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Sequences of Polypeptide Antibiotics Stilboflavins, Natural Peptaibol Libraries of the Mold *Stilbella flavipes*

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Abstract: From the culture broths of the mold *Stilbella flavipes* CBS 146.81, a mixture of polypeptides could be isolated by adsorption on XAD polystyrene resin and purified by Sephadex LH-20 chromatography. Using preparative thin-layer chromatography (TLC) three groups of peptides, named stilboflavins (SF) A, B, and C could be separated. Each of the groups showed microheterogeneity when investigated by high-performance liquid chromatography (HPLC). Employing on-line HPLC-electrospray ionization tandem mass spectrometry in the positive and negative ionization mode, together with gas chromatography-selected ion monitoring mass spectrometry, enantioselective GC and quantitative amino acid analysis, the sequences of stilboflavins A and B could be determined. Exchange of Glu in stilboflavins A peptides (acidic) against Gln in stilboflavins B peptides (neutral) is the rational for different polarity of the peptide groups and their separatability by TLC. Since SF A and B are bioactive *N*-acetylated 20-residue peptides with a high proportion of α-aminoisobutyric acid and *C*-terminal bonded amino alcohols (either leucinol, isoleucinol or valinol) the peptides belong to the group of peptaibol antibiotics. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: α-aminoisobutyric acid (Aib); electrospray ionization mass spectrometry; peptide antibiotics; peptaibol library; sequence analysis; *Stilbella flavipes*; stilboflavins

INTRODUCTION

Polypeptide antibiotics of the peptaibol group are characterized by the presence of a high proportion of the nonprotein α-aminoisobutyric acid and *C*-terminal-bonded amino alcohols. For those lacking the *C*-terminal amino alcohol the name peptaibiotics has been used [1]. Besides their antibiotic activities peptaibols attracted particular attention as a result of their membrane-modifying properties. These include formation of voltage-dependent or independent ion channels or pores in natural and artificial bilayer membranes [2], hemolysis of erythrozytes [3], uncoupling of the oxidative phosphorylation of mitochondria [4], damage of mitochondria of mosquito larvae [5], increasing the permeability of liposome bilayers composed of zwitterionic lipids [6], and influx of calcium ions into bovine adrenal chromaffin cells and catecholamin secretion from

Abbreviations: AAA, amino acid analysis; AA(s), amino acid(s); Ac, acetyl; Aib, α-aminoisobutyric acid, 2-amino-2-methyl-propanoic acid; a.m.u., atomic mass units; DCM, dichlormethane; ESI, electrospray ionization; GC, gas chromatography; HPLC, high-performance liquid chromatography; Ileol, isoleucinol (2-amino-3-methyl-1-pentanol); Leuol, leucinol, (2-amino-4-methyl-1-pentanol); MeCN, acetonitrile; MeOH, methanol; MPLC, medium-pressure liquid chromatography; MS, mass spectrometry or mass spectrum; MS-MS, MS^n, tandem mass spectrometry; NMM, nominal molecular mass; SIM, selected ion monitoring; TCM, trichloromethane; TDM (reagent), chlorine/4,4′-bis(dimethylamino)-diphenylmethane; TFAA, trifluoroacetic acid anhydride; TLC, thin-layer chromatography; Valol, valinol, (2-amino-3-methyl-1-butanol); Xol, leucinol or isoleucinol; common AAs are abbreviated according to three-letter nomenclature.

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the cells [7]. Antithrombic [8] and neuroleptic (antipsychotic) [9] activities were also described.

It was also reported that peptaibols trichorzianins are involved in the mode of action of cellulolytically active fungi used for the biocontrol of plant pathogens [10,11]. Recently it was demonstrated that peptaibols act as highly effective elicitors in beans like *Phaseolus lunatus*, *P. vulgaris*, and *Vigna radiata*. The peptides can induce multiple metabolic activities such as ethylene emission, biosynthesis of volatile substances, and tendril coiling of *Lathyrus* sp. or *Pisum sativum* [12].

Using sensitive and specific chromatographic screening procedures based on the detection of the marker amino acid Aib it was shown that the majority of species and strains of the fungal genus *Stilbella* Lindau, besides *Trichoderma*, *Gliocladium*, and *Hypocrea*, are abundant sources of Aib-peptides [13–22]. The peptide antibiotic Stilbellin from *Stilbella* sp. was shown some time ago to represent the 15-residue peptaibols emerimicins [23]. A chloroform soluble antibiotic from *Stilbella erythrocephala* ATCC 28144 that had been reported to cause inhibition of growth of many other coprophilous fungi, some fungal plant pathogens, and dung bacteria [24], as well as peptides isolated from *S. fimetaria* CBS 548.84, were recently definitely established to represent microheterogeneous 16-residue peptaibols antiamoebins [25].

In the following we report on the sequences and bioactivities of the polypeptides isolated from *S. flavipes* (Peck) Seifert (strain CBS 146.81) that had been recognized to produce Aib-peptides [15,16]. *S. flavipes* is probably a fairly common fungus (Hyphomycete) growing in soil or on decaying angiosperm wood, usually in association with, or growing on, wood decaying Hymenomycetes such as *Sterum* sp. or *Phellinus* sp. [26].

EXPERIMENTAL PROCEDURES

Chemicals

Solvents were of the gradient grade and from Merck (Darmstadt, Germany). For AAA, an AA standard solution (no. AA-S-18) (Sigma, St. Louis, MO, USA) was used and appropriate amounts of Aib, L-Ileuol (from Sigma), L-Leuol and L-Valol (from Fluka, Buchs, Switzerland) were added. The reference dipeptide Pro-Val, serving as a standard, was from Sigma; Pro-Aib was generated by acidic methanolysis from alamethicins F-50 [3,27,28]. For analytical TLC pre-coated plates with silica gel 60 F 254, thickness 0.25 mm (Merck) were used. Preparative separation of stilboflavins A and B was carried out using pre-coated plates with silica gel 60 F 254, thickness 2 mm (Macherey-Nagel, Düren, Germany). The mobile phase TCM–MeOH, 75/25 (v/v) was used exclusively for analytical and preparative TLC. 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 μm (Serva, Heidelberg, Germany), and Sephadex LH-20, particle size 25–100 μm (Pharmacia, Freiburg, Germany) were used. For malt agar medium, 30 g of malt extract (Serva), 15 g of agar (Fluka) and 3 g of soy peptone (Oxoid, Wesel, Germany) were dissolved in 1 l of demineralized water (final pH 6–6.5). For Raulin Thom medium 50.0 g of D-glucose (Riedel-de-Haen, Seelze, Germany), 2.765 g of L(+) tartaric acid (Merck), 2.765 g of diammonium tartrate (Merck), 0.16 g of $(NH_4)_2SO_4$ (Riedel-de-Haen), 0.4 g $(NH_4)_2HPO_4$ (Riedel-de-Haen), 0.4 g of K_2CO_3 (Riedel-de-Haen), 0.33 g of $Mg(HCO_3)_2$ (Merck) and 0.046 g of $FeSO_4 \times 7H_2O$ (Sigma) were dissolved in 1 l of demineralized water and pH 4.0 was adjusted using 0.1 M NaOH.

A mixture of hypelcins A and B [29–31], serving for comparative analysis by HPLC, was a donation to H.B. from Dr T. Fujita, Faculty of Pharmaceutical Sciences, Kyoto University, Japan.

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 were used. The HPLC column was a Superspher® 100 RP-18, 250 × 4 mm i.d., 4 μm particle size (Merck).

HPLC eluents (by volume): A(I) MeOH/water/MeCN, 38/38/24; A(II) MeOH/water/MeCN/TFA, 38/38/24/0.1; B(I), MeOH/MeCN, 1/1; B(II), MeOH/MeCN/TFA, 1/1/0.1; gradient program 0 min 0% B; 10 min 0% B, 35 min 50% B, 48 min 100% B; flow-rate 1.0 ml/min; temperature 35°C; injected amounts 10 μl of a 0.1% methanolic solution of peptides. Eluents A(I)/B(I) were used for gradient elution of SF B peptides (neutral), and eluents A(II)/B(II) were used for SF A peptides (acidic).

