

# New Sequences and New Fungal Producers of Peptaibol Antibiotics Antiamoebins

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**Abstract:** Mixtures of the microheterogeneous 16-mer peptaibol antibiotics called antiamoebins (AAM) have been isolated from the culture broths of strains of the filamentous fungi *Stilbella erythrocephala* ATCC 28144, *Stilbella fimetaria* CBS 548.84 and *Gliocladium catenulatum* CBS 511.66. Sequences were determined using on-line HPLC together with positive- and negative-ion electrospray ionization mass spectrometry. Some characteristic features are recognized in the mass spectrometric fragmentation pattern of AAM. From a sample originally used for sequencing AAM (from Hindustan Antibiotics, Ltd., Pimpri, Poona-411018, India), and a sample of AAM commercially available (from Sigma Chemicals, St. Louis, MO, USA) HPLC elution profiles and sequences were assigned. Further, sequences of AAM previously isolated from *Emericellopsis synnematicola* CBS 176.60 and *Emericellopsis salmosynnemata* CBS 382.62 were determined. The peptide designated AAM I was the most abundant in all isolates and its structure could be confirmed. AAM II was detectable as a minor component (1.9%) only in the original sample of AAM, but not in the other isolates. The structures of AAM III, IV and V, which had previously been partly assigned, were definitely established, and the new sequences AAM VI–XVI were elucidated. AAM showing Phe<sup>1</sup>/Leu<sup>1</sup> or Phe<sup>1</sup>/Val<sup>1</sup> exchange, respectively, are produced in amounts only by *S. erythrocephala*. Sequences, HPLC elution profiles ('fingerprints') and relative amounts of peptides of all isolates were correlated. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

**Keywords:** electrospray ionization mass spectrometry; sequence analysis; peptide antibiotics; microheterogeneous peptides;  $\alpha$ -aminoisobutyric acid (Aib); D-isovaline (Iva)

Abbreviations: AAA, amino acid analysis; AA, amino acid(s); AAM, antiamoebin(s); Ac, acetyl; Aib,  $\alpha$ -aminoisobutyric acid,  $\text{H}_2\text{NC}(\text{CH}_3)_2\text{COOH}$ ; ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures; DCM, dichloromethane; ESI, electrospray ionization; FAB, fast atom bombardment; GC, gas chromatography; HPLC, reversed phase high-performance liquid chromatography; HREIMS, high resolution electron impact mass spectrometry; Hyp, *trans*-4-hydroxy-L-proline; Iva, D-isovaline,  $\text{H}_2\text{NC}(\text{CH}_3)(\text{C}_2\text{H}_5)\text{COOH}$ ; MeCN, acetonitrile; MeOH, methanol; MPLC, medium pressure liquid chromatography; MS, mass spectrometry; MS-MS, tandem mass spectrometry; Pheol, phenylalaninol,  $\text{H}_2\text{NCH}(\text{CH}_2\text{C}_6\text{H}_5)\text{CH}_2\text{OH}$ ; SIM, selected ion monitoring; TCM, trichloromethane; TDM (reagent), chlorine/4,4'-bis-(dimethylamino)diphenylmethane; TFA, trifluoroacetic acid; TFAA, trifluoroacetic acid anhydride; TLC, thin-layer chromatography; u, mass units; common AA are abbreviated according to three-letter nomenclature.

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## INTRODUCTION

The peptide antibiotic antiamoebin was reported to be produced by the filamentous fungi *Emericellopsis poonensis* Thirumalachar, *Emericellopsis synnematicola* Mathur and Thirumalachar and *Cephalosporium pimprina* Thirumalachar [1]. It was realized that this peptide contains, among other amino acids (AA), a high proportion of the non-protein AA  $\alpha$ -aminoisobutyric acid (Aib) as well as the amino alcohol Pheol. A proposed cyclic structure [2] was revised using mainly GC-MS and GC-HREIMS, and sequences were reported for a major and a minor component named antiamoebin (AAM) I and II, accounting for 98 and 2%, respectively [3,4]. Based on these structures for peptides containing Aib and a C-terminal Pheol the name

'peptaibophols' [3], or, 'peptaibols', was proposed and is now generally accepted. A positive-ion FAB-MS and sequence assignment of a peptide named AAM III were reported [5], without presenting details on relative amounts or isolation procedures of the peptide. AAM III was reported to differ from AAM I by replacing Iva<sup>5</sup>/Aib<sup>5</sup>, and Gln<sup>11</sup>/Glu<sup>11</sup> (for sequences of all AAM peptides discussed in the following see Figure 2). The latter exchange, however, is neither supported by the mass differences reported in [5], nor by our data presented here. Thus, the Gln<sup>11</sup>/Glu<sup>11</sup> exchange is assumed to be a printing error and this exchange is not taken into consideration in this paper. Further, investigation by on-line LC-FAB-MS of a trifluoroacetic acid (TFA) partial hydrolysate of AAM I, resulting in a mixture of tetra- and nonapeptides, revealed at least three other AAM to be present, although initially the AAM I sample was assumed to be pure [6]. These three peptides (named AAM III, IV, and V) differ from AAM I by an exchange of Iva<sup>5</sup>/Aib<sup>5</sup> (AAM III; corresponding to the structure reported in [5]), Aib<sup>4</sup>/Ala<sup>4</sup> (AAM IV), or Gly<sup>6</sup>/Ala<sup>6</sup> (AAM V). This work was carried out with original AAM supplied by Hindustan Antibiotics, Ltd, Pimpri, Poona-411018, India. A series of sequence ions for AAM III–V were obtained, however, only for parts of the *N*-terminal sequences. No relative amounts of AAM III–V were reported [6]. Notably, the originally proposed configuration of L-Iva [3–5,7], another non-protein AA residue of AAM, was recognized as D-Iva [8,9]. This was also confirmed recently by X-ray analysis of AAM I [10].

It should be pointed out that sequencing of AAM and related peptaibols zervamicins and emerimicins [11], together with alamethicins [12], was pioneering work, demonstrating convincingly the suitability of mass spectrometric methods for the structure determination of peptaibol antibiotics.

AAM, as well as other peptaibols, attract much attention as a result of their broad range of bioactivities which include inhibition of bacteria, fungi, protozoa and helminths [1]. Broad spectrum anthelmintic activity, however, was not confirmed [13]. Further, insecticidal action on mosquito larvae was reported for AAM and related peptaibols (peptaibiotics) [14].

Notably, the hemolytic action of AAM on erythrocytes is weaker than those of 18-mer trichotoxins and 20-mers alamethicins and paracelsins [15,16]. Membrane channel forming properties and the uncoupling of oxidative phosphoryl-

ation in mitochondria by AAM and the membrane modifying action of individual AAM isolated by HPLC from the natural mixture were recognized [17,18]. There is experimental evidence that AAM is capable of forming ion-gating pores in black bilayer membranes as well as having ion carrier properties [19]. Syntheses of segments of AAM [20–22] and their investigation by X-ray analysis [20,22] and NMR-spectroscopy [17,22] and use for secondary structure predictions [23,24], culminated in the chemo-enzymatic total synthesis of AAM I [25]. Recently pure AAM I, isolated by HPLC from the natural mixture, could be crystallized. Investigation by X-ray crystallographic analyses revealed the particular crystal structure of AAM I, representing a bent molecule, consisting of an *N*-terminal  $\alpha$ -helical part, followed by a  $3_{10}$  helix and ending in a series of *C*-terminal  $\beta$ -turns [10,26]. Helical structures ( $\alpha$ ,  $3_{10}$ , or mixed) are characteristic of natural or synthetic peptides rich in sterically-constrained Aib [27–29]. This bent structure also results in the molecule having a lower dipole moment [10]. The particular structural features, together with the shorter chain length in comparison to 18- to 20-mer peptaibols, might explain the distinct behaviour in interacting with lipid bilayer membranes as well as the characteristic circular dichroism spectra of AAM [10,16].

Application of selective and sensitive screening procedures for peptaibol antibiotics [30–32] on *Stilbella erythrocephala* (Ditm.) Lindau, strain ATTC 28144, *Stilbella fimetaria* (Pers.) Lindau, strain CBS 548.84 [31,33,34], and *Gliocladium catenulatum* Gilman & Abbott, strain CBS 511.66 [35] had already revealed the production of peptaibols, which were assumed to be identical or closely related to AAM. The coprophilous fungus *S. erythrocephala*, which is considered as a synonym of *S. fimetaria* [36], had been reported to produce an antibiotic that causes significant inhibition of the growth of many other coprophilous fungi and dung bacteria [37].

In the following we describe the fermentation, isolation and purification of AAM from the culture broths of these fungi and the determination of their microheterogeneous sequences employing almost exclusively on-line LC-ESI-MS. Further, we determine new, and confirm known, sequences of AAM, correlate structures with HPLC elution profiles and determine relative amounts of peptides in the microheterogeneous mixtures.

