at all input concentrations. As Figure 10 illustrates, introduction of this evaporation effect into our model indeed yields the observed maximum in $[I_2]_{SSI}$. Our calculations give a small but systematic underestimate of the experimentally determined $[I_2]_{SSI}$. One reason for this discrepancy may lie in our neglect of triiodide formation. Particularly in SSI, where $[I^-]$ is relatively high, some of the iodine in our model will actually be present as nonvolatile I_3^-. Equation 7 will then yield an overestimate of the rate of loss of I_2 from the system.

The model employed in our calculations ignores many complexities of the full system. The reverse steps of the component processes are omitted; no note is taken of the complex kinetics of the Roebuck reaction; the formation of triiodide and the reactivity of that species are not considered. In spite of these simplifications, remarkably good agreement between our calculations and the experimentally determined steady-state concentrations, phase diagram, stoichiometry, and dynamics have been achieved. It thus appears that the qualitative picture of the reaction suggested by Eggert and Scharnow[16] some 60 years ago is correct, despite major quantitative differences between their analysis and ours.

We have not attempted to develop a mechanism for the arsenite–iodate system consisting of a complete set of elementary steps. Any such mechanism must pass the stringent tests of consistency not only with the experimental results presented here but also with the considerable body of data available from batch experiments on the Dushman[20] and Roebuck[22,24] reactions as well as on the arsenite–iodate reaction[16] itself. Efforts are now under way to construct such a mechanism.

A number of the "free" parameters in our model were either determined experimentally or were taken from other experimental studies in the literature. The remaining constants could be fixed rather accurately by fitting the observed transition points between the two steady states. We suggest that this procedure of choosing rate constants to reproduce experimentally determined phase diagrams in bistable flow systems may prove a useful addition to the kineticist's array of techniques, particularly when the number of unknown parameters is relatively small.

In addition to forming part of the recently discovered arsenite–iodate–chlorite oscillator, the reaction studied here has been shown to propagate waves of chemical reactivity in an initially homogeneous unstirred solution.[38] To date, only three other isothermal, nonbiological homogeneous bistable systems, the Briggs–Rauscher[39] reaction, the cerium–bromate system,[40] and the reaction of bis(trichloromethyl) trisulfide with aniline in methanol,[41] have been treated in the literature. In further papers in this series we shall examine several other cases of bistability in the CSTR, including the chlorite–iodide and the ferrous–nitrate systems. With this considerably expanded range of experimental examples, it may then prove feasible to elucidate some of the actual relations among the phenomena of autocatalysis, bistability, temporal oscillation, and the propagation of spatial waves.

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Synthesis and Characterization of the Major Component of Alamethicin

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Abstract: Natural alamethicin has been purified on a high-performance liquid chromatography (HPLC) system developed to purify and characterize synthetic peptides corresponding to the proposed structures of alamethicin. Five of the twelve components detectable by UV absorption at 210 nm were isolated in pure form and were characterized for their antibacterial and ionophoric properties. Low-resolution electron-impact mass spectrometry (LREIMS) indicates that the purified major component and a synthetic preparation based on the recently proposed structure are identical in their primary structures. This major component, along with another fraction of the natural alamethicin mixture, closely demonstrates the spectrum of antibiotic activity possessed by the mixture. All fractions are equally potent in producing conductances of 30 $\mu S/cm^2$ in lipid bilayers, but the current–voltage curves induced by the different fractions have different slopes. Thus, their potencies at higher conductance levels can be expected to diverge. The synthetic peptide shows a close correspondence to the major component by all criteria examined.

The recently reported primary structure (Figure 1D) of the major component of alamethicin is based on a combination of techniques emphasizing both electron-impact and field-desorption high-resolution mass spectrometry.[1] Alamethicins fall into a special class of peptide antibiotics along with antiamoebins, emerimicins, and suzukacillin termed as "peptaibophols".[2-5] Peptides of this class have been found to have several aminoisobutyric acid

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A. Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln

B. Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu(Phol)-Gln-OH

C. Ac-Aib-Pro-Aib-Ala-Aib-(Ala/Aib)-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu$^{\gamma}$-Gln$^{\gamma}$-Phol
($^{\alpha}$OH, $^{\alpha}$NH$_2$)

D. Ac-Aib-Pro-Aib-Ala-Aib-(Ala/Aib)-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol

Figure 1. Various structures proposed for alamethicin. A. Cyclic structure originally proposed by Payne et al.[6] and confirmed by Ovchinnikov et al.[7] B. Linear structure proposed by Martin and Williams[8] and Jung et al.[9] C. Tentative structure of Rinehart et al.[10] D. Current structure of alamethicin according to Gisin et al.[15] and Rinehart et al.[1,2]