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

Mass Spectrometry

For ESI-MS a LCQ™ MS (ThermoQuest, Finnigan MAT, San Jose, CA, USA) was used. Peptaibols were analysed by on-line coupling of the HPLC instrument and the LCQ. The sheath gas was nitrogen (purity > 99.5%) from a nitrogen generator Model 75-72 (Whatman, Balston Inc., Haverhill, MA, 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 ± 0.2 a.m.u.

Conditions for positive (negative) ionization mode were: spray voltage 4.25 (4.25) kV, heated capillary temperature 220 (220)°C, capillary voltage +43 (−40) V, tube lens offset +55 (+55) 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 perfluoronated 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 µl/min.

The notation used for sequence determination in the positive-ion mode, referring to a and b fragment ions, is based on the suggestions of Roepstorff and Fohlman [32], modified by Biemann [33]. MS-MS in the negative-ion mode produced the y series, denoted y_N, without protonation [34].

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, Netherlands). For quantitative AAA, dipeptide analysis and identification of isomeric *C*-terminal amino alcohols, the GC-MS instrument (B) was a HP 6890 (Hewlett-Packard, Waldbronn, Germany) with mass selective detector equipped with a Heliflex® AT-5 column (crosslinked 5%-diphenyl–95% dimethyl siloxane), 30 m × 0.25 mm i.d., film thickness 0.25 µm (Alltech Associates, Deerfield, USA). EI-MS on instruments (A) and (B) were recorded at an ionization energy of 70 eV.

Fermentation Procedure and Peptaibol Monitoring

S. flavipes (Peck) Seifert, strain CBS 146.81 [26] ex type culture of *Dendrostilbella hanlinii* Hammill and Shipman [35], was obtained as freeze-dried culture from the Centraalbureau voor Schimmelcultures (CBS), Baarn, Netherlands. The lyophilysate was suspended in sterile water (1.0 ml) and, after soaking for 6 h at 20°C, transferred to Petri dishes (9.5-cm diameter) with malt-agar medium. After 6 days at 21°C intensive growth was observed. Agar discs (1-cm diameter) were used for the inoculation of 12 Erlenmeyer flasks (2 l) each containing 400 ml of Raulin Thom medium. The flasks were shaken at 100 rpm at room temperature (ca. 25°C) using a Model SM-30A rotary shaker (Johanna Otto GmbH, Hechingen, Germany). For daily monitoring of peptaibol production, aliquots (20 ml) of filtered culture broths were passed through Sep-Pak® C-18 cartridges (Waters, Milford, USA). After washing with water (3 × 10 ml) 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 analytical TLC (see the Chemicals section) and peptaibols visualized by spraying with water and, after drying, with TDM reagent. Three spots distinguished by their retention factors (R_F), each consisting of mixtures of microheterogeneous peptides, were observed on TLC and named stilboflavins (SF) A (R_F 0.14), B (R_F 0.51), C (R_F 0.90). The 20-residue reference peptaibol paracelsin [28], serving as a standard, had R_F 0.13.

Isolation and Purification of Stilboflavins

After shaking for 72 h intensive peptide production in the fermentation broth was detected by TLC. Culture broths (pH 2.3) of shake flasks were combined, separated from mycelia (190 g wet weight) by filtration under reduced pressure, and filtrates (4.7 l) were pumped through a MPLC-column (38 × 3.7 cm) filled with XAD-2 resin. The resin was washed with water (1 l) and 40% MeOH (0.5 l). Then the peptides adsorbed were eluted with a linear gradient from 40% to 100% MeOH and 96 fractions (each 25 ml) were collected. Elution of peptides was monitored by TLC. Fraction nos. 37–78 containing peptaibols were combined and evaporated to dryness yielding 2.91 g of a pale yellow powder.

Further purification of the peptides was carried out by chromatography on a Sephadex LH-20 column (100 × 3.1 cm) using MeOH as eluent. The peptide mixture (2.91 g) was dissolved in 20 ml of MeOH and subjected to LH-20 chromatography in four portions of each 5 ml. Fractions were collected and elution of peptides was monitored by TLC. The fractions containing peptaibols were combined and evaporated to dryness, yielding 2.74 g of a colorless peptide mixture consisting of SF A, B and C.

Repetitive preparative TLC made possible the separation and isolation of still microheterogeneous peptides of SF A, B and C. For their isolation approximately 340 mg of the mixture, consisting of SF A, B and C, was dissolved in 3.5 ml of MeOH and subjected in portions (7×0.5 ml) to preparative TLC. Separation was monitored by spraying with water and zones containing separated SF A, SF B and SF C peptaibol groups were marked. Then the plates were dried for 24 h at 60°C and zones containing SF A, B and C were scraped out with a lancet. The peptides were eluted from the silica by treatment with MeOH (25 ml), then the silica was removed by centrifugation (20 min at $3500 \times g$). The precipitate was treated twice with MeOH (25 ml), centrifuged, and the combined supernatants evaporated to dryness using a rotary evaporator. Quantities of SF A (66 mg), SF B (139 mg), and SF C (87 mg) were obtained. In the following we report on the sequencing of the microheterogeneous peptide mixtures named SF A and B.

Amino acid analysis by GC-SIM-MS. For analysis, the peptide mixtures of SF A and SF B (50 μg) were hydrolysed (6 M HCl, 110°C, 24 h). Chirality of AAs and the amino alcohols were determined after derivatization as *N*-pentafluoropropionyl-AA-2-propyl esters and *N*(*O*)-bis(pentafluoropropionyl) amino alcohols by GC-SIM-MS on GC-MS instrument (A) as described previously [25,34,36]. Stoichiometry of components was determined after derivatization as *N*-trifluoroacetyl-AA-*n*-butyl esters and *N*(*O*)-bis(trifluoroacetyl)amino alcohols using GC-MS instrument (B). The stoichiometry was normalized on Gly (1.00) since this amino acid is not exchanged in SF A and B peptides. Since Leuol and minor amounts of its isomer Ileol, as well as Valol, were detected by quantitative AAA in the SF B mixture, the structures of the isomeric amino alcohols had to be assigned Xol by applying ESI-MS that does not distinguish these isomers. In order to assign these alcohols the SF B mixture was subjected to analytical HPLC and peptides containing Xol were collected manually. The isolated peptides were totally hydrolysed, the isomeric amino alcohols analysed by GC-SIM-MS as *N*(*O*)-bis(trifluoroacetyl)amino alcohols and their structures definitively assigned as Leuol or Ileol.

Dipeptide analysis of methanolysates by GC-MS. For acidic methanolysis, aliquots (ca. 30 μg) of peptide mixtures 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 derivates of interest were analysed on GC-MS instrument (B) as described previously [36,37]. For the assignment of the acetylated *N*-termini of SF peptides Ac-Aib-OMe released on partial methanolysis of individual peptides, was directly analysed by GC-SIM-MS [*m*/*z* (%) 100 (100), 128 (3.8)].

Antibiotic and hemolytic activity of stilboflavins. Antibiotic and hemolytic properties of isolated SF A, B and C peptides were measured by applying the agar-diffusion test against *Bacillus subtilis* DSM 10, *Staphylococcus aureus* DSM 20231 T, and *Escherichia coli* DSM 498 (DSM, Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany) as described [36]. The hemolytic activity of SF A and B peptides were tested employing blood Petri dishes comprising ready-to-use Columbia agar plates containing 7–10% defibrinized sheep blood (no. RPP 008B from Oxoid) [36,37]. Aliquots of 1% methanolic solutions were used and hemolysis zones compared with peptaibols paracelsins [28,38] and antiamoebins [25].

