Peptaibiomics: Microheterogeneity, Dynamics, and Sequences of Trichobrachins, Peptaibiotics from *Trichoderma parceramosum* BISSETT (*T. longibrachiatum* RIFAI)

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From the culture broth of the filamentous fungus *Trichoderma parceramosum*, strain CBS 936.69, a mixture of polypeptide antibiotics (pepaibiotics), named trichobrachin (TB), was isolated. Three major groups designated TB I, TB II, and TB III could be separated and isolated by preparative TLC on silica gel. Individual peptides of these three groups were sequenced by on-line LC/ESI-MS^n. The mixture of *N*-acetylated peptides comprises ten 19-residue peptides with a free C-terminal Gln residue (TB I peptides), two 18-residue peptides with a free C-terminal Gln residue (TB II 1 and 2), seven 20-residue peptides with a C-terminal amide-bound phenylalaninol (TB II 3–10), and 34 eleven-residue peptides with either a C-terminal leucinol or isoleucinol or valinol (TB III 1–34). Monitoring production and degradation of peptaibiotics in a pilot experiment revealed that the biosynthesis of TB II and TB III peptides starts two days after the beginning of fermentation. After five days of fermentation, the concentration of TB II decreased, whereas the amount of TB I increased. This observation unequivocally demonstrates that those two 18-residue TB I and TB II peptides with the free carboxy terminus result from enzymatic C-terminal degradation of the 20-residue TB II peptides. In analogy to the technical terms proteome and proteomics, the terms *peptaibiome* and *peptaibiomics* have recently been proposed for the entirety and dynamics of the Aib-containing peptides (comprehensively named peptaibiotics). Consequently, the entire peptaibiome of *T. parceramosum* grown under submerse conditions in shake-flasks for five days comprises at least 54 peptides differing in main-chain length and microheterogeneity, *i.e.*, exchange of amino acids and the C-terminal 1,2-amino alcohol.

Introduction. – It has been shown that filamentous fungi such as *Trichoderma* sp. are capable of producing a group of peptide antibiotics which contain relatively high proportions of the nonproteinogenic α-aminoisobutyric acid (Aib) and, in many cases, isovaline (Iva), in addition to proteinogenic amino acid residues. **Peptaibiotics** are defined as fungal **pept**ides containing **Aib**, which exhibit antib**iotic** or other bioactivities.

The most abundant subgroup of the peptaibiotics, named **peptaibols** [1][2], comprises **pept**ides containing **Aib** and a C-terminal 1,2-amino alcoh**ol**.

In accordance with other technical terms ending in -**omics**, **peptaibiomics** represent the analytical methodology for structural characterization of all peptaibiotics in fungal cells. The **peptaibiome** is defined as the entire expression of fungal peptides containing the characteristic nonproteinogenic amino acid Aib [3–5]. Members of this group consist of 5 up to 21 residues including the C-terminal amino alcohol [6][7].

Peptaibiotics/peptaibols may be classified into groups according to the number of residues: *i*) long-chain peptides with 17–21 residues, *ii*) medium-chain peptides with 11 to 16 residues, *iii*) short-chain peptides with 6–10 residues, and *iv*) the very short-chain peptides with five and less residues.

Members of the group of long-chain peptaibols include, for instance, the 20-residue peptaibols alamethicin [8], suzukacillin [9], and paracelsin [10], the 19-residue peptaibol chrysospermin [11], and the 18-residue peptaibol trichotoxin [10][12]. Examples for medium-chain peptaibols are the 15-residue peptaibols antiamoebin [13] and the eleven-residue peptaibols trichogonin [14] and hypomurocin [15]. Short-chain peptaibiotics are represented by the ten-residue peptaibol trichorozin [16], the nine-residue peptaibiotic helioferin [17], and very short-chain peptaibiotics are represented by the five-residue peptaibol peptaibolin [18].

Notably, alamethicins, trichotoxins, and antiamoebins have been most intensively studied with regard to crystal and solution structure and, in particular, their membrane-modifying properties, which include formation of ionchannels [12][19–23].

Several outstanding bioactivities of peptaibiotics have been described. The 15-residue peptaibol ampullosporin and the 11- and 13-residue trichofumins induce pigment formation in *Phoma destructiva* [24][25] as well as neuroleptic activities including induction of hyperthermia in mice [24–26]. The 16-residue peptaibiotic efrapeptin causes inhibition of mitochondrial ATP-ase and uncoupling of oxidative phosporylation in mitochondria [27]. For the 16-residue integramide peptaibiotics, inhibition of the human immunodeficiency virus (HIV-1) ligase has been reported [28].

Owing to the nonribosomal biosynthesis of peptaibiotics by fungal multi-enzyme complexes, peptaibiotics usually represent groups of peptides differing by main-chain length. Exchange of amino acids and 1,2-amino alcohols within these groups leads to the formation of microheterogeneous peptide mixtures [7][29–31]. These multicomponent mixtures might be considered as natural peptide libraries [32].

From the culture broth of the mold *Trichoderma longibrachiatum* CBS 936.69, we have isolated a mixture of peptaibiotics named trichobrachins I–III (TB I–III). Individual sequences have been determined by fast-atom-bombardment tandem mass spectrometry (FAB-MS/MS). The positions of isobaric amino acids Val/Iva have been determined by automated *Edman* degradation, after selective acidolytic cleavage and HPLC isolation of fragments [33–35]. The stoichiometry and configuration of isolated peptides were determined by quantitative and enantioselective gas-chromatographic amino acid analysis on *Chirasil*-L-*Val*. It was found that trichobrachins consist of different, very microheterogeneous groups of peptides of varying main-chain length. Since Aib-peptides with free carboxy termini had been structurally characterized for the first time, the name *peptaibols* could no longer be applied exclusively, and, consequently the term *peptaibiotics* was proposed [34].

To investigate the reproducibility of our previous experiments [33–35], we have repeated the submerse fermentation of *T. longibrachiatum* and applied our recently described, advanced technique named *'peptaibiomics'* in order to analyze the entirety of peptaibiotics excreted into the culture broth. In this connection, it should be noted that the strain preserved as *T. longibrachiatum* RIFAI (CBS 936.69) was re-described as *T. parceramosum* BISSETT in December 1992. The latter name is still used in the database of the *Centraalbureau voor Schimmelcultures* (*CBS*), NL-Utrecht, and thus

adopted in the following. Recent taxonomic reinvestigation, however, considers CBS 936.69 as *Trichoderma ghanense* (DOI, ABE & SUGIY) (*W. Gams*, *CBS*, personal communication).

Results. – *Dynamics of the Formation of Trichobrachins.* Preliminary experiments in a single shaking flask and daily monitoring of the production of the peptides produced by *T. parceramosum* revealed that production of TB II and TB III could be detected after two days of submerse fermentation. After six days of fermentation, quantities of TB II decreased considerably, whereas TB I was formed. After eight days of fermentation, TB II could no longer be detected.

In contrast, TB III was detected two days after the beginning of fermentation, and quantities did not change very much when having reached a maximum after *ca.* five days. This observation was confirmed by a large-scale experiment providing sufficient TB peptides after five days of fermentation for isolation and sequencing as described in the *Exper. Part*. The dynamics (time dependence) of fermentation is shown in *Fig. 1*. This experiment proves that, at the advanced state of fermentation, the C-terminal Pheol is released from the 20-residue peptide TB II by microbial degradation, leading to the formation of the 19-residue peptide TB I with a C-terminal Gln^{19}. Furthermore, an additional Gln residue can be cleaved from these 19-residue peptides, yielding the 18-residue peptides TB II 1 and 2 which end in a C-terminal Gln^{18} (see sequences presented in *Fig. 5*). Such successive cleavage of the C-terminus has not been observed for the eleven-residue peptides of the TB III group.

Preparative Isolation of Trichobrachins I–III, Characterization of Constituents, and HPLC Elution Profiles. Based on the experiments described above, a separate fermentation was conducted for five days. In total, 309 mg of the crude peptide mixture TB, including TB I–III, have been isolated from 4.8 l of culture broth by applying *XAD-2* and *Sephadex LH 20* column chromatographies.

Parts of this mixture was subjected in portions to preparative TLC. Finally, peptide groups TB I, II, and III could be isolated. The TLC of the isolated peptide groups TB I–III and of the crude TB mixture is also shown in *Fig. 1*. Crude TB and the isolated groups TB I–III were totally hydrolyzed and subjected to enantioselective GC/MS. The presence of Aib, Gly, D-Iva, L-Val, L-Leu, L-Ala, L-Glu (from Gln), and L-Pro could be determined in all peptides, wheras L-Pheol (phenylalaninol) was exclusively detected in TB-II but not in TB-I peptides. In the total hydrolysate of TB III, amino alcohols L-Valol (valinol), L-Leuol (leucinol), and L-Ileol (isoleucinol) have been detected simultaneously, and the presence of D-Iva was established. The chromatograms demonstrating the presence and chirality of the above mentioned constituents are presented in *Fig. 2*.

