Screening the Biosphere: The Fungicolous Fungus *Trichoderma phellinicola*, a Prolific Source of Hypophellins, New 17-, 18-, 19-, and 20-Residue Peptaibiotics\(^1\)

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To investigate the significance of antibiotics for the producing organism(s) in the natural habitat, we screened a specimen of the fungicolous fungus *Trichoderma phellinicola* (syn. *Hypocrea phellinicola*) growing on its natural host *Phellinus ferruginosus*. Results revealed that a particular group of non-ribosomal antibiotic polypeptides, peptaibiotics, which contain the non-proteinogenic marker amino acid, \(\alpha\)-aminoisobutyric acid, was biosynthesized in the natural habitat by the fungicolous producer and, consequently, released into the host. By means of liquid chromatography coupled to electrospray high-resolution time-of-flight mass spectrometry, we detected ten 20-residue peptaibols in the specimen. Sequences of peptaibiotics found *in vivo* were independently confirmed by analyzing the peptaibiome of an agar plate culture of *T. phellinicola* CBS 119283 (*ex-type*) grown under laboratory conditions. Notably, this strain could be identified as a potent producer of 39 new 17-, 18-, and 19-residue peptaibiotics, which

\(^1\) The term *residue* covers both \(\alpha\)-amino acids and the C-terminal \(\beta\)-amino alcohol.

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display the same building scheme as the 20-residue peptaibols found in the specimen. Two of the 19-residue peptaibols are tentatively assigned to carry tyrosinol, a novel C-terminal residue, as deduced from high-resolution tandem mass-spectrometry data. For the new peptaibiotics produced by \textit{T. phellincola}, the name ‘hypophellin(s)’, based on the teleomorph name, is introduced.

\textbf{1. Introduction.} – 1.1. \textit{Fungi as a Prolific Source of Bioactive Natural Products.} The current estimate of the total number of fungal species ranges between 1.0 and 1.5 million [1], whereas the number of those validly described should now exceed only 98,000 [2]. Of the 33,500 bioactive microbial metabolites known to date, the fungal kingdom contributes ca. 15,600. Approximately 10,000 of them were shown to display anti-infective, antitumor, and/or antiviral activities. Microbial-derived drugs on the market comprise ca. 400–500 active pharmaceutical agents [3], including therapeutically relevant antibiotics of fungal origin such as \(\beta\)-lactams, fusidic acid, and griseofulvin, as well as the two immunosuppressants mycophenolic acid and cyclosporine A [4].

Given that less than 1\% of microorganisms visible under the microscope have been cultivated under laboratory conditions so far, microbial diversity provides an enormous, yet underestimated potential for future drug discovery [5] and in the search for new agricultural antibiotics [6].

1.2. \textit{The Potential of \textit{Trichoderma} Species as Biological Control Agents (BCAs).} Species of the ubiquitous fungal genus \textit{Trichoderma} and its \textit{Hypocrea} teleomorphs have attracted considerable interest in the past two decades because of the pivotal role of their secondary metabolites in the antagonistic activities of biocontrol species [7–9]. Most of them occur as opportunistic, plant (endo)symbionts [10], some of which exhibit pronounced antimicrobial activity towards economically important plant pathogens. Recent examples include:

- the hyperparasite \textit{Trichoderma stromaticum} (syn. \textit{Hypocrea stromaticica}), the active agent of ‘Tricovab’ a commercial formulation against \textit{Crinipellis} (syn. \textit{Moniliophthora}) \textit{perniciosa}, the Witches’ broom pathogen of cocoa (\textit{Theobroma cacao}) [11][12];
- \textit{T. paucisporum} and \textit{T. theobromicola}, displaying \textit{in vitro}-activities against frosty pod rot of cocoa, \textit{Moniliophthora roreri} [13];
- \textit{T. martiale}, which, in small-scale in \textit{situ} field trials, proved highly effective against black pod rot of cocoa caused by \textit{Phytophthora palmivora} [14].

The mode of action of phytoprotective \textit{Trichoderma} species is considered rather complex. Depending on the species or even strains investigated, the following mechanisms may contribute to the antagonistic potential towards plant pathogenic fungi:

\begin{itemize}
\item \textit{i}) Competition for nutrients and/or space, \textit{ii}) growth promotion of plants, especially colonization of roots, resulting in improved root and plant growth, \textit{iii}) induction of localized and systemic resistance responses in plants, \textit{iv}) mycoparasitism, \textit{v}) increase of uptake and concentration of nutrients by the plant, including the production of siderophores, and \textit{vi}) production of volatile and non-volatile antibiotics [10].
\end{itemize}
1.3. Peptaibiotics – Non-Ribosomally Biosynthesized Fungal Peptide Antibiotics Containing α,α-Dialkyl-α-amino Acids. During the past two decades, peptaibiotics have regained particular interest because of their unique bioactivities, resulting from their amphipathicity and helical conformations [15]. These are attributed to the presence of high proportions of peptide-bound α-aminoisobutyric acid (Aib), frequently accompanied by α- and/or l-isovaline (Iva) [16], and, in a few sequences, l-α-ethylnorvaline (EtNva), or 1-aminocyclopropane-1-carboxylic acid (Acc) [17]. The presence of these α,α-dialkyl-α-amino acids (Fig. 1, a) has been confirmed in acidic hydrolysates of more than 30 genera of fungi [18].

Peptaibiotics are defined as non-ribosomally biosynthesised, linear or cyclic polypeptide antibiotics of exclusively fungal origin which i) have a molecular weight between 500 and 2,200 Da, thus containing 4–21 residues; ii) show a high content of the marker Aib, as well as further α,α-dialkylamino acids; iii) are characterized by the presence of other non-proteinogenic 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 most of them, consists of a free or O-acetylated, amide-bonded β-amino alcohol. The C-terminus might also be an amine, amide, sugar alcohol, 2,5-diketopiperazine, a heterocyclic residue, or an amino acid with free carboxy terminus. The majority of Aib-containing peptides carry a C-terminal residue representing a β-amino alcohol. Only this group is referred to as peptaibols sensu stricto, whereas for the others the comprehensive name peptaibiotics is used [17].

1.4. Detection of Peptaibiotics in T. phellinicola Growing on Its Natural Host. The genus Trichoderma, which currently consists of ca. 200 validly described species the number of which increases continually [19–28], is generally recognized as the most prolific source of peptaibiotics [17]. However, reports on the detection of peptaibiotics in samples collected in the natural habitat of the producer(s) are rare. Most of the ca.

| Residue | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ac      | Aib | Ala | (Vxx) | (Ser)² | (Ser)² | (Gly)² | (Vxx) |
| Aib     | Ala | Aib | Lxx | Aib | Gly | Lxx | Aib | Pro | Vxx | Aib | Vxx | (Ala) | (Glu) | Gly | Lxx | Aib | Pro | Vxx | Aib | Vxx | (Ala) | (Glu) |

Fig. 1. a) Structures and configurations of α,α-dialkylamino acids found in peptaibiotics. b) Building scheme of subfamily-I (SF1) peptaibiotics, produced by Hypocrea phellinicola. Variable positions are underlined. Minor sequence variations are parenthesized. Deletions of certain amino acid positions are highlighted in different shades: C-terminal deletions are highlighted in dark, deletions of Glu in medium, and deletions of [Aib/Ala]² in light gray. *) Deleted in 17-, 18-, and 19-residues hypophellins. †) Deleted in the 17-residue sequence 29. ‡) Deleted in 18-residue sequences 11, 12, and 28, and in the 17-residue sequence 29. §) Detected with DTU maXis gradient only. ¶) Detected with JLU micrOTOF-Q II gradient only.
1,000 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 isolated from natural specimens were hypelcins A and B obtained from ca. 2 kg of dried, crushed stromata of Hypocrea peltata [29–31]. In 1997 and 1999, three reports were published on the isolation of peptaibiotics from fruiting bodies of Scleroderma texense, Tylopilus neofelleus, and Boletus sp., respectively; all being members of the Boletales [32–34]. However, in 2002, Kiet et al. [35] isolated chrysospermins A–D from the Vietnamese species Xerocomus langbianensis (Boletaceae, Boletales) and attributed the detection of these four 19-residue peptaibols [36] to an unrecognized infection of X. langbianensis with Sepedonium sp. This phenomenon was later commented on by Degenkolb et al. [37]. Finally, Neuhof et al. [39] corroborated the assumption of Kiet et al. [35] by analyzing four fruiting bodies of members of the order Boletales infected by Sepedonium chrysospermum and S. microspermum, respectively. Notably, all samples were screened positive for peptaibiotics of the chrysospermin type. In 2006, Lehr et al. [40] demonstrated that 16-residue peptaibols, the antiamoebins, were solely responsible for antibiosis in herbivore dung naturally colonized by or artificially inoculated with Stilbella fimetaria (syn. S. erythrocephala).