RESULTS AND DISCUSSION

The peptaibol groups named SF A, B and C were isolated by preparative TLC. The elution profile of SF B peptides resolved on an analytical Superspher® column using a neutral eluent is shown in Figure 1(a), and the HPLC of SF A peptides using an acidic eluent is shown in Figure 1(b). The separation of the crude peptide mixture and of groups SF A, B and C peptides isolated therefrom by preparative TLC is shown in the insert of Figure 1(a).

Chirality and ratios of AAs and amino alcohols of the isolated SF B (A) mixture, after total hydrolysis and normalization on Gly, were determined as Gly 1.00 (1.00), L-Ala 1.19 (1.36), Aib 9.62 (9.63), L-Glu 2.85 (2.71), L-Leu 0.92 (1.26), L-Pro 2.06 (2.07), L-Val 1.23 (1.10), L-Leuol 0.67 (0.67), L-Ileol 0.08 (0.06), L-Valol 0.23 (0.15). Non-stoichiometry of AAs and the amino alcohols are the result of microheterogeneity. SF C represents a mixture of peptaibols, the sequences of which have not yet been determined, containing L-Ser among other AAs and Leuol. GC-SIM-MS of the SF A and B mixture established that Val and Leu were constituents, but not their isomers Iva and Ile, respectively. The Gln

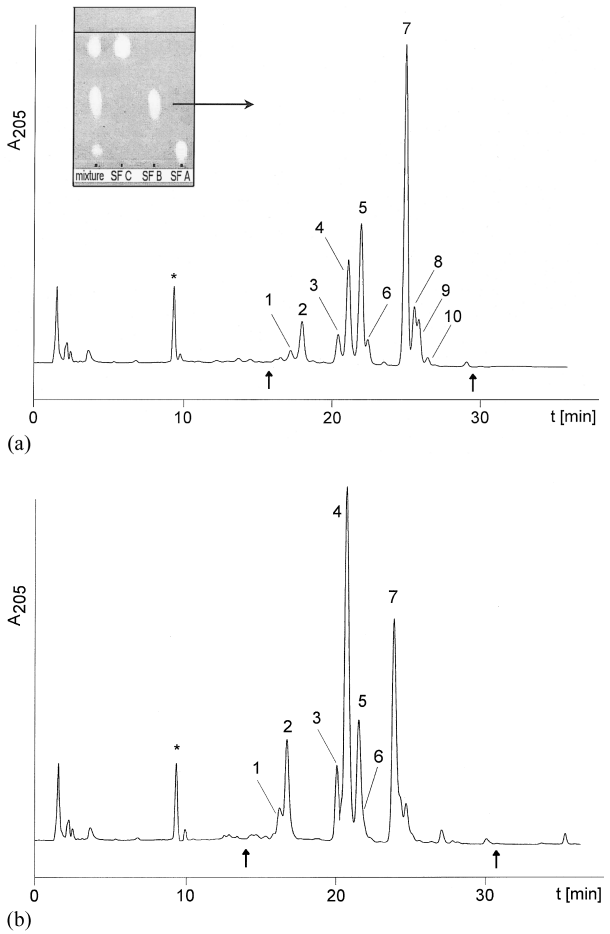

Figure 1 HPLC elution profile of (a) neutral stilboflavin (SF) B peptides and (b) acidic SF A peptides; asterisk refers to non-peptidic impurity; A_{205}, absorption at 205 nm; for correlation of numbers of peptides and sequences see Figure 4. Insert in (a) shows analytical TLC of the SF mixture and of isolated SF peptaibols A, B and C (for chromatographic conditions of TLC and HPLC see 'Experimental procedures' section); arrows indicate elution time in which the sum of peak areas was taken for 100%.

residues originally present in SF B peptides are hydrolysed to Glu by acidic total hydrolysis. However, the mass difference of 1 a.m.u. between Gln (m/z 128) and Glu (m/z 129) in native peptides can be recognized by on-line ESI-MS. This established the presence of Gln^{7}, Gln^{18} and Gln^{19} residues in SF B peptides and the exchange of Gln^{18}/Glu^{18} in SF A peptides. This exchange was also confirmed by ZoomScan experiments (i.e. high resolution MS scan mode) performed by off-line infusion of the SF A peptide mixture. Acidic Glu^{18} residues make the SF A peptides more polar as compared with SF B peptides and enable their separation by analytical and preparative TLC. The $(M\text{-}Ac)^-$ ions were not observed in the mass spectra of peptides using the negative-ion mode as applied. This is in contrast to ESI-MS of peptaibols trichotoxins A-40 [34] and antiamoebins [25]. The presence of *N*-terminal Ac-Aib in SF A and B peptides could be established under conditions of ESI-MS from the difference of $(M)^-$ molecular ions and y_{19N} fragment ions counting for 128 a.m.u. The occurrence of *N*-acetylated Aib in SF A and B was also proven by methanolysis of peptides and determination of the Ac-Aib-OMe released using GC-SIM-MS on instrument (B) [39].

Analysis of SF B components designated B3, B4 and B7 to B10 by HPLC-MS-MS in the positive-ion mode indicated the presence of *C*-terminal Xol (isobaric Leuol or Ileul) as deduced from the mass difference of 103 a.m.u. between the $b_{19}^{\#}$ fragment ion $(b_{19}-H+Na)^+$ and $(M+Na)^+$ pseudo-molecular ion (cf. Figure 6). Quantitative AAA of the SF B mixture had revealed that, besides Valol, major amounts of Leuol and minor amounts of Ileol were present (cf. Figure 2). Whereas the presence of *C*-terminal Valol in SF B1, B2, B5, and B6 was established by on-line HPLC-ESI-MS, the differentiation among the isomeric amino alcohols was not possible under conditions of HPLC-ESI-MS. Therefore, the SF B mixture was subjected to micropreparative HPLC and the peptides SF B3, B4, and B7 to B10 were collected manually. Individual peptides were hydrolysed and amino alcohols released analysed as *N*(*O*)-bis(trifluoroacetyl)amino alcohols by GC-SIM-MS on instrument (B). The presence of Leuol in SF B3, B4, B7 and B8, and of Ileol in SF B9 and B10, could be established. The *C*-terminal amino alcohols of the SF A peptides were determined analogously. As an exception, the amino alcohol of the minor peptide SF A 6 had to be assigned Xol as both Leuol and Ileol were released on hydrolysis. This procedure is illustrated with the GC-SIM-MS elution profiles of the amino alcohols released after hydrolysis of the manually fractionated peptides SF B7–B10 in comparison to an equimolar mixture of the standard L-Leuol and L-Ileol (Figure 3). As a result of the incomplete separation in particular of SF B8 and B9, a decrease of quantities of Leuol and an increase of Ileol can be seen (SF B8 contains some SF B9 and vice versa), whereas SF B7 and SF B10 are almost pure peptides.

The assignment of the structures of amino alcohols together with on-line HPLC-MS-MS in the positive and negative-ion mode, taking into account quantitative AAA and dipeptide analysis (see below), made possible the sequence determination of ten SF

