Stepwise Automated Solid Phase Synthesis of Naturally Occurring Peptaibols Using FMOC Amino Acid Fluorides

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The standard methods of stepwise solid phase synthesis according to Merrifield could not previously be applied to the synthesis of the important naturally occurring peptaibols because of difficulties arising from the pronounced steric hindrance caused by α,α-dialkylated amino acids (incomplete coupling, especially to adjacent similarly constituted units, racemization due to slow coupling to hindered amino acids, etc.), chain degradation due to the presence of acid-labile Aib-Pro linkages, and the lack of any general method for the loading of C-terminal amino alcohols to resin supports. Following recent work on model systems, it is now shown that the adoption of Fmoc amino acid fluorides as coupling reagents makes possible the facile, general assembly of such peptides. The method was demonstrated for alamethicin F30 and F50, saturnisporin SA III, and trichotoxin A50-J. The crude products were of remarkable purity. Amino acid analysis, mass spectral data, and comparison of the synthetic alamethicins with samples of naturally occurring material confirmed the success of the syntheses. No significant amount of racemization (<0.8%) was found for any of the chiral amino acids present. The first step of the synthesis involved a new general method for assembly of C-terminal peptide alcohols via the use of *o*-chlorotrityl resin. In addition, model studies on the question of racemization during the coupling of Fmoc amino acid fluorides are reported.

Introduction

Peptaibols[1,2] are a class of linear, amphipathic peptides with a molecular weight of about 2000 Da. Produced by widespread soil fungi, such as Trichoderma, Hypocrea, or Stilbella,[3] these antibiotic peptides exhibit a wide range of bioactivities which are at least partly due to their membrane-modifying properties.[4] Thus, one of the most common peptaibols, alamethicin, was found to induce characteristic voltage dependent ion-conductivities in lipid bilayer membranes[5] and to cause cell lysis at high concentrations.[6]

Structurally, these compounds are characterized by a number of common features. In addition to proteinogenic amino acids, the peptaibols contain an unusually high proportion (about 50%) of α,α-dialkylamino acids, such as α-aminoisobutyric acid (Aib) or α-ethylalanine (isovaline, EtA, or Iva). The N-terminus is generally acetylated, whereas the C-terminus is generally occupied by an amino alcohol, such as phenylalaninol (Pheol), valinol (Valol), or leucinol (Leuol).

In view of their unique properties the peptaibols have excited intense interest, yet in spite of their relatively short span (20 amino acids) they have only been synthesized by tedious procedures which combine carefully chosen stepwise solution techniques with chemical or enzymatic segment condensations. The solid phase approach, although potentially capable of providing quickly both the natural materials and an infinite variety of analogs for comparison and further evaluation, has not been successful.[7,8] Difficulties arise because of steric effects due to the α,α-disubstituted amino acids (incomplete coupling, racemization, etc.) and degradation inherent in the presence of acid labile Aib-Pro linkages.

A recently described "complete solid phase synthesis" of an alamethicin analog succeeded only by substitution of all Aib units by leucine residues.[9] Recently, Fmoc amino acid fluorides have been shown to be well suited for the coupling of the hindered amino acid Aib. Using these reagents a human corticotropin-releasing factor (h-

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Scheme 1

alamethicin F30:
Ac-**Aib-Pro-Aib**-Ala-Aib-Ala-Gln-**Aib-Val-Aib**-Gly-Leu-**Aib-Pro**-Val-**Aib-Aib**-Glu-Gln-Pheol
alamethicin F50
Ac-**Aib-Pro-Aib**-Ala-Aib-Ala-Gln-**Aib-Val-Aib**-Gly-Leu-**Aib-Pro**-Val-**Aib-Aib**-Gln-Gln-Pheol
saturnisporin SA III
Ac-Aib-Ala-Aib-Ala-**Aib-Aib**-Gln-**Aib-Leu-Aib**-Gly-**Aib-Aib-Pro**-Val-**Aib-Aib**-Gln-Gln-Pheol
trichotoxin A-50:
Ac-Aib-Ala-Aib-Leu-Aib-Gln-**Aib-Aib-Aib**-Ala-**Aib-Aib-Pro**-Leu-**Aib-Iva**-Gln-Valol

CRF) analog bearing four consecutive Aib units was assembled in good yield and purity, and model studies showed that amino acid sequences common to alamethicin, including the C-terminal carboxylic acid, could also be synthesized without problem. Parallel studies showed that other coupling agents which have been recommended for use in the case of highly hindered systems (PyBroP, NCAs, etc.[10]) were not effective.[11] In the present paper it is shown that alamethicin itself (variants F30 and F50) as well as members of the series containing even more highly hindered sequences (saturnisporin SA III,[12] trichotoxin A-50, component J[13]) could be assembled via acid fluorides easily.

Results and Discussion

The peptides examined in this first study are listed in Scheme 1, with particularly hindered sequences in bold type.

