Journal of Peptide Science
J. Peptide Sci. **9**: 799–809 (2003)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.535

JOURNAL OF PEPTIDE SCIENCE

Sequences of Alamethicins F30 and F50 Reconsidered and Reconciled‡

JOCHEN KIRSCHBAUM, CORINA KRAUSE, RUTH K. WINZHEIMER and HANS BRÜCKNER*

University of Giessen, Interdisciplinary Research Center, Department of Food Sciences, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

Abstract: From the culture broth of the mould *Trichoderma viride*, strain NRRL 3199, a microheterogeneous mixture of the membrane active 20-residue peptaibol alamethicin (ALM) could be isolated. ALMs were isolated by XAD-2 column chromatography and separated by silica gel chromatography and trichloromethane/MeOH gradient elution into an acidic and neutral group of peptides, named ALM F30 and ALM F50, respectively, according to their 100 R_f on TLC. Peptides ALM F50 were separated by semi-preparative and analytical HPLC and subjected to ESI-MS. Ten sequences of ALM F30 and their relative quantities could be determined. The major peptides ALM F30/3 (46%) and ALM F30/7 (40%), distinguished by Aib/Ala exchange in position 6, correspond to sequences described as ALM I and II occurring in the original alamethicin from Upjohn Company. Analogously, 13 sequences of the neutral peptide mixture named ALM F50 could be determined. The major peptide ALM F50/5 (75%) and the minor peptide ALM F50/7 (10%) are distinguished from ALM F30/3 and ALM F30/7 by having Gln17 in place of Glu17, the latter occurring in the F30 group. Notably, currently commercially available alamethicins (Fluka, Sigma) represent microheterogeneous mixtures of the neutral ALM F50 peptides with trace amounts of acidic ALM F30 peptides.

Keywords: peptaibols; peptide antibiotics; α-aminoisobutyric acid; isovaline; alamethicin F30 and F50; electrospray ionization mass spectrometry

INTRODUCTION

The first official report on the isolation and partial structural characterization of a polypeptide antibiotic (Upjohn Company, Kalamazoo; U-22,324) from the culture broth of the mould *Trichoderma viride* Pers ex Fries, strain NRRL 3199, was published by Meyer and Reusser in 1967 [1].

It was recognized that, among standard amino acids, the peptide contained a relatively large proportion of the nonprotein amino acid α-methylalanine (2-methylalanine, α-aminoisobutyric acid, Aib). Since this amino acid was listed in the register of Chemical Abstracts under the name '**ala**nine, **meth**yl', in accordance with many antibiotics the ending -**icin** was added and the peptide was named 'alamethicin' (abbreviated ALM in the following). The unique membrane modifying properties of the peptide were soon recognized and reported by Mueller and Rudin in 1968 [2]. As no free *N*- and *C*-terminal amino acids could be detected and the peptide could not be visualized by spraying with ninhydrin, a cyclic structure was proposed based on acidic partial hydrolysates which were sequenced using conventional methods by Payne *et al.* [3]. It was assumed that the γ-carboxy group of Glu17 was peptide bonded to the imino group of Pro1. Using a variety of analytical techniques, including GC-MS of partial hydrolysates and NMR, at about the same time Ovchinnikov *et al.* came to the same structural conclusions [4].

Investigation by TLC on silica H (i.e. acidic silica gel) of the Upjohn ALM by Melling and McMullen in 1975 revealed that this material was composed of two major groups named, according to 100 R_f

*Correspondence to: Dr Hans Brückner, University of Giessen, Interdisciplinary Research Center, Department of Food Sciences, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany;
e-mail: hans.brueckner@ernaehrung.uni-giessen.de
‡ Presented at Peptaibols, October 2002, Jena, Germany.

values, ALM F30 (about 85%), ALM F50 (about 12%), and a minor component ALM F20 (2%) [5]. However, minor or trace components of ALMs, numbered accordingly F40, F60 and F70, were also recognized by TLC [5]. Martin and Williams performed ^{1}H-NMR studies on ALM (Upjohn) and isolated from hydrolysates the hitherto undetected amino alcohol ß-phenylalaninol (Pheol) as well as *N*-acetyl-Aib [6]. They concluded that the peptide is linear and not cyclic and that the *N*-terminal Ac-Aib might be back-linked to Pro thus forming a five-membered ring. Pheol was assumed to be amide-bonded to the γ-carboxyl of Glu at the *C*-terminal end of the peptide. The presence of Pheol was also recognized in 1975 by Jung *et al.* in the ^{13}C-NMR spectra of ALM [7]. Martin and Williams distinguished also between the major component defined as F30 and a minor component F50, the latter assumed to represent the amide analogue of the former [6].

Based on the proposed linear structure an attempt was made to synthesize ALM F30 by Gisin *et al.* in 1977 [8]. Unexpectedly, the peptide was not identical to the natural material. From the pK_a values of Glu model compounds and comparison of data with the natural ALM isolate from Upjohn Company it was concluded that the amino alcohol was amide bonded to the α-carboxy group of *C*-terminal Gln19, rather than to the γ-carboxy group of Glu18 [8, 9].

Applying high resolution MS and field desorption MS on the Upjohn material, Pandey *et al.* in 1977 reported on the structure of a major peptide, named ALM I (85%) and minor component ALM II (2%) [10]. The authors demonstrated convincingly that the Upjohn material indeed represents an acidic 20-residue peptide with a *N*-terminal Ac-Aib-residue and a *C*-terminal Pheol residue α-linked to Gln19, whereas the γ-carboxy group of the preceding Glu18 residue is definitely not substituted. The peptides designated ALM I and ALM II differ by the presence of Aib6 in ALM I and Ala6 in ALM II.

It is worth noting that Pandey *et al.* suggested naming this unique group of peptides, according to its characteristic residues, **peptaibophols** (i.e. **pept**ides containing **Aib** and **ph**enylalanin**ol**). With the detection of valinol in the related peptide trichotoxin [11], the name was generalized to **peptaibols**, a name that has become well established.

Based on the revised structure of ALM three total syntheses of the major component ALM I were reported in 1981 by Gisin *et al.* [12], Balasubramanian *et al.* [13] and Nagaraj and Balaram [14], followed by a total synthesis of Schmitt and Jung in 1985 [15].

Total synthesis of ALM and related peptaibols is still attractive and a synthetic challenge as demonstrated by the solution phase synthesis of ALM by Słomczynska *et al.* performing a chemo-enzymic coupling in the last step [16]. Problems encountered in the coupling of sterically hindered Aib-segments by the use of conventional methods could be overcome by applying solid phase procedures employing highly reactive carboxyfluorides such as FMOC-Aib by Wenschuh *et al.* This method made possible the synthesis of ALMs, as well as other natural peptaibol sequences [17].

