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Structures of Trichovirins II, Peptaibol Antibiotics from the Mold *Trichoderma viride* NRRL 5243

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Abstract: From the culture broth of the mold *Trichoderma viride* NRRL 5243 a mixture of polypeptides, named trichovirins (TV), could be isolated and purified by chromatography on XAD-2 adsorber resin and Sephadex LH-20 gel. Chromatography on silica gel using chloroform/methanol 8:2 as eluent provided a mixture of peptides named TV I. Subsequent elution with chloroform/methanol 1:1 yielded a second group of peptides named TV II. That group could be separated into individual components by repetitive HPLC on an octadecylsilyl and a fluorocarbon stationary phase. The sequences of 12 peptides of TV II could be determined by electrospray ionization tandem mass spectrometry of isolated peptides and gas chromatography-mass spectrometry of methanolysates. The *N*-termini of the 18-mer peptides are acetylated and the *C*-termini consist of leucinol. Owing to the presence of α-aminoisobutyric acid (Aib) residues and the bactericidal and hemolytic activity, the peptides belong to the family of peptaibol antibiotics.

Keywords: α-aminoisobutyric acid; electrospray ionization mass spectrometry; gas chromatography-mass spectrometry; isovaline; peptide antibiotics; sequence analysis; trichotoxin

INTRODUCTION

In 1972 the isolation and preliminary characterization of two peptide mycotoxins, named trichotoxin A and trichotoxin B, from *Trichoderma viride* Pers. ex Fr. strains NRRL 5242 and NRRL 5243, respectively, was reported [1]. It was already realized that these peptides, among proteinogenic AA, also contained non-protein Aib. Trichotoxin B from *T. viride* strain NRRL 5243 was originally reported to be similar in chemical properties to trichotoxin A, however, the yield of material that could be isolated was insufficient to determine its characteristics [1].

Trichotoxin A later turned out to consist of two microheterogeneous groups of 18-mer peptides named trichotoxin A-40 and trichotoxin A-50 according to their R_f-values on TLC [2]. These two groups are distinguished mainly by an exchange of Glu and Gln [2–4] resulting in acidic (A-40) and neutral (A-50) peptides. Since the *N*-acetylated

Abbreviations: AA, amino acid(s); AAA, amino acid analysis; Ac, acetyl; Ac_2O, acetic anhydride; Aib (U), α-aminoisobutyric acid; CID, collision induced dissociation; DCM, dichloromethane; DSM, Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany; EDC, 1-(3-dimethyl-aminopropyl)-3-ethyl-carbodiimide hydrochloride; EI, electron impact; ESI, electrospray ionization; GC, gas chromatography; Gln (Q); HOBt, N-hydroxybenzotriazole; HPLC, reversed phase high-performance liquid chromatography; Iva, isovaline; Leu (L); Leuol, leucinol; MeCN, acetonitrile; MeOH, methanol; MPLC, medium pressure liquid chromatography; MS, mass spectrometry; MS-MS, tandem mass spectrometry; OMe, methyl ester; PFPAA, pentafluoropropionic acid anhydride; Pro (P); 2-PrOH, 2-propanol; SIM, selected ion monitoring; TCM, trichloromethane; TDM (reagent), chlorine/4,4′-bis(dimethylamino)diphenylmethane; TFAA, trifluoroacetic acid anhydride; TLC, thin-layer chromatography; Valol, valinol; Vxx, Val or Iva; Z, benzyloxycarbonyl.

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peptides contain several Aib-residues, a *C*-terminal bonded L-Valol and exhibit antibiotic properties, they belong to the family of peptaibol antibiotics [5].

Besides their antimicrobial activities, peptaibols attracted much attention owing to their capability of forming voltage-gated ion-channels in lipid membranes [6–8]. This is attributed to the formation of helical structures of amphipathic natural peptaibols [9], or Aib-rich synthetic segments [10], as a result of the restricted conformational freedom of Aib-residues.

Further, it was postulated that the parallel formation of peptaibols and hydrolytic enzymes by species of *Trichoderma* might play an important role in its antagonistic actions against fungal pathogens [11].

Since production of Aib-peptides is very common in *Trichoderma* species as well as many other genera of filamentous fungi [12,13], screening of microorganisms for these peptides and the search for new structures and bioactivities is of continuous interest. Sequence determination has already led to structures on which the classical names peptaibols or peptaibophols cannot be applied. Thus, for peptides containing Aib and exerting interesting bioactivities the comprehensive name 'peptaibiotics' has been suggested [13–15] also comprising specifically defined groups such as lipopeptaibols [16] and aminolipopeptides [17].

From the mycelium of a culture of *T. viride* NRRL 5243 grown statically in Fernbach flasks in a complex medium, we could isolate sufficient peptidic raw material for further purification by liquid chromatography. The structures of seven major 14-mer peptides of the microheterogeneous mixture, comprising at least 21 peptides, could be determined [14,18,19]. The originally proposed name trichotoxin B [1] was changed by us to trichovirins I since the sequences of these 14-mer peptides were different from those of the 18-mer trichotoxins A-40 and A-50 [3,4] isolated from *T. viride* strain NRRL 5242.

In order to increase the yield of trichovirins (TV) we carried out large scale submerged fermentation of *T. viride* NRRL 5243. It was realized that under these conditions a second group of microheterogeneous peptides was produced which we named TV II.

