High Resolution and Field Desorption Mass Spectrometry Studies and Revised Structures of Alamethicins I and II[1,2]

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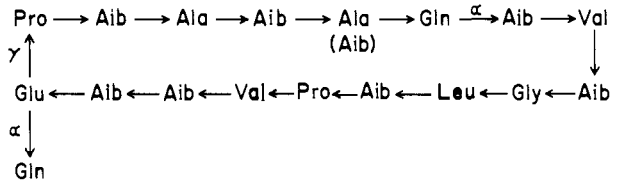
Abstract: Structures **2**-I and **2**-II, revised from those proposed previously by others, have been assigned to alamethicins I and II based on high resolution field desorption and high resolution electron impact mass spectrometry and ^{13}C NMR studies of the intact antibiotics and their hydrolysis products as well as field ionization and high resolution electron impact gas chromatographic studies of the derivatized hydrolysis products. Gas chromatographic analysis on an optically active substrate confirmed that the chiral amino acids in alamethicin have the L configuration.

The peptide antibiotic alamethicin has aroused considerable interest in molecular biology for its ability to alter ionic conductance or permeability across membranes.[3-8] In contrast to those antibiotics which alter conductance by transporting ions across the membrane (mobile ion carriers, inter alia, valinomycin, enniatin B, macrotetrolides),[9] alamethicin, like gramicidin A,[10,11] functions by forming pores or channels in the membrane. More recently, the related antibiotic suzukacillin[12a] has been shown to function by a similar mechanism.[12b]

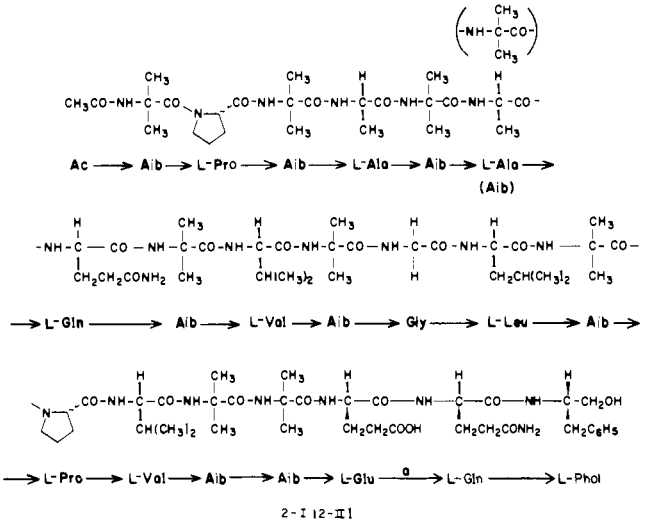
Our attention was drawn initially to alamethicin by our studies with other antibiotics. During the course of our investigations of the antibiotics antiamoebins I and II,[13] emerimicins II, III, and IV,[2b,14a] and zervamicins I and II,[14a] which we have named the peptaibophol class of antibiotics,[15] we observed that this group of antibiotics is characterized by having several (four to eight) α-aminoisobutyric acid units (Aib, cf. following) per mol and an amino alcohol, phenylalaninol (Phol, cf. following), first reported in antiamoebin.[16] A literature search indicated that the reported structures of alamethicins (which we now call alamethicins I and II, **1-I** and **1-II**, containing Ala and Aib,



1-I (1-II)

respectively)[17] contained 7 and 8 Aib units per mol but no Phol. In view of their high content of Aib we felt it likely that the alamethicins also belong to the peptaibophol group of antibiotics and initiated in late 1974 a reinvestigation of their structures. From our initial studies (to be described in the next sections) of the total acid hydrolysate by high resolution field desorption mass spectrometry (HRFDMS) and of the intact antibiotic by ^{13}C NMR spectroscopy, neither of which methods had been employed in the earlier studies,[17] it was immediately apparent that alamethicins I AND II contain phenylalaninol.[1,18a] Consequently, we elected to carry out a complete reinvestigation of their structures,[18b] which we felt would further test the mass spectrometric tools developed in our other peptide studies.[2b,13]

Alamethicin was shown earlier[19] to be a mixture of components and the major component employed in previous structural studies[17] itself contains two homologues. The sample we employed was not separated into its individual components and the present work was carried out on the mixture, principally alamethicins I and II. From our studies, to be detailed below, we assign structures **2-I** and **2-II** to alamethicins I and



2-I 12-II

II, respectively. Following our preliminary report,[1] we learned that others had reached similar, though not identical, conclusions regarding a revised structure for alamethicin I.[20] Their conclusions were based on extensive NMR investigations, ours largely on mass spectrometry. Differences between their structure[20b] and ours will be discussed below.

Amino Acids and Phenylalaninol in Alamethicins I and II. The first indication of a composition for alamethicins I and II different from that reported[17] came from FDMS studies of the total acid hydrolysate of alamethicin. As shown in Figure 1, the FD mass spectrum of the mixture of amino acids from vigorous acidic hydrolysis of alamethicin clearly showed an M + H ion at *m/e* 152 in addition to the M + H ions for the reported amino acids[17,21] at *m/e* 76 (Gly), 90 (Ala), 104 (Aib), 116 (Pro), 118 (Val), 132 (Leu), and 148 (Glu). The compositions of these ions were confirmed by HRFDMS (see Table I), and the peak at *m/e* 152 was assigned to Phol. The precision of these measurements ($\Delta M \leq 0.6$ mmu) demonstrates the utility of high resolution for FDMS studies of compounds of unknown structures. The identifications of these amino acids and Phol in the hydrolysate were confirmed for their *N*-trifluoroacetyl (*N*-TFA) *n*-butyl ester derivatives by gas chromatography (GC) retention times (3% OV-17 and 0.65% EGA columns, Figure 2) compared to those of authentic samples and by gas chromatography-mass spectrometry (GC-MS).

Extensive work was carried out by earlier workers[17,21] on the amino acid composition of alamethicin, employing an amino acid analyzer, and the present amino acid analyzer results (Table II) agree well with the earlier reports,[17,21] indicating 18 to 19 units. On the other hand, quantitation of the amino acids and Phol by GC indicated 20 units (Table II) in alamethicins I and II. The amounts of Aib and Ala, slightly

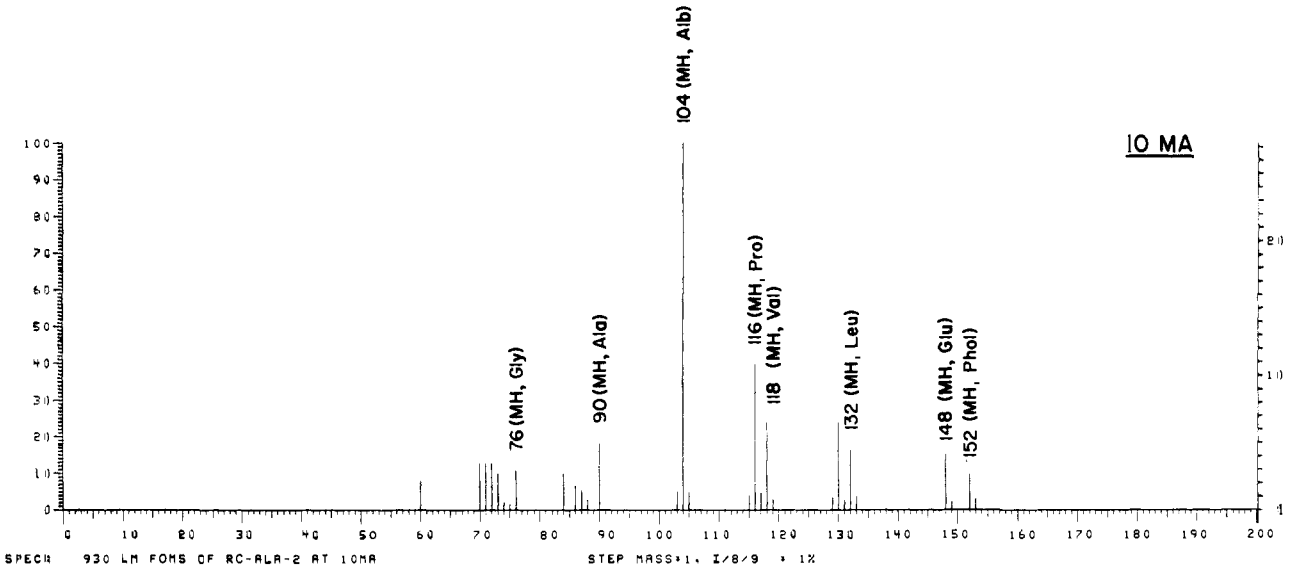


Figure 1. Field desorption mass spectrum of total acid hydrolysate from alamethicin (total mixture).

Table I. HRFDMS Data for the Mixture of Amino Acids from Vigorous Acidic Hydrolysis of Alamethicin

Measured mass	Δ, mmu	Composition	Assignment
76.0401	0.3	$C_2H_6NO_2$	Gly + H
90.0557	0.2	$C_3H_8NO_2$	Ala + H
104.0715	0.3	$C_4H_{10}NO_2$	Aib + H
116.0705	−0.6	$C_5H_{10}NO_2$	Pro + H
118.0864	−0.4	$C_5H_{12}NO_2$	Val + H
132.1028	0.3	$C_6H_{14}NO_2$	Leu + H
148.0613	0.3	$C_5H_{10}NO_4$	Glu + H
152.1077	0.2	$C_9H_{14}NO$	Phol + H

larger or smaller than whole numbers, would agree with reports by earlier workers[17] that the two alamethicins differ from one another in replacement of one Ala by an Aib unit, but the precision of the methods is inadequate to require such a replacement.

Configuration of Amino Acids. It was reported earlier that all the optically active amino acids in alamethicin have the L configuration.[21] we have modified (by employing partial temperature programming, as shown in Figure 3) the recently developed GC technique employing columns packed with an optically active substrate; this allows us to determine the configurations of all optically active amino acids[22,23] in a single GC run. Following this procedure, the amino acids in the above hydrolysate of alamethicin were converted into the methyl esters of their *N*-TFA derivatives and the mixture was injected onto a column of 10% *N*-lauroyl-*N'*-*tert*-butyl-L-valinamide on 60–80 Chromosorb W AW. A comparison of the retention times with those of the derivatives of an authentic mixture of DL amino acids indicated that all the amino acids have the L configuration (Figure 3). Enantiomers of Phol did not separate on this column, but the configuration of Phol in alamethicin was established as L by isolating it and measuring its rotation.

Properties and Molecular Formulas of Alamethicins I and II. Alamethicin, mp 275–279 °C, is acidic (pK_a = 6.04 in 50% ethanol), with a neutralization equivalent of 1960. It forms a monomethyl ester (δ 3.62 ppm) when treated with diazomethane and amide analysis indicated that alamethicin has 2.17 mol of primary amide per mol. The three Glu units of

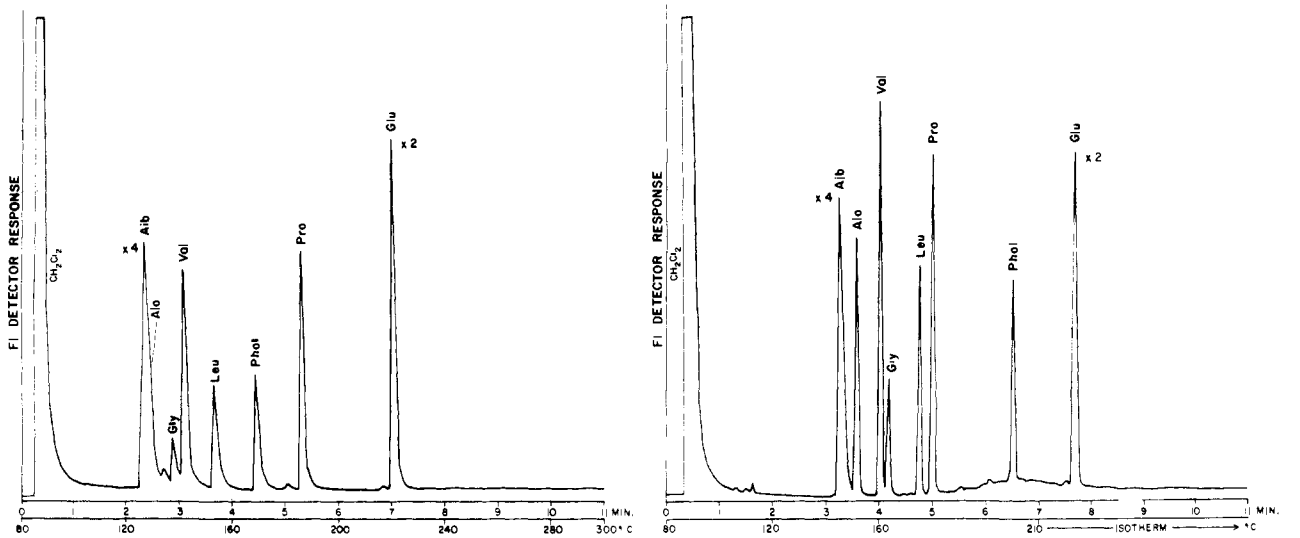


Figure 2. Gas-liquid chromatogram of *N*-TFA *n*-butyl ester derivatives of amino acids in total acid hydrolysate from alamethicin. Left: glass column, 6 ft × 2 mm i.d., 3% OV-17 on 100–120 GCQ, flow, 36 mL/min; attenuation, 1×10^{-10}, 10 mV; temperature program, 20 °C/min (80–300 °C). Right: glass column, 6 ft × 2 mm i.d., 0.65% EGA on 80–100 Chromosorb W AW, flow, 24 mL/min; attenuation, 1×10^{-10}, 10 mV; temperature program, 20 °C/min (80–210 °C).