Thus, the major sequence of ALM was confirmed by many total syntheses and also corroborated by x-ray analysis of the natural peptide [18]. It was realized, however, as early as 1979 by applying HPLC that the Upjohn material was composed of a minimum of 12 components [19].

In the course of their own fermentation and isolation of ALM from *T. viride* NRRL 3199, Irmscher and Jung reported in 1977 that their major product had a R_f of 0.5 and the minor product a R_f of 0.3 on TLC [20]. This is the opposite ratio compared with the Upjohn material. In accordance with the designation used by Melling and McMullen [5] the major group composed of neutral peptides was named ALM F50, whereas the minor group of acidic peptides corresponding to the Upjohn material was named ALM F30. Consequently, the mixture of acidic alamethicins in the literature usually is referred to as alamethicins F30 (ALM F30), whereas the neutral alamethicins are designated alamethicins F50 (ALM F50). Accordingly, alamethicins I and II, as named and sequenced by Pandey *et al.* [10], are referred to as alamethicins F30/I and F30/II.

Investigation of the ALM mixture from Upjohn Company by König and Aydin using derivatized partial hydrolysates by GC-MS revealed at least six exchange positions of amino acids, including the established Ala6/Aib6 exchange [21]. Assignment of complete sequences of individual peptides of the mixtures was not possible by the technique used.

Several fermentations of *T. viride* NRRL 3199 in our laboratory in the complex medium provided, in agreement with the results of Irmscher and Jung, continued the F50 group as the major peptides accompanied by minor amounts of F30 peptides. Fermentation in malt medium, however, yielded a mixture of ALM F30 and F50 peptides. Separation of the F50 peptides by HPLC provided individual peptides designated ALM F50/A–F. Sequencing of these components using FAB-MS, accompanied by gas chromatographic investigations, provided the

complete sequences for peptides F50/C–E [22]. The identity of peptides 'C' and 'E' to ALM F30/I and ALM F30/II was established, with the restriction of ALM F50 having Gln in sequence position 18. Further, the presence of D-isovaline (D-Iva) in minor peptides ALM F50/D and 'F' was detected. The presence of Iva in ALM sequences had not been recognized previously.

In continuation of this work, and in order to characterize commercially available preparations of ALM, the major and minor structures of ALM F30 and F50 peptides are presented and the chromatographic elution profiles, sequences of components, and abbreviations used in the literature are correlated.

MATERIALS AND METHODS

Chemicals

Acetonitrile (MeCN) and methanol (MeOH) (all of gradient grade quality) were purchased from Merck (Darmstadt, Germany); hydrochloric acid (32%, w/v), 1-propanol, aqueous ammonia (25%, w/v), dichloromethane and trichloromethane (TCM) were from Carl Roth (Karlsruhe, Germany); trifluoroacetic acid (TFA), 2,6-di-*tert*-butyl-*p*-cresol (BHT) and trifluoroacetic acid anhydride (TFAA) were from Fluka (Deisenhofen, Germany). For amino acid analysis, an amino acid standard solution (AA-S-18 from Sigma, St Louis, MO, USA) was used and appropriate amounts of α-methylalanine (Aib, U, from Sigma), DL-isovaline (Iva, synthesized in our laboratory via the Strecker procedure) and L-phenylalaninol (Fol, from Sigma) were added.

Sources of Alamethicins

Alamethicins were isolated in our laboratory from *Trichoderma viride* NRRL 3199. A freeze dried sample was received from Northern Regional Research Laboratory, Agricultural Research and Development Division, U.S. Department of Agriculture, Peoria, Ill, USA. Commercial ALMs were from Fluka (product no. 05125), from Sigma (product no. A4665) and compared with a sample from Upjohn Company (antibiotic U-22,324).

Chromatography

For column chromatography, Servachrom XAD-2 polystyrene adsorber resin, particle size 100–200 μm (Serva, Heidelberg, Germany), Sephadex LH 20, particle size 25–100 μm (Pharmacia, Freiburg, Germany) and LiChroprep RP-8, particle size 40–63 μm (Merck) were used. For thin layer chromatography (TLC), pre-coated plates with silica gel 60 (Merck) were used. The mobile phase was trichloromethane/methanol/17% aqueous ammonia (70/35/10, v/v/v). Peptaibols were detected by spraying with water and, after drying, with chlorine/TDM reagent.

For HPLC a HP 1100 series instrument comprising a Model G1322A degasser, G1312A binary pump, G1313A autosampler, G1316A column thermostat, G1314A UV/VIS detector and software HP ChemStation for LC (Rev. A.04.02) was used (all from Agilent, Waldbronn, Germany or Palo Alto, CA, USA). The alamethicins were detected at a wavelength of 205 nm. For analytical HPLC columns (I) Kromasil KR100, 150 mm × 4.6 mm i.d., 3.5 μm particle size (EKA Chemicals, Bohus, Sweden) was employed; for semi-preparative HPLC column (II) was Spherisorb ODS, 250 mm × 8 mm i.d., 3 μm particle size (Grom, Herrenberg, Germany).

Eluents for column (I): for analytical separation of ALM F30 (acidic components) eluent A was a mixture of MeCN/MeOH/water (39/39/22, v/v/v) and eluent B was MeCN/MeOH (1/1, v/v), both containing 0.1% TFA (v/v). For the gradient programme, the column temperature and the flow rate see Table 1a. The analytical separation of neutral ALM F50 eluents A and B were those used for the separation of acidic ALM F30, the difference being that TFA was omitted (see Table 1b). The eluents for column (II), used for the semi-preparative HPLC separation of ALM F50, were identical to those employed for the separation of ALM F50 on column (I) (see Table 1c).

For medium pressure liquid chromatography (MPLC), a MD 80/100 pump, controller PS 1 (Labomatic, Sinsheim, Germany), and a Model FRAC-100 fraction collector (Pharmacia) were used. The adsorber resins XAD-2 and silica gel were packed in individual MPLC glass columns (38 cm × 3.7 cm i.d., Labomatic).

