Synthesis of the Major Component of Alamethicin

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Abstract: Alamethicin (ALA), a natural membrane-active antibiotic, consists of several components. The major component, ALA I, was isolated in pure form and compared with the synthetic peptide acetyl-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol obtained by stepwise segment condensation on a polystyrene support. The synthetic and natural products appeared essentially identical by amino acid analysis, high performance liquid chromatography, 600-MHz proton nuclear magnetic resonance spectroscopy, mass spectrometry, electrical activity in lipid bilayer membranes, and antimicrobial activity. It is concluded that ALA I has the structure indicated above.

Introduction

Alamethicin (ALA)[2] is a peptide antibiotic produced by the fungus *Trichoderma viride*.[3] The peptide (Figure 1) is highly hydrophobic and adsorbs strongly to membranes, a property thought to be responsible for its antimicrobial activity as well as for its mammalian toxicity (hemolysis). The main interest in ALA, however, stems from the unique and specific way in which it affects the electrical properties of artificial lipid bilayer membranes.[4] At very low concentrations ALA induces voltage-dependent conductances that are similar to those observed in nerve membranes.[4,5-7] These effects can be understood by assuming membrane-spanning ALA aggregates that form ion-conducting channels.[8-10]

Natural ALA is a mixture of closely related compounds.[11-16] This explains, in part, why its structure has undergone several revisions. The first proposal, a cyclic structure,[11,17] was found to be incorrect, and an open-chain sequence with an N-terminal Ac-Aib group and the C-terminus R-Glu(Phol)-Gln was proposed.[13,18] An attempted synthesis[19] and preliminary reports[15,16] of the synthesis of this compound (ALA-α) have been published. In each case, however, the synthetic materials were different from the natural product.

One of the differences was found in the acidity of the two products.[15] Thus, in ALA-α a pK_a of 4.4 was measured, as expected for an α-carboxyl group, while in natural ALA it was 5.2. This suggested a γ-carboxyl group for the latter. Accordingly, the isomer R-Glu(OH)-Gln-Phol (ALA I, Figure 1) having a γ-carboxyl group was synthesized. In a preliminary report[20] this preparation was shown to have 60-80% of the antibiotic activity of natural ALA. In contrast, a more recent preliminary report[16] describes a synthetic ALA I preparation with almost no antibiotic activity and the isolated major component of natural ALA with no activity.

Encouraged by independent support for the structure depicted in Figure 1 by mass spectrometry,[14] the final steps of the synthesis of ALA I were repeated and the product was purified to homogeneity. The current contribution contains a detailed description of this synthesis and presents evidence suggesting the identity of the product with the major component of natural alamethicin.

Experimental Section

Amino acid analyses (Beckman Spinco amino acid analyzer Model 121) were performed by Mr. I. C. Chu and Ms. M. LeDoux, elemental analyses by Mr. T. Bella of The Rockefeller University. Melting points were taken in open capillaries and are not corrected. Optical rotations were determined with a Schmidt and Haensch polarimeter. Solid-phase reactions were carried out on a mechanical shaker in screw-cap vessels equipped with a fritted disk and a stopcock.[21] All chemicals and solvents were reagent grade. Methylene chloride (CH_2Cl_2) trifluoroacetic acid (TFA), diisopropylethylamine (DIEA), and acetic anhydride were redistilled prior to use. Hydrolyses of resins and peptides were carried out with propionic acid-12 N HCl (1:1, v/v) at 130 or 140 °C for 4-8 h.[22] Thin-layer chromatography (TLC) was run on Silica Gel G precoated plates (Analtech Inc., Wilmington, Del.) in the following solvent mixtures (v/v): R_f^1 $CHCl_3$-MeOH-AcOH (18:2:1); R_f^2 EtOAc-pyridine-H_2O-AcOH (30:10:5:3); R_f^3 $CHCl_3$-acetone-EtOH (70:30:3); R_f^4 *n*-BuOH-AcOH-pyridine-H_2O (15:10:3:2).

Determination of α-Aminoisobutyric Acid (Aib). Aib gives a low response (ca. 7% that of Leu) when run under standard conditions on an amino acid analyzer. Therefore, radioactive Aib was used. The accuracy of Aib determination by radioisotopic analysis was comparable to that for the other amino acids determined on an amino acid analyzer.

Preparative C_{18} Silica Gel Chromatography.[16] Purification by HPLC (ca. 2 mg per run) was performed on Partisil M9 10/50 ODS-2, 0.94 × 50 cm columns (Whatman) isocratically with methanol/buffer mixtures ranging from 80:20 to 85:15 (v/v) at a flow rate of 3 mL/min. The buffer was 20 mM acetic acid adjusted with triethylamine to pH 5.0.

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(2) Abbreviations according to IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.* **1971**, *242*, 243-247. In addition: Aib = α-aminoisobutyric acid, Phol = L-phenylalaninol, ALA = alamethicin, DIEA = diisopropylethylamine, DCC = *N,N'*-dicyclohexylcarbodiimide, HOSu = N-hydroxysuccinimide, HOBt = 1-hydroxybenzotriazole.