Direct Resin Loading of C-Terminal Alcohols. Since the natural peptaibols terminate in the form of an amino alcohol it would be most convenient to develop a method for direct anchoring of such alcohols onto a solid support. Previously, solid phase syntheses of peptidols have involved indirect ester linkages with release effected by hydrolysis or complex metal hydride reductive release from a simple ester.[14] Neither method is of general applicability. In this work, a new highly convenient approach to the direct preparation of peptidols has been devised based on Barlos' *o*-chlorotrityl resin.[15] Esterification of the 2-chlorotrityl chloride resin with Fmoc-amino acids proceeds quickly without byproduct formation.[16] As described elsewhere,[17] trityl ethers derived from alcohols or Fmoc-hydroxy amino acids can be formed easily in solution, under mild reaction conditions. As expected, a commercial 2-chlorotrityl resin proved to be easily loaded with Fmoc-phenylalaninol and Fmoc-valinol (Figure 1) using a binary mixture of DCM and DMF with pyridine as base. Loadings of about 0.24 mmol/g were obtained after 6 h for both Fmoc-amino alcohols.

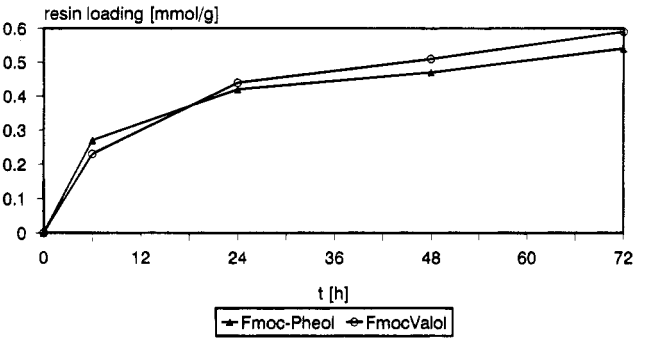

Figure 1. Loading of Fmoc amino alcohols onto the *o*-chloro-Trt resin.

Stepwise Automated Solid Phase Assembly. Initially, in order to develop a standard protocol for automated synthesis, the C-terminal acid analogs of four peptaibols were synthesized (Table 1). The crude peptides showed similar purities to that of alamethicin acid which had previously been assembled via manual synthesis.[11] The results were confirmed by means of ES-MS and amino acid analysis.[18] The two major peaks appearing in the HPLC profile of trichotoxin acid have their origin in the use of racemic Fmoc-Iva-F and the formation of the diastereomeric peptides. Both compounds have been isolated by preparative HPLC and shown to be of identical molecular weight and amino acid content.[18]

In order to synthesize the native peptaibols the *o*-chlorotrityl resins were manually loaded as described with Fmoc-amino alcohols. The loading reaction was stopped after 6 h in order to obtain resin capacities of about the same level as had been used for the synthesis of the acids analogs (about 0.2 mmol/g). The loaded resins were then used for the stepwise automated assembly of the natural sequences of alamethicin F30 and F50, saturnisporin SA III, and trichotoxin A-50 (component J) (Table 1). A single-coupling protocol for 30 min using 3–8 equiv of Fmoc amino acid fluoride was adopted in this work, although neither these conditions, as applied to ordinary amino acids, nor the more prolonged coupling times used for the introduction of the more hindered amino acid Iva were optimized.

Final Peptide Resin Cleavage, Purification, and Characterization. Final peptide resin cleavage of the ether bond was performed using 50% TFA/DCM, 2% triisopropylsilane, 5% water, and 5% phenol for 45 min. Under these conditions all side chain protectants were removed without cleavage of acid labile Aib-Pro bonds. The crude peptaibols obtained by this method showed HPLC purities (Figure 2) similar to the analogous C-terminal acid analogs which had been synthesized on Tenta Gel or PEG-PS resins. After purification by preparative HPLC, single products were obtained, the identity of which was established by amino acid analysis

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(18) Characterizing data for C-terminal acid derivatives of peptaibols. Alamethicin F30 acid: amino acid analysis Glu 3.04 (3); Gly 1.06 (1); Ala 1.97 (2); Aib 7.83 (8); Val 2.10 (2); Leu 1.00 (1); Phe 1.00 (1), Pro 2.03 (2); ES-MS calcd 1977.1 (monoisotopic), found 1978.5 [M + H]$^+$. Saturnisporin SA III acid: amino acid analysis Glu 3.23 (3); Gly 0.98 (1); Ala 1.94 (2); Aib 10.22 (10); Val 0.94 (1); Leu 1.00 (1); Phe 0.93 (1), Pro 0.96 (1); ES-MS calcd 1950.1 (monoisotopic), found 1951.3 [M + H]. Trichotoxin A-50 (component-J) acid: peak 1 Glu 1.90 (2); Ala 2.08 (2); Aib 9.90 (9); Val + Iva n.d. (2); Leu 1.89 (2); Pro 1.00 (1); peak 2 Glu 1.94 (2); Ala 2.04 (2); Aib 9.81 (9); Val + Iva n.d. (2); Leu 1.91 (2); Pro 1.00 (1); ES-MS calcd 1745.1 (monoisotopic), found 1746.4 (peak 1), 1746.7 (peak 2).