The HPLC-elution profiles of the isolated peptide groups TB I, II, and III are shown in *Fig. 3*. As can be seen, analysis of TB I yielded eight peaks, that of TB II ten peaks, and that of TB III 18 peaks, which could be demonstrated to represent peptaibols/peptaibiotics by LC/MS (see below). Peaks designated with an 'x' in the chromatograms are nonpeptidic artefacts from the silica-gel matrix, and non-annotated peaks do not represent peptides.

Sequence Analysis of the TB-I and TB-II Peptides. Sequence determination of isolated TB-I and TB-II peptides was performed by analytical HPLC/ES-MS and direct

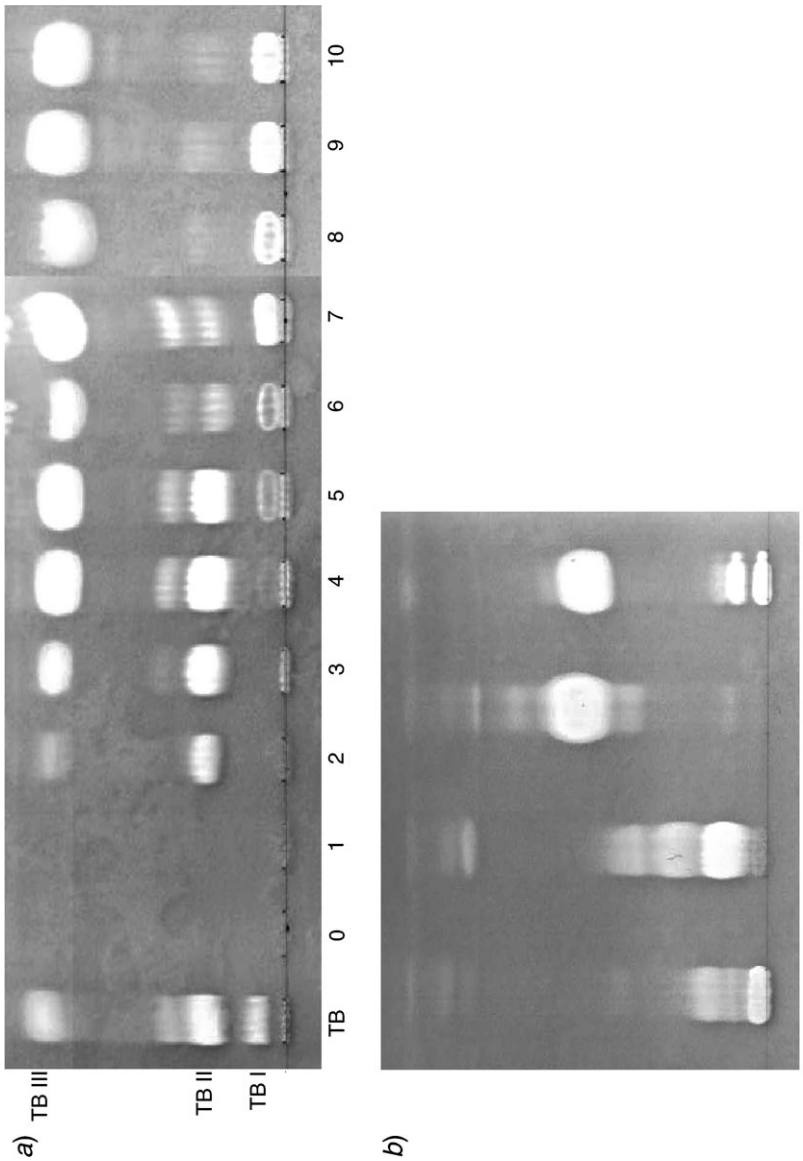

Fig. 1. a) *TLC Monitoring of the production and degradation of trichobrachins from* Trichoderma parceramosum. Numbers 0–10 refer to days of fermentation in malt extract medium. b) *TLC of crude trichobrachin* (TB), *and trichobrachin I* (TB I), *trichobrachin II* (TB II), *and trichobrachin III* (TB III) *resulting from preparative TLC.* Peptides were visualized by spraying with H_2O.

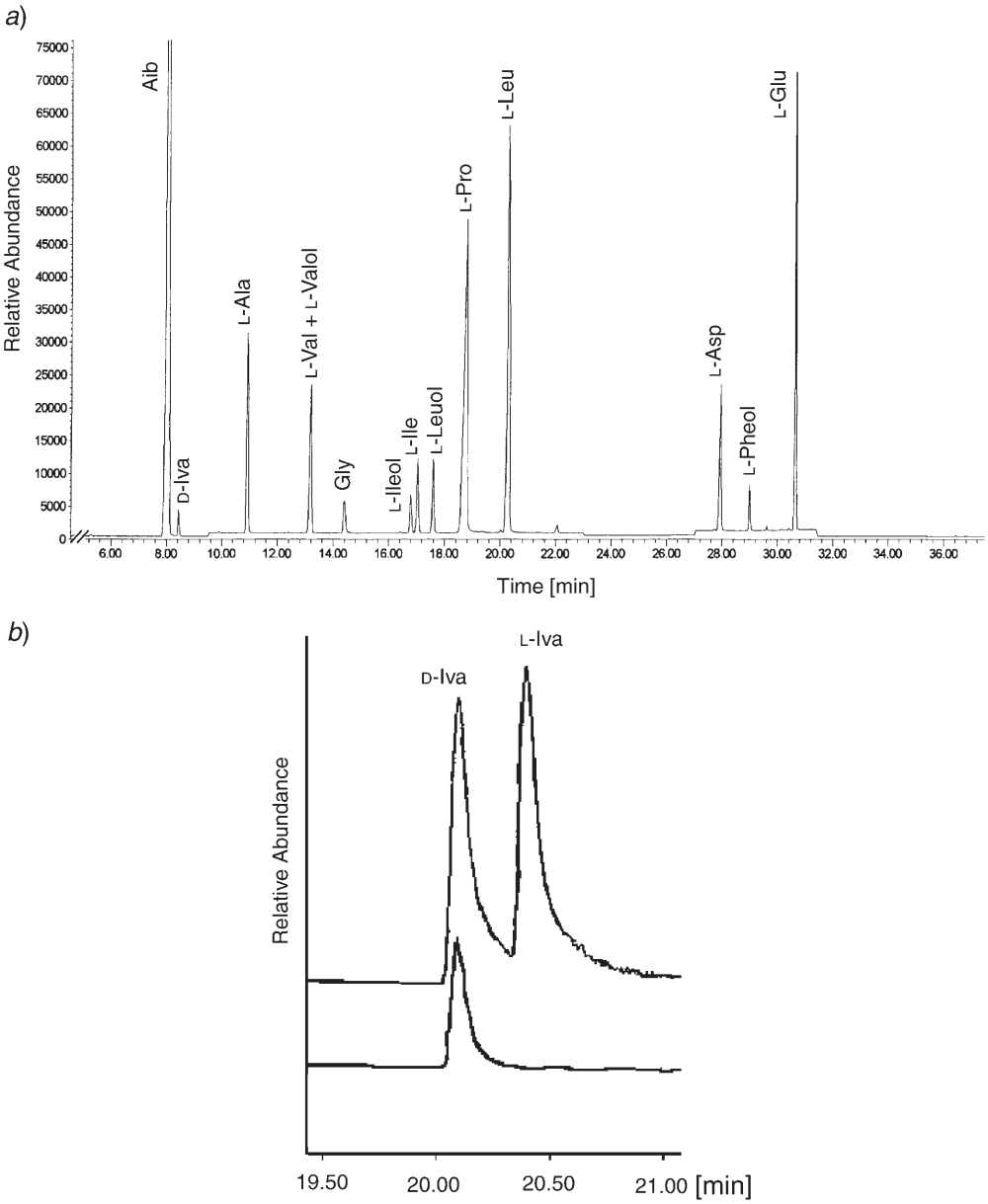

Fig. 2. *Chromatograms on* Chirasil®-L-Val *of* a) *derivatized total hydrolysates of crude trichobrachin* (*N*-trifluoroacetyl amino acid propyl esters and *N*(*O*)-trifluoroacetyl 1,2-amino alcohols), *and* b) *a standard of* N-*acetyl*-DL-*isovaline propyl ester* (upper trace) *and* N-*acetyl*-D-*isovaline propyl ester determined in a hydrolysate of crude trichobrachin* (lower trace). *a*) GC/SIM-MS; *b*) TIC mode.

