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Conformations of Synthetic Alamethicin Fragments. Evidence for 3_{10} Helical Folding from 270-MHz Hydrogen-1 Nuclear Magnetic Resonance and Circular Dichroism Studies†

R. Nagaraj and P. Balaram*

ABSTRACT: ^{1}H NMR studies at 270 MHz on the synthetic alamethicin fragments Z-Aib-Pro-Aib-Ala-Aib-Ala-OMe (1–6), Boc-Gln-Aib-Val-Aib-Gly-Leu-Aib-OMe (7–13), Boc-Leu-Aib-Pro-Val-Aib-OMe (12–16), and Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe (11–16) have been carried out in $CDCl_3$ and $(CD_3)_2SO$. The intramolecularly hydrogen bonded amide hydrogens in these peptides have been delineated by using solvent titration experiments and temperature coefficients of NH chemical shifts in $(CD_3)_2SO$. All the peptides adopt highly folded structures, characterized by intramolecular 4 → 1 hydrogen bonds. The 1–6 fragment adopts a 3_{10} helical conformation with four hydrogen bonds, in agreement with earlier studies (Rao, Ch. P., Nagaraj, R., Rao, C. N. R., & Balaram, P. (1980) *Biochemistry 19*, 425–431]. The 7–13 fragment also appears to be folded in 3_{10} helical fashion, although some intramolecular hydrogen bonds are loosened in $(CD_3)_2SO$. The 11–16 fragment favors a structure with three intramolecular hydrogen bonds of the 4 → 1 and 5 → 1 types. CD studies in trifluoroethanol suggest a helical structure for the 1–13 and 1–17 fragments and alamethicin, while IR studies support a helical structure for the 1–13 peptide, stabilized by intramolecular hydrogen bonding. On the basis of fragment conformations and earlier studies of the stereochemistry of α-aminoisobutyric acid (Aib) containing peptides, a structure is suggested for the alamethicin backbone. A largely 3_{10} helical folding pattern is postulated for the hydrophobic 1–17 segment, with a polar flexible C-terminal tripeptide.

Cation transport across membranes may be mediated by a carrier mechanism involving lipophilic organic ligands capable of chelating metal ions or by formation of transmembrane channels or pores (Ovchinnikov et al., 1974; Pressman, 1976). While considerable effort has been expended on structural studies of carrier ionophores and their metal complexes, relatively little is known about the structure of membrane channels. The polypeptides gramicidin A (Sarges & Witkop, 1964) and alamethicin (Pandey et al., 1977a) are substances that affect the ionic permeabilities of membranes by formation of transmembrane structures (Urry, 1977). Of these, alamethicin also has the ability to induce excitability phenomena in artifical membranes (Mueller & Rudin, 1968). Structural models like the novel π_{LD} (Urry, 1971; Urry et al., 1971) and

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Ac - Aib - Pro - Aib - Ala - Aib - Ala - Gln - Aib - Val - Aib - Gly - Leu - Aib - Pro -
Val - Aib - Aib - Glu - Gln - Phol.

FIGURE 1: Sequence of alamethicin I proposed by Pandey et al. (1977a).

coaxial double helices (Veatch et al., 1974) have been proposed for gramicidin A, which has a large sequence of alternating L and D residues. Both of these models have internal diameters sufficient to accommodate the passage of cations through the interior of the helix. Alamethicin (Figure 1) contains a high proportion of α-aminoisobutyric acid (Aib)[1] a hindered residue that introduces considerable stereochemical constraints. Studies on model peptides have shown that the Aib residue almost exclusively adopts conformations in the right- or left-handed 3_{10} and α-helical regions of conformational space, with $\phi = -60 \pm 20°$ or $+60 \pm 20°$ and $\psi = -30 \pm 20°$ or $+30 \pm 20°$ (Shamala et al., 1977, 1978; Nagaraj et al., 1979; Prasad et al., 1979, 1980; Rao et al., 1979, 1980). The structural models proposed for the gramicidin A transmembrane channel are incompatible with the conformational preferences of Aib residues. Recently, a number of Aib-containing microbial peptides exhibiting alamethicin-like membrane activity have been isolated and sequenced. These include suzukacillin (Jung et al., 1976), trichotoxin A-40 (Bruckner et al., 1979), emerimicins III and IV (Pandey et al., 1977b), antiamoebins (Pandey et al., 1977c), and hypelcins A and B (Fujita et al., 1979). It appears that Aib-containing peptides function by a mechanism distinct from that of gramicidin A. As part of a program to establish the conformations of the Aib-containing membrane-active peptides, we describe in this report 270-MHz ^{1}H NMR and CD studies of synthetic alamethicin fragments. Earlier reports from this laboratory have summarized X-ray studies of amino-terminal peptides (Nagaraj et al., 1979; Prasad et al., 1979) and IR studies of amino-terminal and central fragments of alamethicin (Rao et al., 1979, 1980).

Materials and Methods

The peptides Z-Aib-Pro-Aib-Ala-Aib-Ala-OMe (**1**), Boc-Gln-Aib-Val-Aib-Gly-Leu-Aib-OMe (**2**), Boc-Leu-Aib-Pro-Val-Aib-OMe (**3**), Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe (**4**), the 1–13 and 1–17 alamethicin fragments, and alamethicin I were synthesized by solution-phase methods. Couplings were mediated by dicyclohexylcarbodiimide (DCC) or DCC/1-hydroxybenzotriazole. Acetyl-Aib-Pro-Aib-Ala-Aib-Ala-OMe was synthesized from **1** by hydrogenation followed by acetylation with acetyl chloride and triethylamine (Nagaraj, 1980). All peptides were homogeneous by TLC on silica gel and were characterized by ^{1}H and ^{13}C NMR, elemental analysis, or amino acid analysis. The detailed synthetic procedures will be reported elsewhere (Nagaraj & Balaram, 1981).

