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Hypopulvins, novel peptaibiotics from the polyporicolous fungus *Hypocrea pulvinata*, are produced during infection of its natural hosts

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Abstract

In order to investigate the significance of antibiotics for the producing organism(s) in the natural habitat, we screened specimens of the polyporicolous fungus *Hypocrea pulvinata* growing on its natural hosts *Piptoporus betulinus* and *Fomitopsis pinicola*. Results showed that a particular group of nonribosomally biosynthesised antibiotic polypeptides, the peptaibiotics, which contain the nonproteinogenic marker amino acid α-aminoisobutyric acid (Aib), was produced in the natural

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habitat by the fungicolous producer and, consequently, released into the host. Using liquid chromatography coupled to electrospray high-resolution mass spectrometry we detected especially 19-, but also 11-, 18-, and 20-residue peptaibiotics in the five infected specimens analysed. Structures of peptaibiotics found were confirmed by analysing the peptaibiome of pure agar cultures obtained by single-ascospore isolation from the specimens. The 19-residue peptaibols were determined as deletion sequences of the trichosporins B lacking the Aib residue in position 6. Notably, 26 of the 28 peptaibiotics sequenced were novel; therefore the name 'hypopulvins' was introduced. Considering not only the ubiquity of both the two host species but also the highly specific association between *H. pulvinata* and *P. betulinus*/*F. pinicola*, and the abundance of this fungicolous species in north temperate regions of the world, a decisive role for the peptaibiotics detected in this study is predicted, which may act as mediators of the complex interactions between the basidiomycetous host and its fungicolous ascomycete 'partner'. Structural analogies of the hypopulvins, particularly with other 18-, 19-, and 20-residue peptaibiotics, suggest that the hypopulvins are forming transmembrane ion channels and could thus support the hypothesis of a parasitic lifestyle of the fungicolous producer.

Keywords

Fungicolous fungi; HPLC/QTOF–ESI–HRMS; *Hypocrea pulvinata*; Metabolite profiling; Peptaibiotics; Peptaibols

Introduction

Natural products of fungal origin

As of 2010 approximately half a million natural products were known of which 60 000–80 000 are estimated to be of microbial origin. Approximately half of the latter display some kind of biological activity (Bérdy 2012). The fungal kingdom, which currently comprises more than 98 000 validly described species (Kirk *et al.* 2008) contributes some 30 000 natural products of which 15 000–16 000 are bioactive. Of these, around 11 250 originate from microscopic fungi (Bérdy 2012). Peptide antibiotics constitute a considerable part of those metabolites, including therapeutically important β-lactam antibiotics (penicillins, cephalosporins), which account for more than 65 % of the world antibiotics market. However, the significance of other, nonantibiotic peptide drugs of fungal origin is comparable – the immunosuppressant market is still dominated by the nonribosomal biosynthesised cyclosporine A (Elander 2003; Demain & Sanchez 2009).

Peptaibiotics – nonribosomally biosynthesised fungal peptide antibiotics containing α,α-dialkyl α-amino acids

During the past two decades, a constantly growing group of peptide antibiotics, the peptaibiotics, have started to regain particular interest because of their unique bioactivities, resulting from their amphipathicity and helical conformations. Peptaibiotics are defined as nonribosomally biosynthesised, linear or cyclic polypeptide antibiotics of exclusively fungal origin, which (*i*) have a molecular weight between 500 and 2200 Da, thus containing 4–21 residues; (*ii*) show a high content of the marker α-aminoisobutyric acid (Aib) as well as other α,α-dialkylamino acids; (*iii*) are characterised by the presence of other

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nonproteinogenic amino acids and/or lipoamino acids; (*iv*) possess an acylated N-terminus, and (*v*) in the case of linear peptides, have a C-terminal residue that in large part consists of a free or acetylated amide-bonded β-amino alcohol. The C-terminus might also be an amine, amide, free amino acid, 2,5-diketopiperazine, or sugar alcohol. The majority of Aib-containing peptides carry a C-terminal residue representing a β-amino alcohol, and this subgroup is, therefore, referred to as peptaibols. As a result of the nonribosomal biosynthesis on large multifunctional peptide synthetases, highly complex mixtures of homologous and positionally isomeric peptides are produced. The latter phenomenon is commonly referred to as microheterogeneity, *i.e.*, exchange of single or few amino acids or C-terminal amino alcohols, respectively (Degenkolb & Brückner 2008). Therefore, sophisticated, multidimensional analytical approaches such as HPLC coupled to tandem electrospray ionisation (ESI)-MS^n or Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and chiral capillary gas-chromatography/selected ion monitoring electron impact MS became the methods of choice for structure elucidation of peptaibtiotics. To date, the presence of peptide-bound α,α-dialkyl α-amino acids, *i.e.*, mostly Aib, but also D- and L-isovaline (Iva), L-α-ethylnorvaline (Etnva) as well as 1-aminocyclopropane-1-carboxylic acid (Acc) (for structures, see Fig 1), has been confirmed in acidic hydrolysates of more than 30 genera of fungi (Brückner *et al.* 2009). Among those the ubiquitous genus *Trichoderma*/*Hypocrea*, which currently contains approximately 200 validly described species (Jaklitsch 2009, 2011; Samuels & Ismaiel 2011; Jaklitsch & Voglmayr 2012; Jaklitsch *et al.* 2012; Samuels *et al.* 2012a, b) is generally regarded as the richest source of peptaibiotics (Degenkolb & Brückner 2008). Notably, D- and L-Iva may even occur in different positions of one and the same sequence, as was shown for clonostachin, neoefrapeptins, and integramides, for review see Degenkolb & Brückner (2008). Obviously these results contradict the still widespread belief that α,α-dialkyl α-amino acids do not or rarely occur in the biosphere and, if detected in the environment, are definitely of extraterrestrial origin. In fact fungal species, which contain peptaibiotic-producing strains, are ubiquitous and cosmopolitan, even occurring in marine, Arctic and Antarctic regions (Brückner *et al.* 2009). However, differentiation between biotically and abiotically synthesised Aib and homologues is possible by stable carbon and nitrogen isotopic composition (Elsila *et al.* 2011).

