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Carbon Framework of Valinomycin and Its Metal Ion Complex in Solution†

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ABSTRACT: The solvent-dependent solution conformations of valinomycin and its K^+ complex were determined using proton nuclear magnetic resonance (nmr) spectroscopy and conformational calculations in the previous manuscript (Patel, D. J., and Tonelli, A. E. (1973), *Biochemistry 12*, 486). An extension of this study utilizes ^{13}C nmr spectroscopy to monitor the carbon framework of this depsipeptide and its complex in solution. The C^α, C^β, and C' carbon chemical shifts were assigned to the amino acid and ester residues and found to be a sensitive function of conformation. The ^{13}C chemical shifts reflect the large conformational changes in the depsipeptide on complex formation. The carbonyl carbon resonances involved in complexation move 5 ppm downfield on coordination with diamagnetic univalent metal ions. Potassium exchange between valinomycin and its complex in the uncertainty broadened slow exchange region could be monitored with the larger range of ^{13}C chemical shifts. The rotational correlation time τ_R for valinomycin and its complex in methanol were derived from the measurement of carbon spin–lattice relaxation times in proton-decoupled spectra.

The solution conformations of several cyclic polypeptides have been derived using proton nuclear magnetic resonance (nmr) spectroscopy and conformational energy maps (Hassal and Thomas, 1971). Recently this analysis has been extended to include a computer search to determine the lowest energy conformation of a cyclic polypeptide consistent with the nmr parameters and conformational energy maps (Bovey *et al.*, 1972).

The cyclic depsipeptide valinomycin occupies a special place in these investigations because of its involvement in potassium ion transport. Proton nmr studies on valinomycin and its K ion complex have been reported from several laboratories (Haynes *et al.*, 1969; Ivanov *et al.*, 1969; Ohnishi and Urry, 1969). In the previous paper from this laboratory the conformations of valinomycin in hydrocarbon, hydrogen-bond acceptor, and aqueous media were elucidated using proton nmr spectroscopy and conformational calculations (Patel and Tonelli, 1973). This study reports on the ^{13}C spectra of valinomycin and its metal ion complex in these same solvent systems.

The ^{13}C nmr spectrum of gramicidin S has been reported and the spectral assignments were made by comparison with the amino acid spectra (Gibbons *et al.*, 1970). The ^{13}C resonances of the carbonyl groups participating in intramolecular hydrogen bonds and those exposed to solvent had similar chemical shifts. A preliminary report on the ^{13}C nmr studies of the binding of metal ions to cyclic crown ethers and depsipeptides has appeared (Ohnishi *et al.*, 1972). This investigation of valinomycin was initiated in the expectation that carbonyl group complexation with the metal ion would be reflected in their ^{13}C chemical shifts.

Experimental Section

^{13}C spectra were run on an XL-100 Varian spectrometer with heteronoise proton spin decoupling and variable-temperature facilities. The instrument was interfaced with an F&H pulse program generator and a Fabri-Tek computer for data collection in the Fourier transform mode (Sternlicht and Zuckerman, 1972).

Valinomycin was purchased from Calbiochem. The sample was dissolved in deuterated solvents and the spectrometer locked on deuterium. The carbon resonances of solvent served as an internal standard and were later referenced relative to CS_2. Sample concentrations were in the range 50–100 mg/ml for all nmr runs except T_1 measurements, where the sample concentration was 200 mg/ml.

T_1 measurements were undertaken using the 180°, τ, 90° pulse sequence method (Vold *et al.*, 1968). Measurements in the carbonyl, C^α and $C^\beta + CH_3$ regions were undertaken in separate runs. Repetition rates were 10 sec for carbonyl runs and 3 sec for all other measurements. The estimated errors in the measurements are <10%.

Results and Discussion

Valinomycin is a cyclic depsipeptide with the sequence *cyclo*-(D-HyIv-D-Val-L-Lac-L-Val)$_3$. The amino acid grouping is defined by the atoms ($NC^\alpha(C^\beta)C'$) and the ester grouping

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TABLE I: Carbon Chemical Shifts (ppm) from CS_2 for Valinomycin in Octane–Dioxane (3:1), Dioxane–Water (5:1), and H_4-furan. Comparison of the Carbon Chemical Shifts for Valinomycin and Its K Complex in Methanol and Dimethylformamide.