Table 1. Conditions for the Stepwise Solid-Phase Synthesis of Peptaibols by Means of Fmoc-Amino Acid Fluorides[a]

	(I) alamethicin acid (F30) (II) alamethicin (F30) (III) alamethicin (F50)	(IV) saturnisporin SA III acid (V) saturnisporin SA III	(VI) trichotoxin A-50 acid (component J) (VII) trichotoxin A-50 (component J)
resin	(I) TGSAC (0.24 mmol/g)[b] (II/III) Fmoc-Pheol-*o*-Cl-Trt-resin[c]	(IV) Fmoc-Phe-PEG-PS (0.19 mmol/g) (V) Fmoc-Pheol-*o*-Cl-Trt-resin	(VI) Fmoc-Val-PEG-PS (0.19 mmol/g) (VII) Fmoc-Valol-*o*-Cl-Trt-resin
equiv of Fmoc-amino acid fluoride	(I) 3 (II/III) 4.5	8	8
coupling concentration (M)	0.3	0.3	0.3
base	1 equiv of DIEA	1 equiv of DIEA	1 equiv of DIEA
couplings	(I) single, 15 min (II/III) single, 30 min	single, 30 min	single, 30 min[d]
deprotection	(I) 20% piperidine/DMF, 15 min (II/III) 20% piperidine/DMF, 7 min	20% piperidine/DMF, 15 min	20% piperidine/DMF, 15 min
peptide–resin cleavage	2% triisopropylsilane, 5% water, and 5% phenol in 50% TFA/DCM, 30 min	2% triisopropylsilane, 5% water, and 5% phenol in 50% TFA/DCM, 45 min	2% triisopropylsilane, 5% water, and 5% phenol in 50% TFA/DCM, 45 min
yield of crude peptide[e] (%)	(I) 84 (II) 78 (III) 75	(IV) 74 (V) 71	(VI) 66 (VII) 60

[a] All syntheses, except for alamethicin acid F50, which was manually synthesized, were carried out on a Millipore 9050 peptide synthesizer using standard protocols. [b] The first amino acid was coupled to the TG SA C resin by means of the acid fluoride (2 × 45 min) in DCM in the presence of DIEA. [c] The *o*-Cl-Trt resin was loaded by direct anchoring of the Fmoc-amino alcohol in 50% DCM/DMF for 6 h in the presence of pyridine to give a loading of about 0.24 mmol/g. [d] The introduction of Fmoc-Iva-F and coupling of the following Aib residue were performed using double couplings (2 × 1 h). [e] The yield of crude peptide–alcohol is based on the loading of each alcohol onto the *o*-Cl-Trt resin.

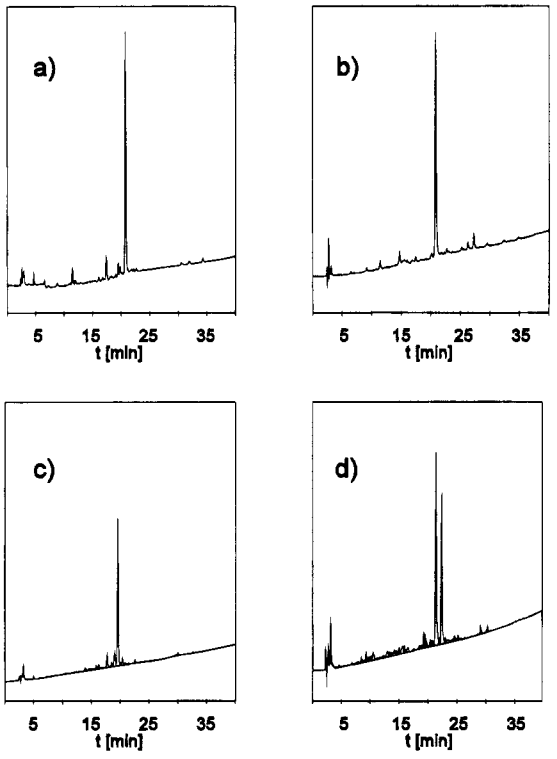

Figure 2. HPLC profiles of crude peptaibols obtained via Fmoc-amino acid fluorides: (a) alamethicin F 50, (b) alamethicin F 30, (c) saturnisporin SA III, and (d) trichotoxin A-50 (component J) (linear gradient: 40–80% B, 40 min, eluent A, 0.1% TFA/water, eluent B, 0.1% TFA in 80% ACN/water, flow rate: 1 mL/min, 220 nm).

and MS determination.[19] The appearance of two major peaks in the HPLC profile of the trichotoxin sample is again due to the efficient separation of the diastereomers containing either L- or D-Iva.

Racemization Studies. All four peptaibols assembled via Fmoc amino acid fluorides were examined for racemization using the method of Kusumoto and coworkers.[20] Hydrolysis in the presence of DCl followed by derivatization and analysis on a chiral GC column showed that racemization could not have exceeded 0.8%[21] for any of the chiral amino acids present.