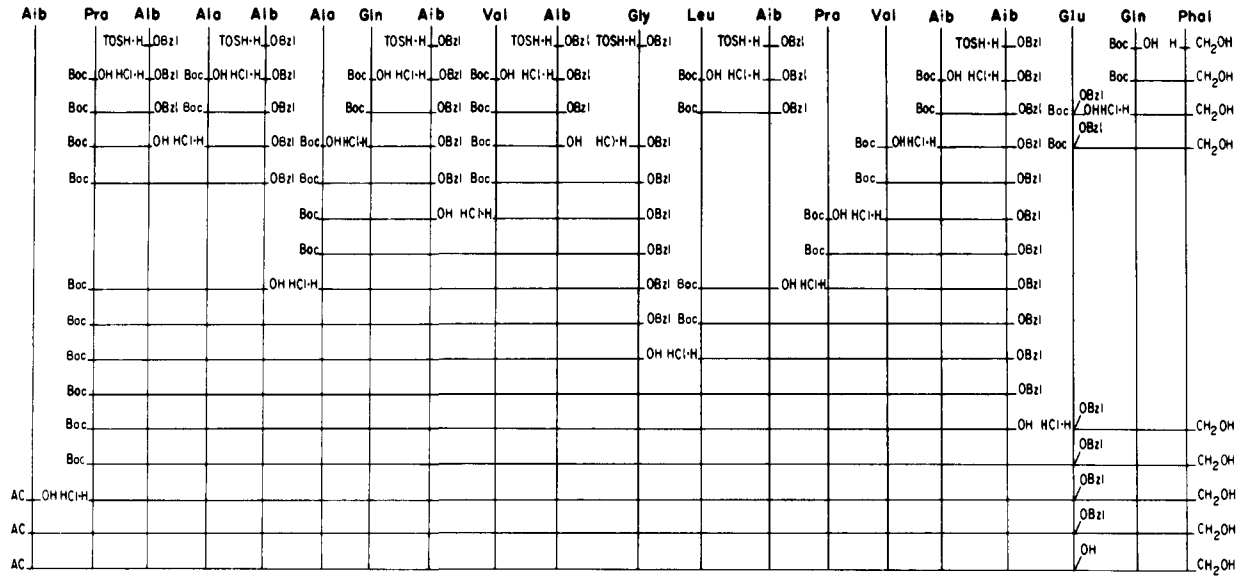

Figure 2. Scheme for the synthesis of the major component of alamethicin by fragment condensation.

(Aib) residues, an acetyl-protected amino terminal, and a phenylalaninol (Phol)-protected carboxyl terminal group in common. Three of the earlier structures proposed for alamethicin (Figures 1A, 1B, and 1C) were found to have significant discrepancies concerning the linearity of the structural sequence and the linkages of residues 18–20.[6-10] Synthetic peptides based on all three of these sequences were prepared in our laboratory and found to have very low membrane activities (less than 10% in comparison to the native mixtures).[11-13] Gisin et al. independently prepared a peptide based on the linear structure (Figure 1B) proposed by Martin and Williams and found the ionophoric activity to be less than 10% that of the native mixture.[14] The above authors also prepared another varient of the possible linkages of residues 18–20 based on the differences in pK_a values of the native mixture and the synthetic peptide shown in Figure 1B. This new peptide (Figure 1D) was prepared by Gisin et al., using fragment condensation on a polymer support, and was found to have 20–30% membrane activity and 60–80% antibiotic activity against B. subtilis.[15] Independent evidence supporting the sequence in Figure 1D was found later upon reinvestigation of the structure of native alamethicin by Rinehart et al.[1]

The discrepancies in the structures reported in literature for alamethicin, the variations in both the antibiotic and ionophoric activities of the various synthetic peptides in comparison with native alamethicin sample, as well as the conflicting reports on concentration-dependent membrane activity data prompted us to separate the various components of the natural alamethicin mixture

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on the HPLC systems we had developed.[16] We herein report the synthesis of the major component of alamethicin and the comparison of its ionophoric and antibacterial activities with that of the HPLC purified major fraction of the native alamethicin.

Experimental Procedures

Melting points are uncorrected and were determined on Thomas-Hoover and Fisher-Johns melting point apparatus. Optical rotations were measured on a Perkin-Elmer Model 241 polarimeter. Amino acid analyses were conducted on a Beckman Model 120C analyzer and are uncorrected. Elemental analyses were performed by Robertson Laboratory, Florham Park, New Jersey, and Huffman Laboratories, Inc., Wheatridge, Colorado. The proton magnetic resonance spectra were obtained on a Varian HR-220 MHz spectrometer and the carbon-13 magnetic resonance spectra were obtained on JEOL FX-60 and JEOL PFT-100 spectrometers.

Thin layer chromatography (TLC) was performed on Silica Gel GHL plates of size 5 × 20 cm (Alatech). The chromatograms were developed with ninhydrin and chlorox-starch reagent. The following solvent systems were used routinely: (I) *n*-butyl alcohol–acetic acid–water, 4:1:1; (II) *n*-butyl alcohol–pyridine–acetic acid–water, 15:10:3:12; (III) chloroform–methanol–acetic acid, 90:8:2; (IV) chloroform–methanol, 7:1; (V) chloroform–acetone, 7:1; (VI) chloroform–acetone, 7:2; (VII) chloroform–methanol, 3:1; (VIII) chloroform–methanol, 1:1; and (IX) chloroform–methanol, 1:3.

High-pressure liquid chromatography (HPLC) was performed on two systems: (1) two Waters 6000 A pumps, a M660 solvent programmer, a Rheodyne injector, μ-Bondapak C_{18} column (or Waters Radial Compression Module, RCM-100 with a radial pak-A cartridge), and a Varichrom UV-Vis detector; (2) a Beckman Model 110A HPLC system with an analytical reverse phase column (spherisorb-ODS). Solvent systems and conditions routinely used are given in Figure 3.

All the Boc-protected amino acids (except Boc-Aib-OH and TOSH·H-Gly-OBzl) and L-phenylalaninol were purchased from Sigma Chemical Co., St. Louis, Mo. Boc-Aib-OH and TOSH·H-Aib-OBzl were prepared in this laboratory.