Here we report on the isolation and sequence determination of the 18-mer peptides of TV II and on its antibiotic and hemolytic properties.

EXPERIMENTAL

Material and Methods

Solvents were of gradient grade and from Merck (Darmstadt, Germany). The synthetic dipeptides serving as standards, i.e. Val-Leu, Gly-Val, Glu-Val, Pro-Leu were from Sigma (Deisenhofen, Germany) and Leu-Val was from Bachem (Heidelberg, Germany). Reference amounts of Gly-DL-Iva-OMe and Leu-DL-Iva-OMe were synthesized in our laboratory using the mixed anhydride method (isobutyl chloroformate) from Z-Gly-OH or Z-Leu-OH (from Bachem) and DL-Iva-OMe, followed by hydrogenolytic cleavage of the Z-protecting group. The protected dipeptides were characterized by GC-MS. DL-Iva was synthesized in our laboratory by the Strecker procedure according to the literature [20], and DL-Iva-OMe was synthesized by heating of DL-Iva in 4 M HCl in MeOH. The reference dipeptides Aib-Val-OMe and Val-Aib-OMe were generated by acidic methanolysis [21] of paracelsin A [4] and Glu-D-Iva-OMe by methanolysis of antiamoebin [5]. For AAA, an AA standard solution (no. AA-S-18) (Sigma) was used and appropriate amounts of Aib (Sigma), DL-Iva and L-Leuol (Sigma) were added. For TLC pre-coated plates with silica gel 60 F 254, thickness 0.25 mm (Merck), were used. Peptaibols were detected by spraying with water and, after drying, with TDM reagent; eluent, TCM-MeOH-AcOH-H_2O (65:25:4:3); ratios of solvent mixtures are by volume throughout the text. 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 silica gel 60, particle size 63–200 μm (Merck), were used.

For malt medium, 30 g of malt extract (Serva) and 3 g of soy pepton (Serva) were dissolved in 1 l demineralized water (final pH 6–6.5). For preparing malt agar medium, 15 g of agar (Fluka, Buchs, Switzerland) was added to the malt medium. For potato dextrose agar, 39 g of potato dextrose agar (Merck) was dissolved in 1 l demineralized water. For standard-1 agar medium, 39 g of standard-1 agar medium (Merck) was dissolved in 1 l demineralized water.

Sheep blood Petri dishes comprised of ready to use Columbia agar plates containing 7–10% defibrinized sheep blood (Oxoid, Wesel, Germany).

Instrumental

For HPLC a HP 1100 instrument (Hewlett-Packard, Waldbronn, Germany) comprising a binary pump, autosampler and UV-detector of the same series and HP ChemStation were used. HPLC columns employed were: HPLC column (A), Superspher® 100 RP-18, 250 × 4 mm i.d., 4 μm particle size (Merck); HPLC column (B), Spherisorb ODS-2, 250 × 8 mm i.d., 3 μm particle size (Grom, Herrenberg, Germany); and HPLC column (C), Fluofix® 1 EW 425, 250 × 4 mm i.d., 5 μm particle size (Maisch High Performance LC, Ammerbuch, Germany).

GC-MS instrument (A) was a HP 6890 (Hewlett-Packard) with mass selective detector equipped with HP-5MS (crosslinked 5%-diphenyl–95% dimethyl siloxane) column 30 m × 0.25 mm i.d., film thickness 0.25 μm (Hewlett-Packard); GC-MS instrument (B) was a Shimadzu 17A/QP 5000 (Shimadzu, Kyoto, Japan) equipped with a Chirasil-L-Val column 25 m × 0.25 mm i.d. (Chrompack, Middelburg, The Netherlands). EI mass spectra on instruments (A) and (B) were recorded at an ionization energy of 70 eV.

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

For ESI-MS a LCQ™ instrument (Finnigan MAT, Bremen, Germany) was used. Peptaibols were analysed by direct injection with a syringe pump, or by on-line coupling of the HP 1100 HPLC and the LCQ instrument. The sheath gas used was nitrogen (purity > 99.5%) from a nitrogen generator Model 75-72 (Whatman, Balston Inc., Haverhill, MA, USA). Spray voltage was 5.3 kV, capillary temperature 230°C, capillary voltage 31 V and tube lens offset 35 V. The collision gas was helium, relative CID energy was set at 25%.

Fermentation Procedure

Trichoderma viride NRRL 5243 was obtained as lyophilized culture from the US Department of Agriculture, Agricultural Research Service, Northern Regional Research Laboratory (NRRL), Peoria, IL, USA. The lyophilisate was suspended in sterile water (0.5 ml) and transferred to Petri dishes (9.5 cm diameter) with malt agar medium. After 10 days at room temperature intensive growth of the mold was observed. Agar discs (1 cm diameter) were used for the inoculation of 40 Erlenmeyer flasks (2 l) each containing 400 ml of malt medium. The flasks were shaken at 100 rpm at ambient temperature on a Model G 25 incubation shaker (New Brunswick Scientific, Edison, NJ, USA). For monitoring peptaibol production aliquots (20 ml) of filtered culture broth were passed through Sep-Pak® C-18 cartridges (Waters, Milford, USA) and the peptides adsorbed eluted with MeOH. The eluate was evaporated to dryness and the residue was dissolved in MeOH (0.5 ml). Aliquots of 20 μl were investigated by TLC and peptaibols visualized by spraying with water and TDM-reagent; R_f (TV I) 0.88 and R_f (TV II) 0.67; the internal standard peptaibol paracelsin [4] had R_f 0.71.