## EXPERIMENTAL

### Material and Methods

Solvents were of the gradient grade and were from Merck (Darmstadt, Germany). For amino acid analysis (AAA), an AA standard solution (no. AA-S-18) (Sigma, St. Louis, Mo, USA) was used and appropriate amounts of Aib (Sigma), DL-Iva (synthesized in our laboratory by the Strecker procedure), *trans*-4-hydroxy-L-proline (Sigma) and L-Pheol (Sigma) were added. For TLC, pre-coated plates with silica gel 60 F 254, thickness 0.25 mm (Merck), were used. The mobile phase was TCM-MeOH 8/2 (v/v); ratios of solvent mixtures are by volume throughout text. Peptaibols were detected by spraying with water and, after drying, with TDM reagent. For column chromatography, Servachrom XAD-2 polystyrene adsorber resin, particle size 100–200  $\mu\text{m}$  (Serva, Heidelberg, Germany), Sephadex LH-20, particle size 25–100  $\mu\text{m}$  (Pharmacia, Freiburg, Germany) and LiChroprep RP-8, particle size 40–63  $\mu\text{m}$  (Merck) were used.

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). For preparing malt agar medium, 15 g agar (Fluka, Buchs, Switzerland) was added to the malt medium.

**Sources of anti amoebins.** An original AAM sample, probably isolated from *E. poonensis* ATCC 16411 [1], was from Hindustan Antibiotics. Reference AAM from Sigma (product no. A 8302, lot. 89F4011) was, according to the suppliers declaration, isolated from *E. synnematicola* ATCC 16540 [18]. Another sample of AAM (identical to peptide antibiotic Tü 165) was isolated from *E. salmosynnemata* CBS 382.62 [8,38]. AAM was also isolated in our laboratory from the culture broth of *E. synnematicola* CBS 176.60 [39]. Isolation of AAM from submers fermenter cultures of the moulds *S. erythrocephala* ATCC 28144, *S. fimetaria* CBS 548.84 and *G. catenulatum* CBS 511.66 is described below.

### INSTRUMENTAL

**Chromatography.** For HPLC a HP 1100 instrument (Hewlett-Packard, Waldbronn, Germany) comprising a binary pump, autosampler and HP ChemStation connected to an UV-detector of the same series was used. HPLC columns employed were: (I) Superspher<sup>®</sup> 100 RP-18, 250 mm  $\times$  4 mm i.d., 4  $\mu\text{m}$  particle size (Merck); (II) Spherisorb ODS-2, 250  $\times$  8 mm i.d. 3  $\mu\text{m}$  particle size (Grom, Herrenberg, Germany).

Eluents for column (I): eluent A, MeOH/water/MeCN 1/1/1; eluent B, MeOH/MeCN 1/1; gradient program 0 min 0% B; 5 min 0% B, 15 min 30% B, 25 min 50% B, 35 min 100% B; flow-rate 0.8 ml/min; temperature 35°C; injected amounts 10  $\mu\text{l}$  of a 0.1% methanolic solution of peptides. Eluents for column (II), used for the micropreparative isolation of peptides from *S. erythrocephala*: eluent A, MeOH/water/MeCN 39/22/39; eluent B, MeOH/MeCN 1/1; gradient program 0 min 0% B, 9 min. 0% B, 12 min. 5% B, 20 min. 15% B, 24 min. 15% B, 25 min. 30% B, 30 min. 70% B, 34 min. 70% B; flow rate 2.7 ml/min; temperature 40°C; injected amounts 100  $\mu\text{l}$  of a 0.1% methanolic solution of peptides.

For MPLC, an MD 80/100 pump, controller PS 1 (Labomatic, Sinsheim, Germany) and a Model FRAC-100 fraction collector (Pharmacia) were used.

**Mass spectrometry.** For ESI-MS an LCQ<sup>™</sup> MS (ThermoQuest, Finnigan MAT, San Jose, CA, USA) was used. Peptaibols were analysed by on-line coupling of the HPLC instrument, equipped with column (I), and the LCQ instrument. The sheath gas was nitrogen (purity > 99.5%) from a nitrogen generator Model 75-72 (Whatman, Balston Inc., Haverhill, Mass., USA); collision gas was helium (Messer-Griesheim, Krefeld, Germany). Sequence analysis was carried out by positive- and negative-ionization, recorded in the centroid mode, providing *m/z* values having an accuracy of one decimal. Values of Table 3 are rounded up or down, respectively. Conditions for positive (negative) ionization mode were: spray voltage 4.25 (4.25) kV, heated capillary temperature 200 (200)°C, capillary voltage +3 (–27) V, tube lens offset +55 (–60) V, maximum ion time 1000 ms. For automatic calibration a mixture of caffeine (*m/z* 195.1), the tetrapeptide Met-Arg-Phe-Ala (*m/z* 524.3) and the perfluorinated mass spectrometric standard 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. In negative-ion mode aqueous 4 M ammonia was added to the HPLC eluate by a syringe pump at a flow rate of 5  $\mu\text{l}/\text{min}$ .

The notation used for sequence determination in the positive-ion mode, referring to a, b and c acylium ions, is based on the suggestions of Roepstorff and Fohlman [40], modified by Biemann [41]. MS-MS in the negative-ion mode produced the y series without protonation [42] which is denoted  $y_N$  in the spectra.

Sequences of AAM peptides from *E. poonensis* ATCC 16411, *E. synnematicola* ATCC 16540 and *S. erythrocephala* ATCC 28144 were investigated by on-line HPLC ESI-MS in positive- and negative-ionization mode. The ions  $(M + Na)^+$  and  $(M)^-$  of

compounds were chosen as precursor ions for MS-MS. The peptides produced by the other fungi investigated were identified by corresponding retention times in HPLC, identical pseudomolecular masses and characteristic fragmentation patterns resulting from labile AA-Hyp(Pro) bonds in positive-ion on-line HPLC-ESI-MS.

For chiral AAA, the GC-MS instrument (A) used was a Shimadzu 17A/QP 5000 (Shimadzu, Kyoto, Japan) equipped with a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) quartz capillary column, 25 m × 0.25 mm i.d. (Chrompack, Middelburg, The Netherlands). For quantitative AAA and dipeptide analysis the GC-MS instrument (B) was a HP 6890 (Hewlett-Packard) with mass selective detector equipped with a Heliflex<sup>®</sup> AT-5 column (crosslinked 5% diphenyl-95% dimethyl polysiloxane), 30 m × 0.25 mm i.d., film thickness 0.25 μm (Alltech Associates, Deerfield, USA). EI mass spectra on instruments (A) and (B) were recorded at an ionization energy of 70 eV.

#### Fermentation Procedures for new Fungal Antiamoebin Producers

*S. erythrocephala* ATCC 28144, *S. fimetaria* CBS 548.84 and *G. catenulatum* CBS 511.66 were obtained as lyophilized cultures from the respective culture collections. The lyophilizates were suspended in sterile water (1.5 ml) and, after soaking for 15 min, transferred to Petri dishes (9.5 cm diameter) with malt-agar medium. After 12 days at 21°C intensive growth was observed. Agar discs (1 cm diameter) were used for the inoculation of 11 Erlenmeyer flasks (2-l) (24 Erlenmeyer flasks for *S. erythrocephala*) each containing 400 ml of malt-medium. The flasks were shaken at 100 rpm at room temperature (25°C). For *S. erythrocephala*, the fermentation procedure was carried out using a rotary shaker Model G 25 (New Brunswick, Edison, USA). For *S. fimetaria* and *G. catenulatum*, a Model SM-30A rotary shaker (Johanna Otto GmbH, Hechingen, Germany) was used. For daily monitoring of peptaibol production, aliquots (20 ml) of filtered culture broths were passed through Sep-Pak<sup>®</sup> C-18 cartridges (Waters, Milford, USA) and the peptides absorbed were eluted with MeOH. The eluates were evaporated to dryness and dissolved in MeOH (0.5 ml). Aliquots of 20 μl were investigated by TLC (see Material and Methods) and peptaibols visualized by spraying with water and, after drying, with TDM reagent. The  $R_f$  of AAM was 0.42; the internal

standard peptaibol paracelsin [16] had an  $R_f$  of 0.13.

#### Isolation and Purification of Antiamoebins

After shaking for 13 days (*S. erythrocephala*), 5 days (*S. fimetaria*) or 3 days (*G. catenulatum*), intensive peptide production in the fermentation broths was detected by TLC. Culture broths were separated from mycelia by filtration under reduced pressure, and filtrates were pumped through a MPLC-column (38 cm × 3.7 cm) filled with XAD-2 resin. The resin was washed with water (1 l) and 40% MeOH (0.5 l). Then the adsorbed peptides were eluted with a linear gradient from 40 to 100% MeOH and fractions (20 ml) were collected. Elution of peptides was monitored by TLC. Fractions containing peptaibols were combined and evaporated to dryness yielding 4.63 g of a dark brown oil (*S. erythrocephala*), 0.88 g pale yellow powder (*S. fimetaria*) and 0.69 g pale yellow powder (*G. catenulatum*).

The peptidic raw material (4.63 g) produced by *S. erythrocephala* was treated with 1:1 TCM-MeOH (50 ml), filtered and evaporated to dryness. The remaining residue (3.15 g) was dissolved in MeOH (12 ml). Peptides were purified in two portions (6 ml each) by chromatography on a MPLC-column (36 cm × 2.0 cm) filled with LiChroprep RP-8 (40–63 μm particle size, Merck) using a linear gradient from 50 to 100% MeOH at a flow rate of 5 ml/min. Fractions (approx. 12 ml) were collected and the elution of peptides was monitored by TLC. Appropriate fractions were combined and evaporated to dryness, providing a total of 1.61 g of colourless crystalline. Microgram amounts of individual peptides were isolated by repetitive HPLC on column (II) (see Chromatography). Elution of peptides was detected at 205 nm and peptide fractions were collected manually. Semipreparative isolation of certain peptides from *S. erythrocephala* was carried out in order to separate and identify those peptides containing isomeric Val and Iva (see Table 2).