^{1}H NMR spectra were recorded on a Bruker WH-270 FT NMR spectrometer at the Bangalore NMR Facility. The ^{2}H resonances of $CDCl_3$ and $(CD_3)_2SO$ were used for internal field frequency locking. Spectra were recorded at concentrations of ~10 mg/mL by using a sweep width of 3012 Hz, with 8K data points, yielding a digital resolution of 0.367 Hz/point. Solvent titration experiments were carried out by adding a peptide solution in $(CD_3)_2SO$ to a peptide solution in $CDCl_3$. Peptide concentrations (10 mg/mL) were maintained throughout. Variable temperature measurements were made over the range 20–60 °C. The probe temperature was regulated with a B-ST 100/700 temperature controller. CD spectra were recorded on a Jasco J-20 spectropolarimeter in 1-mm cells. Peptide concentrations were 0.2–0.5 mM in trifluoroethanol. Spectra were recorded over the range 200–240 nm. Molar ellipticities ($[\theta]_M$) were calculated by using the equation $[\theta]_M = (\theta \times 100)/(lM)$ deg cm^2 dmol^{-1} where θ = observed reading in degrees, l = path length in centimeters, and M = molar concentration of the peptide.

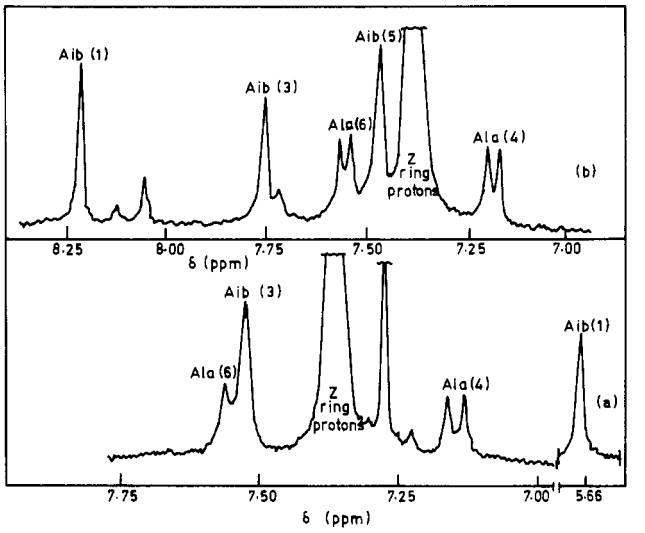

FIGURE 2: Low-field region of the 270-MHz ^{1}H NMR spectrum of Z-Aib-Pro-Aib-Ala-Aib-Ala-OMe (**1**). (a) $CDCl_3$; (b) $(CD_3)_2SO$.

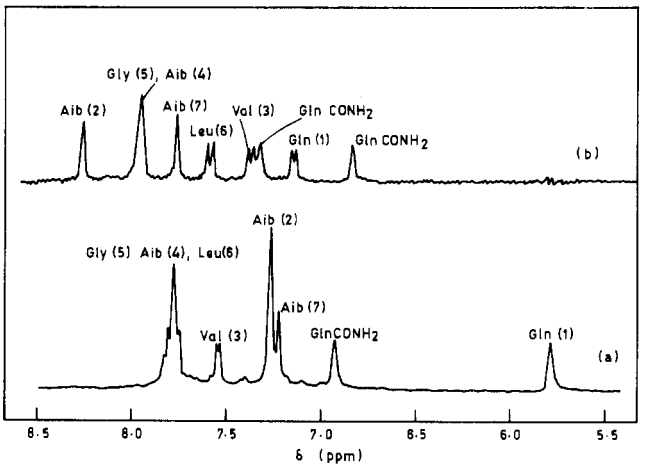

FIGURE 3: Low-field region of the 270-MHz ^{1}H NMR spectrum of Boc-Gln-Aib-Val-Aib-Gly-Leu-Aib-OMe (**2**). (a) $CDCl_3$; (b) $(CD_3)_2SO$.

Results

Assignment of Amide NH Resonances. The NH regions of the 270-MHz ^{1}H NMR spectrum of the alamethicin I amino-terminal (1–6) peptide, Z-Aib-Pro-Aib-Ala-Aib-Ala-OMe (**1**), and the central (7–13) fragment, Boc-Gln-Aib-Val-Aib-Gly-Leu-Aib-OMe (**2**), are shown in Figures 2 and 3. In **1**, the two Ala NH protons appear as doublets at δ 7.14 and 7.53 in $CDCl_3$ and at δ 7.18 and 7.55 in $(CD_3)_2SO$. It is not possible, at present, to distinguish between the Ala(4) and Ala(6) NH resonances. The high-field singlet at δ 5.66 is assigned to the Aib(1) NH since urethane protons invariably

[1] Abbreviations used: Aib, α-aminoisobutyric acid; Boc, *tert*-butyloxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; TFE, trifluoroethanol; OMe, methyl ester; Z, benzyloxycarbonyl; CD, circular dichroism.

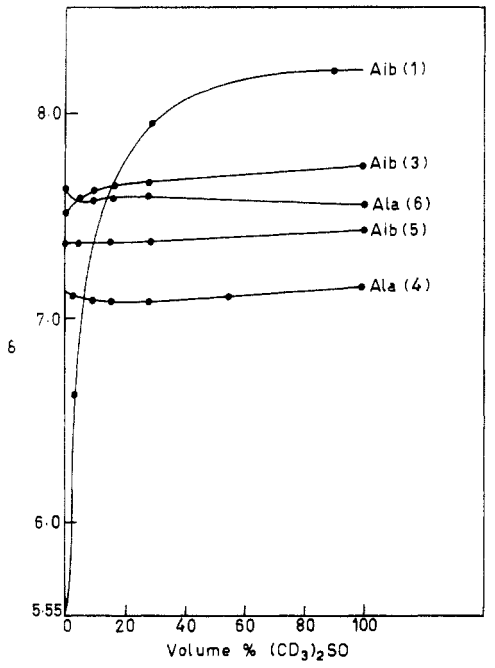

FIGURE 4: Chemical shifts of the NH proton resonances of **1** in $CDCl_3$–$(CD_3)_2SO$ mixtures.

