tadiene is equivalent to the *si-re* face of the trans deuterio substrate, and thus we have previously noted that formation of R(+) epoxide from octadiene requires attack at the *si-si* face (ref 22).
(44) A. M. Jeffrey, H. J. C. Yeh, D. M. Jerina, T. R. Patel, J. F. Davey, and D. T. Gibson, *Biochemistry*, **14**, 575-583 (1975).
(45) E. W. Maynert, R. L. Foreman, and T. Wotabe, *J. Biol. Chem.*, **245**, 5234-5238 (1970).
(46) We recognize that, in general, complex mechanisms involving any number of transitory intermediates can always be postulated, and we have confined our discussions to a few simple mechanistic possibilities.
(47) For the purposes of mechanistic analyses, the situations where either a single enzyme system or several enzyme systems with similar reactivity characteristics are operative represent identical models, and we have no basis on which to distinguish between them. The related question of whether the same "oxygenase" is involved in both the hydroxylation and epoxidation reactions has been considered by us elsewhere (ref 19, 21, 23, **24**).
(48) S. W. May, M. S. Steitenkamp, R. D. Schwartz, and C. J. McCoy, *J. Am. Chem. Soc.*, **98**, 7856 (1976).
(49) The terms fully protonated and cis or trans deuterated are used in this paper with reference to a given epoxide or olefin functionality, without regard to the deuterium content of functionalities at the other end of the molecule. The NMR, of course, looks only at the aggregate of epoxide or olefin sites.
(50) The validity of this assumption for our samples was checked in the following ways. In the first place, the intensities of the epoxide multiplets (which had been estimated from the intensities of the low field exposed lines arising from the fully protonated species) were plotted logarithimically vs. time and in every case these plots were linear. Furthermore, the effective relaxation times obtained from these plots were in good agreement with the values computed from the experimentally measured null times. In addition the calculated $I(0)$ values listed in Table I were used to predict the total intensities of the fully inverted epoxide multiplets, and these agreed within about 5-10% with the experimentally measured intensities.

Raman Spectroscopy of Uncomplexed Valinomycin. 1. The Solid State

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Abstract: The membrane-active antibiotic valinomycin has become an important model compound for studying selective ion transport in biological and synthetic membranes. This paper reports results of the first complete Raman spectroscopic study of uncomplexed valinomycin in the solid state. Splittings in the ester and amide C=O stretch regions of valinomycin samples recrystallized from *n*-octane, CCl_4, $CHCl_3$, $CH_3(CH_2)_2Cl$, CH_3COCH_3, or CH_3CN indicate a structure resembling that obtained by x-ray crystallography. However, valinomycin recrystallized from *o*-dichlorobenzene and *p*-dioxane exhibits a considerably different structure. Comparison is made with the Raman spectra of model compounds in order to facilitate the identification of valinomycin vibrations. These results are extended to valinomycin solutions in an adjoining publication.

The antibiotic valinomycin (VM) produced by the bacteria *Streptomyces fulvissimus* is known to complex with alkali cations selectively[2] in the order $Rb^+ > K^+ > Cs^+ > Na^+ > Li^+$ and to facilitate the transport of alkali cations across mitochondrial membranes with the same selectivity.[3a] Similar results have been obtained in model membrane systems.[3,4] Studies of valinomycin analogues[2] emphasize the importance of structural factors in complex formation, induced ionic permeability, and antimicrobial activity.

Valinomycin is a 12-membered macrocyclic depsipetide in which L-valine, L-lactic acid, D-valine, and D-hydroxyisovaleric acid (HIV) are alternately joined by amide and ester linkages.[5] The primary structure of VM (Figure 1a) suggests how it can facilitate the transport of cations across otherwise impermeable lipid barriers. There are 12 polar C=O groups, some of which can form structurally stabilizing intramolecular hydrogen bonds with the NH groups of the valine subunits, while others are free to bind a cation at the water/lipid interface via ion-dipole interactions. The nonpolar isopropyl and methyl residues can shield the hydrophilic C=O coordinated cation to facilitate its diffusion through the hydrophobic regions of membrane interiors. This structure for the K^+-VM complex has been verified by x-ray crystallography[6,7] and inferred from NMR studies of VM solutions.[2,8,9] It has recently been studied in both the solid state and in solution by laser Raman spectroscopy.[10]

The membrane activity of VM suggests that understanding its complexation mechanisms would greatly further our knowledge of the molecular basis of selective ionic permeability in biological systems. An important first step is the elucidation of the conformations assumed by uncomplexed VM, i.e., the states from which complex formation can be initiated. These states have been the subject of numerous recent investigations utilizing x-ray diffraction,[11-14] infrared spectroscopy,[2,15] nuclear magnetic resonance,[2,8,15-17] circular dichroism,[15] and optical rotary dispersion.[2]

In a preliminary paper[18] we reported the detection of two different forms of VM in the solid state, based on Raman spectroscopic observations in the 1600-1800-cm^{-1} region. We here present the first complete Raman spectra (150-3600 cm^{-1}) of uncomplexed VM recrystallized from several polar and nonpolar solvents and attempt to interpret these results in terms of VM conformations. This work also provides a basis for an accompanying Raman study of VM in solution[19] and a recent study of the VM-KSCN complex.[10]

Materials and Methods

(a) Materials. Valinomycin powder was obtained commercially from Calbiochem, San Diego, Calif. The antibiotic was prepared by the method of MacDonald and Slater,[20] the last stage of which involves slow recrystallization from warm *n*-octane. The resulting white powder is freely soluble in CCl_4, $CHCl_3$, dioxane, and acetone, somewhat soluble in hydrocarbons, and practically insoluble in water. Calbiochem also made available several larger (~1 mm^3) translucent VM crystals; these are monoclinic[12] with a space group of $P2_1$. The crystals readily cleave into thin platelets. We also recrystallized VM samples from a variety of polar and nonpolar solvents in loosely sealed Kimax capillary tubes. The model compounds D- and L-valine, L-lactic acid (lithium salt), D-α-hydroxyisovaleric acid, and poly-L-valine were also obtained from Calbiochem.

(b) Raman Spectroscopic Methods. In Raman spectroscopy, one

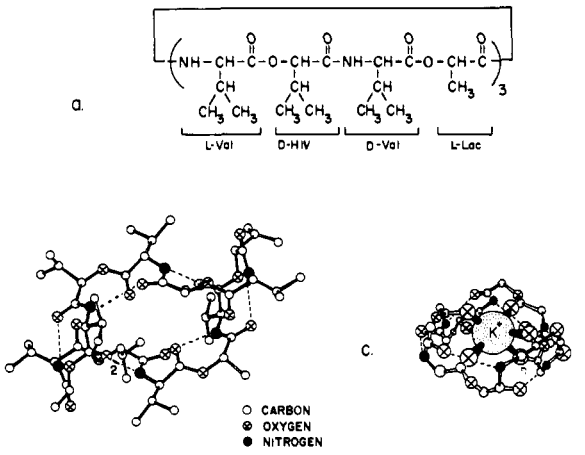

Figure 1. (a) Primary sequence of valinomycin showing alternating amide and ester linkages. (b) Structure of uncomplexed valinomycin crystallized from *n*-octane (from ref 11). (c) Structure of the valinomycin-K^+ ion complex; hydrocarbon side groups omitted for simplicity (from ref 6).