Further purification of the peptides isolated from *S. fimetaria* and *G. catenulatum* was carried out by chromatography on a Sephadex LH-20 column (100 cm × 3.1 cm) using MeOH as eluent. Peptides were dissolved in 10 ml MeOH and subjected to LH-20 chromatography in two portions of 5 ml each. Fractions were collected and elution of peptides was monitored by TLC. The fractions containing peptaibols were combined and evaporated to dryness. Amounts of 0.80 g (from *S. fimetaria*), and 0.55 g (from *G. catenulatum*) of colourless peptides were

obtained, which had already started to crystallize on evaporation.

### Amino Acid Analysis by GC-SIM-MS

The microheterogeneous mixture of peptides (20–50 µg) and fractions of *S. erythrocephala*, obtained from semipreparative HPLC, were hydrolysed (6 M HCl, 110°C, 24 h). Chirality of AA and Pheol were determined after derivatization as *N*-pentafluoropropionyl-AA-(2)-propyl esters (*N*-acetyl 2-propyl esters for Iva) and *N*(*O*)-bis(pentafluoropropionyl)pehol by GC-SIM-MS on instrument (A) as previously described [43]. Stoichiometry of AA and Pheol were determined after derivatization as *N*-trifluoroacetyl-AA-*n*-butyl esters and *N*(*O*)-bis(trifluoroacetyl)pehol on instrument (B); the stoi-

chiometry is normalized on Pheol (1.00) since this amino alcohol is not exchanged in AAM.

### Dipeptide Analysis by GC-MS

For acidic methanolysis aliquots (ca. 30 µg) of peptides of *S. erythrocephala* containing Val and Iva in 4 M HCl in MeOH (200 µl) were heated for 6 h at 100°C. Reagents were removed in a nitrogen stream and the remaining methyl esters were trifluoroacetylated with TFAA (50 µl) in DCM (100 µl). After 15 min at 70°C reagents were removed in a stream of nitrogen and DCM (50 µl) was added. Dipeptide derivatives of interest were analysed on GC instrument (B), and positions of isomeric AA assigned as previously described [43,44].

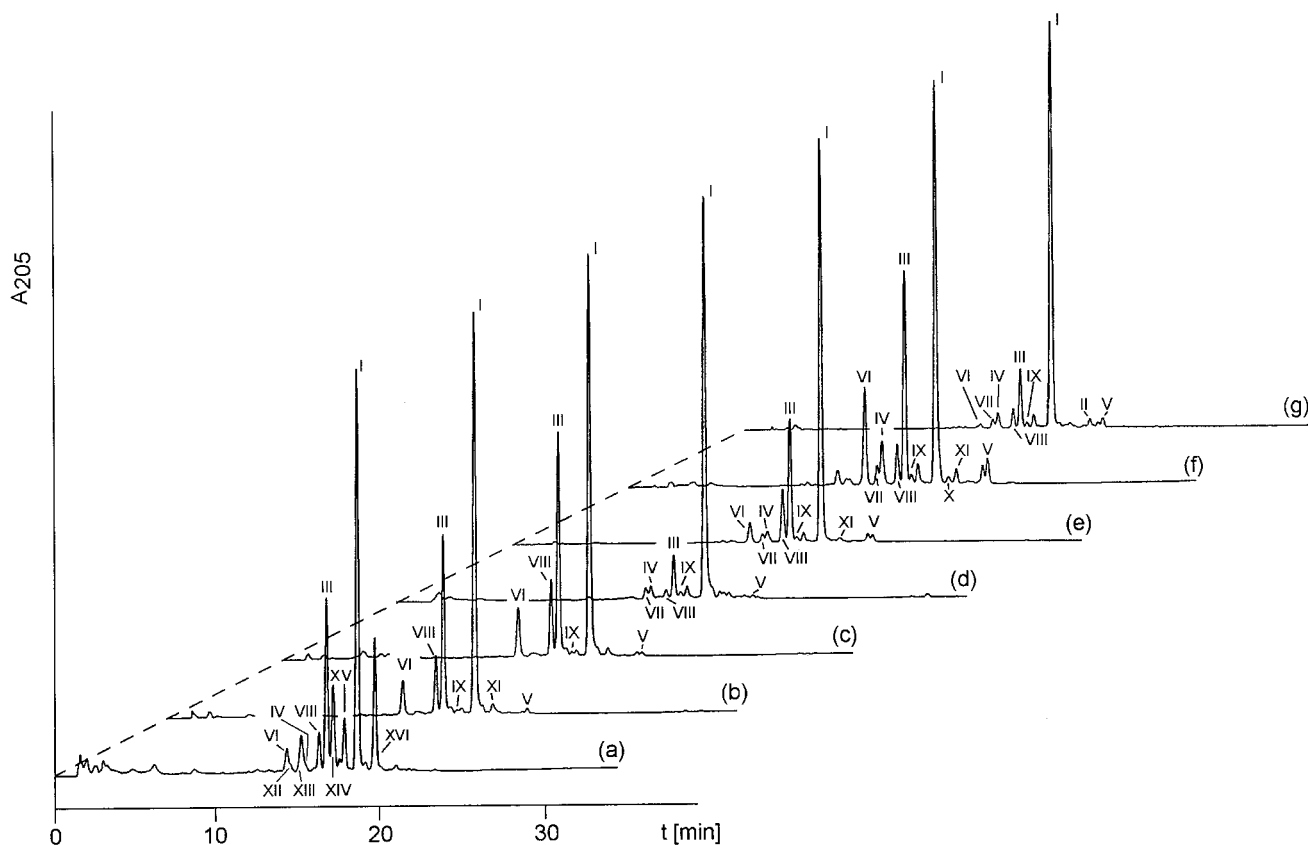


Figure 1 HPLC fingerprints of AAM isolates from (a) *S. erythrocephala* ATCC 28144; (b) *S. finetaria* CBS 548.84; (c) *G. catenulatum* CBS 511.66; (d) *E. salmosynnemata* CBS 382.62; (e) *E. synnematicola* CBS 176.60 in comparison to (f) AAM from Sigma (isolated from *E. synnematicola* ATCC 16540) and (g) AAM from Hindustan Antibiotics (isolated from *E. poonensis* ATCC 16411); the major peak in all chromatograms refers to AAM I; A<sub>205</sub>, absorption at 205 nm; for chromatographic conditions see Experimental; for relative amounts see Table 1; for correlation of roman numbers of peptides and their sequences see Figure 2.

AAM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	NMW	
I*	Ac	<b>Phe</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>D-Iva</b>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1670
II*	Ac	<b>Phe</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>D-Iva</b>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Pro</b>	Aib	Pro	Pheol	1654
III*	Ac	<b>Phe</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1656
IV*	Ac	<b>Phe</b>	<b>Aib</b>	<b>Aib</b>	<b>Ala</b>	<b>D-Iva</b>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1656
V*	Ac	<b>Phe</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>D-Iva</b>	<b>Ala</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1684
VI	Ac	<b>Phe</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>Aib</b>	<b>Hyp</b>	Aib	Pro	Pheol	1642
VII	Ac	<b>Phe</b>	<b>Ala</b>	<b>Aib</b>	<b>D-Iva</b>	<b>Aib</b>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1656
VIII	Ac	<b>Phe</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>D-Iva</b>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>Aib</b>	<b>Hyp</b>	Aib	Pro	Pheol	1656
IX	Ac	<b>Phe</b>	<b>Aib</b>	<b>Ala</b>	<b>Aib</b>	<b>D-Iva</b>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1656
X	Ac	<b>Phe</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>D-Iva</b>	<b>Gly</b>	Leu	<b>D-Iva</b>	Aib	Hyp	Gln	<b>Aib</b>	<b>Hyp</b>	Aib	Pro	Pheol	1670
XI	Ac	<b>Phe</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Ala</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1670
XII	Ac	<b>Phe</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Gly</b>	Leu	<b>Ala</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1642
XIII	Ac	<u><b>Val</b></u>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	<u><b>Gln</b></u>	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1608
XIV	Ac	<u><b>Val</b></u>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<u><b>Val</b></u>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1622
XV	Ac	<b>Leu</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1622
XVI	Ac	<b>Leu</b>	<b>Aib</b>	<b>Aib</b>	<b>Aib</b>	<b>D-Iva</b>	<b>Gly</b>	Leu	<b>Aib</b>	Aib	Hyp	Gln	<b>D-Iva</b>	<b>Hyp</b>	Aib	Pro	Pheol	1636

Figure 2 Sequences of AAM; exchanged positions in bold characters; NMW, nominal molecular weight; Pheol and chiral amino acids are of the L-configuration with the exception of D(=R)-Iva; Hyp refers to *trans*-4-hydroxy-L-proline; sequences of AAM I–V, denoted with an asterisk, were reported partly previously [3–6]; dipeptides used for the assignment of isomeric Val/Iva, generated by methanolysis [43], are underlined; for relative amounts of peptides in various isolates of AAM see Table 1.