Isolation of Trichovirins II

After shaking for 5 days, the culture broth (16 l) was filtered and passed through a MPLC-column (38 × 3.7 cm) packed with XAD-2. The resin was washed with water (1 l) and 40% MeOH (0.5 l). The peptides adsorbed were eluted with a linear gradient from 75% to 100% MeOH at a flow rate of 1.7 ml/min. Elution of peptides was monitored by TLC. Fractions containing peptaibols were combined and evaporated to dryness yielding 2.03 g of crude peptide mixture. The residue was dissolved in MeOH and subjected to chromatography on Sephadex LH-20 column (40 × 10 cm). Elution was monitored by TLC, appropriate fractions were combined and evaporated to dryness, yielding 1.34 g of a pale yellow powder consisting of two groups of peptides named TV I and TV II. The mixture was dissolved in TCM/MeOH (8:2) and subjected to MPLC on silica gel 60 column (38 × 3.7 cm). Using TCM/MeOH (8:2) as eluent, only TV I (650 mg) was eluted. Subsequent elution with TCM/MeOH (1:1) provided TV II (420 mg) after evaporation. Peptides of TV II were analysed by analytical HPLC on column A, and microgram amounts of peptides were isolated by repetitive HPLC of TV II peptides on column B (10 × 100 μl of 1% solutions in MeOH). The same binary gradient program was used for HPLC using columns A and B. Eluent A: MeOH/water/MeCN 39:22:39; eluent B: MeOH/MeCN 50:50; gradient program, 0 min 0% B, 5 min 10% B, 10 min 25% B, 20 min 30% B, 25 min 45% B, 30 min 50% B, 35 min 100% B, 40 min 100% B; temperature 40°C; flow rate 0.8 ml/min (column A) and 2.7 ml/min (column B). Elution of peptides was monitored at 220 nm and peptide fractions numbers 1–6 were collected manually. These peptide fractions were investigated by on-line ESI-MS on HPLC column C using the following conditions: eluent A: MeOH/water/MeCN 39:22:39; eluent B: MeOH/MeCN 50:50; gradient

program was 0 min 30% B, 30 min 100% B, 37 min 100% B; temperature 40°C, flow rate 0.65 ml/min.

Chiral AAA by GC-SIM-MS

Isolated peptaibol TV II 1–6 peptides (20–50 μg) were hydrolysed in 6 M HCl (200 μl) at 110°C for 24 h. After evaporation to dryness in a stream of nitrogen, 2.5 M HCl in 2-PrOH (100 μl) was added and the mixture was heated for 1 h at 110°C. After evaporation to dryness in a stream of nitrogen, acylation was carried out with PFPAA (for protein AA and Leuol) or Ac_2O (for Iva) (50 μl) in DCM (200 μl). Solvents were removed with a stream of nitrogen, DCM (200 μl) was added, samples were subjected to GC-MS instrument (B) and compared to a standard. Carrier gas was helium, split mode; temperature program: 75°C, 8.5 min, 2.5°C/min, 100°C, 3.5°C/min, 145°C, 6.5°C/min, 190°C, 11 min; pressure program: 5 kPa, 8.5 min, 0.5 kPa/min, 7.0 kPa, 0.3 kPa/min, 10.9 kPa, 0.6 kPa/min, 15 kPa, 1 min.

Dipeptide Analysis by GC-MS

Peptides (ca. 50 μg) in 4 M HCl in MeOH (200 μl) were heated for 8 h at 110°C. Reagents were removed in a nitrogen stream and the resulting dipeptide methyl esters were trifluoroacylated with TFAA (50 μl) in DCM (200 μl). After 1 h at 20°C reagents were removed in a stream of nitrogen and DCM (50 μl) was added. For GC-MS instrument A, carrier gas helium, and the following temperature program was used: 80°C, 1 min, 15°C/min, 140°C, 8°C/min, 160°C, 2 min, 25°C/min, 260°C, 2 min, 10°C/min, 280°C, 2 min. For the determination of the *N*-termini of the peptides the *N*-acetyl dipeptide methyl esters (Ac-Aib-Gly-OMe) released on partial methanolysis were directly analysed by GC-MS.

Antibiotic and Hemolytic Activity

Bacteria (*Bacillus subtilis* DSM 347, *Staphylococcus aureus* DSM 797, *Escherichia coli* DSM 498) were incubated for 24 h at 37°C in a test tube containing potato dextrose agar (Merck) and then suspended in 10 ml of sterile water. Aliquots of 1 ml were used to inoculate 25 ml sterile standard-1 medium (Merck) at 45°C on Petri dishes. Paper discs of 6 mm diameter (Schleicher & Schüll, Dassel, Germany) were distributed on the Petri dishes, aliquots (10, 20 and 50 μl) of a 1% methanolic solution of TV II were transferred onto the paper discs and the dishes were incubated for 12 h at 4°C to allow the peptaibols to diffuse into the agar. Then, the dishes were incubated at 37°C for 24 h and the inhibition zones were measured. Hemolysis of erythrocytes was tested on ready to use Petri dishes with sheep blood agar (Oxoid, Wesel, Germany). Aliquots (10, 20 and 50 μl) of a 1% methanolic solution of TV II were applied on 6 mm diameter filter discs which were spread out on the agar. The dishes were incubated at 37°C for 12 h and hemolysis zones were measured. Paracelsin was used as standard.