1.5. Bioactivities of Peptaibiotics from Trichoderma. Peptaibiotics are thus assumed 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 in planar lipid bilayers [41]. Their well-documented membrane activity, however, may also account for other striking bioactivities, such as neurolepsy [42], inhibition of amyloid β-peptide formation [43], inhibition of HIV-1 integrase [44], suppression of tumor cells, targeted calcium-mediated apoptosis, and autophagy in human hepatocellular carcinoma cells [45], as well as induction of defence responses and systemic resistance in tobacco against tobacco mosaic virus [46] and programmed cell death in fungal plant pathogens [47].

1.6. Choice of the Model Organism. Trichoderma phellinicola, a recently described polyporicolous species, which specifically occurs on effused basidiomes of Phellinus spp., was chosen as a model organism. Specimens of H. phellinicola have so far been recorded from Austria, Denmark, Germany [20], and the Czech Republic (see Exper. Part). This species is possibly specific for Phellinus ferruginosus [20].

To confirm the above hypothesis of peptaibiotic production under in vivo conditions, a specimen of Trichoderma phellinicola growing on its host Phellinus ferruginosus, was screened for peptaibiotics. For comparison, the ex-type culture of T. phellinicola, CBS 119283 (=C.P.K. 2137), was investigated. Both morphs were analyzed using a peptaibiomics approach as described in [48–50].

2. Results. – 2.1. General Considerations. All 17-, 18-, 19-, and 20-residue sequences discussed below were obtained from Trichoderma phellinicola [20]. The name ‘hypophellins’ (HPHs), which covers the entirety of long-chain peptaibiotics (>17 residues) produced by this species, is proposed. We base this name on the teleomorph name Hypocrea phellinicola, which used to be the valid name of the holomorph in dual nomenclature [20]. The introduction of a new name for peptaibiotics from a phylogenetically well-defined species is more favorable than earlier names for many
of the 19- and 20-residue peptaibiotics mentioned below, viz. suzukacillins, trichocellins, trichokonins, and longibrachins, which were produced by phylogenetically undefined Trichoderma species with thus highly questionable names. The latter issue is further complicated by the fact that many of the peptaibiotic-producing Trichoderma strains reported in the literature have never been deposited in a public culture collection, or deposition was terminated [51].

Hypophellins are numbered consecutively with Arabic numbers as follows: i) sequences produced by the specimen; ii) sequences produced by the culture CBS 119283 grown and analyzed at JLU; iii) sequences produced by the culture CBS 119283 grown and analysed at DTU.

2.2. Peptaibiotic Pattern of the Teleomorph. Notably, the teleomorph of Trichoderma phellinicola proved to be a prolific source of ten 20-residue peptaibols, compounds 1–10, displaying the characteristic building scheme of subfamily I (SF1), one of the nine ‘peptaibol subfamilies’ (Fig. 1, b, and Tables 1 and 2), as introduced by Chugh and Wallace [52]4).

One Gln residue is found in position 7, and another one towards or at the C-terminus in position 18, whereas position 19 is either occupied by a third Gln or a Glu residue. A highly conserved Pro residue is located in position 14 of the peptide chain. All sequences have a Gly residue in position 11 and terminate in Pheol. At least seven, at most nine, residues are occupied by Aib. Variable amino acid residues are located in positions 2, 6, 17, and 18 (Fig. 1, b).

Most of the peptaibols sequenced resemble previously described compounds (Fig. 1, b, Table 1, and Fig. 2, a) such as longibrachins A and B [53], trichobrachins II [57], trichoaureocins [54], trichokonins [55][62][63], and suzukacillins A [60].

2.3. Peptaibiotic Pattern of the Culture. 2.3.1. General Considerations. As observed before [20], ascospores of T. phellinicola are unstable and die rapidly after collecting. This might have been the reason why no agar culture could be obtained from our specimen. As a substitute, the ex-type culture of T. phellinicola CBS 119283 (= C.P.K. 2137) was provided, and its peptaibiotic pattern was analyzed. Except for the two lipopeptaibols 48 and 49, the remaining compounds 11–47 represent the characteristic building scheme of SF1, resembling the previously described 20-residue peptaibols suzukacillins A, trichosporins B, and trichocellins A [60][61][64–67].

2.3.2. micrOTOF-Q II Screening. In contrast to the specimen analyzed, the ex-type plate culture grown and analyzed at the Justus Liebig University of Giessen (JLU) produced two new 18- and fifteen new 19-residue peptaibols, compounds 11–27, which lacked the [Ala/Aib]6 residue of the 20-residue peptaibols found in the specimen (Tables 3 and 4, and Fig. 2, b). The two truncated 18-residue sequences, compounds 11 and 12, terminated in free Gln. Sequences 14 and 16–27 carry a C-terminal Pheol. For compounds 13 and 15, a C-terminal tyrosinol residue (abbreviated as ‘Tyrol’) was tentatively deduced from HR-ESI-MS/MS data (Tables 3 and 4, Fig. 3).

4) These subfamilies were introduced at a time when the total number of peptaibiotics described did not exceed 200 sequences. As of October 2012, ca. 1,000 individual sequences are known, which also exhibit new building schemes and constituents. Consequently, there is an urgent need to reconsider this classification.
2.3.3. maXis Screening. All SF1 peptaibiotics, compounds 12, 14, 19, 28–47, of the ex-type plate culture grown and analyzed at DTU (Tables 5 and 6, and Fig. 2, c) exhibit the characteristic deletion of the Ala/Aib residue in position 6. However, different positional isomers and homologues were found, e.g., the 17-residue deletion sequence 29, lacking the C-terminal dipeptide [Gln18−Pheol19]. In compound 31, a Ser-residue was found in position 3, whereas compound 30 exhibited a Gly residue in position 4. Overall, the structural diversity of peptaibiotics produced by the two cultures was much higher as compared to the specimen: variable amino acid residues were in positions 2, 3, 4, 5, 6, 17, 18, and 20 (Fig. 1, b).

2.4. Lipopeptaibols as Trace Components in the Plate Cultures. Two lipopeptaibols, compounds 48 and 49, were produced as trace components in the DTU plate culture. Compound 49 probably represents trichogin A IV [68][69] or a positional isomer thereof. The new positionally isomeric compound 48, named ‘lipophellin 1’, is characterized by the deletion of [Gly] of compound 49 (Tables 5 and 6, and Fig. 2, c).

3. Discussion. – 3.1. Hypophellins, Novel Long-Chain Peptaibiotics from T. phellinicola. The most notable result of this investigation is, indeed, the unequivocal confirmation of peptaibiotic biosynthesis in the natural habitat of T. phellinicola growing on its host Phellinus ferruginosus, commonly known as the Rusty Porecrust.
We here describe for the first time the \textit{in vivo} detection of non-ribosomal peptide antibiotics\textsuperscript{5}), which may significantly contribute to the complex interaction of a fungicolous ascomycete growing on its basidiomycetous host.

3.2. The Peptaibiome of the Specimen. The teleomorph produced a microheterogeneous mixture of ten 20-residue HPHs, four of which, 6, 8, 9, and 10, are new (Table 1). Compared to smaller sequences consisting of less than 17 residues, long-chain peptaibiotics display a higher membrane-pore-formation activity by several orders of magnitude \textsuperscript{71}.

