Qualitative Aspects of Hydrogen–Deuterium Exchange in the ^{1}H, ^{13}C, and ^{15}N Nuclear Magnetic Resonance Spectra of Viomycin in Aqueous Solution†

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ABSTRACT: The ^{1}H, ^{13}C, and ^{15}N high field nuclear magnetic resonance spectra of the cyclic peptide viomycin have been fully assigned using homo- and heteronuclear double resonance experiments and pH effects. In addition it is shown how the two- and three-bond H–D isotope effects upon carbonyl resonances may assist in their assignment. The resistance to exchange with solvent water of the amide proton involved in the transannular hydrogen bond is observed directly in the ^{1}H spectra, via the isotope effect on a carbonyl resonance in the ^{13}C spectra, and via the one-bond ^{1}H coupling in the ^{15}N spectra.

Viomycin (I) is a cyclic peptide antibiotic which has been used in the treatment of tuberculosis. Its mode of action is apparently inhibition of protein synthesis at the stage of amino acid transfer from charged tRNA to active ribosome complexes (Alexander et al., 1969, and references therein).

Controversy over the structure of viomycin was resolved by the X-ray analysis by Bycroft (1972) and of the related compound tuberactinomycin O by Yoshioka et al. (1971). An interesting feature of these crystal structures is the intramolecular hydrogen bond between the proton at N-9 and the carbonyl oxygen at C-21 which results in the β-turn conformation for the H-bonded ring, a turn common to many other cyclic peptides. There are indications (Viglino et al., 1972; Wakamiya & Shiba, 1975) from ^{1}H NMR spectra that this hydrogen bond persists in aqueous solution. Our interest lies in the detection of such intramolecular hydrogen bonding in peptides using ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy, and the pH dependence of these spectra. This study uses viomycin as a model compound.

Materials and Methods

The viomycin used was a gift from Professor Franconi (University of Rome) or a gift from Parke, Davis and Co. No differences were detected in the NMR spectra of the samples from these two sources. The pH of the samples was adjusted using aqueous hydrochloric acid and sodium hydroxide, and the pH values (Metrohim Herisau E520 meter) are uncorrected for the H/D isotopic ratio in the samples.

All NMR spectra were recorded in the pulse-Fourier transform mode; deuterium in the samples was used for the field-frequency stabilization signal. ^{1}H spectra were recorded at 270 and 360 MHz (Bruker WH-270 and WH-360 instruments); samples were 0.1–0.2 M in viomycin contained in 5-mm sample tubes. ^{13}C spectra were recorded at 50.3 and 90.52 MHz (Bruker WP-200 and WH-360 instruments); samples were 0.2 M in viomycin contained in 10-mm sample tubes. ^{15}N spectra were recorded at 36.48 MHz (Bruker WH-360 instrument); samples were 0.3 M in viomycin contained in 10-mm sample tubes. For those ^{1}H spectra with solvent supression, the preirradiation method described by Schaefer (1972) and Campbell et al. (1974) was employed. Chemical shifts are referenced as described in Table 1. The temperature of all samples was ca. 28 °C.

Results

Assignment of ^{1}H, ^{13}C, and ^{15}N Spectra. The method used for the assignment of the proton bearing carbon and nitrogen resonances was specific irradiation of the directly bonded ^{1}H resonances (Hawkes et al., 1977). This method requires a full assignment for the ^{1}H region. Such a full assignment has been attempted by Viglino et al. (1972) and Wakamiya & Shiba (1975) using ^{1}H spectra at 220 and 100 MHz, respectively. There were, however, some differences between the assignments made by these two groups, and in addition Wakamiya & Shiba (1975) differentiated between the proton resonances from the two serine residues using arguments which we feel are not completely sound.

We have confirmed most of the ^{1}H assignments made by Wakamiya & Shiba (1975) by employing homonuclear decoupling experiments (^{1}H NMR at 360 MHz). Nevertheless, we cannot on this basis alone unambiguously differentiate between the two serine groups. In addition, at lower pH we confirm the observation by Viglino et al. (1972) of the amino resonances (H-31, H-35) and the resolution of the signals due to H-7, H-15 at ca. 7 ppm. The amino resonances H-31, H-35 were specifically assigned through the ^{15}N spectra by employment of ^{15}N-(^{1}H) double resonance experiments, whereas the differentiation between the resonances due to H-7, H-15 was made with the aid of pH-dependent spectra (see section).

Since one serine residue, that including C-21–C-23, is believed (Bycroft, 1972; Yoshioka et al., 1971; Viglino et al., 1972; Wakamiya & Shiba, 1975) to participate in an intramolecular hydrogen bond, it was felt desirable to specifically assign the serine resonances. The homonuclear decoupling experiments showed that the protons of the two serine residues give rise to two groups of resonances at 3.85, 4.13 ($-CH_2-$), 4.32 (>CH–), and 9.52 ppm (–NH–), and at 3.92, 3.95 ($-CH_2-$), 4.85 (>CH–), and 8.81 ppm (–NH–) which we call serine groups a and b, respectively. The specific assignment was accomplished as follows. McGahren et al. (1977) have