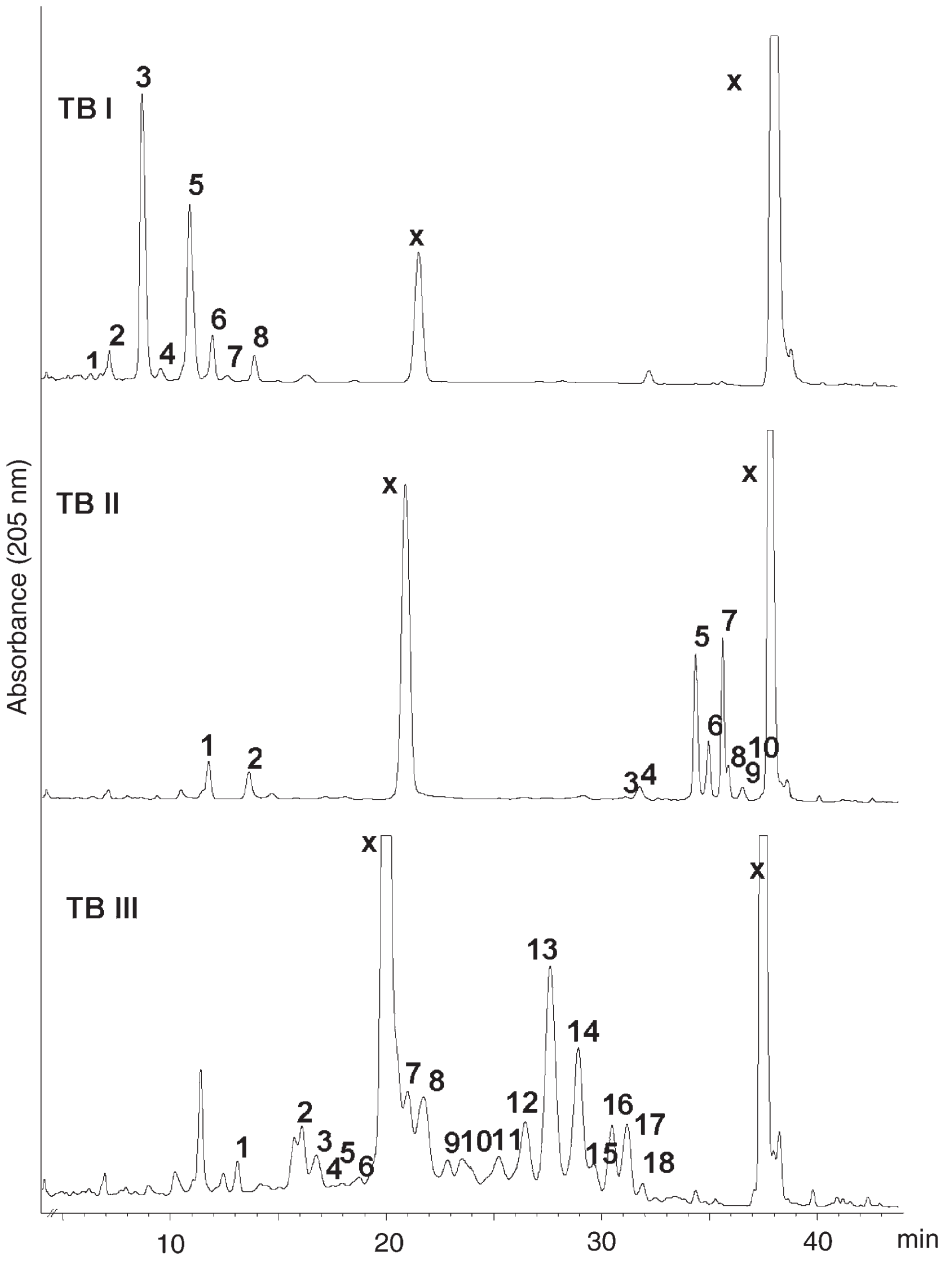

Fig. 3. *HPLC Elution profiles of trichobrachin groups TB I, TB II, and TB III.* Numbers assigned to the peaks in the chromatograms correspond to the peptide sequences presented in *Fig. 5*. Peaks marked with an x represent nonpeptidic artefacts resulting from preparative TLC. For the chromatographic conditions, see the *Exper. Part*.

infusion ES-MS. Furthermore, collision-induced dissociation (CID) experiments were performed in positive- and negative-ionization modes. The diagnostic fragment ions determined for TB I and TB II are listed in *Tables 1* and *2*, respectively. From these data, ten individual sequences of TB I peptides, each containing 19 amino acids, could be deduced. TB II 1 and 2 are 18-amino acid peptides, whereas TB II 3–10 are 20-residue peptides (for sequences discussed in the following, see *Fig. 5*).

Table 1. *Diagnostic Ions* [*m*/*z*] *of Trichobrachin I Peptides*

Diagnostic ions	Trichobrachin I									
	1	2	3	4a	4b	5	6	7	8a	8b
$[M+H]^+$	1790	1790	1804	1804	1818	1818	1832	1818	1818	1832
$[M+Na]^+$	1812	1812	1826	1826	1840	1840	1854	1840	1840	1854
b_3	270	270	284	284	284	284	284	284	284	284
b_4	341	341	355	355	355	355	355	355	355	355
b_5	426	426	440	440	440	440	440	440	440	440
b_6	479	479	511	511	525	511	525	525	511	525
b_7	625	625	639	639	653	639	653	653	639	653
b_8	710	710	724	724	738	724	738	738	724	738
b_9	809	809	823	823	837	823	837	837	823	837
b_{10}	894	894	908	908	922	908	922	922	908	922
b_{11}	951	951	965	965	979	965	979	979	965	979
b_{12}	1064	1064	1078	1078	1092	1078	1092	1092	1078	1092
b_{13}	1149	1149	1163	1163	1177	1163	1177	1177	1163	1177
y_{6P}	641	641	641	641	641	655	655	641	655	655
$(y_{6P}-H_2O)$	623	623	623	623	623	637	637	623	637	637
y_{6P}–AA (19)	495	495	495	495	495	509	509	495	509	509
y_{6P}–AA (19–18)	367	367	367	367	367	381	381	367	381	381
y_{6P}–AA (19–17)	282	282	282	282	282	282	282	282	282	282
y_{6P}–AA (19–16)	197	197	197	197	197	197	197	197	197	197
M^-	1789	1789	1803	1803	1817	1817	1831	1817	1817	1831
y_{2N}	274	274	274	274	274	274	274	274	274	274
y_{3N}	359	359	359	359	359	373	373	359	373	373
y_{4N}	444	444	444	444	444	458	458	444	458	458
y_{5N}	543	543	543	543	543	557	557	543	557	557
y_{6N}	640	640	640	640	640	654	654	640	654	654
y_{12N}	1164	1164	1164	1164	1164	1178	1178	1164	1178	1178
y_{13N}	1292	1292	1292	1292	1292	1306	1306	1292	1306	1306
y_{14N}	1363	1363	1363	1363	1377	1377	1391	1377	1377	1391
y_{15N}	1448	1448	1448	1448	1462	1462	1476	1462	1462	1476
y_{16N}	1519	1519	1519	1519	1533	1533	1547	1533	1533	1547
y_{17N}	1580	1580	1604	1604	1618	1618	1632	1618	1618	1632
y_{18N}	1661	1661	1675	1675	1689	1689	1703	1689	1689	1703

Some of the diagnostic ions, *e.g.*, $[M+Na]^+$, $[M+H]^+$, b_{13}, and y_{5P} (peptides with 18 amino acids), y_{6P} (peptides with 19 amino acids), and y_{7P} (peptides with 20 residues), were generated using on-line HPLC-ES-MS. Positive-ion MS/MS of $[M+H]^+$ precursor ions generated the *b* and y_P series (P for positive-ion mode) of fragment ions. MS/MS of M^- in the negative-ion mode provided fragment ions of the *y* series that were designated as y_N (N refers to the negative-ion mode).

