Peptaibiomics: Screening for Polypeptide Antibiotics (Peptaibiotics) from Plant-Protective *Trichoderma* Species

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Dedicated to the memory of Prof. Dr. *Udo Gräfe* (1941–2003).

Eight strains of *Trichoderma* species (*T. strigosum*, *T. erinaceus*, *T. pubescens*, *T. stromaticum*, and *T. spirale* as well as *T.* cf. *strigosum*, *T.* cf. *pubescens*) were selected because of their antagonistic potential against *Eutypa* dieback and *Esca* which are fungal diseases of grapevine trunks. These isolates were screened for the production of a group of polypeptide antibiotics named *peptaibiotics*, including its subgroups *peptaibols* and *lipopeptaibols*. Fully-grown fungal cultures on potato-dextrose agar were extracted with CH_2Cl_2/MeOH, and these extracts were subjected to SPE using C_{18} cartridges. The methanolic eluates were analyzed by on-line LC/ESI-MS^n coupling – a method which is referred to as '*peptaibiomics*'. New seven-, ten-, and eleven-residue lipopeptaibols, with *N*-terminal alkanoyl, and *C*-terminal leucinol or isoleucinol residues were found and named *lipostrigocins* and *lipopubescins*. Furthermore, new 18-residue peptaibols named *trichostromaticins* and 19-residue peptaibols named *trichostrigocins* were discovered. One peptaibiotic carrying a free *C*-terminal valine (or isovaline) named *trichocompactin XII* was also sequenced. These results corroborate the hypothesis that peptaibiotics might contribute to the plant-protective action of their fungal producers. The data also point out that comparison of peptaibiotic sequences is of limited relevance in order to establish chemotaxonomic relationships among species of the genus *Trichoderma*.

1. Introduction. – *Peptaibiotics* are defined as linear peptide antibiotics which *i*) have a molecular weight between 500 and 2,200 Dalton, *ii*) show a high content of α-aminoisobutyric acid (Aib), *iii*) are characterized by the presence of other non-proteinogenic amino acids and/or lipoamino acids, *iv*) possess an acylated N-terminus, and *v*) have a C-terminal residue that, in most of them, consists of a free or MeO-substituted 2-amino alcohol, but might also be an amine, amide, free amino acid, piperazine-dione, or sugar alcohol [1][2]. Since the majority of Aib-containing peptides carries a C-terminal residue representing a 2-amino alcohol, this subgroup is referred to as '*peptaibols*'. Very lipophilic peptaibols, the N-terminus of which is acylated by octanoic, decanoic, or (*Z*)-dec-4-enoic acid, are named '*lipopeptaibols*'.

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Recently, the terms '*peptaibiome*' and '*peptaibiomics*' have been proposed [3] to describe – in analogy to the proteome and proteomics – the approach to analyze the entirety and dynamics of peptaibiotics produced by a fungal strain under defined conditions. By a rapid and selective solid-phase extraction (SPE) method with *SepPak*® C_{18} cartridges, the peptaibiotic-containing fraction was selectively absorbed, and the eluate was subsequently analyzed by HPLC coupled to an electrospray ion-trap mass spectrometer (ESI-MS^n). The advantage of ion-trap mass spectrometers for screening is the generation of a genealogy of the diagnostic product or daughter ions. For instance, every MS^2 product ion generated by collision with inert gases such as He and Ar, or high-purity N_2, can be fragmented separately, thus generating MS^3 product ions. The further fragmentation of these ions will result in MS^4 product ions and so on [2].

Peptaibiotics show unique physico-chemical and biological activities depending on particular structural properties. Those most intensively investigated are formation of pores in bilayer lipid membranes as well as antibacterial, antifungal, occasionally antiviral, insecticidal, and antiparasitic activities. Furthermore, inhibition of mitochondrial ATPase, uncoupling of oxidative phosphorylation, immunosuppression, and inhibition of platelet aggregation were reported. Recently, induction of fungal morphogenesis and neuroleptic effects have been described (for reviews, see [2][3]).

Species of *Trichoderma* with teleomorphs in *Hypocrea* [4] have attracted much academic and commercial interest as bioprotective agents against fungal pathogens. The mode of action appears to be very sophisticated and complex. It depends on the species/strain studied and may involve competition for nutrients, plant root colonization, biofertilization, stimulation of plant resistance and defence mechanisms, rhizosphere modification, and different types of mycoparasitism. The latter may involve morphological changes such as coiling of parasite hyphae around the host and the formation of specialized appressorium-like structures [5–10].

The species of *Trichoderma* are known as saprotrophs, rarely plant pathogens, or polyphagous mycoparasites which are common in soil ecosystems. During the last years, fungicolous fungi have attracted particular interest because of their bioactivity against economically important fungal diseases of crop plants, which cannot effectively be controlled by methods of classical plant protection [5].

The parallel formation and synergistic action of hydrolytic enzymes and peptaibiotics was attributed an important role in mycoparasitism of *T. harzianum* on its host *Botrytis cinerea* [11][12]. Recent studies clearly indicate the importance of the fungicolous biocontrol agent *T. asperellum* for induction of systemic and local resistance of plants to a variety of plant pathogens [10][13–15]. In addition to that, there is molecular evidence for activity regulation and direct involvement of specific exoenzymes [16] such as endochitinases [17], hexosaminidases [18], and aspartyl proteases [19] during mycoparasitism. Recently, it has been demonstrated that *T. asperellum* CBS 433.97 produces new and recurrent peptaibols of the trichotoxin A-50 family known for their antifungal and membrane activity [3].

More than 400 strains of 30 *Trichoderma* species were investigated in the course of a project aimed at preventive plant protection and biocontrol of two fungal diseases in viticulture: *Eutypa* dieback and *Esca*. These are latent trunk diseases that cause severe economic losses in grapevine production [20][21]. The *in vitro* bioactivity of the *Trichoderma* strains against the causal agents of *Eutypa* dieback, *Eutypa lata*, and *Esca*

disease, *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* has been evaluated in plate assays with crude extracts. In result, some of the most active isolates were classified as *T.* cf. *strigosum*, *T. strigosum*, *T. erinaceus*, *T.* cf. *pubescens*, *T. pubescens*, *T. stromaticum*, and *T. spirale* (*Table 1*).

Table 1. Trichoderma *Species and Strains Included in this Study*

Species	Strains investigated[a])	Habitat	Geographic origin	Yield[b]) [mg]	PAP[c]) production
T. cf. *strigosum*	BBA 69577 = CBS 119777	Compost soil	Berlin, Germany	8.2	d.[d])
T. strigosum	CBS 348.93 (*ex*-type)	Forest soil	Sengletary, North Carolina, USA	2.5	d.
T. erinaceus	DAOM 230019 = CBS 117088 (*ex*-type)	Soil	Coral Island (Koh Lann), Thailand	10.5	n. t.
T. cf. *pubescens*	BBA 66989 = CBS 119776	Soil under pines (nursery)	Rakownia near Posnan, Poland	7.5	d.
T. pubescens	DAOM 166162 = CBS 345.93 (*ex*-type)	Forest soil	Raleigh, North Carolina, USA	3.0 g	n.d.
T. stromaticum	BBA 70638 = CBS 101875 = G.J.S. 97-183 (holotype)	'Witches' broom' of cocoa (*Theobroma cacao*)	Belem, Pará, Brazil	8.0	n.d.
	BBA 70636 = CBS 101730 = G.J.S. 97-180	'Witches' broom' of cocoa (*Theobroma cacao*)	Belem, Pará, Brazil	21.4	
T. spirale	CBS 346.93 (*ex*-type)	Soil	Mekong, Thailand	1.3	n.d.