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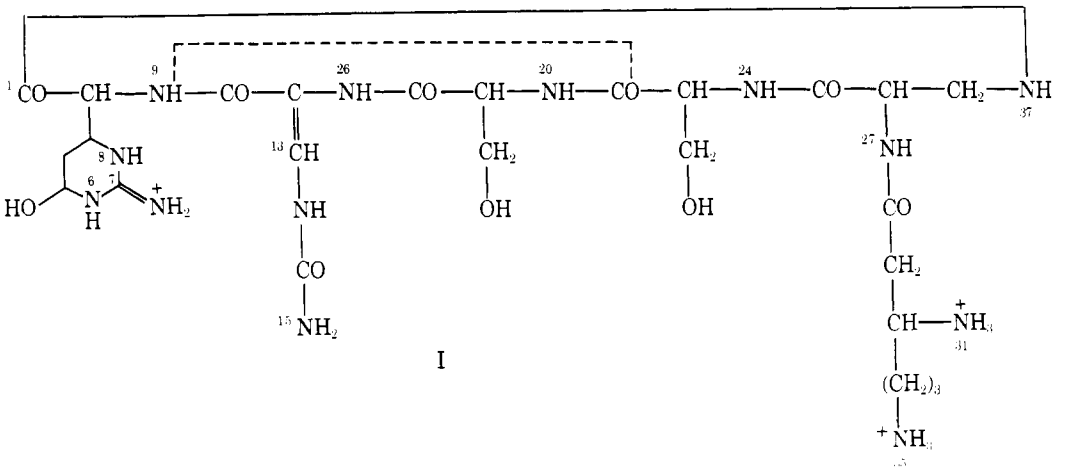

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studied the ^{13}C NMR spectra of a series of tuberactinomycins including viomycin and have been able to specifically assign the methylene ^{13}C resonances for the two serine residues, that due to C23 being the higher frequency (by 3.5 ppm) than the C19 resonance. We have reinvestigated the ^{13}C spectrum (at 50.3 MHz) of viomycin, in particular by employing single frequency ^1H irradiation (200 MHz, γB_2 ca. 500 Hz) to directly correlate the ^{13}C spectrum with the ^1H spectrum by means of the induced variation in the one-bond ^{13}C–^1H coupling. Since the ^1H spectrum had been largely previously assigned, this gave an assignment for the proton bearing carbons which confirmed the assignments of McGahren et al. (1977). With the ^1H irradiation in the region 3.7 to 4.2 ppm, the methylene carbon resonance at 66.0 ppm (C-19) appeared as a doublet of doublets, whereas the signal at 69.3 (C-23) was a triplet. We interpret this to mean that the shift separation for the inequivalent methylene protons at C-19 is greater than for those at C-23, and therefore the group a serine proton resonances are due to the serine residue which includes C-19. This confirms the serine ^1H assignments made by Wakamiya & Shiba (1975). In accord with the work of McGahren et al. (1977), the ^{13}C resonance at 105.8 ppm was assigned to C-11. The remainder of the nonproton-bearing carbon resonances were assigned by specific ^{13}C–(^1H) decoupling experiments in the manner described by Feeney et al. (1974). The required selectivity was obtained by combining maximum dispersion in the proton spectrum (^1H at 360 MHz, ^{13}C at 90.52 MHz) with sufficiently weak ^1H irradiation (35–40 dB below 0.2 W). This high degree of selectivity is required because of the proximity of the proton resonances (particularly in the region 2.5 to 5 ppm) and because the two- and three-bond ^{13}C–^1H couplings being selectively removed are small ($\lesssim$6 Hz). The sample of viomycin used for these latter experiments was in D_2O solution at pH 7.2 in which the exchange N–H → N–D eliminates the complication of long-range coupling between the N protons or deuterons with the carbons under study. In addition these double resonance experiments showed that the resonance at 157.47 ppm was split by H-12 and that at 154.55 ppm by H-5.

The proton and carbon assignments thus obtained are collected in Table I. At this junction the only ambiguities in the ^1H and ^{13}C assignments are between H-7 and H-15 (to be discussed in more detail later) and between C-4 and C-33. This latter ambiguity arises because the proton resonances of H-4 and H-33 were too close to allow selective removal of the one-bond ^{13}C–^1H couplings and because the carbon resonances are also too close to allow differentiation on the basis of ^{13}C shift correlations.

The natural abundance 36.48-MHz ^{15}N spectrum was assigned (with the exception of the $-NH_3$ resonances) by the combination of specific ^{15}N–(^1H) decoupling and off-resonance experiments (γB_2 ca. 1350 Hz) in the manner previously described (Hawkes et al., 1977). These assignments are also collected in Table I. Ambiguities between N-7 and N-15 and between N-13 and N-20 arise because of the proximity of the corresponding ^1H resonances. The amino resonances (N-31 and N-35) were differentiated on the basis of predicted substituent effects on the ^{15}N shifts. Nitrogen-35 has carbon substituents at positions α, β, and γ, whereas N-31 has one α, two β, and two γ carbon substituents. Since the β-carbon substituent effect produces a high-frequency shift of the ^{15}N resonance and the γ effect a smaller low frequency shift (Lichter, 1971; Pregosin et al., 1971; Sogn et al., 1973; Warren & Roberts, 1974; Gattegno et al., 1976), then N-31 is predicted to resonate to higher frequency than N-35. In the selective ^{15}N–(^1H) double resonance experiments described above, irradiation of that broad amino ^1H resonance which appeared at lower frequency resulted in decoupling of the amino ^{15}N resonance which also appeared at lower frequency. Thus these two ^1H signals were assigned.