Table I. Amide Modes

Frequency,[a] cm^{-1}	Amide mode	Primary vibration
206	Amide VII	NC torsion
600	Amide VI	C=O bending (o.p.)[b]
627	Amide IV	NC=O bending (i.p.)[b]
725	Amide V	NH bending (o.p.)
1209	Amide III	NC stretching, NH bending (i.p.)
1567	Amide II	NH bending (i.p.), NC stretching
1653	Amide I	C=O stretching
3090	Amide B	Arise from Fermi resonance between NH stretching and the second harmonic of the amide II mode
3280	Amide A	

[a] For *n*-methylacetamide ($CH_3CONHCH_3$), a simple model compound (ref 21). [b] Bending motions may be either in (i.p.) or out (o.p.) of the NC=O plane.

illuminates a sample with an intense beam of monochromatic light of frequency ν_0 and detects scattered light of various frequencies ν_i produced by inelastic scattering in the sample. The frequency *shifts*, or Stokes "frequencies" $\Delta\nu_i \equiv \nu_0 - \nu_i$ (not the absolute detected frequencies ν_i, as in fluorescence), correspond to the excitation of vibrational normal modes of the sample. The amide modes, which involve nitrogen vibrations, are particularly sensitive to molecular conformation;[21-23] their traditional nomenclature is given in Table I. Since both the incident and detected radiation are visible light (even though the vibrational energies correspond to infrared radiation), Raman spectroscopy is readily applied to aqueous solutions. As in infrared spectroscopy, some normal modes of a molecular system may not be observed because of their symmetry properties.

Although anti-Stokes frequencies ($\nu_0 + \nu_i$) can be observed, their intensities are a factor $\exp(-\nu_i/kT)$ less than the corresponding Stokes ($\nu_0 - \nu_i$) signal. They help confirm low-frequency Stokes observations and provide a measure of the effective temperature of the scattering volume. Raman studies of several homopolypeptides,[23,24] proteins,[22-25] and antibiotics[26,27] have already been reported.

This work was begun at the Northeastern University Solid State Spectroscopy Laboratory and continued at the Massachusetts Institute of Technology. The experimental arrangement is shown in Figure 2a. In the Northeastern system, light from a Coherent Radiation Model 52 argon ion laser was focused on the sample by a 30 mm focal length lens. Narrow band interference filters were used to eliminate laser plasma lines. Raman spectra were taken using 4880 and 5145 Å argon

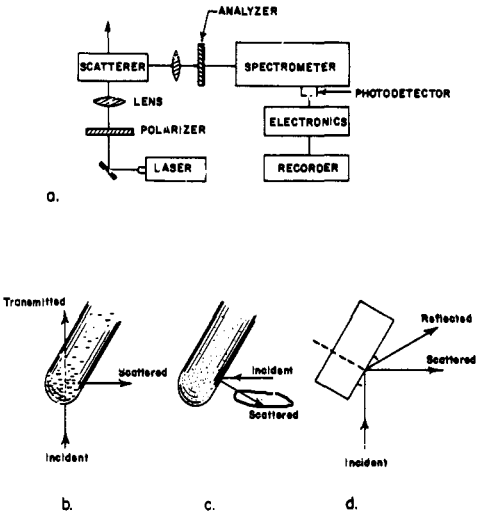

Figure 2. (a) Typical experimental arrangement for laser Raman spectroscopy. (b) Configuration for 90° scattering from liquids in capillaries. For powders the incident beam is close to the capillary wall. (c) Configuration for 180° scattering from powders and liquids in capillaries. (d) Configuration for crystalline scatterer. The scattering is 90° for transparent samples (volume scattering), 180° for opaque samples (surface scattering), and intermediate for translucent samples. The dotted line represents penetration direction of the refracted beam in an opaque sample with a high index of refraction.

laser wavelengths and compared to exclude grating ghosts. The scattered light was analyzed by a Spex 1401 double monochromator with an ITT FW-130 photomultiplier (S-20 photocathode) operated in the photon counting mode.

More recent data (Figures 3-8) were taken at MIT on a Spex Ramalog 4 system with a Spectra-Physics Model 124 argon ion laser. The response of the thermoelectrically cooled RCA-31034 photomultiplier (GaAs photocathode) was comparatively flat from 3000 to 9000 Å. Input powers of 15-140 mW, 3 cm^{-1} slit widths, 0.5-1.0 s time constants, and 30-60 cm^{-1}/min scanning speeds were employed. All spectra were calibrated to the 314-cm^{-1} vibration of CCl_4.

A quartz wedge polarization scrambler placed before the entrance slit of the spectrometer assured equal response of the instrument to all polarizations of the scattered light. Use of a sharply focused laser beam and a periscopic viewer permitted the observation of surface scattering from crystals as small as ~100 μm in diameter, as in previous solid-state studies.[28]

Powdered samples were confined in 1.0-mm i.d. capillary tubes mounted either perpendicular (Figure 2b) or parallel (Figure 2c) to the scattering plane (defined by the direction of the incident and scattered beams). Small crystals were gently supported on sharp needles or mounted on microscope slides (Figure 2d). In nontransparent samples, it was difficult to observe peaks below 200 cm^{-1}.

Raman Spectrum of Uncomplexed Valinomycin (*n*-Octane)

Complete Raman spectra (150-3600 cm^{-1}) of uncomplexed VM crystallized from *n*-octane were obtained (Figure 3-5). Crystalline and powdered samples exhibited identical Raman frequencies, although relative intensities occasionally varied (primarily a polarization/orientation effect, see section F). Averaged Raman frequencies appear in Table II. The accompanying infrared frequencies were measured from a complete infrared absorption spectrum kindly supplied by Calbiochem. To our knowledge only selected regions of the IR spectrum have been previously published.[2,15]

VM hydrocarbon side-chain vibrations were identified by comparisons with VM components (Figures 3-5) and other model compounds. The frequencies of these modes are relatively insensitive to environment. In contrast, vibrations in the vicinity of the peptide linkages are sensitive to molecular conformation.[21-23]