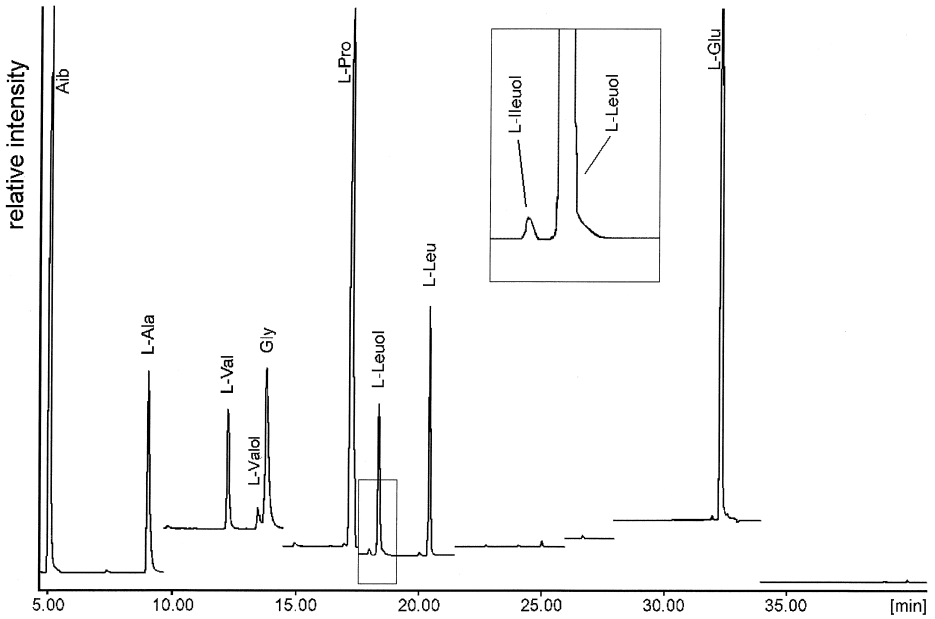

Figure 2 GC-SIM-MS (Chirasil-L-Val) of *N*-pentafluoropropionyl-amino acid-2-propyl esters and *N*(*O*)-bis(pentafluoropropionyl)amino alcohols of a total hydrolysate of SF B peptides; insert shows the expanded section framed in chromatogram. Chirality was assigned by comparison with a standard composed of D- and L-amino acids and D- and L-amino alcohols.

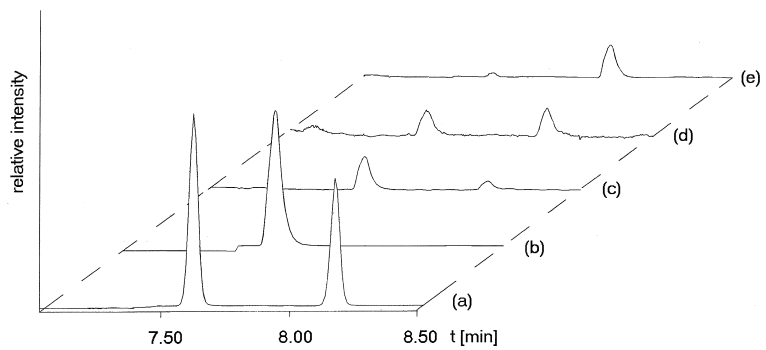

Figure 3 GC-SIM-MS (Heliflex® AT-5 column) of *N*(*O*)-bis(trifluoroacetyl)amino alcohols of (a) an equimolar mixture of L-Leuol (first peak) and L-Ileol (second peak) compared with total hydrolysates of manually collected peptides (b) SF B7, (c) SF B8, (d) SF B9 and (e) SF B10.

B and seven SF A peptaibols (Figure 4). The nominal molecular masses of adducts and diagnostic fragment ions resulting from ESI-MS of SF B and A peptides are presented in Tables 1 and 2, respectively. A scheme of the sequence determination of SF B peptides in the positive- and negative-ion mode, showing the series of diagnostic ions used for the sequence determination of SF B5, is presented in Figure 5. According to this scheme the sequences of the SF B and SF A peptides presented in Figure 4 were determined. Structural characterization by on-line HPLC-ESI-MS is demonstrated in the following with the sequence determination of the peptide SF B5 counting for 17.6% of SF B peptides.

In the positive-ion spectrum of SF B5 the pseudomolecular ions $(M+Na)^+$ at m/z 1937.2 and $(M+H)^+$ at m/z 1915.1 are the most abundant as well as the doubly charged ions at m/z 980.0 and m/z 958.0. In addition, the characteristic and intensive b_{13} fragment ion at m/z 1189.7, resulting from the cleavage of the labile Aib^{13}-Pro^{14} bond, and the internal fragment y_{7P} at m/z 726.5 can be seen (Figure 6(a)).

SF B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	NM	%
1	Ac	Aib	Pro	Aib	Ala	Aib	**Ala**	Gln	Aib	**Val**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Gln**	Gln	**Valol**	1886	2.5
2	Ac	Aib	Pro	Aib	Ala	Aib	**Ala**	Gln	Aib	**Leu**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Gln**	Gln	**Valol**	1900	6.5
3	Ac	Aib	Pro	Aib	Ala	Aib	**Ala**	Gln	Aib	**Val**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Gln**	Gln	**Leuol**	1900	4.4
4	Ac	Aib	Pro	Aib	Ala	Aib	**Ala**	Gln	Aib	**Leu**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Gln**	Gln	**Leuol**	1914	13.7
5	Ac	Aib	Pro	Aib	Ala	Aib	**Aib**	Gln	Aib	**Leu**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Gln**	Gln	**Valol**	1914	17.6
6	Ac	Aib	Pro	Aib	Ala	Aib	**Aib**	Gln	Aib	**Val**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Gln**	Gln	**Valol**	1900	3.3
7	Ac	Aib	Pro	Aib	Ala	Aib	**Aib**	Gln	Aib	**Leu**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Gln**	Gln	**Leuol**	1928	35.1
8	Ac	Aib	Pro	Aib	Ala	Aib	**Aib**	Gln	Aib	**Val**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Gln**	Gln	**Leuol**	1914	6.1
9	Ac	Aib	Pro	Aib	Ala	Aib	**Aib**	Gln	Aib	**Leu**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Gln**	Gln	**Ileol**	1928	4.3
10	Ac	Aib	Pro	Aib	Ala	Aib	**Aib**	Gln	Aib	**Val**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Gln**	Gln	**Ileol**	1914	1.6
SF A																						
1	Ac	Aib	Pro	Aib	Ala	Aib	**Ala**	Gln	Aib	**Val**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Glu**	Gln	**Valol**	1887	3.4
2	Ac	Aib	Pro	Aib	Ala	Aib	**Ala**	Gln	Aib	**Leu**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Glu**	Gln	**Valol**	1901	10.3
3	Ac	Aib	Pro	Aib	Ala	Aib	**Aib**	Gln	Aib	**Val**	Aib	Gly	Aib	**Ala**	Pro	Val	Aib	Aib	**Glu**	Gln	**Leuol**	1901	6.2
4	Ac	Aib	Pro	Aib	Ala	Aib	**Ala**	Gln	Aib	**Leu**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Glu**	Gln	**Leuol**	1915	34.8
5	Ac	Aib	Pro	Aib	Ala	Aib	**Aib**	Gln	Aib	**Leu**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Glu**	Gln	**Valol**	1915	8.3
6	Ac	Aib	Pro	Aib	Ala	Aib	**Ala**	Gln	Aib	**Leu**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Glu**	Gln	**Xol**	1915	6.0
7	Ac	Aib	Pro	Aib	Ala	Aib	**Aib**	Gln	Aib	**Leu**	Aib	Gly	Aib	**Aib**	Pro	Val	Aib	Aib	**Glu**	Gln	**Leuol**	1929	23.6

Figure 4 Sequences of stilboflavins B and A; exchanged AA positions in bold letters; NMM, nominal molecular mass; (%), relative amount of peptides in the natural microheterogeneous mixtures according to HPLC (cf. Figure 1(a) and (b)); amino alcohols and chiral amino acids are of the L-configuration.

As precursor ion for MS-MS the most abundant $(M+Na)^+$ at m/z 1937.2 was chosen. High-collision energy MS-MS (60% relative collision energy corresponding to 2.98 V peak-to-peak of resonance excitation radio frequency voltage applied at the mass analyser) resulted in the generation of the series of sodium adducts, indicated by $^{\#}$, of $b_7^{\#}$–$b_{13}^{\#}$ and $b_{15}^{\#}$–$b_{19}^{\#}$ ions, accompanied by the corresponding $a^{\#}$-series of ions (Figure 6(b)). This fragmentation pattern has also been observed for antiamoebins [25]. These regular series of fragment ions made possible the sequence determination of AA positions Aib^8 to Aib^{13} and Aib^{16} to Valol^{20}. The $b_{14}^{\#}$ and $a_{14}^{\#}$ ions, as a result of the complete and characteristic cleavage of the Aib^{13}-Pro^{14} bond, were not observed. But dipeptide analysis of the methanolysate of the SF B mixture, revealed the presence of Pro-Val, and thus established the Pro^{14}-Val^{15} position.