Depending on the individual sequence, seven to nine Aib residues are present, which strongly promote the formation of helical structures, \textit{i.e.}, $\alpha$- or $3_{10}$-helices, and even mixed forms \textsuperscript{72–74}, which is due to the steric constraints imposed by the geminal Me groups of the C\textsuperscript{a}-atom \textsuperscript{75}. All of them exhibit the structurally important features, which are required for the formation of transmembrane ion channels in artificial lipid bilayer membranes, as compiled by Duclohier \textsuperscript{76}, and Duclohier and CHEMISTRY & BIODIVERSITY – Vol. 10 (2013) 793

\begin{table}[h]
\centering
\begin{tabular}{cccccccccc}
\hline
12 & 13 & 14 & 15 & 16 & 17 & 18 & 19 & 20 & Compound identical or positionally isomeric with Ref.
\hline
Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol Longibrachin A I & [53]
 & Trichoaureocin 3 & [54]
 & Trichokonin VI (= gliodeliquescin A) & [55][56]
 & Trichobrachins II-5, II-6 & [57]
 & Trichobrachin Iib A & [58][59]
Lxx Aib Pro Vxx Aib Aib Glu Gln Pheol Longibrachin B II & [53]
 & Trichokonin VII & [55]
 & Trichoaureocin 4 & [54]
 & Suzukacillin A-10a & [60]
 & Trichobrachins II-7, II-8, II-9 & [57]
 & Trichobrachin Iib B & [58][59]
Lxx Aib Pro Vxx Aib Aib Vxx Glu Gln Pheol Longibrachin B III & [53]
 & Trichokonin VIII (= trichosporin B-IvE) & [55][61]
 & Trichoaureocin V & [54]
 & Trichobrachin Iib C & [58][59]
Lxx Aib Pro Vxx Aib Aib Vxx Glu Gln Pheol New (longibrachin IV: [Gln]$^{18} \rightarrow$[Glu]$^{18}$) & [53]
 & Trichoaureocin VI & [54]
 & Trichobrachin Iib D & [58][59]
Lxx Aib Pro Vxx Aib Vxx Glu Gln Pheol New (homolog of 7) & [53]
 & Trichoaureocin VI & [54]
 & Trichobrachin Iib D & [58][59]
Lxx Aib Pro Vxx Aib Vxx Glu Gln Pheol New (homolog of 8) & [53]
 & Trichoaureocin VI & [54]
 & Trichobrachin Iib D & [58][59]
\hline
\end{tabular}
\caption{Compound identical or positionally isomeric with Ref.}
\end{table}

sequences. This applies to Tables 1, 3, and 5.

\textsuperscript{5}) Hypophellins were simultaneously detected in an LC/MS/MS screening of 15 specimens belonging to nine \textit{Hypocrea} species, which have been collected in their natural habitat. Recently, a manuscript on the \textit{in vivo} detection of hypopulvins, novel peptaibiotics from the polyporicolous fungus \textit{H. pulvinata}, has been published. The results therein corroborate that peptaibiotics are produced by a fungicolous fungus during infection of its natural hosts \textsuperscript{70}. 

\textsuperscript{50}
Table 2. Diagnostic Fragment Ions of 20-Residue Peptaibiotics Detected in the Specimen of Hypoerea phellinicola (micrOTOF-Q II screening)

| Diagnostic fragment ions Peaks [m/z]| 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|--------------------------------------|---|---|---|---|---|---|---|---|----|
| [M + Na]^+                            |   |   |   |   |   |   |   |   |    |
| a1                                    | 12345678910|   |   |   |   |   |   |   |    |
| [M + H]^+                             |   |   |   |   |   |   |   |   |    |
| a1                                    |   |   |   |   |   |   |   |   |    |
| a2                                    | 100.0808 | 100.0808 | 100.0808 | 100.0806 | 100.0809 | 100.0805 | 100.0808 | n.d. | n.d. |
| a3                                    | 171.1191 | 171.1191 | 171.1191 | 171.1191 | 171.1191 | 171.1191 | 171.1191 | 171.1191 | 171.1191 |
| a4                                    | 256.1657 | 256.1657 | 256.1662 | 256.1665 | 256.1668 | 256.1671 | 256.1674 | 256.1677 | 256.1680 |
| a5                                    | n.d.    | n.d.    | 412.2735 | 412.2735 | 412.2735 | 412.2735 | 412.2735 | 412.2735 | 412.2735 |
| b1                                    | 128.0758 | 128.0758 | 128.0762 | 128.0762 | 128.0762 | 128.0762 | 128.0762 | 128.0762 | 128.0762 |
| b2                                    | 199.1102 | 199.1102 | 199.1102 | 199.1102 | 199.1102 | 199.1102 | 199.1102 | 199.1102 | 199.1102 |
| b3                                    | 284.1604 | 284.1604 | 284.1615 | 284.1618 | 284.1621 | 284.1624 | 284.1627 | 284.1630 | 284.1633 |
| b4                                    | 355.1982 | 355.1982 | 355.1982 | 355.1982 | 355.1982 | 355.1982 | 355.1982 | 355.1982 | 355.1982 |
| b5                                    | 440.2121 | 440.2121 | 440.2121 | 440.2121 | 440.2121 | 440.2121 | 440.2121 | 440.2121 | 440.2121 |
| b6                                    | 525.2839 | 525.2839 | 525.2839 | 525.2839 | 525.2839 | 525.2839 | 525.2839 | 525.2839 | 525.2839 |
| b7                                    | 600.3431 | 600.3431 | 600.3431 | 600.3431 | 600.3431 | 600.3431 | 600.3431 | 600.3431 | 600.3431 |
| b8                                    | 675.3937 | 675.3937 | 675.3937 | 675.3937 | 675.3937 | 675.3937 | 675.3937 | 675.3937 | 675.3937 |
| b9                                    | 750.4441 | 750.4441 | 750.4441 | 750.4441 | 750.4441 | 750.4441 | 750.4441 | 750.4441 | 750.4441 |
| b10                                   | 825.4941 | 825.4941 | 825.4941 | 825.4941 | 825.4941 | 825.4941 | 825.4941 | 825.4941 | 825.4941 |
| y7                                    | 774.4598 | 774.4598 | 774.4598 | 774.4598 | 774.4598 | 774.4598 | 774.4598 | 774.4598 | 774.4598 |
| y7 – H2O                              | 756.4345 | 756.4345 | 756.4345 | 756.4345 | 756.4345 | 756.4345 | 756.4345 | 756.4345 | 756.4345 |
| y7 – AA (20)                          | 623.3596 | 623.3596 | 623.3596 | 623.3596 | 623.3596 | 623.3596 | 623.3596 | 623.3596 | 623.3596 |
| y7 – AA (20-19)                       | 495.3197 | 495.3197 | 495.3197 | 495.3197 | 495.3197 | 495.3197 | 495.3197 | 495.3197 | 495.3197 |
| y7 – AA (20-18)                       | 367.2398 | 367.2398 | 367.2398 | 367.2398 | 367.2398 | 367.2398 | 367.2398 | 367.2398 | 367.2398 |
| y7 – AA (20-17)                       | 282.1890 | 282.1890 | 282.1890 | 282.1890 | 282.1890 | 282.1890 | 282.1890 | 282.1890 | 282.1890 |

* a) n.d., Not detected.
A multitude of bioactivities has been described for 20-residue peptaibols of similar structure, which are compiled in Table 7.

3.3. The Peptaibiome of the Ex-Type Plate Culture. In contrast to what has been observed for the specimen, 20-residue peptaibols could not be detected. Instead, fifteen 19-residue peptaibols were detected in the micrOTOF-Q II screening and another eighteen in the maxis screening. Although sequences of 11–47 still exhibit the characteristic building scheme of SF1, they are distinguished from the 20-residue peptaibols of the teleomorph specimen by a deletion of the Aib/Ala residue in position 6 (Δ Ala/Aib6) of the peptide chain. This deletion, however, is predicted not to negatively influence the bioactivity of these long-chain peptaibols, as all important structural features are still present, which comply with the requirements for the formation of transmembrane ion channels in artificial lipid bilayer membranes [76], [77]. The three 18-residue sequences, 11, 12, and 28, exhibit a deletion of the C-terminal amino alcohol, whereas the dipeptide [Gln18–Pheol19] is deleted in 29, a 17-residue sequence. Truncated versions of SF1 peptaibols lacking the C-terminal amino alcohol or even the adjacent Gln residue have been reported before.