Table II. Raman Spectra of Uncomplexed Valinomycin[a] Recrystallized from *n*-Octane, *o*-Cl_2Ph, and *p*-Dioxane[e]

n-Octane[b]	*o*-Cl_2-Ph	*p*-Dioxane	IR	Tentative assignments
158				
(201)				
223 (3)		227		
246 (1)		242		
274 (3)	277	275		Skeletal deformation region
(298-318)	303			
326 (3)	327	324		
346 (2)	348	348		
398 (1)		(396) sh		
412 (1)	(416)	412		
435 (1)		435		Ester OCC bend (?)
(454)				
466 (2)	*c*	465		C-CH_3 rock
474	*c*			
484 (2)	*c*	486		
509				
531				
597 B	(598)			Amide VI
610 B				Amide VI; ester deformation
631				Amide IV; C=O out-of-plane bend
736 (1)	740	740		Amide V
		745		
757 (1)	761	762	764	
804 B			800	
(826)		*c*		
847 (3)	847	847		Ester COC symmetric stretch + CC stretch
867		867		
880 (8)	883	880		CH_2 rock; CC stretch + NC=O bend
912 (1)	914	912	910	C-CH_3 stretch
940 (3)	943	940	935	Ester O-CHR-C symmetric stretch + CH_3 symmetric rock
(945)				
962 (4)	965	959		
981 (2)	986	986	985	
993				
1020	*c*	*c*	1011	Isopropyl stretch + ester skeletal stretch
1039 (1)	*c*	1033	1032	
(1096)	1094		1100	Ester O-CHR-C asymmetric stretch
1127 (6)	*c*		1129	Isopropyl asymmetric stretch
1140 sh				
(1150)			1151	CH_3 asymmetric rock
1178 (5)	1176	1175		
			1193	OCC symmetric stretch
1252 B (2)	1248	1245	1250	Amide III
1271	*c*	1267		C-CH bend (?)
(1294) sh				
1307	1314	1310	1309	Amide mode
1325 (6)	1333	1328	1335	$C^{\alpha}H$ bend
1344 (5)				$C^{\beta}H$ bend
(1353) sh	1350	1351		
1372 (2)	1373	1367	1370	CH_3 symmetric bend
(1391)	1394	1391	1389	
1454 (9)	1453	1450		CH_3 asymmetric bend
(1461)				
1467 (10)	1468	1466	1471	
			1538	Amide II
1649 (7)	1650	1650		Amide I (H bonded)
(1657) sh			1658	
	1663	1663	*d*	Amide I
1675 (5)			1689	Amide I (free)
1742 (6)			1745	Ester C=O stretch (H bonded)
(1747) sh	1756 sh	1757 sh	*d*	
1767 (7)	1763	1767	1769	Ester C=O stretch (free)
			2326	
			2364	
2723				
2731 (2)	2734	*c*		Combination frequencies
(2765)				
2774 (1)	2777	2772	2778	
2875 (22)	2876	2874		Isopropyl $C^{\beta}H$ stretch
2913 (28)	2913	2913	2907	$C^{\alpha}H$ stretch
2938 (28)	2939	2936		CH_3 symmetric stretch

Table II (*continued*)

n-Octane[b]	*o*-Cl_2-Ph	*p*-Dioxane	IR	Tentative assignments
2966 (25)	2972	2966		CH_3 asymmetric stretch
2984 (21)				
			3003	
			3067	Amide B
3312 (2) B	3293 B	3301 B		NH stretch (H bonded)
			3333	Amide A
3406 (1)				Combination frequency (?)
(3426)			3436	

[a] Raman frequencies of VM powder and crystals were identical; numbers in parentheses give the relative heights of the more prominent peaks as measured in VM powder. Frequencies in parentheses are less certain. [b] For VM powder recrystallized from warm *n*-octanes; measured from a spectrum kindly provided by Calbiochem. Wavenumbers are placed adjacent to the nearest Raman entries for convenience only. [c] Residual solvent peaks limit accurate measurement in this region. [d] The tabulated C=O stretch frequencies are from the high-resolution spectra of ref 13. The spectra of footnote *b* display unresolved singlets at 1664 and 1748 cm^{-1}. [e] B = broad; sh = shoulder.

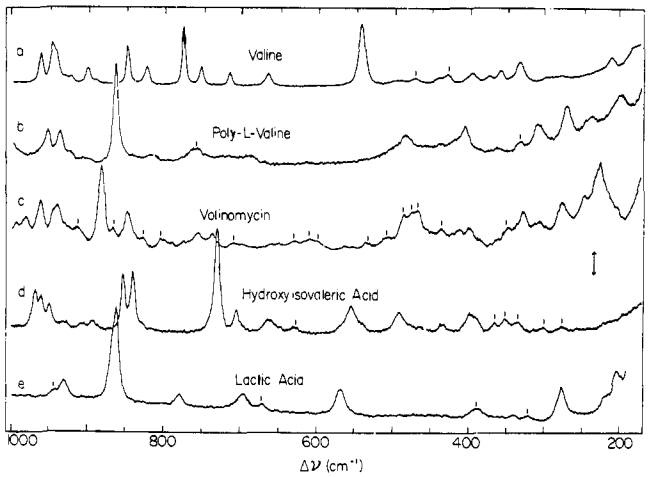

Figure 3. Raman spectrum (170-1000 cm^{-1}) of uncomplexed valinomycin powder crystallized from *n*-octane, compared with its components and poly-L-valine. Spectral resolution is 3 cm^{-1}; laser excitation is 5145 Å in (b) and 4880 Å elsewhere; scanning speed is 5 cm^{-1}/s in (c and d) and 1 cm^{-1} elsewhere. The vertical arrow corresponds to 3×10^3 counts per second (cps) (a, b, e) or 1×10^3 (cps) (c, d). Incident power levels are 60, 120, 24, 40, 28 mW for a-e, respectively. Configuration as in Figure 2b; there is no polarization analyzer in the scattered beam.

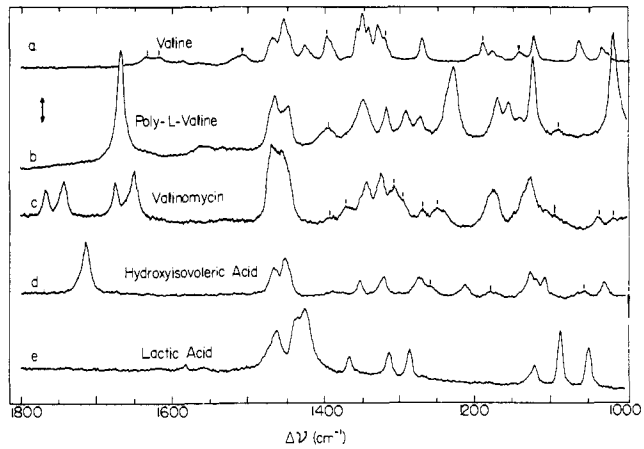

Figure 4. Raman spectrum (1000-1800 cm^{-1}) of uncomplexed valinomycin powder crystallized from *n*-octane, compared with its components and poly-L-valine. Conditions the same as in Figure 3.