Amino Acid Analysis by GC-SIM-MS

Chiral amino acid analysis was performed on a GC-MS Model HP 6890 with a mass selective detector Model HP 5972 (Agilent, Waldbronn, Germany). The instrument was equipped with a Chirasil-L-Val (i.e. *N*-propionyl-L-valine-*tert*-butylamide polysiloxane) quartz capillary column, 25 m × 0.25 mm i.d. (Varian-Chrompack, Darmstadt, Germany). EI mass

Table 1 Gradient Programmes for Analytical Separation of ALM F30, ALM F50 and Semi-preparative HPLC of ALM F50

(a)			(b)			(c)		
Time (min)	Eluent		Time (min)	Eluent		Time (min)	Eluent	
	A (%)	B (%)		A (%)	B (%)		A (%)	B (%)
0	90	10	0	100	0	0	80	20
5	90	10	10	100	0	10	80	20
25	50	50	30	20	80	30	20	80
26	20	80	35	20	80	35	20	80
35	20	80	36	100	0	36	80	20
36	90	10	43	100	0	43	80	20
43	90	10						

(a) Analytical Separation of ALM F30 on Kromasil KR100 column (150 mm × 4.6 mm i.d., 3.5 μm), column temperature 50 °C, flow rate 1.0 ml/min; (b) Analytical Separation of ALM F50, Kromasil KR100 column (150 mm × 4.6 mm i.d., 3.5 μm), column temperature 35 °C, flow rate 1.0 ml/min; (c) Semi-preparative HPLC of ALM F50, Spherisorb ODS column (250 mm × 8 mm i.d., 3 μm), column temperature 50 °C, flow rate 3.5 ml/min. Eluent A, MeCN/MeOH/water 39/39/22, v/v/v; eluent B, MeCN/MeOH 1/1, v/v; for analytical separation of ALM F30 to eluents A and B 0.1% TFA was added.

spectra were recorded at an ionization energy of 70 eV.

Aliquots of 20–100 μg of the microheterogeneous mixture of peptides from ALM F30 and the fractions of ALM F50 (20–100 μg), obtained from semi-preparative HPLC, were hydrolysed with 6 M HCl at 100 °C for 16 h. The chirality of AA was determined after derivatization as *N*-trifluoroacetyl-AA-(1)-propyl esters by GC — selected ion monitoring (SIM) — MS. The chromatographic conditions were: temperature: 70 °C, 1.0 min, 2.5 °C/min; 100 °C, 2.0 min, 3.5 °C/min; 135 °C, 5.0 °C/min, 150 °C, 20 °C/min; 190 °C, 10 min; pressure: 5 kPa, 1 min, 0.2 kPa/min; 7 kPa, 2.0 min, 0.3 kPa/min; 11 kPa, 1.6 kPa/min; 15 kPa, 8.0 min. The split ratio was 30 : 1 at an injection volume of 1.0 μl. The chirality of Pheol and Iva had been analysed previously [22].

Mass Spectrometry

For ESI-MS a LCQ™ MS (Thermo Finnigan MAT, San Jose, CA, USA) was used. Alamethicins were analysed either by online HPLC-MS, using HPLC column (I), or via direct infusion into the MS. Nitrogen served as the sheath and auxiliary gas and helium (purity >99.9990%, Messer-Griesheim, Krefeld, Germany) as the collision gas. Sequence analysis was carried out by positive and negative ionization. The m/z values were recorded in centroid mode and have an accuracy of 0.5 m/z. The values in Tables 2 and 3 are rounded up or down, respectively. Conditions for direct infusion and positive (negative) ionization mode were: spray voltage 4.00 kV (4.00 kV), heated capillary temperature 230 °C (230 °C), capillary voltage +3.0 V (−3.0 V), tube lens offset +30.0 V (−30.0 V), sheath gas 50 units, auxiliary gas 5 units, maximum ion time 1000 ms. For online HPLC-MS the temperature of the heated capillary was set to 250 °C, sheath gas to 65 units, auxiliary gas to 20 units. For automatic mass calibration a mixture of caffeine (m/z 195.1), Met-Arg-Phe-Ala (m/z 524.3) and the perfluoronated Ultramark 1621 (m/z 1022.0, 1122.0, 1222.0, 1322.0, 1422.0, 1522.0, 1622.0, 1722.0, 1822.0, 1921.9) was used. The negative-ion mode was performed via infusion of solutions ($c = 0.1\%$, w/v) of ALM F50, or the fractions of ALM F50 obtained by repetitive semi-preparative HPLC, or ALM F30 in MeOH/2% aqueous ammonia (1/1, v/v). For sequence analysis of ALM peptides $(M + H)^+$ and $(M)^-$ molecular ions were chosen as precursor ions for MS^n.

The notation used for fragment assignments in the positive-ion mode, referring to *a*, *b* and *c* acylium ions, is in accordance to those used previously by us [23–25] and is based on the suggestion of Roepstorff, Fohlman and Biemann. The negative-ion mode produced the *y* series of fragment ions without protonation [25], and is denoted y_N in Tables 2 and 3.

Table 2 Diagnostic Ions (m/z) of ALM F30/1–10

	1	2	3	4	5	6	7	8	9	10
$(M+H)^+$	1950	1950	1964	1964	1978	1965	1978	1992	1992	1992
$(M-H_2O)^+$[a]	1932	1932	1946	1946	1960	1947	1960	1974	1974	1974
$(M+Na)^+$	1972	1972	n.d.	1986	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
b_2[a]	n.d.	225	225	225	225	n.d.	225	n.d.	225	n.d.
b_3[a]	310	310	310	310	310	310	310	310	310	310
b_4[a]	381	381	381	381	381	381	381	381	381	381
b_5[a]	466	466	466	466	466	466	466	466	466	466
b_6[a]	537	537	537	551	537	537	551	551	551	551
b_7[a]	665	665	665	679	665[b]	666	679	679	679[b]	679
b_8[a]	750	750	750	764	750	751	764	764	764	764
b_9[a]	835	849	849	863	849	850	863	877	863	863
b_{10}[a]	920	934	934	948	934	935	948	962	948	948
b_{11}[a]	977	991	991	1005	991	992	1005	1019	1005	1019
b_{12}[a]	1090	1090	1104	1104	1104	1105	1118	1132	1118	1132
b_{13}[a]	1175	1175	1189	1189	1189	1190	1203	1217	1203	1217
b_{16}[a]	1456	1456	n.d.	n.d.	n.d.	1472	n.d.	1485	1485	n.d.
b_{17}[a]	1541	1541	1556	1556	1570	1557	1570	1584	1584	1584
b_{18}[a]	1670	1670	1685	1685	1699	1686	1699	1713	1713	1713
b_{19}[a]	1798	1798	1813	1813	1827	1827	1827	1841	1841	1841
a_7, Na[b]	659	n.d.	n.d.	673	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
y_{7P}[a]	775	775	775	775	789	775	775	775	789	775
$(y_{7P}-H_2O)$[a]	757	757	757	757	771	757	757	757	771	757
y_{7P}–AA (20)[a]	624	624	624	624	638	624	624	624	638	624
y_{7P}–AA (20–19)[a]	496	496	496	496	510	496	496	496	510	496
y_{7P}–AA (20–18)[a]	367	367	367	367	381	367	367	367	381	367
y_{7P}–AA (20–17)[a]	282	282	282	282	282	282	282	282	282	282
y_{7P}–AA (20–16)[b]	197	197	197	197	197	197	197	197	197	197
M^-	1949	1949	1963	1963	1977	1964	1977	1991	1991	1991
y_{4N}[b]	493	493	493	493	n.d.	463	493	493	n.d.	493
y_{5N}[b]	578	578	578	578	n.d.	578	578	578	n.d.	578
y_{6N}[b]	677	677	677	677	n.d.	677	677	677	n.d.	677
y_{7N}[b]	774	774	774	774	n.d.	774	774	774	n.d.	774
y_{13N}[b]	1284	1284	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
y_{14N}[b]	1412	1412	1426	1412	1440	n.d.	1426	n.d.	n.d.	n.d.
y_{15N}[b]	1483	1483	1497	1497	1511	1498	1511	n.d.	n.d.	n.d.
y_{16N}[b]	1568	1568	1582	1582	1596	1583	1596	n.d.	n.d.	n.d.
y_{17N}[b]	1639	1639	1653	1653	1667	1654	1667	1681	1681	1681
y_{19N}[b]	1821	1821	1835	1835	1849	1849	1849	1863	1863	1863

