600 MHz PROTON MAGNETIC RESONANCE STUDIES OF NATURAL AND SYNTHETIC ALAMETHICIN

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1. Introduction

The antibiotic, alamethicin (ALA) occurs in nature as a mixture of several hydrophobic peptides, each differing by the substitution of 1 or 2 residues [1]. While this heterogeneity has not apparently hampered investigations of the effects of ALA on the permeability and electrical properties of lipid bilayer membranes [2–4], it has made the task of elucidating the covalent structure of ALA more difficult. Indeed, descriptions of its structure have progressed through several stages of revision and refinement.

Initially a cyclic structure of 18 residues was proposed [1] then discarded in favor of a 20-residue open but branched sequence terminating in –Glu(Phol)Gln [5]. Synthesis of this molecule [6] gave a compound which was different from the natural material. Its [H^+]- titration properties however suggested that the correct C-terminal sequence might be –Glu–Gln–Phol [7]. Synthesis of this compound [7] and independently, mass spectrometry of the unfractionated natural ALA [8] now indicate that the major component of ALA is a linear peptide of 20 amino acids linked according to scheme (I):

Ac-Aib–Pro–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–
5 10

Gly–Leu–Aib–Pro–Val–Aib–Aib–Glu–Gln–Phol (I)
15 20

Given this history (as well as the reservations in [9]) additional independent evidence bearing on the structure of ALA is desirable.

We have now purified natural ALA to a high degree of homogeneity by reversed phase HPLC. The analysis and comparison of the high-field (600 MHz) ^{1}H-NMR spectra of the major component (~70%) ALA-1 with that of an equally pure synthetic version (syn ALA-I) show that the two compounds are identical and have the structure depicted in (I). Moreover, we have succeeded in assigning almost all the spectral lines to specific protons or groups in the molecule and have learned additional information about its secondary and tertiary structure in solution.

2. Materials and methods

Natural alamethicin (U-22324 8831) was kindly supplied to us by Dr G. B. Whitfield of the Upjohn Company. Its purification as well as the synthesis and purification of syn ALA will be described elsewhere [10]. All other materials were used as obtained from commercial sources.

For the NMR studies ethanol solutions containing ~1 mg chromatographically pure peptide were evaporated to dryness with N_2, redissolved in ~0.5 ml methanol-d_4 (99.9% H, Aldrich Chem. Co.), then filtered through glass wool plugs into NMR tubes (PP-528, Wilmad Glass Co.). A small amount of TMS (~0.5%, v/v) was added as a reference and for ^{1}H-field-frequency locking.

Spectra were taken in the frequency-swept, correlation-mode [11] at 600.2 MHz (14.1 T) on the MPC-600 NMR spectrometer [12]. Typically 800–1000 scans of 2 kHz width, each swept in 3 s periods, were accumulated in 8000 data blocks, digitally filtered then transformed. Frequencies and line intensities were provided by the system software. When double resonance was used, the second (decoupling) field was switched between preset frequencies (generally 100 Hz apart) every 10 scans and the accumulated spectrum was transferred to the appropriate disc file.

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The effects of decoupling were observed by creating difference spectra.

3. Results

Different regions of the ^{1}H-NMR spectra of ALA-I are shown in fig.1. The chemical shifts of resonances that are clearly resolved were evaluated directly. By noting the positions of residual images formed in double resonance difference spectra, the shifts of the overlapping $C_\beta H$ and $C_\gamma H$ lines could also be determined (fig.1B). The latter experiments also established the connectivities among coupled spins.

Line assignments to specific proton groups were accomplished by a combination of methods. These included identification of group-characteristic chemical shifts and spin–spin splitting patterns as well as multiplet connectivities revealed by decoupling or (for $C_\alpha H$) by ^{2}H-exchange of amide NH. Further refinement of the assignments to specific amino acids was effected by comparison of the ALA-I spectra with those of the synthetic 1-17 residue fragment and ALA-II, a minor (~20%) component of natural ALA in which Ala^6 is replaced by an Aib [10].