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 1914.2, together with $(M)^{2-}$ at m/z 957.1 of lower intensity, was detected (Figure 6(c)). It is assumed that the negative charge is the result of the capture of one or two electrons, respectively, since SF B peptides do not contain acidic Glu that can form anions via dissociation. This is also observed for SF A peptides although they contain Glu^{18} residues. Their γ-carboxy groups are obviously completely protonated under the acidic conditions used for on-line HPLC-MS. This behavior in negative-ion MS is in analogy to that of antiamoebins [25] and trichotoxin A-40 peptides [34]. MS-MS of $(M)^-$ at m/z 1914.2 of SF B5 revealed only the abundant y_{19N} ion at m/z 1786.1 (not shown) the difference counting for 128 a.m.u. This is due to the loss of *N*-terminal Ac-Aib^1, the presence of which was also proven by GC-MS of a methanolysate (see above). For further sequence determination MS^3 of the y_{19N} ion was carried out (Figure 6(d)) and made possible the determination of amino acid positions 3–13 of peptides. Notably, in negative-ion MS the characteristic fragmentation pattern as observed in positive-ion MS was not abundant. MS^n of $(M)^-$ provided the y_N series of fragment ions [34]. The y_{8N} (expected from cleavage of Aib^{12}-Aib^{13}) and y_{18N} (expected from cleavage of Pro^2-Aib^3) were not observed. But dipeptide analysis of the SF B peptide methanolysate revealed the presence of TFA-Pro-Aib-OMe exclusively [m/z (%) 154 (100), 114 (13), 267 (11.5), 144 (5.9)] and established the Pro^2-Aib^3 positions. The Aib^{12}-Aib^{13} sequence was identified by MS-MS of $(M+Na)^+$ and also confirmed by MS-MS of y_{7P} (cf. Figure 5).

Table 1 Nominal Molecular Masses and Adducts of Molecular Ions of Stilboflavin Peptides B1–B10 and Diagnostic Fragment Ions Determined by HPLC-ESI-MS Performed in Positive and Negative Ionization Mode (m/z)

Diagnostic ions[a]	Stilboflavins B									
	1	2	3	4	5	6	7	8	9	10
$(M+Na)^{+}$[b]	1909.2	1923.2	1923.1	1937.1	1937.2	1923.1	1951.2	1937.2	1951.1	1937.2
$(M+H)^{+}$[b]	1887.2	1901.2	1901.2	1915.2	1915.2	1901.1	1929.2	1915.2	1929.2	1915.1
$(M+2\ Na)^{2+}$[b]	966.1	973.1	973.0	980.1	980.0	973.0	987.1	980.1	987.1	980.0
$(M+2\ H)^{2+}$[b]	944.1	951.0	951.1	958.1	958.0	951.1	965.0	958.1	965.1	958.1
b_{13}[b]	1161.7	1175.6	1161.6	1175.7	1189.7	1175.7	1189.7	1175.7	1189.7	1175.7
y_{7P}[b]	726.4	726.5	740.5	740.6	726.5	726.5	740.6	740.5	740.5	740.4
$b_{19}^{\#}$[c]	1806.0	1819.9	1805.9	1820.0	1834.0	1819.8	1833.9	1820.0	1833.9	1819.9
$b_{18}^{\#}$[c]	1677.8	1692.0	1677.9	1692.1	1705.9	1692.0	1704.9	1692.0	1705.0	1691.9
$b_{17}^{\#}$[c]	1549.8	1563.8	1549.9	1563.9	1578.0	1563.9	1577.9	1563.9	1577.9	1563.8
$b_{16}^{\#}$[c]	1464.7	1478.8	1464.8	1478.7	1492.7	1478.8	1492.8	1478.7	1492.9	1478.7
$b_{15}^{\#}$[c]	1379.7	1393.7	1379.9	1393.8	1407.7	1393.8	1407.8	1393.8	1407.8	1393.7
$b_{13}^{\#}$[c]	1183.7	1197.7	1183.7	1197.7	1211.7	1197.7	1211.7	1197.6	1211.7	1197.6
$b_{12}^{\#}$[c]	1098.6	1112.6	1098.7	1112.6	1126.6	1112.6	1126.7	1112.7	1126.7	1112.6
$b_{11}^{\#}$[c]	1013.7	1027.5	1013.6	1027.6	1041.7	1027.7	1041.6	1027.6	1041.6	1027.6
$b_{10}^{\#}$[c]	956.5	970.5	956.7	970.6	984.6	970.6	984.6	970.6	984.6	970.6
$b_{9}^{\#}$[c]	871.5	885.6	871.6	885.6	899.6	885.5	899.6	885.5	899.6	885.5
$b_{8}^{\#}$[c]	n.d.	772.4	772.4	772.6	786.5	786.5	786.4	786.4	786.5	786.4
$b_{7}^{\#}$[c]	n.d.	n.d.	687.5	687.4	701.5	701.5	701.4	701.5	701.5	701.4
$(y_{7P}\text{-}H_2O)^{+}$[d]	708.3	708.4	722.4	722.3	708.4	708.4	722.3	722.4	722.4	722.4
6[d]	623.3	623.3	623.3	623.2	623.3	623.3	623.2	623.3	623.3	623.2
5[d]	495.2	495.2	495.2	495.2	495.2	495.2	495.2	495.2	495.2	495.2
4[d]	367.2	367.2	367.1	367.1	367.2	367.2	367.2	367.2	367.1	367.2
3[d]	282.1	282.2	282.1	282.1	282.2	282.2	282.1	282.1	282.1	282.1
2[d]	n.d.	197.1	n.d.	197.1	197.1	n.d.	197.1	197.1	197.1	n.d.
$(M)^{-}$[e]	1886.1	1900.1	1900.2	1914.2	1914.2	1900.2	1928.2	1914.1	1928.1	1914.1
$(M)^{2-}$[e]	943.0	950.0	950.1	957.1	957.1	950.1	964.1	957.1	964.1	957.0
y_{19N}^{f}	1758.1	1772.1	1772.1	1786.1	1786.1	1772.1	1800.1	1786.1	1800.1	1786.1
y_{17N}^{f}	1576.0	1589.9	1590.0	1603.9	1604.0	1590.0	1618.0	1604.0	1618.1	1604.0
y_{16N}^{f}	n.d.	1518.9	1518.9	1533.0	1533.0	1518.9	1547.0	1533.0	1547.0	1533.0
y_{15N}^{f}	1420.0	1433.8	1433.9	1447.9	1447.9	1433.9	1461.9	1447.9	1461.9	1448.0
y_{14N}^{f}	1349.0	1362.8	1362.9	1376.8	1362.8	1348.8	1376.9	1362.8	1376.9	1362.9
$(y_{14N}\text{–}NH_3)^{-}$[f]	1332.0	1344.8	1344.8	1358.8	1344.9	1330.8	1358.9	1344.8	1358.9	1344.9
y_{13N}^{f}	1220.8	1234.8	1234.8	1248.8	1234.8	1220.8	1248.8	1234.8	1248.9	1234.9
y_{12N}^{f}	n.d.	1149.7	1149.8	1163.8	1149.7	1135.8	1163.7	1149.7	1163.8	1149.8
y_{11N}^{f}	n.d.	1036.6	1050.7	1050.7	1036.7	n.d.	1050.6	1050.5	1050.7	n.d.
y_{10N}^{f}	n.d.	n.d.	n.d.	965.6	951.6	n.d.	965.5	n.d.	n.d.	n.d.
y_{9N}^{f}	n.d.	n.d.	n.d.	908.6	894.6	n.d.	908.6	n.d.	n.d.	n.d.
y_{8N}^{f}	n.d.	n.d.	n.d.	823.6	n.d.	n.d.	823.6	n.d.	n.d.	n.d.
y_{7N}^{f}	724.6	724.5	738.6	738.5	724.5	724.6	738.5	738.5	738.5	738.6

[a] Notation of fragments in Tables 1 and 2 based on References [32–34]; subscripts P and N of y-series refer to positive (P) and negative (N) ionization mode, respectively.
[b] Identified via on-line HPLC-MS in the positive-ion mode.
[c] Identified via MS-MS of $(M+Na)^{+}$; the $b^{\#}$ series are accompanied by the corresponding $a^{\#}$ series (see Figure 5(B)).
[d] Identified via MS-MS of y_{7P}; **6**, (H-Pro-Val-Aib-Aib-Gln-Gln)$^{+}$; **5**, (H-Pro-Val-Aib-Aib-Gln)$^{+}$; **4**, (H-Pro-Val-Aib-Aib)$^{+}$; **3**, (H-Pro-Val-Aib)$^{+}$; **2**, (H-Pro-Val)$^{+}$.
[e] Identified via on-line HPLC-MS in the negative-ion mode.
[f] Identified via MS^3 from $(M)^{-}$; n.d., not detected.