					C^α				C^β		
	C′				D-HyIv	L-Lac	L-Val	D-Val	D-HyIv	D-Val	L-Val
Valinomycin, octane–dioxane (3:1), 50°	20.63	21.55	22.43	22.91	114.43	122.78	*132.44*	*133.75*	162.50	*164.68*	*164.78*
Valinomycin, dioxane, 50°	20.98	21.22	22.68	22.82	114.24	122.45	*132.93*	*134.44*	162.55	*163.81*	*164.15*
Valinomycin, dioxane–water (5:1), 50°	21.17	21.27	22.19	22.53	114.04	122.30	*133.90*	*134.49*	162.40	*163.13*	*163.13*
H_4furan, −12°	21.32	*20.40*	22.83	24.14	114.88	123.28	133.18	*136.43*	162.80	*162.21*	164.06
H_4furan, +50°	21.18	*21.47*	22.83	23.12	114.49	122.64	132.99	*134.59*	162.55	*163.82*	164.30
	a	*b*	*a*	*b*	D-HyIv	L-Lac	D-, L-Val		D-HyIv	D-, L-Val	
Valinomycin, CD_3OD, 50°	20.64	*21.12*	21.76	*22.15*	113.61	121.92	*133.65*	*133.85*	162.07	*162.65*	*162.75*
Valinomycin–KSCN, 1:2, CD_3OD, 50°	19.33	*16.51*	20.83	*17.00*	112.64	121.04	*130.55*	*130.65*	162.16	*164.01*	*164.01*
Valinomycin, dimethylformamide, 50°	22.38	*21.89*	23.94	*22.77*	114.77	122.93	*134.43*	*135.06*	162.74	*163.18*	*163.66*
Valinomycin–KSCN, 1:4, dimethylformamide, 50°	20.92	*18.83*	22.38	*19.42*	114.04	112.20	*132.68*	*132.88*	162.93	*164.29*	*164.29*

[a] N-Linked carbonyl chemical shifts. [b] O-Linked carbonyl chemical shifts.

by the atoms ($OC^\alpha(C^\beta)C'$). The carbonyl carbons are designated C′.

I. Solvent-Dependent Valinomycin Conformations

The ^{13}C nmr spectra of valinomycin in octane–dioxane (3:1), dioxane, and dioxane–water (5:1) at 50° are presented in Figure 1. The spectral regions can be subdivided into methyl (173–177 ppm), C^β (161–166 ppm), C^α (113–135 ppm), and carbonyl C′ (20–24 ppm).

The available experimental and theoretical information on ^{13}C chemical shifts suggests that the C^α and C^β carbons of the ester grouping will come downfield from the corresponding amino acid grouping (Stothers, 1965). Further, it has been established that methyl substitution for proton at carbon results in downfield shifts (Grant and Paul, 1964; Lindeman and Adams, 1971) permitting the differentiation between the C^α and C^β resonances of L-Lac from D-HyIv (hydroxyisovaleric acid) in valinomycin. Table I lists the C^α and C^β chemical shifts of the ester residues along with their assignments in these solvent systems.

In cyclic ethers at low temperatures, the predominant valinomycin conformation, designated II-1, exhibits 1–4 type intramolecular hydrogen bonds at D-Val N protons only. With increasing hydrocarbon content, a conformation, designated I, with all valyl protons forming 1–4 type intramolecular hydrogen bonds, participates in equilibrium with II-1. Their rotation angles are summarized (Patel and Tonelli, 1973) as:

	L-Val	D-HyIv	D-Val	L-Lac
I	210, 240	280, 120	*140, 120*	80, 240
II-1	210, 270	280, 120	*320, 280*	80, 270

The temperature dependence of the ^{13}C spectrum of valinomycin in H_4furan is shown in Figure 2 and the chemical shifts are summarized in Table I. One of the valyl C^α and C^β carbon resonances along with a methyl resonance is shifted with temperature while the other valyl and both ester residues remain essentially unaffected (Figure 2). The proton nmr and theoretical calculations in the previous manuscript (Patel and Tonelli, 1973) suggested that the temperature- or solvent-mediated equilibrium between I $\rightleftarrows$ II-1 resulted from rotational angle changes at D-Val only. The C^α and C^β carbon chemical shifts of the D- and L-Val resonances can be differentiated and are summarized in Table I.