For example, the spectra of the C_α protons of synthetic 17-ALA are essentially identical to ALA-I except that the C-terminal Glu–Gln–Phol lines are missing and the $C_\alpha^{Val\text{-}15}$ and $C_\alpha^{Pro\text{-}14}$ lines are shifted ~0.5 and 0.2 ppm, respectively, to lower field. Likewise the C_α-region of the ALA-II spectra shows no high-field Ala C_α-H quartet and the $C_\alpha^{Gln\text{-}7}$ line is shifted upfield by approximately 0.1 ppm. Once the C_α lines were assigned, the positions of the remaining

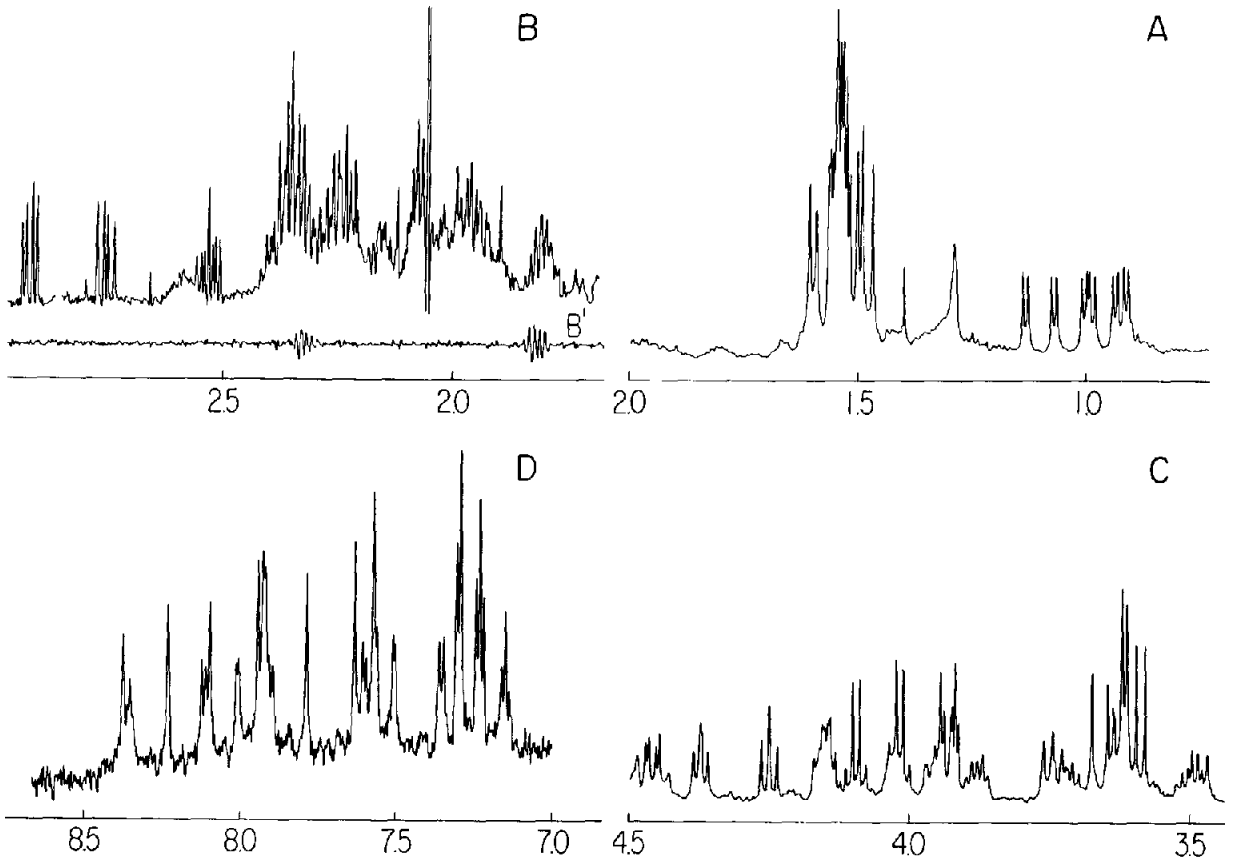

Fig.1. 600 MHz ^{1}H-NMR spectra of ALA-I (2 mg/ml in methanol-d_4, T = 23°C): (A) side chain CH_3 of Aib, Ala-4, 6, Leu-12, Val-9, 14; (B) side chain $C_\beta H$ and $C_\gamma H$ (resolution enhanced, amplification 10 × A); (B) difference spectra upon irradiation of Pro^{14}-$C_\alpha H$ at 2622 Hz; (C) backbone $C_\alpha H$ and Pro 2, 14, $C_\delta H$ (amide NH are deuterated); (D) amide NH and Phol ring protons (7.14–7.30 ppm); spectra are taken ~20 min after dissolving the peptide in methanol-d_4. All scales are in ppm from internal TMS.

lines were found by decoupling techniques. The results are summarized in table 1. Since there are slight variations in the line positions from sample to sample, the entries are average values for 3 different samples of ALA-I. We attribute these variations to differences in the concentration and/or residual H_2O associated with the peptides.