[a] Masses were identified via HPLC/MS^n ($n = 2–5$) from specific ions like $(M+H)^+$, b_{13}, b_8, b_5, and y_{7P} in positive ion mode, and MS^2 from M^- in negative ion mode.
[b] Masses were identified via infusion MS^n ($n = 2–5$) from specific ions like $(M+H)^+$, b_{13}, b_8, and b_5 in positive ion mode, and MS^2 from M^- in negative ion mode. Exceptions were $(M+H)^+$, $(M+Na)^+$, and M^-. n.d. not determined.

Fermentation Procedure for Alamethicin Production

For malt medium, 30 g malt extract (Serva) and 3 g soy peptone (Oxoid, Wesel, Germany) were dissolved in 1 l demineralized water (final pH 6–6.5) followed by sterilization.

Freeze dried *Trichoderma viride* NRRL 3199 was suspended in sterile water (1.5 ml) and, after soaking for 15 min, transferred to Petri dishes (9.5 cm diameter) with potato–glucose–agar medium. This medium was used for storage of *T. viride* NRRL 3199 at 4 °C in the refrigerator. *T. viride* NRRL 3199 was transferred under sterile conditions to Petri dishes

Table 3 Diagnostic Ions[a] (m/z) of ALM F50/2-8c

	2	3a	3b	3c	4a	4b	5	6a	6b	7	8a	8b	8c
$(M + H)^+$	1935	1949	1949	1963	1949	1949	1963	1977	1977	1977	1977	1991	1991
$(M - H_2O)^+$	1917	1931	1931	1945	1931	1931	1945	1959	1959	1959	1959	1973	1973
$(M + Na)^+$	1957	1971	1971	1985	1971	1971	1985	1999	1999	1999	1999	2013	2013
b_2	n.d.	n.d.	225	n.d.	n.d.	225	225	225	225	225	n.d.	n.d.	225
b_3	n.d.	310	310	n.d.	310	296	310	310	310	310	310	310	310
b_4	381	381	381	381	381	367	381	381	395	381	381	381	395
b_5	466	466	452	466	466	452	466	466	480	466	466	466	480
b_6	537	537	523	523	537	523	537	537	551	551	537	551	565
b_7	665	665	651	651	n.d.	n.d.	665	665	679	679	n.d.	679	n.d.
b_8	750	750	736	736	750	736	750	750	764	764	750	764	778
b_9	821	835	835	835	849	835	849	849	863	863	863	863	877
b_{10}	906	920	920	920	934	920	934	934	948	948	948	948	962
b_{11}	963	977	977	977	991	977	991	991	1005	1005	1005	1005	1019
b_{12}	1076	1090	1090	1090	1090	1090	1104	1104	1118	1118	1118	1118	1132
b_{13}	1161	1175	1175	1175	1175	1175	1189	1189	1203	1203	1203	1203	1217
b_{15}	n.d.	1371	1371	n.d.	n.d.	n.d.	1385	1385	n.d.	1399	n.d.	1399	n.d.
b_{16}	1442	1456	1456	n.d.	1456	1456	1470	1470	n.d.	1484	n.d.	1484	n.d.
b_{17}	1527	1541	1541	1555	1541	1541	1555	1569	1569	1569	1569	1583	1583
b_{18}	1655	1669	1669	1683	1669	1669	1683	1697	1697	1697	n.d.	1711	1711
b_{19}	1783	1797	1797	1811	1797	1797	1811	1825	1825	1825	n.d.	1839	1839
y_{7P}	774	774	774	788	774	774	774	788	774	774	774	788	774
$(y_{7P}$ - $H_2O)$	756	756	756	770	756	756	756	770	756	756	756	770	756
y_{7P}–AA (20)	623	623	623	637	623	623	623	637	623	623	623	637	623
y_{7P}–AA (20–19)	495	495	495	509	495	495	495	509	495	495	495	509	495
y_{7P}–AA (20–18)	367	367	367	381	367	367	367	381	367	367	367	381	367
y_{7P}–AA (20–17)	282	282	282	282	282	282	282	282	282	282	282	282	282
M^-	1934	1948	1948	1962	1948	1948	1962	1976	1976	1976	1976	1990	1990
y_{7N}	773	773	773	787	773	773	773	787	773	773	773	787	773
y_{13N}	1269	1283	1297	1311	1283	1297	1297	1311	1297	1297	1311	1311	1297
y_{14N}	1397	1411	1425	1439	1411	1425	1425	1439	1425	1425	1439	1439	1425
y_{15N}	1468	1482	1496	1496	1482	1496	1496	1510	1496	1510	1510	1524	1510
y_{16N}	1553	1567	1567	1581	1567	1581	1581	1595	1581	1595	1595	1609	1595
y_{17N}	1624	1638	1638	1652	1638	1652	1652	1666	1666	1666	1666	1680	1680
y_{19N}	1806	1820	1820	1834	1820	1820	1834	1848	1848	1848	1848	1862	1862

[a] Masses identified via infusion MS^n ($n = 2$–5) from specific ions like $(M + H)^+$, b_{13}, b_8, b_5, and y_{7P} in positive ion mode, and MS^n ($n = 2$–3) from M^- and y_{19n} in negative ion mode. Exceptions were $(M + H)^+$, $(M + Na)^+$, and M^-; n.d., not determined.

with oat-agar medium. After 10–14 days at 25 °C intensive growth was observed. These cultures were used for the inoculation of fermentation broths. Fermentations were repeated several times and typical protocols are described below. Agar discs (1 cm diameter) were used for the inoculation of 18 Erlenmeyer flasks (2 l), each containing 500 ml of malt medium. The flasks were shaken at 100 min^{-1} at 25 °C for 8 days. The fermentation was performed using a rotary shaker Model G 25 (New Brunswick Scientific Co. Inc., Edison, NJ, USA). For daily monitoring of peptaibol production, aliquots (30 ml) of filtered culture broth were passed through Sep-Pak® C-18 cartridges (Waters, Milford, MA, USA) and the peptides adsorbed were eluted with 3 ml MeOH. The eluates were evaporated to dryness and the residues were dissolved in MeOH (0.5 ml). Aliquots of 5–15 µl were investigated by TLC (see Experimental) and spots of about R_f 0.3 and R_f 0.5 could be recognized, corresponding to ALM F30 and ALM F50, respectively.