RESULTS AND DISCUSSION

As described for other peptaibols, submers fermentation of the fungus was followed by isolation of the peptide mixture from the culture broth by adsorption on XAD polystyrene resin. Further, purification was performed by chromatography on Sephadex LH-20. It was realized that under submers conditions two groups of microheterogeneous peptaibols were produced, i.e. 14-mer peptides named TV I and 18-mer peptides named TV II. Structures of TV I peptaibols have been reported previously [14,18,19]. Both groups could be separated and isolated by consecutive silica gel chromatography using TCM/MeOH 8:2 and 1:1, respectively, as eluents. TV I peptides were first eluted and TV II peptides were second eluted. Thus, use of only the first eluent would have resulted in loss of TV II peptides.

From a total of 16 l culture broth, 420 mg of the microheterogenous TV II peptide mixture, uniform on TLC, could be isolated by chromatography following the monitoring and isolation procedures described in 'Experimental'.

For the assignment of the configuration of D-Iva the *N*-acetyl 2-Prp esters of a total hydrolysate of TV II peptides were analysed by GC-SIM-MS on instrument (B). Enantiomers of Ac-DL-Iva-O-(2)-Prp, are baseline resolved on Chirasil-L-Val, in contrast to TFA- or PFP-DL-Iva-O-(2)-Prp esters. No L-Iva was detected. Thus, the D(=*R*)-configuration of Iva [22] in TV peptides was established. The L-configuration of the other chiral AA and Leuol and ratios of isomeric AA were determined by analysing the *N*-PFP-AA-(2)-Prp esters and *N*(*O*)-PFP-Leuol on Chirasil-L-Val.

The elution profile of TV II peptides from the analytical HPLC column A is shown in Figure 1. Repetitive chromatography (HPLC column B) provided six fractions named TV II 1–6; for HPLC see Figure 2. The peptides of these fractions could not

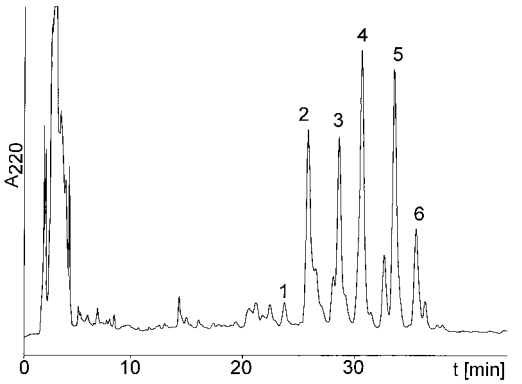

Figure 1 Analytical HPLC (fingerprint) of trichovirins II on column A. For chromatographic conditions of Figures 1–3 see 'Experimental'.

be further purified on this column by varying the chromatographic conditions. However, HPLC of the individual peptaibol fractions 1–6 on a special fluorocarbon-coated silica [23] (HPLC column C) made possible the further separation of peptides (see inserts of Figure 2). Therefore, on-line HPLC/ESI-MS using HPLC column C was used for the investigation of isolated peptides 1–6. As exemplified with the ESI-MS of TV II 5 peptide (Figure 3a; for structure see Figure 4) in all cases sodiated molecular ions were the most abundant, and characteristic fragment ions of the b_2–b_5 and b_7–b_{12} acylium ion series were observed. The b_6 fragment ion cannot be seen in the MS (but in MS-MS) as a result of the stability of the Gln^6-AA^7 bond. Typically, characteristic fragment ions of the b_{12}- and y_6-series arise from cleavage of the labile Aib^{12}-Pro^{13} bonds [4]. The fragment ions b_{12} and y_6 were subjected to MS-MS and sets of diagnostic product ions of the b_4–b_{11} series, including the b_6 fragment and internal fragments of y_6, were generated (Figure 3b,c) and provided sequence information of AA positions 4–12. The b_1 fragment ions, representing *N*-terminal acetylated Aib in all peptides, were not detected under the conditions of positive ESI-MS. The *N*-termini, however, were determined as Ac-Aib^1-Gly^2-OMe directly after acidic partial methanolysis in all peptides by GC-MS [*m*/*z* (%) 100 (100), 157 (73)] of the isolated TV II peptides 1–6. Partial methanolysis of individual peptides [24] was also used for the assignment of the positions of the isomeric AA Val and Iva which could not be distinguished by ESI-MS. Methanolysis of the TV peptides, followed by trifluoroacetylation of the dipeptide methyl esters released, furnished a mixture of TFA dipeptide methyl esters which were resolved on GC. Those containing isomeric AA, or being otherwise of diagnostic interest, were characterized by comparison with retention times of standard dipeptides and by their fragmentation pattern on MS. Analogously, reference dipeptides not available were generated by methanolysis from peptaibols of known sequences. For retention times and mass fragments of selected dipeptide derivatives used for sequencing see Table 2. Under the conditions of EI-MS at 70 eV most