Bioactivities of peptaibiotics from Trichoderma/Hypocrea

In the past decade, many *Trichoderma* species, such as *Trichoderma ovalisporum* (Holmes *et al.* 2004), *Trichoderma koningiopsis* (Samuels *et al.* 2006a), and *Trichoderma citrinoviride* (Maddau *et al.* 2009) have gained increasing interest as potential biocontrol agents in plant protection. Further promising examples include *Trichoderma paucisporum* and *Trichoderma theobromicola*, displaying *in vitro*-activity against frosty pod rot of cocoa, *Moniliophthora roreri* (Samuels *et al.* 2006b), and *Trichoderma martiale*. In small-scale *in situ* field trials the latter proved highly effective against black pod rot of cocoa caused by *Phytophthora palmivora* (Hanada *et al.* 2009). The most successful story in cocoa biocontrol, however, is the recent approval of ‘Tricovab’ (Anonymous Novembro 2011/Fevereiro 2012), a formulation against *Crinipellis* (=*Moniliophthora*) *perniciosa*, the Witches’ broom pathogen, containing the hyperparasite *Trichoderma stromaticum* (Pomella *et al.* 2007). Although this species has been shown to produce five 18-residue peptaibols, trichostromaticins A-E

(Degenkolb *et al.* 2006), the mechanism(s) contributing to its remarkable *in vivo* bioactivity have not been completely resolved yet. Peptaibiotics are prone to play a key role in the infection process of a host by a fungicolous species because of their unique ability of forming voltage-gated ion channels. This phenomenon is best described by the dipole flip-flop gating model (Menestrina *et al.* 1986). Their well-documented membrane activity, however, may also account for other striking bioactivities of peptaibiotics, such as neurolepsy (Berek *et al.* 2009), inhibition of amyloid β-peptide formation (Hosotani *et al.* 2007), inhibition of HIV-1 integrase (Singh *et al.* 2002), suppression of tumour cells, targeted Ca^{2+}-mediated apoptosis and autophagy in human hepatocellular carcinoma cells (Shi *et al.* 2010), as well as induction of defence responses and systemic resistance in tobacco against tobacco mosaic virus (Luo *et al.* 2010), and programmed cell death in fungal plant pathogens (Shi *et al.* 2012).

In vivo-*detection of peptaibiotics*

Reports on the occurrence of peptaibiotics in the natural habitat of the producer(s) have sparsely been published so far. Most of almost 1000 individual sequences of peptaibiotics known to date have been sequenced in extracts of fungal cultures grown under artificial laboratory conditions. The first example of peptaibiotics obtained from natural specimens was the isolation of hypelcins A and B obtained from 2 kg of dried, crushed stromata of *Hypocrea peltata* (Fujita *et al.* 1984a; Matsuura *et al.* 1993, 1994). However, it appears that in recent years only a single contribution directly addressed the detection of peptaibols, *viz.* the 16-residue antiamoebins, under *in vivo*-conditions (Lehr *et al.* 2006) whereas other authors most probably have overlooked the presence of a fungicolous species in the examined material. Thus, the formation of peptaibiotics was accidentally ascribed to basidiomycetous hosts, which have rather been infested by an unrecognised fungicolous fungus, such as *Sepedonium* sp., for review see Degenkolb & Brückner (2008).

The present study is thus aimed at the question as to whether peptaibiotics formation under the conditions of the natural habitat of the producer(s) is either a rather infrequent or a more common phenomenon. If the latter hypothesis is true, peptaibiotics might, indeed, play a decisive role in the antibiotic-based colonisation and defence of the natural substrate, as was recently demonstrated for the antiamoebins from *Stilbella fimetaria* (Lehr *et al.* 2006). Given that this coprophilous species produces fungicidal amounts of peptaibols on herbivore dung, other highly specialised hypocrealean fungi occupying ecological niches must be considered as additional model systems for these investigations.

From an ecophysiological point of view, necrotrophic mycoparasites within the genus *Trichoderma*/*Hypocrea* are best suited for such study. This is due to the fact that a parallel formation and synergistic action of hydrolytic enzymes, above all as β-1,3-glucanase, and peptaibiotics have previously been attributed an important role in mycoparasitism between *Trichoderma atroviride* (originally misidentified as *Trichoderma harzianum*) and its fungal hosts such as *Botrytis cinerea* (Schirmböck *et al.* 1994; Lorito *et al.* 1996).

Choice of the model organism

Hypocrea pulvinata, the Ochre Cushion, a rather common fungus that occurs on decaying basidiomes of polypores in north temperate regions of the world (Jaklitsch 2011), was chosen as a first model organism. Notably, this polyporicolous species is assumed to display host specificity as it has only been unambiguously identified on the Birch Bracket *Piptoporus betulinus* and the Red-banded Bracket *Fomitopsis pinicola* so far. Records from other aphyllophoralean taxa such as *Ganoderma* sp. could not been confirmed in recent years; and the claimed association with *Laetiporus sulphureus* was recently shown to be based on misidentifications of the respective host (Jaklitsch 2011).

To prove our hypothesis of peptaibiotic production by members of the order *Hypocreales* under *in vivo*-conditions, basidiomes of *P. betulinus* and *F. pinicola*, infected with *H. pulvinata*, were collected from different locations. Cultures obtained from the respective teleomorphs were analysed using a peptaibiomics approach as described earlier (Krause *et al.* 2006; Degenkolb *et al.* 2012).

Materials and method

Chemicals

All solvents used, acetonitrile (MeCN), methanol (MeOH), CH_2Cl_2, and formic acid (FA), were of LC–MS grade from Sigma–Aldrich (Steinheim, Germany). Water was purified by a Merck-Millipore Milli-Q system (Schwalbach/Ts., Germany).

Isolation of pure agar cultures of anamorphs

Basidiomes of *Piptoporus betulinus* and *Fomitopsis pinicola* infected with *Hypocrea pulvinata* were collected from five different locations in Austria and Russia (I–V; Table 1). Pure agar cultures were obtained by single-ascospore isolations from the respective specimens as described by Jaklitsch (2009).

Extraction of specimens

Few milligram of teleomorph stromata were extracted with 40 ml of a mixture of CH_2Cl_2–MeOH (1:1v/v), centrifuged, the solvent was evaporated *in vacuo* (Rotavapor R-215, Büchi, Essen, Germany) or using a stream of nitrogen. For screening on the micrOTOF-Q II, the extracts were cleaned up over Sep-Pak Classic C_{18} cartridges (Waters, Eschborn, Germany) as described by Krause *et al.* (2006) whereas the crude extracts (redissolved in MeOH) were analysed directly using the more sensitive maXis QTOF. Hymenophores of apparently uninfected basidiomes of *P. betulinus* were investigated as a control.