Abbreviations routinely used are the following: Boc, *tert*-butyloxycarbonyl; OBzl, benzyl ester; TOSH, p-toluenesulfonic acid; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, *N*-hydroxybenzotriazole; DMF, dimethylformamide; Phol, L-phenylalaninol; MeOH, methanol; EtOAc, ethyl acetate, P_2O_5, phosphorous pentoxide; DPPA, diphenylphosphoryl azide; DEPC, diethylphosphoryl cyanidate; Boc-N_3, *tert*-butyloxycarbonyl azide; $(BOC)_2O$, di-*tert*-butyl dicarbonate; Boc-ON, α-*tert*-butyloxycarbonyloxyimino-2-phenylacetonitrile.

Preparation of H-Aib-OBzl. To a 500-mL round-bottom flask were added 25.8 g (0.25 mol) of Aib, 48.5 g (0.255 mol) of *p*-toluenesulfonic acid, 100 mL of benzyl alcohol, and 50 mL of benzene. The mixture was heated under reflux, with water being removed azeotropically in a Dean-Stark receiver. When the water ceased to distill (after the collection of about 15 mL), the reaction mixture was cooled to room temperature, filtered, diluted with 500 mL of ether, and placed in a 5 °C cold room. The crystalline *p*-toluenesulfonate salt of Aib-OBzl which appeared was filtered, washed with ether, and recrystallized from methanol/ether. Yield, 54.5 g for 70%; mp 150–152 °C.

The *p*-toluenesulfonate salt was dissolved in chloroform and extracted with Na_2CO_3 to obtain the oily free amine. This was treated with HCl to obtain the cryatalline HCl·H-Aib-OBzl as required; mp 160–161 °C. Anal. Calcd for $C_{11}H_{16}NO_2Cl$: C, 57.51; H, 7.03; N, 6.10. Found: C, 57.97; H, 7.13; N, 6.05. R_f, 0.64 (I); R_f, 0.64 (II).

a. General Procedure Employed for the Synthesis and Purification of the Peptides. The synthesis of the di- and tripeptides was carried out on a 10–20 mmol scale and the larger peptides were prepared on a 0.1–3.0 mmol scale. To a stirred and ice cooled solution of the hydrochloride salt of an amino acid (or peptide) benzyl ester derivative (10 mmol) in 25 mL of DMF was added 10 mmol of triethylamine. After about 15 min, 10 mmol of Boc-protected amino acid (or peptide), 20 mmol of HOBt, and 20 mmol of DCC were added successively to this solution. The mixture was allowed to stir for 30 min at 0 °C and for 48 h at 25 °C. The reaction mixture was then filtered, concentrated, diluted with EtOAc (300 mL), and washed successively with 50 mL of 1 N sodium bisulfate (four times), 50 mL of water (3 times), 50 mL of 1 N sodium bicarbonate (4 times), and 50 mL of water (3 times). The EtOAc extract was then dried over anhydrous sodium sulfate and evaporated to dryness in vacuo. The crude peptides were then purified by silica gel column (2 × 100 cm) initially in solvent system V to remove all the ninhydrin-negative impurities followed by solvent system IV at a flow rate of about 30 mL/h. After being checked by TLC for homogeneity the ninhydrin positive fractions were pooled, concentrated to dryness, and dried over P_2O_5 overnight in a vacuum descicator. Syntheses of fragment peptides, using DPPA and DEPC, were carried out according to the previously reported procedure.[17] The peptides were then crystallized from appropriate solvent systems listed in Table I.

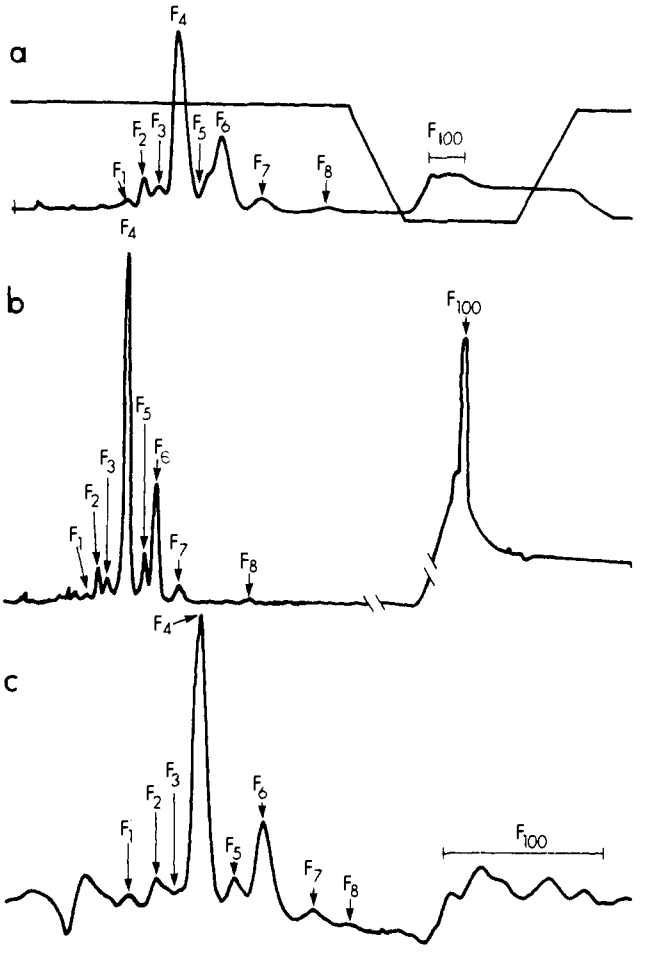

Figure 3. (a) HPLC chromatogram of Upjohn standard alamethicin on a μ-Bondapak C-18 column (Waters Associates). Upper trace shows isocratic elution (49% Mobile Phase B) followed by steep gradient to 100% mobile phase B and return to isocratic elution. Absorption monitored at 210 nm; mobile phase B, THF:CH_3CN:mobile phase A (8:1:2); mobile phase A, $(Et_3NH)_3PO_4$ buffer (0.25 N, pH 3.5); flow rate 1.8 mL/min. (b) Spherisorb-ODS column of Beckman; conditions are as in (a) except $(Et_3NH)_3OOCH$ buffer (0.05 N, pH 3.5) was used instead of the phosphate buffer. (c) Radial compression (cartridge radial-pak A) of Waters Associates; conditions are as in (a); flow rate 3.0 mL/min.