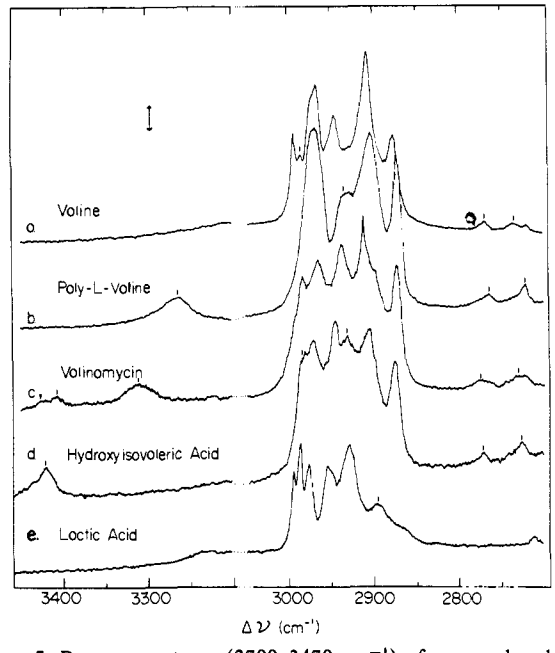

Figure 5. Raman spectrum (2700-3470 cm^{-1}) of uncomplexed valinomycin powder crystallized from *n*-octane, compared with its components and poly-L-valine. Scanning speed is 1 cm^{-1}/s except (c) with is 3200-3470 cm^{-1}/s); the vertical arrow corresponds to 3×10^3 cps except for the 3200-3470-cm^{-1} range of a, c (1×10^3 cps). Incident power levels are respectively 60, 120, 48, 60, 28 mW in the 2600-3050-cm^{-1} region and 120, 120, 60, 28 mW in the 3200-3470-cm^{-1} region. Otherwise conditions are as in Figure 3.

The homopolypeptides poly-L-valine (PLV) and poly-L-alanine (PLA) were used as model compounds for amide linkages; normal mode calculations are available for PLA.[29] Although less Raman data are available for ester linkages, comparison with the macrocyclic nactin antibiotics[27] was of use. *N*-Methylacetamide ($CH_3CONHCH_3$) and methyl acetate (CH_3COOCH_3) were used as comparatively simple compounds with amide and ester linkages, respectively; Miyazawa et al.[30] have calculated the normal modes of the former.

A discussion of the Raman spectrum of octane-crystallized VM follows, divided for convenience into several regions. Sections E, F, and H are particularly relevant to the structure of VM in the solid state.

(A) The 150-500-cm^{-1} Region. Antibiotic-cation coordination bond vibrations are expected below 200 cm^{-1}. Far infrared peaks characteristic of VM···K^+ coordination have been recently reported[31] near 114 and 171 cm^{-1}. However, useful Raman spectra of this region will require the use of a third monochromater because of the high quasielastic background.

The 200-500-cm^{-1} region contains several skeletal deformation and torsion modes; such "backbone" vibrations are usually conformation dependent. The 223-, 274-, 412-cm^{-1} frequencies of VM (Figure 2) closely match the calculated 220-, 277-, 410-cm^{-1} skeletal modes of β-chain polyglycine

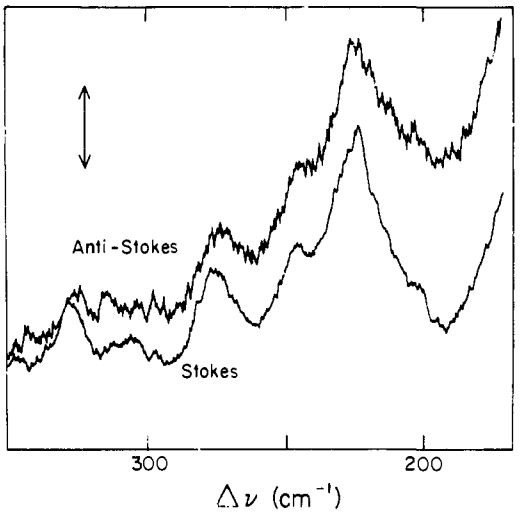

Figure 6. Raman spectra of uncomplexed valinomycin powder crystallized from *n*-octane. Comparison of low-energy (170–300 cm^{-1}) Stokes and anti-Stokes spectra. The ratio of anti-Stokes to Stokes scattering falls exponentially with increasing frequency. Laser excitation 4880 Å; spectral resolution 3 cm^{-1}; incident power level 24 mW; scanning speed 30 cm^{-1}/min. Vertical arrow corresponds to 1×10^3, 3×10^2 counts per second (cps) for Stokes and anti-Stokes spectra, respectively.

I,[32] and similar frequencies (228, 407 cm^{-1}) are observed in β-chain PLV.[33] However, differences in their backbone structure and intermolecular bonding patterns discourage direct comparisons with VM. The richness of the low frequency region of VM may be partially explained by the heterogeneity of its depsipeptide backbone which contains ester linkages as well as peptide bonds. There is apparently some coupling to lattice vibrations in this region (see section I below).

The 435-cm^{-1} VM peak could represent ester OCC bend as in infrared spectra and normal mode calculations of several aliphatic polyesters;[34] it appears at 433 cm^{-1} in methyl acetate. However, it also appears in *n*-methylacetamide (435 cm^{-1}) where it is assigned to C–CH_3 in-plane rock[30] (reference is to the NC=O plane). Although the 484-cm^{-1} VM peak is found in PLV (484 cm^{-1}) but not in valine, Koenig and Sutton[33a] assign it to a residue vibration.

The anti-Stokes spectrum of this region is shown in Figure 6. The ratio of anti-Stokes to Stokes scattering varies as $\exp(-\Delta\nu_i/kT)$ and thus decreases exponentially with the Raman frequency shift $\Delta\nu_i$ from the laser line. Our observations are consistent with a sample temperature near room temperature.

(B) The 500–800-cm^{-1} Region. The Raman spectrum of this region is weak but interesting; it is free of CH and CH_3 modes and is rich in C=O vibrations. A Raman-active amide VI mode (cf. Table I) is observed at 610 cm^{-1} in α-helical PLA[29] (strong intramolecular hydrogen bonding), whereas only a 601-cm^{-1} peak appears between 210 and 880 cm^{-1} in Raman spectra of β-chain polyglycine I.[32] Both 597- and 610-cm^{-1} frequencies appear in VM which may indicate the presence of both tightly and loosely hydrogen bonded amide C=O groups, consistent with several VM structures.[2,11] However, both 595- and 610-cm^{-1} frequencies are *infrared* active in α-helical PLA,[29] but neither is reported in the PLA Raman spectra of ref 24. Furthermore, 610-nm^{-1} ester deformation C=O out-of-plane bend modes are found in nonactin[27] and in esters of propionic acid.

The amide IV mode (cf. Table I) of *n*-methylacetamide[30] occurs at 628 cm^{-1}, near the 632-cm^{-1} frequency of VM. The latter may include similar out-of-plane bending vibrations of the ester C=O groups; it appears near 640 cm^{-1} in methyl acetate.

The amide V mode is highly conformation sensitive;[21] it is infrared active (725 cm^{-1}) and Raman inactive in *n*-methylacetamide (Table I).[30] There are three amide V modes in PLA[29] (728, 757, 772 (IR) cm^{-1}) and two (742, 752 cm^{-1}) in polyglycine II.[32] In Raman spectra of VM, these modes appear at 757 cm^{-1} and possibly 736 cm^{-1}.