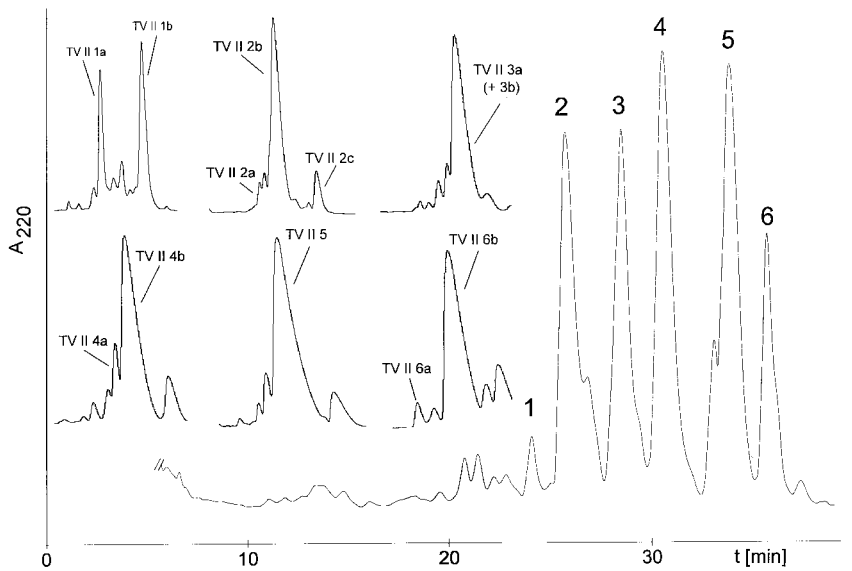

Figure 2 Semipreparative HPLC of trichovirins II on column B. Isolated peptaibol fractions 1–6 were subjected to column C. For resulting chromatograms see inserts.

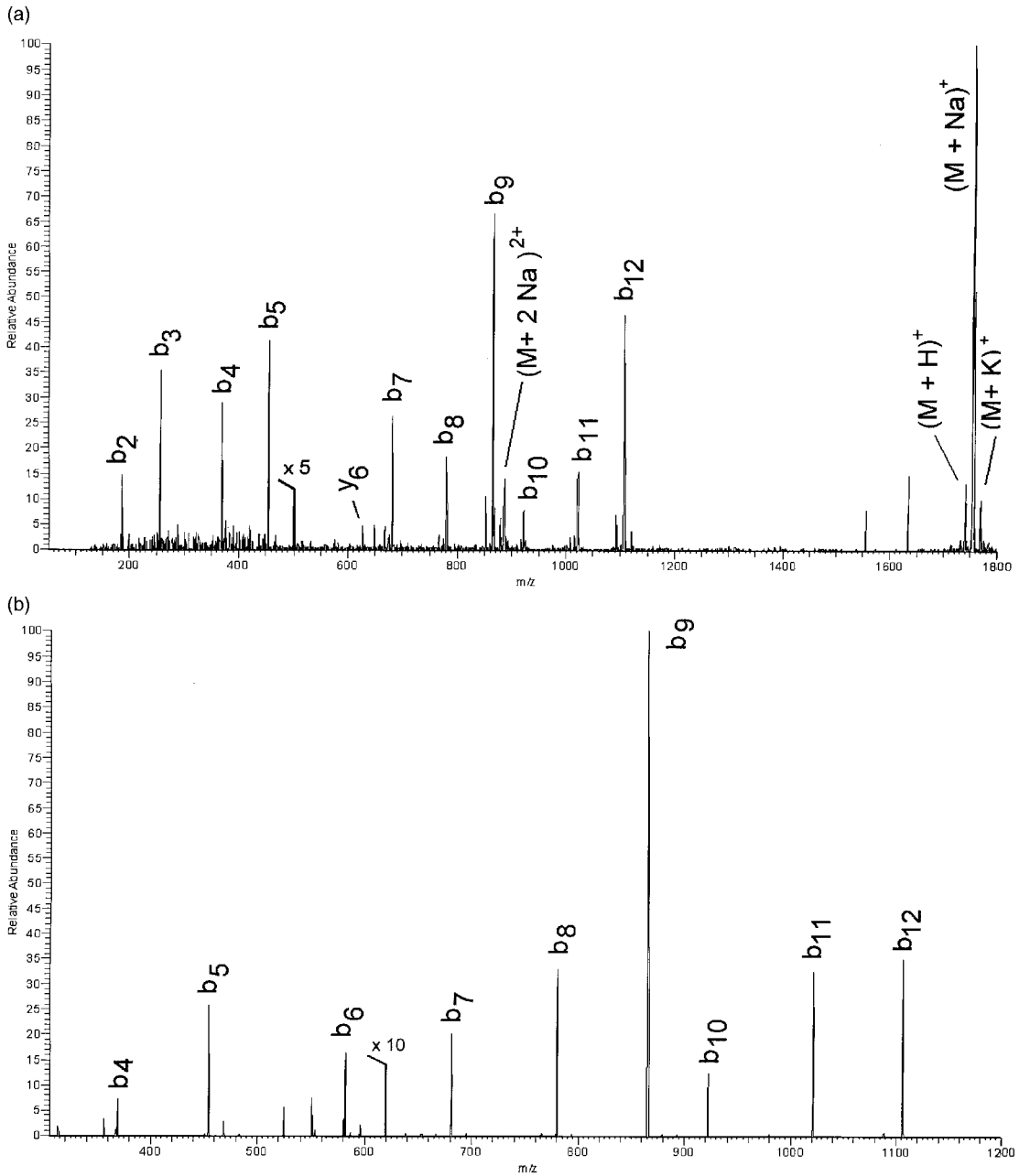

Figure 3 (a) On-line HPLC (column C, see insert in Figure 2) and ESI-MS of TV II 5. (b) ESI-MS-MS of the b_{12} fragment of TV II 5 at m/z 1106. (c) ESI-MS-MS of the y_6 fragment of TV II 5 at m/z 626. For mass spectrometry see 'Experimental'; for corresponding nominal masses and abbreviations see Table 1.