Cultivation and extraction of pure cultures

Cultures of specimens I, II, and III were grown on potato dextrose agar (PDA) (Becton, Dickinson & Co., Heidelberg, Germany) at 23 °C for 6 d in Petri dishes of 9 cm diameter. These subcultures were used for inoculation of the main cultures on PDA. After 10 d of cultivation at 23 °C in the dark, ten fully-grown Petri dishes were extracted, the extracts combined, evaporated to dryness *in vacuo*, and the residues analysed as described below.

LC-MS analysis

Two QTOF systems, both from Bruker Daltonic (Bremen, Germany) controlled by HyStar v. 3.2 were used. Both instruments were equipped with an orthogonal ESI source, and coupled to a Dionex UltiMate 3000 UHPLC (Dionex, Idstein, Germany).

System 1: high-resolution micrOTOF-Q II mass spectrometer. For separation, an Acclaim 120 C_8, 3 µm, 120 Å, 2.1 × 150 mm column (Dionex, Idstein, Germany) at a flow rate of 0.25 ml min^{-1}, and a temperature of 35 °C was used. Eluent A consisted of H_2O + 0.1 % FA, eluent B of 95 % MeCN + 0.1 % FA. Subsamples of 10 µl were injected. The column was held at 80 % A/20 % B for 5 min, then a gradient from 20 % B to 100 % over 55 min was applied. Thereafter, the column was held at 100 % B for 15 min, returned to the start conditions in 1 min, and finally equilibrated for 14 min.

Samples were screened for peptaibiotics in the positive ion mode using the following three-step routine procedure: A full scan was recorded from m/z 50 to 3000. This was followed by an in-source collision-induced dissociation (CID) scan from m/z 50 to 2000, recorded at energy of 150 eV. Finally, results of the in-source CID scan were verified by MS/MS experiments on selected precursor ions. For precursors $<m/z$ 1000, a collision energy of 30 eV was applied, precursor ions in the m/z range from 1000 to 1500 were fragmented at a collision energy of 35 eV and precursor ions $>m/z$ 1500 at a collision energy of 40 eV. The isolation width for MS/MS experiments was set to ±1 Da.

System 2: MaXis 3G QTOF mass spectrometer operated at a resolution of 40 000 full width at half maximum (FWHM). An Acquity UPLC® BEH300 C_{18}, 1.7 µm, 2.1 × 150 mm column was used for separation, using H_2O + 0.1 % FA (eluent A) and B of 100 % MeCN + 0.1 % FA. The flow rate was set to 0.3 ml min^{-1} and the temperature to 40 °C. The gradient started with 90 % A/10 % B at the time: 0 min and was changed to 50 % A/50 % B at time: 7 min, then to 30 % A/70 % B at time: 25 min, then raised to 100% B at time: 38 min and held at 100% B until time: 41 min before set to starting conditions from time 42 min to 46 min. Three microlitres were injected. MS were scanned in the m/z range 100–2000. Auto MS with precursor ion-dependent collision energy optimisation was used for fragmentation in the range of 10–65 eV.

Data interpretation was performed using the DataAnalysis v. 4.0 (Build 281) software (Bruker Daltonic, Bremen, Germany). Use of high-resolution ESI mass spectrometry allows the unequivocal sequencing of fragment ion series according to the Roepstorff/Fohlman/Biemann nomenclature. In cases where the isomeric amino acids (Leu/Ile and Val/Iva, respectively) or the corresponding amino alcohols (Leuol/Ileol) with the same elemental composition could not be distinguished, the abbreviations Lxx, Vxx, and Lxxol were used instead (Degenkolb *et al.* 2006, 2012; Krause *et al.* 2006).

Results

Screening of basidiomes infected by Hypocrea pulvinata

Biomass from the five infected samples investigated (Table 1) was shown to contain intense peaks of novel, especially 19-residue peptaibiotics, which we have named hypopulvins

(HPVs). Analysis of two basidiomes from *Piptoporus betulinus* not infected by *Hypocrea* did not show any indications of peptaibiotics, thus verifying that they are produced by *Hypocrea* during infection of the basidiomes (data not shown). A comparison of base peak chromatograms (BPCs) is given in Fig 2A and B.

The peptaibiome of the teleomorph was dominated by 19-residue peptaibols (Tables 2a and 3a, Table S1a), as well as minor 18-residue deletion analogues (compounds 1 and 2), only detected in isolates IV and V.

All compounds display the typical characters of the peptaibol subfamily 1 (SF1: Table 4, Fig 1) the largest one of the nine subfamilies, as introduced by Chugh & Wallace (2001). Accordingly, in HPV's, one Gln residue is found in position 6, and another two towards the C-terminus, in position 17 and 18. A highly conserved Pro residue is located in position 13 of the peptide chain. Many sequences have a Gly or Ala residue in position 10. Most of the HPV's terminate in phenylalaninol (Pheol), only two in Lxxol. At least four, at most seven, residues are occupied by an Aib residue. Qualitative differences between the teleomorphs observed with the maXis and the micrOTOF-Q II might be explained by the different screening dates, considering that the peptaibiome of a living specimen is subjected to dynamic changes.

Screening of Hypocrea pulvinata *plate cultures*

Pure agar cultures were only obtained from single ascospores of specimens I, II, and III (Table 1), whereas specimens IV and V did not contain viable ascospores anymore. However, BPCs of the three plate cultures screened are highly similar (Fig 2C). Comparing the peptaibiotic pattern of specimens *vs.* plate culture (Tables 2b and 3b, Table S1b), 19-residue peptaibols were also found predominantly in the cultures although their microheterogeneity was less pronounced:

The pattern of 19-residue peptaibols was less diverse, and amino acid exchanges were only found in positions 4, 8, 10, and 14 (Table 2b).

In contrast to what has been observed for the specimens, two new 20-residue minor compounds, (**25**) and (**27**), representing the building scheme of trichosporins B (Iida *et al.* 1990, 1993), were detected. Another two minor compounds, 11-residue peptaibols (**26**) and (**28**), display the structural characteristics of the growing subfamily 4 (SF4: (Chugh & Wallace 2001; Degenkolb *et al.* 2012)).