b. General Procedure for the Removal of *t*-Boc Group. Boc-protected peptide ester (0.5–20 mmol) was dissolved in 10–25 mL of dioxane saturated with hydrogen chloride gas (4–4.5 N) and stirred for about 30 min. The reaction mixture was then taken to dryness in vacuo and the deprotection reaction was repeated. The progress and the completion of the reaction were followed by TLC. After completion of the reaction, the deprotected peptide was taken to dryness in vacuo three times with benzene, dried over P_2O_5 overnight, and used directly in the next coupling reaction.

c. General Procedure for the Removal of the –OBzl Group. The removal of the OBzl group was carried out according to the procedure in the literature.[18] The hydrogenated peptide was filtered to remove all the catalyst and carbon, concentrated and evaporated to dryness with benzene three times in vacuo, and dried over P_2O_5 overnight. The peptide was then crystallized from a suitable solvent system.

Membrane Activity Studies; Voltage–Current Experiments. Five of the twelve fractions of alamethicin obtained after purification of the natural

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Table I. Synthetic, Physical, and Analytical Data of the Intermediate Peptide Derivatives of Alamethicin

Compounds	Recrystallization Solvents	Melting Point (°C)	R_f	$[\alpha]_D^{23}$ (C 1.0, MeOH)	Scale of Synthesis (mmoles)	Yield	Elemental analysis (upper: calc, lower: found) C	H	N	Amino acid analysis (6N HCl, 110°C, 18 hrs)
1. Boc-Aib-OH*†•	CH_2Cl_2/hexone	117-8°	0.76 (III), 0.58 (IIII)	—	100 125 100	30%[a] 47%[b] 65%[c]	53.19 53.10	8.43 8.32	6.89 6.85	—
2. Ac-Aib-OH[e]*	EtOAc	194-5°	0.53 (II), 0.56 (III)	—	25	92%	49.66 49.91	7.59 7.33	9.66 9.61	—
3. Boc-Pro-Aib-OBzl	isopropylether	78°	0.71 (VII), 0.91 (IV)	-70.8°	20	48%	64.62 64.87	7.69 7.95	7.18 7.16	$Pro_{1.10}Aib_{1.00}$
4. Boc-Pro-Aib-OH†•	EtOAc	163-165°	0.71 (I), 0.65 (III)	-64.0°	10	quan	55.99 55.90	8.05 8.14	9.33 9.38	$Pro_{1.05}Aib_{1.00}$
5. Boc-Ala-Aib-OBzl†	—	oil	0.54 (VI), 0.74 (VII)	-29.1°	20	50% (38% MA) (88% EOCl)	62.60 62.40	7.75 7.89	7.69 7.40	$Ala_{1.06}Aib_{1.00}$
6. Boc-Pro-Aib-Ala-Aib-OBzl*†•○	isopropylether	130-1°	0.81 (IV), 0.40 (VI)	-35.4°	10	54%	61.52 61.75	7.74 7.78	10.25 10.29	$Pro_{1.00}Aib_{2.00}Ala_{1.10}$
7. Boc-Pro-Aib-Ala-Aib-OH	MeOH	120° (dec)	0.32 (I), 0.26 (III)	-35.0°	5	95%	55.23 55.25	8.37 8.40	11.72[d] 11.45	$Pro_{1.00}Ala_{2.00}Ala_{0.98}$
8. Boc-Gln-Aib-OBzl*†	ether/pet ether	121-2°	0.62 (IV), 0.86 (II)	-17.6°	20	43% (58% OPPA)	59.81 59.53	7.36 7.29	9.97 9.97	$Glu_{1.00}Aib_{0.96}$
9. Boc-Gln-Aib-OH	EtOAc	183-4°	0.34 (VII), 0.80 (II)	-17.0°	10	97%	50.74 50.50	7.61 7.84	12.68 12.48	$Glu_{1.00}Aib_{0.96}$
10. Boc-Ala-Gln-Aib-OBzl*	MeOH	110° (dec)	0.52 (VII), 0.68 (VIII)	-32.9°	2.35	87% (OPPA)	57.87 57.61	7.48 7.51	11.38[e] 11.79	$Ala_{1.00}Glu_{0.98}Aib_{0.93}$
11. Boc-Ala-Gln-Aib-OH	EtOAc	120-123°	0.40 (VIII), 0.50 (II)	-35.5°	2.0	90%	51.28 51.04	7.69 7.50	11.96[f] 11.90	$Ala_{1.00}Glu_{0.98}Aib_{0.93}$