abundant fragments resulted from the cleavage of C^{α}–CO bonds of the peptide backbone and side chain cleavage. For example, according to AAA, the major peptide TV II 5 contains two residues of Iva and one residue of Val. According to ESI-MS these isomeric AA have to be located as Vxx in positions 7, 8 and 11. Analysis of the methanolysate via GC-MS revealed the release of the peptides Glu-Iva, Val-Aib and Gly-Iva. Consequently, Val had to be put in position 8 and Iva in positions 7 and 11 of TV II 5 (see Figure 4). As a result of the microheterogeneity of the other TV II peptides, ratios of Val and Iva were odd according to AAA, but dipeptide analysis could also be used for the assignment of their positions (see Figure 4). This method, however, did-not allow the unambiguous assignment of presence

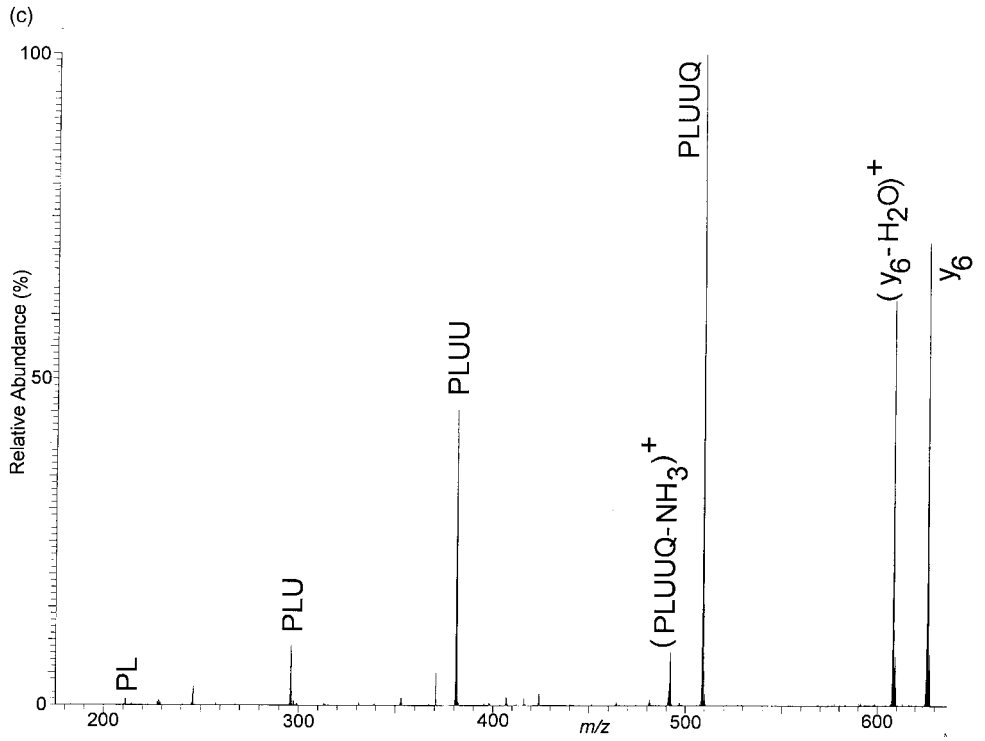

Figure 3 (*Continued*)

and position of Val or Iva in minor peptides TV II 1a and TV II 2c since the dipeptides Glu[6]-Val[7] and Glu[6]-Iva[7] were both detected in the methanolysates of the isolated fractions 1 and 2 of TV II peptides. These peptides were still microheterogeneous as revealed by HPLC on fluorocarbon column C (see inserts of Figure 2). Positions 7 are therefore denoted Vxx in these two sequences (see Figure 4). It should be mentioned that Gln residues in TV peptides are hydrolysed to Glu under the conditions of acidic methanolysis. The presence of two Gln moieties in native peptides was established by ESI-MS.