(C) The 800–1200-cm^{-1} Region. The 800–925 cm^{-1} region of VM is far richer than that of its components and contains many modes which include ester and amide skeletal vibrations. Ester C–O–C symmetric stretch appears near 847, 867 cm^{-1} in Raman spectra of nonactin,[27] and combined ester skeletal stretch/methyl rock appears near 920–960 cm^{-1}. VM displays bands at almost identical frequencies (847, 867, 940–965 cm^{-1}). However, similar frequencies in valine and PLV are assigned[24,33] to CC stretch and CH_3 rock vibrations. The anomalous 940–970-cm^{-1} region of lactic acid (Figure 3) suggests that the latter primarily involves isopropyl CH_3 groups.

The intense VM band at 880 cm^{-1} may represent combined CC stretch and NC=O bend as in *n*-methylacetamide.[30] The 912-cm^{-1} VM peak may represent C–CH_3 stretch as in PLA.[29] The 993-cm^{-1} peak is identified as CH_3 rock in *n*-methylacetamide[30] but is assigned to a complex skeletal mode in PLA.[29]

Simple esters (infrared)[35] and the nactin antibiotics (Raman)[27] have characteristic asymmetric skeletal stretch modes near 1090, 1120, and 1190 cm^{-1}. This suggests assigning the 1096- and 1178-cm^{-1} VM Raman peaks to COC and O–C–C asymmetric stretch, respectively, and assigning the 1193-cm^{-1} infrared peak (Table II) to O–C–C asymmetric stretch. The latter shifts to 1184 cm^{-1} in CCl_4–CH_3CN solution (2:1)[2] and then back to 1194–1197 cm^{-1} upon adding K^+, Rb^+, Cs^+, or even Na^+. Symmetric and asymmetric isopropyl stretch contribute to the 1020, 1127 bands, respectively, as in PLV.[24,33] The 1178-cm^{-1} band of VM (with its 1150-cm^{-1} shoulder) presumably contains methyl rock as in PLV.[24,33]

(D) The 1200–1500-cm^{-1} Region. The most interesting feature of this region is the complex amide III vibration (Table I) whose frequency is highly sensitive to molecular conformation.[21-23,25] For example, it is observed at 1266 cm^{-1} in α-helical glucagon but at 1235 cm^{-1} in the unsolvated random coil conformation.[22] In β-chain polyglycine I,[32] this mode is observed at both 1220 and 1234 cm^{-1}; in 3_1-helical polyglycine II these frequencies shift to 1244, 1283 cm^{-1}. In order to identify the amide III modes of VM, we must first eliminate the numerous CH vibrations also appearing in this region.

The VM frequencies at 1271, 1325, and 1344 cm^{-1} are closely matched by similar frequencies in valine, PLV, and HIV. They are assigned to amide III, $C^{\alpha}H$ bend, and $C^{\beta}H$ bend, respectively, in PLV.[24,33] However, the 1275-cm^{-1} frequency of PLA is identified as primarily C–CH bend;[29] its intensity is not reduced by deuteration.[19]

Such considerations suggest that an amide III mode of VM appears near 1252 cm^{-1} (1250 cm^{-1} infrared), a frequency absent from spectra of its monomeric components. This conclusion is supported by recent N-deuteration studies.[19] There are indications that the 1250-cm^{-1} band is split (Figure 4c). Additional amide vibrations may be buried under the CH peaks of this region. A 1292-cm^{-1} amide III mode is reported for PLV,[24,32] and Raman activity near 1310 cm^{-1} is decreased by N-deuteration.[19]

The 1454, 1467 cm^{-1} antisymmetric CH_3 bend is comparatively insensitive to conformational change and is thus a useful internal standard of relative intensity.

(E) The 1600–1700-cm^{-1} Region. This region is particularly important for determining the structure of uncomplexed VM; it contains prominent amide and ester C=O stretch vibrations which are important indicators of intramolecular hydrogen bonding.[21-23,27]

It is significant that the amide I (amide C=O stretch) band of VM is clearly split (1649, 1675 cm^{-1}). Amide I frequencies are known to be shifted downward by hydrogen bonding.[22,36] For example, glucagon has an amide I frequency of 1660 cm^{-1} in its α-helical conformation (tight hydrogen bonding) but 1685 cm^{-1} in its random coil conformation ("free").[22] Intermediate frequencies are found in polypeptides with β-chain conformations like PLV (1667 cm^{-1})[24,33] and polyglycine I (1674 cm^{-1})[32]. The 1649-cm^{-1} member of the VM doublet thus represents tight intramolecularly hydrogen-bonded amide C=O groups, which contribute to the structural stability of several proposed VM conformations,[2,11-17] whereas the 1675-cm^{-1} band represents comparatively "free" amide C=O groups.

The nature of the mode splittings induced by such interactions as hydrogen bonding involves the symmetry properties of the molecule and its vibrations. For example, β-chain PLV theoretically has four amide I modes, split by both intrachain and interchain interactions.[23] However, only a single Raman-active mode (1666 cm^{-1}; both intrachain and interchain neighbors in-phase) and two infrared-active modes (1625, 1685 cm^{-1}) are observed.[33]

A shoulder is observed near 1657 cm^{-1} on the hydrogen bonded amide I peak of VM (Figure 7a-d), which suggests several different amide C=O hydrogen bond strengths in VM consistent with recent x-ray results.[12,13] In the monoclinic[12] octane-crystallized form considered here, two H···O distances are nearly identical (1.89, 1.90 Å) while the other two are significantly longer (1.97, 2.03 Å). The latter may account for the 1657-cm^{-1} shoulder (weaker hydrogen bonding). Alternately, the shoulder might arise from splitting components of the amide I band with different symmetries.

An amide I doublet has been recently observed at 1658, 1685 cm^{-1} in high resolution infrared absorption spectra of valinomycin[14,15] although the splitting is far less resolved than in the Raman. These frequencies are ~10 cm^{-1} higher than the corresponding Raman frequncies which suggest that they represent amide I modes of different symmetries, a fact borne out by Raman studies of the VM-KSCN complex.[10]

(F) The 1700-1800-cm^{-1} Region. It is most significant that VM displays a clearly split doublet (1742, 1767 cm^{-1}) in the *ester* C=O stretching region (~1750 cm^{-1}). The lower peak (1742 cm^{-1}) represents intramolecular hydrogen bonding of some of the ester C=O groups of VM, a feature found thus far only in the VM structure originally proposed by Duax et al.[11] The slight shoulder on the hydrogen-bonded ester C=O peaks (Figure 7) may arise from the slightly different H···O distances (2.24, 2.33 Å) measured by x-ray crystallography.[12]

The "free" ester C=O stretch vibration of VM powder (1767 cm^{-1}) is observed to be less intense than the corresponding hydrogen-bonded C=O vibration (1742 cm^{-1}). This does not necessarily contradict the Duax model in which two ester C=O groups are intramolecularly hydrogen bonded, while four are comparatively free, nor does it necessarily imply that VM conformations exist in which more than two ester C=O groups are hydrogen bonded. The observed intensity of a given C=O vibration depends upon the Raman activity of that mode, as well as the number of each type of C=O group. For example, the amide III modes corresponding to different conformations of poly-L-lysine vary in Raman scattering efficiency, and thus relative intensity.[38,39]

Patel and Tonnelli[16] have carried out a minimum energy calculation for the Duax structure based on a space-filling molecular mode. They agree that the ester C=O hydrogen bonds are characterized by H···O distances of ~2.0 Å but conclude that these bonds are highly nonplanar, and thus most probably weak relative to the four remaining amide C=O hydrogen bonds. Our observation that the ester C=O splitting (25 cm^{-1}) is identical in magnitude to the amide C=O splitting suggests, on the contrary, that both types of hydrogen bonds are of similar strength. Recent x-ray studies of monoclinic VM[12] report that the H···O distances of the ester and amide C=O hydrogen bonds differ by 10-15%; the NH···O angle is found to be approximately 140-160° and 130° for amide and ester C=O hydrogen bonds, respectively.