Considerable microheterogeneity, including positional isomerism, of long-chain peptaibiotics was observed in both stromata and cultures: Sequences (**4**), (**5**), (**7**), and (**20a**) represent positional isomers of *m*/*z* 1852.0721 $[M + H]^+$, whereas sequences (**8**), (**9**), (**12**), and (**20b**) display *m*/*z* 1866.0909 ($[M + H]^+$). Five sequences (**11**), (**14**), (**16**), (**22**), and (**23**) represent positional isomers of *m*/*z* 1880.1064 ($[M + H]^+$); whereas another three (**17**), (**18**), and (**24**) are of *m*/*z* 1894.1222 ($[M + H]^+$). Further, Ser-containing positional isomers of *m*/*z* 1882.085 $[M + H]^+$ are represented by (**3**) and (**19**), whereas sequences (**6**), (**13**), and (**21**) display *m*/*z* 1896.0874 $[M + H]^+$.

Discussion

General remarks

The most notable result of this investigation is the unequivocal confirmation of peptaibiotic biosynthesis in the natural habitat of a fungicolous fungus, indicating that these are required for infection. We here present the first example of *in vivo*-production of peptaibiotics by a fungicolous fungus growing on either of its natural hosts. Considering (*i*) the ubiquity of both the two host species (*ii*) the highly specific association between *Hypocrea pulvinata* and *Piptoporus betulinus/Fomitopsis pinicola*, and (*iii*) the abundance of this fungicolous species in north temperate regions of the world including Europe, North America, Japan, and Russia (Jaklitsch 2011), it seems reasonable to postulate a decisive role for the peptaibiotics detected in this study:

They are hypothesised to be one of the mediators of the complex interactions between the basidiomycetous host and its fungicolous ascomycete 'partner' (see below).

Twenty-four of the 26 new HPVs produced by *H. pulvinata* are 19-residue peptaibols, basically representing deletion sequences (Δ Aib^{6}) of the 20-residue trichosporins B (Iida *et al.* 1990, 1993) and polysporins A–D (New *et al.* 1996), both isolated from strains of *Trichoderma polysporum*. This deletion, however, is predicted not to negatively influence the bioactivity of these long-chain peptaibols as all important structural features, which comply with the requirements for the formation of transmembrane ion channels in artificial lipid bilayer membranes, are still present. Generally, 18-, 19-, and 20-residue peptaibiotics display a higher membrane pore formation activity by several orders of magnitude in comparison to smaller peptaibols consisting of less than 17 residues (Grigoriev *et al.* 2003).

Importance of different structurally conserved amino acid residues for the bioactivity of peptaibiotics – general remarks

Aib and Iva residues strongly promote the formation of helical structures (α- or 3_{10}-helices, and even mixed forms) (Toniolo & Benedetti 1991), which is due to the steric constraints imposed by the geminal alkyl groups of the C^{α}-atom (Chugh & Wallace 2001).

The significance of glutamine residues

Gln residues in position 6 or 7 were postulated to play a key role for ion channel stabilisation (Fox & Richards 1982), as they are located in the pore lumen, lining it together with $Gln^{17–18}$ and Gly^{10} and Pro^{13} carbonyls of the adjoining transmembrane helices (Duclohier 2004). Molle *et al.* (1996) demonstrated that replacement of Gln^{7} by Ala^{7} resulted in complete loss of channel-forming activity, which supports this hypothesis. This phenomenon was explained by the inability of this nonpolar residue to form side-chain H-bonds. Interhelix H-bonds are thought to play a decisive role in the stabilisation of channel-forming helix bundles. This hypothesis was corroborated by the replacement of Gln^{7} by Asn^{7}, restoring nearly initial functional properties. In contrast, replacement of Gln^{7} by Ser^{7} resulted in a significantly reduced voltage dependence and shorter channel lifetime. This was explained by the formation of much weaker H-bonds by the $–CH_2OH$ group of Ser^{7} (Molle *et al.* 1996).

The decisive role of proline residues

Prolines in position 13 or 14 of the peptide chain create bends of the helical structure, thus affecting the (*i*) probability of ion channel formation, (*ii*) lifetime of the channel, and (*iii*) occurrence of multilevel conductance (Nagaoka *et al.* 1996).

For the 20-residue alamethicins (ALM) F30, proline in position 14 which corresponds to Pro^{13} of the 19-residue HPV's was demonstrated to display optimal channel activity whereas synthetic analogues bearing Pro in positions 11, 12, 13, 15, 16, and 17 formed considerably less stable ion channels (Kaduk *et al.* 1997). A proline residue in position 14 of ALM F30 is essential for (*i*) haemolysis of human erythrocytes, (*ii*) stimulation of catecholamine secretion from bovine adrenal chromaffin cells, and (*iii*) induction of metabolic activity in bovine aortic endothelial cells (Dathe *et al.* 1998). Surprisingly, it was reported that Gln^{7}, Gly^{11}, and Pro^{14} are not essential for channel formation but substitution of any of these residues reduced (*i*) the number of conductance levels and (*ii*) significantly decreased their lifetimes (Kaduk *et al.* 1998).

Role of the C-terminal substituents

Aromatic residues such as the C-terminal Pheol, which are located in the membrane interface between the hydrophilic lipid head groups and the hydrophobic fatty acid chains, have been postulated to stabilise the polypeptide transmembrane segments (Wallace 2000).

The two compounds (**10**) and (**15**) carrying a C-terminal Lxxol residue resemble some of the structural properties of two other 20-residue peptaibol mixtures – hypelcins A from stromata of *Hypocrea peltata* (Fujita *et al.* 1984a; *Matsuura et al.* 1993) and stilboflavins B from *Stilbella flavipes* CBS 146.81 (Jaworski & Brückner 2001). Hypelcins A were shown to (*i*) act as uncouplers of oxidative phosphorylation in rat liver mitochondria (Takaishi *et al.* 1980), (*ii*) exhibit antibacterial and antifungal activity, including growth inhibition of the shiitake mushroom *Lentinus edodes*, (*iii*) exert contractile action on guinea pig ileum (Fujita *et al.* 1984b), and (*iv*) induce ion channels in planar bilayer lipid membranes. Remarkably, the substitution of Pheol for Leuol and Ileol, respectively, resulted in prolonged open channel lifetime (Koide *et al.* 1997). Stilboflavins were reported to exhibit weak antibiotic activity against grampositive bacteria and cause haemolysis of sheep erythrocytes (Jaworski & Brückner 2001).