Pos.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	(%)	MM
1a	Ac	Aib	Gly	Ala	Leu	Ala	Gln	Vxx	Val	Aib	Gly	Aib	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	0.9	1703
1b	Ac	Aib	Gly	Ala	Leu	Aib	Gln	Ala	Val	Aib	Gly	Iva	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	1.6	1703
2a	Ac	Aib	Gly	Ala	Leu	Ala	Gln	Aib	Val	Aib	Gly	Iva	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	1.1	1703
2b	Ac	Aib	Gly	Ala	Leu	Aib	Gln	Aib	Val	Aib	Gly	Aib	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	17.8	1703
2c	Ac	Aib	Gly	Ala	Leu	Aib	Gln	Vxx	Val	Aib	Gly	Aib	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	2.4	1717
3a	Ac	Aib	Gly	Ala	Leu	Aib	Gln	Iva	Val	Aib	Gly	Aib	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	17.9	1717
3b	Ac	Aib	Gly	Ala	Leu	Iva	Gln	Iva	Aib	Aib	Gly	Aib	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	0.7	1717
4a	Ac	Aib	Gly	Ala	Leu	Iva	Gln	Iva	Val	Aib	Gly	Aib	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	2.0	1731
4b	Ac	Aib	Gly	Ala	Leu	Aib	Gln	Aib	Val	Aib	Gly	Iva	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	21.1	1717
5	Ac	Aib	Gly	Ala	Leu	Aib	Gln	Iva	Val	Aib	Gly	Iva	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	24.6	1731
6a	Ac	Aib	Gly	Ala	Leu	Aib	Gln	Iva	Leu	Aib	Gly	Iva	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	1.7	1745
6b	Ac	Aib	Gly	Ala	Leu	Iva	Gln	Iva	Val	Aib	Gly	Iva	Aib	Pro	Leu	Aib	Aib	Gln	Leuol	8.2	1745

Figure 4 Sequences of the peptides of TV II; Pos., position of AA; (%), relative amounts of peptides in the natural microheterogeneous mixture; MM, nominal molecular masses; Leuol and chiral AA are of the L-configuration with the exception of D (=*R*)-Iva [22]; dipeptides used for the assignment of isomeric Val/Iva, generated by methanolysis, are underlined; Vxx means that dipeptides Glu-Val and Glu-Iva both were detected in the methanolysate as a result of microheterogeneity (Gln in natural peptides is hydrolysed to Glu).

Table 1 Detected and Identified Diagnostic Nominal Mass Fragments and Adducts of Molecular Ions of Trichovirins (TV) II Deduced from ESI-MS; Values in m/z

Fragments[a]	TV II											
	1a	1b	2a	2b	2c	3a	3b	4a	4b	5	6a	6b
b_1[b]	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
b_2	185	185	185	185	185	185	185	185	185	185	185	185
b_3	256	256	256	256	256	256	256	256	256	256	256	256
b_4	369	369	369	369	369	369	369	369	369	369	369	369
b_5	440	454	440	454	454	454	468	468	454	454	454	468
b_6[c]	568	582	568	582	582	582	596	596	582	582	582	596
b_7	667	653	653	667	681	681	695	695	667	681	681	695
b_8	766	752	752	766	780	780	780	794	766	780	794	794
b_9	851	837	837	851	865	865	865	879	851	865	879	879
b_{10}	908	894	894	908	922	922	922	936	908	922	936	936
b_{11}	993	993[c]	993[c]	993	1007	1007	1007	1021	1007[c]	1021	1035[c]	1035
b_{12}	1078	1078	1078	1078	1092	1092	1092	1106	1092	1106	1120	1120
y_6	626	626	626	626	626	626	626	626	626	626	626	626
PL[d]	211	211	211	211	211	211	211	211	211	211	211	211
PLU[d]	296	296	296	296	296	296	296	296	296	296	296	296
PLUU[d]	381	381	381	381	381	381	381	381	381	381	381	381
$(PLUUQ\text{-}NH)_3^{+}$[d]	492	492	492	492	492	492	492	492	492	492	492	492
PLUUQ[d]	509	509	509	509	509	509	509	509	509	509	509	509
$(M+H)^+$	1704	1704	1704	1704	1718	1718	1718	1732	1718	1732	1746	1746
$(M+Na)^+$	1726	1726	1726	1726	1740	1740	1740	1754	1740	1754	1768	1768
$(M+K)^+$	1742	1742	1742	1742	1756	1756	1756	1770	1756	1770	n.d	1784
$(M+2Na)^{2+}$	874.5	874.5	874.5	874.5	881.5	881.5	881.5	888.5	881.5	888.5	895.5	895.5

[a] Nomenclature according to Roepstorff and Fohlmann, modified by Biemann [32].
[b] b_1 identified by dipeptide analysis as Ac-Aib-Gly-OMe with GC-MS.
[c] Identification via direct injection of isolated TV II 1–6 fractions and MS-MS of the b_{12} fragment ion.
[d] Identification via direct injection of isolated TV II 1–6 fractions and MS-MS of the y_6 fragment ion.

Selective methanolysis has the advantage that only few micrograms of peptides are required for analysis. This method was also used for the assignment of positions of the isomeric AA Leu and Ile in hypomurocins [24]. In the cases of TV II peptides, however, no Ile was present according to AAA. Further, as demonstrated with Aib[1]-Gly[2] above, and Pro[13]-Leu[14] below, a methanolysate can be screened for any dipeptides suitable for further sequence informations.

The *C*-terminal positions of Leuol[18] were deduced from the differences of 117 mass units from y_6 (m/z 626) and the internal fragments PLUUQ (m/z 509) (Figure 3c). MS-MS of the y_6 fragments at m/z 626 provided a series of diagnostic ions, namely (PL), (PL)U, (PL)UU, and (PL)UUQ establishing AA positions 13–17 (*N*-terminal Pro is protonated). The lowest mass detectable in ESI/MS-MS of y_6 was (PL) at m/z 211, representing Pro[13]-Leu[14]. This was also confirmed by dipeptide analysis after derivatization to TFA-Pro-Leu-OMe [m/z (%) 166 (100), 280 (38), 279 (30), 194 (13), 237 (7)].