Isolation and Purification of Alamethicins

After shaking for 8 days intensive peptide production in the fermentation broth was detected via TLC. Culture broths were separated from the mycelia

by filtration under reduced pressure. The mycelia were washed with distilled water and the combined filtrates were pumped through a MPLC column (380 × 37 mm i.d.) filled with XAD-2 resin at a flow rate of 1 l/h. The resin was washed with water (2.5 l, flow rate 1.5 l/h), then the adsorbed peptides were eluted with a linear gradient from 75% to 100% MeOH at a flow rate of 8 ml/min. The first 100 ml of the eluate was discarded and fractions (15 ml) were collected. Elution of peptides was monitored by TLC. Fractions containing ALMs were combined and evaporated to dryness yielding 3.8 g of a dark brown oil. The raw material was dissolved in 15 ml of a mixture of TCM/MeOH (7/3, v/v) and injected on a MPLC column (380 × 37 mm i.d.) filled with silica gel 60. Elution was performed with an eluent of the above composition at a flow rate of 8 ml/min. After discarding the first 100 ml, fractions (15 ml) were collected and the elution of peptides was monitored by TLC. ALM-containing fractions were combined and evaporated to dryness yielding 1.48 g of a yellow powder. This material was dissolved in 10 ml MeOH and applied to a column (100 cm × 5 cm) filled with Sephadex LH-20 resin. Elution was performed with MeOH at a flow rate of 4 ml/min. Fractions (14 ml) were collected and elution of peptides was monitored by TLC. Fractions containing ALM were combined and evaporated to dryness yielding 0.76 g of a pale yellow powder.

Separation of ALM F30 and ALM F50

For separation of alamethicins in ALM F30 and ALM F50 groups, MPLC on a silica gel 60 column (360 mm × 20 mm) was performed. The mobile phase was TCM/MeOH (7/3, v/v). The crude peptide resulting from XAD-2 chromatography was dissolved in 10 ml mobile phase and the elution was performed at a flow rate of 8 ml/min. For elution of the acidic components of ALM F30 gradient elution (from 50% aqueous MeOH to 100% MeOH) was used. Fractions (15 ml) were collected and analysed by TLC. Fractions containing ALM F30 or ALM F50 were combined separately and evaporated to dryness.

RESULTS AND DISCUSSION

Fermentation and Separation of Alamethicins

It had been recognized that *T. viride* NRRL 3199 in a malt extract fermentation medium produces two microheterogeneous groups of peptaibols, the acidic ALM F30 and the neutral ALM F50 peptides [5,6,20].

Using malt extract medium, the TLC-monitoring revealed that *T. viride* NRRL 3199 produced ALM F50 at the beginning of the fermentation. After about 5 days the ALM F50 concentration decreased, whereas the ALM F30 concentration increased. Fermentation in complex medium yielded ALM F50 almost exclusively.

For comparison of the different alamethicins, TLC and HPLC were performed. Using TLC, our isolations of ALM F30 and ALM F50 were analysed and compared with the original material from Upjohn, and commercially available samples from Fluka and Sigma (10 µl of 0.1% solutions in MeOH were composed). The mobile phase used was TCM/MeOH/17% aqueous ammonia (70/35/10, v/v/v) (Figure 1). This TLC shows ALM components F30 (R_f 0.32) and F50 (R_f 0.58). The TLC demonstrated, that ALM from Upjohn consists almost exclusively of acidic peptides named ALM F30, whereas ALMs from Fluka and Sigma represent definitely neutral ALM F50 peptides. Notably, the sequences presented in catalogues and data sheets of manufacturers represent ALM F30 sequences.

For analysis of the alamethicins with HPLC, different conditions were chosen. For analytical HPLC of acidic ALM F30, amounts of 0.1% TFA were added to eluents and the gradient programme as well as the column temperature were changed (see Table 1a). The separation of ALM F30 (our fermentation) and ALM F30 (Upjohn) are shown in Figure 2. The peak areas of the individual peptides of the Upjohn ALM F30 and our isolated ALM F30 were different. However, all individual peptides analysed were present in our isolated ALM F30 and in the alamethicin from Upjohn. The peptide ALM F30/3

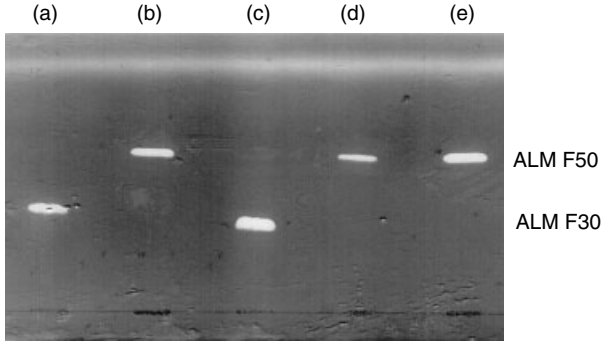

Figure 1 TLC of isolation of (a) ALM F30 and (b) ALM F50 in comparison with alamethicins from (c) Upjohn company, (d) Fluka and (e) Sigma.

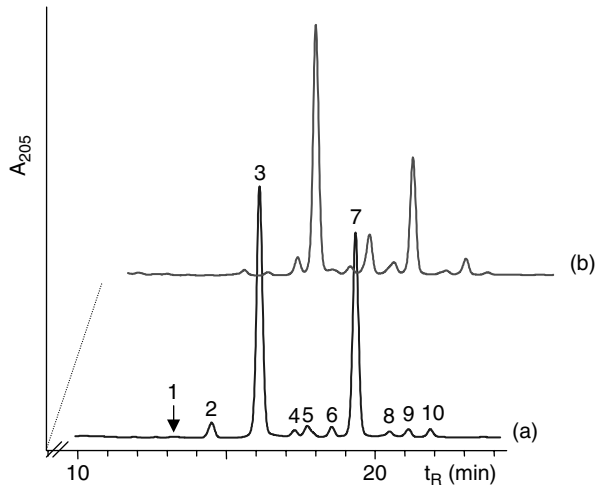

Figure 2 Analytical HPLC of (a) ALM F30 (own isolation) and (b) alamethicin from Upjohn Company; A_{205}, absorption at 205 nm. Numbers refer to peptide sequences displayed in Figure 4.

was the main compound in both alamethicins. The peptide with the second highest abundance was ALM F30/7 in both cases. But in the Upjohn alamethicin the relative content of ALM F30/7 was higher than in our isolated ALM F30.