pH-Dependent ^1H Spectra. The pH dependence (in the pH range 2.6 to 7.5) of the high-frequency region of the 270-MHz ^1H spectrum is shown in Figure 1. The samples were 0.2 M in viomycin in H_2O/D_2O (1:1) and were also used for ^{13}C measurements of the deuterium isotope effects (vide infra). The persistent singlet resonance at 8.03 ppm is due to the grouping =C*H*—ND–, whereas the doublet centered at 8.03 ppm, apparent at low pH, is exchange decoupled (from H-13) at higher pH and is due to =C*H*—NH–. In those spectra determined without elimination of the water signal (see Materials and Methods section for this saturation technique), the onset of exchange between an –NH– proton and the solvent is generally manifested first as a broadening and then as disappearance of the resonance with increasing pH. This effect is greatly amplified in those spectra measured with the water elimination technique, whereby transfer of saturation occurs from the solvent resonance to resonances exchanging with the solvent (Glickson et al., 1974). The resonances at 6.54 and 6.59 ppm are due to H-7 and H-15. The protons causing the lower frequency resonance are exchanging rapidly with the solvent at pH 2.6, but as the pH is increased the rate slows, allowing observation of this resonance, passes through a minimum in the region pH 4.9 to 5.4, then increases again up to pH 7.5. The protons of the resonance at 6.59 ppm are in the slow exchange limit at pH 2.6, and the rate of exchange with the solvent increased with increasing pH up to pH 7.5. The resonance which is the more stable at lower pH is assigned to H-7 of the guanidinium group since Blomberg et al. (1976) have shown that

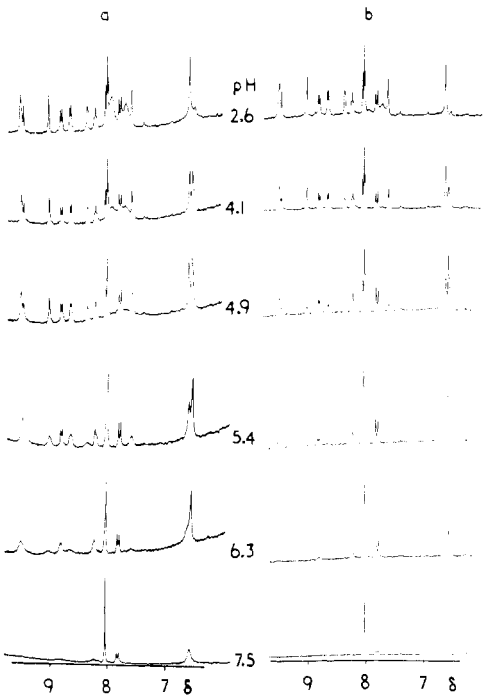


FIGURE 1: High frequency region of the 270-MHz ^1H NMR spectrum of 0.2 M viomycin in 1:1 H_2O/D_2O. (a) Normal spectra (400 scans accumulated in 20 min/spectrum); (b) spectra with solvent supression (64 scans accumulated in 5 min/spectrum).

rapid acid-catalyzed exchange of the protons on the guanidinium group of arginine occurs only at pH <1. The proton resonances of the amino groups are observed only at the lower pHs, whereas the resonances assigned to H-6 (8.34 ppm) and H-8 (7.61 ppm) readily exchange with the solvent as pH is increased.

The remainder of the spectrum due to the amide protons can be separated into three groups of resonances based on qualitative estimates of the rates of exchange with the solvent. As the pH is increased, the most readily exchangeable are the protons H-13 (9.48), H-16 (9.04), and H-27 (8.66 ppm), whereas the second group, which requires a somewhat higher pH for rapid exchange, includes H-20 (9.52), H-24 (8.81), and H-37 (8.21 ppm), and within this second group the triplet due to H-37 appears the most resistant to exchange (see spectrum at pH 6.3 in Figure 1). The third group is just the resonance due to H-9 (7.79 ppm), is highly resistant to exchange with the solvent, and is not completely eliminated from the spectrum (with water saturation) at pH 7.5. It is significant that this N-9-H bond is exactly the one involved in the intramolecular hydrogen bond, as evidenced by the observation of Wakamiya & Shiba (1975) of a low chemical-shift temperature coefficient for this proton resonance.

pH-Dependent ^{13}C Spectra. The pH dependence of the ^{13}C chemical shifts of viomycin (0.2 M in 90% H_2O–10% D_2O) was measured in the region pH 3.3 to 9.1. Between pH 8.2 and 9.1, additional ^{13}C resonances were observed which mostly disappeared upon lowering the pH to 7.3. This indicates that at the higher pHs some fundamental conformational or structural change occurs in viomycin which is not completely reversible. This behavior is the subject of continuing studies and is also the reason why higher pHs than 9.1 were not investigated. The pK_as of viomycin have been measured (Dyer et al., 1965) as 8.2, 10.3, and 12 (due to the two amino and the guanidino groups, respectively), and thus the variation of the pH up to 9.1 reflects partial deprotonation of the ammonium groups. Six resonances shifted significantly (>0.1 ppm relative to the dioxane reference) and these are shown in Table II. Only one carbonyl group (C-28) is in the immediate vicinity of an amino group and thus the resonance at 172.43 ppm is assigned to C-28, corroborating our earlier assignment based on double resonance experiments. The resonance at 30.16 ppm was not significantly shifted with variation in pH, whereas the