Table 2 Nominal Masses and Adducts of Molecular Ions of Stilboflavin Peptides A1–A7 and Diagnostic Fragment Ions Determined by HPLC-ESI-MS Performed in Positive and Negative Ionization Mode (m/z)

Diagnostic ions[a]	Stilboflavins A						
	1	2	3	4	5	6	7
$(M+Na)^{+}$[b]	1910.2	1924.1	1924.2	1938.1	1938.2	1938.1	1952.2
$(M+H)^{+}$[b]	1888.2	1902.2	1902.2	1916.1	1916.2	1916.1	1930.2
$(M+2Na)^{2+}$[b]	966.5	973.6	973.5	980.6	980.6	980.5	987.5
$(M+2H)^{2+}$[b]	944.5	951.5	951.6	958.5	958.6	958.5	965.5
b^{b}_{13}	1161.7	1175.5	1161.7	1175.7	1189.7	1175.8	1189.6
y^{b}_{7P}	727.5	727.4	741.5	741.4	727.5	741.5	741.5
b^{c}_{12}	1076.7	1090.6	1090.6	1090.6	1104.6	1090.7	1104.6
b^{c}_{11}	991.6	1005.6	1005.6	1005.6	1019.6	1005.6	1019.5
b^{c}_{10}	934.5	948.5	948.5	948.5	962.5	948.6	962.5
b^{c}_{9}	849.5	863.5	863.5	863.5	877.5	863.5	877.5
b^{c}_{8}	750.4	750.4	764.4	750.4	764.4	750.4	764.4
b^{c}_{7}	665.3	665.4	679.4	665.3	679.4	665.4	679.4
b^{c}_{6}	n.d.	537.3	551.4	537.3	551.3	537.4	551.3
b^{c}_{5}	n.d.	466.3	n.d.	466.2	466.3	466.3	466.2
$(y_{7P}\text{-}H_2O)^{+}$[d]	709.4	709.4	723.4	723.4	709.5	723.4	723.4
6[d]	624.3	624.3	624.3	624.4	624.4	624.3	624.3
5[d]	496.3	496.3	496.3	496.3	496.4	496.3	496.3
4[d]	367.2	367.2	367.2	367.3	367.3	367.3	367.2
3[d]	282.2	282.1	282.2	282.2	282.3	282.2	282.2
2[d]	n.d.	n.d.	197.2	197.2	197.2	n.d.	197.2
$(M)^{-}$[e]	1887.2	1901.2	1901.1	1915.1	1915.2	1915.2	1929.1
$(M)^{2-}$[e]	943.6	950.6	950.6	957.5	957.6	957.6	964.6
$(M\text{-}H_2O)^{-}$[f]	1869.1	1883.2	1883.2	1897.2	1897.2	1897.2	1911.1
y^{f}_{19N}	1759.1	1773.1	1773.1	1787.1	1787.1	1787.1	1801.0
y^{f}_{17N}	1577.0	1591.0	1589.9	1604.9	1605.0	1604.9	1619.0
y^{f}_{16N}	1506.0	1519.9	1520.0	1533.9	1533.9	1533.9	1548.0
y^{f}_{15N}	1420.9	1435.0	1434.9	1448.9	1449.0	1444.9	1462.9
y^{f}_{14N}	1349.9	1363.9	1349.9	1377.8	1363.9	1377.7	1377.8
$(y_{14N}\text{-}NH_3)^{-}$[f]	1332.9	1346.9	1333.9	1360.8	1346.8	1360.8	1360.8
y^{f}_{13N}	1221.8	1235.9	1221.7	1249.8	1235.8	1259.8	1249.8
y^{f}_{12N}	1136.7	1150.8	n.d.	1164.7	1150.8	1164.7	1164.7
y^{f}_{11N}	n.d.	1037.7	n.d.	1051.6	n.d.	n.d.	1051.6

[a] see Table 1.
[b] Identified via on line HPLC-MS in the positive-ion mode.
[c] Identified via MS-MS of b_{13}.
[d] Identified via MS-MS of y_{7P}; **6**, (H-Pro-Val-Aib-Aib-Glu-Gln)$^{+}$; **5**, (H-Pro-Val-Aib-Aib-Glu)$^{+}$; **4**, (H-Pro-Val-Aib-Aib)$^{+}$; **3**, (H-Pro-Val-Aib)$^{+}$; **2**, (H-Pro-Val)$^{+}$.
[e] Identified via on-line HPLC-MS in the negative-ion mode.
[f] Identified via MS^3 from $(M)^{-}$; n.d., not detected.

On-line HPLC ESI-MS of SF A peptides revealed that the majority of these acidic peptides are distinguished from neutral SF B peptides by exchange of Gln^{18}/Glu^{18} (cf. Figure 4). Consequently, corresponding numbers for SF B and SF A peptides differing only by Gln^{18}/Glu^{18} are used; i.e. SF B1, B2, B4, B5 and B7 correspond to SF A1, A2, A4, A5 and A7 (cf. Figure 4). An exception to this correlation is the peptide eluting just before SF A4 and named SF A3 (cf. Figure 1(b)). It is distinguished from SF B3 by Gln^{18}/Glu^{18} exchange as well as Ala^{6}/Aib^{6} exchange. The minor sequences of SF A peptides with an exchange of Leuol/Ileol (less than 6%), in contrast to SF B peptides, were not