[a]) *Abbreviations:* BBA, Biologische Bundesanstalt für Land- und Forstwirtschaft, Braunschweig and Berlin, Germany; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DAOM, Eastern Cereal and Oilseed Research Centre, Ottawa, Canada; G.J.S., private collection of *Gary J. Samuels*; USDA-ARS, Systematic Botany and Mycology Laboratory, Beltsville, Maryland, USA. [b]) Dry weight of the combined methanolic extracts obtained from 10 *Petri* dishes after clean-up over *SepPak*® C_{18} cartridges (see *Exper. Part*). [c]) PAP = 6-Pentyl-2*H*-pyran-2-on and related structures. [d]) d., detected; n.d. not detected; n. t., not tested.

Compared to the bioactivity of isolates representing well-known biocontrol species (*e.g.*, *T. atroviride*, *T. harzianum*, *T. koningii*, and *T. viride*), crude extracts of the above isolates were found to inhibit the growth of the *Eutypa* and *Esca* pathogens *in vitro* far more effectively [22]. Although secondary metabolism in *Trichoderma* has been studied for decades and reviewed extensively [23][24], comparatively little is known about bioactive metabolites produced by those *Trichoderma* ssp. included in the present study.

In a recent paper, we have reported on the peptaibiome of seven plant-protective strains belonging to the *Trichoderma brevicompactum* complex and described 68 new sequences of peptaibiotics [25]. These findings demonstrate the great potential of the peptaibiomic approach as well as the efficiency and advantages of a systematic search for peptaibiotics in recently discovered *Trichoderma* species.

Some of the *Trichoderma* strains listed in *Table 1* were also shown to produce antimicrobially active volatiles such as α-pentylpyrones (6-PAP; 6-pentyl-2*H*-pyran-2-

one and related structures [22]) the production of which is elicited in the presence of plant-pathogenic fungi such as *Rhizoctonia solani* [26]. However, they were not yet screened for the production of peptaibiotics. If peptaibiotics are, in fact, present, they should considerably contribute to the antifungal activity of these strains, that are already successfully used in biocontrol, or show a great potential for possible use as biofungicides.

To characterize the '*peptaibiome*' of the *Trichoderma* spp. included in this study, we have applied the rapid and selective LC-ESI-MS^n technique defined as '*peptaibiomics*' [3]. The present study was aimed at *i*) investigation of selected plant-protective strains for the production of peptaibols and peptaibol-like antibiotics (peptaibiotics), *ii*) sequencing of new and recurrent peptides found, and *iii*) testing of the hypothesis of a possible use of the peptaibiotic patterns for chemotaxonomy.

2. Results. – *General Remarks.* General information such as habitat and geographic origin of the *Trichoderma* biocontrol strains investigated are compiled in *Table 1*. Diagnostic fragment ions *m/z* of peptaibiotics investigated in this study (excluding lipopeptaibols) are listed in *Table 2*, and their corresponding sequences are presented in *Table 3*. Diagnostic fragment ions *m/z* of all lipopeptaibols are shown in *Table 4*, and their sequences are listed in *Table 5*. Literature references may directly follow the ***italicized bold species names*** at the beginning of every new break in the *Results* section. They refer to literature providing relevant taxonomic data such as description of the *ex*-type strain and its teleomorph, if known.

HPLC Elution profiles of the peptaibiotic-containing fraction from all strains are shown in the *Figure*.

The HPLC/ESI-MS^n-based sequencing and structural characterization of peptaibiotics produced by *T.* cf. *strigosum*, *T. strigosum*, *T. erinaceus*, *T.* cf. *pubescens*, *T. pubescens*, *T. stromaticum*, and *T. spirale* are described in the following section:

***Trichoderma* cf. *strigosum* BBA 69577**. The HPLC elution profile of the strain shown in the *Figure*, *a*, is dominated by three major peaks. According to the series of *b*-type fragments generated during MS^n, the two most prominent $[M+H]^+$ ions represent compound **1**, probably tricholongin BI (*m/z* 1911), and compound **2**, tricholongin BII (*m/z* 1925). These two 19-residue peptaibols have previously been obtained from *Trichoderma longibrachiatum* MNHN 3431. Both exhibit strong membrane activity being more pronounced in the case of tricholongin BII. In contrast to tricholongin BI, the latter carries a more hydrophobic D-Iva residue instead of Aib in position 16 of the peptide chain [27]. A third, new compound **3**, **tricholongin BIII**, is characterized by the exchange of the Ser residue in position 10 against Ala ($[M+H]^+$ at *m/z* 1908) as confirmed by MS^n experiments.

CID-MS analysis of *m/z* 1061, 1075, 1089, and 1103 (all $[M+Na]^+$) and subsequent CID-MS^n analysis of diagnostic *b*-type fragments (CID: 45 eV, CE: 45 eV) indicated the presence of twelve eleven-residue lipopeptaibols, *i.e.*, compounds **4**–**15**. One of them, compound **7**, is probably identical with trikoningin KB I from *Trichoderma koningii* strain No. 90 3589 [28], whereas compound **13** or **14** may represent trichogin GA IV, which has also been isolated from *Trichoderma longibrachiatum* MNHN 3431 [29]. Comparison of sequences and retention times to those of recently reported lipopeptaibols (see *Tables 2* and *3*), revealed that compounds **6**–**14** have been detected

Table 2. *Diagnostic Fragment Ions* m/z *of New Peptaibiotics.* Produced by (**a**) *T.* cf. *strigosum* BBA 69577, (**b**) *T. strigosum* CBS 348.93, (**c**) *T. erinaceus* DAOM 230019, (**d**) *T.* cf. *pubescens* BBA 66989, (**f**) *T. stromaticum* BBA 70638, (**g**) *T. stromaticum* BBA 70636.