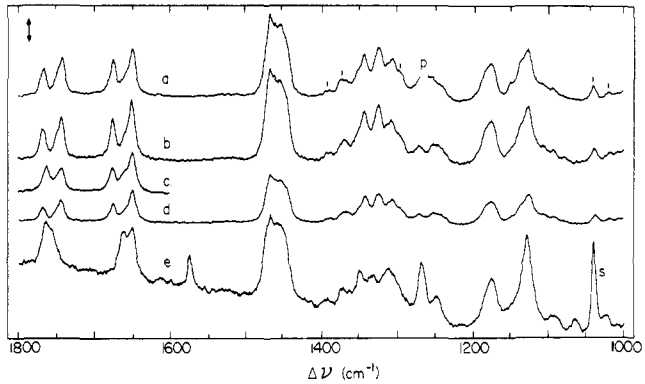

Figure 7. Raman spectra (1000-1800 cm^{-1}) of uncomplexed valinomycin powder crystallized from (a) *n*-octane, (b) carbon tetrachloride, (c) 1-chloropropane, (d) acetonitrile, (e) *o*-dichlorobenzene solution. The letters S, P denote solvent peaks and spurious plasma lines, respectively. Laser excitation 4880 Å; spectral resolution 3 cm^{-1}, except (e) 4 cm^{-1}. The vertical arrow corresponds to 3×10^3 cps (a-d) or 1×10^4 cps (e). Incident power levels are (a) 110 mW, (b-d) 60 mW, (e) 40 mW. Configuration as in Figure 2b; no analyzer in scattered beam. Notice the agreement of Figures 7a and 4c (data taken with different samples and incident power levels).

In preliminary polarization experiments, single VM platelets, cleaved from monoclinic VM crystals, were mounted perpendicular to the scattering plane (Figure 1d) with their flat, cleaved face at ~45° to the incident beam. "Free" and hydrogen-bonded ester C=O vibrations (1742, 1767 cm^{-1}) were observed with roughly equivalent intensities (intensity ratio 1.0-1.5) for most polarization configurations; however, in the ($\perp$, $\perp$) configuration (both incident and collected light polarized perpendicular to the scattering plane) this ratio was ~4.0. In VM powder, in which scattering is simultaneously obtained from all crystallite orientations, this ratio averages 1.4 (Figure 4c).

Other polarization sensitive peaks were observed at 221, 326, 1307, and 1324 cm^{-1}; the splitting of the ~1460-cm^{-1} band was far more prominent in the ($\perp$, $\perp$) configuration than in the ($\parallel$, $\parallel$) configuration. For some crystal orientations, intensity changes in the amide I band could also be observed.

(G) The 2700-3200-cm^{-1} Region. The prominent CH and CH_3 stretch vibrations of this region are readily interpreted from similar frequencies in PLV,[24] PLA[29], and the VM component compounds. The weak VM peaks below 2800 cm^{-1} appear in valine and probably represent overtone or combination frequencies.

(H) The 3200-3600-cm^{-1} Region. This region contains important, conformation-sensitive NH stretch vibrations. The low 3312-cm^{-1} NH frequency of VM indicates that some VM amide groups are hydrogen bonded (compare 3308 cm^{-1} in α-helical PLA). These bonds presumably hold VM in its rigid, bracelet-like solid-state structure.[11-13]

If the 3406-, 3426-cm^{-1} Raman peaks of crystalline VM represent totally free NH stretching, they would be difficult to reconcile with the x-ray structure[11-13] in which four amide C=O groups and two ester C=O groups hydrogen bond to the six available valine NH groups. No such peaks are observed in Raman spectra of VM in CCl_4 solution[19] in which the predominant conformer (conformer A, ref 1) is believed to have all six amide C=O groups hydrogen bonded.[2,15-17] One could postulate that the ester C=O hydrogen bonds are sufficiently weak to cause only minor lowering of the NH stretch fre-

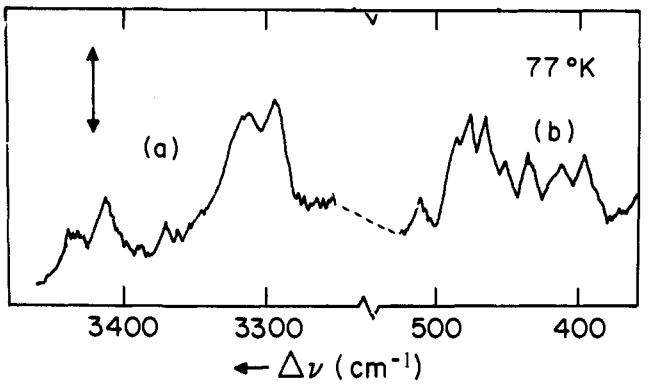

Figure 8. Raman spectrum of valinomycin cooled to 77 K. Note sharpening of the 400–500-cm^{-1} and 3200–3350-cm^{-1} region.

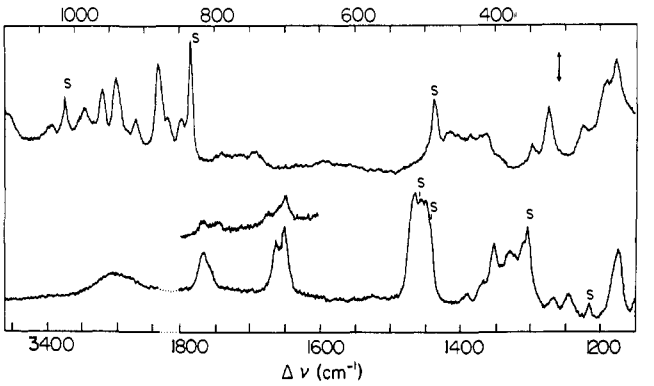

Figure 9. Raman spectrum of uncomplexed valinomycin powder crystallized from *p*-dioxane. Laser excitation 5145 Å, 120 mW; spectral resolution 3 cm^{-1}; no analyzer in scattered beam. The vertical arrow corresponds to 1×10^3 cps; the letter S denotes peaks due to residual solvent. Notice the similarity to the Raman spectrum of valinomycin crystallized from *o*-dichlorobenzene (Figure 7e). Insert: Raman spectrum of *p*-dioxane-grown valinomycin sample allowed to dry for 4 weeks at room temperature. Notice the reemergence of the splittings which characterize *n*-octane-grown valinomycin (Figure 4). Incident power 40 mW; otherwise conditions are as above.

quency. This would be consistent with the greater length of ester C=O hydrogen bonds (H···O distance ~0.4 Å longer than amide C=O hydrogen bonds) but not with the equivalent splittings (25 cm^{-1}) seen in the C=O stretch region. However, no peaks appear above 3350 cm^{-1} in Raman spectra of VM recrystallized from *o*-dichlorobenzene or dioxane in which some NH groups are apparently unbonded or very weakly bound (following section). Thus an alternate, perhaps preferable, explanation of these bands is that they are combination frequencies as in HIV (Figure 5d). (Notice that valine and lactic acid lack both the 1715- and 3422-cm^{-1} peaks.) This is supported by studies of VM recrystallized from *o*-dichlorobenzene (see next section).