The neutral peptides of ALM F50 were separated with a gradient system generated from MeCN/MeOH/water (39/39/22, v/v/v) and MeCN/MeOH (1/1, v/v) (see Table 1b). Under these conditions, separation of ALM F50 (our fermentation), alamethicin from Sigma and alamethicin from Fluka was possible (see Figure 3). By comparison of the HPLC-fingerprints of these three alamethicins differences in peak areas of the single peptides could be observed. All peptides analysed in our isolated ALM F50 were seen in the two other alamethicins. The most abundant peptide (ALM F50/5) represents the main compound in all three isolates.

The fingerprint of the alamethicin from Fluka showed more signals in the first 20 min of the chromatogram than our isolate of ALM F50. The main difference of the alamethicin from Sigma to our isolated ALM F50 was the much higher signal of the component at a retention time of 24 min. This component, which was denoted as ALM F50/7, was in both cases the second most abundant, but in the Fluka alamethicin other peptides showed nearly higher peak areas. The ratio of individual peptides was likely to be influenced by fermentation conditions as well as isolation procedures.

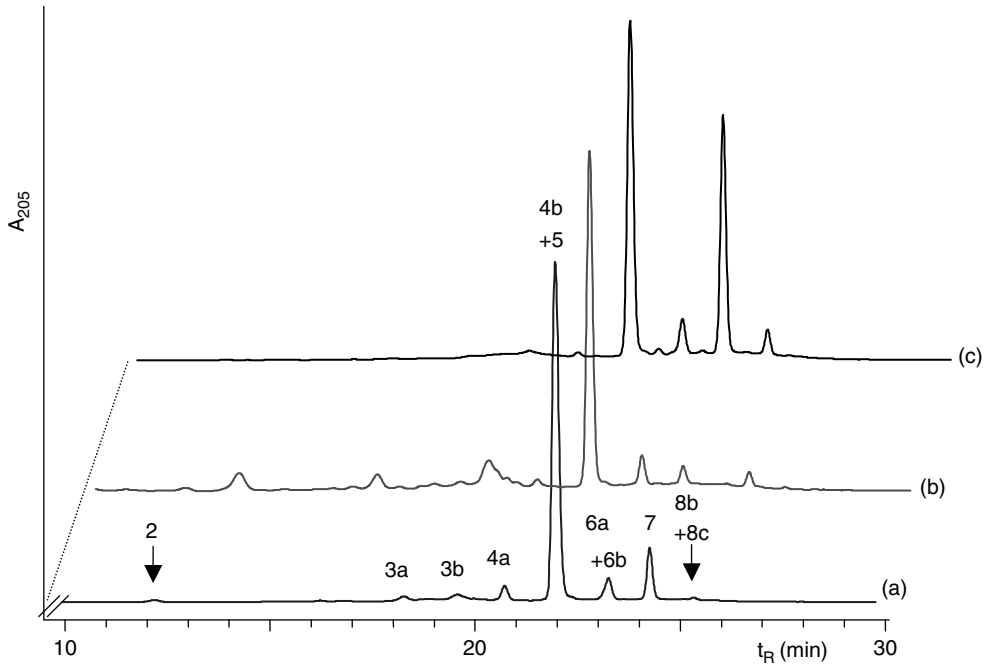

Figure 3 Analytical HPLC of (a) ALM F50 (own isolation), (b) alamethicin from Fluka, and (c) alamethicin from Sigma. A_{205}, absorption at 205 nm. Numbers characterize peptides. Peptides nos. 3c and 8a are minor components of fraction nos. 3 and 8, respectively. They were analysed via direct infusion. Allocation of these peptides to signals was not possible.

Sequence Determination of Alamethicin F30

Chiral amino acids analyses of total hydrolysates of the ALM F30 peptides showed the presence of Aib, L-Ala, L-Pro, Gly, L-Leu, L-Val, L-Glx and L-phenylalaninol (Fol). The L-configuration of Fol had been determined in previous investigations [22].

Using positive and negative ion HPLC-ESI-MS, the sequences of the peptides were analysed along procedures described previously [23–25]. The diagnostic ions are listed in Table 2. Some of the diagnostic ions, e.g. $(M + Na)^+$, $(M + H)^+$, b_{13}, b_8 and y_{7P}, were generated using HPLC-ESI-MS in the collision induced dissociation (CID) mode. The majority of the diagnostic ions was identified via HPLC-ESI-MS^n ($n = 2$–5) in positive ion mode from specific precursor ions such as $(M + H)^+$, b_{13}, b_8, or b_5. For the generation of b_7 ions, resulting from stable Gln-Aib bonds, intensive signals were obtained using direct infusion of ALM F30 dissolved in a mixture of MeOH/water (1/1, v/v) containing 1% formic acid.

In positive ion mode, the formation of b_1 ions could be observed. Signals of b_2 ions were recorded at m/z 225, which corresponds to the fragment Ac-Aib-Pro (43 + 85 + 97 Da). This was confirmed using the negative ionization mode and direct infusion of ALM F30 dissolved in MeOH/2% aqueous ammonia (1/1, v/v). Here a series of negative ions resulting from MS^n of the molecular ion $(M)^-$ was detected. The y_{19N} ion shows a mass difference of m/z 128 to the $(M)^-$ molecular ion, and represents the *N*-terminal fragment Ac-Aib (43 + 85 Da). Taking the data of b_2 and y_{19N} fragments together, Pro in position 2 is assigned definitely. This confirms earlier investigations of ALM F30 [10,22].

Analogously, in positive ionization mode, the ion y_{7P} — AA (20–16) showed m/z 197. Because the y_{7P} ion results from cleavage of the labile Aib-Pro bond, Pro has to be the first AA in the y_{7P} sequence. Therefore, the y_{7P} — AA (20–16) ion results from Pro-Val (97 + 99 Da). This can also be deduced from the series of negative ions y_{7N}, y_{6N} and y_{5N}, providing mass differences of 97 Da (Pro) and 99 Da (Val) were obtained.