12. Boc-Val-Aib-OBzl	EtOAc/pet ether	100-102°	0.80 (VI), 0.84 (IV)	-26.4°	20	79% (53% OEPC)	62.64 62.90	7.69 7.90	7.69 7.58	$Aib_{1.00}Val_{1.05}$
13. Boc-Val-Aib-OH*†	EtOAc	133-135°	0.73 (VII), 0.37 (I)	-22.6°	20	quan	55.85 55.85	8.60 8.86	9.26 9.14	$Aib_{1.00}Val_{1.03}$
14. Boc-Val-Aib-Gly-OBzl*†	ether/pet ether	126°	0.64 (VI), 0.87 (IV)	-2.7°	8.3	79% (72% OPPA)	61.47 61.65	7.80 7.95	9.35 9.77	$Aib_{0.99}Gly_{1.10}Val_{1.00}$
15. Boc-Gln-Aib-Val-Aib-Gly-OBzl*†	ether/pet ether	135-138°	0.32 (IV), 0.77 (VIII)	-10.7°	3.5	49%	57.98 57.93	7.60 7.35	12.68 12.37	$Val_{1.00}Gly_{1.15}Glu_{0.95}Aib_{2.26}$
16. Boc-Ala-Gln-Aib-Val-Aib-Gly-OBzl*†•	ether/pet ether	134° (dec)	0.60 (IV), 0.70 (VII)	-15.9°	2.0	55%	57.30 57.11	7.50 7.55	13.37 13.43	$Aib_{2.19}Glu_{1.16}Gly_{0.99}Ala_{0.92}Val_{1.00}$
17. Boc-Leu-Aib-OBzl *†•	EtOAc/pet ether	108-9°	0.80 (III), 0.50 (IV)	-32.0°	10	43% (41% DEPC)	64.99 64.75	8.45 8.84	6.89 6.79	$Leu_{1.00}Aib_{1.01}$
18. Boc-Leu-Aib-OH	MeOH/H_2O	128-130°	0.48 (I), 0.33 (III)	-23.3°	8	95%	53.89 53.99	8.98 8.58	8.38[g] 8.50	$Leu_{1.00}Aib_{1.02}$
19. Boc-Leu-Aib-Pro-OBzl*†○	ether/pet ether	171-173°	0.55 (V), 0.67 (VI)	-72.5°	6.0	72% (59% DEPC)	64.38 64.32	8.22 8.44	8.34 8.37	$Leu_{1.00}Aib_{0.97}Pro_{1.13}$
20. Boc-Leu-Aib-Pro-OH*†○	ether	134-7°	0.69 (II), 0.63 (VII)	-48.4°	3.6	91%	54.52 54.90	8.70 8.54	9.54[h] 9.49	$Leu_{1.00}Aib_{0.99}Pro_{1.13}$
21. Boc-Aib-Aib-Aib-OBzl†○	EtOAc/pet ether	121-2°	0.86 (I), 0.83 (III)	—	20	63% (62% OPPA)	63.44 63.39	7.93 7.97	7.40 7.48	——
22. Boc-Val-Aib-Aib-OBzl†	EtOAc/pet ether	126-128°	0.85 (III), 0.48 (V)	-75.0°	5.2	52%	62.83 62.68	8.17 8.20	8.79 8.99	$Val_{1.00}Aib_{2.03}$
23. Boc-Pro-Val-Aib-Aib-OBzl†	EtOAc	Semi-solid product	0.92 (I), 0.78 (III)	-15.8°	2.4	92%	62.14 62.30	8.10 8.51	9.06[i] 9.36	$Pro_{1.00}Val_{1.00}Aib_{2.01}$
24. Boc-Leu-Aib-Pro-Val-Aib-Aib-OBzl*†•	EtOAc/pet ether	106°	0.83 (II), 0.79 (III)	-29.9°	1.2	95%	62.18 62.59	8.29 8.56	10.88 10.45	$Leu_{1.00}Pro_{1.06}Val_{1.08}Aib_{2.70}$
25. Boc-Gln-Phol	EtOAc/pet ether	134°	0.61 (I), 0.58 (III)	-38.9°	6	66%	60.09 59.96	7.64 7.67	11.07 11.04	——
26. Boc-Glu-(OBzl)-Gln-Phol*	ether	145°	0.05 (IV), 0.65 (VII)	-36.2°	2.6	44%	62.15 61.82	7.02 7.02	9.36 9.14	——
27. Boc-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OH	EtOAc	156-8°	0.21 (VIII), 0.73 (III)	-17.5°	1.0	57%	53.88 54.51	7.76 7.96	14.40 14.44	$Pro_{1.00}Aib_{4.13}Ala_{1.84}Gln_{0.99}Val_{1.05}Gly_{1.05}$
28. Boc-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OH*	EtOAc	203° (dec)	0.02 (VII), 0.59 (III)	-13.1°	0.6	45%	55.31 55.18	7.93 8.10	——	$Pro_{2.12}Aib_{7.07}Ala_{1.82}Gln_{0.90}Val_{2.15}Gly_{1.00}Leu_{1.00}$
29. Boc-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-(OBzl)-Gln-Phol	ether	155-8°	0.50 (II), 0.69 (II)	-11.6°	0.27	50%	58.10 57.72	7.61 7.93	——	$Pro_{2.42}Aib_{8.73}Ala_{1.86}Glu_{3.19}Val_{2.15}Gly_{1.00}Leu_{1.11}$
30. Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol*••	——	249-255° (259-260°) 275-279° lit	0.32 (II), 0.09 (VII)	—	0.13	38%				$Pro_{2.00}Aib_{8.35}Ala_{1.88}Gln_{2.88}Gly_{1.11}Val_{2.26}Leu_{1.09}$