Conformation II-1, predominant in cyclic ethers at low temperature, exhibits (φ,ψ) D-Val $\neq$ $(-\varphi,-\psi)$ L-Val. This is strikingly brought out in Figure 2 by the difference in D- and L-Val C^α (3.25 ppm) as well as C^β (1.85 ppm) chemical shifts at −12°. Even larger differences are expected on lowering the temperature further. This suggests that the C^α and C^β carbon

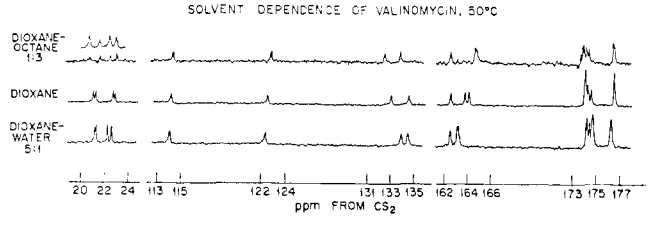

FIGURE 1: The proton-decoupled ^{13}C spectra of valinomycin in dioxane–octane (1:3), dioxane, and dioxane–water (5:1) at 50°

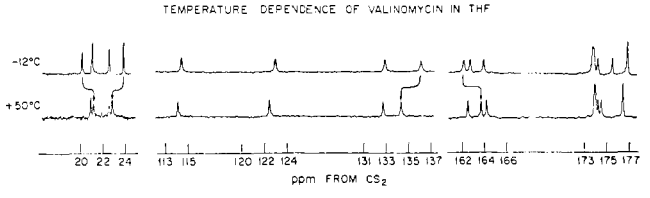

FIGURE 2: The temperature dependence of the proton-decoupled ^{13}C spectrum of valinomycin in H_4furan.

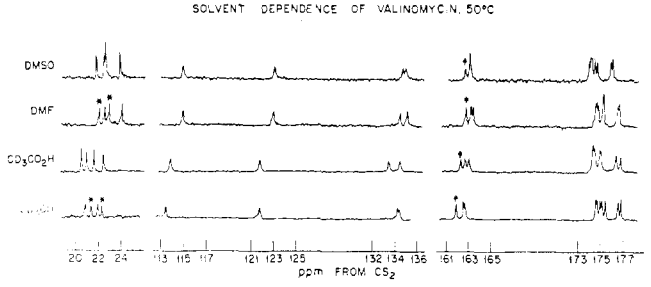

FIGURE 3: The proton-decoupled ^{13}C spectra of valinomycin in Me_2SO, dimethylformamide, CD_3CO_2H, and CD_3OD at 50°.

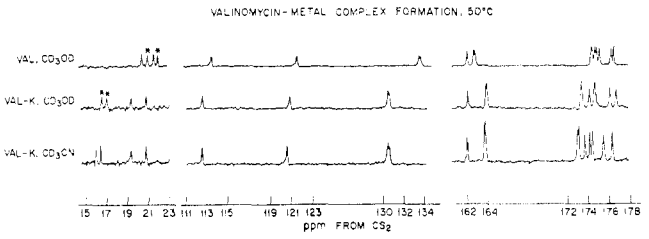

FIGURE 4: The proton-decoupled ^{13}C spectra of valinomycin and its K complex in methanol and acetonitrile at 50°.

chemical shifts along with the H^{α} proton chemical shifts are a sensitive function of conformation.