The two 18-residue sequences, compounds (**1**) and (**2**), exhibit a deletion of the C-terminal amino alcohol residue. Compared to the amount of the major 19-residue peptaibols, their possible contribution to the postulated bioactivity of the mixture of HPVs is regarded negligible. Despite this, truncated sequences of SF1 peptaibols have been reported before:

19-residue peptaibiotics, trichobrachins I (TB I) from *Trichoderma ghanense* CBS 936.69 (syn. *Trichoderma parceramosum*) lacking the C-terminal Pheol residue, were shown to originate from 20-residue trichobrachins II (TB II) by enzymatic degradation (Krause *et al.* 2007). Two minor desPheol-compounds F30 representing 1.3 % of the ALM mixture from *Trichoderma arundinaceum* CBS 123793 (formerly known as *Trichoderma 'viride'* NRRL 3199) have been detected by nonaqueous capillary electrophoresis (NACE) coupled to electrospray mass spectrometry (Psurek *et al.* 2006). Two further 17-residue minor

compounds lacking the Gln residue in position 18 are predicted to be present; however, the intensity of their diagnostic CID-fragment ions was too low for sequencing.

Multiple bioactivities of the structurally related trichosporins B

The structurally similar trichosporins B (Fig 1, Tables 2a and b) were isolated from the culture filtrate of *Trichoderma polysporum* TMI 60146, a destructive mycoparasite of *Lentinus edodes* (Fujita *et al.* 1988; Iida *et al.* 1993). To date, a multitude of biological activities has been reported for trichosporins B, including uncoupling of the respiratory activity of rat liver mitochondria (Fujita *et al.* 1988; Okuda *et al.* 1994), Ca^{2+}-dependent catecholamine secretion from bovine adrenal medullary chromaffin cells (Tachikawa *et al.* 1991, 1995, 1996), formation of voltage-gated ion channels (Nagaoka *et al.* 1995), and antitrypanosomal activity (Iwatsuki *et al.* 2010).

Minor, 11-residue peptaibols coproduced by Hypocrea pulvinata

The two 11-residue compounds (**26**) and (**28**) are typical representatives of the growing SF4 (Chugh & Wallace 2001; Degenkolb *et al.* 2012). Apparently, a complex mixture of homologues and positional isomers was detected in the plate culture of *H. pulvinata* I, whereas only trace amounts were found in isolates II and III. However, 11-residue peptaibols were absent in the teleomorphs. Typical signals, indicative of 11-residue peptaibols, *viz.* pseudomolecular ions *m*/*z* 1147.7/1169.7, 1161.7/1183.7, and 1175.8/1197.8 (all $[M + H]^+$/$[M + Na]^+$) were found. Due to the rather low intensity of these diagnostic ions, which is partly caused by their coelution with major 19-residue peptaibols, only two major peptaibols (**26**) and (**28**) could be sequenced (Table 2b, Table S1b). The huge number of positional isomeric SF4 11-residue sequences described to date (Brückner *et al.* 1993; Krause *et al.* 2007; Mukherjee *et al.* 2011; Degenkolb *et al.* 2012) would require sophisticated methods for chiral sequence analysis (Becker *et al.* 1997; Jaworski & Brückner 2001) in order to evaluate their novelty or recurrence, respectively. Basically, SF4 11-residue sequences may contribute to the bioactivity of *H. pulvinata*. The extent of this contribution cannot be evaluated; however, it is regarded as minor because of the comparatively low abundance of these 11-residue peptaibols. Biological activities reported for homologous and/or positional isomeric SF4 11-residue peptaibols comprise induction of voltage-gated ion channels in lipid bilayers (Iida *et al.* 1995; Wada *et al.* 1995, 1996), haemolysis of rat erythrocytes (Becker *et al.* 1997), cytotoxicity towards KB cancer cells (Ruiz *et al.* 2007), and inhibition of grampositive bacteria (Becker *et al.* 1997; Berg *et al.* 2003; Krause *et al.* 2007).

Thoughts about nonribosomal biosynthesis and module skipping

Only the single anamorphic isolate II has been shown to produce the 20-residue peptaibols (**25**) and (**27**), indicating the presence of a respective 20-module nonribosomal peptide synthetase (NRPS). It is thus surprising to detect both 19- and 20-residue peptaibols in this isolate, indicating that a 20-module NRPS may produce 19-residue peptaibols as major products. The exclusive 19-residue content of the other isolates could be interpreted with the deletion of module 6 to a 19-module multienzyme, or by module skipping during peptide elongation. Module skipping could be related to functional properties of this module with

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respect to binding or processing of Aib intermediates. Likewise *Hypocrea pulvinata* might harbour 19-module synthetases, while isolate II has a gain of function insertion mutagenesis to exclusively arrive at 20 modules. The 19-module NRPS detected by genomic sequencing in *Hypocrea atroviridis* had been associated with 19- and 20-residue atroviridins from *Trichoderma atroviride* (Komon-Zelakowska *et al.* 2007), but the strain used in that study has later been shown to produce only the 19-residue trichorzianins (Stoppacher *et al.* 2007), and this NRPS thus has been identified as trichorzianine synthetase. As in 19-residue HPVs, the first Gln of trichorzianins is found in position 6, clearly resulting from a deletion of the 6th module found in a 20-module NRPS synthetase (as paracelsin synthetase, HvD, unpubl. data). We cannot decide on this without knowledge of the respective gene structures of the anamorphs.

Conclusion

Taken these findings together, we dare to predict a mycoparasitic lifestyle of the host-specific polyporicolous2 *Hypocrea pulvinata*:

It has been demonstrated by *in vitro*-studies, that chitinases and β-1,3-glucanases act synergistically with peptaibiotics in inhibiting spore germination and hyphal elongation of *Botrytis cinerea*. In the strain ATCC 36042 (=CBS 391.92), which was originally identified as *Trichoderma harzianum* (el Hajji *et al.* 1987) but later shown to belong to *Trichoderma atroviride* (Kuhls *et al.* 1996), parallel formation of hydrolytic enzymes and 19-residue antifungal trichorzianins A and B is triggered in the presence of cell walls of plant-pathogenic fungi (Schirmböck *et al.* 1994). Trichorzianins have previously been shown to form voltage-gated ion channels in planar lipid bilayers (Molle *et al.* 1987), modify the membrane permeability of liposomes, and they are active against *Rhizoctonia solani* and *Phythophthora cactorum*. Based on these results, a model of how peptaibiotics such as trichorzianins and hydrolases interact synergistically was proposed: First, the host cell wall is digested enzymatically; thereafter peptaibiotics will penetrate the cell membrane in order to form ion channels. Cell leakage reduces the ability of the host to effectively repair its cell wall. Eventually, inhibition of chitin and β-glucan synthesis further amplifies the destructive effect of chitinases and β-1,3-glucanases (Lorito *et al.* 1996). These mechanisms, however, may also account for the recently published induction of programmed cell death in plant fungal pathogens (Shi *et al.* 2012) caused by the 20-residue peptaibol trichokonin VI (=gliodeliquescin A: (Brückner & Przybylski 1984)), from *Trichoderma koningii*, *Trichoderma pseudokoningii*, and *Trichoderma* (syn. *Gliocladium*) *deliquescens*,3 the anamorph of *Hypocrea lutea* (Jaklitsch 2011). The presence of peptaibiotics was also shown to play a role in the induction of plant defence responses (Viterbo *et al.* 2007).