Table II. Molar Ratios of Amino Acids and Phenylalaninol in Alamethicin

Component	Amino acid analyzer results: Reusser et al.[21]	Hartley et al.[17a]	Ovchinnikov et al.[17b]	Present results	GC results, present work
Aib	8	7.5	7.4	8.2 ± 0.2	8-9
Ala	2	1.6	1.6	1.7	1.8-2
Gly	1	1.0	1	1.0	1
Val	2	1.9	2	2.0	2
Leu	1	1.0	1	1.0	1
Pro	2	2.0	2	1.9	2
Glu	3	3.0	3	3.1	3
Phol				Traces	1

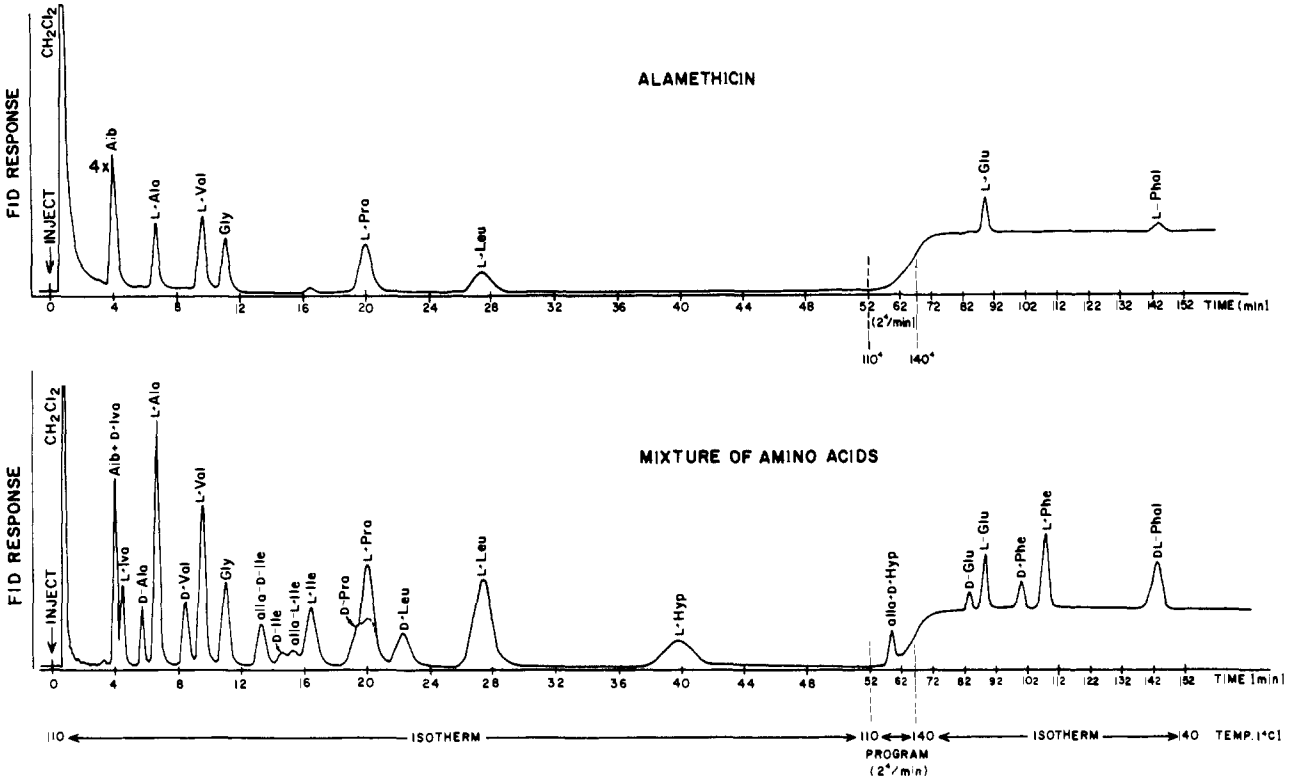


Figure 3. Resolution of enantiomeric amino acids (*N*-TFA methyl ester derivatives) by gas-liquid chromatography. Column conditions: 12 ft × 1/8 in., 10% *N*-lauroyl-*N'*-*tert*-butyl-L-valinamide on 60-80 Chromosorb W AW, 60 mL/min.

alamethicin provide three extra carboxyl groups to go with a possible C-terminal carboxyl (if alamethicin is a linear peptide) for a maximum of four C-terminal groups. Since four C-terminal groups are identified (-COOH, 2-$CONH_2$, -CO → Phol) the molecule must, indeed, be linear. Moreover, since the antibiotic does not react with reagents for amino (N-terminal) groups, the N-terminal amino group must be masked. A singlet at 2.00 ppm in its ^1H NMR spectrum suggests that alamethicin contains an acetyl group, and when the methyl ester of alamethicin was treated with acetic anhydride-pyridine, the product obtained gave a ^1H NMR spectrum in which the methyl ester (3.65 ppm) and the acetyl (2.08 ppm) methyl signals were in the ratio of 1:2. Since alamethicin has only one hydroxyl group (in Phol, Tables I and II) and one carboxyl group (in Glu) this carbomethoxy:acetyl ratio also indicates the presence of an acetyl group in alamethicin itself.

If each alamethicin (I and II) contains 19 amino acids and Phol (those in Table II), an acetyl group, a carboxyl group, two primary amides, and an alcohol group in a linear peptide, and if, as indicated earlier, the difference between the two components is the replacement of an Ala unit by an Aib unit, the molecular formulas should be $C_{92}H_{150}N_{22}O_{25}$ and $C_{93}H_{152}N_{22}O_{25}$, and the molecular weights 1962 (M_I) and 1976 (M_{II}).[24a] We have employed our recently developed cation-exchange technique[24b] to confirm these molecular weights. Alamethicin itself gives "cationated molecular" ions with added potassium chloride at *m/e* 2000 (M_I + K − H), 2014 (M_{II} + K − H), 2039 (M_I + 2K − H), and 2053 (M_{II} + 2K − H) and with added sodium chloride at *m/e* 1966 (M_I + Na − H − H_2O), 1984 (M_I + Na − H), 1989 (M_I + 2Na − H − H_2O), 2007 (M_I + 2Na − H), and corresponding peaks for alamethicin II at *m/e* 1980, 1998, 2003, and 2021 [measured vs. the molecular ion of octafluoropentoxypentakis(dodecafluoroheptoxy)cyclotriphosphazene at *m/e* 2020.9323].[25] Similarly, alamethicin acetate gives "sodiated molecular" ions at *m/e* 2026 (M_I + Na − H), 2040 (M_{II} + Na − H), 2049 (M_I + 2Na − H), and 2063 (M_{II} + 2Na − H).

^{13}C NMR Studies. The ^{13}C NMR spectra of alamethicin and its methyl ester are shown in Figure 4. From these spectra it is clear that alamethicin has a phenyl group, whose carbons (in alamethicin) appear at 139.0 (C-1), 129.1 (ortho), 127.9 (meta), and 125.8 ppm (para),[26] and that the carbonyl region (Figure 5) contains 23 carbonyl carbons, at 176.4, 176.1, 175.8 (2), 175.7, 175.6, 175.4, 175.2, 174.1, 173.9, 173.8, 173.7 (2), 173.6, 173.5, 173.3, 173.1, 173.0, 172.8, 171.9, 171.0, 170.3, 169.7 ppm, in agreement with the molecular formula established by FDMS.

Sequence of Amino Acids. A. By HREIMS. With the mo-

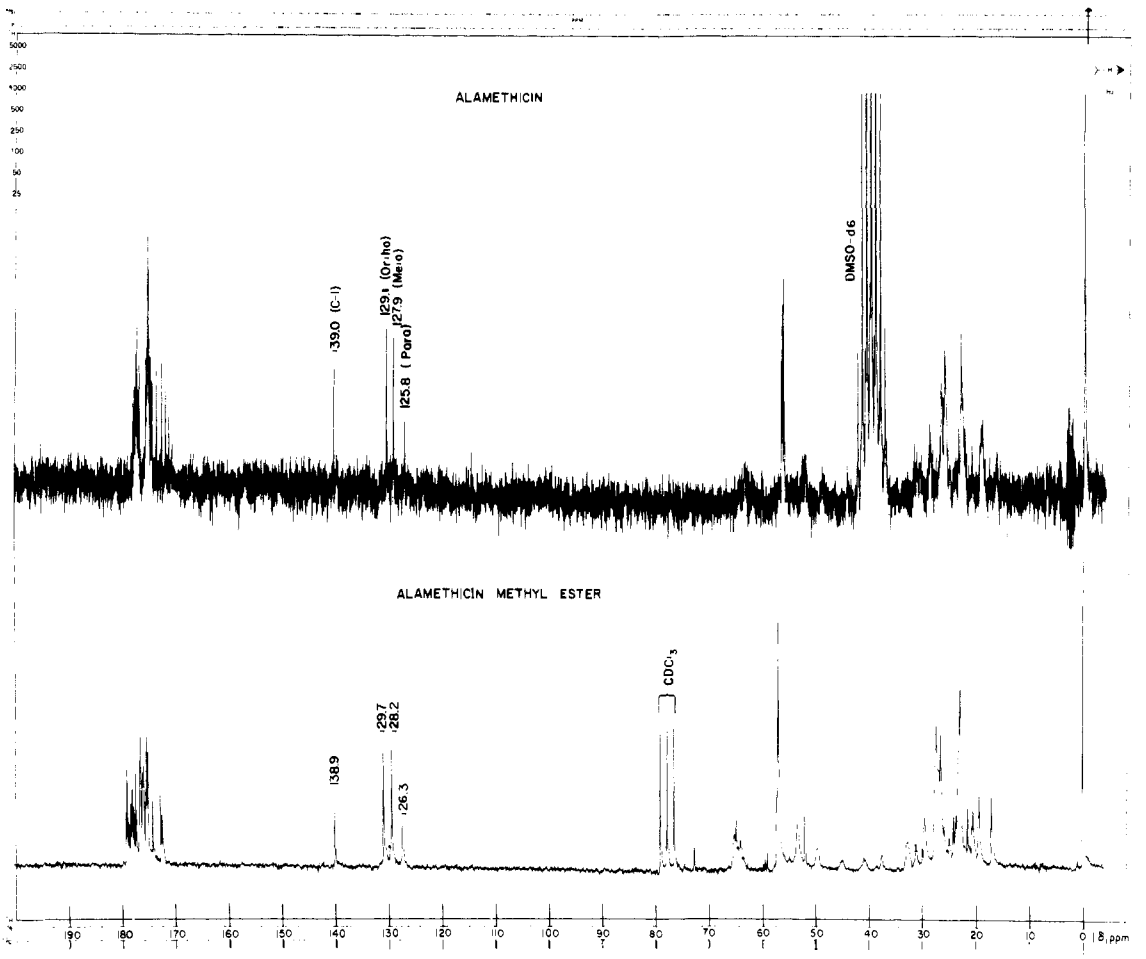


Figure 4. ^{13}C NMR spectra of alamethicin and alamethicin methyl ester.

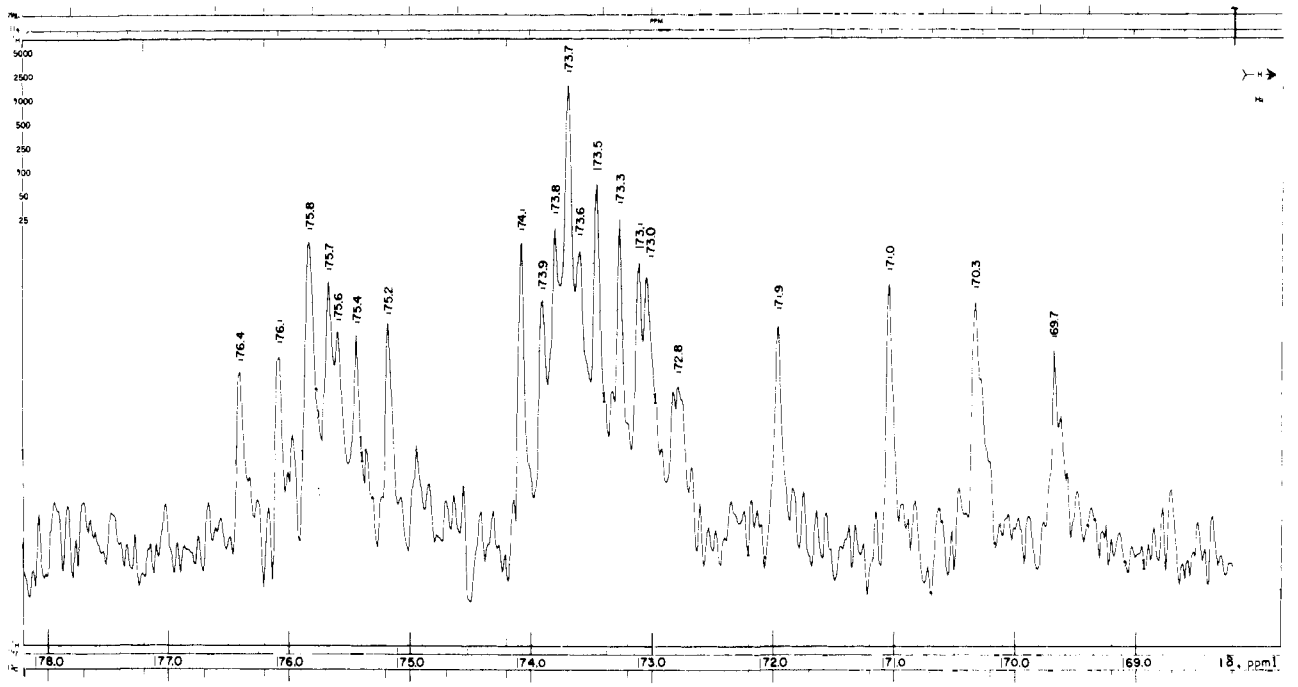


Figure 5. Carbonyl region of the ^{13}C NMR spectrum of alamethicin.

lecular formulas (and component amino acids) of alamethicins I and II defined as $C_{92}H_{150}N_{22}O_{25}$ (8 Aib, 2 Ala, 2 Val, 2 Pro, 2 Gln, 1 Gly, 1 Leu, 1 Glu, 1 Phol) and $C_{93}H_{152}N_{22}O_{25}$ (9 Aib, 1 Ala, 2 Val, 2 Pro, 2 Gln, 1 Gly, 1 Leu, 1 Glu, 1 Phol), respectively, it remains then to assign the sequences of the amino acids in the two antibiotics. Perhaps the first point to settle is the location of the Ala unit in alamethicin I which is replaced by an Aib unit in alamethicin II. This is initially apparent in