The ^{13}C spectrum of valinomycin in dioxane–octane (1:3) at 50° (see Table I and Figure 1) exhibits a 1.3-ppm shift difference between D- and L-Val C^{α} chemical shifts. Since the conformation in hydrocarbon media, I, exhibits D-Val $(\varphi,\psi) \simeq$ L-Val $(-\varphi,-\psi)$, a negligible shift difference would be expected. It must therefore be deduced that the presence of 25% dioxane in the octane results in a considerable proportion of II-1, participating in the equilibrium I $\rightleftarrows$ II-1 in this solvent system.

The structure of valinomycin in aqueous media above room temperature consists of a conformational equilibrium between several structures (including III-1 and III-2). These flexible conformations lacking type 1–4 intramolecular hydrogen bonds are defined by the rotation angles summarized (Patel and Tonelli, 1973) as:

	L-Val	D-HyIv	D-Val	L-Lac
III-1	30–40, 250–320	260–270, 100–120	320–330, 40–110	80–90, 240–260
III-2	90, 290–310	250–260, 0–10	270, 50–70	110–130, 350–360

The C^{α} carbon chemical shifts of L- and D-Val valinomycin resonances in dioxane–water (5:1) at 50° are similar as are the C^{β} chemical shifts, consistent with D-Val $(\varphi,\psi) \simeq$ L-Val $(-\varphi,-\psi)$, for the conformation in aqueous media.

A feature of the valinomycin conformation investigation involving systematic solvent change from hydrocarbon to aqueous environment mediated through dioxane is that while the C^{α} and C^{β} chemical shifts of D- and L-Val vary with solvent polarity, the same carbon resonances of L-Lac and D-HyIv are essentially unaffected (Table I and Figure 1). This result parallels the observation of H^{α} proton chemical shifts in these solvents (Patel and Tonelli, 1973). Clearly, the conformational change with solvent polarity is reflected in the rotation angles of the D- and L-Val residues predominantly. In retrospect, this is not surprising, since the amino acid energy maps show many minima compared to the ester energy maps (Tonelli *et al.*, 1972). Ester residues, however, show more conformational freedom due to the absence of a group anti to the carbonyl oxygen.

It has not been possible to assign the carbonyl resonances (designated C′) in these solvent systems.

The spectra of valinomycin in the hydrogen-bond acceptor solvents dimethylformamide and Me_2SO are very similar (Table I and Figure 3). From proton nmr studies and theoretical calculations, valinomycin in this solvent system was represented by a rapid equilibrium between structures II-1 and II-2, each containing three type 1–4 intramolecular hydrogen bonds. The conformations are defined by the rotation angles (Patel and Tonelli, 1973)

	L-Val	D-HyIv	D-Val	L-Lac
II-1	210, 270	280, 120	320, 280	80, 270
II-2	40, 80	280, 120	140, 120	80, 270

Since the C^{α} chemical shift difference between valinomycin valyl residues in Me_2SO and dimethylformamide is small at 50°, it is proposed that no single conformation predominates at this temperature (Figure 3 and Table I).

Comparison of the valinomycin ^{13}C spectra in dimethylformamide and Me_2SO, on the one hand, with methanol and acetic acid on the other exhibits chemical shift differences throughout the spectrum including the ester C^{α} and C^{β} carbon chemical shifts. These differences result from the predominance of rigid conformation II-1 $\rightleftarrows$ II-2 in dimethylformamide and Me_2SO and flexible conformations III-1 $\rightleftarrows$ III-2 in methanol.

II. Complex Formation

Addition of KSCN to valinomycin in methanol results in complex formation. The spectra and chemical shifts of the depsipeptide and its complex are presented in Figure 4 and Table I. Solution nmr and X-ray crystallographic studies have established that the O-linked carbonyl groups are coordinated to the metal ion while the N-linked carbonyl groups participate in type 1–4 intramolecular hydrogen bonds (Ivanov *et al.*, 1969; Ohnishi and Urry, 1969; Pinkerton *et al.*, 1969).