The data presented here (*i*) substantiate the hypothesis of a mycoparasitic lifestyle for *H. pulvinata*, (*ii*) show the potential for a possible application as a biological control agent, and

[2]Aib has also been detected in an oatmeal agar plate culture of the polyporicolous *Cosmospora berkeleyana* CBS 234.70 (syn. *Acremonium berkleyanum*, syn. *A. butyri*) isolated from the Tinder Polypore *Fomes fomentarius* (Brückner *et al.* 2009).
[3]Gliodeliquescin A was isolated from *Gliocladium deliquescens* NRRL 1086 (Brückner *et al.* 1989) and not from NRRL 3091 (Brückner & Przybylski 1984). According to phylogenetic data (18S rRNA, ITS 1 and 2), *G. deliquescens* NRRL 1086 (=CBS 228.48 = ATCC 10097) has been reidentified as *G. viride* (www.straininfo.net/strains/260309).

(*iii*) indicate the presence of obviously different HPV synthetases (NRPSs) in isolates I, II, and III.

Appendix A. Supplementary data

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

QTOF-MS quadrupole time-of-flight mass spectrometry

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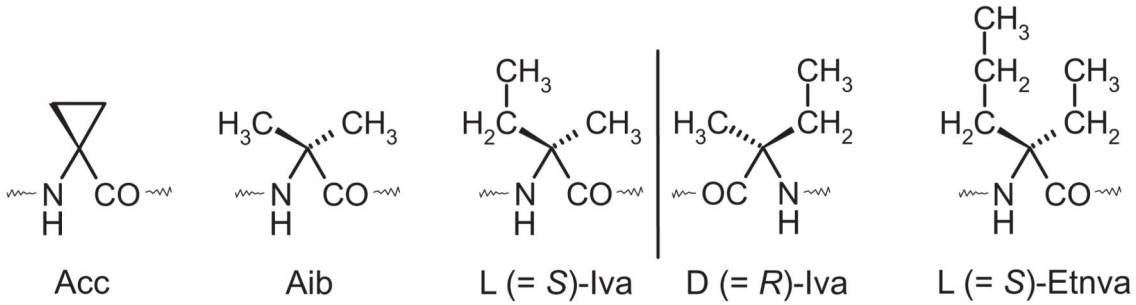

A

B

	Residue																			
	1	2	3[a]	4	5	6[c]	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Ac	Aib	Ala	Gly Ala (Ser)[b] (Aib)	Ala (Aib)	Aib (Ala)	Aib	Gln	Aib	Lxx Ile (Leu) (Val)	Aib (Ala)	Gly (Ala)	Leu Lxx (Vxx)	Aib (Ala)	Pro	Val	Aib	Aib (Vxx) (Iva) (Ala)	Gln	Gln	Pheol

C

	Residue										
	1[a]	2	3	4	5	6	7	8	9	10	11
Ac	Aib Vxx (Iva)[b] (Val)	Gln Asn	Lxx Vxx Leu Val (Ile)	Lxx Vxx Leu Val (Ile)	Aib	Pro	Lxx Leu Ile (Aib) (Iva)	Leu Lxx Vxx Val (Ile)	Aib (Vxx)	Pro	Leuol Valol Lxxol Vxxol (Ileol)

a – variable positions are underlined;

b – minor sequence variations are parenthesised;

c – deleted in 19-residues hypopulvins.

Fig 1. (A) Structures and configurations of α,α-dialkylamino acids found in peptaibiotics. (B) Building scheme of 20-residue SF1 peptaibiotics, produced by *Hypocrea pulvinata*. (C) General building scheme of 11-residue peptaibols found in SF4.

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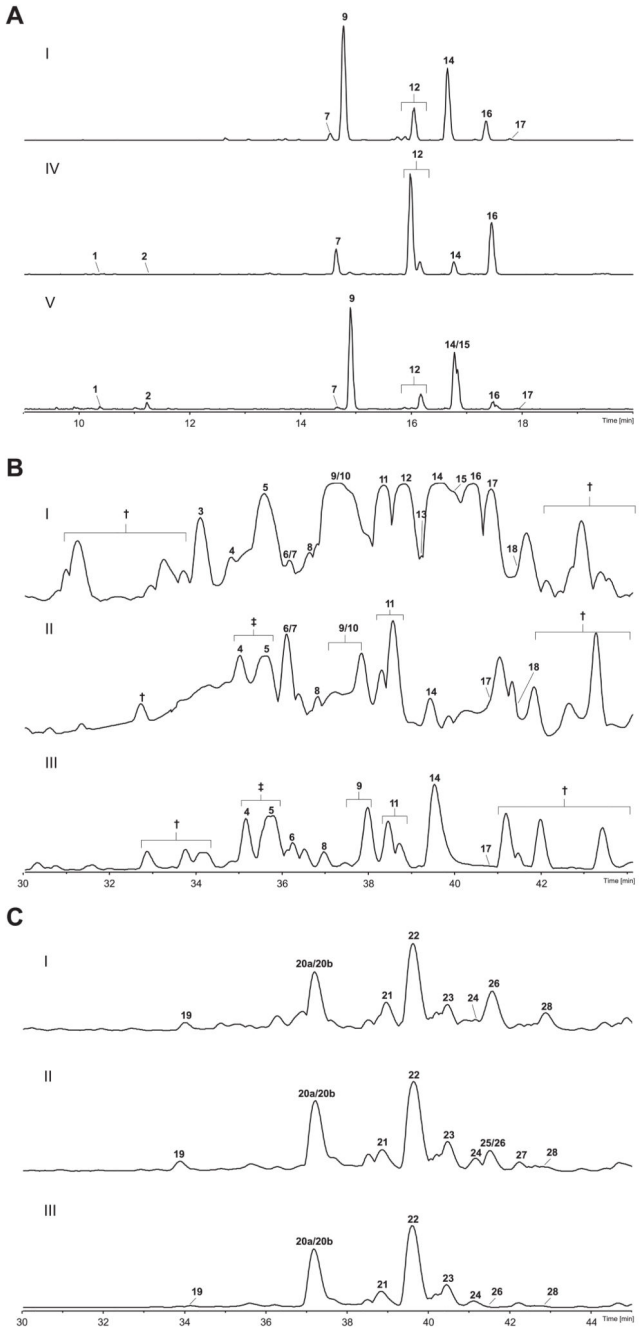

Fig 2. BPCs of (A) specimens I, IV, and V screened with the maxis TOF; (B) specimens I, II, and III screened with the micrOTOF-Q II; (C) plate cultures I, II, and III screened with the micrOTOF-Q II. † – no peptaibiotics; ‡ – coeluting peptaibiotics.