The lack of significant racemization during the use of several protected amino acid fluorides derived from ordinary amino acids has been cited in earlier work.[22,23] In the present study this has been confirmed for the reaction of Fmoc-Leu-F with H-Pro-NH_2 which gave in DMF/DIEA 0.25% of the DL-dipeptide whereas with DMF/collidine no detectable amount of the racemic form was observed. The weakly basic acid scavenger bis(trimethylsilyl)acetamide (BTMSA) in DMF or DMA led to 0.29% and 0.41% racemization, respectively (Table 2).

(19) Characterizing data for peptaibols synthesized via acid fluorides. Alamethicin F30: amino acid analysis Glu 3.10 (3); Gly 1.00 (1); Ala 1.93 (2); Aib 8.82 (8); Val 1.85 (2); Leu 0.93 (1); Pro 2.03 (2); ES-MS calcd 1963.1 (monoisotopic), found 1964.4 $[M + H]^+$. Alamethicin F50: amino acid analysis Glu 3.21 (3); Gly 1.00 (1); Ala 1.99 (2); Aib 8.02 (8); Val 1.93 (2); Leu 1.08 (1); Pro 1.95(2); ES-MS: calcd 1962.1 (monoisotopic), found 1963.8 $[M + H]^+$. Saturnisporin SA III: amino acid analysis Glu 3.21 (3); Gly 1.00 (1); Ala 1.95 (2); Aib 11.52 (10); Val 0.98 (1); Leu 0.95 (1); Pro 1.03 (1); ES-MS calcd 1936.3 (monoisotopic), found 1938.2 $[M + H]^+$. Trichotoxin A-50 (component-J): peak 1 Glu 1.85 (2); Ala 1.92 (2); Aib 9.51 (9); Iva 1.06 (1); Leu 1.80 (2); Pro 1.00 (1); peak 2 Glu 1.90 (2); Ala 2.07 (2); Aib 10.38 (9); Iva 1.08 (1); Leu 1.90 (2); Pro 1.00 (1); ES-MS calcd 1731.1 (monoisotopic), found 1732.5 $[M + H]^+$ (peak 1), 1732.8 (peak 2). Amino acid analysis was performed using standard post column derivatization techniques (ninhydrin). Because of the steric hindrance Aib and Iva showed much lower response factors then ordinary amino acids (about one-tenth), which necessarily leads to higher relative errors. Therefore, amino acid analysis which gave excellent results for determination of peptide content and analysis of proteinogenic amino acids was confirmed by ES-MS and evaluation of the retention behavior of peptaibols.

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(21) The extent of D-amino acid found for the synthetic peptaibols was as follows. Alamethicin F50: Ala, 0.8% (±0.1%); Val, 0.05% (±0.08%); Leu, 0.2% (±0.01%); Pro, 0.25% (±0.01%); Glu, 0.7% (±0.03%). Alamethicin F30: Ala, 0.4% (±0.08%); Val, 0.07% (±0.01%); Leu, 0.18% (±0.02%); Pro, 0.27% (±0.01%); Glu, 0.45% (±0.07%). Saturnisporin SA III: Ala, 0.1% (±0.05%); Val, 0.1% (±0.05%); Leu, 0.3% (±0.1%); Pro, 0% (±0.05%); Glu, 0.8% (±0.2%) Trichotoxin A-50 (component J): Ala, 0.19% (±0.1%); Leu, 0.4% (±0.1%); Pro, 0% (±0.05%); Glu, 0.3% (±0.15%).

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Table 2. Effect of Solvent and Base on Racemization in the Coupling of Protected Amino Acid Fluorides with Proline Amide with or without Preincubation[a,b]

acid fluoride	solvent	base	preincubation time (min)	DL (%)
Fmoc-Leu-F	DMF	DIEA	7	0.25
Fmoc-Leu-F	DMF	TMP	7	<0.1
Fmoc-Leu-F	DMF	BTMSA		0.29
Fmoc-Leu-F	DMA	BTMSA		0.41
Z-Phg-F	DMF	DIEA		19.0
Z-Phg-F	DMF	TMP		11.6
Z-Phg-F	DMF	DIEA	7[c]	49.5
Z-Phg-F	DMF	TMP	7[c]	20.7
Z-Phg-F	DMF	DIEA	7[d]	27.5
Z-Phg-F	DMF	TMP	7[d]	15.9
Z-Phg-F	DMA	DIEA	7[d]	33.4
Z-Phg-F	DMA	TMP	7[d]	28.5
Z-Phg-F	DMF	BTMSA		11.8
Z-Phg-F	DMA	BTMSA		18.5
Z-Phg-F	CH_2Cl_2	TMP		<0.1