a) Boc-N_3 Method; b) $(Boc)_2O$ Method; c) Boc-ON, d) Calculated with 1 mol of MeOH, e) Calculated with 0.5 mol of MeOH, f) Calculated with 0.75 mol of EtOAc, g) Calculated with 1 mol of H_2O, h) Calculated with 1.5 mol of H_2O; i) Calculated with 0.5 mol of EtOAc, j) Calculated with 1 mol of EtOAc; * Purity check by ^1H n.m.r., † Purity check by ^{13}C n.m.r., • Molecular ion and sequence determinations by mass Spectro., ■ Purity check by HPLC; ○ Crystal Structure Solved

product by high-pressure liquid chromatography were used in these studies. Phosphatidyl ethanolamine (PE) from *E-coli* purified by column chromatography was purchased from Avanti Lipids, Inc. (Birmingham, Ala., Cat. No. 810027). We used *n*-pentane and salts from Allinckrodt, Inc. (St. Louis, Mo.). Pentane was passed through an alumina column before use to remove surface active impurities.

The method of membrane formation was similar to that described by Montal and Mueller.[19] We used a Teflon chamber similar to a design by Schindler and Feher.[20] A thin piece of Teflon with a 0.3 mm diameter hole punched by a hypodermic needle was mounted between two chamber halves machined as mirror images of each other. The two halves were forced into a tapered hole in an aluminum block, to clamp the chamber together and to provide an isothermal enclosure.

The temperature was controlled to 0.5 °C by a specially designed bridge amplified circuit driving Peltier thermoelectric elements and was 20 °C in all experiments reported. Lipid solution (10 μL of 10 mg/mL in pentane) was added to the surface of each chamber with use of a glass microliter pipet. Then a membrane was formed by raising the levels of the two aqueous solutions separated by the thin Teflon partition. A small drop of squalene was placed in the hole in this partition before raising the water levels. An alamethicin derivative was added to one side of the chamber after the membrane was formed. By convention, the side opposite the alamethicin was called ground.

We monitored membrane formation by using the current response to a 10 mV amplitude triangular voltage. Membrane specific capacitance was about 0.8 F/cm^2, and membrane area was about 7×10^{-4} cm^2. A four-electrode (chlorided silver wire) system was used for measuring current–voltage and current–time curves. Two electrodes drove an electrometer, and a third electrode was connected to virtual ground, the summing point of an AD 42K operational amplifier. The last electrode

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was connected to a voltage source. Voltage was generated by a computer-controlled 12 bit DAC (AD 5782), buffered by an AD 514 operational amplifier, or a battery driven potentiometer. We used an X-Y recorded (HP 7034A) to record current-voltage curves and current-time curves. For single channel analysis, we used a TIR 115 instrumentation tape recorder (Tandberg), a computer (System 2D, Cromenco, Inc.), and a correlation analyzer SAI-43A (Honeywell, Saicor). We used an unbuffered (pH 5.5) 1 M KCl salt solution.

In order to test the ionophoric activity, current-voltage curves of lipid bilayers are measured with different aqueous concentrations of the separate purified fractions and synthetic derivatives. The I-V curves of each fraction are similar and the I-V curves of each derivative shift in a similar way along the voltage axis when the aqueous concentration of the fraction is changed. In order to estimate the relative activity of the various fractions, the voltage at which the alamethicin-induced conductance reached a value of 30 $\mu S/cm^2$ is measured. This "characteristic" voltage, V_{30}, decreases logarithmically with increasing alamethicin concentration. The lower the value of V_{30} at a given aqueous concentration of a particular fraction, the more effective that fraction.

Results and Discussion

After extensive experiments, two solvent systems were found to give optimum results in resolution and separation of the alamethicins. Figure 3 shows the HPLC scans of the natural product obtained from Upjohn, using three different C-18 reverse-phase columns. It is evident that there are a minimum of twelve components as opposed to the two different components (either Aib or Ala for residue 6 or Gln-NH_2 and Gln in the structure in Figure 1B) reported by others. Reasonable estimates of the percent content based on the relative ratio of the peak areas indicate that the major component (peak 4), the second major component (peak 6), and the third major component (peak 5) are about 45, 20, and 10%, respectively, in the crude mixture. Several minor components account for the rest. Two hundred milligrams of alamethicin was purified on an analytical spherisorb-ODS column, using the conditions shown in Figure 3b. The column was loaded with 10 mg of sample for each run and 20 runs were performed. A buffer system containing triethylammonium formate was preferred since it was found to leave practically no trace of any solid residue after evaporation unlike the triethylammonium phosphate buffer which needs desalting to remove the nonvolatile phosphate salts from the purified fractions. All the components in the isocratic region were carefully collected and fractions corresponding to peaks 5, 6, and 7 were repurified on HPLC. The homogeneity of the peptides thus purified was verified on HPLC and they were used for subsequent characterizations.