The ten 19-residue peptaibiotics, trichobrachins I (TB I), lacking the C-terminal Pheol residue, as well as the two 18-residue trichobrachins II-1 and -2 (TB II), which exhibit a deletion of the C-terminal dipeptide [Gln19–Pheol20], were shown to originate from 20-residue trichobrachins II (TB II) by enzymatic degradation. Two minor desPheol compounds F30, representing 1.3% of the alamethicin (ALM) mixture investigated, have been detected by non-aqueous capillary electrophoresis (NACE) coupled to electrospray mass spectrometry [94].

3.4. 1-Phenylalanyl as Constituent of Natural Products. C-Terminal 1-Phanol is commonly found in peptaibiotics but has also been infrequently reported as a constituent of other plant and fungal secondary metabolites such as N-benzoyl-1-phenylalaninol from Catharanthus pusillus [95] and Diospyros quaesita [96], O-acetyl-N-(N'-benzoyl-1-phenylalanyl)-1-phenylalaninol from Euphorbia fischeriana and E. kansui [97], and N-benzoyl-O-[N'-benzoyl-1-phenylalanyl]-1-phenylalaninol from Penicillium arenicola (syn. P. canadense) [98].

3.5. 1-Tyrosinol as a Constituent of Natural Products. To the best of our knowledge, neither d- nor l-tyrosinol has ever been reported as constituent of either linear or cyclic peptides of microbial origin, including peptaibiotics. However, l-tyrosinol is a ‘cryptic’ building block of the following natural products:

- farinosone C, an amide from Paecilomyces farinosus RCEF 0101 [99];
- cordyceamides A and B from a liquid culture of Cordyceps sinensis [100];
- preoxazinin-7, the linear precursor [101], and cyclic oxazinins from the digestive glands of Mytilus galioprovincialis [102], [103].

3.6. The Lifestyle of Trichoderma phellinicola: Findings and Thoughts. Taken these findings together, we dare predict a mycoparasitic lifestyle of the host-specific polyporicolous Trichoderma phellinicola:

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. Parallel formation of hydrolytic enzymes and 19-residue antifungal

6) C-Terminal β-amino alcohols with the d-configuration have not yet been reported for peptaibiotics.
Fig. 2. Base-peak chromatograms (BPCs) of a) the H. phellinicola specimen screened with the micrOTOF-Q II, b) the H. phellinicola ex-type plate culture screened with the micrOTOF-Q II, and c) the H. phellinicola specimen screened with the maXis. †, co-eluting peptaibiotics, not sequenced; ‡, non-peptaibiotic metabolite.
trichorzianins A and B by the potent mycoparasite *Trichoderma atroviride*\(^7\)) is triggered in the presence of cell walls of plant-pathogenic fungi \[106\]. Trichorzianins have previously been shown to form voltage-gated ion channels in planar lipid bilayers \[107\] and to modify the membrane permeability of liposomes, and they are active against *Rhizoctonia solani* and *Phythophthora cactorum* \[108\]. Based on these findings, 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 to form ion channels. Cell leakage reduces the ability of the host to effectively repair its cell wall. Eventually, inhibition of chitin and \(\beta\)-glucan synthesis further amplifies the destructive effect of chitinases and \(\beta\)-1,3-glucanases \[108\]. These mechanisms, however, may also account for the recently published induction of programmed cell death in plant fungal pathogens \[47\] caused by the 20-residue peptaibol trichokonin VI (= gliodeliquescin A \[56\])\(^8\), from *T. koningii*, *T. pseudokoningii*, and *T. deliquescens* (syn. *Gliocladium deliquescens*) \[20\]. The presence of peptaibiotics was also shown to play a role in the induction of plant defence responses \[110\].

\(7\) The trichorzianin-producing strain ATCC 36042 (= CBS 391.92) was originally identified as *T. harzianum* \[104\] but later shown to belong to *T. atroviride* \[105\]. The high degree of misidentification of *Trichoderma* species prior to introduction of phylogenetic analysis is still regarded a major problem, unless authors describe how their cultures were identified \[17\].

\(8\) Gliodeliquescin A has been isolated from *Gliocladium deliquescens* NRRL 1086 \[109\] and not from NRRL 3091 \[56\]. According to phylogenetic data (18S-rRNA, and ITS 1 and 2), *G. deliquescens* NRRL 1086 (= CBS 228.48 = ATCC 10097) was re-identified as *G. viride* (www.straininfo.net/strains/260309).
Remarks on Non-Ribosomal Biosynthesis and Module Skipping by T. phellinicola. The exclusive production of 20-residue peptaibols by the T. phellinicola teleomorph indicates the presence of a 20-module NRPS. As the culture CBS 119283 has been shown to produce 17-, 18-, and 19-residue peptaibiotics only, it is likely to contain a 19-module NRPS, lacking the 6th module activating Ala or Aib. In addition, modules 3 and 4 show differing substrate specificities, as compared to the teleomorph, thus permitting the incorporation of Ala or Ser in position 3 and of Gly, Ala, or Ser in position 4, respectively. These findings indicate substantial variations in the sequences of the SF1-type peptaibol synthetases of both strains. As has been discussed in the case of SF4-type peptaibols, genes involved in secondary-metabolite products show a much broader sequential variety than housekeeping genes [50]. We here, indeed, find evidence for a significant structural variation within a large gene.

### Experimental Part

#### Chemicals

All solvents used, MeCN (99.9%), MeOH (99.9%), CH₂Cl₂ (99.8%), and HCOOH (98%), were of LC/MS grade from Sigma-Aldrich (D-Steinheim). Water was purified by a Merck-Millipore Milli-Q Synthesis A10 system (D-Schwabach/Ts.).

#### Origin of Specimen

The teleomorphic specimen of Trichoderma phellinicola growing on its host Phellinus ferruginosus was collected in the ‘Národní park Podyji’ (Czech Republic, Moravia), near Hardegg at the bridge across the River Thaya, just across the border between Austria and the Czech Republic.
Origin of Trichoderma phellinicola CBS 119283 (ex-type). All details concerning this new species were given by Jaklitsch [20].

Extraction of Specimens. The teleomorph was extracted with CH$_2$Cl$_2$/MeOH 1:1 (v/v), the solvent was evaporated in vacuo (Rotavapor R-215, B/C-Bchi, D-Essen), and the extract was cleaned up over Sep-Pak Classic C$_18$ cartridges (Waters, D-Eschborn) as described by Krause et al. [48].

Cultivation and Extraction of Pure Cultures. Cultures of the specimen were grown on potato dextrose agar (PDA; Becton Dickinson, D-Heidelberg) at 23°C for 6 d. These subcultures were used for inoculation of the main cultures. After 10 d of cultivation at 23°C in the dark, main cultures were extracted as described for the teleomorph.

LC/MS Analysis. Two QTOF systems, both from Bruker Daltonic (D-Bremen) controlled by HyStar v. 3.2 were used. Both instruments were equipped with an orthogonal ESI source and coupled to a Dionex UltiMate 3000 UPLC (Dionex, D-Idstein).

System 1: high-resolution microTOF Q-II mass spectrometer. For separation, an Acclaim 120 C$_18$, 3 µm, 2.1 × 150 mm, column (Dionex, D-Idstein) at a flow rate of 0.25 ml/min $\pm$ 1 and a temp. of 35°C was used. Eluent A consisted of H$_2$O +0.1% HCOOH and eluent B of 95% MeCN +0.1% HCOOH. 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: first a full scan was recorded from m/z 50 to 3000. In System 1, this was followed by CID measurements from m/z 50 to 2000, recorded at energy of 150 eV. Finally, results of CID-MS were verified by MS/MS experiments on selected precursor ions. For precursors of m/z < 1000, a collision energy of 35 eV and precursor ions of m/z > 1500 at a collision energy of 40 eV. The isolation width for MS/MS experiments was set to ±1 Da.