The sequences deduced from the data in Table 2, for ALM F30 are listed in Figure 4, as well as the

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	%
F30/1	Ac	U	P	U	A	U	A	Q	U	U	U	G	L	U	P	V	U	U	E	Q	Fol	0.4
2	Ac	U	P	U	A	U	A	Q	U	V	U	G	V	U	P	V	U	U	E	Q	Fol	3.7
3	Ac	U	P	U	A	U	A	Q	U	V	U	G	L	U	P	V	U	U	E	Q	Fol	46.2
4	Ac	U	P	U	A	U	U	Q	U	V	U	G	V	U	P	V	U	U	E	Q	Fol	1.1
5	Ac	U	P	U	A	U	A	Q	U	V	U	G	L	U	P	V	U	V	E	Q	Fol	2.6
6	Ac	U	P	U	A	U	A	E	U	V	U	G	L	U	P	V	U	U	E	Q	Fol	1.9
7	Ac	U	P	U	A	U	U	Q	U	V	U	G	L	U	P	V	U	U	E	Q	Fol	39.8
8	Ac	U	P	U	A	U	U	Q	U	L	U	G	L	U	P	V	U	U	E	Q	Fol	1.1
9	Ac	U	P	U	A	U	U	Q	U	V	U	G	L	U	P	V	U	V	E	Q	Fol	1.6
10	Ac	U	P	U	A	U	U	Q	U	V	U	A	L	U	P	V	U	U	E	Q	Fol	1.5
F50/2	Ac	U	P	U	A	U	A	Q	U	A	U	G	L	U	P	V	U	U	Q	Q	Fol	0.8
3a	Ac	U	P	U	A	U	A	Q	U	U	U	G	L	U	P	V	U	U	Q	Q	Fol	1.2
3b	Ac	U	P	U	A	A	A	Q	U	V	U	G	L	U	P	V	U	U	Q	Q	Fol	
3c	Ac	U	P	U	A	U	G	Q	U	V	U	G	L	U	P	V	U	V	Q	Q	Fol	
4a	Ac	U	P	U	A	U	A	Q	U	V	U	G	V	U	P	V	U	U	Q	Q	Fol	5.2
4b	Ac	U	P	A	A	U	A	Q	U	U	U	G	L	U	P	V	U	U	Q	Q	Fol	
5	Ac	U	P	U	A	U	A	Q	U	V	U	G	L	U	P	V	U	U	Q	Q	Fol	75.4
6a	Ac	U	P	U	U	U	A	Q	U	X	U	G	L	U	P	X	U	U	Q	Q	Fol	5.6
6b	Ac	U	P	U	A	U	A	Q	U	X	U	G	L	U	P	X	U	X	Q	Q	Fol	
7	Ac	U	P	U	A	U	U	Q	U	V	U	G	L	U	P	V	U	U	Q	Q	Fol	10.3
8a	Ac	U	P	U	A	U	A	Q	U	L	U	G	L	U	P	V	U	U	Q	Q	Fol	0.6
8b	Ac	U	P	U	U	U	U	Q	U	V	U	G	L	U	P	V	U	U	Q	Q	Fol	
8c	Ac	U	P	U	A	U	U	Q	U	V	U	G	L	U	P	V	U	V	Q	Q	Fol	

Figure 4 Sequences of alamethicins F30/1-10, alamethicins F50/2-8, and relative quantities (%) of peptides; abbreviations according to one-letter nomenclature, Ac = acetyl, U = Aib, X = Val or Iva, Fol = phenylalaninol. Chiral amino acids are of the L-configuration with the exception of D-Iva. For HPLC see Figure 2 and Figure 3; for TLC see Figure 1.

relative quantities of individual peptides in our isolates of ALM F30. The main components, ALM F30/3 and ALM F30/7, make up 86% of ALM F30 peptides. The sequences of these peptides are identical to those reported for the alamethicins originally designated I and II [10,22]. Exchanges of amino acids in ALM F30 occur in certain positions. One special case of exchange shows ALM F30/6, where an Gln/Glu exchange at position no. 7 was recognized. This exchange is the sole difference between ALM F30/3 and ALM F30/6.

Sequence Determination of Alamethicin F50

Chiral amino acids analyses of the total hydrolysates of the ALM F50 peptides showed the presence of Aib, L-Ala, L-Pro, Gly, L-Leu, L-Val, L-Glx (resulting from L-Glu and/or L-Gln), Iva and phenylalaninol (Fol). The configuration of L-Fol and D-Iva had been determined in earlier investigations [22]. Iva was detected exclusively in fraction no. 6.

For further investigations, ALM F50 was separated by HPLC on a semi-preparative Spherisorb ODS column. Using the gradient programme listed in Table 1c, eight fractions were collected [25]. These fractions were further resolved on an analytical Kromasil C8 column using the gradient programme described in Table 1b. The resulting peaks shown in Figure 3 were denoted in accordance with the peptides of the fractions.

Using HPLC-ESI-MS and direct infusion ESI-MS, the sequences of the peptides of ALM F50 were analysed. Diagnostic ions determined are listed in Table 3. Analogously to the sequence analysis of ALM F30, some of the diagnostic ions, e.g. $(M + Na)^+$, $(M + H)^+$, b_{13}, b_8 and y_{7P}, were determined using HPLC-ESI-MS or direct infusion of the fractions with collision induced dissociation (CID) energy. The majority of the diagnostic ions were also identified via HPLC-ESI-MS^n $(n = 2–5)$ from these characteristic ions. As for ALM F30, in positive ion mode no b_1 and sometimes no b_2 ions could be determined. The detected b_2 ions showed m/z 225, corresponding to *N*-terminal Ac-Aib-Pro $(43 + 85 + 97$ Da). This was also confirmed in the negative ion mode using direct infusions of ALM F50 fractions, dissolved in MeOH/2% aqueous ammonia (1/1, v/v). A series of negative ions resulting from MS^n of $(M)^-$ were detected. From the difference of the y_{19N} ion to $(M)^-$ of 128 Da and the results of b_2 from positive ion mode, Pro was assigned at position no. 2. This was also confirmed by earlier investigations of ALM F50 [22]. In analogy to ALM F30, the amino acids at positions nos. 14 and 15 resulting from y_{7P} and y_{7P} –AA (20–16), are Pro and Val, respectively.

The sequences of ALM F50, calculated from the data of Table 3, and the relative quantities of each peptide in our ALM F50 isolates are also shown in Figure 4. Isobaric Iva and Val in the peptide(s) of fraction no. 6 could not be distinguished. Therefore, 'X' stands for Val or Iva. Fraction no. 1 (0.9% of ALM F50 peptides) consists of at least six peptides, which could not be separated by the analytical HPLC used. The structures of these trace peptides could not be analysed and they are not treated in this work.

The peptide ALM F50/5 is identical to the component designated in our previous publication as ALM 'C', whereas ALM F50/6b is equal to ALM 'D', and ALM F50/7 corresponds to ALM F50 'E' [22]. The only difference between ALM F50/6b and the structure of ALM 'D' is the assignment of Iva to position 17 of the latter. Amino acid exchanges of ALM F50 peptides can be depicted from Figure 4.