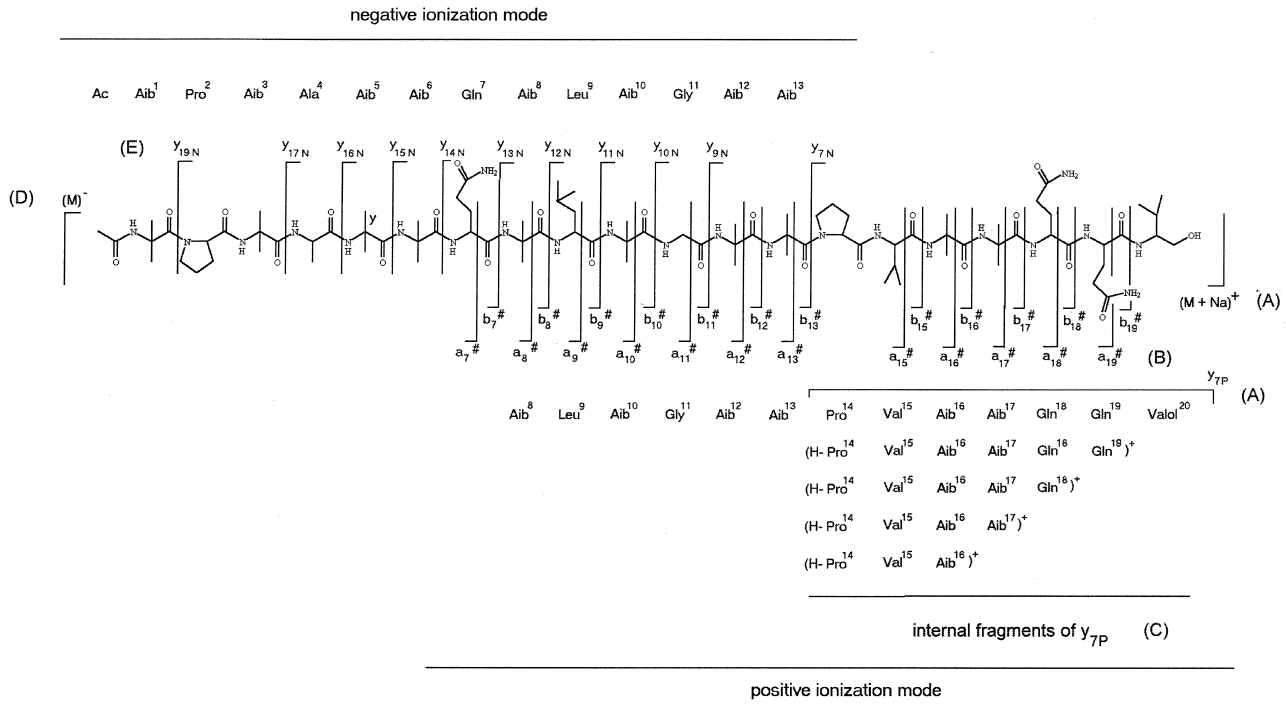

Figure 5 Scheme of sequencing, exemplified with SF B5, using HPLC-ESI-MS^n in positive and negative ionization mode. Mass fragments were generated by (A) positive-ion ESI-MS; (B) MS-MS of $(M + Na)^+$; (C) MS-MS of y_{7p}; (D) negative-ion ESI-MS; (E) MS^3 of $(M)^-$. The dipeptides Pro^2-Aib^3 and Pro^{14}-Val^{15} were identified via GC-MS of SF B methanolysates. Notation of fragments according to the literature [25,32,33]; # refers to sodium adducts and subscript N to negative ionization mode.

determined since SF A peptides are less satisfactorily resolved by HPLC (cf. Figure 1(b)). Note also that the relative amounts of corresponding SF B and SF A peptides are more or less different (cf. Figures 1 and 4).

The corresponding nominal masses of adducts of molecular ions and diagnostic fragment ions used for sequence determination of SF B and SF A are compiled in Tables 1 and 2, respectively. Note that not all observed fragment ions are listed. The minor peaks not denoted in Figure 1(a) and (b), the sum of which accounts for differences to 100% in Figure 4, are still mixtures of co-eluting peptides. On-line sequencing of these minor compounds was ambiguous according to low ion abundances and interfering fragment ions.

It is of interest to note that sequences of SF B 4, B7 and B9 are identical to those reported for hypelcins A-III, A-I, A-V [29,30], whereas SF A4 and A7 are identical to hypelcins B II and B I [31]. This is supported by comparison of the HPLC-elution profiles of SF B peptides and of a mixture of hypelcins A and B using the same HPLC conditions (Figure 7). As can be seen, the above mentioned peptaibols show identical retention times.

It is also worth noting that other peptaibols with *C*-terminal Leuol/Ileol exchange are the 18-residue hypomurocins from *Hypocrea muroiana* [37], the 20-residue hypelcins A and B from *H. peltata* [29–31], and the 18-residue trichokinidins from *Trichoderma harzinanum* [40]. The 10-residue trichorozins from *T. harzinanum* shown an Valol/Leuol exchange [41] and the 11-residue trichorovins from *T. viride* are mixtures of peptaibols with *C*-terminal Valol, Leuol or Ileol [42] (amino alcohols are considered as residues).

The SF A, B, and C peptides exert weak antibiotic activity against *B. subtilis* and *S. aureus*, but are not active against *E. coli*, and also cause hemolysis of sheep erythrocytes (Table 3).

Inspection of the sequences presented in Figure 4 shows that, in accordance with other peptaibols, the stilboflavins exhibit single or multiple amino acid and amino alcohol exchange at certain positions. Consequently, such microheterogeneous mixtures might be also considered as 'natural peptaibol

libraries' or 'metabolite libraries' [43,44]. Such minor structural changes can be recognized already by relatively simple abiotic selectors such as octadecylsilyl [45,46] or fluorocarbon [36,47] stationary phases used in liquid chromatography. Thus, it should be worth investigating the interaction of a library of natural (or synthetic) peptaibols with biological systems. This should reveal structure–bioactivity relationships governed by minor structural features like exchange of acidic, homologous or isomeric residues. Such work has already been started with the chemical synthesis of alamethicin libraries

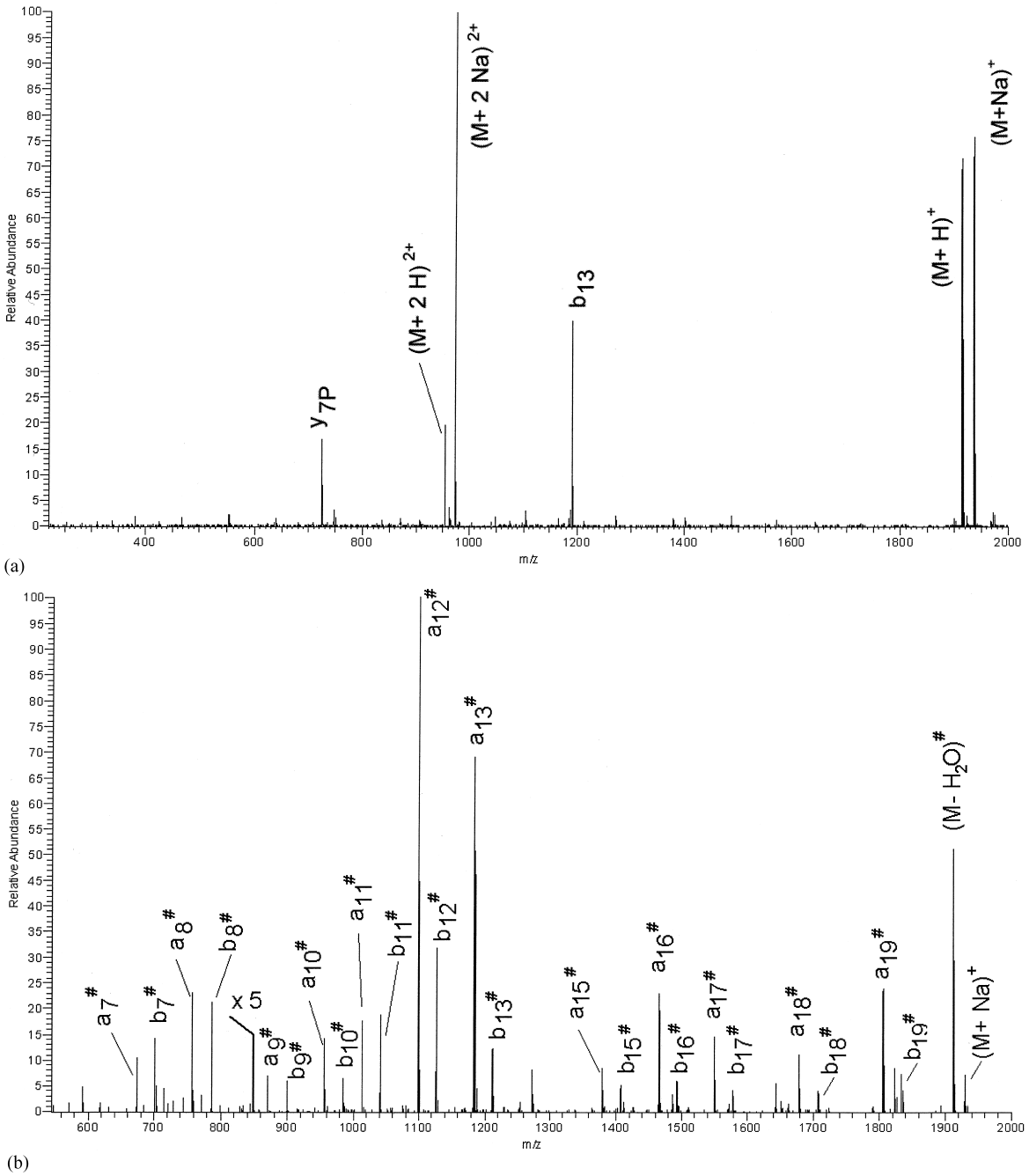

Figure 6 (a) On-line HPLC-ESI-MS of SF B5 positive-ion mode; (b) positive-ion ESI-MS-MS of $(M + Na)^+$ (m/z 1937.2); (c) negative-ion ESI-MS of SF B5; (d) negative-ion ESI-MS^3 of y_{19N} (m/z 1786.1); for chromatography and mass spectrometry see 'Experimental procedures' section; for corresponding nominal molecular masses see Tables 1 and 2.