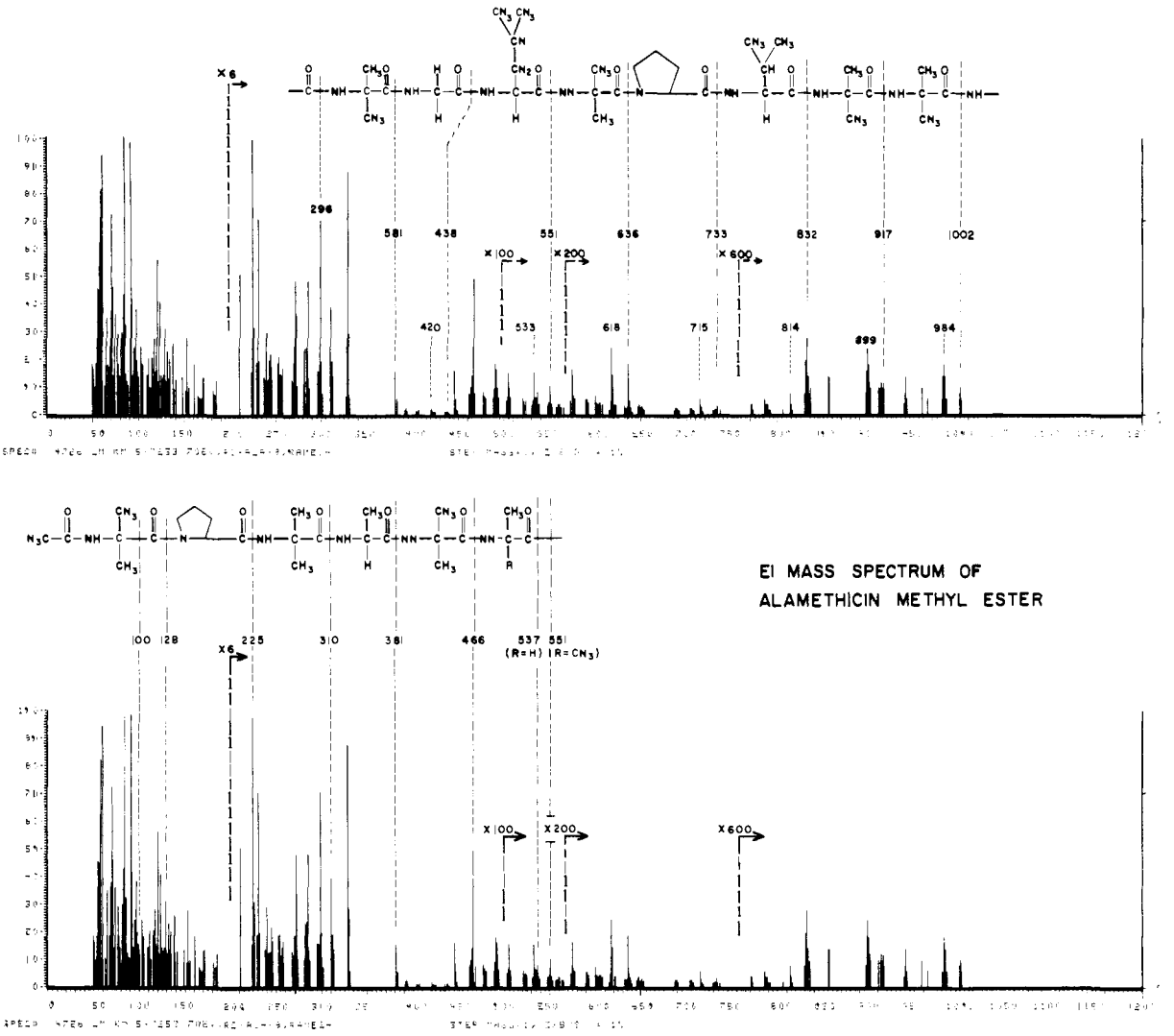


Figure 6. Fragmentation ions observed in the EI mass spectrum of alamethicin methyl ester. Above, sequence a; below, sequence b.

the HREI mass spectrum of alamethicin methyl ester (Figure 6 and Table III), which contains three series of peaks which define the partial sequences a, b, and c.

One of these series (that defining sequence b in Table III) contains a pair of ions at *m/e* 537 and 551, the series' highest masses, which locate the Ala → Aib pair. The remainder of the series indicates successive losses of Aib, Ala, Aib, and Pro, with the residual fragment giving the peak at *m/e* 128.0702 ($C_6H_{10}NO_2$) being formulated as acetyl → Aib →.

The series of highest mass (which defines sequence a) begins at *m/e* 1002 and, accompanied by a second series 18 amu lower beginning at *m/e* 984, shows successive losses of Aib, Aib, Val, Pro, Aib, Leu, Gly, and Aib (Table III). The residual fragment giving the peak at *m/e* 296.1592 ($C_{14}H_{22}N_3O_4$) can be formulated as containing Glu, Val, and Aib and written (based on evidence developed later) as Pyroglu ($C_5H_6NO_2$) → Aib → Val →, though fragment peaks for the tripeptide are lacking.

The third series of (mostly weak) ions is, like that defining b, a double series beginning at *m/e* 306 and 288 (306 − H_2O); this defines the short C-terminal sequence c by successive losses of parts of the terminal pseudodipeptide. Sequences a, b, and c locate all the amino acids of alamethicin except 1 mol of Glu, which must be between sequences a and b or between b and c.

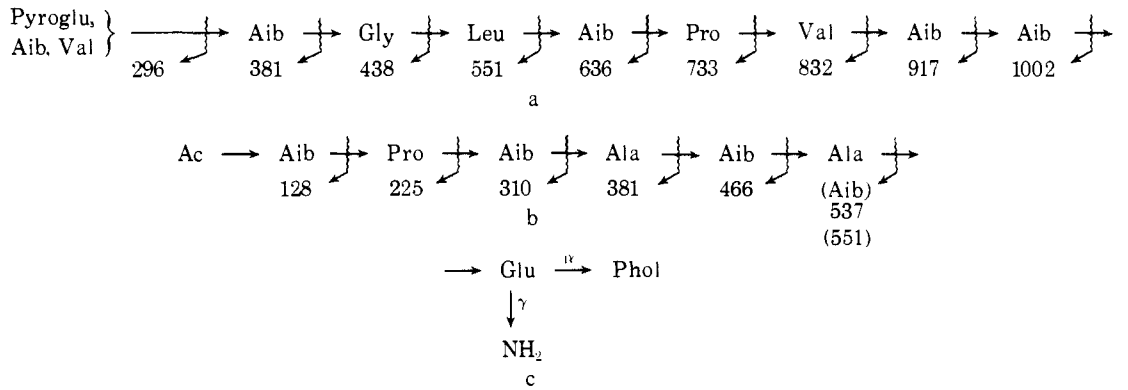


Table III. EIMS and HREIMS Data for Alamethicin Methyl Ester

m/e	Measured mass	Δ, mmu	Composition	Fragment lost	Fragment remaining
a					
1002					
984	984.5936	5.5	$C_{48}H_{78}N_{11}O_{11}$		
917				$-C_4H_7NO$ (Aib)	
899	899.5399	4.5	$C_{44}H_{71}N_{10}O_{10}$		
832				$-C_4H_7NO$ (Aib)	
814	814.4820	−0.6	$C_{40}H_{64}N_9O_9$		
733				$-C_5H_9NO$ (Val)	
715	715.4152	1.0	$C_{35}H_{55}N_8O_8$		
				$-C_5H_7NO$ (Pro)	
636			$C_{30}H_{50}N_7O_8$		
618	618.3608	−0.6	$C_{30}H_{48}N_7O_7$		
				$-C_4H_7NO$ (Aib)	
551	551.3200	0.7	$C_{26}H_{43}N_6O_7$		
				$-C_6H_{11}NO$ (Leu)	
438	438.2352	0.0	$C_{20}H_{32}N_5O_6$		
				$-C_2H_3NO$ (Gly)	
381	381.2146	0.9	$C_{18}H_{29}N_4O_5$		
297	297.1695	0.7	$C_{14}H_{23}N_3O_4$	$-C_4H_7NO$ (Aib)	
296	296.1592	−1.8	$C_{14}H_{22}N_3O_4$		Pyroglu→Aib→Val⁄
b					
551	551.3200	0.7	$C_{26}H_{43}N_6O_7$	$-C_4H_7NO$ (Aib)	
537	537.3041	0.5	$C_{25}H_{41}N_6O_7$		
				$-C_3H_5NO$ (Ala)	
466	466.2666	0.0	$C_{22}H_{36}N_5O_6$		
				$-C_4H_7NO$ (Aib)	
381	381.2146	0.9	$C_{18}H_{29}N_4O_5$		
				$-C_3H_5NO$ (Ala)	
310	310.1766	0.0	$C_{15}H_{24}N_3O_4$		
				$-C_4H_7NO$ (Aib)	
225	225.1224	−1.5	$C_{11}H_{17}N_2O_3$		
				$-C_5H_7NO$ (Pro)	
128	128.0702	−0.9	$C_6H_{10}NO_2$		
				−CO	
100	100.0759	−0.3	$C_5H_{10}NO$		Ac→NHC(CH$_3$)$_2$⁄
c					
306	306.1466	1.3	$C_{15}H_{20}N_3O_4$		⁄CO—NH—CH(CH$_2$—CH$_2$—CO—NH$_2$)—CO—NH—CH(CH$_2$—C$_6$H$_5$)—CH$_2$OH
				$-H_2O$	
288	288.1353	0.5	$C_{15}H_{18}N_3O_3$		
263	263.1405	0.9	$C_{14}H_{19}N_2O_3$		⁄CH(CH$_2$—CH$_2$—CO—NH$_2$)—CO—NH—CH(CH$_2$—C$_6$H$_5$)—CH$_2$OH
262	262.1339	2.2	$C_{14}H_{18}N_2O_3$	$-H_2O$	
245	245.1281	−0.9	$C_{14}H_{17}N_2O_2$		
178	178.0854	−1.4	$C_{10}H_{12}NO_2$		⁄CO—NH—CH(CH$_2$—C$_6$H$_5$)—CH$_2$OH
161	161.0838	−0.2	$C_{10}H_{11}NO$	$-H_2O$	
160	160.0772	1.0	$C_{10}H_{10}NO$		⁄CH(CH$_2$—C$_6$H$_5$)—CH$_2$OH
135	135.0777	−3.2	$C_9H_{11}O$		
134	134.0734	0.2	$C_9H_{10}O$	$-H_2O$	
117	117.0703	−0.1	C_9H_9		
91	91.0541	−0.7	C_7H_7		

In addition, it must be determined whether the thus far unassigned Glu or the Glu unit of a has the second carboxamide function and whether the three Glu units are linked to the peptide chain in α or γ linkages.

B. By GC–HREIMS. Alamethicin on partial acid hydrolysis under different conditions (1:1 12 N HCl:HOAc, room temperature, 72 h; 12 N HCl, 24 °C, 30 min; 12 N HCl, 37 ± 1 °C, 30 min; 12 N HCl, 45 ± 2 °C, 15 h; 12 N HCl, 60 ± 1 °C, 1 h) gave mixtures of different oligopeptides.

In order to confirm and extend the sequences a, b, and c assigned above by HREIMS, one of the above partial acid hydrolysis mixtures of oligopeptides (12 N HCl, 45 ± 2 °C, 15 h) was converted into the corresponding mixture of *N*-TFA *n*-butyl ester derivatives, which was analyzed by GC (Figure 7), GC–MS, and GC–HREIMS (Figure 8). The peaks identified are labeled in Figure 7. These oligopeptide sequences serve mainly to confirm in part (by overlapping) the peptide sequences assigned by HREIMS performed on alamethicin methyl ester. However, the tripeptide Glu → Aib → Val, whose GC mass spectrum is shown in Figure 8g, was especially valuable: in confirming the identities of the three N-terminal amino acids of a, in confirming that Glu is N-terminal and showing it is linked α to the peptide chain (assumed above from the presence of Pyroglu), and in assigning the order → Aib → Val →. Figure 9 summarizes the peptide sequences assigned by GC–HREIMS as well as those assigned by HRFDMS and FDMS in the next section.