[a] A solution of 0.25 mmol of H-Pro-NH_2, 0.125 mmol of the weakly basic scavenger BTMSA, or 0.25 mmol of the base in 1 mL of solvent was treated with 0.275 mmol of protected amino acid fluoride. After 15 min the reaction mixture was worked up as usual. Yields were in the range 94–97%. For runs involving a 7-min preincubation period, the acid fluoride (0.275 mmol) was dissolved in 1 mL of solvent and 0.25 mmol of base or 0.125 mmol of BTMSA added and the solution stored at room temperature for 7 min, at which time 0.25 mmol of H-Pro-NH_2 was added in one portion. Alternatively, the acid fluoride was stirred in the solvent alone for 7 min and then treated with 0.25 mmol of H-Pro-NH_2 along with 0.25 mmol of base. The mixtures were worked up in 1 h as usual. The pure dipeptide, Z-Phg-Pro-NH_2, had mp 89–91 °C. ^{1}H NMR ($CDCl_3$) δ: 2.1–2.2 (m, 4, CH_2), 3.2 (m, 1, CH), 3.7 (m, 1, CH), 4.65 (m, 1, CH), 5.13 (s, 2, CH_2O), 5.6 (m, 2, NH, OH), 6.25 (m, 1, NH), 6.65 (m, 1, NH), 7.25–7.6 (m, 10, aromatic protons). α^{22}_D = 54.5 (*c* = 0.5, EtOAc). Anal. Calcd for $C_{21}H_{23}N_3O_4$: C, 66.14; H, 6.04; N, 11.02. Found: C, 65.96; H, 6.10; N, 10.51. The DL-isomer had mp 87–90 °C. α^{22}_D = −53.9 (*c* = 0.5, EtOAc). Anal. Calcd for $C_{21}H_{23}N_3O_4 \cdot 0.5H_2O$: C, 64.78; H, 5.91; N, 10.79. Found: C, 65.38; H, 6.06; N, 10.35. The LL- and DL-forms were separated by HPLC on a Novapak 4-μm, C-18, 60A, 3.9 × 150 mm column, flow rate 1 mL/min using a Waters 996 PDA detector at 220 nm, isocratic elution with 25% CH_3CN, 0.1% TFA/75% H_2O, 0.1% TFA, t_R 23.06 min (LL-), 28.12 min (DL-). For the dipeptide Fmoc-Leu-Pro-NH_2, mp 89–91 °C. α^{23}_D = −150.8 (*c* = 0.5, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ: 1.05 (d, 6, CH_3CH), 1.3–1.6 (m, 3, $CHCH_2$), 1.8–2.3 (m, 4, 2 × CH_2), 3.5–3.8 (m, 3, CH, CH_2), 4.1–4.5 (m, 4, 2 × CH, CH_2), 5.6 (d, 1, NH), 6.15 (d, 2, NH_2), 7.0–7.8 (m, 8, aromatic protons). Anal. Calcd for $C_{26}H_{31}N_3O_4$: C, 69.49; H, 6.90; N, 9.35. Found: C, 69.26; H, 6.92; N, 9.14. The conditions for HPLC separation were similar to those described above except that the isocratic solvent system 40% CH_3CN/60% H_2O, 0.1% TFA was used. t_R: 13.29 min (LL-) and 15.99 min (DL-). The mixture of diastereomers (Fmoc-Leu-DL-Pro-NH_2, mp 84–86 °C) obtained from acylation of DL-H-Pro-NH_2–HCl with Fmoc-Leu-F was used for the determination of relative retention times. [b] Instrumental methods and purification of reagents were as described in Table 2, ref 26. [c] The acid fluoride was mixed with the solvent and the base and the whole left at room temperature for 7 min prior to addition of H-Pro-NH_2. [d] The acid fluoride was mixed with the solvent alone and the solution left at room temperature for 7 min prior to addition of the base plus H-Pro-NH_2.

Because the automated solid phase syntheses described here were performed on a continuous flow instrument for which the standard protocol starts with a 7-min period of treatment with solvent to dissolve the mixture of coupling reagents, the preliminary model experiments were carried out using the same preincubation period.

Since urethane-protected amino acid fluorides do not undergo conversion to oxazolones in the presence of tertiary amines[23] the observed low racemization could be caused by attack of the base on the α-hydrogen atom of the acid fluoride itself. Such effects are to be expected in the case of amino acids bearing relatively acidic

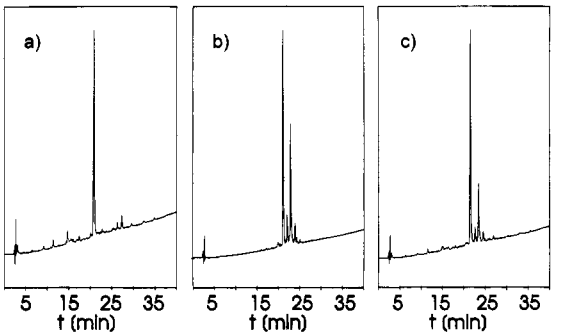

Figure 3. Comparison of HPLC retention behavior of native and synthesized alamethicin F30: (a) synthesized alamethicin F30, (b) native alamethicin F30, (c) coinjected synthetic and native alamethicin F30 (linear gradient: 50–65% B, 40 min, eluent A, 0.1% TFA/water, eluent B, 0.1% TFA in 80% ACN/water, flow rate: 1 mL/min, 220 nm).