TABLE I: ^1H, ^{13}C, and ^{15}N Chemical Shifts[a] for Viomycin.

position	δ ^1H	δ ^{13}C	δ ^{15}N
1		171.63	
2	5.01	55.08	
3	4.62	46.89	
4	1.60, 2.05	30.16	
5	5.58	71.05	
6	8.34		77.9
7	6.59	154.55	51.4
8	7.61		63.0
9	7.79		85.3
10		168.06	
11		105.87	
12	8.03	135.85	
13	9.48		95.2
14		157.47	
15	6.54		62.7
16	9.04		88.4
17		173.06	
18	4.32	57.02	
19	3.85, 4.13	60.03	
20	9.52		96.2
21		173.58	
22	4.85	55.37	
23	3.92, 3.95	63.30	
24	8.81		102.1
25		171.98	
26	4.68	52.90	
27	8.66		102.0
28		172.43	
29	2.71, 2.80	37.31	
30	3.70	49.27	
31	7.95		22.1
32	1.81	23.72	
33	1.81	29.81	
34	3.06	39.98	
35	7.71		12.3
36	3.09, 3.90	41.37	
37	8.21		92.1

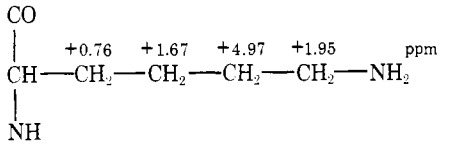
[a] All chemical shifts are to high frequency of the reference. ^1H chemical shifts (measured at 360 MHz on a sample 0.1 M viomycin in 90% H_2O + 10% D_2O at pH 3.5) referenced to 3-(trimethylsilyl)-propionic-*2,2,3,3-d*$_4$ acid, sodium salt; ^{13}C chemical shifts (measured at 90.52 MHz on a sample 0.2 M viomycin in 90% H_2O + 10% D_2O at pH 3.3) referenced to tetramethylsilane; the secondary reference used was internal dioxane taken to be 67.4 ppm from tetramethylsilane; ^{15}N chemical shifts (measured at 36.48 MHz on a sample 0.3 M viomycin in 90% H_2O + 10% D_2O at pH 2.8) referenced to the $^{15}NH_4^+$ resonance from 5 M $^{15}NH_4{}^{15}NO_3$ in 2 N HNO_3.

TABLE II: pH-Dependent ^{13}C Chemical Shifts[a] for Viomycin.

carbon	δ pH 3.3	δ pH 9.1	Δδ
28	172.43	174.18	1.75
29	37.31	40.37	3.06
30	49.27	48.95	−0.32
32	23.72	24.41	0.69
33	29.81	32.51	2.70
34	39.98	40.37	0.39

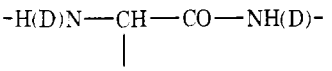
[a] See footnote to Table I.

29.81-ppm signal was strongly affected. This difference removes the ambiguity in the assignment of these two resonances—the C-4 resonance is at 30.16 ppm and C-33 is at 29.81 ppm since it is the one affected by deprotonation at N-35. It is apparent that the shift variations shown in Table II are greater for carbons β to the amino groups (C-29, C-32, C-33) than for carbons α (C-30, C-34). This is in accord with the findings of Saitô & Smith (1973) on the ^{13}C chemical shift changes in poly(lysine) between pD 7.4 and 11.4



where the + sign indicates the ^{13}C resonance is to higher frequency at the higher pD.

Because the amide protons of viomycin exhibit a reasonable range of exchange rates with the solvent, a new method for the assignment of ^{13}C carbonyl resonances becomes feasible. Feeney et al. (1974) have demonstrated the existence of an isotope shift upon carbonyl resonances in small peptides dissolved in 1:1 mixture of H_2O and D_2O, the ^{13}C resonance due to the species -CO—ND- being of lower frequency than CO—NH- by ca. 0.1 ppm. Thus, for slow exchange of the amide protons, the carbonyl resonance appears as a doublet. The pH-dependent high frequency portion of the ^{13}C spectrum of viomycin in a 1:1 mixture of H_2O and D_2O is shown in Figure 2. At low pH the resonances display not only the two-bond isotope effect but also a smaller three-bond effect:



If we label the resonances a to f from low frequency to high frequency and consider first the lowest frequency of the resonances shown (a), there is a two-bond effect of 0.06_7 ppm but no resolution of a three-bond effect. This doublet splitting is the most persistent as the pH is increased, and therefore this resonance is assigned to C-10. Of the resonances b to f, d is the only one not to display the three-bond effect (the two-bond effect is 0.08_1 ppm) and C-28 is the only peptide carbonyl without the appropriate -NH(D)- grouping. Thus resonance d arises from C-28—an assignment that agrees with the results of the ^{13}C-(^1H) double resonance experiments. Of the remaining resonances (b, c, e, f), the two which most readily show collapse of the two-bond effect with increasing pH are d and e, a fact which correlates with our previous observation that the three amide protons, H-13, H-16, and H-27, most readily exchange with the solvent. Since ^{13}C resonance d has already been assigned to C-28 and the resonance due to C-14 appears in a different spectral region, then resonance e must be due to C-17. The remaining three resonances show collapse of the two-bond isotope effect with increasing pH in the order f > c > b which correlates with the observation from the ^1H spectra that the rates of exchange of the three remaining amide protons were H-20 ≈ H-24 > H-37. Therefore ^{13}C resonance b is assigned to C-1, and resonances c and f are due to C-21 and C-25 (although no firm distinction can be made between these two). Confirmation that resonance b is due to C-1 can be found in the persistence of the three-bond effect up to pH 4.9 since the three-bond effect at C-1 is due to the stable -N9—H(D)- group. Thus, firm assignments have been made for four of the six peptide carbonyl ^{13}C resonances which agree with the assignments from the ^{13}C-(^1H) double resonance experiments.

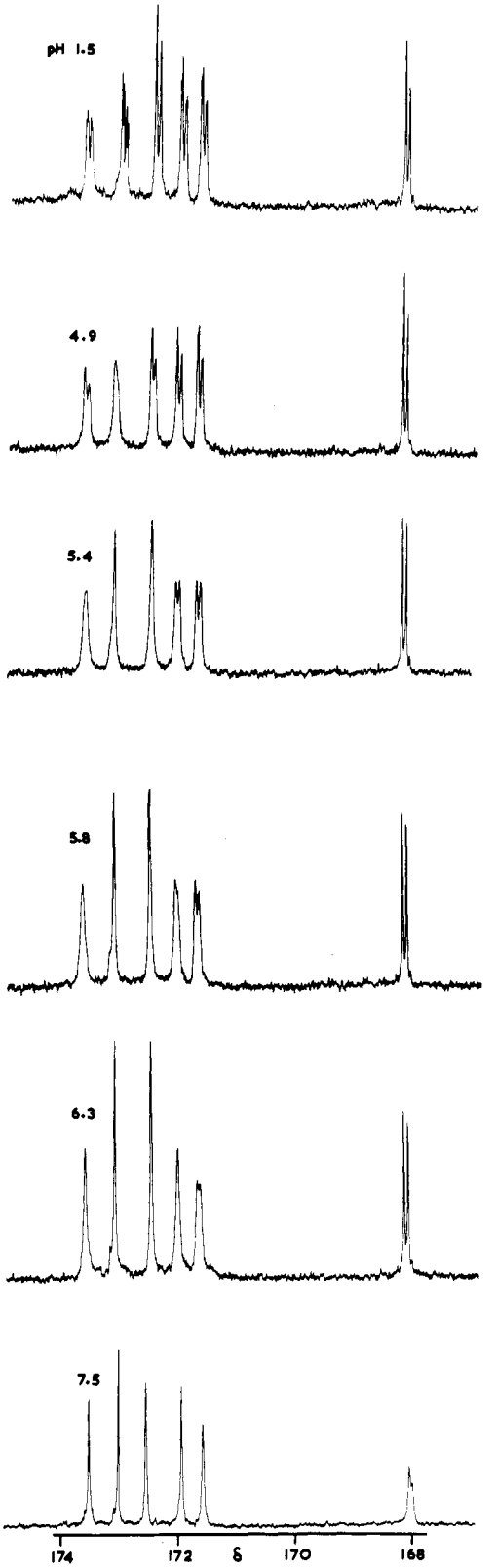


FIGURE 2: High frequency region of the ^1H broad-band decoupled ^{13}C NMR spectrum of 0.2 M viomycin in 1:1 H_2O/D_2O (4000 scans accumulated in 4.5 h/spectrum).

The full ^{13}C spectrum of viomycin in 1:1 H_2O/D_2O at pH 1.45 showed two- and three-bond isotope effects on other resonances. These are collected in Table III. A similar spectrum from viomycin in 2:1 H_2O/D_2O confirmed (Feeney et al., 1974) that the two-bond effect causes the resonance from the species -ND—*C* to be at lower frequency than that from