C. By FDMS. We noted in the preceding section that partial hydrolyses of alamethicin were carried out under a variety of acidic conditions. These hydrolysates were examined by FDMS and, in some cases, by HRFDMS to identify the oligopeptides formed. The peaks observed varied with both the hydrolytic conditions employed and the emitter wire current. Two typical FD mass spectra of these mixtures of oligopeptides are shown in Figure 10. Those peaks for which high resolution data are available (mainly from the spectrum shown at the top

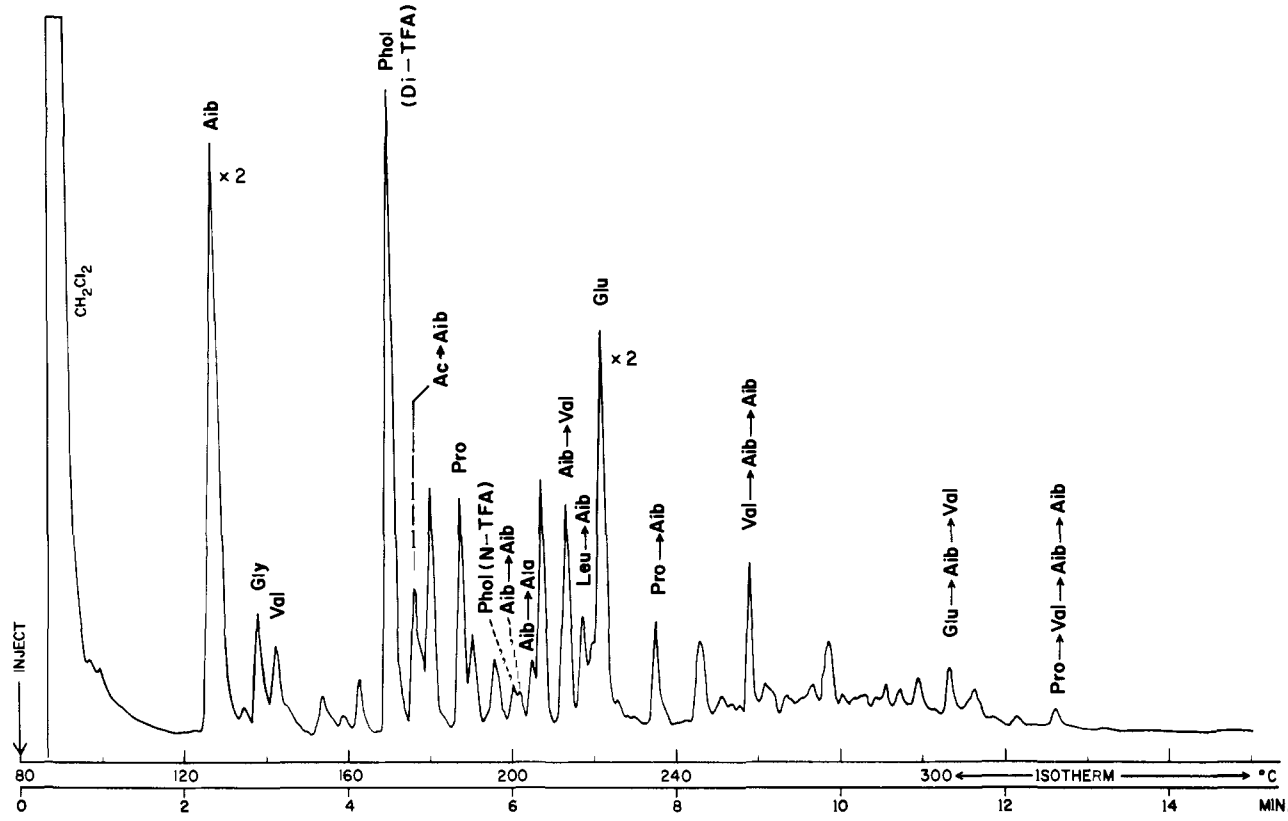


Figure 7. Gas-liquid chromatogram of a partial acid hydrolysate (12 N HCl, 45 ± 2 °C, 15 h) of alamethicin (*N*-TFA *n*-butyl esters, 3% OV-17, 6 ft).

of Figure 10) are the most informative.

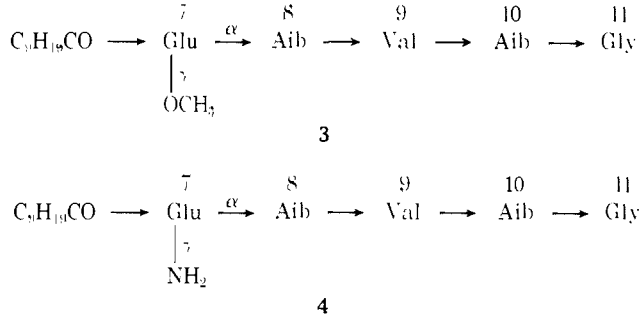
There are, first, a pair of ions (Figure 10, top) at *m/e* 428 and 442 which analyze by HRFDMS for $C_{19}H_{34}N_5O_6$ (428.2529, Δ 2.1 mmu) and $C_{20}H_{36}N_5O_6$ (442.2672, Δ 0.7 mmu), respectively. Thus, these are homologues from alamethicins I and II, respectively, and from the sequence b based on HREIMS and GC-HREIMS results (Figure 9), they can be assigned as Pro → Aib → Ala → Aib → Ala (+ H) and Pro → Aib → Ala → Aib → Aib (+ H). Two other peptides for which high resolution data are available belong to this part of the molecule, those giving the ions at *m/e* 357.2139 [Δ 0.2 mmu, $C_{16}H_{29}N_4O_5$, Pro → Aib → Ala → Aib (+ H)] and 272.1606 [Δ −0.4 mmu, $C_{12}H_{22}N_3O_4$, Pro → Aib → Ala (+ H)], the latter from a spectrum not shown in Figure 10. Other peaks which probably belong to this series, although high resolution data are not available, are the homologous pairs (Figure 10, bottom) 639-653, 737-751, 822-836, 879-893, and 1077-1091.

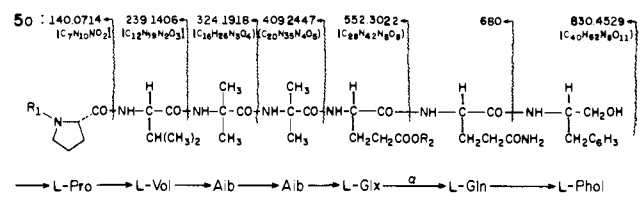
A second important set of peaks in the oligopeptides' FD mass spectra (Figure 10) is that consisting of the peaks at *m/e* 642, 514, and 385. High resolution indicates the composition of the *m/e* 385.2474 (Δ 2.3 mmu) ion to be $C_{18}H_{33}N_4O_5$, which corresponds to Pro → Val → Aib → Aib (+ H) [or, less likely, Aib → Pro → Val → Aib (+ H)] from the sequence a previously assigned by HREIMS. Addition of $C_5H_7NO_3$ (Glu) to give the peak at *m/e* 514.2902 (Δ 2.6 mmu, $C_{23}H_{40}N_5O_8$) can only be at the C-terminal position, corresponding to Pro → Val → Aib → Aib → Glu (+ H), while further extension by $C_5H_8N_2O_2$ to give the peak at *m/e* 642.3434 (Δ −2.8 mmu, $C_{28}H_{48}N_7O_{10}$), corresponds to Pro → Val → Aib → Aib → Glu → Glu (NH_2)(+ H); thus, the third Glu is between sequences a and c.

A final peak of interest is that at *m/e* 280.1443 [Δ 2.0 mmu, $C_{14}H_{20}N_2O_4$, Glu → Phol (+ H)], which confirms sequence c.

In toto, then, the FD mass spectra, taken with the earlier evidence, complete the sequences of alamethicins I and II shown in Figure 9. The only remaining questions are whether the Glu units between Aib and Phol are linked α or γ and whether the final amide function is on Glu-7 or Glu-18.

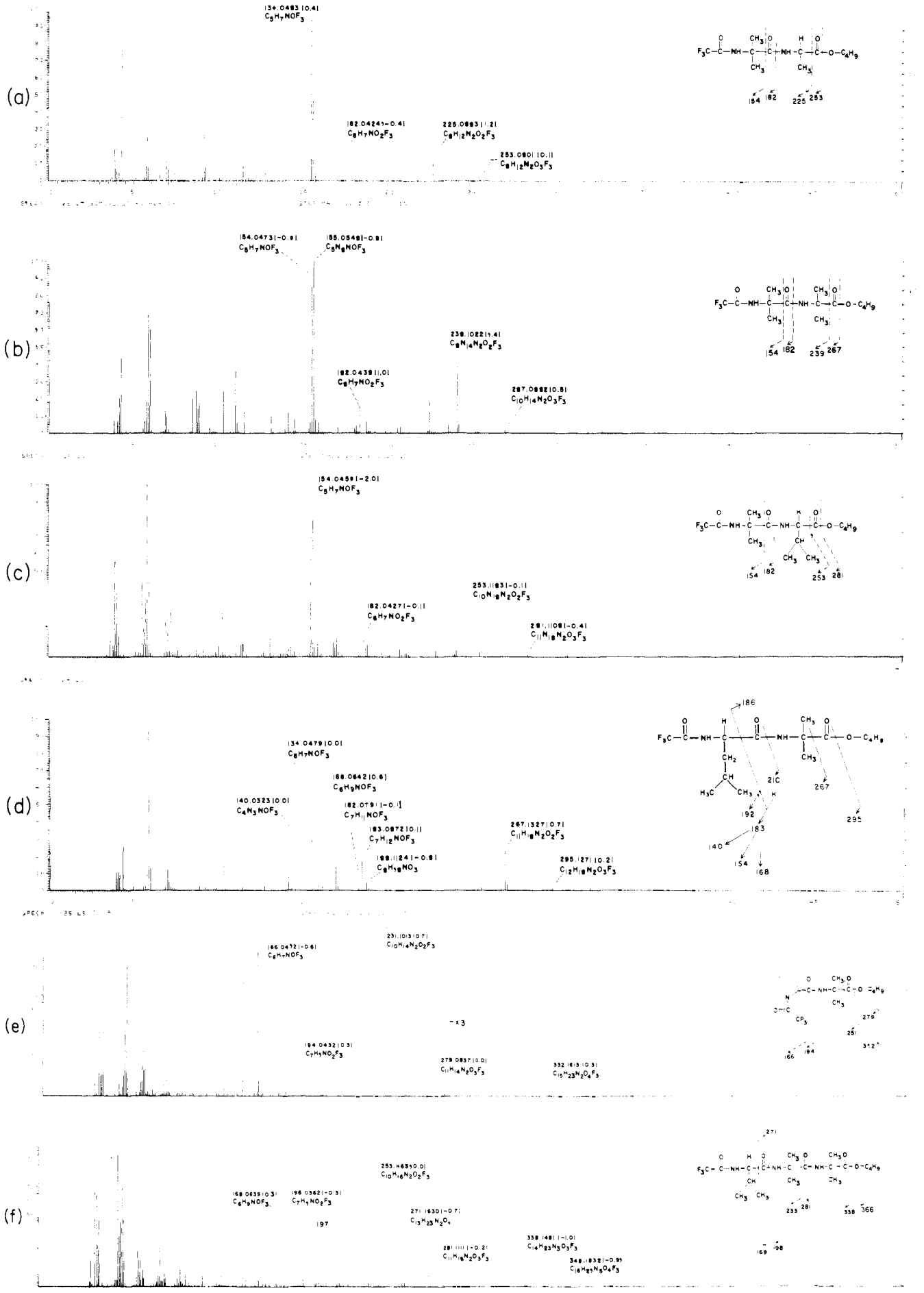
Consideration of Earlier Results. Published spectra from the earlier studies on alamethicin are available and two of these serve to locate the second primary amide group on Glu-7. Two peptides isolated by the Russian workers[17b] contain Glu, Aib, Val, Aib, and Gly and, thus, must come from amino acids 7-11 (Figure 9). These peptides (numbered by them "peptide 15" and "peptide 16") were assigned the structures **3** and **4**, re-





5o : R_1 = Ac, R_2 = CH_3

5b : R_1 = R_2 = H


(a)
(b)
(c)
(d)
(e)
(f)

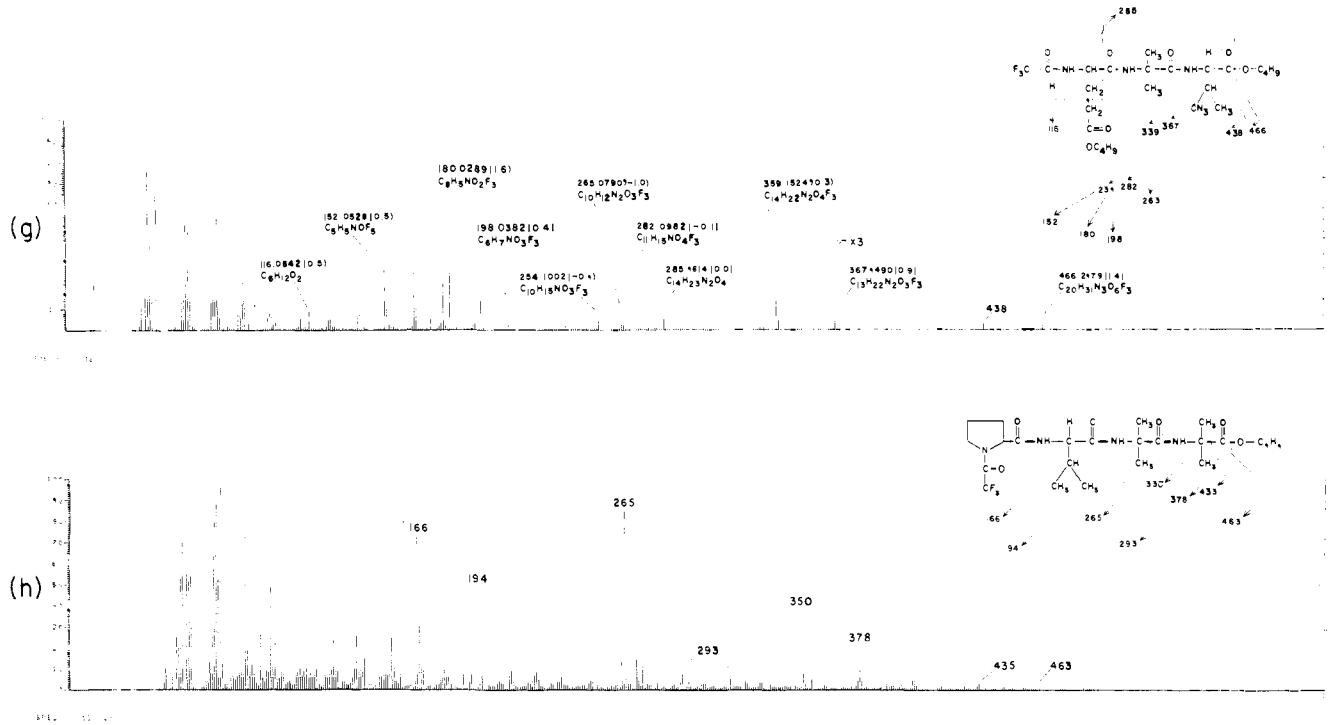


Figure 8. Mass spectra and fragmentation of oligopeptide derivatives (*N*-TFA *n*-butyl esters): (a) Aib → Ala; (b) Aib → Aib;(c) Aib → Val; (d) Leu → Aib; (e) Pro → Aib; (f) Val → Aib → Aib; (g) Glu α → Aib → Val; (h) Pro → Val → Aib → Aib.

spectively, shown here. Since the Glu carboxamide is present in "peptide 16", this locates the final amide group on Glu-7. Moreover, the fragmentation pattern of "peptide 16", shown in Figure 11, clearly assigns the amide to the γ-carboxyl of Glu, in agreement with the free γ-carboxyl assigned from GC-HREIMS (Figure 8g).