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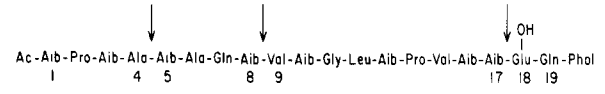

Figure 1. Sequence of alamethicin I. Phol = phenylalaninol; Aib = α-aminoisobutyric acid; Ac = acetyl. The arrows indicate the segments used in the synthesis.

The effluent was monitored at 210 nm. Different columns were used for natural and synthetic materials.

NMR Spectra. The samples for NMR were prepared by evaporating to dryness under a stream of N_2 ethanol or ethanol water solutions containing 1 to 2 mg of peptide. Following overnight storage in a vacuum dessicator, the peptides were redissolved in ca. 0.5 mL of methanol-d_4 (99.99% D Aldrich Chemical Co.) and then filtered through glass wool into 5-mm (O.D.) NMR tubes (PP-528, Wilmad Glass Co.).

Proton NMR spectra were taken in the correlation mode[39] at 600 MHz on the MPC-600 spectrometer (probe ambient temp. ca. 23 °C).[40] Typically 800 to 1000 scans were accumulated in 6 to 8K data blocks at sweep widths and sweep times of 15 000 Hz and 3 s, respectively, stored in memory, and then transformed without any additional digital filtering to give spectra like those shown in Figure 5.

Boc-[*methyl*-^{3}H]Aib-OH. A solution of [*methyl*-^{3}H]Aib (total, 5.0 μCi) in HCl (ICN Isotope & Nuclear Division, Irving Calif.) was evaporated to dryness under reduced pressure. To this residue, unlabeled Aib (30.9 g, 0.3 mol), *N*-methylformamide (300 mL), tetramethylguanidine (103.7 g, 0.9 mol), and *o*-*tert*-butyl *S*-(4,6-dimethyl-2-pyrimidinyl)thiocarbonate[23] (103.6 g, 0.45 mol) were added, and the resulting solution was stirred overnight at 50 °C. Water (450 mL) was then added and the unreacted carbonate was extracted with EtOAc (2 × 450 mL). The EtOAc layers were combined and back-washed with 100 mL of water. The combined aqueous layers were adjusted to pH 2 with 5 N HCl and extracted with EtOAc (3 × 450 mL). The EtOAc layers were combined, dried over Na_2SO_4, filtered, and evaporated. The resulting crystals were collected and recrystallized from ether–petroleum ether: 48.5 g (79.6%); mp 118–119 °C; R_f^4 0.87.

Anal. Calcd for $C_9H_{17}O_4N$: C, 53.19; H, 8.43; N, 6.89. Found: C, 53.14; H, 8.46; N, 6.79.

Phenylalaninol. Similar to the method reported by Karrer et al.[24] Na metal (3.8 g, 16.5 mmol) was dissolved in EtOH (70 mL) and the resulting NaOEt solution was added to a solution of Phe-OEt·HCl (38.0 g, 16.5 mmol) in EtOH (150 mL) under cooling. The resulting white precipitate was filtered and the solvent was evaporated. The residue (Phe-OEt) was dissolved in ether (400 mL), and this solution was added dropwise to a suspension of $LiAlH_4$ (16.0 g) in ether (450 mL) under vigorous stirring. After the addition was completed, the mixture was stirred for 30 min, and then 100 mL of water was added to destroy the excess $LiAlH_4$. The product was then extracted with ether (3 × 500 mL). The ether extract was dried over anhydrous $MgSO_4$ and evaporated to give a white crystalline compound. The crystals were collected and recrystallized from ether: 17.5 g (70.2%); mp 91.5–93 °C, lit.[25] mp 91–93 °C; $[\alpha]^{25}_D$ −25.6° (*c* 1.037, EtOH); R_f^4 0.73.

Anal. Calcd for $C_9H_{13}ON$: C, 71.49; H, 8.67; N, 9.26. Found: C, 71.56; H, 8.82; N, 9.36.

Boc-Glu(Phol)-OBzl. Boc-Glu-OBzl (23.6 g, 70 mmol), HOSu (9.7 g, 84 mmol), and DCC (17.33 g, 84 mmol) were combined in a mixture of EtOAc (125 mL) and dioxane (50 mL). The mixture was stirred for 1 h at 0 °C and for 3 h at room temperature, and then filtered. The filtrate was stirred into a dioxane solution (100 mL) of phenylalaninol (11.3 g, 75 mmol), and the resulting mixture was stirred overnight at room temperature. After evaporation of the solvent, the residue was dissolved in EtOAc. The solution was washed with 1 N HCl, 5% $NaHCO_3$, and saturated NaCl aqueous solution, dried over anhydrous $MgSO_4$, and then evaporated. The residue was recrystallized from EtOAc–ether: 25.4 g (79.4%); mp 72 °C dec; $[\alpha]^{24}_D$ − 29.2° (*c* 1.0, EtOH), R_f^3 0.83.

Anal. Calcd for $C_{26}H_{34}O_6N_2$: C, 66.36; H, 7.28; N, 5.95. Found: C, 66.19; H, 7.43; N, 6.00.

Boc-Glu(Phol)-OH. Boc-Glu(Phol)-OBzl (21.0 g) was hydrogenated with 5% Pd on charcoal for 4 h at 50 psi of H_2 in *t*-BuOH (90 mL). After filtration and evaporation of the solvent, the residue was recrystallized from EtOAc–ether: 14.5 g (85.4%); mp 122–124 °C dec; $[\alpha]^{24}_D$ −15.7° (*c* 1.0, EtOH); R_f^1 0.84.