Diagnostic fragment ions [*m*/*z*]	**Compound number**[a]), (**Producer**), and Peptaibol										
	1 (**a**) LBI[b])	**2/26** (**a**), (**d**) LBII/LBIV	**3** (**a**) LBIII	**23** (**b**) TSG-A	**24** (**b**) TSG-B	**33** (**f**), (**g**) TSM-A	**34** (**f**), (**g**) TSM-B	**35** (**f**), (**g**) TSM-C	**36** (**f**), (**g**) TSM-D	**37** (**f**), (**g**) TSM-E	**40** (**f**), (**g**) TCT XII
$[M+Na]^+$	1933	1947	1931	1913	1913	1783	1797	1811	1825	1839	790
$[M+H]^+$	1911	1925	1909	1891	1891	1761	1775	1789	1803	1817	768
$[M-H_2O+H]^+$	1893	1907	1891	1873	1873	n.d.	1757	1771	1785	1799	750
b_1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	128	n.d.	n.d.	n.d.
b_2	185	185	185	199	199	n.d.	199	199	199	n.d.	184
b_3	332	332	332	284	284	284	284	284	284	284	255
b_4	417	417	417	369	369	355	355	355	355	355	368
b_5	502	502	502	482	482	440	454	454	454	454	467
b_6	n.d.	n.d.	n.d.	610	n.d.	525	539	553	553	553	538
b_7	715	715	715	695	695	n.d.	n.d.	n.d.	n.d.	n.d.	651
b_8	800	800	800	780	780	738	752	766	766	766	
b_9	885	885	885	865	865	837	851	865	n.d.	879	
$b_{10}-H_2O$	n.d.	n.d.	n.d.	n.d.	933	n.d.	n.d.	n.d.	n.d.	n.d.	
b_{10}	972	972	956	952	952	922	936	950	950	964	
b_{11}	1085	1085	1069	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
b_{12}	1171	1171	1154	1150	1150	1135	1149	1163	1163	1177	
$b_{13}-b_{15}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
b_{16}	1538	1551	1535	1517	1517	1515	1529	1543	1557	1571	
b_{17}	1794	1807	1791	1645	1645	n.d.	n.d.	n.d.	n.d.	n.d.	
b_{18}				1773	1773						
y_6						626	626	626	640	640	
y_6-H_2O						n.d.	n.d.	n.d.	622	622	
y_7				741	741						
y_8				n.d.	826						
[Pro-Lxx-Aib-Aib-Gln+H]$^+$						510	510	510	524	524	
[Pro-Lxx-Aib-Aib+H]$^+$						381	381	381	395	395	
[Pro-Lxx-Aib+H]$^+$						296	296	296	296	296	
[Pro-Lxx+H]$^+$						211	211	211	211	211	

[a]) Arabic numbers in bold refer to the consecutive numbering of peptides used throughout the text and in the *Figure.* [b]) *Abbreviations:* LB, tricholongin B; TSG, trichostrigocin; TSM, trichostromaticin; TCT, trichocompactin.

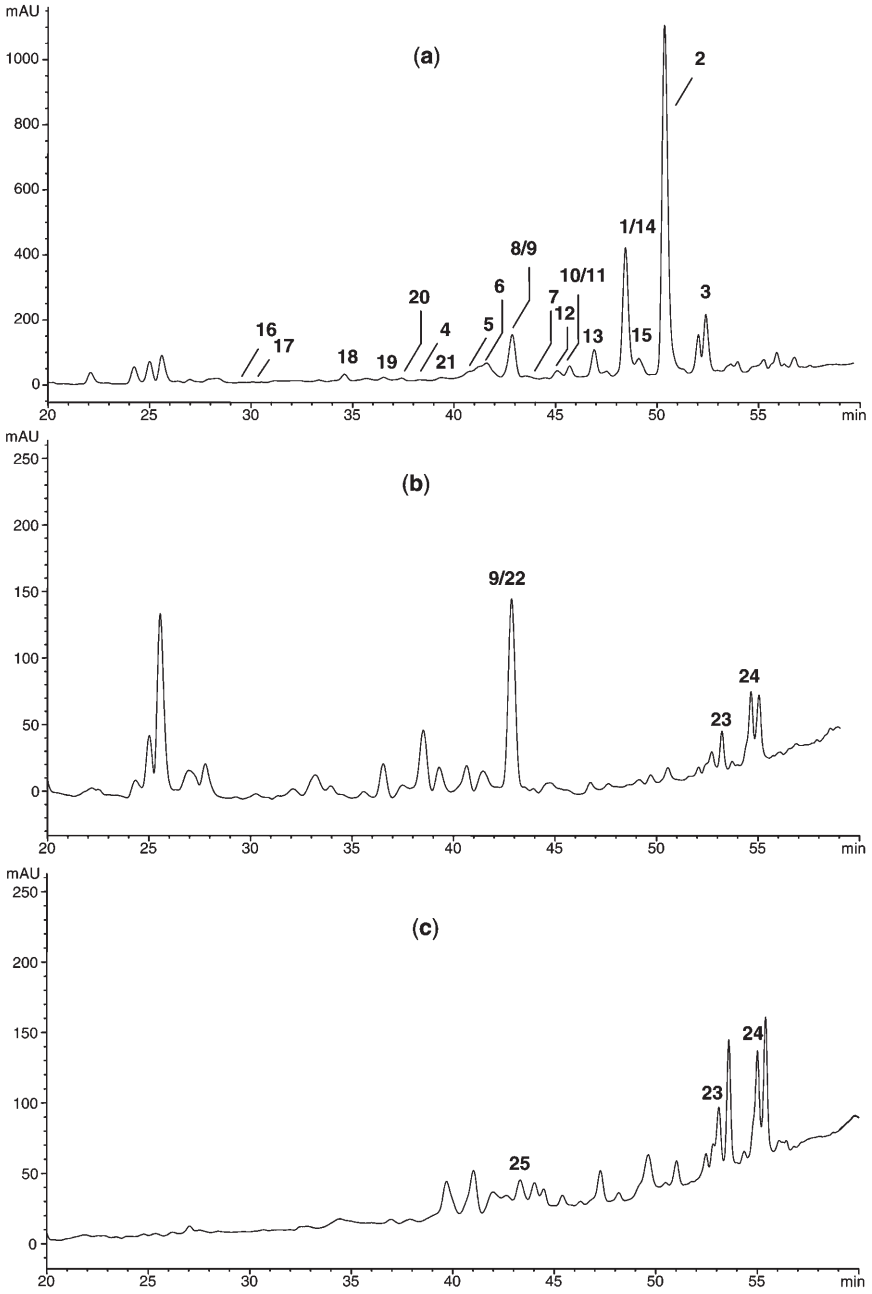

Figure. *HPLC Elution Profiles.* Peptaibiotic-containing fraction of (**a**) *Trichoderma* cf. *strigosum* BBA 69577, (**b**) *Trichoderma strigosum* CBS 348.93, (**c**) *Trichoderma erinaceus* DAOM 230019, (**d**) *Trichoderma* cf. *pubescens* BBA 66989, (**f**) *Trichoderma stromaticum* BBA 70638, (**g**) *Trichoderma stromaticum* BBA 70636, and (**h**) *Trichoderma spirale* CBS 346.93. Note that no HPLC elution profile is available for *Trichoderma pubescens* CBS 345.93 (**e**). Annotations refer to consecutive numbering of peptides used in the text. Numbers separated by a slash refer to co-eluting peptides.