Isolation of a Pseudoheptapeptide Derivative (5a).[27a] In order to obtain a simpler compound containing the C-terminal Glu units, we turned to trifluoroacetic acid cleavage of alamethicin, which we had shown earlier[14b] to cleave antiamoebin selectively on the N-terminal side of Pro. Trifluoroacetolysis of alamethicin followed by N-acetylation, diazomethane methylation, and silica gel chromatography of the products did, in fact, give the *N*-acetyl methyl ester derivative **5a** of the desired heptapeptide **5b**. The EI mass spectrum of **5a** showed peaks at *m/e* 830.4529 (Δ −0.8 mmu, $C_{40}H_{62}N_8O_{11}$, M) and 812.4429 (Δ −0.2 mmu, $C_{40}H_{60}N_8O_{10}$, M − H_2O), and -CO-NH- cleavage peaks at *m/e* 680 ($C_{31}H_{50}N_7O_{10}$), 552.3022 (Δ −1.0, $C_{26}H_{42}N_5O_8$), 409.2447 (Δ −0.3, $C_{20}H_{33}N_4O_5$), 324.1918 (Δ −0.4, $C_{16}H_{26}N_3O_4$), 239.1406 (Δ −1.0, $C_{12}H_{19}N_2O_3$), and 140.0714 (Δ 0.2, $C_7H_{10}NO_2$) for successive losses of Phol, Gln, Glu-OCH_3, Aib, Aib, and Val. Among these the *m/e* 552 and 409 peaks were of special importance in confirming the amide location on Glu-18 and the methyl ester (hence free carboxyl in **5a**) location on Glu-17. Thus, this assignment provides independent evidence for the amide (Gln) location at Glu-7.

To identify the Glu peptide linkages as α or γ, the derivative **5a** was treated with ethylene chlorophosphite in triethyl phosphite, then reduced by sodium–liquid ammonia, a series which has been shown to convert Gln to Orn.[2b,13,27b] In the present study hydrolysis of dehydrated, reduced **5a** gave a mixture of amino acids, which were converted to their *N*-trifluoroacetyl *n*-butyl ester derivatives and subjected to GC, GC–EIMS and GC–HREIMS. As anticipated, the GC peaks for Phol and Glu were absent or much diminished (Figure 12, bottom spectrum). More informative, however, was the new peak for Orn, which establishes the primary amide in Glu-18 as γ, i.e., establishes Gln at position 18.

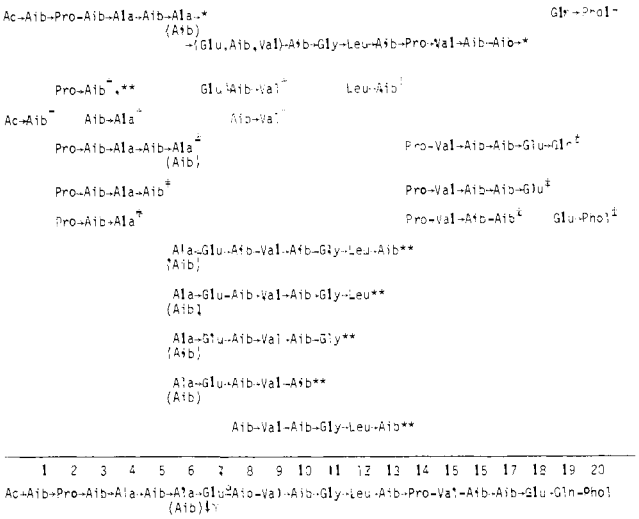


Figure 9. Peptide sequences determined by HREIMS.* GC-HREIMS,† HRFDMS,‡ or FDMS.** HRFDMS and FDMS cannot, of course, assign sequences from protonated molecular ions alone, and the HRFDMS and FDMS sequences shown here are assigned from the partial structures a, b, and c determined by HREIMS as well as sequences assigned by GC-HREIMS.

An additional benefit of the reduction procedure was the serendipitous identification of α-amino-δ-hydroxyvaleric acid (Figure 12, bottom spectrum) among the hydrolysis products. This compound, identified by its mass spectral fragmentation pattern, establishes Glu-17 as having a free γ-carboxyl. Additional evidence for the free γ-carboxyl is found in the ^{13}C NMR shifts of the Glu-17 side chain carbons on treatment with sodium hydroxide (Figure 13). As expected,[26] the carboxylate carbon shifts to lower field (181.32 ppm) in base. In addition, two methylene carbons (β-CH_2 and γ-CH_2, identified by their off-resonance splitting patterns), are found shifted to lower field in base, appearing at 28.9 and 35.7 ppm, respectively (Figure 13). With the linkage of Glu-17 established,

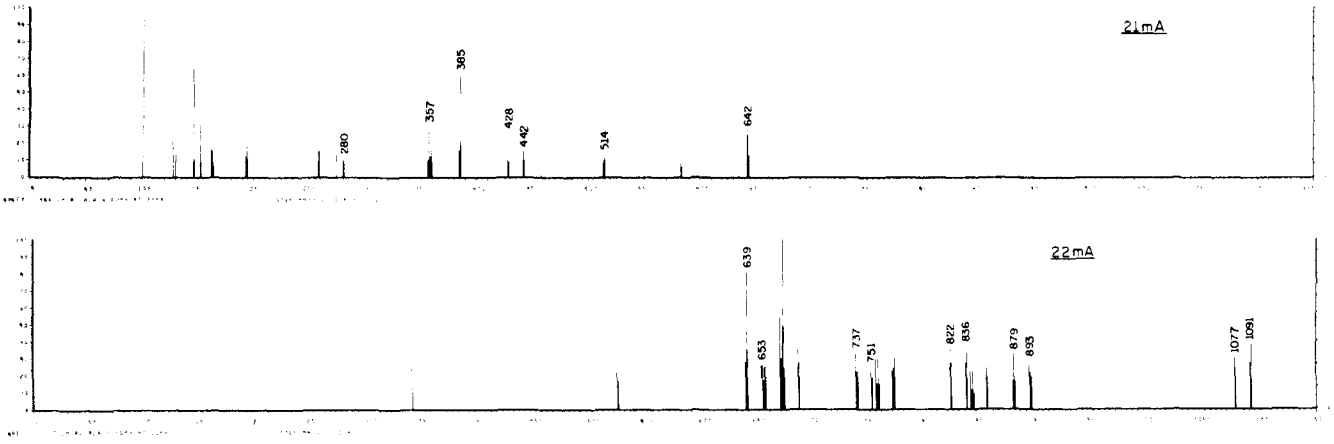


Figure 10. FD mass spectra of the oligopeptides (mixture) from acidic hydrolysis of alamethicin: top, 12 N HCl–glacial AcOH (1:1), 72 h, room temperature; bottom, 12 N HCl, 1 h, 60 ± 1 °C. Other hydrolytic conditions gave different mixtures, and the peaks observed varied with the emitter wire current.

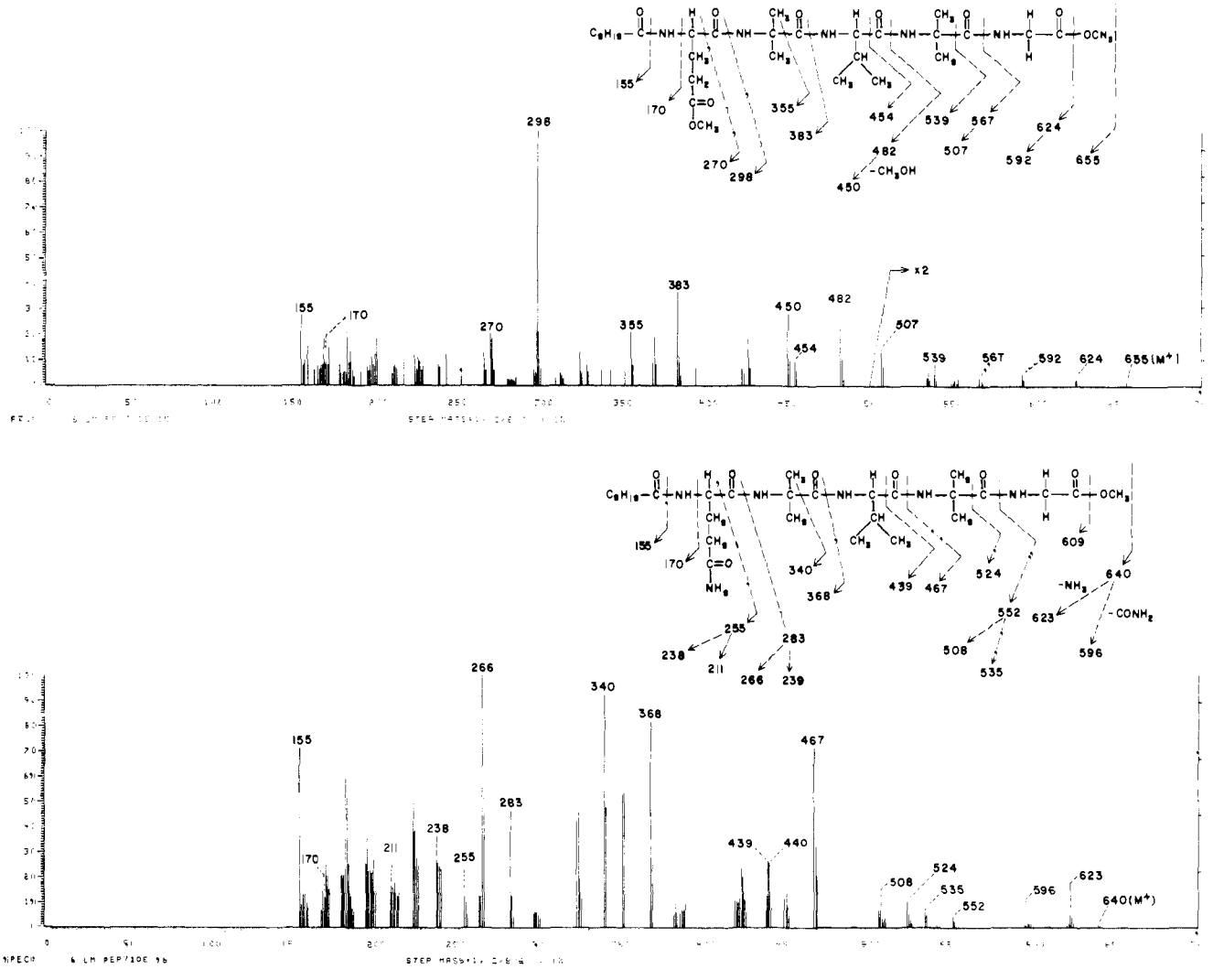
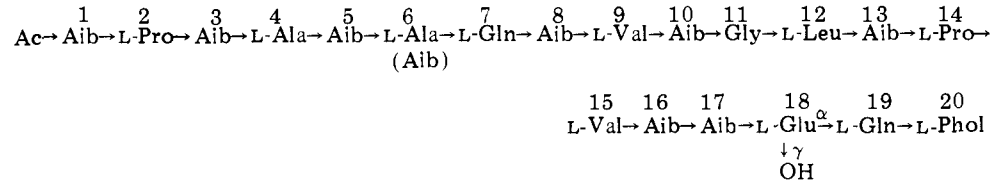


Figure 11. Mass spectra reported for **3** and **4** ("peptides 15 and 16", isolated by Ovchinnikov et al.).[13b]

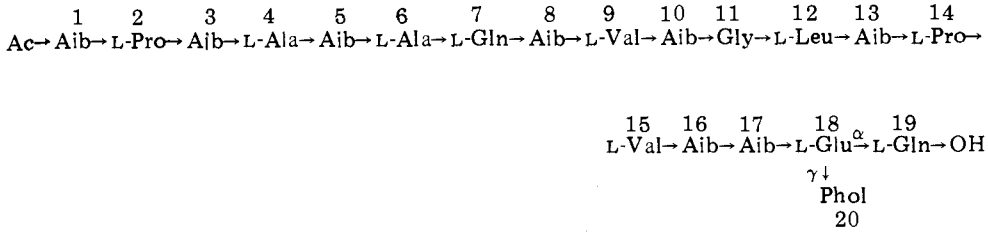
the structures of alamethicins I and II are assigned as **2-I** and **2-II**.

Alamethicin, Suzukacillin, and Related Peptide Antibiotics. The structures which we have assigned to alamethicins I and II are slightly different from that recently assigned by Martin and Williams to alamethicin I (their Component F30).[20,28a] Their structure (**6**) differs (from ours, **2-I, 2-II**) in attaching phenylalaninol to the penultimate glutamic acid residue, on the γ-carboxyl, rather than to the C-terminal glutamine on the α-carboxyl.[28b] Martin and Williams' structure was based largely on high resolution ^{1}H NMR spectral investigations of N-terminal and C-terminal groups and acceptance of amino acid sequences from the earlier work.[17] In particular, those authors[20] were unable to locate the Phol residue from their own

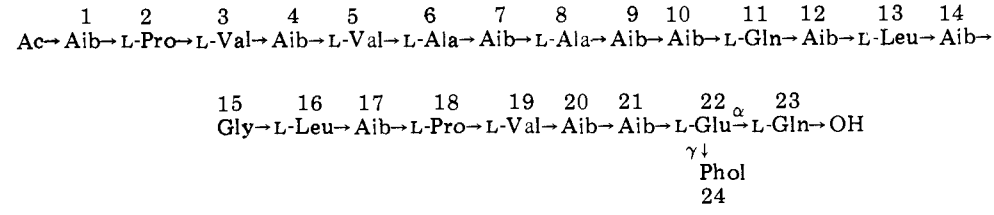
2-I (**2**-II), alamethicin I (II) (our structures)



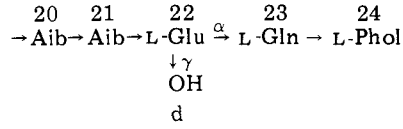
6, alamethicin I (Martin and Williams' structures)[20]



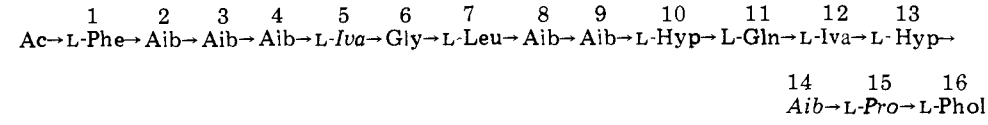
7, suzukacillin A (suggested structure)[12a]



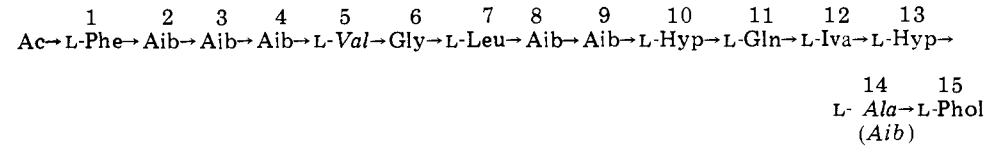
(present suggested structure)



8, antiamoebin I[13]



9 III (**9** IV), emerimicin III (IV)[2b]



data and, thus, accepted the earlier assignment, which we now revise. By contrast, our assignment of structure is essentially independent of earlier work.