The spectra and measured line positions of the synthetic version, syn ALA-I are essentially indistinguishable from those of the natural material. For comparison, spectra of the $C_\alpha H$ resonances are shown in fig.2.

Since the peptides were dissolved in a deuterated, protic solvent, amide NH exchange with solvent-^2H. For ALA-I in methanol we could distinguish several classes of NH according to their relative rates of isotope exchange. Depending upon the apparent pH, the amide resonances of the N-terminal Aib^1 and C-terminal residues in the order Glu < Gln ≃ Phol disappear within a few hours after the peptide is dis-

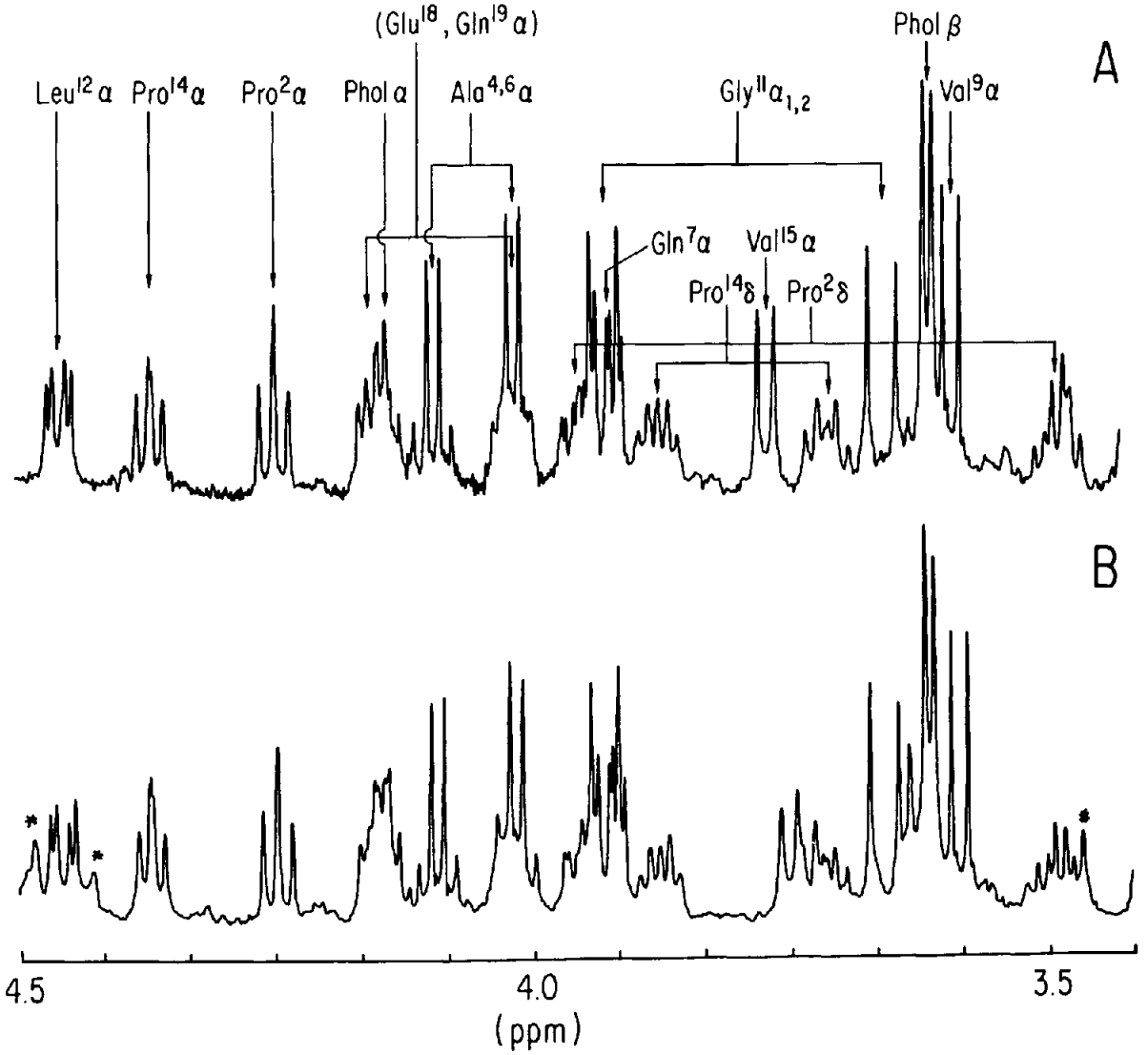

Fig.2. Comparison of the $C_\alpha H$ regions of HPLC-purified (A) synthetic ALA-I and (B) natural ALA-I. Extra lines (*) near the low-field Leu-$C_\alpha H$ and high-field Pro-$C_\delta H$ multiplets in (B) are spinning side bands from residual OH and CH lines of the solvent.