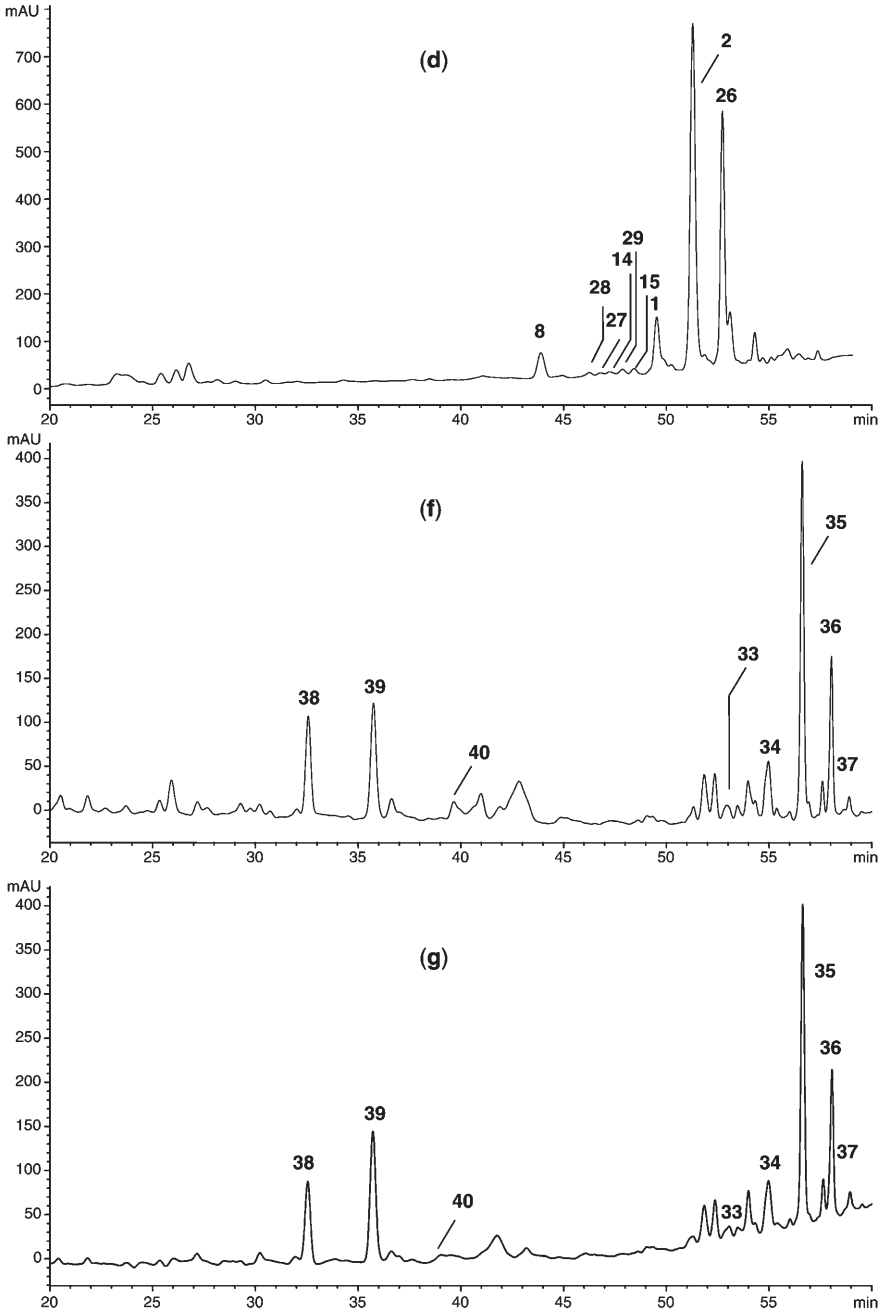

Figure (cont.)

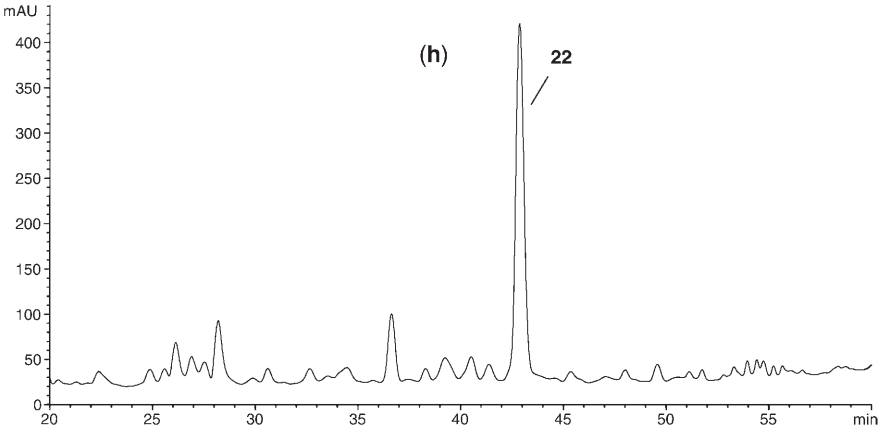

Figure (cont.)

in cultures of *Hypocrea vinosa* CBS 247.63 and *Hyporea lactea* CBS 853.70 [3]. In contrast to that, compounds **4**, **5**, and **15** represent new sequences.

According to structural homologies, the N-terminus of compounds **4** and **5** might be protected by an α- or γ-Me-branched C_6 fatty acid. N-Terminal substitution of the peptide chain with 2- or 4-Me-branched fatty acids was first described for leucinostatins, as compiled in [30], but later defined to be a characteristic feature of all lipoaminopeptides and structurally related peptaibiotics [2].

In addition to the eleven-residue lipopeptaibols **4**–**15**, the strain produced six new, truncated seven-residue lipopeptaibols, *i.e.*, compounds **16**–**21**, lacking the internal pentapeptide Aib-Gly-Gly-Vxx/Lxx-Aib. The slash in the partial sequence refers to the possible amino acid exchange of Vxx against Lxx. We suggest the name ***lipostrigocin*** (**LSG**) **A1**–**A6** for the seven-residue **lipo**peptaibols from *T.* **strigo***sum*, whereas the eleven-residue **lipo**peptaibols, compounds **4**–**6**, **8**–**12**, and **15**, are **lipostrigocin B1**–**B9**.

***Trichoderma strigosum* CBS 348.93** [31–33]. The main peak in the HPLC elution profile (*Fig.*, *b*) consists of at least two different ions. One of these, displaying $[M+Na]^+$ at *m/z* 1075 is probably identical with compound **9** by comparison of retention time and CID-MS fragmentation. The other one, compound **22**, is assumed to represent the eleven-residue peptaibol trichobrevin B-IIIc recently described for *Trichoderma* cf. *brevicompactum* ATCC 90237 (=CBS 119576), IBT 40863 (=CBS 119577), and NRRL 3199 [25].

The two main peaks of four ions displaying $[M+H]^+$ at *m/z* 1891 were analyzed by MS^2 experiments, followed by MS^3 of the MS^2 product ion at *m/z* 1150 from the $[M+H]^+$ ion, and by MS^4 of *m/z* 780 generated from *m/z* 1150. CID-MS Data were used to disclose the structure of the N-termini. The following sequence is proposed for both compounds, **23** and **24**:

Ac-Aib-Ala-Aib-Aib-Lxx-Gln-Aib-Aib-Aib-Ser-Lxx-Aib-Pro-Vxx-Aib-Aib-Gln-Gln-Lxxol

The N-terminal tripeptide, Ac-Aib-Ala-Aib, is very common among *Trichoderma* peptaibiotics [34][35]. The underlined partial sequence might be identical with amino

Table 3. *Sequences of New Peptaibiotics.* Produced by (**a**) *T.* cf. *strigosum* BBA 69577, (**b**) *T. strigosum* CBS 348.93, (**c**) *T. erinaceus* DAOM 230019, (**d**) *T.* cf. *pubescens* BBA 66989, (**f**) *T. stromaticum* BBA 70638, and (**g**) *T. stromaticum* BBA 70636.