As noted at the beginning of this paper, the antibiotic suzukacillin functions by a mode of action[12b] similar to that of alamethicin. Recent studies by Jung et al. on the structure of suzukacillin A[12a] have assigned it the tentative structure **7**, closely related to that proposed by Martin and Williams[20] for alamethicin I, with the linkage of Phol regarded as ambiguous.[12a] Since part of the structure of suzukacillin A was assigned by comparison of spectroscopic data with those for alamethicin, it seems likely that suzukacillin A has a C-terminal end more like those of alamethicins I and II, as in partial structure d.

We have recently assigned structures **8**, **9-III**, and **9-IV** to antiamoebin I,[13] emerimicin III,[2b] and emerimicin IV,[2b] respectively. As seen here, these antibiotics have structures quite similar to one another, differing mainly in chain length. They are also, more distantly, related to alamethicin and suzukacillin in that all are peptaibophol antibiotics, i.e., they contain several Aib units, a C-terminal Phol unit, and an N-terminal acetyl group. Aside from these points of similarity they all contain one Glu unit and mainly neutral, nonpolar amino acids (Gly, Ala, Iva, Val, Leu, Phe, Pro). Considerable additional effort is needed to establish the locus (or loci) of their bioactivities, but it may be noted here that antiamoebin I and emerimicins III and IV also have alamethicin-like ability to alter conductance across membranes.[29]

An additional point of interest regarding suzukacillin A is the appearance of a signal in its ^{13}C NMR spectrum, at 7.2 ppm (in CD_3OD), which was noted as being at unusually high field and unassignable to any of the amino acids reported for suzukacillin A.[12a] The authors also noted that they observed a similar signal (and two peaks at about 60 ppm) in the ^{13}C NMR spectrum of fresh alamethicin isolated from their culture of *T. viride*, which we have not observed in our alamethicin preparations. We suggest that it may be due, in suzukacillin A, to an undetected isovaline (Iva) residue. This suggestion is based on the observation that the methyl carbons of the ethyl groups in the two Iva residues of antiamoebin I[13] are found at 7.0 and 7.4 ppm (in Me_2SO-d_6), while the Iva β-methyl carbon is found at 6.9 ppm (in Me_2SO-d_6) in emerimicin IV (which has one Iva residue).[2b] It is not clear why Iva would not have been found by GC–MS, since it is readily detectable in GC–MS traces of antiamoebins and emerimicins, but it, like Aib, is difficult to detect by standard amino acid analysis. The presence of an Iva unit in suzukacillin A (or in an alamethicin component) would not be surprising in view of that amino acid's occurrence in antiamoebins and emerimicins, other peptaibophol antibiotics.

Experimental Section

General. Melting points, determined on a Kofler micro hot stage apparatus, are uncorrected. Infrared (IR) spectra were recorded on a Beckman IR-12 spectrophotometer and ultraviolet (UV) spectra were taken in methanol on a Beckman Acta MVI spectrophotometer.

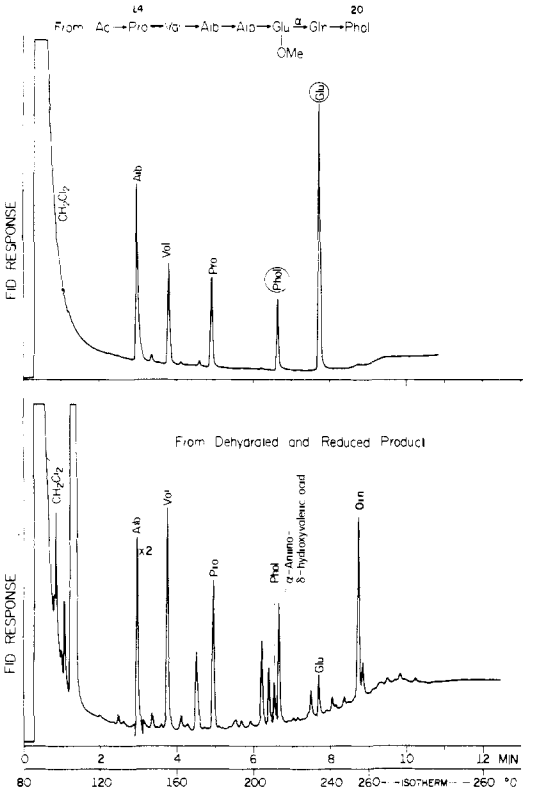


Figure 12. Gas-liquid chromatogram of *N*-TFA *n*-butyl ester derivatives of amino acids in total acid hydrolysate: (a) from Ac → Pro → Val → Aib → Aib → Glu α (γ → OMe) → Gln → Phol (**5a**); (b) after dehydration and sodium-liquid ammonia reduction of **5a**. Conditions: 0.65% EGA on 80-100 Chromosorb W AW, 6 ft × 2 mm i.d. glass column; flow 24 mL/min; attenuation, 1×10^{-10}, 10 mV; temperature program, 20 °C/min (80–260°C).

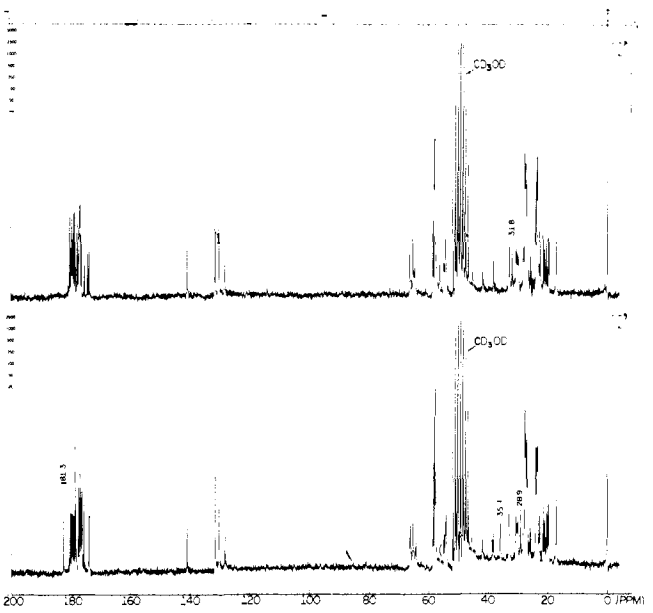


Figure 13. ^{13}C NMR spectra of alamethicin in methanol: before (top) and after (bottom) addition of 1 molar equiv of NaOD.

Optical rotations were measured on a Zeiss polarimeter. Proton magnetic resonance (^1H NMR) spectra were obtained by Mr. M. L. Miller on a Varian HR-220 spectrometer equipped with a Nicolet Instrument Corp. TT220 Fourier transform accessory, employing Me_4Si as the internal standard. Carbon magnetic resonance (^{13}C NMR) spectra were obtained by Mr. S. K. Silber on a Varian XLFT-100 spectrometer with Digilab computer. The spectra were measured in 12-mm sample tubes, employing Me_2SO-d_6 or $CDCl_3$ as the solvent, Me_4Si as internal standard, and a deuterium lock. Chemical shifts are reported in parts per million (ppm) from Me_4Si as internal standard.

Low resolution mass spectra were determined by Mr. J. Wrona with a Varian MAT mass spectrometer, Model CH-5DF, employing the direct probe technique. High resolution electron impact mass spectrometry (HREIMS), gas chromatography–high resolution electron impact mass spectrometry (GC-HREIMS), field desorption mass spectrometry (FDMS), and HRFDMS were measured on a Varian MAT mass spectrometer, Model 731, equipped with a combination EI-FD-field ionization (FI) ion source, a Varian Aerograph gas chromatograph, Model 2700, and a Varian mass spectrometry data system, Model SS100. HREI and HRFD mass spectra were measured either by the photoplate technique or by peak matching at a resolution of 16 000–18 000 for the EI spectra and 8000–12 000 for the FD spectra, using perfluoroalkane 250 (Pierce Chemical Co.) as a reference compound for photoplate and peak matching up to *m/e* 715 and tris(perfluoroheptyl)-*s*-triazine (PCR, Inc.) for peak matching from *m/e* 715 to 984 in the EI spectra. Methyl ethyl ketone ($M^{+\cdot}$ = *m/e*), 2-pentanone (86), methyl isobutyl ketone (100), 3-methylcyclohexanone (112), acetophenone (120), 2-octanone (128), 5-nonanone (142), 2-decanone (156), 2,6-dibromoaniline (249), tris(perfluoromethyl)-*s*-triazine (285), hexachlorocyclotriphosphazene (347), 2,6-diiodo-4-nitrophenol (391), tris(perfluoroethyl)-*s*-triazine (435), and tris(perfluoropropyl)-*s*-triazine (516, M − CF_3; and 585, M) were employed for peak matching FD spectra. The latter compounds have strong FI peaks at convenient masses, do not desensitize the FD emitters, and have suitable volatility for admission to the mass spectrometer source by an available inlet system, i.e., either a gas inlet system or a solid sample probe.[30] All the GC-HREIMS samples were measured by the photoplate technique, with the photoplates (Ionomet, silver halide) being read by the SS100 data system and a Gaertner F-9418M microdensitometer. Preliminary gas chromatography was carried out with a Varian gas chromatograph, Series 1700, using 6 ft × 2 mm i.d. helical glass columns, and the same columns were then used on the gas chromatograph (Varian Aerograph 2700) coupled to the mass spectrometer. Amino acid analyses were carried out by Dr. K. M. Dus, St. Louis University, using a Beckman/Spinco Model 120 amino acid analyzer. Microanalyses were determined by Mr. J. Nemeth and associates. Thin layer chromatography was carried out on precoated thin layer chromatography plates (silica gel GF, 250 μ, Analtech). The spots were visualized either by exposure to iodine vapors or by spraying with sulfuric acid. The spots could also be visualized by hydrochloric acid–ninhydrin spray reagent.[31]

Alamethicin (Upjohn, U-22324, 8831-CEM-93.1), recrystallized from methanol, has mp 275–279 °C (lit.[18] 259–260 °C); R_f 0.09 (major spot), 0.14 and 0.30 (minor spots) in chloroform–methanol (6:2 v/v); R_f 0.46 (major spot), 0.35 (minor) in BAW 415 (upper layer, UL); R_f 0.32 in BEW 415 (UL); pK_a = 6.04 in 50% ethanol (lit.[17] 5.8; lit.[21] 5.5); UV (MeOH) weak absorptions at 267 (ϵ 149), 263 (199), 257 (279), and 252 nm (299) and strong end absorption ϵ_{210} 26 462; IR (KBr) 3450 (sh), 3340, 3070 (sh), 2990, 2945, 2880, 1665, 1540, 1470, 1455, 1415, 1390, 1368, 1300, 1222, 1195, 1128, 1050, 600 (br), 420 cm^{-1}; ^{13}C NMR (Me_2SO-d_6) carbonyl carbons (singlets in off-resonance spectrum) at 176.4 (1 carbon), 176.1 (1), 175.8 (2), 175.7 (1), 175.6 (1), 175.4 (1), 175.2 (1), 174.1 (1), 173.9 (1), 173.8 (1), 173.7 (2), 173.6 (1), 173.5 (1), 173.3 (1), 173.1 (1), 173.0 (1), 172.8 (1), 171.9 (1), 171.0 (1), 170.3 (1), 169.7 (1), phenyl carbons at 139.0 (s, C-1), 129.1 (d, ortho carbons), 127.9 (d, meta carbons), 125.8 ppm (d, para carbon); EIMS (70 eV) major ions at *m/e* 91, 100, 123, 153, 161, 181, 225, 245, 273, 282, 310, 359, 381, 391, 420, 466, 487, 491, 505, 515, 517, 533, 542, 560, 572, 600, 618, 653, 654, 670, 671, 697, 727, 752, 771, 796, 814, 837, 881, 908, 935, 965, 1019; FDMS (23.5 mA) highest ions at *m/e* 1976 ± 1 and 1962 ± 1. Anal. Calcd for $C_{92}H_{150}N_{22}O_{25}\cdot H_2O$: C, 55.74; H, 7.72; N, 15.54; $-CONH_2$, 0.81; neut equiv, 1982.[24a] Calcd for $C_{93}H_{152}N_{22}O_{25}\cdot H_2O$: C, 55.95; H, 7.78; N, 15.44; $-CONH_2$, 0.80; neut equiv, 1996.[24a] Found: C, 55.75, 55.93; H, 7.24, 7.80, N, 15.52, 15.48; $-CONH_2$, 1.79, 1.76; neut equiv, 1960.