Table 1 Relative Amounts (%) of AAM Peptides I–XVI in the Natural Microheterogeneous Mixtures Isolated from Fungi (a)–(g)

AAM	Fungal producers						
	(a)	(b)	(c)	(d)	(e)	(f)	(g)
I*	37.4	52.9	48.9	70.2	60.2	41.6	70.4
II*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.9
III*	16.2	24.1	27.4	8.0	18.5	21.3	9.6
IV*	1.8	n.d.	n.d.	2.7	2.2	5.4	2.9
V*	n.d.	0.9	0.5	1.0	1.1	2.7	1.9
VI	2.6	5.6	6.9	n.d.	3.5	10.8	1.0
VII	n.d.	n.d.	n.d.	2.3	1.3	2.0	1.5
VIII	4.0	8.4	9.6	2.0	8.1	4.3	3.4
IX	n.d.	1.4	1.1	1.6	0.9	1.0	1.0
X	n.d.	n.d.	n.d.	n.d.	n.d.	0.9	n.d.
XI	n.d.	2.5	n.d.	n.d.	1.1	1.7	n.d.
XII	0.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
XIII	4.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
XIV	9.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
XV	5.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
XVI	13.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Σ	95.4	95.8	94.4	87.8	96.9	91.7	93.6

Fungal producers are (a) *S. erythrocephala* ATCC 28144; (b) *S. fimetaria* CBS 548.84; (c) *G. catenulatum* CBS 511.66; (d) *Emericellopsis salmosynnemata* CBS 382.62 (producer of antibiotic Tü 165); (e) *E. synnematicola* CBS 176.60; (f) *E. synnematicola* ATCC 16540 (producer of AAM supplied by Sigma); and (g) *E. poonensis* ATCC 16411 (producer of AAM supplied by Hindustan Antibiotics); n.d., not detected; asterisks indicate previously reported complete or partial sequences of (g) [3–6].

## RESULTS AND DISCUSSION

### Characterization of Microheterogeneous Antiamoebins

Fermentation of *S. erythrocephala*, *S. fimetaria* and *G. catenulatum*, solid-phase extraction of peptides from the culture broths and purification of AAM by chromatographic methods followed the protocols which had been shown to be reliable and effective methods for the isolation other peptaibols [30,44]. These procedures provided several hundred milligrams of microheterogeneous peptide mixtures from individual fungi.

Previous investigations had suggested that the peptides produced are identical, or closely related, to AAM [34]. Therefore, the HPLC elution profiles of the isolated peptides were compared with reference AAM isolated from *E. salmosynnemata* CBS 382.62 [8] and *E. synnematicola* CBS 176.60 [39]. Further, commercially available AAM, isolated from *E. synnematicola* ATCC 16540 according to the suppliers declaration, and an original sample of AAM from *E. poonensis* ATCC 16411 [1] were investigated.

The HPLC fingerprints, taken under identical chromatographic conditions, are presented in Figure 1 and demonstrate the microheterogeneity (i.e. exchange of AA) of AAM. The microheterogeneity of AAM had been previously recognized [18] and is a characteristic of most peptaibols. The use of an analytical Supersphere<sup>®</sup> stationary phase, together with a mixture of MeCN, MeOH and water as eluent, made possible an excellent high performance resolution of the peptide mixtures. As can be seen, the established peptide denoted AAM I [3] is the most abundant, and AAM III [6] is the second most abundant in all isolates (for correlation of roman numbers and sequences cf. Figure 2; for relative amounts of peptides cf. Table 1). The peptide designated AAM II [4] is detectable in low amounts (ca. 2%) only in the original isolate of AAM (cf. Figures 1 and 3).

The chiral and non-chiral AAA of total hydrolysates of individual peptide mixtures provided the configuration and approximate ratios of AA and the amino alcohol in AAM (Table 2). Ratios are normalized on Pheol since the amino alcohol is not the subject of exchange in AAM. Ratios are odd for some AA as a result of microheterogeneities. Chiral AAA established the L-configuration of Pheol and chiral AAA, with the exception of D(=R) Iva as previously reported [8]. Hyp in AAM is present as *trans*-4-hydroxy-L-proline. Notably, all possible

stereoisomers of 3- and 4-hydroxyproline are separable on Chirasil-L-Val [45].

AAA also made the assignment of the presence of Val or Leu and the absence of isomeric AA possible, these are not distinguishable under our routine conditions of ESI-MS as applied. Ile is not present in AAM, whereas Val was detectable only in AAM XIII and XIV produced by *S. erythrocephala*. Since D-Iva is also present in these peptides, methanolysis of Val containing fractions followed by trifluoroacetylation of the dipeptide methyl esters was carried out. The presence of exclusively Glu-Iva and Val-Aib in AAM XIII (Glu-Iva, Val-Aib, Val-Gly and Aib-Val in AAM XIV) was established by GC-MS of TFA-dipeptide methylesters on instrument (B) analogous with procedures previously described [43,44].

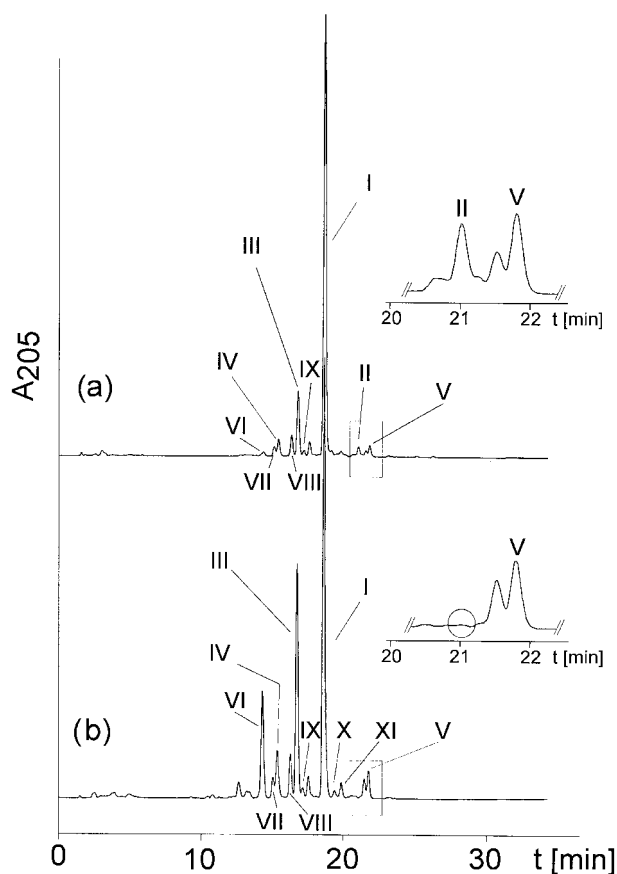


Figure 3 HPLC elution profile of anti-amoebins (AAM) I–XI on Superspher<sup>®</sup>; AAM isolated from (a) *E. poonensis* ATCC 16411 (AAM from Hindustan Antibiotics); and (b) *E. synnematicola* ATCC 16540 (AAM from Sigma); inserts in (a) and (b) are expanded sections of regions framed in chromatograms, circle in (b) shows position at which AAM II should elute;  $A_{205}$ , absorption at 205 nm; for chromatographic conditions see Experimental.

Table 2 Chirality of AA and Pheol and their Ratios in Total Hydrolysates of Microheterogeneous Antiamoebins Isolated from Fungi (a)–(g); Stoichiometry Determined as *N*(*O*)-trifluoroacetyl-*n*-butyl esters by GC-SIM-MS on GC Instrument (B)

AA	Fungal producers						
	(a)	(b)	(c)	(d)	(e)	(f)	(g)
Aib	5.82	6.61	6.18	6.13	6.72	6.45	6.39
Gly	1.22	1.05	1.14	1.16	1.00	0.92	0.97
D-Iva	2.00	1.45	1.58	1.84	1.69	1.60	1.75
L-Leu	1.45	1.16	1.14	0.95	1.09	1.00	0.92
L-Pheol <sup>1</sup>	1.00	1.00	1.00	1.00	1.00	1.00	1.00
L-Pro	1.26	1.07	1.09	1.10	1.00	1.11	0.87
L-Hyp <sup>2</sup>	2.00	2.02	2.06	2.31	1.89	2.18	2.17
L-Phe	0.63	0.91	0.90	0.91	0.96	0.93	0.93
L-Glu <sup>3</sup>	1.01	0.90	0.92	0.91	0.94	0.94	0.88
L-Ala	0.20	0.10	0.16	0.07	0.21	0.19	0.05
L-Val	0.19	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

For names of fungal producers (a)–(g) see Table 1; n.d., not detected (<0.05).

<sup>1</sup> Stoichiometry based on Pheol (1.00).

<sup>2</sup> *trans*-4-hydroxy-L-proline.

<sup>3</sup> Gln, actually present in AAM, hydrolyses to Glu.

The AAA of micropreparatively-isolated peptides AAM XV and XVI show exchange of Phe<sup>1</sup>/Leu<sup>1</sup> (cf. Table 2), which was confirmed by LC-MS. These exchanges are detectable only in AAM isolated from *S. erythrocephala*.

The excellent separation by HPLC and the availability of reference peptides made the confirmation of known, or partly known, and determination of new sequences of AAM possible, using highly sensitive on-line LC-MS-MS as described in the following.