	Peptaibiotic	(**Producer**)	Residue number																			$[M+H]^+$
[a])	[b])		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1	LBI	(**a**)	Ac	Aib	Gly	Phe	Aib	Aib	Gln	Aib	Aib	Aib	Ser	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Lxxol	1911
2	LBII	(**a**)	Ac	Aib	Gly	Phe	Aib	Aib	Gln	Aib	Aib	Aib	Ser	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Lxxol	1925
3	LBIII	(**a**)	Ac	Aib	Gly	Phe	Aib	Aib	Gln	Aib	Aib	Aib	Ala	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Lxxol	1908
26	LBIV	(**d**)	Ac	Aib	Gly	Phe	Aib	Aib	Gln	Aib	Aib	Aib	Ser	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Lxxol	1925
23	TSG-A	(**b**), (**c**)	Ac	Aib	Ala	Aib	Aib	Lxx	Gln	Aib	Aib	Aib	Ser	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Lxxol	1891
24	TSG-B	(**b**), (**c**)	Ac	Aib	Ala	Aib	Aib	Lxx	Gln	Aib	Aib	Aib	Ser	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Lxxol	1891
33	TSM-A	(**f**), (**g**)	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gln	Aib	Pro	Lxx	Aib	Aib	Gln	Lxxol		1761
34	TSM-B	(**f**), (**g**)	Ac	Aib	Ala	Aib	Ala	Vxx	Aib	Gln	Aib	Vxx	Aib	Gln	Aib	Pro	Lxx	Aib	Aib	Gln	Lxxol		1775
35	TSM-C	(**f**), (**g**)	Ac	Aib	Ala	Aib	Ala	Vxx	Vxx	Gln	Aib	Vxx	Aib	Gln	Aib	Pro	Lxx	Aib	Aib	Gln	Lxxol		1789
36	TSM-D	(**f**), (**g**)	Ac	Aib	Ala	Aib	Ala	Vxx	Vxx	Gln	Aib	Vxx	Aib	Gln	Aib	Pro	Lxx	Aib	Vxx	Gln	Lxxol		1803
37	TSM-E	(**f**), (**g**)	Ac	Aib	Ala	Aib	Ala	Vxx	Vxx	Gln	Aib	Lxx	Aib	Gln	Aib	Pro	Lxx	Aib	Vxx	Gln	Lxxol		1817
40	TCT XII	(**f**), (**g**)	Ac	Aib	Gly	Ala	Lxx	Aib	Ala	Lxx	Vxx												768

[a]) Arabic numbers in bold refer to the consecutive numbering of peptides used throughout the text and in the *Figure.* [b]) *Abbreviations:* LB, tricholongin B; TSG, trichostrigocin; TSM, trichostromaticin; TCT, trichocompactin.

acid positions 6–19 of compound **2**, tricholongin BII. We suggest the names **Trichostrigocins** (**TSG**) **A** and **B** for these new 19-residue peptaibols from **Tricho***derma* **strigo***sum*.

***Trichoderma erinaceus* DAOM 230019** [36]. Four pseudomolecular ions *m/z* 1891 were detected, as described above for *Trichoderma strigosum* CBS 348.93. Comparison of CID-MS data and retention times (see *Fig.*, *c*) indicated that the two predominant $[M+H]^+$ ions, could be identical with compounds **23** and **24**.

The strain also produces a mixture of homologous lipopeptaibols. According to CID-MS data, compound **25**, displaying $[M+Na]^+$ at *m/z* 1075, could be identical or positionally isomeric with trikoningin KB II from *Trichoderma koningii* strain No. 90 3589 [28].

***Trichoderma* cf. *pubescens* BBA 66989**. The HPLC elution profile as shown in the *Figure*, *d*, is dominated by three peaks: the first one is at *m/z* 1911, whereas both the second and third one are displayed at *m/z* 1925 (all $[M+H]^+$). Comparison of the CID-MS and MS^n spectra with results obtained for *Trichoderma* cf. *strigosum* BBA 69577 and literature data revealed the presence of compounds **1** and **2**. The third peak, compound **26**, named tricholongin BIV, is a positional isomer of compound **2**, as concluded on the basis of the CID-MS and MS^n data.

The first lipopeptaibol displaying $[M+Na]^+$ at *m/z* 1075 is assumed to be identical with compound **8**. Its positional isomer, compound **27**, displays an exchange of Lxx^3 against Vxx^3, whereas Vxx^7 is replaced by Lxx^7. That lipopeptaibol has previously been reported as sequence 3 from *Hypocrea lactea* CBS 853.70 [3]. Compound **28**, displaying $[M+Na]^+$ at *m/z* 1089, is considered to carry an *N*-terminal Oc-Vxx, whereas Lxx^7 is replaced by Vxx^7. Its positional isomer is probably identical with compound **14**. In contrast to that, compound **29**, the third positional isomer, may also carry an *N*-terminal Oc-Vxx, but Lxx^3 is replaced by Vxx^3, whereas Lxx instead of Vxx is situated in position 7 of the peptide chain. The lipopeptaibol displaying $[M+Na]^+$ at *m/z* 1103 is supposed to be identical with compound **15**. Owing to the positional isomerism with the lipostrigocins B1–B9, compounds **4**–**6**, **8**–**12**, and **15**, we suggest to continue that consecutive numbering. Thus, compounds **27**–**29** are named **lipostrigocins B10**–**B12**.

***Trichoderma pubescens* CBS 345.93** [31][32]. Three novel ten-residue lipopeptaibols displaying modified sequences were preparatively isolated and sequenced from the *ex*-type strain of *T. pubescens*. The general structure of their N-terminal tetrapeptide was elucidated as Oc-Aib-Gly-Vxx/Lxx-Aib, but the remaining sequence, except for the C-terminal Lxxol residue, is slightly different from all other lipopeptaibols described in this study. We propose the names **linopubescin** (**LPB**) **A**, **B**, and **C** for those new compounds **30**–**32**, respectively, from *Trichoderma pubescens* (*Tables 4* and *5*). The presence of the amino acids Gly, Aib, Vxx, Lxx and *C*-terminal Lxxol in the hydrolysates was confirmed by detection of the respective $[M+H]^+$ ions in the positive ionization mode of the LCQ, whereas the negative ionization mode was applied to detect the $[M-H]^-$ ion of the octanoic acid at *m/z* 143.

***Trichoderma stromaticum* BBA 70638 and BBA 70636** [37][38]. The main peak of both HPLC elution profiles shown in the *Figure*, *f* and *g*, respectively, displays a protonated pseudomolecular ion *m/z* 1789. It is accompanied by four homologues displaying $[M+H]^+$ at *m/z* 1761, 1775, 1803, and 1817. These five 18-residue peptaibols, compounds **33**–**37**, represent new sequences, named **trichostromaticins** (**TSM**) **A**–**E**.

Table 4. *Diagnostic Fragment Ions* m/z *of Lipopeptaibols.* Produced by (**a**) *T.* cf. *strigosum* BBA 69577, (**b**) *T. strigosum* CBS 348.93, (**c**) *T. erinaceus* DAOM 230019, (**d**) *T.* cf. *pubescens* BBA 66989, (**e**) *Trichoderma pubescens* CBS 345.93.