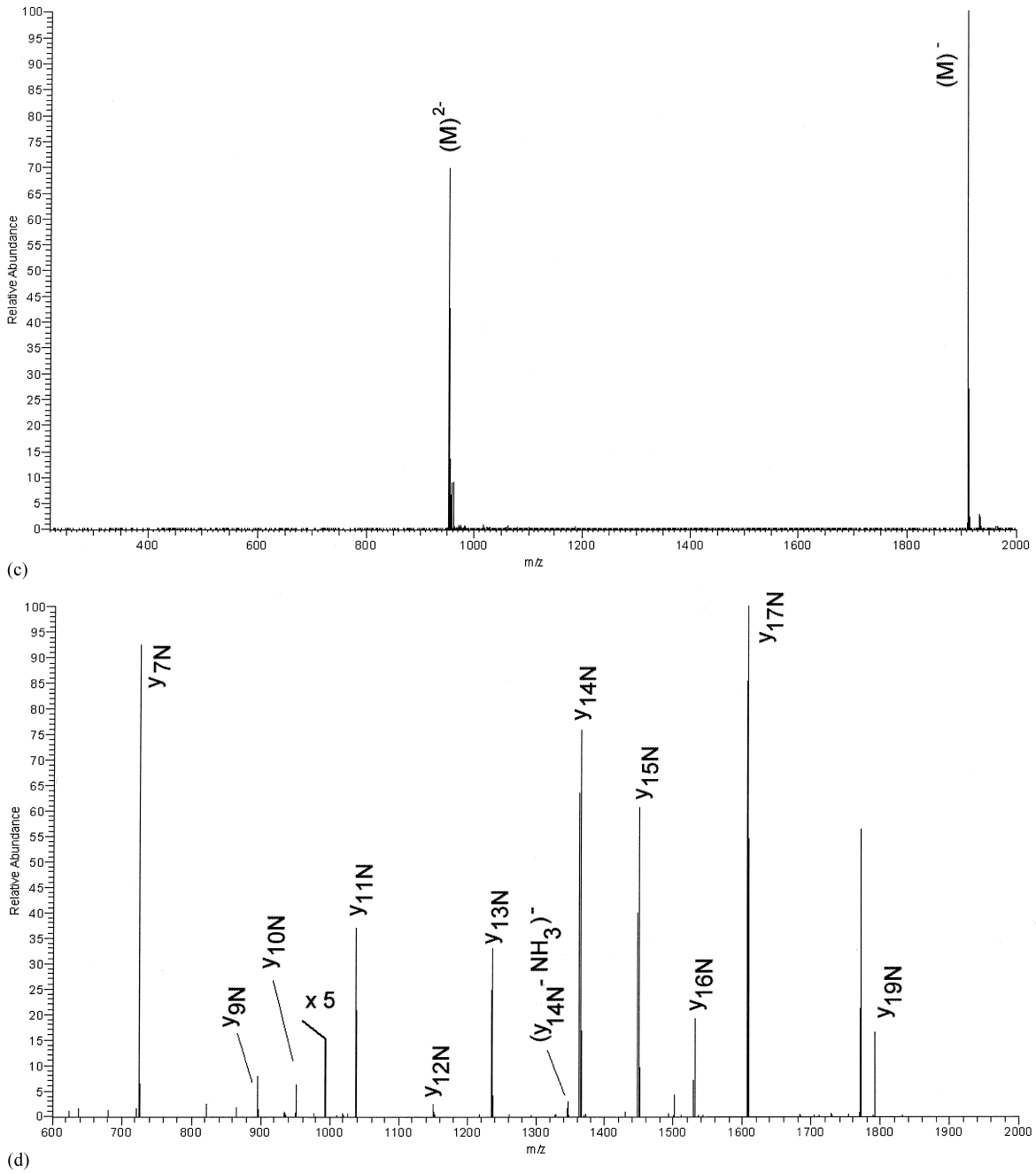

Figure 6 (*Continued*)

[48,49]. Notably, problems encountered with the classical coupling chemistry of sterically constrained Aib residues were recently surmounted using Fmoc amino acid fluorides together with automated stepwise solid phase peptaibol synthesis [48,50].

Production of peptaibol antibiotics by *Stilbella* is also of interest since peptaibol producing species such as *Stilbella aciculosa* have been recognized as potential biocontrol fungi against phytopathogenic fungi like *Rhizoctonia solani* [51]. As to their mode of action there is evidence that peptaibols such as the 19-residue harzianins TA and TB act synergistically with cellulolytic enzymes [10] and inhibit the β-glucanase of plant pathogens like *Botrytis cinerea* [11]. These features make peptaibol producing fungi, in

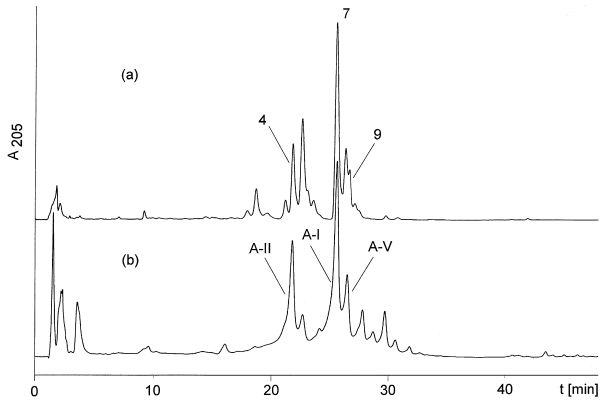

Figure 7 HPLC of (a) stilboflavin B peptides compared to (b) the natural mixture of neutral and acidic hypelcins A and B under the same chromatographic conditions (use of neutral eluent A(I)/B(I), see 'Experimental procedures' section). Note that peak shape in (b) is the result of the presence of both neutral and acidic hypelcins in the analyte. SF B4, B7, and B9 correspond to hypelcin A-II, A-I and A-V.

general, candidates for the biocontrol of plant pathogens.

In conclusion, screening of filamentous fungi for peptaibol production, the search for novel structurally and conformationally diverse peptaibols (peptaibiotics) and their testing for bioactivities not yet known will still continue to be a challenging topic.

Table 3 Antibacterial and Hemolytic Activities ($n = 2$) of Stilboflavin (SF) Groups A/B/C in Comparison to the 20-residue Peptaibols Paracelsins (PC) [38] and 16-residue Antiamoebins (AAM) [25][a]

	B. subtilis	*S. aureus*	Hemolysis
SF A/B/C (10 μl)	0/0/1	0/4/0	0/0.5/0
SF A/B/C (20 μl)	4.5/5/3	1/9.5/6	0/4/2
SF A/B/C (40 μl)	7/9/8	5/13/11	0/8/5
PC (20 μl)	7	12	8
AAM (20 μl)	4	12	2

[a] $c = 1\%$ (MeOH); inhibition zones (mm) of bacteria were measured after 6 h/4°C and 16 h/37°C (*S. aureus* and *E. coli*) and 16 h/30°C (*B. subtilis*); hemolysis zones of erythrocytes (mm) were measured after 6 h/4°C and 16 h/37°C; diameter of discs (6 mm) were substracted from inhibition zones measured.
E. coli was not inhibited.

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