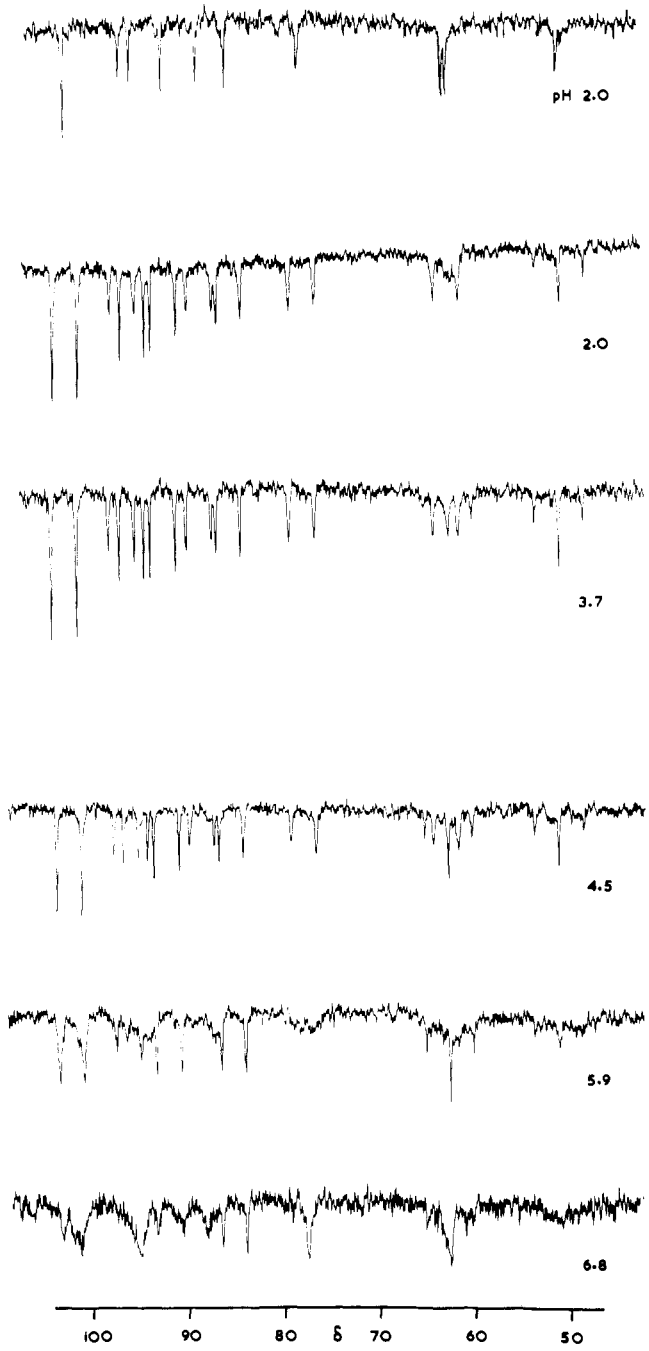


FIGURE 3: High frequency region of the 36.48-MHz natural abundance ^{15}N NMR spectrum of 0.3 M viomycin in 90% H_2O + 10% D_2O. The upper trace is the ^1H broad-band decoupled ^{15}N spectrum (5000 scans accumulated in 4.5 h); for the other spectra, the ^1H decoupler was gated on for 1.6 s between scans to retain both the ^{15}N-(^1H) coupling and the ^{15}N-(^1H) nuclear Overhauser enhancement (15000 scans accumulated in 13.5 h/spectrum).

TABLE III: Deuterium Isotope Effects[a] on the ^{13}C Chemical Shifts of Viomycin.

carbon	2 bond	3 bond
1	-0.07_6	0.01_9
2	-0.08_1	
3	-0.10_2	
5	-0.10_0	
10	-0.06_7	
11	-0.14_3	
12	-0.13_5	0.08_1
14	-0.08_6	
17	-0.07_4	0.02_8
18	-0.10_0	
21	-0.06_8	0.01_9
22	-0.07_1	
25	-0.07_9	0.01_9
26	-0.08_3	0.02_7
28	-0.08_1	
34	-0.08_9	
36	-0.09_5	

[a] Values in ppm; a positive or negative value indicates that the ^{13}C resonance from the deuterated species is to higher or lower frequency, respectively, than the protonated species. Digital resolution in the spectra was ca. 0.003 ppm/point.

-NH—*C*. Because the three-bond effects were so small, it was not possible to conclusively decide whether the species -ND—C—*C* or -NH—C—*C* gave the lower frequency ^{13}C resonance, except in the case of C-12 where the sense of the two- and three-bond effects was different, i.e., the ^{13}C resonance from the deuterated species -*C*H=C—ND- was of higher frequency than the protonated species. Interestingly, the C-34 resonance showed splitting in the 1:1 H_2O/D_2O sample at pH 1.5 (the neighboring amino group exists as $-N^+H_3$, $-N^+H_2D$, $-N^+ND_2$, $-N^+D_3$), whereas the C-30 resonance did not. However, the splitting was removed when the pH was increased to 2.6. The resonance due to C-7 was broadened, but without resolution of the two-bond isotope effect presumably because of extensive overlap of the resonances due to the ten types of variously deuterated guanidinium groups. The C-14 resonance was a well-resolved doublet at pH 1.5 (isotopic substitution at H-13), because the two-bond effect from H-15 was removed by rapid exchange with the solvent at this pH.