Diagnostic fragment ions [*m/z*]	**Compound number**, Lipopeptaibol, and (**Producer**)												
	4[a]) (**a**) LSG B1	**5** (**a**) LSG B2	**6** (**a**) LSG B3	**7** (**a**) KB I	**8** (**a**), (**d**) LSG B4	**9** (**a**), (**b**) LSG B5	**10** (**a**) LSG B6	**11** (**a**) LSG B7	**12** (**a**) LSG B8	**13** (**a**) GA IV	**14** (**a**) GA IV	**15** (**a**) LSG B9	**16** (**a**) LSG A1
$[M+Na]^+$	1061	1061	1061	1061	1075	1075	1075	1075	1089	1089	1089	1103	740
$[M+H]^+$	1039	1039	1039	1039	1053	1053	1053	1053	1067	1067	1067	1081	726
b_1	198	198	212	212	212	212	212	212	226	212	212	226	198
b_2	255	255	269	269	269	269	269	269	283	269	269	283	255
b_3	368	354	368	368	368	382	382	368	396	382	382	396	368
b_4	453	439	453	453	453	467	467	453	481	467	467	481	453
b_5	510	496	510	510	510	524	524	510	538	524	524	538	510
b_6	567	553	567	567	567	581	581	567	595	581	581	595	623
b_7	666	666	666	666	680	680	680	680	694	694	694	708	
b_8	751	751	751	751	765	765	765	765	779	779	779	793	
b_9	808	808	808	808	822	822	822	822	836	836	836	850	
b_{10}	921	921	921	921	935	935	935	935	949	949	949	963	
a_1	n.d.	n.d.	184	184	n.d.	n.d.	n.d.	n.d.	n.d.	184	184	n.d.	
a_2	n.d.	n.d.	n.d.	n.d.	241	241	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
a_3	n.d.	326	n.d.	n.d.	n.d.	354	n.d.	n.d.	n.d.	354	354	n.d.	n.d.
a_7	n.d.	n.d.	638	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	

Diagnostic fragment ions [*m/z*]	**Compound number**, Lipopeptaibol, and (**Producer**)											
	17 (**a**) LSG A2	**18** (**a**) LSG A3	**19** (**a**) LSG A4	**20** (**a**) LSG A5	**21** (**a**) LSG A6	**25** (**c**) KB II	**27** (**d**) LSG B10	**28** (**d**) LSG B11	**29** (**d**) LSG B12	**30** (**e**) LPB-A	**31** (**e**) LPB-B	**32** (**e**) LPB-C
$[M+Na]^+$	740	776	776	790	790	1075	1075	1089	1089	1060	1074	1088
$[M+H]^+$	726	754	754	768	768	1053	1053	1067	1067	1038	1052	1066
b_1	212	212	212	226	226	226	212	226	226	212	212	212
b_2	269	269	269	283	283	283	269	283	283	269	269	269
b_3	368	382	382	396	396	382	368	396	382	368	382	382
b_4	453	467	467	481	481	467	453	481	467	453	467	467
b_5	524	524	524	538	538	524	510	538	524	552	566	566
b_6	623	637	637	651	n.d.	581	567	595	581	665	679	679
b_7						680	680	694	680	750	764	764
b_8						765	765	779	765	807	821	863
b_9						822	822	836	822	920	934	948
b_{10}						935	935	949	935			
a_1	n.d.	n.d.	n.d.	198	n.d.	198	n.d.	n.d.	n.d.			
a_3	340	354	354	368	n.d.	354	n.d.	n.d.	n.d.			

[a]) Arabic numbers in bold refer to the consecutive numbering of peptides used throughout the text and in the *Figure.*

Table 5. *Sequences of Lipopeptaibols* Produced by (**a**) *T.* cf. *strigosum* BBA 69577, (**b**) *T. strigosum* CBS 348.93, (**c**) *T. erinaceus* DAOM 230019, (**d**) *T.* cf. *pubescens* BBA 66989, (**e**) *T. pubescens* CBS 345.93.

[a])	Lipopeptaibol	(**Producer**)	Residue numbers											$[M+Na]^+$
				1	2	3	4	5	6	7	8	9	10	11	
4	LSG B1	(**a**)		198[b])	Gly	Lxx	Aib	Gly	Gly	Vxx	Aib	Gly	Lxx	Lxxol	1061
5	LSG B2	(**a**)		198[b])	Gly	Vxx	Aib	Gly	Gly	Lxx	Aib	Gly	Lxx	Lxxol	1061
6	LSG B3	(**a**)	Oc[c])	Aib	Gly	Vxx	Aib	Gly	Gly	Vxx	Aib	Gly	Lxx	Lxxol	1061
7	KB I	(**a**)	Oc	Aib	Gly	Vxx	Aib	Gly	Gly	Vxx	Aib	Gly	Lxx	Lxxol	1061
8	LSG B3	(**a**), (**d**)	Oc	Aib	Gly	Vxx	Aib	Gly	Gly	Lxx	Aib	Gly	Lxx	Lxxol	1075
9	LSG B4	(**a**), (**b**)	Oc	Aib	Gly	Lxx	Aib	Gly	Gly	Vxx	Aib	Gly	Lxx	Lxxol	1075
10	LSG B5	(**a**)	Oc	Aib	Gly	Lxx	Aib	Gly	Gly	Vxx	Aib	Gly	Lxx	Lxxol	1075
11	LSG B6	(**a**)	Oc	Aib	Gly	Vxx	Aib	Gly	Gly	Lxx	Aib	Gly	Lxx	Lxxol	1075
12	LSG B7	(**a**)	Oc	Vxx	Gly	Lxx	Aib	Gly	Gly	Vxx	Aib	Gly	Lxx	Lxxol	1089
13	GA IV	(**a**)	Oc	Aib	Gly	Lxx	Aib	Gly	Gly	Lxx	Aib	Gly	Lxx	Lxxol	1089
14	GA IV	(**a**)	Oc	Aib	Gly	Lxx	Aib	Gly	Gly	Lxx	Aib	Gly	Lxx	Lxxol	1089
15	LSG B8	(**a**)	Oc	Vxx	Gly	Lxx	Aib	Gly	Gly	Lxx	Aib	Gly	Lxx	Lxxol	1103
16	LSG A1	(**a**)		198[b])	Gly	Lxx	Aib	Gly	Lxx	Lxxol					762
17	LSG A2	(**a**)	Oc	Aib	Gly	Lxx	Aib	Gly	Vxx	Lxxol					762
18	LSG A3	(**a**)	Oc	Aib	Gly	Lxx	Aib	Gly	Lxx	Lxxol					776
19	LSG A4	(**a**)	Oc	Aib	Gly	Lxx	Aib	Gly	Lxx	Lxxol					776
20	LSG A5	(**a**)	Oc	Vxx	Gly	Lxx	Aib	Gly	Lxx	Lxxol					790
21	LSG A6	(**a**)	Oc	Vxx	Gly	Lxx	Aib	Gly	Lxx	Lxxol					790
25	KB II	(**c**)	Oc	Vxx	Gly	Vxx	Aib	Gly	Gly	Vxx	Aib	Gly	Lxx	Lxxol	1075
27	LSG B9	(**d**)	Oc	Aib	Gly	Vxx	Aib	Gly	Gly	Lxx	Aib	Gly	Lxx	Lxxol	1075
28	LSG B10	(**d**)	Oc	Vxx	Gly	Lxx	Aib	Gly	Gly	Vxx	Aib	Gly	Lxx	Lxxol	1089
29	LSG B11	(**d**)	Oc	Vxx	Gly	Vxx	Aib	Gly	Gly	Vxx	Aib	Gly	Lxx	Lxxol	1089
30	LPB-A	(**e**)	Oc	Aib	Gly	Vxx	Aib	Vxx	Lxx	Aib	Gly	Lxx	Lxxol		1060
31	LPB-B	(**e**)	Oc	Aib	Gly	Lxx	Aib	Vxx	Lxx	Aib	Gly	Lxx	Lxxol		1074
32	LPB-C	(**e**)	Oc	Aib	Gly	Lxx	Aib	Vxx	Lxx	Aib	Vxx	Aib	Lxxol		1088

[a]) Arabic numbers in bold refer to the consecutive numbering of peptides used throughout the text and in the *Figure*. [b]) The fragment ion at *m/z* 198 is tentatively assigned to a α- or γ-branched, saturated C_6 fatty acid residue; [c]) Oc: Octanoyl.