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Table 1

Habitat and geographic origin of *Hypocrea pulvinata* isolates included in this study.

Isolate	Host	Collecting information	Culture
I	Old basidiome of *Piptoporus betulinus*	Austria, Styria, Liezen, Untertal S Schladming, bog near Gasthof Tetter, 1020 m s.m., grid square 8648/1, 12 Jun. 2011, H. Voglmayr & Greilhuber	CBS 133228
II	Basidiome of *Piptoporus betulinus* on *Betula pendula*	Austria, Lower Austria, Wien-Umgebung, Mauerbach, 31 Jul. 2011, W.M. Jaklitsch	CBS 133229
III	Basidiome of *Piptoporus betulinus* on *Betula pendula*	Austria, Lower Austria, Wien-Umgebung, Mauerbach, 31 Jul. 2011, W.M. Jaklitsch	CBS 133230
IV	Decaying basidiome of *Piptoporus betulinus* on *Betula pendula*	Russia, Republic of Buryatia, Bargusin district, Lake Baikal, 'Holy Nose' Peninsula; 53° 35'54.9″ N, 108° 52'11.2″ E; 24 Jul. 2011, A. Berg & H. Dörfelt	
V	Decaying basidiome of *Fomitopsis pinicola* on *Pinus sibirica*	Russia, Republic of Buryatia, Severo-Baykalsky district, Nizhneangarsk; 55° 46'39.4″ N, 109° 35'49.9″ E; 24 Jul. 2011, A. Berg & H. Dörfelt	

Table 2a

Sequences of 18- and 19-residue peptaibiotics detected in specimens IeV of *Hypocrea pulvinata*

No.	t_R [min]		Residue																			
			1	**2**	**3**	**4**	**5**	**–**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1[a]	10.5	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	–
2[a]	11.2	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Ala	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	–
3	34.1–34.2	Ac	Aib	Ala	Ser	Ala	Aib	–	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
4	34.7–34.9	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Ala	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
5	35.6–35.9	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Gly	Vxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
6	36.2	Ac	Aib	Ala	Ala	Ser	Aib	–	Gln	Aib	Lxx	Aib	Ala	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
7	36.3	Ac	Aib	Ala	Ala	Ala	Ala	–	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
8	36.7–36.9	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Ala	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Pheol
9	37.2–37.4	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
10	37.3–37.5	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Lxxol
11	38.3–38.4	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Pheol
12	38.7–38.9	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
13	39.2	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Ser	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
14	39.5–39.7	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Ala	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
15	39.9	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Ala	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Lxxol
16	40.4–40.5	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Ala	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
17	40.8–41.1	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Ala	Lxx	Aib	Pro	Vxx	Aib	Vxx	Gln	Gln	Pheol
18	41.3–41.6	Ac	Aib	Ala	Ala	Ala	Vxx	–	Gln	Aib	Lxx	Aib	Ala	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol

No.	Compound identical or positionally isomeric with	References
1[a]	**new** (deletion sequence of **9**)	
2[a]	**new** (deletion sequence of **14**, **15**, and **16**)	
3	**new** (trichosporin B-Ia-[Aib][6])	Iida *et al.* 1990
4	**new** (trichosporin B-IIIa-[Aib][6], [Aib][9] → [Ala][9])	Iida *et al.* 1990
5	**new** (C-terminal pentadecapeptide resembles polysporin A with [Vxx][8] → [Lxx][8], N-terminal: trichosporin B-pentapeptide)	New *et al.* 1996
6	**new** (C-terminal pentadecapeptide resembles trichosporins B-Ia and B-IIIa with [Gly][10] → [Ala][10])	Iida *et al.* 1990
7	**new** (trichosporin B-IIIc-[Aib][6])	Iida *et al.* 1990
8	**new** (C-terminal pentadecapeptide resembles polysporin D with [Aib][9] → [Ala][9], N-terminal: trichosporin B-pentapeptide)	New *et al.* 1996
9	**new** (trichosporin B-IIIa-[Aib][6])	Iida *et al.* 1990
10	**new** (isomer of **9**: [Pheol][19] → [Lxxol][19]; C-terminal octapeptide found in some of the hypelcins A and stilbolflavins B)	Matsuura *et al.* 1993; Jaworski & Brückner 2001
11	**new** (trichosporin B-IVb-[Aib][6])	Iida *et al.* 1990
12	**new** (cf. **9**; trichosporin B-IIIa-[Aib][6])	Iida *et al.* 1990

No.	t_R [min]	Residue																		
		1	2	3	4	5	–	6	7	8	9	10	11	12	13	14	15	16	17	18	19
13	**new** (N-terminal hexadecapeptide: cf. trichosporins B IIIa/IVb; decapeptide [Aib]^{9}-[Gln]^{18}: cf. tricholongin B I)													Iida *et al.* 1990; Rebuffat *et al.* 1991							
14	**new** (trichosporin B-IIIa-[Aib]^{6}, [Gly]^{10} → [Ala]^{10})													Iida *et al.* 1990							
15	**new** (cf. **14**: [Pheol]^{19} → [Lxxol]^{19}; cf. **10**: [Gly]^{10} → [Ala]^{10}; trichosporin B-IIIa-[Aib]^{6}, [Gly]^{10} → [Ala]^{10}, [Pheol]^{19} → [Lxxol]^{19})													Iida *et al.* 1990							
16	**new** (positional isomer of **14**)													Iida *et al.* 1990							
17	**new** (positional isomer of **18**)																				
18	**new** (cf. **14**: [Aib]^{5} → [Vxx]^{5})																				

[a] Detected in specimens IV and V, only.

Variable residues are underlined. Minor sequence variants are underlined in the individual sequences.