The C^{α} D- and L-Val resonances shift downfield by 3 ppm while the L-Lac and D-HyIv C^{α} resonances are downfield shifted by 1 ppm (Figure 4 and Table I). The D-HyIv C^{β} chemical shifts remain unaffected while the D- and L-Val C^{β} chemical shifts move 1 ppm upfield on complexation (Figure 4 and Table I). The larger chemical shift changes for the C^{α} and C^{β} amino acid residues compared to the ester residues observed on complexation reflects a greater conformational change at the amino acid residues and their proximity to the carbonyl groups involved in the complexation.

Figure 5 outlines the effect on the carbonyl (C′) resonances on the gradual addition of KSCN to valinomycin in methanol at 50°. Two C′ resonances move downfield by 5 ppm and since the O-linked carbonyls bind to the metal ion this shift is assigned to them. The remaining two C′ resonances move downfield by 1 ppm on complexation and are assigned to the N-linked carbonyls participating in type 1–4 intramolecular hydrogen bonds in the complex. Knowing the N-linked and O-linked carbonyl assignments in the complex, those in valinomycin in methanol follow directly. An earlier preliminary report suggested alternate assignments based on linewidth measurements of the carbonyl resonances in the depsipeptide and its complex (Ohnishi *et al.*, 1972).

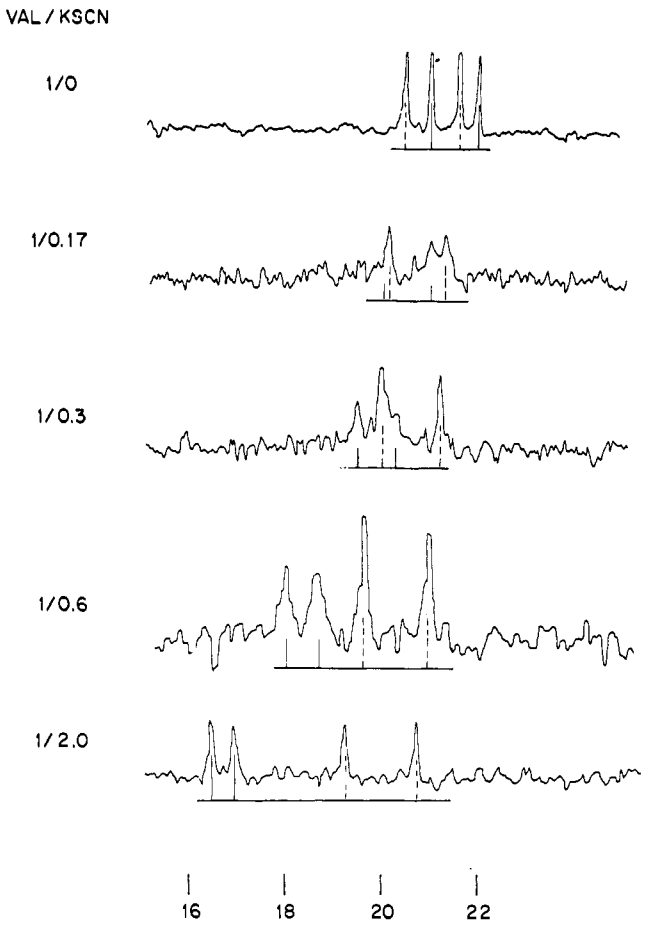

FIGURE 5: The proton-decoupled ^{13}C spectra of the carbonyl region on gradual addition of KSCN to valinomycin in methanol at 50°.

Gradual addition of KSCN to valinomycin in methanol resulted in temperature-dependent line width effects for the ^{13}C resonances. Consider the exchange process: *Val*–K + Val $\rightleftarrows$ Val–K + *Val*, when the concentration of the depsipeptide is greater than KSCN. Cis–trans peptide or ester bond isomerization between valinomycin and its complex would manifest itself in narrow spectra for both structures over the temperature range of interest (−25 to +50°) due to a high barrier of isomerization (~20 kcal/mol). Since this was not experimentally observed in the ^{13}C spectrum of valinomycin plus KSCN (1:0.6), this type of isomerization is ruled out when the depsipeptide bonds metal ion.