Alamethicin Acetate. A mixture of alamethicin (16 mg), pyridine (0.25 mL), and acetic anhydride (0.5 mL) was stirred under anhydrous conditions at room temperature for 20 h, then excess acetic anhydride was decomposed with 2 mL of deionized water at 0 °C. Solvent was removed under vacuum and the residue was dissolved in methanol and precipitated from ether: mp 175–180 °C; R_f 0.10 (major spot), 0.18 and 0.34 (minor spots) in chloroform–methanol

(6:2 v/v); R_f 0.49 (major spot), 0.54 and 0.39 (minor spots) in BAW 415 (UL); R_f 0.35 in BEW (UL); UV (MeOH) very weak absorption at 268, 264, 258, and 253 nm and strong end absorption, ϵ_{210} 26 801; IR (KBr) 3460 (sh), 3360, 3000, 1740 (sh), 1675, 1550, 1475, 1420, 1395, 1372, 1305, 1235, 1200, 1050, 600 (br), 422 cm^{-1}; ^1H NMR (100 MHz, Me_2SO-d_6) 1.98 ppm (-$COCH_3$). Anal. Calcd for $C_{94}H_{152}N_{22}O_{26}$: mol wt, 2004. Calcd for $C_{95}H_{154}N_{22}O_{26}$: mol wt, 2018.[24a] Found: mol wt, 2004, 2018 (FDMS). Calcd for $C_{94}H_{151}N_{22}O_{26}Na$: mol wt, 2026.[24a] Calcd for $C_{95}H_{153}N_{22}O_{26}Na$: mol wt, 2040. Found: mol wt, 2026, 2040 (FDMS).

Alamethicin Methyl Ester. A solution of diazomethane in tetrahydrofuran was added slowly to a solution of alamethicin (492 mg) in 10 mL of methanol until the yellow color persisted. After 30 min at room temperature excess diazomethane was destroyed by a few drops of acetic acid. Solvent removal afforded 484 mg (98%) of the derivative, which was crystallized from chloroform–ethyl ether: mp 275–276 °C (lit.[17b] 240–242 °C); R_f 0.70 (major spot), 0.74 and 0.78 (minor spots) in chloroform–methanol (6:2 v/v); R_f 0.48 (major spot), 0.36 (minor spot) in BAW 415 (UL); R_f 0.50 in BEW 415 (UL); IR (KBr) 3350, 3000, 1740 (sh), 1675, 1550, 1475, 1422, 1390, 1370, 1300, 1228, 1202, 1182, 1050, 600 (br), 422 cm^{-1}; ^1H NMR (100 MHz, $CDCl_3$) 3.62 (-$COOCH_3$), 2.02 ppm (-$COCH_3$) in the ratio of 1:1; ^{13}C NMR ($CDCl_3$) carbonyl carbon singlets at 177.7 (1), 177.4 (2), 177.1 (1), 176.8 (1), 176.5 (1), 176.4 (1), 176.2 (1), 175.8 (1), 175.1 (2), 174.9 (2), 174.6 (1), 174.4 (2), 174.0 (1), 173.8 (2), 173.5 (1), 172.7 (1), 171.2 (1), and 170.9 (1), phenyl carbons at 138.9 (s, C-1), 129.7 (d, ortho carbons), 128.2 (d, meta carbons), 126.2 (d, para carbon), other carbons at 71.7, 64.8, 64.3, 63.3, 59.1, 58.7, 58.3, 56.8, 56.6, 55.8, 52.8, 51.5, 48.7, 44.1, 40.1, 37.0, 32.4, 30.8, 29.7, 29.3, 27.1, 26.0, 25.2, 24.5, 23.7, 23.3, 22.7, 21.3, 20.7, 20.3, 19.1, and 16.8 ppm; the EI mass spectrum (70 eV) is shown in Figure 6 and HREIMS data are given in Table III. Anal. Calcd for $C_{93}H_{152}N_{22}O_{25}\cdot 4H_2O$: C, 54.48; H, 7.87; N, 15.03. Calcd for $C_{94}H_{154}N_{22}O_{25}\cdot 4H_2O$: C, 54.69; H, 7.91; N, 14.93. Found: C, 54.64; H, 7.60; N, 14.41.

Alamethicin Methyl Ester Acetate. A mixture of alamethicin methyl ester (12 mg), pyridine (0.25 mL), and acetic anhydride (0.5 mL) was stirred under anhydrous conditions at room temperature for 20 h. The reaction mixture was then cooled (0 °C) and stirred with 2 mL of deionized water for 1 h. Solvent was removed under vacuum and the residue was dissolved in methanol and precipitated from ether: mp 145–150 °C; R_f 0.73 (major spot), 0.80, 0.88 (minor spots) in chloroform–methanol (6:2 v/v); R_f 0.48 (major spot), 0.54 (minor spot) in BAW 415 (UL); R_f 0.52 (major spot), 0.56 (minor spot) in BEW 415 (UL); UV (MeOH) very weak absorptions at 252, 257, 263, and 267 nm and strong end absorption, ϵ_{210} 26 720; IR (KBr) 3460 (sh), 3350, 2990, 1745, 1670, 1550, 1470, 1420, 1390, 1370, 1300, 1230, 1200, 1180, 1050, 600 (br), 420 cm^{-1}; ^1H NMR (100 MHz, $CDCl_3$) 3.60 (-$COOCH_3$) and 2.04 ppm (-$COCH_3$) in the ratio of 1:2. Anal. Calcd for $C_{95}H_{154}N_{22}O_{26}\cdot H_2O$: C, 55.97; H, 7.71; N, 15.12. Calcd for $C_{96}H_{156}N_{22}O_{26}\cdot H_2O$: C, 56.18; H, 7.76; N, 15.01. Found: C, 56.28; H, 7.60; N, 14.79.

Acidic Hydrolysis of Alamethicin to Amino Acids and Phenylalaninol. A. Amino Acid Analysis. A mixture of 10 mg of alamethicin and 1.0 mL of 6 N aqueous hydrochloric acid sealed in a Pyrex tube (no. 9820, 1 × 10 cm) under nitrogen was heated at 110 ± 1 °C for 24 h in an oven; then the tube was cooled, opened, and dried over potassium hydroxide pellets. The residue was dissolved in 0.5 mL of deionized water and dried again and this was repeated two to three times to remove last traces of hydrogen chloride. FDMS (10 mA, Figure 1) of the mixture showed M + H ions at *m/e* 76 (Gly), 90 (Ala), 104 (Aib), 116 (Pro), 118 (Val), 132 (Leu), 148 (Glu), and 152 (Phol), whose compositions determined by HRFDMS are reported in Table I. The amino acid analyzer indicated the following molar ratios: Aib (8.2 ± 0.2), Ala (1.7), Val (2.0), Gly (1.0), Leu (1.0), Pro (1.9), Glu (3.1), Phol (traces).

B. *N*-Trifluoroacetyl *n*-Butyl Ester Derivatives. A portion (5 mg) of the above hydrolysate from alamethicin and 1 mL of 3 N hydrochloric acid in *n*-butyl alcohol were heated at 100 ± 2 °C for 30 min (oil bath) in a Teflon-lined screw capped Pyrex tube (no. 9826, 1 × 10 cm). The reaction mixture was dried under vacuum to remove all butanolic hydrochloric acid and the residue was heated with 1 mL of methylene chloride and 0.5 mL of trifluoroacetic anhydride at 150 ± 2 °C for 5 min in an oil bath. The tube was cooled (0 °C), solvent was removed at 0 °C (to eliminate loss of low-boiling volatile derivatives) under nitrogen, and the residue was diluted with fresh methylene chloride and analyzed by GC using 3% OV-17 on 100–120 mesh GCQ (6 ft × 2 mm i.d. glass column) and 0.65% EGA on 80–100 mesh Chromosorb W AW (6 ft × 2 mm i.d. glass column) (Figure 2).

C. *N*-Trifluoroacetyl Methyl Ester Derivatives. A portion (5 mg) of the alamethicin hydrolysate above and 1 mL of 3 N hydrochloric acid in methanol were heated at 60 ± 2 °C for 1 h in a Teflon-lined screw capped Pyrex tube (no. 9826, 1 × 10 cm). The reaction mixture was worked up and trifluoroacetylated as in B. The derivatives were analyzed on a column of 10% *N*-lauroyl-*N'*-*tert*-butyl-L-valinamide on 60–80 mesh Chromosorb W AW (12 ft × 2 mm i.d. glass column, helium flow 60 mL/min). A comparison of the derivatives with an authentic mixture of racemic amino acids is shown in Figure 3.

D. Isolation of L-Phenylalaninol. Alamethicin (100 mg) was hydrolyzed as in A above, and the reaction mixture was basified to ~pH 12 and extracted with chloroform (10 mL × 4). The combined chloroform extract was washed twice with water (10 mL) and once with brine (10 mL), then dried over sodium sulfate. Removal of solvent afforded 6.4 mg of crystalline phenylalaninol: mp 87–88 °C; R_f 0.59 (Analtech as well as freshly prepared silica gel plates), 0.70 (Eastman Chromagram sheet, 13181, silica gel) in BAW 415 (UL) (lit.[16] 0.72). Recrystallization from benzene gave fine, long needles, mp 94–95 °C (undepressed in mixture melting point with an authentic sample), $[\alpha]^{25}_D$ −27.06° (*c* 0.32, MeOH) [lit.[16] $[\alpha]^{24}_D$ −25.76° (*c* 3.3, EtOH), lit.[32] $[\alpha]^{24}_D$ −23.3° (*c* 2.01, MeOH)].

One milligram of the above crystalline compound was converted into its *N,O*-bis(trifluoroacetyl) derivative by heating with 1 mL of methylene chloride and 0.2 mL of trifluoroacetic anhydride at 150 °C for 5 min. Solvent was removed and the residue was dissolved in 0.5 mL of fresh methylene chloride and analyzed by GC and GC–MS. The derivative had a retention time of 4.4 min on a 3% OV-17 column (flow rate, 36 mL/min; temperature programmed at 20 °C/min from 80 to 300 °C) and 6.4 min on a 0.65% EGA column (flow rate, 24 mL/min; temperature programmed at 20 °C/min from 80 to 210 °C). GC–EIMS did not show the molecular ion but showed fragment ions at *m/e* 231 (2% of base peak), 230 (27), 229 (97), 215 (5), 211 (3), 200 (2), 198 (7), 197 (5), 187 (1), 177 (5), 170 (1), 162 (3), 161 (6), 160 (3), 152 (1), 145 (3), 142 (4), 141 (1), 140 (1), 138 (5), 137 (47), 133 (5), 132 (30), 131 (4), 118 (20), 117.9 (39), 117 (97), 116 (97), 114 (78), 109 (31), 105 (24), 104 (12), 103 (39), 102 (11), 96 (13), 95 (3), 92 (74), 91 (96), 90 (20), 89 (25), 78 (20), 77 (41), 68 (95), 65 (100), 62 (27); GC–FIMS showed ions at *m/e* 344 (9), 343 (25, $M^{+\cdot}$), 232 (6), 231 (12), 230 (100), 229 (12), 91 (23); GC–HREIMS showed the compositions $C_{11}H_9F_3O_2$ (230.0555, M − CF_3CONH_2), C_4F_3NO (138.0168, $CH_2{=}CNHCOCF_3$), C_9H_9 (117.0698, 230 − CF_3CO_2), C_8H_7 (103.0547, $C_6H_5CH{=}CH$).

Hydrolysis of Alamethicin to Oligopeptides. A. 12 N Hydrochloric Acid, 60 °C, 1 h. A mixture of 29.0 mg of alamethicin and 2 mL of 12 N hydrochloric acid was stirred at 60 ± 1 °C for 1 h. The reaction mixture was cooled, diluted with water (10 mL), and freeze dried. The residue, analyzed by FD, gave major ions (10 mA) at *m/e* 100 (100%, base peak), 101 (94% of base peak), 146 (52), 152 (28), 159 (30), 217 (17), 231 (15), 274 (15), 286 (17), 303 (17), 304 (54), 305 (13), 318 (13), 319 (22), 371 (22), 372 (13), 373 (13), 385 (20), 398 (24), 399 (54), 400 (19), 442 (26), 456 (17), 542 (13); (15 mA) at *m/e* 100 (38% of base peak), 101 (40), 116 (22), 146 (49), 152 (54), 160 (75), 217 (14), 231 (13), 271 (14), 272 (68), 273 (11), 274 (22), 286 (29), 287 (27), 288 (27), 303 (14), 304 (41), 314 (22), 319 (16), 328 (16), 339 (16), 357 (33), 358 (13), 359 (14), 371 (41), 372 (14), 373 (21), 384 (14), 385 (78), 386 (24), 398 (29), 399 (100, base peak), 400 (27), 413 (27), 442 (49), 443 (16), 456 (30), 457 (11), 541 (13), 542 (27), 543 (14), 671 (44), 672 (21), 684 (21), 685 (65), 686 (24), 699 (16); and (22 mA) at *m/e* 339 (32), 523 (22), 524 (16), 638 (27), 639 (81), 640 (35), 653 (27), 654 (16), 655 (24), 656 (24), 669 (54), 670 (30), 671 (100, base peak), 672 (49), 673 (24), 685 (35), 686 (27), 737 (32), 738 (22), 739 (22), 751 (22), 752 (19), 755 (30), 756 (15), 757 (30), 758 (15), 770 (22), 771 (24), 772 (30), 822 (35), 823 (27), 836 (27), 837 (32), 840 (22), 841 (11), 842 (22), 843 (9), 854 (19), 855 (24), 878 (16), 879 (32), 880 (16), 893 (27), 894 (22), 895 (19), 1077 (30), 1078 (24), 1091 (27), 1092 (38).

B. 12 N Hydrochloric Acid, 45 °C, 15 h. A mixture of 37 mg of alamethicin and 3.5 mL of 12 N hydrochloric acid was heated at 45 ± 2 °C for 15 h, solvent was removed, and the residue was analyzed by FDMS (17 mA). Major ions were found at *m/e* 104 (12% of base peak), 115 (6), 116 (8), 118 (4), 130 (4), 132 (4), 148 (6), 152 (100, base peak), 153 (11), 161 (8), 162 (9), 175 (4), 176 (4), 189 (4), 192 (8), 200 (4), 201 (13), 203 (5), 214 (6), 217 (8), 272 (9), 286 (8), 290 (6), 291 (6), 300 (4), 304 (7), 305 (9), 319 (4), 346 (6), 357 (11), 371

(5), 385 (15), 386 (4), 387 (4), 399 (22), 425 (5), 428 (4), 431 (4), 442 (6), 542 (10), 543 (11), 544 (7), 545 (5), 546 (5), 547 (4), 550 (6), 552 (4), 558 (5), 559 (8), 560 (7), 561 (4), 562 (6), 564 (6), 565 (4), 571 (5), 573 (9), 574 (5), 575 (4), 576 (5), 583 (6), 585 (11), 587 (8), 588 (4), 599 (5), 601 (6), 617 (5), 644 (4), 665 (10), 657 (7), 658 (5), 659 (8), 670 (8), 671 (10), 672 (9), 685 (6), 686 (14), 687 (4), 769 (4), 854 (6).