appear at higher field than amide hydrogens in $CDCl_3$ (Bystrov et al., 1965; Pysh & Toniolo, 1977; Nagaraj et al., 1979). When the resonances in $CDCl_3$–$(CD_3)_2SO$ mixtures were monitored, the δ 8.21 singlet in $(CD_3)_2SO$ was assigned to Aib(1) NH. The other two singlets then arise from the Aib(3) and Aib(5) NH groups. An a priori distinction between these groups is again not possible, but tentative assignments have been made in Table I. These are based on comparisons with smaller synthetic fragments. In the heptapeptide (**2**), the triplet amide resonance could be unambiguously assigned to the Gly NH while the Gln, Val, and Leu NH groups were assigned by appropriate spin-decoupling experiments (Wyssbrod & Gibbons, 1973). The Aib NH groups are tentatively assigned by using chemical shifts in smaller fragments and on the basis of solvent exposure studies (described below) which suggest intramolecular hydrogen bonding. Table I summarizes the NMR parameters for the NH groups in **1**, **2**, and the alamethicin fragments, Boc-Leu-Aib-Pro-Val-Aib-OMe (**3**) (12–16) and Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe (**4**) (11–16). The assignments in **3** and **4** were made as described above.

Solvent and Temperature Dependence of NH Chemical Shifts. Amide hydrogens that are involved in intramolecular hydrogen bonds are generally characterized by small changes in chemical shifts on going from a weakly hydrogen bond accepting solvent like $CDCl_3$ to a strong acceptor like $(CD_3)_2SO$ (Pitner & Urry, 1972; Nagaraj et al., 1979). Further, such hydrogens also exhibit low temperature dependence of chemical shifts in solvents like $(CD_3)_2SO$ (Kopple et al., 1969). Figure 4 shows the chemical shifts of the NH protons of **1** as a function of the composition in $CDCl_3$–$(CD_3)_2SO$ mixtures. In **1**, only the Aib(1) NH shows a large change (2.55 ppm) in chemical shift on going from $CDCl_3$ to $(CD_3)_2SO$. The Aib(3), Ala(4), Aib(5), and Ala(6) NH groups are largely unaffected. An examination of the temperature coefficients of chemical shifts ($d\delta/dT$) in $(CD_3)_2SO$ shows that only Aib(1) NH has a large value of 5.5×10^{-3} ppm/°C. The other NH protons show much lower values, ranging from 1.24×10^{-3} ppm/°C to 3.4×10^{-3} ppm/°C.

In the central heptapeptide (**2**), the Gln(1) and one of the Aib NH groups [tentatively assigned to Aib(2)] show large solvent dependences of chemical shifts in $CDCl_3$–$(CD_3)_2SO$ mixtures (Figure 5). One of the other Aib NH groups [tentatively assigned to Aib(7)] does show a steep dependence above 20% $(CD_3)_2SO$, suggesting that a solvent-dependent conformational transition may occur at higher $(CD_3)_2SO$ concentrations, leading to exposure of this group. This NH group may therefore be involved in a relatively weak hydrogen bond in $CDCl_3$, in contrast to Val(3), Gly(5), Leu(6), and one Aib NH [assigned to Aib(4)] which appear to be shielded from the solvent. The $d\delta/dT$ values in $(CD_3)_2SO$ are low for the Val(3) and Leu(6) NH groups ($\sim 2.5 \times 10^{-3}$ ppm/°C), somewhat larger for two Aib NH groups, assigned to Aib(4) and Aib(7) ($\sim 3.7 \times 10^{-3}$ ppm/°C), and large for Gln(1) and Aib(2) NH groups ($\sim 5.68 \times 10^{-3}$ ppm/°C). The Gly(5) NH, however, has a rather high $d\delta/dT$ in $(CD_3)_2SO$, despite exhibiting very little solvent dependence of chemical shift.

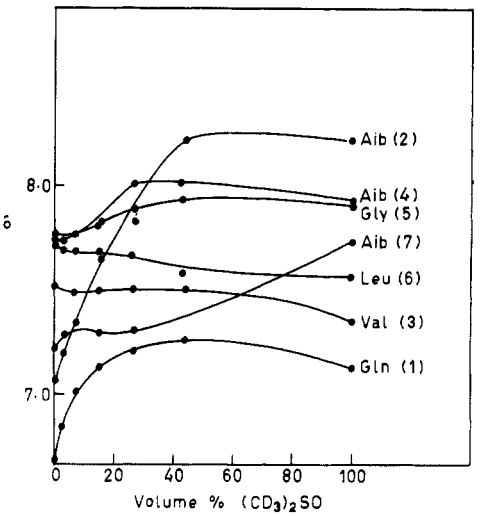

FIGURE 5: Chemical shifts of the NH proton resonances of **2** in $CDCl_3$–$(CD_3)_2SO$ mixtures.

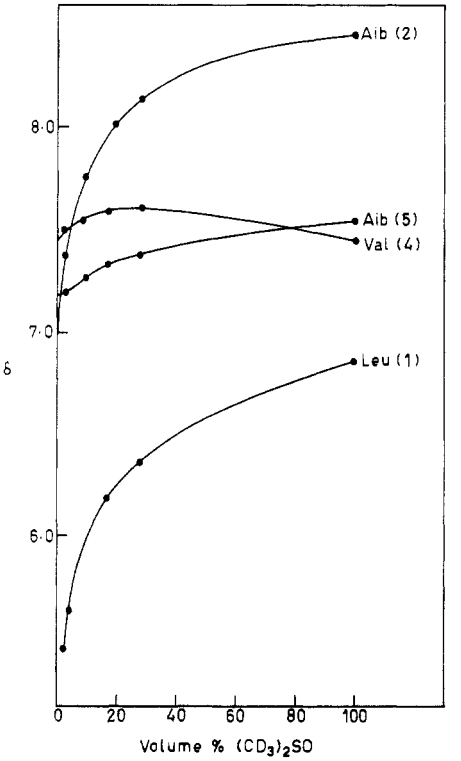

FIGURE 6: Chemical shifts of the NH proton resonances of Boc-Leu-Aib-Pro-Val-Aib-OMe (**3**) in $CDCl_3$–$(CD_3)_2SO$ mixtures.