### Hypocrea phellinicola (microTOF-Q II screening)

| 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|----|----|----|----|----|----|----|----|----|
| Lxx Aib Pro Vxx Aib Vxx Gln Gln | New (trichocellin A-VI – [Aib]$^6$ – Pheol) | [67] |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln | New (trichocellin A-VI – [Ala]$^6$ – Pheol) | [67] |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln Tyrol | New |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
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| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
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| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
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| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | New |
System 2: The maXis 3G QTOF mass spectrometer operated at a resolution of 40,000 FWHM. An Acquity BEH300 C18, 1.7 μm, 2.1 × 150 mm, column (Waters, D-Eschborn) was used for separation, using H2O + 0.1% HCOOH (eluent A) and 100% MeCN + 0.1% HCOOH (eluent B). The flow rate was set to 0.3 ml/min and the temp. to 40°. The gradient started with 90% A/10% B and was changed to 50% A/50% B at 7 min, then to 30% A/70% B at 25 min, then raised to 100% B at 38 min, and held at 100% B until 41 min before setting to starting conditions from time 42 min to 46 min. Three ml were injected. MS were scanned in the m/z range of 100–2,000. Auto MS with precursor ion-dependent collision energy optimization was used for fragmentation in the range of 10–65 eV.

Data interpretation was performed using the DataAnalysis v. 4.0 software (Bruker Daltonic, D-Bremen). Use of high-resolution (HR) ESI-MS allowed 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, resp.) or the corresponding amino alcohols (Leuol/Ileol) with the same

### Table 4. Diagnostic Fragment Ions of 18- and 19-Residue Peptaibiotics Detected in the Ex-Type Culture (CBS)

| Diagnostic fragment ions | Peaks [m/z] | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|--------------------------|-------------|----|----|----|----|----|----|----|----|
| tR [min]                 |             | 30.9–31.1 | 31.8–32.0 | 32.2–32.6 | 32.5–32.7 | 32.8–33.1 | 35.1–35.3 | 37.0–37.2 | 37.7–37.9 |
| [M + Na]⁺                | 1768.9850   | 1783.0115 | 1918.0846 | 1932.0976 | 1932.1017 | 1918.0877 | 1888.0616 | 1902.0891 |
| [M + H]⁺                 | 1747.0135   | 1761.0324 | 1896.0995 | 1910.1131 | 1910.1140 | 1896.1035 | 1866.0928 | 1880.1095 |
| a₁                       | 100.0718    | n.d.     | n.d.     | n.d.     | n.d.     | 100.0720 | 100.0720 | n.d.     |
| a₂                       | 256.1647    | 256.1624 | 242.1508 | 256.1641 | 256.1707 | 242.1511 | 242.1506 | 256.1675 |
| a₃                       | n.d.        | n.d.     | n.d.     | n.d.     | 327.1979 | n.d.     | n.d.     | 327.2046 |
| a₄                       | n.d.        | n.d.     | n.d.     | n.d.     | n.d.     | 398.2312 | n.d.     | n.d.     |
| b₁                       | 128.0687    | 128.0658 | 128.0709 | 128.0708 | 128.0835 | 128.0715 | 128.0719 | 128.0721 |
| b₂                       | 199.1075    | 199.1076 | 199.1109 | 199.1110 | 199.1116 | 199.1118 | 199.1115 | 199.1081 |
| b₃                       | 284.1611    | 284.1617 | 270.1453 | 284.1622 | 284.1637 | 270.1434 | 270.1471 | 284.1634 |
| b₄                       | 355.1972    | 355.1977 | 341.1846 | 355.2032 | 357.1765 | 341.1815 | 355.1988 |
| b₅(H₂O)                  | n.d.        | n.d.     | n.d.     | n.d.     | 353.1758 | n.d.     | n.d.     | n.d.     |
| b₆                       | 426.2340    | 440.2546 | 426.2354 | 456.2441 | 440.2494 | 442.2277 | 426.2314 | 440.2546 |
| b₇                       | 554.2840    | 568.3175 | 554.2840 | 584.3226 | 568.3175 | 570.2870 | 554.2989 | 568.3023 |
| b₈                       | 639.3523    | 653.3691 | 639.3539 | 669.3625 | 653.3679 | 655.3443 | 639.3530 | 653.3685 |
| b₉                       | 752.4400    | 766.4531 | 752.4386 | 782.4408 | 766.4563 | 766.4523 | 766.4519 |
| b₁₀                      | 837.4860    | 851.5024 | 837.4880 | 867.4961 | 851.5028 | 853.4825 | 837.4896 | 851.5066 |
| b₁₁                      | 894.5048    | 908.5271 | 894.5061 | 924.5223 | 908.5250 | 910.5022 | 894.5076 | 908.5242 |
| b₁₂                      | 1007.5856   | 1021.6063 | 1007.5967 | 1037.6039 | 1023.5962 | 1027.6073 | 1007.5917 | 1021.6085 |
| b₁₃                      | 1092.6441   | 1106.6573 | 1092.6442 | 1122.6523 | 1106.6575 | 1108.6413 | 1092.6474 | 1106.6629 |
| b₁₄(H₂O)                 | n.d.        | n.d.     | n.d.     | n.d.     | n.d.     | 1090.6265 | 1074.6077 | 1088.6332 |
| y₁                       | 655.3841    | 655.3841 | –        | –        | –        | –        | –        | –        |
| y₂(–AA (18))             | 509.3130    | 509.3130 | –        | –        | –        | –        | –        | –        |
| y₃(–AA (18-17))          | 381.2540    | 381.2540 | –        | –        | –        | –        | –        | –        |
| y₄(–AA (18-16))          | 282.1709    | 282.1709 | –        | –        | –        | –        | –        | –        |
| y₅                       | –          | –        | 804.4624 | 788.4706 | 804.4669 | 788.4697 | 774.4592 | 774.4593 |
| y₆(H₂O)                  | –          | –        | 786.4472 | 770.4510 | 786.4438 | 770.4510 | 736.4383 | 756.4383 |
| y₇                       | –          | –        | 637.3680 | 637.3708 | 637.3725 | 637.3705 | 623.3566 | 623.3559 |
| y₈(–AA (19))             | –          | –        | 509.3068 | 509.3140 | 509.3085 | 509.3103 | 495.2961 | 495.2962 |
| y₉(–AA (19-18))          | –          | –        | 381.2489 | 381.2515 | 381.2545 | 381.2513 | 367.2370 | 367.2373 |
| y₁₀(–AA (19-16))         | –          | –        | 282.1814 | 282.1814 | 282.1815 | 282.1815 | 282.1815 | 282.1815 |

a) n.d., Not detected.
elemental composition could not be distinguished, the abbreviations Lxx, Vxx, and Lxxol were used instead [48–50].