Anal. Calcd for $C_{19}H_{29}O_5N_3$: C, 59.98; H, 7.42; N, 7.36. Found: C, 60.06; H, 7.52; N, 7.32.

Boc-Gln-Phol. Boc-Gln-OH (4.93 g, 20 mmol), Phol (3.32 g, 22 mmol), HOBt (3.24 g, 24 mmol), and DCC (4.53 g, 24 mmol) were mixed in THF at 0 °C. The mixture was stirred for 5 h at 0 °C and 16 h at room temperature. After filtration and evaporation of the solvent, the residue was dissolved in $CHCl_3$ (50 mL) and aqueous layer was backwashed with $CHCl_3$ (3 × 30 mL). The $CHCl_3$ layers were combined and dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was recrystallized from EtOH–ether: 4.53 g (59.8%); mp 145–146 °C; $[\alpha]^{23}_D$ −30.7° (*c* 1.0, EtOH); R_f^1 0.64.

Anal. Calcd for $C_{19}H_{29}O_5N_3$: C, 60.14; H, 7.70; N, 1107. Found: C, 60.49; H, 7.79; N, 11.15.

Boc-Glu(OBzl)-Gln-Phol. Boc-Gln-Phol (4.00 g, 10.5 mmol) was dissolved in TFA (40 mL) and the solution was stirred for 30 min at room temperature. After evaporation to dryness, the residue and Et_3N (1.46 mL, 10.5 mmol) were dissolved in DMF (80 mL).

Boc-Glu(OBzl)-OH (3.37 g, 10 mmol), HOSu (1.38 g, 12 mmol) and DCC (2.48 g, 12 mmol) were combined in a mixture of EtOAc (20 mL) and dioxane (10 mL) at 0 °C and the mixture was stirred at 0 °C for 2 h, then filtered. The filtrate was added to the DMF solution of Gln-Phol, and the mixture was stirred overnight at room temperature. After evaporation of the solvent, the residue was purified by silica gel column chromatography with $CHCl_3$–MeOH (9:1 v/v) as solvent: 3.25 g (54.3%; mp 145–148 °C; $[\alpha]^{23}_D$ −26.7° (*c* 1.0, EtOH); R_f^3 0.75.

Anal. Calcd for $C_{31}H_{42}O_8N_4$: C, 62.19; H, 7.07; N, 9.36. Found: C, 61.70; H, 6.95; N, 9.30.

Boc-Glu-Gln-Phol. In EtOH (40 mL), Boc-Glu(OBzl)-Gln-Phol (2.69, 4.5 mmol) was hydrogenated with 5% Pd on charcoal for 4 h at 50 psi of H_2. After filtration and evaporation of the solvent, the residue was recrystallized from EtOH: 1.80 g (84.8%); mp 160 °C dec; $[\alpha]^{23}_D$ −43.4° (*c* 1.0, EtOH); R_f^3 0.23.

Anal. Calcd for $C_{24}H_{36}O_8N_4 \cdot {}^1/_2H_2O$: C, 55.69; H, 7.21; N, 10.83. Found: C, 55.48; H, 7.23; N, 10.61.

Boc-Aib-O-Res. Boc-Aib[26] (3.26 g, 16 mmol) was dissolved in EtOH (22.5 mL) and the solution was diluted with water (7.5 mL). The pH of this solution was adjusted to 7.0 by adding aqueous $CsHCO_3$[27] and the solvent was removed. After repeated evaporation to dryness with benzene, Boc-Aib-OCs was obtained as a glassy, hygroscopic solid. This compound was dissolved in DMF (30 mL) and a small amount of undissolved material was filtered off. The filtrate and chloromethylated poly(styrene-*co*-1% divinylbenzene) (Bio-Rad SX-1, Bio-Rad Laboratories, Richmond, Calif.), 11.5 g, 14 mequiv, were placed in a 250-mL three-necked flask equipped with a stirrer and a drying tube. The suspension was stirred gently at 50 °C for 3 days, filtered, and thoroughly washed with DMF, DMF–water (9:1, v/v), DMF, and EtOH, and dried. Hydrolysis of the resin followed by radioisotope analysis indicated a substitution of 968 μmol of Aib per gram of resin.

Boc-Ala-O-Res was prepared as described for Boc-Aib-O-Res from chloromethyl resin (933 μmol/g) and Boc-Ala at 50 °C for 16 h to give a substitution of 589 μmol/g.

Boc-Gln-O-Res was prepared as described for Boc-Aib-O-Res from chloromethyl resin (935 μmol/g) and Boc-Gln at 50 °C for 16 h and room temperature for 48 h to give a substitution of 664 μmol/g.