In the pentapeptide (**3**), the Leu(1) and one of the Aib NH groups [assigned to Aib(2)] show large solvent dependence

Table I: ^{1}H NMR Parameters of Alamethicin Fragments

peptide	proton	NH chemical shifts (δ) $CDCl_3$	NH chemical shifts (δ) $(CD_3)_2SO$	$d\delta/dT \times 10^3$ [a] ppm/100 °C	$J_{HNC^{\alpha}H}$ (Hz) [b] $CDCl_3$	$J_{HNC^{\alpha}H}$ (Hz) [b] $(CD_3)_2SO$
Z-Aib-Pro-Aib-Ala-Aib-Ala-OMe (1)	Aib(1)	5.66	8.21	5.05		
	Aib(3)	7.52	7.55	3.41		
	Ala(4)	7.14	7.18	1.74	8.0	7.7
	Aib(5)	7.37	7.47	2.17		
	Ala(6)	7.53	7.55	1.24	8.0	8.1
Boc-Gln-Aib-Val-Aib-Gly-Leu-Aib-OMe (2)	Gln(1)	6.68	7.13	5.68		
	Aib(2)	7.09	8.25	5.68		
	Val(3)	7.54	7.36	2.50	5.5	7.7
	Aib(4)	7.70	7.94	3.75		
	Gly(5)	7.79	7.94	4.20		
	Leu(6)	7.74	7.57	2.47	8.8	7.7
	Aib(7)	7.23	7.75	3.72		
Boc-Leu-Aib-Pro-Val-Aib-OMe (3)	Leu(1)	4.98	6.87	6.23		
	Aib(2)	7.20	8.43	4.95		
	Val(4)	7.46	7.42	1.88	8.9	9.6
	Aib(5)	7.04	7.55	3.50		
Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe (4)	Gly(1)	5.97	7.06			
	Leu(2)	6.88	7.94	5.56	6.7	7.5
	Aib(3)	7.63	8.29	4.49		
	Val(5)	7.55	7.35	2.56	9.5	9.0
	Aib(6)	7.23	7.51	3.13		

[a] The $d\delta/dT$ values are in $(CD_3)_2SO$. [b] Errors in J values are estimated to be ±0.5 Hz.

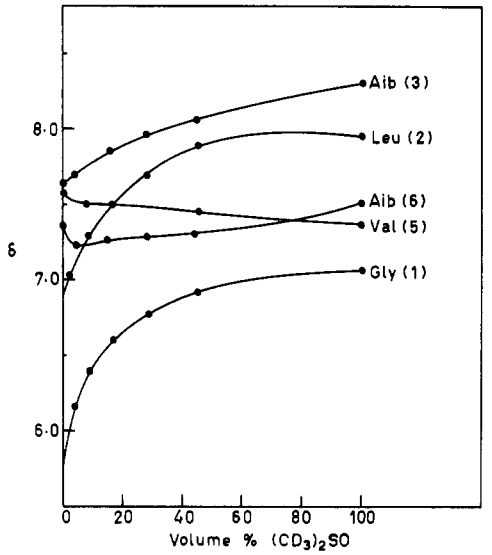

FIGURE 7: Chemical shifts of the NH proton resonances of Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe (4) in $CDCl_3$–$(CD_3)_2SO$ mixtures.

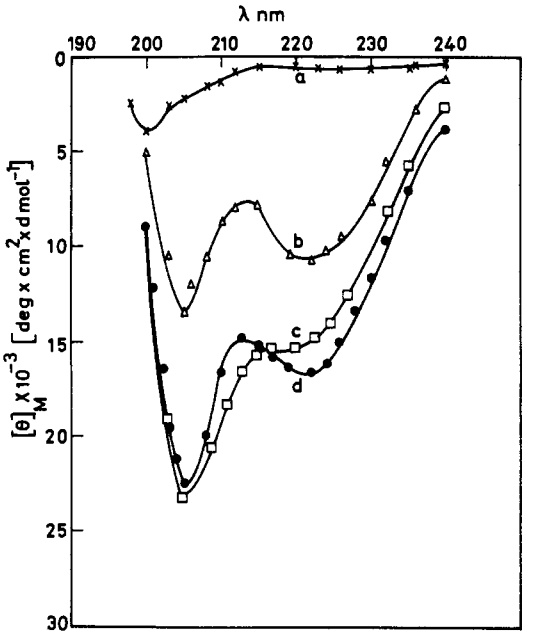

FIGURE 8: CD spectra of alamethicin fragments in trifluoroethanol. (a) 1–6 (1); (b) 1–13; (c) 1–17; (d) synthetic alamethacin.

of chemical shifts (Figure 6), while the changes for Val(4) and Aib(5) NH groups are small. The $d\delta/dT$ values in $(CD_3)_2SO$ are large for the first two NH groups, while they are much lower for Val(4) and Aib(5) NH groups. In the hexapeptide, Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe (4), the Gly(1), Leu(2), and one Aib NH [assigned to Aib(3)] show large solvent dependence of chemical shifts (Figure 7). The Leu(2) and Aib(3) NH groups also have large $d\delta/dT$ values in $(CD_3)_2SO$ (Table I). This is in marked contrast to the Val(5) and Aib(6) NH groups which show small shifts on changing the solvent and have low $d\delta/dT$ values in $(CD_3)_2SO$. The $d\delta/dT$ value for Gly(2) NH could not be determined due to broadening at elevated temperatures. Some NMR results on peptides 3 and 4 have been briefly described earlier, in support of IR studies of their conformations (Rao et al., 1980).