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| 19  | 20  | 21  | 22  | 23  | 24  | 25  | 26  | 27  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 38.3–38.4 | 38.8–39.2 | 39.8–40.1 | 40.6–40.9 | 41.5–41.6 | 42.1–42.3 | 43.4–43.6 | 44.2–44.4 | 45.0–45.6 |
| 1902.0921 | 1916.1081 | 1917.1085 | 1930.1235 | 1931.1236 | 1944.1425 | 1958.1599 | 1958.1548 | 1972.1635 |
| 1880.1136 | 1894.1331 | 1895.1278 | 1908.1474 | 1909.1391 | 1922.1601 | 1936.1738 | 1936.1750 | 1950.1894 |
| 100.0721 | 100.0721 | 100.0747 | 100.0722 | 100.0722 | n.d. | n.d. | n.d. | n.d. |
| 242.1514 | 256.1682 | 256.1682 | 256.1677 | 256.1649 | n.d. | n.d. | n.d. | n.d. |
| 313.1832 | 327.2048 | 327.2049 | 327.2042 | 327.2050 | n.d. | n.d. | n.d. | n.d. |
| n.d. | 412.2533 | 412.2564 | 426.2817 | n.d. | n.d. | n.d. | n.d. | n.d. |
| 128.0722 | 128.0724 | 128.0718 | 128.0708 | 128.0712 | 128.0672 | 128.0701 | 128.0684 | 128.0684 |
| 199.1121 | 199.1081 | 199.1118 | 199.1083 | 199.1141 | 227.1404 | 241.1564 | 241.1564 | 241.1564 |
| 270.1476 | 284.1608 | 284.1608 | 284.1641 | 284.1631 | [255] | [269] | 312.1953 | 326.2055 |
| 341.1814 | 355.1988 | 355.1973 | 355.1972 | 355.1972 | 383.2306 | 397.2427 | 383.2297 | 397.2477 |
| n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 426.2314 | 440.2531 | 440.2513 | 454.2685 | 454.2686 | 468.2836 | 482.3001 | 482.2988 | 496.3148 |
| 554.2989 | 568.3022 | 568.3119 | 582.3249 | 582.3249 | 596.3361 | 610.3531 | 610.3608 | 624.3766 |
| 639.3513 | 653.3673 | 653.3654 | 667.3841 | 667.3836 | 681.3976 | 695.4131 | 695.4110 | 709.4286 |
| 752.4346 | 766.4605 | 766.4489 | 780.4662 | 780.4659 | 794.4802 | 808.4949 | 808.4934 | 822.5109 |
| 837.4888 | 851.5044 | 851.5023 | 865.5205 | 865.5199 | 879.5335 | 893.5492 | 893.5457 | 907.5631 |
| 894.5075 | 908.5234 | 908.5216 | 922.5386 | 922.5395 | 936.5517 | 950.5713 | 950.5659 | 964.5813 |
| 1007.5920 | 1021.6065 | 1021.6039 | 1035.6228 | 1035.6231 | 1049.6347 | 1063.6526 | 1063.6516 | 1077.6661 |
| 1092.6463 | 1106.6606 | 1106.6578 | 1120.6786 | 1120.6785 | 1134.6898 | 1148.7069 | 1148.7051 | 1162.7188 |
| 1074.6284 | 1088.6331 | 1088.6424 | 1102.6441 | 1102.6440 | 1116.6595 | 1130.6997 | 1130.7031 | 1144.7051 |
| 788.4710 | 788.4710 | 789.4647 | 789.4718 | 789.4597 | 788.4710 | 788.4705 | 788.4678 | 788.4668 |
| 770.4509 | 770.4509 | 771.4475 | 770.4508 | 771.4390 | 770.4507 | 770.4507 | 770.1538 | 770.1538 |
| 637.3707 | 637.3707 | 638.3638 | 637.3705 | 638.3574 | 637.3721 | 637.3678 | 637.3649 | 637.3676 |
| 509.3096 | 509.3096 | 510.3014 | 509.3108 | 510.2964 | 509.3105 | 509.3113 | 509.3093 | 509.3082 |
| 381.2513 | 381.2513 | 381.2483 | 381.2524 | 381.2520 | 381.2505 | 381.2508 | 381.2506 | 381.2492 |
| n.d. | n.d. | 282.1837 | 282.1813 | 282.1813 | 282.1813 | 282.1920 | 282.1781 | 282.1917 |
Table 5. Sequences of 10-, 11-, 17-, 18-, and 19-Residue Peptaibiotics Detected in the Ex-Type Culture (CBS)

| No. | $t_{	ext{ex}}$ [min] | $[M + H]^+$ | Residue                  |
|-----|----------------------|-------------|-------------------------|
|     |                      |             | 1 2 3 4 5 6 7 8 9 10 11 |             |
| 28  | 10.8                 | 1747.0131   | Ac Aib Ala Aib Ala Aib – Gln Aib Lxx Aib Gly |
| 12  | 11.2                 | 1761.0273   | Ac Aib Ala Aib Ala Aib – Gln Aib Lxx Aib Gly |
| 14  | 12.2                 | 1911.1213   | Ac Aib Ala Aib Ser Aib – Gln Aib Lxx Aib Gly |
| 29  | 12.6                 | 1632.9708   | Ac Aib Ala Aib Ala Aib – Gln Aib Lxx Aib Gly |
| 30  | 13.0                 | 1880.1000   | Ac Aib Ala Aib Gly Aib – Gln Aib Lxx Aib Gly |
| 31  | 13.2                 | 1882.0784   | Ac Aib Ala Ser Ala Aib – Gln Aib Lxx Aib Gly |
| 19  | 13.5                 | 1880.1008   | Ac Aib Ala Ala Aib – Gln Aib Lxx Aib Gly |
| 32  | 14.1                 | 1896.0964   | Ac Aib Ala Ser Ala Aib – Gln Aib Lxx Aib Gly |
| 33  | 14.9                 | 1880.1035   | Ac Aib Ala Ala Ala Aib – Gln Aib Lxx Aib Gly |
| 34  | 15.5                 | 1866.0863   | Ac Aib Ala Ala Aib – Gln Aib Lxx Aib Gly |
| 35  | 15.9                 | 1880.1012   | Ac Aib Ala Ala Aib – Gln Aib Lxx Aib Gly |
| 36  | 16.2                 | 1867.0706   | Ac Aib Ala Ala Ala Aib – Gln Aib Lxx Aib Gly |
| 37  | 16.4                 | 1880.1007   | Ac Aib Ala Ala Aib – Gln Aib Lxx Aib Gly |
| 38  | 16.7                 | n.d.        | Ac Aib Ala Aib Ala Aib – Gln Aib Lxx Aib Gly |
| 39  | 16.8                 | 1880.1009   | Ac Aib Ala Ala Aib – Gln Aib Lxx Aib Gly |
| 40  | 17.0                 | n.d.        | Ac Aib Ala Ala Aib – Gln Aib Lxx Aib Gly |
| 41  | 17.2                 | 1880.0997   | Ac Aib Ala Ala Aib – Gln Aib Lxx Aib Gly |
| 42  | 17.5                 | 1894.1210   | Ac Aib Ala Aib Ala Vxx – Gln Aib Lxx Aib Gly |
| 43  | 17.7                 | 1895.1007   | Ac Aib Ala Aib Ala Aib – Gln Aib Lxx Aib Gly |
| 44  | 18.0                 | 1894.1177   | Ac Aib Ala Ala Aib Vxx – Gln Aib Lxx Aib Gly |
| 45  | 18.6                 | 1908.1341   | Ac Aib Ala Aib Ala Vxx – Gln Aib Lxx Aib Gly |
| 46  | 20.0                 | 1922.1467   | [227]$^\text{a}$ Aib Ala Aib – Gln Aib Lxx Aib Gly |
| 47  | 21.5                 | 1936.1660   | [241]$^\text{b}$ Aib Ala Aib – Gln Aib Lxx Aib Gly |
| 48  | 22.0                 | 1009.7031   | Oc$^\text{c}$ Aib Gly Lxx Aib – Gly Lxx Aib Gly Lxx Lxxol |
| 49  | 22.1–22.2            | 1066.7242   | Oc Aib Gly Lxx Aib Gly Gly Lxx Aib Gly Lxx Lxxol |