Peptides corresponding to all four proposed structures for the major component of alamethicin (Figure 1) have been synthesized in our laboratory. A fragment condensation approach was found to be the method of choice in the synthesis of all four peptides particularly in view of the fact that the linkage of the C-terminal tripeptide was in question and under repeated investigation. The strategies employed to synthesize both the cyclic (Figure 1A) and the linear structure (Figure 1B) have been previously reported.[12] In our earlier attempts, several fragment peptides, namely Ac-Aib-Pro-Aib-Ala-Aib-OBzl (I), Boc-Ala-Gln-Aib-Val-Aib-Gly-OBzl (II), Boc-Leu-Aib-Pro-Val-Aib-Aib-OBzl (III), Boc-Glu-(Phol)-Gln-OBzl (IV), Boc-Glu(IsoGln-Phol)-OBzl (V), and Boc-Glu(OBzl)-Gln-Phol (VI), were prepared in solution. Since residues 1 through 17 were found to be identical in all the reported linear sequences of the major component of alamethicin, our efforts were aimed at synthesizing Ac-Aib^1 → Aib^{17}-OH. Peptides corresponding to the three linear structures of alamethicin could thus be prepared by a final fragment coupling of this heptadecapeptide with the three different tripeptides (IV, V, and VI). However, several setbacks were encountered in our efforts to synthesize many of these fragment peptides leading to the final product since the unusual amino acid Aib shows steric hindrence in some of the commonly used reactions of peptide chemistry.[21,22]

Excess coupling components (100% excess of DCC/HOBT on the mole basis of the amino and carboxyl components) and longer reaction times (48 h) had to be employed to obtain reasonable yields of these fragment peptides containing Aib residues. Because of considerably low yields of Boc-Aib-OH obtained using the Boc-N_3 method,[23,24] the N-protection of Aib was attempted using $(BOC)_2O$ and Boc-ON to improve the yields (Table I). The synthesis of TOSH·H-Aib-OBzl was prepared according to the azeotropic distillation procedure.[25] However, conversion of the above compound to its hydrochloride salt according to the procedure in ref 25 (addition of triethylamine to generate H-Aib-OBzl followed by treatment with HCl gas) was unsuccessful. Sodium carbonate was found to be a more efficient base than triethylamine in the removal of p-toluenesulfonic acid (see Experimental Procedure).

During the synthesis of Boc-Gln-Aib-OBzl, considerable dehydration (10–15%) of the glutamine side chain residue was found to occur consistantly when DCC/HOBT were used as the coupling components whereas the DPPA method gave better yields with no detectable side reaction. However, the highly reactive phosphorous coupling reagents yielded an equimolar amount of the desired peptide and the undesired Phol ester of Boc-Gln-OH during the synthesis of Boc-Gln-Phol (Phol with an unprotected hydroxyl group was used in the synthesis of all three tripeptides (IV, V, and VI)).

Though the synthesis of Ac-Aib-Pro-Aib-Ala-Aib-OBzl was successful (1 + 4 coupling, crystal structure solved),[26] hydrogenolysis of the peptide gave a mixture of undesired ninhydrin-positive products along with the desired hydrogenated peptide in low yields. Though it may seem unlikely, the acetyl group apparently is quite labile under the conditions of hydrogenation generating ninhydrin-positive fragments. It has been reported that during the attempts to remove the benzyloxycarbonyl group from Z-Aib-Pro-Trp-OH by hydrogenolysis at room temperature or at 0 °C, a mixture of products containing the diketopiperazine of Aib-Pro, free Trp, and minor amounts of the desired unprotected tripeptide were obtained.[27] Similar results were also obtained after the hydrogenolysis of the above peptide containing *cis*- or *trans*-4-fluoroproline residue in place of proline. The steric hindrance of the Aib molecule combined with the cyclization tendencies prevalent when proline or sarcosine are present in the molecule may be a plausible explanation to some of the atypical reactions found in all of the above instances. In addition the two residues Ac-Aib-OH and Phol are found to hydrolyze rapidly from the rest of the alamethicin molecule in acid solution and also the Ac-Aib-OH residue hydrolyses further to acetic acid and Aib.[8] It is quite likely that Ac-Aib-OH hydrolyses from the pentapeptide I during hydrogenation since the pH of the standard hydrogenation solvent mixture is quite low (pH 2.82).

Hence a different fragment approach seemed necessary in which the Ac-Aib-OH residue could be added in the final condensation reaction to synthesize the major component. This will avoid any side reaction of N-Ac-Aib containing peptides during the subsequent hydrogenation steps of the fragment peptides leading to the final target peptide, Ac-Aib^1 → Aib^{17}-OH. Thus the synthesis of the hexadecapeptide, Boc-Pro^2 → Aib^{17}-OH, was attempted in solution by coupling the three fragment peptides, Boc-Pro-Aib-Ala-Aib-OH, the hexapeptides II and III (Figure 2). The synthesis of the hexapeptide II was achieved by two different methods (1 + 5 and 3 + 3 couplings). The conformations of Aib-containing peptides is one of the prime interests of our investigation which led us to prepare this peptide by two different routes. Besides, the tripeptide Boc-Aib^6-Gln^7-Aib^8-OBzl (crystal structure determined)[28] was synthesized during this work with

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Table II. Anitmicrobial Spectrum for Native Mixture, Fractions of Native Alamethicin, and Synthetic Product

	Native Mixture	F_2	F_4	F_5	F_6	F_7	F_{100}	Ala-syn
K. pneumoniae	9	0	0	0	0	0	0	0
E. coli	0	0	0	0	0	0	0	0
P. vulgaris	8	0	0	0	0	tr	0	0
S. aureus	10	tr	13	10	0	11	0	0
S. pyogenes	22	16	22	17	11	17	13	18
P. oxalicum	11	0	10	0	0	17 hazy	0	0
S. faecalis	15	tr	18	11	0	11	0	10

20 μl of 1 mg/ml solucion per 1/4" disc
(20 μg of sample per 1/4" disc)

[a] Each sample was dissolved in 10% DMF, 90% methanol solution to give a concentration of 1 mg/mL. The discs were air dried and spotted on the various organisms.

a view to prepare alamethicin II. However, the yields of this hexapeptide were found to be essentially the same by both methods. The hexapeptide III was synthesized by the 2 + 4 coupling (95% yield), since the yield of 3 + 3 coupling (Pro to Val) was found to be low (33%). The final peptide corresponding to the sequence in Figure 1D was successfully prepared by condensation of the above hexadecapeptide with the tripeptide VI followed by the addition of Ac-Aib-OH and hydrogenolysis. The product was purified by HPLC to homogeneity in order to determine its activity precisely. The major component isolated (F_4) and the synthetic peptide were found to have identical retention time on HPLC and the HPLC scan of a mixture of the two showed a single peak. Mass spectral data and aminc acid analysis showed that the synthetic product was identical with the major component by these criteria. The physiochemical properties of the intermediate peptides are listed in Table II.