Compounds **35** and **36** might be identical with the partial sequences 3 and 4 recently published for *T. stromaticum* CBS 101875 [3]. The N-terminal sequence, Ac-Aib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib, comprising positions 1–10, was first reported for the following 20-residue peptaibols, namely paracelsin C from *T. reesei* QM 9414 [39], trichosporin B-IVc from *T. polysporum* TMI 60146 [40], trichokonin VIII from a strain identified as *T. koningii* [41], and for longibrachins A III and A IV, from *T. longibrachiatum* M 853431 [42]. The isobaric sequence with Iva^8 instead of Val^8 has not yet been published. The C-terminal motif, Aib-Pro-Leu-Aib-Aib-Gln-Leuol, comprising positions 12–18, has been described for the following 18-residue peptaibols, namely trichokindins IIb, IIIa, IIIb, and VI from a strain classified as *T. harzianum* [43], trichorzins HA I and HA III from *T. harzianum* M 903602 and M 922835 [44][45], for hypomurocins B II and B V from *T. atroviride*, formerly described as *Hypocrea muroiana* IFO 31288 [46], and for trichovirins Ia, Ib, IIa, IIb, IIc, IIIa, IVa, IVb, V, and VIb from '*T. viride*' NRRL 5243 [47]. The strain '*T. viride*' NRRL 5243 is currently deposited as *T.* cf. *harzianum*. To date, isobaric C-terminal sequences with Ile instead of Leu and Leuol replaced by Ileol have not been described.

In addition to these 18-residue peptaibols described above, these strains also produced eight-residue, Ser-containing peptaibiotics. They are assumed to be identical with the trichocompactins (TCT) V (compound **38**; $[M+H]^+$ at *m/z* 770) and VI (compound **39**; $[M+H]^+$ at *m/z* 784) recently reported for *T.* cf. *brevicompactum* ATCC 90237, IBT 40863, and NRRL 3199 – as concluded by comparison of the fragmentation pattern and retention times [25]. Another new homologue, compound **40**, carrying Ala^6 instead of Gly^6 was named TCT XII ($[M+H]^+$ at *m/z* 768).

***Trichoderma spirale* CBS 346.93** [31–33]. The main peak in the HPLC elution profile, shown in the *Figure*, *h*, is compound **22**. It displays $[M+Na]^+$ at *m/z* 1157. Comparison of the fragmentation pattern and retention times indicated that it can be assumed identical with eleven-residue peptaibol trichobrevin B-IIIc which has recently described for *T.* cf. *brevicompactum* ATCC 90237, IBT 40863, and NRRL 3199 [25]. Other minor compounds with *m/z* 1127, 1113, and 1143 (all $[M+Na]^+$) were present in the total ion current, but their intensity was not sufficient to perform MS^n experiments in order to confirm the structures of these ions as other members of the trichobrevin B peptaibols.

3. Discussion. – *Correlation of Peptaibiotic Production and Antifungal Activity. Peptaibols.* Long-chain peptaibols are known to form voltage-gated or non-gated ion channels in bilayer lipid membranes. The carboxyfluorescein leakage in small unilamellar egg phosphatidylcholine vesicles with 20–30% cholesterol, induced by tricholongins BI and BII, was demonstrated to be higher than that caused by the acidic trichorzianine A IIIc [27]. In single-channel experiments [48], the mode of action of trichorzianine A IIIc was shown to be consistent with the flip-flop gating model introduced in [49]. The high structural homology of the 19-residue trichorzianine A IIIc with the 19-residue tricholongins BI, BII, and BIII (*i.e.*, compounds **1**–**3**), as well as the 19-residue trichostrigocins A and B (compounds **23**/**24**), suggests a similar mode of membrane activity. The C-terminal fragment of the 18-residue trichostromaticins A–E (*i.e.*, compounds **30**–**35**), represents a six-residue deletion sequence of that of tricholongins BI, BII, and BIII. Obviously, it lacks one of the two Gln residues attached

to the terminal Lxxol (compounds **1**–**3**). Glu/Gln Residues appear to be located in the pore lumen, which is important for conductance of the ion channel [34]. Nevertheless, deletion of one Glu^{18} in a synthetic alamethicin has been demonstrated not to cause a decrease in lifetime values of single ion channels as compared to natural alamethicin [50]. Thus, trichostromaticins A–E may contribute to the potent bioactivity of *T. stromaticum:* Hyperparasitic strains of this species, so far known only as a biologically active cocoa endophyte, have successfully been introduced in field control of the causal agent of *Witches' Broom Disease* of cacao (*Theobroma cacao*) in South America. They were shown to effectively suppress basidioma formation of the plant pathogen *Crinipellis perniciosa* [37][51].

Lipopeptaibols. The eleven-residue trichogin GA IV, the 'classical' paradigm for lipopeptaibols [52], was suggested to form an amphiphilic, right-handed mixed $3_{10}/\alpha$-helical structure in methanolic solution [29]. The same behavior was reported for trikoningins KB I and KB II [53]. Trichogin GA IV exhibits a considerable membrane-perturbing activity which is illustrated by a two-state transition controlled by peptide concentration: at low concentrations, the molecule is situated parallel to the membrane surface in a 'carpet-like' manner with its four polar Gly residues oriented towards the aqueous phase. Hydrophobic residues, including the *N*-octanoyl group as well as $Leu^{3,7}$ and Ile^{10}, face the membrane, thus stabilizing the interaction between the peptide and the lipid bilayer. By increasing peptide concentration until membrane leakage is observed, a cooperative transition occurs. Aggregates of trichogin GA IV are deeply incorporated into the bilayer where they are assumed to form ion channels [54].

Chemotaxonomic Relevance of Peptaibiotics. Peptaibols. As *T. stromaticum* BBA 70638 and BBA 70636 were shown to produce trichocompactins, occurrence of these peptaibiotics is also found in the *T. brevicompactum* complex. Furthermore, the production of trichobrevins B by both *T. strigosum* (section *Trichoderma*, clade *Viride*) and *T. spirale* that has tentatively been grouped into a so-called '*lone lineage*' of section *Pachybasium B* [55] indicated a rather limited relevance of these peptaibiotics for chemotaxonomic purposes.