α-hydrogen atoms. To probe such systems an additional model reaction was examined involving the highly sensitive amino acid α-phenylglycine.[24] The coupling of Z-Phg-F with proline amide is difficult to achieve without racemization[25] although dipeptide formation in dichloromethane with collidine[26] as base occurs cleanly. In DMF, the solvent used for all syntheses described here, DIEA gave 19.0% and collidine gave 11.6% of the DL-form.

Under conditions simulating the operation of the synthesizer (7-min preincubation in DMF in the presence of the base) the figures are 49.5% and 20.9%, respectively. If the 7-min preincubation period involves the solvent alone and proline amide is then added along with 1 equiv of base, racemization levels in DMF are 27.5% (DIEA) and 15.9% (collidine). A similar run in DMA gave 33.4% (DIEA) and 27.5% (collidine). Results are collected in Table 2.

These results clearly demonstrate that acid fluorides may undergo significant racemization when sensitive systems are used. For such systems preincubation with or without base should be avoided to the degree possible.

Additional model studies of some of the more sensitive proteinogenic amino acids (Phe, Asp, Ser, Cys, His), not found in the natural peptaibols, are in progress.

Comparison with Naturally Occurring Peptaibols. In order to confirm the remarkable success of these syntheses, the synthetic alamethicins F50 and F30 were compared with commercially available samples of alamethicin isolated from trichoderma viride broths. According to the HPLC behavior, the synthetic products are identical with the major component of the naturally occurring microheterogeneous mixtures of alamethicin F30 (Sigma) (Figure 3) and alamethicin F50 (Upjohn) (not shown but analogous to that shown in Figure 3). In addition, the synthetic material (F30) showed identical retention behavior with an authentic sample previously synthesized and characterized by Jung et al.[7] The sensitivity of the HPLC system used was established by its ability to effect base line separation of the major components of the coinjected native material comprising

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(25) The coupling of the urethane-protected amino acid Z-Phg-OH to the secondary amino acid proline is particularly sensitive, as was previously observed for analogous segment couplings. For the latter see: Takuma, S.; Hamada, Y.; Shioiri, H. *Chem. Pharm. Bull.* **1982**, *30*, 3147; Weygand, F.; Hoffmann, D.; Prox, A. *Z. Naturforsch.* **1968**, *23b*, 279. König, W.; Geiger, R. *Chem. Rev.* **1970**, *103*, 2024.

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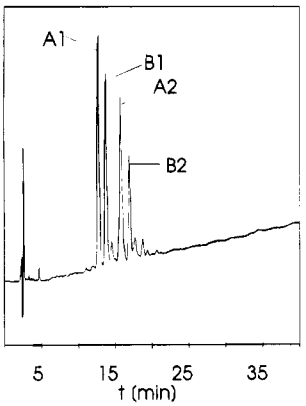

Figure 4. HPLC profiles of coinjected native samples of alamethicin F30 and alamethicin F50: A1, major component of alamethicin F50, A2, minor component of alamethicin F50; B1, major component of alamethicin F30; B2, minor component of alamethicin F30. The major components correspond to the sequences mentioned above for alamethicin F50 and F30, whereas the minor components are related to sequences of analogs involving substitution of one Ala by Aib[7] ((linear gradient: 50–65% B, 40 min, eluent A, 0.1% TFA/water, eluent B, 0.1% TFA in 80% ACN/water, flow rate: 1 mL/min, 220 nm).

Table 3. Antibacterial Activity of Native and Synthesized Alamethicins towards *E. coli* F 515

	F 515 MIC (μg/mL)
alamethicin F30 (synthesis)	15.5
alamethicin F30 (native)	15.5
alamethicin acid F30 (synthesis)	70
alamethicin F50 (synthesis)	68
alamethicin F50 (native)	200

alamethicin F30 and F50. Interestingly, all major components of the two native mixtures are well separated (Figure 4). The polymer-encapsulated silica column (Polyencap A300, Bischoff, Leonberg) used in this study was previously shown to be advantagous in the case of another class of hydrophobic, amphipathic peptides.[27]

Comparative antibacterial tests of natural and synthetic alamethicins have been performed using *Escherichia coli* strains ATCC 11775 and *E. coli* mutants which are increasingly defective in the O-specific side chain, F515 and F516.[28] As described earlier by Balasubramaniam et al.,[7] no activity of alamethicin F30 was observed against mutant ATCC 11775 for either the synthetic or the native compound. The closely related alamethicin F50 analog was also inactive toward this strain. All compounds were also found to be inactive toward the F516 strain, up to an inhibitor concentration of 250 μg/mL. In contrast, all alamethicin samples exhibited activity toward the F515 strain. By using this *E. coli* mutant, synthetic alamethicin F30 and the related native material showed identical activities, whereas for the F50 analog the synthetic material exhibited a higher activity than the native microheterogeneous material (Table 3). Generally, F30-alamethicin was more active than the analogous F50 mixture. The increase in inhibitory activity of alamethicin with increasing core defects (decrease in hydrophilicity) of *E. coli* and *S. Typhimurium* mutants is parallel to observations made with magainin and magainin derivatives.[29,30] In addition, the alamethicin acid analog of alamethicin F30 was shown to be active against the F515 strain although at a level lower than that of the corresponding alcohol (Table 2). This finding raises questions regarding the relationship between the presence of a C-terminal alcohol function and the biological activity of the peptaibols. Details regarding this matter must await further studies.