### Sequence Determination of Antiamoebins

Expanded plots of the HPLC elution profiles of AAM peptides (cf. Figure 1) from an original isolate of AAM (from Hindustan Antibiotics) and from a commercially available sample (from Sigma) are shown in Figure 3(a and b), respectively. Using on-line HPLC ESI-MS in positive- or negative-ionization mode and selecting  $(M + Na)^+$  or  $(M)^-$  as precursor ions for MS-MS, the reported sequences [3,4] of AAM I and II could be confirmed, and sequences of AAM III–V could be completely and definitely established in the original isolate of AAM from Hindustan Antibiotics. As can be seen in the expanded sections of Figure 3(a and b), AAM II occurs only in the original isolate, but not in the commercially available AAM isolated from *E. synnematicola* ATCC

16540. The presence of AAM II in the original isolate, and its absence in all other isolates of AAM investigated, was also corroborated by LC-MS. Application of LC-MS also made the determination of six further peptides possible, designated AAM VI–XI in Figure 3(a and b) (as well as Figure 1). Investigation of all peptide isolates by LC-MS, together with their characterization as described above, made the correlation and assignment of HPLC elution profiles and sequences of AAM possible. Further, the production of (Leu<sup>1</sup>)-AAM I and (Leu<sup>1</sup>) AAM III by *S. erythrocephala* was detected.

Corresponding nominal masses of adducts of molecular ions and generated ion fragments used for sequence determination are compiled in Table 3. Note that not all observed characteristic fragment ions are listed. The minor peaks not denoted in Figure 3, the sum of which account for differences of 100% in Table 1, are still mixtures of coeluting components. On-line sequencing of these compounds was ambiguous due to low abundances and interfering fragment ions. On-line sequencing of these minor peptides might be possible by using a stationary phase of different selectivity as shown recently for trichovirins [43].

The following sequencing of peptides exemplarily demonstrates the suitability and sensitivity of on-line LC-MS, with the hitherto unreported structure of AAM VI, isolated from the mould



Table 3 Nominal Molecular Weights of Adducts of Molecular Ions and Diagnostic Fragment Ions of AAM 1–XVI Deduced from ESI-MS: Values in  $m/z$ 

Ions <sup>a</sup>	AAM	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI
(M+Na) <sup>+</sup> <sup>b</sup>		1693	1677	1679	1679	1707	1665	1679	1679	1679	1693	1693	1665	1631	1645	1645	1659
(M+H) <sup>+</sup> <sup>b</sup>		1671	1655	1657	1657	1685	1643	1657	1657	1657	1671	1671	1642	1609	1623	1623	1637
$\alpha_{15}^{\#c}$		1514	1498	1500	1500	1528	1486	1500	1500	1500	1514	1514	1486	1452	1466	1466	1480
$\alpha_{14}^{\#c}$		1417	1401	1403	1403	1431	1389	1403	1403	1403	1417	1417	1398	1355	1369	1369	1383
$\alpha_{13}^{\#c}$		1332	1316	1318	1318	n.d.	1304	1318	1318	1318	n.d.	n.d.	1304	1270	1284	n.d.	1298
$\alpha_{12}^{\#c}$		1219	1219	1205	1205	1233	1191	1205	1205	1205	1219	1219	1191	1157	1171	1171	1185
$\alpha_{11}^{\#c}$		1120	1120	1106	1106	1134	1106	1106	1120	1106	1134	1120	1092	1058	1072	1072	1086
$c_{10}^{\#c}$		1037	1037	1023	1023	1051	1023	1023	1037	1023	1051	1037	1009	975	989	989	1006
$\alpha_{9}^{\#c}$		879	879	865	865	893	865	865	879	865	893	879	851	817	831	831	845
$\alpha_{8}^{\#c}$		794	794	780	780	808	780	780	794	780	808	794	766	732	746	746	760
$\alpha_{7}^{\#c}$		709	709	695	695	723	695	695	709	695	709	709	695	647	661	661	675
$\alpha_{6}^{\#c}$		596	596	582	582	610	582	582	596	582	596	596	582	n.d.	548	n.d.	562
$\alpha_{5}^{\#c}$		539	n.d.	525	n.d.	539	525	n.d.	539	525	n.d.	525	n.d.	n.d.	n.d.	n.d.	505
(M) <sup>-d</sup>		1670	1654	1656	1656	1684	1642	1656	1656	1656	1670	1670	1642	1608	1622	1622	1636
(M-Ac) <sup>-e</sup>		1627	1611	1613	1613	1641	1599	1613	1613	1613	1627	1627	1599	1565	1579	1579	1593
$y_{15N}^e$		1480	1464	1466	1466	1494	1452	1466	1466	1466	1480	1480	1452	1466	1480	1466	1480
$y_{14N}^e$		1395	1379	1381	1381	1409	1367	1395	1381	1381	1395	1395	1367	1381	1395	1381	1395
$y_{13N}^e$		1310	1294	1296	1296	1324	1282	1310	1296	1310	1310	1310	1282	1296	1310	1296	1310
$y_{12N}^e$		1225	1209	1211	1225	1239	1197	1211	1211	1225	1225	1225	1197	1211	1225	1211	1225
$y_{11N}^e$		1126	1110	1126	1126	1140	1112	1126	1112	1126	1126	1140	1112	1126	1126	1126	1126
$y_{10N}^e$		1069	1053	1069	1069	1069	1055	1069	1055	1069	1069	1069	1055	1069	1069	1069	1069
$y_{9N}^e$		956	940	956	n.d.	n.d.	942	n.d.	942	n.d.	956	956	n.d.	956	956	956	956
$y_{8N}^e$		871	n.d.	871	871	871	857	871	857	871	857	871	n.d.	n.d.	n.d.	871	871
$y_{7N}^e$		786	770	786	786	786	772	786	772	786	772	786	786	786	786	786	786

<sup>a</sup> Notation of series  $\alpha$ ,  $b$ ,  $c$  and  $y$  is based on the literature [40–42].

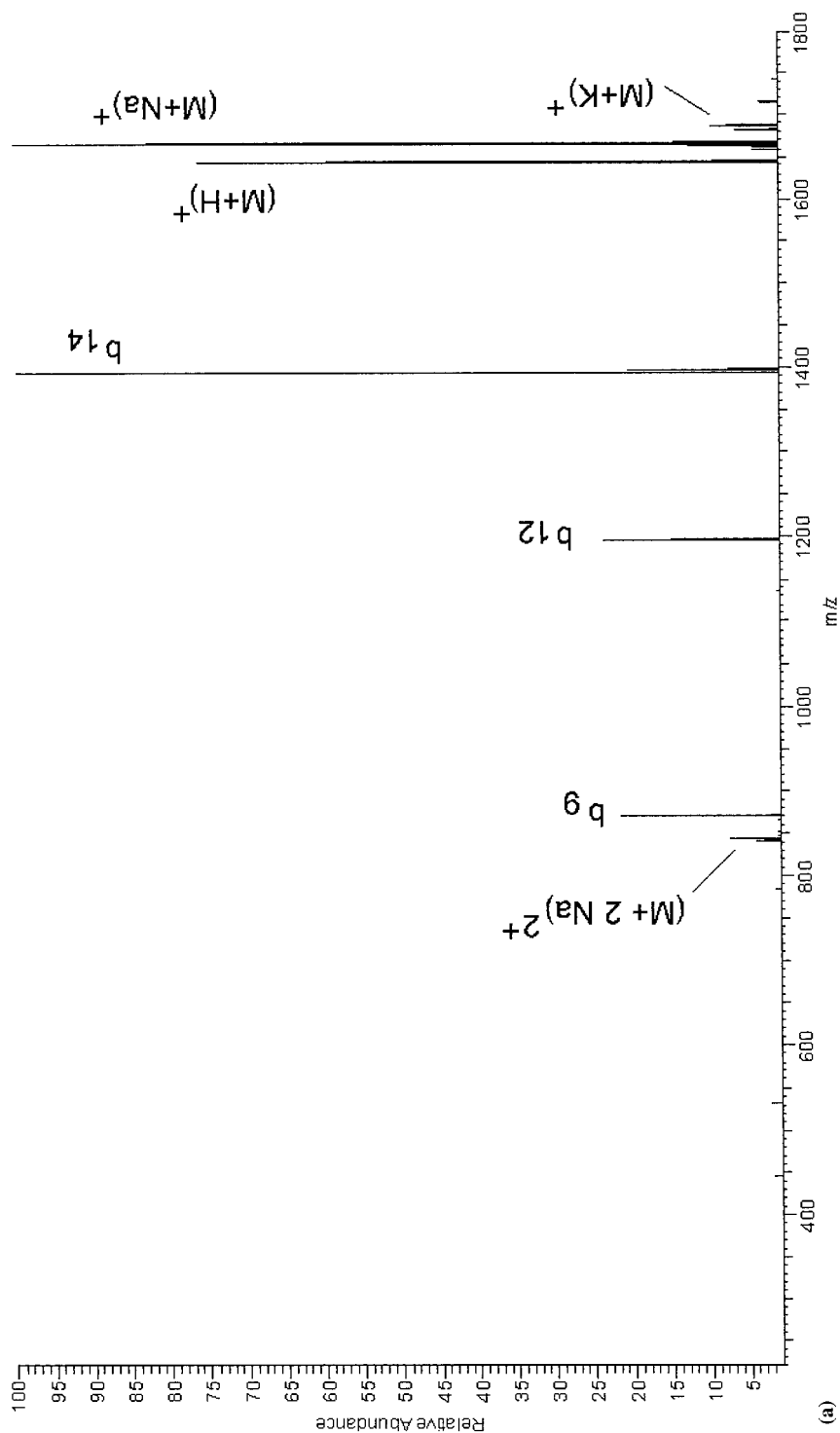
<sup>b</sup> Identified by positive-ion on-line ESI-MS

<sup>c</sup> Identified via MS-MS of (M+Na)<sup>+</sup>

<sup>d</sup> Identified via negative-ion (N) on-line ESI-MS.