The line broadening observed in the ^{13}C spectrum of valinomycin plus KSCN (1:0.6) in methanol (see Figure 6) exhibits the following features: (a) the ^{13}C resonances in valinomycin and the complex with the largest chemical shift differences (C′ and C^{α} amino acid residues) are exchange broadened to the greatest extent, (b) the degree of broadening increases on lowering the temperature, (c) average ^{13}C nmr spectra are observed at 50° for all the resonances, and (d) at −20°, separate exchange broadened ^{13}C resonances for the O-linked C′ and amino acid C^{α} of the depsipeptide and its complex are observed. Analysis of the exchange phenomena yields an activation energy of 12 ± 2.5 kcal/mol for the process: *Val*–K + Val → *Val* + Val–K.

The Na^+ exchange with the cyclic ether dibenzo-18-crown-6 (DBC) exhibits an activation energy of 12 kcal/mol for the process *DBC*–Na + DBC → *DBC* + DBC–Na as determined by proton nmr spectroscopy (Wong *et al.*, 1970).

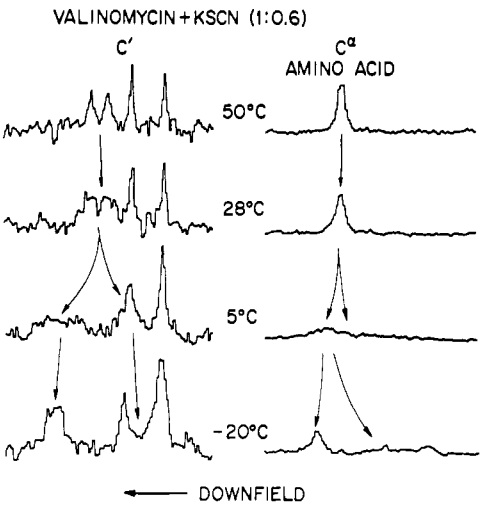

FIGURE 6: The temperature dependence of the proton decoupled ^{13}C spectrum (carbonyl and amino acid C^{α} regions) of valinomycin plus KSCN (1:0.6) in methanol.

The ^{13}C spectra of valinomycin–K in CD_3CN and CD_3OD are very similar (Figure 3). By contrast, the ^{13}C spectra of the complex in dimethylformamide and CD_3OD show striking differences (Table I). These differences are observed throughout the spectrum consistent with the rotation angle differences for the all type 1–4 intramolecularly hydrogen-bonded complex conformation C-I (CD_3OD) and complex conformation C-II (dimethylformamide) lacking these hydrogen bonds, determined from proton nmr and theoretical calculations. Their rotation angles are summarized (Patel and Tonelli, 1973) as:

	L-Val	D-HyIv	D-Val	L-Lac
C-I	110, 290	250, 210	250, 70	110, 150
C-II	20, 340	290, 130	340, 20	70, 230

Valinomycin is known to form 1:1 complexes with K^+, Rb^+, and Cs^+ which possess ionic radii of 2.66, 2.94, and 3.34 Å, respectively. The ^{13}C spectra of these three complexes are similar (Figure 7) suggesting approximately the same conformation for the depsipeptide in the complex. The O-linked carbonyl chemical shifts of the Cs complex are 1 ppm upfield from the corresponding residues in the K and Rb complexes. This reflects a perturbation on the conformation of the complex to accommodate the larger Cs ion. Attempts to generate the complexes with Ba^{2+} (ionic radii 2.68 Å) and Ag^+ (ionic radius 2.52 Å) were unsuccessful.

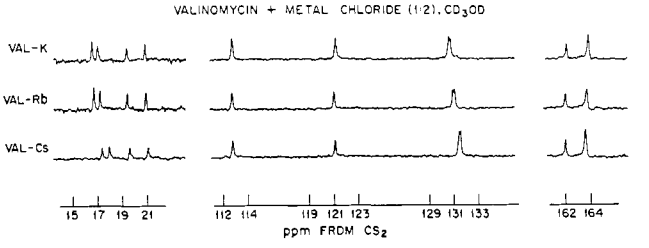

FIGURE 7: The proton-decoupled ^{13}C spectra of the K, Rb, and Cs complexes of valinomycin in methanol at 50°.