Lipopeptaibols. The seven-, ten-, and eleven-residue lipopeptaibols listed in *Tables 4* and *5* were produced by *Trichoderma* species grouped in section *Trichoderma*, *H. rufa* clade (*T. koningii*, *T. strigosum*, *T.* cf. *strigosum*, and *T. erinaceus*), and clade *Pachybasium A* (*T. pubescens*, *T.* cf. *pubescens*). The lipopeptaibol-producing *Hypocrea vinosa* CBS 247.63 [3] is closely related to *T. viride* (*sensu stricto*), which also belongs to the *H. rufa* clade (see above), whereas *Hypocrea citrina* (syn. *H. lactea*) CBS 853.70 [3] belongs to a *Hypocreanum* subclade of section *Pachybasium B.* Production of lipopeptaibols, however, has also been reported for *T. longibrachiatum* MNHN 3431 [29], belonging to section *Longibrachiatum* [56]. Obviously, biosynthesis of lipopeptaibol antibiotics is widespread throughout the genus *Trichoderma*; but has also been described for *Tolypocladium geodes* (*Hypocreales*; *Clavicipitaceae* [57][58]). The genera *Tolypocladium* and *Trichoderma* are morphologically superficially similar as illustrated by the fact that *Tolypocladium inflatum*, the 'principal' source of cyclosporine [59], was originally misidentified as *Trichoderma polysporum* [60].

4. Conclusions. – Generally, it is recommended to thoroughly identify new peptaibiotic-producing isolates. Considering recent progress in molecular taxonomy of

Trichoderma, a critical review and – if necessary – revision of identity of peptaibiotic-producing strains is highly advisable:

To date, *ca.* 90 *Trichoderma* species are characterized by sequencing of ribosomal DNA, and these data indicate that many species recognized exclusively on the basis of morphology have probably been misidentified in the past [4][55]. For instance, the trichobrachin-producing *T. longibrachiatum* CBS 936.69 [61] is now re-classified as *T. ghanense*.

Trichoderma strains that have warted conidia are traditionally identified as *T. viride*, the type species of the genus. Recently, that species was subdivided into two species – *T. viride* and *T. asperellum* – on the basis of ribosomal DNA analysis, but many more species will have to be distinguished in this clade. Antibiotic production was exclusively restricted to *Trichoderma asperellum*, while *T. viride* (*sensu stricto*) produced no antibiotics [62][63]. In a preceding paper, we have shown the affiliation of the alamethicin patent strain, formerly known as '*T. viride*' NRRL 3199, to *T.* cf. *brevicompactum*, and pointed out the necessity to establish a new species that is closely related to *T. brevicompactum* [25]. The trichovirin-producing strain '*T. viride*' NRRL 5243 [47] is currently deposited as *T. harzianum* (*sensu lato*).

Like in other chemotaxonomical studies, it can be concluded that secondary metabolites still seem to be of questionable chemotaxonomic importance. Literature data clearly support this opinion, since fungi belonging to divergent taxonomic groups may produce very similar sequences of peptaibiotics [31][32].

Despite this, the patterns of peptaibiotics produced may occasionally be used as additional markers to distinguish similar species and to supplement results of morphological, molecular, and (bio)chemical approaches in modern taxonomy. As species identification of *Trichoderma* strains was demonstrated to be possible by image analysis of HPLC chromatograms [64], on-line coupling of HPLC and ESI-Ion-Trap-MS^n should, therefore, combine the advantages of both analytical techniques, thus providing a more reliable structural identification of compounds produced by a certain strain. To confirm this hypothesis, we have recently shown that the LC/MS fingerprint of characteristic non-peptidic mycotoxins (trichothecenes [65]) and peptaibiotics (20-residue alamethicins and eight-residue trichocompactins [25]) might be used in addition to morphological and molecular data to separate the '*Brevicompactum* complex' from other taxa of the genus *Trichoderma*.

Financial support by the *Studienstiftung Mykologie* (D-Cologne) and the *Bundesprogramm Ökologischer Landbau* (BLE, D-Bonn) is gratefully acknowledged.

Experimental Part

Chemicals. MeCN (*Chromasolve* for HPLC, far-UV; 99.9%) and CH_2Cl_2 (*ACS* reagent; 99.6%) were obtained from *Sigma-Aldrich* (D-Steinheim), MeOH (99.8%, gradient grade, for HPLC) and CF_3COOH (TFA; 98.0%) were purchased from *Fluka* (D-Steinheim). AcOEt, Na_2SO_4 (all anal. grade), and silica gel were obtained from *Merck-VWR* (D-Darmstadt). Bidistilled water was freshly prepared from demineralized tap water prior to analysis using a quartz distill (*Heraeus*, D-Kleinostheim).

Cultivation of Strains. Extraction of fully-grown agar-plates was performed as described in [25]. Clean-up of extracts by solid-phase extraction was performed according to [3] using *SepPak*® C_{18} cartridges.

Cultivation of Trichoderma pubescens *CBS 345.93, and Extraction of Linopubescins A, B, and C.* The strain was cultivated as surface culture (60 l) at a temp. of 26° in 500-ml conical flasks containing 100 ml of malt medium composed as follows (g/l): malt extract (20), glucose (10), yeast extract (1), $(NH_4)_2SO_4$ (5); pH 6.0. After 20 d of cultivation, the culture broth was harvested and separated by filtration. Thereafter, the culture filtrate and the mycelium were extracted twice with AcOEt. The combined extracts were dried (Na_2SO_4) and evaporated to dryness *in vacuo*. The dry residue (3 g) was subjected to silica-gel chromatography (silica gel *60*, 0.063–0.1 mm; column 600 × 40 mm; $CHCl_3$/MeOH, 9:1 (*v*/*v*)), and 20-ml fractions were collected. Linopubescin-containing samples were detected by ESI-MS. Final purification was achieved by isocratic prep. HPLC (*Spherisorb ODS-2*, 5 μm, RP_{18}, *Promochem*, 250 × 25 mm, MeCN/H_2O 83:17 (*v*/*v*); 12 ml/min, λ 210 nm).

Instruments and Conditions for HPLC and Ion-Trap-ESI-LC-MS Measurements. For HPLC, a *HP 1100* series instrument was used. ESI mass spectra were recorded on a *LCQ* instrument (*Thermo Finnigan MAT*, San José, CA, USA). The gradient used for HPLC and Ion-Trap-ESI-LC-MS measurements has been described in [3]; further details concerning the anal. equipment were given in [66].

As previously reported, a CID energy of 45 and 65 eV was applied to generate sequence-specific *b*- and *y*-type fragments from putative $[M+H]^+$, $[M+Na]^+$, or sequence-specific fragment ions, resp. Collision energy for MS/MS and MS^n measurements was set between 25 and 65 eV, typically at 45 eV [25].

Peptide fragment-ion series were assigned in accordance with the *Roepstorff/Fohlman–Biemann* nomenclature previously used [67][68].

Sequence Determination of Linopubescins A, B, and C. The sequence of amino acids was readily indicated by ESI-MS/MS experiments (LCQ ion trap-instrument, *Thermo Electron*, D-Dreieich) due to the diagnostic *b*-type cleavage of the amide bonds. The amino acid composition was confirmed by hydrolysis. One mg of each peptide was hydrolyzed (6M HCl, 110°, 24 h) in a sealed tube under Ar atmosphere. The residue was evaporated to dryness *in vacuo* and dissolved in MeOH prior to ESI measurements in the positive mode. For isolation of the N-terminal fatty acid, the hydrolysis was extracted twice with $CHCl_3$. The extracts were combined, and the solvent was evaporated to dryness under a cold stream of N_2. Finally, the residue was dissolved in MeOH, and the sample was analyzed in the negative mode of the LCQ.

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Received April 11, 2006