<sup>e</sup> Identified via MS-MS from (M)<sup>-</sup>.

<sup>#</sup> Fragment ions are sodium adducts; n.d., not detected; masses of AA (-H<sub>2</sub>O) are: Ala (71), Aib (85), Gln (128), Gly (57), Hyp/Leu (113), Iva/Val (99), Pro (97), Phe (147); amino alcohol Pheol (151); Ac (43). Sequences can be deduced from mass differences of ions. For a scheme of fragmentation see also Figure 5.



(a) On-line HPLC-ESI-MS of (a)–(d) AAM VI (from AAM supplied by Sigma and isolated from *E. synnematocola* ATCC 16540); (a) positive-ion MS; (b) positive-ion ESI-MS-MS of  $(M + Na)^+$  ( $m/z$  1665); note that a and b series are sodiated ions (marked #) resulting from precursor ion  $(M + Na)^+$ ; (c) negative-ion MS; (d) negative-ion ESI-MS-MS of  $(M)^-$  ( $m/z$  1642) and, for comparison, (e) negative-ion ESI-MS-MS of  $(M)^-$  ( $m/z$  1636) of AAM XV (isolated from *S. erythrocephala* ATCC 28144) demonstrating the Phe<sup>1</sup>/Leu<sup>1</sup> exchange,  $\Delta$  refers to mass differences establishing the exchange. For corresponding nominal molecular weights see Table 3, for chromatography and mass spectrometry see Materials and Methods.

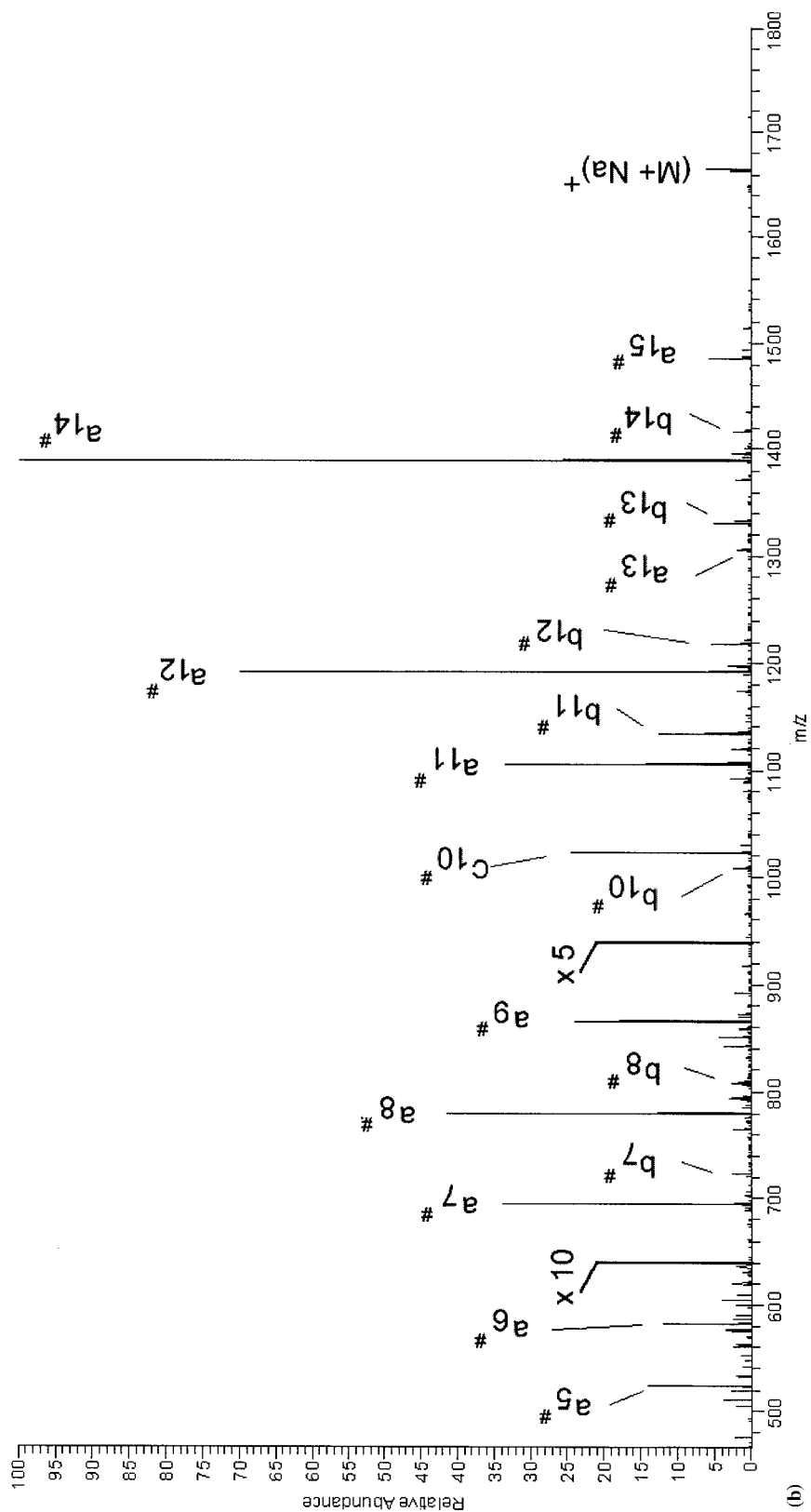


Figure 4 (Continued)