Ac-Aib-Pro-Aib-Ala-O-Res (Residues 1–4). The starting material was 7.65 g of Boc-Ala-O-Res (substitution, 689 μmol/g by amino acid analysis). The peptide chain was built up using the following cycle: (a) deprotection with 50% (v/v) TFA in CH_2Cl_2, 1 × 2 min, and 1 × 25 min; (b) neutralization with 5% (v/v) DIEA in CH_2Cl_2; (c) coupling twice with 1.5 equiv of DCC and acyl component (Boc-Aib, Boc-Pro, Boc-Aib, respectively). The first coupling was carried out for 4 h and the second for 16 h. The resin was washed thoroughly with CH_2Cl_2 after each step, and with DMF and CH_2Cl_2 after each cycle. Because of the loss of peptide through intramolecular aminolysis that was observed when dipeptide resin was exposed to the acyl component, a reversed DCC coupling[21] was used throughout. Boc-Aib-Pro-Aib-Ala-O-resin thus obtained was deprotected and neutralized as described above, and then acetylated with acetic anhydride–pyridine (1:1, v/v), 1 × 1 min and 1 × 15 min. The amounts of the protected peptide found after each coupling as determined by amino acid and radioisotope analyses were as follows: Boc-Aib-Ala-O-resin, 666 μmol/g; Boc-Pro-Aib-Ala-O-resin, 639 μmol/g; Boc-Aib-Pro-Aib-Ala-O-resin, 558 μmol/g. The dried acetyl tetrapeptide resin had a substitution of 540 μmol/g by amino acid and radioisotope analysis, indicating that 86.1% of the peptide chains were retained on the resin. Amino acid analysis: Pro, 1.01; Val, 1.00; Aib, 1.99.

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Boc-Aib-Ala-Gln-Aib-O-Res (Residues 5–8). The starting material was 2.07 g of Boc-Aib-O-Res (substitution, 968 μmol/g by radioisotope analysis), and the same protocol as for residues 1–4 was used. The first coupling was for 8 h and the second for 18 h except for Boc-Ala in which case the first coupling was for 4 h and the second for 16 h. Boc-Gln was coupled in the presence of HOBt (1.5 equiv) to suppress nitrile formation, and two more couplings (overnight) were necessary to complete the reaction. The amounts of the peptide found after each coupling as determined by amino acid and radioisotope analyses were as follows: dipeptide resin, 767 μmol/g; tripeptide resin 691 μmol/g. The dried Boc-tetrapeptide resin had a substitution of 613 μmol/g by amino acid and radioisotope analyses, indicating that 75.2% of the peptide chains were retained on the resin. Amino acid analysis: Glu, 1.06; Ala, 1.07; Aib, 1.89.

Boc-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-O-Res (Residues 9–17). The starting material was 7.75 g of Boc-Aib-O-Res (substitution, 968 μmol/g) and the same protocol as for residues 1–4 was used. The first coupling was for 8 h and the second for 24 h. In order to complete the reaction at the penta- and hexapeptide stages, a third coupling (24 h) was added with 1.0 equiv each of DCC and Boc-Aib and Boc-Leu, respectively. The amounts of the peptide found after each coupling as determined by amino acid and radioisotope analyses were as follows: dipeptide-Res, 875 μmol/g; tripeptide-Res 810 μmol/g; tetrapeptide-Res, 702 μmol/g; pentapeptide-Res, 563 μmol/g; hexapeptide-Res, 574 μmol/g; heptapeptide-Res, 462 μmol/g; octapeptide-Res, 418 μmol/g. The dried Boc-nonapeptide-Res had a substitution of 409 μmol/g by amino acid, and radioisotope analyses, indicating that 51.7% of the peptide chains were retained on the resin. Amino acid analysis: Val, 1.86; Pro, 1.15; Leu, 1.12; Gly, 1.10; Aib, 3.76.

Boc-Glu(Phol)-Gln-O-Res (Residues 18–19, ALA-α). The starting material was 3.85 g of Boc-Gln-O-Res (substitution, 664 μmol/g by amino acid analysis). Boc-Glu(Phol)-OH was coupled to Gln-O-Res in a mixture of CH_2Cl_2 and DMF (5:3, v/v) according to the protocol used for residues 1–4. The dried resin had a substitution of 474 μmol/g by amino acid analysis (Glu), indicating that 72.5% of the peptide chains were retained on the resin.

Boc-Glu(-O-resin)-Gln-Phol (Residues 18–20, ALA I). Boc-Glu-(OH)-Gln-Phol (1.88 g, 3.7 mmol) was converted to its cesium salt and reacted (50 °C, 24 h) with chloromethylresin (3.65 g, 4.4 mmol of Cl) as described for Boc-Aib-O-resin to yield 4.76 g of product: substitution, 452 μmol/g by amino acid analysis (Glu).

Ac-Aib-Pro-Aib-Ala-OH (Residues 1–4). Ac-Aib-Pro-Aib-Ala-O-Res (1.2 g, 648 μmol) was treated with anhydrous HF (ca. 20 mL) at 0 °C for 1 h. After evaporation to dryness, the resin was washed extensively with water. The combined washes were adjusted to pH 4 by adding a few drops of pyridine, treated with Dowex 50W × 2 (200–400 mesh, SO_3H form, 6 mL) to remove ninhydrin-positive material and then evaporated. Crystals (258 mg, mp 203–205 °C) thus obtained were recrystallized twice from MeOH–ether: 131 mg (50.6%), mp 207–209 °C; $[\alpha]^{25}_D$ + 4.0° (*c* 1.0, EtOH); R_f^4 0.55; amino acid analysis: Pro, 1.13; Ala, 1.07; Aib, 1.80.