Table 2. *Diagnostic Ions* [*m*/*z*] *of Trichobrachin II Peptides*

Diagnostic ions	Trichobrachin II									
	1	2	3	4	5	6	7	8	9	10
$[M+H]^+$	1676	1690	1923	1923	1937	1937	1951	1951	1951	1965
$[M+Na]^+$	1698	1712	1945	1945	1959	1959	1973	1973	1973	1987
b_2	199	199	199	199	199	199	199	199	199	199
b_3	284	284	270	284	284	284	284	284	284	284
b_4	355	355	341	355	355	355	355	355	355	355
b_5	440	440	426	440	440	440	440	440	440	440
b_6	511	511	497	511	511	511	511	511	511	525
b_7	639	639	625	639	639	639	639	639	639	653
b_8	724	724	710	724	724	724	724	724	724	738
b_9	823	823	809	823	823	823	823	823	823	837
b_{10}	908	908	894	908	908	908	908	908	908	922
b_{11}	965	965	951	965	965	965	965	965	965	979
b_{12}	1078	1078	1064	1078	1078	1078	1078	1078	1078	1092
b_{13}	1163	1163	1149	1163	1163	1163	1163	1163	1163	1177
y_{5P}	513	527								
$(y_{5P}-H_2O)$	495	509								
Y_{5P}–AA (18)	367	381								
Y_{5P}–AA (18–17)	282	282								
Y_{5P}–AA (18–16)	197	197								
y_{7P}			774	760	774	774	788	788	788	788
$(y_{7P}-H_2O)$			756	742	756	756	770	770	770	770
y_{7P}–AA (20)			623	609	623	623	637	637	637	637
y_{7P}–AA (20–19)			495	481	495	495	509	509	509	509
y_{7P}–AA (20–18)			367	353	367	367	381	381	381	381
y_{7P}–AA (20–17)			282	282	282	282	282	282	282	282
y_{7P}–AA (20–16)			197	197	197	197	197	197	197	197
M^-	1675	1689	1922	1922	1936	1936	1950	1950	1950	1964
y_{2N}	231	245	279	279	279	279	279	279	279	279
y_{3N}	316	330	407	407	407	407	407	407	407	407
y_{4N}	415	429	492	478	492	492	506	506	506	506
y_{5N}	512	526	577	563	577	577	591	591	591	591
y_{6N}			676	662	676	676	690	690	690	690
y_{7N}			773	759	773	773	787	787	787	787
y_{10N}	951	965	1028	1014	1028	1028	1042	1042	1042	1042
y_{11N}	1036	1050	1113	1099	1113	1113	1127	1127	1127	1127
y_{12N}	1164	1178	1212	1198	1212	1212	1226	1226	1226	1226
y_{13N}	1235	1249	1297	1283	1297	1297	1311	1311	1311	1311
y_{14N}	1320	1334	1425	1411	1425	1425	1439	1439	1439	1439
y_{15N}	1391	1405	1496	1482	1496	1496	1510	1510	1510	1524
y_{16N}	1476	1499	1581	1567	1581	1581	1565	1565	1565	1609
y_{17N}	1547	1561	1652	1638	1652	1652	1666	1666	1666	1680
y_{18N}	1632	1646	1723	1723	1737	1737	1751	1751	1751	1765
y_{19N}			1794	1794	1808	1808	1822	1822	1822	1836

The majority of the diagnostic fragment ions of the trichobrachins were identified *via* HPLC/ES-MS^n ($n=2-4$) in the positive-ion mode from specific precursor ions such as $[M+H]^+$, b_{13}, b_8, or b_5.

The Gln[7]–Aib[8] bonds are extremely stable under routine conditions of ES-MS but diagnostic fragment ions b_7 could be generated by addition of 1% HCOOH.

In contrast, the Aib[13]–Pro[14] bond is extremely labile [10][11][36]. Characteristic fragment ions of the b_{13} and y_{7P} (analogously, the y_{6P} for the TB I and y_{5P} for the TB II 1–2 series) were generated by cleavage of these bonds [8].

The b_1 fragment ions, which should be generated from an Ac-Aib sequence occurring in all peptides, were not detected under the conditions of positive-ion ES-MS. In the case of TB I, the b_2 fragment ions that should result from Ac-Aib-Ala were not detected, whereas they were generated from the TB II peptides. Thus, the N-terminal amino acid sequences were determined from fragment ions resulting from the y_N series generated by direct inlet MS-MS of M^- in the negative-ion mode. For the 20-residue peptides TB II 3–10, the mass differences between ions y_{19N} and M^- (128 a.m.u.) represent the N-terminal fragment Ac-Aib. In the cases of the 18-amino acid peptides TB II 1 and TB II 2, this mass difference was observed between ions y_{17N} and M^-. Analogously, the mass difference of ions y_{18N} and M^- established the Ac-Aib terminus for the 19-residue TB I peptides.

The C-terminal position of Pheol in the TB II 3–10 peptides was deduced from the mass differences of 151 a.m.u. between y_{7P} (m/z 760, 774, or 788) and the internal fragments y_{7P}–AA (m/z 609, 623, or 637). Trichobrachins TB I, and TB II 1 and 2, do not contain the amino alcohol.

The C-terminal sequences of the TB I peptides were determined by MS/MS of the y_{6P} fragment ions. Likewise, sequence analyses for TB II 1 and 2 were performed by MS/MS of the y_{5P} fragment and of the y_{7P} fragment for TB II 3–10. These diagnostic fragment ions were generated as a result of the lability of the Aib–Pro bonds [9]. For these internal fragment ions of the C-terminus, the lowest mass detectable by MS/MS was m/z 197, representing Pro[14]–Vxx[15]. Since the fragment ions y_{7P} (from TB II 3–10), as well as y_{6P} (from TB I) and y_{5P} (from TB II 1 and 2), result from cleavage of the labile Aib–Pro bonds, Pro was assigned to be the first amino acid in these sequences. This was also deduced from the series of the corresponding negative ions y_{7N}, y_{6N}, and y_{5N}, providing mass differences of 97 a.m.u. (Pro) and 99 a.m.u. (Vxx).

The TB I, and TB II 1 and 2 peptides with a C-terminal Gln result from microbial degradation of peptides TB II 3–10. This was also validated by the fermentation experiment (see *Exper. Part*). For monitoring of peptaibol production and degradation, see TLC in *Fig. 1*.

Loss of the amino alcohol Pheol from TB II 3–10 resulted in the sequences of TB I. The additional loss of Gln[19] provided the sequences TB II 1 and 2. Characteristic mass spectra of TB I 3 and TB II 5 are shown in *Figs. 4* and *5*.

Sequence Analysis of the TB III Peptides. Application of LC/MS to the mixture of TB III peptides isolated by preparative TLC enabled the determination of 34 sequences of these eleven-residue peptides, designated as TB III 1–18. Diagnostic fragment ions determined are listed in *Table 3*. Many peptides could be resolved by HPLC, but complete sequences could not be elucidated as a result of the presence of the isobaric amino acids Val/Iva and Leu/Ile, and the 1,2-amino alcohols Leuol/Ileol.

In TB III, many sequences of peptides eluting at different retention times seem to be identical. For this group of peptides, the GC/MS analysis clearly demonstrated the presence of isobaric Val/Iva, Leu/Ile, and Leuol/Ileol.