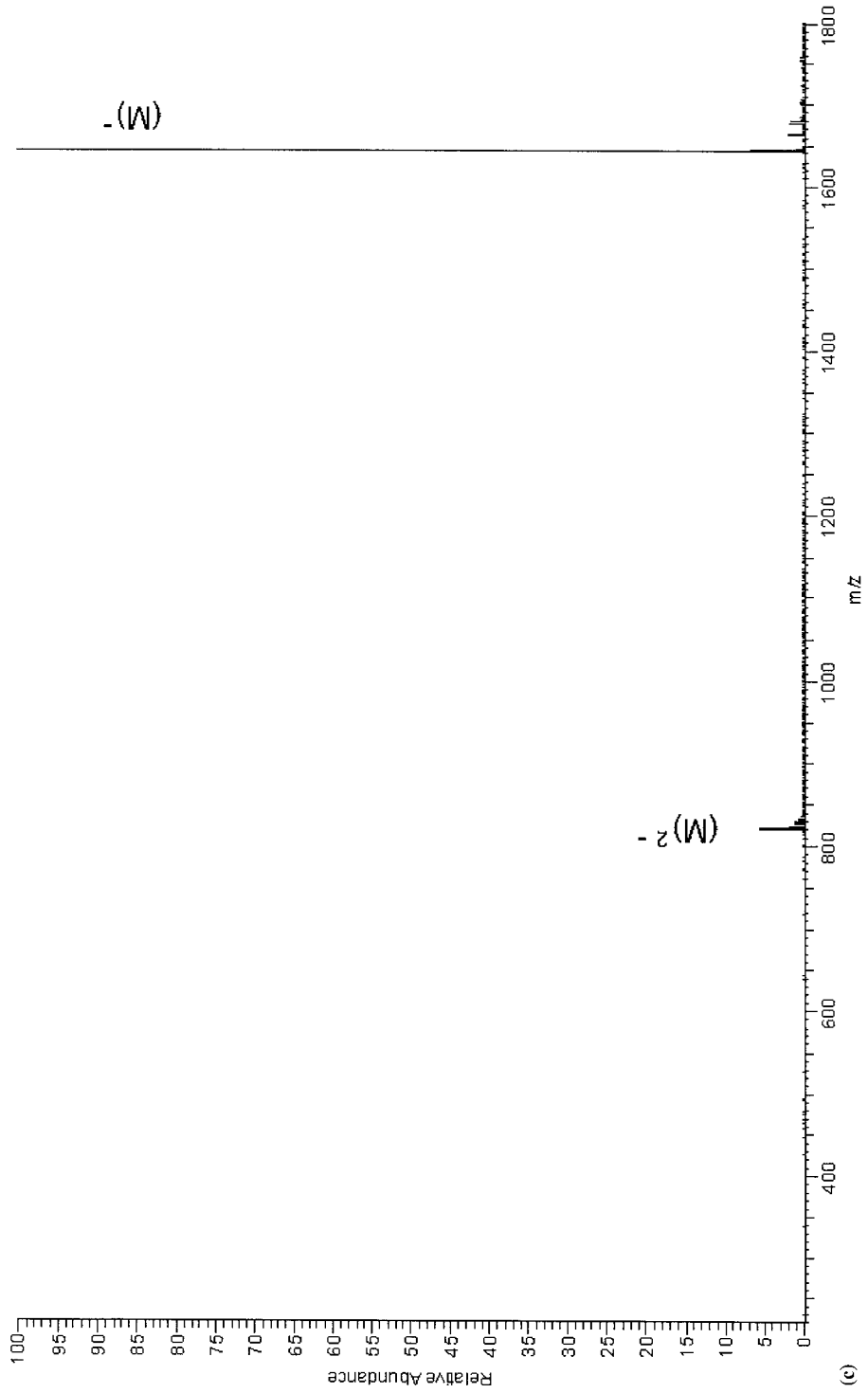


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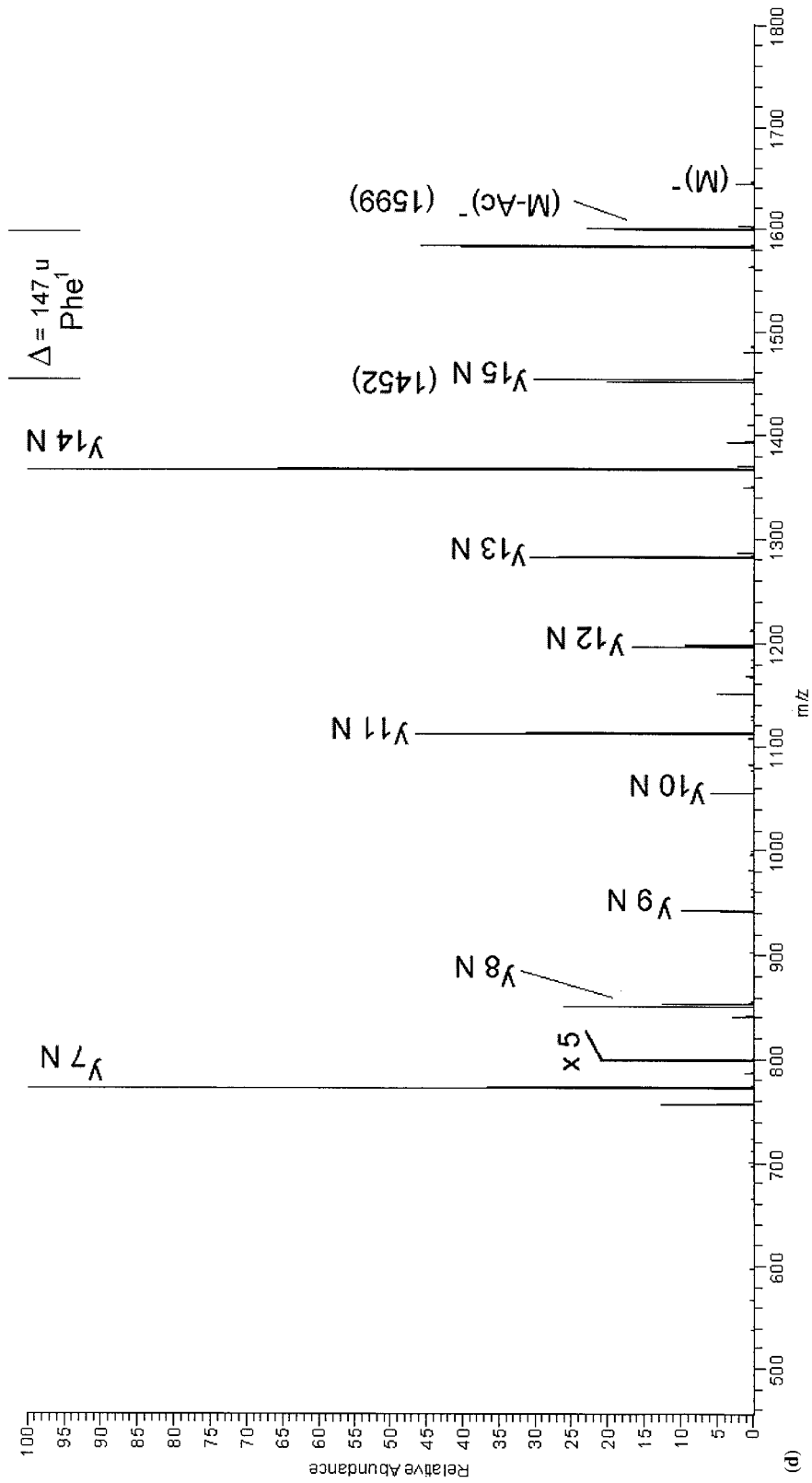


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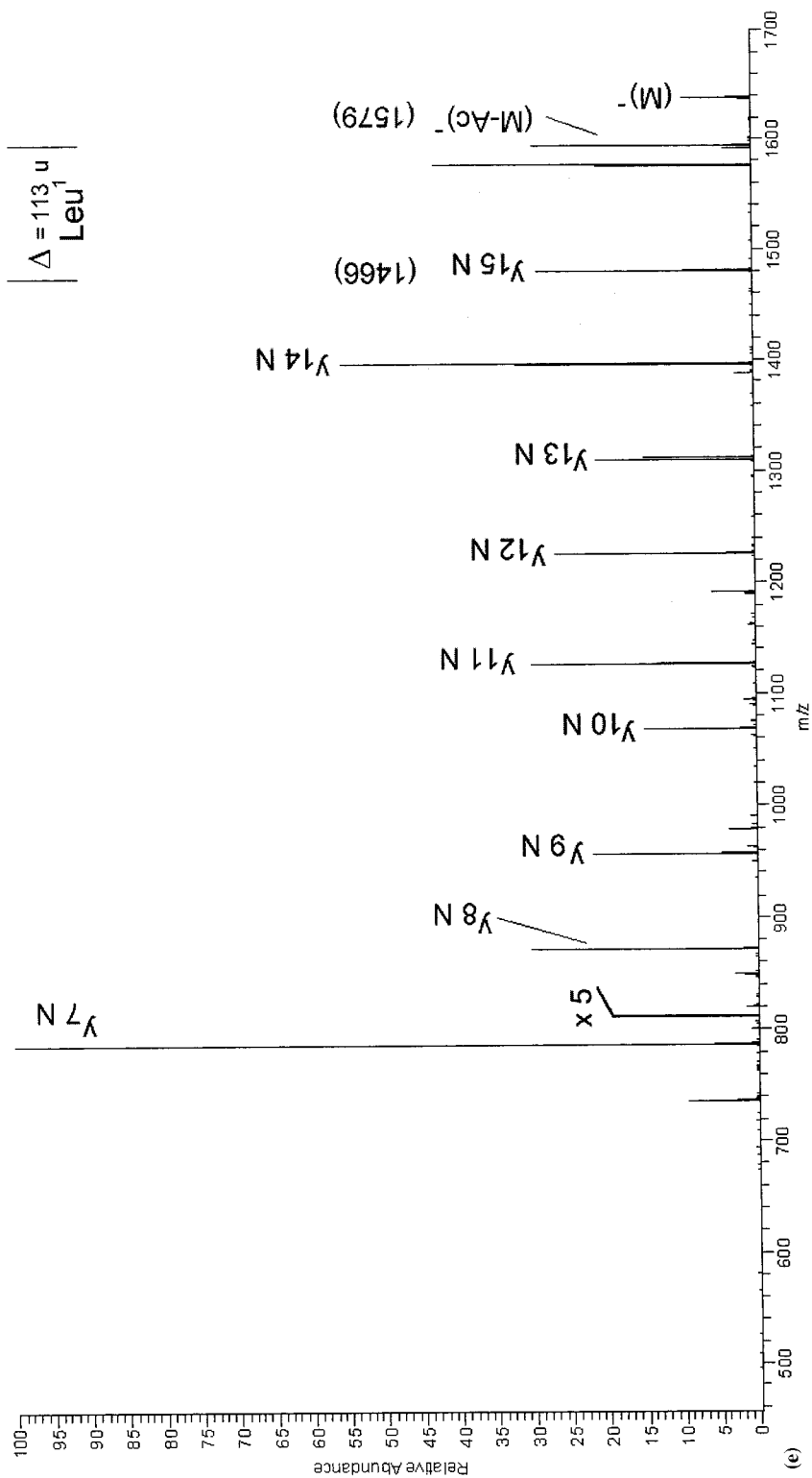


Figure 4 (Continued)

*E. synnematicola*. On-line HPLC ESI-MS and MS-MS in the positive- and negative-ion mode were used. Analogously, the sequences of the AAM peptides presented in Figure 2 were determined.

The pseudomolecular ion  $(M + Na)^+$  at  $m/z$  1665 is most abundant in the positive-ion spectrum, accompanied by  $(M + H)^+$  and  $(M + K)^+$ . In addition, some characteristic fragment ions of the *b* series, resulting from labile Aib<sup>9</sup>-Hyp<sup>10</sup> ( $b_9$ ), Aib<sup>11</sup>-Hyp<sup>12</sup> ( $b_{12}$ ) and Aib<sup>14</sup>-Pro<sup>15</sup> ( $b_{14}$ ) bonds [42] can be seen (Figure 4(a)). As a precursor ion for MS-MS the most abundant  $(M + Na)^+$  was chosen. High-collision energy MS-MS (86%, relative collision energy corresponding to 4.27 V peak-to-peak of resonance excitation radio frequency voltage applied at mass analyzer) resulted in generation of the more abundant *a* series of ions accompanied by the lesser abundant *b* series of ions, and made the sequence determination of AA positions Gly<sup>6</sup> to Pheol<sup>16</sup> possible (Figure 4(b)). Regular series of  $b_7^\#$  to  $b_{15}^\#$  were generated which did not show the characteristic and complete cleavage of Aib(Iva)-Pro(Hyp) bonds [42,43] in positive-ion MS (see Figure 4(a)). Remark-

ably, all generated ions of the *a* and *b* series were sodium adducts, possibly accompanied by hydrogen abstraction [47], and are indicated by # in Figure 4(b). Notably, the abundant sodiated fragment ions of the *a* series were interrupted at AA position 10. At this position, and as an exception, only the  $c_{10}^\#$  ion at  $m/z$  1023 was detected arising from cleavage of the Hyp<sup>10</sup>-Gln<sup>11</sup> bond. Alternatively,  $m/z$  1023 corresponds to  $(b_{10} + 17 + Na)^+$ . It is of interest to note that the fragmentation pattern in positive-ion mode of AAM is different as compared to those observed at sequencing trichotoxin A-40 peptides [42]. These peptides, which contain Glu residues, required acidification of the HPLC eluent in order to be eluted from the column. Use of this acidic eluent resulted in the formation of most abundant  $(M + H)^+$  pseudomolecular ions. In trichotoxins A-40, MS-MS of  $(M + H)^+$  generated an intensive series of *b* ions proceeding to, and ending with, the Aib-Pro bond [42].