The synthetic peptide and several of the HPLC purified fractions were screened for their antibiotic activity as shown in Table II. The major component (F4) and fraction 7 are found to show similar antibiotic spectra of activities in comparison to the native mixture. Although the synthetic product does not show an identical antibiotic spectrum to that of the major component, it does have comparable activity against *S. pyogenes* and *S. faecalis*. None of the fractions showed any significant activity like the native mixture against *K. pneumonia* and *P. vulgaris*. It is our opinion that these discrepancies may simply reflect contamination of F4 by minor amounts of other components and that significant synergistic effects may be involved in antibiotic activity. Experiments to test this hypothesis await purification of additional quantities of the minor components.

A hemolysis assay involving erythrocyte suspensions was used to determine the hemolytic activities of the native alamethicin, the HPLC-purified major component (F4), and the synthetic product.[29] With use of native alamethicin as a standard, the major component and the synthetic alamethicin were found to be 100 ± 7% and 83 ± 7% active respectively in the hemolysis assays.

Figure 4 shows the dependence of V_{30} on antibiotic concentration for six purified fractions, the synthetic derivative apparently similar to fraction 4 and the mixture supplied by Upjohn (Kalamazoo, Michigan) as U-22324. The membrane system was bacterial phosphatidyl ethanolamine (Avanti Biochemicals, Birmingham, Ala.) prepared by the technique described[30] using squalene instead of vaseline. The slope of the logarithmic dependence is about 40 mV per e-fold change in concentration of antibiotic for all fractions and all fractions have approximately the same activity as V_{30}.

At a fixed antibiotic concentration, the conductance depends exponentially on voltage with the form $G = G_{0e}V/V_0$. V is the voltage, V_0 is the voltage required for an e-fold change in conductance, and G_0 is the conductance at zero voltage. V_0 ranges from 3.5 to about 15 mV for the different fractions. This implies that at high levels of conductance, differences in activity between the different fractions would become more apparent. Our finding of relatively equivalent activities using 30 μS/cm² as the test conductance thus shows that all of the compounds are equally effective at inducing a small number of conducting sites per square centimeter. But the finding of different current–voltage curve slopes implies that the potencies of the different analogues can be expected to diverge at higher conductance levels.

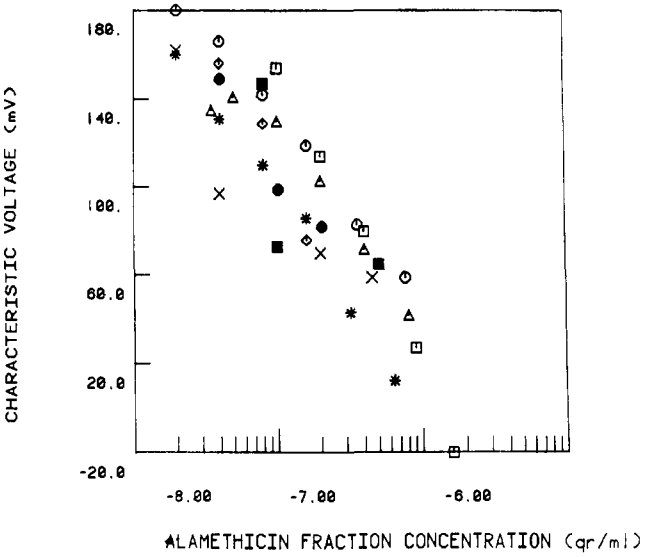

Figure 4. The voltage at which an alamethicin derivative induces a conductance of 30 μS/cm² V_{30} plotted against the aqueous concentration of the alamethicin derivative. Phosphlidyl ethanolamine membrane in 1 M KCl, unbuffered (pH 5.5); □ = fraction 4, ○ = fraction 6, ▲ = fraction 5, ◇ = fraction 7, * = native alamethicin mixture (Upjohn), × = synthetic peptide (presumed same as fraction 4), ■ = F-100, ● = fraction 2.

We have also found clear differences between the single-channel conductances induced by the different fractions.[31] (These will be described in detail elsewhere.[32]) Fraction 4, for example, induces well-defined single channels with sharp conductance levels, but fraction 6 shows no well-defined single channels at all in the PE membrane system. Nevertheless, the gross conductance properties induced by the two fractions are reasonably similar.

We conclude that natural alamethicin is a heterogeneous mixture containing at least 12 components. Most of these components can induce voltage-dependent conductances in lipid bilayers. The induced conductances are grossly similar, but differ in the degree of voltage dependence and in single channel properties. Similarly, most of the components have antibiotic activity. The activity spectrum, however, differs in degree from fraction to fraction. The major component of the mixture, fraction 4, has properties very close to those of the synthetic alamethicin of Figure 1D by bilayer, antibiotic, and mass spectroscopic criteria. We conclude that fraction 4 is thus very likely a material identical with the formula of Figure 1D.

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