The detected and identified diagnostic mass fragments and adducts of molecular ions of TV II peptaibols deduced from ESI-MS are summarized in Table 1. The AA sequences are deducible from the differences of nominal masses between the consecutive ions of any particular series. The resulting sequences of TV II: 1a,b; 2a,b,c; 3a,b; 4a,b; 5; and 6a,b peptides, determined by on-line-HPLC/ESI-MS, direct injection ESI-MS, dipeptide analysis by GC-MS, and chiral AAA are shown in Figure 4. Relative amounts of peptaibols were calculated from their peak areas determined by HPLC. In principle, the HPLC elution pattern ('fingerprint') and relative amounts of individual peptaibols should be reported together with sequences.

Table 2 Retention Times (t_R), Diagnostic Mass Fragments (m/z), and Relative Intensities (%) of Reference TFA-Dipeptide-Methyl Esters Used for the Assignment of Positions of Isomeric Val and Iva by GC-MS (See 'Experimental' for Conditions)

t_R (min)	Reference dipeptide derivatives	m/z (%)						
10.09	Val-Aib[b]	169 (100)	253 (37)	116 (17)	168 (10)	144 (3)	126 (2)	
10.15	Aib-Val[b]	154 (100)	253 (13)	169 (10)	130 (4)	210 (2)		
10.30	Gly-DL-Iva[a]	225 (100)	130 (45)	126 (40)	158 (6)	209 (4)	154 (4)	99 (4)
11.18	Gly-Val[a]	225 (100)	126 (31)	210 (18)	182 (4)			
11.43	Leu-DL-Iva[a]	140 (100)	183 (62)	168 (34)	281 (31)	102 (20)	130 (12)	253 (3)
11.67	Leu-Val[a]	140 (100)	183 (62)	168 (33)	281 (36)	283 (23)		
11.88	Val-Leu[a]	169 (100)	154 (52)	281 (33)	144 (20)	126 (10)	238 (8)	126 (8)
13.01	Glu-D-Iva[c]	213 (100)	152 (38)	311 (30)	181 (30)	279 (15)	115 (2)	
13.21	Glu-Val[a]	213 (100)	152 (94)	311 (47)	181 (45)	212 (34)	267 (3)	

[a] Synthetic dipeptides.
[b] Generated by methanolysis of paracelsin A [4].
[c] Generated by methanolysis of antiamoebin [5,22]; Gln in TV peptides is hydrolysed to Glu on methanolysis.
For diagnostic dipeptides Pro-Leu and Ac-Aib-Gly see 'Results and Discussion'.

The mixture of TV II peptides exerts antibiotic activity against *Bacillus subtilis* and *Staphylococcus aureus* and causes hemolysis of sheep erythrocytes (Table 3).

The TV II peptides are related to the 18-mer trichorzin HA peptaibols [25]. They differ by AA exchange mainly in positions 4 and 11. As a result of related structures the antibiotic and hemolytic properties of TV II are similar to 18-mer peptaibols trichotoxins containing Valol [2–4] and hypomurocins B [24]. The latter is a mixture of peptides containing either *C*-terminal Leuol or Valol.

Other microheterogeneous peptaibols containing *C*-terminal Leuol are, for example, the 20-mer hypelcins [26], the 14-mer harzianins [27] the 19- and 11-mer trikoningins [28], the 18-mer trichokindins [29], the 11-mer trichogin [16] and trichorozins [30], the 7-mer trichodecenins [31], and the 7- and 6-mers detected in the trichobrachin III mixture [19].

CONCLUSIONS

Using submerged fermentation, *Trichoderma viride* strain NRRL 5243 produces two groups of microheterogeneous peptaibol antibiotics, named trichovirins (TV) I and II. Under the conditions of static fermentation only TV I are produced. As demonstrated previously, TV I consist of 14-mer peptaibols, whereas TV II, as described here, represent a mixture of 18-mer peptaibols. For the separation of individual peptides of TV II, HPLC on an

Table 3 Antibacterial and Hemolytic Activities ($n = 2$) of the Trichovirin II Mixture in Comparison to the 20-mer Peptaibol Paracelsin (PC) [4]; $c = 1\%$ (MeOH); Inhibition Zones (mm) of Bacteria were Measured After 24 h, Hemolysis Zones of Erythrocytes (mm) were Measured After 12 h, Diameter of Discs were Subtracted in Both Cases

	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	Hemolysis
TV II (10 μl)	12	14	0	0
TV II (20 μl)	17	19	0	5
TV II (50 μl)	22	25	0	16
PC (20 μl)	12	14	0	11

octadecylsilyl (ODS) stationary phase, followed by the use of a special fluorocarbon stationary phase with different selectivity, was found to be highly suitable. The latter phase is also considered as being of advantage for the complementary resolution of other microheterogeneous peptaibols. This also demonstrates the limits of separating peptaibols on most frequently used ODS phases. TV II were subjected to partial methanolysis, and diagnostic dipeptides formed were characterized by GC-EI-MS. This method is capable of distinguishing positions of isomeric Val/Iva and Leu/Ile in peptaibols and can be used for the generation of any other dipeptides of interest. This approach can be used alternatively, or complementary, to multidimensional NMR techniques.

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