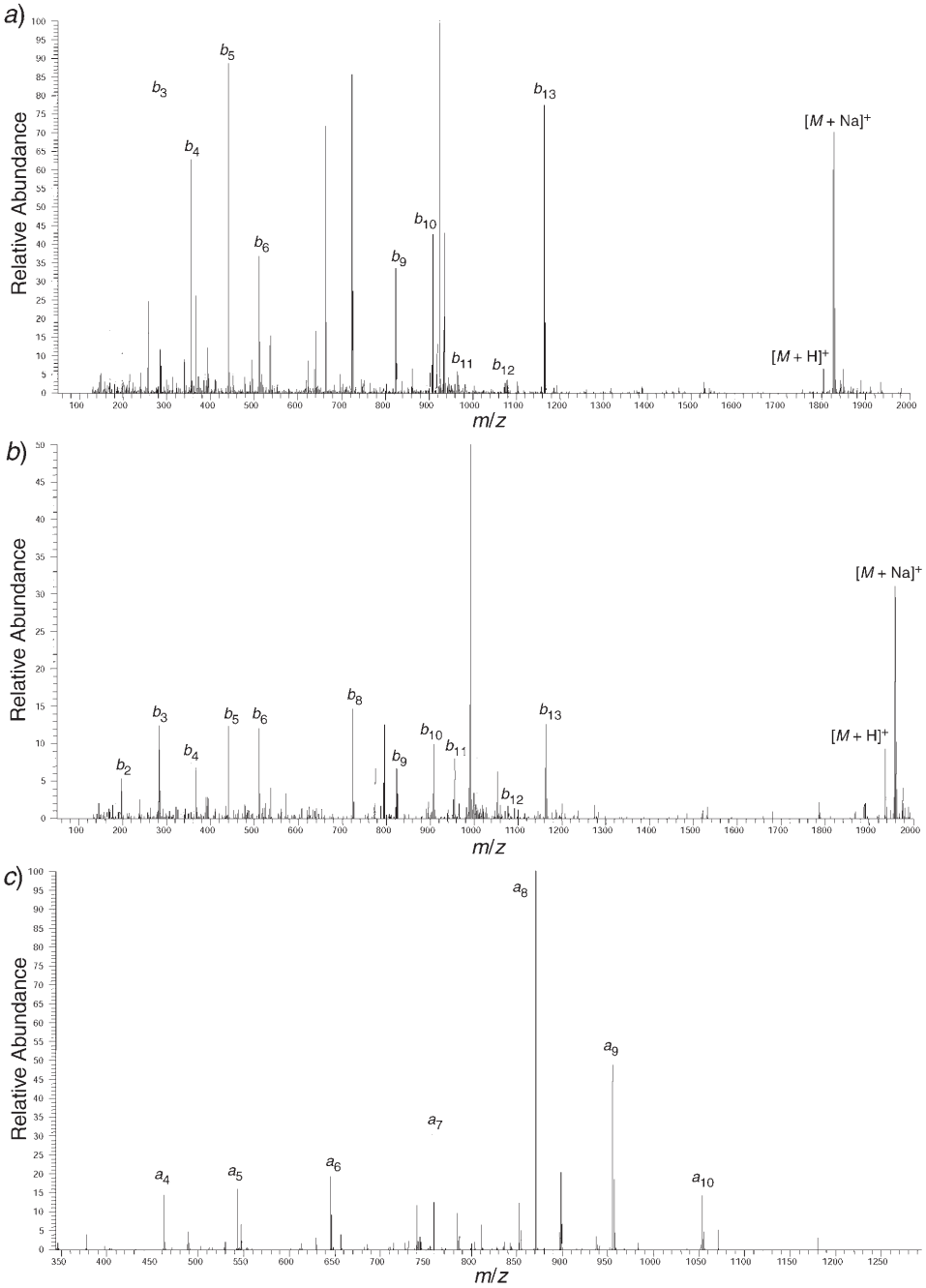

Fig. 4. *Characteristic mass spectra* (positive-ion mode) *resulting from HPLC/ES-MS analysis of* a) *trichobrachin I 3,* b) *trichobrachin II 5, and* c) *of fragment ions of* $[M+Na]^+$ *of trichobrachin III 10a*

a)

TB I		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	Ac	Aib	Ala	Ala	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	Aib	Gln	Gln
2	Ac	Aib	Ala	Ala	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	Aib	Gln	Gln
3	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	Aib	Gln	Gln
4a	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	Aib	Gln	Gln
4b	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	Aib	Gln	Gln
5	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln
6	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln
7	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	Aib	Gln	Gln
8a	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln
8b	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Leu	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln

b)

TB II		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln		
2	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln		
3	Ac	Aib	Ala	Ala	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
4	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Ala	Gln	Gln	Pheol
5	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
6	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
7	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Pheol
8	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Pheol
9	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Pheol
10	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Pheol

Fig. 5. *Sequences of trichobrachin peptides of group TB I (1–8), group TB II (1–10), and group TB III (1–18)*. Abbreviations of protein amino acids are according to the three-letter code; Ac = acetyl; Aib = α-aminoisobutyric acid; Vxx = Val or Iva (isovaline), Lxx = Leu or Ile, Lxxol = leucinol or isoleucinol; Valol = valinol. Chiral amino acids are of the L-configuration with the exception of D-Iva. For the HPLC elution profiles of TB I–III, see *Fig. 3*.

Analogously to the sequence analyses of TB I and TB II, some of the diagnostic TB III ions, such as $[M+\mathrm{Na}]^+$, $[M+\mathrm{H}]^+$, the *a* series in the positive-ion mode, and the *y* series in the negative-ion mode were analyzed by HPLC/ES-MS or direct inlet with CID. Positive MS/MS of the $[M+\mathrm{Na}]^+$ precursor ions generated the *a* series of product ions. MS/MS of $[M-\mathrm{H}]^-$ in the negative-ion mode generated the *y* series, designated as y_N. The majority of the diagnostic fragment ions was identified *via* HPLC/ES-MS^n ($n=2-4$) from the specific precursor ions $[M+\mathrm{Na}]^+$, and the fragment ions a_9 as well as a_7.

The mass spectrum of the diagnostic fragment ions of TB III 10a after HPLC/ES-MS in the positive-ion mode is shown as an example in *Fig. 4*,*c*.

The a_2 fragment ions, which should be generated from the *N*-Ac-Aib sequence occurring in all peptides, have not been detected under the conditions of the positive-ion ES-MS. Thus, the N-terminal amino acids were determined from fragments resulting from the *y* series of negative ions generated by direct inlet MS/MS of $[M-$

c)

TB III		1	2	3	4	5	6	7	8	9	10	11
1a	Ac	Aib	Asn	Vxx	Vxx	Aib	Pro	Lxx	Vxx	Aib	Pro	Lxxol
1b	Ac	Aib	Asn	Vxx	Lxx	Aib	Pro	Lxx	Vxx	Aib	Pro	Valol
2a	Ac	Aib	Asn	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
2b	Ac	Aib	Asn	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Valol
3a	Ac	Aib	Asn	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
3b	Ac	Aib	Asn	Lxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Valol
4	Ac	Aib	Gln	Vxx	Lxx	Aib	Pro	Lxx	Vxx	Aib	Pro	Lxxol
5	Ac	Aib	Gln	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
6	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Valol
7a	Ac	Aib	Asn	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
7b	Ac	Aib	Asn	Lxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
8a	Ac	Aib	Asn	Lxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
8b	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Valol
8c	Ac	Aib	Gln	Lxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Valol
9a	Ac	Aib	Asn	Lxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
9b	Ac	Aib	Gln	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
9c	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Valol
9d	Ac	Aib	Gln	Lxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Valol
9e	Ac	Aib	Asn	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Valol
10a	Ac	Aib	Gln	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
10b	Ac	Aib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Valol
11a	Ac	Aib	Gln	Lxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
11b	Ac	Aib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Valol
12a	Ac	Aib	Gln	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
12b	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
13	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
14	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
15a	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
15b	Ac	Aib	Gln	Vxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
15c	Ac	Aib	Asn	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
16a	Ac	Aib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
16b	Ac	Aib	Asn	Vxx	Vxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
17	Ac	Aib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol
18	Ac	Aib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol

Fig. 5 (cont.)

$H]^-$ in the negative-ion mode. The mass differences of 128 a.m.u. between y_{10N} and $[M-H]^-$ represent the N-terminal fragment Ac-Aib.

Diagnostic product ions y_{10N} to y_{4N} were also analyzed by negative-ion MS/MS from $[M-H]^-$ and MS^3 of the fragment ion y_{6N} (*Table 3*).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
TB I A	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	
TB I B	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Iva	Gln	Gln	
TB I C	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	
TB I D	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Iva	Gln	Gln	
TB IIa A	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val					
TB IIa B	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val					
TB IIa C	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Iva	Gln		
TB IIa D	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Iva	Gln		
TB IIb A	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	Pheol
TB IIb B	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Iva	Gln	Gln	Pheol
TB IIb C	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	Pheol
TB IIb D	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Val	Aib	Iva	Gln	Gln	Pheol
TB III A a	[N^{211}]	Gly	Lxx	Aib	Gly	Val	Leuol														
TB III A b	[N^{143}]	Lxx	Pro	Lxx	Gly	Val	Leuol														
TB III B a	[N^{210}]	Gly	Lxx	Aib	Gly	Lxx	Leuol														
TB III B b	[N^{144}]	Lxx	Pro	Lxx	Gly	Lxx	Leuol														
TB III C a	Ac	Aib	Gln	Aib	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Leuol									
TB III C b	Ac	Aib	Gln	Val	Lxx	Aib	Pro	Lxx	Lxx	Aib	[C^{199}]										
TB III D a	Ac	Aib	Asn	Val	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Leuol									
TB III D b	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	[C^{199}]										
TB III E a	Ac	Aib	Gln	Val	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Leuol									
TB III E b	Ac	Aib	Gln	Lxx	Val	Aib	Pro	Lxx	Lxx	Aib	Pro	Leuol									
TB III E c	Ac	Aib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	[C^{199}]										
TB III F a	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Leuol									
TB III F b1	Ac	Aib	Gln	Lxx	Val	Aib	Pro	Lxx	Lxx	Aib	Pro	Leuol									
TB III F b2	Ac	Aib	Gln	Val	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Leuol									
TB III F c	Ac	Aib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	[C^{199}]										
TB III G a	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Leu	Leu	Aib	Pro	Leuol									
TB III G b	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Ile	Leu	Aib	Pro	Leuol									
TB III H a	Ac	Aib	Asn	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Leuol									
TB III H b	Ac	Aib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	[C^{199}]										
TB III I	Ac	Aib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Leuol									
TB III J	Ac	Aib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Leuol									

Fig. 6. *Sequences and designations of the trichobrachin III peptides determined in previous works* [30–32]. Lxx, Leu, or Ile; [$N^{143/144}$] and [$N^{210/211}$] stand for unknown N-terminal of *m*/*z* 143 or 144 and 210 or 211; [C^{199}] was established as C-terminal Pro-Valol of *m*/*z* 199 in the work presented here (see text). The TB III A and TB III B peptides could not be detected in the experiments described here.