Conclusions

The first successful stepwise syntheses of the naturally occurring peptaibols using standard solid phase protocols by means of Fmoc amino acid fluorides have been described. The outstanding effectiveness of the method is confirmed by the high purity of the crude products and the low extent of racemization. In combination with the introduction of a new convenient method for direct anchoring of Fmoc-amino alcohols onto a commercial solid support the acid fluoride technique has been established as a powerful tool for rapid access to an important class of peptides, not previously readily available.

Experimental Section

General. Analytical HPLC characterization of all synthesized peptides was carried out on a Polyencap A300 column, 250 × 4 mm i.d., Bischoff Analysentechnik GmbH, Germany, with a Bischoff HPLC system consisting of a Lambda 100 detector, pumps, central processor, and Knauer mixing chamber. Preparative isolation of peptides was carried out by HPLC on a 10 μm Polyencap A300 preparative column from the same company using a Shimadzu LC-8A system including LC-8A pumps, an SCL-8A system controller, an SPD-6A UV detector operated at 220 nm, an injector, and a C-R4A Chromatopac recording unit. Peptides were eluted using a linear gradient: eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in 80% ACN/20% water (v/v).

ES-MS was performed on a TSQ 700, Finnigan MAT (sample flow 1 μL/min methanol/water (1:1), 3–5 kV, 80 °C). High-resolution mass spectroscopy was carried out on a Finnigan MAT 95 (Bremen, Germany) with electrospray ionization in the positive mode. IR spectra (KBr) of fluorides were recorded on an Impact 400 instrument (Nicolet). ^{1}H-NMR determinations were carried out on a Varian Gemini 200 instrument. Amino acid analysis was performed using ion-exchange chromatography and post column derivatization with ninhydrin (Biotronik-Eppendorf LC 3000). Peptides were hydrolyzed in 6 N HCl at 120 °C for 48 h. Because valine and isovaline were not separated by the system used, these amino acids could not be determined when present together. Fmoc-L-Val-PEG-PS and Fmoc-L-Phe-PEG-PS resins were provided by Millipore, Burlington, MA. TG SA C-resin was purchased from Rapp Polymere, Tübingen, Germany, and *o*-chlorotrityl resin from Novabiochem, Bad Soden/Ts., Germany. Valinol was obtained from Advanced ChemTech, Louisville, KY, and phenylalaninol from Aldrich, Steinheim, Germany. Alamethicin F50 was purchased from Sigma, Deisenhofen, Germany, and alamethicin F30 was a gift from the Upjohn Company, Kalamazoo, MI.

Loading of *o*-Chlorotrityl Resin with Fmoc-amino Alcohols. Linkage of the Fmoc-amino alcohol to the *o*-Cl-Trt-resin (Novabiochem, 1.6 mmol/g) was performed by stirring 250 mg of the resin (0.32 mmol), 0.96 mmol of the Fmoc-amino alcohol, and 1.92 mmol of pyridine for 6, 24, 48, and 72 h in 1.5 mL of dry DCM, 1.5 mL of dry DMF. Methanol (4 mL) was added, and the mixture stirred for 30 min to terminate

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the reaction and to remove any remaining reactive chloro functionality. The loaded resin was washed, the Fmoc-group cleaved by treatment of the resin with 20% piperidine/DMF for 15 min, and the loading determined by UV analysis (see Figure 2). Resins of moderate loading were chosen for this work.

Amino Acid Derivatives, Activating Reagents. The fluorides were prepared using a standard procedure.[23]

Fmoc-phenylalaninol. Fmoc-phenylalaninol was prepared by stirring 20 mmol of the amino alcohol together with 24 mmol of 9-fluorenylmethyl chloroformate and 20 mmol of TEA in 50 mL of dry DCM at ambient temperature overnight. The DCM was evaporated in vacuo and the precipitate carefully washed with ether, 10% citric acid, and water. The solid was dissolved in ethyl acetate and dried over $MgSO_4$. Removal of solvent in vacuo followed by precipitation with hexane gave in 76% yield the pure alcohol as a white solid, mp 163 °C. ES-MS: calcd 396.1576 $[M + Na]^+$, found 396.1566 $[M + Na]^+$. ^{1}H-NMR ($CDCl_3$) δ: 2.2 (s, 1, OH), 2.85–2.88 (d, 2, CH_2), 3.6–3.65 (d, 2, CH_2), 3.92–3.93 (m, 1, CH), 4.15–4.49 (m, 3, CH, CH_2), 5.00 (s, 1, NH), 7.19–7.78 (m, 13, aromatic protons). Anal. Calcd for $C_{24}H_{24}NO_{3.5}$ ($M + 0.5H_2O$): C, 75.37; H, 6.32; N, 3.66. Found: C, 75.36; H, 6.13; N, 3.63.