Table 1
Chemical shifts of proton groups in alamethicin[a–c]

	N	C_α	C_β	C_γ	C_δ
Pro[2]	–	4.428	1.80	2.08	3.955
			2.35	1.99	3.490
Ala[4]	7.560	4.095	1.496	–	–
Ala[6]	7.912	4.018	1.538		
Glu[7]	8.002	3.926	2.27	2.540	–
			2.13	2.35	
Val[9]	7.500	3.588	2.24	1.136	
				1.069	
Gly[11]	8.375	3.932	–	–	–
		3.661			
Leu[12]	8.110	4.457	1.96	1.91	0.937
					0.914
Pro[14]	–	4.373	1.82	2.08	3.881
			2.33	1.83	3.725
Val[15]	7.593	3.747	2.38	1.069	–
				0.981	
Glu[18]	7.91[d]	4.148	2.00		
			2.08		
Gln[19]	7.899[d]	4.026	2.25	–	–
Phol[20]	7.351[d]	4.150	2.932	3.616	–
			2.746		

[a] In ppm from TMS
[b] Accuracy is ±0.003 (4 sig. fig., directly observed) or ±0.01 (3 sig. fig., via double resonance)
[c] Aib-1NH occurs 8.622 ppm, the remaining at 8.272; 8.229; 8.094; 7.941; 7.785; 7.631; 7.570 ppm. The Aib CH_3 occur between 1.60–1.46 ppm
[d] Tentative assignment

solved. After 24 h, ~50% of the Gly[11] NH has exchanged while those of Leu[12] and Val[15] are ~20% exchanged. In this time period the NH of the remaining residues show little if any evidence of exhange. At somewhat higher pH, and generally faster exchange rates, of Gln[7], Val[9] and two Aib (tentatively 8 and 10) remain particularly refractory to exchange.

Torsional angles, ϕ about the N–C_α bonds were estimated from the $^3J_{NC_\alpha}$ vicinal coupling constants between NH and C_αH pairs. For Ala[6], Gln[7], Val[9] and Gly[11], $^3J \simeq 4$–6 Hz giving a ϕ of $\sim -65 \pm 10°$; for Ala[4], Leu[12], Val[15], Phol[20], $^3J \simeq 7$–9 Hz and ϕ is $\sim -80 \pm 10°$.

4. Discussion and conclusions

^{1}H-NMR studies of partially purified Ala at 270 MHz [5] were instrumental in establishing its open-chain structure and the presence of the hitherto unsuspected phenylalaninol residue (see also [13]). The extent to which the spectra could be analyzed was limited by the low field and low resolution.

From the studies reported here on well-purified natural and synthetic samples at more than twice the field strength, much more can be learned. Almost every proton or proton group in the peptide has been assigned to specific resonance lines. Moreover the line-by-line correspondence between the spectra of ALA-I and its synthetic version shows that they are identical molecules having the structure depicted in scheme (I).

With regard to higher levels of structural organization we note that under the solvent conditions used, the rate constants for NH-solvent–^{2}H exchange of all but the N-terminal Aib and C-terminal tripeptide ($t_{1/2} \sim 1$–2 h) are typical of solvent-shielded, H-bonded NH groups [14]. Moreover we can distinguish two classes of solvent shielded groups:

(i) A 'very slow' ($t_{1/2} \simeq 10^2$ h) exchanging set in the sequence Aib[3]–Aib[10];
(ii) A 'slowly' ($t_{1/2} \simeq 10$ h) exchanging set from Gly[11]–Aib[16].

If the differences in the rate constants for the solvent-shielded groups are interpreted in terms of the relative flexibility or degree of fluctuation in the conformations of the backbone regions containing these groups then the segment containing Gln[7], Val[9] and tentatively Aib[8], Aib[10] is especially rigid, such as one might expect for a helical backbone or β-sheet configuration. In fact, the torsional angles, ϕ for Ala[6], Gln[7] and Val[9] are in the correct range for a helix (60°). From CD studies in methanol [13], it was estimated that 40% of the ALA backbone was in a helix. This degree of helicity is consistent with the observation that the 8 NHs in the segment from Aib[3]–Aib[10] exchange 'very slowly', and that residues 6, 7 and 9 have helical ϕ-values.

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