The C-terminal positions of Valol and Lxxol in the TB III peptides were deduced from the mass difference of 103 and 117 a.m.u. between a_{10} and $[M+\mathrm{Na}]^+$.

Since the previously determined sequences of the trichobrachins have only been published in Proceedings volumes [34][35], they are presented here for comparison (*Fig. 6*). Note that the original numbering and designation of the peptides is used in

Table 3. *Diagnostic Ions* [*m*/*z*] *of Trichobrachin III Peptides*

Diagnostic ions	Trichobrachin III																
	1a	1b	2a	2b	3a	3b	4	5	6	7a	7b	8a	8b	8c	9a	9b	9c
$[M+H]^+$	1134	1134	1148	1148	1148	1148	1162	1162	1162	1162	1162	1162	1162	1162	1162	1162	1162
$[M+Na]^+$	1156	1156	1170	1170	1170	1170	1184	1184	1184	1184	1184	1184	1184	1184	1184	1184	1184
a_2	n.d.[a])	n.d.	n.d.	237	n.d.	237	251	n.d.	n.d.	n.d.	n.d.	n.d.	237	251	n.d.	n.d.	237
a_3	336	336	336	336	336	350	350	350	350	336	350	350	350	364	350	350	350
a_4	435	449	435	449	435	449	463	449	463	449	449	449	463	463	449	449	463
a_5	520	534	520	534	520	534	548	534	548	534	534	534	548	548	534	534	548
a_6	617	631	617	631	617	631	645	631	645	631	631	631	645	645	631	631	645
a_7	730	744	730	744	730	744	758	744	758	744	744	744	758	758	744	744	758
a_8	829	843	843	857	843	857	857	857	871	857	857	857	871	871	857	857	871
a_9	914	928	928	942	928	942	942	942	956	942	942	942	956	956	942	942	956
a_{10}	1011	1025	1025	1039	1025	1039	1039	1039	1053	1039	1039	1039	1053	1053	1039	1039	1053
$[M-H]^-$	1132	1132	1146	1146	1146	1146	1160	1160	1160	1160	1160	1160	1160	1160	1160	1160	1160
y_{2N}	n.d.	n.d.	213	n.d.	213	199	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
y_{3N}	n.d.	n.d.	298	n.d.	298	n.d.	n.d.	298	n.d.	n.d.	298	298	n.d.	284	298	298	284
y_{4N}	n.d.	383	411	397	411	397	397	411	397	411	411	411	397	397	411	411	397
y_{5N}	510	496	524	510	524	510	510	524	510	524	524	524	510	510	524	524	510
y_{6N}	607	593	621	607	621	607	607	621	607	621	621	621	607	607	621	621	607
y_{7N}	692	678	n.d.	n.d.	n.d.	692	692	706	n.d.	706	706	706	n.d.	n.d.	706	706	n.d.
y_{8N}	791	791	805	805	805	791	805	805	805	819	805	805	805	791	805	805	805
y_{9N}	890	890	904	904	904	904	904	904	918	918	918	918	918	904	918	904	918
y_{10N}	1004	1004	1018	1018	1018	1018	1032	1032	1032	1032	1032	1032	1032	1032	1032	1032	1032

[a]) n.d. = Not detected.

Fig. 6, and that the positions of the isomeric amino acids Val/Iva and of Leu could be assigned in TB-I and TB-II peptides as a result of the sequencing methods previously used. As can be seen, the data are in excellent agreement, but many more sequences could be determined in the work presented here. However, those degradation products previously assigned as TB IIa A–C [33][35] could not be detected in the experiments described here. Notably, several sequences of TB II might be identical with *longibrachins A* [36][37].

The sequences of 34 TB III peptides could be assigned, but isomeric amino acids Val/Iva and Leu/Ile, and isomeric 1,2-amino alcohols Leuol/Ileol could not be distinguished. However, the presence of a C-terminal Valol in many of these peptides could be established. Notably, C-terminal fragments of *m*/*z* 199, which could not be assigned in several TB III peptides in previous work [33], were demonstrated to represent C-terminal Pro-Valol sequence. Minor peptides, termed TB III A and TB III B, could not be detected in the experiments described in this work (see *Fig. 6*).

Discussion. – The group of trichobrachins represents a microheterogeneous mixture of fungal peptides belonging to the group of peptaibiotics. These microheterogeneous mixtures are generated by peptide synthetases during nonribosomal biosynthesis [7][29][30][37][38]. TLC Methods have successfully been applied to separate the three TB groups according to main-chain length and hydrophobicity. HPLC enables the

Table 3 (cont.)

9d	9e	10a	10b	11a	11b	12a	12b	13	14	15a	15b	15c	16a	16b	17	18
1162	1134	1176	1176	1176	1176	1176	1176	1176	1176	1176	1176	1148	1190	1148	1190	1190
1184	1156	1198	1198	1198	1198	1198	1198	1198	1198	1198	1198	1170	1212	1170	1212	1212
n.d.	237	251	251	n.d.	251	251	237	237	n.d.	n.d.	251	n.d.	251	n.d.	251	251
364	336	350	364	364	364	350	350	350	350	350	350	336	364	336	364	364
463	435	463	477	463	477	463	463	463	463	463	463	435	477	435	477	477
548	520	548	562	548	562	548	548	548	548	548	548	520	562	520	562	562
645	617	645	659	645	659	645	645	645	645	645	645	617	659	617	659	659
758	730	758	772	758	772	758	758	758	758	758	758	730	772	730	772	772
871	843	871	885	871	885	871	871	871	871	871	871	843	885	843	885	885
956	928	956	970	956	970	956	956	956	956	956	956	928	970	928	970	970
1053	1025	1053	1067	1053	1067	1053	1053	1053	1053	1053	1053	1025	1067	1025	1067	1067
1160	1132	1174	1174	1174	1174	1174	1174	1174	1174	1174	1174	1146	1188	1146	1188	1188
n.d.	n.d.	n.d.	199	n.d.	199	n.d.	213	213	213	213	n.d.	213	213	213	213	213
284	284	n.d.	284	n.d.	284	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	298	298	298	298	298
397	397	411	397	411	397	411	411	411	411	411	411	411	411	411	411	411
510	510	524	510	524	510	524	524	524	524	524	524	524	524	524	524	524
607	607	621	607	621	607	621	621	621	621	621	621	621	621	621	621	621
n.d.	692	706	692	706	692	706	706	706	706	706	706	n.d.	n.d.	n.d.	n.d.	n.d.
791	791	819	805	805	805	819	819	819	819	819	819	805	819	805	819	819
904	890	918	918	918	918	918	932	932	932	932	918	904	932	904	932	932
1032	1004	1046	1046	1046	1046	1046	1046	1046	1046	1046	1046	1018	1060	1018	1060	1060

separation of individual peptides of these groups. On-line HPLC and a set of ES-MS experiments provided, in combination with enantioselective GC/MS, the sequences of the peptides. By these methods, even sequences of co-eluting peptides could be determined. Note that on-line HPLC/ES-MS/MS is restricted to sequencing of peptides, whereas isobaric amino acids and 1,2-amino alcohols cannot be distinguished by this approach. Assignment of the positions requires isolation of sufficient amounts of homogeneous peptides to characterize the C-terminal 1,2-amino alcohols. Alternatively, alignment of the position and chemical structure of isomeric amino acids is possible using the method of methanolysis/acylation of peptaibiotics, followed by GC/MS characterization of the small peptides formed [15][32]. Assignments of the positions of the isomeric amino acids have been performed in previous experiments for the TB I and TB II peptides (*Fig. 6*).

Monitoring of the production and degradation of trichobrachins during fermentation revealed that endogeneous enzymatic degradation of the C-terminus of the 20-residue peptides representing TB II leads to the loss of the C-terminal Pheol, thus generating the 19-amino acid peptides of TB I. Further cleavage of the Gln residue resulted in the 18-amino acid peptides TB II 1 and 2 with a free C-terminus.