The band appearing near 3430 cm^{-1} in infrared spectra of solid state VM (Table II, ref 15) may also represent combination frequencies. In some infrared spectra,[37] this band is split and appears at frequencies (3408, 3423 cm^{-1}) almost identical with those observed in Raman spectra. In contrast, the weak ~3390-cm^{-1} infrared band appearing in CCl_4 solutions[2,37] probably represents a free NH stretch mode; it disappears when the K^+ complex is formed.[2] The 3390-cm^{-1} band presumably reflects the presence of the minority conformer (conformer B, ref 2) with three free NH groups known to be present in substantial amounts even in nonpolar solutions.[2,16] The corresponding Raman vibration is either forbidden or too weak to be seen.[19] A NH stretch band appears near 3382 cm^{-1} in infrared spectra of VM dissolved in *n*-hexane but is not observed in dioxane solution.[15]

In infrared absorption spectra of monosubstituted amides and polypeptides, the NH stretching mode (~3300 cm^{-1}) interacts with the nearby second harmonic of the amide II vibration (~3100 cm^{-1}) via anharmonic terms in the vibrational potential (Fermi resonance). This shifts the frequencies of these modes (called amide A and B, respectively) somewhat further apart.[21] For example, the amide A, B bands of *n*-methylacetamide are observed[30] at 3300, 3110 cm^{-1} rather than at 3236, 3134 cm^{-1}. These bands occur at 3333 cm^{-1} (amide A) and 3067 cm^{-1} (amide B) in infrared spectra of uncomplexed VM powder.

(I) Low-Temperature Spectra. Single crystals of VM were cooled to 77 K by immersion in liquid nitrogen using a system described elsewhere.[28] Although most of the spectrum is unaffected by cooling, considerable sharpening is observed in the 400–500- and 3300–3450-cm^{-1} regions (Figure 8). In particular, the broad NH stretch band near 3312 cm^{-1} splits into a resolvable 3295-, 3315-cm^{-1} doublet. This is consistent with the presence of hydrogen bonds of differing strengths (sections E and F). This preliminary experiment suggests that cryogenic techniques may be useful in further elucidating spectral features in hard-to-resolve regions.

Uncomplexed VM Recrystallized from Other Solvents

The results of the preceding section were obtained with VM samples crystallized from warm *n*-octane. Although crystal structures may differ, recent x-ray studies[12,13] show that the molecular conformations of uncomplexed VM recrystallized from octane, methanol–water, and acetone are highly similar. Preliminary reports suggest that orthorhombic ($P2_12_12_1$) crystals of VM grown from DMSO solution appear to represent a larger, distinct conformation.[13] In this section, we discuss the Raman spectra of VM samples recrystallized from a variety of solvents of increasing polarity: CCl_4 (0.00), *p*-dioxane (0.0), $CHCl_3$ (1.02), $CH_3(CH)_2Cl$ (2.05), *o*-dichlorobenzene (2.52), CH_3COCH_3 (2.89), and CH_3CN (3.84) (the numbers in parentheses represent the dipole moment of the corresponding vapor in 10^{18} esu).

The VM Raman spectra are found to be similar for most solvents (Figure 7a–d). However, the "free" ester C=O stretching frequency is shifted ~4 cm^{-1} downward in VM recrystallized from CH_3CN (Figure 7c) which may indicate loose bonding between the exposed ester C=O groups and the highly polar residual solvent. It is not uncommon for VM to retain disordered solvent in its lattice.[12,18]

In contrast, spectra of VM samples recrystallized from *o*-dichlorobenzene show dramatic changes in the C=O stretch region (Figure 7d). The H-bonded C=O stretch vibration at 1742 cm^{-1} disappears, and the free C=O vibration is shifted 4 cm^{-1} downward to 1763 cm^{-1}, with a shoulder ~7 cm^{-1} lower. Although the hydrogen-bonded amide I frequency (1649 cm^{-1}) remains unchanged, the free amide I frequency is shifted from 1675 to 1663 cm^{-1}. This may represent the loose bonding of the "free" amide C=O groups to residual solvent in this new conformation.

When moist crystals of *o*-dichlorobenzene-grown VM are dried to a white powder, the Duax-like conformation described in the preceding section reappears; however, the moderate size of the solvent peaks in Figure 7d suggest that the new solid state conformation is stable in even slightly solvated crystals. Measurements on crystalline samples indicate that the intensity of VM peaks in this region (1600–1800 cm^{-1}) is still highly orientation and polarization dependent.

Our observations suggest that both semifree and strongly hydrogen-bonded amide C=O groups exist in this VM conformation, although the former appear to be in more exposed positions which encourage interaction with the solvent. There

are no strongly intramolecularly hydrogen-bonded ester C=O groups as in the Duax conformation. Rather, the ester C=O groups seem to occupy at least two different positions which vary in their availability to interact with the solvent. These interpretations are consistent with the large, relatively open "polar solvent" structures[2,16] which are throught to have three "free" and three hydrogen-bonded NH groups.

The broad hydrogen-bonded NH stretch band of *o*-dichlorobenzene-recrystallized VM (3293 cm^{-1}) is ~19 cm^{-1} lower than in *n*-octane-grown VM. The clear absence of 3406-, 3426-cm^{-1} frequencies further indicates that those modes are unrelated to the free NH stretch vibrations of VM, which are apparently Raman inactive or too weak to be observed. The simultaneous absence of the 1742-cm^{-1} frequency (H-bonded ester C=O stretch) in *o*-dichlorobenzene-grown VM supports the assignment of the 3406-, 3426-cm^{-1} peaks of *n*-octane-grown VM to combination frequencies (3406 $\simeq$ 1657 + 1747 and 3426 $\simeq$ 1675 + 1747).

Other differences between the two conformations are: the appearance of a 303-cm^{-1} vibration; the merging of the 2966-, 2984-cm^{-1} methyl stretch vibrations (2972 cm^{-1} as in HIV); and 4- to 7-cm^{-1} shifts in the 736, 757 (amide V), 1252 (amide III), and 1307, 1324, 1344 cm^{-1} (CH vibration) peaks.