$^\text{a}$) The N-terminal sequence of compound 46, which is represented by a mass difference of 227 Da, could 241 Da, could not be assigned. $^\text{b}$) Oc, Tentatively assigned as n-octanoyl residue.
of Hypocrea phellinicola (maXis screening)

| 12  | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|-----|----|----|----|----|----|----|----|----|
| Lxx Aib Pro Vxx Aib Aib Gln Gln | **New** (trichocellin A-V − [Ala]⁶ − Pheol) [67] |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln | **New** (trichocellin A-VI − [Ala]⁶ − Pheol) [67] |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol | **New** |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln | **New** (12 − [Gln]³⁹) |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol | **New** (17: [Ala]⁴ − [Gly]³) |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | **New** |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol | **New** (trichosporin B-IVb − [Aib]⁶, trichosporin B-VIb − [Aib]⁶) [61] |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol | **New** |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | **New** (positional isomer of 19, 37, and 41) |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | **New** (positional isomer of 17) |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | **New** (trichosporin B-Vla − [Aib]⁶, trichosporin B-VIb − [Aib]⁶) [61][66] |
| Lxx Aib Pro Vxx Aib Aib Glu Gln Pheol | **New** (35: [Gln]³⁹ − [Glu]³⁹) |
| Lxx Aib Pro Vxx Aib Vxx Glu Gln Pheol | **New** (positional isomer of 19, 33, and 41) |
| Lxx Aib Pro Vxx Aib Aib Glu Gln Pheol | **New** (positional isomer of 35) |
| Lxx Aib Pro Vxx Aib Vxx Glu Gln Pheol | **New** (trichosporin B-VIIa − [Aib]³) [66] |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol | **New** (positional isomer of 19, 33, and 37) |
| Lxx Aib Pro Vxx Aib Aib Gln Gln Pheol | **New** |
| Lxx Aib Pro Vxx Aib Vxx Glu Gln Pheol | **New** (positional isomer of 40) |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol | **New** (positional isomer of 45) |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol | **New** (positional isomer of 44) |
| Lxx Aib Pro Vxx Aib Vxx Gln Gln Pheol | **New** |

Trichogin A IV

Sequence 13 or 14 from *Trichoderma* cf. *strigosum* CBS 119777 [49]
Partial sequence 4 from *Hypocrea citrina* CBS 853.70 [48]
Partial sequence 4 from *Hypocrea vinosa* CBS 247.63 [48]

not be assigned. b) The N-terminal sequence of compound 47, which is represented by a mass difference of
Table 6. Diagnostic Fragment Ions of 10-, 11-, 17-, 18-, and 19-Residue Peptaibiotics Detected in the Ex-

| Diagnostic fragment ions Peaks [m/z]⁺ | 28  | 12  | 14  | 28  | 30  | 31  |
|--------------------------------------|-----|-----|-----|-----|-----|-----|
| t_R [min]                            | 10.8| 11.2| 12.2| 12.6| 13.0| 13.2|
| [M + H]⁺                             | 1747.0131 | 1761.0273 | 1911.1213 | 1632.9708 | 1880.1000 | 1882.0784 |
| b₁                                  | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| b₂                                  | 284.1601 | 284.1607 | 284.1613 | 284.1609 | 284.1604 | 286.1389 |
| b₃                                  | 355.1989 | 355.1980 | 371.1938 | 355.1975 | 341.1819 | 357.1760 |
| b₁ – H₂O                            | n.d. | n.d. | 438.2353 | n.d. | 412.2541 | 424.2167 |
| b₅                                  | 440.2512 | 440.2509 | 456.2470 | 440.2506 | 426.2347 | 442.2296 |
| b₇                                  | 568.3097 | 568.3098 | 584.3039 | 568.3096 | 554.2926 | 570.2869 |
| b₉                                  | 653.3615 | 653.3626 | 669.3571 | 653.3619 | 639.3458 | 655.3404 |
| b₉ – H₂O                            | n.d. | n.d. | n.d. | 438.2353 | n.d. | 412.2541 |
| b₁₀                                 | 908.5192 | 908.5208 | 924.5190 | 908.5199 | 894.5026 | 910.4971 |
| b₁₁                                 | 1021.6077 | 1021.6046 | 1038.5981 | 1021.6053 | 1007.5901 | 1023.5860 |
| b₁₂                                 | 1106.6578 | 1106.6578 | 1122.6537 | 1106.6590 | 1092.6412 | 1108.6356 |
| b₁₂ – H₂O                           | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| y₁                                  | – | – | – | 527.3191 | – | – |
| y₁ – AA (17)                        | – | – | – | 381.2497 | – | – |
| y₁ – AA (17-16)                     | – | – | – | 282.1814 | – | – |
| y₁ – AA (17-15)                     | – | – | – | 197.1287 | – | – |
| y₁ – AA (17)                        | 641.3626 | 655.3768 | – | – | – | – |
| y₁ – AA (18)                        | 495.2923 | 509.3095 | – | – | – | – |
| y₁ – AA (18-17)                     | 367.2353 | 381.2500 | – | – | – | – |
| y₁ – AA (18-16)                     | 282.1812 | 282.1816 | – | – | – | – |
| y₁ – AA (18-15)                     | 197.1274 | 197.1288 | – | – | – | – |
| y₁ – H₂O                            | – | – | 788.4676 | – | 788.4676 | 774.4501 |
| y₁ – AA (19)                        | – | – | 637.3673 | – | 637.3673 | 623.3515 |
| y₁ – AA (19)                        | – | – | 509.3117 | – | 509.3117 | 495.2926 |
| y₁ – AA (19-18)                     | – | – | 381.2509 | – | 381.2509 | 367.2344 |
| y₁ – AA (19-17)                     | – | – | 282.1814 | – | 282.1814 | 282.1813 |
| y₁ – AA (19-16)                     | – | – | 197.1284 | – | 197.1284 | 197.1270 |

* n.d., Not detected.
| Type | Culture (CBS 119283) of Hypocrea phellinicola (maXis screening) |
|------|---------------------------------------------------------------|
|      | 19 | 32 | 33 | 34 | 35 | 36 |
|      | 13.5 | 14.1 | 14.9 | 15.5 | 15.9 | 16.2 |
| 1880.1008 | 1896.0964 | 1880.1035 | 1866.0863 | 1880.1012 | 1867.0706 |
| n.d. | n.d. | n.d. | 128.0697 | n.d. | n.d. |
| 199.1123 | 199.1123 | 199.1123 | 199.1074 | 199.1078 | 199.1078 |
| 270.1449 | 286.1389 | 270.1449 | 270.1449 | 284.1605 | 270.1438 |
| 341.1826 | 357.1760 | 341.1826 | 341.1819 | 355.1975 | 341.1816 |
| n.d. | 424.2191 | 408.2242 | 408.2280 | 422.2402 | n.d. |
| 426.2349 | 442.2296 | 426.2349 | 426.2349 | 440.2506 | 426.2354 |
| 554.2934 | 570.2869 | 554.2934 | 554.2933 | 568.3087 | 554.2932 |
| 639.3463 | 655.3404 | 639.3463 | 639.3465 | 653.3621 | 639.3461 |
| 752.4301 | 768.4257 | 752.4301 | 752.4303 | 766.4461 | 752.4295 |
| 837.4813 | 853.4789 | 837.4813 | 837.4833 | 851.4992 | 837.4824 |
| 894.5075 | 910.4971 | 894.5075 | 894.5044 | 908.5203 | 894.5037 |
| 1007.5825 | 1023.5860 | 1007.5825 | 1007.5891 | 1021.6041 | 1007.5911 |
| 1092.6420 | 1108.6370 | 1092.6440 | 1092.6432 | 1106.6582 | 1092.6413 |
| n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
|      | 788.4661 | 788.4667 | 788.4668 | 774.4504 | 774.4503 | 775.4366 |
| 637.3669 | 637.3683 | 637.3683 | 623.3499 | 623.3499 | 624.3356 |
| 509.3092 | 509.3078 | 509.3078 | 495.2931 | 495.2931 | 496.2769 |
| 381.2498 | 381.2500 | 381.2500 | 367.2337 | 367.2337 | 367.2337 |
| 282.1806 | 282.1815 | 282.1815 | 282.1812 | 282.1812 | 282.1814 |
| 197.1288 | 197.1286 | 197.1286 | 197.1284 | 197.1284 | 197.1287 |
Table 6 (cont.)