Circular Dichroism and Infrared Studies. Earlier NMR and IR studies (Nagaraj et al., 1979; Rao et al., 1980) suggested that Aib peptides adopt highly folded conformations in solution. It was therefore of interest to examine the CD spectra of alamethicin fragments, with a view toward correlating the observed spectral characteristics with the folding of the peptide backbone. The CD spectra of the alamethicin fragments, Ac-Aib-Pro-Aib-Ala-Aib-Ala-OMe (1–6), Z-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-OMe (1–13), Z-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OMe (1–17), and synthetic alamethicin, in trifluoroethanol (TFE) are shown in Figure 8. The benzyloxycarbonyl (Z) group in 1 was replaced by an acetyl group to minimize absorption effects. TFE was chosen as the solvent since it promotes the formation of helical secondary structures, is a good solvent for peptides, and is transparent to 190 nm. Two negative bands at 205 and 220 nm, assigned to the amide π–π^* and n–π^* transitions, re-

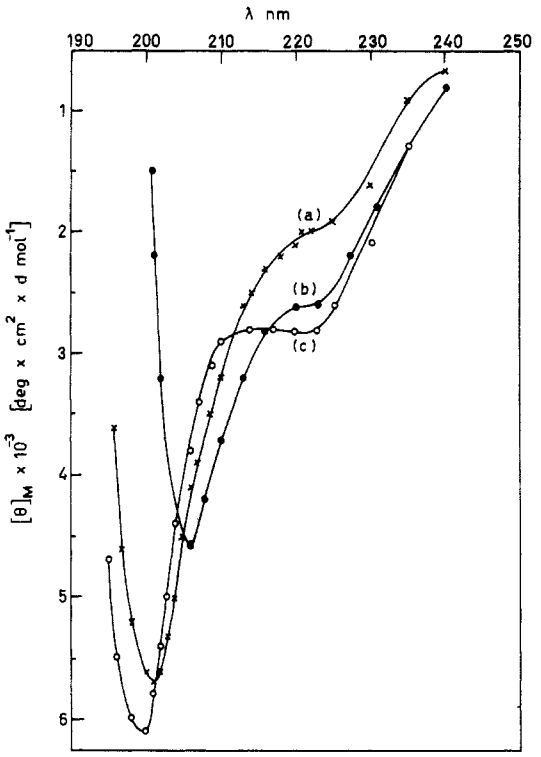

FIGURE 9: CD spectra of alamethicin fragments in trifluoroethanol. (a) 7–13 (**2**); (b) 12–16 (**3**); (c) 11–16 (**4**). Note ordinate has been expanded as compared to Figure 8.

spectively, are clearly observed in the 1–13 and 1–17 fragments and in alamethicin. There is a considerable increase in ellipticity on going from the 13-residue peptide to the longer sequences. The amino-terminal hexapeptide shows only very weak circular dichroism as compared to the longer fragments. The spectra of the central alamethicin fragments **2** (7–13), **3** (12–16), and **4** (11–16) are shown in Figure 9. Again, two negative bands at 205 and 220 nm are observed. In these peptides, the long-wavelength band is significantly weaker. The ellipticity values for both bands are lower than those obtained for the larger peptides (Figure 8), suggesting an increase in ordered helical conformations with peptide chain elongation.

Figure 10 shows the NH stretching bands (ν_{NH}) in the solution IR spectra of the 1–6 (**1**), 7–13 (**2**), and 1–13 fragments of alamethicin. The peak at 3440–3420 cm^{-1} is due to free NH groups (ν_{NH} free) while the peak at 3340–3320 cm^{-1} is due to intramolecularly hydrogen bonded NH groups (ν_{NH} bonded). It can be clearly seen that the ν_{NH} bonded intensity increases dramatically with peptide chain length, suggesting that a large fraction of the newly introduced amide groups are intramolecularly hydrogen bonded. In earlier studies (Rao et al., 1979, 1980), we have established a quantitative relationship between the integrated intensities of the ν_{NH} bands and the number of intramolecular hydrogen bonds. Such quantitation is, however, difficult in the case of large peptides like the 1–13 fragment due to the broad ν_{NH} bonded peak. However, the spectra in Fgure 10 suggest that the 1–13 fragment of alamethicin is indeed highly folded, with a large number of stabilizing intramolecular hydrogen bonds.

Discussion

Conformation of Alamethicin Fragments. Low temperature coefficients ($d\delta/dT$) of chemical shifts of NH protons in $(CD_3)_2SO$ and insensitivity of chemical shifts to changes in solvent have generally been considered as diagnostic of solvent-shielded or intramolecularly hydrogen bonded NH groups.

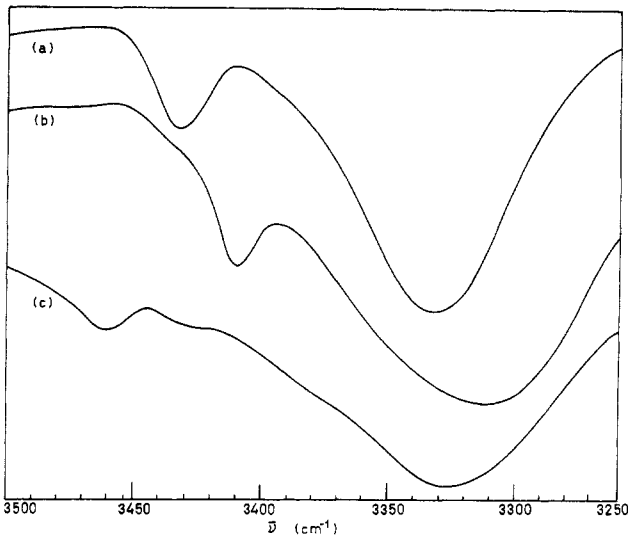

FIGURE 10: NH stretching bands in alamethicin fragments. (a) 1–6 (**1**); (b) 7–13 (**2**); (c) 1–13. Peptide solutions in $CHCl_3$.