Anal. Calcd for $C_{18}H_{30}O_6N_4 \cdot {}^1/_2H_2O$: C, 53.05; H, 7.67; N, 13.75. Found: C, 52.76; H, 7.49; N, 13.60.

The residue from the mother liquor was chromatographed on Sephadex G-10 (1.6 × 90 cm) in water. The product was lyophilized to give an additional 71.0 mg (27.5%), total yield, 202 mg (78%).

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-OH (Residues 1–8). Boc-Aib-Ala-Gln-Aib-O-Res (759 mg, 465 μmol) was allowed to swell in CH_2Cl_2 for 10 min and was treated with 50% (v/v) TFA in CH_2Cl_2 for 20 min at room temperature. After neutralization with 5% (v/v) DIEA in CH_2Cl_2 (3 × 3 min), the resin was washed with CH_2Cl_2 (8 × 3 min) and then with DMF (3 × 3 min).

Ac-Aib-Pro-Aib-Ala-OH (123.5 mg, 310 μmol) and HOBt (125.7 mg, 930 μmol) were mixed in 10% (v/v) DMF in CH_2Cl_2 (1.5 mL). To this suspension was added DCC (76.8 mg, 372 μmol) at 0 °C. Immediately after the addition of DCC, the suspension became clear and then a white precipitate, dicyclohexylurea (DCU), began to appear. After stirring for 30 min at 0 °C and for 2.5 h at room temperature, the DCU was filtered off and a 0.5-mL wash with DMF-CH_2Cl_2 (1:9, v/v) was added to the Aib-Pro-Aib-Ala-O-Res. The reaction mixture was agitated for 20 h at room temperature. DCC (32.0 mg, 15.5 μmol) was then added and the mixture was shaken for another 20 h at room temperature. After filtration, the resin was washed extensively with 10% (v/v) DMF in CH_2Cl_2, DMF, 10% (v/v) DMF in CH_2Cl_2, CH_2Cl_2, and EtOH, and dried: yield 750 mg. Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-O-Res (650 mg) thus obtained was treated with anhydrous HF (ca. 20 mL) in the presence of anisole (0.5 mL) at 0 °C for 20 min. After the volatile components had been removed, the residue was washed with ether, and then with 1% aqueous AcOH. The AcOH washings were combined and lyophilized to give the crude product (237 mg) which was dissolved in water (15 mL). The solution was adjusted to pH 4.5 by adding a few drops of pyridine, treated with Dowex 50W × 2 (200–400 mesh, SO_3H form, 2 mL) to remove ninhydrin-positive material, and evaporated. The crystalline residue was recrystallized from water: 102 mg (43%); mp 220–221 °C; $[\alpha]^{25}_D$ +24.8° (*c* 1.0, EtOH); R_f^4 0.55; amino acid analysis, Glu, 0.97; Pro, 1.03; Ala, 1.98; Aib, 4.02.

Anal. Calcd for $C_{34}H_{57}O_{11}N_9 \cdot 2H_2O$: C, 50.80; H, 7.65; N, 15.69. Found: C, 50.88; H, 7.32; N, 15.56.

The residue from the recrystallization mother liquor was chromatographed on Sephadex G-10 (1.6 × 90 cm) in water. The product (fractions 55–66 mL) was lyophilized to give an additional 46 mg (19%) of pure material, total yield, 148 mg (62%).

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OH (Residues 1–17). Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-OH (143 mg, 187 μmol) and Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-O-Res (684 mg, 260 μmol) were coupled for 24 h as described for the synthesis of residues 1–8 to yield 697 mg of acetyl heptadecapeptide resin; 233 mg of this material (50.4 μmol by Aib analysis) was treated with anhydrous HF (ca. 14 mL) in the presence of anisole (0.4 mL) at 0 °C for 25 min. After evaporation to dryness the residue was washed with ether, and then with 50% EtOH. The 50% EtOH washings were combined, concentrated, and lyophilized. The residue, dissolved in 50% EtOH, was adjusted to pH 6 by adding pyridine and treated with Dowex 50W × 2 (200–400 mesh, SO_3H form). After evaporation and lyophilization, the residue was chromatographed on Sephadex G-25 (1.6 × 90 cm) in 50% EtOH. The product (fractions 51 to 57 mL) was lyophilized: 46 mg (59%); $[\alpha]^{23}_D$ +13.3° (*c* 1.0, EtOH); R_f^4 0.67; amino acid analysis, Glu, 0.93; Pro, 2.08; Gly, 1.09; Ala, 1.96; Val, 1.96; Leu, 1.06; Aib, 7.91.