Comparison of the Structures of the Peptides Constituting ALMs F30 and ALMs F50

By comparing the structures of the acidic peptides ALM F30 and the neutral peptides ALM F50 (cf. Figure 4), common and deviating domains can be recognized. All peptides start with the *N*-terminal sequence Ac-Aib-Pro, and end with *C*-terminal Gln-Fol. Further, Aib in positions 8, 10, 13 and 16, and Pro in position 14 are not exchanged.

The sequences of the major peptides ALM F30/3 and ALM F50/5, as well as minor peptides ALM F30/7 and ALM F50/7, differ from each other only by Glu/Gln exchange at position 18. Other minor components, such as ALM F30/1 and ALM F50/3a, ALM F30/2 and ALM F50/4a, as well as ALM F30/9 and ALM F50/8c, are also distinguished by this characteristic replacement. Notably, in combined ALMs F50/6a and b, the presence of Iva could be detected, but in none of the ALM F30 peptides.

Acknowledgements

We thank Dr Michael Kieß for fermentation and isolation of alamethicin F30 and F50 mixtures in our Hohenheim laboratories. We are indebted to Professor J. Raap from Leiden University, The Netherlands, for providing a sample of alamethicin (purchased from Sigma) for comparison with our isolates.

REFERENCES

1. Meyer CE, Reusser F. A polypeptide antibacterial agent isolated from *Trichoderma viride*. *Experientia* 1967; **23**: 85–86.
2. Mueller P, Rudin DO. Action potentials induced in biomolecular lipid membranes. *Nature* 1968; **217**: 713–719.
3. Payne JW, Jakes R, Hartley BS. The primary structure of alamethicin. *Biochem. J.* 1970; **117**: 757–766.
4. Ovchinnikov YA, Kiryushkin AA, Kozhevnikova IV. Mass-spectrometric determination of the amino acid sequence in peptides. Structure of alamethicin. *J. Gen. Chem. USSR* 1971; **41**: 2105–2116.
5. Melling J, McMullen AJ. Separation, purification and characterisation of alamethicins produced from *Trichoderma viride*. *ISC-IAMS Proc. Science Council of Japan* 1975; **5**: 446–452.
6. Martin DR, Williams RJP. The nature and function of alamethicin. *Biochem. J.* 1976; **153**: 181–190.
7. Jung G, Dubischar N, Leibfritz D. Conformational changes of alamethicin induced by solvent and temperature. *Eur. J. Biochem.* 1975; **54**: 395–409.
8. Gisin BF, Kobayashi S, Hall JE. Synthesis of a 19-residue peptide with alamethicin-like activity. *Proc. Natl Acad. Sci. USA* 1977; **74**: 115–119.
9. Gisin BF, Kobayashi S, Davis DG. Synthesis of biologically active alamethicin. In *Peptides* 1977, Proc. 5th Am. Peptide Symp, Goodman M, Meienhofer J (eds). Wiley: New York, 1977; 215–217.
10. Pandey R, Cook JC, Rinehart KL. High resolution and field desorption mass spectrometry studies and revised structures of alamethicins I and II. *J. Am. Chem. Soc.* 1977; **99**: 8469–8483.
11. Brückner H, König WA, Greiner M, Jung G. Sequences of membrane modifying trichotoxin A-40. *Angew. Chem. Int. Ed. Engl.* 1979; **18**: 476–477.
12. Gisin BF, Davis DG, Borowska ZK, Hall JE, Kobayashi S. Synthesis of the major component of alamethicin. *J. Am. Chem. Soc.* 1981; **103**: 6373–6377.
13. Balasubramanian TM, Kendrick NCE, Taylor M, Marshall GR, Hall JE, Vodyanov I, Reusser FJ. Synthesis and characterization of the major component of alamethicin. *J. Am. Chem. Soc.* 1981; **103**: 6127–6132.
14. Nagaraj R, Balaram P. Solution phase synthesis of alamethicin I. *Tetrahedron* 1981; **37**: 1263–1270.
15. Schmitt H, Jung G. Total synthesis of the α-helical eicosapeptide antibiotic alamethicin. *Liebigs Ann. Chem.* 1985; 321–344.
16. Słomczynska U, Zabrocki J, Kaczmarek K, Leplawy MT, Beusen DD, Marshall GR. Facilitated synthesis of peptaibols: alamethicin via enzymatic segment condensation. *Biopolymers* 1992; **32**: 1461–1470.
17. Wenschuh H, Beyermann M, Haber H, Seydel JK, Krause E, Bienert M. Stepwise automated solid phase synthesis of naturally occurring peptaibols using Fmoc amino acid fluorides. *J. Org. Chem.* 1995; **60**: 405–410.
18. Fox OR, Richard MR. A voltage-gated channel model inferred from the crystal structure of alamethicin at 1.5-Å resolution. *Nature* 1982; **300**: 325–330.
19. Marshall GR, Balasubramanian TM. Alamethicin: purification, characterization, conformational and synthetic studies. In *Peptides 1979, Structure and Biological Function*, Gross E, Meienhofer J (eds). Pierce Chemical Co.: Rockford IL, 1979; 639–646.
20. Irmscher G, Jung G. Die hämolytischen Eigenschaften der membranmodifizierenden Peptidantibiotika Alamethicin, Suzukacillin und Trichotoxin. *Eur. J. Biochem.* 1977; **80**: 165–174.
21. König WA, Aydin M. Glass capillary gas chromatography — chemical ionization mass spectrometry in peptide sequence analysis. In *Peptides* 1980, Brundfeld K (ed.). Kopenhagen, 1980; 711–718.
22. Brückner H, Bokel M, Przybylski M. Detection and sequential characterization of Aib-containing polypeptides ('Peptaibols') and utilization of their natural microheterogeneity for an assignment of ^{13}C NMR signals. In *Peptides 1985, Structure and Function*. Proceedings of the Ninth American Peptide Symposium, Deber CM, Hruby VJ, Kopple KD (eds). Pierce Chemical Company: Rockford, Illinois, 1985; 109–112.
23. Jaworski A, Brückner H. Sequences of polypeptide antibiotics stilboflavins, natural peptaibol libraries of the mold *Stilbella flavipes*. *J. Peptide Sci.* 2001; **7**: 433–447.
24. Jaworski A, Kirschbaum J, Brückner H. Structures of trichovirin II, peptaibol antibiotics from the mould *Trichoderma viride* NRRL 5243. *J. Peptide Sci.* 1999; **5**: 341–351.
25. Kirschbaum J, Winzheimer RK, Brückner H. Alamethicin sequences reconsidered. In *Peptides* 2002. Proc. 27th Eur. Peptide Symp, Benedetti E, Pedone C (eds). Edizioni Ziino: Napoli, Italy, 2002; 360–361.