pH-Dependent ^{15}N Spectra. The high frequency region of the natural abundance 36.48-MHz ^{15}N spectrum of viomycin is shown in Figure 3, as a function of pH. The resonances are proton coupled and appear inverted since the ^1H broad-band decoupler was gated to yield coupled spectra while retaining the favorable negative nuclear Overhauser effect. Let us first consider the two triplet resonances at 51.4 and 62.7 ppm for which definite assignments could not be made from the ^{15}N-(^1H) double resonance experiments. At pH 2.0 the lower frequency of these two resonances is a well-defined triplet; as the pH is increased the components of the triplet first broaden (pH 5.9) and then disappear (pH 6.8). This indicates that the exchange rate of the protons directly bonded to the nitrogen observed increases with pH. On the other hand, the triplet at 62.7 ppm is broadened at pH 2.0, sharpens in the region pH 4.5 to 5.9, and begins to broaden again by pH 6.8. This behavior indicates that the exchange rate of the directly bonded protons passes through a minimum. These qualitative conclusions about the proton exchange rates exactly match our previous qualitative conclusions from the pH dependence of the ^1H resonances at 6.54 and 6.59 ppm. Therefore we assign the lower frequency of these two ^{15}N triplets to the guanidinium nitrogen N-7. The ^{15}N-(^1H) double resonance experiments do not allow a distinction in assignment for N-13 and N-20 between the two resonances at 95.2 and 96.2 ppm. It was established from the pH-dependent ^1H spectra that H-13 undergoes exchange with the solvent more readily than does H-20. If the ^{15}N spectra at pH 2.0 and 5.9 are compared, it can be readily seen that the ^{15}N doublet centered at 96.2 ppm persists, whereas that centered at 95.2 ppm is significantly broadened. This indicates that the directly bonded proton is exchanging more rapidly on the nitrogen whose resonance is at 95.2 ppm, and it is assigned to N-13. Finally, at pH 6.8 only one amide nitrogen doublet is still well defined, indicating a

relatively slow exchange rate for the directly bonded proton. This doublet, centered at 85.3 ppm, is the resonance assigned by the ^{15}N-(^1H) double resonance experiments to N-9, the nitrogen involved in the intramolecular hydrogen bond.

A possible complication in the interpretation of the ^{15}N spectra would be the complexation of viomycin with any paramagnetic impurities present in the sample. This would be expected to have more effect at higher pHs when broadening of the resonances by a paramagmetic could obscure the effects of chemical exchange. However, there is no evidence to suggest that broadening effects in these pH-dependent ^{15}N spectra are the result of paramagnetic impurities since there were no anomalous effects in the ^1H, ^{13}C, or ^{15}N spectra at the pHs investigated. In addition Irving & Lapidot (1975) have studied the pH-dependent effect of paramagnetic impurities upon the ^{15}N spectrum of glycylglycine and did not observe any effect upon the peptide nitrogen resonance due to the paramagnetic in the pH region 0 to 10.

Discussion

Conformational Considerations. The relative stabilities of the peptide N-H bonds may be summarized as $9 \gg 37 > (20, 24) > (27, 16)$. Inspection of a space-filling model, constructed according to the conformation given by Bycroft (1972), shows that the bond N-37-H is directed toward the center of the ring and is thus shielded from the solvent, while N-27-H is particularly exposed to solvent interactions. This readily explains our qualitative observations from the pH-dependent ^1H spectra. The model also shows that the serine N-20-H is directed away from the ring, whereas the N-H bonds 16 and 24 are more or less perpendicular to the ring plane. However, we feel that a rationalization of the stabilities of these latter three bonds from arguments based upon solvent accessibility is not justified.

Wakamiya & Shiba (1975) have concluded that most dihedral angles in the backbone, calculated from α—C*H*—N*H*- three-bond couplings are in substantial agreement with the X-ray data for tuberactinomycin O. Our limited data on the relative stabilities of the peptide N-H bonds add support to the conclusion that the backbone conformation of the related viomycin is also similar in both the crystal and solution.

Conclusion

Evidence that the intramolecular hydrogen bond in viomycin, present in the crystal, persists in solution has been obtained from three sets of NMR spectra: (a) in ^1H spectra, the resistance of this hydrogen bonded proton to exchange with solvent water as the pH is increased; (b) in ^{13}C-(^1H) spectra, the resistance to collapse of the two-bond isotope shift (-NH—*C*O- $\rightleftharpoons$ -ND—*C*O-) observed on carbonyl resonances as the pH is increased; (c) in ^{15}N spectra, the resistance to collapse of the one-bond ^{15}N-^1H coupling as the pH is increased.

The two-bond isotope effect upon carbonyl ^{13}C resonances suggests another application to peptide chemistry. If it is possible to assign the carbonyl resonance to a specific amino acid residue, e.g., by ^{13}C-(^1H) double resonance (Feeney et al., 1974) or by isotopic substitution (Sogn et al., 1974) and if there exists quantitative information on the peptide proton exchange rates (Campbell et al., 1977), then the pH dependence of the two-bond effect can provide sequence information. This is because the two-bond effect is an interaction between the NH(D) group and a carbonyl group on different adjacent amino acid residues.

It has been demonstrated that the three-bond isotope effect -*C*O—CH—NH(D)- can assist in the assignment of peptide carbonyl resonances. In this study the absence of a three-bond effect on the ^{13}C resonances at 168.06 and 172.43 ppm suggests that one of these two resonances must be due to C-28.

