

^a The disordered atoms, C^γ, for the first molecule and C3, C3' in the ethyl acetate molecule, have population parameters of 0.5 which were not refined. In general, there are two sets of parameters for each name and these refer to the molecules called first and second, respectively, in the text. For C^γ of proline, the first two sets of parameters refer to the two disordered positions and the third set refers to the second molecule. The U value is the geometric mean of the diagonal terms of the vibration tensor, multiplied, in the case of the disordered C^γ and C2, C3, C3', C4 of the ethyl acetate and the water molecule, by 100.

likely given the instability in air, several molecules of ethyl acetate per molecule of peptide.

X-ray intensity data were collected, but consistent with the supposition that there were many molecules of solvent of crystallization, the quality of the data was poor. Only 150 reflections with θ angles greater than 40° proved to be as much as 1 standard deviation above background, and it did not seem profitable to further pursue the structure determination.

It had been observed that there was a color change to a deeper yellow on heating solutions of the peptides, and a saturated ethyl acetate solution was heated in a closed capillary to about 100 °C. Thin deep-yellow platelets were observed to crystallize, but it was not possible to isolate a clean specimen large enough for crystallographic examination.

Crystals suitable for structural work were finally obtained by very slow evaporation of an ethyl acetate solution. These were orthorhombic prisms

of a lighter yellow tint than the original tetragonal crystals. There were very few clean single crystals and the density was not measured. The cell dimensions were $a = 12.891$ (1) Å, $b = 21.740$ (2) Å, and $c = 24.909$ (2) Å, and the space group was $P2_12_12_1$. All experimental work was carried out on a crystal $0.3 \times 0.1 \times 0.1$ mm³ encapsulated in a sphere of epoxy resin to impede possible loss of solvent. The crystal did not diffract as strongly as one might expect, but reflections were observed to the θ limit of the diffractometer (74 °C) with Cu K α x-radiation. The X-ray intensity data were collected by standard methods with an Enraf-Nonius CAD4 diffractometer, and only about 50% of the 7747 measured reflections were greater than 1 standard deviation above background. The calculated density was 1.207 g cm⁻³ for eight molecules of peptide in the unit cell (the final asymmetric unit of two peptides + one molecule ethyl acetate + one molecule water and a unit weight of

1377.44 [2 (632.66) + 88.11 + 18.02] corresponds to a calculated density of 1.310 g cm⁻³.

Initial attempts to solve the phase problem did not succeed when using MULTAN⁷⁸ with 500 *E* values derived by standard methods from the full experimental data set. Since the smallest *E* value allowed by an essentially standard version of MULTAN would be 1.575 for the present structure, an expanded version of the program was built allowing 800 reflections and 9000 triplet equations, but it did not produce better results. One obvious problem with the data was the large fraction of "unobserved" intensities which caused the Wilson curve approach of the NORMAL component of MULTAN to be unreliable. A data set with only 2σ data above a θ angle of 50° was scaled by using the inflection points of the *K* curve (a process which appears to have arisen independently in many laboratories) to position a straight line, and a temperature factor of 3.8 was indicated (more likely than the much larger value arising from an automatic application). With nine general variable phases and the magic integer approach, there was a unique solution showing two essentially identical sets of 46 atoms. The model structure was a large ring with apparently a long chain of attached atoms. A weighted Fourier map approach expanded the model to 97 atoms and defined the expected protecting group fairly well in both independent molecules.

Refinement was carried out by using the programs of XRAY72⁴¹ with a data set containing all reflections up to a θ angle of 50° but only those with *I* > σ(*I*) at larger angles. The data set had 4686 X-ray observations, with 1325 corresponding to measurements with *I* < σ(*I*). The maximum sin θ/λ was 0.6237 Å⁻¹, the weighting scheme was that given by Peterson and Levy,⁴² the scattering factors used were taken from ref 43, the function minimized was ΣwΔ², and the anisotropic temperature factor used had the form $-\exp[-(2\pi^2 \sum_i \sum_j (U_{ij} h_i h_j a_i^* a_j^*))]$. With the scale and temperature factors of NORMAL, the initial *R* factor was 34.4%. Some atoms in the model were incorrect and some were missing, and also some extramolecular peaks were found in a difference map which were assigned to a molecule of ethyl acetate and successfully refined. It was possible to identify all atoms by using chemical knowledge and temperature factors from the refinement. At this point in the refinement, with isotropic temperature factors, the conventional *R* factor was 17%. Introduction of anisotropic thermal parameters reduced the *R* factor to 12.3%, but the values for the atoms of the proline ring of one molecule were extremely large and analysis of the results indicated that a disorder model might be appropriate although only the two sites for the apical atom of the ring would be resolved. A persistent single peak unattached to any others was also found and appeared best explained as a water molecule. No special pains were taken to maintain dryness during crystallization, and it is entirely possible that some atmospheric moisture was absorbed. Statistical tests⁴⁴ indicated that both the proline disorder

and the assignment of a water molecule were justified. A difference map showed plausible locations for all H atoms in the peptides and for the non-methyl H atoms of the ethyl acetate molecule. No indications were apparent for H atoms of the water molecule (not surprising in view of the high O thermal parameters).

The large size of the problem caused difficulties in refinement but after several experiments with different partitionings of the normal matrix, the most successful method proved to be division into blocks consisting of the parameters of a heavy atom and its attached H atoms and refining only the positional parameters of the H atoms. H atoms on disordered atoms and the atoms of the ethyl acetate molecules were not refined, and those of the water molecule, although predictable from possible hydrogen bonding, were not included. Towards the end of the refinement, it became apparent that a single site did not explain the electron density near C3 of the ethyl acetate molecule. The introduction of two half atoms removed the residual electron density, and the refined thermal parameters were comparable with those of the rest of the molecule. In the final cycles of refinement, 1/3 parameter shifts were applied and the process converged at a conventional *R* factor of 0.0636. The final positional parameters for the heavier atoms are given in Table VI, and full refinement parameters for all atoms and the listing of observed and calculated structure factors have been deposited (see paragraph at end of paper regarding supplemental material). The bond angles for the heavier atoms are given in Table V.

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Supplementary Material Available: Tables of observed and calculated structure factors and full X-ray refinement parameters (26 pages). Ordering information is given on any current masthead page.

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