In order to increase ionization of peptides for ESI-MS, in the negative-ion mode aqueous ammonia was added to the eluate before entering the MS interface. A most intensive  $(M)^-$  ion at  $m/z$  1642, together with  $(M)^{2-}$  at  $m/z$  821.5 of low intensity, were detected (Figure 4(c)). It is assumed that the negative charge is the result of the capture of one or two electrons, respectively, since AAM do not contain acidic AA which can form anions via dissociation. Notably, the characteristic fragmentation pattern as observed in positive-ion mode, was not abundant. MS-MS of  $(M)^-$  provided the  $y_N$  series of fragment ions [42]. At a relative collision energy of 64% of the instrument (corresponding to 3.24 V applied at mass analyzer) the regular series, starting with  $y_{15N}$  and ending at  $y_{7N}$ , were detected (see Figure 5) and established AA positions Phe<sup>1</sup> to Aib<sup>9</sup> (Figure 4(d)). Acetylation of peptides was deduced from the differences of 43 mass units from  $(M)^-$  ( $m/z$  1642) and  $y_{15N}$  ( $m/z$  1599). The *N*-terminal exchange of Phe<sup>1</sup>/Leu<sup>1</sup> is demonstrated with AAM XV, detectable only in the MS of the peptide mixture of *S. erythrocephala* ATTC 28144 (cf. Figure 4(e)). It is supposed that deacetylation is followed by formation of an aziridinolate anion, as also postulated for the peptaibols trichotoxins [42]. For a proposed mechanism showing the formation of the  $y_N$  fragment series see Figure 5. Loss of 3-alkylaziridinones from peptides in positive-ion mode has been reported [46,47]. Sequence determination of AAM peptides in the positive- and negative-ion mode, showing series of diagnostic ions used for the

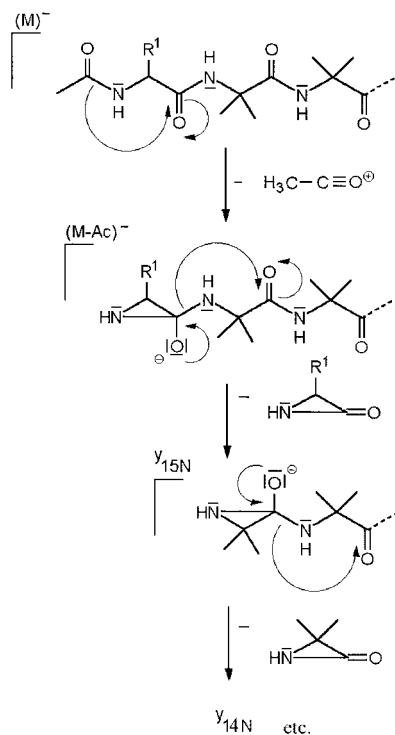
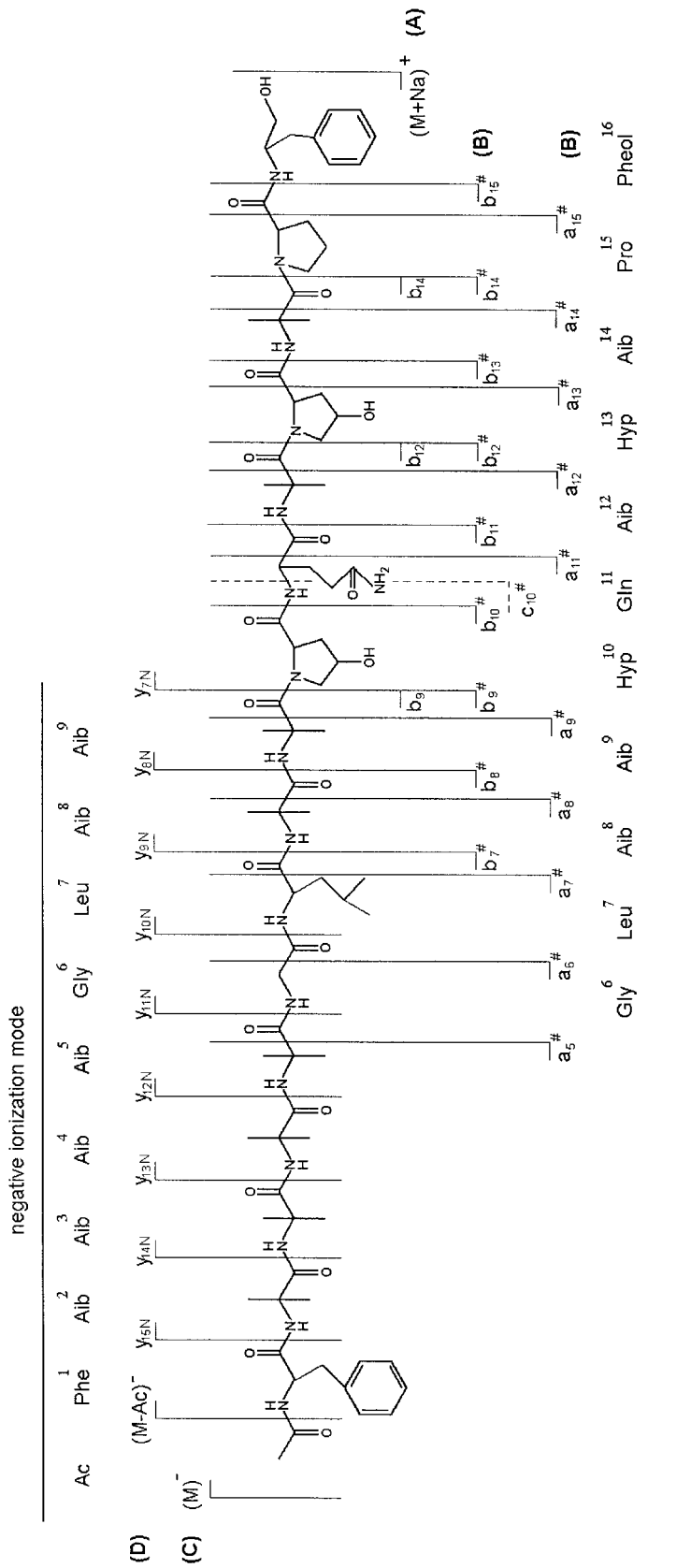


Figure 5 Proposed mechanism for the formation of the  $y_{15N}$  to  $y_{7N}$  series (negative-ion mode) via deacetylation of  $(M)^-$  and release of an acetylum ion, followed by repetitive formation of aziridinolate anions and release of aziridinones ( $R^1$ , benzyl for Phe<sup>1</sup>).



positive ionization mode

Figure 6 Overview of sequencing AAM, demonstrated with the example AAM VI, using positive- and negative-ion ESI-MS; mass fragments were generated by (A), positive ion ESI-MS; (B) MS-MS of  $(M + Na)^+$ ; (C) negative ion ESI-MS; and (D) MS-MS of  $(M)^-$ .  $Y_N$  refers to negative-ion mode; # indicates sodium adducts of  $\alpha$ ,  $b$  and  $c$  series.



sequence determination of AAM VI, is summarized in Figure 6.

## CONCLUSIONS

It is demonstrated that species of the filamentous fungi *Stilbella* and *Gliocladium*, beside *Emericellopsis* and *Cephalosporium*, are capable of producing micro-heterogeneous mixtures of AAM. On-line LC-ESI-MS run in the positive- and negative-ion mode is well suited for the rapid and sensitive determination of sequences of these peptaibol antibiotics. Some specific features are recognized in their mass spectrometric fragmentation pattern in comparison to peptides having exclusively proteinogenic AA as constituents. The most abundant peptides in all AAM are components I and III, which are accompanied by varying amounts of AAM IV–XI. Low amounts (1.9%) of AAM II occur only in a sample of AAM distributed originally by Hindustan Antibiotics, whereas AAM XV and XVI, showing a characteristic Phe<sup>1</sup>/Leu<sup>1</sup> exchange, are only produced by *S. erythrocephala*. Establishing HPLC fingerprints, individual sequences and relative amounts of AAM isolated from various fungi is of importance since experiments described in the literature were partly carried out with micro-heterogeneous mixtures of AAM from various sources.

Proof of the production of relatively high amounts of AAM by *Stilbella* and *Gliocladium*, or the telomorph *Trichoderma* of the latter, are of interest since their use for the biocontrol of phytopathogens is the subject of intensive investigations [48–50]. As synergistic actions of peptaibols and fungal cellulases in this context have been postulated [51], the bioactive AAM produced might be involved in the mode of action.

## Acknowledgements

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