A comparison of the peptide sequences reported here shows that they are in excellent agreement with those previously determined [33–35]. In particular, deletion of Pheol and formation of 19- and 18-amino acid peptides could be confirmed. Obviously, further C-terminal degradation occurs in the course of the fermentation,

thus leading to 15-amino acid peptides with a C-terminal Val. The sequences of TB I 1 and 2, and TB II 1, 3, and 4, shown in *Fig. 5*, are described for the first time.

The formation of the very microheterogeneous TB III peptides comprising *N*-acetylated eleven-residue peptides with a C-terminal 1,2-amino alcohol could also be confirmed, and many more sequences could be determined. In particular, the structure of the C-terminal residue *m*/*z* 199 could be assigned as Pro-Valol.

The microheterogeneous peptide mixtures, as described here, might also be considered as natural peptaibol libraries [32][39][40].

Testing of the antibiotic activity of the mixture of all trichobrachin (TB) peptides and isolated, but still microheterogeneous, groups TB I, TB II, and TB III, against *Bacillus subtilis* using a microtiterplate photometric assay revealed that, at concentrations of 50 μg/ml, the 20-residue peptaibol alamethicin F 50, serving as standard, showed the highest activity, that the TB II and TB III peptides were about equal but less active, and that the mixture of the TB peptides, as well as TB I, were about equal, but each group less active in comparison to the TB-II and TB-III peptides. It should be noted, however, that, on a molar basis, the concentration of the eleven-residue TB-III peptides is about twice that of the 20-residue peptides. A comparison of TB IIb A and TB I demonstrates that the C-terminal alcohol is of importance for the antibacterial activity against the *Gram*-positive test organism. The concentration-dependent activity curves, measured in terms of change of the optical density of the bacterial suspension, is presented in *Fig. 7*.

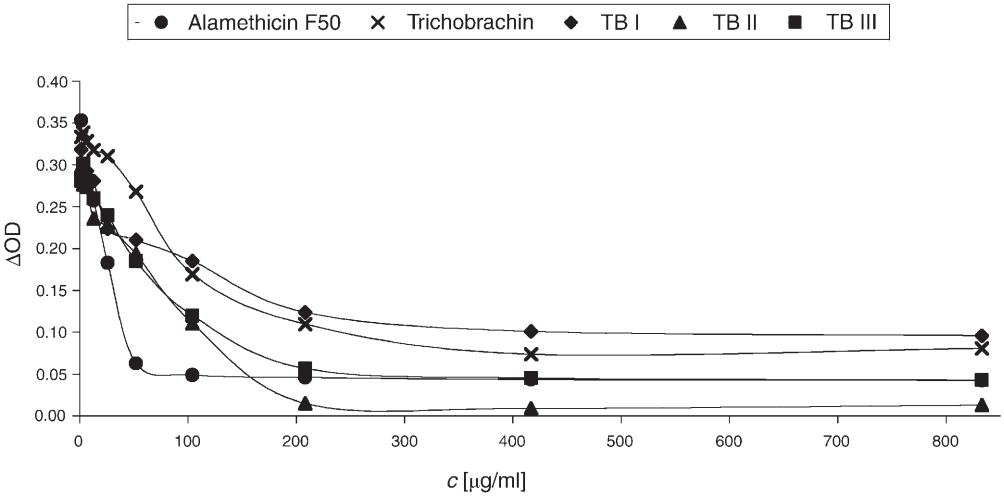

Fig. 7. *Concentration dependence* ($c = 1.6–833$ μg peptide/ml) *of the change of the optical density* (Δ OD) *at 540 nm for a suspension of* Bacillus subtilis *subsp.* subtilis, *incubated for 5 h at 30°*. Crude trichobrachin (TB), and isolated peptide groups TB I, TB II, and TB III were tested in comparison to the standard peptaibol alamethicin F50. Data are the average of three experiments.

It should be emphasized that the previous fermentation experiments [33] and those described here for the first time are separated by a time period of *ca.* 15 years. They have been conducted under similar, but somewhat different, conditions. This concerns

materials, location, instrumentation, and personnel. The data presented here prove, however, that the production of peptaibiotics and their structural features are of very good reproducibility. It is worth noting in this context that eleven-residue trichobrachins were isolated recently from a marine strain of *Trichoderma longibrachiatum* grown on a medium prepared with sea water [41].

The results also corroborate our concepts of the 'peptaibiome' and 'peptaibiomics': it is defined as the analysis of the entirety of Aib-containing peptides (peptaibiotics) produced by a fungus under defined conditions by applying analytical state-of-the-art techniques. The data also demonstrate that both the time domain and the resulting dynamics of the formation of peptaibiotics have to be considered.

Experimental Part

Chemicals. Acetonitrile (MeCN; *Chromasolve* for HPLC) and MeOH (99.8%; gradient grade, for HPLC) were purchased from *Merck-VWR* (D-Darmstadt); propan-1-ol, aq. NH_3 (25%), CH_2Cl_2, and $CHCl_3$ were from *Carl Roth* (D-Karlsruhe), CF_3COOH (TFA), $(CF_3CO)_2O$, AcOH, and Ac_2O were from *Fluka* (D-Deisenhofen). Bidistilled H_2O was used.

For enantioselective amino acid analysis, a DL-amino acid standard containing all proteinogenic amino acids of TB peptides was prepared, and suitable amounts of Aib (U, from *Sigma*), DL-Iva (synthesized in our laboratory *via* the *Strecker* procedure), L-Pheol (from *Sigma*), L-Ileol (from *Sigma*), L-Leuol (from *Fluka*), and D- and L-Valol (from *Fluka*) were added.

Malt Extract Medium. 30.0 g of light malt extract (*Serva*, D-Heidelberg) and 3.0 g of soy peptone (*Oxoid*, D-Wesel) were dissolved in 1 l of demineralized H_2O and adjusted to give a final pH of *ca.* 6.3. All media were sterilized at 121° for 20 min. For the preparation of malt extract agar (MEA), 15.0 g/l of powdered agar (*Fluka*) were added to the liquid medium.

TLC. For anal. TLC plates, pre-coated with silica gel *60*, of 0.25 mm thickness (*Merck-VWR*) were used. The mobile phase consisted of $CHCl_3$/MeOH/AcOH/H_2O 72 : 25 : 3 : 2 (*v*/*v*/*v*/*v*). About 10 µl of 1% MeOH solns. of peptides were spotted onto the plates. Peptides were visualized by spraying with H_2O and, after drying, with Cl_2/potassium iodine/*N*,*N*,*N*′,*N*′-tetramethyl-4,4′-diaminodiphenylmethane (TDM) reagent (*Fluka*) [42].

HPLC. A *Hewlett-Packard 1100* instrument, comprising a model *G1322A* degasser, a *G1312A* binary pump, a *G1313A* autosampler, a *G1316A* column thermostat, a *G1314A* UV/VIS detector, and the software *HP* ChemStation for LC (Rev. A.04.02), was used (from *Agilent*, D-Waldbronn). Trichobrachins were analyzed on a *Kromasil KR100 C_8* column, 150 mm × 4.6 mm i.d., 3.5 µm particle size (*EKA Chemicals*, S-Bohus) using a binary gradient at 35°, a flow rate of 1 ml/min, and a detection wavelength of 205 nm. Eluent *A* consisted of MeCN/MeOH/H_2O 32 : 32 : 36 (*v*/*v*/*v*) and eluent *B* of MeCN/MeOH 1 : 1 (*v*/*v*); 0.1% TFA (*v*/*v*) was added to both eluants. The gradient program was as described in *Table 4*.

Table 4. *Gradient Program for HPLC*

Time [min]	Eluent *A* [%]	Eluent *B* [%]
0	100	0
25	100	0
30	70	30
35	50	50
45	0	100
50	0	100
51	100	0
56	100	0

GC/SIM-MS. For enantioselective amino acid analysis, a GC/MS model *HP 6890* with a mass selective detector model *HP 5972* (*Agilent*) was used, together with a *Chirasil*-L-*Val*™ (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) quartz cap. column, 25 m × 0.25 mm i.d., film thickness 0.12 μm (*Varian–Chrompack*, D-Darmstadt). EI Mass spectra were recorded at ionization energy of 70 eV in the total-ion-current (TIC) and selected-ion-monitoring (SIM) mode. Aliquots of 10–30 μg of the microheterogeneous mixture of peptides from crude trichobrachin, and groups of trichobrachins I, II, and III isolated by prep. TLC, or 100–300 μg of peptides isolated from filtered cultures broths by chromatography on *LiChroprep RP-8* (*Merck*), were analyzed. Peptides were hydrolyzed with 6M HCl at 100° for 16 h. The configuration of α-amino acids and 1,2-amino alcohols was determined after conversion into *N*-trifluoroacetyl amino acid propyl esters by GC/SIM-MS [43][44]. Iva was analyzed as *N*-acetyl-isovaline propyl ester, since the enantiomers of these derivatives of DL-Iva are much better resolved on *Chirasil-Val* in comparison to the corresponding *N*-perfluoroacyl esters.