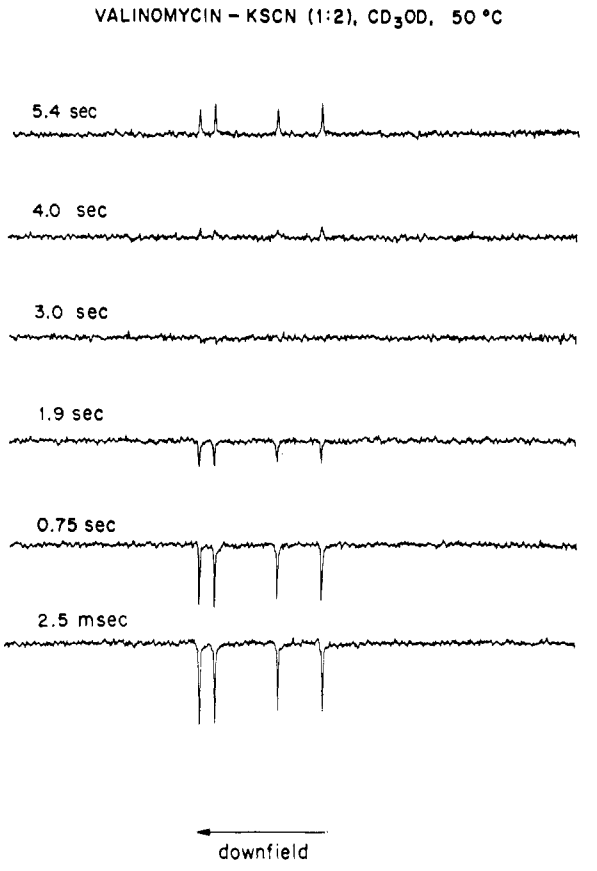

FIGURE 8: The proton-decoupled ^{13}C spectra of the carbonyl region of valinomycin–K in methanol at 50°.

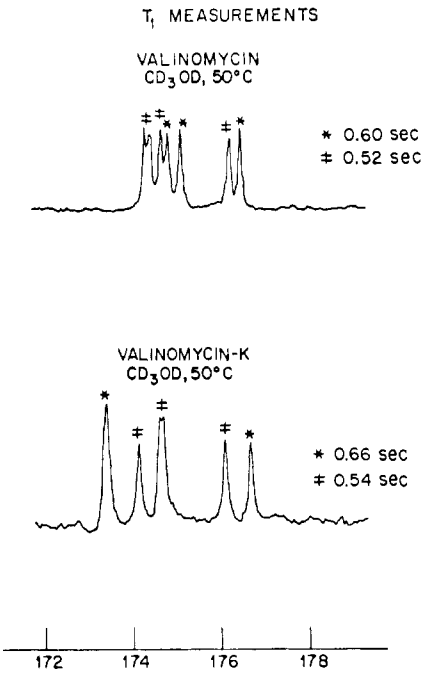

FIGURE 9: T_1 values for methyl carbons in valinomycin and its K complex in methanol.

III. T_1 Measurements

Spin–lattice relaxation time (T_1) measurements were undertaken on the heteronoise proton-decoupled ^{13}C spectra of valinomycin and its K^+ complex in methanol using the 180°, τ, 90° pulse sequence method (Vold *et al.*, 1968). The value of the spin–lattice relaxation time is given by

$$A = A_e\left[1 - 2\exp\left(\frac{-\tau}{T_1}\right)\right]$$

where A and A_e are observed and equilibrium intensities and τ is the interval between 180 and 90° pulses (Abragam, 1961).

A typical experiment covering the carbonyl C′ region of valinomycin–K in methanol at 50° is depicted in Figure 8. The data suggest that all the carbonyls have the same spin–lattice relaxation time.