By use of these criteria, the Aib(3), Ala(4), Aib(5), and Ala(6) NH groups in the hexapeptide **1** are involved in hydrogen bonding. The NMR data thus support a structure for **1** having four intramolecular hydrogen bonds. This conclusion is in agreement with earlier IR studies (Rao et al., 1980). It is likely that the incipient 3_{10} helical structure formed in the amino-terminal tetrapeptide Z-Aib-Pro-Aib-Ala-OMe (Nagaraj et al., 1979) continues to generate a longer helical segment leading to four C_{10} (4 → 1) hydrogen bonds in **1**. This is schematically illustrated in Figure 11a. The vicinal coupling constants ($J_{HNC^{\alpha}H}$) values of ~8 Hz obtained for the Ala(4), and Ala(6) residues are consistent with a ϕ value of ~−75° (Bystrov, 1976), which is compatible with the requirement of a right-handed 3_{10} helical fold ($\phi \sim -60°$, $\psi \sim -30°$).

The $d\delta/dT$ values for the NH groups in the central alamethicin fragment **2** and the solvent titration curves in Figure 5 clearly show that the Val(3) and Leu(6) NH groups are intramolecularly hydrogen bonded. Two Aib NH groups, tentatively assigned to Aib(4) and Aib(7), have $d\delta/dT \sim 3.7 \times 10^{-3}$ ppm/°C. Of these, Aib(4) NH (tentative assignment) shows little solvent dependence of chemical shift. Aib(7) is insensitive below 20% $(CD_3)_2SO$ but moves downfield at higher concentrations. This behavior supports a conformational transition on going from $CDCl_3$ to $(CD_3)_2SO$. However, both Aib NH groups show significantly smaller solvent shifts than the Gln(1) and Aib(2) NH protons. The latter also have large $d\delta/dT$ values. The data support the conclusion that while the Aib(4) and Aib(7) NH groups are intramolecularly hydrogen bonded, the Gln(1) and Aib(2) NH groups are free. The solvent dependence of the Gly(5) NH chemical shift is low but the $d\delta/dT$ value of 4.2×10^{-3} ppm/°C is rather high for a hydrogen bonded group. However, values in the range $\sim 4 \times 10^{-3}$ ppm/°C have been interpreted as indicative of weak intramolecular hydrogen bonds (Khaled et al., 1976).

The data support a population of conformations for **2**, which involve five intramolecular hydrogen bonds fromed by the Val(3), Aib(4), Gly(5), Leu(6), and Aib(7) NH groups. This would imply a 3_{10} helical structure involving five consecutive type III β turns. Of these, the third β turn with Val(3)-Aib(4) at the corners is likely to be the weakest since the Val residue has a low preference for helical conformations (Chou & Fasman, 1979). This would be consistent with the observed parameters for the Gly(5) NH. The slightly enhanced sensitivity of Aib(7) NH to solvent composition at higher $(CD_3)_2SO$ levels suggests that the Gly(5)-Leu (6) β turn is de-

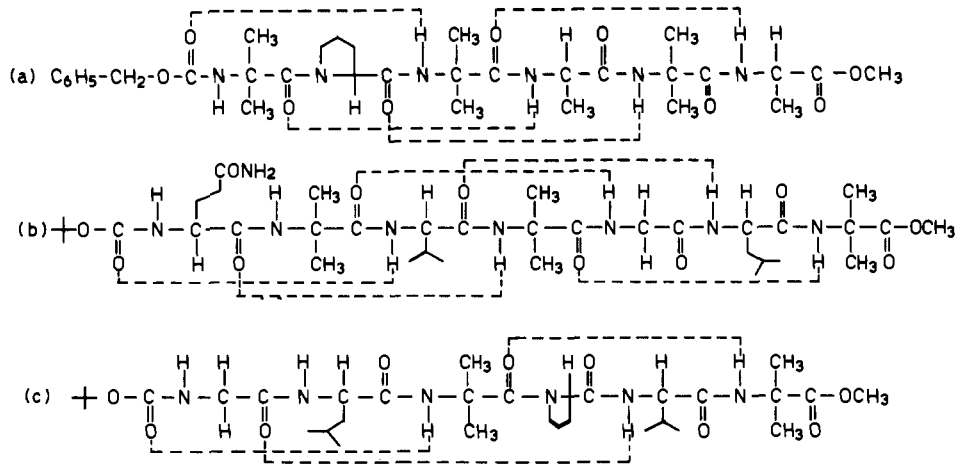

FIGURE 11: Schematic structures indicating the hydrogen bonding patterns in alamethicin fragments. (a) 1–6 (**1**); (b) 7–13 (**2**); (c) 11–16 (**4**).

stabilized. The β turns with Gln(1)-Aib(2) and Aib(4)-Gly(5) are expected to be significantly stronger in view of the known preference of all three residues to occur in β-turn conformations (Chou & Fasman, 1977; Rao et al., 1980). This expectation is borne out by the observation that the Val(3) and Leu(6) NH groups have NMR parameters characteristic of strongly hydrogen bonded protons. A schematic representation of the postulated hydrogen bonding scheme is given in Figure 11b. The preceding argument is based on the distinction between the Aib(2), Aib(4), and Aib(7) NH groups. The tentative assignments are chosen to be consistent with the proposed helical folding of the oligopeptide. From known hydrogen bonding patterns in peptides, it is generally observed that NH groups of succeeding residues are bonded to CO groups of preceding residues (Ramachandran & Sasisekharan, 1968). We have therefore assigned the solvent-exposed NH to the Aib(2) residue, and the assignments of the Aib(4) and Aib(7) NH protons are based on the expected stabilities of the β-turn hydrogen bonds. The $J_{HNC^{\alpha}H}$ values for Val(3) and Leu(6) are 5.5 and 8.8 Hz in $CDCl_3$ and 7.7 Hz for both groups in $(CD_3)_2SO$. These differences in the two solvents suggest differing backbone conformations at least for the ϕ values for the Val and Leu residues. However, both sets of values are reasonably compatible with $\phi \sim -60 \pm 20°$ required for a gross 3_{10} or α-helical fold of the peptide chain. It is likely that the folded structures obtained for oligopeptides in solution may depart quite considerably from the values expected for regular polypeptide structures. In conformations composed of consecutive β turns, the occurrence of type I turns (Venkatachalam, 1968) would lead to higher ϕ values ($\sim -90°$) for the $i + 2$ residues. Such features have been observed in oligopeptide segments of proteins (Chou & Fasman, 1977). Further, the $J_{HNC^{\alpha}H}$ values for L-amino acids have a very steep dependence on ϕ in the vicinity of $-60°$ (Bystrov, 1976). $J_{HNC^{\alpha}H}$ values of $\sim$7 Hz have been suggested to indicate dynamically averaged conformations (Gibbons et al., 1970). However, in the heptapeptide (**2**), it is certain, from the $d\delta/dT$ values and the solvent dependence of NH chemical shifts, that the structure is highly folded. The difference in J values in $CDCl_3$ and $(CD_3)_2SO$ may reflect a loosening of intramolecular hydrogen bonds and an opening out of the structure in the hydrogen bond accepting solvent $(CD_3)_2SO$. Such effects have been noted in an earlier study of Z-Aib-Pro-Aib-Ala-OMe (Nagaraj et al., 1979).