Two milligrams of the above hydrolysate in 1 mL of 3 N hydrochloric acid in *n*-butyl alcohol was heated at 100 ± 1 °C for 30 min. Solvent was removed and the residue was treated with 1 mL of methylene chloride and 0.5 mL of trifluoroacetic anhydride and heated at 150 °C for 5 min. Solvent was removed and the residue was dissolved in fresh methylene chloride and analyzed by GC (Figure 7), GC-EIMS, and GC-HREIMS (Figure 8).

C. 12 N Hydrochloric Acid, 37 °C, 5 h. A mixture of 37 mg of alamethicin and 3.5 mL of 12 N hydrochloric acid was heated at 37 ± 1 °C for 5 h, then solvent was removed under high vacuum. The residue, analyzed by FDMS (21 mA), had major ions at *m/e* 79 (59% of base peak), 80 (40), 84 (26), 100 (100, base peak), 101 (47), 120 (23), 146 (37), 152 (43), 160 (81), 272 (20), 273 (13), 286 (12), 287 (20), 339 (14), 357 (16), 371 (16), 385 (21), 398 (15), 399 (24), 442 (21), 456 (15), 527 (12), 528 (13), 542 (15), 683 (21), 684 (51), 685 (24), 698 (12).

D. Hydrochloric Acid–Acetic Acid, 72 h. A mixture of 40 mg of alamethicin and 4 mL of 12 N hydrochloric acid–glacial acetic acid (1:1) was stirred at room temperature for 72 h, then the reaction mixture was dried under high vacuum. The residue, analyzed by FDMS (21 mA), had intense ions at *m/e* 101 (100%, base peak), 128 (22% of base peak), 130 (13), 146 (64), 147 (10), 152 (31), 153 (9), 162 (17), 163 (16), 164 (9), 193 (12), 194 (19), 195 (9), 258 (24), 259 (14), 274 (13), 280 (10), 281 (9), 356 (10), 357 (31), 358 (12), 359 (17), 360 (9), 384 (16), 385 (62), 386 (19), 428 (27), 429 (9), 442 (16), 443 (10), 458 (10), 513 (8), 514 (14), 515 (10), 583 (9), 584 (7), 642 (28), 643 (22), 644 (11). These ions were analyzed by HRFDMS (see text).

E. 12 N Hydrochloric Acid, 24 °C, 30 min. Alamethicin (30.4 mg) was stirred in 2 mL of 12 N hydrochloric acid at 24 °C for 30 min, then the mixture was cooled, diluted with 10 mL of deionized water, and freeze dried. The product analyzed by FDMS (28 mA), had intense ions at *m/e* 70 (44% of base peak), 84 (97), 86 (12), 91 (19), 100 (26), 101 (29), 128 (25), 129 (17), 130 (39), 149 (100, base peak), 150 (16), 153 (22), 169 (21), 171 (16), 244 (15), 245 (30), 262 (17), 263 (26), 281 (15), 282 (48), 299 (14), 373 (34), 374 (15), 465 (11), 492 (12), 493 (21), 505 (21), 506 (14), 537 (32), 538 (13), 590 (14), 618 (29), 619 (15), 635 (10), 636 (18), 637 (13), 786 (14), 898 (24), 899 (20), 916 (25), 917 (15), 982 (13), 983 (47), 984 (34), 985 (20), 1001 (35), 1002 (22).

Trifluoroacetolysis of Alamethicin to Pseudoheptapeptide 5a.[27a] A solution of 1.0 g of alamethicin (dried under high vacuum for 24 h) in 10 ml of trifluoroacetic acid was stirred at 24 °C for 26 h. Solvent was removed and the residue was taken up in methanol (20 mL) and esterified with ethereal diazomethane. Excess diazomethane was decomposed with a few drops of glacial acetic acid, solvent was removed, and the residue was N-acetylated with acetic anhydride (1 mL) in 50 mL of methanol at 24 °C for 12 h. Solvent was removed and the residue was chromatographed on a column (2.4 × 40 cm) of E. Merck silica gel (particle size less than 0.08 mm); 300 3-mL fractions were collected. Elution of the mixture with 10%, 20%, and 40% methanol in chloroform gave fractions 1 to 192, 193 to 270, and 271 to 300, respectively. About 0.5 mg of material from every fifth tube containing product was hydrolyzed with 0.2 mL of 6 N hydrochloric acid at 110 ± 1 °C for 24 h, and the hydrolyzed material was derivatized (*N*-TFA *n*-butyl esters) and analyzed by GC using 0.65% EGA on 80–100 Chromosorb W AW (6 ft × 2 mm i.d. glass column). Fractions 116 to 220 were found to contain 1 Pro, 1 Val, 2 Aib, 2 Glu, and 1 Phol (Figure 12, upper chromatogram). Other fractions, which gave amino acids from other peptides, were not investigated. The combined fractions 116 to 220 afforded 58.5 mg (~15%) of white crystalline product, mp 100–104 °C, R_f 0.66 in chloroform–methanol (6:2, v/v); R_f 0.31 in BAW 415 (UL); IR (KBr) 3460, 3360, 2980, 1740, 1670 (s), 1548, 1458, 1390, 1370, 1300, 1220, 1180, 1050 cm^{-1}; ^1H NMR (220 MHz, $CDCl_3$) 0.95 (3, d, J = 7 Hz), 1.47 (6, s), 1.50 (3, s), 1.56 (3, s), 2.19 (2, s, $-COCH_3$), 3.64 (3, s, $-COOCH_3$), 7.26, 7.23, 7.19 (5, Ar); EIMS (70 eV) *m/e* 830 (M^+), 812, 800, 781, 769, 754, 739, 721, 680, 646, 552, 523, 492, 474, 438, 426, 408, 368, 353, 325, 296, 268, 241, 240, 239, 211, 197, 185, 171, 169, 149, 140, 129, 120, 112, 100, 91, 84, 74, 71, 60, 58; HREIMS data are discussed in the text. Anal. Calcd for $C_{40}H_{62}N_8O_{11}$: mol wt, 830.4537. Found: mol wt, 830.4529 (HREIMS).

Dehydration and Sodium–Ammonia Reduction of Pseudoheptapeptide 5a.[27a] In a thick-walled test tube (1.5 × 15 cm) with a tightly closed screw top, 10.4 mg of pseudoheptapeptide **5a**, 0.25 mL of ethylene chlorophosphite, and 1.25 mL of triethyl phosphite were heated in an oil bath at 100 ± 2 °C for 22 h. The tube was cooled and its contents allowed to stand at room temperature for 30 min with 1.0 mL of absolute ethanol to decompose excess reagent, then concentrated under high vacuum at ~70 °C to a yellow, syrupy mass. Absolute methanol (0.2 mL) and condensed ammonia gas were added and the reaction mixture was stirred at −75 °C while ca. 50 mg of sodium was added, in small pieces, until the solution turned blue. After 2 min more of stirring, 80 mg of ammonium chloride was added and a very slow stream of nitrogen was applied to remove ammonia. The product was extracted with 1-butanol and washed with aqueous ammonium chloride and water. Solvent was removed under high vacuum and the residue was hydrolyzed with 1.5 mL of 6 N HCl at 110 ± 1 °C for 24 h.

The hydrolyzed product was converted into a mixture of *N*-TFA *n*-butyl ester derivatives as described above and analyzed by GC (Figure 12, lower chromatogram), GC-EIMS, and GC-HREIMS. In addition to the peaks present in the starting material (Figure 12, top chromatogram), two new GC peaks were identified. The peak with retention time 6.76 min (Figure 12, lower chromatogram) was identified by coinjection of an authentic sample and by GC-EIMS as the *N,O*-bis-TFA *n*-butyl ester derivative of **α-amino-δ-hydroxyvaleric acid**, whose mass spectrum contained ions at *m/e* 39 (9% of base peak), 41 (85), 42 (6), 43 (11), 53 (15), 54 (6), 55 (9), 56 (13), 57 (83), 58 (4), 68 (5), 70 (5), 71 (57), 72 (3), 91 (3), 96 (14), 98 (8), 99 (7), 114 (3), 126 (7), 128 (12), 139 (43), 148 (6), 149 (5), 152 (3), 165 (3), 166 (100, base peak), 167 (27), 212 (3), 280 (16), and 281 (7). The GC-FI mass spectrum had ions at *m/e* 381 (15% of base peak, M^+), 308 (25), 281 (24), 280 (55), 268 (27), 267 (100, base peak), and 209 (80). The peak with retention time 8.9 min was identified by coinjection of an authentic sample and by GC-EIMS as the *N,N'*-bis-TFA *n*-butyl ester derivative of **ornithine**, whose mass spectrum contained ions at *m/e* 39 (11% of base peak), 41 (84), 42 (8), 43 (20), 53 (15), 54 (6), 56 (22), 57 (86), 58 (6), 68 (8), 70 (12), 71 (28), 72 (9), 78 (8), 86 (7), 96 (15), 98 (6), 114 (11), 126 (31), 127 (5), 128 (7), 138 (4), 139 (27), 140 (7), 141 (7), 148 (4), 152 (6), 153 (17), 154 (12), 164 (7), 165 (15), 166 (100, base peak), 167 (71), 168 (5), 171 (10), 181 (6), 193 (4), 209 (4), 211 (6), 227 (3), 261 (7), 279 (7), 306 (16), and 307 (2). The GC-FI mass spectrum had ions at *m/e* 380 (40% of base peak), 305 (22), 278 (100, base peak), 223 (70), and 154 (39). GC-HREIMS indicated the following elemental compositions for the important ions at *m/e* 307.0480 (Δ 0.7 mmu, $^{13}CC_8H_8F_6N_2O_3$), 306.0475 (Δ 3.6 mmu, $C_9H_8F_6N_2O_3$), 279.0590 (Δ 2.2 mmu, $C_8H_9F_6N_2O_2$), 267.1085 (Δ 0.3 mmu, $C_{11}H_{16}F_3NO_3$), 166.0466 (Δ −1.3 mmu, $C_6H_7F_3NO$), 154.0488 (Δ 0.8 mmu, $C_5H_7F_3NO$), 140.0274 (Δ −4.9 mmu, $C_4H_5F_3NO$) and 126.0187 (Δ2.0 mmu, $C_3H_3F_3NO$).

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(24) (a) Low resolution (nominal mass) mass spectrometric molecular weights are used throughout. These follow the convention of assigning unit masses to the most abundant isotopes. Thus, $C_{92}H_{150}N_{22}O_{25}$ has the nominal mass molecular weight 1962, although its accurate (high resolution) mass spectrometric molecular weight would be 1963.1142. Formula molecular weights (reflected, for example, in neutralization equivalent calculations) are based on atomic weights, of course. (b) K. L. Rinehart, Jr., J. C. Cook, Jr., H. Meng, K. L. Olson, and R. C. Pandey, *Nature (London)*, **269**, 832–833 (1977).
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(27) (a) This section, added in proof, describes experiments carried out in July and August, 1977; (b) C. Ressler, and D. V. Kashelikar, *J. Am. Chem. Soc.*, **88**, 2025–2035 (1966).
(28) (a) A peptide having the structure **6** assigned alamethicin I by Martin and Williams[20] has very recently been synthesized by two groups (B. F. Gisin, S. Kobayashi, and J. E. Hall, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 115–119 (1977); T. M. Balasubramanian, N. C. E. Kendrick, and G. R. Marshall, personal communication to K. L. Rinehart, Jr.). Both groups reported far lower pore-forming activity for **6** than for alamethicin. (b) Gisin et al.,[28a] actually synthesized compound **2**-I after they found no activity for synthetic **6**. They reported synthetic **2**-I to have 60–80% of the antibacterial activity of alamethicin vs. *B. subtilis* but only 30–40% of its pore-forming activity and concluded **2**-I was not the structure of alamethicin though it must be closely related. In view of the present structural assignment, these bioactivity data need to be redetermined. Other possible sources of quantitative differences are the occurrence of **2**-II (of possibly higher bioactivity) in crude alamethicin and, similarly, of potential impurities in the synthetic material.
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^1H Nuclear Magnetic Resonance Relaxation of Water on Lysozyme Powders

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Abstract: Nuclear magnetic resonance transverse and longitudinal relaxation rates are reported at 30 MHz for water protons adsorbed on lysozyme powders from the gas phase as a function of temperature and water content. The free induction decay amplitude accounts for all of the water protons in the sample over the range of water concentrations studies. Two types of model are considered to interpret the transverse NMR relaxation data. It is shown that a multisite fast exchange model which assumes water concentration independent protein binding sites is inconsistent with the data. A crude model that includes the possibility that water molecule motion at all sites may be influenced by subsequent addition of water does account for the observations. A chemical exchange model and a cross-relaxation model are considered as sources of the nonexponential decay observed in the longitudinal relaxation data throughout the temperature and concentration range studied. It is shown that when cross-relaxation between protein protons and water protons is included, long water molecule residence times at protein sites are not required and that the usual approaches to the analysis of water relaxation at surfaces must be altered. The data demonstrate that the rate of water molecule motion decreases with decreasing water content, even though some distribution of motional correlation times may be appropriate. The water in the immediate vicinity of the protein surface appears to be best characterized as a viscous liquid but not as a solid.

An understanding of the interactions between water and proteins is central to an understanding of the many relationships between protein structure, catalysis, activation, function, and decomposition. In spite of the importance of water in maintaining the structural integrity of proteins, the details of the water–protein interaction are poorly understood. Even in the relatively dry state, the water–protein interaction appears to be of practical importance for problems such as the storage of foods, organism drought survival, cold or heat hardiness in plants, and the mechanical properties of natural products derived from primarily protein sources. The present study was undertaken to investigate the water–protein interaction in dry protein systems. For physical reasons we have chosen to examine lyophilized lysozyme powders rehydrated through the gas phase.

Most previous work relevant to the present study has been summarized in reviews.[1-3] Papers of particular importance to protein powder systems were contributed by the laboratories