| Diagnostic fragment ions | Peaks [m/z]a) | 37 | 38 | 39 | 40 | 41 | 42 |
|--------------------------|--------------|----|----|----|----|----|----|
| tR [min]                 | 16.4         | 16.7| 16.8| 17.0| 17.2| 17.5|
| [M + H]+                 | 1880.1007    | n.d.| 1880.1009 | n.d.| 1880.0997 | 1894.1210|
| b1                       | n.d.         | n.d.| 128.0701 | 128.0713 | n.d.| n.d.|
| b2                       | 199.1075     | 199.1075 | 199.1077 | 199.1075 | 199.1075 | 199.1080|
| b3                       | 270.1444     | 284.1603 | 284.1602 | 284.1599 | 270.1444 | 284.1604|
| b4                       | 341.1819     | 355.1974 | 355.1975 | 355.1973 | 341.1819 | 355.1974|
| b1 – H2O                 | n.d.         | n.d.| 422.2399 | n.d.| n.d. | 436.2493|
| b5                       | 426.2350     | 440.2504 | 440.2499 | 440.2504 | 426.2350 | 454.2659|
| b6                       | 554.2935     | 568.3091 | 568.3080 | 568.3086 | 554.2935 | 582.3240|
| b7                       | 639.3462     | 653.3619 | 653.3613 | 653.3615 | 639.3462 | 667.3770|
| b8                       | 752.4307     | 766.4459 | 766.4450 | 766.4452 | 752.4307 | 780.4612|
| b9                       | 837.4843     | 851.4983 | 851.4983 | 851.4987 | 837.4843 | 865.5140|
| b10                      | 894.5019     | 908.5197 | 908.5205 | 908.5230 | 894.5019 | 922.5363|
| b12                      | 1007.5901    | 1021.6066 | 1021.6041 | 1021.6054 | 1007.5901 | 1035.6190|
| b12 – H2O                | 1092.6420    | 1106.6569 | 1106.6577 | 1106.6577 | 1092.6420 | 1120.6761|
| y1                       | –            | –      | –        | –        | –        | –        |
| y1 – AA (17)             | –            | –      | –        | –        | –        | –        |
| y1 – AA (17-16)          | –            | –      | –        | –        | –        | –        |
| y1 – AA (17-15)          | –            | –      | –        | –        | –        | –        |
| y1 – AA (18)             | –            | –      | –        | –        | –        | –        |
| y1 – AA (18-17)          | –            | –      | –        | –        | –        | –        |
| y1 – AA (18-16)          | –            | –      | –        | –        | –        | –        |
| y1 – AA (18-15)          | –            | –      | –        | –        | –        | –        |
| y1 – H2O                 | 788.4660     | 775.4348 | 774.4505 | 788.4664 | 788.4664 | 774.4522|
| y1 – H2O                 | 637.3704     | 624.3348 | 623.3515 | 637.3670 | 637.3670 | 623.3499|
| y1 – AA (19)             | 509.3084     | 469.2766 | 495.2929 | 509.3079 | 509.3079 | 495.2931|
| y1 – AA (19-18)          | 381.2504     | 367.2338 | 367.2338 | 381.2493 | 381.2493 | 367.2337|
| y1 – AA (19-17)          | 282.1808     | 282.1808 | 282.1807 | 282.1807 | 282.1807 | 282.1812|
| y1 – AA (19-16)          | 197.1288     | 197.1283 | 197.1274 | 197.1282 | 197.1282 | 197.1284|

a) n.d., Not detected.
|   | 43     | 44     | 45      | 46      | 47      | 48      | 49  |
|---|--------|--------|---------|---------|---------|---------|-----|
|   | 17.7   | 18.0   | 18.6    | 20.0    | 21.5    | 22.0    | 22.1–22.2  |
| n.d. | 128.0684 | 128.0684 | n.d. | n.d. | n.d. | n.d. | n.d. |
| 1895.1007 | 1894.1177 | 1908.1341 | 1922.1467 | 1936.1660 | 1009.7031 | 1066.7242 |
| 199.1084 | 199.1074 | 199.1080 | 227.1386 | 241.1536 | 212.1663 | 212.1644 |
| 284.1606 | 270.1440 | 284.1604 | 312.1916 | 326.2076 | 269.1858 | 269.1850 |
| 355.1969 | 341.1818 | 355.1974 | 383.2288 | 397.2443 | 382.2698 | 382.2695 |
| n.d. | 422.2401 | 436.2550 | n.d. | n.d. | – | – |
| 1992.4998 | 440.2501 | 454.2659 | 468.2807 | 482.2975 | 467.3234 | 467.3230 |
| 568.3077 | 568.3087 | 582.3240 | 596.3410 | 610.3540 | 524.3442 | 524.3428 |
| 653.3609 | 653.3614 | 667.3770 | 681.3925 | 695.4084 | 637.4289 | 581.3654 |
| 766.4466 | 766.4453 | 780.4612 | 794.4774 | 808.4926 | 722.4814 | 694.4498 |
| 851.4985 | 851.4983 | 865.5140 | 879.5284 | 893.5450 | 779.5027 | 779.5029 |
| 908.5184 | 908.5202 | 922.5363 | 936.5518 | 950.5672 | 892.5860 | 836.5243 |
| 1021.6039 | 1021.6067 | 1035.6190 | 1049.6372 | 1063.6524 | – | 949.6064 |
| 1106.6577 | 1106.6590 | 1120.6744 | 1134.6878 | 1148.7083 | – | – |
| 1088.6389 | n.d. | 1102.6586 | n.d. | n.d. | – | – |
| 789.4503 | 788.4660 | 788.4670 | 788.4660 | 788.4650 | – | – |
| 638.3516 | 637.3677 | 637.3670 | 637.3677 | 637.3678 | – | – |
| 510.2927 | 509.3076 | 509.3079 | 509.3076 | 509.3077 | – | – |
| 381.2498 | 381.2495 | 381.2493 | 381.2495 | 381.2492 | – | – |
| 282.1814 | 282.1807 | 282.1807 | 282.1807 | 282.1814 | – | – |
| 197.1292 | 197.1284 | 197.1282 | 197.1284 | 197.1277 | – | – |
Fig. 3. Sequencing of compounds 13 and 15 containing a new C-terminal residue with a peak at m/z 804.46, tentatively assigned as tyrosinol (Tyrol)
Table 7. Biological Activities of Selected 20-Residue Peptaibols Structurally Closely Related to Hypophellins

| Peptaibols     | Bioactivities reported                                      | Ref. |
|----------------|------------------------------------------------------------|------|
| Longibrachins  | Ion-channel formation in BLM, antimycoplasmic              | [53] |
| Suzukacillins  | Antibacterial, antifungal                                  | [78] |
|                | Ion-channel formation in BLM                               | [79] |
|                | Haemolysis of human erythrocytes                           | [80] |
| Trichoauracins | Haemolysis of sheep erythrocytes, antibacterial (g⁻)        | [54] |
| Trichobrachins | Antibacterial (g⁻), antifungal                             | [57] |
| Trichocellins  | Induction of Ca²⁺-dependent catecholamine secretion         | [67] |
|                | from bovine adrenal medullary chromaffin cells             |      |
|                | Ion-channel formation in BLM                               | [81] |
| Trichokonins   | Agonist towards Ca²⁺-channels in bullfrog cardiac myocytes | [55] |
|                | Antibacterial (g⁻), antifungal                             | [82] |
|                | Induction of defense responses and systemic resistance in   | [46] |
|                | tobacco against tobacco mosaic virus                        |      |
|                | Induction of apoptotic programmed cell death in fungal     | [47] |
|                | plant pathogens                                            |      |
| Trichosporins  | Uncoupling of the respiratory activity of rat liver        | [64] |
| B              | mitochondria                                              | [84] |
|                | Induction of Ca²⁺-dependent catecholamine secretion         | [85–87]|
|                | from bovine adrenal medullary chromaffin cells             |      |
|                | Ion-channel formation in BLM                               | [88] |
|                | Antitrypanosomal                                            | [66] |
| Paracelsins     | Antibacterial (g⁻)                                         | [89] |
|                | Increasing digestibility of starch and cellulose in         | [90] |
|                | ruminants; haemolysis of human erythrocytes; acutely toxic|      |
|                | in mice (LD₅₀ 5 mg/kg, i.p.)                                |      |
|                | Mosquitocidal (larvae of Culex pipiens)                     | [91] |
|                | Toxic against aquatic invertebrates (Daphnia magna,        | [92] |
|                | Artemisia salina)                                           | [93] |
|                | Ion-channel formation in BLM                               | [71] |
|                | Antifungal                                                  | [93] |

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