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Table 2b

Sequences of 11-, 19-, and 20-residue peptaibiotics detected in plate cultures of *Hypocrea pulvinata* I–III.

No.	t_R [min]		Residue																		
			1	**2**	**3**	**4**	**5**	**–**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
19	34.0–34.2	Ac	Aib	Ala	Ala	Ser	Aib	–	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
20a	37.0–37.2	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
20b	37.0–37.3	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
21	38.9–39.0	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Ser	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
22	39.6–39.7	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Ala	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
23	40.2–40.5	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Ala	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
24	41.0–41.1	Ac	Aib	Ala	Ala	Ala	Aib	–	Gln	Aib	Lxx	Aib	Ala	Lxx	Aib	Pro	Lxx	Aib	Aib	Gln	Gln	Pheol

			Residue																			
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
25	41.3–41.6	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Lxx	Aib	Aib	Gln	Gln	Pheol
26	41.5	Ac	Aib	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol									
27	42.3–42.5	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Vxx	Aib	Gly	Lxx	Aib	Pro	Lxx	Aib	Aib	Glu	Gln	Pheol
28	42.9	Ac	Vxx	Gln	Lxx	Lxx	Aib	Pro	Lxx	Lxx	Aib	Pro	Lxxol									

No.	Compound identical or positionally isomeric with	References
19	**new** (cf. **6**: $[Ala]^{10} \rightarrow [Gly]^{10}$)	
20a	**new** (trichosporin B-IIId -$[Aib]^6$)	Iida *et al.* 1990
20b	**new** (cf. **9**)	
21	**new** (cf. **13**)	
22	**new** (cf. **14**)	
23	**new** (cf. **14**)	
24	**new** (cf. **22**, $[Vxx]^{14} \rightarrow [Lxx]^{14}$)	
25	**new** (trichosporin B-IIId: $[Vxx]^{15} \rightarrow [Lxx]^{15}$)	Iida *et al.* 1990
26	cf. trichorovins XIII, XIV; hypomurocins A-V, A-Va;	Iida *et al.* 1995; Wada *et al.* 1995; Becker *et al.* 1997
	trichorozin IV; trichobrachins III: I, J; trichobrachins III: 16a, 17, 18;	Brückner *et al.* 1993; Krause *et al.* 2007
	trichobrachins C-1, C-2; Tv29-11-V b	Ruiz *et al.* 2007; Mukherjee *et al.* 2011
27	**new**	
28	cf. Trichofumin B, Tv29-11-VI, hypojecorin A-22 and A-23	Berg *et al.* 2003; Mukherjee *et al.* 2011
		Degenkolb *et al.* 2012

Variable residues are underlined in the table header. Minor sequence variants are underlined in the individual sequences.

Table 3a
Comparison of the peptaibiotic pattern of specimens I–V.

	Compound								
	1[a]	**2**[a]	**3**	**4**	**5**	**6**	**7**	**8**	**9**
t_R [min]	10.5	11.2	34.1–34.2	34.7–34.9	35.6–35.9	36.2	36.3	36.7–36.9	37.2–37.4
$[M + Na]^+$	n.d.	n.d.	1904.0666	1874.0586	1874.0624	1918.0835	1874.0788	1888.0757	1888.0711
$[M + H]^+$	1732.9987	1747.0138	1882.0893	1852.0790	1852.0776	1896.0999	1852.0743	1866.0911	1866.1009
I	n.d.	n.d.	+	+	+	+	+	+	+
II	n.d.	n.d.	n.d.	(+)	(+)	+	(+)	+	+
III	n.d.	n.d.	n.d.	n.d.	n.d.	(+)	n.d.	+	+
IV[b]	(+)	(+)	(+)	(+)	+	(+)	+	n.d.	+
V[b]	+	+	(+)	(+)	+	(+)	+	n.d.	+

	Compound								
	10	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
t_R [min]	37.3–37.5	38.3–38.4	38.7–38.9	39.2	39.5–39.7	39.9	40.4–40.5	40.8–41.1	41.3–41.6
$[M + Na]^+$	1854.0799	1902.0918	1888.0783	1918.1024	1902.0898	1868.0893	1902.0974	1916.1049	1916.1051
$[M + H]^+$	1832.1076	1880.1072	1866.0997	1896.1048	1880.1187	1846.1231	1880.1177	1894.1248	1894.1233
I	+	+	+	+	(+)	(+)	+	+	+
II	+	+	+	n.d.	n.d.	(+)	+	+	(+)
III	n.d.	+	+	n.d.	n.d.	n.d.	+	(+)	n.d.
IV[b]	n.d.	+	+	n.d.	+	+	+	(+)	(+)
V[b]	+	+	+	+	+	+	+	(+)	n.d.

n.d.: not detected.

+: positive.

(+): weakly positive.

[a]Detected by DTU maXis gradient, only.

[b]Interpolated from DTU maXis gradient using higher injection volume.

Table 3b
Comparison of the peptaibiotic pattern of plate cultures I–III.

	Compound										
	19	**20a**	**20b**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
t_R [min]	34.0–34.2	37.0–37.2	37.0–37.3	38.9–39.0	39.6–39.7	40.2–40.5	41.0–41.1	41.3–41.6	41.5	42.3–42.5	42.9
$[M + Na]^+$	1904.0757	n.d.	1888.0737	n.d.	1902.0868	1902.0864	1916.1030	1987.1402	1189.7938	1988.1451	1203.8111
$[M + H]^+$	1882.0812	1852.0721	1866.0909	1896.0874	1880.1063	1880.1065	1894.1222	1965.1576	1211.7767	1966.1576	1225.7929
I	+	(+)	+	(+)	+	+	+	n.d.	+	n.d.	+
II	+	(+)	+	(+)	+	+	+	+	+	+	+
III	+	(+)	+	(+)	+	+	+	n.d.	(+)	n.d.	(+)

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Table 4

Structural variation of 19-residue peptaibiotics produced by the five *H. pulvinata* isolates investigated.

	Residue																		
	1	**2**	**3**[a]	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Ac	Aib	Ala	Ala	Ala	Aib	Gln	Aib	Lxx	Aib	Gly	Lxx	Aib	Pro	Vxx	Aib	Aib	Gln	Gln	Pheol
			(Ser)[b]	(Ser)	(Ala)			(Vxx)	(Ala)	Ala	(Vxx)			(Lxx)		(Vxx)			(Lxxol)
					(Vxx)					(Ser)									

[a] Variable positions are underlined.

[b] Minor sequence variations are parenthesised.