The C^α, C^β, and C′ carbon T_1 values of valinomycin in methanol and valinomycin–K in methanol at 50° are summarized in Table II while the CH_3 carbon T_1 values are depicted in Figure 9.

The data suggest that the carbon T_1 values increase in the order $C^\alpha < C^\beta < CH_3 < C'$. For valinomycin and its complex, the value of the spin–lattice relaxation time for a particular carbon (C^α, C^β, or C′) is approximately independent of its origin (amino acid or ester residue). A T_1 value of 4.0–4.3 sec for the N- and O-linked carbonyl resonances is measured for valinomycin and its complex in methanol. By contrast for the amino acid and ester C^α and C^β carbons, the spin–lattice relaxation times are larger for the complex as compared to the depsipeptide in methanol.

TABLE II: Spin–Lattice Relaxation Time, T_1, for C′, C^α, and C^β Carbons in Valinomycin and Its K Complex in Methanol at 50°.

	T_1 Measurements (sec)	
	Valinomycin, CD_3OD, 50°	Valinomycin–K, CD_3OD, 50°
C′ D-Val	4.0	4.3
L-Val	4.0	4.3
D-HyIv	4.0	4.3
L-Lac	4.0	4.3
C^α D-Val	0.24	0.29
L-Val	0.24	0.29
D-HyIv	0.25	0.32
L-Lac	0.25	0.32
C^β D-Val	0.27	0.35
L-Val	0.27	0.35
D-HyIv	0.28	0.40

For proton decoupled ^{13}C spectra, the carbon spin–lattice relaxation time is dominated by the carbon–proton dipolar interaction given by the relationship (Abragam, 1961)

$$\frac{1}{T_1} = \frac{N\hbar^2\gamma_C^2\gamma_H^2}{10r_{CH}^6}\left[\frac{1}{1 + (\omega_C - \omega_H)^2\tau_R^2} + \frac{3}{1 + \omega_C^2\tau_R^2} + \frac{6}{1 + (\omega_C + \omega_H)^2\tau_R^2}\right]\tau_R$$

where γ is the gyromagnetic ratio, ω the Larmor frequency, r_{CH} the carbon proton distance, N the number of protons attached to carbon, and τ_R the rotational correlation time. For

the depsipeptide valinomycin, $\omega^2\tau_R^2 \ll 1$, in which case the equation simplifies to

$$\frac{1}{T_1} = \frac{N\hbar^2\gamma_C^2\gamma_H^2}{r_{CH}^6}\tau_R$$

The C^α and C^β carbons are each bonded to a proton and they should exhibit shorter relaxation times than the carbonyl carbons as observed experimentally.

The C^α carbons are part of the rigid peptide backbone while the side-chain C^β and methyl carbons are capable of rotation around the C^α—C^β and C^β—CH_3 bonds, respectively. This results in a decrease in the effective value of τ_R and an increase in T_1 on proceeding away from the peptide backbone. Thus, the experimentally observed increase in T_1 in the order $C^\alpha < C^\beta$ (Table II) is consistent with the greatest internal motion at the terminal carbon atoms.

The C^α and C^β carbon spin–lattice relaxation times are longer in the complex as compared to the depsipeptide in methanol. The complex conformation, C-I, is a compact structure held together by six type 1–4 intramolecular hydrogen bonds and its correlation time τ_R should be shorter (resulting in longer T_1) as compared to the τ_R for the flexible conformations III-1 $\rightleftarrows$ III-2 of the depsipeptide in methanol. Using the T_1 values for the C^α carbons (Table II), the following correlation times are measured:

	τ_R (nsec)
valinomycin, methanol, 50°	0.19
valinomycin-K, methanol, 50°	0.15

It is clear that the N- and O-linked carbonyl T_1 values cannot be used to differentiate between carbonyls exposed to solvent (valinomycin in methanol, N- and O-linked C′ carbons), carbonyls forming type 1–4 intramolecular hydrogen bonds (valinomycin-K in methanol, N-linked C′ carbons) and carbonyls bonded to diamagnetic metal ion (valinomycin-K in methanol, O-linked C′ carbons).

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