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu(Phol)-Gln-OH(ALA-α). This compound was obtained from Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OH (31 mg, 19.7 μmol) and Boc-Glu(Phol)-Gln-O-Res (450 mg, 213 μmol) using the techniques described in the preceding paragraph (residues 1–17): 26.3 mg (68.6%); $[\alpha]^{22}_D$ −4.7° (*c* 1.0, EtOH); amino acid analysis, Glu, 2.98; Pro, 2.06; Gly, 0.94; Ala, 1.87; Val, 2.03; Leu, 1.06; Aib, 8.22 by radioisotopic analysis, 8.10 by amino acid analysis. The properties of ALA-α have been described in previous publications.[15,20]

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol (ALA I). This compound was obtained from Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OH (31.5 mg, 20 μmol) and Boc-Glu(O-Res)-Gln-Phol (442 mg, 200 μmol) in the ways described for the synthesis of segments 1–17: yield 19.9 mg (51%); $[\alpha]^{23}_D$ −5.5° (*c* 1.0, EtOH). Further purification was by C_{18} silica gel and Sephadex G-10 chromatography to give 6 mg of ALA I (30%). See Table I for amino acid analysis and Figure 3 for homogeneity by HPLC.

Natural ALA I and ALA II. Natural alamethicin (U-22324 8831-CEM-93.3, The Upjohn Co., Kalamazoo, Mich.) was fractionated into components I and II (Figure 3) by preparative C_{18} silica gel chromatography followed by sephadex G-10 chromatography in ethanol–water. See Table I for amino acid analyses of ALA I and II and Figure 3 for homogeneity of ALA I by HPLC.

Table I. Amino Acid Analyses[a] of Crude Natural Alamethicin,[b] the Isolated Components I and II, and Synthetic Alamethicin I

amino acid	natural[c]			synthetic[c]
	crude	I	II	I
Glu	2.98 (3)	3.03 (3)	2.89 (3)	2.99 (3)
Pro	2.12 (2)	2.00 (2)	2.01 (2)	1.99 (2)
Gly	0.94 (1)	1.04 (1)	0.99 (1)	1.06 (1)
Ala	1.68 (1–2)	1.88 (2)	1.18 (1)	1.90 (2)
Val	2.13 (2)	1.95 (2)	2.07 (2)	2.01 (2)
Leu	1.02 (1)	1.01 (1)	1.05 (1)	1.00 (1)
Aib	8.15 (8)	7.74 (8)	9.20 (9)	8.33 (8)

[a] Determined on amino acid analyzer. [b] U-22324 8831-CEM-93.3, The Upjohn Co., Kalamazoo, Mich. [c] Numbers in parentheses are the integral values.

Results and Discussion

Synthesis. The synthesis of ALA I was performed by the solid-phase method[28] using a combined strategy of stepwise elongation and segment condensation. This approach was chosen because of the uncertainties that still existed in the exact structure of the N- and C-terminal portions when this work was begun. It

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Scheme I

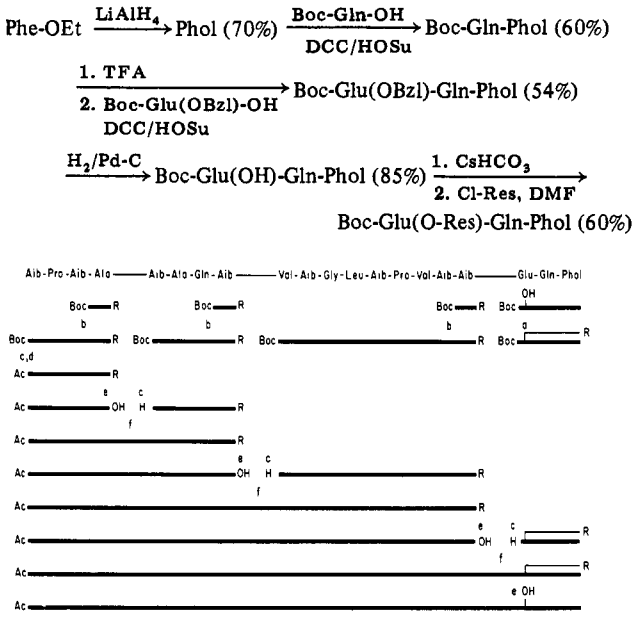

Figure 2. Scheme of the synthesis of alamethicin I by solid-phase segment condensation (R = resin). Procedures and reagents (see Experimental Section for details): (a) $CsHCO_3$/DMF; (b) stepwise chain elongation; (c) (1) TFA, (2) DIEA; (d) Ac_2O; (e) HF followed by purification; (f) DCC-HOBt.

allowed the preparation of analogs without requiring synthesis of the whole sequence. Moreover, purification of the segments was possible before their condensation and consequently, purification of the final product was expected to be simpler.

Three of the four protected segments (Figures 1 and 2) used were prepared starting with chloromethylated poly(styrene-*co*-1% divinylbenzene) resin[28,29] to which the Boc-protected C-terminal amino acid was esterified with the cesium salt procedure.[27] Trifluoroacetic acid (50%) in methylene chloride was used for deprotection and 5% diisopropylethylamine in the same solvent for neutralization. Coupling was with 1.5 equiv each of Boc-amino acid and DCC[30] in methylene chloride for 4 h followed by another 16-h period of coupling with fresh reagents. This protocol was normally sufficient to give coupling yields greater than 99% as determined with picrate.[31] However, when either the carboxyl component was Boc-Aib or when the amine component ended in an Aib residue, longer reaction times were necessary. In these cases, coupling was repeated until the picrate test indicated a yield greater than 98%. The C-terminal tripeptide resin was prepared as shown in Scheme I.