In the 12–16 alamethicin fragment, Boc-Leu-Aib-Pro-Val-Aib-OMe (**3**), Val(4) NH and one Aib NH [δ 7.04 $CDCl_3$; δ 7.55 $(CD_3)_2SO$] exhibit NMR parameters characteristic of hydrogen bonded protons. Leu(1) NH and the second Aib NH [δ 7.20 $CDCl_3$; δ 8.43 $(CD_3)_2SO$] are exposed to the solvent (Table I). As discussed for **2** above, the Aib(2) NH is stereochemically unlikely to participate in forming strong intramolecular hydrogen bonds and is therefore assigned as the low-field NH singlet. The NMR data would then be compatible with a structure involving Aib(2)-Pro(3) and Pro(3)-Val(4) β turns. The higher $d\delta/dT$ value for Aib(5) NH may then reflect a lower tendency for Pro-Val β-turn formation, which would be consistent with the conformational preferences of the Val residue (Chou & Fasman, 1977). Similarly, in the 11–16 alamethicin fragment, Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe (**4**), the Val(5) and one Aib NH [δ 7.23 $CDCl_3$, δ 7.51 $(CD_3)_2SO$] appear to be intramolecularly hydrogen bonded while the Gly(1), Leu(2), and one Aib NH are exposed. By analogy with the pentapeptide (**3**), the hydrogen bonded proton is assigned to Aib(6). Again, a structure involving consecutive Aib(3)-Pro(4) and Pro(4)-Val(5) β turns is consistent with the data. However, in both **3** and **4** the Pro residue occupies a central position in the sequence, preventing a 4 $\rightarrow$ 1 hydrogen bond stabilized Leu-Aib β turn. This disrupts the 3_{10} helix at the amino-terminal end of these molecules. It is necessary in these cases to also consider the possibility of 5 $\rightarrow$ 1 (C_{13}) hydrogen bond formation. For both **3** and **4**, structures involving C_{10} and C_{13} hydrogen bonds (two C_{10}, one C_{10} + one C_{13}, or two C_{13}), with the Val and C-terminal Aib NH groups participating, are possible. These cannot be distinguished on the basis of ^1H NMR data alone. However, from an analysis of solution IR spectra, a structure has been proposed involving an amino-terminal 3_{10} helical conformation (C_{10}) expanding to one turn of an α helix (C_{13}), which then tightens to a 3_{10} structure (Rao et al., 1980). This is schematically illustrated in Figure 11c. While a Gly-Leu β turn involving Aib(3) NH in a hydrogen bond has been proposed in **4**, from IR data in $CHCl_3$, the $d\delta/dT$ value in $(CD_3)_2SO$ is rather high (4.48×10^{-3} ppm/°C). This presumably follows from the loosening of the Gly-Leu β turn in $(CD_3)_2SO$. In both **3** and **4**, the $J_{HNC^{\alpha}H}$ values observed for the Val residue are high in $CDCl_3$ and $(CD_3)_2SO$ (9–9.5 Hz). This would favor ϕ values of $\sim -100°$, which is moderately close to the values expected in types I and III β turns (Venkatachalam, 1968). In interpreting $d\delta/dT$ values for alamethicin fragments, the possibility of intermolecular association merits consideration. Studies of model $(Aib\text{-}Pro)_n$ sequences in $(CD_3)_2SO$ suggest that aggregation is unimportant in this solvent (Venkatachalapathi & Balaram, 1981). The excellent agreement obtained between IR results for the 1–6

peptide (**1**) in dilute $CHCl_3$ solution (Rao et al., 1979, 1980) and the present NMR studies in $(CD_3)_2SO$ support the view that in these fragments intramolecular interactions determine conformation.

The preceding discussion of the NMR data leads us to conclude that the 1–6 (**1**), 7–13 (**2**), 12–16 (**3**), and 11–16 (**4**) fragments of alamethicin adopt highly folded structures in $CDCl_3$ and $(CD_3)_2SO$, largely stabilized by $4 \rightarrow 1$ (C_{10}) hydrogen bonds. This 3_{10} helical conformation appears to be strongly favored over a major portion of the alamethicin sequence and is modified only by the presence of Pro(14). In the sequence 11–16, an expansion of the 3_{10} fold to a single turn of an α helix is possible. The conformational analysis of the 11–16 fragment is, however, complicated by the following factors: (i) flexibility at the Gly-Leu segment leading to dynamic averaging of NMR parameters; (ii) the strategic location of Pro at the center, leading to the possibility of both $5 \rightarrow 1$ and $4 \rightarrow 1$ hydrogen bonds, without any alteration in the number of intramolecular hydrogen bonds; (iii) the low preference of the Val residue for β-turn conformations, resulting in the formation of partially opened structures in hydrogen bond accepting solvents like $(CD_3)_2SO$.