Similarly, Raman spectra of VM recrystallized from *p*-dioxane (Figure 9), a nonpolar hydrogen bond accepting solvent, correspond to those of *o*-dichlorobenzene-VM, not *n*-octane-VM. This indicates that the ability of a solvent to accept hydrogen bonds and to be incorporated into the VM crystal lattice is more essential than polarity in determining the stability of a given VM conformation in the solid state. Again, thoroughly dried crystals are observed to revert to the Duax conformation (Figure 9, insert).

Although the amide I frequencies (1650, 1663 cm^{-1}) of VM recrystallized from *o*-dichlorobenzene and *p*-dioxane are identical, their free ester C=O stretch frequencies differ (1763 and 1767 cm^{-1}, respectively). Similarly, the hydrogen-bonded NH stretch frequency of *p*-dioxane-VM is somewhat higher (3301 cm^{-1}). This may indicate that *o*-dichlorobenzene interacts more strongly with VM.

An additional peak (1267 cm^{-1}) is observed in the amide III region of *p*-dioxane-VM; it cannot be seen in *o*-dichlorobenzene-VM because of a prominent solvent peak near 1275 cm^{-1}. Other differences include minor shifts (~5 cm^{-1}) in the 959-, 1310-, 1328-, 1367-, 2772-, 2966-cm^{-1} CH vibrations (perhaps related to crystalline packing).

The observations presented in this section would imply that the Duax structure is not a major conformation in polar (*o*-dichlorobenzene) or hydrogen-bonding (*p*-dioxane) solutions. Conversely, no conformer equivalent to the "nonpolar" solution structure of VM[2,16] has yet been observed in the solid state, which suggests it is unsuitable for forming a VM lattice. Our discovery of a new solid state conformer of VM in crystalline samples of *o*-dichlorobenzene-VM and *p*-dioxane-VM opens the possibility of obtaining a full three-dimensional x-ray crystallographic structure for this conformation, which resembles one observed previously only in solution.[2,16]

Conclusion

We have presented complete Raman spectra (150–3600 cm^{-1}) of two distinct forms of uncomplexed valinomycin (VM) in the solid state. Their spectra were analyzed in detail using several component and model compounds in an attempt to elucidate their structure. Although considerable Raman information is available for polypeptides, this is the first such study of a depsipeptide.

Splittings observed in the amide I and ester C=O stretch regions indicate that VM samples recrystallized from *n*-octane, CCl_4, $CHCl_3$, $CH_3(CH_2)_2Cl$, and CH_3CN represent a VM conformation whose x-ray crystallographic structure has been recently determined.[12,13] In that conformation (Figure 1b) VM has two hydrogen-bonded ester C=O groups; the four free ester C=O groups are highly exposed and thus available to initiate coordination of nearby K^+ ions. A different conformation, lacking strongly hydrogen-bonded ester C=O groups, is inferred from Raman spectra of uncomplexed VM recrystallized from *o*-dichlorobenzene and dioxane. This new solid state conformer resembles a VM conformation which has been previously observed only in solution.[2,16]

Such studies underline the growing usefulness of Raman spectroscopy in the study of ion-selective processes[10,26,27,40] and in particular suggest several avenues of future research including the x-ray crystallography of the new solid-state conformer of VM. They also lay the basis for Raman spectroscopic studies of VM in solution[19] and in lipid/water systems.

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Raman Spectroscopy of Uncomplexed Valinomycin. 2. Nonpolar and Polar Solution

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Abstract: The molecular conformations of uncomplexed valinomycin in CCl_4, CS_2, CH_2Cl_2, $CHCl_3$, CH_3OH, C_2H_5OH, C_3H_7Cl, *p*-dioxane ($C_4H_8O_2$), and dioxane/D_2O have been studied using laser Raman spectroscopy. The stretching frequencies of the ester and amide carbonyl groups are found to be affected by both the polarity of the solvent and its ability to form hydrogen bonds. Results in nonpolar solvents are consistent with the presence of hydrogen bonding ester carbonyl groups, reopening the question of whether the conformation found in valinomycin recrystallized from *n*-octane can exist in nonpolar solution. In polar solvents, a conformation is detected that contains fewer hydrogen bonds. As the dielectric constant of the solvent increases, the stretching frequency of the amide carbonyl groups increases (perhaps reflecting a reduction of intramolecular hydrogen bonding), while the stretching frequency of the ester carbonyl groups decreases.

The macrocyclic dodecadepsipeptide valinomycin (hereafter abbreviated VM; Figure 1a) was among the first ion-specific antibiotics to be used as a model of ionic transport in biological membranes.[2-4] Although the three-dimensional structure of crystals can often be obtained with x-ray or neutron diffraction, these techniques cannot provide information about whether conformations found in the solid state persist in solution. In contrast, Raman spectroscopy can be applied to both solids and liquids, permitting one to compare structural characteristics of molecules in a variety of environments. Raman spectroscopic investigations of the molecular conformations of VM in the solid state have recently been reported,[5] and in this work we report studies of VM in a variety of solvents. These studies, which include deuteration of the NH groups, reveal new information about the dependence of VM conformation on environment.

X-ray methods have been used to reveal[6,7] the complete structure of one form of uncomplexed VM; all six NH groups are intramolecularly hydrogen bonded, four to amide C=O groups and two to ester C=O groups (conformation D, Figure 1b). Structural similarities between crystalline uncomplexed VM and the VM-K^+ complex have led to the suggestion[6] that this form may be involved in ion complexation at the membrane–water interface. However, nuclear magnetic resonance (NMR), infrared absorption (IR), and optical rotatory dispersion (ORD) studies[4,8-11] reveal no evidence of conformation D in solution. One question we address is whether or not there is Raman spectroscopic evidence that conformation D persists in solution.

A mixture of several VM conformations exists in solution.[4,8-11] It is believed that the predominant conformation of VM in nonpolar solvents contains six hydrogen-bonded amide C=O groups and six unbonded ester C=O groups (conformation A, Figure 1c), while the predominant conformation in polar solvents is believed to contain only three hydrogen bonded amide C=O groups (conformation B, Figure 1d). These conclusions are based primarily on NMR data,[9] which indicate a threefold equivalence of L- and D-valine protons. Conformation D lacks this symmetry; however, Patel and Tonelli[9] have pointed out that asymmetric structures could exist in solution if they were in "rapid" equilibrium with each other (rapid on an NMR time scale) thereby appearing to be symmetric in NMR measurements.

The Raman spectra of VM recrystallized from *n*-octane (known[7] to be in conformation D) exhibit a hydrogen-bonded ester C=O group mode near 1742 cm^{-1}, 25 cm^{-1} lower in frequency than the corresponding mode of the free C=O groups.[5] The discovery of a similar downshift (or splitting) in solution would support the presence of hydrogen bonded ester C=O groups (and thus conformation D) in such environments.

Materials and Methods

Valinomycin (VM) was obtained from Calbiochem (San Diego, Calif.) and Sigma Chemicals (St. Louis, Mo.) and was used without

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