The segments were cleaved from the resin with HF[32] for 20–60 min at 0 °C. In each instance, this procedure gave rise to some ninhydrin-positive side products. They could be removed by treatment in water or ethanol/water (pH ~4–6) with Dowex 50W (H form). The peptides were purified further by crystallization or by Sephadex G-25 chromatography. Homogeneity of each segment was established by thin layer chromatography. One single, ninhydrin-negative I_2/tolidine-positive spot was observed in each case.

Condensation of the segments (Figure 2) was effected by preactivating the carboxyl component with DCC and HOBt prior to addition to the resin-bound amine component in DMF. The reaction was allowed to proceed at room temperature and was monitored by measuring the radioactivity remaining in the solution

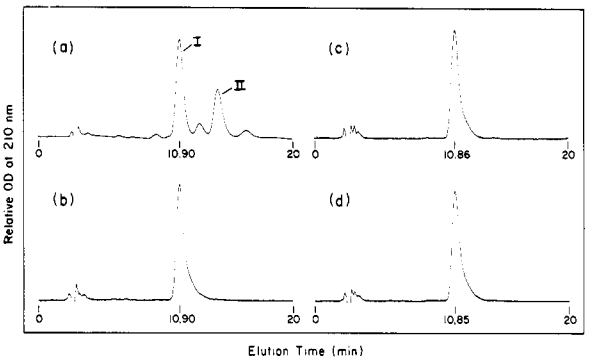

Figure 3. Reversed phase high performance liquid chromatography of natural and synthetic alamethicins. Column, 0.46 × 25 cm Partisil 5 ODS (Whatman); flow rate, 1.2 mL/min; eluent, methanol (85%)/2 × 10^{-2} M aqueous triethylammonium acetate pH ~5 (15%); samples ca. 10 μg in ethanol–water (1:1). (a) Natural alamethicin (Upjohn) containing the two major components I and II. (b) Isolated natural alamethicin I. (c) Synthetic alamethicin I. (d) 1:1 mixture of natural and synthetic alamethicins I.

Table II. Antimicrobial Activity of Alamethicin as Determined on Agar Plates by a Filter Disk Assay[a]

		diameter (mm) of inhibition zones at amounts[b] indicated			
organism	peptide tested	2 μg	5 μg	10 μg	20 μg
Bacillus subtillis	crude natural ALA		7	13	13
	natural ALA I		4	8	14
	synthetic ALA I		4	8	13
Staphylococcus aureus	crude natural ALA	10	13	15	
	natural ALA I	5	8	12	
	synthetic ALA I	5	7	12	

[a] The peptide solutions (concn, 1 mg/mL) in ethanol-water 1:1 were applied to moist 1-cm filters disks. The disks were then placed on inoculated agar plates and removed after 3 h at 4 °C. Incubation of the plates was for 16 h at 37 °C. No inhibition zones were observed with solvent alone. [b] By amino acid analysis of stock solution.

phase. The molar equivalents used for each segment, coupling times and yields of the cleaved purified products were as follows:

$$1.0\ \text{Ac-(1–4)-OH} + 1.5\ \text{H-(5–8)-O-R} \xrightarrow{40\ \text{h}} \text{Ac-(1–8)-OH (71\%)}$$

$$1.0\ \text{Ac-(1–8)-OH} + 1.3\ \text{H-(9–17)-O-R} \xrightarrow{17\ \text{h}} \text{Ac-(1–17)-OH (33\%)}$$

$$1.0\ \text{AC-(1–17)-OH} + 10\ \text{H-(18–20)-O-R} \xrightarrow{24\ \text{h}} \text{Ac-(1–20)-OH (30\%)}$$

The acetyl-octapeptide was tested for racemization of alanine (data not shown) by the Manning–Moore[33] procedure. Less than 2% D-Ala was found in this product (and <1% in the tetrapeptide) indicating excellent chiral homogeneity.

Evaluation. Homogeneity of the synthetic product and identity with natural ALA I by analytical HPLC are shown in Figure 3. This powerful technique readily separates[16] ALA I and II which differ only by one CH_2 group.[14] It has been suggested previously[12,13,18] that the less abundant components (at 13 and 17 min, Figure 3a) may represent the amides of ALA I and II.

The amino acid composition of synthetic ALA I (Table I) agrees with the expected values and with that of the purified natural component. The heterogeneity of natural alamethicin[11–16] is reflected in the values for crude ALA. Consistent with the HPLC pattern (Figure 3a), component II containing the substitution of one Ala by Aib appears to account for about 30% of the peptide

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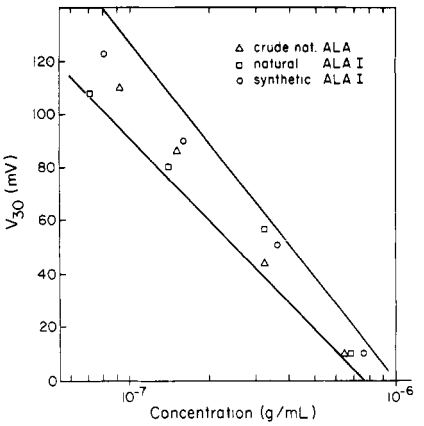

Figure 4. Semilog plot of the effect of alamethicins on the electric conductance of lipid bilayers membranes. V_{30} denotes the voltage required to produce a membrane conductance of 30 μS/cm^2. Membrane composition was bacterial phosphatidylethanolamine in squalene formed by the monolayer technique described in ref 36. Aqueous solutions were 1.0 M KCl buffered to pH 7.0 with 5 mM HEPES. Membrane diameter was 0.3 mm. Both aqueous compartments were stirred and temperature was controlled at 20 °C. The symbols are averages of several readings over a 15-min period and the lines denote the upper and lower limits of observed values.

chains. It has been proposed[11,14,34] that the residue in question is Ala^6.