ES-MS/MS. A *LCQ*™ ion-trap MS instrument (*Thermo Electron*, D-Dreieich) was used. Trichobrachins were analyzed either by on-line HPLC/MS or *via* direct inlet (infusion). N_2 served as sheath and auxiliary gas, and He (purity >99.9990%, *Messer–Griesheim*, D-Krefeld) as collision gas. Sequence analysis was carried out by positive and negative ionizations. The *m/z* values were recorded in the centroid mode and have an accuracy of 0.5 a.m.u. Values in *Tables 2* and *3* are rounded up or down, resp. Conditions for direct infusion and the positive (negative) ionization mode were: spray voltage 4.00 kV (−4.00 kV), heated capillary temp. 230° (230°), capillary voltage +3.0 V (−3.0 V), tube lens offset +30.0 V (−30.0 V), sheath gas 50 (relative) units, auxiliary gas 5 units, maximum ion time 1000 ms. For on-line HPLC/MS, the temp. of the heated capillary was set at 250°, sheath gas at 65 units, auxiliary gas to 20 units. For automatic mass calibration, a mixture of caffeine (*m/z* 195.1), the tetrapeptide Met-Arg-Phe-Ala (*m/z* 524.3), and the perfluorinated *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.

Direct inlet ES-MS in the positive-ion mode was performed by infusion of solns. ($c = 0.1\%$ (*w/v*)) of trichobrachins in MeOH/1% AcOH 1:1 (*v/v*), and analysis in the negative-ion mode was performed *via* infusion of solns. ($c = 0.1\%$ (*w/v*)) of trichobrachins in MeOH/1% aq. NH_3 1:1 (*v/v*). For the sequence analysis of TB peptides, the $[M+H]^+$ and M^- ions were selected as precursor ions for MS^n.

The annotation used for fragment assignment in the positive-ion mode, referring to *a*, *b*, and *c* acylium ions, is in accordance to that used previously by us [3][6][13][45][46]. The negative-ion mode produced the *y* series of fragment ions without protonation [14], and is denoted y_N in *Tables 2* and *3*.

Culture and Fermentation of T. parceramosum *CBS 936.69. Trichoderma parceramosum* CBS 936.69 was obtained as freeze-dried culture from the *Centraalbureau voor Schimmelcultures* (*CBS*), Utrecht. The lyophilisate was suspended in sterile demineralized H_2O (1.5 ml) and, after soaking for 15 min, streaked out with a *Drigalski* spatula onto *Petri* dishes (9.5-cm diameter) under sterile conditions. Malt extract agar was used for cultivation. After 8 d at r.t. under diffuse daylight, rapid growth and sporulation of the mold were observed. For subculture, agar plugs (1-cm diameter) were used as an inoculation for three conical flasks (2 l), each containing 400 ml of malt extract medium. The flasks were shaken at 80 rpm at r.t. for 4 d using a rotary shaker model *G 25* (*New Brunswick*, Edison, NJ).

These cultures were used for the inoculation of fermentation broths. Inoculation of twelve conical flasks (2 l), each containing 400 ml of malt extract medium, was performed by adding 20 ml of the subculture. The flasks were shaken at 80 rpm at r.t. for 4 d.

For daily monitoring of peptaibol production, aliquots (25 ml) of the filtered culture broth were passed through a glass cartridge packed with *LiChroprep RP-8* (*Merck*), particle size 40–63 μm, cartridge size 50 × 15 mm i.d., and the peptides adsorbed were eluted with MeOH (15 ml). The eluates were evaporated to dryness, and the residues were dissolved in MeOH to give a final concentration of 1% (*w/v*). Aliquots of 20 μl were spotted onto TLC plates, and peptaibols were visualized by spraying with H_2O and TDM reagent [42].

Fermentation Experiments for Monitoring of Peptaibol Production and Degradation. For monitoring of the production and degradation of trichobrachins during the fermentation, a separate experiment with 400 ml of culture broth in 1 l conical flask was performed under the same conditions as described for the large-scale fermentation above. Aliquots of 25 ml were sampled from the culture broths every 24 h for a

period of 14 d under sterile conditions. The culture broths were cleaned up with *LiChroprep RP-8* and analyzed by TLC as described above.

Isolation of Trichobrachins from the Culture Broth and Purification of Peptides. After shaking for 4 d, intensive peptide production in the fermentation broth was detected by TLC. Culture broths (4.8 l) were separated from the mycelia by vacuum filtration. The mycelia were rinsed with distilled H_2O, and the combined filtrates were subjected to medium-pressure liquid chromatography (MPLC). Heavy-wall glass columns (380 × 37 mm i.d.) filled with *XAD-2* polystyrene adsorber resin (*Supelco*, Belefonte, PA) of 0.3–1.0 mm particle size were used together with an *MD 80/100* pump and a controller *PS 1* (*Labomatic*, D-Sinsheim). The resin was washed at flow rates of 2.5 l/h with H_2O (1 l) and MeOH/H_2O 30 : 70 (*v*/*v*) (500 ml). Peptides adsorbed were eluted with a linear gradient increasing from 30% to 100% MeOH at a flow rate of 7.5 ml/min (900 ml) and 100% MeOH (500 ml). The first 100 ml of the eluate were discarded, and fractions (15 ml) were collected, using a model *FRAC-100* fraction collector (*Pharmacia*, D-Freiburg). Elution of peptides was monitored by TLC and HPLC. Peptide-containing fractions were combined and evaporated to dryness to yield 471 mg of trichobrachins. The crude material was dissolved in MeOH (9.5 ml) and applied onto a column (100 cm × 5 cm i.d.) filled with *Sephadex LH-20* resin, particle size 25–100 μm (*Pharmacia*, D-Freiburg). Peptides were eluted with MeOH at a flow rate of 2.5 ml/min. Fractions (15 ml) were collected, and elution of peptides was monitored by TLC. Fractions containing TB were combined and evaporated to dryness yielding 309 mg of a pale yellow powder consisting of a mixture of the TB I, TB II, and TB III peptide fractions.

Separation of TB I, II, and III. Repetitive prep. TLC allowed the separation and isolation of still microheterogeneous peptide mixtures named TB I, TB II, and TB III. For their isolation, *ca.* 63 mg of the trichobrachin resulting from *Sephadex-LH 20* chromatography were dissolved in MeOH to yield a 5% methanolic soln. (*w*/*v*). Portions of 200 μl were subjected to six prep. TLC plates pre-coated with silica gel *SIL G-200* (*Macherey-Nagel*). A mixture $CHCl_3$/MeOH/AcOH/H_2O 80 : 20 : 2 : 1 (*v*/*v*/*v*/*v*) was used as the mobile phase. Trichobrachins were monitored by spraying with H_2O, and zones containing the TB I, TB II, and TB III peptide groups were marked. Then, the six plates were dried for 24 h, and the zones containing TB I, II, and III were scraped out with a lancet. The peptides were eluted from the silica gel by treatment with MeOH, then the silica gel was removed by centrifugation. The precipitate was treated (3 ×) with 10 ml of a mixture CH_2Cl_2/MeOH 1 : 1 (*v*/*v*), the silica gel was removed by centrifugation (1.600 × *g*), and the combined org. extracts were evaporated to dryness using a rotatory evaporator. These procedures provided the trichobrachin groups TB I (5.7 mg), TB II (11.7 mg), and TB III (45.1 mg).

Antibiotic Assay. Assays were conducted using 96-well microtiter plates. To aliquots of a 100-μl standard-1 nutrient broth (*Merck*), amounts of a 50 μl bacterial suspension ($c = 10^7$ colony forming units, harvested at log phase) and a 20 μl methanolic soln. of peptides ($c = 1.6$–833 mg peptide/ml) were added and incubated for 5 h at 30°. The optical densities (OD) were measured at 540 nm with a microplate reader *Titertek Multiskan MCC/340* (*Labsystems*, Espoo, Finland) spectrophotometer before and after incubation. If a turbity was observed on addition of analytes to bacterial suspensions, the difference of the optical density ($\Delta(OD) = OD_{5h} - OD_{0h}$) was used. Three independent experiments were performed.

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