In their investigation of H-D exchange of amide protons in polymixin B, Perkins et al. (1978) have concluded that slow exchange is not alone indicative of intramolecular hydrogen bonding. Instead they adopt the criterion of a small temperature coefficient on the ^1H chemical shift. As Perkins et al. (1978) indicate, the factors to consider in interpretation of exchange data of an amide proton in a peptide include inductive effects arising from the nature of the amino acid itself and its nearest neighbors, solvent accessibility which will largely be determined by the conformation the peptide adopts in solution, and intramolecular hydrogen bonding. In cyclic peptides with a ring size similar to that of viomycin, it is reasonable that the formation of a strong transannular hydrogen bond precludes that N-H bond involved being accessible to the solvent; therefore, these two effects reinforce each other and together can be the primary cause of slow proton exchange, as has been proposed (Stern et al., 1968) for the intramolecular hydrogen bonding in gramicidin S.

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Structure of Iturine A, a Peptidolipid Antibiotic from *Bacillus subtilis*†

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ABSTRACT: A mixture of iturines extracted from *Bacillus subtilis* gave, on column chromatography, iturine A, iturine B, and iturine C. Iturine A has the entire antifungal activity. It is a mixture of two homologous peptidolipids $C_{48}H_{74}N_{12}O_{14}$ and $C_{49}H_{76}N_{12}O_{14}$ (mp 177 °C, $[\alpha]_D$ −1.7° in methanol (*c* 0.05 g/mL); mol wt 1042 and 1056). The lipid moiety is a mixture of 3-amino-12-methyltridecanoic acid and 3-amino-12-methyltetradecanoic acid. The peptide moiety contains 7 mol of amino acids: D-Asn_2, L-Asn, L-Gln, L-Pro, L-Ser, and D-Tyr. A cyclic structure for iturine A with the serine residue linked to the fatty amino acids through a peptide bond has been demonstrated. By mild HCl hydrolysis, lipid-soluble and water-soluble peptides were obtained. They were analyzed by chemical methods and by mass spectrometry. Permethylated and perdeuteriomethylated derivatives of iturine A were also subjected to mass spectrometric analysis. Both chemical analysis and mass spectrometry led to the cyclic structure I for iturine A.

A polypeptide antibiotic named iturine was isolated from a culture medium of a strain of *Bacillus subtilis* collected in Ituri, Belgian Congo (presently Zaire). It has a high antifungal activity against various strains of yeasts and fungi (Delcambe & Devignat, 1957). The purification and characteristics of this antibiotic were previously described (Delcambe, 1965). A reinvestigation of iturine revealed it to be a mixture of iturine A, iturine B, and iturine C which were separated by column chromatography. Iturine A is the major constituent (30% of the crude iturine) having the entire antifungal activity; iturine B (5%) and iturine C (10%) have no antifungal activity. The lipid part of iturine A was shown to be a mixture of two long-chain β-amino acids (Peypoux et al., 1973). The present paper reports the complete structural determination of iturine A.

Materials and Methods

Solvent systems used for chromatography are: (A) chloroform–methanol–acetic acid (92:5:3); (B) chloroform–methanol–pyridine–water (40:14.5:14:2.5); (C) phenol–10% sodium citrate (1:1); (D) butanol–acetic acid–water (65:10:25); (E) chloroform–methanol–water (65:25:4); (F) pyridine–*tert*-amyl alcohol–water (35:35:30); (G) butanol–acetic acid–water (5:1:2); (H) benzene–pyridine–acetic acid (20:5:1); (I) benzene–pyridine–formic acid–acetic acid (75:25:1:0.2); (J) propanol–34% ammonia (7:3).

Amino Acid Analysis. Total hydrolyses of iturine A and lipopeptides with 6 N HCl were carried out at 150 °C for 8 h and similar hydrolyses of water-soluble peptides were performed at 105 °C for 18 h. The quantitative analysis of amino acids (ca. 150–200 nmol) was performed on a Technicon amino acid analyzer following the procedure of Spackman et al. (1958) modified by Piez & Morris (1960). A different procedure described by Ghuysen et al. (1966) was also used with smaller quantities of amino acids (ca. 50 nmol): the amino acids were dinitrophenylated, and the DNP derivatives were separated by thin-layer chromatography in solvents A or B, collected from the silica gel, and quantitatively estimated by spectrophotometry at 360 nm.

Configuration of amino acids was determined enzymatically on 25 nmol of each amino acid. Pro, Ser, and Tyr were incubated for 2 h at 37 °C with D-amino acid oxidase (Worthington; 170 μg) in a final volume of 50 μL of 0.1 M $K_4P_2O_7$ (pH 8.3) (Ishii & Witkop, 1963). Glu was incubated for 2 h at 37 °C in the presence of L-glutamate decarboxylase (Sigma) (10 μg) in a final volume of 40 μL of 0.2 M $NaCOOCH_3$ (pH 4.5; Gale, 1965). After enzymatic digestion, DNP derivatives of residual amino acids were estimated by spectrophotometry. The optical configuration of Asp was determined with L-glutamate–oxalacetate transaminase (Sigma; 20 μg) by incubation in a sodium arsenate buffer, 0.05 M, pH 7.6 (35 μL) with α-ketoglutarate (50 nmol). DNP derivatives of residual Asp and released Glu were estimated by spectrophotometry.

Identification of Asparaginyl and Glutaminyl Residues. The method of Ressler & Kashelikar (1966) was used. A solution of iturine A (6 mg) in triethyl phosphite (0.5 mL) was added to ethylene chlorophosphite (0.2 mL) at 100 °C for 18 h. After elimination of the excess reagent and solvent, the

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