CD and IR Evidence. The gross appearance of the CD spectra of alamethicin and its synthetic fragments is reminiscent of the patterns obtained for poly(L-lysine) (Greenfield & Fasman, 1969) and poly(L-glutamic acid) (Pflumm & Beychok, 1969) in the α-helical form. By use of a value of $[\theta]_M$ at 220 nm of $-38\,825$ deg cm^2 dmol^{-1} for poly(L-Glu) in 100% α-helical form, values of 27%, 39%, and 40% "helical content" are obtained for the 1–13, and 1–17 fragments and alamethicin, respectively, in TFE (Figure 8). Even though the 1–6 hexapeptide (**1**) shows a highly folded structure in NMR studies as well as in earlier IR studies (Rao et al., 1979, 1980), the θ_M values are low. It therefore appears that estimates of helicity in small peptides, using polypeptide standards, are unlikely to be useful. Similarly, while the 12–16 (**3**) and 11–16 (**4**) fragments show evidence for helical folding in their CD spectra, more definitive conclusions cannot be drawn at present. Earlier CD studies using natural alamethicin suggested 20–40% α-helical content in a variety of solvents (McMullen et al., 1971; Jung et al., 1975). These estimates agree well with our results for synthetic alamethicin but would appear to be lower than the expected helicity. From the NMR studies reported in this paper and earlier results on the stereochemistry of Aib-containing peptides (Nagaraj et al., 1979; Prasad et al., 1979, 1980; Shamala et al., 1977, 1978; Rao et al., 1979, 1980), it appears reasonable to expect a predominantly 3_{10} helical conformation over the largely hydrophobic 1–17 segment of alamethicin. The lower estimates obtained from comparisons of polypeptide CD spectra would imply that such correlations are of doubtful validity, in this case. The computed CD spectrum for a 3_{10} helix (Woody & Tinoco, 1973) also does not agree well with the observed CD spectra of the larger alamethicin fragments. The CD results reported here and in other studies of polymer-bound Aib oligopeptides (Mayr et al., 1979) clearly indicate a pronounced tendency of Aib peptides to adopt highly folded helical structures. However, a detailed correlation between CD spectra and secondary structure is not feasible as the chiroptical distinction between 3_{10} and α-helical structures and the effect of α-alkylation on peptide Cotton effects remain to be firmly established.

Detailed ^{1}H NMR studies on the conformation of the 1–13 and 1–17 alamethicin fragments have not been carried out due to considerable spectral overlap of amide NH and C^αH resonances of different residues at 270 MHz. But a comparison of the CD spectra of **1** and the 1–13 fragment suggests that helical folding is maintained in the larger peptide. The IR spectra in Figure 10 clearly support this conclusion. The dramatic increase in the ν_{NH} bonded intensity in the 1–13 fragment suggests that a folded structure, stabilized by a large number of intramolecular hydrogen bonds, is favored in this peptide. Thus, the 3_{10} helical conformations suggested for the 1–6 (**1**) and 7–13 (**2**) fragments, from NMR data, appear to be maintained in the 1–13 fragment. From Figure 8 it can be seen that the 1–17 fragment and synthetic alamethicin yield very similar CD spectra, suggesting that the polar tripeptide tail Glu-Gln-Phol (18–20) may not affect the conformation of the preceding hydrophobic segment.

Alamethicin Conformation. The steric constraints introduced by Aib residues (Marshall & Bosshard, 1972; Nagaraj et al., 1979) appear to dictate the folding of short segments, which are largely unperturbed by further chain elongation. It is therefore reasonable to expect that studies on synthetic fragments would yield useful conclusions about the conformation of alamethicin itself. On the basis of the studies discussed here and in earlier reports (Nagaraj et al., 1979; Rao et al., 1980), we wish to suggest a structure for the alamethicin backbone involving a 3_{10} helical segment from residues 1–10, followed by a slight expansion at residues 11–14 to one turn of an α helix, which is then tightened to a 3_{10} helix from residues 14–17. The flexibility noted earlier for the Gly-Leu segment could provide the necessary "structural hinge" allowing the molecule to interconvert between two conformational states, involving different spatial orientations of the rigid helical segments 1–10 and 13–17. The possibility of limited conformational flexibility may be useful in explaining the voltage-dependent conductance characteristics of alamethicin channels in artificial membranes (Boheim & Kolb, 1978). In this context, it is pertinent to note that the intramolecular hydrogen bonded structures detected in $CDCl_3$ solutions of small fragments tended to open in the more polar solvent $(CD_3)_2SO$. These changes may also occur in alamethicin, with the structures at the aqueous–lipid interface and in the phospholipid bilayer differing in the number of stabilizing intramolecular hydrogen bonds. The polar charged tail, Glu-Gln-Phol (18–20), is likely to be flexible and should facilitate proper orientation of alamethicin in the lipid bilayer. ^{1}H NMR studies of Boc-Glu-Gln-Phol in $(CD_3)_2SO$ suggests that no particular conformation is favored in this fragment (unpublished results). Alamethicin is likely to adopt a highly folded, largely 3_{10} helical conformation over the hydrophobic 1–17 segment with a flexible C-terminal tripeptide.

All the suggested helical structural possibilities (like 3_{10} and α-helical conformations) have very small internal diameters that cannot account for the passage of ions through the helix interior, as postulated for gramicidin A channels (Urry, 1977). Membrane channels of alamethicin must therefore be built up of aggregates, and there is considerable evidence for a hexameric aggregate as the functional alamethicin channel (Boheim & Kolb, 1978; Mueller, 1976). An association of 3_{10} or α helices to form a channel structure has been suggested earlier (Urry, 1977). Preliminary studies on fluorescent-labeled Aib-containing fragments of emerimicin provided evidence for aggregation of larger peptides in aqueous solution (Nagaraj & Balaram, 1979). Currently, studies are under way in this laboratory on the synthesis of fluorescent alamethicin fragments to study modes of aggregation. Preliminary evidence is also available that, while the small fragments have no effect on the cation permeability of phospholipid liposomes,

the 1–13 fragment weakly enhances permeability while the 1–17 peptide and both synthetic and natural alamethicin have a strong effect (Nagaraj et al., 1980). Further studies on the conformation and modes of aggregation of the 1–17 peptide and alamethicin are necessary before a satisfactory molecular model of the alamethicin transmembrane channel can be evolved.

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