Fmoc-valinol. The method described for Fmoc-Pheol was used. Yield: 72%. Mp: 127 °C. ES-MS: calcd 348.1576 $[M + Na]^+$, found 348.1611 $[M + Na]^+$. ^{1}H-NMR ($CDCl_3$) δ: 0.91–0.99 (m, 6, 2 × CH_3), 1.81–1.92 (m, 2, CH_2), 3.47 (s, 1, OH), 3.64–3.7 (m, 2, 2 × CH), 4.19–4.48 (m, 3, CH, CH_2), 5.0 (s, 1, NH), 7.26–7.78 (m, 8, aromatic protons). Anal. Calcd for $C_{20}H_{23}NO_3$: C, 73.87; H, 7.12; N, 4.31. Found: C, 73.85; H, 7.22; N, 4.09.

Fmoc-Iva-OH. Racemic α-ethylalanine was prepared by the procedure of Levene.[31] The Fmoc-group was introduced by stirring 5 mmol of Iva, 5.5 mmol of 9-fluorenylmethyl chloroformate, and 15 mmol of Na_2CO_3 in 100 mL of 50% acetone/water overnight. Acetone was evaporated and the aqueous solution washed with ether and acidified with 10% citric acid. The precipitated acid was washed with water, dissolved in ethyl acetate, dried ($MgSO_4$), and evaporated, and hexane was added to precipitate the pure acid. After being dried in vacuo the acid was obtained in a yield of 79% as a white solid, mp 154–155 °C. ES-MS: calcd 362.1368 $[M + Na]^+$, found 362.1376 $[M + Na]^+$. ^{1}H-NMR ($CDCl_3$) δ: 0.7–1.2 (m, 3, CH_3); 1.6 (s, 3, CH_3); 1.8–2.1 (m, 2, CH_2); 4.19–4.4 (m, 3, CH, CH_2); 5.57 (s, 1, NH); 7.26–7.78 (m, 8, aromatic protons); 8.53 (s, 1, OH). Anal. Calcd for $C_{20}H_{21}NO_4$: C, 70.78; H, 6.24; N, 4.13. Found: C, 70.87; H, 6.34; N, 3.98.

Fmoc-Iva-F. Yield: 78%. MP: 131 °C. IR: 1839 cm^{-1}. ES-MS: calcd 364.1325 $[M + Na]^+$, found 364.1306 $[M + Na]^+$. ^{1}H-NMR (DMSO-d_6) δ: 0.82 (t, 3, CH_3); 1.37 (s, 3, CH_3); 1.69–1.82 (m, 2, CH_2); 4.23–4.4 (m, 3, CH, CH_2); 6.16 (s, 1, NH); 7.29–8.10 (m, 8, aromatic protons). Anal. Calcd for $C_{20}H_{20}NO_3F$: C, 70.36; H, 5.91; N, 4.10. Found: C, 69.93; H, 5.66; N, 4.26.

Racemization Tests on Peptaibols. Gas chromatographic separation of enantiomers was carried out on a FISONS TRIO 1000 gas chromatographic/mass spectrometric data system using permabond L-Chirasil-Val (MACHERY NAGEL) as the chiral column (50 m × 0.25 mm i.d.). To determine racemization levels the peptides were hydrolyzed in 6 N DCl/D_2O for 46 h at 110 °C and the amino acids were converted to the N-(trifluoroacetyl) isopropyl esters as described earlier.[32] Racemization during the hydrolysis procedure was accompanied by complete deuterium labeling. Derivatized samples were analyzed using ultrapure helium as carrier gas. The column head pressure was 16 psi, the injector temperature was held at 190 °C, and the interface and the ion source temperature were maintained at 200 °C. The splitless injection mode was used with the purge valve turned on 90 s after injection, with a split flow of 25 mL/min during the GC run. The gas chromatograph oven temperature was held at 80 °C for 1 min and then programmed at 4 °C/min to 190 °C for 30 min. Electron-impact ionization mass spectra were recorded in the selective-ion mode (SIM). Molecular ions and typical fragment ion peaks of the derivatized amino acids were monitored.

Antibiotic Tests. The *E. coli* strain ATCC 11775 was purchased from the American Type Culture Collection. The mutant strains F 516 and F 515 with defects in core oligosaccharides were a gift from G. Schmidt, Institute of Experimental Biology and Medicine, Borstel, Germany.

The bacteria were maintained on agar slants. The culture broth was that described by Anton.[33] Serial dilution techniques following standard procedures were used for MIC-determination.

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Abbreviations: DMF = dimethylformamide; DIEA = diisopropylethylamine; DCl = deuterated hydrogen chloride; TEA = triethylamine; TLC = thin layer chromatography; ES-MS = electrospray mass spectrometry; MIC = minimal inhibitory concentration; BTMSA = bis(trimethylsilyl)acetamide; Iva = isovaline, α-ethylalanine; TMP = 2,4,6-trimethylpyridine = collidine; DCM = dichloromethane; DMA = dimethylacetamide; nd = not determined.

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