The antibiotic activity of synthetic ALA I was found to be equal to that of natural ALA I against *Bacillus subtilis* and *Staphyloccus aureus* (Table II). This finding is at variance with a report showing no antibiotic activity against several microorganisms including *S. aureus* of the major component of natural alamethicin purified by HPLC and almost no activity of synthetic ALA I.[16] Note that the crude natural product is a slightly more potent antibiotic than ALA I. This indicates that components of higher activity were removed in the purification process. Obvious candidates for such components are ALA II and the uncharged ALA I and II amides.

Alamethicin influences the electric properties of artificial bilayers membranes in a unique way; i.e., it induces voltage-dependent conductances. An assay based on this property is presented in Figure 4. This plot shows the voltage required to produce the same conductance (30 μS/cm^2) for different peptide concentrations. Within experimental limits, natural and synthetic ALA I gave the same response as the reference sample of crude natural product.

Natural and synthetic ALA I were subjected to Cf^{252} fission fragment mass spectrometry[36] a method that is characterized by its ability to produce molecular ions of large mass and a relatively small number of fragments. In this assay (data not shown) both compounds had the same molecular mass (1963 daltons). Moreover, there was no discrepancy in the meaningful regions of their fragmentation patterns.[37]

Figure 5 shows the C_α proton regions of the 600-MHz NMR spectra of natural and synthetic ALA I. Clearly, there is excellent agreement in the line positions and intensities of the two products and most of the lines have been assigned to specific protons or groups.[38] One small but significant difference, however, is seen in the position and line width of the valine-15 C_α doublet. In the natural ALA I spectrum this doublet is broadened and shifted upfield. We attribute this to differences in the peptide concentration and the amount of residual water in the two samples. Thus, dilution and the addition of H_2O caused this line to sharpen and shift to lower fields.

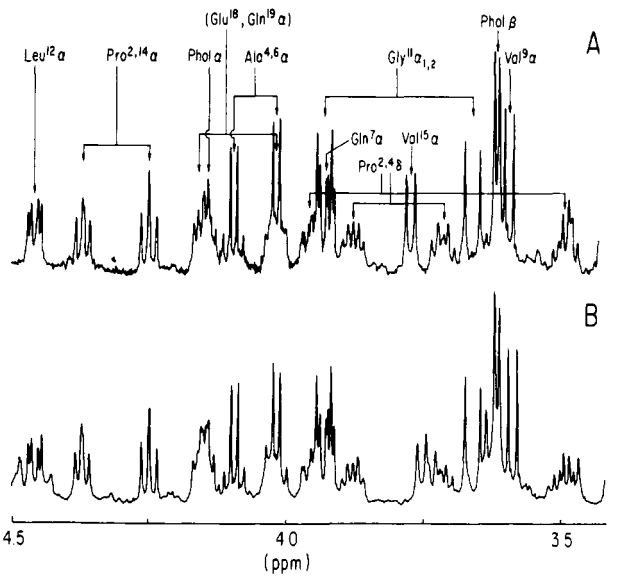

Figure 5. The 600-MHz ^{1}H NMR spectra of the C_α-H region of synthetic (A) and natural (B) alamethicin I. The spectra were taken in methanol-d_4 at a concentration of 1–2 mg/mL and at a temperature of 23 °C. Line assignments (in ppm relative to TMS) are based on splitting patterns, comparison with spectra of synthetic segments, and double resonance difference spectra (cf. ref 38). The upfield shift (0.02 ppm) and twofold broadening of the Val^{15} C_α-H signal at 3.75 ppm in B is accounted for by differences in concentration and water contents of the two samples. Thus, dilution and addition of D_2O (~2%) to B caused the line to sharpen and shift downfield.

In other regions of the spectrum (not shown) there was also close agreement between the two samples. However, in these rather complex regions (side-chain protons) the signals are generally less sensitive to differences in structure and conformation than are those of C_α protons. Thus, for purposes of illustration the latter are more suitable.

Like the C_α protons the amide protons are part of the backbone and their lines are equally sensitive to structural differences. In the present case, however, observation of the amide proton resonances was complicated by the proton–solvent deuterium exchange reaction, which gave time-dependent differences in intensities. Nonetheless, amide-region spectra of natural and synthetic ALA I taken shortly after dissolving the peptides show the same degree of agreement in line positions as shown in Figure 5 for the C_α protons.

Conclusion

Synthesis of Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol (ALA I) has yielded a product that corresponded within experimental error to the main component of natural alamethicin in several assays. Because of their high sensitivity, 600-MHz NMR spectroscopy and high performance liquid chromatography provided the most convincing evidence for identity of the two products. In addition, functional identity was demonstrated in lipid bilayer membranes and in an antimicrobial assay. The conclusion that above structure represents the main component of alamethicin seems therefore justified.

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